BIOSYNTHESIS OF PYRROLIZIDINE ALKALOIDS AND ANALOGUES

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

by

Alastair Alexander Denholm

Department of Chemistry
The University,
Glasgow.

November 1990

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGEMENTS

I would like to offer my thanks to Professor D.J. Robins for his help and guidance throughout the course of this work including the presentation of this thesis.

The support of the technical staff of the Chemistry Department is gratefully acknowledged, in particular the following: Dr. D.S. Rycroft, Mr. J. Gall and Mr. J. McIver for NMR spectra; Mr. A. Ritchie for mass spectra; Mrs. K. Wilson for elemental analyses; Mrs. F. Lawrie and Mr. G. McCulloch for i.r. spectra; Mrs. P. Tait and her staff for radioactive counting; and Miss Isabel Freer for tissue culture feeding experiments.

I am grateful to Dr. R.J. Molyneaux, U.S.D.A., California, USA for the donation of mother liquors from recrystallisations of riddelliine from *Senecio riddellii*, and to Dr. A.R. Mattocks, MRC Toxicology Unit, Surrey for the gift of seeds of *Crotalaria lachnosema*.

Financial support from the S.E.R.C. is gratefully acknowledged.

Thanks also to Liz Hughes for the excellent typing of this thesis.

To my friends and colleagues in the Henderson Lab., I extend my gratitude. Their constant humour and appetite for living life to the full has made the past

three years all the more tolerable.

Finally, I wish to give my warmest thanks to my Mum and Dad who have been a constant source of encouragement and support throughout my time at University.

TABLE OF CONTENTS.

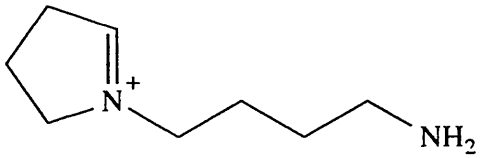
SUMMARY

The work presented in this thesis is divided into four sections : a) Synthesis of analogues of a known intermediate in pyrrolizidine alkaloid biosynthesis and the study of their incorporation into various pyrrolizidine alkaloid-producing plant species; b) Studies on the kinetics of enzymic oxidation of these analogues by pea seedling diamine oxidase; c) Further biosynthetic studies on the pyrrolizidine necine bases; d) Biosynthesis of the 3-hydroxy-3-methylglutarate portion of dicrotaline.

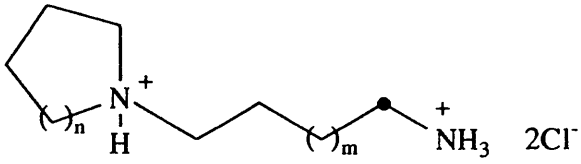
a)Analogue Studies

Radiolabelled analogues of N-(4-aminobutyl)-1,2-didehydropyrrolidinium (A) were synthesised and fed to various plant species which produce pyrrolizidine alkaloids. ^{14}C-Labelled analogues (B) and (C) were synthesised by the extension of an established route to the natural precursor (A). These compounds were of low specific activity (2-30 μCi $mmol^{-1}$). A new synthetic route was developed which furnished a series of analogues (D) and (E) labelled with tritium. These compounds were of higher specific activity (150-250 μCi $mmol^{-1}$), and higher incorporations were observed in the feeding experiments (*Senecio pleistocephalus*, *S. isatideus*, and *Cynoglossum australe spp.*). All feeding experiments used radiolabelled putrescine as an internal standard. Iminium ion precursors (E) were utilised more efficiently than the corresponding saturated salts (D). The saturated salts (D) appeared to inhibit the

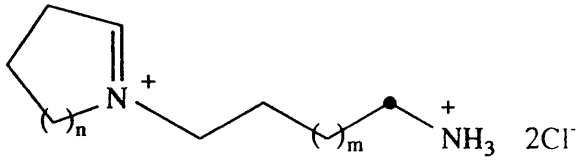
incorporation of putrescine into pyrrolizidine alkaloids. The higher homologues were generally more poorly utilised than putrescine.



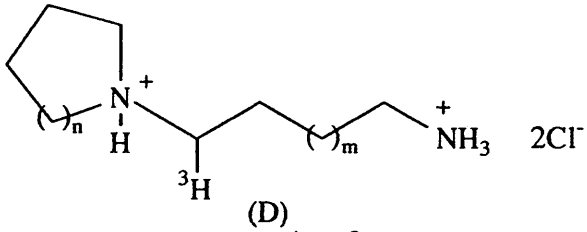
(A)



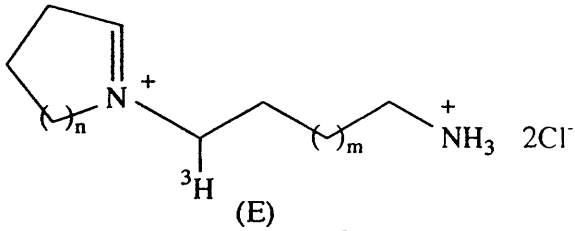
(B)
m=1 or 2,
n=1 or 2
● = ^{14}C



(C)
m=1 or 2,
n=1 or 2
● = ^{14}C



(D)
m=1 or 2,
n=1 or 2
● = ^{14}C



(E)
m=1 or 2,
n=1 or 2
● = ^{14}C

b) Enzyme Kinetics

The enzymic oxidation of non-labelled analogues of (B) and (C) by pea seedling diamine oxidase was studied using a spectrophotometric assay which allowed determination of K_M and V_{max} data for each substrate. Saturated analogues could be classified as putrescine or cadaverine analogues on the basis of binding affinities, as indicated by K_M values. Iminium ions could similarly be divided into sub-groups. The strong binding affinities of the iminium ions are of particular note. This, along with the relatively low catalytic rate constants, makes the iminium ions potential candidates as inhibitors of the enzymic oxidation of putrescine.

c) Biosynthesis of Necine Bases

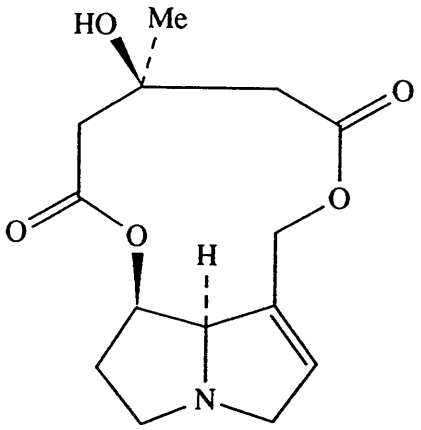
Platynecine and rosmarinecine were identified as intermediates on the biosynthetic pathway to rosmarinine by an intermediate trapping experiment.

N-Acetylputrescine was incorporated more efficiently than putrescine into the alkaloids of *Cynoglossum australe*, while putrescine was the better precursor of rosmarinine in *Senecio pleistocephalus*.

d) Biosynthesis of the 3-Hydroxy-3-methylglutarate (HMG) portion of Dicrotaline

Acetate, mevalonate, and 3-hydroxy-3-methyl-glutarate were not specific precursors for the necic

acid portion of dicrotaline (F) in *Crotalaria lachnosema*. The C-5 methyl group of isoleucine was specifically incorporated into the methyl group of HMG. This was shown by the specific incorporation of [4,5-^{3}H]isoleucine into the necic acid portion of dicrotaline and degradation to establish the site of labelling as the methyl group of dicrotaline.



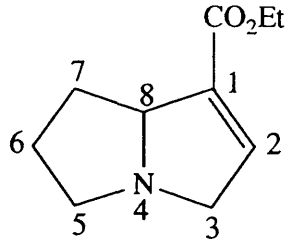
(F)

ABBREVIATIONS.

AFRC	-	Agriculture and Food Research Council.
Boc	-	tertiary butoxycarbonyl
br	-	broad
CBZ	-	benzyloxycarbonyl
d	-	doublet
DAO	-	Diamine oxidase
DEPT	-	Distortionless Enhancement by Polarisation Transfer
DIBAL	-	di-isobutylaluminium hydride
DMAB	-	3-(dimethylamino)benzoic acid
DMF	-	N,N-dimethylformamide
DMSO	-	dimethylsulphoxide
Hz	-	Hertz
i.r.	-	infra red
m	-	multiplet
MBTH	-	3-methyl-2-benzothiazolinone
MRC	-	Medical Research Council
NMR	-	Nuclear Magnetic Resonance
ODC	-	Ornithine decarboxylase
s	-	singlet
t	-	triplet
TBDMS	-	tertiary butyldimethylsilyl
THF	-	tetrahydrofuran
TLC	-	Thin Layer Chromatography
USDA	-	United States Department of Agriculture

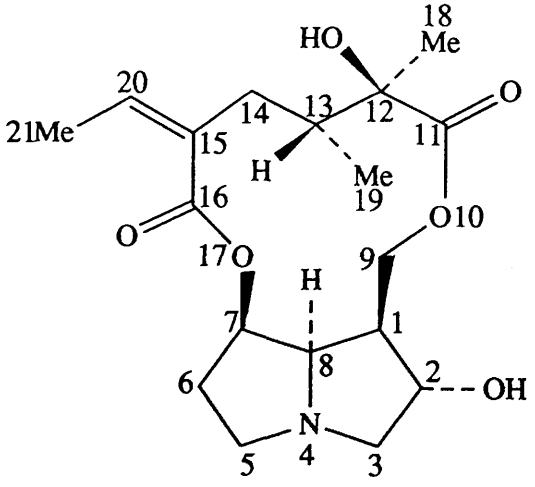
NOTES ON NOMENCLATURE

Pyrrolizidine compounds with one or two double-bonds are named as derivatives of 1H- or 3H-pyrrolizine in accordance with Chemical Abstracts nomenclature, e.g. ethyl 5,6,7,8-tetrahydro-3H-ethyl -pyrrolizine-1-carboxylate.

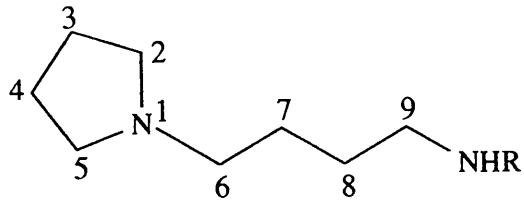


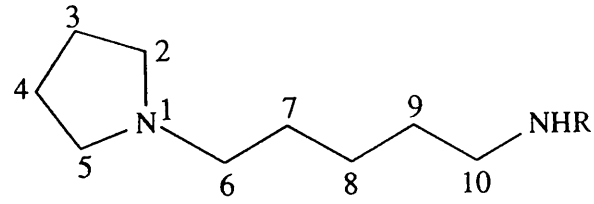
Fully saturated compounds are named as pyrrolizidine derivatives. The stereochemistry of substituents is indicated by the α and β nomenclature.

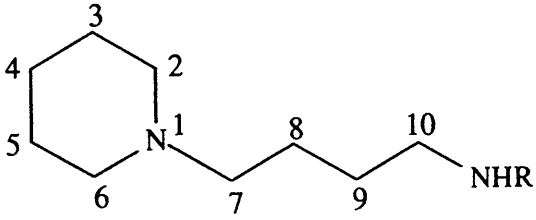
For macrocyclic diester alkaloids, the numbering scheme proposed by Culvenor et al is used (C.C.J. Culvenor, D.H.G. Crout, W. Klyne, W.P. Mose, J.D. Renwick, and P.H. Scopes, J.Chem.Soc.(C), 1971, 3653). For example, rosmarinine is shown.

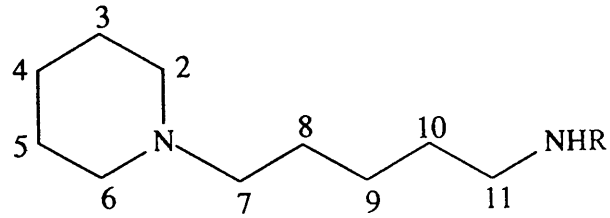


The following numbering schemes are used for pyrrolidine and piperidine derivatives:-









PUBLICATIONS

Some of the work described in this thesis has been presented for publication:-

"Biosynthesis of the 3-Hydroxy-3-methylglutarate Portion of the Pyrrolizidine Alkaloid Dicrotaline,"

J.Chem.Soc.,Chem.Commun., in the Press.

Chapter 1

INTRODUCTION

1.1 Pyrrolizidine Alkaloids.

In the area of natural product chemistry the many unique features of pyrrolizidine alkaloids[1] have been a source of considerable interest. These compounds have been the subject of many elegant synthetic and biosynthetic studies, and their toxicology has been characterised.[2]

Pyrrolizidine alkaloids are found in 14 unrelated plant families.[3] The most examined families are Compositae (eg tribes Senecioneae and Eupatorieae); Leguminosae, in which pyrrolizidine alkaloids are found only in the genus *Crotalaria*; and Boraginaceae, in which every genus examined has been shown to contain pyrrolizidine alkaloids, eg *Heliotropium*. The plant species are geographically widespread and may be present in most environments. It has been estimated that up to 8000 plant species may contain pyrrolizidine alkaloids, representing 3% of the world's flowering plants.[4] *Senecio* is thought to have more species, ca. 1450, than any other genus of plants.[1]

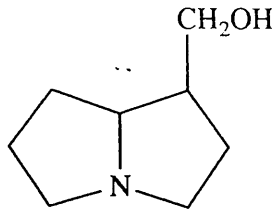
Most pyrrolizidine alkaloids are derivatives of 1-hydroxymethylpyrrolizidine (1) rather than of pyrrolizidine (2). An exception is 1-methylene-pyrrolizidine (3), present in *Crotalaria damarensis*.[5] The aminoalcohol moiety, known as a necine, may be further hydroxylated, and a wide range of necines is known, differing in the position and stereochemistry of the hydroxyl groups, eg. rosmarinecine (4).

Furthermore, many necines contain a 1,2-double bond, eg retronecine (5). Most necines are found in nature as esters, with notable exceptions such as loline (6). The esterifying acids, known as necic acids, are often of an oxygenated, highly branched nature, and some have not been found in any other plant or animal source. Pyrrolizidine alkaloids may be monoesters, such as heleurine (7), diesters, eg heliosupine (8), or macrocyclic diesters, such as fulvine (9).

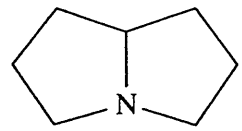
Recently, a series of polyhydroxylated pyrrolizidines has been isolated, exemplified by alexine (10). These differ from the usual pyrrolizidine alkaloid structures, in that they have a carbon substituent at C-3 rather than at C-1. Alexine has been tested for biological activity because of its structural resemblance to the glucosidase inhibitor (11), but it has been shown to be a poor inhibitor of a series of glucosidases.[6]

1.2 Effects of Pyrrolizidine Alkaloids on Humans and Animals.

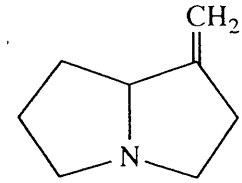
Many pyrrolizidine alkaloids are toxic, and have often been responsible for poisoning of livestock and humans. As long ago as 1787 farmers in Britain suspected the common ragwort *Senecio jacobaea* of being harmful to livestock. The poisoning of cattle has become a severe economic problem: *S. jacobaea* is said to cause more livestock losses than all other poisonous plants put together.[7] Humans have come into contact with pyrrolizidine alkaloids by deliberate use or in mistake for other species, either as medicines or as


CH2OH
N

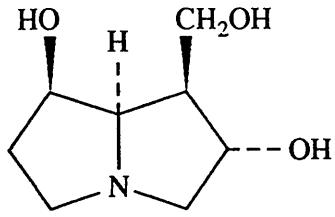
(1)


N

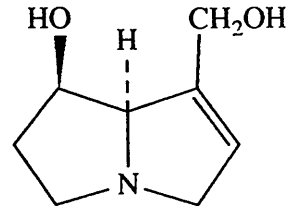
(2)


CH2
N

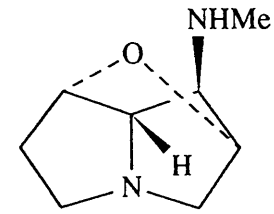
(3)


HO
H
CH2OH
OH
N

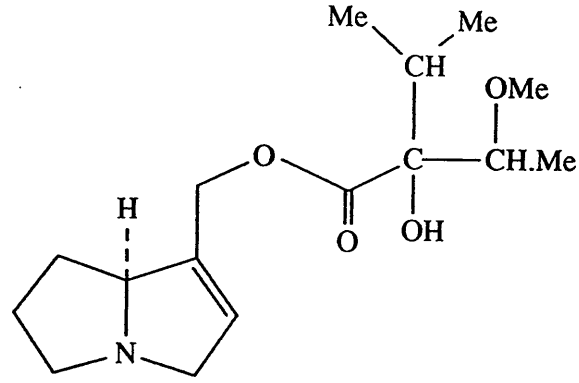
(4)


HO
H
CH2OH
N

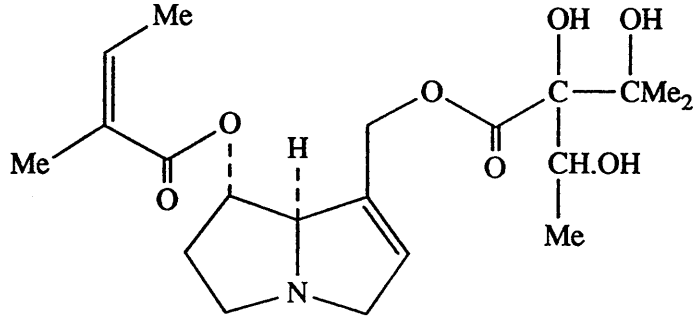
(5)


NHMe
O
H
N

(6)


Me
Me
CH
OMe
O
C
CH.Me
H
O
OH
N

(7)


Me
OH
OH
O
C
CMe2
Me
O
H
O
CH.OH
Me
N

(8)

food. Herbal teas sold in 'health food' shops may contain pyrrolizidine alkaloids, eg comfrey (*Symphytum sp.*).

Human poisoning by consumption of pyrrolizidine alkaloids is well documented. "Bread poisoning", consisting of abdominal pain and the development of ascites occurred in Cape Province, South Africa when seeds of *Senecio ilicifolius* and *S. burchelli* were harvested along with the wheat.[8] Veno-occlusive disease, characterised by centrilobular necrosis of the liver leading to occlusion of central and sublobular hepatic veins and congestion of the liver has been ascribed to ingestion of pyrrolizidine alkaloids via bush teas in Jamaica in the 1950's, and most seriously in Afghanistan in 1974.[9] Around 1600 people were affected by abdominal distention and emaciation, and many died.

Ingestion of pyrrolizidine alkaloids results in tissue damage primarily to the liver, although the lungs, kidneys and sometimes the heart may be affected. Many organic compounds exert toxic effects on liver cells, eg halogenated hydrocarbons, dialkylnitrosamines and aflatoxins. However, progressive and irreversible chronic liver damage, characterised by inhibition of cell division and megalocytosis of the liver is virtually restricted to pyrrolizidine alkaloids.

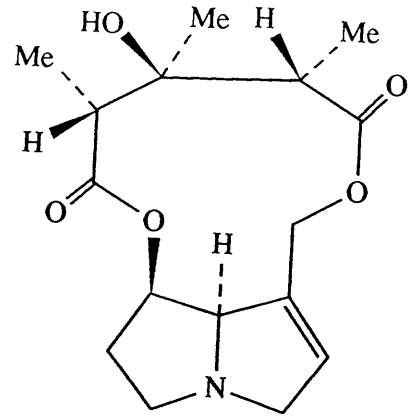
Furthermore, pyrrolizidine alkaloids include some of the relatively few substances occurring in higher plants known to be carcinogenic. Indeed, the

carcinogenic effects are of a chronic and progressive character resembling the effects of mycotoxins rather than those of most known alkaloids.

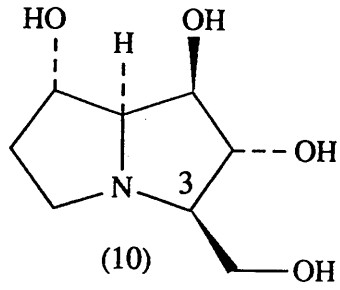
Although pyrrolizidine alkaloids are poisonous to most animals they can be stored by specialised insects, which use them to their advantage. For example, ingestion of *Senecio vulgaris* by the tiger moth *Arctica caja* renders it unpalatable to predators such as birds and spiders.[10] Male danaid butterflies, eg *Danaus plexippus*, transform pyrrolizidine alkaloids into male sex pheromones such as danaidone (12).[11] It has also been suggested that female butterflies will preferentially mate with males which can pass on the alkaloids since her eggs will then become distasteful to predators.[12]

1.3 Metabolism and Cytotoxicity.

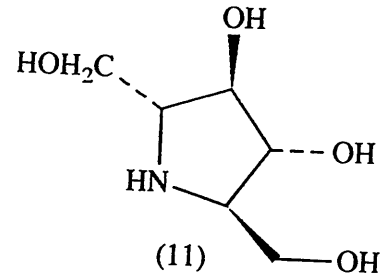
The principal routes of metabolism are ester hydrolysis, conversion into N-oxides and dehydrogenation. The first two are detoxication routes, which result in more water-soluble species and hence facilitate excretion in the urine. The last metabolic pathway is the one associated with cytotoxicity. Various observations point to the fact that it is metabolites rather than the alkaloids themselves which are responsible for the toxic action. For example, the main site of tissue damage is the liver, regardless of the site of administration of the alkaloid. It is known that pyrrolizidine alkaloids can be oxidised by hepatic microsomal enzymes to pyrrolic



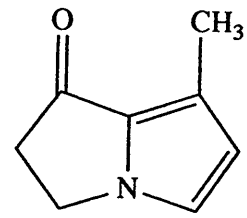
(9)



(10)



(11)



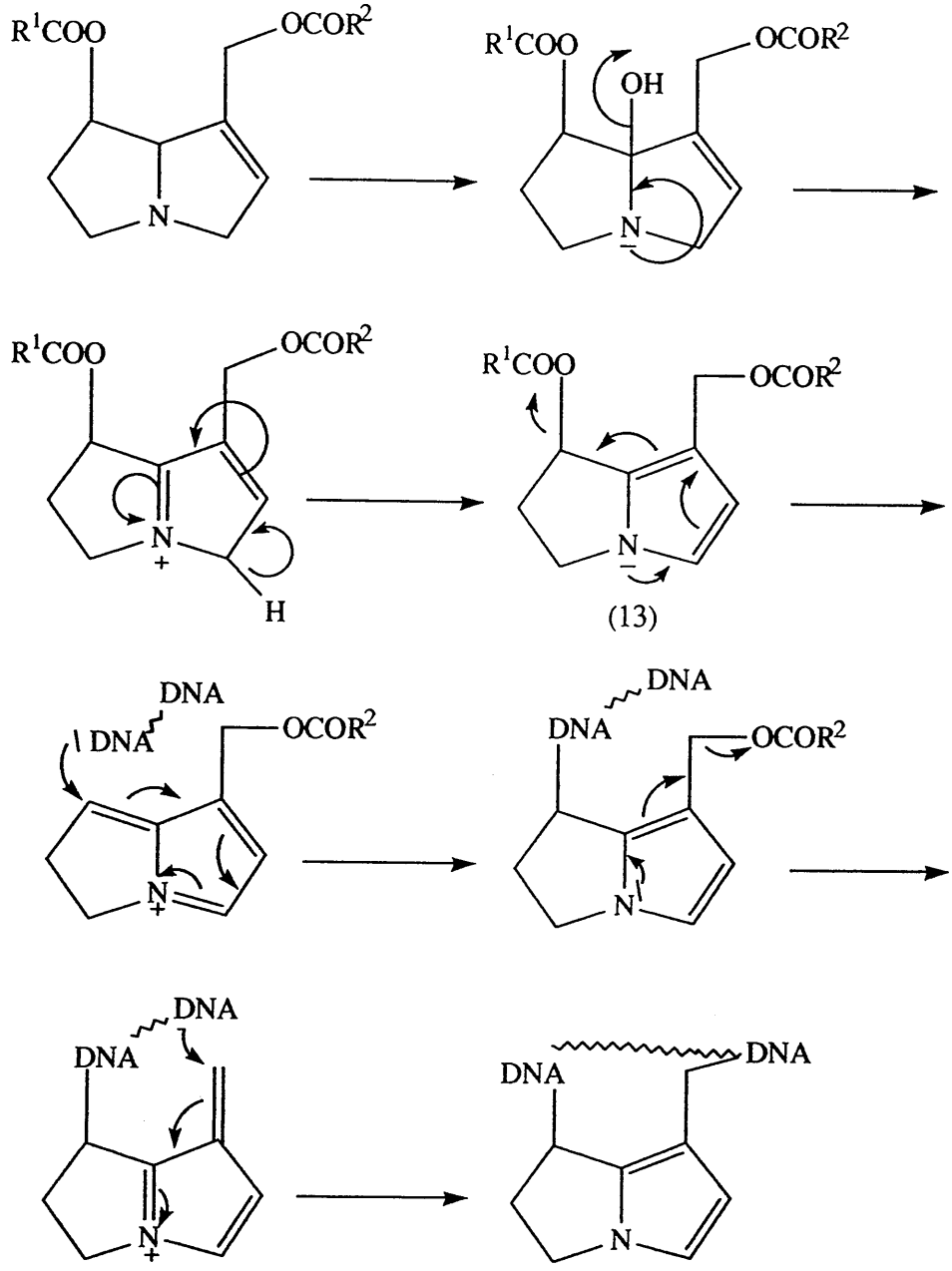
(12)

derivatives (13) (Scheme 1). Pyrrolizidine alkaloids are themselves chemically rather unreactive whereas the pyrrolic metabolites are highly reactive. Mattocks has shown, by measuring the levels of pyrrolic metabolites in the livers of rats, that there is a direct relationship between the amounts of such metabolites and acute hepatotoxicity.[13] Such derivatives can act as bifunctional alkylating agents which can be attacked by nucleophilic moieties on DNA molecules, resulting in cross-linking of the DNA (Scheme 1).

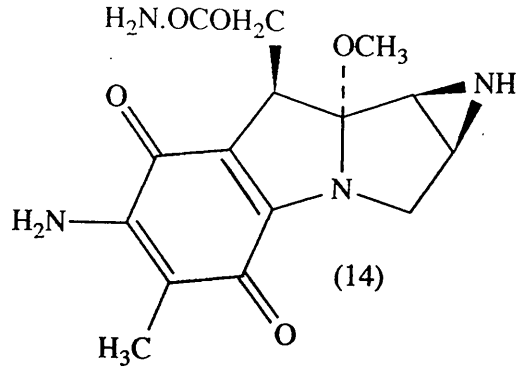
Support for the metabolites of pyrrolizidine alkaloids acting as DNA-interactive ligands has come from the isolation and characterisation of adducts of mitomycin C (14) (which is structurally related to pyrrolizidine alkaloids) and DNA.[14] Adduct (15) demonstrates the cross-linking of a DNA molecule *via* two deoxyguanosine residues.

1.4 Structure-Activity Relationships.

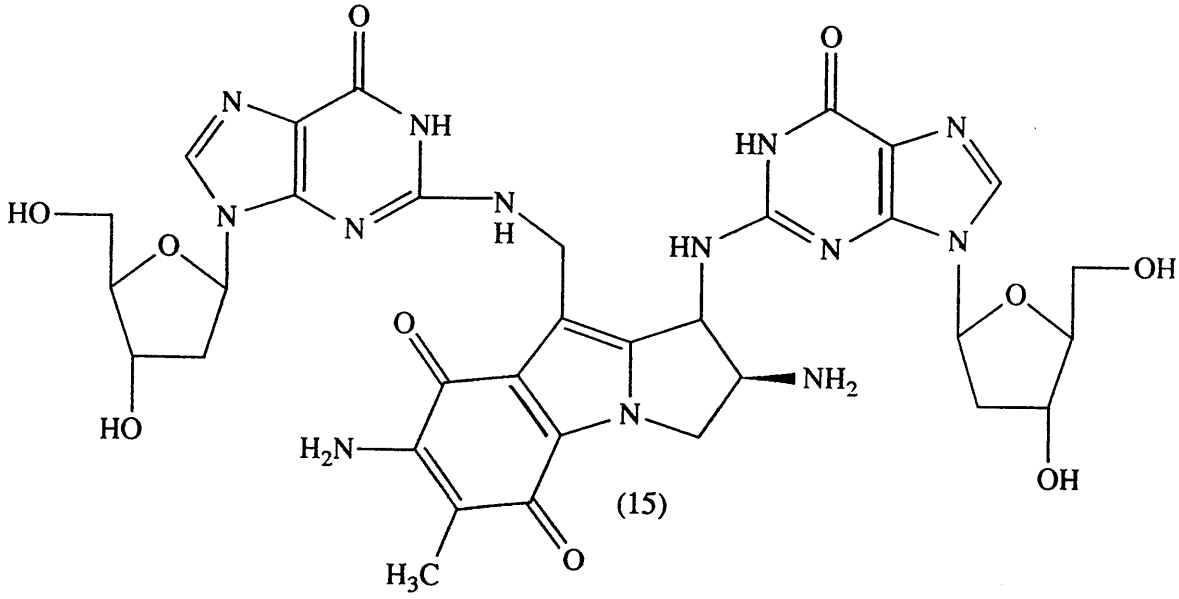
Schoental noted that all known hepatotoxic pyrrolizidine alkaloids were esters of retronecine (5), heliotridine (16), or supinidine (17). Conversely, Grebennik and Zaharova showed that platyphylline (18), which lacks the allylic ester function, produced no trace of liver damage after prolonged exposure in dogs.[15] This led Schoental to propose that the 1,2-double bond was necessary for hepatotoxicity.[16] Furthermore, Culvenor *et al* proposed the mechanism for tissue damage *via* alkylation as shown in Scheme 1.[17]



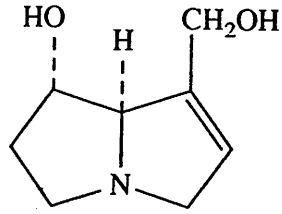
Scheme 1



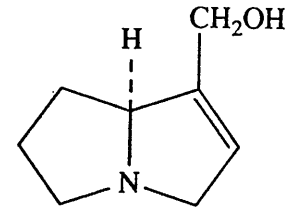
(14)



(15)



(16)



(17)

Detoxication can occur by ester hydrolysis to an aminoalcohol which is not hepatotoxic. Bulky substituents at the α-carbon of the acid portion can inhibit hydrolysis by esterases and hence increase the toxicity of the alkaloids.[18,19] Hence, macrocyclic diesters are usually more toxic than similar diesters.

Toxicity is also affected by the lipophilicity and base strength of the alkaloids.[2] Pyrrolizidine alkaloids which are highly lipophilic are more susceptible to oxidation by hepatic microsomal enzymes whereas those which are more water soluble can be more readily excreted and form lower levels of toxic metabolites. Alkaloids of lower base strength tend to be more lipophilic and hence more toxic. A higher proportion of the more basic alkaloids is ionised at physiological pH and can therefore be excreted.

The toxicity of many synthetic analogues of pyrrolizidine alkaloids has been tested. Synthanecine A bis-diethyl carbamate (19) is more toxic than monocrotaline (20), demonstrating that the second (pyrrolidine) ring is not required for toxicity.

1.5 Clinical Uses of Pyrrolizidine Alkaloids.

Due to their pronounced effects on both animals and humans, such as cytotoxicity, mutagenicity, and antimitotic action, many pyrrolizidine alkaloids have been tested for anti-cancer activity. This anti-tumour activity may be due to two unrelated mechanisms:

1. The antimitotic effect of pyrrolic metabolites.
2. An unknown mechanism involving N-oxides.

To date, indicine N-oxide (21) is the only pyrrolizidine alkaloid to have undergone clinical trials as an anti-cancer drug. A course of indicine N-oxide was given to 10 patients with advanced acute leukemia, with the result that two patients had complete remissions, while one had a partial remission.[20]

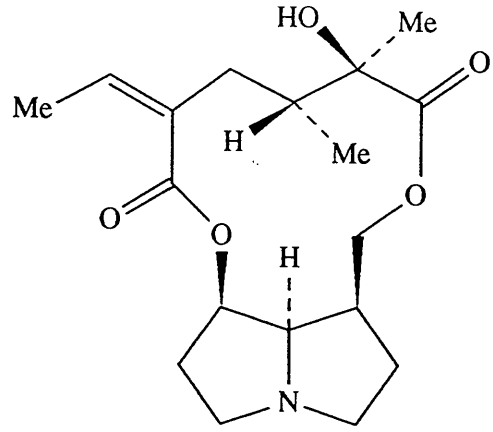
Also of interest is the use of the non-hepatotoxic platyphylline (18) in the USSR as an antispasmodic and mydriatic.[21]

Further information on pyrrolizidine alkaloids can be found in annual reviews.[22]

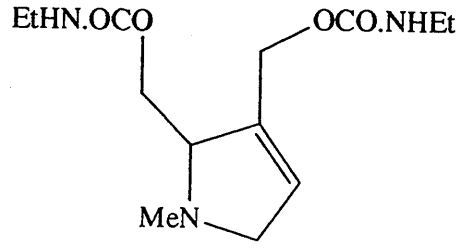
1.6 Aims of Project.

The biosynthesis of pyrrolizidine alkaloids has stimulated much interest in our research group.[23] This work is reviewed in Chapter 2. The biosynthetic pathway to necines involves the coupling of two molecules of 1,4-diaminobutane (putrescine) *via* the iminium ion (22) to give the necine (23). A number of steps in this biosynthetic pathway involve oxidation of primary amines to the corresponding aldehydes.

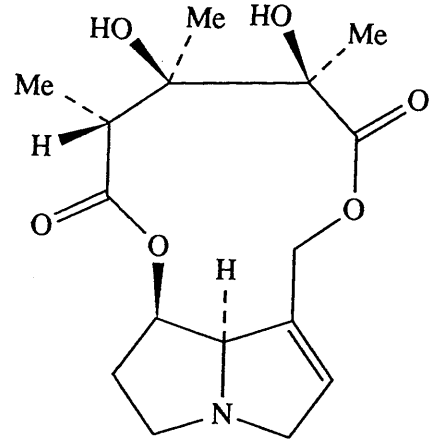
These primary amine oxidations are thought to be catalysed by a diamine oxidase (DAO). It was decided to test whether DAO would accept unnatural substrates. A two-fold strategy was adopted to probe this proposal.


HO
Me
O
Me
H
Me
O
O
O
H
N

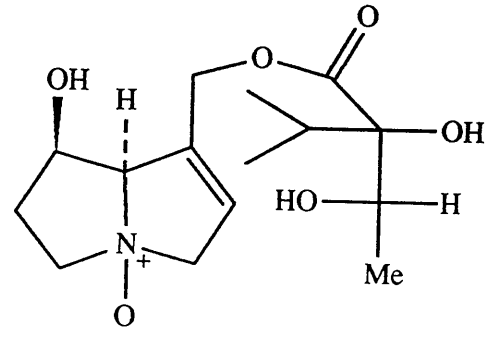
(18)


EtHN.OCO
OCO.NHEt
MeN

(19)


HO
Me
HO
Me
Me
H
O
O
O
O
H
N

(20)


O
O
OH
H
OH
HO
H
N+
O
Me

(21)

Firstly, radiolabelled analogues of N-(4-aminobutyl)-1,2-didehydropyrrolidinium (22), an established intermediate in pyrrolizidine alkaloid biosynthesis, were prepared and their incorporation into plant species which produce pyrrolizidine alkaloids was studied (Chapter 3). Assessment of the biological activity of biosynthetically-derived analogues should be instructive in the study of structure-activity relationships of pyrrolizidine alkaloids. Secondly, determination of kinetic parameters (K_M and V_{max}) for the analogues of iminium ion (22) by a spectrophotometric assay should give an insight into the enzyme specificity of DAO. These results are discussed in Chapter 4.

Chapter 5 contains a description of various biosynthetic studies on pyrrolizidine necine bases. It was deemed necessary to provide further evidence for the intermediacy of the iminium ion (22) in pyrrolizidine alkaloid biosynthesis. Accordingly, intermediate trapping experiments were carried out with the aim of isolating derivatives of (22) labelled with either ^{3}H or ^{2}H.

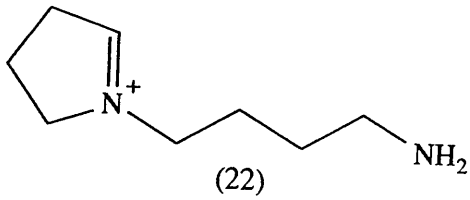
The order of hydroxylation in the transformation of isoretronecanol (23) into rosmarinecine (4) has not been firmly established, although platynecine (25) is an efficient precursor of rosmarinecine. An intermediate trapping experiment was carried out to detect whether or not platynecine is present in *S. pleistocephalus*.

No studies have been published on the enzyme stereospecifities of the biosynthesis of necine bases having H-8β stereochemistry. The biosynthesis of

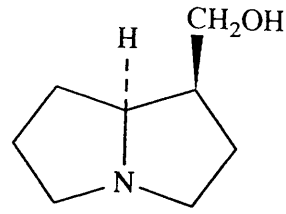
cynaustine (26) and cynaustraline (27) in *C. australe* R.Br. was investigated in feeding experiments with putrescine enantiomerically labelled with deuterium.

The possible role of N-acetylputrescine (24) in pyrrolizidine alkaloid biosynthesis was investigated in *Senecio pleistocephalus* S. Moore and *Cynoglossum australe* R.Br.

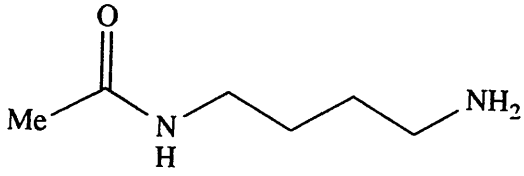
It was decided to widen the scope of the study to encompass the acid portions of pyrrolizidine alkaloids. An investigation of the biosynthesis of the diacid portion of dicrotaline (28), the trivial name of which is dicrotalic acid, was carried out with the plant species *Crotalaria lachnosema* Stapf. This diacid has a deceptively simple structure. The findings of this work are presented in Chapter 6.



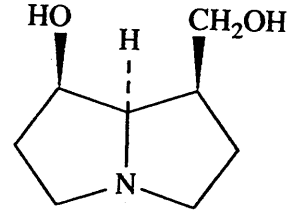
(22)



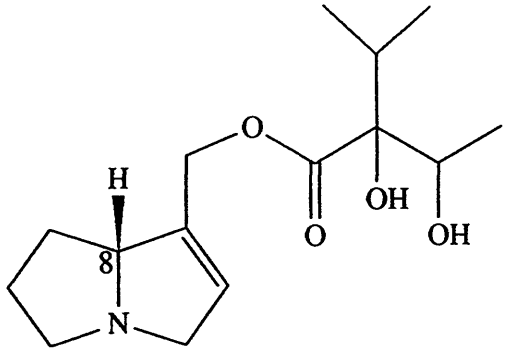
(23)



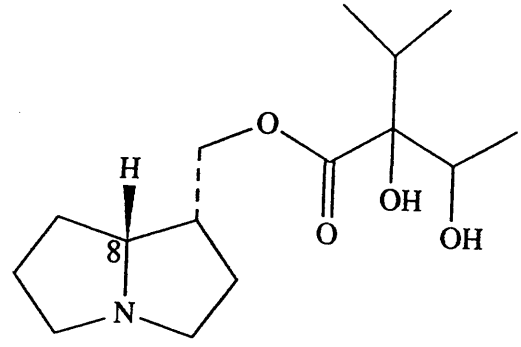
(24)



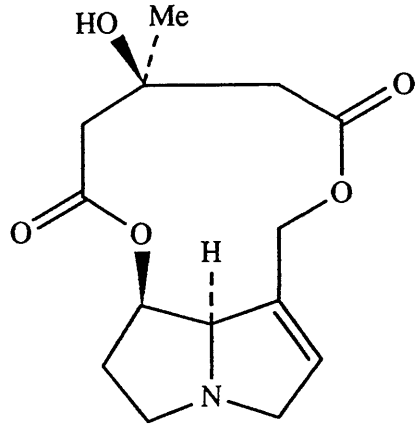
(25)



(26)



(27)



(28)

Chapter 2

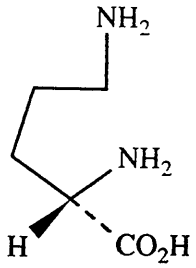
BIOSYNTHESIS OF PYRROLIZIDINE ALKALOIDS

2.1 Biosynthesis of Necine Bases.

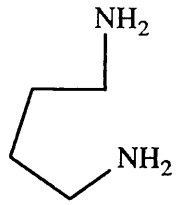
In the past three decades much effort has been expended on the elucidation of the biosynthesis of various derivatives of 1-hydroxymethylpyrrolizidines (necines). The original postulate of Sir Robert Robinson[24] that the pyrrolizidine ring system is derived from two molecules of ornithine (29) via putrescine (30) has been confirmed, and in the ensuing period a vast array of information been unearthed, leading to the identification of biosynthetic intermediates, and to an understanding of the stereochemical aspects of many of the enzymic processes.

Initial experiments utilised radioisotopes (^{3}H, ^{14}C), although the greatest advances have been achieved by the use of stable isotopes (^{2}H, ^{13}C) in conjunction with nuclear magnetic resonance spectroscopy. Complete labelling patterns were obtained by the use of specifically labelled ^{13}C-putrescines, and the stereochemistry of many enzymic processes were determined by the use of putrescines enantiomerically labelled with deuterium.

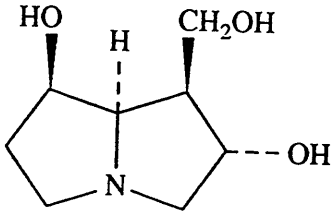
The most widely studied pyrrolizidine base is retronecine (5), the most common necine. Much detailed research has also been carried out on rosmarinecine (4), and the scope of biosynthetic studies has been further widened by elucidation of aspects of the biosynthesis of heliotridine (16) and otonecine (31).[23]


NH2
NH2
H
CO2H

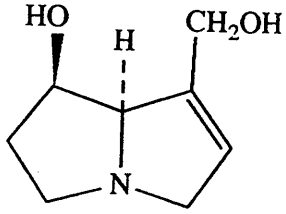
(29)


NH2
NH2

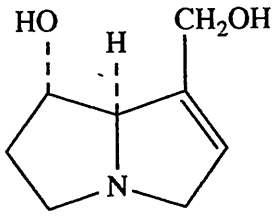
(30)


HO
H
CH2OH
OH
N

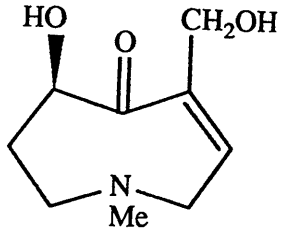
(4)


HO
H
CH2OH
N

(5)


HO
H
CH2OH
N

(16)

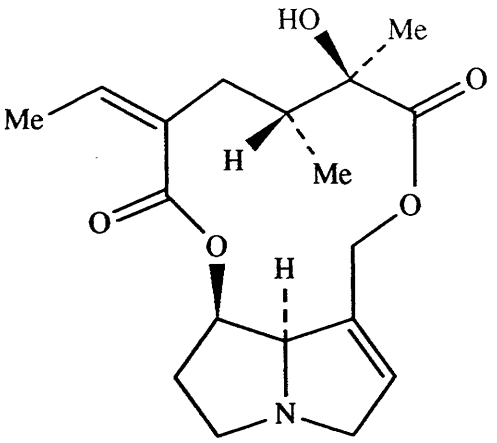

HO
O
CH2OH
N
Me

(31)

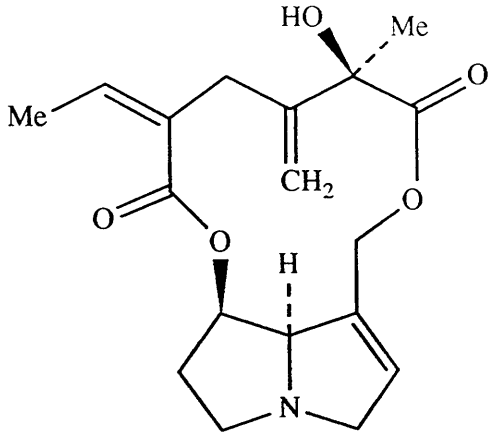
Biosynthetic studies on the necine bases were initiated in 1962 when Nowacki and Byerrum demonstrated that [2-^{14}C]ornithine was specifically incorporated into retronecine (5), the base portion of monocrotaline (20) in *Crotalaria spectabilis*.[25] They also fed [1-^{14}C]acetate and [1-^{14}C]propionate to this species, resulting in labelling mainly in the monocrotalic acid portion.

Senecio douglasii produces four alkaloids: senecionine (32), seneciphylline (33), retrorsine (34) and riddelliine (35), all of which contain retronecine as the base portion. Bottomley and Geissman[26] carried out feeding experiments with [1,4-^{14}C]putrescine, [2-^{14}C]ornithine and [5-^{14}C]ornithine which produced total incorporations into *S.douglasii* of 0.18, 0.30, and 0.75% respectively. It was shown that 94-98% of the radio-activity resided in retronecine (5) by basic hydrolysis of each alkaloid mixture. Oxidative degradation with osmium tetroxide-sodium periodate yielded C-9 of retronecine as formaldehyde, isolated as its dimedone derivative (36) (Scheme 2). This derivative contained one-quarter of the total activity of retronecine, suggesting that C-2 and C-5 of ornithine become equivalent in the formation of ring B of retronecine.

