Biosynthesis of Pyrrolizidine Alkaloids

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

by

Iain Robert Stirling

Department of Chemistry,
The University,
Glasgow.

July 1993

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Our business in life is not to succeed, but to continue to fail in good spirits.

Robert Louis Stevenson

ACKNOWLEDGEMENTS

I would like to thank Professor D. J. Robins for his support and guidance throughout my time in the laboratory, and his help in the presentation of this thesis.

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I am particularly grateful to Miss I. K. A. Freer for her work involving the preparation and feeding experiments with the transformed root cultures.

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Finally, I would like to thank Jane and my mother for their support and assistance over the past years.

TABLE OF CONTENTS

Publications

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

"Assignment of the ^{1}H and ^{13}C NMR Spectra and Conformational Analysis of the Pyrrolizidine Alkaloid 13-*O*-Acetyldicrotaline." D.S. Rycroft, I. R. Stirling and D. J. Robins, *Magn. Reson. Chem.*, 1992, 30, 42.

Abbreviations

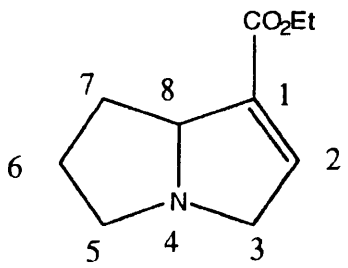
The following abbreviations are used in the text.

br	broad
DIBAL	diisobutylaluminium hydride
DMSO	dimethylsulfoxide
HMG	3-hydroxy-3-methylglutaric acid
Hz	hertz
IR	infrared
m	multiplet
MHz	megahertz
MS	mass spectrum
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect
q	quartet
s	singlet
t	triplet
THF	tetrahydrofuran
TLC	thin layer chromatography

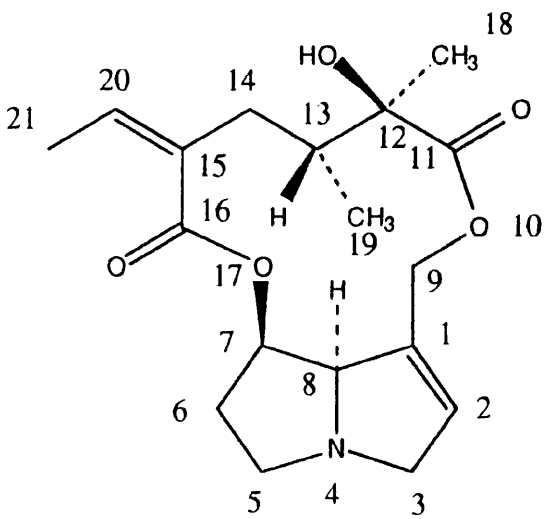
Notes on Nomenclature

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

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



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

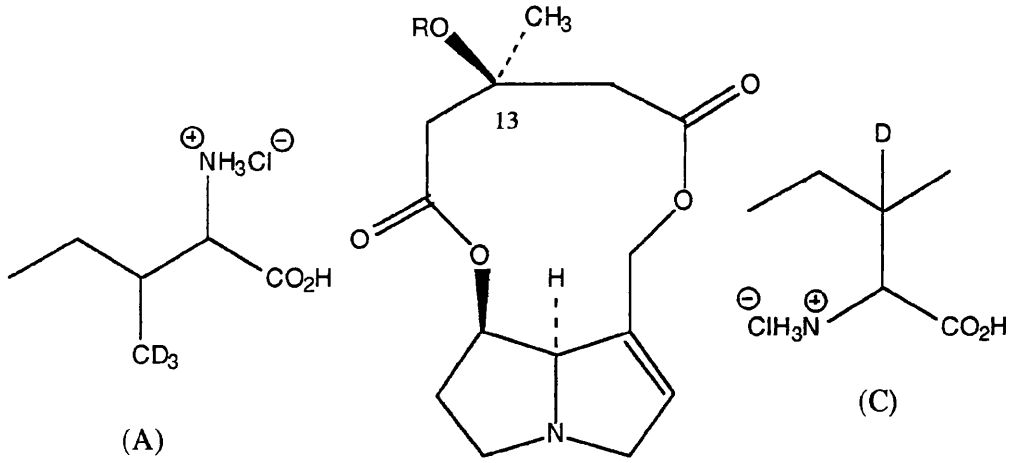


SUMMARY

The work presented in this thesis is concerned with the biosynthesis of the necic acid portions of pyrrolizidine alkaloids and has been divided into five main areas: (a) the development of a general route to a variety of ^{13}C- and ^{2}H- labelled isoleucines; (b) the synthesis of a specifically labelled isoleucine; (c) the synthesis of labelled 2-aminobutanoic acid; (d) feeding experiments with plants and transformed root cultures which produce pyrrolizidine alkaloids; (e) the synthesis of new and known synthanecines for biosynthetic studies and investigation of their anti-tumour activity.

(a) Development of a General Route to Isoleucine

Isoleucine has been shown to be specifically incorporated into the acid portions of several pyrrolizidine alkaloids. Some acids with ten carbon atoms are formed from two isoleucine units. In the development of a synthetic route to isoleucine it would be desirable to assemble the target compound by the stepwise addition of one and two carbon units. This would allow the same route to furnish a range of isoleucines, isotopically labelled with ^{2}H or ^{13}C at different positions. We decided to modify a published route to valine. However this synthesis required many stages, a few of which were low yielding, making this scheme uneconomic as an all-purpose route to labelled isoleucines. DL-[6-$^{2}H_3$]Isoleucine hydrochloride (A) together with the allo-racemate was the only labelled amino acid prepared using this procedure.



(A)

(B) R = H
(F) R = Ac

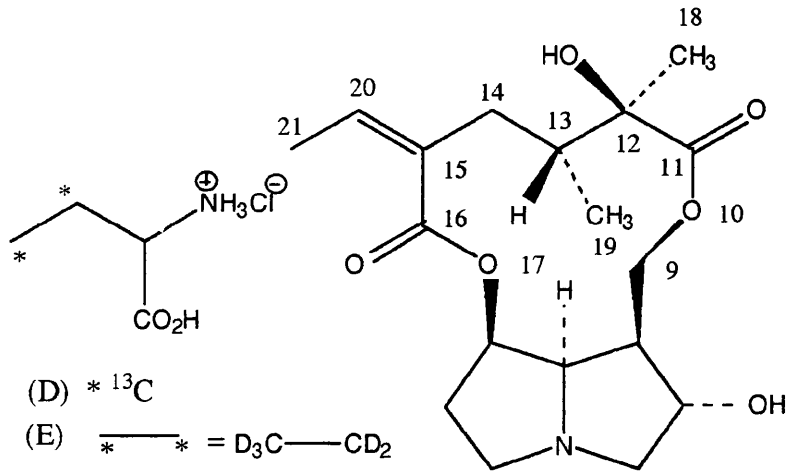
(C)

(b) Synthesis of a Specifically Labelled Isoleucine

In the biosynthesis of the acid portion of dicrotaline (B) in *Crotalaria lachnosema*, isoleucine labels four out of the six carbons. A route was developed to [3-^{2}H]isoleucine hydrochloride (C) and the allo-racemate in an attempt to discover which of the two methylene groups of 3-hydroxy-3-methylglutaric acid is provided by isoleucine.

(c) Synthesis of Labelled 2-Aminobutanoic acid

2-Aminobutanoic acid is also a biosynthetic precursor to several necic acids. A short synthesis of this precursor from diethyl acetamidomalonate was developed from literature procedures. Samples of DL-[3,4-$^{13}C_2$]-2-aminobutanoic acid hydrochloride (D) and DL-[3,4-$^{2}H_5$]-2-aminobutanoic acid (E) were made.



(D) * ^{13}C

(E) $\overline{\quad}$ * = D_3C—CD_2

(G)

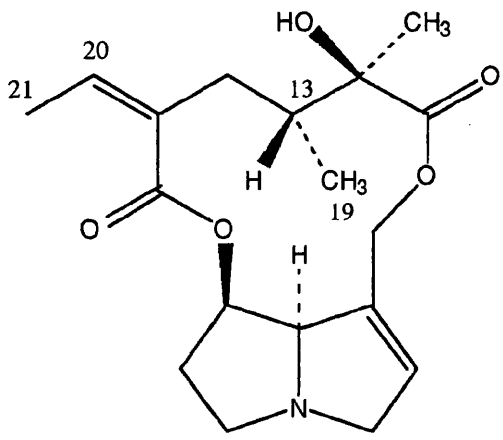
(d) Feeding Experiments

Feeding experiments were carried out on two plants, *Senecio pleistocephalus* and *Crotalaria lachnosema*, and on two transformed root cultures, *Senecio vulgaris* and *Emilia flammea*. *C. lachnosema* produces dicrotaline (B) and 13-*O*-acetyldicrotaline (F). The crude alkaloid mixture isolated from this plant was converted wholly into 13-*O*-acetyldicrotaline, and a full ^{1}H and ^{13}C NMR assignment and conformational analysis of this compound (F) were performed. However all feeding experiments on this plant species failed to show any incorporation of stable isotopes into the alkaloid above natural abundance.

Successful feeding experiments were carried out with *S. pleistocephalus* which produces rosmarinine (G). Deuterium (E) and ^{13}C-^{13}C doubly labelled 2-aminobutanoic acid (D) were incorporated into rosmarinine (G) labelling two pairs of carbon atoms, namely C-13 and C-19 plus the C-20 and C-21 positions and equal labelling was observed in both halves of the acid portion. Senecionine (H) from *S. vulgaris* was also labelled after feeding experiments with 2-aminobutanoic acid. Again the C-13 and C-19 plus C-20 and C-21

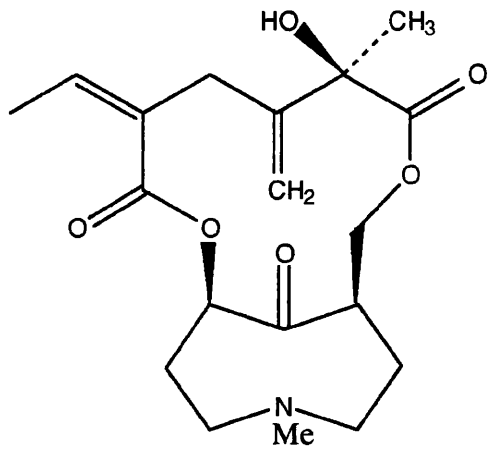
positions were labelled with the amino acid incorporated into both halves of the necic acid equally.

The root culture *E. flammea* produces two alkaloids, senecionine (H) and emiline (I). However in the feeding experiment with 2-aminobutanoic acid, only senecionine and a small amount of its geometrical isomer integerrimine (J) were observed. The labelling patterns for senecionine were the same as previously observed with material isolated from *S. vulgaris.*



(H)

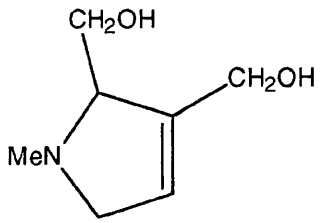
(J) *E*-isomer



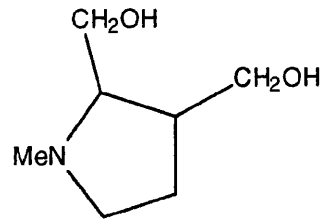
(I)

(e) Formation of Synthanecines

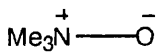
Synthanecine A (K) and synthanecine B (L) were prepared using known procedures. These synthanecines were fed to *S. vulgaris* root cultures, but no new alkaloid analogues could be detected. The use of 1,3-dipolar cycloaddition reactions was investigated as a method for the manufacture of synthanecines. The use of trimethylamine *N*-oxide (M) and lithium diisopropylamide with a variety of alkenes proved unsuccessful. In an alternative procedure *N*-benzyl-*N*-(trimethylsilylmethyl)aminomethyl methylether (N) did undergo the desired cycloaddition with diethyl fumarate, and the product was subsequently converted into a novel synthanecine (O).


CH2OH
CH2OH
MeN

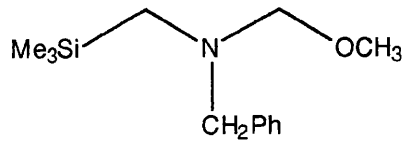
(K)


CH2OH
CH2OH
MeN

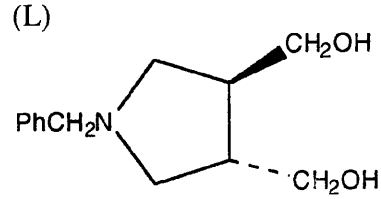
(L)


Me3N
O

(M)


Me3Si
N
OCH3
CH2Ph

(N)


CH2OH
PhCH2N
CH2OH

(O)

CHAPTER 1

Introduction

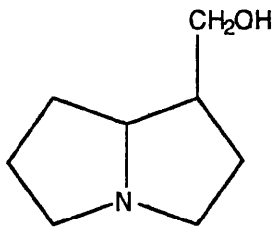
1.1 Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids are secondary metabolites produced by certain higher plants, and they make up a large and interesting area of natural product chemistry.[1] There have been many studies into the synthesis and biosynthesis of these alkaloids. The toxicology of pyrrolizidine alkaloids has also been extensively investigated.[2]

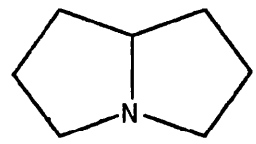
The widespread occurrence of pyrrolizidine alkaloids is well documented and they have been found in 14 unrelated plant families.[3] Over 200 different alkaloids have been isolated and identified from over 300 plant species.[4] It has been estimated that approximately 3% of the world's flowering plants contain pyrrolizidine alkaloids.[5] The plant families studied include Asteraceae (formerly Compositae), e.g. tribes Senecioneae and Eupatorieae, and Fabaceae (formerly Leguminosae) of which the genus *Crotalaria* is the only one known to produce pyrrolizidine alkaloids.

Most pyrrolizidine alkaloids are derivatives of 1-hydroxymethylpyrrolizidine (1), rather than of pyrrolizidine (2) itself. They are comprised of an acid portion (necic acid) and a base portion, called a necine. A wide range of necines has been identified, differing in the degree of hydroxylation and the positions and stereochemistry of the hydroxyl groups, e.g.

rosmarinecine (3). Another important variation is the 1,2-unsaturation, shown here in retronecine (4).



(1)



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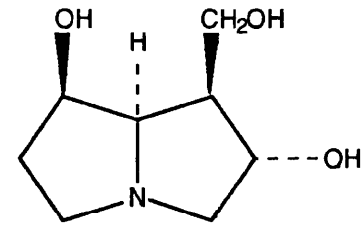

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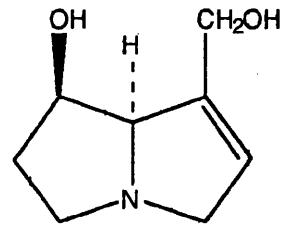
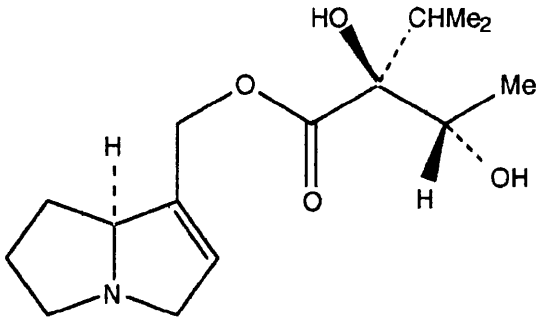


(4)

The esterifying acids are branched chain mono- or di-carboxylic acids. They vary in degree of chain branching, hydroxylation and unsaturation. The alkaloids can be monoesters e.g. viridiflorine (5); diesters, like symlandine (6); or macrocyclic diesters, as retrorsine (7).



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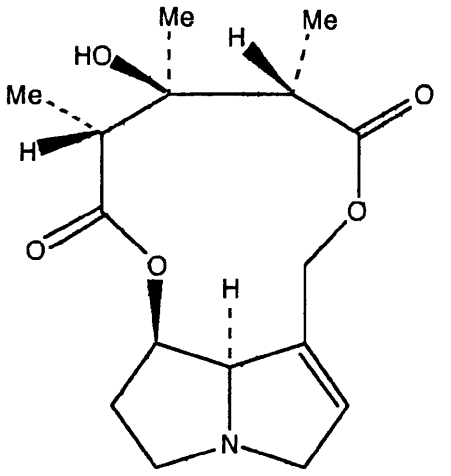

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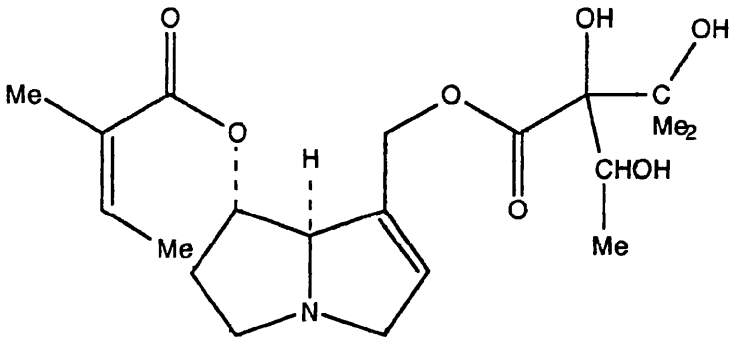


(6)

1.2 Effects of Pyrrolizidine Alkaloids on Humans and Animals

The importance of pyrrolizidine alkaloids is due to their wide spectrum of biological action.[3] Alkaloids with 1,2-unsaturation are hepatotoxic[3] and there have been many instances of the poisoning of both livestock and humans. Indeed ragworts are said to have caused more livestock losses than any other poisonous plant species.[6]

As early as 1787, British farmers suspected that the common ragwort, *Senecio jacobaea*, was harmful to grazing cattle. Ingestion of pyrrolizidine alkaloids has either been by deliberate use or by mistake for other species. Some herbal remedies or foods are plants containing pyrrolizidine alkaloids, for example comfrey (*Symphytum* sp.).

In South Africa seeds of *Senecio ilicifolius* and *Senecio burchelli* were harvested with the wheat, resulting in bread poisoning[7] and one of the most serious examples was in Afghanistan in 1974 when around 1600 people were affected by abdominal distension and emaciation resulting in many deaths.[8]

The main target organ of pyrrolizidine alkaloids is the liver but the lungs, kidneys and the heart may also be affected. Although other organic compounds are known to be toxic to liver cells, e.g. halogenated hydrocarbons and dialkylnitrosoamines. The progressive and irreversible chronic liver damage, illustrated by inhibition of cell division and megalocytosis of the liver is diagnostic for pyrrolizidine alkaloid poisoning.

Some species can use pyrrolizidine alkaloids to their advantage. Various butterflies (Lepidoptera) feed on plants containing alkaloids and store the alkaloids as a defence against predators.[9] It is also known that some butterflies convert the alkaloids into volatile aldehydes or ketones which act as pheromones.[10]

1.3 Metabolism and Cytotoxicity

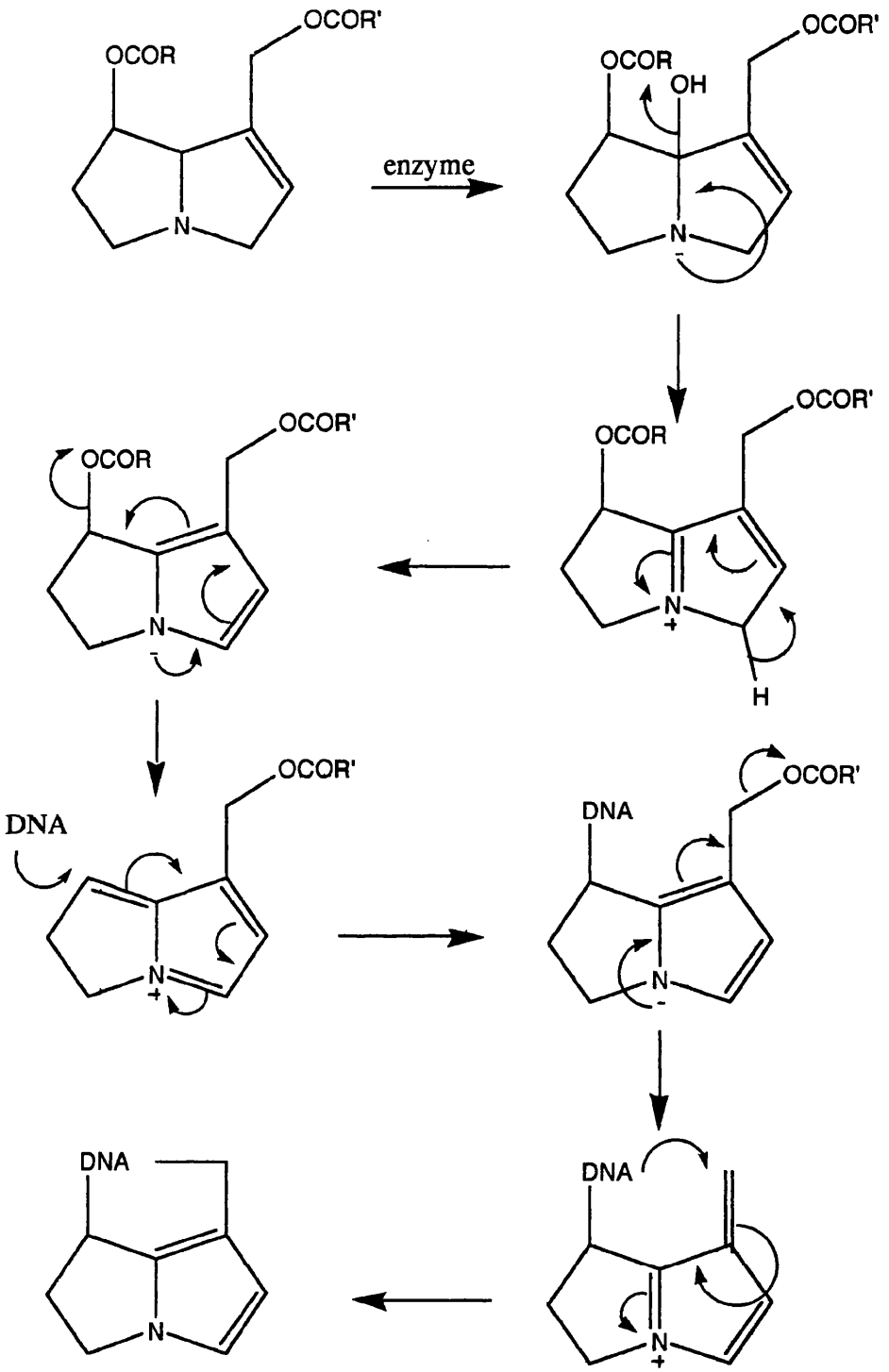
There are three main routes for metabolism of pyrrolizidine alkaloids. These are ester hydrolysis and *N*-oxidation, which are

detoxification pathways that produce water soluble species which can be excreted, and dehydrogenation. It is the dehydrogenation pathway which leads to the cytotoxicity.

The fact that liver damage is the main effect of pyrrolizidine alkaloid poisoning, regardless of the site of administration,[11] indicates that it is the metabolites rather than the alkaloids which are toxic. The liver is the organ where pyrrolizidine alkaloid metabolism occurs. Hepatic microsomal enzymes oxidise those alkaloids which contain 1,2-unsaturation to pyrrolic intermediates (Scheme 1). Cytochrome P450 is the major enzyme catalysing pyrrole formation[12] producing highly reactive species compared to the chemically unreactive alkaloids. The pyrrole formation is proposed to be via oxidation at C-8.

Toxicology studies by Mattocks[13] have shown that there is a direct relationship between the amounts of pyrrolic metabolites found in rat livers and acute hepatotoxicity. The toxic action of the pyrroles is associated with alkylation processes due to the activation of the ester functions by the nitrogen[14] (Scheme 1).

Scheme 1

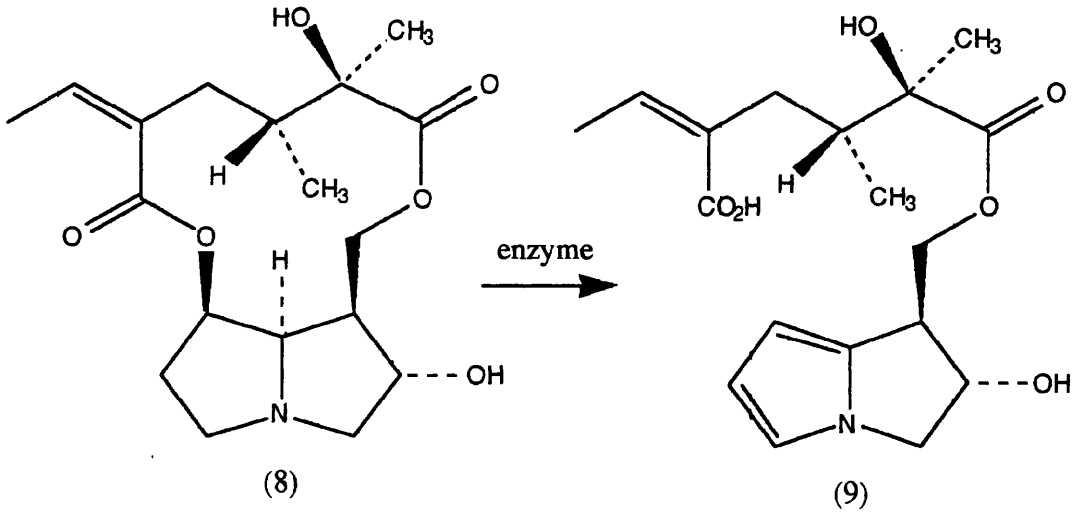


The pyrrolic metabolites formed from pyrrolizidine alkaloids can have one or two reactive ester sites or one ester function and a less reactive hydroxyl group. They can, therefore, act as

bifunctional alkylating agents when they are attacked by nucleophilic sites of DNA, resulting in crosslinking[15] (Scheme 1).

Pyrrolizidine alkaloids containing saturated necines are not cytotoxic. Although they are also metabolised to pyrrolic species, these are different to those formed by hepatotoxic pyrrolizidine alkaloids. Mattocks and White investigated the pyrrolic species formed from rosmarinine[15] (8) and found that it is the left hand ring which is converted into the pyrrole (Scheme 2). This produces a metabolite (9) which does not have an ester group activated by the nitrogen and hence cannot act as an alkylating agent.

Scheme 2

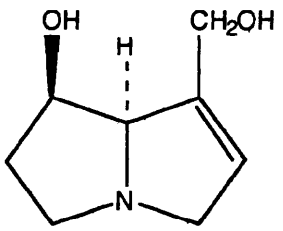


(8) (9)

1.4 Structure Activity Relationships

The toxicity of pyrrolizidine alkaloids is closely associated with their structures. Schoental observed that all known hepatotoxic alkaloids were esters of retronecine (4), supinidine (10) or heliotridine (11) and concluded that the 1,2-unsaturation

was necessary.[16] This agreed with the findings of Grebenuik and Zaharova, who observed that platyphylline (12), which does not possess the allylic ester function, produced no trace of liver damage in dogs.[17] Subsequently, Culvenor *et al.* proposed the mechanism shown in Scheme 1.[18]



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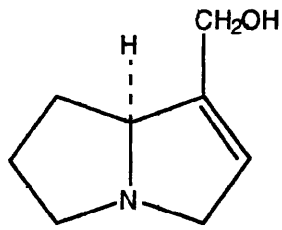

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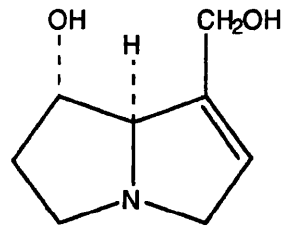

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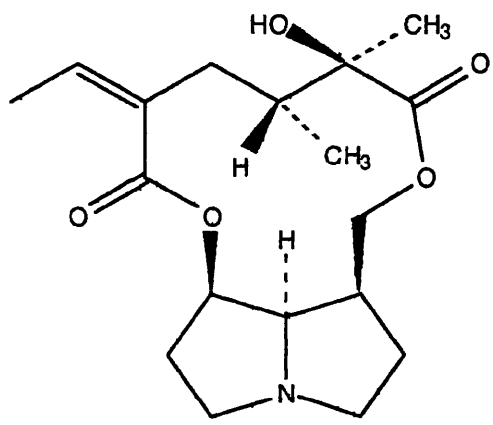


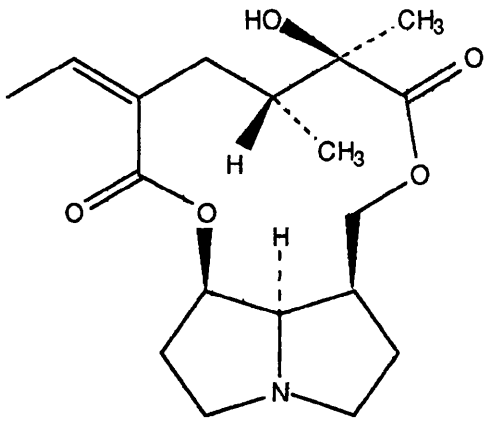
(12)

Lipophilicity and base strength of the alkaloid are two factors which affect toxicity.[2] Higher lipophilicity means that the alkaloids are more susceptible to oxidation by hepatic microsomal enzymes, and hence are more toxic. Alkaloids of higher base strength are usually less lipophilic and therefore less toxic. The reduced toxicity is due to the fact that proportionally more of the alkaloid is protonated at physiological pH and thus can be excreted.

The ester hydrolysis detoxification pathway is less favourable if access to the esters is restricted. For example bulky substituents at the α-carbon of the acid will increase the toxicity due to steric hindrance of the hydrolysis.[19,20]

1.5 Clinical Uses of Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids have also been studied for anticancer activity. Two mechanisms are possible for anti-tumour activity: (i) anti-mitotic effect of pyrrolic metabolites; and (ii) an unknown mechanism involving *N*-oxides. In the USA indicine *N*-oxide (13) has undergone clinical trials as an anti-cancer agent[21] and platyphylline[22] (12), which has the benefit of not being hepatotoxic, has been examined for antispasmodic and mydriatic affects.



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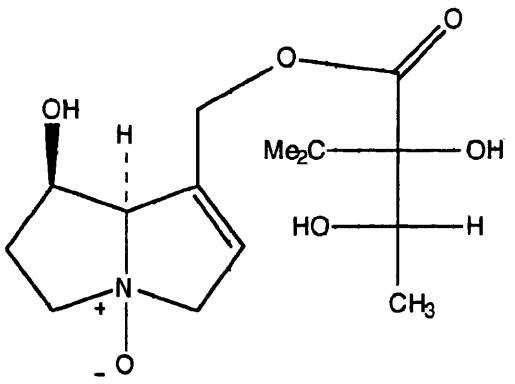


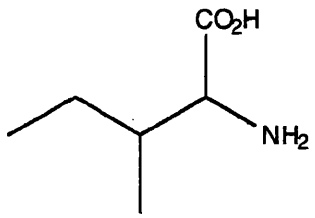
(13)

Further information on pyrrolizidine alkaloids is available in the books by Mattocks,[2] and by Bull *et al.*,[1] and in annual reviews.[23]

1.6 Aims of Project

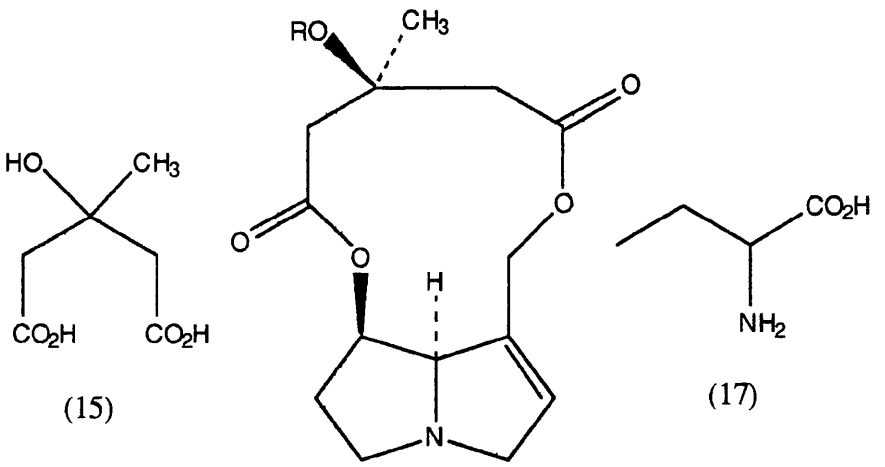
The biosynthesis of pyrrolizidine alkaloids has been at the centre of a tremendous amount of research in our group.[24] This work and the work of others is reviewed in Chapter 2. The biosynthetic pathways to necines are now well established and many of the intermediates have been identified. However the biosynthesis of the necic acids is not so clear. Mevalonate and

acetate have been shown not to be precursors.[25,26] Work by Crout[27,28] identified the specific incorporation of α-amino acids into the acid portions of some of the alkaloids. All previous investigations of necic acid biosynthesis have involved radiotracers. Our intention was to provide the first study of the biosynthetic pathways to the necic acids using stable isotopes. The attempts to develop a general route to a range of labelled isoleucines (14) are described in Chapter 3.



(14)

Isoleucine has been shown to be a precursor for 3-hydroxy-3-methylglutaric acid (15), the acid portion of dicrotaline (16) which is produced, along with 13-*O*-acetyldicrotaline (18), by *Crotalaria lachnosema*. In order to investigate the biosynthesis of this necic acid a specifically labelled isoleucine was required. The synthesis of this amino acid is detailed in Chapter 4.



(15)

(16) R=H
(18) R=Ac

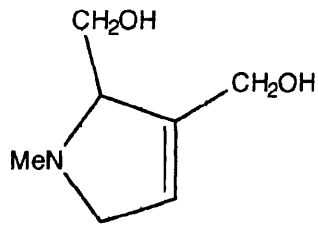
(17)

2-Aminobutanoic acid is another known precursor of some necic acids. The formation of ^{2}H- and ^{13}C-doubly labelled 2-aminobutanoic acid (17) and the feeding experiments performed are described in Chapter 5.

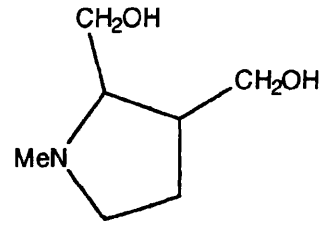
To examine the biosynthesis of pyrrolizidine alkaloids using stable isotopes the NMR spectral analysis of the alkaloids under study is required. The conformational analysis and NMR assignments performed on 13-*O*-acetyldicrotaline (18) are described in Chapter 6.

Chapter 7 is concerned with the synthesis of novel and known synthanecines, which are analogues of naturally occurring necines. These synthetic analogues are simpler to prepare than the corresponding pyrrolizidine bases and have been used in toxicology studies. Synthanecines A (19) and B (20) were prepared by known methods for feeding experiments, in an attempt to form new alkaloid analogues. The synthesis of new synthanecines using dipolar cycloaddition reactions was

investigated in order to provide material for tests of possible anticancer activity.



(19)



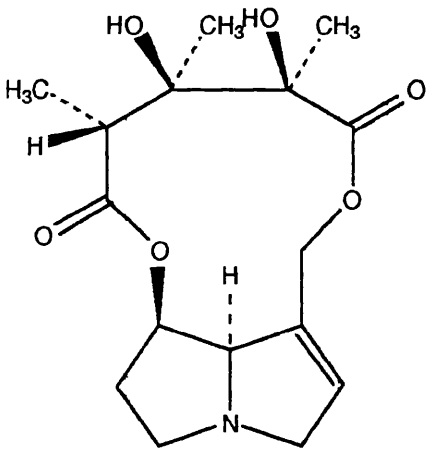
(20)

CHAPTER 2

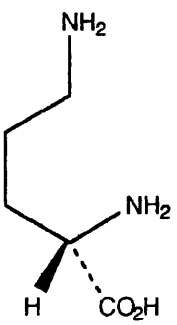
Biosynthesis of Pyrrolizidine Alkaloids

2.1 Biosynthesis of Necine Bases

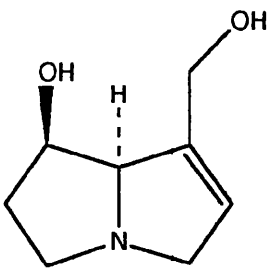
The study of the biosynthesis of necines began over 30 years ago when Nowacki and Byerrum[29] used *Crotalaria spectabilis* to examine the pathway to monocrotaline (21). These initial experiments demonstrated the incorporation of ornithine (22) into the base portion retronecine (4). Similar results were observed by Warren and co-workers[30] with feeding experiments using *Senecio isatideus* and *S. sceleratus.*



(21)



(22)

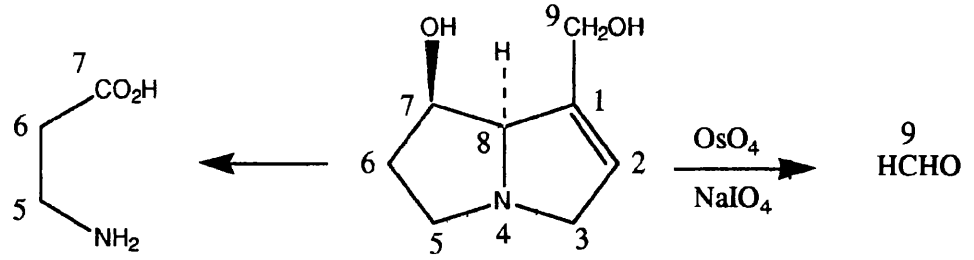


(4)

Retronecine (4) is the base portion of all the alkaloids produced from *Senecio douglasii.*. Bottomley *et al.*[31] fed ^{14}C labelled ornithines to this species, which were incorporated to a reasonable extent (0.3-0.75%), and approximately 94% of the activity was located in the base portion. The retronecine (4) obtained was subjected to further degradation with osmium tetroxide and sodium periodate, liberating

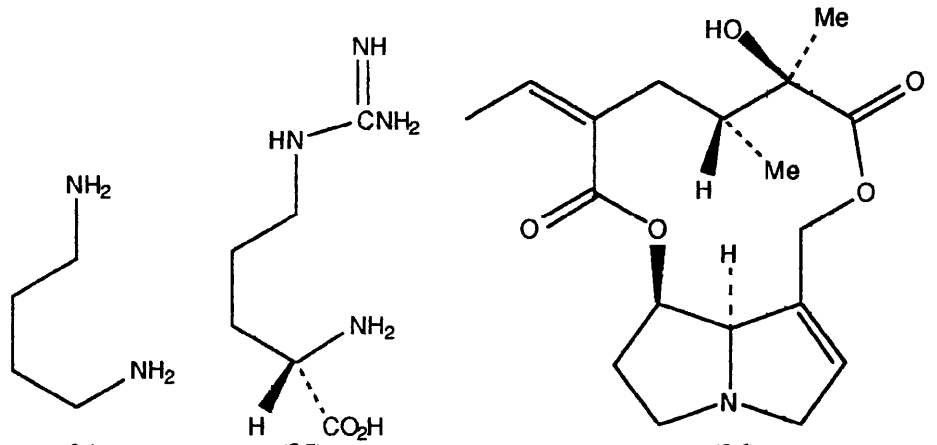
formaldehyde (23) which was trapped as the dimedone derivative (Scheme 3).

Scheme 3



(31) (23)

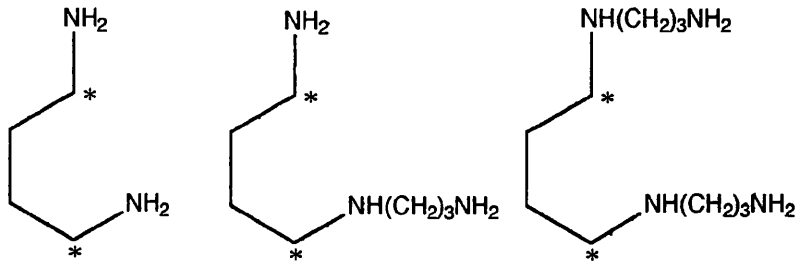
This corresponds to C-9 of retronecine and, after feeding [2-^{14}C]ornithine and [5-^{14}C]ornithine to *S. douglasii*, 25% of the base portion activity was found in the formaldehyde dimedone. This suggested that C-2 and C-5 of ornithine become equivalent, possibly by decarboxylation to putrescine (24). When Bottomley and Geismann[31] investigated this possibility, they found that [1,4-^{14}C]putrescine was incorporated fairly well (0.18%) into the alkaloids produced by *S. douglasii*, with 98% of the activity in the base portion. Again 25% of this radioactivity was found in the formaldehyde dimedone derivative.



(24) (25) (26)

Bale and Crout observed the incorporation of arginine (25) into senecionine (26) in *Senecio magnificus*.[32] Due to erratic results with plants growing under hydroponic conditions, they developed a

double isotope technique, measuring the ratio of $^{3}H/^{14}C$ isotopes in the precursor compared to the final alkaloid. Feeding L-[3-^{3}H]arginine and L-[U-^{14}C]arginine (initial ratio 4.84) to *S. magnificus* yielded senecionine (26) with an average $^{3}H/^{14}C$ ratio of 3.0. Experiments with L-[3-^{3}H]arginine and L-[U-^{14}C]ornithine showed a fall in the $^{3}H/^{14}C$ ratio in the resulting senecionine samples, indicating that ornithine is a more efficient precursor than arginine for retronecine in *S. magnificus.*[32]



(27) (28) (29)

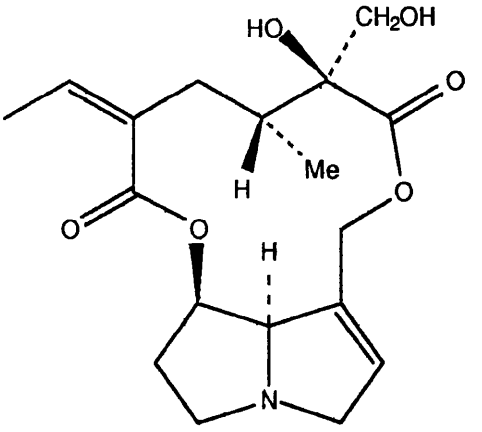
A better technique for carrying out feeding experiments was devised by Robins and Sweeney[33] By dropping aqueous solutions of the precursors on the stems of the plant and puncturing the stems, large incorporations were obtained for [1,4-^{14}C]putrescine (27), [1,4-^{14}C]spermidine (28) and [1,4-^{14}C]spermine (29) into retrorsine (30) with *Senecio isatideus.* Using the degradation techniques previously described (Scheme 3), 25% of the activity was found in the dimedone derivative and 25% of the activity was found in the β-alanine (31). This suggested that two molecules of putrescine combine to give retronecine (4) , with spermine (32) and spermidine (33) probably acting as a source of putrescine.

$H_2N(CH_2)_3NH(CH_2)_4NH_2$

(32)

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

(33)

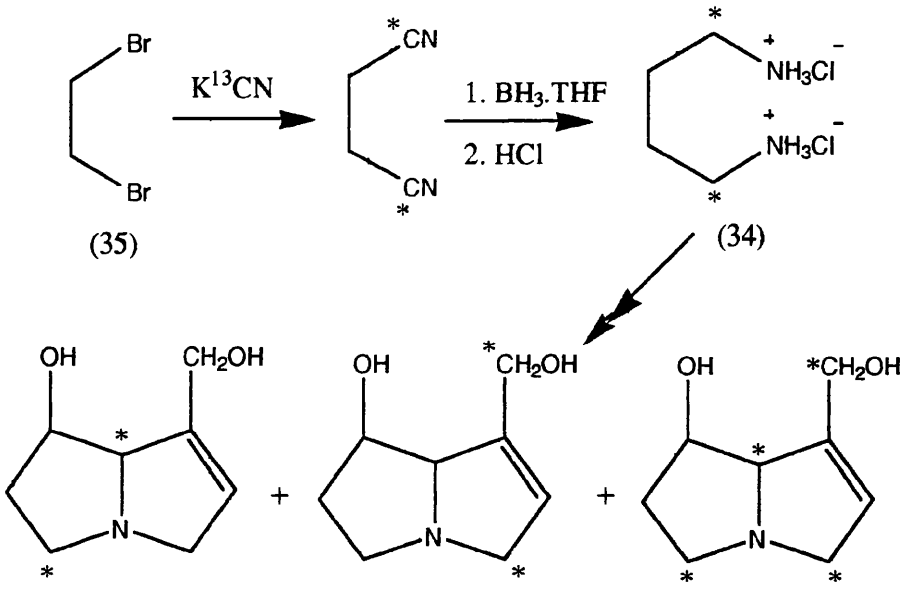


(30)

Hartmann and co-workers[34] reported that putrescine (24) was formed only from arginine (25) in *Senecio vulgaris*, while Birecka *et al.* found that putrescine is formed from arginine in *Heliotropium* species[35] but in *Senecio* and *Crotalaria* species it is ornithine that is converted into putrescine[36]. The contradiction of the origin of putrescine in *Senecio* species has not yet been resolved.

