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BIOSYNTHESIS OF PYRROLIZIDINE ALKALOIDS AND BIO-TRANSFORMATIONS OF DIAMINES

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

by

Jane Reid Matheson

Department of Chemistry,
The University,
Glasgow.

March 1993

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The fundamental fact about the Greek was that he had to use his mind. The ancient priests had said, 'Thus far and no further. We set the limits on thought.' The Greeks said, 'All things are to be examined and called into question. There are no limits on thought.'

Edith Hamilton

A science career for women is now almost as acceptable as being cheerleader.

Myra Barker

ACKNOWLEDGEMENTS

I would like to thank Professor D. J. Robins for his support and guidance throughout the course of this work, and in the presentation of this thesis.

The support of the technical staff of the Chemistry Department is gratefully acknowledged: 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 and Mr G. McCulloch for IR spectra. I would also like to thank Mr A. Burns and his colleagues for all their technical assistance.

I am particularly grateful to Ms I. K. A. Freer for all her work with the transformed root cultures.

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

To my friends in the Henderson Lab, thanks for making the last three years so enjoyable.

A special thank you to Iain for proof reading this thesis and his endless support.

Finally, I want to thank my family and friends, in particular my mum and dad for their support and encouragement throughout my university career. It is to them that I dedicate this thesis.

TABLE OF CONTENTS

PUBLICATIONS

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

"Pyrrolizidine Alkaloids. Stereochemistry of the Enzymic Processes Involved in the Biosynthesis of Otonecine." I. K. A. Freer, J. R. Matheson, M. Rodgers and D. J. Robins, *J. Chem. Res. (S)*, 1991, 46.

"Pyrrolizidine Alkaloids from *Gynura sarmentosa*." J. R. Matheson and D. J. Robins, *Fitoterapia*, 1992, **63**, 557

"*In Vitro* Culture and the Production of Secondary Metabolites in *Emilia flammea*." I. K. A. Freer, J. R. Matheson and D. J. Robins, in Biotechnology of Medicinal and Aromatic Plants, Vol. 26, Ed. Y. P. S. Bajaj, Springer-Verlag, 1993, in Press.

ABBREVIATIONS

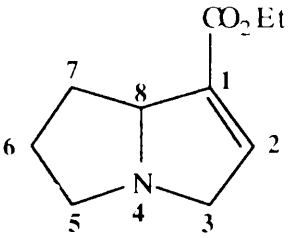
The following abbreviations are used in the text.

ADC	arginine decarboxylase
alk.	alkaloid
ANL	*Aspergillus niger* lipase
b r	broad
conc.	concentrated
CRL	*Candida rugosa* lipase
d	doublet
d. e.	diastereomeric excess
DEPT	Distortionless Enhancement by Polarisation Transfer
DIBAL	diisobutylaluminium hydride
DMAP	*N,N*-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
e. e.	enantiomeric excess
ext.	extract
FMO	Frontier Molecular Orbital
FTIR	Fourier Transform Infrared
HEH	2-hydroxyethylhydrazine
HOMO	Highest Occupied Molecular Orbital
Hz	Hertz
IR	infrared
LUMO	Lowest Unoccupied Molecular Orbital
m	multiplet
MEM	methoxyethoxymethyl
MHz	megahertz
MOM	methoxymethyl
MS	mass spectrum
NMR	nuclear magnetic resonance
ODC	ornithine decarboxylase
PCL	*Pseudomonas cepacia* lipase

PPL	porcine pancreatic lipase
q	quartet
s	singlet
SEM	2-(trimethylsilyl)ethoxymethyl
t	triplet
TBAF	tetra-*n*-butylammonium fluoride
TBDMS	*t*-butyldimethylsilyl
THF	tetrahydrofuran
THP	tetrahydropyranyl
TLC	thin layer chromatography
UK	United Kingdom
USDA	United States Department of Agriculture
wt.	weight

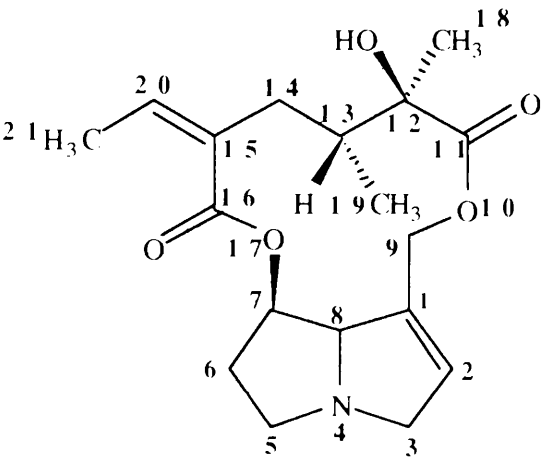
NOTES ON NOMENCLATURE

Pyrrolizidine compounds with one or two double bonds are named in accordance with Chemical Abstracts nomenclature, e.g. 5,6,7,8-tetrahydro-3H-ethylpyrrolizine-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. P. Mose, J. D. Renwick and P. H. Scopes, *J. Chem. Soc. (C)*, 1971, 3653). For example, senecionine is shown.

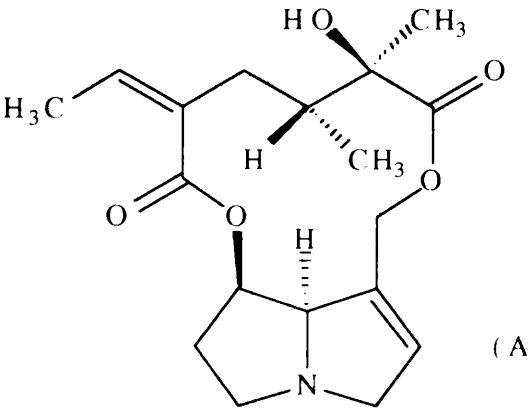


SUMMARY

The work presented in this thesis is divided into four sections: (a) examination of the pyrrolizidine alkaloid content of plants and transformed root cultures; (b) further biosynthetic studies on the pyrrolizidine necine bases; (c) biosynthesis of analogues of pyrrolizidine alkaloids; (d) lipase-catalysed acylation of diamines.

(a) Pyrrolizidine Alkaloid Isolations

Certain species of plant and root culture were examined to see which, if any, alkaloids they produced. *Emilia flammea* transformed root cultures produced the alkaloids senecionine (A), integerrimine (B) and emiline (C).



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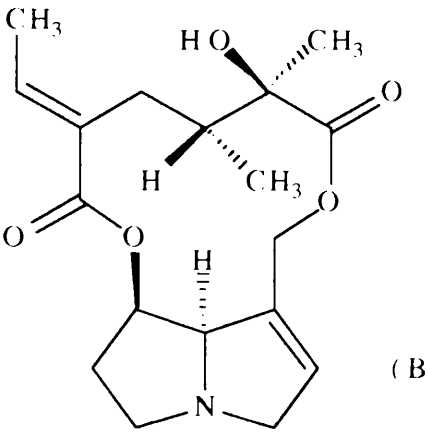

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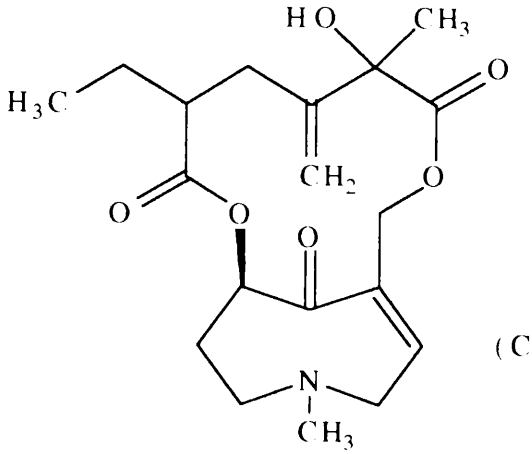
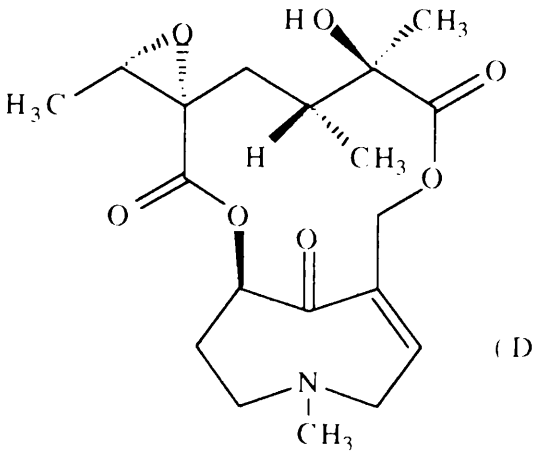


(C)

The proportions of these alkaloids varied with the age of the culture. Senecionine was the predominant alkaloid until the culture was 21 days old when emiline became the major alkaloid. High total incorporations (*ca.* 11%) were observed when [1,4-^{14}C]-1,4-diaminobutane (putrescine) dihydrochloride was fed to the cultures. *Senecio vulgaris* transformed root cultures contained mainly senecionine (A) with a small amount (< 5%) of integerrimine (B). When radiolabelled putrescine was fed total incorporations of about 10% were achieved. Otosenine (D), senkirkine (E) and senecionine (A) were found in the common house plant *Gynura sarmentosa*.



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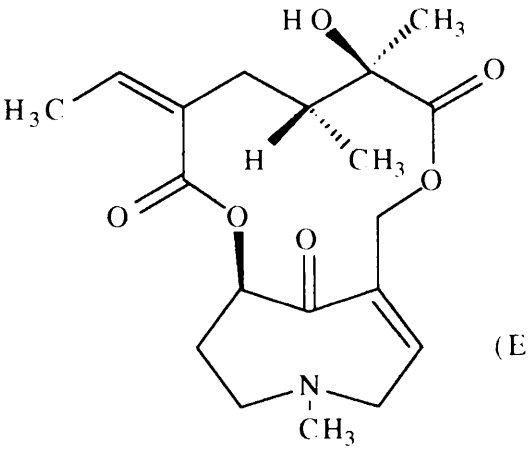
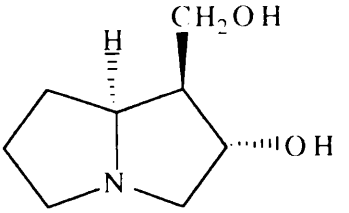


(E)

(b) Biosynthesis of Necine Bases

N-Acetyl[1,4-^{14}C]putrescine hydrochloride was synthesised and fed along with [1,4-^{3}H]putrescine dihydrochloride to *Senecio pleistocephalus* and *Cynoglossum australe* plants, and *E. flammea* and *S. vulgaris* transformed root cultures. In all cases putrescine was incorporated more efficiently than *N*-acetylputrescine into the pyrrolizidine alkaloids.

Efforts were made to synthesise diol (F) which is required to probe the biosynthesis of the pyrrolizidine alkaloid rosmarinine.

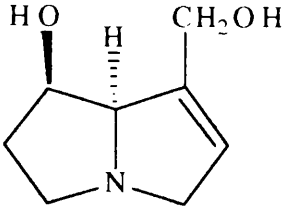


(F)

(c) Biosynthesis of Pyrrolizidine Alkaloid Analogues

The biosynthesis of pyrrolizidine alkaloids in *S. vulgaris* transformed root cultures was shown to be inhibited when 2-hydroxyethylhydrazine was present in the culture medium above a concentration of 1.6 mM. Retronecine (G) and trachelanthamidine (H) were taken up by the inhibited roots

and transformed into senecionine. It was not clear whether the 3β-methyl analogue of trachelanthamidine (I) was converted into an alkaloid analogue.



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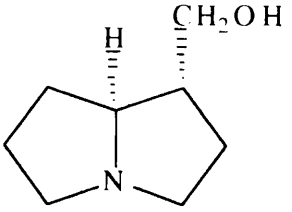

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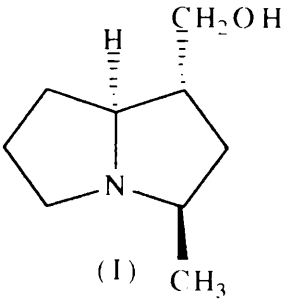


(I)

(d) Lipase-catalysed Acylation of Diamines

Putrescine, cadaverine and 1,2-diaminopropane were monoacetylated using ethyl acetate and porcine pancreatic lipase. The acetylation of 1,2-diaminopropane was regiospecific with *N*-acetyl-1,2-diaminopropane hydrochloride being the product. This reaction showed some stereoselectivity with the R-enantiomer predominating in the product. A number of methods were used to try and improve the stereoselectivity of the lipase-catalysed acylation of 1,2-diaminopropane.

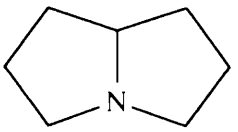
CHAPTER 1

Introduction

1.1 Pyrrolizidine Alkaloids

The pyrrolizidine alkaloids are a group of secondary metabolites produced by certain higher plants. It is estimated that 3% of all flowering plants may contain these alkaloids. To date, in excess of 300 plant species have been found to contain pyrrolizidine alkaloids and from these species over 200 different alkaloids have been isolated and identified.[1,2]

Pyrrolizidine alkaloids were first discovered in *Senecio* plant species (family Asteraceae, formerly Compositae) and were hence named the *Senecio* alkaloids.[3] Since then these alkaloids have been found in a number of other, unrelated, plant families, e.g. Boraginaceae and Fabaceae (formerly Leguminosae).[2,4] The alkaloids were then renamed after the pyrrolizidine ring system (1) which is the basis of their structure.



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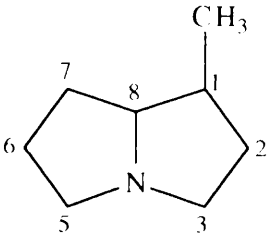

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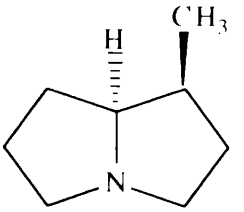
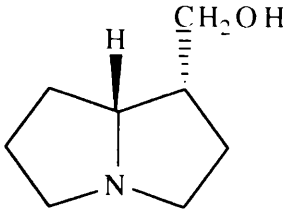


(3)

Most pyrrolizidine alkaloids are derivatives of 1-methylpyrrolizidine (2) rather than of pyrrolizidine (1) itself, e.g. heliotridane (3). The ester pyrrolizidine alkaloids consist of a base component and an acid component. Hydroxylated derivatives of 1-methylpyrrolizidine form the base. This amino alcohol moiety is known as the necine and it can be either saturated or unsaturated. The unsaturation is typically at the 1,2-positions of the necine.

Figure 1.1: Some saturated pyrrolizidine bases



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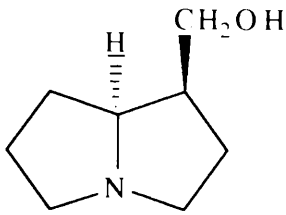

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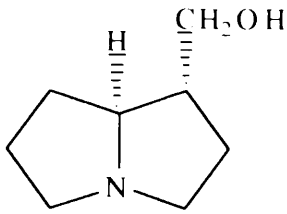

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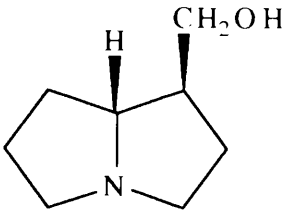

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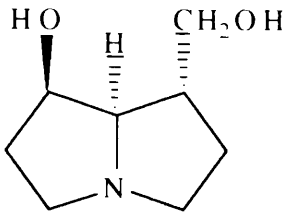

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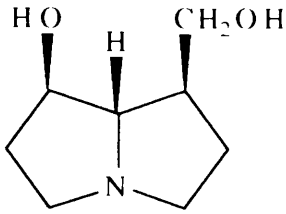

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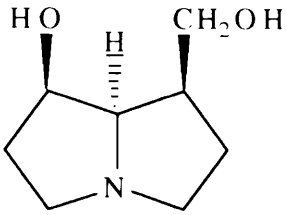

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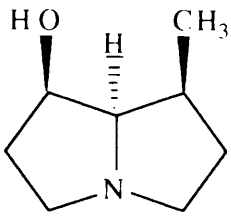

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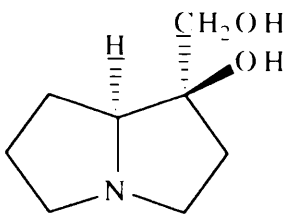

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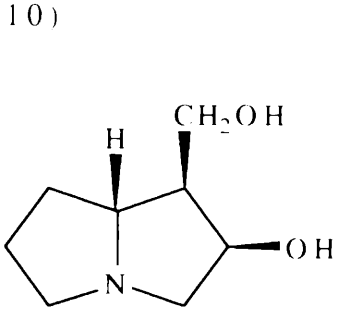

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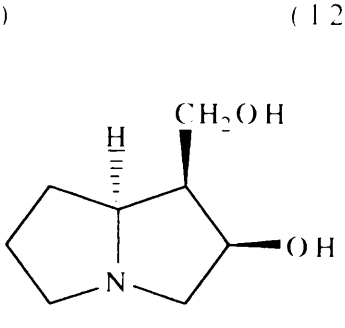

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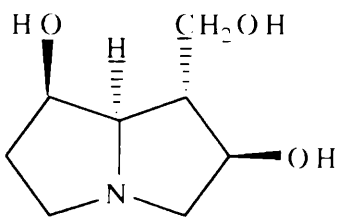

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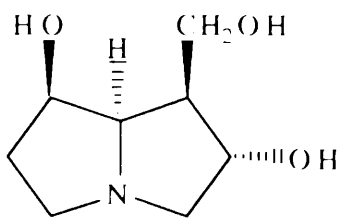
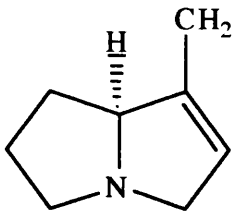


(16)

A good example is retronecine (18) which is the most common necine. A selection of necines is shown in Figures 1.1 and 1.2.

Figure 1.2 : Some unsaturated pyrrolizidine bases



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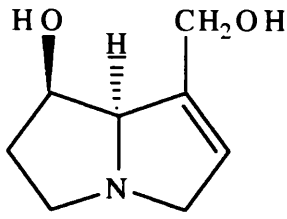

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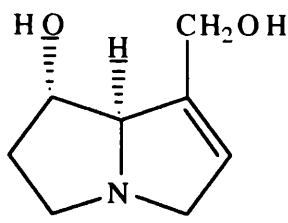

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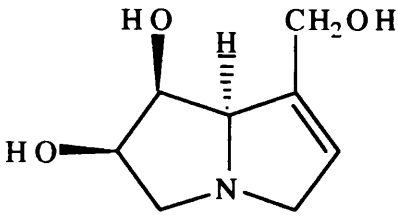

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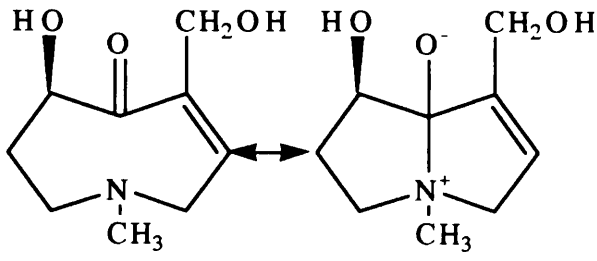
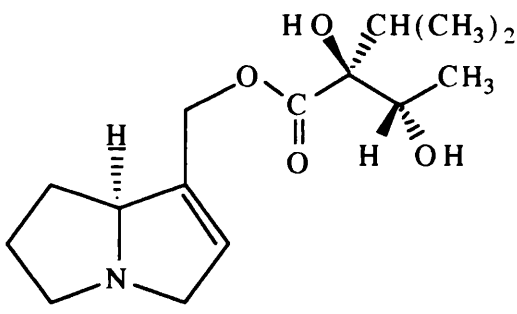


(21)

The esterifying acids, called necic acids, have unusual structures. They contain five to ten carbon atoms and differ in the degree of chain branching, hydroxylation and unsaturation. The alkaloids usually occur as monoesters such as viridiflorine (22), diesters such as symlandine (23), or macrocyclic diesters such as retrorsine (24).



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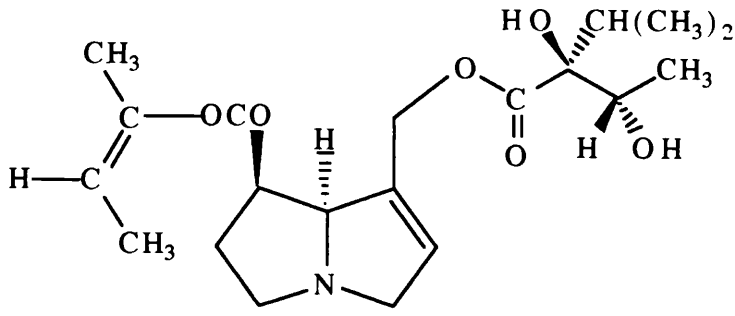

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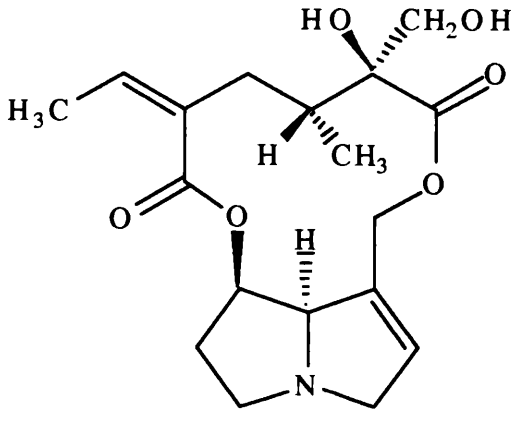


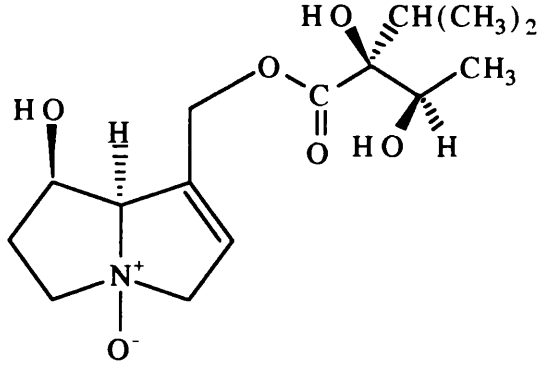
(24)

Pyrrolizidine alkaloids are of great importance because of the wide spectrum of biological action that they display.[5] Alkaloids which contain 1,2-unsaturation in the necine are hepatotoxic.[5] Ingestion of plants containing these alkaloids by livestock and by people is a major health problem. For instance, *Senecio jacobaea* (ragwort), a plant which is abundant in the U.K. is said to cause more livestock losses than all other poisonous plants put together.[6] Many herbal remedies are made from these plants, e.g. comfrey (*Symphytum* species - Boraginaceae) and are known to contribute towards human liver disease. The toxicity of these alkaloids is discussed in further detail later.

Some species of Lepidoptera use pyrrolizidine alkaloids to their advantage. They feed on plants which produce pyrrolizidine alkaloids and store the alkaloids as a defence against potential predators.[7] The same is true of some types of

aphids and ladybird beetles.[8] Certain species of butterflies and moths convert the necine part of the alkaloid into volatile ketones which act as pheromones.[9]

One of the most interesting biological actions that some pyrrolizidine alkaloids display is antitumour activity. The best known example is indicine *N*-oxide (25) which has undergone clinical trials in the USA.[10] In addition, this alkaloid shows less of the usual pyrrolizidine alkaloid toxicity.



(25)

More information on the structures, sources, chemistry and toxicology of pyrrolizidine alkaloids can be found in the books by Mattocks,[5] and Bull *et al.*,[11] and in annual reviews.[2]

1.2 Metabolism and Cytotoxicity

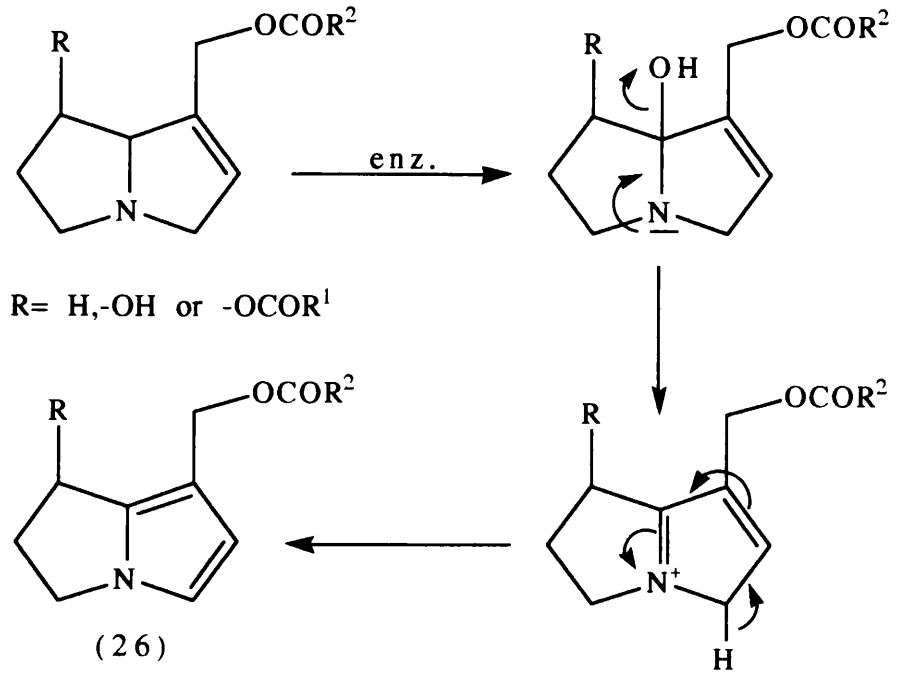
There is a close link between the cytotoxic activity of pyrrolizidine alkaloids and their metabolism. A number of facts point to the metabolites, rather than the alkaloids themselves, being responsible for the toxic action. For instance, the main organ damaged, regardless of the site of administration, is the liver.[12] The liver is where most pyrrolizidine alkaloid metabolism occurs.

The main routes by which the alkaloids are metabolised are ester hydrolysis, *N*-oxidation and dehydrogenation. Hydrolysis and *N*-oxidation are detoxification routes which produce species that are more water soluble and can therefore

be excreted in the urine. Dehydrogenation is the pathway associated with cytotoxicity.

Hepatic microsomal enzymes convert unsaturated pyrrolizidine alkaloids into pyrrolic derivatives such as (26). Cytochrome P450 is the major enzyme catalysing pyrrole formation and *N*-oxide formation of senecionine in human liver.[13] Pyrrole formation probably proceeds via oxidation of a carbon adjacent to the nitrogen.[14] Overall oxidation would be expected to result from hydroxylation at either C-3[15] or C-8[16] of the unsaturated necine moiety followed by the loss of the elements of water. Evidence supports oxidation at C-8. A probable mechanism for metabolic dehydrogenation of an unsaturated pyrrolizidine alkaloid is shown in Scheme 1.1.[1]

Scheme 1.1

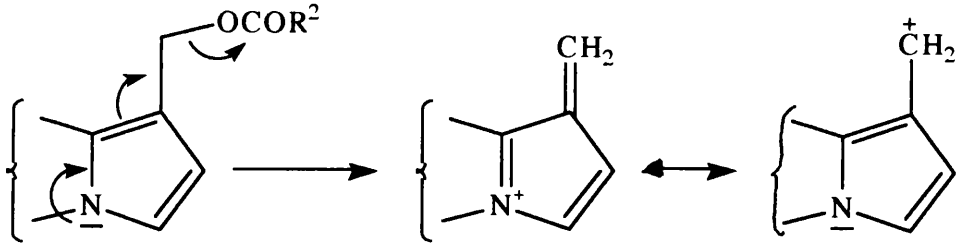


(26)

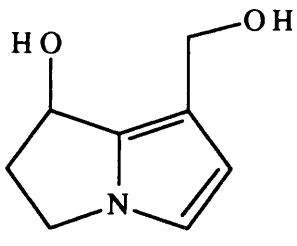
A great deal of evidence exists linking these pyrrolic metabolites with the toxic action. For example, Mattocks has shown that there is a direct relationship between the amounts of pyrrolic metabolites found in rat livers and acute

hepatotoxicity.[17] The toxic actions of the pyrrole metabolites are associated with their chemical reactivity. This reactivity is due to the oxygen function being activated by its relationship to nitrogen (Scheme 1.2).[18] This enables these pyrroles to alkylate tissue constituents.

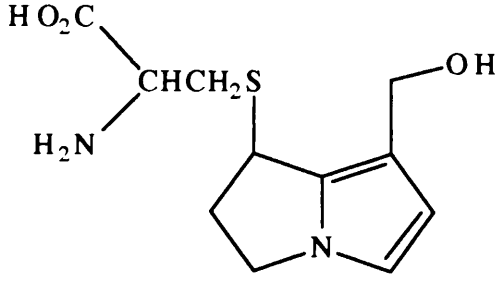
Scheme 1.2



Pyrrolic metabolites formed from pyrrolizidine alkaloids can have one or two reactive (i.e. easily displaced) ester groups or one ester group plus one less reactive hydroxyl group. These pyrroles can therefore act as mono- or bifunctional alkylating agents. Dehydroretronecine (27) has been shown to react with the thiol group of cysteine to produce a covalent adduct (28).[19]



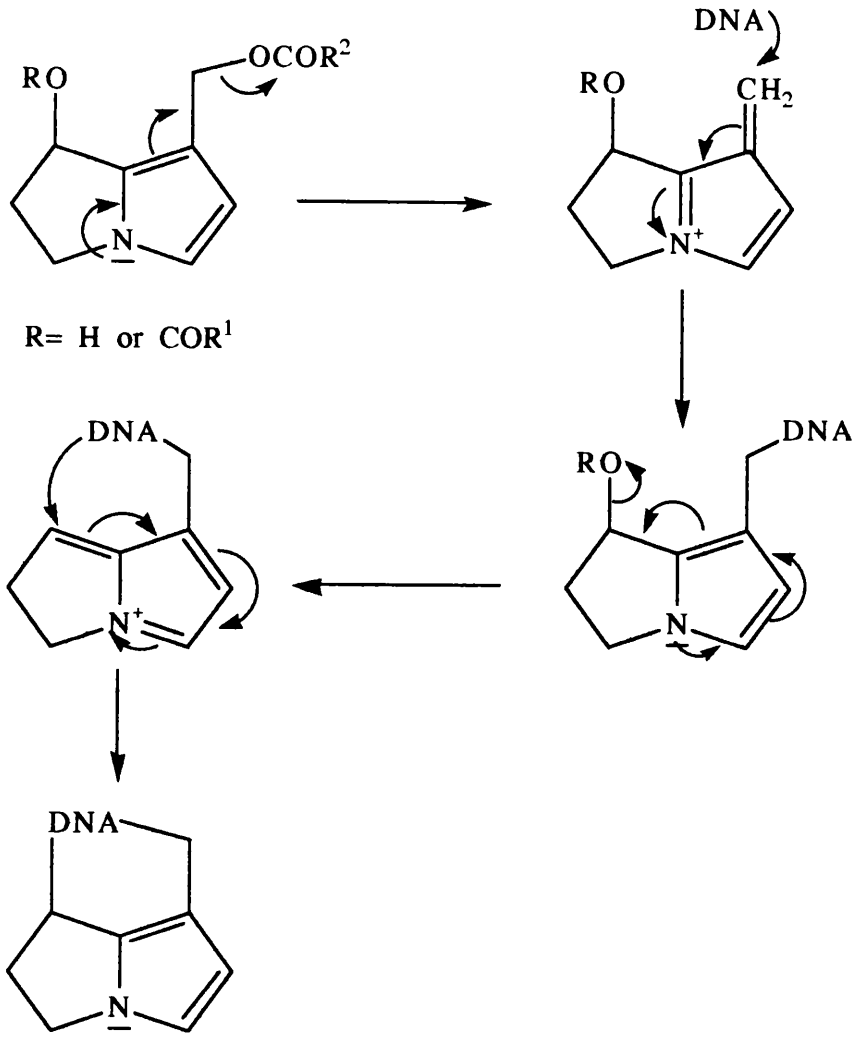
(27)



(28)

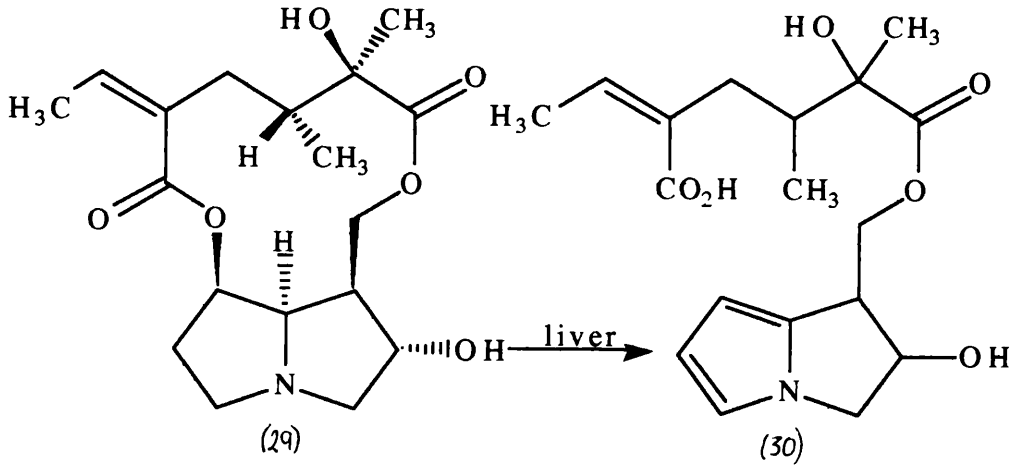
Bifunctional pyrroles can crosslink macromolecules such as DNA.[20] A mechanism for crosslinkage is shown in Scheme 1.3. The nucleophiles on DNA are likely to be nucleophilic nitrogens on the bases of DNA (e.g. N-7 of guanine).

Scheme 1.3



Pyrrolizidine alkaloids which possess a saturated necine are not cytotoxic. These alkaloids are also metabolised to pyrrolic materials but these differ from those formed by hepatotoxic pyrrolizidine alkaloids.[21] Mattocks and White investigated the structure of the pyrrolic metabolites formed from rosmarinine (29).[15,22] In rosmarinine, which contains a saturated necine, it is the left-hand ring that is dehydrated and dehydrogenated to form a pyrrole, and not the right-hand one as with hepatotoxic pyrrolizidine alkaloids. In the metabolite (30) formed, the ester group cannot be activated by the

nitrogen and so this metabolite cannot act as an alkylating agent and the alkaloid is therefore not cytotoxic.



(29) (30)

1.3 Structure Activity Relationships

The toxicity of pyrrolizidine alkaloids is closely linked to their structure. The first structural feature ascertained to be necessary for hepatotoxicity was 1,2-unsaturation in the necine. Schoental proposed this after noticing that all known hepatotoxic pyrrolizidine alkaloids were esters of supinidine (17), retronecine (18) or heliotridine (19).[23]

Toxicity is affected by the lipophilicity and base strength of pyrrolizidine alkaloids.[5] Alkaloids with a high lipophilicity are more susceptible to oxidation by hepatic microsomal enzymes. Alkaloids of higher base strength are less lipophilic and hence usually less toxic. This decreased lipophilicity is due to a larger proportion of the alkaloid being protonated at physiological pH.

Ester hydrolysis and *N*-oxidation are the two major detoxification pathways. Ester hydrolysis produces an aminoalcohol which because of its water solubility is not hepatotoxic. Hydrolysis is reduced if access to the ester groups is restricted. For example, in esters which have bulky substituents at the α-carbon of the acid, hydrolysis is decreased because of steric hindrance.[24,25] With α,β-

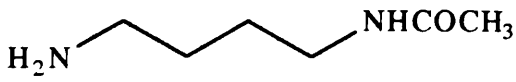
unsaturated esters and macrocyclic diesters hydrolysis is reduced because the greater rigidity of the necic acid again hinders access to the ester linkage. Hence, macrocyclic diesters are usually more toxic than similar 'open' diesters.

The relative amounts of pyrrolizidine alkaloid *N*-oxide and pyrrolic metabolite produced also depends on the type of ester present. The greatest amount of *N*-oxide is produced with 'open' diesters because they give the most steric hindrance at C-8 of the necine moiety but have little effect at the nitrogen or C-3.[16] It is because the ester has a large influence on the *N*-oxide: pyrrole ratio that it is proposed that pyrrole formation occurs via oxidation at C-8 of the necine and not C-3.

1.4 Aims of Project

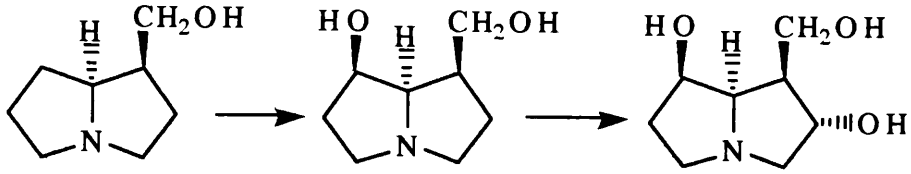
The examination of new plant species to determine if they contain pyrrolizidine alkaloids is of great importance. *Gynura sarmentosa* DC. and transformed root cultures of *Emilia flammea* and *Senecio vulgaris* were investigated for pyrrolizidine alkaloid content and the results are given in Chapter 3. The root cultures were also examined for their suitability as systems for use in biosynthetic studies.

The biosynthesis of pyrrolizidine alkaloids has stimulated much interest in our research group.[1] This work, and the work of others, is reviewed in Chapter 2. Further studies on the biosynthesis of the pyrrolizidine bases are described in Chapter 4. The possible role of *N*-acetylputrescine (31) in pyrrolizidine alkaloid biosynthesis was investigated in *Senecio pleistocephalus* S. Moore and *Cynoglossum australe* R.Br. plants, and in *E. flammea* and *S. vulgaris* transformed root cultures.

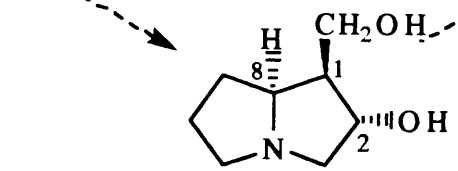


(31)

The order of oxidation in the transformation of isoretronecanol (5) into rosmarinecine (16) has not been firmly established, although platynecine (10) is an efficient precursor of rosmarinecine. The other diol that might lie on the biosynthetic pathway is (32). Efforts have been made towards the synthesis of this diol in labelled form.



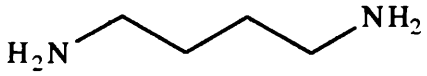
(5) (10) (16)



(32)

Analogues of pyrrolizidine alkaloids are useful for the study of biological pathways and may also show biological activity. The use of transformed root cultures of *S. vulgaris* to produce these analogues is investigated in Chapter 5.

N-Acetylputrescine (31) was synthesized from putrescine (33) using porcine pancreatic lipase (PPL) and ethyl acetate.



(33)

The use of lipases to monoacetylate diamines was thought worthy of further investigation. The results, which examine questions about regioselectivity and stereoselectivity, are given in Chapter 6.

CHAPTER 2

Biosynthesis of Pyrrolizidine Alkaloids

2.1 Introduction

The biosynthesis of pyrrolizidine alkaloids has been the subject of much study for over thirty years. In this time many of the intermediates have been determined and much of the pathway, including the stereochemistry of some of the enzyme processes, has been elucidated.[1]

The means of studying alkaloid biosynthesis, and indeed the formation of any natural product, is through the feeding of potential precursors that are suitably labelled, to plants that produce alkaloids.

Originally the only label that was readily available for such studies was ^{14}C, the radioactive isotope of carbon with a half life of 5 770 years. This was followed by tritium (^{3}H) labelling, which was used both on its own and in conjunction with ^{14}C. The feeding of two different precursors, one labelled with ^{14}C and the other with ^{3}H, allows for the determination of the relative incorporation of one precursor with respect to the other. These radiolabels are detected by liquid scintillation counting. The total incorporation of a radioactive precursor is defined as:- total activity in the isolated natural product/ total activity in the administered precursor x 100%. The advantage of using radioactive isotopes is the sensitivity of the technique, in that small incorporations can be observed.

The major problem with radiolabelling is that once a radioactive natural product has been formed, the location of the radioactive isotope has to be determined. This process of degradation can be long and involved and mistakes can be made.

In more recent times the use of stable isotopes (^{13}C, ^{15}N, ^{2}H) has come to the fore. These labels can be detected by mass spectrometry and by NMR spectroscopy. ^{13}C is magnetic with a

spin of 1/2 and has a natural abundance of 1.11%. It is this abundance that allows ^{13}C NMR spectra to be obtained with Fourier-transform spectrometers. If precursors have been enriched in ^{13}C at a particular site this can be detected in the ultimate natural product by enhancement of the ^{13}C-NMR signal of that specifically labelled atom. Because of variability in the intensity of the ^{13}C NMR peaks, to be sure of observing an enrichment of a certain carbon signal, the percentage specific incorporation must be greater than 0.5%.

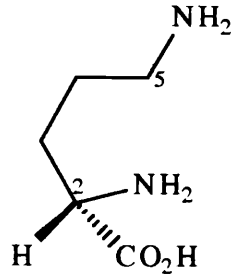
This problem can be reduced by the feeding of precursors labelled with contiguous ^{13}C atoms. The natural abundance of adjacent ^{13}C is only 1.11 x 1.11% = 0.0123% i.e. negligible. In sample enriched with ^{13}C at adjacent positions the ^{13}C atoms couple to each other giving rise to satellite peaks of J = 30-80 Hz in the ^{13}C NMR spectrum, located about the central natural abundance signal.

Precursors can also be synthesized with adjacent ^{13}C and ^{15}N atoms. ^{15}N is magnetic with spin 1/2 and therefore ^{13}C-^{15}N spin-spin coupling, J = 0-20 Hz, can be observed around the ^{13}C natural abundance signals in the ^{13}C NMR spectrum.

Deuterium (^{2}H) NMR has been used to examine details of the stereochemistry of biosynthetic mechanisms. The signals obtained in ^{2}H NMR spectra have the same chemical shifts as those observed with ^{1}H NMR spectra but are usually broader. The natural abundance of ^{2}H is very low (0.0156%) and therefore small specific incorporations of deuterium-labelled precursors can be detected by ^{2}H NMR spectroscopy.

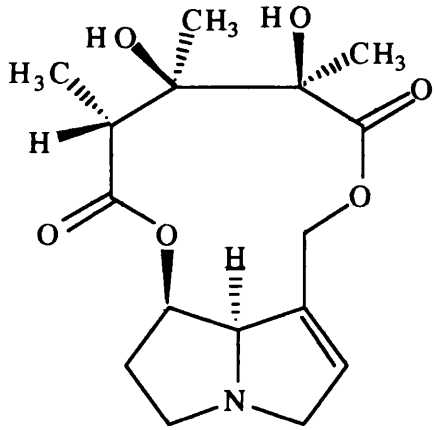
2.2 Biosynthesis of Pyrrolizidine Alkaloid Necine Bases

It was Sir Robert Robinson who postulated that the 1-methylpyrrolizidine skeleton was derived from two ornithine (34) molecules.[26] This postulate was confirmed by early biosynthetic work using radiolabelled precursors.[27,28]



(34)

The first investigation was carried out by Nowacki and Byerrum in 1962.[27] They fed [2-^{14}C]ornithine, [1-^{14}C]propionate and [1-^{14}C]acetate to *Crotalaria spectabilis*. The monocrotaline (35) isolated was hydrolysed with barium hydroxide to give retronecine (18) and monocrotalic acid. [2-^{14}C]Ornithine was incorporated almost totally into retronecine whereas radiolabelled propionate and acetate were incorporated almost exclusively into the necic acid.



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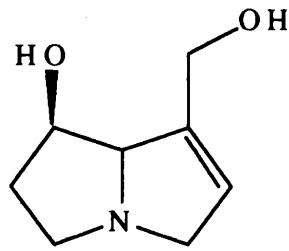
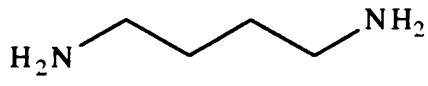


(18)

This was followed in 1964 by two studies that were partially contradictory in their content.[28,29] Bottomley and Geissman[28] examined the incorporation of [1,4-^{14}C]putrescine (33), [2-^{14}C]ornithine and [5-^{14}C]ornithine into the alkaloids of *Senecio douglasii*.



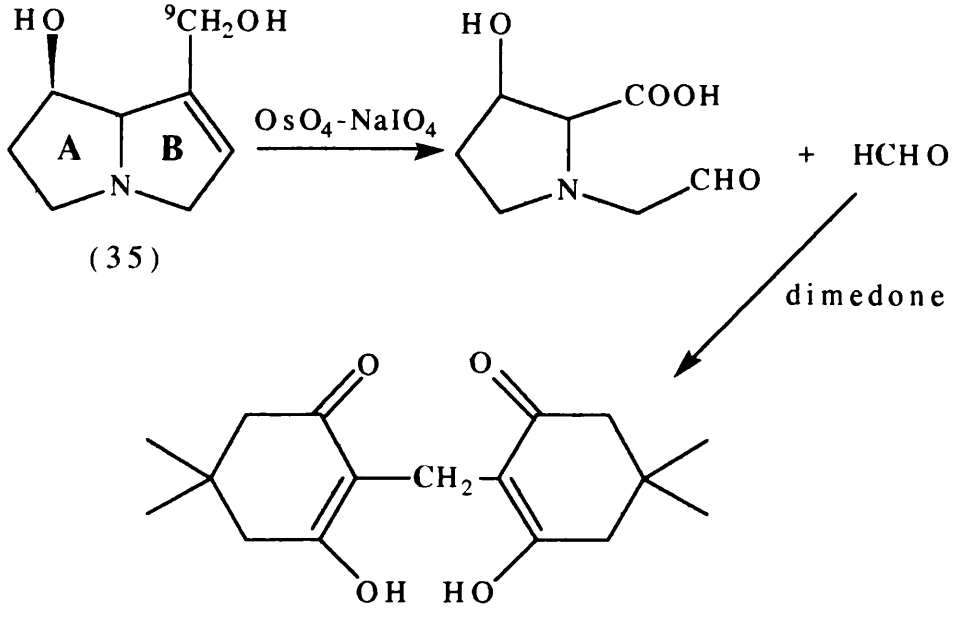
(33)

These alkaloids are all cyclic esters of C_{10}-dicarboxylic acids with the base retronecine (18). Basic hydrolysis of the alkaloid mixture gave retronecine which contained nearly all the total activity in all three cases (Table 2.1).

Table 2.1 : Incorporation of radioactive precursors into the alkaloids of *S. douglasii.*

Precursor	Total Inc (%)	% of Total activity of alkaloid found in acids , retronecine		% of necine activity in C-9
[1,4-^{14}C]-putrescine	0.18	5.0	98	25
[2-^{14}C]-ornthine	0.30	1.4	94	24
[5-^{14}C]-ornithine	0.75	2.4	94	25

The labelled retronecine was partially degraded by treatment with osmium tetroxide followed by sodium periodate. **Scheme 2.1**

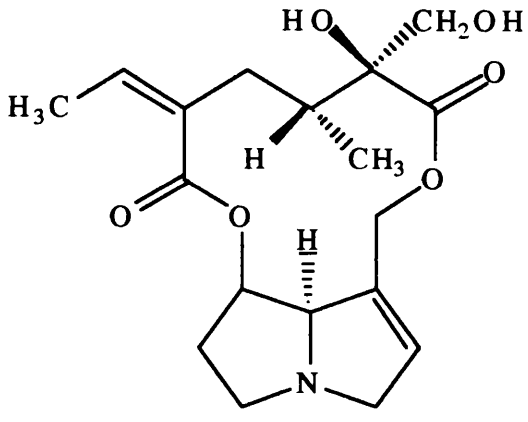


(35)

(36)

This converted C-9 of retronecine into formaldehyde which was isolated as its dimedone derivative (36) (Scheme 2.1). In each case, one quarter of the activity in retronecine was found in the side-chain carbon atom (Table 2.1). These results indicated that in the biosynthesis of ring B of retronecine, C-2 and C-5 of ornithine become equivalent probably by the formation of putrescine (33).

Hughes *et al.*[29] fed [2-^{14}C]ornithine to *Senecio isatideus* to give retrorsine (24) with a total incorporation of 0.98%. Retrorsine was hydrogenolysed to retronecanol (11) which contained 97% of the total activity.



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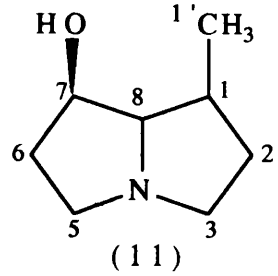
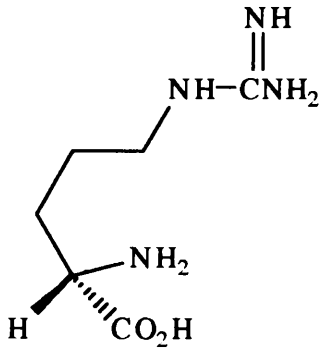


(11)

A complex series of degradations on retronecanol indicated that 26% of the activity was at C-1'. This was in agreement with the previous results.[27,28] However 71% of the activity was found at C-7 and C-8. This would indicate that no radiolabel was present at C-3 or C-5 which could not be the case if an ornithine molecule forms a symmetrical intermediate to form ring B of retronecine. This contradicted the results of Bottomley and Geissman.[28] The result of Hughes *et al.*[29] was negated by Grue-Sorensen and Spenser[30] who suggested that this erroneous result had its basis in the chemistry of the degradation reactions.

The first use of a double isotope (^{3}H /^{14}C) technique in this field came from Bale and Crout.[31] Using L-[3-^{3}H]arginine as standard, they showed that in *Senecio magnificus* arginine (37)

and ornithine (34) are both specifically incorporated into retronecine, the base portion of senecionine (38). When a mixture of L-[U-^{14}C]arginine and L-[3-^{3}H]arginine was fed the ^{3}H /^{14}C ratio fell from 3.0 in the precursor mixture to 2.7 in the isolated senecionine. In the case where L-[U-^{14}C]ornithine and L-[3-^{3}H]arginine were fed the ^{3}H /^{14}C ratio dropped from 11.5 to 5.5. Hence, in the biosynthesis of senecionine in *S. magnificus*, both arginine and ornithine are incorporated with ornithine being the more efficient precursor.



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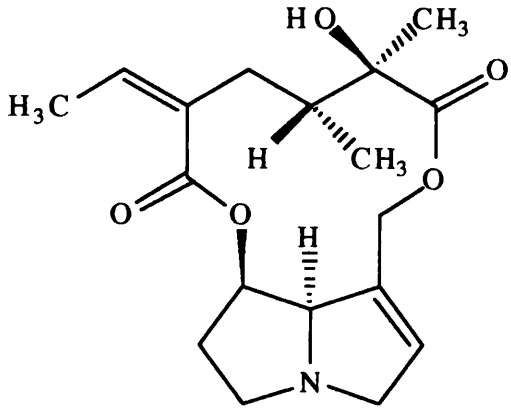
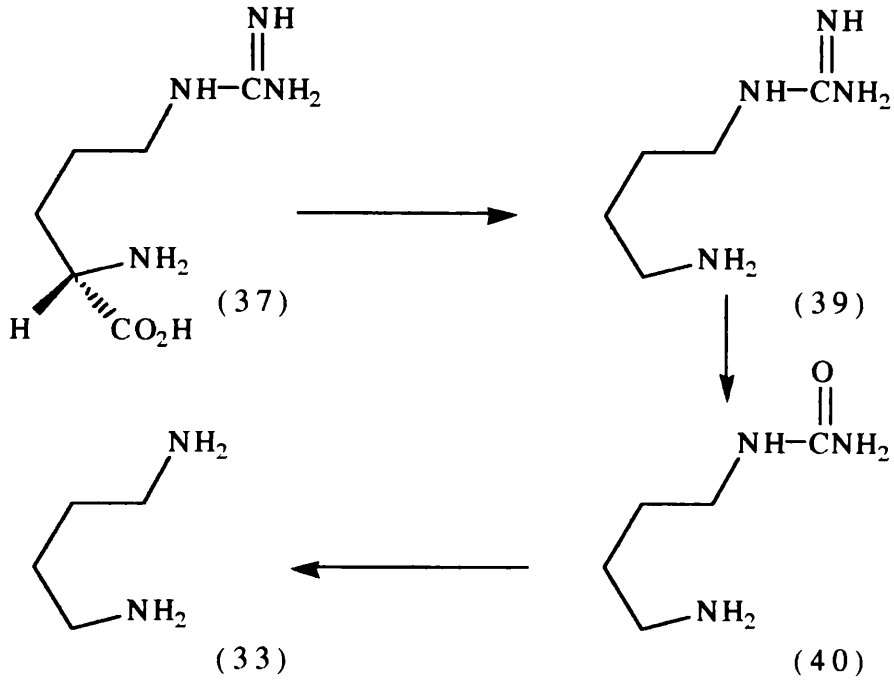


(38)

Different groups of workers have contrasting views on whether arginine (37) and/or ornithine (34) are utilised in the formation of putrescine for necine biosynthesis. Birecka *et al.*[32] exposed shoots of *Heliotropium* spp. to ^{14}C-labelled CO_2 achieving an incorporation into retronecine of 0.15%. Treatment of the shoots with a specific ornithine decarboxylase inhibitor had no effect on ^{14}C incorporation into the necine. However, the use of a specific arginine decarboxylase inhibitor prevented ^{14}C incorporation. Thus, arginine was the only endogenous precursor of putrescine channelled into pyrrolizidine alkaloid biosynthesis in *Heliotropium* spp. However, exogeneous ornithine and arginine were incorporated into retronecine. When these workers carried out a similar study on certain *Senecio* and *Crotalaria* species, ornithine was discovered to be the only precursor of putrescine converted into retronecine.[33]

On the other hand, Hartmann and co-workers[34] established that the incorporation of [^{14}C]arginine and [^{14}C]ornithine into senecionine (38) from *S. vulgaris* root cultures was completely prevented by the presence of a specific inhibitor of arginine decarboxylase. A specific ornithine decarboxylase inhibitor had no effect on the flow of radioactivity into the alkaloids. They therefore postulated that putrescine for necine biosynthesis was derived exclusively from arginine. Ornithine was rapidly converted into arginine in the roots. Agmatine (39), the decarboxylation product of arginine was incorporated into senecionine with the same efficiency as putrescine (33). Agmatine is probably converted into putrescine *via* *N*-carbamoylputrescine (40). This pathway is well characterised in several higher plants (Scheme 2.2).[35]

Scheme 2.2



The ^{3}H /^{14}C double-label strategy was also used by Robins and Sweeney[36] to study the relative incorporations of D-, L- and DL-isomers of [^{14}C]arginine and [^{14}C]ornithine into retrorsine (24) from *Senecio isatideus*, using L-[5-^{3}H]arginine

as internal standard. Retronecine (18) was shown to be derived from L-arginine and L-ornithine.

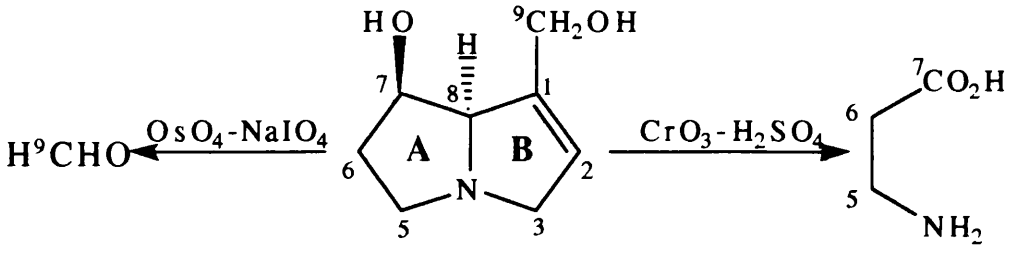
In an attempt to improve the total incorporation figures, Robins and Sweeney[37] examined a wide variety of methods for the feeding of precursors to *S. isatideus*. The best method was to feed the precursor as an aqueous solution into the xylems of plants in soil, through stem punctures. Various ^{14}C-labelled precursors were fed by this method, along with L-[5-^{3}H]arginine as reference, to *S. isatideus*.[37] [1,4-^{14}C]Putrescine, [1,4-^{14}C-tetramethylene]spermine (41) and [1,4-^{14}C-tetramethylene]spermidine (42) were all efficient precursors for retrorsine (24) in *S. isatideus* with total incorporations ranging from 1.6% to 5.2%.

$$H_2N(CH_2)_3NH^{14}CH_2(CH_2)_2{}^{14}CH_2NHR$$

(41) R = $(CH_2)_3NH_2$
(42) R = H

These were all significantly higher than those obtained previously.[28] Spermidine and spermine are probably utilised *via* putrescine (33), and these three precursors were incorporated into retrorsine about ten times more efficiently than L-[U-^{14}C]arginine and DL-[5-^{14}C]ornithine. ^{14}C-Labelled retronecine, which was obtained by the basic hydrolysis of retrorsine, was degraded in the following manner (Scheme 2.3).

