BIOSYNTHESIS OF THE PYRROLIZIDINE ALKALOID ROSMARININE

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

by

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September 1987

To: My Mum and Dad

ACKNOWLEDGEMENTS

I should like to extend to Dr. D.J. Robins my sincere gratitude, for his unfailing support and direction during the course of this work, and ultimately, in the presentation of this thesis.

I also wish to express my appreciation to Dr. G.N. Sheldrake and to Dr. A.M. Fraser for their assistance and helpful discussions.

Thanks are also due to the following people: to Dr. D.S. Rycroft, Mr. J. Gall and Dr. I. Sadler (University of Edinburgh), for n.m.r. spectra; to Mr. A. Ritchie and Mr. P. Stewart for mass spectra; to Mrs. W. Harkness and Mrs. K. Wilson for microanalyses ; to Mrs. F. Lawrie for i.r. spectra; to Mrs. P. Tait for radioactive counting; and to Dr. A.A. Freer for X-ray analyses.

I am grateful to the staff of the Edinburgh Royal Botanic Garden for identifying and supplying Senecio pleistocephalus.

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

Thanks also go to Mrs. J. Anthony for the excellent typing of this thesis.

Finally, I want to say 'thank-you' to my Mum, Dad, and brother Andrew for their endless encouragement and support throughout this period of my career.

TABLE OF CONTENTS

SUMMARY

The work presented in this thesis is divided into three sections: (a) Biosynthesis of rosmarinecine; (b) Synthesis of macrocyclic pyrrolizidine diester analogues; and (c) Structural studies.

(a) Biosynthesis of Rosmarinecine

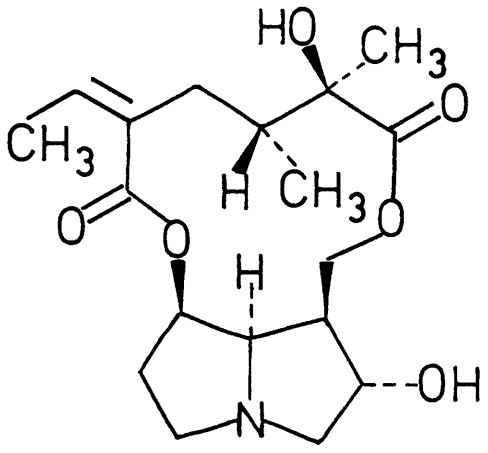
The biosynthesis of rosmarinecine, the base portion of the pyrrolizidine alkaloid rosmarinine (A), has been studied in Senecio pleistocephalus (family Compositae) plants. Samples of [1-^{13}C]- and [2,3-$^{13}C_2$]putrescine dihydrochloride were incorporated with very high specific incorporations (up to 25% per C_4 unit) into rosmarinine. ^{13}C N.m.r. spectroscopy established complete labelling patterns and showed that two molecules of putrescine were incorporated to about the same extent into rosmarinine. The incorporation of [1-amino-^{15}N, 1-^{13}C]-putrescine dihydrochloride into rosmarinine produced a labelling pattern which was indicative of the conversion of two putrescine molecules into a C_4-N-C_4 symmetrical intermediate. Intact incorporation of [1,9-$^{13}C_2$]-homospermidine trihydrochloride into rosmarinecine, was consistent with this intermediate being homospermidine.

The stereochemistry of the enzymic processes in the pathway was investigated by feeding (R)-[1-^{2}H]- and (S)-[1-^{2}H]putrescine dihydrochloride to S. pleistocephalus. The labelling patterns obtained in rosmarinine, as determined by ^{2}H n.m.r. spectroscopy, were consistent with the following. The oxidation of putrescine to 4-aminobutanal occurs with the loss of the pro-S hydrogen. The aldehyde and another

molecule of putrescine condense to give the corresponding imine, which after reduction (on the C-si face) affords homospermidine. Two further oxidations each take place with loss of the pro-S hydrogens generating the dialdehyde, which after Mannich cyclisation produces 1β-formyl-8α-pyrrolizidine. Reduction to isoretronecanol proceeds by the delivery of a hydride equivalent on the C-re face of the carbonyl group. Feeding experiments with (R)-[2-^{2}H]- and (S)-[2-^{2}H]putrescine dihydrochloride established that the two hydroxylations in rosmarinecine occur with retention of configuration and that formation of the pyrrolizidine ring involves the stereospecific removal of the pro-R hydrogen on the carbon which becomes C-1 of rosmarinecine.

The proposed product from the first oxidation of homospermidine, N-(4-aminobutyl)-1,2-didehydropyrrolidinium, was shown to be an intermediate in the biosynthetic pathways to several pyrrolizidine alkaloids, by ^{14}C-labelling experiments and an intermediate trapping experiment.

Platynecine was shown to be an efficient precursor for rosmarinecine biosynthesis using ^{3}H-labelling experiments. Similarly, rosmarinecine was found to be an efficient precursor for rosmarinine biosynthesis.



(A)

(b) Synthesis of Macrocyclic Pyrrolizidine Diester Analogues

A number of optically active 12-membered macrocyclic dilactones containing (+)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine were prepared by lactonisation *via* the pyridine-2-thiolesters with different anhydrides. These are the first synthetic macrocyclic pyrrolizidine diester analogues which contain a saturated pyrrolizidine base.

(c) Structural Studies

The plant *Lindelofia longiflora* (family Boraginaceae) was shown, by spectroscopic studies, to contain one pyrrolizidine alkaloid, echinatine. Similarly, *Cynoglossum macrostylum* (family Boraginaceae) was found to contain two major alkaloidal components. Echinatine and heliosupine were identified in the ratio 3:1.

ABBREVIATIONS

br - broad

CDI - N,N'-carbonyl diimidazole

COSY - correlation spectroscopy

d - doublet

DBN - 1,5-diazabicyclo[4.3.0]non-5-ene

DBU - 1,8-diazabicyclo[5.4.0]undec-7-ene

DCC - Dicyclohexylcarbodiimide

DEPT - distortionless enhancement by polarisation transfer

DIBAL - di-isobutylaluminium hydride

DMAP - 4-N,N -dimethylamino pyridine

DMF - N,N -dimethylformamide

DME - 1,2-dimethoxyethane

Im - imidazole

i.r. - infra red

MEM - methoxyethoxy methyl

MOM - methoxy methyl

MTM - methane thio methyl

n.m.r. - nuclear magnetic resonance

s - singlet

t - triplet

TBDMS - tertiary butyldimethylsilyl

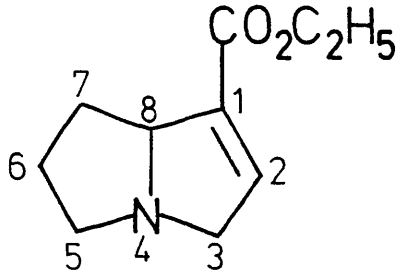
THF - tetrahydrofuran

t.l.c. - thin layer chromatography

u.v. - ultra violet

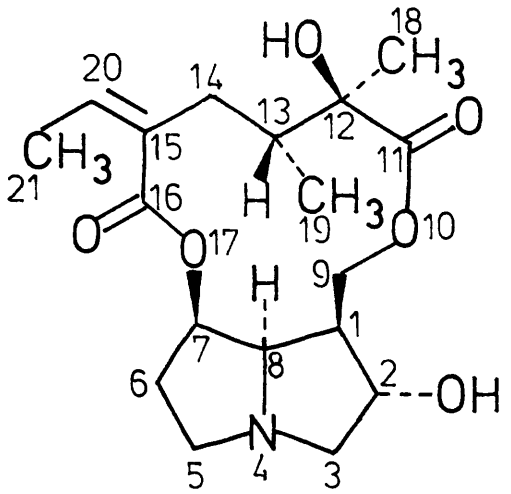
NOTES ON NOMENCLATURE

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



Fully saturated compounds are named as pyrrolizidine derivatives. The stereochemistry of substituents is indicated by the α and β nomenclature conforming with the usual practice in this field.

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.M. Scopes, J. Chem. Soc. (C), 1971, 3653). For example, rosmarinine is shown.



PUBLICATIONS

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

1. "Incorporation of ^{13}C-Labelled Precursors into Rosmarinine", H.A. Kelly and D.J. Robins, J. Chem. Soc., Perkin Trans. 1, 1987, 177.

2. "Rosmarinine: A Pyrrolizidine Alkaloid", A.A. Freer, H.A. Kelly, and D.J. Robins, Acta Crystallogr., 1986, C42, 1348.

3. "Pyrrolizidine Alkaloids. Stereochemistry of the Enzymic Processes Involved in the Biosynthesis of Rosmarinecine", H.A. Kelly and D.J. Robins, J. Chem. Soc., Perkin Trans. 1, 1987, in the Press.

4. "Platynecine: A Pyrrolizidine Necine", A.A. Freer, H.A. Kelly, and D.J. Robins, Acta Crystallogr., 1987, in the Press.

5. "Evidence for an Immonium Ion Intermediate in Pyrrolizidine Alkaloid Biosynthesis", H.A. Kelly and D.J. Robins, J. Chem. Soc., Chem. Commun., in the Press.

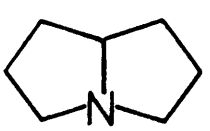
Chapter 1

INTRODUCTION

1.1 Pyrrolizidine Alkaloids

The pyrrolizidine alkaloids constitute a large group of secondary metabolites which are world-wide in distribution.[1] Their occurrence in the plant kingdom is also extensive;[1] over 200 pyrrolizidine alkaloids have been isolated from 60 genera. The three most important genera are Senecio (family Compositae), Heliotropium (Boraginaceae) and Crotalaria (Leguminosae).[2] Indeed it has been estimated that 3% of the world's flowering plants contain pyrrolizidine alkaloids.[3] Many of these alkaloids are toxic, acting specifically on the liver.[1] This presents a health hazard to both humans and livestock. In fact, the loss of livestock due to pyrrolizidine alkaloid poisoning is a growing economic problem.

Most pyrrolizidine alkaloids are derivatives of 1-methylpyrrolizidine exemplified by heliotridane, (2), rather than of pyrrolizidine (1-azabicyclo[3.3.0]octane) (1) itself. Frequently, the pyrrolizidine component of an alkaloid contains a diol system, as in retronecine (3).



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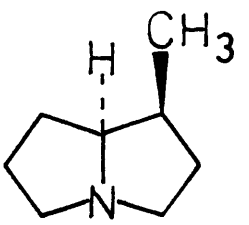

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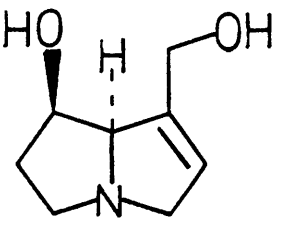


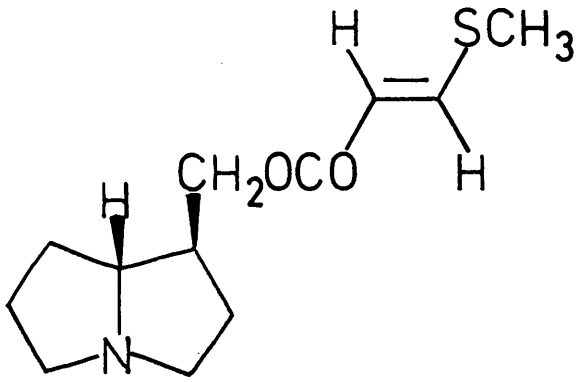
(3)

Pyrrolizidine alkaloids usually exist as esters.[2] These may be monoesters such as planchonelline (4), diesters such as echimidine (5), or macrocyclic diesters like jacobine (6). The hydroxylated pyrrolizidines which constitute the amino alcohol moieties of pyrrolizidine alkaloid esters are called necines. An extensive range of necines exists because the hydroxyl substituents can vary in number, position and stereochemistry. Additionally, the necines may contain 1,2-unsaturation as in (5). The acid portions of the alkaloids, the necic acids, usually contain five or ten carbon atoms and include mono- and dicarboxylic acids. A small group of substituted amino pyrrolizidines such as loline (7) have also been isolated.

Books by Mattocks[4] and Bull et al.,[1] both provide further information on the occurrence, chemistry and pharmacology of pyrrolizidine alkaloids. The reviews in the Specialist Periodical Reports[5] and Natural Product Reports[6] on pyrrolizidine alkaloids, are also recommended.

1.2 Effects of Pyrrolizidine Alkaloids on Humans

People from all regions of the world have deliberately or unintentionally employed a wide range of plant material containing pyrrolizidine alkaloids, either medicinally or for food.[4] On several occasions, this has had disastrous consequences. In 1974, the largest known outbreak of pyrrolizidine poisoning occurred in Afghanistan.[7] Due to a prolonged drought, food was scarce, and the diet consisted mainly of bread made from wheat which had Heliotropium popovii growing alongside it. This resulted in the incorporation of heliotrine N-oxide (8) in the bread. Around 1600 people then suffered from an acute



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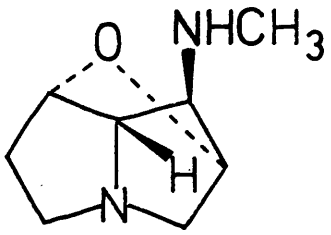

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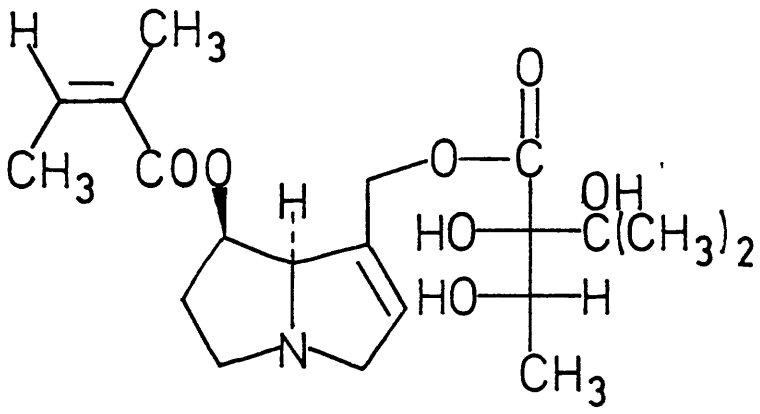

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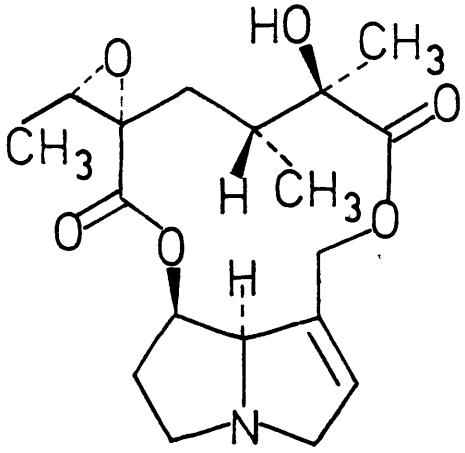

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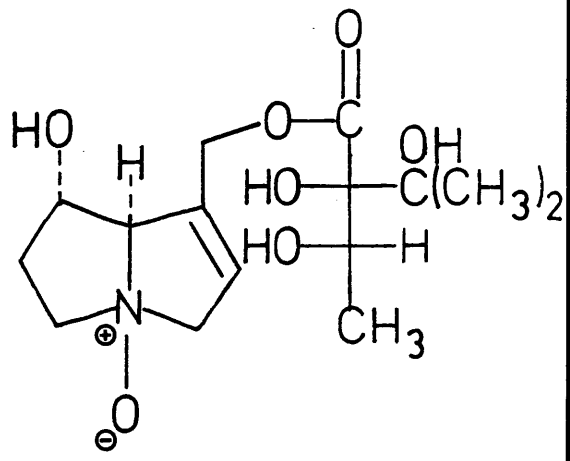


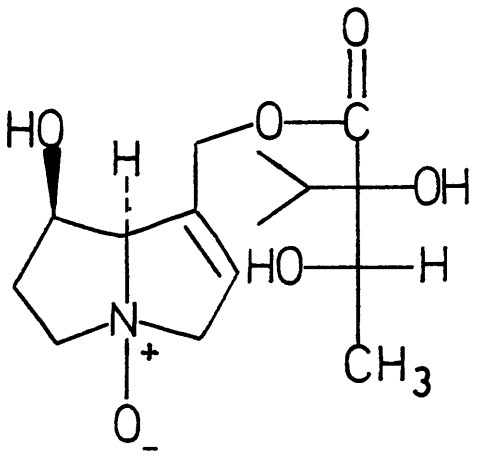
(8)

liver disease (veno-occlusive disease), and many of them died. Another instance was in Jamaica in the late 1950's, when many people suffered from a liver disease resulting from the ingestion of teas prepared from the leaves of bushes, especially Crotalaria fulva.[8]

Evidence of toxicity may not become apparent until some time after the ingestion of pyrrolizidine alkaloids. The characteristic feature of pyrrolizidine alkaloid poisoning is a megalocytosis of the liver in which the liver contains a small number of giant cells. Early clinical signs include nausea and acute epigastric pains. Sometimes fever and vomiting occur in the later stages. Death may ensue from two weeks to over two years following poisoning,[9] but it is possible for patients to recover almost completely from acute veno-occlusive disease if the alkaloid intake discontinues.[10]

Apart from the liver, other organs which can be affected by the consumption of pyrrolizidine alkaloids are the lungs, heart, and occasionally the kidneys.[4]

Indicine N-oxide (9) is the only pyrrolizidine alkaloid which has been deliberately administered to people under clinically controlled conditions, as a potential anti-cancer drug.[11] The effects of this N-oxide (9) are not typical of those due to hepatotoxic pyrrolizidine alkaloids. Indicine N-oxide has the advantages of being relatively low in toxicity and highly water soluble. Recently, a semi-synthetic pyrrolizidine alkaloid (10) was shown to be a more active antitumour agent than indicine N-oxide (9).[12]



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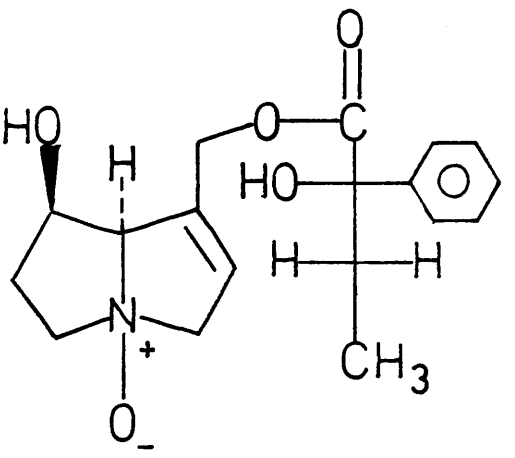

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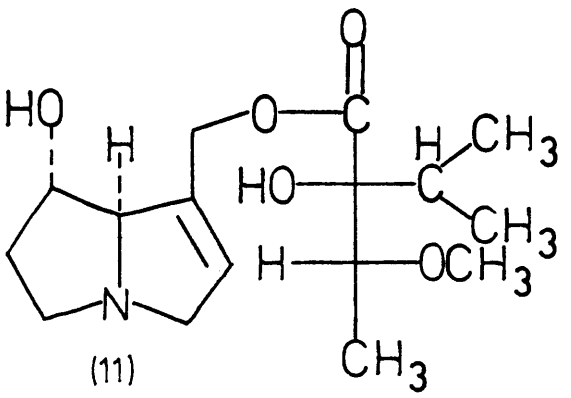
1.3 Metabolism of Pyrrolizidine Alkaloids

Certain pyrrolizidine alkaloids such as heliotrine (11) and retrorsine (12) have been known to cause chronic liver poisoning in animals since the 1900's,[13] while others do not. This led Schoental[14] to suggest that the 1,2-double bond in the pyrrolizidine moiety of the alkaloids is essential for hepatotoxicity. Support for this theory came when Chen et al. reported that injection of rats with platyphylline (13) caused no liver damage, even when the alkaloid was given in lethal doses.[15] In addition, the same group of workers administered the products of hydrolysis of retrorsine (12) to rats and discovered that retronecine (3) and isatinecic acid (14) had no effects on the liver. They thus concluded that the intact allylic ester structure was required for hepatotoxic activity.



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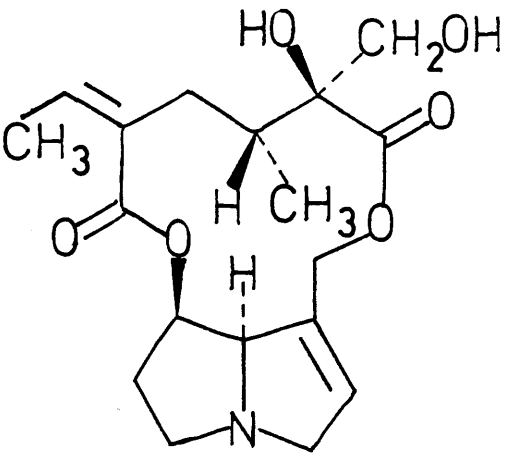

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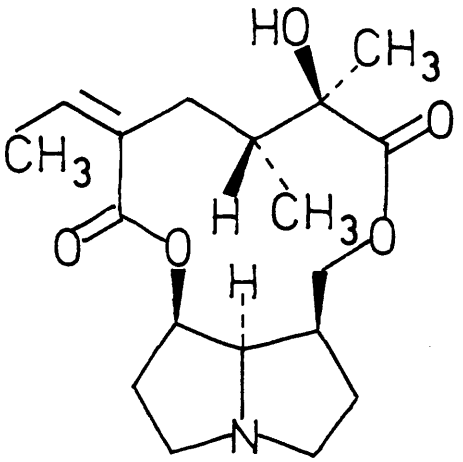

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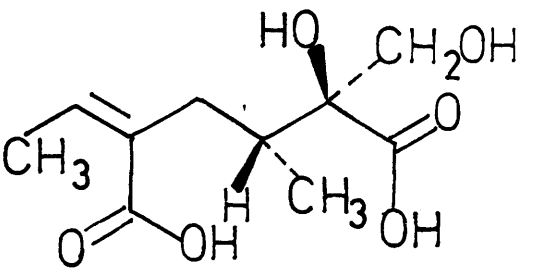
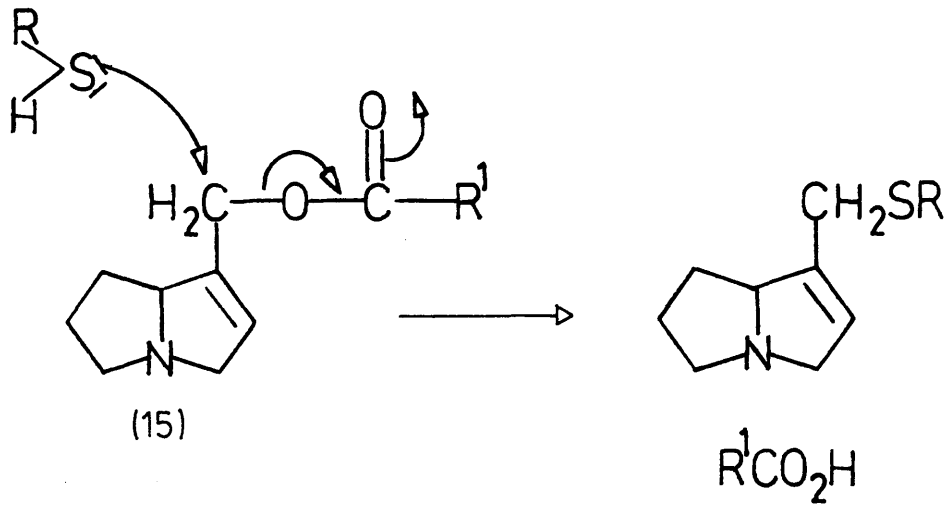


(14)

Culvenor[16] noticed that the effects of pyrrolizidine alkaloids on cell nuclei are analogous to biological alkylating agents. Consequently, he proposed that such effects on cell nuclei are due to the ability of the pyrrolizidine alkaloids to act as alkylating agents within the cell. Alkaloids such as retrorsine (12) are allylic esters and by a mechanism involving alkyl-oxygen fission of the ester linkages, they can function as alkylating agents. For example, an alkaloid such as (15) may become covalently bound to a protein molecule that contains a nucleophilic sulphur atom (such as cysteine), by such a mechanism (Scheme 1).

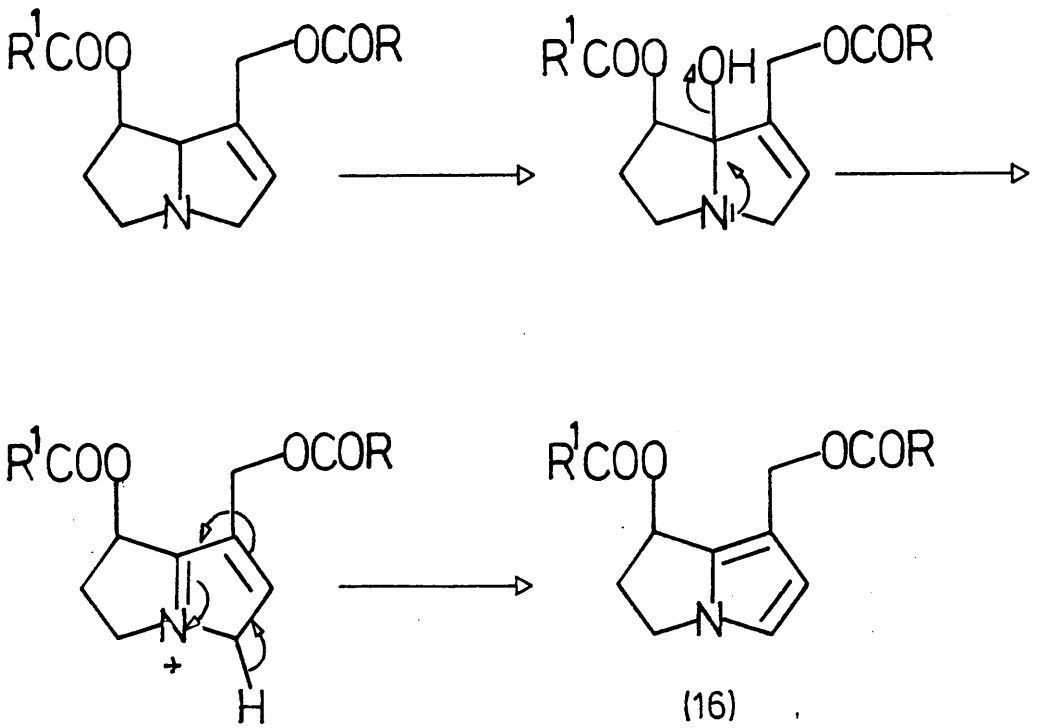


Scheme 1

However, it was Mattocks[17,18] who provided the most convincing evidence for the mechanism of toxicity. He proposed that the toxicity is not attributable to the alkaloids themselves, but is due to pyrrolic derivatives (16). After he injected rats with pyrrolizidine alkaloids, Mattocks found evidence for these metabolic pyrroles (16) in several organs, especially the liver and in the excreted urine. Substantial quantities of these metabolites were also located in the lungs; the metabolites could have been transported from the liver in the bloodstream.

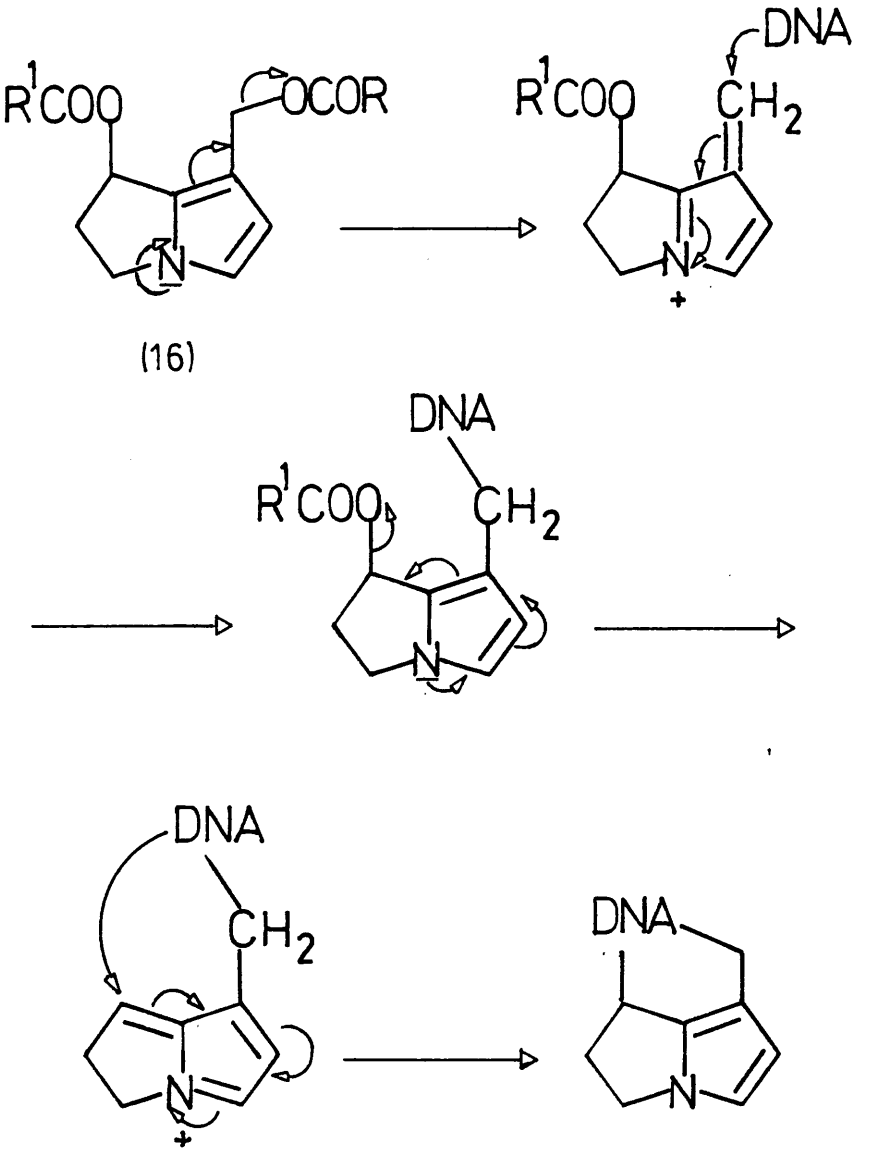
The production of dihydropyrrolizines (16) from pyrrolizidine alkaloids in vitro using liver slices gave additional evidence that the major site of metabolic activation is the liver.

It has been suggested that the production of the pyrroles involves the action of a hepatic microsomal oxidase enzyme on the alkaloid. This process is believed to occur by a hydroxylation-dehydration process as shown in Scheme 2.



Scheme 2

The pyrrole (16) can then be regarded as a bifunctional alkylating agent, because both of the ester groups have been activated as a result of conjugation with the nitrogen lone pair of electrons. Thus nucleophiles on a DNA molecule can covalently bond to the pyrrolizidine alkaloid metabolite which can lead to cross-linking of the DNA (Scheme 3).



