SYNTHESIS OF ALKALOIDS AND KINETIC STUDIES WITH DIAMINE OXIDASE.

A thesis in part fulfilment of the requirement for the Degree of Doctor of Philosophy.

by

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TABLE OF CONTENTS

ABBREVIATIONS

NMR	Nuclear magnetic resonance
TLC	Thin layer chromatography
IR	Infra red
DMF	*N,N*-Dimethylformamide
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
DFMO	α-Difluoromethylornithine
DAO	Diamine oxidase
K_M	Michaelis Menten constant
SDS	Sodium dodecylsulphate
PQQ	Pyrroloquinoline quinone
HPLC	High performance liquid chromatography
EPR	Electron paramagnetic resonance
BSA	Bovine serum albumin
V	Reaction rate
S	Substrate
E	Enzyme
ES	Enzyme-substrate complex
P	Product
V_{max}	Maximum reaction rate
I	Inhibitor
MBTH	3-Methyl-2-benzothiazolinone hydrazone
DMAB	3-(Dimethylamino)benzoic acid
THF	Tetrahydrofuran
UV	Ultra violet

SUMMARY

This thesis covers two main topics of research: (1) the use of 1-pyrroline and 1-piperideine aimed towards the synthesis of alkaloids and analogues; and (2) the inhibition of diamine oxidase.

(1) The Use of 1-Pyrroline and 1-Piperideine aimed towards the Synthesis of Alkaloids and Analogues.

1-Pyrroline is an unstable oil but can be complexed with zinc iodide to form a stable solid. This complex can be reacted, as a source of 1-pyrroline, with a range of β-ketoacids to give acylpyrrolidines in high yield. 1-Pyrroline was also found to form stable solid complexes with zinc chloride, zinc bromide and cadmium iodide. Attempts to use the zinc iodide complex as a source of 1-pyrroline in cycloaddition reactions proved unsuccessful. The complexation of 1-piperideine with zinc iodide also proved unsuccessful, though 1-piperideine was reacted with β-ketoacids to form acylpiperidines. 1-Piperideine and piperidine have been used in the construction of quinolizidine and tetracyclic quinolizidine alkaloids.

(2) The Inhibition of Diamine Oxidase.

A range of cyclic diamines was synthesised and tested as inhibitors of diamine oxidase using a spectrophotometric assay. This is a peroxidase-coupled assay which monitors the production of hydrogen peroxide during diamine oxidation. Although many of the compounds synthesised lack a primary amino group they are substrate analogues and could bind well to the enzyme. The majority

of the compounds synthesised, inhibited the diamine oxidase catalysed oxidation of cadaverine, and they were shown to be competitive inhibitors. A range of K_i values was obtained for the compounds tested.

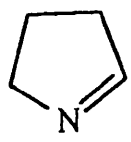
CHAPTER 1

REVIEW OF CYCLIC IMINES IN ORGANIC SYNTHESIS

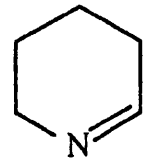
1.1 The Importance Of Imines

The use of imines and enamines in organic synthesis has been well documented.[1,2] The electrophilic nature of the carbon atom of the imine provides a ready site for nucleophilic attack. Most of the work in imine chemistry has focused on finding limits of chemical reactivity and structure of imines, but the applications to specific synthetic goals has established the use of imines in organic chemistry.

Imines also have an important role in the biosynthesis of a wide range of natural products. For example, 3,4-dihydro-2*H*-pyrrole (1-pyrroline) (1) has proved of interest to chemists for many years due to its proposed role in the biosynthesis of phenanthroindolizidine alkaloids such as septicine (2) and tylophorine (3).[3]



(1)



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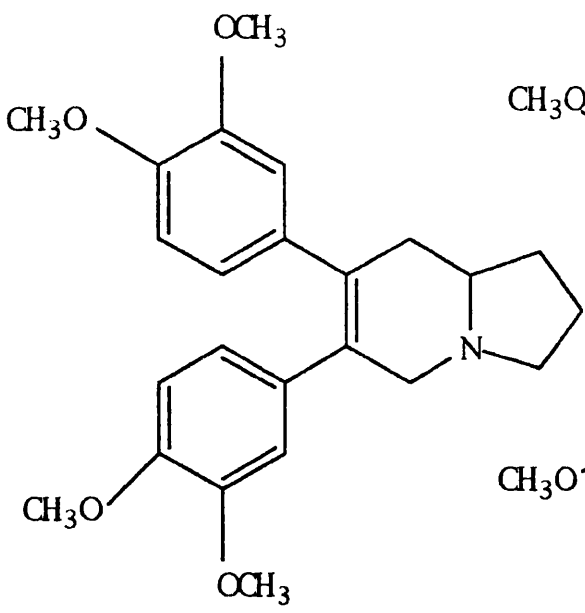

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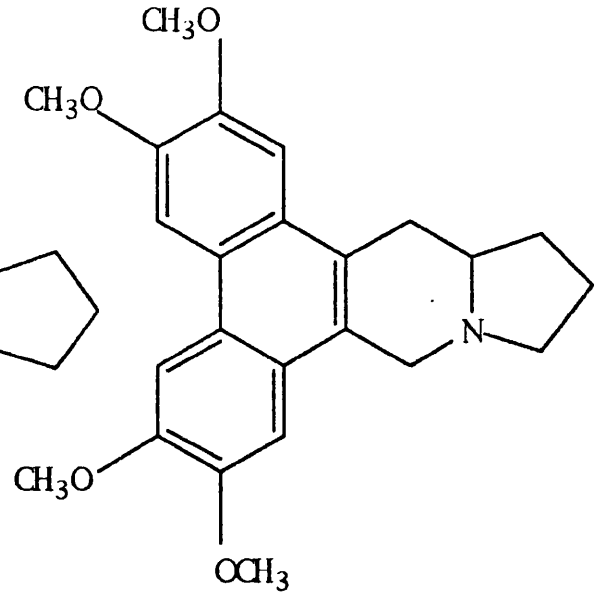
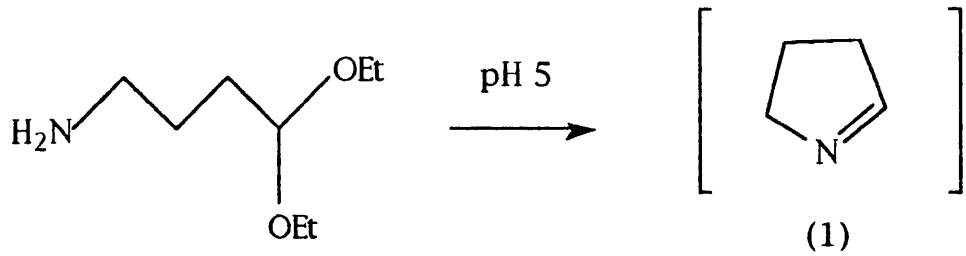


(3)

1.2 Synthesis of Cyclic Imines

Generally, the use of these reactive compounds in organic synthesis has suffered from the limitations present in the methods of generation of the iminium ion intermediates. These include low yields and lack of regiospecificity.[4]

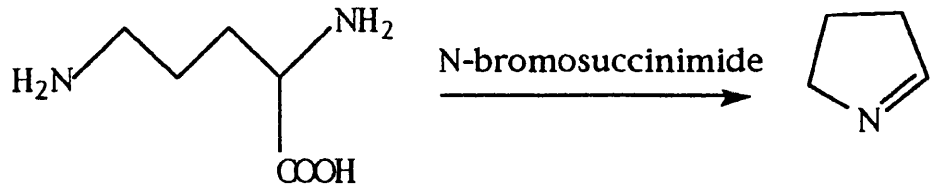
Cyclic imines such as 1-pyrroline (1) and 3,4,5,6-tetrahydropyridine (1-piperideine) (4) are potentially very useful synthetic imines, but their use has been hampered by their lack of stability and tendency to polymerise. Schopf carried out some of the earliest work on the preparation of 1-pyrroline.[5] Schopf developed one method which involved the hydrolysis of 4-aminobutanal diethyl acetal (5) at pH 5 which generated the imine *in situ* which was further condensed with *ortho*-aminobenzaldehyde (Scheme 1.1).[5] 1-Pyrroline can also be generated from ornithine (6) following treatment with *N*-bromosuccinimide [6] (Scheme 1.2) and by dehydrohalogenation of *N*-chloropyrrolidine (7) (Scheme 1.3).[7]



(5) (1)

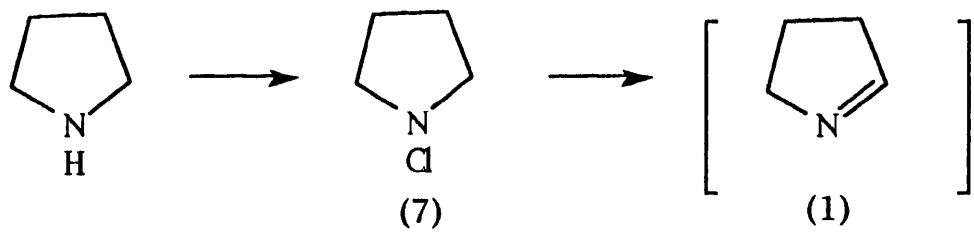
Not isolated

Scheme 1.1[5]



(6) (1)

Scheme 1.2[6]



(7) (1)

Not isolated

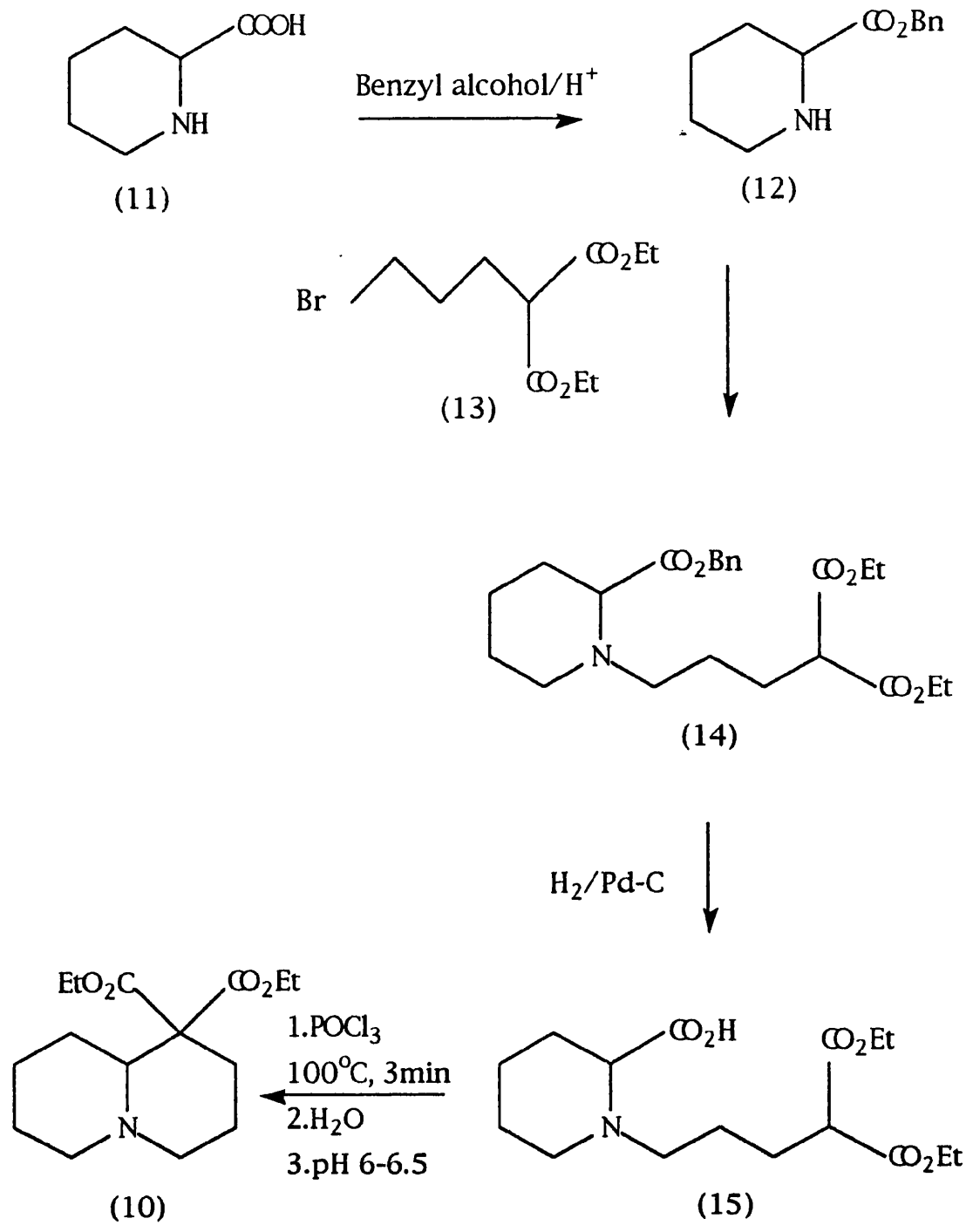
Scheme 1.3[7]

The apparently simple procedures of partial dehydrogenation of pyrrolidines and partial hydrogenation of pyrroles do produce 1-pyrroline, however, the reactions are complex and of little preparative value.[7] A better method involving an enzyme is also available. The action of pea-seedling diamine oxidase on 1,4-diaminobutane (putrescine) (8) and 1,5-diaminopentane (cadaverine) (9) produces, respectively, 1-pyrroline and 1-piperideine.[8] This is discussed in more detail later.

$H_2N(CH_2)_4NH_2$ (8)

$H_2N(CH_2)_5NH_2$ (9)

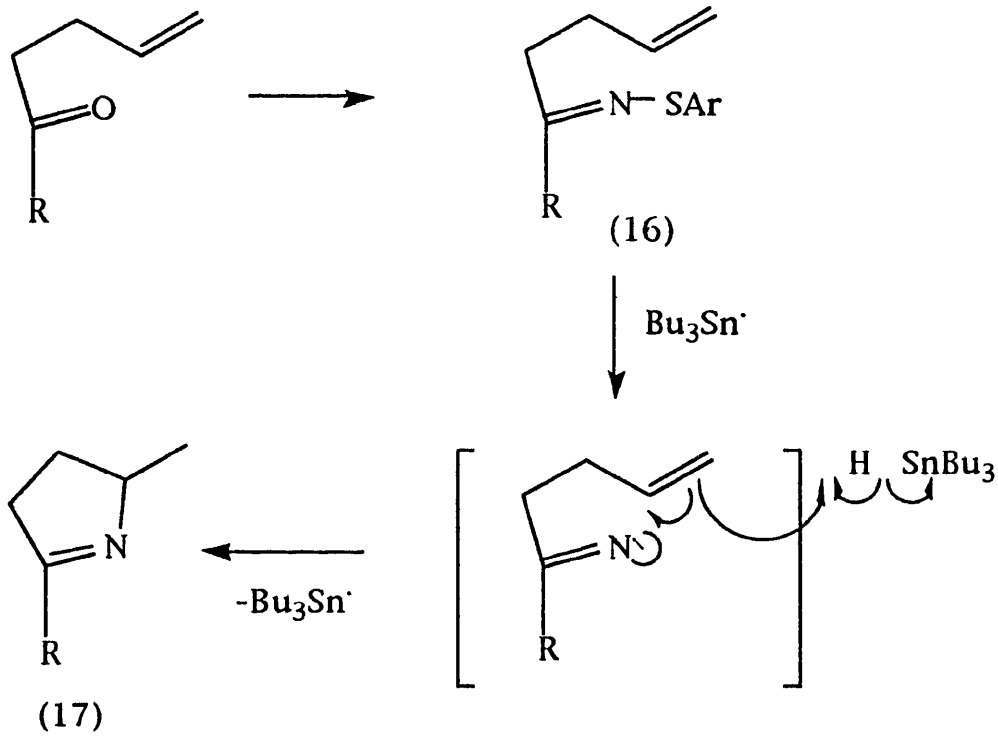
Rapoport and co-workers developed a method of generating cyclic iminium salts in high yields.[9] Generation of the iminium ion was achieved by decarbonylation of readily available α-(tertiaryamino)acids. The acids were mixed with an excess of phosphorus oxychloride and heated briefly to complete decarbonylation. Proline, pipecolic acid and hexahydroazepine-2-carboxylic acid served as the starting materials for the synthesis of pyrrolizidines, indolizidines, quinolizidines and 1-azabicyclo[5.4.0.]undecanes. One example of this synthetic strategy is the formation of diethyl 1-azabicyclo[4.4.0]decane-5,5-dicarboxylate (10) which is shown in Scheme 1.4. Pipecolic acid (11) was converted into the α-amino acid benzyl ester (12) which was then alkylated with diethyl 3-bromopropylmalonate (13) to give the triester (14). The benzyl group was removed by hydrogenation to give the free amino acid (15) as a crystalline solid. To effect cyclisation, the amino acid was treated with phosphorus oxychloride and heated to form the iminium ion. The mixture was then quenched with water and the pH adjusted to 6-6.5 which resulted in cyclisation to the corresponding 1-azabicycloalkane diester (10).



(11) (12) (13) (14) (10) (15)

Scheme 1.4[9]

A more recent approach to the synthesis of 1-pyrrolines has been developed by Zard and co-workers (Scheme 1.5).[10] A range of sulphenylimines (16) containing a double bond γ,δ to an imine were treated with tri-n-butylstannane to produce the corresponding 1-pyrroline derivatives (17) in good yield. Sulphenylimines are accessible from the corresponding carbonyl derivative or, in some cases, from the oxime.



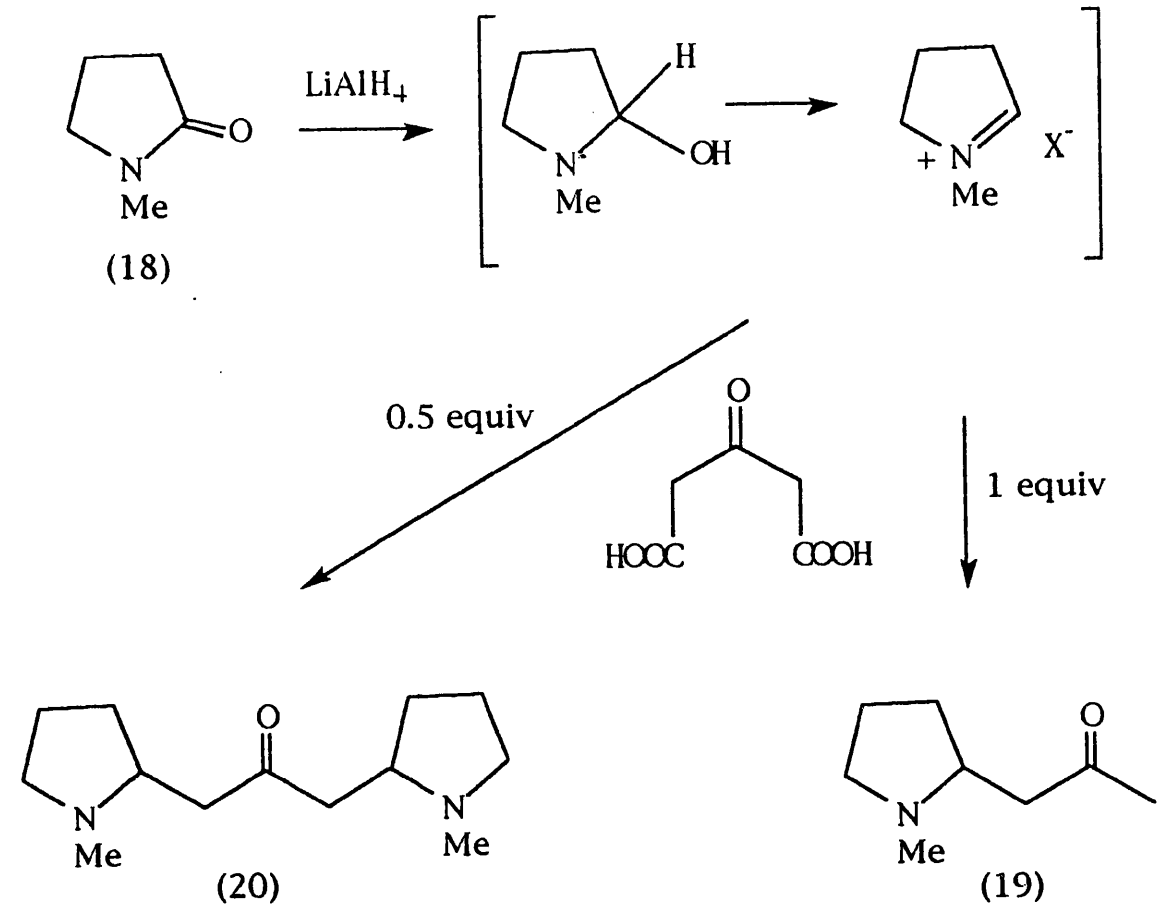
Scheme 1.5[10]

1.3 Cyclic Imines in Alkaloid Synthesis

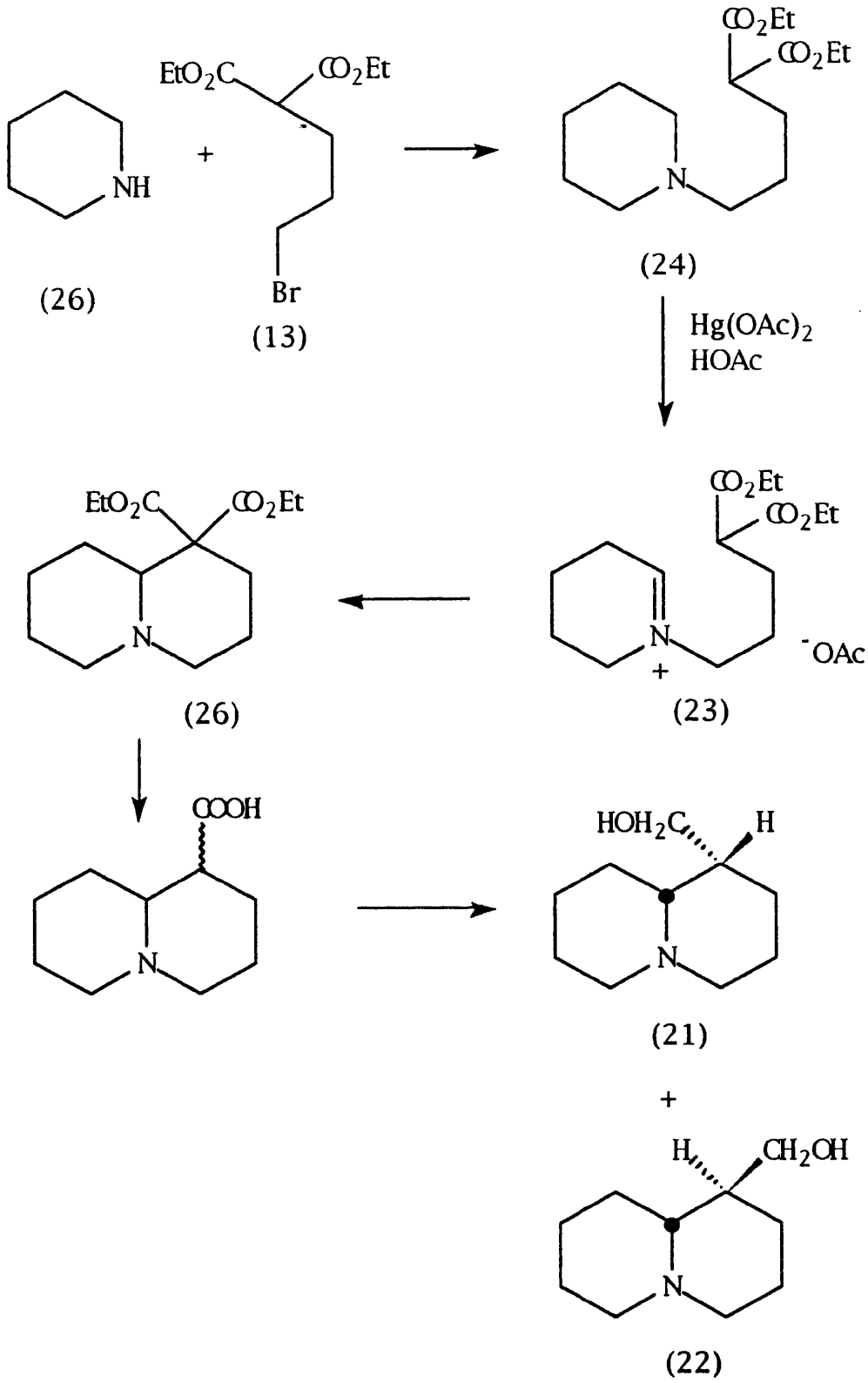
The earliest uses of cyclic imines in natural product synthesis were electrophilic reactions with the iminium salts and a number of syntheses are based on the reaction of these iminium salts with reactive methylene compounds under physiological conditions.

The reaction between 2-oxo-*N*-methylpyrrolidine (18) and lithium aluminium hydride, shown in Scheme 1.6, produces the corresponding iminium ion which combines with one equivalent of acetonedicarboxylic acid to give hygrine (19)[11] or 0.5 equivalents of acetonedicarboxylic acid to give cuscohygrine (20).[12]

Tamelen and Foltz reported a synthesis of lupinine (21) and its epimer (22) using a Mannich-type cyclisation of a malonate derivative involving an iminium ion intermediate (23) (Scheme 1.7).[13] *N*-(4,4-Dicarbethoxybutyl)piperidine (24) was obtained by the reaction of diethyl 3-bromopropylmalonate (13) with 2 equivalents of piperidine in benzene. Mercuric acetate dehydrogenation of (24) yielded 1,1-dicarbethoxyquinolizidine (26). Treatment of this product with hydrochloric acid to affect hydrolysis and decarboxylation followed by reduction with lithium aluminium hydride afforded the mixture of epimers (21) and (22) in a ratio of 1:1.



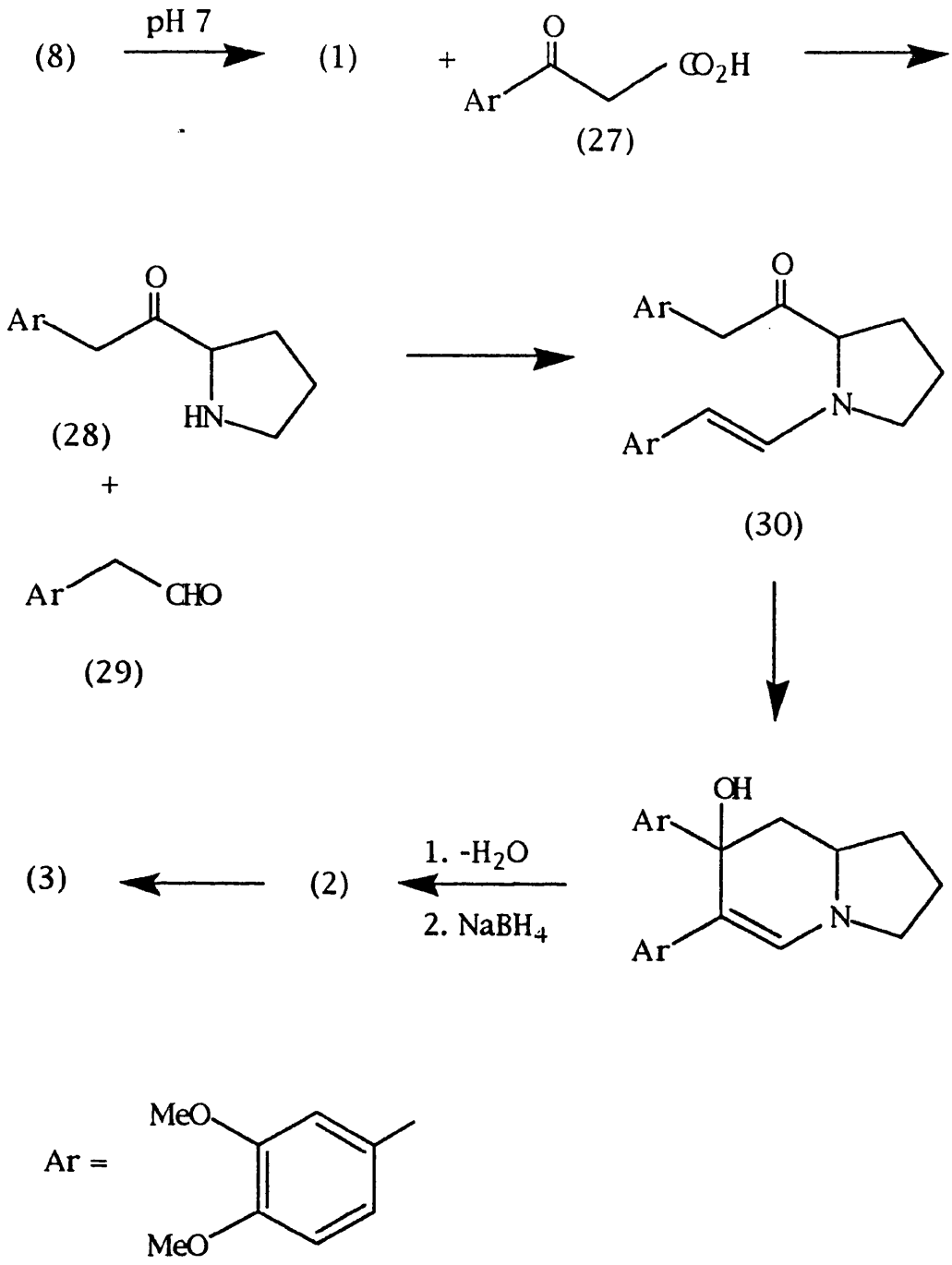
Scheme 1.6[11,12]


EtO2C
CO2Et
NH
Br
(26)
(13)
(24)
Hg(OAc)2
HOAc
(23)
-OAc
(26)
COOH
HOH2C
H
(21)
+
H
CH2OH
(22)

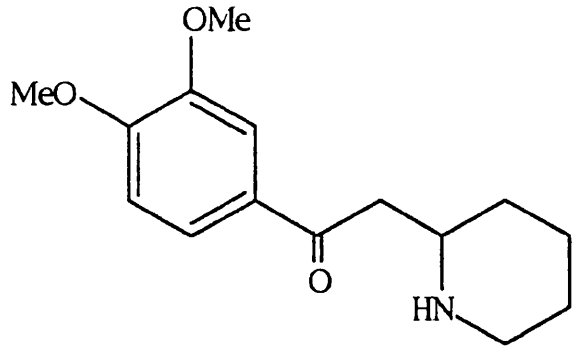
Scheme 1.7[13]

As previously mentioned 1-pyrroline (1) has been proposed as an intermediate in the biosynthesis of alkaloids such as septicine (2) and tylophorine (3). The use of an enzymic method to generate 1-pyrroline and 1-piperideine in the synthesis of these alkaloids has been employed by Herbert and co-workers.[14,15] They approached the synthesis of these compounds by a biogenetically patterned sequence, as shown in Scheme 1.8. 1-Pyrroline was prepared *in situ* by the oxidation of putrescine (8) using partially purified diamine oxidase and condensed with (3,4-dimethoxybenzoyl)acetic acid (27). Condensation of the product (28) with 3,4-dimethoxyphenylacetaldehyde (29) in benzene produced the enamine intermediate (30) which then underwent intramolecular cyclisation and dehydration. Reduction with sodium borohydride yielded septicine (2) which, on oxidation with thallium(III) trifluoroacetate, produced tylophorine (3).

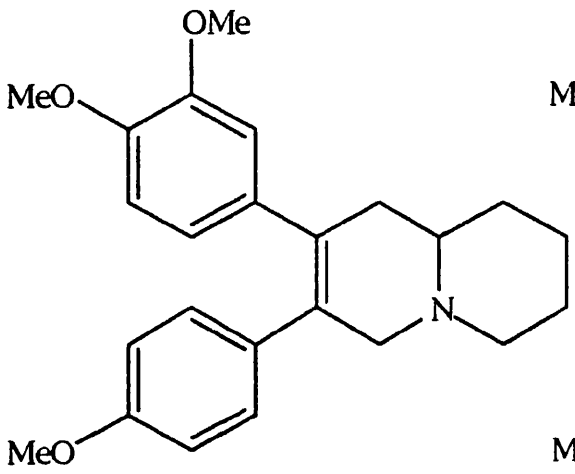
A similar reaction sequence using 1-piperideine, generated *in situ* from cadaverine using pea seedling diamine oxidase, resulted in the synthesis of 3',4'-dimethoxy-2-(2-piperidyl)acetophenone (31), julandine (32) and cryptopleurine (33).


(8)
pH 7
(1)
+
Ar
O
CO_2H
(27)
Ar
O
HN
(28)
+
Ar
CHO
(29)
Ar
O
N
Ar
(30)
OH
Ar
N
Ar
1. $-H_2O$
2. $NaBH_4$
(2)
(3)
Ar =
MeO
MeO

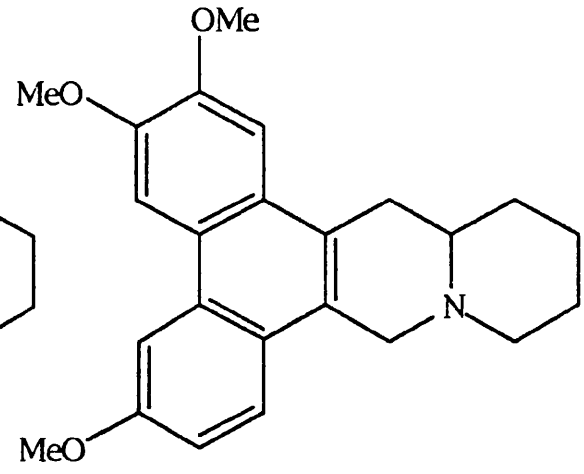
Scheme 1.8[14]



(31)

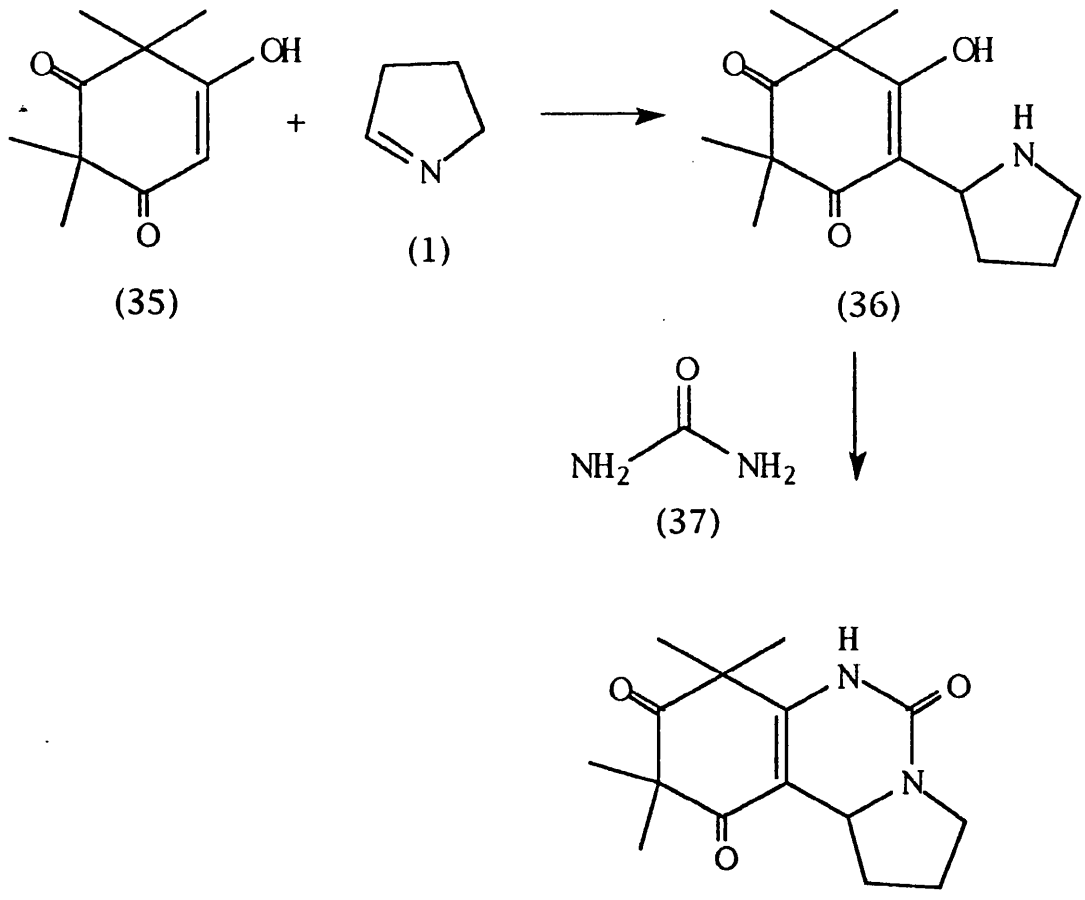


(32)

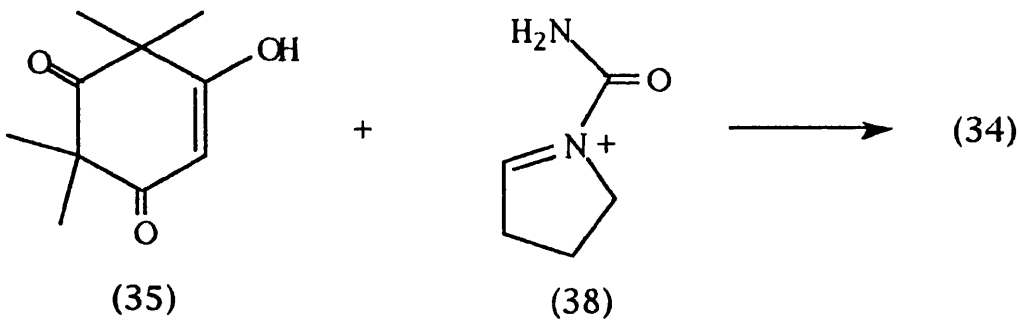


(33)

Syncarpurea (34), a minor metabolite of *Uvaria afzelii*, has shown interesting antibiotic and antitumour properties.[16] Clardy and Okamoto have reported two relatively simple syntheses of this natural product.[17] The first approach, shown in Scheme 1.9, involved the generation of 1-pyrroline from the reaction between ornithine hydrochloride and *N*-bromosuccinimide. The imine was then reacted with syncarpic acid (35), prepared from 2,4,6-trihydroxyacetophenone, to give (36). This product was then heated with urea (37) to produce syncarpurea (34). In the second approach to the synthesis, citrulline was treated with *N*-bromosuccinimide to yield the 1-carboxamido-Δ^1-pyrrolidinium (38) ion *in situ*. Further reaction with syncarpic acid produced syncarpurea (34) in good yield (Scheme 1.10).