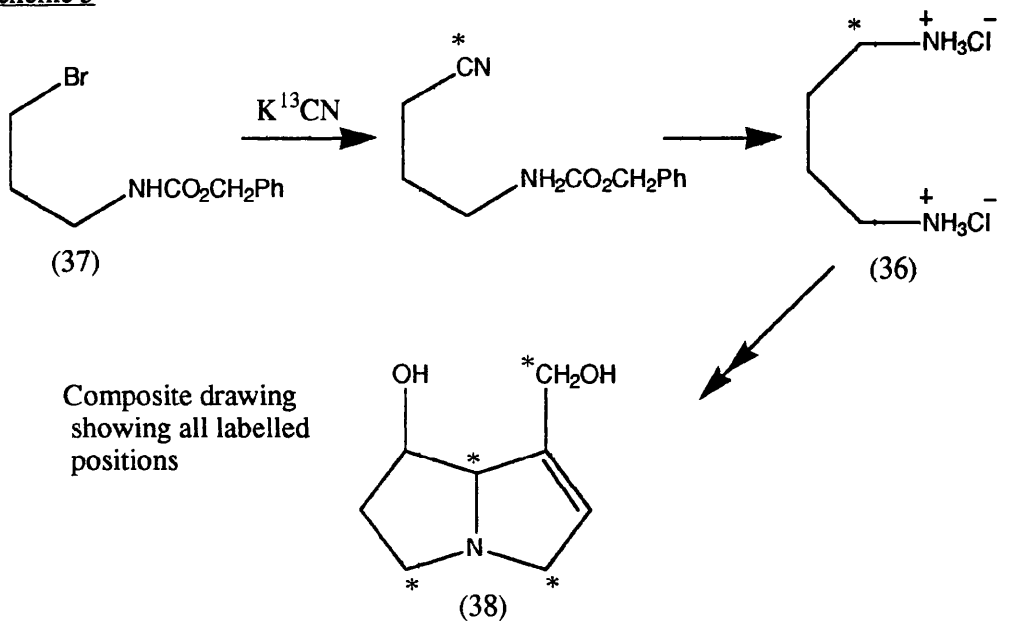
The lack of suitable degradations meant that complete labelling patterns were impossible to obtain with radiotracers. Using stable isotopes, complete labelling patterns can be determined by NMR spectroscopy. Khan and Robins[37] prepared [1,4-$^{13}C_2$]putrescine dihydrochloride (34) from 1,2-dibromoethane (35), and fed it to *Senecio isatideus* (Scheme 4). Enrichment of the C-3, C-5, C-8 and C-9 positions of retronecine (4) was observed. This result verified that two molecules of putrescine combine in the biosynthesis of retronecine.

Scheme 4



Use of this precursor led to broadening of the signals in the ^{13}C NMR spectrum due to ^{13}C-N-^{13}C and ^{13}C-C-^{13}C species. Therefore [1-^{13}C]putrescine (36) dihydrochloride was synthesised from *N*-protected 3-bromopropylamine (37). The retronecine produced by feeding to *S. isatideus* showed approximately 100% enhancement of the C-3, C-5, C-8 and C-9 positions of retronecine (38) (Scheme 5).

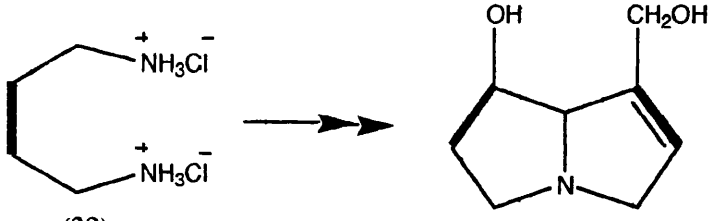
Scheme 5



The ^{13}C labelling patterns obtained in some experiments with [1-^{13}C]putrescine dihydrochloride were not easy to establish, as the enhancements were difficult to measure and the conditions had to be carefully controlled to limit the amount of unlabelled material present. A more effective method is to use ^{13}C-^{13}C doubly labelled precursors resulting in pairs of doublets around the natural abundance ^{13}C signal in the alkaloid.

Khan and Robins[37] used the procedure described in Scheme 4 to synthesis [2,3-$^{13}C_2$]putrescine dihydrochloride (39). After feeding this precursor to *S. isatideus* the retronecine hydrochloride (40) sample had two pairs of doublets of approximately equal intensity, corresponding to C-1 and C-2 and the C-6 and C-7 positions, with coupling constants of 71 and 34 Hz, respectively (Scheme 6).

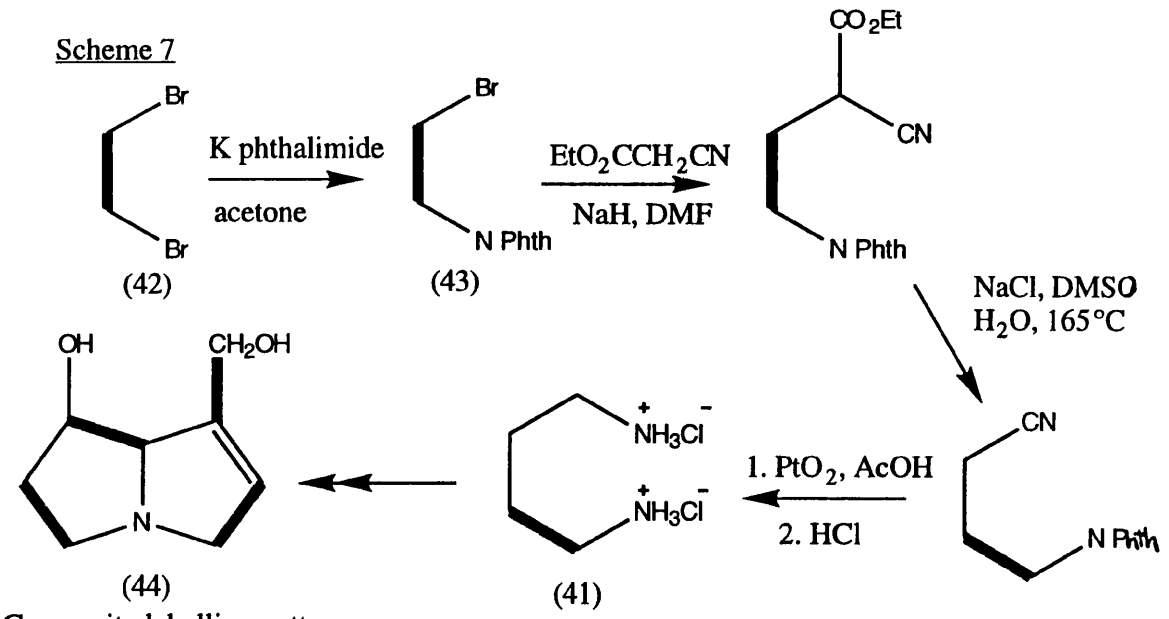
Scheme 6



(39) (40)

Robins also prepared [1,2-$^{13}C_2$]putrescine dihydrochloride (41) as shown in Scheme 7.[38] [1,2-$^{13}C_2$]-1,2-Dibromoethane (42) was converted into the mono-*N*-phthalimide derivative (43), and the other bromine was displaced with ethyl cyanoacetate. The ester function was removed, the nitrile was reduced and the phthalimide was hydrolysed to give the doubly labelled putrescine hydrochloride (41). Incorporation studies with *S. isatideus* gave retronecine (44) containing four pairs of doublets in the ^{13}C NMR spectrum with different coupling constants (Scheme 7).

Scheme 7

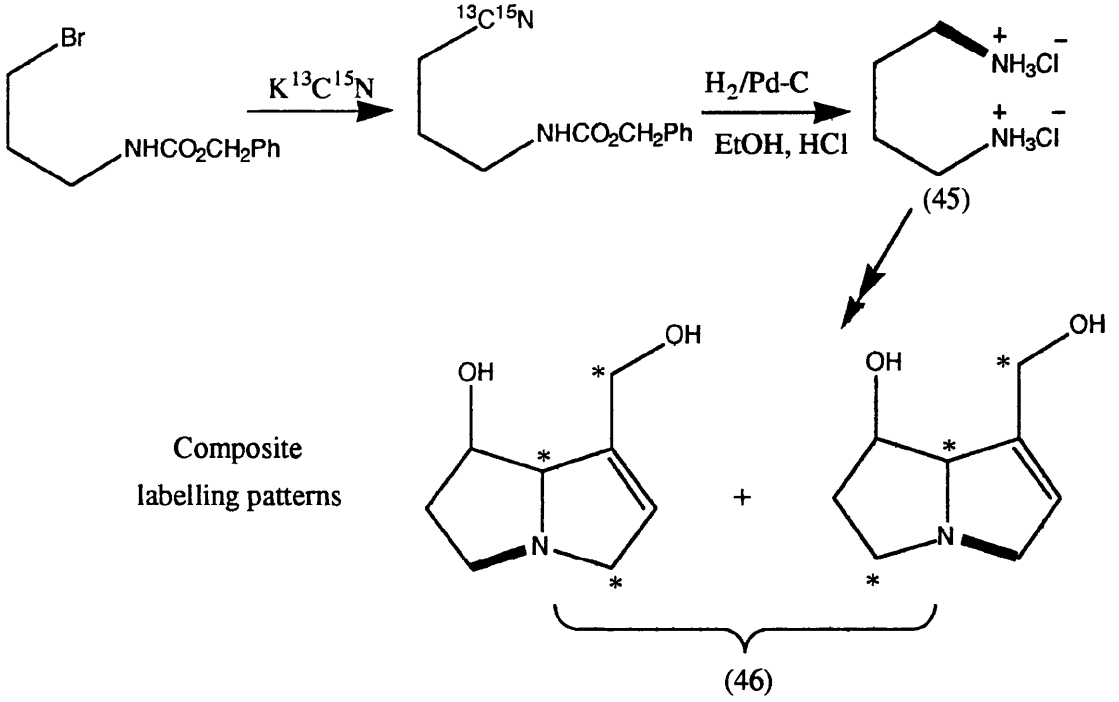


(42) (43) (41) (44)

Composite labelling pattern

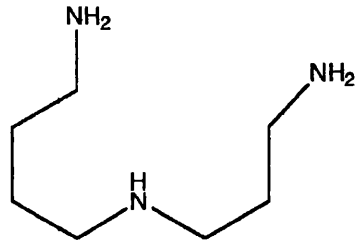
All the evidence indicated that two putrescine molecules form the necine via a later symmetrical intermediate. To determine which C-N bonds remain intact in retronecine, Robins and Khan[39] used the route described in Scheme 5 to prepare ^{13}C-^{15}N labelled putrescine (45). The ^{13}C NMR spectrum of the biosynthetically enriched retronecine (46) showed two doublets around the C-3 and C-5 positions of equal intensity. The asterisks indicate the enriched positions. This agreed with similar experiments by Grue-Sorensen and Spenser[40] with retronecine (46) obtained from *Senecio vulgaris* (Scheme 8).

Scheme 8



This is further evidence that there is a C_4-N-C_4 symmetrical intermediate produced during the biosynthesis of retronecine.

The identity of the C_4-N-C_4 intermediate seemed likely to be *N*-(4-aminobutyl)-1,4-diaminobutane (homospermidine) (47), which has been found in other plants.[41]

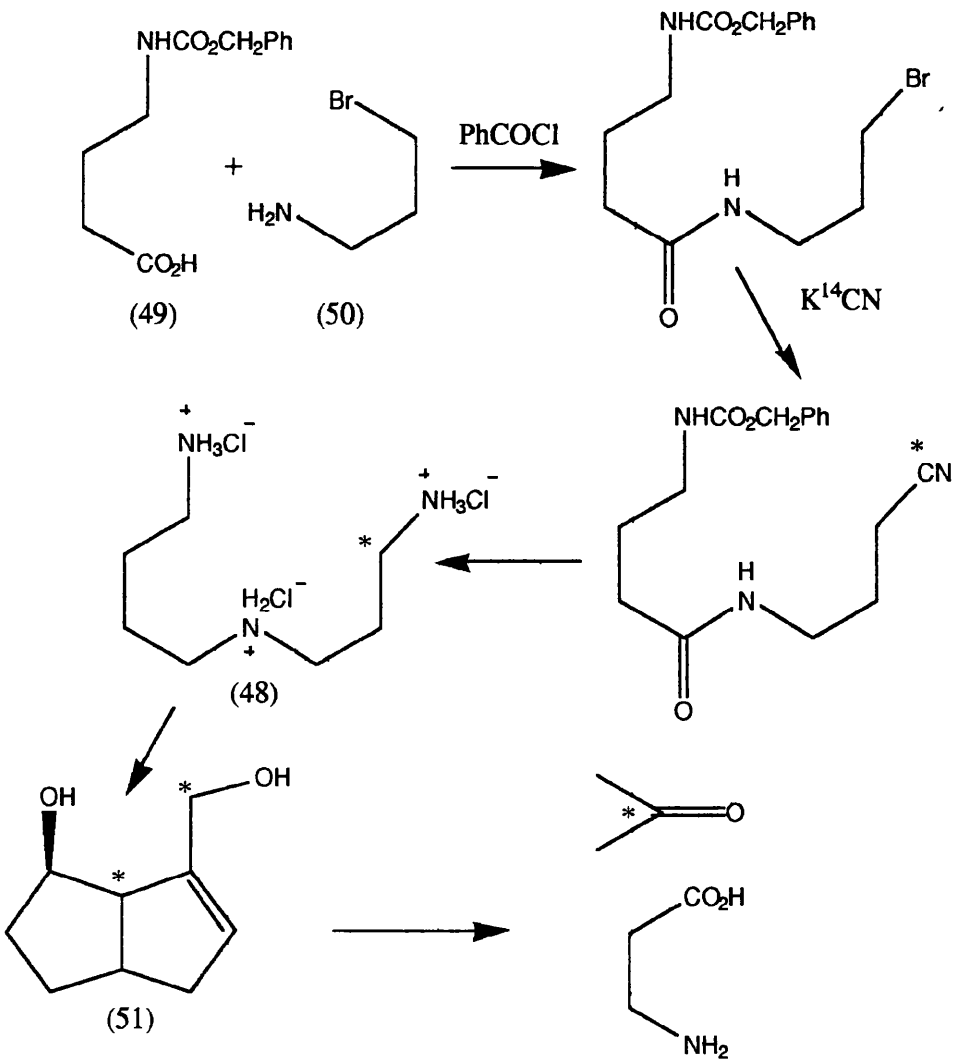


(47)

Khan and Robins[42] prepared [1,9-^{14}C]homospermidine trihydrochloride (48) from *N*-protected 4-aminobutanoic acid (49) and 3-bromopropylamine (50) via the mixed anhydride method (Scheme 9). The radiolabel was introduced by ^{14}C-cyanide displacement of bromine. Removal of the protecting group, reduction of the nitrile and amide, and acidification gave the labelled

homospermidine trihydrochloride (48). The precursor was incorporated (0.5%) into retrorsine which was hydrolysed to retronecine (51) which contained most of the radioactivity. After the degradations described earlier were carried out (Scheme 3), 44% of the activity was found to be at the C-9 position and virtually none in the C-(5+6+7) fragment, consistent with the labelling pattern shown (51).

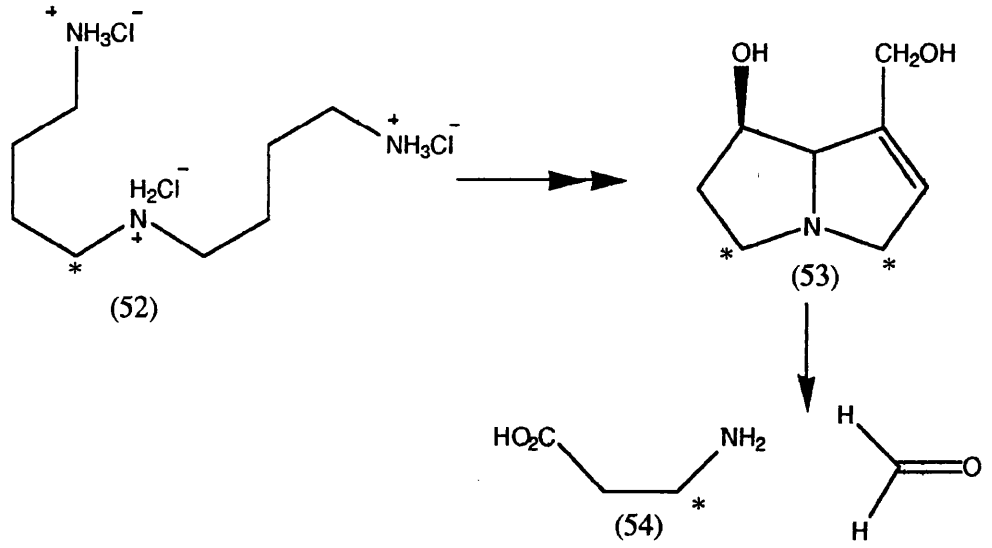
Scheme 9



[4,6-^{14}C]Homospermidine trihydrochloride (52) was also prepared by Khan and Robins[42] and was incorporated into retrorsine (0.7%). The alkaloid was hydrolysed to retronecine (53) containg most of the radioactivity and, after carrying out the degradations previously described, 46% of the activity was located in the C-(5+6+7) fragment

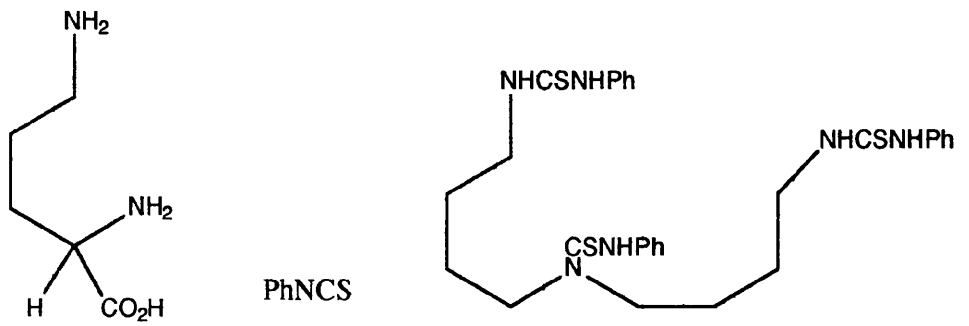
(54), with little being found in the C-9 position of retronecine. This agrees with the labelling pattern (53) proposed (Scheme 10).

Scheme 10



(52) (53) (54)

Khan and Robins[42] proved the intermediacy of homospermidine (47) with an intermediate trapping experiment. DL-[5-^{14}C]Ornithine (55) was fed to *S. isatideus*, and the plant was extracted after one day and inactive homospermidine was added to the extract. The extract was derivatised with isothiocyanatobenzene (56) and the derivative (57) was recrystallised to constant activity (0.5% of the precursor activity). This proves that homospermidine (47) is present in the plant and can be formed from ornithine (22). Birecka *et al.*[43] have also identified homospermidine in *Heliotropium indicum.*

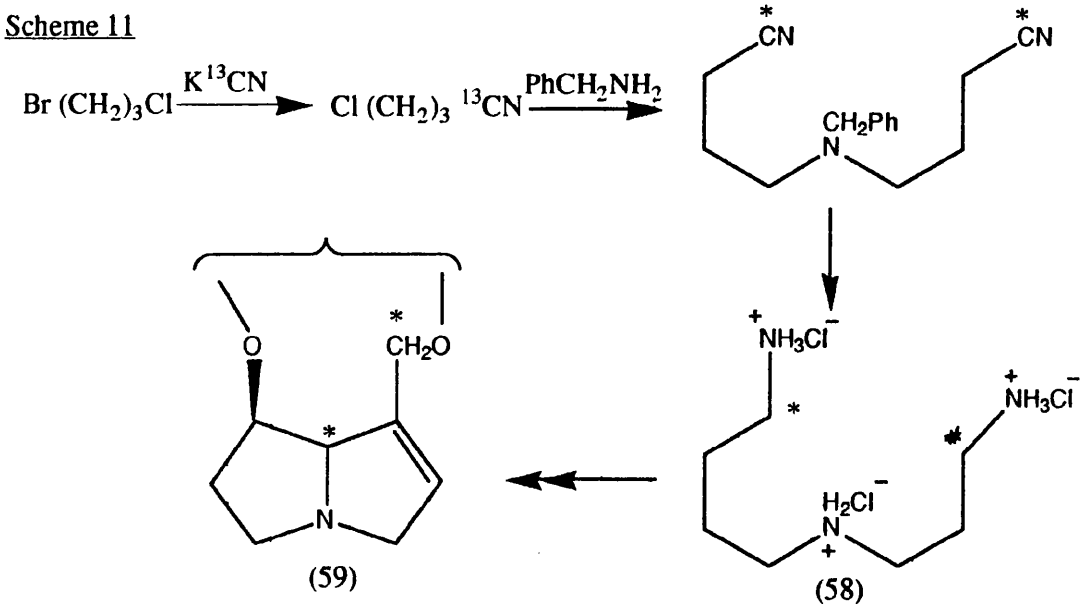


(55) (56) (57)

Studies with stable isotopes have also supported the role of homospermidine (47) as a precursor to necines. [1,9-

$^{13}C_2$]Homospermidine trihydrochloride (58) was prepared from 1-bromo-3-chloropropane (59) by Rana and Robins[44] (Scheme 11).

Scheme 11

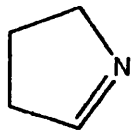


The precursor was fed to *S. isatideus* and retrorsine was extracted. The ^{13}C NMR spectroscopic analysis on the alkaloid showed doublets of approximately equal intensity around the signals for C-8 and C-9. The geminal coupling constant of 6 Hz and lack of any other enriched signals proves that homospermidine is incorporated intact into retronecine.

Kelly and Robins prepared [1,9-$^{13}C_2$]homospermidine trhydrochloride (58) and fed it to *Senecio pleistocephalus* and observed about 100% enrichment of C-8 and C-9 of rosmarinine (59).[45]

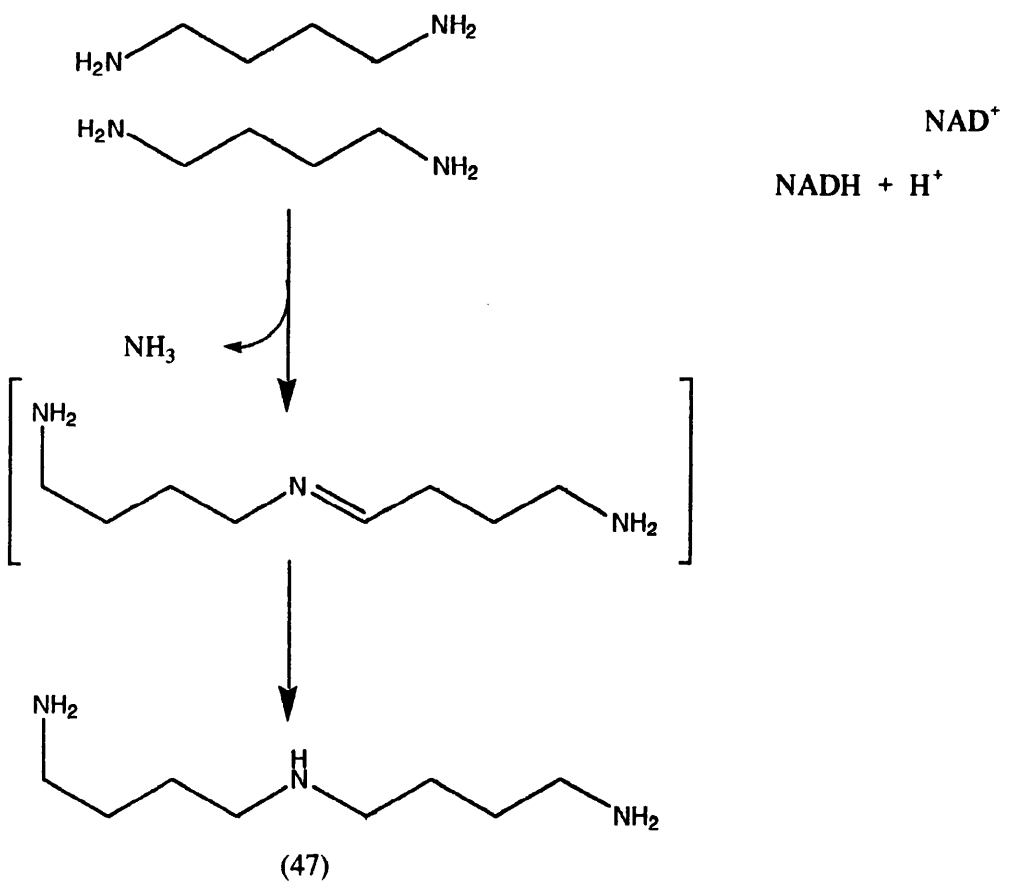
The formation of homospermidine from putrescine could involve three possible known enzymes: (a) diamine oxidase; (b) diamine transaminase; or (c) homospermidine synthase. Hartmann and co-workers have isolated (c) from root cultures of *S. vulgaris* and *Eupatorium cannabinum*.[46] They also eliminated the possibility of diamine oxidase and diamine transaminase being involved in the formation of homospermidine (47), and demonstrated that 1-

pyrroline (60) is not an intermediate in the formation of homospermidine (Scheme 12).



(60)

Scheme 12

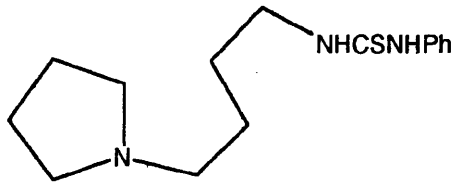


(47)

It is likely, however, that diamine oxidases are involved in the formation of the pyrrolizidine ring system from homospermidine. Robins[47] demonstrated that this was possible by incubating homospermidine with pea seedling diamine oxidase. After one week, reduction of the mixture by enzymic or chemical means yielded trachelanthamidine (61), showing that conversion of homospermidine into 1-hydroxymethylpyrrolizidine (1) is possible under physiological conditions. The likely pathway is shown in Scheme 13.

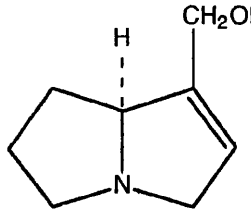
and 4.5% respectively). The $^{3}H/^{14}C$ ratios of the precursor compared to the alkaloid decreased in both experiments, indicating that the iminium ion is incorporated more efficiently than putrescine.

The iminium ion was also shown to be formed from putrescine in *S. pleistocephalus* by an intermediate trapping experiment, performed by Kelly and Robins.[48] One day after feeding [1,4-^{14}C]putrescine dihydrochloride to one plant, extraction of the amines, reduction of the mixture with sodium borohydride and reaction with isothiocyanatobenzene (56), gave the derivative (66) which was recrystallised and shown to contain 0.4% of the activity fed.

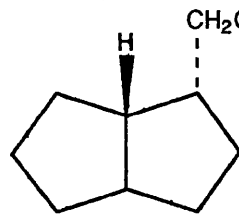


(66)

The intermediacy of the iminium ion (62) has also been demonstrated for heliotridine in *Cynoglossum officinale*, and for (+) isoretronecenol (67) and (+) supinidine (68) from *C. australe*.[49]



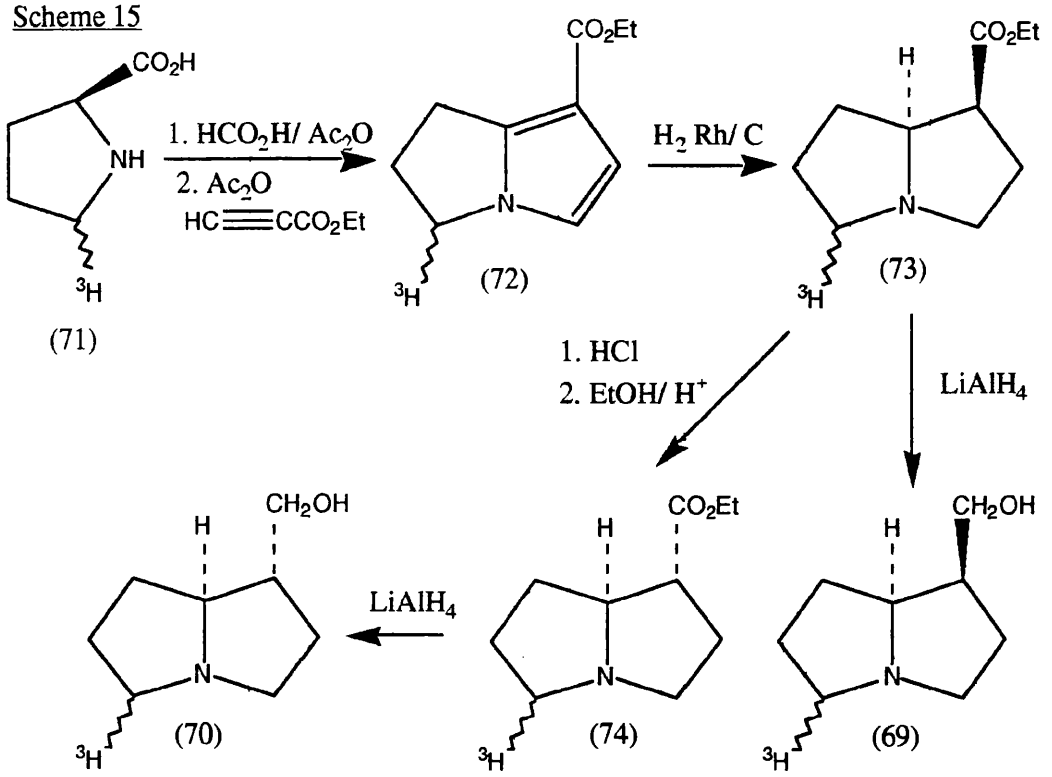
(67)



(68)

Kunec and Robins prepared ^{3}H-labelled 1-hydroxymethylpyrrolizidines (69), (70) (Scheme 15), starting from L-[5-^{3}H]-proline (71).[50]

Scheme 15



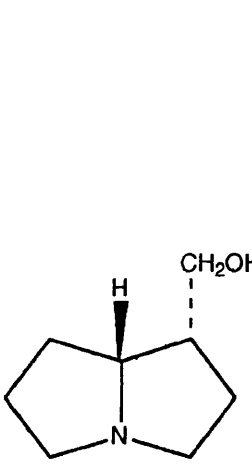
The proline (71) was *N*-formylated, and then the product underwent a 1,3-dipolar cycloaddition with ethyl propiolate. Hydrogenation of the pyrrole (72) gave the *endo*-pyrrolizidine ester (73), which was treated with lithium aluminium hydride to give [5-^{3}H]isoretronecacanol (69). [5-^{3}H]Trachelanthamidine (70) was prepared by epimerisation of the *endo*-ester (74), under acidic conditions, to the *exo*-acid which is thermodynamically more stable, then re-esterified. Reduction gave the ^{3}H- labelled precursor (70).

Various feeding experiments showed that trachelanthamidine (61) is a good precursor for retronecine (4) and heliotridine (11) but isoretronecanol (75) was incorporated far better into rosmarinine (8).[50, 51] Leete and Rana[52] also demonstrated the incorporation of trachelanthamidine into riddelline (76) in *Senecio riddelli.*

[5-^{3}H]Isoretronecanol (69) has also been shown to be incorporated well into cynaustraline (77) and cynaustine (78) in *Cynoglossum australe*, but trachelanthamidine (61) showed no incorporation into these alkaloids.[53] Hagan and Robins obtained

radioactive samples of (+)-isoretronecanol (67) and (+)-supinidine (68) by feeding [1,4-^{3}H]putrescine dihydrochloride to *C. australe*.[53] In turn, feeding experiments showed that (+)-isoretronecanol (67) is incorporated is incorporated into cynaustraline (77) and cynaustine (78) but (+)-supinidine (68) is incorporated only into cynaustine (78).

These results suggest that the biosynthetic pathway diverges in the formation of the necine bases. (+)-Supinidine (68) is formed from isoretronecanol after the pathway has split.



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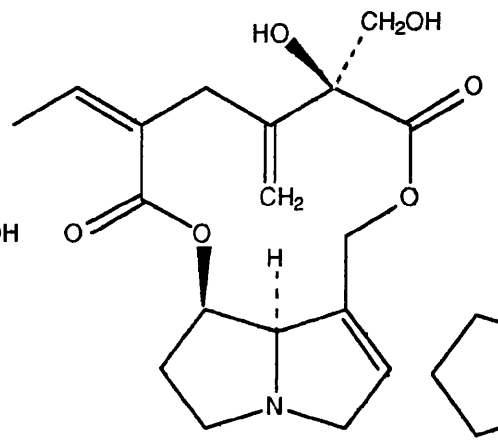
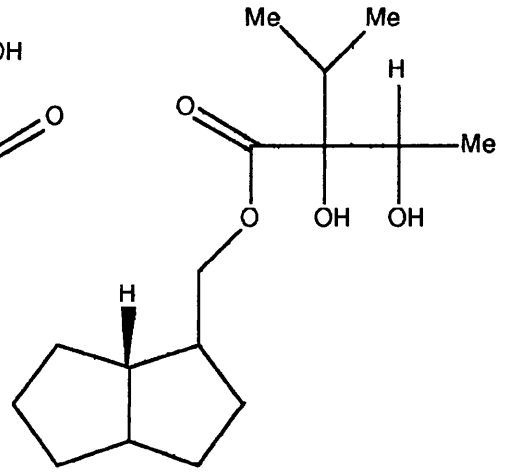
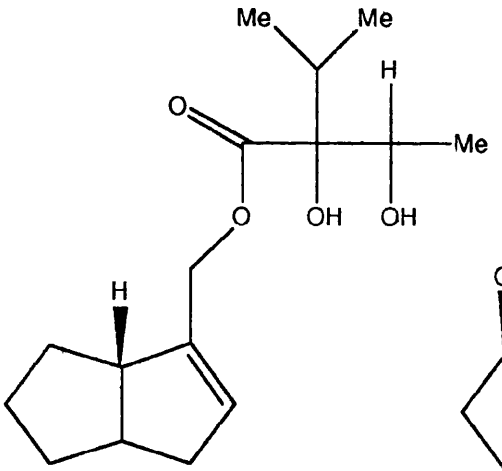


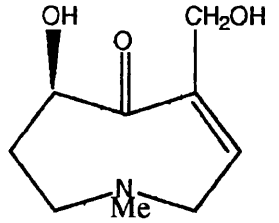
(76)



(77)

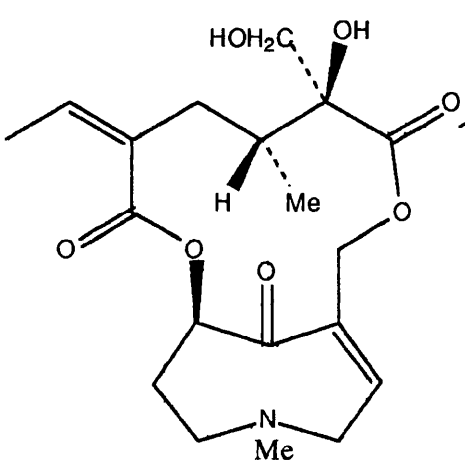


(78)

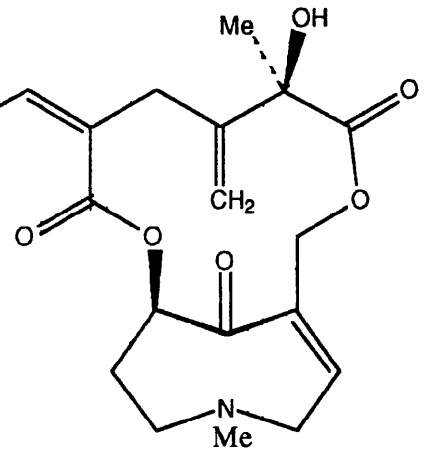


(79)

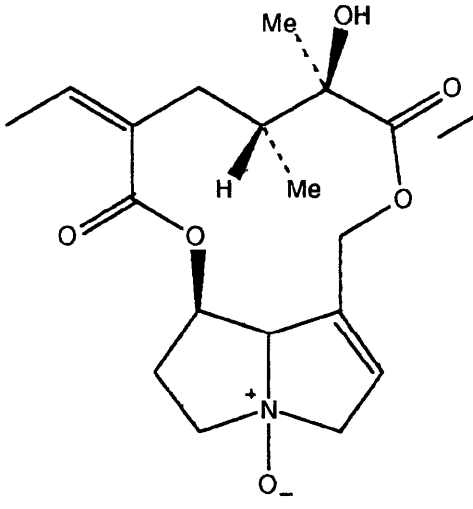
Another common base portion of pyrrolizidine alkaloids is otonecine (79), present in senkirkine (80), emiline (81) and syneilesine (82). Hartmann and co-workers[54] observed that the amount of senkirkine present in *Senecio vernalis* root cultures increased as the amounts of senecionine *N*-oxide (83) present decreased. Robins and co-workers showed that putrescine (24), homospermidine (47), the iminium ion (62), trachelanthamidine (61) and retronecine (4) were all precursors to otonecine (79).[55] The C-8 to N-4 band must be broken late in the biosynthetic pathway, possibly after hydroxylation at C-8 and methylation of the nitrogen, allowing cleavage of the bicyclic ring (Scheme 16).



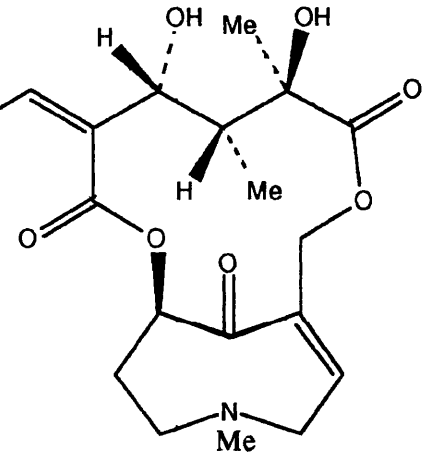
(80)



(81)

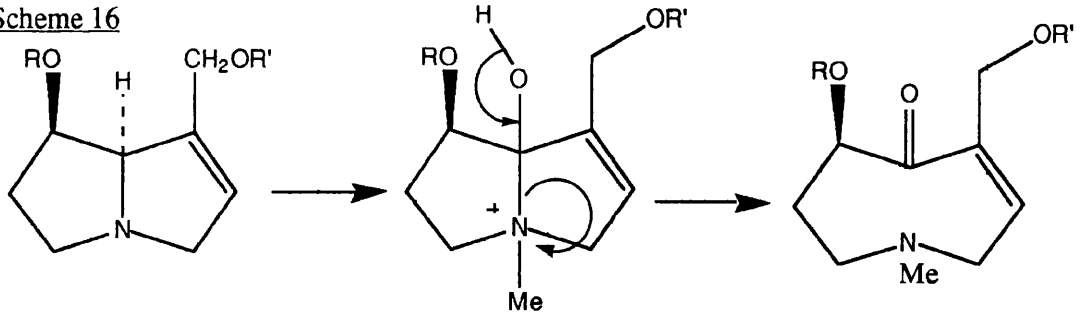


(83)



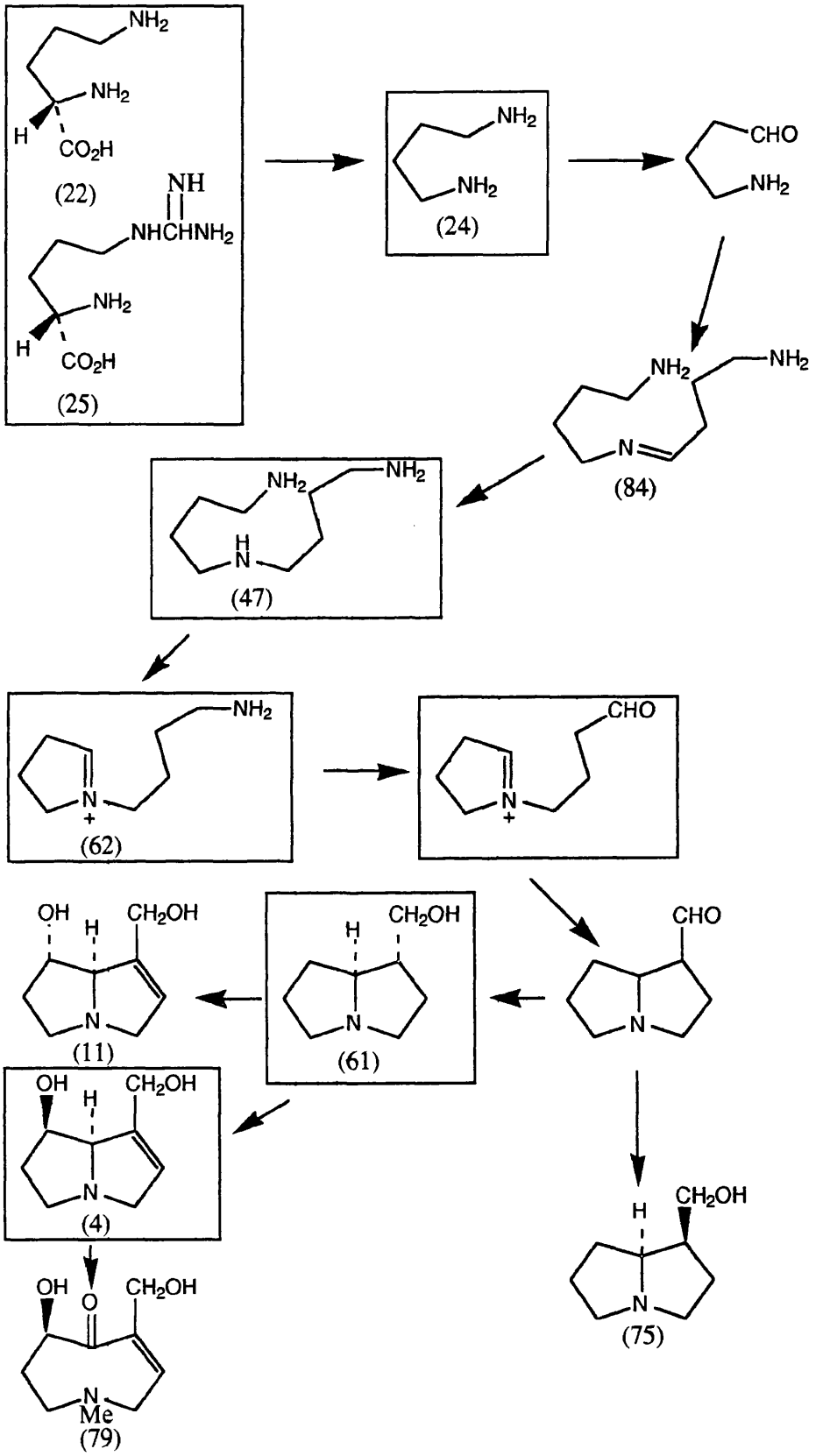
(82)

Scheme 16



As a result of the comprehensive studies of necine biosynthesis the pathways have been established. These are shown in Scheme 17 with the known intermediates in boxes. L-Ornithine (22) undergoes decarboxylation to putrescine (24). Homospermidine synthase oxidises one of the primary aldehyde groups of putrescine to the aldehyde, which combines with another molecule of putrescine to give the imine (84), which is reduced to homospermidine (47). Oxidation of a primary amine function of homospermidine, again yields an aldehyde which undergoes cyclisation to the iminium ion (62). The remaining primary aldehyde is oxidised to the aldehyde and this iminium ion can undergo cyclisation to give either isoretronecanol (75) or trachelanthamidine (61). Hydroxylation of trachelanthamidine and elimination forms retronecine (4) and heliotridine (11). Otonecine (79) can be formed from retronecine (4) as previously described.