(16)

Scheme 3

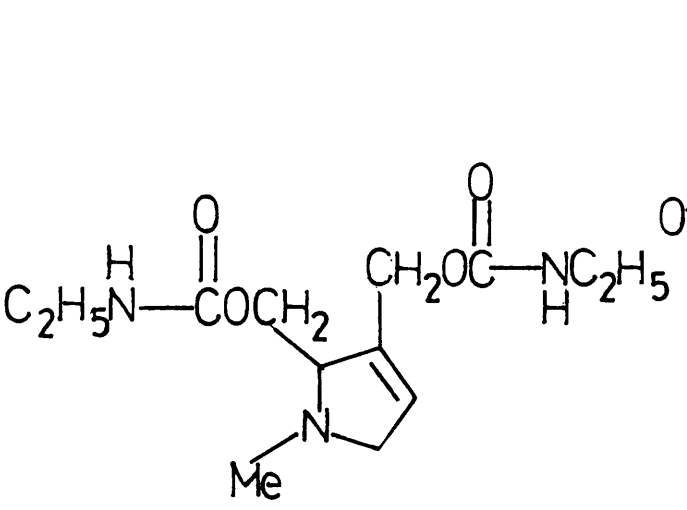
Schoental and Mattocks initially believed that the necic acid portion of an alkaloid had to possess a highly branched carbon chain to exhibit hepatotoxicity.[19] This was supported by their observations that several synthetic esters of retronecine (3) with branched chain acids were hepatotoxic, whereas those with straight chain acids were not.

Nevertheless, it has since been shown that the nature of the acid moiety only has a secondary effect on toxicity.[20]

Bulky carbon or hydroxy substituents on the α-position of the acid portions create steric hindrance around the ester groups.[21] This inhibits esterase hydrolysis, which in turn increases the toxicity of the alkaloids.[22] For this reason, macrocyclic diesters are generally more toxic than simple diesters.

The solubility in water and the base strength[4] of an alkaloid are two other factors which affect hepatotoxicity. Generally, the more water soluble the alkaloid, the greater is the tendency for it to be excreted and hence the toxicity is lower. Also, it has been observed that the more weakly basic is the alkaloid, the greater is the toxicity.[4]

It has been demonstrated that a synthetic compound with the appropriate substitution as in (17), can display hepatotoxic properties.[23] In fact, the biscarbamate ester (17) is more toxic than the natural alkaloid monocrotaline (18). This indicates that the second (pyrrolidine) ring is not a structural requirement for toxicity.



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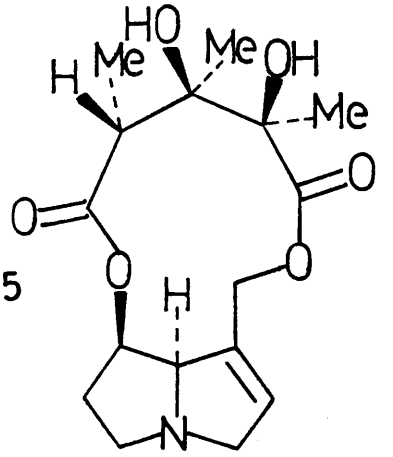
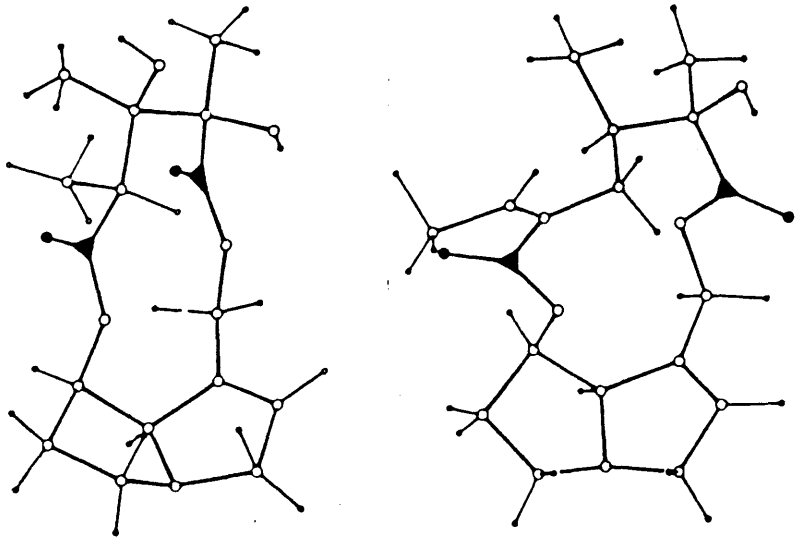


(18)

1.4 Conformational Aspects of Macrocyclic Diester Pyrrolizidine Alkaloids

The determination of conformations of pyrrolizidine macrocyclic diesters may lead to a better understanding of structure-activity relationships involved in their metabolism. Both X-ray crystallographic data and ^{1}H n.m.r. spectroscopic studies have provided information on the conformation of these alkaloids in the solid state and organic solutions, respectively.[1,24] For the few examples studied, it is believed that the conformation of the alkaloid is similar in organic solutions and in the solid state, e.g. retrorsine (12).[25,26]

A considerable number of X-ray crystal structures of pyrrolizidine alkaloids have been determined and some interesting conformational patterns have emerged. X-Ray data on 11-membered macrocyclic diesters of retronecine (3) have shown that in most the ester carbonyl groups are synperiplanar, as shown in Figure 1 for monocrotaline (18).[24] On the other hand 12-membered dilactones have ester carbonyl groups that are antiperiplanar, e.g. senecionine (19) (Figure 1).[24]



(18) (19)

Crystal structures of monocrotaline (18) and senecionine (19)

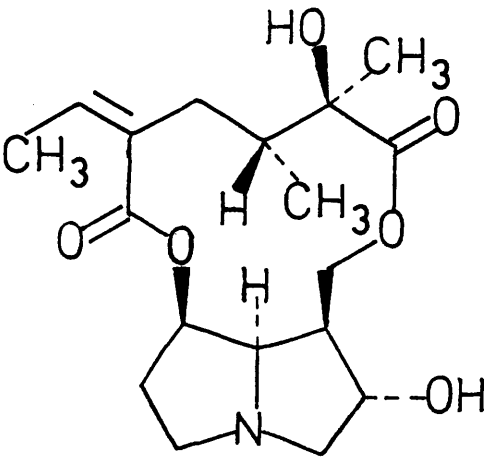
Figure 1

The chemical shift differences of the C-9 diastereotopic protons ($\Delta\delta$H-9) of these alkaloid systems can also be indicative of conformational properties. When $\Delta\delta$H-9 is large (i.e. 1.1-1.5 p.p.m.), the downfield proton is subjected to maximum deshielding as a consequence of being in the plane of the ester carbonyl and 1,2-double bond. Alternatively, when $\Delta\delta$H-9 is small (i.e. 0.2-0.9 p.p.m.) the C-9 protons are more symmetrically situated about the plane of the carbonyl and the more deshielded proton is closer to the 1,2-double bond. The $\Delta\delta$H-9 values of 0.16 and 1.47 p.p.m. were observed for monocrotaline (18) and senecionine (19), respectively. Thus, the conformations deduced from the ^{1}H n.m.r. spectral data for monocrotaline (18) and senecionine (19) appeared to be consistent with the X- ray crystal structures depicted in Figure 1.

It should be pointed out however, that the conformation adopted by an alkaloid in the solid state and organic solution may not be the same as that when the alkaloid is metabolised in vivo by enzyme systems.

1.5 Aims of Project

The way in which pyrrolizidine alkaloids are formed in plants (biosynthesis) is of fundamental interest. Previously retronecine (3) was the only necine whose biosynthesis had been studied in depth.[27,28] This area is reviewed in Chapter 2. It was considered necessary to widen the scope of these biosynthetic studies. Accordingly, this research project was principally directed towards an investigation into the biosynthesis of rosmarinecine (21), the base portion of the pyrrolizidine alkaloid rosmarinine (20). The results of these investigations are presented in Chapters 3 and 4.



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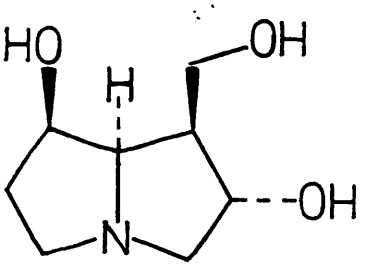
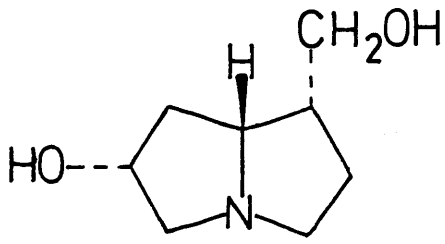


(21)

The synthesis of macrocyclic pyrrolizidine alkaloids has received limited attention (Chapter 5). Yet, these are the most toxic of the pyrrolizidine alkaloids, and good routes to these alkaloids and selected analogues are required to provide material for the study of structure-biological activity relationships. Thus, a series of macrocyclic diesters was prepared from the optically active diol (22), derived from 4-hydroxy-L-proline. This work is discussed in Chapter 6.



(22)

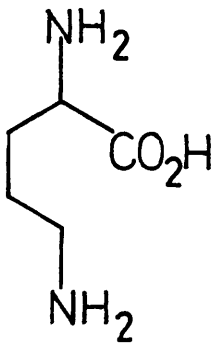
There is considerable interest in the occurrence of pyrrolizidine alkaloids for two main reasons. Firstly, knowledge of the chemical constituents of plants is being increasingly used to assist in the classification of plant species. Secondly, the structural relationships between alkaloids occurring together in the same species may help in determining the biogenesis of these compounds. Therefore, the alkaloidal content of Cynoglossum macrostylum and Lindelofia longiflora was investigated. This work is described in Chapter 7.

Chapter 2

BIOSYNTHESIS OF PYRROLIZIDINE ALKALOIDS

2.1 Biosynthesis of Pyrrolizidine Necine Bases

Sir Robert Robinson first postulated that the pyrrolizidine ring system is derived biosynthetically from the condensation of two units of ornithine (23).[29] Support for this observation has only been available after the advent of radiotracers. Extensive research has been carried out recently using stable isotopes and n.m.r. spectroscopy on the biosynthesis of necines. Initial studies concentrated on the feeding of radioactive precursors to plants which produce pyrrolizidine alkaloids. The labelled alkaloid was isolated and the labels were located by chemical degradation. Nowacki and Byerrum[30] provided one of the first pieces of experimental evidence to substantiate Robinson's theory, when they fed [2-^{14}C]ornithine, [1-^{14}C]acetate and [1-^{14}C]propionate to Crotalaria spectabilis, a plant which produces monocrotaline (18). It was found that ornithine was primarily used for the synthesis of the retronecine part of monocrotaline, whereas acetate and propionate were incorporated principally into the monocrotalic acid portion of the molecule.



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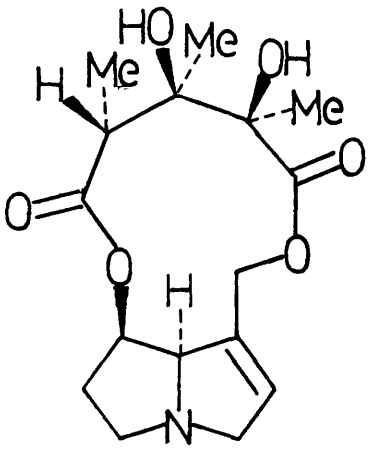

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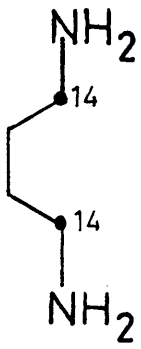
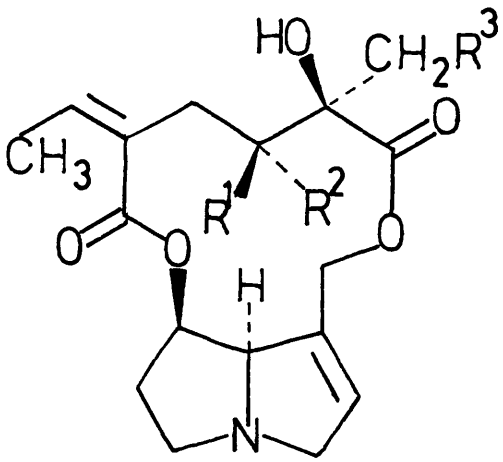


(24)

In 1963, Bottomley and Geissman[31] fed [1,4-^{14}C]putrescine (24), [2-^{14}C]ornithine and [5-^{14}C]ornithine to Senecio douglasii. This plant contains four alkaloids, namely, senecionine (19), seneciphylline (25), retrorsine (12), and riddelline (26), all of which have retronecine (3) as the base portion.



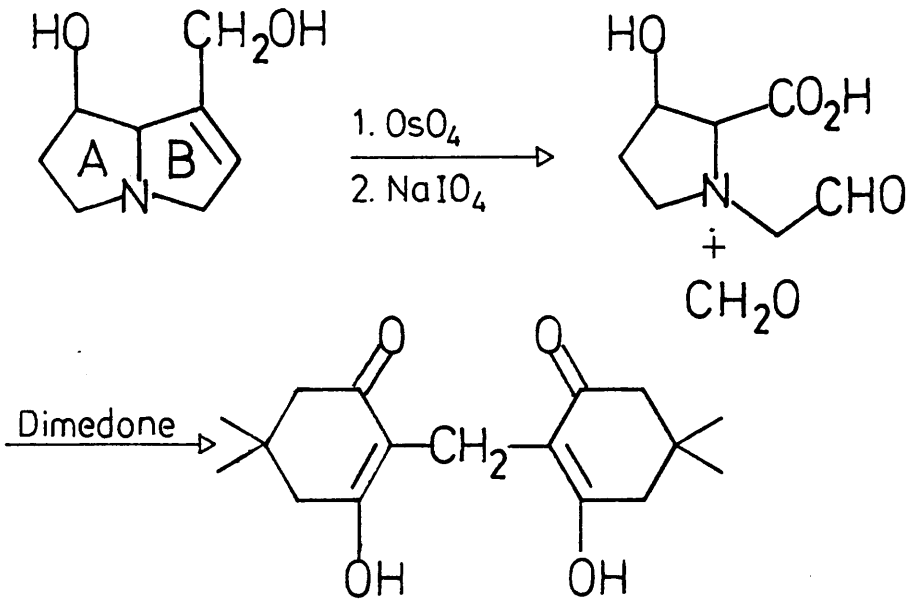
(19) $R^1 = R^2 = H$, $R^3 = CH_3$
(25) $R^1, R^2 = CH_2$, $R^3 = H$
(12) $R^1 = H$, $R^2 = CH_3$, $R^3 = OH$
(26) $R^1, R^2 = CH_2$, $R^3 = OH$

One week after administration of the precursors, the plants were extracted to give a mixture of ^{14}C-labelled alkaloids. Alkaline hydrolysis of each mixture of alkaloids gave retronecine (3) and the corresponding acids. Table 1 shows the relative amounts of radioactivity incorporated into retronecine, and the C_{10}-acids.

Table 1. Incorporation of Radioactive Precursors into the Alkaloids of S. douglasii

Precursor	Total Incorporation into alkaloid (%)	Percentage of total activity found in:	
		Acids	Retronecine
[1,4-^{14}C]putrescine	0.18	5.0	98
[2-^{14}C]ornithine	0.30	1.4	94
[5-^{14}C]ornithine	0.75	2.4	94

It was therefore apparent that feeding ^{14}C-labelled putrescine or ornithine resulted in the incorporation of radioactivity into the retronecine portions of the alkaloids. Furthermore, the labelled retronecine, derived from each feeding experiment, was treated with osmium tetroxide and sodium periodate. This converted the primary carbinol carbon atom into formaldehyde, which was then trapped as the dimedone derivative (Scheme 4).



Scheme 4

The radioactivity of the dimedone derivatives from each experiment was measured and these derivatives were found to contain one quarter of the total activity of retronecine. This showed that during biosynthesis, C-2 and C-5 of the molecule of ornithine used to form ring B of retronecine became equivalent.

Evidence for the involvement of other precursors in the biosynthesis of retronecine (3) was reported by Bale and Crout.[32] Using a double isotope technique, they showed that arginine (30) was a specific precursor for retronecine (3) in Senecio magnificus, although not as effective as ornithine (23). Robins and Sweeney also observed that spermidine (28) and spermine (29) were efficient precursors of retronecine in Senecio isatideus plants, in addition to arginine, ornithine and putrescine (27).[33] The principal alkaloidal constituent of the plant is retrorsine (12), which on hydrolysis yields retronecine and isatinecic acid (14).

(27) $H_2N(CH_2)_4NH_2$

(28) $H_2N(CH_2)_3NH(CH_2)_4NH_2$

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

(30)

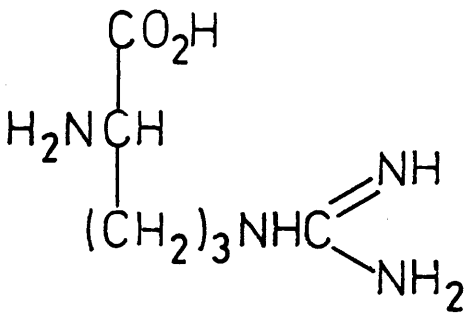


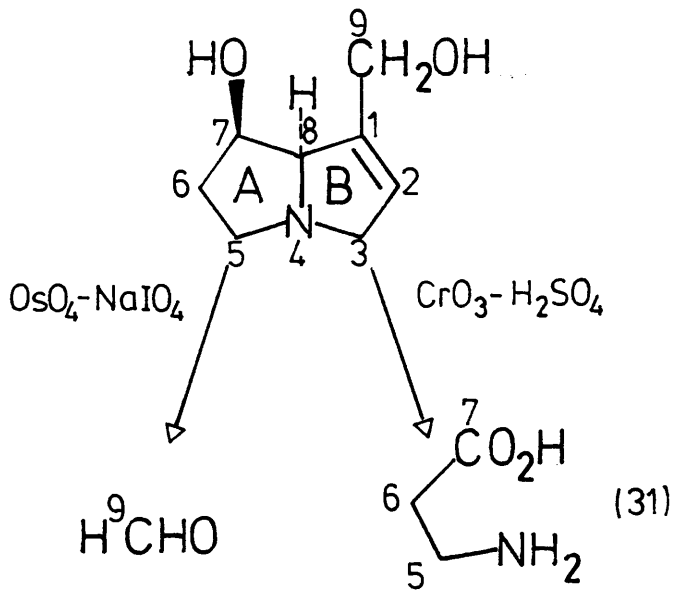
Table 2 shows the incorporation of the aforementioned precursors (27)-(30) into the base and acid components. Thus, putrescine (27) is a much better precursor than ornithine (23) in retronecine biosynthesis which supports the theory proposed by Geissman and Crout,[34] that putrescine follows ornithine in the biosynthetic pathway.

Table 2. Incorporation of Precursors into Retrorsine (12) in S. isatideus

Experiment	% Incorporation	% ^{14}C Radioactivity in (3)	(14)
1. L-[U-^{14}C]arginine	0.46	99	6
2. DL-[5-^{14}C]ornithine	0.25	97	5
3. [1,4-^{14}C]putrescine	1.6	94	1
4. [1,4-^{14}C-tetramethylene] spermine	5.2	103	1
5. [1,4-^{14}C-tetramethylene] spermidine	2.0	95	1

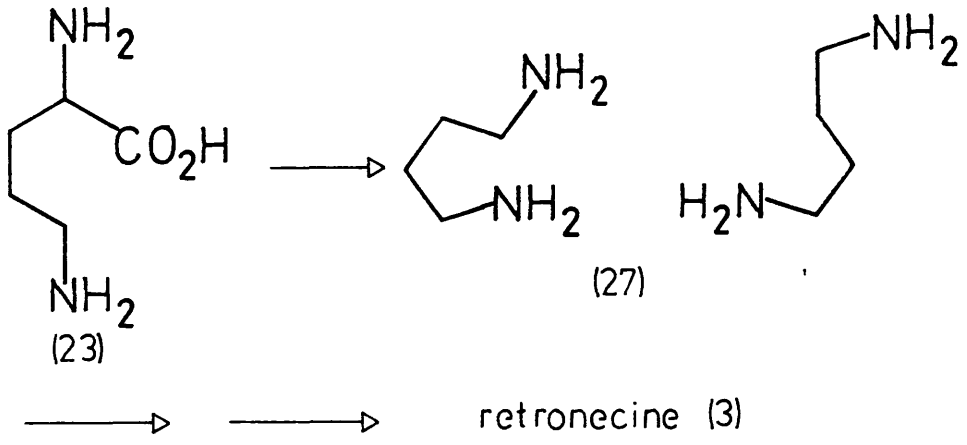
Robins and Sweeney were also successful in partially locating the labels within retronecine for experiments 2-5. Oxidation of retronecine with osmium tetroxide and sodium periodate gave formaldehyde, isolated as its dimedone derivative, and this corresponded to C-9 of retronecine. One quarter of the total ^{14}C base activity was located at C-9 in each experiment, which compared well with the observations of

Bottomley and Geissman.[31] Robins and Sweeney also reported the first chemical degradation which produced a fragment from ring A of the necine base. A modified Kuhn-Roth oxidation procedure[35] used on retronecine, gave β-alanine (31), isolated as its 2,4-dinitrophenyl derivative. This corresponded to C-(5+6+7) from ring A of retronecine (Scheme 5). The degradations were carried out on the samples of retronecine specifically labelled with ^{14}C from experiments 2-5. This proved that 22-24% of the total ^{14}C base activity was located in this fragment in each experiment.



Scheme 5

The result of experiment 2 (Table 2), demonstrated that C-2 and C-5 or ornithine (23) become equivalent in the formation of ring A of retronecine. Because putrescine (27) is a more efficient precursor than ornithine, it was deduced that retronecine (3) is formed from two molecules of ornithine via putrescine (Scheme 6).



Scheme 6

Although considerable insight into the biosynthetic pathway to retronecine had been gained through radioactive feeding experiments, further progress was hindered by the lack of degradative experiments to give complete labelling patterns. This obstacle was surmounted using ^{13}C nuclear magnetic resonance spectroscopy.

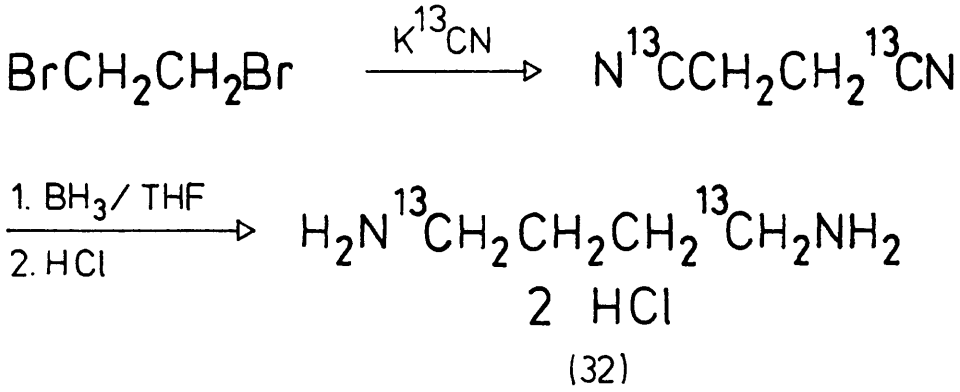
Khan and Robins[36] were the first to observe complete labelling patterns when they fed specifically ^{13}C-labelled putrescines to S. isatideus, and examined the biosynthetically derived retronecine (after hydrolysing retrorsine) by ^{13}C n.m.r. spectroscopy. Reaction of 1,2-dibromoethane with potassium [^{13}C]cyanide gave [1,4-$^{13}C_2$]succinonitrile, which on reduction with borane in tetrahydrofuran (THF), and acidification of the product, yielded [1,4-$^{13}C_2$]putrescine (32)

dihydrochloride (Scheme 7(a)).

Using the xylem pricking technique, the ^{13}C-labelled putrescine (32) was fed to S. isatideus and one week after administration, retrorsine was isolated. Basic hydrolysis afforded ^{13}C-labelled retronecine. Four equally enhanced signals were evident in the ^{13}C-{^{1}H} n.m.r. spectrum corresponding to enrichment of ^{13}C at C-3, C-5, C-8 and C-9 of retronecine (Scheme 8).

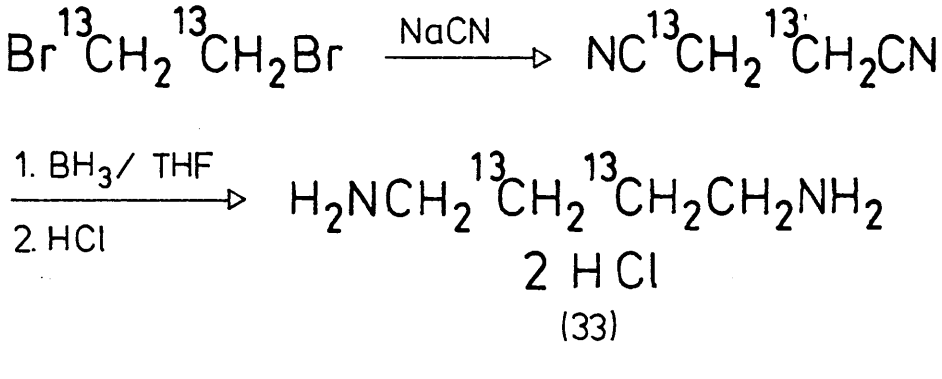
A complementary labelling pattern in retronecine was produced by feeding [2,3-$^{13}C_2$]putrescine (33) dihydrochloride. This was prepared from [1,2-$^{13}C_2$]-1,2-dibromoethane by treatment with sodium cyanide, followed by reduction of the dinitrile and acidification of the product (Scheme 7(b)). The ^{13}C-{^{1}H} n.m.r. spectrum of the labelled retronecine showed a pair of doublets at δ137.4 and 122.1 (J 71 Hz) corresponding to C-1 and C-2 of retronecine and a pair of doublets at δ35.9 and 70.1 (J 34 Hz) corresponding to C-6 and C-7. The four labelled sites displayed nearly equal enrichment factors. The labelling patterns were consistent with the formation of a later symmetrical C_4-N-C_4 intermediate in retronecine biosynthesis (Scheme 8).

Further confirmation of the rôle of putrescine (27) in the construction of the necine portion of retrorsine (12) was obtained by synthesising [1,2-$^{13}C_2$]putrescine (34) dihydrochloride and feeding it to S. isatideus.[37] [1,2-$^{13}C_2$]-1-Bromo-2-phthalimidoethane (35) was treated with the anion of ethyl cyanoacetate to give the ester (36). The ester was de-ethoxycarbonylated in dimethyl sulphoxide (DMSO) containing sodium chloride and water to give the nitrile (37), which was hydrogenated, and the phthalimide was hydrolysed in acid to afford the ^{13}C-labelled precursor (34) (Scheme 9).



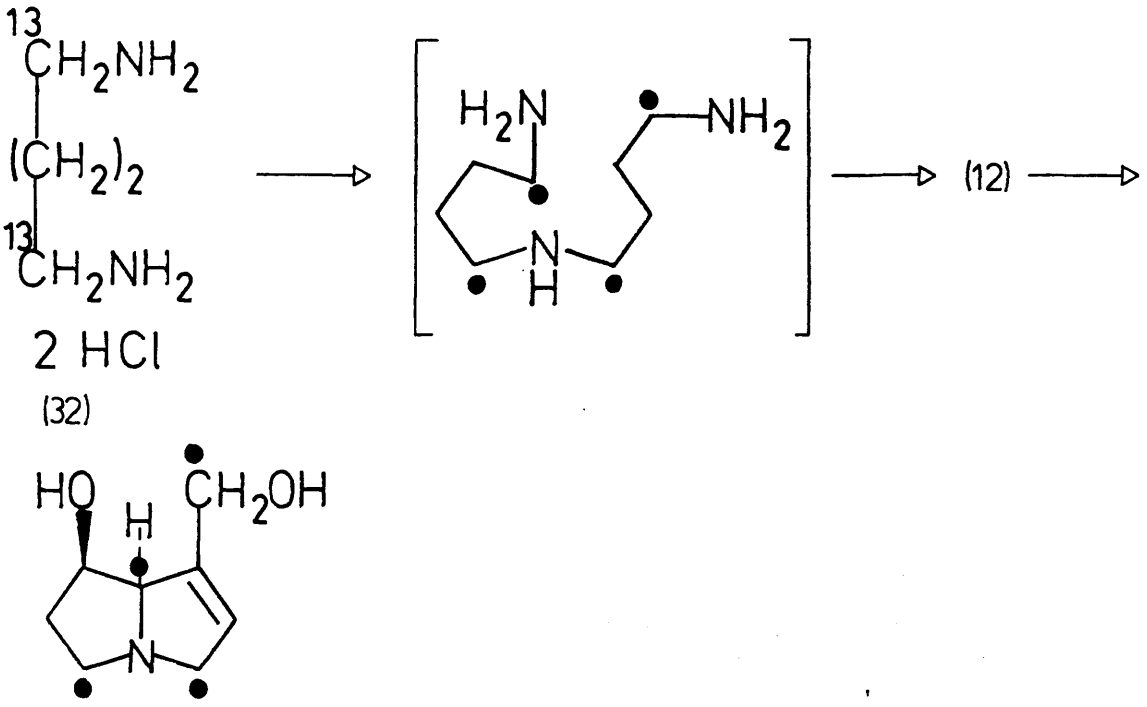
(32)

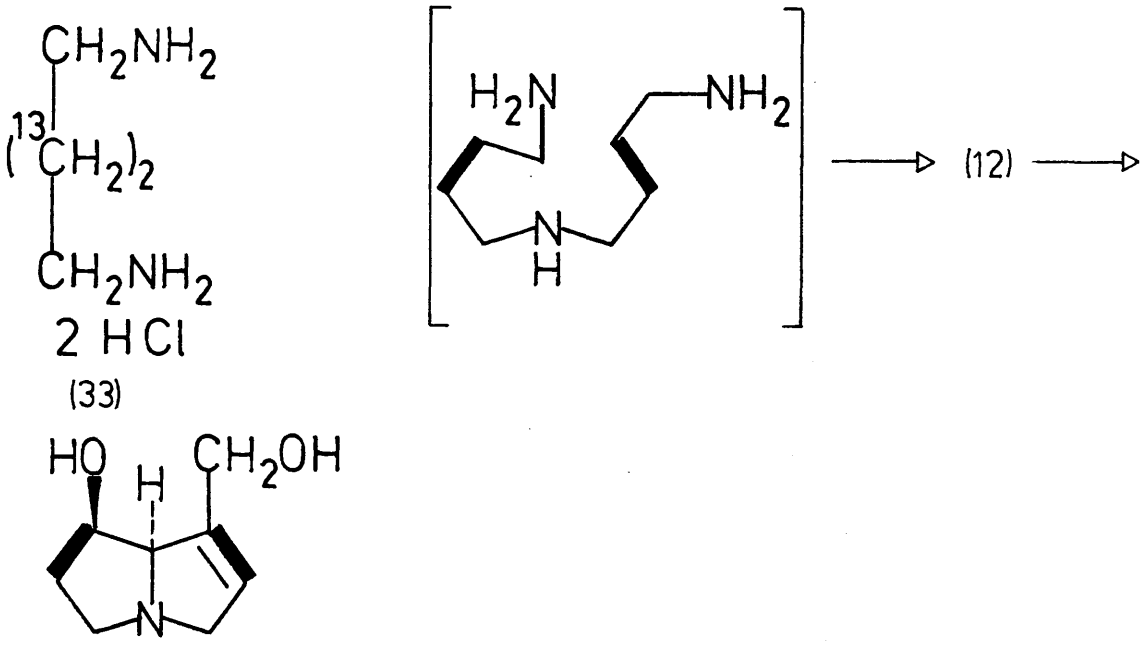
Scheme 7(a)



