SYNTHESIS AND BIOSYNTHESIS OF QUINOLIZIDINE ALKALOIDS WITH ENZYME WORK

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

by

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Finally, I would like to dedicate this thesis to my Mum.

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Abbreviations

Ac	-	acetyl
br	-	broad
d	-	doublet
D	-	deuterium (^{2}H)
DIAD	-	di-isopropyl axodicarboxylate
DIBAL	-	di-isobutyl aluminium hydride
DMF	-	*N*,*N*-dimethylformamide
i.r.	-	infra red
J	-	coupling constant
m	-	multiplet
m.s.	-	mass spectrometry
NMR	-	nuclear magnetic resonance
q	-	quartet
s	-	singlet
t	-	triplet
THF	-	tetrahydrofuran
TLC	-	thin layer chromatography
u.v.	-	ultraviolet
DAO	-	diamine oxidase
DNA	-	deoxyribonucleic acid
RNA	-	ribonucleic acid
PDAO	-	pea seedling diamine oxidase
PQQ	-	pyrroloquinoline quinone
SDS	-	sodium dodecyl sulphate
HPLC	-	high performance liquid chromatography
GC-MS	-	gas chromatography- mass spectrum
MBTH	-	3-methyl-2-benzothiazolinone

DMAB	-	3-(dimethylamino)benzoic acid
BSA	-	bovine serum albumin
E	-	enzyme
S	-	substrate
V	-	reaction rate
$[E_T]$	-	total enzyme concentration
V_{max}	-	maximum rate
K_M	-	Michaelis Menten constant
I	-	inhibitor
K_i	-	dissociation constant of enzyme-inhibitor complex
PMT	-	putrescine-*N*-methyltransferase

Summary

The research presented in this thesis can be divided into two main areas: a) the biosynthesis of quinolizidine alkaloids; b) enzyme work involving the synthesis and testing of diamines and polyamines as substrates and inhibitors of pea seedling diamine oxidase, and experiments involving the feeding of *N*-alklycadaverines to transformed root cultures of *Nicotiana rustica* and *Datura stramonium.*

Biosynthesis of Quinolizidine Alkaloids

Previous work has revealed that cadaverine is a good precursor of quinolizidine alkaloids. (*R*)- and (*S*)-[1-^{2}H]Cadaverines were synthesised by a known route and isolated as their dihydrochloride salts. Samples of these labelled precursors were fed with a radioactive tracer, by the wick method, to *Sophora microphylla* plants in order to study the biosynthesis of matrine. After ten to fourteen days the plants were macerated and the alkaloids extracted. The alkaliod extract contained matrine, anagyrine, *N*-methylcytisine and cytisine. Levels of incorporation, established by ^{2}H and NMR spectroscopy, were comparable to those obtained in a similar experiment by a previous researcher.

Attempts were made to synthesise (*R*)- and (*S*)-[2-^{2}H]cadaverine dihydrochlorides by the route devised by a previous researcher. Problems were experienced in the early stages of the synthesis and repeated attempts were made to obtaine pure pentane-1,2,5-triol for use in the later stages of the synthesis. Various methods were attempted with varying degrees of success. An alternative synthesis, avoiding the need for extraction and purification of triol from the aluminium complex is proposed.

The Synthesis and Testing of Diamines and Polyamines as Substrates and Inhibitors of Diamine Oxidase

Diamine oxidase catalyses the oxidative deamination of diamines to their corresponding aminoaldehydes. Cadaverine and putrescine have been shown to be the best substrates of diamine oxidase. The cadaverine and putrescine analogues *N*-methylcadaverine and *N*-methylputrescine have also been shown to be good substrates. A series of *N*-alkylcadaverines were synthesised by a new, improved route and were tested as substrates of diamine oxidase. Also tested were a series of *N*-alkylputrescines. The assay used in the testing was Stoner's spectrophotomeric assay which involves the measurement of hydrogen peroxide produced as a by-product of the enzymic reaction. From this assay K_M and V_{max} values were obtained for the oxidation of these substrates using diamine oxidase. The K_M is a measure of the strength of the enzyme-substrate complex and determines the binding efficiency of the substrate to the enzyme. The V_{max} is the maximal rate and is related to the turnover number of an enzyme. Analysis of results provided information on the steric constraints of the active site and showed that as the steric bulk of the alkyl group increases the V_{max} decreases.

Several of these compounds were also tested as inhibitors of diamine oxidase, and K_i values obtained. The K_i is the dissociation constant of the enzyme-inhibitor complex. Most of the compounds did inhibit the diamine oxidase catalysed reaction. The best inhibitors of the compounds tested were *N*-cyclohexylcadaverine and *N*-benzylputrescine, both of which were shown to be apparent competitive inhibitors.

A series of *N*-ethylpolyamines were also synthesised and tested as inhibitiors and substrates of diamine oxidase using the same assay as before. Since *N*-ethylpolyamines can interfere with polyamine metabolisism and may have useful biological activity, selected compounds were tested by other workers for biological activity against cancer and fungi. Two compounds were also used to confirm the

identity of certain natural compounds extracted by other workers from plants and fungi. The synthesised compounds proved to be the same as the natural compounds.

The Feeding of *N*-Alkylcadaverines to Transformed Root Cultures of *N. rustica* and *D. stramonium.*

It has been established that feeding certain modified precursors of a natural precursor to biological systems may result in new or modified alkaloids being produced. Transformed root cultures of *Nicotiana rustica* have been shown to produce mainly nicotine, with smaller quantities of anabasine, nornicotine and anatabine. *Datura stramonium* produces hygrine and hyoscamine. *N*-Alkylcadaverines were fed to both species with the aim of discovering whether any new alkaloid analogues were formed. No new alkaloid analogues appeared to be produced from the compounds fed to *D. stramonium*. *N*-Ethylanabasine appeared to be a new alkaloid analogue formed in response to the feeding of *N*-ethylcadaverine to *N. rustica*. The *N*-propylcadaverine feed to *N. rustica* appeared to stimulate the formation of the new alkaloid analogue, *N*-propylanabasine.

CHAPTER ONE

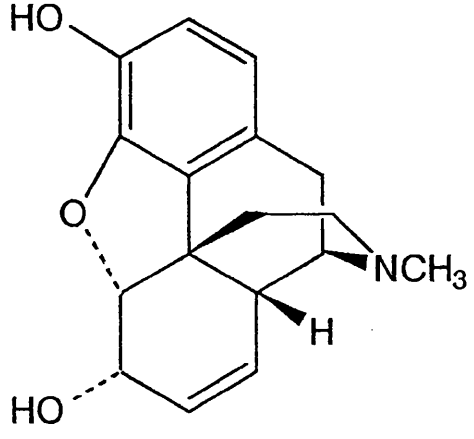
INTRODUCTION

The work described here is in two separate but related areas. The first involved biosynthetic work on quinolizidine alkaloids. The second involved work on diamine oxidase and polyamines.

1.1 INTRODUCTION TO WORK ON QUINOLIZIDINE ALKALOIDS

The term alkaloid was first invented in the 19th century.[1] Since that time alkaloids have been the subject of considerable study. Some 7000 different alkaloids have been identified and, in many cases, their occurrence, structures and uses have been well documented.[2] Pelletier has defined an alkaloid as a cyclic organic compound containing nitrogen in a negative oxidation state which is of limited distribution among living organisms.[3] Alkaloids are found rarely in the more primitive or non-flowering species of plants but club mosses, one of the lowest forms of plant life, are abundant in alkaloids. Alkaloids are plentiful in such families as the Asteraceae, which includes plants such as groundsel and ragwort; the Fabaceae, which includes lupins and broom; and the Papaveraceae or poppy family.

Alkaloids often have a complex molecular structure, contain a nitrogen atom, usually as part of a heterocyclic system, and many have been found to have pharmacological activity.[3] Morphine (1), for example, was the first pure alkaloid isolated from *Papaver somniferum,* the opium poppy.



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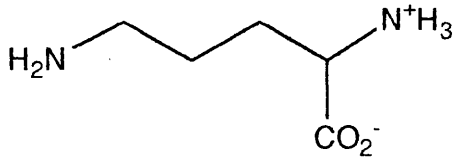
Although the structures of alkaloids are often complex, it has been demonstrated that their origins can be traced back to five of the common α-amino acids, ornithine (2), lysine (3), phenylalanine (4), tyrosine (5) and tryptophan (6). In this work we are concerned with one particular group of alkaloids, the quinolizidine alkaloids, which have their origin in lysine (3).



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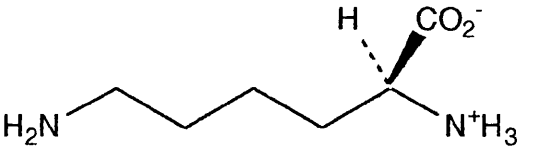

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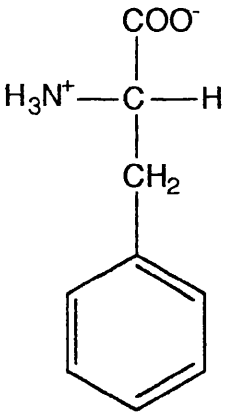

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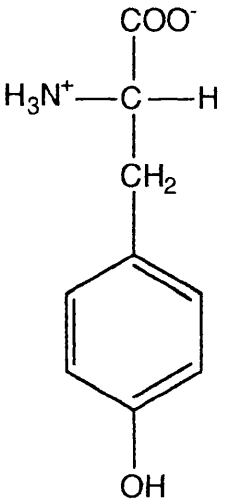

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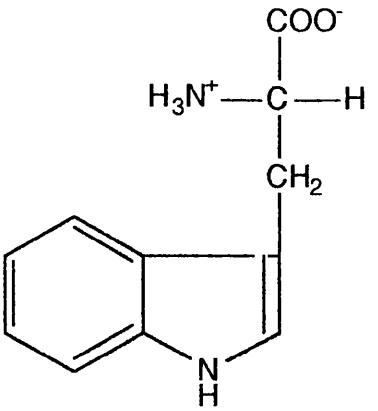

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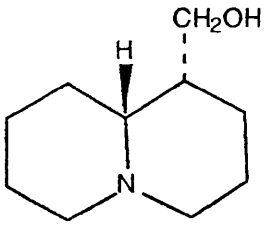
1.2 QUINOLIZIDINE ALKALOIDS

Quinolizidine alkaloids are found in the Papilionaceae, a sub-family of the Fabaceae. They are often referred to as lupin alkaloids. They represent the largest group of legume alkaloids and 2% of all alkaloids from plants. Although the quinolizidine alkaloids are not found outside the Fabaceae, Wink and Witte[4] were able to induce the biosynthesis of quinolizidine alkaloids in cell cultures of plants in which different alkaloids or no alkaloids are usually found. This suggests that the genes for the production of quinolizidine alkaloids are present in other plant families although the alkaloids themselves are restricted to the Fabaceae. The ability of

species of other plant families to synthesise quinolizidine alkaloids seems to be the result of an accumulation of the necessary genes.[5]

1.3 MAIN STRUCTURAL TYPES OF QUINOLIZIDINE ALKALOIDS

The main structural types of quinolizidine alkaloids are the bicyclic alcohol (-)-lupinine (7), tricyclic pyridones such as cytisine (8) and (-)-*N*-methylcytisine (9) and tetracyclic alkaloids such as (-)-sparteine (10), anagyrine (11) and matrine (12). The biosynthesis of these alkaloids will be discussed in more depth in later Chapters.



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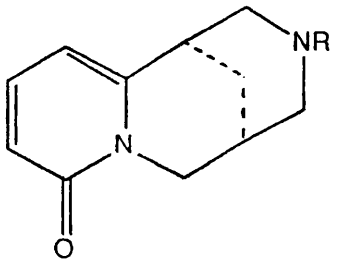

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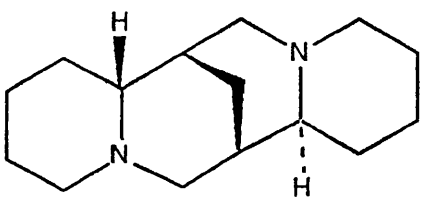
(9) R = Me



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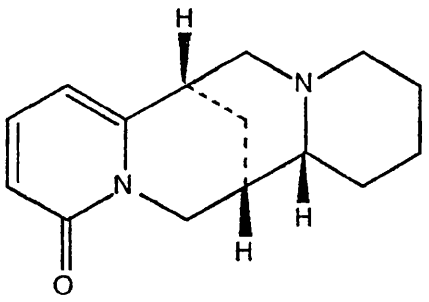

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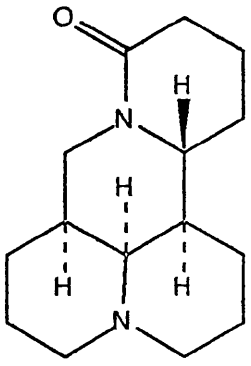

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1.4 THE IMPORTANCE OF QUINOLIZIDINE ALKALOIDS

The Fabaceae represents an important source of food for humans and animals. Traditionally farmers have used plants like clover, a member of the legume family, to

replace the nitrogen in soil lost through continuous crop production. Quinolizidine alkaloids serve as a means of nitrogen storage in plants, and at one time it was thought they were merely metabolic waste products or forms of nitrogen storage. It has now been demonstrated that they have several other significantly important functions. These include:

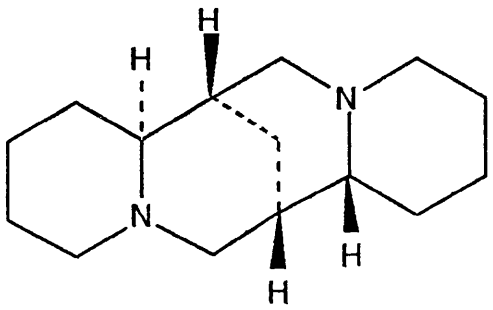
a) serving as an anti-predator defence mechanism;[6] and being part of a more generalised chemical defence function;[7]

b) having potentially useful pharmacological properties;[3]

c) having an anti-microbial function, enabling the plant to recover from trauma.[8,9]

1.5 ANTI-PREDATOR AND DEFENCE FUNCTIONS

Quinolizidine alkaloids are toxic to animals when ingested in very large quantities but moderate quantities may be safely eaten on a regular basis since their toxicity is not cumulative.[6] The pyridone alkaloids such as anagyrine (11) and cytisine (8) are more toxic than the saturated alkaloids such as (+)-sparteine (13). Anagyrine and cytisine can both cause foetal malformations in higher animals like chickens and rabbits. Anagyrine is the cause of crooked calf disease in cattle.[10] Only a few cases of poisoning in humans have been reported.[11]



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It has been shown that when lupins abundant in quinolizidine alkaloids and lupins with no quinolizidine alkaloids are both available for grazing, herbivores show a definite preference for the latter.[13] Moreover, Wink has shown that the accumulation of these alkaloids in plants increases markedly when plant leaves are

wounded.[12] This suggests that the plant is manufacturing the alkaloids to increase its toxicity and therefore deter animals from further grazing.

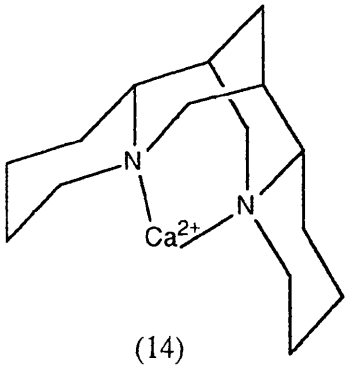
In addition, the concentrations of quinolizidine alkaloids found in plants are not constant. There is an increase in daylight when herbivores are more active in grazing and decreased accumulation at night. However, Wink found that when wounded leaves were kept in the dark they manufactured similar quantities of quinolizidine alkaloids to those in wounded leaves which were exposed to daylight, thus increasing their toxicity and providing a deterrent for any night grazing which might occur.[12] Quinolizidine alkaloids, therefore, appear to have a strong anti-predator function and form part of the defence mechanism in legumes that do not have thorns or stinging hairs with which to defend themselves.

Wippich and Wink have argued that quinolizidine alkaloids also act as chemical defence compounds and are important for the biological fitness of plants.[14] It has been shown that quinolizidine alkaloids can protect lupins from the pea aphid by deterring it from feeding.[15] Other aphids have developed the ability to metabolise alkaloids and these, in turn, act as a deterrent to their predators such as the beetle.[16]

Quinolizidine alkaloids are produced in the aerial green parts of lupins. They are not inert end products of metabolism but dynamic compounds which are synthesised in daylight, translocated via the phloem to the other plant organs and stored in the epidermal tissue.[17] As a result all parts of the plant contain alkaloids but the highest concentrations occur in those areas of the plants most likely to be eaten, namely the ripe fruits, leaves and shoots, and in parts that are important to ensure the reproduction of the species such as seeds and roots.[18] Moreover, young, healthy leaves produce high concentrations of alkaloids, but as leaves age their level of alkaloid concentration drops markedly until it is very low in old, dry leaves. This, too, is consistent with alkaloids playing a role in helping to ensure survival of the species.[19]

1.6 PHARMACOLOGICAL AND ANTI-MICROBIAL FUNCTIONS

The pharmacological activity of some quinolizidine alkaloids may be due to their ability to bind to divalent cations such as calcium, e.g. the α-isosparteine complex (14), and they are able to function as ligands in a way similar to that of other diamines.[20]



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Quinolizidine alkaloids have been found to assist plants to recover from the effects of wounding.[13] They have been shown to inhibit the growth of bacteria and fungi, and to inhibit the germination of powdery mildew in cereal crops.[14]

Some of the pharmacological properties of quinolizidine alkaloids which are currently known are listed below.[21]

Alkaloid	Type of Activity
Sparteine	Antiarrhythmic, Diuretic
	Uterotonic, Oxytocic
Lupanine	Antiarrhythmic
	Hypotensive, Hypoglycaemic
Anagyrine	Teratogenic (calves)
Cytisine	Teratogenic (chicks; rabbits)
	Hallucogenic
	Uterotonic, Oxytocic
	Respiratory stimulant

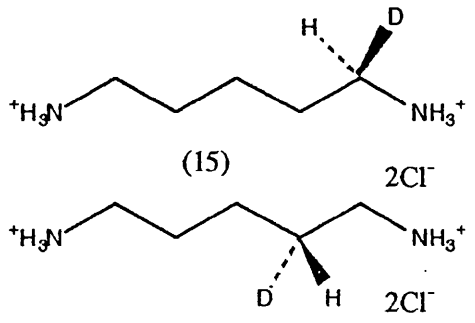
Since quinolizidine alkaloids have such potentially useful pharmacological and ecological properties, it is important to obtain as much information about them as

possible. One way of doing so is through research on their biosynthesis. This Chapter has dealt in broad, general terms with the occurrence and importance of quinolizidine alkaloids for plants and humans. In the next Chapter current research findings on the biosynthesis of quinolizidine alkaloids are reviewed.

1.7 AIMS IN RELATION TO QUINOLIZIDINE ALKALOIDS

The research findings reviewed in Chapter Two demonstrate that although the biosynthesis of some of the quinolizidine alkaloids is well understood there are still gaps in our knowledge, especially in relation to the tetracyclic alkaloids and, in particular, anagyrine (11) and matrine (12) which are discussed in Chapter Three.

One of the aims of this work was to synthesise (*R*)- and (*S*)-[1-^{2}H]cadaverine dihydrochloride, (15) and (16) respectively, and to feed these labelled precursors to *Sophora microphylla* in order to extend the biosynthetic studies on matrine. Relatively few studies have been performed on matrine to date and labelling patterns have not been fully established. The aim of the feeding experiments in the present study was to repeat some of the earlier studies in order to obtain complete labelling patterns for matrine. This work, and the results obtained are described in Chapter Three.



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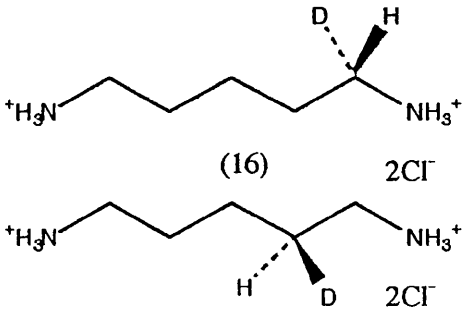

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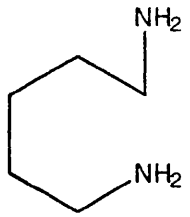
(18)

Chapter Four describes attempts to synthesise (*R*)- and (*S*)-[2-^{2}H]cadaverine dihydrochloride, (17) and (18) respectively, with the aim of carrying out feeding experiments on *Anagyris foetida*, a plant which produces anagyrine. Like matrine, the biosynthesis of anagyrine has not been fully established. The aims of the feeding experiments were to establish complete labelling patterns for anagyrine and to

provide information about some of the stereochemical details of the enzymic processes in anagyrine biosynthesis.

1.8 AIMS IN RELATION TO DIAMINE OXIDASE

Cadaverine (19) is a known substrate of diamine oxidase. One of the aims of this work was to synthesise *N*-alkylcadaverines to be tested as substrates for pea seedling diamine oxidase using the Stoner spectrophotometric assay.[22] Also tested were *N*-alkylputrescines. This testing was carried out to discover more about the steric requirements at the active site of the pea seedling diamine oxidase enzyme. After analysis of the results of the enzymic oxidation of the synthetic diamines, some of these compounds were tested as inhibitors of the oxidative deamination of cadaverine and putrescine (20) by diamine oxidase. This was carried out using the same assay as before with the addition of a known concentration of potential inhibitors.



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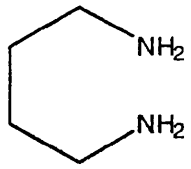

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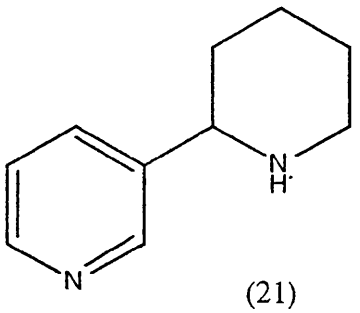
Chapter Five provides a brief introduction to this work and reviews some of the current research findings on the study of diamine oxidase. Chapters Six and Seven describe the synthesis and testing of *N*-alkylcadaverines and *N*-alkylputrescines as substrates or inhibitors of pea seedling diamine oxidase.

Another aim of this work was to devise a route for the synthesis of a series of *N*-ethylpolyamines. These were also tested as substrates or inhibitors of diamine oxidase. As *N*-alkylpolyamines can interfere with polyamine metabolism and may have useful biological activity, selected compounds were tested for anti-fungal and anti-cancer activity.

Transformed root cultures of *Nicotiana rustica* are known to produce alkaloids when cadaverine is fed, while similar cultures of *Nicotiana tabacum* produce nicotine and other alkaloids, including anabasine (21). Some of the *N*-alkylcadaverines were fed to transformed root cultures of *N. rustica* with the aim of discovering whether any analogues of anabasine (21) were formed. They were also fed to *Datura stramonium* to see whether any new alkaloid analogues were formed. All of the work on diamine oxidase, and the feeding experiments carried out, is discussed in detail in Chapters Six, Seven, Eight and Nine.



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CHAPTER TWO

BIOSYNTHESIS OF QUINOLIZIDINE ALKALOIDS

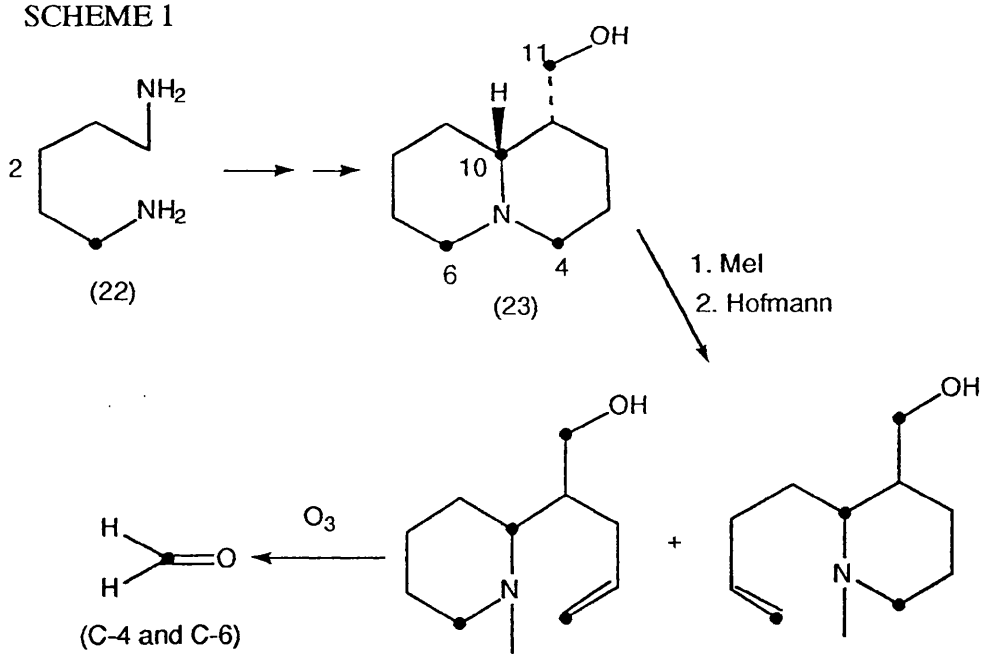
2.1 INTRODUCTION

A knowledge of the biosynthesis of alkaloids is important as it enables alkaloids to be divided up into manageable groups of biosynthetically related compounds. By the 1950's radiotracer methods were being used to study natural product biosynthesis. These laid the foundation for many of the subsequent studies but further progress only became possible with the advent of compounds labelled with stable isotopes (^{2}H, ^{13}C, and ^{15}N), together with the ability to determine complete labelling patterns in alkaloids by the use of NMR spectroscopy. This development enabled studies to be undertaken which produced information that could never have been obtained by using radioactive precursors.

2.2 BIOSYNTHESIS OF LUPININE

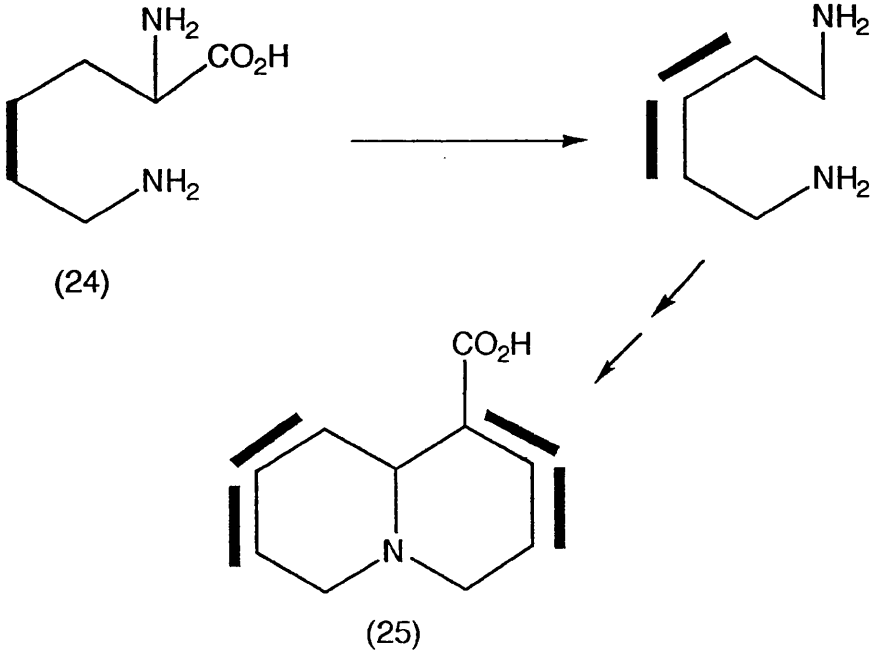
The simplest of the quinolizidine alkaloids, and one which has received considerable attention, is lupinine (7). Schutte and co-workers carried out the first biosynthetic studies on lupinine.[23] Their results demonstrated that [1,5-^{14}C]cadaverine (22) and ^{14}C-labelled lysine were incorporated into lupinine (23) in *Lupinus luteus* plants. By degradation it was found that half of the total radioactivity was at positions C-4 and C-6 and a quarter in the hydroxymethylene carbon, C-11. It was assumed that the final quarter was located at the bridgehead carbon C-10 (Scheme 1).[24]

SCHEME 1



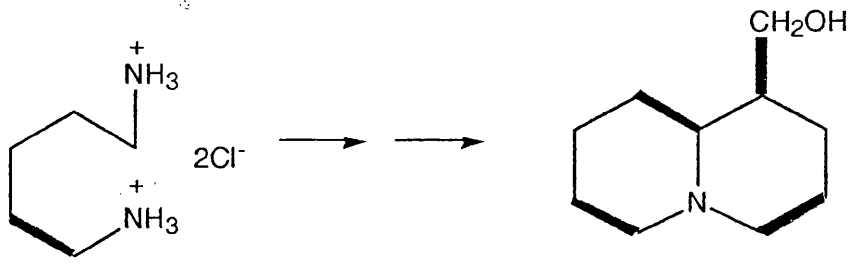
A complete labelling pattern for lupinine was obtained by Rana and Robins[25] and subsequently confirmed in studies by Robins and Sheldrake.[26] When Rana and Robins fed DL-[4,5-$^{13}C_2$]lysine (24) to *L. luteus* plants (Scheme 2) the labelling pattern obtained was consistent with the formation of lupinine (25) from two molecules of lysine via the symmetrical intermediate cadaverine, thus confirming a much earlier postulate put forward by Sir Robert Robinson.[27]

SCHEME 2



This was further confirmed when Robins and Sheldrake[26] fed [1,2-$^{13}C_2$]cadaverine dihydrochloride (26) to *L. luteus* (Scheme 3). Feeding this precursor gave the distinctive labelling pattern (27) for lupinine.

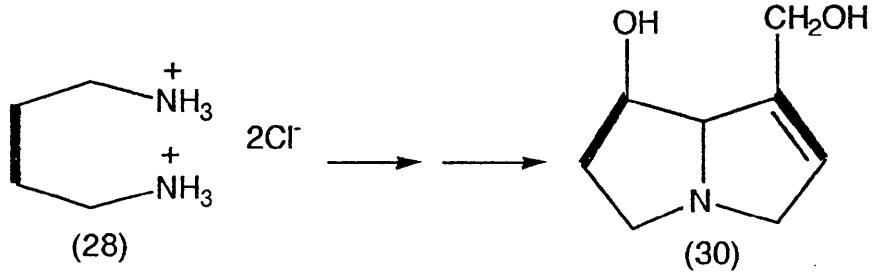
SCHEME 3



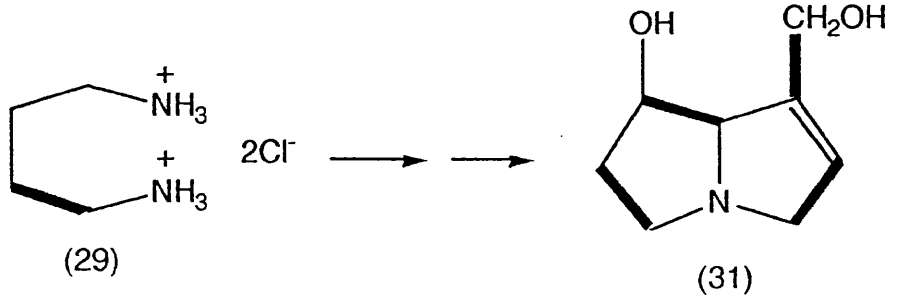
(26) (27)

Work on the biosynthesis of another group of alkaloids, the pyrrolizidine alkaloids, had demonstrated that retronecine (30) is formed from two molecules of putrescine[28] (28), (29) and that a later intermediate with C_{2v} symmetry formed by a combination of two putrescine molecules, could be involved in the biosynthetic pathway to retronecine. Groups led by Robins[29] and by Spenser[30] subsequently produced good evidence to show that a C4-N-C4 intermediate is involved in the biosynthesis of retronecine. When the labelling patterns for lupinine (Schemes 2 and 3) had been established it appeared that they were analogous to those for retronecine (30), (31) (Scheme 4).[31,32]

SCHEME 4



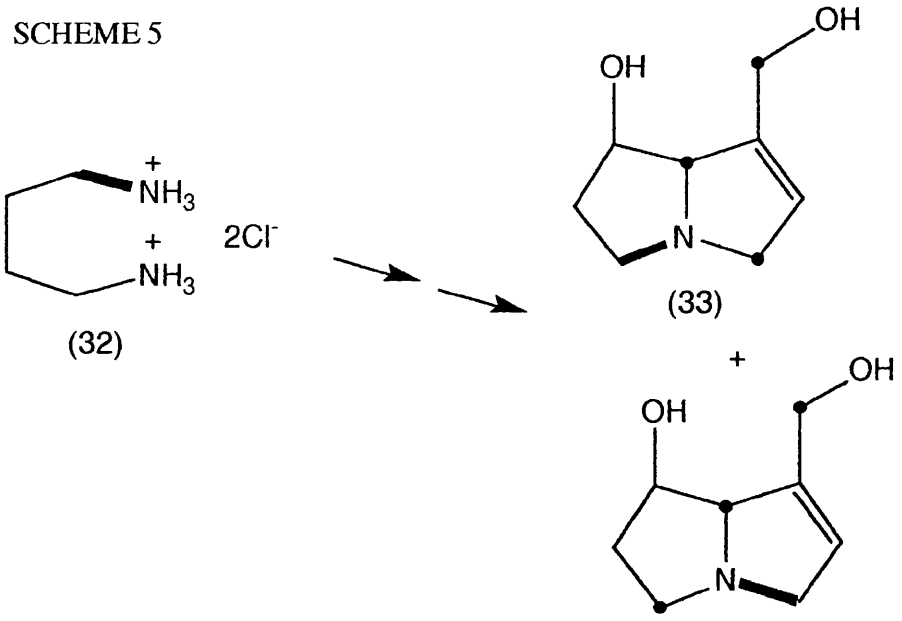
(28) (30)



(29) (31)

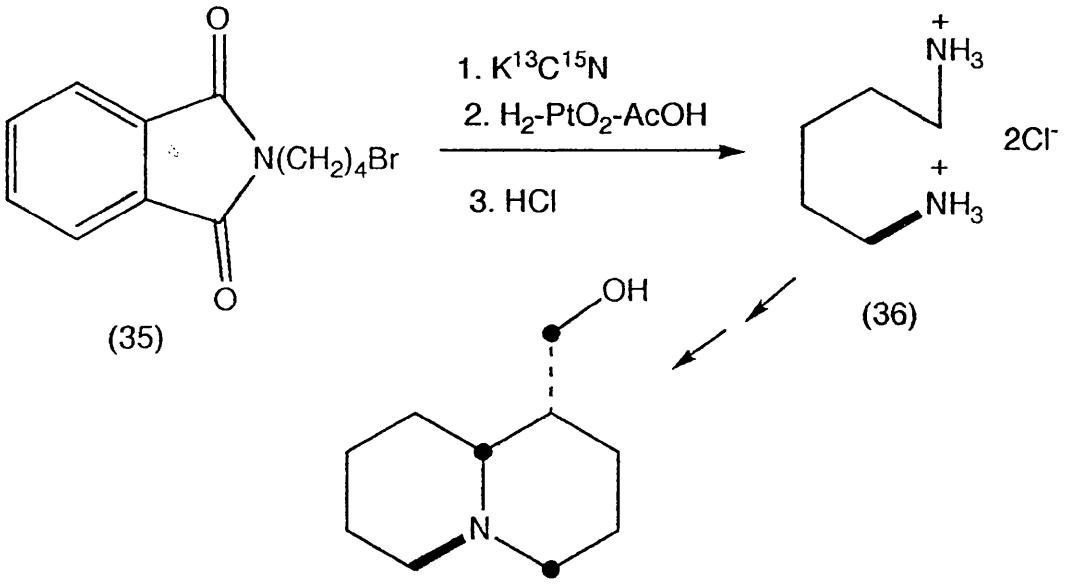
It was demonstrated that a later intermediate with C_{2v} symmetry was involved in the biosynthetic pathway to retronecine by feeding [^{13}C-^{15}N]putrescine (32) to obtain equal amounts of the two labelled species (33) and (34) (Scheme 5). It was postulated that a similar situation might be true in the biosynthesis of lupinine.

SCHEME 5



In order to test the validity of this postulate two research groups, led by Robins[29] and by Spenser[30] carried out independent but parallel studies. Both groups synthesized [^{13}C-^{15}N]-labelled cadaverine dihydrochloride (36) from 1-phthalimido-4-bromobutane (35) by treatment with $K^{13}C^{15}N$ followed by catalytic hydrogenation and hydrolysis (Scheme 6).

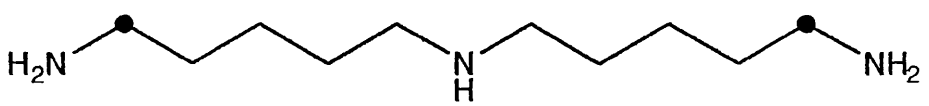
SCHEME 6



(35) (36) (37)

Feeding [^{13}C-^{15}N]cadaverine dihydrochloride (36) to *L. luteus* gave a sample of lupinine (37) which showed four enriched carbon signals for C-4, C-6, C-10, and C-11 in its ^{13}C {^{1}H} NMR spectrum. The resolution enhanced spectrum of lupinine (37) showed that only one signal (for C-6) was flanked by a doublet (J 3.2 Hz) due to the presence of a ^{13}C-^{15}N species, indicating that there is no later symmetrical intermediate of the type C_5-N-C_5 involved in lupinine biosynthesis. This result provided a distinct contrast with retronecine biosynthesis.

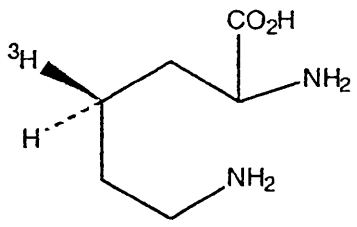
In a subsequent experiment *N*-(5-aminopentyl)-1,5-diaminopentane (38) was prepared by Rana and Robins[29] with ^{14}C at each terminal carbon. This material was incorporated very poorly (0.04% specific incorporation) into lupinine thus indicating that this triamine is not likely to be an intermediate in lupinine biosynthesis. An intermediate trapping experiment for this triamine (38) also gave a negative result.[35]



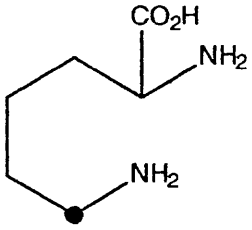
(38)

2.3 STEREOCHEMISTRY OF ENZYMIC PROCESSES INVOLVED IN LUPININE BIOSYNTHESIS

By feeding a mixture of L-[4-^{3}H]lysine (39) and DL-[6-^{14}C]lysine (40) to *L. luteus*, Golebiewski and Spenser[36] were able to demonstrate that the biosynthesis of lupinine starts from L-lysine (3). The ^{3}H/^{14}C ratio in lupinine (7) increased from 4.1 to 8.4 indicating that lupinine is derived entirely from L-lysine. The next step in the pathway, the decarboxylation of L-lysine to form cadaverine (19) is known to proceed with retention of configuration.[33,34,37,38,39]



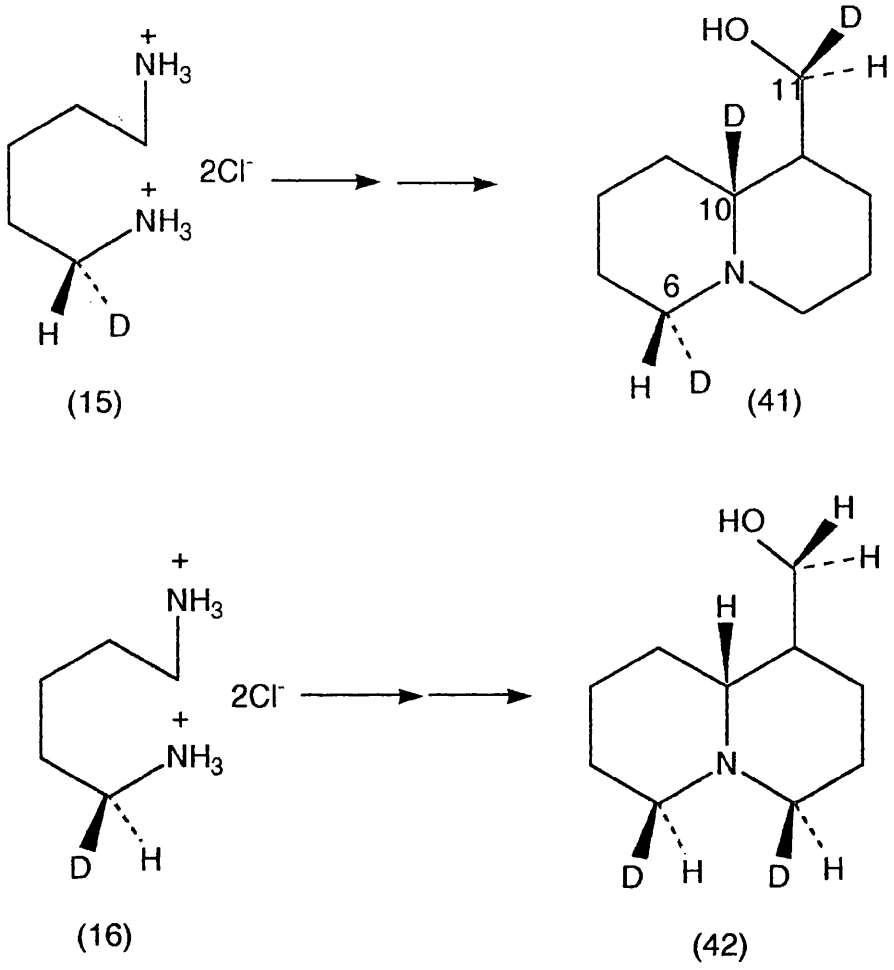
(39)



(40)

Golebiewski and Spenser[40] also provided answers to the stereochemical questions concerning the steps from cadaverine (19) to lupinine (7) involving retention or removal of hydrogen from the terminal carbons of cadaverine. (*R*)- and (*S*)-[1-^{2}H]cadaverine dihydrochlorides, (15) and (16) respectively, were made and fed to *L. luteus*. Complete labelling patterns in lupinine were determined by ^{2}H NMR spectroscopy. The assignment of the ^{2}H NMR spectrum of lupinine (7) was carried out by Golebiewski[41] but some revisions were made by Rycroft and co-workers[42] after more detailed examination. Samples of the (*R*)- and (*S*)-[1-^{2}H]cadaverines were made enzymically.[43] Feeding of these precursors to *L. luteus* gave ^{2}H-labelled lupinine samples (41) and (42).

SCHEME 7



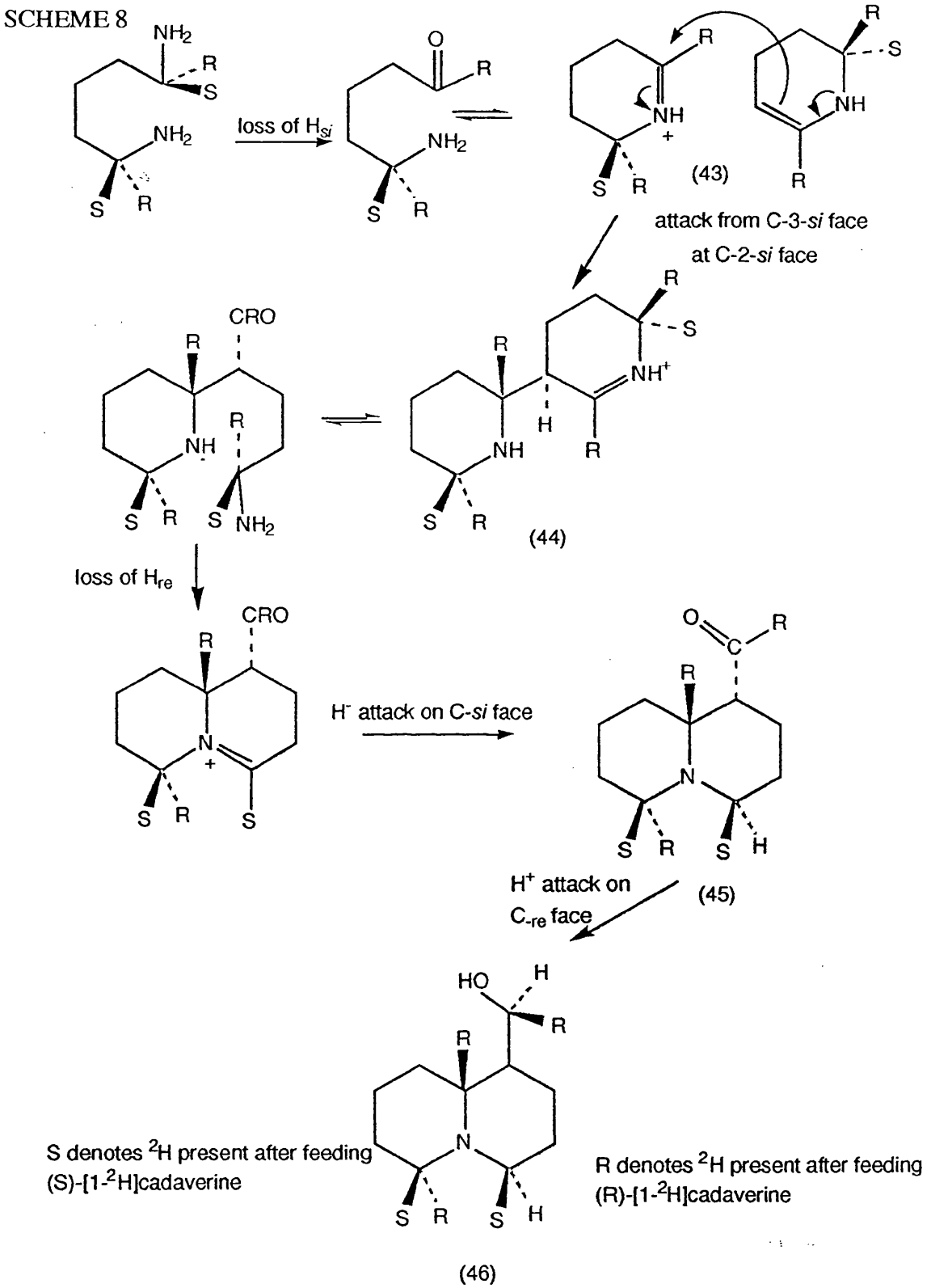
The ^{2}H NMR spectrum of lupinine obtained after feeding the (*R*)-isomer (15) showed that lupinine (41) contained ^{2}H at C-6α, C-10, and C-11 *pro-S*, whereas the (*S*)-isomer (16) produced a sample of lupinine (42) labelled at C-4β and C-6β. The labelling patterns (41) and (42) show that ^{2}H is retained with no change of stereochemistry at C-6 of lupinine after feeding each ^{2}H-labelled precursor. This accords with the results of feeding [^{13}C-^{15}N]cadaverine (36) which established that the N(5)-C(6) bond remains intact (Scheme 6).

After feeding the (S)-[1-^{2}H]cadaverine, the carbons destined to become C-10 and C-11 in lupinine show a loss of ^{2}H. The loss of ^{2}H and the proposed ring closure of cadaverine to form 1-piperideine (43) could occur via transamination or by oxidative deamination. The 1-piperideine formed then couples with another

piperideine molecule obtained from cadaverine to form tetrahydroanabasine (44) in the pathway to lupinine (46) (Scheme 8).[40]

This scheme can be used to explain the loss of the *pro-R* hydrogen from the carbon of cadaverine which becomes C-4 of lupinine and the retention and overall inversion of configuration of the *pro-S* hydrogen at this centre. Reduction of the aldehyde (45) to give lupinine must occur by attack of a hydride donor on the C-*re* face of the carbonyl group which is the usual stereospecificity observed for a coupled dehydrogenase reaction.

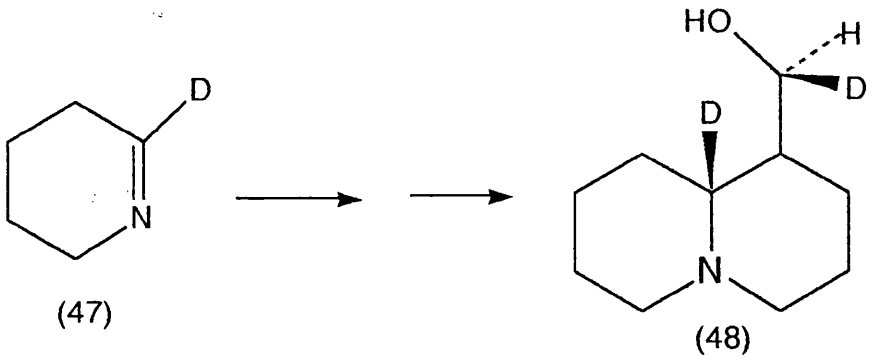
SCHEME 8



Golebiewski and Spenser[36] provided support for the proposed biosynthesis of lupinine (7) via 1-piperideine by feeding [2-^{2}H]-1-piperideine (47) to *L. luteus*

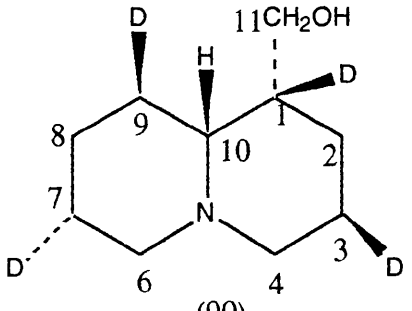
(Scheme 9). The ^{2}H-labelled lupinine (48) contained ^{2}H at C-10 and C-11 *pro-S* as predicted by the route shown in Scheme 8.

SCHEME 9

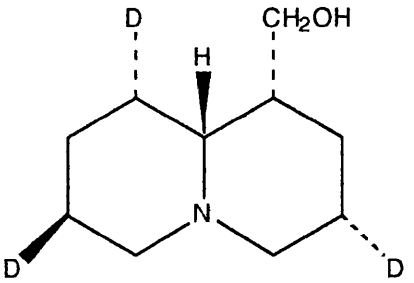


(47) (48)

(*R*)- and (*S*)-[2-^{2}H]cadaverine, (17) and (18), were fed to *L. luteus* and the labelling patterns for lupinine, (90) and (91) respectively, were obtained. Although the chemical shifts for C-7α, C-7β and C-3β are similar it was concluded that the deuterium atoms which appeared at these positions have stayed intact during the biosynthesis with their original stereochemistry. It appears that the quinolizidine ring system is formed by the loss of the *pro-S* hydrogen and retention of the *pro-R* hydrogen at the carbon which becomes C-1 of lupinine. The presence of ^{2}H at C-1 and C-3 of lupinine indicates that no imine-enamine equillibria are involved in the biosynthetic pathway to remove ^{2}H from these positions in lupinine.[78]



(90)



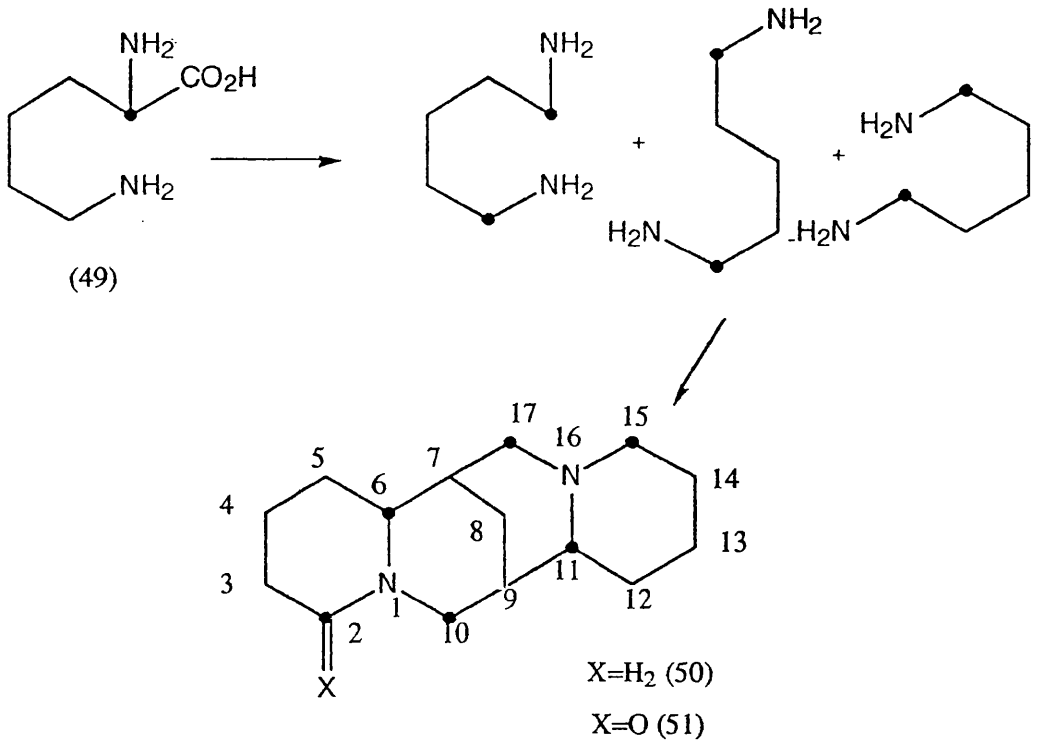
(91)

2.4 BIOSYNTHESIS OF TETRACYCLIC QUINOLIZIDINE ALKALOIDS

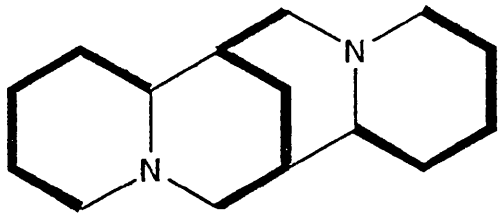
In the 1950's Robinson had proposed that sparteine (50) and (-)-lupanine (51) were produced from three C_5 chains derived from lysine or cadaverine.[27] In addition, he proposed that the nitrogen atoms in the alkaloids were also derived from these same precursors. Robinson's proposals were confirmed by the work of Schutte

and co-workers after feeding [2-^{14}C]lysine (49) in the first tracer experiments carried out on these alkaloids (Scheme 10).[44]

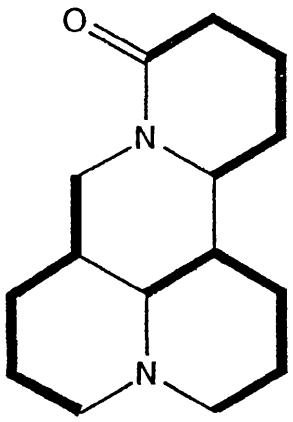
SCHEME 10



Subsequent studies by Leeper and co-workers[45] showed that cadaverine appeared to be incorporated in a symmetrical fashion and with equal efficiency into each of the three parts of the tetracyclic quinolizidine alkaloids sparteine (52) and matrine (53).



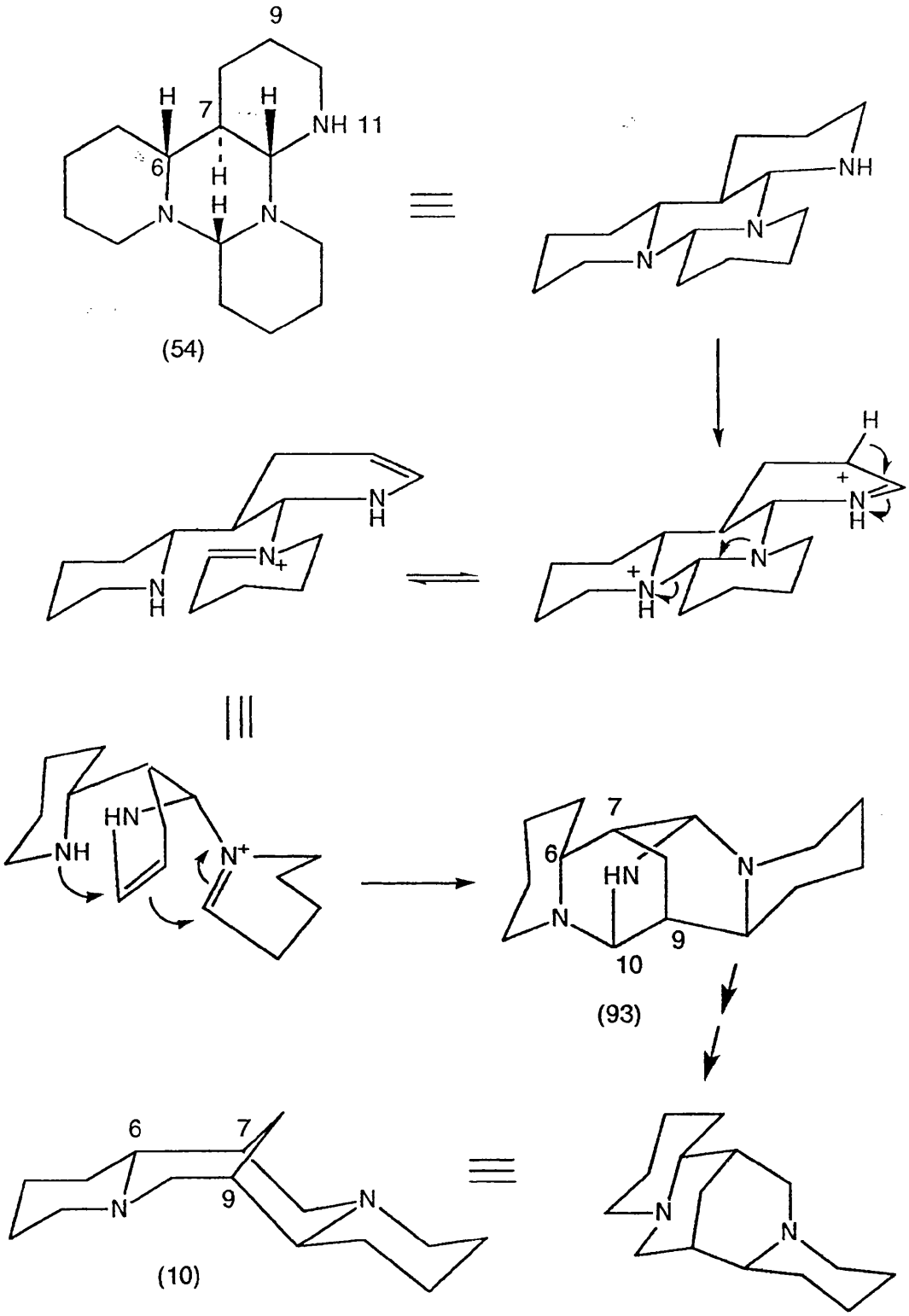
(52)



(53)

The way in which three units of cadaverine combine to form the tetracyclic quinolizidine alkaloids has been the subject of speculation for many years. Golebiewski and Spenser[46] put forward the hypothesis that cadaverine is oxidised to 5-aminopentanal which is in equilibrium with 1-piperideine (first two steps of Scheme 8). 1-Piperideine is known to trimerise readily and the tetracyclic quinolizidine alkaloids might be modified trimers of 1-piperideine (Scheme 11).

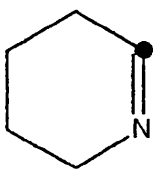
SCHEME 11



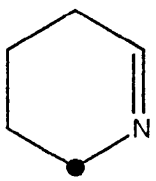
It had been proposed that the stereochemistry of the tetracylic quinolizidine alkaloids was a result of the involvement of the favoured all-*trans* stereoisomer of isotripiperideine (54) in a four step sequence. The stereochemistry of the

"prealkaloid" trimer (93) at three sites, C-6, C-7, and C-9, is determined by the stereochemistry of the trimer of 1-piperideine from which it originates (Scheme 11). The stereochemistry at C-11 is determined by the intramolecular mode of ring closure - i.e. whether attack is on the *re* face or the *si* face at C-11.

This proposal was tested by labelling experiments carried out with [2-^{14}C]-(55) and [6-^{14}C]-1-piperideine (56) on *Lupinus angustifolius*. Partial labelling patterns established by degradation were in accord with this theory.[46] C-17, C-11 and, by inference, C-6 of lupanine (50) are derived from C-2 of 1-piperideine. Likewise C-2, C-15 and, again by inference, C-10 of lupanine are derived from C-6 of 1-piperideine.



(55)



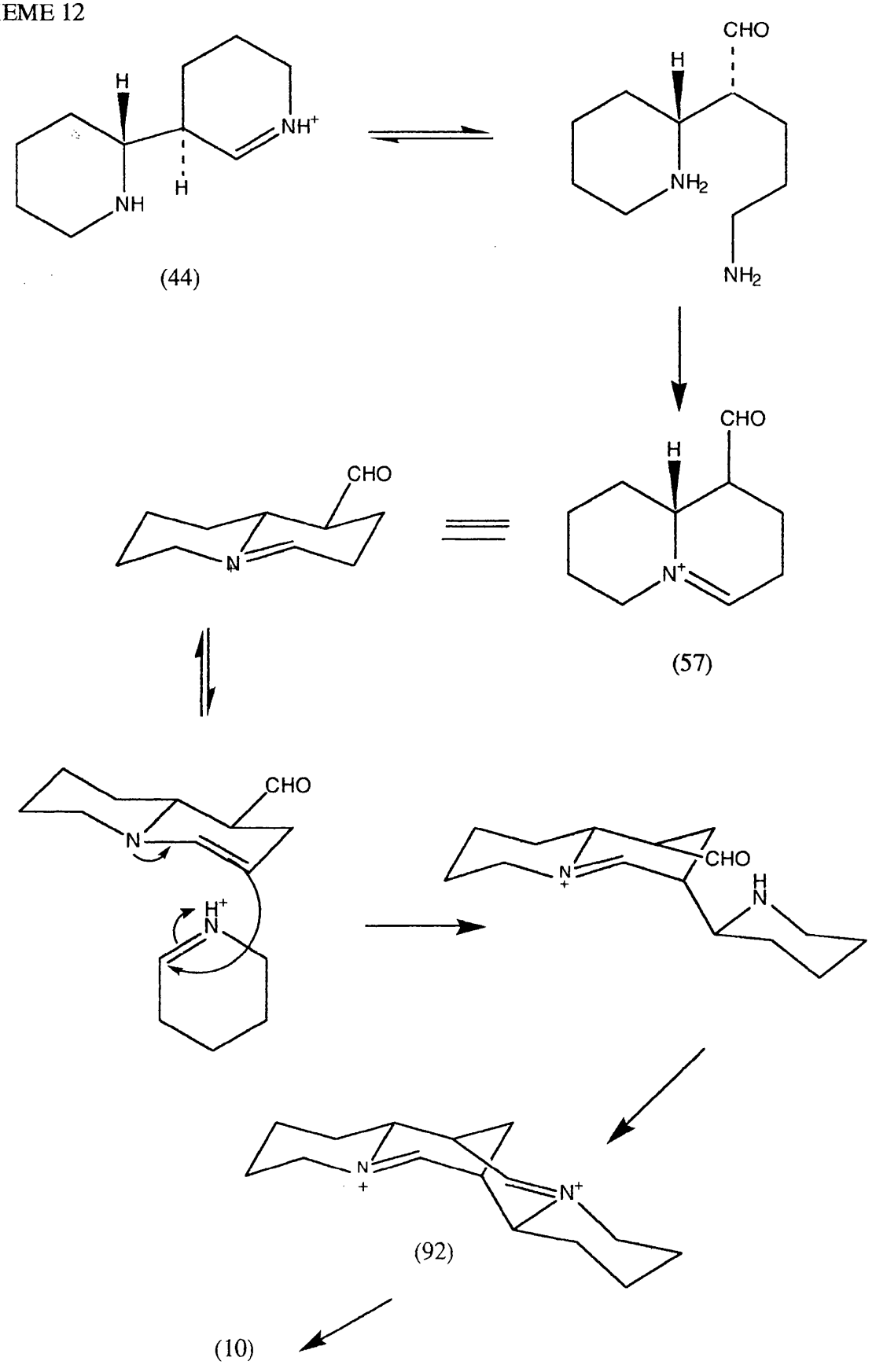
(56)

Spenser and co-workers also fed [6-^{14}C]-1-piperideine (56) to *Sophora tetraptera* and to *S. microphylla* to obtain labelled matrine. A partial labelling pattern was established by degradation which could be explained by a modification of the trimer theory.[45]

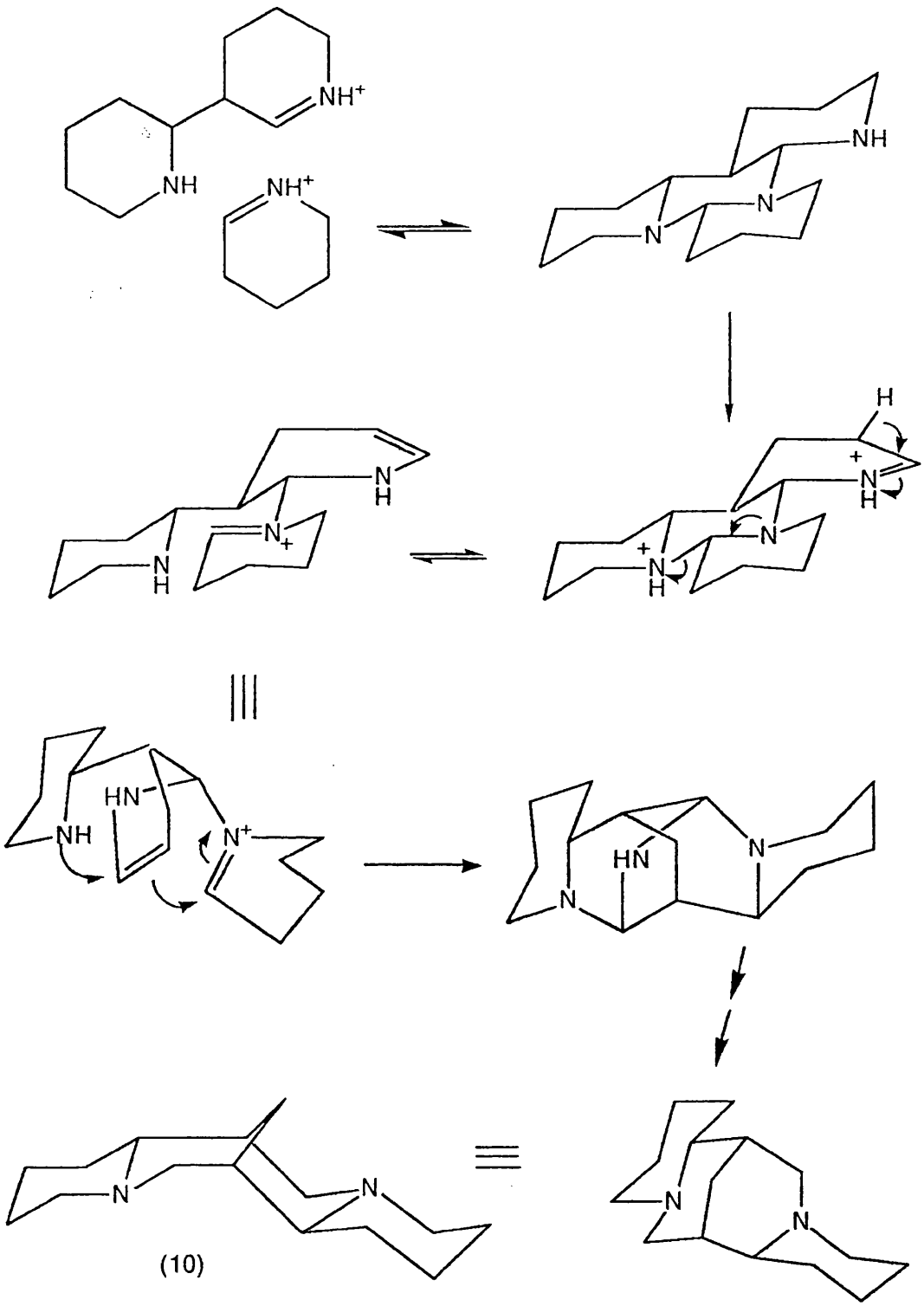
In 1988 a new biogenetic model was proposed by Golebiewski and Spenser[48] based on results of experiments in which they fed DL-[6-^{14}C]lysine (40) and [6-^{14}C]-piperideine (56) to *Lupinus angustifolius*.[49] Chemical degradation of the lupanine extracted showed that the levels of incorporation into two of the C5 monomers were identical. The third C5 unit, however, showed higher incorporation levels. This pattern of incorporation is not possible if three C5 units simultaneously form a trimer on the way to lupanine. Consequently, they put forward a proposal which would incorporate these new findings. This involved modification of the tripiperideine theory based on the initial involvement of two units of piperideine to give tetrahydroanabasine (44), as in the route to lupinine shown in Scheme 8, followed by combination with the third C5 unit.

The way in which the third C_5 unit is combined, however, is the subject of some controversy. Two possible hypotheses have been advanced. The first is that tetrahydroanabasine forms the lupinine aldehyde (57) which then condenses with a third piperideine moiety and goes on to form the tetracyclic product (92) which then forms sparteine (10). The second theory involves the tetrahydroanabasine dimer condensing directly with the third piperideine moiety. This then loses nitrogen, rearranges and eventually forms the tetracyclic product sparteine (10) (Schemes 12 and 13).[50]

SCHEME 12



SCHEME 13

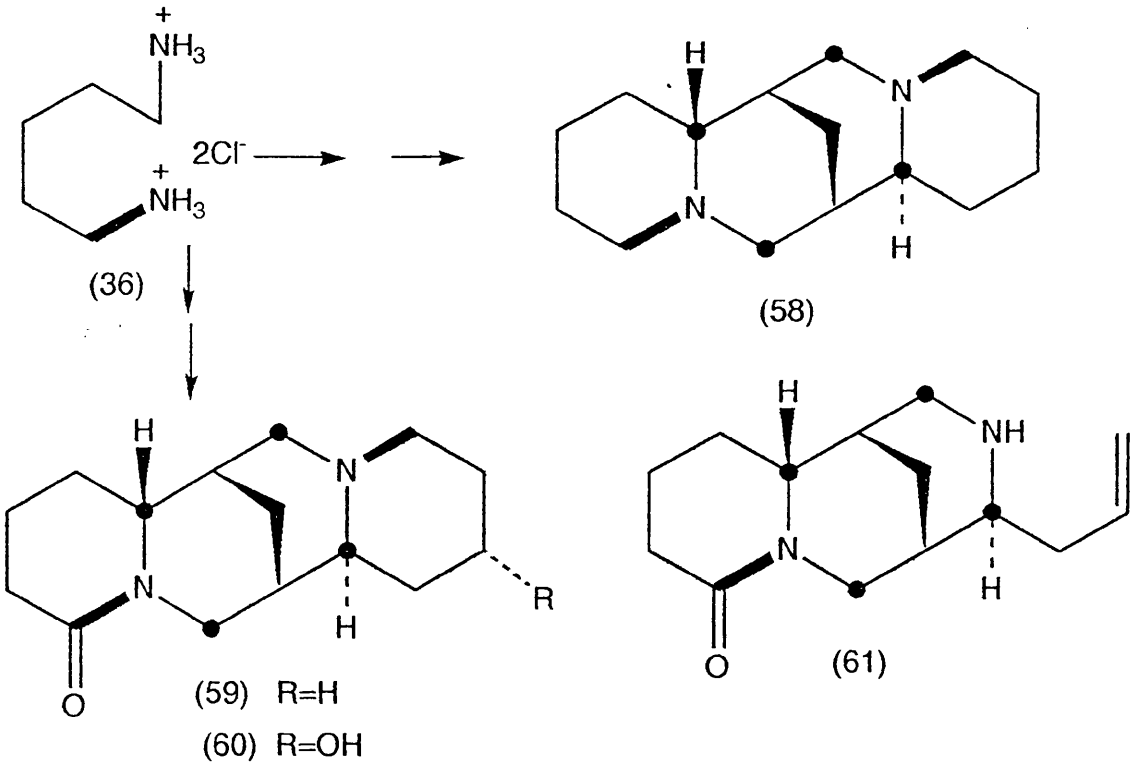


Neither of these theories has, as yet, been fully substantiated and there is still some doubt as to whether 1-piperideine is an intermediate in quinolizidine alkaloid

biosynthesis. In short term experiments, cadaverine was found to be a much better precursor than 1-piperideine or the trimer α-tripiperideine for lupanine in leaf discs of *Lupinus polyphyllus.*[51] Cadaverine was also found to be a much better precursor in similar experiments for sparteine in *L. arboreus.*[52] In these experiments ^{14}C-labelled 1-piperideine and α-tripiperideine, as well as cadaverine, were fed to *L. polyphyllus,* and similarly for sparteine in *L. arboreus.*[53] The incorporation of cadaverine into lupanine was good but incorporation of the other two precursors was up to 60 times less than that of cadaverine. This suggests that 1-piperideine and α-tripiperideine may not be intermediates of lupanine and sparteine although they are incorporated to some degree.

Additional key experiments carried out by the groups of Robins and Spenser[34,54] have contributed to the debate. They fed [^{13}C-^{15}N]cadaverine (36) to *L. luteus* to obtain labelled sparteine (58). Complete labelling patterns for sparteine were obtained by ^{13}C NMR spectroscopy. They found six enriched carbon signals. Only the two ^{13}C NMR signals for C-2 and C-15 adjacent to the nitrogen in sparteine and lupanine showed intense doublets due to ^{13}C-^{15}N coupling (Scheme 14). These results confirmed that three cadaverine units are required to form sparteine and indicated that two of these units are incorporated into the outer rings of (-)- sparteine (58) in a specific manner as shown (Scheme14).