Scheme 2.3

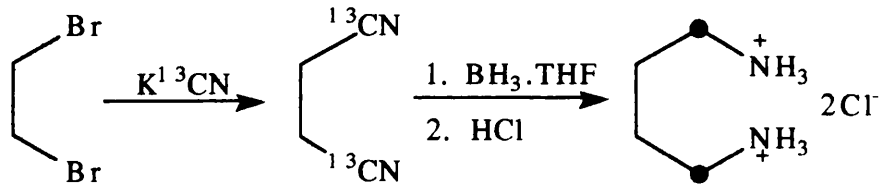


(18) (43)

As before,[28] formaldehyde was obtained by the oxidative cleavage of retronecine to give C-9. For all the precursors mentioned, one quarter of the total ^{14}C base activity was located at C-9. This was in agreement with previous results.[28,29] A fragment of ring A of the necine base was produced for analysis by a modified Kuhn-Roth oxidation of retronecine. This led to the production of β-alanine (43) which was isolated as its 2,4-dinitrophenyl derivative and corresponds to C-(5 + 6 + 7) of retronecine. In every case *ca.* 25% of the total ^{14}C base activity was found in this fragment. These results supported the view of Geissman and Crout[38] that retronecine (18) is derived from two molecules of putrescine (33) formed from ornithine (34) and/or arginine (37).

The improved incorporations obtained[37] brought in the possibility of using ^{13}C-labelled precursors. Accordingly, [1,4-$^{13}C_2$]putrescine (46) dihydrochloride was synthesized by Khan and Robins[39] as shown in Scheme 2.4. The ^{13}C label was introduced by S_N2 displacement of bromide from 1,2-dibromoethane (44) with potassium [^{13}C]cyanide. Reduction of the resultant [1,4-$^{13}C_2$]succinonitrile (45) with borane in tetrahydrofuran (THF) gave [1,4-$^{13}C_2$]putrescine (46) which was isolated as its dihydrochloride salt. [2,3-$^{13}C_2$]Putrescine dihydrochloride (47) was produced in a similar manner starting from [1,2-$^{13}C_2$]-1,2-dibromoethane (50).[39]

Scheme 2.4

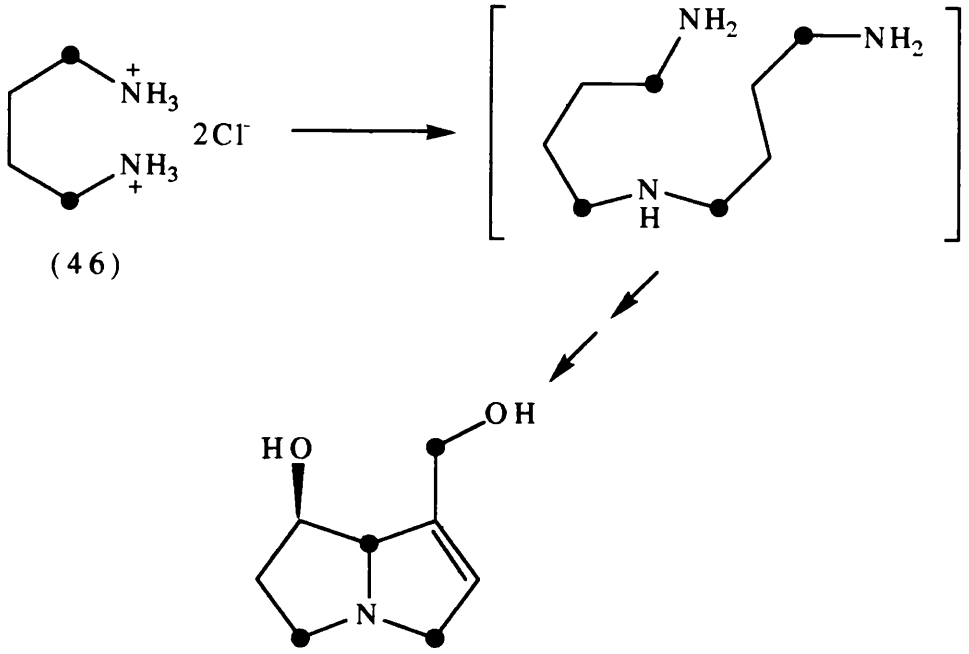


(44) (45) (46)

Precursors (46) and (47) were fed separately by the xylem pricking method[37] to *S. isatideus* plants.[39] The isolated ^{13}C-labelled retrorsine samples were hydrolysed to give

retronecine samples which were examined by ^{13}C NMR spectroscopy.

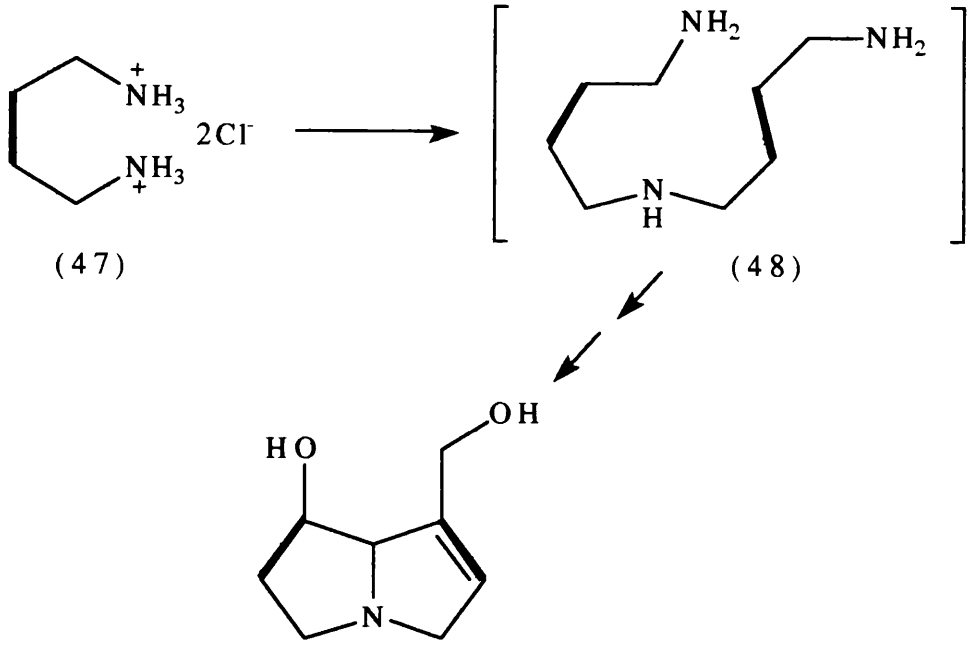
Scheme 2.5



(46)

Retronecine derived from [1,4-$^{13}C_2$]putrescine (46) showed nearly equal enrichment of the ^{13}C NMR signals corresponding to C-3, C-5, C-8 and C-9 (Scheme 2.5). Considerable broadening of these signals was observed due to for example, ^{13}C-N-^{13}C coupling from C-5 to C-8, arising from combination of two molecules of ^{13}C-labelled putrescine in the formation of retronecine. The ^{13}C NMR spectrum of retronecine derived from the feeding of [2,3-$^{13}C_2$]putrescine (47) showed a pair of doublets straddling the natural abundance signals for C-6 and C-7 (J 34 Hz) and a pair of doublets corresponding to C-1 and C-2 (J 71 Hz) (Scheme 2.6). All four labelled sites in the ^{13}C-labelled retronecine showed almost equal enrichment. These findings suggested that two molecules of putrescine (33) combine together to form a symmetrical C_4-N-C_4 intermediate, such as (48), which is then converted into retronecine.

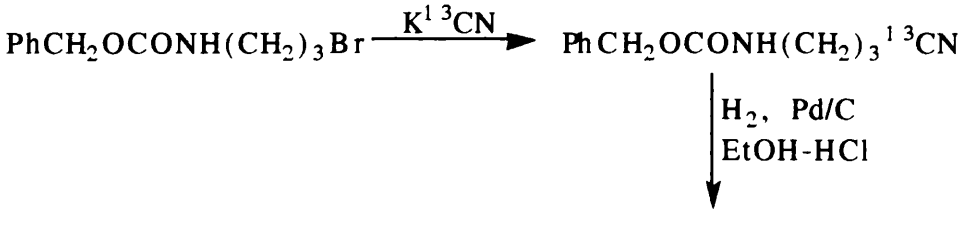
Scheme 2.6



(47) (48)

In order to reduce the extra couplings observed in the ^{13}C NMR spectrum of retronecine from the [1,4-$^{13}C_2$]putrescine feed, [1-^{13}C]putrescine (49) was synthesized as outlined in Scheme 2.7.[39] As expected, the ^{13}C NMR spectrum of the retronecine produced showed four equally enriched carbon signals corresponding to C-3, C-5, C-8 and C-9 of retronecine.

Scheme 2.7



(49)

In another report, Robins synthesized and fed [1,2-$^{13}C_2$]putrescine dihydrochloride (54) to *S. isatideus* plants (Scheme 2.8).[40] The starting point for the synthesis was [1,2-

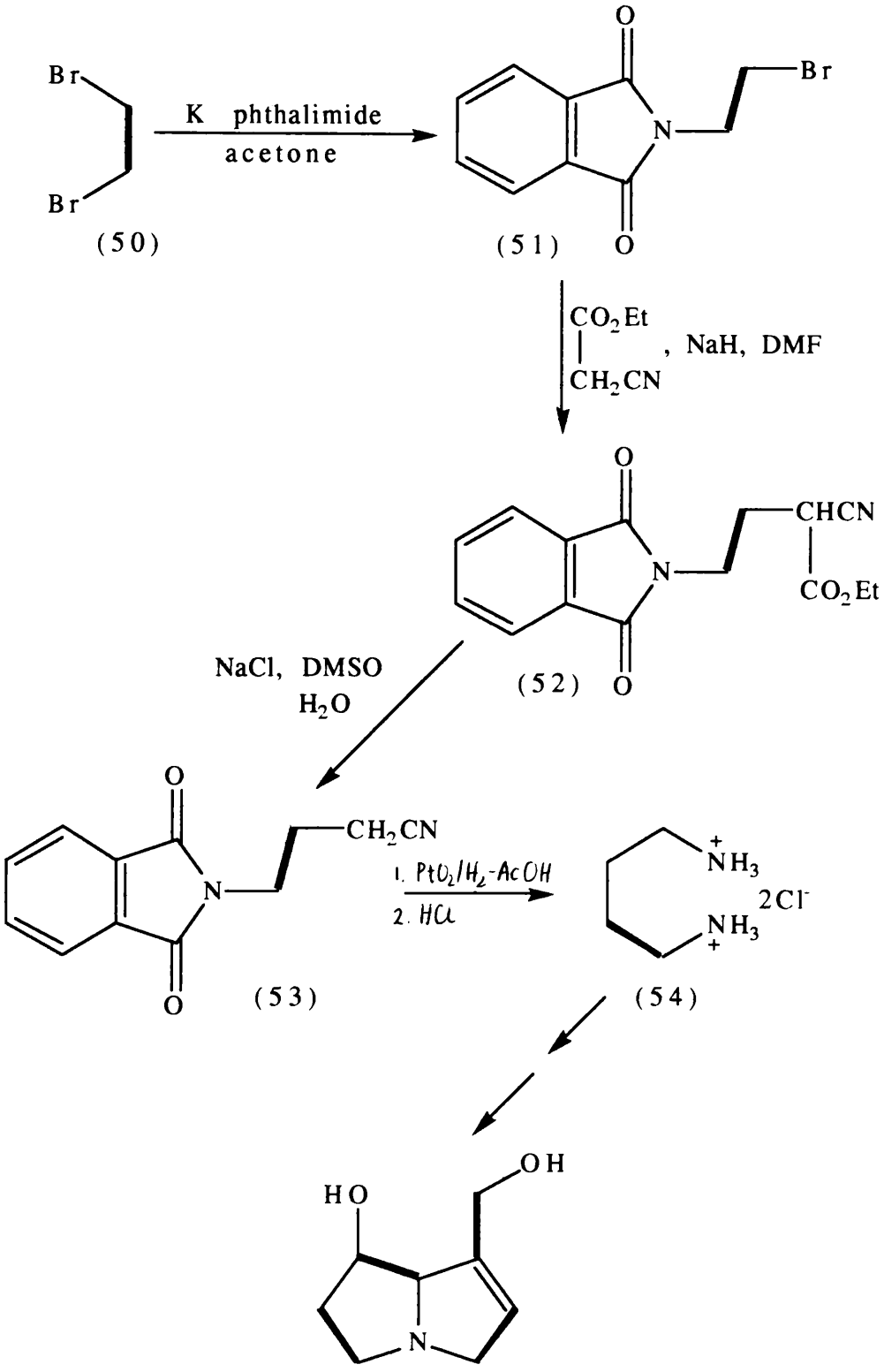
$^{13}C_2$]-1,2-dibromoethane (50). This was converted into [1,2-$^{13}C_2$]-1-bromo-2-phthalimidoethane (51) by treatment with potassium phthalimide. The bromide (51) was substituted with ethyl cyanoacetate to give ester (52). Removal of the ester group, hydrogenation of the nitrile and hydrolysis of the protecting group afforded [1,2-$^{13}C_2$]putrescine dihydrochloride (54).

The ^{13}C NMR spectrum of retronecine derived from the feeding of this precursor showed the presence of eight doublets flanking the natural abundance carbon singlets (Scheme 2.8). All eight carbon signals were enriched supporting the theory that two molecules of putrescine come together to form a symmetrical intermediate.

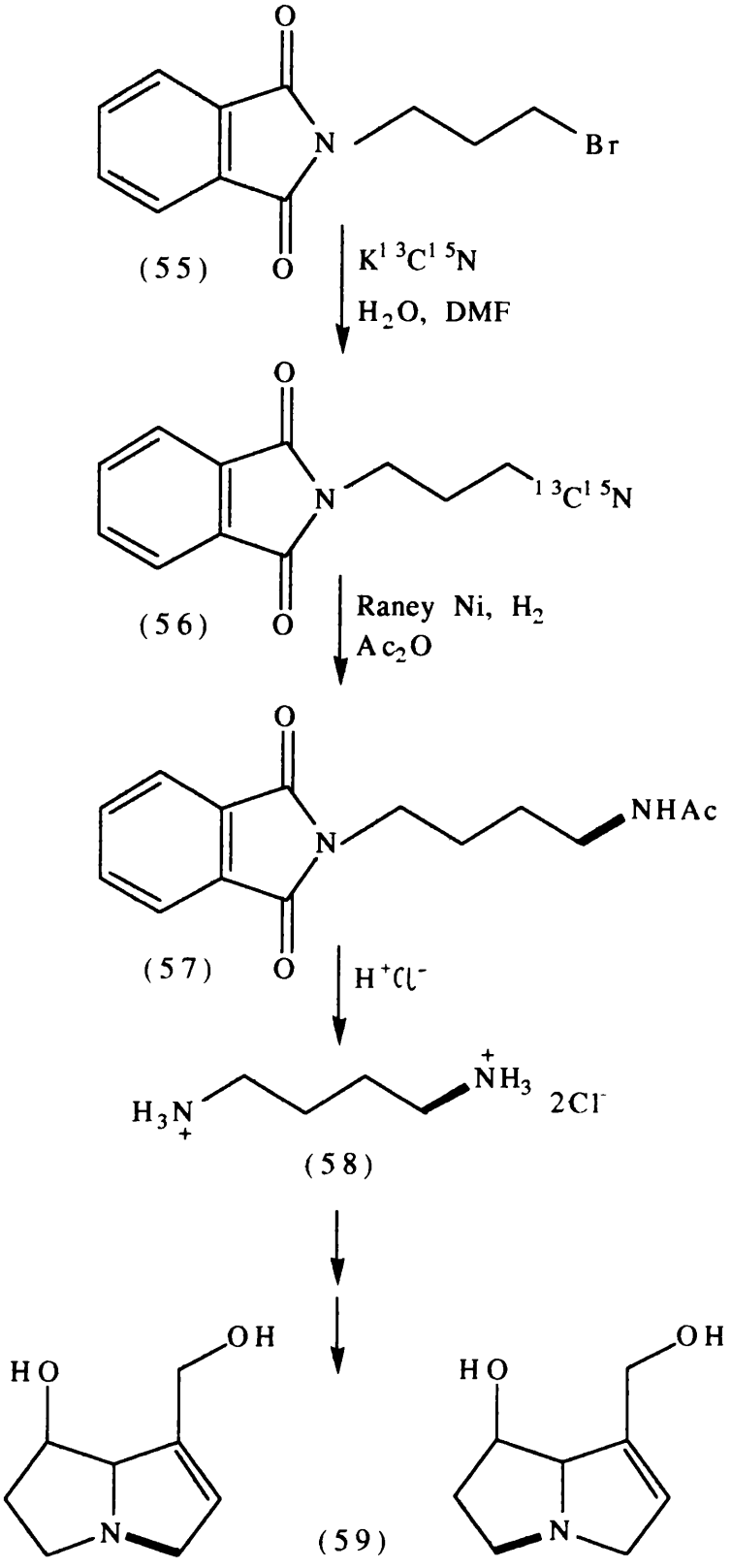
Proof of the involvement of a symmetrical intermediate in pyrrolizidine alkaloid biosynthesis came from independent reports by Grue-Sorensen and Spenser[30] and by Khan and Robins.[41]

Grue-Sorensen and Spenser[30] synthesized and fed [1-^{13}C, 1-^{15}N]-1,4-diaminobutane (putrescine) dihydrochloride (58) to *Senecio vulgaris* plants. This synthesis is outlined in Scheme 2.9. The labels were introduced by the treatment of 1-bromo-3-phthalimidopropane (55) with $K^{13}C^{15}N$. Reduction of nitrile (56) followed by acid hydrolysis gave ^{13}C-^{15}N-doubly-labelled putrescine (58) as its dihydrochloride salt. This precursor was fed and the alkaloid mixture extracted from the plants was hydrolysed to give labelled retronecine. The distribution of label within retronecine was revealed by ^{13}C NMR spectroscopy. Four positions within retronecine, C-3, -5, -8 and -9 were all equally enriched in ^{13}C. 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 ^{13}C-^{14}N species. This corresponds to labelling pattern (59). Since the doublets at C-3 and C-5 were of almost equal intensity, retronecine was likely to be produced *via* a C_4-N-C_4 intermediate.

Scheme 2.8


Br
Br
K phthalimide
acetone
O
N
Br
O
(50)
(51)
CO2Et
CH2CN
, NaH, DMF
CHCN
CO2Et
(52)
NaCl, DMSO
H2O
CH2CN
1. PtO2/H2-AcOH
2. HCl
NH3
NH3
2Cl-
(53)
(54)
HO
OH

Scheme 2.9


O
N
Br
(55)
O
$K^{13}C^{15}N$
H_2O, DMF
$^{13}C^{15}N$
(56)
Raney Ni, H_2
Ac_2O
NHAc
(57)
H^+Cl^-
H_3N^+
NH_3^+
$2Cl^-$
(58)
HO
OH
(59)

Khan and Robins[41] carried out analogous experiments on *S. isatideus* and found similar labelling pattern in their labelled retronecine. These workers proposed that the later symmetrical intermediate was the triamine homospermidine (60), a known plant constituent.[42]

$$H_2N(CH_2)_4NH(CH_2)_4NH_2$$

(60)

To test this hypothesis ^{14}C-labelled homospermidine was synthesized (Scheme 2.10).[41,43]

Scheme 2.10

$PhCH_2OCONH(CH_2)_3CO_2H$ (61)

+

$H_2N(CH_2)_3Br$ (62)

↓ PhCOCl

$PhCH_2OCONH(CH_2)_3CONH(CH_2)_3Br$ (63)

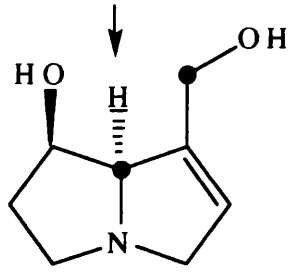
↓ $K^{14}CN$

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

↓ (i) H_2, Pd-C
(ii) BF_3-THF
(iii) HCl

$H_3\overset{+}{N}(CH_2)_4\overset{+}{N}H_2(CH_2)_3{}^{14}CH_2\overset{+}{N}H_3$ $3Cl^-$ (64)

↓



(65)

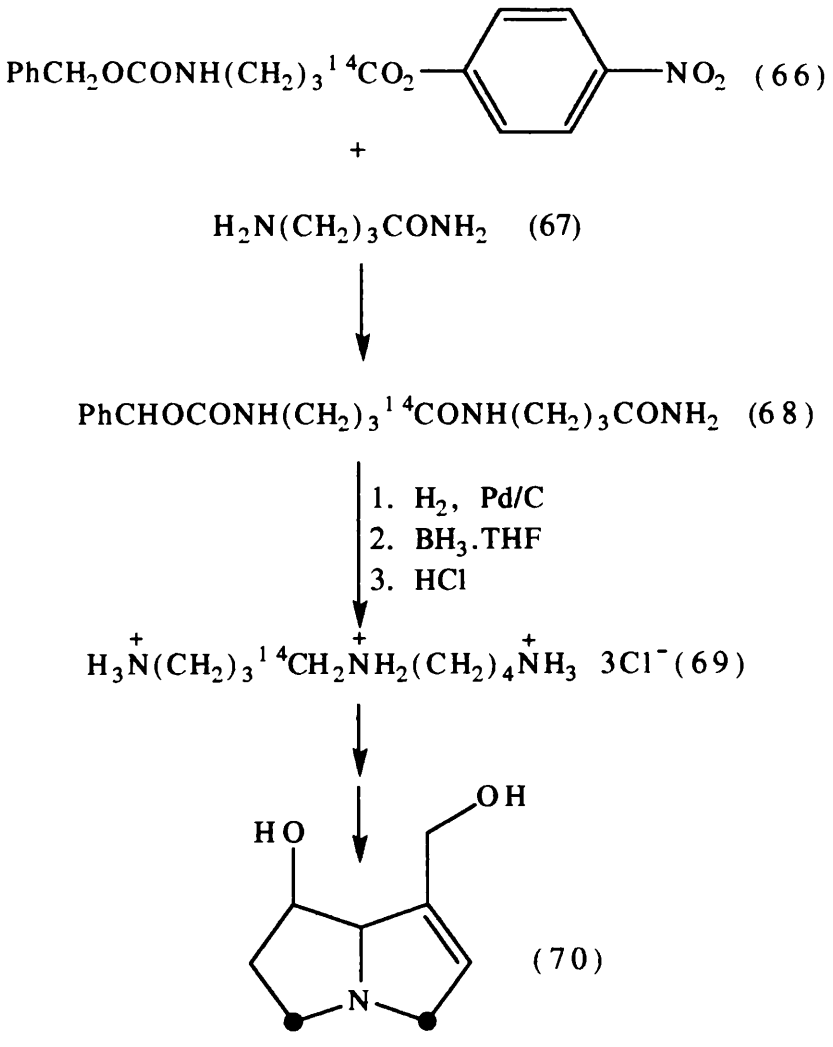
The *N*-benzyloxycarbonyl derivative (61) of 4-aminobutanoic acid was coupled with 3-bromopropylamine (62) by the mixed anhydride method. Treatment of the protected bromoamide (63) with $K^{14}CN$, followed by hydrogenation and reduction with borane in THF yielded [1,9-^{14}C]homospermidine (64) which was isolated as its trihydrochloride salt. This was fed to *S. isatideus* in the usual manner.[37] Total incorporations of 0.48-0.97% were observed in the isolated retrorsine.[43] Hydrolysis of the alkaloid followed by degradation of the retronecine produced, showed that 44% of the radioactivity was present at C-9 and 2% was in C-(5 + 6 + 7). These results are consistent with labelling pattern (65).

Further proof of the intermediacy of homospermidine in retronecine biosynthesis came from the feeding of [4,6-^{14}C]homospermidine trihydrochloride (69).[43] This ^{14}C-labelled precursor was synthesized from the *N*-benzyloxycarbonyl derivative of [1,4-^{14}C]-4-aminobutanoic acid. The acid (61) was converted into its *p*-nitrophenyl ester (66), and this was coupled with 4-aminobutamide (67) to give the *N*-protected diamide (68). Reduction of the amide function, removal of the protecting group and treatment with HCl gas yielded [4,6-^{14}C]homospermidine trihydrochloride (69) (Scheme 2.11). A total incorporation of 0.7% was achieved in retrorsine after feeding this material to *S. isatideus*. Degradation showed that 3% of the radioactivity was at C-9 and 46% was present in C-(5 + 6 + 7). This was indicative of labelling pattern (70) in retronecine.

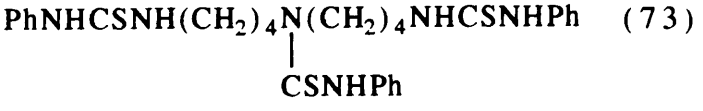
Complementary labelling patterns (65) and (70) are consistent with the intact conversion of homospermidine (60) into retronecine.

The conversion of putrescine (33) into homospermidine (60) probably occurs *via* the oxidation of putrescine to 4-aminobutanal (71). Coupling of aldehyde (71) to another putrescine molecule gives imine (72), reduction of which leads to homospermidine (Scheme 2.12).

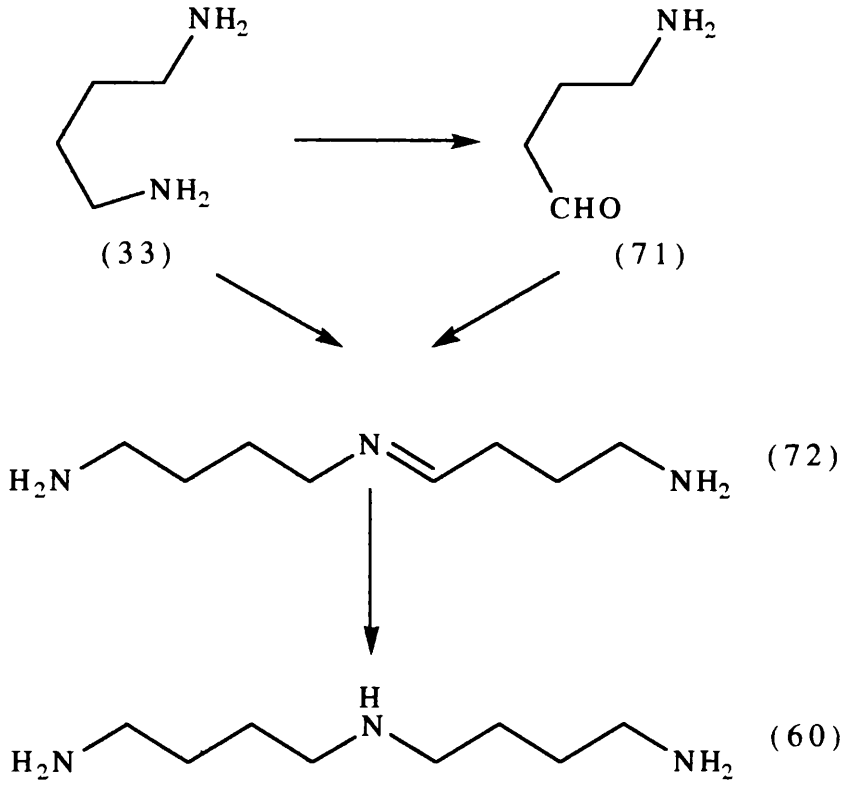
Scheme 2.11



The presence of homospermidine in *S. isatideus* plants was demonstrated by an intermediate trapping experiment.[41,43] DL-[5-^{14}C]Ornithine was fed to one plant of *S. isatideus*. After one day the *N*-phenylamino(thiocarbonyl) derivative (73) of homospermidine was isolated and shown to contain *ca.* 0.5% of the radioactivity originally fed. Homospermidine was therefore formed from ornithine in *S. isatideus*.



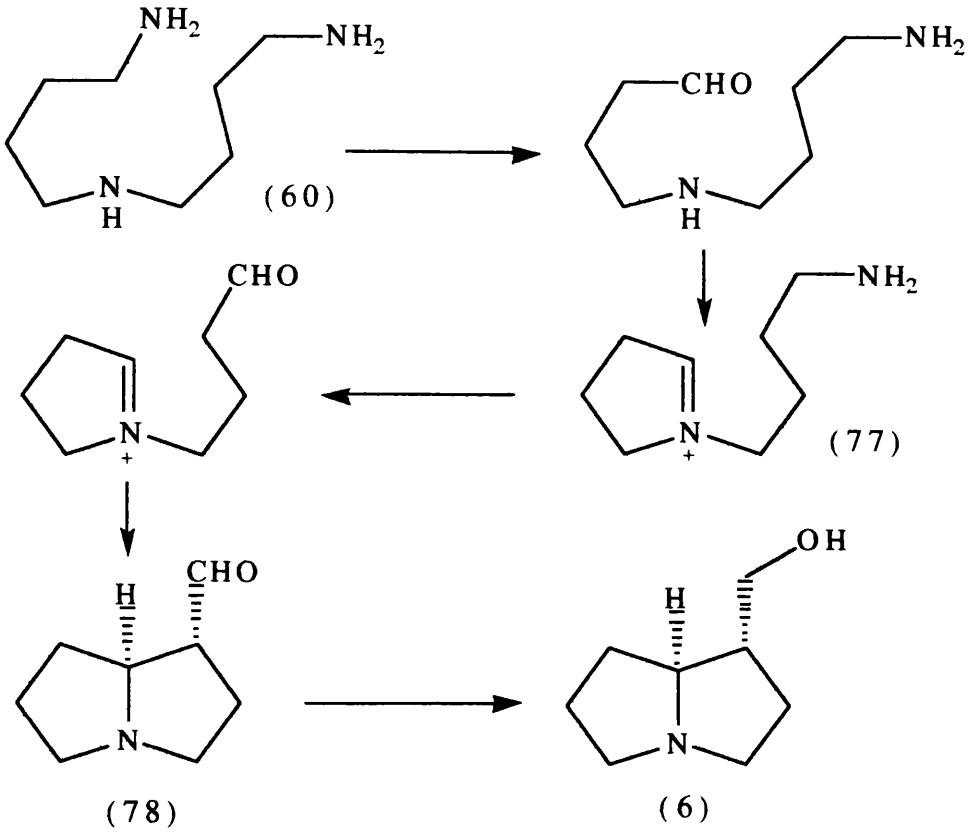
Scheme 2.12



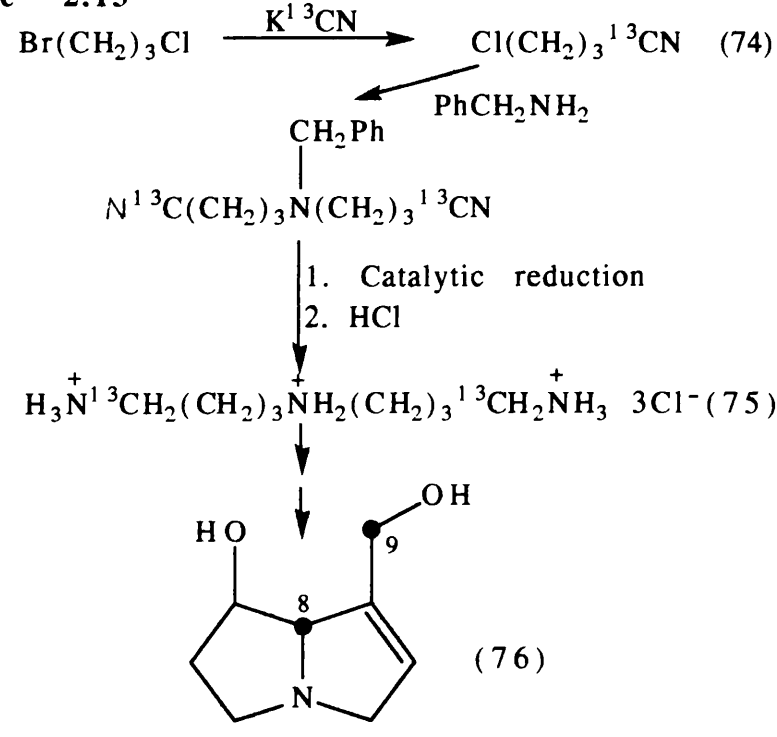
To provide further evidence for the intact incorporation of homospermidine into retronecine and to obtain a complete labelled pattern, [1,9-$^{13}C_2$]homospermidine (75) was prepared and fed to *S. isatideus*.[44] This precursor was synthesized by the reaction of benzylamine with two equivalents of 4-chloro[1-^{13}C]butanenitrile (74), followed by catalytic reduction (Scheme 2.13). This precursor produced a geminal coupling of *ca.* 6 Hz between C-8 and C-9 in the ^{13}C NMR spectrum of the biosynthetically derived retronecine (76). This was convincing evidence for intact incorporation of homospermidine into retronecine.

Further evidence for the intermediacy of homospermidine (60) came from the work of Robins[47] when, using enzymes and physiological conditions, he converted homospermidine into the known necine (±)-trachelanthamidine (6). A probable route for this conversion is shown in Scheme 2.14.

Scheme 2.14

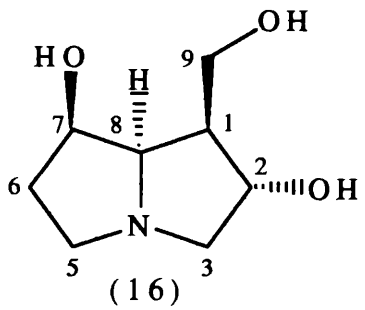


Scheme 2.13

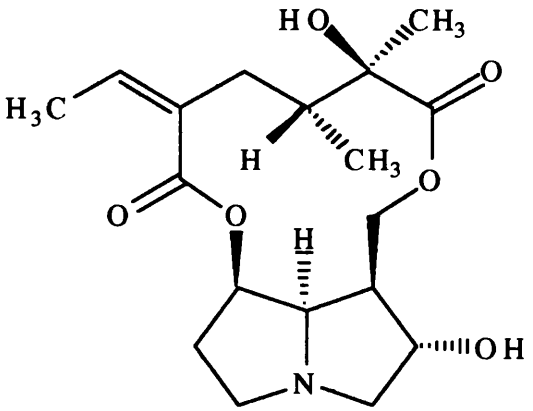


Oxidation of one primary amino group in homospermidine with diamine oxidase would give an aldehyde in equilibrium with the iminium ion (77). Oxidation of the remaining amino group would afford an aldehyde which could cyclise to give the thermally more stable *exo*-aldehyde (78). Reduction of this aldehyde with a dehydrogenase enzyme would then yield trachelanthamidine (6).

All work up to this point had concentrated on the biosynthesis of retronecine. Kelly and Robins extended the field of study to include the necine base rosmarinecine (16).[48]



(16)

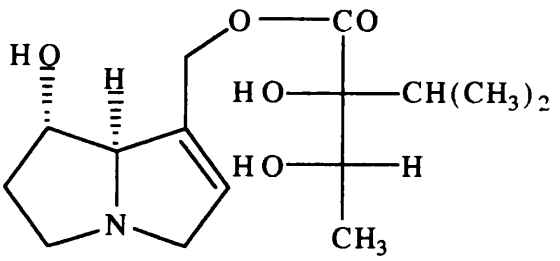


(29)

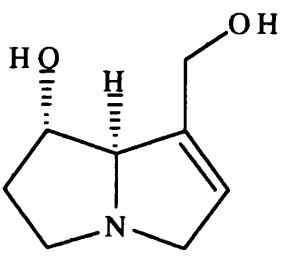
Studies were carried out on *Senecio pleistocephalus*, the main alkaloidal constituent of which is rosmarinine (29). Highest incorporations were achieved when feeding precursors by the wick method.[48] When [1-^{13}C]putrescine dihydrochloride (49) was fed the enriched signals were, as expected, associated with C-3, C-5, C-8 and C-9. The use of [2,3-$^{13}C_2$]putrescine dihydrochloride (47) led to the appearance of doublets around the natural abundance signals for C-1, C-2, C-6 and C-7. Feeding of [^{13}C-^{15}N]putrescine dihydrochloride (58) gave rise to doublets around the signals for C-3 (J 4 Hz) and C-5 (J 2-3 Hz). When [1,9-$^{13}C_2$]homospermidine trihydrochloride (75) was fed to *S. pleistocephalus*, no doublets were observed around the C-8 and C-9 signals i.e. the geminal coupling constant between these carbons was zero. However, it was clear that the C-8 and C-9 signals were the only ones which were enriched.

These results all indicated that, as with retronecine, rosmarinecine (16) was biosynthesised from two molecules of putrescine (33) *via* homospermidine (60).[48]

The formation of trachelanthamidine (6) from homospermidine (60) using enzymes[47] suggested that this simple 1-hydroxymethylpyrrolizidine and its stereoisomers might be biosynthetic precursors of the more complex necines. To establish if this was true, Kunec and Robins[49] examined the incorporation of (±)-[5-^{3}H]isoretronecanol (81) and (±)-[5-^{3}H]trachelanthamidine (83) into the alkaloids from *S. isatideus, S. pleistocephalus* and *C. officinale.* This last species produces the alkaloid echinatine (84), the base portion of which is heliotridine (19).[49]



(84)



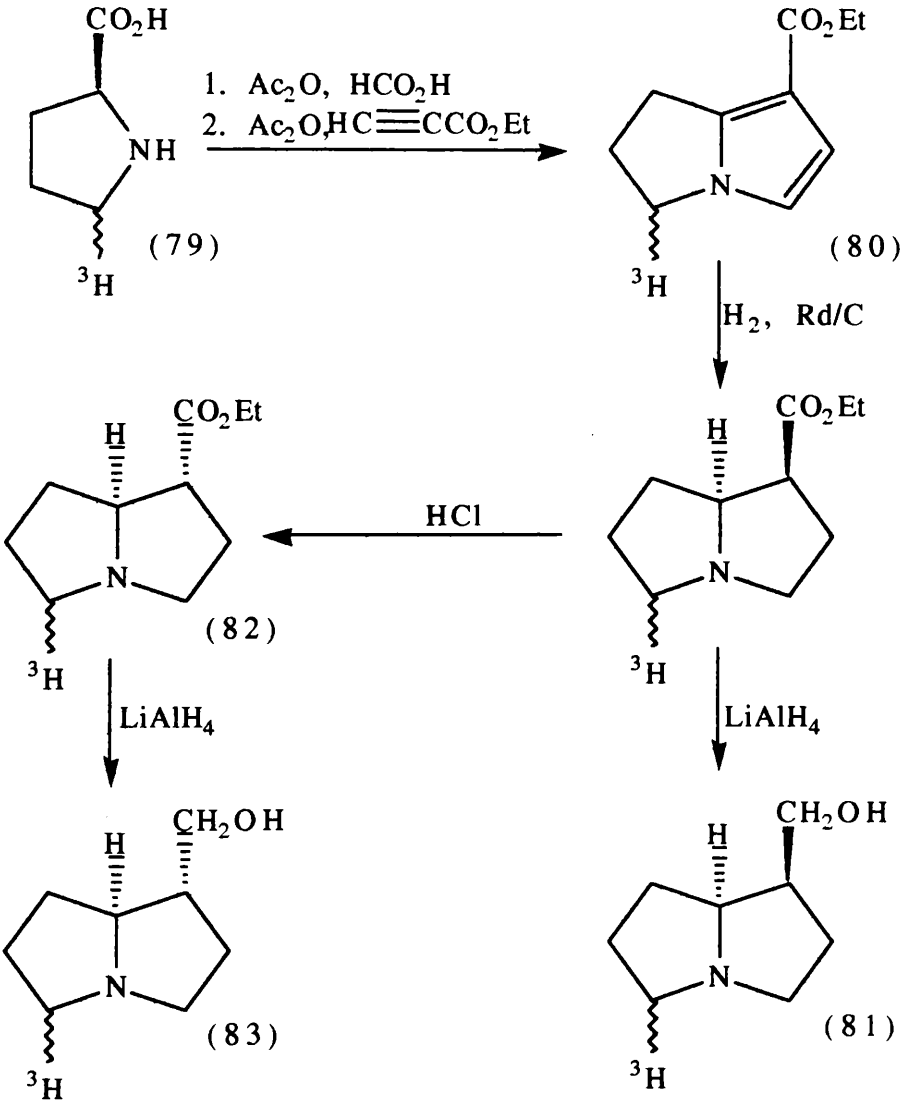
(19)

The synthesis of ^{3}H-labelled isoretronecanol and ^{3}H-labelled trachelanthamidine is outlined in Scheme 2.15.[49] [5-^{3}H]-L-Proline (79) was converted into its *N*-formyl derivative, and this was transformed into dihydropyrrolizine ester (80) by a 1,3-dipolar cycloaddition with ethyl propiolate. Hydrogenation of this ester (80) followed by reduction yielded (±)-[5-^{3}H]-isoretronecanol (81). Epimerisation of the 1β-ester (80) to the thermodynamically more stable 1α-ester (82) followed by reduction with lithium aluminium hydride generated (±)-[5-^{3}H]trachelanthamidine (83).

The ^{3}H-labelled necines (81) and (83) were fed to the two different *Senecio* plant species along with [1,4-^{14}C]putrescine dihydrochloride. The ^{3}H /^{14}C ratios from each feeding experiment therefore provided a measure of the relative

efficiency of each ^3H-labelled necine as a precursor with respect to putrescine. The results are summarised in Table 2.2.

Scheme 2.15



The $^3H/^{14}C$ ratios indicated that (±)-isoretronecanol was incorporated 30 times more efficiently than (±)-trachelanthamidine into rosmarinine (29) from *S. pleistocephalus* whereas, (±)-trachelanthamidine was incorporated 20 times more efficiently into retrorsine (24) from *S. isatideus* and 17 times more efficiently into echinatine (84) from *C. officinale* than (±)-isoretronecanol. In each case the preferred necine precursor was incorporated more efficiently than putrescine, in support of the theory that

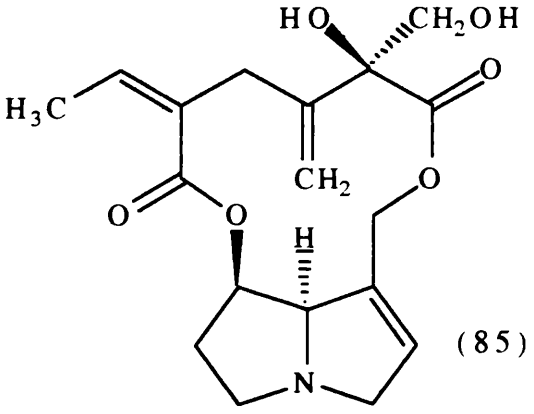
putrescine occurs further back in the biosynthetic pathway. Basic hydrolysis of rosmarinine and retrorsine showed that the radioactivity was confined to the base portion and degradation proved that almost all of this activity was found in C-(5 + 6 + 7) as expected.

Table 2.2 : Incorporation of (±)-isoretronecanol (81) and (±)-trachelanthamidine (83) into alkaloids from various *Senecio* species.

Precursor	Alkaloid	^{3}H /^{14}C ratio*	^{3}H Specific Inc. (%)
(81)	retrorsine	0.7	0.3
(83)	retrorsine	14.3	2.8
(81)	echinatine	1.0	0.04
(83)	echinatine	17.0	0.35
(81)	rosmarinine	17.0	2.4
(83)	rosmarinine	< 0.5	< 0.1

*Initial ^{3}H /^{14}C ratio 10.0.

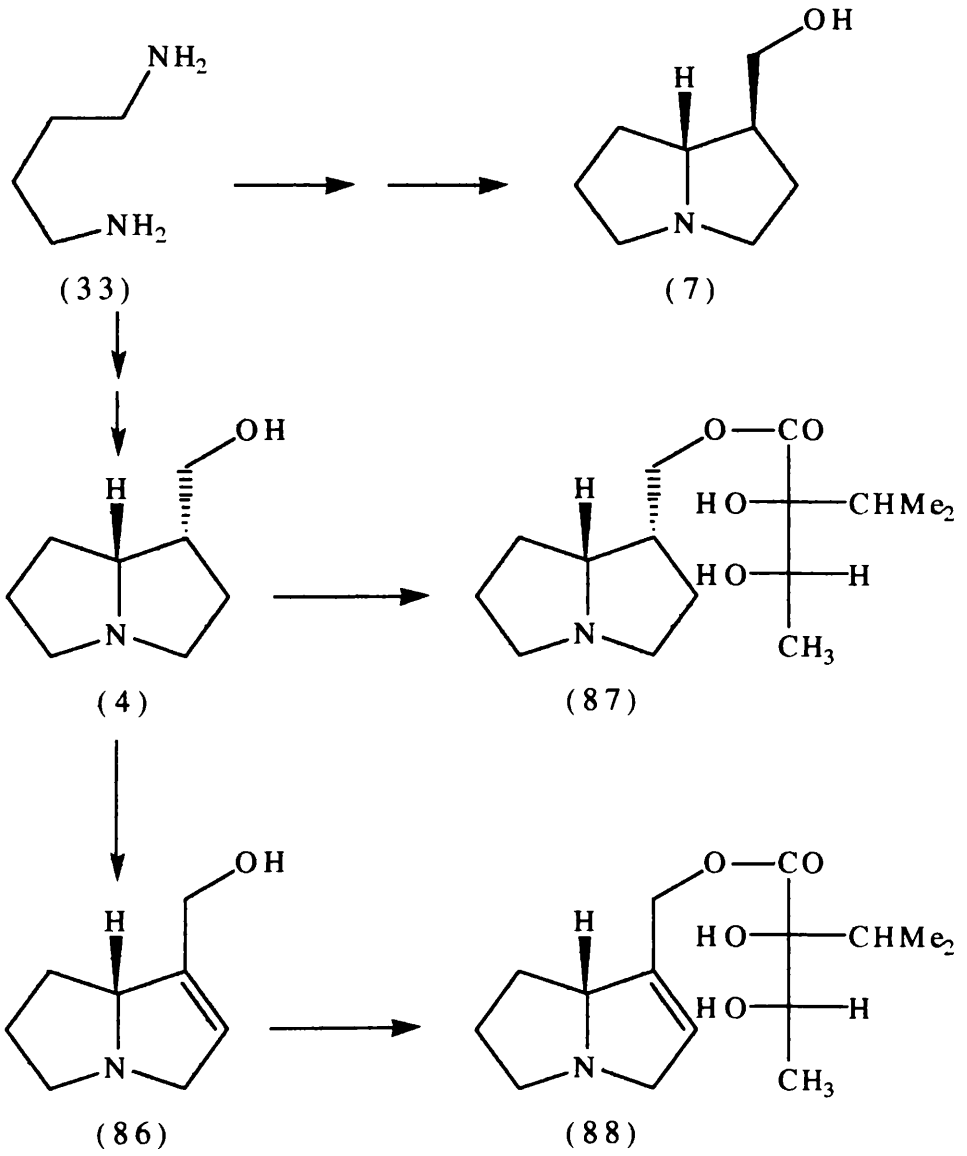
Leete and Rana[50] carried out similar experiments on *Senecio riddellii* corroborating the results of Kunec and Robins.[49] ^{3}H-Labelled trachelanthamidine was incorporated into riddelliine (85) from *S. riddellii*, rather that isoretronecanol. The base portion of riddelliine is retronecine (18).



(85)

Work by Hagan and Robins,[51] using similar methodology, established that (±)-isoretronecanol (81) is a good precursor for the base portions of cynaustraline (87) and cynaustine (88) in *Cynoglossum australe* R. Br., whereas (±)-trachelanthamidine was not incorporated into the two alkaloids.

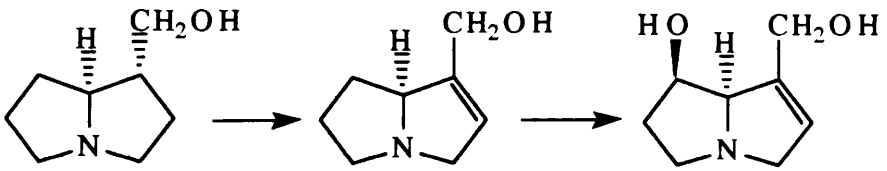
Scheme 2.16



Basic hydrolysis of cynaustraline and cynaustine yielded (+)-isoretronecanol (4) and (+)-supinidine (86) which contained over 97% of the alkaloid radioactivity. Optically active (+)-

isoretronecanol and (+)-supinidine were prepared biosynthetically and fed to *C. australe*.[51] (+)-Isoretronecanol was incorporated into both alkaloids whereas (+)-supinidine was an efficient precursor only for cynaustine. These results indicated that (+)-isoretronecanol (4) is converted into (+)-supinidine (86) in the biosynthetic pathway to cynaustine (88) (Scheme 2.16).

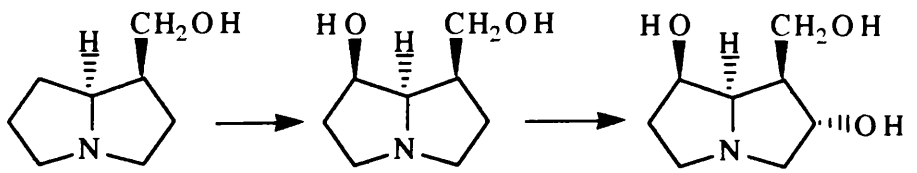
The incorporation of trachelanthamidine into retronecine is consistent with results obtained from the $^{14}CO_2$ pulse labelling of *Heliotropium spathulatum*.[45] By exposing the plants to $^{14}CO_2$ for different lengths of time and examining the specific activities of the radiolabelled products, Birecka and Catalfamo[45] established that *H. spathulatum* produce three necine bases in the sequence: (-)-trachelanthamidine (6) then (-)-supinidine (17) then (+)-retronecine (18).



(6) (17) (18)

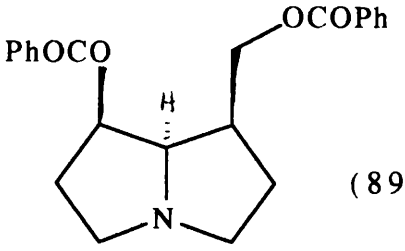
Retronecine might be formed from trachelanthamidine *via* two hydroxylations at C-2 and C-7, followed by loss of the elements of water.

The order in which hydroxylation occurs to convert isoretronecanol (5) into rosmarinecine (16) has not been established. However, hydroxylation may occur first at C-7 because ^{3}H-labelled playnecine (10) was incorporated into rosmarinine (29) in *S. pleistocephalus*.[52]



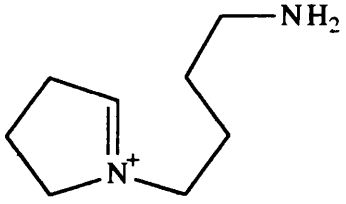
(5) (10) (16)

In an intermediate trapping experiment, platynecine isolated as its dibenzoyl derivative (89) was shown to contain 0.37% of the fed radioactivity.[53] This is good evidence for the presence of platynecine on the biosynthetic pathway to rosmarinine.



(89)

No intermediates had so far been identified between homospermidine (60) and the various 1-hydroxymethylpyrrolizidines. Homospermidine had been converted into trachelanthamidine by enzymic oxidation followed by reduction.[47] The question that had to be answered was does the oxidation of homospermidine take place in two discrete steps? If this was the case, a probable intermediate was the iminium ion *N*-(4-aminobutyl)-1,2-didehydropyrrolidinium (77).[54]



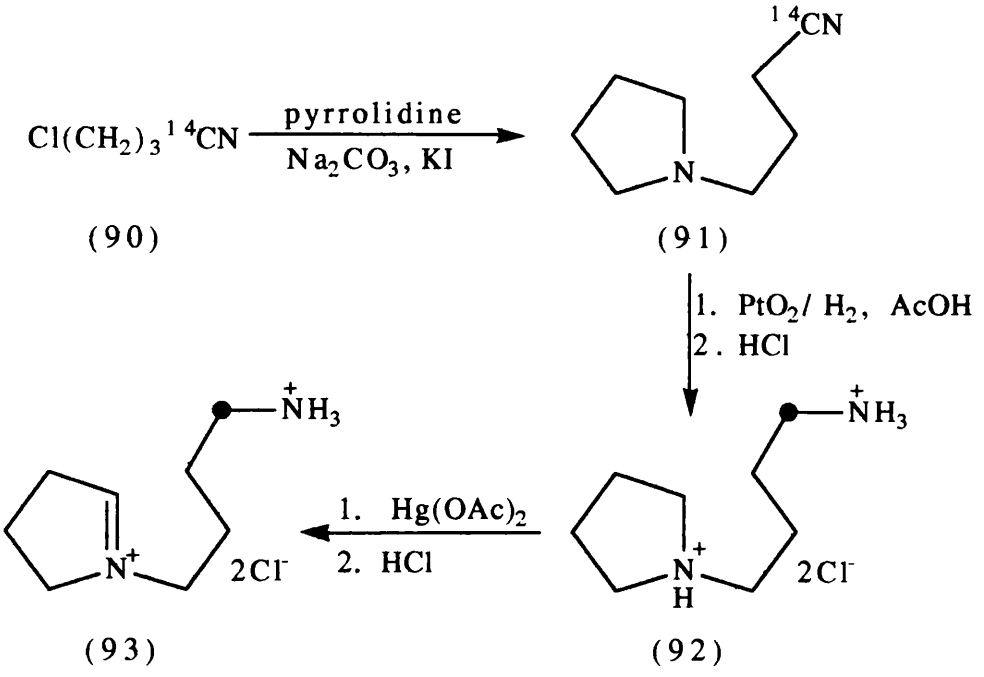
(77)

Kelly and Robins[54] synthesized this iminium ion in labelled form (93) and fed it to *S. pleistocephalus* and *S. isatideus*. The synthesis is shown in Scheme 2.17.

The mesylate of 3-chloropropanol was treated with $Na^{14}CN$ to give [1-^{14}C]-4-chlorobutanenitrile (90). Displacement of the chloro group with pyrrolidine gave pyrrolidinenitrile (91). Reduction of the nitrile followed by acidification of the product yielded diamine salt (92). Oxidation of this with mercuric

acetate led to the formation of the desired ^{14}C-labelled iminium salt (93).

Scheme 2.17



In a $^{3}H/^{14}C$ double-labelling experiment,[54] iminium ion (93) was shown to be an efficient precursor for rosmarinecine (16) from rosmarinine (29) and for retronecine (18) from retrorsine (24). The iminium ion (93) was incorporated into these necines more efficiently than putrescine (33).

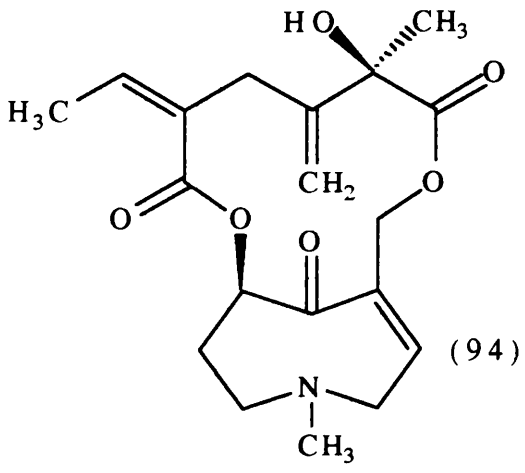
Evidence for the presence of the iminium ion (93) in *S. pleistocephalus* was obtained from an intermediate trapping experiment.[54] [1,4-^{14}C]Putrescine dihydrochloride was fed to one plant and after one day the plant was harvested. Inactive iminium ion (77) was added to the methanolic extract, followed by sodium borohydride. The *N*-phenylthiourea derivative of the reduced iminium ion, i.e. the *N*-phenylthiourea derivative of diamine salt (92), was shown to contain 0.4% of the original radioactivity. In a similar experiment,[55] when the methanolic extract was treated with sodium [^{3}H]borohydride, the *N*-phenylthiourea derivative contained a similar amount of ^{14}C as

before and about 5% of the ^{3}H activity. This confirmed that iminium ion (77) is present in *S. pleistocephalus.*

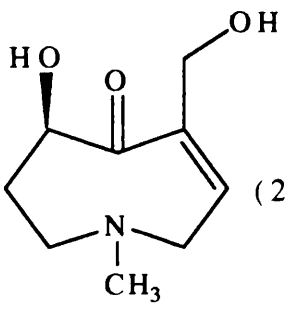
N-(4-Aminobutyl)pyrrolidine dihydrochloride (92) is also a reasonably good precursor of both retronecine and rosmarinecine, but an intermediate trapping experiment gave a derivative containing <0.017% of the radioactivity fed.[54,55] The most likely explanation for this result is that saturated salt (92) is not actually on the biosynthetic pathway to the necines but it can be oxidised to iminium ion (77) by enzymes present in *S. pleistocephalus.*

This work was extended by Denholm *et al.*[55] to show that iminium ion (77) is an efficient precursor of the necines heliotridine (19) from echinatine (84) in *C. officinale*, and (+)-isoretronecanol (4) and (+)-supinidine (86) from the alkaloids in *C. australe.*

The biosynthesis of the *seco*-pyrrolizidine base otonecine (21) was studied by Kelly and co-workers.[56] In summary, putrescine (33), homospermidine (60), *N*-(4-aminobutyl)-1,2-didehydropyrrolidinium (77), trachelanthamidine (6) and retronecine (18) are all efficient precursors for the otonecine (21) portion of emiline (94) from *Emilia flammea.* The incorporation of trachelanthamidine and retronecine into emiline established that the N(4)-C(8) bond is broken during the formation of the otonecine portion.



(94)



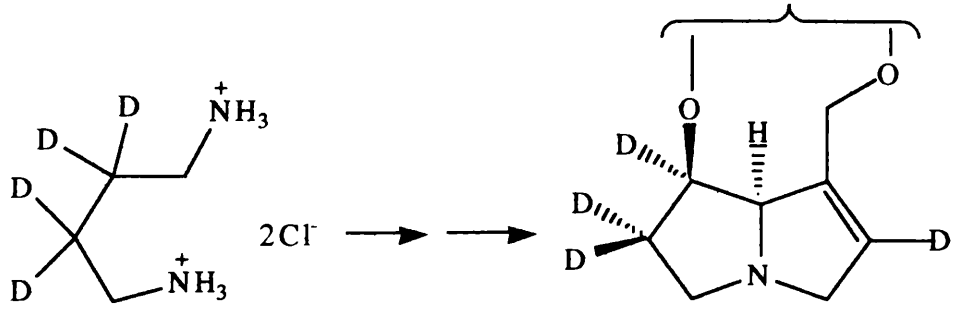
(21)

The foregoing discussion has not touched on the stereochemical aspects of necine biosynthesis. The first steps to receive attention were the decarboxylation of L-ornithine (34) and L-arginine (37) by ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) respectively. It was shown, by three different groups of workers, that these decarboxylations take place with retention of configuration.[57]

The stereochemistry of other enzymic processes involved in necine biosynthesis was established by feeding precursors specifically labelled with deuterium and then establishing the complete labelling pattern in the isolated alkaloid by ^2H NMR spectroscopy.