Scheme 1.9[17]

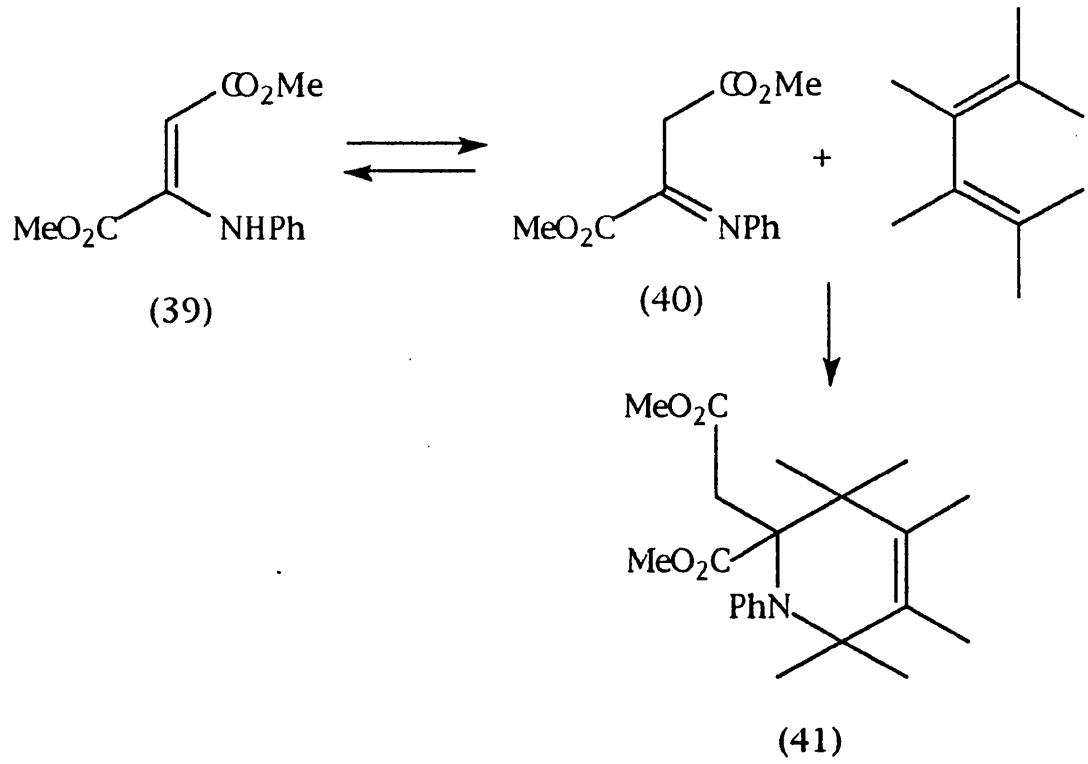


Scheme 1.10[17]

1.4 Imines In Diels-Alder Cycloadditions

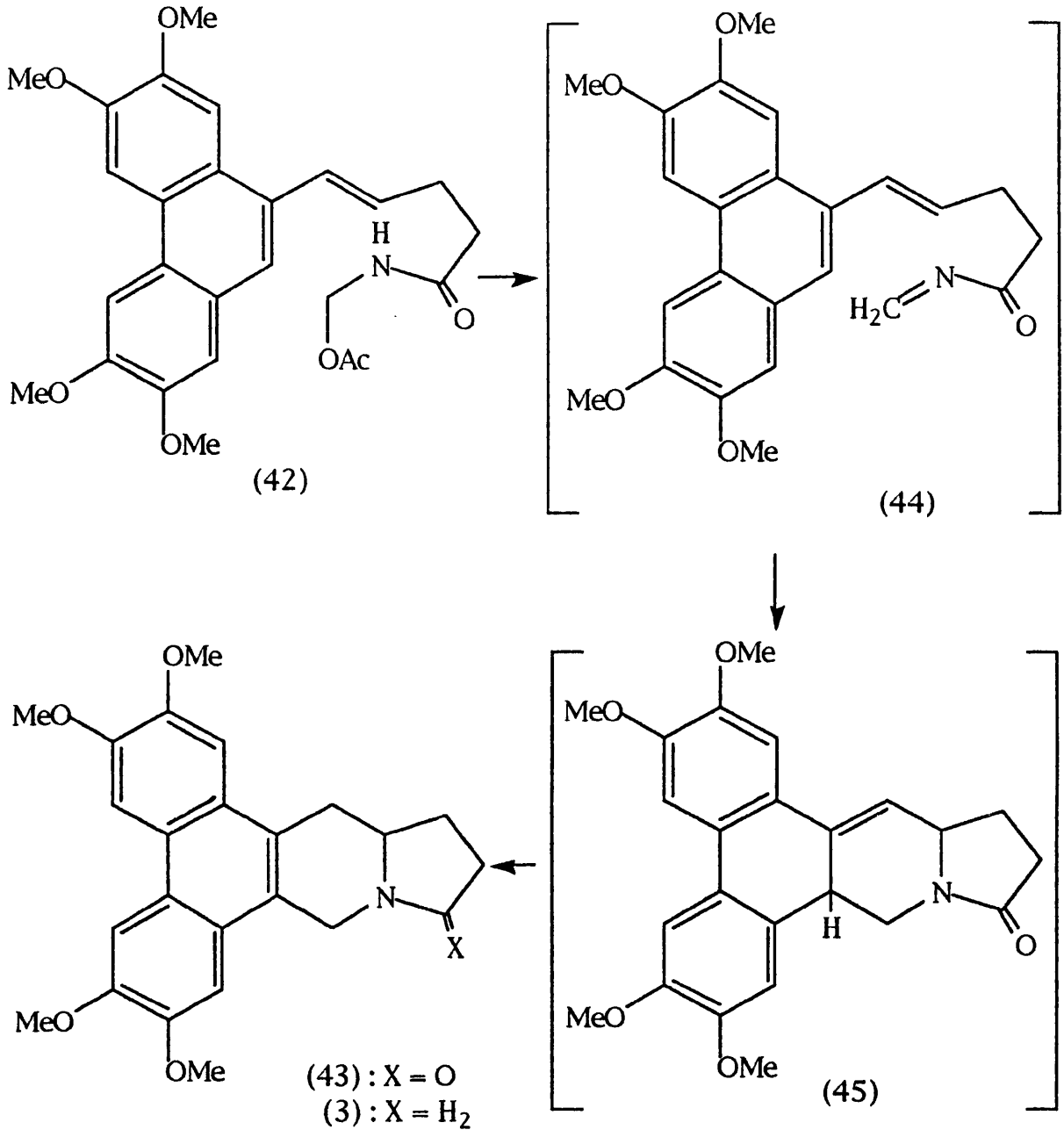
The Diels-Alder reaction is undoubtedly one of the most useful methods for the construction of carbocyclic systems. Alder wrote a brief description of the first example of a [4+2] cycloaddition reaction with an imine as the dienophile in a review in 1943.[18] The reaction between the enamine (39) and a number of dienes did not produce the expected carbocyclic systems. Instead the reaction proceeded by means of the imine tautomer (40) as the reactive species to produce a range of tetrahydropyridines (41) (Scheme 1.11). It has therefore been known for decades that reactive species can be generated, in which one or both of the atoms of the dienophile have been replaced by heteroatoms. There have been many reviews since, on the use of imines as dienophiles [19,20,21]

Generally, free imines make poor dienophiles, but do take part in Diels-Alder reactions in the presence of a Lewis acid catalyst.[22] Electron deficient imines have particularly proved to be the most effective dienophiles. Simple Schiff bases have been shown to be unreactive in [4+2] cycloadditions unless exceptionally reactive dienes are employed.[23,24] The generality of the Diels-Alder reaction with electron deficient species was highlighted by work using *N*-acylimines and their iminium salts[25] and *N*-sulfonylimines.[26] *N*-Acylimines are good electrophiles[27] and can also react as dienes in Diels-Alder cycloadditions[28] and *N*-sulfonylimines make good enophiles.[29] However, the scope of these reactions are limited since both types of imines are rapidly hydrolysed in the presence of traces of water.



Scheme 1.11[18]

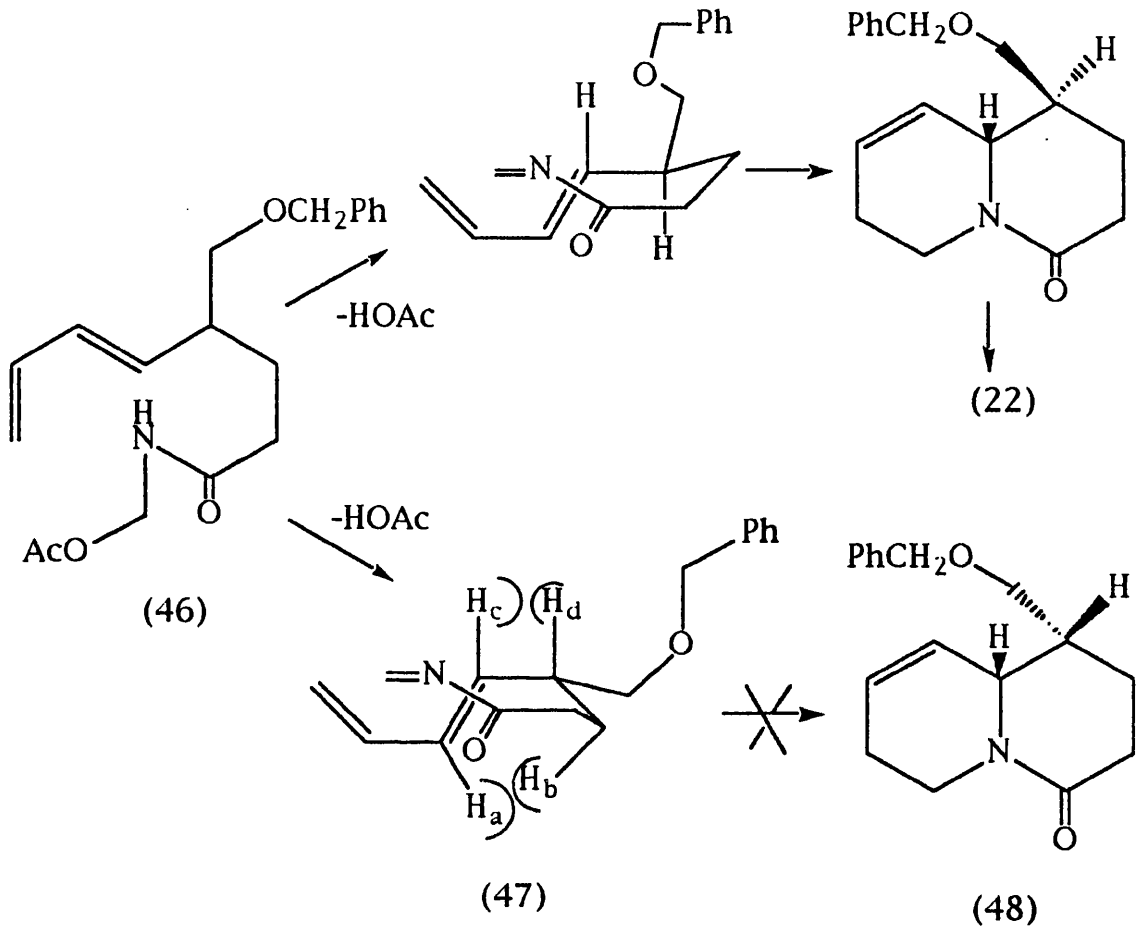
Weinreb has reported the synthesis of a number of alkaloids by intramolecular imino Diels-Alder cycloadditions.[30] In a different approach to the synthesis of tylophorine (3), shown in Scheme 2.12, Weinreb heated the acetate (42) in a sealed tube to give the pentacyclic lactam (43). The *N*-acylimine intermediate (44) undergoes a [4+2] cycloaddition to afford (45) followed by a 1,3-hydrogen shift to produce (43). Reduction of (43) with lithium aluminium hydride gave tylophorine (3).



Scheme 1.12[30]

Weinreb also investigated the application of the intramolecular imino Diels-Alder reaction to the synthesis of epilupinine (22) (Scheme 1.13).[30] The acetate (46) was prepared from methyl sorbate and heated to reflux in *o*-dichlorobenzene. The product formed was one epimer only of the bicyclic lactam. Catalytic hydrogenation of this epimer, followed by borane reduction yielded racemic epilupinine (22). It was thought that unfavourable interactions between H_a/H_b

and H_c/H_d in the intermediate (47) could account for the absence of the second lactam (48).



Scheme 1.13[30]

1.5 Natural Product Biosynthesis Involving Iminium Ions

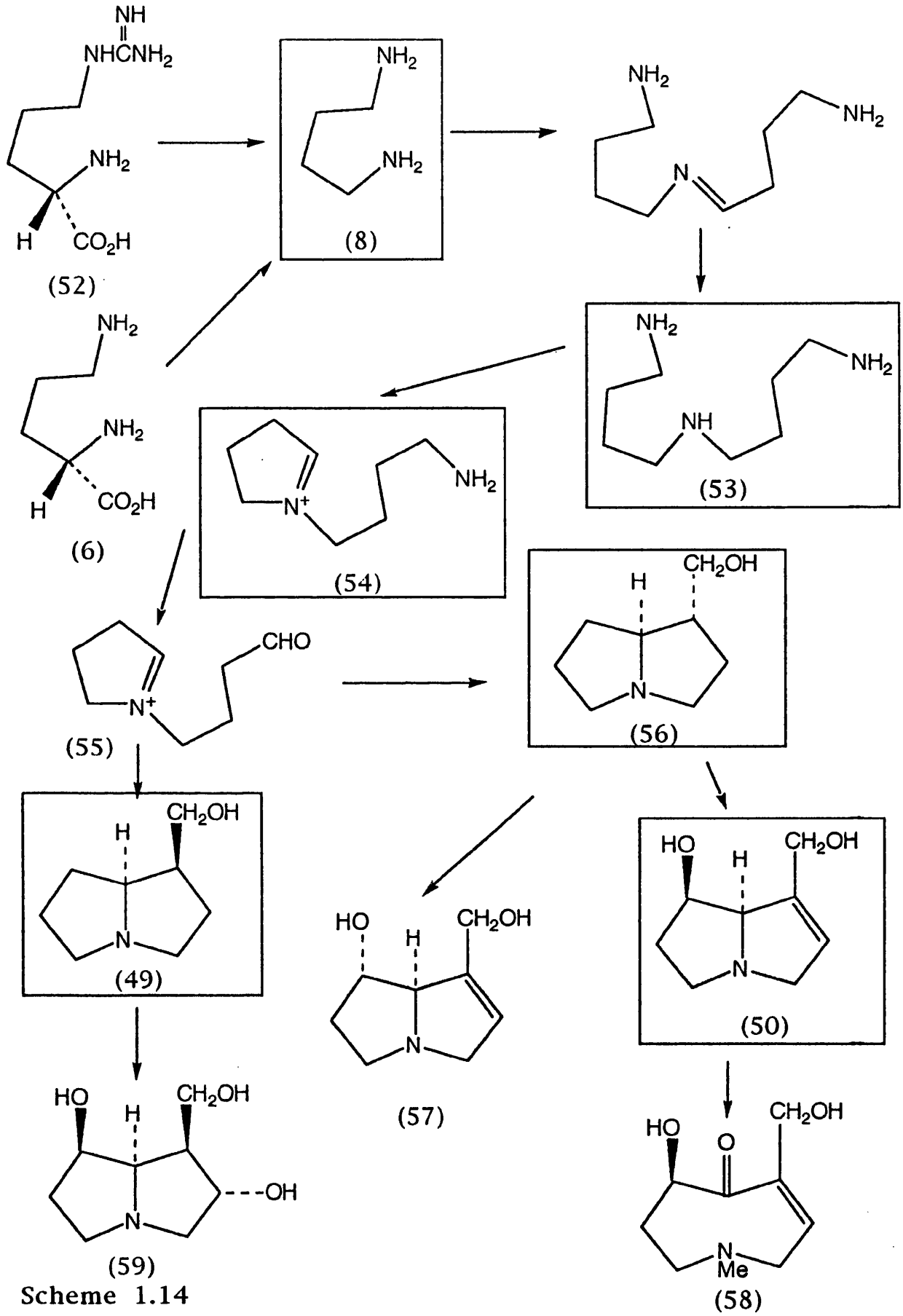
It was first suggested in 1955 that the biosynthetic pathways to the pyrrolizidine alkaloids isoretronecanol (49) and retronecine (50), and the quinolizidine alkaloid lupinine (21), could be very similar [31] It was suggested that isoretronecanol was derived from two molecules of ornithine (6), and lupinine from two molecules of lysine (51). However, with the advent of highfield NMR spectroscopy for isotopic labelling studies this theory has been proven incorrect and the pathways are in fact quite different.

1.5a The Biosynthesis of Retronecine

The biosynthesis of retronecine involves a symmetrical intermediate formed from two molecules of putrescine (8) as shown in Scheme 1.14.[32] The putrescine is derived from either L-arginine[33] (52) or L-ornithine (6).[34,35] Oxidation of putrescine, catalysed by homospermidine synthase, produces an enzyme-bound intermediate which reacts with a second molecule of putrescine. The resulting imine is reduced by NADH to give homospermidine (53).[36] Oxidation of a primary amino group of (53) yields the iminium ion (54)[37] which is converted into (55) by oxidative deamination of the other primary amino group. There are two possible cyclisations of the iminium ion. Following reduction of the aldehydes an *endo*-alcohol or an *exo*-alcohol are formed.[38] The *exo*-alcohol, trachelanthamidine (56), is the likely precursor of retronecine[39] and heliotridine (57), and otonecine (58) is probably formed from retronecine. The *endo*-alcohol, isoretronecanol, is converted by two hydroxylations into rosmarinecine (59).

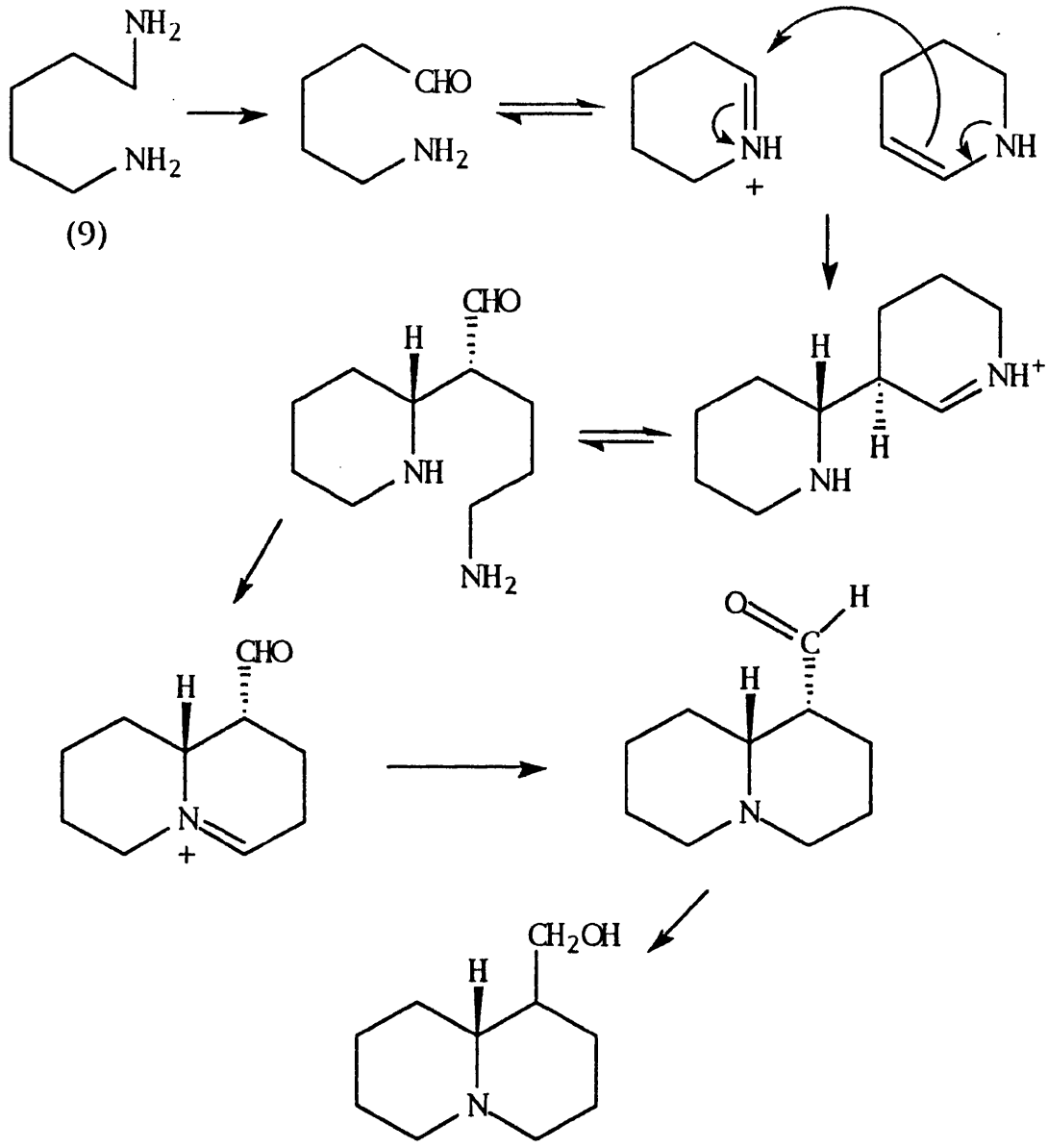
1.5b The Biosynthesis of Lupinine

As mentioned earlier it was proposed that lupinine (21) is derived from two molecules of lysine. By analogy with the proposed pathway to isoretronecanol this would suggest that the route to lupinine would proceed via cadaverine (9). L-Lysine and cadaverine have both been shown to be precursors of lupinine.[40] However evidence from feeding [^{13}C-^{15}N] labelled cadaverine indicates that there is no later symmetrical intermediate in the lupinine pathway, in contrast to the biosynthesis of retronecine.[41]


NH
NHCNH2
NH2
H
CO2H
(52)
NH2
NH2
(8)
NH2
NH2
N
NH2
NH2
H
CO2H
(6)
NH2
NH2
NH
(53)
N+
NH2
(54)
CHO
N+
(55)
CH2OH
H
N
(56)
CH2OH
H
N
(49)
HO
H
CH2OH
N
(57)
HO
H
CH2OH
N
(50)
HO
H
CH2OH
N
---OH
(59)
HO
O
CH2OH
N
Me
(58)

Scheme 1.14

It has been proposed that two molecules of 1-piperideine are generated from cadaverine which may combine to form tetrahydroanabasine.[42] The proposed pathway to lupinine is shown in Scheme 1.15

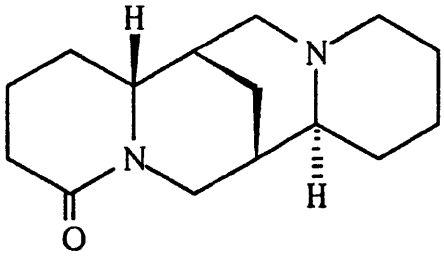


Scheme 1.15

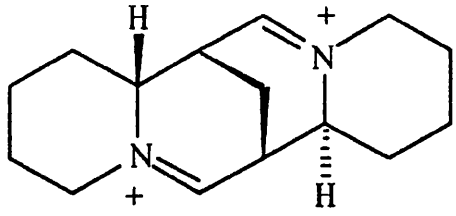
1.5c The Biosynthesis of Tetracyclic Quinolizidine Alkaloids

Labelling studies have indicated that tetracyclic quinolizidine alkaloids such as (-)-sparteine (60) and (+)-lupanine (61) are derived from three molecules of lysine and cadaverine.[43] Golebiewski and Spenser suggested the cadaverine is oxidized to 5-aminopentanal which is in equilibrium with 1-piperideine. It was thought that the alkaloids are formed from a modified form of the trimer of 1-piperideine.[44] Later evidence then suggested that tetrahydroanabasine was involved as an intermediate and the tetracyclic system is formed by combination with a third five carbon unit.[45] However, no intermediates between cadaverine and tetracyclic quinolizidine alkaloids have been firmly established. The proposed pathway to (-)-sparteine from tripiperideine is shown in Scheme 1.16. One of the intermediates postulated in this pathway is the bis-(iminium) ion (62).[45] Sparteine and lupanine could both be formed by stereospecific attack of a hydride donor on these iminium ions.

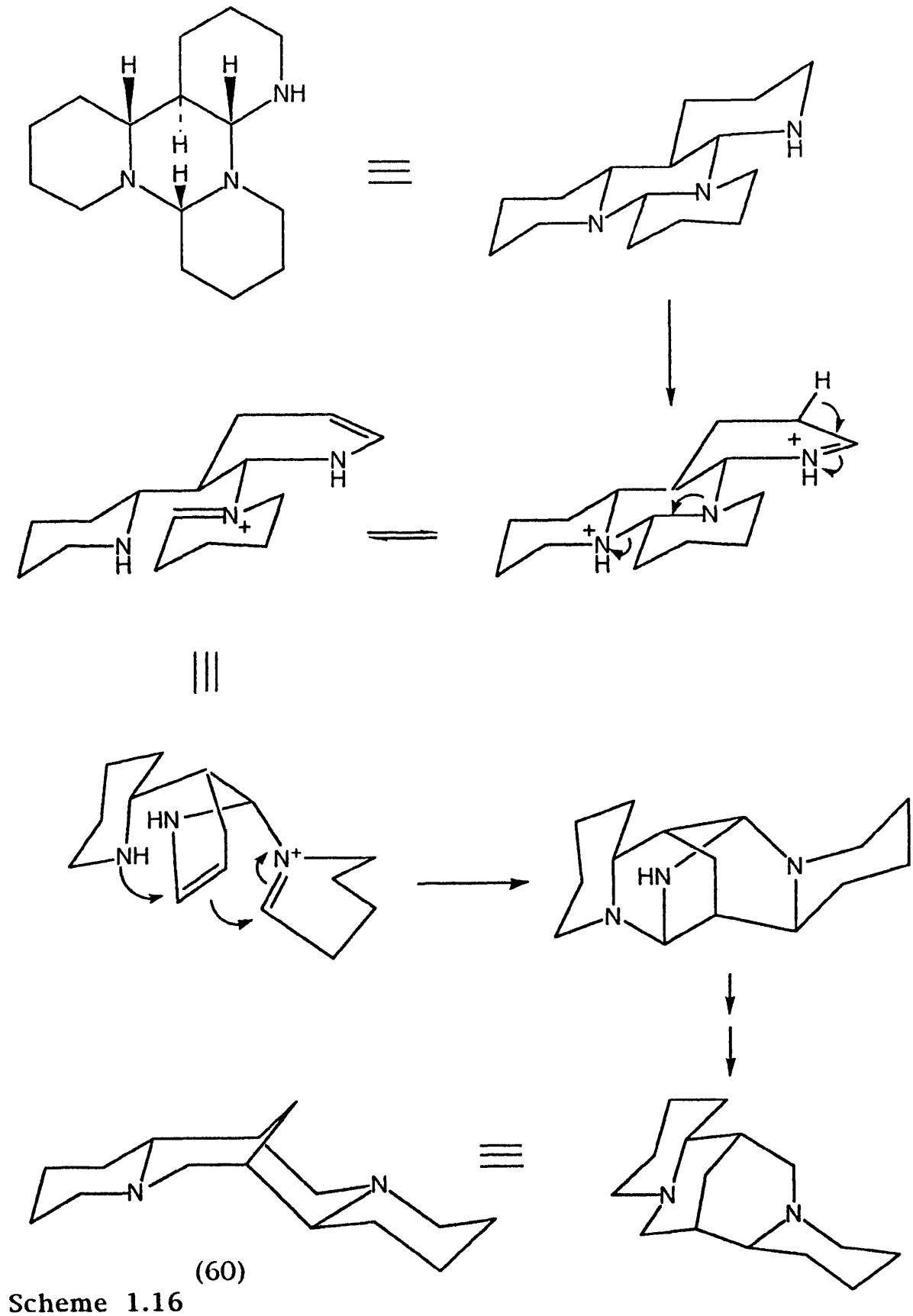
The synthesis of the tetracyclic quinolizidine alkaloid 8-ketosparteine is discussed in Chapter 2.



(61)



(62)



(60)

Scheme 1.16

CHAPTER 2

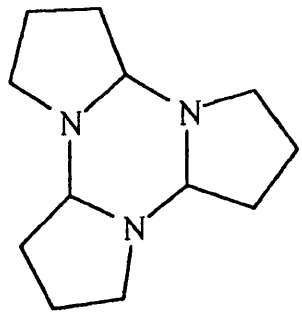
THE USE OF 1-PYRROLINE AND 1-PIPERIDEINE IN ALKALOID SYNTHESIS

2.1 Introduction

The generation and the use of imines in organic synthesis, and their role in natural product biosynthesis was discussed in detail in Chapter 1. In this chapter the following topics will be covered: the synthesis and stabilisation of 1-pyrroline (1) as a complex with a variety of metal halides; the use of 1-pyrroline complexes in alkaloid synthesis and cycloaddition reactions; and finally the synthesis and use of 1-piperideine (4) in alkaloid synthesis and cycloaddition reactions.

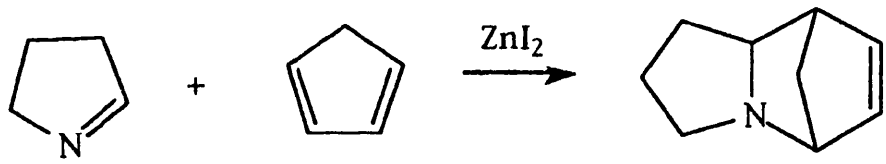
2.2 Synthesis of 1-Pyrroline Complexes

As previously mentioned, 1-pyrroline is a useful sython for the construction of 5-membered systems, but its usage is limited due to its instability and tendency to trimerise to (63) in basic solution.[7,46] The work of Schopf revealed that the acidic hydrolysis of 4-aminobutanal diethylacetal (5) does not yield the free aldehyde, instead it produces 1-pyrroline which is stable up to 100 °C in acid.[5]



(63)

It was recently discovered at the end of a Ph.D. project by George Baxter[47] that 1-pyrroline forms a stable crystalline complex with zinc iodide. The discovery was made while attempting to react a solution of 1-pyrroline in ether with cyclopentadiene (64) using zinc iodide as a Lewis acid catalyst as a route to 1,3-disubstituted pyrrolizidines (65) (Scheme 2.1).

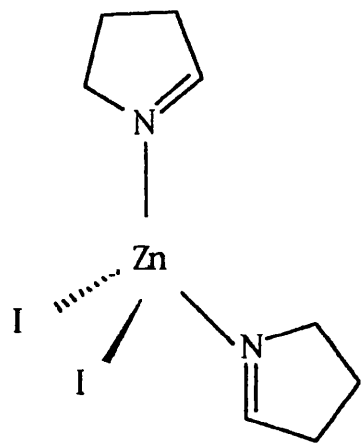


(1) (64) (65)

Scheme 2.1[47]

Instead of the desired product a white solid was obtained with the empirical formula $C_8H_{14}N_2I_2Zn$ which suggests a molecular formula of $(C_4H_7N)_2ZnI_2$ which was assigned the tetrahedral structure (66) from knowledge of other zinc complexes. Compared to the free imine, all the signals in the ^1H NMR spectrum were shifted downfield as a result of the electron withdrawing effect of the zinc. The infra-red spectrum shows the imine stretch at 1635 cm^{-1} compared to 1620 cm^{-1} for the free

imine. Also present in the infra-red spectrum is a zinc-iodine stretch at 420 cm^{-1}.



(66)

It was our aim to synthesise the complex and obtain good crystals in order to obtain confirmation of the structure by X-ray crystallography. It was also of interest to investigate whether the reaction of 1-pyrroline with zinc iodide is unique or if other metal halides yield similar complexes.

4-Aminobutanal diethylacetal (5) underwent acid hydrolysis at 0 °C in ether. On treatment with base, 1-pyrroline could be extracted with cold ether. Addition of zinc iodide to ethereal extracts at 0 °C precipitated the complex and recrystallisation from chloroform gave the complex in 61% yield. Cold ether is used in the work-up since 1-pyrroline is very volatile.

Structure (66) was confirmed for the complex by X-ray crystal structure analysis, shown in Figure 2.1. This was carried out by Dr. A. A. Freer in the University of Glasgow. The molecule straddles a mirror plane with the zinc atom sitting on the plane. One of the 1-pyrroline rings lies in the mirror plane and the other lies perpendicular to it.[48]

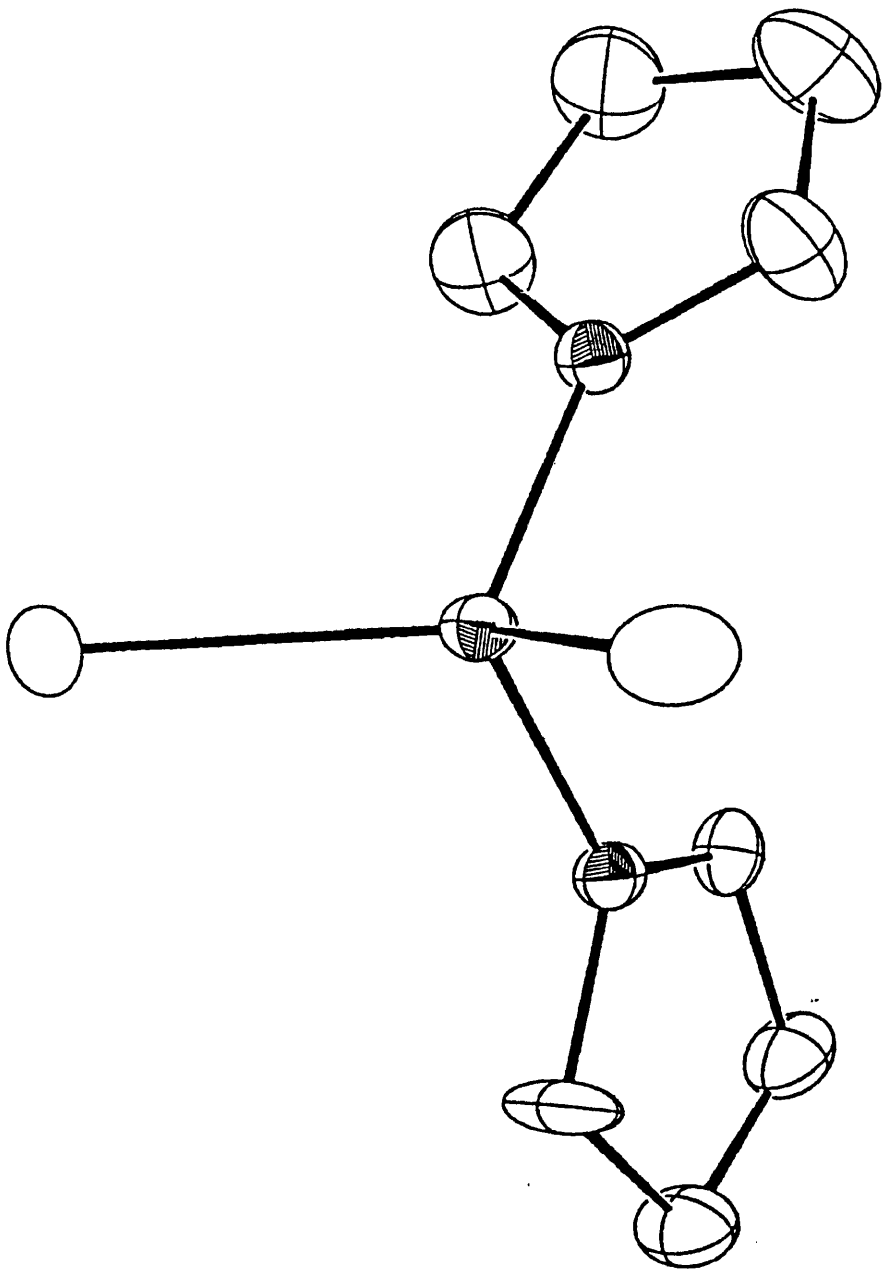


Figure 2.1

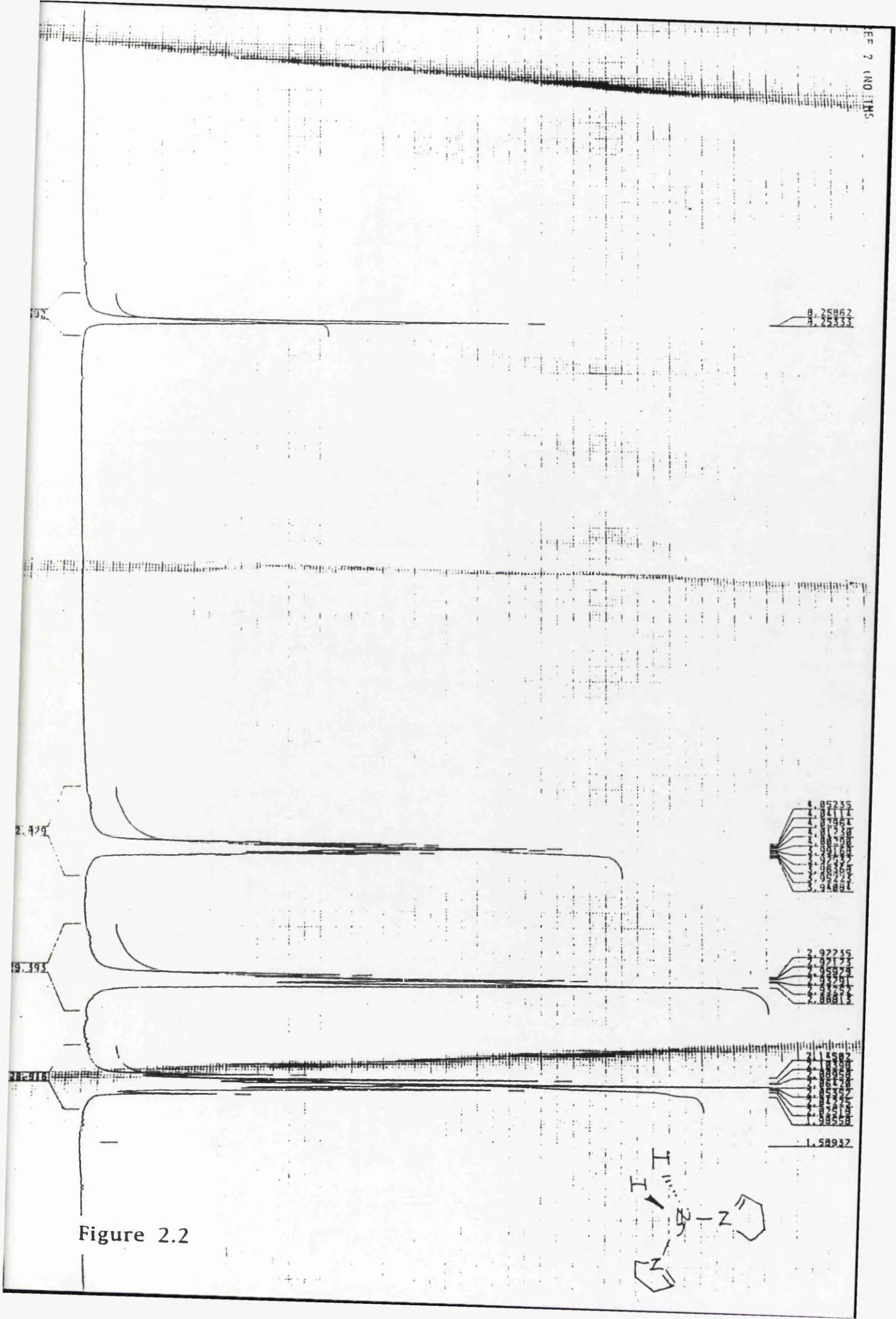


Figure 2.2

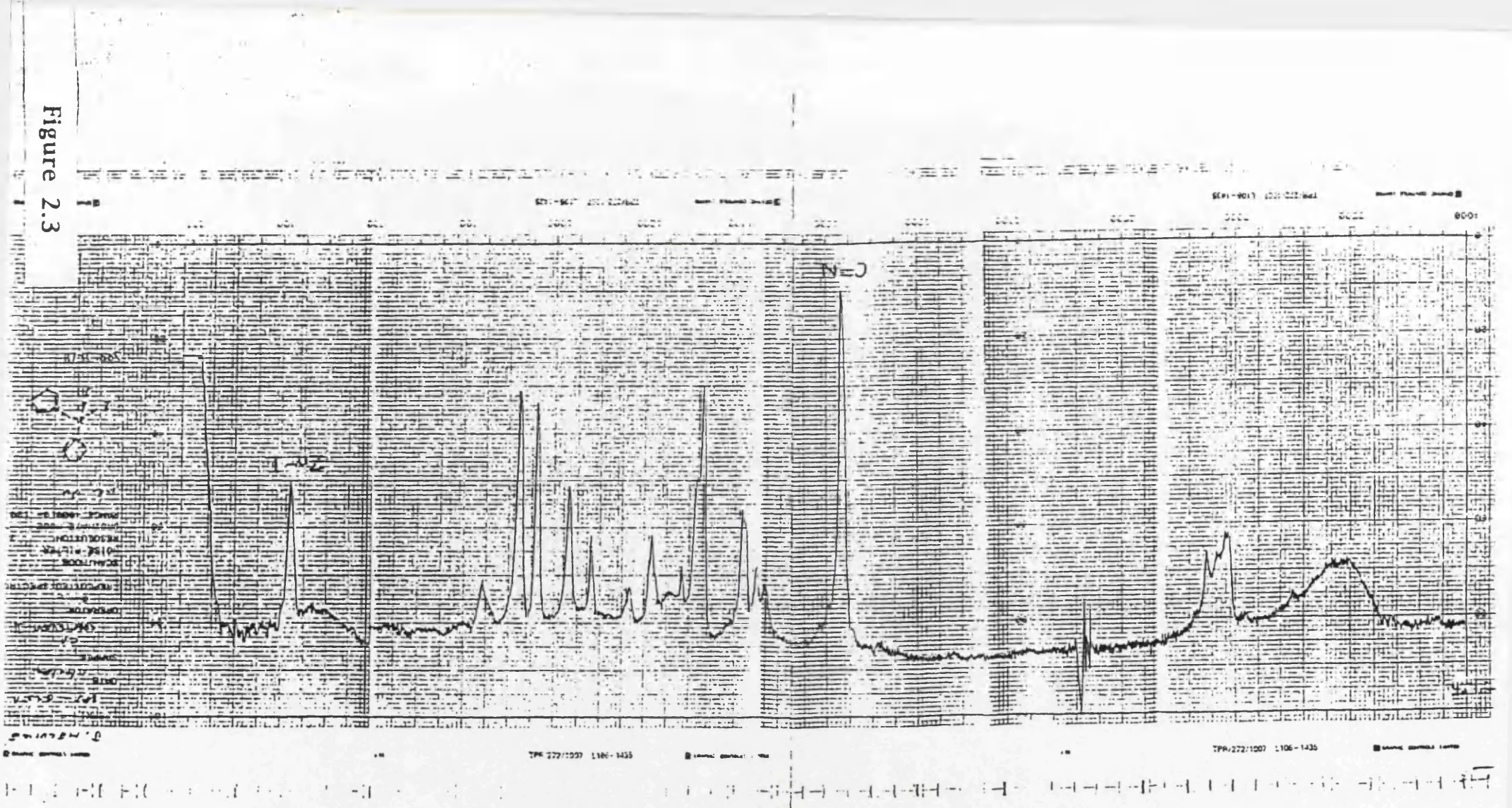
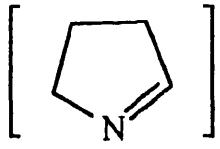


Figure 2.3

1-Pyrroline was also found to react with zinc chloride, zinc bromide and cadmium iodide to give compounds (67)-(69) (Scheme 2.2) Both the zinc chloride and zinc bromide complexes gave elemental analyses similar to the zinc iodide complex. However the cadmium iodide complex gave an elemental analysis indicating an empirical formula of $C_4H_7NI_2Cd$. These three additional complexes were formed in rather lower yields compared to the zinc iodide complex. For that reason, combined with the possibly high toxicity of the cadmium complex, the zinc iodide complex was used as the starting material for the following syntheses.