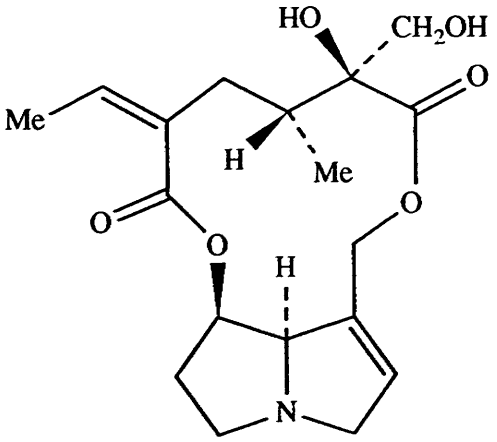
In 1975, Bale and Crout[27] suggested that the use of a double isotope (^{3}H/^{14}C) technique would be of value, since this would allow the relative efficiencies of different precursors to be measured. Using L-[3-^{3}H]arginine as a standard, they showed that in *S.*



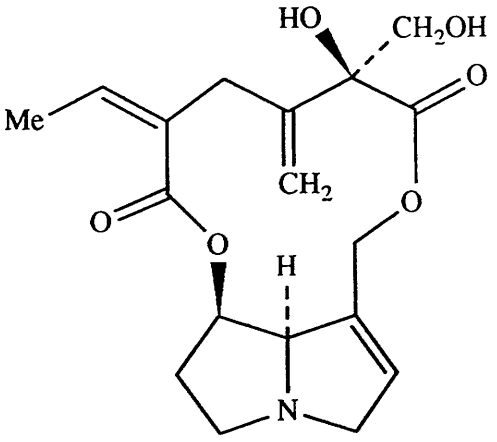
(32)



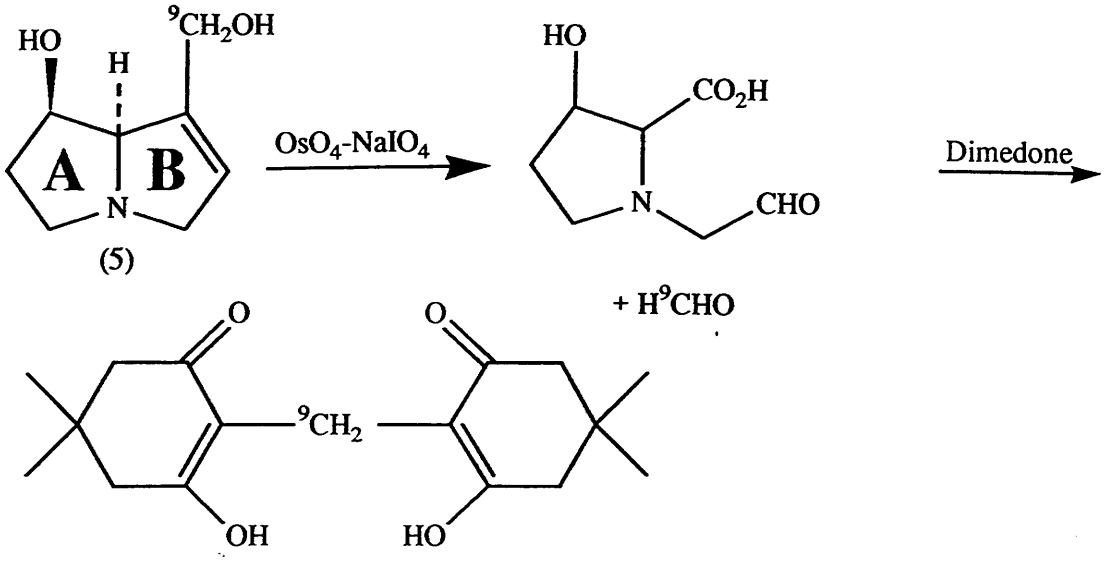
(33)



(34)



(35)



(5)

Scheme 2

magnificus arginine (37) and ornithine (29) are both specifically incorporated into retronecine, the base portion of senecionine (32). Feeding a mixture of L-[U-^{14}C]arginine and L-[3-^{3}H]arginine led to a decrease from the initial $^{3}H/^{14}C$ ratio of 4.84 to 3.0 whereas the $^{3}H/^{14}C$ ratio fell from 3.62 to 2.2 in senecionine when a mixture of L-[U-^{14}C]ornithine and L-[3-^{3}H]arginine was fed. Hence, in the biosynthesis of retronecine in *S. magnificus*, ornithine is marginally more efficiently incorporated than is arginine.

Different groups of workers have contrasting views on the role of amino acids in necine biosynthesis via putrescine. Birecka et al suggest that in *Heliotropium* spp. arginine (37) acts as the precursor for putrescine (30)[28] whereas ornithine (29) is the progenitor in *Senecio* and *Crotalaria* spp.[29] On the other hand, Hartmann et al[30] are of the opinion that arginine alone is the source of putrescine in *S. vulgaris*.

The first of a vast range of feeding experiments with *Senecio isatideus*, which produces retrorsine (34), was carried out by Robins and Sweeney in 1978.[31] Total incorporations of 1.6-5.2% were obtained on feeding putrescine (30), spermidine (38) and spermine (39), the latter two of which are likely to be utilised via putrescine. This work also confirmed the role of ornithine (29) and arginine (37) in necine biosynthesis. Labelled forms of compounds (30), (38) and (39) were incorporated specifically into retronecine and incorporations were ten times higher than for ornithine or arginine, providing further evidence for the theory

of Geissman and Crout that putrescine (30) follows ornithine (29) in the biosynthetic pathway.

Specific incorporation of [5-^{14}C]ornithine, [1,4-^{14}C]putrescine, spermine and spermidine, both labelled [1,4-^{14}C] in the tetramethylene portion was demonstrated by degradative work. One quarter of the ^{14}C activity from retronecine (5) was shown to be located at C-9 by osmium tetroxide-sodium periodate oxidation, confirming the results of Bottomley and Geissman.[26] In addition, C-(5+6+7) could be isolated by a modified Kuhn-Roth oxidation, yielding β-alanine (40) as its 2,4-dinitrophenyl derivative, containing 22-24% of the total radioactivity (Scheme 3). This demonstrates that C-2 and C-5 of ornithine become equivalent in the formation of ring A of retronecine (5).

Further progress in this work was hindered by the lack of applicable degradations to locate all the ^{14}C labels. The next advance came with the advent of ^{13}C-labelling experiments. The use of specifically labelled ^{13}C-putrescines allowed full labelling patterns to be established by means of ^{13}C nmr spectroscopy.[32]

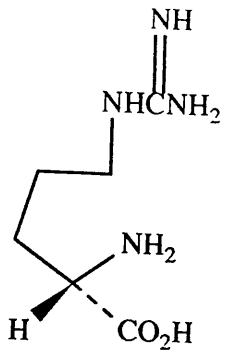
The first precursor employed was [1,4-$^{13}C_2$]putrescine dihydrochloride (43), synthesised by Khan and Robins as shown in Scheme 4. The ^{13}C label was introduced by S_N2 displacement of bromide from 1,2-dibromoethane (41) with potassium [^{13}C]cyanide. The resultant [1,4-$^{13}C_2$]succinonitrile (42) was reduced with borane in tetrahydrofuran (THF), and acidification gave

the dihydrochloride (43). [2,3-$^{13}C_2$]Putrescine dihydrochloride (44) was produced in a similar manner, starting from reaction of [1,2-$^{13}C_2$]-1,2-dibromoethane with sodium cyanide. Reduction to the diamine with borane in THF and acidification gave the desired product (44).

These two samples were administered individually to *S. isatideus* by the xylem pricking method[31], and after a suitable period of time, the plants were harvested, and the ^{13}C-labelled retrorsine was isolated. After basic hydrolysis, ^{13}C NMR spectroscopy revealed complementary labelling patterns in the two retronecine samples.[32]

Retronecine derived from [1,4-$^{13}C_2$]putrescine (43) showed equal enrichment of the ^{13}C NMR signals corresponding to C-3, C-5, C-8 and C-9 (Scheme 5), although signal broadening resulted from geminal coupling (C-3 to C-5 and C-8 to C-9) in molecules where combination of two ^{13}C labelled putrescines had occurred. Feeding of [2,3-$^{13}C_2$]putrescine (44) produced a pair of doublets straddling the natural abundance signals of C-1 and C-2 (J 71Hz) and another pair of doublets corresponding to C-6 and C-7 (J 34Hz) (Scheme 6). There was almost equal enrichment at all four sites, consistent with the involvement of a later symmetrical C_4-N-C_4 intermediate in the biosynthetic pathway.

Further evidence for this later intermediate was produced when almost equal enrichment at C-3, C-5, C-8

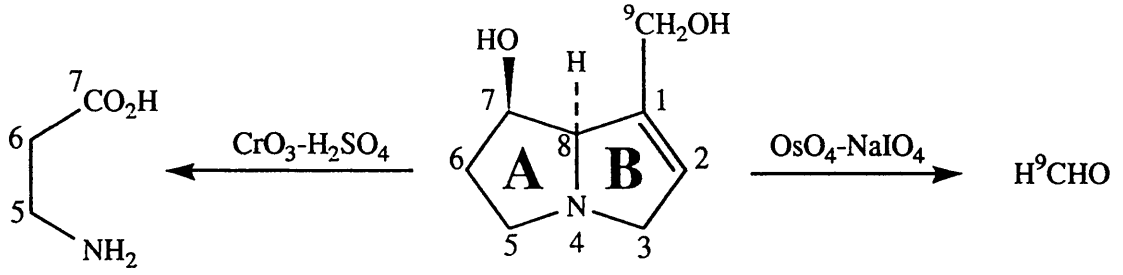


(37)

$H_2N(CH_2)_3NH(CH_2)_4NHR$

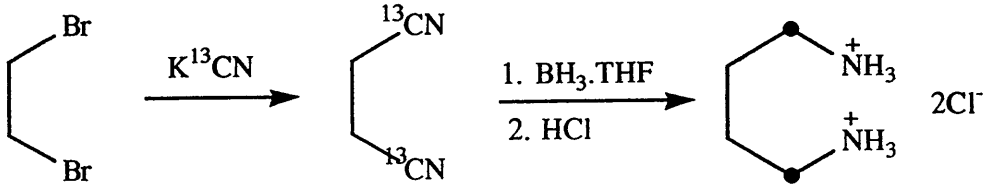
(38) R=H

(39) $R=(CH_2)_3NH_2$



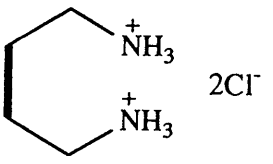
(40) (5)

Scheme 3



(41) (42) (43)

Scheme 4

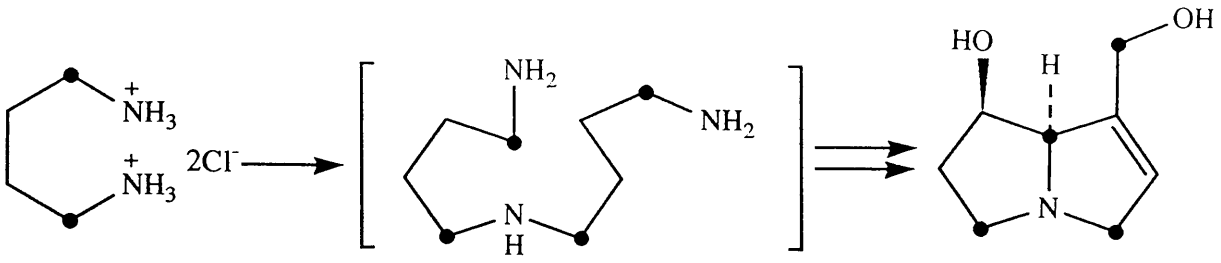


(44)

and C-9 was observed on feeding [1-^{13}C]putrescine dihydrochloride to *S. isatideus*.[32]

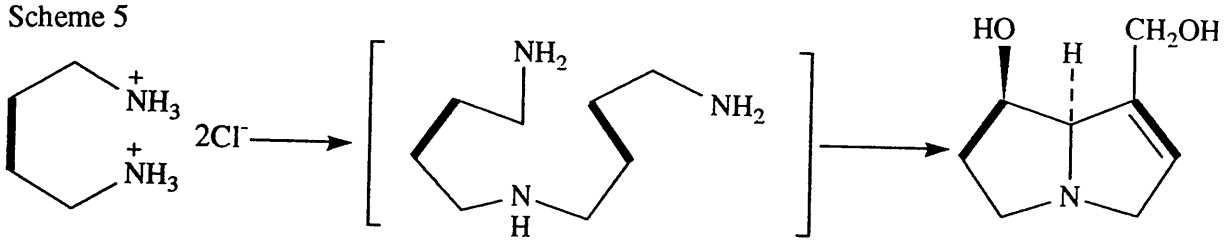
Labelling of all eight carbon atoms in retronecine was achieved by feeding [1,2-$^{13}C_2$]putrescine dihydrochloride (47).[33] This was synthesised as shown in Scheme 7. [1,2-$^{13}C_2$]-1-Bromo-2-phthalimidoethane (45) was reacted with ethyl cyanoacetate in the presence of sodium hydride in DMF to furnish the ester (46). De-ethoxycarbonylation in DMSO with sodium chloride/water followed by catalytic hydrogenation gave the mono-protected diamine which was deprotected by acid hydrolysis to yield [1,2-$^{13}C_2$]putrescine dihydrochloride (47). Retronecine (48) derived biosynthetically from (47) showed four pairs of doublets around the natural abundance resonances of the eight carbon atoms in the ^{13}C NMR spectrum. These doublets were of almost equal intensity which again points to a later symmetrical intermediate.

Khan and Robins synthesised and fed [^{13}C-^{15}N]putrescine dihydrochloride (49) to *S. isatideus*.[34] This yielded a sample of retronecine having ^{13}C enrichment at C-3, C-5, C-8 and C-9. More importantly, the signals corresponding to C-3 and C-5 in the ^{13}C NMR spectrum each consisted of a doublet arising from retronecine containing intact ^{13}C-^{15}N species superimposed on a singlet representing a ^{13}C-^{14}N species. This corresponds to the labelling pattern (50). Retronecine is likely to be produced <u>via</u> a C_4-N-C_4 intermediate since the doublets at C-3 and C-5 were of almost equal intensity. This finding was



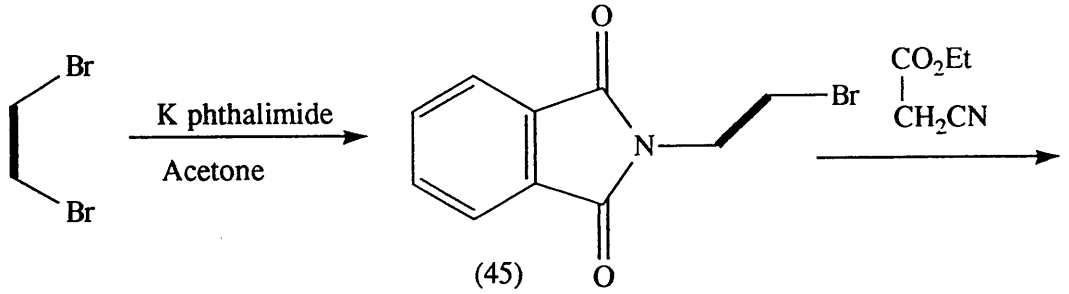
(43)

Scheme 5

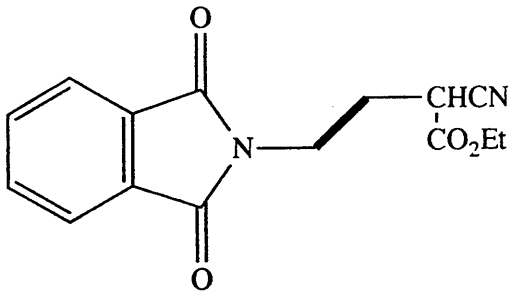


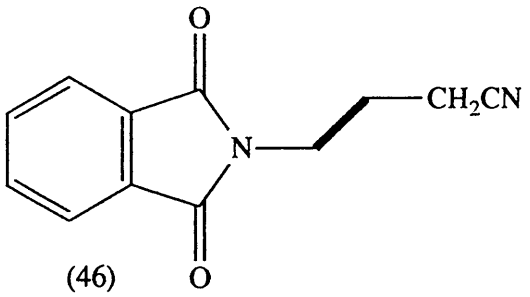
(44)

Scheme 6

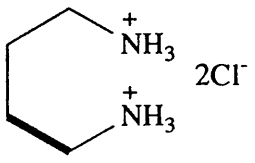


(45)





(46)



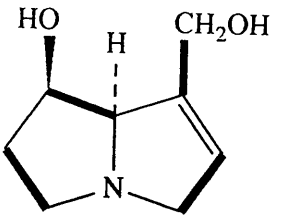
(47)

Scheme 7

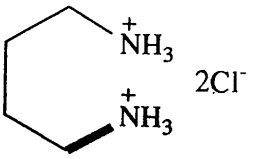
corroborated by Grue-Sorensen and Spenser.[35]

Khan and Robins proposed that the later symmetrical intermediate was the triamine homospermidine (51), which has been isolated from sandalwood trees *Santalum album* by Kuttan et al.[36] Feeding experiments demonstrated that arginine (37) and ornithine (29) are efficiently converted into homospermidine.[37] Moreover, in 1980 Srivenugopal and Adiga produced homospermidine from putrescine using a partially purified enzyme from *Lathyrus sativus* (grass pea) seedlings and from sandalwood leaves.[38] Homospermidine is also known to be present in *Heliotropium indicum*.[39]

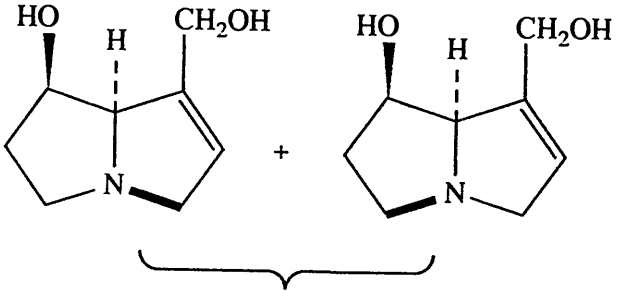
Schopf et al[40] and Robinson[24] postulated the biogenesis of lupinine (55) as being from lysine (52) and cadaverine (53) via the dialdehyde (54) (Scheme 8). Similarly, they proposed that 1-hydroxymethyl-pyrrolizidine was biosynthesised from ornithine (29) via dialdehyde (57). Leonard and Blum[41] carried out a biomimetic synthesis of 1-hydroxymethylpyrrolizidine by double ring closure of dialdehyde (57), derived from the bis-ketal (56), in aqueous phosphate buffer at pH7, followed by sodium borohydride reduction. This produced the racemates of isoretronecanol (23), (1β-hydroxymethyl-(8α)-pyrrolizidine) and trachelanthamidine (58), (1α-hydroxymethyl-(8α)-pyrrolizidine). The latter predominated, corresponding to formation of the thermodynamically more stable exo-aldehyde (Scheme 9).



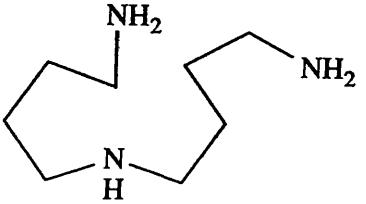
(48)



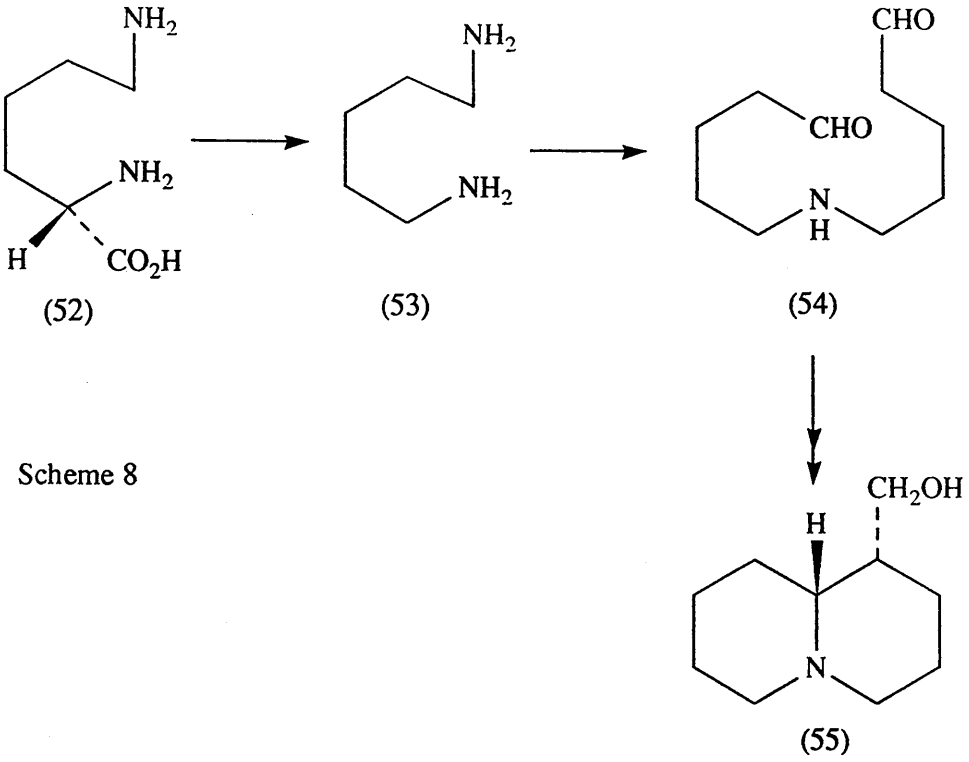
(49)



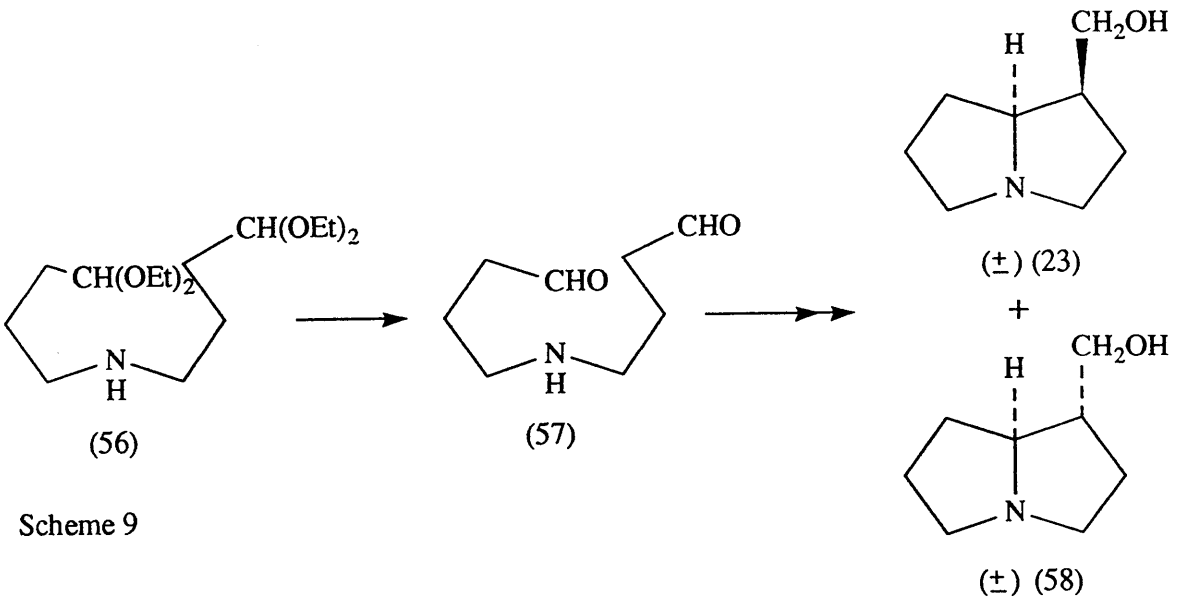
(50)



(51)



Scheme 8



Scheme 9

Initial experiments on the role of homospermidine (51) in pyrrolizidine alkaloid biosynthesis utilised [1,9-^{14}C]homospermidine trihydrochloride (61).[34] As shown in Scheme 10, amide formation by reaction of N-benzyloxycarbonyl-4-aminobutanoic acid (59) with 3-bromopropylamine in the presence of benzoyl chloride followed by bromide displacement with potassium [^{14}C]cyanide gave a nitrile (60). Catalytic hydrogenation, reduction of the amide with borane in THF and acidification afforded [1,9-^{14}C]homospermidine as its trihydrochloride (61). A total incorporation of 0.5% was obtained on feeding this species to *S. isatideus*. Basic hydrolysis and degradative work indicated that 44% of the ^{14}C activity resided at C-9 whereas only 2% of the radioactivity was located at C-(5+6+7) of retronecine (5). This is consistent with labelling pattern (62). In a complementary experiment, [4,6-^{14}C]homospermidine trihydrochloride (63) was synthesised and fed to *S. isatideus*.[42] The total incorporation of ^{14}C was 0.7% and as in the case of [1,9-^{14}C]homo-spermidine, greater than 95% of the radioactivity was found in the necine base. Retronecine was degraded and it was shown that 1-4% of the ^{14}C activity was present at C-9 while 45-47% was at C-(5+6+7). This is consistent with the labelling pattern (64).

A trapping experiment also demonstrated the intermediacy of homospermidine (51) in the biosynthetic pathway.[42] The N-phenylamino (thiocarbonyl) derivative (65) of homospermidine was isolated and shown to contain

$PhCH_2OCONH(CH_2)_3CO_2H$ + $H_2N(CH_2)_3Br$

(59)

↓ PhCOCl

$PhCH_2OCONH(CH_2)_3CONH(CH_2)_3Br$

↓ $K^{13}CN$

$PhCH_2OCONH(CH_2)_3CONH(CH_2)_3{}^{14}CN$

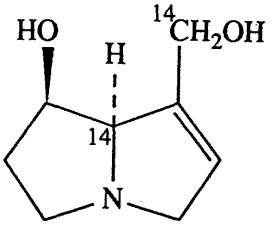
(60)

↓ 1. Pd/C, H_2
2. BH_3.THF
3. HCl

$H_3\overset{+}{N}(CH_2)_4\overset{+}{N}H_2(CH_2)_3{}^{14}CH_2\overset{+}{N}H_3$ $3Cl^-$

(61)

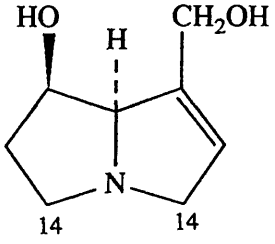
Scheme 10



(62)

$H_3\overset{+}{N}(CH_2)_3{}^{14}CH_2\overset{+}{N}H_2(CH_2)_4\overset{+}{N}H_3$ $3Cl^-$

(63)



(64)

$PhNHCSNH(CH_2)_4N(CH_2)_4NHCSNHPh$
|
$CSNHPh$

(65)

ca. 0.5% of the original radioactivity when DL-[5-^{14}C]ornithine was fed to *S. isatideus*.

Further convincing evidence was obtained when the incorporation of [1,9-$^{13}C_2$]homospermidine into retronecine in *S. isatideus* was studied. 1-Bromo-3-chloropropane was reacted with potassium [^{13}C]cyanide to afford 4-chloro-[1-^{13}C]butanenitrile. The synthesis of homospermidine by Bergeron et al[43], involving reaction of benzylamine with two equivalents of 4-chlorobutane-nitrile, gave the dinitrile (66). Catalytic hydrogenation of the nitrile groups, with concomitant hydrogenolysis of the N-benzyl group furnished [1,9-$^{13}C_2$]homospermidine which was purified as its trihydrochloride salt (67) (Scheme 11). The biosynthetically derived retronecine hydrochloride (68) from *S. isatideus* had a resolution-enhanced $^{13}C\{^1H\}$ NMR spectrum indicating a pair of doublets (J 6Hz) of equal intensity straddling the natural abundance signals for C-8 and C-9. This geminal coupling is indicative of intact incorporation of homospermidine into retronecine.[44]

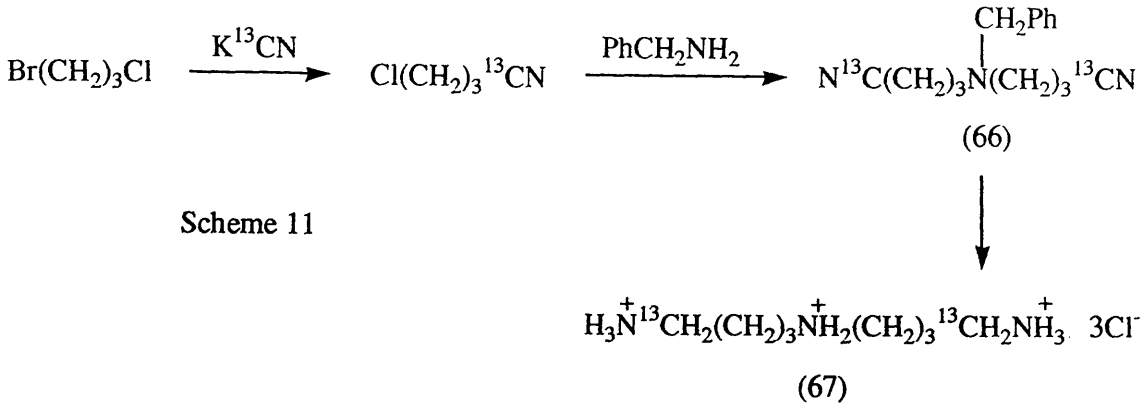
The feasibility of the Robinson-Schopf scheme for pyrrolizidine synthesis was demonstrated by Robins.[45] Facile transformation of homospermidine into 1-hydroxymethylpyrrolizidine was achieved using pea seedling diamine oxidase under physiological conditions. This was presumably achieved by enzymic oxidation of one primary amine to an aldehyde (69) in equilibrium with iminium ion (22). Further oxidation and Mannich cyclisation afforded 1-formylpyrrolizidine which could

be reduced either chemically or enzymically to (±)trachelanthamidine (58). This is shown in Scheme 12.

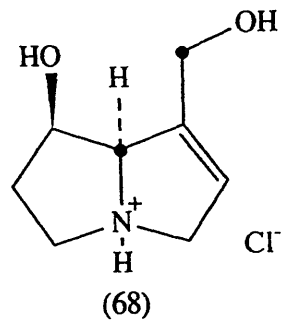
Biosynthetic studies on rosmarinine (70), the sole alkaloidal constituent of *Senecio pleistocephalus* demonstrated that the early stages of retronecine (5) and rosmarinecine (4) biosynthesis follow the same pathway.

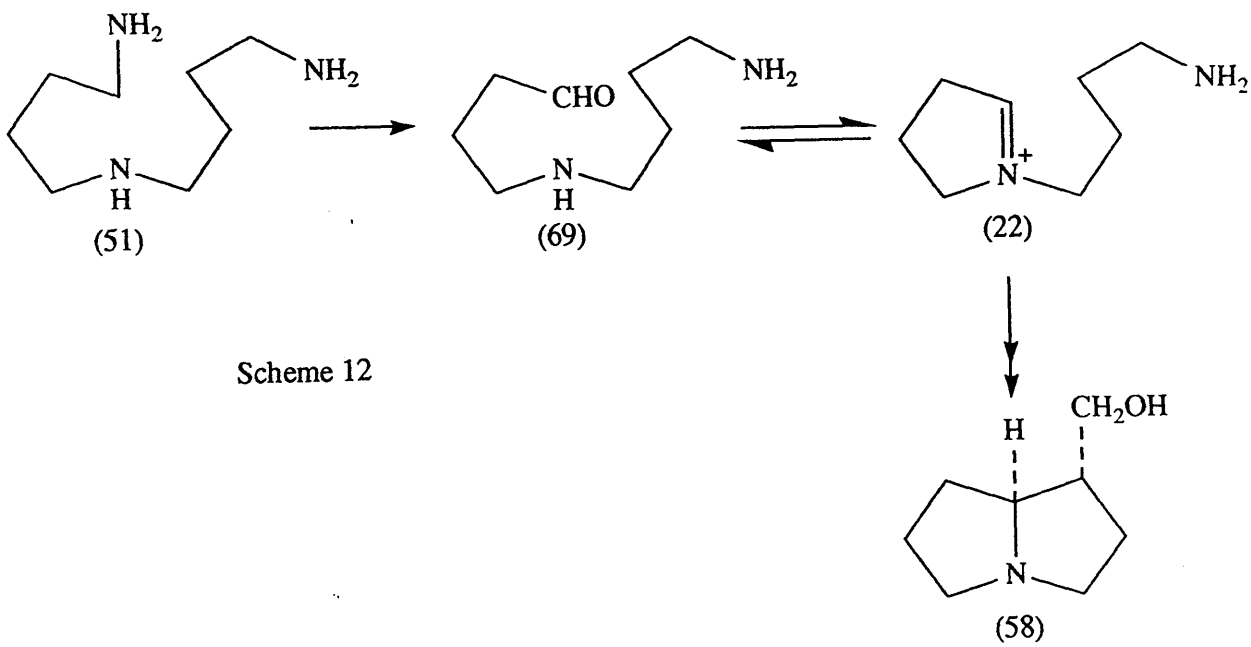
Feeding of [1-^{13}C]putrescine dihydrochloride (71) to freshly rooted cuttings of *S. pleistocephalus* resulted in an exceedingly high ^{14}C specific incorporation of 22% per C_4 unit. The ^{13}C specific incorporation was estimated at 24.4%, with the signals corresponding to C-3, C-5, C-8 and C-9 equally enriched over natural abundance (Scheme 13).[46] Next, a sample of [2,3-$^{13}C_2$]putrescine (44) was produced using the procedure of Khan and Robins,[34] although catalytic hydrogenation of the dinitrile was preferred to hydroboration (see Scheme 5).

The biosynthetically derived rosmarinine had doublets around the natural abundance ^{13}C NMR signals corresponding to C-1, C-2, C-6 and C-7, with almost equal enrichment at each site (Scheme 14).[46] Feeding of [^{13}C-^{15}N]putrescine dihydrochloride (49) gave rise to ^{13}C enrichment at C-3 and C-9 of 0.3±0.05% and at C-5 and C-8 of 0.4±0.05%. The C-3 and C-5 resonances appeared to exhibit doublets due to ^{13}C-^{15}N coupling which unfortunately were partially obscured by the natural abundance ^{13}C signals. Coupling constants were estimated to be 4Hz for C-3 and 2-3Hz for C-5 and

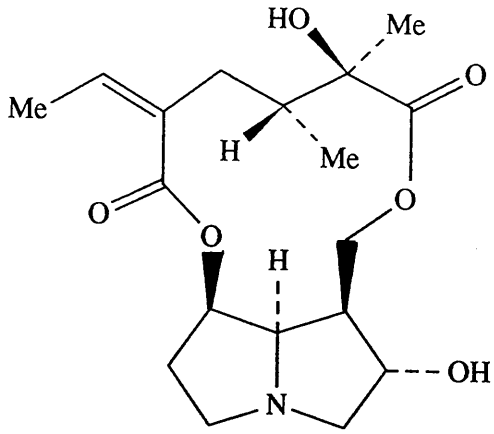


Scheme 11

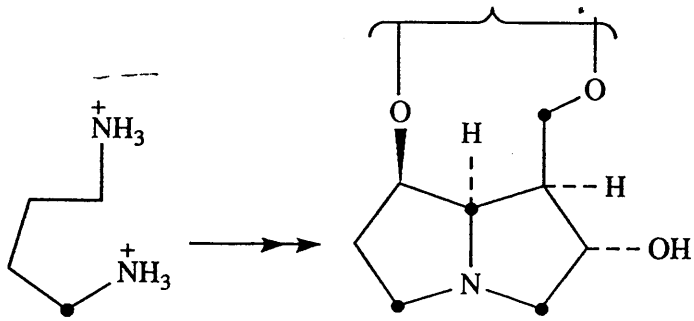




Scheme 12

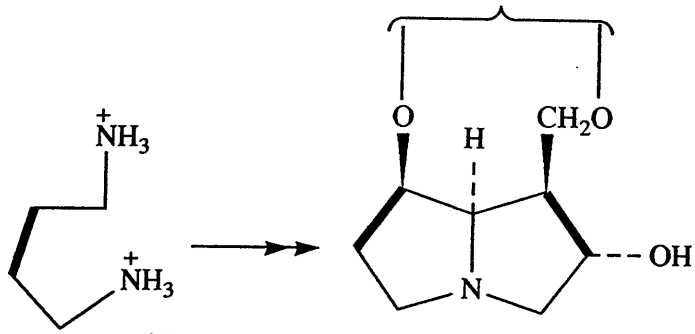


(70)



(71)

Scheme 13



(44)

Scheme 14

hence the doublets do not arise from C-3 to C-5 geminal coupling.[46]

[1,9-$^{13}C_2$]Homospermidine trihydrochloride (67), on feeding to *S.pleistocephalus*, led to enrichment of the signals due to C-8 and C-9 of rosmarinine in the $^{13}C\{^1H\}$ NMR spectrum. It was noted earlier that in the corresponding experiment in *S. isatideus*, the sample of retronecine indicated a geminal coupling constant between C-8 and C-9 of 6Hz. In this case the geminal coupling constant is zero.[46] The feeding of ^{13}C-labelled precursors to *S. pleistocephalus* has shown that rosmarinecine (4) is derived from putrescine (30) via homospermidine (51), which is in agreement with the findings for retronecine.

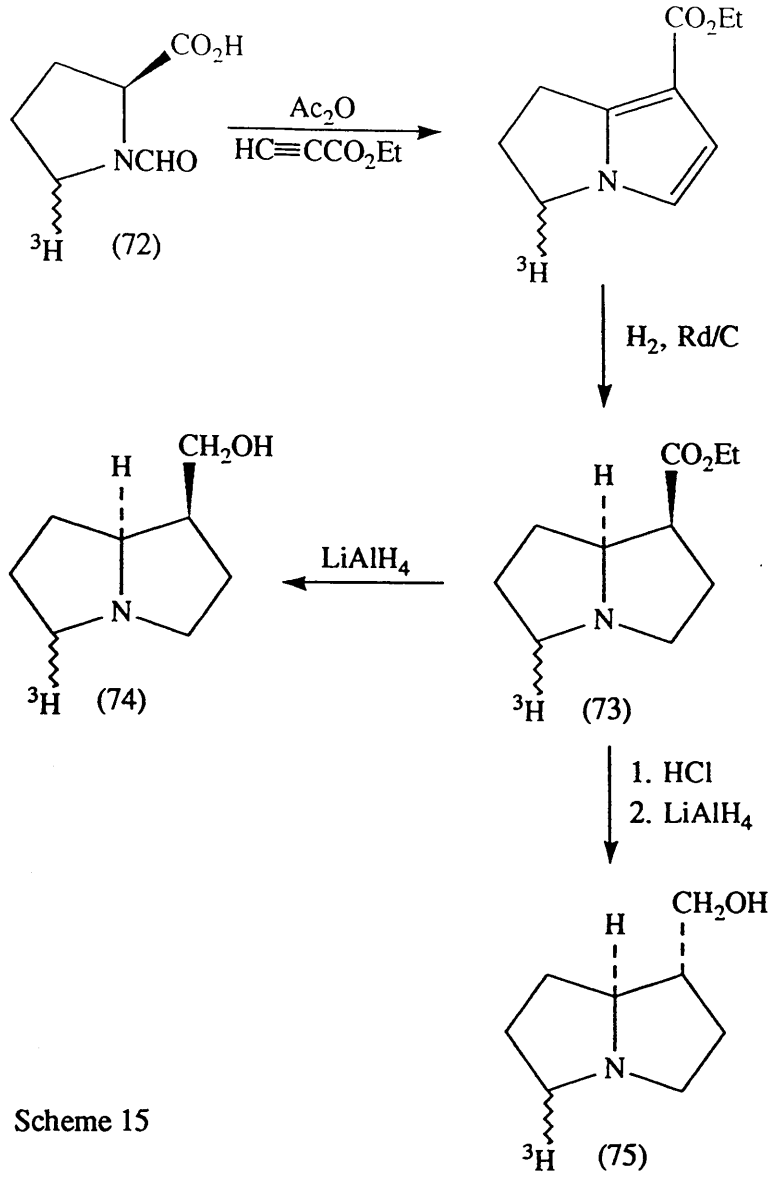
However, the work of Robins and co-workers led to the conclusion that there was a divergence in the biosynthetic pathways by feeding the epimeric 1-hydroxymethylpyrrolizidines trachelanthamidine (58) and isoretronecanol (23). Kunec and Robins[47] carried out a radiolabelled synthesis of trachelanthamidine and isoretronecanol (Scheme 15) starting from N-formyl-[5-^3H]-L-proline (72), using the method of Pizzorno and Albonico.[48] 1,3-Dipolar cycloaddition with ethyl propiolate followed by hydrogenation yielded the endo-ester (73) which was reduced with lithium aluminium hydride ($LiAlH_4$) to (±)-[5-^3H]isoretronecanol (74). The thermodynamically more stable exo-ester was obtained by epimerisation of the endo-ester (73) in acid, and $LiAlH_4$ reduction furnished (±)-[5-^3H] trachelanthamidine (75). Feeding experiments using [1,4-^{14}C]putrescine as

an internal standard are summarised in Table 1.

Thus, isoretronecanol (23) is the preferred diastereoisomer utilised in rosmarinecine biosynthesis since it is incorporated 34 times more efficiently than is trachelanthamidine (58) in *S. pleistocephalus*, whereas in the formation of retronecine (5) in *S. isatideus* and heliotridine (16) in *C. officinale*, trachelanthamidine is a much better precursor than isoretronecanol. In all cases, the 1-hydroxymethyl-pyrrolizidines are incorporated more efficiently than putrescine, consistent with the hypothesis that they are further along the biosynthetic pathway, strengthened by the likely assumption that only one enantiomer of each racemate is utilised. Similar findings were presented by Rana and Leete, showing that trachelanthamidine (58) is a much better precursor than isoretronecanol (23) for riddelliine (35) the base portion of which is retronecine, in *Senecio ridellii*.[49]

It can be concluded that there is a divergence of the pathways to retronecine and rosmarinecine. This may occur either on cyclisation of the iminium ion (22), or epimerisation of the resultant aminoaldehyde may occur.

Further support for the proposed sequence of events came from the work of Birecka and Catalfamo.[50] Using *Heliotropium spathulatum*, which produces trachelanthamidine (58), supinidine (17), and retronecine (5), short term feeding of $^{14}CO_2$ produced samples with specific activities consistent with the



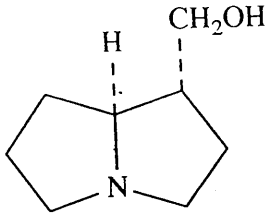
Scheme 15

Table 1

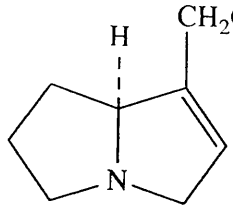
	Initial $^3H/^{14}C$	Final $^3H/^{14}C$	^3H Specific incorporation
		S. pleistocephalus	
Trachelanthamidine (75)	10.0	< 0.5	< 0.1%
Isoretronecanol (74)	10.0	17.0	2.4%
		S. isatideus	
Trachelanthamidine (75)	10.0	14.3	2.8%
Isoretronecanol (74)	10.0	0.7	0.3%
		C. Officinale	
Trachelanthamidine (75)	10.0	17.0	0.35%
Isoretronecanol (74)	10.0	1.0	0.04%

sequence (58) → (17) → (5). Elaboration of isoretronecanol (23) to rosmarinecine (4) requires oxidations at C-2 and C-7. Efficient incorporation of platynecine (25) labelled with ^{3}H into rosmarinine (70) in *S. pleistocephalus* suggests that hydroxylation at C-7 may occur first.[51]

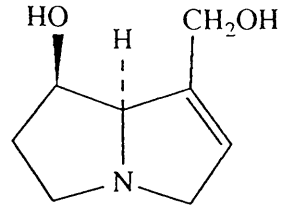
Kelly and Robins have provided strong evidence for the intermediacy of the iminium ion (22) in the biosynthetic pathway.[52] ^{14}C-Labelled iminium ion was synthesised as shown in Scheme 16. Coupling of [1-^{14}C]-4-chlorobutanenitrile (76) with pyrrolidine followed by catalytic hydrogenation in the presence of Adams' catalyst furnished the saturated salt (77). Regiospecific oxidation with mercury (II) acetate then yielded the desired ^{14}C-labelled iminium ion (78). The results of feeding experiments with *S. isatideus* and *S. pleistocephalus* are shown in Table 2. These results indicate that the iminium ion (22) is a better precursor for retronecine (5) and rosmarinecine (4) than is putrescine. An intermediate trapping experiment allowed the N-phenylamino (thiocarbonyl) derivative (79) of the reduced iminium ion to be isolated in radioactive form, containing 0.4% of the activity fed as [1,4-^{14}C]putrescine dihydrochloride. Curiously, the saturated salt (77) was also incorporated reasonably well into the necines (4) and (5). However, in an intermediate trapping experiment, the derivatised saturated salt contained a very low level of radioactivity (less than 0.017% of the original ^{14}C activity). Thus, the iminium ion is the likely biosynthetic intermediate, although *in vivo* oxidation



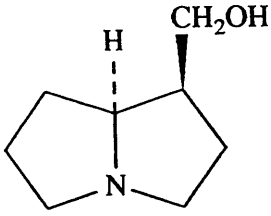
(58)



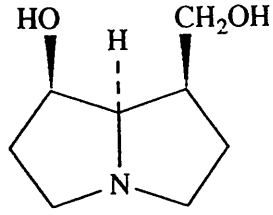
(17)



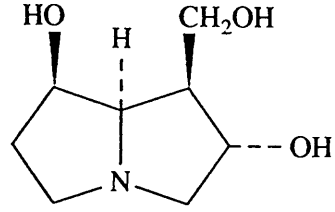
(5)



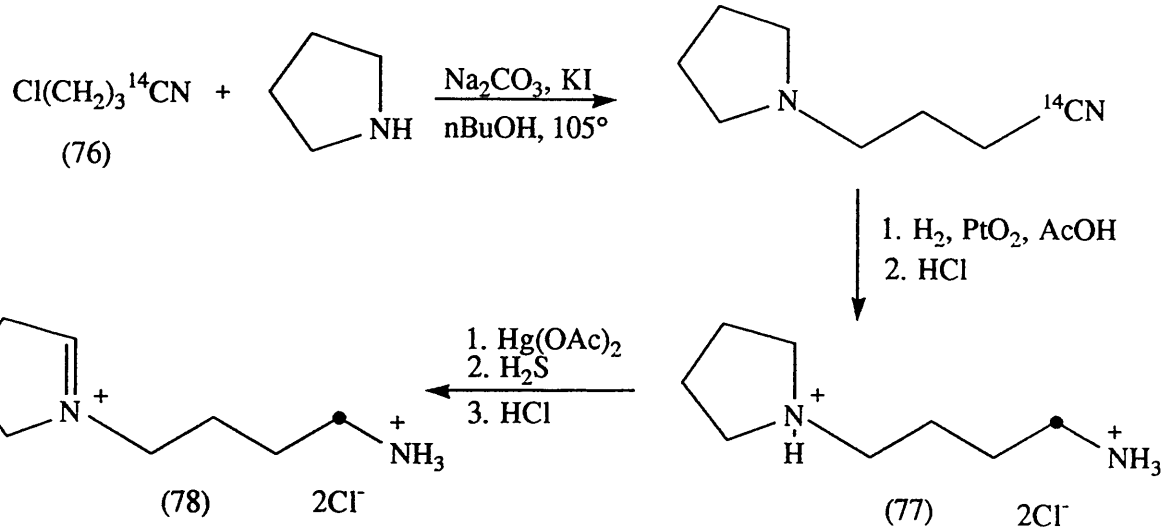
(23)



(25)



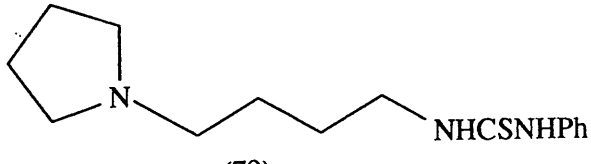
(4)



Scheme 16

Table 2

	Initial $^{3}H/^{14}C$	Final $^{3}H/^{14}C$	^{14}C Specific incorporation
S. pleistocephalus	5	2.9	6.5%
S. isatideus	12.3	9.8	4.5%

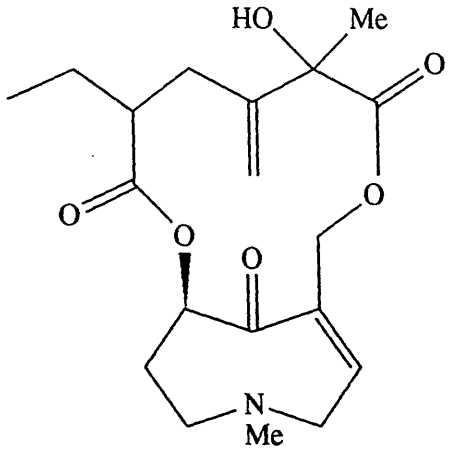


(79)

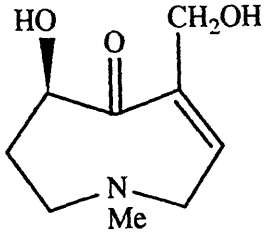
can apparently introduce the saturated compound into the biosynthetic scheme.