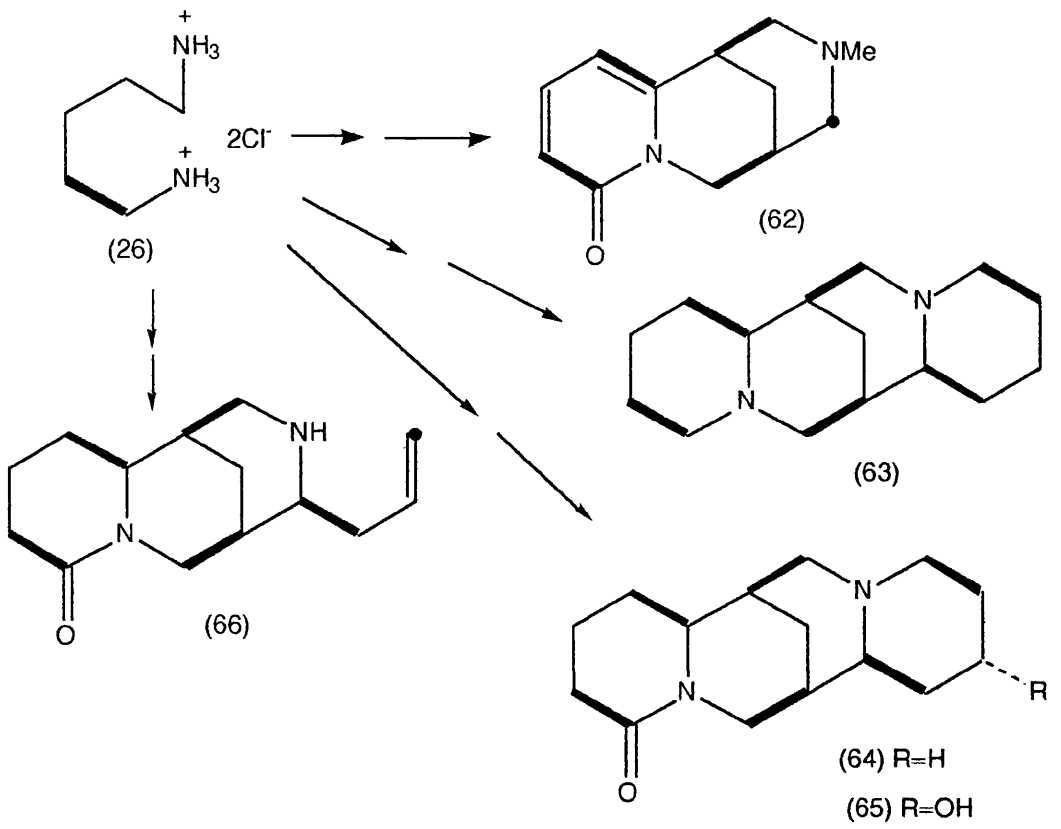
SCHEME 14



Similar labelling patterns were observed in (+)-lupanine (59), (+)-13-hydroxylupanine (60) and (+)-angustifoline (61) when Rana and Robins fed [^{13}C-^{15}N]cadaverine dihydrochloride (27) to *L. polyphyllus* (Scheme 14).[55] The three cadaverine units are incorporated to roughly the same extent in each of these three alkaloids. Angustifoline, unlike the other two, has only one ^{13}C-^{15}N doublet corresponding to position C-2, thus providing further evidence that the two cadaverine units are converted into the outer rings of the tetracyclic quinolizidine alkaloids. The labelling pattern in angustifoline is therefore consistent with its formation from three units of cadaverine *via* a tetracyclic intermediate.[55]

Robins and Sheldrake[26] fed [1,2-$^{13}C_2$]cadaverine dihydrochloride (26) to *L. luteus* and *L. polyphyllus*, plants which produce labelled examples of sparteine (63), lupanine (64), (+)-13-hydroxylupanine (65) and (+)-angustifoline (66).

SCHEME 15



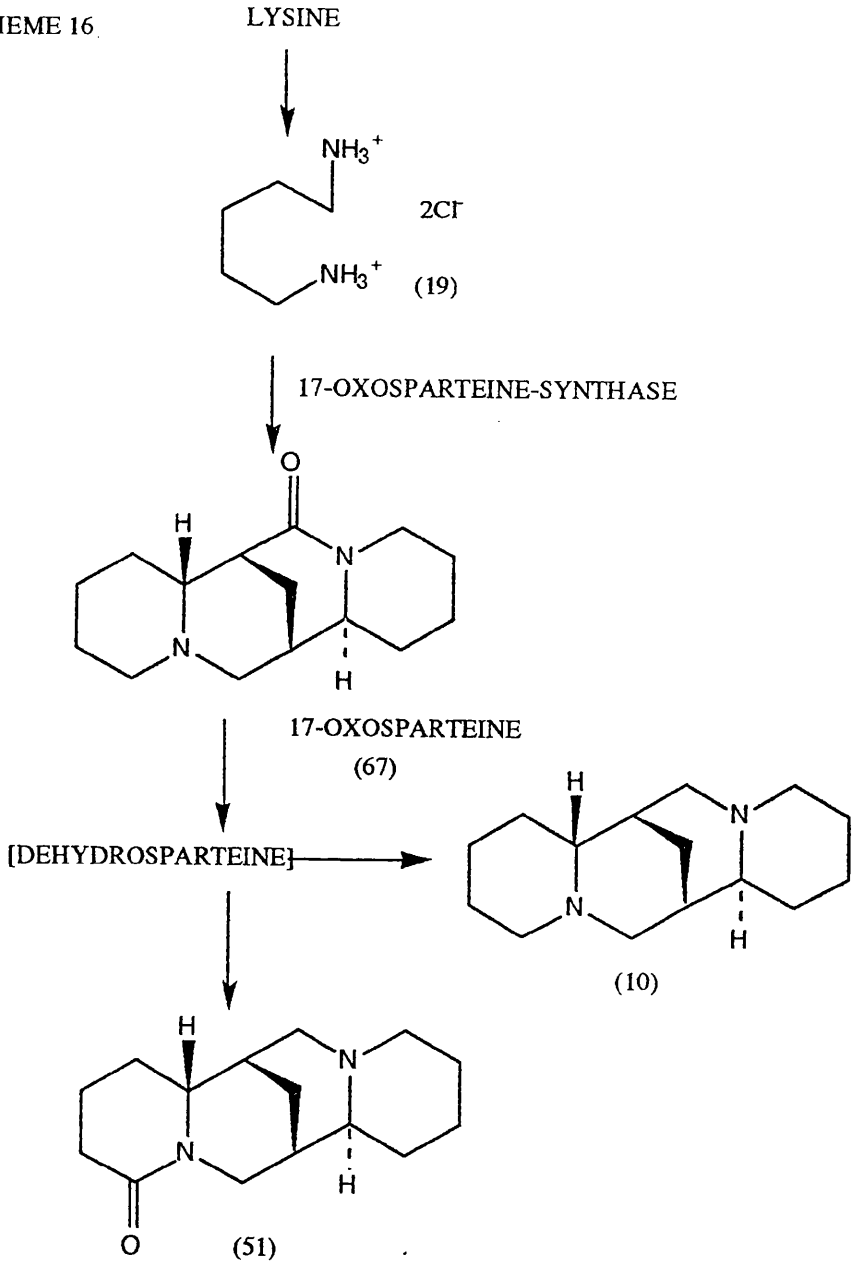
Again it was found that the cadaverine units were incorporated to about the same extent into each part of the individual quinolizidine alkaloids. The labelling pattern in angustifoline was again consistent with the theory that the allyl group is formed by degradation of one ring of a tetracyclic precursor without rearrangement. Finally, Robins and co-workers were able to isolate labelled *N*-methylcytisine (62) by feeding [1,2-$^{13}C_2$]cadaverine dihydrochloride to *B. australis*. Once more the labelling pattern (62) was consistent with degradation of a tetracyclic precursor.[57]

2.5 STEREOCHEMISTRY OF THE ENZYMIC PROCESSES INVOLVED IN THE BIOSYNTHESIS OF TETRACYCLIC QUINOLIZIDINE ALKALOIDS.

As indicated earlier in this Chapter, no intermediates have been firmly established between cadaverine and tetracyclic quinolizidine alkaloids. However, in 1979, Wink and co-workers had postulated an alternative biosynthetic route. They had demonstrated that cadaverine was a substrate for crude enzyme preparations isolated from cell suspension cultures of *L. polyphyllus* and that, in the presence of

pyruvic acid, 17-oxosparteine (67) was isolated.[56,51] Consequently, they concluded that transamination reactions were occurring with pyruvic acid acting as a receptor for the amino groups in cadaverine which were undergoing transamination. As they detected no intermediates in the enzymic process they postulated a series of enzyme-linked intermediates in the enzyme complex, and suggested that (-)-sparteine (10) and lupanine (51) are derived from 17-oxosparteine (67) (Scheme 16).

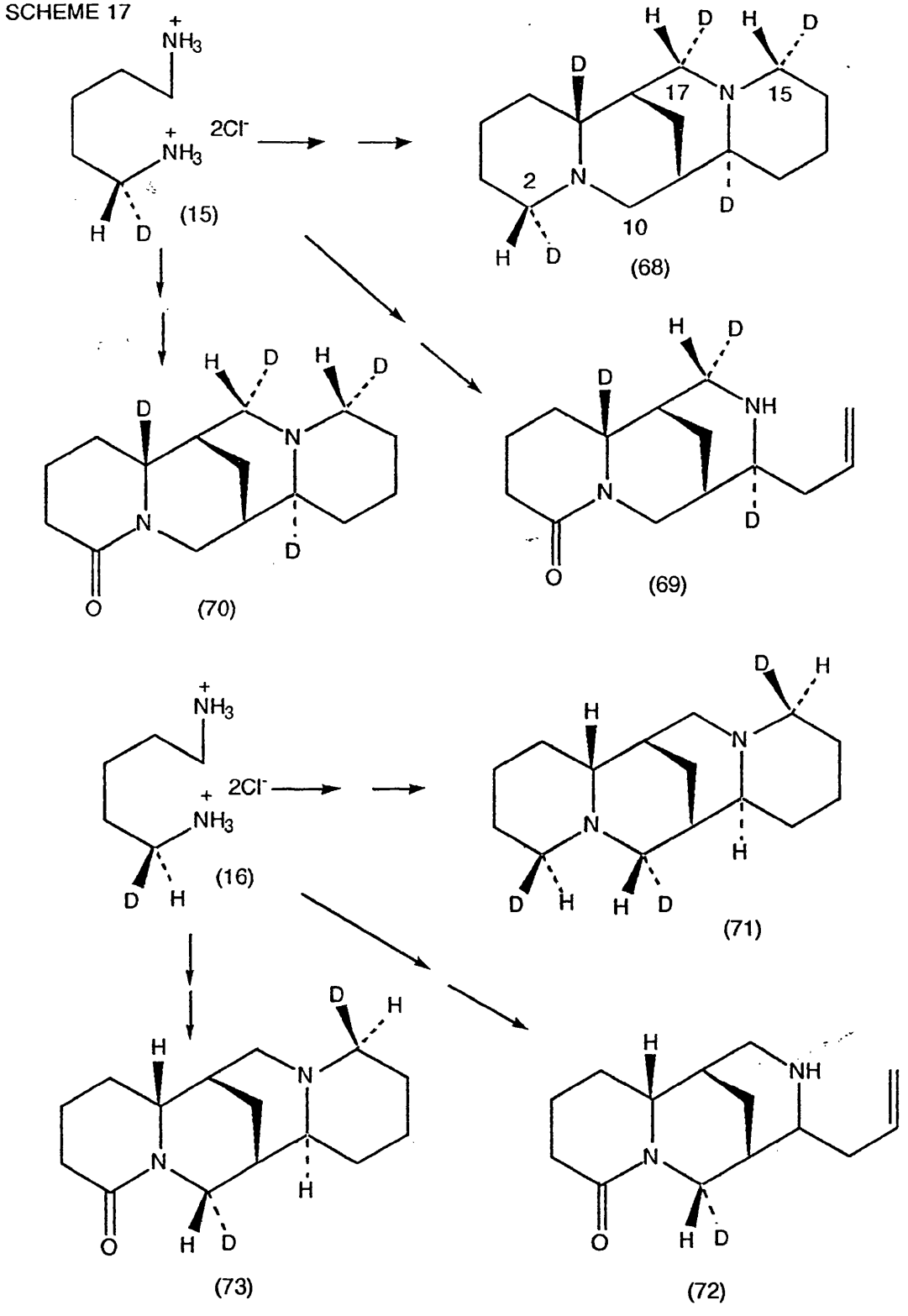
SCHEME 16



Subsequent work by Fraser, and Robins[58] demonstrated that this postulation was not tenable. Fraser and Robins fed samples of (*R*)- (15) and

(*S*)-[1-^{2}H]cadaverine dihydrochloride (16) to *L. luteus* to obtain (-)-sparteine, and to *L. polyphyllus* to obtain lupanine and (+)-angustifoline. Golebiewski and Spenser fed the same precursors to *L. angustifolius* to obtain (+)-lupanine.[52] In both groups the ^{2}H-labelling patterns for sparteine (68) and (71) lupanine (70) and (73) and angustifoline (69) and (72) were obtained by ^{2}H NMR spectroscopy.

SCHEME 17



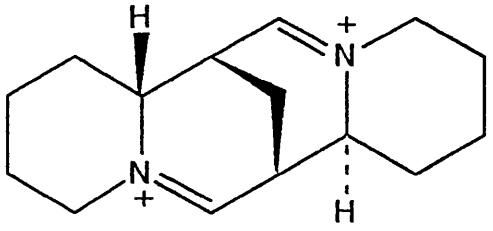
From the composite labelling pattern for (-)-sparteine (68) and (71) it could be seen that where the C-N bonds remain intact, the ^{2}H labels are retained with their original stereochemistry on C-2 and C-15 adjacent to the nitrogen atoms. At the other end of these two cadavarine units oxidation had occurred with removal of the

pro-S hydrogens. In the cadaverine unit providing the central portion of (-)-sparteine the *pro-R* hydrogen is retained at C-17α and the *pro-S* at C-10α. The appearance of ^{2}H at C-17α in sparteine after feeding the (*R*)-isomer (15) disproves the theory that 17-oxosparteine (67) is a key intermediate in sparteine biosynthesis.[58]

The postulate that lupanine is a precursor for sparteine in quinolizidine alkaloid biosynthesis is also disproved because ^{2}H appears at C-2α and C-2β in sparteine, (68) and (71), after feeding both (*R*)- and (*S*)-[1-^{2}H]cadaverine dihydrochlorides.[59]

The mechanisms by which these three units are joined is still not clear. However, the labelling patterns shown in Scheme 17 are consistent with the suggestion that tetracyclic quinolizidine alkaloids are formed by extensive modification of a trimer of 1-piperideine as earlier discussed.

The bis(iminium)ion (74) has been postulated as a late intermediate in this pathway.[48] Stereospecific attack of a hydride donor on the C-*re* faces of both iminium ions would lead to the three quinolizidine alkaloids sparteine, lupanine, and angustifoline labelled with ^{2}H at C-17α after feeding the *(R)*-isomer (15) and with ^{2}H at C-10α from the *(S)* - precursor (16).



(74)

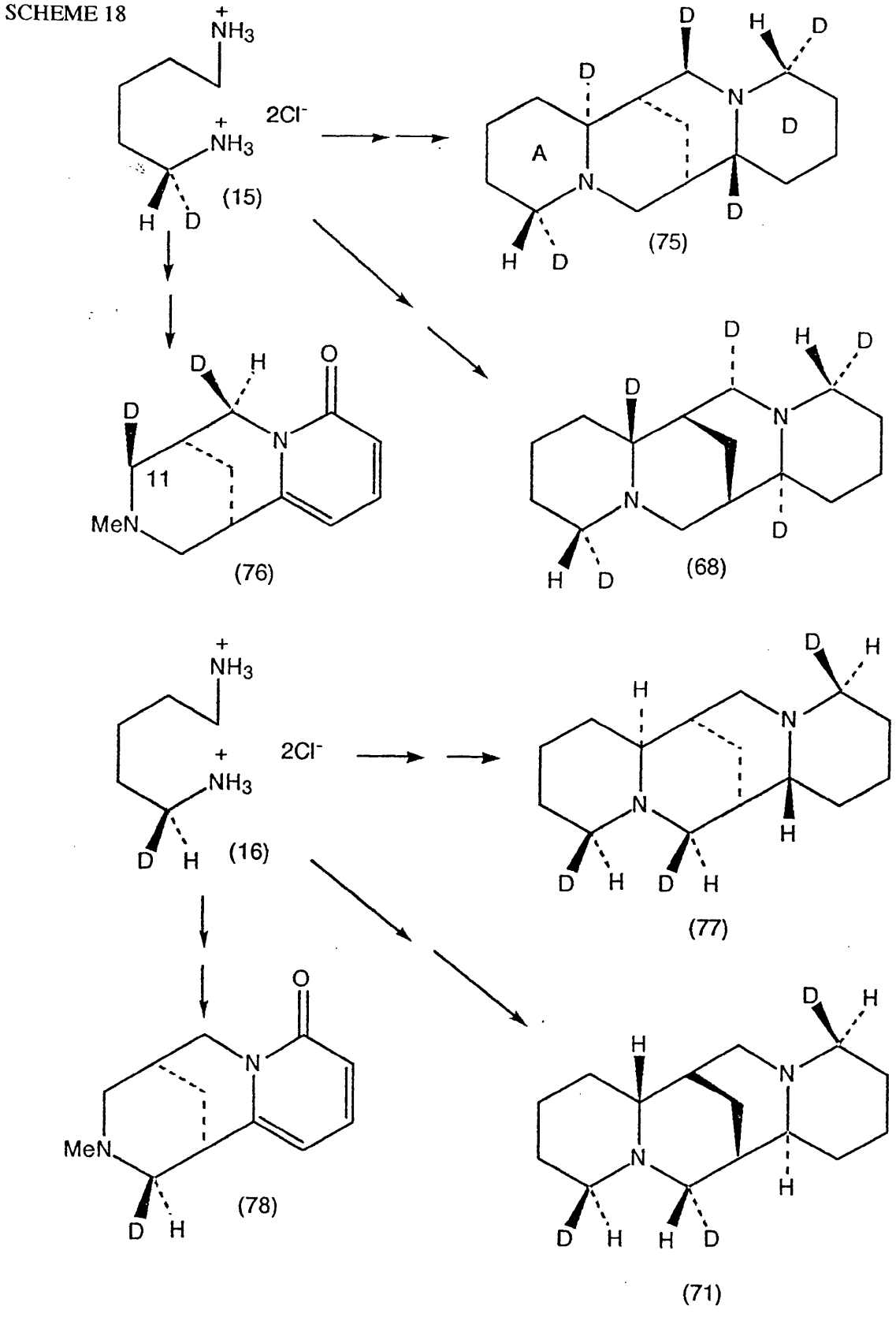
Fraser and Robins[60] fed (*R*)- and (*S*)-[1-^{2}H]cadaverine dihydrochloride to *Baptisia australis* to obtain ^{2}H-labelled (+)-sparteine (75) and (77) and (-)-*N*-methylcytisine (76) and (78).[59] Scheme 18 shows the labelling patterns obtained for these alkaloids after feeding the (*R*)- and (*S*)-[1-^{2}H]cadaverines and shows a comparison with the labelling patterns for (-)-sparteine (68) and (71). Comparison of the labelling patterns for (+)-sparteine and (-)-sparteine showed that the stereochemistry of the deuterium atoms at C-2 and C-15 was preserved as these

C-N bonds remain intact, whereas the rest of the labels are in mirror image positions. The overall labelling patterns in (+)-and (-)-sparteine are not mirror images.

Comparison of the labelling patterns for (+)-sparteine and (-)-*N*-methylcytisine (Scheme 18) suggest that it is ring A of a tetracyclic precursor that must be degraded and ring D that is converted into a pyridone to account for the observed position and stereochemistry of the ^{2}H labels at C-10 and C-17. The ^{2}H present at C-11 of (-)-*N*-methylcytisine (76) after feeding the (*R*)-isomer (15) is retained on the cleavage of ring A but its stereochemistry is inverted. This could arise by reduction of an intermediate C(11)-N(12) iminium ion stereospecifically from the *C-re* face.

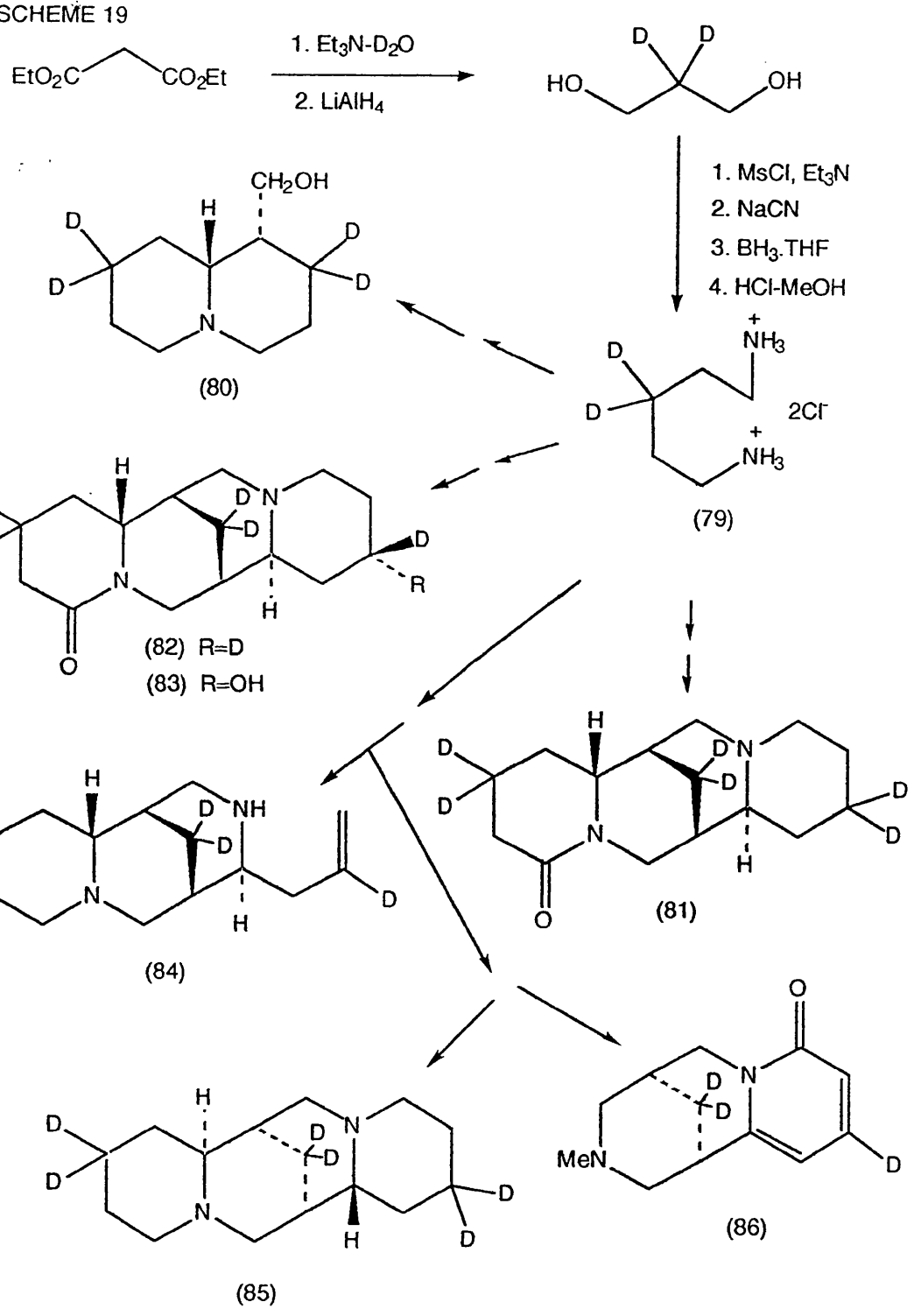
No ^{2}H label is present in the *N*-methyl group of (-)-*N*-methylcytisine (76), (78) after feeding the (*R*)- and (*S*)-precursors (Scheme 18). This indicates that the *N*-methyl group is not formed from C-1 of a cadaverine precursor and is consistent with the existence of an *N*-methyltransferase in *Laburnum anagyroides* which can convert (-)-cytisine (8) into (-)-*N*-methylcytisine (9).[24]

SCHEME 18


+
NH3
+
NH3
2Cl-
(15)
H
D
D
H
D
A
N
D
D
H
D
(75)
D
H
O
D
D
N
11
MeN
(76)
D
H
D
D
N
D
N
D
H
D
(68)
+
NH3
+
NH3
2Cl-
D
H
(16)
H
N
D
H
D
H
H
(77)
O
N
MeN
D
H
(78)
D
H
H
N
D
H
H
D
H
(71)

Robins and Sheldrake[61] also prepared [3,3-$^2H_2$]cadaverine dihydrochloride (79) as shown in Scheme 19. This was incorporated into a range of quinolizidine alkaloids and the ^2H labelling patterns were established by ^2H NMR spectroscopy.

SCHEME 19



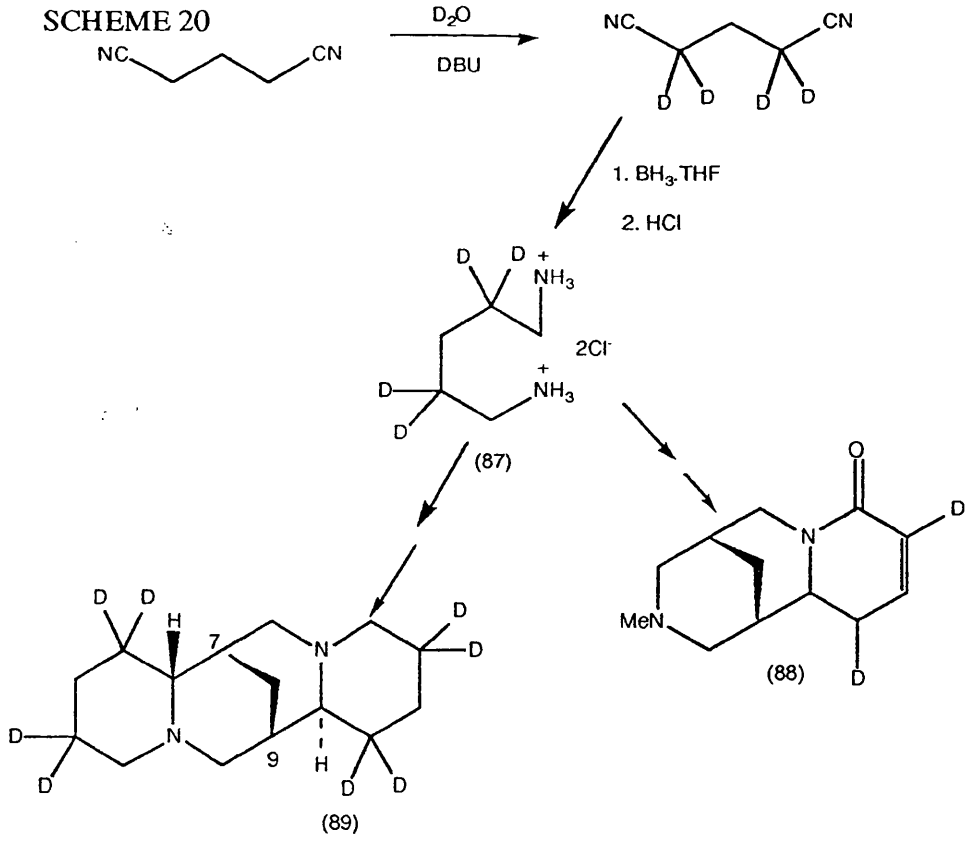
Four ^{2}H atoms were retained in lupinine (80) and ^{2}H was retained at C-8α and C-8β in (-)-sparteine (81), lupanine (82), 13-hydroxylupanine (83), angustifoline (84),[61] (+)-sparteine (85), and (-)-*N*-methylcytisine (86).[62] This shows that no hydrogen atoms are removed from this position in the biosynthesis.

The presence of ^{2}H at C-13 in 13-hydroxylupanine (83) shows that introduction of oxygen at this position does not involve keto or enol intermediates, and it is again consistent with its derivation from a tetracyclic precursor. The ^{2}H label present at C-4 of (-)-*N*-methylcytisine (86) shows that no keto or enol intermediate is involved in the formation of the double bond at C-4 in the pyridone.

Hemscheidt and Spenser[63] prepared [3,3-$^{2}H_2$]cadaverine by a different route and obtained similar labelling patterns in lupanine (82) and 13-hydroxylupanine (83) after carrying out feeding experiments with *L. angustifolius.*

When [2,2,4,4,-$^{2}H_4$]cadaverine dihydrochloride (87) was prepared and fed to *Baptisia australis*[62] (Scheme 20), (-)-*N*-methylcytisine (88) contained ^{2}H labels with equal enrichment at C-3 and C-5 only. Again this demonstrates that no keto or enol units are involved during the formation of the pyridone at these carbon atoms. Moreover, in the ^{2}H NMR spectra of (+)-sparteine (89) and (-)-*N*-methylcytisine (88) no ^{2}H is present at the bridgehead positions C-7 and C-9. This suggests that enamine-imine equilibria are involved in the biosynthetic pathway to remove ^{2}H from these positions in both alkaloids

SCHEME 20



2.6 SUMMARY

The use of precursors labelled with stable isotopes and the advent of technological advances such as highfield NMR spectroscopy has greatly enhanced our understanding of the biosynthesis of quinolizidine alkaloids. Nevertheless there are still gaps in our knowledge. It is now clear that the quinolizidine alkaloids are formed from lysine via cadaverine and the biosynthetic pathway to lupinine has been established. In the case of the tetracyclic alkaloids which have been discussed there are still areas of ambiguity. It is not yet clear, for example, whether a piperideine intermediate is involved in the transformation from cadaverine to alkaloid.

Two alkaloids which have not been discussed in this review are matrine (12) and anagyrine (11). Although these have been the subject of considerable research interest in the last few years, they are still relatively poorly understood. In the next Chapter the research dealing with matrine and anagyrine biosynthesis will be reviewed. Also discussed are the feeding experiments on matrine undertaken in this work.

CHAPTER 3

THE BIOSYNTHESIS OF MATRINE AND ANAGYRINE

3.1 INTRODUCTION

Many of the biosynthetic studies which have already been carried out on the quinolizidine alkaloids have concentrated on lupinine (7), lupanine (51) and (+)-sparteine (13). Some work has been done on the pyridone alkaloids (-)-*N*-methylcytisine (9) and (-)-cytisine (8). Much less work has been carried out on matrine (12) and anagyrine (11).

Both matrine (12) and anagyrine (11) have been shown to have cadaverine as a precursor in their biosynthesis. This Chapter reviews what is currently known about the biosynthesis of anagyrine (11) and matrine (12). It discusses the synthesis and feeding of (*R*)- and (*S*)-[1-^{2}H]cadaverine dihydrochlorides, (15) and (16) respectively, to *Sophora microphylla,* and indicates the results obtained. The aim of the feeding experiments was to obtain good labelling patterns for matrine (12) by use of ^{2}H NMR spectroscopy, and hence to gain information about the stereochemistry of the enzymic processes involved in matrine biosynthesis.

3.2 THE BIOSYNTHESIS OF ANAGYRINE

Anagyrine occurs in several species of *Lupinus* and *Genista* and has been extracted from gorse. To date, relatively little research has been conducted on anagyrine. In 1961 Okudo and co-workers[64] determined its absolute configuration, but it was the late 1980's before Brown[65] carried out the first biosynthetic studies on anagyrine, and Rycroft and co-workers[66] established the complete assignment of the ^{1}H NMR spectrum of (-)-anagyrine (11).

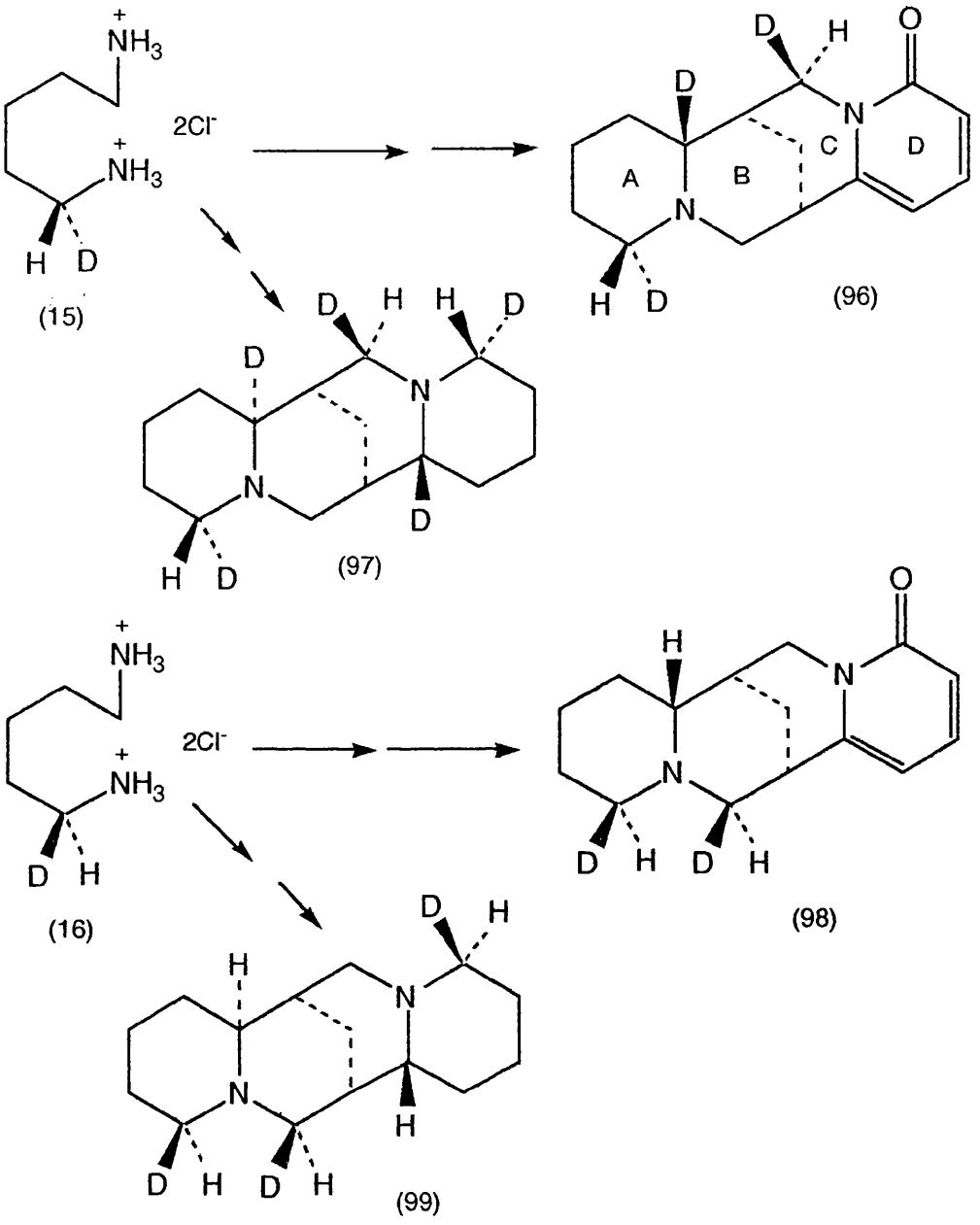
As indicated in Chapter Two (Scheme 20), previous work by Robins and Sheldrake had established labelling patterns in (+)-sparteine (89) and (-)-*N*-methylcytisine (88) by feeding [2,2,4,4-^{2}H$_4$]cadaverine (87) to *Baptisia australis.*[62] They found that ^{2}H is retained at each of the pyridone carbons, C-3 and C-5 in (-)-*N*-methylcytisine, and that ^{2}H is completely lost from the carbons which become bridgeheads, C-7 and C-9, in both alkaloids. The labelling patterns are

consistent with formation of (-)-*N*-methylcytisine by cleavage of ring A and conversion of ring D of a tetracyclic precursor into a pyridone. Fraser and Robins[60] were able to demonstrate that (+)-sparteine and (-)-*N*-methylcytisine are formed from a common tetracyclic intermediate, and they suggested that this might be the bis(iminium)ion (74).

Robins and Brown[57] compared the structure and stereochemistry of (-)-anagyrine (11) with that of (+)-sparteine (13) and postulated that if they are formed from a tetracyclic intermediate with the same absolute configurations at C-6 and C-11, then it is likely that ring A of the tetracyclic intermediate is converted into a pyridone in order to form anagyrine (11).

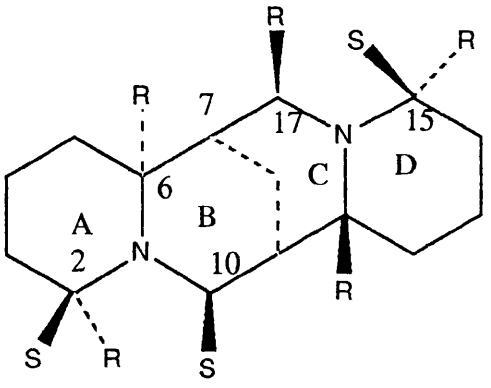
Robins and Brown tested this theory by feeding (*R*)- and (*S*)-[1-^{2}H]cadaverine dihydrochlorides, (15) and (16), to *Anagyris foetida*, a plant which produces (-)-anagyrine (11) as the major alkaloid together with (-)-*N*-methylcytisine (9). The ^{2}H-labelling patterns for anagyrine, (96) and (98), were established by ^{2}H NMR spectroscopy and compared with those obtained for (+)-sparteine, (97) and (99), (Scheme 22).

SCHEME 22

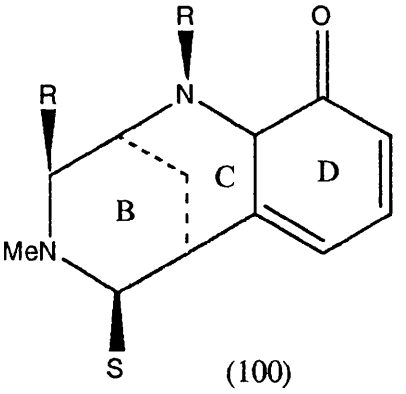


From these labelling patterns it appeared that if (+)-sparteine and (-)-anagyrine are formed from the same tetracyclic intermediate, then it must be ring D and not ring A that is converted into a pyridone. This is the same orientation as that required for the formation of the pyridone in (-)-*N*-methylcytisine (100) and is the opposite result to that postulated. This is because conversion of ring D of the tetracyclic intermediate required to form (+)-sparteine (101) into a pyridone would require inversion of configuration at C-6. Thus Robins and Brown have suggested that the tetracyclic intermediate involved in (-)-anagyrine formation has different

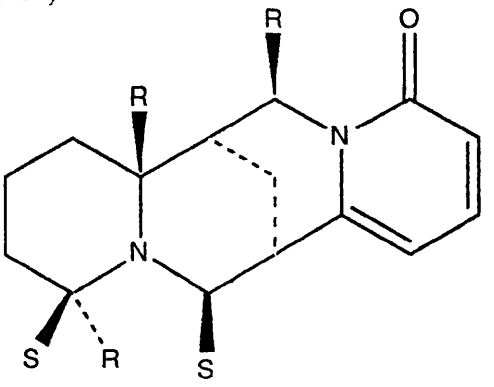
stereochemistry from (+)-sparteine at C-6. What has not been established is whether (-)-anagyrine (102) and (-)-*N*-methylcytisine (100) are both formed from the same tetracyclic intermediate and different to that used in the formation of (+)-sparteine (101), or whether the formation of (-)-anagyrine from a tetracyclic intermediate with the stereochemistry of the A-B ring junction opposite to that shown in (+)-sparteine prevents further metabolism of (-)-anagyrine to (-)-*N*-methylcytisine. Composite labelling patterns (101), (100) and (102) are shown below.



(101)

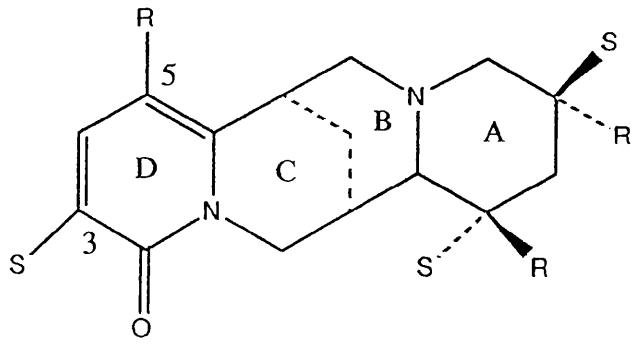


(100)



(102)

Brown also fed (*R*)- and (*S*)-[2-^{2}H]cadaverine dihydrochlorides, (17) and (18) respectively, to *Anagyris foetida* to establish the proton removal at positions C-3 and C-5 of (-)-anagyrine.[65] The signals obtained were very weak and mainly due to the natural abundance deuterium. The spectrum obtained from anagyrine fed with the (*R*)- precursor showed three small enriched signals at δ 5.97, 1.64 and 1.20, corresponding to positions 5, 12β and 14α, while that from the (*S*)-precursor feed contained slightly enriched signals at δ 6.41, 1.85 and 1.16, corresponding to positions 3, 12α and 14β. The composite labelling pattern is shown below (103).

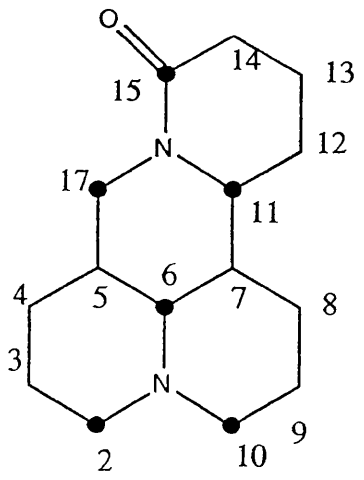


(103)

It is the *pro-R* proton that is retained at the carbon atom which becomes C-5 in anagyrine, and it is the *pro-S* proton that is retained at the C-3 position in the formation of the pyridone ring. However, the weakness of the signals obtained make this a tentative rather than a definitive assignment. Clearly there is much still to be done in establishing complete labelling patterns for anagyrine.

3.3 THE BIOSYNTHESIS OF MATRINE

Interest in the synthesis of matrine has been aroused because of its known antiulcer properties.[68] Schutte and co-workers were among the first to investigate the biosynthesis of matrine.[69] On feeding [1,5-^{14}C]cadaverine (22) and [2-^{14}C]lysine (49) to *Sophora tetraptera* they found that they were both incorporated. This enabled a sample of matrine to be obtained, degraded and the labelling pattern (104) deduced.

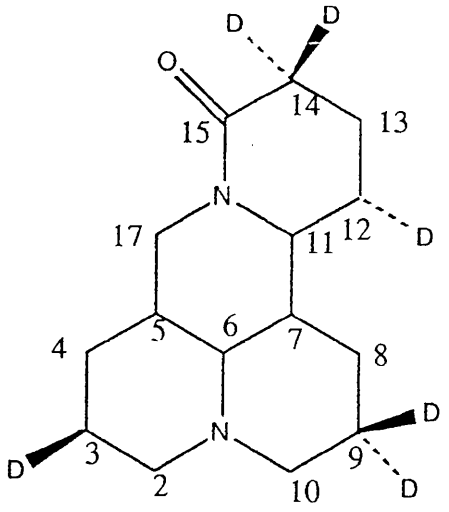


● ^{14}C

(104)

In 1965 the first synthesis of racemic matrine in low yields was carried out by Mandell and co-workers.[70] In 1986 a stereoselective synthesis in reasonable yields was reported by Chen and co-workers.[71] The same group also used NMR spectroscopy to analyse the stereochemistry and structure of matrine (104).[72]

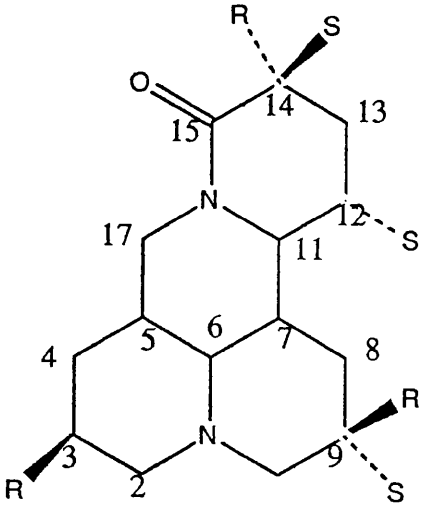
Brown subsequently undertook a study of the processes involved in matrine biosynthesis by preparing labelled cadaverines and feeding them to *Sophora microphylla*, a plant which produces matrine.[65] When [2,2,4,4-$^{2}H_4$]cadaverine dihydrochloride (87) was fed to *S. microphylla*, the sample of matrine obtained was found to contain ^{2}H labels at 14β, 14α, 3β, 9β, 9α and 12α. This corresponds to the labelling pattern (105).



(105)

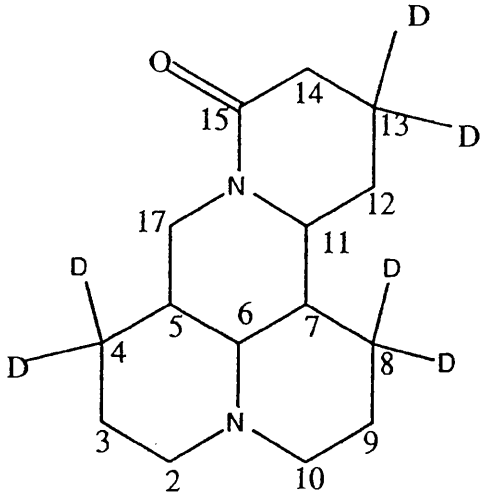
Thus, there are processes occurring in the biosynthetic pathway which result in the removal of the deuterium labels at 12β and 3α specifically, and the removal of the bridgehead labels at C-5 and C-7.

In the same study Brown also fed (*R*)- (17) and (*S*)-[2-^{2}H]cadaverine dihydrochloride (18) to *S. microphylla.* The (*R*)-[2-^{2}H]cadaverine produced a sample of matrine with signals at δ 2.23, 2.07 and 1.64, corresponding to positions 14α, 9β and 3β of matrine. The (*S*)-[2-^{2}H]cadaverine feed gave signals at δ 2.57, 1.82 and 1.51, corresponding to positions 14β, 9α, and 12α of matrine (106).



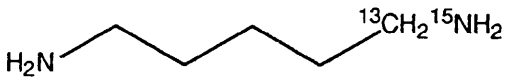
(106)

Brown[65] also fed [3,3,-$^{2}H_2$]cadaverine (79) to the same plants and obtained a ^{2}H NMR spectrum consistent with matrine labelled at 4β, 13α, 8β, 8α, 13β and 4α (107). None of the deuterium atoms are lost, therefore enzymic processes involving proton removal and replacement do not occur at these positions.



(107)

A further feeding experiment was carried out by Brown[65] using [1-amino-^{15}N, 1-^{13}C]cadaverine (108) in an attempt to establish a complete labelling pattern in matrine using ^{13}C NMR spectroscopy, and to show which C-N bonds from cadaverine remain intact during the biosynthetic pathway. However only spectra with poor ^{13}C enrichment were obtained, possibly due to dilution of labelled alkaloid with endogenous unlabelled material.

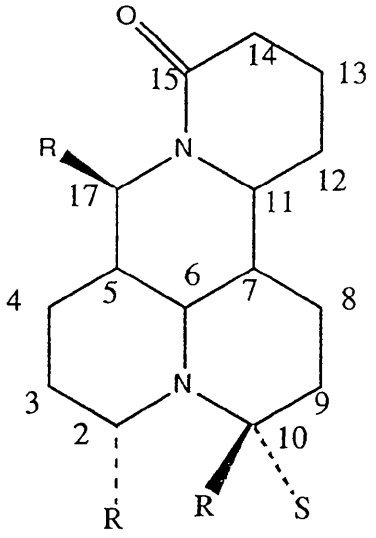


2HCl

(108)

Brown[65] also prepared (*R*)- (15) and (*S*)-[1-^{2}H]cadaverines (16) which were fed to *S. microphylla* with a view to establishing complete labelling patterns for matrine. However the ^{2}H NMR spectra of matrine gave very small signals. Those from the (*R*)-[1-^{2}H]cadaverine run in chloroform produced small enriched signals at δ 3.24, 2.28, and 2.01, corresponding to positions 2α, 17β and 10β of matrine (109). The ^{2}H NMR spectrum of matrine from the (*S*)-precursor feed, which was run in both

benzene and chloroform contained only one small deuterium signal. This appeared at δ 1.65 in benzene and δ 1.99 in chloroform. In published data this peak corresponds to position 10α of matrine (109).



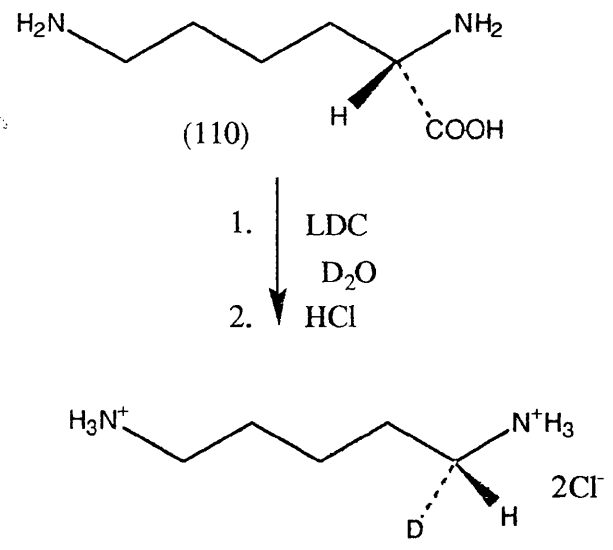
(109)

Because of the lack of clarity in the results obtained, Brown subsequently repeated these experiments. On this occasion specific ^{2}H incorporations were even lower and this was reflected in poorer deuterium NMR spectra.

3.4 SYNTHESIS OF (*R*)- AND (*S*)-[1-^{2}H]CADAVERINE DIHYDROCHLORIDES

Because of the inconclusive nature of Brown's findings it was desirable to repeat the feeding experiments in an attempt to obtain samples of matrine with higher enrichments of deuterium. (*R*)- (15) and (*S*)-[1-^{2}H]cadaverine dihydrochlorides (16) were synthesised by a known route.[43] (*R*)-[1-^{2}H]Cadaverine was synthesised by placing (L)-Lysine (110) in a flask with D_2O, a phosphate buffer, and lysine decarboxylase (LDC) in a constant temperature 37 ^{o}C shaking bath for two days before working up, involving acidification and purification, to give the deuteriated (*R*)-[1-^{2}H]cadaverine dihydrochloride (15) (Scheme 23).

SCHEME 23

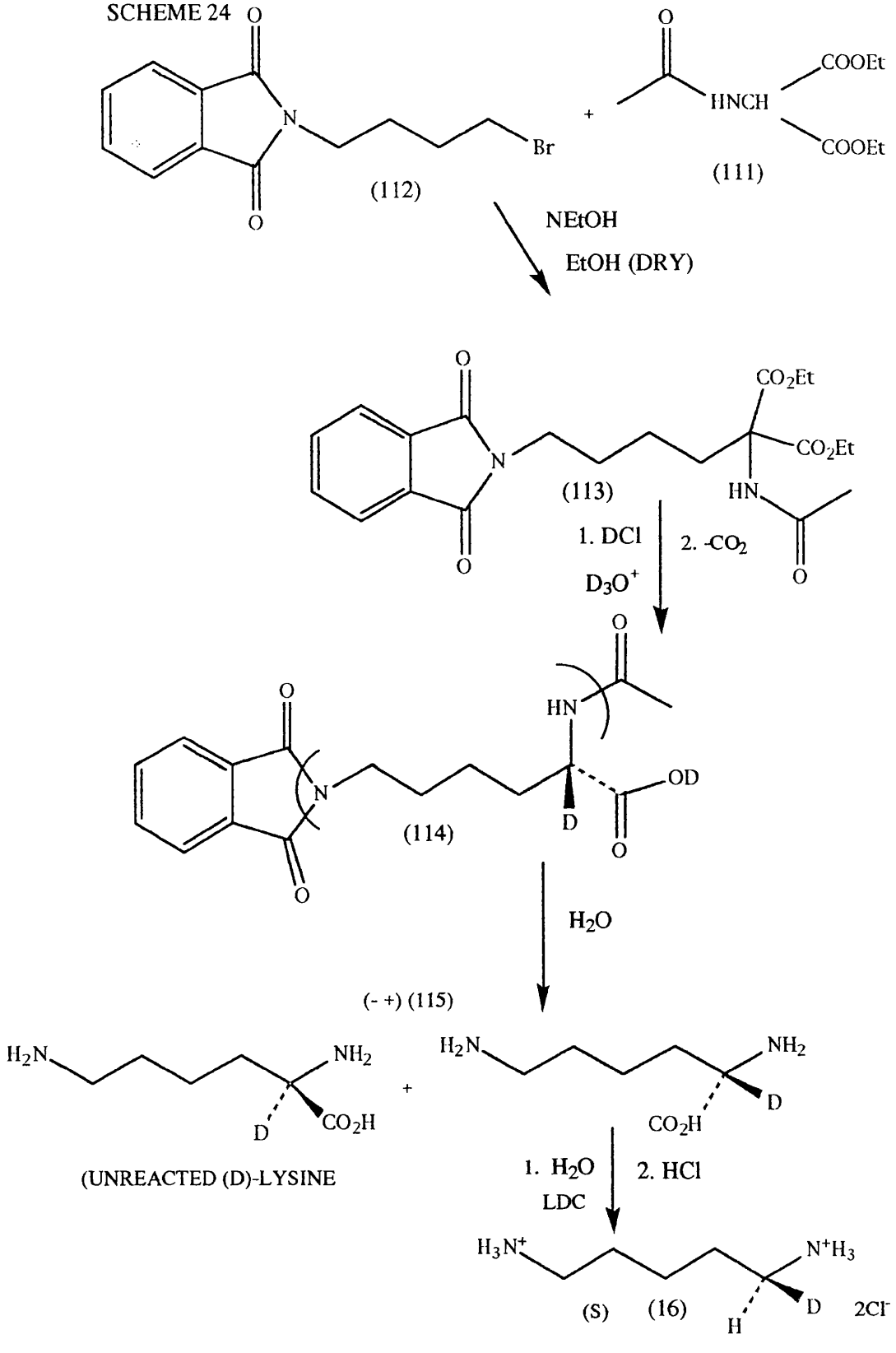


(15)

Synthesis of (*S*)-[1-^{2}H]cadaverine dihydrochloride (16) is shown in Scheme 24. Diethyl acetamidomalonate (111) was treated with *N*-(4-bromobutyl)phthalimide (112) in ethanol to give diethyl 2-acetamido-2-(4-phthalimidobutyl)malonate (113) which was treated with deuterium hydrochloride 37% w/w in D_2O to give (114) and then hydrolyzed to give (DL)-[2-^{2}H]lysine (115). This racemate was then incubated with lysine decaboxylase, in water this time, again at 37 ^{o}C for three days. After work-up and acidification (*S*)-[1-^{2}H]cadaverine dihydrochloride (16) was obtained. (D)-Lysine is left after the reaction also since the enzyme only catalyses the decarboxylation of the (L)-lysine.

Radioactive cadaverine was also made. [U-^{14}C]-L-Lysine was diluted with unlabelled material and incubated with lysine decarboxylase in water. The cadaverine produced was divided into fifteen sample phials and left to dry in a desiccator with phosphorus pentoxide. This gave equal amounts of ^{14}C cadaverine in each phial (123333 Bq).

SCHEME 24


(112)
(111)
NEtOH
EtOH (DRY)
(113)
1. DCl
2. -CO2
D3O+
(114)
H2O
(- +) (115)
(UNREACTED (D)-LYSINE
1. H2O
LDC
2. HCl
(S)
(16)
2Cl-

3.5 FEEDING OF [1-^{2}H]CADAVERINES TO *SOPHORA MICROPHYLLA*

The (*R*)- and (*S*)-[1-^{2}H]cadaverine dihydrochlorides were split into separate batches of approximately 20 mg each and dissolved in water. These solutions were fed to two sets of *S. microphylla* plants by the wick method, each plant receiving 2.5 mg of feed on alternate days for ten days. On the third feeding day both sets of plants were also given ^{14}C cadaverine. After a further 10-14 days the leaves and roots of the *Sophora* plants were extracted and the alkaloids isolated.

During the feeding period some of the plants grew sickly and died. For these plants feeding was discontinued before the end of the feeding period, especially those fed with the (*S*)-cadaverine, as their poor condition made it pointless to continue feeding. This was probably due to giving too much cadaverine too quickly. In subsequent trials the same quantity of feed was given, but over a considerably longer feeding period.

Due to the extraction being predominantly of dead leaves, little alkaloid was obtainable (Wink and Hartmann[19] have established that the alkaloid content of leaves is considerably reduced as the plant ages and moves towards death).

Incorporations of cadaverine in the extracted alkaloid mixture were determined by liquid scintillation counting. This showed a total incorporation of 3.9% of the ^{14}C cadaverine in the (*R*)- feed and a total incorporation of 3.3% in the (*S*)- feed. The lower incorporation of the (*S*)-isomer was probably due to the greater number of plant deaths in that feeding group. These incorporations were only slightly better than the values obtained by Brown in her second feed with (*R*)- and (*S*)-[1-^{2}H]cadaverine.

^{2}H NMR spectra for both alkaloid mixtures in both chloroform and benzene as the solvents were obtained. Both show deuterium incorporation, with the solutions in benzene giving slightly clearer spectra. TLC of the two mixtures showed four spots with Rf values corresponding to the alkaloids cytisine (8), *N*-methylcytisine (9), matrine (12), and anagyrine (11). Separation of the alkaloids using preparative TLC gave too low a quantity of matrine to characterise.

The experiment was repeated the following year but again the plants grew sickly, feeding was discontinued, and no further work was attempted in this area.

3.6 A POSSIBLE BIOSYNTHESIS OF MATRINE

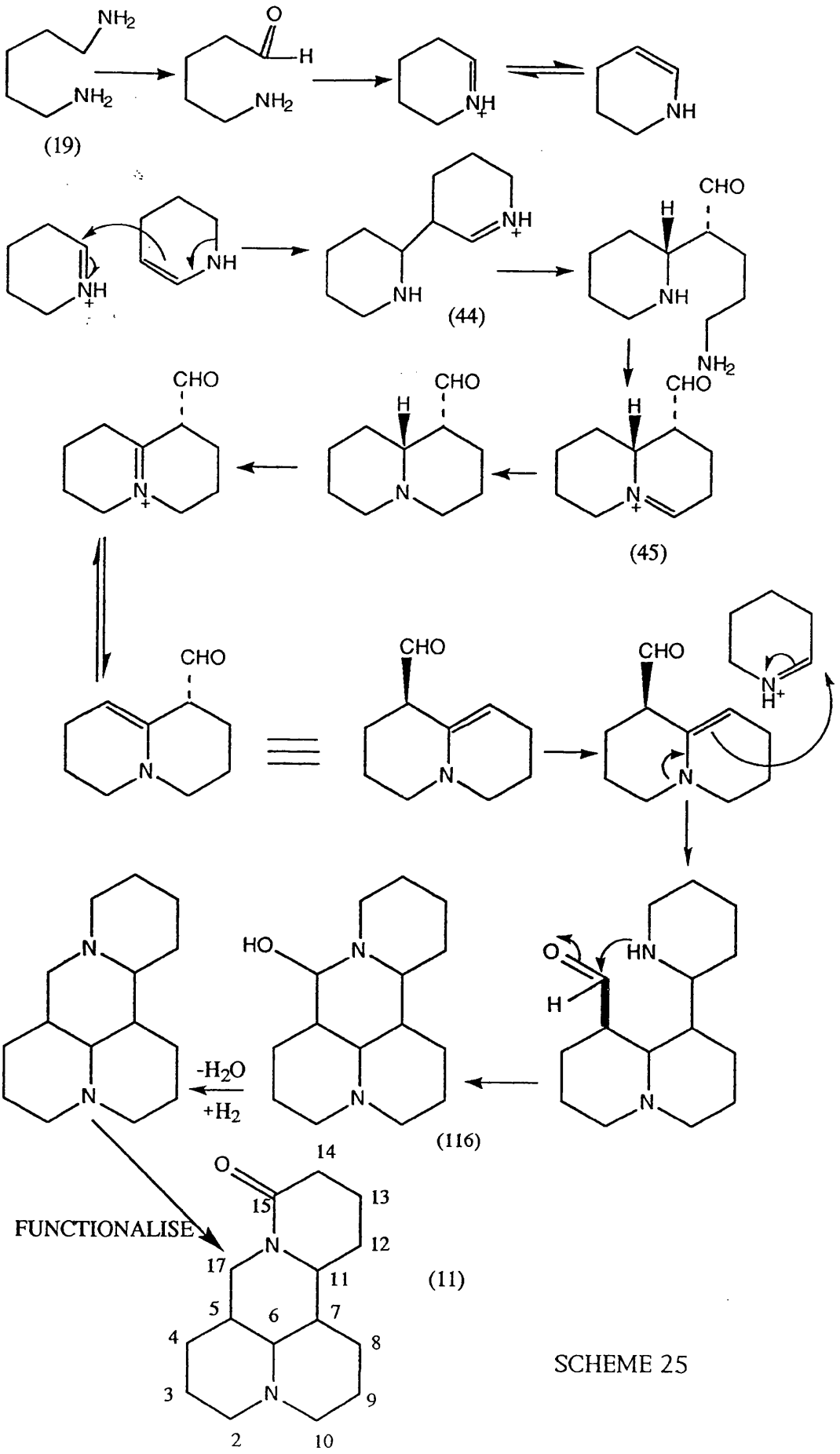
Although there are still ambiguities in the biosynthesis of matrine, enough has been established currently to postulate a possible biosynthetic pathway which takes into account all the labelling patterns obtained from the various studies conducted to date. Schemes 25, 26 and 27 show the proposed biosynthetic pathway.[65]

Two piperideine moieties, derived from cadaverine, couple to form tetrahydroanabasine (44). The *pro-R* hydrogen is retained when cadaverine is oxidised and the *pro-S* hydrogen lost. The oxidised cadaverine is in equilibrium with 1-piperideine and its enamine tautomer. These couple to form tetrahydroanabasine. The tetrahydroanabasine imine hydrolyses and ring closure produces a lupinine-type precursor. Implied after the ring closure is attack by a hydride donor on an intermediate N-1, C-10 iminium ion which occurs from the C-*Si* face of the intermediate.

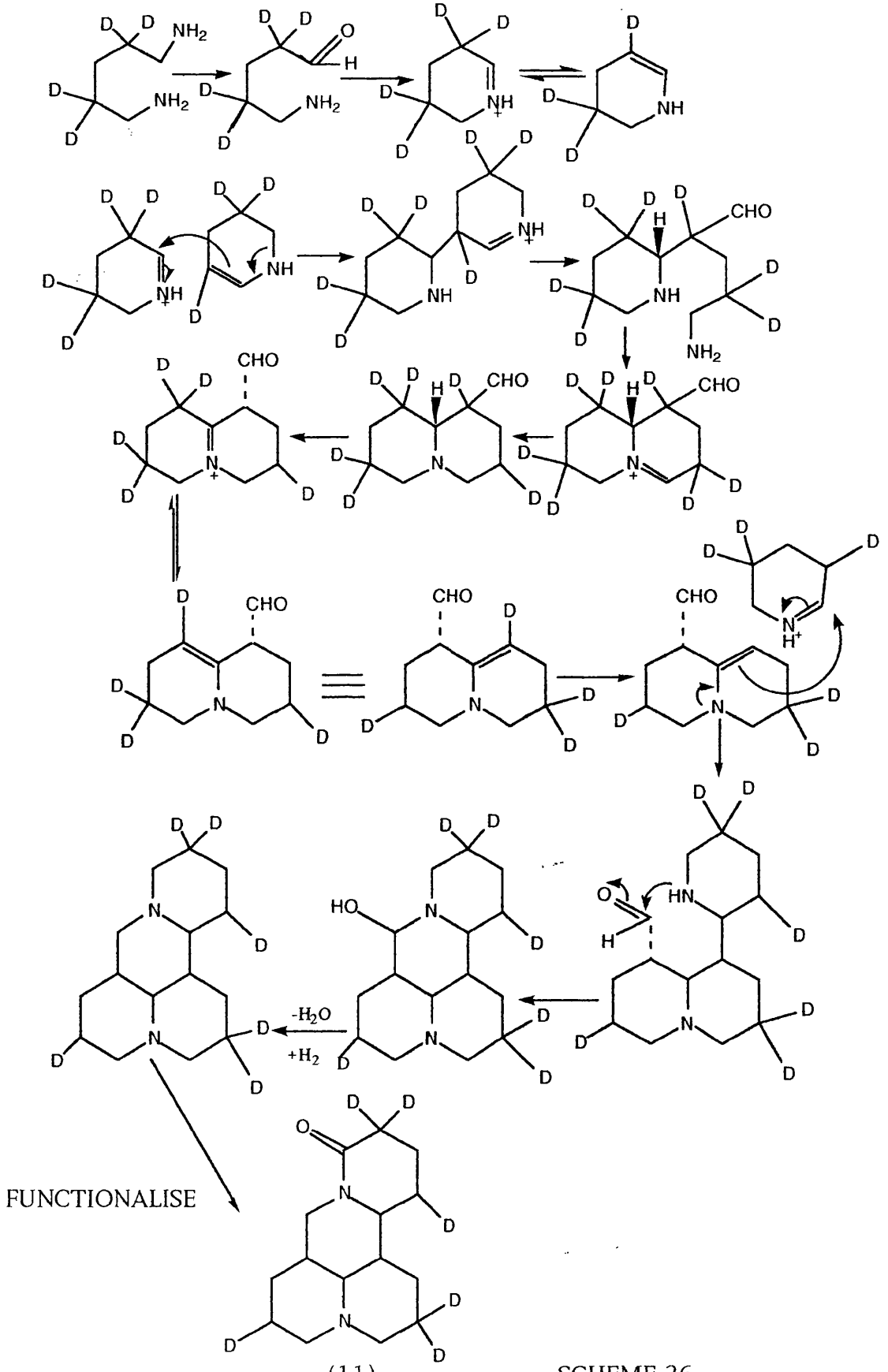
Next, the key intermediate combines with a third piperideine moiety to generate the matrine skeleton (116). All the deuterium labels destined to become bridgehead hydrogens are lost, probably due to enamine-imine tautomerisation. The deuterium destined to be 6α is probably lost during the processes taking place at the lupinine intermediate stage.

Deuterium atoms appeared at 10β and 10α respectively after feeding the (*R*)- and (*S*)-[1-^{2}H]cadaverines. Thus the C-N bond must remain intact in the formation of the N-1, C-10 bond of matrine. *Pro-S* protons which are destined to be C-2 and C-17 of matrine were lost, and *pro-R* protons at C-2 and C-17 were retained with retention of stereochemistry.

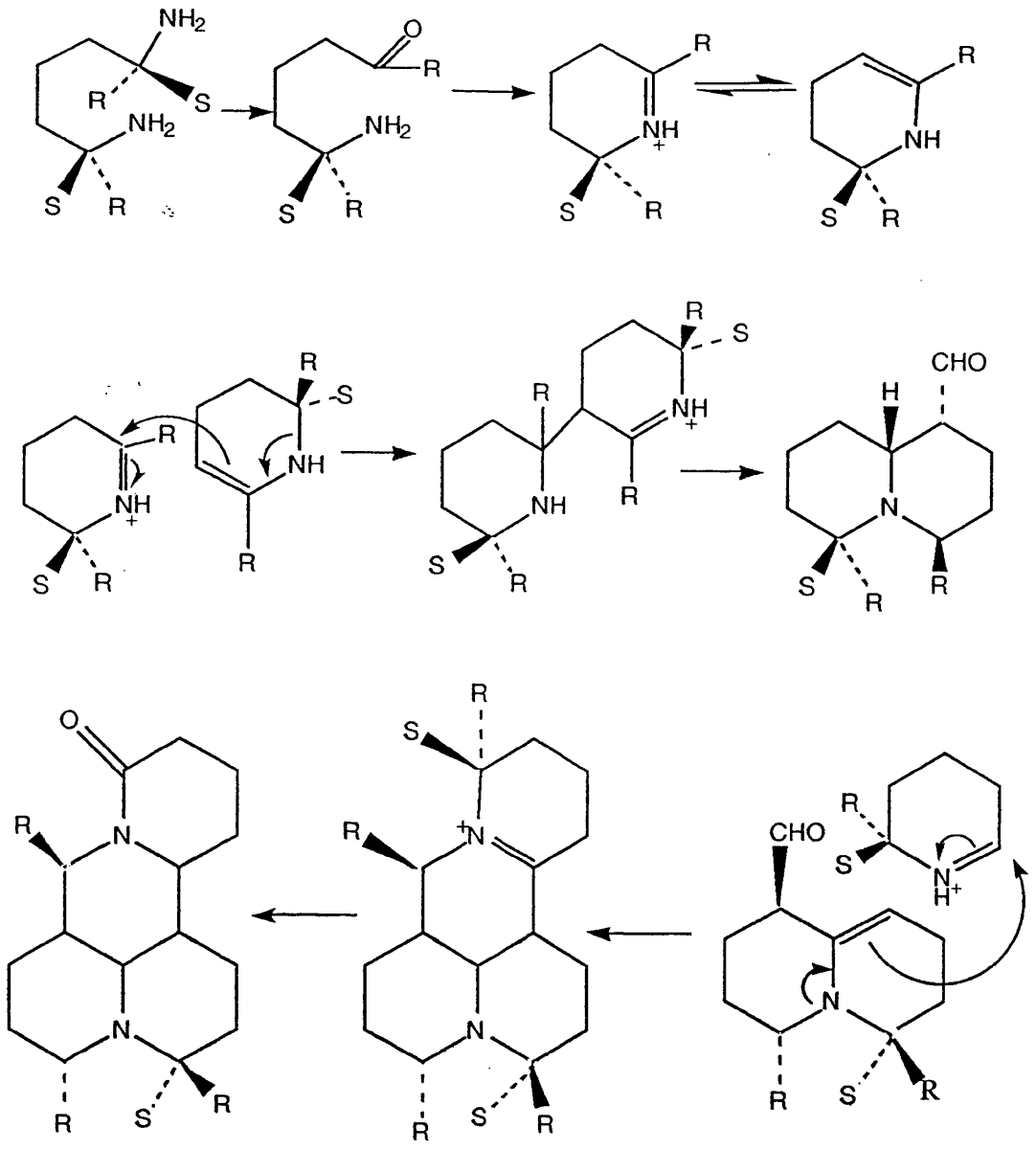
In the feed with (*R*)- and (*S*)-[2-^{2}H]cadaverine[65] deuterium was retained where no enzymic processes involving proton removal have taken place - i.e. deuterium was retained at 9β, 14α and 3β from the (*R*) precursor and at 9α, 14β and 12α from the (*S*) precursor. However, deuterium atoms at 12β and 3α, C-5 and C-7


NH2
NH2
O
H
NH2
NH
NH
(19)
NH
NH
NH
(44)
CHO
H
NH
NH2
CHO
H
N
(45)
CHO
N
CHO
H
N
CHO
H
N
CHO
N
CHO
N
N
H+
HO
N
N
O
H
HN
N
-H2O
+H2
14
(116)
FUNCTIONALISE
O
15
13
N
12
17
11
5
6
7
4
8
3
N
9
2
10
(11)

SCHEME 25



(11) SCHEME 26


NH2
R
S
NH2
S
R
O
R
NH2
S
R
R
NH
S
R
R
NH
S
R
R
S
R
NH
S
R
R
NH
S
R
R
S
R
NH
S
R
CHO
H
N
S
R
R
O
N
R
N
R
S
R
R
S
N
R
N
S
R
R
CHO
R
S
N
H
N
R
S
R

(11)

SCHEME 27

CHAPTER FOUR

SYNTHESIS OF (*R*)-AND (*S*)-[2-^{2}H]CADAVERINE DIHYDROCHLORIDES

4.1 INTRODUCTION

As indicated in Chapter Three, complete labelling patterns for anagyrine at reasonable levels of enrichment have not yet been established. In particular, the stereochemistry of proton removal at C-3 and C-5 is not yet known for certain. This Chapter deals with the attempted synthesis of (*R*)- and (*S*)-[2-^{2}H]cadaverine dihydrochlorides, (17) and (18) respectively, with the aim of feeding them to *Anagyris foetida* in order to obtain good labelling patterns for anagyrine (11).

Synthesis of (*R*)- and (*S*)-[2-^{2}H]cadaverine dihydrochlorides, (17) and (18), had been carried out previously by Sheldrake[78] using the route shown in Scheme 30, but these had been used up in previous feeding experiments carried out by Sheldrake and Brown.[65,78] Repetition of Sheldrake's route would therefore serve the dual purpose of providing ^{2}H-labelled precursors for the proposed feeding experiments and perhaps enable the route to be improved.

4.2 INTRODUCTION TO SYNTHESIS OF (*R*)- AND (*S*)-[2-^{2}H]CADAVERINE DIHYDROCHLORIDES

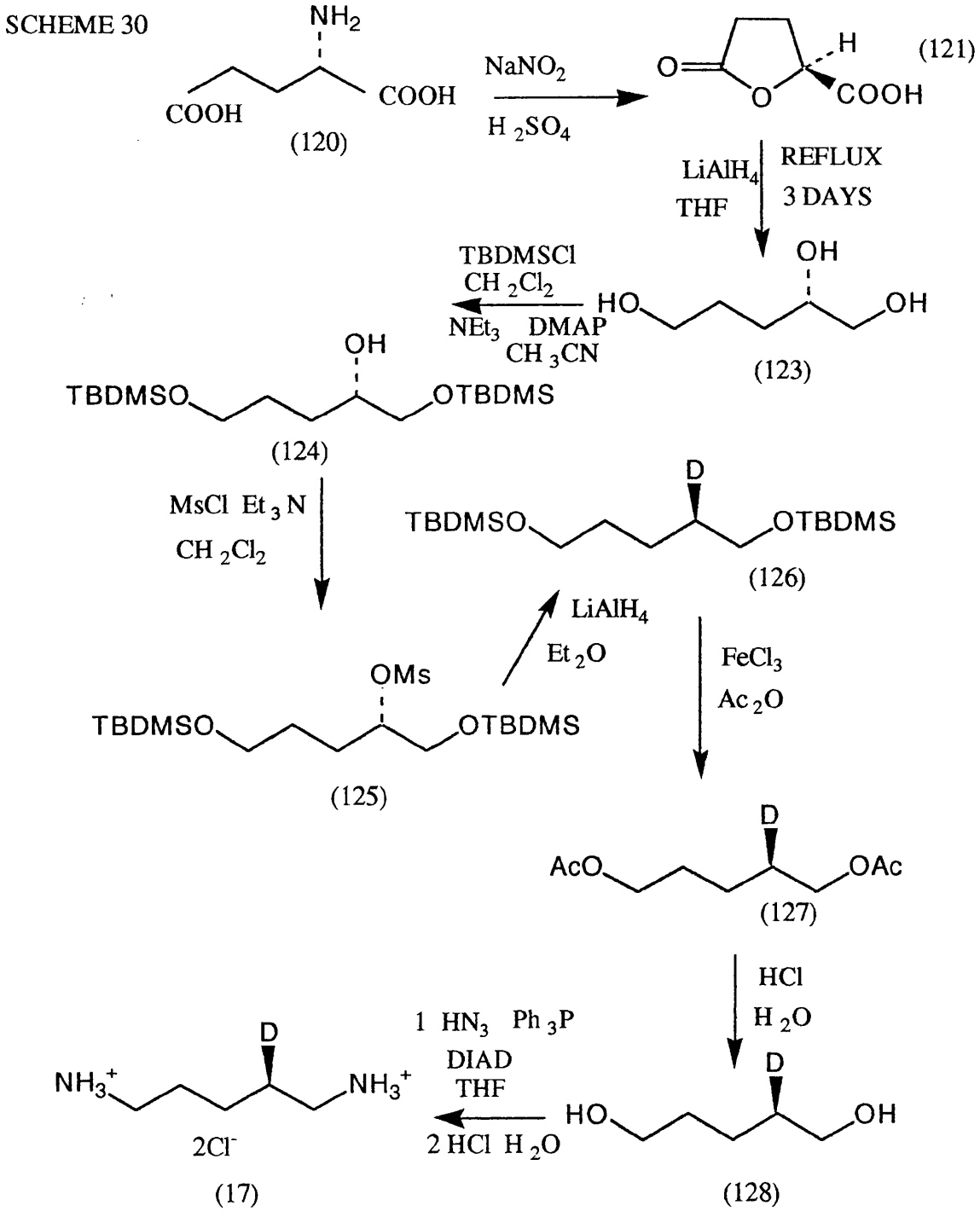
Sheldrake's route for the synthesis of (*R*)- (17) and (*S*)-[2-^{2}H]cadaverine (18) dihydrochlorides involves seven intermediates as shown in Scheme 30. Although the procedures in Scheme 30 appear to be straightforward, Sheldrake noted that there were practical problems, particularly in the first few stages of the route. In this study repetition of the route prove considerably more troublesome and time-consuming than anticipated. All intermediates in the route were synthesised, but long reaction times and low yields on purification made it impossible to accumulate sufficient quantities of pure pentane-1,2,5-triol (123), to complete the synthesis and to carry out the feeding experiments.