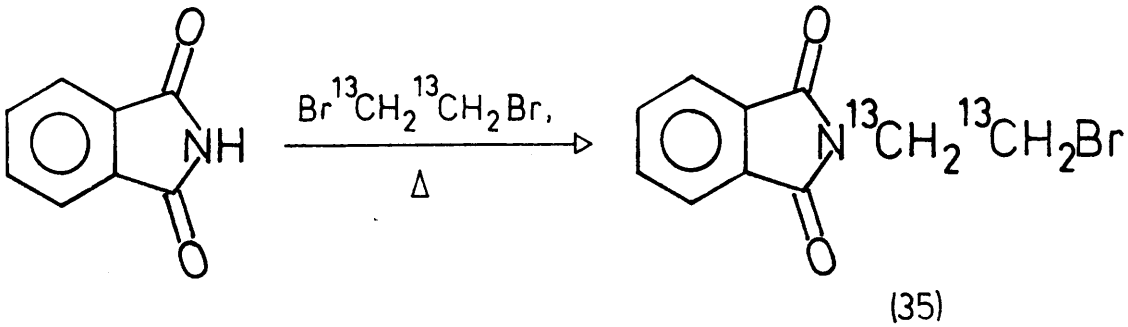
(33)

Scheme 7(b)

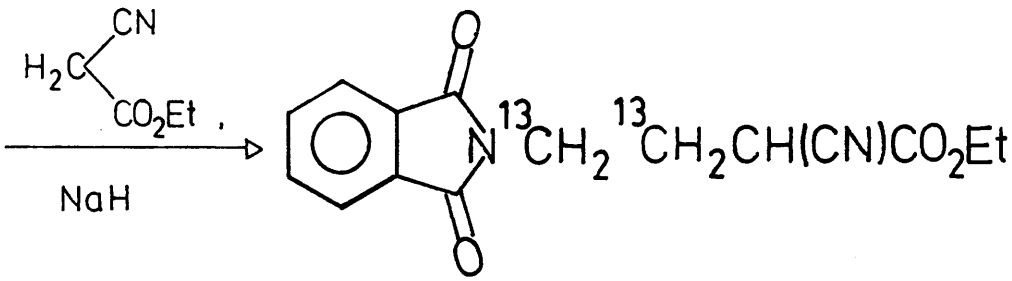

13
CH2NH2
(CH2)2
13
CH2NH2
2 HCl
(32)
H2N
NH2
N
H
(12)
HO
H
CH2OH
N


CH2NH2
13
(CH2)2
CH2NH2
2 HCl
(33)
H2N
NH2
N
H
(12)
HO
H
CH2OH
N

Scheme 8



(35)



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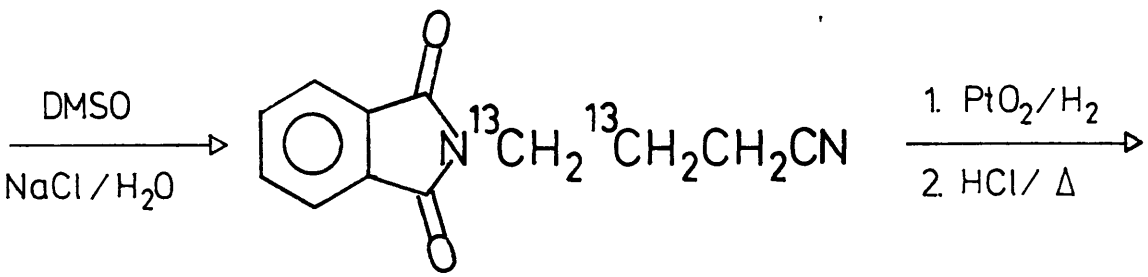

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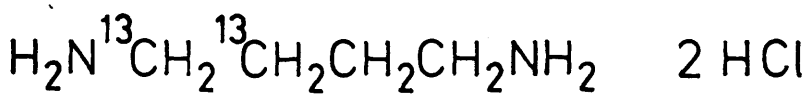
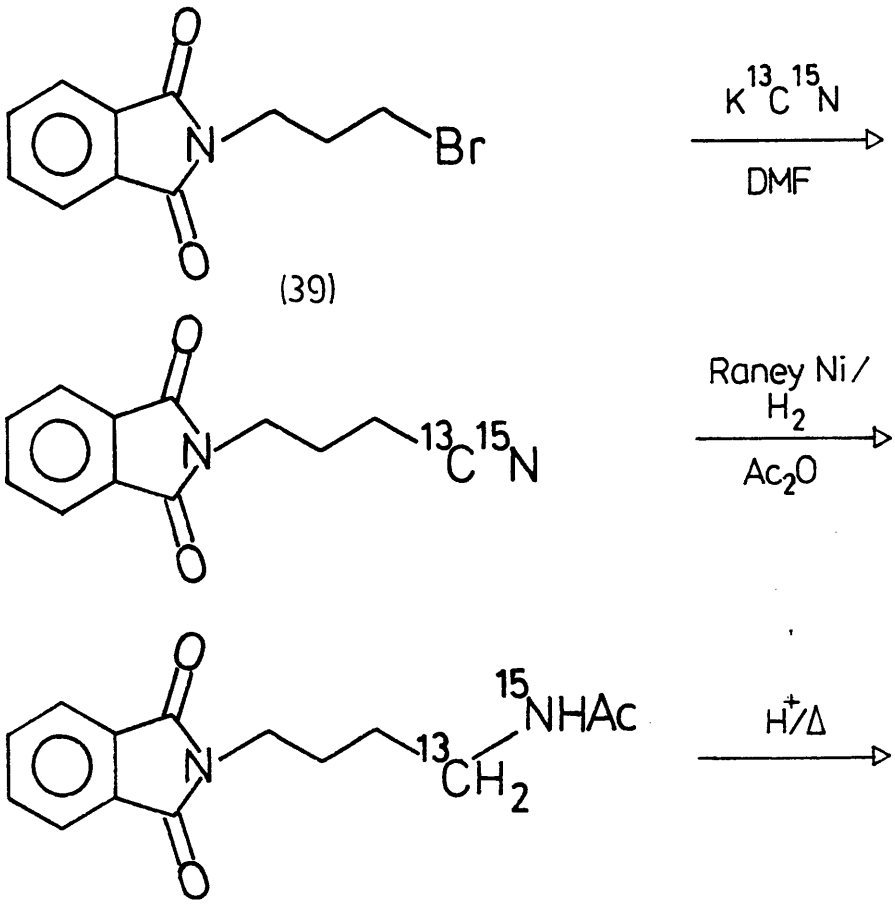


(34)

Scheme 9

The ^{13}C-{^{1}H} n.m.r. spectrum of the biosynthetically formed retronecine exhibited eight pairs of doublets flanking the eight carbon singlets. Once again the idea of a symmetrical intermediate was supported, because all the doublets were of equal intensity.

Grue-Sørensen and Spenser provided unequivocal evidence that a symmetrical C_4-N-C_4 intermediate is involved in retronecine biosynthesis when they determined the mode of incorporation of [1-amino-^{15}N,1-^{13}C]putrescine dihydrochloride (38) into Senecio vulgaris.[38] This plant contains a mixture of pyrrolizidine alkaloids, all of which have retronecine as their necine base. A sample of this precursor (38) was prepared by treatment of N-(3-bromopropyl)phthalimide (39) with potassium [^{13}C-^{15}N]cyanide, followed by hydrogenation using Raney nickel and acid hydrolysis (Scheme 10). On completion of the feeding experiment, the total alkaloidal mixture extracted was hydrolysed and the distribution of labels within the labelled retronecine was analysed by ^{13}C n.m.r. spectroscopy. Four equally enhanced signals were observed corresponding to enrichment of ^{13}C at C-3, C-5, C-8 and C-9 of retronecine. More importantly however, examination of the difference spectrum (^{13}C-labelled - natural abundance), showed that both sets of signals due to C-3 and C-5 comprised a doublet, superimposed on a singlet. The doublets were indicative of ^{13}C-^{15}N species and the singlets were a consequence of ^{13}C-^{14}N species. Furthermore, the doublets were of roughly equal intensity, which was entirely consistent with the concept that the biosynthesis of retronecine proceeded by way of a symmetrical C_4-N-C_4 intermediate.

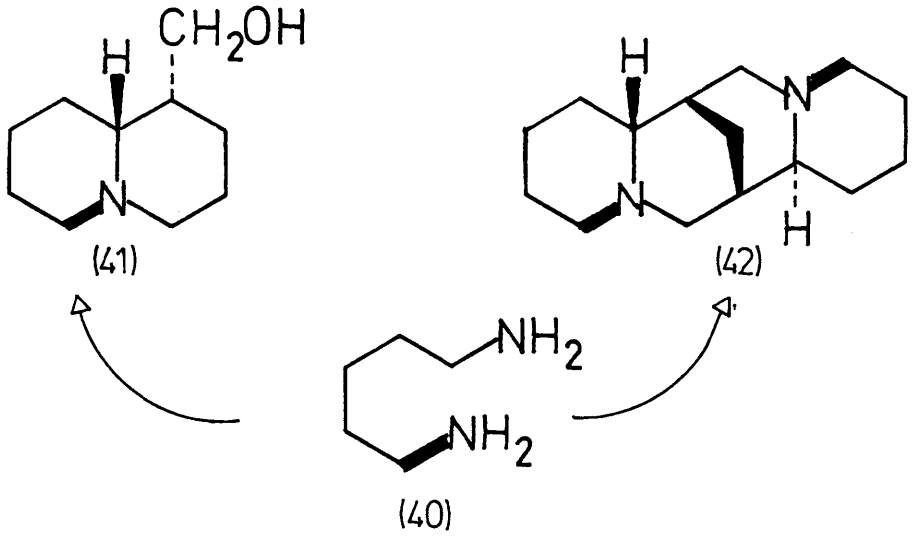


$H_2NCH_2CH_2CH_2{}^{13}CH_2{}^{15}NH_2$ 2 HCl

(38)

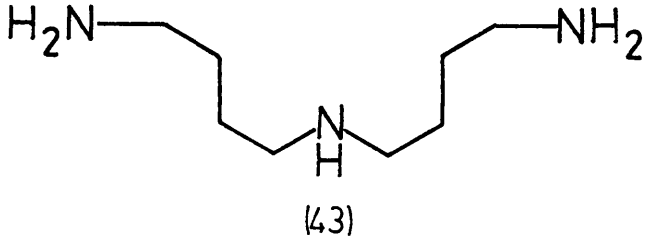
Scheme 10

By contrast, feeding experiments with [^{13}C-^{15}N]cadaverine (40) on Lupinus species showed that no later symmetrical intermediate is involved in the biosynthesis of quinolizidine alkaloids, such as lupinine (41)[39] and sparteine (42)[40] (Scheme 11)



Scheme 11

Khan and Robins corroborated the findings of Grue-Sørensen and Spenser when they fed [1-amino-^{15}N,1-^{13}C]putrescine (38) dihydrochloride to S. isatideus.[41] However, of greater significance was the identification of the symmetrical intermediate as 1,6,11-triazundecane (homospermidine) (43).



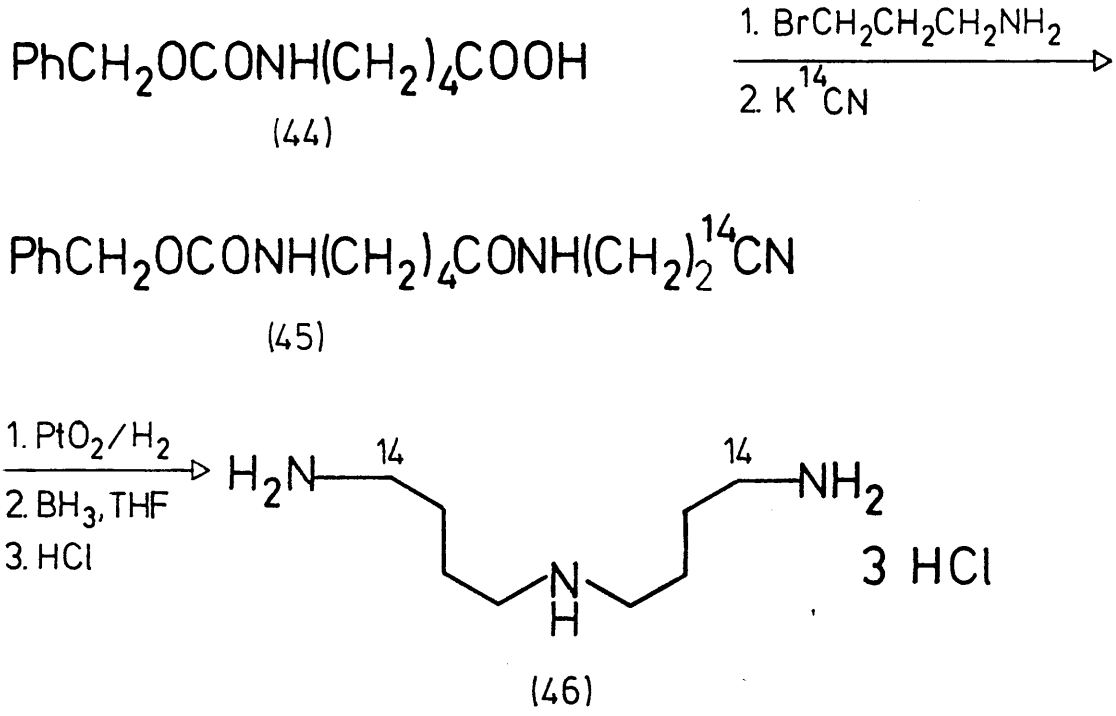
(43)

One of the principal reasons that Khan and Robins considered homospermidine as the C_4-N-C_4 intermediate was because it was a known constituent in many plants.[42] Accordingly, ^{14}C-labelled homospermidine was synthesised as follows. The N-benzyloxycarbonyl derivative of 4-aminobutanoic acid (44) was condensed with 3-bromopropylamine. Treatment of the resultant protected amide with potassium [^{14}C]cyanide gave the corresponding nitrile (45), which was hydrogenated and the amide was reduced with borane in THF to afford ^{14}C-labelled homospermidine (46), isolated as its trihydrochloride (Scheme 12).

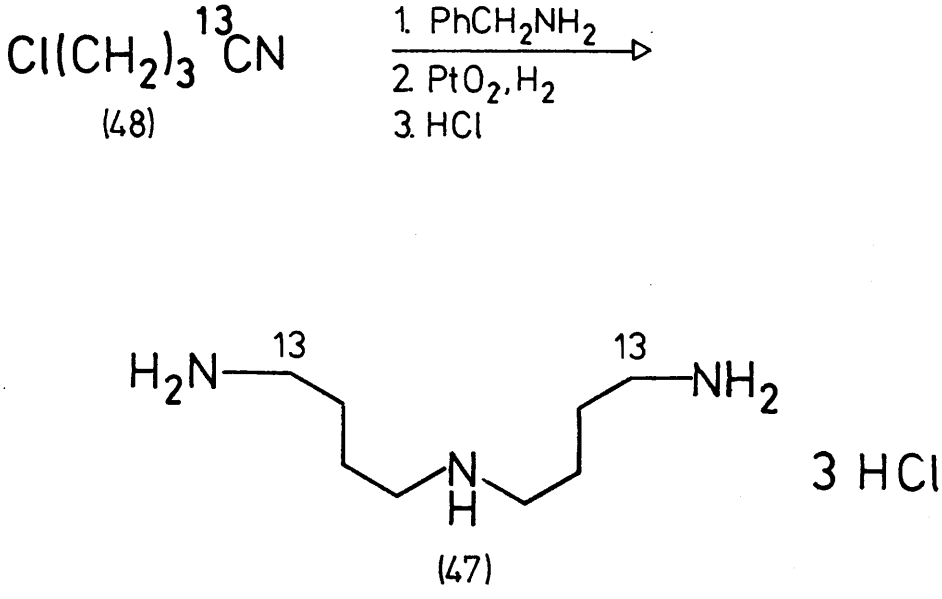
Feeding ^{14}C-labelled homospermidine to S. isatideus gave specifically ^{14}C-labelled retronecine, after alkaline hydrolysis of retrorsine. The base was treated with OsO_4-HIO_4 to give formaldehyde, isolated as its dimedone derivative, and this contained 44% of the ^{14}C base activity. Modified Kuhn-Roth oxidation produced β-alanine (corresponding to C-5+C-6+C-7 of retronecine), isolated as its N-2,4-dinitrophenyl derivative with ca. 1% of the total ^{14}C base activity. Both observations were consistent with the intact incorporation of homospermidine (43) into retronecine. The presence of homospermidine within the plant was also shown by an intermediate trapping experiment.

A complete labelling pattern in retronecine (3) was provided after administering [1,9-$^{13}C_2$]homospermidine (47) trihydrochloride to S. isatideus,[43] Preparation of this precursor involved the reaction of benzylamine with two equivalents of 4-chloro-[1-^{13}C]butanenitrile (48) followed by catalytic hydrogenation and acidification[44] (Scheme 13).

The ^{13}C-{^{1}H} n.m.r. spectrum of retronecine showed the presence of two doublets (J 6 Hz), flanking the singlets corresponding to



Scheme 12

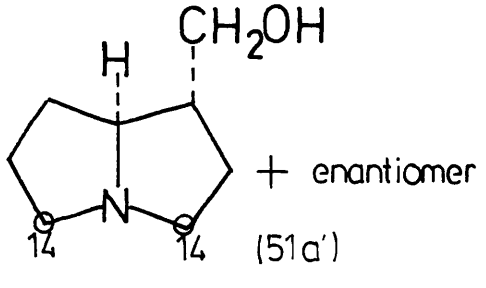


Scheme 13

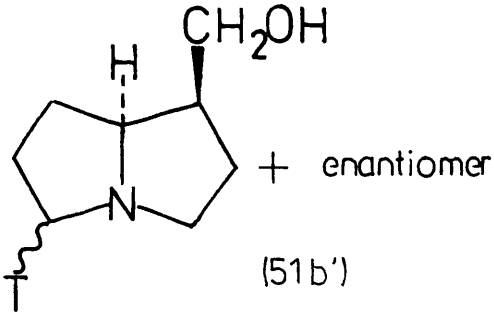
C-8 and C-9. The observation of a geminal coupling constant (between C-8 and C-9 of retronecine) was excellent evidence for the intact incorporation of homospermidine into retronecine.

With the likelihood that homospermidine is the intermediate with C_{2v} symmetry, an explanation of how it is converted into pyrrolizidine alkaloids was necessary. It is possible that diamine oxidase enzymes oxidises the two primary amino groups, thus generating a dialdehyde (49a) in equilibrium with an immonium ion (49b). Cyclisation of the immonium ion would afford 1-formylpyrrolizidine (50) from which 1-hydroxymethyl-pyrrolizidine (51) would be obtained after reduction (Scheme 14).

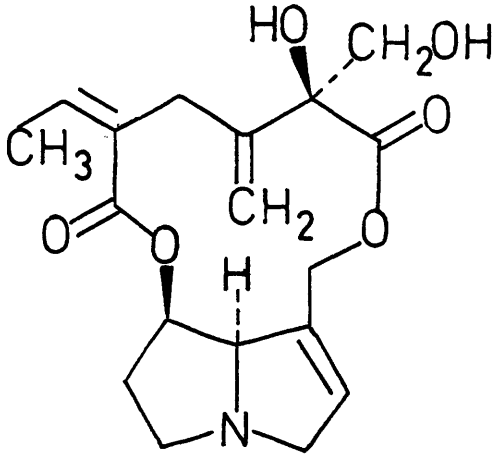
The feasibility of the proposed pathway was demonstrated by the conversion of homospermidine (43) into (±)-trachelanthamidine (51a), using diamine oxidase and dehydrogenase enzymes and physiological conditions.[45] Additional support came from the incorporation of (±)-[3,5-^{14}C]trachelanthamidine (51a') and (±)-[5-^{3}H]isoretronecanol (51b') into riddelline (26) in Senecio riddelli.[46]



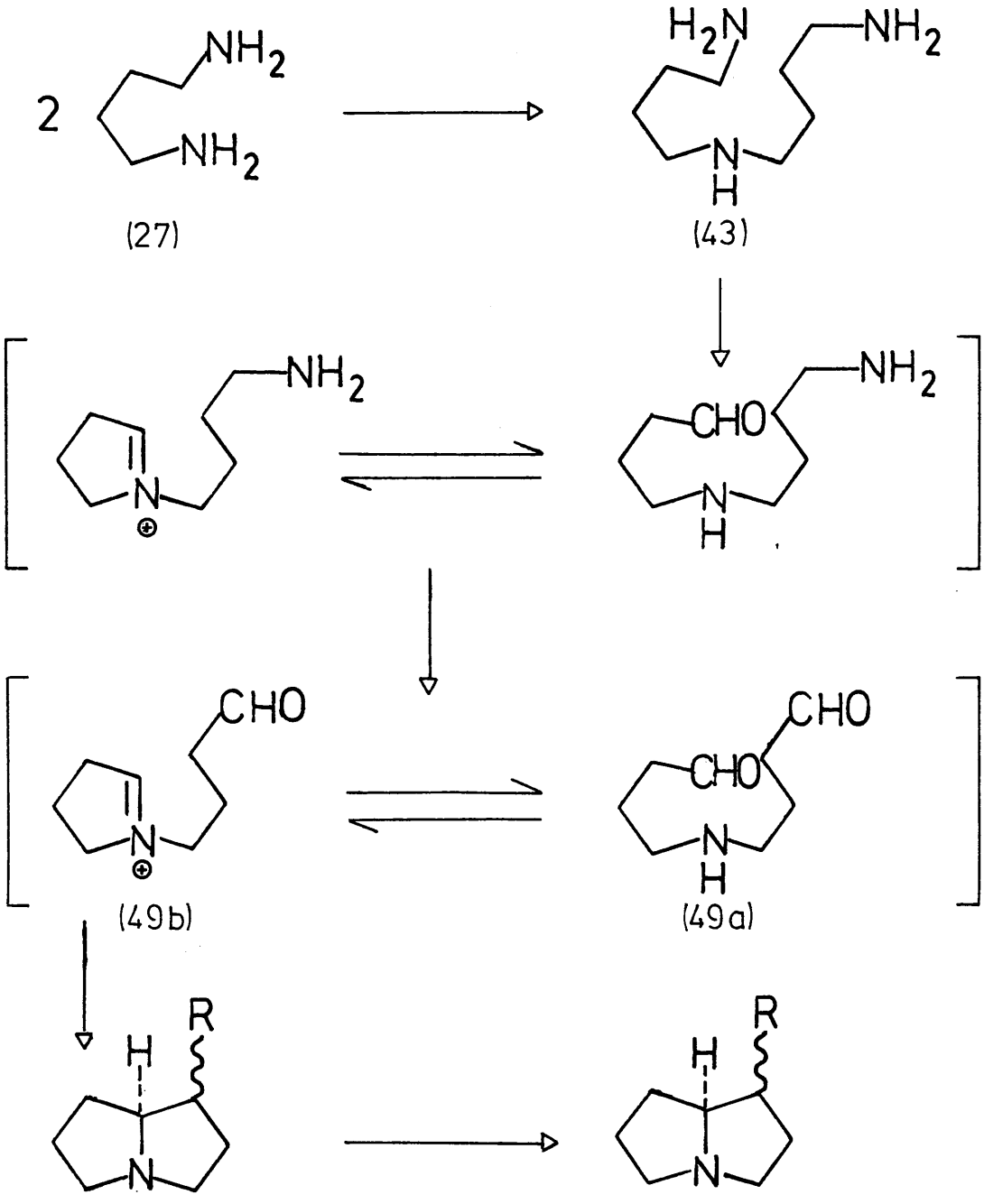
(51a')



(51b')



(26)



(27) (43)

(49b) (49a)

(50a) R = α-CHO
(50b) R = β-CHO

(51a) R = α-CH_2OH
(51b) R = β-CH_2OH

⟶ pyrrolizidine alkaloids

Scheme 14

Degradation studies indicated that both diastereoisomers (51a') and (51b') were incorporated specifically into the retronecine moiety of riddelline (26). Trachelanthamidine was also a more efficient precursor than isoretronecanol, although which enantiomer was involved was not established.

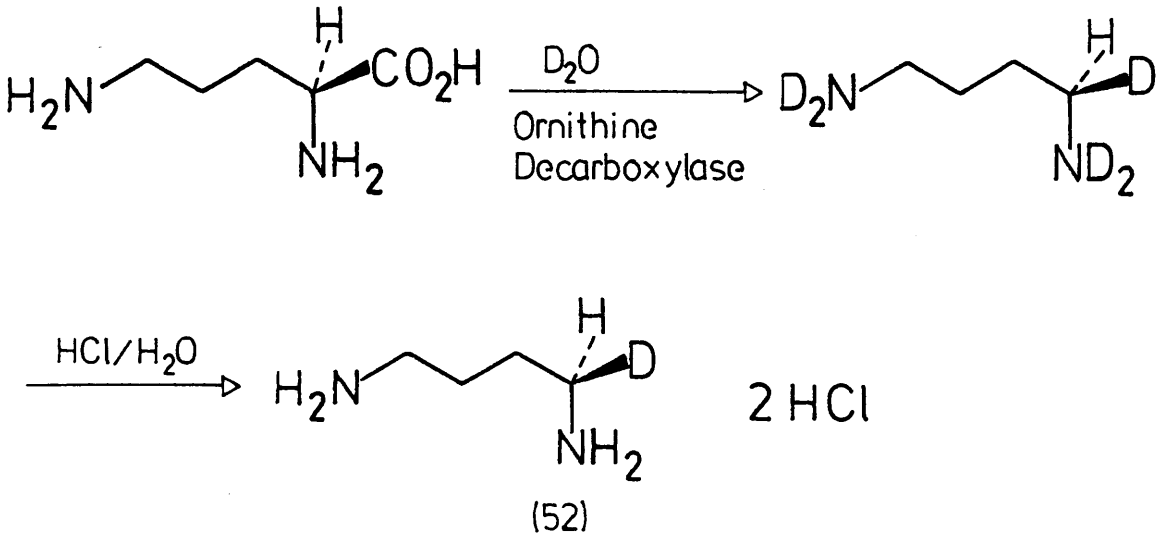
In a similar set of experiments (±)-[5-^{3}H]trachelanthamidine and (±)-[5-^{2}H]isoretronecanol were fed to S. isatideus and S. pleistocephalus, the latter of which produces rosmarinine (20).[47] It was concluded that isoretronecanol (51b) is incorporated into rosmarinine 34 times more efficiently than trachelanthamidine (51a) and ca. four times more efficiently than putrescine (27). On the other hand, trachelanthamidine was found to be 20 times better as a precursor for retronecine biosynthesis than isoretronecanol. Combining results from both groups of workers suggests that trachelanthamidine (51a) is a later intermediate in retronecine biosynthesis, which is in good agreement with the postulated pathway.

Until 1983, details on the stereochemical aspects of retronecine biosynthesis had not been published. This changed when it was shown that retronecine is derived from the L-enantiomers of arginine or ornithine, with little or no contribution from the corresponding D-isomers.[48]

A wealth of stereochemical information was obtained when putrescines, specifically labelled with deuterium were fed to plants producing pyrrolizidine alkaloids, and complete labelling patterns were established by ^{2}H n.m.r. spectroscopy. Key stereochemical deductions were made after (R)-[1-^{2}H]-(52) and (S)-[1-^{2}H]-putrescine (53) dihydrochloride were fed to S. isatideus.[49] Both precursors were first

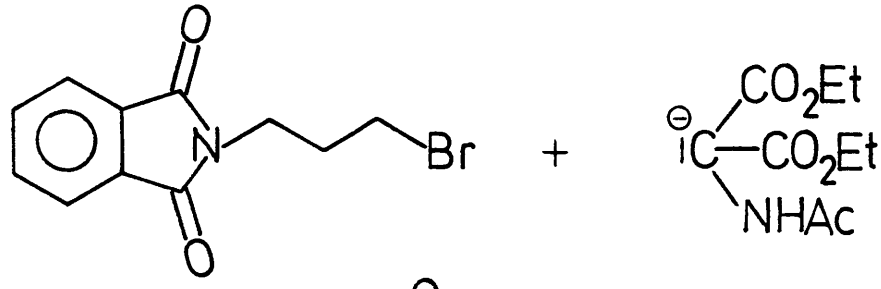
prepared by Richards and Spenser.[50] (R)-[1-^{2}H]Putrescine (52) was prepared by decarboxylation of L-ornithine in ^2H_2O with ornithine decarboxylase (Scheme 15). This is an enzyme reaction which is known to proceed with retention of configuration.[51] Preparation of the (S)-isomer involved reacting the carbanion of diethyl acetamidomalonate with N-(3-bromopropyl)phthalimide (39) to give intermediate (54). On heating with DCl at reflux the phthalimide (54) was decarboxylated and hydrolysed to form DL-[2-^{2}H]ornithine monohydrochloride (55), after work up. Incubation of the racemate with ornithine decarboxylase in water provided (S)-[1-^{2}H]-putrescine dihydrochloride (53) leaving D-[2-^{2}H]ornithine unchanged (Scheme 16).

When (R)-[1-^{2}H]putrescine (52) was fed to S. isatideus, four main signals were evident in the ^{2}H-{^{1}H} n.m.r. spectrum of retrorsine and these corresponded to deuterium at C-3β, C-5α, C-8α and C-9 pro-S. The distribution of labels was interpreted as follows. The initial step involves the enzymatic oxidation of putrescine to 4-aminobutanal (56). It is known that diamine oxidases stereospecifically removes the pro-S hydrogens from the methylene groups of primary amines on oxidation,[52] and so this initial oxidation occurs with complete retention of deuterium. Coupling of 4-aminobutanal with another molecule of putrescine gives an imine (57), which undergoes stereospecific reduction, with the hydride equivalent being delivered on the C-si face to form labelled homospermidine (58). Two further oxidations on the terminal carbon atoms of homospermidine take place with retention of the pro-R and loss of the pro-S hydrogens to give the dialdehyde (59). Intramolecular attack on the C-re face of the immonium ion (60) gives 8α-formylaldehyde by delivery of the

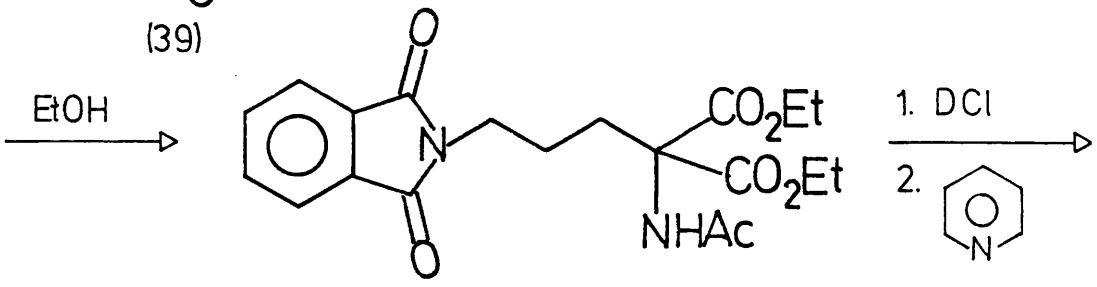


(52)

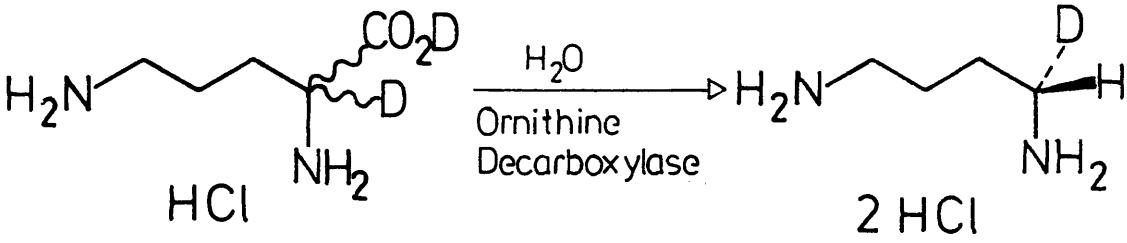
Scheme 15



(39)



(54)



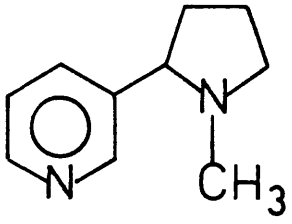
(55) (53)

Scheme 16

hydride equivalent on the C-re face of the carbonyl group leads to retrorsine (62) with labels shown (Scheme 17).