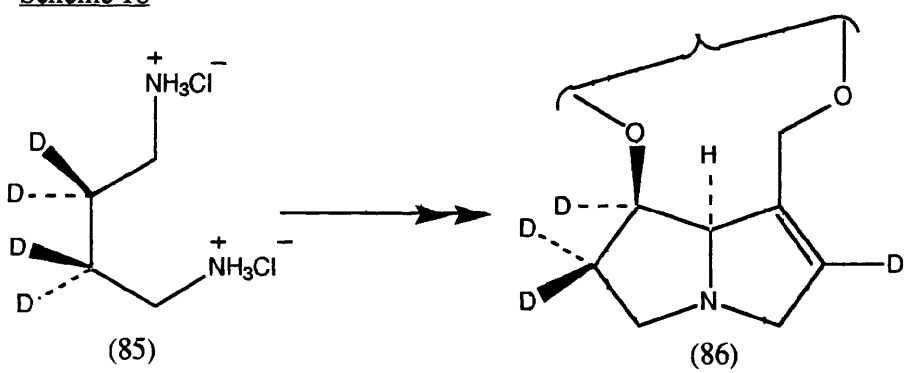
Scheme 17


NH2
NH2
H
CO2H
(22)
NH
NHCHNH2
NH2
H
CO2H
(25)
NH2
NH2
(24)
CHO
NH2
NH2
NH2
N
(84)
NH2
NH2
H
N
(47)
NH2
N+
(62)
CHO
N+
OH
H
CH2OH
N
(11)
H
CH2OH
N
(61)
CHO
N
OH
H
CH2OH
N
(4)
H
CH2OH
N
(75)
OH
O
CH2OH
N
Me
(79)

The stereochemistry of the biosynthesis of necines has also been investigated. Firstly Robins found that only the L-isomers of ornithine (22) and arginine (25) are precursors of necines,[56] and several groups showed that the decarboxylation step proceeds with retention of configuration for both amino acids.[57, 58, 59]

[2,2,3,3-$^2H_4$]Putrescine hydrochloride (85) was fed to *S. isatideus* by Rana and Robins.[60] ^2H-Labelling patterns were established by ^2H NMR spectroscopy. The H-2, H-6α, H-6β and H-7α positions of retrorsine (86) were labelled equally. The retention of ^2H at H-7α excludes the involvement of keto or enol intermediates during the hydroxylation process at C-7 (Scheme 18).

Scheme 18

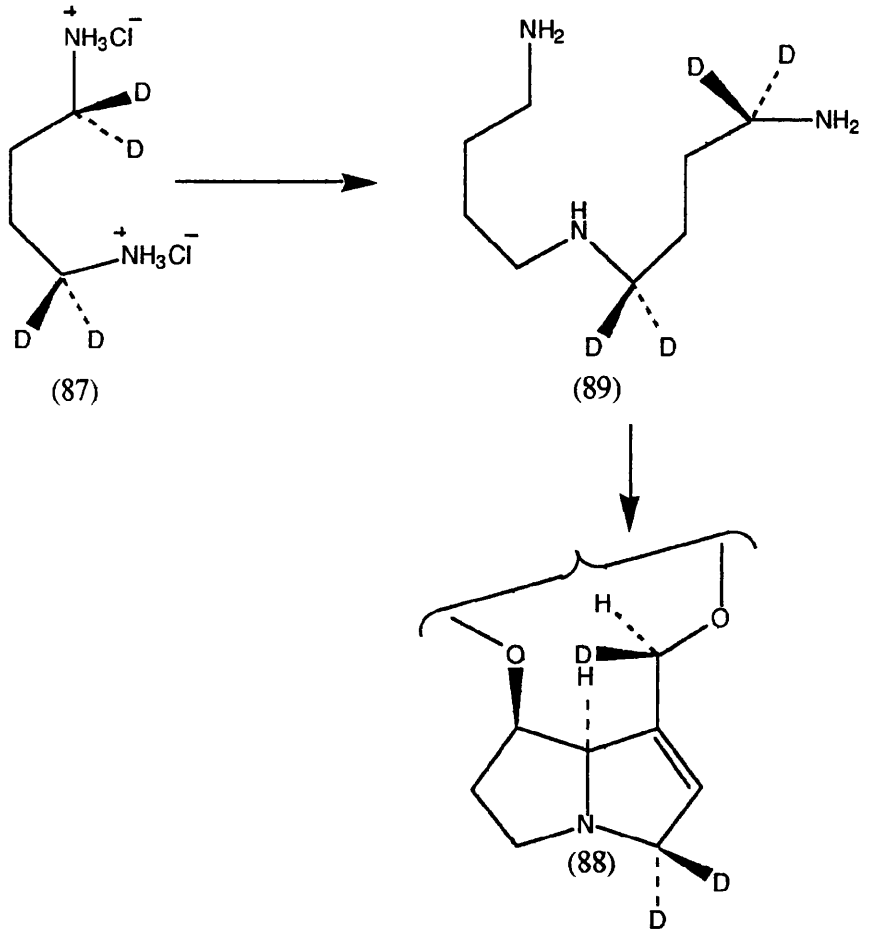


(85) (86)

A similar experiment with [1,1,4,4-$^2H_4$]putrescine dihydrochloride (87) showed almost exclusive labelling of the right hand ring of retrorsine (88), in the H-3α, H-3β, and H-9 *pro-S* positions. This can be explained via the following route (Scheme 19). The combination of the labelled putrescine (87) with unlabelled material would give the $^2H_4$-labelled homospermidine (89). Intramolecular ^2H isotope effects during oxidation of (89) to the aldehyde would favour the formation of the left hand ring from the unlabelled half, with the labelling mostly in the right hand portion. Also the retention of deuterium in the H-9 *pro-S* position of retrorsine (88) indicates that the reduction of the pyrrolizidine aldehyde to the alcohol must

proceed with the hydride addition to the *re*-face of the carbonyl function (Scheme 19).

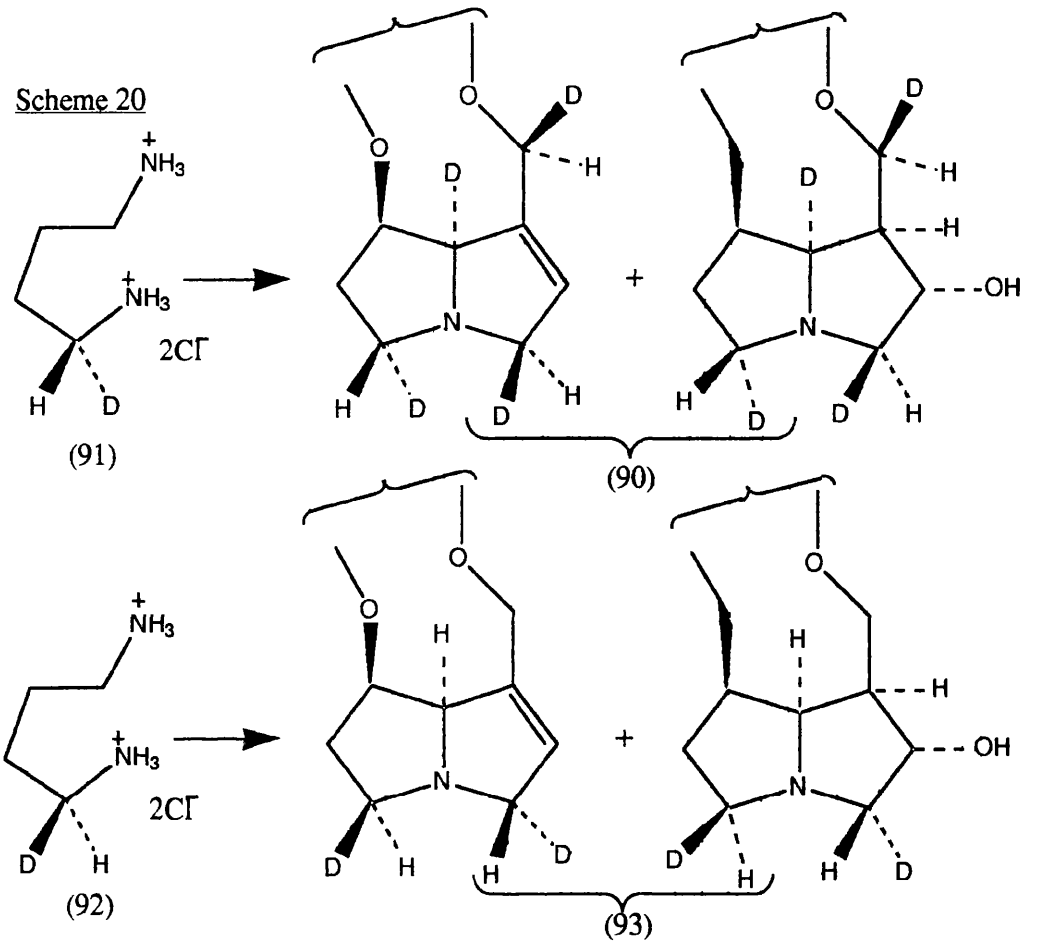
Scheme 19



Independently the groups of Spenser[61] and Robins[62] prepared *(R)*- and *(S)*-[1-^{2}H]putrescine dihydrochloride. Robins and co-workers fed this labelled putrescine to *S. isatideus*. The sample of retrorsine (90) obtained from feeding the *(R)*-isomer (91) had equal labelling in the H-3β, H-5α, H-8α and H-9 *pro-S* positions (Scheme 20). Feeding of the *S*-isomer (92) resulted in the labelling of only the H-3α and H-5β of retrorsine (93). These results show that the oxidation of the primary amines to aldehydes in putrescine and homospermidine occur with stereospecific loss of the *pro-S* hydrogens. The reduction of the imine (84) to homospermidine must proceed via hydride addition to the C-*si*-face of the imine. Incorporation studies with the same precursor into*S. vulgaris* by

Grue-Sorensen and Spenser gave results which agreed with these findings (Scheme 20).

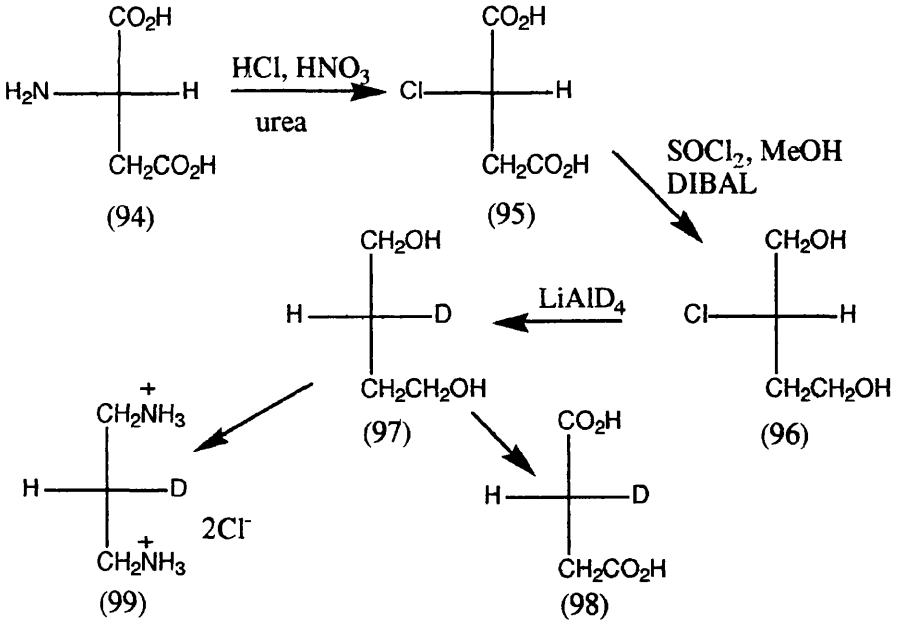
Scheme 20



Composite labelling patterns

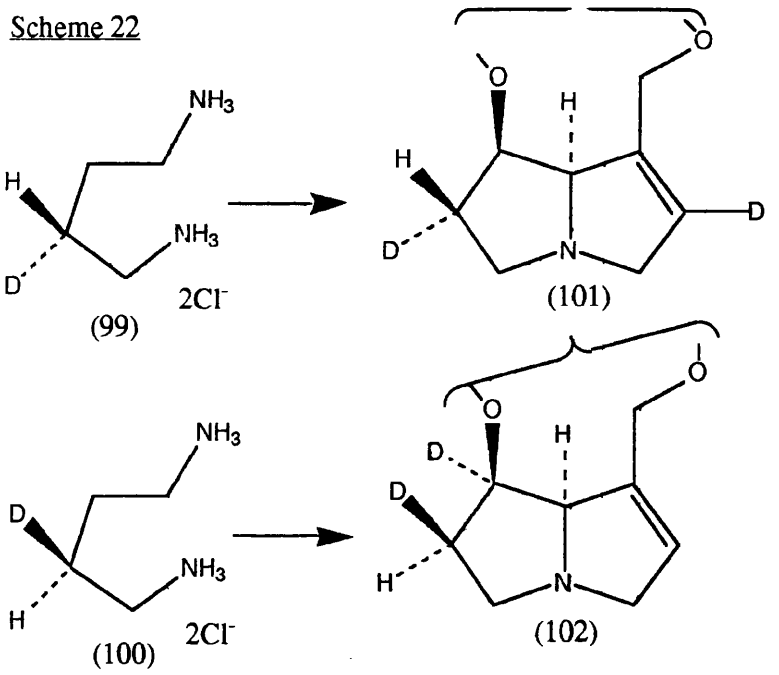
Kunec and Robins[63] also prepared *(R)*- and *(S)*-[2-^{2}H]putrescine dihydrochloride (Scheme 21). Starting from L-aspartic acid (94), *(S)*-2-chlorosuccinic acid (95) was formed with retention of configuration. The diacid was esterified then selectively reduced to the diol (96). Deuteride displacement of the chloride gave the diol (97). Chromic acid oxidation of this material resulted in *(R)*-[2-^{2}H]succinic acid (98) for literature comparison, while the diol was also converted into (*R*)-[2-^{2}H]putrescine dihydrochloride (99). The corresponding *(S)*-isomer (100) was prepared from D-aspartic acid (Scheme 21).

Scheme 21



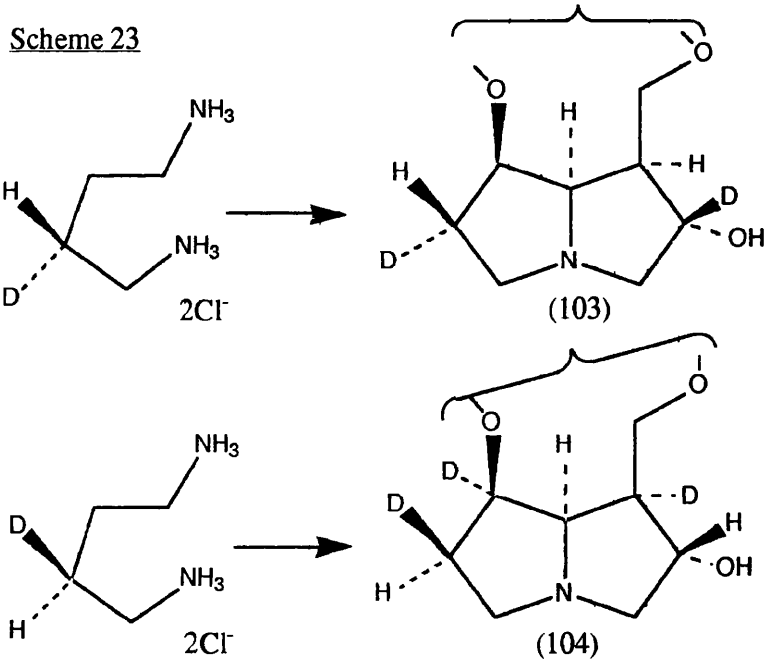
These two precursors were fed to *S. isatideus*. Feeding of the *(R)*-isomer resulted in labelling of the H-2 and H-6α positions of retrorsine (101), while the H-6b and H-7a positions of the alkaloid (102) were labelled from the *(S)*-isomer (Scheme 22). This implies that hydroxylation at C-7 occurs with retention of configuration, and that the unsaturation must be introduced with the retention of the *pro-R* hydrogen at position C-2 of retrorsine.

Scheme 22



Kelly and Robins[64] also fed *(R)*- and *(S)*-[2-^{2}H]putrescine dihydrochloride to *S. pleistocephalus*. Deuterium was incorporated into H-2β and H-6α of rosmarinine (103) after feeding the *(R)*-isomer, while the *(S)* isomer labelled the H-1α, H-6β, and H-7α positions in rosmarinine (104) (Scheme 23). These results show that hydroxylation at C-2 also proceeds with the retention of configuration. Furthermore, at the C-1 position the *pro-S* hydrogen is retained and the *pro-R* is lost when the pyrrolizidine ring is formed.

Scheme 23



Robins also studied the base portion otonecine, produced by *Emilia flammea*.[65] With the exception of the necessary loss of deuterium when the keto group is formed at C-8, the labelling pattern was exactly analogous to that observed for retronecine. Introduction of the keto group and cleavage of the bicyclic ring does not involve the loss of deuterium from the surrounding sites.

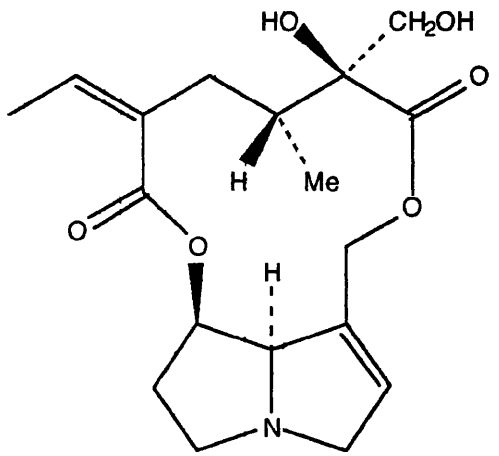
As a result of the extensive research on the necine bases most of the biosynthetic pathways and the stereochemistry of the enzymic processes have been established, and thus the labelling investigations into the biosynthesis of necines are nearly complete.

Chapter 2.2 Biosynthesis of Necic Acids

Although the study of the biosynthesis of the necine bases has been extensive, less work on the necic acid portions has been reported. All the research has involved the use of radiotracers. There have been no studies reported using stable isotopes.

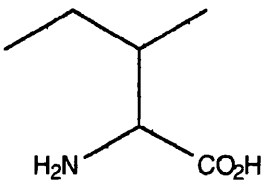
Necic acids are mono- or di-carboxylic acids containing between five and, most commonly, ten carbon atoms. They normally have branched chains and highly oxygenated structures. The fact that many consist of ten carbon atoms led to the original suggestion that they were terpene derived, despite the highly oxygenated nature of the necic acids.

However this theory was dispelled when Hughes and Warren showed that [2-^{14}C]mevalonolactone was not a specific precursor for the acid portion of retrorsine (7) in *Senecio isatideus*.[25] There were also many attempts to feed radiolabelled acetates to other *Senecio* species, but no conclusions could be drawn from the incomplete labelling patterns obtained.[25,26,66]

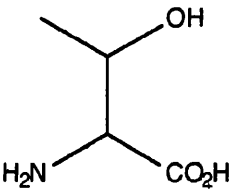


(7)

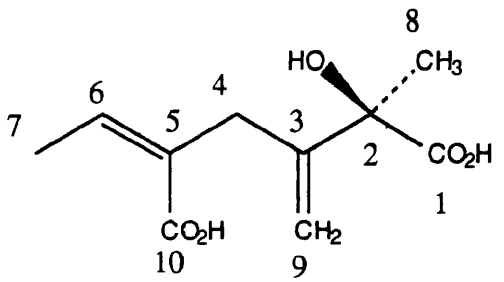
The next area to be investigated was the possibility that the necic acids are derived from α-amino acids. Crout *et al.* [26] demonstrated that isoleucine (14) and threonine (104) were incorporated into seneciphyllic acid (105), produced by *Senecio douglasii.*



(14)



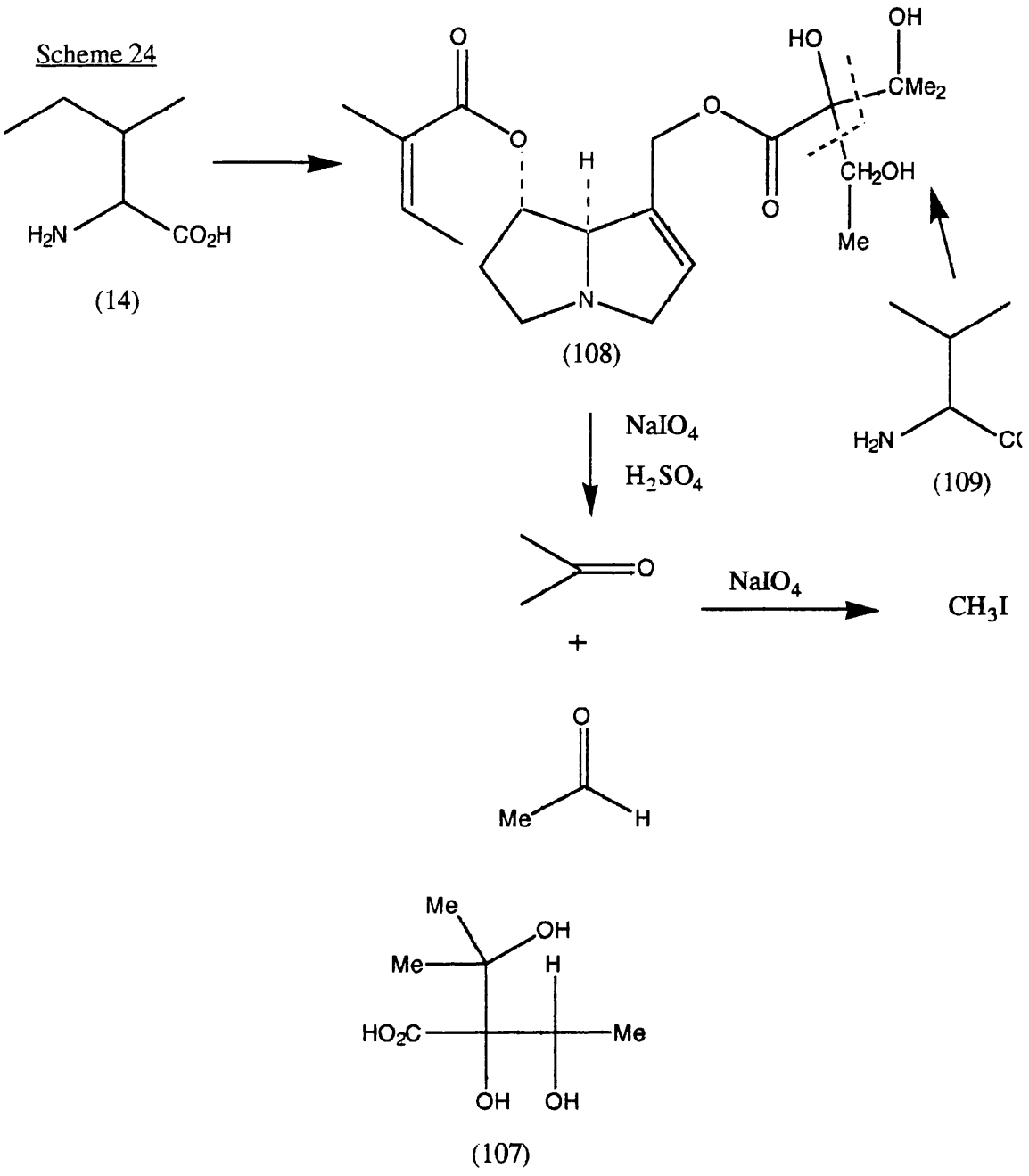
(104)



(105)

Crout also showed that DL-[4-^{14}C]-valine was incorporated to the extent of 0.246% into echimidinic acid (107), compared to 0.019% for [2-^{14}C]-acetate,[27] when fed to *Cynoglossum officinale.* The high rate of incorporation for valine indicates that it may be a precursor, and in support of this 85% of the radioactivity was found in the acid portion. Degradation studies on the heliosupine (108) showed that almost all of this activity was present in the acetone and iodoform (Scheme 24). It was concluded, therefore, that echimidinic acid (107) is formed from valine (109) with the incorporation of an additional C_2 unit.

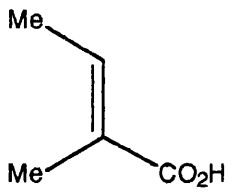
Scheme 24



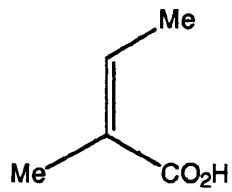
It was also demonstrated by Crout that the angelic acid portion of heliosupine (108) is derived from isoleucine.[28] L-[U-^{14}C]-Isoleucine was fed to *C. officinale* and was incorporated to the extent of 0.205%. This compared favourably with the 0.019% incorporation achieved with [2-^{14}C]-acetate. As 98% of this activity was found in the angeloyl ester portion of heliosupine

(108), isoleucine was shown to be a specific precursor for angelic acid.

Tiglic acid (110) is known to be formed from isoleucine in *Datura meteloides*.[67] McGaw and Wooley[68] found that [1-^{14}C]tiglic acid was specifically incorporated into the angelic acid portion of heliosupine. This suggests that tiglic acid (110) is converted into angelic acid (111) via a *cis-trans* isomerisation mechanism.



(110)

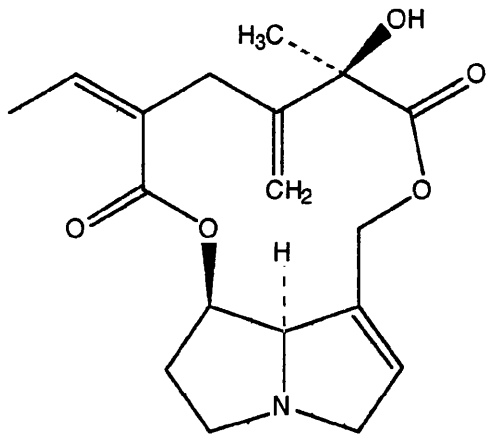


(111)

The feeding of [1-^{14}C]- and [2-^{14}C]-acetate to *Senecio douglasii* resulted in randomisation of the label between the acid and base portions,[26] ruling out the possibility of there being a direct route from acetate to seniciphyllic acid. [2-^{14}C]Mevalonate was incorporated to a small extent (0.025%), although more than half of the activity was incorporated into the acid portion. If [2-^{14}C] mevalonate was incorporated directly, half of the activity would be located in the C-1 and C-8 positions of seneciphyllic acid. Degradations showed that these positions contained 0.35% and 0.17% respectively of the total activity. Although any further degradation was impossible due to the low activity of the acid, the results obtained meant that the possibility of an acetate-mevalonate pathway could be dismissed.

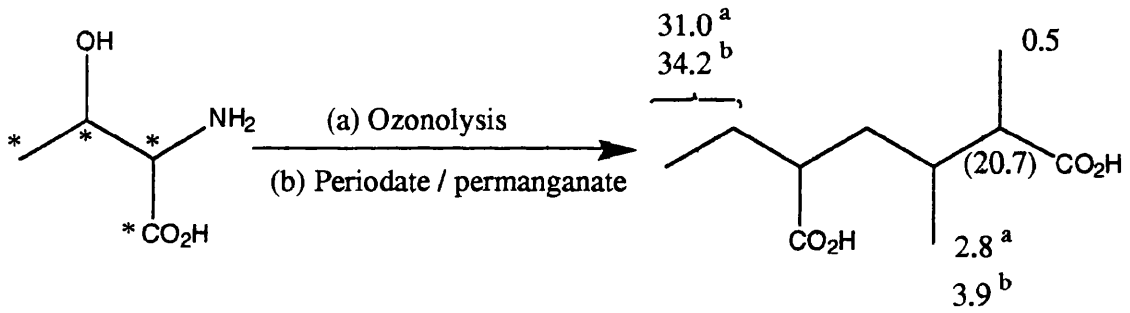
The incorporations of L-[U-^{14}C]isoleucine and L-[U-^{14}C]threonine were significantly higher into seneciphylline (112) than for acetate. Moreover virtually all the activity was located

in the acid portion. The seneciphyllic acid obtained from L-[U-^{14}C]-threonine (113) was degraded as shown (Scheme 25).



(112)

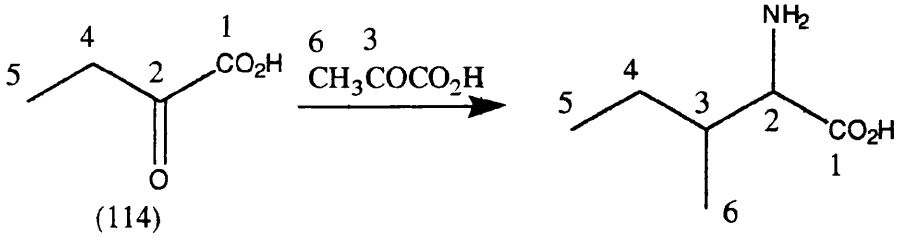
Scheme 25



The results show that the threonine is not incorporated in an equal fashion into each half of seneciphyllic acid. When it was discovered that [1-^{14}C]isoleucine was incorporated with one tenth of the efficiency of [U-^{14}C]isoleucine, and with dramatically reduced specificity, it became clear that the carboxyl group of isoleucine is lost during biosynthesis.

A major pathway in the metabolism of threonine leads to α-ketobutyric acid (114),[69] which is also involved in isoleucine biosynthesis with the other two carbons coming from pyruvate (Scheme 26).

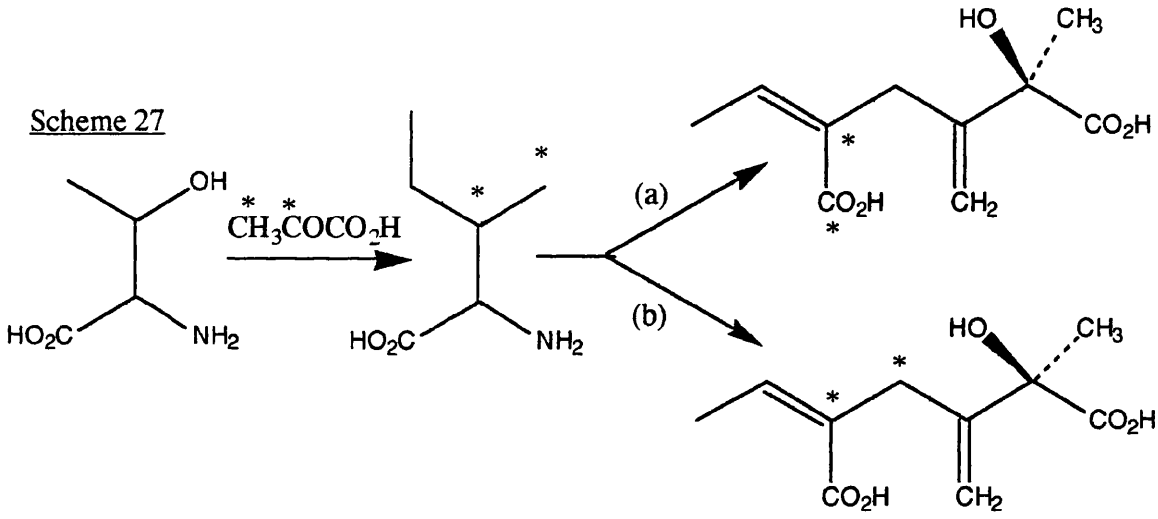
Scheme 26



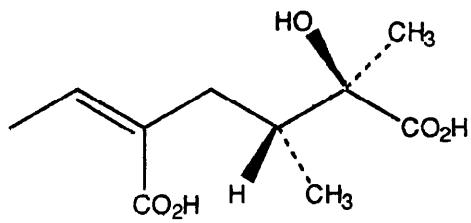
(114)

If threonine is incorporated via isoleucine two pathways to seneciphyllic acid are possible (Scheme 27).

Scheme 27



Studies on the structurally similar senecic acid (115) indicated an even distribution of label between both halves of the necic acids.[70]



(115)

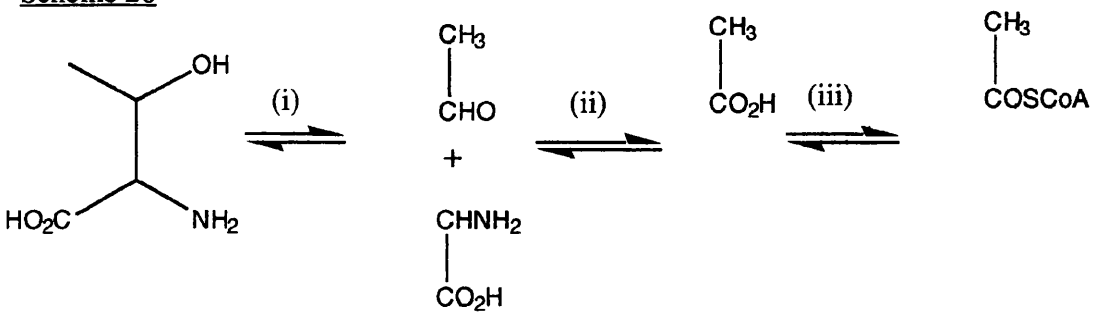
Also, [2-^{14}C]- and [6-^{14}C]-isoleucine were shown to be precursors of senecic acid (115). The former labelled C-1 and C-10 exclusively and equally, and 58% of the activity from the

latter isoleucine feed was located at C-8 of senecic acid. This is consistent with path (b) of Scheme 27. Path (a) was previously preferred due to increased labelling of C-6/C-7 over C-10 of seneciphyllic acid after feeding [U-^{14}C]threonine to *S. douglasii.*

It is known, however, that a uniformly labelled threonine can be modified *in vivo* to introduce an inequality of labelling at C-1/C-2 and C-3/C-4.

Threonine aldolase is known to exist in certain higher plants.[71] The action of threonine aldolase, acetaldehyde dehydrogenase and acetyl CoA synthase is shown in Scheme 28. If any degree of reversibility can exist in the system, the distribution of the label will depend on the equilibria and the existing amounts of glycine and acetyl CoA. The observed uneven distribution of labelling could be explained by equilibration of the C-1 and C-2 portion with a pool of unlabelled glycine greater than the pool of acetyl CoA.

Scheme 28

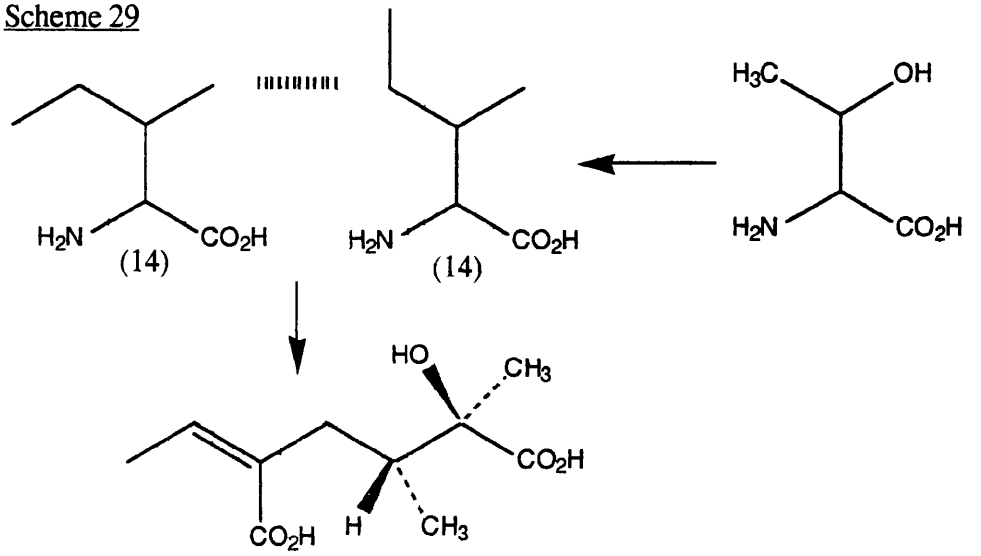


(i) threonine aldolase
(ii) acetaldehyde dehydrogenase
(iii) acetyl CoA synthetase

Crout and co-workers[70] completed an extensive study of the biosynthesis of senecic acid (115), the acid portion of senecionine

(26) produced by *Senecio magnificus*. Various labelled isoleucines and threonines were synthesised containing ^{14}C. The labelling patterns obtained by degradations demonstrated that senecic acid was formed by the combination of two C-5 units derived from isoleucine (14), with the loss of the carboxyl groups from each isoleucine moiety, in the manner shown in Scheme 29.

Scheme 29



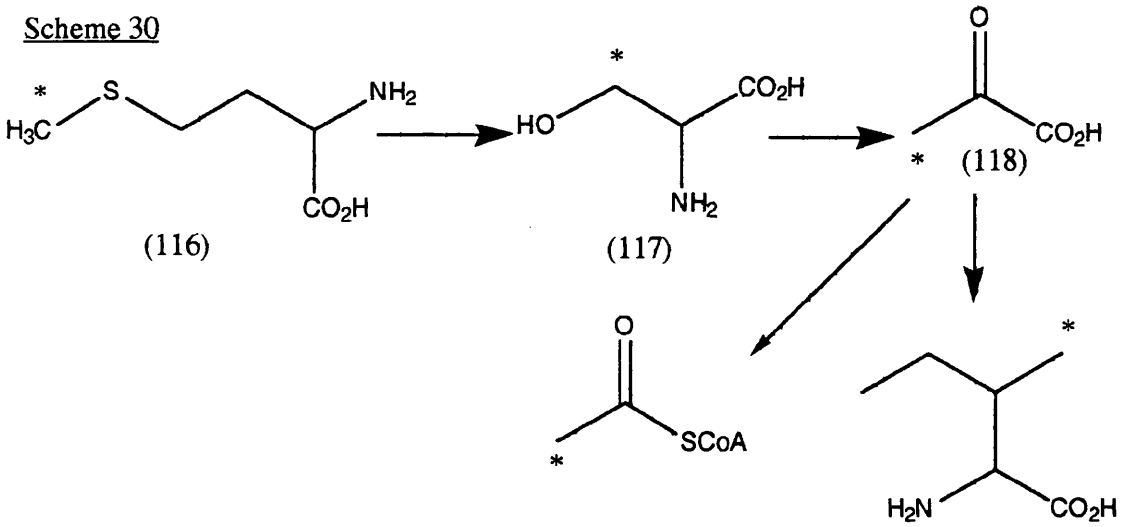
(115)

It was also shown that of the four stereoisomers of isoleucine, only L-isoleucine is incorporated in senecic acid biosynthesis.[72]

The *S*-methyl group of L-[Me-^{14}C]methionine is known to be preferentially incorporated into the necic acid portion of seneciphylline (112),[70] with between 22 and 26% of the activity located in the C-8 position of seneciphyllic acid (106), although the total incorporation is low. The C-8 atom of the necic acids is derived from C-6 of isoleucine, which arises from the methyl carbon of pyruvate.

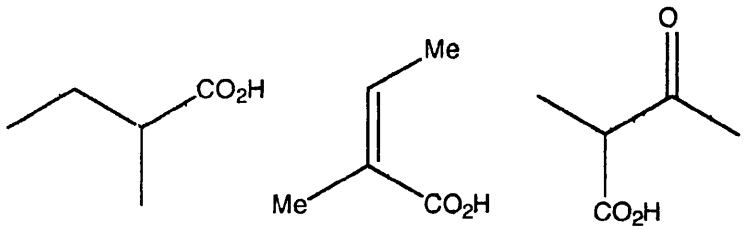
As there is evidence[73] that the S-methyl of methionine (116) can be converted into the hydroxymethyl group of serine (117), which is in turn transformed into pyruvate (118) *in vivo*, it seems possible that the methyl group of methionine enters the biosynthetic pathway to isoleucine by conversion into the hydroxymethyl group of serine (Scheme 30).

Scheme 30



It is also known that the label from [Me-^{14}C]methionine is incorporated specifically into the hydroxymethyl group of serine (118) in pea seedlings.[70]

The mode of coupling between the C-4 and C-6 carbons was then examined. Crout and co-workers[74] attempted to identify the five carbon intermediate formed from isoleucine in the biosynthesis of senecic acid. However feeding experiments with 2-methylbutanoic acid (119), angelic acid (111) and 2-methyl-3-oxobutanoic acid (120) showed that these compounds were not specific precursors for senecic acid.



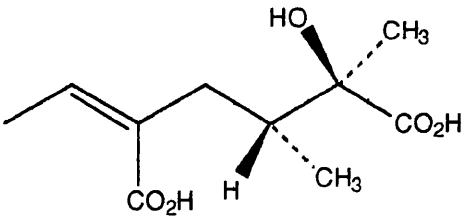
(119) (111) (120)

The coupling mechanism was also studied, through feeding experiments with [6-^{3}H]- and [4-^{3}H]-isoleucine on *S. magnificus* and *S. isatideus.* [74]

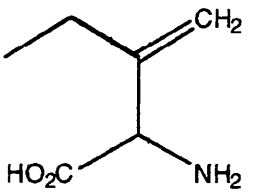
[6-^{3}H, 6-^{14}C]Isoleucine was prepared and fed to *S. magnificus.* When the ^{3}H:^{14}C ratio of the labelled alkaloid was compared with that of the precursor, it was observed that approximately 5/6 of the tritium was retained.

The evidence cannot be unambiguously interpreted due to possible isotope effects, but it is clear that one hydrogen of C-6 in isoleucine is retained during conversion into C-14 of senecionine (26) and it is probable that two are retained.

When L-[4-^{3}H, U-^{14}C]isoleucine was fed to *S. isatideus,* the results showed that half the tritium was retained in isatinecic acid (121). This limits the oxidation level to which C-4 of the isoleucine precursor is raised to that of a carbinol or a vinylic methine group. It definitely excludes the involvement of a carbonyl function in the coupling mechanism, and dismisses the intermediacy of 2-methyl-3-oxobutanoate. The vinyl methine functionality appeared particularly attractive, as the corresponding amino acid, β-methylene norvaline (122) is known in nature, as a metabolite of the fungus *Lactarius helvus.*[75]



(121)



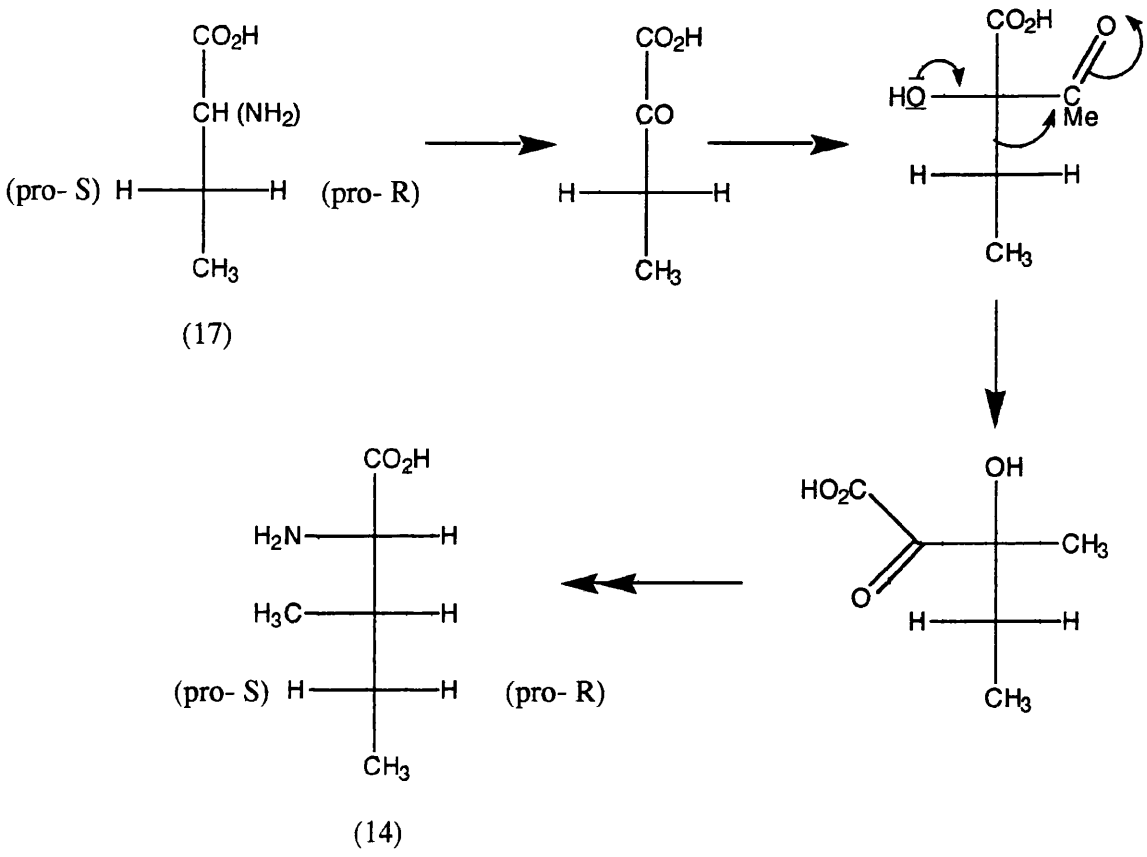
(122)

The feeding of DL-β-[$^3H_2$]methylenenorvaline resulted in exclusive incorporation into senecic acid although the low activity of the derived alkaloid prevented any degradation studies.

The stereochemistry of the coupling mechanism was also addressed by Crout and his co-workers.[76, 77] Incorporation studies with (2*S*, 4*S*)-[3,4-^{3}H]- and (2*S*,4*R*)-[4-^{3}H]-isoleucine showed that the H-4 *pro-R* is retained and H-4 *pro-S* is lost from both labelled isoleucines in the formation of the ten carbon necic acids in the *Senecio* species. This result again demonstrates that the introduction of functionality into C-4 of isoleucine is limited to a maximum two electron oxidation and excludes any ketonic precursors.

2-Aminobutanoic acid (17) was also shown to be specifically incorporated into senecic acid, with the ethyl migration step, in the conversion into L-isoleucine, taking place with retention of stereochemistry at the migrating centre (Scheme 31).