Robins and co-workers further widened the scope of biosynthetic studies on necine bases by using the species *Emilia flammea*. This produces emiline (80) as the major alkaloidal component, containing the seco-pyrrolizidine otonecine (31) as the base portion. In summary, experiments with radiolabelled precursors have shown that putrescine (30), homospermidine (51), the iminium ion (22), trachelanthamidine (58) and retronecine (5) are all incorporated efficiently into otonecine. Cleavage of the N-4 to C-8 bond is likely to occur late in the biosynthetic sequence, as shown by the incorporation of trachelanthamidine and retronecine into otonecine (31).[53]

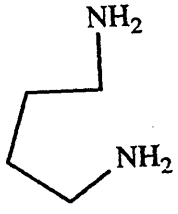
The main intermediates in necine biosynthesis have now been well established. These intermediates are enclosed in boxes in Scheme 17. Putrescine (30) is derived from the amino acids ornithine (29) and/or arginine (37). Oxidation of putrescine to an aminoaldehyde and condensation with another putrescine molecule gives an imine (81) which is reduced to homospermidine (51). Oxidation of one primary amine produces an aldehyde in equilibrium with the iminium ion (22). Further oxidation and cyclisation leads to aminoaldehydes which are reduced to trachelanthamidine (58) or isoretronecanol (23). After the divergence in the pathways, trachelanthamidine (58) can be elaborated to retronecine (5), heliotridine (16) or otonecine (31) whereas isoretronecanol (23) is transformed into



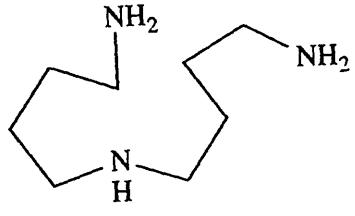
(80)



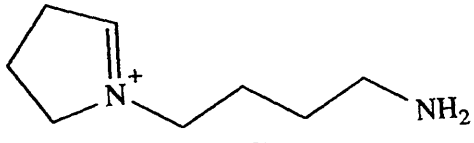
(31)



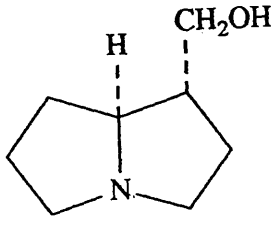
(30)



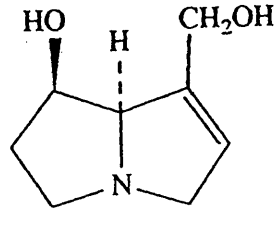
(51)



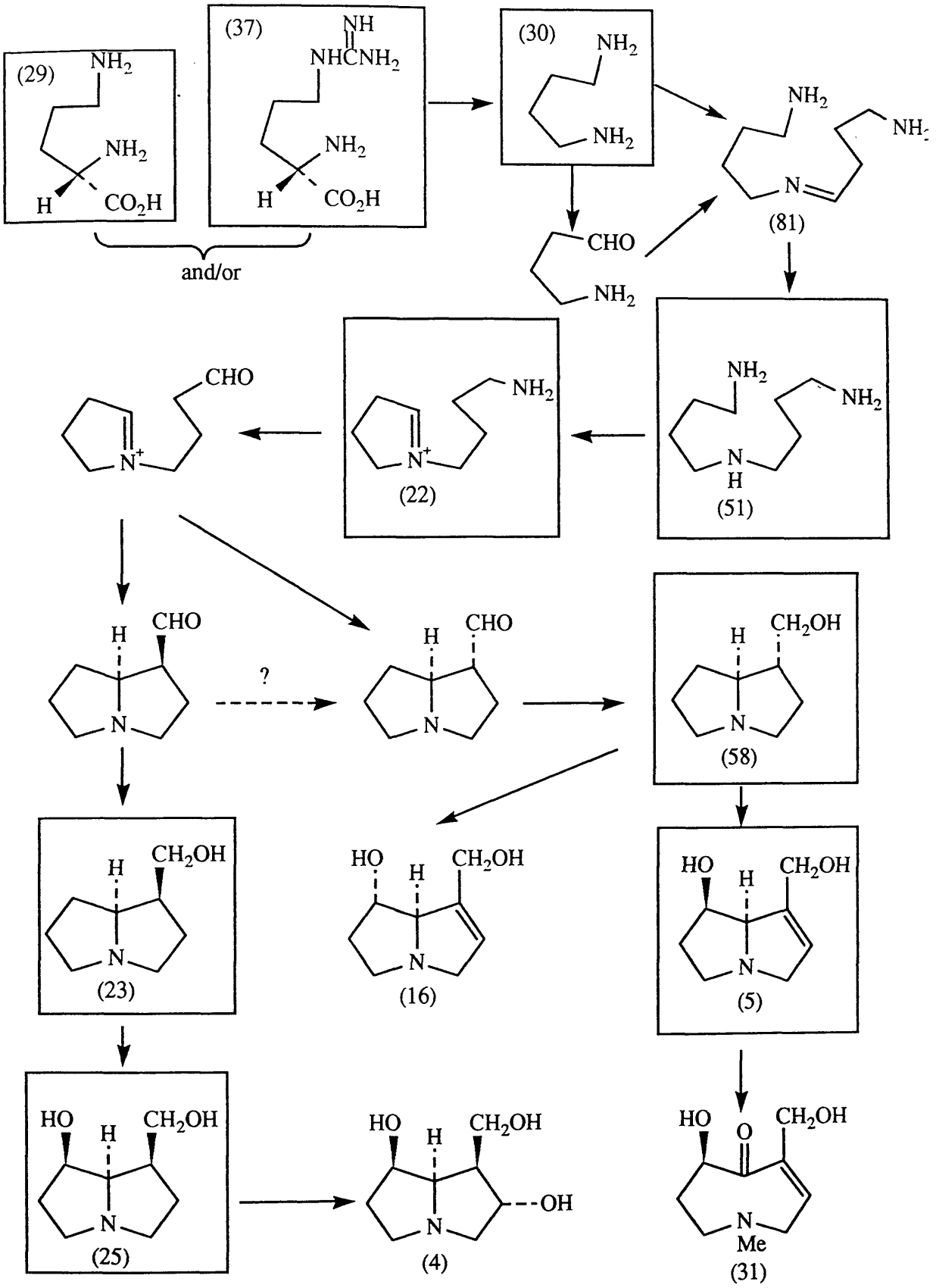
(22)



(58)



(5)



Scheme 17

rosmarinecine (4) via platynecine (25).

The foregoing discussion has not touched on stereochemical aspects of necine biosynthesis. The first foray into this field was the observation by Robins and Sweeney that only the (natural) L-enantiomers of ornithine and arginine are utilised in retronecine biosynthesis.[54]

The next major advance in the elucidation of the stereochemistry of the biosynthesis of pyrrolizidine alkaloids came with the use of putrescines specifically labelled with deuterium. Much information concerning the stereospecificity of enzymic processes has been obtained by feeding experiments with a series of deuteriated putrescines, originally with *Senecio isatideus* and subsequently with *S. pleistocephalus* and *Emilia flammea*.

The first experiment involved the use of [2,3-$^2H_4$] putrescine dihydrochloride (82). Succinonitrile was heated at reflux in deuterium oxide, followed by catalytic hydrogenation and acidification to give compound (82). This precursor was fed to *S. isatideus*, leading to a specific incorporation of deuterium into retronecine (5) of 3-5% per C_4 unit. The $^2H\{^1H\}$ NMR spectrum of retrorsine (83) showed equal enrichment at H-2,H-6α,H-6β, and H-7α. The presence of deuterium at H-7α indicates that hydroxylation at C-7 does not involve keto or enol intermediates.[55]

[1,4-$^2H_4$]Putrescine dihydrochloride (84) was

produced by reduction of succinonitrile under a deuterium atmosphere, followed by acidification. When this sample was fed to *S. isatideus*, it resulted in labelling of retrorsine (86), at H-3α, H-3β, and H-9 pro-S. The presence of the label mainly in one half of the molecule was explained by a deuterium isotope effect. An intramolecular isotope effect (K_H/K_D) of 3.5-4 has been observed in the oxidation of [1-$^2H_2$]putrescine by hog kidney diamine oxidase.[56] If a homospermidine molecule (85) is formed from one molecule of deuteriated putrescine and one molecule of endogenous, unlabelled putrescine, preferential oxidation will occur at the unlabelled end of homospermidine, resulting in the observed labelling pattern (86). The labelling of 9-H pro-S with deuterium indicates that reduction to trachelanthamidine (58) occurs by delivery of a hydride equivalent to the C-re face of the aldehyde. This is the expected stereospecificity for coupled dehydrogenase enzyme systems.[57]

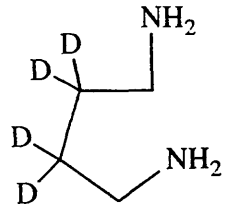
Decarboxylation of L-ornithine is known to occur with retention of configuration.[58] Samples of (R)-[1-^{2}H]- and (S)-[1-^{2}H]putrescine were produced using the method of Richards and Spenser.[59] Decarboxylation of L-ornithine in deuterium oxide, catalysed by L-ornithine decarboxylase furnished (R)-[1-^{2}H]putrescine (87) whereas decarboxylation of the L-component of DL-[2-^{2}H]ornithine (88) in H_2O yielded (S)-[1-^{2}H]putrescine (89) (Scheme 18).

After feeding (R)-[1-^{2}H]putrescine to *S.isatideus*, equal labelling was observed at H-3β, H-5α, H-8α, and H-9

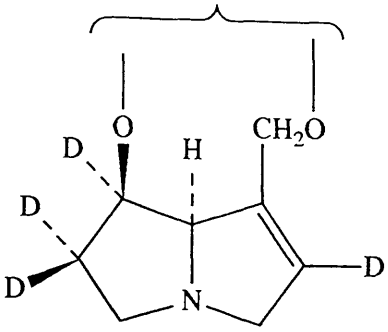
pro-S of retrorsine (90). This indicates that the pro-S hydrogen is removed from the primary amine groups in all three oxidations on the pathway, in agreement with previous findings for the stereochemical course of primary amine oxidation with pea seedling amine oxidase by Battersby et al.[60]

There was substantial loss of deuterium on feeding (S)-[1-^{2}H]putrescine to *S. isatideus*. Deuterium was present only at H-3α and H-5β of retrorsine (91) and it was inferred that reduction of imine (81) to homospermidine (51) occurs on the C-si face.

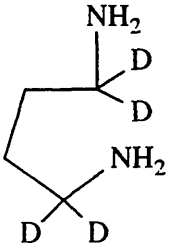
(R)-[2-^{2}H]Putrescine was synthesised from (S)-aspartic acid (92) by a modification of the unpublished synthesis of Arigoni and Eliel[61] of (R)-[2-^{2}H]succinic acid (95) (Scheme 19). Treatment of (92) with hydrochloric and nitric acids in the presence of urea replaced the amino group with a chlorine with retention of configuration. Conversion into the diester followed by DIBAL reduction gave the diol (93). S_N2 displacement of chloride introduced the deuterium, and the resultant diol was converted into (R)-[2-^{2}H]putrescine (94) via the dibromide and the diazide. Similarly (S)-[2-^{2}H]putrescine (96) was formed from (R)-aspartic acid.[62] In feeding experiments with *S. isatideus*, (R)-[2-^{2}H]putrescine labelled retrorsine (97) at H-2 and H-6α whereas the labels were present at H-6β and H-7α in retrorsine (98) on feeding (S)-[2-^{2}H] putrescine. From these results it was concluded that there is retention of the pro-R hydrogen and loss of the pro-S hydrogen at the carbon which becomes C-2 of



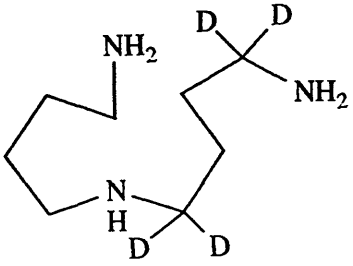
(82)



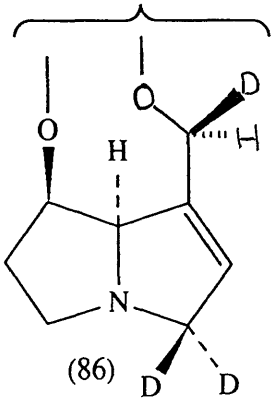
(83)



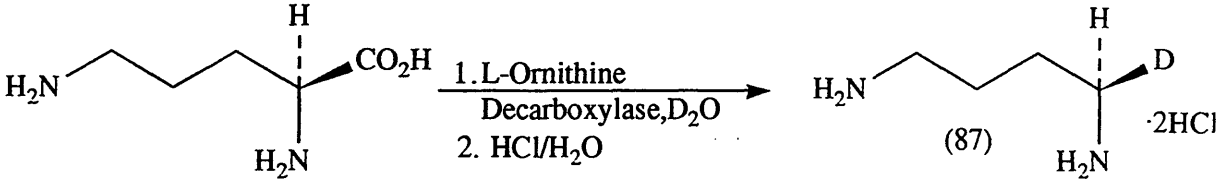
(84)



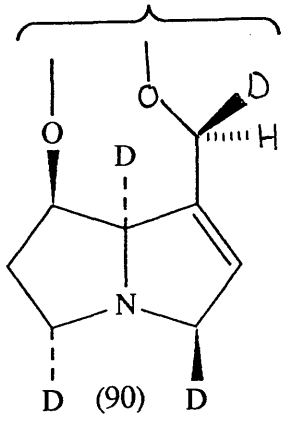
(85)



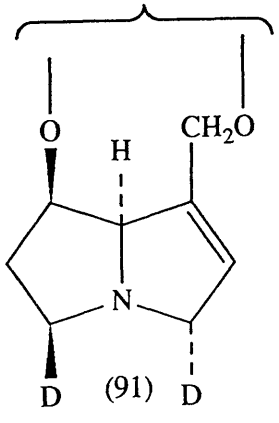
(86)



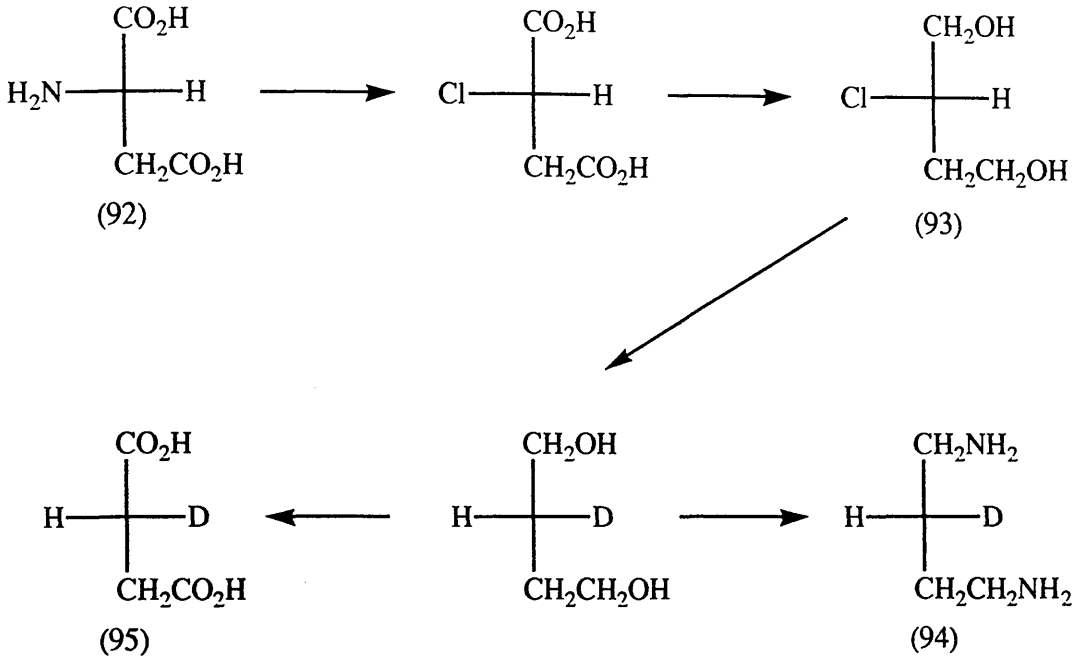
Scheme 18



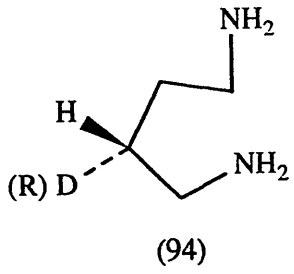
(90)



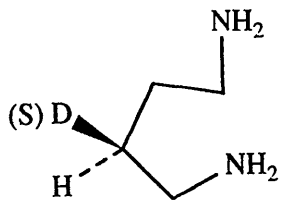
(91)



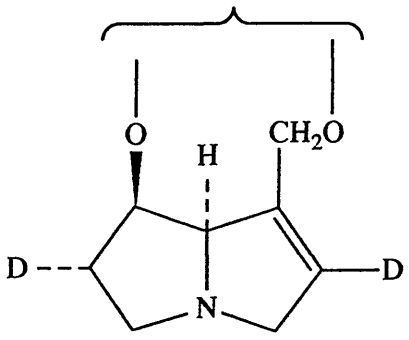
Scheme 19



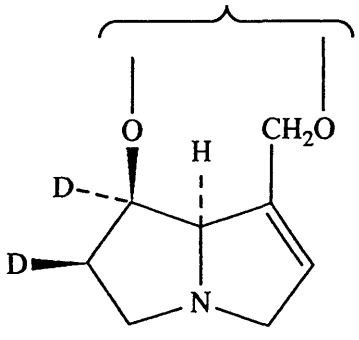
(94)



(96)



(97)

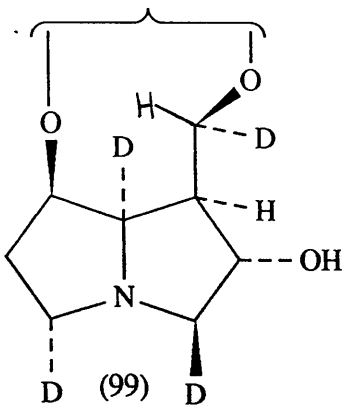


(98)

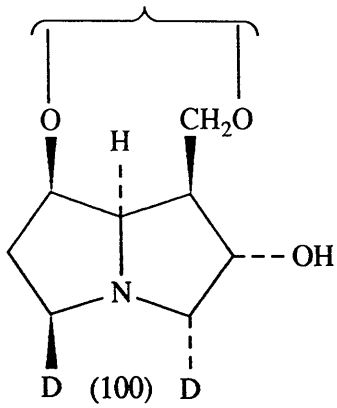
retronecine. Also, hydroxylation at C-7 occurs with normal retention of configuration at an sp^3 carbon.[57]

The outcome of a number of enzymic processes in *S.pleistocephalus* has also been determined.[63] When (R)- and (S)-[1-^{2}H]putrescine were fed, labelling patterns in rosmarinine, (99) and (100) respectively, were observed, which were comparable to those in retrorsine. The stereospecificity of a number of enzymic processes was demonstrated in further experiments using (R)- and (S)-[2-^{2}H]putrescine[63]; these resulted in rosmarinine (101) labelled at H-2β and H-6α from the (R)-isomer and rosmarinine (102) labelled at H-1α, H-6β, and H-7α from the (S)-isomer. From these results it was inferred that hydroxylation at C-2 and C-7 to form rosmarinine occurs with retention of stereochemistry. Of particular note is the presence of deuterium at C-1 in rosmarinine (102) after feeding (S)-[2-^{2}H]putrescine. This indicates that the pro-R hydrogen is removed during Mannich cyclisation to the pyrrolizidine ring system, and also that aldehyde (103) is formed directly from the iminium ion (22), rather than by epimerisation of the exo-aldehyde (104).

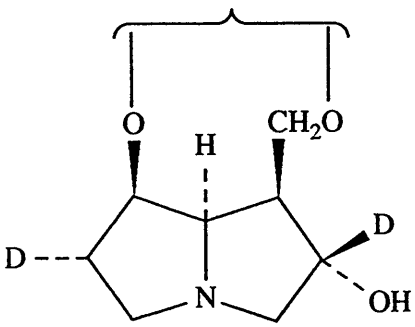
The most recent work on enzyme stereospecificity was carried out by Rodgers and Robins.[64] Feeding of (R)-[1-^{2}H]-, (R)-[2-^{2}H]-, and (S)-[2-^{2}H]putrescine to *Emilia flammea* demonstrated the following: the three primary amine oxidations occur with the normal stereospecificity[60]; reduction of imine (81) to homospermidine (51) occurs on the C-si face; reduction of aldehyde (104) to trachelanthamidine (58) occurs on



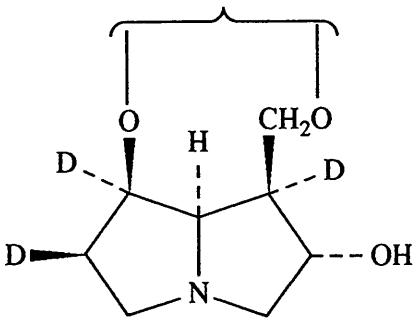
(99)



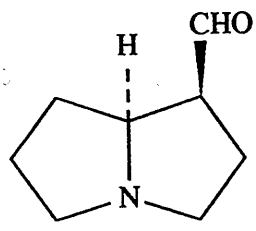
(100)



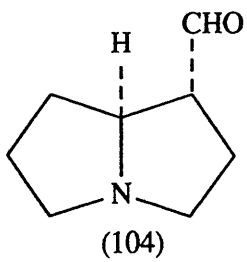
(101)



(102)



(103)



(104)

the C-re face of the carbonyl group; hydroxylation at C-7 of otonecine (31) occurs with retention of configuration; and introduction of the carbonyl at C-8 causes loss of ^{2}H at C-8 but at no other positions on the necine. These stereospecifities are in accordance with those observed for both retrorsine and rosmarinine.

2.2 Biosynthesis of Necic Acids.

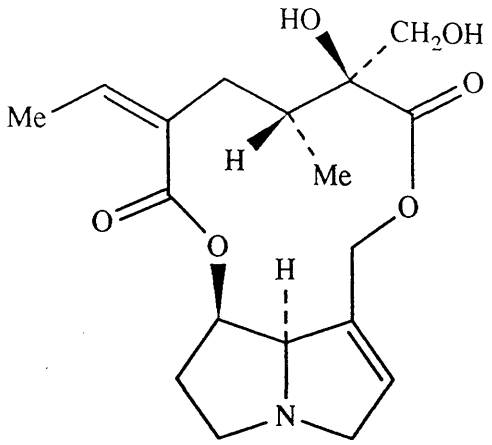
In contrast to the wealth of information which has been obtained on the biosynthesis of necine bases, the study of the derivation of the necic acids has been less extensive. Work has concentrated on the use of radiotracer techniques : no stable isotope studies have been reported.

The necic acids are branched-chain aliphatic mono- or dicarboxylic acids with 5,6,7,8,9 or (most commonly) 10 carbon atoms. On first inspection, many look to be terpene-derived, but acetate and mevalonolactone have been shown not to be specific precursors for necic acids. For example, when [2-^{14}C]mevalonolactone was fed to *Senecio isatideus*, inactive retrorsine (34) was isolated.[65] ^{14}C-Labelled acetates have been fed to *Senecio* species, but lack of suitable degradations meant that incomplete labelling patterns were obtained for the necic acids.[65-67]

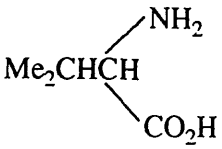
Attention was next directed to the possibility that the necic acids may be derived from α-amino acids such as valine (105), leucine (106), isoleucine (107)

and threonine (108). Support for this hypothesis came from the work of Crout, who studied the biosynthesis of echimidinic (109) and angelic(110) acid, the esterifying acids of heliosupine (8) in *Cynoglossum officinale*. DL-[4-^{14}C]Valine was shown to be incorporated into echimidinic acid to the extent of 0.246% whereas the incorporation of [2-^{14}C]acetate was only 0.019%.[68] Furthermore, 85% of the activity was found in echimidinic acid after feeding DL-[4-^{14}C]valine and virtually all of this activity was present in the acetone and iodoform after degradation (Scheme 20). The angelic acid portion was shown to be derived from L-[U-^{14}C]isoleucine.[69] In separate experiments, L-[U-^{14}C]isoleucine and [2-^{14}C]acetate were fed to *C. officinale*, the total incorporations of which were 0.205 and 0.019% respectively. Isoleucine is a specific precursor for angelic acid since 98% of the activity was located in the angeloyl ester portion of heliosupine (8).[69] It is known that tiglic acid (111) is derived from L-isoleucine in *Datura meteloides*.[70-72] McGaw and Woolley demonstrated that [1-^{14}C]tiglic acid was specifically incorporated into the angelic acid portion of heliosupine, suggesting that angelic acid may be formed from tiglic acid by a cis-trans isomerisation.[73]

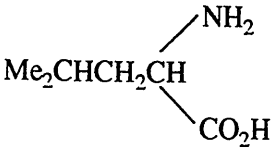
Crout et al[66] studied the biosynthesis of seneciphyllic acid (112) with *Senecio douglasii* which produces seneciphylline (33) as the major alkaloid component. The label from [1-^{14}C]- and [2-^{14}C]-acetate was randomised between the acid and base portions, indicating that there is no direct route from acetate to seneciphyllic acid. [2-^{14}C]Mevalonate was



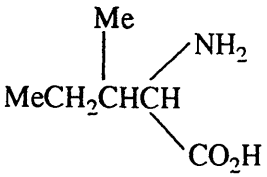
(34)



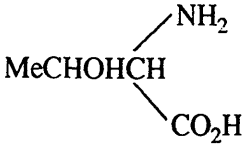
(105)



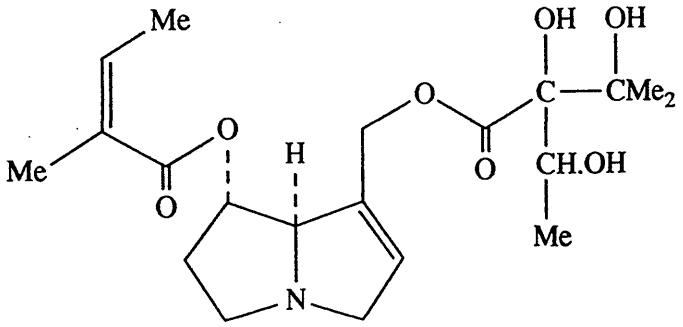
(106)



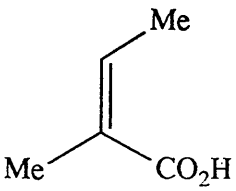
(107)



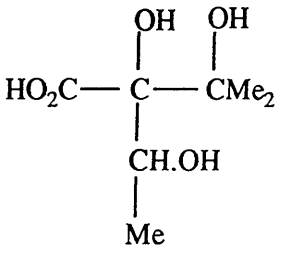
(108)



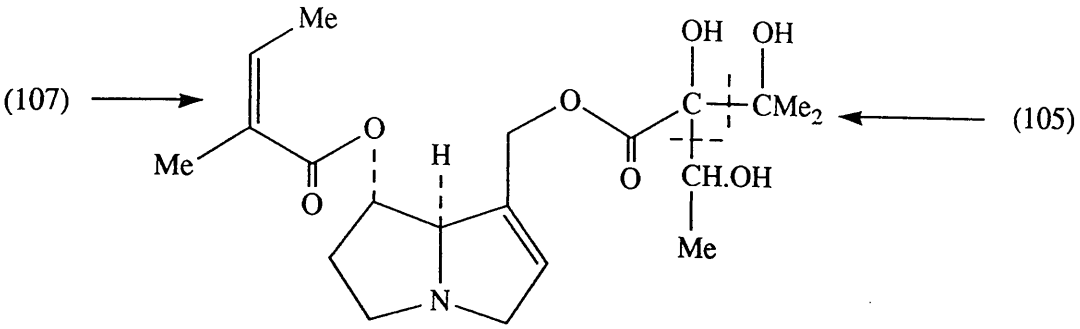
(8)



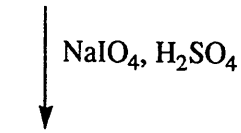
(110)

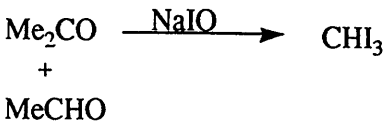


(109)

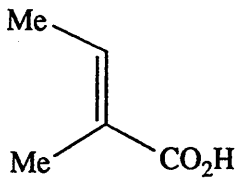


(8)

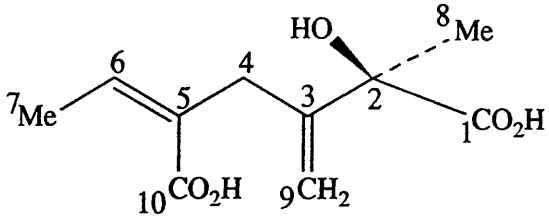




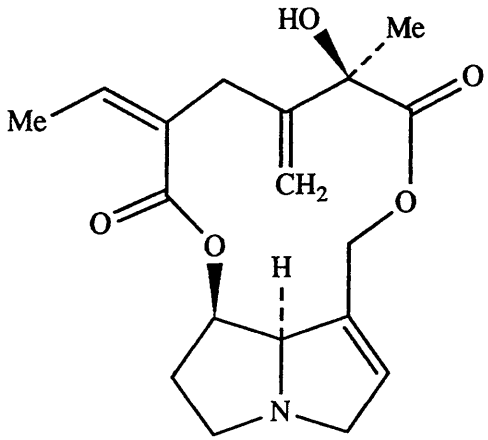
Scheme 20



(111)



(112)



(33)

incorporated to about the same extent as ^{14}C-acetate, although greater specificity for seneciphyllic acid was observed. Direct utilisation of [2-^{14}C]mevalonate would have been expected to label C-1 or C-8 of one half of seneciphyllic acid, but degradation showed that these positions carried respectively 0.35 and 0.17% of the total activity. The operation of an acetate-mevalonate pathway to seneciphyllic acid was therefore excluded.

High specific incorporations were observed for L-[U-^{14}C]isoleucine and its biogenetic precursor L-[U-^{14}C]threonine into seneciphyllic acid (112).[66] In the case of threonine, >99% of the total activity was found in the acid portion, and the total incorporation was 20-30 times greater than that for acetate. Degradation of seneciphyllic acid derived from L-[U-^{14}C]threonine shown in Scheme 21 seemed to indicate that threonine is not incorporated in a symmetrical manner. It should be noted that the figure for activity at C-2 is not reliable. Kuhn-Roth degradation of seneciphyllic acid liberated two equivalents of acetic acid, isolated as barium acetate, corresponding to (C-2/C-8) + (C-6/C-7). The acetic acid was degraded by the Schmidt reaction to methylamine (C-7 and C-8), isolated as 5-methylamino-2,4 dinitrotoluene (113), and carbon dioxide (C-2 and C-6), isolated as barium carbonate. The activity at C-2 was estimated by subtracting from the barium acetate activity the activities for (C-6,C-7) and C-8 (obtained by separate degradations).

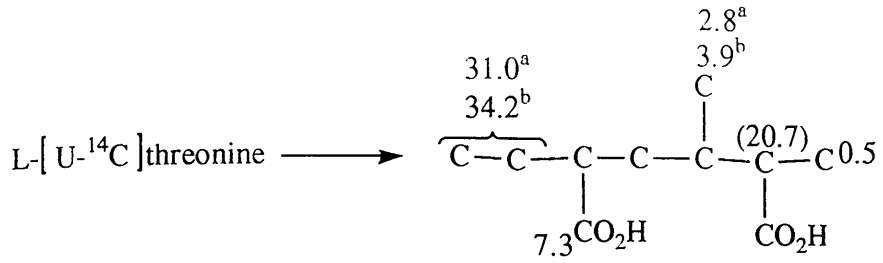
[1-^{14}C]Isoleucine was incorporated with one-tenth

of the efficiency of [U-^{14}C]isoleucine into seneciphyllic acid and with significantly lower specificity. It is apparent that the carboxyl group of isoleucine is lost on incorporation into seneciphyllic acid.[66]

A major pathway in threonine biosynthesis leads to α-ketobutyric acid (114), which is used in isoleucine biosynthesis.[74] The biosynthetic transformation of α-ketobutyric acid into isoleucine is shown in Scheme 22. In the transformation of threonine into isoleucine, C-1, -2,-3, and -4 of threonine become C-1, -2, -4 and -5 of isoleucine respectively, with the side chain C-3 and -6 being derived from pyruvate.

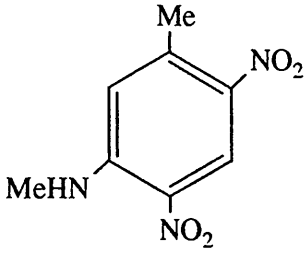
Assuming incorporation <u>via</u> isoleucine, threonine could provide either C-4, C-6 and C-7 (path A) or C-6 C-7 and C-10 (path B) of seneciphyllic acid (Scheme 23). Incorporation of [U-^{14}C]threonine <u>via</u> isoleucine would give a ratio of the activities of (C-6/C-7) and C-10 of greater than 2 by path A whereas if path B was operative, the ratio would be just two. It was observed that the C-6/C-7 unit of seneciphyllic acid derived from [U-^{14}C]threonine contained more than four times the activity of C-10, implying that path A was preferred.[66] Subsequently, a re-evaluation of the evidence suggested that the entire C_{10} skeleton of seneciphyllic and related acids may be derived from isoleucine and hence from threonine and pyruvate.

It appeared that isoleucine was incorporated into the (C-1,C-2,C-3,C-8 and C-9) unit of seneciphyllic acid

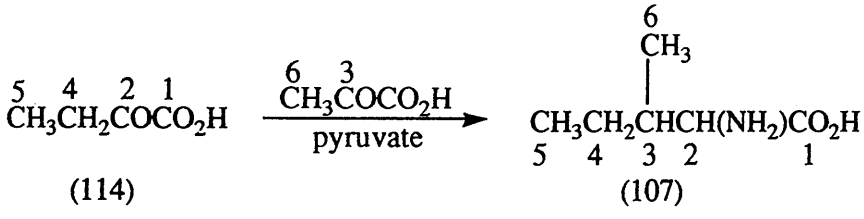


a. Ozonolysis
b. Periodate/Permanganate

Scheme 21

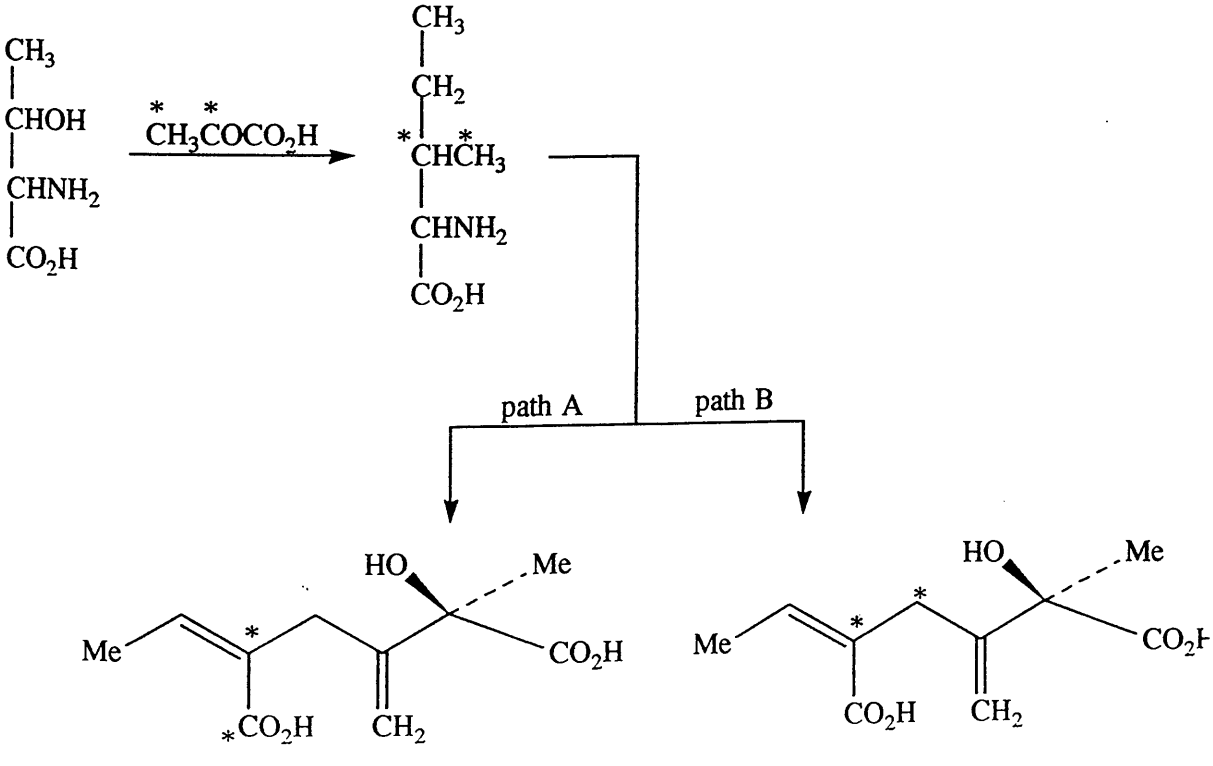


(113)



(114) (107)

Scheme 22



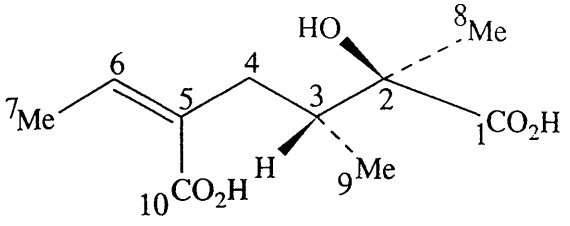
Scheme 23

(112) at only half the rate at which it was incorporated into the other half. However, with L-[U^{14}C]isoleucine as a precursor, C-1, C-8 and C-9 were equally labelled[66], so the C-1,-2,-3,-8, and -9 unit of seneciphyllic acid might also be derived from isoleucine.

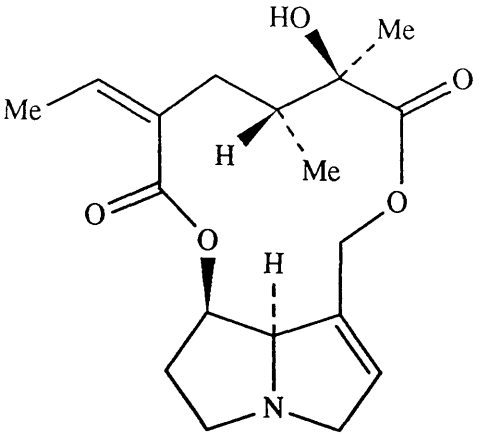
Studies on the structurally related senecic acid (115), the diester portion of senecionine (32) in *Senecio magnificus*, indicated an even distribution of activity between the two C_5 units of senecic acid derived from [U-^{14}C]isoleucine.[75]

Furthermore, [2-^{14}C]- and [6-^{14}C]-isoleucine were specifically incorporated into senecic acid. [2-^{14}C] Isoleucine labelled C-1 and C-10 of senecic acid equally and exclusively, whereas 58% of the label appeared at C-8 of senecic acid derived from [6-^{14}C]-isoleucine.[75] This is in accord with the biosynthesis shown in Scheme 24. From this it is apparent that path B (p. 56) is operative. Previously, path A was proposed due to greater labelling at C-6/ C-7 than at C-10 of seneciphyllic acid derived from [U-^{14}C]threonine.[66]

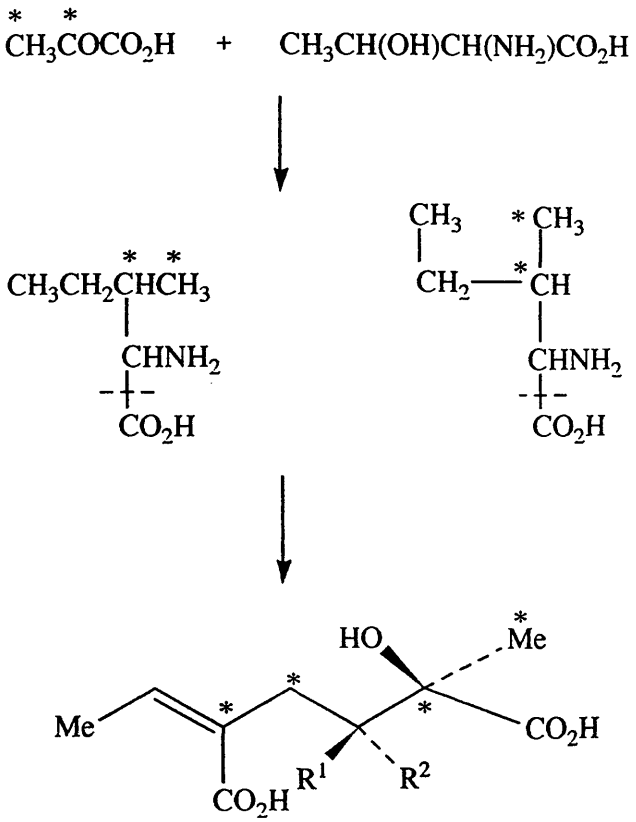
However, uniformly labelled threonine can be modified <u>in vivo</u> to introduce an inequality in the labelling of its C-1,2 and C-3,-4 components. Threonine adolase is widely distributed in higher plants.[76] Sequential action of threonine aldolase, acetaldehyde dehydrogenase and acetyl CoA synthetase is shown in Scheme 25. If this is reversible to some



(115)



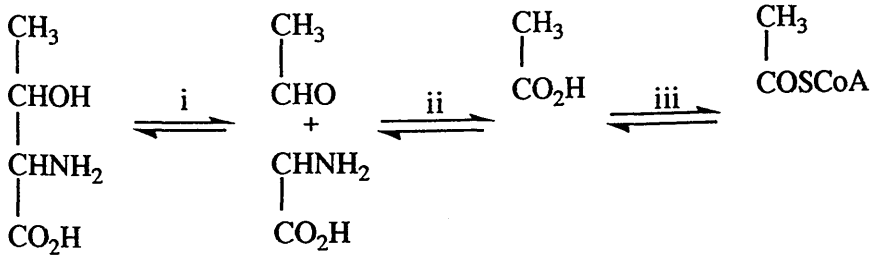
(32)



$R^1, R^2 = CH_2$ Seneciphyllic acid

$R^1 = H, R^2 = Me$ Senecic acid

Scheme 24



(108)

i) Threonine aldolase

ii) Acetaldehyde dehydrogenase

iii) Acetyl CoA synthetase

Scheme 25

degree, then the distribution of label in threonine will depend on the extent to which the system moves to equilibrium and on the relative sizes of existing pools of glycine and acetyl CoA. Thus the observed distribution of labelling in seneciphyllic acid derived from [U-^{14}C]threonine might have resulted from dilution of the label of the C-1,-2 portion of threonine by equilibration with a pool of unlabelled glycine considerably larger than that of acetyl CoA.