(1)

$\xrightarrow{ZnCl_2}$ $ZnCl_2(C_4H_7N)_2$ (67)

$\xrightarrow{ZnBr_2}$ $ZnBr_2(C_4H_7N)_2$ (68)

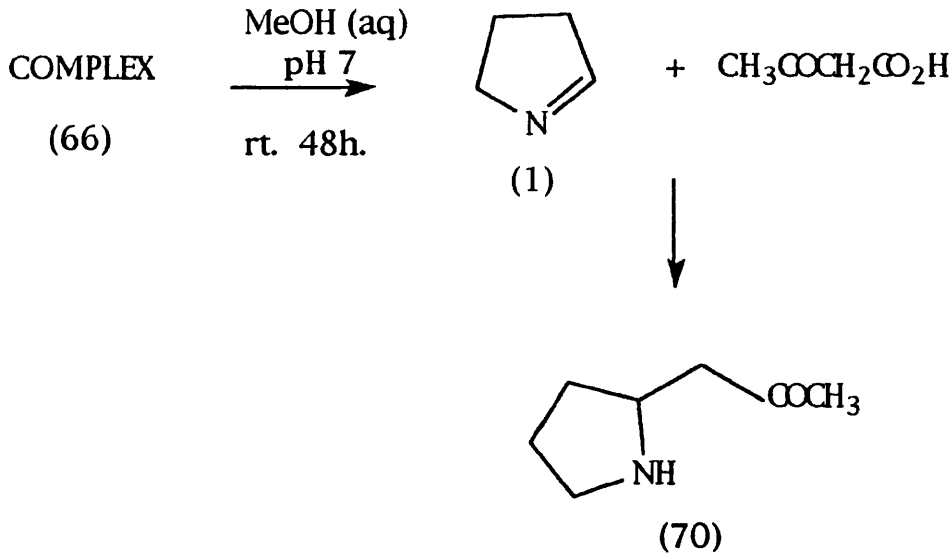
$\xrightarrow{CdI_2}$ $CdI_2(C_4H_7N)$ (69)

Scheme 2.2

2.3 The Use of the Complex with β-Ketoacids

George Baxter investigated the ease with which 1-pyrroline could be released from the complex. He found that the free imine could be obtained using ethylene diamine, diethylamine, ammonia, ethylamine or triethylamine, by displacement of 1-pyrroline as the ligand. The use of the complex as a direct source of 1-pyrroline was also investigated. As mentioned in Chapter 1

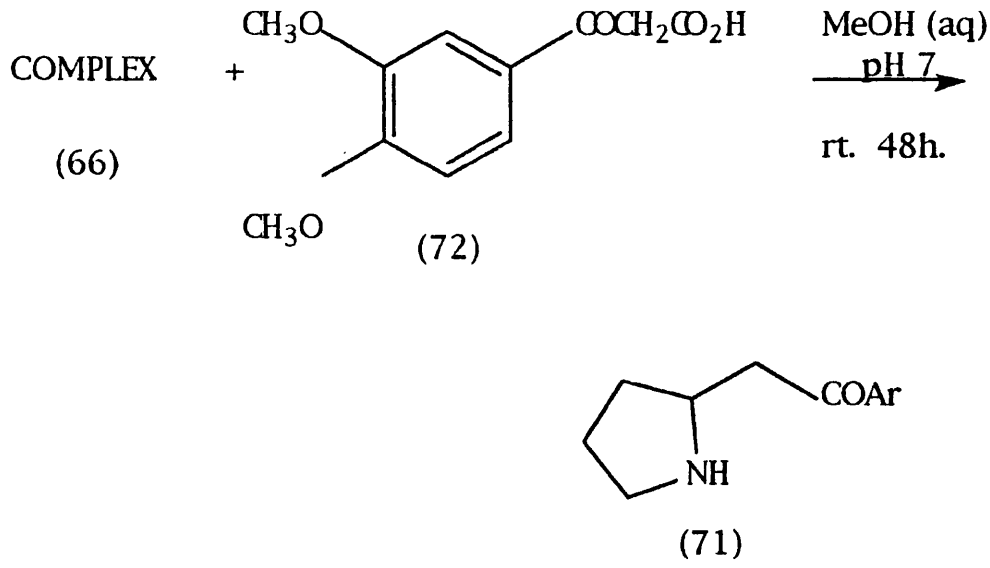
Herbert and co-workers generated 1-pyrroline from putrescine enzymically for the synthesis of phenanthroindolizidine alkaloids.[14,15] George Baxter used the complex directly and added it to a solution of acetoacetic acid and phosphate buffer in methanol to give norhygrine (70) (Scheme 2.3).



Scheme 2.3[47]

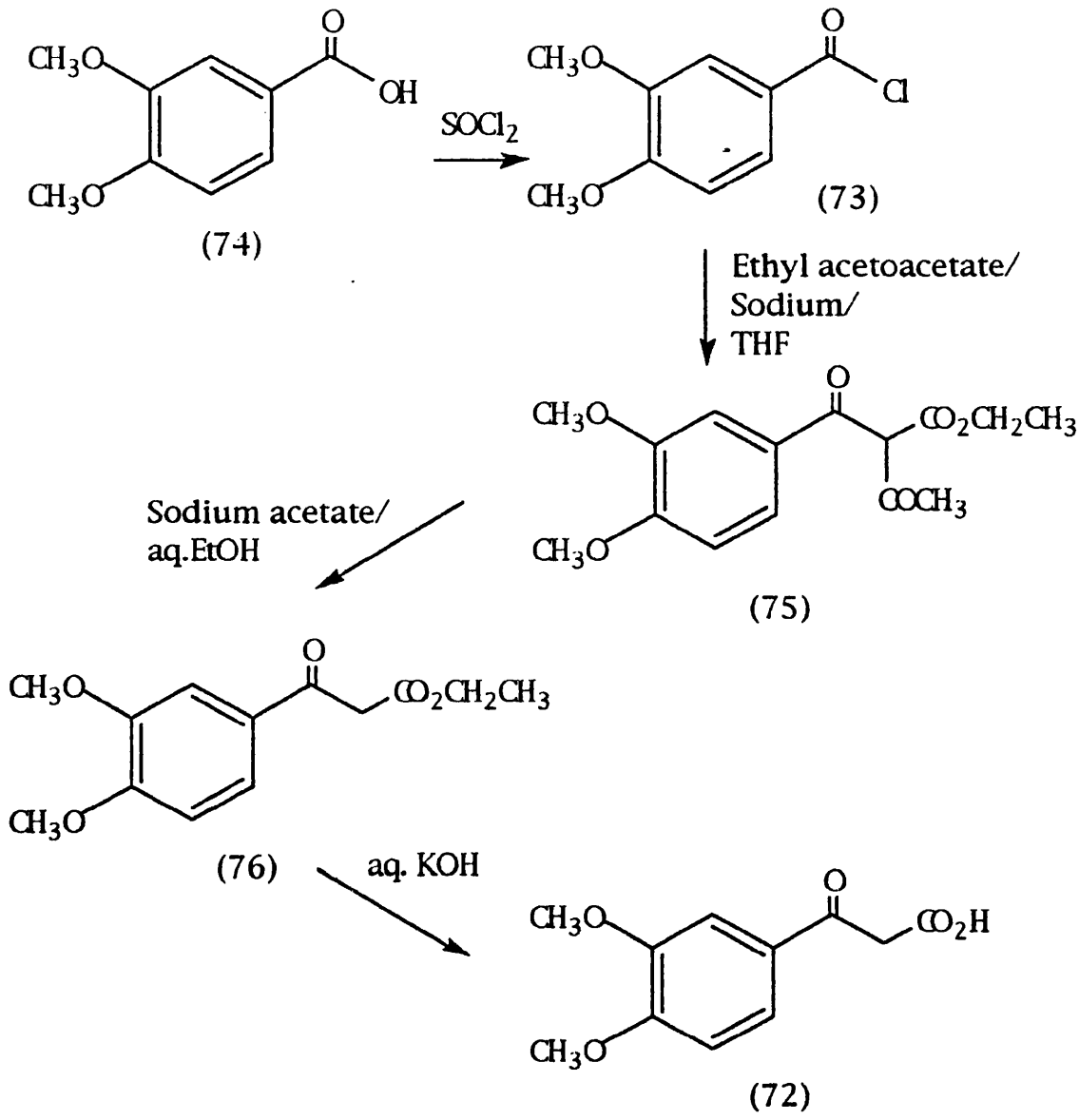
In our work, we repeated this synthesis of norhygrine and adapted it to the synthesis of ruspolinone (71). Ruspolinone is a pyrrolidine alkaloid isolated from *Ruspolia hypercrateriformis* a plant of the Acanthaceae family.[49] The zinc iodide complex in methanol was added directly to 3,4-dimethoxybenzoylacetic acid (72) in phosphate buffer. After 48 hours reaction time, ruspolinone was isolated in 92% yield (Scheme 2.4). Preparative TLC gave the purified product in 81% yield. The IR spectrum showed the ketone carbonyl absorption at 1675 cm^{-1} and high resolution mass spectrometry gave a molecular weight of 249.1356 in accordance with a theoretical value of 249.1365. The ^{1}H and ^{13}C NMR spectra were both in accordance with literature

spectra of ruspolinone.[50] This work on the preparation and use of the zinc complex has been published.[51]



Scheme 2.4

3,4-Dimethoxybenzoyl acetic acid (72) is not available commercially and was synthesised as shown in Scheme 2.5.[52] 3,4-Dimethoxybenzoyl chloride (73) was prepared from the acid (74) by stirring with excess thionyl chloride. The thionyl chloride was removed by evaporation and the acid chloride was used immediately in the next stage without purification. The reaction of the acid chloride, ethyl acetoacetate and sodium gave the product, ethyl 3,4-dimethoxybenzoylacetoacetate (75) in 86% yield. Removal of the acetyl group using a catalytic amount of sodium acetate yielded ethyl 3,4-dimethoxybenzoylacetate (76). Base hydrolysis of the β-keto ester yielded the β-keto acid (72). The acid decarboxylates readily above 25 °C, so the hydrolysis stage was not carried out until 2 days before the acid was required and it was then stored in the freezer.



Scheme 2.5[52]

2.4 Diels-Alder Reactions of the 1-Pyrroline Complex

The next stage in our work was to investigate the use of the complex as a source of 1-pyrroline for cycloaddition reactions. As mentioned in Chapter 1, free imines are poor dienophiles, but in the presence of a Lewis acid catalyst will take part in Diels-Alder cycloaddition reactions. It was hoped that the zinc iodide present

in the complex would provide a source of this catalysis. The reaction of the complex with the trimethylsilyl ether of methyl vinyl ketone has already been unsuccessfully tested under a variety of reaction conditions.[47] In our work a range of dienes was used in reaction with the complex and the results are shown in Table 2.1.

Diene	Solvent	Conditions	Temp	Time	Result
2,3-Dimethyl-	neat	sealed tube	150°C	18h	tar
1,3-butadiene	DMF	under N_2	RT	18h	trimer
	H_2O	open	RT	18h	trimer
	H_2O	open	0°C	18h	trimer
Methyl 2,4-hex-	neat	sealed tube	140°C	24h	tar
adienoate (77)	DMF	under N_2	RT	24h	trimer
Methyl 2,4-pen-	neat	sealed tube	150°C	24h	tar
tadienoate (78)	DMF	under N_2	RT	24h	trimer
1-Acetoxy-1,3-	neat	sealed tube	70°C	24h	tar
butadiene					

Table 2.1

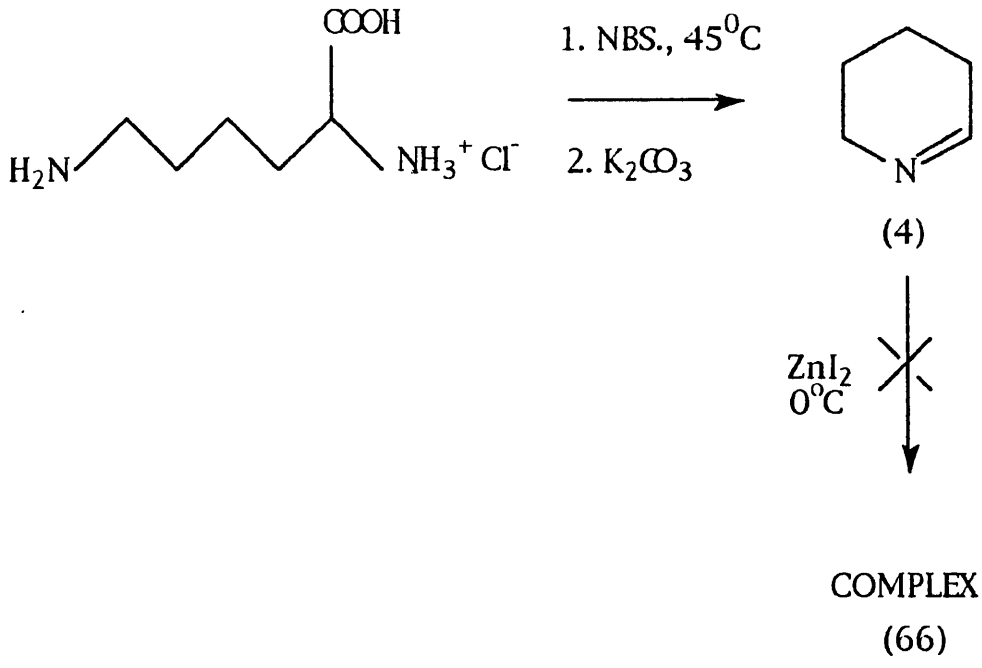
No success was achieved with the complex despite the variety of reaction conditions tested. It is possible that the complex is simply unsuitable as a source of 1-pyrroline for these types of reactions.

The next logical step in this work was the consideration of 1-piperideine (4) in complexation with zinc iodide and comparison of its behaviour to 1-pyrroline in parallel experiments leading to heterocyclic compounds of biological interest.

2.5 Synthesis of 1-Piperideine

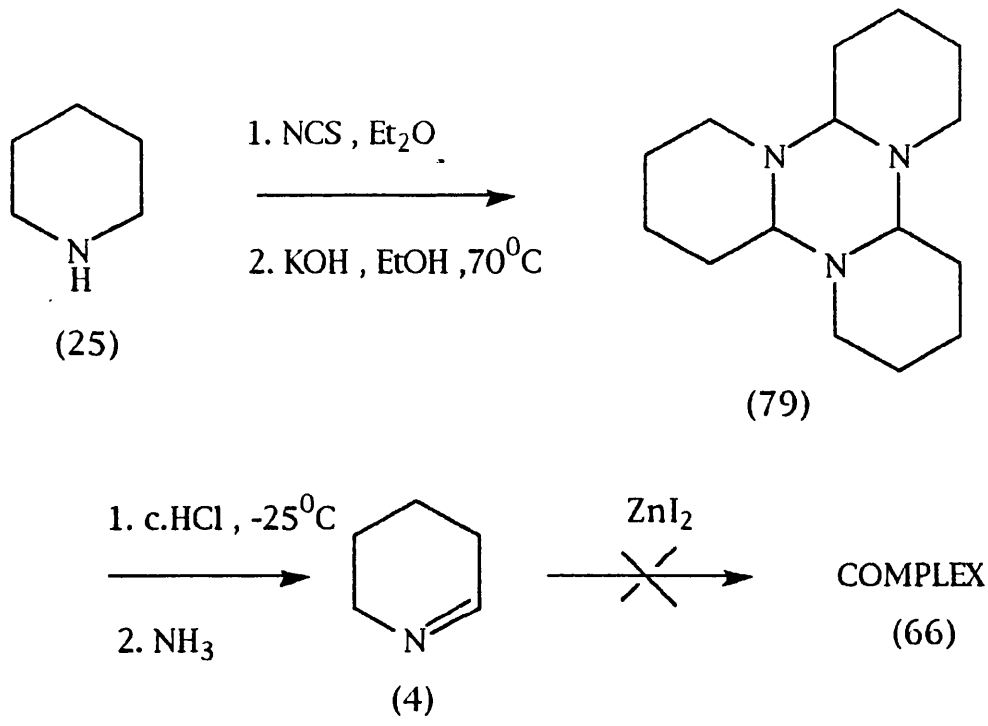
The formation of 1-piperideine from a number of starting materials was investigated.

1-Piperideine can be easily prepared in a convenient one step reaction from lysine (51) (Scheme 2.6).[53] An aqueous solution of L-lysine monohydrochloride was treated with *N*-bromosuccinimide. The hydrogen bromide formed was removed by evaporation and addition of base to the aqueous solution allowed extraction of the free imine into cold ether. Addition of zinc iodide to the ethereal extracts failed to precipitate the desired product. In the absence of zinc iodide the organic extracts were evaporated to give a clear oil. The ^{1}H NMR spectrum of the product indicated that 1-piperideine was the major component, though traces of unreacted lysine and succinimide were present.



Scheme 2.6

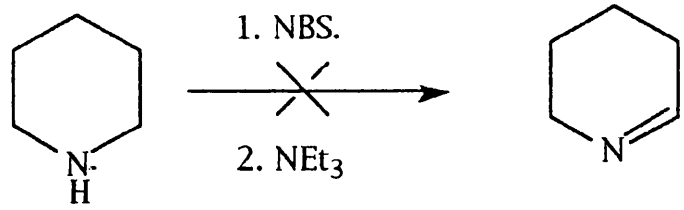
Like 1-pyrroline, 1-piperideine is known to trimerise in basic solution.[54] This tripiperideine (79) can be 'cracked' in acid as an efficient route to the monomer imine. Tripiperideine was prepared by the treatment of piperidine (25) with *N*-chlorosuccinimide to form *N*-chloropiperidine. Addition of potassium hydroxide in ethanol to the mixture effects dehydrohalogenation to give tripiperideine (79) in 71% yield, following recrystallisation.[115] Treatment of the trimer with concentrated hydrochloric acid, followed by adjustment of the pH to 9 allowed the extraction of 1-piperideine into cold ether. Addition of zinc iodide to the extracts failed to precipitate the desired product (Scheme 2.7).



Scheme 2.7

At this stage it seemed unlikely that 1-piperideine formed a complex with zinc iodide. It was decided therefore to find a route to 1-piperideine which might be extended to the corresponding 7-membered ring compound and its zinc complex.

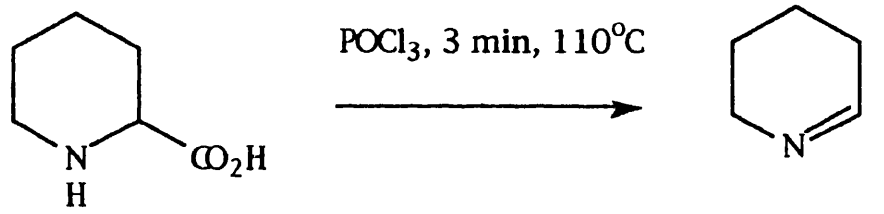
The reaction of piperidine, triethylamine and *N*-bromosuccinimide in dichloromethane gave a mixture of products by TLC (Scheme 2.8). However, the ^1H NMR spectrum showed no proton signal for the imine. Similarly, the reaction with hexahydroazepine gave the same result.



(25) (4)

Scheme 2.8

In an adaptation of the work by Rapoport and co-workers[9], pipecolinic acid (11) was heated briefly with phosphorus oxychloride. Extraction of the basified solution with cold ether gave a yellow solid with spectral properties identical to tripiperideine (79) (Scheme 2.9).

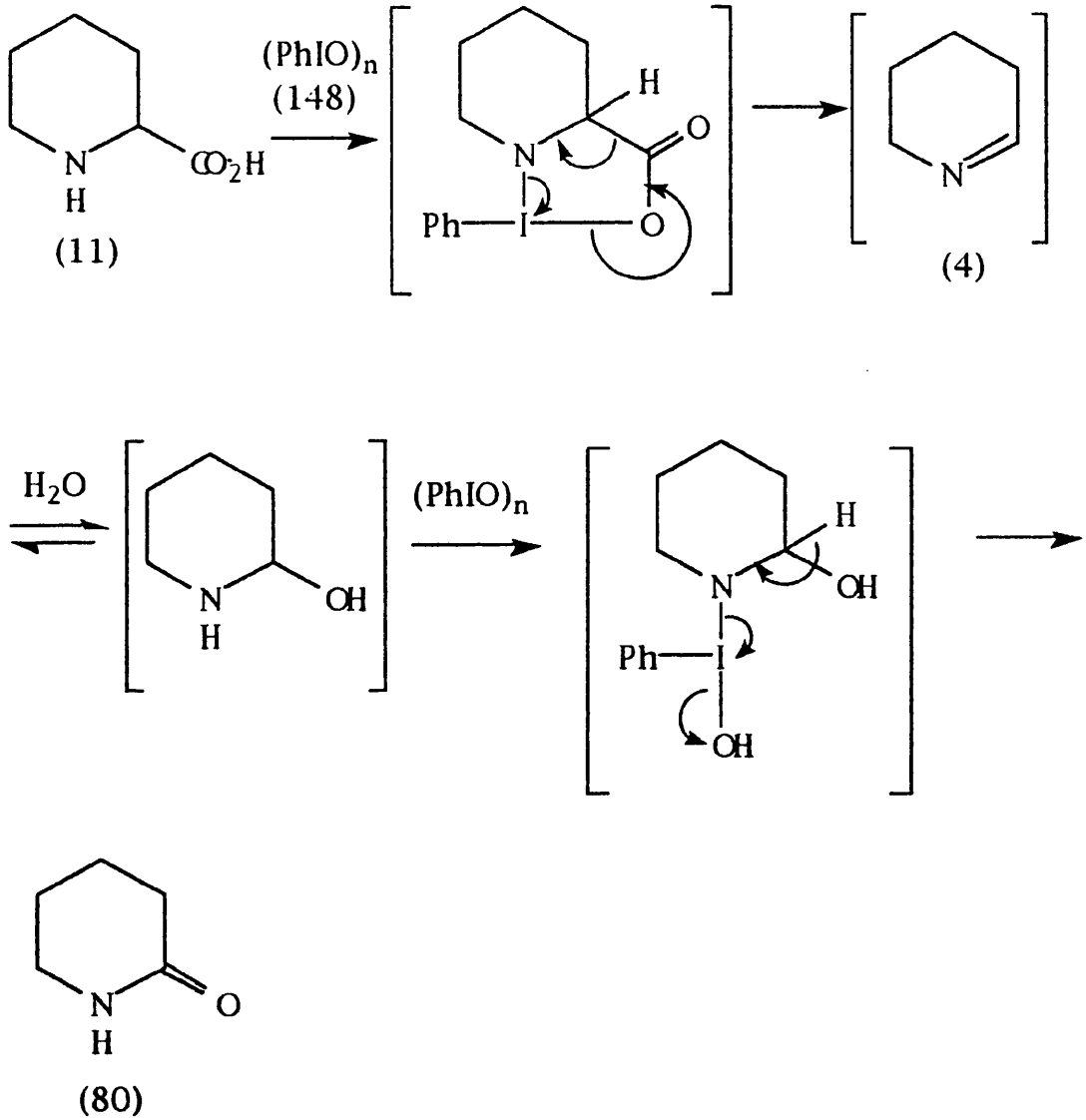


(11)

Not isolated
(4)

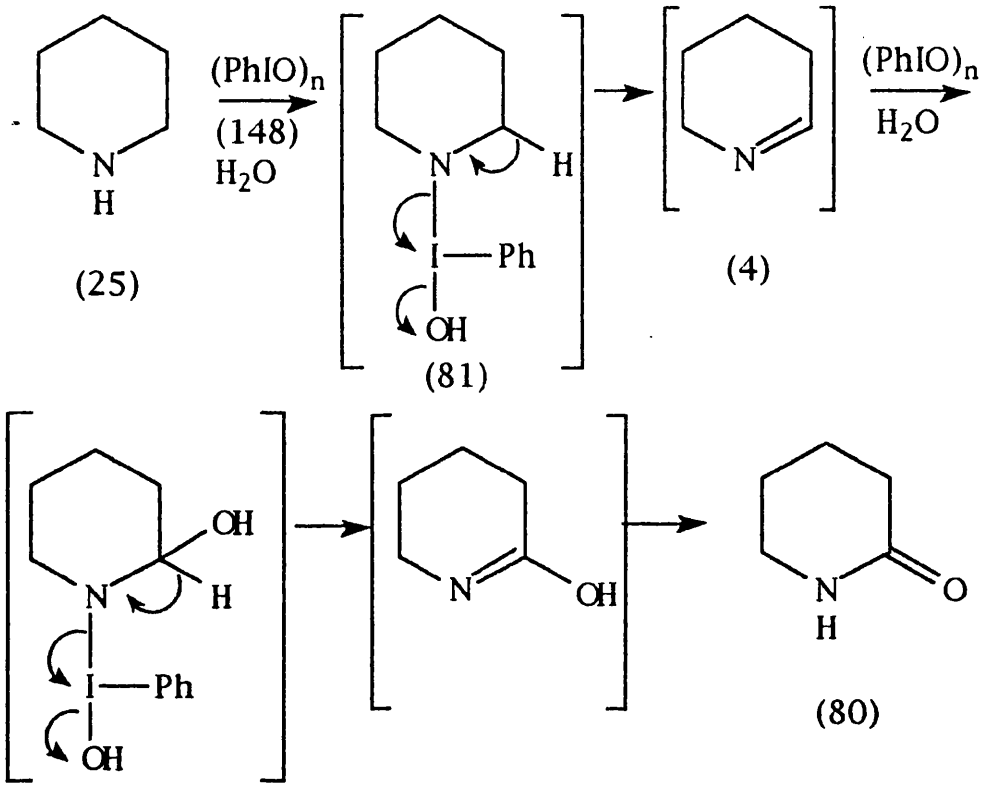
Scheme 2.9

Moriarty and co-workers reported the use of iodosobenzene (148) in the synthesis of δ-valerolactam (80) from pipecolinic acid and piperidine.[55] Oxidative decarboxylation of the amino acid under neutral conditions, in dichloromethane or water, was thought to proceed via initial imine formation, as shown in Scheme 2.10.



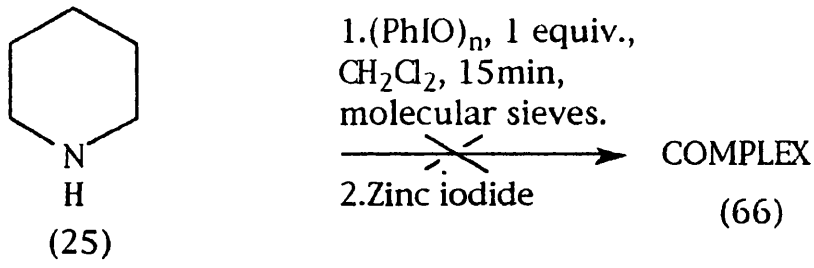
Scheme 2.10[55]

The oxidation of piperidine is thought to proceed via an initial nitrogen-iodine type intermediate (81) which dissociates to give 1-piperideine (Scheme 2.11). The imine is then believed to react with a second equivalent of iodosobenzene and water to give δ-valerolactam (80). It was thought that if the oxidation of pipecolic acid and piperidine could be undertaken in non-aqueous conditions with one equivalent of iodosobenzene it might be possible to isolate 1-piperideine from the reaction mixture.

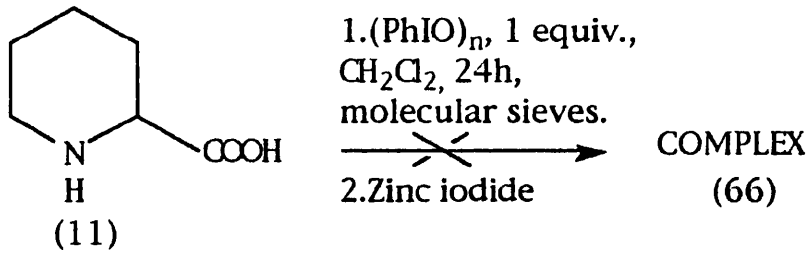


Scheme 2.11[55]

Iodosobenzene (148) was prepared by the treatment of iodosobenzene diacetate with aqueous sodium hydroxide.[56] Iodosobenzene and one equivalent of piperidine were mixed together in dry dichloromethane in the presence of crushed molecular sieves (Scheme 2.12). The mixture was filtered and zinc iodide added to the filtrate. No complex was formed. In the absence of zinc iodide the organic solution was evaporated to dryness. The ^{1}H NMR spectrum showed no imine proton signal. The reaction between pipecolic acid and iodosobenzene gave the same result (Scheme 2.13).



Scheme 2.12

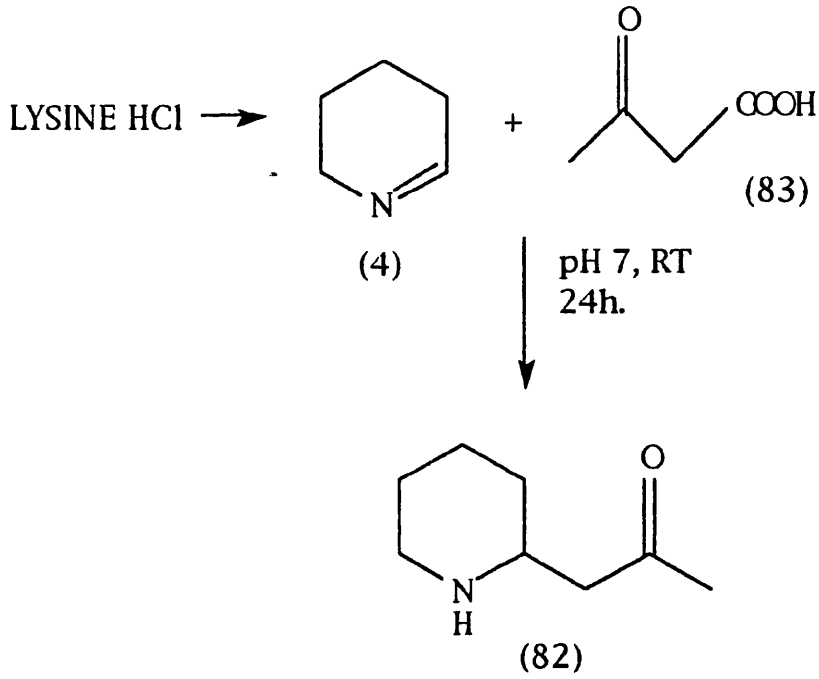


Scheme 2.13

Since all attempts at complexing 1-piperideine with zinc iodide proved unsuccessful, the imine was used directly in the synthesis of pelletierine (82) and 3',4'-dimethoxy-2'-(2-piperidyl)acetophenone (31).

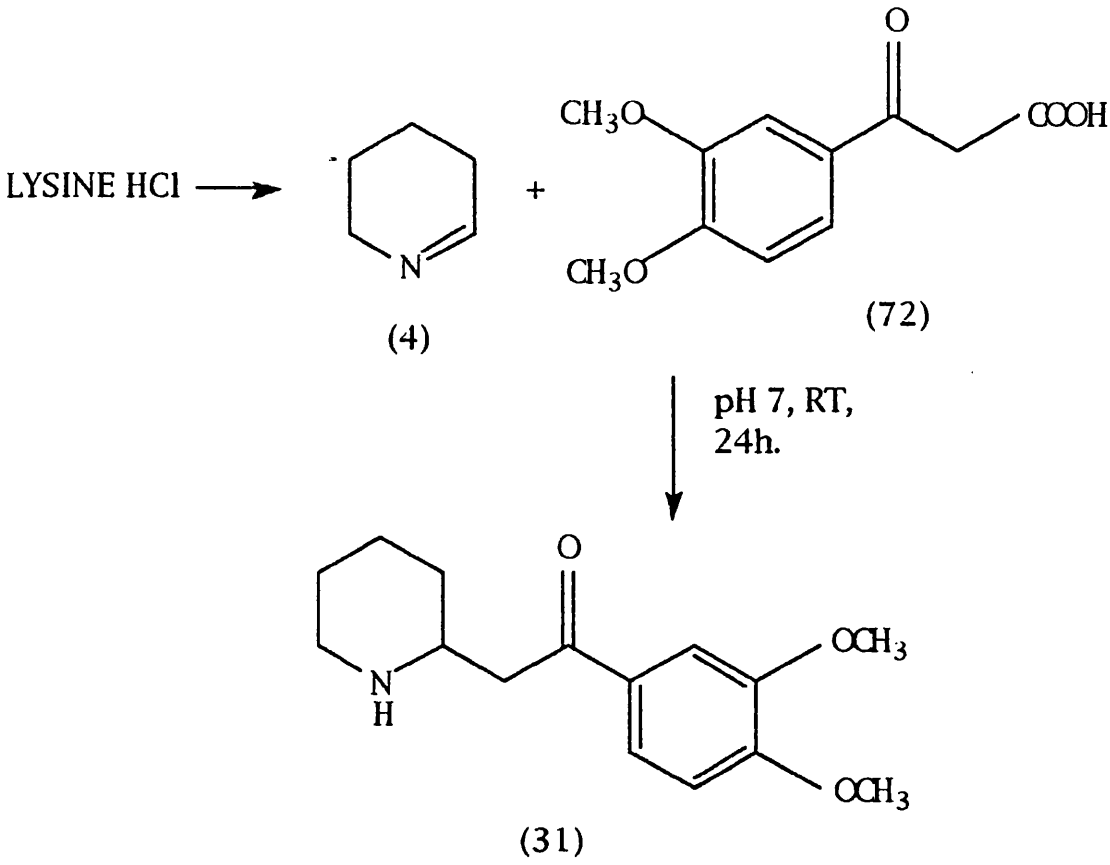
2.6 The Use of 1-Piperideine with β-Ketoacids

In a synthesis of pelletierine (82) adapted from that of Gupta and Spenser[57], 1-piperideine was prepared from L-lysine monohydrochloride and *N*-bromosuccinimide. The aqueous solution of the imine was added to lithium acetoacetate (83) in phosphate buffer to give pelletierine in 68% yield (Scheme 2.14). The ^{1}H NMR spectrum of the product showed a singlet at δ 2.14 which integrated to three protons, for the methyl group, and in the IR spectrum the carbonyl stretch appeared at 1720 cm^{-1}.



Scheme 2.14

3',4'-Dimethoxy-2'-(2-piperidyl)acetophenone (31) was prepared in a similar manner from lysine and 3',4'-dimethoxybenzoylacetic acid (72) in 70% yield (Scheme 2.15). The IR spectrum showed the carbonyl absorption at 1675 cm^{-1} and the high resolution mass spectrum gave a molecular ion at 263.1515 in accordance with a theoretical value of 263.1521. These values, along with the other spectroscopic properties are in accordance with literature values.[15]



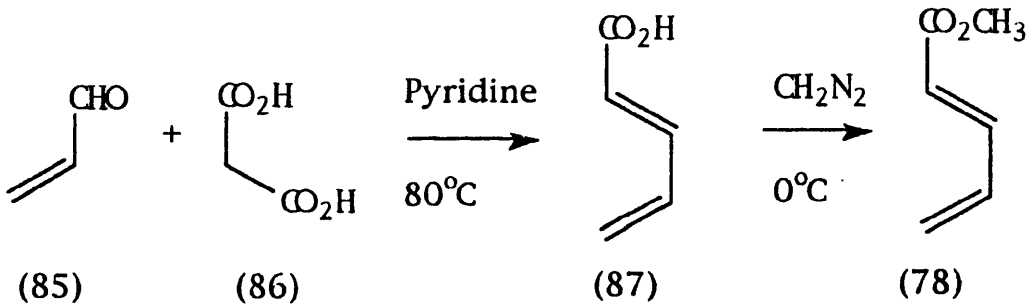
Scheme 2.15

2.7 The Use of 1-Piperideine in Diels-Alder Cycloaddition Reactions

1-Piperideine is known to react with methyl 2,4-pentadienoate (78) to give methyl 1,2-didehydro-quinolizidine-1-carboxylate (84).[58] It was of interest to us to see if this reaction could be adapted for a variety of dienes as a convenient route to a range of quinolizidine structures.

Methyl 2,4-pentadienoate (78) was prepared from acrolein (85) and malonic acid (86) in pyridine followed by treatment of the resultant acid (87) with an excess of diazomethane (Scheme 2.16).[59] Tripiperideine (79) and methyl 2,4-pentadienoate were

heated together in a sealed tube with a catalytic amount of potassium hydrogen sulphate to give (88), which then underwent a double-bond isomerisation to give the α,β-unsaturated ester (84) (Scheme 2.17). Stereoselective hydrogenation of (84), in the presence of platinum oxide catalyst gave the saturated ester (89). Reduction of the ester with lithium aluminium hydride afforded (±)-lupinine (21). The ^{1}H NMR spectrum of the product showed a doublet at δ 3.68 for the methylene of the alcohol group, and a broad singlet at δ 5.15 for the -OH signal. The high resolution mass spectrum gave a value for the molecular ion of 169.1463 in accordance with a theoretical value of 169.1466.



(85) (86) (87) (78)

Scheme 2.16[59]

For 1H NMR spectrum of lupinine (21) see Figure 2.4.

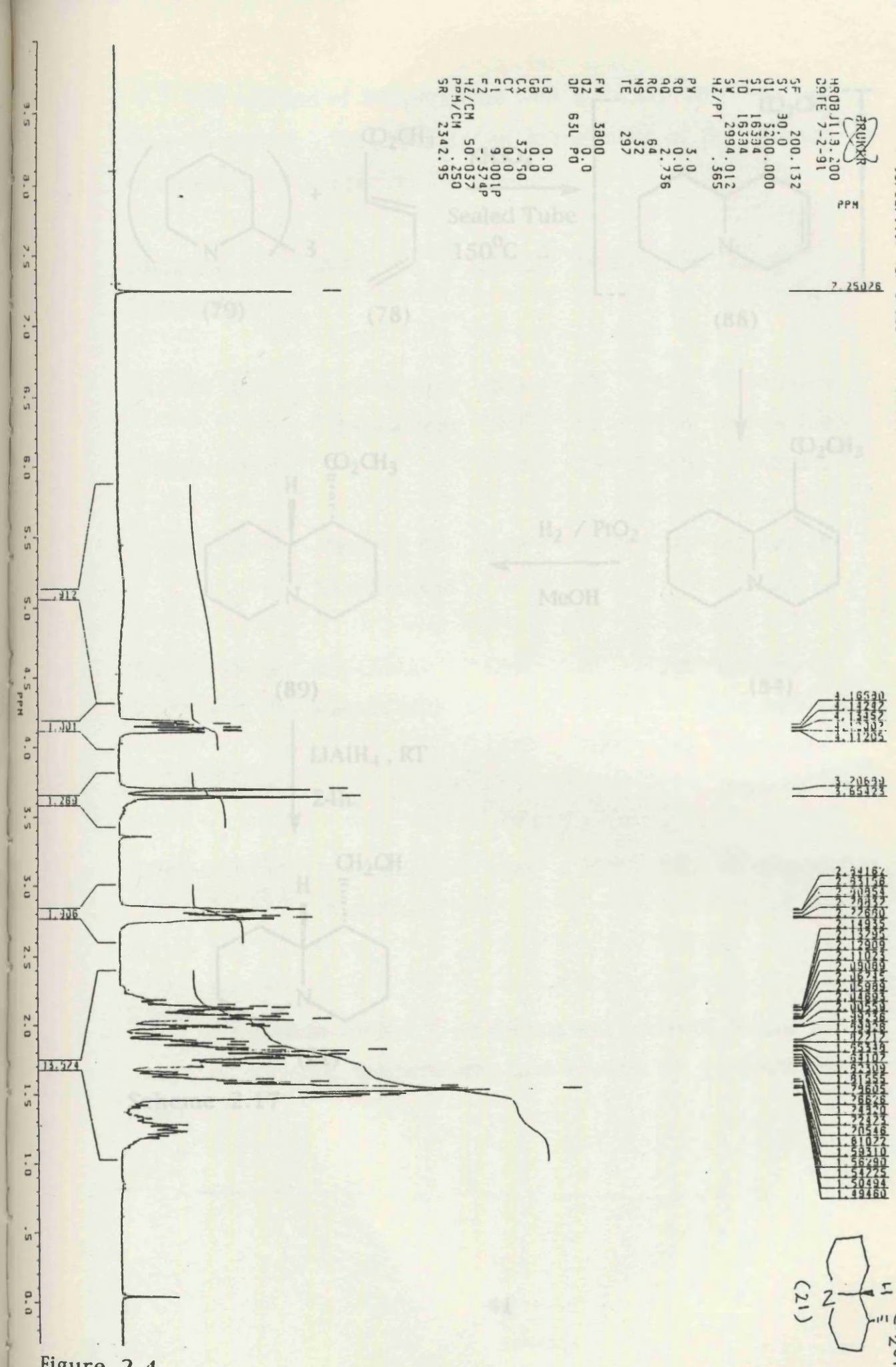
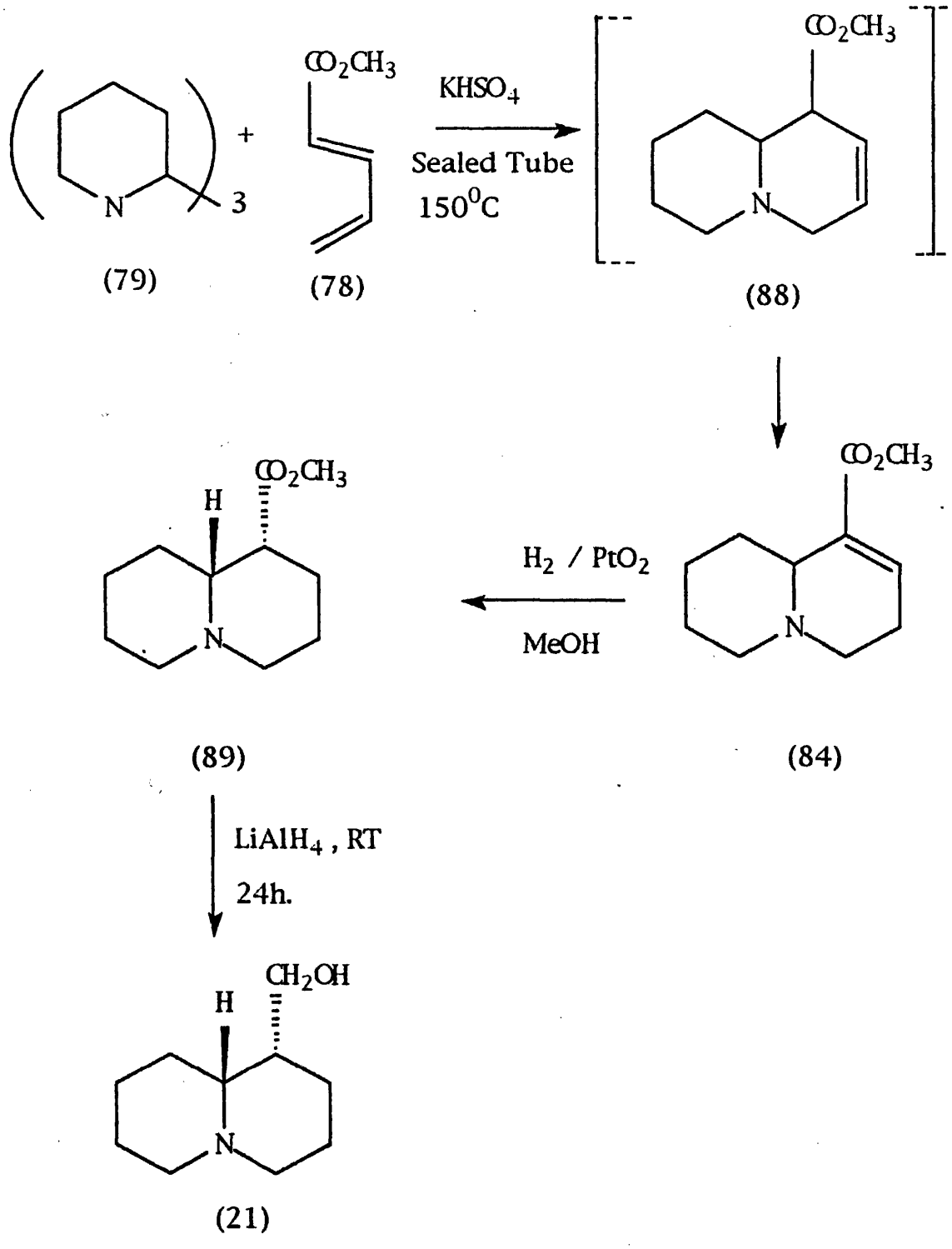


Figure 2.4


CO2CH3
KHSO4
Sealed Tube
150°C
3
N
(79)
(78)
(88)
(84)
H2 / PtO2
MeOH
(89)
LiAlH4 , RT
24h.
CH2OH
(21)

Scheme 2.17

The reaction of 1-piperideine with a variety of dienes was then attempted. Table 2.2 shows a summary of the reaction conditions tried and the results obtained.