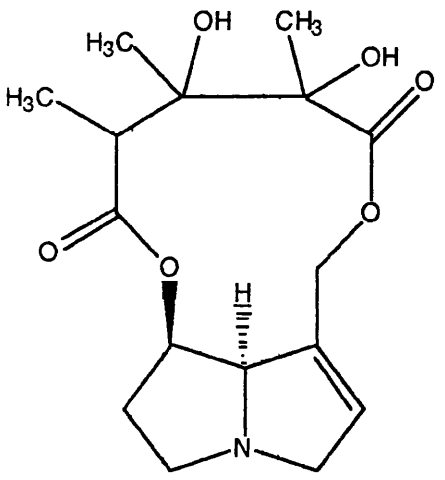
Scheme 31



The high incorporation of 2-aminobutanoic acid into retrorsine (7) was also noteworthy (up to 3.6%). The incorporation was higher than for any other precursor, and it was also observed that both stereoisomers were incorporated with nearly equal efficincies.[76]

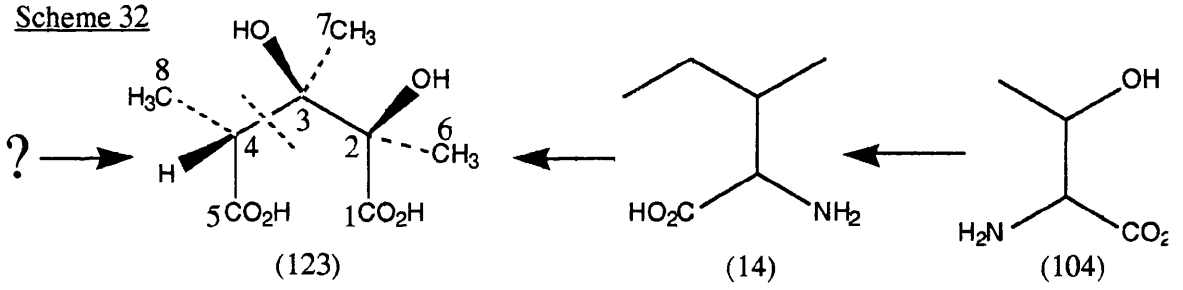
Necic acid biosynthesis has also been studied in *Crotalaria* species. L-[U-^{14}C]Isoleucine and L-[U-^{14}C]threonine were both incorporated into monocrotalic acid (123), the acid portion of monocrotaline (21), produced by *C. retusa*.[78] By degradation studies the partial labelling pattern was obtained, and it was found that the right hand C_5 unit was most heavily labelled. It seems that this C_5 unit is derived from isoleucine (Scheme 32).

The exact nature of the biosynthesis of the C_3 unit comprising C-4, C-5 and C-8 of monocrotalic acid is still uncertain.



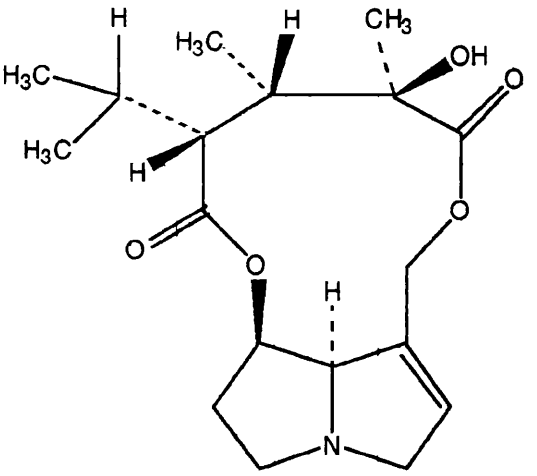
(21)

Scheme 32



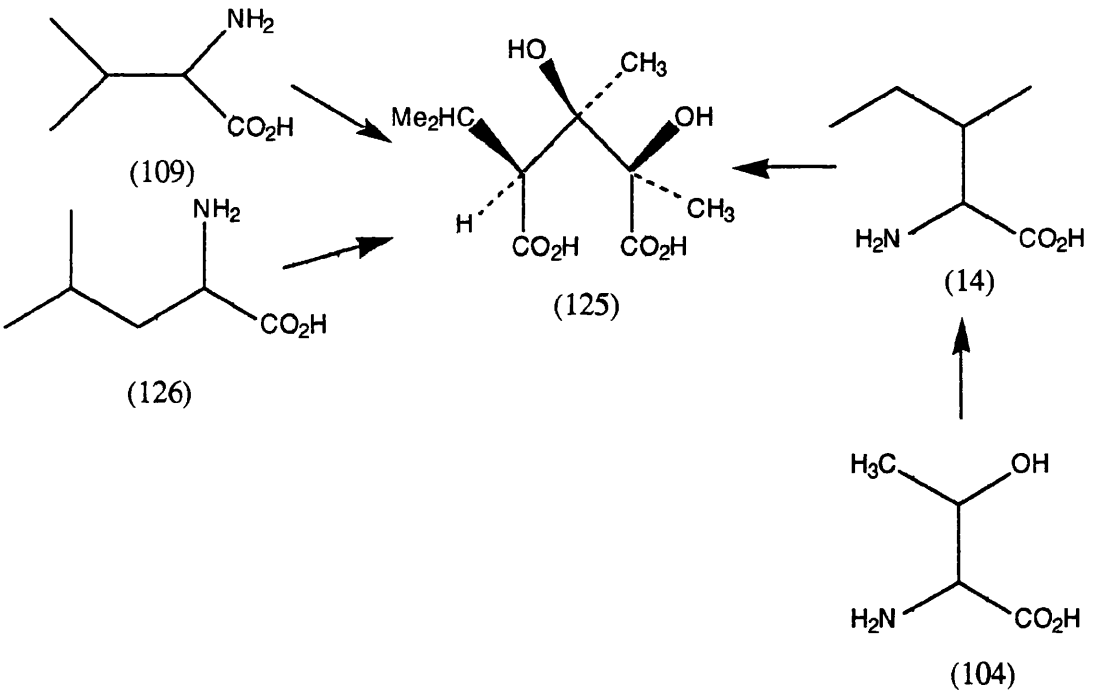
(123) (14) (104)

The major alkaloid produced by *Crotalaria globifera* is trichodesmine (124). The acid portion, trichodesmic acid (125), is structurally similar to monocrotalic acid. Work by Devlin and Robins[79] with ^{14}C-labelled amino acids showed that threonine (105) and isoleucine (14) are incorporated to a greater extent into the right hand C_5 unit, with valine (109) and leucine (126) mainly labelling the left hand portion (Scheme 33).



(124)

Scheme 33



(109)

(126)

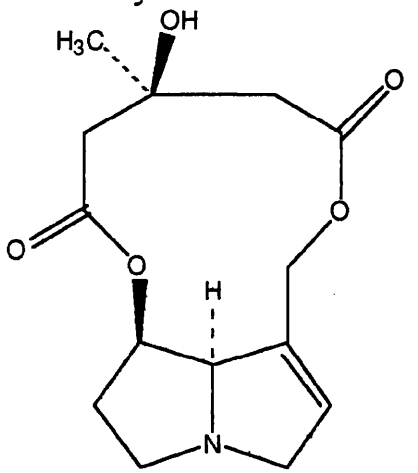
(125)

(14)

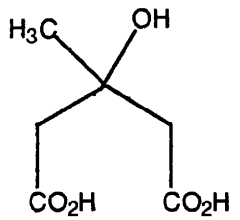
(104)

Dicrotaline (16) contains 3-hydroxy-3-methylglutaric acid (HMG) (15), the simplest necic acid known. Possible precursors of HMG are acetate and mevalonate but Denholm and Robins[80] showed that neither these, nor dicrotalic acid itself, were incorporated to any degree. However L-[U-^{14}C]threonine and L-[U-^{14}C]isoleucine were found to be specific precursors of

dicrotalic acid. Indeed [4,5-^{3}H]isoleucine was found to label the methyl group of the diacid specifically. It was shown therefore, that isoleucine provides four out of the six carbons of HMG, with loss of the C-6 methyl and carboxyl group in one of two possible ways (Scheme 34). The origin of the other two carbons of dicrotalic acid is as yet unknown.

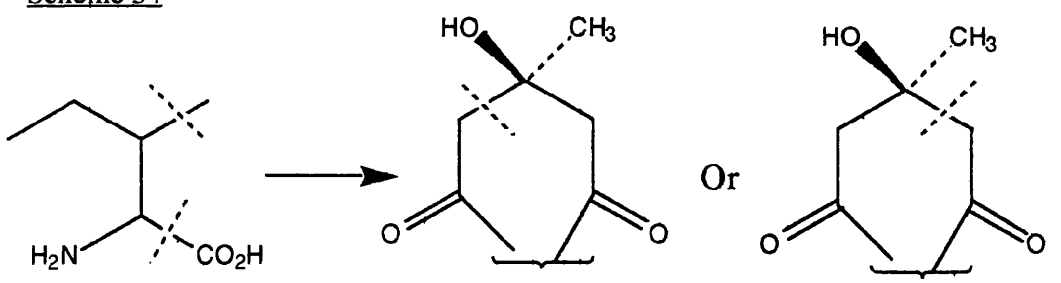


(16)



(15)

Scheme 34



In summary, all of the necic acids studied so far have been shown to be derived from α-amino acids. It is hoped that through the use of NMR spectroscopy complete labelling patterns in pyrrolizidine alkaloids can be obtained by carrying out feeding experiments with precursors labelled with stable isotopes. Using this approach some of the remaining mysteries of

necic acid biosynthesis may be unravelled. Some studies with ^{2}H- and ^{13}C-labelled forms of isoleucine and 2-aminobutanoic acid are discused in the following chapters.

CHAPTER 3

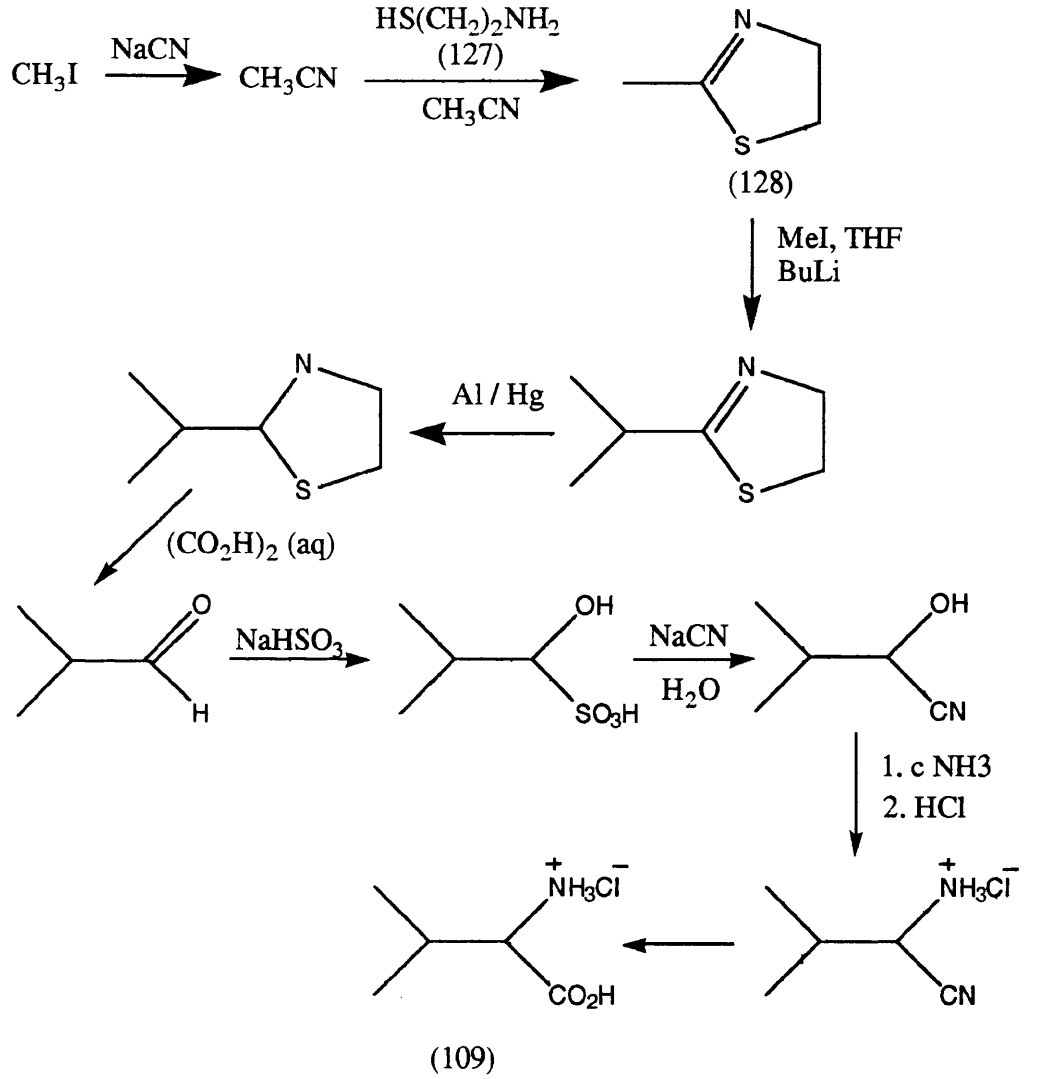
Development of a General Route to Isoleucine

3.1 Introduction

The majority of the work described in this thesis is concerned with the synthesis of precursors labelled with stable isotopes for the studies of the biosynthesis of the acid portion of pyrrolizidine alkaloids. The previous investigations into the pathway have been detailed in Chapter 2.2. The necic acids have been shown to be formed from α-amino acids, in particular threonine and isoleucine.[26] As all of the previous experiments have involved radiotracers the labelling patterns produced have been incomplete due to the lack of suitable degradations. The aim was to prepare isoleucines labelled at specific sites with ^{2}H and ^{13}C isotopes. The cost of commercially available isoleucines labelled with stable isotopes was prohibitively expensive (e.g. [6-$^{2}H_3$]isoleucine costs £7500 g^{-1}). Feeding experiments with these precursors and NMR degradation studies on the resulting alkaloids could give valuable information on necic acid biosynthesis. As a range of labelled isoleucines, labelled in various positions, would be required for incorporation studies, a route which involves the build up of the isoleucine molecule by a stepwise addition of one and two carbon units would be advantageous. This would allow greater flexibility in the positions of the label and the same route might be used for all the target compounds. It appeared that a modification of the

route published by Whaley *et al.*,[81] to labelled valine, would provide the best procedure (Scheme 35).

Scheme 35



(109)

3.2 Development of a General Route to Labelled Isoleucines

The starting point of the synthesis was the condensation of aminoethanethiol (127) and acetonitrile to give 4,5-dihydro-2-methylthiazole (128) in moderate yield. The mass spectrum of the didehydrothiazole showed a molecular ion peak at *m/z* 101.

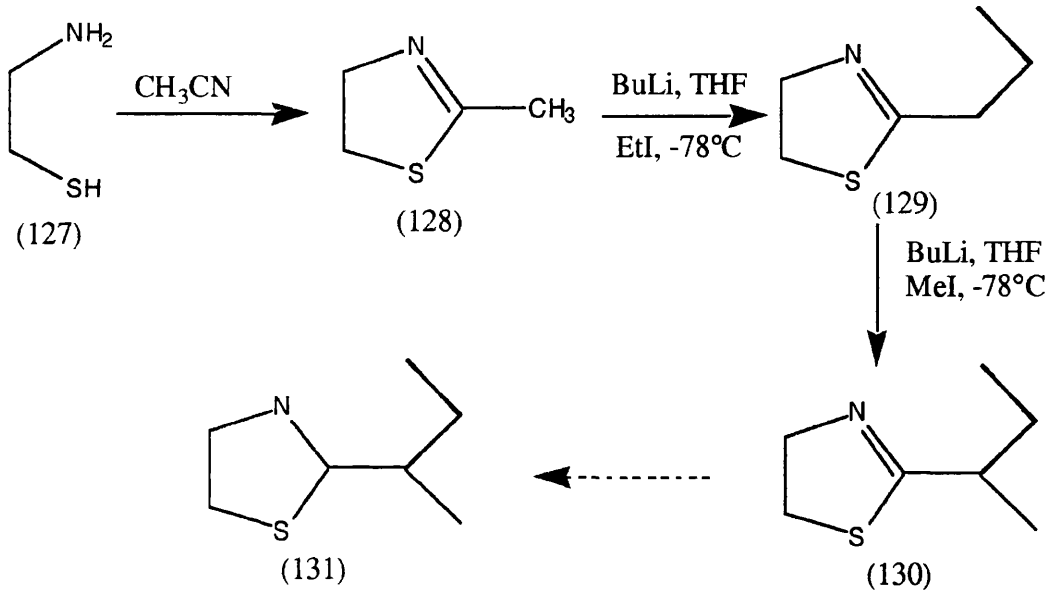
^{1}H NMR spectroscopic analysis showed the methyl group as a triplet, coupled to the methylene adjacent to the nitrogen, with a coupling constant of 1.6 Hz. This reaction was equally successful starting from either the free base or the hydrochloride salt, or when the free base was generated *in situ*.

The next step was alkylation of the methyl group. Using butyl lithium the thiazole was alkylated, firstly with ethyl iodide then with methyl iodide. Isolation and distillation of 2-propylthiazoline (129) before the second alkylation was preferable, as attempting both alkylations in a single pot led to a mixture of products. Again the mass spectrum of 2-propylthiazoline (129) showed a strong molecular ion peak, at *m/z* 129. In the ^{1}H NMR spectrum of (129) the long range coupling through the C=N bond between the two methylene groups was visible with a coupling constant of 1.4 Hz. The second alkylation was performed in the same manner, although it resulted in a slightly lower yield. Distillation of the dialkylated material gave the pure *sec*-butylthiazoline (130). The ^{1}H NMR spectrum showed the methyl group as a doublet at δ 1.10 with a coupling constant of 6.9 Hz.

Reduction of the 4,5-dihydro-2-*sec*-butylthiazole (130) to the thiazolidine (131) was then required (Scheme 36). The literature procedure used an aluminium/mercury amalgam in moist ether. After a number of attempts at this reaction, including increasing the amount of amalgam and reflux time, only partial reduction was observed. This was indicated by the appearance of the H-2 signal in the ^{1}H NMR spectrum at δ 4.0. This method of reduction

was deemed not to be satisfactory and a more reliable method was sought.

Scheme 36

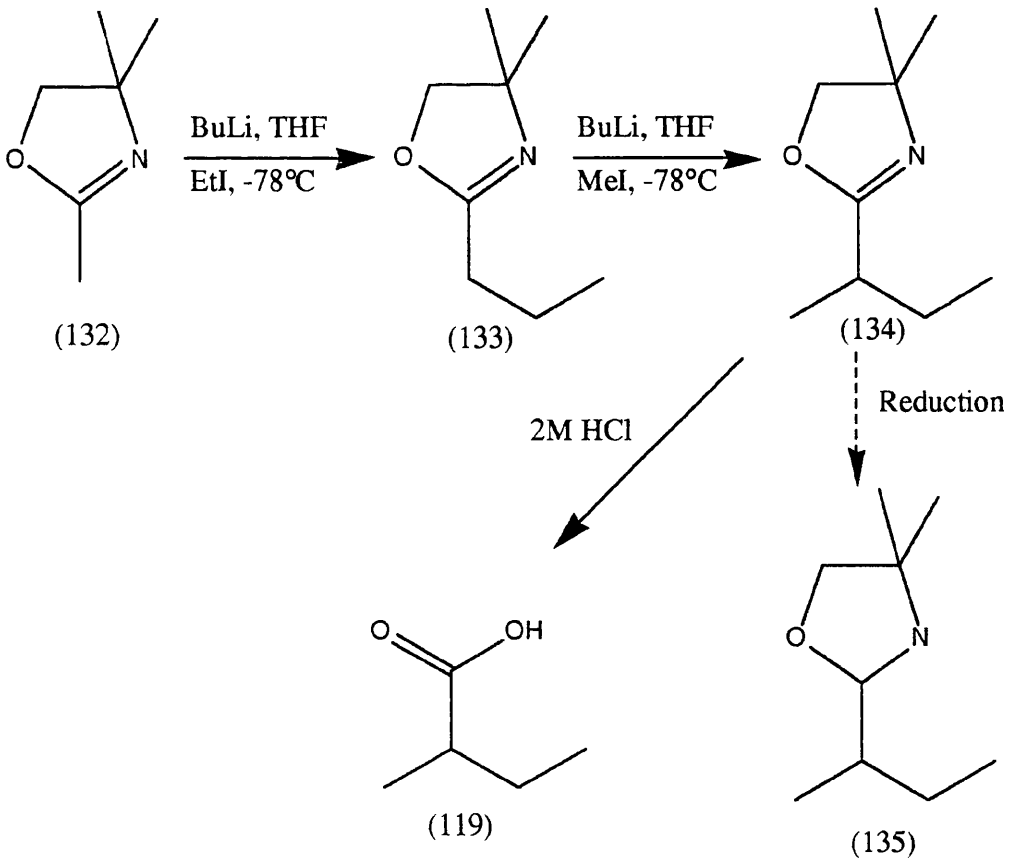


In an attempt to achieve reduction of (130) a variety of reducing agents was tried. Sodium borohydride, sodium cyanoborohydride and lithium aluminium hydride were all unsuccessful. Hydrogenation at atmospheric pressure only returned starting material. As the prospect of reducing the thiazoline did not look promising, an alternative procedure was sought. Attention turned to an analogous route using oxazoles.[82]

The route began with 2,4,4-trimethyl-2-oxazoline (132). This was alkylated using the same procedure as before, with iodoethane to give 4,4-dimethyl-2-propyl-2-oxazoline (133), and then with iodomethane, to give 4,4-dimethyl-2-*sec*-butyloxazoline (134), in good yield (Scheme 37). The two methyl groups appeared in the ^{1}H NMR spectrum at δ 0.88 and 1.08 as a triplet and a doublet respectively. However the reduction stage again proved troublesome. No oxazolidine (135) could be

obtained by direct reduction. All the methods used with the thiazoles were also unsuccessful with the oxazolidines. Hydrogenation, using palladium on carbon (10%), in ethanol and glacial acetic acid only resulted in the recovery of starting material at atmospheric pressure. When this reaction was repeated in 2M hydrochloric acid, hydrolysis to 2-methylbutanoic acid (119), rather than reduction occurred. The acid proton was observed at δ 10.89 in the ^{1}H NMR spectrum. Initially it was thought that this compound might be of value in feeding experiments, but the literature revealed that 2-methylbutanoic acid (119) was not a precursor to necic acids[74] (Scheme 37).

Scheme 37

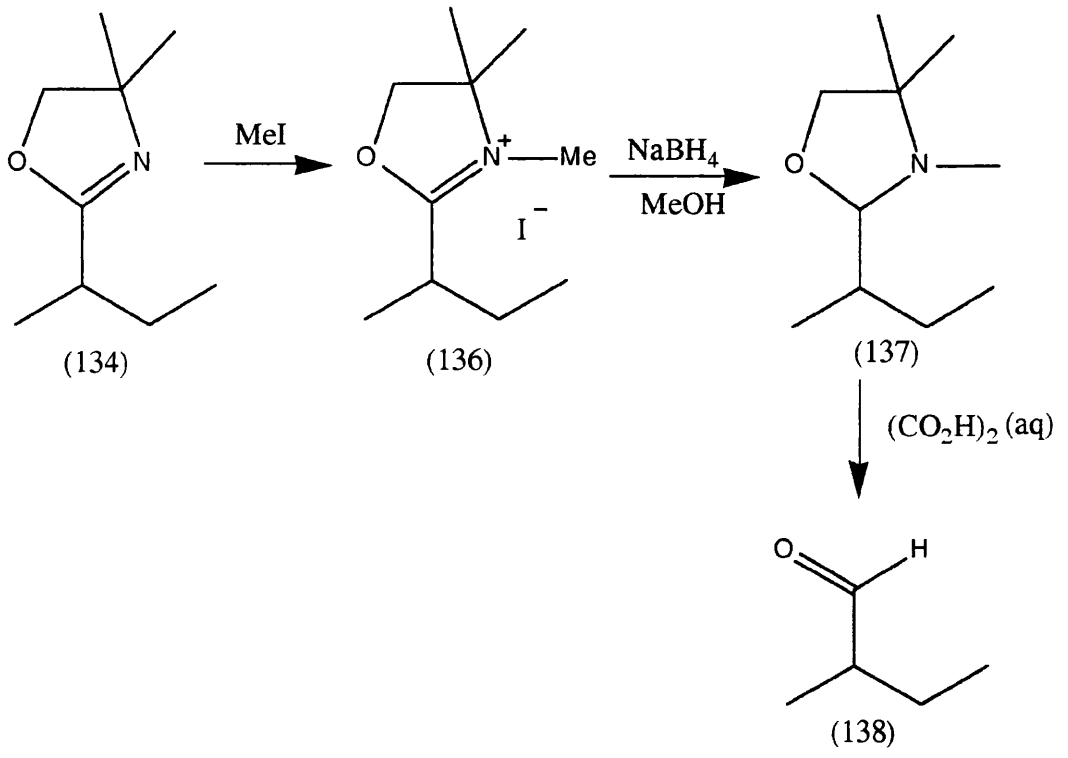


Reduction was achieved, albeit in low yield, using the procedure of Nordin (Scheme 38).[83,84] The oxazoline (134) was first methylated to give the oxazolinium iodide (136). This reaction proceeded in good yield (92%), resulting in a crystalline material which could be recrystallised from isopropanol to give pure 3,4,4-trimethyl-2-*sec*-butyloxazolinium iodide (136). The *N*-methyl signal was observed at δ 3.24 in the ^{1}H NMR spectrum.

Reduction of the oxazolinium salt (136) was achieved with sodium borohydride, giving the oxazolidine (137) in low yield. Sodium cyanoborohydride also accomplished the reduction, but with no improvement in yield. Purification of the oxazolidine was by column chromatography on basic alumina.

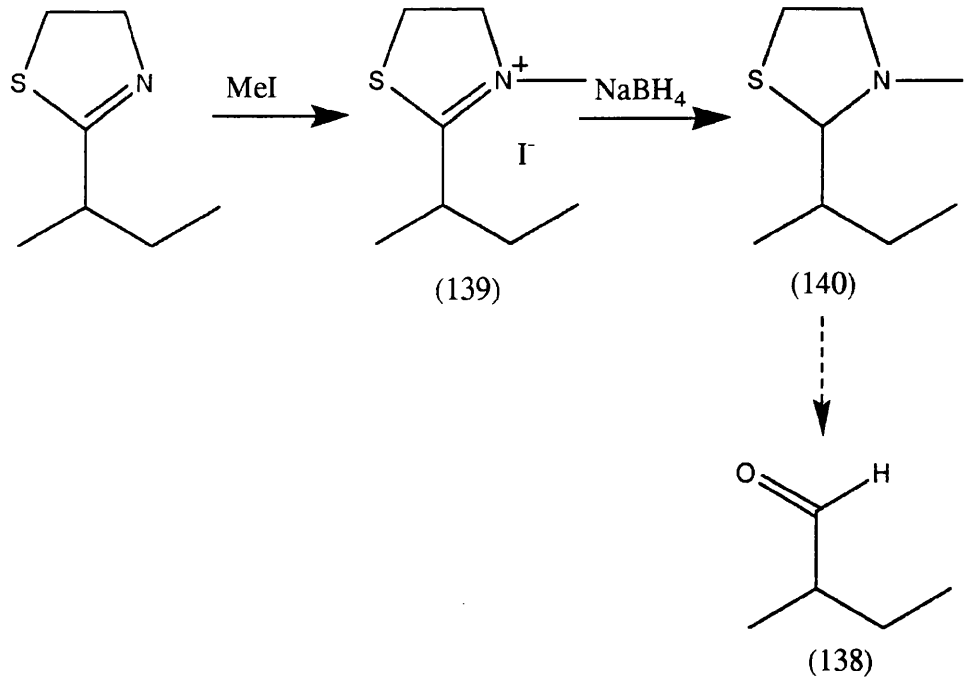
The hydrolysis of the oxazolidine (137) was also low yielding, giving 2-methylbutanal (138) in 22% yield at best (Scheme 38). The aldehyde proton occurred as a doublet in the ^{1}H NMR spectrum, with coupling constant of 2 Hz, at δ 9.53. The IR spectrum indicated the presence of the aldehyde carbonyl group at 1725 cm^{-1}.

Scheme 38



This procedure (Scheme 38) was also repeated for the thiazoline route (Scheme 39). A good yield was achieved for the formation of the thiazolinium iodide (139). The reduction stage, however, was again low yielding. The best yield obtained was less than for the oxazolidine (33%). The hydrolysis of the thiazoline (140) to the aldehyde (138) was not achieved.

Scheme 39



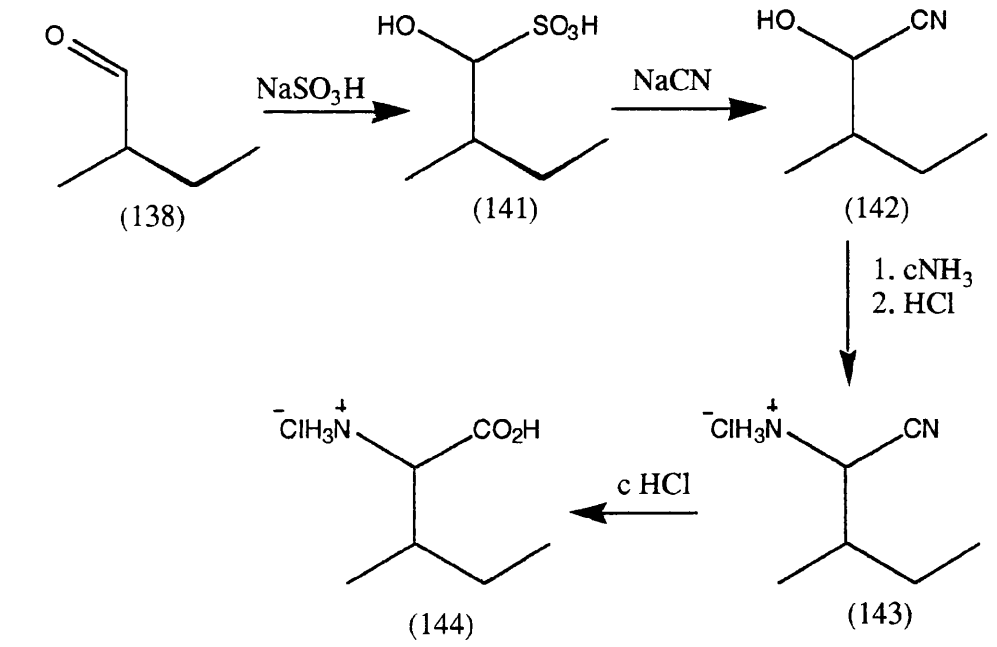
(139) (140) (138)

2-Methylbutanal (138) was a useful relay point, as this material is commercially available, and the remainder of the route had actually been accomplished before the problems with reduction and hydrolysis steps were encountered. A modified version of the Strecker synthesis was used (Scheme 40). The aldehyde (138) was converted into the crystalline bisulfite addition product (141) in good yield. There was a broad peak at 1170 cm^{-1} in the IR spectrum corresponding to the bisulfite functionality.

The cyanohydrin (142) was prepared from the bisulfite addition product (141) by treatment with an aqueous solution of sodium cyanide. A good yield (85%) was achieved, yielding the product as an oil. There was a weak band at 2250 cm^{-1} in the IR spectrum corresponding to the nitrile group of the cyanohydrin. The carbon of the nitrile function was observed at δ 119 in the ^{13}C NMR spectrum.

A solution of the cyanohydrin (142) in concentrated ammonia solution was then stirred at room temperature. Subsequent extraction of the organic extracts of this reaction with 10% hydrochloride acid gave the aminonitrile hydrochloride (143). A peak in the mass spectrum at *m/z* 112 was observed corresponding to M^+ minus HCl. The product gave a white solid when recrystallised from water. The final stage involved the hydrolysis of the nitrile group. Treatment of the aminonitrile hydrochloride (143) with concentrated hydrochloric acid, for 24 hours at room temperature and then seven hours at reflux temperature gave the required DL-isoleucine hydrochloride (144) and the allo-racemate (Scheme 40). The ^{13}C NMR spectrum showed the carboxylic acid carbon at δ 175, and the C=O stretch in the IR spectrum showed an absorption at 1700 cm^{-1}.

Scheme 40



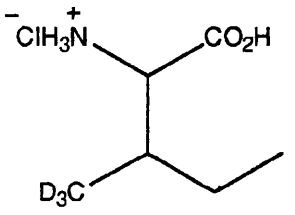
This final product was analysed by ^{13}C NMR spectroscopy, and shown to be an approximately 1: 1 ratio of DL-isoleucine and DL-allo-isoleucine. It is known that only L-isoleucine is incorporated in the necic acid biosynthesis. Separation of the mixture was not necessary as the other isomers have no adverse effects, but it meant that only one quarter of the mixture of hydrochlorides prepared was useful for incorporation studies. In combination with the fact that the overall yield was low, the result was that this route was not economically viable as a general route for the synthesis of a range of isoleucines labelled with stable isotopes.

3.3 Synthesis of [6-$^{2}H_3$]-Isoleucine and Allo-isoleucine Hydrochloride

The route described above was used to prepare [6-$^{2}H_3$]-isoleucine and [6-$^{2}H_3$]-allo-isoleucine hydrochloride (145). 2,4,4-Trimethyloxazoline was alkylated with ethyl iodide, then the deuterium label was introduced via alkylation with [$^{2}H_3$]-iodomethane. The remainder of the route was as previously described. All yields obtained were comparable to those achieved with unlabelled material. The deuterium labelled products were examined by ^{2}H NMR spectroscopy and mass spectroscopy. A broad singlet at δ 0.70 in the deuterium NMR spectrum of the amino acid hydrochloride (145) showed the presence of the ^{2}H labelled methyl group.

The crucial stages of this synthesis were the reduction of the oxazoline and hydrolysis of the product. Although these steps were eventually accomplished, the poor yields of these reactions drastically reduced the overall yield. The procedure was only

used once, to prepare a few milligrams of [6-$^{2}H_3$]-isoleucine hydrochloride (145), and the allo isomer, but this material was not used in feeding experiments as an insufficient amount could be prepared.



(145)

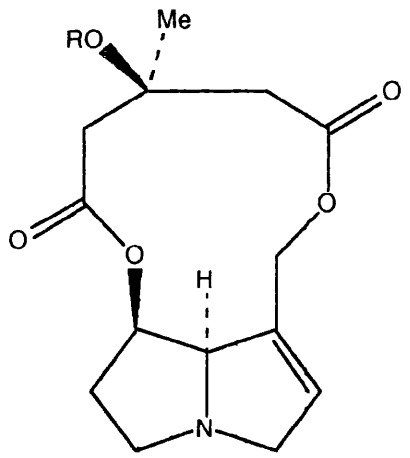
CHAPTER 4

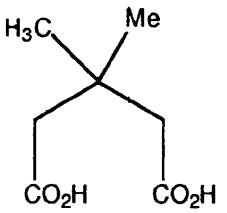
Synthesis of a Specifically Labelled Isoleucine

4.1 Introduction

As it was not possible to develop a general synthetic route to a range of labelled isoleucines (Chapter 3), it was necessary to devise a synthesis for each individual precursor labelled in the required positions.

Dicrotaline (16) was first isolated by Marais from *Crotalaria dura* and *Crotalaria globifera*,[85] and contains 3-hydroxy-3-methylglutaric acid (HMG) (15) which is the simplest diacid found in pyrrolizidine alkaloids. 3-Hydroxy-3-methylglutaric acid is a well known metabolite in biosynthesis, and is usually formed from acetate on the pathway to mevalonate and terpenes. However tracer studies by Denholm and Robins[80] demonstrated that acetate and mevalonate were not incorporated into the acid portion of dicrotaline. It was found however, that isoleucine and threonine are precursors for HMG. Feeding experiments with *Crotalaria lachnosema* indicated that isoleucine contributes four out of six carbons of the 3-hydroxy-3-methylglutaric acid portion. The carboxyl group and the C-6 methyl group of isoleucine are both lost. The origin of the remaining two carbons is still unknown. The isoleucine precursor appears to label four of the six carbons of HMG, as shown in Scheme 34.

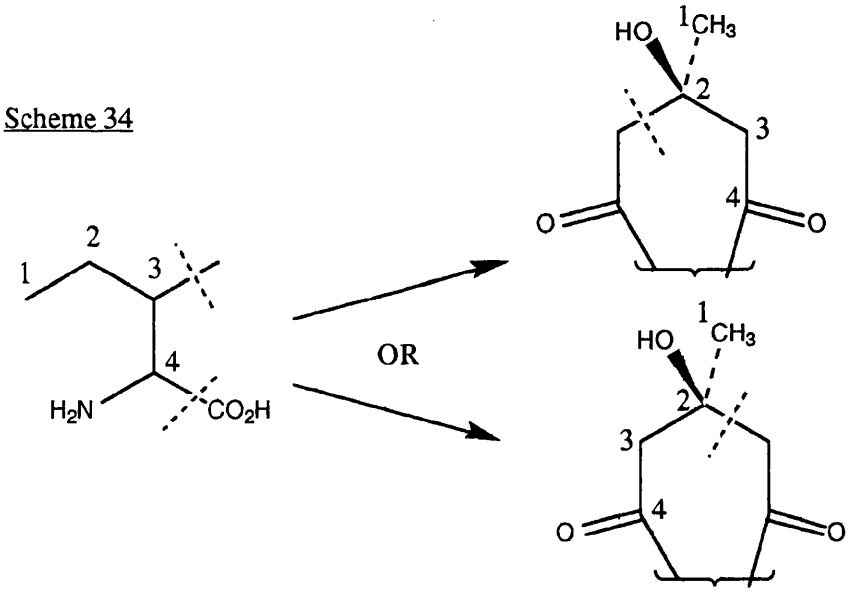




(15)

(16) R = H
(18) R = Ac

Scheme 34

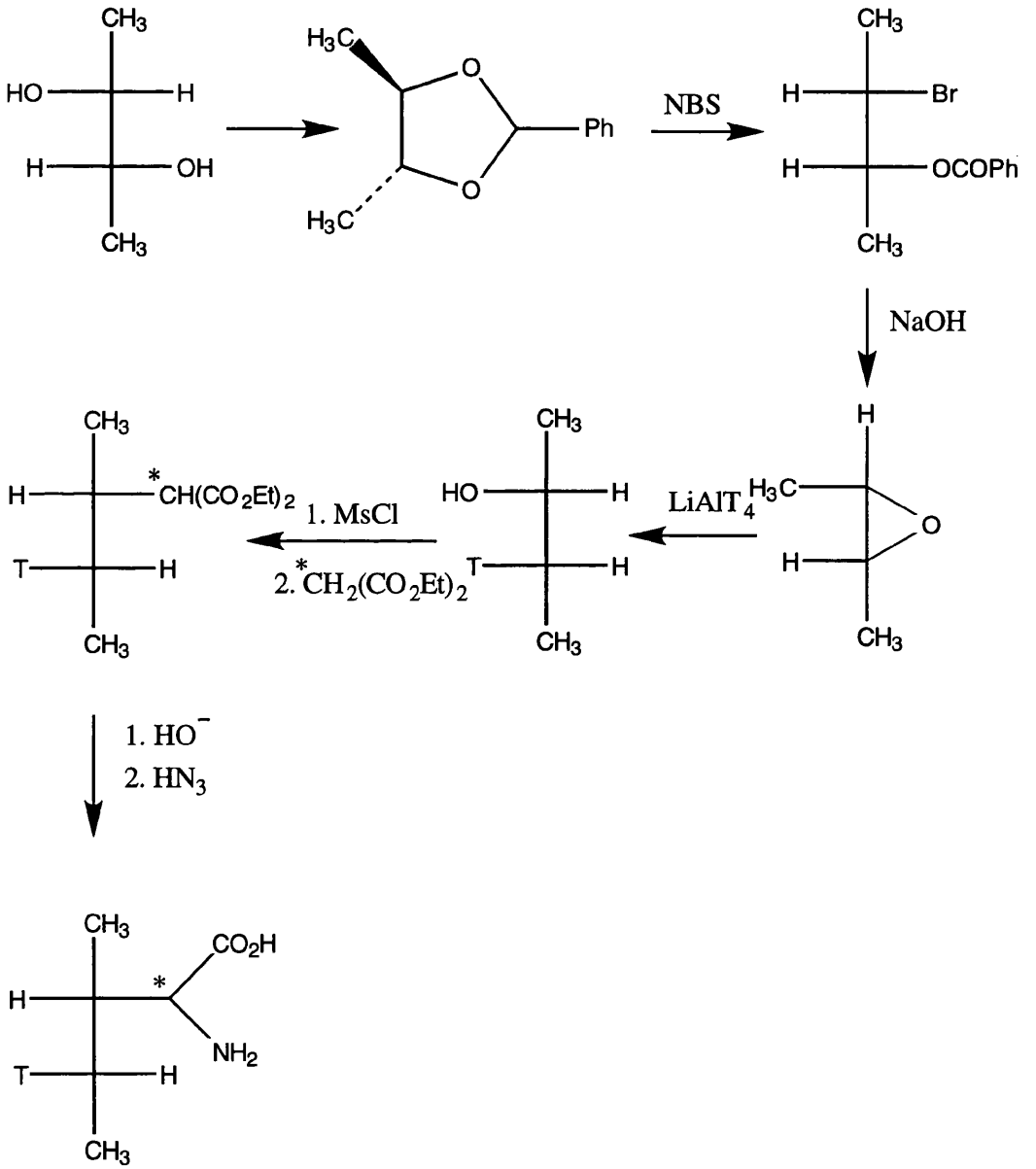


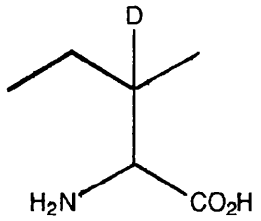
The dicrotaline extracted from *C. lachnosema* consists of a mixture of dicrotaline and 13-*O*-acetyldicrotaline[86] (18). For ease of analysis the mixture was converted wholly into the acetylated alkaloid. A full NMR spectroscopic analysis showed that, unlike in HMG itself, the two methylene groups in the acid portion of 13-*O*-acetyldicrotaline have distinct chemical shifts and can be distinguished[87] (Chapter 6).

In an attempt to identify which side of the necic acid is incorporated into isoleucine a route to [3-^{2}H]isoleucine (146) was

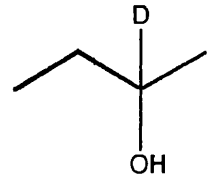
required. As [2-^{2}H]-2-butanol (147) is commercially available the route used by Hill *et al.* to prepare radiolabelled isoleucine was developed[88] (Scheme 41). They used lithium aluminium tritiide to introduce ^{3}H and [2-^{14}C]malonate to incorporate ^{14}C.

Scheme 41





(146)



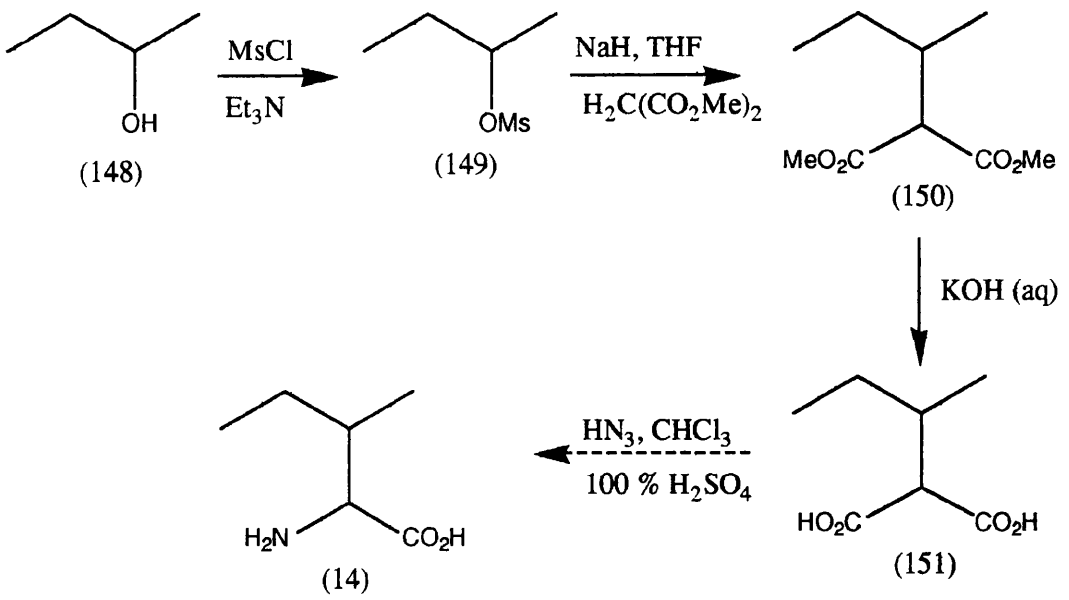
(147)

4.2 Development of a Synthetic Route to [3-^{2}H]Isoleucine

In accordance with the synthesis used by Hill *et al.*, 2-butanol (148) was converted into 2-butylmethanesulfonate (149) by reaction with mesyl chloride and triethylamine (Scheme 42). The reaction proceded in good yield (90%). The methyl signal of the sulfonate (149) appeared at δ 3.0 in the ^{1}H NMR spectrum and the sulfonate was detected by bands at 1350 and 1170 cm^{-1} in the IR spectrum. The mesylate (149) could be distilled to give the product as a colourless liquid.