Initial experiments were carried out on *S. isatideus.* The first precursor prepared was [2,3-$^2H_4$]putrescine dihydrochloride (95).[58] Catalytic hydrogenation of [2,3-$^2H_4$]succinonitrile furnished this precursor, which was isolated as its dihydrochloride salt. After the feeding experiment the $^2H\{^1H\}$ NMR spectrum of the isolated retrorsine (96) showed equal enrichment of the signals at four sites. These were the expected ones of C-2, C-6α, C-6β and C-7α (Scheme 2.18). All deuterium labelling patterns are composite representations of the various deuterium labelled species present. The presence of deuterium at C-7α shows that the introduction of the hydroxyl group at this position does not involve a keto or enol intermediate.[58]

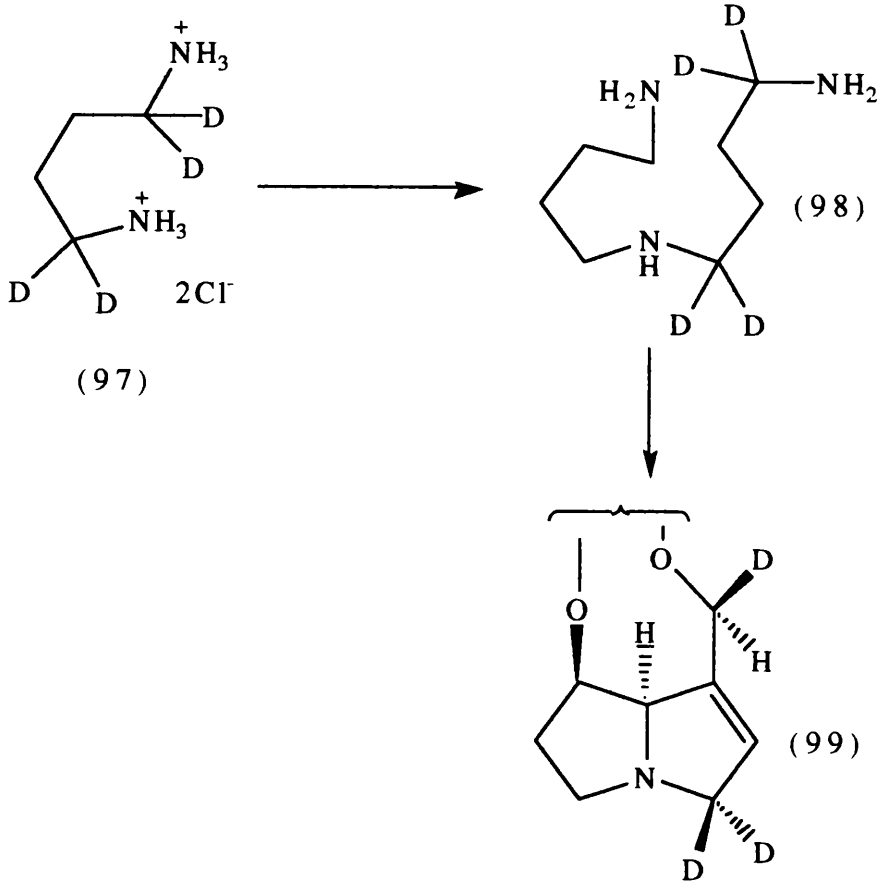
Scheme 2.18



(95) (96)

[1,4-$^2H_4$]Putrescine dihydrochloride (97) was formed by the catalytic hydrogenation of succinonitrile under a deuterium atmosphere, followed by acidification.[58] The $^2H\{^1H\}$ NMR spectrum of retrorsine derived from this feed showed the enrichment of signals corresponding to 3β-H, 3α-H and 9-H *pro* -S in retrorsine (99) (Scheme 2.19).[58]

Scheme 2.19



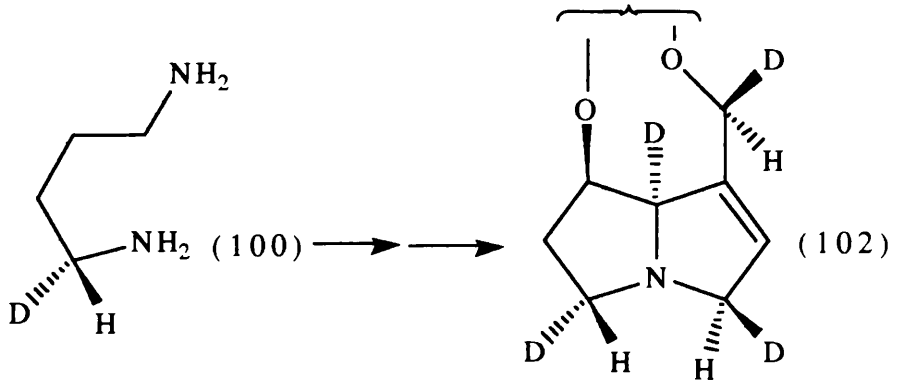
This result was surprising as most of the deuterium was located in one half of the base portion. This can be explained in terms of a substantial deuterium isotope effect. The most likely homospermidine intermediate (98) will be formed from the reaction of labelled putrescine with endogenous unlabelled material. A deuterium isotope effect would lead to the unlabelled end of homospermidine (98) being oxidised preferentially and hence give a preponderance of deuterium in

one half of the base portion.[58] This theory is supported by the finding that the oxidation of [1,4-$^2H_4$]putrescine to [1,4-$^2H_3$]-4-aminobutanal catalysed by hog kidney diamine oxidase is subject to an intermolecular deuterium isotope effect of 1.26.[59] The ^2H-labelling of the 9 *pro*-S hydrogen established that in the reduction of aldehyde (78) to give trachelanthamidine (6) a proton is added to the *re*-face of the carbonyl group.[58]

The ability to produce enantiomerically deuteriated precursors allowed more detailed study on the stereochemistry of the enzymic processes. (R)-[1-^2H]Putrescine (100) was prepared by the decarboxylation of L-ornithine (34) in ^2H_2O with ODC, whereas similar decarboxylation of [2-^2H]-DL-ornithine in H_2O yielded (S)-[1-^2H]putrescine (101).[60]

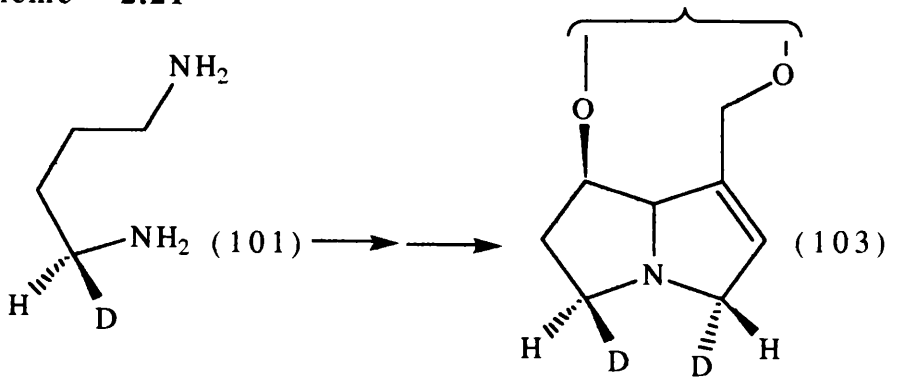
Grue-Sorensen and Spenser[61] studied the mode of incorporation of these precursors into the mixture of alkaloids obtained from *S. vulgaris*. All the alkaloids contained retronecine as their base portion. The retronecine part (102) derived from (R)-[1-^2H]putrescine (100) was labelled with deuterium equally at positions 3β, 5α, 8α and 9 *pro*-S (Scheme 2.20).

Scheme 2.20



The feeding of (S)-[1-^2H]putrescine (101) gave a retronecine portion (103) labelled only at the 3α and 5β positions (Scheme 2.21). Similar labelling patterns were observed in retronecine (24) when precursors (100) and (101) were fed to *S. isatideus* by Rana and Robins.[62,63]

Scheme 2.21



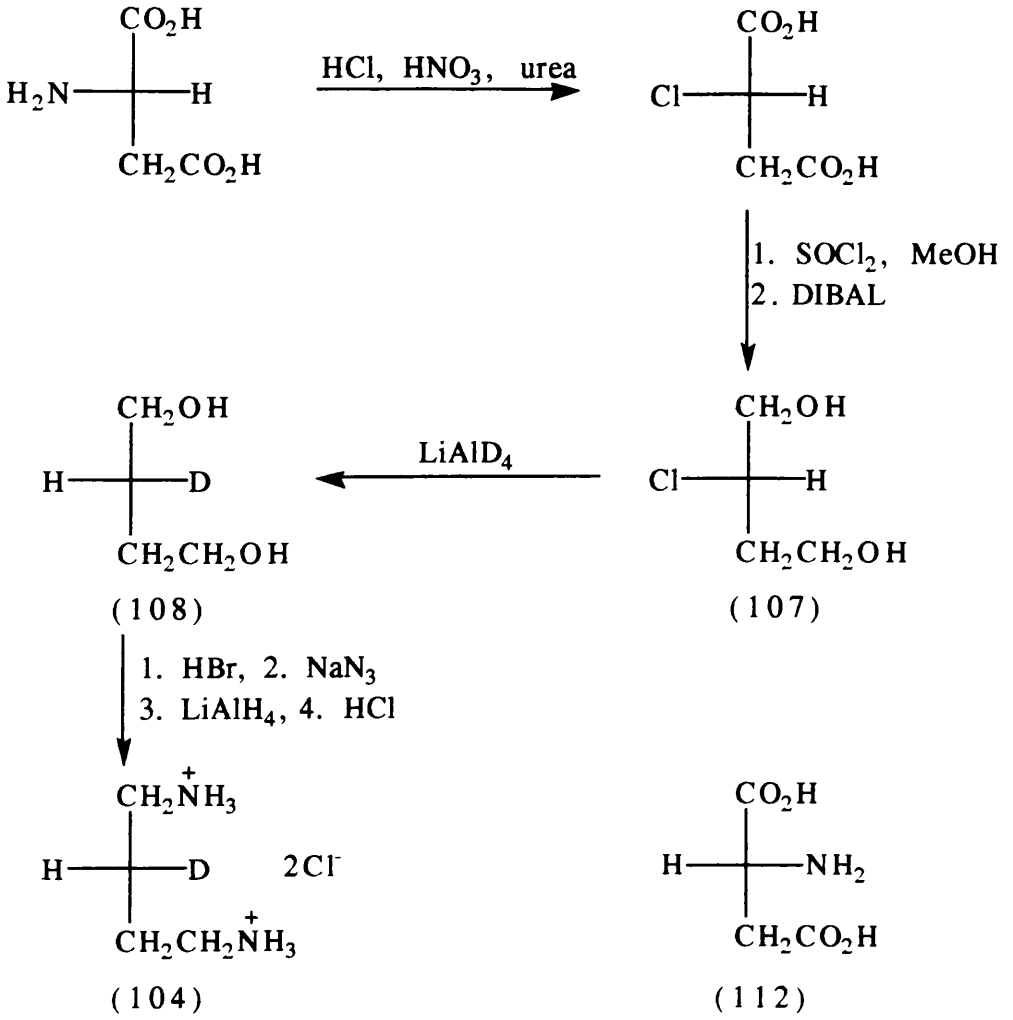
These labelling patterns [(102) and (103)] are consistent with the following stereochemical details in retronecine biosynthesis. Initial oxidation of putrescine (33) to 4-aminobutanal (71) takes place with loss of the *pro*-S hydrogen. This is the usual stereochemistry observed with diamine oxidases. Reduction of imine (72) formed by the coupling of putrescine and 4-aminobutanal occurs by hydride attack on the *si*-face, to give homospermidine (60). The oxidations of homospermidine and iminium ion (77) each result in the removal of the *pro*-S hydrogen. These results also confirm that reduction of aldehyde (78) takes place on the *re*-face of the carbonyl group.[63] This is the usual stereochemistry obtained with dehydrogenases.

The synthesis and feeding of (R)-[2-^{2}H]putrescine (104) dihydrochloride and (S)-[2-^{2}H]putrescine (109) dihydrochloride to *S. isatideus* allowed the stereochemical consequences of enzymic processes involving removal of hydrogen from the 2- and 3- positions of putrescine (33) as it is converted into retronecine (18) to be determined.[64]

(R)-[2-^{2}H]Putrescine (104) dihydrochloride was prepared from (2S)-aspartic acid (105) (Scheme 2.22).[64] The acid (105) was treated with a mixture of hydrochloric and nitric acids in the presence of urea, which led to replacement of the amino group by chlorine with retention of configuration. Diacid (106) was then converted into its methyl ester and the ester groups were selectively reduced with diisobutylaluminium hydride

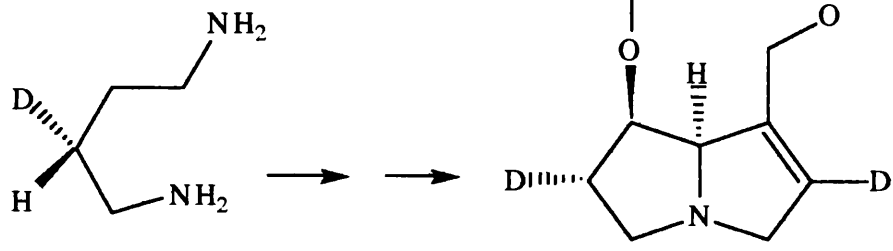
(DIBAL). Treatment of 2-chlorobutane-1,4-diol (107) with lithium aluminium deuteride introduced one deuterium atom with inversion of configuration to yield (S)-[2-^{2}H]butane-1,4-diol (108). This diol (108) was converted into a diamine by formation of the dibromide, conversion into the diazide and reduction of the diazide. (R)-[2-^{2}H]Putrescine (104) was isolated as its dihydrochloride salt. (S)-[2-^{2}H]Putrescine (109) dihydrochloride was prepared from (2R)-aspartic acid (112) in an analogous fashion.[64]

Scheme 2.22



After feeding the (R)-isomer (104) to *S. isatideus*, ^{2}H was present at H-2 and H-6α, corresponding to labelling pattern (110) in retrorsine (Scheme 2.23).

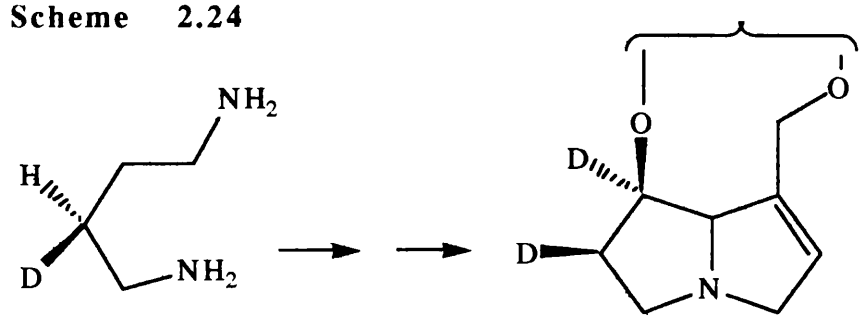
Scheme 2.23



(104) (110)

Incorporation of the (S)-isomer (109) led to retrorsine (111) labelled with deuterium at H-6β and H-7α (Scheme 2.24).

Scheme 2.24



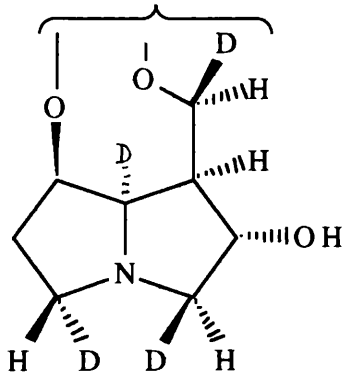
(109) (111)

These results show that hydroxylation at C-7 of retronecine occurs with retention of configuration. They also indicate that formation of the 1,2-double bond involves removal of the *pro*-S hydrogen at C-2 of retronecine.[64]

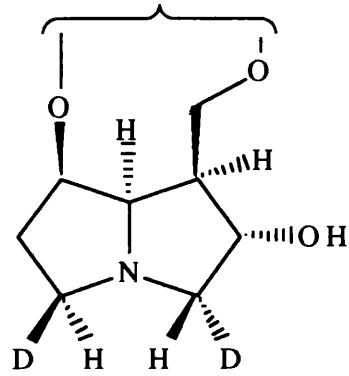
The availability of specifically deuteriated putrescines allowed examination of the stereochemical processes involved in the biosynthesis of the rosmarinecine (16) portion of rosmarinine (29) from *S. pleistocephalus*,[65] and otonecine (21) derived from emiline (94) in *E. flammea* transformed root cultures. (Root cultures were used instead of plants because

more alkaloid was produced and higher incorporations were obtained.)

Kelly and Robins[65] fed (R)-[1-^{2}H]putrescine (100) dihydrochloride and (S)-[1-^{2}H]putrescine (101) dihydrochloride to *S. pleistocephalus* and obtained labelling patterns (113) and (114) respectively, for rosmarinine. These were exactly analogous to those observed for retronecine.

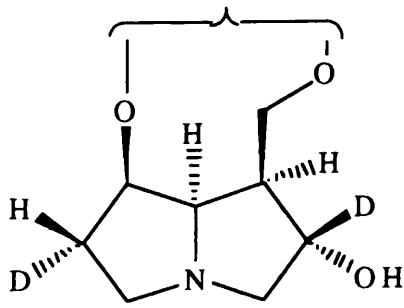


(113)

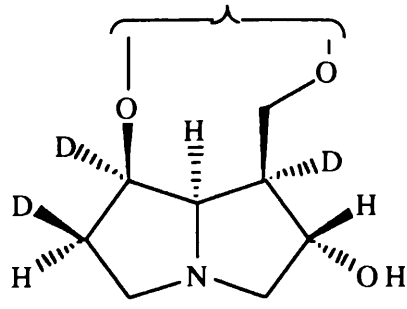


(114)

Feeding experiments with (R)-[2-^{2}H]- (104) and (S)-[2-^{2}H]-putrescine (109) dihydrochloride gave rosmarinine (115) labelled with deuterium at C-2β and C-6α from the former, and rosmarinine (116) with deuterium present at C-1α, C-6α and C-7α from the latter precursor.[65]

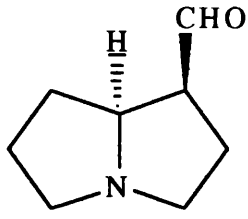


(115)

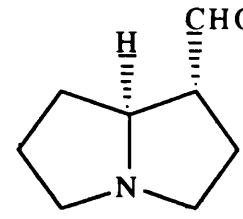


(116)

Thus, on formation of the pyrrolizidine ring the *pro*-R hydrogen on the carbon destined to be C-1 in rosmarinecine is stereospecifically removed. The presence of deuterium at C-1 in rosmarinine after feeding the (S)-isomer is consistent with the direct formation of aldehyde (117) from iminium ion (77) rather than *via* the exoaldehyde (78). These results also indicate that the hydroxylations at C-2 and C-7 of isoretronecanol (5) proceed with retention of configuration.[65]

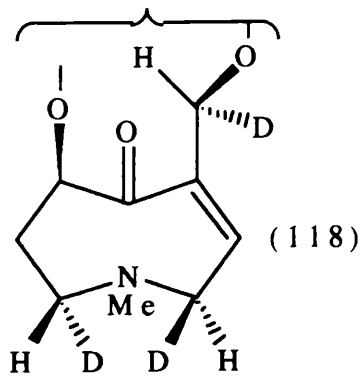


(117)

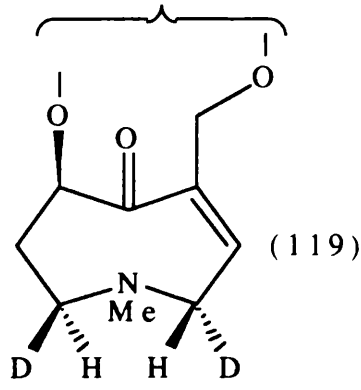


(78)

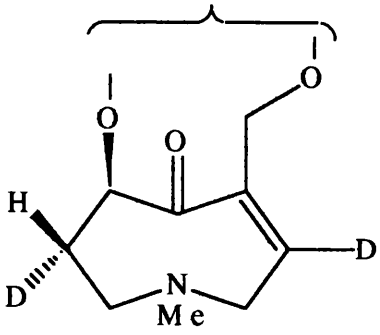
Investigations into the stereochemistry of the enzymic processes involved in otonecine (21) biosynthesis were carried out by Rodgers *et al.*[66] The feeding of (R)-[1-^{2}H]- (100), (S)-[1-^{2}H]- (101), (R)-[2-^{2}H]- (104) and (S)-[2-^{2}H]-putrescine (109) dihydrochloride to transformed root cultures of *E. flammea* gave labelling patterns (118), (119), (120) and (121) respectively for the emiline isolated. These patterns were analogous to those obtained for retrorsine. The main point of note was that introduction of the carbonyl group at C-8 of retronecine (18) and cleavage of the bicyclic system to form otonecine (21) did not involve loss of deuterium at any site on the pyrrolizidine ring apart from 8-H.[66]



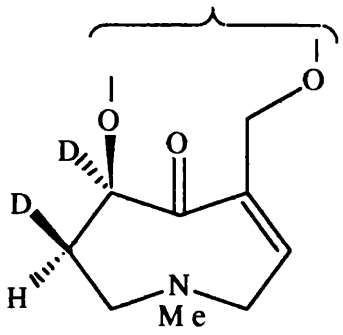
(118)



(119)



(120)



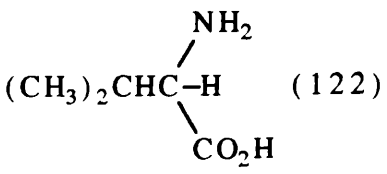
(121)

2.3 Biosynthesis of Pyrrolizidine Alkaloid Necic Acids

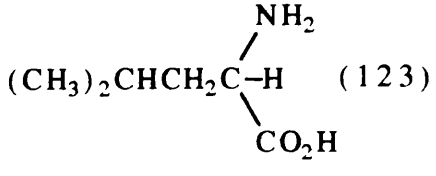
No work on the biosynthesis of necic acids is contained in this volume and therefore only a brief overview of the literature is presented.

The majority of necic acids are C_{10} diacids, e.g. senecic acid (126). It was originally believed that these acids were of terpenoid origin. However, it was shown that [2-^{14}C]-mevalonolactone was not incorporated into retrorsine (24) from *S. isatideus*.[67] [^{14}C]-Acetate was incorporated into necic acids from alkaloids in various *Senecio* species but it was not a specific precursor and the partial labelling patterns obtained by degradation techniques were difficult to interpret.[67-69]

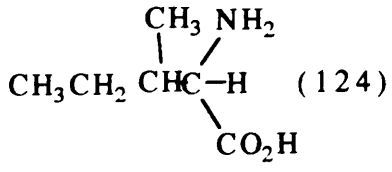
Studies by Crout and others[70-72] have shown that the necic acids originate from α-amino acids, namely valine (122), leucine (123), isoleucine (124) and threonine (125).



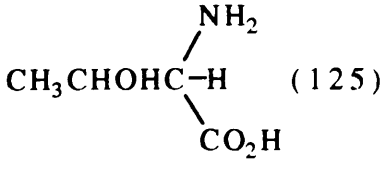
(122)



(123)



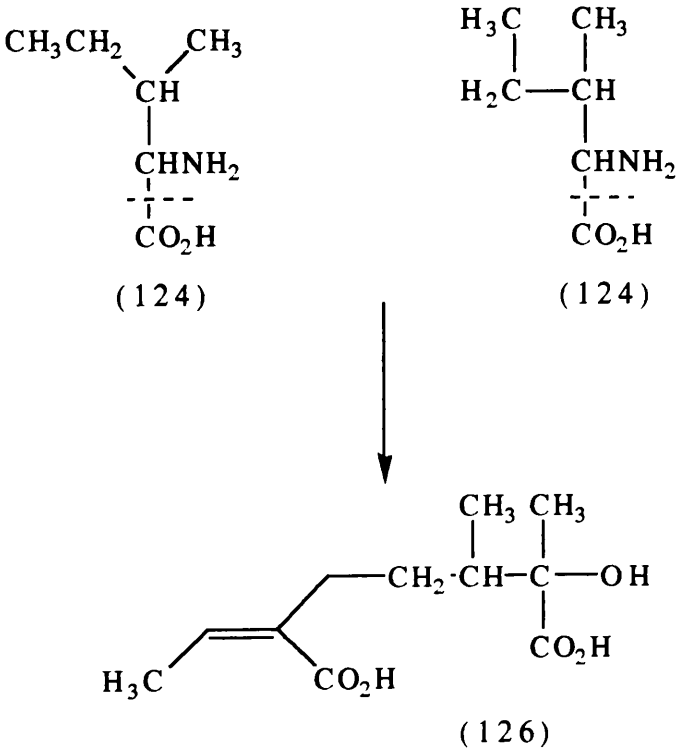
(124)



(125)

The majority of the work in this field has been carried out on senecic acid (126), the acid portion of senecionine (38) from *S. magnificus.* It has been shown that senecic acid is formed from two molecules of L-isoleucine with loss of both carboxyl carbons of isoleucine (Scheme 2.25).[73] No other stereoisomer was utilised in senecic acid biosynthesis.[74] The method by which these L-isoleucine molecules are coupled together is not yet known. However it has been shown that the H-4 *pro*-S is lost and the H-4 *pro*-R is retained from both molecules during this coupling reaction.[75]

Scheme 2.25



No stable isotope studies have been carried out in the examination of necic acid biosynthesis.

2.4 Summary

The overall biosynthesis of pyrrolizidine alkaloid necine bases is given in Scheme 2.26. Proven intermediates are shown in boxes.

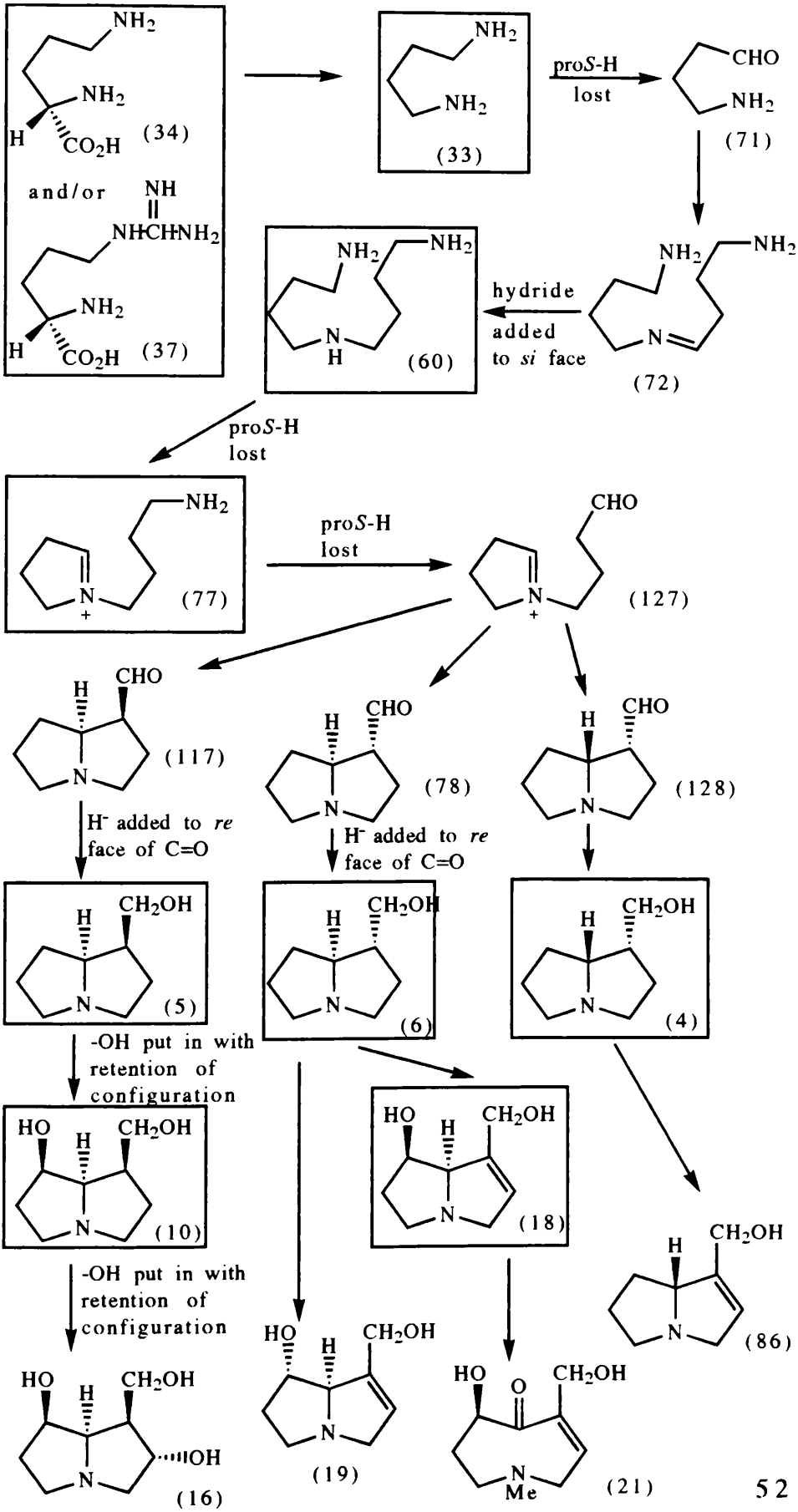
The aminoacids L-ornithine (34) and/or arginine (37) are the starting point for necine biosynthesis. Decarboxylation of ornithine yields putrescine (33) which is probably oxidised to 4-aminobutanal (71) with loss of the C-1 *pro*-S hydrogen. 4-Aminobutanal can then couple to another molecule of putrescine to give imine (72). Reduction of the imine by addition of hydride to its *si*-face furnishes homospermidine (60). Oxidation of this diamine (60) again proceeds with loss of the *pro*-S hydrogen. Cyclisation of the aldehyde formed produces iminium ion (77) which is oxidised again with loss of the *pro*-S hydrogen.

It is at this stage that divergence in the necine biosynthetic pathways occurs. Aldehyde (127) undergoes completion of a Mannich-type cyclisation to produce the stereoisomers (117), (78) and (128). Reduction of these aldehydes produces (-)-isoretronecanol (5), (-)-trachelanthamidine (6) and (+)-isoretronecanol (4) respectively. In the case of aldehydes (117) and (78) it is known that hydrogen is added to the *re*-face of the carbonyl during reduction. (-)-Isoretronecanol (5) is converted into rosmarinecine (16) *via* platynecine (10) by hydroxylation at C-2 and C-7. These hydroxylations both occur with retention of configuration. Trachelanthamidine (6) is transformed into retronecine (18) by hydroxylation, with retention of configuration, at C-7 and introduction of a 1,2-double bond in which the *pro*-S hydrogen at C-2 of trachelanthamidine is lost. Retronecine (18) is the direct precursor of otonecine (21).

(-)-Trachelanthamidine is a precursor of (+)-heliotridine (19) and (+)-isoretronecanol is a precursor of (+)-supinidine (86). The stereochemistry of the processes involved in the biosynthesis of necines (19) and (86) is not known.

The precursors of the pyrrolizidine alkaloid necic acids are the α-amino acids valine (122), leucine (123), isoleucine (124) and threonine (125).

Scheme 2.26


(34)
and/or
(37)
(33)
proS-H lost
(71)
(72)
hydride added to si face
(60)
proS-H lost
(77)
proS-H lost
(127)
(117)
(78)
(128)
H⁻ added to re face of C=O
H⁻ added to re face of C=O
(5)
(6)
(4)
-OH put in with retention of configuration
(10)
(18)
-OH put in with retention of configuration
(86)
(19)
(16)
(21)

CHAPTER 3

Isolation of Pyrrolizidine Alkaloids from Plants and Root Cultures

3.1 Introduction

There are a number of reasons for examining new plant species to determine if they contain pyrrolizidine alkaloids. It is necessary to ascertain which plants contain toxic pyrrolizidine alkaloids. This should allow for a reduction in the consumption of these plants by humans and livestock. New alkaloids discovered can be biologically tested and the results used to enhance our understanding of their useful biological activity and also their toxicity.

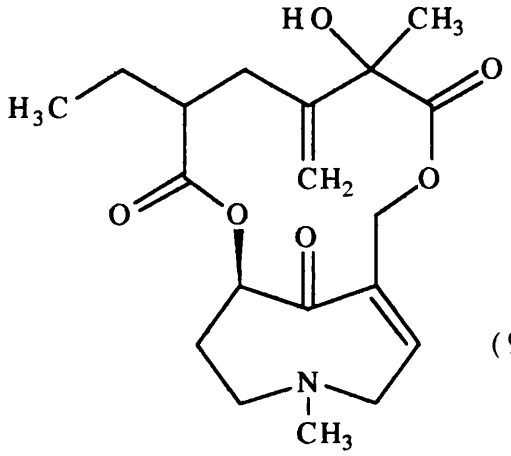
It has to be noted that nature often provides template models for biologically active compounds for synthetic work, leading to the production of natural products and analogues with useful biological activity. The pyrrolizidine ring structure was not known before its discovery in these alkaloids.[3] New alkaloids, enhancing the diversity of structure in the necine and necic acid, are likely to provide further inspiration for the synthesis of new analogues.

Unstudied species are therefore suitable for investigation to determine which, if any, alkaloids they produce.

In the last few years our research has turned to the study of root cultures derived from plants which produce pyrrolizidine alkaloids. Originally root cultures were very slow growing but recently they have been genetically engineered to increase the rate of growth.[76] The bacterial plant pathogen *Agrobacterium rhizogenes* infects plant cells by transferring a segment of DNA contained in its root-inducing plasmid into the plant cell nucleus. This piece of transferred DNA expresses bacterial genes which cause the infected plant cell to behave like a root cell. When a plant is infected with *A. rhizogenes* 'hairy roots' arise at the site of inoculation. These can be

removed and established in culture after antibiotic treatment to kill the remaining bacteria. These re-engineered roots are termed transformed roots.

Root cultures of *Senecio vulgaris* are known to synthesize and store pyrrolizidine alkaloids.[77] Of the transformed root cultures established for our group by Dr N. J. Walton of the Institute of Food Research, Norwich, no pyrrolizidine alkaloids could be detected in cultures of *Senecio pleistocephalus, Crotalaria lachnosema, Cynoglossum australe* or *C. officinale* grown under a variety of conditions.[78] However, cultures of *Emilia flammea* did produce pyrrolizidine alkaloids.[78] Emiline (94) was isolated and identified by comparison with authentic material.



(94)

The yield of emiline was increased from *ca.* 0.005% in fresh plant leaves[79] to approx. 0.02% in fresh transformed root cultures after 15-20 days growth.[78] A specific incorporation of ^{14}C of 6% per C_4 unit was obtained when [1,4-^{14}C]putrescine was added to the cultures after 7 days growth and the cultures were left for a further 10-15 days.[78] (Specific radiolabel incorporation per C_4 unit for a putrescine precursor was calculated from {[molar activity of emiline x 0.5]/[molar activity of precursor]} x 100%.) This incorporation figure was much higher than the value obtained from intact plants of 1.9% per C_4 unit.[56] The high specific incorporation achieved allowed for the use of precursors labelled with stable isotopes to study

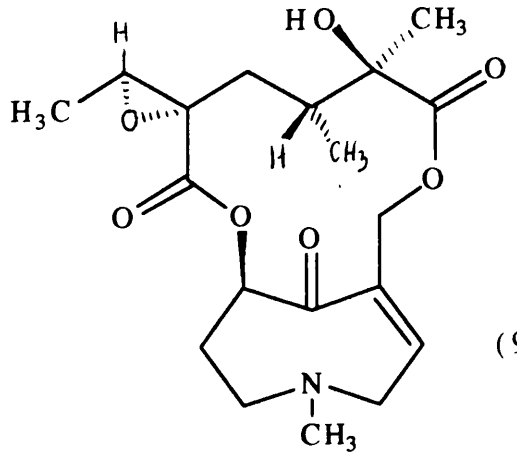
details of the biosynthesis of emiline.[66] This work would have been more difficult to carry out using the intact plants.

As transformed root cultures looked promising for biosynthetic studies we wished to obtain cultures of other species which produce pyrrolizidine alkaloids, to check that they produce pyrrolizidine alkaloids and to discover if these cultures also give high incorporations of precursors.

3.2 *Emilia flammea* Transformed Root Cultures

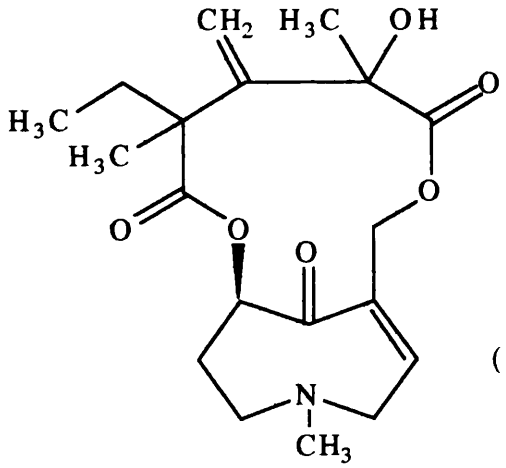
Transformed root cultures of *Emilia flammea* Cass. (Asteraceae, formerly Compositae) have been examined[78] and used in biosynthetic studies[66] by our group. Towards the end of these studies changes were observed in the alkaloid content of the culture which called for it to be re-examined.

The species *E. flammea* is a native of India and China and has been cultivated in Europe. It was originally investigated[80] for its pyrrolizidine alkaloid content because of its use in folk medicine in India, Burma, Indonesia and South Africa.[81] In this first study of the species by Polish workers,[80] a number of alkaloids were isolated from the aerial parts and roots of the plant in *ca.* 0.045% yield based on the dry weight of plant material. The only alkaloid identified was otosenine (129) which was found in all parts of the plant except the leaves.



(94)

Further work[82] extended these studies and a new alkaloid, which was named emiline, was found in the above ground parts of the plant. The structure (130) proposed for emiline based on IR, ^{1}H NMR and mass spectral data was that of an 11-membered macrocyclic diester of otonecine (21). The structure was re-examined[79] when an anomaly came to light. The original emiline structure put forward requires four methyl signals in the ^{1}H NMR spectrum but there were only three present. Barbour and Robins[79] ascertained that (94) was the correct structure of emiline.



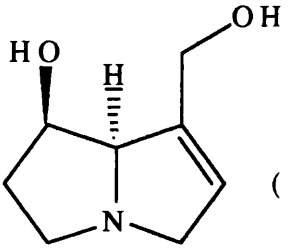
(130)

Emilia flammea hairy root cultures were established by Dr N. Walton.[83] They were derived from *E. flammea* plants in which the major alkaloidal constituent was emiline.[79]

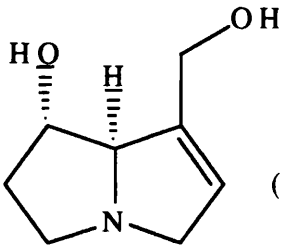
In our work, after 21 days growth the roots were ground up and extracted with methanol. The extract was then taken up in dilute acid solution. After washing with dichloromethane, the solution was stirred with zinc powder to remove any *N*-oxides, then filtered, basified and extracted with dichloromethane. The crude alkaloid extract obtained was examined by thin layer chromatography (TLC) on silica, eluting with chloroform/methanol/conc. ammonia (85:14:1, v/v/v), and visualising with the modified Dragendorff reagent.[84] This is the standard system for the TLC analysis of pyrrolizidine alkaloid mixtures.[5] The extract was shown to be a mixture of

two components with R_f values of 0.54 and 0.30. These were separated on a neutral alumina gravity column.

The component of R_f 0.54 was shown by 200 MHz ^1H NMR spectroscopy to be a 3:1 mixture of two alkaloids. Repeated recrystallisation from dichloromethane/acetone (1:1, v/v) gave the major alkaloid in 0.004% yield based on the weight of fresh roots. The mass spectrum for this alkaloid showed peaks at *m / z* 111 and 80 indicating that the necine was either retronecine (18) or heliotridine (19).[4]

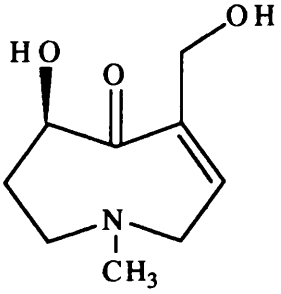


(18)



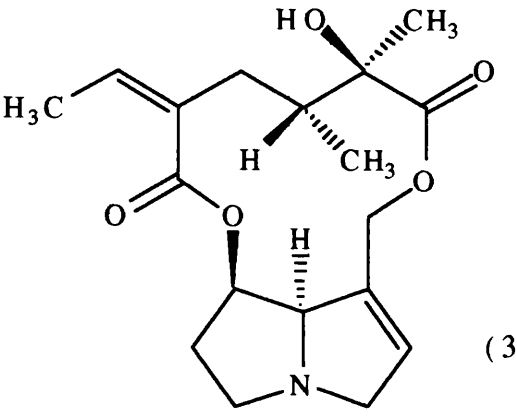
(19)

This ruled out emiline and otosenine, the known plant alkaloids, as the unidentified alkaloid because they both contain the necine otonecine (21). A high resolution mass spectrum showed the molecular formula to be $C_{18}H_{25}NO_5$.



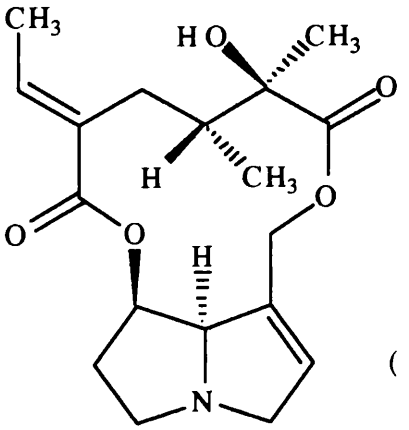
(21)

A search of the literature showed that only two other species of *Emilia* had been studied. *E. sonchifolia* DC. produces the pyrrolizidine alkaloid senecionine (38).[11] This alkaloid is a diester of retronecine and has the required molecular formula. A comparison of the ^1H and ^{13}C NMR spectra of senecionine[85] with those of this alkaloid showed that it was indeed senecionine. The melting point and optical rotation were the same as those for senecionine[3] confirming this identification.



(38)

A re-examination of the data for the alkaloid mixture of R_f 0.54 suggested that the minor alkaloid might be very similar to senecionine. The 200 MHz ^1H NMR spectrum showed one major difference between the two alkaloids. The spectrum for senecionine showed a quartet at δ 5.73 which corresponds to the vinylic proton at position C-20. In the spectrum of the mixture, the quartet for the minor alkaloid appeared at δ 6.53. From this it seemed probable that the minor alkaloid was the C-20 geometrical isomer of senecionine, called integerrimine (131). A comparison of the literature ^1H and ^{13}C NMR data of integerrimine with that for the minor alkaloid proved unambiguously its identity. The deshielding influence of the C-16 carbonyl group of integerrimine on the 20-H means that the proton on C-20 shows resonance at 1 ppm lower field than in senecionine.[86]



(131)

The third alkaloid present was easily identified. The mass spectrum showed peaks at *m / z* 168, 151, 150, 122, 110 and 94

which indicated that the alkaloid was an ester of otonecine (6).[4] It had a melting point of 106-109 °C which is the same as the melting point of emiline.[82] Other spectral data confirmed that it was emiline (94), and it was obtained in 0.006% yield.

A study was carried out to examine the changes in the alkaloid content of the root cultures with age. Forty 250 ml flasks each containing several root tips and 100 ml of culture medium were set up. Batches of five flasks were taken off at intervals over a one month period. The roots were extracted as before and the crude alkaloid extracts were analysed by 200 MHz ^{1}H NMR spectroscopy. From this, the types and relative amounts of alkaloids present could be determined (Table 3.1).

Table 3.1: The alkaloid content of *E. flammea* transformed root cultures.

Five flasks per batch. Each flask contained 100 ml of medium.

Age of batch (d)	Fr. wt. roots(g)	Wt. alk. ext. (mg)	Alkaloids s : e : i*
5	4.07	2.7	none
9	5.84	8.4	trace
15	58.6	21.5	4.3: 2.1: 1
17	45.4	21.2	4.8: 3.5: 1
21	69.3	37.5	5.1: 5.5: 1
23	62.1	53.6	5.9: 6.1: 1
26	66.6	24.0	5.3: 5.4: 1
29	76.1	34.0	5.7: 6.3: 1

*s = senecionine, e = emiline, i = integerrimine.

From the figures it can be seen that the roots do not start to produce alkaloids until they have been in culture for about nine days. Senecionine (38) is the predominant alkaloid in the early days of growth but after 21 days in culture, emiline (94) is the major alkaloid.

Transformed root cultures are regarded as very stable systems so the production of another alkaloid was quite unexpected. The yield of emiline was originally 0.02% based on the weight of fresh roots.[78] The total alkaloid yield was now 0.01%. Therefore the synthesis of pyrrolizidine alkaloids by the cultures has been considerably reduced. The emiline yield at 0.006% is equivalent to that found in fresh plant leaves.[79] The reason for these changes is not known at present.

The precursor administered to the root cultures to determine the incorporations was [1,4-^{14}C]putrescine dihydrochloride. Feeding was carried out by dividing the sample among the flasks containing the root cultures. When the roots were five days old, approximately 5 μCi of [1,4-^{14}C]putrescine dihydrochloride were added into the culture medium of 20 flasks. The flasks were left for varying times after feeding to investigate the effect of time on the incorporation of label. So, five, nine, twelve and fifteen days after feeding the roots were harvested and the alkaloids were extracted (Table 3.2).

Table 3.2: Incorporation of [1,4-^{14}C]putrescine dihydrochloride into alkaloids in *Emilia flammea* transformed root cultures.

Five flasks per batch. 4.8 μCi of precursor were fed to 20 flasks containing five day old roots.

Age of batch (d)	10	14	17	20
Fresh wt. roots (g)	50.1	92.1	88.7	76.6
Crude alk. ext. Wt. (mg)	14.9	17.7	26.9	21.7
Crude alk. ext.(mg/g fr. wt.)	0.30	0.19	0.30	0.28
Activity recovered in alkaloid extract (μCi)	0.119	0.126	0.114	0.160
Total incorporation(%)	10.0	10.6	9.6	13.4

The cultures were fed after five days growth because this is the start of their rapid growth phase.

The total incorporation was constant at *ca.* 11% throughout the time of the study. This seems to indicate that the [1,4-^{14}C]putrescine dihydrochloride was transformed into the alkaloids within five days after feeding.

$$\left(\text{Total incorporation} = \frac{\text{Amount of radioactivity in alk.}}{\text{Amount of radioactivity fed}} \times 100\%\right)$$

Radioscanning of the crude alkaloid extracts on TLC plates eluted as before gave two bands coincident with emiline and senecionine. In all four cases the band corresponding to emiline had a higher activity than the one for senecionine.

The alkaloids from the ten day old batch were separated and their specific activities were found to be 1.88 μCi mmol^{-1} for emiline and 0.54 μCi mmol^{-1} for senecionine.

$$\left(\text{Specific activity} = \frac{\text{No. of μCi radioactivity in alk.}}{\text{No. of mmol of alkaloid}} \text{ in μCi mmol}^{-1}\right)$$

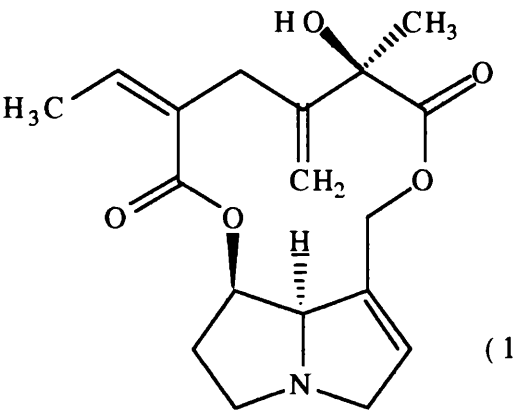
Although the *E. flammea* root cultures now produce less alkaloid, the high total incorporation and specific activities achieved mean that they are still good systems for biosynthetic studies.

This work has been published.[66,98]

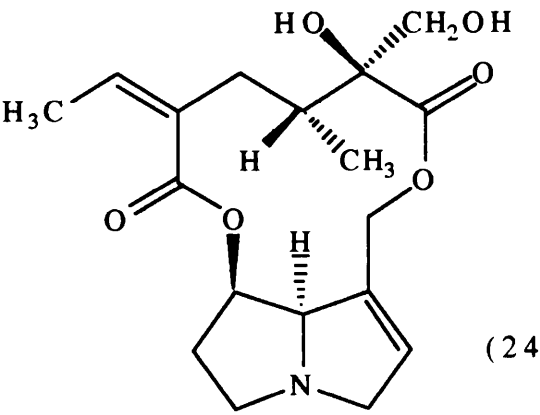
3.3 *Senecio vulgaris* Transformed Root Cultures

Senecio vulgaris, or the common groundsel as it is more usually known, is the species in which the first pyrrolizidine alkaloid was discovered.[87] This species has since been shown to contain the four alkaloids senecionine (38), integerrimine (131), seneciphylline (132) and retrorsine (24).[88] *S. vulgaris* is a self-pollinating species and therefore the alkaloid composition in the plant varies greatly from one location to the

next. This is especially true for the relative abundances of senecionine and seneciphylline.[89]



(132)



(24)

Normal root cultures of *S. vulgaris* have been established and studied by Hartmann and Toppel.[77] They found the same alkaloid profile in the root cultures as in the roots of the intact plants from which the cultures were derived. All four alkaloids were present in the form of their *N*-oxides. In the 14 day old root cultures senecionine *N*-oxide (86%) and its geometrical isomer integerrimine *N*-oxide (14%) accounted for over 99% of the total alkaloids. After 22 days in culture both seneciphylline *N*-oxide (13%) and retrorsine *N*-oxide (4%) could be detected.

On feeding [1,4-^{14}C]putrescine dihydrochloride to the normal root cultures, total incorporations into the alkaloids of between 20 and 30% were obtained.[77] Other biosynthetic

precursors such as L-[U-^{14}C]arginine, L-[U-^{14}C]ornithine, [^{14}C]spermidine and L-[U-^{14}C]isoleucine were also incorporated into the alkaloids although surprisingly [^{14}C]spermine was not.[77]

We set out to establish whether transformed root cultures of *S. vulgaris* produce pyrrolizidine alkaloids. If so, we intended to study the incorporations of precursors to see if they were as high as those found for the normal root cultures.

S. vulgaris transformed root cultures were given to us by Dr. Walton. They were established as described before.[83]

At three weeks old the roots were filtered from the medium, washed with tap water and extracted as reported for *E. flammea* root cultures. The total amount of alkaloid obtained was *ca.* 0.7 mg per g fresh weight of roots which is the same amount as found in normal root cultures.[77] Examination of the alkaloid extract by TLC showed it to have one alkaloidal component of R_f 0.54. Analysis by 200 MHz ^{1}H NMR spectroscopy showed the extract to contain more than 95% senecionine with the rest being integerrimine. Seneciphylline and retrorsine were not present. Senecionine was purified by repeated recrystallisation from dichloromethane/acetone (1:1, v/v).

The investigation to determine the total incorporation of [1,4-^{14}C]putrescine dihydrochloride into the alkaloids was carried out as for *E. flammea* root cultures (Table 3.3).

Again high incorporations were observed. They reached a peak ten days after feeding and then started to decline slowly. In the 11 day old batch some contamination occurred which resulted in poor growth and a lower than expected total incorporation. Radioscanning on TLC plates of the crude alkaloid extracts, showed one band coincident with authentic senecionine. (Integerrimine and senecionine are not separable by TLC.)

Recrystallised senecionine showed a specific activity of *ca.* 1.3 μCi mmol^{-1}.

Table 3.3: Incorporation of [1,4-^{14}C]putrescine dihydrochloride into alkaloids in *Senecio vulgaris* transformed root cultures.

Five flasks per batch. 5 μCi of precursor were fed to 20 flasks containing five day old roots.

Age of batch (d)	11	15	19	21
Fresh wt. roots (g)	16.6*	45.8	55.6	106.8
Crude alk. ext. Wt. (mg)	17.2	42.8	55.6	70.2
Crude alk. ext. (mg/g fr. wt.)	1.04	0.93	0.70	0.66
Activity recovered in alkaloid extract (μCi)	0.089	0.149	0.130	0.126
Total incorporation(%)	7.2	11.9	10.4	10.1
Specific activity senecionine (μCi mmol^{-1})	-	1.30	1.13	1.52

* poor growth

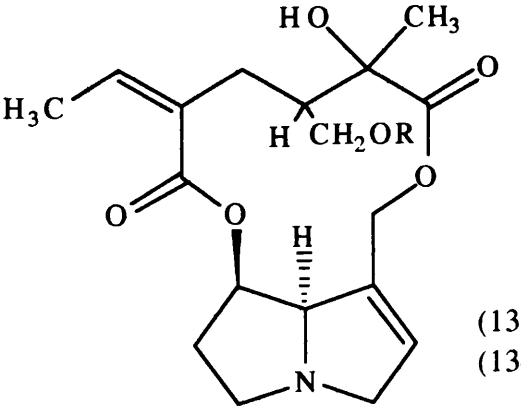
Further studies, examining the timing of feeding of the precursor and the concentration at which the precursor is fed, are required to try and increase the incorporation levels to those achieved in the normal root cultures.[77]

The high yield of senecionine of about 0.03% based on fresh weight of roots, makes these cultures a good alkaloid source. The cultures possess a simpler alkaloid profile than that found in *S. vulgaris* plants. This makes analysis at the end of a biosynthetic study easier as separation problems are eliminated. Again total incorporations and specific activities much higher than those found in plants were observed, making the cultures excellent for the examination of alkaloid biosynthesis.

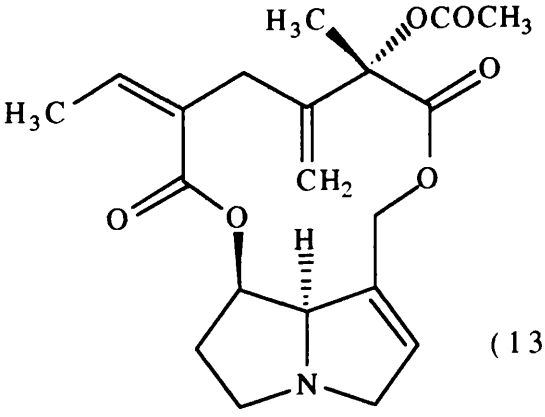
3.4 *Gynura sarmentosa* Plants

The *Gynura* genus has been largely unstudied with respect to its pyrrolizidine alkaloid content. This is despite it being related to *Senecio* species and belonging to the same tribe Senecioneae which is a major source of pyrrolizidine alkaloids. There are about 20 known species of *Gynura*, all of which come from India or the Far East.

The first species studied was *G. scandens*.[90] Wiedenfeld discovered that this species contained two new pyrrolizidine alkaloids.[90] These he named gynuramine (133) and acetylgynuramine (134). The only other species that has been examined is *G. segetum*.[91,92] This was studied because of its use in China for the treatment of malaria and cancer. It was ascertained that this species produced the alkaloids senecionine (38), seneciphyllinine (135), seneciphylline (132) and its geometrical isomer E-seneciphylline (136).[92]



(133) R = H
(134) R = $COCH_3$



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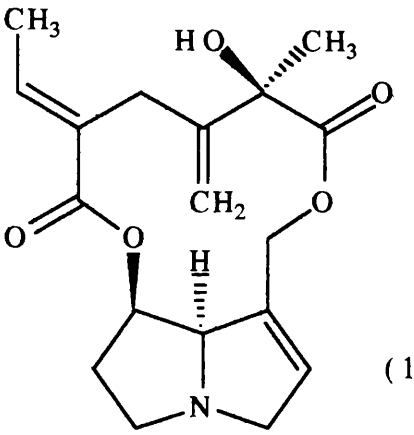


(136)

Gynura sarmentosa DC. is used as a medicinal plant in East and Southeast Asia.[93] In the United Kingdom *G. sarmentosa*, which is a common house plant, is more usually known as the velvet plant because of its purple colour and the texture of its leaves.

The plants were grown from cuttings in a greenhouse and harvested while in flower. Methanolic extraction of the macerated plant material was followed by extraction of the alkaloids using the method employed with *E. flammea* transformed root cultures.