These results indicate that senecic acid is formed from two molecules of isoleucine, or its biological precursor threonine with loss of both carboxyl groups of isoleucine. Furthermore, of the four possible stereoisomers of isoleucine, only L-isoleucine is utilised in senecic acid biosynthesis.[77]

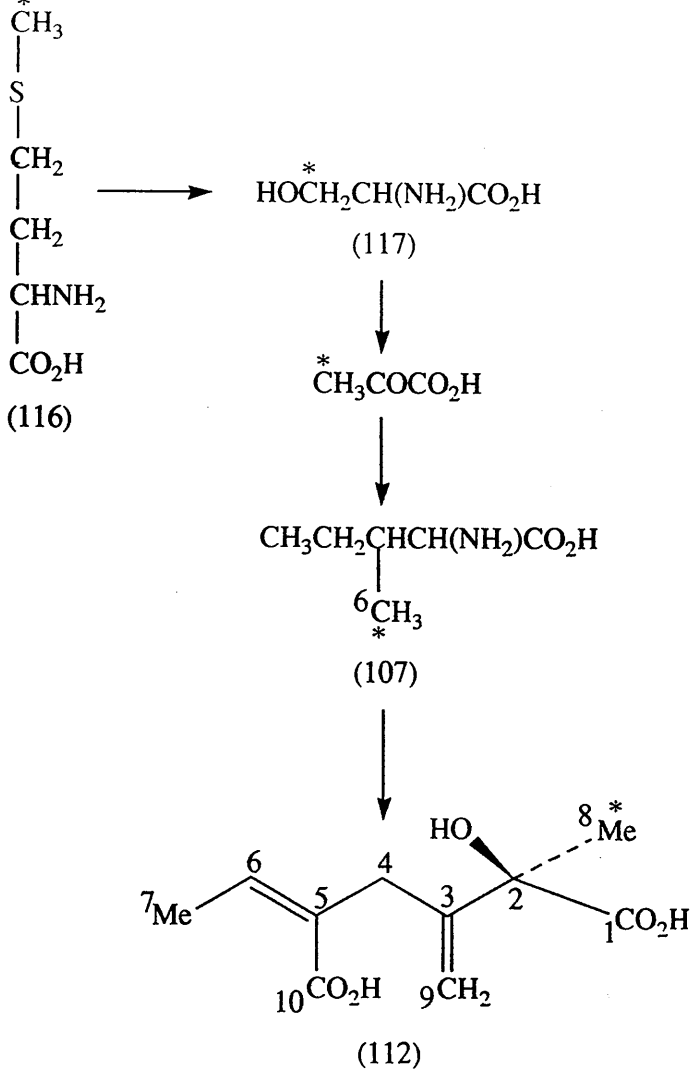
The S-methyl group of L-[Me-^{14}C]methionine has been shown to be a relatively specific precursor for C-8 of seneciphyllic acid, though the total incorporation was low.[75] Indeed, the label was somewhat randomised between the necic acid and the necine base. There is evidence[78] that the S-methyl of methionine (116) can be transformed into C-3 of serine (117) *via* a one-carbon pool. Serine can be converted into pyruvate *in vivo* and hence the S-methyl group of methionine could be incorporated into C-8 of seneciphyllic acid *via* C-6 of isoleucine (Scheme 26). The label from [Me-^{14}C]methionine is incorporated specifically into the hydroxylmethyl group of serine in pea seedlings.[75]

The next point of interest was the mode of

coupling between C-4 and C-6 of the two isoleucine molecules[79](Scheme 27). Five carbon intermediates of isoleucine metabolism, 2-methylbutanoic acid (118), angelic acid (110) and 2-methyl-3-oxobutanoic acid (119) have been excluded as precursors of senecic acid (115) in *Senecio magnificus*.[79] The mechanism of the coupling reaction was investigated through incorporation experiments with [6-^{3}H]- and [4-^{3}H]-isoleucine in *S. magnificus* and *S. isatideus*.[79]

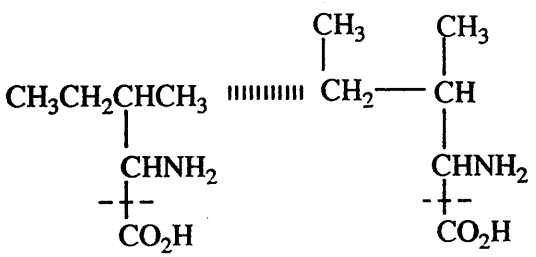
Feeding of [6-^{3}H, 6-^{14}C]isoleucine to *S. magnificus* resulted in retention of five-sixths of the tritium activity. Thus, it is probable that one of the H-atoms at C-6 of isoleucine is retained during conversion into C-14 of senecionine, and it is likely that two are retained. L-[4-^{3}H, U-^{14}C]Isoleucine was a specific precursor for isatinecic acid (120) in *S. isatideus*, and half of the tritium was retained. This limits the oxidation level to which C-4 of isoleucine is raised during incorporation into C-13 of retrorsine to that of a carbinol or a vinylic methine. This provides further evidence against the involvement of 2-methyl-3-oxobutanoate (119) since clearly a carbonyl function is not introduced for the coupling of two isoleucine-derived units. The possibility of a -C=CH$_2$ functionality being introduced into the C-3, C-6 side chain of isoleucine was pursued since the corresponding amino acid, β-methylenenorvaline (121) is known, as a metabolite of the fungus *Lactarius helvus*.[80]

β-[^{3}H$_2$]Methylenenorvaline was incorporated specifically into senecic acid. Unfortunately, the low

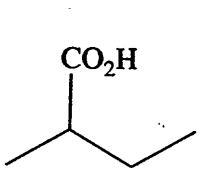


(116) (117) (107) (112)

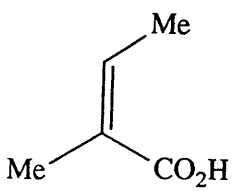
Scheme 26



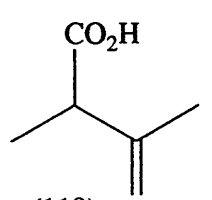
Scheme 27



(118)



(110)



(119)

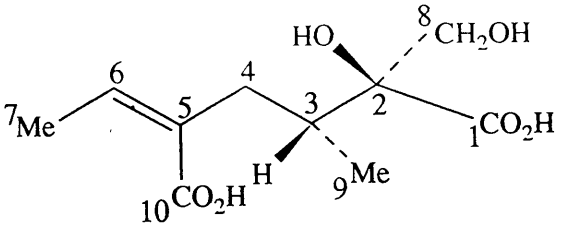
activity of the derived alkaloid prevented degradative work to establish whether this was incorporated into one or both halves of senecic acid. It was suggested that incorporation was into one half only, since feeding of [6-^{3}H]isoleucine had shown that probably only one of the six H-atoms of the two molecules of isoleucine is lost. Furthermore, feeding experiments with (2S,4S)-[3,4-^{3}H]- and (2S,4R)-[4-^{3}H]isoleucine demonstrated that during conversion into senecic acid, both C_5 units are formed with loss of H-4 pro-S and retention of H-4 pro-R of isoleucine.[81]

The basic C_5 unit present in many necic acids can be formulated as (122). Further modification of this unit and coupling with a similar C_5 unit derived from isoleucine can then lead to senecic and seneciphyllic acids, by the introduction of unsaturation. Further hydroxylation of this C_5 unit may occur on the pathway to monocrotalic acid (123). [U-^{14}C]Threonine and [U-^{14}C]isoleucine are specifically incorporated into monocrotalic acid in *Crotalaria spectabilis* and *Crotalaria retusa*.[82] The C_5 unit comprising C-1,-2,-3, -6 and -7 of monocrotalic acid was most heavily labelled, in support of the proposed biogenesis (Scheme 28). Significant labelling was also present at C-4, -5 and -8 after feeding L-[U-^{14}C]threonine and L-[U-^{14}C]isoleucine, both of which can be metabolised to propionyl CoA.[83] However, the exact nature of the C_3 unit comprising C-4, -5 and -8 of monocrotalic acid has yet to be established.

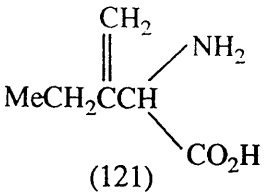
Trichodesmic acid (124) is the diester portion of

trichodesmine, the major alkaloid of *Crotalaria globifera*. Both the 'left' and 'right' hand C_5 units are known to be derived from amino acids.[84] Threonine and isoleucine mainly label the 'right hand' C_5 unit, while valine and leucine are incorporated to a greater extent into the 'left hand' unit, as shown in Scheme 29.

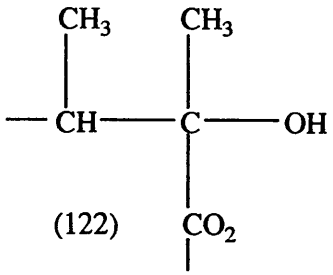
In conclusion, all necic acids studied so far are derived from the common branched-chain α-amino acids threonine, isoleucine, valine and leucine. The biosynthesis of dicrotalic acid, the diacid portion of dicrotaline (28), in *Crotalaria lachnosema* is discussed in Chapter 6.



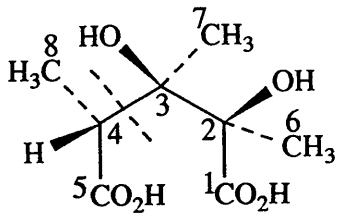
(120)



(121)

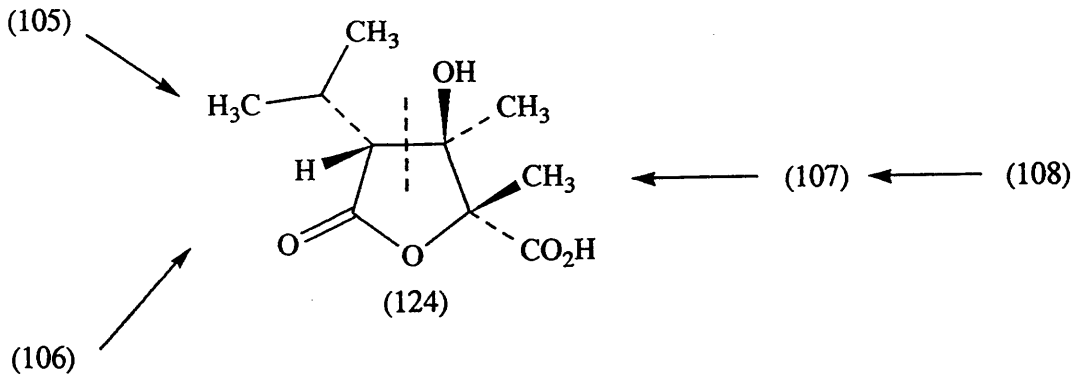


(122)

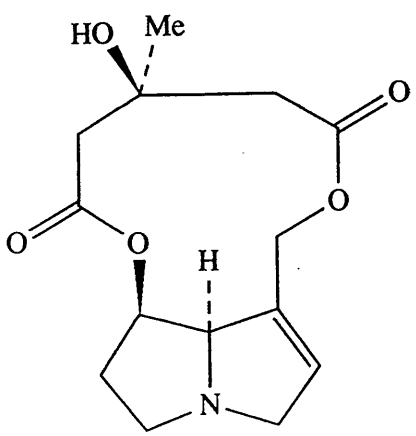


(123)

Scheme 28



Scheme 29



(28)

Chapter 3

BIOSYNTHESIS OF PYRROLIZIDINE ALKALOID ANALOGUES

3.1 Introduction

The biosynthetic pathways leading to the necine bases are now well-established and a number of intermediates have been identified. A further challenge is to make use of these biological systems in plants for the production of novel compounds, as an alternative to total synthesis, by the feeding of unnatural precursors.

Studies with analogues of known intermediates on a biosynthetic pathway can have a number of uses. These could include (i) further elucidation of the pathway; (ii) the blocking of the biosynthesis of a natural product; (iii) a means of producing unnatural products. In the first instance, the observation that an enzyme system can transform one unnatural precursor but not another may shed light on the mechanism of a particular enzymic reaction. Figure 1 illustrates the latter two points. A modified precursor A^{1} may be carried through a given number of steps in the biosynthetic route but is then the cause of the blocking of a specific transformation. This type of enzyme inhibitory action is the basis of much of the research carried out in the pharmaceutical and agrochemical industries. Alternatively, administration of an unnatural precursor A^{11} to an organism may result in the production of a new compound D^{11}, the biological activity of which can be assessed.

The first example of the transformation of an unnatural precursor into an unnatural product was provided by Rueppel and Rapoport.[85] Feeding of 1,3-dimethyl-1-pyrrolinium (125) to *Nicotiana glutinosa* resulted in the biosynthesis of a substituted nicotine which was identified as 3′-methylnicotine (126). The absolute configuration at C-2′ was tentatively assigned to be the same as in nicotine (127) on the basis of circular dichroism and optical rotatory dispersion measurements. Only one of the possible diastereomers seemed to be formed since only one methyl doublet was present in the ^{1}H NMR spectrum, although the absolute configuration at C-3′ was not clear.

Further examples of the biosynthesis of unnatural products came from the work of Kirby et al.[86] It is known that codeine (128) is the biosynthetic precursor of morphine (129).[87] Efficient demethylation of unnatural dihydrodeoxycodeine (130) was observed in *Papaver somniferum*, and (130) was converted into dihydrodeoxymorphine (131) with a total incorporation of 9.27%.[86] On the other hand, only 0.15% incorporation of 1-bromo[2-^{3}H]codeine (132) into 1-bromo[2-^{3}H]morphine (133) occurred, indicating that introduction of bromine into the aromatic ring of codeine (128) inhibits the demethylation.[86]

Halogenated precursors have been much-favoured in the study of aberrant biosynthesis, particularly in the use of precursors labelled with fluorine. The van der Waals radii of hydrogen and fluorine are very similar, 1.20 Å and 1.35 Å respectively, and it was proposed that

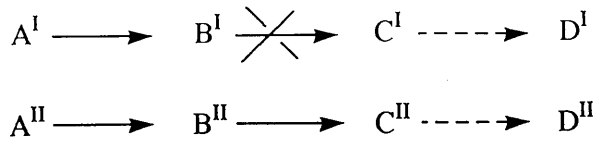
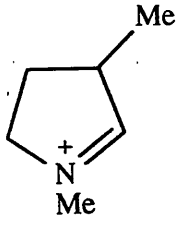
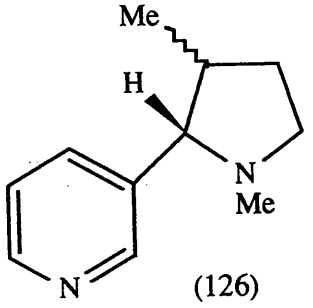


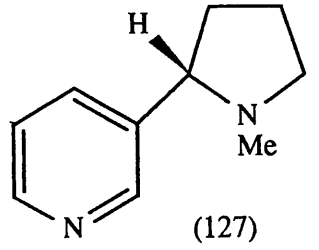
Figure 1



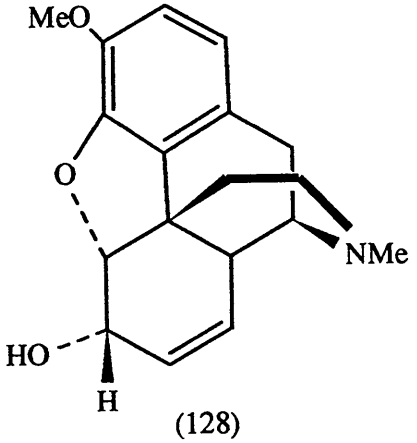
(125)



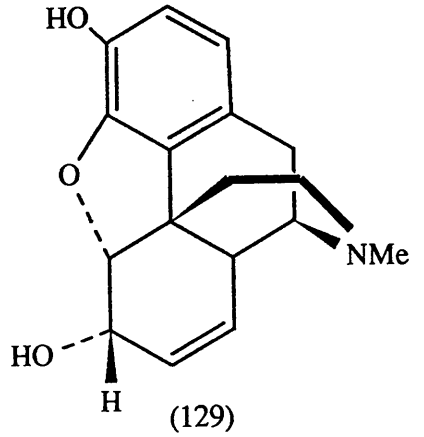
(126)



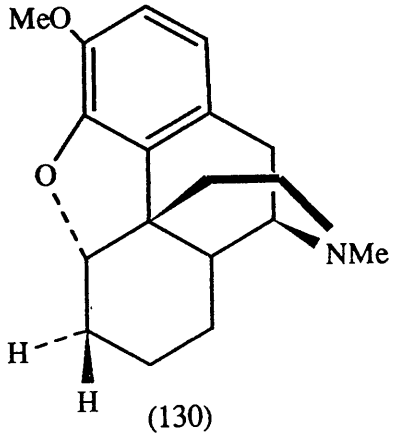
(127)



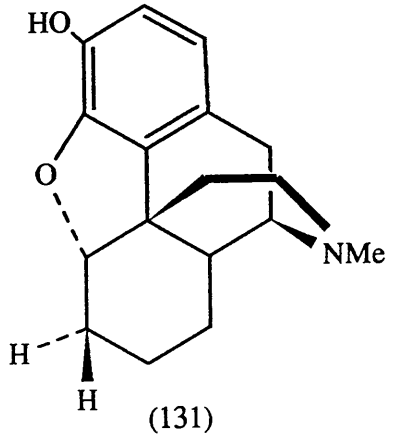
(128)



(129)



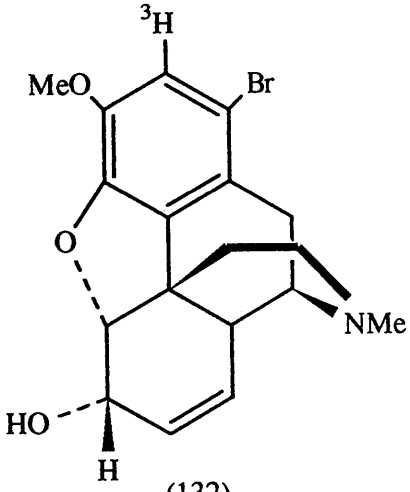
(130)



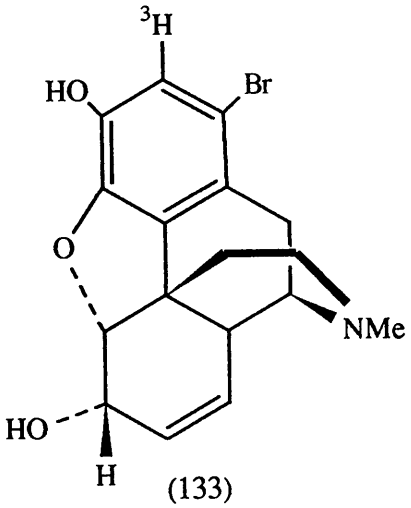
(131)

fluorine would closely mimic hydrogen with respect to steric requirements at enzyme receptor sites. Leete et al[88] fed 5-fluoronicotinic acid (134) to *Nicotiania tabacum* and this was converted (incorporation 0.15%) into 5-fluoronicotine (135). Organofluorine compounds have been used medicinally in areas such as cancer chemotherapy, antibiotics and anti-inflammatory agents. They are often more bioactive than the corresponding hydrogen-containing compounds probably because they are usually more lipophilic, which results in enhanced rates of absorption and transport of drugs in vivo.

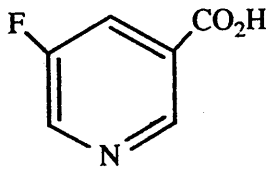
In this chapter, the synthesis of, and feeding experiments with, analogues of an intermediate in pyrrolizidine alkaloid biosynthesis are described. Just prior to the start of this project the iminium ion N-(4-aminobutyl)-1,2-didehydropyrrolidinium (22) had been identified as a natural precursor of the pyrrolizidine necines rosmarinecine (4) and retronecine (5).[52] It was decided to undertake a synthesis of radiolabelled analogues of (22) and to study their incorporation into various plant species which produce pyrrolizidine alkaloids. Using the route described by Kelly and Robins to produce the natural precursor (22)[52], compounds with a larger heterocyclic ring size (136) or a homologated N-alkyl chain (137), or both of the above (138), were readily synthesised in ^{14}C-labelled form.



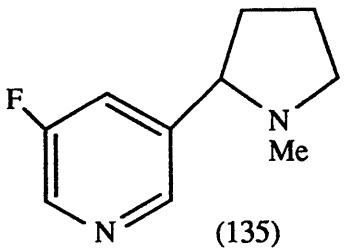
(132)



(133)



(134)

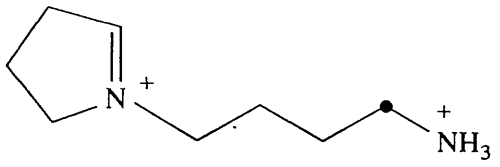


(135)

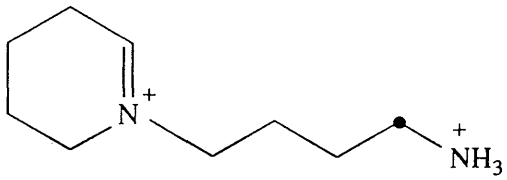
3.2 Synthesis and Feeding of ^{14}C-Labelled Analogues

The general route to the ^{14}C-labelled analogues of the iminium ion (22) is shown in Scheme 30. The ^{14}C label was introduced by S_N2 displacement of a mesylate with sodium [^{14}C]cyanide to yield [1-^{14}C]-4-chlorobutanenitrile (76) or [1-^{14}C]-5-chlorovalero-nitrile (139). Unfortunately, the yields for these two reactions were not particularly high [39% for (76) and 61% for (139)], and the radiochemical yields were lower, at 35% and 42% respectively, probably due to the presence of NaOH in the labelled cyanide. These nitriles and subsequent compounds were of low specific activity (2-30μCi/mmol) which was to prove deleterious in attempts to achieve explicable results from feeding experiments. The chloro-nitrile compounds (76) and (139) were coupled with either pyrrolidine or piperidine in the presence of a catalytic amount of potassium iodide, followed by catalytic hydrogenation using Adams' catalyst to afford the saturated salts (77) and (142)-(144).

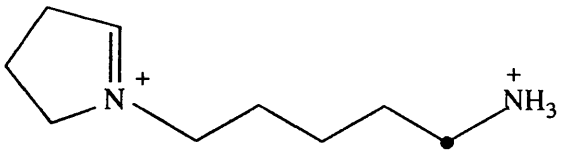
The crucial step in the sequence was the final step, involving oxidation of the saturated salts with mercury (II) acetate. Precedent suggested that this oxidation would result in an endocyclic double bond. Leonard and Hauck reacted the substituted piperidines (145) and (147) with mercury (II) acetate. In the former case, unsaturated amine (146) was obtained whereas (147) was resistant to mercury (II) acetate oxidation[89] (Scheme 31). Similarly, oxidation of substituted pyrrolidines occurs with formation of an



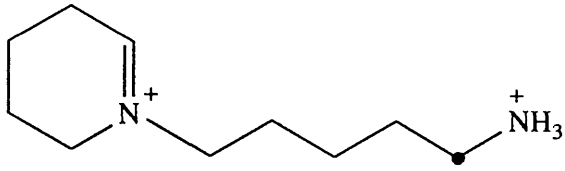
(78) $2Cl^-$



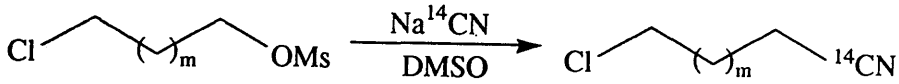
(136) $2Cl^-$



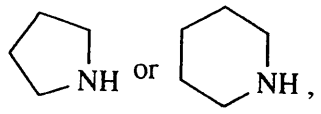
(137) $2Cl^-$

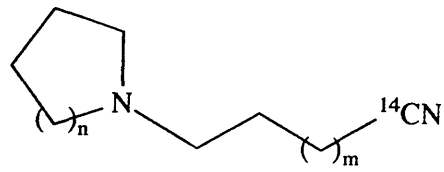


(138) $2Cl^-$



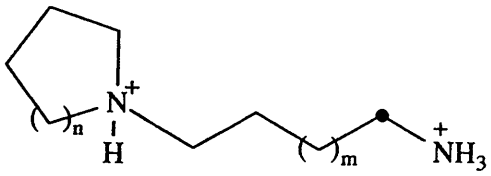
(76) m=1
(139) m=2





(140a) m=1, n=1
(140b) m=1, n=2
(141a) m=2, n=1
(141b) m=2, n=2

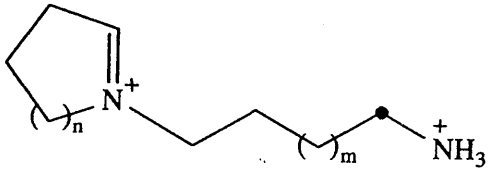
i) PtO_2, H_2,
AcOH
ii) HCl



$2Cl^-$

(77) m=1, n=1
(142) m=1, n=2
(143) m=2, n=1
(144) m=2, n=2

i) $Hg(OAc)_2$, 5% aq AcOH
ii) H_2S
iii) HCl



$2Cl^-$

(78) m=1, n=1
(136) m=1, n=2
(137) m=2, n=1
(138) m=2, n=2

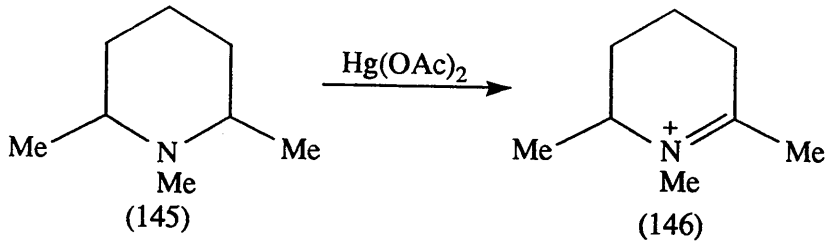
• = ^{14}C

Scheme 30

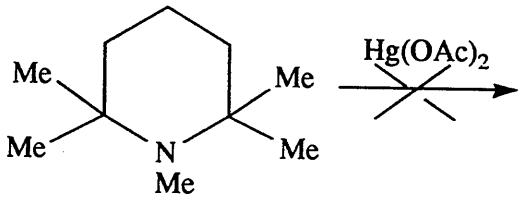
endocyclic double bond, as shown by the reaction of N-(2-hydroxyethyl)pyrrolidine (148) with mercury (II) acetate to produce the tetrahydro-1,3-oxazine (149)[90] (Scheme 32). Kelly and Robins demonstrated[52] that the oxidation of saturated salt (77) occurs with formation of an endocyclic double bond. A sample of iminium ion (78) was reduced with sodium cyanoborodeuteride to furnish the corresponding deuteriated salt (150). A 200 MHz ^{1}H NMR spectrum of the ^{2}H-labelled salt (150) contained a three proton multiplet at δ2.80 whereas the nondeuteriated salt (77) showed a four proton multiplet at δ2.80 for the pyrrolidine ring protons α to the nitrogen, which is consistent with the assignment of an endocyclic double bond to the iminium ion (78).

Nevertheless, it was felt necessary to establish that, in this work, oxidation of an N-alkylpiperidine compound such as (144) would occur with formation of an endocyclic double bond. Reduction of a sample of the iminium ion (138) with sodium cyanoborodeuteride gave a monodeuteriated salt (151) having a three proton multiplet at δ2.82 in the 200 MHz ^{1}H NMR spectrum. The nondeuteriated salt (144) had a four proton multiplet at δ2.82. This indicates regiospecific formation of an endocyclic double bond as in the case for the pyrrolidine ring system.

With the synthesis of ^{14}C-labelled precursors (78) and (136-138) complete a series of feeding experiments was carried out in the summer of 1988 with plant species which produce pyrrolizidine alkaloids, viz *Senecio pleistocephalus*, *Emilia flammea*, and *Cynoglossum*

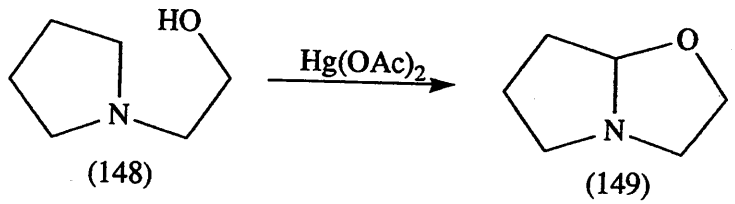


(145) (146)



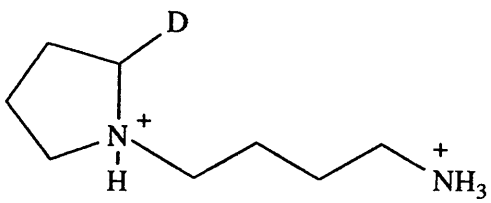
(147)

Scheme 31

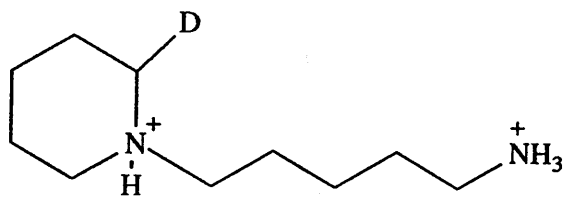


(148) (149)

Scheme 32



(150) $2Cl^-$



(151) $2Cl^-$

australe. Each precursor was mixed with [1,4-^{3}H]putrescine dihydrochloride as an internal standard to give an initial $^{3}H/^{14}C$ ratio of approximately 2:1. The precursors were fed on one day and after 14 days the alkaloids from each plant species were extracted and a $^{3}H/^{14}C$ ratio of each crude alkaloid mixture was measured. These radioactive counts were taken on 1% of each alkaloid mixture and in many cases gave very low readings. Recounts were then taken using 10% of each sample, and again the results proved inconclusive. After tabulating the results (Tables 3-5) no evident pattern of results emerged. These inconclusive results may be partly explained by the low specific activity of the radiolabelled synthetic precursors which together with low incorporations gave rise to a large margin of error on the radioactive counting of the alkaloid extracts.

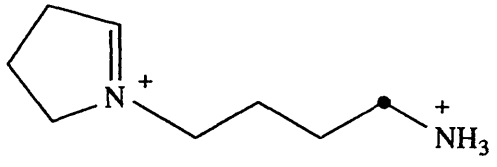
Since the saturated salt (77) had also been reasonably well incorporated into rosmarinecine (4) and retronecine (5)[52], it was decided to study the incorporation of (77) and the ^{14}C-labelled analogues (142)-(144) into the aforementioned plant species. However, as with the iminium ions (78) and (136)-(138), ^{14}C incorporations were disappointingly low.

Since the low levels of incorporation into the crude alkaloid mixtures made it impractical to investigate further at this point, a new synthetic route was considered which would lead to radiolabelled precursors of higher specific activities.

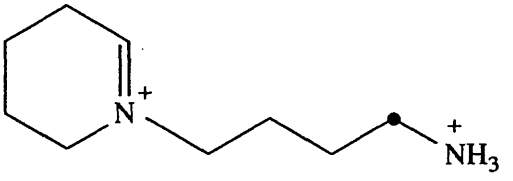
Table 3

S. pleistocephalus.

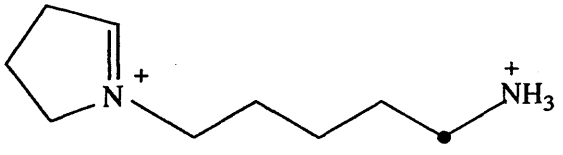
Precursor	Amount fed		Amount isolated			
	^{14}C μCi	mg	mg	Initial $^{3}H/^{14}C$	Final $^{3}H/^{14}C$	^{14}C Total incorporation
(78)	0.57	4.8	143	1.4	0.80	0.75
(136)	0.27	13.6	187	1.8	4.3	0.23
(137)	0.43	3.0	117	2.1	1.6	0.92
(138)	0.54	4.6	160	1.6	0.87	0.83
(77)	0.60	5.9	132	1.5	0.81	0.70
(142)	0.24	2.7	173	4.4	5.4	0.94
(143)	0.11	0.7	190	1.5	3.8	0.37
(144)	0.51	4.2	172	1.7	4.5	0.32



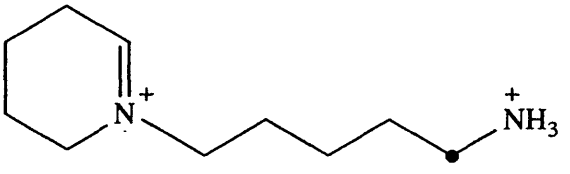
(78) $2Cl^-$



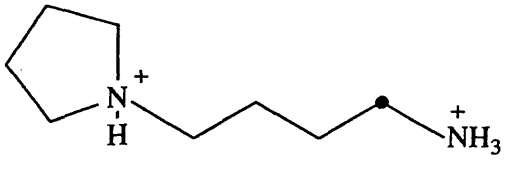
(136) $2Cl^-$



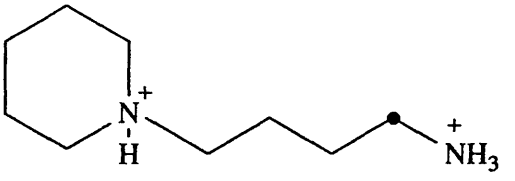
(137) $2Cl^-$



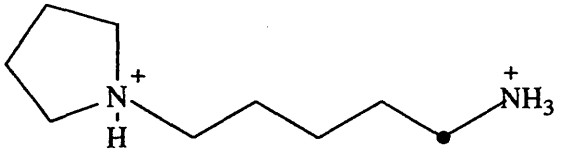
(138) $2Cl^-$



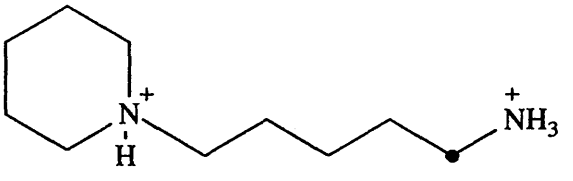
(77) $2Cl^-$



(142) $2Cl^-$



(143) $2Cl^-$

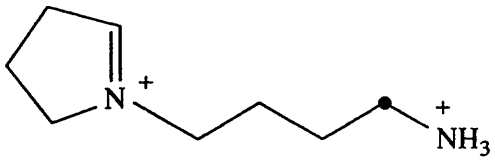


(144) $2Cl^-$

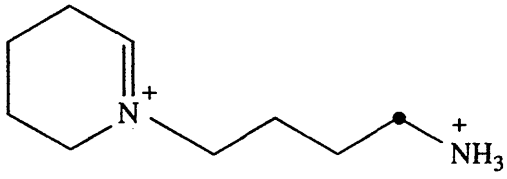
Table 4

E. flammea

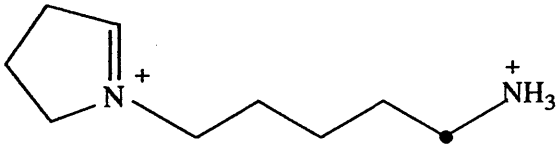
Precursor	Amount fed		Amount isolated			
	^{14}C μCi	mg	mg	Initial $^{3}H/^{14}C$	Final $^{3}H/^{14}C$	^{14}C Total incorporatior
(78)	0.47	3.9	9	1.5	0.43	0.42
(136)	0.53	26.6	6	1.7	1.5	0.29
(137)	0.42	2.9	7	2.1	0.57	0.88
(138)	-	-	-	-	-	-
(77)	0.51	5.0	19	1.4	0.53	0.68
(142)	0.19	2.1	8	4.9	2.3	0.39
(143)	0.56	3.9	11	1.6	1.0	0.74
(144)	0.64	5.4	7	2.1	0.06	0.76



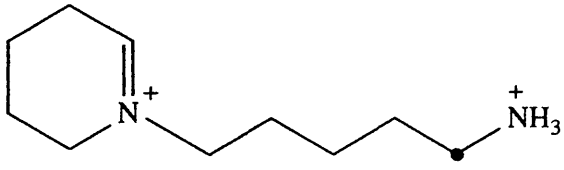
(78) $2Cl^-$



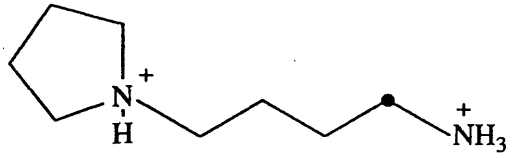
(136) $2Cl^-$



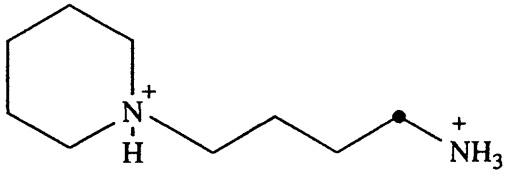
(137) $2Cl^-$



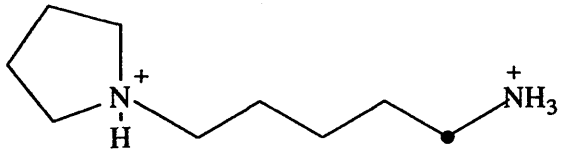
(138) $2Cl^-$



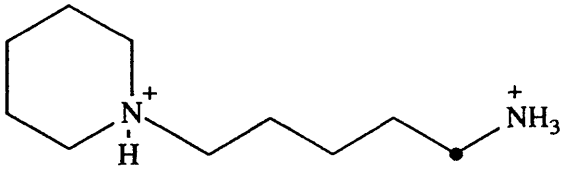
(77) $2Cl^-$



(142) $2Cl^-$



(143) $2Cl^-$

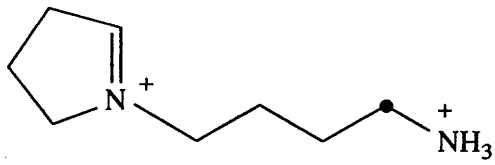


(144) $2Cl^-$

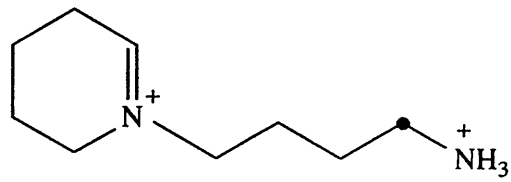
Table 5

C. australe

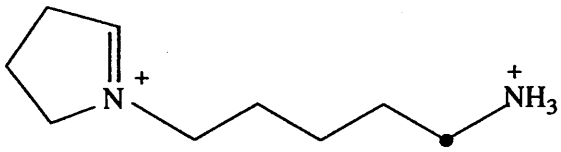
Precursor	Amount fed		Amount isolated			
	^{14}C μCi	mg	mg	Initial $^{3}H/^{14}C$	Final $^{3}H/^{14}C$	^{14}C Total incorporation
(78)	0.44	3.7	7	1.6	1.2	0.45
(136)	0.55	27.6	26	1.7	0.18	0.38
(137)	0.45	3.1	10	2.2	0.6	0.32
(138)	0.51	4.3	9	1.7	0.15	0.82
(77)	0.59	5.8	21	1.1	0.23	0.84
(142)	0.11	1.2	30	3.4	1.8	0.99
(143)	0.30	2.1	19	1.6	1.8	0.23
(144)	0.38	3.2	27	1.6	1.5	0.25



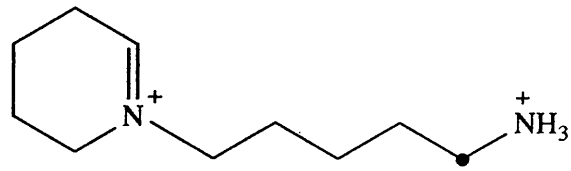
(78) $2Cl^-$



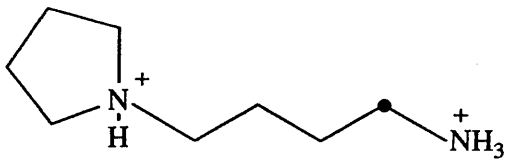
(136) $2Cl^-$



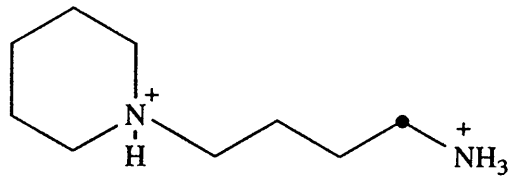
(137) $2Cl^-$



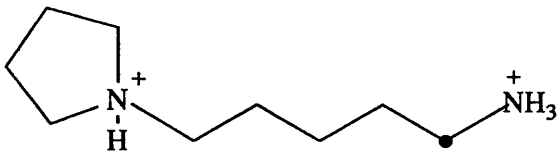
(138) $2Cl^-$



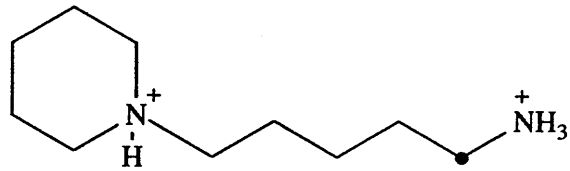
(77) $2Cl^-$



(142) $2Cl^-$



(143) $2Cl^-$



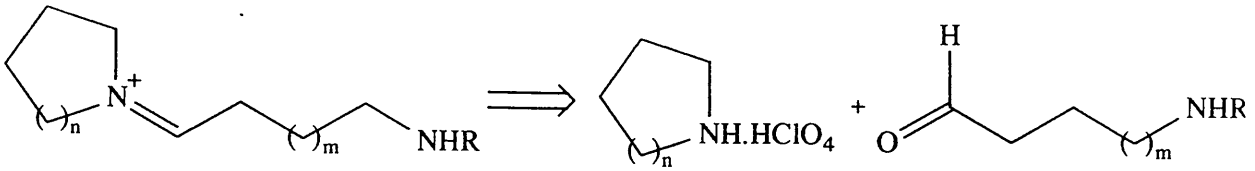
(144) $2Cl^-$

3.3 Synthesis and Feeding of ^{3}H-Labelled Analogues

In this case, the general strategy adopted was to synthesise the iminium species (152)-(155) which could be reduced with a tritide (^{3}H) reducing agent. Leonard and Paukstelis reported[91] the direct synthesis of such iminium salts by reaction of secondary amine salts with aldehydes and ketones. Synthetic equivalents (156) and (157) of, respectively, 4-aminobutanal and 5-aminopentanal were required for reaction with pyrrolidine perchlorate (158) or piperidine perchlorate (159) (Scheme 33). N-Protection was required to prevent inter- or intramolecular reactions between the amine and aldehyde groups. Thus, 4-aminobutanol and 5-aminopentanol were converted into the corresponding N-benzyloxycarbonyl (CBZ) derivatives (160) and (161). Swern oxidation[92] of the primary alcohols of (160) and (161) gave the corresponding aldehydes (162) and (163) in quantitative yield. These aldehydes were unstable and had to be used immediately.

Reaction of aldehydes (162) and (163) with either pyrrolidine perchlorate (158) or piperidine perchlorate (159) under a variety of reaction conditions failed to produce the desired iminium salts (152)-(155). For example, reaction at room temperature in the presence of dehydrating agents such as molecular sieves or anhydrous magnesium sulphate, or in a Dean-Stark apparatus with refluxing benzene all proved unsuccessful.

It was therefore decided to synthesise the iminium salts *via* the corresponding enamines. Reaction of

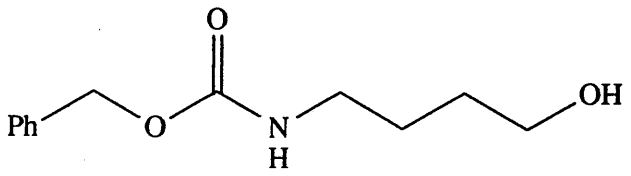


(152) m=1, n=1
(153) m=1, n=2
(154) m=2, n=1
(155) m=2, n=2

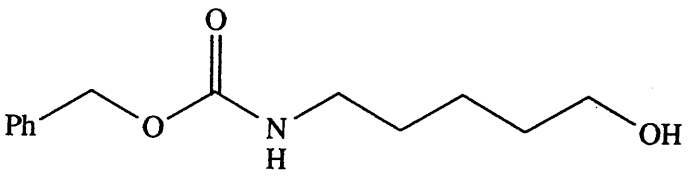
(158) n=1
(159) n=2

(156) m=1
(157) m=2

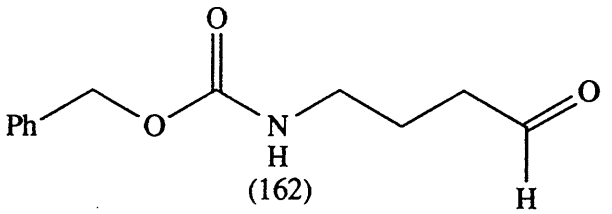
Scheme 33



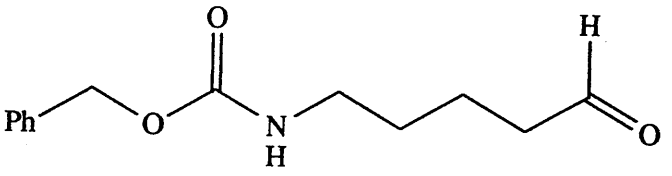
(160)



(161)



(162)



(163)

aldehydes (162) and (163) with either pyrrolidine or piperidine in the presence of various dehydrating agents, eg. K_2CO_3,[93] $CaCl_2$,[94] $TiCl_4$,[95] or molecular sieves[96] was studied in order to maximise the yield of the reaction. It was found that the highest yield of iminium salt, formed by acidification of an ether solution of the enamine with 70% perchloric acid ($HClO_4$)[97], was achieved by reaction in diethyl ether in the presence of anhydrous K_2CO_3. It was also noted that enamine formation was faster in diethyl ether than in dichloromethane.

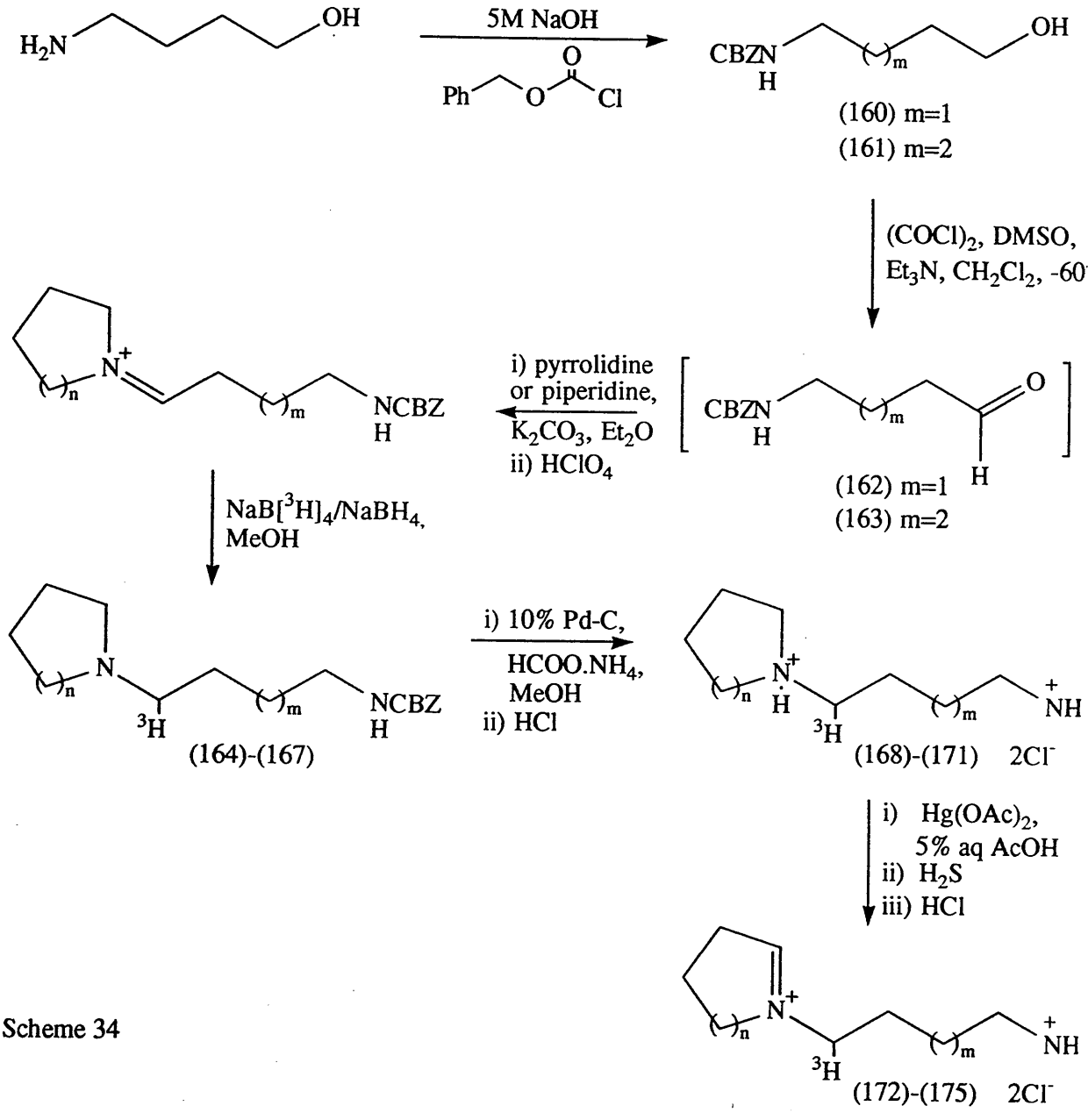
The iminium salts (152)-(155) were not particularly stable and in general were used without purification in the next step, in which the ^3H label was introduced by sodium boro[^3H]borohydride/$NaBH_4$ reduction, yielding the ^3H-labelled compounds (164)-(167).