The mesylate was treated with dimethyl malonate to yield dimethyl2-*sec*-butylmalonate (150). Sodium hydride was used as the base and the mixture was heated at reflux temperature for around 48 hours. Distillation of the product gave the diester (150) in good yield (86%). The ^{1}H NMR spectrum of (150) showed a doublet at δ 3.7 corresponding to the H-2 proton α to the ester functions. Basic hydrolysis of the diester (150) with aqueous sodium hydroxide gave the *sec*-butylmalonic acid (151), which crystallised in a desiccator overnight. The signal for the H-2 proton was observed as a doublet in the ^{1}H NMR spectrum at δ 3.36 and the acid protons appeared as a broad singlet at δ 11.04. The mass spectrum showed a peak for the molecular ion at *m/z* 160.

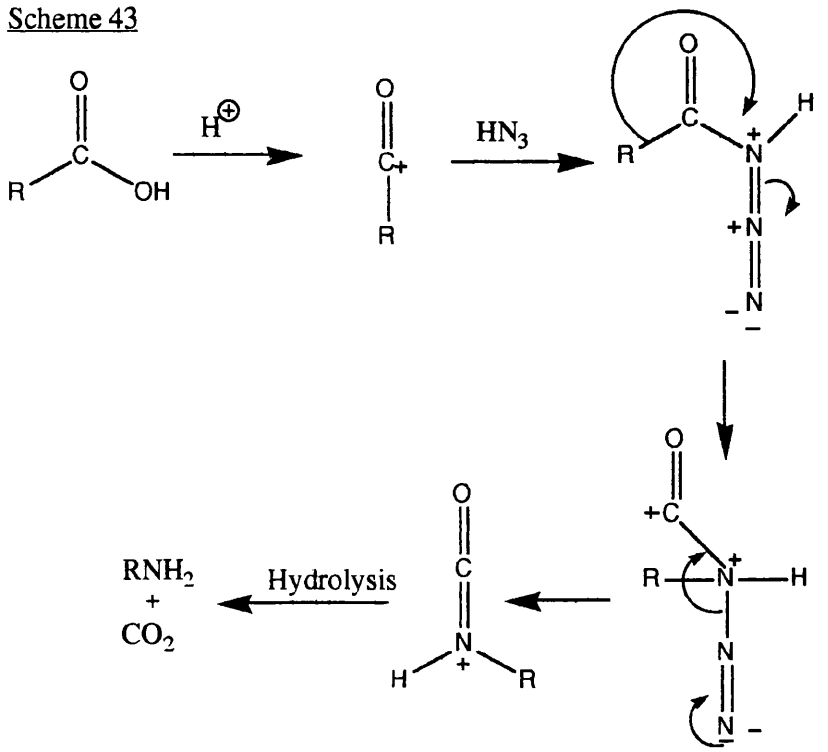
Scheme 42



(148) (149) (150) (151) (14)

The preferred reaction of Hill *et al.* was a direct Schmidt reaction, using hydrazoic acid solution on the diacid (151) to give isoleucine (14). This reaction is analogous to the Curtius rearrangement except it is the protonated azide that undergoes the rearrangement[89] (Scheme 43).

Scheme 43



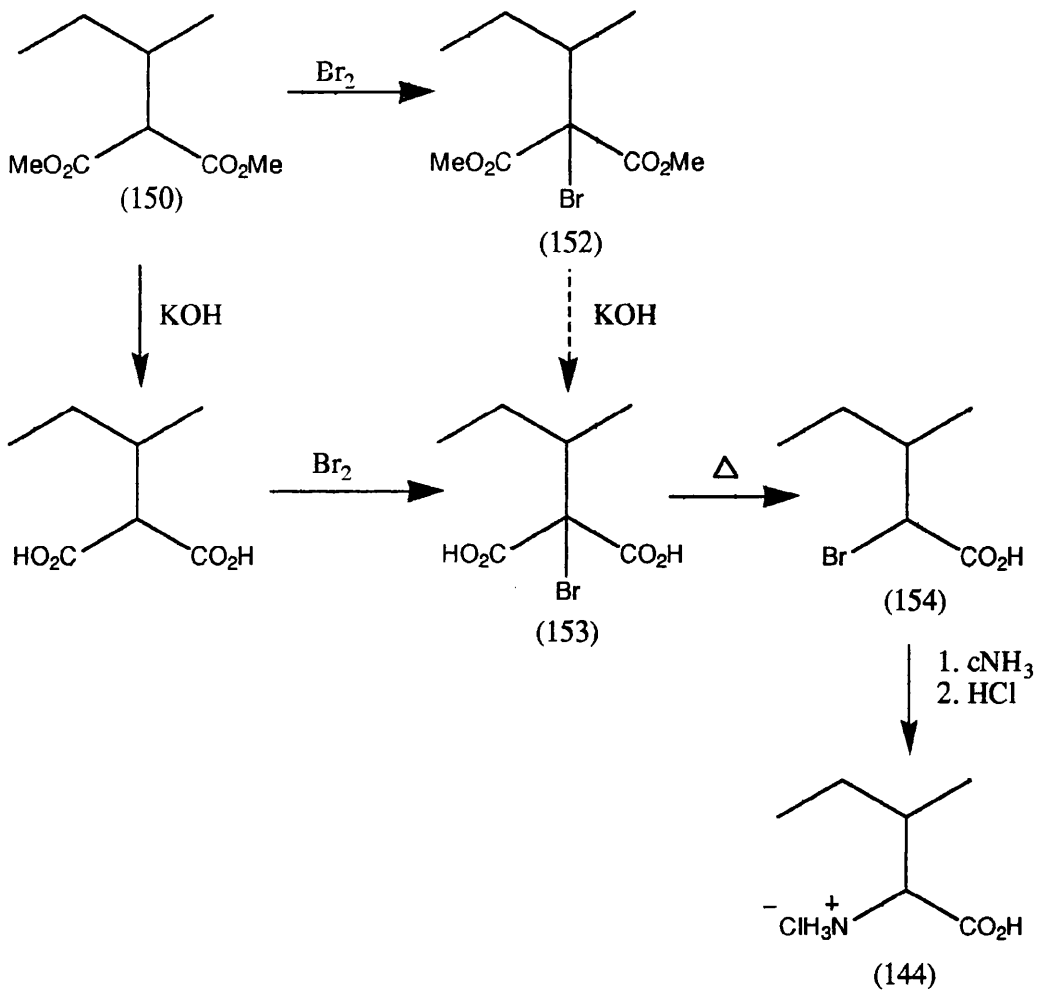
However this procedure proved unsuccessful. Attempted Schmidt reactions on the diacid (151) with hydrazoic acid in benzene or toluene gave a mixture of substituted aromatic species, and no isoleucine could be isolated. Using a solution of hydrazoic acid in chloroform, with 100% sulfuric acid, DL-isoleucine and DL-allo-isoleucine could be prepared but results were erratic and yields were very low when the reaction was successful (Scheme 42).

A more reliable procedure was required and an alternative method involving bromination, decarboxylation and displacement was used. The diester (150) was brominated according to known methods.[90] A good yield (94%) of the bromodiester (152) was achieved. The quaternary carbon signal in the ^{13}C NMR spectrum was observed at δ 70.1. Unfortunately basic hydrolysis did not give the diacid.

However, reversal of these two steps gave the desired bromomalonic acid (153). The quaternary carbon at δ 73.5 was seen in the ^{13}C NMR spectrum. Again, crystallisation of the diacid was by leaving overnight in a desiccator.

When the malonic acid (153) was subjected to heat, decarboxylation occurred.[94] There were two doublets at δ 4.2 in the ^{1}H NMR spectrum due to the α-H in the diastereomers. The ratio of diastereomers was approximately 1:1. Unfortunately the yield of this reaction was less than the previous stages (42%). The α-bromo-β-methylvaleric acid (154) was added to concentrated aqueous ammonia solution and stirred overnight. The isoleucine and allo-isoleucine were isolated as the hydrochloride salts (144) (Scheme 44).

Scheme 44



The overall yield of this synthesis was 10.3% which was satisfactory for the preparation of [3-^{2}H]isoleucine hydrochloride (146).

4.3 Synthesis of [3-2H]Isoleucine and Allo-isoleucine Hydrochloride

A mixture of [3-^{2}H]isoleucine and [3-^{2}H]alloisoleucine hydrochloride was prepared, using the procedure described above, starting from [2-^{2}H]butan-2-ol. Again, examination of the ^{2}H products obtained was by ^{2}H NMR spectroscopy. The yields for the deuterium labelled route compared favourably for the yields

obtained for the unlabelled material. A signal at δ 1.63 in the ^{2}H NMR spectrum corresponds to the deuterium label at the 3-H position of the isoleucine hydrochloride.

4.4 Feeding of [3-^{2}H]Isoleucine hydrochloride

The labelled precursor mixture was dissolved in sterile water and fed to medium sized *Crotalaria lachnosema* plants by the wick method. The feeding was continued over 14 days and the plants were harvested in the usual manner after a further four days. The alkaloid mixture extracted was converted wholly into 13-*O*-acetyldicrotaline (18), using acetyl chloride in a refluxing solution of acetic anhydride.

4.5 Results and Discussion

As a result of previous biosynthetic studies and NMR spectroscopic analysis, it was expected known that feeding of the [3-^{2}H]isoleucine would label one of the four positions at either 12-*pro-R* or 12-*pro-S* proton, at δ 3.26 and 2.41 respectively, or the 14-*pro-R* or 14-*pro-S* proton at δ 2.93 and 2.78. Unfortunately the ^{2}H NMR spectrum showed no signals at these positions nor any of the other chemical shifts known for the alkaloid although the ^{1}H NMR spectrum showed that 13-*O*-acetyldicrotaline was present. There were however strong signals at δ 0.90 and 1.12 in the ^{2}H NMR spectrum. The cause of these peaks is unknown.

So, although the *C. lachnosema* plants appeared healthy at the time of feeding and harvesting, there appeared to be no incorporation of the isoleucine into the necic acid portion of the 13-O-acetyldicrotaline sample obtained.

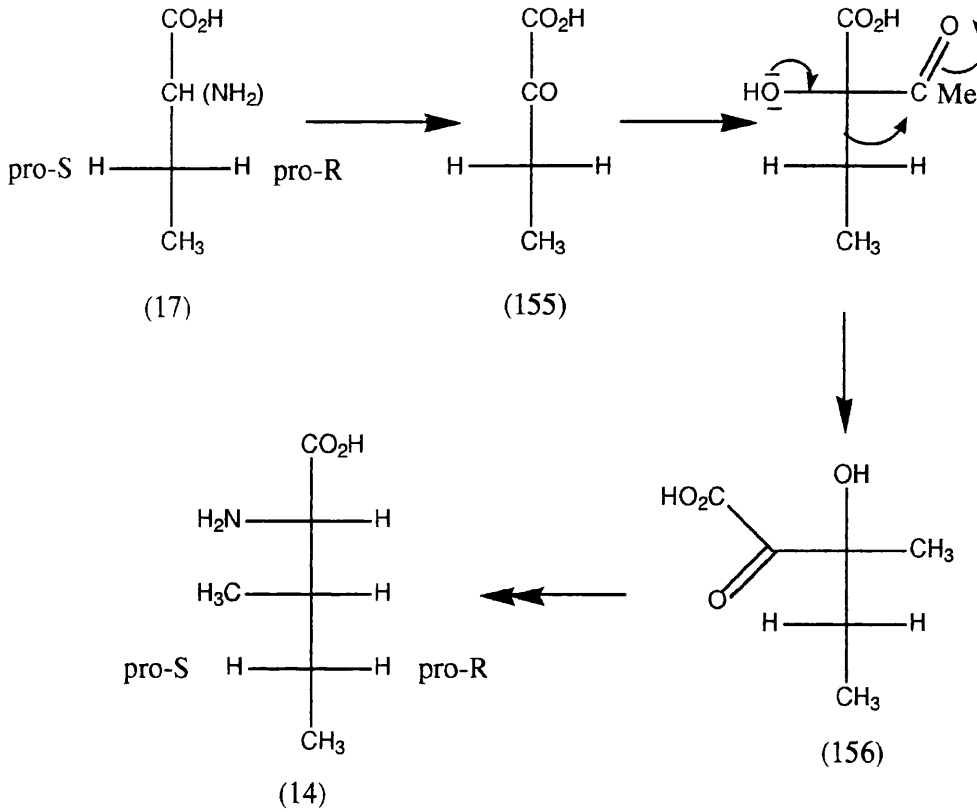
CHAPTER 5

Synthesis of Labelled 2-Aminobutanoic Acid

5.1 Introduction

A known biosynthetic precursor of necic acids is 2-aminobutanoic acid.[16] Crout and co-workers[77] fed radioactively labelled forms of 2-aminobutanoic acid to *Senecio isatideus*, and found that the incorporations were high (up to 3.6%), and that the label was exclusively in the acid portion. It was also observed that both L- and D-2-aminobutanoic acid were incorporated into the acid portion. It was proposed that isoleucine (14) was formed from 2-aminobutanoic acid after conversion by the appropriate enzyme (amino acid aminotransferases or oxidases) into 2-oxobutanoic acid (155) (Scheme 45).

Scheme 45



Notably, Crout and co-workers also showed that in the generation of both of the five carbon units of the C_{10} necic acid the C-3 *pro-S* hydrogen of the 2-aminobutanoic acid is lost and the C-3 *pro-R* hydrogen is retained. Corresponding experiments with isoleucine found that the C-4 *pro-S* hydrogen is lost and the C-4 *pro-R* hydrogen is retained. This demonstrated that the ethyl migration step takes place with retention of configuration in (156), agreeing with previous hypotheses based on orbital symmetry arguments.[92]

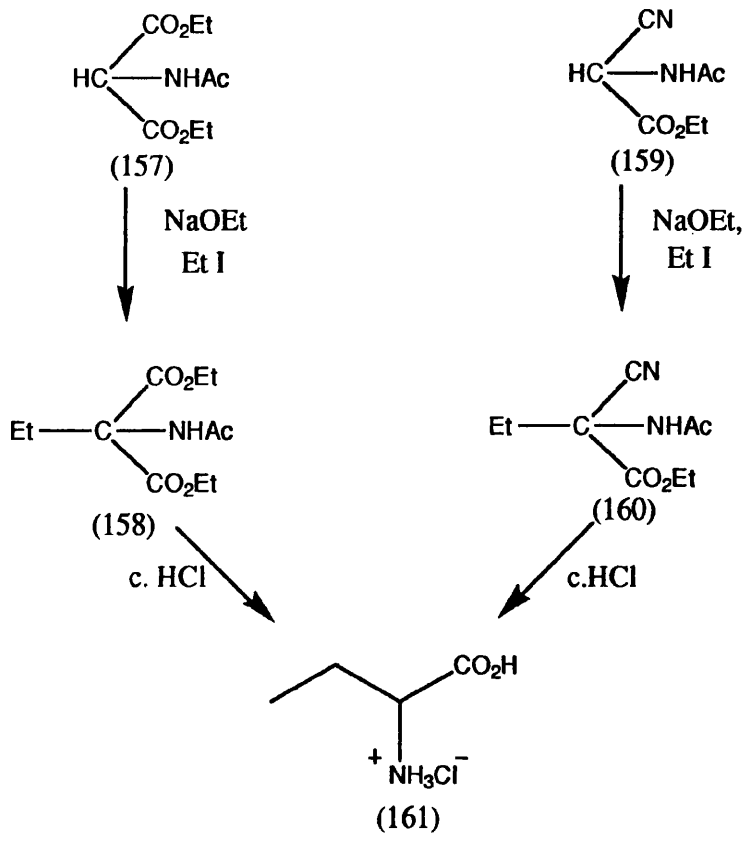
There have also been reports of the occurrence of 2-aminobutanoic acid in a number of higher plants.[93] Most importantly, the reported finding of the *R*-isomer in legume seeds[94] and of a 2-oxobutanoate aminotransferase in pea seedlings[95] seem to suggest that the conversion of 2-aminobutanoic acid (17) into 2-oxobutanoate (155) may be a normal metabolic process.

These results suggested that 2-aminobutanoic acid would be an interesting and useful probe for investigations of the necic acid biosynthetic pathway using precursors labelled with stable isotopes.

5.2 Preparation of 2-Aminobutanoic Acid

A short and efficient synthesis of 2-aminobutanoic acid was known.[96] Starting from diethyl acetamidomalonate (157), and using sodium ethoxide as a base, the first step is an alkylation with ethyl iodide (Scheme 47). This gave diethyl 2-ethylacetamidomalonate (158) in a yield of 75%. The ethyl group appeared in the ^{13}C NMR spectrum with signals at δ 7.7 and 13.9, and the ^{1}H NMR spectrum showed a triplet and a quartet at δ 0.77 and 2.36 respectively. The coupling constants for both these signals was 7.6 Hz. An alternative alkylation was also achieved, starting from ethyl acetamidocyanoacetate (159), but with no improvement in the yield. The alkylated products (158) and (160) from these reactions could be recrystallised to white crystalline material. Hydrolysis of these compounds in concentrated hydrochloric acid gave 2-aminobutanoic acid as the hydrochloride salt (161). Recrystallisation of the salt from 95% ethanol gave the product in good yield. The C-4 and C-3 positions of the amino acids corresponded to the signals at δ 9.3 and 24.0 respectively in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum showed triplets at δ 0.82 and 3.79 corresponding to the methyl group and the proton at H-2 respectively. The methylene protons were observed as a multiplet at δ 1.78. Microanalysis of the salt also gave satisfactory data (Scheme 46).

Scheme 46

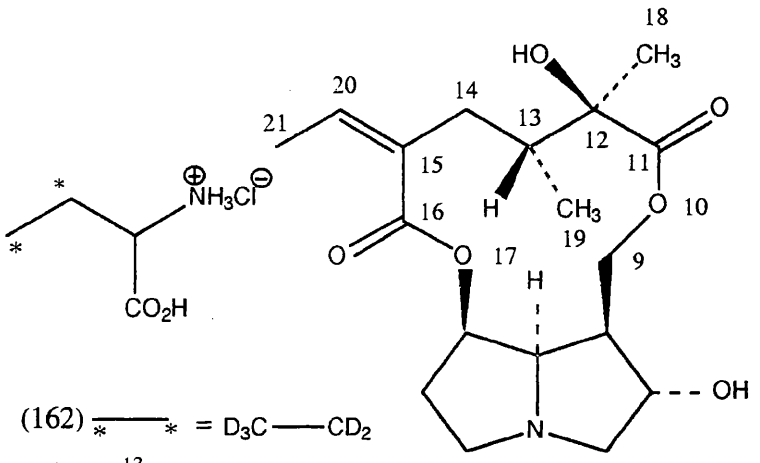


5.3 Synthesis of [3,4-$^{2}H_5$]-2-Aminobutanoic Acid and [$^{13}C_2$]-2-Aminobutanoic Acid Hydrochloride

The synthesis was then carried out with labelled material. The ^{2}H and ^{13}C labels were introduced at the alkylation stage. Using $^{2}H_5$-iodoethane afforded [3,4-$^{2}H_5$]-2-aminobutanoic acid (162) and alkylation with [$^{13}C_2$]-iodoethane gave [3,4-$^{13}C_2$]-2-aminobutanoic acid (163). The production of these labelled amino acids was verified by NMR spectroscopy. The ^{2}H NMR spectrum of (162) showed two broad singlets at δ 1.20 and 2.16 of relative intensities 3: 2. The deuterium content was greater than 95 %, estimated from the NMR spectra.

The ^{13}C NMR spectrum of (163) contained two large doublets at δ 9.3 and 24.2, with a coupling constant of 34 Hz, along with very

small natural abundance signals. Again around 99.9 % labelled material was obtained. The yields obtained with the labelled material were equivalent to those achieved with the unlabelled material.



(162) * —— * = D_3C——CD_2

(163) * ^{13}C

(8)

5.4 Feeding Experiments with 2-Aminobutanoic acid

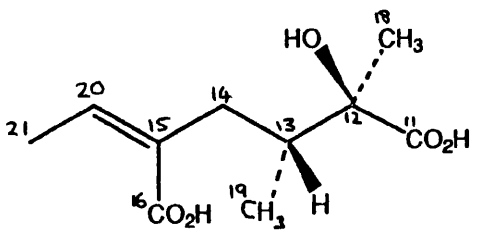
The ^{2}H-labelled (162) and ^{13}C-doubly labelled 2-aminobutanoic acids (163) were fed to two plants and two transformed root cultures which produce pyrrolizidine alkaloids. The labelled amino acid salts were dissolved in distilled water and fed by the wick method to *Senecio pleistocephalus* and *Crotalaria lachnosema* plants. The $^{13}C_2$-labelled material was fed to small plants to limit the amount of endogenous unlabelled material. As the ^{2}H signals should be easier to observe over natural abundance by NMR spectroscopy, the deuterium labelled 2-aminobutanoic acid was fed to older, larger plants.

For feeding to *Senecio vulgaris* and *Emilia flammea* root cultures the precursors were dissolved in water, sterilised and divided among ten flasks containing seven day old roots. The roots were filtered off

and extracted after a further 14 days. The alkaloids were purified by preparative TLC.

5.5 Results and Discussion

The acid portion of rosmarinine (8) produced by *Senecio pleistocephalus* is senecic acid (115). After feeding [3,4-$^{13}C_2$]-2-aminobutanoic acid the alkaloid was extracted and examined by ^{13}C NMR spectroscopy. Compared with the ^{13}C NMR spectrum of unlabelled romarinine run under the same conditions the ^{13}C NMR spectrum of the labelled rosmarinine showed four doublets of approximately equal intensity at δ 11.7, 15.1, 37.8 and 134.6. The corresponding coupling constants for these doublets were 36.4, 42.0, 36.3 and 42.0 Hz respectively. The labelling corresponds to the C-13 and C-19 positions, plus the C-20 and C-21 positions of rosmarinine. There was equal labelling in both halves of the necic acid. The incorporation of the 2-aminobutanoic acid was 0.43%. The ^{13}C NMR spectrum of the labelled romarinine is shown in figure 1.



(115)

The ^2H NMR spectrum of the rosmarinine sample obtained, after feeding [3,4-$^2H_5$]-2-aminobutanoic acid to *S. pleistocephalus* was also examined. Three signals were observed at δ 0.88, 1.75 and 5.79. The relative intensities of these signals were approximately 3: 4: 1, respectively. The signal at δ 0.88 corresponds to the methyl group at C-19, and the C-20 vinylic proton has the chemical shift

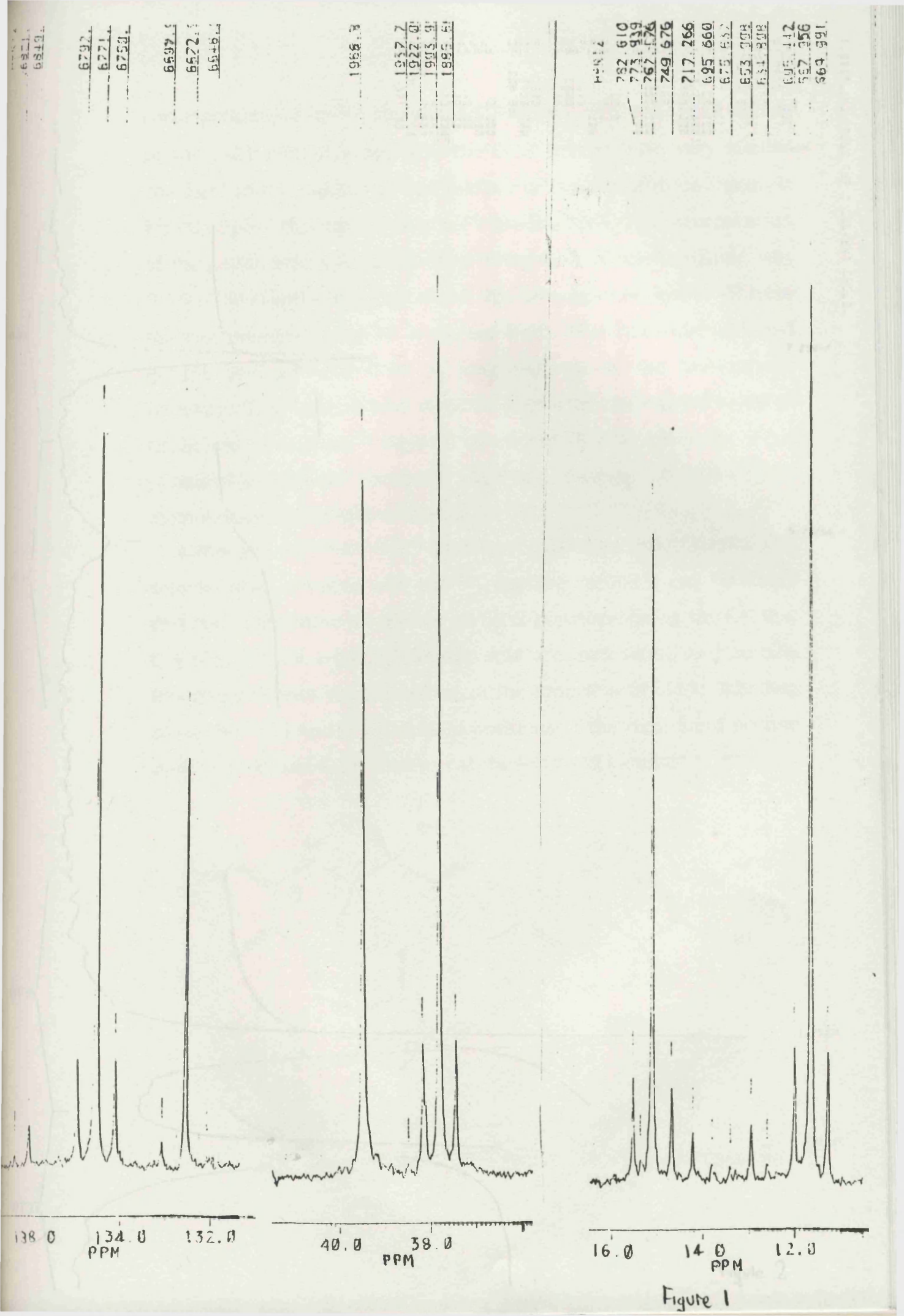

6792.
6721.
6758.
6697.
6622.
1966.3
1922.0
1903.9
1895.5
782.610
773.939
767.526
749.676
717.266
695.660
653.994
631.898
596.142
587.356
569.991
138.0
134.0
132.0
PPM
40.0
38.0
PPM
16.0
14.0
12.0
PPM

Figure 1

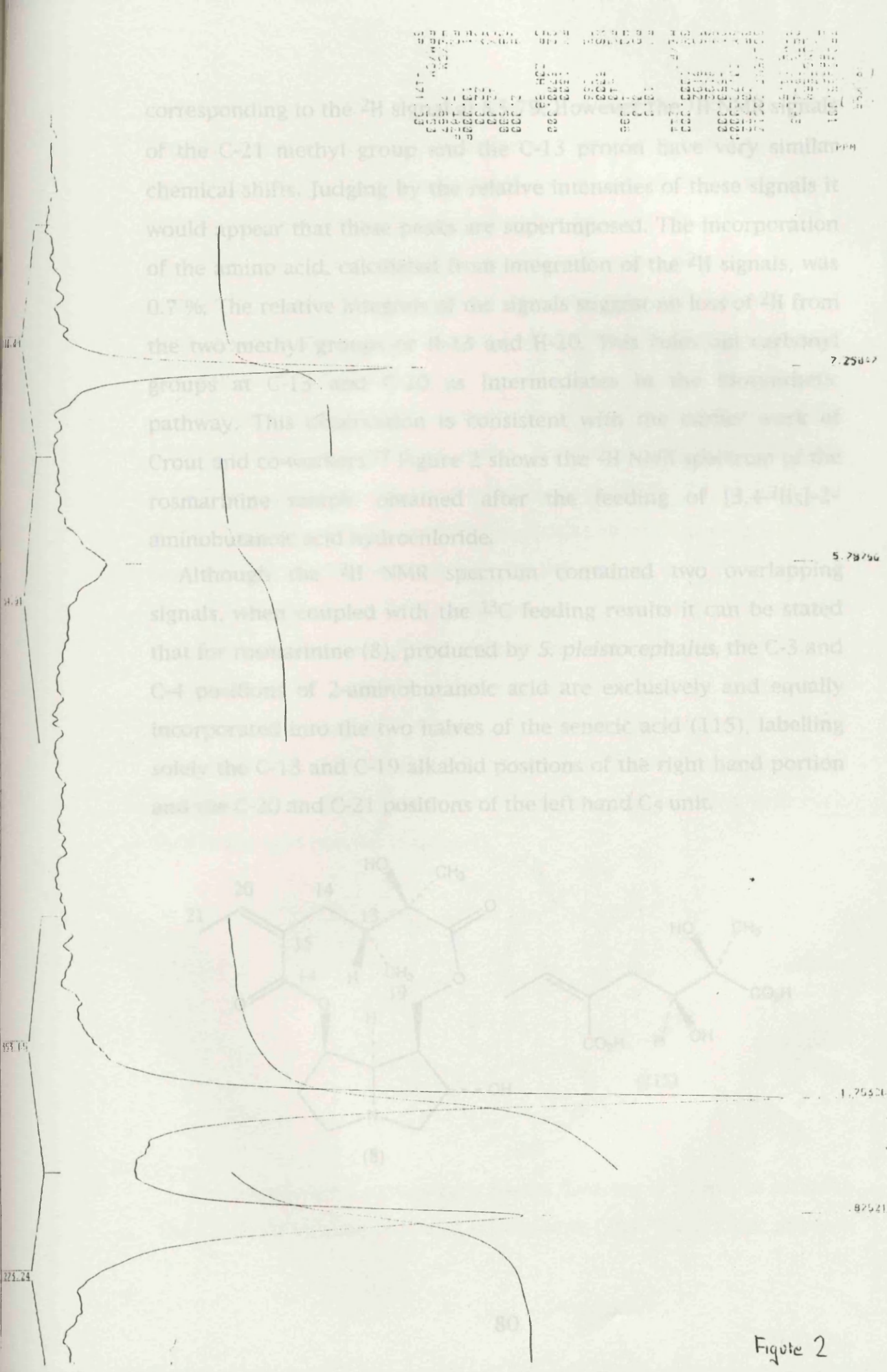
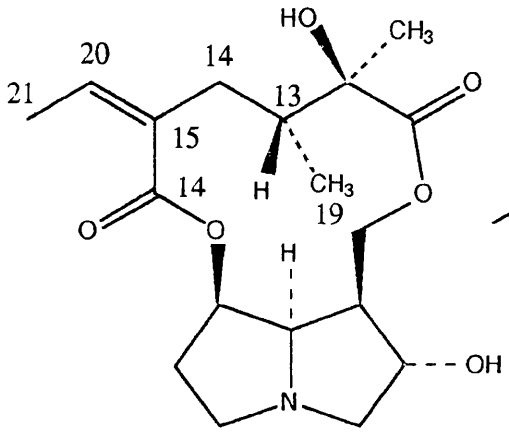


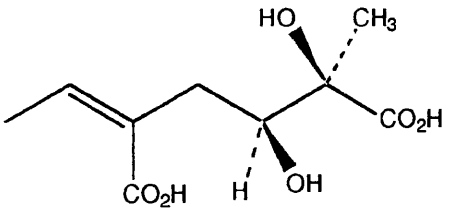
Figure 2

corresponding to the ^{2}H signal at δ 5.79. However the ^{1}H NMR signals of the C-21 methyl group and the C-13 proton have very similar chemical shifts. Judging by the relative intensities of these signals it would appear that these peaks are superimposed. The incorporation of the amino acid, calculated from integration of the ^{2}H signals, was 0.7 %. The relative integrals of the signals suggest no loss of ^{2}H from the two methyl groups or H-13 and H-20. This rules out carbonyl groups at C-13 and C-20 as intermediates in the biosynthetic pathway. This observation is consistent with the earlier work of Crout and co-workers.[77] Figure 2 shows the ^{2}H NMR spectrum of the rosmarinine sample obtained after the feeding of [3,4-$^{2}H_5$]-2-aminobutanoic acid hydrochloride.

Although the ^{2}H NMR spectrum contained two overlapping signals, when coupled with the ^{13}C feeding results it can be stated that for rosmarinine (8), produced by *S. pleistocephalus*, the C-3 and C-4 positions of 2-aminobutanoic acid are exclusively and equally incorporated into the two halves of the senecic acid (115), labelling solely the C-13 and C-19 alkaloid positions of the right hand portion and the C-20 and C-21 positions of the left hand C_5 unit.



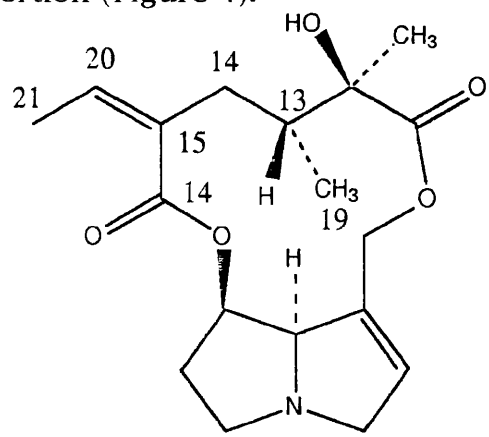
(8)



(115)

Senecic acid is also the acid portion of senecionine (26), from *Senecio vulgaris*. Although the incorporation of the precursor (163) was not as good as for rosmarinine (8), in the ^{13}C NMR of senecionine sample doublets could be observed around the signals at δ 11.1, 15.1 38.4 and 134.3. The respective coupling constants were 36, 42, 36, and 42 Hz. Again, the amounts of label in each half of senecic acid were approximately equal. Figure 3 shows the ^{13}C NMR spectrum of the senecionine obtained.

After feeding [3,4-$^{2}H_5$]-2-aminobutanoic acid to *S. vulgaris* the ^{2}H NMR spectrum showed three peaks, appearing at δ 0.91, 1.81, and 5.78. These signals correspond to labels at H-19, H-13 plus H-21 and H-20. The ratios of the relative intensities (approximately 3: 4: 1) indicate that, as for rosmarinine, the H-13 signal and the signal for the methyl group at H-21 positions are co-incident. The specific incoporation was 0.5%. These experiments show that in senecionine, produced by *S. vulgaris*, 2-aminobutanoic acid is a biosynthetic precursor. The C-3 and C-4 positions of the amino acid are converted into C-20 and C-21 of the left hand unit and the C-13 and C-19 positions of the right hand unit. There is equal labelling into each half of the acid portion (Figure 4).



(26)

The transformed root culture *Emilia flammea* is known to produce the alkaloids emiline (81) and senecionine (26). The emiline appears

HERTZ
6822.24
6815.59
6722.55
6256.65
6245.58
6095.15
6610.79
6591.95
[STIRLING IRSSS REF 2 SOLVENT CDCL3.
HERTZ
1988.06
1949.20
1931.26
1924.00
1912.11
1876.14
[STIRLING IRSSS REF 2 SOLVENT
HERTZ
270.615
252.259
230.124
684.206
665.279
526.825
558.410
539.955
494.695
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
PPM

Figure 3

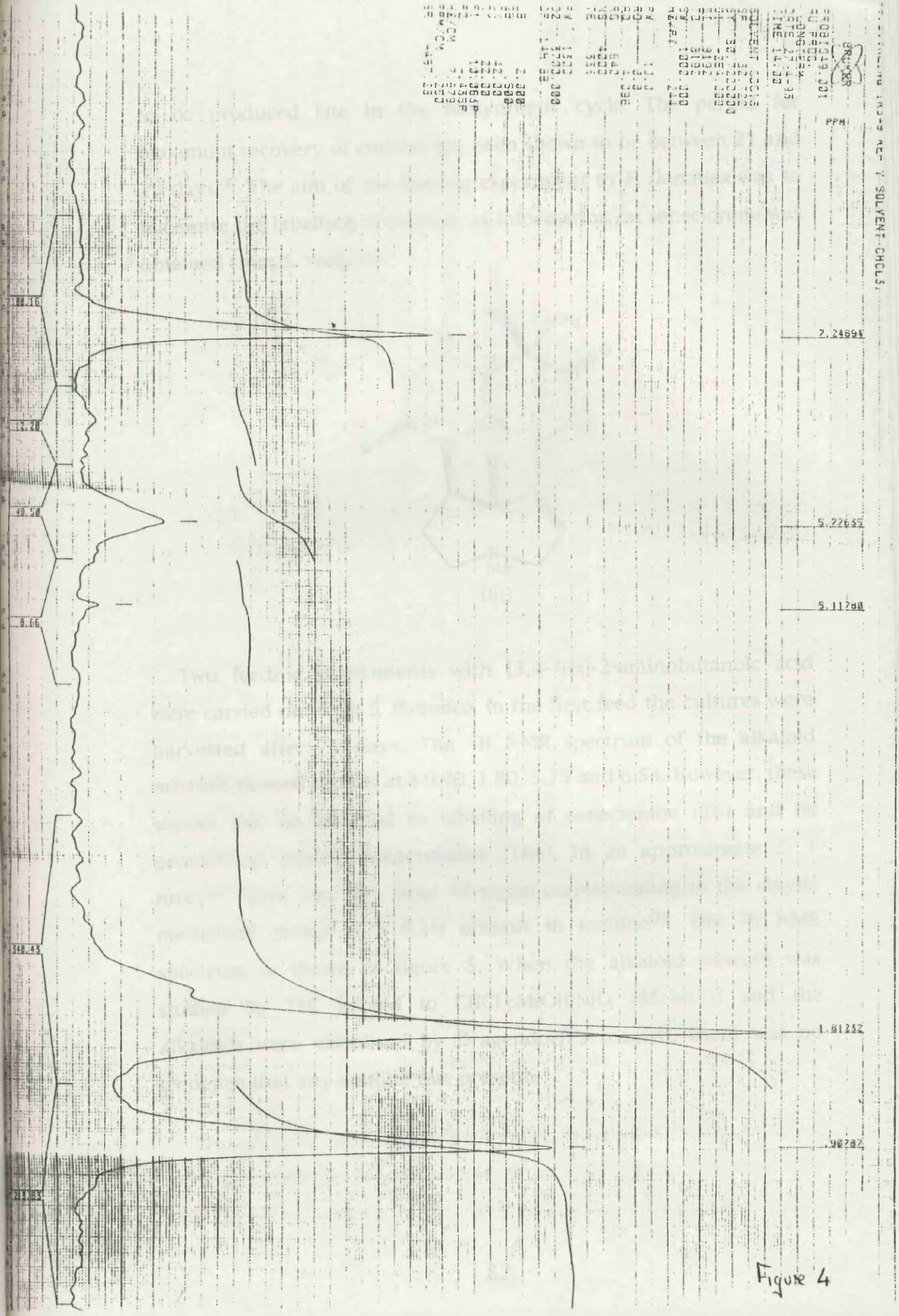
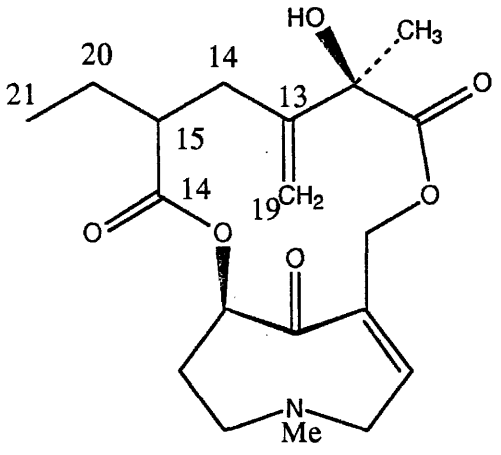


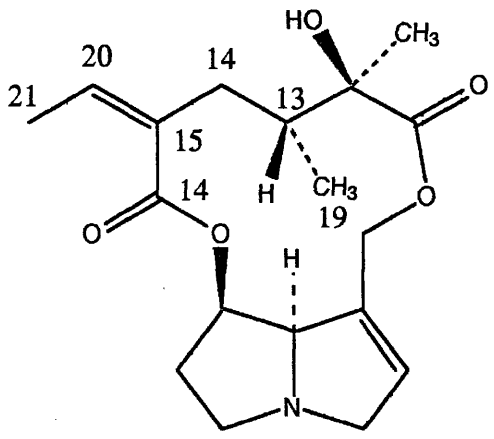
Figure 4

to be produced late in the biosynthetic cycle. The period for maximum recovery of emiline has been shown to be between 21 and 29 days.[97] The aim of the feeding experiment to *E. flammea* was to maximise the labelling of emiline, as information on senecionine was obtained from *S. vulgaris.*

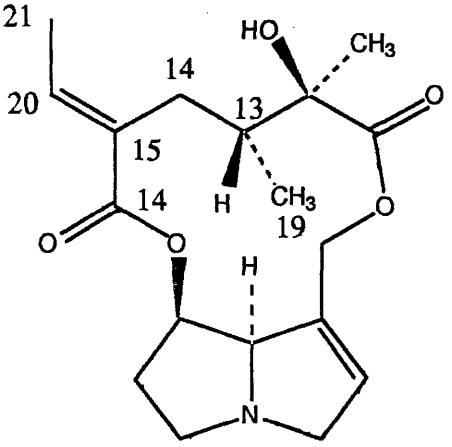


(81)

Two feeding experiments with [3,4-$^2H_5$]-2-aminobutanoic acid were carried out with *E. flammea.* In the first feed the cultures were harvested after 19 days. The ^2H NMR spectrum of the alkaloid mixture showed signals at δ 0.90, 1.80, 5.75 and 6.54. However, these signals can be assigned to labelling of senecionine (26) and its geometrical isomer integerrimine (164), in an approximate 2: 1 ratio.[98] There was very little ^2H signal corresponding to the vinylic methylene group at δ 5.10 present in emiline[99]. The ^2H NMR spectrum is shown in figure 5. When the alkaloid mixture was studied by TLC [eluted in $CHCl_3$:MeOH:NH_3 (85:14:1)] and the alkaloids were visualised by Dragendorff's reagent, there was no evidence that any emiline was present.



(26)



(164)

This feeding experiment was repeated with *E. flammea* and harvesting was carried out after 26 days. Again, examination by TLC and ^{1}H NMR spectroscopy showed that no emiline was present. When feeding experiments were carried out with *E. flammea* with ^{13}C-labelled 2-aminobutanoic acid the alkaloid extract was examined by NMR spectroscopy. Unfortunately only a small amount of alkaloid mixture was isolated and no signals could be observed in the ^{13}C NMR spectrum.

Feeding experiments with both ^{2}H- and ^{13}C-labelled 2-aminobutanoic acid were carried out with young plants of *Crotalaria lachnosema*. On this occasion the plants suffered from severe withering and very little alkaloid could be extracted. No incorporation of label was observed.

When the experiments with *C. lachnosema* were repeated the daily amount of precursor fed was reduced and the feeding was continued over a longer period. At harvesting time the plants appeared healthy and the plants were extracted according to the normal procedure.