The second stage in the route involves a lithium aluminium hydride reduction. Such reductions play a major role in many organic synthetic procedures but occasionally difficulties are encountered in the separation of intractable products.[92]

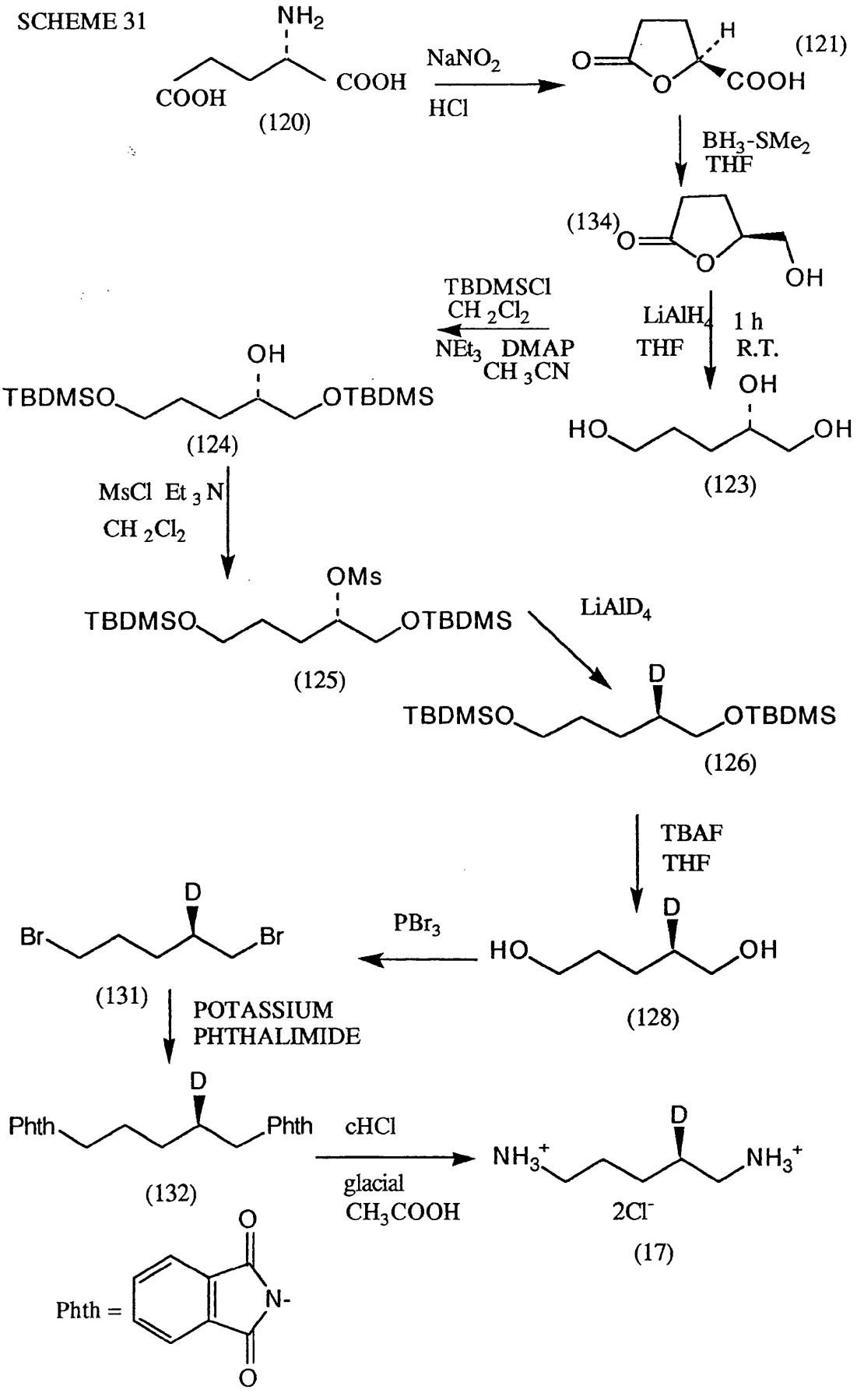
This is particularly the case when the required organic product can function as a bidentate or multidentate ligand,[93,94,95] and consequently remains strongly bound to the aluminium cations following hydrolysis.[96] As a result reactions are often low yielding[97] as was found to be the case in this study.

In the course of the study minor modifications were made to simplify the route to intermediates (121), (127), and (128). The conversion into the final product (17) was also modified to comply with more stringent Health and Safety Regulations introduced. These modifications are shown in Scheme 31 and discussed later in the Chapter. At the end of the Chapter a possible scheme for an alternative synthesis is proposed.

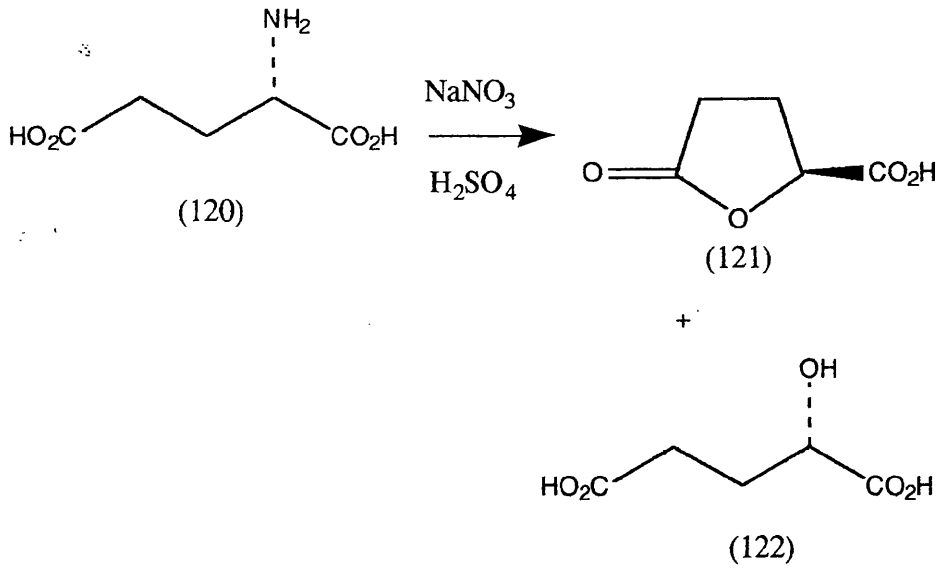
SCHEME 30


NH2
COOH
COOH
(120)
NaNO2
H2SO4
O
O
H
COOH
(121)
LiAlH4
THF
REFLUX
3 DAYS
OH
HO
OH
(123)
TBDMSCl
CH2Cl2
NEt3 DMAP
CH3CN
OH
TBDMSO
OTBDMS
(124)
MsCl Et3N
CH2Cl2
D
TBDMSO
OTBDMS
(126)
LiAlH4
Et2O
FeCl3
Ac2O
OMs
TBDMSO
OTBDMS
(125)
D
AcO
OAc
(127)
HCl
H2O
1 HN3 Ph3P
DIAD
THF
2 HCl H2O
D
HO
OH
(128)
D
NH3+
NH3+
2Cl-
(17)

SCHEME 31


NH2
COOH
COOH
(120)
NaNO2
HCl
H
O
O
COOH
(121)
BH3-SMe2
THF
(134)
O
O
OH
TBDMSCl
CH2Cl2
NEt3 DMAP
CH3CN
LiAlH4
THF
1 h
R.T.
OH
HO
OH
(123)
OH
TBDMSO
OTBDMS
(124)
MsCl Et3N
CH2Cl2
OMs
TBDMSO
OTBDMS
(125)
LiAlD4
D
TBDMSO
OTBDMS
(126)
TBAF
THF
D
HO
OH
(128)
PBr3
D
Br
Br
(131)
POTASSIUM
PHTHALIMIDE
D
Phth
Phth
(132)
cHCl
glacial
CH3COOH
D
NH3+
NH3+
2Cl-
(17)
Phth =
O
N-
O

4.3 SYNTHESIS OF (*S*)-(+)-TETRAHYDRO-5-OXO-2-FURANCARBOXYLIC ACID (121)



(120) (121) (122)

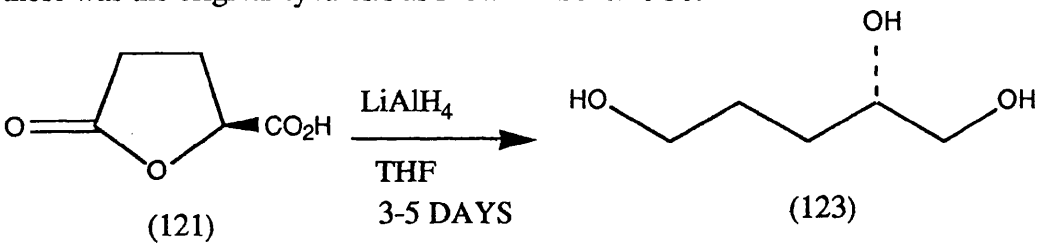
L-Glutamic acid (120) was converted into lactonic acid (121).[90] The lactone is evident at 1790 cm^{-1} in the IR spectrum. Distillation was necessary to remove the straight chain product (122) and, as Sheldrake found, distillation had to be carried out in small volumes of 7 ml or less to avoid major problems. Distillation occurred at 190 ^{o}C and 0.3 mmHg. Under these conditions the material darkened to brown or black, probably due to traces of sulphuric acid. The product solidified in the receiver, even with the flow of water disconnected. Recrystallisation had to be carried out instantly using ethanol-free chloroform to avoid esterification. The melting point of 70-72 ^{o}C was comparable to the 72-73 ^{o}C obtained by Sheldrake. The optical rotation of the recrystallised acid was found to be $[\alpha]_D^{20}$ +11.6^{o} which is slightly higher than that of $[\alpha]_D^{20}$ +10.6^{o} quoted in the literature[90] and slightly lower than Sheldrake's finding of $[\alpha]_D^{20}$ +14.4^{o} (c 5, MeOH). The yield of 65% was comparable to that of Sheldrake (63%).

Subsequently it was found that charring could be avoided by using hydrochloric acid in preference to sulphuric acid (undistilled yield 72%), (lit.[74] 75%). The HCl was added at 0 ^{o}C over four hours as opposed to one hour at room temperature. At a much later stage in this work distillation was rendered unnecessary by using a procedure for crystallisation of the lactonic acid (121) which involved stirring the acid in ethyl acetate

with sodium sulphate, concentration and then drying for several hours on an oil pump. This method gave solid lactonic acid (121) in 63% purified yield (lit.[76] 56%). These modifications are shown in Scheme 31.

4.4 SYNTHESIS OF PENTANE-1,2,5-TRIOL (123)

Sheldrake indicated that there were a number of problems associated with this stage in his route, primarily in relation to low yields and long reaction times. A study of his lab reports shows that he gives varying yields around the 30-35% level and a best yield of 66%. In this work average yields of only 10% were achieved, with a best yield of 36.7%. The major source of difficulty lay in the extraction of the polar triol (123) from the slurry of aluminium salts. A number of different methods were used in repeated attempts to achieve pure samples of pentane-1,2,5-triol (123). The first of these was the original synthesis as shown in Scheme 30.

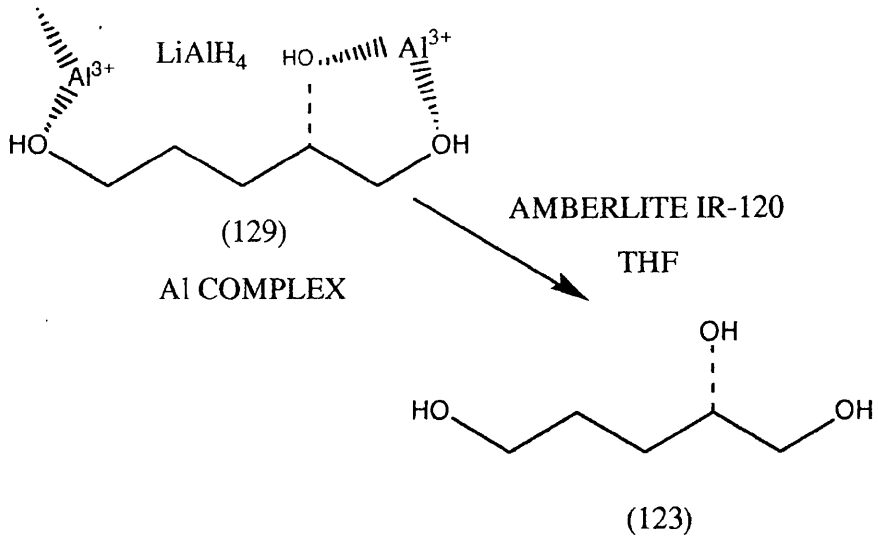


(121) (123)

Reduction of the lactonic acid (121) by lithium aluminium hydride ($LiAlH_4$) in tetrahydrofuran (THF) was carried out using 3 mole equivalents of $LiAlH_4$ in THF and heating at reflux for 3-5 days. Shorter times gave incomplete reaction. Extraction of the product was difficult, probably due to incomplete break-up of the aluminium complex. Extraction with boiling THF yielded around 10% triol (123). Treatment of the complex with hydrochloric acid (HCl) and continuous extraction of the product with ethyl acetate yielded only acetic acid and a black tar. When the scale of the reaction was increased the yield went down even further.

To free the triol (123) trapped in the aluminium complex (129) an ion exchange resin was used (Figure 4.1).

Figure 4.1



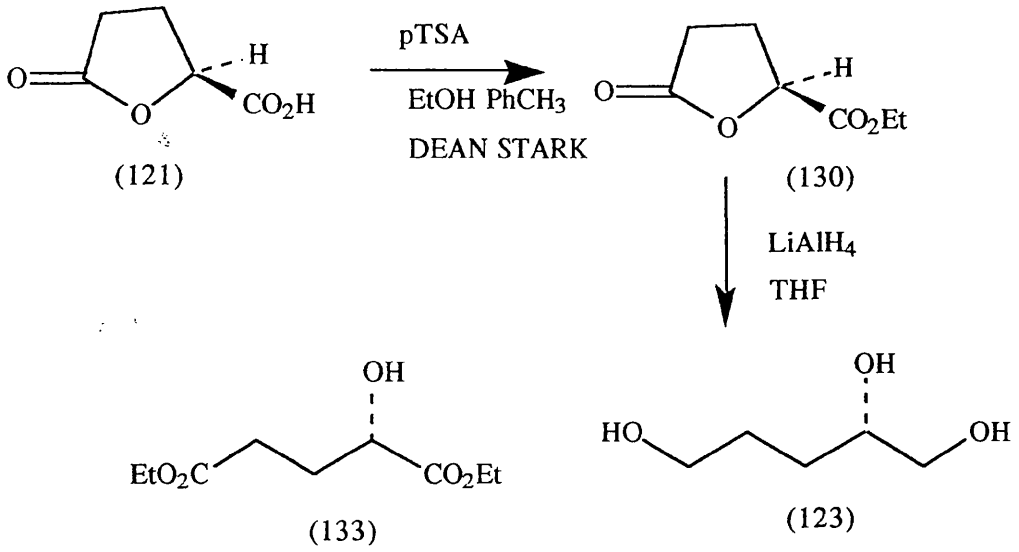
(123)

The assumption was that the lithium (Li^+) and aluminium (Al^{3+}) ions would complex with the resin and leave the triol free to be extracted. As a strong cation exchanger resin was needed, Amberlite IR-120, a cross-linked polystyrene with a hydrogen sulphite functional group, was used.

The aluminium complex was placed in a flask with THF and Amberlite IR-120 and stirred at room temperature overnight. After filtration and concentration, a clear oil was formed. This proved to be mainly THF with only a minor trace of triol. The ^1H NMR spectrum of triol (123) had two multiplets at δ_H 1.58 and 3.16 and a broad singlet at δ_H 10.33.

In an attempt to improve yields, an alternative route to the triol (123) was attempted as shown in Scheme 32.

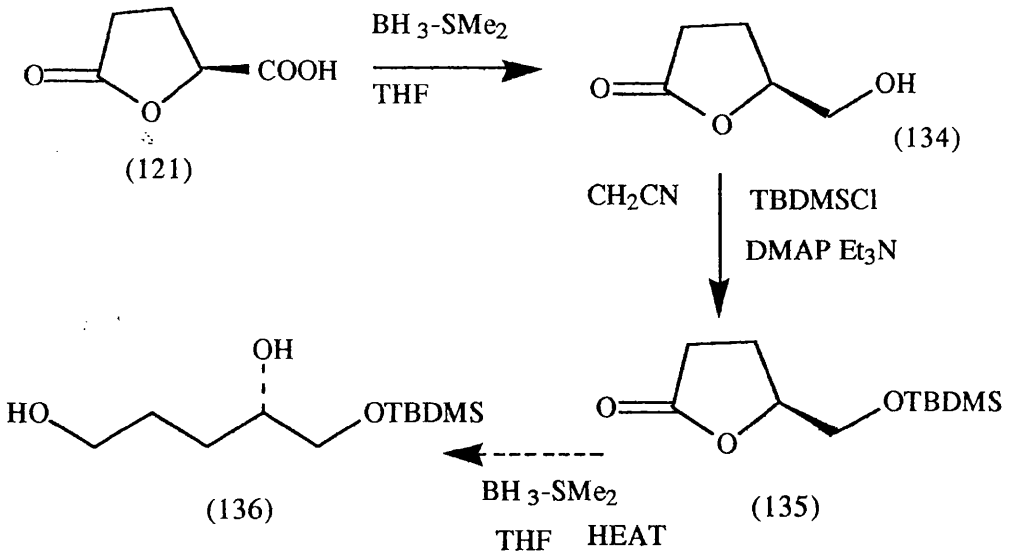
SCHEME 32



This route involved esterification of the acid (121) by means of a Dean Stark apparatus. The ester (130) distilled cleanly. The distillation removed the straight chain ester diethyl 2-hydroxyglutarate (133), identified in the mass spectrum by an M^+ of 204. Complete removal of this was necessary to get an accurate optical rotation measurement. The optical rotation measurement obtained was $[\alpha]_D^{25}$ +14.3° (Lit.[86] $[\alpha]_D^{25}$ 15.3°). Again, problems in extracting the triol (123) from the Al^{3+} complex were experienced and average yields of around 18% were not significantly greater than those achieved by the method described in Scheme 30.

An alternative approach, based on papers by Hanessian,[76] and by Brown and co-workers,[77] is shown in Scheme 33.

SCHEME 33

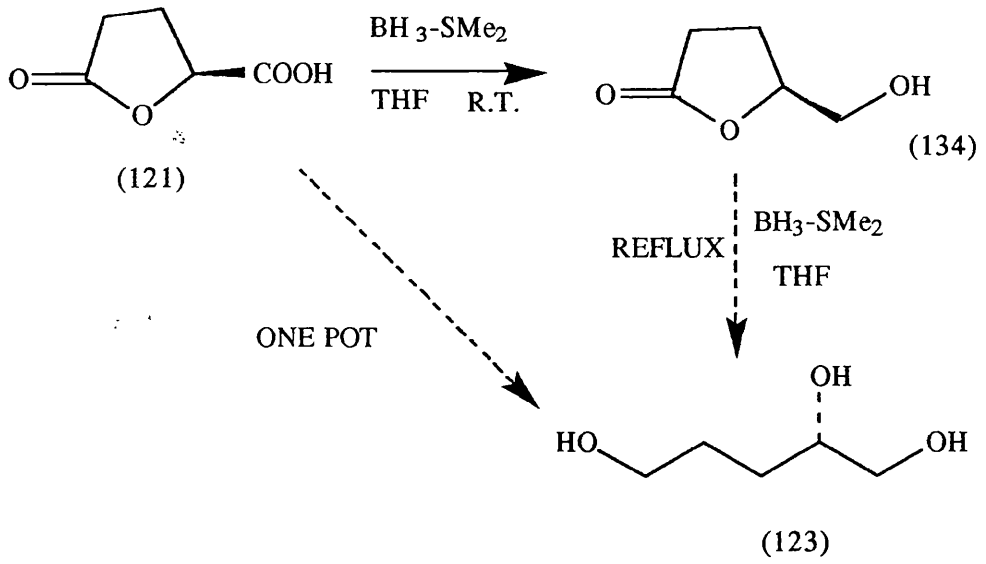


This method involved reduction of lactonic acid (121) to (*S*)-γ-(hydroxymethyl)-γ-butyrolactone (134) using borane dimethyl sulphide complex (BH_3-SMe_2) in THF at room temperature. This gave lactonic alcohol (134) in 100% crude yield, which was immediately treated with TBDMS-Cl. Hanessian's procedure (TBDMSCl / imadazole / CH_2Cl_2) gave starting material only. However, the protected alcohol (135) was obtained by using triethylamine, acetonitrile and DMAP.

The silylated lactone (135) was again treated with borane dimethyl sulphide in THF but in more forcing conditions by distilling off the dimethyl sulphide as the reduction proceeded.[75] This reaction yielded a very crude mixture which, when distilled, seemed to contain only starting material, as evidenced by the absence of a multiplet at δ_H 1.58 (straight chain) for (136) and the appearance of a multiplet at δ_H 2.2-2.6 (lactone) in the ^1H NMR spectrum.

A selective silyl protection was not necessary for our purposes.[76] Thus, there seemed to be no need to protect the alcohol (134) with a silyl group prior to reduction since there appeared to be no reason why the reduction should work better with a TBDMS group present. Consequently, it was decided to use a one-pot reaction so that the two steps could be tried at varying temperatures since the alcohol (134) is used unpurified (Scheme 34).

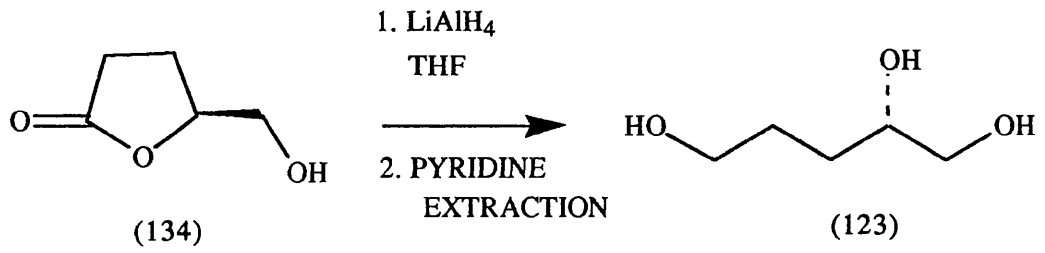
SCHEME 34



This method involved reducing the acid (121) at room temperature in THF using one equivalent borane dimethyl sulphide; then, after stirring for two hours, more borane dimethyl suphide (1 equivalent) was added and the mixture was heated at reflux as before to reduce the lactone alcohol to form the triol (123). Only the intermediate alcohol (134) was formed.

An alternative approach involved reduction of the lactonic alcohol (134), using two mole equivalents of LiAlH4 and heating at reflux overnight in THF (Scheme 35).

SCHEME 35



It appeared that the LiAlH4 reduction was successful and that extraction was the only problem area. Therefore an attempt was made to find another viable extraction method.

This involved destroying the LiAlH4 using water only and removing all the solvent from the reaction mixture under vacuum. The residual solid was extracted repeatedly using pyridine. The pyridine extracts were then concentrated and residual

traces of pyridine were removed on an oil pump. After distillation 40% of optically active triol (123) was obtained $[\alpha]_D^{24}$ -20.365° (lit.[87] $[\alpha]_D^{24}$ -19.6°). Although this yield was not great, it was higher than any of the yields obtained by the methods previously outlined and high enough to make this a viable method of extraction.

It was therefore decided to repeat the original method shown in Scheme 30 but using the pyridine extraction method outlined above. The yields were again greater - 33% distilled yield, as opposed to the 10% average yields originally obtained. Even so, these yields are still smaller than those obtained by using the method shown above in Scheme 35.

However the method shown in Scheme 35 was carried out on a small scale (1.2 g $LiAlH_4$). When this was was scaled up to 4 g of $LiAlH_4$, the yields dropped, as was the case in all of the other methods. The optimal quantity of $LiAlH_4$ for this method proved to be around 2 g. The reason for the drop in yield when using greater quantities of $LiAlH_4$ is perhaps the greater amount of aluminium residues present causing inorganic salts to be brought through in the product (123). The presence of inorganic salts hinders distillation.

Nevertheless, at this point in time, this method appeared to offer the best way of producing sufficient triol (123) to attempt the later stages of the route.

4.5 SYNTHESIS OF (*S*)-(-)-1-*O*,5-*O*-DI(*t*-BUTYLDIMETHYLSILYL)PENTANE-1,2,5-TRIOL (124)

Pentane-1,2,5-triol (123) was treated with *t*-butyldimethylsilyl chloride (TBDMSCl) in a solution of dimethylaminopyridine (DMAP), triethylamine and acetonitrile in dichloromethane and stirred for 24 hours at room temperature to give the diprotected alcohol (124). The mass spectrum shows no parent ion but does show an M^+ of 291 corresponding to $C_{13}H_{31}O_2Si_2$ with loss of one tertiary butyl group. Slightly less than two equivalents of TBDMSCl were used under dry conditions to prevent *t*-butyldimethylsilanol being present.

Purification of the protected triol (124) by column chromatography gave poor recovery of product. Possible reasons could be that either the TBDMS groups were

being removed on the column or that the triol (123), even after distillation, had chelated Al^{+3} and Li^{+} ions which masked the hydroxyl groups and thus only allowed a small amount of TBDMS protection.

4.6 SYNTHESIS OF (*S*)-(-)-1-*O*,5-*O*-DI(*t*-BUTYLDIMETHYLSILYL)-2-*O*-METHANESULPHONYLPENTANE-1,2,5-TRIOL (125)

Mesylation was carried out on the diprotected alcohol (124) using methanesulphonylchloride in dichloromethane and stirring at room temperature for three hours to give the triol (125), which was unstable and used immediately without further purification.

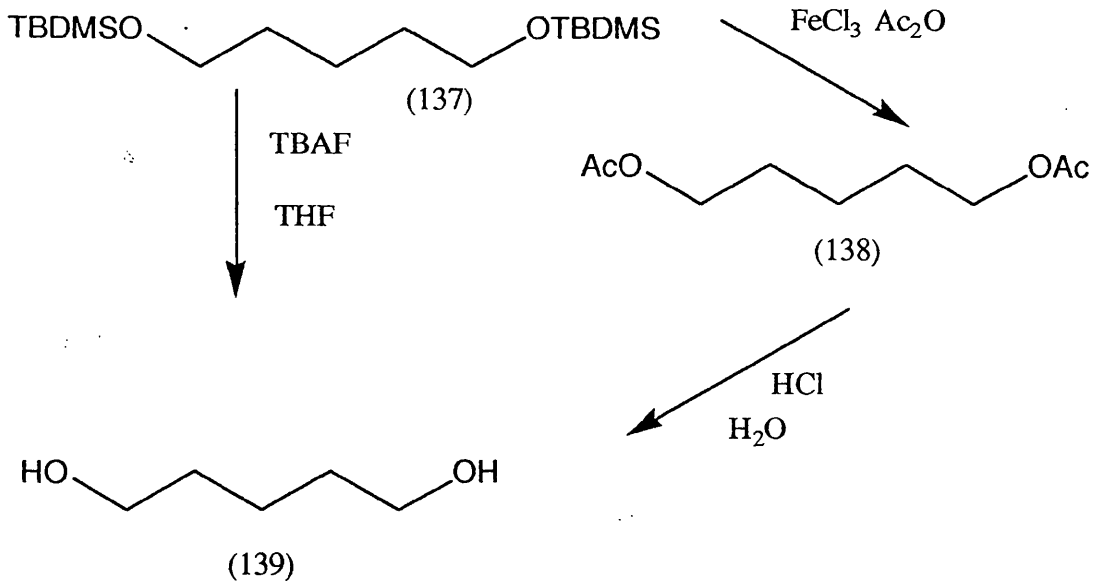
4.7 SYNTHESIS OF (*R*)-[2-^{2}H]-1-*O*,5-*O*-DI(*t*-BUTYLDIMETHYLSILYL)PENTANE-1,5-DIOL (126)

The next stage, the lithium aluminium deuteride ($LiAlD_4$) reduction of the mesylate to form the deuteriated diprotected alcohol (126) gave a small amount of material which was found to contain none of the desired product (126). The triol (123) used had been obtained by the method shown in Scheme 35, which used unpurified lactonic alcohol (134) formed from borane dimethy lsulphide reduction. The failure to obtain deuteriated diprotected alcohol (126) was probably due to the small scale of the reaction, and to traces of dimethyl sulphide and pyridine left in the starting material. Also traces of borane dimethyl sulphide or aluminium complexed with triol (123) and remained even after distillation.

4.8 SYNTHESIS OF (*R*)-[2-^{2}H]-1,5-DIACETOXYPENTANE (127) and (*R*)-[2-^{2}H]-PENTANE-1,5-DIOL (128)

Since compounds (127) and (128) were first synthesised in undeuteriated form, they are shown in Scheme 36 as (138) and (139) respectively.

SCHEME 36

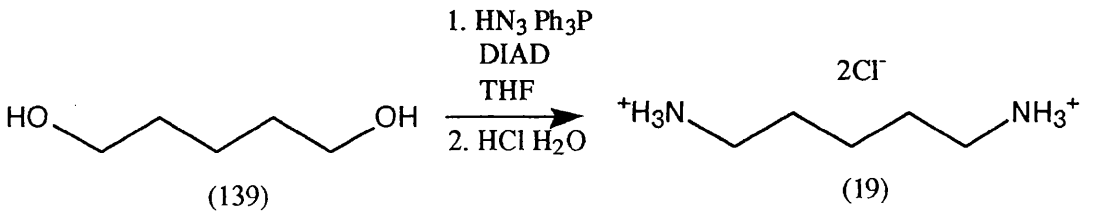


Compound (137) had been prepared by TBDMSCl protection of the pentane-1,5-diol. Deprotection was carried out using iron III chloride and acetic anhydride to give the diacetate (138)[91] with a six hydrogen singlet at δ_H 1.76 in the NMR spectrum. This diacetate was then stirred overnight with 1M HCl to give pentane-1,5-diol (139), M^+ 104. Some of the pentane-1,5-diol was also formed from the column chromatography purification carried out on the diacetate (138).

This method is effective but it is time-consuming due to the difficulty in removing the acetic anhydride. The volatility of the diacetate (138) causes a problem in that acetic anhydride can not be removed under vacuum, and has to be removed using column chromatography.

An easier and less time-consuming method of deprotection involved stirring the disilyl compound (137) in THF with tetrabutylammonium fluoride (TBAF) at 45 ^oC to form the diol (139) in a one-step reaction.

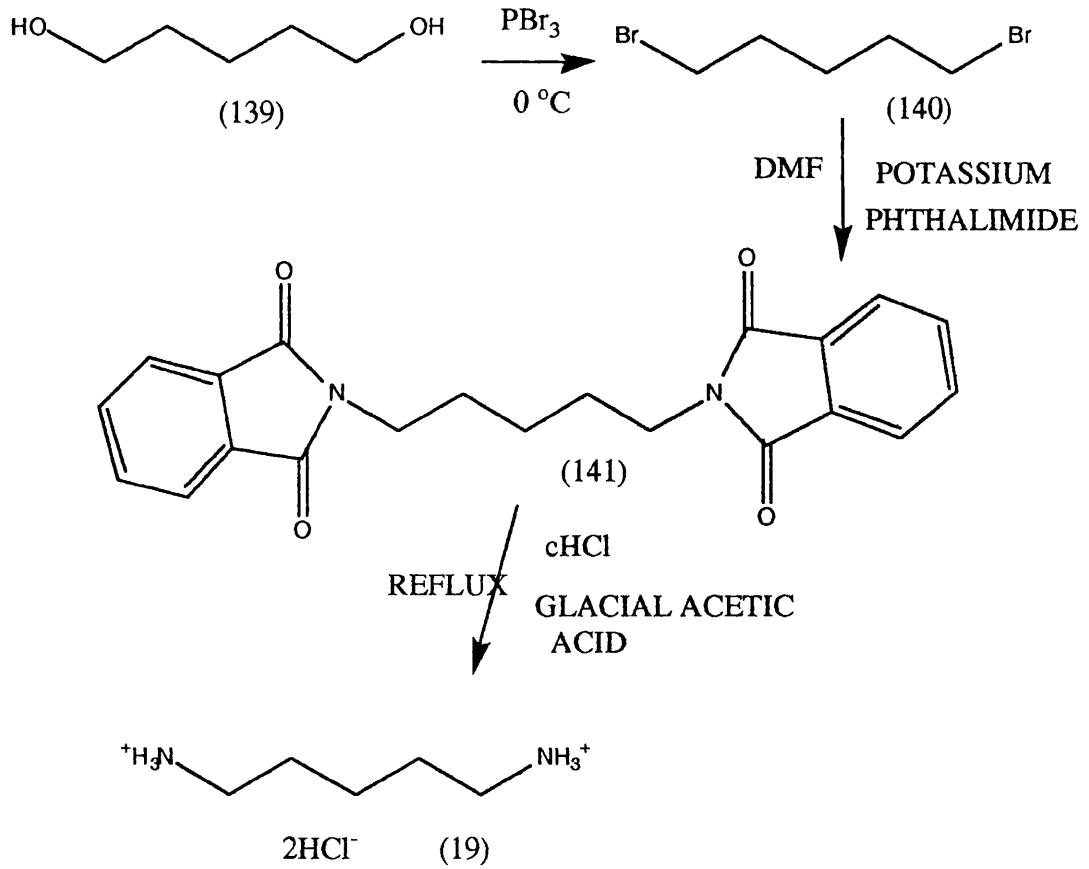
4.9 SYNTHESIS OF 1,5-DIAMINOPENTANE (CADAVERINE) DIHYDROCHLORIDE



The conversion of pentane-1,5-diol (139) into cadaverine dihydrochloride (19)[88] involved a one-pot reaction with di-isopropyl azodicarboxylate (DIAD), triphenylphospine (Ph_3P) and the very toxic hydrazoic acid (HN_3)[89] which has to be prepared in advance. The conversion was effective (48% yield) but because of more stringent Health and Safety restrictions being brought into operation, it became necessary to use a less toxic method for the conversion.

This method uses three steps to get to the cadaverine instead of one, but reagents are far less toxic and intermediates are easy to handle since the diphthalimide intermediate is a solid (Scheme 37).

SCHEME 37



The diol (139) was converted into the dibromide (140) using phosphorus tribromide at 0 °C to give a 52% yield. This was then converted in the diphthalimide (141) using potassium phthalimide in DMF to give the diphthalimide (141) in 80%

yield with M^+ 362.1259. It was then heated at reflux in strong acid (concentrated HCl and glacial acetic acid) to give the cadaverine dihydrochloride (19).

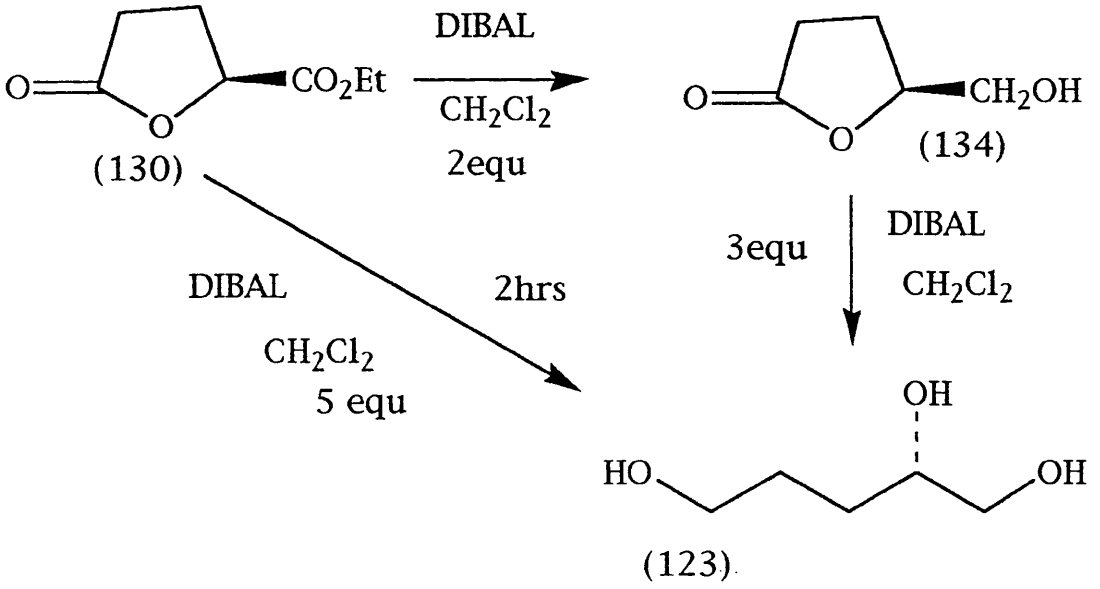
A Mitzunobu-type reaction can also be used to convert the diol (139) directly into the diphthalimide (141). In practice, this reaction took longer and gave no better yields.

4.10 FURTHER WORK ON SYNTHESIS OF PENTANE-1,2,5-TRIOL

In order to avoid the use of borane dimethylsulphide and pyridine, and thus obtain a purer triol (123) for use in the synthesis of intermediates (124), (125) and (126), the synthesis of pentane-1,2,5-triol (123) was again attempted using another common reducing agent, DIBAL (Scheme 38).

Since DIBAL is known to reduce esters, a reduction from the lactonic ester (130) using DIBAL to give the alcohol (134) could be carried out. The alcohol (134) could then be reduced again with DIBAL to give the triol (123). Thus, there appeared to be no good reason why the ester (130) could not be taken directly to the triol (123) by adding DIBAL as shown in Scheme 38. Earlier in the work DIBAL was inappropriate since it did not reduce the lactonic acid (121).

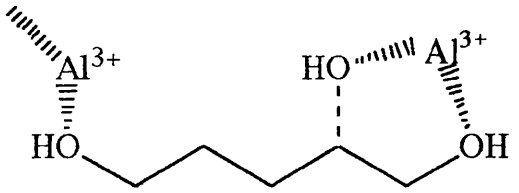
SCHEME 38



This procedure worked effectively, giving a 46% crude yield of (123).

On purification the yield was reduced due to the chelated triol product (142) and inorganic impurities which were present. A suggested structure is given in Figure 4.2.

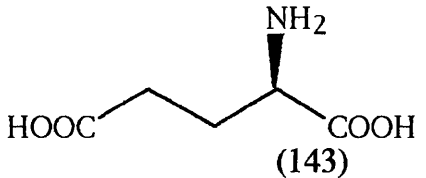
Figure 4.2



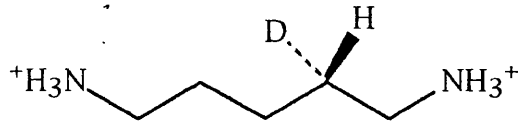
(142)

The chelated product (142), when used to sythesise intermediates (124), (125) and (126) still gave poor yields.

By this time the work had been on-going for over two years with little or no productive results. It has to be borne in mind that the aim of repeating Sheldrake's route was primarily to obtain the (*R*)- and (*S*)-[2-^{2}H]cadaverine dihydrochlorides (17) and (18) in order to feed them to *Anagyris foetida* to obtain good labelling patterns for anagyrine (11), as previous work had used up all the available [2-^{2}H]cadaverines. Moreover, the work described above was on the synthesis of the (*R*)-[2-^{2}H]cadaverine (18) only. The whole process would require to be repeated using D-glutamic acid (143) as a starting material for the synthesis of the (*S*)-[2-^{2}H]cadaverine (18). This would also mean an increase in the overall time-scale.



(143)



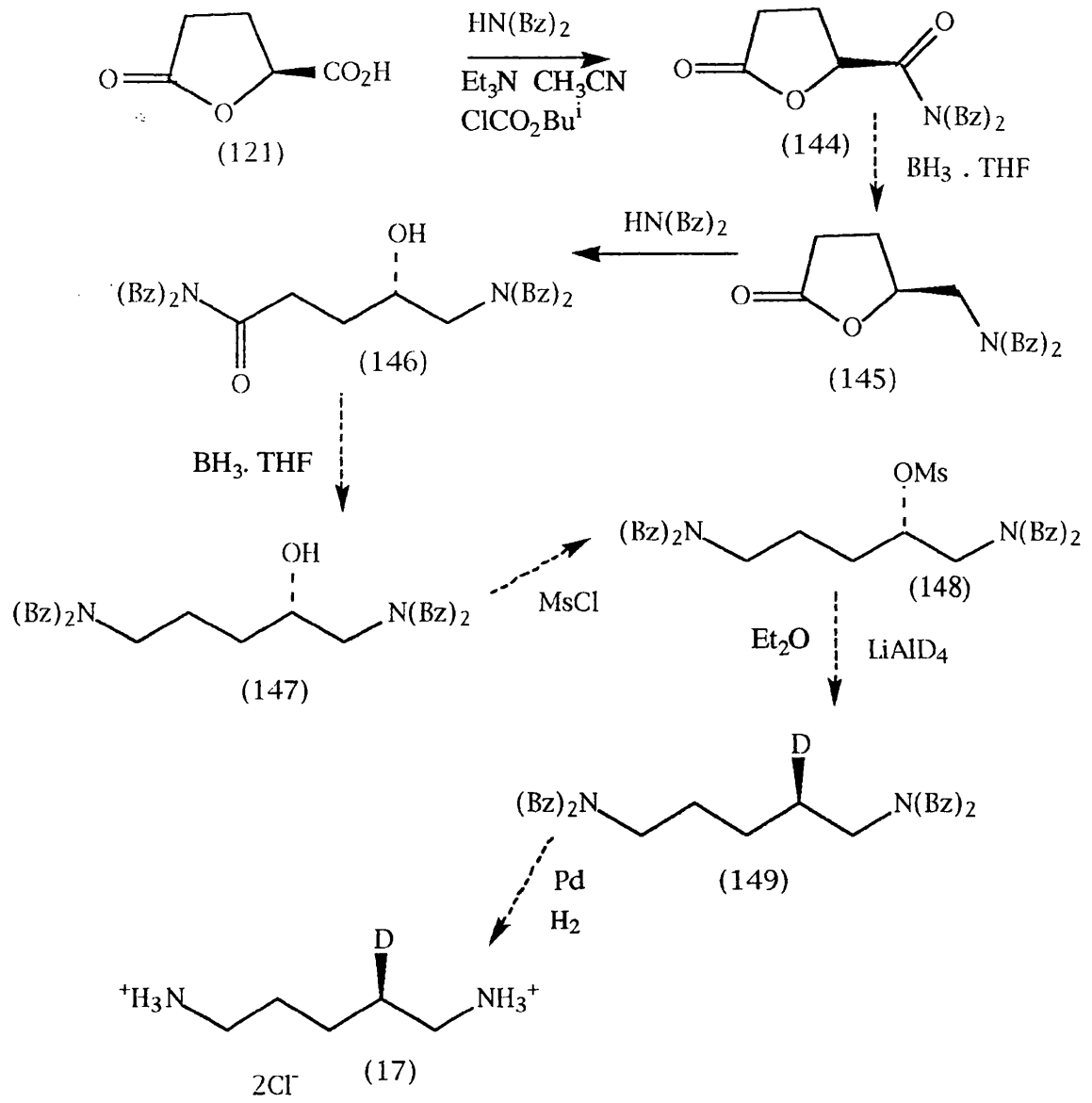
(18)

Consequently, a decision was reached to try to achieve the synthesis of the (*R*)-[2-^{2}H]cadaverine (17) by an alternative approach. This new approach was based on findings in recent literature[79,80] and on ideas arising out of work on diamine oxidases which had just begun. The new methods are discussed and described below.

4.11 ALTERNATIVE SYNTHESIS OF (*R*)- AND (*S*)-[2-^{2}H]CADAVERINE DIHYDROCHLORIDES

Since the problems in the original route were isolation and purification of pentane-1,2,5-triol (123) and associated difficulties in the protection stages, a route containing no pentane-1,2,5-triol and no TBDMS protections seemed desirable. Results obtained from the synthesis of *N*-alkylcadaverines had shown that a mixed anhydride[79] reaction works well. From recent papers it was clear that neat dibenzylamine could open up lactones,[80] and there are several papers documenting catalytic hydrogenation[81,82,83,84] for removal of the protecting dibenzyl groups. Thus the following route (Scheme 39) was devised and tried.

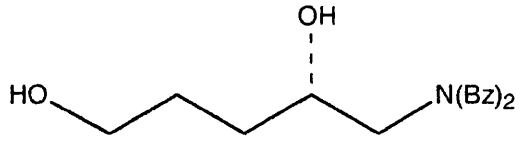
SCHEME 39



This Scheme envisaged a mixed anhydride reaction on lactonic acid (121) using neat dibenzylamine to produce dibenzylamide lactone (144), followed by a borane reduction to give dibenzylamine lactone (145). The dibenzylamine lactone (145) would be ring-opened using neat dibenzylamine to give a straight chain alcohol amide (146). The amide (146) would then be reduced and the alcohol (147) protected by mesylation (148). The mesyl group would be replaced by deuterium to give deuteriated pentane-1,5-dibenzylamine (149) using LiAlD4. Finally the dibenzyl protecting groups would be removed using catalytic hydrogenation to give the (*R*)-[2-^{2}H]cadaverine dihydrochloride (17).

The mixed anhydride reaction gave a 65% yield of dibenzylamide lactone (144) with M^+ 309.1366.

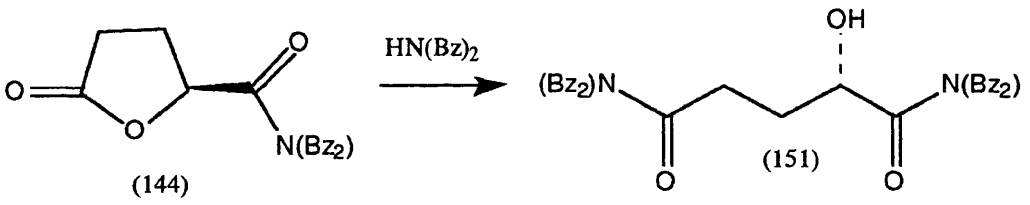
On reduction of amide (144) with 1 M borane in THF solution the completely reduced straight chain alcohol (150) was obtained. This was identified by a large alcohol peak at 3630 cm^{-1} and the absence of a carbonyl. Work needs to be done to determine the quantity of borane needed to give a controlled reduction which will only reduce the carbonyl of the amide.



(150)

An alternative approach is to attempt to open up the unreduced amide dibenzylamine directly as shown in Scheme 40.

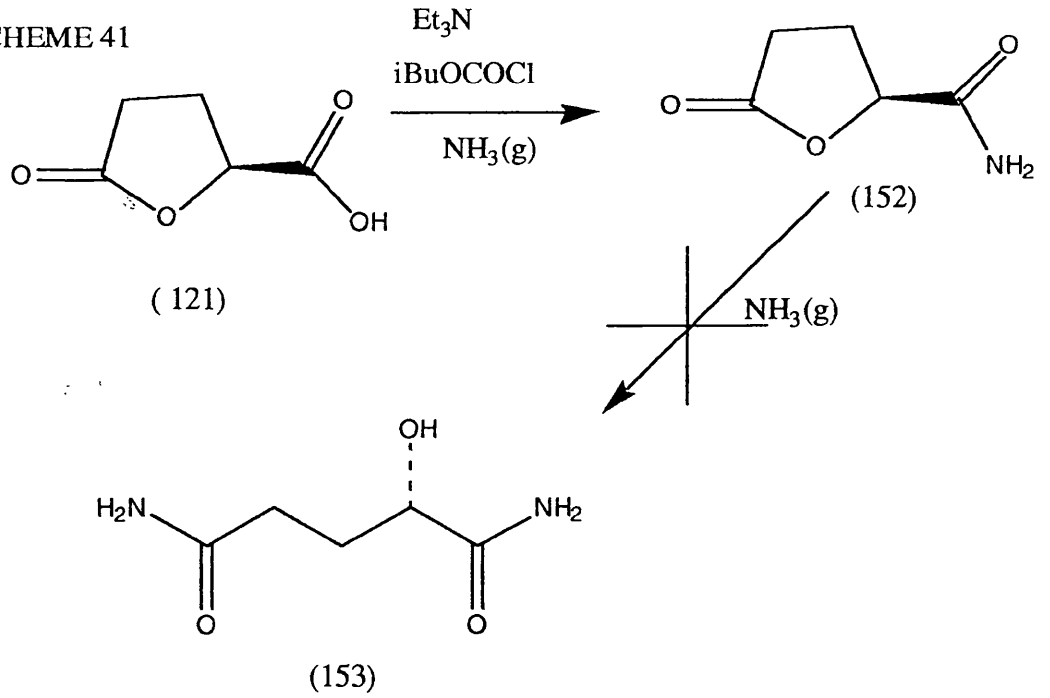
SCHEME 40



(144) (151) + UNIDENTIFIED PRODUCT

This was done in neat dibenzylamine at 50 °C and excess dibenzylamine was precipitated out as the HCl salt by ether. The precipitate was filtered off to leave the product in organic phase. This reaction gave a mixture of products: the straight chain diamide (151), some unreacted starting material and an unidentified product. Purification of this mixture proved difficult due to the high boiling point of the impure product. To reduce the boiling point of the product and to avoid removal of the benzyl groups at the end of the route the mixed anhydride reaction was attempted with ammonia gas to give amide (152) as shown in Scheme 41.

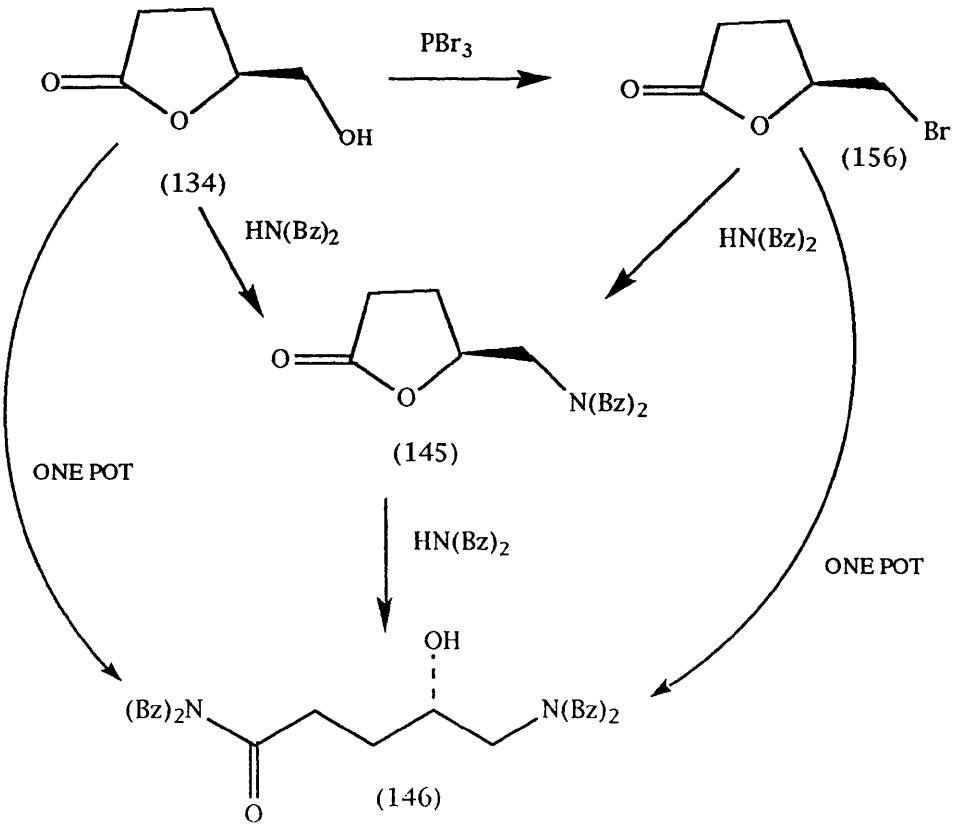
SCHEME 41



This gave a 45% yield of amide lactone (152) with M^+ 129.042. Opening of the lactone to form the straight chain diamide (153) with ammonia gas failed.

One final method was devised. This is shown in Scheme 42.

SCHEME 42



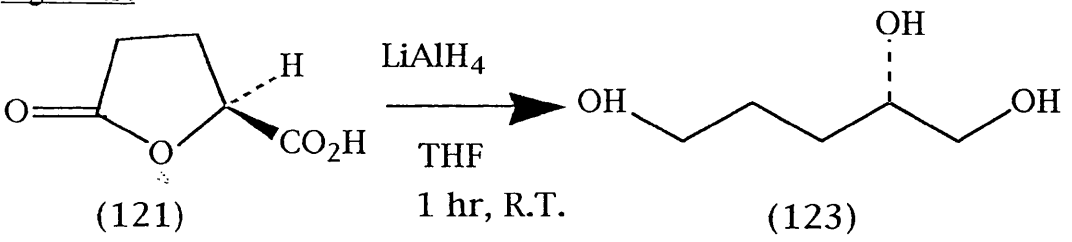
The acid (121) was reduced to the alcohol (134) using borane dimethyl sulphide as before. The alcohol (134) was then treated with phosphorus tribromide in order to replace the hydroxyl group with the better leaving group bromine and to form the bromolactone (156) with a C-bromine stretch at 720 cm^{-1} in the IR specrtum. This reaction was low yielding and requires some work in order to establish conditions which will give a more favourable yield.

The alcohol (134) and bromolactone (156) were both treated in a one pot reaction with neat dibenzylamine to afford the dibenzyl lactone (145). This then reacted further by opening the lactone (145) to afford the dibenzylamine-protected straight chain amide alcohol (146). This was evident by the loss of the lactone at 1740 cm^{-1} in the IR and the formation of an amide peak at 1600 cm^{-1}.

Finally, a new method for the synthesis of pentane-1,2,5-triol was devised by Professor Gani's research group.[85] This method required only 2 mole equivalents of $LiAlH_4$ and reduction was done over 1 hour at room temperature, as opposed to heating at reflux for 3 days. The work-up procedure was also different. $LiAlH_4$ was destroyed with a water/THF solution and filtered. The residue was redissolved in acetone and after removal of solvent *in vacuo* the colourless pentane-1,2,5-triol was obtained in 56% crude yield.

On an initial trial this procedure worked effectively. However it must be emphasised that Gani's procedure deals only with the formation of pentane-1,2,5-triol in crude yield. On distillation, the yield dropped to a level which was not markedly superior to other methods tried (yield 25%). The two major advantages of this method, however, are the short reaction time - one hour as opposed to several days - and the fact that the product appears to contain fewer impurities. The method is shown in Figure 4.3.

Figure4.3.



(121) (123)

This route, and the alternative route using dibenzylamine, look promising. However, a considerable amount of time and effort is likely to be required in order to optimise the conditions, especially those for the catalytic removal of the protecting groups. As these routes were only attempted in the final months of this work, it was not possible, in the time available, to optimise conditions. Moreover, by this time it had become necessary to start on the second phase of the work, the synthesis of *N*-alkylcadaverines for testing with the enzyme diamine oxidase. A decision was therefore taken to discontinue further work on the synthesis of the (*R*)- and (*S*)-[2-^{2}H]cadaverine dihydrochlorides (17) and (18). While this meant that it was not possible to carry out the envisaged feeding experiments, it did mean that other, more productive areas of the work could be completed.

CHAPTER 5

REVIEW OF DIAMINE OXIDASE AND POLYAMINES

5.1 INTRODUCTION

Early work on enzymes was mainly concerned with hydrolases and oxidoreductases,[99] but over the past decade recognition of the wider application of enzymes has increased considerably.[100] This is due to their ability to carry out easily chemical reactions that are highly specific and sometimes very difficult to achieve synthetically - probably because nature has had millennia in which to evolve effective techniques. Not only do they allow possible control of stereochemistry and regiochemistry, they also enable the use of very mild reaction conditions.

Enzymes are exceptionally versatile catalysts, being able to catalyse a broad range of reactions usually at room temperature and in aqueous solutions at neutral pH. They are also extremely efficient in that they can increase the rate of reactions by over a million times.

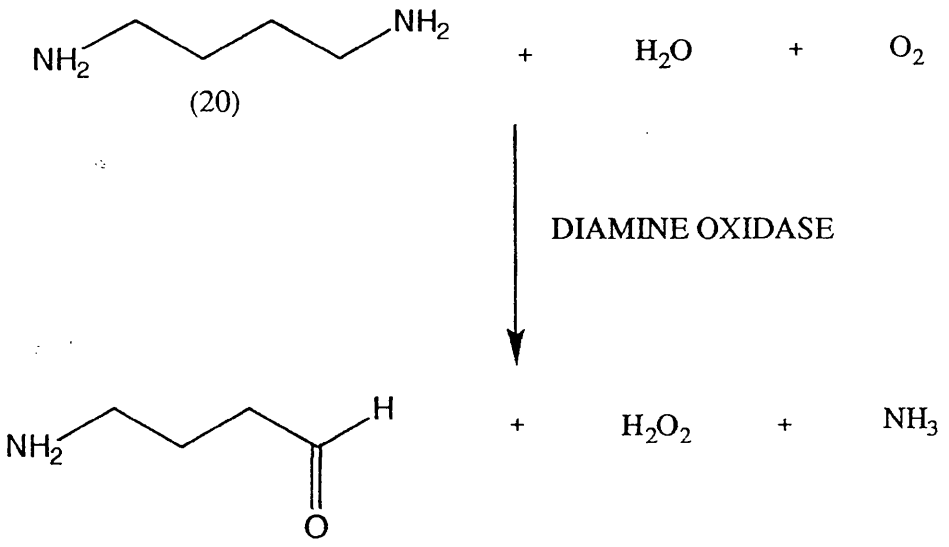
Drug manufacturers find enzymes particularly beneficial in the production of optically active materials since they wish to limit the synthesis to the single enantiomer that will have the desired biological activity.[101] This helps minimise possible detrimental effects caused by the other enantiomer as happened in the case of thalidomide.

5.2 DIAMINE OXIDASES

In this section we are limiting consideration to the diamine oxidases (DAO, diamine: oxygen oxidoreductase EC 1.4.3.6), as the particular focus in this study was the synthesis of a range of *N*-alkyl analogues of putrescine and cadaverine and the testing of these as substrates or inhibitors of pea seedling diamine oxidase (PDAO).

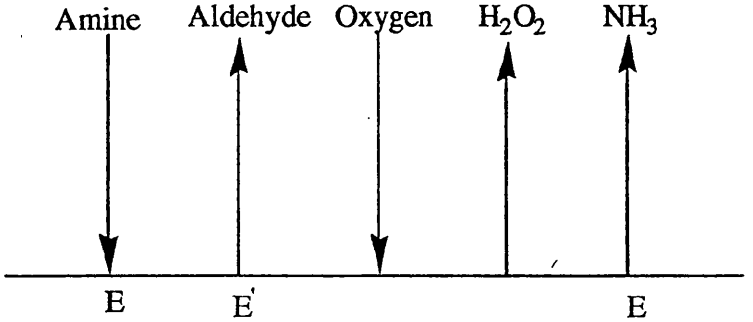
Diamine oxidases are copper containing proteins found in many living organisms.[102,103] Diamine oxidases catalyse the oxidative deamination of a range of primary diamines to the corresponding aminoaldehydes (Scheme 45), in reactions for which there are often no really reliable or convenient non-enzymic methods available.

SCHEME 45



Cleland[170] has shown that PDAO has a double-displacement mechanism (bi-ter, ping-pong kinetics) in which one equivalent of amine is transformed to the corresponding aldehyde under anaerobic conditions simultaneously with the formation of an intermediate reduced form of the enzyme (E'). E' is then oxidised by reacting with oxygen and the native enzyme is restored by the elimination of hydrogen peroxide and ammonia (Figure 5.1). Pec and co-workers have subsequently found the same kinetic sequence in pig kidney DAO.[105]

Figure 5.1. Schematic Representation of the reaction mechanism, which involves only binary enzyme-substrate complexes, the catalytic sequence being amine on, aminoaldehyde and ammonia off, oxygen on and hydrogen peroxide off.[105]



5.3 SOURCES OF DIAMINE OXIDASE

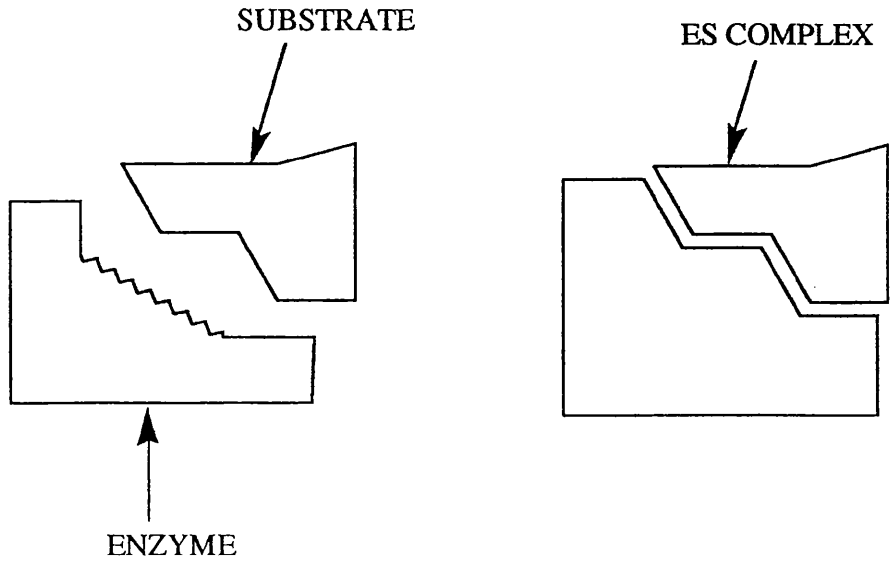
Two particularly convenient sources of diamine oxidase enzymes are the pig kidney enzyme, which is commercially available in an impure form, and pea

seedlings. Pea seedlings are the most active source of diamine oxidase and the enzyme is particularly stable during long-term storage and in assay.[128] Enzymic activity appears mainly in the cotyledons of pea seedling three to four days after germination and is at its maximum level at around ten days. Diamine oxidase is readily extracted from ten day old pea seedlings by methods developed by Hill, Tabor and, others.[116,117,118] In 1993 Wimmerova and co-workers outlined an improved and simplified procedure.[98]

5.4 SUBSTRATES AND FEATURES OF ACTIVE SITES

The first step in enzymatic catalysis is usually the formation of an enzyme-substrate (ES) complex. The active site of the enzyme binds the substrate to a specific area in the enzyme. The active site contributes the catalytic groups that are directly involved in the making and breaking of bonds and most enzymes are highly selective in their binding of substrates. The catalytic specificity of enzymes is largely dependent on the specificity of the binding process. This, in turn, depends on the arrangement of atoms in an active site. In many cases it is the complementarity of the shape of the substrate to the site that enables binding to take place. In other cases the active sites of enzymes are not so rigid, but are modified by the binding of the substrate and only take up their complimentary shape after the substrate is bound (Figure 5.2).[101]

Figure. 5.2



The active site of an enzyme takes up a relatively small part of the total. Most enzymes are made up of more than 100 amino acid groups, many of which will not be in contact with the substrate. It is the presence of these amino acids that contributes to the size and overall conformation of enzymes. In a study carried out by Yanagisawa and co-workers,[106] sodium dodecylsulphate (SDS) slab gel electrophoresis (a denaturing gel) gave a single band of molecular weight 85,000 for the pea seedling diamine oxidase. As the molecular weight of the enzyme, was estimated by gel filtration to be *ca* 180,000, this suggested that the enzyme consists of two identical subunits.[106] In more recent chromatographic and crystallographic studies the molecular weight has been estimated to be somewhat lower at 142,000 (± 10,000)[98] and 131,200 (± 13,000).[109] In both studies it was confirmed that the enzyme comprises two identical subunits.

Diamine oxidase has been shown to have wide substrate specificity, being able to oxidise a wide range of substrates including aromatic and aliphatic monoamines.[119] Two particularly good substrates are cadaverine and putrescine. In the present study one of the aims was to synthesise a range of *N*-alkyl cadaverines to establish whether these are substrates of pea seedling diamine oxidase as it has been shown that substrate analogues may act as substrates for DAO.

5.5 METALLOENZYMES

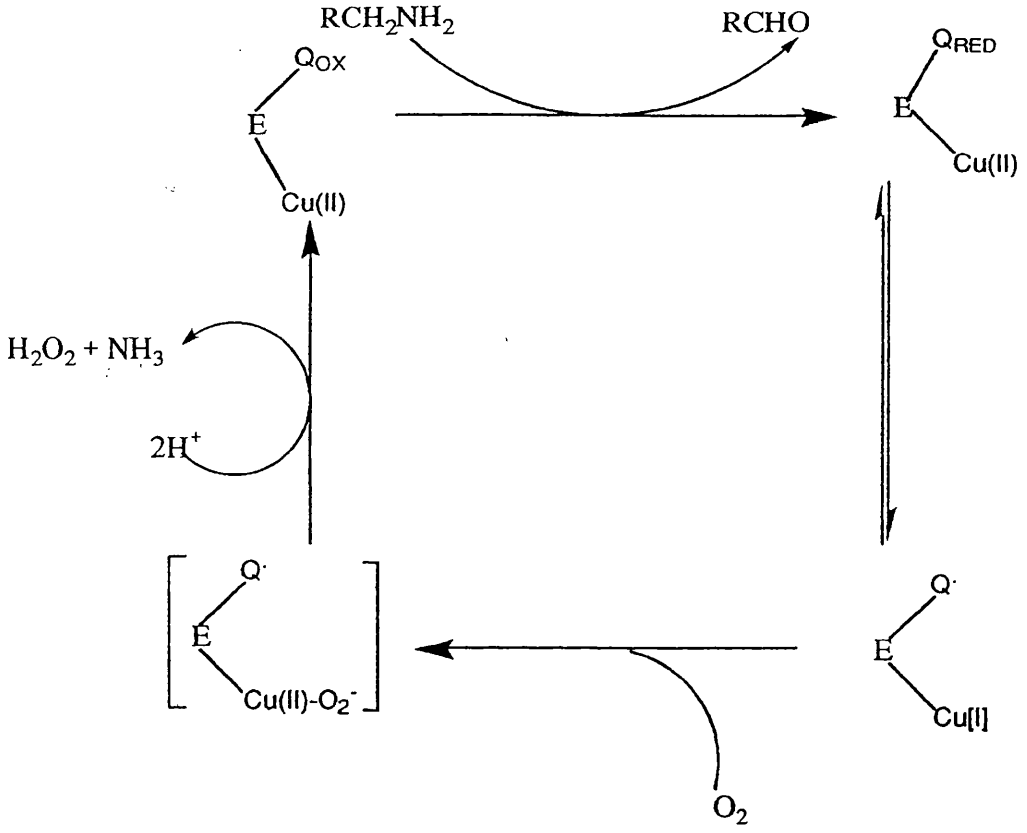
Some enzymes incorporate one or more metal atoms in their normal structure. The metal ion is a permanent part of the enzyme and its participation is not limited only to the time that the enzyme-substrate complex exists. A metal atom occurs very close to, or directly at the active site and plays a role in the activity of the enzyme. Such enzymes are called metalloenzymes, and the most commonly occurring metals in these are zinc, iron and copper. Most of the copper-containing enzymes are oxidases, with diamine oxidase being a typical example. Although more than 15 copper containing enzymes have been isolated, their structures and functions are not yet fully understood.[107]

The diamine oxidase enzyme consists of two identical subunits containing two copper ions (Cu^{2+}) per dimer.[108] In diamine oxidase the two copper ions are firmly bound and the removal of copper by dialysis against a chelating agent causes deactivation. Activity can be restored by the addition of Cu^{2+}. Other metal ions do not reactivate the copper free enzyme.[105]

Historically it was thought that the protein only contained Cu^{2+} ions. More recent research by Dooley and co-workers has produced evidence of the generation also of a Cu(I)-semiquinone state by substrate reduction of amine oxidase under anaerobic conditions.[167] They suggest that copper acts as a mediator in a catalytic cycle whereby a diamine is oxidised to a corresponding amino aldehyde with a quinone moiety in the enzyme acting as an electron acceptor. The reduced quinone is then partly re-oxidised by the transfer of one electron to the cupric ion (Cu^{2+}) which then changes to the cuprous ion (Cu^{+}). The cupric state is restored by the bonding of oxygen to the enzyme complex, co-ordinating to the reduced copper Cu(1) ion and leading finally to the fully oxidised quinone and thus the fully oxidised enzyme (Scheme 46).

The reductive potential acquired by oxygen is used to produce hydrogen peroxide and release ammonia. In Scheme 46 the species shown in brackets is the hypothesised intermediate, Q_{ox} is the oxidised quinone, $Q\cdot$ the semiquinone, and Q_{red} the reduced quinone.

SCHEME 46

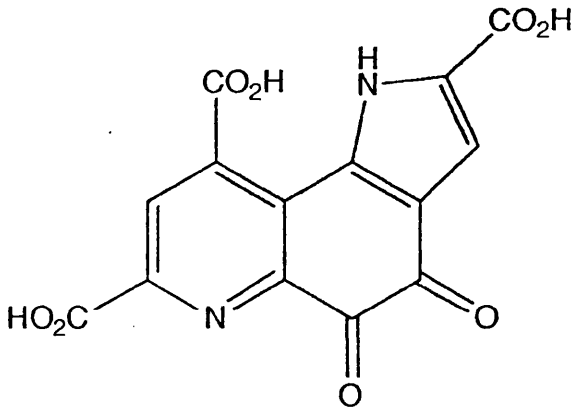


The Cu(1)-semiquinone state of amine oxidase had not been detected in earlier work because this state is in thermal equilibrium with Cu(II)-reduced quinone. Internal electron transfer from copper to semiquinone is favoured by low temperatures. Since previous work had used low temperature electron paramagnetic resonance spectroscopy to study the copper centre, the semiquinone form was missed.[109] It is not yet known if the quinone is bonded to the copper, or whether the substrates interact at the metal site.[109] There is also some doubt as to whether both, or only one of the copper sites, are required for the enzyme reaction.[110]

5.6 THE ORGANIC COFACTOR IN DIAMINE OXIDASE

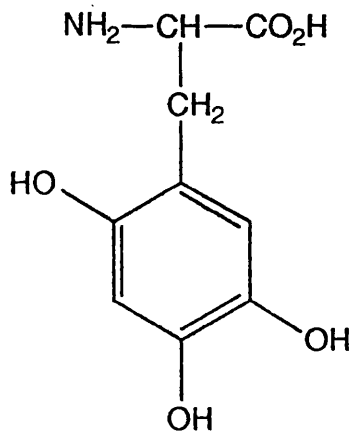
The identity of the organic co-factor in amine oxidases was unknown for many years. In 1984 the presence of covalently bound pyrroloquinoline quinone (PQQ) (160) was reported in copper-containing plasma amine oxidase.[111] The PQQ was extracted in the form of a hydrazone after treatment of the enzyme with a hydrazine, followed by hydrolysis with proteases.[112] Using this method, known as the hydrazine method, Glatz and co-workers appeared to have established the

presence of covalently bound PQQ in amino oxidases of pea seedlings and pig kidney.[113]



(160)

Recently, however, this finding has been strongly challenged.[109] Although PQQ can be extracted from amine oxidases, a three dimensional structure elucidation of the enzyme showed that the electron density map of the cofactor in the active site could not be fitted with PQQ.[114] It was concluded that the hydrazine method and the hexanol extraction method were unsuited to reveal the real structure of the co-factor. It has subsequently been claimed by James and co-workers that 6-hydroxydopa (Topa) (161), and not PQQ, is the co-factor in diamine oxidases.[115]



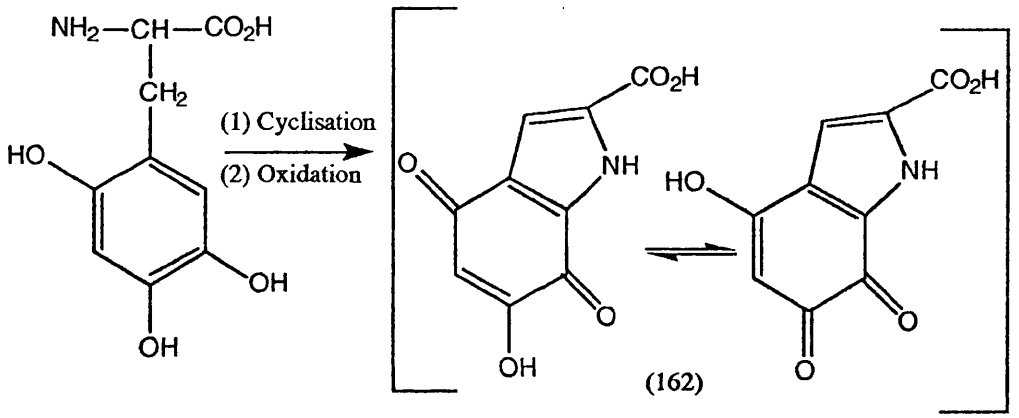
(161)

James and co-workers isolated an active site cofactor-containing peptide from bovine serum amine oxidase in sufficiently high yield to enable them to achieve a full structural characterisation. Because only one amino acid was detected at each round

of peptide sequencing it was concluded that the cofactor had a single stable point of attachment to the protein. They also postulated that a derivative of the topa phenylhydrazone formed during proteolysis may co-elute with the phenylhydrazone of PQQ.