The penultimate stage in the reaction scheme involved removal of the CBZ protecting group. This group proved resistant to cleavage using catalytic hydrogenation (10% palladium on charcoal, glacial acetic acid, H_2) so an alternative method using catalytic transfer hydrogenation was adopted. Carrying out the reaction in methanol containing 10% Pd-C, both cyclohexene[98] and 1,4-cyclohexadiene[99] proved to be ineffective as hydrogen donors. However, smooth hydrogenolysis of the CBZ group could be achieved in each case using ammonium formate[100] as the hydrogen donor, furnishing the ^3H-labelled precursors (168)-(171). The final step then involved, as in the

synthesis of ^{14}C-labelled analogues, oxidation to iminium ions (172)-(175) with mercury (II) acetate. The overall synthetic route to ^{3}H-labelled precursors is shown in Scheme 34.

This synthetic route had a number of advantages over that used to synthesise ^{14}C-labelled analogues. These were (i) overall yields were higher; (ii) the radiolabel was introduced at a later stage; and (iii) precursors had higher specific activities, in the region of 150-250 μCi/mmol, compared with values of 2-30 μCi/mmol for the ^{14}C-labelled analogues. Of particular note is the fact that the specific activity of each precursor remained reasonably constant after oxidation with mercury (II) acetate, indicating little or no loss of tritium during the oxidation.

With the ^{3}H-labelled iminium ions (172)-(175) as well as the corresponding saturated salts (168)-(171) now in hand, a series of feeding experiments was carried out in the summer of 1989 using the plants species *Senecio pleistocephalus, S. isatideus* and *Cynoglossum australe*. Each ^{3}H-labelled precursor was mixed with [1,4-^{14}C]putrescine as an internal standard to give an initial $^{3}H/^{14}C$ ratio of approximately 10:1. The precursors were administered on one day and after fourteen days the plants were harvested and the alkaloids were extracted. This time, the results from radioactive counting of the crude alkaloid mixtures were more encouraging (Tables 6-8), with total incorporations of tritium into the crude alkaloid mixture of 2-15% for *S.pleistocephalus*. A few general points emerged from

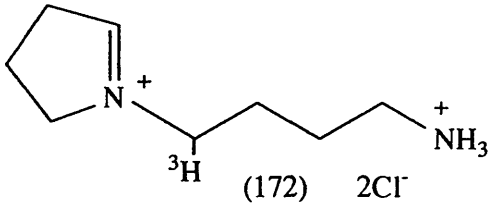

5M NaOH
(160) m=1
(161) m=2
(COCl)2, DMSO,
Et3N, CH2Cl2, -60
i) pyrrolidine
or piperidine,
K2CO3, Et2O
ii) HClO4
(162) m=1
(163) m=2
NaB[3H]4/NaBH4,
MeOH
i) 10% Pd-C,
HCOO.NH4,
MeOH
ii) HCl
(164)-(167)
(168)-(171) 2Cl-
i) Hg(OAc)2,
5% aq AcOH
ii) H2S
iii) HCl
(172)-(175) 2Cl-

Scheme 34

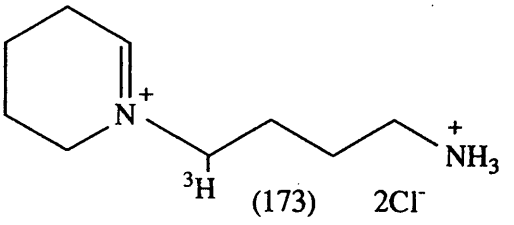
Table 6

S. pleistocephalus

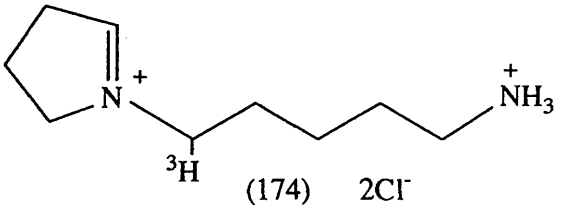
Precursor	Amount fed		Amount isolated			
	^3H μCi	mg	mg	Initial $^3H/^{14}C$	Final $^3H/^{14}C$	^3H Total incorporation
(172)	3.0	3.1	235	4.9	7.8	14.2
(173)	8.9	9.4	118	14.1	10.5	6.4
(174)	6.3	5.9	202	13.1	14.5	14.1
(175)	10.1	10.8	240	10.1	7.4	5.9
(168)	4.6	6.1	222	8.8	7.5	5.0
(169)	7.5	11.2	379	10.9	9.5	3.4
(170)	9.2	8.0	255	9.2	8.8	3.9
(171)	10.8	14.0	255	12.7	8.3	2.9



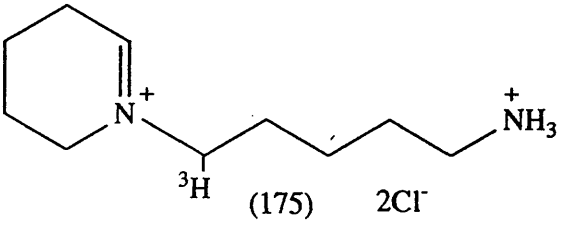
(172) $2Cl^-$



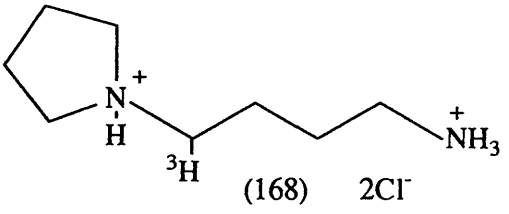
(173) $2Cl^-$



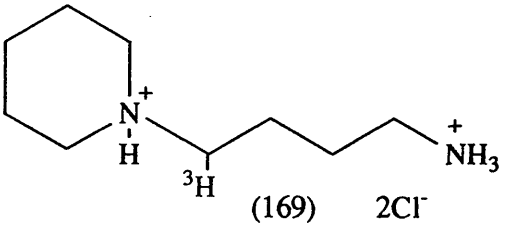
(174) $2Cl^-$



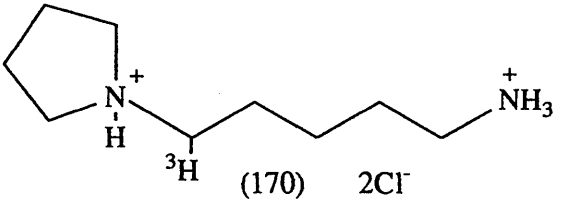
(175) $2Cl^-$



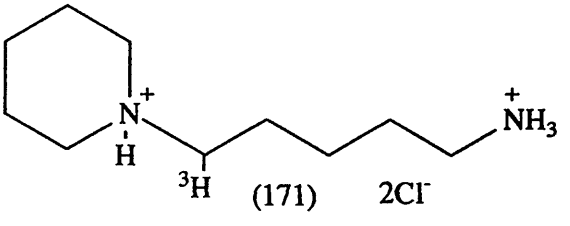
(168) $2Cl^-$



(169) $2Cl^-$



(170) $2Cl^-$

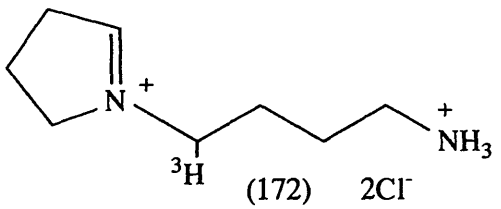


(171) $2Cl^-$

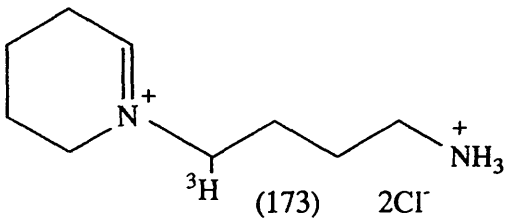
Table 7

C. australe

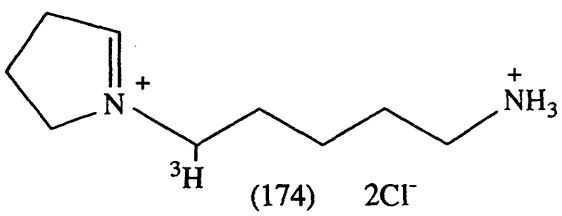
Precursor	Amount fed		Amount isolated			
	^{3}H μCi	mg	mg	Initial $^{3}H/^{14}C$	Final $^{3}H/^{14}C$	^{3}H Total incorporation
(172)	2.9	3.0	90	10.8	11.1	0.97
(173)	8.5	9.0	71	6.6	4.5	0.53
(174)	10.8	10.2	41	8.5	7.3	0.71
(175)	9.5	10.2	87	9.5	6.4	0.41
(168)	5.2	6.9	49	14.1	11.2	0.65
(169)	5.4	8.0	65	6.9	4.3	0.31
(170)	9.6	8.4	75	9.6	8.2	0.36
(171)	10.0	13.0	45	8.4	5.0	0.26



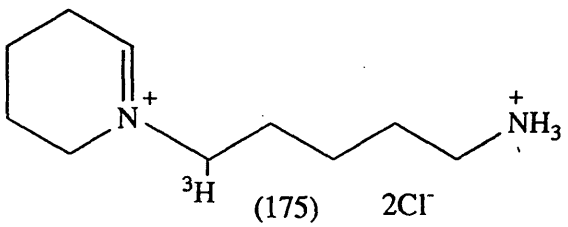
(172) $2Cl^-$



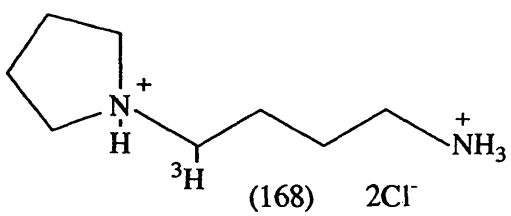
(173) $2Cl^-$



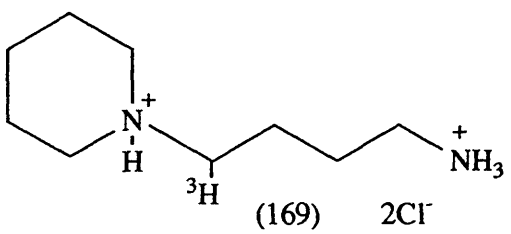
(174) $2Cl^-$



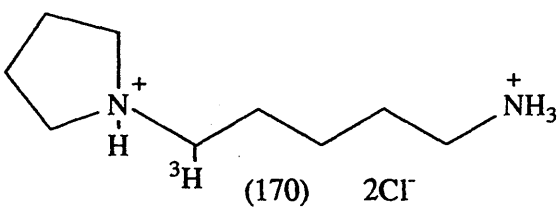
(175) $2Cl^-$



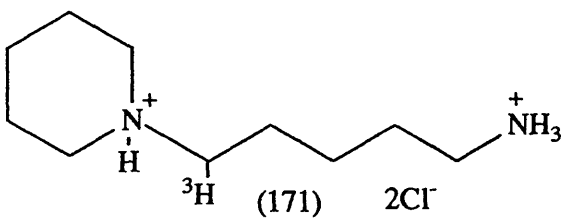
(168) $2Cl^-$



(169) $2Cl^-$



(170) $2Cl^-$

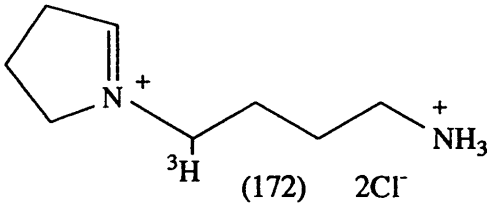


(171) $2Cl^-$

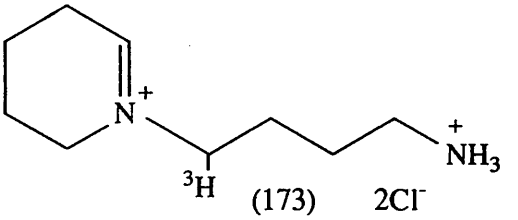
Table 8

S. isatideus

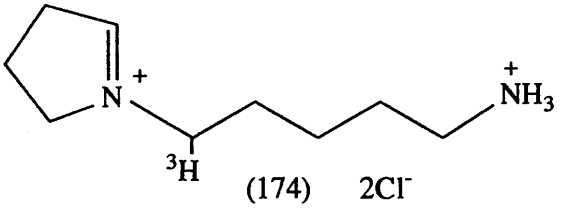
Precursor	Amount fed		Amount isolated			
	^3H µCi	mg	mg	Initial $^3H/^{14}C$	Final $^3H/^{14}C$	^3H Total incorporation
(172)	3.1	3.2	54	5.2	5.9	1.1
(173)	10.0	10.6	75	9.6	10.3	0.84
(174)	9.6	9.0	89	10.1	11.2	0.91
(175)	9.7	10.4	84	9.7	8.6	0.71



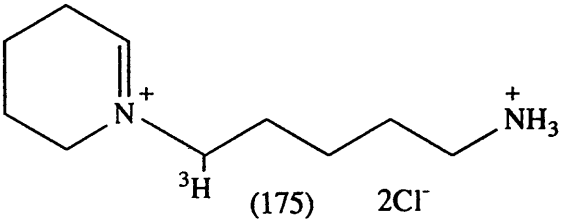
(172) $2Cl^-$



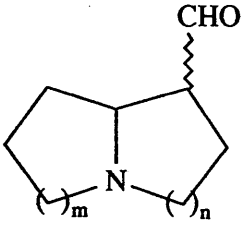
(173) $2Cl^-$



(174) $2Cl^-$



(175) $2Cl^-$



(176) m=2, n=1

(177) m=1, n=2

(178) m=2, n=2

these feeding experiments: (i) the highest total incorporations were achieved in experiments with *S. pleistocephalus*; (ii) on the whole, iminium ion precursors (172)-(175) were incorporated more efficiently than the corresponding saturated compounds (168)-(171) - the latter probably have to undergo an additional in vivo oxidation in order to become involved in the biosynthetic pathway; (iii) the higher homologues tend to be incorporated less efficiently than putrescine (30) since there was usually a decrease in the $^{3}H/^{14}C$ ratio in these cases; (iv) the incorporation figures for putrescine were generally lower in feeding experiments with saturated precursors (168)-(171), with smaller decreases in the $^{3}H/^{14}C$ ratios. This last point is of particular note since it suggests that the saturated analogues may be inhibiting enzymic oxidation of putrescine. Incorporation figures for experiments with *S. pleistocephalus* were particularly high, and so most effort was expended on determination of the composition of each crude extract from this species. All eight samples derived from feeding experiments with *S. pleistocephalus* were subjected to TLC analysis using chloroform-methanol-conc. ammonia (85:14:1) as the eluting mixture, and a radioscan of each chromatogram was used to determine the location of the radioactivity. The samples derived from feeding (168) and (172) each showed one radioactive band coincident with authentic unlabelled rosmarinine (70). Of particular interest was the presence of a broad band at R_F 0.15-0.28 on the chromatogram of each of the other six samples, derived from (169)-(171) and (173)-(175) along with a band corresponding to radiolabelled rosmarinine (70) at R_F

0.34. Each of these bands was shown to be due to the presence of one or more tertiary amines by spraying each of the tlc plates with the modified Dragendorff reagent.[101] Next, the rosmarinine (70) in each sample was recrystallised to constant specific activity from dichloromethane-acetone (1:1). Rosmarinine derived from precursors (168) and (172) gave constant $^{3}H/^{14}C$ ratios. It was found that after five recrystallisations almost all the ^{3}H activity of each of the remaining six samples was concentrated in the mother liquors whereas most of ^{14}C activity was in the recrystallised rosmarinine. The residue from each of these six mother liquors was purified by preparative tlc to remove residual amounts of ^{14}C-labelled rosmarinine, allowing isolation of virtually all of the ^{3}H activity. Unfortunately, in each case the amount of material isolated was insufficient to characterise any analogue produced, save to say that each appeared to be a tertiary amine. The initial product of the action of a diamine oxidase on analogues such as (173)-(175) would be of the general structure (176)-(178). At present, the degree of any further elaboration of such compounds has yet to be determined.

3.4 Conclusions

It appears from this work that the enzymes present in plant species which produce pyrrolizidine alkaloids can accept unnatural substrates. The use of precursors of high specific activity has been of particular value. The highest incorporations were achieved with *S. pleistocephalus*, so future work should concentrate on

feeding experiments with quantities of unnatural precursors using a number of *S. pleistocephalus* plants. This should enable milligram quantities of analogues to be isolated, facilitating their characterisation and perhaps biological evaluation.

Chapter 4

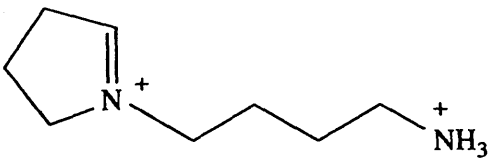
OXIDATION OF DIAMINES BY DIAMINE OXIDASE

4.1 Introduction

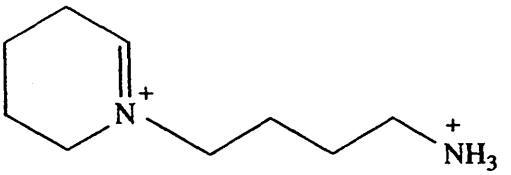
The previous chapter contains a discussion of biosynthetic experiments carried out with a series of analogues of a known intermediate in pyrrolizidine alkaloid biosynthesis. In this chapter the results of a biophysical study of these analogues are presented. Synthesis of the iminium ion (22) and non-labelled analogues (179)-(185) was carried out as outlined in Scheme 30 (Chapter 3), substituting sodium cyanide for sodium [^{14}C]cyanide. These compounds were then assayed as substrates for the diamine oxidase (DAO,EC 1.4.3.6) from pea seedlings, using a kinetic assay procedure which allowed determination of K_M and V_{max} data for each substrate. This provides an opportunity for a more rigorous study of the enzymic specificity of the diamine oxidase.

4.2 Enzyme Kinetics

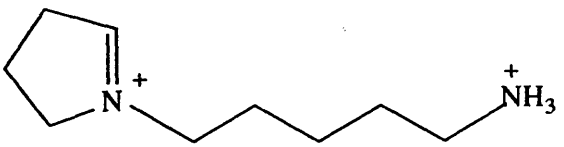
Much of the catalytic power of enzymes comes from their bringing substrates together in favourable orientations in enzyme-substrate (ES) complexes. High-resolution images of substrates and substrate analogues bound to the active sites of enzymes have been provided by X-ray crystallography. These non-covalently bound complexes were first postulated by Michaelis in 1913.[102] It was noted that at constant enzyme concentration, the rate of reaction increases with



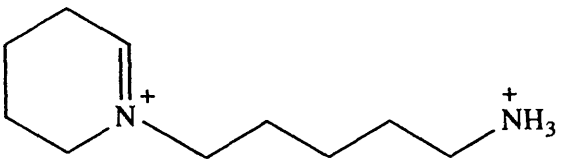
(22) $2Cl^-$



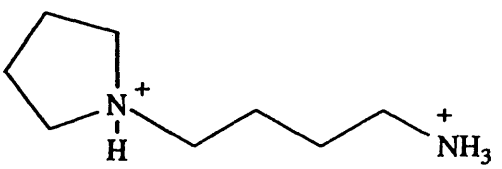
(183) $2Cl^-$



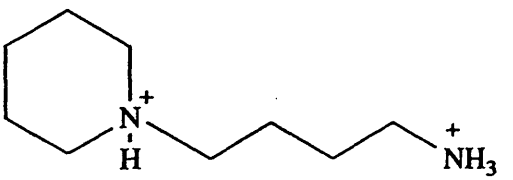
(184) $2Cl^-$



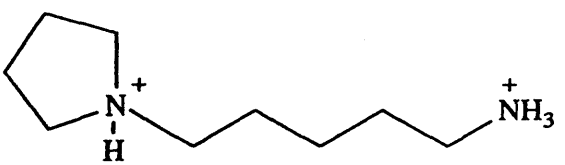
(185) $2Cl^-$



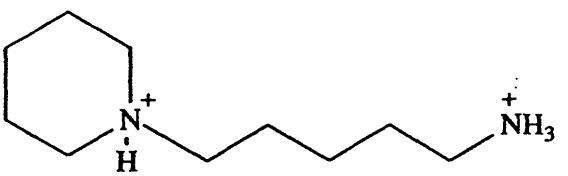
(179) $2Cl^-$



(180) $2Cl^-$



(181) $2Cl^-$



(182) $2Cl^-$

increasing substrate concentration until a maximal velocity is reached. There is no such saturation effect in un-catalysed reactions. In enzymic reactions the maximal velocity is reached when all the catalytic sites are filled. The Michaelis-Menten model is the simplest one that can account for the kinetic properties of many enzymic reactions, in which the rate of catalysis, V, varies with substrate concentration, [S]. At a fixed enzyme concentration, V is almost linearly proportional to [S] when [S] is small. At high [S], V is nearly independent of [S], and at a saturating [S], V reaches a limiting value termed V_{max}.

In deriving the kinetic expressions of the Michaelis-Menten model, it is assumed that the enzyme concentration is negligible compared to that of the substrate. The critical feature of the model is that a specific enzyme-substrate complex is a necessary intermediate in catalysis (equation 1).

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

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

An expression is required that relates the rate of catalysis to the concentrations of substrate and enzyme and the rates of the individual steps. The catalytic rate, V, is equal to the product of the concentration of the ES complex and k_3:

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

We now need to express [ES] in known quantities:

Rate of formation of ES = $k_1[E][S]$ [3]

Rate of breakdown of ES = $(k_2+k_3)[ES]$ [4]

since ES can either dissociate to E and S or can proceed to form product P.

Under steady state conditions, when the concentration of ES is constant while the concentrations of S and P are changing, the rates of formation and breakdown of ES are equal:

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

$$\text{i.e.} \quad [ES] = \frac{[E][S]}{(k_2+k_3)/k_1} \quad [6]$$

This expression can be simplified by defining the Michaelis constant, K_M, as :

$$K_M = \frac{k_2+k_3}{k_1} \quad [7]$$

$$\text{i.e.} \quad [ES] = \frac{[E][S]}{K_M} \quad [8]$$

Provided the enzyme concentration is much less than the concentration of substrate, then the concentration of uncombined substrate is effectively equal to the total substrate concentration. The concentration of uncombined enzyme [E] is equal to the total enzyme concentration $[E_T]$ minus the concentration of the enzyme-substrate complex [ES]

ie $[E] = [E_T]-[ES]$ [9]

Substituting this expression for [E] into equation 8 gives:

$$[ES] = \frac{([E_T]-[ES])[S]}{K_M} \qquad [10]$$

$$[ES] = [E_T]\frac{[S]/K_M}{1+[S]/K_M} \qquad [11]$$

$$[ES] = [E_T]\frac{[S]}{[S]+K_M} \qquad [12]$$

Substituting this expression for [ES] into equation 2 gives:

$$V = k_3[E_T]\frac{[S]}{[S]+K_M} \qquad [13]$$

The limiting rate, V_{max}, is attained when the enzyme active sites are saturated with substrate, ie when [S] is much greater than K_M, so $[S]/[S]+K_M$ approaches 1. Thus,

$$V_{max} = k_3[E_T] \qquad [14]$$

The Michaelis-Menten equation is obtained by substituting equation 14 into equation 13:

$$V = V_{max}\frac{[S]}{[S]+K_M} \qquad [15]$$

At very low [S], when [S] is much less than K_M,

$$V = V_{max}\frac{[S]}{K_M} \qquad [16]$$

and the rate is directly proportional to the substrate concentration. At high [S], when [S] is much greater than K_M, it can be seen from equation 15 that $V = V_{max}$, ie the rate is maximal, and independent of enzyme concentration. Also, when $[S] = K_M$, $V = V_{max}/2$, ie K_M is equal to the substrate concentration at which the reaction rate is half its maximal value. K_M is of significance since it gives an indication of the binding

efficiency of a given substrate since it is the concentration at which half the enzyme active sites are filled.

The turnover number of an enzyme is the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate. The kinetic constant k_3 denotes the turnover number. The maximal rate, V_{max}, reveals the turnover number if the concentration of active sites is known since.

$$V_{max} = k_3 [E_T] \qquad [14]$$

A plot of the reaction velocity, V, as a function of the substrate concentration, [S], for an enzyme that obeys Michaelis-Menten kinetics is shown in Figure 2.

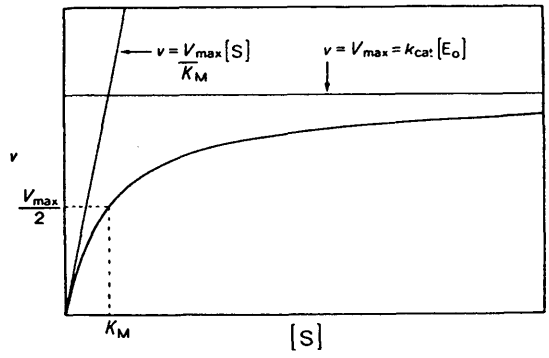
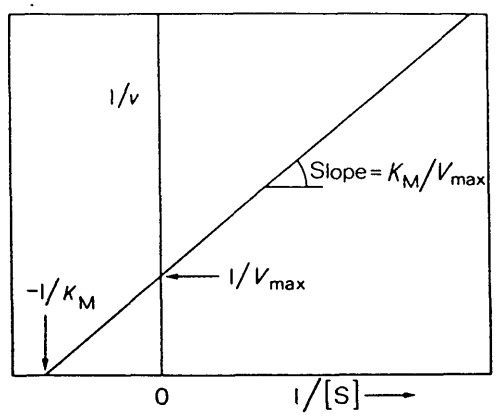


Figure 2 [103].

The Michaelis-Menten equation can be transformed into a straight line plot by taking the double reciprocal:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} \qquad [17]$$

A plot of 1/V versus 1/[S], called a Lineweaver-Burk plot, gives a straight line with intercept $1/V_{max}$ and slope K_M/V_{max} (Figure 3).



The Lineweaver–Burk plot

Figure 3 [103].

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

$$V = V_{max} - K_M V/[S] \qquad [18]$$

The graphic representation of V versus V/[S] is an Eadie-Hofstee plot (Figure 4).

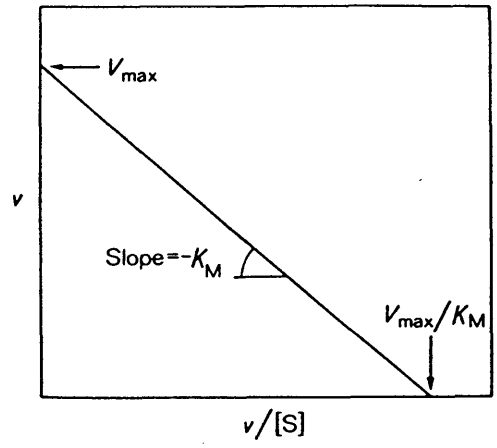


Figure 4 [103].

The Eadie–Hofstee plot

This plot gives an intercept of V_{max} on the y axis as V/[S] tends to zero. The slope of the line is equal to $-K_M$.

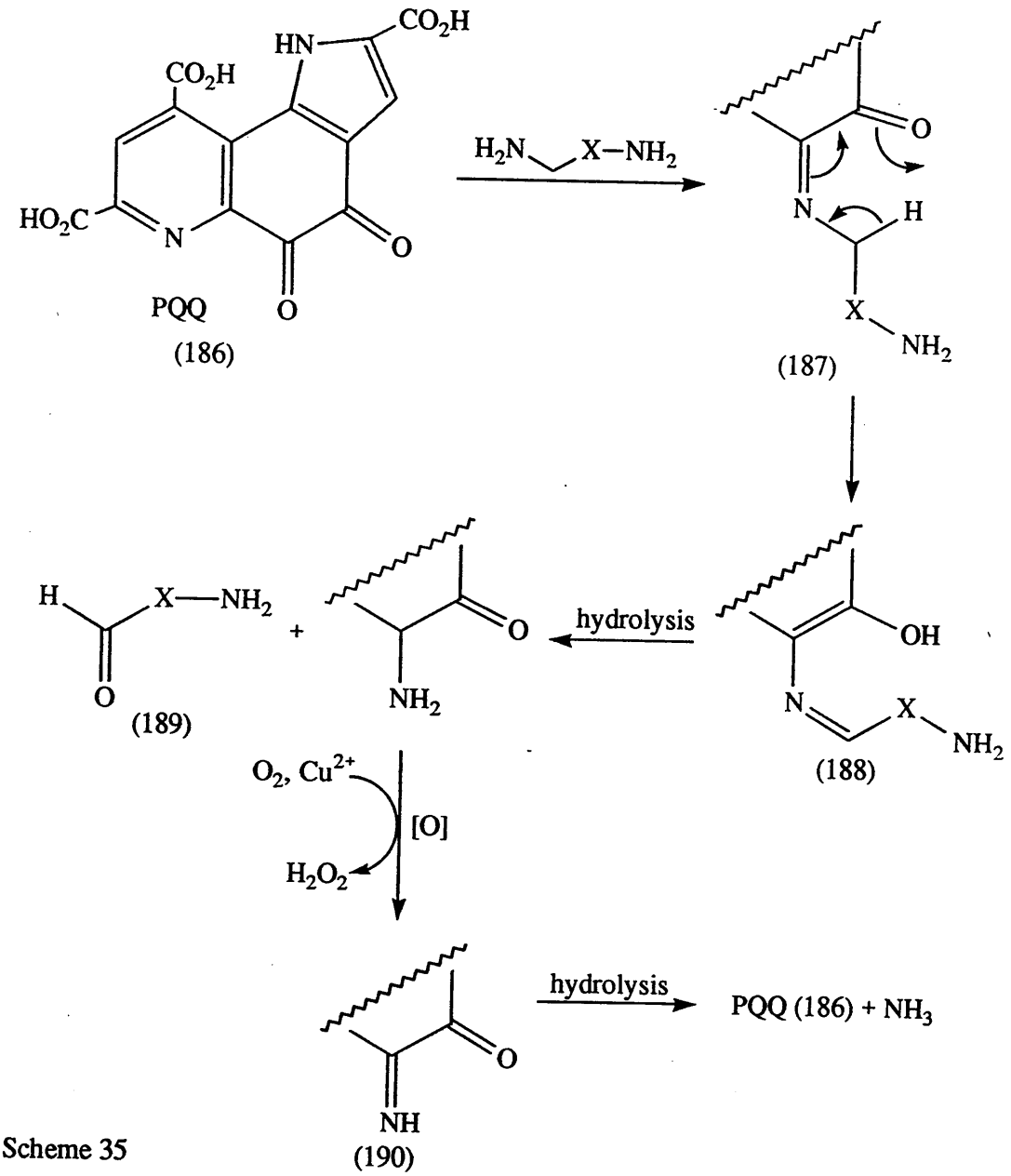
The Lineweaver-Burk plot compresses the data points at high substrate concentrations into a small region and emphasises the points at low concentration.

The Eadie-Hofstee plot does not compress the higher values and is considered to be more accurate and generally superior.[103]

4.3 The Oxidation of Diamines by DAO.

There is much interest in diamine oxidases due to their role in the metabolism of polyamines in most organisms.[104] They are required for the regulation of cellular levels of polyamines which are essential for the growth and proliferation of all living cells.[105] Although the precise function of the cellular polyamines is uncertain, it has been proposed that the polyamines serve to stabilise DNA through electrostatic and hydrogen-bonding interactions and, in this way, may play a role in cell replication.[106]

The majority of diamine oxidases are dimeric proteins with subunits of molecular weight ca. 90,000 containing Cu^{2+} and pyrroloquinolinequinone (PQQ) (186) as co-factors.[107] The proposed mechanism for DAO oxidation involving PQQ is shown in Scheme 35. Kinetic and spectroscopic evidence[108] suggest that PQQ forms an imine (187) with the diamine substrate. Subsequent tautomerisation and hydrolysis of the resultant imine (188) liberates the aldehyde (189). The co-factor is oxidised by O_2 in a process requiring Cu^{2+} and H_2O_2 is released. Hydrolysis of imine (190) regenerates PQQ and liberates NH_3.


HN
CO2H
CO2H
HO2C
N
O
O
PQQ
O
(186)
H2N
X—NH2
O
N
H
X
NH2
(187)
OH
N
X
NH2
(188)
hydrolysis
H
X—NH2
O
(189)
+
O
NH2
O2, Cu2+
H2O2
[O]
O
NH
(190)
hydrolysis
PQQ (186) + NH3

Scheme 35

4.4 The Spectrophotometric Assay

The kinetic data for DAO-catalysed oxidation of substrates (22) and (179)-(185) were determined by the procedure of Stoner.[109] This involves a peroxidase-coupled assay (horseradish peroxidase, EC 1.11.1.7, from Sigma) to monitor continuously the hydrogen peroxide released during diamine oxidation (Scheme 36) at 25°C, 70mM phosphate buffer, pH 6.3, in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH) (191) and 3-(dimethylamino)benzoic acid (DMAB) (192). Oxidative coupling (Scheme 37) generates stoichiometric quantities of an indamine dye (193) with a characteristic absorbance maximum at 595nm, and rates of reaction can be determined directly in the spectrophotometer. Initial rates were determined over a range of substrate concentrations from the linear absorbance changes during the first minute of reaction, and Michaelis-Menten 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]) and Lineweaver-Burk (1/V vs 1/[S]) plots.[103] Data quoted are the means of at least three determinations.

4.5 Results and Discussion

The eight compounds (22) and (179)-(185) were tested as substrates for pea-seedling DAO using the aforementioned peroxidase-coupled assay.[109] This provided Michaelis-Menten parameters for each enzyme/substrate pair (Tables 9 and 10). Published data[110] for putrescine (30), N-methylputrescine (194), N-ethylputrescine (195), N-propylputrescine (196), and cadaverine (197) are also shown for comparison (Table 11).

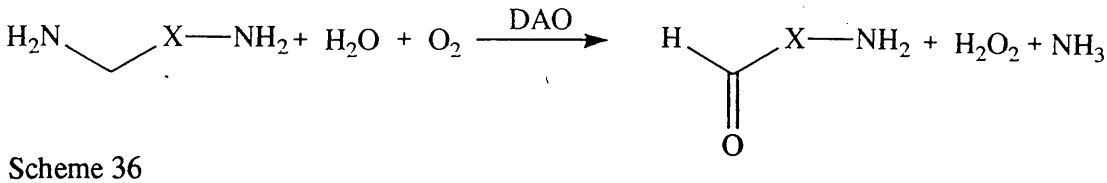
Analysis of the parameters from Table 9 reveals that the K_M values can be classified into two distinct groups. The substrate binding affinities, as indicated by K_M values, for compounds (179) and (180) are 0.94 mM and 1.19mM respectively, which is in good agreement with the value for putrescine (1.18mM). Furthermore, the binding affinities for substrates (181) and (182), 0.20mM and 0.37mM respectively, correlate well with that for cadaverine (0.24mM). Thus, it can be said that compounds (179) and (180) are substrates comparable in binding affinity to N-alkyl putrescines whereas compounds (181) and (182) behave like cadaverine analogues. In both of these groups of substrates there is a large decrease in the catalytic rate constant (V_{max}) compared with putrescine or cadaverine. Smaller decreases in V_{max} are observed with increasing bulk of the N-alkyl substituent. This is in accord with the observed trend of rate constants for N-alkylputrescines (194)-(196).[110] It is clear from this work, as with the study on N-alkylputrescines, that molecular

differentiation by pea-seedling DAO for these substrates lies in the rate of catalysis rather than in substrate binding affinity. The catalytic rate constant (V_{max}) is lower for substrates with bulkier substituents, but the similar binding affinities, as shown by K_M values, for each group of substrates suggests that the active site of the enzyme is relatively uncrowded. It remains to be seen what role N-alkyl substituents have in reducing the rate of catalysis, but it has been suggested[110] that such substituents may inhibit conformational changes required in the enzymic process. Another possible effect of bulky substituents is to restrict access of other reagents (H_2O and O_2) to the active site. The differences in K_M and V_{max} values observed for 5- and 6-membered rings may be due to differences in conformations of these cyclic systems.

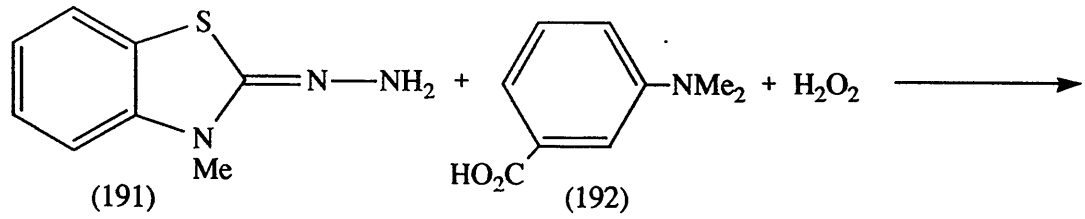
Next, the unsaturated amines (22) and (183)-(185) were tested as substrates for pea-seedling DAO. As previously discussed, (22) is a known intermediate in pyrrolizidine alkaloid biosynthesis, and so an opportunity to study its interaction with isolated DAO was particularly valuable. As shown in Table 10, all four unsaturated amines have high binding affinities, as indicated by K_M values, for DAO. The four compounds can be subdivided into two groups on the basis of K_M values: (22) and (183) have similar binding affinities while (184) and (185) can be classed together on this criterion. This is in line with the treatment of saturated compounds (179)-(182) in that the substrates can be sub-divided into putrescine analogues and cadaverine derivatives with related kinetic data.

Similarly, within each sub-group the catalytic rate constant (V_{max}) decreases as the bulk of the N-alkyl substituent increases. Finally it is clear that unsaturated amines such as (22) have a much higher binding affinity for pea-seedling DAO than the corresponding saturated compounds, eg (179).

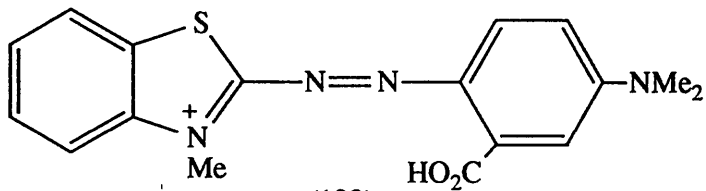
It appears that the presence of a $C=\overset{+}{N}<$ double bond has a profound effect on the binding affinity. It is not clear yet how this relates to the observed binding affinities. Possible consequences of the introduction of the double bond include changes in pK_a and/or polarity of the substrate molecule. An important effect is probably due to the different hybridisation of the orbitals around the nitrogen atom of the cyclic system. Favourable binding interactions between the π-system and the enzyme are possible. The presence of a double bond will also alter the conformation of the compounds, particularly in the case of the 6-membered ring iminium ions.



Scheme 36



(191) (192)

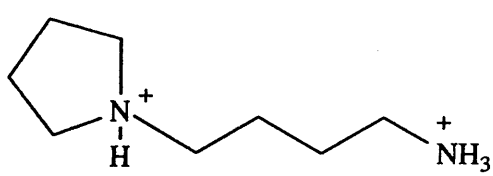


(193)

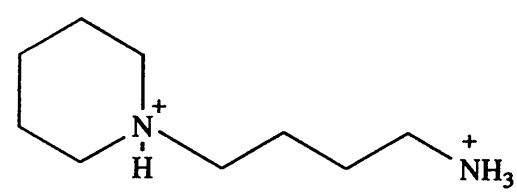
Scheme 37

Table 9

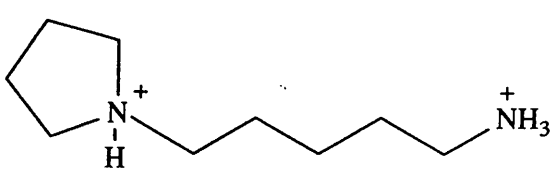
Substrate	K_M (mM)	V_{max} (μmol mg^{-1} h^{-1})
(179)	0.94 (±0.04)	31.9 (±0.85)
(180)	1.19 (±0.11)	5.43 (±0.05)
(181)	0.20 (±0.02)	17.2 (±0.42)
(182)	0.37 (±0.03)	3.57 (±0.25)



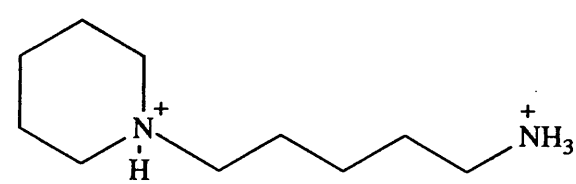
(179) $2Cl^-$



(180) $2Cl^-$



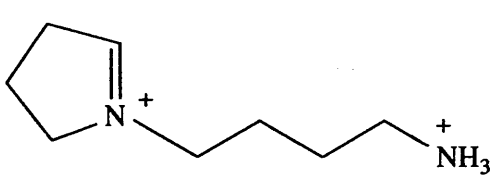
(181) $2Cl^-$



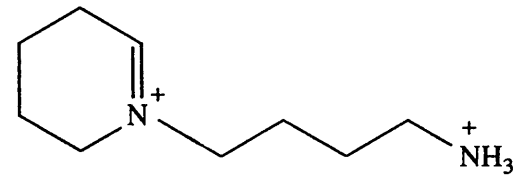
(182) $2Cl^-$

Table 10

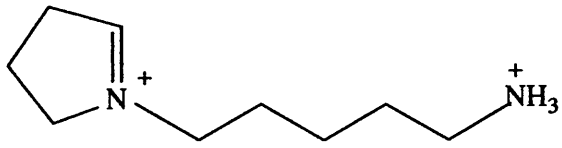
Substrate	K_M (mM)	V_{max} (μmol mg^{-1} h^{-1})
(22)	0.052 (±0.005)	15.7 (±0.78)
(183)	0.056 (±0.003)	7.69 (±0.46)
(184)	0.035 (±0.003)	25.3 (±0.92)
(185)	0.037 (±0.004)	7.96 (±0.53)



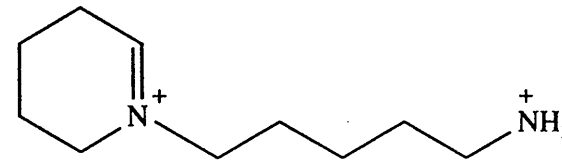
(22) $2Cl^-$



(183) $2Cl^-$



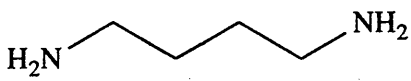
(184) $2Cl^-$



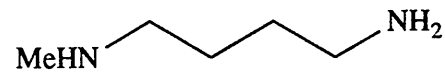
(185) $2Cl^-$

Table 11

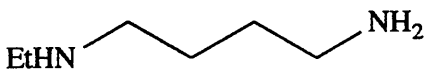
Substrate	K_M (mM)	V_{max} (μmol mg^{-1} h^{-1})
(30)	1.18 (±0.37)	1170 (±220)
(194)	1.12 (±0.14)	211 (±24)
(195)	1.17 (±0.40)	3.1 (±0.3)
(196)	0.68 (±0.22)	0.26 (±0.06)
(197)	0.24 (±0.07)	2680 (±410)



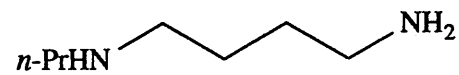
(30)



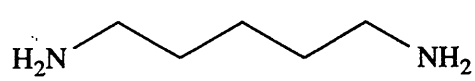
(194)



(195)



(196)



(197)

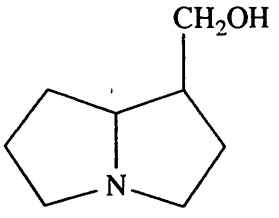
4.6 Conclusions

The results presented here for the oxidation of a series of amines by pea-seedling DAO are valuable for two reasons. Firstly, as a contribution to the understanding of the substrate specificity of an enzyme which has great potential for use in biotransformations and as a target for drug design. There is no reliable or convenient non-enzymic method for the oxidative deamination of primary amines, so the use of DAO for this reaction is attractive.

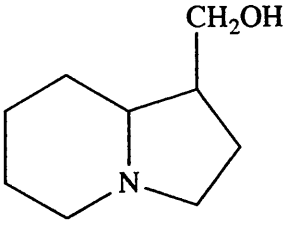
In the second instance, this study can be linked with the biosynthesis of pyrrolizidine alkaloids. This work has shown that the iminium ion (22), a known precursor of pyrrolizidine alkaloids, has a greater binding affinity for DAO than has the corresponding saturated compound (179).

The finding of a set of compounds (22) and (183)-(185) with strong binding affinities and relatively low catalytic rate constants for DAO suggests that these compounds should be tested as inhibitors of DAO in experiments with putrescine. Good inhibitors should be tested for useful biological activity. Inhibitors of DAO possess a broad range of biological activities, eg as antimalarial, antibacterial, and antifungal agents. The design, synthesis and testing of further compounds containing the $C{=}\overset{+}{N}{<}$ system should be a high priority for further research.