The alkaloid mixtures were acetylated and samples of 13-*O*-acetyldicrotaline (18) were obtained. Considering the feed with $^{13}C_2$-labelled aminobutanoic acid first, the alkaloid extract yielded only 3

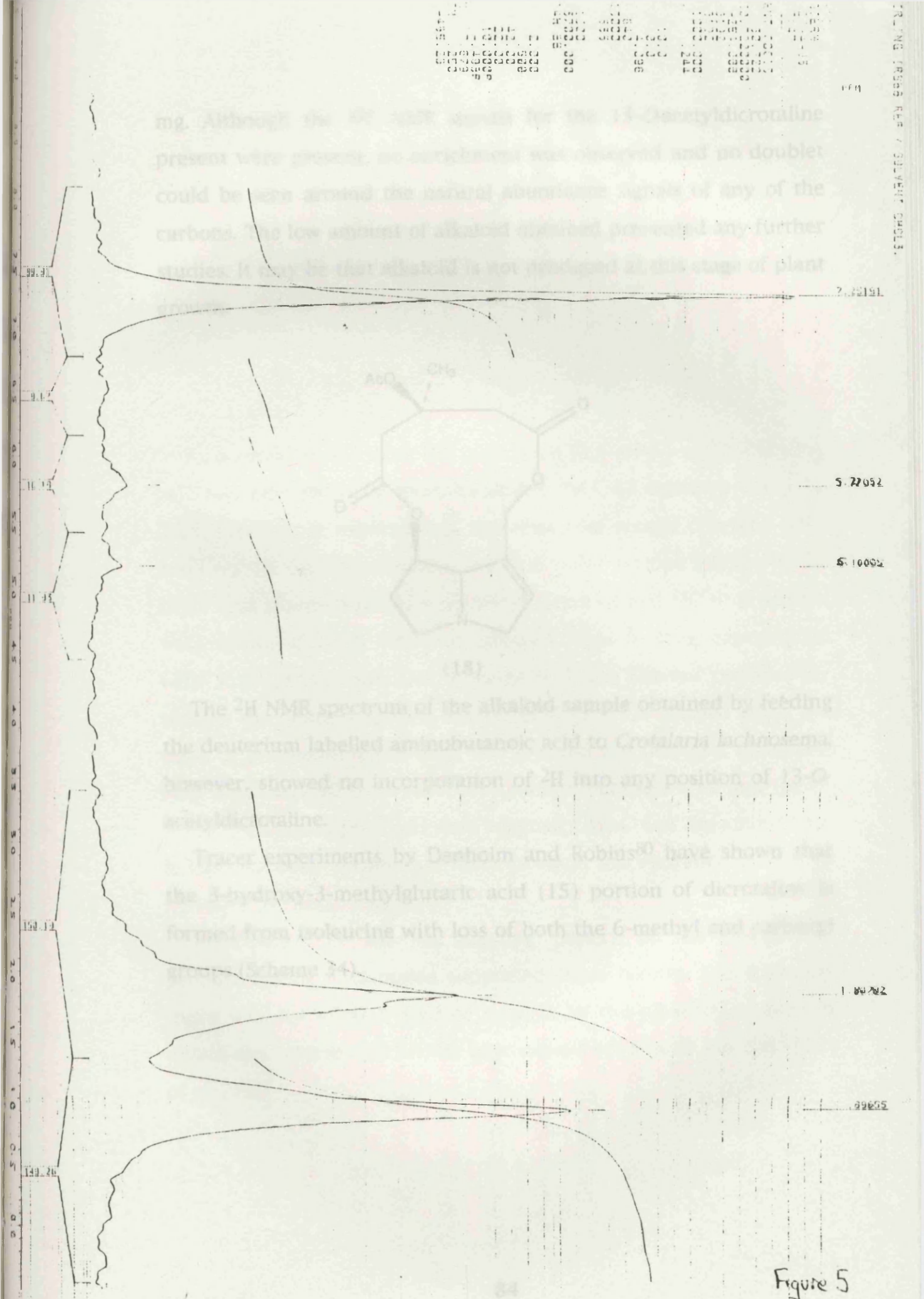
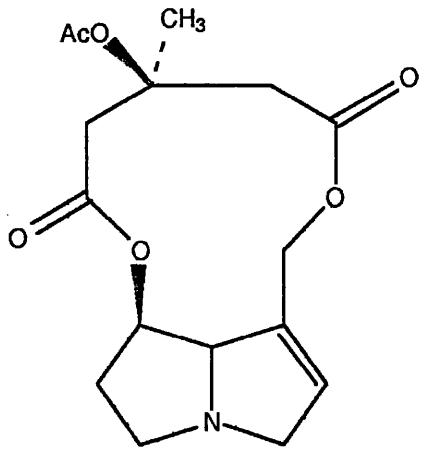


Figure 5

mg. Although the ^{13}C NMR signals for the 13-*O*-acetyldicrotaline present were present, no enrichment was observed and no doublet could be seen around the natural abundance signals of any of the carbons. The low amount of alkaloid obtained prevented any further studies. It may be that alkaloid is not produced at this stage of plant growth.

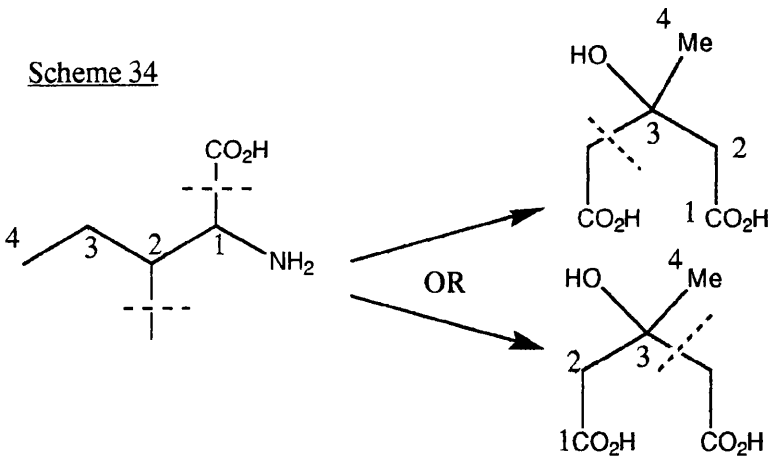


(18)

The ^{2}H NMR spectrum of the alkaloid sample obtained by feeding the deuterium labelled aminobutanoic acid to *Crotalaria lachnosema*, however, showed no incorporation of ^{2}H into any position of 13-*O*-acetyldicrotaline.

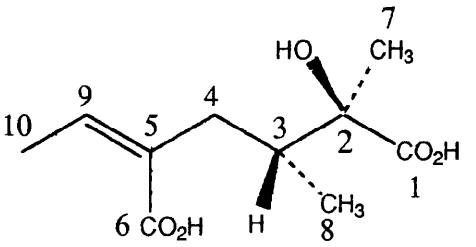
Tracer experiments by Denholm and Robins[80] have shown that the 3-hydroxy-3-methylglutaric acid (15) portion of dicrotaline is formed from isoleucine with loss of both the 6-methyl and carboxyl groups (Scheme 34).

Scheme 34

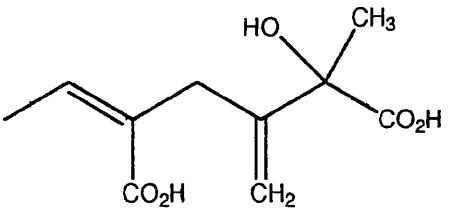


As a result of this work the label from [3,4-$^{2}H_5$]-2-aminobutanoic acid was expected to be solely located in the C-13 methyl group at δ_H 1.72. From these experiments, therefore, the results obtained have been mixed. On the plus side, the first stable isotope studies of the necic acid biosynthesis were achieved. Good ^{2}H and ^{13}C NMR spectra were obtained from alkaloids isolated from feeding experiments with *S. pleistocephalus* and *S. vulgaris*. These showed conclusively that in the *Senecio* species senecic acid (115) is formed from two units of 2-aminobutanoic acid. The C-3 and C-9 positions of senecic acid are generated from the C-3 of 2-aminobutanoic acid, and the C-8 and C-10 diacid positions were originally the C-4 of the amino acid. It has also been shown that there is equal incorporation into both halves of the necic acid.

Examination of the ^{2}H labelling pattern of the senecionine produced from *E. flammea* supported these results. Unfortunately there was not enough emiline present in the alkaloid mixture to obtain any information on the biosynthesis of the acid portion (165) of emiline.



(115)



(165)

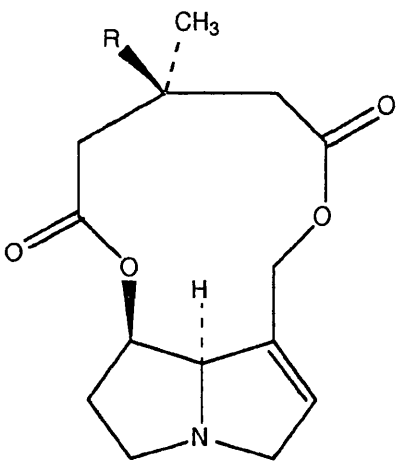
Unfortunately the feeding experiments with *C. lachnosema* did not give any data on the biosynthesis of the 3-hydroxy-3-methylglutaric acid portion. Despite careful feeding conditions no incorporation of 2-aminobutanoic acid was observed. These African plants are slow growing and do not thrive in our northern climate. It is clear that alkaloid levels are low in these plants and very little new alkaloid was produced during the course of the experiments.

CHAPTER 6

Assignment of NMR Spectra and Conformational Analysis of 13-*O*-Acetyldicrotaline

6.1 Introduction

Dicrotaline (16) is known to be the major pyrrolizidine alkaloid produced by *Crotalaria lachnosema*.[86] As 13-*O*-acetyldicrotaline (18) is also present, the most convenient method of analysis is to convert all the alkaloidal material isolated to the acetylated product.



(16) R = H
(18) R = OAc
(166) R = Me

Work by Denholm and Robins[80] identified isoleucine as a precursor. For stable isotope studies on 13-*O*-acetyldicrotaline a full assignment of the ^{1}H and ^{13}C NMR spectra was required. Particular problems to be resolved were: (1) the distinction of the two halves of the symmetrical 3-acetoxy-3-methylglutarate portion (including the *pro*-R and *pro*-S assignments of the protons); (2) the stereochemical assignments of the methylene protons of the necine; and (3) the relative conformation of

the carbonyl ester groups of 13-*O*-acetyldicrotaline for use in possible structure activity relationships.

6.2 Results and Discussion

The mixture of dicrotaline (16) and 13-*O*-acetyldicrotaline (18) was extracted from *C. lachnosema* and converted into 13-*O*-acetyldicrotaline.[100] The ^{1}H and ^{13}C NMR spectra of this sample were studied at 4.7T with the help of Dr. D. S. Rycroft. The signals are named from A to Q in increasing order of shielding for the purpose of this discussion. The chemical shifts and coupling constants are listed in table 1.

The proton on the double bond (H_A) was identified at δ 5.90, coupling to three methylene protons, vicinally to F and G at C-3, and allylically to E at C-9. These two methylene positions were identified by the greater deshielding of B and E relating them to 9-H_2. The fact that no coupling was observed between A and B implies that B lies in the plane of the double bond. Similarly, homoallylic coupling of 3-H_2 with E but not with B supports this. The 3-H_2 protons also couple with the methylene proton at C-8 (D). The signal D also couples with allylically to proton A and the methylene proton C, which is C-7. Signal C correlated with the combined signal for the resonances of N and O, assigned to 6-H_2. The methylene protons at C-5, signals H and L, also correlated to 6-H_2.

Furthest upfield was the doublet, Q, at δ 1.72, corresponding to the methyl group attached to C-13. The signal P was due to the acetate methyl group. The two halves of the glutarate portion were distinguished by a 2D δ_C/δ_H long range correlation experiment.[101]

The furthest downfield carbonyl signal was at δ 168.7. As this correlated with B and E (9-H_2) and with I and M, assigned to 12-H_2 on

the acid portion, this signal was assigned to C-11. The most deshielded carbonyl signal correlated with the methyl protons (P) of the acetate. The remaining carbonyl signal (δ 169.5) must therefore be C-15, as it correlated with J and K which were assigned to 14-H_2. However no correlation was observed with H-7.

To determine the stereochemical questions, NOE difference experiments were used. The results obtained are shown in Table 2.

The protons at C-8 and C-7 must be on the same side of the molecule because of the NOEs between A and B. As the proton B has already been deduced to be planar to the double bond, the conformation around the C-9 bond must be as shown in Figure 6.

Other NOE findings agreed with this conclusion. Arising from the three spin effect[102] there was a negative NOE at E after irradiation of A, and a negative NOE at D after irradiation of B. Signal B was therefore assigned as the H-9 *pro*-S proton, and E as the *pro*-R proton of H-9. Substantial NOEs were observed between G and L but not between F and H. Signals G and L were therefore assigned as the β-protons of C-3 and C-5 respectively. on irradiating at F, a negative NOE was observed at L and *vice versa*. This was consistent with the assignment.

A more complex analysis was necessary to determine the stereochemistry of the acid portion. Long range proton-proton couplings around C-12, C-13 and C-14 indicated that one conformation was preferred.

The C-13 methyl group (Q) couples only to K, whereas irradiation of Q caused positive NOEs at the methylene protons J, I and M, but a small negative NOE at K. This implies that Q is *gauche* to J, I and M, but *antiperiplanar* to K, as shown in figure 6. Signal J was assigned to the H-14-*pro*-R and K to the H-14-*pro*-S proton. A coupling constant of 1.2 Hz between I and J was observed due to the near W-coupling path

Table 1. NMR parameters[a] of 13-*O*-acetyldicrotaline (2) ($CDCl_3$ solution)

Site	δ_C	δ_H	Label	Multiplicity	^2J (Hz)	^3J (Hz)[b]	^4J (Hz)[b]	^5J (Hz)[b]
1	132.5							
2	131.4	5.90	A	quin.		2.0 (3α), 2.1 (3β)	1.6 (8), 1.5 (7)	
3α	61.9	3.88	F	dtd	15.8	2.0 (2)	2.9 (8)	2.9 (9-*pro-R*)
3β		3.40	G	ddt	15.8	2.1 (2)	5.6 (8)	1.7 (9-*pro-R*)
5α	53.7	3.28	H	dm	8.9	Σ = 8.8 (6α, 6β)		
5β		2.55	L	dm	8.9	Σ = 18.0 (6α, 6β)		
6α, 6β	34.0	2.07	N, O	m				
7	75.1	5.16	C	dt		4.4 (8), Σ = 5.0 (6α, 6β)		
8	77.0	4.30	D	br m				
9-*pro-R*	60.2	4.09	E	ddq	12.2		1.5 (2), 1.3 (8)	2.9 (3α), 1.7 (3β)
9-*pro-S*		5.31	B	d	12.2			
11	168.7							
12-*pro-R*	41.3	3.26	I	dd	15.6		1.2 (14-*pro-R*)	
12-*pro-S*		2.41	M	d	15.6			
13	79.5							
14-*pro-R*	43.7	2.93	J	dd	13.4		1.2 (12-*pro-R*)	
14-*pro-S*		2.78	K	dq	13.4		0.8 (13-Me)	
15	169.5							
13-Me	26.1	1.72	Q	d			0.8 (14-*pro-S*)	
13-*O*-Ac	22.3	2.00	P	s				
13-*O*-CO	170.2							

[a] Absolute values of coupling constants are shown. δ_C is relative to $CDCl_3$ at δ 77.0 and δ_H is relative to $CHCl_3$ at δ 7.25.
[b] The site number of the coupling partner is given in parentheses.

Table 2. NOEs observed[a] in 13-*O*-acetyldicrotaline (2)

	Proton irradiated.[b] label and percentage saturation													
	2	3α	3β	5α	5β	6α, β	7	8	9-*pro-R*	9-*pro-S*	12-*pro-R*	12-*pro-S*	14-*pro-R*	13-Me
Site of	A	F	G	H	L	N, O	C	D	E	B	I	M	J	Q
NOE	63	32	19	34	34	17[c]	50	48	28	40	37	41	30	66
2		5	4						−2	7				
3α	3		23		−1									
3β	1	25			6									
5α					31	2								
5β		−1	5	21		−2								
6α, β[d]				2			2							
7					0.5	13		10				2		0.4
8						2	10		3	−1				
9-*pro-R*	−1							2		34				
9-*pro-S*	3								33					
12-*pro-R*												40		2
12-*pro-S*							2				28			5
14-*pro-R*														6
14-*pro-S*														−0.4
13-Me											0.4	1	1	

[a] The NOEs reported are the result of scaling up the observed NOEs in inverse proportion to the degree of saturation in order to obtain values equivalent to the result of complete saturation.
[b] No NOEs were observed when H-14-*pro-S* or the acetate protons were irradiated.
[c] Average saturation of the combined signal. In reporting the NOEs in this column it has been assumed that the two protons were equally saturated.
[d] Scaled-up percentage enhancements of the combined signal.

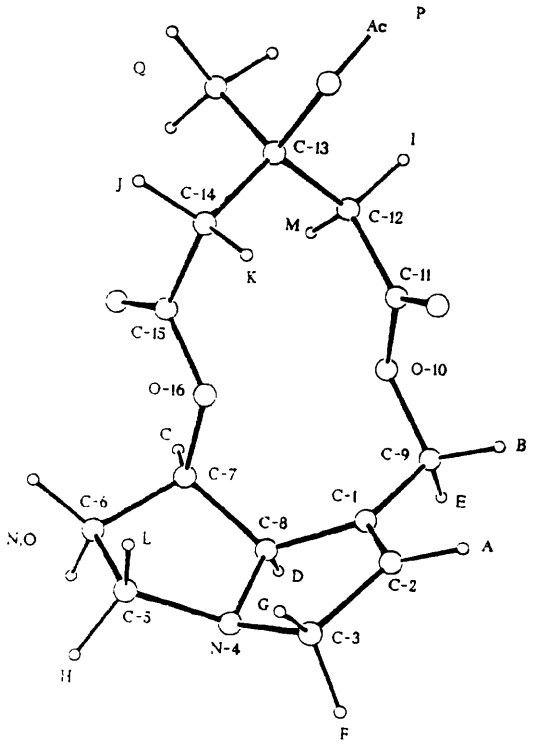


Figure 5 Drawing of the proposed preferred conformation of 13-*O*-acetyldicrotaline.

between J and H-12-*pro*-R. Therefore I was attributed to the latter proton and M to H-12-*pro*-S.

The X-ray crystal structure results on studies with 13,13-dimethyl-1,2-didehydrocrotaline picrate, the picrate salt of (166),[103] combined with the assignments deduced above suggested that the preferred conformation of 13-O-acetyldicrotaline must be similar to that shown in figure 6.

The ester carbonyl functions are antiparallel with the C-15 carbonyl group on the α-face, and the C-11 carbonyl group on the β-face of the molecule. In conformations where the C-11 carbonyl group is directed below the plane of the macrocycle H_B is displaced out of the plane of the double bond. Also, in conformations when the C-15 carbonyl group is directed above the plane of the macrocycle it was impossible to achieve the correct relationship around the C-12, C-14 and C-15 region.

Further evidence in support of the conformation shown in figure 6 came from analysing the effect of changing the C-11 carbonyl group on the chemical shifts of the 9-H_2 protons. In 13,13-dimethyl-1,2-didehydrocrotaline picrate (166) these protons were observed at δ 4.08 and 5.32, very similar to the shifts in 13-*O*-acetyldicrotaline. Dicrotaline (16) shows the chemical shift of the 9-H_2 protons as δ 4.18 and 5.41, possibly due to hydrogen bonding of the hydroxyl group. Naturally occurring pyrrolizidine alkaloids derived from retronecine, and containing an 11-membered ring, mainly have the ester groups *syn*-parallel and directed below the plane of the macrocycle.[2] These conformations normally have the substituent on the α-face at C-13 approximately antiperiplanar to the β-substituent of both C-13 and C-14. This was not observed in the analysis of 13-O-acetyldicrotaline.

A few significant NOEs were observed between necine and acid portion protons. Irradiation of the H-12-*pro*-S proton caused a NOE at

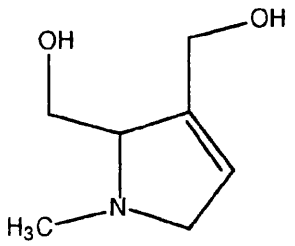
C (H-7), and *vice versa*. Irradiation of the methyl protons Q also caused a NOE at C. The conformation shown in figure 6 does not explain these NOEs, but they may be accounted for by a contribution from a conformational equilibrium from other conformations where the C-13 end of the macrocycle is folded down, The NOEs also required that the methyl protons Q, and the proton M are on the underface of the molecule as shown in figure 6. This confirmed that the configuration of C-13 is *S*, agreeing with previous degradation experiments.[100] If the configuration were *R*, there could be no possible conformation which would give the NOEs observed. Thanks are due to Dr. D. S. Rycroft and Miss I. Freer for their help in carrying out this work. This work has been published.

CHAPTER 7

Formation of Synthanecines

7.1 Introduction

Synthetic analogues of pyrrolizidine alkaloids are required for metabolic and toxicological studies. The first synthesis of the most common necine retronecine (4) was reported in 1962,[105] but the route was lengthy and the overall yield was low (<1%). This led to the preparation of a range of monocyclic analogues of pyrrolizidine bases, called synthanecines.[106,107] For example, synthanecine A is 2,3-bis(hydroxymethyl)-1-methyl-3-pyrroline (19) and synthanecine B (20) is the corresponding saturated derivative. The structural similarity of synthanecines A and B to retronecine (4) and platynecine (167) respectively, is shown.



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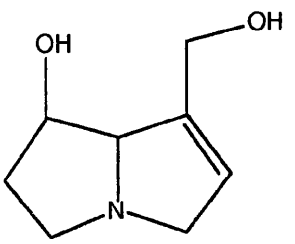
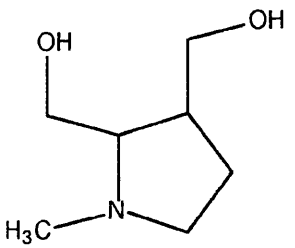
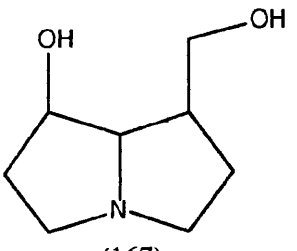


(4)



(20)



(167)

Synthanecines A and B were required for feeding experiments and a route for the synthesis of novel synthanecines for the investigation of possible anti-tumour activity was developed.

7.2 Formation of Synthanecines A and B for Feeding Experiments

The synthesis of synthanecines A and B has been well documented.[106,108,109] Mattocks originally prepared a range of analogues for toxicology studies, while possible anti-tumour activity was investigated by Barbour[108] and by Baxter.[109] It was intended that synthanecines A and B would be prepared, using known procedures, for feeding experiments with *S. vulgaris*. The root cultures would then be extracted and the extracts analysed for the appearance of any new alkaloid analogues.

A modified version of the procedure initially developed by Mattocks[106] was used to form the synthanecines (Scheme 47). Additional spectroscopic data have also been reported. Methylamine was added to diethyl maleate (168) to give diethyl 2-methylaminosuccinate (169) in good yield (81%). The *N*-methyl signal appeared at δ 2.42 in the ^{1}H NMR spectrum. The single C-H proton was observed as a doublet of doublets at δ 3.55.

The diethyl 2-methylaminosuccinate (169) was then alkylated by treatment with ethyl bromoacetate to yield the triester (170). There were three triplets at δ 1.25, 1.27, and 1.30 in the ^{1}H NMR spectrum. All these signals had coupling constants of 7.1 Hz. The spectrum also showed a singlet at δ 3.43 ppm corresponding to the methylene position adjacent to the nitrogen.

Dieckmann cyclisation of the triester (170) with 95% sodium hydride resulted in the formation of diethyl 1-methyl-4-

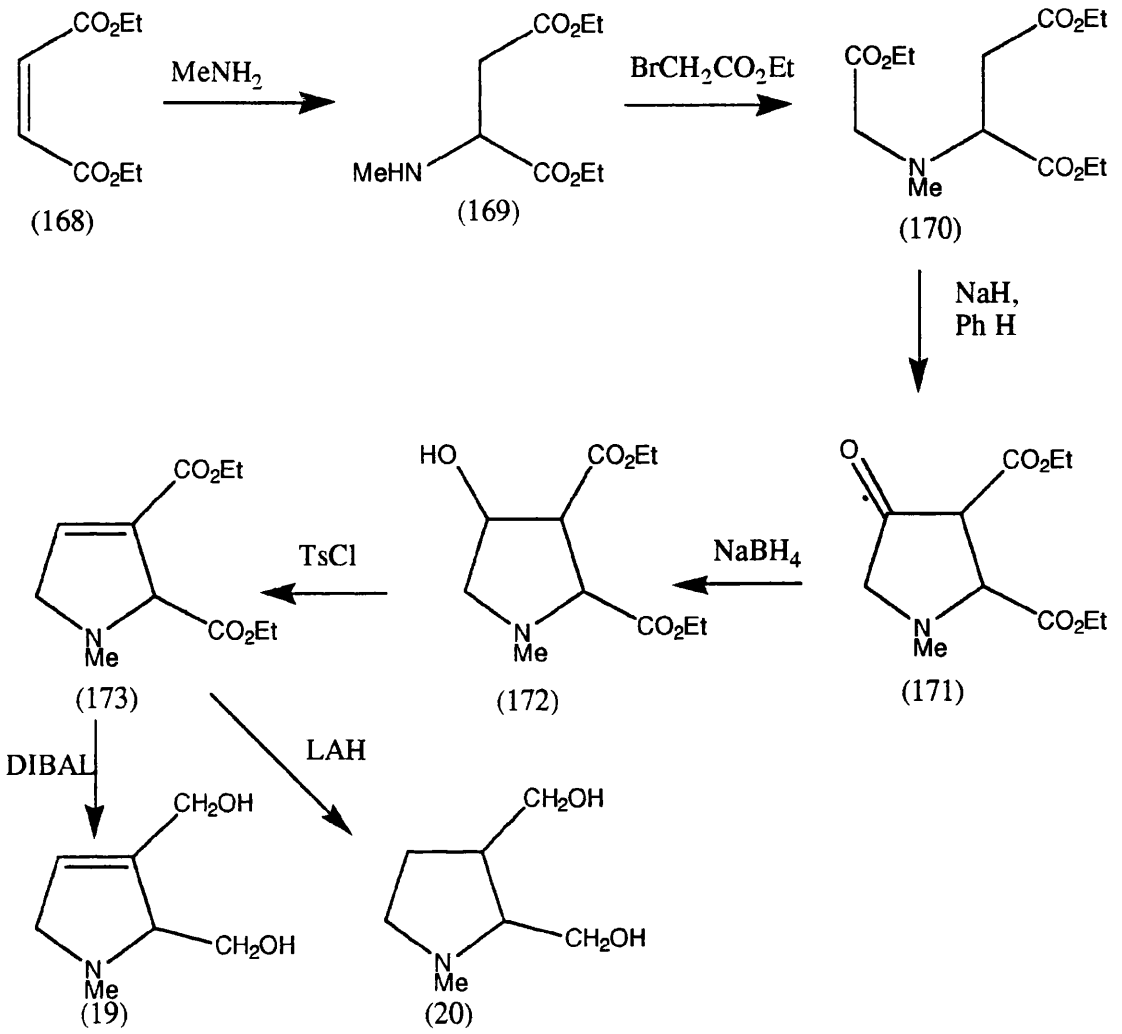
oxopyrrolidine-2,3-dicarboxylate (171). The structure of the cyclised product was verified by an absorption at 1770 cm^{-1} in the IR spectrum, due to the cyclic ketone. In the ^{1}H NMR spectrum the ring methylene group appeared as AB systems with geminal coupling constants of 17 Hz.

Reduction of the ketone with sodium borohydride gave the hydroxypyrrolidine (172). There was a broad signal at 3450 cm^{-1} in the IR spectrum for the O-H function. There were three peaks in the ^{13}C NMR spectrum corresponding to the different diastereomers.

This hydroxypyrrolidine (172) was dehydrated in pyridine using *p*-toluenesulfonyl chloride to the pyrroline diester (173). Any pyrrolic material (non-basic) formed in this reaction was easily removed by an acid-base wash of the products. The vinylic proton appeared at δ 6.88 in the ^{1}H NMR spectrum. The C=C stretching frequency was observed at 1645 cm^{-1} in the IR spectrum, and the unsaturated C-H stretch showed at 3020 cm^{-1}.

Synthanecine A and synthanecine B could both be formed from this intermediate. The correct choice of reducing agent for reduction of the pyrroline diester (173) was crucial. Using the fact that di-isobutylaluminium hydride does not reduce C=C bonds, reduction with lithium aluminium hydride gave synthanecine B (20), while reduction with DIBAL gave mainly synthanecine A (19) (Scheme 47). The ^{1}H NMR spectrum of synthanecine A showed the hydroxyl protons as a broad signal at δ 4.65. The vinylic proton appeared as a broad peak at δ 5.62. Synthanecine B was formed as a mixture of diastereomers. The O-H groups appeared as a broad singlet at δ 4.90 in the ^{1}H NMR spectrum.

Scheme 47



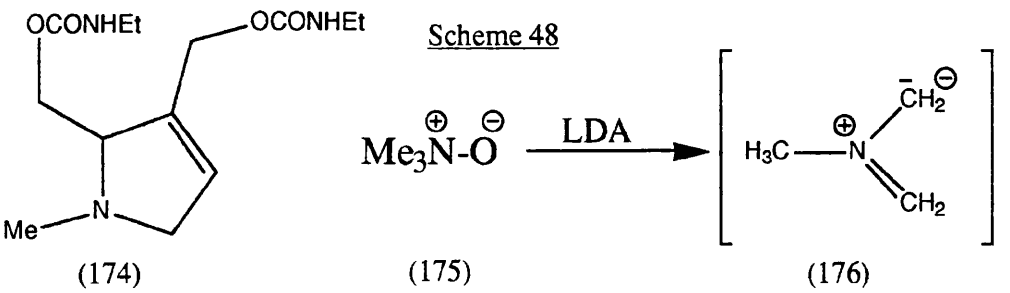
Synthanecines A and B were thick gums and purification was a problem. The normal acid-base wash procedure was impossible due to the water solubility of the amino diols. Mattocks' method using buffer solution proved unsuccessful.[106] The formation of crystalline hydrochloride and oxalate salts of the synthanecines was not achieved either. Column chromatography on basic alumina was found to be the best purification method. Synthanecines A and B were then fed to the transformed root culture *S. vulgaris.*

7.3 Results of Feeding Experiments with Synthanecines A and B

Synthanecines A and B were fed to *Senecio vulgaris* root cultures. The cultures were extracted after 21 days and the extracts were studied by TLC and mass spectroscopy. There was no evidence of any material other than senecionine present in either mixture.

7.4 Preparation of Synthanecines for Testing of Anti-tumour Activity

The variety of the biological action of pyrrolizidine alkaloids has encouraged a detailed investigation of their properties. As synthanecine A biscarbamate (174) has shown anti-mitotic activity,[2] the preparation of synthetic analogues of pyrrolizidine alkaloids for similar studies is an attractive proposition. In an attempt to develop a short, efficient synthesis to synthanecines the 1,3-dipolar cycloaddition reaction published by Roussi and co-workers[110] was studied. This suggested the possibility of using trimethylamine *N*-oxide (175), to generate the azomethine ylid (176), which should undergo 1,3-dipolar cycloaddition with a range of alkenes, to furnish a range of pyrrolidines. Roussi *et al.*, observed cycloadditions with allyl alcohol and a series of unfunctionalised alkenes (Scheme 48).

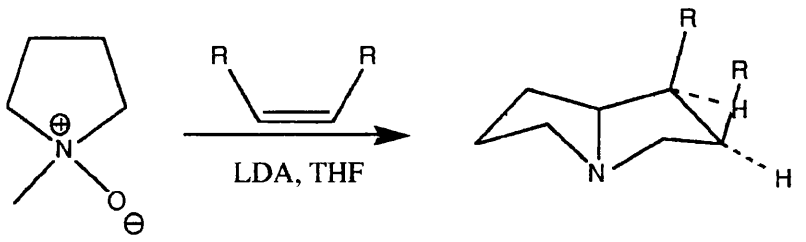


Scheme 48

(174) (175) (176)

The pyrrolizidine ring structure was also constructed using this procedure, with the R groups being phenyl, alkyl or acetal functions[111] (Scheme 49).

Scheme 49



This 1,3-dipolar cycloaddition reaction is important, because previously azomethine ylids were known only to be formed from precursors bearing electron withdrawing or conjugating substituents, formed, for example, by thermal ring opening of aziridine or by the deprotonation of iminium salts.[112] The ylid produced from trimethylamine *N*-oxide is an extremely reactive species and can be quenched by simple alkenes (hex-1-ene, cyclopentene or cyclohexene). The stabilised ylids only react with activated dipolarophiles (ethylene dicarboxylic esters, phenylmaleimide, etc.).

Roussi and co-workers[110] reported the cycloaddition of ylid (176) with allyl alcohol (177). A repeat of this reaction was attempted, together with further cycloadditions with propargyl alcohol (178), dimethyl acetylenedicarboxlate (179), diethyl fumarate (180), diethyl malonate (181) and *cis*-4,7-dihydro-1,5-dioxepin (182) (Scheme 50).

However, many attempts at repeating the cycloaddition with allyl alcohol were unsuccessful in forming 3-hydroxymethyl-1-methylpyrrolidine (183). Visualisation of the TLC plate with Dragendorff's reagent, specific for tertiary amines, showed that no product had been formed. The reaction was attempted at temperatures between 0 and -78°C, and reaction times up to 24 hours. A similar reaction with propargyl alcohol (178) also did not appear to yield any of the expected pyrroline (184).

avoid the possible ring opening to the water soluble amino diol species, TLC monitoring of the reaction mixture did not reveal a spot with the Dragendorff reagent. It was obvious after the failure of the dioxepin to undergo cycloaddition, that this method was not practical as a route to synthanecines and that some other procedure was required. The reactions which were attempted are summarised in table 3.

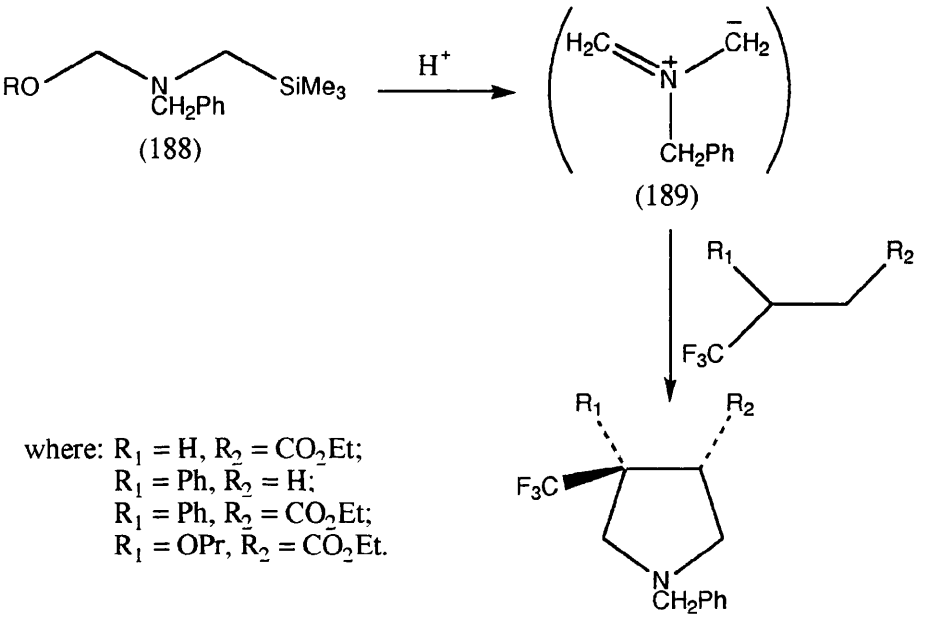
Table 3

Dipolarophile	Reaction Temp.	Reaction Time
Allyl alcohol	-78 °C	4 h and 6 h
	0 °C	4 h and 20 h
Propargyl alcohol	-78 °C	4 h
Diethyl maleate	-78 °C	4 h and 8 h
	0 °C	4 h and 20 h
Diethyl fumarate	-78 °C	4 h and 6 h
	0 °C	3 h and 6 h
Dimethyl acetylene-dicarboxylate	-78 °C	6 h
	0 °C	6 h
cis-4,7-Dihydro-1,5-dioxopin	-78 °C	4 h
	0 °C	4 h

7.5 Alternative Procedure for 1,3-Dipolar Cycloaddition Reactions

When the procedure used by Roussi and co-workers[110] could not be repeated to give the synthanecines an alternative method was needed. A recent publication by Begue *et al.*[113] noted the formation of fluorinated pyrrolidine rings via 1,3-dipolar cycloaddition. The method had been developed from a procedure where non-fluorinated, disubstituted alkenes activated by a carbonyl group reacted with an azomethine ylid to provide pyrrolidines in good yields[114] (Scheme 52).

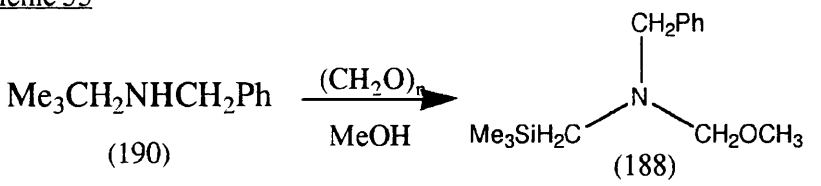
Scheme 52



where: $R_1 = H$, $R_2 = CO_2Et$;
$R_1 = Ph$, $R_2 = H$;
$R_1 = Ph$, $R_2 = CO_2Et$;
$R_1 = OPr$, $R_2 = CO_2Et$.

This procedure used *N*-benzyl-*N*-(trimethylsilylmethyl)-aminomethyl methyl ether (188) to generate the azomethine ylid (189). The required aminomethyl methyl ether was prepared in good yield from *N*-(trimethylsilylmethyl)benzylamine (190), with formalin in methanol (Scheme 53).

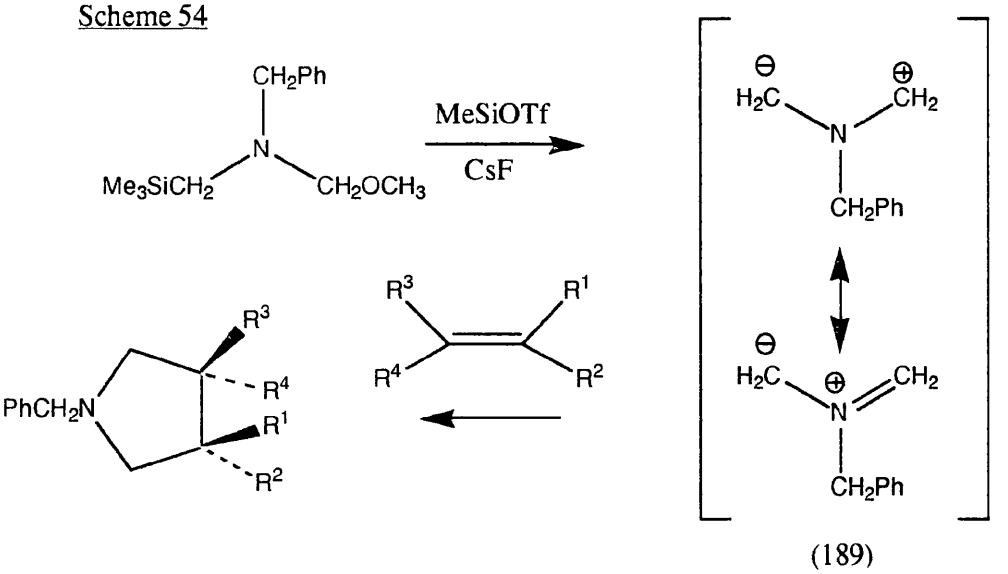
Scheme 53



The ^1H NMR spectrum of the product showed a multiplet corresponding to the benzyl group, and five singlets at δ 0.0, 2.1, 3.2, 3.7 and 4.0. The trimethylsilyl group appeared at δ 0.0 and the methylene group adjacent to the silicon at δ 2.1. The signal at δ 3.2 is indicative of the methyl ether group, and the two other singlets are due to the benzyl methylene and the methylene group between the nitrogen and the oxygen.

Decomposition of the amino methyl ether (188), catalysed by trimethylsilyl trifluoromethanesulfonate and caesium fluoride, generates the unstabilised azomethine ylid (189), which can react with electron deficient alkenes. The cycloaddition proceeds stereospecifically in good yield (Scheme 54).

Scheme 54



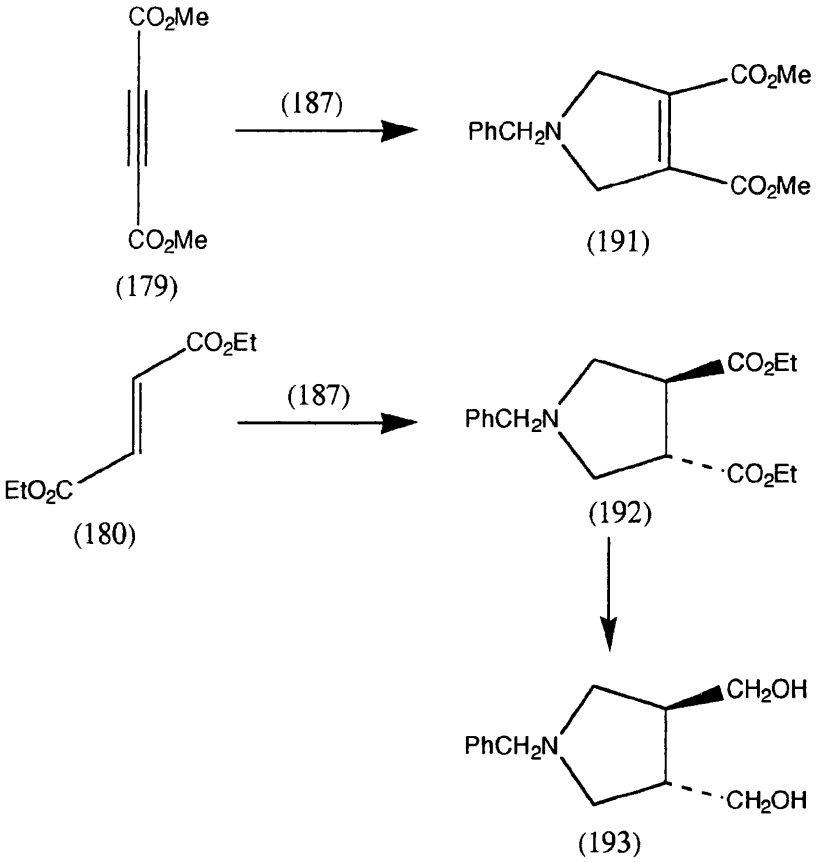
This cycloaddition method was successful with dimethyl acetylenedicarboxylate (179) and diethyl fumarate (180) (Scheme

55). Reaction with the alkyne (179) gave the pyrroline (191). The presence of the cyclised product was demonstrated by the appearance of a molecular ion peak in the mass spectrum. ^{1}H NMR spectroscopy showed three singlets in a ratio of 3:1:2, corresponding to the methyl ester groups and the two different methylene functions. All these signals had similar chemical shifts, around δ 3.8. The ^{13}C NMR spectrum showed both olefinic carbons appearing co-incidently at δ 137.1.

1,3-Dipolar cycloaddition of the ylid (189) with diethyl fumarate (180) also gave the desired product in good yield (84%). The pyrrolidine (192) also showed a molecular ion peak in the mass spectrum at *m/z* 305. The product appeared to be formed stereospecifically, with only one racemate observed in the ^{13}C NMR spectrum. ^{1}H NMR spectroscopic analysis of the pyrrolidine showed a triplet at δ 1.18 and a quartet at δ 4.09, with coupling constants of 7.2 Hz, corresponding to the ethyl ester groups. The benzylic methylene position appeared as a singlet at δ 3.54, with the pyrrolidine ring protons identified as multiplets at δ 2.78 and δ 3,39. Purification of the cyclised products was by preparative TLC, eluted with ethyl acetate.

The pyrrolidine (192) was taken one step further and reduced with di-isobutyl aluminiumhydride, to yield the diol (193). The product is a crystalline material, with a melting point of 82°C. The ^{13}C NMR spectrum of (193) showed the C-3 and C-4 positions at δ 44.6. The three different methylene groups corresponded to signals at δ 57.7, 60.2, and 65.7. A peak at *m/z* 221 in the mass spectrum also indicated the formation of the synthanecine (193) (Scheme 55).

Scheme 55



Unfortunately lack of time prevented further elaboration of this route.

7.6 Conclusions

Repeated attempts at using the procedure of Roussi and co-workers[110] to furnish a range of synthanecines were unsuccessful. The 1,3-dipolar cycloaddition reaction with electron deficient alkenes appeared to undergo side reactions readily, but there is no explanation as to why the cycloaddition with allyl alcohol, as reported by Roussi, could not be repeated. However from *N*-(trimethylsilyl methyl)benzylamine it was possible to achieve 1,3-dipolar cycloaddition, after conversion into the methyl ether, using the procedure described by Begue *et. al.*[113] It should be possible to

prepare a variety of synthanecines using this short and efficient synthesis.

CHAPTER 8

Experimental

8.1 General

Melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Mass spectra were obtained with A.E.I. MS 12 or 902 spectrometers. Nuclear magnetic resonance (NMR) 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 30.72 MHz (δ_D). 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 an internal standard. Infrared spectra (IR) 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. Plates were visualised with Dragendorff's reagent unless otherwise stated. The TLC plates were eluted with the solvent system CH_3Cl:MeOH:NH_3 (85:14:1) unless otherwise stated.

Tetrahydrofuran (THF) was dried by distillation from sodium-benzophenone under nitrogen prior to use. Other solvents and reagents were purified by standard techniques. Acetic anhydride was distilled from calcium hydride and stored over 4Å sieves.