Topa quinones undergo a rapid intramolecular cyclisation reaction (Scheme 47). The compound formed (162) bears a very close resemblance to PQQ which may help explain why PQQ had been postulated as the cofactor and apparently demonstrated to be so in so many studies.

SCHEME 47

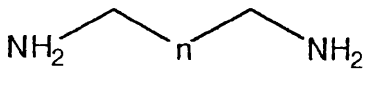


(162)

As a result of the discovery of topa as a possible cofactor, Vignevich and co-workers have suggested that amine oxidases may be the first examples of what may prove to be a new class of enzyme, where the cofactor is a post-translationally modified amino acid side-chain of the protein itself.[109] They also suggest that crystal structure analyses are now required to solve remaining long-standing problems. These include determining the structure of the active enzyme site, discovering the relationship between the copper atom and the topa cofactor and obtaining details of the enzyme-substrate interactions. Moreover, it is not yet clear whether the cofactor of PDAO is identical to that of pig kidney, and other diamine oxidases, as it has been shown that there are differences in the substrate specificity of their active sites.[121]

Diamine oxidases have a wide substrate specificity.[119,120] Research has shown, however, that the diamine oxidases obtained from pig kidney and from pea seedlings are not identical.[121] There are important differences in their ability to

oxidise compounds of different chain lengths. Although both have similar catalytic functions, there are structural differences in the enzyme active sites. For example, although both can readily oxidise 1,4- and 1,5-diamines, (20) and (19) respectively, only the pig kidney enzyme can oxidise 1,3-diaminopropane (165).[122]



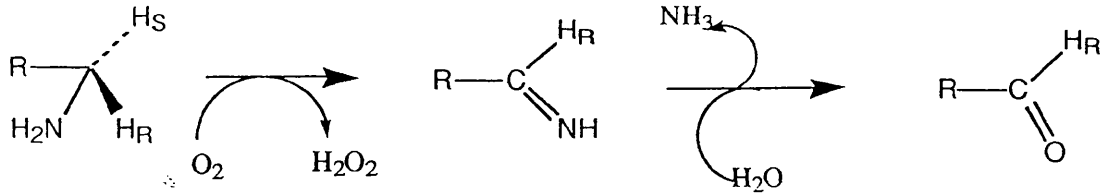
$n = CH_2$ (165)
$n = CH_2CH_2$ (20)
$n = CH_2CH_2CH_2$ (19)

5.7 STEREOCHEMISTRY OF DIAMINE OXIDASES

The synthetic usefulness of enzymes is due, in part, to their ability to discriminate between structural and stereochemical features of their substrates. The most useful enzymes will accept a wide range of substrates while retaining the ability to act stereospecifically on each. As indicated earlier, diamine oxidases are of low substrate specificity, acting upon a wide range of amines. Pea seedling diamine oxidase has been shown to oxidise a variety of substrates including aromatic and aliphatic monoamines, lysine and ornithine.[128] In an inhibition study carried out in 1991 Pec and Frebort[169] concluded that a protonated diamine with a five carbon chain length is required for substantial substrate binding with the pea seedling diamine oxidase. They argued that the problem with studying the interaction of amines and diamines with pea seedling diamine oxidase was in attempting to decide between their behaviour as substrates or inhibitors since it has been found that some relatively good substrates such as *N*- and *C*-alkylputrescines may also act as inhibitors.

Among the best substrates found to date are cadaverine and putrescine. The oxidation of the methylene group adjacent to the nitrogen atom is of vital importance in the metabolism of primary amines in a wide range of organisms (Scheme 48).

SCHEME 48

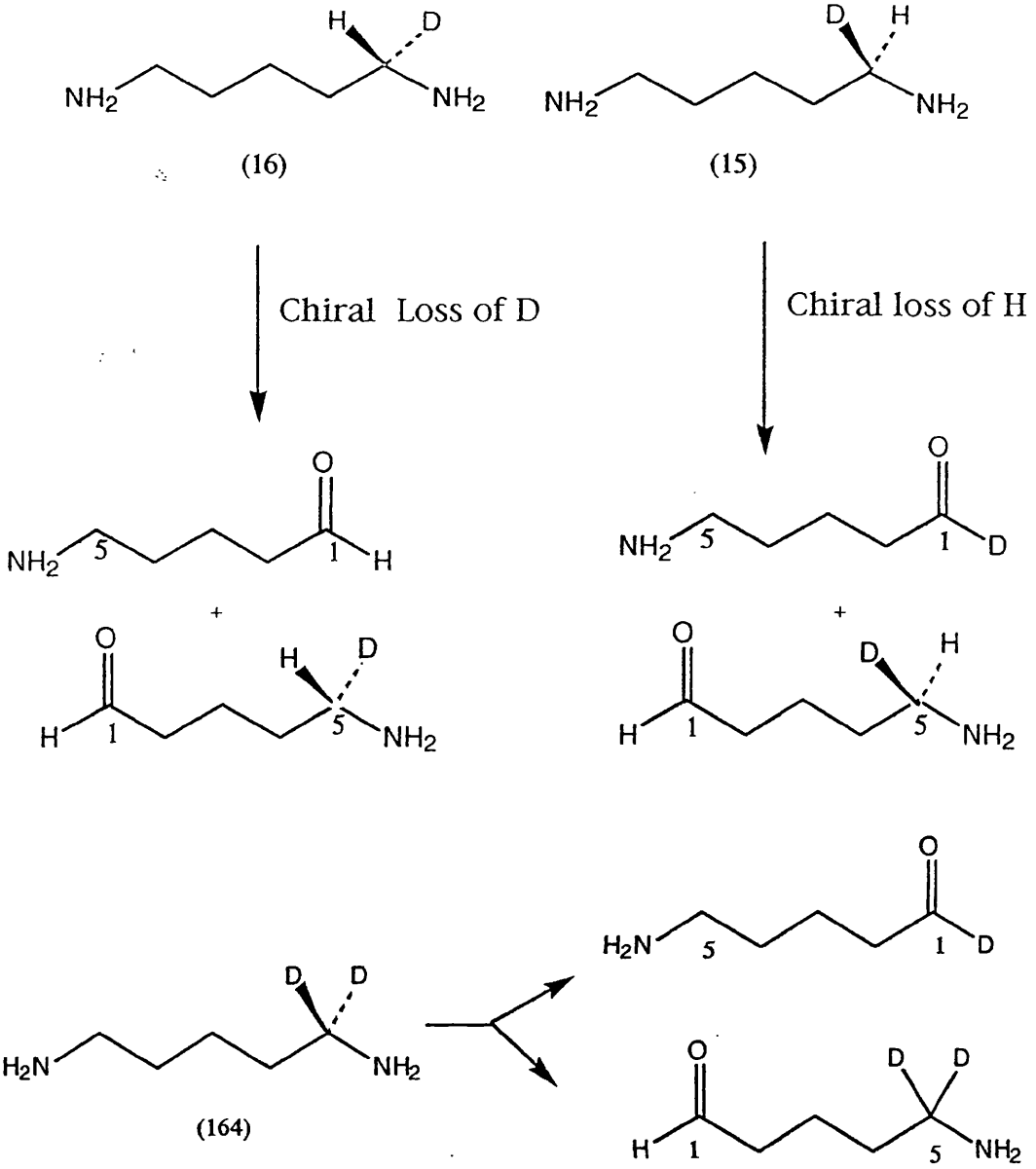


This process is also a key step in the biosynthesis of many alkaloids. It is therefore important to understand the stereochemistry of the transformation.

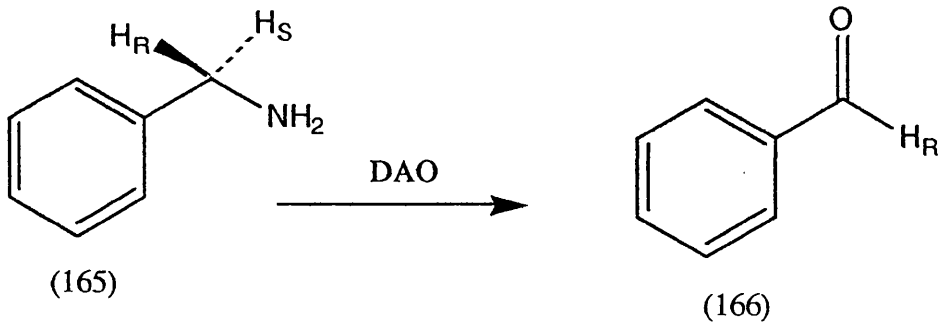
In a number of different studies a wide range of substrates has been examined using substrates enantiomerically labelled with deuterium or tritium whose absolute stereochemistry had been determined by correlation with compounds of known absolute configuration. For example, the stereochemistry of the deamination of cadaverines catalysed by pig kidney DAO was investigated by Richards and Spenser.[123] They found that it is the *pro-R* hydrogen which is retained while the *pro-S* hydrogen is removed by action of the enzyme (Scheme 48).

Deuterium NMR spectroscopy was carried out on the products obtained from enzyme catalysed oxidations using (*S*)-[1-^{2}H]- (16), (*R*)-[1-^{2}H]- (15) and [1,1-^{2}H$_2$]-cadaverines (164) to locate deuterium in the samples. The spectroscopic studies showed that the *pro-S* hydrogen from C-1 of cadaverine was lost in the oxidative deamination (Scheme 49).

SCHEME 49



This stereospecificity corresponds to that of the oxidative deamination of benzylamine (165) to benzaldehyde (166) catalysed by pea seedling diamine oxidase.

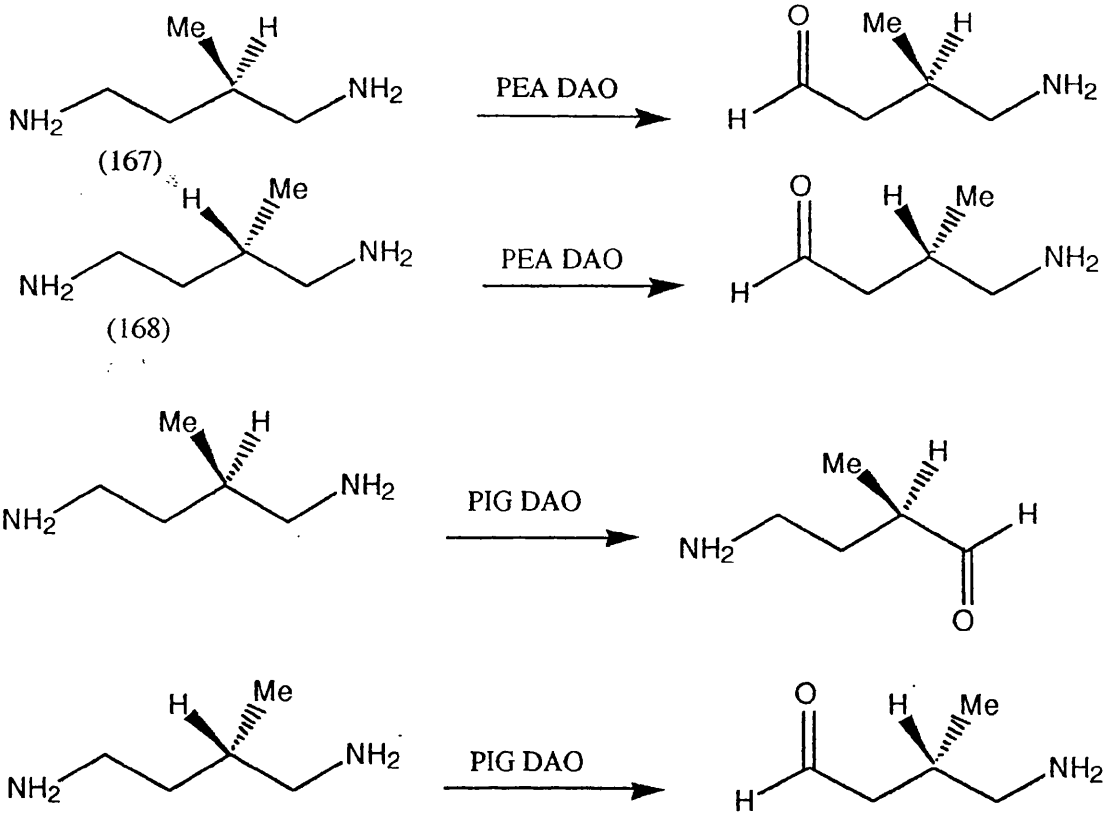


Pea seedling diamine oxidase was also used by Battersby and co-workers[124] to study the stereochemistry of cadaverine oxidation. They confirmed the earlier findings on the loss of the *pro-S* hydrogen and concluded that, in a symmetrical molecule, either of the two amino groups can be oxidised initially by the enzyme.

5.8 REGIOSELECTIVITY AND STEREOSELECTIVITY

The regioselectivity in the oxidative deamination of 2-methylputrescine catalysed by pea seedling and pig kidney DAO's has been studied by Santaniello and co-workers.[125,126] They aimed to provide more information on the differences in regioselectivity between the two DAO enzymes. They found that the pea seedling DAO catalysed the oxidation of both (*R*)- and (*S*)-2-methylputrescine, (167) and (168) at the less hindered C-4 position in a regioselective manner independent of the configuration of the substrate. The pig kidney DAO, however, showed two different oxidation patterns dependent on the stereochemistry of the substrate used. Oxidation of the (*R*)-isomer occurs at the C-1 primary amine, and the (S)-isomer is oxidised at the less hindered C-4 position. The pig kidney DAO is thus shown to be more sensitive to the configuration of the substrate (Scheme 50).

SCHEME 50



5.8 INHIBITION OF DIAMINE OXIDASES

The inhibition of enzymic activity by specific small compounds and ions is important since it can act as a control mechanism in biological systems, and can provide insight into the mechanisms of enzyme action.

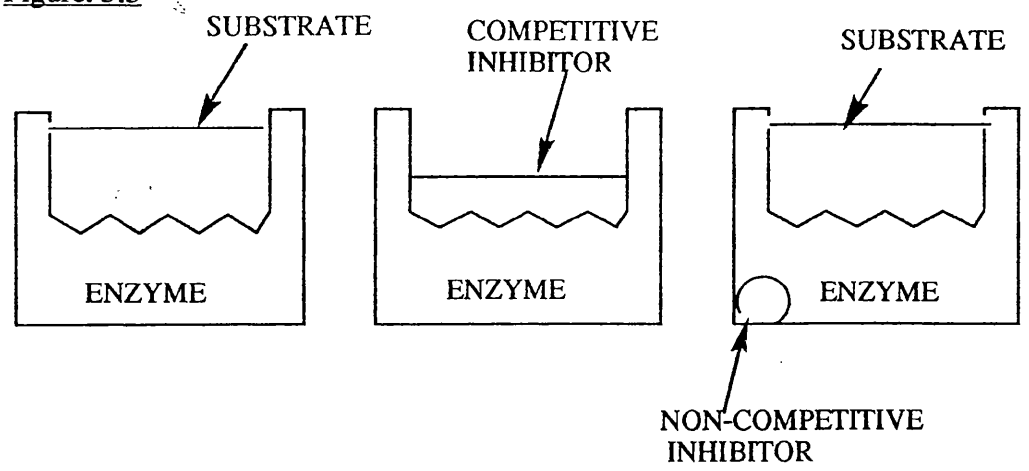
Inhibition may be either reversible or irreversible. In irreversible inhibition the inhibitor is either covalently linked to the enzyme or is so tightly bound in some other way that it releases very slowly. Reversible inhibition, on the other hand, involves a rapid binding/dissociation equilibrium of the inhibitor and enzyme.

The simplest form of reversible inhibition is competitive inhibition, in which the inhibitor mimics the substrate and binds to the active site of the enzyme thus preventing the substrate from binding to the same active site. A competitive inhibitor diminishes the rate of catalysis by reducing the proportion of enzymic molecules that have a bound substrate.[101]

The other simple form of reversible inhibition is non-competitive inhibition. In this form the inhibitor and substrate can both bind to the same enzyme molecule.

As their binding sites do not overlap, a non-competitive inhibitor decreases the turnover number of an enzyme rather than reducing the proportion of enzyme molecules that have a bound substrate (Figure 5.3).

Figure. 5.3



More complex situations may also be encountered. For example: "mixed inhibition", which shows features of both competitive and non-competitive models; coupling (or acompetitive) inhibition which is a specific form of mixed inhibition which occurs when the inhibitor is bound into the same subsite as the substrate but the inhibitor binds only with the enzyme-substrate complex and not with the free enzyme; and partial non-competitive in which the presence of the inhibitor does not affect the binding of the substrate, but the rate of the breakdown of the enzyme-inhibitor-substrate complex is slower than the breakdown of the enzyme-substrate complex.[147]

Measurement of the rates of catalysis at different concentrations of substrate and inhibitor is the means whereby competitive and non-competitive inhibition may be distinguished. The kinetics of these and the other specific types of inhibition are dealt with in Chapter Seven.

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

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

5. product inhibition;

6. suicide substrates/inhibitors.

1. Enzyme inactivators.

These usually act by irreversible inhibition and lead to partial or total loss of enzyme activity. Known examples of enzyme inactivators are phenylhydrazine, hydroxylamine, aminoguanidine and semicarbazide. They operate by reacting with the carbonyl function on the enzyme at the active site.

2. Copper chelating agents.

These inhibitors act by chelating to the Cu(11) present in the enzyme. Examples of copper chelating agents are the azide and cyanide groups and compounds such as 8-hydroxyquinoline and 1,10-phenanthroline.

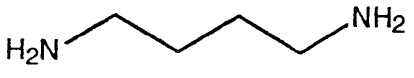
Inhibition studies carried out by Pec and Havinger[134] in 1988 demonstrated that sodium azide inhibited PDAO acompetitively (coupling). They concluded that the azide forms only an inactive complex with the enzyme substrate and suggested that the binding of the substrate to the active site of the enzyme gives access to the central Cu^{2+} ion on which the azide, as ligand, is bound. They argued that there was little possibility of the azide being bound to another site of the molecule because the character of the inhibition mitigated against this.

3. Substrate analogues.

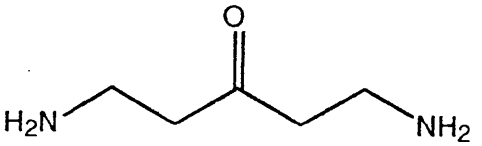
As indicated earlier, compounds that resemble the structure of the substrate can make effective competitive inhibitors by diminishing the rate of catalytic action. Comparisons of structure and activity relationships between substrates and inhibitors of DAO have shown that a change in structure may bring about major changes in reactivity with the enzyme. The rate at which the substrate analogue is enzymically converted into product is one way whereby possible inhibitor activity may be predicted.

The structural relationships between DAO inhibitors and the two substrates, putrescine (20) and histamine (176) were the subject of study in the 1980's.[127] Earlier work had suggested that pea seedling diamine oxidase was only inhibited by

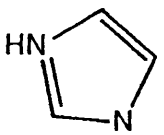
aliphatic diamines such as putrescine (20) and 1,5-diaminopentan-3-one (177), whereas pig kidney diamine oxidase was inhibited by imidazole (178) and its derivatives such as histamine (176). The more recent work shows that structures combining histamine with a reactive amino group, such as compounds (179) and (180), can inhibit both enzymes. It appears that the presence of the imidazole ring could be the inhibiting agent in the plant enzyme. Compound (181) inhibits the pea seedling enzyme selectively. This has an aliphatic diamine on the side chain of the ring and it is this which may be acting as the effective inhibitor.



(20)



(177)



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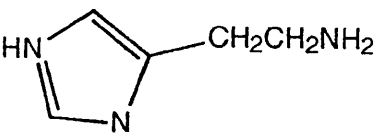

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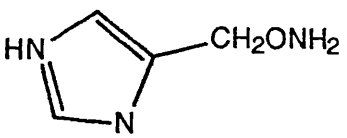

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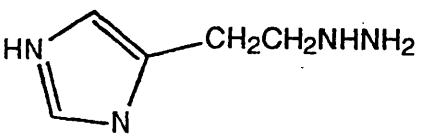

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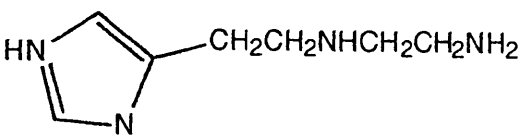


(181)

4. Substrate inhibition.

In many enzymes the rate of oxidation increases to maximum value over a limited lower range of substrate concentrations but at higher concentrations the rate decreases with the expected maximal rate not being obtained. At very high

concentrations the rate may actually drop to virtually zero. This phenomenon is termed substrate inhibition. Many enzymes display this characteristic, among them diamine oxidase.[165]

Several mechanisms may be responsible. For example, the substrate at high concentrations may reduce the water concentration and slow the rate if water is one of the reactants. Alternatively, the substrate may interfere with the binding of a co-enzyme in transfer reactions, or increasing substrate concentration may increase the ionic strength of the reaction mixture. This could modify the rate independently of any direct effects of the substrate.

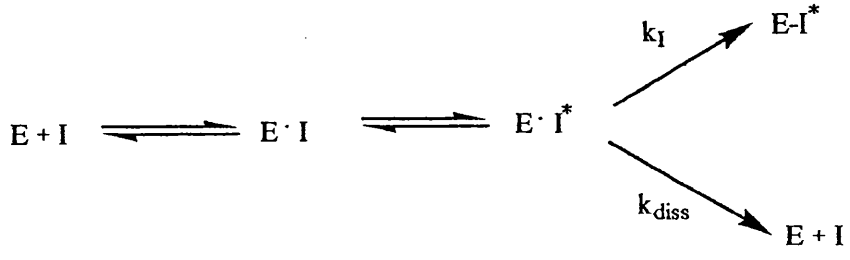
5. Product inhibition.

In one form of product inhibition the reverse reaction, whereby the products are transformed into substrate, competes with the forward reaction so that the rate of substrate disappearance or product formation is reduced. In another form of product inhibition the product of the reaction can combine with the enzyme, or other component of the system, so that the forward rate is inhibited.

6. Suicide substrates.

This is a class of inhibitors which not only use the binding specificity of their target enzyme but also use its catalytic appparatus for chemical activation. The result is that a normally harmless reversible inhibitor is converted into a powerful irreversible inhibitor. The characteristics of suicide substrates are that they must be chemically unreactive in the absence of enzyme, they must be specifically activated by their target enzymes and, in activated form, react more rapidly with the target enzymes than they dissociate ($k_I >> k_{diss}$) (Figure 5.4).[169,165]

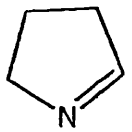
Figure 5.4



5.10 ASSAY SYSTEMS FOR DAO

There are many assay procedures for the measurement of diamine oxidase. The particular assay used in any instance will be the one which is judged to be most appropriate for the specific application required. Early assay techniques for diamine oxidase involved manometric measurement of oxygen uptake.[122] Techniques involving oxygen uptake are still regarded as useful as they can be applied for a wide variety of substrates.

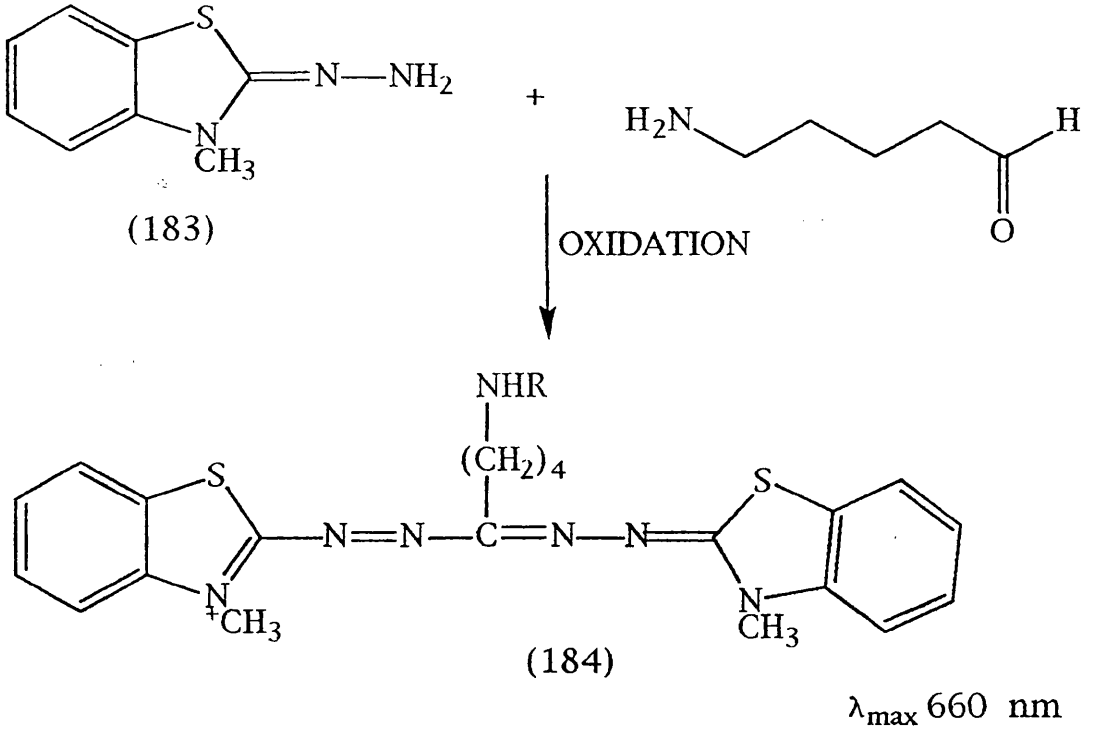
Other methods developed involved the production of 1-pyrroline (182) to form coloured complexes for the spectrophotometric determination of DAO activity.[129,130] These, however, are limited to applications involving putrescine as the substrate.



(182)

Frydman and co-workers[131] developed an assay for determining the rates of oxidation of *N*-alkylputrescines by diamine oxidase. This involved trapping the oxidation products, aminoaldehydes, with 3-methyl-2-benzothiazolinone hydrazone (MBTH) (183), and then measuring the absorbance of the resulting bishydrazone (184) at 660nm (Scheme 51). Although this procedure is convenient for routine screening of potential DAO substrates, it is unsuitable for detailed kinetic studies and the relative rates are of qualitative value only (Scheme 51).

SCHEME 51



Hydrogen peroxide is a common product in all diamine oxidase reactions. Thus assays to determine the rates of hydrogen peroxide production should have wide application. One such assay was developed in the 1950's by Booth and Saunders[132] and involved peroxidase coupling. This reaction was used in the 1970's to determine the activity of polyamine and diamine oxidases.[175] This assay proved reliable but had many potential complications. Thus, when in 1985 Stoner reported an improved spectrophotometric assay using hydrogen peroxide for the measurement of diamine oxidase activity, it gained widespread acceptance.[22] Subsequent workers have found that Stoner's assay system is not only a rapid and sensitive method for measuring diamine oxidase activity but is also a particularly reliable method for determining activity with a wide range of substrates.[135,136] As this is the assay method used in the present research, it will be discussed in more detail in subsequent Chapters.

5.11 THE RELATIONSHIP BETWEEN DIAMINE OXIDASES AND POLYAMINES

Diamine oxidases are central to polyamine metabolism in most organisms.[104,137,108] DAO is the key enzyme in the metabolism of polyamines, and inhibitors of DAO have been shown to have antimalarial, antibacterial and antifungal activities. This makes DAO a potentially invaluable resource for combined chemical and biophysical study and as a means of developing specifically designed drugs and inhibitors.

Diamine oxidases play an important role in regulating the cellular level of natural polyamines. Polyamines are metabolised by two main pathways. The first, the interconversion pathway, is a cyclic process which controls polyamine turnover. The second pathway involves terminal polyamine catabolism. This is catalysed by Cu^{2+} dependent amine oxidases, of which only diamine oxidase has been well defined. Each diamine intermediate can be converted into the corresponding aminoaldehyde by the oxidative deamination of a primary amino group. By further oxidation it is then taken to the amino acid or γ-lactam.

5.12 POLYAMINES

Polyamines are a group of simple, aliphatic compounds. They are vital to life since all living things contain at least one polyamine: for example, putrescine (20), spermidine (185) or spermine (186).

$N_2H(CH_2)_3NH(CH_2)_4NH_2$ $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

(185) (186)

Polyamines have many key roles to play in nature but it is their involvement in cell growth and replication that gives them their particular importance. Antoni van Leeuwenhoek is credited with the first observation of crystals of a polyamine in 1678, but it was over two hundred years later before the crystals were idendified as an organic base and given the name spermine (186).[139] The growth promoting properties of polyamines were first recognised in 1949 when a factor in orange juice

was found to be essential for the growth of a particular bacterium *Hemophilus parainfluenzae.* It was subsequently established that the factor involved was putrescine. Since that time there has been an increase in research and an accumulation of evidence showing the link between polyamines and the growth of living cells.[139]

5.13 POLYAMINES AND CELL GROWTH

The importance of polyamines in cell growth is due to their ionic binding to nucleic acids. Spermine and spermidine, for example, bind strongly with the acidic phosphate groups of nucleic acids. Polyamines stabilise DNA and RNA, and they also speed up every step in the transcription-translation sequence, that is the process whereby information coded by genes is used in the manufacture of proteins.[139]

Polyamine levels in dividing cells such as cancer cells have been found to be much higher than in resting cells, although the precise reasons for this have not yet been fully established.[140] This has important implications for chemotherapy,[141,142,143,144] and has encouraged a number of workers to consider the effects of inhibiting polyamine biosynthesis as a means of limiting cell proliferation in cancer treatments.[145]

5.14 THE INHIBITORY PROPERTIES OF POLYAMINES

By examining the roles that compounds play in cell physiology, it is possible to analyse the effects on the system when the concentrations of the compound is reduced or depleted. In the early 1970's a search for specific inhibitors of polyamine biosynthesis was initiated. The synthesis of a compound called α-difluoromethylornithine (DFMO) by Merrell-Dow Pharmaceuticals enabled researchers to confirm the role of polyamines in cell proliferation in many systems. It also had exciting spin-offs in relation to the inhibition of cell proliferation.

The first step in the sythesis of polyamines is the decarboxylation of the amino acid ornithine by the enzyme ornithine decarboxylase (ODC). DFMO binds specifically and irreversibly to ODC, thus inactivating it. It is therefore a very effective inhibitor of ODC.[146] Tumour cells proliferate rapidly and thus have a

greater requirement for polyamines. DFMO was shown to have a strongly inhibiting effect on the proliferation of cells such as leukaemia cells, and has proved to have anti-tumour activity when used on its own or in combination with other anti-tumour drugs like interferon.[139] Similarly, inhibitors of diamine oxidase may also have possible roles in anti-cancer activity.

One of the aims of the present research was to synthesise a number of polyamines, not previously synthesised, and to test them as substrates or inhibitors of DAO and also as possible anti-cancer or anti-fungal agents. This work will be discussed in some detail in Chapter Eight.

CHAPTER SIX

SUBSTRATES OF DIAMINE OXIDASE

6.1 INTRODUCTION

In the previous Chapter some general characteristics of enzymes and diamine oxidase were discussed. It was noted that enzymes have a powerful catalytic role in nature, being able to accelerate and control reactions in living cells. In this Chapter we examine the methods available for measuring the rate of catalysis and the relationship between catalytic rate and substrate. Also discussed will be a range of *N*-alkylcadaverines as substrates of pea seedling DAO.

The rate of catalysis (V) in many enzymes varies according to the substrate concentration [S]. When there is a fixed concentration of enzyme the rate of catalysis is almost linearly proportional to the substrate concentration, providing that the substrate concentration is small. When substrate concentration is high, the rate of catalysis is virtually independent of it (See graph 6.1).

As early as 1913 Michaelis and Menten proposed a simple model to account for these kinetic characteristics. This model is still used to explain the kinetic properties of many enzymes.[147]

6.2 THE MICHAELIS AND MENTEN MODEL

The crucial element in this model is that a specific enzyme-substrate (ES) complex is a necesssary intermediate in catalysis. The model may be presented as:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1)$$

The enzyme (E) combines with (S) to form an (ES) complex with a rate constant k_1. The ES complex can either dissociate to E and S with a rate constant k_2, or it can go on to form the product (P) with a rate constant k_3, usually in a process involving several intermediate steps.

The next requirement is an expression which relates the rate of catalysis to the concentrations of substrate and enzyme and to the rates of the individual steps. The

model starts with the assumption that the catalytic rate is equal to the product of the concentration of the ES complex and k_3.

$$V = k_3[ES] \qquad (2)$$

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

$$\text{Rate of formation of ES} = k_1[E][S] \qquad (3)$$

$$\text{Rate of breakdown of ES} = (k_2 + k_3)[ES] \qquad (4)$$

Under steady state conditions, when the rates of formation and breakdown of the ES complex are equal, the concentrations of intermediates remain constant while the concentration of starting materials and products are changing. Thus:

$$k_1[E][S] = (k_2 + k_3)[ES] \qquad (5)$$

This equation may be further rearranged as shown in (6), simplified by defining a new constant, the Michaelis constant, (K_M) as shown in (7), and substituting it into equation 6 which then takes the form shown in (8).

$$[ES] = [E]][S] / [(k_2 + k_3)/k_1] \qquad (6)$$

$$K_M = (k_2 + k_3) / (k_1) \qquad (7)$$

$$[ES] = [E][S] / K_M \qquad (8)$$

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

$$[E] = [E_T] - [ES] \qquad (9)$$

Substituting equation 9 into equation 8 produces:

$$[ES] = ([E_T] - [ES])[S] / K_M \qquad (10)$$

This implies that:

$$[ES] = [E_T][S] / ([S] + K_M) \qquad (11)$$

Substitution of the expression for [ES] into equation 2 gives:

$$V = k_3[E_T][S] / ([S] + K_M) \qquad (12)$$

The maximal rate of catalysis, V_{max}, is achieved when the enzyme sites are saturated with substrate, that is when [S] is much greater than K_M and [S] / ([S] $+K_M$) is close to 1. Thus:

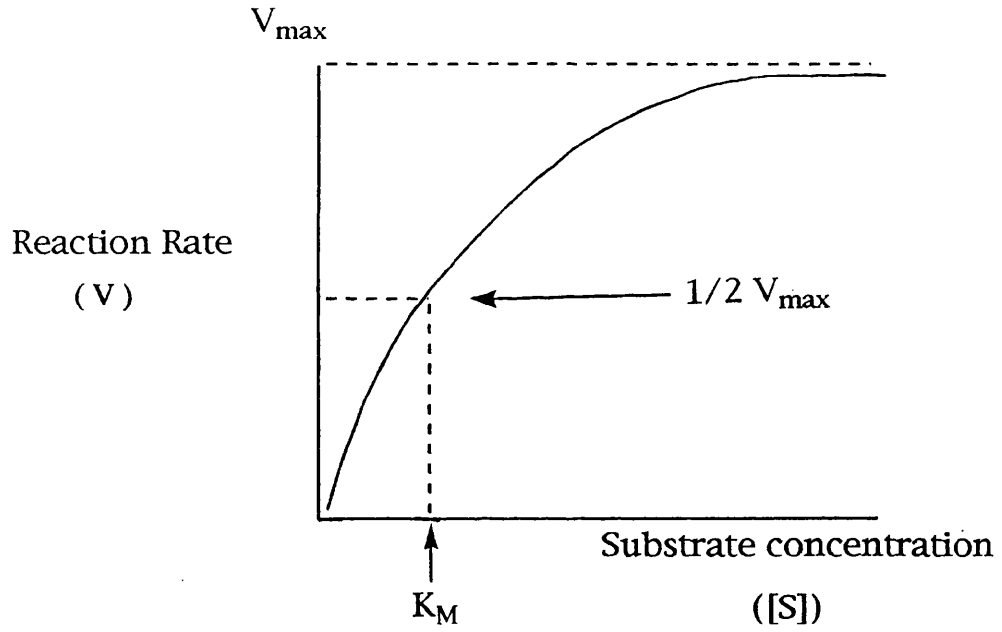
$$V_{max} = k_3[E_T] \qquad (13)$$

By substituting this equation into equation 12 we get the Michaelis-Menten equation:

$$V = V_{max}[S] / ([S] + K_M) \qquad (14)$$

This equation explains the kinetic data given in Graph 6.1.

Graph 6.1: Michaelis-Menten Plot: Reaction rate (V) *vs* Substrate concentration ([S])



At low substrate concentration, when [S] is much less than K_M, the rate of catalysis is directly proportional to the substrate concentration and equation 14 becomes:

$$V = [S]V_{max} / K_M \qquad (15)$$

At high substrate concentration, when [S] is much greater than K_M, the rate of catalysis is maximal and independent of substrate concentration. Thus;

$$V = V_{max} \qquad (16)$$

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

In the individual steps in equation 1, K_M is related to the rate constants. When k_2 is much greater than k_3, K_M is equal to the dissociation constant of the ES complex. Under these conditions, therefore

$$K_M = k_2/k_1 \qquad (17)$$

and the dissociation constant of the ES complex is given by

$$K_{ES} = [E][S]/[ES] = k_2/k_1 \qquad (18)$$

Under the conditions described in equations 17 and 18 the K_M is a measure of the strength of the ES complex. A high K_M indicates weak binding and a low K_M indicates strong binding.

The V_{max} is the maximal rate and reveals the turnover number of an enzyme if the concentration of the active sites $[E_T]$ is known as shown in equation 19.

$$V_{max} = k_3[E_T] \qquad (19)$$

where $[E_T]$ = the total enzyme concentration.

k_3 is called the turnover number of the enzyme which is the number of substrate molecules converted into product per unit of time when the enzyme is fully saturated with substrate.

6.3 THE LINEWEAVER-BURK PLOT

K_M and V_{max} are not difficult to derive from rates of catalysis at different substrate concentrations if an enzyme operates according to the scheme given in equation 1. The graph given by the Michaelis/Menten equation, however, is less useful if there are not three consistent points on the plateau of the curve at different substrate concentrations. In this situation an accurate value of V_{max} is impossible to obtain, and this, in turn, makes it impossible to obtain an accurate value for K_M. Moreover, the curve of the graph makes it difficult to extrapolate accurately upwards from non-saturating values of substrate concentrations.

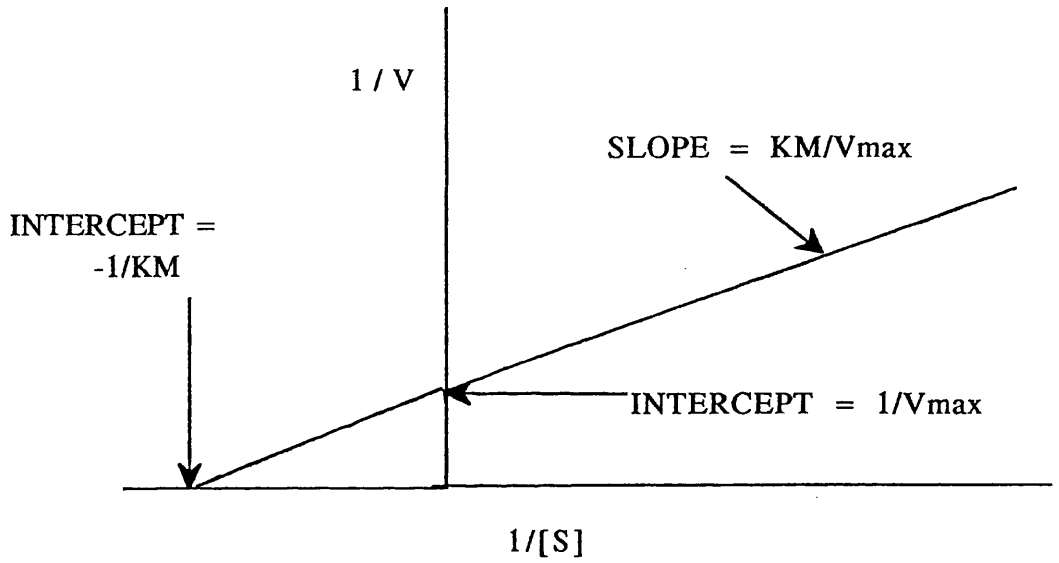
These difficulties were overcome ingeniously by Lineweaver and Burk.[148] By inverting the original Michaelis/Menten equation they were able to produce an

equation which allowed the graph to be drawn as a straight line and made it possible to obtain more accurate estimates of values for K_m and V_{max}. Thus the equation becomes:

$$(1/V) = K_M/V_{max}(1 / [S]) + 1/V_{max} \quad (20)$$

A plot of 1/V versus 1/[S] gives a straight line with an intercept of $1/V_{max}$ and a slope of K_M/V_{max} as shown in the Graph 6.2.

Graph 6.2: Lineweaver-Burk Plot



The Lineweaver-Burk plot is widely used by enzymologists, but is not without its critics.[149] It has been argued, for example, that it gives too much weight to low substrate concentrations and that these values are therefore not as accurate as they might be. Moreover, graphs often have to be re-drawn when extrapolations turn out to be unacceptably long, and departures from linearity may not be immediately obvious in this plot. A modification proposed by Eadie and Hofstee seeks to overcome this problem.[150]

Since it is generally necessary to obtain good data covering a wide range of substrate concentrations, enzymologists often find it more satisfactory to use both the Lineweaver-Burk and the Eadie-Hofstee plots together as a means of obtaining the best values for V_{max} and K_M.

6.4 THE EADIE AND HOFSTEE PLOT

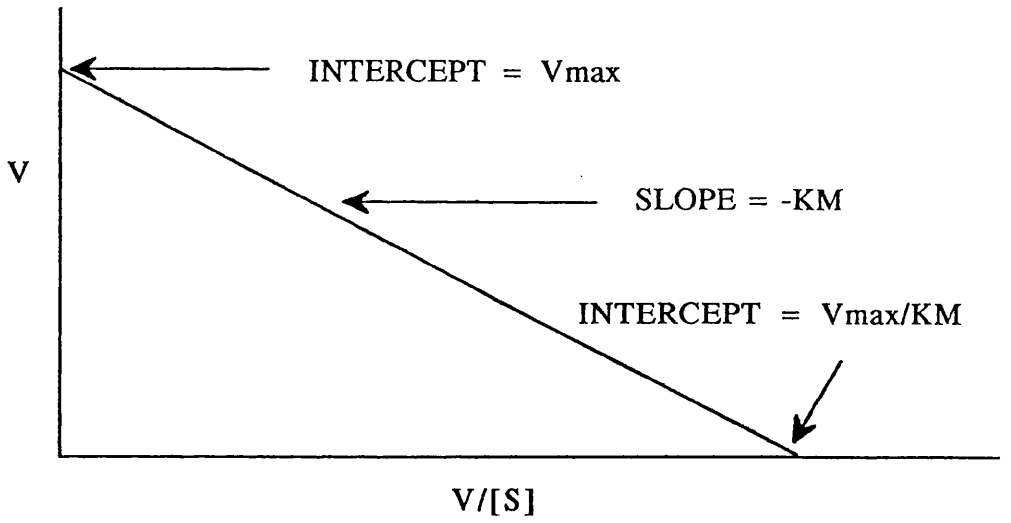
This plot modifies the Lineweaver-Burk plot by multiplying both sides of the equation by $V.V_{max}$. Thus:

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

$$\therefore V = -K_M(V/[S]) + V_{max} \qquad (21)$$

Like the previous equation this also gives a straight line graph. Plotting V against V[S] gives the slope, $-K_M$ and the intercept on the y-axis, V_{max}.

Graph 6.3: Eadie-Hofstee Plot

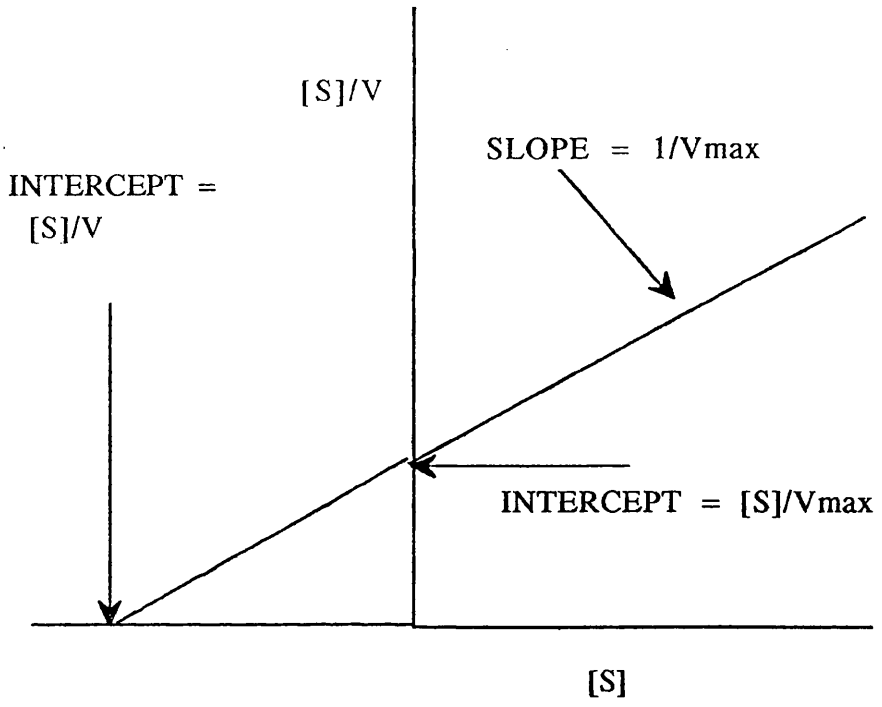


6.5 THE HANES PLOT

The Hanes Plot[154] provides an additional check on the accuracy of plotted data. This is obtained by plotting [S]/V against [S]. This gives a straight line with slope $1/V_{max}$ and intercept [S]/Vmax. The equation is

$$[S]/V = [S]/V_{max} + K_M/V_{max} \qquad (22)$$

Graph 6.4: The Hanes Plot



6.6 EXTRACTION OF PEA SEEDLING DAO

As indicated earlier, the DAO used in this research study is pea seedling DAO. This was extracted and purified using the method developed by Hill.[116] The protein obtained was taken up in phosphate buffer and stored in the freezer at around -20 ^{o}C in 0.5 ml aliquots. The enzyme was found to be stable for several months with very little loss of activity.

The protein concentration was determined using the Sedmak and Grossberg method.[151] This depends on the conversion of Coomassie brilliant blue G in dilute perchloric acid from a brownish-orange into an intense blue colour with the addition of the protein, using bovine serum albumin (BSA) or the protein lysozyme for standard calibration. Although the colour was found to be stable for about an hour at

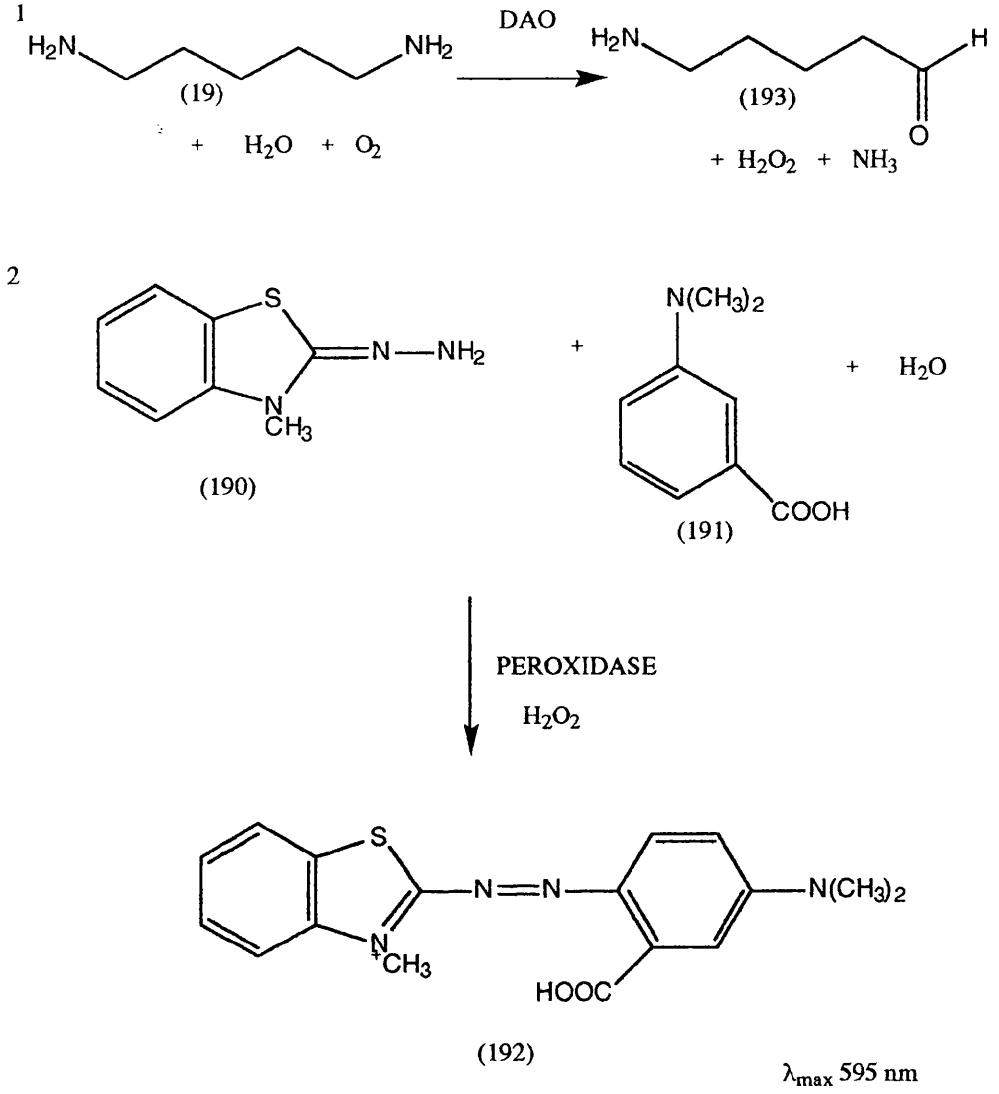
room temperature, the absorbance of the mixture was usually measured immediately after the addition of the protein.

The absorbances of the mixture at A_{620} (blue) and A_{465} (brownish-orange) were recorded for various concentrations of the proteins. Determination of the concentration of protein samples was obtained by measuring the A620/A465 ratio as plotted on standard graphs against the protein concentration. The results of this assay for the determination of protein concentrations can be easily reproduced and have been shown to detect less than 1mg of albumin. The protein concentration of the sample of pea seedling DAO used in this study was found to be 5.6 mg/ml of enzyme solution. (See Appendix 1.)

6.7 THE ASSAY PROCEDURES

The assay system used in this research was the spectrophotometric assay developed by Stoner.[22] Stoner's assay involves a coupled reaction with peroxidase and 3-methyl-2-benzothiazolinone hydrazone (MBTH) (190) with the acceptor 3-(dimethylamino)benzoic acid (DMAB) (191). In the presence of hydrogen peroxide and peroxidase, the chromogen MBTH is oxidatively coupled to DMAB, forming a purple indamine dye (192) having an absorption maximum at 595 nm (Scheme 54).

SCHEME 54



The rates of reaction were determined directly from the spectrophotometer. The relationship between dye formation and the extent of the reaction was initially calibrated using standard solutions of hydrogen peroxide.

Stoner noted that there could be potential problems with his assay due to the possibility of MBTH acting as an inhibitor of DAO. His suggested method for minimising this difficulty was to control the concentration of MBTH. Inhibiting effects can be further decreased if enzyme solution is added to the thermally equilibrated reaction mixture, followed immediately by the addition of substrate.

Equi[157] tested the validity of Stoner's assay system and found that her results corresponded with those obtained by Stoner. In addition she was able to show that

the oxygen level in the reaction vessel is not a limiting factor in the rate of reaction. Thus the validity of Stoner's assay procedures was confirmed.

In the present study, using the spectrophotometric assay, Michaelis/Menten behaviour was observed in every case. Lineweaver-Burk (1/V *vs.* 1/[S]), Eadie-Hofstee (V *vs.* V/[S]) and Hanes ([S]/V *vs.* [S]) plots were also used to analyse rate data for obtaining V_{max} and K_M values. The experiments were carried out at least three times with each substrate and the values quoted are a mean of the results obtained.

6.8 INTRODUCTION TO SYNTHESIS AND TESTING OF *N*-ALKYLCADAVERINES AND *N*-ALKYLPUTRESCINES

Several cadaverine and putrescine analogues have been shown to possess anti-fungal activity when tested on plant rusts.[156] Strong anti-fungal activity was demonstrated by the unsaturated *cis*- and *trans*-1,4-diaminobut-2-enes.[156] 3,3-Dimethylcadaverine and 2-ketoputrescine were also shown to be toxic to plant cultures by Dr. N. Walton (Institute of Food Research, Norwich).

Cadaverine and putrescine analogues have also been shown to have some substrate and some inhibitory activity in both mammalian and pea seedling DAO.[161] In 1987 Frydman and co-workers[131] carried out a study in which they found that *N*-methylputrescine (194) showed little substrate activity with DAO. *N*-Ethylputrescine (195) and other higher homologues were oxidised to the corresponding aminoaldehydes quite efficiently. This was an unexpected finding in that *N*-methylputrescine is oxidised readily in a number of plants such as *Nicotiana tabacum* and *Datura stramonium*.[160] A subsequent study by Ner,[135] using the same assay as Frydman, found *N*-methylputrescine to be a reasonable substrate for DAO. Equi[136] assayed *N*-methylputrescine as a substrate of DAO using Stoner's improved peroxidase coupled assay system described above (Scheme 54). She was able to verify that the oxidation of *N*-methylputrescine is catalysed by DAO. In another study Walton and McLaughlan[177] also confirmed *N*-methylputrescine as a substrate but with a somewhat better rate of oxidation.

N-Ethylputrescine (195) and *N*-propylputrescine (196), were also tested by Equi[136] and found to be fairly poor substrates of DAO. These compounds were again tested in the present study. The K_M values for both compounds were found to be virtually identical to those obtained by Equi,[136] while the V_{max} values obtained for both compounds in the present study were found to be somewhat higher. The reason for the differences in oxidation rates of putrescine analogues found in different studies is not entirely clear. However the values obtained in the present study were reproduced over several trials with substantially the same results on each occasion. It is possible, therefore, that the better oxidation rates shown in the present study, coupled with the comparable binding affinities, may indicate that compounds (195) and (196) are better substrates than Equi found them to be. The raw data curve and the Lineweaver-Burk, Eadie-Hofstee and Hanes plots for (195) and (196) are shown in Appendix 2.

Boswell has extended the synthesis of putrescine analogues but has not tested these with DAO.[79] A logical follow-on from Equi's and Boswell's work is to complete the testing of the *N*-alkylputrescine series of analogues in relation to DAO to see if comparison of results obtained provides any common pattern of substrate activity across the whole series of compounds.

Synthesis and testing of a range of *N*-alkylcadaverines would enable further comparison between the substrate activity of putrescine and cadaverine analogues. As some of these *N*-alkylcadaverines are known to be novel compounds which have not been tested previously, this work should also give further information about the structure and binding characteristics of the active sites of DAO enzymes.

6.9 SYNTHESIS OF *N*-ALKYLCADAVERINES

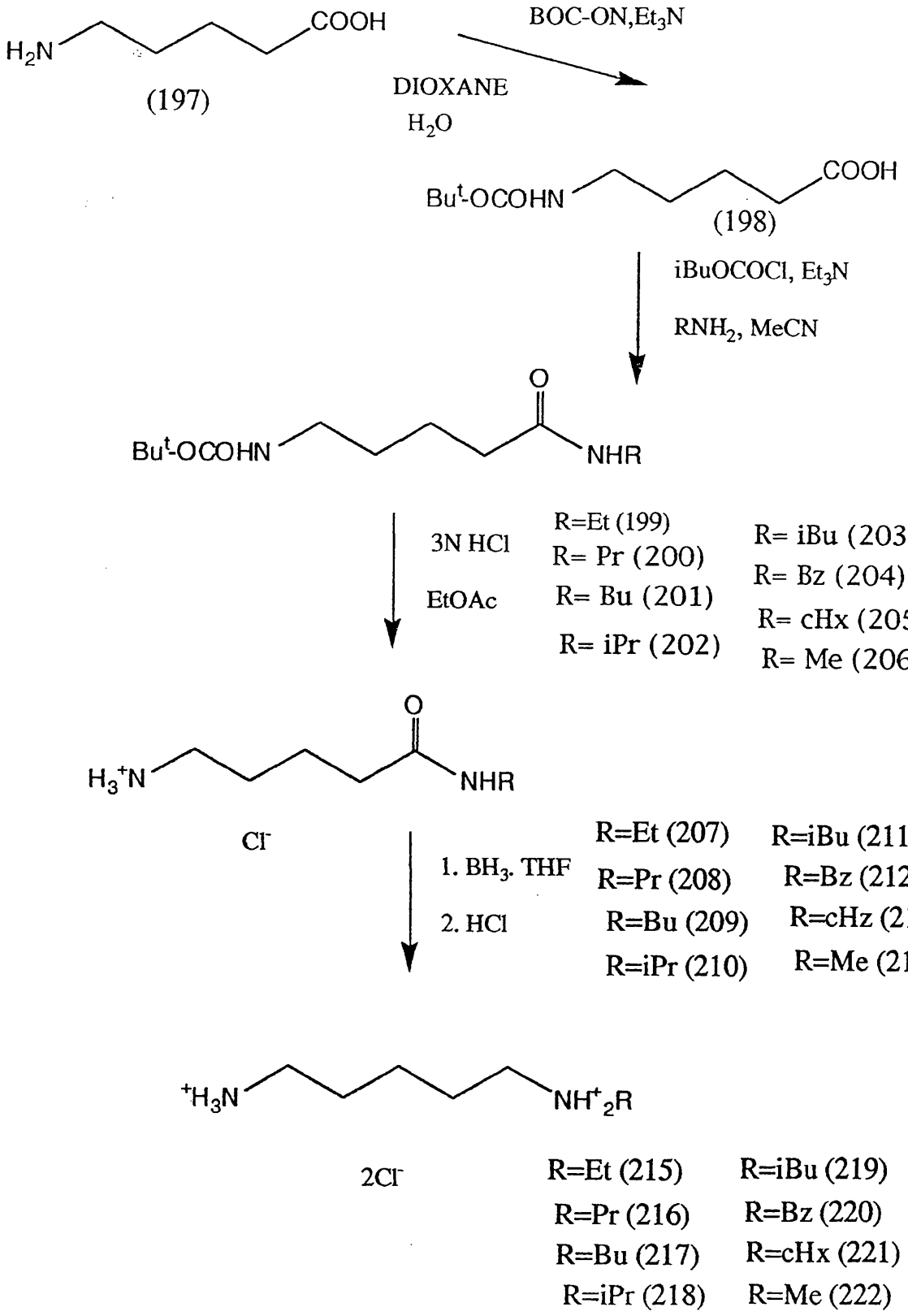
The synthesis of various *N*-alkylcadaverines was carried out using a modification of a route devised by Boswell for the synthesis of *N*-alkylputrescines.[79] The route for the synthesis of the *N*-alkylcadaverines is shown in Scheme 55. 5-Aminovaleric acid (197), a five carbon amino acid, was protected at the amine end using 2-*t*-butoxycarbonyloxyimino-2-phenyl acetonitrile (BOC-ON). This reaction

gave an average purified yield of 98%. The acid portion of 5-*t*-butoxycarbonylaminopentanoic acid (198) was converted into an amide with the appropriate amine via a mixed anhydride reaction. Reaction with an excess of neat alkylamine afforded the various *N*-alkyl-amides with yields varying from 76% for the *N*-propyl compound (200) to 99.5% for the *N*-ethyl compound (199). Loss of the acid group was monitored by the loss of the acidic proton at 10.49 in the ^{1}H NMR spectrum. Removal of the *t*-butoxycarbonyl (BOC) protecting group using 3M hydrochloric acid and ethyl acetate afforded *N*-alkyl-5-aminopentanamide hydrochloride salts (207) - (214) as oils.

The oils obtained, after being dried *in vacuo*, were triturated to give white crystals. Trituration times for this reaction can vary from one week to a few hours depending on the purity of the protected amide and on the dryness of the oil. The purer and drier the oil before trituration the faster it crystallises. Yields obtained were 72% for the *N*-propyl salt (208) and 99% for the *N*-cyclohexyl salt (213).

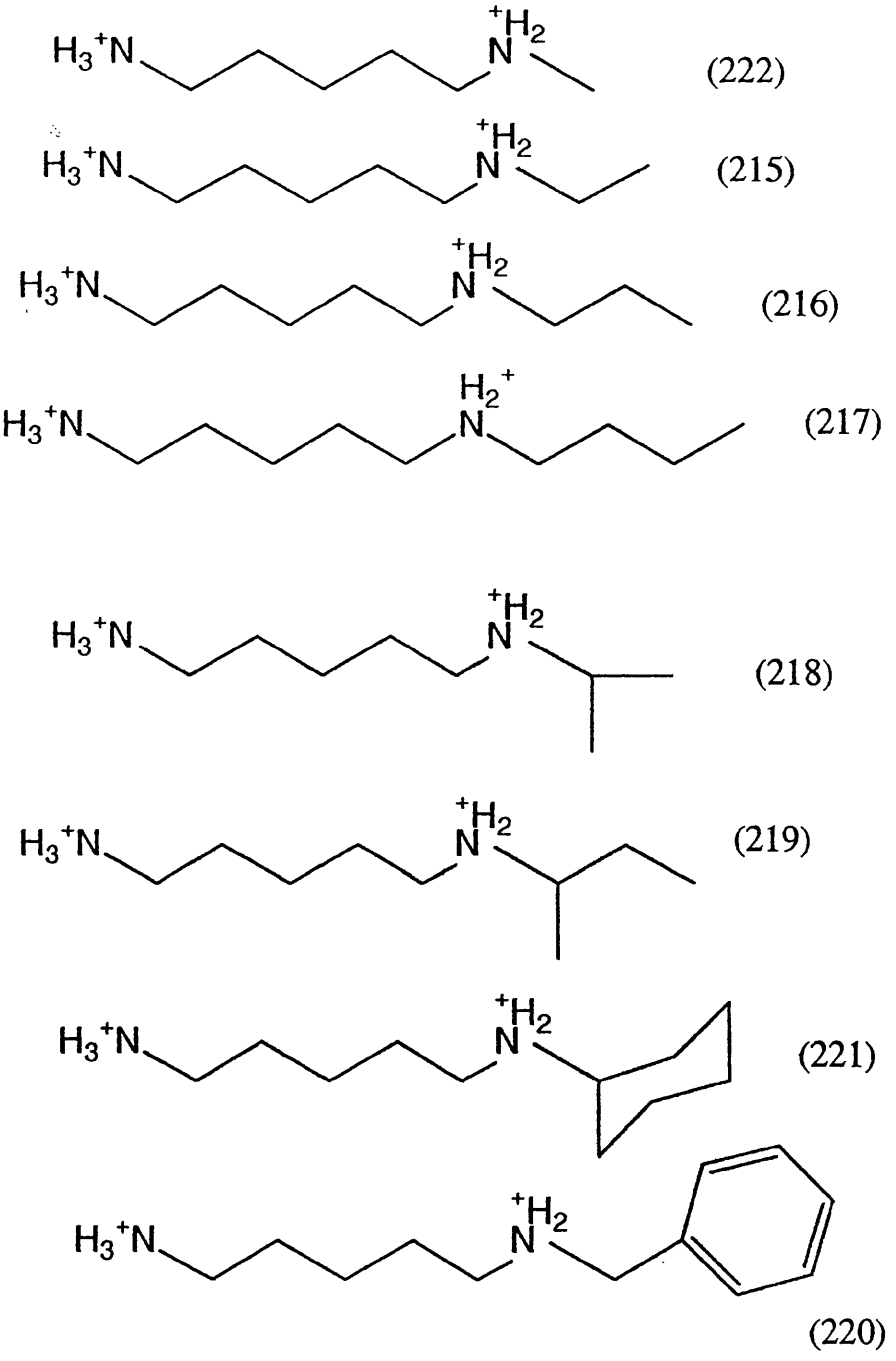
Finally, reduction of the amides to the *N*-alkylcadaverines was carried out using an excess of a 1M solution of borane in tetrahydrofuran (THF) heated at reflux overnight. The amides do not dissolve in THF. They only dissolve as the reaction proceeds. Thus long reaction times of around 24 hours were needed. An excess of borane was used during the long reaction period to help the reaction to go to completion. THF was distilled off at atmospheric pressure to prevent a build up of the hydrogen gas given off, and to help break down any chelated borane complex that formed. Methanol was repeatedly added and the mixture evaporated to dryness under reduced pressure until the weight of the product remained constant, i.e. no borate ester was left. The products were then recrystallised from methanol to give pure *N*-alkyl-cadaverines in yields of 45% for *N*-propylcadaverine (216) and 88% for *N*-methylcadaverine (222) (Scheme 55).

SCHEME 55


H2N
COOH
(197)
BOC-ON,Et3N
DIOXANE
H2O
But-OCOHN
COOH
(198)
iBuOCOCl, Et3N
RNH2, MeCN
O
But-OCOHN
NHR
3N HCl
EtOAc
R=Et (199)
R= Pr (200)
R= Bu (201)
R= iPr (202)
R= iBu (203)
R= Bz (204)
R= cHx (205)
R= Me (206)
O
H3+N
NHR
Cl-
1. BH3. THF
2. HCl
R=Et (207)
R=Pr (208)
R=Bu (209)
R=iPr (210)
R=iBu (211)
R=Bz (212)
R=cHz (213)
R=Me (214)
+H3N
NH+2R
2Cl-
R=Et (215)
R=Pr (216)
R=Bu (217)
R=iPr (218)
R=iBu (219)
R=Bz (220)
R=cHx (221)
R=Me (222)

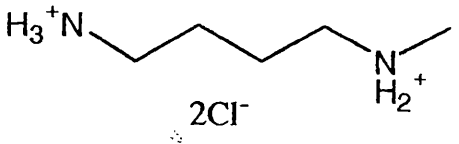
The full structures of the final compounds are shown in Figure 6.1.

Figure 6.1

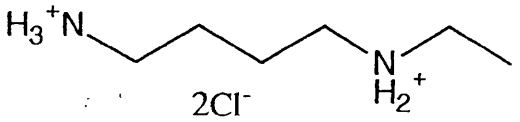


All of the compounds shown above were tested as substrates of pea seedling DAO. Also tested were the *N*-alkylputrescines which were kindly donated by H. Boswell.[79] The structures and compound numbers of these are shown in Figure 6.2.

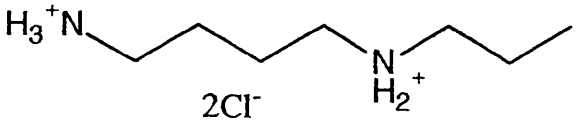
Figure 6.2



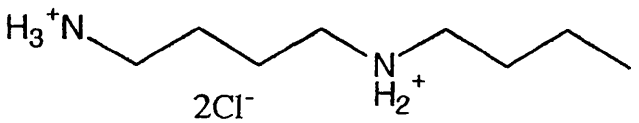
(194)



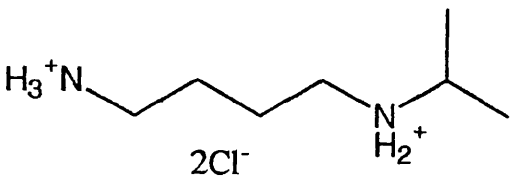
(195)



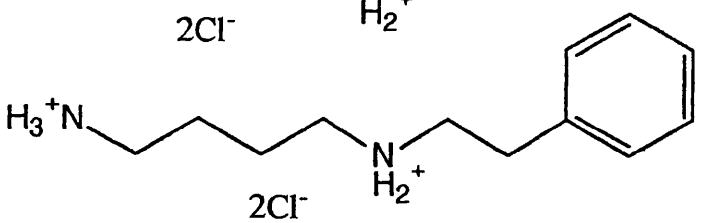
(196)



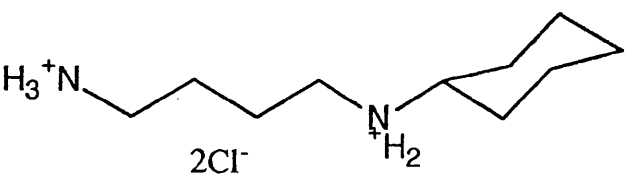
(223)



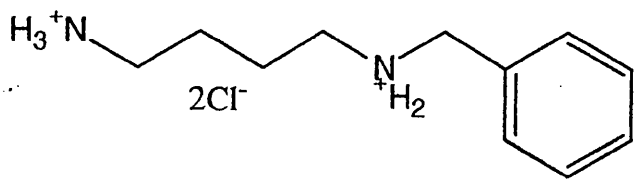
(224)



(225)



(227)



(226)

6.10 RESULTS AND DISCUSSION

The *N*-alkylcadaverines and putrescines tested in this study are listed in Table 6.1, which also gives the K_M and V_{max} figures obtained. In order to compare K_M and V_{max} values across the series, the figure Equi obtained for *N*-methylputrescine (194) is also given.

Table 6.1: K_M and V_{max} values for oxidation of *N*-alkyl- cadaverines and putrescines catalysed by diamine oxidase from pea seedlings at 25 °C and pH 6.3.

SUBSTRATE	K_M (mM)	V_{max} (μmol $mg^{-1}h^{-1}$)
Cadaverine (19)	0.14	1831
N-methylcadaverine (222)	1.18	650
N-ethylcadaverine (215)	1.21	619
N-propylcadaverine (216)	0.29	372
N-butylcadaverine (217)	0.21	352
N-isopropylcadaverine (218)	1.04	215
N-benzylcadaverine (220)	0.14	161
N-isobutylcadaverine (219)	0.90	73
N-cyclohexylcadaverine (221)	0.12	66
Putrescine (20)	1.18	1055
N-methylputrescine (194)	1.12	211*
N-ethylputrescine (195)	1.18	178
N-propylputrescine (196)	0.35	174
N-butylputrescine (223)	0.26	125
N-isopropylputrescine (224)	1.29	38
N-methylbenzylputrescine (225)	0.21	103

* Equi's figures μmol $mg^{-1}h^{-1}$

As can be seen from Table 6.1, the most effective substrates for pea seedling diamine oxidase in the series of analogues tested are *N*-methyl- (222) and *N*-

ethylcadaverine (215). Their K_M values are similar to putrescine. *N*-Propyl- (216) and *N*-butylcadaverine (217) are also good substrates. These compounds bind more effectively to the enzyme but have a slightly lower rate of oxidation.

The least effective substrate in the cadaverine series appears to be *N*-cyclohexylcadaverine (221). Its V_{max} is the lowest in the cadaverine series (66 $\mu mol\ mg^{-1}h^{-1}$), possibly due to its steric bulk. Its tight ES binding (K_M = 0.12 mM) suggests that it may be a good inhibitor.

Also tested at the highest substrate concentration of 2.86 mM were *N*-benzylputrescine (226) and *N*-cyclohexylputrescine (227). They showed very low specific activity at this concentration. Thus they are not substrates of DAO but may be good inhibitors.

An interesting feature of the results is that the whole series of compounds tested shows a progressive decrease in V_{max} as the steric bulk of the *N*-alkyl substituents increases. This could be due to inhibition of important conformational changes in the catalytic step of the enzyme by the bulky substituents. It could also be that, due to their bulk, the substituents limit the access of other reactants to the active site of the enzyme. They might, for example, impede the access of water to the active site and thus hinder the necessary Schiff base hydrolysis reaction.

Pec and co-workers[134] have argued that a protonated diamine, or monoamine with a protonated amine group, and a five carbon chain or aromatic ring is required in substrates for substantial binding in the active site of DAO. Gavin and co-workers[153] also found that the highest V_{max} values occurred in compounds with chain lengths of four to six. As all of the compounds shown in Table 6.1 have four or five carbon chain lengths, chain length is not a significant variable in this study. It would appear, therefore, that there must be a different explanation for the decreasing pattern of V_{max} values found in this study. A possible explanation could be the mechanism proposed by Equi and co-workers,[164] who argue that the catalytic process may be a classic two-stage equilibrium model as shown below.

$$E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES \overset{K_2}{\rightleftharpoons} ES^* \xrightarrow{k_{cat}} E + P$$

In this equation k_1 (= [E][S]/[ES] and k_2 (= [ES]/[ES*]) are the dissociation constants for substrate binding and intermediate formation respectively, and k_{cat} is the rate constant for the irreversible catalytic step. The first step in the catalysis of diamine oxidase may represent the initial attachment of the substrate molecule to the enzyme. The second step may involve formation of a cyclic conformation by the substrate to permit attachment of the second amino group to the protein.

If it proved to be the case that cyclisation is the rate-limiting step, it may be that the primary nitrogen end of the substrate binds to the enzyme first and that the bulkier groups hinder the cyclisation reaction, thus lowering V_{max}. Less bulky groups allow the cyclisation to proceed more easily thus showing a relatively higher V_{max}.

However close inspection of the K_M values shown in Table 6.1 does indicate the possibility that there may be two differentiated groups of compounds within the series; the first being all the compounds with K_M values approximating to 1 mM; the second showing somewhat lower K_M values which also reduce systematically, albeit slightly, with increasing steric bulk. The two categories of compounds observed are shown in Tables 6.2 and 6.3. From analysis of their K_M and V_{max} values some hypotheses have been proposed regarding the relationship between binding affinity, type of binding and structure.

Table 6.2: Compounds in which the K_M value remains relatively stable at around 1 mM.

	K_M (mM)		K_M (mM)
Putrescine (20)	1.18		
N-methylputrescine (194)	1.12	*N*-methylcadaverine(222)	1.18
N-ethylputrescine (195)	1.18	*N*-ethylcadaverine(215)	1.21
N-isopropylputrescine(196)	1.29	*N*-isopropylcadaverine(218)	1.04
		N-isobutylcadaverine(219)	0.90

Table 6.3 Compounds in which the K_M value shows a systematic decrease with increasing steric bulk.

	K_M (mM)		K_M (mM)
N-propylputrescine(196)	0.35	*N*-propylcadaverine(216)	0.29
N-butylputrescine(223)	0.26	*N*-butylcadaverine(217)	0.21
N-methylbenzylputrescine (225)	0.21	*N*-benzylcadaverine(220)	0.14
		N-cyclohexylcadaverine(221)	0.12

The binding affinities of analogues in Table 6.3 indicate that as steric bulk increases the binding affinity also increases, albeit by a relatively small amount. The cadaverine analogues show lower K_M values than the corresponding putrescine analogues. This is in line with cadaverine being a better substrate of diamine oxidase. It may be that in these compounds the bulkier alkyl group is stabilising the initial binding to the enzyme.

The binding affinities of analogues in Table 6.2 do not show this relationship suggesting that steric bulk linked to increasing binding affinity is not a characteristic of these analogues. The compounds in Table 6.2 have a relatively constant binding affinity (K_M around 1 mM) but their V_{max} values vary from 1055 to 38 μmol $mg^{-1}h^{-1}$. These results suggest that the distal end of the active site of pea seedling diamine oxidase is relatively uncrowded. As the binding affinity does not vary significantly in the group tested, despite variations in oxidation rates, it could be argued that the substrate selectivity in the enzyme exists in the catalytic mechanism rather than the binding affinity.