Source of 1-Piperideine	Diene	Solvent	Temp	Time	Result
L-Lysine HCl	2,3-Dimethyl-	H_2O	RT	18h	no reaction
	1,3-butadiene	DMF	RT	18h	no reaction
Tripiperideine		neat	150°C	24h	no reaction
Tripiperideine	Methyl 2,4-hexadienoate	neat	140°C	24h	mixture of products
Tripiperideine	2,4-penta-dienoic acid	DMF	RT	24h	mixture of products
		DMF	70°C	24h	mixture of products
Tripiperideine	1-Acetoxy-1,3 -butadiene	neat	70°C	24h	no reaction

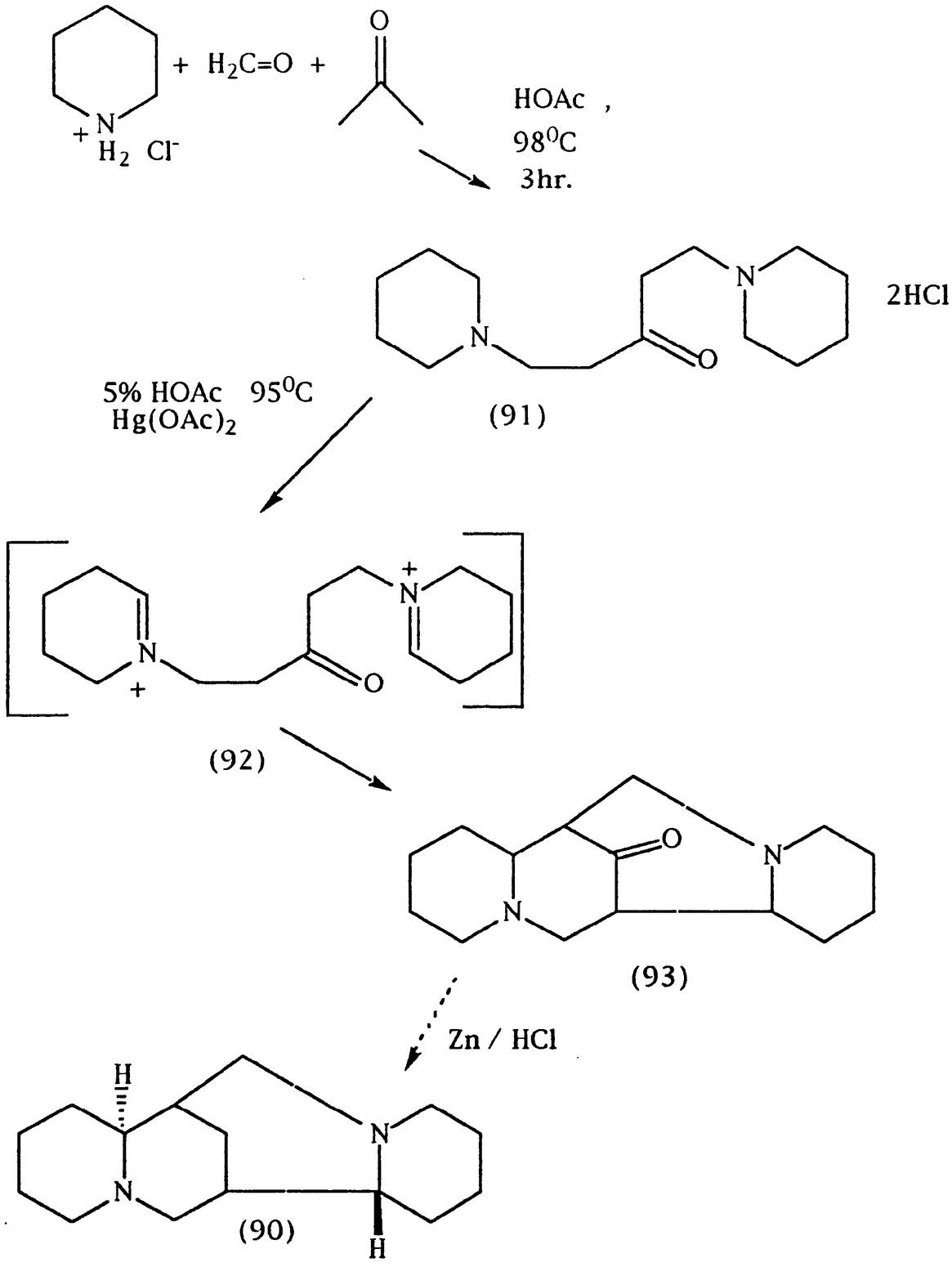
Table 2.2

As shown in Table 2.2, no success was achieved despite the range of solvents, temperatures and sources of 1-piperideine tried.

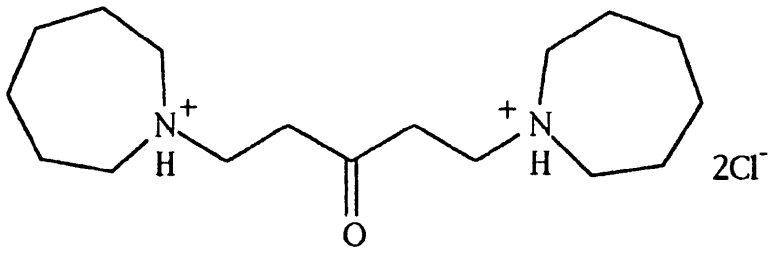
2.8 Synthesis of Tetracyclic Quinolizidine Alkaloids

Sparteine (90) is an alkaloid of the lupin family (Fabaceae) and is one of the more common tetracyclic quinolizidine alkaloids. The synthesis of sparteine was attempted through initial formation of 8-ketosparteine by an application of the Mannich reaction.[13] Piperidine hydrochloride, acetone and paraformaldehyde react by a symmetrical bis-Mannich condensation to give 1,5-bis(*N*-piperidyl)-3-pentanone dihydrochloride (91). Mercuric acetate oxidation of (91) gave the di-iminium ion (92) which cyclised to 8-ketosparteine (93) (Scheme 2.18). The final step of Wolff-Kishner reduction to (±)-sparteine was not undertaken due to the low yields of (93) obtained. 8-Ketosparteine was characterised by the IR spectrum carbonyl stretching frequency at 1725 cm^{-1}. The high resolution mass spectrum gave a value for the molecular ion of 248.1879 compared to 248.1888.

It was of interest to synthesise the corresponding five and seven membered compounds as sparteine analogues. 1,5-bis(*N*-Hexahydroazepinyl)-3-pentanone dihydrochloride (94) was prepared from hexahydroazepine hydrochloride, acetone and paraformaldehyde as above. The attempted oxidation of (94) produced a mixture of products. Column chromatography failed to isolate the desired product.



Scheme 2.18[13]



(94)

Pyrrolidine hydrochloride, paraformaldehyde and acetone were treated in a similar manner. The product formed was extremely hydroscopic and was found to be a mixture of compounds. The desired product could not be isolated.

The testing of 1,5-bis(*N*-piperidyl)-3-pentanone (91), 1,5-bis(*N*-hexahydroazepinyl)-3-pentanone (94) and analogues as inhibitors of pea seedling diamine oxidase is discussed in Chapter 4.

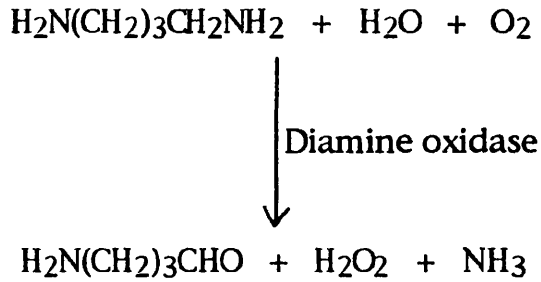
CHAPTER 3

REVIEW OF DIAMINE OXIDASE

3.1 The Importance of Diamine Oxidase

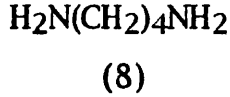
Diamine oxidases are important due to the role that they play in polyamine metabolism in most organisms. These enzymes regulate cellular levels of polyamines. It is known that polyamines are essential for the growth and replication of all living cells.[60]

Diamine oxidases catalyse the oxidative deamination of a range of primary diamines to the corresponding amino aldehydes (Scheme 3.1).[61]



Scheme 3.1[61]

In general, there is no reliable or convenient method available for the above reaction which does not involve enzymes. The best substrates for diamine oxidase have been shown to be putrescine (8) and cadaverine (9).



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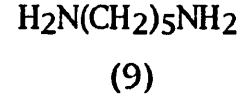


(9)

Diamine oxidases are found in many biological tissues. Pig kidney diamine oxidase is commercially available, but a more active form of diamine oxidase can be isolated from 10 day old pea seedlings.[61] Diamine oxidases have been found to contain copper, which is easily removed from the enzyme by dialysis against chelating agents resulting in deactivation of the enzyme. The enzyme activity can be restored on addition of small quantities of Cu^{2+} salts.[62]

3.2 Polyamine Metabolism and Cell Growth

Polyamines such as putrescine (8), spermidine (95) and spermine (96) are present in all animals and higher plants and they have many important physiological roles.

$H_2N(CH_2)_3NH(CH_2)_4NH_2$ (95)

$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ (96)

Rapidly proliferating cells and tissues usually have greater concentrations of putrescine and spermidine than non-growing tissues. It has been suggested that this link between polyamines and growth is due to electrostatic interactions which serve to stabilise DNA, RNA and proteins. At physiological pH at least one amino group is protonated, which allows these groups to bind strongly to the phosphate groups of nucleic acids. Polyamines also affect the rate of each step in the transcription-translation sequence, whereby genetic information is coded for the manufacture of proteins.[60]

Polyamines are metabolised via the interconversion pathway and the so-called terminal polyamine catabolism pathway. The interconversion pathway regulates polyamine levels in the cell using a cyclic process to control polyamine turnover. Decarboxylation of

ornithine produces putrescine which in turn is the precursor of spermidine and spermine. Spermine is formed from spermidine, which itself is produced from putrescine using spermine synthase. In the catabolic branch of the interconversion cycle spermine is degraded to spermidine and spermidine to putrescine. This sequence starts with *N'*-acetylation followed by oxidative splitting of the acetylated polyamines. Terminal polyamine catabolism is catalysed by amine oxidases which are Cu^{2+} dependent, of which only diamine oxidase has been well defined. Each intermediate of the interconversion cycle can serve as a substrate for a Cu^{2+} containing amine oxidase. The intermediates can then be transformed into the corresponding amino aldehydes following oxidative deamination of a primary amino group followed by further oxidation to an amino acid or a γ-lactam. Since these products cannot be directly reconverted into polyamines these reactions are termed terminal catabolism. All products of terminal catabolism can be found in the urine.

Examining the consequences when the internal concentration of polyamines is depleted gives an insight into the workings of a biological system.

In the early 1970's a compound called α-difluoromethylornithine (DFMO) was synthesised by Merrell Dow Pharmaceuticals. Since DFMO is a close analogue of ornithine it reacts in the same manner with ornithine decarboxylase. Subsequently, DFMO was shown to possess anti-tumour activity and significant anti-fungal activity.[60]

In order to control the growth of rapidly proliferating cells such as tumours which have a higher demand for polyamines it is necessary to inhibit polyamine biosynthesis. It is possible that inhibitors of diamine oxidase may have interesting anti-cancer and anti-fungal properties.

3.3 Purification of Diamine Oxidase

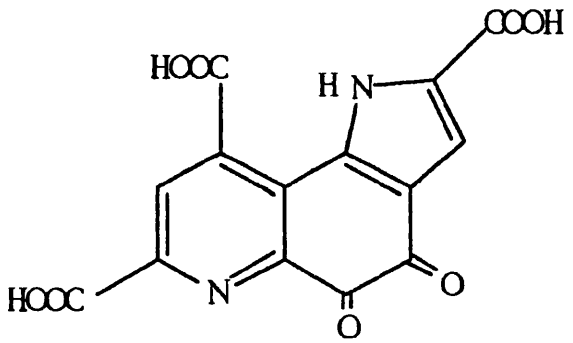
Diamine oxidases [DAO, EC 1.4.3.6; diamine: O_2-oxidoreductase (deaminating; copper containing)] occur widely throughout living organisms. Pea seedlings and pig kidneys are the main sources of these enzymes. Diamine oxidase can be extracted and purified from pea seedlings after a period of 8-16 days germination.[63] Diamine oxidase has been purified separately from cotyledons and embryos of pea seedlings germinated for 6 days. These enzymes have been shown to differ in K_M, with respect to putrescine and cadaverine, thermal stability and most significantly in electrophoretic mobility.[64]

The enzyme has also been purified to homogeneity from pea epicotyls by the use of polyacrylamide gel electrophoresis. The purified enzyme (yield 35%) showed absorption maxima at 280 and 500 nm and ornithine was not amongst the amino acids present. The molecular weight estimated by gel filtration was ca. 180,000 and the enzyme contained one mole of carbonyl group per mole. Sodium dodecylsulphate (SDS) gel electrophoresis gave a single band at a molecular weight of 85,000 suggesting that the enzyme is made up of two identical subunits.[65]

Purified pea seedling DAO contains 0.08-0.09% copper. Hill and Mann reported that copper is present in the Cu^{2+} form and does not change oxidation state during the enzymatic reaction. Copper is firmly bound to the protein but could be removed when dialysed with sodium diethyldithiocarbamate rendering the enzyme inactive. The activity could be restored by addition of Cu^{2+} ions.[62]

3.4 Cofactors for Diamine Oxidase

It is well known that diamine oxidases are sensitive to chelating agents of copper and carbonyl compounds such as hydrazine, phenylhydrazine and semicarbazide.[66] This suggested that the active site of the enzyme contains a carbonyl group. Some researchers have proposed a role for pyroxidal phosphate as the cofactor in copper amine oxidases. Alternative theories have included the postulated role of a ring modified flavin[67] or a sulphenic acid derivative.[68] The perspective on cofactor structure changed in 1984 with reports from two independent groups that bovine plasma amine oxidase may contain pyrroloquinoline quinone (PQQ) (97) as the cofactor.

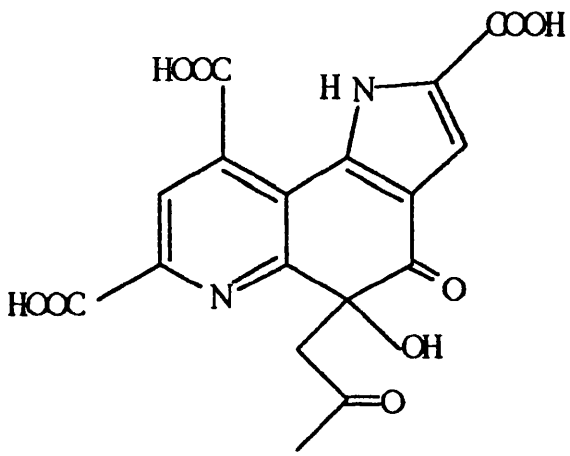


(97)

3.5 Evidence for Pyrroloquinoline Quinone as the Cofactor in Copper Amine Oxidases

Duine and co-workers prepared a dinitrophenylhydrazone derivative of the bovine plasma amine oxidase before hydrolysing the protein.[69] The adduct was compared with an authentic derivative of PQQ using HPLC retention times for structural identification. The second approach by Ameyama *et al.* used native enzyme which, when digested, yielded a chromophoric material with a fluorescence spectrum similar to PQQ.[70]

The earliest indication of PQQ as an enzymatic cofactor was by Hauge in 1964 from studies of a bacterial glucose dehydrogenase which indicated the presence of a dissociable cofactor which was neither a flavin nor a nicotinamide.[71] He suggested that the structure was a substituted 1,4-naphthoquinone. At the same time Anthony and Zatman postulated a pteridine structure as the cofactor following studies on methanol dehydrogenase from *Hyphomicrobium* X.[72] However, Duine and co-workers, using EPR found a hyperfine structure which was not compatible with a pterine cofactor.[73] This structure was thought to contain quinone and two nitrogens which, following further investigation using X-ray crystallography was found to be the acetone adduct of PQQ (98).



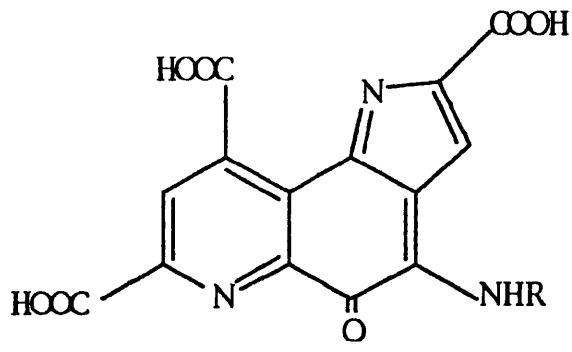
(98)

Glatz *et al.* investigated the effect of known ligands which form complexes with PQQ.[74] The addition of borate ions to a solution of native enzyme resulted in fluorescence and absorbance properties similar to those of the mixture of PQQ and borate ions.

The precise biological function and evolutionary significance of PQQ in micro-organisms are as yet unclear, since the reactions catalysed by enzymes containing PQQ can also be catalysed by enzymes using flavins and nicotinamides.

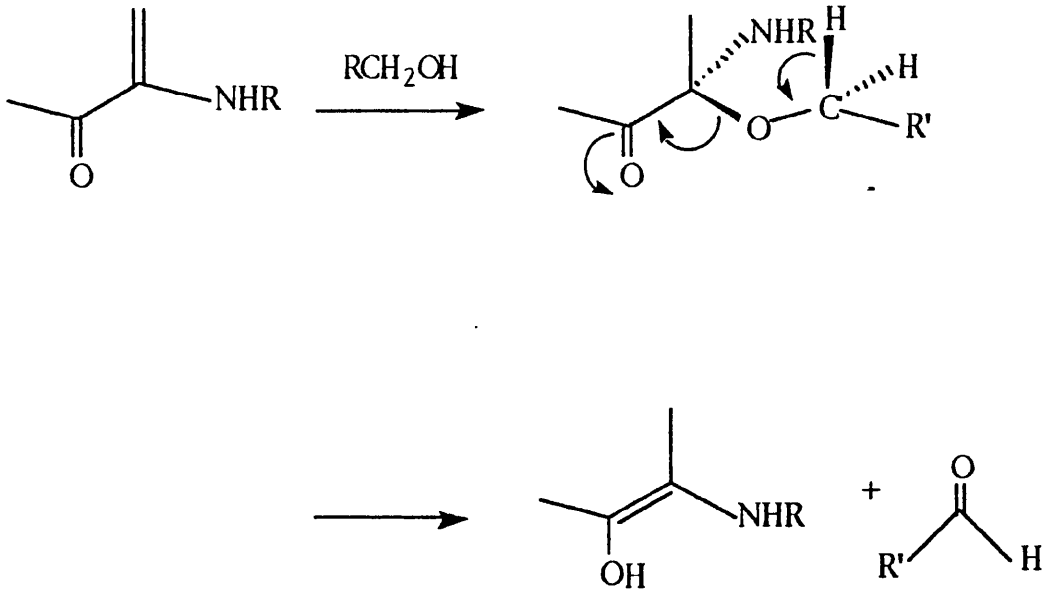
3.6 The Mechanism of Oxidation Involving PQQ

Various mechanisms have been proposed for the reaction of the PQQ-containing enzyme. The mechanism of substrate oxidation by PQQ in methanol dehydrogenase was thought to be activated by ammonia or primary amines. Forrest et al suggested that the C-4 position of the cofactor interacts with amines to generate the compound (99) shown below.[75]



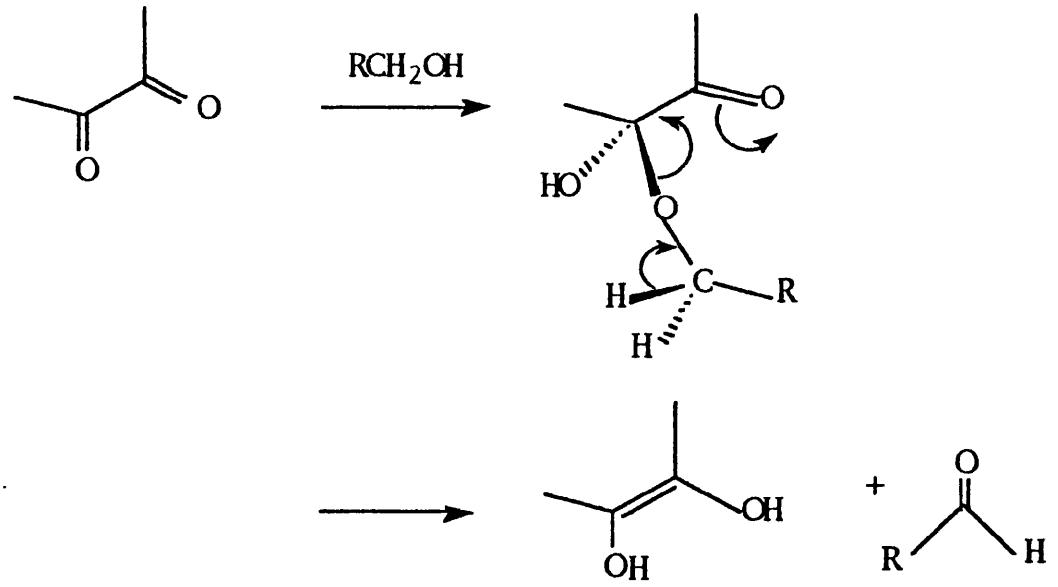
(99)

Attack by one amino group of the substrate leads to a carbinolamine derivative which followed by oxidation produces the reduced aminoquinol of PQQ (Scheme 3.2).



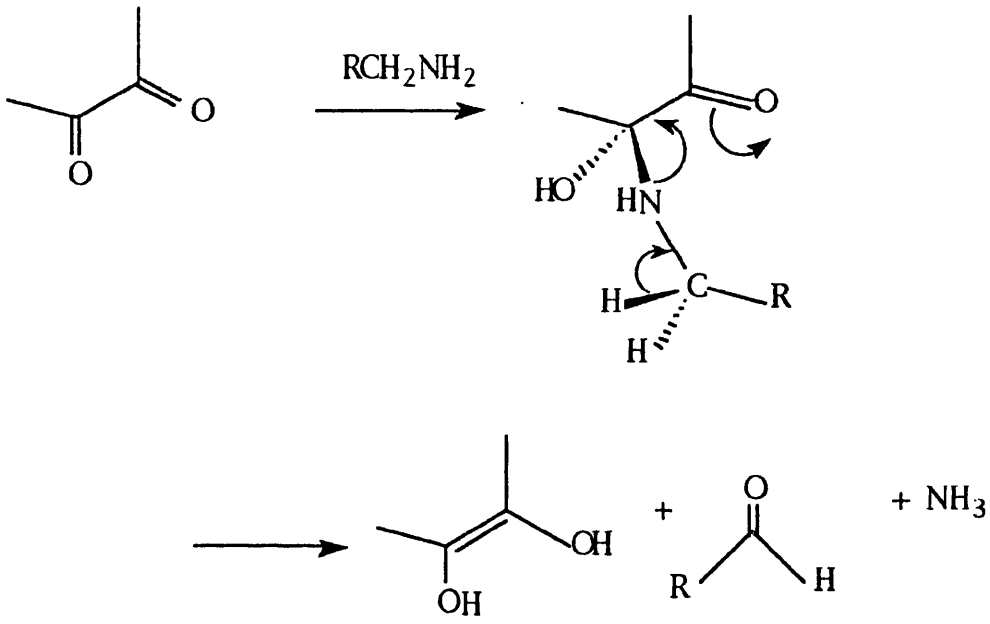
Scheme 3.2

However, literature concerning PQQ indicates that the C-5 position has an unusually high reactivity towards nucleophilic attack. Subsequently, a simple mechanism can be argued involving initial formation of a hemiacetal followed by oxidation of the substrate to yield the reduced quinol of the cofactor (Scheme 3.3).



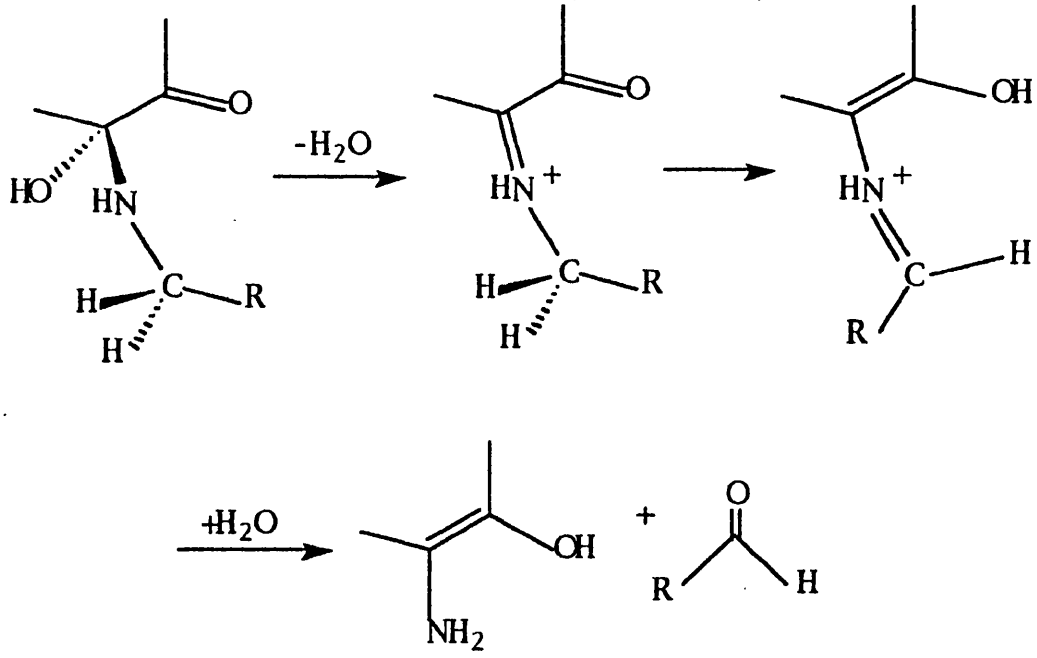
Scheme 3.3

The reduced quinol form of PQQ is a major product in amine oxidation, which allows an analogous mechanism to be suggested (Scheme 3.4).



Scheme 3.4

Since amines, unlike alcohols, can generate stable Schiff base intermediates with PQQ, a minor pathway has been reported by Bruice and co-workers[76] and Itoh *et al.* [77] (Scheme 3.5).



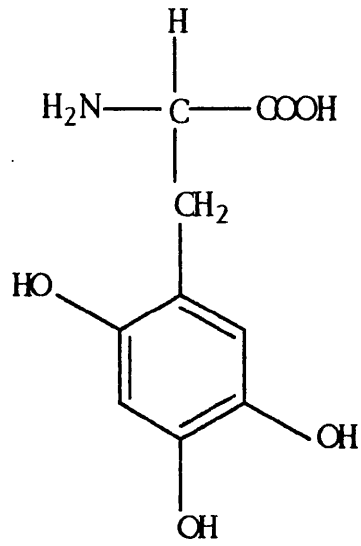
Scheme 3.5

Hartmann and Klinman have reported a large amount of evidence indicating that the enzymatic reaction proceeds by the latter aminotransferase mechanism.[78] This evidence includes reductive covalent binding of the substrate to the enzyme with the reagent $NaCNBH_3$ which specifically reduces imines. They have also examined the stoichiometric transfer of nitrogen from substrate to cofactor following reduction of the cofactor. Only under conditions of imine formation is a covalent transfer of nitrogen to the reduced cofactor expected. However, under anaerobic conditions to prevent cofactor reoxidation, Knowles and co-workers observed a burst of ammonia production with porcine plasma amine oxidase.[79] This result appeared to rule out an aminotransferase mechanism. However, a subsequent study by Linstrom and Pettersson which compared native and inhibited enzyme showed no difference in ammonia release.[80]

3.7 Evidence Against Pyrroloquinoline Quinone (PQQ) as the Cofactor

Despite the vast amount of research undertaken on PQQ disagreement still exists on its distribution. This is due to the fact that there is no agreement with respect to the reliability of some detection methods for PQQ. Recent developments have shown that the finding PQQ covalently bound to the enzyme is unlikely in a number of cases. X-Ray diffraction studies on methylamine dehydrogenase have shown that the electron density map of the cofactor in the active site does not correspond to PQQ.[81] Plasma amine oxidase has been reported to form a hydrazone derivative under mild conditions which was hydrolysed with thermolysin to produce the hydrazone of 6-hydroxydopaquinone or topaquinone (topa) (100).[82] This suggests that the original

hydrazine method in fact converted the hydroxydopaquinone into PQQ by an as yet unknown sequence of reactions.



(100)

Both topa and dopaquinone are very reactive compounds leading to a lack of knowledge about their properties. The absorption maxima of topaquinone however have been reported.[83] There is disagreement about the occurrence of topa in mammalian systems.[84]

Falk and co-workers have reported the effects of plasma amine oxidase inhibited with fluorinated phenylhydrazines and acridinylhydrazines.[85] From ^{19}F-NMR and fluorescence spectroscopy respectively they have shown that no direct interaction could take place between the Cu^{2+} ion and topaquinone or the substrate.

However ESR spectroscopy revealed that electron transfer takes place from the reduced topaquinone to Cu^{2+} since an organic free radical was observed, which is likely to derive from the cofactor.[86] It is thought that this Cu-semiquinone state may be involved in the reoxidation of the substrate-reduced enzyme by O_2. This observation only occurs in plant amine oxidases but can be induced in others by addition of substrate and cyanide.[87]

In view of the similarity in the type of inhibitors and spectroscopic properties of copper-containing amine oxidases, it is thought likely that topaquinone is the cofactor for all of them. The most conclusive evidence for topaquinone as the cofactor in pea seedling diamine oxidase was reported by Klinman and co-workers.[88] They isolated an active site, cofactor-containing peptide of pea seedling diamine oxidase by derivatisation with *p*-nitrophenylhydrazone. This peptide was characterised by resonance Raman spectroscopy. This spectrum showed identical peak positions and intensities relative to a model *p*-nitrophenylhydrazone derivative of topaquinone hydantoin establishing topaquinone as the cofactor. This approach was also used for enzymes from porcine plasma and porcine kidney. In each instance topaquinone was established as the cofactor.

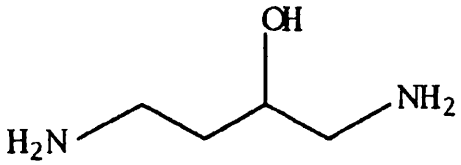
This year the amine oxidase from pea seedlings has been crystallised and has undergone preliminary crystallographic characterisation.[89] These results show that the unit cell is orthorhombic and that the mass of the asymmetric unit is 131(±13) kDa which is in agreement with evidence that the molecule consists of two identical subunits of approximately 66 kDa. The detailed X-ray structure should reveal the nature of the cofactor.

3.8 Substrate Specificity In Pea Seedling and Pig Kidney Diamine Oxidase

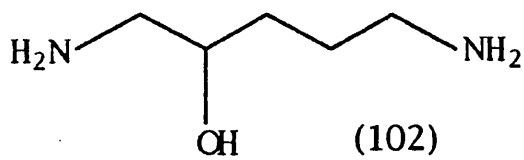
In 1952 Kenten and Mann reported that both mono- and di-amines were oxidised by extracts of pea seedlings accompanied by a release of hydrogen peroxide.[63] Of the mono amines tested, tyramine, tryptamine and mono- and di-β-phenylethylamine were the best substrates. Kenten and Mann concluded that, from their evidence, one

enzyme catalysed the oxidation of all the amines tested. Of the diamines tested 1,6-diaminohexane was a poorer substrate than putrescine and cadaverine and no activity at all was observed with 1,3-diaminopropane or 1,2-diaminoethane. The diamines were more readily oxidised than the mono-amines, indicating the importance of the second amino group. With the animal enzyme the rate of oxidation increases with the length of the carbon chain up to cadaverine, including 1,3-diaminopropane.[114] This would seem to suggest that the active sites of the plant and animal enzymes are different. However, Robins and co-workers reported that both 1,3-diaminopropane and 1,2-diaminoethane are in fact substrates of pea seedling diamine oxidase, though poor.[90] They also reported the significant chain length dependance of both the substrate binding affinity and the catalytic rate of oxidation. As the carbon chain length is increased to C_5-C_7 the binding affinity of the substrate rises, but diminishes for longer diamines. The catalytic rate has a maximum activity for C_5 and again decreases for longer diamines.

Macholan and co-workers investigated the oxidation of 2-hydroxyputrescine (101) and 2-hydroxycadaverine (102) with partially purified pea seedling and pig kidney diamine oxidase.[91]



(101)



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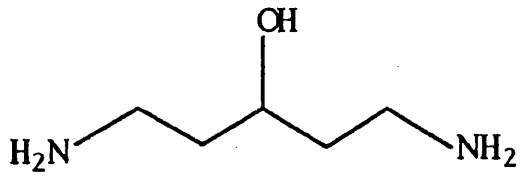


(103)

They found that neither was as good a substrate as putrescine or cadaverine but that 2-hydroxyputrescine was a better substrate for pig kidney diamine oxidase that 2-hydroxycadaverine and vice versa for the plant amine oxidase.

In a later paper Macholan again tested compounds (101) and (102) along with 3-hydroxycadaverine (103).[92] Putrescine and cadaverine were oxidised with comparable rates by pea seedling and pig kidney diamine oxidase at pH 7.[62,93] In the series of hydroxyamines tested the optimal pH values for pig kidney diamine oxidase were found in general to be shifted to the alkaline region in contrast to the plant enzyme. Macholan also found that 3-hydroxycadaverine (103) had the lowest binding affinity to pea seedling diamine oxidase. However, it had the highest affinity to the animal enzyme. These differences in behaviour suggest that certain distinctions in the structure of the active sites of these enzymes do exist.

3.9 Inhibitors of Diamine Oxidase

Virtually all modern drug treatments are based on the concept of inhibition of enzymatic activity. Drugs are designed to inhibit a specific enzyme in a particular metabolic pathway. Product inhibition in a metabolic pathway provides a means of controlling or modulating the flow of substrate through the pathway.

Enzyme inhibition can be divided into two main types; reversible and irreversible.

In reversible inhibition the enzyme-inhibitor complex dissociates very rapidly, whereas an irreversible inhibitor binds very tightly to the enzyme and the complex formed dissociates very slowly.

Within reversible inhibition there are two main classes of inhibitor; competitive and noncompetitive. In competitive inhibition the enzyme can bind to the substrate and the inhibitor but not at the same time. The inhibitor binds at the same active site of the enzyme, and competes with the substrate for the enzyme, and in so doing slows the rate of reaction. The enzyme-bound inhibitor then either lacks the appropriate reactive group and cannot be processed by the enzyme, or it is held in an unfavourable position with respect to the catalytic site resulting in a dead-end complex. The inhibitor can then be displaced from the enzyme and replaced by substrate. Many competitive inhibitors are analogues of substrates.

A noncompetitive inhibitor binds at a different active site from the substrate. The enzyme can therefore bind to the inhibitor and the substrate at the same time. The inhibitor acts as if it were removing active enzyme from the solution. Thus the inhibitor decreases the turnover number of the enzyme, hence decreasing the V_{max}, rather than decreasing the number of substrate molecules bound to the enzyme (Figure 3.1).

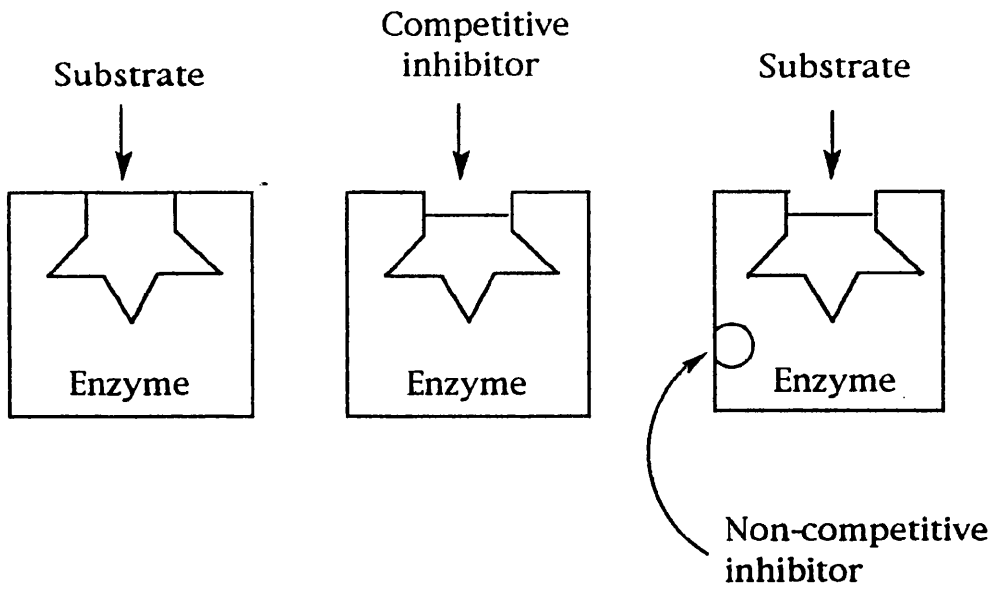


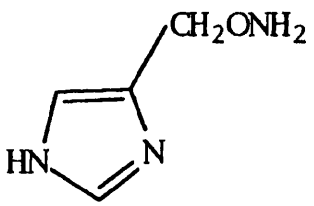
Figure 3.1

Kenten and Mann investigated the effect of a number of inhibitors on the oxidation of β-phenylethylamine and putrescine by pea seedling diamine oxidase.[63] The oxidation of both the mono- and di-amine were inhibited strongly by a 0.01M solution of cyanide, semicarbazide and iso-amylamine. However the oxidation of the monoamine but not that of the diamine was inhibited by *p*-benzoquinone indicating the possibility that specific enzymes are present for mono- and di-amine oxidation.

Other types of inhibitors are copper (II) chelating agents such as 8-hydroxyquinoline, 2,2'-bipyridyl, 1,10-phenanthroline and sodium diethyldithiocarbamate.[61] Diamine oxidases are also sensitive to many carbonyl reagents and especially to hydrazine.[66]

Substrate analogues are often found to be very good inhibitors. The competitive inhibition of pea seedling and pig kidney diamine oxidase with 1,5-diaminopentan-3-one has been reported.[94] The ketodiamine proved to be a more effective inhibitor of the plant enzyme than the mammalian enzyme. The duration of inhibition depended on the amount of enzyme present, indicating that an enzymic degradation of the inhibitor was occurring.

The structural relationships between the diamine oxidase substrates histamine and putrescine and some inhibitors with respect to the plant and animal enzymes were investigated by Buganski and co-workers.[95] They were interested in whether the imidazole derivatives would be more inclined to inhibit the pig kidney enzyme rather than the plant enzyme. Compounds (104) and (105) were found to be potent inhibitors of diamine oxidase with no particular bias to either enzyme.



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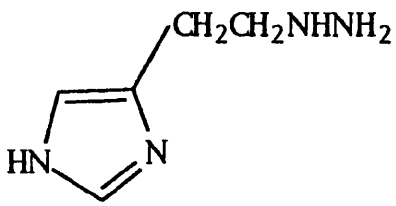
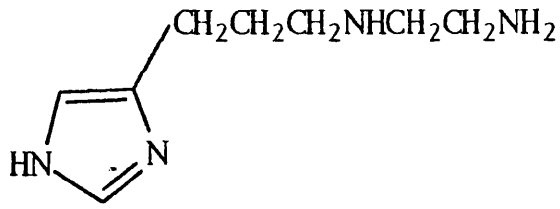
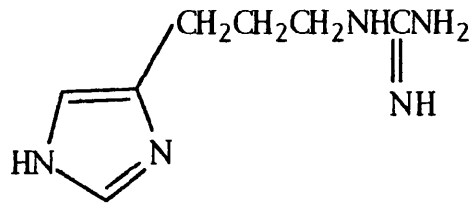


(105)

Compound (106) showed some selectivity towards inhibiting the pea seedling diamine oxidase in contrast to the two guanidine derivatives (107) and (108) which contain the imidazole ring structure but are not amines. These compounds only inhibited the animal enzyme.



(106)



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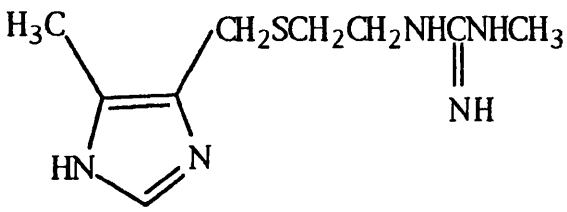


(108)

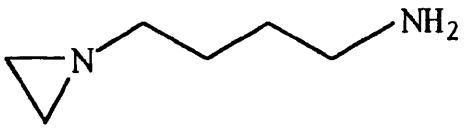
Therefore the possession of an imidazole ring by a substrate analogue does not appear to cause a differential inhibition of pig kidney diamine oxidase with respect to the substrates histamine or putrescine.

Pec and co-workers investigated a range of lysine derivatives including L-lysine hydrazide as inhibitors of pea seedling diamine oxidase.[96] All the compounds tested were competitive inhibitors. In the case of L-lysine hydrazide the inhibition was found to be time dependent and partly reversible. The enzyme can be protected by an excess of substrate by addition of a hydrazide sensitive compound such as sodium pyruvate.