Triethylamine was distilled from, and stored over, potassium hydroxide.

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

8.2 Experimental for Chapter 3

Synthesis of 4,5-Dihydro-2-methylthiazole (128)

Triethylamine (4.45 ml, 3.172 g, 31.3 mmol) was added to aminoethanethiol (3.569 g, 31.3 mmol) in methanol (40 ml) and stirred for 1 h. Acetonitrile (1.280 g, 31.3 mmol) was then added and the mixture was heated at reflux temperature for 24 h. The methanol was distilled off and the residue was partitioned between ether (30 ml) and water (30 ml). The ether layer was separated and the aqueous layer was extracted with ether (8 x 30 ml). The combined ether layers were dried over anhydrous potassium carbonate and filtered. The ether was then removed by distillation yieding the title compound as a pale yellow oil (3.168 g, 41%); υ_{max} 2980 (C-H), 1680 (C=N), 1480, 1180 (N=C-S) 940, and 750 cm^{-1}; δ_H (200 MHz) 2.07 (3H, t, J 1.6 Hz, CH_3), 3.17 (2H, t, J 8.4 Hz, 5-H_2), 4.06 (2H, tq, J 8.4, 1.6 Hz, 4-H_2); δ_C 19.6 (CH_3), 34.3 (C-5), 64.4 (C-4), 166.4 (C-2); *m/z* 101 (M^+, 58.6%), 73, 60 (100%), 59, 55; (Found: M^+, 101.0302. C_4H_7NS requires *M*, 101.0299).

Synthesis of 4,5-Dihydro-2-propylthiazole (129)

A three-neck flask was equipped with a magnetic stirrer and two dropping funnels under a nitrogen atmosphere. The flask was charged with 2-methyl-2-thiazoline (9.386 g, 0.093 mol) and anhydrous THF (100 ml). The reaction mixture was cooled to -78 °C and butyl lithium (85 ml, 1.0 M in hexanes, 0.085 mol) was added slowly. The reaction mixture was stirred at -78 °C for an additional 1 h before ethyl iodide (13.3 ml, 0.085 mol) was added dropwise. The

reaction mixture was again stirred at -78 °C for 1 h before the temperature was raised to approximately 10 °C and the stirring was continued for 2 h. After this time, the reaction mixture was poured onto ice (200 g). The aqueous layer was adjusted to pH 2 with 1M HCl and the organic layer was separated. The aqueous layer was extracted with hexane (50 ml) and was then adjusted to pH 10 with 20% NaOH. The thiazoline was extracted from the aqueous phase with hexane (4 x 40 ml) and the combined organic extracts were dried over anhydrous potassium carbonate. Filtration and evaporation of the solvent gave the crude 4,5-dihydro-2-propylthiazole. Distillation of the crude product gave the title compound as a colourless liquid (8.552 g, 78%); υ_{max} 2960 (C-H), 1630 (C=N), 1460, and 1150 (N=C-S) and 983 cm^{-1}; δ_H (200 MHz) 0.88 (3H, t, J 7.3 Hz, CH_3), 1.53-1.64 (2H, m, $C\underline{H}_2CH_3$), 2.40 (2H, tt, J 7.3, 1.4 Hz, $C\underline{H}_2C{=}N$), 3.18 (2H, t, J 8.4 Hz, 5-H_2), 4.12 (2H, tt, J 8.4 Hz, 1.4 Hz, 4-H_2); δ_C 13.5 (CH_3), 20.7 ($\underline{C}H_2CH_3$), 33.5 ($\underline{C}H_2C{=}N$), 36.0 (C-5), 64.3 (C-4), 171.3 (C-2); *m/z* 129 (M^+, 12.5 %), 114, 101, 70, 60 (100%); (Found: M^+ 129.0613. $C_6H_{11}NS$ requires *M*, 129.0612).

Synthesis of 4,5-Dihydro-2-*sec*-butylthiazole (130)

The procedure was carried out as previously described for 4,5-dihydro-2-propylthiazole using 2-propylthiazoline (4.1017 g, 31.8 mmol); THF (80 ml); butyl lithium (1.0 M in hexanes, 30 ml, 30 mmol); and methyl iodide (1.88 ml, 4.25 g, 30 mmol).

Distillation of the crude mixture yielded the title compound as a colourless liquid (2.7456 g, 64%); υ_{max} 2980-2860 (C-H), 1630 (C=N), 1460, 1200 (N=C-S), 990, 925 cm^{-1}; δ_H (200 MHz) 0.84 (3H, t, J 5.8 Hz, $C\underline{H}_3CH_2$), 1.10 (3H, d, J 6.9 Hz, $C\underline{H}_3CH$), 1.33-1.62 (2H, m, $C\underline{H}_2CH_3$), 2.53 (1H, m, CH), 3.14 (2H, t, J 8.3 Hz, 5-H_2), 4.11 (2H, dt, J 8.3, 0.84

Hz, 4-H); δ_C 11.4 ($C\underline{H}_3CH_2$), 18.5 ($C\underline{H}_3CH$), 28.3 ($C\underline{H}_2CH_3$), 32.8 (CH_2S), 40.6 (CH), 64.1 (CH_2N). 176.4 (C); *m/z* 143 (M^+, 3.9%), 128, 115 (100%), 114, 86, 83, 57; (Found: M^+, 143.0769 : $C_7H_{13}NS$ requires *M*, 143.0769).

Attempted Synthesis of Tetrahydro-*sec*-butylthiazole (131)

Aluminium foil (35 g) was treated with 5% KOH (200 ml) for 10 min after which time the basic solution was removed and the metal was washed with water (200 ml). The aluminium was then treated with 0.5% mercuric chloride solution (200 ml). After 5 min the solution was removed and a second treatment with aqueous mercuric chloride was carried out. The foil was washed successively with water (100 ml), 95% ethanol (2 x 50 ml) and diethyl ether (2 x 50 ml). The flask containing the foil was fitted for reflux with an efficient condenser and a solution of 4,5-dihydro-2-*sec*-butylthiazole (0.575 g, 4.01 mmol) in moist ether (30 ml) was added. The reaction mixture was heated at reflux temperature for 5 d and, after cooling, the ether solution was filtered. The aluminium amalgam was washed with ether (2 x 20 ml), and the ether solutions were combined. After removal of the ether, the residual oily product was dried over anhydrous potassium carbonate. The drying agent was separated and washed with small amounts of ether. Removal of the ether yielded mainly starting material and only a very small amount of product.

Attempted Synthesis of Tetrahydro-2-*sec*-butylthiazole (131)

The thiazoline (0.1451 g, 1 mmol) was dissolved in methanol (5 ml) and cooled to 0 °C. Sodium borohydride (0.152 g, 4 mmol) was dissolved in 50% methanol (5 ml) and added dropwise. The mixture was then stirred for 1 h. Cold 1M HCl was added until the solution reached pH 2. The solvent was then removed under reduced pressure and the residue was taken up in 1M NaOH (10 ml). The alkaline solution was then extracted with chloroform (3 x 10 ml) and the combined organic extracts were washed with brine (3 x 10 ml), dried, filtered and concentrated *in vacuo*. No thiazolidine was obtained.

Attempted Synthesis of Tetrahydro-2-*sec*-butylthiazole (131)

The thiazoline (0.101 g, 1 mmol) was dissolved in 50% ethanol (10 ml) and 3M HCl was added to adjust the pH to 5. The flask was then cooled to 0°C and a slightly alkaline solution of sodium cyanoborohydride (0.189 g, 3 mmol in 5ml H_2O containing 2 drops of 30% NaOH) was added dropwise. The temperature was kept at 0 °C and the pH was maintained between 4 and 6 by the addition of 3M HCl. After hydride addition was complete the mixture was stirred for 30 min, then diluted with water (30 ml).The pH was then adjusted to 9 - 10 and the solution was extracted with ether (4 x 30 ml). The combined extracts were dried and concentrated *in vacuo*. No thiazolidine was observed in the ^1H NMR spectrum.

Attempted Synthesis of Tetrahydro-2-*sec*-butylthiazole (131)

A solution of the thiazoline (0.100, 1.0 mmol) in dry THF (10 ml) was added dropwise over 30 min to a solution of lithium aluminium hydride (114 mg, 3 mmol) in THF (10 ml). The mixture was heated at reflux for 48 h. Again, there was no thiazolidine observed.

Attempted Synthesis of Tetrahydro-2-*sec*-butylthiazole (131)

To a stirred solution of sodium borohydride (0.7182 g, 18.9 mmol) and 2-methylthiazole (0.6757 g, 4.73 mmol) in 1,4 dioxan (20 ml) was added acetic acid (10 ml) in dioxan (20 ml) dropwise at 0 °C. The resulting mixture was stirred at reflux temperature for 2 h. The reaction mixture was concentrated *in vacuo* and excess reagent was decomposed with water and 1M hydrochloric acid (20 ml) and ethyl acetate (20 ml) was added. The aqueous layer was basified with potassium carbonate. The organic layer was separated and the aqueous layer was extracted with ethyl acetate (3 x 20 ml). The combined organic layers were dried, filtered and concentrated under reduced pressure. Only starting material was recovered.

Attempted Synthesis of Tetrahydro-2-*sec*-butylthiazole (131)

2-*sec*-Butylthiazoline (0.2467 g, 6.49 mmol) was added to a suspension of palladium on carbon (10 % w/w) in absolute ethanol and hydrogenated at 1 atm for 48 h. The suspension was then filtered through Celite and the ethanol was removed under reduced pressure. This reaction yielded only starting material.

Synthesis of 4,4-Dimethyl-2-propyl-2-oxazoline (133)

The procedure was carried out as previously described for 4,5-dihydro-2-propylthiazole using 2,4,4-trimethyl-2-*sec*-butyl-oxazoline (4.60 ml, 4.083 g, 0.036 mol); butyl lithium (1.6M in hexanes, 45 ml, 0.035 mol); THF (50 ml); and ethyl iodide (5.4610 g, 0.035 mol).

Distillation of the crude residue gave the title compound as a colourless liquid (4.769 g, 94%); b.p. 145 °C ; υ_{max} (thin film) 2980-2880 (C-H), 1670 (C=N), 1190 (C-O), 990, 975, and 930 cm^{-1}; δ_H (200 MHz) 0.88 (3H, t, J 7.3 Hz, $C\underline{H}_3CH_2$), 1.18 (6H, s, $(\underline{Me})_2C$), 1.55 (2H, m, $C\underline{H}_2CH_3$), 2.15 (2H, t, J 7.2 Hz, $N{=}CC\underline{H}_2$), 3.82 (2H, s, 5-H_2); δ_C 13.5 ($\underline{C}H_3CH_2$), 19.3 ($\underline{C}H_2CH_3$), 28.3 ($\underline{Me}_2C$), 29.8 ($N{=}C\underline{C}H_2$), 66.7 (C-4), 78.7 (C-5), 165.8 (C=N); *m/z* 141 (M^+, 1.5%), 126, 113 (100%), 98, 70, 55; (Found: M^+, 141.1152: $C_8H_{15}NO$ requires *M*, 141.1154).

Synthesis of 4,4-Dimethyl-2-*sec*-butyl-2-oxazoline (134)

The procedure was carried out as previously described for 4,5-dihydro-2-*sec*-butylthiazole using 4,4-dimethyl-2-propyl-2-oxazoline (3.229 g, 22.9 mmol); THF (60 ml); butyl lithium (1.6M in hexanes, 13.75 ml, 22.0 mmol); and methyl iodide (1.37 ml, 3.142 g, 22 mmol). Distillation of the crude reaction product gave the title compound as a colourless liquid. (2.1881 g, 64%); υ_{max} (thin film) 2970 - 2940 (C-H), 1665 (C=N), 1160 (C-O), 1005, 975, and 925 cm^{-1}; δ_H (200 MHz) 0.88 (3H, t, J 7.0 Hz, $C\underline{H}_3CH_2$), 1.08 (3H, d, J 7.0 Hz, $C\underline{H}_3CH$), 1.23 (6H, s, $\underline{Me}_2C$), 1.62 (2H, m, $C\underline{H}_2CH_3$), 2.32 (1H, m, C$\underline{H}$), 3.85 (5-H_2); δ_C 14.1 ($\underline{C}H_3CH_2$), 17.4 ($\underline{C}H_3CH$), 26.9 ($\underline{C}H_2CH_3$), 28.4 ($\underline{Me}_2C$), 35.0 (CH), 66.6 ($\underline{C}Me_2$), 78.8 (C-5), 169.2 (C=N); *m/z* 155 (M^+, 0.5%), 140, 127, 102, 58 (100%), 57; (Found: 155.1325: $C_9H_{17}NO$ requires *M*, 155.1340).

Formation of [9-$^2H_3$]-4,4-Dimethyl-2-*sec*-butyl-2-oxazoline

The procedure was carried out as for unlabelled material but using d_3-iodomethane in 62% yield, δ_D 1.07 (CD_3CH)

Attempted Synthesis of 4,4-Dimethyl-2-*sec*-butyloxazolidine (135)

4,4-Dimethyl-2-*sec*-butyl-2-oxazoline (1.552, 10 mmol) was added to a suspension of palladium on carbon (10% w/w) in ethanol under a nitrogen atmosphere at 1 atm for 48 h. The suspension was then filtered through Celite and the ethanol was distilled off. The clear liquid obtained was shown to be starting material.

The reaction was repeated in glacial acetic acid and again, only starting material was found.

When the above procedure was carried out in 2M HCl the reaction gave 2-methylbutanoic acid as a colourless liquid. υ_{max} (thin film) 3020 (br O-H), 2965-2940 (C-H), 1710 (C=O), 1230 (C-O), 940 cm^{-1}; δ_H (200 MHz) 0.91 (3H, t, J 7.4 Hz, $\underline{CH_3}CH_2$), 1.14 (3H, d, J 7.0 Hz, $\underline{CH_3}CH$), 1.43-1.72 (2H, m, CH_2), 2.34 (1H, m, CH), 10.89 (1H, br s, CO_2H); δ_C 11.5 ($\underline{CH_3}CH_2$), 16.3 ($\underline{CH_3}CH$), 26.5 (CH_2), 40.9 (CH), 163.5 (CO_2H); *m/z* 102 (M^+, 0.4%), 87, 74 (100%), 57; (Found: M^+, 102.0697: $C_5H_{10}O_2$ requires *M*, 102.0681).

Synthesis of 3,4,4-Trimethyl-2-*sec*-butyloxazolinium iodide (136)

4,4-Dimethyl-2-*sec*-butyloxazoline (4.1919 g, 0.027 mol) was dissolved in acetonitrile (50 ml) and heated to reflux temperature. Iodomethane was added in portions (5 x 2 ml) during the period of reflux, around 8 h. After this time the solvent was removed *in vacuo*, and the crude oxazolinium iodide was recrystallised from isopropanol to give the oxazolinium salt as a white, crystals (7.377 g, 92%); υ_{max} (KBr disc) 2980-2880 (C-H), 1385 (CMe_2), 1700 ($C=N^+$), and 1080 cm^{-1}; δ_H (200 MHz) (CD_3CN ref CHD_2CN @ 1.93) 0.94 (3H, t, J 7.4 Hz, $C\underline{H}_3CH_2$), 1.35 (3H, d, J 6.9 Hz, $C\underline{H}_3CH$), 1.49 (6H, s, $\underline{Me}_2C$), 1.68 (2H, m, CH_2CH_3), 3.00 (1H, m, CH), 3.24 (3H, s, NMe), 4.68 (2H, s, 5-H_2); δ_C 11.5 ($\underline{C}H_3CH_2$), 16.5 ($\underline{C}H_3CH$), 23.2 ($\underline{Me}_2C$), 26.6 ($\underline{C}H_2CH_3$), 28.2 (N-Me), 40.9 (CH), 53.1 ($\underline{C}Me_2$), 68.6 (C-5), 176.3 (C=N); *m/z* 170 (M^+, 45.1%), 154, 126, 86, 72, 57, 56, 41 (100%).

Formation of [9-$^2H_3$]-3,4,4-Trimethyl-2-*sec*-butyloxazolinium Iodide

The procedure was carried out as described for unlabelled material using [9-$^2H_3$]-4,4-dimethyl-2-*sec*-butyl-2-oxazoline in 94% yield, δ_D 1.40 (CD_3CH).

Synthesis of 3,4,4-Trimethyl-2-*sec*-butyloxazolidine (137)

The oxazolinium iodide (1.518 g, 5.11 mmol) was dissolved in dry methanol (10 ml) and charged into a flame dried 3-necked flask under a nitrogen atmosphere. Sodium borohydride (0.772, 20.4 mmol) was added, in portions, at 0 °C and the mixture was stirred for a further 2 h after the completion of the addition of borohydride. Purification by column chromatography on basic alumina (eluted

with $CHCl_3$), yielded the title compound as a colourless oil (41%); υ_{max} (thin film) 3460 (br w), 2960-2880 (C-H), 1380, 1360 (CMe_2), 1230 (C-O), and 1115 cm^{-1}; δ_H 0.83-1.10 (6H, m, $C\underline{H}_3CH_2$, $C\underline{H}_3CH$), 0.10 (6H, s, $\underline{Me}_2C$), 1.33-1.57 (2H, m, $C\underline{H}_2CH_3$), 1.60-1.82 (1H, m, $C\underline{H}CH_3$), 2.12 (3H, s, NMe), 3.55 (2H, s, 5-H_2), 3.98 (1H, m, 2-H); δ_C 11.5, 12.2 ($\underline{C}H_3CH_2$), 16.6, 16.8 ($\underline{C}H_3CH$), 23.7 ($\underline{Me}_2C$), 26.3, 26.4 ($\underline{C}H_2CH_3$), 29.9, 30.3 (NMe), 37.4, 37.9 ($\underline{C}HCH_3$), 59.3, 59,6 (CMe_2), 78.3, 78.7 (5-H_2), 98.2, 99.6 (2-H); *m/z* 170, 154, 126, 100, 86, 72, 57, 55, 41 (100 %).

Formation of [9-$^2H_3$]-3,4,4-Trimethyl-2-*sec*-butyloxazoline

The procedure was carried as previously described for unlabelled material using [9-$^2H_3$]-3,4,4-trimethyl-2-sec-butyloxazolinium iodide in 34% yield, δ_D 1.10 ($C\underline{D}_3CH$).

Synthesis of 4,5-Dihydro-*N*-methyl-2-*sec*-butyl-thiazolidine (140)

The procedure was carried out as previously described for 3,4,4-trimethyl-2-*sec*-butyloxazolidine in 21% yield, δ_H (200 MHz) 0.90 (3H, t, J 7.8 Hz, $C\underline{H}_3CH_2$), 1.01 (3H, d, J 6.6 Hz, $C\underline{H}_3CH$), 1.18 (2H, m, $C\underline{H}_2CH_3$), 1.41-1.72 (1H, m, $C\underline{H}CH_3$), 2.26, 2,27 (3H, s, NMe), 2.88 (2H, m, 5-H_2), 3.09 (2H, m, 4-H_2), 3.93, 3.98 (1H, d J 4.3 Hz, 2-H); δ_C 11.0, 11.1 ($\underline{C}H_3CH_2$), 16.2, 16.8 ($\underline{C}H_3CH$), 27.0, 27.2 ($\underline{C}H_2CH_3$), 29.5, 29.6 (C-5), 39.7, 40.0 ($CHCH_3$), 58.5, 58.7 (C-4), 83.6, 84.2 (C-2); *m/z* 159 (M^+, 0.3%), 144, 112, 102 (100%), 98, 84, 71 ,57: (Found: M^+, 159.1068: $C_8H_{17}NS$ requires *M*, 159.1081).

Synthesis of 2-Methylbutanal (138)

The tetrahydro-oxazole (0.180 g, 1.05 mmol) was added to a solution of 1M oxalic acid (5 ml) and heated at reflux temperature for 2 h. The reaction mixture was then distilled and fractions boiling below 100 °C were collected. The reaction mixture was extracted with diethyl ether (3 x 5 ml), and the extracts were washed with 5% $NaHCO_3$ solution (5 ml) and dried. Filtration and solvent evaporation gave the desired aldehyde in low yield (0,020 g, 22%); υ_{max} (thin film) 2980-2860 (C-H), 2800, 2700 (O=C-H), 1725 (C=O), 1460, 970 cm^{-1}; δ_H (200 MHz) 0.85 (3H, t, J 7.5 Hz, 4-H_3), 1.00 (3H, d, J 7.0 Hz, 5-H_3), 1.27-1.69 (2H, m, CH_2), 2.19 (1H, dq, J 7.0, 1.9 Hz, 2-H), 9.53 (1H, d, J 1.9 Hz, 1-H); δ_C 11.1 (C-4, 12.6 (C-5), 23.3 (C-3), 47.6 (C-2), 205.2 (C-1); *m/z* 86 (M^+, 14.0%), 74, 58, 57, 41 (100%), 29; (Found: M^+ 86.0728: $C_5H_{10}O$ requires *M*, 86.0732).

Formation of [5-$^2H_3$]-2-Methylbutanal

The procedure was carried out as described above using [9-$^2H_3$]-3,4,4-trimethyl-2-*sec*-butyloxazoline in 20% yield, δ_D 0.98.

Synthesis of 2-Methylbutanal Bisulfite Addition Product (141)

A solution of 2-methylbutanal (5.110 g, 0.06 mol) in acetonitrile (20 ml), was added to a stirred solution of sodium bisulfite (6.120 g, 0.06 mol) in water (10 ml). The bisulfite addition separated as a colourless solid, and the mixture was stirred overnight. The addition product was collected, washed with acetonitrile (10 ml) and dried to

give 2-methylbutanal bisulfite addition product. (8.164 g, 81%); m.p. >230°C; υ_{max} (KBr disc) 3550-3400 (br, O-H), 2960-2880 (C-H), 1170 (br, -SO_2-), and 640 cm^{-1}; δ_H (200 MHz) (D_2O ref. HOD @ 4.63) 0.76, 0.77 (3H, t, J 7.4 Hz, 4-H_3), 0.84, 0.88 (3H, d, J 6.9 Hz, 5-H_3), 1.11-1.36 (2H, m, 3-H_2), 1.40-1.88 (1H, m, 2-H), 4.10 (1H, d, J, 1-H); δ_C 11.2, 11.7 (C-4), 13.4, 16.3 (C-5), 24.3, 27.7 (C-3), 36.7, 37.3 (C-2), 87.3, 88.9 (C-1).

Synthesis of DL-2-Hydroxy-3-methylpentanenitrile (142)

A solution of sodium cyanide (2.548 g, 0.052 mol) in water (5 ml) was added dropwise to a solution of 2-methylbutanal bisulfite addition product (4.934 g, 0.026 mol) in water (10.3 ml) at 0 °C. After addition was complete the reaction mixture was stirred at room temperature for 2 h. The cyanohydrin was extracted from the aqueous solution with dichloromethane (5 x 20 ml), The combined organic extracts were dried over anhydrous $MgSO_4$ and filtered. The solvent was removed *in vacuo* to give the cyanohydrin as a colourless oil (2.497 g, 85%); υ_{max} (thin film) 3445 (br O-H), 2970-2880 (C-H), 2250 (w, CN), 1065 (C-O), and 1022 cm^{-1}; δ_H (200 MHz) 0.94, 0.95 (3H, t, J 7.4 Hz, 5-H_3), 1.05, 1.06 (3H, d, J 3.3 Hz, 6-H_3), 1.21-1.36 (2H, m, 4-H_2), 1.57-1.81 (1H, m, 3-H), 2.86 (1H, s, OH), 4.34, 4.37 (1H, d, J 3.2 Hz, 2-H); δ_C 11.1, 11.2 (C-5), 14.1, 14.3 (C-6), 24.4, 24.8 (C-4), 39.3, 39.4 (C-3), 65.6, 65.9 (C-2), 119.2, 119.5 (C-1); *m/z* 87, 86, 71, 57, 29 (100%): (Found: M^+-CN, 87.0777: $C_5H_{11}O$ requires *M*-CN, 87.0765).

Formation of DL-[6-$^2H_3$]-2-Hydroxy-3-methyl-pentanenitrile

The procedure was carried out as described for unlabelled material using [5-$^2H_3$]-2-methylbutanal in 79% yield, δ_D 1.10.

Synthesis of DL-2-Amino-3-methylpentanenitrile Hydrochloride (143)

The cyanohydrin (2.493 g, 0.022 mol) was added dropwise to concentrated aqueous ammonia (7 ml) in a conical flask, with stirring. The reaction mixture was stirred at room temperature for 6 h, then the mixture was extracted with dichloromethane (5 x 30 ml). The combined organic extracts were extracted with 10% HCl (4 x 50 ml). The aqueous extracts were combined and the solvent was evaporated *in vacuo* to give the aminonitrile hydrochloride which was recrystallised from 95% ethanol (1.960 g, 60%); υ_{max} (KBr disc) 3400 (br N-H), 2960-2880 (C-H), 2500, 2100 ($^+NH_3$) 2260 (CN), 1450, and 1385 cm^{-1}; δ_H (200 MHz) (D_2O ref HOD @ 4.64), 0.75 (3H, t, J 7.4 Hz, 5-H_3), 0.93, 0.92 (3H, d, J 6.7 Hz, 6-H_3), 1.28 (2H, m, 4-H_2), 1.88 (1H, m, 3-H), 4.26, 4.35 (1H, d, J 5.1 Hz, 2-H); δ_C 11.1 (C-5), 14.4 (C-6), 26.4 (C-4), 36.6 (C-3), 47.0 (C-2), 115.6 (C-1); *m/z* 112 (M^+-HCl, 0.1%), 97, 83, 70, 57 (100%), 56; (Found M^+-HCl, 112.0995: $C_6H_{12}N_2$ requires *M*-HCl, 112.1000).

Formation of DL-[6-$^2H_3$]-2-Amino-3-methyl-pentanenitrile hydrochloride

The procedure was carried out as described for unlabelled material using DL-[6-$^2H_3$]-2-hydroxy-3-methylpentanenitrile in 61% yield, δ_D (H_2O ref HOD @ 4.63) 1.18.

Synthesis of DL-Isoleucine and Allo-isoleucine Hydrochloride (144)

A solution of the aminonitrile hydrochloride (1.093 g, 7.36 mmol) in concentrated HCl (5 ml) was allowed to stand at room temperature for 24 h. The reaction mixture was then heated at reflux temperature for 7 h and then allowed to cool to room temperature, at which point crystals began to form. On further cooling to 0 °C more crystals formed. The crystals were collected, washed with cold concentrated HCl and dried to give the required DL-isoleucine hydrochloride. The filtrate and washings were combined and the solvent was removed *in vacuo*. The solid was extracted with hot ethanol and filtered. The ethanol was removed *in vacuo*. The isoleucine and allo-isoleucine hydrochloride were recrystallised from 95% ethanol. (0.5792, 47%); υ_{max} (KBr disc) 3440 (br O-H), 2965-2880 (C-H), 1700 (C=O), ; δ_H (200 MHz) (D_2O ref HOD @ 4.63) 0.55 (3H, t, J 7.3 Hz, 5-H_3), 0.62 (3H, d, J 7.0 Hz, 6-H_3), 0.80-1.15 (2H, m, 4-H_2), 1.60 (1H, m, 3-H), 3.27 (1H, d, J 3.9 Hz, 2-H); δ_C 11.9, 12.2 (C-5), 15.5, 15.8 (C-6), 25.3, 25.5 (C-4), 36.7, 37.0 (C-3), 60.3, 60.6 (C-2), 175.0, 175.3 (C-1); *m/z* 86, 69, 57, 28 (100%).

Formation of [6-$^2H_3$]Isoleucine and Allo-isoleucine Hydrochloride (146)

The procedure was carried out as described above for unlabelled material using DL-[6-$^2H_3$]-2-amino-3-methylpentanenitrile hydrochloride in 46% yield, δ_D (H_2O) 0.70.

8.3 Experimental for Chapter 4

Synthesis of 2-Butyl Methanesulfonate (149)

A mixture of 2-butanol (30.5 ml, 24.67 g, 0.33 mol), and methanesulfonyl chloride (25.5 ml, 37.801 g, 0.33 mol) in THF (150 ml) was cooled to -78 °C. Triethylamine (46 ml, 33.393 g, 0.33 mol) was added slowly. After addition was complete the mixture was left overnight to attain room temperature. The mixture was poured onto ice-water and then extracted with dichloromethane (3 x 75 ml). The organic extracts were dried, filtered and concentrated *in vacuo*. Distillation of the residue yielded the title compound as a colourless oil (45.012 g, 90%); b.p. 70 °C / 25 mmHg; υ_{max} (thin film) 2970-2880 (C-H), 1350, 1170 (O-SO_2), and 910 cm^{-1}; δ_H (200 MHz) 0.99 (3H, t, 7.4 Hz, 4-H_3), 1.42 (3H, d, J 6.3 Hz, 1-H_3), 1.70 (2H, m, 3-H_2), 3.01 (3H, s, 5-H_3), 4.74 (1H, m, 2-H); δ_C 9.4 (C-4), 20.6 (C-1), 29.5 (C-3), 38.4 (C-5), 81.6 (C-2); *m/z* 137, 123 (100%), 79, 59, 57; (Found M^+-Me, 137.0231 : $C_4H_9SO_3$ requires 137.0227).

Formation of [2-^{2}H]-2-Butyl Methylsulfonate

The procedure was carried out as described above for unlabelled material but using [2-^{2}H]butanol in 91% yield, δ_H 0.98 (3H, t, J 7.4 Hz, 4-H_3), 1.40 (3H, s, 1-H_3), 1.70 (2H, m, 3-H_2), 2.97 (3H, s, 5-H_3); δ_D 4.76 (s, 2-D).

Synthesis of Dimethyl *sec*-Butylmalonate (150)

To a suspension of NaH (95%, 1.895 g, 0.075 mol), in THF (60 ml) was added, dropwise with cooling, and under a nitrogen atmosphere, dimethyl malonate (8.44 ml, 9.702 g, 0.0735 mol) over a period of 10 min. The mixture was stirred at room temperature for 1 h, then heated to 80 °C to bring the contents into solution. 2-Butyl methanesulfonate (11.172 g, 0.0735 mol) was added and the mixture was heated at reflux temperature for 44 h. The cooled mixture was distributed between water (100 ml) and ether (250 ml). The ether layer was washed with 100 ml portions of water until neutral. The solution was dried and concentrated under reduced pressure to give the diester. Distillation of the crude product yielded the desired compound as a colourless liquid (11.883 g, 86%) b.p. 217 °C; υ_{max} (thin film) 2965-2880 (C-H), 1755, 1740 (C=O), 1265 (C-O), 1150, and 1130 cm^{-1}; δ_H (200 MHz) 0.91 (3H, t, J 7.3 Hz, $C\underline{H}_3CH_2$), 0.97 (3H, d, J 6.8 Hz, $C\underline{H}_3CH$), 1.15-1.53 (2H, m, CH_2), 2.18 (1H, m, CH), 3.28 (1H, d, J 8.2 Hz, $C\underline{H}(CO_2Me)_2$), 3.73 (6H, s, $\underline{Me}O_2C$); δ_C 11.0 ($\underline{C}H_3CH_2$), 16.3 ($\underline{C}H_3CH$), 26.9 (CH_2), 34.9 ($\underline{C}HMe$), 52.0, 52.1 (2 x $CO_2\underline{Me}$), 57.0 ($\underline{C}H(CO_2Me)_2$), 169.1, 169.3 (2 x $\underline{C}O_2Me$); *m/z* 157, 132 (100%), 101, 100, 74, 69, 59, 57; (Found M^+-OMe, 157.0856: $C_8H_{13}O_3$ requires 157.0856).

Formation of [5-^{2}H]-Diethyl *sec*-butylmalonate

The procedure was as described above for unlabelled material but using [2-^{2}H]-2-butyl methanesulfonate in 83% yield, δ_D 2.18 (s).

Synthesis of *sec*-Butylmalonic Acid (151)

The diester (6.166 g, 0.0328 mol), potassium hydroxide (6.081 g), and water (50 ml) were stirred at 70 °C for 4 h and then at room

temperature overnight. The solution was concentrated to dryness at reduced pressure and the residue was washed with ether (10 ml) and then carefully acidified with ice cold 6M HCl, keeping the temperature below 10 °C. The solution was extracted with ether (6 x 15 ml), and the organic extracts were dried ($MgSO_4$), filtered and concentrated *in vacuo*. On being kept overnight in a desiccator the malonic acid solidified as colourless crystals (4.089 g, 78%); m.p. 92 °C (Ref. 93 °C); υ_{max} ($CHCl_3$ soln.) 3050 (br O-H), 3000-2920 (C-H), 1700 (C=O), 1290, 1270 (C-O), and 930 cm^{-1}; δ_H (200 MHz) 0.92 (3H, t, J 7.3 Hz, $C\underline{H}_3CH_2$), 1.04 (3H, d, J 6.8 Hz, $\underline{Me}CH$), 1.16-1.62 (2H, m, CH_2), 2.19 (1H, m, $C\underline{H}Me$), 3.36 (1H, d, J 7.6 Hz, $C\underline{H}(CO_2Me)_2$), 11.04 (2H, br s, 2 x $CO_2\underline{H}$); δ_C 11.2 ($\underline{C}H_3CH_2$), 16.3 ($\underline{C}H_3CH$), 26.9 ($\underline{C}H_2$), 35.2 (CHMe); 56.9 ($CH(CO_2Me)_2$), 174.7, 174.9 (2 x CO_2H); *m/z* 160 (M^+, 4.6%), 114, 104 (100%), 98, 87, 69; (Found: M^+, 160.0718; C, 52.75; H, 7.39%: $C_7H_{12}O_4$ requires *M*, 160.0700; C, 52.50, H 7.50%).

Formation of [4-^{2}H]-*sec*-Butyl Malonic Acid

The procedure was carried out as described for unlabelled material but using [5-2H]-diethyl *sec*-butylmalonate in 75% yield, δ_D 2.20 (s, CDMe).

Preparation of Hydrazoic Acid Solution

A three-neck flask was equipped with a dropping funnel, thermometer and a mechanical stirrer. Sodium azide (13 g, 0.2 mol) and water (13 ml) were added to the flask forming a paste. Chloroform (80 ml) was added and the mixture was cooled to 0 °C. While the mixture was efficiently stirred and cooled concentrated sulfuric acid (11 ml) was added dropwise over 1 h. The temperature

was maintained below 10 °C. After addition of sulfuric acid was complete the reaction was stirred at 0 °C for a further 1 h. The organic layer was then decanted and dried over anhydrous sodium sulfate. The hydrazoic acid solution was then titrated with standard sodium hydroxide solution.

Attempted Synthesis of Isoleucine and Allo-isoleucine

A solution of the malonic acid (0.527 g, 3.3 mmol), in 100% (3 ml), and $CHCl_3$ (3 ml) was vigorously stirred while hydrazoic acid solution in $CHCl_3$ (1.23 M, 2.85 ml, 3.5 mmol) was added dropwise over 1 h. The mixture was stirred at 55 °C for 2 h, and then for another 4 h at 25 °C. The $CHCl_3$ layer was decanted and the acid layer was poured onto ice-water (15 ml) and washed with ether (3 x 10 ml). Solid $Ba(OH)_2$. 8 H_2O (8 g) was added, bringing the pH to 2, the mixture was filtered through Celite and the precipitate was washed with water (40 ml). The combined filtrate and washings were concentrated *in vacuo* to yield a mixture of products containing a small amount of the required isoleucine and allo-isoleucine.

Synthesis of Dimethyl 2-Bromo-2-*sec*-butylmalonate (152)

A three necked flask was fitted with a stirrer, dropping funnel and a reflux condenser with a tube leading to a flask of water for absorption of HBr. The flask was charged with dimethyl *sec*-butylmalonate (11.518 g, 0.061 mol) in dichloromethane (300 ml). Dry bromine (4.03 ml, 12.579 g, 0.08 mol) was placed in the dropping funnel. A few drops of bromine were added, with stirring. A large spotlight was shone on the flask until the reaction started.

The remainder of the bromine was then added at such a rate as to keep the reaction mixture boiling gently. The reaction mixture was then heated at reflux temperature for 2 h. The mixture was cooled and washed with 5% Na_2CO_3 solution (5 x 200 ml). The organic layer was dried ($MgSO_4$), filtered and the solvent was evaporated *in vacuo* (15.2265 g, 94%), υ_{max} (thin film) 2970-2880 (C-H), 1745 (C=O), 1250, 1210 (C-O), and 665 cm^{-1}; δ_H (200 MHz) 0.97 (3H, t, J 7.2 Hz, $C\underline{H}_3CH_2$), 1.07 (3H, d, J 6.6 Hz, $C\underline{H}_3CH$), 1.41-1.75 (2H, m, CH_2), 1.68 (1H, m, CH), 3.81 (6H, s, 2 x $CO_2\underline{Me}$); δ_C 12.2 ($\underline{C}H_3CH_2$), 15.3 ($\underline{C}H_3CH$), 26.3 (CH_2), 42.2 (CH), 53.7 (2 x $CO_2\underline{Me}$), 70.1 (C-Br), 167.1 (2 x $\underline{C}O_2CH_3$); *m/z* 212, 210, 198, 196, 180, 179, 127; (Found: M^+- Bu, 211.9496, 209.9529 : requires 211.9507, 209.9527).

Synthesis of 2-Bromo-2-*sec*-butylmalonic Acid (153)

The malonic acid (10.710 g, 66.9 mmol) was dissolved in ether (20 ml) and added to a 3-necked flask fitted with a reflux condenser and a dropping funnel. Bromine (5 x 1 ml) was added in portions. If the initial addition of bromine did not decolourise the solution the mixture was heated. The remainder of the bromine was added and the mixture was refluxed gently for 1 h. Water (20 ml) was added and the organic layer was separated. The solvent was removed *in vacuo* to give a yellow oil which crystallised overnight in a vacuum desiccator (12.77 g, 80%); υ_{max} (KBr disc) 3400-2500 (br O-H), 2980-2880 (C-H), 1725 (C=O), 1385, 1265, 1240 (C-O), and 781 cm^{-1}; δ_H (200 MHz) (CD_3OD ref @ 4.10) 0.76 (3H, t, J 7.3 Hz, CH_3CH_2), 0.87 (3H, d, J 6.5 Hz, $C\underline{H}_3CH$), 1.00-1.57 (2H, m, CH_2), 1.98 (1H, m, CH); δ_C 13.1 ($\underline{C}H_3CH_2$), 16.2 ($\underline{C}H_3CH$), 28.0 (CH_2), 43.5 (CH), 73.5 (CBr), 170.2, 170.3 (2 x CO_2H); *m/z* 212, 210, 184, 182, 167, 166, 165, 164, 149, 147,

140, 138 (100%); (Found: M^{+}-Et , 211.9533, 209.9514: $C_5H_7O_4Br$ requires 211.9507, 209.9527).

Formation of [5-^{2}H]-2-Bromo-2-*sec*-butylmalonic acid

The procedure was carried out as described for unlabelled material but using [4-^{2}H]-*sec*-butyl malonic acid in 77% yield, δ_D 2.23 (s, CD).

Synthesis of α-Bromo-β-methylvaleric Acid (154)

The bromomalonic acid (9.602 g, 0.04 mol) was heated for 3 h at 130 °C, after which time the title compound was separated from the water formed and distilled to give a colourless oil (3.276 g, 42%); b.p. 140 °C (20 mmHg); υ_{max} (thin film) 3400-3000 (br O-H), 2970-2880 (C-H), 1715 (C=O), 1203 (C-O), and 665 cm^{-1}: δ_H (200 MHz) 0.93 (3H, t, J 7.4 Hz, 5-H_3), 1.05 (3H, d, J 6.7 Hz, 6-H_3), 1.27-1.78 (2H, m, 4-H_2), 2.04 (1H, m, 3-H), 4.18 (1H, d, J 6.2 Hz, 2-H), 11.23 (1H, br s, CO_2H); *m/z* 168, 166, 140, 138, 81, 79, 69, 57, 55, 41 (100 %), 28.

Synthesis of Isoleucine and Allo-isoleucine Hydrochloride (144)

The α-bromo-β-methylvaleric acid (2.071 g, 0.011 mol) was added to concentrated aqueous ammonia and stirred in a stoppered flask overnight. The aqueous solution was then concentrated *in vacuo*. The residue was taken up in ethanol and ethereal hydrochloric acid solution was added dropwise. The solvent was removed under reduced pressure and crude isoleucine and allo-isoleucine hydrochloride were recrystallised from hot water (0.9396

g, 51%), 3440 (br O-H), 2960-2880 (C-H), 2580 (H_3N^+), 1590 (C=O), 1330, 1185 (C-O), and 540 cm^{-1}; δ_H (200 MHz) (D_2O ref HOD @ 4.64) 0.80 (3H, t, J 7.3 Hz, 5-H_3), 1.02 (3H, d, J 7.0 Hz, 6-H_3), 1.05-1.40 (2H, m, 4-H_2), 1.59 (1H, m, 3-H), 3.28 (1H, d, J 3.9 Hz, 2-H); δ_C 11.6, 11.9 (C-5), 15.3, 15.5 (C-6), 25.3, 25.4 (C-4), 36.4, 36.7 (C-3), 60.2, 60.3 (C-2), 174.7, 175.0 (C-1); *m/z* 86, 69, 57, 28 (100%).

Formation of DL-[3-^{2}H]Isoleucine and Allo-isoleucine Hydrochloride (146)

The procedure was carried out as described for the unlabelled material but using [5-^{2}H]-2-bromo-2-*sec*-butylmalonic acid in 44% yield, δ_D 1.63 (s, CD).

Feeding of [3-^{2}H]Isoleucine and Allo-isoleucine Hydrochloride

[3-^{2}H]Isoleucine mixture (370 mg) was dissolved in distilled water and fed by the wick method method to three one-year-old *Crotalaria lachnosema* plants over 14 d. The plants were harvested 4 d after feeding was complete and the standard alkaloid extraction procedure was followed.

General Conditions for Growth of *Crotalaria lachnosema*

Seeds of *Crotalaria lachnosema* Stepf. were obtained from Dr. A. R. Mattocks. They were germinated in January and grown in a standard compost in 4" pots in a greenhouse. Feeding experiments were carried out with six-month-old plants for ^{13}C-labelled precursors and plants more than a year old for ^{2}H-labelled precursors.

General Procedure for Alkaloid Extraction

After harvesting, the plants were macerated repeatedly in methanol. The methanolic extract was filtered and the solid residue was washed with methanol until the washings were colourless. The combined organic washings were concentrated *in vacuo* leaving a green residue. This residue was taken up in dichloromethane (20 ml) and extracted with 1M hydrochloric acid (2 x 20 ml). The combined acid extracts were washed with dichloromethane (6 x 20 ml) and then stirred at room temperature with zinc dust (2 g) for 2 h. The mixture was then filtered through Celite and basified with concentrated ammonia solution. The aqueous solution was extracted with dichloromethane (4 x 30 ml) and the combined organic extracts were dried (Na_2SO_4), filtered and concentrated under reduced pressure to give the crude alkaloids which were purified by chromatography or crystallisation.

8.4 Experimental for Chapter 5

Synthesis of Diethyl 2-Ethylacetamidomalonate (158)

Ethyl iodide (0.38 ml, 0.756 g, 4.85 mmol) was added dropwise to a solution of sodium (0.110 g, 4.78 mmol) and diethyl acetamidomalonate (0.9440 g, 4.35 mmol) in absolute ethanol (20 ml). After addition was complete the solution was heated at reflux temperature for 1 h. The mixture was then cooled to room temperature and the solution was decanted from the sediment. The ethanol was removed *in vacuo*. Recrystallisation of the crude product from 95% aqueous acetone gave the title compound as white crystals (0.878 g, 75%); R_f 0.53 (EtOAc); m.p. 82 °C (lit., 83°C); υ_{max} (KBr disc) 3480-3300 (N-H), 2980-2880 (C-H), 1650 (N-HCO), 1760, 1745 (C=O), 1520 (NHCO), 1245, 1200 (C-O), and 860 cm^{-1}; δ_H (200 MHz) 0.77 (3H, t, J 7.6 Hz, $C\underline{H}_3CH_2C$), 1.26 (6H, t, J 7.1 Hz, $C\underline{H}_3CH_2O$), 2.05 (3H, s, $C\underline{H}_3CO$), 2.36 (2H, q, J 7.6 Hz, $CH_2\underline{C}$), 4.25 (4H, q, J 7.1 Hz, $CH_2\underline{O}$), 6.81 (1H, s, NHCO); δ_C 7.71 ($\underline{C}H_3CH_2C$), 13.9 ($\underline{C}H_3CH_2O$), 22.9 ($\underline{C}H_3CO$), 25.3 ($CH_3CH_2\underline{C}$), 62.3 (CH_2O), 67.0 (EtC), 160.1 (NHCO), 168.9 (2 x CO_2Et); *m/z* 245, (M^+, 0.8%), 200, 172, 130 (100%), 102; (Found M^+, 245.1267; C, 53.38; H, 7.83; N, 5.78: $C_{11}H_{19}NO_5$ requires *M*, 245.1263; C, 53.88; H, 7.76; N, 5.71).