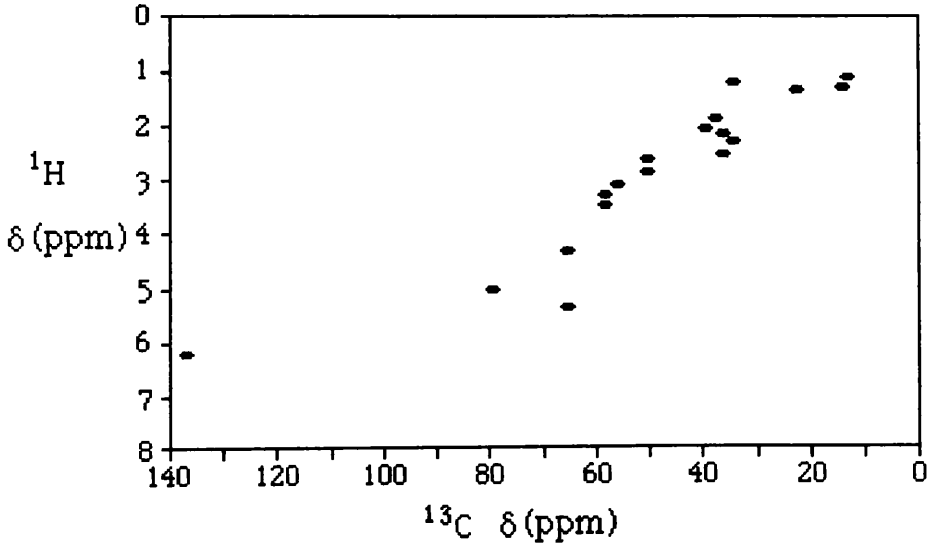
TLC analysis of the crude alkaloid extract showed the presence of three alkaloids with R_f values of 0.38, 0.45 and 0.54. These alkaloids were separated by TLC on a silica plate.

The alkaloid at R_f 0.38 was the most abundant with a yield, based on the weight of fresh plant material, of 0.005%. The mass spectrum of this alkaloid showed peaks at *m / z* 168, 151, 122 and 110 indicating that otonecine (21) was the necine present.[4] High resolution mass spectrometry gave the molecular formula as $C_{1\,9}H_{2\,7}NO_7$. The 200 MHz ^1H NMR spectrum showed the presence of only one vinylic proton. This peak at δ 6.10 corresponded to the proton at the two position of the necine. Therefore the necic acid had to be saturated.

To aid identification of the alkaloid, in addition to 600 MHz ^1H- and 150 MHz ^{13}C-NMR spectra, an HMQC 2D spectrum which gave a one bond δ_H/δ_C correlation, was obtained (Figure

3.1). *I am grateful to Dr I. H. Sadler and the Edinburgh University Ultra High Field NMR Service for these spectra.*

Figure 3.1: HMQC 2D spectrum of otosenine run in $CDCl_3$ showing one bond δ_C/δ_H correlation. 150 MHz ^{13}C and 600 MHz ^1H NMR spectra

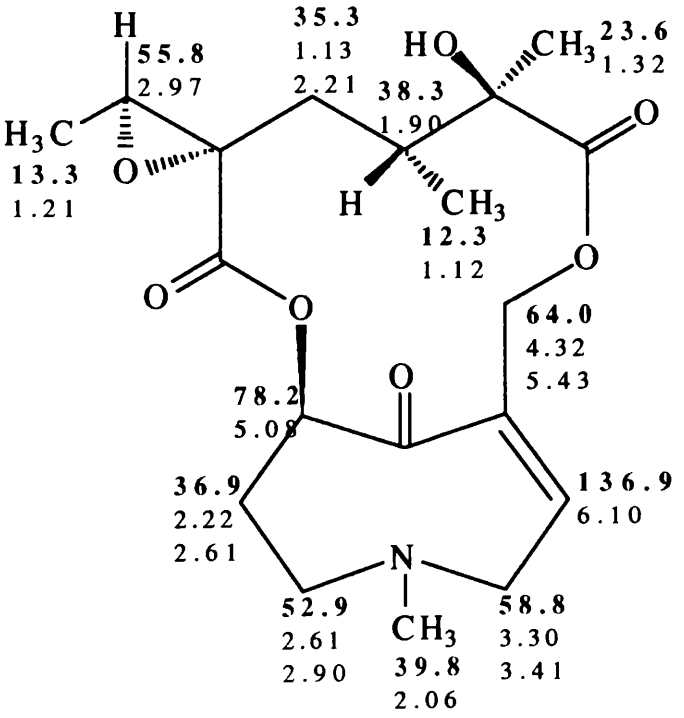


The ^{13}C NMR spectrum showed a CH at δ 55.8 and a quaternary carbon at δ 63.5. These values correspond to the carbons being attached to the oxygen of an epoxide. Further analysis of all the spectra available indicated that the alkaloid was otosenine (129).[94] Petasitenine, which is the β-epoxide isomer of otosenine, was ruled out because it has a melting point of 129-130 °C.[95] The alkaloid isolated had a melting point of 202-204 °C. Some literature assignments for otosenine have been revised on the basis of the HMQC 2D spectrum.[96,97] The ^{13}C and ^1H NMR assignments are shown in Figure 3.2.

The second alkaloid, of R_f 0.45, was obtained in 0.002% yield. The mass spectrum showed that it was also an ester of otonecine (21).[4] The 200 MHz ^1H NMR spectrum was similar to that of otosenine (129), except that two vinylic protons were present. The high resolution mass spectrum gave a molecular

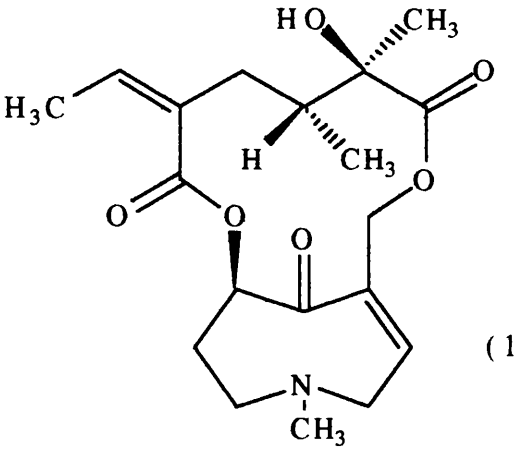
formula of $C_{19}H_{27}NO_6$, i.e. otosenine minus one oxygen. These facts pointed to this alkaloid being senkirkine (137), the parent alkene of otosenine. Comparison of the ^1H NMR, IR and mass spectra with those in the literature corroborated this identification.[96]

Figure 3.2 : 150 MHz ^{13}C and 600 MHz ^1H NMR spectral assignments for otosenine (129), based on HMQC 2D spectrum



^{13}C assignments in **bold**

The final alkaloid, of R_f 0.54, was only found when the plants were harvested while in flower. The structure was elucidated with the aid of 200 MHz ^1H NMR, IR and mass spectra. The alkaloid was identified as senecionine (38) by comparison with authentic material. It was present in 0.001% yield.



(137)

This work has been published.[99]

3.5 Conclusions

Transformed root cultures which produce pyrrolizidine alkaloids are ideal for use in biosynthetic studies. They hold several advantages over plant systems. First of all they are fast growing. A few root tips in fresh medium can reach a biomass of over 150 g l^{-1} in three weeks. The root cultures give higher yields of alkaloid per gram of fresh plant material than plants of the same species. Finally, the total incorporation of biosynthetic precursors is increased substantially. This aids the use of precursors labelled with stable isotopes in biosynthetic studies.

In the longterm it may be possible, through genetic engineering, to increase the production of alkaloids in the root cultures and thus to use plant roots as chemical factories.

Gynura sarmentosa contains hepatotoxic pyrrolizidine alkaloids in low concentration. For this reason the ingestion of this plant because of its beneficial medicinal properties should be discouraged as it may cause liver damage.

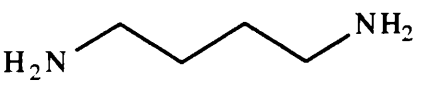
CHAPTER 4

Further Studies on the Biosynthesis of the Pyrrolizidine Alkaloid Necine Bases

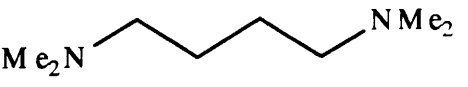
4.1 On the Role of *N*-Acetylputrescine Hydrochloride in Necine Biosynthesis

4.1.1 Introduction

Many derivatives of putrescine (33) are found in nature. For example, tetramethylputrescine (138) has been isolated from *Hyoscyamus muticus.*[100]

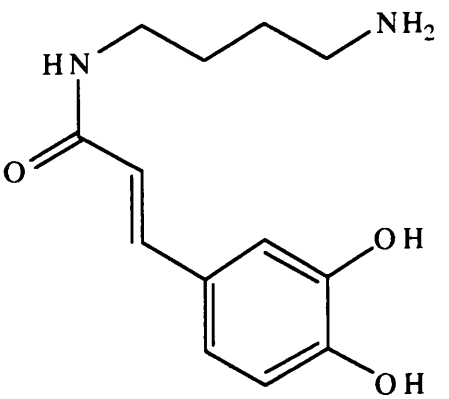


(33)

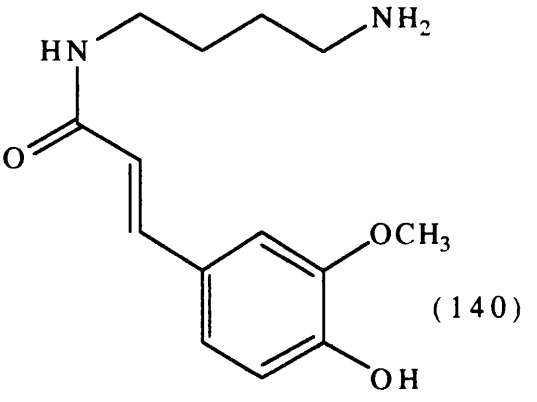


(138)

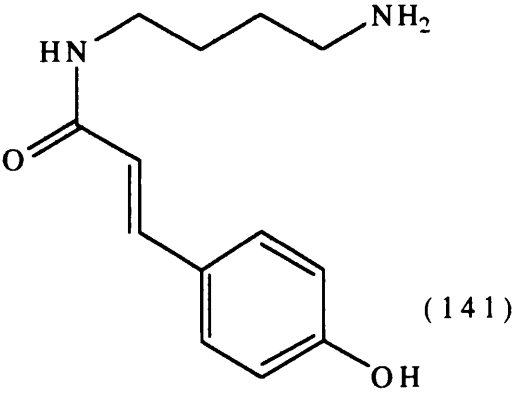
A number of putrescine analogues with amide linkages have been found in plant species. Caffeoyl- (139), feruloyl- (140) and *p*-coumaroyl- (141) putrescine have been reported in callus tissue cultures of *Nicotiana tabacum.*[101]



(139)

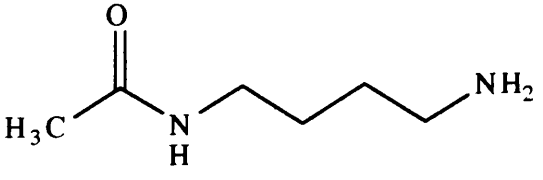


(140)



(141)

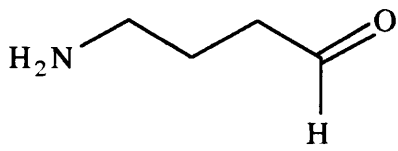
A study of the possible role of the simple amide *N*-acetylputrescine (31) in the biosynthesis of pyrrolizidine alkaloid necine bases was undertaken by Denholm.[53] This putrescine analogue has been isolated many times from animal and bacterial sources.[102]



(31)

Transient derivatisation of putrescine (33) *in vivo* could have a number of purposes. The transport of putrescine within the plant might be improved because *N*-acetylputrescine is not as basic as putrescine and is therefore less likely to act as a

strong positively charged interactive ligand with cell constituents (particularly phosphate residues on DNA). A further consequence of derivatisation may be to prevent the cyclisation of the aminoaldehyde (71), formed when putrescine is oxidised. Amide hydrolysis could then occur further along the biosynthetic pathway.



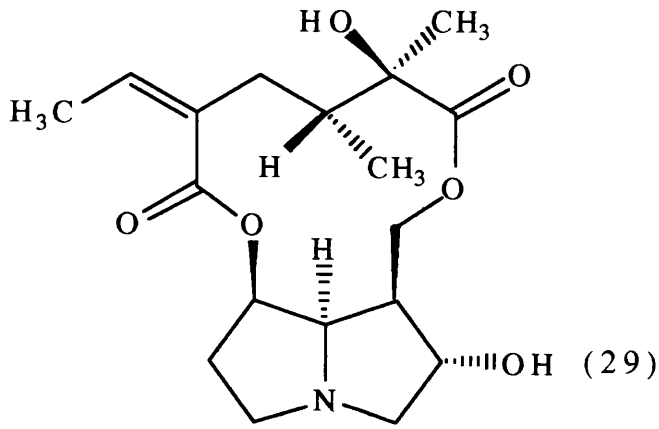
(71)

In initial experiments,[53] *N*-acetyl[1,4-^{3}H]putrescine hydrochloride was fed, along with [1,4-^{14}C]putrescine dihydrochloride as internal standard, to *Senecio pleistocephalus* and *Cynoglossum australe* plants. *N*-Acetylputrescine hydrochloride was formed by treating putrescine in acetic acid with acetic anhydride (0.8 equiv.), followed by acidification. It had previously been shown that when mixtures of [1,4-^{3}H]- and [1,4-^{14}C]-putrescine dihydrochloride were fed to plants which produce pyrrolizidine alkaloids, there was little change to the ^{3}H:^{14}C ratios of the precursor mixture and the alkaloid extract.[65] The results from these experiments are given in Table 4.1.

Table 4.1: Incorporation of *N*-Acetyl[1,4-^{3}H]putrescine hydrochloride into the alkaloids of (i) *S. pleistocephalus* and (ii) *C. australe* with [1,4-^{14}C]putrescine dihydrochloride as reference.

	(i)	(ii)
Amount precursor fed ^{3}H μCi	0.773	0.628
" " " mg	25	20
Amount of alkaloid isolated (mg)	58	17
Initial ^{3}H:^{14}C	1.52	0.707
Final ^{3}H:^{14}C	0.24	12.2
Total ^{3}H incorporation (%)	1.81	1.58

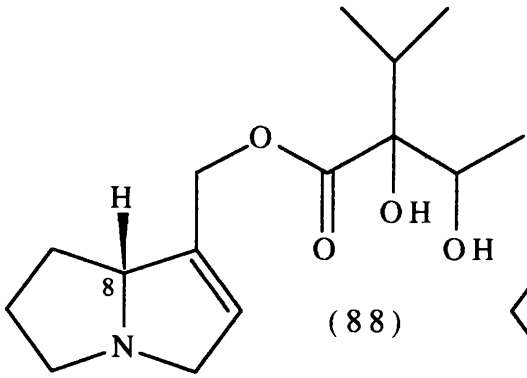
The results indicate that putrescine is a better precursor than *N*-acetylputrescine for rosmarinine (29) in *S. pleistocephalus*, whereas *N*-acetylputrescine is a more efficient precursor than putrescine for alkaloids in *C. australe.*



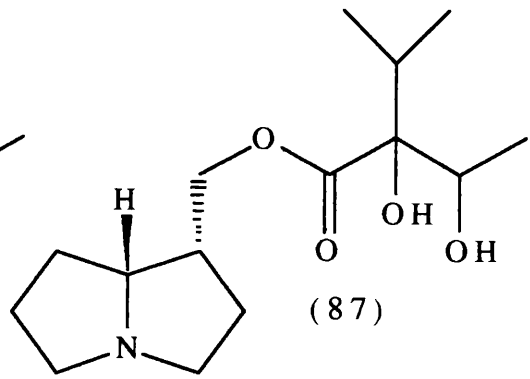
(29)

It was our aim to repeat this study and then extend the work as described below.

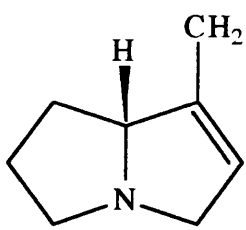
Cynoglossum australe produces two alkaloids,[103] cynaustine (88) and cynaustraline (87), which are ester derivatives of (+)-supinidine (86) and (+)-isoretronecanol (4), respectively.



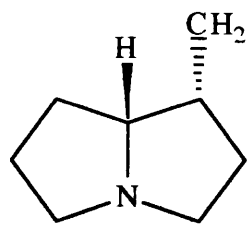
(88)



(87)

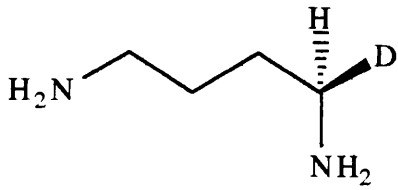


(86)



(4)

Denholm had attempted to study the stereochemical aspects of the biosynthesis of these necines, which have unusual H-8β stereochemistry.[53] Feeding experiments with (1*R*)-[1-^{2}H]putrescine (100) were carried out in an attempt to determine the stereochemical outcome of some of the enzymic processes involved in the biosynthesis of these necines. Unfortunately, no deuterium incorporation into the alkaloids was observed. Since *N*-acetylputrescine seemed to be incorporated more efficiently than putrescine into the alkaloids of *C. australe*, it might be possible to repeat this experiment using an acetylated form of (1*R*)-[1-^{2}H]putrescine (100). This in turn might improve the level of deuterium incorporation into the alkaloids.



(100)

4.1.2 Synthesis of *N*-Acetyl[1,4-^{3}H]putrescine Hydrochloride

The monoacetylation of putrescine was carried out by two different methods. In the first chemical method, putrescine (33) was treated with acetic anhydride (0.8 equiv.) in glacial acetic acid. After acidification with hydrochloric acid, *N*-acetylputrescine (31) hydrochloride was obtained in 23.4% yield.

The second method involved the use of the enzyme porcine pancreatic lipase (PPL). This enzyme can catalyse the acylation of amines in organic solvents. PPL and other lipase enzymes are considered in greater detail in Chapter 6. *N*-Acetylputrescine (31) hydrochloride was prepared by the reaction of putrescine (33) with ethyl acetate catalysed by PPL, followed by acidification. In this reaction ethyl acetate acted as both acyl donor and solvent. Monoacetylation was achieved in

35.2% yield after five days. Diacetylation could be observed, by TLC of the reaction mixture, after six days.

A sample of *N*-acetylputrescine labelled with tritium was prepared by the enzymic method as this gave higher yields. The reaction of [1,4-^{3}H]putrescine dihydrochloride and putrescine with ethyl acetate in the presence of PPL unfortunately gave a chemical yield of only 16.3%. Radioscanning of the product showed two bands, one corresponding to the desired product and the other corresponding to an impurity. *N*-Acetylputrescine hydrochloride was recrystallised twice to remove this impurity further lowering the yield to 5.2%.

4.1.3 Results of Feeding *N*-Acetyl[1,4-^{3}H]putrescine Hydrochloride

Feeding experiments were carried out using [1,4-^{14}C]putrescine dihydrochloride as an internal standard.

A. *S. pleistocephalus*

A mixture of the two precursors was fed, by the wick method, to one plant on one day. After eight days the plant was harvested and the alkaloid was extracted. *S. pleistocephalus* produces rosmarinine (29).[48] The results of this experiment are shown in Tables 4.2 and 4.3. It is apparent from the decrease in the ^{3}H:^{14}C ratio in the alkaloid mixture as compared with the precursor mixture, that putrescine dihydrochloride is a better precursor than *N*-acetylputrescine hydrochloride for rosmarinine (29).

B. *C. australe*

A mixture of the two precursors was fed, by the wick method, to three plants on one day. The plants were harvested eight days later and the crude alkaloid extract was obtained. Separation of cynaustine (88) and cynaustraline (87) was

achieved by preparative TLC. The results for this experiment are given in Tables 4.2 and 4.3.

From the $^{3}H:^{14}C$ ratio for the crude alkaloid extract from *C. australe* it would seem that *N*-acetylputrescine hydrochloride is incorporated to a greater extent than putrescine dihydrochloride. However, once the alkaloids were purified it can be seen that this is not the case. The $^{3}H:^{14}C$ ratio has again decreased and therefore putrescine dihydrochloride is incorporated more efficiently than *N*-acetylputrescine hydrochloride into the alkaloids from *C. australe.*

Table 4.2: Incorporation of *N*-Acetyl[1,4-^{3}H]putrescine hydrochloride into the alkaloids of *S. pleistocephalus* and *C. australe*, with [1,4-^{14}C]putrescine dihydrochloride as reference.

	S. pleistocephalus	*C. australe*
Fresh Wt. Plant (g)	149.4	72.5
Wt. crude alk. ext. (mg)	286	62.7
Amount of precursor fed (mg)	6.5	6.5
" " " ^{3}H (μCi)	8.0	8.0
$^{3}H:^{14}C$ of precursor	5.6	5.6
$^{3}H:^{14}C$ of crude alk. ext.	0.56	9.5
Total ^{3}H incorporation (%)	1.77	0.16

Table 4.3: Incorporation of *N*-Acetyl[1,4-^{3}H]putrescine hydrochloride into the purified alkaloids of (i) *S. pleistocephalus* and (ii) *C. australe*, with [1,4-^{14}C]putrescine dihydrochloride as reference.

	(i)	(ii)	(ii)
Alkaloid	rosmarinine	cynaustraline	cynaustine
R_f	0.32	0.33	0.47
Wt. of alkaloid (mg)	137	19.8	2.3
Specific activity ^{3}H ($\mu Ci\ mmol^{-1}$)	0.223	0.021	0.140
$^{3}H:^{14}C$ of precursor	5.6	5.6	5.6
$^{3}H:^{14}C$ of alkaloid	0.50	3.0	3.4

C. *S. vulgaris* Transformed Root Cultures

Feeding was carried out by dividing a mixture of the two precursors, dissolved in sterile water, among 20 flasks containing five day old roots. The roots were harvested at regular intervals after feeding and senecionine (38) was extracted. The results of this study are given in Table 4.4.

Table 4.4: Incorporation of *N*-Acetyl[1,4-^{3}H]putrescine hydrochloride into senecionine from *S. vulgaris* transformed root cultures, with [1,4-^{14}C]putrescine dihydrochloride as reference.

Five flasks per batch. Precursors fed to 20 flasks containing five day old roots.

Age of batch (days)	11	15	18	21
Fresh Wt. Roots (g)	35.7	62.6	70.9	101.9
Wt. crude alkaloid extract (mg)	25.9	43.5	38.1	42.9
Amount of precursor fed (mg)	2.6	2.6	2.6	2.6
" " " ^{3}H (μCi)	3.23	3.23	3.23	3.23
^{3}H:^{14}C of precursor mixture	10.75	10.75	10.75	10.75
^{3}H:^{14}C of crude alkaloid extract	0.59	0.60	0.85	0.80
Total ^{3}H incorporation (%)	0.31	0.31	0.39	0.43
Wt. of purified senecionine (mg)	3.7	5.2	11.8	9.8
^{3}H:^{14}C of pure senecionine	0.35	0.39	0.17	0.21
Specific activivty of pure senecionine (μCi mmol^{-1})	0.22	0.13	0.17	0.21

The ^{3}H:^{14}C ratio in the alkaloid is lower than that of the precursor mixture and hence it can be deduced that putrescine dihydrochloride is a better precursor than *N*-acetylputrescine hydrochloride for senecionine (38) in *S. vulgaris* transformed root cultures.

D. *E. flammea* Transformed Root Cultures

Feeding was carried out as for *S. vulgaris* transformed root cultures. The results are given in Table 4.5. Separation of emiline (94) and senecionine (38) by preparative TLC proved unsuccessful. The ^{3}H:^{14}C ratios show that putrescine dihydrochloride is a more efficient precursor than *N*-acetylputrescine hydrochloride into the alkaloids from *E. flammea* transformed root cultures.

Table 4.5: Incorporation of *N*-Acetyl[1,4-^{3}H]putrescine hydrochloride into the alkaloids from *E. flammea* transformed root cultures, with [1,4-^{14}C]putrescine dihydrochloride as reference.

Five flasks per batch. Precursors fed to 20 flasks containing five day old roots.

Age of batch (days)	9	14	19	22
Fresh Wt. Roots (g)	14.3	48.0	46.8	46.1
Wt. crude alkaloid extract (mg)	9.6	11.9	13.9	35.4
Amount of precursor fed (mg)	0.93	0.93	0.93	0.93
" " " ^{3}H (μCi)	1.14	1.14	1.14	1.14
^{3}H:^{14}C of precursor mixture	3.44	3.44	3.44	3.44
^{3}H:^{14}C of crude alkaloid extract	0.90	2.00	2.02	1.86
Total ^{3}H incorporation (%)	6.5	14.0	14.6	6.9

4.1.4 Conclusions

Putrescine (33) dihydrochloride was incorporated more efficiently than *N*-acetylputrescine (31) hydrochloride into the alkaloids in all the plant systems studied. In the case of *C. australe* there is obviously another ^{3}H-species present in the crude alkaloid extract which affected the ^{3}H:^{14}C ratio. The erroneous result of Denholm[53] probably occurred because the

separation and purification of cynaustine (88) and cynaustraline (89) could not be achieved.

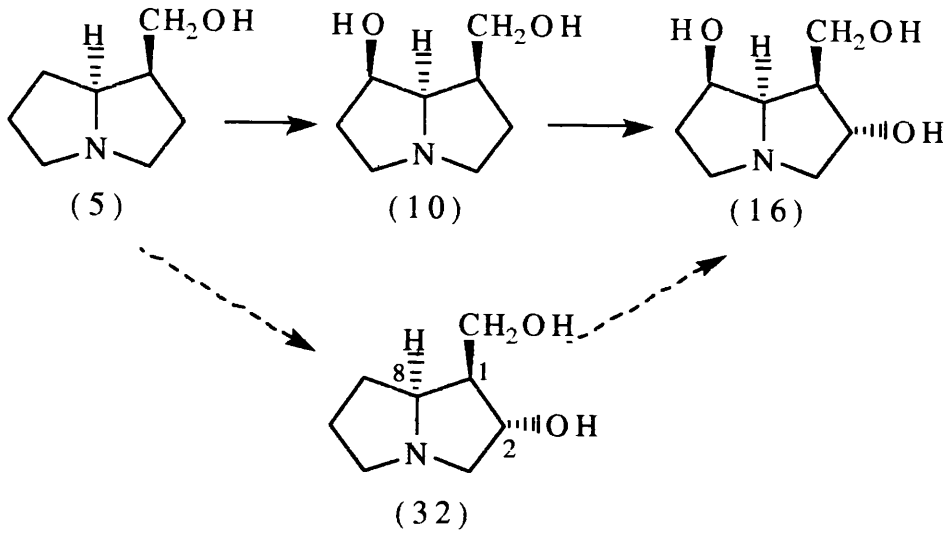
It is clear that *N*-acetylputrescine (31) is not immediately hydrolysed to putrescine (33) on administration to the plants and root cultures. If this occurred then the $^{3}H:^{14}C$ ratio in the alkaloids would be the same as that in the precursor mixture. However, it could be the case that some, if not all, of the incorporation of ^{3}H seen is due to hydrolysis of *N*-acetylputrescine after a period of time in the plant or root culture.

Accordingly feeding of enantiomerically deuteriated samples of *N*-acetylputrescine hydrochloride into *Cynoglossum australe* was not carried out.

4.2 Synthetic Studies Towards a New Diol for Use in Biosynthetic Work

4.2.1 Introduction

The diol 1β-hydroxymethyl-2α-hydroxy-8α-pyrrolizidine (32) was required to probe the biosynthesis of the pyrrolizidine alkaloid rosmarinine (29).[49] Isoretronecanol (4) is an efficient precursor for rosmarinecine (16),[49] the base portion of rosmarinine. The order of oxidations to produce rosmarinine, at C-2 and C-7 of isoretronecanol (4), is not known. It has been shown that platynecine (10) is a precursor for rosmarinine in *S. pleistocephalus*.[52,53] The objective of this work was to synthesize the diol (32) in labelled form in order to feed it to *Senecio pleistocephalus* plants. The result from this feeding experiment should show whether the oxidations of isoretronecanol (4) occur in a specific order or not.



(5) (10) (16)

(32)

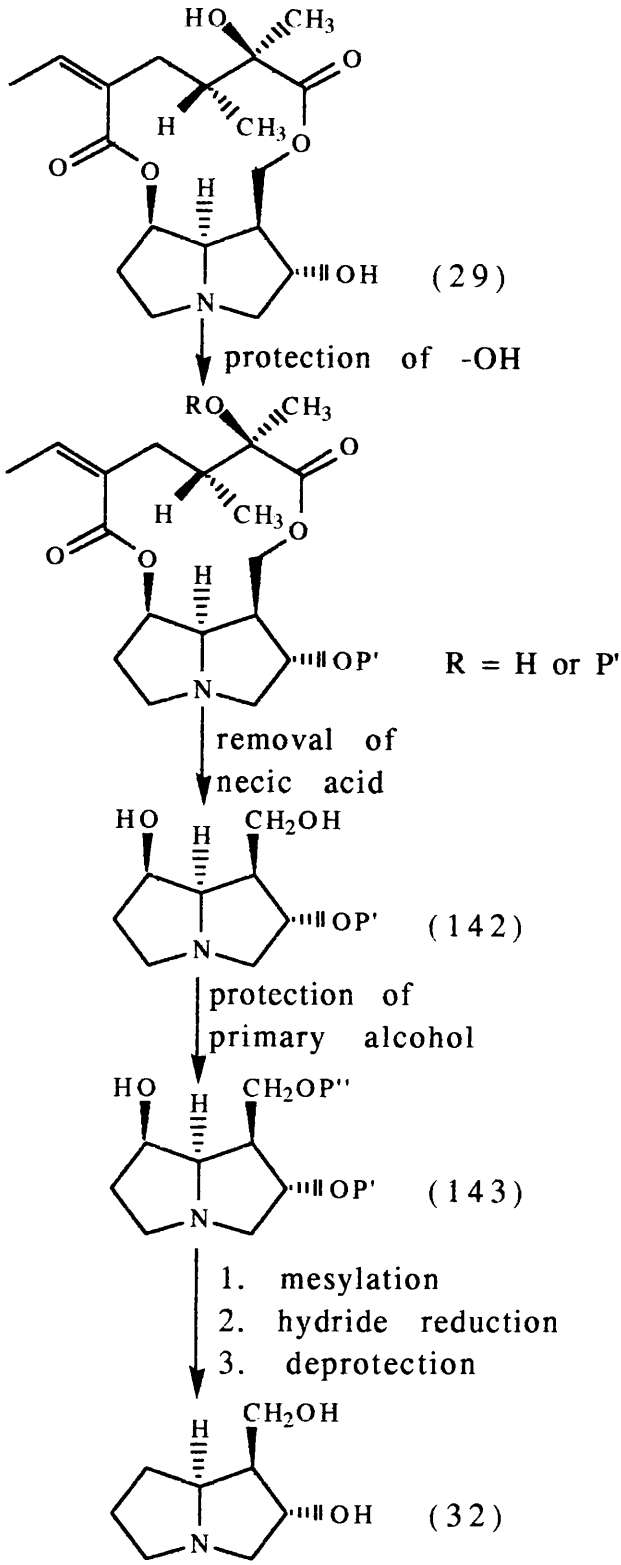
4.2.2 Synthesis of 1β-hydroxymethyl-2α-hydroxy-8α-pyrrolizidine (32)

The synthesis of diol (32) had to allow for the introduction of a radiolabel or a stable isotope label. We possessed a large supply of rosmarinine (29) and for two reasons this made a good starting point for the synthesis. First of all rosmarinine already had the correct stereochemistry at the 1-, 2- and 8-positions. The other advantage of using rosmarinine is that it could be produced in labelled form by feeding a radioactive precursor, such as [1,4-^{3}H]putrescine, to *S. pleistocephalus.*

The overall synthetic plan envisaged is shown in Scheme 4.1. The first step involves protection of the hydroxyl group at the 2-position of rosmarinine. This is followed by removal of the necic acid to give diol (142). Selective protection of the primary alcohol of (142) should allow for elimination of the unwanted hydroxyl at C-7 by mesylation followed by reduction. Deprotection of the alcohol formed would then give the required product (32).

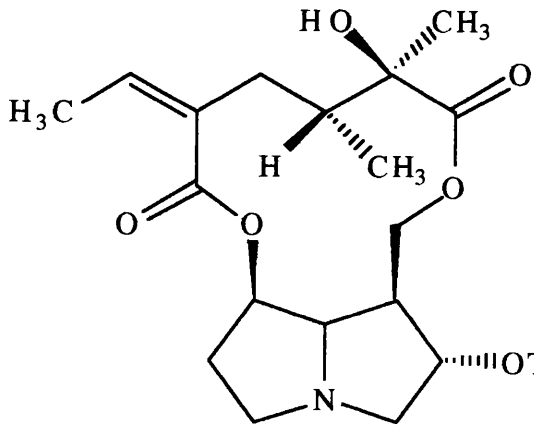
An added advantage of this synthetic scheme is that it is possible to introduce a stable isotope into the product. Reduction of the mesylate with a deuteride source would lead to the introduction of deuterium at the 7α-position of diol (32). It is known that in the biosynthesis of rosmarinine (29),

Scheme 4.1 : Synthetic plan for production of 1β-hydroxy-methyl-2α-hydroxy-8α-pyrrolizidine (32)



hydroxylation at C-7 occurs with retention of configuration.[65] Therefore, if diol (32) is a precursor of rosmarinine, the deuterium would be retained when hydroxylation occurs and should be detected by ^2H NMR spectroscopy.

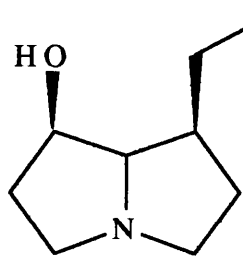
Kelly[52] and Rodgers[104] have both carried out work towards the synthesis of diol (32). In the first stage of the synthesis they looked at protecting the C-2 hydroxyl with groups that were base stable because the necic acid is usually removed by hydrolysis with barium hydroxide. Attempts to form the tetrahydropyranyl (THP) ether and the methoxymethyl (MOM) ether gave back only starting material.[52] The treatment of rosmarinine with methoxyethoxymethyl (MEM) chloride resulted in a mixture of products.[52] However, reacting *tert*-butyldimethylsilyl (TBDMS) chloride with rosmarinine in dimethylformamide (DMF) in the presence of imidazole gave the desired product of 2-*O*-TBDMS-rosmarinine (144).[52,104]



(144)

Unfortunately when the protected rosmarinine (144) was hydrolysed the TBDMS group was removed.[52] This problem was avoided when the ester groups of 2-*O*-TBDMS-rosmarinine were reduced with diisobutylaluminium hydride (DIBAL) to give 2-*O*-TBDMS-rosmarinecine (145).[104] The next stage was the protection of the primary hydroxyl of 2-*O*-TBDMS-rosmarinecine. The reagent chosen for this step had to protect only the primary hydroxyl without affecting the secondary hydroxyl or the tertiary amine. Chaudray and Hernandez had

reported that their silylating reagent of TBDMS chloride with 4-dimethylaminopyridine (DMAP) selectively protected primary alcohols in the presence of other alcohols and amines.[105] Rodgers attempted the protection of 2-*O*-TBDMS-rosmarinecine under these conditions but no reaction occurred.[104] Even using the highly reactive TBDMS-triflate reagent, which is known to silylate a wide range of hydroxyl groups,[106] neither the hydroxyl at C-7 nor C-9 was protected.[104] From this it would seem that both the hydroxyl groups in 2-*O*-TBDMS-rosmarinecine (145) are unreactive. The lack of reactivity shown by the primary hydroxyl might be due to steric hindrance caused by the TBDMS ether.

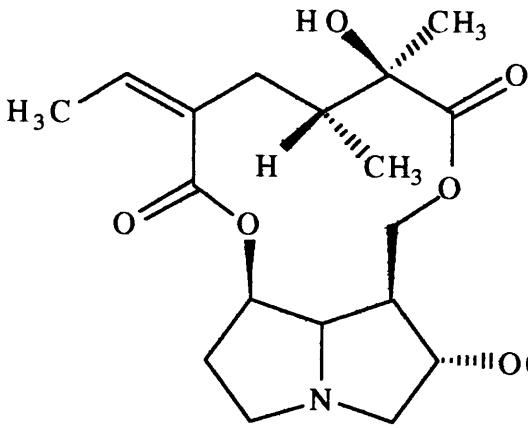


(145)

The starting point for this work was to find an alternative protecting group for the hydroxyl at the two position of rosmarinine as the TBDMS ether was obviously unsuitable. It was thought that methyl ether protection might be appropriate. This ether would be more difficult to remove at the end of the synthesis than the TBDMS ether. However it was hoped that using methyl ether protection would alleviate the problem of protecting the primary hydroxyl in the third stage of the synthesis (see Scheme 4.1) as steric hindrance would be reduced.

Formation of 2-*O*-methylrosmarinine (146) was firstly attempted by adding rosmarinine and methyl iodide to a mixture of powdered potassium hydroxide in dimethylsulphoxide (DMSO). Unfortunately this reaction gave a complex mixture of products. This was possibly due to the fact that rosmarinine reacted with the potassium hydroxide and the solution turned brown before the methyl iodide was added.

Obviously milder conditions were required to carry out this protection to avoid destroying the starting material. An examination of the various methods available to convert an hydroxyl group into a methyl ether showed that one of the mildest and highest yielding involved the use of diazomethane.[107] A catalyst is necessary in cases such as this one, where the hydroxyl is not acidic.



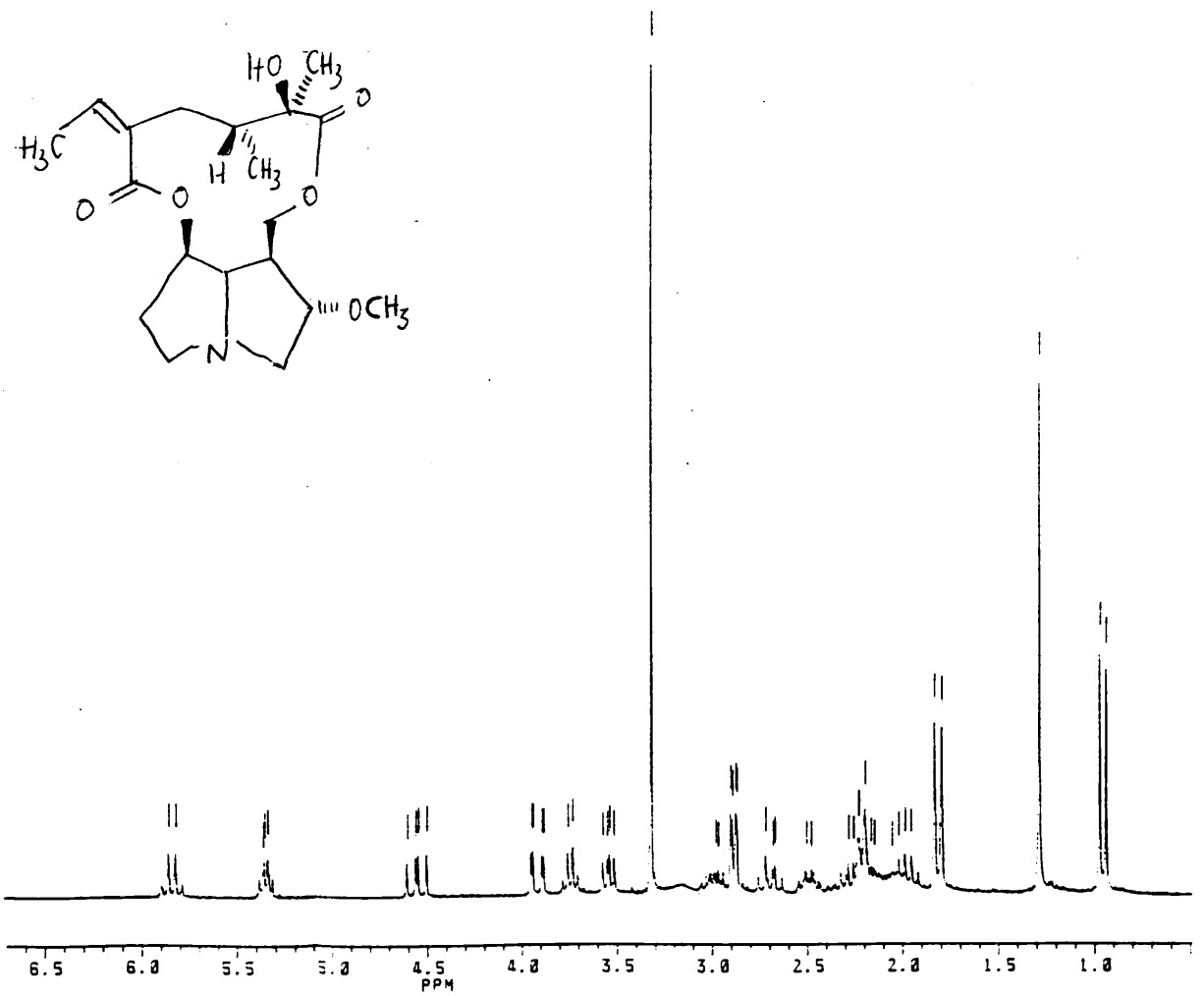
(146)

Rosmarinine, dissolved in dichloromethane, was treated with diazomethane in the presence of a catalytic amount of boron trifluoride etherate.[107] Analysis of the white solid obtained showed it to be starting material.

Another compound that has been used to catalyse this reaction is silica gel.[108] When rosmarinine in dichloromethane was treated with diazomethane in the presence of silica gel, a mixture of two compounds was obtained. These were separated by column chromatography on basic alumina.

The first compound eluted from the column was crystallised to give fine white needles. High resolution mass spectrometry showed it to have a molecular formula of $C_{19}H_{29}NO_6$. Microanalytical data confirmed this formula. This indicated that the compound formed was a monomethylether of rosmarinine. Corroboration of this came from the ^1H NMR spectrum where only one methyl ether singlet was present. The 200 MHz ^1H NMR spectrum of 2-*O*-methylrosmarinine (146) is shown in Figure 4.1. Of note is the extent to which the

Figure 4.1 : 200 MHz ^{1}H NMR Spectrum of 2-*O*-Methylrosmarinine (146)



^1H NMR spectrum had changed with the conversion of the hydroxyl group into a methyl ether. These changes indicated that, in solution, the conformation of both the acid and base portions had altered because of the methylation. For example, the protons at the five position in rosmarinine showed resonance at δ 2.54 and δ 3.25 whereas in the product they came at δ 2.72 and δ 3.04. The 9-H protons in 2-*O*-methylrosmarinine came at δ 3.93 and δ 4.96 while in rosmarinine they were at δ 4.11 and δ 4.88.

The proton at the two position of the product came at δ 3.76 as compared to δ 4.20 in rosmarinine. This indicated that methylation had occurred on the 2-hydroxyl group. Confirmation that the methyl protecting group had become attached to the secondary hydroxyl and not the tertiary hydroxyl came from the ^{13}C NMR spectrum. The C-2 signal in this spectrum appeared at δ 73.8 compared to δ 69.0 for rosmarinine itself. In contrast, the signal for C-12 at δ 75.7 was largely unchanged.

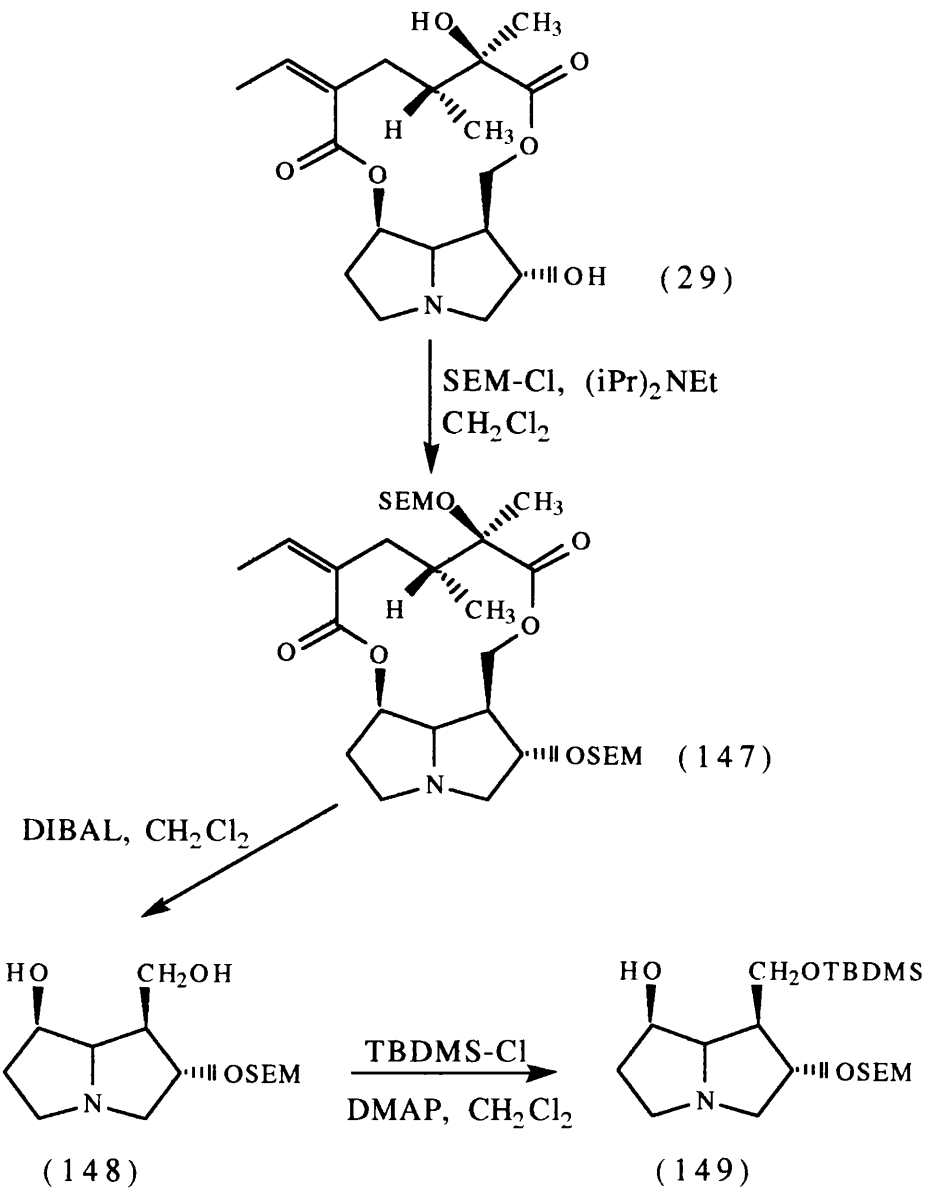
The product was therefore the desired one of 2-*O*-methylrosmarinine (146) but it was obtained in only 6% yield. The other compound isolated from the reaction mixture was shown to be starting material.

The low yield and loss of material (only 60% of the original material was recovered at the end of the reaction) were thought to be due to attachment of the basic pyrrolizidine nitrogen found in both the product and starting material to the silica gel. Attempted removal of any 2-*O*-methylrosmarinine or starting material from the silica gel by stirring with chloroform/methanol/conc. ammonia (85:14:1, v/v/v) gave no success.

As well as silica gel, Ohna and coworkers had used neutral alumina as the catalyst for the methylation of alcohols with diazomethane.[108] It was hoped that using neutral alumina would eliminate any problems of basic compounds sticking to the catalyst. Unfortunately, when neutral alumina was used in place of silica gel no reaction took place.

The methyl ether was therefore not going to be viable as a means of protection because it could not be formed in sufficient quantities.

Scheme 4.2 :
Synthesis of 9-*O*-TBDMS-2-*O*-SEM-rosmarinecine



At this point the protection of hydroxyl groups as the 2-(trimethylsilyl)ethoxymethyl (SEM) ether came to our notice.[109] SEM-ethers can be formed from primary, secondary and tertiary alcohols.[109] This protecting group is not as bulky

as the TBDMS group. Thus it was hoped that, unlike the TBDMS ether, the SEM ether would not hinder protection of the 9-hydroxyl of 2-*O*-protected rosmarinecine (142) (Scheme 4.2).

Protection was achieved by treating rosmarinine with SEM chloride in the presence of diisopropylethylamine.[109] Examination of the yellow oil obtained after work up by TLC , showed it to contain two Dragendorff active compounds with R_f values of 0.32 and 0.15. These were separated by column chromatography on basic alumina.

The less polar compound, which was obtained as a yellow oil, had the same R_f as rosmarinine and was at first thought to be starting material. However examination of this oil by ^1H NMR spectroscopy indicated the presence of two SEM groups. This product was therefore probably 2,12-*O*-diSEM-rosmarinine (147). Attempted crystallisation of this oil from various solvents proved unsuccessful.

Confirmation that both hydroxyl groups in rosmarinine had been protected came from a number of sources. The IR spectrum showed no absorption in the 3 100 - 3 600 cm^{-1} region indicating that no hydroxyl groups were present. Mass spectrometry gave the molecular ion at *m / z* 613 which corresponds to the presence of two SEM groups on rosmarinine. The mass spectrum had peaks at *m / z* 540 (30%) and *m / z* 467 (90%) corresponding to the loss of one $Si(CH_3)_3$ group and two $Si(CH_3)_3$ groups respectively. The 200 MHz ^1H NMR spectrum was too complex for detailed analysis but it could be seen that the singlet for the 18-methyl group was at δ 1.42 as compared to δ 1.32 in rosmarinine itself. This indicated that the hydroxyl group on C-12 was indeed protected. The protection of the 2-hydroxyl group was ratified by the ^{13}C NMR spectrum. The resonance for C-2 had changed from δ 69.0 in rosmarinine to δ 77.2 in the product. All the data indicated that the product was indeed 2,12-*O*-diSEM-rosmarinine (147). The product was furnished in 31% yield. This compound was optically active with an $[\alpha]_D$ of +9.2° as compared with an $[\alpha]_D$ of -85.3° for rosmarinine.[48]

The more polar component of the reaction mixture was not the expected one of monoSEM-rosmarinine. ^1H NMR spectroscopy showed that there was a SEM group present but it was not attached to rosmarinine. The identity of this compound was not pursued due to time restraints.

The next stage of the synthesis was to remove the necic acid to yield 2 -*O*-SEM-rosmarinecine (148). Hydrolysis of the ester functions with barium hydroxide was not attempted as this might have led to loss of the SEM protection. The use of DIBAL to reduce the esters, as had been used in the 2-*O*-TBDMS-rosmarinine (144) case, seemed feasible here. The reduction was carried out at 0 °C in dichloromethane solution and, after work up, the product was purified by neutral alumina dry-column flash chromatography. 2-*O*-SEM-rosmarinecine (148) was obtained as an oil in 90% yield.

High resolution mass spectrometry showed the mass peak at M^+ 303.1865, corresponding to the formula $C_{14}H_{29}NO_4Si$. The base peak at m/z 139 was due to the loss of the SEM ether and an hydroxyl function. There was no sign of a carbonyl ester absorption in the IR spectrum confirming that the esters had been reduced.

The ^1H NMR spectrum showed the presence of only one SEM group with peaks for this at δ 0.01 [$Si(CH_3)_3$], δ 0.92 ($SiCH_2$) and δ 4.63 (OCH_20). The ^{13}C NMR spectrum was similar to that of rosmarinecine (16) except that the C-2 absorption was slightly higher in the protected version and that peaks for the SEM group carbons were present. The compound retained its optical activity with an $[\alpha]_D$ of -58.3° as compared with the rosmarinecine value of -118.6°.[110]

The next step was to protect the primary hydroxyl of 2-*O*-SEM-rosmarinecine (148). In an attempt to simplify the later deprotections the TBDMS group was chosen for this protection.

Using the method of Chaudray and Hernandez[105] on diol (148) gave, in 59% yield, a pale yellow oil. The 90 MHz ^1H NMR spectrum of this oil showed the presence of three singlets in the Si-CH_3 region around δ 0.1 and a singlet at δ 0.93 which

corresponds to a *tert*-butyl group. This pointed to one SEM and one TBDMS group being present in the product. Three Si-CH_3 signals were present because the two Si-CH_3 groups from the TBDMS ether are diastereotopic. They can therefore be nonequivalent and in fact appear as two singlets in the ^1H NMR spectrum.

The IR spectrum was very similar to that of the starting material except the hydroxyl absorption at 3 200 cm^{-1} was reduced. The product was confirmed as being 9-*O*-TBDMS-2-*O*-SEM-rosmarinecine (149) from the mass spectrum which gave the molecular ion at *m / z* 360. This correlates to the diprotected species (149) minus the *t*-butyl group.

Unfortunately the sample had deteriorated before 200 MHz ^1H- and ^{13}C- NMR spectra could be run and therefore the site of the TBDMS group could not be verified. However, as the reaction conditions used were selective for the primary hydroxyl group[105] it can be assumed that it was the 9-hydroxyl, and not the secondary hydroxyl at C-7, that was protected.

The mesylation of alcohol (149) was carried out under standard conditions. 9-*O*-TBDMS-2-*O*-SEM-rosmarinecine (149) was treated with methanesulphonyl (mesyl) chloride and triethylamine at -78 °C. Analysis of the brown residue acquired after work up by ^1H NMR spectroscopy indicated that the TBDMS protecting group had been lost during the reaction. This loss was probably due to traces of acid present in the methanesulphonyl chloride.

Unfortunately at this stage work had to be stopped due to lack of time.

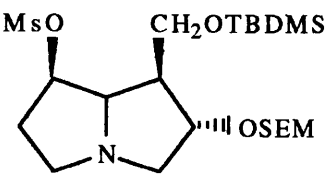
4.2.3 Conclusions

Most of the steps of a useful route have been established. It was a great pity that it was not possible to complete this work due to the nonavailability of 200 MHz ^1H NMR, ^{13}C NMR

and mass spectrometry facilities for three months, due to refurbishment work.

The loss of the TBDMS protection during mesylation of 9-*O*-TBDMS-2-*O*-SEM-rosmarinecine (149) should be avoided by the distillation of the mesyl chloride to remove any acid, prior to its use in the reaction.

After formation of the mesylate, completion of the synthesis should be straightforward. Removal of the 7-hydroxyl would be completed by displacement of the mesylate in alcohol (150) with a hydride donor such as lithium aluminium hydride ($LiAlH_4$). Deprotection to give the desired diol (32) would be accomplished in one step by using tetra-*n*-butylammonium fluoride (TBAF).



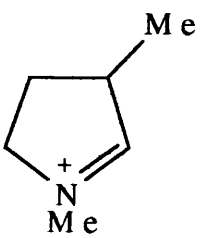
(150)

CHAPTER 5

Biosynthesis of Pyrrolizidine Alkaloid Analogues

5.1 Introduction

The use of plant systems for the production of novel compounds, as an alternative to total synthesis, by the feeding of precursor analogues of natural products has been accomplished by a number of workers. The first transformation of this type was carried out by Rueppel and Rapoport.[111] When 1,3-dimethyl-1-pyrrolinium (151) was fed to *Nicotiana glutinosa* a substituted nicotine was produced. This was identified as 3'-methylnicotine (152). The absolute configuration at C-2' was tentatively assigned to be the same as in nicotine (153) on the basis of circular dichroism and optical rotatory dispersion measurements. The ^{1}H NMR spectrum of 3'-methylnicotine (152) possessed only one methyl doublet indicating that only one of the possible diastereoisomers was present. The absolute configuration at C-3' was not clear.



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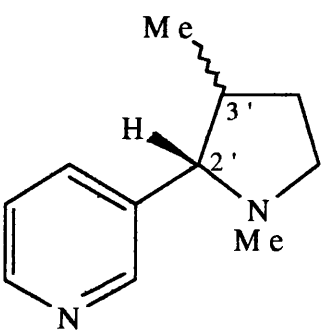

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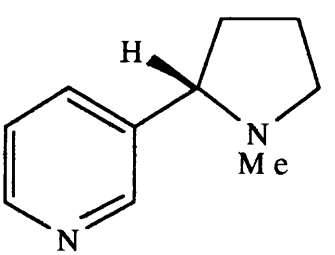
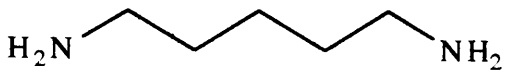


(153)

In unpublished work from our group, Watson[112] and Boswell[113] have formed anabasine analogues by feeding analogues of cadaverine (154) to *Nicotiana rustica* transformed root cultures which produce anabasine (155).



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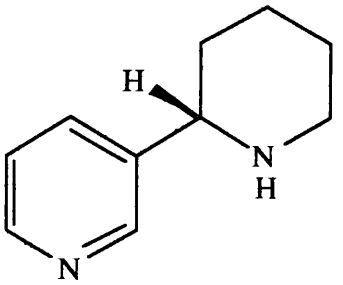


(155)

The feeding of an analogue of a known intermediate on a biosynthetic pathway to a biological system can have a number of outcomes. The unnatural precursor may be carried through the biosynthetic pathway resulting in the production of a natural product analogue, the biological activity of which can be assessed. The modified precursor might be carried through a number of steps on the biosynthetic route and then cause the blocking of a specific transformation and the build up of an analogue of a particular intermediate, which may be identified. However the modified precursor may not be transformed at all. The observation that an enzyme system can transform one unnatural precursor but not another may shed light on the mechanism of a particular enzyme reaction.

Most of the biosynthetic pathways to the necine bases are now established and a number of intermediates have been identified. The challenge now is to try and use these biological pathways for the production of new pyrrolizidine alkaloid analogues which would be difficult to synthesize.

A problem that often arises in this type of work is that although a new compound is produced it is usually accompanied by substantial amounts of the normal natural products. Separation of these is often difficult and tedious.

The object of this work was to use transformed root cultures which produce pyrrolizidine alkaloids to synthesize novel compounds and at the same time to stop the roots producing the normal alkaloids.