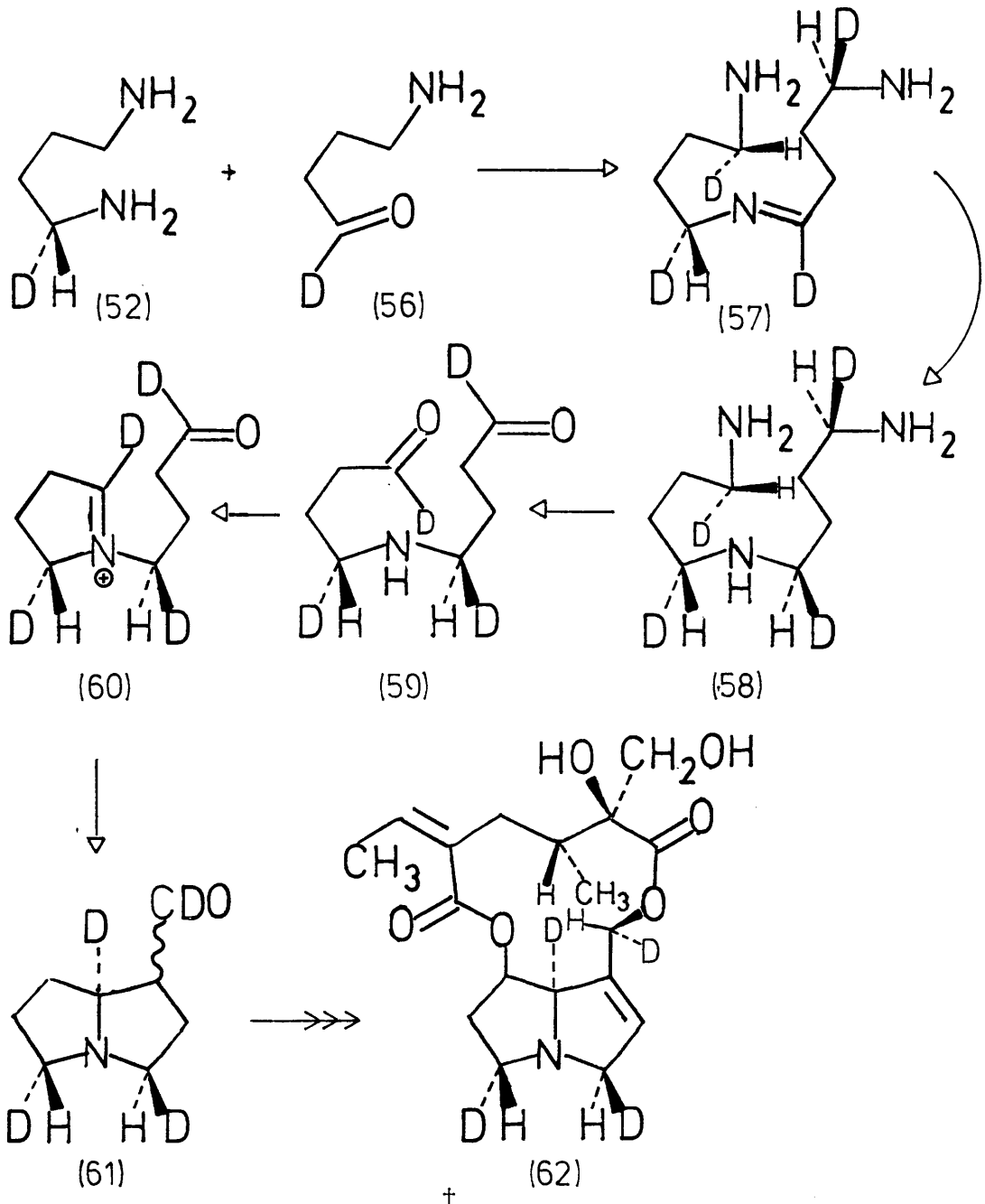
Additional support for this explanation came from feeding (S)-[1-^{2}H]putrescine dihydrochloride (53). The ^{2}H-{^{1}H} n.m.r. spectrum showed two signals corresponding to deuterium at C-3α and C-5β of retrorsine. Fewer deuterium labels were present because of the removal of the (S)-[1-^{2}H] atoms during the three oxidation steps of amine to aldehyde (63) (Scheme 18).

Comparable results were obtained by Spenser who looked at the incorporation of enantiomeric [1-^{2}H]putrescines into a mixture of alkaloids.[53] Spenser et al., also used (R)-[1-^{2}H]putrescine dihydrochloride in conjunction with ^{2}H n.m.r. spectroscopy in a study of the stereochemistry of nicotine (64) biosynthesis.[53]



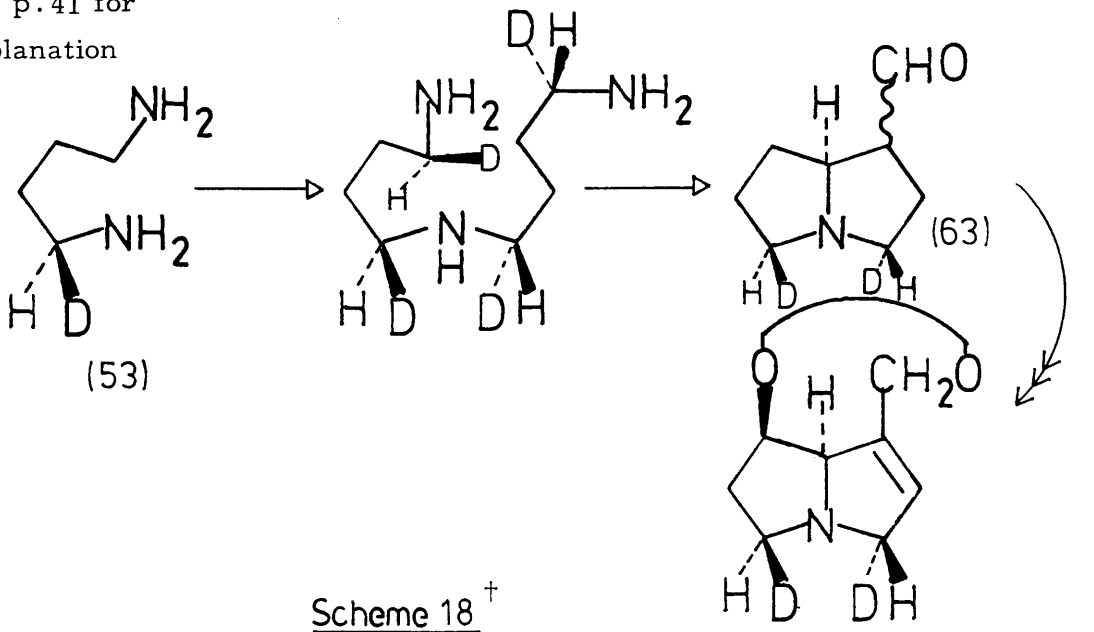
(64)

More stereochemical details emerged from a sample of retrorsine derived biosynthetically from feeding S. isatideus with (2R)-[2-^{2}H]- (65) and (2S)-[2-^{2}H]putrescine (66) dihydrochloride.[54] Kunec and Robins prepared these by extending and slightly modifying the unpublished route of Arigoni and Eliel[55] to the enantiomeric [2-^{2}H]-succinic acids. Treatment of (2S)-aspartic acid (67) with a mixture of hydrochloric and nitric acids in the presence of urea, resulted in the



Scheme 17 †

† See p. 41 for explanation



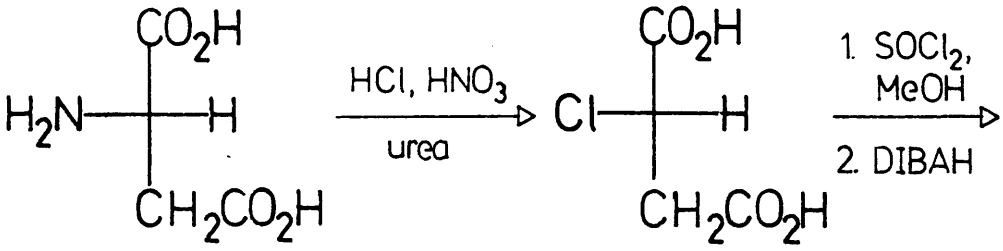
Scheme 18 †

replacement of the amino group by chlorine with retention of configuration. Subsequent diester formation and selective reduction with diisobutylaluminium hydride afforded (2S)-2-chlorobutane-1,4-diol (68). Then, treatment of the diol with lithium aluminium deuteride resulted in displacement of the chlorine by a deuterium atom with inversion of configuration to yield (2R)-[2-^{2}H]butane-1,4-diol (69). (A portion of this sample was oxidised to (2R)-[2-^{2}H]succinic acid for comparison of its o.r.d. data with literature values). Finally, formation of the dibromide from the diol (69), conversion into the diazide and reduction of the diazide gave (2R)-[2-^{2}H]putrescine (65) dihydrochloride (Scheme 19). A sample of (2S)-[2-^{2}H]putrescine (66) dihydrochloride was prepared from (2R)-aspartic acid in a similar fashion.

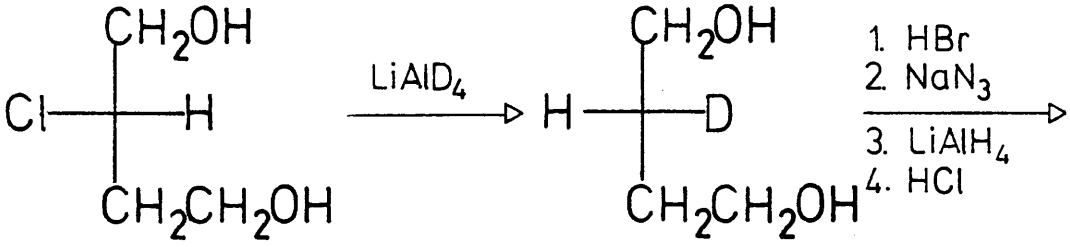
The ^{2}H-{^{1}H} n.m.r. spectrum of retrorsine derived from the (2R)-precursor (65) showed ^{2}H to be present at C-2 and C-6α (Scheme 20), and from the (2S)-isomer (66), ^{2}H was located at C-6β and C-7α (Scheme 21).

These labelling patterns revealed firstly that hydroxylation at C-7 of retronecine occurs with retention of configuration. Since there is a deuterium label at C-7α after feeding (2S)-[2-^{2}H]putrescine, this indicates that the introduction of oxygen does not involve a keto or enol intermediate. Secondly, the formation of the 1,2-double band involves the removal of the pro-S hydrogen at the carbon which becomes C-2 of retronecine, and the retention of the pro-R hydrogen.

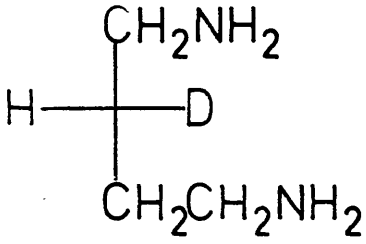
Conclusions - A number of intermediates in the biosynthetic pathway to retronecine have been identified and most of the stereochemical details have been elucidated. This detailed knowledge should now be extended to encompass more of the known pyrrolizidine bases.



(67)

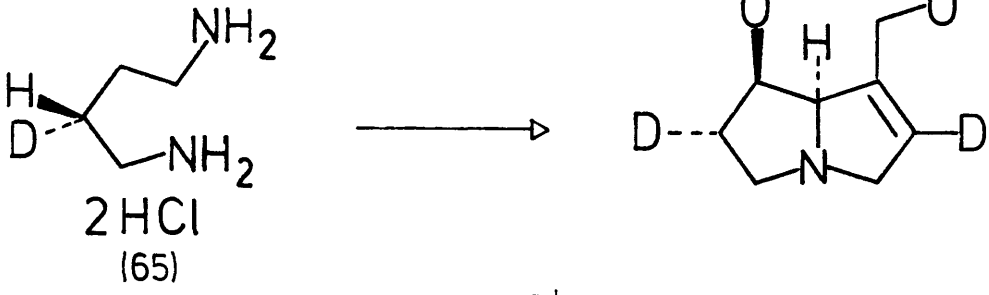


(68) (69)



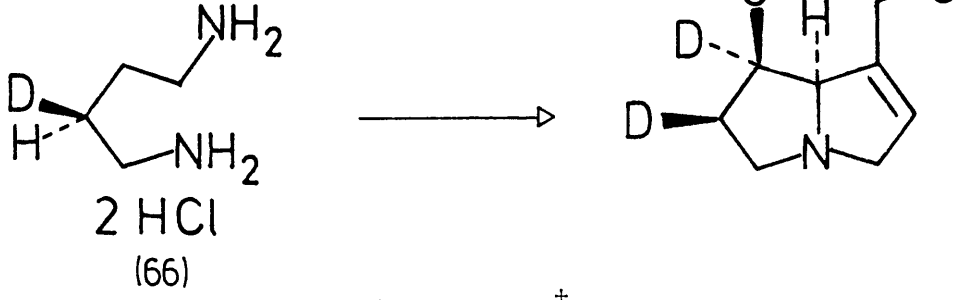
2 HCl (65)

Scheme 19



2 HCl
(65)

Scheme 20†



2 HCl
(66)

Scheme 21†

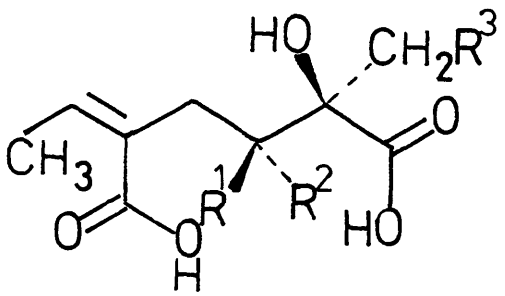
† No molecules of putrescine precursors (52), (53), (65), and (66) can contain more than one ^{2}H atom. The labelling patterns for retrorsine depicted in Schemes 17, 18, 20 and 21 are therefore composite representations of all the ^{2}H-labelled species that were present.

2.2 Biosynthesis of Pyrrolizidine Necic Acids

Although work on necic acid biosynthesis does not form part of this thesis, the biosynthetic routes to these acids are of considerable interest. Therefore, the research in this area is summarised here.

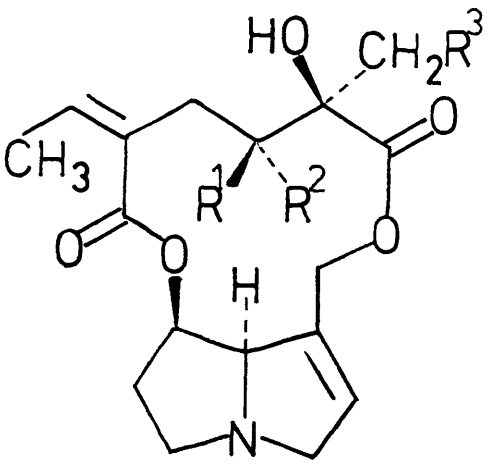
Pyrrolizidine alkaloid acid portions demonstrate several structural types. They can be dibasic, as well as monocarboxylic, and show variations in degrees of unsaturation, hydroxylation and stereochemistry (Table 3).

Studies on necic acid biosynthesis have concentrated mainly on the structurally related acids of Group 1 (Table 3); in particular, senecic (70) and seneciphyllic (71) acids.



(70) $R^1 = R^3 = H$, $R^2 = CH_3$

(71) $R^1, R^2 = CH_2$, $R^3 = H$

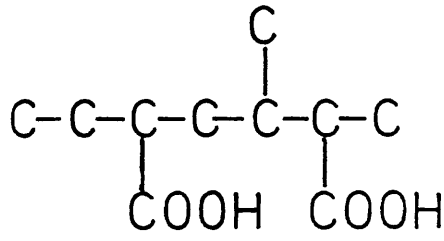
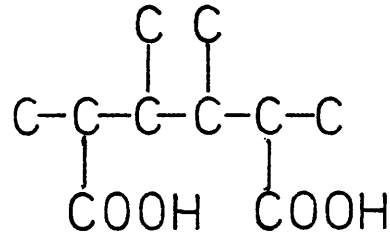
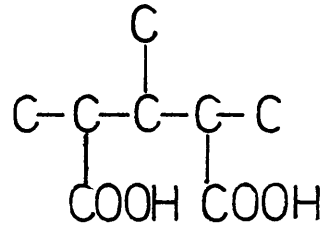
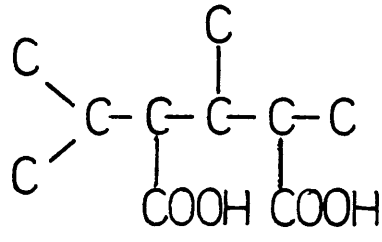
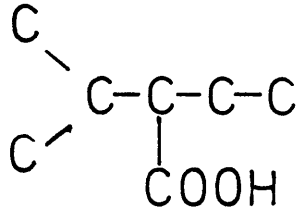
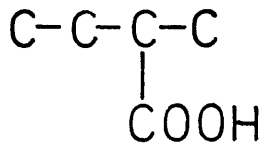


(19) $R^1 = R^3 = H$, $R^2 = CH_3$

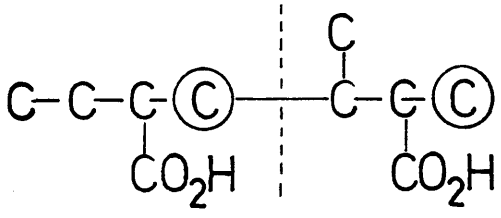
(25) $R^1, R^2 = CH_2$, $R^3 = H$

Biosynthetic investigations began when Hughes and Warren[56] fed [1-^{14}C]and [2-^{14}C]acetate to Senecio isatideus. Chemical degradations showed two complementary labelling patterns in the

Table 3. Variation in Structure of the Acids from the Pyrrolizidine Alkaloids

GROUP	CARBON SKELETON	NATURAL ACIDS
1.		Senecic (70), senecic-phyllic (71), riddellic, isatinecic (14)
2.		Sceleranecic, sceleratinic
3.		Monocrotalic (86)
4.		Incaninic, trichodesmic (87), grantianic
5.		Trachelanthic, viridifloric (208), echimidinic, lasiocarpic
6.	Simple acids 	Angelic (83), tiglic, sarracinic

isatinecic acid. To account for their observations, they proposed a biogenetic route which involved the condensation of acetate to aceto-acetate, insertion of a one-carbon unit and the combination of the two resultant C_5-units, as shown below.

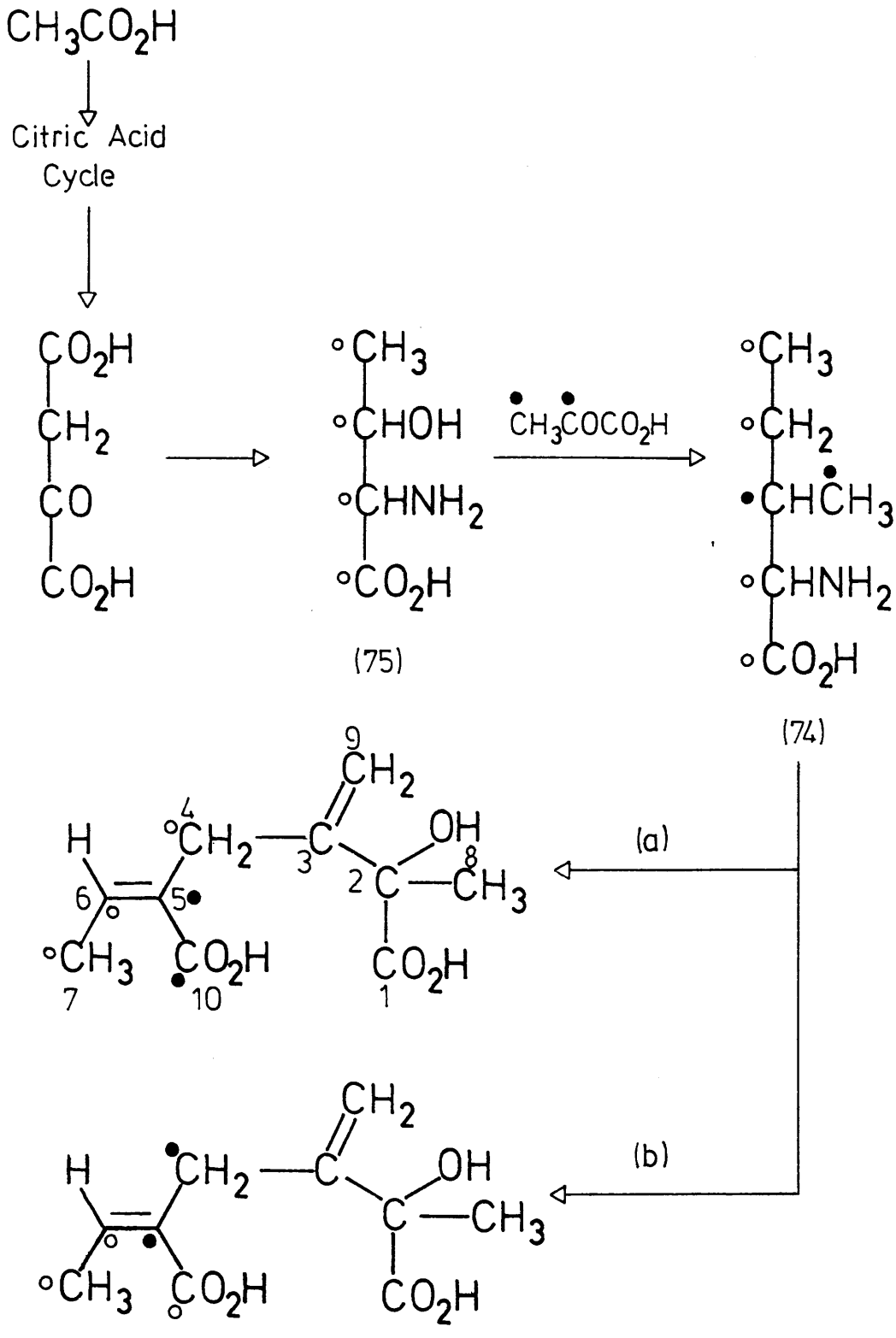


Ⓒ— : from a one-carbon donor

Related studies were carried out by Crout et al. on Senecio douglasii DC, a plant which produces four known alkaloids.[57] However, investigations were based only on seneciphyllic acid (71), since the parent alkaloid seneciphylline (25), is the major component of the mixture (65%). Feeding experiments with labelled acetate (^{14}C) resulted in a complete randomisation of activity between the acid and base portions. Crout et al. attributed this to the incorporation of acetate by an indirect route and thus it was not directly condensing to form immediate precursors of the necic acid. The possibility of an acetate-mevalonate pathway was subsequently explored. One of the reasons for considering this pathway was the apparent isoprenoid skeleton of seneciphyllic acid (71). [2-^{14}C]Mevalonate was fed, but the incorporation figures were no higher than for acetate, and so the acetate-mevalonate pathway was not considered likely in necic acid biosynthesis.

During these preliminary attempts to discover the pathways to necic acids, various ^{14}C-labelled amino acids were screened as possible precursors, and it was found that isoleucine (73) and its biological precursor threonine (72) were efficient and specific precursors of seneciphyllic acid (71) in Senecio douglasii DC.[58] Feeding [U-^{14}C]-L-isoleucine (74) resulted in a five-carbon component comprising (C-4, -5, -6, -7, and -10) of seneciphyllic acid being most heavily labelled (ca. 2/3 of total activity). When [1-^{14}C]-L-isoleucine was administered, it was incorporated with less than one tenth of the efficiency of [U-^{14}C]-L-isoleucine, indicating that C-1 of isoleucine is not incorporated. These results allowed Crout et al. to suggest that isoleucine is incorporated into the left hand C_5-unit of seneciphyllic acid as shown in Scheme 22(a). Another mode of incorporation was also considered, (Scheme 22(b)), but this was rejected on the following evidence. If pathway (b) is the correct route, then after feeding [U-^{14}C]-L-threonine (75), there should have been equal labelling at C-6, C-7 and C-10 of the necic acid. However, degradation experiments showed that the C-6, -7 unit derived from [U-^{14}C]-L-threonine contained more than four times as much activity as C-10. Thus, route (a) was preferred.

When Crout et al. administered [U-^{14}C]-L-isoleucine (74) to Senecio douglasii, it was discovered that C-1, C-8 and C-9 of seneciphyllic acid (71) were equally labelled. Consequently, it appeared possible that the C-1, -2, -3, -8, and -9 unit might also be derived from isoleucine. This was confirmed when [U-^{14}C]-L-threonine (75) was

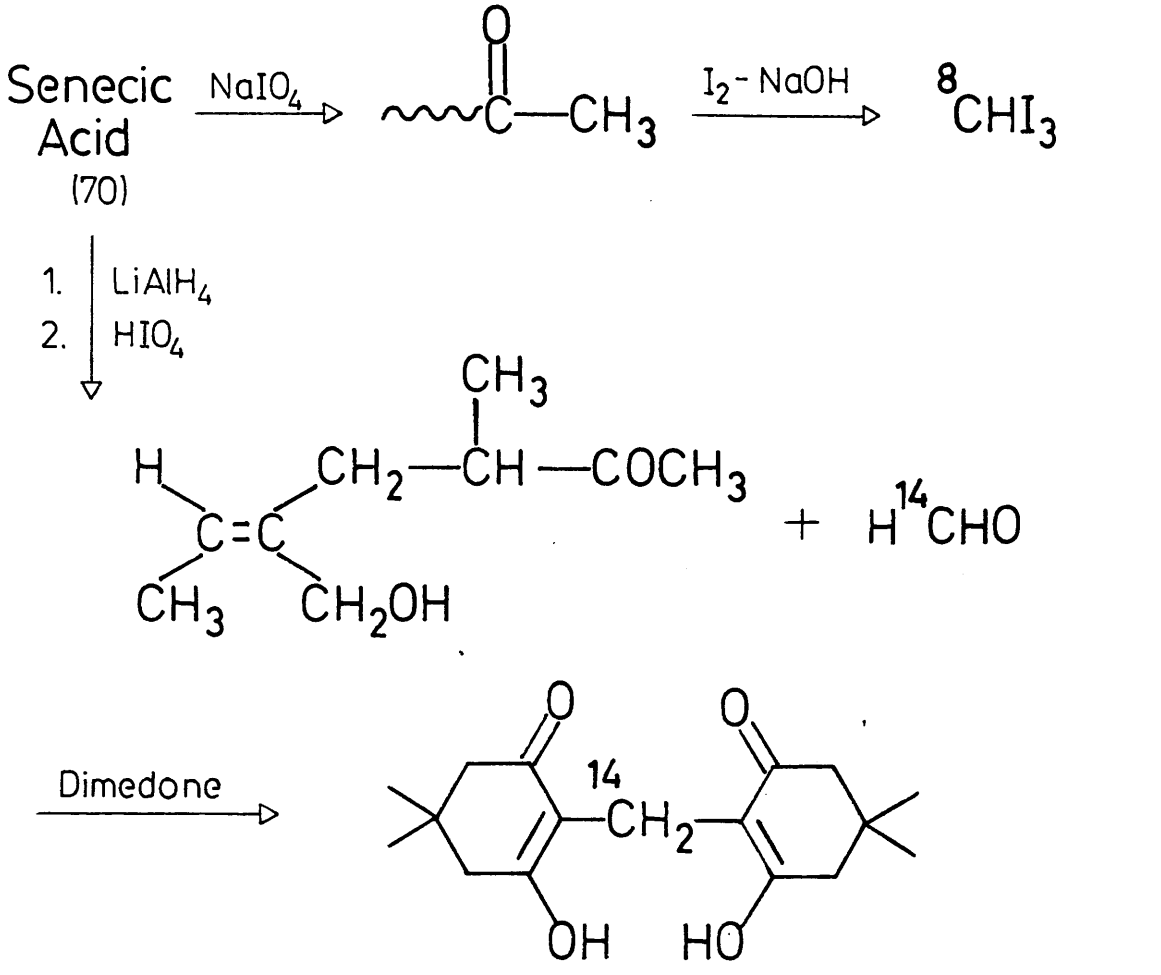

CH3CO2H
Citric Acid Cycle
CO2H
CH2
CO
CO2H
CH3
CHOH
CHNH2
CO2H
(75)
CH3COCO2H
CH3
CH2
CHCH3
CHNH2
CO2H
(74)
(a)
(b)
9
CH2
4
CH2
H
C
OH
3
2
8
6
5
CH3
CH3
CO2H
CO2H
7
10
1

Scheme 22

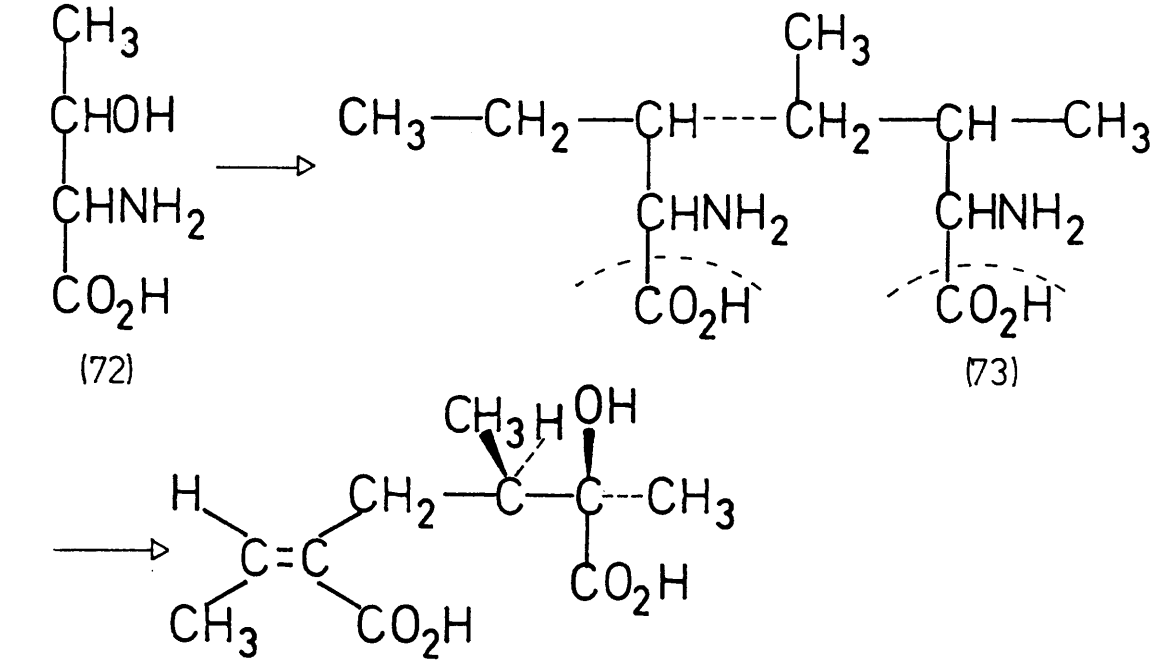
used in a feeding experiment with Senecio magnificus.[58] This plant produces senecionine (19) as its major alkaloid and hydrolysis yields senecic acid (70) and retronecine (3). Specific chemical degradation of the molecule showed C-8 to be essentially unlabelled, whereas C-1 was heavily labelled (Scheme 23).