It has also been shown[161] that structurally different compounds can be bound into the same active subsite of the pea enzyme and it has been deduced that the active site of the enzyme is on the surface or in an exposed open pocket. If this is the case, it could be postulated that the accessibility and open-ness of the active site enables the less bulky groups to be more easily accommodated but to bind rather less tightly

because of their lack of steric bulk. This can only be a very tentative postulate as so little is known, as yet, about the catalytic mechanism, the role of the cofactor and the characteristics of the active site.

CHAPTER SEVEN

INHIBITORS OF DIAMINE OXIDASE

7.1 INTRODUCTION

Various *N*-alkylcadaverines and *N*-alkylputrescines were synthesised as described in Chapter Six. Some of the analogues synthesised showed little or no substrate activity, while others were found to bind well but to be oxidised at a slower rate than cadaverine. The latter compounds therefore appear to fulfil some of the conditions necessary for inhibitors of diamine oxidase. Consequently, testing was carried out in order to determine whether they were inhibitors and, if so, the type of inhibition shown.

In addition, other compounds which did show some substrate activity were tested to establish whether they too might have some inhibitory effects because there are cases in which a compound can be both a substrate and inhibitor, as, for example, ethane-1,2-diamine.[166,169]

The compounds tested are listed in Table 7.1.

Table 7.1.

N-ethyldiaminopropane (228)

N-ethylcadaverine (215)

N-ethylputrescine (195)

N-propylcadaverine (216)

N-isopropylcadaverine (218)

N-butylcadaverine (217)

N-cyclohexylputrescine (227)

N-benzylcadaverine (220)

N-cyclohexylcadaverine (221)

N-benzylputrescine (226)

N-isobutylcadaverine (219)

N-butylputrescine (223)

7.2 ASSAY PROCEDURES TO TEST FOR INHIBITION

For the inhibition studies Stoner's spectrophotomeric assay procedure was used.[22] A fixed concentration of inhibitor was added for each experiment. A typical

typical inhibition study consisted of three or four experiments in which the conditions were varied as shown:

(i) Substrate only

(ii) Substrate + [I]

(iii) Substrate + [I]/2

(iv) Substrate + [I]/4

where [I] = inhibitor concentration.

As experimental values for each oxidation can vary slightly from day to day, it is necessary to provide a standard for comparison of experimental values. Each inhibition experiment was therefore accompanied by one with no inhibitor present in order to provide the standard against which to compare the experimental values of inhibitors.

The reaction without inhibitor present was initiated by adding the enzyme and immediately adding the substrate. In experiments in which the inhibitor was present the inhibitor was added after the enzyme and immediately before the addition of the substrate. This is a necessary precaution to avoid the faster oxidation occurring before the addition of the inhibitor, and also to minimise breakdown of inhibitors which might also be poor substrates.

7.3 THE KINETICS OF INHIBITION

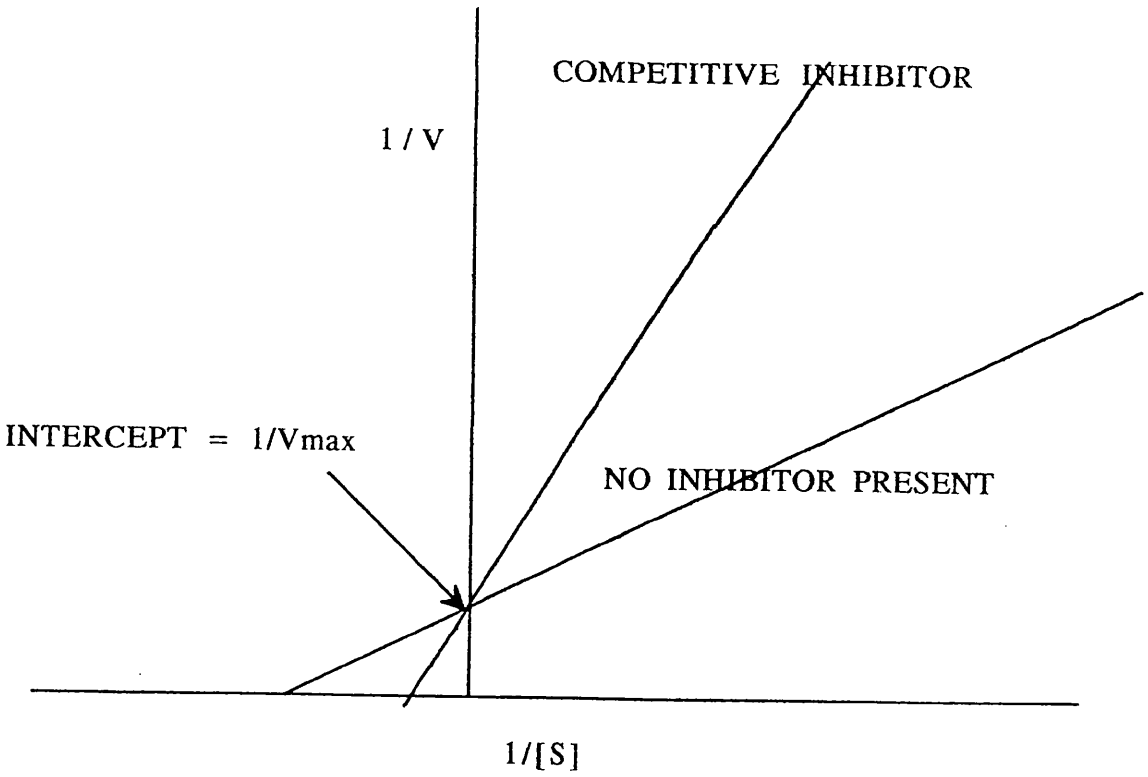
Enzymes may be irreversibly inactivated by heat or chemical reagents. They may be reversibly inhibited by the noncovalent binding of inhibitors. The main types of reversible inhibition are: competitive and non-competitive, plus sub-types such as, mixed and coupling, which are described in more detail later in the Chapter.

The better the competitive inhibitor the more easily it is bound into the active site of the enzyme. Conversely, the less good the inhibitor the less easily it is bound into the active site due to steric and other effects. It is possible to distinguish between the inhibition types by measurements of the rates of catalysis at different concentrations of substrate and inhibitor.

7.4 COMPETITIVE INHIBITION

If an inhibitor binds reversibly at the active site of an enzyme and prevents the substrate from binding, or *vice versa*, the inhibitor and substrate are in competition for the same active site and the inhibitor is designated as a competitive inhibitor. The enzyme is fully operative when there is a sufficiently high substrate concentration to fill virtually all the active sites. Competitive inhibition has the classic characteristic of K_M changing while V_{max} remains the same with varying inhibitor concentrations. This can be shown graphically (Graph 7.1.). From the graph it can be seen that the intercept of the plot of 1/V *vs.* 1/[S] is the same regardless of inhibitor concentration. The slope, however, does differ. The steeper the slope of the line the stronger the binding of the competitive inhibitor to the substrate.

Graph 7.1: Illustrating the Lineweaver-Burk plot for competitive inhibition.



V_{max} is not altered by the presence of a competitive inhibitor. The crucial factor in identifying competitive inhibition is the fact that inhibition can be overcome at a sufficiently high substrate concentration. Thus, in the presence of a competitve inhibitor, the Lineweaver/Burk equation

$$1/V = 1/V_{max} + K_M/V_{max}(1/[S]) \qquad (23)$$

becomes

$$1/V = 1/V_{max} + K_M/V_{max}(1 + [I]/K_i)/[S] \qquad (24)$$

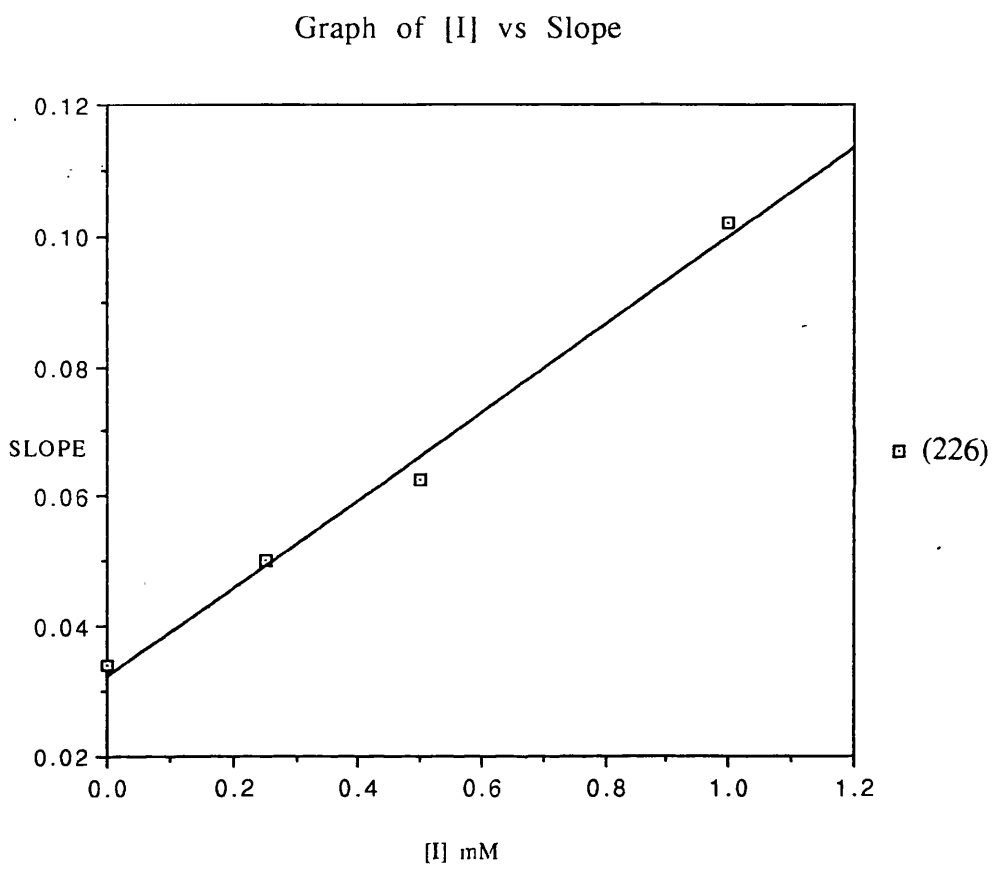
where [I] is the concentration of inhibitor and K_i is the dissociation constant of the enzyme inhibitor complex.

In other words:

$$(slope)^1/(slope) = 1 + [I]/K_i \qquad (25)$$

where $(slope)^1$ equals the slope of the line when the competitive inhibitor is present and (slope) is the slope of the line without inhibitor. In situations where there are two or more concentrations of inhibitor tested, another method for calculating the inhibition constant K_i is used. A plot of the slopes from the competitive inhibition graph versus concentrations of inhibitors gives a straight line, and its intercept on the x axis gives K_i. The lower the K_i value the better the inhibitor and *vice versa.*

GRAPH 7.2: Example illustrating the effect of different inhibitor concentrations in the determination of K_i. Each point is a mean of the slopes at a specific inhibitor concentration ([1 mM], [0.5 mM] and [0.25 mM]).



Of the compounds tested in this study, those listed below were demonstrated to be competitive inhibitors.

N-isopropylcadaverine (218)

N-isobutylcadaverine (219)

N-cyclohexylcadaverine (221)

N-benzylputrescine (226)

N-cyclohexylputrescine (227)

N-ethyldiaminopropane (228)

These compounds were tested as inhibitors because they were either shown not to be substrates, (226), (227), (228), for DAO or showed very poor substrate activity, (219) and (221). *N*-Isopropyicadaverine (218), which was a relatively poor

substrate, did prove to be an inhibitor, albeit less strong than the other five analogues shown. The inhibition constants (K_i) for these compounds, and all other compounds tested, will be discussed later in this Chapter.

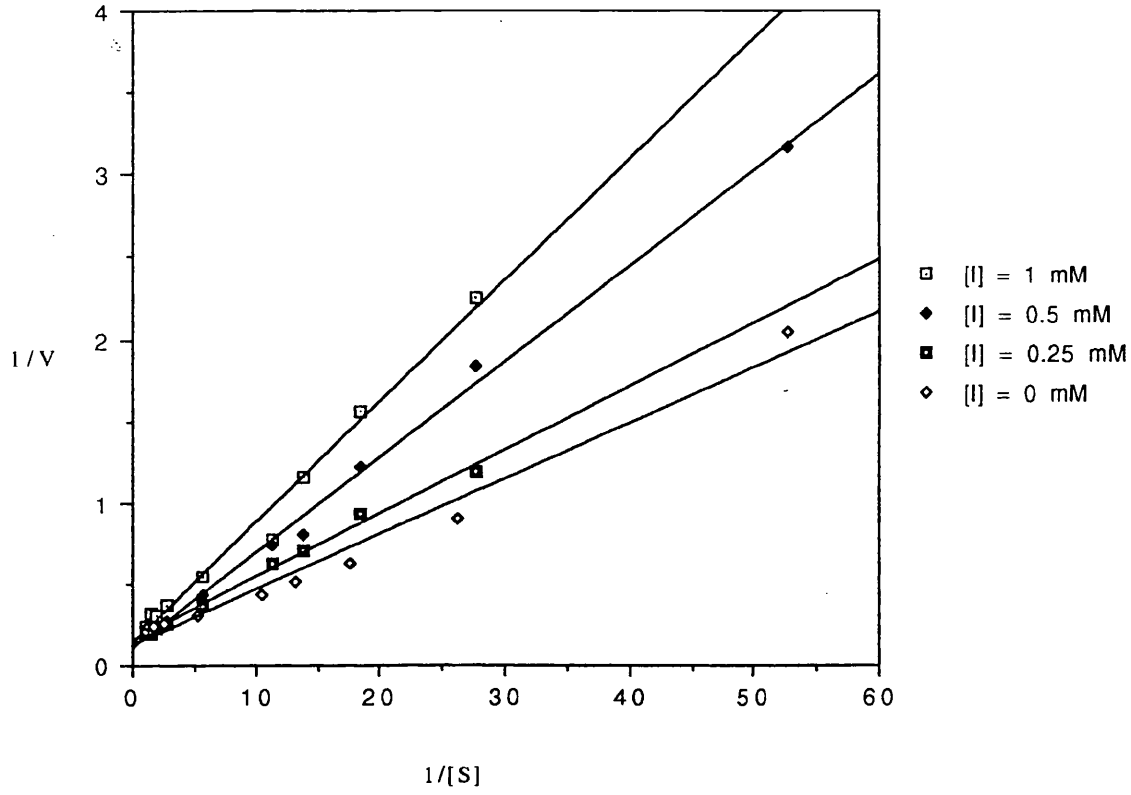
For each of the compounds data were analysed by three graphical methods: the Lineweaver-Burk plot, the Hanes plot and the Eadie-Hofstee plot. All three plots change with different types of inhibition but when one plot is used in isolation it may be ambiguous as to which type of inhibition applies. Comparison and analysis of all three provides a more accurate indication of the type of inhibition present. An example of all three plots is shown for *N*-benzylputrescine (226) in Graphs 7.3, 7.4 and 7.5.

For comparison purposes, the K_i values obtained from the various graphs of N-benzylputrescine (226) are shown below.

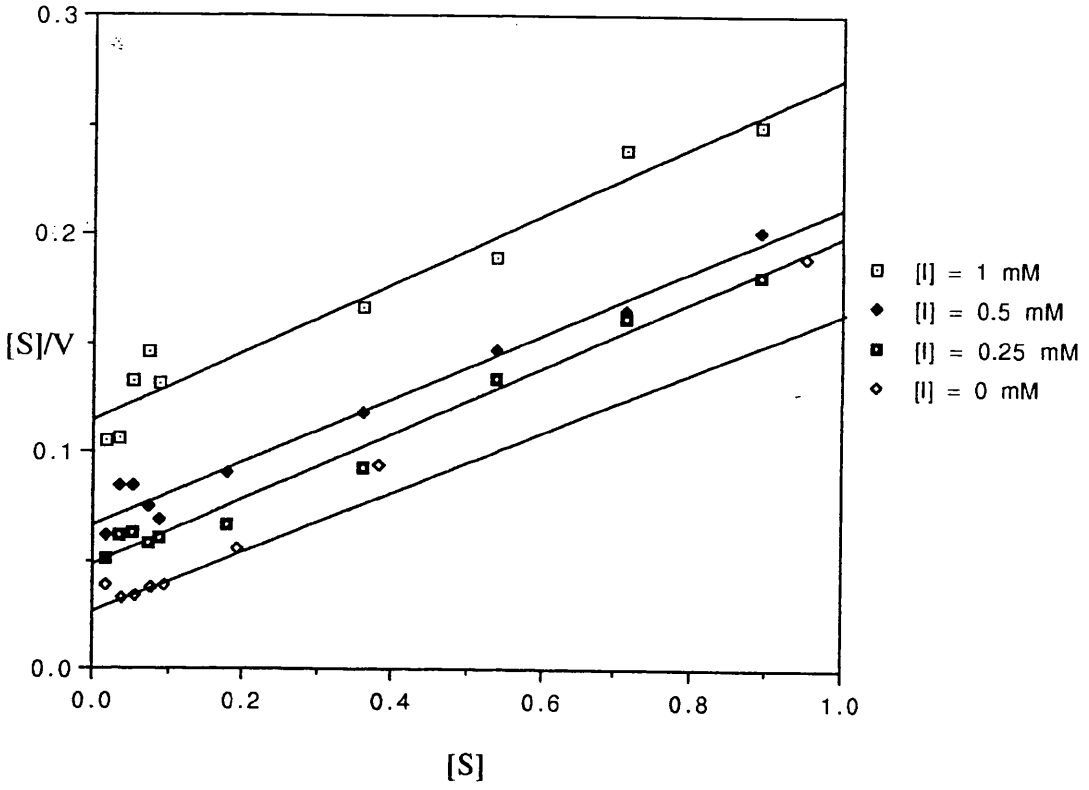
Lineweaver-Burk	$K_i = 0.45$ mM
Hanes	$K_i = 0.28$ mM
Eadie-Hofstee	$K_i = 0.33$ mM

The mean of these values gives a K_i of 0.35 mM.

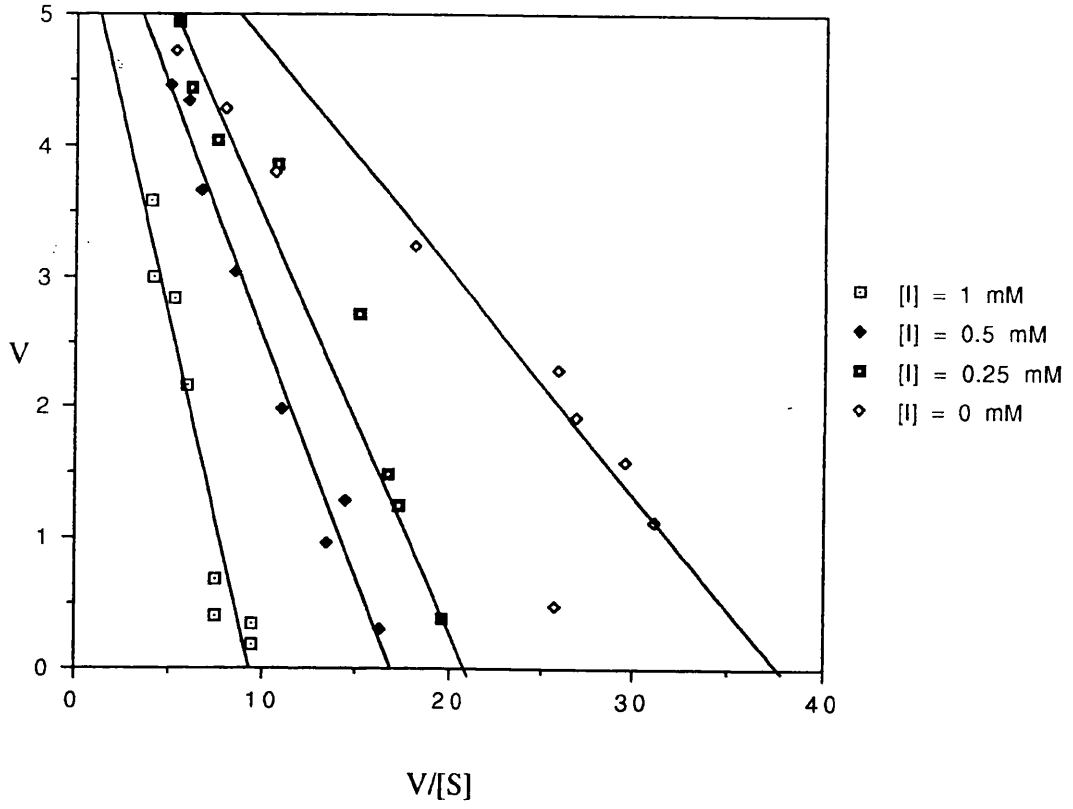
Graph 7.3 Lineweaver-Burk Plot for Kinetics of Cadaverine Oxidation with *N*-Benzylputrescine (226) as Inhibitor.



Graph 7.4 Hanes Plot for Kinetics of Cadaverine Oxidation with *N*-Benzylputrescine (226) as Inhibitor.



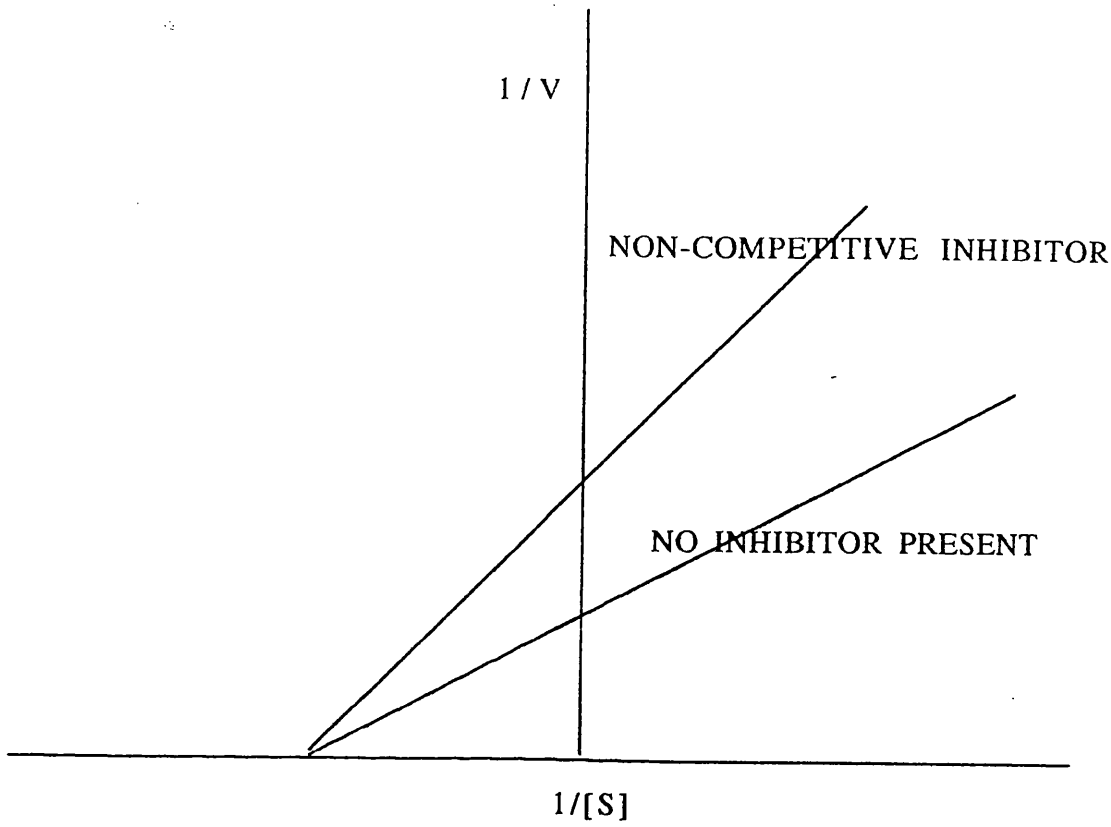
Graph 7.5 Eadie Hofstee Plot for Kinetics of Cadaverine Oxidation with *N*-Benzylputrescine (226) as Inhibitor.



7.5 NON-COMPETITIVE INHIBITION

By contrast, non-competitive inhibition is not affected by high substrate concentrations and cannot be overcome by increasing the substrate concentration. In non-competitive inhibition, inhibitor and substrate bind simultaneously to the enzyme rather than competing for the same binding site. Inhibitors are bound into different sites from that of the substrate in another place than the active site of the enzyme. The inhibitor does not necessarily alter the binding of the substrate but affects its rate of transformation into products.

Graph 7.6: Graph illustrating the Lineweaver-Burk plot for non-competitive inhibition.

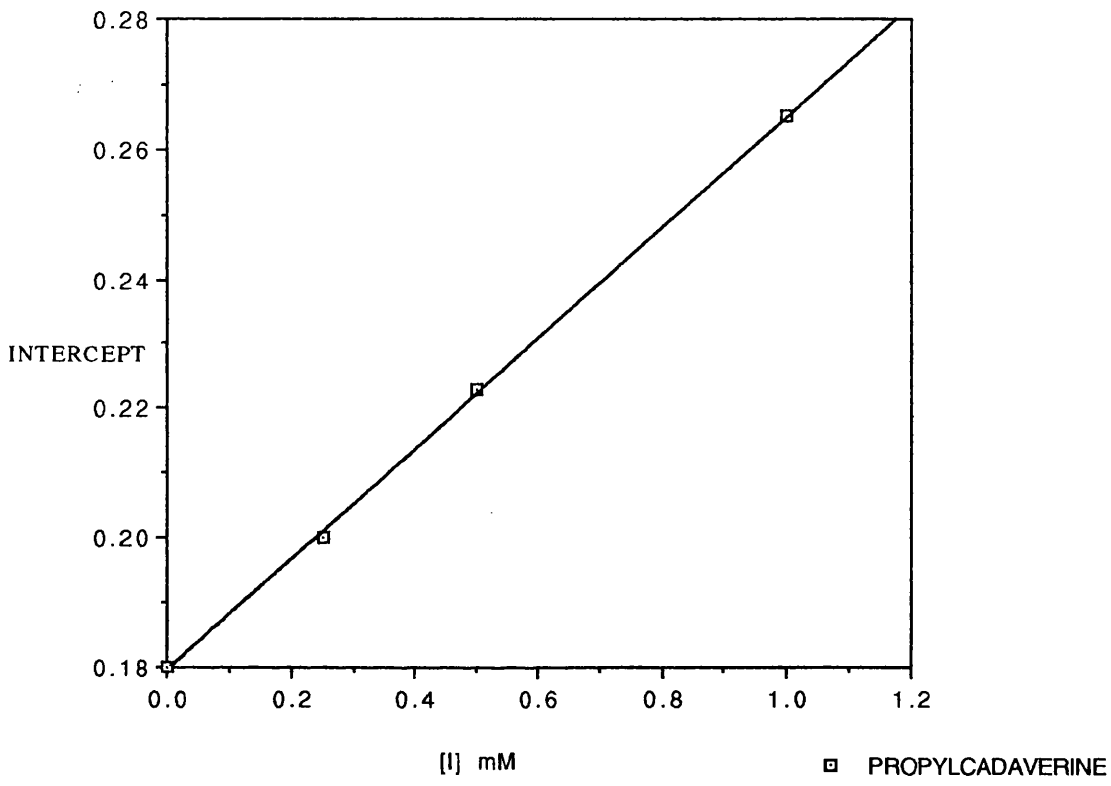


In non-competitive inhibition V_{max} is decreased and so the intercept on the y-axis of the graph is increased. The slope, which is equal to K_M/V^I_{max} , is increased by the same factor. In simple non-competitve inhibition K_M is usually unaffected. The maximum velocity in the presence of a non-competitive inhibitor is given by the equation

$$V^I_{max} = V_{max}/(1 + [I]/K_i) \qquad (26)$$

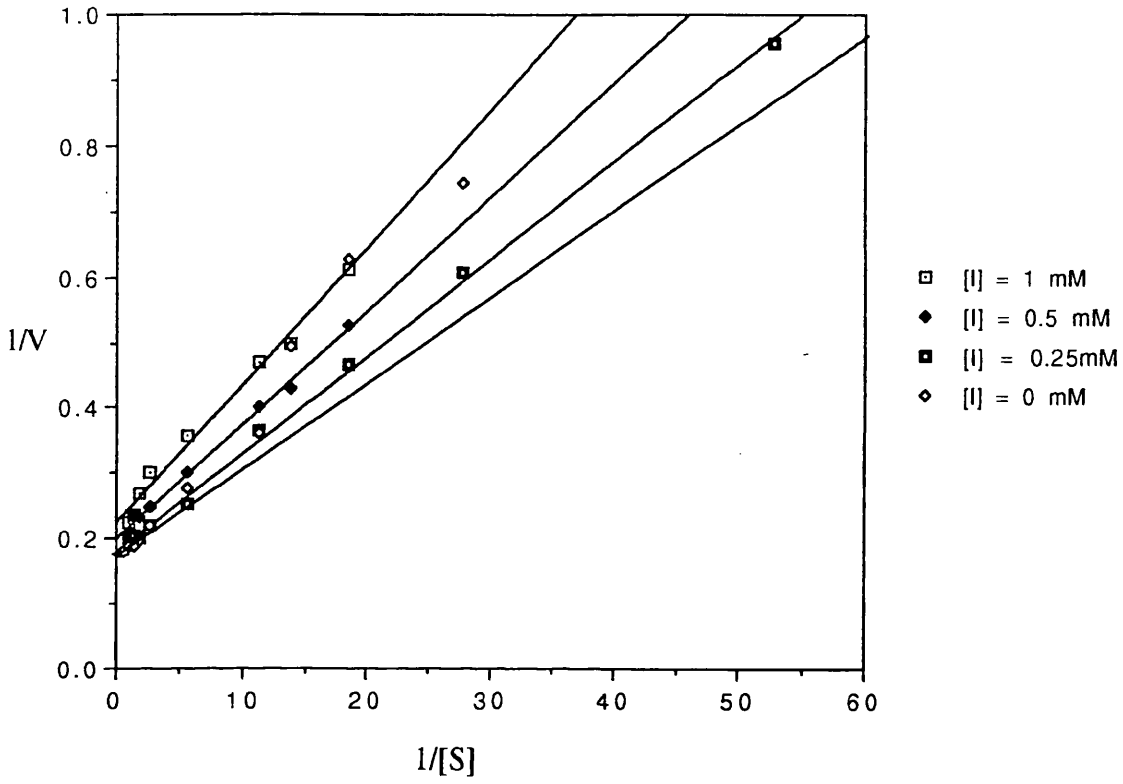
When more than one inhibitor concentration is being tested, a graph of the slopes versus inhibitor concentration, or intercepts of the y axis versus inhibitor concentration, gives K_i as the intercept on the x axis.

Graph 7.7: Example of determination of K_i, [I] *v* Intercepts, using the Lineweaver-Burk Plot for *N*-Propylcadaverine (216)

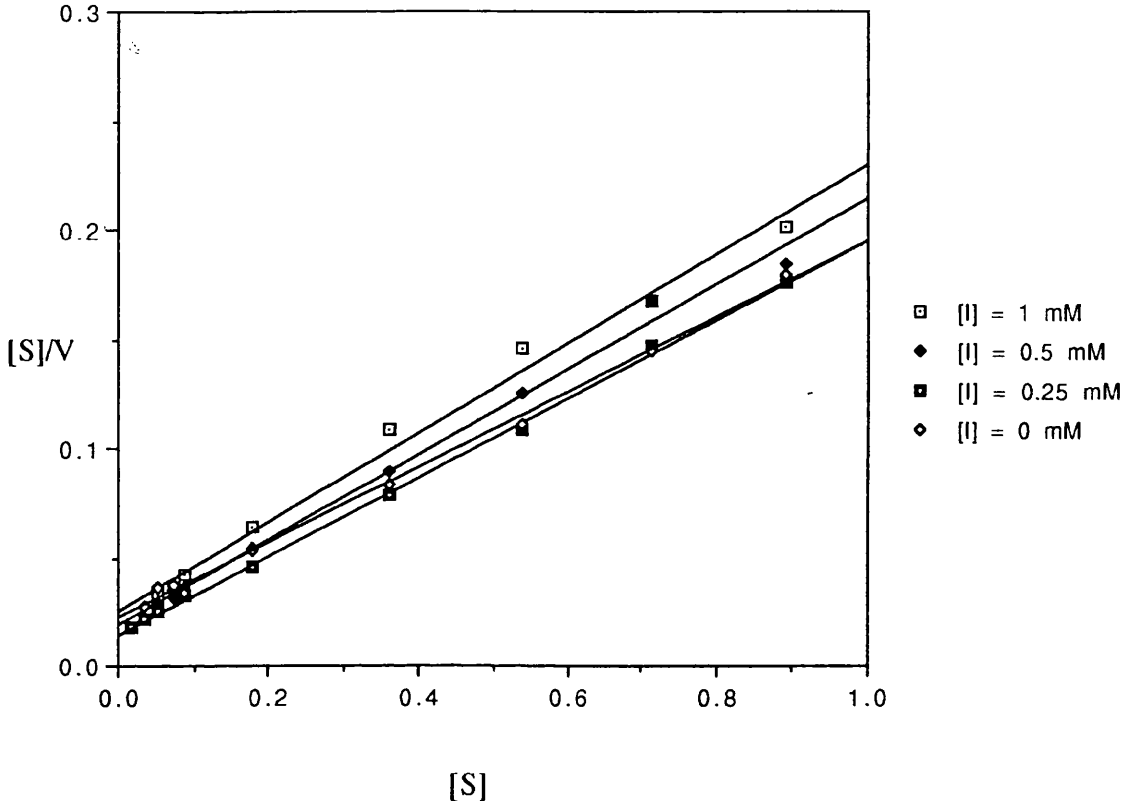


Of the compounds tested only *N*-propylcadaverine (216) appears to exhibit the characteristics of non-competitive inhibition. However, *N*-propylcadaverine is also a substrate for DAO. It may be that simultaneous oxidation of both substrate and inhibitor is complicating the analysis. The three plots for this compound are shown on Graphs 7.8, 7.9 and 7.10.

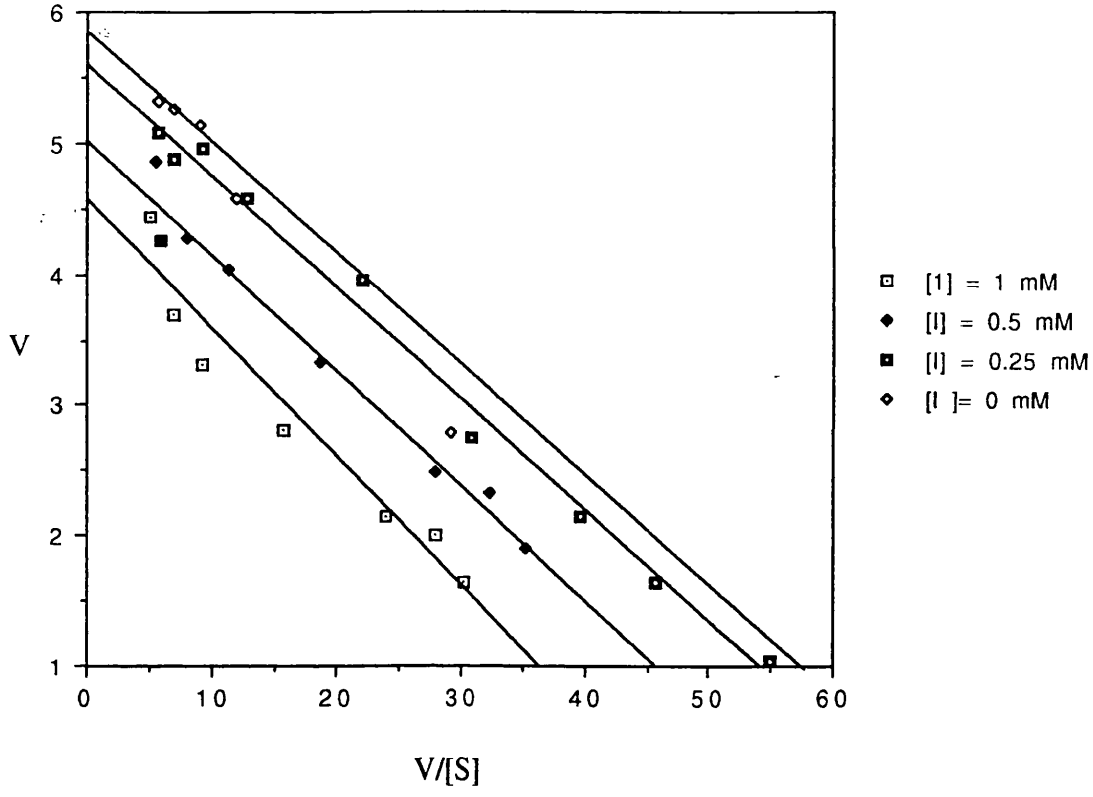
Graph 7.8 Lineweaver-Burk Plot for Kinetics of Cadaverine Oxidation with *N*-propylcadaverine (216) as Inhibitor.



Graph 7.9 Hanes Plot for Kinetics of Cadaverine Oxidation with *N*-Propylcadaverine (216) as Inhibitor.



Graph 7.10 Eadie-Hofstee Plot for Kinetics of Cadaverine Oxidation with *N*-Propylcadaverine (216) as Inhibitor.

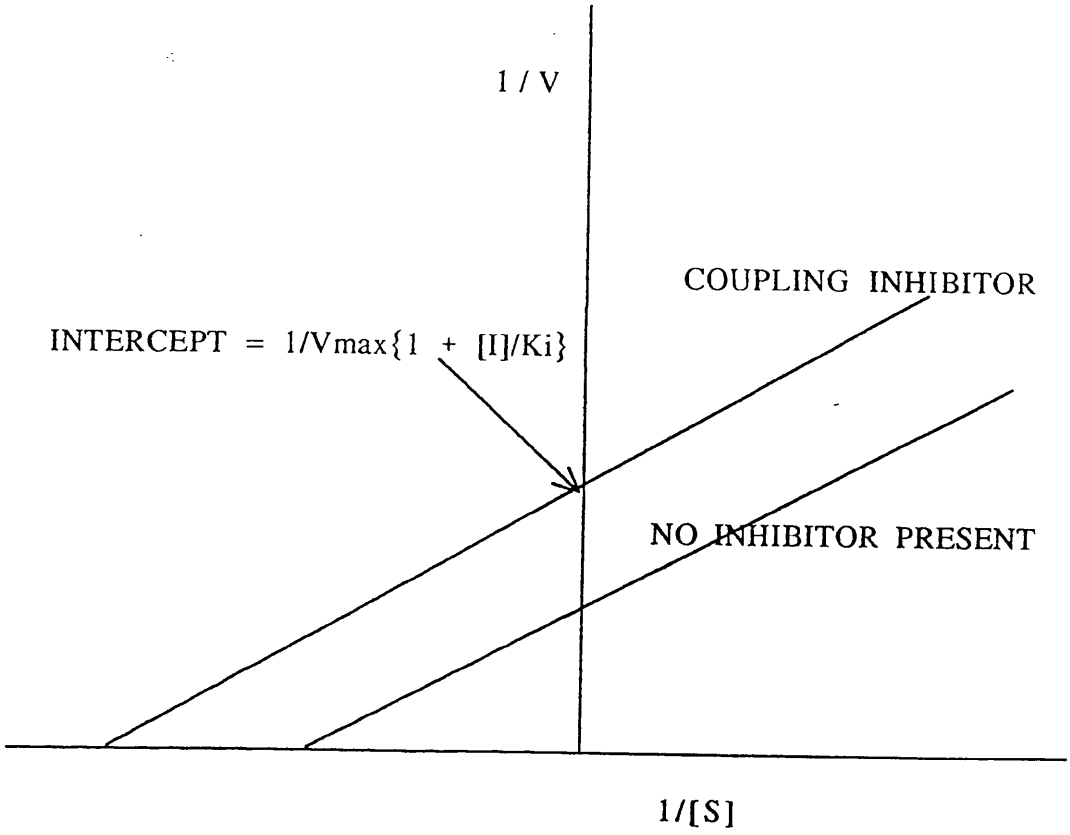


7.6 COUPLING INHIBITION:

Coupling is a form of inhibition which occurs when the inhibitor increases the coupling of enzyme and substrate. Various names have been attributed to this type of inhibition. These include "uncompetitive" and "acompetitive". The inhibitor is bound into the same subsite as the substrate but the inhibitor binds only with the enzyme-substrate complex and not with the free enzyme. The ability to bind to the same subsite may be a result of structural similarities in the substrate and inhibitor, as shown in Graph 7.11.

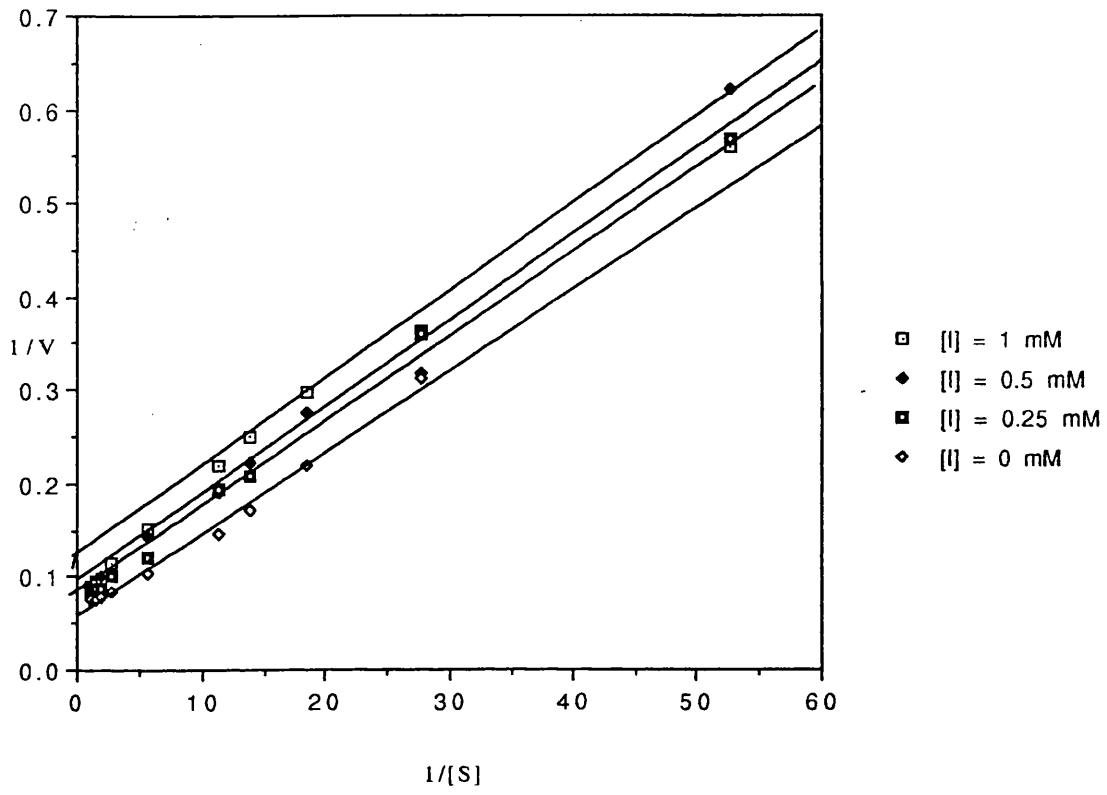
The graph, obtained by plotting 1/V against 1/[S], gives a set of parallel lines and is characterised by having K_M and V_{max} both reduced as inhibitor concentration increases. The slope stays the same but the intercept changes with different inhibitor concentrations. The graph to determine the inhibitor constant K_i is obtained by plotting intercepts against inhibitor concentration and is not obtained by plotting slopes. This was illustrated in Graph 7. 7.

Graph 7.11 Example of Lineweaver-Burk plot for determining Coupling inhibition

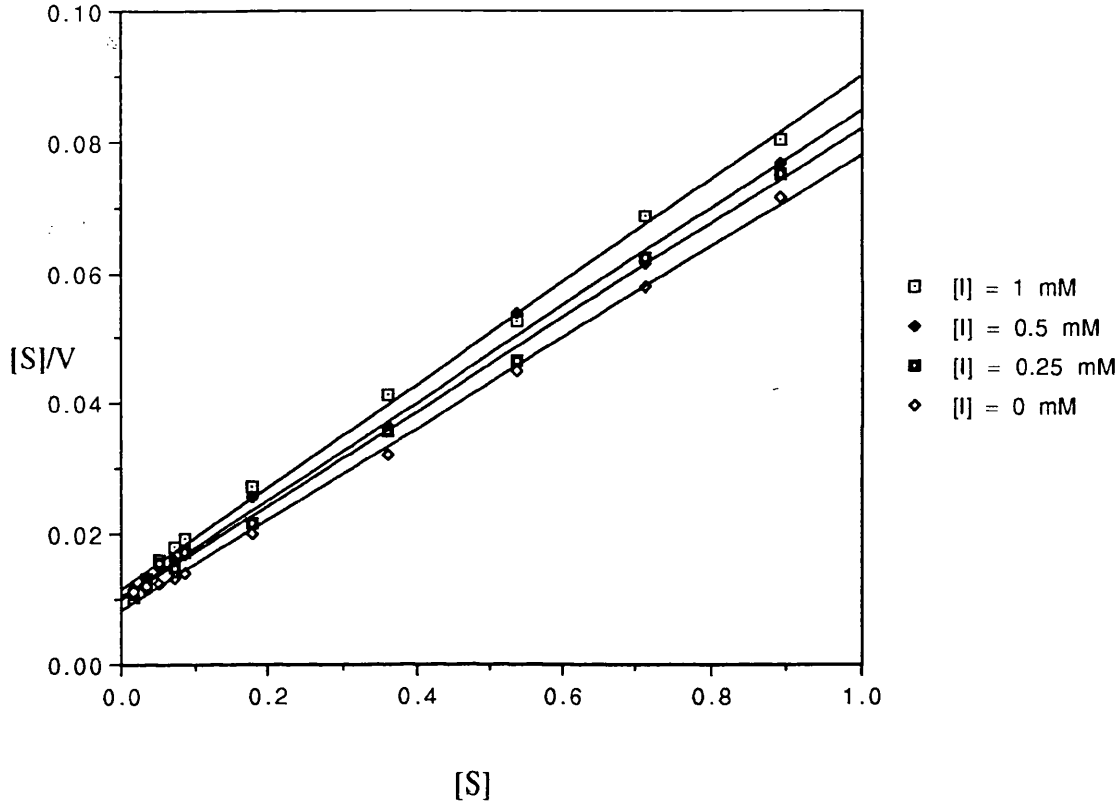


N-Ethylputrescine (195) was shown to be a fairly good inhibitor, and *N*-ethylcadaverine (215) less good. Both appeared to show the characteristics of coupling inhibition. As indicated above, the determination of K_i is via the plot of the intercept rather than the slope. The K_i values of these compounds could not be calculated from the graph of inhibitor concentration versus slope since the slope is constant for all inhibitor concentrations, thus providing further evidence of coupling inhibition. The plots for *N*-ethylputrescine (195) are shown in Graphs 7.12 7.13 and 7.14.

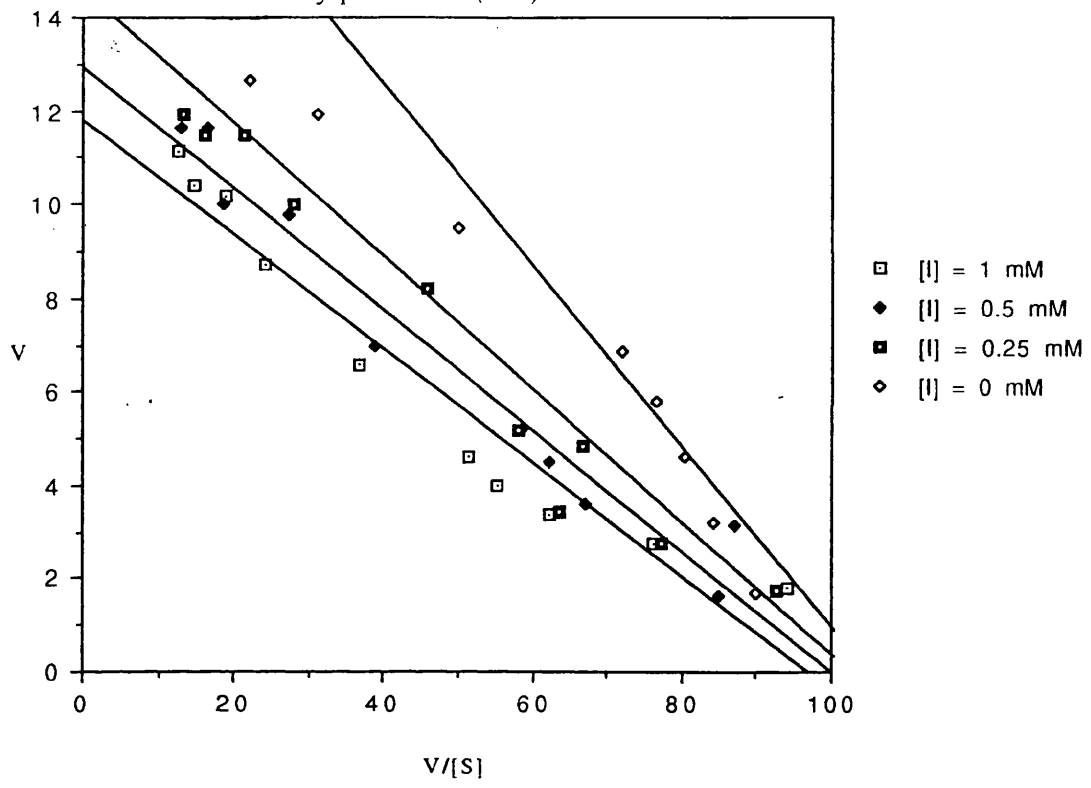
Graph 7.12 Lineweaver-Burk Plot for Kinetics of Cadaverine Oxidation with *N*-Ethylputrescine (195) as Inhibitor.



Graph 7.13 Hanes Plot for Kinetics of Cadaverine Oxidation with *N*-Ethylputrescine (195) as Inhibitor.



Graph 7.14 Eadie-Hofstee Plot for Kinetics of Cadaverine Oxidation with *N*-Ethylputrescine (195) as Inhibitor.

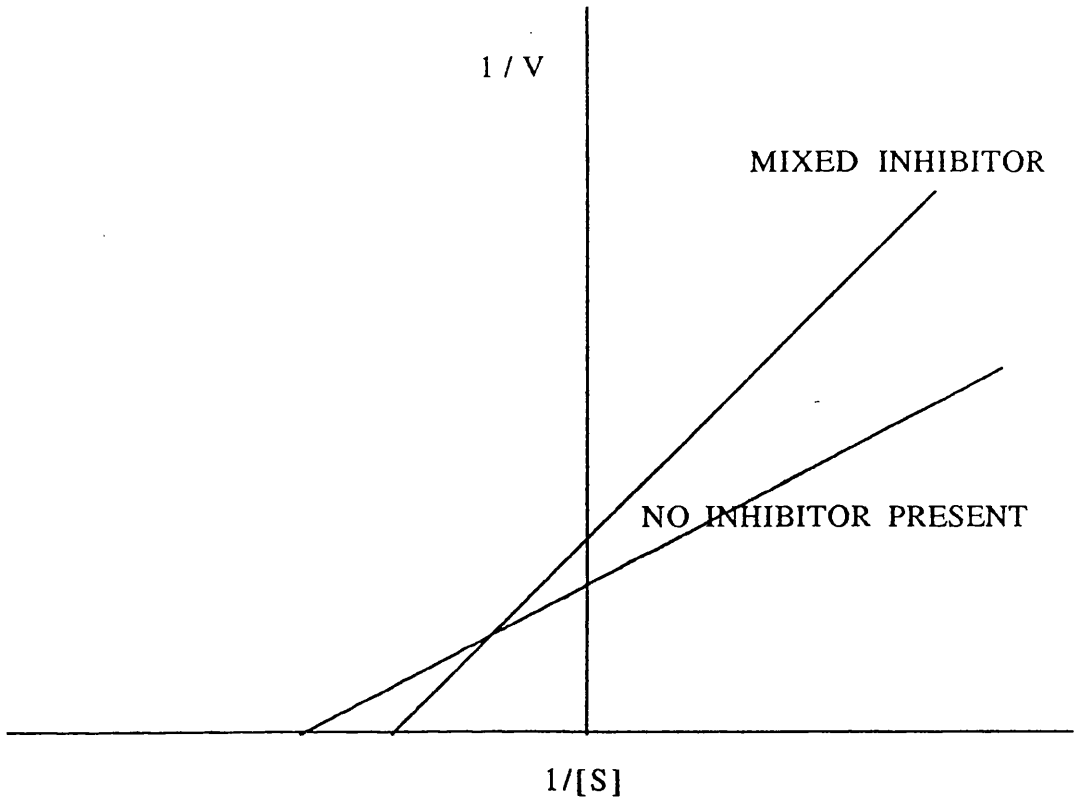


In this type of inhibition, the inhibitor cannot bind to the free enzyme, only to the ES complex. It could therefore be postulated that the mechanism of inhibition is that in the first stage of catalysis the enzyme binds to cadaverine. This forms an ES complex to which the inhibitor can bind. In doing so it increases the affinity of the enzyme for the substrate (coupling) and may form an inactive complex which prevents the formation of product, thus inhibiting the reaction.

7.7 MIXED INHIBITION

When the presence of the inhibitor on the enzyme prevents the breakdown of the active complex and also interferes to some extent with the binding of the substrate, both K_M and V_{max} are altered and the inhibitor is termed mixed. (Coupling, when both K_M and V_{max} are reduced, is a special form of mixed inhibition.)

GRAPH 7.15: Example of Lineweaver-Burk plot illustrating mixed inhibition



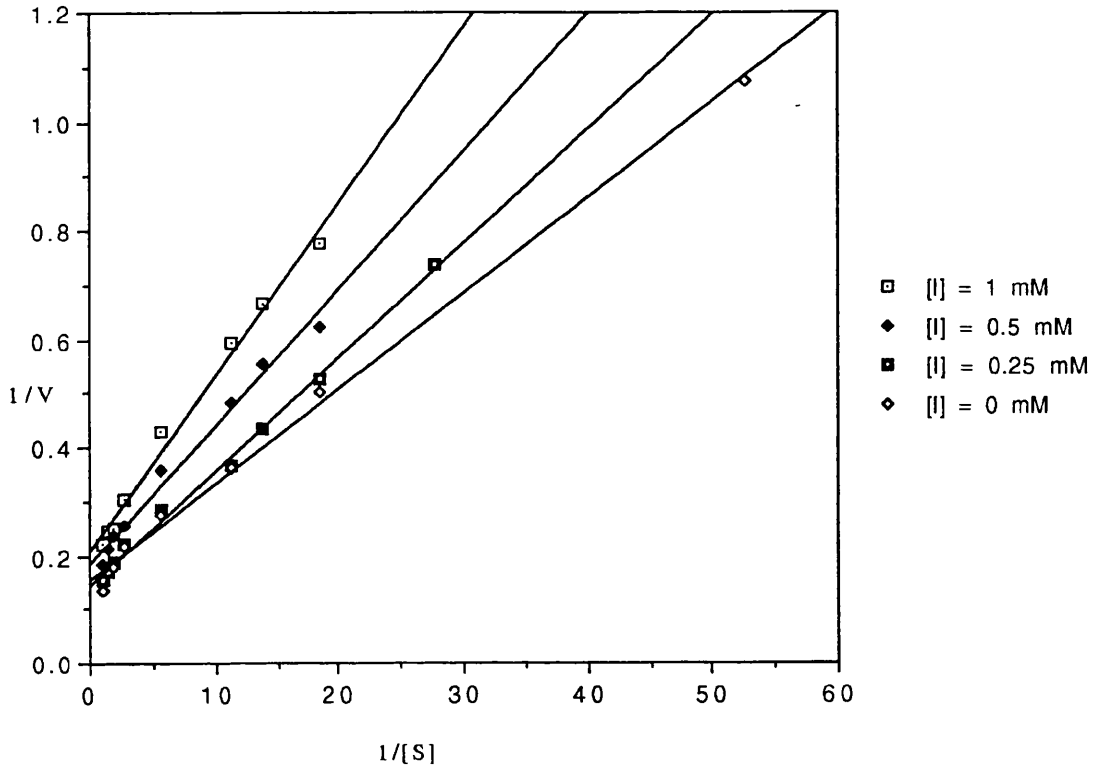
The graph of 1/V against 1/[S] shows a common intercept between the axes rather than at either axis. Thus both slope and intercept change with different inhibitor concentrations. The extent of the competitive or non-competitive nature of a mixed inhibition is determined by how close the intercept is to a particular axis. However, the usual method of determining K_i from the graph of slope against inhibitor concentration is still applicable.

It was difficult to determine from the plots the exact type of inhibition shown by *N*-butylcadaverine (217), *N*-butylputrescine (226) and *N*-benzylcadaverine (220). From the low K_M and relatively low V_{max} data for these analogues it was anticipated that they would show fairly good competitive inhibition. The plotted data do show some competitive inhibition but also some non-competitive inhibition, suggesting that these may mixed inhibitors. This would suggest that these can be accommodated into the same subsite as cadaverine causing competitive inhibition, but

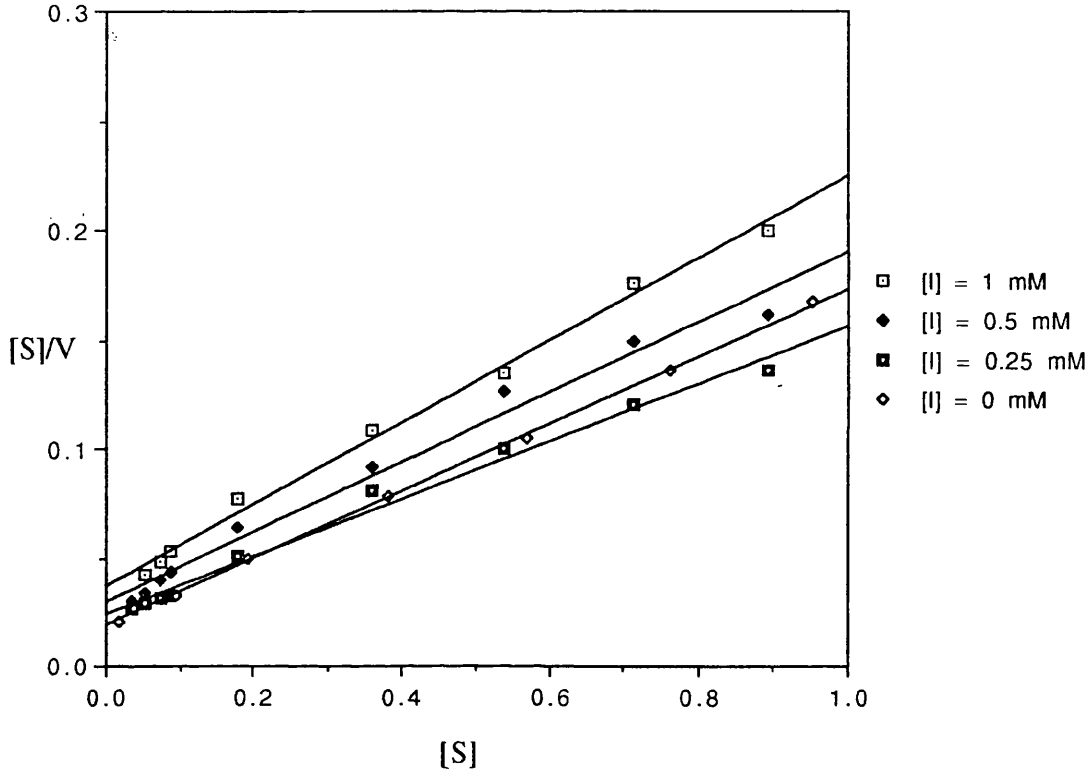
may also be capable of binding into a different subsite from cadaverine. However, it may be that all of these compounds are competitive inhibitors and it is simply that poorer data for low substrate concentrations is complicating the analysis.

The plots for *N*-butylputrescine (226) are shown in Graphs 7.16, 7.17 and 7.18.

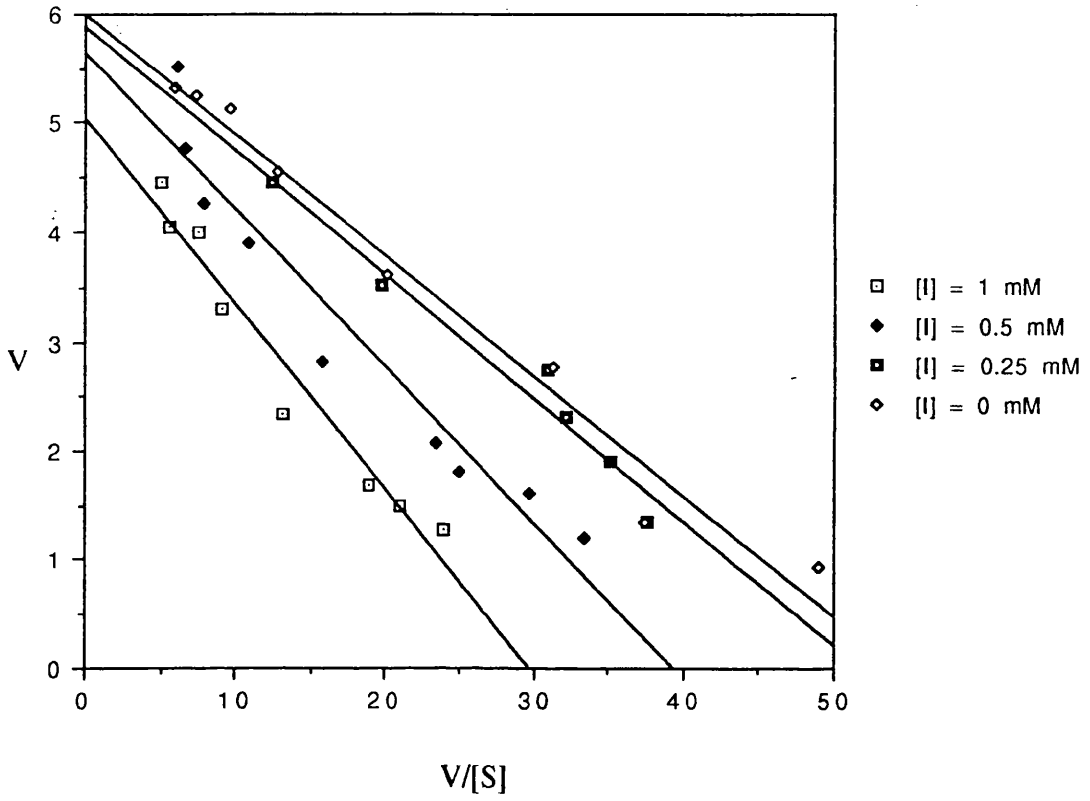
Graph 7.16 Lineweaver-Burk Plot for Kinetics of Cadaverine Oxidation with *N*-Butylputrescine (223) as Inhibitor.



Graph 7.17 Hanes Plot for Kinetics of Cadaverine Oxidation with *N*-Butylputrescine (223) as Inhibitor.



Graph 7.18 Eadie-Hofstee Plot for Kinetics of Cadaverine Oxidation with *N*-Butylputrescine (223) as Inhibitor.



7.8 DISCUSSION AND SUMMARY OF RESULTS

Table 7.2 shows the K_i values for the compounds tested. The lower the inhibition constant K_i the better the inhibitor and the higher the K_i the poorer the inhibitor. Also shown are the apparent types of inhibition found for each compound.

Table 7.2: Inhibition constants (K_i) and apparent type of inhibition found for compounds tested as inhibitors of pea seedling diamine oxidase.

INHIBITOR	K_i (mM)	APPARENT TYPE OF INHIBITION
N-ethyldiaminopropane (228)	1.63	Competitive
N-ethylcadaverine (215)	3.09	Coupling
N-ethylputrescine (195)	1.52	Coupling
N-propylcadaverine (216)	2.09	Non-Competitive
N-isopropylcadaverine (218)	2.85	Competitive
N-butylcadaverine (217)	6.27	Mixed
N-butylputrescine (223)	0.99	Mixed
N-isobutylcadaverine (219)	1.76	Competitive
N-benzylcadaverine (220)	1.05	Mixed
N-cyclohexylcadaverine (221)	0.26	Competitive
N-benzylputrescine (226)	0.35	Competitive
N-cyclohexylputrescine (227)	0.83	Competitive

Cadaverine is a better substrate for DAO than putrescine and a much poorer inhibitor. Therefore it was anticipated that the cadaverine analogues, which are generally better substrates than the corresponding putrescine analogues, would be less good inhibitors. From Table 7.2 it can be seen that, with the exception of *N*-cyclohexylcadaverine, the cadaverine analogues were less good inhibitors and had higher K_i values than the putrescine analogues. *N*-cyclohexylcadaverine (221) was the poorest substrate in the cadaverine series. It proved to be the best inhibitor with

the lowest K_i value of 0.26 mM. The compounds which were not substrates, *N*-benzylputrescine (226), *N*-cyclohexylputrescine (227) and *N*-ethyldiaminopropane (228), were all fairly good inhibitors with K_i values of 0.35 mM, 0.83 mM, and 1.63 mM respectively. *N*-Benzylputrescine was the second best inhibitor of all the compounds tested.

Of the compounds tested in this study, the best substrates were *N*-ethylcadaverine (215), *N*-propylcadaverine (216), *N*-butylcadaverine (217) and *N*-isopropylcadaverine (218). As expected, these were the poorest inhibitors of DAO.

Table 7.2 also shows that, with the exception of *N*-butylcadaverine (217) and *i*-propylcadaverine (218), as the steric bulk increases the K_i values decrease. Again this was in line with expectations since the steric bulk reduces the substrate activity (V_{max} and K_M are both lowered).

When relating the K_i values to types of inhibition it can be seen that the competitive inhibitors were fairly good inhibitors. All of the putrescine and cadaverine analogues which showed good competitive inhibition had alkyl substituents which were bulkier than a butyl group, suggesting that this may one of the conditions for competitive inhibition in pea seedling diamine oxidase. Moreover, it is interesting that, when all of the plotted data had been analysed, it was found that in three cases there appeared to be some relationship between the particular alkyl substituent, the K_M value and the type of inhibition demonstrated.

Both of the *N*-cyclohexyl compounds, (221) and (227), showed low substrate activity and both were good competitive inhibitors and, contrary to what might have been expected from the other results obtained, the cadaverine derivative was the better of the two. Both of the *N*-butyl compounds, (217) and (223), had very similar K_M values around 0.2 mM and both appear to display characteristics of mixed inhibition. In this case the cadaverine derivative, a better substrate, was the poorer inhibitor and showed a much higher K_i value.

Neither of the two *N*-ethyl compounds, (215) and (195), were particularly good inhibitors. The putrescine derivative was rather better than the cadaverine.

However both of these compounds had very similar K_M values around 1.2 mM and both appear to be coupling inhibitors which do not bind directly with the free enzyme but with the ES complex.

It may be recalled that, at the end of Chapter Six, it was tentatively postulated that if the active site of the pea seedling enzyme is on the surface or an open exposed pocket, the *N*-ethyl compounds (215) and (195), when acting as substrates, may be easily accommodated due to their lack of steric bulk but their initial binding may be less tight than that of the bulkier groups. However, they show better oxidation rates than the bulkier groups. It could therefore be further postulated that they fulfil the classic requirements for transition-state binding in which weak initial ES binding is followed by stonger binding in the transition state. This increase in binding energy is used to lower the activation energy and provides an increased V_{max}.[162,163,147]

The maximum binding energy between an enzyme and a substrate occurs when each binding group on the substrate is matched by a binding site on the enzyme, giving enzyme-substrate complementarity. However, the structure of the substrate changes throughout the reaction, becoming first the transition state and then the products. As the structure of the undistorted enzyme can be complementary to only one form of the substrate, there may be a catalytic advantage for the enzyme to be complementatry to the structure of the transition state of the substrate rather than the original substrate structure. The presence on the enzyme of a group that can bind only to the transition state of the substrate (transition state stabilisation) decreases the activation energy for a chemical step and does not have to distort the substrate to do so.[147]

Clearly, as indicated earlier, the possibility that the *N*-ethyl derivatives demonstrate transition-state binding characteristics can only be a very tentative hypothesis. However it could be a fruitful line of further research to retest the two *N*-ethyl derivatives to determine unambiguously whether they are coupling inhibitors. Moreover, since both the *N*-methyl derivatives have similar K_M values around 1.1 mM, and since both show relatively good oxidation rates, it would be interesting to

discover whether they also show coupling inhibition (albeit weak as they are good substrates) and, if they do, to investigate what this may tells us about their mechanism when acting as substrates.

CHAPTER 8

THE SYNTHESIS AND TESTING OF POLYAMINES

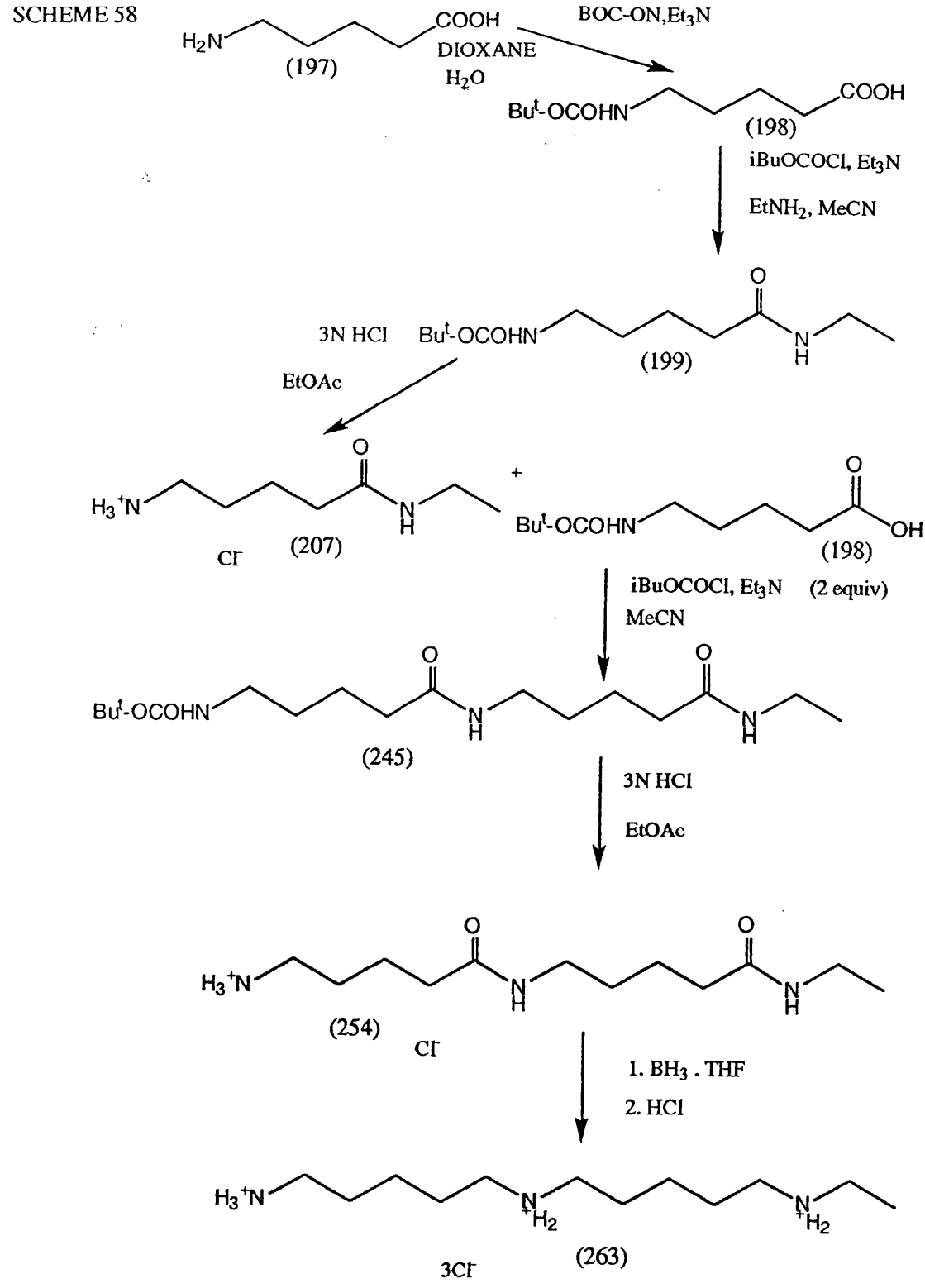
8.1 INTRODUCTION

As indicated in Chapter Five, polyamines have an important role in growth and cell replication. The effect of inhibiting polyamine synthesis in plants can have herbicidal properties, while their use in the development of anti-cancer and other drugs is well recognised. In this study the aim was the synthesis and testing of polyamines as substrates or inhibitors of pea seedling diamine oxidase. A selection of the compounds synthesised was tested for anti-fungal, anti-cancer or anti-malarial activity. It was established that these were novel compounds which had not previously been synthesised or tested.