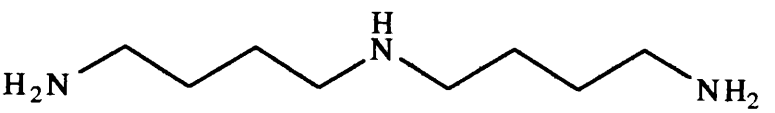
Adolf and Hartmann noticed that when they grew root cultures of *Senecio vulgaris* in the presence of 2-

hydroxyethylhydrazine (HEH) (156), which is an inhibitor of diamine and polyamine oxidases, pyrrolizidine alkaloid biosythesis was strongly inhibited.[114]

$H_2NNHCH_2CH_2OH$

(156)

There was a concomitant accumulation of homospermidine (60) within the roots. This indicated that HEH was inhibiting the oxidation of homospermidine and hence preventing pyrrolizidine alkaloid biosynthesis.



(60)

This system looked ideal for study because the production of pyrrolizidine alkaloids had been stopped but the roots were still growing. Feeding analogues of precursors that occur after homospermidine (60) in pyrrolizidine alkaloid biosynthesis to the inhibited roots might allow for the production of new compounds without any of the undesired normal pyrrolizidine alkaloids being present.

The plan was to repeat the inhibition of pyrrolizidine alkaloid biosythesis by HEH in transformed root cultures of *Senecio vulgaris*. Natural precursors would then be fed to the inhibited root cultures to discover which ones could still be transformed into the major alkaloidal constituent senecionine (38). Finally, analogues of those precursors which were turned into alkaloids could be fed and might be transformed into senecionine analogues. The formation of analogues of senecionine is desirable because it is alkaloids with 1,2-unsaturation in the necine that possess the most interesting biological activity.

5.2 Inhibition of Pyrrolizidine Alkaloid Biosynthesis in *Senecio vulgaris* Transformed Root Cultures.

It was our aim to repeat the work of Adolf and Hartmann.[114] However their paper gave no indication of the concentration at which HEH (156) should be present in the culture medium to stop pyrrolizidine alkaloid production. Therefore a broad range of HEH concentrations was tested as follows.

At subculture of the roots, HEH was added to the culture medium of *S. vulgaris* transformed root cultures. The roots were grown for three weeks then harvested and the crude alkaloid mixtures were extracted. These extracts were examined for alkaloid content using TLC analysis and ^{1}H NMR spectroscopy.

The results from the first experiment (Table 5.1A) indicated that the desired HEH concentration lay somewhere between 10^{-3} M and 10^{-4} M. A further experiment was carried out to ascertain the HEH concentration which would give no pyrrolizidine alkaloids but maximum growth of roots. This study looked at HEH concentrations between 1.6 mM and 8.0 mM (Table 5.1B). The optimum HEH concentration was narrowed down to between 1.6 mM and 3.2 mM. The final study looked at concentrations between these two values (Table 5.1C). The HEH in this experiment which gave maximum root growth with almost no pyrrolizidine alkaloid production was 1.6 mM.

It has to be noted however that the effect of HEH on the root cultures was not totally constant. In the last two experiments the growth of the roots at the HEH concentration of 3.2 mM was markedly different. Also, in the second study there was alkaloid production at the 1.6 mM HEH concentration whereas in the third study there was no alkaloid production at this concentration.

Table 5.1: Effect of HEH concentration on the production of pyrrolizidine alkaloids in *Senecio vulgaris* transformed root cultures.

5 Flasks of each HEH concentration.

Table 5.1A:

Concentration (M)	Fresh weight roots (g)	Wt. alkaloid extract (mg)	Alkaloids
0 (control)	80.04	48.0	+ + +
10^{-1}	0.20	1.1	no
10^{-2}	1.54	2.5	trace
10^{-3}	15.66	17.5	+
10^{-4}	60.48	31.8	+ +
10^{-5}	70.73	32.7	+ +

Table 5.1B:

Concentration (mM)	Fresh weight roots (g)	Wt. alkaloid extract (mg)	Alkaloids
0 (control)	66.74	30.4	+ + +
1.6	62.85	9.3	+
3.2	14.30	8.6	trace
4.8	0.57	1.3	trace
6.4	0.54	0.6	trace
8.0	0.79	0.3	trace

Table 5.1C:

Concentration (M)	Fresh weight roots (g)	Wt. alkaloid extract (mg)	Alkaloids
0 (control)	35.67	47.9	+ + +
1.6	38.87	21.2	trace
2.0	27.18	8.6	trace
2.4	4.40	17.8	trace
2.8	0.65	12.8	trace
3.2	0.50	13.2	trace

+ + + = alkaloid easily seen on 90 MHz ^{1}H NMR spectrum

+ + = alkaloid visible on 90 MHz ^{1}H NMR spectrum

+ = alkaloid just visible on 90 MHz ^{1}H NMR spectrum

trace = alkaloid not visible on 90 MHz ^{1}H NMR spectrum but spot present on TLC.

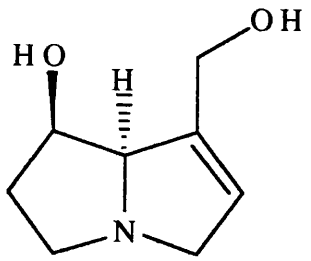
The concentration of HEH chosen for the feeding experiments was 1.6 mM. To account for any variation in the effects of HEH on the root cultures a standard with only HEH present was run in each experiment. *S. vulgaris* transformed root cultures grown in the presence of HEH are termed 'inhibited' because pyrrolizidine alkaloid biosynthesis was inhibited.

5.3 Synthesis and Feeding of Precursors to Inhibited *S. vulgaris* Transformed Root Cultures.

5.3.1 (+)-Retronecine (18)

(+)-Retronecine (18) is the base portion of senecionine (38), the alkaloid normally produced by *S. vulgaris* transformed root

cultures. It was wished to feed this to the cultures to examine whether, in the presence of HEH, the roots were still able to synthesize senecic acid (126) and to carry out the dilactonisation between this necic acid and retronecine.



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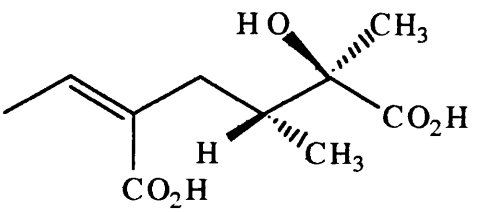
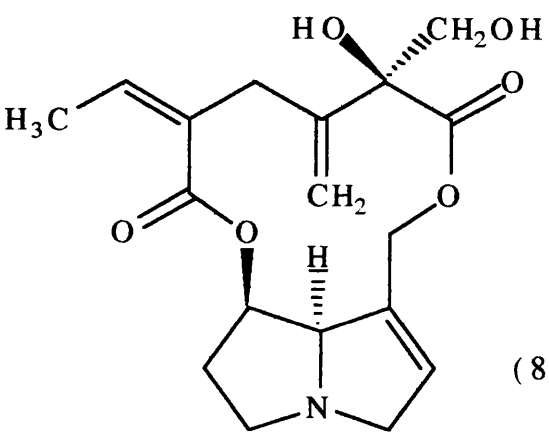


(126)

(+)-Retronecine (18) was formed by the basic hydrolysis of mother liquors from recrystallisations of riddelliine (85) from *Senecio riddellii* kindly given to us by Dr R. J. Molyneux, USA.



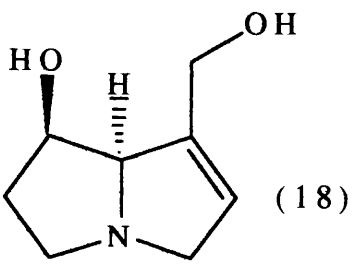
(85)

S. vulgaris transformed root cultures were set up with HEH present at a concentration of 1.6 mM. Retronecine was dissolved in sterile water and divided among the flasks to give a final retronecine concentration in the medium of 1.0 mM. After 21 days the roots were harvested and the alkaloids were extracted. The results are given in Table 5.2.

Table 5.2: Feeding of precursors to inhibited *S. vulgaris* transformed root cultures.

3 Flasks per precursor. HEH 1.6 mM in medium. Precursors 1.0 mM in medium. Roots grown for 21 days.

Precursor	Fresh wt. roots (g)	Wt. crude alk. ext. (mg)	Amount precursor fed (mg)	Alkaloid
none	5.10	4.7	-	trace
(18)	21.35	73.6	46.5	+ + +
(16)	7.32	39.3	51.9	trace
(169)	2.12	22.5	42.6	trace



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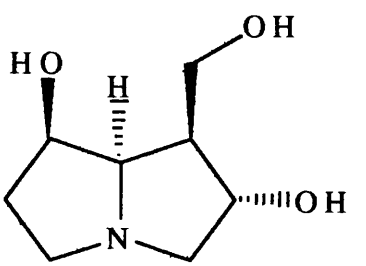

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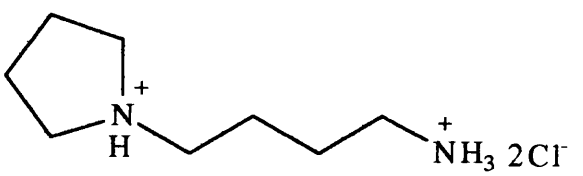


(169)

The addition of (+)-retronecine to the inhibited root cultures caused the rate of root growth to increase fourfold. The crude alkaloid extract contained one alkaloidal component of R_f 0.54. Spectral analysis showed this to be senecionine (38).

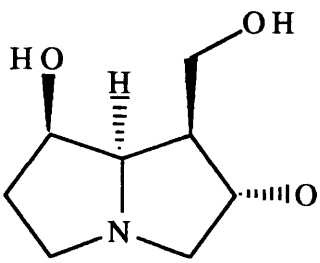
These results indicated that the presence of HEH in the culture medium did not affect either the formation of senecic

acid (126) or the coupling of the necine and necic acid by the roots.

5.3.2 (-)-Rosmarinecine (16)

To determine whether the lactonisation of senecic acid and retronecine was selective for retronecine, another necine was fed to the cultures.

(-)-Rosmarinecine (16) was the necine chosen. It was formed by the basic hydrolysis of rosmarinine (29), an alkaloid which was readily available. Rosmarinine is biosynthesised from rosmarinecine and senecic acid (126). This is the necic acid produced by *S. vulgaris* transformed root cultures.



(16)

The feeding of (-)-rosmarinecine to *S. vulgaris* transformed root cultures was carried out as for (+)-retronecine. The results are shown in Table 5.2.

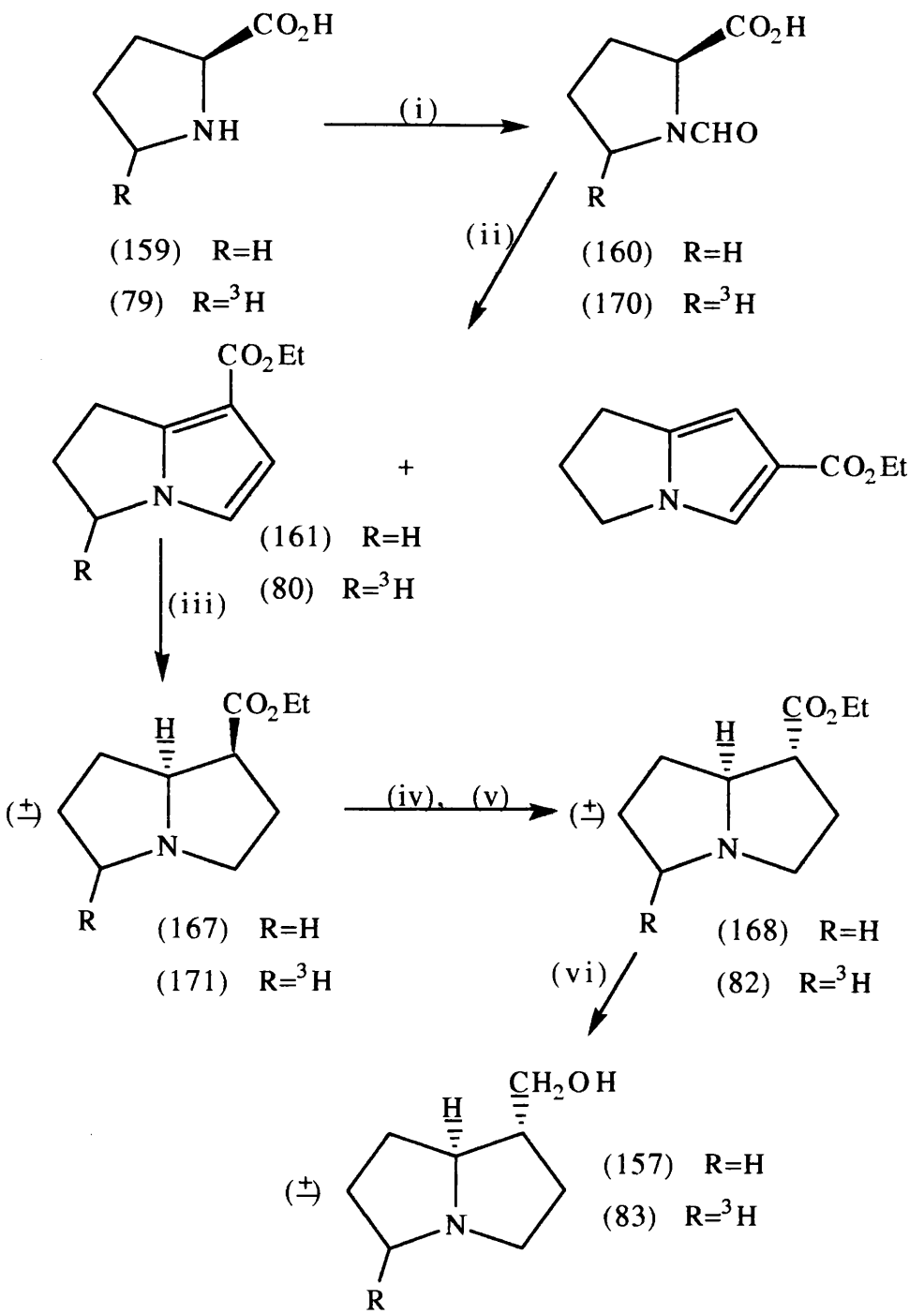
Not surprisingly, rosmarinecine was not converted by the root cultures into anything of an alkaloidal nature.

5.3.3 (±)-Trachelanthamidine (157)

As discussed in Chapter Two, (-)-trachelanthamidine (6) is a precursor of retronecine (18) in pyrrolizidine alkaloid biosynthesis. If trachelanthamidine was transformed into senecionine (38) in inhibited *S. vulgaris* transformed root cultures, then this would indicate that HEH has no effect on the conversion of trachelanthamidine (6) into retronecine (18).

Racemic trachelanthamidine was synthesised by the method of Kunec and Robins,[49] with some minor modifications.

Scheme 5.1: Synthesis of (±)-Trachelanthamidine



Reagents: (i) HCO_2H, Ac_2O; (ii) $HC{\equiv}CCO_2Et$, Ac_2O;

(iii) H_2, Pd/C, AcOH; (iv) conc. HCl

(v) EtOH, SO_2Cl_2; (vi) $LiAlH_4$, THF.

This method was an extension of the synthesis of (±)-isoretronecanol (158) performed by Pizzorno and Albonico.[115] The overall route is shown in Scheme 5.1.

The first step was a simple *N*-formylation of the readily available L-proline (159) and this proceeded in high yield. The ^{1}H- and ^{13}C-NMR spectra were more complex than expected due to the existence of *N*-formyl-L-proline (160) as two rotameric forms in solution.

The key step in the synthesis of (±)-trachelanthamidine (157) is the conversion of *N*-formyl-L-proline into the pyrrole ester (161). Following the method of Kunec and Robins,[49] a solution of *N*-formyl-L-proline and ethyl propiolate (163) in acetic anhydride was heated under reflux for three hours. No product was obtained. Increasing the reaction time and altering the temperature had no effect. However, when the concentration of the solution was increased tenfold by reducing the amount of acetic anhydride present, a 6:1 mixture of two isomeric, pyrrolic products was obtained. These were separated by dry-column flash chromatography on silica.

The mass spectral data for both compounds showed an M^{+} at m/z 179 corresponding to a molecular formula of $C_{10}H_{13}NO_2$, and a large peak at m/z 106 corresponding to loss of CO_2Et. The IR spectra of both isomers showed an ester carbonyl absorption at 1690 cm^{-1}.

The ^{1}H NMR spectra of the two isomers provided the key to their identity. The spectra were similar except that in the spectrum of the major isomer the two pyrrolic protons appeared as an AB system at δ 6.53 and δ 6.57 (Figure 5.1) whereas with the minor isomer these protons appeared as a broad multiplet at δ 6.25 (Figure 5.2). The AB system had a coupling of 2.9 Hz which is of the order expected for a vicinal coupling in a pyrrole system such as that in ethyl 2,3-dihydro-1H-pyrrolizine-7-carboxylate (161). Therefore the major isomer was pyrrole ester (161). The minor isomer was its regioisomer, ethyl 2,3-dihydro-1H-pyrrolizine-6-carboxylate

Figure 5.1 : 90 MHz ^1H NMR Spectrum of Ethyl 2,3-dihydro-1H-pyrrolizine-7-carboxylate (161)

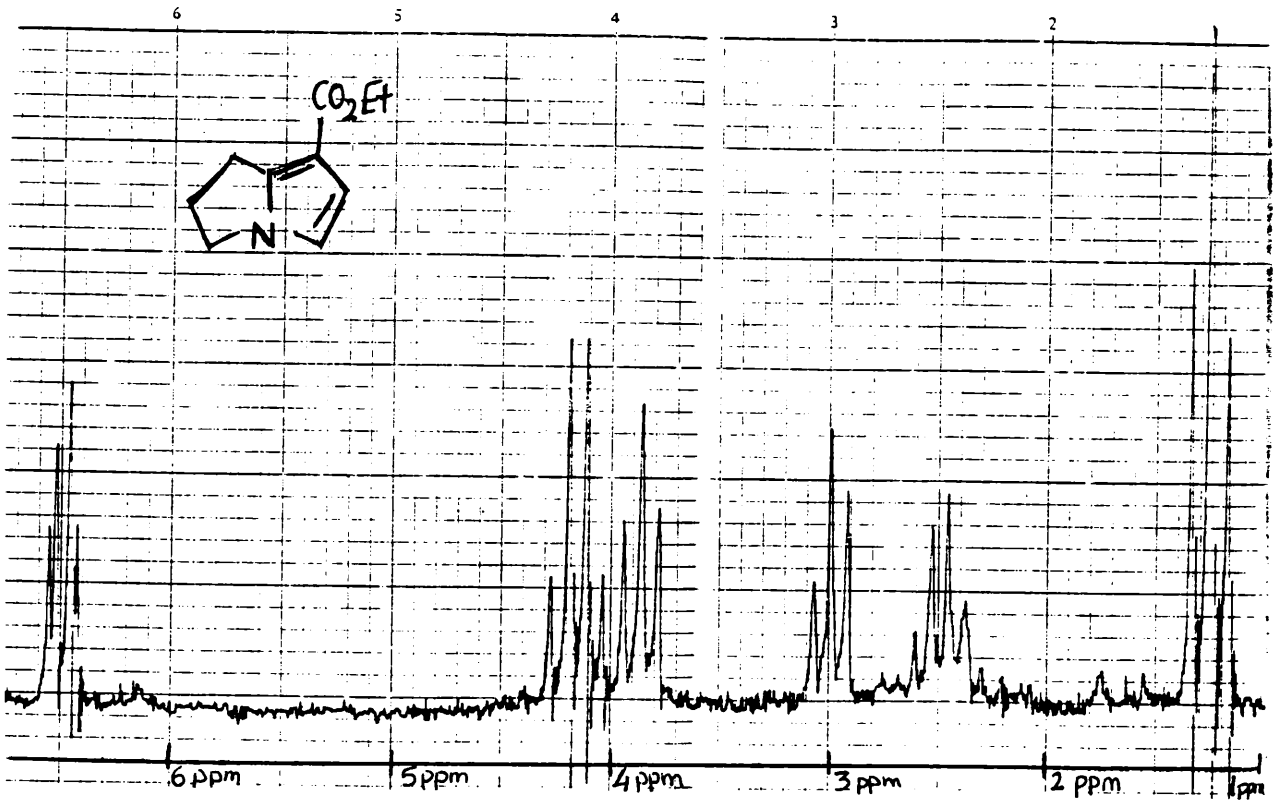
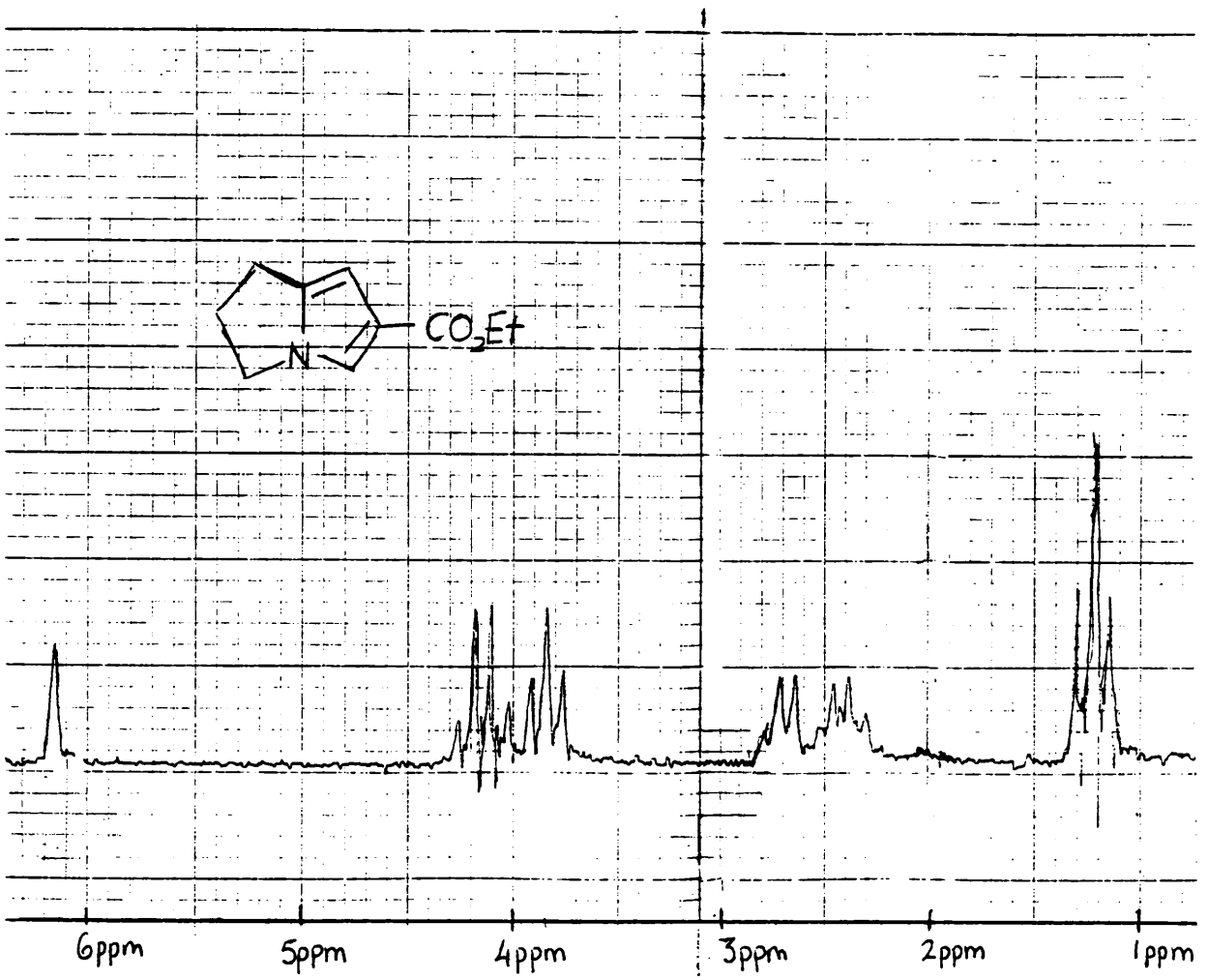


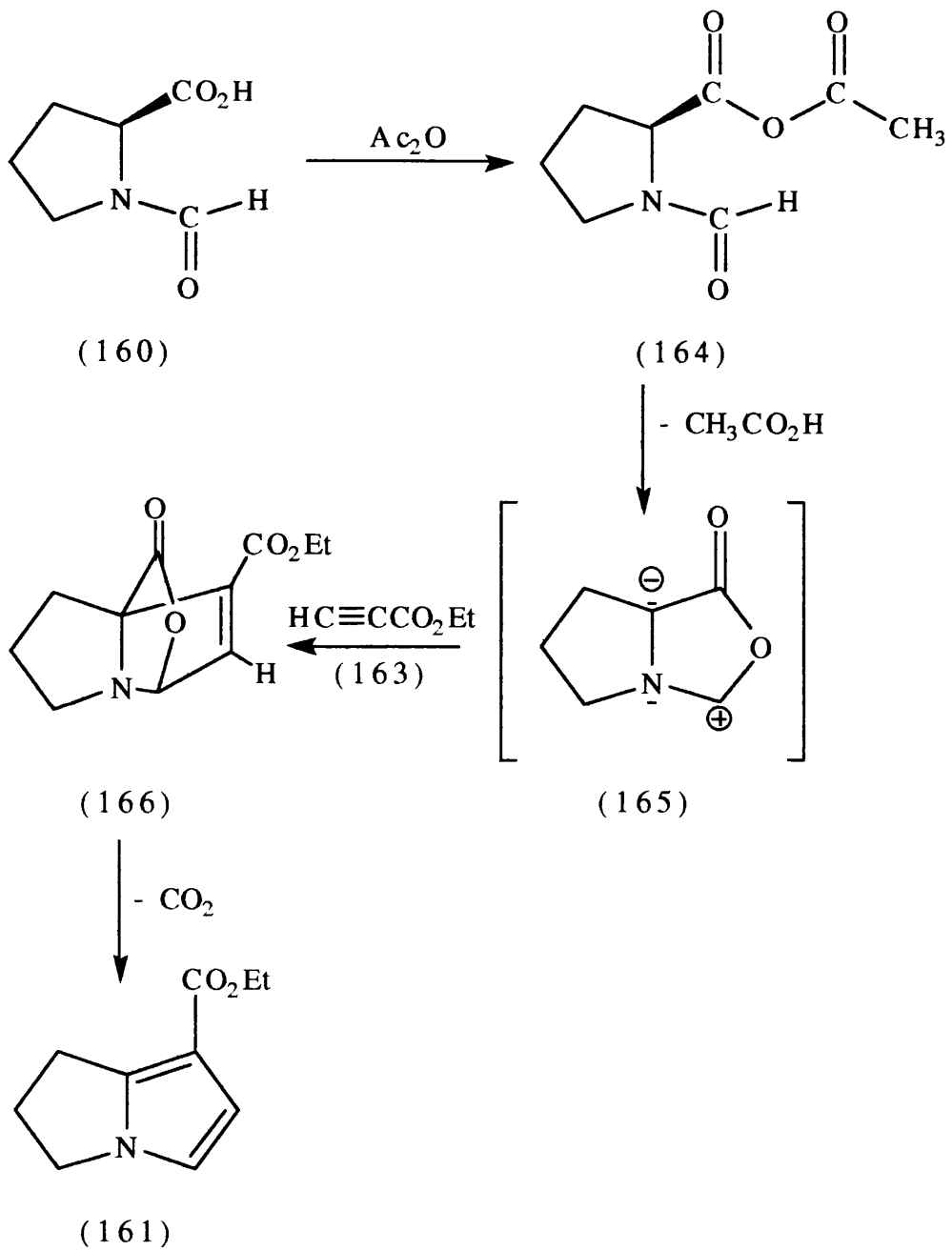
Figure 5.2 : 90 MHz ^1H NMR Spectrum of Ethyl 2,3-dihydro-1H-pyrrolizine-6-carboxylate (162).



(162). The minor isomer has never previously been reported from this reaction.[49,115]

The probable mechanism for formation of the major isomer is shown in Scheme 5.2.[116]

Scheme 5.2



(161)

Initially, the *N*-acylaminoacid (160) forms a mixed anhydride (164) by acylation with acetic anhydride. This

species (164) spontaneously cyclises to afford the azomethine ylid (165) which contains a 1,3-dipole. Cycloaddition of the ylid (165) and ethyl propiolate (163) gives the initial adduct (166) which, under the reaction conditions, loses CO_2 *via* a retro-Diels Alder process to give the pyrrole ester (161).

The regioselectivity of the addition of ethyl propiolate to the azomethine ylid (165) was thought to be completely specific,[49,115] but this is obviously not the case.

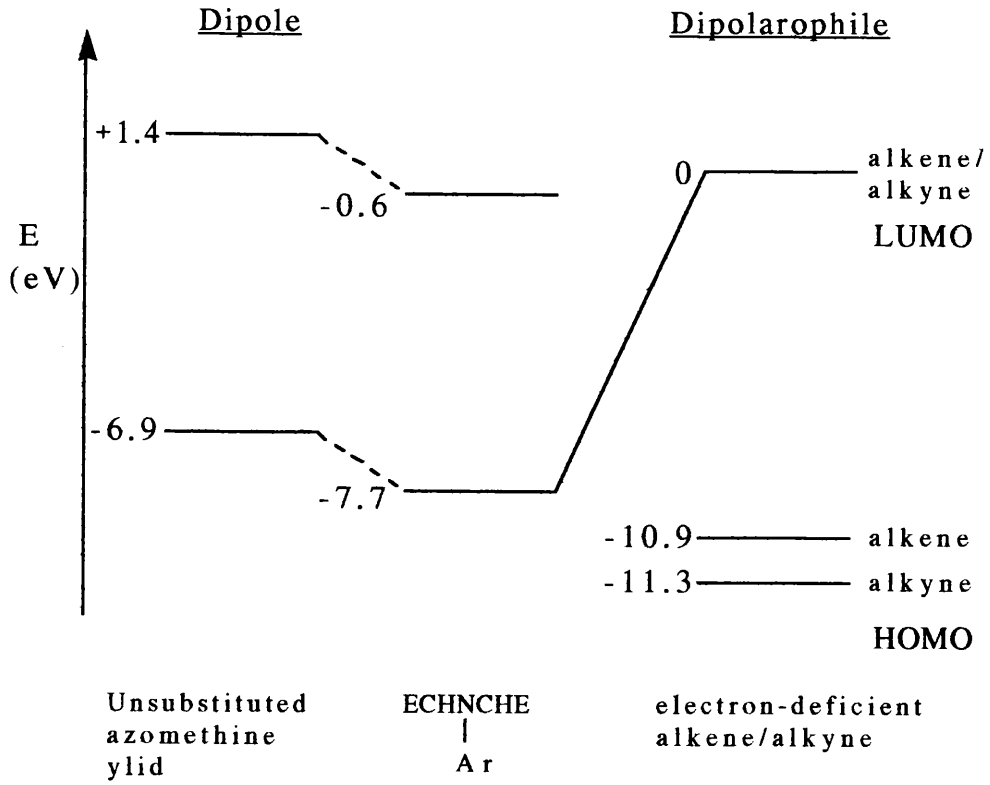
The regiochemistry of this 1,3-dipole-dipolarophile cycloaddition is controlled by the Frontier Molecular Orbital (FMO) interactions. Two factors have to be considered to determine the regiochemistry. These are (i) which HOMO-LUMO pair are closer in energy and hence involved in the reaction and, (ii) the orbital coefficients have to be aligned such that large/large and small/small coefficient interactions are maximised. However, in this case both regioisomers are produced and therefore the energy difference between the two HOMO-LUMO pairs is very small.

The 1,3-dipole involved in this reaction is azomethine ylid (165). A full FMO treatment is not possible due to lack of data on the energies of the orbitals of the azomethine ylids that are involved. However it is possible to give a quantitative description of the system.

The unsubstituted azomethine ylid has a highest occupied molecular orbital (HOMO) energy of -6.9 eV and a lowest unoccupied molecular orbital (LUMO) energy of +1.4 eV.[117] These values are average values obtained from different calculations.[117]

The effect of substituents on the energies of the frontier molecular orbitals of 1,3-dipoles is not fully understood. Electron-withdrawing groups cause a pronounced lowering of the LUMO energy and a lesser lowering of the HOMO energy. Electron-releasing substituents on the other hand raise both the HOMO and the LUMO energies with the LUMO energy increasing more slowly.

Figure 5.3 : HOMO and LUMO energies of azomethine ylids and an electron-deficient alkene/alkyne.



Energy values from Refs 117 and 118. E = ester

In this case one end of the azomethine ylid is attached to an electron-withdrawing group (-C=O), and the other end to a weakly electron-releasing group (-O-), while the central nitrogen has a lone pair and an alkyl substituent which is regarded as weakly electron releasing. The combination of these effects results in a lowering of the LUMO energy and a smaller lowering of the HOMO energy.

Putting exact figures on these energy changes is not possible. Figure 5.3 gives the values of a studied azomethine ylid with two ester groups attached.[118] These values will be similar to those for azomethine ylid (165).

Photoelectron spectroscopy indicates that the HOMO energy of an alkyne is 0.4 to 0.9 eV lower than that of the corresponding alkene.[117] The LUMO energy of an alkyne with

an electron withdrawing substituent, such as ethyl propiolate is virtually identical to the LUMO energy of the analogous alkene.[117]

The HOMO-LUMO combination which gives the smaller energy difference, and hence is involved in the reaction, is the HOMO of the dipole and the LUMO of the dipolarophile.

The regiochemistry is controlled by the overlap of the frontier orbitals which maximise the large/large and small/small orbital coefficient interactions. Here it is the unequal magnitude of the terminal coefficients in the HOMO and the LUMO of the substituted azomethine ylid that control the regiochemistry and therefore the orbital coefficients of the central nitrogen are not considered.

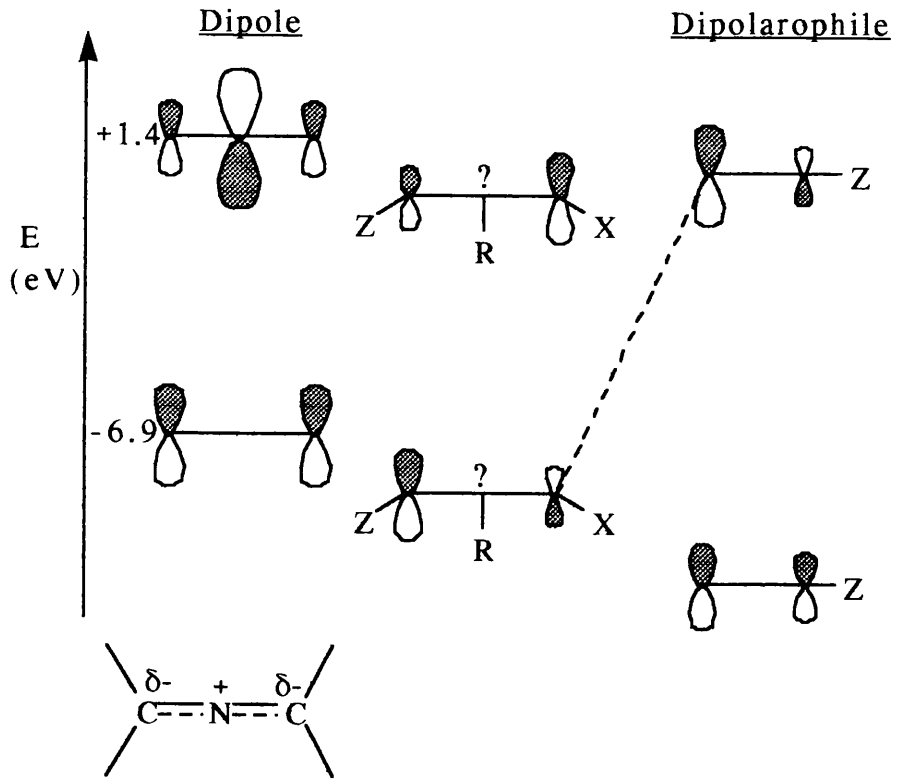
The effects of the three different substituents on the orbital coefficients of the azomethine ylid are such that in the LU orbital the largest coefficient is at the site of attachment of the electron-releasing group (-O-) and in the HO orbital the largest coefficient is at the site of the electron-withdrawing substituent (C=O).[117]

For an alkyne with an electron-withdrawing group attached, the larger terminal coefficient is on the substituted carbon in the HOMO and on the unsubstituted carbon in the LUMO. However, the ester group is also conjugating and this effect diminishes the coefficient at the site of attachment in both the HO orbital and the LU orbital. The combination of these two effects results in the largest coefficient being at the unsubstituted end in both the HOMO and the LUMO of the alkyne. (Figure 5.4).

This reasoning however gives the regiochemistry of the minor isomer (162) not the major isomer (161).

This inconsistency is due to lack of accurate information for this specific azomethine ylid. Clearly more data on the coefficients and energy levels are required to explain this phenomenon fully.

Figure 5.4 : Relative orbital coefficients for azomethine ylids and an electron-deficient alkyne.



Z = electron-withdrawing group
X = electron-releasing group
R = alkyl group

The pyrrole ester (161) was hydrogenated at a pressure of six atmospheres in the presence of rhodium-on-carbon catalyst. Delivery of the hydrogen atoms occurs from one side only and therefore the product was exclusively ester (167). It should be noted that this ester was formed as a racemate. The ^{1}H NMR spectrum showed no pyrrolic proton signals confirming that the pyrrole ring had been reduced. The ester carbonyl stretching frequency in the IR spectrum was at 1730 cm^{-1} indicating that the carbonyl was part of a saturated system. Ethyl (±)-8α-pyrolizidine-1β-carboxylate (167) was furnished in 58% yield.

Reduction of ester (167) would give (±)-isoretronecanol (158).[49] Trachelanthamidine is a diastereoisomer of

isoretronecanol, having the opposite stereochemistry at the 1-position. Therefore epimerisation of ester (167), to give the thermodynamically more stable *exo*-ester, followed by reduction would give the desired necine (157).

Epimerisation was carried out at 150 °C in a sealed tube under acidic conditions. The resulting oil was re-esterified by stirring with ethanol in the presence of thionyl chloride. The 1α-epimer (168) was obtained in reasonable yield. Corroboration that epimerisation was complete came from the ^{1}H NMR spectrum. The signal for the 8-H in the starting material (167) came at δ 3.80. In the ^{1}H NMR spectrum of the product (168) there was no sign of a signal at δ 3.80. The 8-H in the 1α-epimer (168) appeared at δ 3.63.

The final stage of the synthesis was the reduction of ethyl (±)-8α-pyrrolizidine-1α-carboxylate (168) to give (±)-trachelanthamidine (157). Lithium aluminium hydride reduction of ester (168) gave a colourless oil in high yield. The IR spectrum of this oil contained a large hydroxyl absorption at 3 420 cm^{-1}. No carbonyl absorption was present. Therefore ester (168) had been completely reduced to the corresponding alcohol.

(±)-Trachelanthamidine (157) was obtained in 13% overall yield from L-proline (159).

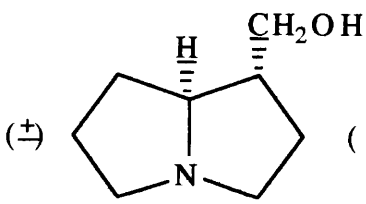
The feeding of (±)-trachelanthamidine to inhibited *S. vulgaris* transformed root cultures was carried out as for retronecine (18). Only one enantiomer of trachelanthamidine can be utilised by the roots for alkaloid synthesis and therefore the actual precursor concentration used was 0.5 mM. The results are given in Table 5.3.

The first point to note here is that HEH had a stronger effect than desired. The roots with HEH present produced only one third of the growth shown by the roots from the feeding experiment with retronecine.

Table 5.3: Feeding of (±)-trachelanthamidine to inhibited *Senecio vulgaris* transfromed root cultures.

3 Flasks per batch. HEH 1.6 mM in medium. Precursor 1.0 mM in medium. Roots grown for 21 days.

Precursor	Fresh wt. roots (g)	Wt. crude alk. ext. (mg)	Amount precursor fed (mg)	Alkaloid
none	1.54	3.6	-	trace
(157)	3.47	10.4	42.3	+



(157)

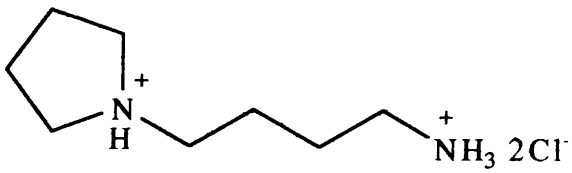
The introduction of trachelanthamidine into the medium of the inhibited root cultures caused the growth of roots to double. TLC analysis of the crude alkaloid extract indicated the presence of one alkaloid of R_f 0.54 in very small amounts. Purification of this alkaloid by preparative TLC proved unsuccessful.

It is probable that trachelanthamidine is taken up by the roots and transformed into senecionine (38). However, the strong effect of HEH on the roots was detrimental to the experiment, possibly causing less trachelanthamidine to be converted into alkaloid by the roots.

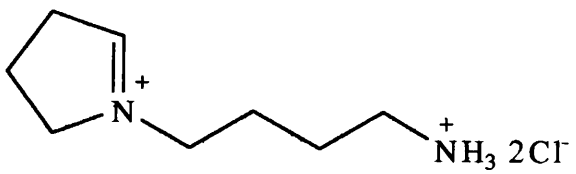
5.3.4 *N*-(4-Aminobutyl)pyrrolidinium dihydrochloride (169)

The saturated salt (169) has been shown to be incorporated reasonably well into retronecine (18) during pyrrolizidine alkaloid biosythesis.[54] This compound is not thought to lie on the biosynthetic pathway itself, but is oxidised

in vivo to the *N*-(4-aminobutyl)-1,2-didehydropyrrolidinium ion (77). This iminium ion (77) is an intermediate in pyrrolizidine alkaloid biosynthesis between homospermidine (60) and trachelanthamidine (6).[54]



(169)



(77)

A sample of saturated salt (169) was provided by Dr A.A. Denholm and fed to the inhibited root cultures as described for retronecine. The results are given in Table 5.2.

In this case the roots grew more poorly than those in the flasks with only HEH present. No alkaloid could be detected in the crude alkaloid extract.

It is not surprising that this compound was not transformed by the roots into alkaloid because HEH, as a polyamine oxidase inhibitor, probably inhibits the oxidation of the iminium ion (77) which is formed *in vivo* from saturated salt (169).

There is the possibility that HEH inhibits the oxidation of saturated salt (169) to iminium ion (77). This could be checked by carrying out the feeding of *N*-(4-aminobutyl)-1,2-didehydropyrrolidinium dichloride (77) to the inhibited root cultures. Unfortunately time did not allow for the synthesis of iminium ion (77).

5.4 Synthesis and Feeding of Radiolabelled Precursors to Inhibited *S. vulgaris* Transformed Root Cultures.

In an attempt to confirm that the retronecine (18) and trachelanthamidine (6) fed to the roots were transformed

directly into senecionine (38), radiolabelled precursors were synthesized and administered to the inhibited root cultures.

Radiolabelled retronecine was produced biosynthetically. *N,N*-Bis-3-(aminopropyl)-[1,4-^{14}C]-tetramethylene-1,4-diamine (spermine) (41) was dissolved in sterile water and distributed among flasks containing seven day old roots of *S. vulgaris*. After a further 14 days growth the roots were harvested and senecionine (38) was extracted. The senecionine produced had a specific activity of 10.2 μCi mmol^{-1}. The total incorporation of [^{14}C]-spermine into senecionine was 16.5%.

^{14}C-Labelled senecionine was formed by the basic hydrolysis of the radiolabelled senecionine. Although crystallisation of retronecine proved unsuccessful, the retronecine was shown to be pure by TLC and ^{1}H NMR spectroscopy and was therefore fed as an oil. The specific activity of ^{14}C-labelled retronecine was 11.1 μCi mmol^{-1}.

Radiolabelled trachelanthamidine was formed from [5-^{3}H]-L-proline (79) *via* the same route as used to prepare unlabelled material. The (±)-[5-^{3}H]-trachelanthamidine (83) furnished had a specific activity of 11.6 μCi mmol^{-1}.

Feeding of [^{14}C]-retronecine and [5-^{3}H]-trachelanthamidine to inhibited *S. vulgaris* transformed root cultures was carried out as for unlabelled retronecine (Scheme 5.1). (±)-Trachelanthamidine was fed at 2.0 mM concentration so that the naturally occurring enantiomer was present at a 1.0 mM concentration. The [^{14}C]-retronecine was diluted with unlabelled retronecine to achieve the required concentration of 1.0 mM. The specific activity of the ^{14}C-labelled retronecine was therefore reduced to 5.3 μCi mmol^{-1}. The results of these feeds are given in Table 5.4.

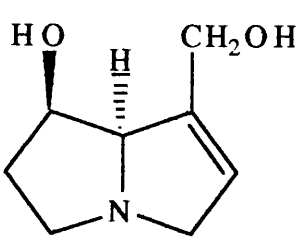
Two major problems were encountered in this experiment. The first was that the roots were not growing well even under normal conditions i.e. when no HEH or precursor present. Instead of fine roots being produced the roots showed callous formation. The second problem that arose was that, yet again, HEH was having too much of an effect on root growth.

Table 5.4: Feeding of radiolabelled precursors and analogues to inhibited *Senecio vulgaris* transformed root cultures.

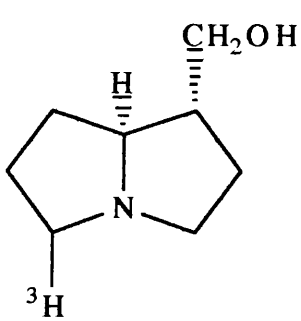
2 Flasks per batch. HEH 1.6 mM in medium. Roots grown for 22 days.

Batch	Control	HEH only	(18)	(83)	(182)
Fresh wt roots (g)	6.24	0.91	0.85	1.09	0.94
Weight crude alk. ext. (mg)	7.5	1.7	21.6	18.8	1.1
Amount precursor fed (mg)	-	-	31	54*	30*
Amount precursor fed (μCi)	-	-	1.06	4.44*	1.99*
Total ^{3}H or ^{14}C incorp. (%)	-	-	0.24	0.04	0.55
S. act. of precursor (μCi $mmol^{-1}$)	-	-	5.3	11.6*	10.3
S. act. of alk. ext. (μCi $mmol^{-1}$)	-	-	0.04	0.02	1.66
Alkaloid	+ + +	trace	+ +	trace	trace
Conc. of precurs. in medium (mM)	-	-	1.0	2.0	1.0

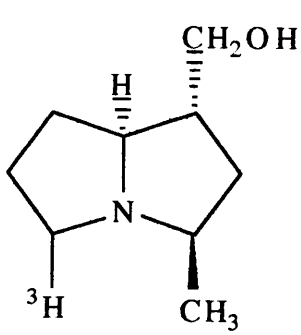
* Trachelanthamidine (83) and its methyl analogue (182) were fed as racemic mixtures. Only one enantiomer is utilised by the roots for alkaloid synthesis.



(18)



(±) (157)



(±) (182)

The roots to which the precursors were fed grew only to the same extent as those with HEH present. This was in complete contrast to the feeding of unlabelled retronecine where there was a fourfold increase in root growth. However, both the retronecine and trachelanthamidine fed roots did produce small amounts of alkaloid. TLC analysis and mass spectra of the crude alkaloid extracts indicated that this alkaloid was senecionine (38).

The total incorporations of [^{14}C]-retronecine and (±)-[5-^{3}H]-trachelanthamidine were very low, at 0.24% and 0.038% respectively. This compares with *ca.* 70% incorporation of unlabelled retronecine when the roots were growing reasonably in the presence of HEH.

These feedings were also carried out at 1.4 mM concentration of HEH. Again the roots grew poorly and the results were almost identical to those for the 1.6 mM HEH concentration.

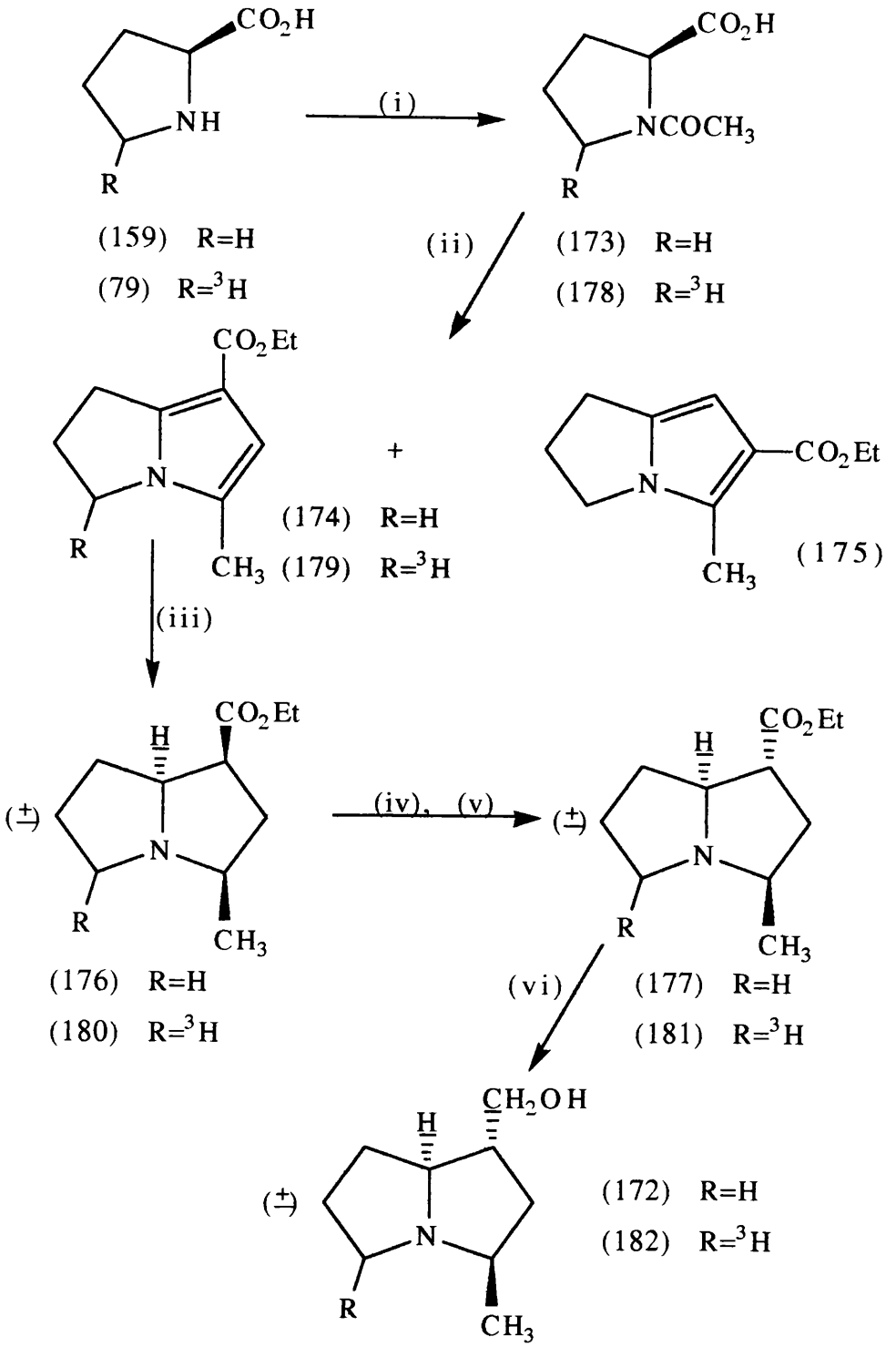
Attempts were again made to find an HEH concentration which would give good root growth and inhibit pyrrolizidine alkaloid biosynthesis. These were unsuccessful and due to time restraints no further studies could be carried out.

5.5 Synthesis and Biosynthesis of a Precursor Analogue to Inhibited *S. vulgaris* Transformed Root Cultures.

The next stage was to feed a precursor analogue to the inhibited *S. vulgaris* root cultures to see if it could be transformed into a pyrrolizidine alkaloid analogue. The only biosynthetic precursors turned into senecionine (38) by the roots were retronecine (18) and trachelanthamidine (6) and hence an analogue of one of these was chosen.

The 3β-methyl analogue (172) of trachelanthamidine was selected because it could be synthesized by a method similar to that used to prepare (±)-trachelanthamidine (Scheme 5.3).

Scheme 5.3: Synthesis of (±)-1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (182)



Reagents: (i) AcOH, Ac_2O;(ii) $HC{\equiv}CCO_2Et$, Ac_2O;

(iii) H_2, Pd/C, AcOH; (iv) conc. HCl

The extra methyl group was introduced by acetylation of L-proline (159), rather than formylation as in the (±)-trachelanthamidine synthesis.

Acetylation of L-proline (159) was carried out by the procedure outlined by Price *et al.*[119] Racemic *N*-acetylproline (173) was obtained as a white crystalline solid in 72% yield.

Heating this *N*-acetylated aminoacid (173) and ethyl propiolate at reflux in acetic anhydride for several hours produced a brown oil which solidified when left at 0 °C overnight. This solid was shown by 200 MHz ^{1}H NMR spectroscopy to be a 3:2 mixture of two isomeric pyrrole esters. Separation of these was achieved by neutral alumina column chromatography.

The spectral data for the two pyrrole esters were very similar. The IR spectra possessed ester carbonyl absorptions at *ca.* 1 684 cm^{-1}. High resolution mass spectrometry gave the molecular formulae as $C_{11}H_{15}NO_2$. All data indicated that these esters were the expected products of ethyl 2,3-dihydro-5-methyl-1H-pyrrolizine-7-carboxylate (174) and ethyl 2,3-dihydro-5-methyl-1H-pyrrolizine-6-carboxylate (175).

The 200 MHz ^{1}H NMR spectra were again the key to identifying which pyrrole ester was the major isomer and which was the minor isomer. In the spectrum of the major isomer a coupling of 0.8 Hz was observed between the pyrrolic proton and the protons of the methyl group attached to the pyrrole ring. These proton signals were both singlets in the ^{1}H NMR spectrum of the minor isomer. The major isomer was therefore pyrrole ester (174) where these protons are only four bonds apart and a $^4J_{HH}$ long range coupling can be observed. In pyrrole ester (175) these protons are five bonds apart and therefore no coupling was observed.

Again a FMO treatment is not possible due to lack of accurate information for the azomethine ylid involved in this reaction. However the extra electron-releasing methyl group attached to the ylid alters the orbital energies such that the regioisomers are formed in nearly equal amounts.

Catalytic hydrogenation of pyrrole ester (174) at six atmospheres pressure and 60 °C gave ethyl (±)-3β-methyl-8α-pyrrolizidine-1β-carboxylate (176) in 57% yield. The yield was only 13% if the reaction mixture was not heated during the hydrogenation process.

Hydrogenation was shown to have been successful from the ^{1}H NMR spectrum where the pyrrolic proton signal of the starting material at δ 6.25 was no longer present. Confirmation came from the IR spectrum where the ester carbonyl absorption was at 1 723 cm^{-1}.

Epimerisation of ester (176) under acidic conditions resulted in the formation of ethyl (±)-3β-methyl-8α-pyrrolizidine-1α-carboxylate (177) in 62% yield. Verification that epimerisation was complete came from the ^{1}H NMR spectrum. The 8-H of the product came at δ 3.95 whereas in the starting material it was at δ 3.50-δ 3.70. The ^{1}H NMR spectrum for ester (177) had no peaks in the δ 3.50-δ 3.70 region.

The reduction of ester (177) with lithium aluminium hydride proceeded in 88% yield. Proof of complete reduction came from the IR spectrum where no carbonyl absorption was present and a large hydroxyl absorption was present at 3 350 cm^{-1}.

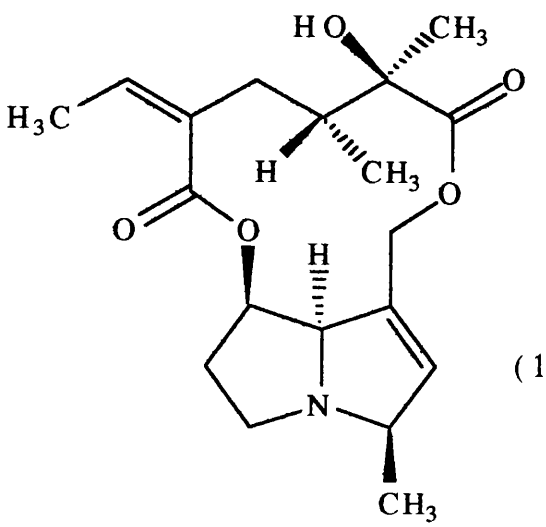
(±)-1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (172) was synthesized in an overall yield of 6.3% from L-proline (159).

This precursor analogue (172) was synthesised in radioactive form for feeding to the root cultures (Scheme 5.3). [5-^{3}H]-L-Proline (79) was converted by the above method into (±)-[5-^{3}H]-1α-hydroxymethyl-3β-methyl-8α-pyrrolizidine (182) in 7.3% yield. This had a specific activity of 10.3 μCi $mmol^{-1}$.

(±)-[5-^{3}H]-3β-Methyltrachelanthamidine (182) was fed to the inhibited *S. vulgaris* cultures at the same time as [^{14}C]-retronecine and (±)-[5-^{3}H]-trachelanthamidine had been fed. It therefore suffered from the same growth problems as those

feeds did. The results in Table 5.4 testify to this with the roots from the feeding of the analogue only growing to the same extent as those with just HEH present.

The crude alkaloid extract weighed just 1.1 mg and TLC analysis indicated that there was no alkaloidal material present in this extract. However the extract showed the highest radiolabel total incorporation of any of the radiolabelled precursor feeds at 0.55%. A mass spectrum of the crude alkaloid extract had no peaks at *m / z* 349 or 335 corroborating that none of the expected alkaloid analogue 3β-methylsenecionine (183) or senecionine (38) was present. Therefore some of the tritium label was in the crude alkaloid extract but this was not present in the form of senecionine or an alkaloid analogue.