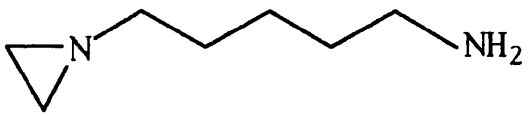
Pec and Frebort reported the inhibition of pea seedling diamine oxidase by a range of piperidine alkaloids and aromatic aminoketones and compared the binding sites for inhibition.[97] The alkaloids tested were L-lobeine, (-)-norsedamine and cinchonine and the aromatic ketones were 1-amino-3-phenylpropan-3-one and 1-amino-3-phenylpropan-2-one. In all cases they obtained evidence to indicate

that all the compounds are bound into the same subsite of the enzyme. They concluded that for compounds so structurally different to be bound at the same subsite, the active site of the enzyme is on the surface or in an exposed opened pocket.

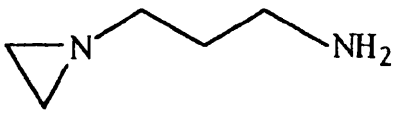
Recently a set of aminoalkylaziridine analogues of putrescine, cadaverine and 1,3-diaminopropane were found to inhibit pig kidney diamine oxidase irreversibly.[98]



(109)



(110)



(111)

N-(4-Aminobutyl)aziridine (109) and *N*-(5-aminopentyl)aziridine (110) were found to be, respectively, 35 and 15 times more effective as inhibitors than the 1,3-diaminopropane derivative (111). The differences in reactivity of the aziridinylalkylamines may be related to an association of the inhibitors with specific sites on the enzyme. These results suggest that the aziridinylalkylamines tested are site-directed agents which form irreversible complexes at the active site of pig kidney diamine oxidase. *N*-(4-Aminobutyl)aziridine (109) is a potent reversible inhibitor of polyamine uptake[99] and has been evaluated as a potential chemotherapeutic agent for prostatic cancer.[100]

3.10 Uses of Diamine Oxidase

As disscussed in Chapter 1 Herbert and co-workers have described convenient synthetic routes to tylophorine (3) and cryptopleurine (33) using the enzymic action of diamine oxidase to generate 1-pyrroline and 1-piperideine.[14,15]

For example, 3,4-dimethoxybenzoylacetic acid (72) was condensed with 1-piperideine, generated from the oxidation of cadaverine by pea seedling diamine oxidase, to give 3',4'-dimethoxy-2-(2-piperidyl)acetophenone (31) which is an intermediate in the synthesis of cryptopleurine (33).

CHAPTER 4

THE INHIBITION OF DIAMINE OXIDASE

4.1 Isolation and Purification of Diamine Oxidase (DAO).

There are two main sources from which the enzyme, diamine oxidase, can easily be isolated. These are pig kidneys and peas. Pea seedling diamine oxidase was found to be preferable for our work as the enzyme isolated from pig kidneys is too impure as supplied commercially and it would be difficult and expensive to produce enzyme with sufficient activity to carry out kinetic determinations. Also, the pea seedling enzyme is very stable during long-term storage.

Pea seedling diamine oxidase was extracted and partially purified using the method of Hill.[61] The extraction and purification procedure was carried out by Miss I. Freer in the University of Glasgow. Pea seedlings from the 'Fillbasket' variety were grown for ca. 10 days. Precipitation with a mixture of chloroform and ethanol (2:1) removed unwanted material from the crude extract. Subsequent saturation with ammonium sulphate precipitated the enzyme after one hour. Unlike Hill's preparation, complete separation of the enzyme was not obtained by us after one hour of standing, and it was decided to improve separation by allowing the mixture to stand overnight. Further precipitation by ammonium sulphate, then dialysis, led to the isolation of the partially purified enzyme. There was no need to undertake the chromatographic purification steps described by Hill, as the enzyme obtained was pure enough for our purposes. The enzyme was then taken up in phosphate buffer (pH 7) and stored in 0.5 ml aliquots in the freezer at ca. -20 °C. The enzyme was found to be stable, under these conditions, for several months.

4.2 Determination of the Protein Concentration

The method of Sedmark and Grossberg[101] was used to determine the protein concentration of the enzyme. This was carried out by Dr. A. M. Tierney in the University of Glasgow. This method works on the principle that Coomassie brilliant blue G in dilute acid changes from a brownish/orange to an intense blue colour with the addition of protein. This method was carried out using bovine serum albumin (BSA) as standard (1 mg of BSA is equivalent to 1 mg of protein). For various concentrations of BSA the absorbances of the mixture at 620 nm (A_{620})(blue) and 465 nm (A_{465})(brownish/orange) were recorded immediately after the protein was added. A standard graph was obtained by plotting A_{620}/A_{465} against the protein concentration. The A_{620} and A_{465} absorbances were then measured for a range of enzyme samples of varying dilutions. The A_{620}/A_{465} absorbance ratios then allowed the determination of the protein concentrations in the enzyme samples, by using the standard graph.

This assay has been shown to be highly reproducible for the determination of protein concentration.

4.3 Enzyme Kinetics

4.3a Michaelis-Menten Kinetics

Generally the initial rate (V) of an enzyme-catalysed reaction increases with increasing substrate concentration ([S]) as shown in Figure 4.1. The rate of product formation is directly in proportion to the substrate concentration. As the substrate concentration increases

further, the enzyme becomes saturated with substrate, and a maximal rate is reached.

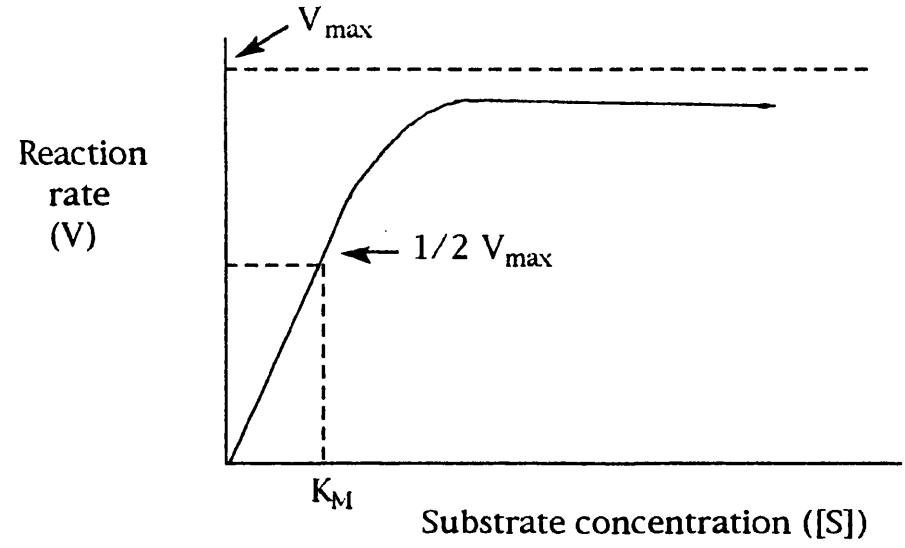


Figure 4.1 Reaction Rate (V) against Substrate Concentration ([S])

The Michaelis-Menten model is a simple one which accounts for the kinetic properties of most enzymic reactions.[102] Here the mechanism of enzyme action must involve the formation of a specific complex between the enzyme and the substrate as an intermediate (Scheme 4.1).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

where E = Enzyme, S = Substrate, ES = Enzyme-Substrate Complex, P = Product.

Scheme 4.1

The rate constant for formation of this ES complex is defined as k_1, and the rate constant for dissociation is defined as k_2. The rate

constant for the conversion of the complex into the products is defined as k_{cat}.

Firstly, a rate equation is required which relates the rate of catalysis to the substrate and enzyme concentrations and the rates of the individual steps. The rate of catalysis (V) can be expressed as being equal to the product of k_{cat} and the concentration of the ES complex (Equation 4.1).

$$V = k_{cat}[ES]$$

Equation 4.1

The ES complex is assumed to be under steady state conditions, that is the rates of formation and breakdown of ES are equal.

$$\text{Rate of formation of ES} = k_1[E][S]$$

Equation 4.2

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

Equation 4.3

therefore,

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

Equation 4.4

i.e. $$[ES] = [E][S] / \{(k_2+k_{cat})/k_1\}$$

Equation 4.5

By defining the Michaelis-Menten constant, K_M as,

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

Equation 4.6

Equation 4.5 can be simplified to,

$$[ES] = [E][S] / K_M$$

Equation 4.7

Provided that the enzyme concentration is much smaller than the substrate concentration, the concentration of free substrate [S] is effectively equal to the total substrate concentration. The concentration of free enzyme [E] must be expressed in terms of the total enzyme concentration $[E_T]$ minus the enzyme concentration in the [ES] complex:

i.e. $$[E] = [E_T] - [ES]$$

Equation 4.8

Substituting this expression for [E] into equation 4.7 gives,

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

Equation 4.9

i.e. $$[ES] = [E_T][S] / ([S]+K_M)$$

Equation 4.10

Substituting this expression for [ES] into equation 4.1 gives,

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

Equation 4.11

The maximal rate V_{max}, is observed when the enzyme active sites are saturated with substrate, that is when [S] is far greater than K_M, so [S]/[S]+K_M approaches 1. Therefore,

$$V_{max} = k_{cat}[E_T]$$

Equation 4.12

Substituting this expression for $k_{cat}[E_T]$ in equation 4.11 gives the Michaelis-Menten equation,

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

Equation 4.13

At low substrate concentrations, when [S] is much smaller than K_M,

$$V = V_{max}[S] / K_M$$

Equation 4.14

The rate is therefore directly proportional to the substrate concentration. At high substrate concentrations, when [S] is much greater than K_M,

$$V = V_{max}$$

Equation 4.15

The rate is therefore maximal and independent of the substrate concentration.

When the substrate concentration is equal to the K_M,

$$V = V_{max} / 2$$

Equation 4.16

The K_M is therefore equal to the substrate concentration at which the reaction rate is half its maximal value.

4.3b The Determination of K_M and V_{max}

Both the Michaelis constant K_M, and the maximal rate V_{max}, can be determined by the measurement of the rate of catalysis at various substrate concentrations, for an enzyme catalysed reaction which obeys Michaelis-Menten kinetics.

However, in practice the plot of reaction rate (V) against substrate concentration [S] is not satisfactory for the determination of K_M and V_{max}, since V_{max} is approached asymptotically.

The reciprocal of equation 4.13 separates the variables into an equation consistent with the familiar y = mx+c format for a straight line,

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

Equation 4.17

A plot of the reciprocal of the reaction rate (1/V) against the reciprocal of the substrate concentration (1/[S]), gives a straight line whose gradient of the slope is K_M/V_{max} and whose y intercept is $1/V_{max}$ (Figure 4.2). This is known as the Lineweaver-Burk plot.[103]

The accuracy of this double reciprocal plot is however widely criticised due to the undue weight given to low substrate concentrations, and these values are not as accurate.

The Michaelis-Menten equation can be rearranged to give V as a function of V/[S],

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

Equation 4.18

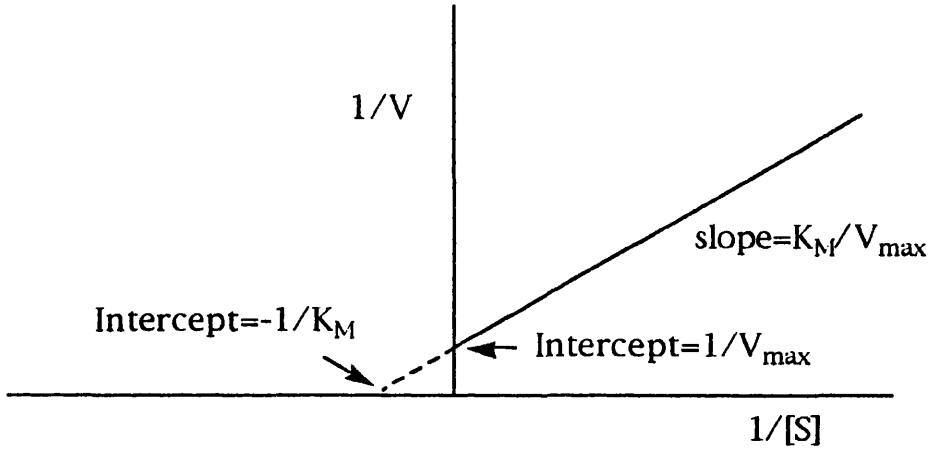


Figure 4.2 The Lineweaver-Burk Plot

A plot of V against V/[S], known as the Eadie-Hofstee plot, is also a straight line with the gradient equal to $-K_M$ and the y intercept equal to V_{max} (Figure 4.3).[104]

The Eadie-Hofstee plot is generally considered to be more accurate than the Lineweaver-Burk plot.

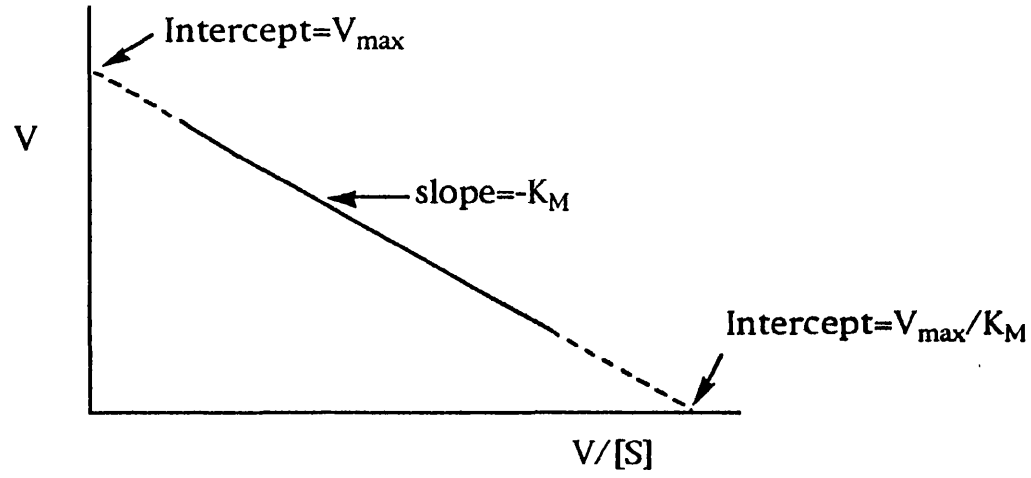


Figure 4.3 The Eadie-Hofstee Plot

4.3c The Importance of K_M and V_{max}

The K_M is related to the rate constants of the formation and dissociation of the ES complex. If k_2 is much greater than k_{cat} then,

$$K_M = k_2 / k_1$$

Equation 4.19

and the dissociation of the ES complex is given by,

$$K_{ES} = [E][S] / [ES] = k_2 / k_1$$

Equation 4.20

That is the K_M is equal to the dissociation constant of the ES complex if $k_2 \gg k_{cat}$.

Therefore under this condition the K_M is a measure of the strength of the ES complex. A high K_M indicates weak binding of the substrate to the enzyme and a low K_M indicates strong binding of the substrate to the enzyme.

The V_{max} is the maximal rate and is related to the rate of formation of the products, k_{cat}. The V_{max} is a measure of the turnover number of the enzyme.

4.4 Enzyme Kinetics for Inhibitors of Diamine Oxidase

It is possible to distinguish between competitive and noncompetitive inhibitors by studying the rates of catalysis in the presence and absence of an inhibitor at different substrate concentrations.

In the presence of a competitive inhibitor the Lineweaver-Burk plot has the same appearance as that in the absence of inhibitor. A competitive inhibitor does not affect the turnover number of the enzyme so the V_{max} does not change. The plot, therefore, has the same y intercept ($1/V_{max}$). Since the substrate and the inhibitor are competing for the same active site of the enzyme, the K_M shows an apparent increase in the presence of inhibitor. Therefore both the x intercept ($-1/K_M$) and the slope of the line (K_M/V_{max}) change (Figure 4.5).

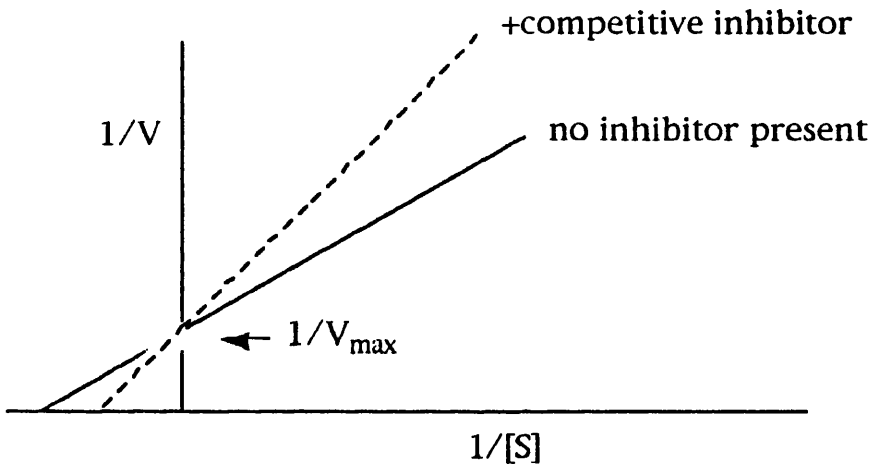


Figure 4.5 Double Reciprocal Plot for Competitive Inhibition.

The difference in the slopes of the lines indicates the difference in binding. The Lineweaver-Burk equation,

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

Equation 4.21

becomes,

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

Equation 4.22

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

$$(slope)^I/(slope) = 1 + [I]/K_i$$

Equation 4.23

Where $(slope)^I$ is the slope of the line with competitive inhibitor present, and (slope) is the slope of the line without inhibitor.

In noncompetitive inhibition the V_{max} is decreased and the K_M remains unchanged. This effect is seen graphically in the double reciprocal plot (Figure 4.6).

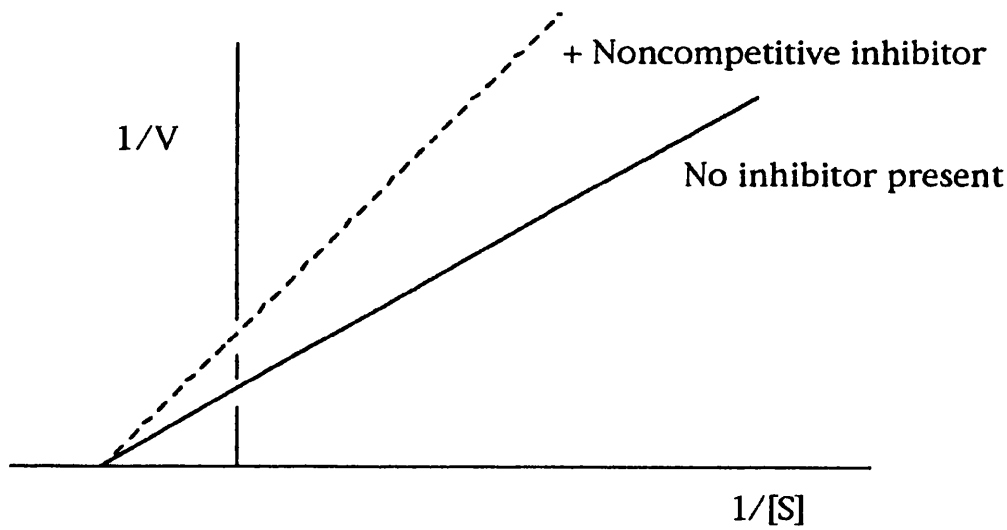


Figure 4.6 Double Reciprocal Plot for Noncompetitive Inhibition.

Since K_M remains unchanged, the maximum velocity in the presence of a noncompetitive inhibitor is given by,

$$V_{max}^I = V_{max}/(1+[I]/K_i)$$

Equation 4.24

4.5 The Spectrophotometric Assay

The kinetic data for diamine oxidase catalysed oxidation of a substrate in the presence of an inhibitor were determined using the procedure of Stoner.[105] This involved a peroxidase-coupled assay (horseradish peroxidase, EC 1.11.1.7, from Sigma) to monitor continuously the production of hydrogen peroxide during diamine oxidation at 25 °C, in 70 mM phosphate buffer (pH 6.3), in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH) (112) and 3-(dimethylamino)benzoic acid (DMAB) (113). Oxidative coupling generated stoichiometric quantities of an indamine dye (114) with a characteristic absorbance at 595 nm (Scheme 4.2). The rates of reaction could be determined directly in the spectrophotometer. Initial rates were determined over a range of substrate concentrations from the linear absorbance changes measured during the first thirty seconds of reaction. For inhibition studies, four separate experiments were carried out. A constant amount of inhibitor was added to each experiment. Typically,

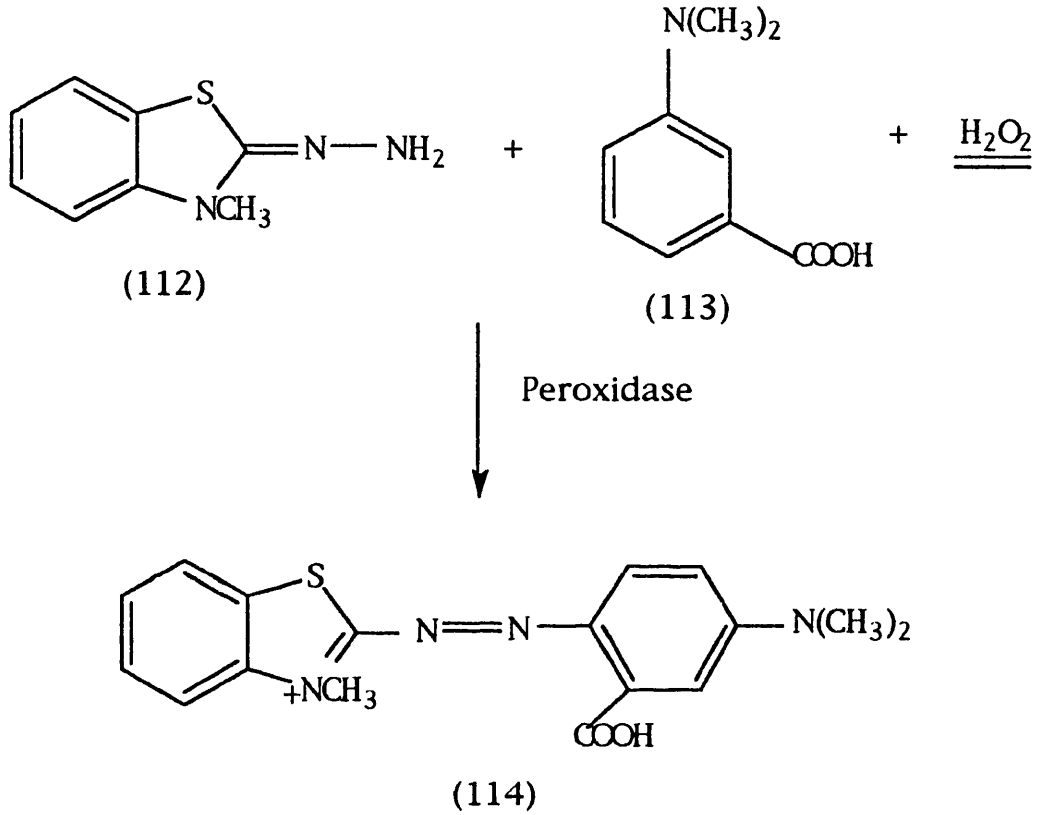
(1) Substrate only
(2) Substrate + [I]
(3) Substrate + 2[I]
(4) Substrate + 4[I]

where [I] is the inhibitor concentration.

Since experimental values for the oxidation can vary slightly from day to day it was necessary to obtain rate data for the substrate alone for every inhibition study. The oxidation was initiated by the addition of the enzyme to the thermally equilibrated reaction mixture. The same concentration of inhibitor was added in each experiment

followed immediately by the substrate. The reaction mixture was shaken, before both the inhibitor and the substrate were added (see Experimental section for more details of assay system).

Michaelis-Menten behaviour was observed in most cases. Rate data were analysed for K_M and V_{max} by least squares fitting of Lineweaver-Burk (1/V vs 1/[S]) and Eadie-Hofstee (V vs V/[S]) plots.



(112) (113) (114)

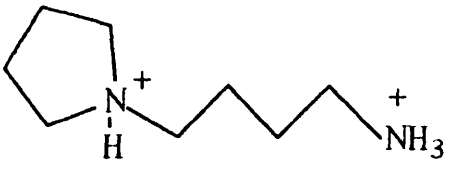
Scheme 4.2 The Spectrophotometric Assay[105]

4.6 *N*-Alkylated Amines as Inhibitors of Pea Seedling Diamine Oxidase

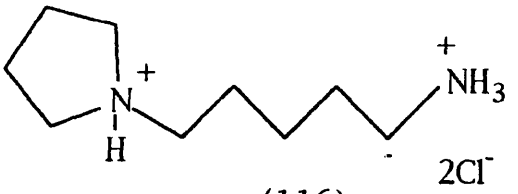
4.6a Introduction

A range of diamines (115)-(118), (54) and (121)-(123) were tested as substrates of pea-seedling diamine oxidase by Alastair Denholm[106] using the peroxidase coupled assay system. Compounds (115)-(120) and (121)-(123) are analogues of a known intermediate (54) in pyrrolizidine alkaloid biosynthesis.[37]

The five membered saturated compounds (115) and (117) were found by Denholm to have a similar binding affinity, as shown by the K_M values, to putrescine and the K_M values of the six membered compounds (116) and (118) were similar to that of cadaverine. However, there was a large decrease in the V_{max} for all four compounds, indicating a lower activity compared to the natural substrates putrescine and cadaverine (see Table 4.1). The unsaturated amines (54), (121) and (122)-(123) were shown to have a far higher binding affinity for pea seedling DAO than putrescine and cadaverine. These compounds also showed a lower rate of oxidation, indicated by a decrease in the V_{max} (see Table 4.2). This stronger binding effect may be due to a number of changes produced by the presence of the $C{=}N^+$ double bond. Changes in the pK_a of the remaining amino group and/or polarity of the molecule may be important, as well as the change in conformation of the ring system and the presence of the π system. Due to the good binding affinities and low rates of oxidation shown by these compounds it was decided to test them as inhibitors of pea seedling diamine oxidase with cadaverine as the substrate.



(115)



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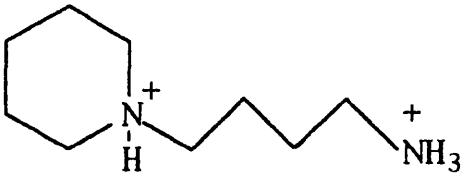

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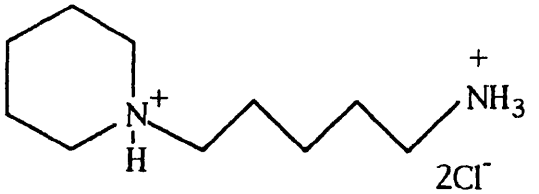

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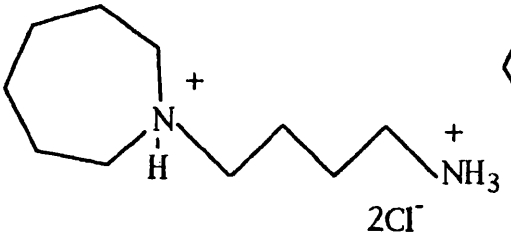

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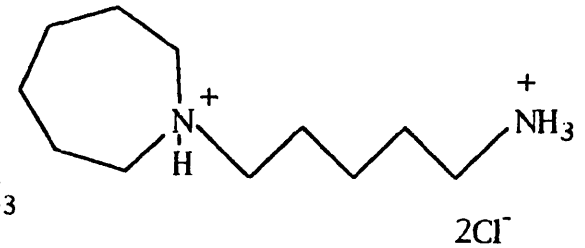

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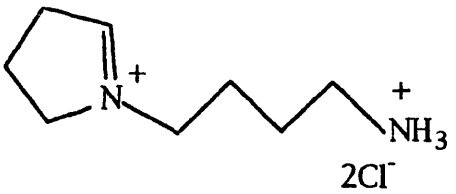
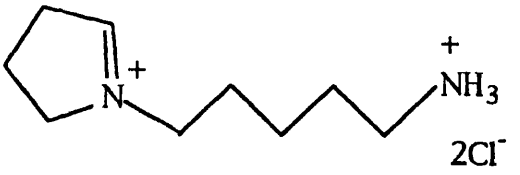


(54)



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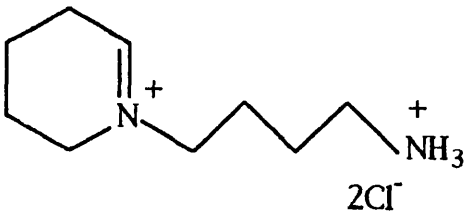

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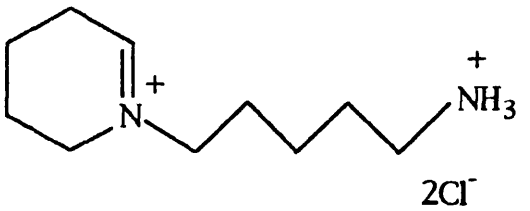


(123)

Substrate	K_M(mM)	V_{max}(μmol $mg^{-1}h^{-1}$)
(115)	0.94(±0.04)	31.9(±0.85)
(117)	1.19(±0.11)	5.43(±0.05)
(116)	0.20(±0.02)	17.2(±0.42)
(118)	0.37(±0.03)	3.57(±0.25)

Table 4.1[106]

Substrate	K_M(mM)	V_{max}(μmol $mg^{-1}h^{-1}$)
(54)	0.052(±0.005)	15.7(±0.78)
(122)	0.056(±0.003)	7.69(±0.46)
(121)	0.035(±0.003)	25.3(±0.92)
(123)	0.037(±0.004)	7.96(±0.53)

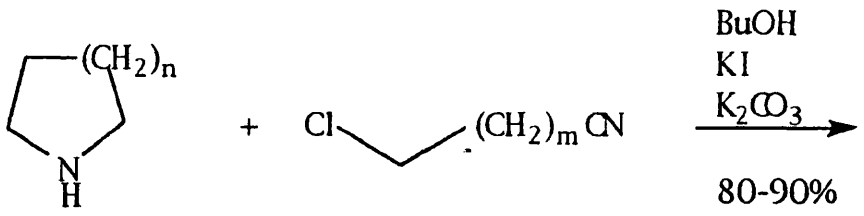
Table 4.2[106]

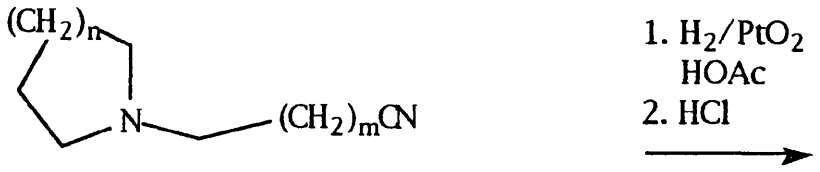
4.6b Synthesis of *N*-Alkylated Amines

For our work, compounds (115)-(118), (54) and (121)-(123) were prepared by the method of Alastair Denholm (see Scheme 4.3).[106] Heating piperidine or pyrrolidine with 4-chlorobutyronitrile or 5-chlorovaleronitrile in dry butanol at reflux, in the presence of base and a catalytic amount of potassium iodide, produced the *N*-alkylated aminonitriles (149)-(154) in good yield. The nitriles were characterised by the nitrile stretching frequency in the IR spectra at 2240 cm^{-1}. Reduction by catalytic hydrogenation in aqueous acid over Adams' catalyst gave the saturated diamines. The iminium salts were obtained by mercuric acetate oxidation of the saturated diamines in aqueous acetic acid. In each case the product was obtained as a viscous gum which did not solidify. The iminium salts were characterised by ^1H NMR spectra showing signals around δ 8.5 for the imine proton. Synthesis of the seven membered cyclic iminium ions was attempted. The saturated diamines (119) and (120) were obtained in good yield, however oxidation with mercuric acetate returned only starting material. This is possibly due to the seven membered ring having to adopt an unfavourable conformation for imine formation.

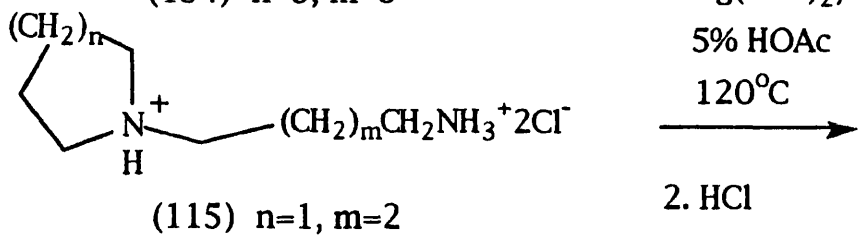
4.6c Results and Discussion

Using the peroxidase-coupled spectrophotometric assay[105] the saturated compounds (115)-(120) were tested as inhibitors of pea seedling diamine oxidase with cadaverine as the substrate. The results obtained obeyed Michaelis-Menten kinetics over inhibitor concentrations of 0.25 mM, 0.5 mM and 1.0 mM and were characteristic of competitive inhibition (see Table 4.3).

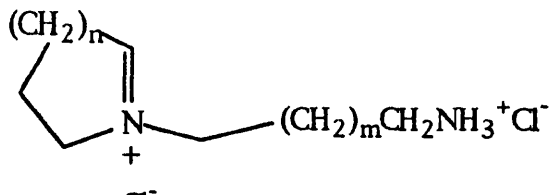




(149) n=1, m=2
(150) n=1, m=3
(151) n=2, m=2
(152) n=2, m=3
(153) n=3, m=2
(154) n=3, m=3



(115) n=1, m=2
(116) n=1, m=3
(117) n=2, m=2
(118) n=2, m=3
(119) n=3, m=2
(120) n=3, m=3



(54) n=1, m=2
(121) n=1, m=3
(122) n=2, m=2
(123) n=2, m=3

Scheme 4.3[106]

Inhibitor	Inhibitor Concentration	K_i
N-(4-Aminobutyl)pyrrolidinium dihydrochloride (115)	0.5	6.50
	1.0	--
N-(4-Aminobutyl)piperidinium dihydrochloride (117)	0.5	--
	1.0	8.50
N-(4-Aminobutyl)hexahydro-azepinium dihydrochloride (119)	0.5	0.27
	1.0	0.27
N-(5-Aminopentyl)pyrrolidinium dihydrochloride (116)	0.5	6.50
	1.0	4.30
N-(5-Aminopentyl)piperidinium dihydrochloride (118)	0.5	1.30
	1.0	1.86
N-(5-Aminopentyl)hexahydro-azepinium dihydrochloride (120)	0.5	0.52
	1.0	0.81

Table 4.3 Units; mM

It is clear from these results that the seven membered ring compounds (119) and (120) are better inhibitors of pea seedling DAO than the five and six membered ring compounds. Also, in the case of the six and seven membered ring compounds increasing the chain length causes a decrease in the inhibition. Therefore, increasing ring size appears to increase the inhibitory effect, on the other hand, increasing the substituent chain length causes the opposite effect. The

inhibition observed with *N*-(5-aminopentyl)piperidinium dihydrochloride (118) is not as great as that of the seven membered ring compounds although it is still significant. Compounds (115)-(117) showed no significant inhibition of pea seedling DAO.

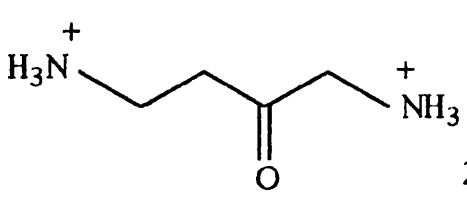
The saturated amines with chain length between the two nitrogens the same as cadaverine were stronger inhibitors than those with four carbon atoms between the nitrogens as in putrescine. Further work is required to test the above compounds as inhibitors of pea seedling DAO with putrescine as the substrate.

The unsaturated compounds (54) and (121)-(123) were also tested as inhibitors of pea seedling diamine oxidase with cadaverine as the substrate. However, the results obtained did not obey Michaelis-Menten kinetics, therefore it was not possible to determine values for K_M and V_{max}. It was thought that the imines were in some way interfering with the assay in the production of hydrogen peroxide. If this is correct it may be possible to test these compounds by means of another assay system which does not involve the formation of hydrogen peroxide.

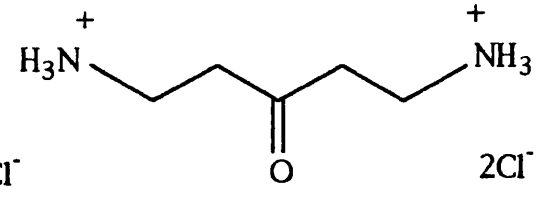
4.7 Diamino Carbonyl Compounds as Inhibitors of Diamine Oxidase

4.7a Introduction

In 1973 Macholan compared the inhibitory effects of 1,4-diamino-2-butanone and 1,5-diamino-3-pentanone.[94] The 3-ketocadaverine dihydrochloride (124) proved to be 14 times more potent a competitive inhibitor of pea seedling diamine oxidase, but a far weaker inhibitor of the animal enzyme than 2-ketoputrescine dihydrochloride (125), indicating that a β-amino-keto grouping plays a greater inhibitory role than an α-amino-keto group.



(125)



(124)

The previous chapter contains a discussion of synthetic approaches to sparteine analogues. 1,5-bis(Piperidyl)-3-pentanone dihydrochloride (91) and 1,5-bis(hexahydroazepinyl)-3-pentanone dihydrochloride (94) are intermediates used in this work. It was thought that these diamino carbonyl compounds might be inhibitors of diamine oxidase and it would be of interest to see the effect of increasing substituent size rather than chain size. Although compounds (91) and (94) lack a primary amino group, and therefore are not expected to be substrates for diamine oxidase, they are substrate analogues and could still bind well to the enzyme. As discussed in Chapter 2 the pyrrolidine compound could not be synthesised.

4.7b Results and Discussion

Both compounds (91) and (94) were initially tested as substrates of pea seedling diamine oxidase. No oxidation was observed in either case.

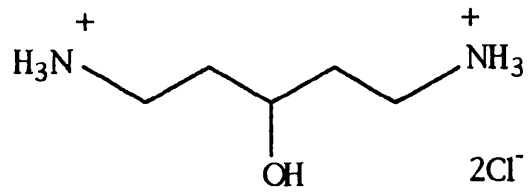
The compounds were then tested as inhibitors of pea seedling diamine oxidase with cadaverine as the substrate. Both compounds have five carbon atoms between the two nitrogens as in cadaverine. The results are shown in Table 4.4.

Inhibitor	Inhibitor Concentration	K_i
1,5-bis(piperidyl)-3-pentanone dihydrochloride (91)	0.5	0.47
	1.0	0.38
1,5-bis(hexahydroazepinyl)-3-pentanone dihydrochloride (94)	0.5	0.35
	1.0	0.21

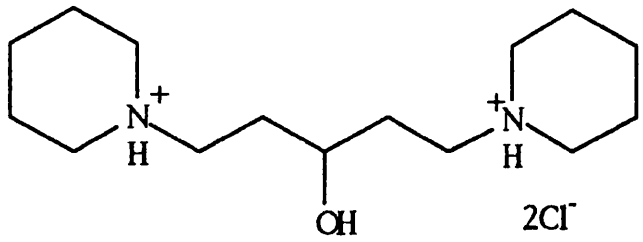
Table 4.4 Units: mM

Both compounds (91) and (94) assayed behaved as competitive inhibitors and obeyed Michaelis-Menten kinetics. As with the *N*-alkylated amines (115)-(120) discussed in the previous section the hexahydroazepinyl derivative (94) proved to be a better inhibitor than the six membered analogue. This suggests that the active site of the enzyme is relatively uncrowded to allow such large molecules to affect the rate of substrate oxidation so significantly.

Macholan concluded that the keto group was essential for enzyme inhibition since catalytic hydrogenation of 1,5-diaminopentan-3-one to the corresponding alcohol (126) resulted in the loss of its inhibitory properties.[94] This appears to be true also for 1,5-bis(piperidyl)-3-pentanone (91). 1,5-bis(Piperidyl)-3-pentanol (127) was prepared by sodium borohydride reduction in methanol of the diaminoketone (91) in quantitative yield. The alcohol (127) was tested as a substrate of diamine oxidase using the peroxidase-coupled assay system.[105] No oxidation was observed. The compound (127) was then tested as an inhibitor with cadaverine as the substrate. No inhibition was observed at concentrations of 0.25, 0.5 and 1.0 mM of alcohol. This suggests that the inhibition may be due to interactions such as hydrogen bonding between the lone pairs of the carbonyl oxygen and the active site, or it may be due to the different geometry of the sp^2 carbonyl and the sp^3 hydroxy groups.



(126)

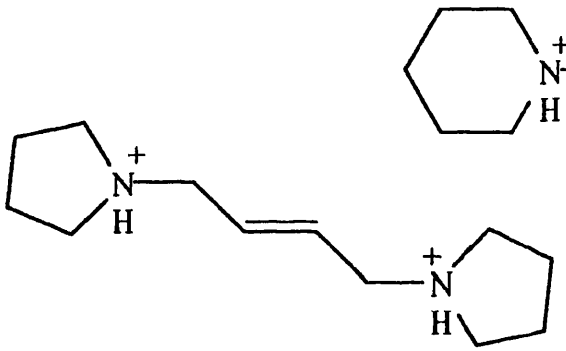


(127)

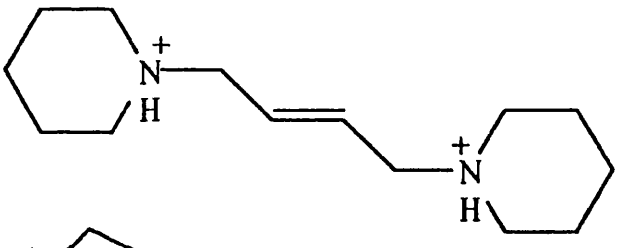
4.8 Diaminobutenes as Inhibitors of Diamine Oxidase

4.8a Introduction

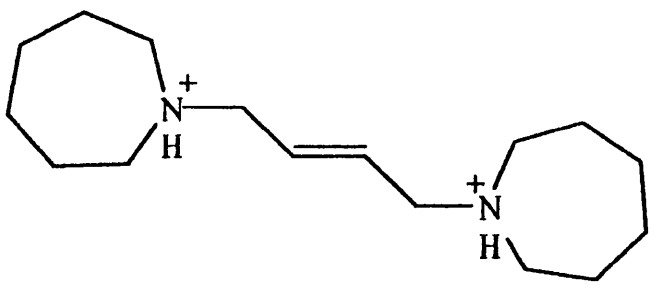
It has been reported that 1,4-diaminobut-2-ene is a substrate of diamine oxidase and undergoes oxidative deamination to yield pyrrole.[107] It has also been shown to inhibit the enzyme at high concentration.[107] It was thought to be of interest to compare both the *cis*- and *trans*-forms of analogues of 1,4-diaminobut-2-ene (128)-(135) as potential inhibitors of diamine oxidase.