Additionally, examination of senecic acid derived from [U-^{14}C]-L-isoleucine (74) showed that C-1 and C-8 were nearly equally labelled and that the activity was evenly distributed throughout the necic acid.

When feeding experiments were carried out with specifically labelled isoleucines on S. magnificus, the manner in which isoleucine is incorporated into the C-4, -5, -6, -7, and -10 component of senecic acid was determined. Further verification was also provided that isoleucine is a specific precursor of the C-1, -2, -3, -8, and -9 component. For instance, [2-^{14}C]isoleucine labelled C-1 and C-10 of senecic acid equally and exclusively, and the labels from [5-^{14}C]isoleucine were located at C-7 and C-9 only. Intact incorporation of isoleucine was also demonstrated by feeding doubly labelled (^{14}C) isoleucine. All of these experiments demonstrated conclusively that senecic acid is biosynthesised from two C_5 units derived from isoleucine (73) (Scheme 24). More specifically, it has been experimentally established that of all the four stereoisomers of isoleucine, only L-isoleucine is efficiently incorporated into senecic acid.[59]

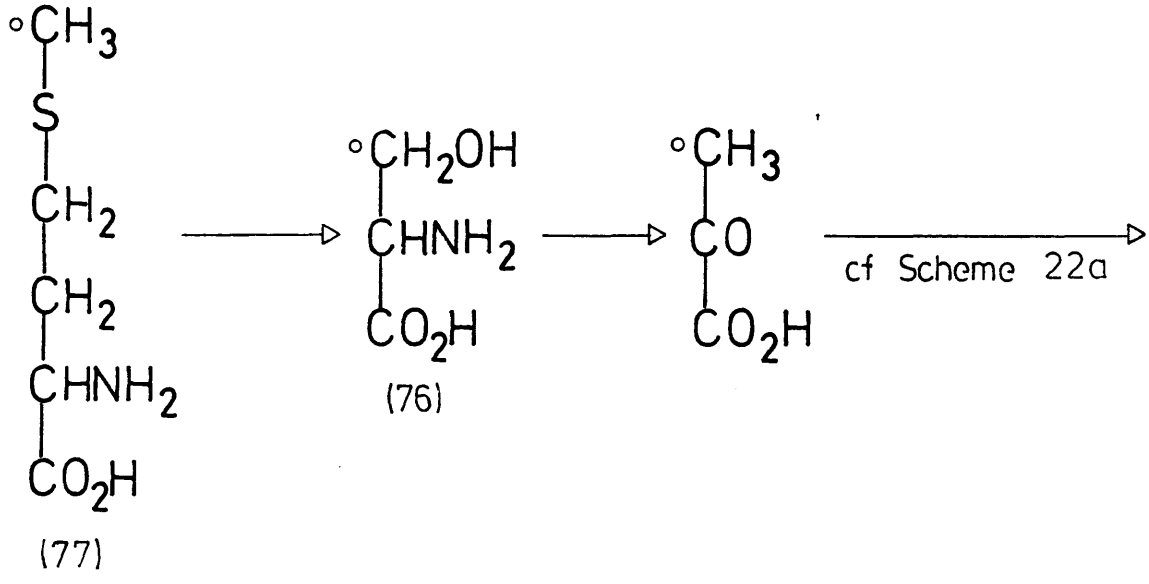


Scheme 23



Scheme 24

During the earlier investigations into the biosynthesis of seneciphyllic acid (71), in S. douglasii DC, Crout et al. observed that the S-methyl group of methionine was a relatively efficient specific precursor of C-8.[58] This can be explained by assuming that the methyl group of methionine is converted in Senecio species into the methyl group of pyruvate via serine (76). Support for this theory rests in the conversion of serine into pyruvate in vivo (Scheme 25).[60]

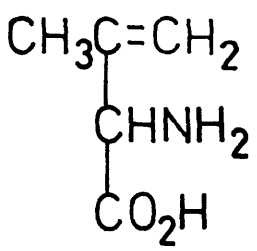


Scheme 25

Confirmation of this observation was provided by feeding pea seedlings with [Me-^{14}C]-L-methionine (77). This gave an incorporation of 0.14% into serine, and degradation studies proved that the radioactivity was solely located in the hydroxymethyl group.

Having established the amino acid basis of necic acid biosynthesis, Crout and co-workers decided to explore the mechanism of coupling of the two isoleucine derived units.[61] Initially, attention was

focussed on the changes taking place at the coupling positions C-4 and C-6 of isoleucine. Accordingly, [6-^{3}H, 6-^{14}C]isoleucine was prepared and administered to a Senecio magnificus plant. Approximately five sixths of the tritium was retained in the senecic acid and thus it was certain that one of the hydrogen atoms at C-6 in isoleucine (73) is retained in its conversion into senecionine (19). To determine the fate of the hydrogen atoms at C-4 in isoleucine, [4-$^{3}H_{2}$]isoleucine was fed to S. isatideus. Half of the tritium label was retained in the isatinecic acid (14). These results were informative for two reasons. Firstly, the possibility of a carbonyl function being used to couple the two isoleucine units was eliminated. Secondly, the type of functionality introduced into the C-3, C-6 side chain of isoleucine prior to coupling might be C-CH_2OH or C=CH_2. The latter appeared to be the better choice because the corresponding amino acid β-methylenenorvaline (78) (2-amino-3-methylenepentanoic acid), occurs in nature. Once a suitable synthesis of this acid had been established, radiolabelled material was fed to Senecio magnificus. Despite being able to show that the radioactivity was located entirely in the necic acid, it was not possible to state whether the precursor was incorporated into only one or both halves of senecic acid.



(78)

During L-isoleucine biosynthesis, 2-ethyl-2-hydroxy-3-oxobutanoic acid (81) undergoes a tertiary ketol rearrangement to 3-hydroxy-3-methyl-2-oxopentanoic acid (82). The stereochemistry of this rearrangement was elegantly revealed by feeding 2-aminobutanoic acid (79), stereospecifically labelled with tritium at C-3, to S. isatideus and S. magnificus.[62] It was assumed that enzymes such as amino acid transferases or oxidases would operate on 2-aminobutanoic acid to generate 2-oxobutanoate (80) in situ. The results from feeding (2RS ,3S)-[3-$^{3}H_{2}$]-, (2RS)-[3-$^{3}H_{2}$]-, (2S)-[3-$^{3}H_{2}$] - and (2R)-[3-$^{3}H_{2}$]-2-aminobutanoic acid, together with [3-^{14}C]-labelled material are shown in Table 4. They indicate that during the generation of both five carbon units of the necic acid, the C-3 pro-S hydrogen of 2-aminobutanoic acid is lost, and the C-3 pro-R hydrogen is retained. For comparison, the corresponding changes in stereochemistry at C-4 in L-(2S)-isoleucine were determined after feeding (2RS , 4RS)-[3-$^{3}H_{2}$]-, (2S, 4S)-[3,4-$^{3}H_{2}$]-(2S)-[4-$^{3}H_{2}$]-, and (2S,4R)-[4-$^{3}H_{1}$]-isoleucine, together with L-[U-^{14}C]-isoleucine.

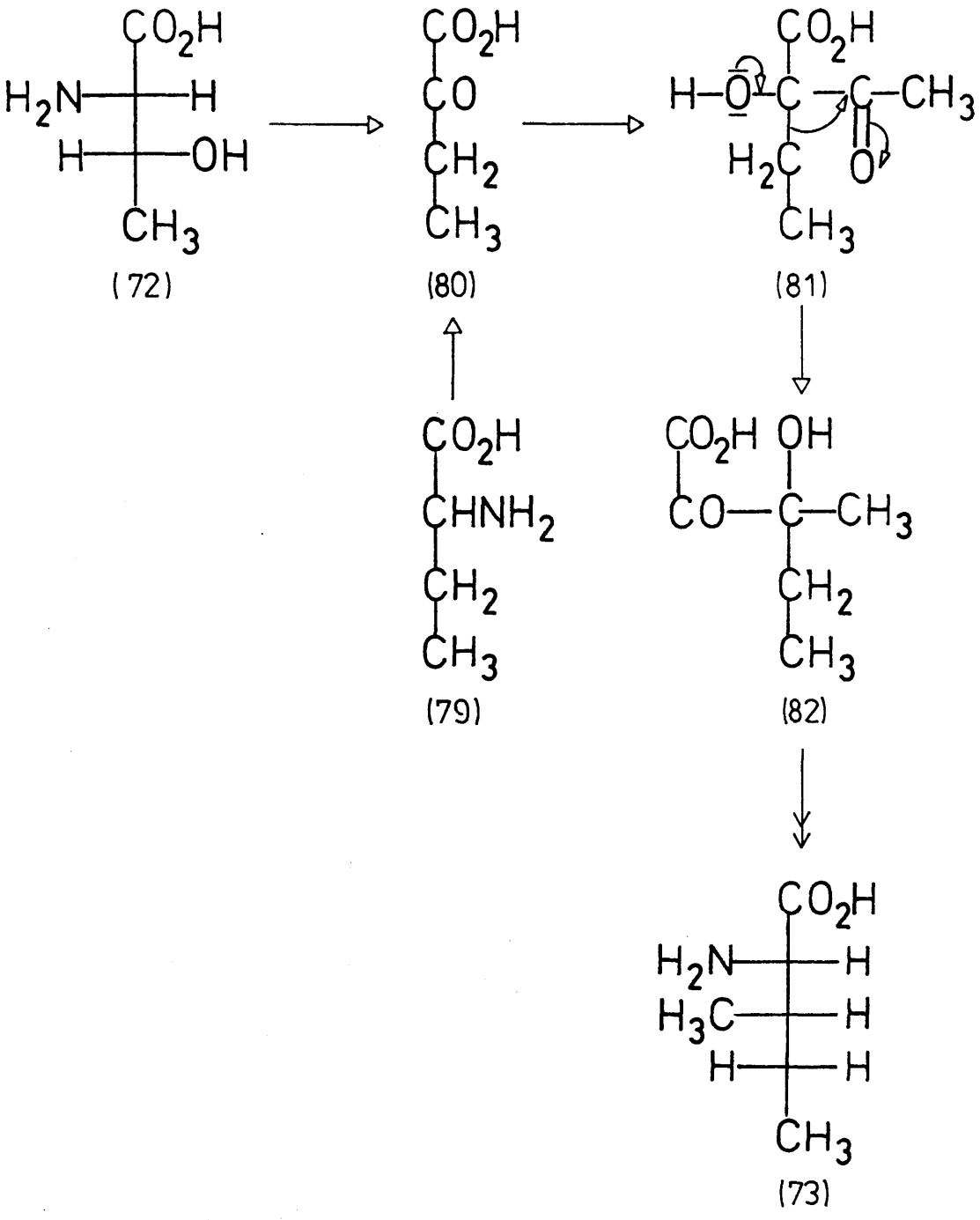
From the table of results it is evident that the C-4 pro-S hydrogen of isoleucine is lost, whereas the C-4 pro-R hydrogen is retained during incorporation into both halves of the necic acid. In other words, the hydrogen atoms at C-13 and C-20 of retrorsine (12) and senecionine (19) are derived from the 4-pro-R hydrogen atom of L-isoleucine. Combination of the results from both sets of experiments, indicated that the migration of the ethyl group during isoleucine biosynthesis (Scheme 26) occurs with retention of configuration, which is in accord with orbital symmetry calculations.[63]

Table 4. Incorporation of 2-aminobutanoic acid and isoleucine into alkaloids (19) and (12) in Senecio species

Precursor	$^3H/^{14}C$ ratio			% Tritium in
	Precursor	Alkaloid (19)or(12)	Necic Acid	Necic Acid
2-Aminobutanoic acid				
(2RS,3S)-[3-^{3}H, 3-^{14}C][a]	2.85	0.8	-	-
(2RS)-[3-$^3H_2$, 3-^{14}C][b]	8.23	3.85	3.78	-
(2S)-[3-$^3H_2$, 3-^{14}C][b]	7.9	3.63	3.72	47.1
(2R)-[3-$^3H_2$, 3-^{14}C][b]	7.97	3.77	3.88	48.7
Isoleucine				
2S-[U-^{14}C] plus:				
(2RS,4RS)-[3,4-$^3H_2$][a]	1.44	0.014	-	(5.1)
(2S,4S)-[3,4-$^3H_2$][a]	1.84	0.12	-	-
(2S)-[4-$^3H_2$][b]	2.36	1.42	1.60	56
(2S,4R)-[4-$^3H_1$][b]	2.72	2.59	-	95.2

a. Incorporation experiments in S. magnificus. Alkaloid: senecionine (19); necic acid: senecic acid (70).

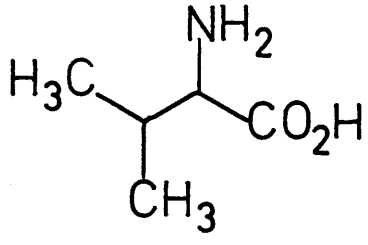
b. Incorporation experiments in S. isatideus. Alkaloid: retrorsine (12); necic acid: isatinecic acid (14).


CO2H
H2N
H
H
OH
CH3
(72)
CO2H
CO
CH2
CH3
(80)
CO2H
CHNH2
CH2
CH3
(79)
CO2H
H–O
C
C
CH3
H2C
O
CH3
(81)
CO2H
OH
CO
C
CH3
CH2
CH3
(82)
CO2H
H2N
H
H3C
H
H
H
CH3
(73)

Scheme 26

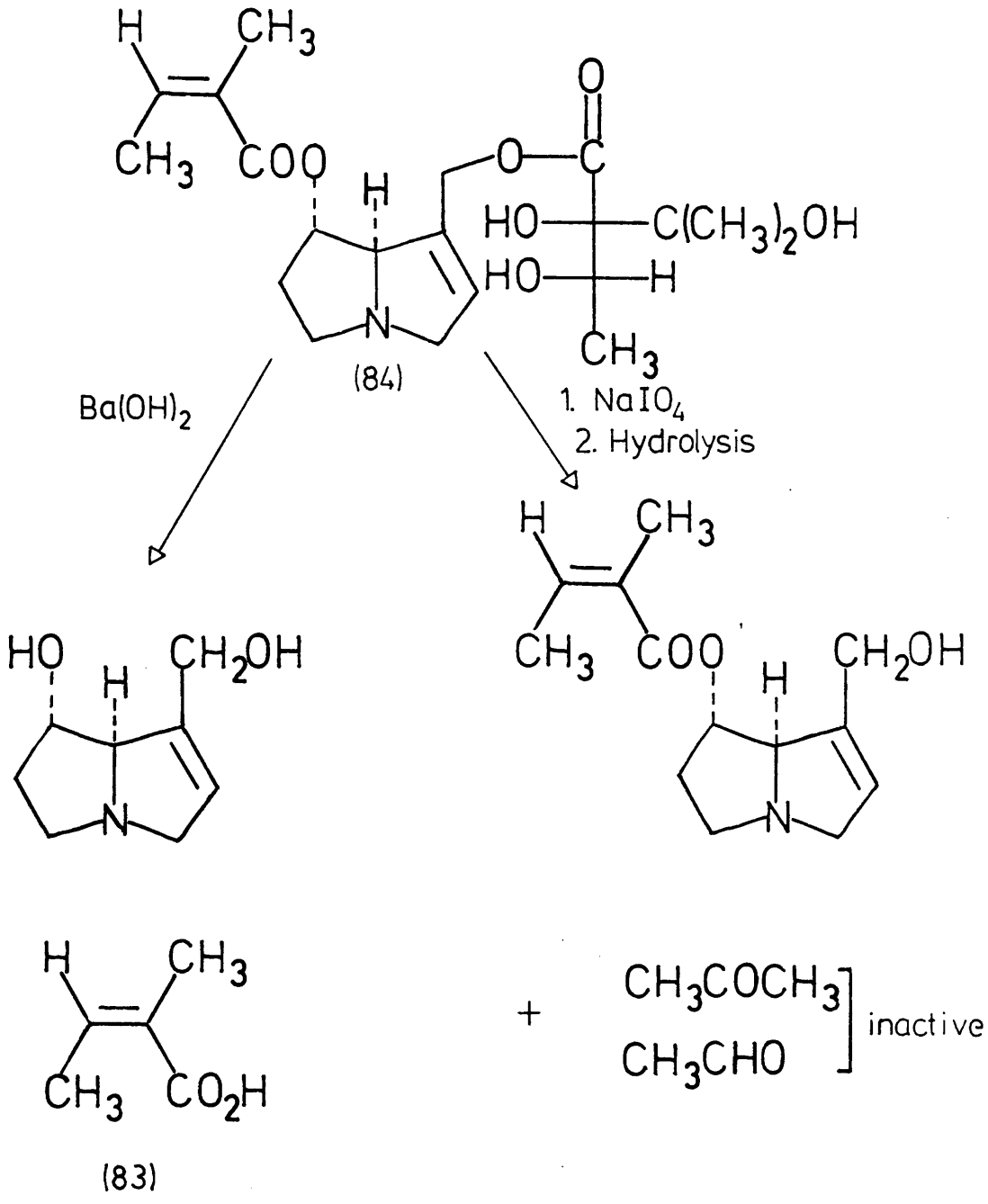
Isoleucine has also been shown to be a good precursor for the angelic acid (83) component of heliosupine (84)[64] in C. officinale L. plants (family Boraginaceae). Degradation of heliosupine derived from [U-^{14}C]-L-isoleucine proved that the activity was almost entirely (98%) located in the angeloyl portion of the alkaloid (Scheme 27).

On the other hand, echimidinic acid, the 'right hand' necic component of heliosupine (84) is derived from valine (85).[65] Feeding [4-^{14}C]-DL-valine resulted in the biosynthesis of labelled heliosupine, with the echimidinic acid containing 85% of the total activity of the alkaloid. In addition, most of the activity of the acid was located in the geminal methyl atoms.



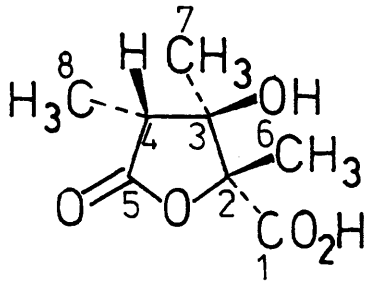
(85)

Monocrotalic acid (86) is the necic acid component of monocrotaline (18), which is produced by Crotalaria spectabilis. A biosynthetic study on monocrotalic acid began by testing ^{14}C-labelled acetate as a precursor, since it had been claimed that high incorporations of [1-^{14}C]-acetate into monocrotalic acid were obtained.[66] However, despite

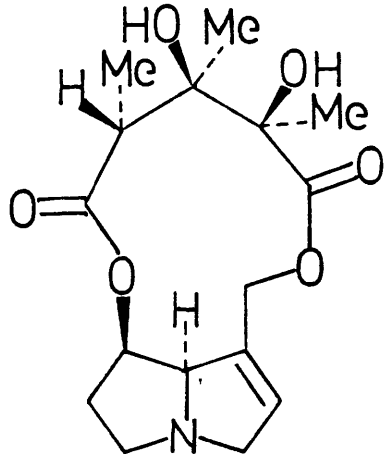


Scheme 27

repeated attempts to confirm this finding, it was concluded that acetate was not an efficient precursor.



(86)



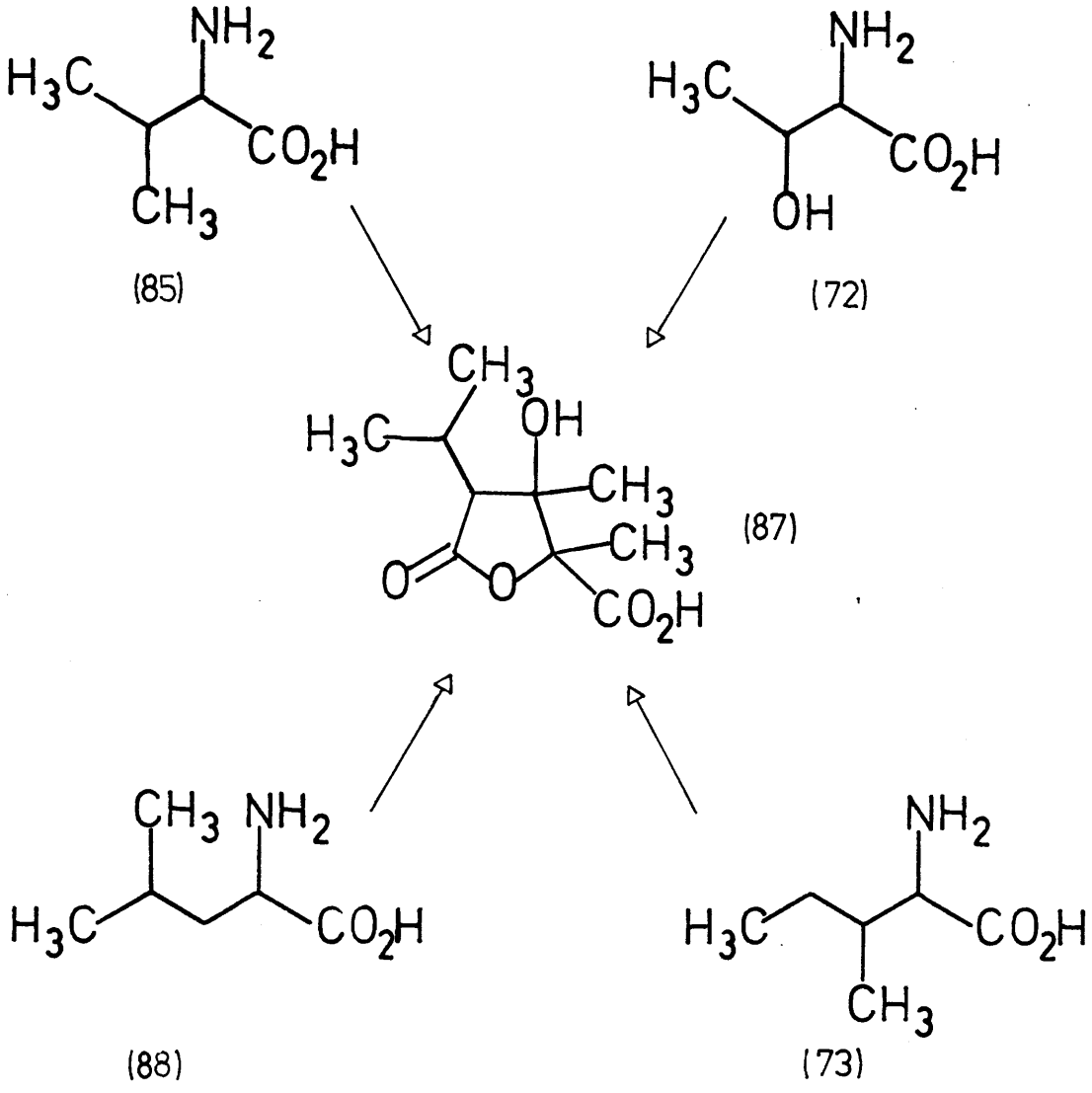
(18)

By analogy with the biosynthesis of senecic acid, it appeared probable that the five-carbon unit consisting of C-1,-2,-3,-6, and -7 of monocrotalic acid was similarly derived from isoleucine. Labelling experiments with [U-^{14}C]-L-isoleucine (74) and its precursor [U-^{14}C]-L-threonine (75) found this to be true. The origin of the three-carbon unit comprising C-4,-5, and -8 of monocrotalic acid has still not been established. Structurally, it looks as if it could be derived from propionate. Indeed, it had been reported by Nowacki and Byerrum that propionate was incorporated into the necic acid component of monocrotaline in Crotalaria spectabilis, but this evidence is not conclusive.[30]

The involvement of branched-chain amino acids in necic acid biosynthesis is exemplified further by the work of Devlin and Robins. They looked at the biosynthesis of trichodesmic acid (87) [parent

alkaloid trichodesmine], in Crotalaria globifera.[67] Labelling experiments showed that the 'right hand' C_5 unit is derived from threonine (72) or isoleucine (73), whereas the 'left hand' C_5 unit is formed from valine (85) or leucine (88) (Scheme 28).

Conclusions - Much information has been obtained concerning the biosynthesis of necic acids using radiotracers. However, experiments with precursors labelled with stable isotopes (^{13}C, ^{2}H) have not been carried out yet. Such experiments may throw more light on the biosynthesis of the wide range of necic acids; in particular, the intriguing mode of coupling of the two C_5 units to form the C_{10} acids.


NH2
H3C
CO2H
CH3
(85)
NH2
H3C
CO2H
OH
(72)
CH3
H3C
OH
CH3
CH3
O
O
CO2H
(87)
CH3 NH2
H3C
CO2H
(88)
NH2
H3C
CO2H
CH3
(73)

Scheme 28

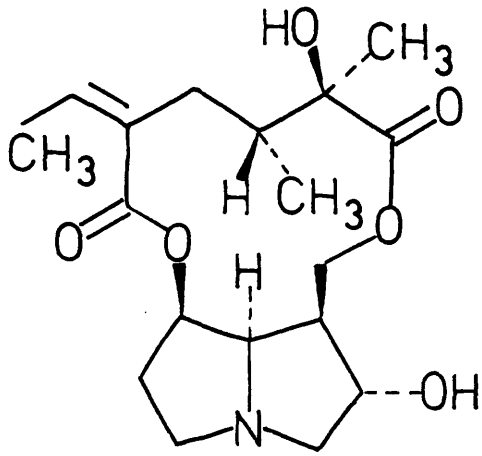
Chapter 3

BIOSYNTHESIS OF ROSMARINECINE

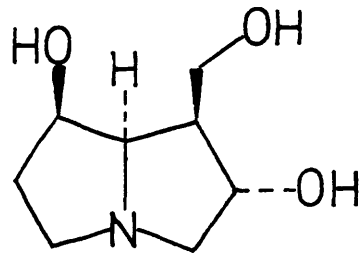
3.1 Introduction

Retronecine (3) was the only necine base whose biosynthesis had been studied in detail until 1986. This was rather curious, considering the extensive range of necines which had been isolated, and the powerful spectroscopic techniques which the organic chemist can utilise to investigate biosynthetic pathways. It was therefore decided to widen the scope of biosynthetic studies in this area.

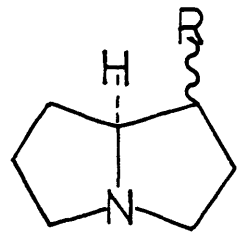
To start our work, rosmarinine was isolated as the major alkaloidal constituent of Senecio pleistocephalus S. Moore plants (family Compositae). Rosmarinecine (21) is the base portion of rosmarinine (20). It had been shown by Kunec and Robins that (±)-isoretronecanol (51b) is incorporated specifically and efficiently into rosmarinecine[47] whereas the diastereoisomer (±)-trachelanthamidine (51a) is an efficient precursor for retronecine biosynthesis.[46,47] Thus, it is clear that the two biosynthetic pathways diverge prior to the formation of alcohols (51a) and (51b). These observations therefore made an investigation of the earlier steps in the biosynthetic pathway to rosmarinecine essential.



(20)



(21)



(51a) R= α-CH_2OH
(51b) R= β-CH_2OH

3.2 Identification of Rosmarinine (20)

A methanolic extraction of Senecio pleistocephalus provided one crystalline alkaloid, m.p. 204°C in 0.15% yield. When a thin layer chromatogram of the compound was sprayed with o-chloranil followed by Ehrlich's reagent,[68] it was evident that the alkaloid did not contain a 1,2-double bond. A molecular formula of $C_{18}H_{27}NO_6$ was assigned to the alkaloid on the basis of high resolution mass spectrometry and analytical data. This molecular formula corresponds to two known pyrrolizidine alkaloids containing saturated necines, hygrophylline and rosmarinine. The ^{1}H and ^{13}C n.m.r. spectroscopic data of the alkaloid were consistent with those reported for rosmarinine (20).[69] Non-depression of a mixed melting point with an authentic sample of rosmarinine (supplied by Dr. C.C.J. Culvenor) confirmed the identity, along with X-ray crystal structure analysis (Figure 2)[70] carried out by Dr. A.A. Freer. This showed that the ester carbonyl groups are

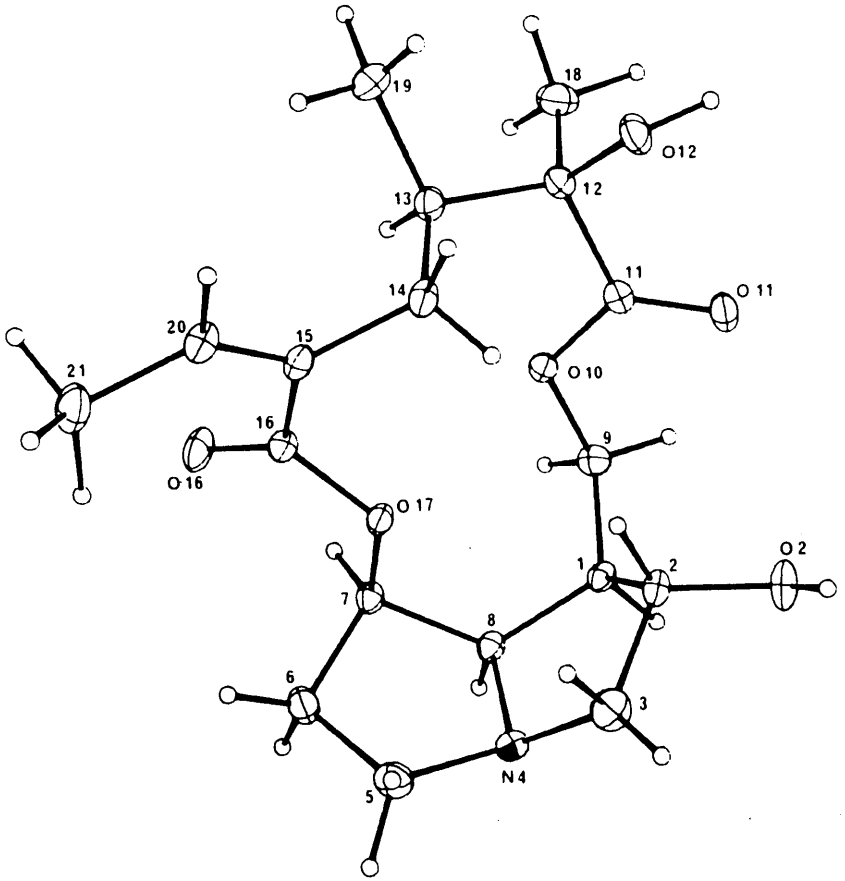
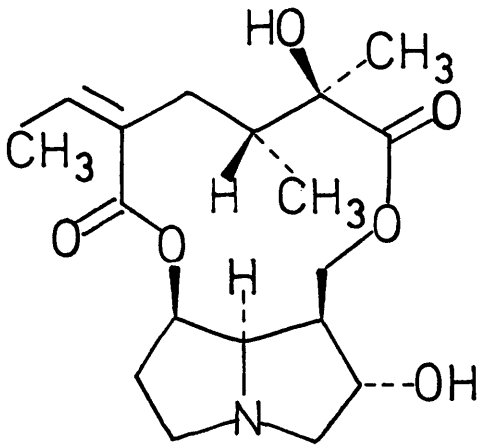


Figure 2

X-Ray Crystal Structure of Rosmarinine (20).[70]



antiparallel, as with all the 12-membered pyrrolizidine alkaloids which have been studied by X-ray crystallography. Furthermore, it is apparent that there is an intermolecular hydrogen bond between the C-2 hydroxyl and the N atom.