(183)

5.6 Conclusions

The biosynthesis of pyrrolizidine alkaloids in *S. vulgaris* transformed root cultures was stopped by the addition of HEH to the culture medium. Both retronecine and trachelanthamidine were taken up by these inhibited roots and transformed into senecionine efficiently. However, the percentage of fed precursor transformed into alkaloid was very much dependent on how much growth was exhibited by the

inhibited roots. Unfortunately, although an HEH concentration of 1.6 mM always stopped pyrrolizidine alkaloid biosynthesis its effect on root growth was not constant. The ideal situation occurred in the feed of unlabelled retronecine where the inhibited roots showed a reasonable amount of growth and about 70% of the fed retronecine was transformed into senecionine. In all of the other feeding experiments the roots hardly grew at all and this was detrimental to the transformation of the precursors into alkaloids.

The 3β-methyl analogue of trachelanthamidine was not turned into an alkaloid analogue by the inhibited roots. However it cannot be said whether this was due to the biosynthetic enzymes not being able to transform this precursor analogue or the poor root growth giving the precursor analogue no chance of being transformed into an alkaloid analogue.

For this system to be useful for the production of pyrrolizidine alkaloid analogues the problem of variable root growth will have to be eliminated.

Due to lack of time no further studies could be undertaken.

CHAPTER 6

Lipase-Catalysed Biotransformations of Diamines

6.1 Use of Lipases in Organic Synthesis

6.1.1 Introduction

Enzymes have always had great potential as catalysts for particular steps in organic synthesis.[120,121] However, as their use seemed to be limited to reactions in aqueous media, organic chemists were often reluctant to employ them. This reluctance stemmed from a number of factors. Most organic compounds are insoluble in water and some functional groups are unstable in an aqueous environment. In addition, having to recover products from water is far from an ideal situation.

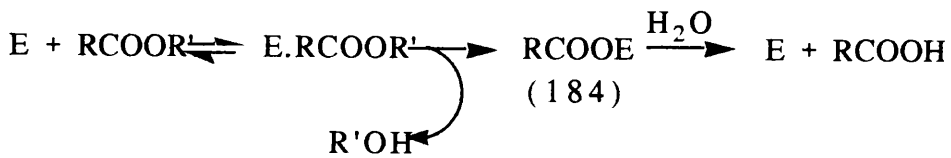
This whole field was revolutionised in 1984 with the discovery by Klibanov and Cambou[122] that some enzymes (particularly proteases and lipases) retained their catalytic activity when used as heterogeneous catalysts in organic solvents. This finding led to the range of reactions which enzymes could catalyse being extended to include esterification, amide formation and transacylation,[123] as well as the conventional hydrolyses.

The three types of enzyme commonly used with organic compounds are esterases (e.g. Pig Liver Esterase), proteases (e.g. α-Chymotrypsin, Subtilisin) and lipases. Lipases are considered to be one of the most versatile groups of enzymes for chemical conversions.[124] They are stable in nonpolar organic solvents and have the ability to accommodate a diverse range of substrate structures.[125]

The natural role of lipases is to catalyse the hydrolysis of triacylglycerols in the low-water environment of an oil-globule/water interface. This *in vivo* working environment must contribute, at least in part, to their stability in organic solvents.

The mechanism for the hydrolysis of an ester by a hydrolytic enzyme, such as a lipase, can be considered as shown in Scheme 6.1.[126]

Scheme 6.1



E - hydrolytic enzyme
RCOOR' - hydrolysable ester
E.RCOOR' - noncovalent enzyme-substrate complex
RCOOE - covalent acyl-enzyme intermediate

From Ref. 126

In an aqueous environment the acyl-enzyme intermediate (184) is hydrolysed giving back the free enzyme and producing the acid. In the case where organic solvent is the reaction medium no water is present and intermediate (184) is not hydrolysed. This intermediate can therefore be treated with another nucleophile (e.g. an alcohol) and hydrolysis can be replaced by a number of alternative reactions (e.g. transesterification).

6.1.2 Lipase-catalysed Reactions of Amines and Aminoalcohols in Organic Synthesis.

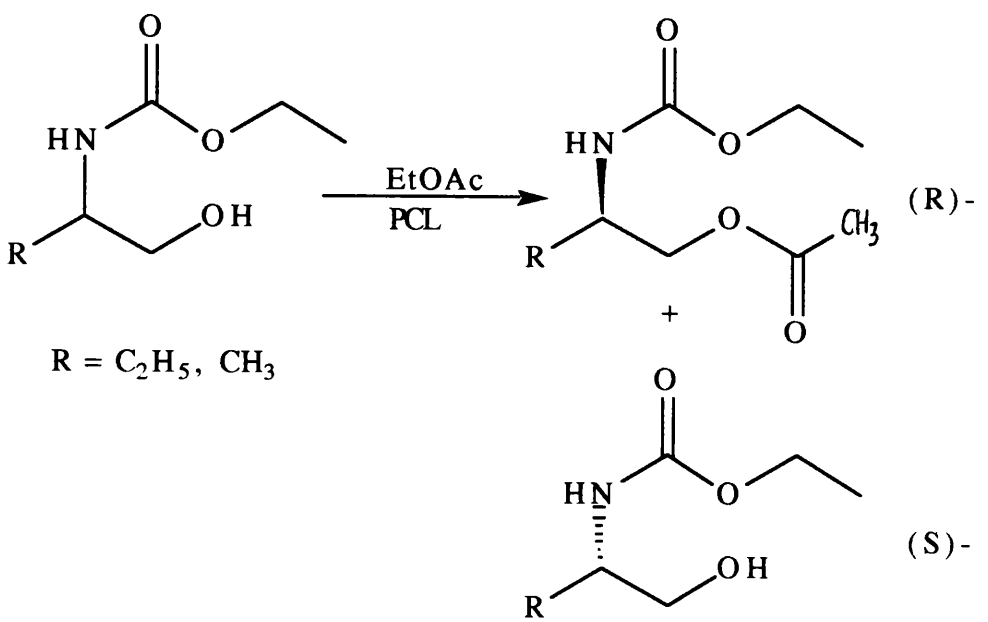
The use of lipases as catalysts in organic chemistry has increased dramatically in recent years. Lipase substrates range from simple alcohols[122,127] to complex molecules such as steroids,[128] ferrocene-alcohols[129] and chromium-benzyl alcohol complexes.[130] This brief review will concentrate on the use of lipases for the asymmetric transformation of substrates with amine functionality i.e. amines and aminoalcohols. A comprehensive examination of the use of lipases in organic synthesis can be found in several recent reviews.[125,126,131,132]

A. Aminoalcohols

Aminoalcohols are of considerable interest because of their use as synthetic intermediates,[133] chiral auxiliaries and catalysts.[134,135]

Francalanci *et al.*[136] found that 2-amino-1-alcohols could be resolved by transesterification catalysed by *Pseudomonas cepacia*[137] (PCL), using ethyl acetate as both acyl donor and solvent, provided that the amino group was protected (Scheme 6.2). Enzymic transesterification of the unprotected amino alcohol resulted in the nonstereospecific acylation of the more nucleophilic amino group.

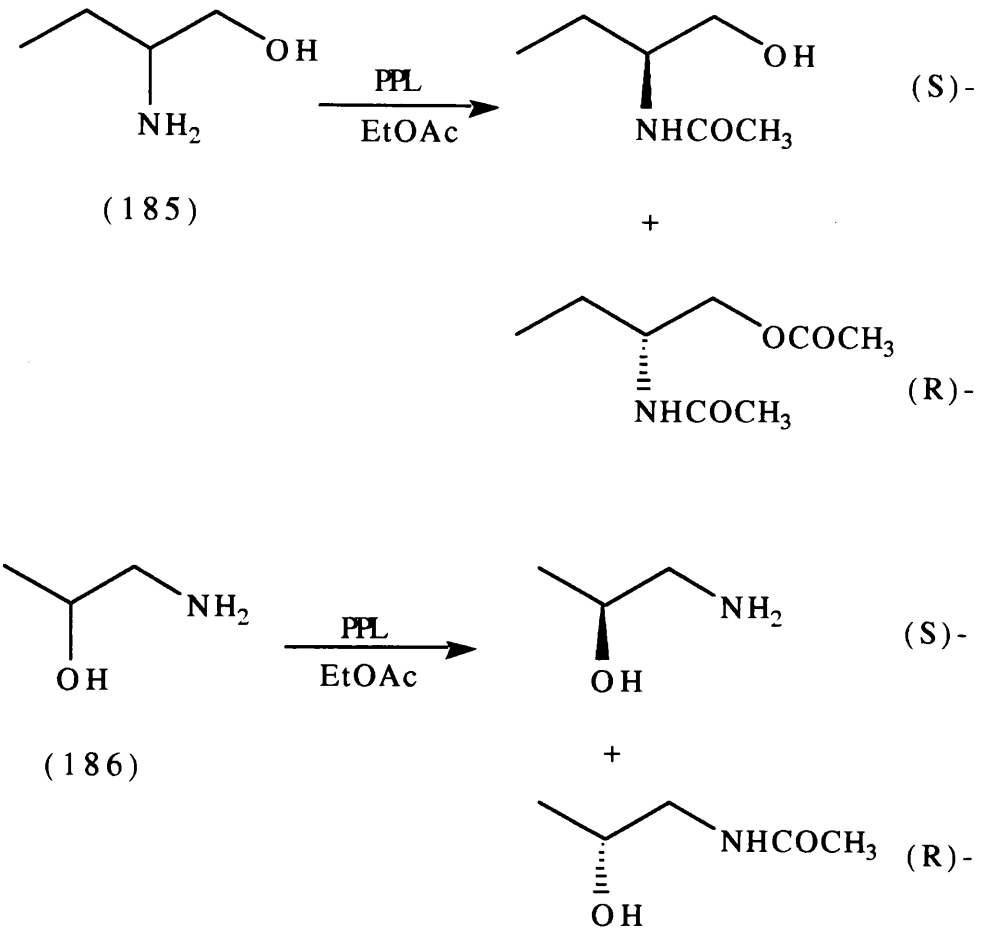
Scheme 6.2



$R = C_2H_5, CH_3$

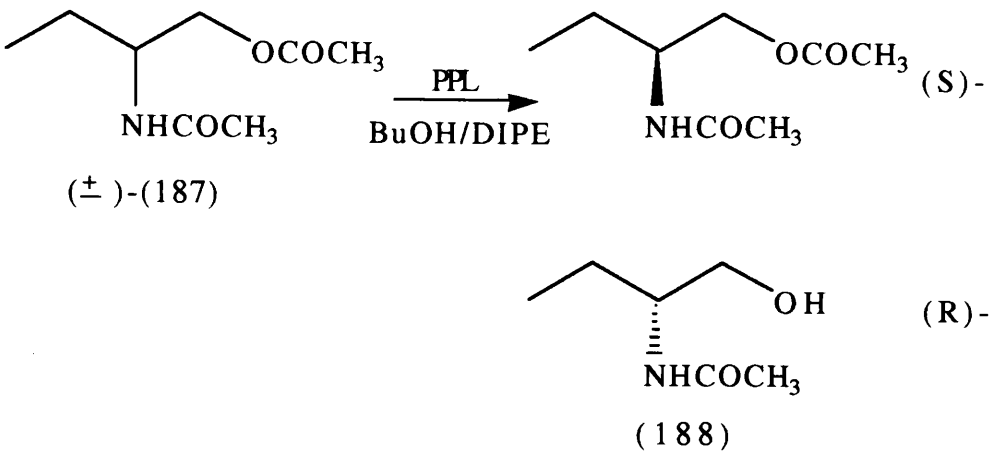
In direct contrast, Gotor *et al.*[138] obtained acylated derivatives of (±)-1-amino-2-propanol (186) and (±)-2-amino-1-butanol (185) in over 95% enantiomeric excess *via* PPL catalysed transacylation in ethyl acetate (Scheme 6.3).

Scheme 6.3



Bevinakatti and Newadkar[139] found that a large amount of crude lipase powder had to be used in order to repeat the work of Gotor *et al.* Bevinakatti and Newadkar resolved (±)-2-amino-1-butanol (185) by performing a PPL-catalysed transesterification between the diacetyl derivative (187) of 2-amino-1-butanol and 1-butanol in diisopropylether (DIPE). The (R)-enantiomer of the ester reacted faster to give (R)-2-acetamido-1-butanol (188) with reasonable optical purity (Scheme 6.4).

Scheme 6.4

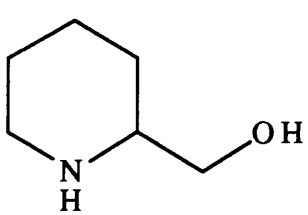


(±)-(187)

(188)

Chinsky *et al.*[140] reported that the relative reactivities of the hydroxyl and amino groups in an aminoalcohol depends on the acyl donor used. 6-Amino-1-hexanol was used as the model substrate and *Aspergillus niger* lipase (ANL) as the biocatalyst. This phenomenon was also observed using PPL or PCL in place of ANL. Acylation occurred on the oxygen when 2-chloroethyl butanoate was acyl donor and on the nitrogen when the acyl donor was 2-chloroethyl-*N*-acetyl-L-phenylalaninate. These workers found that shorter aliphatic aminoalcohols e.g. 4-amino-1-butanol, were acylated on the oxygen but subsequently underwent non-enzymic oxygen to nitrogen migration of the acyl moiety.[141] Chinsky *et al.*[140] postulated that this migration might be responsible for the *N*-acetylation observed in the work of Gotor *et al.*[138]

Asensio *et al.*[142] in their work, concluded that the observed acylation of the cyclic 1,2-aminoalcohol (189) using PPL and ethyl acetate was due to initial *O*-acylation followed by acyl migration.



(189)

B Amines

Few studies have been carried out on the enzymic preparation of amides. Much of the early work was concerned with the synthesis of peptides catalysed by proteases.[143] However, Margolin and Klibanov[144] discovered that PPL in organic solvents could also catalyse peptide synthesis.

Gotor *et al.*[145,146] formed optically active amides from the *Candida rugosa* lipase[147] (CRL) catalysed reaction of ethyl (±)-2-chloropropionate (190) with aliphatic and aromatic amines (Scheme 6.5).

Scheme 6.5

$$\underset{(\pm)\text{-(190)}}{CH_3CHCl\text{-}CO_2C_2H_5} + RNH_2 \xrightarrow{CRL} \underset{(S)\text{-}}{CH_3CHCl\text{-}CONHR}$$

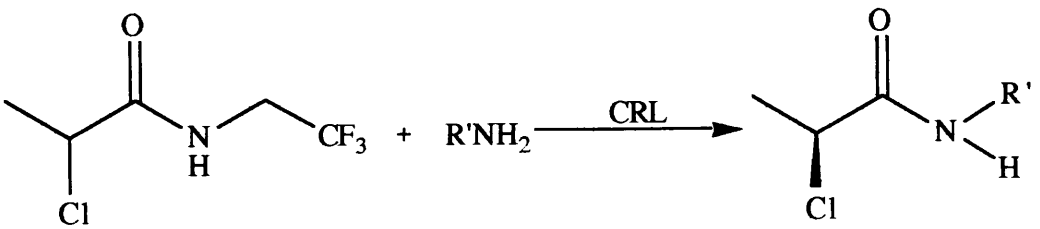
The enantiomeric excesses ranged from 30% to 95% with the S-isomer predominating in all cases. This reaction was carried out at 2 °C because racemic aliphatic amide was slowly formed in the absence of enzyme at room temperature.

Ethyl (±)-2-chloropropionate was also used by Gotor and coworkers[148] for the preparation of optically active, straight-chain diamides. CRL catalysed the formation of the (S,S) isomer with high enantiomeric excess, whereas PCL exhibited an opposite and lower selectivity, yielding a mixture of the (R,R) and (R,S) isomers.

CRL has been shown to catalyse the transamidation reaction between *N*-trifluoroethyl-2-chloropropionamide and various amines (Scheme 6.6).[146] The amides were obtained in moderate enantiomeric excess.

Djeghaba *et al.*[149] found that various lipases could catalyse amide synthesis from primary amines in the presence of ethyl butyrate.

Scheme 6.6



Clearly lipases can catalyse the *N*-acylation of both aminoalcohols and amines. Stereoselectivity can be achieved in these reactions under suitable conditions.

6.2 Introduction

Our work with lipases was initiated by the need for a good method for the production of *N*-acetylputrescine hydrochloride (31) for use in biosynthetic work (see Chapter 4.1). A search of the literature indicated that the use of porcine pancreatic lipase (PPL) in association with ethyl acetate[138] might allow the monoacetylation of the diamine putrescine (33) with the production of a minimal amount of diacetylputrescine (191). The behaviour of a number of diamines with PPL in ethyl acetate was studied.

6.3 PPL-Catalysed Monoacetylation of Diamines

The diamines were acetylated using ethyl acetate in the presence of PPL. Ethyl acetate acts as both acylating agent and solvent.

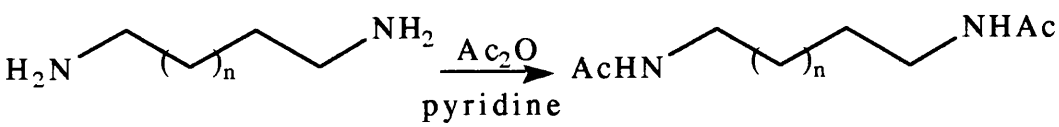
In this reaction, an amine reacts with an ester to form an amide and releases an alcohol (Equation 6.1).

$$R^1NH_2 + R^2CO_2R^3 \rightleftharpoons R^1NHCOR^2 + R^3OH \quad \text{(Equation 6.1)}$$

It is possible for the liberated alcohol (R^3OH) to participate in the reverse reaction. This is clearly undesirable as the yield of amide (R^1NHCOR^2) is decreased. More importantly, when an optically active product could be produced such reversible reactions serve to lower the enantiomeric excess of the final product.

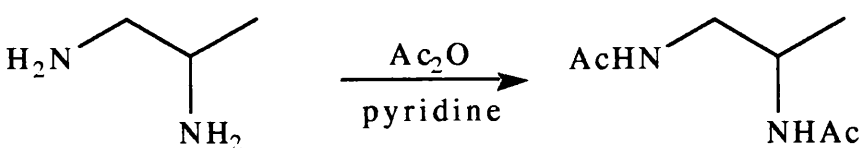
In this example, with ethyl acetate present in such a large excess this reverse reaction is suppressed. The results of this are increased product yield and in the case of stereoselective reactions enhanced selectivity.

Scheme 6.7

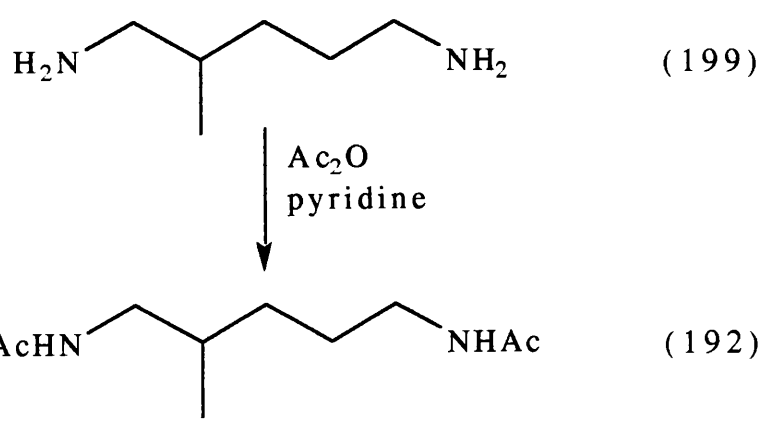


n=1 (33)
n=2 (154)

n=1 (191)
n=2 (194)



(195) (196)



(199)

(192)

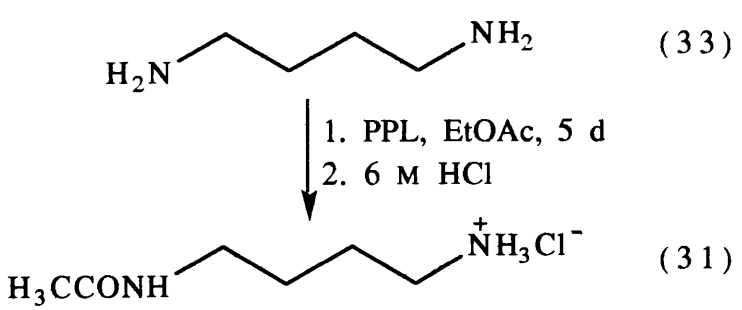
For reference purposes the diacetyldiamines were prepared by chemical means. These were produced by the reaction of each diamine with acetic anhydride in the presence of pyridine (Scheme 6.7). The diacetyldiamines were prepared without difficulty. Diacetyl-2-methyl-1,5-diaminopentane (192) could not be crystallised. However, the waxy solid obtained was pure by TLC and 90 MHz ^{1}H NMR spectroscopy and hence suitable for our purposes.

Each PPL-catalysed acetylation was monitored with time by TLC using ethyl acetate/ isopropanol/ conc. ammonia (9:7:4) as eluant. The reaction mixtures were examined after 1 hour, 2 hours, 4 hours, 8 hours, 24 hours and every 24 hours thereafter.

6.3.1 Putrescine (33)

After 24 hours *N*-acetylputrescine was visible by TLC. The amount of *N*-acetylputrescine increased slowly until six days after the start of the reaction. At this point TLC indicated that diacetylputrescine (191) was being produced. Hence the reaction was worked up after five days. At this point a significant amount of putrescine was still present. After recrystallisation the yield of *N*-acetylputrescine hydrochloride (31) was 25.9% (Scheme 6.8).

Scheme 6.8

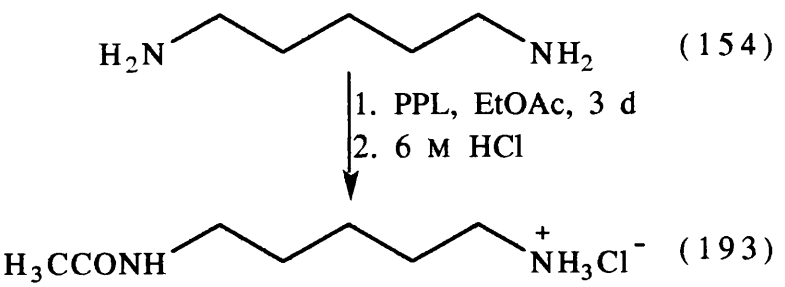


Since this reaction had proved successful further studies on the acylation of diamines catalysed by lipases were carried out.

6.3.2 Cadaverine (154)

Monoacetylated cadaverine was present in the reaction mixture after 24 hours. Diacetylcadaverine (194) appeared after four days. Therefore the reaction was worked up after three days (Scheme 6.9). About 40% (by TLC) of the starting material was still present.

Scheme 6.9



The yield of *N*-acetylcadaverine hydrochloride (193) at 13% was very low. ^{1}H and ^{13}C NMR spectral data showed the product to be free of cadaverine dihydrochloride impurities. This was corroborated by the microanalytical data on the *N*-acetylcadaverine hydrochloride sample.

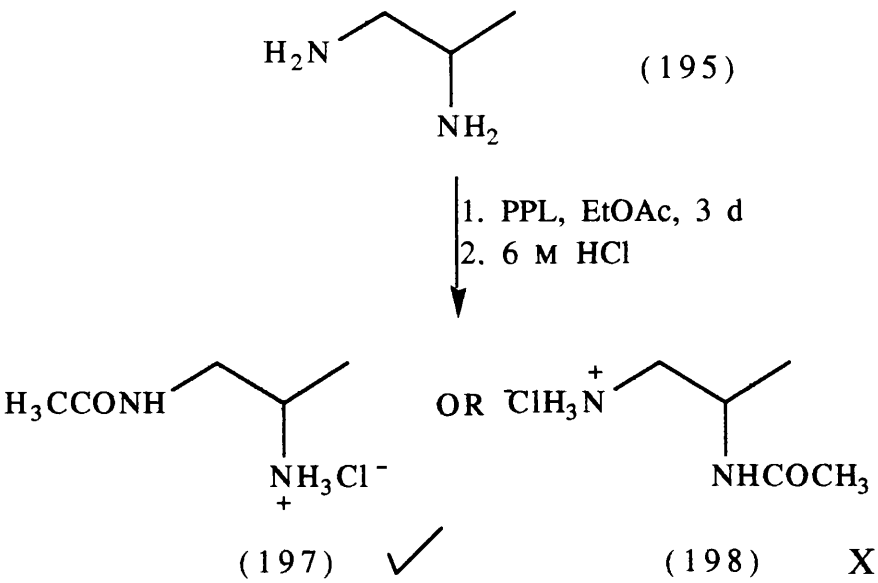
6.3.3 1,2-Diaminopropane (195)

With 1,2-diaminopropane (195) as substrate there are two possible monoacetylated products: (197) and (198). However when 1,2-diaminopropane was treated with ethyl acetate in the presence of PPL only one monoacetylated product was produced (Scheme 6.10). Diacetyl-1,2-diaminopropane (196) was never observed on a TLC of the reaction mixture even after 30 days.

The reaction was worked up after three days because after this point the ratio of product to starting material stayed approximately the same. The yield of product was low at 19%

but this was due to the repeated recrystallisations required to remove all impurities of 1,2-diaminopropane dihydrochloride.

Scheme 6.10



To ascertain which monoacetyl-1,2-diaminopropane had been produced, the 200 MHz ^{1}H NMR spectrum of the product was examined with respect to the ^{1}H NMR spectrum of diacetyl-1,2-diaminopropane (196) and compared with average chemical shift values from the literature.[150] The literature[150] gives the following values:

R-CH_2-N^+	δ 3.3
R-CH_2-NHCOR	δ 3.2
R_2-CH-NHCOR	δ 4.0

If the CH of the product was attached to the amide i.e. -NHCOR, this proton would resonate at *ca.* δ 4.0. In the ^{1}H NMR spectrum of diacetyl-1,2-diaminopropane this proton appears at δ 3.98. However, the CH proton in the product appears at *ca.* δ 3.2 and hence the CH must be attached to the N^+. Therefore the product of this reaction is *N*-acetyl-1,2-diaminopropane hydrochloride (197). Thus the acetylation of 1,2-diaminopropane using ethyl acetate and PPL is completely

regiospecific with the only product being *N*-acetyl-1,2-diaminopropane hydrochloride (197).

6.3.4 2-Methyl-1,5-diaminopentane (199)

In the case of the acetylation of 2-methyl-1,5-diaminopentane two products were observed by TLC after 24 hours. These had R_f values of 0.28 and 0.32 and were probably the two different monoacetylated products since diacetyl-1,2-diaminopropane (192) had an R_f of 0.55.

The reaction was stopped after 5 days . Separation of the two products from each other and the starting material was attempted by preparative TLC. This proved unsuccessful and since no regioselectivity was apparent, the reaction was abandoned.

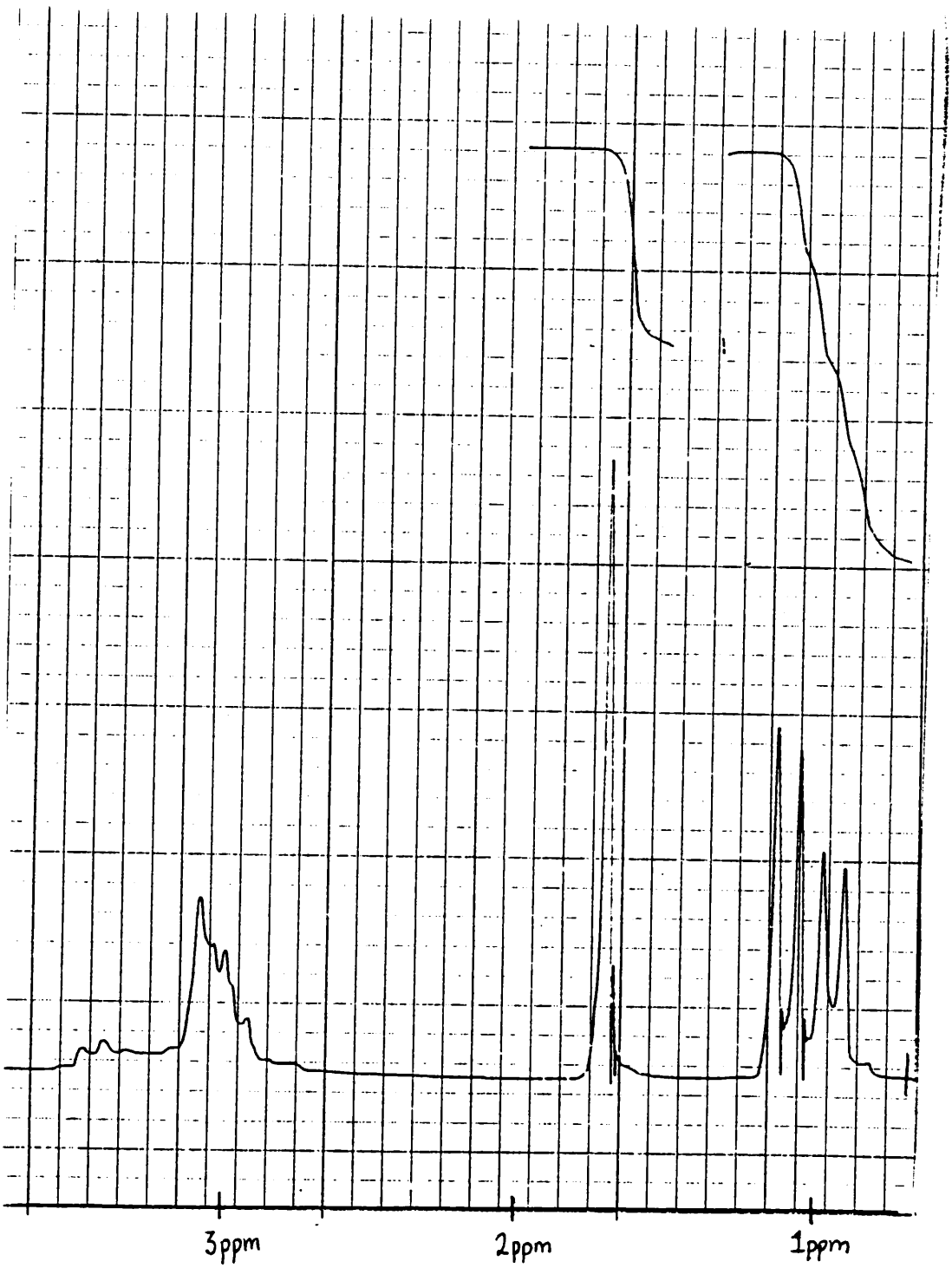
6.4 Stereoselective Monoacetylation of 1,2-Diaminopropane (195)

As described before, the PPL/ ethyl acetate system regiospecifically acetylates 1,2-diaminopropane (195) on the *N*-1 nitrogen to give *N*-acetyl-1,2-diaminopropane hydrochloride (197). Since 1,2-diaminopropane possesses a chiral centre it was chosen as the substrate to examine the stereoselectivity of this PPL-catalysed acetylation.

Four identical reactions were set up in separate vessels and worked up at daily intervals. The percentage conversion at each time was calculated from the ^1H NMR spectrum of the reaction mixture. For example, the 90 MHz ^1H NMR spectrum for the 48 hour reaction is shown in Figure 6.1. The percentage conversion was calculated from the following equation.

$$\text{Percentage conversion} = \frac{[\text{integral of s @ } \delta\ 1.7\ (CH_3CO \text{ of product})] \times 100\%}{[\text{integral from } \delta\ 0.85 \text{ to } \delta\ 1.2\ (CH_3CH \text{ of SM and product})]}$$

Figure 6.1: 90 ^{1}H NMR Spectrum of PPL-Catalysed Monoacetylation of 1,2-Diaminopropane (195) after 48 hours.



Optical rotations for each *N*-acetyl-1,2-diaminopropane hydrochloride sample were recorded in water. The results are given in Table 6.1.

Table 6.1: Stereoselective Monoacetylation of 1,2-Diaminopropane (195) Using PPL and EtOAc.

Experiment Number	1A	1B	1C	1D
Time (h)	24	48	72	96
% Conversion	31	47	49	85
Wt. Product (mg)	20.0	21.6	40.7	26.5
Yield (%)	4.9	5.2	9.9	6.4
$[\alpha]_D$ of (197) (H_2O)	-15.5° (c 2.0)	-10.4° (c 2.16)	-6.5° (c 2.24)	-0.3° (c 0.93)
E. excess (%)	51%	-	21%	-

As expected the percentage conversion increased with time. Since the percentage conversion was over 50%, PPL catalysed the acetylation of both enantiomers of 1,2-diaminopropane. In an ideal situation the enzyme would catalyse the reaction of only one enantiomer hence giving an enantiomeric excess approaching 100%.

The amount of *N*-acetyl-1,2-diaminopropane hydrochloride isolated was very small. This resulted from the combination of a difficult work up procedure and the repeated recrystallisation of *N*-acetyl-1,2-diaminopropane which was necessary to obtain a pure product sample for optical activity analysis.

All of the *N*-acetyl-1,2-diaminopropane hydrochloride samples possessed optical rotations. The optical activity decreased with increasing conversion. PPL was therefore preferentially acetylating one enantiomer of 1,2-diaminopropane.

The 1,2-diaminopropane dihydrochloride which was reclaimed at the end of the 24 hour reaction had a small optical

rotation of -1.35°. None of the other 1,2-diaminopropane dihydrochloride samples isolated at the end of the reactions possessed any optical activity.

6.5 Estimation of Enantiomeric Excess

The PPL-catalysed acetylation of 1,2-diaminopropane using ethyl acetate showed stereoselectivity. The next problem was to ascertain the degree of selectivity i.e. the enantiomeric excess (e.e.).

The first technique looked at to resolve the two enantiomers of *N*-acetyl-1,2-diaminopropane hydrochloride was to use a chiral shift reagent in conjunction with 200 MHz ^1H NMR spectroscopy. However, hydrochloride salt (197) only dissolved in D_2O and no chiral shift reagents were available for use in that solvent. Formation of the free amine and then dissolution of that in $CDCl_3$ was another option as chiral shift reagents for use in deuteriochloroform were available. This was not attempted because it was felt that the reagents required to form the free amine e.g. strong base, would probably destroy the optical activity if not *N*-acetyl-1,2-diaminopropane itself.

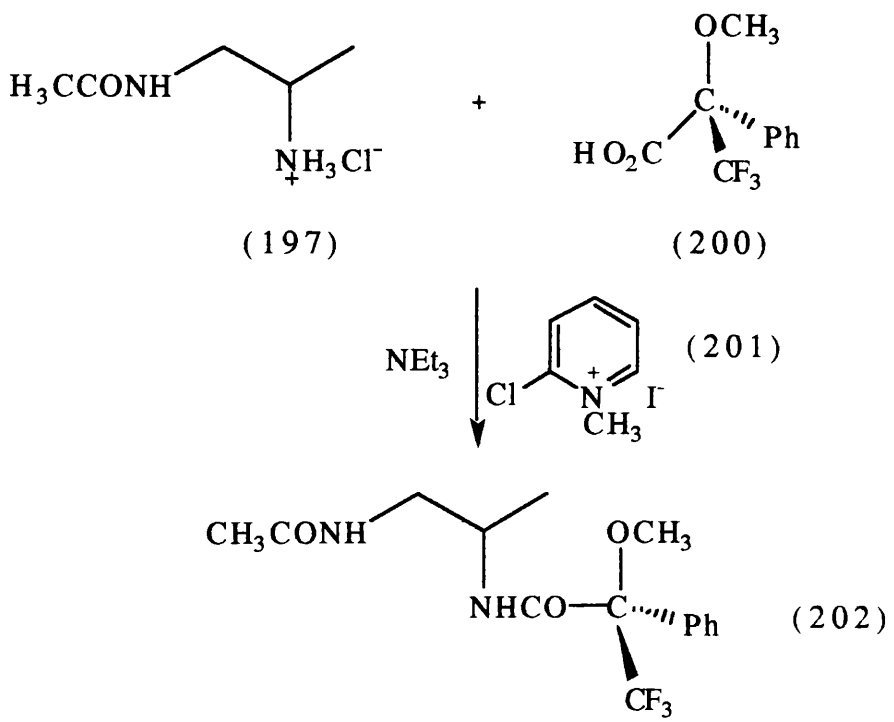
The formation of a diastereomeric salt with for example D-camphorsulphonic acid was not feasible because the hydrochloride salt would again have had to be converted into the free amine.

Dale and coworkers[151] used α-methoxy-α-trifluoromethylphenylacetic acid (known as Mosher's Acid) for the determination of the enantiomeric composition of amines. The amides prepared showed significantly different chemical shifts for each diastereomer in both the proton and fluorine NMR spectra. These workers formed diastereomeric amides by treating α-methoxy-α-trifluoromethylphenylacetyl chloride with amines.[151] Again the problem arose of converting *N*-acetyl-1,2-diaminopropane hydrochloride into the free amine.

This problem was circumvented by the use of a coupling agent which worked in conjunction with triethylamine.

2-Chloro-1-methylpyridinium iodide (Mukaiyama's reagent) (201) is used to couple carboxylic acids with alcohols.[152] This reagent was used to couple (R)-Mosher's acid (200) and *N*-acetyl-1,2-diaminopropane hydrochloride (31). The addition of an extra 1.5 equivalents of triethylamine on top of that required for the coupling reaction resulted in the formation of the free amine *in situ.* The amine then reacted with (R)-Mosher's acid to produce *N*-acetyl-*N*'-α-methoxy-α-trifluoromethylphenylacetyl-1,2-diaminopropane (202) (Scheme 6.11).

Scheme 6.11



This process was first carried out on a racemic sample of *N*-acetyl-1,2-diaminopropane hydrochloride. This use of a racemic sample allowed the coupling reaction to be examined for diastereoselection within the reaction. The product (202) was purified by column chromatography. The white solid

obtained had a melting point range of 10 °C but this was not surprising since it was a mixture of two diastereomers.

Proof that the coupling reaction had been successful came from the ^{1}H and ^{13}C NMR spectra where the expected signals were present. The ^{13}C NMR spectrum indicated the presence of two diastereomers since many of the peak were doubled.

Usually with diastereomers based on Mosher's acid the diastereomer ratio can be calculated from the ^{19}F NMR spectrum because there should only be one peak present for each diastereomer.[151] Unfortunately here the two peaks were not baseline separated and could not be used to calculate the diastereomeric excess.

The most significant chemical shift difference was in the 200 MHz ^{1}H NMR spectrum. As can be seen from Figure 6.2 the singlets for the acetyl group protons resonate at δ 1.74 and 1.92. These peaks integrated for 1.5 protons each and hence the product was a 50:50 mixture of diastereomers. Thus although the yield of diamide (202) was low at 35%, the diastereomeric mixture at the end of the reaction was representative of the mixture of *N*-acetyl-1,2-diaminopropane hydrochloride enantiomers that was present at the start.

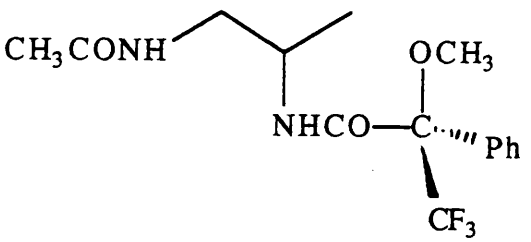
Other differences noticed for the two diastereomers in the ^{1}H NMR spectrum included a broadening of the methoxyl proton singlet and the appearance of the H-3 protons as two doublets.

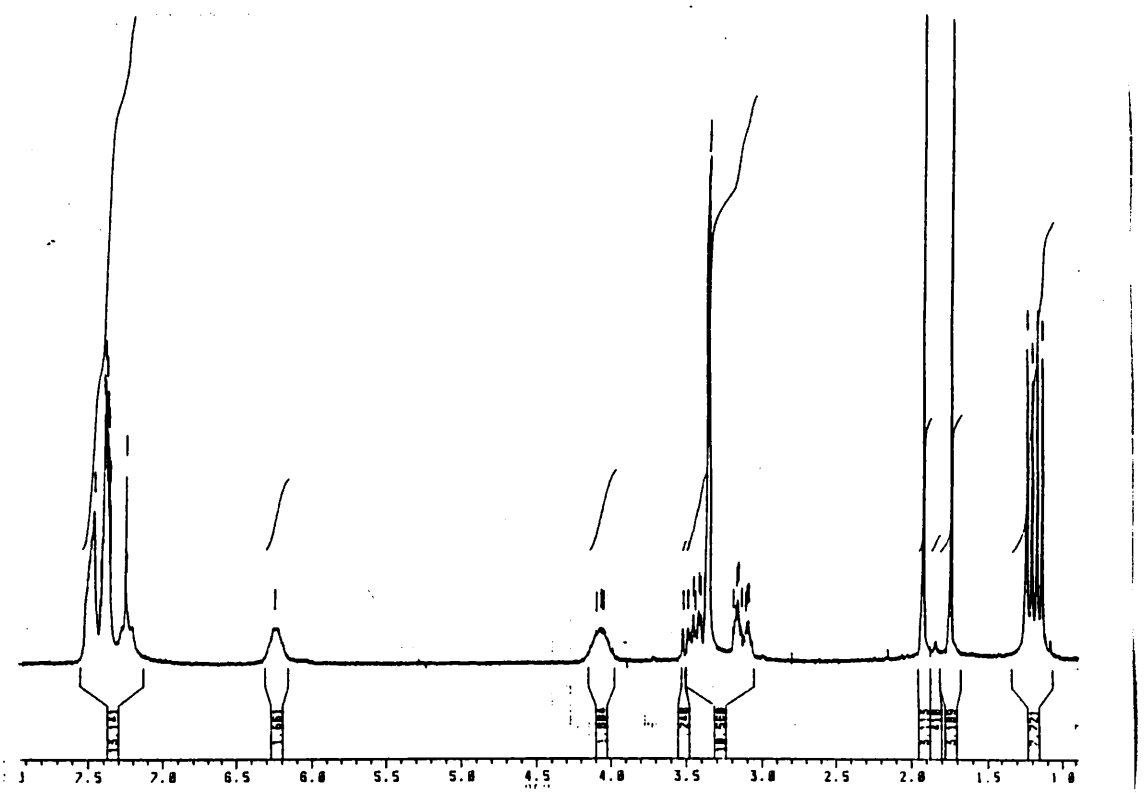
The acetyl proton singlets are ideal for estimation of the diastereomeric ratio in the *N*-acetyl-*N'*-α-methoxy-α-trifluoromethylphenylacetyl-1,2-diaminopropane samples because no other protons resonate in that area and the two peaks are completely baseline separated.

It is surprising that the major chemical shift differences between the diastereomers is seen with the acetyl group protons because these are the protons furthest away from the new chiral centre in the molecule.

Molecular modelling gave the following conformations for the (R,R)- [Figure 6.3] and (S,R)- [Figure 6.4] isomers.

Figure 6.2 : 200 MHz ^1H NMR Spectrum of 50:50 Mixture of *N*-Acetyl-*N'*-α-methoxy-α-trifluoromethyl-phenylacetyl-1,2-diaminopropane diastereomers.





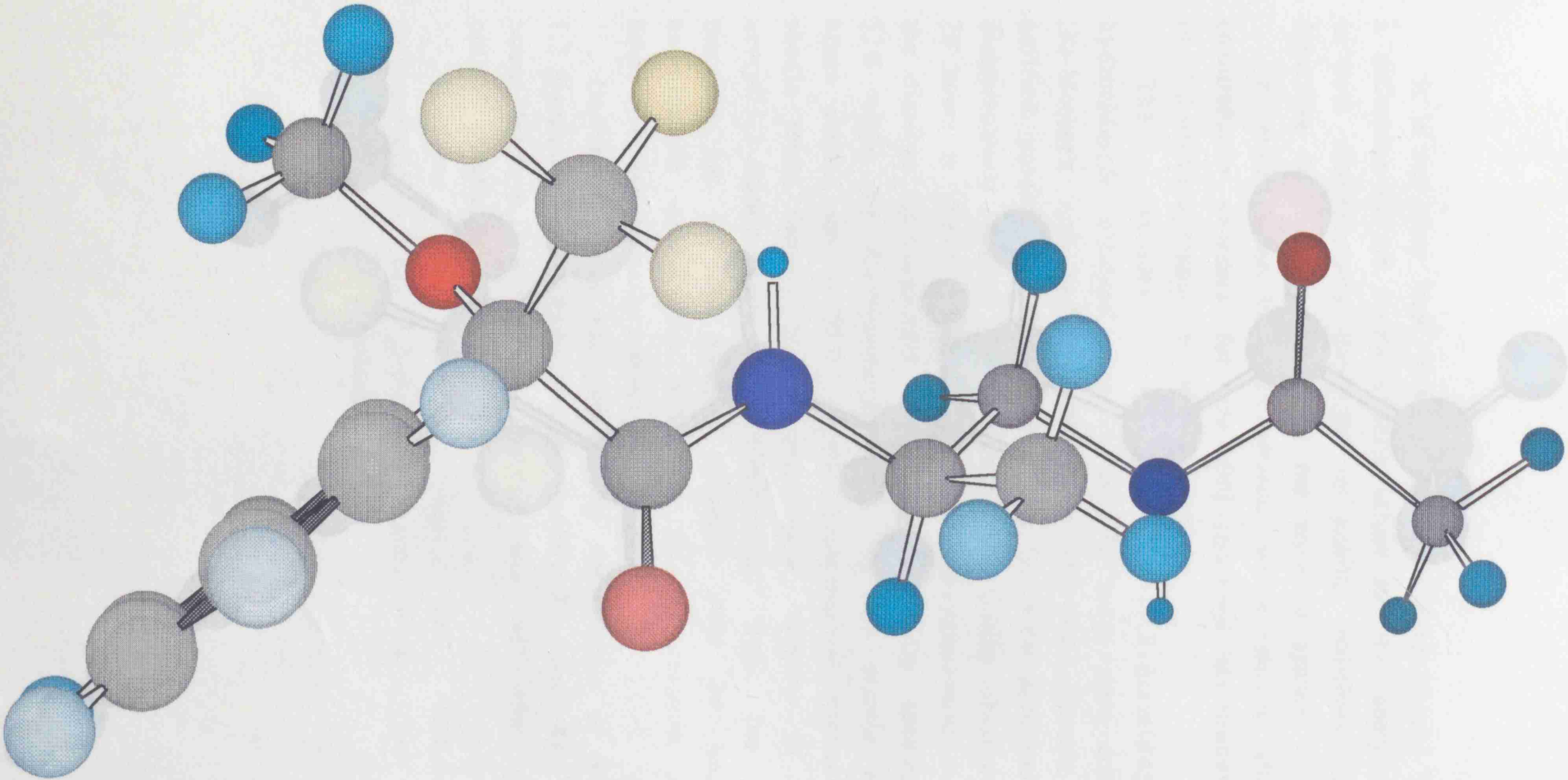


Figure 6.3: (R,R) isomer

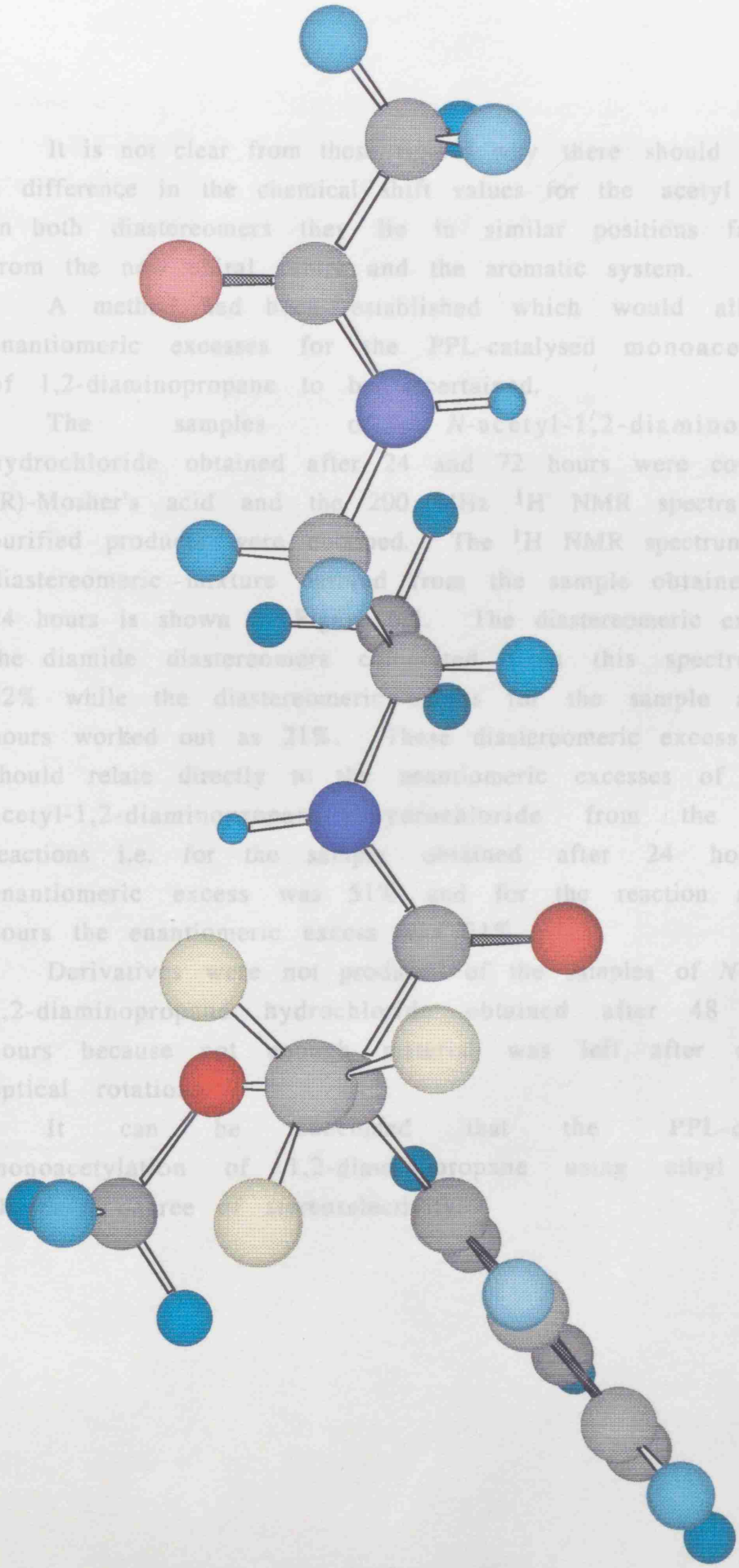


Figure 6.4: (S,R) isomer

It is not clear from these figures why there should be such a difference in the chemical shift values for the acetyl groups. In both diastereomers they lie in similar positions far away from the new chiral centre and the aromatic system.

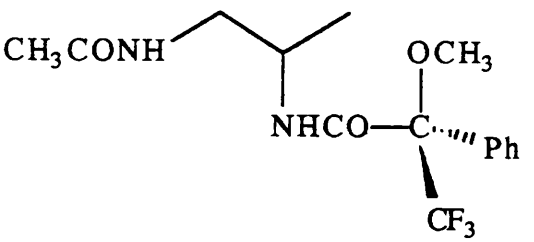
A method had been established which would allow the enantiomeric excesses for the PPL-catalysed monoacetylation of 1,2-diaminopropane to be ascertained.

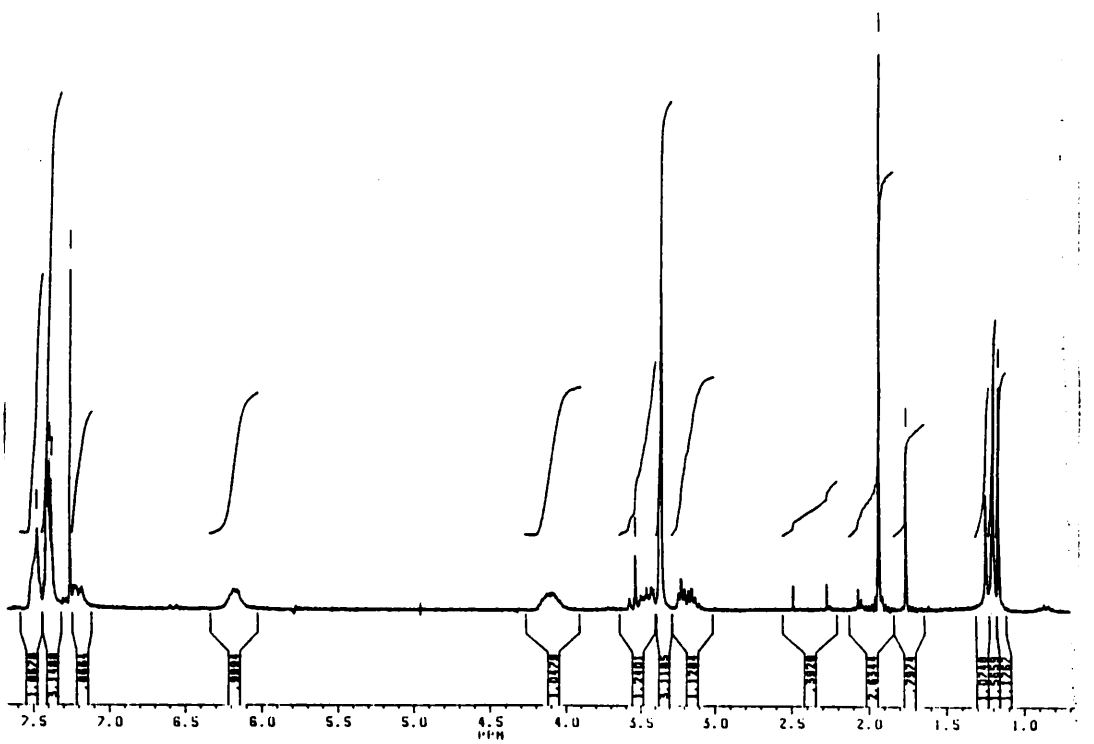
The samples of *N*-acetyl-1,2-diaminopropane hydrochloride obtained after 24 and 72 hours were coupled to (R)-Mosher's acid and the 200 MHz ^{1}H NMR spectra of the purified products were obtained. The ^{1}H NMR spectrum of the diastereomeric mixture derived from the sample obtained after 24 hours is shown in Figure 6.5. The diastereomeric excess of the diamide diastereomers calculated from this spectrum was 52% while the diastereomeric excess for the sample after 72 hours worked out as 21%. These diastereomeric excess values should relate directly to the enantiomeric excesses of the *N*-acetyl-1,2-diaminopropane hydrochloride from the enzyme reactions i.e. for the sample obtained after 24 hours the enantiomeric excess was 51% and for the reaction after 72 hours the enantiomeric excess was 21%.

Derivatives were not produced of the samples of *N*-acetyl-1,2-diaminopropane hydrochloride obtained after 48 and 96 hours because not enough material was left after obtaining optical rotations.

It can be concluded that the PPL-catalysed monoacetylation of 1,2-diaminopropane using ethyl acetate shows a degree of stereoselectivity.

Figure 6.5 : 200 MHz ^{1}H NMR Spectrum of *N*-Acetyl-*N*'-α-methoxy-α-trifluoromethyphenylacetyl-1,2-diaminopropane derived from *N*-Acetyl-1,2-diaminopropane sample obtained after 24 hours.





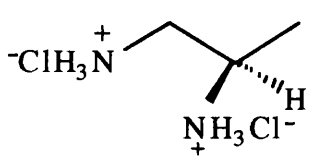
6.6 Determination of Major Enantiomer of *N*-Acetyl-1,2-diaminopropane Hydrochloride Produced

Two approaches were used to determine which enantiomer of 1,2-diaminopropane (195) was being acetylated preferentially by PPL i.e. which enantiomer was the major one present in the *N*-acetyl-1,2-diaminopropane hydrochloride samples.

There were no optical rotation values in the literature for the enantiomers of *N*-acetyl-1,2-diaminopropane hydrochloride.

The recovered 1,2-diaminopropane dihydrochloride from the reaction after 24 hours had an optical rotation of -1.35°. Optical rotation values for the two enantiomers of 1,2-diaminopropane (195) were available[153] but since no adequate method existed to transform the dihydrochloride salt into the free amine these figures were of no use. The only clue from the literature was that (R)-1,2-diaminopropane dihydrochloride had an optical rotation that was positive in value.[153]

At this point a sample of (R)-(-)-1,2-diaminopropane tartrate was donated to us by Dr I. Fallis, University of Glasgow. This was converted into (R)-1,2-diaminopropane dihydrochloride (203) by the treatment of the tartrate salt with potassium chloride.[154] The optical rotation of (R)-1,2-diaminopropane dihydrochloride was +3.70°. This was in agreement with what was known in the literature.[153]



(203)

From this evidence it seemed that in the 1,2-diaminopropane dihydrochloride sample from the reaction after 24 hours the (S)-isomer predominated. It is therefore

likely that the *N*-acetyl-1,2-diaminopropane hydrochloride sample contains more of the (R)-isomer.

No other samples of 1,2-diaminopropane hydrochloride possessed optical activity. It was for this reason that another method was sought to corroborate that the (R)-enantiomer was the major isomer present in the samples of *N*-acetyl-1,2-diaminopropane hydrochloride.

N-Acetyl-*N*'-α-methoxy-α-trifluoromethylphenylacetyl-1,2-diaminopropane (202) was a white solid. Therefore if a crystal of one diastereomer of diamide (202) could be produced then its absolute configuration could be established by X-ray diffraction.