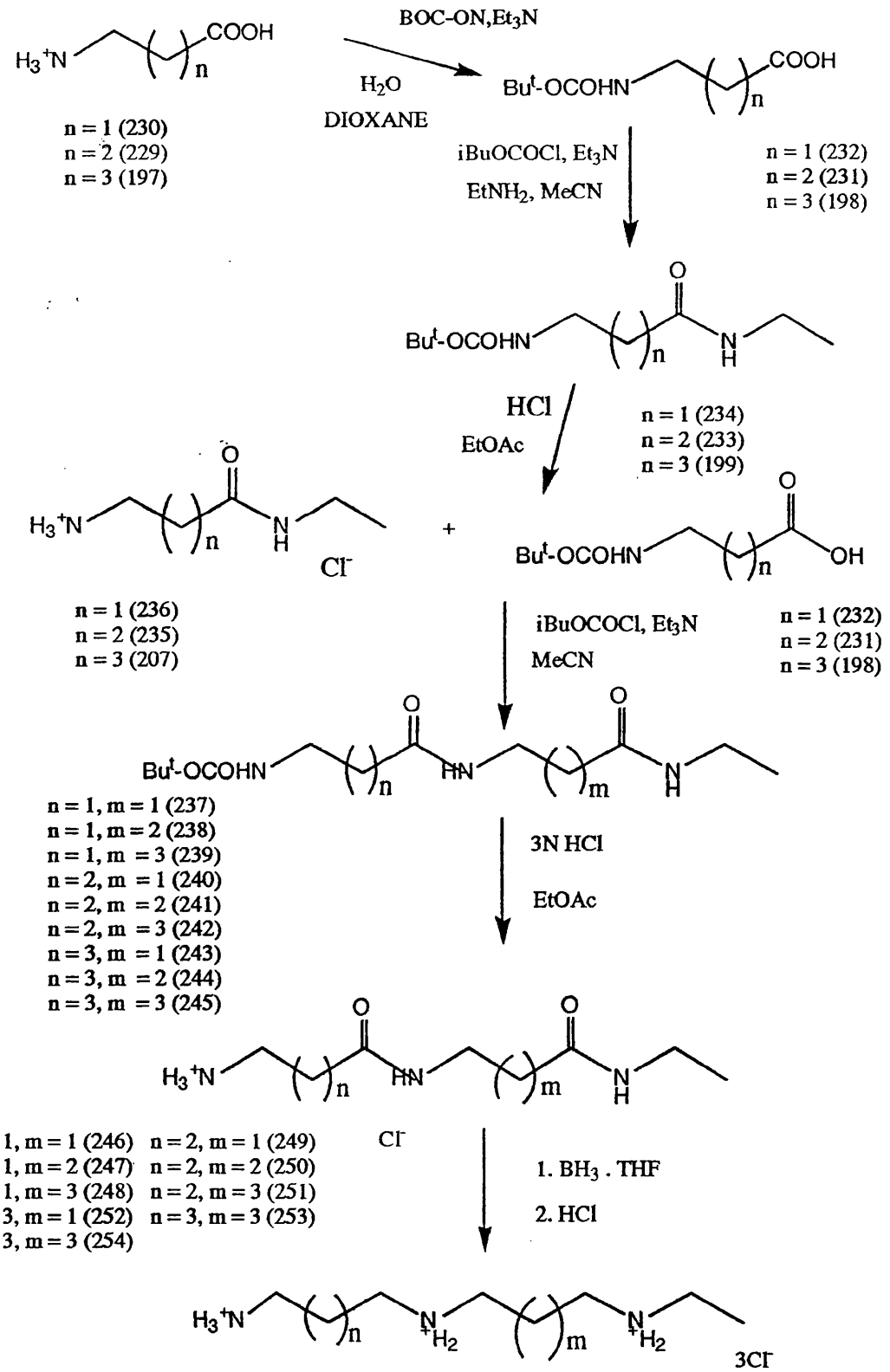
8.2 SYNTHESIS OF POLYAMINES

Polyamines were synthesised by a method devised as a part of this study (Scheme 58). The method is exemplified for the compound *N*-5-aminopentyl-*N'*-ethyl-1,5-pentanediamine trihydrochloride (263).

SCHEME 58


H2N
COOH
(197)
DIOXANE
H2O
BOC-ON,Et3N
Bu^t-OCOHN
COOH
(198)
iBuOCOCl, Et3N
EtNH2, MeCN
3N HCl
Bu^t-OCOHN
(199)
EtOAc
H3+N
Cl-
(207)
Bu^t-OCOHN
(198)
iBuOCOCl, Et3N (2 equiv)
MeCN
Bu^t-OCOHN
(245)
3N HCl
EtOAc
H3+N
(254)
Cl-
1. BH3 . THF
2. HCl
H3+N
(263)
3Cl-

SCHEME 59



n = 1 (230)
n = 2 (229)
n = 3 (197)

n = 1 (232)
n = 2 (231)
n = 3 (198)

n = 1 (234)
n = 2 (233)
n = 3 (199)

n = 1 (236)
n = 2 (235)
n = 3 (207)

n = 1 (232)
n = 2 (231)
n = 3 (198)

n = 1, m = 1 (237)
n = 1, m = 2 (238)
n = 1, m = 3 (239)
n = 2, m = 1 (240)
n = 2, m = 2 (241)
n = 2, m = 3 (242)
n = 3, m = 1 (243)
n = 3, m = 2 (244)
n = 3, m = 3 (245)

n = 1, m = 1 (246) n = 2, m = 1 (249)
n = 1, m = 2 (247) n = 2, m = 2 (250)
n = 1, m = 3 (248) n = 2, m = 3 (251)
n = 3, m = 1 (252) n = 3, m = 3 (253)
n = 3, m = 3 (254)

n = 1, m = 1 (255) n = 2, m = 1 (258) n = 3, m = 1 (261)
n = 1, m = 2 (256) n = 2, m = 2 (259) n = 3, m = 2 (262)
n = 1, m = 3 (257) n = 2, m = 3 (260) n = 3, m = 3 (263)

The valeric acid (197) was protected at the amine end with 2-*t*-butoxycarbonyloxyimino-2-phenyl acetonitrile (BOC-ON) as described previously. The product, 5-*t*-butoxycarbonylaminopentanoic acid (198) was divided into two portions. One portion of the BOC protected acid (198) was then subject to a mixed anhydride reaction with a three fold excess of ethylamine to give *N*-ethyl-5-*t*-butoxycarbonylaminopentanamide hydrochloride (199). This was followed by careful acid hydrolysis to remove selectively the *N*-*t*-butoxycarbonyl protection and to afford (207). The other 5-*t*-butoxycarbonyl pentanoic acid (198) portion was reacted with 3 equivalents of base plus 1.1 equivalents of *N*-ethylaminopentanamide salt (207) via a mixed anhydride reaction. The BOC-protected diamine (245) was formed in 20% yield. This was then deprotected by acid hydrolysis followed by trituration to afford the bis amide amine salt (254) with a 40% yield. This was reduced using BH_3 in THF to give *N*-5-aminopentyl-*N*'-ethyl-1,5-pentanediamine trihydrochloride (263) with a 99% yield.

The BOC protected acids of 4-aminobutanoic acid (229) and 3-aminopropanoic acid (230) were formed by the same procedure as for (263) above (Scheme 59). Some of each was subject to a mixed anhydride reaction with Et_3N followed by deprotection of the amine end as above.

N-Ethyl-4-*t*-butoxycarbonylaminobutanamide (233) and propanamide (236) salts were formed. All the reactions were high yielding (93% on average).

This procedure enabled us to create a bank of C_3, C_4 and C_5 BOC protected acids and C_3, C_4 and C_5 *N*-ethylamide salts.

Reactions were attempted with each of the acids reacting with each of the salts in a mixed anhydride reaction. Potentially nine products can be formed (255) - (263), (Scheme 59).

The mixed anhydride reaction took place between the acid portion of the BOC protected acid followed by attack of the nitrogen of the chosen amide salt. The free base of the amide salts was unstable and difficult to isolate. However, the reaction took place with the amine, formed in situ using 3 equivalents of base. An excess of

the amide salt was used. Only a slight excess (1.1 equivalents) should be used since larger excesses do not increase the yields of these reactions.

The degree of success and the yield for the mixed anhydride reaction depends on the combination of BOC acid and amine salt chosen. The best yields were obtained between acid (197) and amide (235) to produce (244) (41% yield). The reaction between C_3 acid and C_3 salt was tried repeatedly but gave a maximum yield of only 10%.

The next stage involves the selective acid hydrolysis to remove the BOC protecting group, followed by trituration to yield white crystals of the hygroscopic *N*-ethyl mixed amide salts. The trituration sometimes yielded crystals, but on other occasions an oily residue was left even after two to three weeks trituration. The best yield (99%) for the deprotection was obtained for compound (247).

The final stage in the synthesis is the borane reduction using BH_3 in THF. This is described in some detail in Chapter Six and the same procedure applies here. Provided the reduction involved solid mixed amide salts the reduction worked well. If the oily residue left after trituration was used it was impossible to purify the reduction of products and a mixture of products was obtained. The best yield for the reduction was for compound (263) (99% yield).

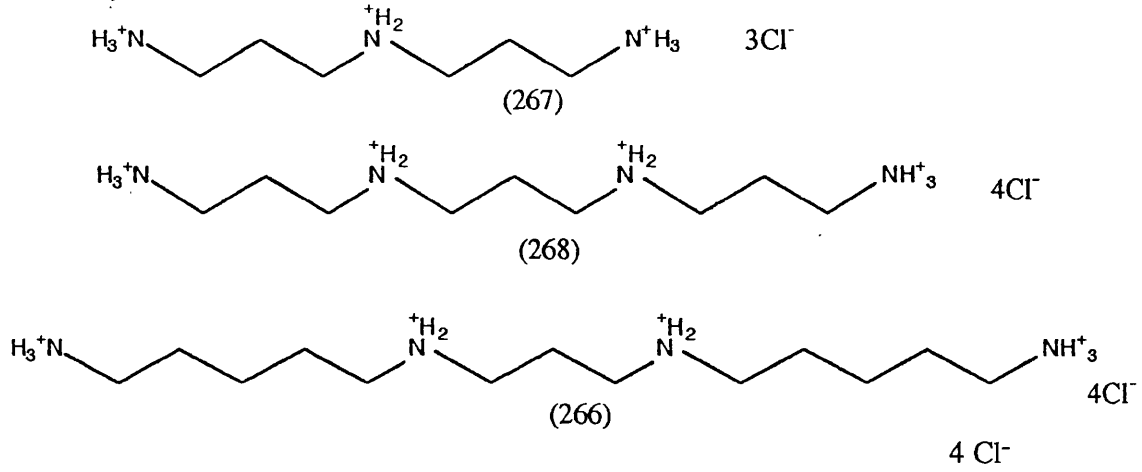
8.3 THE VERSATILITY OF SCHEMES 58 and 59

One of the important aspects of the synthesis shown in Schemes 58 and 59 is its versatility. The compounds shown are all *N*-ethyl derivatives. However the synthesis is not only applicable to *N*-ethyl substituents. Theoretically, any *N*-alkyl substituent can be used in this synthesis to create a range of *N*-alkyl polyamines. The formation of products is not limited to compounds of three, four and five carbon chain lengths. Again, theoretically, the synthesis makes it possible to form polyamines with higher carbon chain lengths. Moreover, the synthesis is not restricted to triamines. Tetraamines and higher polyamines may also be synthesised. A tetraamine (268) has been synthesised and is discussed at the end of this Chapter.

8.4 ASSESSMENT OF POLYAMINES FOR SUBSTRATE ACTIVITY

Table 8.1 shows the kinetic data for the cadaverine and putrescine analogues tested for substrate activity. Where relevant, the K_M and V_{max} values obtained are given. Their structures are given in Scheme 59 and Figure 8.1. With the exception of compounds (267) and (268), all were synthesised in the present study. The assay method used was Stoner's peridoxase coupled assay as described in Chapter Seven.[22]

Figure 8.1



Some of the compounds tested had very low specific activity at the highest substrate concentrations of 2.86 mM. Since there was only a limited quantity of the polyamines available, a decision had to be taken as to whether to use the synthetic products to test for inhibition or to test for substrate activity. The initial activity of all compounds was tested at the highest substrate concentration of 2.86 mM. At this concentration very low specific substrate activity was shown by the trihydrochloride salts: (267), (268), (256), (257) and (262). Consequently K_M values were not obtained for these compounds. As they did not appear to be substrates, they were tested as inhibitors.

A decision to test for inhibition was also made in respect of compounds (258) and (261), since initial testing indicated that they were substrates but of a lower order of substrate activity than the other compounds tested.

From the initial testing at high substrate concentrations a rough approximation of the V_{max} values can be estimated by the equation $V = V_{max}$. For an accurate estimate for K_M a full range of substrate concentrations is needed. For the other

compounds in Table 8.1 full substrate testing was carried out and their K_M and V_{max} values shown.

Table 8.1: K_M and V_{max} values for oxidation of analogues catalysed by diamine oxidase from pea seedlings at 25 °C and pH 6.3.

SUBSTRATE	V_{max}* (μ mol $mg^{-1}h^{-1}$)	K_M (mM)	V_{max} (μ mol $mg^{-1}h^{-1}$)
3 + 3 (267)	21.5		not substrate
3 + 3 + 3 (268)	59.1		
3 + 4 - Et (256)	5.5		not substrate
3 + 5 - Et (257)	9.2		not substrate
4 + 3 - Et (258)	65.3		
4 + 4 - Et (259)		0.53	262
4 + 5 - Et (260)		0.62	226
5 + 3 (264)	161.3		
5 + 3 - Et (261)	112.9		
5 + 4 - Et (262)	49.1		
5 + 5 - Et (263)		0.98	517
3 + 5 + 3 (265)		1.35	355
5 + 3 + 5 (266)	101.4		

Units K_M: mM V_{max}: μ mol $mg^{-1}h^{-1}$

V_{max}* = specific activity when tested as a substrate at the highest concentration of 2.86 mM. At high [S], $V = V_{max}$.

8.5 RESULTS AND DISCUSSION

From Table 8.1 it can be seen that some of the compounds tested showed reasonable substrate activity with *N*-ethyl-5-(5-aminopentanamino)-1-aminopentane, trihydrochloride (263) being a particuarly good substrate, with K_M of 0.98 mM and

V_{max} of 517 μmol $mg^{-1}h^{-1}$, around the same value as *N*-ethylcadaverine. As the number of methylene units increases the K_M values also increase. A less obvious pattern is shown for V_{max}.

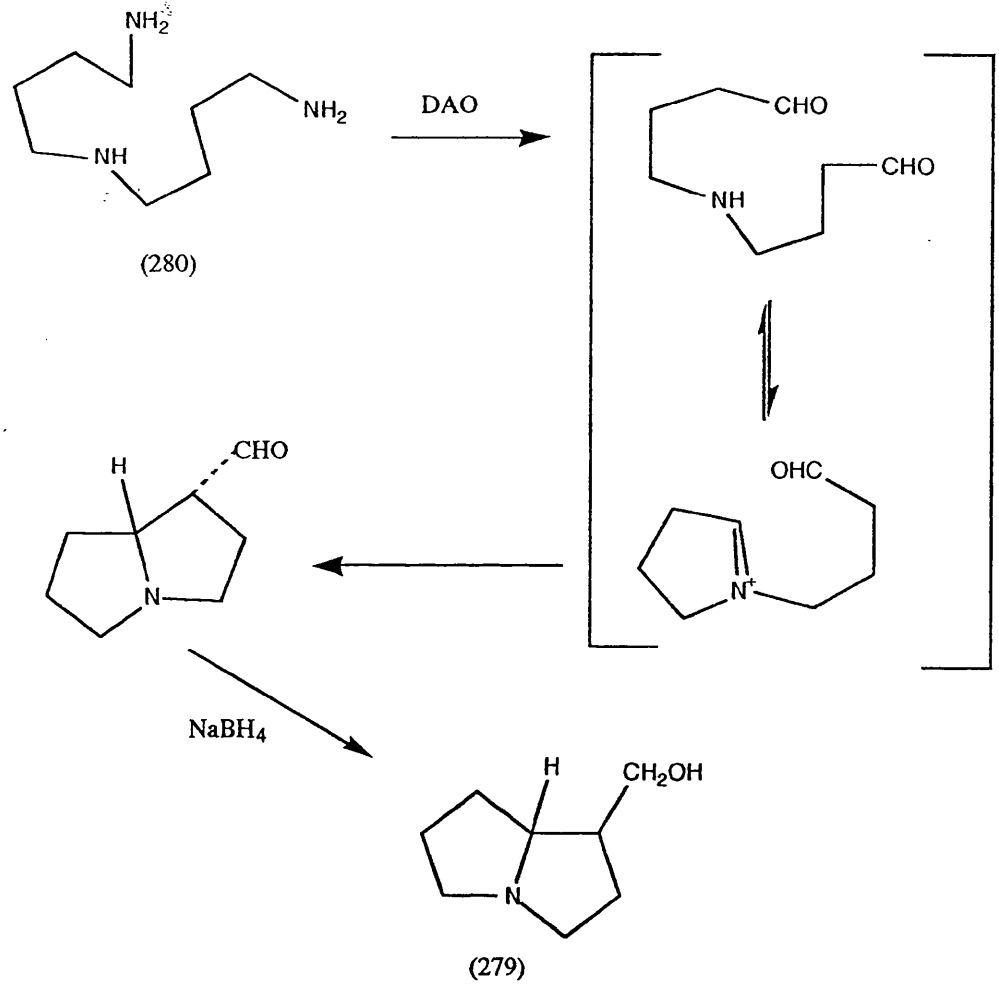
The lowest substrate activity occurs when the 1,3-diaminopropane group has a primary amine. The compounds (259) and (260), with a 1,4-diaminobutane group with a primary amine give V_{max} values around 262 and 226 μmol $mg^{-1}h^{-1}$ respectively, and K_M values around 0.6 mM characteristic of medium substrate activity. The polyamine which showed the highest rate of oxidation has a 1,5-diaminopentane group with a primary amine.

The important factor for substrate activity in polyamines therefore seems to be similar to the factors which affect the mode of action of the simple diamines, cadaverine, putrescine and 1,3-diaminopropane. It is already well known that cadaverine is a very good substrate of DAO, followed by putrescine. 1,3-Diaminopropane is a very poor substrate. Neither of the conditions necessary for binding in the active site are fulfilled by 1,3-diaminopropane. This suggests that only the group with the primary amine is involved in cyclisation with the remainder of the molecule acting as an *N*-alkyl group.

The *N*-alkyl groups attributed to the polyamine substrates are considerably larger than those on the diamines discussed in Chapter Six. They do not, however, show the decreasing V_{max} and K_M values expected from an alkyl group of this magnitude and do not appear to be causing steric hindrance. It could be postulated that the lack of steric hindrance might be due to the formation of a bicyclic system caused by the *N*' nitrogen attacking the double bond next to the protonated nitrogen in the cyclic system. This would speed up the rate of the reaction and explain the higher V_{max} values obtained. This cyclisation would not contribute any extra binding to the subsite (hence the relative stability of the K_M values), and might explain the kinetic data obtained for (259), (260) and (263).

Robins[152] carried out an experiment in which homospermidine (280) was reacted with diamine oxidase. After reduction with sodium borohydride the alkaloid 1-hydroxy-methylpyrrolizidine (279) was obtained (Scheme 60).

SCHEME 60



(280)

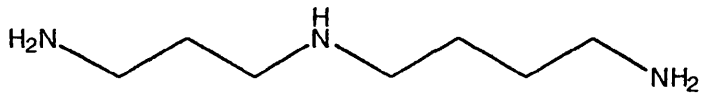
(279)

It could be of value for a future researcher to carry out a similar procedure using the polyamines found to be good substrates to see if their reaction with diamine oxidase produces any new alkaloid analogues. If analogues were found it could help to establish whether the postulate above is valid.

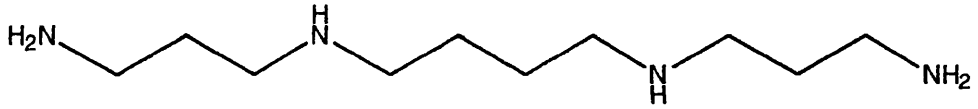
For compounds with the 1,3-diaminopropyl primary amine unit the formation of a bicyclic ring is unlikely to occur since the initial cyclisation is hindered, thus preventing any secondary cyclisation and explaining why these compounds are not substrates.

Another possible explanation is that cyclisation of the oxidised primary amine with *N* ' nitrogen forms a very large ring structure. If this were the case, the rings formed would be equivalent to a chain length in excess of nine carbons. For a chain length of this magnitude one would expect V_{max} values considerably lower than those obtained, making this an unlikely mechanism for any of the compounds tested.

These compounds were originally synthesised as analogues of spermidine (185), which is a natural substrate of DAO. The mechanism involved in spermidine may be an initial cyclisation of the four carbon units since this is energetically favoured. Thus the primary nitrogen attached to this may bind to the enzyme first. In the analogues synthesised where the three carbon chain is next to the primary nitrogen this unit must cyclise first. However, this is hindered in DAO reactions due to the short chain length.



(185)



(186)

Spermine (186) is not a natural substrate of DAO but is a substrate of polyamine oxidase. Since two analogues of spermine were available, (268) and (265), they were tested as substrates. Compound (268) showed no substrate activity - not perhaps surprising in view of the results obtained from other compounds with a three carbon chain. Compound (265) showed surprisingly good substrate activity with a K_M of 1.35 mM and a V_{max} of 355 μmol $mg^{-1}h^{-1}$. Only the primary nitrogens can be oxidised to form an aldehyde, but secondary nitrogens may bind into a different part of the active site. Since the K_M and V_{max} values are both relatively high this may suggest transition state binding. The three carbon side chains normally hinder substrate activity. If, however, they bind weakly in the initial stage of ES

binding they may form a more favourable binding in the transition state, thus overcoming the initial steric hindrance and increasing the rate.

A similar situation may be happening in (263) since this also has K_M and V_{max} values similar to those of *N*-ethylcadaverine, which was also postulated as showing transition state binding.

8.6 INHIBITION OF DAO BY POLYAMINES

Table 8.2 shows the polyamines tested as inhibitors, the apparent type of inhibition found and their corresponding K_i values. As in the previous Chapter all compounds were analysed by the three graphical methods Lineweaver-Burk, Hanes and Hofstee.

Table 8.2: Polyamines tested as inhibitors of pea seedling diamine oxidase, K_i values and apparent type of inhibition found. Inhibitor concentrations of 1 mM, 0.5 mM and 0.25 mM were used.

INHIBITOR	K_i (mM)	APPARENT TYPE OF INHIBITION
3 + 3 (267)	2.29	Competitive
3 + 3 + 3 (268)	2.43	Competitive
3 + 4 - Et (256)	0.67	Competitive
3 + 5 - Et (257)	0.71	Competitive
4 + 3 - Et (258)	0.57	Competitive
4 + 4 - Et (259)	1.89	Mixed
5 + 3 (261)	3.29	Noncompetitive
5 + 4 -Et(262)	1.96	Coupling

8.7 RESULTS AND DISCUSSION

Competitive inhibition.

It can be seen from Table 8.2 that one requirement for competitive inhibition appears to be the presence of a 1,3-diaminopropane unit. With the exception of (267)

and (268) all of the competitive inhibitors showed fairly low K_i values, but the strongest inhibitors were those with the N^9-ethyl group attached. Although these competitive inhibitors are not themselves oxidised, they do compete for the same active site as cadaverine, thus inhibiting the reaction between the cadaverine and the enzyme. This may be due to the fact that they are substrate analogues of the natural substrate spermidine.

Non-competitive inhibition.

Compound (261) was the only one of the series tested to show apparent non-competitive inhibition and it also had the highest K_i value. This is consistent with the results discussed in Chapter Seven, where the only apparent non-competitive inhibitor was *N*-propylcadaverine (216). Neither compound binds to the same active site as cadaverine, but both structures have a cadaverine portion and a propane portion and it is not known whether they both bind to a common sub-site.

Coupling inhibition.

Compound (262) appeared to be a coupling inhibitor where K_i must be determined from the plot of the intercept against the inhibitor concentration and not from the slope. This compound binds to the cadaverine/enzyme complex, inactivating it. It has a cadaverine base portion with an *N*-ethylputrescine attached. Interestingly, *N*-ethylputrescine was one of the compounds which, earlier, was disscussed as an apparent coupling inhibitor (Chapter Seven). It may be that this polyamine gains its inhibitory activity from the *N*-ethylputrescine side portion rather than the cadaverine base portion.

Mixed inhibition.

Compound (259) appears to be a mixed inhibitor which may be binding to the same subsite as cadaverine and simultaneously to a different site. Its binding to the same subsite as cadaverine is not surprising as it is a substrate. Its noncompetitive characteristics may be due to its having some structural similarities to *N*-butylputrescine, which also appeared to display mixed inhibition. There may be a

common subsite, separate from the cadaverine subsite, to which both of these compounds can bind.

8.8 TESTING OF POLYAMINES FOR ANTI-FUNGAL, ANTI-CANCER AND ANTI-MALARIAL ACTIVITY

Some of the polyamines, (256) - (265), were also sent for testing as anti-fungal, anti-cancer and anti-malarial agents. None of the polyamines proved to have significant anti-cancer activity. Two compounds showed strong anti-fungal activity. A third showed moderate anti-fungal activity. Results from the other tests are not yet available.

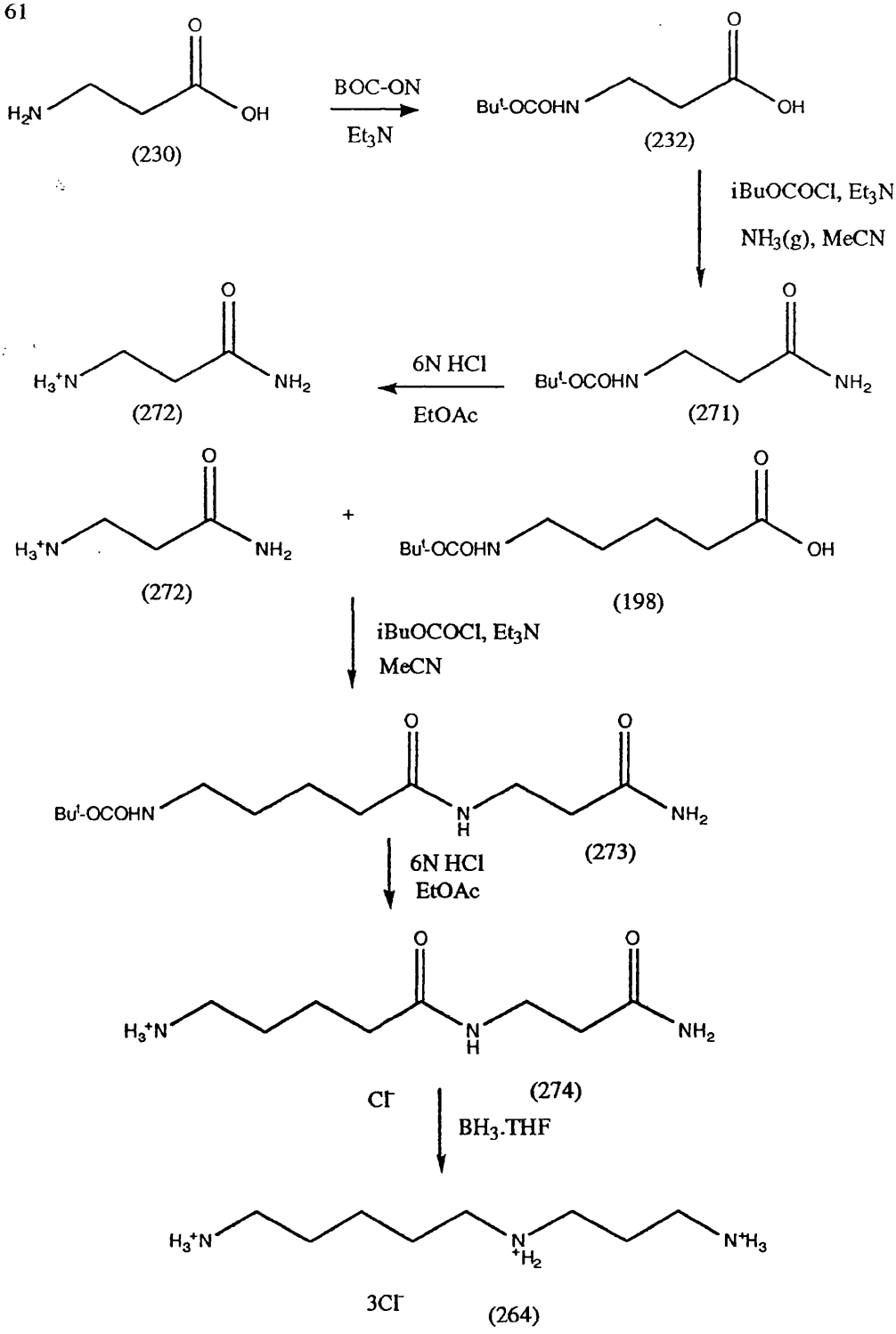
8.9 INTRODUCTION TO SYNTHESIS OF COMPOUNDS (264) AND (265)

A study carried out by Walters and co-workers[176] showed that if fungi or plants are fed labelled lysine, the label appears in compounds which co-migrate with spermidine (185) and spermine (186) on TLC plates. These compounds appear to be *N*-(3-aminopropyl)cadaverine (264) and its homologue *N*,*N*'-bis(3-aminopropyl)cadaverine (265). Formation of these compounds has been shown in cancer cells and in certain bacteria, but never in fungi, and only very rarely in plants. Walters wished to confim that the compounds which he had extracted were in fact (264) and (265) and to quantify their changes in fungal and plant tissue. The aim of this synthesis, therefore, was to synthesise two compounds which could be used to determine the identity of the compounds extracted by Walters from six plants and two fungi. The compounds synthesised (264) and (265) were tested by Walters and were found to be the same as the natural compounds.

8.10 SYNTHESIS OF COMPOUNDS (264) AND (265)

Compounds (264) and (265) were synthesised by the methods shown in Schemes 61 and 62 respectively.

SCHEME 61

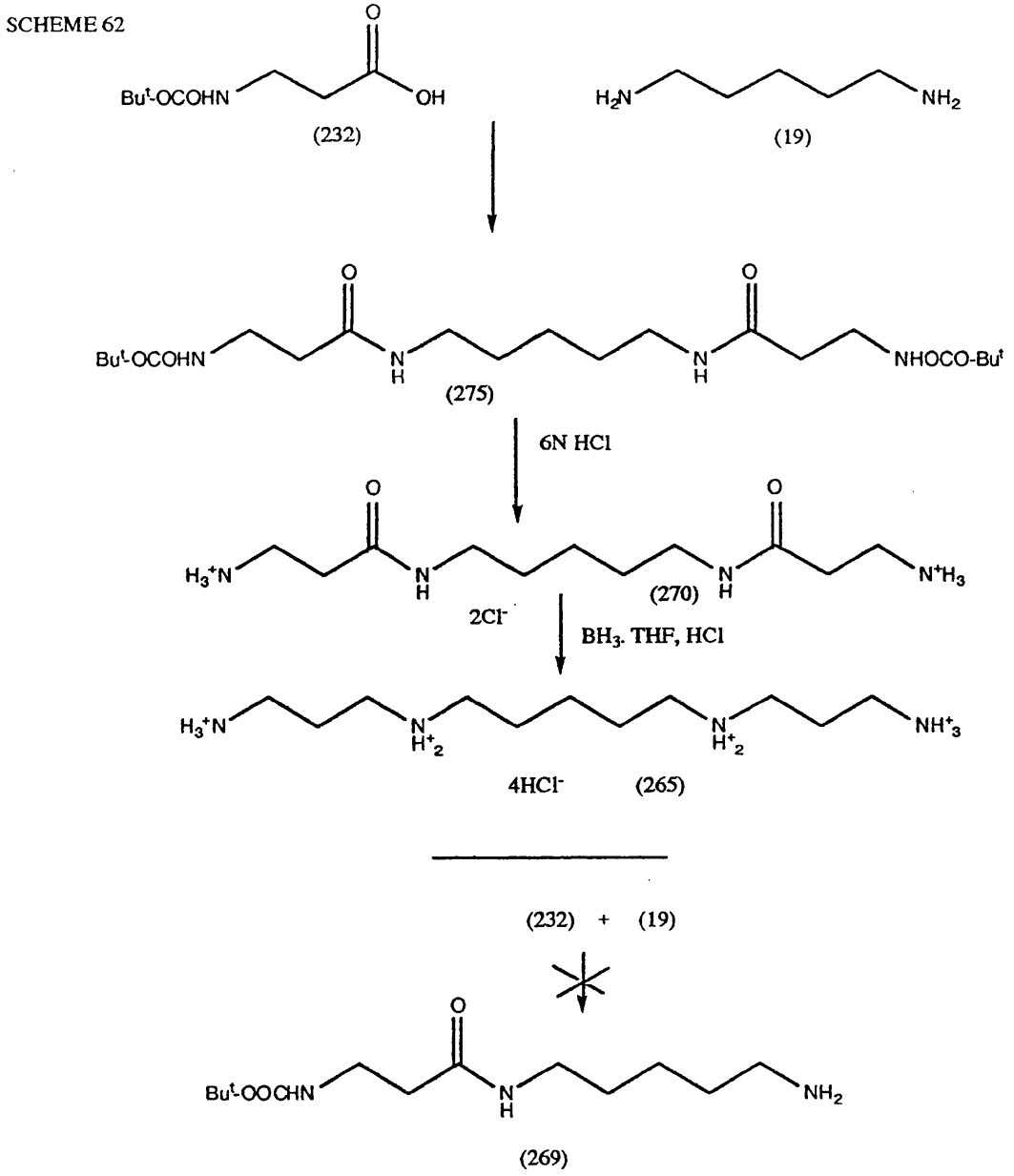


3-Aminopropanoic acid (230) was protected with BOCON (99.7% crude yield) (232) at the amine end, then reacted with ammonia gas in a mixed anhydride reaction to form (271) (92% yield). It was then deprotected using 6N HCl to afford 3-aminopropanamide hydrochloride(272) (89% yield). This was then reacted with the BOC protected valeric acid (198) in a mixed anhydride reaction followed by

deprotection, again with 6N HCl, and finally a borane reduction to yield the product (264) in 96% yield.

Due to the hygroscopic nature of the intermediate (274), it was difficult to obtain a crystalline product, even after trituration. The resulting product was also hygroscopic and an oil, It proved difficult to crystallise. A slightly brown solid of (264) was obtained in 96% yield.

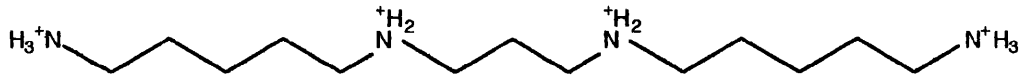
SCHEME 62



Scheme 62 was used to form compound (265). The BOC protected acid (198) was reacted with 1,5-diaminopentane (19) in a mixed anhydride reaction to form the Di-BOC protected product (275) selectively in 32% yield. This selectivity was surprising since it had been anticipated that the synthesis might yield a mixture of (269) and (275). In subsequent trials the diBOCprotected product was consistently formed selectively, even when a five-fold excess of 1,5-diaminopentane (19) was used.

The diBOCprotected product (275) was deprotected with HCl, followed by a borane reduction to produce pure (265) in 48% yield. This was then used to test for the natural product. The results of testing (265) showed that the product formed by the plant is in fact the same compound as compound (265).

One final point of interest is that, while the reaction to form (265) was selective, a reaction with BOC protected valeric acid (198) and 1,3-diaminopropane (163) gave a mixture of the mono- (264) and the di-product (266). The ratio of the mixture varied depending on conditions.



4Cl⁻ (266)

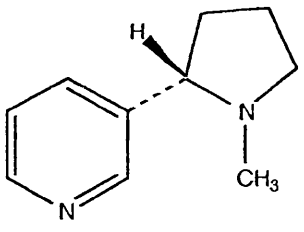
CHAPTER NINE

FEEDING EXPERIMENTS USING TRANSFORMED ROOT CULTURES

9.1 INTRODUCTION

The tobacco alkaloids have been the subject of a great number of scientific investigations, and there is a large volume of literature available.[73] The main species for the commercial production of tobacco is *Nicotiana tabacum*, but much of the biosynthetic work on the tobacco alkaloids has been carried out with other *Nicotiana* species such as *N. glauca* and *N. rustica*.[89] One of the main alkaloids found in almost all species of *Nicotiana* is nicotine (281), although nicotine has been isolated from many other species.[89] Natural products such as nicotine (281) often have useful biological activity. Nicotine, for example, is both an insecticide and feeding deterrent for many animals.[79] It is biosynthesised from nicotinic acid (282) and *N*-methylputrescine (194).[73] In *N. tabacum* nicotine (281) synthesis occurs mainly in the roots and the alkaloid is translocated to stems and leaves. This has led to biosynthetic investigations in which the excised roots of the plant are grown like a micro-organism in a sterile medium, and tracer is added to this solution.

Transformed root cultures of *N. rustica* have also been shown to produce mainly nicotine, with smaller quantities of anabasine (21), nornicotine and anatabine. Transformed root cultures of *D. stramonium* contain very small quantities of nicotine[173] but produce other alkaloids, hygrine and hyoscyamine, in greater quantities. This Chapter describes experiments based on the feeding of *N*-alkylcadaverines (215) to (218) and (220) to *N. rustica* and to *D. stramonium*, with the aim of discovering whether any new alkaloid analogues are formed.



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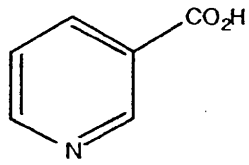

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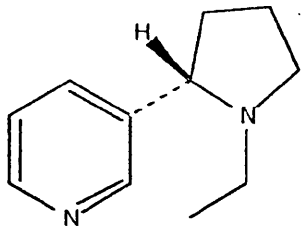
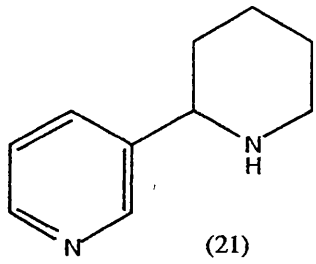


(283)



(21)

9.2 THE USE OF ANALOGUES IN BIOSYNTHETIC STUDIES

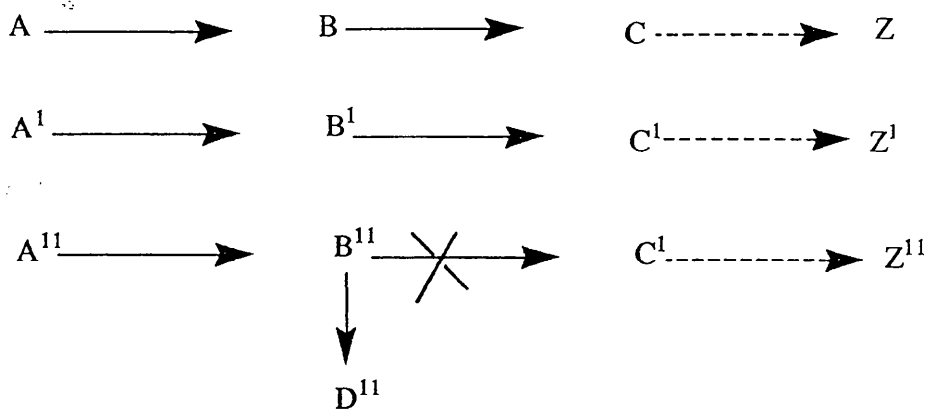
It has been established that feeding certain modified precursors of a natural precursor to biological systems may result in new or modified alkaloids being produced. For example, the feeding of *N*-ethylputrescine dihydrochloride (195) to root cultures of *Nicotiana rustica* stimulated the formation of a new metabolite, *N'*-ethylnornicotine (283), in optically active form and in reasonable yield.[79] Previously *N'*-ethylnornicotine has only been detected in tobacco and tobacco smoke.[73] Identification of the biosynthesised alkaloid as *N'*-ethylnornicotine was carried out by spectroscopic analysis. The ORD and CD data obtained showed that the biosynthesised product had the same absolute configuration as the natural product.

The formation of *N'*-ethylnornicotine is an example of aberrant synthesis, the term that is applied to abnormal synthetic reactions that occur in biological systems. Analogues of known precursors can be of assistance in clarifying biological pathways, and the ability of living systems to convert chemically modified precursors into analogues of their normal metabolites may be helpful in elucidating the transformations which are occurring.

For example, an organism may be capable of dealing successfully with one modified precursor but unable to deal with another; analogue substrates may be

metabolized in an abnormal way, giving rise to new structural types; or the metabolism may be changed to such an extent that growth is inhibited and the cells die. A model of a biogenetic pathway is shown in Figure 9.1.

Figure 9.1



In a biogenetic pathway precursor A is transformed into B and then to C, and so on until the biosynthesis is complete and a natural product Z is produced. If a modified precurser A^1 is fed it may be mistaken by the plant for a normal metabolite and transformed into a modified natural product, Z^1. If, however, a modified precursor A^{11} is only recognised by some of the enzymes on the biogenetic pathway to Z^{11}, the biosynthesis may come to halt. and may result in the formation of a new product D^{11}. The most drastic outcome of non-recognition is that the modified precursor may completely block one or more steps in the biosynthesis which may have a detrimental effect on the whole system.

As indicated in previous Chapters, *N*-ethylputrescine is a reasonable substrate for pea seedling diamine oxidase. In the biosynthesis of *N'*-ethylnornicotine it appears that *N*-ethylputrescine competes with the normal metabolite *N*-methylputrescine for the enzyme, *N*-methylputrescine oxidase, which is involved in nicotine biosynthesis. Since no *N'*-ethylnornicotine was found in cultures grown in media without the addition of *N*-ethylputrescine, it was assumed that the new metabolite was formed in response to the addition of *N*-ethylputrescine.

The enzyme putrescine-*N*-methyltransferase (PMT; EC 2.1.1.53) catalyses the initial reaction in the biosynthesis of tropane and pyrrolidine alkaloids from

putrescine in the roots of plants of a number of solanaceous genera, including *Datura* and *Nicotiana*, and it is thought that all *N*-methylations in tropane and pyrrolidine alkaloid biosynthesis are mediated by this enzyme.[174]

A study by Walton and co-workers[172] has shown that the enzyme is active with some derivatives and analogues of putrescine, but that 1,3-diaminopropane (163) and 1,5-diaminopentane (19) showed no substrate activity. They suggest, therefore, that a requirement for substrate activity in cyclic compounds is the presence of two amino groups in a *trans* conformation, separated by four carbon atoms. Cadaverine and certain cadaverine analogues,which showed no substrate activity, proved to be good inhibitors of the enzyme.

Certain species of *Nicotiana* synthesise the cadaverine-derived alkaloid anabasine (21) as a major product, in addition to the putrescine-derived alkaloid nicotine, and the production of anabasine can be stimulated at the expense of that of nicotine by supplying exogenous cadaverine.[171] Unlike nicotine, anabasine is not *N*-methylated, which is consistent with the substrate requirements of the PMT enzyme and its inability to methylate cadaverine. Walton and co-workers found that the production of anabasine could be stimulated at the expense of that of nicotine in transformed root cultures of *Nicotiana* by supplying exogenous cadaverine. The effect of exogenous cadaverine in suppressing nicotine formation could not be ascribed completely to its effect as a competitive inhibitor of *N*-methylputrescine oxidase. Walton and co-workers therefore postulated that inhibition of the PMT enzyme by cadaverine may be an additional factor promoting the biosynthesis of anabasine from cadaverine, and recommended that the production of cadaverine-derived alkaloids in *Datura* and *Nicotiana* species should be further explored.[172]

Brown[65] fed 3,3-dimethylcadaverine dihydrochloride to *D. stramonium* cultures in an attempt to produce an anabasine analogue. She found that administration of this precursor at a concentration of 2 mM completely inhibited growth, whereas 3-methylcadaverine and 2,4-methylcadaverine only partially inhibited growth.

9.3 FEEDING EXPERIMENTS WITH *D. STRAMONIUM* AND *N. RUSTICA*

The compounds fed were the *N*-alkylcadaverines (215) - (218) and (220). Each of the precursors were fed at 1 mM concentrations to both the root cultures, *N. rustica* and *D. stramonium.* After ten days the transformed root cultures were chopped and finely extracted. Once extraction was complete the extract was dried, weighed and analysed by TLC against control cultures which were grown simultaneously but to which no precursor was added. If any new metabolites appeared they were separated by preparative HPLC and analysed by GC and GC / MS to establish their structures. The root extracts obtained from feeding the five precursors to the two cultures were analysed by Dr. N. Walton and Dr. A. Parr at the Institute of Food Research, Norwich.

All of the root cultures produced some alkaloids as shown by TLC. No new alkaloid analogues appeared to be produced from the compounds fed to *D. stramonium.* Cadaverine is only partially metabolised to hygrine and hyoscyamine in *D. stramonium,* and does not result in the production of tropane alkaloids. This appears to be the case with the *N*-alkylcadaverines also.

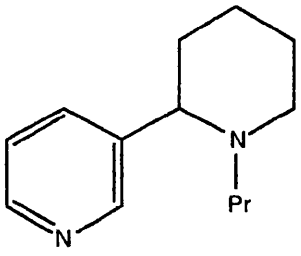
Table 9.1 shows the the percentages of the total peak areas of the GC-chromatogram, together with a peak area value for nicotine for each compound fed.

Table 9.1 Amines Fed to *N. rustica.*

Cadaverine Fed	Nicotine GC Peak Area (units) x 10^9	% of Total Chromatogram Peak Areas				
		Nicotine	Nornicotine	Anabasine	Anatabine	New
N-ethyl- (215)	2.54	57.9	2.5	2.2	19.6	9.2
Control	3.66	68.4	4.6	2.1	15.2	0
N-propyl- (216)	1.66	69.8	1.9	0.9	13.0	1.5
N-isopropyl- (218)	3.01	64.2	3.4	1.5	19.1	0
Control	2.01	62.8	3.4	1.6	18.9	0
N-benzyl- (220)	4.78	51.0	7.7	2.1	12.4	0
Control	4.37	63.2	6.1	1.5	16.0	0

The ratio of nicotine to total chromatogram peak areas was roughly 60% in each case, with approximately one third of this amount of anatabine present, and minor amounts of nornicotine and anabasine.

GC-Chromatograms for feeds on *N. rustica* with *N*-ethylcadaverine and *N*-propylcadaverine, and a control experiment, are shown in Figures 9.2 to 9.4. Both *N*-ethylcadaverine (215) and *N*-propylcadaverine (216) appeared to produce a new alkaloid analogue, namely the corresponding anabasine derivative, when fed to *N. rustica*. *N*-Ethylcadaverine produced *N*-ethylanabasine (285). *N*-Propylcadaverine (286), a poorer precursor, produced *N*-propylanabasine. *N*-Isopropylcadaverine (218) and *N*-benzylcadaverine (220) did not appear to be substrates and produced no new alkaloid analogues.



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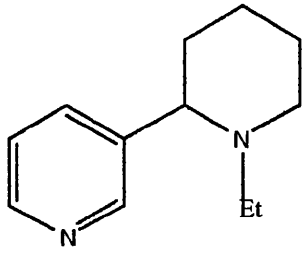
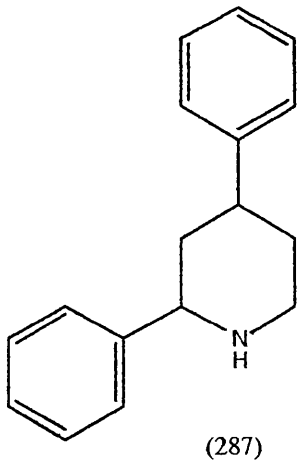


(286)

The GC/MS of the extract from the *N*-ethylcadaverine feed showed major peaks at 20.28, 24.70, 24.41, 26.28, 41.71, and 42.95 (Figure 9.5). These were identified from their mass spectra as the alkaloids nicotine (281) (M^+ 162), anabasine (21) (M^+162), *N*-ethylanabasine (285) (M^+ 192), anatabasine (288) (M^+ 160), plus possibly acetovanillone (which sometimes appears in extracts of transformed roots of the Solanaceae), an unidentified impurity at M^+ 279, and possibly anatalline (287), a minor *Nicotiana* alkaloid, (M^+ 238). *N*-Ethylanabasine appears to be a new alkaloid analogue formed in response to the feeding of *N*-ethylcadaverine.



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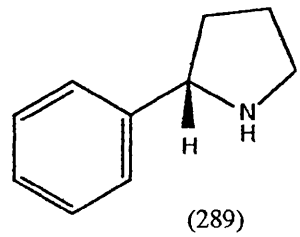

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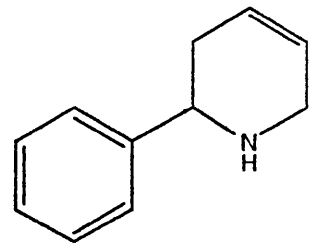


(288)

The extract from the *N*-propylcadaverine feed showed fewer alkaloids on the GC/MS (Figure 9.7). Peaks at 19.75, 23.28, 25.60 and 26.40 corresponded to nicotine, (281) nornicotine (289) (M^+ 148), *N*-propylanabasine (286) (M^+ 204) and anatabasine. The mass spectra for compounds (285) and (286) are shown in Figures 9.6 and 9.8 respectively. The mass spectrum of (285) is consistent with the formation of a new alkaloid analogue in response to the feeding of *N*-propylcadaverine.

FIGURE 9.2 THE GC TRACE FOR THE *N*-ETHYLCADAVERINE FEED TO *N. RUSTICA*

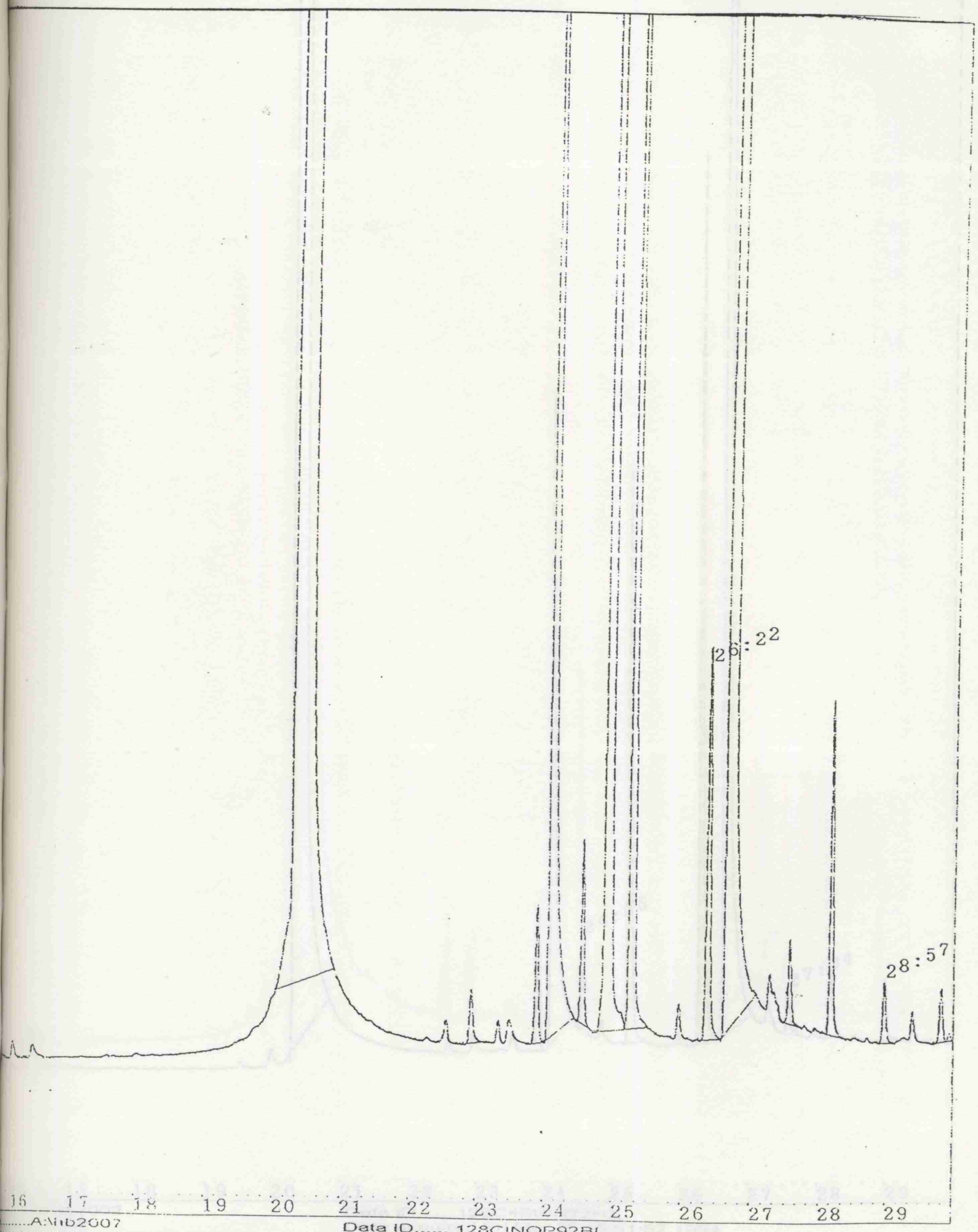


FIGURE 9.3 THE GC TRACE FOR THE *N*-PROPYLCADAVERINE FEED TO *N.*

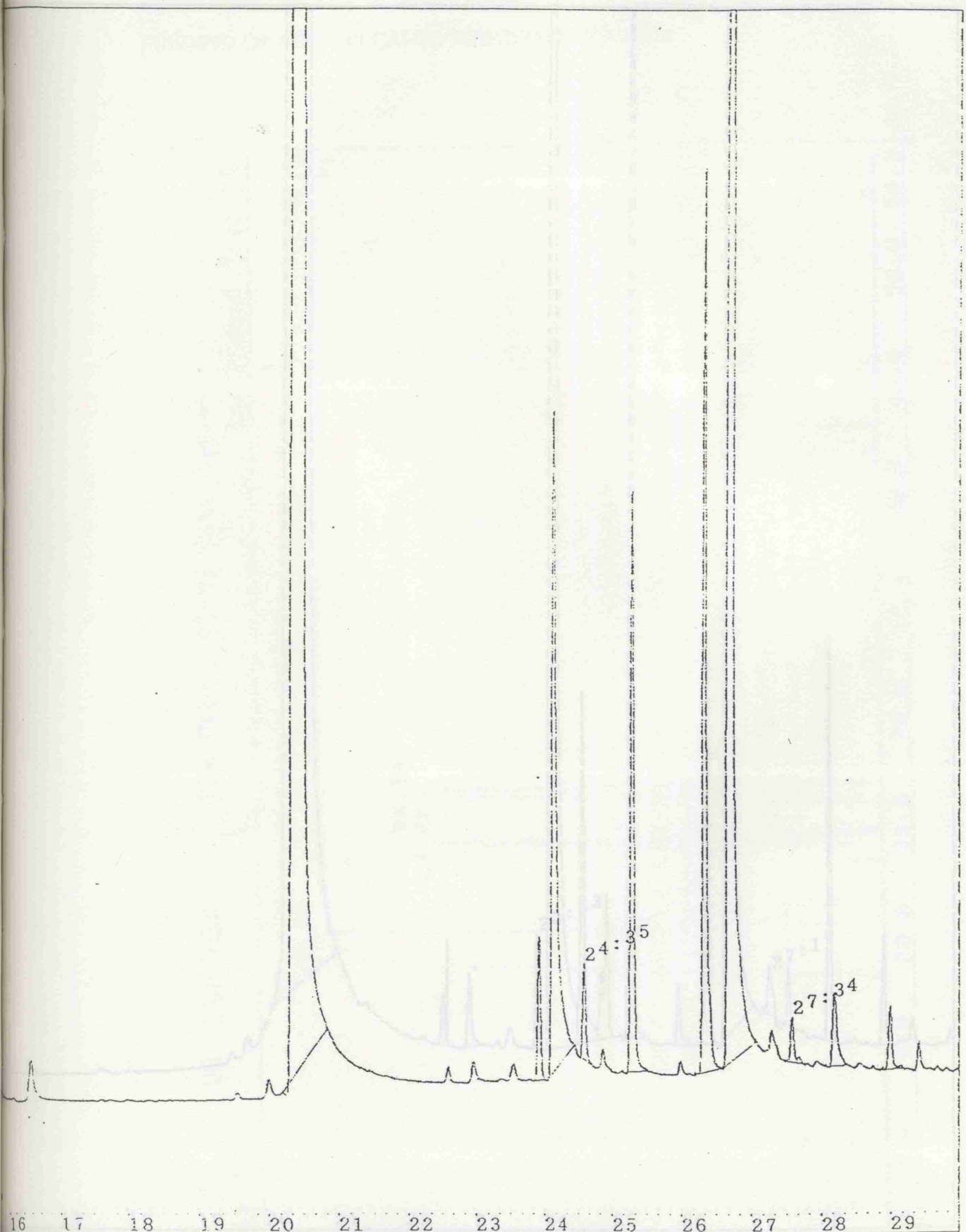
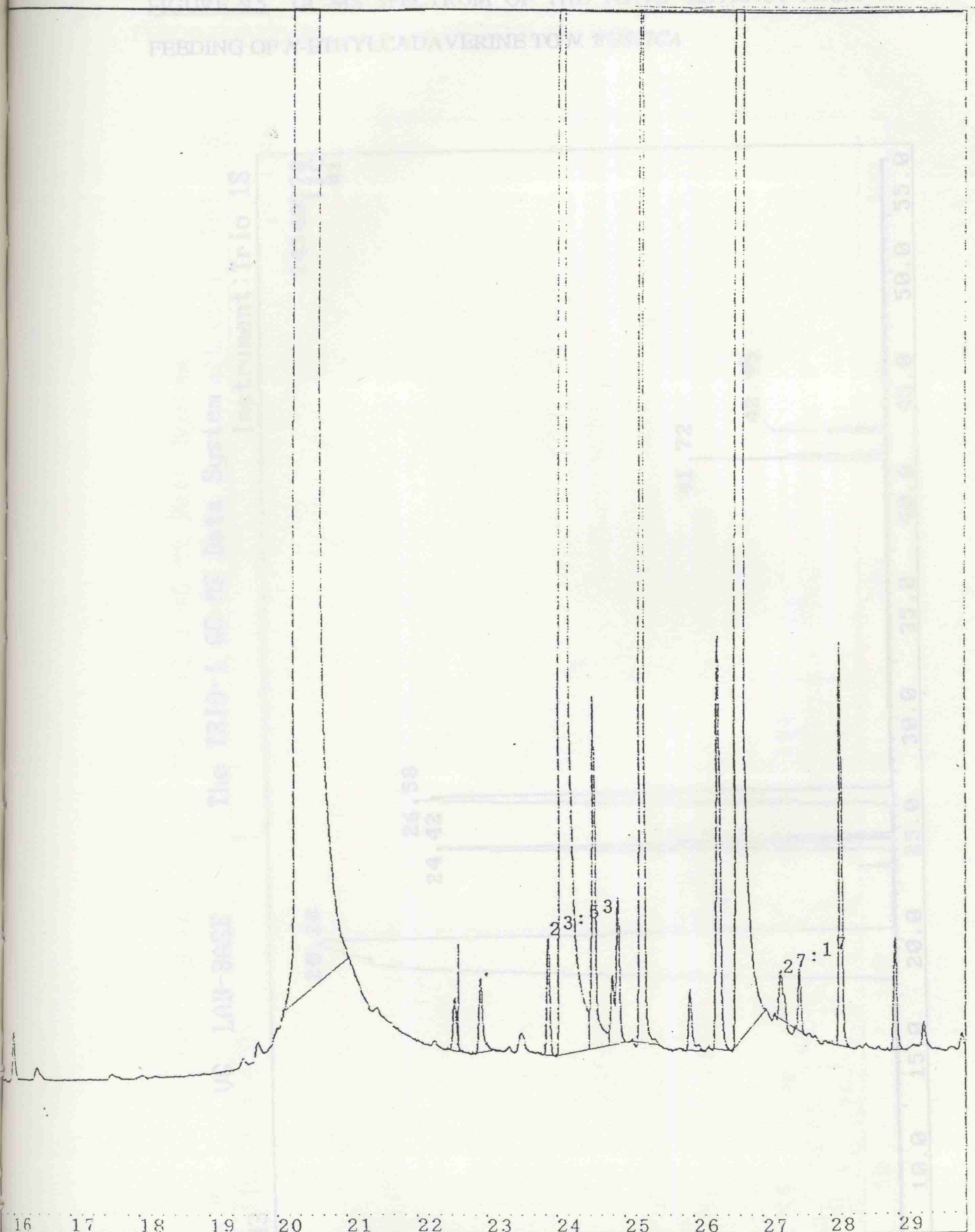


FIGURE 9.4 THE GC TRACE FOR THE CONTROL WITH *N.RUSTICA*



.....A:\hb2008
header..HBZ
method...c:\methods\tropanes
sequence.c:\methods\rjr
run ptr..8
comment..
method...c:\methods\tropanes

Data ID.....128CLFJ8WU2
Date acquired.Fri Mar 25 21:51:03 1994
Method ID.....116V4EEP1D4
Sequence ID...11JSHLGA0XP
Run time.....49:59
Study number..
Method ID.....12DDB0FOP29R

FIGURE 9.5 GC-MS SPECTRUM OF THE TOTAL EXTRACT FROM THE FEEDING OF *N*-ETHYLCADAVERINE TO *N. RUSTICA*

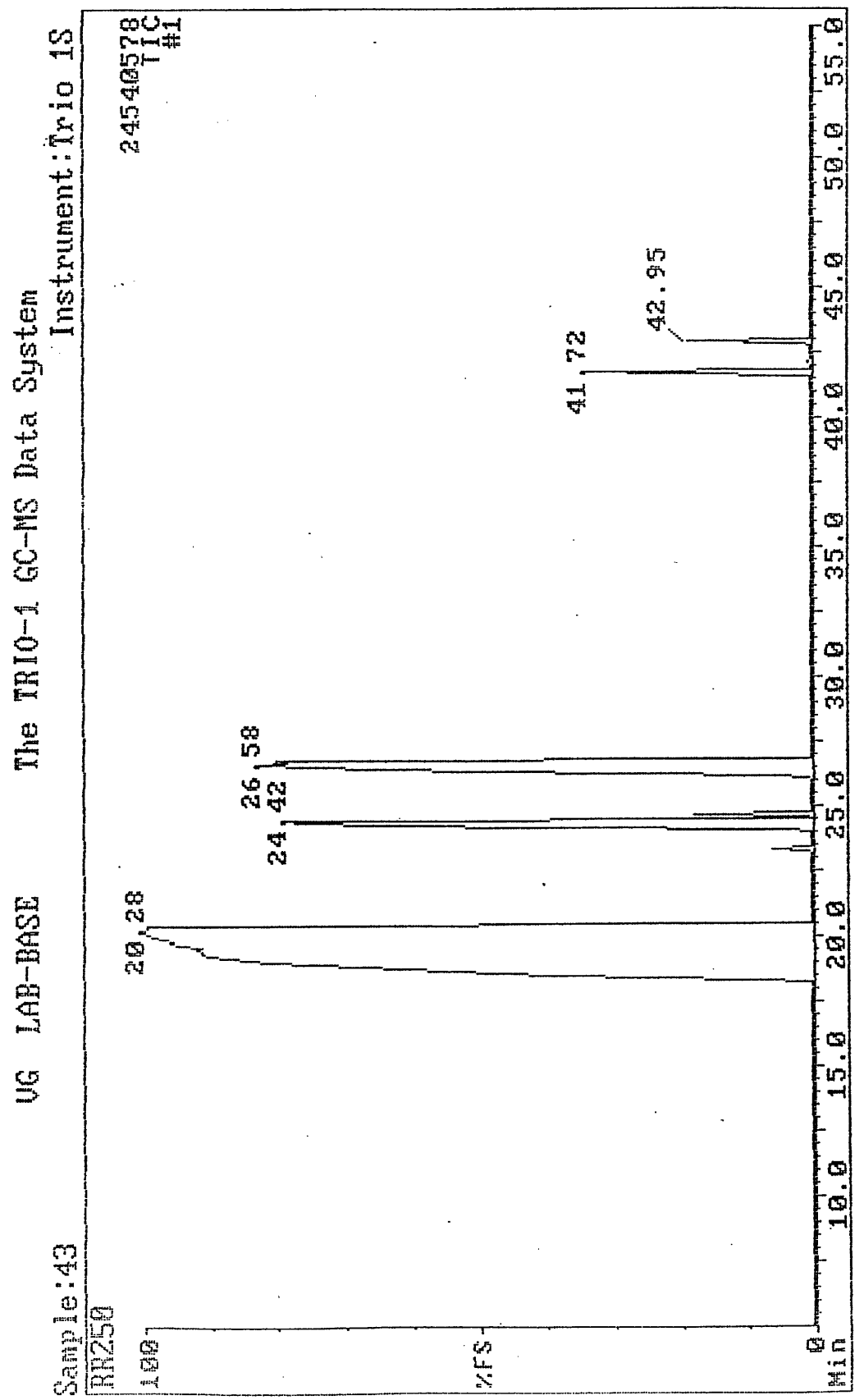


FIGURE 9.6 GC-MS SPECTRUM OF *N*-ETHYLANABASINE DERIVED FROM FEEDING *N*-ETHYLCADAVERINE TO *N. RUSTICA*

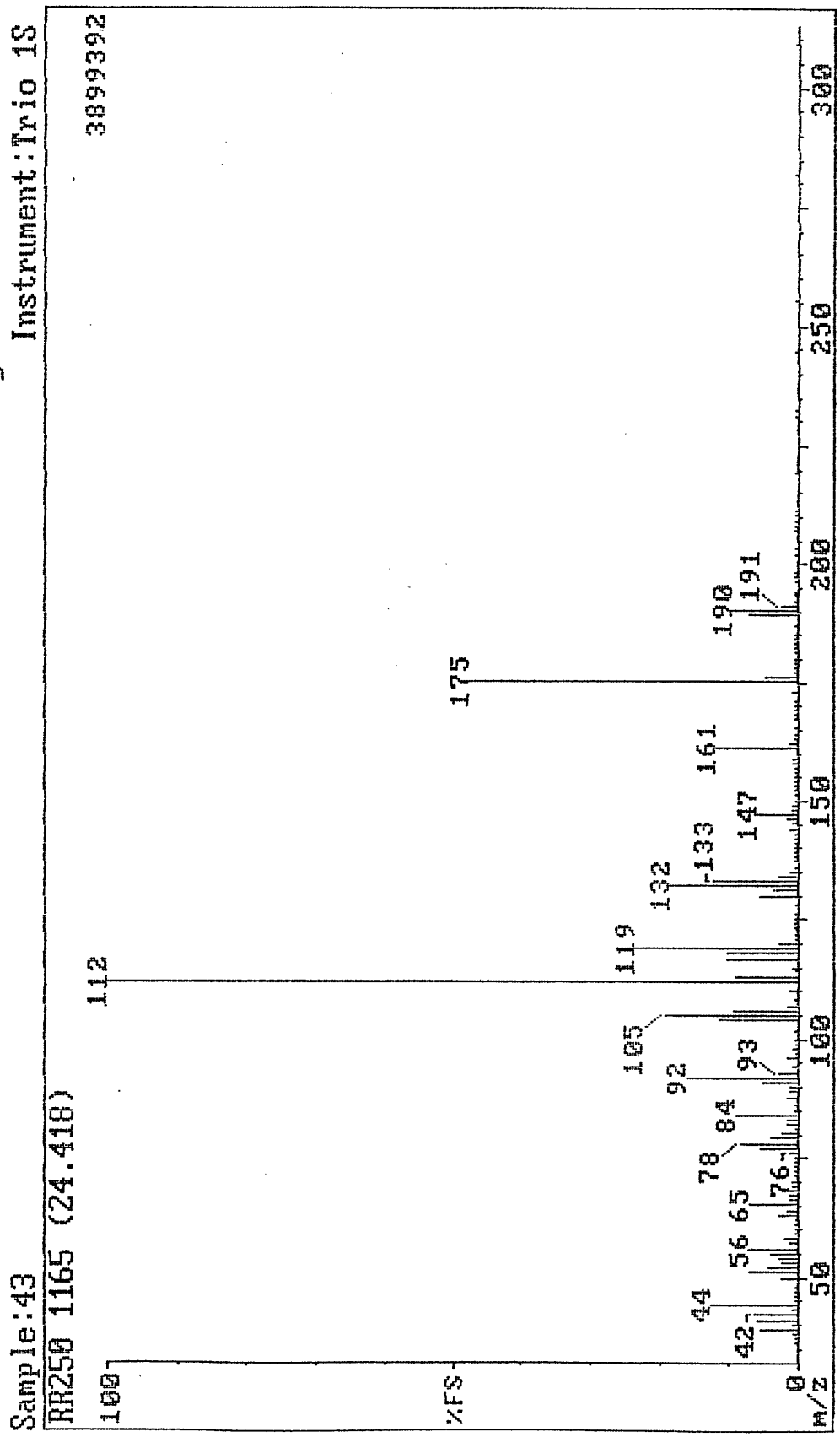


FIGURE 9.7 GC-MS SPECTRUM OF THE TOTAL EXTRACT FROM THE FEEDING OF *N*-PROPYLCADAVERINE TO *N. RUSTICA*

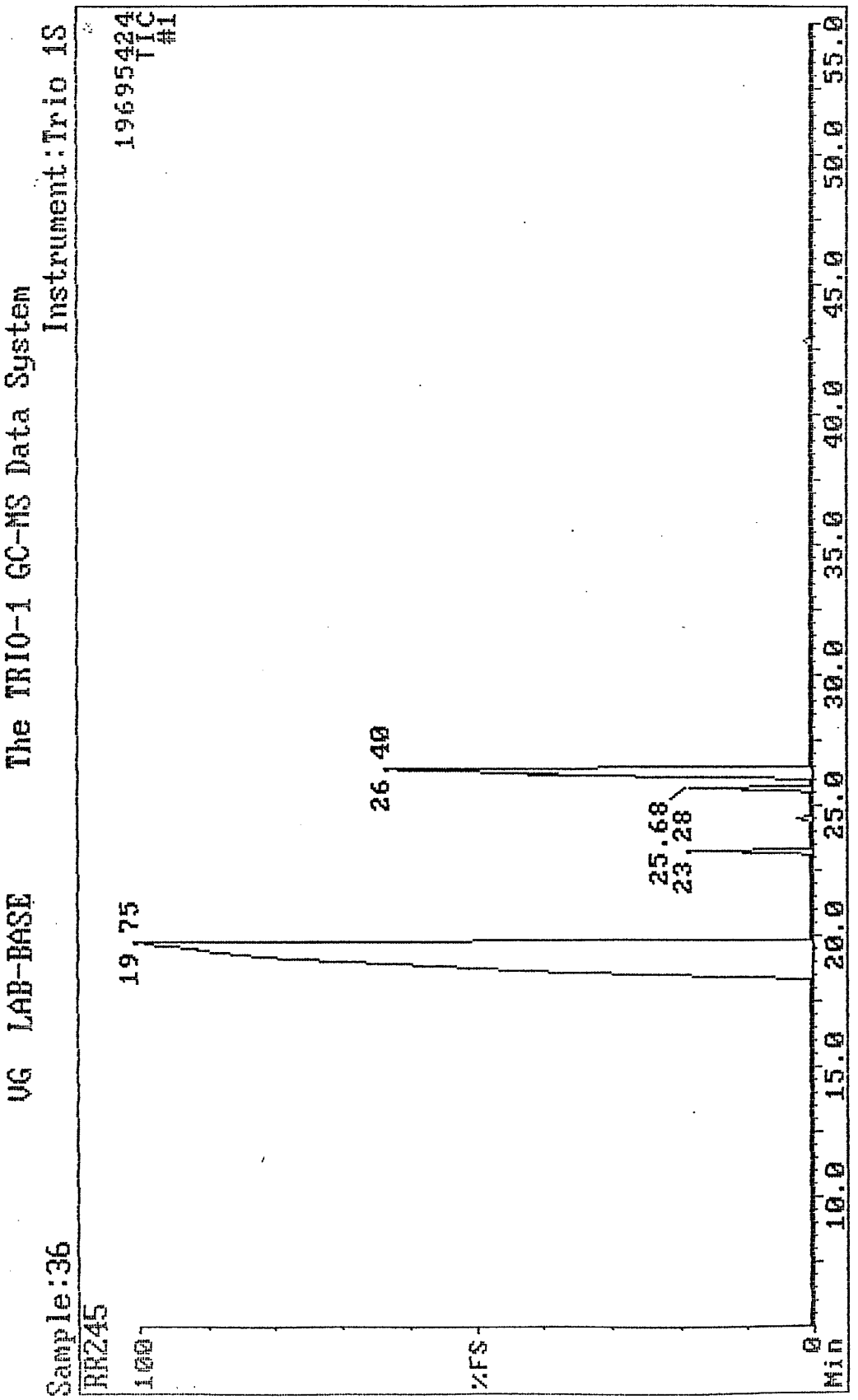
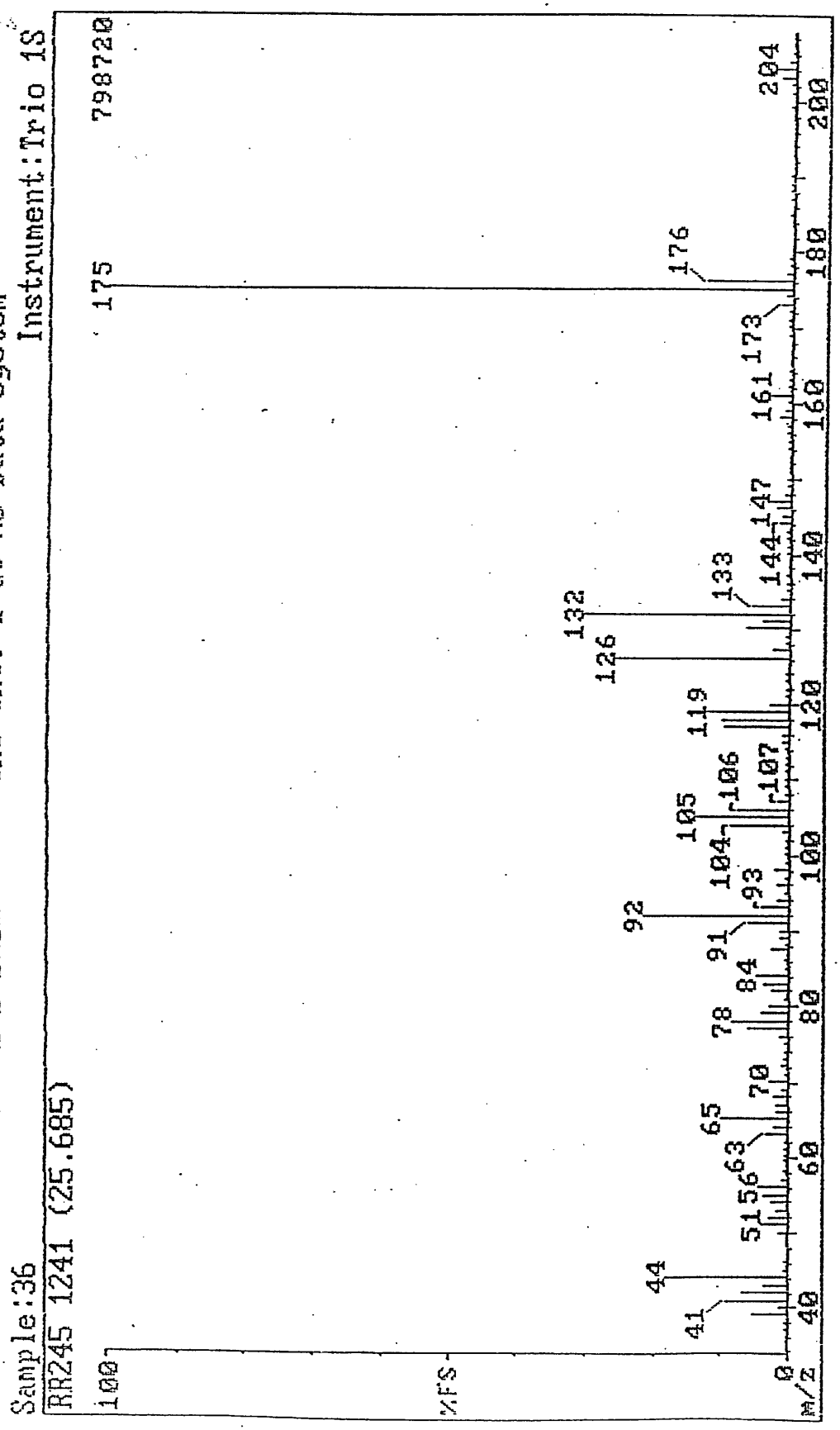


FIGURE 9.8 GC-MS SPECTRUM FOR *N*-PROPYLANABASINE DERIVED FROM FEEDING *N*-PROPYLCADAVERINE TO *N. RUSTICA*



CHAPTER TEN

EXPERIMENTAL

10.1 GENERAL

Melting points were measured on a Kofler hot-stage apparatus. Boiling points refer to the oven temperature using a Kugelrohr apparatus. Optical rotations were measured with an Optical Activity Ltd. AA 10 Polarimeter. Infra red spectra were obtained on a Perkin Elmer 500 spectrometer. Nuclear magnetic resonance spectra were recorded with a Perkin Elmer R 32 spectrophotometer operating at 90 MHz (δ_H), or a Bruker WP200-SY spectrophotometer operating at 200 MHz (δ_H), 50.3 (δ_C), or 30.72 MHz (δ_D). The multiplicities of the ^{13}C NMR spectra were determined using DEPT spectra with pulse angles of $\theta = 90^\circ$ and $\theta = 135^\circ$. Spectra were recorded with either tetramethylsilane at 0 p.p.m. or the NMR solvent as the internal standard. Mass spectra were obtained using A.E.I. MS 12 or 902 spectrometers. Elemental analyses were performed with a Carlo-Erba 1106 elemental analyser.