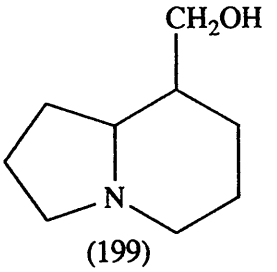
Finally, there is the potential to carry out biomimetic syntheses of various heterocyclic systems by incubation of the iminium ions (22) and (183)-(185) with pea-seedling DAO. The resultant amino-aldehydes could be reduced to the corresponding amino-alcohols to produce pyrrolizidine (1), indolizidine (198) and (199), and quinolizidine (200) structures. The first example has already been carried out by Robins.[45]



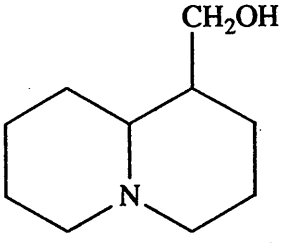
(1)



(198)



(199)



(200)

Chapter 5

FURTHER STUDIES ON THE BIOSYNTHESIS OF THE PYRROLIZIDINE NECINE BASES.

5.1 Introduction

A description of experiments concerning various aspects of pyrrolizidine alkaloid biosynthesis is presented in this section. The intention was, in some cases, to corroborate previous findings, while in other instances new areas were investigated.

The iminium ion (22) has been identified as an intermediate in necine biosynthesis.[52] It was deemed necessary to provide further evidence for the presence of this species on the biosynthetic pathway. The two approaches adopted are described in Sections 5.2 and 5.3.

Isoretronecanol (23) is an efficient precursor for rosmarinecine (4).[47] The order of hydroxylation, at C-2 and C-7 of isoretronecanol (23) is not known, although platynecine (25) is incorporated well into rosmarinecine (4) in *Senecio pleistocephalus*.[51] An intermediate trapping experiment was carried out to ascertain the status of platynecine (25) in the biosynthesis of rosmarinecine (4) (Section 5.4).

Stereochemical aspects of necine biosynthesis can be elucidated by the use of putrescine specifically labelled with deuterium. Feeding experiments with (1R)-[1-^{2}H]putrescine (87) to *Cynoglossum australe* are

described in Section 5.5.

Finally, Section 5.6 is a discussion of the possible role of N-acetylputrescine (24) in pyrrolizidine alkaloid biosynthesis.

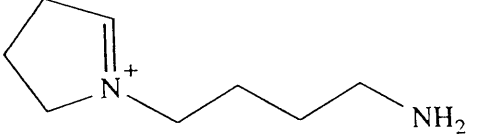
5.2 Intermediate Trapping Experiments I

Support for iminium ion (22) as an intermediate in pyrrolizidine biosynthesis came in part from an intermediate trapping experiment.[52] A derivative (79) of the reduced iminium ion (22) contained ca. 0.4% of the original radioactivity (fed as [1,4-^{14}C]putrescine), which was good evidence for the presence of iminium ion (22) on the biosynthetic pathway. In contrast, a trapping experiment to ascertain the status of the saturated salt (179) yielded a derivative containing very little radioactivity (<0.017%).

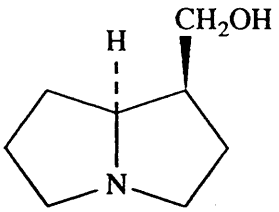
It was envisaged that further support for the role of iminium ion (22) in necine biosynthesis could be obtained by a intermediate trapping experiment in which the iminium ion was isolated in ^{3}H-labelled form by reduction with a tritide reducing agent. Accordingly, [1,4-^{14}C]putrescine dihydrochloride (50 μCi) was fed to one *S. pleistocephalus* plant. After 24 h the plant was macerated repeatedly in methanol. To this extract was added unlabelled saturated salt (179) (28 mg), $NaBH_4$ (49 mg), and $NaB[^{3}H]_4$ (ca. 200 μCi), and the mixture was stirred at room temperature overnight. Phenyl isothiocyanate (1 ml) was then added to the basified

solution and stirring was continued for a further 3 d. The derivatised amines were isolated as described by Golding and Nassereddin.[111] A radioscan of the mixture indicated radioactive bands coincident with unlabelled derivatives (201) and (79) of, respectively, putrescine (30) and diamine (179). These derivatives were separated by preparative TLC. It was anticipated that ^{14}C activity would be present in both derivatives while ^{3}H label appear only in the derivative of diamine (179). Radiolabelled forms of (201) and (79) contained respectively 0.18% and 0.44% of the original ^{14}C activity. Derivative (79) contained a high level of ^{3}H activity (ca. 9.6 μCi). Unfortunately, there was also a significant amount of ^{3}H label present in the putrescine derivative (201) (ca. 2.5 μCi). This may be due to tritium exchange of the N-H protons.

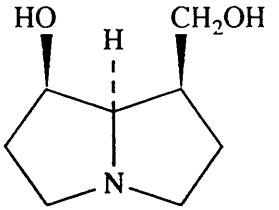
To overcome this difficulty, it was decided to effect reduction of the iminium ion with sodium cyanoborodeuteride ($NaCNBD_3$) in place of sodium borotritide. In this way, spectroscopic evidence could be used in support of the proposal. To this end, a feeding experiment was carried out in a similar manner to that described above. Spermidine trihydrochloride, acting as a source of putrescine, (labelled [1,4-^{14}C] in the tetramethylene portion) (50 μCi) was fed to one *S. pleistocephalus* plant. After 24 h the plant was macerated repeatedly in methanol. To this extract was added unlabelled saturated salt (179) (31 mg) and sodium cyanoborodeuteride (42 mg), and the mixture was stirred at room temperature overnight. The amines present in the reaction mixture were derivatised and isolated as



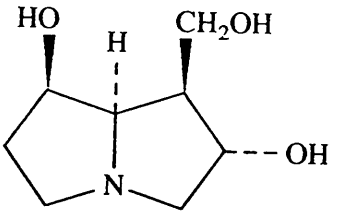
(22)



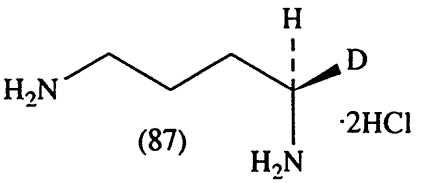
(23)



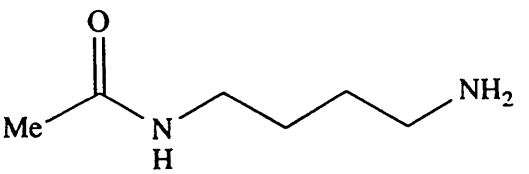
(25)



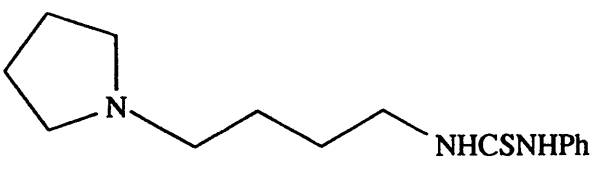
(4)



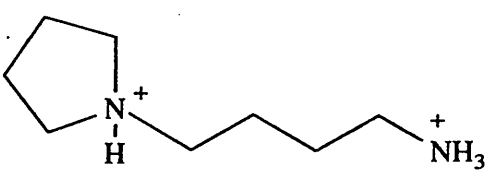
(87)



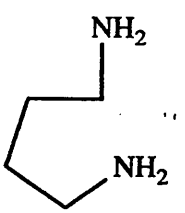
(24)



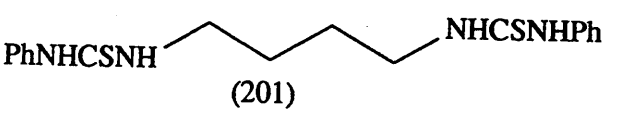
(79)



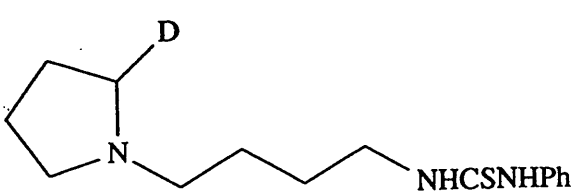
(179) $2Cl^-$



(30)



(201)

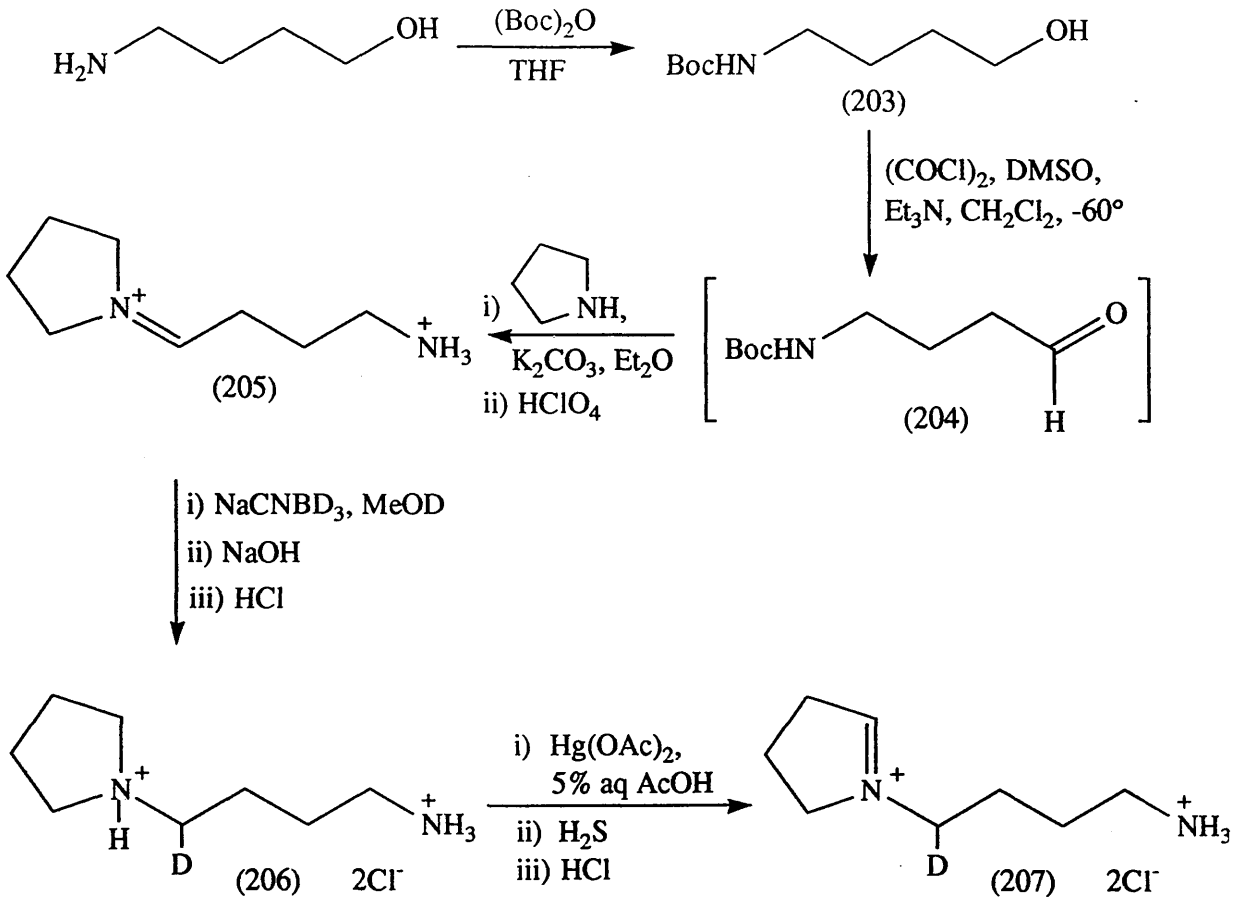


(202)

before. To our disappointment, it was not possible to isolate a derivative (202) labelled with deuterium. Decomposition of the reducing agent in a reaction mixture with a considerable water content may explain the lack of success.

5.3 Synthesis and Feeding of a Deuterium-labelled Iminium Ion.

Due to the inconclusive outcome of the two intermediate trapping experiments it was decided to attempt to provide direct evidence by the synthesis and feeding of the iminium ion in ^{2}H-labelled form. The synthesis (Scheme 38) was carried out by a modification of the route to ^{3}H-labelled iminium ions (172)-(175) (Scheme 34, Chapter 3). 4-Aminobutanol was converted into its N-Boc derivative (203) since this protecting group is more easily removed than the CBZ group. Swern oxidation gave the unstable aldehyde (204) which was reacted with pyrrolidine in the presence of K_2CO_3 in diethyl ether to give an enamine which was converted into the iminium salt (205) by acidification of an ether solution of the enamine with 70% $HClO_4$. The Boc protecting group was also removed under the acidic conditions, thereby making this route shorter and more efficient than that shown in Scheme 34. Reduction of iminium ion (205) with sodium cyanoborodeuteride in CH_3OD gave the ^{2}H-labelled compound, isolated as its dihydrochloride salt (206). Oxidation of the tertiary amine with mercury (II) acetate then yielded the ^{2}H-labelled iminium ion (207) which showed 1 peak at δ3.81


H2N
OH
(Boc)2O
THF
BocHN
OH
(203)
(COCl)2, DMSO,
Et3N, CH2Cl2, -60°
BocHN
O
H
(204)
i) NH,
K2CO3, Et2O
ii) HClO4
N+
NH3
(205)
i) NaCNBD3, MeOD
ii) NaOH
iii) HCl
N+
H
D
NH3
(206) 2Cl-
i) Hg(OAc)2,
5% aq AcOH
ii) H2S
iii) HCl
N+
D
NH3
(207) 2Cl-

Scheme 38

in its ^{2}H NMR spectrum.

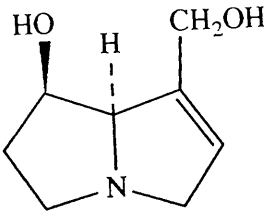
This compound (207) was fed, along with [1,4-^{14}C]putrescine dihydrochloride to one *S.pleistocephalus* plant, and also to transformed 'hairy' root cultures[112] of *S.vulgaris*, which produces senecionine (32) as the major alkaloid. In both instances, the total incorporation of ^{14}C was encouraging (21.6% and 19.4% respectively). However, ^{2}H NMR spectroscopy indicated no deuterium incorporation into either rosmarinine (70) or senecionine (32). Also, no deuterium-labelled alkaloid was obtained on feeding the saturated salt (206) to either *S.pleistocephalus* or *S.vulgaris*.

5.4 Intermediate Trapping Experiments II

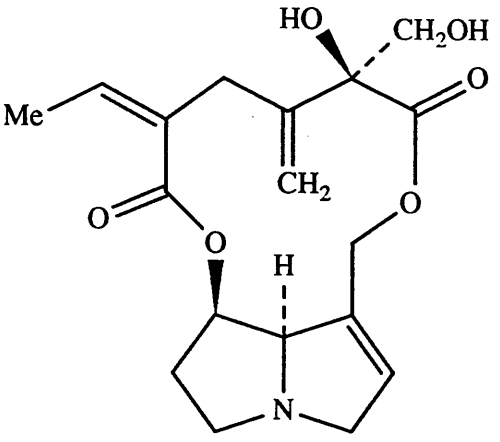
The biosynthetic pathway to rosmarinecine (4) is now well understood. An intriguing point which has yet to be resolved is the order of hydroxylation at C-2 and C-7 of (-)-isoretronecanol (23) in the elaboration to rosmarinecine (4). It has been suggested that hydroxylation at C-7 occurs first since platynecine (25) is an efficient precursor for rosmarinecine (4) in *S. pleistocephalus*.[51] It was decided to carry out an intermediate trapping experiment in order to provide further support for the role of platynecine in the biosynthesis of rosmarinecine.

Since we had no readily available source of platynecine, this was prepared by stereospecific catalytic hydrogenation of retronecine (5).

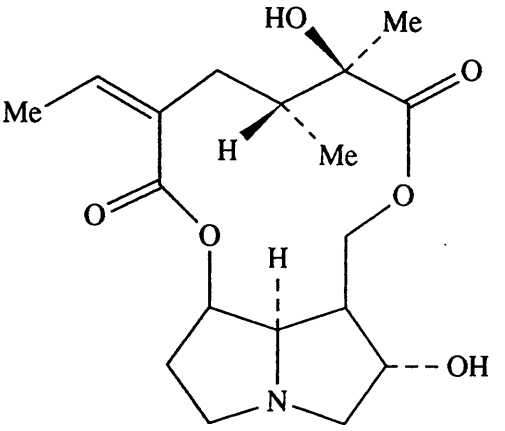
Retronecine was obtained from the mother liquors from crystallisations of riddelliine (35), provided by Dr. R.J. Molyneaux. Alkaline hydrolysis of the crude extract yielded retronecine (5), after extensive chromatography. Rosmarinecine (4) was obtained by alkaline hydrolysis of rosmarinine (70). Platynecine and rosmarinecine are both highly polar, and so they were derivatised to make them easier to handle. Treatment of platynecine with benzoyl chloride/pyridine furnished dibenzoylplatynecine (208). Tribenzoyl-rosmarinecine (209) was produced by the same reaction conditions from rosmarinecine. One *S.pleistocephalus* plant was used for the intermediate trapping experiment. Spermine tetrahydrochloride (labelled [1,4-^{14}C] in the tetramethylene portion) (50 μCi) was fed to this plant and after 24 h the plant was harvested and macerated repeatedly in methanol. The general procedure for alkaloid extraction (see Chapter 7, Section 7.2) was employed up to the stage of basifying the aqueous solution. This was extracted once with chloroform (60 ml) to remove most of the rosmarinine (70). It is noteworthy that even after only 24 h a total incorporation of ^{14}C into rosmarinine of 2.5% was observed. The aqueous layer was then saturated with Na_2CO_3 and extracted continuously with chloroform for 3 d. The chloroform extracts were concentrated <u>in vacuo</u> and to the residue was added platynecine (25) (32 mg) and rosmarinecine (4) (29 mg). This mixture was dissolved in pyridine (500 μl), cooled to O°C, treated with benzoyl chloride (300 μl), and the reaction mixture was stirred at room temperature overnight. After work-up, TLC ($CHCl_3$/MeOH/c.NH_3, 85:14:1) indicated the



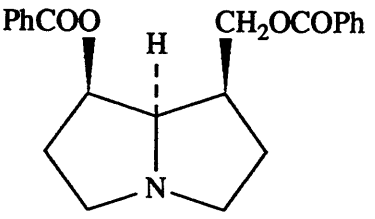
(5)



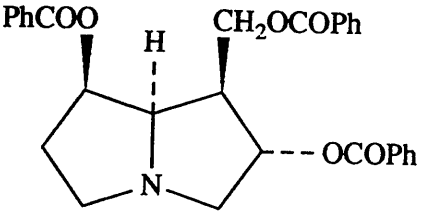
(35)



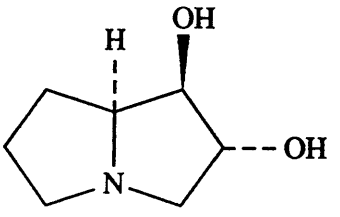
(70)



(208)



(209)



(210)

presence of dibenzoylplatynecine (208), R_F 0.39, and tribenzoylrosmarinecine (209), R_F 0.65, which were readily separable by preparative TLC. These derivatives contained, respectively 0.37% and 0.18% of the total ^{14}C activity fed as ^{14}C-labelled spermine. This is good evidence for the presence of both platynecine (25) and rosmarinecine (4) on the biosynthetic pathway to rosmarinine (70). It also suggests that hydroxylation at C-7 of (-)-isoretronecanol (23) may occur before hydroxylation at C-2. Further work should aim to ascertain the status of the alternative diol (210) in the biosynthesis of rosmarinine.

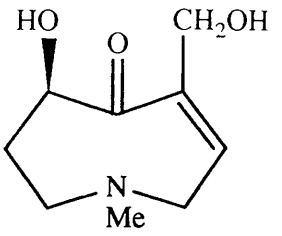
5.5 Feeding of (1R)-[1-^{2}H]Putrescine to *C.australe*.

Much of the information relating to the biosynthesis of the pyrrolizidine necine bases has been obtained through feeding experiments utilising putrescine specifically labelled with deuterium. Stereochemical aspects of the biosynthesis of retronecine (5)[55,61,113], rosmarinecine (4)[63], and otonecine (31)[64] have been determined. As yet, no results have been published on enzymic specificity concerning the biosynthesis of necines with H-8β stereochemistry.

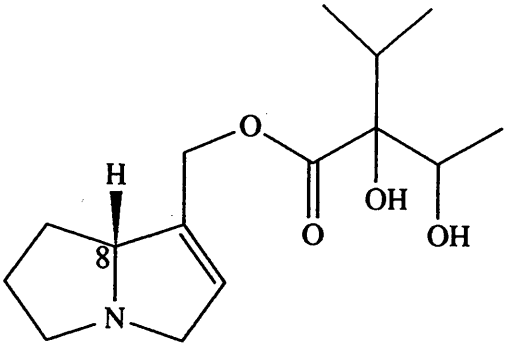
Cynoglossum australe produces two alkaloids[114], cynaustraline (27) and cynaustine (26), which are ester derivatives of, respectively, (+)-isoretronecanol (211) and (+)-supinidine (212). Feeding experiments with (1R)-[1-^{2}H]putrescine (87) were carried out in an attempt to determine the outcome of some of the enzymic processes involved in the biosynthesis of (+)-

isoretronecanol (211) and (+)-supinidine (212).

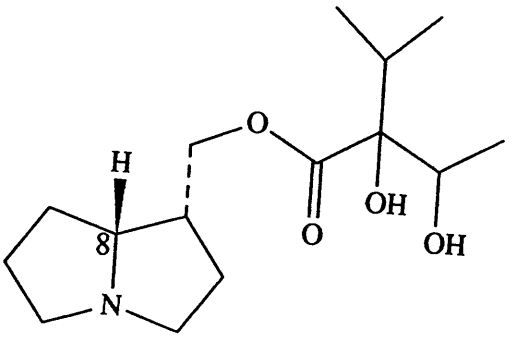
(1R)-[1-^{2}H]Putrescine (87) was prepared by the method of Richards and Spenser.[59] This involves decarboxylation of L-ornithine in D_2O with ornithine decarboxylase. The first feeding experiment was carried out in the summer of 1989.(1R)-[1-^{2}H]putrescine dihydrochloride (180mg) was mixed with [1,4-^{14}C]putrescine dihydrochloride (6.8 μCi). This mixture was fed over 5 days to 30 *C. australe* plants by the wick method. 14 days after completion of the feeding period the plants were harvested and the alkaloids were extracted. The total incorporation of ^{14}C-putrescine into the crude alkaloid mixture was 3.2%. A similar incorporation of deuterium would be sufficient to allow a ^{2}H NMR spectrum to be obtained. Unfortunately, ^{2}H NMR spectroscopy indicated no enrichment over the natural abundance signals in the mixture of alkaloids. This may be due to dilution of the deuterium label by a large pool of endogenous, unlabelled alkaloid. The feeding experiment was then repeated, in summer 1990, using a smaller number of plants. (1R)-[1-^{2}H]putrescine dihydrochloride (172 mg) was fed along with [1,4-^{14}C]putrescine dihydrochloride(5.4μCi) to 6 *C. australe* plants by the wick method. The precursors were fed on 5 consecutive days and after a further 10 days the alkaloids were extracted from the plants. Again, the total incorporation of ^{14}C-putrescine into the crude alkaloid mixture was encouraging (2.9%). However, to our disappointment, no deuterium incorporation was observed into the mixture of alkaloids. There was no time available to repeat the



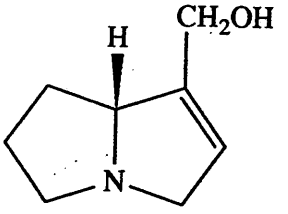
(31)



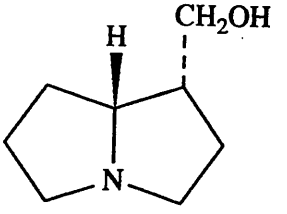
(26)



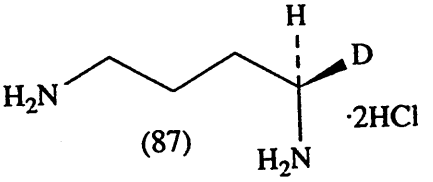
(27)



(212)



(211)



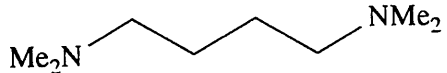
(87)

feeding experiment using another set of experimental conditions.

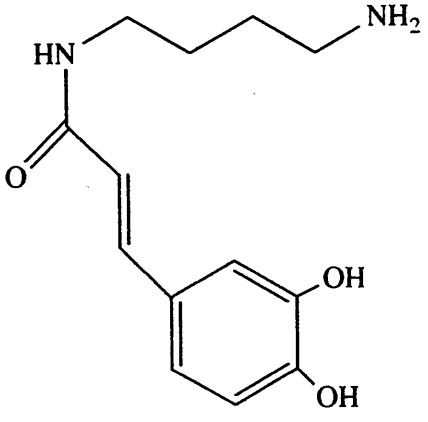
5.6 On The Role of N-Acetylputrescine in Necine Biosynthesis.

Many derivatives of putrescine (30) are found in nature. For example, tetramethylputrescine (213) has been isolated from *Hyoscyamus muticus*.[115] A number of putrescine analogues with amide linkages are known. Caffeoylputrescine (214) has been found in seeds of *Pentaclethra macrophylla*[116] while caffeoyl-(214), feruloyl-(215), and p-coumaroyl-(216) putrescine have been reported in callus tissue cultures of *Nicotiana tabacum*.[117]

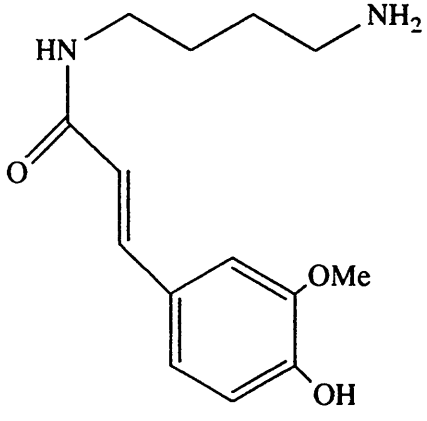
We decided to undertake a study of the possible role of the simple amide derivative N-acetylputrescine (24) in the biosynthesis of pyrrolizidine necine bases.Transient derivatisation of putrescine (30) *in vivo* could have a number of purposes. N-Acetyl-putrescine (24) is less strongly basic than putrescine (30) and is less likely to act as a strong interactive ligand with cell constituents. Hence, there may be greater ease of transport to the site of enzymic oxidation. A further consequence of derivatisation may be to prevent over-oxidation of putrescine since only one free amine group would be available. This would also prevent the possibility of polymerisation of putrescine. Amide hydrolysis could then occur further along the biosynthetic pathway.



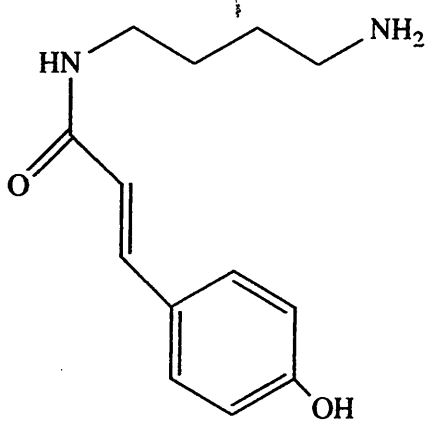
(213)



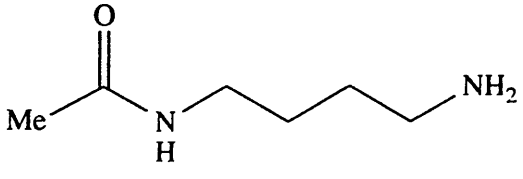
(214)



(215)



(216)



(24)

N-Acetylputrescine (24) hydrochloride was prepared by reaction of putrescine (30) with acetic anhydride (1.4 equivalents) in glacial acetic acid followed by addition of hydrochloric acid Mono-acetylation could be achieved in 40.1% yield. A sample of N-acetyl putrescine labelled with tritium was prepared by reaction of [1,4-^{3}H]putrescine dihydrochloride and putrescine with acetic anhydride in glacial acetic acid. Unfortunately, in this instance the chemical yield was only 20.4%.

Feeding experiments were carried out with *S.pleistocephalus* and *C.australe* plants, using [1,4-^{14}C]putrescine dihydrochloride as an internal standard in each case. Each mixture of precursors was fed to the two plant species on the same day by the wick method, using one plant per experiment. After 10 days the plants were harvested and the alkaloids were extracted. The results of these experiments are shown in Table 12. It is apparent from the decrease in the $^{3}H/^{14}C$ ratio that putrescine is a better precursor than N-acetylputrescine for rosmarinine (70) in *S.pleistocephalus*. Conversely, N-acetylputrescine is incorporated 17 times more efficiently than putrescine into the alkaloids of *C.australe*. Satisfactory separation of cynaustine (26) and cynaustraline (27) could not be achieved.

In both experiments it is clear that N-acetyl-putrescine is not immediately hydrolysed to putrescine on administration to the plants. If this had occurred

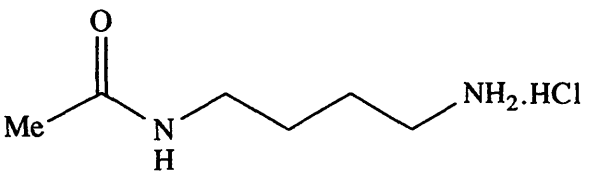
then the $^{3}H/^{14}C$ ratio in the alkaloids would be the same as that in the precursor mixture. The contrasting results of the experiments with *S.pleistocephalus* and *C.australe* is certainly intriguing, although it is not clear why this outcome should arise.

One consequence of these results is immediately apparent. As discussed in Section 5.5, it was not possible to achieve deuterium incorporation into the alkaloids of *C.australe* by feeding (1R)-[1-^{2}H]putrescine (87). Since N-acetylputrescine is incorporated much more efficiently into these alkaloids than is putrescine, it may be possible to repeat the feeding experiment using an acylated form of (1R)-[1-^{2}H] putrescine. This may improve the level of deuterium incorporation into cynaustine (26) and cynaustraline (27).

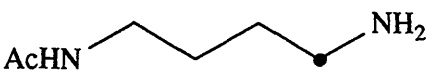
Further feeding experiments with N-acetyl-putrescine to *C.australe* would be of interest. For example, feeding of 1-acetamido-4-amino-[4-^{13}C]butane (217) to *C.australe* could produce either (a) (218), or (b) (219) and/or (220) (Scheme 39). Labelling pattern (218) would indicate that acetylation of putrescine is important for transport *in vivo*, but that the pathway is *via* putrescine. If (219) and/or (220) were obtained it would indicate that removal of the acetyl group occurs further along the biosynthetic pathway.

Table 12 Incorporation of ^{3}H-Labelled *N*-Acetylputrescine into the alkaloids of i) *S. pleistocephalus* and ii) *C. australe* with [1,4-^{14}C]putrescine as Reference

Species	Amount fed		Amount isolated			
	^{3}H µCi	mg	mg	Initial $^{3}H/^{14}C$	Final $^{3}H/^{14}C$	^{3}H Total incorporatior
i)	0.773	25	58	1.52	0.24	1.81
ii)	0.628	20	17	0.707	12.2	1.58

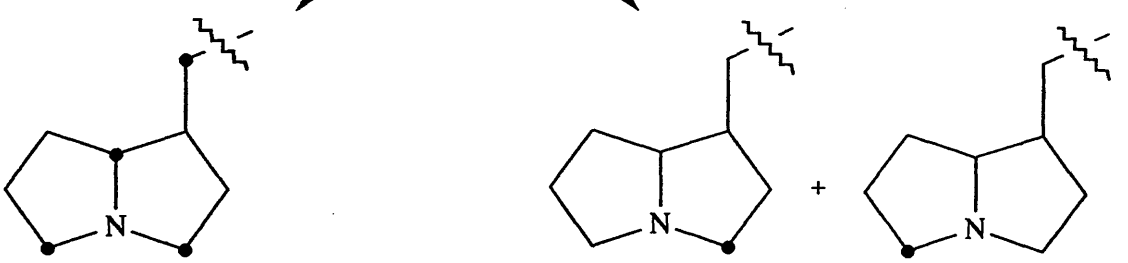


(24)



(217)

or



(218) (219) (220)

Scheme 39

Chapter 6

BIOSYNTHESIS OF THE 3-HYDROXY-3-METHYLGLUTARATE PORTION OF DICROTALINE.

6.1 Introduction

The work described so far in this thesis has concentrated on the chemistry of the necine bases. The biosynthesis of the necic acids is equally fascinating, and it was decided to undertake a study of the mode of formation of the necic acid portion of dicrotaline (28), which is 3-hydroxy-3-methylglutaric acid (221) (HMG). The recent discovery[118] that dicrotaline is the major constituent (86% of the total alkaloids) of *Crotalaria lachnosema* provided an opportunity to study this aspect of pyrrolizidine alkaloid biosynthesis. The work presented in this chapter describes a series of feeding experiments designed to elucidate features of the biosynthesis of HMG.

6.2 Incorporation of Radiolabelled Precursors into HMG.

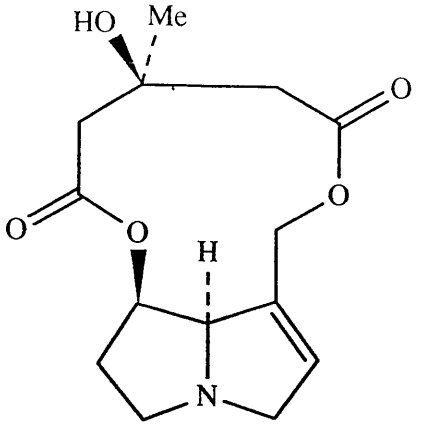
It is generally known that HMG is derived biosynthetically from three molecules of acetate. As its coenzymeA ester it is on the pathway to mevalonate and terpenoid compounds. With this in mind, initial experiments in this work involved feeding acetate and mevalonate in ^{14}C-labelled form to *C.lachnosema* plants. Seeds of *C. lachnosema* were provided by Dr. A.R. Mattocks, MRC Toxicology Unit, Surrey.

Each ^{14}C-labelled precursor was mixed with [1,4-^{3}H]putrescine dihydrochloride as an internal standard to give an initial ^{3}H:^{14}C ratio of ca. 2. The precursors were fed on one day by the wick method, using one six-month old plant per experiment. After 10 days the plants were harvested and the alkaloids were extracted. Dicrotaline (28) was readily separated from the minor alkaloid component, acetyldicrotaline (222), by preparative TLC. As shown in Table 13 (Expts. 1 and 3), acetate and mevalonate are incorporated into dicrotaline (28) much less efficiently than putrescine, which is a known precursor of (+)-retronecine (5), the necine portion of dicrotaline (28). Moreover, the distribution of the ^{3}H and ^{14}C activity was revealed by alkaline hydrolysis of dicrotaline to give (+)-retronecine (5) and 3-hydroxy-3-methylglutaric acid (221). This showed that putrescine almost exclusively labelled the (+)-retronecine portion (100-101% of the ^{3}H activity in the base; 2-4% in the acid) whereas the ^{14}C label from acetate and mevalonate was almost equally distributed between the acid and base portions. Feeding experiments were also carried out using only the ^{14}C-labelled precursors. Again, incorporations of ^{14}C into dicrotaline (28) were low and the radiolabel was evenly split between the acid and base portions (Table 13, Expts. 2 and 4).

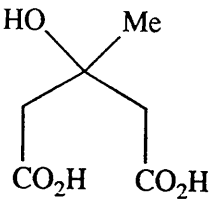
The next feeding experiments investigated the fate of HMG itself when fed to *C.lachnosema*. [3-^{14}C]HMG was fed along with [1,4-^{3}H]putrescine dihydrochloride, resulting in a reasonable incorporation of ^{14}C with only a small increase in the ^{3}H:^{14}C ratio. However,

alkaline hydrolysis of the dicrotaline sample showed that a greater proportion of the ^{14}C label was present in the necine base (Table 13, Expt. 5). This experiment was repeated using only [3-^{14}C]HMG, and the resultant sample of dicrotaline was subjected to a Kuhn-Roth degradation. This produced a sample of barium acetate containing only 5.2% of the total alkaloid activity, demonstrating that HMG is not a specific precursor for the diacid portion of dicrotaline. Bachhawat et al[119] have identified an enzyme that catalyses the cleavage of HMG-CoA to acetoacetate and acetyl-CoA, and incorporation of the ^{14}C label from [3-^{14}C]HMG is likely to occur via this route.

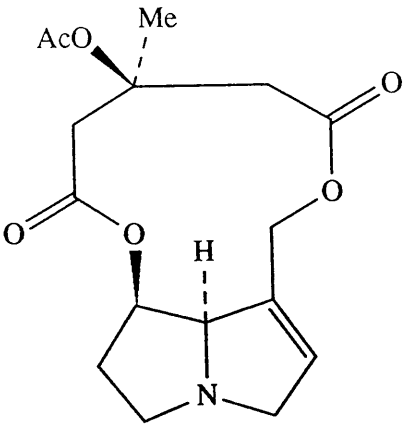
Attention was next focussed on the possible role of the branched-chain α-amino acids valine (105), leucine (106), isoleucine (107) and threonine (108) in HMG biosynthesis in *C.lachnosema*. Interestingly, leucine can be converted into HMG in vivo, as shown in Scheme 40.[120] However, leucine was poorly incorporated into dicrotaline with a large increase in the ^{3}H:^{14}C ratio and the ^{14}C activity was split between the acid and base portions (Table 13, Expt. 7). Feeding of valine to *C.lachnosema* yielded a similar result to that for leucine (Table 13, Expt. 8). On the other hand, threonine and isoleucine were each efficiently incorporated into dicrotaline with relatively small increases in the ^{3}H:^{14}C ratios (Table 13, Expts. 9 and 10). Furthermore, alkaline hydrolyses of these two samples showed that most of the ^{14}C label was present in the acid portions. In all four experiments with amino acids (Table 13, Expts. 7-10), the ^{3}H-activity from



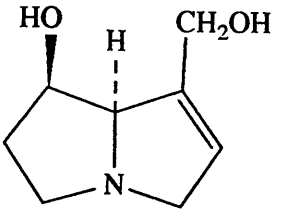
(28)



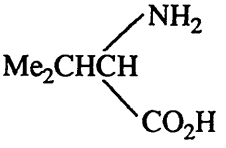
(221)



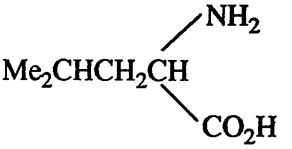
(222)



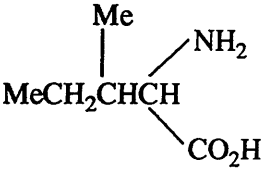
(5)



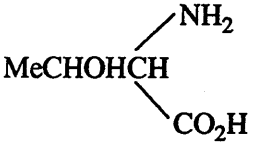
(105)



(106)



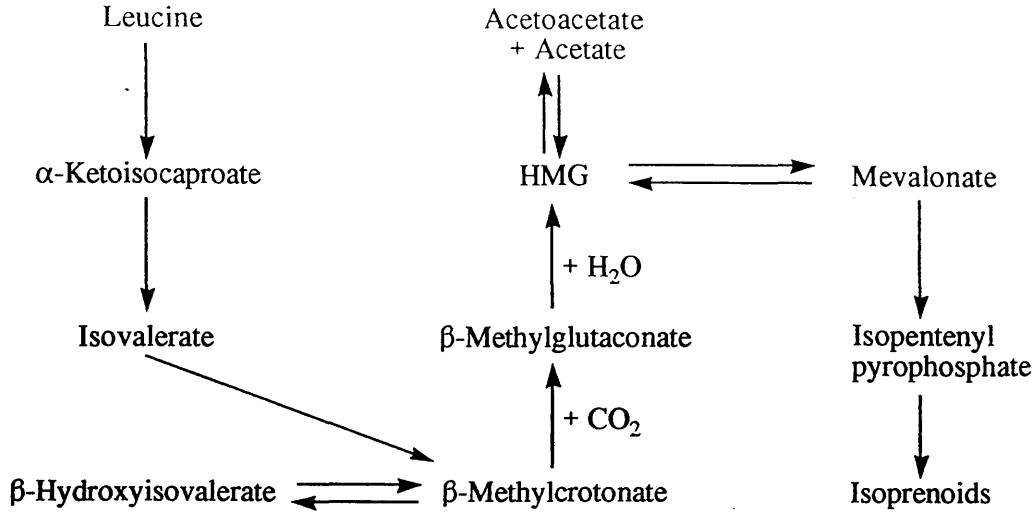
(107)



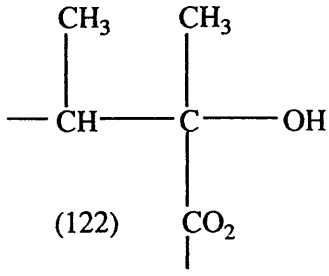
(108)

putrescine was located mainly in the base portion (94-102%) with very little radioactivity in the acid moiety (2-5%).

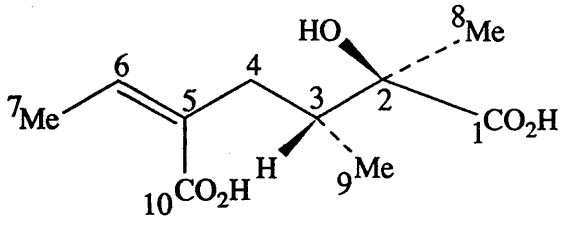
The next point of interest was the manner in which isoleucine is incorporated into HMG (221). Since isoleucine and HMG have the same carbon skeleton, one possibility is that isoleucine is converted into HMG by a series of reactions involving no changes to the carbon framework. This would entail removal of the amino group, oxidation of the C-5 methyl group to a carboxyl group, and hydroxylation at C-3. However, a more intriguing mode of incorporation is also apparent. A basic C_5 unit (122), utilised in the biosynthesis of the 'right-hand' halves of senecic acid (115) and trichodesmic acid (124) was discussed in Chapter 2. By analogy, a similar unit, formulated as $MeC(OH)CH_2CO_2H$ (223), may be involved in the conversion of isoleucine into HMG, with loss of the C-6 methyl group and the carboxyl group. In order to distinguish between the two possibilities, L-[4,5-^{3}H]isoleucine (224) was fed to *C.lachnosema*. A reasonable total incorporation of 0.57% was observed (no allowance was made for any loss of ^{3}H). The labelled dicrotaline was subjected to a Kuhn-Roth oxidation which produced a sample of barium acetate containing 90.5% of the total alkaloid radioactivity. This result demonstrates that isoleucine is not converted into HMG by reactions not affecting the carbon skeleton. It is extremely unlikely that the carbon framework of isoleucine undergoes the unprecedented rearrangement necessary to form HMG with C-4 or C-5 of isoleucine becoming the



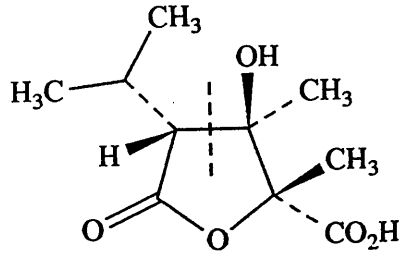
Scheme 40



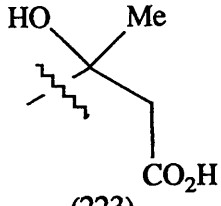
(122)



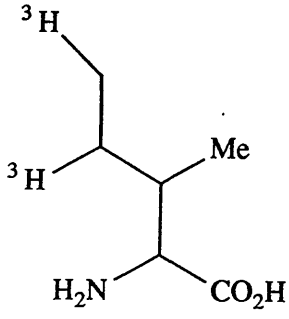
(115)



(124)



(223)

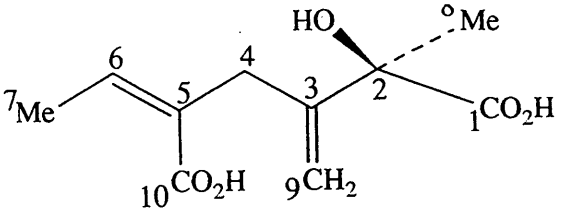


(224)

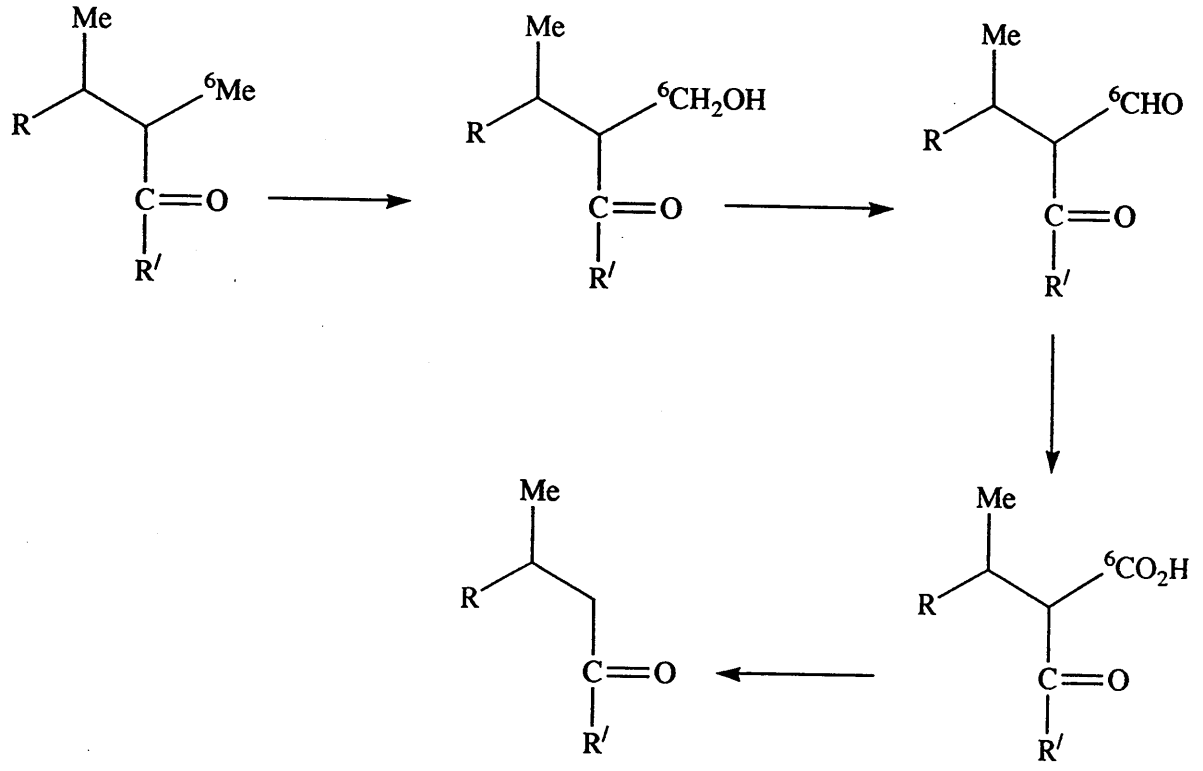
methyl group of HMG. The most likely explanation for this result is that incorporation of ^{3}H occurs from the C-5 position of isoleucine into the methyl group of HMG and loss of ^{3}H necessarily takes place from the C-4 position. The carboxyl group and the methyl group corresponding to C-6 of isoleucine must also be removed. The carboxyl group of isoleucine is known to be lost on incorporation into seneciphyllic acid (112)[66]. However, the loss of the C-6 methyl group of isoleucine is worthy of further discussion. One possible mechanism for the loss of the methyl group would be through oxidation to a carboxyl group followed by decarboxylation from a β-oxo acid of some description (Scheme 41). Whether this would occur as the final step in the biosynthetic pathway or at some intermediate stage is open to discussion. The presence of a hydroxylmethyl group at the equivalent position, ie β to the 'right-hand' ester group, in pyrrolizidine alkaloids such as retrorsine (34) provides a precedent for the oxidation of such a methyl group. The proposed biogenesis of HMG (221) is shown in Scheme 42. Isoleucine would thus contribute four of the six carbons of HMG. The origin of the remaining two carbon atoms in HMG has yet to be determined.