Recrystallisation seemed a reasonable approach for separating the two diastereomeric forms of diamide (202). All samples of *N*-acetyl-*N*'-α-methoxy-α-trifluoromethylphenyl-acetyl-1,2-diaminopropane were pooled together and repeatedly recrystallised. After three recrystallisations a pure sample of one diastereomer was furnished as clear, needle-like crystals. This diastereomer was shown by ^{1}H NMR spectroscopy to be the major isomer. These crystals were submitted for X-ray diffraction analysis. Unfortunately the results of this were not obtained.

6.7 Improvement of Stereoselectivity

Many different experimental procedures have been developed to increase the efficiency of lipase catalysed resolutions. A number of these were employed by us to try and improve the enantioselectivity of 1,2-diaminopropane monoacetylation.

6.7.1 Alternative Lipases

The first method examined to improve the stereoselectivity was to use an alternative lipase to PPL. *Candida rugosa* lipase (CRL) and Lipozyme (*Mucor miehei* lipase immobilised on a macroporous anion exchange resin) had been successfully used before to catalyse amide formation[145,146,148] and were readily available to us.

When CRL was used *N*-acetyl-1,2-diaminopropane (197) was formed although the rate of the reaction was slower that with PPL (Table 6.2). The product (197) isolated had no optical activity. It was therefore concluded that CRL did not catalyse the monoacetylation of 1,2-diaminopropane stereoselectively.

Table 6.2: Monoacetylation of 1,2-Diaminopropane (195) Using CRL and EtOAc.

Experiment Number	2A	2B	2C	2D
Time (h)	24	72	168	192
% Conversion	7	14	11.9	2.9
Wt. Product (mg)	6.3	11.9	39.3	-
Yield (%)	1.5	2.9	9.5	-
$[\alpha]_D$ of (197) (H_2O)	0° (c 0.63)	0° (c0.53)	0° (c 1.26)	-

With Lipozyme catalysed acetylation of 1,2-diaminopropane, *N*-acetyl-1,2-diaminopropane was observed by TLC in small amounts. However, no *N*-acetyl-1,2-diaminopropane hydrochloride could be isolated. The Lipozyme catalysis was therefore not pursued.

6.7.2 Use of Various Alkyl Acetates

It was felt that the replacement of ethyl acetate with a different alkyl acetate might lead to improved stereoselectivity. The alkyl acetate acts as the lipase substrate and it seemed plausible that providing a substrate closer in structure to the natural one might lead to an increased reaction rate and perhaps higher enantioselectivity. The natural lipase substrates are triacylglycerols and therefore an alkyl group similar in structure to glycerol would be expected to show the largest effects. A range of available alkyl acetates were examined.

When methyl acetate was the alkyl acetate, the rate of monoacetylation of 1,2-diaminopropane was comparable to that when ethyl acetate was used (Table 6.3).

Table 6.3: Monoacetylation of 1,2-Diaminopropane (195) Using PPL and CH_3OAc.

Experiment Number	4A	4B	4C	4D
Time (h)	24	48	72	96
% Conversion	31	50	54	73
Wt. Product (mg)	25.3	20.3	28.8	78.1
Yield (%)	6.1	4.9	7.0	19.0
$[\alpha]_D$ of (197) (H_2O)	-9.1° (c 1.43)	-5.3° (c 0.85)	-3.3° (c 0.95)	-1.6° (c 0.75)
E. excess (%)	-	24.7%	14.5%	4.9%

All of the *N*-acetyl-1,2-diaminopropane hydrochloride samples obtained were optically active. The enantiomeric excess values were estimated for the samples obtained after 48, 72 and 96 hours. by forming Mosher's acid derivatives.

After 24 hours both the reaction with methyl acetate and ethyl acetate had reached 31% conversion. The optical rotation of the sample using ethyl acetate was higher than the sample involving methyl acetate. Therefore it could be said that

higher stereoselectivity was observed when ethyl acetate was the acyl donor. However if the figures at close to 50% conversion of 1,2-diaminopropane into *N*-acetyl-1,2-diaminopropane are examined, i.e. the sample after 72 hours from the experiment using ethyl acetate and the sample after 48 hours from the experiment with methyl acetate, a different story emerges. In this instance the optical rotations corroborate the above argument but the enantiomeric excess values indicate that the methyl acetate reaction had slightly higher stereoselectivity.

The small contradiction seen here (and in later experiments) between the optical rotation figures and estimated enantiomeric excess values is due to an error in one of the figures. The more accurate figure is probably that for the enantiomeric excess because most of the samples were slightly coloured and therefore gave brown solutions when the optical rotations were taken.

It can be concluded that when ethyl acetate and methyl acetate are used as acyl donors the stereoselectivities observed are approximately equal.

The substitution of ethyl acetate with *n*-propyl acetate gave a much slower rate of reaction (Table 6.4).

Table 6.4: Monoacetylation of 1,2-Diaminopropane (195) Using PPL and *n*-PrOAc.

Experiment Number	5A	5B	5C
Time (h)	72	168	240
% Conversion	30	31	39
Wt. Product (mg)	17.6	50.4	-
Yield (%)	4.3	12.2	-
$[\alpha]_D$ of (197) (H_2O)	-	-1.3° (c 1.54)	-
E. excess (%)	-	36%	-

After 72 hours the reaction rate reduced considerably. This is probably caused by deactivation of the enzyme with time. *N*-Acetyl-1,2-diaminopropane hydrochloride from the experiment that ran for one week was optically active with an e.e. calculated at 31%. Comparison of this value to that for the reaction in ethyl acetate after 24 hours which also went to 31% conversion, indicated that using *n*-propyl acetate gave a reaction of lower steroselectivity.

When *i*-propyl acetate was used as the alkyl donor no *N*-acetyl-1,2-diaminopropane was formed.

In the case of *n*-butyl acetate, the reaction was extremely slow and the tiny amount of product isolated possessed no optical activity.

With *n*-octyl acetate in place of ethyl acetate no reaction of 1,2-diaminopropane occurred.

When phenyl acetate, an activated acetate, was used the diamine (195) and phenyl acetate reacted on contact to produce diacetyl-1,2-diaminopropane (196).

In conclusion, shorter *n*-alkyl chains in the alkyl portion of the alkyl acetate led to increasing reaction rates and higher stereoselectivities. The branched alkyl acetate gave no reaction and was probably not a PPL substrate.

A summary of these results is given in Table 6.5.

6.7.3 Use of Various Ethyl Acylates

Conventional wisdom has it that in a lipase-catalysed reaction the initial rate of acylation increases as the alkanoate moiety of the ester increases in chain length from C_2- C_4.[155,156]

Using another alkyl acylate in place of ethyl acetate leads to a different product but these alternatives were worth examining because of the potential for increasing enantioselectivity. Substitution of ethyl acetate with ethyl formate, ethyl propionate and ethyl butyrate gave the following results.

Table 6.5 Summary of Monoacetylation of 1,2-Diaminopropane (195) Using PPL and Various Alkyl acetates.

Alkyl acetate	Reaction rate[$]	E. excess[£]
methyl acetate	* * *	* * *
ethyl acetate	* * *	* * *
n-propyl acetate	* *	* *
n-butyl acetate	*	0
n-octyl acetate	0	-
i-propyl acetate	0	-

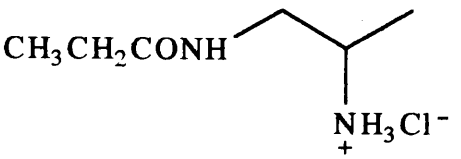
$ Reaction rate
* * * = *ca.* same rate as EtOAc reaction
* * = noticable after 24 h but slower than EtOAc reaction
* = just noticable after *ca.* 5 days
0 = no reaction even after *ca.* 7 days

£ Enantiomeric excess
* * * = *ca.* same as EtOAc reaction
* * = not as high as EtOAc reaction but still reasonable
* = small amount of stereoselectivity
0 = no stereoselectivity

Ethyl formate reacted exothermically with 1,2-diaminopropane (195) on contact. This reaction was therefore not pursued.

The PPL-catalysed reaction of ethyl propionate and 1,2-diaminopropane proceeded at a slower pace than that when ethyl acetate was the acylating agent (Table 6.6). *N*-propionyl-1,2-diaminopropane hydrochloride (204) was formed and could be identified in the 90 MHz ^{1}H NMR spectrum of the reaction mixture. However attempts to isolate this compound proved fruitless. Only brown sticky oils were obtained and these

usually contained a high proportion of starting material. It was not clear whether the product (204) was unstable in some way or whether the work up procedure used to isolate *N*-acetyl-1,2-diaminopropane hydrochloride (197) was not suitable for isolating higher homologues in the series.

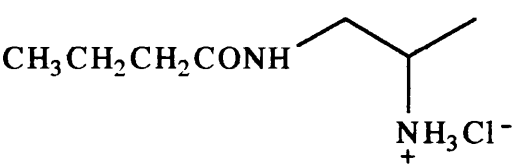


(204)

Table 6.6: Monoacetylation of 1,2-Diaminopropane (195) Using PPL and Ethyl propionate.

Experiment Number	11A	11B	11C	11D
Time (h)	24	48	72	168
% Conversion	34	22	32	46

When ethyl butyrate was used in place of ethyl acetate the fastest reaction rate should have been observed. In fact the exact opposite was the case (Table 6.7). After 24 hours only a 5% conversion had occurred and the maximum conversion observed was 26%. This maximum conversion occurred after 48 hours after which time the yield of *N*-butyryl-1,2-diaminopropane hydrochloride (205) tailed off. Therefore again it looked like the product might be unstable. Isolation of the product, as in the previous case, proved unsuccessful.



(205)

Table 6.7: Monoacetylation of 1,2-Diaminopropane (195) Using PPL and Ethyl butyrate.

Experiment Number	12A	12B	12C	12D
Time (h)	24	48	72	168
% Conversion	5	26	11	10

In summary, in terms of percentage conversion ethyl acetate was the best acylating agent tried (Table 6.8). Since no *N*-acyl-1,2-diaminopropane hydrochloride other than *N*-acetyl-1,2-diaminopropane hydrochloride could be isolated, the relative stereoselectivities could not be compared.

Table 6.8: Summary of Monoacetylation of 1,2-Diaminopropane (195) Using PPL and Various Ethyl Acylates.

Ethyl acylate	Reaction Rate[$]
Ethyl acetate	* * *
Ethyl propionate	* *
Ethyl butyrate	*

$ For key see Table 6.5

6.7.4 Alteration of Reaction Temperature

Increasing the reaction rate of the PPL-catalysed acetylation of 1,2-diaminopropane was desirable for two reasons. It could lead to an increase in the enantioselectivity of the reaction. Also the usefulness of this method of monoacetylating diamines would be greatly enhanced if the reaction time could be substantially decreased.

The reaction was carried out at 35 °C rather than at 25 °C as before. This temperature is closer to the natural working

temperature of PPL and might therefore give a higher rate of reaction. The results are shown in Table 6.9.

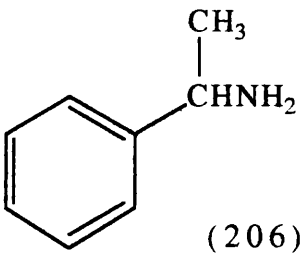
Table 6.9: Monoacetylation of 1,2-Diaminopropane (195) Using PPL and EtOAc at 35 °C.

Experiment Number	13A	13B	13C	13D
Time (h)	24	29	48	78
% Conversion	43	37	49	70
Wt. Product (mg)	13.4	35.8	38.0	44.0
Yield (%)	3.3	8.7	9.2	10.7
$[\alpha]_D$ of (197) (H_2O)	-10.5° (c 0.77)	-3.5° (c 1.30)	-4.2° (c 0.38)	-2.1° (c 0.74)
E. excess (%)	-	45%	-	25%

As can be seen the rate was approximately the same as at 25 °C. The *N*-acetyl-1,2-diaminopropane hydrochloride samples were all optically active. However from these results it could not be said whether the reaction showed higher stereoselectivity at 25 °C or 35 °C.

6.7.5 Alteration of Solvent

Solvent has been shown by a number of researchers to have a dramatic effect on the enantioselectivity of lipase catalysed reactions.[125,157,158]



(206)

Klibanov and coworkers examined the reaction between α-methyl benzylamine (206) and trifluoroethyl butyrate in

octane with different lipases acting as catalyst.[158] For no enzyme was any substantial enantioselectivity observed. However, when different solvents were examined changes were seen in the enantioselectivity of the reaction. The initial rate of acylation of R- and S-α-methylbenzylamine in various solvents was studied using subtilisin as the catalyst (Table 6.10).

Table 6.10: Enantioselectivity of Subtilisin in the acylation of α-Methylbenzylamine (206) as a Function of the Solvent.

Solvent	Initial reaction rate (υ) (mM h^{-1})		υ_S/υ_R
	S-amine	*R*-amine	
toluene	0.38	0.40	0.95
cyclohexane	1.1	0.87	1.3
acetonitrile	1.8	1.4	1.3
octane	1.3	0.9	1.4
ethyl acetate	1.4	0.88	1.6
pyridine	8.3	3.3	2.5
tetrohydrofuran	2.6	0.75	3.5
3-methyl-3-pentanol	23	3.0	7.7

From Ref. 158

As can be seen from from the figures, 3-methyl-3-pentanol was the best solvent to achieve high enantioselectivity because the S-amine was acylated at a much faster rate than the R-amine. Under these conditions an amide product with an e.e. of 85% was obtained. Due to these results we wished to try 3-methyl-3-pentanol in our system to see if enhanced enantioselectivity could be achieved.

The first method investigated used a minimal amount of ethyl acetate (two equivalents) in 3-methyl-3-pentanol. However in this case the reaction rate was totally negligible.

Therefore various volumes of ethyl acetate in 3-methyl-3-pentanol were tried in an attempt to increase the rate of reaction but still retain the effect of 3-methyl-3-pentanol (Table 6.11).

Table 6.11: Monoacetylation of 1,2-Diaminopropane (195) Using PPL.

Experiment Number	14A	14B	14C	14D
Volume EtOAc (ml)	5	2.5	2	1
Vol 3-methyl-3-pentanol (ml)	5	7.5	8	9
Time (h)	91	96	216	216
Wt. Product (mg)	75.8	48.7	61.3	25.8
Yield (%)	18.4	11.8	14.9	6.3
% Conversion	40	30	42	43
$[\alpha]_D$ of (197) in H_2O	-5.8° (c 1.4)	-7.3° (c 1.5)	-3.9° (c 0.6)	-1.6° (c 0.3)
E. excess (%)	42%	75%	19%	35%

These reactions were left running until they reached *ca.* 40% conversion. All the reactions were extremely slow in comparison with the standard in which ethyl acetate acted as both acyl donor and solvent. All the *N*-acetyl-1,2-diaminopropane hydrochloride samples were optically active. Unfortunately in this case there seemed to be no correlation between calculated enantiomeric excess values and the optical rotations obtained. No conclusions could therefore be drawn from this experiment. However as the reactions were very slow these systems possessed very little potential.

6.7.6 Alternative Acetylating Agents

In this lipase catalysed acetylation the amine reacts with an ester to form an amide and an alcohol is released. Unless

some factor interferes it is possible for the liberated alcohol to react leading to a reversible reaction (Equation 6.1). As discussed before this leads to lower product yields and more importantly to a decrease in the enantioselectivity of the reaction.

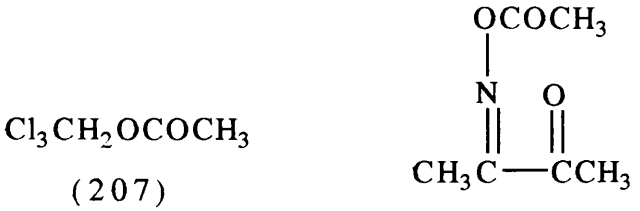
One way to suppress this reverse reaction is by reducing or removing the nucleophilic character of the alcohol. A way of achieving this is to have electronegative substituents on the α-carbon of the alcohol. This can be done by using 2,2,2-trichloro- and 2,2,2-trifluoroethyl esters as the acyl donors.[156]

Another way of reducing the nucleophilicity of the liberated alcohol is to use an oxime ester as the acylating agent. The resultant oxime has an hydroxyl group which is significantly less nucleophilic than normal.[159]

Alternatively the reverse reaction can be reduced by removing the problem hydroxyl group. With an enol ester as acyl donor, the alcohol formed tautomerises to the corresponding carbonyl compound.[160] A commonly used enol ester in vinyl acetate. However there are problems associated with this acetylating agent. Polymerisation of vinyl acetate sometimes occurs and the byproduct of the reaction, acetaldehyde, can in sufficient quantities deactivate the enzyme.

These three methods were assessed to see if they could be utilised in the PPL-catalysed monoacetylation of 1,2-diaminopropane.

2,2,2-Trichloroethyl acetate (207) was synthesised from 2,2,2-trichloroethanol and acetyl chloride in the presence of triethylamine. The oxime ester chosen was 2,3-butanedione monoxime acetate (208). This was prepared simply by the reaction of 2,3-butanedione monoxime and acetyl chloride. Unfortunately when tried both of these acetylating agents reacted with 1,2-diaminopropane without the enzyme being present.



(207)

(208)

It was a similar story when vinyl acetate was tried. *N*-Acetyl-1,2-diaminopropane was formed without the lipase being present in a number of different reaction solvents

6.8 Conclusions

The monoacetylation of a diamine is a difficult task to achieve by chemical means.

The monoacetylation of certain diamines was accomplished effectively using PPL and ethyl acetate but this useful technique was limited by the low yields of product isolated. For this technique to be viable another method would have to be found to purify the *N*-acetylated diamine. Preparative TLC was tried in the case of the monoacetylation of 2-methyl-1,5-diaminopentane but proved unsuccessful. Column chromatography on neutral or basic alumina may be the answer but time did not allow for this to be attempted. The use of HPLC is another possibility.

The PPL/ethyl acetate reaction system showed some stereoselectivity when presented with a racemic substrate with the (R)-isomer probably being preferentially acetylated. All attempts to improve reaction rates and the stereoselectivity failed.

The use of an alternative ethyl acylate as a means of improving enantioselectivity would be worth further examination once an improved work up procedure had been instigated.

The lipase catalysed monoacylation of diamines shows potential and with an improved purification method could be a useful technique.

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. Mass spectra were obtained with A.E.I. MS 12 or 902 spectrometers. Nuclear magnetic resonance spectra were recorded with a Perkin Elmer R32 spectrophotometer operating at 90 MHz (δ_H), a Varian EM 390 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 188.3 MHz (δ_F). The multiplicities of the ^{13}C NMR resonances were determined using DEPT spectra with pulse angles of $\theta = 90°$ and $\theta = 135°$. Spectra were recorded for solutions in deuteriochloroform unless otherwise stated, with either $CHCl_3$ or tetramethylsilane (TMS) as internal standard. Infrared spectra were obtained on either a Perkin Elmer 983 spectrophotometer or a Philips PU 9800 FTIR spectrophotometer. Elemental analyses were performed using a Carlo-Erba 1106 Elemental analyser.

Thin layer chromatography (TLC) was carried out on Merck Kieselgel G (silica) plates of 0.25 mm thickness. This was developed with chloroform-methanol-conc. ammonia (85:14:1) and visualised with iodine unless otherwise stated.

Radiochemicals were purchased from Amersham International. Radioactivity was measured with a Philips PW 4700 Liquid Scintillation Counter using solutions in 'Ecoscint'. 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 TLC plates.

Tetrahydrofuran (THF) was dried by distillation from sodium-benzophenone under nitrogen prior to use. Dichloromethane was distilled from phosphorus pentoxide and stored over 4 Å molecular sieves. Other solvents and reagents were purified by standard techniques. Dimethyl sulphoxide (DMSO) and acetic anhydride were distilled from calcium hydride and stored over 4 Å sieves. Triethylamine was distilled from, and stored over, potassium hydroxide.

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

Establishment and Propagation of Root Cultures

Hairy root cultures of *Emilia flammea* Cass. and *Senecio vulgaris* L. transformed with *Agrobacterium rhizogenes* were established as described for *Nicotiana* sp.[83] These cultures, provided by Dr N. Walton, were grown on Gamborg's B5 basal medium with 80 m M sucrose added. At subculture every 3 weeks, roots (*ca.* 0.1 g) were transferred into medium (100 ml) in each of thirty, 250 ml conical flasks. The cultures were grown at 25 °C with shaking at 90 rpm in normal laboratory light for 21 d before the roots were filtered off.

Extraction of Alkaloids from *Emilia Flammea* Transformed Root Cultures.

The roots from *Emilia flammea* (*ca.* 450 g) were chopped and macerated with methanol. The blended mixture was filtered and the residue was rinsed with methanol until the washings were colourless. The filtrate was concentrated *in vacuo* to leave a green residue. This was extracted with 1 M hydrochloric acid (2 x 20 ml) and the combined acid layers were washed with CH_2Cl_2 (6 x 20 ml). Zinc was added and the mixture was stirred for 2 h at room temperature. This suspension was filtered through Celite, basified with concentrated ammonia and extracted with CH_2Cl_2 (6 x 30 ml). The combined organic layers were dried, filtered and concentrated to give a crude alkaloid extract (*ca.* 80 mg). A TLC plate visualised with the modified Dragendorff reagent[84] showed two spots with R_f values of 0.30 and 0.54. These were separated by column chromatography on basic alumina. Elution with ethyl acetate/methanol (99:1, v/v) gave firstly a 3:1 mixture of senecionine (38) and integerrimine (131) (analysed by 200 MHz ^1H NMR spectroscopy), R_f 0.54. Recrystallisation from CH_2Cl_2/acetone (1:1, v/v) gave

senecionine (38) (*ca.* 20 mg); m.p. 228-230 °C (lit.,[163] 232-233 °C); $[\alpha]_D$ -57.3° (c 1.3, CH_2Cl_2) [lit.,[163] -56° ($CHCl_3$)]; all spectral data were identical to those of an authentic sample; (Found: M^+, 335.1723; C, 64.26; H, 7.62; N, 4.06%: $C_{18}H_{25}NO_5$ requires M, 335.1733; C, 64.47; H, 7.46; N, 4.18%). Further elution with ethyl acetate/methanol (98:2 v/v) yielded emiline (94) which was recrystallised from hexane (*ca.* 25 mg); R_f 0.30; m.p. 106-109 °C (lit.,[82] 105-107 °C); $[\alpha]_D$ -14.3° (c 0.35, CH_2Cl_2) [lit.,[82] -13.1° ($CHCl_3$)]; MS, IR, ^1H and ^{13}C NMR spectra were identical to literature[79,82]; (Found: M^+, 365.1838; C, 62.21; H, 7.38; N, 3.92%: $C_{19}H_{27}NO_6$ requires M, 365.1838; C, 62.47; H, 7.40; N, 3.84%).

Study of Alkaloid Content of *Emilia flammea* Transformed Root Cultures with Respect to Age.

Flasks (40 x 250 ml) were prepared and roots were propagated as before. Batches of five flasks were taken off at regular intervals over a one month period. They were extracted in the usual manner and the crude alkaloid extracts were analysed by 200 MHz ^1H NMR spectroscopy. The types of alkaloid present were determined by analysing the ^1H NMR spectrum for signals specific to each alkaloid i.e. δ 6.53 [20-H of integerrimine (131)], δ 6.18 [2-H of senecionine (38)] and δ 6.02 [2-H of emiline (94)]. Integration of these signals gave an estimate of the relative amounts of each alkaloid present. The results are shown in Table 3.1, Chapter 3.

Extraction of Alkaloids from *Senecio vulgaris* Transformed Root Cultures.

S. vulgaris cultures (*ca.* 430 g) were extracted as for *E. flammea* to give a cream coloured solid (*ca.* 0.24 g). TLC analysis of this crude alkaloid extract, visualising with the modified Dragendorff reagent,[84] showed one spot of R_f 0.54. Recrystallisation from CH_2Cl_2/acetone (1:1, v/v) gave

senecionine (38) (*ca.* 0.150 g) as white crystals, m.p. 229-232 °C (lit.,[163] 232-233 °C). All spectra and data were identical to those for senecionine from *E. flammea.*

Feeding of [1,4-^{14}C]Putrescine Dihydrochloride to Transformed Root Cultures.

[1,4-^{14}C]Putrescine dihydrochloride (5 μCi) was dissolved in sterile water and divided among 20 flasks each containing five day old roots. At regular intervals after feeding, batches of five flasks were removed and the crude alkaloid extracts were obtained. A sample of each extract was taken for scintillation counting. TLC was run of every extract and these plates were examined by radioscanning.

The various incorporation figures for *E. flammea* are given in Table 3.2, Chapter 3. The alkaloids from the ten day old root culture batch were separated by TLC on a 20 x 20 cm silica plate. From this 9.2 mg of senecionine (38) and 5.2 mg of emiline (94) were obtained.

The data for *S. vulgaris* are given in Table 3.3, Chapter 3. The crude alkaloid extract was recrystallised a number of times to give senecionine of constant specific activity.

Extraction of Alkaloids from *Gynura sarmentosa*

G. sarmentosa DC. (Asteraceae) plants were grown in pots in a greenhouse from cuttings supplied and identified by Staff of the Royal Botanic Garden, Edinburgh. The plants were harvested while in flower (*ca.* 100 g) and extracted as for *E. flammea* root cultures. The crude alkaloid extract (*ca.* 20 mg) was shown by TLC analysis to contain three alkaloids. These were separated on a silica preparative TLC plate. The following alkaloids were obtained. Otosenine (129) (*ca.* 5 mg); R_f 0.38; m.p. 202-204 °C (lit.,[94] 221-223 °C); υ_{max} ($CHCl_3$) 3 525, 3 018, 3 010, 2 935, 1 755, 1 730, 1 620, 1 270 and 1115 cm^{-1}; δ_H (600 MHz) 1.12 (3H, d, J 7Hz, 19-H_3), 1.13 (1H, dd, J 15 Hz and

11 Hz, 14-H), 1.21 (3H, d, J 5.5Hz, 21-H_3), 1.32 (3H, s, 18-H_3), 1.79 (1H, br s, OH), 1.90 (1H, m, 13-H), 2.06 (3H, s, N-CH_3), 2.11 (1H, d, J 15 Hz, 14-H), 2.22 (1H, m, 6-H), 2.61 (2H, m, 5- and 6-H), 2.90 (1H, m, 5-H), 2.97 (1H, q, J 5.5 Hz, 20-H), 3.30 (1H, ddd, J 18 Hz, 2 Hz and 2 Hz, 3-H), 3.41 (1H, d, J 18 Hz, 3-H), 4.32 and 5.43 (2H, AB system, J_{AB} 11 Hz, 9-H_2), 5.08 (1H, m, 7-H) and 6.10 (1H, m, 2-H); δ_C (150 MHz) 12.3 (C-19), 13.3 (C-21), 23.6 (C-18), 35.3 (C-14), 36.9 (C-6), 38.3 (C-13), 39.8 (N-CH_3), 52.9 (C-5), 55.8 (C-20), 58.8 (C-3), 63.5 (C-15), 64.0 (C-9), 76.7 (C-12), 78.2 (C-7), 134.0 (C-1), 136.9 (C-2), 167.8 (C-11), 177.6 (C-16) and 190.8 (C-8); *m / z* 381(M^+, 4.6%), 266, 250, 168, 151 (100%), 123, 110, 96, 82 and 70; (Found: M^+, 381.1771: $C_{19}H_{27}NO_7$ requires *M*, 381.1787). The 600 MHz ^1H-, 150 MHz ^{13}C- and two-dimensional ^1H-detected one bond δ_H/δ_C correlation (HMQC) NMR spectra were recorded on a Varian VXR600S spectrometer. The HMQC 2D spectrum is shown in Figure 3.1, Chapter 3.

Senkirkine (137) (*ca.* 2 mg); R_f 0.45; m.p. 195-198 °C (lit.,[96] 197-198 °C); υ_{max} ($CHCl_3$) 3 520, 3 020, 2 960, 2 920, 2 840, 1 730, 1 650, 1 140, 1 110 and 1 090 cm^{-1}; δ_H (200 MHz) 0.89 (3H, d, J 6 Hz, 19-H_3), 1.32 (3H, s, 18-H_3), 1.67 (1H, m, 13-H), 1.77 (1H, d, J 12 Hz, 14-H_β), 1.89 (3H, dd, J 7 Hz and 2 Hz, 21-H_3), 2.08 (3H, s, N-CH_3), 2.29 (1H, br d, J 12 Hz, 14-H_α), 2.37 (1H, m, 6-H_β), 2.53 (1H, m, 6-H_α), 2.71 (1H, m, 5-H_β), 2.86 (1H, ddd, J 13 Hz, 6 Hz and 2 Hz, 5-H_α), 3.21 (1H, ddd, J 18 Hz, 3 Hz and 3 Hz, 3-H_β), 3.42 (1H, dm, J 18 Hz, 3-H_α), 4.34 and 5.41 (2H, AB system, J_{AB} 11 Hz, 9-H_2), 4.97 (1H, t, J 3.5 Hz, 7-H), 5.86 (1H, qd, J 7 Hz and 1 Hz, 20-H) and 6.12 (1H, m, 2-H); *m / z* 365 (M^+, 6.0%), 266, 250, 168, 153 (100%), 151, 123, 110, 82 and 70; (Found: M^+ 365.1808: $C_{19}H_{27}NO_6$ requires *M*, 365.1838).

Senecionine (38) (*ca.* 1 mg); R_f 0.54; m.p. 227-229 °C (lit.,[163] 232-233 °C). IR, 200 Mz ^1H NMR and mass spectra were identical to those of an authentic sample.

No optical rotations were obtained for these alkaloids because not enough material was available.

7.3 Experimental to Chapter 4

Synthesis of *N*-Acetylputrescine Hydrochloride (31)

Chemical Method:

Putrescine (33) (1.00 g, 11.4 mmol) was added dropwise to a stirred solution of glacial acetic acid (10 ml). The solution was kept at 50-60 °C during the stepwise addition over 1 h of acetic anhydride (0.90 g, 8.80 mmol, 0.78 equiv.). The reaction mixture was allowed to cool to room temperature and was then stirred overnight. Concentration under reduced pressure gave a yellow oil which was dissolved in a mixture of hot water (5 ml) and 6 M hydrochloric acid (3.3 ml). The solution was evaporated to dryness to give a cream coloured solid. TLC analysis, eluting with EtOAc/isopropanol/conc. NH_3 (9:7:4), showed this to be a mixture of the title compound and putrescine dihydrochloride. Extraction of the mixture with isopropanol (50 ml) dissolved the desired product, and putrescine dihydrochloride was filtered off. The volume of the solution was reduced to *ca.* 10 ml and the solution was kept at 0 °C overnight. The white solid that formed was collected by filtration to afford *N*-acetylputrescine hydrochloride (31) (0.444 g, 23.4%); R_f 0.24 (EtOAc/isopropanol/conc. NH_3, 9:7:4); m.p. 134-137 °C (lit.,[164] 136-138 °C); υ_{max} (KBr) 3 270, 3 070, 3 000, 2 980, 2 890, 1 650, 1 555, 1 470 and 1 308 cm^{-1}; δ_H (200 MHz) (D_2O, ref. HOD @ 4.63) 1.34-1.66 (4H, complex, 2- and 3-H_2), 1.62 (3H, s, CH_3), 2.66 (2H, t, J 7.1 Hz, 4-H_2) and 3.05 (2H, t, J 6.7 Hz, 1-H_2); δ_C (D_2O, ref. dioxan @ 67.8) 23.1 (CH_3), 25.4 and 26.6 (C-2 and -3), 39.9 and 40.3 (C-1 and -4) and 175.3 (C=O); *m / z* 130 (M^+-HCl, 3.3%), 86, 73, 58, 43 and 30 (100%); (Found: C, 43.27; H, 9.06; N, 16.73%: $C_6H_{15}N_2OCl$ requires C, 43.24; H, 9.09; N, 16.80%).

Enzymic Method:

Putrescine (33) (0.5 g, 5.7 mmol) was dissolved in ethyl acetate (10 ml) and to this was added porcine pancreatic lipase

(PPL) (0.5 g, activity 13.3 units per mg of solid*). (*One unit will hydrolyse 1.0 microequvalent of fatty acid from a triglyceride in one hour at pH 7.4 at 37 °C.) This mixture was shaken at 100 rpm in a constant temperature bath at 27 °C. The reaction was monitored by TLC using EtOAc/isopropanol/conc. NH_3 (9:7:4) as eluant. The reaction was worked up after 5 d because this was the point of maximum monoacetylputrescine production, i.e. before diacetylputrescine was formed. The solvent was decanted and the enzyme was stirred for 1 h with $CHCl_3$/MeOH (9:1) (20 ml). The mixture was filtered through Celite. The combined solutions were then dried, filtered and concentrated *in vacuo* to give a yellow oil. This oil was dissolved in a mixture of hot water (5 ml) and 6 M HCl (3.3 ml). The solution was evaporated to dryness to give a solid. This solid was extracted with isopropanol (50 ml) to dissolve the desired product and putrescine dihydrochloride was filtered off. The filtrate was reduced in volume to *ca.* 5 ml and left at 0 °C overnight. The desired compound (31) solidified as a white powder (0.335 g, 35.2%); m.p. 135-136 °C (lit.,[164] 136-138 °C); all other data were identical to those for *N*-acetylputrescine hydrochloride formed by the chemical method.

Synthesis of *N*-Acetyl[1,4-^{3}H]putrescine Hydrochloride

A mixture of putrescine (0.5 g, 5.7 mmol) and [1,4-^{3}H]putrescine hydrochloride (1 mCi) was converted, by the enzymic method, into the title compound (0.143 g, 16.3%). A radioscan (eluting the TLC plate with isopropanol/conc. NH_3, 5:3) indicated radioactive bands corresponding to the title compound at R_f 0.37 and an unknown impurity at R_f 0.64. Recrystallisation twice from isopropanol/diethyl ether afforded *N*-acetyl[1,4-^{3}H]putrescine hydrochloride as a white solid (0.046 g, 5.2%, 206 μCi mmol^{-1}); m.p. 135-138 °C (lit.,[164] 136-138 °C. All spectra and physical properties were identical to authentic unlabelled material. Radioscanning showed one band

at R_f 0.37 coincident with that of authentic *N*-acetylputrescine hydrochloride.

Feeding Methods and Results

A. *S. pleistocephalus*

^{3}H-Labelled *N*-acetylputrescine hydrochloride (6.5 mg, 206 μCi mmol^{-1}) was mixed with [1,4-^{14}C]putrescine dihydrochloride to give an initial ^{3}H:^{14}C ratio of 5.6. The mixture was dissolved in sterile water and fed, by the wick method, to one plant on one day. Nine days later rosmarinine (29) was extracted by the standard procedure. The total incorporation of ^{3}H into the crude rosmarinine (286 mg) was 1.77%, with a ^{3}H:^{14}C ratio of 0.56. Rosmarinine (29) was recrystallised from CH_2Cl_2/acetone (1:1) to constant specific activity (137 mg, 0.223 μCi mmol^{-1} for ^{3}H). The ^{3}H:^{14}C ratio for the purified alkaloid was 0.50. Radioscanning showed one band coincident with authentic, unlabelled rosmarinine. For full results see Tables 4.2 and 4.3, Chapter 4.

B. *C. australe*

N-Acetyl[1,4-^{3}H]putrescine hydrochloride (6.5 mg, 206 μCi mmol^{-1}) was mixed with [1,4-^{14}C]putrescine dihydrochloride to give an initial ^{3}H:^{14}C ratio of 5.6. The mixture was dissolved in sterile water and fed, by the wick method, to three plants on one day. Eight days later the alkaloids were extracted (63 mg). The total incorporation of ^{3}H into the crude extract was 0.16%, with a ^{3}H:^{14}C ratio of 9.5. Radioscanning showed no radioactive bands. The two alkaloids present in the extract were separated by preparative TLC. Cynaustine (88) (2.3 mg), R_f 0.47, had a ^{3}H:^{14}C ratio of 3.4. Cynaustraline (87) (19.8 mg), R_f 0.33, showed a ^{3}H:^{14}C ratio of 3.0. For full results see Tables 4.2 and 4.3, Chapter 4.

C. *S. vulgaris* Transformed Root Cultures

^{3}H-Labelled *N*-acetylputrescine hydrochloride (10.4 mg, 206 μCi mmol^{-1}) was mixed with [1,4-^{14}C]putrescine dihydrochloride to give an initial ^{3}H:^{14}C ratio of 10.75. The mixture was dissolved in sterile water and divided among 20 flasks each containing five day old roots. At regular intervals after feeding, batches of five flasks were removed and crude senecionine (38) was obtained. Senecionine was recrystallised to constant specific activity from CH_2Cl_2/acetone (1:1). A radioscan of each extract showed one radioactive band coincident with authentic, unlabelled senecionine. The results are given in Table 4.4, Chapter 4.

D. *E. flammea* Transformed Root Cultures

N-Acetyl[1,4-^{3}H]putrescine hydrochloride (3.7 mg, 206 μCi mmol^{-1}) was mixed with [1,4-^{14}C]putrescine dihydrochloride to give an initial ^{3}H:^{14}C ratio of 3.44. The feeding was carried out as for *S. vulgaris* transformed root cultures. Radioscanning of the crude alkaloid extracts showed two radioactive bands, corresponding to emiline (94) and senecionine (38). Separation of the alkaloids by preparative TLC proved unsuccessful. The data for this experiment are given in Table 4.5, Chapter 4.

Attempted Synthesis of 2-*O*-Methylrosmarinine (146)

Powdered KOH (0.257 g, 4.53 mmol) was added to distilled DMSO (2 ml) and stirred for 5 min. To this was added rosmarinine (29) (0.200 g, 0.567 mmol) followed by methyl iodide (0.322 g, 0.141 ml, 2.27 mmol). When rosmarinine was added the KOH dissolved and the reaction mixture turned brown. Stirring was continued for 30 min, after which the mixture was poured into water (20 ml) and extracted with CH_2Cl_2 (3 x 20 ml). The combined organic extracts were dried, filtered and concentrated *in vacuo*. Analysis, by TLC and 90

MHz ^1H NMR spectroscopy, of the brown oil obtained showed it to be a complex mixture of products.

Attempted Synthesis of 2-*O*-Methylrosmarinine (146)

Rosmarinine (0.104 g, 0.29 mmol) was dissolvd in CH_2Cl_2 (100 ml) and the solution was cooled to 0 °C. To this was added, with stirring, BF_3 etherate (5 mg, 5 µl, 0.030 mmol) followed by a solution of diazomethane in ether until the solution had a permanent yellow coloration. The solution was allowed to reach room temperature and stirring was continued for a further 24 h. Then the reaction mixture was filtered to remove any polymer that had formed. The reaction mixture was washed with 5% sodium bicarbonate solution (2 x 50 ml) and water (50 ml). The aqueous washes were extracted with CH_2Cl_2 (2 x 100 ml). Finally, the combined organic extracts were dried, filtered and the solvent was removed under reduced pressure to leave a white solid (0.102 g). Analysis showed this to be rosmarinine (29).

Synthesis of 2-*O*-Methylrosmarinine (146)

Rosmarinine (0.200 g, 0.57 mmol) was dissolved in CH_2Cl_2 (100 ml) and silica gel (1 g) was added. This was cooled to 0 °C and, with stirring, a solution of diazomethane in ether was added until the reaction mixture turned yellow. Stirring was continued for a further 24 h and then the solution was filtered to remove the silica gel. The solvent was removed *in vacuo* to leave a yellow oil which was shown by TLC to be a mixture of product (146) and starting material. These were separated by column chromatography on basic alumina eluting with CH_2Cl_2 followed by MeOH/CH_2Cl_2 (1%, 2% and 100% MeOH). The product appeared in the fraction eluted with 1% MeOH and rosmarinine was in the 2% MeOH fraction. Recrystallisation from CH_2Cl_2/hexane gave the title compound (146) as fine white needles (0.012 g, 6%); R_f 0.73; m.p. 132-134 °C; $[\alpha]_D$

-66.3° (0.3, MeOH); υ_{max} ($CHCl_3$) 3 540, 3 010, 2 940, 1 720, 1 450, 1 170 and 1 120 cm^{-1}; δ_H (200 MHz) 0.97 (3H, d, J 6.8 Hz, 19-H_3), 1.30 (3H, s, 18-H_3), 1.83 (3H, d, J 7.2 Hz, 21-H_3), 1.98 (1H, dq, J 13.2 and 6.8 Hz, 13-H), 2.10-2.40 (4H, complex, 6- and 14-H_2), 2.51 (1H, dddd, J 8.9, 7.3, 5.3 and 2.0 Hz, 1-H), 2.72 (1H, dt, J 9.8 and 7.5 Hz, 5-H), 2.91 (2H, m, 3-H_2), 3.04 (1H, ddd, J 4.9, 7.6 and 9.7 Hz, 5-H), 3.10 (1H, br s, -OH), 3.33 (3H, s, $-OCH_3$), 3.58 (1H, dd, J 7.0 and 4.8 Hz, 8-H), 3.76 (1H, q, J 5.2 Hz, 2-H), 3.93 (1H, dd, J 11.5 and 2.0 Hz, 9-H), 4.56 (1H, dd, J 11.5 and 9.0 Hz, 9-H), 5.36 (1H, dt, J 5.0 and 3.3 Hz, 7-H) and 5.86 (1H, q, J 7.2 Hz, 20-H); δ_C 13.5 (C-19), 15.5 (C-21), 26.2 (C-18), 35.7 (C-6), 37.1 (C-13), 39.2 (C-14), 44.9 (C-1), 51.9 (C-5), 57.4 (OCH_3), 58.3 (C-3), 65.0 (C-9), 68.2 (C-8), 73.8 (C-2), 75.7 (C-12), 84.8 (C-7), 131.6 (C-15), 135.8 (C-20), 167.5 (C-16) and 178.6 (C-11); *m / z* 367 (M^+, 11.3%), 241, 170, 152 (100%), 122 and 82; (Found: M^+, 367.1997; C, 62.28; H, 7.89; N, 3.72%: $C_{19}H_{29}NO_6$ requires *M*, 367.1995; C, 62.13; H, 7.91; N, 3.81%). Unreacted rosmarinine (29) was obtained as a white solid (0.107 g, 54%) and identified by comparison with an authentic sample.

The silica gel, which had been filtered off, was stirred with $CHCl_3$/MeOH/conc. NH_3 (85:14:1) overnight to try and remove any product or rosmarinine which might be attached to it. The silica gel was removed by filtration and the solvent was removed *in vacuo*. TLC analysis of the oil obtained (5.1 mg) showed no spots for 2-*O*-methylrosmarinine or rosmarinine itself.

Attempted Synthesis of 2-*O*-Methylrosmarinine (146)

Rosmarinine (0.200 g, 0.57 mmol) was dissolved in CH_2Cl_2 (100 ml) and neutral alumina (1 g) was added. The mixture was cooled to 0 °C and, with stirring, a solution of diazomethane in diethyl ether was added until the reaction mixture turned yellow. Stirring was continued for a further 24 h. The solution was filtered, dried and the solvent was removed to leave a

yellow solid. TLC analysis showed this solid to contain only starting material.

Synthesis of 2,12-*O*-Di-[2-(trimethylsilyl)-ethoxymethyl]rosmarinine (147)

To a solution of rosmarinine (29) (0.250 g, 0.71 mmol) in dry CH_2Cl_2 (7.5 ml) under an N_2 atmosphere, was added, diisopropylethylamine (0.730 g, 986 µl, 5.66 mmol) and 2-(trimethylsilyl)ethoxymethyl (SEM) chloride (0.710 g, 750 µl, 4.25 mmol). This mixture was heated at 40 °C for 3 h. The reaction mixture was then washed with water (2.5 ml), saturated ammonium chloride solution (2 x 2.5 ml) and brine (2.5 ml). The organic solution was dried, filtered and concentrated under reduced pressure to give a yellow oil. TLC analysis of this mixture showed it to contain two components both of which were Dragendorff active.[84] These were separated by column chromatography on basic alumina eluting with EtOAc/MeOH (9:1, v/v). The title compound (147) was eluted from the column first and obtained as a yellow oil (0.136 g, 31.2%); R_f 0.32; $[\alpha]_D$ +9.2° (c 0.79, CH_2Cl_2); υ_{max} ($CHCl_3$) 2 960, 2 920, 2 890, 1 730 (br), 1 145, 1 110 and 1 030 cm^{-1}; δ_H (200 MHz) 0.01 (9H, s, $Si(CH_3)_3$), 0.05 (9H, s, $Si(CH_3)_3$), 0.70-1.10 (7H, complex, 19-H_3 and 2 x $SiCH_2$), 1.42 (3H, s, 18-H_3), 1.81 (3H, d, J 7 Hz, 21-H_3), 2.00-5.50 (23H, complex) and 5.87 (1H, q, J 7 Hz, 20-H); δ_C -1.43 and 1.00 (2 x $Si(CH_3)_3$), 13.3 (C-19), 15.9 (C-18), 18,1 and 18.4 (2 x $SiCH_2$), 26.3 (C-21), 31.2 (C-6), 37.0 (C-13), 39.3 (C-14), 47.6 (C-1), 58.9 (C-5), 60.8 and 61.2 (C-3 and C-9), 66.3 ($O\underline{C}H_2CH_2Si$), 69.2 (C-8), 70.6 ($O\underline{C}H_2CH_2Si$), 73.2 (C-7), 76.2 (C-12), 77.2 (C-2), 90.1 and 94.3 (2 x OCH_2O), 131.2 (C-15), 139.8 (C-20), 166.5 (C-16) and 179.0 (C-11); *m / z* 613 (M^+, 7%), 540, 512, 496, 467, 438, 368 (100%), 268 and 122; (Found: M^+, 613.3434: $C_{30}H_{55}Si_2NO_8$ requires *M*, 613.3466).

The other component from the reaction mixture, R_f 0.17, was not identified.

Synthesis of 2-*O*-[2-(trimethylsilyl)ethoxymethyl] rosmarinecine (148)

2,12-*O*-Di-[2-(trimethylsilyl)ethoxymethyl]rosmarinine (147) (0.100 g, 0.163 mmol) was dissolved in dry CH_2Cl_2 (10 ml) under an N_2 atmosphere and cooled to 0 °C. A solution of DIBAL in CH_2Cl_2 (1.33 ml, 1.0 M) was added with stirring while maintaining the temperature at 0 °C. After stirring at 0 °C for 0.5 h and 1 h at room temperature, ethyl acetate (0.5 ml) was added and the mixture was poured onto a suspension of Celite (2.6 g) in acetone (16 ml). Methanol (1 ml) was added with vigorous stirring until the mixture formed a gel and this was then allowed to stand for 0.5 h. Water (2.6 ml) was added to break up the gel and the mixture was filtered. The solid was washed with water (4 x 5 ml) and methanol (10 x 5 ml) and the solvent was removed *in vacuo* to leave a clear oil. This was purified on a neutral alumina flash column eluting with $CHCl_3$/MeOH mixtures (1%, 2%, 3%, 4%, 5%, 100% MeOH). The product (148) was found in the fractions of 2% MeOH and above and obtained as a colourless oil (0.045 g, 90.3%); R_f 0.25 (EtOAc/*i*-PrOH/conc NH_3, 9:7:4); $[\alpha]_D$ -58.3° (c 0.6, CH_2Cl_2); υ_{max} ($CHCl_3$) 3 200 (br), 2 970, 2 940, 2 860 and 1 070 cm^{-1}; δ_H (200 MHz) 0.01 (9H, s, $Si(CH_3)_3$), 0.92 (2H, t, J 8 Hz, CH_2Si), 2.00-4.50 (16H, complex) and 4.68 (2H, s, OCH_2O); δ_C -1.43 ($Si(CH_3)_3$), 18.1 ($SiCH_2$), 34.5 (C-6), 47.0 (C-1), 54.6, 57.0 and 59.8 (C-3, C-5 and C-9), 66.0 ($O\underline{C}H_2CH_2Si$), 70.7 (C-8), 72.2 (C-7), 76.1 (C-2) and 95.7 (OCH_2O); *m / z* 303 (M^+, 1.2%), 230, 157, 139 (100%), 121, 107, 95, 81 and 75; (Found: M^+, 303.1865: $C_{14}H_{29}NO_4Si$ requires *M*, 303.1866).

Synthesis of 9-*O*-(*t*-butyldimethylsilyl)-2-*O*-[2-(trimethylsilyl)ethoxymethyl]rosmarinecine (149)

2-*O*-[2-(Trimethylsilyl)ethoxymethyl]rosmarinecine (148) (42 mg, 0.138 mmol) in dry CH_2Cl_2 (3 ml) and triethylamine

(31 mg, 43 µl, 0.30 mmol) were added to a stirred solution of *t*-butyldimethylsilyl (TBDMS) chloride (19 mg, 0.125 mmol, 0.9 equiv) and 4-dimethylaminopyridine (DMAP) (*ca.* 1 mg, catalytic) in dry CH_2Cl_2 (3 ml) under an N_2 atmosphere. Stirring was continued for 24 h at room temperature. The reaction mixture was washed with water (2 ml), saturated ammonium chloride solution (2 x 2 ml) and brine (2 ml). The organic layer was dried, filtered and concentrated to give the title compound (149) as a pale yellow oil (34 mg, 59.1%); R_f 0.28; $[\alpha]_D$ -13.3° (c 0.3, CH_2Cl_2); υ_{max} ($CHCl_3$) 3 650, 3 200 (br), 2 940, 2 920, 2 880, 1 110 and 910 cm^{-1}; δ_H (90 MHz) 0.06, 0.08 and 0.13 (15H, 3 s, 2 x $SiCH_2$ and $Si(CH_3)_3$), 0.93 (9H, s, $C(CH_3)_3$), 1.30 (2H, m), 2.00-5.00 (15H, complex) and 4.63 (2H, s, OCH_2O); *m / z* 360 (M^+-$C(CH_3)_3$, 2.4%), 332, 316, 288, 186, 157, 141, 99, 82 and 73 (100%); (Found: M^+-$C(CH_3)_3$, 360.2010: $C_{16}H_{34}Si_2NO_4$ requires *M*, 360.2026).

Attempted Synthesis of 7-*O*-Methanesulphonyl-9-*O*-TBDMS-2-*O*-SEM-rosmarinecine (150)

Methanesulphonyl chloride (4 µl, 0.053 mmol, 1.1 equiv) and 9-*O*-TBDMS-2-*O*-SEM-rosmarinecine (149) (20 mg, 0.048 mmol) were dissolved in dry CH_2Cl_2 (3 ml) under an N_2 atmosphere and cooled to -78 °C. Triethylamine (7 µl, 0.053 mmol, 1.1 equiv) was added and the mixture was stirred at -78 °C for 1 h. The mixture was allowed to warm to room temperature, poured into water (15 ml) and extracted with CH_2Cl_2 (3 x 15 ml). The combined organic extracts were washed with brine (10 ml), dried and concentrated *in vacuo* to give a brown oil. TLC analysis (EtOAc/*i*-PrOH/conc NH_3, 9:7:4) showed this oil to be a mixture of products. In the 90 MHz NMR spectrum of this oil there was no singlet present for the *t*-butyl group indicating that the TBDMS group had been lost.

7.4 Experimental to Chapter 5

Study of the Effect of 2-Hydroxyethylhydrazine on Pyrrolizidine Alkaloid Biosynthesis in *Senecio vulgaris* Transformed Root Cultures.

S. vulgaris transformed root cultures were established and propagated as described in Section 7.2. At subculture the roots were transferred into medium which contained 2-hydroxyethylhydrazine (HEH) (156) at various concentrations. The roots were grown for a further 21 d before they were filtered off. The roots were subjected to the standard work up which gave the crude alkaloid extracts. These extracts were analysed for alkaloid content by ^{1}H NMR spectroscopy and TLC, visualising with the modified Dragendorff reagent.[84]

In the first study batches of five flasks were set up at HEH concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} M. The results are shown in Table 5.1A, Chapter 5. The second experiment looked at HEH concentrations of 1.6 mM, 3.2 mM, 4.8 mM, 6.4 mM and 8.0 mM. The results for this are given in Table 5.1B, Chapter 5. The final experiment studied HEH concentrations between 1.6 mM and 3.2 mM. The results are given in Table 5.1C, Chapter 5.

A control batch of five flasks with no HEH present was run in conjunction with each of the above experiments.

Formation of (+)-Retronecine (18)

Retronecine was obtained by alkaline hydrolysis of mother liquors from recrystallisations of riddelliine (85) from *Senecio riddellii*, supplied by Dr. R.J. Molyneux, U.S.D.A., California, U.S.A. A dark brown gum (*ca.* 24 g) was partially dissolved in water (100 ml). Barium hydroxide octahydrate (30 g) was added, and the mixture was heated at reflux 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 4 d. The chloroform extracts were dried, filtered and concentrated *in vacuo* to give a brown syrup which solidified on standing overnight. Recrystallisation from acetone/light petroleum (b.p. 60-80 °C) afforded retronecine (18) as white crystals (1.55 g); R_f 0.73 (isopropanol/conc. ammonia, 5:3); m.p. 118-121 °C (lit.,[37] 118-120 °C); $[\alpha]_D$ -50.3° (c 1.05, EtOH) [lit.,[37] $[\alpha]_D$ -50.2°]; ν_{max} (KBr) 3 330 (br), 2 940, 2 920, 2 860, 2 840, 2 640, 1 660, 840 and 745 cm^{-1}; δ_H (90 MHz) 2.15 (2H, m, 6-H_2); 2.80 (1H, m, 5-H), 3.20-3.60 (2H, complex, 3-H and 5-H), 3.96 (1H, m, 3-H), 4.15-4.75 (4H, complex, 7-H, 8-H and 9-H_2) and 4.91 (1H, s, 2-H); *m / z* 155 (M^+, 33%), 138, 111, 94 and 80 (100%); (Found: M^+, 155.0945; C, 61.74; H, 8.52; N, 9.05%: $C_8H_{13}NO_2$ requires *M*, 155.0946; C, 61.94; H, 8.39; N, 9.03%).

Formation of (-)-Rosmarinecine (16)

To a solution of rosmarinine (29) (0.50 g, 1.42 mmol) in water (30 ml) was added barium hydroxide octahydrate (1.78 g, 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. The filtrate was saturated with sodium carbonate and extracted continuously with chloroform for 48 h. The organic extracts were dried, filtered and concentrated to afford (-)-rosmarinecine (16) (66 mg, 26.9%); m.p. 170-172 °C (from acetone/light petroleum, b.p. 60-80 °C) (lit.,[110] 171-172 °C); $[\alpha]_D$ -135.2° (c 0.46, EtOH) (lit.,[110] $[\alpha]_D$ -118.5°); ν_{max} ($CHCl_3$) 3 650, 2 960, 2 840 and 1 025 cm^{-1}; δ_H (90 MHz) (CD_3OD) 1.80 (2H, m, 6-H_2), 2.21 (1H, m, 1-H), 2.55-3.50 (5H, complex, 8-H, 3-H_2 and 5-H_2), 3.86 (2H, m, 9-H_2), 4.09 (1H, m, 7-H) and 4.35 (1H, m, 2-H); *m / z* 173 (M^+, 3.9%), 128, 98 (100%), 82 and 68; (Found: C, 55.31; H, 8.76; N, 8.22%: $C_8H_{15}NO_3$ requires C, 55.49; H, 8.82; N, 8.09%).

Synthesis Of (±)-Trachelanthamidine (157)

This was prepared according to the work of Kunec and Robins[49], but some modifications were made and additional spectral data is presented. Therefore the preparation is given in full.

N-Formyl-L-proline (160)

A mixture of 98% formic acid (6.6 ml, 0.174 mol, 20 equiv) and acetic anhydride (8.2 ml, 0.087 mol, 10 equiv) was heated with stirring at 40-45 °C for 2 h, after which time the reaction mixture was allowed to cool to room temperature. To this was added, with stirring at 0 °C, a solution of L-proline (159) (1.00 g, 8.7 mmol) in formic acid (14 ml). The resulting mixture was stirred for 18 h at room temperature. Iced water (18 ml) was added and the solution was concentrated to give an oil. This oil was taken up in ethyl acetate, dried over sodium sulphate, and concentrated. The product (160) crystallised as a white solid which was shown by ^{1}H and ^{13}C NMR spectroscopy to exist in two rotameric forms (0.94 g, 75.6%); m.p. 91-96 °C (lit.,[165] 88-90 °C); $[\alpha]_D$ -124° (c 2.2, EtOH) [lit.,[165] $[\alpha]_D$ -125° (c 1, EtOH)]; υ_{max} (KBr) 3 000, 2 940, 2 890, 2 500 (br), 1 730, 1 630 (br), 1 380, 1 180 and 680 cm^{-1}; δ_H (200 MHz) 1.80-2.40 (4H, complex, 3- and 4-H_2), 3.50-3.68 (2H, complex, 5-H_2), 4.40-4.51 (1H, complex, 2-H), 8.26 and 8.30 (1H, 2s, CHO), and 12.06 (1H, s, COOH); δ_C [major isomer] 23.9 and 29.0 (C-3 and C-4), 46.9 (C-5), 57.0 (C-2), 162.2 (CHO) and 174.4 (COOH), [minor isomer] 22.7 and 29.6 (C-3 and C-4), 44.3 (C-5), 59.0 (C-2), 163.0 (CHO) and 173.9 (COOH); *m/z* 143 (M^+, 15%), 98 (100%) and 70; (Found: M^+, 143.0583; C, 50.22; H, 6.16; N, 9.72%: $C_6H_9NO_3$ requires *M*, 143.0582; C, 50.35; H, 6.29; N, 9.79%).