The biosynthesis of rosmarinine (20) was to be explored using ^{13}C and ^{1}H n.m.r. spectroscopy. Consequently, the chemical shifts of each carbon in the natural abundance ^{13}C spectrum and of each proton in the ^{1}H n.m.r. spectrum of rosmarinine had to be determined, prior to starting the biosynthetic work.

The ^{13}C n.m.r. spectrum of rosmarinine (20) run in deuteriochloroform was assigned with the aid of a Distortionless Enhancement by Polarisation Transfer (DEPT) experiment. The ^{13}C assignments were in good agreement with those published by Roitman.[69] However, it was also necessary to carry out a Heteronuclear (^{13}C-^{1}H) Chemical Shift Correlation Spectroscopy (COSY) experiment because the chemical shifts of C-2 and C-8 could not be established, due to their closeness in value i.e., 69.1 and 69.3 p.p.m. Since the chemical shifts of the protons on C-2 and C-8 in the ^{1}H n.m.r. spectrum of rosmarinine are markedly different, the exercise of assigning the C-2 and C-8 resonances from the COSY spectrum is straightforward. Figure 3 shows part of the heteronuclear (^{13}C-^{1}H) COSY spectrum of rosmarinine.

Most of the proton signals in rosmarinine were assigned using high field n.m.r. spectroscopy. On examination of the ^{1}H n.m.r. spectrum of rosmarinine (Figure 4) some characteristic peaks corresponding to macrocyclic diesters of pyrrolizidine alkaloids were obvious. For instance, the C-9 non-equivalent protons appeared as an

2D (^{13}C-^{1}H) COSY OF ROSMARINININE (20)

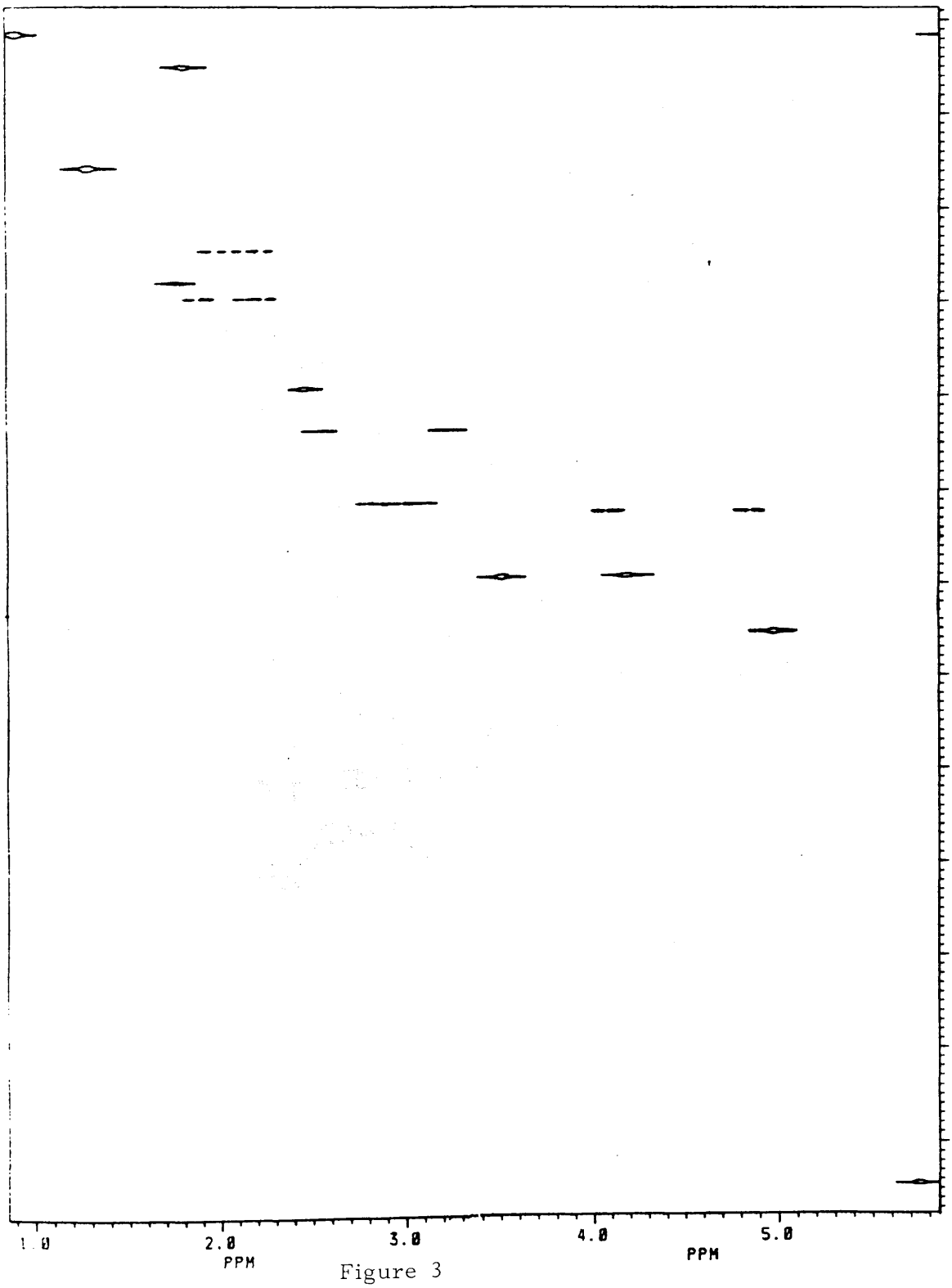
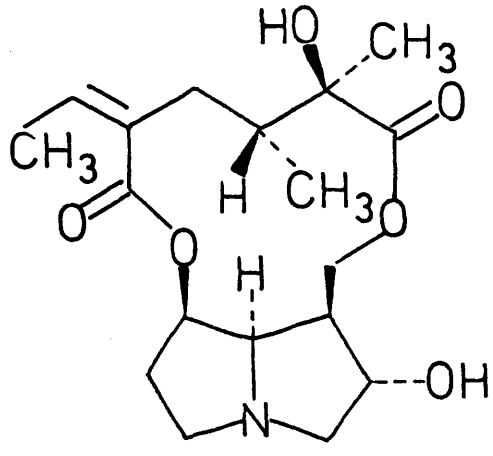


Figure 3

Figure 4. 200.13 MHz ^{1}H N.m.r. spectrum of rosmarinine (20) in $CDCl_3$



AB system at δ4.87 and 4.08, making the chemical shift difference between these two diastereotopic protons ca. 0.8 p.p.m. However it was not immediately apparent, which of the two C-9 protons was the more downfield in the spectrum. From previous ^{1}H n.m.r. spectroscopic studies[25] and X-ray crystallographic data[26] on retrorsine (12), it is believed that the conformation of the alkaloid is similar in organic solution and in the solid state. The H-9 pro-S of retrorsine is deshielded because it lies in the plane of the adjacent carbonyl and double bond. The chemical shift difference between these C-9 protons is ca. 1.4 p.p.m. X-Ray crystallographic data on rosmarinine (20) suggested that a similar situation exists.[70] Therefore the H-9 pro-S was assigned as the more deshielded of the prochiral hydrogens at δ4.87. The deshielding effect in this case was less than with retrorsine because there is no 1,2-double bond in rosmarinine. Additional support for this assignment came from nuclear Overhauser enhancement (n.O.e.) experiments on rosmarinine using the NEOMULT.AU programme.[71] (Figure 5 presents some of the results of a series of n.O.e. experiments on rosmarinine). The 8% n.O.e. at δ4.88 (H-9 pro-S) of rosmarinine, upon irradiation at δ2.48 (1-H), compared to 5% n.O.e. at δ4.09 (H-9 pro-R), provided additional justification for attributing the most downfield AB system to H-9 pro-S. Inserting the coupling constants $J_{1,9\ \text{pro-R}}$ = 1.2 Hz and $J_{1,9\ \text{pro-S}}$ = 5.5 Hz into the Karplus equation, indicated dihedral angles of ca. 70° and 40°, respectively, between the C-9 protons and the 1-proton. Further n.O.e. measurements, confirmed the assignments of the remaining protons of the necine component of rosmarinine. For example, irradiation at δ4.20 (2-H) produced an n.O.e.

on the signal at δ 2.90 (ca. 5%), indicating that this should be assigned to 3β-H. Finally for completeness, these assignments were further confirmed by a Homonuclear (^{1}H-^{1}H) COSY experiment, which corroborated our assignments and previous work.[69]

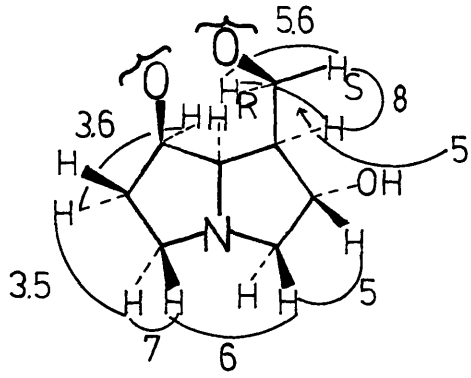
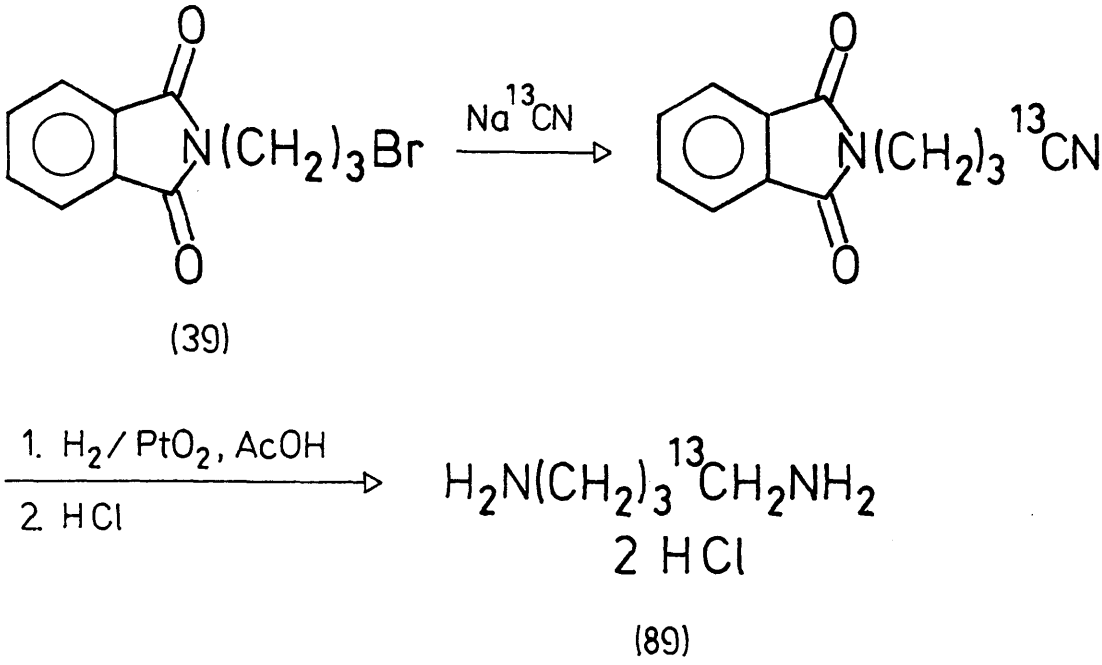


Figure 5

3.3 Incorporation of ^{13}C-Labelled Precursors into Rosmarinine (20)

A strategy, similar to that adopted in the study of retronecine biosynthesis in S. isatideus plants, was employed in the investigation of rosmarinecine biosynthesis in Senecio pleistocephalus. Initial feeding experiments on S. pleistocephalus plants were carried out with [1-^{13}C]putrescine (89) dihydrochloride. Preparation of this precursor was achieved by modification of a published route[41] and involved treatment of N-(3-bromopropyl)phthalimide (39) with sodium [^{13}C]cyanide followed by catalytic reduction of the nitrile and acid hydrolysis (Scheme 29).



Scheme 29

In order to obtain an additional measure of specific incorporation, radioactive [1,4-^{14}C]putrescine dihydrochloride was added to this ^{13}C-labelled precursor (89) before being fed to the plants. The wick feeding technique was used to administer the precursors. The stems of the plants were threaded with needle and cotton twice and the cotton ends were immersed in a vial containing an aqueous solution of the labelled compound. This method gave higher incorporations than those obtained by the xylem pricking procedure.[33]

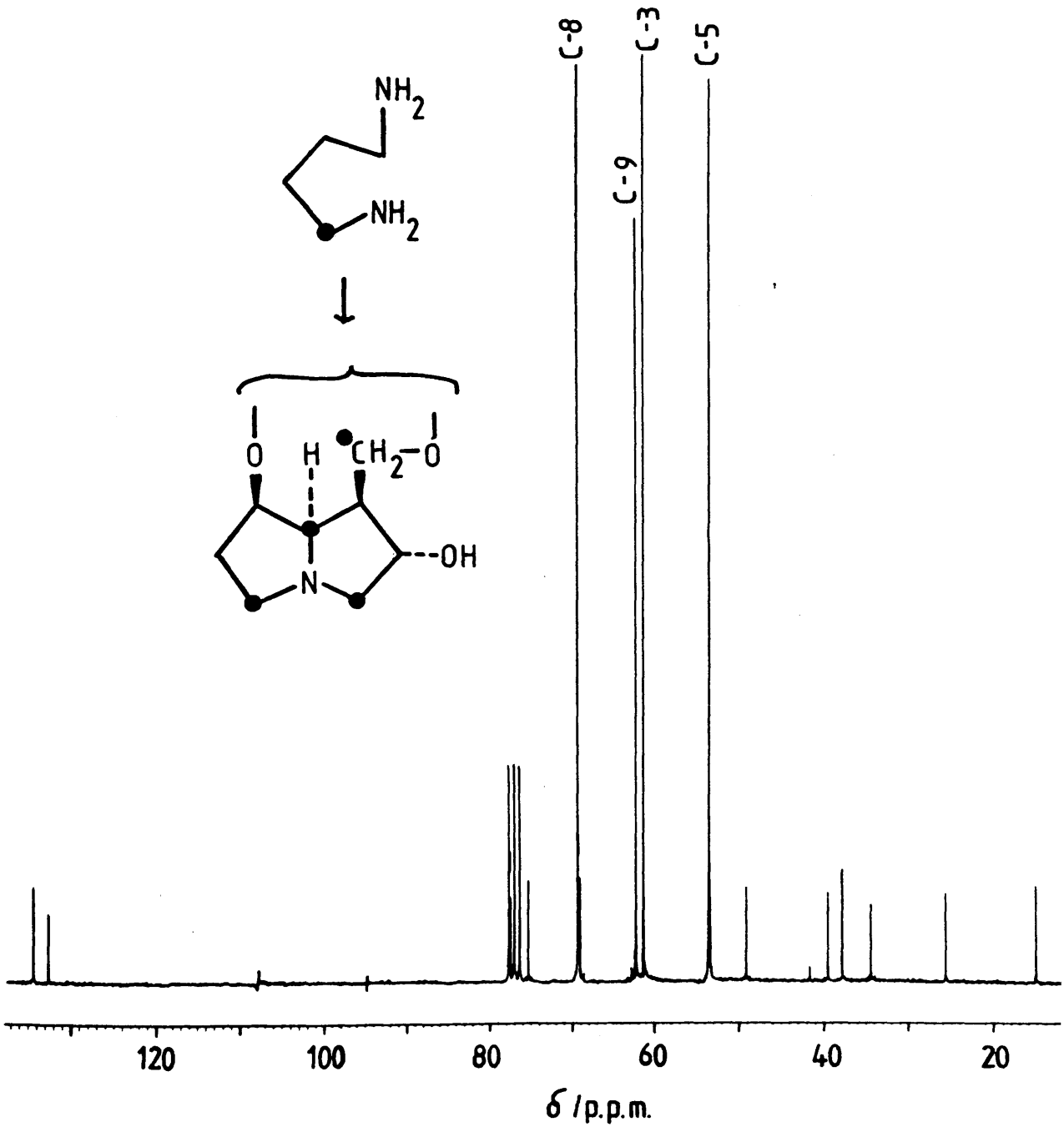
When freshly rooted cuttings were fed on each day for one week with the precursor (89), by the wick method, astonishingly high incorporations were obtained. One week later, the plants were extracted and rosmarinine was isolated and recrystallised to a constant specific

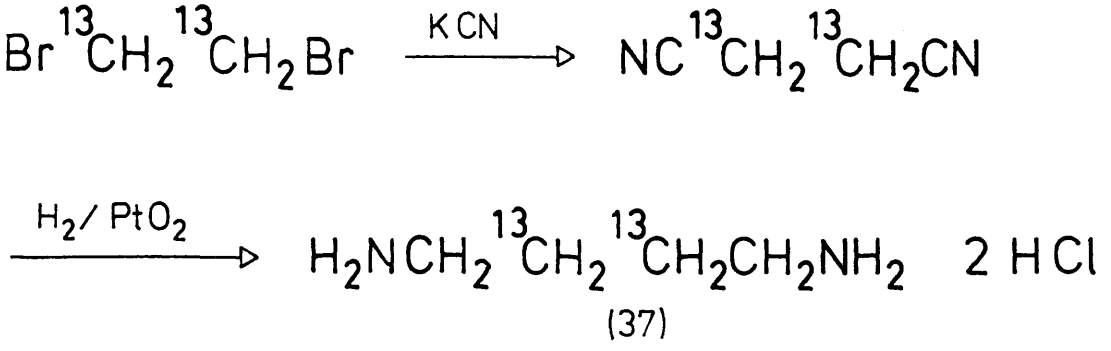
radioactivity† of 22% per C_4 unit of putrescine. The ^{13}C-{^1H} n.m.r. spectrum of the ^{13}C-labelled rosmarinine (Figure 6) displayed four enhanced peaks, when compared to the ^{13}C natural abundance spectrum. These peaks correspond to ^{13}C labels in rosmarinine at C-3, C-5, C-8 and C-9 with enrichment factors* for each position of 12.0, 12.2, 10.0 and 10.0 (all ± 1.0)% ^{13}C, respectively, making the average enrichment factor for each labelled site 11.1% ^{13}C. The estimated ^{13}C specific incorporation is therefore 11.1 x $^{2}/_{91}$ x 100 = 24.4% per C_4 unit of putrescine where $^{91}/_{2}$ atom % ^{13}C was the average enrichment at each labelled position of putrescine (89). On close inspection of the ^{13}C n.m.r. spectrum, some broadening at the base of each enhanced peak was noticeable. This can be attributed to geminal coupling (e.g. C_3 with C_5 or C_8). This requires combination of two ^{13}C-labelled putrescines (89) which is likely as seen from the high incorporation figures.

The next objective was to feed a ^{13}C-labelled putrescine with the aim of providing a complementary labelling pattern in rosmarinine (20). Thus [2,3-$^{13}C_2$]putrescine (37) dihydrochloride was prepared by the method of Khan and Robins (Scheme 7(b)),[41] with a modification to the reduction step (Scheme 30). This, together with [1,4-^{14}C]putrescine dihydrochloride was fed to one well-established Senecio pleistocephalus plant.

† * - see p.76 for explanation.

Figure 6. 50 MHz ^{13}C-{^{1}H} N.m.r. spectrum of rosmarinine (20) in $CDCl_3$ enriched with [1-^{13}C]putrescine dihydrochloride (89).



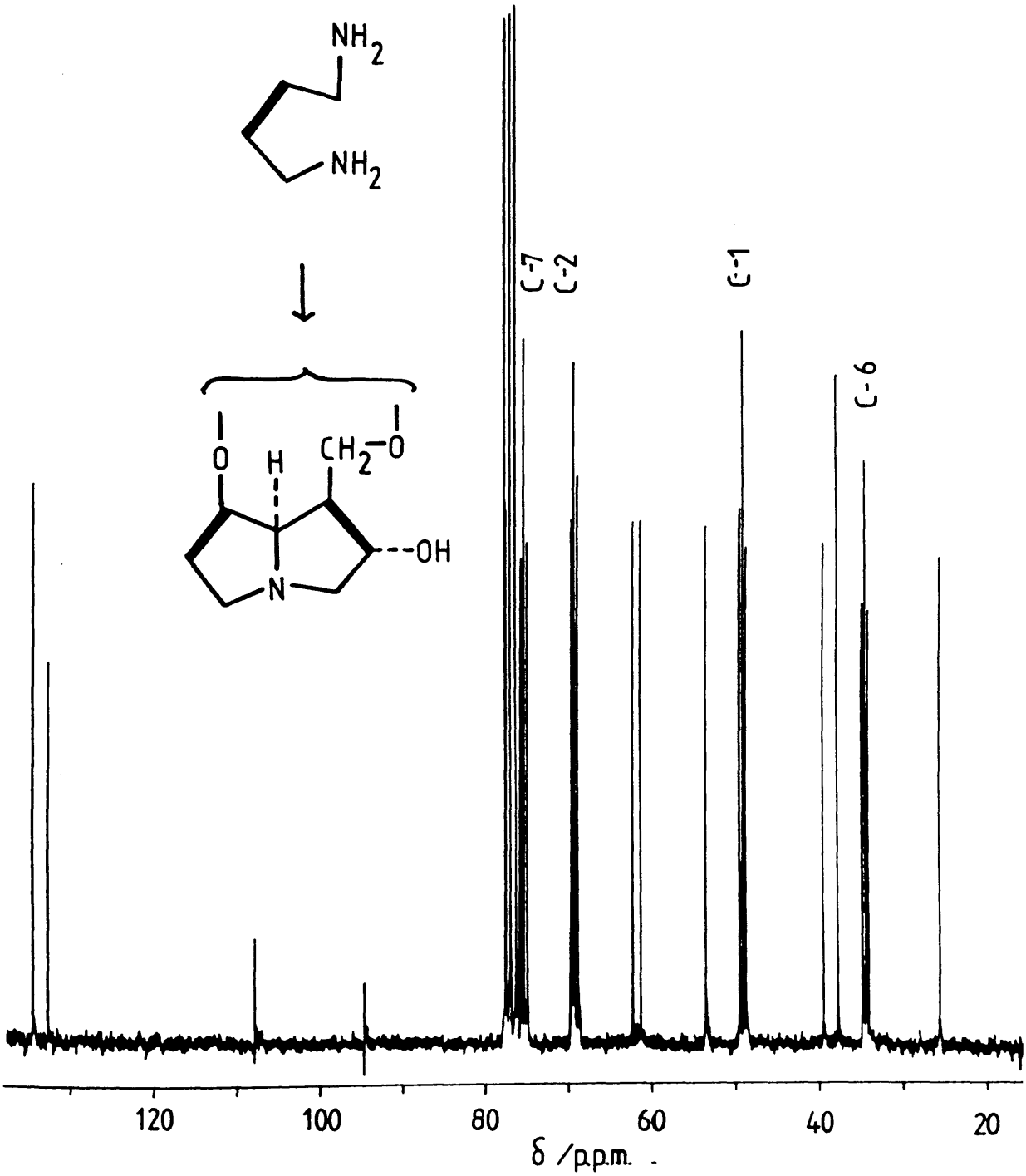


(37)

Scheme 30

With this experiment there was the bonus of being able to feed smaller amounts of precursor and lower enrichments of labelled sites were identified by observation of ^{13}C-^{13}C couplings in the ^{13}C-{^{1}H} n.m.r. spectrum of rosmarinine. A ^{14}C specific incorporation† of 2.4% was measured. This value was much lower than that obtained in the previous experiment. This may be explained by the fact that the plants used in each experiment were of different ages. The ^{13}C-{^{1}H} n.m.r. spectrum of the biosynthetically derived rosmarinine contained a pair of doublets at δ49.1 (J 35.2 Hz) and 69.1 (J 35.2 Hz) corresponding to C-1 and C-2, and another pair of doublets at δ34.4 (J 35.4 Hz) and 75.3 (J 35.5 Hz) corresponding to C-6 and C-7 of rosmarinine, respectively (Figure 7). The enrichment factors* for the labelled sites at C-1, C-2, C-6 and C-7 were 1.69, 1.93, 1.76 and 1.63 (all ± 0.1)%, respectively. This gave an average enrichment factor for each labelled site of 1.75% ^{13}C, which corresponds to an estimated ^{13}C specific incorporation of $1.75 \times {}^{1}/_{81} \times 100\% = 2.2\%$ per C_4 unit of putrescine [81 atom % ^{13}C was the average enrichment factor due to $^{13}C_2$ species at each labelled position of putrescine (37)]. The fact that all the enrichment factors are nearly equal is consistent with the formation of a symmetrical

Figure 7. 50 MHz ^{13}C-{^{1}H} N.m.r. spectrum of rosmarinine (20) in $CDCl_3$ enriched with [2,3-$^{13}C_2$]putrescine (37) dihydrochloride.



C_4-N-C_4 intermediate in pyrrolizidine alkaloid biosynthesis,[36] from two molecules of putrescine.

The existence of a later symmetrical intermediate in the biosynthetic pathway to rosmarinine was demonstrated by feeding [1-amino-^{15}N, 1-^{13}C]putrescine (38) dihydrochloride to one Senecio pleistocephalus plant. The preparation of this precursor involved treating the N-benzyloxycarbonyl derivative (90) of 1-amino-3-bromopropane with potassium [^{13}C,^{15}N]cyanide, followed by hydrogenation of the resultant nitrile and acidification (Scheme 31).[41]

$$PhCH_2OCONH(CH_2)_3Br \xrightarrow{Na^{13}C^{15}N} PhCH_2OCONH(CH_2)_3{}^{13}C^{15}N$$

(90)

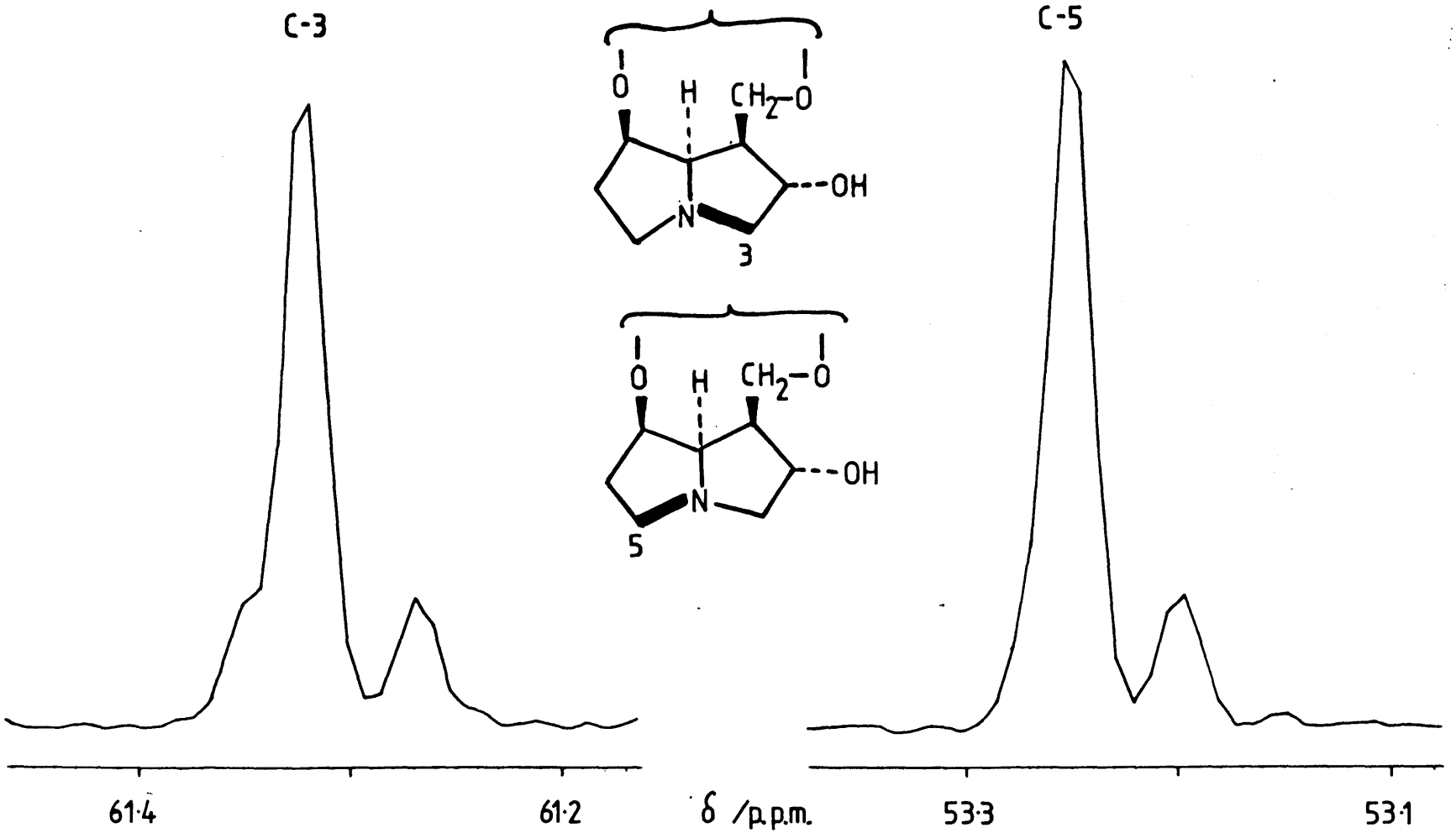
$$\xrightarrow[2.\ HCl]{1.\ H_2,\ Pd/C} H_2{}^{15}N{}^{13}CH_2(CH_2)_3NH_2 \quad 2\,HCl$$

(38)

Scheme 31

On completion of the feeding experiment rosmarinine was isolated with a ^{14}C specific incorporation† of 1.1% per C_4 unit of putrescine. Figure 8 shows part of the ^{13}C-{^{1}H} n.m.r. spectrum of rosmarinine obtained from a Senecio pleistocephalus plant administered with the doubly labelled precursor (38). The signals due to C-3 and C-9 at δ61.3 and 62.2, respectively, had enrichment factors* of 0.3 ± 0.05% ^{13}C, and the signals for C-5 and C-8 at δ53.5 and 69.3,

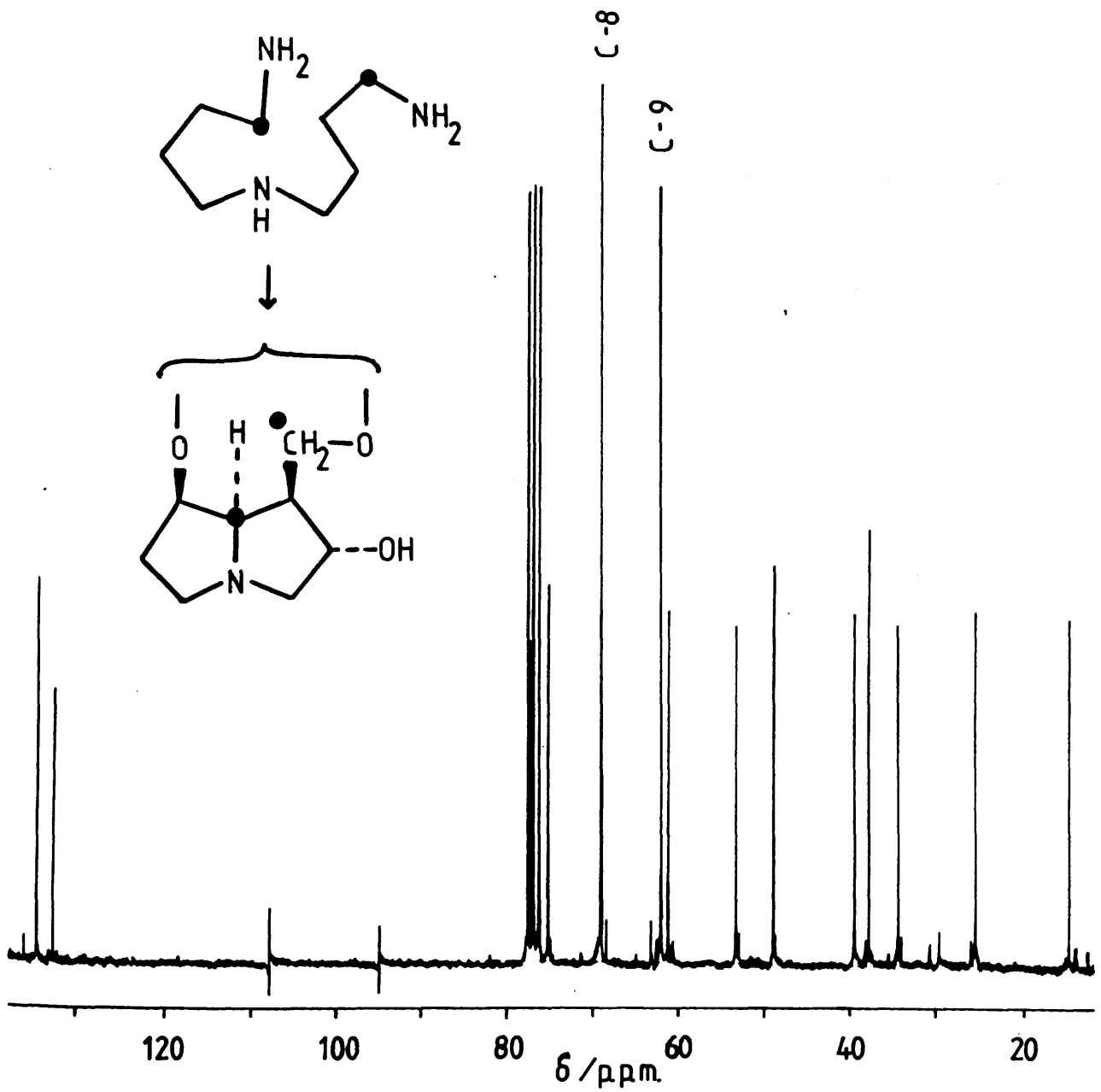
Figure 8. Part of the resolution-enhanced $^{13}C-\{^{1}H\}$ n.m.r. spectrum of rosmarinine (20) in $CDCl_3$ enriched with [1-amino-^{15}N,1-^{13}C]putrescine (38) dihydrochloride.



respectively, had values of 0.4 ± 0.05% ^{13}C. This corresponds to an average enrichment factor of 0.35% ^{13}C for each labelled site and a specific ^{13}C incorporation per C_4 unit of (0.35 x $^{2}/_{90.6}$ x 100) = 0.77%. [$^{90.6}/_{2}$ was the average enrichment due to ^{13}C at each labelled position of putrescine (38)]. More significantly however, the two carbon atoms adjacent to nitrogen, C-3 and C-5, appeared as doublets superimposed on singlets. The doublets were not fully resolved, which made calculations of coupling constants difficult. The coupling constants were estimated to be 4 Hz for C-3 and 2-3 Hz for C-5. Because the coupling constants appeared to have different values, this indicated that the coupling was not due to geminal coupling (i.e., C_3 coupling with C_5) but was a result of ^{13}C-^{15}N species being present. Moreover, the ^{13}C-^{15}N doublets at C-3 and C-5 were of equal intensity which was in keeping with the existence of a later symmetrical intermediate in the biosynthetic pathway to rosmarinine.