6.3 Conclusions

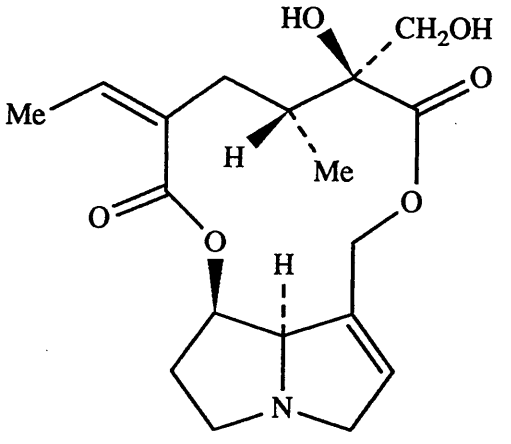
It would appear that the necic acid portion of dicrotaline (28), 3-hydroxy-3-methylglutaric acid (221), is not formed specifically from acetate, mevalonate, or 3-hydroxy-3-methylglutarate. Isoleucine (107), and



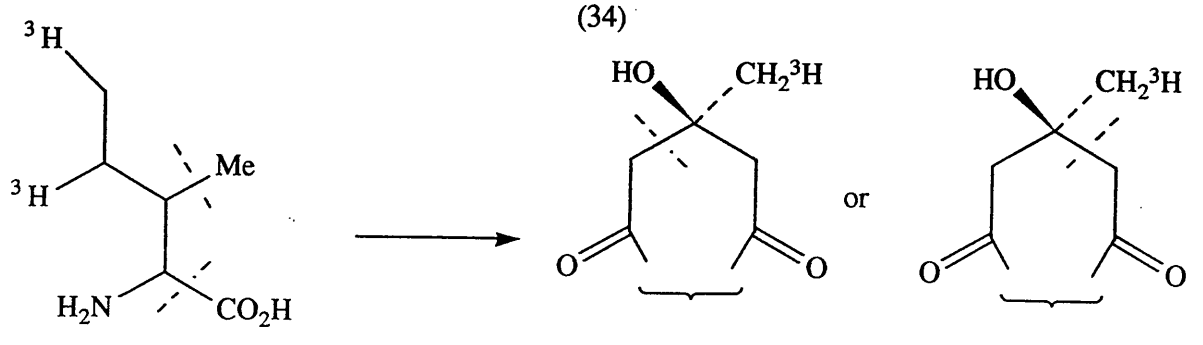
(112)



Scheme 41



(34)



(224)

Scheme 42

its biological precursor threonine (108), are incorporated specifically into HMG in a novel and unusual manner.

The use of precursors specifically labelled with stable isotopes such as ^{2}H and ^{13}C should be the next aim in this work. This is likely to provide confirmation of the intermediacy of amino acids in the biosynthesis of HMG and other necic acids and may shed light on unresolved stereochemical aspects of necic acid biosynthesis.

Attention should also be focussed on providing more information about the intriguing process by which the two C_5 units are joined to form the C_{10} necic acids.

Table 13 Incorporation of ^{14}C- Labelled Precursors into Dicrotaline (28) in *C. lachnosema* plants with [1,4-^{3}H]putrescine as Reference

Expt.	Precursor	^{3}H:^{14}C ratio		^{14}C %	% ^{14}C	% ^{14}C
		Start	End	Incorpn.	in base	in acid
1	[1-^{14}C]acetate	1.88	30.5	0.02	60	45
2	"	-	-	0.012	55	45
3	DL-[2-^{14}C]-mevalonate	1.98	18.6	0.04	43	58
4	"	-	-	0.034	54	49
5	[3-^{14}C]-3-hydroxy-3-methylglutarate	1.9	3.55	0.40	56	42
6	"	-	-	0.23	59	42
7	L-[U-^{14}C]valine	1.67	13.6	0.068	65	29
8	L-[U-^{14}C]leucine	1.66	10.9	0.079	49	49
9	L-[U-^{14}C]threonine	0.65	1.44	0.25	14	88
10	L-[U-^{14}C]isoleucine	1.61	3.09	0.26	5	94

Chapter 7

EXPERIMENTAL

7.1 General

Melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with an Optical Activity Ltd. AA 10 Polarimeter. Infra red spectra were obtained on a Perkin Elmer 580 spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Perkin Elmer R32 spectrophotometer operating at 90 MHz (δ_H), or a Bruker WP200-SY spectrophotometer operating at 200 MHz (δ_H), 50.3 MHz (δ_C), or 30.72 MHz (δ_D). The multiplicities of the ^{13}C NMR resonances were determined using DEPT spectra[121] with pulse angles of $\theta = 90^o$ and $\theta = 135^o$. Spectra were recorded for solutions in deuteriochloroform unless otherwise stated, with either tetramethylsilane at 0 p.p.m., or the NMR solvent as internal standard. Mass spectra were obtained with A.E.I. MS 12 or 902 spectrometers. Elemental analyses were performed using a Carlo-Erba 1106 elemental analyser.

Radiochemicals were purchased from Amersham International. Radioactivity was measured with a Philips PW 4700 Liquid Scintillation counter using toluene-methanol solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. Where appropriate, radioactive samples were recrystallised to constant specific activity, and they were counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for radioscanning of

the TLC plates.

TLC was carried out on Merck Kieselgel G plates of 0.25mm thickness and developed with chloroform-methanol-conc. ammonia (85:14:1) unless otherwise stated. The alkaloids were detected by the modified Dragendorff reagent.[101] Chromatographic purification refers to dry-column flash chromatography[122] with either Kieselgel 60 (Merck, 70-230 mesh) or neutral alumina (Riedel De-Haen, 400 mesh). Boiling points refer to the oven temperature using a Kugelrohr apparatus.

All organic solvents were distilled prior to use. Tetrahydrofuran (THF) and diethyl ether were dried by distillation from sodium-benzophenone under argon immediately prior to use. Other solvents and reagents were purified by standard techniques.[123] Benzoyl chloride was distilled and stored over 4Å molecular sieves. Dimethyl sulphoxide and dichloromethane were distilled from calcium hydride and stored over 4Å molecular sieves. Pyridine and triethylamine were distilled from, and stored over, potassium hydroxide. Piperidine was pre-dried with potassium hydroxide and then distilled from phosphorus pentoxide. Methane-sulphonyl chloride was distilled from phosphorus pentoxide. Pyrrolidine was distilled from sodium under nitrogen. Oxalyl chloride was distilled under nitrogen immediately prior to use. Mercury (II) acetate was recrystallised from glacial acetic acid.

Reactions were normally carried out under an atmosphere of dry nitrogen. Organic solvents were

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

7.2 Experimental to Chapter 3

7.2.1 3-Chloro-1-propanol methanesulphonate[124]

To a solution of methanesulphonyl chloride (3.8ml, 49 mmol) and 3-chloro-1-propanol (3.7ml, 44.5 mmol) in dry dichloromethane (30ml) at -78°C was added dropwise triethylamine (6.8ml, 49 mmol). The mixture was stirred at -78°C for 30 min and was then allowed to reach room temperature. The resultant slurry was poured onto ice/water (100ml) and extracted with dichloromethane (3 x 100ml). The organic extracts were combined, washed with brine (3 x 100ml), dried, filtered, and concentrated to give an oil which was distilled; yield 6.88 g, 89.6%; b.p. 165°C (14 mm Hg) (lit.[125], b.p. 143-146°C at 9 mm Hg); ν_{max} (CCl_4) 1355 and 1175 cm^{-1}; δ_H (90 MHz) 1.90 (2H, m, 2-H_2), 3.00 (3H, s, $OSO_2C\underline{H}_3$), 3.55 (2H, t, $\underline{J}$ 6 Hz, 4-H_2), and 4.25 p.p.m. (2H, t, $\underline{J}$ 4 Hz, 1-H_2).

4-Chloro-1-butanol methanesulphonate[124]

Using the previous method the title compound was obtained as a clear oil; yield 1.82 g, 81%; b.p. 175°C (0.8 mm Hg) (lit.[125], b.p. 115°C at 0.5 mm Hg); ν_{max} (CCl_4) 1340 and 1160 cm^{-1}; δ_H (200 MHz) 1.89 (4H, m, 2- and 3-H_2), 2.99 (3H, s, $OSO_2C\underline{H}_3$), 3.56 (2H, t, $\underline{J}$ 6.1 Hz, 4-H_2), and 4.23 p.p.m. (2H, t, $\underline{J}$ 3.8 Hz, 1-H_2); δ_C (50 MHz) 26.4 (C-2 and -3), 28.3 (C-4), 37.3 ($OSO_2\underline{C}H_3$), and 44.0 p.p.m. (C-1).

[1-^{14}C]-4-Chlorobutanenitrile(76)

Sodium cyanide (588mg, 12.0mmol) and sodium [^{14}C]cyanide (500 μCi) were dissolved in dry dimethylsulphoxide (40ml) at 90°C. 3-Chloro-1-propanol methanesulphonate (2.0g, 11.1mmol) was added, with stirring, and heating was continued at 85-90°C for 2 h. The reaction mixture was then allowed to cool to room temperature and was then stirred for a further 18 h. The mixture was diluted with diethyl ether (100ml), and water (50ml) was added. The layers were separated and the aqueous fraction was extracted with an additional portion of diethyl ether (75 ml). The combined organic extracts were washed with water (4 x 100ml) and brine (2 x 100ml), dried, filtered, and concentrated under reduced pressure. Chromatographic purification (silica), eluting with diethyl ether afforded the title compound as a pale yellow oil; yield 461mg, 38.4%, 32.6 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material, e.g. ν_{max} (CCl_4) 2250 and 665 cm^{-1}; δ_H (90 MHz) 2.10 (2H, m, 3-H_2), 2.50 (2H, t, J 7 Hz, 2-H_2), and 3.60 p.p.m. (2H, t, J 7 Hz, 4-H_2).

[1-^{14}C]-5-Chloropentanenitrile (139)

This was obtained by a similar procedure. Dry-column flash chromatography (silica), eluting with diethyl ether, gave a pale yellow oil; yield 830 mg, 61.3%, 28.4 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material, e.g. ν_{max} (CCl_4) 2250 and 660 cm^{-1}; δ_H (90 MHz) 2.15 (4H, m, 3- and 4-H_2), 2.55 (2H, t, J 7Hz, 2-H_2), and 3.55 p.p.m. (2H, t, J 7 Hz, 5-H_2).

General Procedure for N-Alkylations

A solution of the chloro-nitrile (76) or (139) in dry butanol (1ml per 2 mmol of chloro-nitrile) was added to a mixture of pyrrolidine or piperidine (1.1 equivalents), anhydrous sodium carbonate (1 equivalent) and a catalytic amount of potassium iodide (0.17 equivalents), and was stirred at 105°C for 18 h. The solution was cooled and filtered through Celite. The filtered solid was washed well with diethyl ether, and the combined washings and filtrate were extracted with 4M hydrochloric acid (3 x 50 ml). The combined aqueous extracts were washed with diethyl ether (2 x 100 ml), basified with solid sodium carbonate, and extracted with diethyl ether (3 x 100ml). The organic extracts were dried, filtered, and concentrated under reduced pressure to give an oil which was purified by distillation.

N-([4-^{14}C]-3-Cyanopropyl)pyrrolidine (140a)

This was obtained as a clear oil; yield 303 mg, 74.9%, 23.7 μCi mmol^{-1}); b.p. 105°C (0.6 mm Hg). All physical properties were identical to authentic unlabelled material : ν_{max} ($CHCl_3$) 2250 cm^{-1}; δ_H (200 MHz) 1.57 (6H, m) and 2.27 p.p.m. (8H, m); δ_C (50 MHz) 14.3 (C-7), 22.8 (C-3 and -4), 24.2 (C-8), 53.3 (C-2 and -5), 53.7 (C-6), and 119.2 p.p.m. (C-9); m/z 138 (M^+, 8.6%) and 84 (100%); (Found: M^+, 138.1147. $C_8H_{14}N_2$ requires M, 138.1156).

N-([4-^{14}C]-3-Cyanopropyl)piperidine (140b)

This was obtained as a clear oil; yield 186 mg, 61.1%, 24.8 μCi mmol^{-1}; b.p. 35°C (0.15 mm Hg). All physical properties were identical to authentic unlabelled material : ν_{max} (thin film) 2245 cm^{-1}; δ_H (200 MHz) 1.26-1.68 (8H, complex) and 2.23 p.p.m. (8H, m); δ_C (50 MHz) 14.4 (C-8), 22.4 (C-4 or -9), 23.9 (C-4 or -9), 25.5 (C-3 and -5), 54.0 (C-2 and -6), 56.6 (C-7), and 119.4 p.p.m. (C-10); m/z 152 (M^+, 5.4%) and 98 (100%); (Found : M^+, 152.1320. $C_9H_{16}N_2$ requires M, 152.1313).

N-([5-^{14}C]-4-Cyanobutyl)pyrrolidine (141a)

This was obtained as a clear oil; yield 311 mg, 57.9%, 33.0 μCi mmol^{-1}; b.p. 33°C (0.07 mm Hg). All physical properties identical to authentic unlabelled material : ν_{max} (CCl_4) 2240 cm^{-1}; δ_H (90 MHz) 1.79 (8H, m) and 2.51 p.p.m. (8H, m); δ_C (50 MHz) 16.1 (C-8), 22.6 (C-3 and -4), 22.7 (C-9), 27.0 (C-7), 53.2 (C-2 and -5), 54.4 (C-6), and 118.8 p.p.m. (C-10); m/z 152 (M^+, 3.5%) and 84 (100%); (Found : M^+, 152.1319. $C_9H_{16}N_2$ requires M, 152.1313).

N-([5-^{14}C]-4-Cyanobutyl)piperidine (141b)

This was obtained as a clear oil; yield 352 mg, 60.1%, 28.4 μCi mmol^{-1}; b.p. 75°C (0.6 mm Hg). All physical properties were identical to authentic unlabelled material : ν_{max} (thin film) 2245 cm^{-1}; δ_H (200 MHz) 1.39-1.67 (10H, m) and 2.31 p.p.m. (8H, m);

δ_C (50 MHz) 16.3 (C-9), 23.0, 23.8, and 25.1 (C-4, -8, and -10), 25.4 (C-3 and -5), 53.9 (C-2 and -6), 57.5 (C-7), and 119.1 p.p.m. (C-11); m/z 166 (M^+, 3.3%) and 98 (100%); (Found : M^+, 166.1455. $C_{10}H_{18}N_2$ requires M, 166.1469).

General Procedure for Nitrile Reductions

The nitrile was added to a suspension of Adams' catalyst (15% w/w) in glacial acetic acid (5 ml per mmol of nitrile) and was hydrogenated at 1 atmosphere for 18 h. The catalyst was filtered off through Florisil and the Florisil was washed well with glacial acetic acid (25 ml). Conc. hydrochloric acid (10 ml) was added to the filtrate. Concentration under vacuum gave a solid which was recrystallised from 95% aqueous ethanol-ethyl acetate (1:1).

N-([4-^{14}C]-4-Aminobutyl)pyrrolidinium dihydrochloride (77)

This was obtained as a white powder; yield 364 mg, 77.9%, 21.8 μCi $mmol^{-1}$. All physical properties were identical to authentic unlabelled material (179) : m.p. 214-215°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); ν_{max} (KBr disc) 2980 and 1405 cm^{-1}; δ_H (200 MHz) (D_2O) 1.70 (4H, m,7- and 8-H_2), 1.93 (4H, m, 3- and 4-H_2), 2.90 (4H, m, 2- and 5-H_2), 3.09 (2H,m, 9-H_2), and 3.55 p.p.m. (2H, m, 6-H_2); δ_C (50 MHz) (D_2O) 20.8, 22.8, 22.9 (C-3, -4, -7, and -8), 47.0 (C-9), 52.3 (C-2 and -5), and 54.2 p.p.m. (C-6).

N-([4-^{14}C]-4-Aminobutyl)piperidinium dihydrochloride (142)

This was obtained as a white powder; yield 253 mg, 93.4%, 21.1 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material (180) : m.p. 282-284°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); ν_{max} (KBr disc) 2940 and 1405 cm^{-1}; δ_H (200 MHz) (D_2O) 1.67 (10H, m, 3-,4-,5-,8-, and 9-H_2), 2.81 (4H, m, 2- and 6-H_2), 2.97 (2H,m, 10-H_2), and 3.37 p.p.m. (2H, m,7-H_2); δ_C (50 MHz) (D_2O) 21.6, 22.0, 23.7 (C-3, -4, -5, -8, and -9), 47.7 (C-10), 54.1 (C-2 and -6), and 56.8 p.p.m (C-7).

N-([5-^{14}C]-5-Aminopentyl)pyrrolidinium dihydrochloride (143)

This was obtained as a white powder; yield 415 mg, 88.8%, 32.4 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material (181) : m.p. 196-198°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); ν_{max} (KBr disc) 2940 and 1410 cm^{-1}; δ_H (200 MHz) (D_2O) 1.36 (2H, m, 8-H_2), 1.65 (4H, m, 7- and 9-H_2), 1.96 (4H, m, 3- and 4-H_2), 2.91 (4H, m, 2- and 5-H_2), 3.08 (2H, m, 10-H_2), and 3.55 p.p.m. (2H, m, 6-H_2); δ_C (50 MHz) 23.6, 23.8, and 23.9 (C-3, -4, -7, -8, and -9), 48.1 (C-10), 54.9 (C-2 and -5), and 55.5 p.p.m. (C-6).

N-([5-^{14}C]-5-Aminopentyl(piperidinium dihydrochloride (144)

This was obtained as a white powder; yield 510 mg, 99.0%, 28.9 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material (182) : m.p. 192-193°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); ν_{max} (KBr disc) 2940 and 1410 cm^{-1}; δ_H (200 MHz) (D_2O) 1.32 (4H, m, 9- and 10-H_2), 1.65 (8H, m, 3-,4-,5-, and 8-H_2), 2.80 (4H, m, 2- and 6-H_2), 2.93 (2H, m, 11-H_2), and 3.40 p.p.m. (2H, m, 7-H_2); δ_C (50 MHz) (D_2O) 21.3, 23.0, 23.1, 25.2 (C-3, -4, -5, -8, -9, and -10), 47.3 (C-11), 53.3 (C-2 and -6), and 56.6 p.p.m. (C-7).

[2-^{2}H]-N-(5-Aminopentyl)piperidinium dihydrochloride (151)

To a solution of iminium salt (180) (26.7 mg, 0.11 mmol) in d_1-methanol (CH_3OD) (2 ml) was added sodium cyanoborodeuteride (14.6 mg, 0.22 mmol), and the reaction mixture was stirred at room temperature for 4 h. Excess sodium cyanoborodeuteride was destroyed by the slow addition of 10% sodium hydroxide (ca. 3 ml) and the resultant solution was extracted with chloroform (4 x 5 ml). The combined chloroform extracts were dried, filtered, and concentrated under vacuum to give an oil which was taken up in conc. hydrochloride acid. Evaporation under reduced pressure furnished the title compound; yield 20.8 mg, 76.9%; δ_H (200 MHz) (D_2O) 1.32 (4H, m, 9- and 10-H_2), 1.65 (8H, m, 3-, 4-, 5-, and 8-H_2), 2.82 (3H, m, 2-H and 6-H_2), 2.97 (2H, m, 11-H_2),

and 3.45 p.p.m. (2H, m, 7-H_2).

General Procedure for Mercury (II) Acetate Oxidations

Mercury (II) acetate (4 equivalents) was stirred in 5% aqueous acetic acid (6 ml/mmol of tertiary amine) at 120°C until completely dissolved (ca. 2 min). To this solution was added the diamine dihydrochloride (1 equivalent) and this mixture was heated at 120°C for 4 h. The reaction mixture was then cooled and filtered to remove precipitated mercury (I) salts. The filtrate was saturated with hydrogen sulphide gas, filtered through Celite, and the process was repeated. This removed mercury (II) salts. The aqueous solution was basified with 40% aqueous sodium hydroxide and then extracted with chloroform (3 x 20 ml). The chloroform extracts were dried and filtered. Addition of dry hydrogen chloride gas precipitated the iminium salt, which was extracted into water (10 ml). Lyophilisation of the separated aqueous layer yielded the iminium salt. In each case a viscous yellow gum was obtained, which did not solidify.

N-([4-^{14}C]-4-Aminobutyl)-1,2-didehydropyrrolidinium chloride hydrochloride (78)

Yield 76 mg, 33.4%, 25.1 μCi $mmol^{-1}$. All physical properties were identical to authentic unlabelled material (22), e.g. ν_{max} (nujol) 3000, 2025, and 1685 cm^{-1}; δ_H (200 MHz) (D_2O) 1.69-2.08 (6H, complex, 4-, 7-, and 8-H_2), 2.26 (2H, m, 3-H_2), 2.90-3.20

(4H, complex, 5- and 9-H_2), 3.95 (2H, m, 6-H_2), and 8.65 p.p.m. (1H, br s, 2-H); δ_C (50 MHz) (D_2O) 19.3, 23.9, and 24.5 (C-4, -7, and -8), 37.8 (C-3), 47.3 (C-9), 52.9 and 58.1 (C-5 and -6), and 181.7 p.p.m. (C-2).

N-([4-^{14}C]-4-Aminobutyl)-1,2-didehydropiperidinium chloride hydrochloride (136)

Yield 111 mg, 56.0%, 4.5 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material (183), e.g. ν_{max} (nujol) 3000, 2025, and 1650 cm^{-1}; δ_H (200 MHz) (D_2O) 1.65-2.12 (8H, complex, 4-, 5-, 8-, and 9-H_2), 2.30 (2H, m, 3-H_2), 2.93-3.24 (4H, complex, 6- and 10-H_2), 4.01 (2H, m, 7-H_2), and 8.62 p.p.m. (1H, br s, 2-H); δ_C (50 MHz) (D_2O) 21.4, 23.6, and 25.1 (C-4, -5, -8, and -9), 39.8 (C-3), 47.2 (C-10), 55.2 and 57.9 (C-6 and -7), and 182.5 p.p.m. (C-2).

N-([5-^{14}C]-5-Aminopentyl)-1,2-didehydropyrrolidinium chloride hydrochloride (137)

Yield 180 mg, 52.8%, 32.5 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material (184), e.g. ν_{max} (nujol) 3000, 2025, and 1645 cm^{-1}; δ_H (200 MHz) (D_2O) 1.38 (2H, m, 8-H_2), 1.63-2.08 (6H, complex, 4-, 7-, and 9-H_2), 2.22 (2H, m, 3-H_2), 2.94-3.26 (4H, complex, 5- and 10-H_2), 3.96 (2H, m, 6-H_2), and 8.65 p.p.m. (1H, br s, 2-H); δ_C (50 MHz) (D_2O) 19.8, 20.4, 21.6, and 23.2 (C-4, -7, -8, and -9), 38.2 (C-3), 47.9 (C-10), 54.1 and 54.7 (C-6 and -7), and 182.6 p.p.m. (C-2).

N-([5-^{14}C]-5-Aminopentyl)-1,2-didehydropiperidinium chloride hydrochloride (138)

Yield 159 mg, 46.7%, 28.2 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material (185), e.g. ν_{max} (nujol) 3000, 2025, and 1650 cm^{-1}; δ_H (200 MHz) (D_2O) 1.33 (4H, m, 9- and 10-H_2), 1.66 - 2.15 (6H, complex, 4-, 5-, and 8-H_2), 2.19 (2H, m, 3-H_2), 2.90-3.29 (4H, complex, 6- and 11-H_2), 3.92 (2H, m, 7-H_2), and 8.64 p.p.m. (1H, br s, 2-H); δ_C (50 MHz) (D_2O) 19.6, 22.3, 23.6, 24.5, and 24.7 (C-4, -5, -8, -9, and -10), 39.7 (C-3), 46.9 (C-11), 53.8 and 56.4 (C-6 and -7), and 182.7 p.p.m. (C-2).

7.2.2 General Procedure for N-Benzyloxycarbonylamino-alcohol Formation.

A solution of the aminoalcohol in 5M sodium hydroxide (1ml per 2 mmol of alcohol) was cooled to O^{o}C. Benzyl chloroformate (1.1 equivalents) was added dropwise and the solution was stirred at O^{o}C for 1 h then allowed to warm to room temperature. Much of the product had precipitated as a white solid. Ethyl acetate was added to dissolve this material and the aqueous layer was neutralised with 1M hydrochloric acid. The two layers were separated and the aqueous layer was extracted a further two times with ethyl acetate. The combined organic extracts were washed with water (3 times), dried, filtered, and concentrated *in vacuo* to give a clear oil which crystallised on standing. This was recrystallised from chloroform-diethyl ether (1:1).

4-Benzyloxycarbonylamino-1-butanol (160)

This was obtained as white plates; yield 6.06 g, 92.1%; m.p. 84-85°C (from $CHCl_3$-Et_2O, 1:1); ν_{max} ($CHCl_3$) 3620, 3450, 3050, 2940, 1715, 1515, and 1230 cm^{-1}; δ_H (90 MHz) 1.55 (4H, m, 2- and 3-H_2), 3.20 (2H, m, 4-H_2), 3.64 (2H, m, 1-H_2), 5.08 (2H, s, ArCH_2), and 7.31 p.p.m. (5H, s, ArH); δ_C (50 MHz) 26.4 (C-3), 29.5 (C-2), 40.7 (ArCCH_2), 62.1 (C-4), 66.5 (C-1), 128.0 and 128.4 (ArC), 136.5 (ArCCH_2), and 156.5 p.p.m. (C=O); m/z 223 (M^+, 3.0%), 132, 108, 107, 92, 91 (100%), 88, and 71; (Found : M^+, 223.1210; C, 64.58; H, 7.69; N, 6.27%. $C_{12}H_{17}NO_3$ requires M, 223.1208; C, 64.54; H, 7.68; N, 6.28%).

5-Benzyloxycarbonylamino-1-pentanol (161).

This was obtained as white plates, yield 10.04 g, 89.8%; m.p. 45-46°C (from $CHCl_3$-Et_2O, 1:1); ν_{max} ($CHCl_3$) 3620, 3450, 3050, 2940, 1715, 1515, and 1230 cm^{-1}; δ_H (200 MHz) 1.49 (6H, m, 2-, 3-, and 4-H_2), 2.49 (1H, br, O-H), 3.15 (2H, m, 5-H_2), 3.57 (2H, t, J 6.3 Hz, 1-H_2), 5.06 (2H, s, ArCH_2), and 7.31 p.p.m. (5H, s, ArH); δ_C (50 MHz) 22.7 (C-3), 29.5 and 32.0 (C-2 and -4), 40.8 (ArCCH_2), 62.2 (C-5), 66.4 (C-1), 127.9 and 128.4 (ArC), 136.5 (ArCCH_2) and 156.5 p.p.m. (C=O); m/z 237 (M^+, 1.3%), 146, 108, 107, 102, 92, 91 (100%), and 85; (Found : M^+, 237.1368; C, 65.96; H, 8.07; N, 6.01%. $C_{13}H_{19}NO_3$ requires M, 237.1365; C, 65.78; H, 8.07; N, 5.90%).

General Procedure for Swern Oxidations

A mixture of dichloromethane (25 ml) and oxalyl chloride (1.0 ml, 11 mmol) was cooled to -60°C. DMSO (1.7 ml, 22 mmol) diluted with dichloromethane (5 ml) was added slowly to the stirred solution. The reaction mixture was stirred for 2 min and the alcohol (10 mmol in 10 ml dichloromethane) was added within 5 min. Stirring was continued at -60°C for an additional 15 min and then triethylamine (7.0 ml, 50 mmol) was added. The reaction mixture was stirred for 5 min and then allowed to warm almost to room temperature (ca. 15 min). Water (50 ml) was added and the layers were separated. The aqueous layer was re-extracted with dichloromethane (50 ml) and the combined organic layers were washed well with brine (4 x 100ml), dried, filtered, and concentrated in vacuo to give a pale yellow oil. To remove traces of triethylamine hydrochloride, the oil was taken up in diethyl ether (15 ml) and the resultant cloudy solution was filtered through Celite. Concentration of the filtrate under reduced pressure furnished the aldehyde.

4-Benzyloxycarbonylamino-1-butanal (162)

This was obtained as a pale yellow oil; yield 1.11g, 100%; δ_H (90 MHz) 1.82 (2H, m, 3-H_2), 2.45 (2H, m, 2-H_2), 3.15 (2H, m, 4-H_2), 5.05 (2H, s, ArCH_2), 7.30 (5H, s, ArH), and 9.70 p.p.m. (1H, s, CHO).

5-Benzyloxycarbonylamino-1-pentanal (163)

This was obtained as a pale yellow oil; yield 2.35 g, 100%; δ_H (90 MHz) 1.55 (4H, m, 3- and 4-H_2), 2.42 (2H, m, 2-H_2), 3.15 (2H, m, 5-H_2), 5.05 (2H, s, Ar-CH_2), 7.30 (5H, s, ArH), and 9.68 p.p.m. (1H, s, CHO).

General Procedure for Introduction of the ^3H Label

To a slurry of pyrrolidine or piperidine (2.4 equivalents) and potassium carbonate, cooled to O°C, was added slowly dropwise a solution of aldehyde (162) or (163) in diethyl ether (0.5 ml/mmol of aldehyde). The reaction mixture was stirred at O°C for 0.5 h and then allowed to warm to room temperature over 3 h. Filtration through Celite and concentration in vacuo gave an oil which was taken up in diethyl ether (10ml/mmol of enamine). The solution was cooled to O°C and acidified to Congo red with 70% perchloric acid/ethanol (1:1), resulting in precipitation of the iminium salt as a viscous oil. This was immediately dissolved in methanol (1ml/mmol of iminium salt), and the solution was cooled to O°C. Sodium boro[^3H]hydride (ca. 200 μCi) and excess sodium borohydride was added and the reaction mixture was stirred at O°C for 0.5 h. Further sodium borohydride was added, the cooling bath was removed, and the reaction mixture was stirred for an additional 0.5 h. Cold 1M hydrochloric acid was added until the solution reached pH2. The solvent was then removed under reduced pressure to leave a solid residue which was taken up in 1M sodium hydroxide (25

ml). The alkaline solution was extracted with chloroform (3 x 20ml) and the combined organic extracts were washed with brine (3 x 50ml), dried, filtered, and concentrated *in vacuo* to afford the ^{3}H-labelled compounds (164)-(167). These were purified by dry-column flash chromatography (neutral alumina).

N-([1-^{3}H]-4-Benzyloxycarbonylaminobutyl) pyrrolidine (164)

The title compound was eluted with 80-90% chloroform in dichloromethane and obtained as a pale yellow oil; yield 914 mg, 66.9% [from aldehyde (162)], 182.6 μCi $mmol^{-1}$. All physical properties were identical to authentic unlabelled material: ν_{max} ($CHCl_3$), 1715, 1515, and 1235 cm^{-1}; δ_H (200 MHz) 1.54 (4H, m, 3- and 4-H_2), 1.71 (4H, m, 7- and 8-H_2), 2.45 (6H, m, 2-, 5-, and 6-H_2), 3.15 (2H, m, 9-H_2), 5.06 (2H, s, ArCH_2), and 7.32 p.p.m. (5H, m, ArH); δ_C (50 MHz) 23.3 (C-3 and -4), 26.4 and 28.1 (C-7 and -8), 40.9 (Ar-CH_2), 53.9 (C-2 and -5), 55.9 (C-6), 66.3 (C-9), 127.9 and 128.4 (ArC), 136.8 (ArCCH_2), and 156.5 p.p.m. (C=O); m/z 276 (M^+, 2.1%), 112, 91, and 84 (100%); (Found : M^+, 276.1829. $C_{16}H_{24}N_2O_2$ requires M, 276.1838)

N-([1-^{3}H]-5-Benzyloxycarbonylaminopentyl) pyrrolidine (166)

The title compound was eluted with 80-90% chloroform in dichloromethane and obtained as a pale yellow oil; yield 900 mg, 62.0% [from aldehyde (163)], 250.1 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material: ν_{max} ($CHCl_3$) 1715, 1515, and 1235 cm^{-1}; δ_H (200 MHz) 1.39-1.54 (6H, complex, 3-, 4-, and 8-H_2) 1.74 (4H, m, 7- and 9-H_2), 2.45 (6H, m, 2-, 5- and 6-H_2), 3.15 (2H, m, 10-H_2), 5.07 (2H, s, ArCH_2), and 7.32 p.p.m. (5H, m, ArH); δ_C (50 MHz) 23.3 (C-3 and -4), 24.7 (C-8), 28.6 and 29.7 (C-7 and -9), 41.0 (ArCCH_2), 54.1 (C-2 and -5), 56.3 (C-6), 66.5 (C-10), 128.0 and 128.4 (ArC), 136.6 (ArCCH_2), and 156.4 p.p.m. (C=O); m/z 290 (M^+ 2.8%), 126, 91, and 84 (100%); (Found : M^+ 290.1983. $C_{17}H_{26}N_2O_2$ requires M, 290.1994).

N-([1-^{3}H]-4-Benzyloxycarbonylaminobutyl) piperidine (165)

The title compound was eluted with 80-90% chloroform in dichloromethane and obtained as a pale yellow oil; yield 934 mg, 65.1% [from aldehyde (162)], 167.4 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material: ν_{max} ($CHCl_3$) 1715, 1515, and 1235 cm^{-1}; δ_H (200 MHz) 1.31-1.62 (10H, complex, 3-, 4-, 5-, 8-, and 9-H_2), 2.31 (6H, m, 2-, 6-, and 7-H_2), 3.15 (2H, m, 10-H_2), 5.06 (2H, s, ArCH_2), and 7.32 p.p.m. (5H, m, ArH); δ_C (50 MHz)

23.9, 24.3, and 24.7 (C-4, -8, and -9), 25.7 (C-3 and -5), 40.0 (ArC$\underline{C}H_2$), 54.2 (C-2 and -6), 59.2 (C-7), 66.5 (C-10), 127.9 and 128.2 (ArC), 136.6 (Ar$\underline{C}CH_2$), and 156.3 p.p.m. (C=O); $\underline{m}/\underline{z}$ 290 ($\underline{M}^+$, 3.2%), 126, 98 (100%), and 91; (Found $\underline{M}^+$, 290.1980. $C_{17}H_{26}N_2O_2$ requires $\underline{M}$, 290.1994).

N-([1-^{3}H]-5-Benzyloxycarbonylaminopentyl) piperidine (167)

The title compound was eluted with 80-90% chloroform in dichloromethane and obtained as a pale yellow oil; yield 810 mg, 53.4% [from aldehyde (163)] 201.4 μCi mmol^{-1}. All physical properties were identical to authentic unlabelled material : ν_{max} ($CHCl_3$) 1720, 1515, and 1230 cm^{-1}; δ_H (200 MHz) 1.28-1.59 (12H, complex, 3-, 4-, 5-, 8-, 9-, and 10-H_2), 2.28 (6H, m, 2-, 6-, and 7-H_2), 3.14 (2H, m, 11-H_2), 5.07 (2H, s, ArC$\underline{H}_2$), and 7.32 p.p.m. (5H, m, ArH); δ_C (50 MHz) 24.2 and 24.5 (C-4 and -9), 25.4 (C-3 and -5), 26.2 (C-8), 29.6 (C-10), 40.7 (ArC$\underline{C}H_2$), 54.4 (C-2 and -6), 59.1 (C-7), 66.2 (C-11), 127.8 and 128.2 (ArC), 136.5 (Ar$\underline{C}$-CH_2), and 156.3 p.p.m. (C=O); $\underline{m}/\underline{z}$ 304 ($\underline{M}^+$, 2.8%), 140, 98 (100%), and 91; (Found $\underline{M}^+$, 304.2172. $C_{18}H_{28}N_2O_2$ requires $\underline{M}$, 304.2151).

General Procedure for Removal of the CBZ Protecting Group

A mixture of N-protected diamine (164)-(167), ammonium formate (0.3 g), 10% palladium on charcoal [0.35 g per mmol of (164)-(167)], and methanol (15 ml) was heated at reflux for 3 h. The reaction mixture was cooled, and the catalyst was filtered off through Florisil. The filtrate was evaporated to give an oil which was taken up in conc. hydrochloric acid. The resultant solid was recrystallised from 95% aq. EtOH-EtOAc (1:1). The four compounds (168)-(171) had physical properties identical to authentic unlabelled compounds (179)-(182) (see pp. 138 - 140 for characterisation data).

N-([1-^{3}H]-4-Aminobutyl)pyrrolidinium dihydrochloride (168)

This was obtained as an off-white solid; m.p. 214-216°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); yield 596 mg, 90.9%, 161.7 μCi mmol^{-1}.

N-([1-^{3}H]-4-Aminobutyl)piperidinium dihydrochloride (169)

This was obtained as an off-white solid; m.p. 280-282°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); yield 638 mg, 87.1%, 153.0 μCi mmol^{-1}.

N-([1-^{3}H]-5-Aminopentyl)pyrrolidinium dihydrochloride (170)

This was obtained as a white solid; m.p. 194-196°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); yield 510 mg, 67.9%, 263.0 μCi mmol^{-1}.

N-([1-^{3}H]-5-Aminopentyl)piperidinium dihydrochloride (171)

This was obtained as a white solid; m.p. 191-193°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); yield 394 mg, 84.3%, 186.0 μCi mmol^{-1}.

The ^{3}H-labelled saturated salts (168)-(171) were oxidised with mercury(II) acetate, as described on p to furnish the ^{3}H-labelled iminium ions (172-175). These four compounds had physical properties identical to authentic unlabelled material (22) and (183)-(185), (see pp. 141 - 143 for characterisation data).

N-([1-^{3}H]-4-Aminobutyl)-1,2-didehydropyrrolidinium chloride hydrochloride (172)

Yield 122 mg, 31.0%, 203.0 μCi mmol^{-1}.

N-([1-^{3}H]-4-Aminobutyl)-1,2-didehydropiperidinium chloride hydrochloride (173)

Yield 174 mg, 36.4%, 211.7 μCi mmol^{-1}.

N-([1-^{3}H]-5-Aminopentyl)-1,2-didehydropyrrolidinium chloride hydrochloride (174)

Yield 186 mg, 45.8%, 239.5 μCi mmol^{-1}.

N-([1-^{3}H]-5-Aminopentyl)-1,2-didehydropiperidinium chloride hydrochloride (175)

Yield 110 mg, 38.9%, 223.6 μCi mmol^{-1}.

7.2.3 Feeding Methods

Each ^{14}C-labelled precursor was mixed with [1,4-^{3}H]putrescine dihydrochloride to give an initial ^{3}H:^{14}C ratio of ca. 2:1. Each ^{3}H-labelled precursor was mixed with [1,4-^{14}C]putrescine dihydrochloride to give an initial ^{3}H:^{14}C ratio of ca 10:1. The precursors were dissolved in distilled water and were fed on one day. One well-established plant was used for each experiment. After 10 d the plants were harvested and the alkaloids were extracted.

7.2.3 General Procedure for Alkaloid Extraction

After harvesting, the plant was macerated repeatedly in methanol. The methanolic extract was filtered and concentrated *in vacuo* to give a green residue. This was taken up in dichloromethane (100ml) and then extracted with 1M hydrochloric acid (2 x 100ml) The combined acid extracts were washed with dichloromethane (6 x 100ml) followed by stirring with zinc dust (2g) for 2 h. The mixture was then filtered through Celite, basified to pH 9 with conc. ammonia, and extracted with chloroform (4 x 100ml). The combined organic extracts were dried, filtered, and concentrated under reduced presure to give the crude alkaloids which were purified by chromatography or crystallisation.

In experiments with ^{14}C-labelled analogues (fed together with [1,4-^{3}H]putrescine), a ^{3}H:^{14}C ratio was measured for each crude alkaloid mixture. The low level of radioactivity present in these crude extracts precluded any further investigation (see Tables 3-5).

Higher total incorporations were achieved using the ^{3}H-labelled precursors (see Tables 6-8). Each extract from *Senecio pleistocephalus* was analysed by TLC.

Autoradiography indicated the presence of a band coincident with authentic unlabelled rosmarinine (70) at R_F 0.34. Furthermore, in experiments with ^{3}H-labelled precursors (169)-(171) and (173)-(175), a broad band was observed at R_F 0.15-0.28. Partial separation of the

products was achieved by recrystallisation from dichloromethane-acetone (1:1). After 5 recrystallisations most of the ^{14}C activity was associated with the recrystallised rosmarinine (70) while almost all of the ^{3}H activity was present in the mother liquors. Residual amounts of rosmarinine (70) were removed from the mother liquors by preparative TLC. It was not possible to isolate sufficient amounts of material to allow characterisation of any analogues which may have been produced.

In feeding experiments with ^{3}H-labelled precursors (168) and (172) to *S. pleistocephalus*, each extract was shown by autoradiography to contain one radioactive species, coincident with authentic unlabelled rosmarinine (70).

In all eight feeding experiments rosmarinine was recrystallised to constant specific activity, m.p. 203-204°C (decomp.) (from dichloromethane-acetone, 1:1) (lit.[126], 202-204°C).

7.3 Experimental to Chapter 4

7.3.1

Substrates (22) and (179)-(185) for the diamine oxidase (DAO) assay were synthesised by the route outlined in Scheme 30 (Chapter 3) for the synthesis of ^{14}C-labelled analogues. Sodium cyanide was used in place of sodium [^{14}C]cyanide. Higher yields were obtained in the mercury (II) acetate oxidations by a modification of the work-up described on p 141.

After passing dry hydrochloric acid through the chloroform extracts, water (10ml) was added to dissolve the precipitated iminium ion, and the layers were separated. The chloroform layer was extracted with a further portion of water (10ml). Lyophilisation of the combined aqueous extracts afforded the iminium ions (22) and (183)-(185) in yields of 58.8%-72.5% (cf. yields of 33-53% for Scheme 30). See pp 138 - 143 for characterisation data for compounds (22) and (179)-(185).

N-(4-Aminobutyl)pyrrolidinium dihydrochloride (179)

This was obtained as a white powder; m.p. 210-212°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); yield 2.12 g, 89.9%.

N-(4-Aminobutyl)piperidinium dihydrochloride (180)

This was obtained as a white powder; m.p. 283-284°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); yield 1.98g, 91.9%.

N-(5-Aminopentyl)pyrrolidinium dihydrochloride (181)

This was obtained as a white powder; m.p. 198-200°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); yield 2.40g, 85.1%

N-(5-Aminopentyl)piperidinium dihydrochloride (182)

This was obtained as a white powder; m.p. 192-193°C (decomp.) (from 95% aq. EtOH-EtOAc, 1:1); yield 1.83g, 95.1%

N-(4-Aminobutyl)-1,2-didehydropyrrolidinium (22) chloride hydrochloride

This was obtained as a viscous yellow gum which failed to solidify; yield 115 mg, 58.2%.

N-(4-Aminobutyl)-1,2-didehydropiperidinium chloride hydrochloride (183)

This was obtained as a viscous yellow gum which failed to solidify; yield 139mg, 69.9%.

N-(5-Aminopentyl)-1,2-didehydropyrrolidinium chloride hydrochloride (184)

This was obtained as a viscous yellow gum which failed to solidify; yield 142mg, 71.7%.

N-(5-Aminopentyl)-1,2-didehydropiperidinium chloride hydrochloride (185)

This was obtained as a viscous yellow gum which failed to solidify; yield 144 mg, 72.5%.

7.3.2 The Spectrophotometric Assay

Pea seedling diamine oxidase was extracted from 10 day old seedlings as described by Hill and was purified up to Step 4.[127] Enzyme activity[128] was 1200 units per mg solid (at 25°C), with a yield of ca. 30 mg per kg seedlings. Protein concentrations were determined by the Coomassie Blue method of Sedmak and Grossberg.[129]
I am grateful to Angela Equi, Stephen Barr and Isabel Freer for carrying out the aforementioned extractions and determinations.

The kinetics of DAO-catalysed oxidation of substrates (22) and (179)-(185) were determined by the procedure of Stoner.[109] A typical reaction mixture in a 1 cm pathlength cuvette comprised 3 ml of 70mM phosphate buffer (pH 6.3) containing horseradish peroxidase (EC 1.11.1.7) (6μg/ml), MBTH (20μM), DMAB (1mM), and DAO (EC 1.4.3.6) (0.03-0.06mg/ml). Substrate concentrations ranged up to 30 mM. Reaction was initiated by addition of standard enzyme solution to the thermally equilibrated reaction mixture, followed immediately by substrate addition. Initial rates were determined over a range of substrate concentrations from the linear absorbance changes during the first minute of reaction, and Michaelis-Menten 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]) and Lineweaver Burk (1/V vs. 1/[S]) plots.[103] See Tables 9 and 10 for results.