Cycloaddition Of *N*-Formyl-L-proline With Ethyl Propiolate

Ethyl propiolate (163) (1.71 g, 1.77 ml, 17.5 mmol, 5 equiv) was added to a solution of *N*-formyl-L-proline (160) (0.50 g, 3.49 mmol) in acetic anhydride (4 ml) and heated at reflux under N_2 for 3 h. The solution was cooled and concentrated *in vacuo* to leave a brown oil. ^1H NMR spectroscopy showed this oil to contain a mixture of two isomeric products. These were separated by dry-column flash chromatography on silica eluting with pentane/diethyl ether (2:1 v/v) giving the following products:-

Ethyl 2,3-dihydro-1H-pyrrolizine-7-carboxylate (161) was obtained as a pale yellow oil (0.360 g, 57.3%); R_f 0.67 ($CHCl_3$/diethyl ether 1:1); υ_{max} ($CHCl_3$) 3 030, 2 990, 2 940, 2 900, 1 690 (br), 1 570, 1 380 and 1 050 cm^{-1}; δ_H (200 MHz) 1.32 (3H, t, J 7.1 Hz, CH_3), 2.52 (2H, m, 2-H_2), 3.06 (2H, t, J 7.7 Hz, 1-H_2), 3.92 (2H, t, J 7.1 Hz, 3-H_2), 4.27 (2H, q, J 7.1 Hz, OCH_2), 6.53 and 6.57 (2H, AB system, J 2.9 Hz, 5-H and 6-H); δ_C 14.5 (CH_3), 25.5 (C-2), 27.1 (C-1), 46.8 (C-3), 59.2 (OCH_2), 113.2 (C-5 or C-6), 114.6 (C-5 or C-6), 143.9 (C-7), 149.4 (C-8) and 166.4 (C=O); *m/z* 179 (M^+, 63%), 150, 134 (100%) and 106; (Found: M^+, 179.0947: $C_{10}H_{13}NO_2$ requires *M*, 179.0946).

Ethyl 2,3-dihydro-1H-pyrrolizine-6-carboxylate (162) was obtained as a white solid which was crystallised from diethyl ether (0.031 g, 5%); R_f 0.67 (diethyl ether/chloroform 1:1 v/v); m.p. 70-73 °C; υ_{max} ($CHCl_3$) 3 010, 2 980, 2 890, 1 690 (br), 1 515, and 1 375 cm^{-1}; δ_H (90 MHz) 1.31 (3H, t, J 7 Hz, CH_3), 2.51 (2H, m, 2-H_2), 2.82 (2H, t, J 7 Hz, 1-H_2), 3.94 (2H, t, J 7 Hz, 3-H_2), 4.24 (2H, q, J 7 Hz, OCH_2) and 6.25 (2H, br m, 5- and 7-H); *m/z* 179 (M^+, 45.5%), 164, 150, 134 (100%), 106 and 77; (Found: M^+, 179.0930 ; C, 67.13; H, 7.30; N, 7.78%: $C_{10}H_{13}NO_2$ requires *M*, 179.0946 ; C, 67.04; H, 7.26; N, 7.82%).

Ethyl (±) 8α-Pyrrolizidine-1β-carboxylate (167)

Ethyl 2,3-dihydro-1H-pyrrolizine-7-carboxylate (161) (0.274 g, 1.54 mmol) in glacial acetic acid (20 ml) was hydrogenated using 5% rhodium-on-carbon (0.274 g) at 6 atm for 18 h at room temperature. The catalyst was filtered off through Celite and the filtrate was concentrated. The residual oil was partitioned between 1 M HCl (25 ml) and diethyl ether (25 ml). The acid layer was washed with ether (4 x 25 ml), basified with conc. ammonia, and then extracted with ether (6 x 30 ml). The combined ether extracts were dried, filtered and concentrated to give the ester (167) as a colourless oil (0.164 g, 58.3%); R_f 0.50; υ_{max} (neat) 2 960, 2 880, 1 730 (br), 1 455, 1 380 and 1 180 cm^{-1}; δ_H (200 MHz) 1.20 (3H, t, J 7.1 Hz, CH_3), 1.25-2.20 (6H, complex, 2-, 6- and 7-H_2), 2.45-2.60 (1H, complex, 1-H), 2.70-3.23 (4H, complex, 3-H_2 and 5-H_2), 3.80 (1H, complex, 8-H) and 4.09 (2H, dq, J 2.2 and 7.1 Hz, OCH_2); δ_C 14.1 (CH_3), 26.1, 26.4 and 28.2 (C-2, -6 and -7), 46.9 (C-1), 53.2 and 55.3 (C-3 and C-5), 60.6 (OCH_2) and 172.8 (C=O); *m/z* 183 (M^+, 13.6%), 182, 169, 154, 138, 108, 96, and 83 (100%); (Found: M^+, 183.1250 : $C_{10}H_{17}NO_2$ requires M, 183.1259).

Ethyl (±) - 8α-Pyrrolizidine-1α-carboxylate (168)

Ethyl (±) - 8α-pyrrolizidine-1β-carboxylate (167) (0.134 g, 0.74 mmol) was dissolved in conc. HCl (2 ml) and left for 2 h at room temperature. The solution was heated in a sealed tube at 150 °C for 18 h. The tube was then cooled and the contents were removed and evaporated to dryness. The residue was dissolved in ethanol (10 ml) at 0 °C and thionyl chloride (0.3 ml) was added. This mixture was stirred for 18 h at room temperature. The solution was concentrated to an oil which was partitioned between 1 M HCl (25 ml) and diethyl ether (25 ml). The aqueous layer was washed with ether (3 x 25 ml), then basified with conc. ammonia and extracted with diethyl

ether (5 x 30 ml). The combined ether extracts were dried, filtered and concentrated to give the product (168) as a colourless oil (0.089 g, 66%); R_f 0.45; υ_{max} ($CHCl_3$) 2 970, 2 940, 2 910, 2 880, 1 725, 1 380 and 1 095 cm^{-1}; δ_H (200 MHz) 1.21 (3H, t, J 7.1 Hz, CH_3), 1.35-2.25 (6H, complex, 2-, 6- and 7-H_2), 2.40-3.30 (5H, complex, 1-H, 3- and 5-H_2), 3.63 (1H, complex, 8-H) and 4.12 (2H, q, J 7.1 Hz, OCH_2); δ_C 14.2 (CH_3), 25.6 , 30.5 and 31.4 (C-2, -6 and -7), 50.1 (C-1), 54.5 and 54.9 (C-3 and C-5), 60.5 (OCH_2), 68.2 (C-8) and 174.1 (C=O); *m / z* 183 (M^+, 10.9%), 154, 138, 108, 83 (100%) and 74; (Found: M^+, 183.1246: $C_{10}H_{17}NO_2$ requires *M*, 183.1259).

(±) - Trachelanthamidine (157)

Lithium aluminium hydride (26 mg, 0.73 mmol) was added to a solution of ethyl (±)-8α-pyrrolizidine-1α-carboxylate (168) (89 mg, 0.49 mmol) in dry THF (10 ml) at 0 °C under N_2. The mixture was stirred for 1 h at 0 °C, then wet THF was added, followed by 20% aqueous NaOH (0.7 ml). The mixture was filtered through anhydrous Na_2SO_4 and the filtrate was concentrated *in vacuo* to give (±)-1α-hydroxymethyl-8α-pyrrolizidine (157) as a colourless oil (52 mg, 76%); R_f 0.09; υ_{max} ($CHCl_3$) 3 620, 3 460 (br), 2 960, 2 880, 1 450 and 910 cm^{-1}; δ_H (200 MHz) 1.00-2.00 (6H, complex, 2-, 6-, and 7-H_2), 2.20-3.25 (6H, complex, 1- and 8-H, 3- and 5-H_2), 3.55 (2H, d, J 6.3 Hz, 9-H_2) and 4.70 (1H, br s, OH); δ_C 25.6, 29.9 and 31.8 (C-2, -6 and -7), 48.3 (C-1), 54.4 and 54.6 (C-3 and C-5), 64.8 (C-9) and 67.8(C-8); *m / z* 141 (M^+, 23.4%), 124, 113, 110, 97, 83 (100%), 80 and 70; (Found: M^+, 141.1153: $C_8H_{15}NO$ requires *M* 141.1154).

Synthesis of *N*-(4-Aminobutyl)pyrrolidinium dihydrochloride (169)

This was prepared by Dr. A.A. Denholm employing the method of Kelly and Robins.[54]

Feeding of Precursors to Inhibited *Senecio vulgaris* Transformed Root Cultures

Twelve flasks of *S. vulgaris* were set up as described previously, with HEH present in the medium at a concentration of 1.6 mM. Each precursor [(+)-retronecine (18) (46.5 mg), (-)-rosmarinecine (16) (51.9 mg) and the pyrrolinium salt (169) (42.6 mg)] was dissolved in sterile water and divided among three flasks giving a final precursor concentration of 1 mM. The other three flasks were used as a standard. After 21 d the roots were harvested and the alkaloids were extracted. These crude extracts were examined by ^{1}H NMR spectroscopy and TLC visualising with the modified Dragendorff reagent,[84] to determine how much alkaloidal material, if any, they contained. The results are given in Table 5.2, Chapter 5.

In the case of trachelanthamidine, six flasks of *S. vulgaris* were set up with an HEH concentration of 1.6 mM. (±)-Trachelanthamidine (157) (42.3 mg) was distributed among three flasks giving a 1.0 mM concentration while the other three flasks were used as a standard. The roots were grown for 21 d then harvested. The alkaloids were extracted and examined as described before. Table 5.3 in Chapter 5 gives the results of this feeding.

All spectral data for senecionine (38) from the alkaloid extract from the retronecine feeding were identical to those for an authentic sample.

Purification of the alkaloid, R_f 0.54, in the extract from the (±)-trachelanthamidine feeding was carried out by preparative

TLC on a 20 x 20 cm plate. However, no alkaloid was recovered from the plates.

Formation of ^{14}C-Labelled (+)-Retronecine (18)

N,N'-Bis-3-(aminopropyl)-[1,4-^{14}C-tetramethylene]-1,4-diamine (spermine) (41) tetrahydrochloride (50 μCi) was dissolved in sterile water and fed to 7 day old *S. vulgaris* transformed root cultures (40 flasks). After a further 14 d the roots (326 g) were harvested and crude ^{14}C-labelled senecionine (38) was extracted (0.273 g, 10.2 μCi mmol^{-1}).

The crude senecionine (0.273 g, 0.815 mmol) was partially dissolved in water (20 ml) and the mixture was heated at reflux with barium hydroxide octahydrate (1.03 g, 3.26 mmol, 4 equiv) for 4 h. Solid carbon dioxide was added to the cooled solution and the precipitated barium carbonate was filtered off. The filtrate was saturated with sodium carbonate and continuously extracted with chloroform for 66 h. The chloroform extracts were dried, filtered and concentrated *in vacuo* to give ^{14}C-labelled retronecine (18) as a yellow oil (0.044 g, 35%). Attempted crystallisation of this oil from acetone/light petroleum (b.p. 60-80 °C) proved unsuccessful. TLC and ^{1}H NMR spectroscopic analysis indicated that the retronecine was pure. It was therefore used in this form. Retronecine had a specific activity of 11.1 μCi mmol^{-1}.

Formation of [5-^{3}H]-(±)-Trachelanthamidine (83)

[5-^{3}H]-(±)-Trachelanthamidine (83) was prepared in an analogous fashion to unlabelled (±)-trachelanthamidine (157). All the compounds had physical properties identical to authentic unlabelled material. For experimental detail and characterisation data see synthesis of (±)-trachelanthamidine (157).

_N_-Formyl-[5-^{3}H]-L-proline (170)

A mixture of L-proline (159) (1.00 g, 8.60 mmol) and [5-^{3}H]-L-proline (79) (500 μCi) was converted, by treatment with formic acid and acetic anhydride, to the title compound (170). Yield 0.91 g, 73%, 12.0 μCi mmol^{-1}.

Ethyl [3-^{3}H]-2,3-dihydro-1H-pyrrolizine-7-carboxylate (80)

Yield 0.598 g, 53.3%, 8.77 μCi mmol^{-1}.

Ethyl (±)-[5-^{3}H]-pyrrolizidine-1β-carboxylate (171)

Yield 0.389 g, 63.2%, 9.89 μCi mmol^{-1}.

Ethyl (±)-[5-^{3}H]-pyrrolizidine-1α-carboxylate (82)

Yield 0.334 g, 87.7%, 8.87 μCi mmol^{-1}.

(±)-[5-^{3}H]-Trachelanthamidine (83)

Yield 0.162 g, 69.2%, 11.6 μCi mmol^{-1}.

Feeding of Radiolabelled Precursors to Inhibited *Senecio vulgaris* Transformed Root Cultures

S. vulgaris transformed root cultures (6 flasks) were set up with an HEH (156) concentration of 1.6 mM as described previously. (+)-[^{14}C]-Retronecine (18) (31 mg, 5.3 μCi mmol^{-1}) and (±)-[5-^{3}H]-trachelanthamidine (83) (54 mg, 11.6 μCi mmol^{-1}) were dissolved in sterile water and each was fed to two flasks of roots. The final two flasks were used as a standard. After 21 d the roots were harvested and the crude alkaloid extracts were obtained. These extracts were examined

by TLC, visualising with the modified Dragendorff reagent,[84] and mass spectrometry, to determine if they contained senecionine (38). The results are given in Table 5.4, Chapter 5. Both crude alkaloid extracts had small TLC spots at R_f 0.54 and the mass spectra of the extracts had peaks at *m / z* 335. A 10% sample of the extracts was taken for scintillation counting.

Synthesis Of (±) - 1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (172)

N-Acetyl-DL-proline (173)

L-Proline (159) (3.45 g, 0.03 mol) was dissolved in boiling glacial acetic acid (30 ml) and to this was added acetic anhydride (6.72 g, 6.2 ml, 0.066 mol). The resulting solution was stirred at room temperature for 2 h then the solvent was removed *in vacuo* to leave a yellow syrup. This was crystallised from acetone/diethyl ether to give *N*-acetyl-DL-proline (173) (3.42 g, 72%); m.p. 103 °C (lit.,[166] 106 °C); υ_{max} (KBr) 2 990, 2 890, 2 710, 2 580, 2 500, 1 730, 1 600 1 470 and 1 190 cm^{-1}; δ_H (200 MHz) 2.12 (3H, s, CH_3), 1.80-2.40 (4H, complex, 3- and 4-H_2), 3.30-3.70 (2H, complex, 5-H_2), 4.53 (1H, complex, 2-H) and 11.74 (1H, s, CO_2H); δ_C 22.0 (CH_3), 24.6 and 28.3 (C-3 and C-4), 48.3 (C-5), 59.3 (C-2), 171.9 ($COCH_3$) and 173.4 (CO_2H); *m / z* 157 (M^+, 1.6%), 154, 143, 113, 98, 85, 70 (100%), and 56; (Found: M^+, 157.0742; C, 53.71; H, 7.12; N, 8.82%: $C_7H_{11}NO_3$ requires *M*, 157.0739; C, 53.50; H, 7.01; N, 8.92%).

Cycloaddition Of *N*-Acetyl-DL-proline With Ethyl Propiolate

A solution of *N*-acetyl-DL-proline (173) (0.50 g, 3.18 mmol) and ethyl propiolate (1.6 ml, 15.9 mmol) in acetic anhydride (4 ml) was heated at 140 °C for 3.5 h under N_2. Removal of the

solvent under reduced pressure gave a red-brown oil which ^1H NMR spectroscopy showed to be a mixture of two isomeric products. Separation of these by flash chromatography on neutral alumina eluting with pentane/dichloromethane (3:1, v/v) gave the following products:

Ethyl (±)-2,3-dihydro-5-methyl-1H-pyrrolizine-6-carboxylate (175) was obtained as a white solid which was crystallised from diethyl ether (0.134 g, 22%); R_f 0.68 (diethyl ether/chloroform 1:1, v/v); m.p. 39-41 °C; υ_{max} ($CHCl_3$) 3 050, 3 010, 2 990, 1 685, 1 520 and 900 c m^{-1}; δ_H (200 MHz) 1.31 (3H, t, J 7.1 Hz, CH_3CH_2O), 2.44 (3H, s, CH_3C=), 2.45 (2H, complex, 2-H_2), 2.79 (2H, t, J 7.4 Hz, 1-H_2), 3.81 (2H, t, J 7.0 Hz, 3-H_2), 4.23 (2H, q, J 7.1 Hz, OCH_2) and 6.16 (1H, br s, 7-H); δ_C 11.8 (CH_3CH_2O), 14.5 (CH_3C=), 23.9 (C-2), 27.3 (C-1), 44.2 (C-3), 58.9 (OCH_2), 100.3 (C-7), 125.7 (C-5), 130.5 (C-6), 134.4 (C-8) and 165.4 (C=O); *m / z* 193 (M^+, 55%), 164 (100%), 148, 120, 106 and 91; (Found: M^+, 193.1104; C, 68.21; H, 7.72; N, 7.35%: $C_{11}H_{15}NO_2$ requires *M*, 193.1103; C, 68.39; H, 7.77; N, 7.25%).

Ethyl (±)-2,3-dihydro-5-methyl-1H-pyrrolizine-7-carboxylate (174) was obtained as a white solid which was also crystallised from diethyl ether (0.173 g, 28.3%); R_f 0.68 (diethyl ether/chloroform 1:1, v/v); m.p. 54-56 °C; υ_{max} ($CHCl_3$) 3 050, 3 010, 2 980, 1 683, 1 570, 1 530, 1 430 and 1 080 c m^{-1}; δ_H (200 MHz) 1.29 (3H, t, J 7.1 Hz, CH_3CH_2O), 2.15 (3H, d, J 0.8 Hz, CH_3C=), 2.50 (2H, complex, 2-H_2), 3.04 (2H, t, J 7.2 Hz, 1-H_2), 3.82 (2H, t, J 7.1 Hz, 3-H_2), 4.23 (2H, q, J 7.1 Hz, OCH_2) and 6.25 (1H, d, J 0.8 Hz, 6-H); δ_C 11.7 ($\underline{C}H_3CH_2O$), 14.5 ($\underline{C}H_3C$=), 25.9 and 26.9 (C-1 and C-2), 44.9 (C-3), 59.1 (OCH_2), 106.2 (C-5), 109.9 (C-6), 124.0 (C-7), 142.5 (C-8) and 165.3 (C=O); *m/z* 193 (M^+, 48.5%), 164 (100%), 148, 120, 106 and 91; (Found: M^+, 193.1107; C, 68.52; H, 7.75; N, 7.35%: $C_{11}H_{15}NO_2$ requires *M*, 193.1103; C, 68.39; H, 7.77; N, 7.25%).

Ethyl (±)-3β-methyl-8α-pyrrolizidine-1β-carboxylate (176)

A solution of ethyl 2,3-dihydro-5-methyl-1H-pyrrolizine-7-carboxylate (174) (0.074 g, 0.38 mmol) in glacial acetic acid (7 ml) was hydrogenated in the Cook hydrogenation apparatus at 6 atm pressure and 60 °C in the presence of a 5% rhodium-on-carbon catalyst (0.074 g) for 18 h. The catalyst was removed by filtration through Celite and the filtrate was concentrated to give a brown oil. The oil was taken up in 1 M HCl (5 ml) and washed with CH_2Cl_2 (4 x 5 ml). The aqueous layer was basified with conc. ammonia and extracted with CH_2Cl_2 (6 x 6 ml). The combined organic extracts were dried, filtered and concentrated to give the desired product (176) as a colourless oil (0.043 g, 57%); R_f 0.31; υ_{max} ($CHCl_3$) 2 970, 2 940, 2 880, 1 723, 1 450 (br), 1 380 and 1 040 cm^{-1}; δ_H (200 MHz) 1.23 (3H, t, J 7.1 Hz, CH_3CH_2O), 1.27 (3H, d, J 6.8 Hz, CH_3CH), 1.55-2.10 (6H, complex, 2-, 6- and 7-H_2), 2.55-2.75 (1H, complex, 1-H), 2.95-3.45 (3H, complex, 3-H and 5-H_2), 3.95 (1H, complex, 8-H) and 4.14 (2H, dq, J 2.9 and 7.2 Hz, OCH_2); δ_C 14.3 (CH_3CH_2O), 15.5 (CH_3CH), 25.7, 28.6 and 32.9 (C-2, -6 and -7), 46.9 (C-5), 47.7 (C-1), 56.7 (C-3), 60.5 (OCH_2), 65.3 (C-8) and 173.2 (C=O); *m / z* 197 (M^+, 16.5%), 182, 168, 152, 124, 108 and 97 (100%); (Found: M^+, 197.1406: $C_{11}H_{19}NO_2$ requires *M*, 197.1416).

Ethyl (±)-3β-methyl-8α-pyrrolizidine-1α-carboxylate (177)

This was prepared in an analogous fashion to ethyl (±)-8α-pyrrolizidine-1α-carboxylate (168) starting from ethyl (±)-3β-methyl-8α-pyrrolizidine-1β-carboxylate (176) (81.3 mg, 0.42 mmol). The title compound (177) was obtained as a colourless oil (50.6 mg, 62%); R_f 0.49; υ_{max} ($CHCl_3$) 2 960, 2 940, 2 880, 1 720 (br), 1 450 and 1 370 cm^{-1}; δ_H (200 MHz) 1.24 (3H, d, J 7.0 Hz, CH_3CH), 1.26 (3H, t, J 7.2 Hz, CH_3CH_2O), 1.50-2.00 (5H,

complex), 2.05-2.90 (4H, complex), 3.30-3.50 (1H, complex), 3.50-3.70 (1H, complex, 8-H) and 4.16 (2H, q, J 7.1 Hz, OCH_2); δ_C 14.2 ($\underline{C}H_3CH_2$), 16.8 ($\underline{C}H_3CH$), 25.2, 30.8, and 34.9 (C-2, -6 and -7), 45.6 (C-5), 50.4 (C-1), 57.3 (C-3), 60.5 (OCH_2), 69.7 (C-8) and 175.3 (C=O); *m / z* 197 (M^+, 5.6%), 195, 189, 182, 156, 122, 86, 71 and 43 (100%); (Found: M^+, 197.1408: $C_{11}H_{19}NO_2$ requires *M*, 197.1416).

(±) - 1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (172)

This was synthesised from ethyl (±)-3β-methyl-8α-pyrrolizidine-1α-carboxylate (177) (35 mg, 0.18 mmol) by the same method as used to convert ethyl-8α-pyrrolizidine-1α-carboxylate (168) into (±)-trachelanthamidine (157). The alcohol (172) was obtained as a pale yellow oil (24 mg, 88%); R_f 0.10; υ_{max} ($CHCl_3$) 3 620, 3 350 (br), 2 960, 2 940, 2 880, 2 500, 1 450 and 1 380 cm^{-1}; δ_H (200 MHz) 1.17 (3H, t, J 6.7 Hz, CH_3), 1.40-2.30 (6H, complex, 2-, 6- and 7-H_2), 2.40-2.90 (3H, complex, 3-H and 5-H_2), 3.05-3.35 (2H, complex, 1- and 8-H), 3.52 (2H, d, J 7.0 Hz, CH_2O) and 3.91 (1H, br s, OH); δ_C 16.9 (CH_3), 25.9, 31.6 and 34.6 (C-2, -6 and -7), 45.8 (C-5), 48.2 (C-1), 56.3 (C-3), 65.3 (C-9) and 68.3 (C-8); *m / z* 155 (M^+, 22.6%), 140, 122, 108, 97 (100%) and 69; (Found: M^+, 155.1290: $C_9H_{17}NO$ requires *M*, 155.1310).

Synthesis of (±)-[5-^{3}H]-1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (182)

(+)-[5-^{3}H]-1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (182) was prepared in an analogous fashion to the unlabelled material (172). Full experimental detail and compound characterisation data are given in that synthesis. All radiolabelled compounds had physical properties identical to those of authentic unlabelled material.

_N_-Acetyl-[5-^{3}H]-DL-proline (178)

A mixture of L-proline (159) (1.00 g, 8.60 mmol) and [5-^{3}H]-L-proline (79) (500 μCi) was converted into the desired compound (41) by treatment with Ac_2O in acetic acid. Yield 0.99 g, 72.6%, 10.8 μCi mmol^{-1}.

Ethyl [3-^{3}H]-2,3-dihydro-5-methyl-1H-pyrrolizine-7-carboxylate (179)

Yield 0.378 g, 31%, 8.65 μCi mmol^{-1}.

Ethyl (±)-[5-^{3}H]-3β-methyl-8α-pyrrolizidine-1β-carboxylate (180)

Yield 0.234 g, 56.2%, 9.08 μCi mmol^{-1}.

Ethyl (±)-[5-^{3}H]-3β-methyl-8α-pyrrolizidine-1α-carboxylate (181)

Yield 0.143 g, 65.2%, 10.8 μCi mmol^{-1}.

(±)-[5-^{3}H]-1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (182)

Yield 0.097 g, 88.2%, 10.3 μCi mmol^{-1}.

Feeding of (±)-[5-^{3}H]-1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (182) to Inhibited *Senecio vulgaris* Transformed Root Cultures

(+)-[5-^{3}H]-1α-Hydroxymethyl-3β-methyl-8α-pyrrolizidine (182) (30 mg, 10.3 μCi mmol^{-1}) was fed to inhibited *Senecio*

vulgaris transformed root cultures as described for [^{14}C]-retronecine and [5-^{3}H]-trachelanthamidine. The results are given in Table 5.4, Chapter 5.

A TLC of the crude alkaloid extract, visualised with the modified Dragendorff reagent,[84] showed no spots. The mass spectrum of the crude alkaloid extract had no peak at *m / z* 349 or 335, indicating that 3β-methylsenecionine (183) and senecionine (38) were not present.

7.5 Experimental to Chapter 6

General Procedure for Diacetylation of Diamines

The diamine (6.8 mmol) was dissolved in CH_2Cl_2 (15 ml) and pyridine (13.6 mmol, 2 equiv) was added. This solution was cooled to 0 °C then Ac_2O (20.4 mmol, 3 equiv) in CH_2Cl_2 (10 ml) was added dropwise. The reaction was stirred for 2 h at room temperature; the solvent was removed *in vacuo* and the residue was azeotroped twice with toluene. The product was recrystallised from CH_2Cl_2.

Diacetylputrescine (191) was formed from putrescine (33) and obtained as white crystals (0.84 g, 72%); R_f 0.55 (EtOAc/isopropanol/conc. NH_3, 9:7:4); m.p. 142 °C (lit.,[167] 137 °C); ν_{max} (KBr) 3 300, 3 070, 2 940, 2 880, 1 640 (br), 1 545, 1 475, 1 361, 1 295 and 600 cm^{-1}; δ_H (200 MHz) (D_2O, ref. HOD @ 4.67) 1.34 (4H, complex, 2- and 3-H_2), 1.80 (6H, s, 2 x CH_3) and 3.00 (4H, t, 1- and 4-H_2); δ_C (D_2O, ref. dioxan @ 67.4) 22.6 (CH_3), 26,5 (C-2 and C-3), 39.8 (C-1 and C-4) and 174.7 (C=O); *m / z* 172 (M^+, 18.2%), 129, 112, 110, 87 and 70 (100%); (Found: M^+, 172.1215; C, 55.82; H, 9.34; N, 16.28%: $C_8H_{16}N_2O_2$ requires *M*, 172.1212; C, 55.78; H, 9.38; N, 16.25%).

Diacetylcadaverine (194) was produced from cadaverine (154) and obtained as white crystals (0.63 g, 50%); R_f 0.54 (EtOAc/isopropanol/conc. NH_3, 9:7:4); m.p. 123-128 °C; ν_{max} (KBr) 3 260, 3 080, 2 940, 2 880, 1 630 (br), 1 550, 1 375 and 610 cm^{-1}; δ_H (200 MHz) (D_2O, ref. HOD @ 4.63) 1.15 (2H, m, 3-H_2), 1.30 (4H, complex, 2- and 4-H_2), 1.76 (6H, s, 2 x CH_3) and 2.96 (4H, t, J 6.6 Hz, 1- and 5-H_2); δ_C (D_2O, ref. dioxan @ 67.4) 22.8 (CH_3), 24.2 (C-3), 28.2 (C-2 and C-4), 40.1 (C-1 and C-5) and 174.6 (C=O); *m / z* 186 (M^+, 21.6%), 143, 126, 114, 101, 84 and 72 (100%); (Found: M^+, 186.1385; C, 57.80; H, 9.70; N, 15.03%: $C_9H_{18}N_2O_2$ requires *M*, 186.1368; C, 58.03; H, 9.76; N, 14.96%).

Diacetyl-1,2-diaminopropane (196) was synthesised from 1,2-diaminopropane (195) and obtained as white needles (0.74 g, 69%); R_f 0.42 (EtOAc/isopropanol/conc. NH_3, 9:7:4); m.p. 148-150 °C (lit.,[153] 138-139 °C); ν_{max} (KBr) 3 300, 3 090, 2 940, 2 880, 1 654 (br), 1 552, 1 370, 1 292 and 1 148 cm^{-1}; δ_H (200 MHz) 1.10 (3H, d, J 5.5 Hz, 3-H_3), 1.91 (3H, s, $COCH_3$), 1.93 (3H, s, $COCH_3$), 3.18 (2H, m, 1-H), 3.98 (1H, m, 2-H), 6.69 (1H, br d, J 7.2 Hz, D_2O exch., NHCH) and 6.92 (1H, br t, D_2O exch., $NHCH_2$); δ_C 18.1 (C-3), 23.0 and 23.2 (2 x $CO\underline{C}H_3$), 45.5 (C-1), 46.2 (C-2) and 170.8 and 171.4 (2 x C=O); *m / z* 158 (M^+, 0.4%), 101, 99 (100%), 87, 86, 73 and 72; (Found: M^+, 158.1061; C,53.12; H, 8.79; N, 17.62%): $C_7H_{14}N_2O_2$ requires *M*, 158.1055; C, 53.10; H, 8.94; N, 17.59%).

Diacetyl-2-methyl-1,5-diaminopentane (192) was synthesised from 2-methyl-1,5-diaminopentane (199) and obtained as a waxy solid (0.68 g, 50%). Crystallisation of this solid proved unsuccessful. R_f 0.58 (EtOAc/isopropanol/conc. NH_3, 9:7:4); ν_{max} ($CHCl_3$) 3 460, 3 330, 2 940, 2 880, 1 665 (br), 1 530 (br) and 1 380 cm^{-1}; δ_H (200 MHz) 0.83 (3H, d, J 6.7 Hz, $CHCH_3$), 0.90-1.70 (5H, complex, 2-H, 3-H_2 and 4-H_2), 1.92 (3H, s, $COCH_3$), 1.94 (3H, s, $COCH_3$), 2.95-3.33 (4H, complex, 1- and 5-H_2) and 6.50 (2H, br s, 2 x NH); δ_C 17.6 ($CH\underline{C}H_3$), 23.0 (2 x $CO\underline{C}H_3$), 26.3 (C-3), 30.7 (C-4), 32.6 (C-2), 39.3 (C-5), 45.0 (C-1) and 170.7 and 170.8 (2 x C=O); *m / z* 200 (M^+, 21%), 157, 141, 128, 114, 101, 87 and 72 (100%); (Found: M^+, 200.1529: $C_{10}H_{20}N_2O_2$ requires *M*, 200.1525).

Standard Enzyme Reaction Work Up

The solvent was decanted and the coagulated enzyme was stirred for 1 h with $CHCl_3$/MeOH (9:1) (20 ml). This mixture was filtered through Celite and the filtrate was combined with the reaction solvent. Removal of the solvent *in vacuo* gave an oil which was dissolved in a mixture of hot water (5 ml) and 6M HCl (3.3 ml). The solution was evaporated to dryness to

leave a solid. Extraction of this solid with isopropanol (50 ml) dissolved the desired product and the dihydrochloride salt of the starting diamine was filtered off. The filtrate was reduced in volume to *ca.* 1 ml and left at 0 °C overnight. The product was crystallised as a white solid.

PPL-Catalysed Monoacetylation of Diamines

The diamine (0.200 g) was dissolved in ethyl acetate (20 ml) and to this was added porcine pancreatic lipase (PPL) (0.200 g, 13.3 units of activity per mg of solid). This mixture was shaken at 100 rpm in a constant temperature bath at 25 °C. The reaction was monitored by TLC using EtOAc/ isopropanol/ conc. NH_3 (9:7:4) as the solvent system and worked up when the maximum amount of monoacetylated product was formed i.e. before dicaetylation occurred.

N-Acetyputrescine Hydrochloride (31)

The title compound was formed from putrescine (33). The reaction mixture was worked up by the standard procedure after 5 d. *N*-Acetylputrescine hydrochloride (31) was recrystallised from isopropanol/diethyl ether (0.097 g, 25.9%). All physical and spectral data were identical to those of an authentic sample of *N*-acetylputrescine hydrochloride (see Section 7.3).

N-Acetycadaverine Hydrochloride (193)

The lipase-catalysed acetylation of cadaverine (154) was stopped after 3 d and worked up by the standard procedure. *N*-Acetylcadaverine hydrochloride (193) was recrystallised from isopropanol/diethyl ether (0.046 g, 13%); R_f 0.29 (EtOAc/isopropanol/conc. NH_3, 9:7:4); m.p. 136-140 °C; ν_{max} (KBr) 3 250, 3 050 (br), 2 960, 2 860, 1 650, 1 554 and 1 470

c m^{-1}; δ_H (200 MHz) (D_2O, ref. HOD @ 4.63) 1.18-1.52 (6H, complex, 2-, 3- and 4-H_2), 1.73 (3H, s, CH_3), 2.80 (2H, t, J 7.6 Hz, 5-H_2) and 2.98 (2H, t, J 6.7 Hz, 1-H_2); δ_C (D_2O, ref. dioxan @ 67.4) 22.7 (CH_3), 23.8 (C-3), 27.2 and 28.6 (C-2 and C-4), 39.9 and 40.2 (C-1 and C-5) and 174.8 (C=O); *m / z* 145 (M^+-Cl, 2.1%), 127, 115, 100, 87, 72 (100%) and 59; (Found: C, 46.52; H, 9.48; N, 15.57%: $C_7H_{17}N_2OCl$ requires C, 46.49; H, 9.50; N, 15.50%).

N-Acetyl-1,2-diaminopropane Hydrochloride (197)

1,2-Diaminopropane (195) was reacted with PPL in EtOAc for 3 d before being worked up in the usual manner. Even after 30 d no diacetyl-1,2-diaminopropane was observed by TLC. The reaction was taken off after 3 d because the maximum amount of product had been formed. *N*-Acetyl-1,2-diaminopropane hydrochloride (197) was recrystallised from isopropanol/diethyl ether (0.080 g, 19%); R_f 0.34 EtOAc/isopropanol/conc. NH_3, 9:7:4); m.p. 185-190 °C; ν_{max} (KBr) 3 265, 2 980, 1 635 (br), 1 550, 1 510, 1 375 and 1 290 c m^{-1}; δ_H (200 MHz) (D_2O, ref. HOD @ 4.63) 1.10 (3H, d, J 7.5 Hz, 3-H_3), 1.83 (3H, s, $COCH_3$) and 3.03-3.39 (3H, complex, 1-H_2 and 2-H); δ_C (D_2O, ref. dioxan @ 67.4) 16.2 (C-3), 22.7 ($CO\underline{C}H_3$), 43.1 (C-1), 48.5 (C-2) and 176.1 (C=O); *m / z* 117 (M^+-Cl, 1.8%), 101, 87 and 72 (100%); (Found: M^+-Cl, 117.1010: $C_5H_{13}N_2OCl$ requires *M*-Cl, 117.1028).

Monoacetylation of 2-Methyl-1,5-diaminopentane (199)

In the case of the acetylation of 2-methyl-1,5-diaminopentane (199) two products with R_f values of 0.28 and 0.32 were observed by TLC. After 5 d the enzyme and solvent were separated and the enzyme was stirred for 1 h with $CHCl_3$/MeOH (9:1) (20 ml). The mixture was filtered through Celite and the filtrate was added to the reaction solvent. The combined solutions were dried, filtered and concentrated *in*

vacuo to give a yellow oil. Separation of the starting material and products was attempted by preparative TLC (isopropanol/conc. NH_3, 5:3). However, only small amounts of material were obtained from the plate and these were not pure. This reaction was not pursued further.

Stereoselective Monoacetylation of 1,2-Diaminopropane (195) Using PPL and EtOAc (Experiment 1)

Four flasks were set up as described in the 'PPL-catalysed monoacetylation of 1,2-diaminopropane' experiment. These flasks were taken off at daily intervals and worked up as explained previously.

The percentage conversion was calculated from the 90 MHz ^1H NMR spectrum of the hydrochloride salt mixture in D_2O before the isopropanol extraction of *N*-acetyl-1,2-diaminopropane hydrochloride. After running the spectrum, the D_2O was removed *in vacuo* to leave a solid which was extracted as before.

The percentage conversion was calculated from the ratio of the integral of the CH_3CO singlet in product (δ 1.7) / integral of the doublets for CH_3CH in starting material and product x 100%.

The 90 MHz ^1H NMR spectrum for the 48 h reaction is shown in Figure 6.1, Chapter 6. The optical rotations for each *N*-acetyl-1,2-diaminopropane hydrochloride (197) sample were recorded. The results for this experiment are given in Table 6.1, Chapter 6.

A small optical rotation $[\alpha]_D$ -1.35° (c 1.11, H_2O) was obtained for the 1,2-diaminopropane dihydrochloride reclaimed from the 24 h reaction. No optical rotations were observed for any of the other starting material dihydrochloride salts isolated.

Formation of *N*-Acetyl-*N'*-α-methoxy-α-trifluoromethyl phenylacetyl-1,2-diaminopropane (202)

To a suspension of 2-chloro-1-methylpyridinium iodide (201) (92 mg, 0.39 mmol) and racemic *N*-acetyl-1,2-diaminopropane hydrochloride (31) (50 mg, 0.30 mmol) in dry CH_2Cl_2 (2 ml) under an N_2 atmosphere, was added a mixture of (R)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid [(R)-Mosher's acid] (200) (70 mg, 0.30 mmol) and distilled triethylamine (106 mg, 146 µl, 1.05 mmol, 3.5 equiv) in dry CH_2Cl_2 (2 ml). The resulting mixture was heated at reflux for 3 h, then the solvent was removed under reduced pressure to leave a yellow solid. This solid was shown by 90 MHz ^1H NMR spectroscopy to contain the desired product (202) and 1-methyl-2-pyridone which is a by-product of the reaction. These were separated by dry-column flash chromatography on silica, eluting with ethyl acetate. *N*-acetyl-*N'*-α-methoxy-α-trifluoromethylphenylacetyl-1,2-diaminopropane (202) was eluted from the column first. As the two diastereomers formed came off the column at slightly different rates each fraction was examined carefully by 90 MHz ^1H NMR spectroscopy to ensure that all of the diasteromeric products were isolated. The title compound was obtained as a white solid (35.3 mg, 35%); R_f 0.69 (EtOAc/isopropanol/conc. NH_3, 9:7:4); m.p. 104-114 °C; ν_{max} (KBr) 3 360, 3 320, 3 260, 3 100, 2 980, 2 940, 2 850, 1 660 (br), 1 540 (br), 1 180, 1 160, 1 120, 740 and 698 cm^{-1}; δ_H 1.16 (1.5 H, d, J 6.7 Hz, 3-H_3), 1.20 (1.5 H, d, J 6.7 Hz, 3-H_3), 1.74 (1.5 H, s, $COCH_3$), 1.92 (1.5 H, s, $COCH_3$), 3.08-3.18 (1H, complex, 2 x 1-H), 3.35 (3H, br s, 2 x OCH_3), 3.40-3.52 (1H, complex, 2 x 1-H), 4.06 (1H, br m, 2 x 2-H), 6.25 (1H, br d, D_2O exch., 2 x NHCH), 7.24 (1H, br t, D_2O exch., $NHCH_2$), 7.38 (3H, complex, aromatic ortho/para CH) and 7.46 (2H, complex, aromatic meta CH); δ_C 18.0 and 18.2 (C-3), 22.91 and 22.96 ($COCH_3$), 45.0 and 45.2 (C-1), 46.2 and 46.3 (C-2), 54.81 and 54.85 (OCH_3), 120.9 (C-Ph), 126.7 (aromatic 'C'), 127.51 and 127.54 (aromatic meta CH), 128.6 and 128.7 (aromatic ortho

CH), 129.4 and 129.5 (aromatic para CH), 132.3 and 132.5 (CF_3), 167.0 and 167.1 ($\underline{C}OCH_3$) and 171.0 and 171.1 ($\underline{C}OCF_3$); δ_F (Ref. $CFCl_3$ @ δ 0.00) -69.25 and -69.20 (3F, 2 x s, CF_3); *m / z* 333 (MH^+, 0.1%), 273, 260, 189, 143, 101 (100%) and 77; (Found: MH^+, 333.1426: $C_{15}H_{19}N_2O_3F_3$ requires MH^+, 333.1426).

Molecular modelling was carried out using the Macintosh Chem3D programme.

Formation of Diastereomers between (R)-Mosher's Acid and *N*-Acetyl-1,2-diaminopropane Hydrochloride Samples from Experiment 1A and 1C.

This was performed as described above. The results are given in Table 7.1 and Table 6.1, Chapter 6. The *N*-acetyl-*N'*-α-methoxy-α-trifluoromethylphenylacetyl-1,2-diaminopropane samples formed had almost identical analytical data to the diastereomeric mixture formed from racemic *N*-acetyl-1,2-diaminopropane hydrochloride. The following signals in the NMR spectra could be ascertained as being from either the major diastereomer or minor diastereomer. δ_H [major isomer] 1.16 (3H, d, J 6.7 Hz, 3-H_3) and 1.92 (3H, s, $COCH_3$); [minor diasteromer] 1.20 (3H, d, J 6.7 Hz, 3-H_3) and 1.74 (3H, s, $COCH_3$); δ_C [major isomer] 18.0 (C-3) and 22.90 ($CO\underline{C}H_3$); [minor isomer] 18.2 (C-3) and 22.85 ($CO\underline{C}H_3$). With all other NMR spectroscopic data, signals corresponding to the major and minor isomer could not be distinguished.

Formation of (R)-1,2-Diaminopropane Dihydrochloride (203)

A sample of resolved (R)-(-)-1,2-diaminopropane tartrate was kindly donated by Dr I. Fallis, University of Glasgow. This was converted into (R)-1,2-diaminopropane dihydrochloride (203) by the method of Dwyer, Garvann and Shulman.[154]

The tartrate salt (1.25 g, 3.33 mmol) was dissolved in boiling water (10 ml) and to this was added KCl (0.5 g, 6.67 mmol). On cooling, potassium tartrate precipitated out and this was removed by filtration. The filtrate was reduced in volume (*ca.* 3 ml), refiltered and the solution was evaporated to dryness to give crude (R)-1,2-diaminopropane dihydrochloride (203). This was recrystallised from 95% aqueous ethanol/acetone several times to remove all traces of potassium tartrate (0.169 g, 34.5%); m.p. 235-240 °C; $[\alpha]_D$ +3.70° (c 3.32, H_2O); ν_{max} (KBr) 3 426 (br), 2 924 (br), 1 562, 1 522 and 1 487 cm^{-1}; δ_H (90 MHz) (D_2O, ref. HOD @ 4.63) 1.30 (3H, d, J 7 Hz, 3-H_3) and 3.1-3.4 (3H, complex, 1-H and 2-H_2); *m / z* 74 (M^+-2HCl, 38.9%), 58 and 56 (100%); (Found: M^+-2HCl, 74.0844: $C_3H_{12}N_2Cl_2$ requires M^+-2HCl, 74.0844).

Isolation and Determination of Stereochemistry of Major Diastereomer of *N*-Acetyl-*N'*-α-methoxy-α-trifluoromethylphenylacetyl-1,2-diaminopropane.

The *N*-acetyl-*N'*-α-methoxy-α-trifluoromethylphenylacetyl -1,2-diaminopropane samples prepared were collected together and recrystallised from dichloromethane/pentane. A 90 MHz ^1H NMR spectrum of the recrystallised sample showed it to be 80% major diastereomer. After a further two recrystallisations a pure sample of the major diastereomer was obtained as white, needlelike crystals; m.p. 152-153 °C; $[\alpha]_D$ +23.5° (c 0.26, CH_2Cl_2). These crystals were suitable for X-ray analysis. Unfortunately the results of this were not obtained.

Monoacetylation of 1,2-Diaminopropane Using CRL and EtOAc (Experiment 2)

This was performed in an analogous fashion to Experiment 1 except that CRL (16 mg, 690 units of activity per mg of solid)

was substituted for PPL. The amount of enzyme used was equivalent to the number of units of activity used in Experiment 1. The results are given in Table 6.2, Chapter 6. In all the *N*-acetyl-1,2-diaminopropane hydrochloride samples looked at, the optical rotations were 0° and therefore no (R)-Mosher's acid derivatives were synthesised.

Monoacetylation of 1,2-Diaminopropane Using Lipozyme and EtOAc (Experiment 3)

1,2-Diaminopropane (0.200 g, 2.70 mmol) was dissolved in ethyl acetate (10 ml) and to this was added Lipozyme (0.5 g, 12.2 units of activity per gram of solid). This suspension was shaken at 100 rpm in a temperature-controlled bath at 25 °C. TLC analysis (EtOAc/isopropanol/conc. NH_3, 9:7:4) of the reaction mixture with time indicated that *N*-acetyl-1,2-diaminopropane was formed after 24 h with the maximum yield (*ca.* by TLC) occurring after 5 d. Analysis of the reaction mixture by TLC from 5 d onwards showed no change in the ratio of starting material to product.

The reaction was worked up by the standard procedure. However no product was isolated from isopropanol. Removal of isopropanol *in vacuo* gave an oil which was shown by ^1H NMR spectroscopy (D_2O) to consist mainly of 1,2-diaminopropane.

This reaction was repeated but again no product could be isolated.

Monoacetylation of 1,2-Diaminopropane Using PPL and Various Alkyl Acetates

(i) Methyl acetate (Experiment 4)

This was set up in an analogous fashion to Experiment 1 with ethyl acetate substituted by methyl acetate. The results

are given in Table 6.3, Chapter 6. The (R)-Mosher's acid derivatives of *N*-acetyl-1,2-diaminopropane hydrochloride from the 2 d, 3 d and 4 d reaction were synthesised as described before. The results of this are given in Table 7.1 and Table 6.3, Chapter 6.

(ii) *n*-Propyl acetate (Experiment 5)

This was set up as described in Experiment 1 except that ethyl acetate was replaced by *n*-propyl acetate. Only three flasks were put on and these were taken off and worked up over a longer time span because the reaction was slower than that in Experiment 1. The results of this experiment are given in Table 7.1 and Table 6.4, Chapter 6.

(iii) *i*-Propyl acetate (Experiment 6)

1,2-Diaminopropane (0.200 g, 2.70 mmol) was dissolved in *i*-propyl acetate (10 ml) and to this was added PPL (0.200 g, activity as before). This mixture was shaken at 100 rpm in a temperature controlled water bath at 25 °C. The reaction was monitored by TLC (EtOAc/isopropanol/conc. NH_3, 9:7:4). No *N*`-acetyl-1,2-diaminopropane was formed even after 14 d.

(iv) *n*-Butyl acetate (Experiment 7)

This experiment was carried out in an analogous fashion to (iii) with *n*-butyl acetate in place of *i*-propyl acetate. The reaction was worked up by the standard procedure after 14 d since a small amount of product had been formed. The *N*-acetyl-1,2-diaminopropane hydrochloride isolated (15 mg, 3.6%) possessed no optical activity {$[\alpha]_D$ 0° (c 0.68, H_2O)}.

(v) *n*-Octyl acetate (Experiment 8)

This experiment was also carried out in an analogous fashion to (iii) but this time *n*-octyl acetate was used in place of *i*-propyl actate. No reaction was observed by TLC after 9 d.

(vi) Phenyl acetate (Experiment 9)

When phenyl acetate (10 ml) was added to 1,2-diaminopropane (0.200 g, 2.70 mmol) an exothermic reaction took place with diacetyl-1,2-diaminopropane being produced.

Monoacetylation of 1,2-Diaminopropane Using PPL and Various Ethyl Acylates

1,2-Diaminopropane (0.200 g, 2.70 mmol) was dissolved in the indicated ethyl acylate (10 ml) and to this was added PPL (0.200 g, activity as before). This mixture was shaken at 100 rpm in a constant temperature bath at 25 °C.

(i) Ethyl formate (Experiment 10)

When this acylating agent was added to 1,2-diaminopropane an exothermic reaction occurred. TLC analysis using EtOAc/isopropanol/conc. NH_3 (9:7:4) as eluant indicated that partial acylation had taken place.

(ii) Ethyl propionate (Experiment 11)

Four flasks were set up as described. These were taken off at daily intervals and worked up by the standard method. The percentage conversions were calculated from the 90 MHz ^1H NMR spectra of the hydrochloride salt mixtures of starting material and *N*-propionyl-1,2-diaminopropane (204) (Table 6.6, Chapter 6). δ_H (D_2O), ref. HOD @ 4.8) 0.95 (t, J 8 Hz, CH_3CH

of product), 1.15 (d, J 7 Hz, CH_3CH of product), 1.35 (d, J 7 Hz, CH_3CH of starting material), 2.20 (q, J 8 Hz, CH_3CH_2 of product) and 3.0-4.0 (complex, CH-N and CH_2-N of starting material and product).

Percentage conversion =

$$\frac{\text{[integral of q @ } \delta \text{ 2.20] x 3/2 x 100\%}}{\text{[integral from } \delta \text{ 0.8 to } \delta \text{ 1.45] - 3/2[integral of q @ } \delta \text{ 2.20]}}.$$

The product came out of isopropanol as a dark-brown oily solid. This could not be crystallised. No accurate analytical data could be obtained on the oily solids.

(iii) Ethyl butyrate (Experiment 12)

Four flasks were set up as described. At intervals the flasks were worked up in the usual manner. The percentage conversions were again calculated from the 90 MHz ^1H NMR spectra of the hydrochloride salt mixtures of *N*-butyryl-1,2-diaminopropane (205) and 1,2-diaminopropane (Table 6.7, Chapter 6). δ_H (D_2O, ref. HOD @ 4.75) 0.93 (t, J 7 Hz, CH_3CH_2 of product), 1.35 (d, J 7 Hz, CH_3CH of product), 1.50 (d, J 7 Hz, CH_3CH of starting material), 1.50-1.90 (m, $CH_3CH_2CH_2$ of product), 2.32 (t, J 7 Hz, $CH_3CH_2CH_2$ of product) and 3.30-4.00 (complex, CH-N and CH_2-N of starting material and product).

Percentage conversion =

$$\frac{\text{[integral of t @ } \delta \text{ 2.32] x 3/2 x 100\%}}{\text{[integral from } \delta \text{ 1.2 to } \delta \text{ 2.0] - [integral of t @ } \delta \text{ 2.32]}}.$$

Only brown sticky solids were isolated from isopropanol and these were too impure for any analyses.

Monoacetylation of 1,2-Diaminopropane Using PPL and EtOAc at 35 °C (Experiment 13)

This study was performed in a similar fashion to Experiment 1 except that this experiment was carried out in a temperature-controlled water bath at 35 °C rather that 25 °C. The flasks were taken off and worked up over a 78 h timespan. The results are given in Table 6.9, Chapter 6. The (R)-Mosher's acid derivatives were prepared of *N*-acetyl-1,2-diaminopropane hydrochloride from the 29 h and 78 h experiments as previously described. Results are in Table 7.1 and Table 6.9, Chapter 6.

Monoacetylation of 1,2-Diaminopropane Using PPL and EtOAc in 3-Methyl-3-pentanol (Experiment 14)

!,2-Diaminopropane (0.200 g, 2.70 mmol) was dissolved in 3-methyl-3-pentanol (10 ml) and to this was added PPL (0.200 g, activity as before) and ethyl acetate (0.410 g 5.40 mmol, 2 equiv). This mixture was shaken at 100 rpm in a temperature-controlled water bath at 25 °C. The reaction was followed by TLC (EtOAc/isopropanol/conc. NH_3, 9:7:4). After 6 d no product had been formed and after 11 d only a tiny amount of *N*-acetyl-1,2-diaminopropane was visible on the TLC. Therefore the reaction was abandoned.

Another set of experiments was set up as described but using varying volumes of ethyl acetate and 3-methyl-3-pentanol (5 ml: 5 ml, 2.5 ml: 7.5 ml, 2 ml: 8 ml, 1 ml: 9 ml). These were left running until *ca.* 30-40% conversion as indicated by TLC. The results are given in Table 6.10, Chapter 6. The (R)-Mosher's acid dervative of every *N*-acetyl-1,2-diaminopropane hydrochloride sample was prepared. The procedure used was as described earlier and the results are given in Table 7.1 and Table 6.10, Chapter 6.

Synthesis of 2,2,2-Trichloroethyl acetate (207)

To a solution of 2,2,2-trichloroethanol (4.0 g, 26.8 mmol) and triethylamine (4.0 ml, 28.8 mmol) in CH_2Cl_2 (20 ml) at 0 °C was added, over 1 h, acetyl chloride (2.51 g, 32.0 mmol). The mixture was brought to room temperature and stirred for a further 3 h. The reaction mixture was washed with water, 1 M hydrochloric acid and water (each 30 ml), dried, filtered and concentrated to give a yellow oil. This was purified by dry-column flash chromatography on silica eluting with CH_2Cl_2. The product (207) was obtained as a colourless oil (3.78 g, 74%); υ_{max} ($CHCl_3$) 2 957, 1 765, 1 440, 1 380 and 1 050 c m^{-1}; δ_H (200 MHz) 2.17 (3H, s, CH_3) and 4.70 (2H, s, 1-H_2); δ_C 20.5 (CH_3), 73.9 (C-1), 94.6 (C-2) and 169.2 (C=O); *m / z* 189 (M^+, 0.1%), 157, 155, 131, 117, 95, 82, 72 (100%) and 61; (Found: M^+, 189.9337; C, 25.18; H, 2.64%: $C_4H_5O_2Cl_3$ requires *M*, 189.9355; C, 25.05; H, 2.64%).

Synthesis of 2,3-Butanedione monoxime acetate (208)

Acetyl chloride (2.0 g, 25.7 mmol) in CH_2Cl_2 (10 ml) was added slowly over 0.5 h to a solution of 2,3-butanedione monoxime (2.0 g, 19.8 mmol) and triethylamine (4.0 ml, 28.8 mmol) in CH_2Cl_2 (20 ml) at 0 °C. The mixture was allowed to warm to room temperature and stirring was continued for 2 h. The reaction mixture was washed with H_2O, 1 M HCl and H_2O (each 25 ml), dried, filtered and the solvent removed *in vacuo.* The pale yellow oil obtained was subjected to dry-column flash chromatoraphy on silica eluting with CH_2Cl_2. The title compound (208) was furnished as a clear oil (2.03 g, 72%); R_f 0.69; υ_{max} ($CHCl_3$) 3 020, 1 783, 1 712, 1 375 and 1 005 cm^{-1}; δ_H (200 MHz) 2.00 (3H, s,1-H_3), 2.21 (3H, s, CH_3CO_2) and 2.41 (3H, s, 4-H_3); δ_C 10.0 (C-1), 19.4 ($\underline{C}H_3CO_2$), 25.5 (C-4), 160.5 (C-2), 167.7 ($CH_3\underline{C}O_2$) and 196.0 (C-3).

Control Experiments

A control in which all components except the enzyme were present was run alongside each reaction described in this chapter. This gave a measure of how much, if any, non-enzymic catalysed acylation was occurring. In the majority of experiments no non-enzymic acylation was observed within the time span of the experiment. In each of the following cases this non-enzymic acylation occurred.

(i) Using 2,2,2-trichloroethyl acetate (1 equiv) as acyl donor with CH_2Cl_2 as solvent.

After 24 h a significant (*ca.* 20%) amount of *N*-acetyl-1,2-diaminopropane was visible by TLC analysis (EtOAc/isopropanol/conc. NH_3, 9:7:4). After 6 d, 1,2-diaminopropane was completely monoacetylated.

The racemic *N*-acetyl-1,2-diaminopropane isolated after the standard work up was used as the racemic substrate for Mosher's acid derivative formation (see earlier).

(ii) Using 2,3-butanedione monoxime acetate (1 equiv) as acyl donor with CH_2Cl_2 as solvent.

N-Acetylation of 1,2-diaminopropane was visible within 6 h. After 24 h the conversion was *ca.* 40% (by TLC).

(iii) Using vinyl acetate (1 equiv) as acyl donor with either CH_2Cl_2, diethyl ether or 3-methyl-3-pentanol as solvent.

When CH_2Cl_2 was the solvent 70% of 1,2-diaminopropane had been monoacetylated after 24 h. The percentage conversion was calculated as described in Experiment 1.

In the cases where diethyl ether and 3-methyl-3-pentanol were the solvents the control runs gave after 24 h, conversions of 54% and 30% respectively.

Table 7.1 : Synthesis of *N*-acetyl-1,2-diaminopropane hydrochloride/(R)-Mosher's Acid Diastereomers

Experiment No.	Wt. starting material.(mg)	Wt. Product (mg)	Yield (%)	d. e. (%)
1 A	20	26	66%	51%
1 C	18	19	55%	21%
4 A	9	-	-	-
4 B	10	17	86%	25%
4 C	17	19	58%	14%
4 D	35	44	58%	5%
5 B	25	26	48%	36%
13 B	20	22	57%	45%
13 D	35	42	55%	25%
14 A	56	59	54%	42%
14 B	35	20	27%	-
14 C	42	60	73%	19%
14 D	22	25	57%	33%

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