In retronecine biosynthesis, the symmetrical C_4-N-C_4 intermediate was shown to be homospermidine (43). Thus, to ascertain whether this was also the case in rosmarinecine (21) biosynthesis, the dihydrochlorides of [1,9-$^{13}C_2$]homospermidine (47) and [1,9-^{14}C]-homospermidine were prepared as described (Scheme 13).[43,72] The mixture of precursors was fed to one S. pleistocephalus plant and rosmarinine was isolated with a ^{14}C specific incorporation† of 1.4%. Only two signals in the ^{13}C-{^{1}H} n.m.r. spectrum of rosmarinine at δ62.2 and 69.3 showed enhancement, due to enrichment of ^{13}C at C-9 and C-8, respectively (Figure 9). Enrichment factors* of 1.58% ^{13}C for C-8 and 1.50% ^{13}C for C-9 were measured, making the ^{13}C specific

Figure 9. 50 MHz ^{13}C-{^{1}H} N.m.r. spectrum of rosmarinine (20) in $CDCl_3$ enriched with [1,9-$^{13}C_2$]homospermidine (47) trihydrochloride.



incorporation 1.54 x $^{1}/_{96}$ x 100 = 1.6%, where 96 atom % was the average enrichment at each labelled site in homospermidine (47). The geminal coupling between C-8 and C-9 was zero. [This contrasted with a 6 Hz coupling in retronecine[43]]. Nevertheless, only the signals for C-8 and C-9 of rosmarinine were enriched with ^{13}C, as evident from the $^{13}C-\{^{1}H\}$ n.m.r. spectrum, and this is reasonable evidence for the intact incorporation of homospermidine into rosmarinecine.

In summary, the above feeding experiments have served to demonstrate that rosmarinecine (21), the necine component of rosmarinine (20), in Senecio pleistocephalus is biosynthesised from two putrescine (27) units via homospermidine (43).

† Specific ^{14}C incorporation per C_4 unit for a putrescine precursor was calculated from [(molar activity of rosmarinine (20) x 1/2)/(molar activity of precursor)] x 100%.

* The enrichment factor for each labelled site in rosmarinine (20) is the excess of ^{13}C above natural abundance and was calculated [(integral of labelled site - natural abundance integral or integral of doublet signals)/(natural abundance integral)] x 1.1%.

3.4 Incorporation of ^{2}H-Labelled Putrescines into Rosmarinine

An insight into the stereochemistry of the enzymic processes involved in rosmarinecine (21) biosynthesis was provided using ^{2}H-labelled putrescines in conjunction with ^{2}H n.m.r. spectroscopy. The objective was to investigate the fate of all the hydrogen atoms on the four carbon atoms of putrescine.

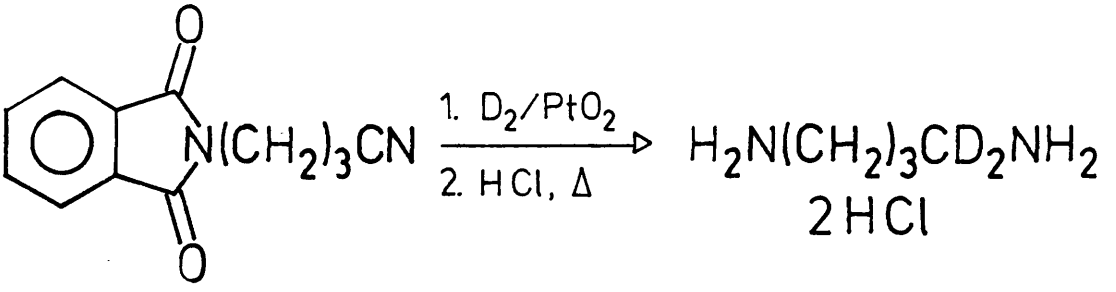
Initially, attention was focussed on the hydrogen atoms on the two end carbon atoms of putrescine. This required the synthesis of [1,1,4,4-$^2H_4$]putrescine (91) dihydrochloride, which was prepared by D.B. Hagan by catalytic reduction of succinonitrile under an atmosphere of deuterium gas, followed by acidification of the product with hydrochloric acid (Scheme 32).[49] This precursor has also been prepared by Callery et al., using a similar procedure.[73]

$$NC(CH_2)_2CN \xrightarrow[2.HCl]{1.\ D_2/PtO_2} H_2NCD_2(CH_2)_2CD_2NH_2 \cdot 2\,HCl$$

(91)

Scheme 32

[1,1-$^2H_2$]Putrescine (92) dihydrochloride was also prepared for this study by D.B. Hagan. Catalytic reduction of 4-phthalimidobutyronitrile (93) under an atmosphere of deuterium gas followed by acid hydrolysis, afforded the ^{2}H-labelled putrescine (92) (Scheme 33).[74] The synthesis of this material has also been reported by Callery et al.[73]



(93) (92)

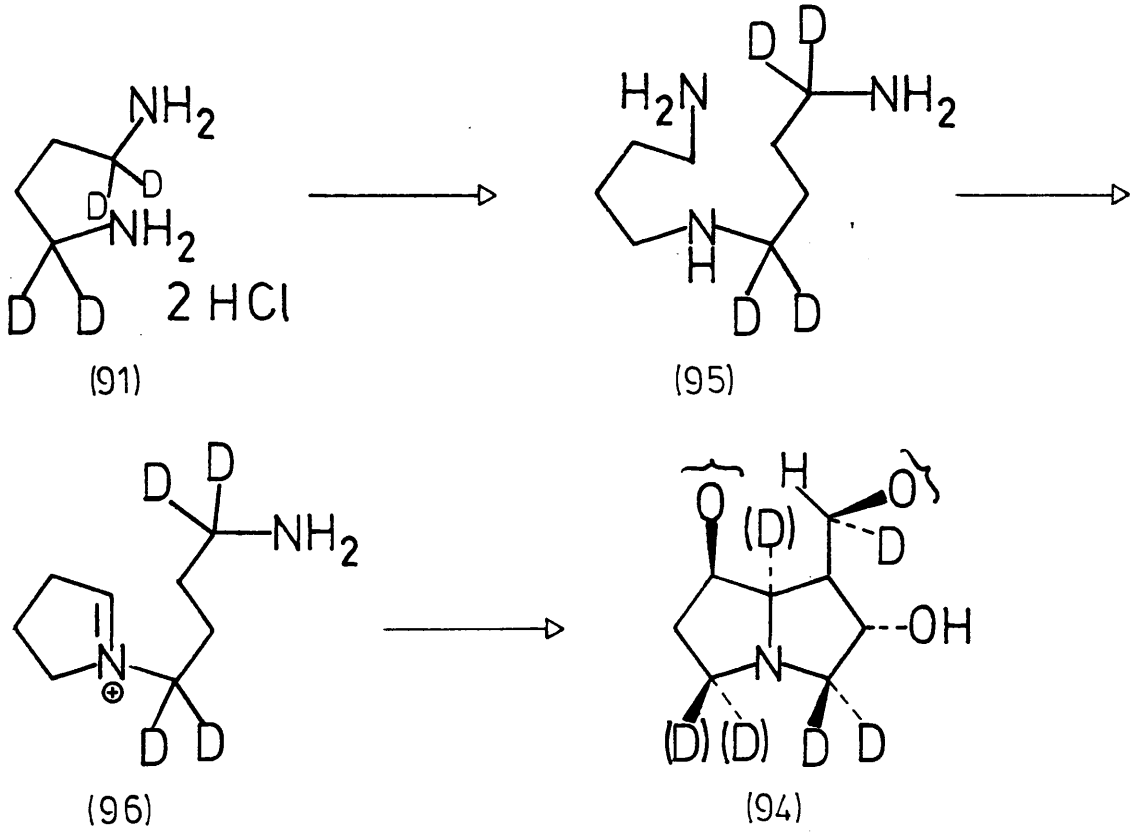
Scheme 33

Furthermore, enantiomeric (R)-[1-^{2}H]-(52) and (S)-[1-^{2}H]putrescine (53) dihydrochloride were made by enzymatic decarboxylation of L-ornithine in ^2H_2O and of DL-[2-$^2H_1$]ornithine in ^1H_2O, respectively, using L-ornithine decarboxylase (Schemes 15 + 16).[50] The ^{2}H content of each putrescine sample was estimated by analysing the ^{1}H and ^{2}H n.m.r. and mass spectral data of the dihydrochlorides.

Wick feeding of each precursor with [1,4-^{14}C]putrescine dihydrochloride (10 μCi) was carried out on well established Senecio pleistocephalus plants. One plant was used per experiment, and each precursor was administered over a period of ten days. Ten days after completion of feeding, the plants were macerated, and rosmarinine was isolated and recrystallised to constant specific radioactivity. Table 5 shows the ^{14}C specific incorporation per C_4 unit for each precursor.

The ^{2}H-{^{1}H} n.m.r. spectra of the ^{2}H-labelled samples of rosmarinine in chloroform at room temperature were very broad. However, increasing the temperature to 60°C resulted in a narrowing of the signals and so the spectra were always taken at this elevated temperature.[25]

A sample of rosmarinine derived from [1,1,4,4-$^{2}H_{4}$]putrescine (91) dihydrochloride displayed three main peaks in the ^{2}H-{^{1}H} n.m.r. spectrum at δ2.93, 3.08 and 4.88 (Figure 10(a)) corresponding to rosmarinine (94) with ^{2}H-labels at C-3β, C-3α and C-9pro-S, respectively (Scheme 34).



Scheme 34

The three sites were labelled to about the same extent with enrichment factors$^{\Theta}$ of 3.5 ± 0.3% ^{2}H. It was also possible that signals due to ^{2}H at C-5β, C-5α and C-8β were present at δ2.62, 3.28 and 3.58, respectively, with much lower enrichment factors. The specific incorporation of ^{2}H for the C_{4} unit in which most of the ^{2}H appears (C-1,-2,-3 and -9 of rosmarinine) is 3.5 x $^{1}/_{96}$ x 100 = 3.6% where 96 atom % ^{2}H was

the average content of $^2H_4$ species in the sample of the precursor (91). These observations can be explained in terms of ^2H isotope effects during the biosynthetic pathway to rosmarinine. Homospermidine (43), a key intermediate in the biosynthetic pathway to rosmarinine (20) is believed to be formed by the coupling of putrescine with 4-aminobutanal, followed by reduction of the corresponding imine. Therefore, on feeding [1,1,4,4-$^2H_4$]putrescine (91) dihydrochloride it is expected that [1,1,4,4-$^2H_4$]homospermidine is formed by the coupling of unlabelled 4-aminobutanal from the plant with precursor (91), since it is known that oxidation of [1,1,4,4-$^2H_4$]putrescine (91) to [1,4,4-$^2H_3$]-4-aminobutanal by hog kidney diamine oxidase is subject to an intermolecular isotope effect of 1.26.[73] This intermediate (95) is probably then involved in an intramolecular isotope effect during its conversion into the immonium ion (96), because it has also been shown that hog kidney diamine oxidase has a four times greater preference for oxidation of the unlabelled end of [1,1-$^2H_2$]putrescine (92).[73] Thus, the non-deuteriated end of homospermidine is preferentially oxidised to an aldehyde, which ultimately leads to rosmarinine (94) containing most of the ^2H at C-3 and C-9.

Additional information regarding the existence of ^2H isotope effects in the biosynthetic pathway to rosmarinecine (21) was gained after feeding [1,1-$^2H_2$]putrescine (92) dihydrochloride to S. pleistocephalus. The ^2H-{^1H} n.m.r. spectrum of the resultant rosmarinine (97) (Figure 10(b)) showed five signals at δ 2.55 (5β-H), 2.90 (3β-H), 3.02

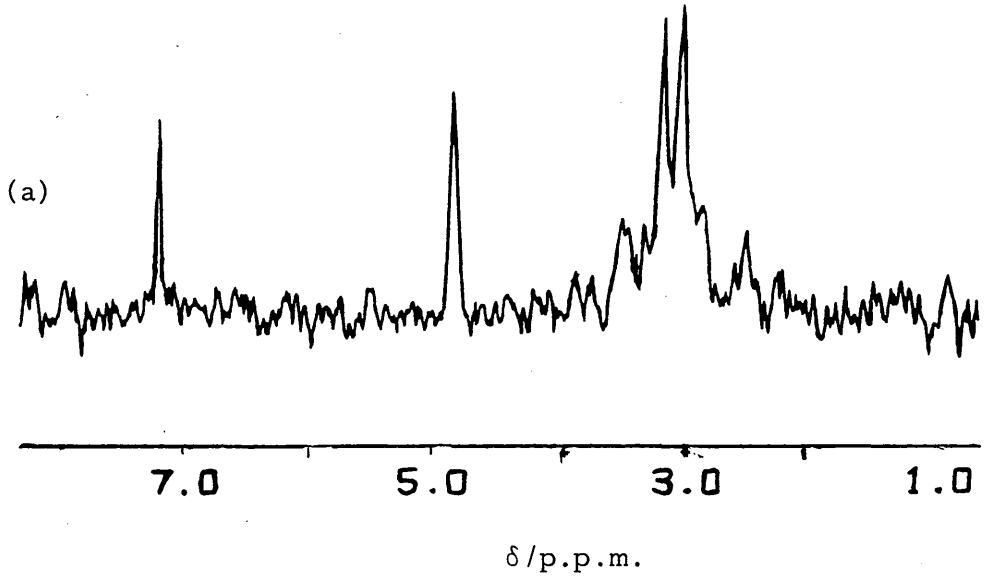
Θ see p.93 for explanation.

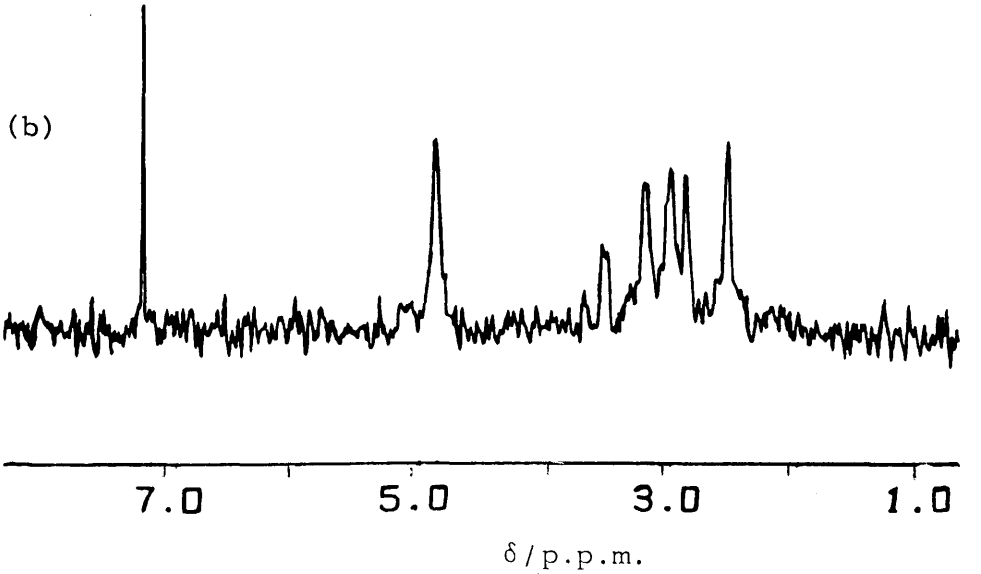
Figure 10. 55.28 MHz $^{2}H-\{^{1}H\}$ N.m.r. spectra of rosmarinine in $CHCl_3$ at 60°C:

(a) sample of rosmarinine (94) derived from [1,1,4,4-$^{2}H_4$]putrescine (91);

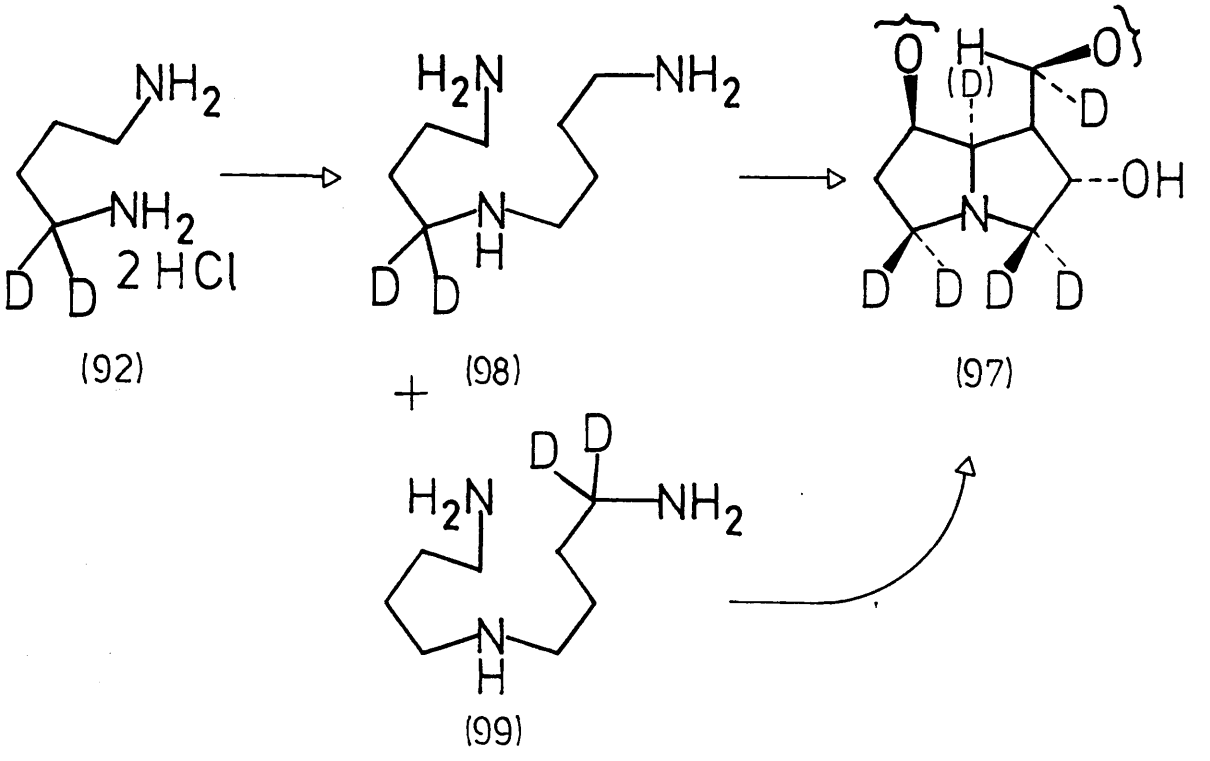
(b) sample of rosmarinine (97) derived from [1,1-$^{2}H_2$]-putrescine (92);

The signal at δ 7.25 is natural abundance ^{2}H in $CHCl_3$.





(3α-H), 3.23 (5α-H) and 4.88 (9-H*pro*-*S*). All the signals had equal enrichment factors[θ] of *ca.* 2.6 ± 0.3 % ^2H. A smaller signal for 8-H at δ3.56 was also observed. The ^2H specific incorporation per C_4 unit is therefore 2.6 x $^2/_{95}$ x 100 = 5.5%, where $^{95}/_2$ atom % ^2H was the average enrichment of $^2H_2$ species at the 1- and 4- positions of putrescine (92). The observed labelling pattern in rosmarinine can be explained as follows. Feeding [1,1-$^2H_2$]putrescine (92) dihydrochloride can produce two differently labelled $^2H_2$-homospermidine species (98) and (99) (Scheme 35). The former (98), arises by the coupling of precursor (92) with 4-aminobutanal and the latter (99) by combination of [4,4-$^2H_2$]-4-aminobutanal [ex. (92)] with unlabelled putrescine. Taking homospermidine (98) along the proposed biosynthetic pathway leads to rosmarinine equally labelled with ^2H at C-3 and C-5, while homospermidine (99) affords rosmarinine labelled with more ^2H at C-9 than C-8, since oxidation probably occurs preferentially at the non-deuteriated primary amino group. It is also conceivable that [1,1,6,6-$^2H_4$]- and [1,1,9,9-$^2H_4$]homospermidines are formed in the biosynthetic pathway. The involvement of [1,1,6,6-$^2H_4$]homospermidine would lead to rosmarinine with most of the ^2H at C-5, whereas [1,1,9,9-$^2H_4$]homospermidine would be a poor substrate for the enzyme system to act on. In short, intramolecular and intermolecular ^2H isotope effects can account for the labelling patterns in rosmarinine samples (94) and (97).



Scheme 35

The enantiomeric precursors, (R)-[1-^{2}H]-(52) and (S)-[1-^{2}H]-putrescine (53) dihydrochloride, when fed to Senecio pleistocephalus shed light on the stereochemical course of some of the enzymic reactions of rosmarinecine biosynthesis. The ^{2}H-{^{1}H} n.m.r. spectrum of rosmarinine derived from the (R)-isomer showed four signals at δ 2.90 (C-3β), 3.24 (C-5α), 3.55 (C-8α) and 4.87 (C-9pro-S) (Figure 11(a)); all with nearly equal enrichment factors$^{\Theta}$ of 4.4 ± 0.4% ^{2}H. The specific incorporation of ^{2}H per C_4 unit is therefore 4.4 x $^{2}/_{97}$ x 100 = 9.1%, where $^{97}/_{2}$ atom % was the average content of ^{2}H at the terminal carbons of the putrescine precursor (52). This value agreed well with the ^{14}C specific incorporation, indicating that no ^{2}H was lost from the precursor on its conversion into rosmarinine (101) (Scheme 36). With (S)-[1-^{2}H]-putrescine as precursor the ^{2}H-{^{1}H} n.m.r. spectrum of rosmarinine (103)

showed two signals at δ2.58 and 3.05, which corresponds to ^{2}H labels in rosmarinine at C-5β and C-3α, respectively (Figure 11(b)). The enrichment factors[θ] were calculated as 1.2 ± 0.1% ^{2}H for C-5β and 1.0 ± 0.1% ^{2}H for C-3β making the specific incorporation of ^{2}H per C_4 unit $^{1}/_{2}(1.2 + 1.0) \times {}^{2}/_{90} \times 100 = 2.4\%$, where $^{90}/_{2}$ atom % was the average enrichment of ^{2}H at the terminal carbons of labelled putrescine (53) (Scheme 37).

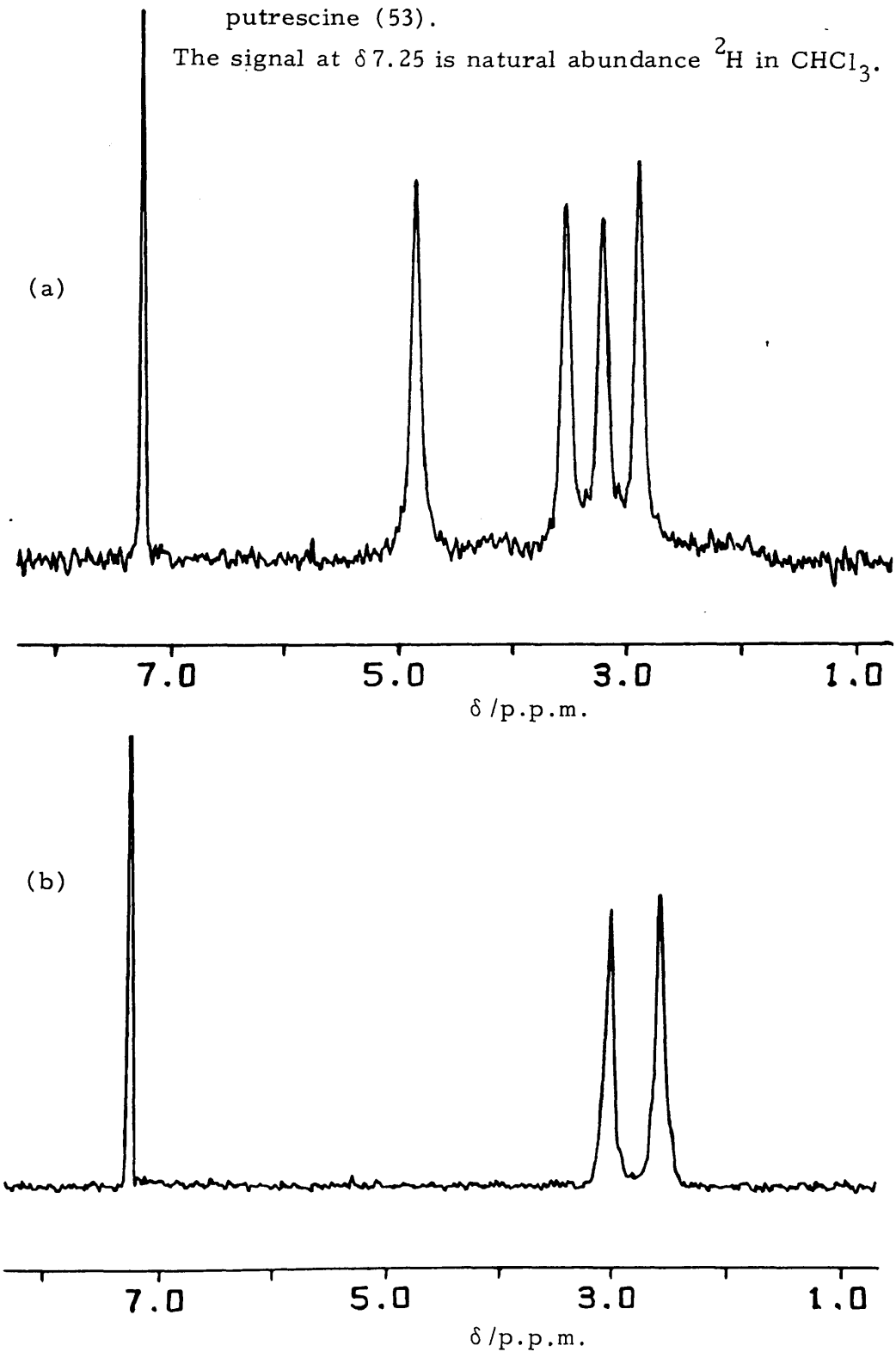
The observed labelling patterns in rosmarinine after feeding samples (52) and (53) can be explained thus. The oxidation of putrescine (27) to 4-aminobutanal takes place stereospecifically with the retention of the pro-R and loss of the pro-S hydrogens. (It has been reported that diamine oxidases selectively remove the pro-S hydrogen from the methylene group adjacent to the amino group of other primary amines.[52]) Coupling of 4-aminobutanal with putrescine affords the corresponding imine, reduction of which gives homospermidine, labelled as shown [(100) + (102)]. Since the (R)-isomer gave rosmarinine labelled as in (101), it may be deduced that the reduction of the imine occurs by a hydride equivalent being delivered on the C-si face of the imine. Support for this theory comes from the complementary labelling pattern at C-3 and C-5 in rosmarinine (103) after feeding the (S)-isomer. Two further oxidations on homospermidine result in the loss of the pro-S hydrogens at C-1 and C-9 giving the presumed immonium ion intermediate (49b). Cyclisation of the immonium ion occurs by attack on the C-re face of the double bond, generating 8α-pyrrolizidine aldehyde (50). Finally, reduction of the aldehyde to isoretronecanol

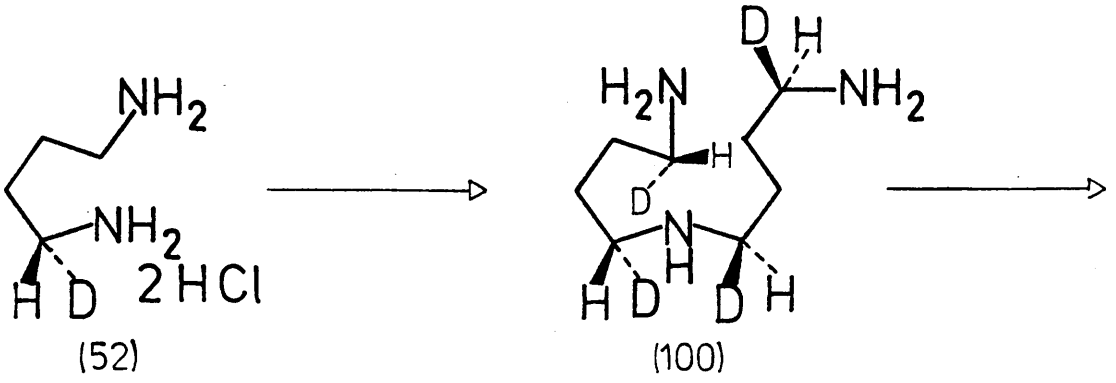
Figure 11. 55.28 MHz $^{2}H-\{^{1}H\}$ N.m.r. spectrum of rosmarinine in $CHCl_3$ at 60°C:

(a) Sample of rosmarinine (101) derived from (R)-[1-^{2}H]-putrescine (52);

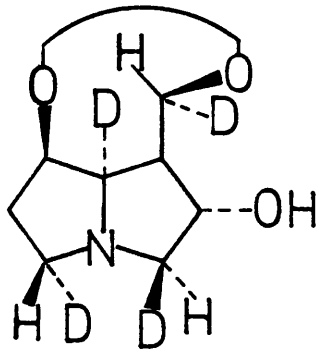
(b) Sample of rosmarinine (103) derived from (S)-[1-^{2}H]-putrescine (53).