7.4 Experimental to Chapter 5

7.4.1 Intermediate Trapping Experiments I

(i) [1,4-^{14}C]Putrescine dihydrochloride (50 μCi) was fed to one well-established *Senecio pleistocephalus* plant. After 24 h the plant was harvested and macerated repeatedly in methanol. To this extract was added unlabelled saturated salt (179) (28 mg), sodium borohydride (49 mg), and sodium boro[^{3}H]hydride (ca. 200 μCi), and the mixture was stirred at room temperature overnight. Triethylamine (1ml), followed by phenyl-isothiocyanate (1ml) was added, and the mixture was stirred for a further 3 d. Brine (150ml) was added, and the solution was extracted with dichloromethane (3 x 150ml). The organic extracts were dried, filtered, and concentrated under vacuum to yield an oil (566 mg). Autoradiography of a TLC plate (with dichloromethane-acetonitrile, 9:1 as the eluting mixture) indicated the presence of bands at R_F 0.24 and R_F 0.46 coincident with unlabelled derivatives (201) and (79) of, respectively, putrescine (30) and diamine (179). (Most of the radioactivity was present at the baseline). Derivatives (201) and (79) were separated by preparative TLC (eluting with dichloromethane-acetonitrile, 9:1), and were shown to contain respectively 0.18% and 0.44% of the total ^{14}C activity fed as [1,4-^{14}C]putrescine dihydrochloride. The tritium content of (201) and (79) was ca. 2.5 μCi and ca. 9.6 μCi respectively.

(ii) Spermidine trihydrochloride (labelled [1,4-^{14}C] in the tetramethylene portion) (50 μCi) was fed to one *S. pleistocephalus* plant. After 24 h the plant was

harvested and macerated repeatedly in methanol. To this extract was added unlabelled saturated (179) (31mg) and sodium cyanoborodeuteride (42 mg), and the mixture was stirred at room temperature for 24 h. Derivatisation and isolation was carried out as described above. No deuterium-labelled derivative was isolated by preparative TLC (^2H NMR spectroscopic data).

7.4.2 4-t-Butoxycarbonylamino-1-butanol (203)

A solution of di-t-butyldicarbonate (2.53g, 11.6mmol) in THF (15ml) was cooled to 0^oC, and a solution of 4-amino-1-butanol (1ml, 11.6 mmol) in THF (2ml) was added dropwise. The solution was allowed to warm to room temperature, and was then stirred for 18 h. The solution was concentrated in vacuo to give a clear oil which was purified by chromatography (silica; eluted with 40% diethyl ether in ethyl acetate); yield 2.11g, 96.4%; ν_{max} ($CHCl_3$) 3620, 3460, 1710, and 1510 cm^{-1}; δ_H (200 MHz) 1.44 (9H, s, t-Bu), 1.55 (4H, m, 2- and 3-H_2), 3.11 (2H, m, 4-H_2), and 3.62 p.p.m. (2H, m, 1-H_2); δ_C (50 MHz) 26.5 (C-3), 28.3 [$C(\underline{C}H_3)_3$], 29.6 (C-2) 40.3 [$\underline{C}(CH_3)_3$], 62.2 (C-4), 66.9 (C-1), and 156.1 p.p.m. (C=O). The O-TBDMS derivative had M^+, 303.2239. $C_{15}H_{33}NO_3Si$ requires M, 303.2230.

4-t-Butoxycarbonylamino-1-butanal (204)

The title compound was obtained by Swern oxidation of alcohol (203) (see p 145 for experimental details); yield 913 mg, 100%; δ_H (90 MHz) 1.40 [9H, s, $C(C\underline{H}_3)_3$], 1.78 (2H, m, 3-H_2), 2.47 (2H, m, 2-H_2), 3.11 (2H, m, 4-H_2), and 9.78 p.p.m. (1H, s, C$\underline{H}$O).

N-([1-^{2}H]-4-Aminobutyl)pyrrolidinium dihydrochloride (206)

A solution of aldehyde (204) (913 mg, 4.9 mmol) in dry diethyl ether (5 ml) was added to a slurry of pyrrolidine (985 µl, 11.8 mmol) in dry diethyl ether (2ml) containing anhydrous potassium carbonate at O°C. The reaction mixture was stirred at O°C for 0.5 h and then allowed to warm to room temperature overnight. Filtration through Celite and concentration under vacuum gave a pale yellow oil which was taken up in diethyl ether (10ml). The solution was cooled to O°C and acidified to Congo red with 70% perchloric acid - ethanol (1:1). The diethyl ether was decanted to leave the iminium salt (205) which was immediately taken up in d_1-methanol (CH_3OD) (10ml). To this solution was added sodium cyanoborodeuteride (646mg, 9.8mmol) and the reaction mixture was stirred at room temperature for 4 h. Excess sodium cyanoborodeuteride was destroyed by the slow addition of 10% sodium hydroxide (10ml). The resultant solution was extracted with chloroform (4 x 20ml), and the chloroform extracts were washed with brine (2 x 30ml), dried, filtered, and concentrated under

reduced pressure to give a pale yellow oil. This was taken up in conc. hydrochloric acid, and concentration *in vacuo* gave the title compound; yield 472mg, 44.8% from aldehyde (204); m.p. 212-214°C (from 95% aq. EtOH-EtOAc, 1:1); δ_H (200 MHz) (D_2O) 1.74 (4H, m, 7- and 8-H_2), 1.98 (4H, m, 3- and 4-H_2), 2.90 (4H, m, 2- and 5-H_2), 3.18 (2H, m, 9-H_2), and 3.58 p.p.m. (1H, m, 6-H); δ_D (30.72 MHz) (H_2O) 3.40 p.p.m; δ_C (50 MHz) (D_2O) 23.3, 23.4 and 24.7 (C-3, -4, -7, and -8), 39.6 (C-9), 54.8 (C-2 and -5), and 54.6 p.p.m. (t, *J* 21.8 Hz, C-6).

N-([1-^{2}H]-4-Aminobutyl)-1,2-didehydropyrrolidinium chloride hydrochloride (207)

This was obtained by mercury (II) acetate oxidation of ^{2}H-labelled saturated salt (206) (see p 141 and pp 154-155 for experimental procedure); yield 145.9mg, 73.6%; δ_H (200 MHz) (D_2O) 1.59-2.02 (6H, complex, 4-, 7-, and 8-H_2), 2.16 (2H, m, 3-H_2), 2.93-3.17 (4H, complex, 5- and 9-H_2), 3.87 (1H, m, 6-H), and 8.64 p.p.m. (1H, m, 2-H); δ_D (30.72 MHz) (H_2O) 3.81 p.p.m; δ_C (50 MHz) (D_2O) 20.1, 23.5, and 24.1 (C-4, -7, and -8), 36.6 (C-3), 39.7 (C-9), 53.7 (t, *J* 22.2 Hz, C-6), 54.9 (C-5), and 182.2 p.p.m. (C-2).

Feeding Methods

1. *S.pleistocephalus.*

One well-established plant was used per feeding experiment. Both ^{2}H-labelled precursors were mixed with [1,4-^{14}C]putrescine dihydrochloride (ca. 5μCi), dissolved in distilled water, and administered to the plant by the wick method.

(i) ^{2}H-labelled saturated salt (206) (44.6mg) was fed to one plant over 4 d. After a further 10 d, rosmarinine (70) was extracted and recrystallised to constant specific activity (422mg, 1.31 μCi $mmol^{-1}$, 0.24% of the fresh weight of the plant). Total incorporation of ^{14}C = 25.2%.

(ii) ^{2}H-labelled iminium ion (207) (68mg) was fed to one plant over 4 d. After a further 10 d, rosmarinine (70) was extracted and recrystallised to constant specific activity (323mg, 1.12 μCi $mmol^{-1}$, 0.27% of the fresh weight of the plant). Total incorporation of ^{14}C = 21.6%.

2. *S. vulgaris*

Hairy root cultures were propagated from cultures provided by Dr. N. Walton at the A.F.R.C. Food Research Institute, Norwich, and were grown in standard medium with a 200 r.p.m. shake rate. Extraction of a trial batch showed that the roots contained mainly senecionine (32), m.p. 230-231°C (from dichloromethane) (lit.[130],

232-233°C) (Jane Matheson, unpublished results). At one week old, a batch of 5 flasks each containing 50 ml medium was inoculated with the precursor in sterile aqueous solution. After a further 9 d growth, the cultures were drained and the roots blended in methanol. *I am grateful to Isabel Freer for carying out this part of the feeding experiments.* The standard alkaloid extraction procedure was then followed.

(i) ^{2}H-labelled saturated salt (206) (22.8mg) was fed. The resultant sample of senecionine (32) was purified by dry-column flash chromatograhy (neutral alumina). Senecionine (32) was eluted with 2% methanol in dichloromethane. Recrystallisation to constant specific activity from dichloromethane gave senecionine (41.2 mg, 6.10 μCi mmol^{-1}, 1.55% of the weight of the root extract). Total incorporation of ^{14}C = 14.6%.

(ii) ^{2}H-labelled iminium ion (207) (24.1 mg). The resultant sample of senecionine was purified as described above to yield: 30.4mg, 12.2 μCi mmol^{-1}, 1.41% of the weight of the root extract. Total incorporation of ^{14}C = 19.4%.

In all four feeding experiments, a radioscan of a TLC plate indicated the presence of one band coincident with authentic unlabelled material (rosmarinine, R_F 0.34, from *S. pleistocephalus*, and senecionine, R_F 0.54, from *S. vulgaris*.

7.4.3 (+)-Retronecine (5)

This was obtained by alkaline hydrolysis of mother liquors from recrystallisations of riddelliine (35) from *Senecio riddellii*, supplied by Dr. R.J. Molyneaux, U.S.D.A., California, USA. A dark brown gum (ca. 400 mg) was partially dissolved in water (30ml). Barium hydroxide octahydrate (2g) was added, and the mixture was heated at 100°C for 18 h. The cooled solution was filtered, solid carbon dioxide was added, and the precipitated barium carbonate was filtered off. The filtrate was saturated with sodium carbonate and extracted continuously with chloroform for 72 h. The chloroform extracts were dried, filtered, and concentrated under reduced pressure to give a brown gum. TLC indicated the presence of retronecine (5) along with other tertiary amines (visualised by the modified Dragendorff reagent). The oil was partially purified by preparative TLC (with chloroform-methanol-conc. ammonia, 85:14:1, as the eluent). This process was carried out twice. Final purification was achieved by dry-column flash chromatography (neutral alumina). The title compound was eluted with 4-6% methanol in chloroform.

Recrystallisation from acetone-light petroleum (b.p. 60-80°C) gave (+)-retronecine (5), 83 mg ; m.p. 117-119°C (lit.[31], 118-120°C).

(-)-Platynecine (25)

A solution of retronecine (5) (84mg, 0.54 mmol) in absolute ethanol (4.2ml) containing 10% palladium on charcoal (12.6mg, 15% w/w) was hydrogenated at 1 atmosphere for 4 h. The catalyst was filtered off through Celite, and the filtrate was concentrated *in vacuo* to yeild an oil. This oil was taken up in boiling acetone (1ml) which, on cooling, deposited platynecine (25) as a white powder; yield 79mg, 92.8%; m.p. 149-151°C (from acetone) (lit.[131], 148-149°C); $[\alpha]_D^{19}$ -58° (*c* 1.1, EtOH) (lit.[131], -56.8°C); ν_{max} (KBr disc) 3350, 2940, 2870, and 1470 cm^{-1}; δ_H (200 MHz) (CD_3OD) 1.69-2.03 (4H, complex, 2- and 6-H_2), 2.43 (1H, m, 1-H_2), 2.73-2.91 (2H, complex 3- or 5-H_2), 3.02 (1H, m, 8-H), 3.19 (2H, m, 3- or 5-H_2), 3.92 (1H, m, 9-H), 3.94 (1H, m, 9-H), and 4.24 p.p.m. (1H, m, 7-H); δ_C (50 MHz) (CD_3OD) 28.9 (C-2), 37.3 (C-6), 45.1 (C-1), 54.8 and 58.6 (C-3 and -5), 61.7 (C-9), 72.7 (C-8), and 73.1 p.p.m. (C-7); *m/z* 157 (M^+, 9.5%), 113, 82 (100%), and 81; (Found : M^+, 157.1078. $C_8H_{15}NO_2$ requires *M*, 157.1103).

(-)-Rosmarinecine (4)

To a solution of rosmarinine (70) (500mg, 1.42 mmol) in water (30ml) was added barium hydroxide octahydrate (1.78g, 5.65 mmol) and the solution was heated at 100°C for 4 h. Solid carbon dioxide was added to the cooled solution, which was then filtered through Celite. The filtrate was saturated with

sodium carbonate and extracted continuously with chloroform for 48 h. The organic extracts were dried, filtered, and concentrated under reduced pressure to afford (-)-rosmarinecine; yield 149mg, 60.6%; m.p. 170-172°C (from acetone-light petroleum, b.p. 60-80°C) (lit.[132a], 171-172°C); $[\alpha]_D^{23}$ - 119.1° (c 0.94, EtOH) (lit.[132b], -118.5°); ν_{max} (KBr disc) 3340, 2930 and 1435 cm^{-1}; δ_H (200 MHz) (CD_3OD) 1.84 (2H, m, 6-H_2), 2.29 (1H, m, 1-H), 2.73-3.34 (4H, complex, 3-and 5-H_2), 3.40 (1H, dd, J 8.1 Hz and 2.7 Hz, 8-H), 3.91 (1H, dd, J 11.0 Hz and 3.7 Hz, 9-H), 4.03 (1H, dd, J 11.0Hz and 6.6 Hz, 9-H), 4.24 (1H, m, 7-H), and 4.39 p.p.m. (1H, m, 2-H); δ_C (50 MHz) (CD_3OD) 36.2 (C-6), 51.7 (C-1), 55.2 (C-5), 59.6 (C-3), 64.6 (C-9), 71.0 (C-8), 72.4 and 72.7 p.p.m. (C-2 and -7); m/z 173 (M^+, 7.6%), 129, 99, 98 (100%), 82, 81, and 68; (Found : M^+, 173.1056. $C_8H_{15}NO_3$ requires M, 173.1052).

General Procedure for Benzoylation

A solution of either rosmarinecine (4) or platynecine (25) in dry pyridine (2ml/mmol of necine) was cooled to 0°C. Benzoyl chloride (2.2 equivalents/hydroxyl) was added dropwise, the solution was allowed to warm to room temperature and then stirred overnight. The reaction mixture was diluted with cold water (2ml), acidified with 1M hydrochloride acid, and the resultant solution was washed with diethyl ether (3 x 5ml). The aqueous layer was basified with conc. ammonia and extracted with diethyl ether (3 x 5ml). The combined ethereal extracts were dried,

filtered, and concentrated *in vacuo*. Excess pyridine was removed azeotropically under reduced pressure using toluene (x 4) and then CCl_4 (x 2). The resultant oil was puified by dry-column flash chromatography (neutral alumina).

(-)-2,7-O,O-*(Dibenzoyl)platynecine* (208)

This was purified by chromatography (neutral alumina). The title compound was eluted with 1.5% methanol in dichloromethane and obtained as a clear oil; yield 27.4mg, 57.0%; $[\alpha]_D^{20}$ -190.6° (*c* 0.96, $CHCl_3$); ν_{max} ($CHCl_3$), 1720, 1600, and 1450 cm^{-1}; δ_H (200 MHz) 2.00 (2H, m, 2-H_2), 2.29 (3H, m, 1-H and 6-H_2), 2.94-3.23 (2H, complex, 3- or 5-H_2), 3.67 (2H, m, 3- or 5-H_2), 3.89 (1H, m, 8-H), 4.27-4.44 (1H, complex, 9-H), 4.61 (1H, dd, *J* 11.1 and 7.2 Hz, 9-H), 5.75 (1H, t, *J* 3.2 Hz, 7-H), 7.37-7.62 (6H, complex, *o*- and *p*-ArH), and 7.93-8.07 p.p.m. (4H, complex, *m*-ArH); δ_C (50 MHz) 29.0 (C-2), 34.6 (C-6), 40.1 (C-1), 53.4 and 54.2 (C-3 and C-5), 63.1 (C-9), 69.0 (C-8), 75.5 (C-7), 127.9, 128.4, 128.9, 129.2, 129.5,and 129.6 (ArC), 133.3 and 133.8 (Ar*C*CO), 165.1 and 166.0 p.p.m. (C=O); *m/z* 244, 243, 138, 122, 121, 82, and 81; (Found : M^+-$PhCO_2$, 244.1325.

$C_{22}H_{23}NO_4$ requires *M*-$PhCO_2$, 244.1338).

(-)-2,7,9-O,O,O-*(tribenzoyl)rosmarinecine* (209)

This was purified by chromatography (neutral

alumina). The title compound was eluted with 0-0.5% methanol in dichloromethane and obtained as a clear oil; yield 23.6mg, 36.1%; $[\alpha]_D^{20}$ -289.2° (c 0.12, $CHCl_3$); ν_{max} ($CHCl_3$) 1720, 1600, and 1450 cm^{-1}; δ_H (200 MHz) 2.31 (2H, m, 6-H_2), 2.96 (1H, m, 1-H), 3.42-3.68 (4H, m, 3- and 5-H_2), 4.10 (1H, dd, J 8.3 Hz and 3.1 Hz, 8-H), 4.59 (1H, m, 9-H), 5.63 (1H, m, 7-H), 5.80 (1H, dd, J 15.1 Hz and 8.2 Hz, 9-H), 7.25-7.57 (9H, complex, o- and p-ArH), and 7.86-8.10 p.p.m. (6H, complex, m-ArH); δ_C (50 MHz) 34.0 (C-6), 44.6 (C-1), 53.8 (C-5), 60.9 (C-3), 62.5 (C-9), 68.4 (C-8), 74.8 and 75.6 (C-2 and -7), 128.1, 128.3, 128.4, 129.0, 129.2, 129.3, 129.4, 129.6, and 129.8 (ArC), 133.1, 133.4 and 133.7 (ArCCO), 165.5, 166.1, and 166.3 p.p.m. (C=O); m/z 364, 363, 243, 242, 241, 138, 137, 136, 107, 106, 105 (100%), 82, and 81; (Found : M^+-$PhCO_2$, 364.1540. $C_{29}H_{27}NO_6$ requires M-$PhCO_2$, 364.1549).

Intermediate Trapping Experiments II

Spermine tetrahydrochloride (50 μCi) was administered to one well-established *S. pleistocephalus* plant. After 24 h the plant was harvested and macerated repeatedly in methanol. The methanolic extract was filtered and concentrated in vacuo to give a green residue. This was taken up in dichloromethane (100ml) and extracted with 1M hydrochloric acid (2 x 100ml). The combined acid extracts were washed with dichloromethane (6 x 100ml) followed by stirring with zinc dust (2g) for 2 h. The mixture was then filtered through Celite, basified to pH 9 with conc. ammonia, and extracted with chloroform (60ml). The aqueous layer was then saturated with sodium carbonate and extracted

continuously with chloroform for 72 h. The chloroform extracts were dried, filtered, and concentrated under reduced pressure to give a residue (ca 80 mg). To this residue was added platynecine (25) (31.7mg, 0.20 mmol) and rosmarinecine (4) (29.3mg, 0.17mmol). The mixture was dissolved in dry pyridine (500 µl) and cooled to $0^{o}C$ Excess benzoyl chloride (300 µl) was added dropwise and the reaction mixture was allowed to warm to room temperature and then stirred for a further 18 h. The reaction mixture was diluted with water (10 ml) and acidified with conc. hydrochloric acid. The acid solution was washed with diethyl ether (3 x 10ml), basified with conc. ammonia, and extracted with chloroform (3 x 15ml). The chloro-form extracts were dried, filtered, and concentrated in vacuo. Excess pyridine was removed azeotropically under reduced pressure using toluene (x 4) and CCl_4 (x 2). This gave a brown gum (36.3mg). TLC (chloroform-methanol-conc. ammonia, 85:14:1) indicated the presence of both dibenzoyl platynecine (208) (R_F 0.39) and tribenzoyl rosmarinecine (209) (R_F 0.65). Auto-radiography indicated the presence of bands coincident with authentic unlabelled material. Separation of (208) and (209) was achieved by preparative TLC with chloroform-methanol-conc. ammonia(85:14:1) as the eluting mixture. This furnished samples of dibenzoyl platynecine (208) (12.8mg) and tribenzoyl rosmarinecine (209) (7.4mg). These contained respectively 0.37% and 0.18% of the total ^{14}C activity fed as ^{14}C-labelled spermine.

7.4.4 (1R)-[1-^{2}H]Putrescine (87) dihydrochloride

The title compound was prepared according to the method of Richards and Spenser[59]; yield 416mg, 86.8%; m.p. >300° (from 95% aq. ethanol-acetone); δ_D (30,72 MHz) (H_2O) 2.88 p.p.m; δ_H (200 MHz) (D_2O) 1.98 (4H, m, 2- and 3-H_2), and 2.91 p.p.m. (3H, m, 1-H and 4-H_2).

Feeding Methods

(i) A sample of (1R)-[1-^{2}H]putrescine dihydrochloride (180mg) was mixed with [1,4-^{14}C]putrescine dihydrochloride (6.8 μCi). The mixture was divided into 5 equal portions and was fed on consecutive days to 30 *Cynoglossum australe* plants by the wick method. After a further 14 d the alkaloids were extracted as described previously. This yielded a yellow oil (2.64g, 0.44% of the fresh weight of the plant material). The total incorporation of ^{14}C into the crude extract was 3.2%. Approximately one-tenth of the sample was analysed by ^{2}H NMR spectroscopy : no incorporation of deuterium was observed into the alkaloid mixture.

(ii) Using 6 *C. australe* plants, (1R)-[1-^{2}H]putrescine dihydrochloride (172mg) and [1,4-^{14}C]putrescine dihydrochloride (5.4 μCi) were fed on 5 consecutive days. After a further 10 d, extraction of the plants yielded an oil (531mg, 0.41% of the fresh weight of plants) containing 2.9% of the total ^{14}C activity fed as [1,4-^{14}C]putrescine dihydrochloride. Again no incorporation of deuterium was observed.

7.4.5 Monoacetylputrescine (24) hydrochloride

Putrescine (30) (1.42g, 16.2mmol) was added to a cooled solution of glacial acetic acid (10ml). The solution was kept at 50-60°C during the stepwise addition over 1 h of acetic anhydride (1.16ml, 12.3mmol). The reaction mixture was allowed to cool to room temperature and was then allowed to stand overnight. Concentration under reduced pressure gave an oil which was taken up in a mixture of water (5ml) and 6M hydrochloric acid (4ml). The solution was evaporated to dryness to give a mixture of the title compound and putrescine dihydrochloride. Extraction of the mixture with isopropanol (50 ml) dissolved the desired product, and the unreacted putrescine dihydrochloride was filtered off. The volume of the solution was reduced to 15ml, and it was stored in the fridge overnight. The crystals that were formed were collected by filtration to afford monoacetylputrescine (24) hydrochloride; yield 1.64g, 54.3%; m.p. 134-136°C (from isopropanol-diethyl ether) (lit.[132], 136-139°C); ν_{max} (KBr disc) 3280, 3260, 3070, 1650, and 1555 cm^{-1}; δ_H (200 MHz) (D_2O) 1.41-1.60 (4H, m, 2- and 3-H_2), 1.85 (3H, s, NHCOC$\underline{H}_3$), 2.88 (2H, t, $\underline{J}$ 7.3 Hz, 4-H_2), and 3.06 p.p.m. (2H, t, $\underline{J}$ 6.5 Hz, 1-H_2); δ_C (50 MHz) (D_2O) 22.7 (NHCO$\underline{C}H_3$), 24.9 and 26.1 (C-2 and -3), 39.4 (C-4), 39.9 (C-1), and 174.7 p.p.m. (NH$\underline{C}OCH_3$); (Found : C, 43.19; H, 9.14; N, 16.90; Cl, 20.90%. $C_6H_{15}ClN_2O$ requires C, 43.35; H, 9.10; N, 16.86; Cl, 21.07%).

Check on the Radiochemical Purity of [1,4-^{3}H]Putrescine dihydrochloride

A sample of [1,4-^{3}H]putrescine dihydrochloride was applied to a cellulose-coated TLC plate which was then eluted with isopropanol-conc. ammonia (5:3). A radioscan of the developed TLC plate showed one radioactive band coincident with authentic unlabelled material of R_F 0.67.

Monoacetyl [1,4-^{3}H]Putrescine hydrochloride

A mixture of putrescine (109mg, 1.24 mmol) and [1,4-^{3}H]putrescine dihydrochloride (2.64 μCi) was stirred in glacial acetic acid (700 μl) at room temperature for 18 h. The temperature was then raised to 50-60°C and acetic anhydride (98 μl, 1.04 mmol) was added dropwise. The reaction mixture was allowed to cool to room temperature and was then left standing overnight. The mixture was concentrated in vacuo and the oily residue was dissolved in hot water (400 μl) and 6M hydrochloric acid (260 μl), then the mixture was evaporated to dryness. The resultant white solid was extracted with isopropanol (3.6 ml), with warming, and the solution was filtered and reduced in volume to 1.5 ml. The solution was kept in the fridge overnight, and it deposited a white solid (97mg). A radioscan (eluting the TLC plate with ethyl acetate-isopropanol-conc. ammonia, 9:7:4) indicated radioactive bands corresponding to unreacted [1,4-^{3}H]putrescine dihydrochloride, at the baseline, and to the title

compound at R_F 0.24. Separation by preparative TLC using the above solvent system afforded monoacetyl[1,4-^{3}H]putrescine hydrochloride as a white solid; yield 42 mg, 20.3%, 5.14 μCi mmol^{-1}; m.p. 138-140°C (from iso-propanol-diethyl ether) (lit.[133], 136-139°C). All physical properties were identical to authentic unlabelled material.

Feeding Methods

1. *S. pleistocephalus*

^{3}H-Labelled N-acetylputrescine hydrochloride (25 mg, 5.14 μCi mmol^{-1}) was mixed with [1,4-^{14}C]putrescine dihydrochloride to give an initial ^{3}H:^{14}C ratio of 1.52. The mixture was dissolved in sterile water and fed to one plant on one day. Ten days later, rosmarinine (70) was extracted and recrystallised to constant specific activity (97mg, 0.051 μCi mmol^{-1} for ^{3}H). Total incorporation of ^{3}H was 1.81% and the ^{3}H:^{14}C ratio was 0.24. Autoradiography indicated one band coincident with authentic unlabelled rosmarinine.

2. *C. australe*

^{3}H-Labelled N-acetylputrescine hydrochloride (20mg, 5.22 μCi mmol^{-1}) was mixed with [1,4-^{14}C]putrescine dihydrochloride to give an initial ^{3}H:^{14}C ratio of 0.71, and a feeding experiment was carried out as described above, using one *C. australe* plant. The total incorporation of ^{3}H into the crude extract (11mg) was 1.58%, with a ^{3}H:^{14}C ratio of 12.2. Satisfactory chromatographic separation of cynaustine (26) (R_F 0) and

cynaustraline (27) (R_F 0.1) could not be achieved.

7.5 Experimental to Chapter 6

Feeding Methods

In double label ($^3H/^{14}C$) experiments each ^{14}C-labelled precursor (ca. 5 μCi) was mixed with [1,4-^3H]putrescine dihydrochloride to give an initial $^3H:^{14}C$ ratio of ca. 2. In all feeding experiments, the precursors were dissolved in distilled water and fed to one *Crotalaria lachnosema* plant by the wick method. The alkaloids were extracted after 10 d and purified by preparative TLC. Dicrotaline (28), the major alkaloid was readily separated from the minor alkaloid, acetyldicrotaline (222) : the respective R_F values were 0.64 and 0.81. ^1H NMR, infra-red and mass spectral properties were in accord with the published data[134] for dicrotaline. The acid-base balance of the ^3H and ^{14}C activity was determined by barium hydroxide hydrolysis of the purified dicrotaline samples (isolated weights of the dicrotaline samples ranged from 3.7 - 11.7 mg, representing on average 0.71% of the fresh weight of the plant material).

General Procedure for Kuhn-Roth Oxidations

The following solutions were prepared:

A) Chromium trioxide (168g) in water (1l). 20ml of this solution was mixed with 5 ml conc. sulphuric acid immediately prior to use.

B) 0.01M standard $Ba(OH)_2$

C) 5M sodium hydroxide

D) 50% hydrazine hydrate in water

Method : The purified dicrotaline sample was dissolved in cooled reagent (A) (5ml) and was then heated at 100°C for 0.5 h. The reaction mixture was cooled and excess chromic acid was destroyed by dropwise addition of 50% aq. hydrazine hydrate (D) until the first tinge of green appeared in the solution (ca. 10-11 drops). 5M Sodium hydroxide (C) (5ml) was added and the solution was the just acidified with phosphoric acid (ca. 1 ml). The acetic acid was distilled off in 5 x 10ml portions and titrated against the standard 0.01M barium hydroxide solution (B). After estimation of the acetic acid the neutralised solution was concentrated to 0.5 ml and filtered. The filtrate was reduced to 0.2ml and treated dropwise with cold ethanol until a permanent turbidity was obtained. On standing, the solution deposited barium acetate as a white powder, m.p. > 300°C.

References

1. L.B. Bull, C.C.J. Culvenor, and A.T. Dick, "The Pyrrolizidine Alkaloids", North-Holland, Amsterdam, 1968.
2. A.R. Mattocks, "Chemistry and Toxicology of Pyrrolizidine Alkaloids", Academic Press, 1986.
3. D.J. Robins, *Fortschr. Chem. Org. Naturst.*, 1982, **41**, 115.
4. C.C.J. Culvenor, in "Toxicology in the Tropics", (Eds. R.L. Smith and E.A. Bababunmi), pp. 124-141, Taylor and Francis, London, 1981.
5. C.C.J. Culvenor and L.W. Smith, *Aust. J. Chem.*, 1962, **15**, 328.
6. R.J. Nash, L.E. Fellows, J.V. Dring, G.W.J. Fleet, A.E. Derome, T.A. Hamor, A.M. Scofield, and D.J. Watkin, *Tetrahedron Lett.*, 1988, **29**, 2487.
7. A.A. Forsyth, "British Poisonous Plants", H.M. Stationery Office, London, 1968.
8. F.C. Willmot and G.W. Robertson, *Lancet*, 1920, **2**, 848.
9. O. Mohabbat, R.N. Srivastava, M.S. Younos, G.G. Sediq, A.A. Merzad, and G.N. Aram, *Lancet*, 1976, **2**, 269.
10. M. Rothschild, in "Phytochemical Ecology", (Ed. J.B. Harborne), p.1, Academic Press, London, 1972.
11. J.A. Edgar and C.C.J. Culvenor, *Experientia*, 1975, **31**, 393.
12. see *Chem. Br.*, 1989, **25**, 1192.
13. A.R. Mattocks, *Chem.-Biol. Interact.*, 1972, **5**, 227.
14. M. Tomasz, R.Lipman, D. Chowdary, J. Pawlak, G.L. Verdine, and K. Nakanishi, *Science (Washington, D.C.)*, 1987, **235**, 1204.
15. L.I. Grebennik and Zh.F. Zaharova, *Farmakol. i. Toksikol.*, 1952, **15**, 25.
16. R. Schoental, *Nature*, 1957, **179**, 361.

17. C.C.J. Culvenor, A.T. Dann, and A.T. Dick, *Nature*, 1962, **195**, 570.

18. R. Schoental, *Israel J. Med. Sci.*, 1968, **4**, 1133.

19. A.R. Mattocks, *Toxicology Lett.*, 1982, **14**, 111.

20. L. Letendre, W.A. Smithson, G.S. Gilchrist, E.O. Burgret, C.H. Hoagland, H.H. Ames, G. Powis, and J.S. Kovach, *Cancer*, 1981, **37**, 437.

21. S.V. Anichkov and M.L. Belenky, in "Textbook of Pharmacology", Medgiz, Leningrad, 1955.

22. D.J. Robins, *Nat. Prod. Rep.*, 1984, **1**, 235; 1985, **2**, 213; 1986, **3**, 297; 1987, **4**, 577; 1989, **6**, 221; 1989, **6**, 577.

23. D.J. Robins, *Chem. Soc. Rev.*, 1989, **18**, 375.

24. R. Robinson, "The Structural Relations of Natural Products", The Clarendon Press, Oxford, 1955.

25. E. Nowacki and R.U. Byerrum, *Life Sci.*, 1962, **1**, 157.

26. W. Bottomley and T.A. Geissman, *Phytochemistry*, 1964, **3**, 357.

27. N.M. Bale and D.H.G. Crout, *Phytochemistry*, 1975, **14**, 2617.

28. H. Birecka, M. Birecki, and M.W. Frohlich, *Plant Physiol.*, 1987, **84**, 42.

29. H. Birecka, M. Birecki, E.J. Cohen, A.J. Bitonti, and P.P. McCann, *Plant Physiol.*, 1988, **86**, 224.

30. T. Hartmann, H. Sander, R. Andolph, and G. Toppel, *Planta,* 1988, **175**, 82.

31. D.J. Robins and J.R. Sweeney, *J. Chem. Soc., Chem. Commun.*, 1979, 120; *idem, J. Chem. Soc., Perkin Trans. 1*, 1981, 3083.

32. H.A. Khan and D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 146; *idem, J. Chem. Soc., Perkin Trans. 1*, 1985, 101.

33. D.J. Robins, *J. Chem. Res.(S)*, 1983, 326.

34. H.A. Khan and D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 554.

35. G. Grue-Sorensen and I.D. Spenser, *J. Am. Chem. Soc.*, 1981, **103**, 3208; *idem*, *Can. J. Chem.*, 1982, **60**, 643.

36. R. Kuttan, A.N. Radhakrishnan, T. Spande, and B. Witkop, *Biochemistry*, 1971, **10**, 361.

37. R. Kuttan and A.N. Radhakrishnan, *Biochem. J.*, 1972, **127**, 61.

38. K.S. Srivenugopal and P.R. Adiga, *Biochem. J.*, 1980, **190**, 461.

39. H. Birecka, T.E. DiNolfo, W.B. Martin, and M.W. Frohlich, *Phytochemistry*, 1984, **23**, 991.

40. C. Schopf, E. Schimdt, and W. Braun, *Ber.*, 1931, **64**, 683.

41. N.J. Leonard and S.W. Blum, *J. Am. Chem. Soc.*, 1960, **82**, 503.

42. H.A. Khan and D.J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1985, 819.

43. R.J. Bergeron, P.S. Burton, K.A. McGovern, and S.J. Kline, *Synthesis*, 1981, 732.

44. J. Rana and D.J. Robins, *J. Chem. Res.(S)*, 1983, 146.

45. D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1982, 1289.

46. H.A. Kelly and D.J. Robins, *J. Chem. Soc., Perkin Trans 1*, 1987, 177.

47. E.K. Kunec and D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1986, 250; *idem, J. Chem. Soc., Perkin Trans. 1*, 1989, 1437.

48. M.T. Pizzorno and S.M. Albonico, *J. Org. Chem.*, 1974, **39**, 731.

49. J. Rana and E. Leete, *J. Chem. Soc., Chem. Commun.*, 1985, 1742; E. Leete and J. Rana, *J. Nat. Prod.*, 1986, **49**, 838.

50. H. Birecka and J.L. Catalfamo, *Phytochemistry*, 1982, **21**, 2645.

51. H.A. Kelly and D.J. Robins, unpublished results.

52. H.A. Kelly and D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1988, 329.

53. H.A. Kelly, E.K. Kunec, M. Rodgers, and D.J. Robins, *J. Chem. Res.(S)*, 1989, 358.

54. D.J. Robins and J.R. Sweeney, *Phytochemistry*, 1983, **22**, 457.

55. J. Rana and D.J. Robins, *J. Chem. Soc.,Chem. Commun.*, 1983, 1222.

56. P.S. Callery, M.S.B. Nayar, E.M. Jakubowski, and M. Stogniew, *Experientia*, 1982, **38**, 431; J.C. Richards and I.D. Spenser, *Tetrahedron*, 1983, 3549.

57. R. Bentley, "Molecular Asymmetry in Biology", Vol. 2, Academic Press, New York, 1970.

58. G.R. Orr and S.J. Gould, *Tetrahedron Lett.*, 1982, **23**, 3139; I.D. Wigle, L.J.J. Mestichelli, and I.D. Spenser, *J. Chem. Soc., Chem. Commun.*, 1982, 662; D.J. Robins, *Phytochemistry*, 1983, **22**, 1133.

59. J.C. Richards and I.D. Spenser, *Can. J. Chem.*, 1982, **60**, 2810.

60. A.R. Battersby, J. Staunton, and M.C. Summers, *J. Chem. Soc., Perkin Trans. 1*, 1976, 1052.

61. D. Arigoni and E.L. Eliel, *Top. Stereochem.*, 1969, **4**, 200.

62. E.K. Kunec and D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1985, 1450; *idem, J. Chem. Soc., Perkin Trans. 1*, 1987, 1089.

63. H.A. Kelly and D.J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1987, 2195.

64. M. Rodgers and D.J. Robins, unpublished results.

65. C. Hughes and F.L. Warren, *J. Chem. Soc.*, 1962,34.

66. D.H.G. Crout, M.H. Benn, H. Imaseki, and T.A. Geissman, *Phytochemistry*, 1966, **5**, 1.

67. C.G. Gordon-Gray and F.D. Schlosser, *J. S. Afr. Chem. Inst.*, 1970, **23**, 13.

68. D.H.G. Crout, *J. Chem. Soc.(C)*, 1966, 1968.

69. D.H.G. Crout, *J. Chem. Soc.(C)*, 1967, 1233.

70. W.C. Evans, *Abhandl. Deut. Akad. Wiss. Berlin Kl. Chem. Geol. Biol.*, 1966, **3**, 507.

71. J.G. Wooley, *Abhandl. Deut. Akad. Wiss. Berlin Kl. Chem. Geol. Biol.*, 1966, **3**, 531.

72. E. Leete and J.B. Murrill, *Tetrahedron Lett.*, 1967, 1727.

73. B.A. McGaw and J.G. Wooley, *Phytochemistry*, 1979, **18**, 1647.

74. H.E. Umbarger and E.A. Adelburg, *J. Biol. Chem.*, 1951, **192**, 883.

75. D.H.G. Crout, N.M. Davies, E.H. Smith, and D. Whitehouse, *J. Chem. Soc., Chem. Commun.*, 1970, 635; *idem, J. Chem. Soc., Perkin Trans. 1,* 1972, 671.

76. Tsung-ya-Lo and Yu-Wei Tang, *Chem. Abs.*, 1965, **62**, 15004.

77. N.M. Davies and D.H.G. Crout, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2079.

78. R.L. Kisliuk, W. Sakami, and M.V. Patwardham, *J. Biol. Chem.*, 1956, **221**, 885; W.E. Splittstosser and M. Mazelis, *Phytochemistry*, 1967, **3**, 39.

79. N.M. Bale, R. Cahill, N.M. Davies, M.B. Mitchell, E.H. Smith, and D.H.G. Crout, *J. Chem. Soc., Perkin Trans. 1*, 1978, 101.

80. B. Levenberg, *J. Biol. Chem.*, 1968, **243**, 6009.

81. R. Cahill, D.H.G. Crout, M.B. Mitchell, and U.S. Muller, *J. Chem. Soc., Chem. Commun.*, 1980, 419; R. Cahill, D.H.G. Crout, M.V.M.

Gregorio, M.B. Mitchell, and U.S. Muller, *J. Chem. Soc., Perkin Trans. 1*, 1983, 173.

82. D.J. Robins, N.M. Bale, and D.H.G. Crout, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2082.

83. V.W. Rodwell, in "Metabolic Pathways", (Ed. D.M. Greenberg), Vol.3, Academic Press, New York and London, 1969.

84. J.A. Devlin and D.J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1329.

85. M.L. Rueppel and H. Rapoport, *J. Am. Chem. Soc.*, 1970, **92**, 5528.

86. G.W. Kirby, S.R. Massey, and P. Steinreich, *J. Chem. Soc., Perkin Trans. 1*, 1972, 1642.

87. H. Rapoport, F.R. Stermitz, and D.R. Baker, *J. Am. Chem. Soc.*, 1960, **82**, 2765; A.R. Battersby and B.J.T. Harper, *Tetrahedron Lett.*, 1960, 21.

88. E. Leete, G.B. Bodem, and M.F. Manuel, *Phytochemistry*, 1971, **10**, 2687.

89. N.J. Leonard and F.P. Hauck, Jr., *J. Am. Chem. Soc.*, 1957, **79**, 5279.

90. N.J. Leonard and W.K. Musker, *J. Am. Chem. Soc.*, 1960, **82**, 5148.

91. N.J. Leonard and J.V. Paukstelis, *J. Org. Chem.*, 1963, **28**, 3021.

92. K. Omura and D. Swern, *Tetrahedron*, 1978, **34**, 1651.

93. C. Mannich and H. Davidsen, *Chem. Ber.*, 1936, **69**, 2106.

94. E.P. Blanchard, Jr., *J. Org. Chem.*, 1963, **28**, 1397.

95. W.A. White and H. Weingarten, *J. Org. Chem.*, 1967, **32**, 213.

96. E.P. Kyba, *Org. Prep. Proced.*, 1970, **2**, 149.

97. N.J. Leonard and K. Jann, *J. Am. Chem. Soc.*, 1962, **84**, 4806.

98. A.E. Jackson and R.A.W. Johnstone, *Synthesis*, 1976, 685.

99. A.M. Felix, E.P. Heimer, T.J. Lambros, C. Tzougraki, and J. Meienhofer, *J. Org. Chem.*, 1978, **43**, 4194.

100. T. Bieg and W. Szeja, *Synthesis,* 1985, 76.

101. R. Munier and M. Macheboef, *Bull. Soc. Chim. Biol.*, 1951, **33**, 846.

102. L. Michaelis and M.L. Menten, *Biochem. Z.*, 1913, **49**, 333.

103. A.R. Fersht, "Enzyme Structure and Mechanism", (2nd. Edition), W.H. Freedman and Co., 1985.

104. T.A. Smith, *Biochem. Soc. Trans.*, 1985, **13**, 319.

105. C.W. Tabor and H.Tabor, *Ann. Rev. Biochem.*, 1984, **53**, 749; D.R. Walters, *Biologist,* 1987, **34**, 73.

106. E.J. Gabbay, B.L. Gaffney, and R. Glasser, *Ann. N.Y. Acad. Sci.*, 1970, **171**, 810.

107. Z. Glatz, K. Kovar, L. Macholan, and P. Pec, *Biochem. J.*, 1987, **242**, 603.

108. C. Hartmann and J.P. Klinman, *Biofactors*, 1988, **1**, 41; J.A. Duine and J.A. Jongejan, *Ann. Rev. Biochem.*, 1989, **58**, 403.

109. P. Stoner, *Agents and Actions,* 1985, **17**, 5.

110. A. Cooper, A.M. Equi, S.K. Ner, A.B. Watson, and D.J. Robins, *Tetrahedron Lett.*, 1989, **30**, 5167; A.M. Equi, A.M. Brown, A. Cooper, S.K. Ner, A.B. Watson, and D.J. Robins, *Tetrahedron*, in the Press.

111. B.T. Golding and I.K. Nassereddin, *J. Chem. Res.,* 1981, *(S)*, 342;*(M)*, 3931.

112. J.D. Hamill, A.J. Parr, M.J.C. Rhodes, R.J. Robins, and N.J. Walton, *Biotechnology*, 1987, **5**, 800.

113. J. Rana and D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1984, 517; *idem, J. Chem. Soc., Perkin Trans. 1*, 1986, 983.

114. C.C.J. Culvenor and L.W. Smith, *Aust. J. Chem.*, 1967, **20**, 2499.

115. R. Willstatter and W. Heubner, *Ber. Dtsch. Chem. Ges.*, 1907, **40**, 3869.

116. E.I. Mbadiwe, *Phytochemistry*, 1973, **12**, 2546.

117. S. Mizusaki, Y. Tanabe, M. Noguchi, and E. Tamaki, *Phytochemistry*, 1971, **10**, 1347.

118. A.R. Mattocks and N. Nwude, *Phytochemistry*, 1988, **27**, 3289.

119. B.K. Bachhawat, W.G. Robinson, and M.J. Coon, *J. Biol. Chem.*, 1955, **216**, 727.

120. G. MacKinney, in "Metabolic Pathways", (Ed. D.M. Greenberg), Vol.2, p. 251, Academic Press, New York and London, 1968.

121. M.R. Bendell, D.T. Pegg, D.M. Doddrell, and D.H. Williams, *J. Org. Chem.*, 1982, **47**, 3021.

122. L.M. Harwood, *Aldrichimica Acta*, 1985, **18**, 25.

123. D.D. Perrin, W.L.F. Armarego, and D.R. Perrin, "Purification of Laboratory Chemicals", 2nd. Edition, Permagon Press, Oxford, 1980.

124. D.J. Aberhart, S.J. Gould, H.-J. Lin, T.K. Thiruvengadam, and B.H. Weiller, *J. Am. Chem. Soc.*, 1983, **105**, 5461.

125. F.L.M. Pattison and J.E. Millington, *Can. J. Chem.*, 1956, **34**, 757.

126. J.N. Roitman, *Aust. J. Chem.*, 1983, **36**, 1203.

127. J.M. Hill, *Methods in Enzymology*, 1971, **17B**, 730.

128. One standard unit will oxidise 1.0 μmol of putrescine per hour at 37 °C and pH 7.2.

129. J.J. Sedmak and S.E. Grossberg, *Anal. Biochem.*, 1977, **79**, 544.

130. Dictionary of Organic Compounds, (J. Buckingham, Exec. Ed.), 5th. Edition, 1982, Vol. 5, p. 4982.

131. R. Adams and E.F. Rogers, *J. Am. Chem. Soc.*, 1941, **63**, 537.

132. Dictionary of Organic Compounds, (J. Buckingham, Exec. Ed.), 5th. Edition, 1982, Vol. 3, (a) p. 3151; (b) p. 2527.

133. C.W. Tabor, H. Tabor, and U.Bachrach, *J. Biol. Chem.*, 1964, **239**, 2201.

134. K. Brown, J.A. Devlin, and D.J. Robins, *J. Chem. Soc., Perkin Trans 1*, 1983, 1819.