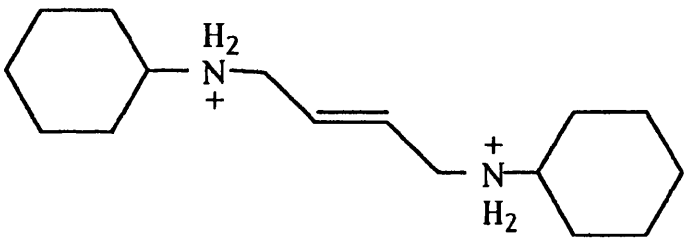
(128) $2Cl^-$



(129) $2Cl^-$



(130) $2Cl^-$

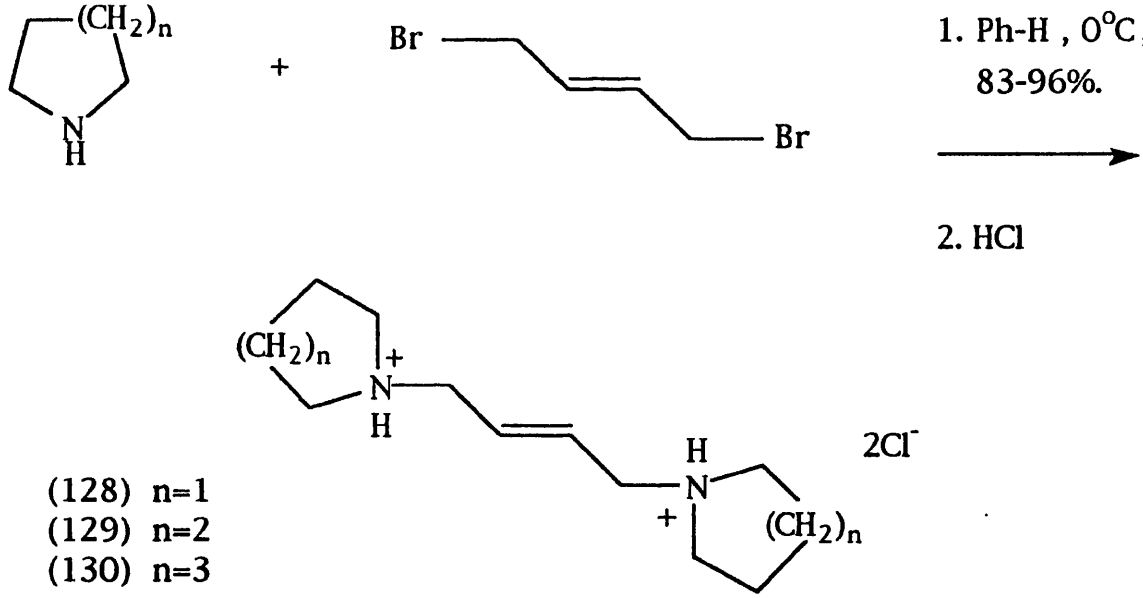


(131) $2Br^-$

(132)-(135): corresponding *cis*-isomers

4.8b Synthesis of Diaminobutenes

The *trans*-diaminobutenes (128)-(131) were prepared according to the method of Roberts *et al.* (Scheme 4.4).[108] This involves the addition of eight equivalents of amine to a cooled concentrated solution of *trans*-1,4-dibromobut-2-ene to give the free base of the diamino product in good yields. The dihydrochloride salts were then formed by addition of aqueous HCl to an organic solution of the free base. In the case of the di-pyrrolidyl compound (128), addition of aqueous HCl resulted in the precipitation of a pale yellow hygroscopic solid. The salt was obtained more cleanly by adding diethyl ether saturated with dry HCl gas to an ethereal solution of the free base. These compounds are characterised by a $X_2AA'X_2'$ system in their ^1H NMR spectra.



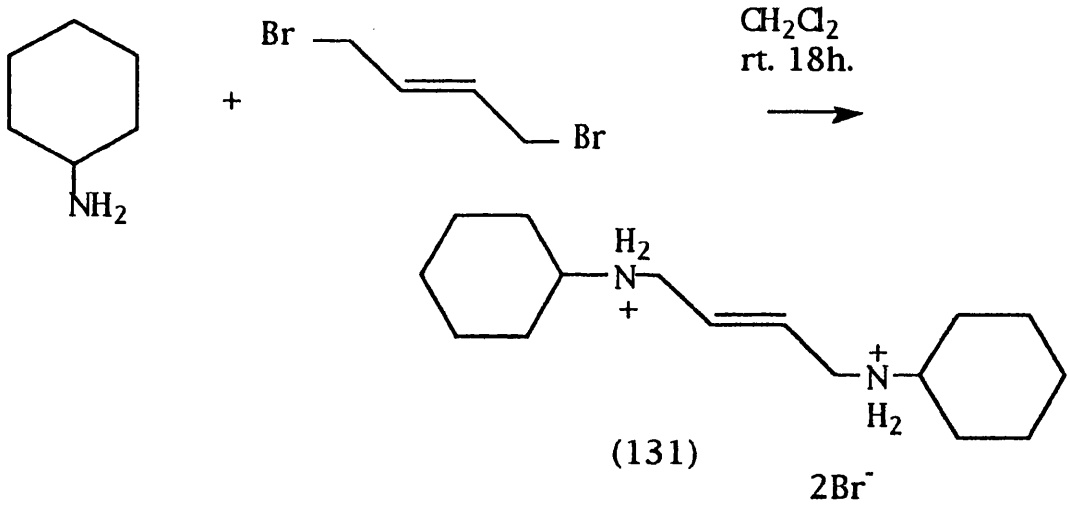
(128) n=1
(129) n=2
(130) n=3

Scheme 4.4[108]

trans-1,4-Dibromobut-2-ene in dichloromethane was added to two equivalents of cyclohexylamine at room temperature to form the

trans diaminobutene (131) directly as the hydrobromide salt (Scheme 4.5).

The formation of the free base of (131) was attempted as for compounds (128)-(130) however this gave a mixture of products which was difficult to purify by fractional distillation. The method of formation of the dihydrobromide of (131) also gave a mixture of products but the salt was fairly easy to purify by crystallisation.



Scheme 4.5

Synthesis of the corresponding *cis*-compounds (132)-(135) was attempted using both the above methods with *cis*-1,4-dibromobut-2-ene (155) [prepared by treatment of *cis*-but-2-ene-1,4-diol with phosphorus tribromide] and *cis*-1,4-dichlorobut-2-ene. These methods yielded only the *trans*-compounds. This result is perhaps not surprising due to the bulky substituents. There is no evidence to suggest that a 1,4-elimination has taken place, since there is no terminal methylene signal visible in the ^1H NMR spectrum. However, since the double bond is already *cis*, it is surprising that no

intramolecular reaction occurs after monosubstitution to form the quaternary ammonium salt.

(155)

4.8c Results and Discussion

Compounds (128)-(131) were initially tested as substrates of pea seedling diamine oxidase using the spectrophotometric assay.[105] No oxidation was observed in any case.

The compounds were then tested as inhibitors of pea seedling diamine oxidase with cadaverine as the substrate. The results are shown below (see Table 4.5).

Inhibitor	Inhibitor Concentration	K_i
trans-1,4-bis(pyrrolidyl)but-2-ene dihydrochloride (128)	0.25	3.75
	0.5	3.75
	1.0	2.14
trans-1,4-bis(piperidyl)but-2-ene dihydrochloride (129)	0.25	0.32
	0.5	0.40
	1.0	0.59
trans-1,4-bis(hexahydroazepinyl) but-2-ene dihydrochloride (130)	0.25	--
	0.5	6.0
	1.0	1.09
trans-*N,N'*-bis(cyclohexyl)-1,4-but-2-ene diamine dihydrobromide (131)	0.25	0.15
	0.5	0.31
	1.0	0.23

Table 4.5 Units: mM

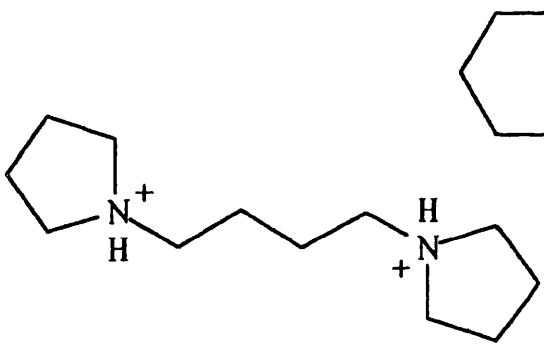
Previously, in general, the compounds with seven membered rings were proving to be the better inhibitors, but it was thought that the introduction of a double bond which would reduce the flexibility of the chain would cause a significant change in inhibitory properties. Both compounds (128) and (130) were shown to be poor inhibitors, whereas, (129) was shown to inhibit more significantly. The arrangement of electron density around the nitrogens and the π electrons of the double bond may be favourable to the binding site. *trans*-*N,N'*-bis(Cyclohexyl)-1,4-but-2-ene diamine (131) proved to be the best inhibitor of the four. This compound is similar in size and

ring shape to (129) but has the probable added bonus of good binding to the enzyme through the more accessible secondary nitrogen.

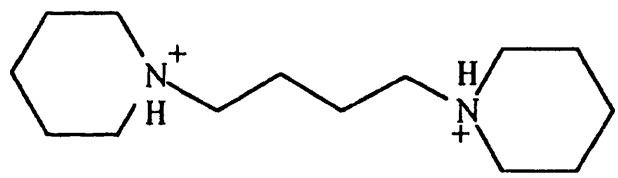
4.9 Diaminobutanes as Inhibitors of Diamine Oxidase

4.9a Introduction

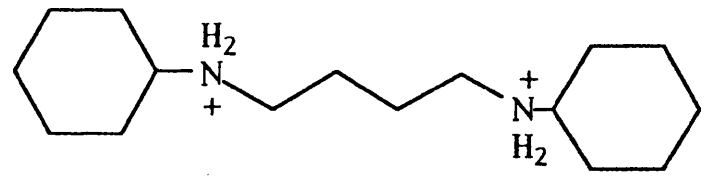
The obvious next step was to investigate the inhibitory properties of the saturated butanes corresponding to compounds (128)-(131) to see the effect of increasing the chain flexibility. Compounds (136)-(139) were synthesised from the butenes (128)-(131) to be tested as inhibitors of pea seedling diamine oxidase.



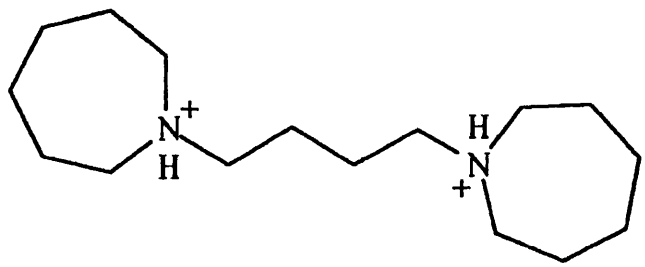
(136) $2Cl^-$



(137) $2Cl^-$



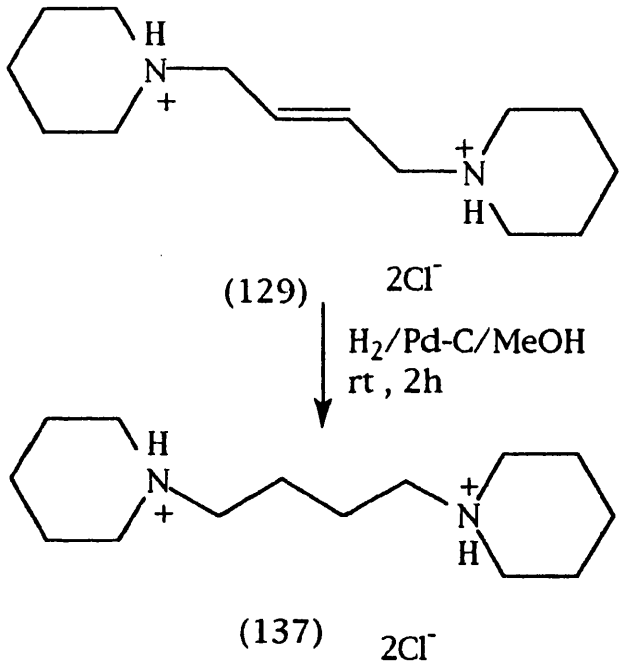
(139) $2Br^-$



(138) $2Cl^-$

4.9b Synthesis of Diaminobutanes

The double bond was reduced in all cases, in good yields, by hydrogenation of the dihydrochloride or dihydrobromide salt in methanol in the presence of palladium on charcoal (Scheme 4.6).



(129)

(137)

Scheme 4.6

4.9c Results and Discussion

Compounds (136)-(139) were tested as substrates of pea seedling diamine oxidase using the spectrophotometric assay.[105] No oxidation was observed.

Compounds (136)-(139) were then examined as inhibitors of diamine oxidase with cadaverine as the substrate. The results are shown in Table 4.6. Assays in the presence of these compounds obeyed Michaelis-Menten kinetics and were characteristic of competitive inhibition.

Inhibitor	Inhibitor Concentration	K_i
1,4-bis(pyrrolidyl)butane dihydrochloride (136)	0.5	1.07
	1.0	0.94
1,4-bis(piperidyl)butane dihydrochloride (137)	0.5	0.90
	1.0	1.25
1,4-bis(hexahydroazepinyl) butane dihydrochloride (138)	0.5	0.35
	1.0	0.54
N,N'-bis(cyclohexyl)-1,4-butane diamine dihydrobromide (139)	0.5	0.62
	1.0	0.54

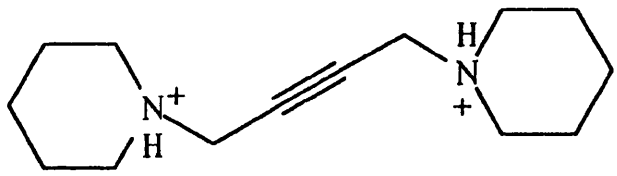
Table 4.6 Units: mM

These results show that again the seven membered ring compound (138) is the best inhibitor in the comparison of ring sizes. However, the inhibition shown by *N,N'*-bis(cyclohexyl)-1,4-butane diamine (139) is also significant.

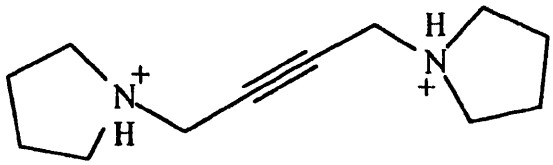
4.10 Diaminobutynes as Inhibitors of Diamine Oxidase

4.10a Introduction

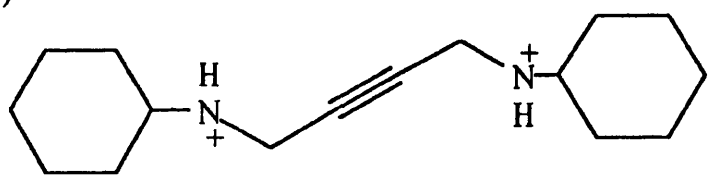
It has been reported that 1,4-diaminobut-2-ene is a mechanism-based inhibitor of pea seedling diamine oxidase.[109] Following on from these studies it was decided that the next target molecules should be the corresponding butyne analogues. Compounds (140)-(143) were synthesised to be tested as inhibitors of diamine oxidase.



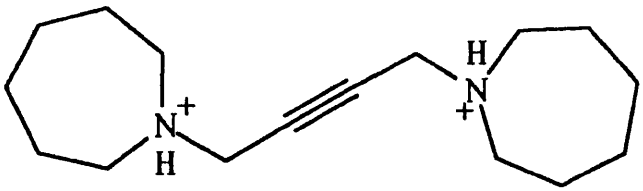
(141) $2Cl^-$



(140) $2Cl^-$



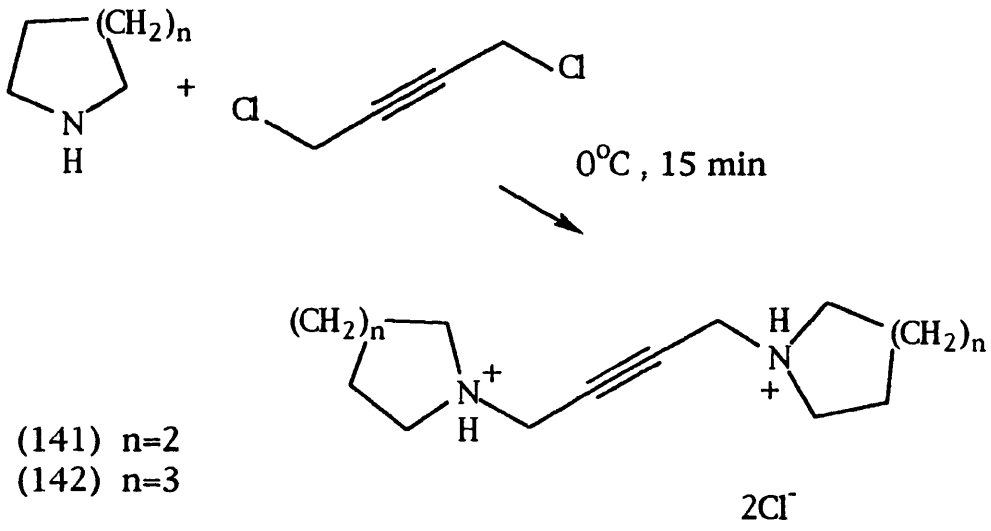
(143) $2Cl^-$



(142) $2Cl^-$

4.10b Synthesis of Diaminobutynes

Compounds (141)-(143) were synthesised following the method of Biel *et al.* (Scheme 4.7).[110] Four equivalents of the amine were added to cooled, neat 1,4-dichlorobut-2-yne to give the products in the form of the free base in excellent yields. Previous experience has shown that pyrrolidyl compounds tend to be hygroscopic and are difficult to extract from aqueous solution. The method of Roberts *et al.* [108] used previously for the synthesis of the butenes was adapted for the preparation of 1,4-bis-(pyrrolidyl)but-2-yne (140). Subsequent treatment with dry HCl gas in diethyl ether gave the dihydrochloride salt in 98% yield.



(141) n=2
(142) n=3

Scheme 4.7[110]

4.10c Results and Discussion

Compounds (140)-(143) were tested as substrates of pea seedling diamine oxidase. No oxidation was observed.

The butynes (140)-(143) were then assayed as inhibitors of diamine oxidase with cadaverine as the substrate. All obeyed Michaelis-Menten kinetics and data were characteristic of competitive inhibition. The results are shown in Table 4.7

Inhibitor	Inhibitor Concentration	K_i
1,4-bis(pyrrolidyl)but-2-yne dihydrochloride (140)	0.5	2.50
	1.0	1.15
1,4-bis(piperidyl)but-2-yne dihydrochloride (141)	0.5	3.75
	1.0	1.87
1,4-bis(hexahydroazepinyl) but-2-yne dihydrochloride (142)	0.5	1.17
	1.0	1.07
N,N'-bis(cyclohexyl)-1,4-but-2-yne diamine dihydrobromide (143)	0.5	0.37
	1.0	0.39

Table 4.7 Units: mM

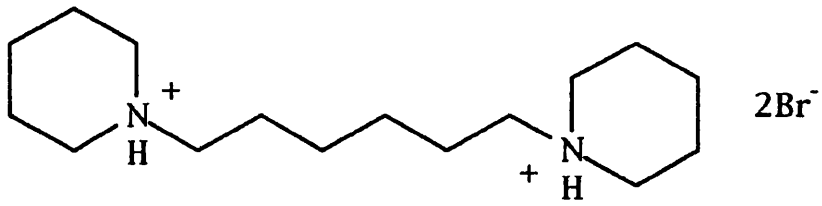
These results show that *N,N'*-bis(cyclohexyl)-1,4-but-2-yne diamine (143) was the best inhibitor of the butynes tested. This may again be due to a favourable arrangement of the π electrons of the

triple bond and the charge density around the two nitrogen atoms for the active site. Reducing the degrees of freedom in the chain has decreased the inhibitory properties of the seven membered ring compound (142).

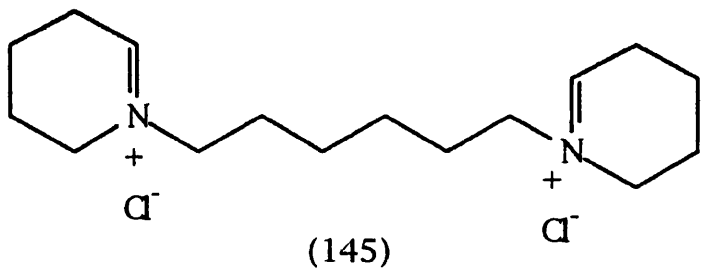
4.11 Di-*N*-Alkylated Diamines as Inhibitors of Diamine Oxidase

4.11a Introduction

1,6-bis(Piperidyl)hexane dihydrobromide (144) is a compound which is easily synthesised[111] and has previously been tested as an inhibitor of diamine oxidase.[112] Although not a particularly good inhibitor, it was thought that the di-iminium salt (145) might be a good inhibitor if it obeyed Michaelis-Menten kinetics, unlike the previous imines (54) and (121)-(123) tested. 1,6-bis(Piperidyl)hexane (144) and its di-iminium salt (145) were synthesised to be tested as inhibitors of pea seedling diamine oxidase. Attempts were also made to synthesize the corresponding pyrrolidine analogues.



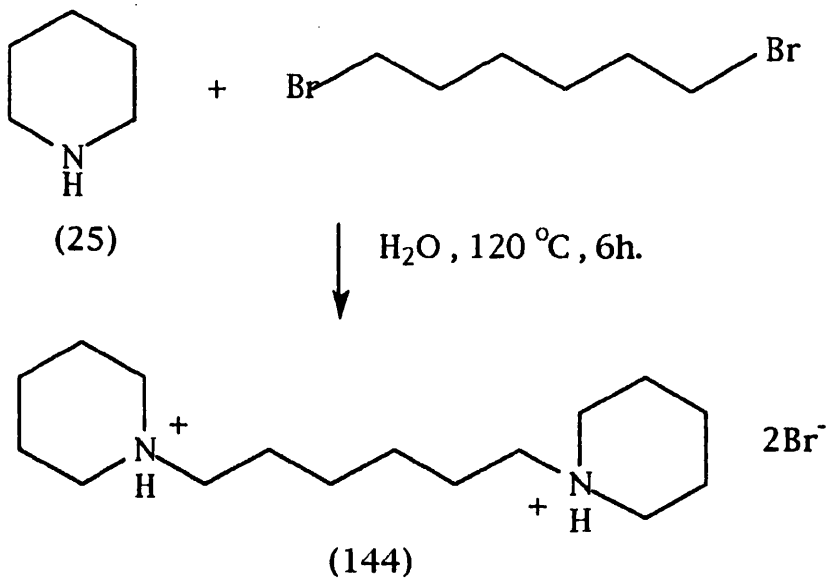
(144)



(145)

4.11b Synthesis

1,6-bis(Piperidyl)hexane (144) was formed by the addition of piperidine to an aqueous solution of 1,6-dibromohexane (Scheme 4.8).[111] Synthesis of 1,6-bis(pyrrolidyl)hexane was attempted in the same manner but gave a brown tar from which the product could not be crystallised. This is not surprising due to previous results when using pyrrolidine in aqueous conditions.



Scheme 4.8[111]

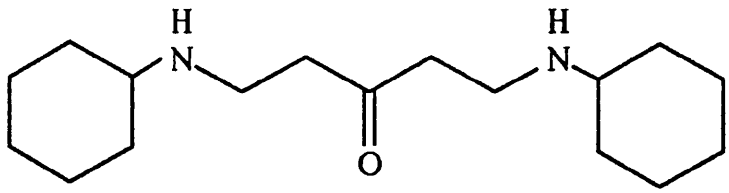
The di-iminium salt (145) was synthesised from pipecolic acid and 1,6-dibromohexane (Scheme 4.9). The methyl ester of pipecolic acid (156) was prepared to protect the amino acid carboxyl function. Treatment of the diester product (146) with aqueous hydrochloric acid to hydrolyse the ester groups to give (157) was followed by phosphorus oxychloride to complete decarbonylation. The ^1H NMR spectrum of (145) showed a broad singlet at δ 8.45 for the imine proton Despite attempted purification using a chloride ion-exchange

column, the product was too contaminated with phosphate ions to be tested as an inhibitor of diamine oxidase.

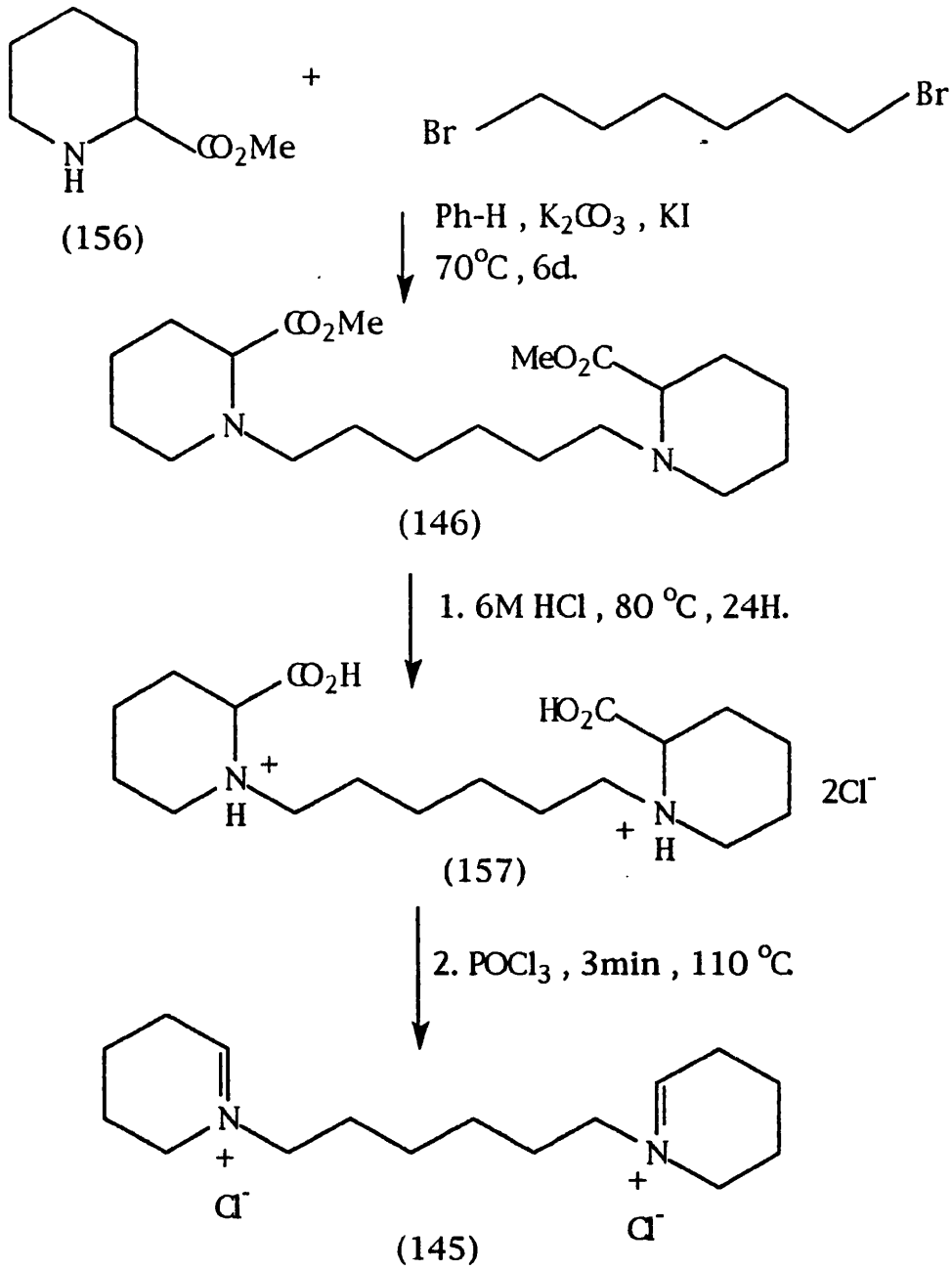
Synthesis of the corresponding pyrrolidyl compound was attempted using methyl prolinate and 1,6-dibromohexane. Under various reaction conditions, involving slow addition of the amino ester to the alkyl dibromide to avoid diketopiperazine formation, the product was not formed. This precaution would however be negated by the long reaction time required for substitution to occur.

4.12 Conclusions

It is apparent from these results that the most effective inhibitors of pea seedling diamine oxidase, from the compounds tested, were those containing a β-amino keto moiety or a secondary amine substituent. Time constraints prevented the synthesis and testing of other potential inhibitors such as *N,N'*-bis(cyclohexyl)-pentan-3-one-1,5-diamine (147). Further investigation is recommended into compounds of this type.



(147)



(156)

(146)

(157)

(145)

Scheme 4.9

CHAPTER 5

EXPERIMENTAL

5.1 General

Melting points were measured on a Kofler hot-stage apparatus, and boiling points refer to the oven temperature using a Kugelrohr apparatus. Infra red spectra (IR) were obtained on a Perkin Elmer 580 spectrometer. Nuclear magnetic resonance spectra (NMR) were recorded on a Perkin Elmer R32 spectrophotometer operating at 90 MHz, or a Bruker WP200-SY spectrophotometer 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 θ = 90° and θ = 135°. Spectra were recorded with either tetramethylsilane at 0 ppm or the NMR solvent as the internal standard. Mass spectra were obtained using A.E.I. MS 12 or 902 spectrometers. Elemental analyses were obtained with a Carlo-Erba 1106 elemental analyser.

Thin layer chromatography (TLC) was carried out on Merck Kieselgel G plates of 0.25 mm thickness in chloroform-methanol-ammonia (85:14:1) unless otherwise stated. Secondary amines were detected with ninhydrin, tertiary amines with Dragendorff's reagent and all other compounds with iodine or by ultra violet radiation (UV).

All solvents were purified by standard techniques.[113] Tetrahydrofuran (THF) and diethyl ether were dried by distillation from sodium-benzophenone under nitrogen immediately before use.

5.2 Experimental to Chapter 2

General Procedure for Complex Formation with 1-Pyrroline

4-Aminobutanal diethylacetal (5) (2.34 g, 14.5 mmol) was dissolved in 2M HCl (20 ml) and diethyl ether (50 ml) was added. The mixture was stirred at 0 °C for 20 min, then basified with potassium carbonate. The aqueous layer was extracted with cold diethyl ether (3 x 50 ml). The combined organic extracts were cooled to 0 °C, dried, filtered and the zinc or cadmium dihalide (0.5 equivalent) was added. Each mixture was stirred at 0 °C for 30 min. The precipitate was filtered off and washed well with diethyl ether and hexane leaving a solid which was recrystallised from chloroform and hexane.

bis(3,4-Dihydro-2*H*-pyrrol-1-yl)di-iodozinc (66)[47]

The zinc iodide was flame-dried under vacuum before use. The product (66) was obtained as white crystals (2.0 g, 61%), m.p. 203-205 °C; ν_{max} (KBr disc) 2970, 1635, 1330, 960, 925 and 420 cm^{-1}; δ_H (200 MHz) [$(CD_3)_2CO$] 1.99-2.15 (4H, complex, 4-H_2), 2.89-2.98 (4H, complex, 3-H_2), 3.94-4.05 (4H, complex, 5-H_2) and 8.26 (2H, m, 2-H); δ_C (50 MHz) [$(CD_3)_2CO$] 20.7 (t, C-4), 37.4 (t, C-3), 59.9 (t, C-5) and 178.6 (d, C-2). (Found: C, 20.94; H, 2.84; N, 6.01; I, 55.56; Zn,14.86%. $C_8H_{14}N_2I_2Zn$ requires C, 21.00; H, 3.09; N, 6.12; I, 55.49; and Zn, 14.29%.). Spectroscopic data were identical to those reported by G. Baxter.[47]

bis(3,4-Dihydro-2*H*-pyrrol-1-yl)dichlorozinc (67)

This was obtained as a white solid (67) (0.37 g, 18%), m.p. 135-140 °C; ν_{max} (KBr disc) 2970, 1635, 1330, 960, 925 and 420 cm^{-1}; δ_H (200 MHz) [$(CD_3)_2CO$] 1.94-2.10 (4H, complex, 4-H_2), 2.83-2.94 (4H, complex, 3-H_2), 3.88-3.99 (4H, complex, 5-H_2) and 8.11 (2H, m, 2-H); δ_C (50 MHz) [$(CD_3)_2CO$] 20.6 (t, C-4), 37.2 (t, C-3), 59.9 (t, C-5) and 177.9 (d, C-2). (Found: C, 34.80; H, 4.81; N, 9.98%. $C_8H_{14}N_2Cl_2Zn$ requires C, 35.00; H, 5.14; and N, 10.20%.)

bis(3,4-Dihydro-2*H*-pyrrol-1-yl)dibromozinc (68)

This was obtained as a yellow solid (68) (0.46 g, 17%), m.p. 162-165 °C; ν_{max} (KBr disc) 2960, 1635, 1330, 960, 925 and 420 cm^{-1}; δ_H (200 MHz) [$(CD_3)_2CO$] 1.96-2.12 (4H, complex, 4-H_2), 2.85-2.96 (4H, complex, 3-H_2), 3.90-4.02 (4H, complex, 5-H_2) and 8.16 (2H, m, 2-H); δ_C (50 MHz) [$(CD_3)_2CO$] 20.6 (t, C-4), 37.3 (t, C-3), 59.9 (t, C-5) and 178.2 (d, C-2). (Found: C, 26.35; H, 3.38; N, 7.52; Br, 44.33; Zn, 18.42%. $C_8H_{14}N_2Br_2Zn$ requires C, 26.44; H, 3.88; N, 7.71; Br, 43.97; and Zn, 18.00%.)

bis(3,4-Dihydro-2*H*-pyrrol-1-yl)di-iodocadmium (69)

This complex was precipitated by the addition of cadmium iodide (5.31 g, 14.5 mmol, 1 equivalent) to the dried ethereal extracts. Recrystallisation from acetone gave the complex (69) as white crystals (1.26 g, 20%), m.p. 108-109 °C; ν_{max} (KBr disc) 2950, 1625, 1330, 955, 920 and 415 cm^{-1}; δ_H (200 MHz) [$(CD_3)_2CO$] 1.98-2.06 (2H, complex, 4-H_2), 2.84-2.96 (2H, complex,

3-H_2), 3.93-4.05 (2H, complex, 5-H_2) and 8.17 (1H, m, 2-H); δ_C (50 MHz) [$(CD_3)_2CO$] 20.3 (t, C-4), 37.2 (t, C-3), 60.5 (t, C-5) and 178.0-178.2 (s, C-2). (Found: C, 11.20; H, 1.53; N, 3.37; I, 58.58; Cd, 25.32%. $C_4H_7NI_2Cd$ requires C, 11.04; H, 1.62; N, 3.22; I, 58.30; and Cd, 25.82%.)

Preparation of Norhygrine (70)[47]

bis(3,4-Dihydro-2*H*-pyrrol-1-yl)di-iodozinc (66) (0.97 g, 2.13 mmol) in methanol (50 ml) and 1M phosphate buffer (pH 7, 4 ml) were added to a solution of acetoacetic acid (0.5 g, 4.9 mmol) in water (10 ml) and the pH was adjusted to 7 with 1M KOH. The mixture was stirred at room temperature for 60 h then acidified with 2M HCl and heated at reflux for 3 h. The cooled solution was washed with diethyl ether (3 x 50 ml), and saturated with solid potassium carbonate. The basic solution was extracted with chloroform (3 x 80 ml). The combined organic extracts were dried, filtered and concentrated to yield norhygrine (70) as an unstable oil (0.30 g, 55.6%), R_F 0.52 (*n*-BuOH/acetic acid, 4:1, saturated with water); ν_{max} ($CHCl_3$) 2940, 1700 and 1535 cm^{-1}; δ_H (90 MHz) 1.3-3.8 (10H, complex) and 2.20 (3H, s, CH_3-).

Preparation of 3,4-Dimethoxybenzoyl Chloride (73)[52]

3,4-Dimethoxybenzoic acid (74) (5 g, 0.027 mol) in thionyl chloride (5 ml) was heated at reflux overnight. The excess thionyl chloride was removed by concentration under reduced pressure leaving the acid chloride (73) in quantitative yield (The excess thionyl chloride was quenched with $NaHCO_3$). The acid

chloride was used immediately in the next stage; m.p. 73-75 °C; δ_H ($CDCl_3$) 4.00 (6H, 2s), 6.95 (1H, d, *J* 9 Hz), 7.55 (1H, br s) and 7.85 (1H, d with fine splitting, *J* 9 Hz).

Preparation of Ethyl 3,4-Dimethoxybenzoylacetoacetate (75)[52]

The acid chloride (73) (5.45 g, 0.0272 mol) in anhydrous THF (40 ml) was added dropwise with good mixing to a suspension of ethyl acetoacetate (10.66 g, 0.0815 mol) and sodium (1.64 g, 0.0713 mol) in anhydrous THF (75 ml) under a nitrogen atmosphere. The solution was heated at reflux for 5 h and left to stand overnight. The precipitate formed was filtered and washed with diethyl ether. The sodium salts were dissolved in cold water (1.5 l) and decomposed with 10% hydrochloric acid. The diketo ester separated as an oil which was extracted with diethyl ether. The combined organic extracts were dried, filtered and evaporated to dryness under reduced pressure to yield the product (75) (6.90 g, 86%), m.p. 79-80 °C; ν_{max} ($CHCl_3$) 2980, 1740, 1710, 1670, 1595, 1510, 1460, 1270, and 1150 cm^{-1}; δ_H ($CDCl_3$) 1.15 (3H, t), 2.40 (3H, s), 3.98 (6H, 2s), 4.17 (2H, q), 5.35 (1H, s), 6.92 (1H, d), 7.30 (1H, br s) and 7.50 p.p.m. (1H, d with fine splitting); *m/z* 295 (M^+ + 1), 294 (M^+), 248 and 165 (100 %).

Preparation of Ethyl 3,4-Dimethoxybenzoylacetate (76)[52]

Ethyl 3,4-dimethoxybenzoylacetoacetate (75) (6.89 g, 0.023 mol) was dissolved in aqueous ethanol (90%, 30 ml). To this solution sodium acetate (96 mg, 1.17 mmol) was added and the mixture was heated at 100 °C for 6 h. After a long period of

cooling the product (76) crystallised out of solution in quantitative yield; m.p. 44-45 °C; ν_{max} ($CHCl_3$) 3020, 2940, 2840, 1735, 1680, 1600, 1510, 1420, 1270, 1150 and 1025 cm^{-1}; δ_H ($CDCl_3$) 1.30 (3H, t, *J* 7 Hz), 4.00 (6H, s), 4.25 (2H, q, *J* 7 Hz), 6.90 (1H, d, *J* 9 Hz), 7.56 (1H, br s) and 7.65 (1H, d with fine splitting); *m/z* 253 (M^+ + 1), 252 (M^+), 165 (100 %) and 137.

Preparation of 3,4-Dimethoxybenzoylacetic Acid (72)[52]

This stage was not carried out until 2 d before the acid was required. The β-keto acid decarboxylates readily.

Ethyl 3,4-dimethoxybenzoylacetate (76) (2.02 g, 8.0 mmol) in 2.5% aqueous potassium hydroxide (140 ml) was stirred for 48 h at room temperature. The solution was then washed with diethyl ether (3 x 40 ml), acidified at 10 °C with 2M sulphuric acid and extracted with diethyl ether (5 x 50 ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated to dryness under reduced pressure at room temperature to yield 3,4-dimethoxybenzoylacetic acid (72) (1.57 g, 87%) which was stored at < 0 °C; m.p. 29 °C (decarb.); δ_H ($CDCl_3$) 3.92 (6H, 2s), 4.02 (2H, s), 6.95 (1H, d, *J* 9 Hz), 7.55 (1H, br s) and 7.65 (1H, d with fine splitting).

Preparation of Ruspolinone (71)

bis(3,4-Dihydro-2*H*-pyrrol-1-yl)di-iodozinc (66) (0.91 g, 1.98 mmol) in methanol (60 ml) and 1M phosphate buffer (pH 7, 5 ml) was added to a solution of 3,4-dimethoxybenzoylacetic acid

(72) (1.12 g, 5 mmol, 2.5 equivalents) in water (12 ml) and the pH was adjusted to 7 with 1M potassium hydroxide. The mixture was stirred at room temperature for 48 h then acidified with 2M hydrochloric acid to pH 2 and heated at reflux for 3 h. The cooled solution was washed with diethyl ether (3 x 50 ml), saturated with solid potassium carbonate and then extracted with chloroform (3 x 100 ml). The combined organic extracts were dried, filtered and evaporated to dryness to yield ruspolinone (0.91 g, 92%). The product (71) was purified (81%) by preparative TLC on silica gel and elution with $CHCl_3$-MeOH-conc. ammonia (85:14:1), R_F 0.23; ν_{max} (KBr disc) 3430, 2930, 1670, 1640, 1600, 1260, 1145 and 1020 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.41-2.10 (4H, complex, 3-and4-H_2), 2.96 (2H, m, 5-H_2), 3.21 (2H, d, *J* 6 Hz, 6-H_2), 3.5 (1H, br s, NH), 3.57-3.74 (1H, m, 2-H), 3.90 (3H, s), 3.92 (3H, s), 6.84 (1H, d, *J* 9 Hz, 12-H), 7.49 (1H, d, *J* 2 Hz, 9-H), 7.56 (1H, dd, *J* 9 and 2 Hz, 13-H); δ_C (50 MHz) ($CDCl_3$) 24.5 (t, C-4), 31.2 (t, C-3), 43.8 (t, C-6), 45.9 (t, C-5), 55.0 (d, C-2), 55.9 and 56.0 (2q, C-14 and -15), 110.0 (2d, C-9 and -12), 122.9 (d, C-13), 130.1 (s, C-8), 149.0 (s, C-10), 153.3 (s, C-11) and 198.0 (s, C-7); *m/z* 249 (M^+), 180, 165, 137, 77 and 70 (100%). (Found: M^+ 249.1356. $C_{14}H_{19}O_3N$ requires 249.1365). Spectroscopic data were identical to those reported.[50]

Attempted Formation of Complex with 1-Piperideine (4)

Using L-Lysine Monohydrochloride[53]

L-Lysine monohydrochloride (0.184 g, 1 mmol) was dissolved in water (20 ml) and freshly crystallised *N*-

bromosuccinimide (0.356 g, 2 mmol) was added to the solution. The mixture was rotated on a rotary evaporator under mild suction at 40 °C until colourless (ca. 15 min). The solution was basified with aqueous potassium carbonate (20% w/v, 4 ml) and then extracted with cold diethyl ether (3 x 10 ml). The combined organic extracts were washed with water (3 x 30 ml), dried, filtered and zinc iodide (0.482 g, 1.6 mmol) was added. The mixture was stirred at 0 °C for 30 min. No complex was formed.