TLC was carried out on Merck Kieselgel G plates of 0.25 mm thickness in the solvent stated. The alkaloids were detected by the modified Dragendorff reagent.[159] Diamine dihydrochlorides were detected using ninhydrin and all other compounds by iodine. Chromatographic purification was carried out by dry column flash chromatography using Kieselgel 60 (Merck, 70-230 mesh).

Radiochemicals were purchased from Amersham International. Radioactivity was measured with a Philips PW 4700 Liquid Scintillation Counter using Ecoscint solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. Radioactive samples were counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for radioscanning of TLC. plates.

All solvents were purified by standard techniques.[168] Tetrahydrofuran (THF) and diethyl ether were dried by distillation from sodium - benzophenone under nitrogen immediately before use. Organic solvents were dried using either anhydrous

sodium sulphate or anhydrous magnesium sulphate and solvents were evaporated off under reduced pressure below 50 °C.

10.2 EXPERIMENTAL TO CHAPTER 3

(*R*)-[1-^{2}H]-1,5-Diaminopentane dihydrochloride (15)

The title compound was prepared by the method of Richards and Spenser.[43] employing enzymatic decarboxylation of L-lysine in D_2O by L-lysine decarboxylase. From ^{1}H n.m.r. spectroscopic data, the deuterium content was estimated to be *ca.* 88%.

(*S*)-[1-^{2}H]-1,5-Diaminopentane dihydrochloride (16)

The enantiomer was prepared by enzymatically decarboxylating DL-[2-^{2}H]lysine in water using L-lysine decarboxylase.[43] The ^{1}H n.m.r. spectrum was identical to that of (*R*)-[1-^{2}H]-1,5-diaminopentane dihydrochloride (15). The deuterium content was estimated to be *ca.* 83%.

Feeding Methods and Extraction of the Alkaloids

Sophora microphylla plants were grown in pots in standard compost in a greenhouse. Eight plants were used for most of the experiments. To each precursor was added a sample of ^{2}H-labelled cadaverine dihydrochloride, and the mixtures were divided into equal portions. Each portion was dissolved in sterile water and fed to the plants by the wick method, on alternate days for a period of 10-14 days. After a further period of 10-14 days, the plants were harvested, and the alkaloids were isolated by a standard method[65], described below.

Fresh leaves, stems and roots [43 g from (*S*)- precursor (16) and 72 g from the (*R*)- precursor (15)] of the plants were finely chopped and blended with methanol. The blended extracts were filtered and the methanolic filtrates were concentrated under reduced pressure. The resulting green residue was dissolved in

dichloromethane (100 ml) and extracted with 1.5 M sulphuric acid (3 x 100 ml). The combined aqueous solutions were cooled in ice-water and basified with concentrated ammonia solution. The resultant alkaline solution was extracted with dichloromethane (4 x 300 ml), and the combined extracts were dried, filtered and evaporated to dryness to leave a viscous oil (200 mg). Incorporation figures for each experiment are provided in the relative sections of Chapter Three.

The total alkaloidal mixtures from *S. microphylla* were separated using Kiesgel G silica plates of 0.25 mm thickness. The plates were developed in chloroform / methanol / ammonia (85:14:1) and visualized with u.v. or Dragendorff's reagent. Four bands were evident corresponding to the alkaloids matrine, anagyrine, *N*-methylcytisine and cytisine with the corresponding Rf values 0.88, 0.80, 0.74 and 0.53 (lit.[65] 0.88, 0.80, 0.75 and 0.53). The bands were scraped off and eluted with methanol containing a few drops of ammonia. The solutions were then filtered and evaporated to dryness to yield the isolated alkaloids, matrine, anagyrine, *N*-methylcytisine and cytisine, but in quantites too low to enable characterisation.

10.3 EXPERIMENTAL TO CHAPTER 4

10.3(a) Experimental for Schemes 30 and 31

(S)-(+)-5-Oxotetrahydrofuran-2-carboxylic acid (121)[90]

Sulphuric acid (1M, 120 ml, 0.12 mol) and a solution of sodium nitrite (16.8 g, 0.24 mol) in distilled water (120 ml) were added simultaneously and dropwise to a suspension of L-(+)-glutamic acid **(120)** (29.4 g, 0.2 mol) in distilled water (200 ml) over 1 h. A smooth evolution of nitrogen, accompanied by a trace of NO_2, was observed and after 2 h a clear, colourless solution resulted. Stirring was continued at room temperature for a further 16 h. The solution was then concentrated at <50 °C *in vacuo* to leave a sticky white solid. This was washed with boiling acetone (3 x 150 ml), and the combined washings were dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a viscous yellow oil, which was distilled in a Kugelrohr apparatus

connected to a high vacuum pump protected by a Dreschel bottle filled with soda lime. The product distilled as a clear colourless oil (190 °C, 0.3 mmHg) which solidified to a waxy solid on standing. This was then immediately recrystallised from ethanol-free chloroform to afford white, granular crystals of the lactone acid **(121)** (16.9 g, 65%); m.p. 72-73.5 °C; $[\alpha]_D^{24}$ +11.6° (c 5, MeOH); ν_{max}/cm^{-1} ($CHCl_3$) 3 500 - 2 400 (br), 1 792, 1 730 and 1 215; δ_H (200 MHz, d_6-acetone) 2.03 - 2.74 (4H, m), 4.99 - 5.05 (1H, m); δ_C (d_6-acetone) 26.33 (t), 27.19 (t), 76.09 (d), 171.81 (s, C=O lactone), 177.10 (s, C=O acid); *m/z* 130 (M^+, 0.7%) and 85 (100.0) [Found: M^+, 130.0269. $C_5H_6O_4$ requires M^+, 130.0266].

Ethyl (*S*)-(+)-2-oxotetrahydrofuran-2-carboxylate **(130)**[86]

To a solution of concentrated HCl (42 ml) in water (45 ml) was added (*S*)-glutamic acid (30 g; 0.2 mol). The resulting suspension was cooled to 0 °C and stirred vigorously. A solution of sodium nitrite (21 g, 0.2 mol) in water (8 ml) was added over 4 h at such a rate that the temperature of the reaction mixture did not exceed 5 °C. The clear solution thus obtained was stirred at room temperature overnight, then concentrated under high vacuum. Ethyl acetate (200 ml) was added to the residue, and the insoluble material was removed by filtration. The organic solution was dried ($MgSO_4$) and concentrated *in vacuo* to give crude *(S)*-(+)-5-oxotetrahydrofuran-2-carboxylic acid **(121)**. The crude product was dissolved in toluene (200 ml) and ethanol (87 ml), along with *p*-toluenesulphonic acid (1.3 g). The solution was heated to reflux in a Dean-Stark apparatus for 14 h, then concentrated *in vacuo*. Toluene (600 ml) was added to the residue, and the whole was washed with 10% aq. Na_2CO_3 solution (50 ml portions) until the washings were alkaline, then with saturated brine until the washings were neutral. The toluene solution was dried (Na_2SO_4) and concentrated *in vacuo*. Fractional distillation of the residue to remove diethyl 2-hydroxyglutarate **(133)** gave ethyl *(S)*-(+)-2-oxotetrahydrofuran-2-carboxylate **(130)** as a clear oil (17.94 g, 69%); b.p. 125-135 °C (1 mm Hg). $[\alpha]_D^{24}$ +15.3° (c 1.5, EtOH); ν_{max}/cm^{-1} (neat) 1780, 1745 and 1530 cm^-

^{1}; δ_H (200 MHz, $CDCl_3$) 1.29 (3H, t, *J* 7Hz, $CH_2\underline{CH_3}$), 2.30-2.65 (4H, m, $\underline{CH_2}$-$\underline{CH_2}$), 4.25 (2H, q, *J* 7Hz, $\underline{CH_2}CH_3$), 4.94 (1H, m, CH-O); δ_C ($CDCl_3$) 13.87 (q, $\underline{CH_3}CH_2$), 25.63 and 26.54 (2 x t, C-3 and C-4), 61.75 (t, $CH_3\underline{CH_2}O$), 75.57 (d, C-5), 169.72 (s, C=O ethyl ester) and 175.99 (s, C=O lactone); *m/z* 158 (M^+, 0.8%),86 and 85 (100.0) [Found: M^+, 158.0581. $C_7H_{10}O_4$ requires M^+, 158.0579].

(S)-Pentane-1,2,5-triol **(123)**[78]

A solution of *(S)*-(+)-5-oxotetrahydrofuran-2-carboxylic acid **(121)** (0.98 g, 7.5 mmol) in anhydrous THF (25 ml) was added dropwise to a stirred suspension of lithium aluminium hydride (0.95 g, 25 mmol) in anhydrous THF (25 ml) at room temperature under an atmosphere of dry nitrogen. When addition was complete, the mixture was brought to reflux and heating continued for 3 days. The mixture was allowed to cool, and the reaction mixture was then subjected to one of the following work-up procedures:

Procedure A : Saturated Na_2SO_4 solution (1.1 ml) was cautiously added with vigorous stirring under nitrogen. Solid anhydrous Na_2SO_4 (1.1 g) was then added, and the mixture again brought to reflux for 3 h. The mixture was filtered hot through a small pad of Celite and the solids were washed with boiling THF (3 x 15 ml). The combined organic phases were concentrated *in vacuo* to give a pale yellow oil which was distilled on a Kugelrohr apparatus to afford the triol **(123)** as a clear, colourless, viscous oil (0.10 g, 10%); b.p. 160 °C (0.15 mm Hg); $[\alpha]_D^{21}$ -21.5° (c 5.4, abs. EtOH); ν_{max}/cm^{-1} ($CHCl_3$) 3 420 (br), 3 010, 2 950, 2 890, 2 510 (br); δ_H (200 MHz, D_2O) 0.72 - 1.68 (5H, m), 3.16 - 3.80 (4H, m); δ_C (D_2O) 29.57 (t), 31.86 (t), 62.49 (t), 66.20 (t), 72.41 (d); *m/z* 90 (M^+ - CH_2O, 6.6 %), 89 and 71 (100.0) [Found: M^+ - CH_2O, 90.0670. $C_5H_{12}O_3$ - CH_2O requires M^+, 90.0681].

Procedure B : Saturated Na_2SO_4 solution (1.1 ml) was cautiously added with vigorous stirring under nitrogen. The reaction mixture was stirred at room

temperature for 1 h, and then the mixture was filtered hot through a small pad of Celite. The solids were broken down using HCl and continuously extracted with EtOAc for 48 h. The EtOAc was then evaporated to dryness *in vacuo*, to yield an intractable black tar and acetic acid.

Procedure C : Saturated Na_2SO_4 solution (1.1 ml) was cautiously added with vigorous stirring under nitrogen. The reaction mixture was stirred at room temperature for 30 min, and then Amberlite IR-120 ion exchange resin (H^+ form) was added (1.0 g). The reaction flask was fitted with a silica gel drying tube, and the reaction mixture was stirred overnight. The reaction mixture was then filtered and evaporated *in vacuo* to give only a slight trace of the desired triol product.

Procedure D : Sufficient water was carefully added to the reaction mixture to destroy unreacted metal hydride. The reaction mixture was then evaporated to dryness *in vacuo*, and the resulting yellow solid was repeatedly washed with pyridine until the washings were colourless. The combined washings were then evaporated *in vacuo* and the residue distilled to give the desired triol product as a thick, pale yellow oil (33%); b.p. 160 °C (0.15 mm Hg). This compound showed identical spectroscopic behaviour to an authentic sample

(S)-Pentane-1,2,5-triol **(123)** [From **(130)**][86]

A solution of ethyl *(S)*-(+)-2-oxotetrahydrofuran-2-carboxylate **(130)** (1.0 g, 6.3 mmol) in anhydrous THF (25 ml) was added dropwise to a stirred suspension of lithium aluminium hydride (0.95 g, 25.0 mmol) in anhydrous THF (25 ml) at room temperature under an atmosphere of dry nitrogen. When addition was complete, the mixture was brought to reflux and heating continued for 3 days. The mixture was allowed to cool, and the reaction mixture was then subjected to one of the above work-up procedures to give a pale yellow oil (procedure A - 18%), (procedure D -

32%); b.p. 160 °C (0.15 mm Hg). This compound showed identical spectroscopic behaviour to an authentic sample.

(S)-5-(Hydroxymethyl)tetrahydrofuran-2-one **(134)**[76]

To a solution of *(S)*-5-oxotetrahydrofuran-2-carboxylic acid **(121)** (5.8 g, 45 mmol) in dry THF (27 ml) was added borane-dimethyl sulphide complex (4.7 ml, 47 mmol) over 40 min. During the addition, the temperature of the reaction mixture was kept below 5 °C by ice-water cooling. After addition was complete, the reaction mixture was stirred at room temperature for an additional 2 h, and then quenched by the careful addition of methanol (8.9 ml). The solvent was removed *in vacuo*, methanol (4.5 ml) was added to the residue and the resulting solution was again evaporated *in vacuo*. This procedure was repeated 3 times in order to remove any trimethyl borate formed. The residue was dried by co-evaporation with toluene to afford crude *(S)*-5-(hydroxymethyl)tetrahydrofuran-2-one **(134)**, which was used without further purification (5.17 g, 100%); ν_{max}/cm^{-1} ($CHCl_3$) 3 470, 2 935, 1 770 and 1 210; δ_H 2.2-2.9 (4H, m), 3.8-4.1 (2H, m), 5.0 (1H, m), 5.2 (1h, br s); *m/z* 117 (M^+, 1.7%), 99 (6.1), 85 (100.0), 68 (3.1) [Found: M^+, 117.0562. $C_5H_9O_3$ requires M^+, 117.0551].

(S)-5-(*t*-Butyldimethylsilyloxymethyl)tetrahydrofuran-2-one **(135)**[76]

This compound was prepared by one of two procedures:

Procedure A: To a mixture of crude *(S)*-5-(hydroxymethyl)tetrahydrofuran-2-one **(134)** (5.9 g, 0.05 mol) and imidazole (4.4 g, 0.07 mol) in CH_2Cl_2 (25 ml), cooled to 0-5 °C, was added *t*-butyldimethylsilyl chloride (9.85 g, 0.06 mol). The mixture was stirred at 0 °C for 15 min, and then at room temperature for 2 h. The mixture was then poured into water and extracted with CH_2Cl_2. The organic layer was washed with brine, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was then distilled under reduced pressure to give only starting material **(134)** and *t*-butyldimethyl silanol.

Procedure B: To a solution of lactone alcohol **(134)** (2.0 g, 0.02 mmol), triethylamine (1.3 ml, 9.2 mmol) and 4-dimethylaminopyridine (40 mg, cat.) in dry CH_2Cl_2 (20 ml), cooled under N_2 in an ice bath, was added a solution of *t*-butyldimethylsilyl chloride (3.07 g, 19.7 mmol) in dry acetonitrile (1 ml). The reaction mixture was allowed to warm gradually to room temperature, and stirring was continued overnight. The reaction mixture was poured into water (30 ml) and extracted with CH_2Cl_2 (3 x 50 ml). The combined organic phases were dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a clear oil, which was distilled under reduced pressure to yield *(S)*-5-(*t*-butyldimethylsilyloxymethyl)tetrahydrofuran-2-one **(135)** (3.3 g, 88%); b.p. 88-92 °C (0.05 mm Hg); $[\alpha]_D^{24}$ +11.1° (c 0.92, $CHCl_3$); ν_{max}/cm^{-1} ($CHCl_3$) 1770; d_H (200 MHz, $CDCl_3$) 0.06 (6H, s), 0.88 (9H, s), 2.17 (1H, m), 2.25 (1H, m), 2.46 (1H, m), 3.86 (1H, dd, *J* 10.5 Hz and 2.5 Hz), 4.58 (1H, m), 3.67 (1H, dd, *J* 10.5 Hz and 2.5 Hz); δ_C ($CDCl_3$) -3.79 (q), -3.04 (q), 17.89 (s), 25.59 (q), 25.71 (t), 26.67 (t), 52.73 (t), 75.39 (d), 176.25 (s); *m/z* 173 (M^+ - C_4H_2, 0.1%) and 85 (100.0) [Found: C, 57.4%; H, 10.0; Si, 12.5. $C_{11}H_{22}O_3Si$ requires C, 57.4; H, 9.6; Si ,12.2%].

(S)-1-(*t*-Butyldimethylsilyloxy)pentane-2,5-diol **(136)** (BH_3/DMS procedure)

To a stirred solution of borane-dimethyl sulphide complex (2M, 0.73 ml, 0.015 mmol) in dry THF (10 ml), under an atmosphere of N_2, was added *(S)*-5-(*t*-butyldimethylsilyloxymethyl)tetrahydrofuran-2-one (135) (2.0 g, 8.7 mmol). Once addition was complete, the reaction mixture was heated to reflux and dimethyl sulphide was collected as it distilled through a Vigreux column. When no more DMS distilled, the reaction mixture was allowed to cool to room temperature. Water (5 ml) was added, followed by anhydrous K_2CO_3 (0.6 g). The resulting mixture was stirred at room temperature for 30 min, after which time the aqueous phase was saturated with K_2CO_3. Ether (10 ml) was added, the organic phase was separated, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give no trace of the desired product.

(S)-1-(*t*-Butyldimethylsilyloxy)pentane-2,5-diol (**136**) (LAH procedure)

To a stirred suspension of lithium aluminium hydride (LAH) (0.66 g, 17.4 mmol) in dry THF (30 ml), in a nitrogen atmosphere, was added carefully a solution of *(S)*-5-(*t*-butyldimethylsilyloxymethyl)tetrahydrofuran-2-one (**135**) (2.0 g, 8.7 mmol) in THF (2 ml). The resulting mixture was heated to reflux for 48 h. ^1H N.m.r. spectroscopic analysis of the crude reaction mixture showed that reduction had occurred. However, standard aqueous work-up of the reaction mixture failed to yield any of the desired product, no doubt due to the polar nature of the diol product.

(S)-Pentane-1,2,5-triol (**123**) (One-pot procedure)

To a stirred solution of *(S)*-5-oxotetrahydrofuran-2-carboxylic acid (**121**) (4 g) in dry THF (22 ml), under an N_2 atmosphere, was added dropwise borane-dimethyl sulphide (2 M, 4.5 ml 0.09 mmol). During the addition, the temperature of the reaction mixture was maintained below 5 °C by means of an ice-bath. Once addition was complete, the reaction mixture was allowed to warm to room temperature, and stirring was continued for 2 h. The reaction mixture was then heated to reflux, and dimethyl sulphide was distilled off through a Vigreux column and collected. When distillation of DMS had ceased, the reaction mixture was allowed to cool to room temperature. Water (17 ml) was added, followed by anhydrous K_2CO_3 (3 g). The reaction mixture was stirred for 30 min at room temperature, and the aqueous phase was then saturated with K_2CO_3. Ether (11 ml) was added, the organic phase was separated and fractionally distilled to give an intractable mixture of compounds, of which (**134**) was the only recognisable component.

(S)-Pentane-1,2,5-triol (**123**) (LAH procedure)

A mixture of lithium aluminium hydride (1.2 g) and crude *(S)*-5-(hydroxymethyl)tetrahydrofuran-2-one (**134**) (1.60 g) in dry THF (50 ml) was heated to reflux under an N_2 atmosphere for 48 h. The reaction mixture was cooled to room temperature, and sufficient water was added carefully to destroy unreacted metal

hydride. The reaction mixture was then evaporated to dryness *in vacuo*, and the resulting yellow solid was repeatedly washed with pyridine until the washings were colourless. The combined washings were then evaporated *in vacuo*, and the residue distilled to give the desired product as a thick, pale yellow oil (0.50 g, 40%); b.p. 160 °C (0.15 mm Hg); $[\alpha]_D^{24}$ -20.4° (c 5, EtOH) [lit.[87] $[\alpha]_D^{24}$ -19.6° (c 5, EtOH)]. This compound displayed identical spectroscopic behaviour to the previously described samples.

(S)-(-)-1-*O*, 5-*O*-Bis(*t*-butyldimethylsilyl)pentane-1,2,5-triol **(124)**[78]

To a stirred solution of *t*-butyldimethylsilyl chloride (6.0 g, 40 mmol), triethylamine (4.0 ml, 40 mmol) and 4-dimethylaminopyridine (120 mg, 1 mmol) in dry CH_2Cl_2 (90 ml), under N_2, was added dropwise a solution of *(S)*-pentane-1,2,5-triol (2.14 g, 18 mmol) in dry acetonitrile (3 ml). The reaction mixture was stirred at room temperature for 8 h, and then washed sequentially with water (50 ml), sat. NH_4Cl (2 x 50 ml) and brine (50 ml). The organic phase was dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a yellow oil. This was subjected to column chromatography, eluting with ether-light petroleum (1:4), to give the desired product as a clear, colourless mobile oil (1.39 g, 20%); $[\alpha]_D^{21}$ -8.1° (c 20.6, MeOH); ν_{max}/cm^{-1} ($CHCl_3$) 3 400 (br); δ_H (200 MHz, $CDCl_3$) 0.0 (12H, s), 0.81 (18H, s), 1.2-1.7 (4H, m), 2.7 (1H, br s), 2.9-3.8 (5H, m); δ_C ($CDCl_3$) -5.43 (q), -3.62 (q), 17.98 (s), 18.24 (s), 25.65 (q), 25.81 (q), 28.83 (t), 29.62 (t), 63.18 (t), 67.09 (t), 71.63 (d); *m/z* 348 (M^+, 0.4%), 347 (1.7) and 132 (100.0) [Found: M^+, 348.2169. $C_{17}H_{40}O_3Si_2$ requires M^+, 348.2518].

(S)-(-)-1-*O*, 5-*O*-Bis(*t*-butyldimethylsilyl)-2-*O*-(methanesulphonyl)pentane-1,2,5-triol **(125)**[78]

To a stirred solution of *(S)*-(-)-1-*O*, 5-*O*-bis(*t*-butyldimethylsilyl)pentane-1,2,5-triol **(124)** (1.25 g, 3.65 mmol) and triethylamine (0.45 ml, 3.25 mmol) in dry CH_2Cl_2 (20 ml), in an argon atmosphere, was added methanesulphonyl chloride

(0.49 g, 4.3 mmol). The reaction mixture was stirred at room temperature for 3 h, then washed with water (7 ml) and brine (2 x 7 ml). The organic phase was then dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a yellow oil, which was further purified by column chromatography (Florisil, ether-light petroleum (1:4) to give the desired product as a clear colourless oil which was unstable to prolonged storage (0.57 g, 37%); $[\alpha]_D^{20}$ -2.6° (c 52, MeOH); δ_H ($CDCl_3$) 0.09 (6H, s), 0.14 (6H, s), 0.98 (18H, 2 x s), 1.5-1.9 (4H, m), 3.31 (3H, s), 3.80 (4H, m), 4.39 (1H, m).

(R)-[2-^{2}H]-Pentane-1,5-diol **(126)**[78]

To a suspension of lithium aluminium deuteride (0.06 g, 1.50 mmol) in dry Et_2O (2.5 ml), under N_2, was added dropwise a solution of the disilyl mesylate **(125)** (0.45 g, 1.1 mmol) in dry Et_2O (2.5 ml). The reaction mixture was stirred at room temperature for 24 h, and then quenched by the addition of sat. Na_2SO_4 solution (0.1 ml). The reaction mixture was stirred at room temperature for 10 min, and then a further 0.1 g of anhydrous Na_2SO_4 was added. The reaction mixture was filtered through a short pad of Celite, and the solids were washed with ether (3 x 5 ml). The combined ethereal layers were evaporated *in vacuo* to give no trace of the desired product.

1-*O*, 5-*O*-Bis(*t*-butyldimethylsilyl)pentane-1,5-diol **(137)**[78]

Pentane-1,5-diol (1.04 g, 10 mmol), triethylamine (2.2 ml, 22 mmol), 4-dimethylaminopyridine (70 mg) and *t*-butyldimethylsilyl chloride (3.32 g, 22 mmol) were reacted together in dry CH_2Cl_2 (50 ml) and dry acetonitrile (1.3 ml) according to the procedure described for compound **(124)** to give, after work-up, the di-TBDMS protected diol **(137)** (2.7 g, 82%); δ_H (90 MHz, $CDCl_3$) 0.05 (12H, S), 0.9 (18H, S), 1.3 - 1.7 (6H, m), 3.6 (4H, m).

Pentane-1,5-diol **(139)** ($FeCl_3/Ac_2O$ procedure)[91]

Anhydrous iron (III) chloride (0.117 g, 0.7 mmol) was added to an ice-cold stirred suspension of 1-*O*, 5-*O*-bis(*t*-butyldimethylsilyl)pentane-1,5-diol **(137)** (0.8 g, 2.4 mmol) in acetic anhydride (5.5 ml), under an argon atmosphere. After stirring for 15 min, the resulting deep red solution was extracted with light petrol (3 x 7 ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a clear, colourless oil with a fruity aroma. This was subjected to flash column chromatography (Et_2O-light petrol 1:4) to give 1-*O*, 5-*O*-diacetyl-1,5-pentanediol **(138)** as a clear colourless oil (0.36 g, 70%) υ_{max} ($CHCl_3$) 3 000, 1 720, 1 250 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.31-1.58 (6H, m), 2.00 (6H, s), 3.19 - 4.10 (4H, m); *m/z* 174 ($M^+ - CH_3$, 0.2%), 126, 114, 71 (100.0), 59; [Found: $M^+ - CH_3$, 174.0882. $C_8H_{14}O_4$ requires $M^+ - CH_3$ 174.0892]

This diacetate was stirred overnight in 1M HCl (4.3 ml) and then concentrated *in vacuo* to afford an oil, which was distilled in a Kugelrohr apparatus to give pentane-1,5-diol as a clear, colourless oil (0.18 g, 75%), whose spectroscopic behaviour was identical in all respects to an authentic sample.

Pentane-1,5-diol **(139)** (TBAF procedure)

To a stirred solution of 1-*O*, 5-*O*-bis(*t*-butyldimethylsilyl)pentane-1,5-diol **(137)** (1.00 g) in dry THF (6 ml), in a N_2 atmosphere, was added a 1.0M solution of tetrabutylammonium fluoride (TBAF) in THF (1.74 ml, 2 equiv.). The reaction mixture was stirred at room temperature and monitored periodically by t.l.c. (Et_2O-pet. ether, 1:4). When no more starting material was evident by t.l.c. analysis (20 h), the reaction mixture was washed with saturated brine solution (5 ml), dried ($MgSO_4$), filtered and evaporated *in vacuo* to give the crude product. This was further purified by column chromatography to give 1,5-pentanediol as a clear, colourless oil (0.33 g, 91%) whose spectroscopic behaviour was identical in all respects to an authentic sample.

Preparation of Hydrazoic Acid[89]

Sodium azide (32.5 g, 1 equiv.) and water (32.5 ml) were mixed to a paste, and benzene (200 ml) was added with stirring. The reaction mixture was cooled to <10 °C, and conc. H_2SO_4 (13.4 ml, 0.5 equiv.) was added dropwise, maintaining the temperature of the reaction mixture below 10 °C. The mixture was then cooled to 0 °C and the organic layer was decanted and dried with anhydrous sodium sulphate. The reaction mixture was then filtered and titrated with 1.0M NaOH solution. The resulting benzene solution of hydrazoic acid was sealed and stored at 0-5 °C, at which temperature it was stable for several days.

Cadaverine dihydrochloride (19) (Method 1)[88]

To a solution of pentane-1,5-diol (734 mg, 7.00 mmol) in anhydrous THF (30 ml) was added hydrazoic acid (0.96M in benzene, 17.5 ml, 16.8 mmol). Di-isopropylazodicarboxylate (DPAD) (3.11 g, 14.4 mmol) was then added, followed by the slow addition of a solution of triphenylphosphine (8.80 g, 33.6 mmol) in anhydrous THF (70 ml). During the first half of this addition the orange colour of the DPAD was discharged, the evolution of gas was observed and the temperature of the mixture rose to 30 °C. After addition was complete, the mixture was stirred at room temperature for 1 h and then at 50 °C for a further 3 h. During this time a yellow, oily substance was deposited on the walls of the flask. Water (2.5 ml) was added, and stirring was continued for 3 h at 50 °C. The reaction mixture was then concentrated *in vacuo* at <40 °C to give a thick yellow oil. This residue was partitioned between CH_2Cl_2 (50 ml) and 1M HCl (50 ml). The aqueous fraction was separated, washed thoroughly with CH_2Cl_2 (4 x 30 ml) and then concentrated *in vacuo* to leave a semi-solid residue. After drying under vacuum over P_2O_5 for 3 h, the solid was recrystallised from 96% EtOH to afford cadaverine dihydrochloride as an off-white powdery solid (674 mg, 55%); *m/z* 103 (M^+ - HCl_2, 0.1%), 85 (5.6) , 56 (21.7) and 30 (100.0). This compound displayed identical spectroscopic behaviour to an authentic sample of cadaverine dihydrochloride.

1,5-Dibromopentane **(140)** (Method 2)

Pentane-1,5-diol (5.0 g) was added slowly to ice-cold PBr_3 (4.3 ml) , giving a dark brown solution which was stirred overnight (15 h). Ice-water (25 ml) was added slowly to hydrolyse the excess PBr_3, and the organic layer was separated. To the organic layer was added ether (50 ml), and the resulting solution was washed with $NaHCO_3$ solution (3 x 10 ml) and water (10 ml). The organic layer was then dried (Na_2SO_4), filtered and evaporated *in vacuo* to give 1,5-dibromopentane as a yellow oil (5.43 g, 52%). This compound gave identical spectroscopic data to an authentic sample.

1,5-Diphthalimidopentane **(141)**

To a solution of 1,5-dibromopentane (3 g) in DMF (75 ml) was added potassium phthalimide (5.25 g), and the resulting solution was stirred at room temperature for 48 h. The reaction mixture was then poured onto water (75 ml) and extracted with CH_2Cl_2 (5 x 150 ml). The combined organics were concentrated *in vacuo* to leave a residue, from which the product crystallised on standing. The product was isolated by filtration and washed with Et_2O (10 ml) to remove the last traces of DMF and gave 1,5-diphthalimidopentane as a fine white solid (3.79 g, 80%); ν_{max}/cm^{-1} (KBr) 2 970, 2 951, 1 773, 1 727; δ_H (200 MHz, $CDCl_3$) 1.17-1.38 (2H, m), 1.57-1.77 (4H, m), 3.59 (4H, t, *J* 7.1 Hz), 7.53-7.77 (8H, m); δ_C ($CDCl_3$) 23.99 (t), 28.04 (t), 37.58 (t), 123.10 (d), 132.03 (s), 133.80 (d), 168.33 (s); *m/z* 362 (M^+, 43.0%), 229 (2.1), 162 (4.0) and 160 (100.0) [Found: C, 69.5%; H, 5.0; N, 7.7; M^+, 362.1259. $C_{21}H_{18}N_2O_4$ requires C, 69.6; H, 5.0; N, 7.7%; M^+, 362.1266].

Cadaverine dihydrochloride **(19)**

To a mixture of conc. HCl (20 ml) and glacial acetic acid (20 ml) was added 1,5-diphthalimidopentane (2 g). The reaction mixture was heated to reflux until all the phthalimide had dissolved. Heating was continued at 140 °C for 24 h. The

the phthalimide had dissolved. Heating was continued at 140 °C for 24 h. The resulting solution was then cooled to room temperature, and the precipitated phthalic acid was removed by filtration and washed with glacial acetic acid (2 x 10 ml). The combined washings and filtrate were reduced in volume *in vacuo* to ~10 ml and cooled in an ice bath. The resulting precipitate of phthalic acid was removed by filtration and washed with glacial acetic acid (2 x 10 ml). The combined washings and filtrate were again reduced in volume *in vacuo*, cooled and filtered. Ether (10 ml) was added to the filtrate, and the cadaverine dihydrochloride which precipitated was collected by filtration (0.68 g, 88.6%). This compound displayed identical spectroscopic behaviour to an authentic sample of cadaverine dihydrochloride.

1,5-Diphthalimidopentane **(141)** (Mitsunobu procedure)

In a N_2 atmosphere, diethyl azodicarboxylate (DEAD) (1.6 ml, 10 mmol) was added over 5 min to an ice-cold solution of pentane-1,5-diol (0.52 g, 5 mmol), triphenylphosphine (2.2 g, 10 mmol) and phthalimide (1.47 g, 10 mmol) in dry THF (50 ml). The solution was allowed to warm to room termperature and stirred for 12 h. When the reaction was complete, the solution was concentrated *in vacuo*, diluted with ethyl acetate (200 ml) and washed with brine (150 ml). The combined aqueous washings were extracted with ethyl acetate (3 x 100 ml). The combined organic phases were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (ethyl acetate-hexane, 2:8) to give 1,5-diphthalimidopentane (0.09 g, 50%). This compound gave identical spectroscopic data to a sample prepared *via* the dibromide.

(S)-Pentane-1,2,5-triol **(123)** (DIBAL procedure)

A solution of di-isobutylaluminium hydride (DIBAL) in CH_2Cl_2 (1.0M, 70 ml) was added, with stirring and cooling, to a solution of ethyl *(S)*-5-

oxotetrahydrofuran-2-carboxylate **(130)** (2.125 g, 0.01 mmol) in dry CH_2Cl_2 (18 ml). The reaction mixture was stirred at room temperature for 1 h, and then quenched by the addition of EtOAc (9 ml). The reaction mixture was stirred for 15 min, after which time sat. Na_2SO_4 solution was added dropwise until a precipitate appeared. This precipitate was removed by filtration and washed with several portions of CH_2Cl_2. The combined filtrate and washings were then dried ($MgSO_4$), filtered and evaporated *in vacuo* to give an oil. This was fractionally distilled to give the desired triol product as a clear oil (0.48 g, 30%). This compound displayed identical spectroscopic behaviour to an authentic sample of cadaverine dihydrochloride.

(S)-Pentane-1,2,5-triol **(123)** (Gani's procedure)[85]

A solution of *(S)*-(+)-5-oxotetrahydrofuran-2-carboxylic acid **(121)** (2.0 g, 15.38 mmol) in anhydrous THF (20 ml) was added dropwise to a stirred suspension of lithium aluminium hydride (1.17 g, 30.8 mmol) in anhydrous THF (20 ml) at room temperature under an atmosphere of dry nitrogen. When addition was complete, the mixture was stirred at room temperature for 1 h. Water (3 ml) in THF (15ml) was cautiously added with vigorous stirring under nitrogen. The reaction mixture was stirred at room temperature for 30 min. The resulting mixture was then filtered and the residue repeatedly dissolved in acetone (50ml), refiltered and evaporated *in vacuo* and distilled to give triol **(123)** (25%) which showed identical spectroscopic behaviour to a previously prepared sample.

10.3(b) Experimental for Schemes 39 - 42

(S)-N,N-Dibenzyl-5-oxotetrahydrofuran-2-carboxamide **(144)**

A solution of the lactonic acid **(121)** (2.8 g, 21.5 mmol) and distilled triethylamine (3.5 ml, 24.0 mmol) in acetonitrile (56 ml) was cooled to -5 °C. To this was added dropwise isobutylchloroformate (3.5 ml, 24 mmol), followed by neat dibenzylamine (15 ml, 48.5 mmol). The resulting solution was stirred for 2 min at -5

organic phases were dried (Na_2SO_4), filtered and evaporated *in vacuo* to give a yellow solid, which was recrystallised from ethyl acetate to give the desired product as a white solid (4.32 g, 65%); m.p. 114-115 °C; ν_{max}/cm^{-1} (KBr) 1 771, 1 736, 1 646, 1 609, 1 584, 1 443 and 1 167; δ_H ($CDCl_3$) 2.34 - 2.88 (4H, m), 4.42 - 4.77 (4H, m), 5.20 (1H, m) and 7.15 - 7.43 (10H, m); δ_C ($CDCl_3$) 24.86 (t), 27.32 (t), 48.87 (t), 49.56 (t), 74.72 (d), 126.49 (d), 127.76 (d), 128.03 (d), 128.23 (d), 128.81 (d), 129.15 (d), 135.67 (s), 136.26 (s), 168.89 (s) and 176.49 (s); *m/z* 309 (M^+ + 1, 3.1%), 218 (68.4), 150 (1.0), 91 (100), 85 (31.1) and 69 (2.1) [Found: C, 72.5%; H, 6.4; N, 4.4; M^+, 309.1366. $C_{19}H_{19}NO_3$ requires C, 73.8; H, 6.2; N, 4.5%; M^+, 309.1365].

1-Dibenzylamino-2,5-pentanediol **(150)**

To a solution of amidolactone **(144)** (1.7 g, 0.55 mmol) in dry THF (57 ml), cooled in an ice-bath and in an N_2 atmosphere, was added a solution of BH_3 in THF (1.0M, 25.4 ml). The reaction mixture was heated to reflux and maintained thereat for 24 h. The reaction mixture was then cooled to room temperature, and 6M HCl was slowly added. THF was removed by distillation at atmospheric pressure, and the residue was then evaporated *in vacuo* to give 1-dibenzylamino-2,5-pentanediol **(150)** as a thick oil (0.17 g, 10%); ν_{max}/cm^{-1} ($CHCl_3$) 3 630, 3 020, 2 960, 1 500, 1 425, 1 220; δ_H (200 MHz, $CDCl_3$) 1.58 (4H, m, 3-H_2 and 4-H_2), 3.5 (3H, m, 5-H_2 and 2-H), 4.0 (4H, s, 2 x $ArCH_2N$), 4.4 (2H, m, 1-H_2), 7.26-7.33 (6H, unres. m, 2 x Ph), 7.49-7.58 (4H, unres. m, 2 x Ph), 10.44 (2H, br s, OH); δ_C ($CDCl_3$) 27.97 and 29.01 (2 x t, C-3 and C-4), 45.00 (t, C-1), 61.46 (t, $ArCH_2N$), 61.60 (t, C-5), 65.26 (d, C-2), 128.65 (s), 128.79 (s), 129.24 (d), 129.89 (d), 130.00 (d), 131.35 (d), 131.61 (d); *m/z* 281 (M^+ - H_2O, 0.1%), 240 (0.5), 210, (45.0) 118 (1.5) and 91 (100%) [Found: M^+ - H_2O, 281.1753. $C_{19}H_{25}NO_2$ - H_2O requires M^+, 281.1780].

N,N,N',N'-Tetrabenzyl-2-hydroxypentanedicarboxylic acid diamide **(151)**

Amidolactone **(144)** (0.06 g, 0.19 mmol) was stirred overnight with neat dibenzylamine (2 ml, 10.4 mmol, 50 equiv.). Excess dibenzylamine was removed by

vacuum distillation (160 °C, 5 mm Hg) to leave a solid residue, which proved on analysis to be an intractable mixture of compounds, containing a little of the desired product.

(S)-5-Oxotetrahydrofuran-2-carboxamide **(152)**

This amide was prepared from *(S)*-5-oxotetrahydrofuran-2-carboxylic acid **(121)** (0.2 g, 1.54 mmol) in the same manner as compound **(144)**, except that gaseous ammonia (excess) was used in place of dibenzylamine, yielding the desired product as a white solid (0.67 g, 37%); ν_{max}/cm^{-1} (KBr) 3 440, 3 340, 2 970, 1 700, 1 690, 1 680, 1 420; *m/z* 129 (M^+, 0.5%), 86 (4.3), 72, (5.4) 59 (1.0) and 56 (100.0) [Found: M^+, 129.0421. $C_5H_7NO_3$ requires M^+, 129.0426].

(S)-5-(Methylamine)tetrahydrofuran-2-one **(153)**

(R)-5-Oxotetrahydrofuran-2-carboxamide **(152)** (0.60 g, 0.47 mmol) in THF (15 ml) was reacted with NH_3 (g) and stired over night, then concentrated *in vauo.* The residue proved on analysis to be an intractable mixture of compounds, containing none of the desired product.

(S)-5-(Bromomethyl)tetrahydrofuran-2-one **(156)**

(S)-5-(Hydroxymethyl)tetrahydrofuran-2-one **(134)** (5.56 g, 4.8 mmol) was added slowly to ice-cold PBr_3 (4.3 ml), giving a dark brown solution which was stirred overnight (15 h). Ice-water (25 ml) was added slowly to hydrolyse the excess PBr3, and the organic layer was separated. To the organic layer was added ether (50 ml), and the resulting solution was washed with $NaHCO_3$ solution (3 x 10 ml) and water (10 ml). The organic layer was then dried (Na_2SO_4), filtered and evaporated *in vacuo* to give mainly starting material (134) as yellow oil and only a trace of *(S)*-5-(bromometyl)tetrahydrofuran-2-one **(156)**; ν_{max}/cm^{-1} 3 010, 1 710, 1 220, 720.

N,N-Dibenzyl-5-(*N',N'*-Dibenzylamino)-4-hydroxypentanamide **(146)**

A mixture of dibenzylamine (8 ml, 6 equiv) and *(S)*-5-(hydroxymethyl)tetrahydrofuran-2-one **(134)** (0.70 g, 0.58 mmol)) was heated at 60 °C for 1 h, and then cooled to room temperature. Ether was added and a solid immediately precipitated. This precipitate was removed by filtration, and the filtrate was evaporated *in vacuo* to yield the straight chain dibenzyl amide **(147)** as a thick oil; (1.28 g, 45%) ν_{max}/cm^{-1} ($CHCl_3$) 3 300 (br), 3 010, 1610, 1 595, 1 500, 1 480, 1 210; δ_H (200 MHz, $CDCl_3$) 1.23-1.79 (2H, m), 2.36-2.43 (1H, m), 2.60-2.67 (2H, unres. m), 3.30-3.54 (2H, m), 3.74 (8H, s), 7.11-7.36 (20H, m); δ_C ($CDCl_3$) 29.38 (t), 31.70 (t), 45.12 (t), 53.07 (t), 67.18 (d), 127.07 (d), 128.34 (d), 128.45 (d), 140.01 (s); *m/z* 240 (M^+ - $N(Bz)_2C_3H_4O$, 0.2%), 208, (0.4) 198 (0.4), 192 (0.4), 91 (100.0) [Found: M^+- $N(Bz)_2C_3H_4O$, 240.1409. $C_{16}H_{18}NO$ requires 240.1388]

The above reaction also worked using (*S*)-5-(bromomethyl)tetrahydrofuran-2-one **(156)** and the product gave the same spectroscopic data.

10.4 EXPERIMENTAL FOR CHAPTER 6

Extraction of Diamine Oxidase (EC 1.4.3.6) from Pea Seedlings[116]

Step 1

Pea seeds (500g), variety 'Fillbasket', were soaked in tap water for 24 h. The water was changed *ca.* 4 times. The pea seeds were then sown thickly in Perlite (4-6 cm deep) and covered in Perlite (1-2 cm). They were allowed to germinate and grow in the dark for 10-14 days until the shoots were 5-10 cm tall. The Perlite was kept moist throughout but not too wet as this reduced germination. The shoots were stripped of their rooots, washed free of growing medium, drained and weighed (1-1.5 kg). The harvested shoots were kept cool throughout the following operation. The peas were minced using a precoooled Waring blender. They were then strained through cotton mesh and the juice was squeezed out. The solid residue was mixed with 0.1 m potassium phosphate buffer (pH 7, 1 ml / g of material) and the juice was squeezed out as before. A second extraction using the same potassium phosphate buffer (0.5 ml / g of material) was performed. The total extract was cooled to < 5 °C.

Step 2

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

Step 3

The dialysis tubing was pre-soaked in distilled water for *ca.* 2 h. The solution was stirred for 1.5 h at 15-18 °C and the precipitate was removed by centrifugation at 3000-4000 g for 20 min. The supernatant was again saturated with ammonium sulphate (200 -300 g) and left for 1.5 h at 8-10 °C. It was then centrifuged at 3000-4000 g for 20 min. The curd was mixed with 0.2 M phosphate buffer (pH 7, 20 ml). The solution was dialysed in a 30 cm tube (diameter 15 mm) for 2-3 h with cold running water. Dialysis was then carried out with 0.005 M phosphate buffer (pH 7, 1 1) over 36 h at 0-4 °C. The buffer was changed twice during this period.

Step 4

The dialysed material was centrifuged at 3000 g for 10-20 min to remove inactive precipitate. The supernatant liquid was adjusted to pH 5 by slow addition of 0.05 M acetic acid at *ca.* 5 °C then allowed to stand for 1 h at 0-4 °C. The precipitate was collected by centrifugation and triturated with water (20 ml). The pH was adjusted to pH 7 using 0.05 M potassium hydrochloride to dissolve the precipitate and

then to pH 5 with 0.05 M acetic acid. The solution was left for 1 h and centrifuged to collect the precipitate. The precipitate obtained was taken up in 0.01 M phosphate buffer (pH 7, 1 ml / 100 g of seedlings harvested). It was stored in the freezer (in 0.5 ml batches) at *ca.* -20 °C and was stable for many months.

Protein concentration was *ca.* 5.6 mg per ml of enzyme solution. [See Appendix 1 for calculation]

Determination of Protein Concentration[133]

Coomassie brilliant blue G was prepared as a 0.06% (w/v) solution in 3% perchloric acid. The solution was stirred overnight and filtered to remove any undissolved material. The stock dye was diluted to A 465 1.3-1.5 (the absorbance maximum for the leuko form of the dye). The standard graph was determined using Bovine Serum Albumin (BSA, 1 mg / ml phosphate buffer pH 6.3).

A typical cuvette contained :

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

x = 50 to 5 µl.

The experiment was carried out twice and the average plot was used to determine the protein concentration of unknown DAO samples (replacing BSA with DAO in the cuvette). [See Appendix 1]

Spectrophotometric Assay[22]

The kinetics of DAO-catalysed oxidation of putative substrates were determined by the procedure of Stoner.[22] This involved peroxidase-coupled assay (horseradish peroxidase, EC 1.11.17, from Sigma) to monitor continuously the hydrogen peroxide released during diamine oxidation at 25 °C, in 70 mM phosphate buffer (pH 6.3), in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH)

and 3-(dimethylamino)benzoic acid (DMAB). Oxidative coupling generated stoichiometric quantities of an indamine dye with characteristic absorbance maximum at 595 nm. Rates (abs s^{-1} x m 10^{-3}) were determined directly in the spectrophotometer. Using the extinction Coefficient (ε = 2.6335 x 10^4) and the concentration of DAO solution (mg/ml) used in the assay, the units of rate can be converted from abs s^{-1} to μmol $mg^{-1}h^{-1}$.

Stock solutions were prepared as follows:

DMAB	18 mM (29.7 mg / 10 ml phosphate buffer pH 6.3)
MBTH	0.6 mM (12.9 mg / 100 ml distilled water)
Peroxidase	0.34 mg / ml phosphate buffer pH 6.3 (150-200 units per mg solid. One unit will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH at 20 °C)
Pea seedling DAO	0.03-0.06 mg / ml phosphate buffer pH 6.3

A typical reaction mixture in a 1 cm pathlength cuvette comprised:

2.5 ml	Phosphate buffer pH 6.3
100 µl	MBTH
170 µl	DMAB
50 µl	peroxidase
25 µl	pea seedling DAO
300 µl	substrate (concentration range up to 3 mM)

The production of indamine dye was calibrated using standard solutions of hydrogen peroxide. The hydrogen peroxide was standardised using potassium permanganate.[168]

The reaction was initiated by addition of standard enzyme solution to the thermally equilibrated reaction mixture, followed immediately by substrate addition, therefore minimizing the possibly inhibiting effects of extensive preincubation of DAO with the chromogenic agents.[22] Initial rates were determined over a range of substrate concentrations from the linear absorbance changes during the first minute of reaction. Michaelis-Menten kinetic behaviour was observed in all cases. Rate data were analysed for K_M and V_{max} by least squares fitting of Eadie-Hofstee (V *vs.* V[S]), Lineweaver-Burk (1/V *vs.* 1[S]) and Hanes ([S]/V *vs* [S]) plots. [See Appendix 2]

All experiments were carried out at least three times with the respective substrate. Therefore all data quoted are the means of three determinations.

Inhibition Studies

Inhibition studies were performed by the same method as above and by incorporating various concentrations of inhibitor or co-substrate into the initial reaction mixture. A separate experiment was carried out for each concentration of inhibitor or co-substrate. The potential inhibitor was added to the reaction mixture after DAO addition but before the addition of substrate.

GENERAL PROCEDURES FOR THE SYNTHESIS OF DI- AND POLYAMINES

10.4 (a) General procedure for the synthesis of N-BOC amino acids .

A solution of 2-*t*-butoxycarbonyloxyimino-2-phenylacetonitrile (BOC-ON) (1.2 equiv.) in 1,4-dioxane (1 ml / mmol amino acid) and deionised water (1 ml / mmol amino acid) was added to a stirred solution of the relevant amino acid (1 equiv.) in distilled Et_3N (3 equiv.). The resultant mixture quickly became homogeneous. Stirring was continued at R.T. for 2 h. Water (1.3 ml / mmol amino acid) was added, then EtOAc (1.6 ml / mmol amino acid). The aqueous layer was separated and washed with EtOAc. The aqueous layer was acidified with 5% citric acid solution. then extracted with EtOAc. The organic extracts were combined, dried

(Na_2SO_4), filtered and the solvent removed *in vacuo* to give the *N*-alkyl-*t*-butoxycarbonylamino acid.

5-*t*-Butoxycarbonylamino)pentanoic acid **(198)**

This compound was prepared from δ-aminovaleric acid (2.24 g, 19.1 mmol) and was obtained as a white crystaline solid (4.9 g, 98%); m.p. 139-140 °C; ν_{max}/cm^{-1} (KBr) 3 380, 3 000, 2 950, 1 710, 1 690, 1 520, 1 370, 1 280, 1 170; δ_H ($CDCl_3$) 1.40 (9H, s), 1.70 -1.49 (4H, m), 2.32 (2H, t, *J* 7.2 Hz), 2.88 (2H, m), 4.79 (1H, br s), 11.0 (1H, br s); δ_C ($CDCl_3$) 21.8 (q), 28.3 (q), 29.2 (t), 33.5 (t), 40.0 (t), 79.2 (s), 156.1 (OCONH), 178.6 (COOH); *m/z* 217 (M^+, 0.1%), 160 (38.6), 158 (10.5), 144 (48.9), 126 (71.3), 116 (100.0), 100 (60.0)and 98 (46.4) [Found M^+ 217.1313, $C_{10}H_{219}N_1O_4$ calc M^+ 217.1314]

10.4 (b) General procedure for the amidation of N-BOC amino acids **(199) - (206).**

A solution of the relevant N-BOC amino acid (1 equiv.) and Et_3N (1.2 equiv.) in acetonitrile (8 ml / mmol of N-BOC amino acid) was cooled to -5 °C. 2-Butylchloroformate (1.2 equiv.) was added dropwise with stirring, and the mixture was left for 3 min. The neat amine (5 equiv.) was added dropwise to the resultant mixture, and the solution was stirred for 2 min at 0 °C. The resulting solution was left at 0 °C for 2 h. Solvent was removed *in vacuo*, and the resulting solid was partitioned between EtOAc and water. The organic phase was separated, and the aqueous layer was extracted with several portions of EtOAc. The combined organic phases were then dried (Na_2SO_4), filtered and evaporated *in vacuo* to yield the crude product, which was then recrystallised from EtOAc.

N-Ethyl-5-(*t*- butoxycarbonylamino)pentanamide **(199)**

This compound was prepared from 5-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (2.24 g, 10.32 mmol) and ethylamine (3.3 ml, 50 mmol), and was obtained as a white crystalline solid (2.5 g, 99.4%); m.p. 85-86.5 °C; ν_{max}/cm^{-1} (KBr) 3 320, 3 290, 1 670, 1 640, 1 520, 1 280 and 1 170; δ_H ($CDCl_3$) 1.13 (3H, t, *J* 7.2Hz), 1.44

(9H, s) 1.69 - 1.40 (4H, m), 2.19 (2H, t, *J* 7.4 Hz), 3.14 (2H, m), 3.28 (2H, m), 4.68 (1H, br s), 5.72 (1H, br s); δ_C ($CDCl_3$) 14.9 (q), 22.7 (t), 28.4 (q), 29.5 (t) , 34.3 (t), 36.0 (t), 39.8 (t), 79.1 (s), 156.2 (OCONH), and 172.7 (CONH); *m/z* 244 (M^+, 0.7 %), 188 (10.8), 144 (11.3), 128 (13.8), 115 (25.0), 100 55.8), 87 (100.0) and 72 (53.6) [Found: C, 59.1%; H, 9.6; N, 11.5; M^+, 244.1778. $C_{12}H_{24}N_2O_3$ requires C, 59.0; H,9.8; N,11.5%; M^+, 244.1781].

N-(*n*-Propyl)-5(-*t*-butoxycarbonylamino)pentanamide **(200)**

This compound was prepared from 5-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (2.24 g, 10.32 mmol) and propylamine (2.9 g, 4.03ml, 50 mmol), and was obtained as a white crystalline solid (1.51 g, 57%); m.p. 79-80 °C; ν_{max}/cm^{-1} (KBr) 3 330, 3 290, 2 980, 2 950, 1 680, 1 643, 1 550, 1 480, 1 280 and 1 170; δ_H ($CDCl_3$) 0.88 (3H, t, *J* 7.2 Hz) 1.39 (9H,s) 1.69 - 1.42 (6H, m), 2.18 (2H, t, *J* 7.2Hz), 3.17 (4H, m), 4.80 (1H, br s), 6.16 (1H, br s); d_C ($CDCl_3$) 11.3 (q) 22.7 (t) 22.7 (t) 28.3 (q), 29.4 (t), 35.8 (t), 39.7 (t), 41.1 (t), 79.0 (t), 156.2 (OCONH) and 173.1 (CONH); *m/z* 258 (M^+, 1.0%), 202 (14.7), 185 (31.8), 157 (2.6); 129 (35.8), 114 (59), 101 (100.0), 98 (39.9) and 82 (7.9) [Found: M^+, 258.1941. $C_{13}H_{26}N_2O_3$ requires M^+, 258.1937].

N-(*n*-Butyl)-5-*t*-butoxycarbonylaminopentanamide **(201)**

This compound was prepared from 5-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (2.24 g, 10.32 mmol) and butylamine (3.65 g, 5.3 ml, 50 mmol), and was obtained as a white crystalline solid (2.51 g, 85.2%); m.p. 70-71 °C; ν_{max}/cm^{-1} (KBr) 3 260, 3 220, 2 980, 1 640, 1 530, 1 280 and 1 170; δ_H ($CDCl_3$) 0.83 (3H, t, *J* 7.0 Hz), 1.36 (9H, s) 1.66- 1.15 (8H, m), 2.12 (2H, t, *J* 7.0 Hz), 3.20 - 3.00 (4H, m), 4.88 (1H, br s), 6.22 (1H, br s); δ_C ($CDCl_3$) 13.6 (q), 19.8 (t), 22.7 (t), 29.3 (q), 29.4 (t), 31.6 (t), 35.8 (t), 39.1 (t), 39.7 (t), 78.9 (s), 156 (OCONH) and 172.9 (CONH); *m/z* 216 (M^+, 17.1%), 199 (39.0), 171 (62.5), 157 (5.8), 128 (65.3), 115 92.6), 100 (72.9), 74 (100.0) and 72 (36.8) [Found: C, 61.6%; H, 10.4; N, 10.2; M^+, 216.1460. $C_{10}H_{20}N_2O_3$ requires C, 61.8; H, 10.3; N, 10.3%; M^+, 216.1469].

N-(*i*-Propyl)-5-*t*-butoxcarbonylaminopentanamide (202)

This compound was prepared from 5-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (2.24 g, 10.32 mmol) and isopropylamine (3.02 g, 4.56 ml, 50 mmol), and was obtained as a white crystalline solid (1.86 g, 70%); m.p. 102 - 103 °C; ν_{max}/cm^{-1} (KBr) 3 310, 3 070, 2 980, 1 690, 1 650, 1 550, 1 365, 1 280, 1 460; δ_H ($CDCl_3$) 1.08 (3H, s), 1.11 (3H, s), 1.39 (9H, s), 1.69 - 1.46 (4H, m), 2.14 (2H, t, *J* 7.2 Hz), 3.07 (2H, m), 4.10 - 3.84 (1H, m), 4.77 (1H, br s), 5.78 (1H, br s); δ_C ($CDCl_3$) 22.6 (q), 22.7 (t), 28.3 (t), 29.4, (t), 35.9 (t), 39.7 (t), 41.2 (t), 79.O (s), 156.1, (OCONH), 172.0 (CONH); *m/z* 258 (M^+, 1.6 %), 202 (11.8), 185 (23.8), 158, (10.5) 114, (38.7) 98, (25.9) 60 (49.4) and 57 (100.0) [Found: C, 60.3%; H, 10.0; N, 11.0; M^+, 258.1936. $C_{13}H_{26}N_2O_3$ requires C, 60.5; H 10.1; N, 10.9%; M^+, 258.1937].

N-(*i*-Butyl)-5-*t*-butoxcarbonylaminopentanamide (203)

This compound was prepared from 5-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (2.24 g, 10.32 mmol) and isobutylamine (3.85 g, 5ml, 50 mmol), and was obtained as a white crystalline solid (0.51g, 18%); m.p. 82-83 °C; ν_{max}/cm^{-1} (KBr disc) 3 290, 2 970, 2 934, 1 690, 1 640, 1 550, 1 460, 1 365, 1 170; δ_H ($CDCl_3$) 0.88 (3H, t, *J* 7.4 Hz), 0.9 (2H,m); 1.1 (3H, t, *J* 6.6 Hz), 1.42 (9H, s), 1.73 - 1.47 (4H, m), 2.17 (2H, t, *J* 7.2 Hz), 3.13 (2H, m), 3.8 (1H,m); 4.72 (1H, br s) 5.49 (1H, br s); δ_C ($CDCl_3$) 8.8 (q), 18.8 (q), 21.1 (t), 26.8 (q) 27.8 (t), 28.0 (t), 34.5 (t), 38.1 (t), 44.8 (d), 77.5 (s), 154.5 (OCONH), 170.6 (CONH); *m/z* 272 (M^+, 1.3%), 199 (50.1), 171 (58.8) and 100 (100.0) [Found: C, 60.1%; H, 10.0; N, 10.3; M^+, 272.2117. $C_{14}H_{28}N_2O_3$ requires C, 61.8; H, 10.3; N, 10.3%; M^+, 272.2100].

N-Benzyl-5-*t*-butoxycarbonylaminopentanamide (204)

This compound was prepared from 5-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (2.24 g, 10.32 mmol) and benzylamine (5.3 g, 5.4 ml, 50 mmol), and was obtained as a white crystalline solid (2.2 g, 70%); m.p. 111-113 °C; ν_{max}/cm^{-1} (KBr) 3 320, 2 980, 1 690, 1 660, 1 530, 1 460, 1 370, 1 280; δ_H ($CDCl_3$) 1.4 (9H, s), 1.65

(4H, m), 2.22 (2H, m), 3.09 (2H, m), 4.37 (2H, m), 4.75 (1H, br s), 6.38 (1H, br s), 7.28 (5H, m); δ_C ($CDCl_3$) 22.7 (t), 28.3 (q), 29.5 (t), 35.8 (t), 39.7 (t), 43.4 (t), 79.0, (s), 127.3 (s), 127.6 (d), 128.6 (d), 138.3 (d), 156.2 (OCONH) and 172.8 (CONH); *m/z* 250 (M^+, 9.0), 233 (10.1), 206 (20.7), 177 (10.4), 149 (1.5), 100 (9.2), 91 (100.0) and 77 (7.3) [Found: M^+-*t*-Bu, 250.1324. $C_{13}H_{18}N_2O_3$ requires 250.1313); C, 66.7%, H,8.6; N, 9.1; $C_{17}H_2N_2O_3$ requires C, 66.7; H, 8.5; N, 9.2%].

N-Cyclohexyl-5-*t*-butoxycarbonylaminopentanamide (205)

This compound was prepared from 5-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (2.24 g, 10.32 mmol) and cyclohexylamine (4.8 g, 5.54 ml, 50 mmol), and was obtained as a white crystalline solid (1.54 g, 50%); m.p. 129-130 °C; ν_{max}/cm^{-1} (KBr) 3 360, 3 320, 2 940, 2 860, 1 690, 1 640, 1 530, 1 170; δ_H ($CDCl_3$) 1.30 - 1.07 (5H, m), 1.41 (9H, s), 1.63 - 1.48 (6H, m), 1.89 - 1.84 (4H, m), 2.16 (2H, m), 3.09 (2H, m), 4.74 (1H, br s), 5.72 (1H, br s); δ_C ($CDCl_3$) 22.8 (t), 24.1 (t), 24.6 (t), 24.8 (t), 25.4 (t), 28.4 (q), 29.4 (t) 30.9 (t) 33.1 (t), 36.1 (t), 48.2 (d), 79.0 (s), 156.1 (OCONH), 172.0 (CONH); *m/z* 298 (M^+, 3.6%), 241 (7.7), 197 (56.7), 169, (39.2) 144 (1.4), 141 (56.1), 100 (100.0), 98 (75.9) and 74 (7.9) [Found: M^+, 298.2246. $C_{16}H_{30}N_2O_3$ requires M^+, 298.2249].

N-Methyl-5-*t*-butoxycarbonylaminopentanamide (206)

This compound was prepared from 5-(*t*-butoxycarbonylaminopentanoic acid **(198)** (2.24 g, 10.32 mmol) and methylamine solution in EtOH (1.55 g, 50 mmol) and was obtained as a white crystalline solid (1.50 g, 46%); m.p. 67-69 °C; ν_{max}/cm^{-1} (KBr) 3 330, 3 290, 2 930, 1 680, 1 650, 1 530, 1 390, 1 170; δ_H ($CDCl_3$) 1.36 (9H, s), 1.56 (4H, br m), 2.15 (2H, t, *J* 7.2 Hz), 2.70 (3H, s), 3.00 (2H, br m), 4.80 (1H, br s, NH), 6.15 (1H, br s, NH); δ_C ($CDCl_3$) 22.69 (t), 26.15 (q), 28.32 (q), 29.44 (t), 35.67 (t), 39.67 (t), 79.01 (s), 156.16 (s), 173.63 (s); *m/z* 230 (M^+, 0.2%), 175,(1.3); 144 (1.4), 130,(17.7); 87 (36.8), 73 (100.0) and 71 (2.7) [Found: C, 57.2%; H, 9.5; N,

12.2; M^+, 230.1635. $C_{11}H_{22}N_2O_3$ requires C, 57.4; H, 9.6; N, 12.2%; M^+, 230.1630].

10.4 (c) General Procedure for Removal of BOC Group in Compounds (207) - (214)

The *N*-BOC compound was stirred for 30 min in 3 M HCl / EtOAc (1:1). The solvents were removed under high vacuum to give a clear oil, which was repeatedly triturated with Et_2O to give the desired amide as the hydrochloride salt.

N-Ethyl-5-aminopentanamide hydrochloride (207)

This compound was prepared from *N*-ethyl-5-(*t*-butoxycarbonylamino)pentanamide **(199)** (0.45 g, 1.84 mmol) and was obtained as a white crystalline solid (0.22 g, 65%); m.p. 79-80 °C; ν_{max}/cm^{-1} (KBr) 3 360, 3 210, 2 980, 1 620, 1 380, 1 190; δ_H (D_2O) 0.82 (3H, t, *J* 7.2 Hz), 1.41 (4H, m), 2.01 (2H, s), 2.73 (2H, s) 2.90 (2H, m); δ_C ($CDCl_3$) 14.3 (q), 23.1 (t), 26.9 (t) 35.3 (t), 35.8 (t), 39.9 (t), 176.5 (c-1); *m/z* 144 (M^+ - HCl, 2.8%), 115 (33.0), 100 (20.2), 87 (100.0), 72 (75.4) and 56 (3.5) [Found: M^+ - HCl, 144.1259. $C_7H_{17}N_2OCl$ - HCl requires M^+, 144.1259].

N-(*n*-Propyl)-5-aminopentanamide hydrochloride (208)

This compound was prepared from *N*-(*n*-propyl)-5-(*t*-butoxycarbonylaminopentanamide **(200)** (1.24g, 4.80 mmol) and was obtained as a white crystalline solid (0.67 g, 72%); m.p. 134-135 °C; ν_{max}/cm^{-1} (KBr) 3 420, 3 030, 2 970, 2 880, 1 640, 1 630, 1 360; δ_H (D_2O) 0.75 (3H, t, *J* 7.2 Hz), 1.3 (2H, m), 1.45 (4H, m), 2.15 (2H, unres. m), 3.00 (4H, unres. m and t, *J* 7.0Hz); δ_C (D_2O) 11.43 (q), 22.61 (t), 23.16 (t), 26.98 (t), 35.90 (t), 39.91 (t), 41.97 (t), 176.78 (s); *m/z* 158 (M^+ - HCl, 0.1%), 101 (1.4), 98 (45.6), 84,(43.2); 72 (100.0) and 58 (11.2) [Found: M^+ - HCl, 158.1420. $C_8H_{20}N_2OCl$ - HCl requires M^+, 158.1419].

N-(*n*-Butyl)-5-aminopentamide hydrochloride (209)

This compound was prepared from *N*-(*n*-butyl)-5-(*t*-butoxycarbonylamino)pentanamide (201) (1.04 g, 3.82 mmol) and was obtained as a white crystalline solid (0.74 g, 71%) m.p. 129.5 - 131 °C; ν_{max}/cm^{-1} (KBr) 3 290, 3 200, 2 960, 1 660, 1 630, 1 560, 1 470, 1 200; δ_H (D_2O) 0.66 (3H, t, *J* 7.2 Hz), 1.32 - 1.00 (4H, m), 1.44 (4H, s), 2.00 (2H, s), 2.78 (2H, s), 2.95 (2H, t, *J* 6.8 Hz); δ_C (D_2O) 13.8 (q), 20.2 (t), 23.1 (t), 26.9 (t) 31.2 (t), 35.9 (t), 39.9 (t) and 176.7 (CONH); *m/z* 172 (M^+ - HCl, 9.0%), 143 (33.7), 128 (20.8), 115 (100.0), 100 (51.0), 74 (4.3) and 71 (4.9) [Found: M^+ - HCl, 173.1574. $C_9H_{21}N_2OCl$ - HCl requires M^+, 172.1571].

N-(*i*-Propyl)-5-aminopentamide hydrochloride (210)

This compound was prepared from *N*-(*i*-propyl)-5-(*t*-butoxycarbonylamino)pentanamide (202) (1.52 g, 5.89 mmol) and was obtained as a white crystalline solid (1.02 g, 89%); m.p. 163-164 °C; ν_{max}/cm^{-1} (KBr) 3 275, 3 065, 2 980, 1 630, 1 555, 1 470, 1 460, 1 205; δ_H (D_2O) 0.97 (6H, d), 1.50 (4H, m), 2.1 (2H, m), 2.83 (2H, m), 3.67 (1H, m); δ_C (D_2O) 22.19 (q), 22.93 (q), 26.90 (t), 23.14 (t), 35.90 (t), 42.52 (t), 43.07 (d), 175.63 (s); *m/z* 158 (M^+ - HCl, 11.8%), 129 (54.1), 101 (100.0), 86 (44.5) and 70 (2.2) [Found: C, 48.5%; H, 9.5; N, 14.2; M^+ - HCl, 158.1428. $C_8H_{19}N_2OCl$ - HCl requires C, 49.4; H, 9.8; N, 14.4; M^+, 158.1423].

N-(*i*-Butyl)-5-aminopentamide hydrochloride (211)

This compound was prepared from *N*-(*i*-butyl)-5-(*t*-butoxycarbonylamino)pentanamide (203) (0.48 g, 1.77 mmol) and was obtained as a white crystalline solid (0.223 g, 65%); m.p. 109-110 °C; ν_{max}/cm^{-1} (KBr disc) 3 430, 3 280, 3 060, 2 970, 2 940, 1 630, 1 550; δ_H (D_2O) 0.60 (3H, t, *J* 7.2Hz), 0.83 (3H, d, *J* 6.6 Hz), 1.20 (2H,m), 1.50 (4H,m), 2.00 (2H, m), 2.76 (2H, br s), 3.56- 3.40 (1H, m); δ_C (D_2O) 10.6 (q), 17.6 (q), 21.2 (t) 26.9 (t), 29.4 (t), 35.0 (t), 39.9 (t), 48.0 (d), 176.0 (CONH); *m/z* 173 (M^+, 1.7%), 128, (19.4) 114, (34.6) 100 (100), 60 (62.2) and

56 (76.6) [Found: C, 50.4%; H, 9.8; N, 12.4; M^+, 173.1643. $C_9H_{21}N_2O$ requires C, 51.8; H, 10.7; N, 13.4%; M^+, 173.1649].

N-Benzyl-5-aminopentanamide hydrochloride (212)

This compound was prepared from *N*-benzyl-5-(*t*-butoxycarbonylamino)pentanamide **(204)** (0.91 g, 2.96 mmol) and was obtained as a white crystalline solid (0.63 g, 87%); m.p. 122.6 - 123.6 °C; ν_{max}/cm^{-1} (KBr) 3 290, 3 050, 3 000; 2 980, 2 890, 2 870, 1 660, 1 630, 1 550; d_H (D_2O) 1.48 (4H, m), 2.15 (2H, t, *J* 6.6 Hz), 2.80 (2H, t, *J* 6.8 Hz), 4.17 (2H, s), 7.28 -7 .12 (5H, m); δ_C (D_2O) 23.1 (t), 27.0 (t), 35.8 (t), 39.9 (t), 43.8 (t), 130.1 (d), 129.7 (d), 129.6 (d), 138.8 (s), 176.8 (CONH); *m/z* 206 (M^+ - HCl, 1.5%), 189 (0.3), 132 (0.1), 106 (100.0), 104 (6.0), 100 (0.9)and 91 (29.9) (Found: C, 59.2%; H, 7.6; N, 11.2; M^+ - HCl, 206.1426. $C_{12}H_{19}N_2OCl$ - HCl requires C, 59.4; H, 7.8; N,11.5%; M^+, 206.1415].

N-Cyclohexyl-5-aminopentanamide hydochloride (213)

This compound was prepared from *N*-cyclohexyl-5-(*t*-butoxycarbonylamino)pentanamide **(205)** (0.80 g, 2.70 mmol) and was obtained as a white crystalline solid (0.62 g, 98%); m.p. 163.5-165.5 °C; ν_{max}/cm^{-1} (KBr) 3 290,3 000,2 940, 2 860, 1 660, 1 630, 1 550, 1 480, 1 470, 1 460; δ_H (D_2O) 0.96 - 1.28 (6H, m), 1.53 - 1.46 (9H, m), 2.12 (2H, m), 2.84 (2H, m); δ_C (D_2O) 23.2, (t) 24.6 (t), 25.2 (t), 25.7 (t), 26.9 (t), 31.3 (t), 32.8 (t), 35.9 (t), 39.9 (t), 49.6 (q), 512.2 (d) and 175.6 (CONH); *m/z* 198 (M^+ - HCl, 7.8%), 181 (1.9), 169 (19.7), 141 (23.6), 116 (9.1), 98 (2.8), 83 (6.7), 60 (35.7) and 56 (100.0) [Found M^+ - HCl, 198.1719. $C_{11}H_{23}N_2OCl$ - HCl requires M^+, 198.1727].

N-Methyl-5-aminopentanamide hydochloride (214)

This compound was prepared from *N*-methyl-5-(*t*-butoxycarbonylamino)pentanamide **(206)** (1.89 g, 14.54 mmol) and was obtained as a white crystalline solid (1.33 g, 97%); m.p. 73-74 °C; ν_{max}/cm^{-1} (KBr) 3 360, 3 000,

2 980, 1 660, 1 620, 1 550; δ_H (D_2O) 1.41 (4H, m), 2.06 (2H, m), 2.48 (3H, s) 2.94 (2H, m); δ_C ($CDCl_3$) 21.7 (t), 26.9 (q) 35.3 (t), 35.6 (t), 39.9 (t), 176.5 (CONH); *m/z* 130 (M^+ - HCl, 3.5%), 113 (2.5); 99 (37.3), 58 (100.0) [Found: M^+ - HCl, 130.1109 $C_6H_{14}N_2OCl$ - HCl requires M^+, 130.1106].

10.4 (d) General Procedure for the BH_3-Reduction of Amides (214) - (222)

To a suspension of the relevant amide hydrochloride salt (1 equiv.) in dry THF (cooled to 0 °C),was added a solution of BH_3 in THF (1.0 M, 4 equiv). The resultant mixture was heated to reflux for 24 h, and then cooled to R.T. 6M HCl was added slowly to the solution until no more H_2 was evolved. THF was removed by distillation at atmospheric pressure, and MeOH and conc. HCl (2 drops) were added to the residue. Solvents were evaporated *in vacuo*, and the crude product was then recrystallised from MeOH to afford the desired *N*-alkylamine hydrochloride salt.