Synthesis of Ethyl Acetamidocyanoacetate (160)

The procedure was carried out as described above but using acetamidocyanoacetate (69%); R_f 0.36 (EtOAc); m.p. 132°C; υ_{max} (nujol) 3440, 3410 (N-HCO), 2950 (C-H), 1755 (OC=O), 1700 (NHC=O), 2250 (CN), 1230 (C-O), and 795 cm^{-1}; δ_H (200 MHz) 1.09 (3H, t, J 7.4

Hz, $\underline{CH_3}CH_2C$), 1.29 (3H, t, J 7.1 Hz, CH_3CH_2O), 1.99 (3H, s, CH_3CO), 2.07 (2H, q, J 7.4 Hz, CH_2C), 4.26 (2H, dq, J 1.2 Hz, 7.1 Hz, CH_2O), 7.39 (1H, s, NHCO); δ_C 7.9 ($\underline{C}H_3CH_2C$), 13.3 ($\underline{C}H_3CH_2O$), 21.1 (CONH), 29.3 ($\underline{C}H_2C$), 58.4 ($\underline{C}CH_2$), 62.8 (CH_2O), 117.3 (CN), 166.6 (NHCO), 170.4 (CO_2); m / z 198 (M^+, 0.6%), 153, 125, 111, 97, 83, 73, 43 (100%); (Found M^+, 198.000; C, 54.60; H, 7.16; N, 14.11: $C_9H_{14}N_2O_3$ requires *M*, 198.0996; C, 54.54; H, 7.67; N, 14.14).

Synthesis of 2-Aminobutanoic Acid Hydrochloride (161)

Diethyl 2-ethylacetamidomalonate (0.904 g, 3.69 mmol) was mixed with concentrated HCl (35 ml, 37%) and heated at reflux temperature for 4 h. The reaction mixture was then cooled and concentrated *in vacuo*. The reaction mixture was washed with acetone, filtered and the filtrate was evaporated to dryness. Recrystallisation from 95% ethanol gave the desired amino acid hydrochloride as white crystals (0.407 g, 79%); m.p. >230 °C; υ_{max} (KBr disc) 3420 (br O-H), 3000 (br N-H), 1730 (C=O), 1210 (C-O), and 780 cm^{-1}; δ_H (200 MHz) (D_2O ref HOD @ 4.63) 0.82 (3H, t, J 7.5 Hz, 4-H_3), 1.78 (2H, m, 3-H_2), 3.79 (1H, t, J 6.0 Hz, 2-H), δ_C 9.3 (C-4), 24.0 (C-3), 54.8 (C-2), 173.0 (C-1); *m/z* 74, 58 (100%); (Found: C, 34.25; H, 7.03; N, 10.10: $C_4H_{10}NO_2Cl$ requires: C, 34.41; H, 7.17; N, 10.04).

Formation of [3,4-$^2H_5$]-2-Aminobutanoic acid (162)

The procedure was carried out as previously described but using d_5-iodoethane. δ_D (H_2O) 1.20 (3D, br s, CD_3), 2.16 (2D, br s, CD_2).

Formation of [3,4-$^{13}C_2$]-2-Aminobutanoic acid (163)

The procedure was repeated as described above but with $^{13}C_2$-iodoethane δ_H (200 MHz) (0.81 (3H, dm, J 130Hz, $^{13}CH_3$), 1.76 (2H, dm, J 130 Hz, $^{13}CH_2$), 3.76 (1H, m, CH); δ_C 9.3 (d, J 34 Hz, $^{13}CH_3$-$^{13}CH_2$), 24.2 (d, J 34 Hz, $^{13}CH_2$-$^{13}CH_3$).

Feeding Experiments with 2-Aminobutanoic Acid Hydrochloride

1. *Senecio pleistocephalus* S. Moore;

Plants were obtained from the Royal Botanic Gardens, Edinburgh, and were propagated by stem cuttings and grown in 5" pots in a standard compost in a greenhouse. The labelled amino acids were dissolved in distilled water and fed by the wick method.

(i) [3,4-$^{13}C_2$]-2-Aminobutanoic acid (11 mg) was fed to three six month old plants over 4 d. After a further 10 d rosmarinine was extracted, using the standard alkaloid extraction procedure, and recrystallised (DCM: Acetone; 1: 1).

(ii) [3,4-$^{2}H_5$]-2-Aminobutanoic acid (150 mg) was fed to two well established plants over 10 d. After a further 5 d the plants were harvested and, using standard methods the alkaloid sample was extracted. The rosmarinine obtained was purified by recrystallisation; m.p. 205-207°C (lit. 202-204°C).

2. *Crotalaria lachnosema*

The labelled amino acids were fed by the wick method as solutions in distilled water.

(i) [3,4-$^{13}C_2$]-2-Aminobutanoic acid (20 mg) was fed over 4 d to eight six month old plants. The plants were harvested after a further 6 d. Extraction was by the normal procedure.

(ii) [3,4-$^{2}H_5$]-2-Aminobutanoic acid (150 mg) was fed over 14 d to one large plant. After a further 4 d the *C. lachnosema* plant was harvested and the standard alkaloid procedure was followed.

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.[115] These cultures, provided by Dr. N Walton at the A.F.R.C. Food Research Institute, Norwich, were grown on Gamborgs B5 basal medium with 80 mM of sucrose added, with a 90 r.p.m. shake rate.

Feeding of 2-Aminobutanoic Acid Hydrochloride to Transformed Root Cultures

1. *Senecio vulgaris*

(i) [3,4-$^{2}H_5$]-2-Aminobutanoic acid (150 mg) was dissolved in sterile water (1 ml) and divided among 10 flasks each containing seven day old roots. The flasks were shaken for a further 14 d before the cultures were drained and the roots were blended in methanol.

(ii) [3,4-$^{13}C_2$]-2-Aminobutanoic acid (15 mg) was dissolved in sterile water and divided among 10 flasks containing six day old roots. After a further 14 d the cultures were drained and the roots blended in methanol.

The standard alkaloid procedure was followed and senecionine isolated.

2. *Emilia flammea*

(i) [3,4-$^{2}H_5$]-2-Aminobutanoic acid (150 mg) was dissolved in sterile water and divided among 10 flasks containing seven day old roots. After a further 15 d shaking, the cultures were harvested.

(ii) The above feeding experiment was repeated and the cultures were harvested after a total of 26 d.

(iii) [3,4-$^{13}C_2$]-2-Aminobutanoic acid (15 mg) was dissolved in sterile water and divided among 10 flasks, each containing seven day old roots. After a further 14 d shaking the cultures were drained and the roots blended in methanol.

The standard alkaloid procedure was then followed.

I am grateful to Isabel Freer for the preparation and maintenance of the root cultures and for carrying out the feeding experiments to these root cultures .

8.5 Experimental for Chapter 6

Preparation of 13-*O*-Acetyldicrotaline (18)

The alkaloid extract from *C. lachnosema* was shown to be a mixture of dicrotaline and the minor product 13-*O*-acetyldicrotaline. For ease of analysis this mixture was converted into solely 13-*O*-acetyldicrotaline. The mixture was dissolved in acetic anhydride (5 ml) and acetyl chloride (5 ml) was added. The reaction mixture was heated at reflux temperature for 2 h, then the solvent was removed *in vacuo*. The residue was taken up in dichloromethane (20 ml) and extracted with 1M HCl (2 x 20 ml). The acidic layer was basified with conc. aqueous ammonia and extracted with dichloromethane (6 x 20 ml). The combined organic layers were dried, filtered and the solvent removed *in vacuo* to give 13-*O*-acetyldicrotaline as an oil δ_H (200 MHz) 1.72 (3H, d, J 0.8 Hz, 13-Me), 2.00 (3H, s, 13-O-Ac), 2.07 (2H, m, 6α, 6β), 2.41 (1H, d, J 15.6 Hz 12-*pro-S*), 2.55 (1H, dm, J 8.9 Hz, 5β), 2.78 (1H, dq, J 13.4, 0.8 Hz, 14-*pro-S*), 2.93 (1H, dd, J 13.4, 1.2 Hz,, 14-pro-R), 3.26 (1H, dd, J 15.6, 1.2 Hz, 12-*pro-R*), 3.28 (1H, dm, J 8.9 Hz, 5α), 3.40 (1H, ddt, J 15.8, 2.1, 5.6, 1.7 Hz, 3β), 3.88 (1H, dtd, J 4.4, 5.5 Hz, 3α), 4.09 (1H, ddq, J 12.2, 1.5, 1.2, 3.9, 1.7 Hz, 9-*pro-R*), 4.30 (1H, br m, H-8), 5.16 (1H, dt, J 4.4, 5.0 Hz, H-7), 5.31 (1H, d, J 12.2 Hz, 9-*pro-S*), 5.90 (1H, quin, J 2.0, 2.1, 1.6, 1.5 Hz, H-2); δ_C 22.3 (13-O-Ac), 26.1 (13-Me), 34.0 (6α, 6β), 41.3 (C-12), 43.7 (C-14), 53.7 (C-5), 60.2 (C-9), 61.9 (C-3), 75.1 (C-7), 77.0 (C-8), 79.5 (C-13), 131.4 (C-2), 132.5 (C-1), 168.7 (C-11), 169.5 (C-15), 170.2 (13-OCO).

8.6 Experimental for Chapter 7

Synthesis of Diethyl 2-Methylaminosuccinate (169)

Methylamine in ethanol (33%, 30.8 ml, 0.30 mol) was added, in portions at 0°C to diethyl maleate (44.130 g, 0.257 mol) with occasional stirring and ice-bath cooling. After addition was complete, the flask was stoppered and kept in the ice-bath for a further 30 min then the mixture was allowed to stand at room temperature for 48 h. The ethanol was then removed under reduced pressure and the colourless residue was dissolved in sufficient 4M HCl to give an acidic solution. This acidic solution was washed with hexane (3 x 150 ml), basified with concentrated ammonia and extracted with dichloromethane (3 x 150 ml). The combined extracts were dried, filtered and concentrated *in vacuo* to give the title compound as a colourless oil (42.258 g, 81%), b.p. 60 °C (0.6 mmHg); υ_{max} (thin film) 3340 (N-H), 2980 (C-H), 2800 (N-Me), 1730 (C=O), 1180 (C-O), ; δ_H (200 MHz) 1.26 (3H, t, J 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 1.29 (3H, t, J 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 2.42 (3H, s, NMe), 2.68 (2H, ddd, J 10.4, 6.2, 6.9 Hz, $C\underline{H}_2CH$), 3.55 (1H, dd, J 6.9, 6.2 Hz, CH), 4.15 (2H, q, J 7.1 Hz, $COC\underline{H}_2$), 4.22 (2H, q, J 7.1 Hz, $COCH_2$); δ_C 14.0, 14.1 (2 x $CO_2CH_2\underline{C}H_3$), 34.9 (NMe), 37.5 (CH_2CH), 59.2 (CH), 60.5, 60.8 (2 x $CO_2\underline{C}H_2$), 170.7, 173.3 (2 x $\underline{C}O_2$); *m/z* 203 (M^+, 0.6%), 158, 130 (100%), 116, 84, 29, 28; (Found: M^+, 203.1151: $C_9H_{17}NO_4$ requires *M*, 203.1158).

Synthesis of Diethyl (*N*-Ethoxycarbonylmethyl)-2-methylamine (170)

Ethyl bromoacetate (6.37 ml, 58.1 mmol) was added to a mixture of diethyl 2-methylaminosuccinate (11.4317 g, 56.2 mmol) and anhydrous potassium carbonate (11.6 g, 84.3 mmol) in aqueous acetone (7% H_2O) (45 ml). The mixture was heated at reflux temperature for 24 h, filtered and the filtrate was concentrated *in vacuo*. The oily residue was dissolved in sufficient 2 M HCl to give an acidic solution. The acidic solution was washed with ether (3 x 10 ml), basified with concentrated ammonia solution and extracted with ether (3 x 15 ml). The combined extracts were dried, filtered and concentrated under reduced pressure to give the desired product (11.894 g, 73%), b.p. 115 °C (0.2 mmHg); υ_{max} 2980 (C-H), 2800 (NMe), 1730 (C=O), 1180 (C-O), 1030 cm^{-1}; δ_H (200 MHz) 1.25, 1.27, 1.30 (9H, t, J 7.1 Hz, 3 x $CO_2CH_2C\underline{H}_3$), 2.44 (3H, s, NMe), 2.76 (2H, ddd, J 16.1, 9.0, 5.9 Hz, $C\underline{H}_2CH$), 3.43 (2H, s, NCH_2), 3.86 (1H, dd, J 9.0, 5.9 Hz, CH), 4.22 (6H, m, 3 x CO_2CH_2); δ_C 14.0, 14.1, 14.2 (3 x $CO_2CH_2CH_3$), 35.6 ($\underline{C}H_2CH$), 38.9 (NMe), 56.0 (NCH_2), 60.4, 60.5, 60.6 (3 x CO_2CH_2), 62.4 (CH), 170.6, 170.7, 170.9 (3 x CO_2); *m/z* 289 (M^+, 4.8%), 216 (100 %), 202, 170, 142, 70; (Found: M+, 289.1524: $C_{13}H_{23}NO_6$ requires *M*, 289.1525).

Synthesis of Diethyl 1-Methyl-4-oxopyrrolidine-2,3-dicarboxylate (171)

The triester (3.538 g, 12.5 mmol) in dry benzene (25 ml) was stirred with sodium hydride (oil free, 95%, 0.336 g, 12.9 mmol)

at room temperature, under a nitrogen atmosphere. After 5 h the reaction mixture was extracted with water (3 x 20 ml). The aqueous extracts were combined, washed with diethyl ether (3 x 20 ml) and acidified with concentrated HCl, with ice cooling, to pH 1. The acidic solution was washed with ether (3 x 30 ml) and then the pH adjusted to 6 with concentrated aqueous ammonia solution. The combined organic extracts were dried, filtered and concentrated *in vacuo* to give the title compound as a colourless oil (1.869 g, 62 %); υ_{max} (thin film) 2980 (C-H), 2790 (NMe), 1770 (C=O), 1730 (OC=O), 1200 (C-O), and 1030 cm^{-1}; δ_H (200 MHz) 1.28 (6H, t, J 7.2 Hz, 2 x $CO_2CH_2CH_3$), 2.54 (3H, s, NMe), 3.02 (1H, d, J 17.0 Hz, NCH_A), 3.59 (1H, d, J 17.0 Hz, NCH_B), 3.60 (1H, d, J 9 Hz, NCH), 3.85 (1H, d, J 9,0 Hz, CHCO), 4.24 (4H, q, J 7.2 Hz, 2 x CO_2CH_2); δ_C 14.1 (2 x $CO_2CH_2CH_3$), 40.7 (NMe), 58.6 (NCH), 61.6, 62.1 (OCH_2), 62.5 (NCH_2), 67.6 (CHCO), 166.2, 170.2 (CO_2Et), 203.7 ($COCH_2$); *m/z* 243 (M^+, 1.5 %), 198, 170, 142, 124, 98, 29 (100 %); (Found: M^+, 243.1108: $C_{11}H_{17}NO_5$ requires *M*, 243.1107).

Synthesis of Diethyl 4-Hydroxy-1-methylpyrrolidine-2,3-dicarboxylate (172)

A solution of sodium borohydride (0.381 g, 0.44 mmol) in water (0.5 ml) was added to an ice-cold solution of the crude pyrrolidone (0.2155 g, 0.88 mmol) in aqueous sodium hydroxide (2%) (1.6 ml). The mixture was stirred at 0 °C for 1.5 h, and then carefully acidified with 2M HCl. The acidic solution was washed with ether (3 x 5 ml), then basified with concentrated ammonia solution. The aqueous layer was then extracted with ether (3 x 5 ml). The combined organic extracts were dried, filtered and

concentrated *in vacuo.* to give title compound as an oil (70 mg, 65%); υ_{max} 3450 (O-H), 2980 (C-H), 2800 (NMe), 1770, 1730 (C=O), 1205, 1180 (C-O), and 1030 cm^{-1}; δ_H (200 MHz) 1.23-1.35 (6H, m, 2 x $CO_2CH_2\underline{C}H_3$), 2.44 (3H, s, NMe), 2.50-3.65 (6H, m, 2-H, 3-H, 4-H, 5-H_2, OH), 4.14-4.39 (4H, m, 2 x $CO_2\underline{C}H_2CH_3$); δ_C 14.0 (2 x $CH_2\underline{C}H_3$), 39.5, 40.6, 41.0 (NMe), 53.1, 53.6, 57.8 (<u>C</u>HOH), 61.3 (2 x $CO_2\underline{C}H_2$), 63.2, 63.6, 63.8 (NCH_2), 66.5, 67.8, 69.0 (<u>C</u>HCHOH), 70.5, 71.9, 73.8 (CHN), 172.0 (2 x $\underline{C}O_2Et$); *m/z* 247 (M^+, 0.1 %), 245, 172 (100 %), 144, 126, 82; (Found M^+-H_2, 245.1268: $C_{11}H_{19}NO_5$ requires *M*-H_2, 245.1263).

Synthesis of Diethyl 1-Methyl-3-pyrroline-2,3-dicarboxylate (173)

A solution of *p*-toluenesulfonyl chloride (8.20 g, 0.44 mol), in dry pyridine (13 ml), was added to a solution of the hydroxypyrroline (2.729 g, 0.11 mol) in pyridine (6 ml). The mixture was heated on a steam bath in a stoppered flask for 2 h, after which time the solvent was removed under reduced pressure. The residue was dissolved in sufficient ice-cold 2M HCl to give an acidic suspension. The suspension was washed with ether (4 x 25 ml), basified with concentrated ammonia solution, and extracted with ether (3 x 65 ml). The combined organic extracts were dried, filtered and concentrated *in vacuo* to give a dark red oil. The oil was azeotroped with water (3 x 10 ml) and then benzene (3 x 10 ml) to remove any residual pyridine. The product was then redissolved in ether, dried, filtered and concentrated under reduced pressure to give the desired product as dark red oil (0.998 g, 40%); υ_{max} ($CHCl_3$ soln) 3020 (C=C-H),

2980 (C-H), 2800 (NMe), 1730 (C=O), 1645 (C=C), 1280, 1215 (C-O), 1035, and 980 cm^{-1}; δ_H (200 MHz) 1.26, 1.27 (6H, t, J 7.1 Hz, 2 x $CO_2CH_2C\underline{H}_3$), 2.59 (3H, s, NMe), 3.52 (2H, ddd, J 16.6, 4.4, 2.1 Hz, $NC\underline{H}_2$), 4.01 (1H, ddd, J 16.6, 5.4, 2.1, Hz, NCH_2) 4.11-4.26 (5H, m, 2-H and 2 x $CO_2C\underline{H}_2$), 6.88 (1H, m, C=CH); δ_C 14.1, 14.2, (2 x $CO_2CH_2\underline{C}H_3$), 40.9 (NMe), 60.6, 61.0 (2 x $CO_2C\underline{H}_2$), 62.0 (NCH_2), 73.0 ($N\underline{C}H_2CO_2$), 133.2 ($\underline{C}$=CH), 141.7 (CH=C), 162.7 ($CHCO_2Et$), 171.3 (CH=C-CO_2Et); *m/z* 227 (M^+, 1.5 %), 180, 154, 126, 108, 82 (100 %); (Found: 227.1148; $C_{11}H_{17}NO_4$ requires M^+, 227.1158).

Synthesis of 2,3-bis(Hydroxymethyl)-1-methyl-3-pyrroline (Synthanecine A) (19)

A solution of DIBAL in dichloromethane (1.0M, 33.5 ml, 33.5 mmol) was added, with stirring and cooling to 20-25 °C, over 30 min to a solution of the diester (0.710 g, 2.8 mmol) in dry toluene (5 ml) under argon. The reaction mixture was stirred at room temperature for 1 h. Ethyl acetate (5 ml) was then added to consume the excess DIBAL. After a further 5 min acetone (11 ml) followed by Celite (2.2 g) were added. Methanol was then added slowly and with cooling. The mixture was shaken vigorously until gelling occurred (5 min), then water (22 ml) was added. The mixture was shaken vigorously again to break up the gel, then stirred at room temperature for 1.5 h. The resulting suspension was filtered, and the solid residue was washed, firstly with hot water (2 x 10 ml) then with hot methanol (2 x 10 ml). The combined filtrates were concentrated under reduced pressure, and then azeotroped with benzene (4 x 5 ml). Finally a

solution of the product in chloroform was dried, filtered and concentrated under reduced pressure to give the title compound as a thick oil (0.151 g, 42 %); υ_{max} ($CHCl_3$) 3380 (br, O-H) 3020 (C=C-H), 2975- 2880 (C-H), 2800 (NMe), 1215 (C-O), and 1010 cm^{-1}; δ_H (200 MHz) 2.33 (3H, s, NMe), 3.08 (1H, m, 5-H), 3.36 (1H, m, 2-H), 3.55 (2H, m, 6-H_2), 4.03 (2H, br s, 7-H_2), 4.65 (2H, br s, 2 x OH), 5.62 (1H, br s, 7-H_2); δ_C 41.0 (NMe), 59.1 (C-6), 61.0 (C-5 and C-7), 73.8 (C-2), 124.0 (C-4), 141.7 (C-3); *m/z* 143 (M^+, 0.9%), 141, 112, 94, 82 (100%), 67, 59, 57; (Found: M^+, 143.0936; $C_7H_{13}NO_2$ requires 143.0946).

Synthesis of 2,3-bis(Hydroxymethyl)-1-methyl-pyrrolidine (Synthanecine B) (20)

The pyrroline (0.736 g, 3.24 mmol) and lithium aluminium hydride (1.49 g) in ether (28 ml) were heated at reflux temperature for 1 h. 1M NaOH solution was added to the cooled mixture. The ether layer was decanted and the wet solids were stirred with hot ethanol. Chloroform (5 ml) and Celite (1.5 g) were added and the mixture was filtered. The filtrate was concentrated to an oil which was re-extracted with hot chloroform. This extract was combined with the original ether layer and the combined organic layers were dried, filtered, and the solvent was evaporated *in vacuo* to give synthanecine B as a thick oil (282 mg, 60 %), υ_{max} ($CHCl_3$) 3380 (br, O-H), 2950-2880 (C-H), 2795 (NMe), 1220-1210 (C-O), and 1035 cm^{-1}; δ_H (200 MHz) 2.35 (3H, s, NMe), 2.12-2.51 (3H, m, 3-H, 4-H_2), 3.39-3.90 (7H, m, 6-H_2, 7-H_2, 2-H, 5-H), 4.90 (2H, br s, 2 x OH); δ_C 26.0 (C-4), 40.5, 40.9 (NMe), 44.5 (C-3), 72.1, 73.5 (C-2), 55.7, 56.0 (C-5),

59.3, 60.5 (C-7), 60.9, 61.7 (C-6); *m/z* 111, 97, 85, 71, 57 (100 %).

Feeding of Synthanecine A and Synthanecine B to *Senecio vulgaris*

(i) Synthanecine A (150 mg) was dissolved in distilled water and divided among 10 flasks containing seven day old roots. The roots were shaken for a further 14 d before the cultures were drained and the roots were blended in methanol.

(ii) Synthanecine B (150 mg) was fed as described above.

Root culture preparation was as previously described. Once again thanks are due to Isabel Freer for carrying out this part of the work. The standard alkaloid procedure was then followed. Senecionine (20-30 mg) was isolated in both cases.

Attempted Synthesis of 3-Hydroxymethyl-1-methylpyrrolidine (183)

Trimethylamine *N*-oxide (0.400 g, 5.29 mmol) was heated under vacuum at 40 °C for 2 h. THF (50 ml) and allyl alcohol (0.36 ml, 307 mg, 5.29 mmol) were added and the temperature was lowered to -78 °C for 4 h. Excess LDA was quenched with water (20 ml). The layers were separated and the aqueous layer was extracted with ether (2 x 20 ml). The combined organic layers were extracted with 1M HCl (2 x 30 ml). The acidic layers were basified with concentrated aqueous ammonia and extracted

with dichloromethane (3 x 50 ml). The organic layers were dried, filtered and the solvent was evaporated. No pyrrolidine was observed and the starting material could not be recovered. The reaction was repeated at 0 °C and with stirring continued up to 18 h.

Attempted Synthesis of Dimethyl 1-Methyl-3-pyrroline-3,4-dicarboxylate

The procedure was carried out as described above except that dimethyl acetylenedicarboxylate was used. The reaction was attempted at 0 °C and -78 °C and with stirring for up to 6 h. No pyrroline was observed and the product was a dark brown oil which was a mixture of materials.

Attempted Synthesis of Diethyl 1-Methylpyrrolidine-3,4-dicarboxylate

The procedure was carried out as before but using diethyl fumarate and diethyl maleate at 0 °C and -78 °C. The reaction was stirred for up to 20 h but a mixture of products were obtained as a brown oil. There was no pyrrolidine present.

Attempted Synthesis of 3-Hydroxymethyl-1-methylpyrroline (184)

The reaction procedure was carried out as previously described but using propargyl alcohol. With a temperature of -78 °C and stirring for 4 h there was no pyrroline detected. A

dark oil was obtained and no starting material could be recovered.

Attempted Synthesis of 1-Methyl-4,5,6,7-tetrahydrodioxepinpyrrolidine (186)

The procedure was carried out as before except with *cis*-4,7-dihydro-1,3-dioxepin at 0 °C and -78 °C and stirring for 4 h. No pyrrolidine was observed and the starting material could not be recovered.

Synthesis of *N*-Benzyl-*N*-(trimethylsilylmethyl)-aminomethyl methyl Ether (188)

To a solution of formalin and methanol (0.092 ml. 0.073 g, 2.28 mmol) was added *N*-(trimethylsilylmethyl)benzylamine (0.366 g, 1.90 mmol) dropwise at 0 °C. The mixture was then stirred for an additional 3 h. Potassium carbonate was added and the oily layer was separated. The residue was washed with ether and the combined organic layers were dried over anhydrous potassium carbonate. Filtration and solvent evaporation gave the title compound as a colourless liquid (0.3762 g, 84 %); b.p. 77 °C υ_{max} 3030 (Ar C-H), 2950 (C-H), 2840 (O-CH_3), 1500 (Ar C-C), 1250 (Me_3Si), 1070 (C-OC), 850 (Me_3Si), 740, and 700 cm^{-1}; δ_H (200 MHz) 0.00 (9H, s, Me_3Si), 2.11 (2H, s, $SiCH_2$), 3.18 (3H, s, OCH_3), 3.71 (2H, s, OCH_2), 3.95 (2H, s, CH_2Ph), 7.12 (5H, m, Ph); δ_C -1.44 (Me_3Si) 42.6 ($SiCH_2$), 55.3 (OCH_3), 59.5 (OCH_2), 88.4 (CH_2Ph), 126.8, 128.0, 128.7 (CH), 139.7 (C); *m/z* 237 (M^+, 0.5 %),

222, 206, 192, 164, 134, 116, 91, 89, 77, 30 (100 %); (Found: M^+ 237.1537; $C_{13}H_{23}NOSi$ requires *M*, 237.1526).

Synthesis of Diethyl 1-Benzylpyrrolidine-3,4-dicarboxylate (192)

Caesium fluoride (84 mg, 0.55 mmol) was heated under vacuum at 40°C for 2 h, then THF (10 ml), aminomethyl methyl ether (647 mg, 2.73 mmol) and diethyl fumarate (470 mg, 2.73 mmol) were added, under a nitrogen atmosphere. To this solution, trimethylsilyl triflate (109 mg, 0.55 mmol) was added and the reaction mixture was stirred at 60 °C for 18 h. The flask was cooled to 0 °C and 15% NaOH (10 ml) added. The organic layer was separated and the aqueous phase was extracted with ether (3 x 10 ml). The combined organic layers were dried (K_2CO_3), filtered and the solvent was removed under reduced pressure. The pyrrolidine was isolated as an oil by preparative TLC (84%); R_f 0.85 (Et_2O) υ_{max} 3020 (ArC-H), 2980-2800 (C-H), 1730 (C=O), 1300, 1220 (C-O), and 740 cm^{-1}; δ_H (200 MHz) 1.18 (6H, t, J 7.2 Hz, 2 x CH_3), 2.78 (4H, m, NCH_2), 3.39 (2H, m, 2 x $CHCH_2$), 3.54 (2H, s, $PhCH_2$), 4.09 (4H, q, J 7.2 Hz, 2 x CO_2CH_2), 7.23 (5H, m, Ph); δ_C 14.1 (2 x CH_3), 45.4 (2 x $CHCO_2$), 56.5 (2 x CH_2CH), 59.3 (NCH_2), 60.9 (2 x CO_2CH_2), 127.0, 128.2, 128.5 (Ar CH), 138.3 (CCH_2), 173.5 (2 x CO_2Et); δ_C 14.1 (CH_3), 45.4 (CH_2), 56.5 (CH_2CH), 59.3 (NCH_2), 60.9 (CO_2CH_2), 127.0, 128.2, 128.5 (C-H), 138.3 (CCH_2), 173.5 (2 x CO_2); *m/z* 305 (M^+, 2.2%), 276, 260, 228, 214, 168, 140, 91 100%), 77; (Found: M^+, 305.1626; $C_{17}H_{23}NO_4$ requires *M*, 305.1626).

Synthesis of Dimethyl 1-Benzyl-3-pyrroline-3,4-dicarboxylate (191)

The procedure described above was carried out using caesium fluoride (0.2580 g, 1.7 mmol), dimethyl acetylene dicarboxylate (0.98 ml, 1.137g, 8 mmol), aminomethyl methylether (1.896 g, 8 mmol), and trimethylsilyl triflate (0.328 ml, 0.334 g, 0.17 mmol), and the mixture was heated at 55 °C for 20 h to give the title compound as a colourless oil (61 %); υ_{max} 3020 (C-H), 2960 (C-H), 1735 (C=O), 1725 (C=O), 1600, 1580, 1500 (Ar C-C), 1270, 1210 (C-O), and 780 cm^{-1}; δ_H (200 MHz) 3.79 (6H, s, 2 x CH_3), 3.81 (2H, s, $PhCH_2$), 3.92 (4H, s, 2 x CH_2C), 7.33 (5H, m, CH); δ_C 52.2 (CH_3), 59.7 ($PhCH_2$), 60.6 (CH_2C), 127.3, 128.5, 128.6 (CH), 137.1 (C=C), 138.4 (ArC), 164.0 (2 x CO_2); *m/z* 275 (M^+, 3.5%), 243, 184, 157, 91 (100%), 77; (Found: 275.1166; $C_{15}H_{17}NO_4$ requires *M*, 275.1158).

Synthesis of 3,4-bis-(Hydroxymethyl)-1-benzylpyrrolidine (193)

The diester (228 mg, 0.748 mmol) was dissolved in dry toluene (5 ml) in a three-necked flask under a nitrogen atmosphere. A solution of DIBAL (1.0M in toluene, 90 ml, 9.0 mmol) was added over 10 min, with stirring and cooling in an ice-bath. After addition was complete the reaction mixture was stirred at room temperature for 1 h. Ethyl acetate (2 ml) was added to consume the excess DIBAL, followed after 5 min by acetone (10 ml) and Celite (2 g). Methanol (2 ml) was then added, slowly and with cooling. The mixture was then shaken

vigorously until gelling occurred (5 min), before water (20 ml) was added, to break up the gel, and the mixture was stirred for 1.5 h. The suspension was then filtered and the solid residue was washed with hot water (2 x 10 ml) then hot methanol (2 x 10 ml). The combined filtrates were concentrated *in vacuo*. The crude product was extracted with chloroform (10 ml), dried, filtered and concentrated to give the desired product which was recrystallised from dichloromethane/ acetone (1:1) (70 mg, 70 %); m.p. 82 °C; υ_{max} ($CHCl_3$) 3350 (br, O-H), 3020 (Ar C-H), 2955-2870 (C-H), 1600, 1580 (Ar C-C), 1220, 1215, 1213 (C-O), and 760 cm^{-1}; δ_H (200 MHz) 2.15 (2H, m, 2 x C<u>H</u>CH_2), 2.30 (2H, dd, J 8.5, 5.6 Hz, C<u>H</u>CH_2OH), 2.72 (2H, dd, J 9.0, 7.3 Hz, C<u>H</u>$_2$CH), 3.54 (4H, m, 2 x C<u>H</u>$_2$OH), 3.55 (2H, s, PhC<u>H</u>$_2$), 3.81 (2H, br s, 2 x OH), 7.27 (5H, m, Ph); δ_C 44.6 (<u>C</u>HCH_2), 57.1 (N<u>C</u>H$_2$CH), 60.2 (Ph<u>C</u>H$_2$), 65.7 (<u>C</u>H$_2$OH), 127.1, 128.3, 128.8 (Ar CH), 138.3 (Ar C); *m / z* 221 (M^+, 3.4%), 202, 191, 144, 130, 112, 91 (100%), 77; (Found: 221.1403; $C_{13}H_{19}NO_2$ requires *M*, 221.1416).

REFERENCES

1. L.B. Bull, C. C. J. Culvenor and A.T. Dick, " *The Pyrrolizidine Alkaloids* ", North Holland, Amsterdam, 1968.
2. A. R. Mattocks, " *Chemistry and Toxicology of Pyrrolizidine Alkaloids* ", Academic Press, London, 1986.
3. D. J. Robins, *Fortschr. Chem. Org. Naturstoffe*, 1982, 41, 115.
4. C. C. J. Culvenor, in " *Toxicology in the Tropics* ", Eds. R. L. Smith and E. A. Bababunmi, pp 124-141, Taylor and Francis, London, 1981.
5. A. A. Forsyth, " British Poisonous Plants ", H.M. Stationery Office, London, 1968.
6. F. C. Willmot and G. W. Robertson, *Lancet*, 1920, 2, 848.
7. O. Mohabbat, R. N. Srivastava, M. S. Younos, G. G. Sediq, A.

A. Merzad, and G. N. Aram, *Lancet*, 1976, 2, 269.

8. see *Chem. Br.*, 1989, 25, 1192.
9. J. A. Edgar and C. C. J. Culvenor, *Experientia*, 1975, 31, 393.
10. A. R. Mattocks, " *Phytochemical Ecology* ", Ed. J. B. Harborne, Academic Press, New York, 1972, p 179.
11. C. L. Miranda, R. L. Reed, F. P. Guengerich and D. R. Buhler, *Carcinogenesis*, London, 1991, 12, 515.
12. A. R. Mattocks, *Nature*, 1968, 219, 480.
13. A. R. Mattocks, *Chem.-Biol. Interact.*, 1972, 5, 227.
14. A. R. Mattocks and H. E. Driver, *Toxicology*, 1983, 27, 159.
15. I. N. A. White and A. R. Mattocks, *Biochem. J.*, 1972, 128, 291.
16. R. Schoental, *Nature*, 1957, 179, 361.

17. L. I. Grebenuik and Z. F. Zaharova, *Farmacol. i. Toksikol*, 1952, 15, 25.
18. C. C. J. Culvenor, A. T. Dann and A. J. Dick, *Nature*, 1962, 195, 570.
19. R. Schoental, *Israel J. Med. Sci.*, 1968, 4, 1133.
20. A. R. Mattocks, *Toxicology Lett.*, 1982, 14, 111.
21. L. Letendre, W. A. Smithson, G. S. Gilchrist, E. O. Burgret, C. H. Hoagland, H. H. Ames, G. Powis and J. S. Kovach, *Cancer*, 1981, 37, 437.
22. S. V. Anichkov and M. L. Belenky, " *Textbook of Pharmacology*", Medgiz, Leningrad, 1955.
23. D. J. Robins, *Nat. Prod. Rep.*, 1984, 1, 235; 1985, 2, 213; 1986, 3, 297; 1987, 4, 577; 1989, 6, 221; 1990, 7, 377; 1991, 8, 213; 1992, 9, 313; 1993, 10, 487.
24. D. J. Robins, *Chem. Soc. Rev.*, 1989, 18, 375.
25. C. Hughes and F. L. Warren, *J. Chem. Soc.*, 1962, 34.
26. D, H. G. Crout, M. H. Benn, H. Imaseki and T. A. Geissman, *Phytochemistry*, 1966, 1, 5.
27. D. H. G. Crout, *J. Chem. Soc. (C)*, 1966, 1968.
28. D. H. G. Crout, *J. Chem. Soc. (C)*, 1967, 1233.
29. E. Nowacki and R. U. Byerrum, *Life Sci.*, 1962, 1, 157.
30. C. A. Hughes, R. Letcher and F. L. Warren, *J. Chem. Soc.*, 1964, 4974
31. N. Bottomley and J. A. Geissman, *Phytochemistry*, 1964, 3, 357.
32. N. M. Bale and D. H. G. Crout, *Phytochemistry*, 1975, 14, 2617.
33. D. J. Robins and J. R. Sweeney, *J. Chem. Soc., Perkin Trans I*, 1981, 3083.

34. T. Hartmann, H. Sander, R. Adolph and G. Toppel, *Planta*, 1988, 175, 82.

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

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

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

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

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

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

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

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

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

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

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

46. F. Bottcher, R-D. Adolph, and T. Hartmann, Phytochemistry, 1993, 32, 679..

47. D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1986, 250.

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

49. A. A. Denholm, H. A. Kelly, and D. J. Robins, *J. Chem. Soc., Perkin Trans.I*, 1981, 2003.

50. E. K. Kunec and D. J. Robins, *J. Chem. Soc. Chem. Commun.*, 1986, 250.

51. E. K. Kunec and D. J. Robins, *J. Chem. Soc. Perkin Trans. I*, 1989, 1437.

52. J. Rana and E. Leete, *J. Chem. Soc., Chem. Commun.*, 1983, 1742; E. Leete and J. Rana, *J. Nat. Prod.*, 1986, 49, 838.

53. D. B. Hagan and D. J. Robins, *J. Chem. Res. (S)*, 1990, 292.

54. G. Toppel, L. Witte, B. Riebesehl, K. V. Borstel and T. Hartmann, *Plant Cell Rep.*, 1987, 6, 466.

55. H. A. Kelly, E. K. Kunec, M. Rodgers and D. J. Robins, *J. Chem. Res. (S)*, 1989, 292.

56. D. J. Robins and J. R. Sweeney, *Phytochemistry*, 1975, 14, 2617.

57. G. R. Orr and S. J. Gould, *Tetrahedron Lett.*, 1982, 23, 3139.

58. I. D. Wigle, L. J. J. Mestichelli and I. D. Spenser, *J. Chem. Soc., Chem. Commun.*, 1982, 662.

59. D. J. Robins, *Phytochemistry*, 1983, 22, 1133.

60. J. Rana and D. J. Robins, *J. Chem. Soc. Chem. Commun.*, 1983, 122.

61. G. Grue-Sorensen and I. D. Spenser, *J. Chem. Soc., Chem. Commun.* 1982, 662.

62. J. Rana and D. J. Robins, *J. Chem. Soc., Perkin Trans. I*, 1986, 983.

63. E. K. Kunec and D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1985, 1450; *J. Chem. Soc., Perkin Trans. I*, 1986, 983.

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

65. I. K. A. Freer, J. R. Matheson, M. Rodgers and D. J. Robins, *J. Chem. Res. (S)*, 1991, 46.

66. C. G. Gordon-Gray and F. D. Schlosser, *J. S. Afr. Chem. Inst.*, 1970, 2313.

67. W. C. Evans and J. G. Wooley, *J. Pharm. Pharmacol.*, 1965, 17, suppl., 375.

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

69. H. E. Umbarger and E. A. Adelberg, *J. Biol. Chem.*, 1951, 192, 883.

70. D. H. G. Crout, N. M. Davies, E. H. Smith and D. Whitehouse, *J. Chem. Soc., Perkin Trans. I*, 1972, 671.

71. Tsung-ya-Lo and Yo-Wei Tong, *Chem. Abs.*, 1965, 62, 5004.

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

73. W. E. Splittstosser and M. Mazales, *Phytochemistry*, 1967, 3, 39.

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

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

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

77. R. Cahill, D. H. G. Crout, M. V. M. Gregorio, M. B. Mitchell, and U. S. Muller, *J. Chem. Soc., Perkin Trans. I*, 1983, 173.

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

79. J. A. Devlin and D. J. Robins, *J. Chem. Soc., Perkin Trans. I*, 1984, 1329.

80. A. A. Denholm and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1991, 19.

81. T. W. Whaley, G. H. Daub, V. N. Kerr, T. A. Lyle, and E. S. Olson, *J. Lab. Compd. Radiopharm.*, 1979, 16, 809.
82. S-S. Yuan, *J. Lab. Compd. Radiopharm.*, 1983, 20, 173.
83. I. C. Nordin, *J. Heterocyclic Chem.*, 1966, 3, 531.
84. P. Allen Jnr. and J. Ginos, *J. Org. Chem.*, 1963, 28, 2759.
85. J. S. C. Marais, *Onderstepoort J. Vet. Sci. Anim. Ind.*, 1944, 20, 61.
86. A. R. Mattocks and N. Nwude, *Phytochemistry*, 1988, 27, 3289.
87. D. J. Robins, D. S. Rycroft and I. R. Stirling, *Magn. Reson. Chem.*, 1993, 30, 42.
88. R. K. Hill, S-W. Rhee, C. Leete and B. A. McGaw, *J. Am. Chem. Soc.*, 1980, 102, 7344.
89. J. March, *Advanced Organic Chemistry*, 4th Edn., Wiley, 1992.
90. C. S. Palmer and P. W. McWhirter in " *Organic Synthesis* ", Coll. Vol. 1, Wiley, 245.
91. C. S. Marvel in " *Organic Synthesis* ", Coll. Vol. 3, Wiley, 495.
92. S. Komatsubara, M. Kiumi, I. Chibata, M. V. M. Gregorio, U. S. Muller, and D. H. G. Crout, *J. Chem. Soc. Chem. Commun.*, 1979, 839.
93. A. S. Chahal and M. S. Kang, *J. Res. (Punjab Agric. Univ.)*, 1979, 16, 57; R. M. Kluba, L. R. Mattrick and L. R. Hacker, *Am. J. Enol. Vitic*, 1978, 29, 102;
94. T. Ogawa, N. Bando and K. Sasaoka, *Agric. Biol. Chem.*, 1976, 40, 1661.
95. F. Scheffer, R. Kickuth and E. Schlimme, *Naturwiss enschaften*, 1967, 54, 118.

96. V. W. Rodwell, " *Methods Enzymol.* ", Vol 17, Issue Pt. B, 188-189.

97. J. R. Matheson, Ph. D. Thesis, University of Glasgow, 1993 .

98. R. H. Barbour and D. J. Robins, *Phytochemistry*, 1987, 26, 2430.

99 .G. G. Habermeld, *Toxicon*, 1988, 26, 275.

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

101. J. C. Tchouankev, E. Tsamo, B. L. Sandengam, J. D. Connolly and D. S. Rycroft, *Tetrahedron Lett.*, 1990, 31, 4505.

102. J. H. Noggle and R. E. Schirmer, *The Nuclear Overhauser Effect: Chemical Applications*, p.59, Academic Press, New York, 1971.

103. H. Stoecki-Evans and D. J. Robins, *Acta Crystallogr., Sect. C*, 1987, 43, 1638.

104. J. A. Devlin, D. J. Robins and S. Sakdarat, *J. Chem. Soc., Perkin Trans. I*, 1982, 1117.

105. T. A. Geismann and A. C. Weiss, *J. Org. Chem.*, 1962, 27, 139.

106. A. R. Mattocks, *J. Chem. Soc., Perkin Trans I*, 1974, 707.

107. A. R. Mattocks, *J. Chem. Soc., Perkin Trans I*, 1978, 896.

108. R. Barbour, Ph. D. Thesis, University of Glasgow, 1986.

109. G. Baxter, Ph. D. Thesis, University of Glasgow, 1989.

110. R. Beugelmans, L. Benadjila-Iguertsira, J. Chastanet, G. Negron, and G. Roussi, *Can. J. Chem.*, 1984, 63, 725.

111. J. Chastenet and G. Roussi, Heterocycles, 1985, 23, 653.

112. R. Huigsen in " *1,3-Dipolar Cycloaddition Chemistry* ", General Heterocyclic Chemistry Series, Ed. A. Padwa, Wiley Interscience Publication, New York, 1984.

113. J-P. Begue, D. Bonnet-Delpon and T. Lequeux, *Tetrahedron Lett.*, 1993, 34, 3279.

114. A. Hosomi, Y. Sakata, and H. Sakurai, *Chem. Lett.*, 1984, 1117.

115. N. J. Walton and N. J. Belshaw, *Plant Cell Rep.*, 1988, 7, 115; N. J. Walton, D. J. Robins and M. J. C. Rhodes, *Plant Sci.*, 1988, 54, 125.

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