The reaction was repeated in the absence of zinc iodide. The dried ethereal extracts were evaporated to dryness at room temperature leaving a clear oil (0.095 g). A small portion of the oil was dissolved in 2M hydrochloric acid. TLC (silica gel, *n*-BuOH/HOAc/water, 2:1:1) showed the presence of 1-piperideine (4) (R_F 0.50) as the major component, together with traces of unreacted L-lysine (51) (0.22); δ_H (D_2O) 7.5 (s), 6.9 (s), 6.3 (s), 3.5 (br m), 3.0 (m) and 2.6 (s, $[C\underline{H}_2CO]_2NH$, succinimide).

Preparation of α-Tripiperideine (79)[115]

Freshly distilled piperidine (5 ml, 50.6 mmol) was added by syringe to a stirred suspension of *N*- chlorosuccinimide (12.0 g, 89.9 mmol) in anhydrous diethyl ether (250 ml) at room temperature. Stirring was continued for 90 min. The mixture was then filtered directly into a dropping funnel and added dropwise over 1 h to a stirred solution of potassium hydroxide (3 g, 53.5 mmol) in ethanol (30 ml) at 70 °C. The mixture was stirred at 70 °C for 2 h then left to stand at room temperature overnight. The precipitate formed was filtered off and the filtrate was

evaporated to dryness under reduced pressure leaving a yellow solid (3.36 g, 80%). Recrystallisation from acetone yielded the product (79) as yellow crystals (3.0 g, 71%), m.p. 60 °C (lit.,[115] 59-61 °C); R_F 0.52; ν_{max} (KBr disc) 2930, 2850, 1455, 1240 and 1150 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.25-2.30 (12H, complex), 2.20 (6H, m), 2.87 (3H,m), 3.05 (3H, m) and 3.50 (3H, m); δ_C (50 MHz) [$(CD_3)_2CO$] 23.0 (t, C-4), 26.6 (t, C-5), 29.8 (t, C-3), 46.9 (t, C-6) and 82.6 (d, C-2); *m/z* 249 (M^+), 83, 68, 55 (100%) and 41. (Found: C, 72.12; H, 10.79; N, 16.76%. $C_{15}H_{27}N_3$ requires C, 72.29; H, 10.84; N, 16.87%.)

Using α-Tripiperideine (79)

Conc. hydrochloric acid (0.5 ml) was added slowly to α-tripiperideine (79) (0.5 g, 2 mmol) at -20 °C and then water (16.5 ml) was added. The solution was basified with conc. ammonia then extracted with cold diethyl ether (3 x 20 ml). The combined organic extracts were then dried, filtered, and zinc iodide (1.9 g, 6 mmol) was added and the solution was stirred at 0 °C for 30 min. No complex was formed.

Using Piperidine

A solution of piperidine (0.45 ml, 4.5 mmol) and triethylamine (1.89 ml, 13.6 mmol) in dry CH_2Cl_2 (7 ml) was cooled to 0 °C and *N*-bromosuccinimide (1.34 g, 7.5 mmol) was added with stirring. After 30 min triethylamine (5.65 ml, 40.7 mmol) was added. After 16 h in a slowly warming ice bath, the mixture was extracted with cold 1M hydrochloric acid (3 x 15 ml). Potassium iodide (0.5 g, 3.0 mmol) and sodium thiosulphate (1 g)

were added to the solution which was then basified with conc. ammonia and extracted with cold diethyl ether (3 x 50 ml). The combined organic extracts were dried, filtered and evaporated under reduced pressure leaving a mixture of products; δ_H ($CDCl_3$) no C<u>H</u>=N.

<u>Using Pipecolinic Acid</u>

Pipecolinic acid (0.258 g, 2 mmol) was stirred at 100 °C with phosphorus oxychloride (18 ml, 20 mmol) for 3 min. The solution was then cooled and water (20 ml) was added very slowly. The solution was then saturated with solid potassium carbonate and extracted with diethyl ether (3 x 20 ml). The combined organic extracts were dried, filtered and evaporated to dryness under reduced pressure leaving a yellow solid (0.028 g); δ_H ($CDCl_3$) no C<u>H</u>=N; all spectral properties were identical to α-tripiperideine.

<u>Preparation of Iodosobenzene (148)</u>[56]

3M Sodium hydroxide (50 ml) was added over 5 min with vigorous stirring to finely ground iodosobenzene diacetate (10.7 g, 0.033 mol) at room temperature. The solid formed was broken up using a stirring rod and then the mixture was left to stand for 45 min. Water (35 ml) was added and the mixture was stirred vigorously for 30 min. The crude solid was filtered off and triturated with water (65 ml). The solid was again filtered, washed well with water and then triturated with chloroform (25

ml). The product was then filtered off and dried leaving a yellow solid (148) (6.47 g, 88.5%); m.p. 210 °C (explosive) (lit.,[56] 210 °C).

Using Iodosobenzene (148) and Piperidine

Iodosobenzene (148) (0.83 g, 3.76 mmol), crushed molecular sieves and anhydrous dichloromethane (10 ml) were stirred together at in a round-bottomed flask equipped with reflux condenser and drying tube containing Drierite. Piperidine (0.43 ml, 3.76 mmol) was added and the mixture was stirred for 1 h at 0 °C. The mixture was then filtered and the filtrate was transferred to a flask containing zinc iodide (0.51 g, 1.6 mmol). The mixture was stirred at 0 °C for 30 min. No complex was formed. In the absence of zinc iodide the organic solution was evaporated under reduced pressure. δ_H (CDCl3) No C<u>H</u>=N.

Using Iodosobenzene (148) and Pipecolic acid

Iodosobenzene (148) (0.81 g, 3.68 mmol), crushed molecular sieves and anhydrous dichloromethane (10 ml) were stirred together at in a round-bottomed flask equipped with reflux condenser and drying tube containing Drierite. Pipecolinic acid (0.475 g, 3.68 mmol) was added and the mixture was stirred for 24 h at room temperature. The mixture was then filtered and the filtrate was transferred to a flask containing zinc iodide (1.18 g, 3.7 mmol). The mixture was stirred at 0 °C for 30 min. No complex was formed. In the absence of zinc iodide the organic solution was evaporated under reduced pressure. δ_H (CDCl3) No C<u>H</u>=N.

Preparation of Pelletierine (82)[57]

1-Piperideine (4) [from L-lysine monohydrochloride (0.046 g, 0.25 mmol) and *N*- bromosuccinimide (0.089 g, 0.5 mmol) in water (5 ml)] was added dropwise over 24 h to a solution of lithium acetoacetate (0.106 g, 1 mmol) in 1M phosphate buffer (pH 7, 2.5 ml) at room temperature under nitrogen. The dropping funnel was washed with water (0.5 ml) and the washings were allowed to drop into the reaction mixture. The mixture was then cooled to 5 °C, saturated with solid potassium carbonate and extracted with chloroform (3 x 10 ml). The combined organic extracts were dried, filtered and evaporated to dryness leaving a clear oil (0.032 g, 90%). The product (82) was purified (68%) by preparative TLC on silica gel, R_F 0.46; ν_{max} ($CHCl_3$) 3490, 2940, 1720, 1590, 1265, 1170 and 920 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.54-1.85 (6H, complex, 3-, 4- and 5-H_2), 2.14 (3H, s, 9-H_3), 2.70 (2H, m, 6-H_2), 2.79 (2H, d, *J* 6 Hz, 7-H_2), 3.12 (2H, m, 2-H and NH); δ_C (50 MHz) ($CDCl_3$) 23.6 (t, C-4), 24.3 (t, C-5), 30.5 (q, C-9), 30.6 (t, C-3), 46.0 (t, C-7), 48.6 (t, C-6) and 56.2 (d, C-2); *m/z* 141 (M^+), 126, 98, 84, 56, 43 and 28 (100%).

Preparation of 3', 4'-Dimethoxy-2'-(2-piperidyl)acetophenone (31)

1-Piperideine (4) [from L-lysine monohydrochloride (0.092 g, 0.5 mmol) and *N*-bromosuccinimide (0.18 g, 1 mmol) in water (10 ml)] was added dropwise over 24 h to a solution of 3,4-dimethoxybenzoylacetic acid (72) (0.22 g, 1 mmol) in 1M phosphate buffer (pH 7, 6 ml) at room temperature under nitrogen. The dropping funnel was washed with water (1 ml) and the washings were added to the reaction mixture. The mixture was then cooled to 5 °C, basified with cold 10% potassium hydroxide and extracted with chloroform (3 x 10 ml). The combined organic extracts were dried, filtered and evaporated to dryness leaving a yellow oil (0.141 g). The product (31) was purified (70%) by preparative TLC on silica gel and elution with $CHCl_3$-MeOH-conc. ammonia (85:14:1), R_F 0.49; ν_{max} (CHCl3) 3440, 2940, 1675, 1635, 1600, 1265, 1150 and 1020 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.25-1.82 (6H, complex, 3-, 4- and 5-H_2), 2.72 (2H, m, 6-H_2), 2.98 (2H, d, *J* 6 Hz, 7-H_2), 3.5 (1H, br s, NH), 3.10 (1H, m, 2-H), 3.91 (3H, s), 3.92 (3H, s), 6.85 (1H, d, *J* 9 Hz, 12-H), 7.49 (1H, d, *J* 2 Hz, 9-H), 7.59 (1H, dd, *J* 9 and 2 Hz, 13-H); δ_C (50 MHz) ($CDCl_3$) 25.9 (t, C-4), 29.7 (t, C-5), 32.8 (t, C-3), 45.0 (t, C-7), 46.6 (t, C-6), 53.2 (d, C-2), 55.9 and 56.0 (2q, C-15 and -16), 110.0 (d, C-10 and -13), 122.9 (d, C-14), 130.3 (s, C-9), 149.0 (s, C-11), 153.4 (s, C-12) and 198.0 (s, C-8); *m/z* 264 (M^++ 1), 263 (M^+), 180, 165 (100%), 137, 84 and 55. (Found: M^+ 263.1515. $C_{15}H_{21}O_3N$ requires 263.1521.). Spectroscopic data are in accordance with literature values.[15]

Preparation of 2,4-Pentadienoic Acid (87)[59]

Acrolein (10 ml, 0.15 mol) was added dropwise over 15 min to malonic acid (25 g, 0.24 mol) in pyridine (35 ml) at 80 °C. After 15 min stirring another portion of acrolein (10 ml, 0.15 mol) was added. The solution was then cooled and quenched by adding to ice (150 g), rinsing the reaction flask with CH_2Cl_2 (50 ml). The mixture was acidified with conc. sulphuric acid (17.5 ml) at 0 °C. The layers were then separated and the aqueous layer was extracted with more CH_2Cl_2 (3 x 50 ml). The combined organic extracts were dried, filtered and evaporated under reduced pressure. The residue was washed with ice/water (10 ml), filtered, dissolved in water (70 ml) containing hydroquinone (0.15 g) at 75 °C, cooled, filtered and dried *in vacuo*. The product (87) was obtained as white crystals (7.7 g, 53%); m.p. 71-72 °C (lit.[59], 71.5-72.5 °C); R_F 0.57 [EtOAc-HOAc, (9:1)]; ν_{max} (KBr disc) 3100-2400, 2930, 1690, 1640, 1600, 1445, 1380, 1240, 1105, 1115, 970, 960 and 800 cm^{-1}; δ_H (200 MHz) 5.47-5.72 (2H, ABCDD' system, J_{5A} 10.02, 1.65, 0.82 and 0.71, J_{5B} 16.98, 1.65, 0.81, 0.76, 5A and 5B-H), 5.92-6.00 (H, ABCDD' system, J 15.40, 0.81, 0.72 and 0.71, 2-H), 6.47-6.61 (H, ABCDD' system, J 16.98, 10.88, 10.02 and 0.72, 4-H) and 7.19-7.32 (H, ABCDD' system, J 15.40, 10.88, 0.82 and 0.76, 3-H); ($CDCl_3$) δ_C (50 MHz) ($CDCl_3$) 123.13 (d, C-2), 125.93 (t, C-5), 135.81 (d, C-4), 145.80 (d, C-3) and 168.07 (s, C-1); *m/z* 99 (M^++ 1), 98 (M^+), 97, 81, 70, 69, 55, 53, 52, 51, 50, 42, 41, 28, 27 (100%) and 18.

Preparation of Methyl 2,4-Pentadienoate (78)[59]

2,4-Pentadienoic acid (87) (4.0 g, 0.041 mol) partially dissolved in ether (150 ml) was treated with an excess of diazomethane at 0 °C. Nitrogen gas was bubbled gently through the solution for 2 h and then aqueous acetic acid (5% v/v, 10 ml) was added. The organic layer was separated, washed with aqueous sodium bicarbonate (5% w/v, 100 ml) and water (100 ml), dried ($MgSO_4$), filtered and evaporated under reduced pressure to leave a pale yellow oil which was purified by distillation. The product (78) was obtained as a clear oil (2.0 g, 44%); b.p. 76 °C (20 mmHg); R_F 0.56 (Et_2O); ν_{max} ($CHCl_3$) 3010, 2950, 1715, 1645, 1600, 1440, 1275, 1150, 1115, 965, 930 and 870 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 3.69 (3H, s, $COOC\underline{H}_3$), 5.49-5.73 (2H, ABCDD' system, J_{5A} 10.02, 1.65, 0.82 and 0.71, J_{5B} 16.98, 1.65, 0.81, 0.76, 5A and 5B-H), 5.94-6.02 (H, ABCDD' system, J 15.40, 0.81, 0.72 and 0.71, 2-H), 6.45-6.62 (H, ABCDD' system, J 16.98, 10.88, 10.02 and 0.72, 4-H) and 7.18-7.31 (H, ABCDD' system, J 15.40, 10.88, 0.82 and 0.76, 3-H); δ_C (50 MHz) ($CDCl_3$) 51.62 (q, $-CH_3$), 122.70 (d, C-2), 126.08 (t, C-5), 135.77 (d, C-4), 145.44 (d, C-3) and 167.25 (s, C-1); *m/z* 113 (M^++ 1), 112 (M^+), 111, 97, 81, 79, 67, 55.5, 54.5, 53.5 (100%), 52, 51, 50, 43, 41, 28 and 27.

Preparation of Methyl 1,2-Didehydro-quinolizidine-1-carboxylate (84)[58]

α-Tripiperideine (79) (0.60 g, 2.4 mmol), potassium hydrogen sulphate (152 mg, 1.1 mmol) and methyl 2,4-

pentadienoate (78) (1.5 g, 13.3 mmol) were heated together in a sealed tube at 150 ^{o}C for 24 h. After cooling the mixture was extracted with 2M hydrochloric acid (5 x 10 ml). The combined aqueous extracts were washed with diethyl ether (3 x 50 ml), basified with conc. ammonia and then extracted with dichloromethane (3 x 50 ml). The combined organic extracts were dried, filtered and evaporated under reduced pressure to give a brown oil which was purified by column chromatography on basic alumina and elution with ethyl acetate-methanol (95:1). The product (84) was obtained as a pale yellow oil (0.37 g, 26%); R_F 0.59; ν_{max} ($CHCl_3$) 2940, 2810, 2760, 1710, 1645, 1445, 1270 and 980 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.38-1.85 (4H, complex), 2.30-2.55 (4H, complex), 2.77-3.08 (4H, complex), 3.70 (3H, s), 4.10 (1H, m) and 6.85 (1H, m); δ_C (50 MHz) ($CDCl_3$) 24.48 (t, C-8), 25.01 (t, C-7), 26.48 (t, C-9), 28.68 (t, C-3), 48.85 (t, C-6), 51.37 (q, C-12), 55.98 (t, C-4), 60.29 (d, C-10), 133.71 (s, C-1), 137.24 (d, C-2) and 166.95 (s, C-11); *m/z* 196 (M^++ 1), 195 (M^+), 180, 166, 164, 136 (100%), 106, 80, 53, 41, 28 and 18. (Found: M^+ 195.1260. $C_{11}H_{17}O_2N$ requires 195.1259.)

Preparation of Methyl Quinolizidine-1-carboxylate (89)[58]

Methyl 1,2-didehydro-quinolizidine-1-carboxylate (84) (0.11g, 0.56 mmol) was added to a suspension of Adams' catalyst (10% w/w) in methanol (10 ml) and was hydrogenated at 1 atmosphere for 12 h. The catalyst was filtered off through Florisil and the Florisil was washed well with methanol. Evaporation of the solvent under reduced pressure gave the product (89) (0.093 g, 84%) which was purified (55%) by preparative TLC on silica gel

and elution with $CHCl_3$-MeOH-conc. ammonia (85:14:1), R_F 0.62; ν_{max} ($CHCl_3$) 2950, 1730, 1620, 1435, 1270 and 1170 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.38-1.65 (5H, complex), 1.70-2.20 (8H, complex), 2.58 (1H, m), 2.94 (2H, m) and 3.66 (3H, s); δ_C (50 MHz) ($CDCl_3$) 22.13 (t, C-3), 24.49 (t, C-8), 24.92 (t, C-7), 26.30 (t, C-9), 29.69 (t, C-2), 44.54 (d, C-1), 51.25 (q, C-12), 55.04 (t, C-6), 57.16 (t, C-4), 62.74 (d, C-10) and 174.01 (s, C-11); *m/z* 197 (M^+), 182, 166, 164, 136, 110, 106, 97, 83 (100%), 53, 41, 28 and 18. (Found: M^+ 197.1410. $C_{11}H_{19}O_2N$ requires 197.1416.)

Preparation of Lupinine (21)

Methyl quinolizidine-1-carboxylate (89) (0.06 g, 0.30 mmol) in anhydrous diethyl ether (2 ml) was added dropwise to a stirred suspension of lithium aluminium hydride (0.40 g, 0.01 mol) in anhydrous ether (4 ml) at room temperature under nitrogen. The mixture was stirred for 18 h then water (2.5 ml) was added at 0 °C and after 10 min potassium hydroxide (10% w/v, 2.5 ml) was added. The mixture was stirred for a further 30 min then extracted with CH_2Cl_2 (3 x 10 ml). The combined organic extracts were dried, filtered and concentrated to leave the product (21) as a pale yellow oil (0.04 g, 78%) which failed to solidify; R_F 0.57; ν_{max} ($CHCl_3$) 3250, 2930, 1600, 1490 and 1145 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.25 (2H, m), 1.55 (6H, m), 1.80 (3H, m), 2.09 (2H, m), 2.81 (2H, m), 3.68 (2H, d, *J* 10 Hz, 11-H_2), 4.13 (1H, m, 10-H) and 5.15 (1H, br s, -O<u>H</u>); δ_C (50 MHz) ($CDCl_3$) 22.93 (t, C-3), 24.60 (t, C-8), 25.59 (t, C-7), 29.88 (t, C-9), 31.48 (t, C-2), 38.07 (d, C-1), 57.04 and 57.14 (2t, C-4 and -6), 65.08 (d, C-10) and 66.04 (t, C-11); *m/z* 170 (M^++ 1), 169 (M^+), 168, 152, 138, 110, 98, 97,

96, 83, 82, 55 and 41 (100%). (Found: M^+ 169.1463. $C_{10}H_{19}ON$ requires 169.1466.) The sample of lupinine (21) gave similar spectroscopic and chromatographic data to an authentic sample of lupinine and to literature values.[116]

Preparation of Methyl 2,4-Hexadienoate (77)

2,4-Hexadienoic acid (11.2 g, 0.1 mol) in methanol (250 ml) was stirred at 0 °C. Thionyl chloride (9 ml, 0.12 mol) was added and the solution was then allowed to warm to room temperature overnight. The solvent was then removed under reduced pressure leaving the product (77) as a pale yellow liquid which was purified by distillation (8.6 g, 68%); b.p. 160 °C (20 mm Hg); R_F 0.63 ($CHCl_3$); ν_{max} ($CHCl_3$) 3020, 2950, 1710, 1645, 1435, 1330, 1275, 1250, 1145, 1000 and 870 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.75 (3H, d with fine splitting, *J* 5Hz, 6-H_3), 3.64 (3H, s), 5.70 (1H, d with fine splitting, *J* 15 Hz, 5-H), 5.98-6.20 (2H, complex, 2- and 4-H), 7.12-7.25 (1H, complex, 3-H); δ_C (50 MHz) ($CDCl_3$) 18.4 (q, C-6), 51.3 (q, -CO$\underline{C}H_3$), 118.4 (d, C-2), 129.6 (d, C-5), 139.2 (d, C-4), 145.0 (d, C-3) and 192.2 (s, C-1); *m/z* 127 (M^++ 1), 126 (M^+), 111, 95, 67 (100%), 66, 65, 51, 40, 39, 38, 31 and 29.

Preparation of 1,5-bis(*N*- Piperidyl)-3-pentanone Dihydrochloride (91)[13]

Piperidine hydrochloride (3.05 g, 25 mmol) and paraformaldehyde (0.75 g, 25 mmol) in glacial acetic acid (5 ml) were stirred at 98 °C. Acetone (0.92 ml, 12.5 mmol) was added and the mixture was heated at reflux for 3 h. The solvent was

then removed under reduced pressure and the residue was triturated with hot acetone (6 ml). The white solid formed was filtered off then triturated with hot chloroform (5 ml) and filtered again to leave the product (0.98 g, 24%). Evaporation of the mother liquors and trituration of the residue gave a further yield of product (0.12 g). Recrystallisation from methanol gave the product (91) as white crystals (0.10 g, 25%); m.p. 216-219 °C (lit.,[13] 217-219 °C); R_F 0.63 [ethyl acetate-*i*-propanol-ammonia, (9:7:4)]; ν_{max} (KBr disc) 2950, 2640, 1725, 1465, 1395, 1230, 1105 and 1070 cm^{-1}; δ_H (200 MHz) (D_2O) 1.27 (4H, m), 1.37-1.78 (8H, complex), 2.72 (4H, m), 2.91 (4H, t, 2- and 4-H_2), 3.15 (4H, t, 1- and 5-H_2) and 3.29 (4H, m); δ_C (50 MHz) (D_2O) 21.60 (t, C-9 and -15), 23.57 (t, C-8, -10, -14 and -16), 37.06 (t, C-2 and -4), 51.77 (t, C-1 and -5), 54.38 (t, C-7, -11, -13 and -17) and 206.90 (s, C-3); *m/z* 168, 167, 154, 136, 98 (100%), 85, 84, 70, 56, 55, 42, 41 and 28.

Preparation of 8-Ketosparteine (93)[13]

Freshly crystallised mercuric acetate (4.4 g, 13.9 mmol) was dissolved in aqueous acetic acid (5% v/v, 15 ml) at 95 °C. 1,5-bis(*N*-Piperidyl)3-pentanone dihydrochloride (91) (0.50 g, 1.5 mmol) was added and the mixture was heated at 95 °C for 2 h. A white precipitate was formed after 5 min. The mixture was then cooled, filtered and the precipitate was washed well with aqueous acetic acid. Hydrogen sulphide gas was bubbled through the filtrate until the solution was saturated and the mixture was then filtered through Celite. This process was repeated until the solution no longer changed colour on treatment with hydrogen

sulphide. The filtrate was then saturated with potassium carbonate and extracted with diethyl ether (3 x 50 ml). The combined organic extracts were dried, filtered and evaporated under reduced pressure to leave a yellow oil (0.11 g, 29%) which was found to be a mixture of seven products. The mixture was purified by column chromatography on basic alumina and elution with toluene-diethyl ether (5:1). 8-Ketosparteine (93) was obtained as a pale yellow semi-solid (0.020 g, 5%) which failed to solidify; R_F 0.62; ν_{max} ($CHCl_3$) 3040, 2960, 1725, 1270, 1230 and 1130 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.05-1.67 (12H, complex), 1.95-2.10 (4H, complex), 2.31 (1H, dd, *J* 3 and 11 Hz), 2.45 (1H, m), 2.63-2.98 (5H, complex) and 3.15 (1H, dd, *J* 5 and 10 Hz); δ_C (50 MHz) ($CDCl_3$) 23.28 and 23.58 (2t, C-4 and -13), 25.37 and 25.48 (2t, C-3 and -14), 29.83 (t, C-5), 34.88 (t, C-12), 51.86 (d, C-9), 54.35 (t, C-17), 54.59 (d, C-7), 55.10 (t, C-15), 55.92 (t, C-2), 62.13 (t, C-10), 66.63 and 66.75 (2d, C-6 and -11) and 213.62 (s, C-8); *m/z* 249 (M^++ 1), 248 (M^+), 193, 150, 137, 110, 98 (100%), 84, 55, 41 and 28. (Found: M^+248.1879. $C_{15}H_{24}ON_2$ requires 248.1888.)

Preparation of 1,5-bis(*N*- Hexahydroazepinyl)-3-pentanone Dihydrochloride (94)

This was prepared by the same method as 1,5-bis(*N*-piperidyl)-3-pentanone dihydrochloride (91). The product (94) was obtained as a white solid (2.93 g, 66%), which was recrystallised from methanol to give colourless crystals (0.96 g, 22%); m.p 144-146 °C; R_F 0.71 (ethyl acetate-*i*-propanol-ammonia, 9:7:4); ν_{max} (KBr disc) 2940, 2700, 1715, 1455, 1405, 1190, 1170 and 1040 cm^{-1}; δ_H (200 MHz) (D_2O) 1.64 (8H, m), 1.82 (8H, m),

3.05 (4H, t, 2- and 4-H_2), 3.17 (4H, m), 3.34 (4H, t, 1- and 5-H_2) and 3.40 (4H, m); δ_C (50 MHz) (D_2O) 23.94 (t, C-9, -10, -16 and -17), 26.77 (t, C-8, -11, -15 and -18), 38.09 (t, C-2 and -4), 52.33 (t, C-1 and -5), 55.74 (t, C-7, -12, -14 and -19) and 211.05 (s, C-3); *m/z* 169, 168, 140, 126, 112 (100%), 98, 84, 70, 58, 55, 42, 41 and 28.

Attempted Oxidation of 1,5-bis(*N*-Hexahydroazepinyl)-3-pentanone Dihydrochloride (94)

1,5-bis(*N*-hexahydroazepinyl)-3-pentanone dihydrochloride (94) (0.342 g, 0.98 mmol) was oxidised with mercuric acetate (2.82 g, 8.85 mmol) by the same method as 1,5-bis(*N*-piperidyl)-3-pentanone. Evaporation of the organic extracts gave a yellow viscous gum (0.101 g) which was found to be a mixture of 6 products. Column chromatography on basic alumina failed to isolate the desired product.

Attempted Preparation of 1,5-bis(*N*-Pyrrolidyl)-3-pentanone Dihydrochloride

Pyrrolidine hydrochloride (0.54 g, 5 mmol) and paraformaldehyde (0.15 g, 5 mmol) in glacial acetic acid (1.5 ml) were stirred at 98 °C. Acetone (0.2 ml, 2.7 mmol) was added and the mixture was heated at reflux for 3 h. The solvent was then removed under reduced pressure and the residue was triturated with diethyl ether (1.5 ml). The solvent was decanted off and the residue was triturated with chloroform (1 ml). The solvent was decanted off and the hydroscopic residue was found to be a

mixture of products which could not be purified by trituration or recrystallisation.

5.3 Experimental to Chapter 4

Spectrophotometric Assay[105]

The kinetic data of inhibition studies on diamine oxidase catalysed reactions were determined by the procedure of Stoner.* This involved a peroxidase coupled assay (horseradish peroxidase EC 1.11.1.7, from Sigma) to monitor the production of hydrogen peroxide from the oxidation reaction at 25 °C, 70 mM phosphate buffer (pH 6.3), and in the presence of 3-(dimethylamino)benzoic acid (DMAB) (113) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) (112). This assay generates stoichiometric quantites of an indamine dye (114) with an absorbance maximum at 595 nm. The rates were measured directly in the spectrophotometer.

The solutions for the assay were prepared as follows;

Phosphate buffer	70 mM (sodium dihydrogen phosphate 5.46 g/500 ml distilled water adjusted to pH 6.3 with disodium hydrogen phosphate 4.97 g/500 ml distilled water)
MBTH (112)	0.6 mM (12.9 mg/100 ml distilled water)
DMAB (113)	18 mM (59.4 mg/20 ml phosphate buffer pH 6.3)

Peroxidase	1.36 mg/4 ml phosphate buffer pH 6.3
Pea seedling DAO	20/980µl or 40/960µl phosphate buffer pH 6.3 (ie. 1/50 or 1/25 dilute)

The reaction was initiated by addition of the enzyme then inhibitor to the thermally equilibrated reaction medium, followed immediately by the substrate. The substrate was added last to minimise the possibility of extensive preincubation of the enzyme with the chromogenic agents producing additional inhibiting effects. The initial rates were measured from the linear absorbance changes during the first thirty seconds of reaction for a range of substrate concentrations. A separate experiment was carried out for each inhibitor concentration with every substrate concentration. A control experiment with no inhibitor present was also carried out before testing as kinetic data could vary slightly from day to day. Michaelis-Menten behaviour was observed in most cases. Rate data were analysed for K_M and V_{max} by least squares fitting of Lineweaver-Burk (1/V vs 1/[S]) and Eadie-Hofstee (V vs V/[S]) plots. [See Appendix 1]

General Procedure for *N*-Alkylations[106]

A solution of 4-chlorobutyronitrile or 5-chlorovaleronitrile in dry *n*-butanol (1 ml per 2 mmol of chloronitrile) was added to a mixture of pyrrolidine, piperidine or hexahydroazepine (1.1 equivalents), anhydrous sodium carbonate (1 equivalent) and a catalytic amount of potassium iodide (0.17 equivalent), and was stirred at 105 °C for 18 h. Each solution was cooled and filtered through Celite. The filtered solid was washed well with diethyl ether, and the combined filtrate and washings were extracted with 4M hydrochloric acid (3 x 50 ml). The combined aqueous extracts were washed well with diethyl ether (2 x 100 ml), basified with solid sodium carbonate and extracted with diethyl ether (3 x 100 ml). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give an oil which was purified by distillation.

N-(3-Cyanopropyl)pyrrolidine (149)

This was obtained as a clear oil (1.76 g, 60%); b.p. 40 °C (0.02 mm Hg); ν_{max} (thin film) 2960 and 2240 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.79 (6H, m) and 2.50 (8H, m); δ_C (50 MHz) ($CDCl_3$) 15.07 (t, C-7), 23.50 (t, C-3 and -4), 24.79 (t, C-8), 54.02 (t, C-2 and -5), 54.45 (t, C-6) and 119.8 (t, C-9); *m/z* 138 (M^+), 137, 98, 84, 55, 41 and 28 (100%). (Found: M^+ 138.1155. $C_8H_{14}N_2$ requires 138.1156.)

N-(3-Cyanopropyl)piperidine (151)

This was obtained as a clear oil (2.33 g, 64%); b.p. 30 °C (0.02 mm Hg); ν_{max} (thin film) 2930 and 2240 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.30-1.92 (8H, complex) and 2.33-2.44 (8H, m); δ_C (50 MHz) ($CDCl_3$) 14.94 (t, C-8), 22.82 and 24.30 (2t, C-4 and -9), 25.88 (t, C-3 and -5), 54.46 (t, C-2 and -6), 57.11 (t, C-7) and 119.7 (t, C-10); *m/z* 152 (M^+), 98 (100%), 84, 55, 41 and 28. (Found: M^+ 152.1313. $C_9H_{16}N_2$ requires 152.1313.)

N-(3-Cyanopropyl)hexahydroazepine (153)

This was obtained as a clear oil (3.45 g, 87%); b.p. 55 °C (0.04 mm Hg); ν_{max} ($CHCl_3$) 2930 and 2240 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.48-1.65 (8H, complex), 1.74 (2H, tt, 9-H_2), 2.40 (2H, t, *J* 7.5 Hz, 10-H_2), 2.54 (2H, t, *J* 7.5 Hz, 8-H_2) and 2.50-2.63 (4H,m, 2- and 7-H_2); δ_C (50 MHz) ($CDCl_3$) 14.84 (t, C-9), 23.80 (t, C-10), 26.91 (t, C-4 and -5), 28.29 (t, C-3 and -6), 55.30 (t, C-2 and -7), 55.92 (t, C-8) and 120.04 (t, C-11); *m/z* 166 (M^+), 154, 141, 126, 112 (100%), 98, 84, 55 and 41. (Found: M^+ 166. $C_{10}H_{18}N_2$ requires 166.)

N-(4-Cyanobutyl)pyrrolidine (150)

This was obtained as a clear oil (4.25 g, 58%); b.p. 41 °C (0.18 mm Hg); ν_{max} (thin film) 2960 and 2240 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) ; δ_C (50 MHz) ($CDCl_3$) 15.07 (t, C-7), 23.50 (t, C-3 and -4), 24.79 (t, C-8), 54.02 (t, C-2 and -5), 54.45 (t, C-6) and 119.8 (t, C-

9); *m/z* 152 (M^+), 110, 84 (100%), 70, 55, 41 and 28. (Found: M^+ 152.1315. $C_9H_{16}N_2$ requires 152.1313.)

N-(4-Cyanobutyl)piperidine (152)

This was obtained as a clear oil (2.47 g, 62%); b.p. 45 °C (0.06 mm Hg); ν_{max} (thin film) 2930 and 2240 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.39-1.75 (10H, complex) and 2.35 (8H,m); δ_C (50 MHz) ($CDCl_3$) 16.25 (t, C-9), 22.87, 23.68 and 24.98 (3t, C-4, -8 and -10), 25.62 (t, C-3 and -5), 54.04 (t, C-2 and -6), 57.41 (t, C-7) and 119.5 (t, C-11); *m/z* 166 (M^+), 98 (100%), 84, 55, 41 and 28. (Found: M^+ 166.1460. $C_{10}H_{18}N_2$ requires 166.1469.)

N-(4-Cyanobutyl)hexahydroazepine (154)

This was obtained as a clear oil (3.69 g, 85%); b.p. 60 °C (0.04 mm Hg); ν_{max} ($CHCl_3$) 2940 and 2260 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.48-1.75 (12H, complex), 1.74 (2H, tt, 9-H_2), 2.35 (2H, t, *J* 7.5 Hz, 11-H_2), 2.45 (2H, t, *J* 7.5 Hz, 8-H_2) and 2.51-2.63 (4H,m, 2- and 7-H_2); δ_C (50 MHz) ($CDCl_3$) 16.99 (t, C-10), 23.37 (t, C-9), 26.51 (t, C-11), 26.90 (t, C-4 and -5), 28.08 (t, C-3 and -6), 55.43 (t, C-2 and -7), 56.96 (t, C-8) and 119.80 (t, C-12); *m/z* 180 (M^+), 112 (100%), 98, 84, 55 and 41. (Found: M^+ 180.1622. $C_{11}H_{20}N_2$ requires 180.1626.)

General Procedure for Nitrile Reductions[106]

The *N*-alkylnitriles (149)-(154) were added to a suspension of Adams' catalyst (15% w/w) in glacial acetic acid (5 ml per mmol of nitrile) and were hydrogenated at 1 atmosphere for 18 h. The catalyst was filtered off through Florisil and the Florisil was washed well with glacial acetic acid (25 ml). Conc. hydrochloric acid (10 ml) was added to the filtrates. Evaporation of the solvent under reduced pressure gave a solid which was recrystallised from 95% aqueous ethanol-acetone (1:1).

N-(4-Aminobutyl)pyrrolidinium Dihydrochloride (115)

This was obtained as a white powder (1.52 g, 75%); m.p. 206-208 °C (decomp.) (from 95% aq. EtOH-acetone, 1:1); ν_{max} (KBr disc) 3130, 2980 and 1400 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.63 (4H, m, 7- and 8-H_2), 1.90 (4H, m, 3- and 4-H_2), 2.94 (4H, m, 2- and 5-H_2), 3.09 (2H, m, 9-H_2) and 3.52 (2H, m, 6-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 21.46, 23.47 and 23.65 (3t, C-3, -4, -7 and-8), 47.70 (t, C-9) and 54.95 (t, C-2, -5 and-6); *m/z* 142 (M^+), 126, 84 (100%), 55, 41 and 28.

N-(4-Aminobutyl)piperidinium dihydrochloride (117)

This was obtained as a white powder (1.52 g, 67%); m.p. 282-284 °C (decomp.) (from 95% aq. EtOH-acetone, 1:1); ν_{max} (KBr disc) 3130, 2970 and 1405 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.67 (10H, m, 3-, 4-, 5-, 8- and 9-H_2),

2.81 (2H, m, 10-H_2), 2.99 (4H, m, 2- and 6-H_2) and 3.37 (2H, m, 7-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 21.69, 22.02 and 23.74 (3t, C-3, -4, -5, -8 and -9), 47.75 (t, C-10), 54.11 (t, C-2 and -6) and 56.87 (t, C-7); *m/z* 156 (M^+), 126, 98 (100%), 84, 55, 41, 36 and 28.

N-(4-Aminobutyl)hexahydroazepinium Dihydrochloride (119)

This was obtained as a white powder (2.09 g, 86%); m.p. 230-232 °C (decomp.) (from 95% aq. EtOH-acetone, 1:1); ν_{max} (KBr disc) 3130, 2960 and 1400 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.17-1.69 (12H, complex, 3-, 4-, 5-, 6-, 9- and 10-H_2), 2.79-3.05 (6H, complex, 2-, 7-, 11-H_2) and 3.27 (2H, m, 8-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 23.82 (t, C-10), 24.26 (t, C-4 and -5), 25.94 (t, C-9), 26.67 (t, C-3 and -6), 48.07 (t, C-11), 55.53 (t, C-2 and -7) and 57.78 (t, C-8); *m/z* 170 (M^+), 112 (100%), 98, 84, 55, 41 and 36.

N-(5-Aminopentyl)pyrrolidinium Dihydrochloride (116)

This was obtained as a white solid (3.91 g, 85%); m.p. 195-197 °C (decomp.) (from 95% aq. EtOH-acetone, 1:1); ν_{max} (KBr disc) 3130, 2950, 1620 and 1455 cm^{-1}; δ_H (200 MHz) (D_2O) 1.12 (2H, m, 8-H_2), 1.44 (4H, m, 7- and 9-H_2), 1.59-1.83 (4H, m, 3- and 4-H_2), 2.63-2.76 (4H, complex, 2- and 5-H_2), 2.83 (2H, m, 10-H_2) and 3.31 (2H, m, 6-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 23.50, 23.67, 25.78 and 27.12 (4t, C-3, -4, -7, -8 and -9), 48.09 (t, C-10), 54.90 (t, C-2 and -5) and 55.47 (t, C-6).

N-(5-Aminopentyl)piperidinium Dihydrochloride (118)

This was obtained as a white solid (3.06 g, 96%); m.p. 192-193 °C (decomp.) (from 95% aq. EtOH-acetone, 1:1); ν_{max} (KBr disc) 3130, 2945 and 1410 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.32 (4H, m, 9- and 10-H_2), 1.65 (8H, m, 3-, 4-, 5- and 8-H_2), 2.80 (4H, m, 2- and 6-H_2), 2.93 (2H, m, 11-H_2) and 3.40 (2H, m, 7-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 21.29, 23.00, 23.14 and 25.17 (t, C-3, -4, -5, -8, -9 and -10), 47.32 (t, C-11), 53.34 (t, C-2 and -6) and 56.65 (t, C-7); *m/z* 170 (M^+), 140, 124, 98 (100%), 84, 70, 55, 41 and 36.

N-(5-Aminopentyl)hexahydroazepinium Dihydrochloride (120)

This was obtained as a white powder (2.11 g, 82%); m.p. 214-217 °C (decomp.) (from 95% aq. EtOH-acetone, 1:1); ν_{max} (KBr disc) 2940 and 1470 cm^{-1}; δ_H (200 MHz) (D_2O) 1.46-1.75 (14H, complex, 3-, 4-, 5-, 6-, 9-, 10- and 11-H_2), 2.70-3.05 (6H, complex, 2-, 7- and 12-H_2) and 3.20-3.32 (2H, complex, 8-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 22.02 (t, C-4 and -5), 23.70 (t, C-10), 24.26 (t, C-3 and -6), 26.65 (t, C-9 and -11), 47.70 (t, C-12), 55.59 (t, C-2 and -7) and 57.26 (t, C-8); *m/z* 184 (M^+), 112 (100%), 98, 84, 55, 41 and 36.

General Procedure for Mercury (II) Oxidations[106]

Mercury (II) acetate (4 equivalents) was stirred in 5% aqueous acetic acid (6 ml/mmol of tertiary amine) at 120 °C until completely dissolved (ca. 2 min). To the solution was added the diamine dihydrochloride (1 equivalent) and the mixture was heated at 120 °C for 4 h. The reaction mixture was then cooled and filtered to remove precipitated mercury (I) salts. The filtrate was saturated with hydrogen sulphide gas, filtered through Celite, and the process was repeated. This removed mercury (II) salts. The aqueous solution was basified with 40% aqueous sodium hydroxide and was then extracted with chloroform (3 x 40 ml). The combined organic extracts were dried and filtered. Addition of dry hydrogen chloride gas precipitated the iminium salt. The solvent was decanted off and the residue was dried *in vacuo*. In each case a viscous yellow gum was obtained which did not solidify.