N-Ethyl-1,5-diaminopentane dihydrochloride (215)

This compound was prepared from *N*-ethyl-5-aminopentanamide hydrochloride (207) (0.48 g, 2.76 mmol) and was obtained as a white crystalline solid (0.32 g, 59%) m.p. 212-214 °C; ν_{max}/cm^{-1} (KBr) 3 220, 2 960, 2 930, 2 830, 2 800, 2 760, 1 460; δ_H (D_2O) 1.08 (3H, t, *J* 7.2 Hz), 1.12 - 1.31 (2H, m), 1.33 - 1.61 (4H, m), 2.79 - 3.00 (6H, m); δ_C (D_2O) 11.4 (q), 23.6 (t), 26.0 (t), 27.12 (t), 40.0 (t), 43.7 (t), 47.6 (t); *m/z* 131 (M^+ - 2HCl, 2.6%), 101 (0.5), 85 (4.8); 73 (6.9), 58 (100.0) and 56 (11.5) [Found: C, 41.3%; H, 9.7; N, 13.74; M^+ - 2HCl, 130.1479. $C_7H_{20}N_2Cl_2$ - 2HCl requires C, 41.4; H, 9.8; N, 13.8%; M^+, 130.1466].

N-(*n*-Propyl)-1,5-diaminopentane dihydrochloride (216)

This compound was prepared from *N*-(*n*-propyl)-5-aminopentanamide hydrochloride (208) (1.18 g, 6.07 mmol) and was obtained as a white crystalline solid (0.52 g, 40%); m.p. 256-257 °C; ν_{max}/cm^{-1} (KBr) 2 960, 2 810, 2 540, 2 500; δ_H (D_2O) 0.85 (3H, t, *J* 7.4 Hz), 1.31 (2H, m), 1.58 (6H, m), 2.87 (6H, m); δ_C (D_2O)

10.98 (q), 19.97 (t), 23.59 (t), 25.88 (t), 27.05 (t), 39.93 (t), 47.95 (t), 49.96 (t); *m/z* 145 (M^+ - 2HCl, 0.3%), 106 (0.2), 102 (0.1), 86 (6.5), 72 (100.0) and 59 (2.0) [Found: M^+ - 2HCl, 145.1700. $C_8H_{22}N_2Cl_2$ - 2HCl requires M^+, 145.1626].

N-(*n*-Butyl)-1,5-diaminopentane dihydrochloride (217)

This compound was prepared from *N*-(*n*-butyl)-5-aminopentanamide hydrochloride (**209**) (0.40 g, 1.92 mmol) and was obtained as a white crystalline solid (0.34 g, 77%); m.p. 285 °C; ν_{max}/cm^{-1} (KBr) 3 430, 2 960, 2 930, 2 870, 2 770, 1 480; δ_H (D_2O) 0.76 (3H, t, *J* 7.2 Hz), 1.33 (4H, m), 1.57 (6H, m), 2.90 (6H, m); δ_C (D_2O) 13.72 (q), 20.05 (t), 22.39 (t), 23.63 (t), 25.89 (t), 27.08 (t), 40.01(t), 47.99 (t), 48.20 (t); *m/z* 158 (M^+ - 2HCl, 5.3%), 142 (4.7), 115 (21.3), 86 (100.0), 72 (20.3) and 55 (7.9) [Found: C, 45.9%; H, 10.4; N, 11.4; M^+ - 2HCl, 158.1784. $C_9H_{24}N_2Cl_2$ - 2HCl requires C, 46.8, H, 10.4, 12.1%; M^+, 158.1783].

N-(*i*-Propyl)-1,5-diaminopentane dihydrochloride (218)

This compound was prepared from *N*-(*i*-propyl)-5-aminopentanamide hydrochloride (**210**) (0.9 g, 4.63 mmol) and was obtained as a white crystalline solid (0.69 g, 69%); m.p. 219 °C (dec.); ν_{max}/cm^{-1} (KBr) 2 980, 2 870, 2 650, 1 460, 1 390; δ_H (D_2O) 1.2 (6H, d, *J* 6.6 Hz), 1.32 (2H, m), 1.50 (4H, m), 2.9 (4H, m), 3.28 (1H, septet, *J* 6.6 Hz); δ_C (D_2O) 19.05 (q), 23.68 (t), 26.17 (t), 27.08 (t), 39.98 (t), 45.22 (t), 51.45 (d); *m/z* 144 (M^+ - 2HCl, 3.3%); 129 (8.3), 101 (7.0), 72 (100.0) and 60 (2.1) [Found: M^+ - 2HCl, 144.1626. $C_8H_{22}N_2Cl_2$ - 2HCl requires M^+, 145.1626].

N-(*i*-Butyl)-1,5-diaminopentane dihydrochloride (219)

This compound was prepared from *N*-ethyl-5-aminopentanamide hydrochloride (**211**) (0.15 g, 0.72 mmol) and was obtained as a white crystalline solid (0.12 g, 71%); m.p. 220 °C (dec.); ν_{max}/cm^{-1} (KBr) 2 970, 2 940, 2 870, 2 830; δ_H (D_2O) 0.75 (3H, t, *J* 7.4 Hz), 1.06 (3H, d, *J* 6.6 Hz), 1.20 - 1.38 (2H, m), 1.65 - 1.43 (6H, m), 2.88 (4H, m), 3.00 (1H, m); δ_C (D_2O) 9.5 (q), 15.6 (q), 23.7 (t) 26.1 (t), 26.3

(t), 27.1 (t), 39.9 (t), 45.1 (t), 56.5 (d); *m/z* 158 (M^+, 0.7%), 129 (52.2), 86 (100) and 71 (2.1) [Found: C, 45.0%; H, 9.8; N, 11.0; M^+, 158.1798. $C_9H_{22}N_2$ requires C, 46.7; H, 10.4; N, 12.1%; M^+, 158.1778].

N-Benzyl-1,5-diaminopentane dihydrochloride (220)

This compound was prepared from *N*-benzyl-5-aminopentanamide hydrochloride (212) (1.01 g, 4.17 mmol) and was obtained as a white crystalline solid (0.55 g, 50%) ; m.p. 260-261 °C; υ_{max}/cm^{-1} (KBr) 3 030, 2 940, 2 800, 2 770, 2 580, 1 610, 1 510; d_H (D_2O) 1.61 (6H, m), 3.52 (4H, m), 4.07 (2H, s), 7.31 (5H, m); d_C (D_2O) 23.51 (t), 25.32 (t), 27.21 (t) 39.78 (t), 49.1 (t), 67.18 (t), 128.11 (s), 128.21 (s), 129.54 (d), 129.74 (s); m/z 192 (M^+-2HCl, 0.5%), 132 (0.8), 101 (10.7), 91 (100.0) and 77 (1.6) [Found: M^+ - 2HCl, 192.1616. $C_{12}H_{22}N_2Cl_2$ - 2HCl requires M^+, 192.1626]

N-Cyclohexyl-1,5-diaminopentane dihydrochloride (221)

This compound was prepared from *N*-cyclohexyl-5-aminopentanamide hydrochloride (213) (0.60 g, 2.63 mmol) and was obtained as a white crystalline solid (0.31 g, 48%) ; m.p. 238 °C (dec.); υ_{max}/cm^{-1} (KBr) 2 940, 2 860, 2 670, 2 590, 1 625, 1 465; δ_H (D_2O) 1.21 - 1.66 (17H, m), 2.89 (4H, m); δ_C (D_2O) 23.69 (t), 24.75 (t), 25.35 (t) 26.14 (t), 27.09 (t), 29.70 (t), 39.98 (t), 44.87 (t), 57.93 (d); m/z 185 (M^+ - 2HCl, 1.5%), 155 (1.0), 112 (100.0) 110 (2.8), 98 (67.1), 84 (70.9) and 83 (7.4) [Found:C, 50.54%; H, 10.64; N, 10.28; M^+ - 2HCl, 184.1927. $C_{11}H_{26}N_2Cl_2$ - 2HCl requires C, 51.36; H, 10.89; N, 10.11%; M^+, 184.1939].

N-Methyl-1,5-diaminopentane dihydrochloride (222)

This compound was prepared from *N*-methyl-5-aminopentanamide hydrochloride (214) (0.66 g, 3.96 mmol) and was obtained as a white crystalline solid (0.66 g, 88%); m.p. 126-127 °C; ν_{max}/cm^{-1} (KBr) 3 420, 2 960, 2 870, 1 470; δ_H (D_20) 1.62 - 1.4 (6H, m), 2.52 (3H, s), 2.86 (4H, m), δ_C (D_2O) 23.51 (t), 25.82 (t),

27.11 (t), 33.52 (q), 40.10 (t), 49.53 (t); *m/z* 117 (M^+ - 2HCl, 2.6%), 100, (16.6) 86 (22.1), 70 (95.0), 56 (100.0) and 53 (5.5) [Found: C, 37.9%; H, 9.6; N, 13.8; M^+ - 2HCl, 117.1377. $C_6H_{12}N_2Cl_2$ - 2HCl requires C, 38.1; H, 9.5; N, 14.8; M^+, 117.1388]

10.5 EXPERIMENTAL TO CHAPTER 8

4-(*t*-Butoxycarbonylamino)butanoic acid (231)

This compound was prepared from **(329)** (3.20 g, 37.6 mmol) using the general method 10.4 (a) and gave identical spectroscopic data to the literature compound.[79]

3-(*t*-Butoxycarbonylamino)propanoic acid (232)

This compound was prepared from 3-aminopropanoic acid **(230)** (8.28 g, 93.03 mmol) using the general method 10.4 (a), (17.50 g, 99.7%); m.p. 68 - 69 °C; υ_{max}/cm^{-1} (KBr) 3 420, 3 050, 2 980, 1 720, 1 710, 1 510, 1 390 and 1 210; δ_H ($CDCl_3$) 1.45 (9H, s), 2.58 (2H, m), 3.42 (2H, m), 5.19 (1H, br s), 6.39 (1H, br s), 10.71 (1H, br s); δ_C ($CDCl_3$) 28.3 (q), 34.38 (t), 35.79 (t), 79.7 (s), 156.0 (NCOO) and 177.4 (COOH); *m/z* 189 (M^+, 0.1%), 135 (0.4), 131 (0.3), 98 (8.1); 59 (0.1), 57 (100.0), [Found: C, 49.94%; H, 7.78; N, 7.10; M^+, 189.1001. $C_8H_{15}N_1O_4$ requires C, 50.79; H, 7.94; N, 7.41%; M^+, 189.1001].

N-Ethyl-4-(*t*-butoxycarbonylamino)butanamide (233)

This compound was prepared from **(231)** (1.30 g, 6.41 mmol) using the general method 10.4 (a) and gave identical spectroscopic data to the literature compound.[79]

N-Ethyl-3-(*t*-butoxycarbonylamino)propanamide (234)

This compound was prepared from 3-(*t*-butoxycarbonylamino)propanoic acid (232) (4 g, 21.16 mmol) and ethylamine (6.8 ml) using the general method 10.4 (b) and was obtained as a white crystalline solid (234) (3.20g, 70%); m.p. 122-123 °C; υ_{max}/cm^{-1} (KBr) 3 360, 3 300, 2 980, 1 700, 1 660, 1 550, 1 390 and 1 180; δ_H ($CDCl_3$) 1.11 (3H, t, *J* 7.2), 1.40 (9H, s,), 2.36 (2H, t, *J* 6.0), 3.22 (2H, t, *J* 6.0), 3.38 (2H, t, *J* 6.0), 5.29 (1H, br s) and 6.09 (1H, br s); δ_C ($CDCl_3$) 14.7 (q), 28.3 (q), 34.2 (t), 36.2 (t), 36.6 (t), 79.2 (s), 156.1 (OCONH) and 171.1 (CONH); *m/z* 216 (M^+, 2.3%), 161 (19.4), 143 (100.0), 131 (1.7), 116 (21.5), [Found: C, 55.5%; H, 9.1; N, 13.0; M^+, 216.1475. $C_{10}H_{20}N_2O_3$ requires C, 55.6; H, 9.2; N, 13.0%; M^+, 216.1469].

3-t-Butoxycarbonylaminopropanamide (271).

This compound was prepared from 3-(*t*-butoxycarbonylamino)propanoic acid (232) (6.23 g, 32.94 mmol) and NH_3(g) using the general method 10.4 (a) and was obtained as a white crystalline solid of (271) (5.71 g, 92%); mp 156 - 157 °C; υ_{max}/cm^{-1} (KBr) 3 360, 2 980, 1 690, 1 655, 1 540, 1 290, 625; δ_H ($CDCl_3$) 1.42 (9H, s,), 2.45 (2H, t, *J* 6.00), 3.13 (2H, m), 5.25 (1H, br s), 5.96 (1H, br s). δ_C ($CDCl_3$) 28.32 (q), 35.60 (t), 36.39 (t), 154.75 (OCONH), 175.79 (CONH). *m/z* 133 (M^+,- HCl, 1.7%), 132 (100), 115 (71.3), 88 (36.4), 72 (38.9) [Found: C, 50.99%; H, 8.50; N, 14.80; O, 25.83.; M^+- HCl, 132.0539. $C_8H_{16}N_2O_3$ - HCl, requires C, 51.06; H, 8.51; N, 14.89; O, 25.54% ; M^+ - HCl, 132.0533]

N-Ethyl-4-aminobutanamide hydrochloride salt (235)[79]

This compound was prepared from *N*-ethyl-4-(*t*-butoxycarbonylamino)butanamide (233) (0.86 g, 3.70 mmol) using the general method 10.4 (c) and was obtained as a white crystalline solid (235) (6.07 g, 99%) m.p. 79-80 °C; ν_{max}/cm^{-1} (KBr) 3 242, 3 092, 2 977, 2 894, 1 636, 1 561, 1 647, 1 618 and 1 294; δ_H (D_2O) 0.92 (3H, t, *J* 7.2), 1.76 (2H, m), 2.17 (2H, m), 2.84 (2H,

t, *J* 7.6) and 3.03 (2H, q, *J* 7.2); δ_C (D_2O) 14.29 (q), 23.94 (t), 33.42 (t), 35.34 (t), 39.67 (t) and 175.30 (s); *m/z* 131 (M^+ - Cl, 0.4%), 100 (15.8), 88 (8.7) and 72 (100) [Found: M^+ - Cl, 131.1125. $C_6H_{15}N_2OCl$ - Cl requires M^+, 131.1184].

N-Ethyl-3-aminopropanamide hydrochloride (236)

This compound was prepared from *N*-ethyl-3-(*t*-butoxycarbonylamino)propanamide **(234)** (15.00 g, 64.44 mmol) using the general method 10.4 (c) and was obtained as a white crystalline solid **(236)** (9.38 g, 89%) m.p. 81.5 - 83 °C; ν_{max}/cm^{-1} (KBr) 3 275, 3 030, 2 970, 1 660, 1 650, 1 560, 1 390, 1 260; δ_H (D_2O) 1.03 (3H, t, *J* 7.2), 2.51 (2H, t, *j* 6.8), 3.01 (4H, m); δ_C (D_2O) 14.24 (q), 32.92 (t), 35.33 (t), 36.71(t), 172.58 (CONH); *m/z* 116 (M^+ - HCl, 35.0%), 100 (13.1); 86 (3.8) and 72 (100) [Found: M^+ - Cl, 116.0955. $C_5H_{13}N_2OCl$ - HCl requires M^+, 116.0949].

3-Aminopropanamide hydrochloride (272)

This compound was prepared from 3-*t*-butoxycarbonylaminopropanamide **(271)** (5 0 g, 26.60 mmol) using the general method 10.4 (c) and was obtained as a white crystalline solid **(272)** (3.12 g, 82%); mp 124 - 126 °C; ν_{max}/cm^{-1} (KBr) 3 280, 3 130, 3 050, 2 980, 1 670, 1 600, 1 510, 670. δ_H (D_2O) 2.52 (2H, t, *J* 6.6Hz, H-2), 3.07 (2H, t, *J* 6.6Hz, H-3). δ_C (D_2O) 32.12 (t), 36.37 (t), 175.79 (t); *m/z* 89 (M^+, 12.6 %) 87 (5.8), 70 (63.2), 59 (100), 51 (4.3) [Found: M^+, 88.0651. $C_3H_8N_2O_1$requires: M^+, 88.0635].

N-Ethyl-1,4-diaminobutane dihydrochloride salt (195)[79]

This compound was prepared from *N*-ethyl-4-aminobutanamide hydrochloride salt **(235)** (8.40 g, 50.45 mmol) using the general method 10.4 (d) and was obtained as a white crystalline solid **(195)** (5.82 g, 61%) m.p. 2.18 - 219 °C; ν_{max}/cm^{-1} (KBr) 3 430, 2 970, 2 950 and 670; δ_H (D_2O) 1.08 (3H, t, *J* 7.2), 1.56 (4H, m) and 2.88 (6H, m); δ_C (D_2O) 11.36 (q), 23.62 (t), 24.76 (t), 39.63 (t), 43.73 (t) and 47.16 (t); *m/z* 116

(M^+, 0.3%), 86 (0.6), 73 (18.6) and 58 (100) [Found: M^+ - 2Cl, 116.1316. $C_6H_{16}N_2Cl_2$ - 2Cl requires M^+, 116.1313].

N-Ethyl-1,3-diaminopropane dihydrochloride (228)

This compound was prepared from *N*-ethyl-3-aminopropanamide hydrochloride salt **(236)** (0.367 g 2.41 mmol) using the general method 10.4 (d) and was obtained as a white crystalline solid **(228)** (0.42 g, 99.9%); m.p. 219 °C; ν_{max}/cm^{-1} (KBr) 2 970, 2 955, 2 950, 2 830, 2 760; δ_H (D_2O) 1.09 (3H, t, *J* 7.2 Hz), 1.56 (2H, m), 2.91 (6H, m); δ_C (D_2O) 11.3 (q), 24.6 (t), 37.4 (t), 43.9 (t), 44.8 (t); *m/z* 102 (M^+ - 2HCl, 1.1%), 73 (1.2) and 58 (100) (Found: C, 33.6%; H, 9.4; N, 15.1; M^+ - 2HCl, 102.1165. $C_5H_{16}N_2Cl_2$ - 2HCl requires C, 34.3; H, 9.1; N, 16.0%; M^+ - 2HCl, 102.1154).

10.5 (a) General procedure for the coupling of N-BOC amino acids with alkylamide hydrochloride salts (238) - (245).

A solution of the relevant N-BOC amino acid (1 equiv.) and Et_3N (2.4 equiv.) in acetonitrile (8 ml / mmol of N-BOC amino acid) was cooled to -5 °C. 2-Butylchloroformate (1.2 equiv.) was added dropwise with stirring, and the mixture was left for 3 min. The ethylamide hydrochloride salt (1.1 equiv.) was added dropwise to the resultant mixture, and the solution was stirred for 2 min at 0 °C. The resulting solution was left at 0 °C for 2 h. Solvent was removed *in vacuo*, and the resulting solid was partitioned between EtOAc and water. The organic phase was separated, and the aqueous layer was extracted with several portions of EtOAc. The combined organic phases were then dried (Na_2SO_4), filtered and evaporated *in vacuo* to yield the crude product, which was then recrystallised from EtOAc.

N-Ethyl-4-[3-(*t*-butoxycarbonylamino)propanamido]butanamide (238)

This compound was prepared from 3-(*t*-butoxycarbonylamino)propanoic acid **(232)** (1.54 g, 8.15mmol) and *N*-ethyl-4-aminobutanamide hydrochloride **(235)** (1.36

g, 8.17mmol), and was obtained as a white crystalline solid **(238)** (0.825 g, 34%); m.p. 115-117 °C; ν_{max}/cm^{-1} (KBr) 3 347, 3 299, 3 092, 2 977, 2 934, 1 686, 1 638, 1 560, 1 389, 1 366, 1 279 and 1 252; δ_H ($CDCl_3$) 1.11 (3H, t, *J* 7.2), 1.40 (9H, s), 1.79 (2H, m), 2.19 (2H, m), 2.38 (2H, t, *J* 6.0), 3.20 - 3.37 (6H, m), 5.35 (1H, br s), 6.58 (1H, br s) and 6.98 (1H, br s); δ_C ($CDCl_3$) 14.66 (q), 25.44 (t), 28.31 (q), 33.76 (t), 34.34 (t), 36.24 (t), 36.76 (t), 38.83 (t), 156.13 (s), 171.96 (s) and 172.73 (s); *m/z* 301 (M^+, 1.7%), 245 (12.0), 202 (2.2), 183 (1.2), 159 (11.3), 140 (3.1) and 87 (100) [Found: M^+, 301.1987. $C_{14}H_{27}N_3O_4$ requires M^+, 301.1995].

N-Ethyl-5-[3-(*t*-butoxycarbonylamino)propanamido]pentanamide **(239)**

This compound was prepared from 3-(*t*-butoxycarbonylamino)propanoic acid **(232)** (2.6 g, 13.76 mmol) and *N*-ethyl-5-aminopentanamide hydrochloride **(207)** (2.8 g, 15.51 mmol) and was obtained as a white crystalline solid **(239)** (0.83 g, 19.2%); m.p. 131-132 °C; ν_{max}/cm^{-1} (KBr) 3 351, 3 314, 1 682, 1 638, 1 541 and 1 252; δ_H (200 MHz, $CDCl_3$) 1.37 (3H, unres. t), 1.36 - 1.58 (4H, m), 2.15 (1H, unres. m), 2.35 (2H, unres. m), 3.21 (6H, unres. m), 5.39 (1H, br s), 6.14 (1H, br s) and 6.66 (1H, br s); δ_C ($CDCl_3$) 14.70 (q), 22.59 (t), 28.27 (t), 28.65 (q), 34.20 (t), 35.60 (t), 36.16 (t), 36.72 (t) and 38.62 (t); *m/z* 315 (M^+, 0.7 %), 260 (0.2), 242, (8.0) 155 (7.3), 143 (35.2), 114 (27.1) and 100 (100%) [Found: C, 57.0%; H, 9.3; N, 13.1; M^+, 315.2151. $C_{15}H_{29}N_3O_4$ requires C, 57.1; H, 9.3; N, 13.3%; M^+, 315.2151].

N-Ethyl-3-[4-(*t*-butoxycarbonylamino)butanamido]propanamide **(240)**

This compound was prepared from 4-(*t*-butoxycarbonylamino)butanoic acid **(231)** (1.65 g, 8.05 mmol) and *N*-ethyl-3-aminopropanamide hydrochloride **(236)** (1.40 g, 9.18 mmol) and was obtained as a white crystalline solid **(240)** (0.95 g, 41%); m.p. 138-138.5 °C; ν_{max}/cm^{-1} (KBr) 3 300, 2 980, 1 690, 1 640, 1 530, 1 370, 1 280; ; δ_H ($CDCl_3$) 1.1 (3H, t, *J* 7.2 Hz), 1.40 (9H, s), 1.76 (2H, m), 2.19 (2H, t, *J* 7.2 Hz), 2.39 (2H, t, *J* 6.0 Hz), 3.05 - 3.30 (4H, m), 3.49, (2H, m), 5.08 (1H, br s), 6.55 (1H, br s), 7.05 (1H, br s); δ_C ($CDCl_3$) 14.6 (q), 26.1 (t), 28.33 (q), 33.58 (t), 34.28 (t),

35.67 (t), 35.76 (t), 39.70 (t), 79.14 (s), 156.26 (NCOO), 171.36 (CONH), 173.07 (CONH); *m/z* 301 (M^+, 1.4%), 228, (21.9) 185 (6.4); 158 (5.8), 116 (27.1), 115 (100.0) [Found: C, 55.19%; N, 9.32; H, 13.77; M^+, 301.2009. $C_{14}H_{27}N_3O_4$ requires C, 55.45; N, 9.57; H, 13.86%; M^+, 301.2002].

N-Ethyl-4-[4-(*t*-butoxycarbonylamino)butanamido]butanamide **(241)**

This compound was prepared from 4-(*t*-butoxycarbonylamino)butanoic acid **(231)** (1.52 g, 7.41 mmol) and *N*-ethyl-4-aminobutanamide hydrochloride **(235)** (1.20 g, 7.21 mmol), and was obtained as a white crystalline solid **(241)** (0.71 g, 30%); m.p. 134-136 °C; ν_{max}/cm^{-1} (KBr) 3 310, 2 980, 2 940, 1 690, 1 660, 1 640, 1 540, 1 390, 1 250 and 1 180; δ_H ($CDCl_3$) 1.14 (3H, t, *J* 7.2), 1.43 (9H, s), 1.83 (4H, m), 2.23 (4H, 2 x t), 3.14 - 3.30 (6H, m), 4.94 (1H, br s), 6.59 (1H, br s) and 6.87 (1H, br s); δ_C ($CDCl_3$) 14.73 (q), 25.63 (t), 28.34 (q), 31.15 (t), 33.51 (t), 33.75 (t), 34.35 (t), 38.71 (t), 39.52 (t), 79.09 (s), 173.55 (s) and 174.10 (s); *m/z* 316 ($M^+ + 1$, 0.4%), 315 (1.7), 230 (0.7), 216 (1.0), 197 (14.6), 172 (74.4), 129 (48.3); 114 (30.4), 100 (44.4), 87 (100) and 86 (2.0) [Found: M^+, 315.2154. $C_{15}H_{29}N_3O_4$ requires M^+, 315.2158].

N-Ethyl-5-[4-(*t*-butoxycarbonylamino)butanamido]pentanamide **(242)**

This compound was prepared from 4-(*t*-butoxycarbonylamino)butanoic acid **(231)** (1.40 g, 6.83 mmol) and *N*-ethyl-5-aminopentanamide hydrochloride **(207)** (1.47 g, 8.14 mmol), and was obtained as a white crystalline solid **(242)** (0.92 g, 40%); m.p. 99-100 °C; ν_{max}/cm^{-1} (KBr) 3 302, 2 975, 2 936, 1 690, 1 638, 1 541 and 1 177; δ_H ($CDCl_3$) 1.10 (3H, t, *J* 7.2 Hz), 1.40 (9H, s), 1.47 - 1.84 (6H, m), 2.19 (4H, m), 3.07 - 3.26 (6H, m), 5.05 (1H, br s), 6.13 (1H, br s) and 6.74 (1H, br s); δ_C ($CDCl_3$) 14.73 (q), 22.69 (t), 26.30 (t), 28.31 (q), 28.73 (t), 33.53 (t), 34.22 (t), 35.71 (t), 38.67 (t), 39.61 (t), 79.14 (s), 156.42 (s) and 172.64 (s); *m/z* 329 (1.0%), 256, 230 (0.3), 213 (1.0), 169 (6.9), 100 (100) and 87 (86.5) [Found: $M^+ - C_4H_9O$, 256.1665. $C_{16}H_{31}N_3O_4 - C_4H_9O$ requires M^+, 256.1661].

N-Ethyl-3-[5-(*t*-butoxycarbonylamino)pentanamido)propanamide (243)

This compound was prepared from 5-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (1.50 g, 6.85 mmol) and *N*-ethyl-3-aminopropanamide hydrochloride **(236)** (1.2 g, 7.87 mmol), and was obtained as a white crystalline solid **(243)** (0.83 g, 38%); m.p. 132-133 °C; ν_{max}/cm^{-1} (KBr) 3 347, 3 312, 2 975, 2 934, 1 684, 1 644, 1 541, 1 368 and 1 250; δ_H ($CDCl_3$) 1.10 (2H, t, *J* 7.3), 1.40 (9H, s), 1.46 - 1.71 (4H, m), 2.16 (2H, t), 2.35 (2H, t, *J* 6.0), 3.03 - 3.30 (4H, m), 3.48 (2H, q, *J* 6.0), 4.85 (1H, br s), 6.57 (1H, br s) and 6.82 (1H, br s); δ_C ($CDCl_3$) 14.62 (q), 22.69 (t), 28.34 (q), 29.32 (t), 34.28 (t), 35.64 (t), 39.94 (t), 45.66 (t), 79.02 (s), 156.10 (s), 171.48 (s) and 173.27 (s); *m/z* 315 (M^+, 1.8%), 242 (20.2), 197 (53.3), 171 (27.5), 158 (91.6) and 115 (100) [Found: M^+, 315.2162. $C_{15}H_{29}N_3O_4$ requires M^+, 315.2158].

N-Ethyl-4-[5-(*t*-butoxycarbonylamino)pentanamido)butanamide (244)

This compound was prepared from 5-(-*t*-butoxycarbonylamino)pentanoic acid **(198)** (2.00 g, 9.22 mmol) and *N*-ethyl-4-t-butoxycarbonylaminobutanamide **(235)** (1.74g, 10.45 mmol) and was obtained as a white crystalline solid **(244)** (1.30 g, 41% yield); m.p. 92 - 93 °C ν_{max}/cm^{-1} (KBr) 3 310, 2 970, 2 940, 1 680, 1 640, 1 540, 1 460, 1 370, 1 250, 1 110; δ_H ($CDCl_3$) 0.86 (3H, t), 1.36 (9H, s), 1.43 - 1.81 (6H, m), 2.11 - 2.18 (4H, m), 3.00 - 3.40 (6H, m), 4.81 (1H, br m), 6.68 (2H, br m); δ_C ($CDCl_3$) 14.65 (q), 22.70 (t), 25.53 (t), 28.34 (q), 29.40 (t), 33.73 (t), 34.36 (t), 35.82 (t), 38.80 (t), 39.74 (t), 60.38 (s), 158.21 (s), 172.88 (s), 173.50 (s); *m/z* 329 [M^+, 5.6%), 256 (14.1), 211 (36.6), 172 (33.3), 143 (42.0) and 87 (100.0) [Found: M^+, 329.2321. $C_{16}H_{31}N_3O_4$ requires M^+, 329.2315].

N-Ethyl-5-[5-(*t*-butoxycarbonylamino)pentanamido)pentanamide **(245)**

This compound was prepared from *N*-ethyl-aminopentanamide hydrochloride **(198)** (2.00 g, 11.0 mmol) and *N*-ethyl-5-t-butoxycarbonylaminopentanamide **(207)** (1.77 g, 8.16 mmol) and was obtained as a white crystalline solid **(245)** (0.56 g, 20%); m.p. 133-134 °C; ν_{max}/cm^{-1} (KBr) 3 310, 2 940, 1 690, 1 660, 1 650, 1 540, 1 390,

1 250; δ_H ($CDCl_3$) 1.09 (3H, t, *J* 7.2 Hz), 1.39 (9H, s), 1.55 (8H, m), 2.19 (4H, m), 3.09 (4H, m), 3.22 (2H, m), 4.83 (1H, br s), 6.03 (1H, br s), 6.28 (1H, br s); δ_C ($CDCl_3$) 14.74 (q), 22.62 (t), 22.75 (t), 28.35 (t), 28.79 (t), 28.41 (t), 34.25 (t), 35.67 (t), 35.90 (t), 38.56 (t), 39.79 (t), 79.02 (s), 156.15 (s), 172.87 (s), 173.11 (s); *m/z* 343 (M^+, 0.4%), 270 (2.1), 243, (2.4) 199 (3.1), 186 (3.2), 143 (21.3) and 100 (100.0) [Found: M^+, 343.2446. $C_{17}H_{33}N_3O_4$ requires M^+, 343.2471].

10.5 (b) General Procedure for Removal of BOC Group in Compounds (247) - (254)

The N-BOC protected amine was stirred for 30 min in 3 M HCl / EtOAc (1:1). The solvents were removed under high vacuum to give a clear oil, which was repeatedly triturated with Et_2O to give the desired amino amide hydrochloride salt.

N-Ethyl-4-(3-aminopropanamido)butanamide hydrochloride salt (247)

This compound was prepared from *N*-ethyl-4-[3-(*t*-butoxycarbonylamino)propanamido)butanamide **(238)** (0.74 g, 2.46 mmol) and was obtained as a white crystalline solid **(247)** (0.58 g, 99%); m.p. 167°C (dec.); ν_{max}/cm^{-1} (KBr) 3 414, 3 291, 3 087, 2 977, 2 936, 1 644, 1 557, 1 509 and 1 267; δ_H (200 MHz, D_2O) 0.89 (3H, t, *J* 7.2), 1.58 (2H, dt, *J* 7.0), 1.85 (2H, t, *J* 6.8), 2.61 (2H, t, *J* 6.8) and 3.02 (6H, m); δ_C (D_2O) 14.31 (q), 25.62 (t), 33.95 (t), 35.28 (t), 36.59 (t), 36.83 (t), 39.42 (t), 172.83 (s) and 176.40 (s); *m/z* 202 (M^+- Cl, 2.4%), 172 (33.2), 140 (7.1), 115 (100) and 72 (85.3) [Found: M^+ - Cl, 202.1543. $C_9H_{20}N_3O_2Cl$ - Cl requires M^+, 202.1551].

N-Ethyl 5-(3-aminopropanamido)pentanamide hydrochloride salt (248)

This compound was prepared from *N*-ethyl-5-[3-(*t*-butoxycarbonylamino)propanamido)pentanamide **(239)** (0.78 g, 2.48 mmol) and was obtained as a white crystalline solid **(248)** (0.51 g, 85%); m.p. 162-163°C; ν_{max}/cm^{-1} (KBr) 3 272, 2 949, 1 647 and 1 553; δ_H (200 MHz, D_2O) 0.88 (3H, t, *J* 7.2), 1.32 (4H, m), 2.02 (2H, t, *J* 6.8), 2.59 (2H, t, *J* 6.8) and 2.98 (6H, m); δ_C (D_2O) 14.30 (q),

25.50 (t), 28.40 (t), 31.66 (t), 32.80 (t), 35.20 (t), 36.10 (t), 36.60 (t), 39.72 (t), 172.71 (s) and 177.05 (s); *m/z* 215 [M^+ - HCl, 4.2%), 186 (11.3), 145 (25.5), 128 (67.0), 100 (88.7) and 72 (100) (Found: M^+ - HCl, 215.1628. $C_{10}H_{22}N_3O_2Cl$ - HCl requires M^+, 215.1629].

N-Ethyl 3-(4-aminobutanamido)propanamide hydrochloride salt **(249)**

This compound was prepared from *N*-ethyl-3-[4-(*t*-butoxycarbonylamino)butanamido)propanamide **(240)** (1.0 g, 3.17 mmol) and was obtained as a white crystalline solid **(249)** (0.48 g, 63%); m.p. 159°C (dec.); ν_{max}/cm^{-1} (KBr) 3 077, 2 975, 2 936, 1 636, 1 557, 1 505, 1 480, 1 385 and 1 149; δ_H (200 MHz, D_2O) 0.98 (3H, t, *J* 7.3), 1.83 (2H, m), 2.22 - 2.36 (4H, 2 x t), 2.91 (2H, t, *J* 7.7), 3.10 (2H, q, *J* 7.3) and 3.35 (2H, t, *J* 6.4); δ_C (D_2O) 14.34 (q), 23.86 (t), 33.30 (t), 35.32 (t), 36.29 (t), 36.87 (t), 39.66 (t), 174.41 (s) and 175.62 (s); *m/z* 202 (0.9), 171 (1.8), 158 (100%), 115 (30.5), 100 (29.2) and 87 (25.8) [Found: M^+ - Cl, 202.1547. $C_9H_{20}N_3O_2Cl$ - Cl requires M^+, 202.1556].

N-Ethyl-4-(4-aminobutanamido)butanamide hydrochloride salt **(250)**

This compound was prepared from *N*-ethyl-4-[4-(*t*-butoxycarbonylamino)butanamido)butanamide **(241)** (0.63 g, 1.99 mmol) and was obtained as a white crystalline solid **(250)** (0.46 g, 91%); m.p. 140-141°C (dec.); ν_{max}/cm^{-1} (KBr) 3 320, 3 059, 2 969, 1 638, 1 547, 1 383 and 1 223; δ_H (D_2O) 1.00 (3H, t, *J* 7.3), 1.62 - 1.92 (4H, m), 2.12 - 2.30 (4H, m), 2.92 (2H, t, *J* 7.5) and 3.10 (4H, q, *J* 7.2); δ_C (D_2O) 14.31 (q), 23.88 (t), 25.67 (t), 33.37 (t), 35.30 (t), 39.48 (t), 39.67 (t), 175.62 (s) and 176.43 (s); *m/z* 216 (M^+ - Cl, 1.4%), 185 (1.7), 172 (73.2), 129 (21.0), 114 (25.9), 100 (53.6), 87 (100) and 72 (54.5) [Found: M^+ - Cl, 216.1698. $C_{10}H_{22}N_3O_2Cl$ - Cl requires M^+, 216.1707].

N-Ethyl-5-(4-aminobutanamido)pentanamide hydrochloride salt (**251**)

This compound was prepared from *N*-ethyl-5-(4-aminobutanamido)pentanamide, hydrochloride salt (**242**) (0.6 g, 1.82 mmol) and was obtained as a white crystalline solid (**251**) (0.33 g, 69%); m.p. 138 °C (dec.); ν_{max}/cm^{-1} (KBr) 3 410, 3 270, 2 980, 1 640, 1 560, 1 550; δ_H (D_2O) 0.87 (3H, t, *J* 7.2 Hz), 1.32 (4H, m), 1.75 (2H, m), 1.98 - 2.17 (4H, m), 2.79 (2H, t, *J* 7.6 Hz), 2.98 (4H, m); δ_C (D_2O) 14.29 (q), 23.49 (t), 23.97 (t), 28.42 (t), 33.33 (t), 33.58 (t), 36.05 (t), 39.58 (t), 39.78 (t), 175.50 (s), 177.04 (s); *m/z* 196 (M^+ - HCl - CH_2CH_3,- $2H_2$, 0.8%), 143 (2.6), 129 (1.0), 101 (17.3) and 86 (100.0) [Found: M^+ - HCl - CH_2CH_3, - $2H_2$, 196.1110. $C_{11}H_{24}N_3O_2Cl$ - HCl - CH_2CH_3 - $2H_2$ requires M^+, 196.083].

N-Ethyl-3-(5-aminopentanamido)propanamide hydrochloride salt (**252**)

This compound was prepared from *N*-ethyl-3-(5-aminopentanamido)propanamide, hydrochloride salt (**243**) (0.65 g, 2.06 mmol) and was obtained as a white crystalline solid (**252**) (0.47 g, 91.5% yield); m.p. 141 °C (dec.); ν_{max}/cm^{-1} (KBr) 3 280, 3 080, 3 040, 2 980, 1 640, 1 560, 1 370 and 1 200; δ_H (D_2O) 0.94 (3H, t), 1.48 (4H, m), 1.93 (2H, m), 2.26 (2H; t), 2.85 (2H, m), 3.01 (2H, q) and 3.39 (2H, t); δ_C (D_2O) 14.29 (q), 23.02 (t), 26.92 (t), 35.28 (t), 35.73 (t), 36.28 (t), 36.77 (t), 39.86 (t), 174.36 (s) and 176.85 (s); *m/z* 215 (M^+ - HCl, 3.2%), 199 (1.5), 171 (21.3), 158 (100) and 112 (3.4) [Found: M^+ - HCl, 215.1636. $C_{10}H_{22}N_3O_2Cl$ - HCl requires M^+, 215.1634].

N-Ethyl-4-(5-aminopentanamido)butanamide hydrochloride salt (**253**)

This compound was prepared from *N*-ethyl-4-[5-(*t*-butoxycarbonylamino)pentanamido]butanamide (**244**) (0.49 g,1.49 mmol) and was obtained as a white crystalline solid (**253**) (0.30 g, 77% yield); m.p. 157-159 °C; ν_{max}/cm^{-1} (KBr) 3 260, 2 977, 1 642, 1 551, 1 458 and 1 381; δ_H (D_2O) 0.88 (3H, t, *J* 7.2), 1.41 - 1.64 (6H, m), 2.03 (4H, m), 2.79 (2H, unres. m) and 2.98 (4H, m); δ_C (D_2O) 14.27 (q), 23.04 (t), 25.66 (t), 26.97 (t), 33.94 (t), 35.28 (t), 35.83 (t), 39.37 (t)

and 39.89 (t); *m/z* 230 (0.2%), 200 (16.0), 185 (10.7), 172 (29.7), 114 (56.4), 100 (82.2) and 87 (100) [Found: M^+ - CH_4NCl, 200.1520. $C_{11}H_{24}N_3O_2Cl$ - CH_4NCl requires M^+, 200.1526].

N-Ethyl-5-(5-aminopentanamido)pentanamide hydrochloride salt **(254)**

This compound was prepared from *N*-ethyl-4-[5-(*t*-butoxycarbonylamino)pentanamido]butanamide **(245)** (0.47 g, 1.37 mmol) and was obtained as a white crystalline solid (0.14 g, 40%); m.p. 123-125 °C; ν_{max}/cm^{-1} (KBr) 3 260, 3 040, 1 660, 1 560, 1 250; δ_H (D_2O) 0.92 (3H, t, *J* 7.2 Hz), 1.24 - 1.42 (8H, m), 2.02 (4H, m), 2.75 (2H, m), 2.96 (4H, m); δ_C (D_2O) 14.27 (q), 23.09 (t), 23.50 (t), 26.91 (t), 28.44 (t), 35.23 (t), 35.79 (t), 36.01 (t), 39.66 (t), 39.85 (t), 176.75 (s), 177.04 (s); *m/z* 243 (M^+ - HCl, 3.3%), 227 (1.3), 199 (15.0), 171 (2.1), 143 (40.6), 128 (28.6) and 100 (100.0) [Found: M^+ - HCl, 243.1953. $C_{12}H_{26}N_3O_2Cl$ - HCl requires M^+, 243.1947].

10.5 (c) General Procedure for the BH_3-Reduction of Polyamides **(256)-(263)**

To a suspension of the relevant amino amide hydrochloride salt (1 equiv.) in dry THF (cooled to 0 °C) was added a solution. of BH_3 in THF (1.0 M, 8 equiv). The resultant mixture was heated to reflux for 24 h, and then cooled to R.T. 6M HCl was added slowly to the solution until no more H_2 was evolved. THF was removed by distillation at atmospheric pressure, and MeOH and conc. HCl (2 drops) were added to the residue. Solvents were evaporated *in vacuo*, and the crude product was then recrystallised from MeOH to afford the desired *N*-alkylpolyamine hydrochloride salt.

N-Ethyl-4-(3-aminopropanamino)-1-aminobutane trihydrochloride salt **(256)**

This compound was prepared from *N*-ethyl-4-(3-aminopropanamido)butanamide hydrochloride salt **(247)** (0.51 g, 2.10 mmol) and was obtained as a white crystalline solid (0.418 g, 70.8%); m.p. 284 °C (dec.); ν_{max}/cm^{-1} (KBr) 2 955, 2 828, 2 805, 2 774 and 1 460; δ_H (D_2O) 1.08 (3H, t, *J* 7.2), 1.56 (4H,

unres. m), 1.90 (2H, m) and 2.91 (10H, m); δ_C (D_2O) 11.35 (q), 23.67 (t), 23.71 (t), 24.96 (t), 37.52 (t), 43.72 (t), 45.48 (t), 47.14 (t) and 47.91 (t); *m/z* 173 (M^+- 3HCl, 2.1%), 154, (1.0) 130 (11.9), 113 (16.2), 102 (7.5), 98 (61.3) and 84 (100) [Found: M^+ - 3HCl, 173.1895. $C_9H_{26}N_3Cl_3$ - 3HCl requires M^+, 173.1887].

N-Ethyl-5-(3-aminopropanamino)-1-aminopentane trihydrochloride salt (257)

This compound was prepared from *N*-ethyl-5-(3-aminopropanamido)pentanamide **(248)** (0.49 g, 3.14 mmol) and was obtained as a white crystalline solid (0.33 g, 57%); m.p. 280-281 °C (dec.); ν_{max}/cm^{-1} (KBr) 2 959, 2 932, 2 870, 2 857, 1 385 and 1 165; δ_H (D_2O) 1.08 (3H, t, *J* 7.2), 1.34 (2H, m), 1.53 (4H, m), 1.90 (2H, m) and 2.81 - 3.00 (10H, m); δ_C (D_2O) 11.38 (q), 23.69 (t), 24.58 (t), 25.96 (t), 37.39 (t), 43.67 (t), 45.30 (t), 47.53 (t), 48.28 (t) and 67.27 (t); *m/z* 187 (M^+ - 3HCl, 1.5%), 141 (13.5), 98 (80.3), 87 (25.3) and 84 (100) [Found: M^+ - 3HCl, 187.2041. $C_{10}H_{28}N_3Cl_3$ - 3 HCl requires M^+, 187.2043].

N-Ethyl-3-(4-aminobutanamino)-1-aminopropane trihydrochloride salt **(258)**

This compound was prepared from *N*-ethyl-3-(4-aminobutanamido)propanamide hydrochloride salt **(249)** (0.37 g, 1.54 mmol) and was obtained as a white crystalline solid **(258)** (0.39 g, 89.5%); m.p. 270 °C (dec.); ν_{max}/cm^{-1} (KBr) 2 957, 2 780, 2 822, 1 518, 1 489, 1 460 and 1 391; δ_H (D_2O) 1.06 (3H, t, *J* 7.2), 1.56 (4H, m), 1.93 (2H, m) and 2.83 - 2.97 (10H, m); δ_C (D_2O) 11.34 (q), 19.00 (t), 23.64 (t), 24.74 (t), 33.34 (t), 39.61 (t), 43.89 (t), 44.74 (t) and 45.33 (t); *m/z* 173 (M^+ - 3HCl, 0.7%), 154 (1.3), 144 (2.0), 130 (6.9), 101 (10.7), 84 (44.5) and 70 (100) [Found: M^+ - 3HCl, 173.1887. $C_9H_{26}N_3Cl_3$ - 3HCl requires M^+, 173.1887].

N-Ethyl-4-(4-aminobutanamino)-1-aminobutane trihydrochloride salt (259)

This compound was prepared from *N*-ethyl-4-(4-aminobutanamido)butanamide hydrochloride salt **(250)** (0.63 g, 2.0 mmol) and was obtained as a white crystalline solid **(259)** (0.46 g, 91%); m.p. 269 °C (dec.);

ν_{max}/cm^{-1} (KBr) 2 960, 2 800, 1 580, 1 450, 1 390, 1 370, 1 170; δ_H (D_2O) 1.08 (3H, t, *J* 7.2 Hz), 1.57 (8H, m), 2.88 (10H, m); δ_C (D_2O) 11.4 (CH_3), 23.6 (C-4), 23.5 (C-9), 24.7 (C-3), 39.6 (CH_2CH_3), 43.7 (C-2), 47.1 (C-5), 47.7 (C-10 and C-7); *m/z* 187 (M^+ - 3HCl, 1.1%), 144, (5.9) 115 (3.3), 88 (7.7); 58 (100.0) [Found: M^+ - 3HCl, 187.2059. $C_{10}H_{28}N_3Cl_3$ - 3HCl requires M^+, 187.2049].

N-Ethyl-5-(4-aminobutanamino)-1-aminopentane trihydrochloride salt (**260**)

This compound was prepared from *N*-ethyl-5-(4-aminobutanamido)pentanamide hydrochloride salt (**251**) (0.42 g, 1.58 mmol) and was obtained as a white crystalline solid (**260**) (0.26 g, 53%); m.p. 264 °C (dec.); ν_{max}/cm^{-1} (KBr) 3 430, 2 960, 2 930, 2 870, 1 460; δ_H (D_2O) 1.11 (3H, t, *J* 7.2 Hz), 1.36 (4H, m), 1.43 (6H, m), 2.97 (8H, m), 3.54 (2H, m); δ_C (D_2O) 11.37 (q), 23.60 (t), 23.73 (t), 24.78 (t), 25.98 (t), 28.68 (t), 39.64 (t), 43.67 (t), 47.53 (t), 47.68 (t), 48.13 (t); *m/z* 201 (M^+ - 3HCl, 0.6%), 186 (0.4), 158 (1.7), 143 (4.6), 101 (24.1) and 84 (100.0) [Found: M^+ - 3HCl, 201.2213. $C_{11}H_{30}N_3Cl_3$ - 3HCl requires M^+, 201.2205].

N-Ethyl-3-(5-aminopentanamino)-1-aminopropane trihydrochloride salt (**261**)

This compound was prepared from *N*-ethyl-3-(5-aminopentanamido)propanamide hydrochloride salt (**252**) (0.40 g, 1.59 mmol) and was obtained as a white crystalline solid (0.15 g, 32%); ν_{max}/cm^{-1} (KBr) 3 432 (br), 2 955, 2 818, 2 787, 2 768; δ_H (D_2O) 1.08 (3H, t, *J* 7.3 Hz), 1.25 - 1.32 (2H, m), 1.43 - 1.62 (4H, m), 1.82 - 1.98 (2H, m), 2.77 - 2.99 (10H, m); δ_C (D_2O) 11.33 (q), 23.56 (2 x t), 25.91 (t), 27.06 (t), 39.94 (t), 43.90 (t), 44.71 (t), 45.23 (t), 48.30 (t); *m/z* 187 [M^+ - 3HCl, 1.2%), 158 (1.1), 144 (4.3) and 70 (100.0) [Found: M^+ - 3HCl, 187.2066. $C_{10}H_{28}N_3Cl_3$ - 3HCl requires M^+ , 187.2048].

N-Ethyl-4-(5-aminopentanamino)-1-aminobutane trihydrochloride salt **(262)**

This compound was prepared from *N*-ethyl-4-(5-aminopentanamido)butanamide hydrochloride salt **(253)** (0.13 g, 0.49 mmol) and was obtained as a white crystalline solid (0.08 g, 51%); ν_{max}/cm^{-1} (KBr) 3 433 (br), 2 957, 2 814, 2 773; δ_H (D_2O) 0.86 - 1.68 (13H, m), 2.79 - 2.98 (10H, m); δ_C (D_2O) 11.34, 23.61, 23.66, 25.92, 27.08, 31.47, 39.94, 43.72, 47.10, 47.62, 48.13, 62.12; *m/z* 201 (M^+, 1.3%), 58 (100) 72 (28.1) and 100 (16.4) [Found: M^+ - 3HCl, 201.2204. $C_{11}H_{30}N_3Cl_3$ - 3HCl requires M^+, 201.2205].

N-Ethyl-5-(5-aminopentanamino)-1-aminopentane trihydrochloride salt **(263)**

This compound was prepared from *N*-ethyl-5-(5-aminopentanamido)pentanamide hydrochloride salt **(254)** (0.07 g, 0.25 mmol) and was obtained as a white crystalline solid (0.08 g, 98.4%) m.p. 123-124 °C; ν_{max}/cm^{-1} (KBr) 3 222, 2 953, 1 456 and 1 194; δ_H (D_2O) 1.04 (3H, t, *J* 7.2), 1.21 - 1.48 (12H, m), 2.85 (6H, m), 3.38 (4H, m); δ_C (D_2O) 11.34 (q), 23.63 (t), 23.74 (t), 25.94 (t), 27.09 (t), 28.66 (t), 39.98 (t), 43.65 (t), 47.52 (t), 48.05 (t) and 62.34 (t); *m/z* 215 (M^+ - 3HCl, 3.0%), 172 (1.2), 129 (8.5), 98 (100) and 84 (99.5) [Found: M^+ - 3HCl, 215.2356. $C_{12}H_{32}N_3Cl_3$ - 3HCl requires M^+, 215.2355].

N-3-[5-(*t*-Butoxycarbonylamino)pentanamido]propanamide **(273)**

This compound was prepared from 5-t-butoxycarbonylaminopentanoic acid **(198)** (4 g, 18.43 mmol) and 3-aminopropanamide hydrochloride **(272)** (2.6 g, 20.88 mmol) and was obtained as a white crystalline solid **(273)** (1.25 g, 22%); m.p. 130-131 °C; ν_{max}/cm^{-1} (KBr) 3 360, 3 320, 2 980, 2 950, 2 874, 1 684, 1 655, 1 578, 1 524, 1 390, 1 365 and 1 250; δ_H (D_2O) 1.29 (9H, s), 1.36 - 1.51 (4H, m), 2.04 (2H, t, *J* 7.2), 2.27 (2H, t, *J* 6.8), 2.90 (2H, t, *J* 6.8) and 3.19 (2H, m); δ_C (CD_3OD) 23.64 (t), 28.70 (q), 29.88 (t), 35.66 (t), 36.41 (t) and 40.49 (t); *m/z* 273 [M^+, 3.4%), 226, 216 (3.3), 200 (6.0), 183 (16.4), 101 (100) and 98 (27.9) (Found: M^+, 273.2063. $C_{13}H_{27}N_3O_3$ requires M^+, 273.2052].

N-3-(5-Aminopentanamido)propanamide hydrochloride salt (**274**)

This compound was prepared from *N*-3-[5-(*t*-butoxycarbonylamino)pentanamido]propanamide (**273**) (0.80 g, 2.78 mmol) using the general method 10.5(b) and was obtained as a white crystalline solid (**274**) (0.23 g, 92%); ν_{max}/cm^{-1} (KBr) 3 390, 3 190, 2 970, 2 940, 1 660, 1 650, 1 630 and 1 250; δ_H (D_2O) 1.56 (4H, m), 2.32 (2H, t, *J* 6.6), 2.44 (2H, t, *J* 6.4) and 2.89 (4H, unres. m); δ_C (D_2O) 19.07 (t), 21.89 (t), 22.04 (t), 26.91 (t), 33.84 (t), 39.92 (t), 176.83 (s) and 178.89 (s); *m/z* 187 (M^+ - HCl, 1.4%), 172 (1.0), 113 (1.1), 99 (100) and 71 (9.5) [Found: C, 36.9%; H, 7.8; N, 8.8; M^+ - HCl, 187.1220. $C_8H_{18}N_3O_2Cl$ requires C, 36.9; H, 7.3; N, 16.2%; $C_8H_{18}N_3O_2Cl$ - HCl, 187.1321].

N-3-(5-Aminopentanamino)-1-aminopropane trihydrochloride salt or *N*-(3-aminopropyl)cadaverine (**264**)

This compound was prepared from *N*-3-(5-aminopentanamido)propanamide hydrochloride salt (**274**) (1.0 g, 3.37 mmol) using the general method 10.5 (c) and was obtained as a white crystalline solid (**264**) (0.86 g, 96%); m.p. 181 °C (dec.); ν_{max}/cm^{-1} (KBr) 3 600 - 2 600 (br), 1 736, 1 406; δ_H (D_2O) 1.20 - 1.49 (8H, m), 2.07 - 2.14 (2H, m), 2.63 - 2.91 (4H, m), 3.23 - 3.39 (2H, m); δ_C (D_2O) 21.96 (t), 22.92 (t), 23.10 (t), 26.98 (t), 27.34 (t), 31.58 (t), 33.92 (t), 39.91 (t); *m/z* 159 (M^+ - 3HCl, 5.5%), 158 (1.4), 114 (12.8), 100 (88.7), 98 (29.1) and 84 (100.0) [Found: M^+ - 3HCl, 159.1722. $C_8H_{24}N_3Cl_3$ - 3HCl requires M^+, 159.1735].

N-[3-(*t*-Butoxycarbonylamino)propanamido]-5-[3-(*t*-butoxycarbonylamino)propanamido]pentanamide (**275**)

This compound was prepared from 1-(-*t*-butoxycarbonylamino)propanoic acid (**232**) (1.50 g, 7.93 mmol) and 1,5-diaminopentane (4.4 ml, 43.14 mmol) using the general method 10.5 (a) and was obtained as a white crystalline solid (0.71 g, 32%); m.p. 138-140 °C; ν_{max}/cm^{-1} (KBr) 3 339, 2 934, 2 867, 1 692, 1 644, 1 541, 1 389, 1 366 and 1 250; δ_H ($CDCl_3$) 1.44 (18H, 2 x s), 1.54 (6H, m), 2.42 (4H, 2 x t), 3.22 -

3.46 (8H, m), 5.42 (2H, br s) and 6.30 (2H, br s); δ_C ($CDCl_3$) 23.70 (t), 28.35 (2 x q), 28.86 (2 x t), 36.37 (2 x t), 36.80 (t), 38.96 (2 x t), 79.27 (2 x s), 156.23 (2 x s) and 171.57 (2 x s); *m/z* 314(M^+ - C_4H_9O and C_4H_9, 0.4%), 297 (0.4), 270 (4.1), 184 (7.2), 115 (100) [Found: C, 56.2%; H, 8.8; N, 12.4. M^+ - C_4H_9O and C_4H_9, 314.1602. $C_{21}H_{40}N_4O_6$ - C_4H_9O and C_4H_9 requires C, 56.7; H, 9.0; N, 12.6%].

N-(3-Aminopropanamido)-5-(3-aminopropanamido)pentanamide dihydrochloride (270)

This compound was prepared from *N*--[3-(*t*-butoxycarbonylamino)propanamido]-5-[3-(*t*-butoxycarbonylamino)propanamido]pentanamide **(275)** (0.68 g, 1.53 mmol) using general method 10.5(b) and was obtained as a white crystalline solid (0.45 g, 93%); m.p. 92 °C (dec.); ν_{max}/cm^{-1} (KBr) 3 266, 3 025, 2 938, 1 647, 1 561 and 1 250; δ_H (D_2O) 1.13 - 1.57 (6H, m), 2.51 (4H, t, *J* 6.8) and 3.07 (6H, m); δ_C (D_2O) 24.23 (t), 28.68 (t), 32.86 (t), 36.66 (t), 40.07 (t) and 172.71 (s); *m/z* 244 (M^+ - 2HCl, 1.2%), 228 (6.0), 174 (18.5), 130 (64.9), 115 (50.9), 102 (100), 100 (72.2) [Found: M^+ - 2HCl, 244.1883. $C_{11}H_{26}N_4O_2Cl_2$ - 2HCl requires M^+, 244.1899].

N-(3-Aminopropanamino)-5-(3-aminopropanamino)-1-aminopentane trihydrochloride or *N,N'*-bis(3-aminopropyl)cadaverine (265)

This compound was prepared from *N*-(3-aminopropanamido)-5-(3-aminopropanamido)pentanamide dihydrochloride **(270)** (0.40 g, 1.26 mmol) using the general method 10.5 (c) and was obtained as a white crystalline solid **(265)** (0.22 g, 48%); m.p. 291-292 °C (dec.); ν_{max}/cm^{-1} (KBr) 2 960, 2 930, 2 830, 2 800, 1 650, 1 460, 1 400 and 1 160; δ_H 1.15 - 1.39 (6H, m), 1.85 (4H, m) and 2.77 - 3.14 (2H, m); δ_C 24.68 (t),, 28.66 (t), 36.65 (t), 40.05 (t), 45.32 (t) , 48.27 (t); *m/z* 216, 172 (8.1), 160 (1.5), 98 (93.1) and 84 (100.0) [Found: C, 32.7%; H, 8.1; N, 12.5; M^+ - 4HCl, 216.2319. $C_{11}H_{32}N_4Cl_4$ requires C, 36.5; H,8.8; N, 15.5%; $C_{11}H_{32}N_4Cl_4$ - 4HCl requires M^+, 216.2308].

N-3-[5-(*t*-Butoxycarbonylamino)pentanamido]-1-aminopropane (278)

This compound was prepared from 1-(*t*-butoxycarbonylamino)pentanoic acid **(198)** (0.5 g, 2.30 mmol) and 1,5 diaminopropane (1.2 ml, 16.22 mmol) using the general method 10.5(a) and was obtained as a white crystalline solid (0.59 g, 89%); m.p. 85-87 °C; ν_{max}/cm^{-1} (KBr) 3 335, 3 320, 2 938, 1 686, 1 644, 1 530, 1 391, 1 366 and 1 173; δ_H ($CDCl_3$) 1.44 (9H, s), 1.52 - 1.75 (4H, m), 2.28 (2H, t, *J* 7.3), 3.09 - 3.30 (4H, m), 4.87 (1H, br s), 5.38 (1H, br s), 6.70 (2H, br s); δ_C ($CDCl_3$) 18.99 (t), 22.75 (t), 27.94 (t), 28.35 (q), 29.42 (t), 35.67 (t), 35.93 (t), 39.10 (t), 79.07 (s), 156.16 (s) and 173.80 (s); *m/z* 273 (M^+, 6.3%), 226, 181, 57 (100.0) [Found: C, 57.3%; H, 9.3; N, 11.8; M^+, 273.2032. $C_{13}H_{27}N_{43}O_3$ requires C, 58.4; H, 9.3; N, 11.8%; M^+, 273.2046]. A trace of *N*-[5-(*t*-butoxycarbonylamino)pentanamido]-3-[5-(*t*-butoxycarbonylamino)pentanamido]propanamide contaminated the product *m/z* 300 (M^+- $C_9H_{18}NO_2$, 4.8%), 273 , 226, 181, 57 (100.0) [Found: M^+- $C_9H_{18}NO_2$, 300.1915. $C_{23}H_{44}N_4O_6$ - $C_9H_{18}NO_2$ requires M^+, 300.1923].

N-3-(5-Aminopentanamido)propanamine dihydrochloride (284)

This compound was prepared from *N*-3-[5-(*t*-butoxycarbonylamino)pentanamido]-1-aminopropane **(278)** (3.2 g, 7.21 mmol) and was obtained as a white crystalline solid (1.18 g, 94.6%); ν_{max}/cm^{-1} (KBr) 3 408, 3 264, 3 029, 2 963, 1 634, 1 551; δ_H (200 MHz, D_2O) 1.41 - 1.52 (6H, m), 2.04 - 2.13 (2H, br m), 2.76 - 2.80 (2H, unres. m), 2.89 - 3.02 (4H, m); δ_C (D_2O) 23.08 (t), 29.96 (t), 28.37 (t), 28.60 (t), 35.84 (t), 37.39 (t), 39.89 (t), 176.87 (s); *m/z* 173 (M^+ - 2HCl, 4.9%), 157, 144, 130 and 99 (100.0) [Found: M^+ - 2HCl, 173.1284. $C_8H_{21}N_3OCl_2$ - 2HCl requires M^+, 173.1498]. A trace of *N*-(5-aminopentanamido)-3-[5-(*t*-butoxycarbonylamino)pentanamido]-1-aminopropane contaminated the product.

N-3-(5-Aminopentanamino)-1-aminopropane trihydrochloride salt or (*N*-(3-aminopropyl)cadaverine (**264**)

This compound was prepared from *N*-3-(-5-aminopentanamido)propanamine hydrochloride salt (**284**) (1.0 g, 4.77 mmol) using the general method 10.5 (c) and was obtained as a white crystalline solid (**264**) (1.23 g, 96%); m.p. 181 °C (dec.); ν_{max}/cm^{-1} (KBr) 3 600 - 2 600 (br), 1 736, 1 406; δ_H (D_2O) 1.20 - 1.49 (8H, m), 2.07 - 2.14 (2H, m), 2.63 - 2.91 (4H, m), 3.23 - 3.39 (2H, m); δ_C (D_2O) 21.96 (t), 22.92 (t), 23.10 (t), 26.98 (t), 27.34 (t), 31.58 (t), 33.92 (t), 39.91 (t); *m/z* 159 (M^+ - 3HCl, 5.5%), 158 (21.0), 114 (8.6), 100 (12.5), 98 (61.6) and 84 (100.0) [Found: M^+ - 3HCl, 159.1722. $C_8H_{24}N_3Cl_3$ - 3HCl requires M^+, 159.1735].

10.6 EXPERIMENTAL TO CHAPTER 9

General procedure for feeding experiments to *N. rustica* and *D. stramonium*

Root cultures from *N. rustica* and *D. stramonium*, transformed with *Agrobacterium rhizogenes*, were set up and maintained according to the method described by Walton *et al.*[171]

N-Alkylcadaverine dihydrochlorides, (215) - (218) and (220), (1 mmol) were fed at 1 mM concentrations to flasks of *N. rustica* and *D. stramonium* transformed root cultures, 4 days after subculture. The alkaloids were then harvested 10 days after feeding.

General procedure for extraction of alkaloids

The transformed root cultures were chopped finely and the dry weight of roots recorded. They were then extracted with MeOH at room temperature. The MeOH solution was concentrated *in vacuo* and the weight recorded. The residue was taken up in CH_2Cl_2 (20 ml) and extracted with 1M HCl (2 x 20 ml). The acid layers were combined and washed with CH_2Cl_2 (6 x 20 ml). The acqueous layer was basified with conc. NH_3 and extracted with CH_2Cl_2 (4 x 30 ml). The organic extracts were

dried (Na_2SO_4), filtered and concentraed *in vacuo* to yield the relevant alkaloidal extract.

Separation and identification of alkaloids carried out by Drs. Walton and Parr.

Separation was performed using capillary gas chromatography on a DB-17 column (0.32 mm i.d., 30 m long, 0.25 μm film thickness; J & W Scientific, Folson, U.S.A.) fitted to a Mega HRGC5160 (Carlo Erba, Milan, Italy) gas chromatograph using cold on-column injection. Chromatography was performed at a flow rate of 1.5 $ml.min^{-1}$ He with an initial temperature of 65 °C and a ramp of 6 $°C.min^{-1}$ to 300 °C. Detection was by a phosphorous-nitrogen detector and quantification was by reference to external standards of the relevant alkaloids. The identity of products was confirmed by GC/MS using the same column in a VG TRIO-Is mass spectrometer (VG Masslab, Ltd., Manchester,) linked to a Hewlett Packard 5890 Series II gas chromatograph. Separation conditions were as described by Robins *et al.*[174]

The compounds fed, the number of root cultures, the dry weight of roots, the weight of residue and the resultant weight of extract produced are shown in Tables 10.1 and 10.2.

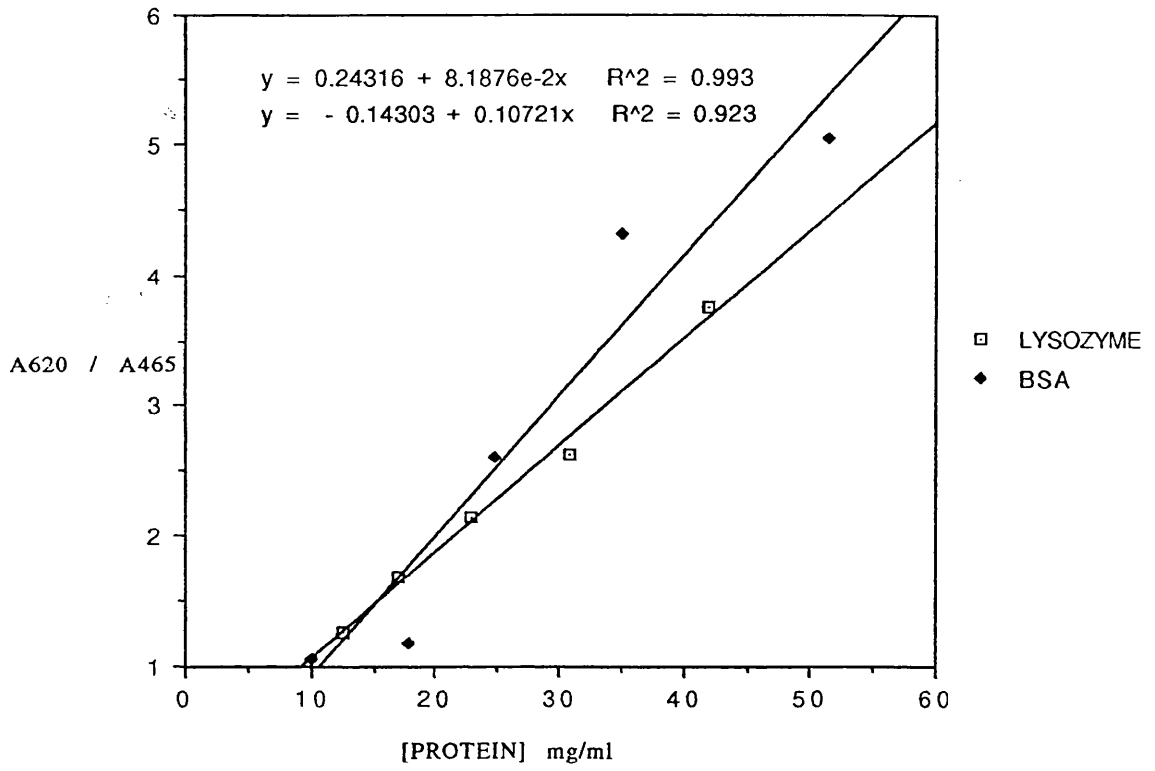
Table 10.1 *N.rustica* feed

Cadaverine Precursor	Number of root cultures	Dry roots (g)	Residue (g)	Extract (g)
N-Ethyl- (215)	10	130.06	3.68	0.10
Control	10	133.04	2.60	0.05
N-Propyl- (216)	5	35.76	1.32	0.01
N-Isopropyl- (218)	5	62.70	1.84	0.03
Control	5	61.45	1.34	0.03
N-Benzyl- (220)	10	92.66	2.83	0.06
Control	10	109.72	2.96	0.05

Table 10.2 *D. srramonium* feed

Cadaverine Precursor	Number of root cultures	Dry roots (g)	Residue (g)	Extract (g)
N-Ethyl- (215)	10	32.35	1.76	0.08
N-Propyl- (216)	4	10.77	0.50	0.02
Control	3	6.19	0.40	0.01
N-Isopropyl- (218)	5	11.01	0.53	0.02
Control	5	9.52	0.46	0.02
N-Butyl- (217)	3	8.51	0.50	0.04
N-Benzyl- (220)	4	11.50	0.63	0.03
Control	3	6.75	0.48	0.02

GRAPH: PROTEIN DETERMINATION FOR LYSOZYME AND BSA



Calculation of Protein Determination

From the graph: $y = -0.14303 + 0.10721x$

Enzyme $A_{620}/A_{465} - A_{620}/A_{465}$ (blank) = 2.85

Thus: $2.85 = -0.14303 + 0.10721x$, giving $x = 27.9$ abs/s

C_ε = concentration of the DAO solution used = 1/200 = 0.005

protein concentration = x/C_ε

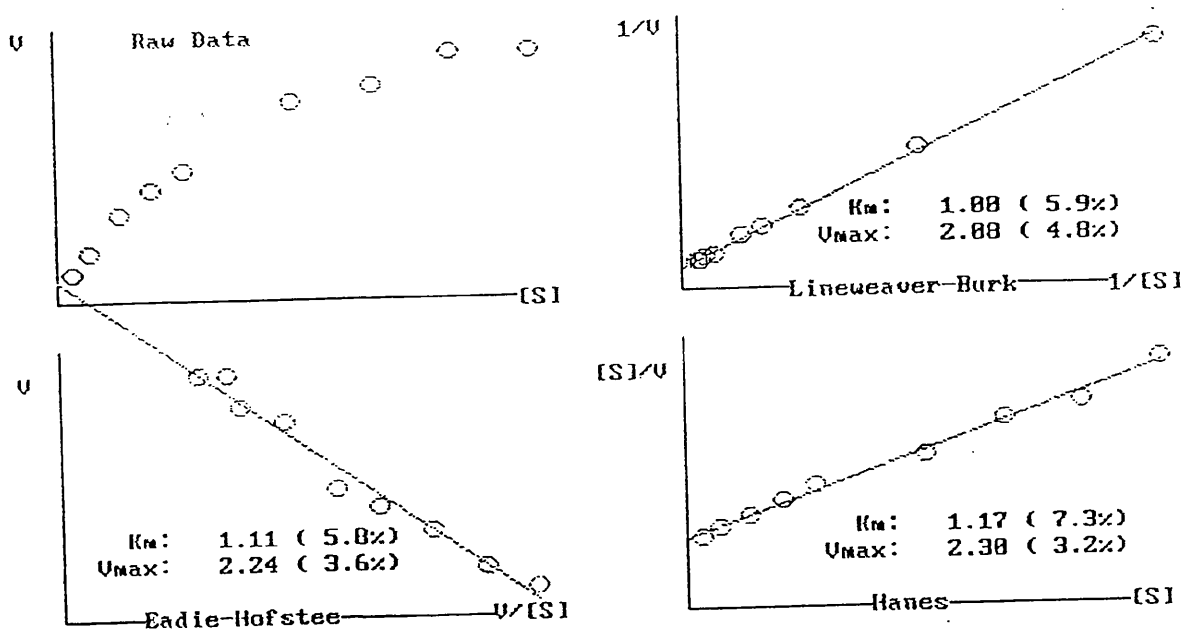
Thus the protein concentration using the BSA calibration curve is 5.6 mg per ml of enzyme solution.

The equivalent calculation using the Lysozyme calibration curve is 6.4 mg /ml.

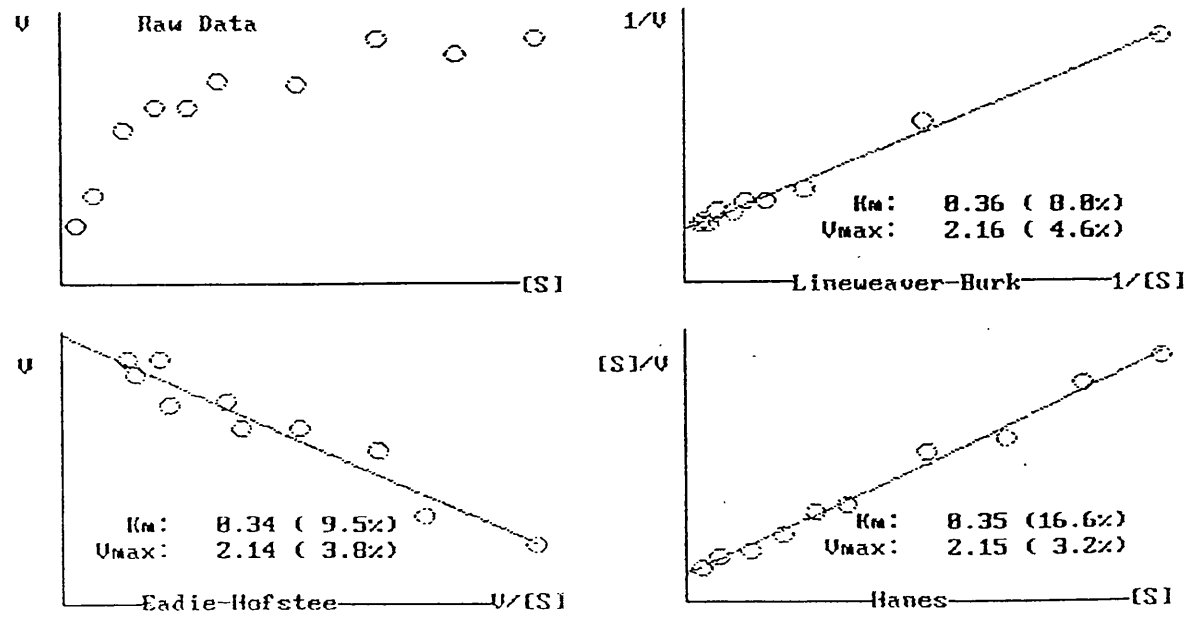
BSA figures have been used throughout.

APPENDIX 2

1.Lineweaver-Burk, Eadie-Hofstee and Hanes plots for the substrate *N*-ethylputrescine, showing comparison of the K_M (mM) and V_{max} (μ mol $mg^{-1}h^{-1}$) values obtained.



2. Lineweaver-Burk, Eadie-Hofstee and Hanes plots for the substrate *N*-propylputrescine, showing comparison of the K_M and V_{max} values obtained.



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