N-(4-Aminobutyl)-1,2-didehydropyrrolidinium Chloride Hydrochloride (54)

This was obtained as an oil 0.11 g, 46%; ν_{max} (nujol mull) 2920, 2080 and 1625 cm^{-1}; δ_H (200 MHz) (D_2O) 1.69-2.08 (6H, complex, 4-, 7- and 8-H_2), 2.26 (2H, m, 3-H_2), 2.90-3.20 (4H, complex, 5- and 9-H_2), 3.95 (2H, m, 6-H_2) and 8.65 (1H, br s, 2-H); δ_C (50 MHz) (D_2O) 19.3, 23.9 and 24.5 (3t, C-4, -7 and -8), 37.8 (t, C-3), 47.3 (t, C-9), 52.9 and 58,1 (2t, C-5 and -6) and 169.0 (s, C-2).

N-(4-Aminobutyl)-1,2-didehydropiperidinium Chloride Hydrochloride (122)

This was obtained as an oil 0.24 g, 70%; ν_{max} (nujol mull) 2910, 2020 and 1640 cm^{-1}; δ_H (200 MHz) (D_2O) 1.41-1.85 (8H, complex, 4-, 5-, 8- and 9-H_2), 2.35 (2H, m, 3-H_2), 2.70-3.03 (4H, complex, 6- and 10-H_2), 3.58-3.85 (2H, complex, 7-H_2) and 8.50 (1H, br s, 2-H); δ_C (50 MHz) (D_2O) 20.48, 23.66 and 25.04 (3t, C-4, -5, -8 and -9), 39.59 (t, C-3), 47.70 (t, C-10), 50.80 and 54.87 (2t, C-6 and -7) and 170.03 (s, C-2).

N-(4-Aminobutyl)-1,2-didehydrohexahydroazepinium Chloride Hydrochloride

Addition of hydrogen chloride gas produced only starting material in 35% yield; δ_H (90 MHz) (D_2O) no C<u>H</u>=N present.

N-(5-Aminopentyl)-1,2-didehydropyrrolidinium Chloride Hydrochloride (121)

This was obtained as an oil 0.35 g, 58%; ν_{max} (nujol mull) 2950, 2150 and 1640 cm^{-1}; δ_H (200 MHz) (D_2O) 1.21 (2H, m, 8-H_2), 1.41-1.89 (6H, complex, 4-, 7- and 9-H_2), 2.28 (2H, m, 3-H_2), 2.78-3.06 (4H, complex, 5- and 10-H_2), 3.58-3.73 (2H, complex, 6-H_2) and 8.47 (1H, br s, 2-H); δ_C (50 MHz) (D_2O) 20.02 (t, C-8), 23.43, 25.89 and 26.46 (3t, C-4, -7 and -9), 36.45 (t, C-3), 48.03 (t, C-10), 54.83 and 59.62 (2t, C-5 and -6) and 169.54 (s, C-2).

N-(5-Aminopentyl)-1,2-didehydropiperidinium Chloride Hydrochloride (123)

This was obtained as an oil 0.275 g, 57%; ν_{max} (nujol mull) 2950, 2050 and 1645 cm^{-1}; δ_H (200 MHz) (D_2O) 1.27 (4H, m, 9- and 10-H_2), 1.38-2.05 (6H, complex, 4-, 5- and 8-H_2), 2.35 (2H, m, 3-H_2), 2.84 (4H, m, 6- and 11-H_2), 3.58-3.70 (2H, complex, 7-H_2) and 8.23 (1H, br s, 2-H); δ_C (50 MHz) (D_2O) 20.42 (t, C-9), 23.38, 23.64, 25.95 and 27.09 (4t, C-3, -4, -8 and -10), 39.98 (t, C-3), 48.10 (t, C-11), 53.97 and 61.05 (2t, C-6 and -7) and 163.63 (s, C-2).

N-(5-Aminopentyl)-1,2-didehydrohexahydroazepinium Chloride Hydrochloride

Addition of hydrogen chloride gas produced only starting material in 29% yield; δ_H (90 MHz) (D_2O) no C<u>H</u>=N present.

Preparation of 1,5-bis(*N*-Piperidyl)-3-pentanol (127)

1,5-bis(*N*-Piperidyl)3-pentanone dihydrochloride (91) (0.24 g, 0.73 mmol) in methanol (10 ml) was stirred at room temperature. Sodium borohydride (0.11 g, 2.92 mmol) was added over 2 h in small portions. The solvent was then removed under reduced pressure and the residue was triturated with CH_2Cl_2 (20 ml). Evaporation of the CH_2Cl_2 gave the product (127) as a clear oil (0.187 g) in quantitative yield; R_F 0.66 [Ethyl acetate-*i*-propanol-ammonia, (9:7:4)]; ν_{max} ($CHCl_3$) 3150, 2920, 1460, 1435 and 1115 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.32-1.73 (16H, complex),

2.20-2.58 (12H, complex) and 3.77 (1H, m); δ_C (50 MHz) ($CDCl_3$) 24.29 (t, C-9 and -15), 25.94 (t, C-8, -10, -14 and -16), 33.29 (t, C-2 and -4), 54.61 (t, C-7, -11, -13 and -17), 57.33 (t, C-1 and -5) and 72.95 (d, C-3); *m/z* 255 (M^++ 1), 254 (M^+), 170, 142, 98 (100%), 84, 55, 41 and 28. (Found: M^+ 254.2357. $C_{15}H_{30}N_2$ requires 254.2358.)

General Procedure for Preparation of *trans*-Diaminobutenes[108]

Pyrrolidine, piperidine or hexahydroazepine (37.6 mmol, 8 equivalents) was added over 15 min to a cooled solution of *trans*-1,4-dibromobut-2-ene (1.00 g, 4.68 mmol) in benzene (5 ml). A white slurry was formed almost immediately in all three cases. In each case the solution was allowed to warm to room temperature, diluted with chloroform (40 ml) and then washed with water (40 ml). The organic layer was separated, dried (solid potassium carbonate) and filtered. The solvent was then removed under reduced pressure.

trans-1,4-bis-(*N*-Pyrrolidyl)but-2-ene

This was obtained as a pale yellow oil (0.75 g, 83%); R_F 0.54 [ethyl acetate-*i*-propanol-ammonia, (9:7:4)]; ν_{max} (thin film) 2960, 1675 (weak), 1345 and 970 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.78 (8H, m), 2.50 (8H, m, 4 x CH_2-N) 3.06 (4H, $X_2AA'X_2'$ system, 1- and 4-H_2) and 5.71 (2H, $X_2AA'X_2'$ system, 2- and 3-H); δ_C (50 MHz) ($CDCl_3$) 23.43 (t, 4 x CH_2CH_2N), 53.99 (t, 4 x CH_2N), 58.00 (t, C-1 and -4) and 130.37 (d, C-2 and -3); *m/z* 195 (M^++ 1), 194 (M^+), 124, 123, 110, 84 (100%), 55, 41 and 28.

trans-1,4-bis-(*N*-piperidyl)but-2-ene

This was obtained as a white crystalline solid (1.00 g, 96%); m.p. 49 °C; R_F 0.68 [ethyl acetate-*i*-propanol-ammonia, (9:7:4)]; ν_{max} (KBr disc) 2930, 1300, 1130 and 970 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.38 (4H, m), 1.52-1.68 (8H, complex), 2.37 (8H, m, 4 x CH_2-N) 2.96 (4H, $X_2AA'X_2'$ system, 1- and 4-H_2) and 5.67 (2H, $X_2AA'X_2'$ system, 2- and 3-H); δ_C (50 MHz) ($CDCl_3$) 24.21 (t, 2 x $CH_2CH_2CH_2N$), 25.79 (t, 4 x CH_2CH_2N), 54.39 (t, 4 x CH_2N), 61.28 (t, C-1 and -4) and 130.39 (d, C-2 and -3); *m/z* 222 (M^+), 137 (100%), 122, 98, 84, 55, 41 and 28.

trans-1,4-bis-(*N*-hexahydroazepinyl)but-2-ene

The solvent was evaporated to leave a heterogeneous residue, which was triturated with diethyl ether. The solution was then evaporated to leave a pale yellow oil (1.05 g, 90%); R_F 0.69 [ethyl acetate-*i*- propanol-ammonia, (9:7:4)]; ν_{max} (thin film) 2930, 1355 and 975 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.52-1.74 (16H, complex), 2.58 (8H, m, 4 x CH_2-N) 3.09 (4H, $X_2AA'X_2'$ system, 1- and 4-H_2) and 5.64 (2H, $X_2AA'X_2'$ system, 2- and 3-H); δ_C (50 MHz) ($CDCl_3$) 26.79 (t, 4 x $CH_2CH_2CH_2N$), 27.90 (t, 4 x CH_2CH_2N), 55.45 (t, 4 x CH_2N), 60.42 (t, C-1 and -4) and 130.78 (d, C-2 and -3); *m/z* 250 (M^+), 151, 138, 136, 112 (100%), 98, 84, 55, 41 and 28.

trans-1,4-bis-(*N*-Pyrrolidyl)but-2-ene Dihydrochloride (128)

trans-1,4-bis-(*N*-Pyrrolidyl)but-2-ene (0.50 g, 2.6 mmol) was dissolved in diethyl ether (15 ml). An excess of diethyl ether saturated with dry hydrochloride gas was added to give a white precipitate. The solvent was decanted off and the product (128) (0.66 g, 96%) was dried *in vacuo*; m.p. 270 °C (decomp.). (Found: C, 53.74; H, 8.91; N, 10.30%. $C_{12}H_{24}N_2Cl_2$ requires C, 53.93; H, 8.99; N, 10.49%.)

trans-1,4-bis-(*N*-Piperidyl)but-2-ene Dihydrochloride (129)

trans-1,4-bis-(*N*-Piperidyl)but-2-ene (0.58 g, 2.61 mmol) was dissolved in acetone (25 ml). 6M Hydrochloric acid was added dropwise to the solution, precipitating the product. The solid was filtered off, washed well with acetone and then recrystallised from 95% aqueous ethanol-acetone (1:1). The product (129) was obtained as a white crystalline solid (0.75 g, 97%). (Found: C, 57.19; H, 9.59; N, 9.40%. $C_{14}H_{28}N_2Cl_2$ requires C, 56.95; H, 9.49; N, 9.49%.)

trans-1,4-bis-(*N*-Hexahydroazepinyl)but-2-ene Dihydrochloride (130)

This was prepared in the same way as *trans*-1,4-bis-(*N*-piperidyl)but-2-ene dihydrochloride. The product (130) was recrystallised from 95% aqueous ethanol-acetone (1:1) and was obtained as a white crystalline solid (0.77 g, 93%); m.p. 320 °C

(decomp.). (Found: C, 59.30; H, 10.00; N, 8.58%. $C_{16}H_{32}N_2Cl_2$ requires C, 59.44; H, 9.91; N, 8.67%.)

trans-N,N'-bis(Cyclohexyl)-1,4-but-2-ene diamine Dihydrobromide (131)

A solution of *trans*-1,4-dibromobut-2-ene (2.14 g, 0.01 mol) in dichloromethane (15 ml) was added over 1 h to freshly distilled cyclohexylamine (1.98 g, 0.02 mol) in dichloromethane (30 ml) at room temperature. The solution was stirred for 18 h during which time a white precipitate appeared. The solid was filtered off and washed well with dichloromethane. Recrystallisation from methanol gave the product (131) as white crystals (1.04 g, 25%); m.p. 230 °C (decomp.); R_F 0.70 [ethyl acetate-*i*-propanol-ammonia, (9:7:4)]; ν_{max} (KBr disc) 2940, 2550-2250, 1570, 1450 and 995 cm^{-1}; δ_H (200 MHz) (D_2O) 0.88-1.31 (10H, complex), 1.46 (2H, m), 1.65 (4H, m), 1.89 (4H, m),2.95 (2H, m, C<u>H</u>-N), 3.57 (4H, <u>X</u>$_2$AA'<u>X</u>$_2$' system, 1- and 4-H_2) and 5.85 (2H, X$_2$<u>AA'</u>X$_2$' system, 2- and 3-H); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 24.70 (t, 4 x <u>C</u>H_2CH_2CH-N), 25.28 (t, <u>C</u>H_2CH_2CH_2CHNH), 29.67 (t, 4 x <u>C</u>H_2CHNH), 45.78 (t, C-1 and -4), 57.49 (d, 2 x <u>C</u>HNH) and 130.78 (d, C-2 and -3); *m/z* 207, 151 (100%), 138, 136, 122, 112, 98, 81, 70, 56, 41 and 28. (Found: C, 45.66; H, 7.31; N, 6.48%. $C_{16}H_{32}N_2Br_2$ requires C, 46.60; H, 7.77; N, 6.80%.)

Preparation of *cis*-1,4-Dibromobut-2-ene (155)

cis-But-2-ene-1,4-diol (6.0 g, 0.068 mol) was added over 3 h to phosphorus tribromide (5 ml) with stirring at 0 °C. The slowly warming solution was stirred for 15 h. Ice/water (25 ml) was added slowly to hydrolyse excess phosphorus tribromide. The organic layer was separated, dissolved in diethyl ether (50 ml) then washed with aqueous sodium bicarbonate (5%, 3 x 10 ml) and water (10 ml). The organic layer was dried, filtered and evaporated under reduced pressure to give a yellow oil (12.9 g, 88%). Distillation gave a clear oil (12.4 g, 85%); b.p. 81 °C (14 mmHg); ν_{max} (thin film) 3040, 2970, 1450, 1390 1200 and 775 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 3.95 (4H, $\underline{X}_2AA'\underline{X}_2'$ system, 1- and 4-H_2) and 5.80 (2H, $X_2\underline{AA}'X_2'$ system, 2- and 3-H); *m/z* 213 (M^+), 135, 133 (100%), 53, 39 and 28.

First Attempted Preparation of *cis*-1,4-bis-(*N*-Piperidyl)but-2-ene (133)

Piperidine (3.8 ml, 37.6 mmol, 8 equivalents) was added over 15 min to a cooled solution of *cis*-1,4-dibromobut-2-ene (155) (1.00 g, 4.68 mmol) in benzene (5 ml). The solution was allowed to warm to room temperature, diluted with chloroform (40 ml) and then washed with water (40 ml). The organic layer was separated, dried (solid potassium carbonate) and filtered. The solvent was removed under reduced pressure to give a pale yellow oil. All spectral properties were identical to *trans*-1,4-bis-(*N*-piperidyl)but-2-ene (129).

Second Attempted Preparation of *cis*-1,4-bis-(*N*-Piperidyl)but-2-ene (133)

A solution of *cis*-1,4-dibromobut-2-ene (155) (2.14 g, 0.01 mol) in dichloromethane (15 ml) was added over 1 h to freshly distilled piperidine (1.98 ml, 0.02 mol) in dichloromethane (30 ml) at room temperature. The solution was stirred for 18 h during which time a white precipitate appeared. The solid was filtered off and washed well with dichloromethane. All spectral properties were identical to *trans*-1,4-bis-(*N*-piperidyl)but-2-ene (129).

Third Attempted Preparation of *cis*-1,4-bis-(*N*-Piperidyl)but-2-ene (133)

Piperidine (3.8 ml, 37.6 mmol, 8 equivalents) was added over 15 min to a cooled solution of *cis*-1,4-dichlorobut-2-ene (1.00 g, 4.68 mmol) in benzene (5 ml). The solution was allowed to warm to room temperature, diluted with chloroform (40 ml) and then washed with water (40 ml). The organic layer was separated, dried (solid potassium carbonate) and filtered. The solvent was removed under reduced pressure to give a pale yellow oil. All spectral properties were identical to *trans*-1,4-bis-(*N*-piperidyl)but-2-ene (129).

General Procedure for Alkene Reductions

Each *trans*-diaminoalkene dihydrochloride (128)-(131) was added to a suspension of palladium on charcoal (20% w/w) in methanol (5 ml per mmol of alkene) and was hydrogenated at 1 atmosphere for 2 h. The catalyst was filtered off through Celite and the Celite was washed well with methanol (25 ml). Evaporation of the filtrate under reduced pressure gave a solid which was recrystallised from 95% aqueous ethanol-acetone (1:1).

1,4-bis-(*N*-Pyrrolidyl)butane Dihydrochloride (136)

This was obtained as a white solid (0.235 g, 88%); m.p. 275 °C (decomp.)(from 95% aqueous ethanol-acetone, 1:1); R_F 0.31 ($CHCl_3$-methanol-ammonia, 90:9:1); ν_{max} (KBr disc) 2940, 1630 and 1450 cm^{-1}; δ_H (200 MHz) (D_2O) 1.67 (4H, m, 2- and 3-H_2), 1.82 (8H, m, 4 x $\underline{C}H_2\underline{C}H_2$-N), 2.97-3.10 (8H, complex, 4 x $C\underline{H}_2$-N) and 3.52 (4H, m, 1- and 4-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 23.50 (2t, 4 x $\underline{C}H_2\underline{C}H_2$-N and C-2 and -3) and 54.91 and 54.97 (2t, 4 x $C\underline{H}_2$-N and C-1 and -4).

1,4-Bis-(*N*-piperidyl)butane Dihydrochloride (137)

This was obtained as a white solid (0.264 g, 89%); m.p. 315 °C (decomp.)(from 95% aqueous ethanol-acetone, 1:1); R_F 0.22 ($CHCl_3$-methanol-ammonia, 90:9:1); ν_{max} (KBr disc) 2940, 1635 and 1455 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.21-1.76 (16H, complex), 2.64-2.78 (4H, m), 2.88-2.95 (4H, complex) and 3.30 (4H, m, 1- and 4-H_2); δ_C (50 MHz) (D_2O

with dioxan as reference at 67.4 ppm) 21.65 and 21.93 (2t, 2 x $\underline{C}H_2CH_2CH_2$-N and C-2 and -3), 23.64 (t, 4 x $\underline{C}H_2CH_2$-N), 54.04 (t, 4 x $C\underline{H}_2$-N) and 56.77 (t, C-1 and -4); *m/z* 224 (M^+), 140, 124, 111, 98 (100%), 84, 70, 55, 41 and 28. (Found: C, 56.65; H, 10.00; N, 9.21%. $C_{14}H_{30}N_2Cl_2$ requires C, 56.57; H, 10.10; N, 9.43%.)

1,4-bis-(*N*-Hexahydroazepinyl)butane dihydrochloride (138)

This was obtained as a white solid (0.276 g, 85%); m.p. 285 °C (decomp.) (from 95% aqueous ethanol-acetone, 1:1); R_F 0.24 ($CHCl_3$-methanol-ammonia, 90:9:1); ν_{max} (KBr disc) 2940, 1630 and 1450 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.45-1.68 (20H, complex) and 2.95-3.30 (12H, complex); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 22.02 (t, C-2 and -3), 24.24 (t, 4 x $\underline{C}H_2CH_2CH_2$-N), 26.59 (t, 4 x $\underline{C}H_2CH_2$-N), 55.60 (t, 4 x $C\underline{H}_2$-N) and 57.22 (t, C-1 and -4); *m/z* 252 (M^+), 209, 154, 112 (100%), 98, 84, 70, 55, 41 and 28.

N,N'-Bis(cyclohexyl)-1,4-butane Diamine Dihydrobromide (139)

This was obtained as a white solid (0.387 g, 94%); m.p. 280 °C (decomp.) (from 95% aqueous ethanol-acetone, 1:1); R_F 0.29 ($CHCl_3$-methanol-ammonia, 90:9:1); ν_{max} (KBr disc) 3430, 2935, 2710-2250, 1630 and 1450 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.10 (10H, m), 1.53 (10H, m), 1.84 (4H, m), 2.88 (4H, m, 2- and 5-H_2) and 3.51 (2H, m, C$\underline{H}$-NH); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 23.95 (t, 2 x $\underline{C}H_2CH_2CH_2CH$-N), 24.75 (t, 4 x $\underline{C}H_2CH_2CH$-N), 25.33 (t, C-3 and -4),

29.71 (t, 4 x $C\underline{H}_2CH$-N), 44.49 (t, C-2 and -5) and 58.06 (d, 2 x $C\underline{H}$-N); *m/z* 153, 124, 110 (100%), 97, 84, 55, 41 and 28.

General Procedure for Preparation of Diaminobutynes (141)-(143)[110]

Piperidine, hexahydroazepine or cyclohexylamine (0.08 mol, 4 equivalents) was added over 15 min at 0 °C to 1,4-dichlorobut-2-yne (2.46 g, 0.02 mmol). The solution was allowed to warm to room temperature over 1 h and diluted with water (30 ml). The aqueous solution was then saturated with solid potassium carbonate and extracted with diethyl ether (3 x 10 ml). The combined organic extracts were dried, filtered and evaporated under reduced pressure to give an oil which was purified by distillation. The oil was then dissolved in acetone (35 ml). 6M Hydrochloric acid was added dropwise to the solution, precipitating the product. The solid was filtered off, washed well with acetone and then recrystallised from 95% aqueous ethanol-acetone (1:1).

1,4-bis-(*N*-Piperidyl)but-2-yne Dihydrochloride (141)

This was obtained as a white solid (5.49 g, 94%); m.p. 275 °C (decomp.) (from 95% aqueous ethanol-acetone, 1:1); R_F 0.43 ($CHCl_3$-methanol-ammonia, 90:9:1); ν_{max} (KBr disc) 2935, 2800, 1450 and 1110 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.22-1.67 (12H, complex), 2.91 (4H, m), 3.47 (4H, m) and 3.98 (4H, s, 1- and 4-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 21.68 (t, $\underline{C}H_2CH_2CH_2$-N), 23.76 (t, 4 x

$\underline{C}H_2CH_2$-N), 46.72 (t, C-1 and -4), 53.81 (t, 4 x $C\underline{H}_2$-N) and 79.65 (s, C-2 and -3); *m/z* (free base) 220 (M^+), 137, 136, 135, 134, 106, 98 and 85 (100%). (Found: M^+ 220.1937. $C_{14}H_{24}N_2$ requires 220.1939.) (Found: C, 57.31; H, 9.23; N, 9.39%. $C_{14}H_{26}N_2Cl_2$ requires C, 57.34; H, 9.56; N, 9.56%.)

1,4-bis-(*N*-Hexahydroazepinyl)but-2-yne Dihydrochloride (142)

This was obtained as a white solid (6.12 g, 95%); m.p. 285 °C (decomp.) (from 95% aqueous ethanol-acetone, 1:1); R_F 0.48 ($CHCl_3$-methanol-ammonia, 90:9:1); ν_{max} (KBr disc) 2910, 1450 and 1315 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.54-1.78 (16H, complex), 3.17 (4H, m), 3.39 (4H, m) and 4.01 (4H, m, 1- and 4-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 24.63 (t, 2 x $\underline{C}H_2CH_2CH_2$-N), 26.30 (t, 4 x $\underline{C}H_2CH_2$-N), 47.55 (t, C-1 and -4), 55.89 (t, 4 x $C\underline{H}_2$-N) and 79.61 (s, C-2 and -3); *m/z* (free base) 249 (M^++ 1), 248 (M^+), 151, 150, 149, 148, 134 and 99 (100%). (Found: M^+ 248.2270. $C_{16}H_{28}N_2$ requires 248.2253.) (Found: C, 59.25; H, 9.46; N, 8.33%. $C_{16}H_{30}N_2Cl_2$ requires C, 59.81; H, 9.35; N, 8.72%.)

N,N'-bis(Cyclohexyl)-1,4-but-2-yne diamine Dihydrobromide (143)

This was obtained as a white solid (6.10 g, 94%); m.p. 240 °C (decomp.) (from 95% aqueous ethanol-acetone, 1:1); R_F 0.48 ($CHCl_3$-methanol-ammonia, 90:9:1); ν_{max} (KBr disc) 3430, 2940, 2780-2240, 1640 and 1500 cm^{-1}; δ_H (200 MHz) (D_2O) 1.20 (12H, m), 1.55 (4H, m), 1.92 (4H, m), 3.12 (2H, m, C$\underline{H}$-NH) and 3.89 (4H,

s, 2- and 5-H_2); δ_C (50 MHz) (D_2O) 24.73 (t, 4 x $\underline{C}H_2CH_2CH$-N), 25.28 (t, 2 x $\underline{C}H_2CH_2CH_2CH$-N), 29.54 (t, 4 x $C\underline{H}_2CH$-N), 34.32 (t, C-2 and -5), 57.45 (d, 2 x $C\underline{H}$-N) and 79.15 (s, C-3 and -4); *m/z* 99, 70, 56 (100%), 43, 36 and 28.

Preparation of 1,4-bis-(*N*-Pyrrolidyl)but-2-yne Dihydrochloride (140)

Pyrrolidine (3.00 ml, 37.6 mmol, 8 equivalents) was added over 15 min to a cooled solution of 1,4-dichlorobut-2-yne (0.58 g, 4.68 mmol) in benzene (5 ml). The solution was allowed to warm to room temperature, diluted with chloroform (40 ml) and washed with water (40 ml). The organic layer was separated, dried (solid potassium carbonate) and filtered. The solvent was removed under reduced pressure to give a yellow oil which was purified by distillation; b.p.95 °C (0.1 mm Hg) (lit.,[110] 93-95°C, 0.1 mm Hg). The oil was then dissolved in diethyl ether (15 ml). An excess of diethyl ether saturated with dry hydrochloride gas was added to give a white precipitate. The solvent was decanted off and the product was obtained as a white solid (1.22 g, 98%); m.p. 235 °C (decomp.); R_F 0.43 ($CHCl_3$-methanol-ammonia, 90:9:1); ν_{max} (KBr disc) 2960, 1630 and 1450 cm^{-1}; δ_H (200 MHz) (D_2O with dioxan as reference at 3.53 ppm) 1.93 (8H, m), 3.08 (4H, m), 3.52 (4H, m) and 4.04 (4H, s, 1- and 4-H_2); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 23.90 (t, 4 x $\underline{C}H_2CH_2$-N), 44.06 (t, C-1 and -4), 54.60 (t, 4 x $C\underline{H}_2$-N) and 79.20 (s, C-2 and -3); *m/z* (free base) 193 (M^++ 1), 192 (M^+), 123, 122, 121, 120, 84 and 71 (100%). (Found: M^+ 192.1607. $C_{12}H_{20}N_2$ requires 192.1626.)

(Found: C, 54.53; H, 8.26; N, 9.74%. $C_{12}H_{22}N_2Cl_2$ requires C, 54.34; H, 8.30; N, 10.57%.)

Preparation of 1,6-bis(*N*-Piperidyl)hexane Dihydrobromide (144)[111]

Piperidine (3.48 ml, 34.8 mmol) was added to water (30 ml) and 1,6-dibromohexane (1.34 ml, 8.7 mmol) was added with stirring. The mixture was heated at reflux for 6 h and then evaporated under reduced pressure to give a yellow solid which was recrystallised from methanol and acetone to give a white crystalline solid (1.82g, 51%); m.p. 280 °C; ν_{max} (KBr disc) 2960, 2940, 2640 and 1450 cm^{-1}; δ_H (200 MHz) (D_2O) 1.08-1.65 (20H, complex) and 2.55-3.24 (12H, complex); δ_C (50 MHz) (D_2O with dioxan as reference at 67.4 ppm) 22.39 (t, 2 x $\underline{C}H_2CH_2CH_2$-N), 24.05 (t, 4 x $\underline{C}H_2CH_2$-N), 24.41 (t, C-3 and -4), 26.52 (t, C-2 and -5), 54.31 (t, 4 x $\underline{C}H_2$-N) and 57.93 (t, C-1 and -6); *m/z* 252 (M^+), 123, 168, 154, 98 (100%) and 84. (Found: C, 46.16; H, 8.30; N, 6.63%. $C_{16}H_{34}N_2Br_2$ requires C, 46.38; H, 8.21; N, 6.76%.)

Preparation of Methyl pipecolate Hydrochloride (156)

To a suspension of pipecolic acid (4.0 g, 0.031 mol) in anhydrous 2,2-dimethoxypropane (60 ml) stirred while heated at reflux was added conc. hydrochloric acid (17.6 ml). Once all the acid had dissolved (ca. 20 min), the solution was cooled and was then stirred at room temperature for 72 h. Evaporation of the solvent at 35 °C gave a dark brown solid (6.3 g). Recrystallisation from methanol and ether gave the product as a white solid (5.3 g,

96%); m.p 144-146 °C; R_F 0.70 [ethyl acetate-*i*-propanol-ammonia, (9:7:4)]; ν_{max} (KBr disc) 3440, 2920, 2800, 2720, 1750, 1580, 1280, 1230 and 1030 cm^{-1}; δ_H (200 MHz) (D_2O) 1.42-1.73 (5H, complex), 2.10 (1H, m), 2.86 (1H, m), 2.29 (1H, m), 3.64 (3H, s) and 3.87 (1H, dd, *J* 4 and 11 Hz); δ_C (50 MHz) (D_2O) 22.34 and 22.46 (t, C-3 and -4), 26.71 (t, C-5), 45.20 (t, C-2), 54 70 (q, C-8), 57.86 (d, C-6) and 171.27 (s, C-7); *m/z* 143 (M^+), 84 (100%), 82, 56, 41 and 28.

Preparation of Methyl 1,6-bis(*N*- Pipecolate)hexane (146)

Methyl pipecolate hydrochloride (156) (0.905 g, 5 mmol) in anhydrous benzene (6 ml) was stirred overnight with triethylamine (0.7 ml, 5 mmol) at room temperature. The mixture was filtered and the filtrate was added over 90 min to 1,6-dibromohexane in anhydrous benzene (6 ml) under nitrogen at 70 °C. The reaction flask also contained anhydrous potassium carbonate (2.16 g, 15.6 mmol) and potassium iodide (0.16 g, 0.96 mmol). The mixture was stirred at 70 °C for 4 d, cooled, then poured into water (15 ml). The aqueous layer was separated then extracted with diethyl ether (3 x 25 ml). The organic extracts were extracted with 1M phosphoric acid (3 x 50 ml). The combined acidic extracts were saturated with potassium carbonate and then extracted with CH_2Cl_2 (5 x 150 ml). The combined organic extracts were dried, filtered and evaporated under reduced pressure to leave a yellow oil (0.545 g, 59%). The product (146) was purified (19%) by column chromatography on basic alumina and elution with pentane-toluene (2:1); R_F 0.67; ν_{max} ($CHCl_3$) 2940, 1735, 1440, 1190 and 1160 cm^{-1}; δ_H (200 MHz)

($CDCl_3$) 1.20-1.95 (16H, complex), 2.15-2.62 (8H, complex), 3.20 (6H, m) and 3.73 (6H, s); δ_C (50 MHz) ($CDCl_3$) 22.43 (t, C-3 and -4), 25.00 (t, C-10 and -16), 26.23 (t, C-2 and -5), 27.37 (t, C-9 and -15), 29.39 (t, C-11 and -17), 50.59 (t, C-1 and -6), 51.75 (q, C-20 and -22), 56.60 (t, C-12 and -18), 65.11 (t, C-8 and -14) and 173.84 (s, C-19 and -21); *m/z* 369 (M^++ 1), 368 (M^+), 353, 309 (100%), 291, 166, 128, 110, 98, 84, 55, 41 and 28. (Found: M^+ 368.2683. $C_{20}H_{36}O_4N_2$ requires 368.2675.)

Preparation of 1,6-bis(*N*-Pipecolic acid)hexane (157)

Methyl 1,6-bis(*N*-pipecolate)hexane (146) (0.088 g, 0.23 mmol) in 4M hydrochloric acid was heated at reflux for 48 h. The solvent was then removed under reduced pressure to leave a clear oil (0.098 g, 99%); ν_{max} ($CHCl_3$) 3200-2400, 2940, 1735, 1450, 1380, 1280, 1210 and 1050 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.17 (4H, m), 1.35-2.00 (16H, complex), 2.06 (2H, m), 2.75-3.13 (6H, complex), 3.51 (2H, m) and 3.78 (2H, dd, *J* 4 and 11 Hz); δ_C (50 MHz) ($CDCl_3$) 21.51 (t, C-3 and -4), 22.75 (t, C-10 and -16), 24.02 (t, C-2 and -5), 25.94 (t, C-9 and -15), 28.29 (t, C-11 and -17), 52.78 (t, C-1 and -6), 57.02 (t, C-12 and -18), 65.88 (t, C-8 and -14) and 172.30 (s, C-19 and -21); *m/z* 248, 246, 202 (100%), 114, 98, 84, 55, 41 and 28.

Oxidation of 1,6-bis(*N*-Pipecolic acid)hexane (145)

1,6-bis(*N*-Pipecolic acid)hexane (157) (0.058 g, 0.14 mmol) and phosphorus oxychloride (0.22 g, 1.44 mmol) were heated together at 100 °C for 3 min. The solution was cooled and

ice/water (10 ml) was added carefully. Evaporation of the solvent gave a clear oil which was taken up in deionised water and passed down an Amberlite IRA-400 ion-exchange column (in the chloride form). The elutate was concentrated to give the product (145) as a clear oil (0.21 g); ν_{max} (thin film) 2950, 2320, 1680 and 1000 cm^{-1}; δ_H (200 MHz) ($CDCl_3$) 1.28 (4H, m), 1.46-2.00 (12H, complex), 3.77 (4H, m), 3.53-3.82 (8H, complex) and 8.45 (2H, br s); δ_C (50 MHz) ($CDCl_3$) 21.09 (t, C-3, -4, -10 and -16), 25.91 (t, C-2, -5, -11 and -17), 27.14 (t, C-9 and -15), 51.53 (t, C-1 and -6), 62.97 (t, C-12 and -18), 179.82 (t, C-8 and -14).

Appendix 1

Spectrophotometric Assay

Calculation of Results for Cadaverine as a Substrate of DAO

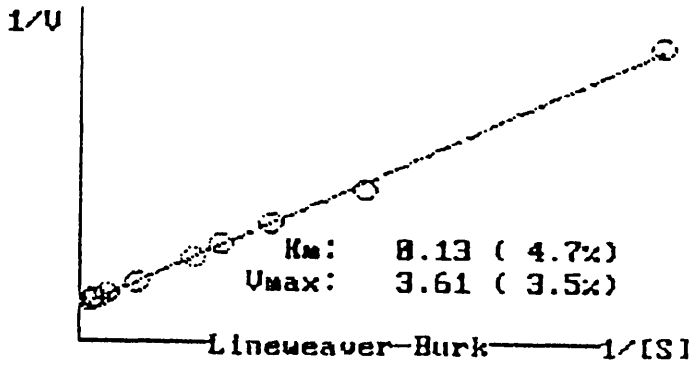
Assay:	2500 µl	Phosphate buffer pH 6.3
	170µl	DMAB
	100µl	MBTH
	50µl	Peroxidase
	25µl	Pea seedling DAO (1/50 dilute)
	300µl	Cadaverine (range of concentrations)

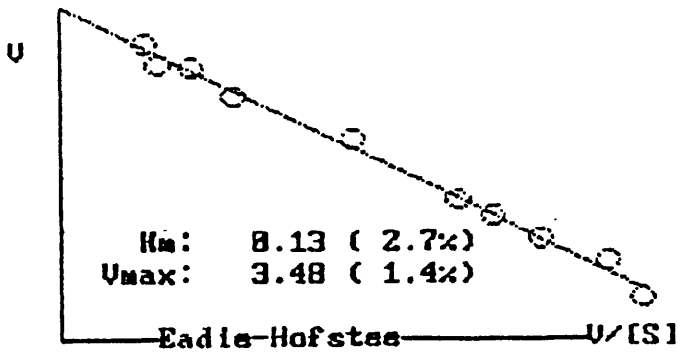
Substrate Concentrations and Rates

Substrate Concentration (mM)	Rate (conc/sec x 10^{-7})	Rate (μmol/mg/hr)
0.92	3.10	3018
0.74	2.88	2804
0.55	2.87	2794
0.37	2.55	2482
0.18	2.10	2044
0.092	1.46	1421
0.074	1.28	1246
0.055	1.06	1032
0.037	0.81	789
0.018	0.42	409

Results from Lineweaver Burk and Eadie Hofstee Plots.

	K_M (mM)	V_{max} (μmol/mg/hr)
Lineweaver Burk Plot	0.13	3514
Eadie Hofstee Plot	0.13	3388


1/V
Km: 8.13 (4.7%)
Vmax: 3.61 (3.5%)
Lineweaver-Burk
1/[S]


V
Km: 8.13 (2.7%)
Vmax: 3.48 (1.4%)
Eadie-Hofstee
V/[S]

Appendix 2

Inhibition of Diamine Oxidase

Calculation of Results for *N*-(5-Aminopentyl)hexahydroazepinium dihydrochloride (120) as an Inhibitor of DAO with Cadaverine as a Substrate.

Assay:	2500 µl	Phosphate buffer pH 6.3
	170µl	DMAB
	100µl	MBTH
	50µl	Peroxidase
	25µl	Pea seedling DAO (1/50 dilute)
	300µl	Cadaverine (range of concentrations)
	100µl	*N*-(5-Aminopentyl)hexahydro-azepinium dihydrochloride (120) (inhibitor)

Substrate Concentrations and Rates with Inhibitor Concentration of 0 mM.

Substrate Concentration (mM)	Rate (conc/sec x 10^{-7})	Rate (μmol/mg/hr)
0.93	3.75	3650
0.74	3.55	3456
0.56	3.43	3339
0.37	3.46	3368
0.19	2.75	2677
0.093	1.64	1597
0.074	1.61	1567
0.056	1.12	1090
0.037	0.91	886
0.019	0.39	380

Substrate Concentrations and Rates with Inhibitor Concentration of 1 mM.

Substrate Concentration (mM)	Rate (conc/sec x 10^{-7})	Rate (μmol/mg/hr)
0.93	2.50	2434
0.74	2.40	2336
0.56	2.29	2229
0.37	1.86	1811
0.19	1.14	1110
0.093	0.67	652
0.074	0.53	516
0.056	0.42	409
0.037	0.28	272
0.019	0.15	146

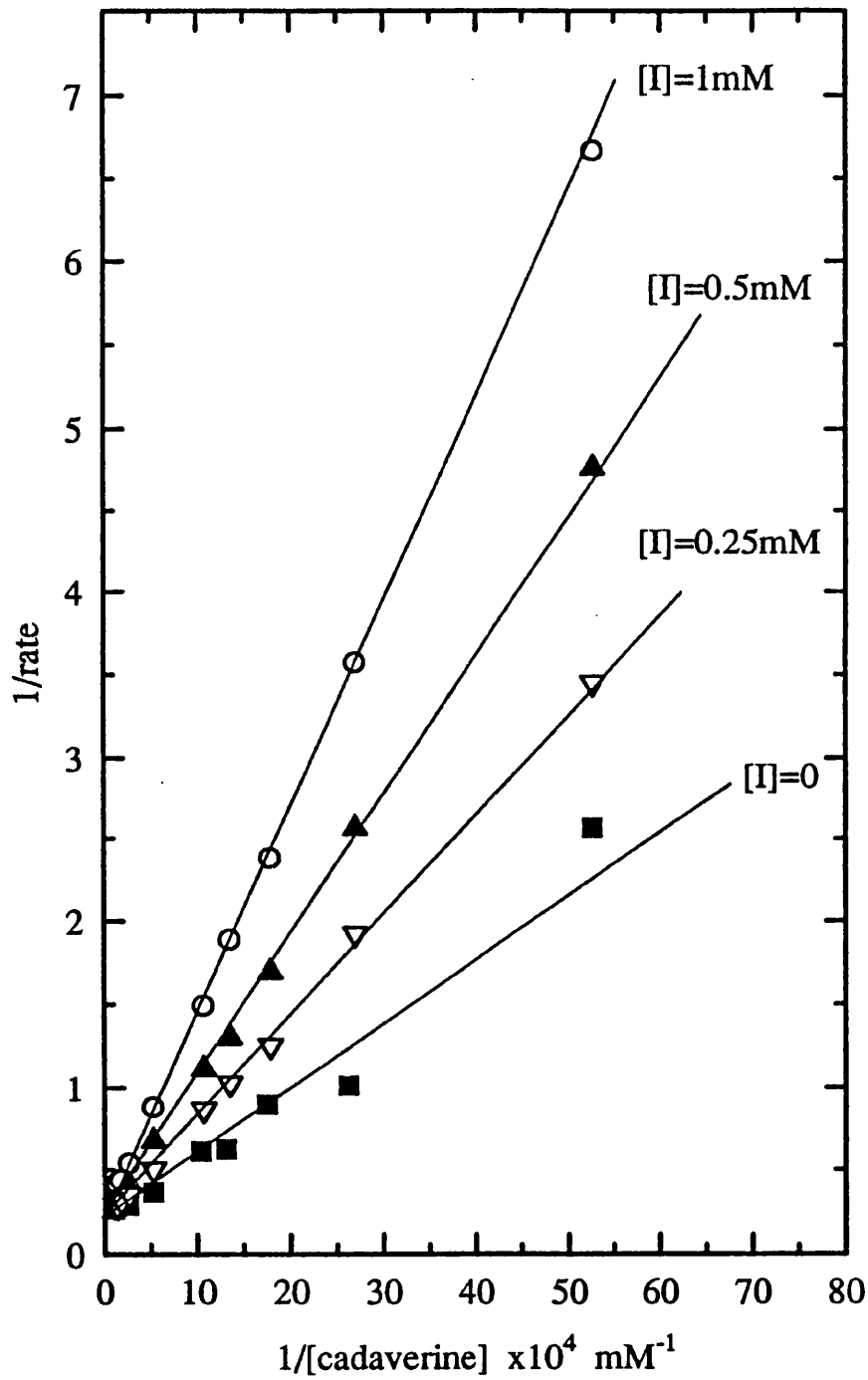
Substrate Concentrations and Rates with Inhibitor Concentration of 0.5 mM.

Substrate Concentration (mM)	Rate (conc/sec x 10^{-7})	Rate (μmol/mg/hr)
0.93	3.33	3242
0.74	3.14	3057
0.56	2.91	2833
0.37	2.46	2395
0.19	1.48	1440
0.093	0.90	876
0.074	0.77	750
0.056	0.59	574
0.037	0.39	380
0.019	0.21	204

Substrate Concentrations and Rates with Inhibitor Concentration of 0.25 mM.

Substrate Concentration (mM)	Rate (conc/sec x 10^{-7})	Rate (μmol/mg/hr)
0.93	3.49	3398
0.74	3.54	3446
0.56	3.19	3105
0.37	2.84	2765
0.19	2.00	1947
0.093	1.16	1129
0.074	0.98	954
0.056	0.80	779
0.037	0.52	506
0.019	0.29	282

Inhibition of Diamine Oxidase activity
by N-(5-Aminopentyl)hexamethylene imine.



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