The signal at δ 7.25 is natural abundance ^{2}H in $CHCl_3$.



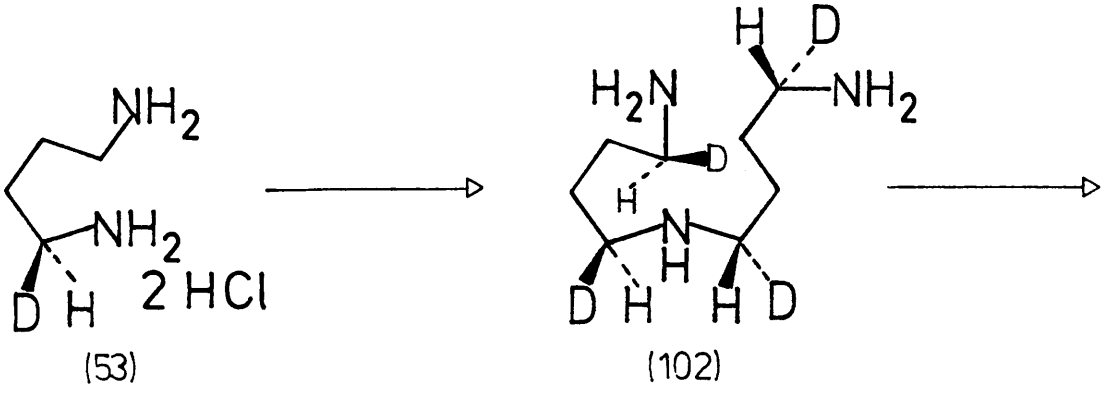


(52) (100)

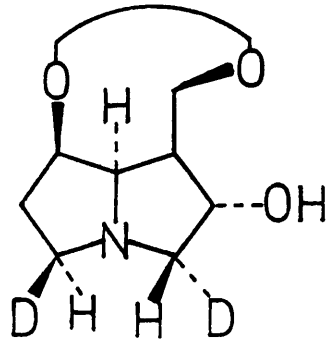


(101)

Scheme 36†



(53) (102)



(103)

Scheme 37†

(51b) occurs by attack of the hydride equivalent on the C-re face of the carbonyl group. The labelling patterns for rosmarinine samples (101) and (103) were similar to those observed for retrorsine after feeding the enantiomeric putrescines (52) and (53), and a similar explanation to that above was used to account for the distribution of labels (Chapter 2.1).

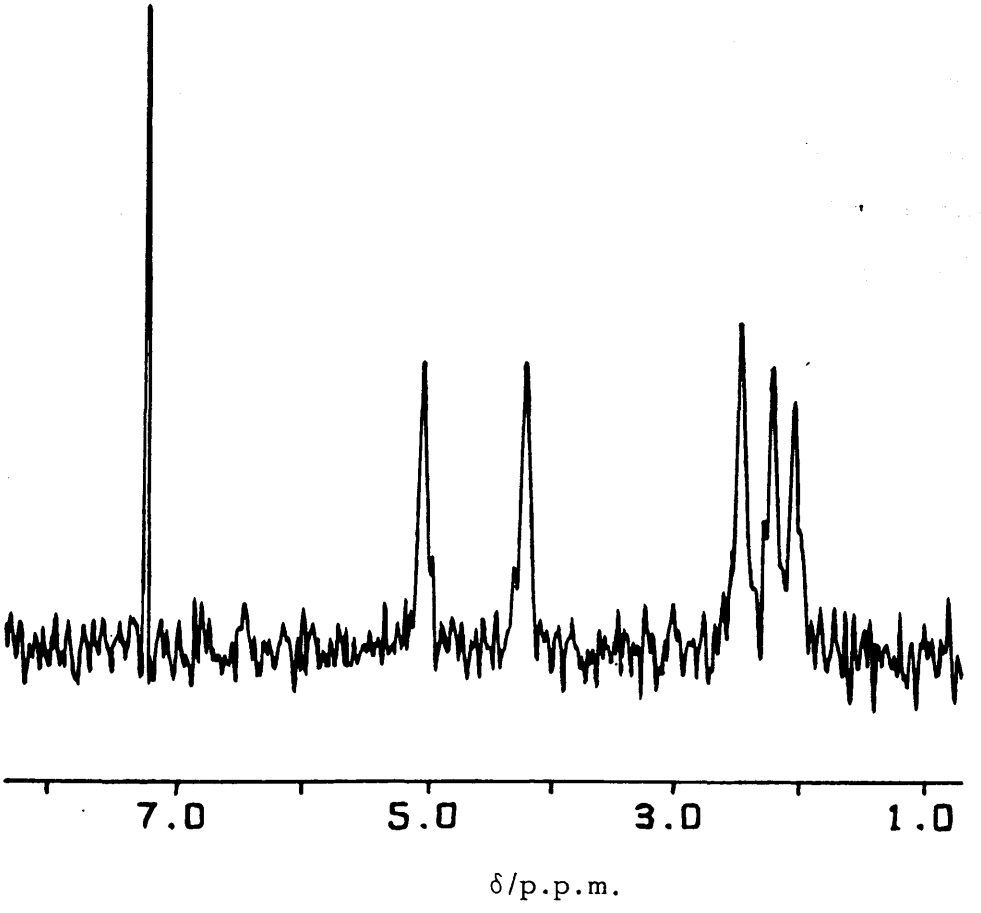
In order to continue the investigation of what happens to the hydrogen atoms of putrescine during the biosynthesis of rosmarinine, [2,2,3,3-$^2H_4$]putrescine (104) dihydrochloride was synthesised and fed to S. pleistocephalus. The synthesis of this precursor involved exchanging the acidic hydrogens of succinonitrile for deuterium by heating succinonitrile in deuterium oxide, followed by catalytic reduction of the [2,2,3,3-$^2H_4$]succinonitrile and acidification of the product with hydrochloric acid (Scheme 38).[49]

$$NC(CH_2)_2CN \xrightarrow[2.\ H_2/PtO_2]{1.\ D_2O,\ \Delta} H_2NCH_2CD_2CD_2CH_2NH_2 \cdot 2\ HCl$$
$$3.\ HCl$$
(104)

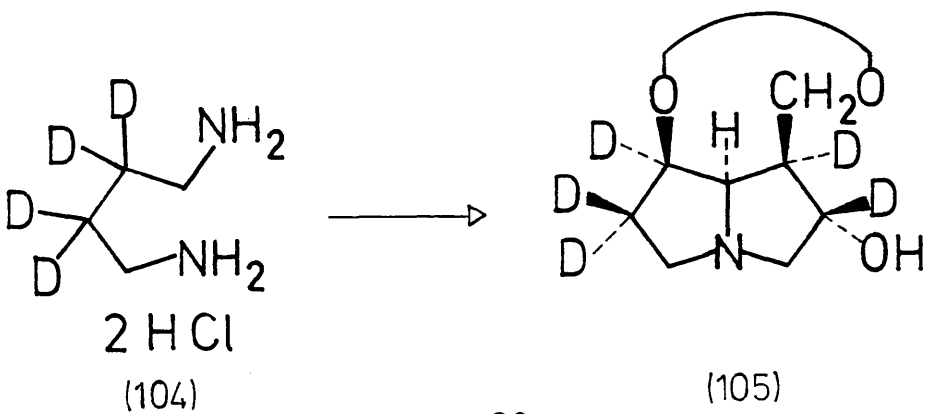
Scheme 38

Examination of the ^2H-{^1H} n.m.r. spectrum of rosmarinine derived from (104) indicated five ^2H signals at δ 2.02 (6α-H), 2.2 (6β-H), 2.46 (1α-H), 4.20 (2β-H), and 5.00 (7α-H) (Figure 12). The five sites were labelled to about the same extent, with enrichment factors[Θ]

Figure 12. 55.28 MHz $^{2}H-\{^{1}H\}$ N.m.r. spectrum of rosmarinine (105) in $CHCl_3$ at 60°C derived from [2,2,3,3-$^{2}H_4$]putrescine (104). The signal at δ 7.25 is natural abundance ^{2}H in $CHCl_3$.



of 7.0 ± 0.5% ^2H, making the specific incorporation of ^2H per C_4 7.0 x $^1/_{99}$ x 100 = 7.1%. [The precursor (104) contained 99% $^2H_4$ species]. From consideration of the ^2H-labelling pattern in rosmarinine, it is clear that the conversion of two molecules of labelled putrescine (104) into rosmarinine (105) occurs by loss of three ^2H atoms. Secondly, it is apparent that the introduction of the hydroxyl groups at C-2 and C-7 does not involve a keto or enol intermediate. Furthermore, the presence of ^2H at C-1α means that it is unlikely that the biosynthesis proceeds <u>via</u> the formation of the 1α-aldehyde (50a), followed by epimerisation at the 1-position. (The alternative formation of the 1β-aldehyde (50b) followed by epimerisation leading to retronecine in <u>S. isatideus</u> cannot be ruled out because of the formation of the 1,2-double bond).



Scheme 39

The stereochemistry of the enzymic processes involving protons at C-2 and C-3 of putrescine in the biosynthetic pathway to rosmarinine was explored by feeding (2<u>R</u>)-[2-^2H]-(65) and (2<u>S</u>)-[2-^2H]putrescine (66) dihydrochloride. These precursors were prepared by Dr. E.K. Kunec.[54] The ^2H-{^1H} n.m.r. spectrum of rosmarinine derived from

the (R)-isomer (65) showed two main signals at δ2.05 and 4.20 (Figure 13(a)) corresponding to rosmarinine (106) labelled with ^2H at C-6α and C-2β, respectively (Scheme 40). The enrichment factors[θ] for both signals were 2.5 ± 0.2% ^2H, which results in a ^2H specific incorporation per C_4 unit of 2.5 x $^{2}/_{98}$ x 100 = 5.1% [$^{98}/_{2}$ atom % ^2H was the average enrichment of ^2H at each labelled site of (65)].

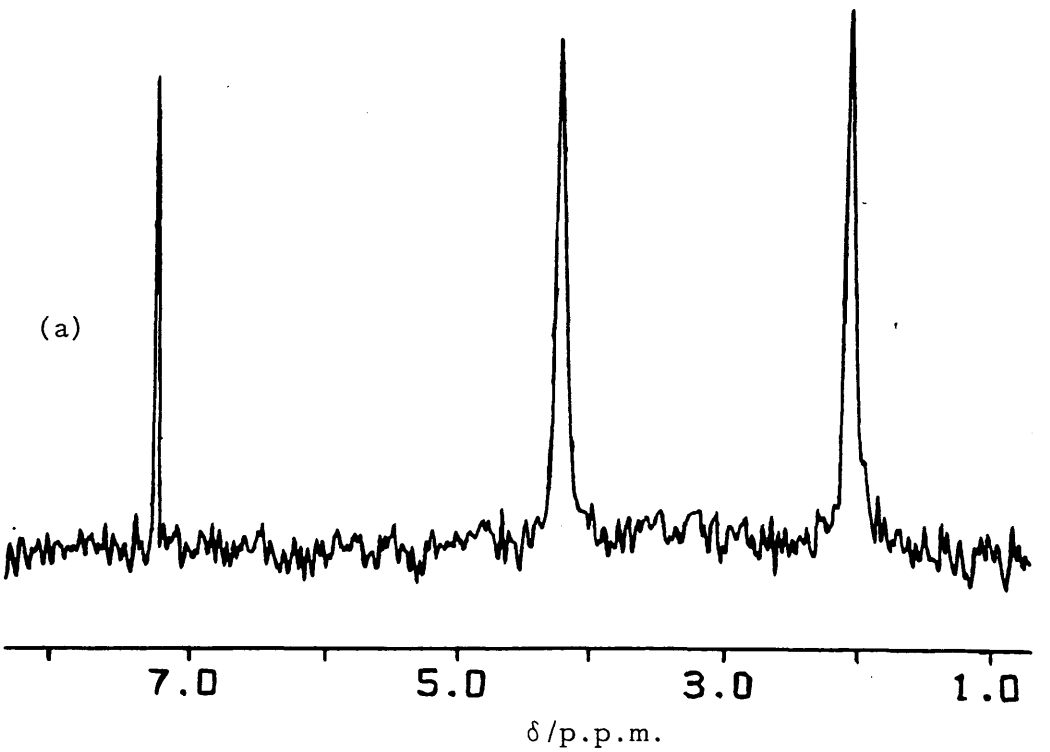
When (2S)-[2-^2H]putrescine (66) was fed to S. pleistocephalus, the ^2H-{^1H} n.m.r. spectrum of the extracted rosmarinine contained three peaks at δ2.23, 2.45 and 5.06 (Figure 13(b)). This revealed that rosmarinine was labelled with ^2H at C-6β, C-1α, and C-7α (Scheme 41). Because all the enhanced signals had an enrichment factor of ca. 2.9 ± 0.3% ^2H, this gives a ^2H specific incorporation of 2.9 x $^{2}/_{83}$ x 100 = 6.5% per C_4 unit, where $^{83}/_{2}$ atom % ^2H was the average content of ^2H species at the central carbon atoms of precursor (66). Unfortunately, the (2S)-putrescine (66) contained ca. 6% impurity of [1,1-$^2H_2$]putrescine (92) dihydrochloride,[54] which resulted in some additional small peaks in the ^2H-{^1H} n.m.r. spectrum.

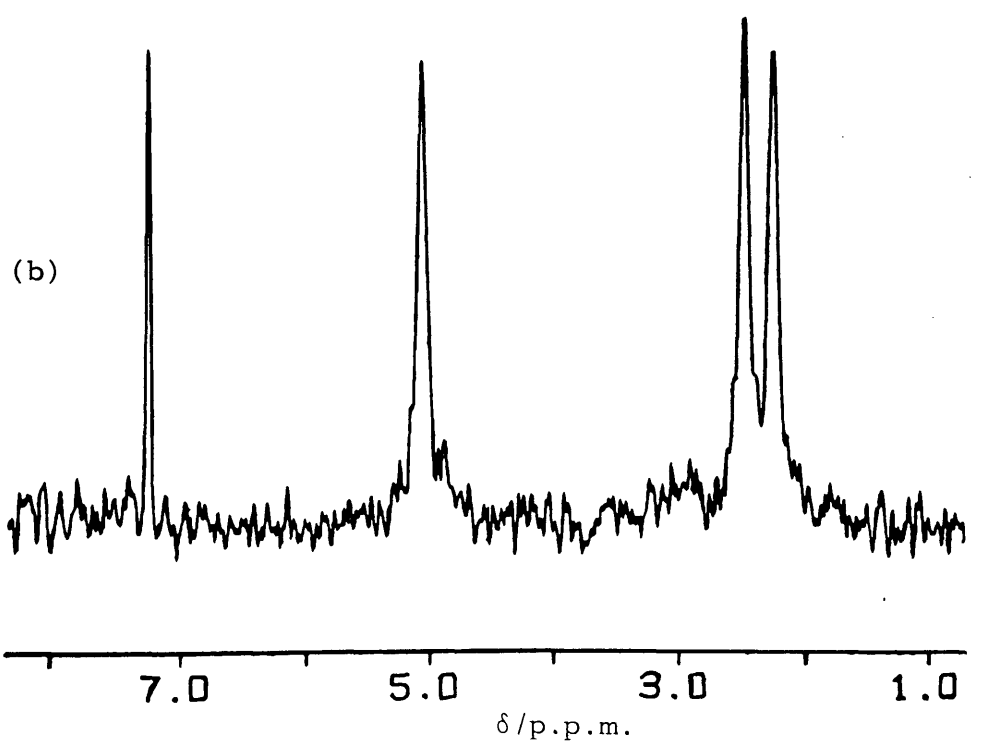
Figure 13. 55.28 MHz ^{2}H-$\{^{1}H\}$ N.m.r. spectra of rosmarinine in $CHCl_3$ at 60°C:

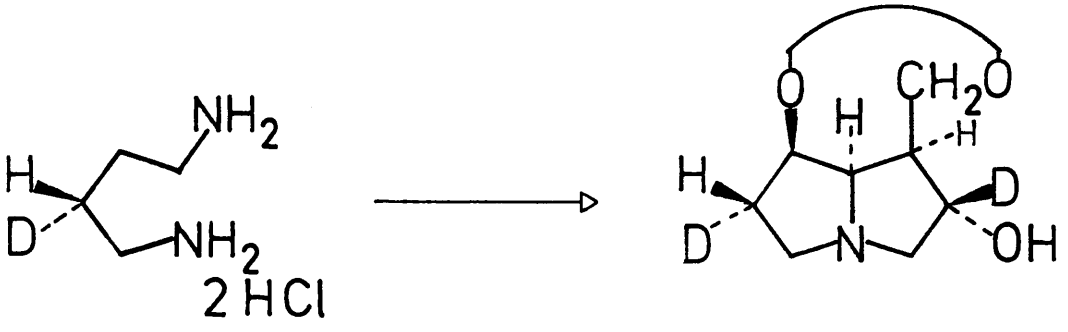
(a) Sample of rosmarinine (106) derived from (2R)-[2-^{2}H]-putrescine (65);

(b) Sample of rosmarinine (107) derived from (2S)-[2-^{2}H]-putrescine (66).

The signal at δ 7.25 is natural abundance ^{2}H in $CHCl_3$.

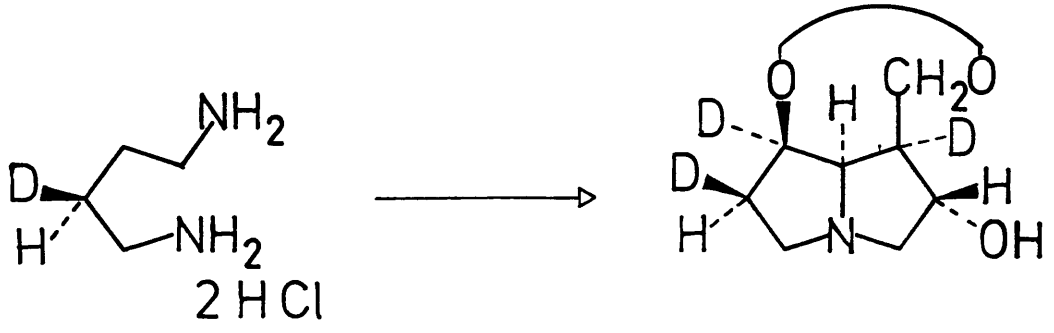






(65) (106)

Scheme 40†



(66) (107)

Scheme 41†

Two interesting stereochemical details emerged from these experiments. Formation of the pyrrolizidine ring system involves stereospecific removal of the pro-R hydrogen and retention of the pro-S hydrogen on the carbon which becomes C-1 of rosmarinecine (21). Such an observation was not possible when studying retronecine biosynthesis because of the presence of the 1,2-double bond. Additionally, the hydroxylations at C-2 and C-7 both proceed with retention of configuration which is consistent with the observed stereospecificity for hydroxylations at sp^3 carbons.[75]

1,2-Unsaturation is a common structural feature in pyrrolizidine alkaloids. The 1,2-double bond in retronecine (3) may result

from hydroxylation at C-2α of trachelanthamidine (51a), followed by a trans-elimination of the elements of water. On the other hand, the formation of a 1,2-double bond in rosmarinecine (21) would necessitate a cis-elimination of the elements of water. Furthermore, the formation of rosmarinecine by dehydrogenation and hydration of the double bond is unlikely because of the observed ^{2}H-labelling patterns.

The stereochemistry of the enzymic reactions in the biosynthesis of rosmarinecine in Senecio pleistocephalus has been elucidated by employing enantiomeric [1-^{2}H]- and [2-^{2}H]putrescines in conjunction with ^{2}H n.m.r. spectroscopy. This approach of investigating biosynthetic pathways could be extended to the study of other plants which produce pyrrolizidine alkaloids with different necine components.

θ The enrichment factor for a labelled site in rosmarinine (20) was calculated from (integral of unlabelled site in rosmarinine/concentration of rosmarinine)/(natural abundance integral of ^{2}H in $CHCl_3$ at δ7.25/concentration of $CHCl_3$) x 0.0156%.

† No molecules of putrescine precursors (52), (53), (65), and (66) can contain more than one ^{2}H atom. The labelling patterns for rosmarinine depicted in Schemes 36, 37, 40 and 41 are therefore composite representations of all the ^{2}H-labelled species that were present.

Table 5. Incorporation of ^{2}H-Labelled Putrescines into Rosmarinine in Senecio pleistocephalus plants

Precursor	Quantity fed (mg)	Amount of rosmarinine (20) isolated (mg)	% ^{14}C Specific incorporation in rosmarinine (20)[a]	% ^{2}H Specific incorporation in rosmarinine (20) per C_4 unit
90	100	400	5.1	3.6
92	100	238	6.3	5.5
52	100	157	9.2	9.1
53	50	234	3.7	2.5
104	100	194	9.1	7.1
65	100	130	4.9	5.1
66	100	282	6.6	6.5

[a] Specific ^{14}C incorporation per C_4 unit is calculated from [(Molar activity of product x 0.5)/ (Molar activity of precursor)] x 100%

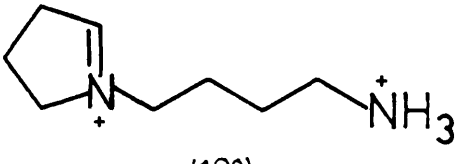
Chapter 4

IDENTIFICATION OF INTERMEDIATES IN ROSMARININE BIOSYNTHESIS

4.1 Introduction

As discussed in Chapter 2, the immonium ion (108) has been proposed as a key intermediate in the biosynthesis of retronecine (3) and rosmarinecine (21). Indeed, the interpretation of many feeding experiment results on pyrrolizidine alkaloid biosynthesis has relied upon the intermediacy of such a species. One such experiment was the intact incorporation of homospermidine (43) into retronecine (3).[43] Thus, it was considered necessary to provide experimental evidence supporting the involvement of the immonium ion in the biosynthetic pathway to pyrrolizidine alkaloids.

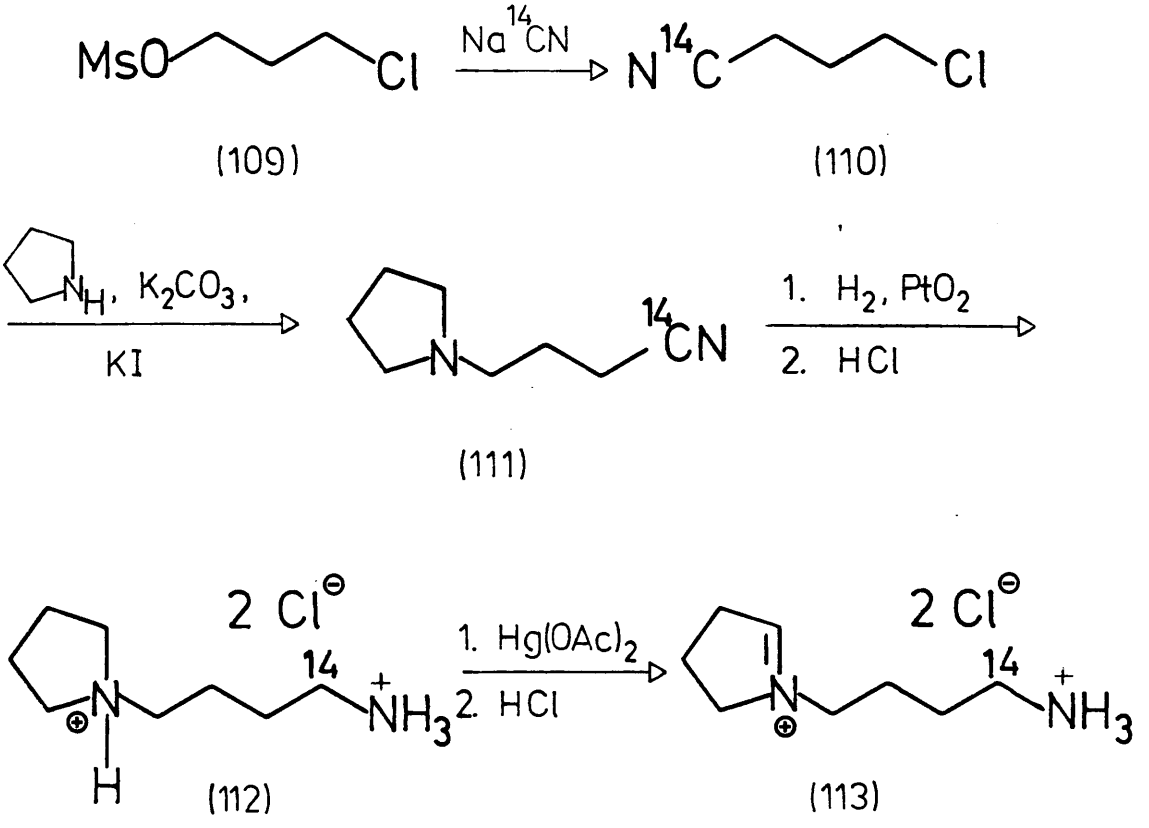
The biosynthetic pathways to retronecine (3) and rosmarinecine (21) diverge at the 1-hydroxymethylpyrrolizidine stage and no intermediates have been identified between homospermidine and these bases. Information about the intermediacy of immonium ions in these pathways should help to narrow down the point at which these two pathways diverge. The experiments concerning the participation of immonium ions in rosmarinine biosynthesis are described first.



(108)

4.2 Preparation and Feeding of N-([4-^{14}C]-4-aminobutyl)-1,2-didehydropyrrolidinium chloride hydrochloride (113) to S. pleistocephalus

A synthesis of the title compound was carried out as shown in Scheme 42.

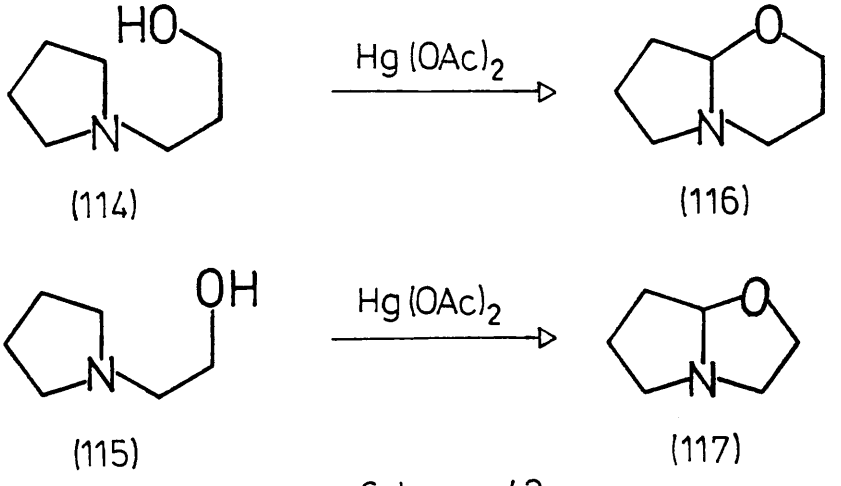


Scheme 42

3-Chloro-1-propanol methanesulphonate (109) was treated with sodium [^{14}C]cyanide to yield the chloronitrile (110). Displacement of the chloride was carried out to produce the pyrrolidinenitrile (111). Catalytic hydrogenation of the nitrile (111), followed by mercury(II) acetate oxidation of the resultant dihydrochloride (112), afforded (113).

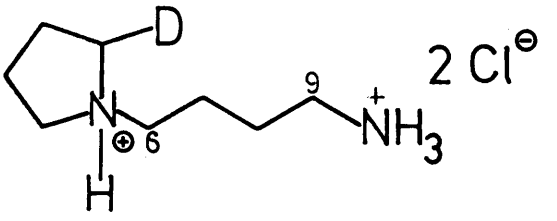
The i.r. spectrum of the ^{14}C-labelled immonium ion (113) contained an absorption at 1688 cm^{-1} due to $>C=\overset{+}{N}<$. The ^{1}H n.m.r. spectrum in deuterium oxide exhibited a characteristic broad singlet at δ8.25 associated with the olefinic proton at C-2.

In most cases reported,[76] the mercuric acetate oxidation of cyclic tertiary amines to immonium salts, resulted in the formation of an endocyclic double bond. For instance, Leonard and Musker[77] reacted N-(3-hydroxypropyl)pyrrolidine (114) and N-(2-hydroxyethyl)-pyrrolidine (115) with mercury(II) acetate to yield, respectively, the tetrahydro-1,3-oxazines (116) and (117) via immonium ion intermediates (Scheme 43).



Scheme 43

To check that the immonium salt (113) had an endocyclic rather than an exocyclic double bond, a sample was reduced with sodium cyanoborodeuteride to give the corresponding deuteriated saturated salt (118).



(118)

A 200 MHz ^{1}H n.m.r. spectrum of the unlabelled salt (112) run in deuterium oxide contained a four proton multiplet at δ2.95 corresponding to the four equivalent protons of the pyrrolidine ring, on the carbons adjacent to the nitrogen. [The methylene protons on C-9 and C-6 each appeared as a two proton multiplet at δ3.05 and 3.55, respectively]. On the other hand, the ^{2}H-labelled salt (118), showed only a three proton multiplet at δ2.95, which was a good indication that the original immonium ion had an endocyclic double bond.

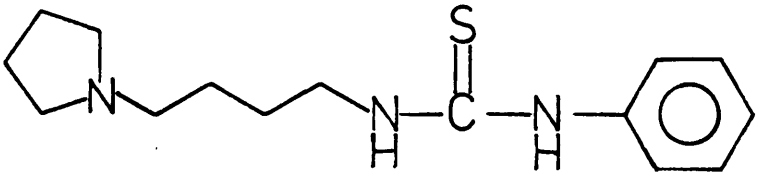
The ^{14}C-labelled immonium ion (113) was mixed with [2,3-^{3}H]-putrescine dihydrochloride to give an initial $^{3}H/^{14}C$ ratio of 5.1. The mixture of isotopically labelled species were fed to one Senecio pleistocephalus plant by the wick method over five days. After a further ten days, rosmarinine (20) was isolated and recrystallised to constant specific radioactivity. The ^{14}C specific incorporation‡ for rosmarinine (20) was ca. 6.5% with a $^{3}H/^{14}C$ ratio of 2.9. Furthermore, a sample of this radiolabelled rosmarinine was hydrolysed to give rosmarinecine containing 94% of the specific radioactivity ($^{3}H/^{14}C$ ratio of 2.8) and the acid portion with 3% of the specific radioactivity. Thus, it appears from these experiments that the immonium ion (113) is incorporated specifically and efficiently into rosmarinecine. The

‡ see p.102 for explanation.

$^{3}H/^{14}C$ ratios also suggest that the immonium ion is more efficiently incorporated into rosmarinine than putrescine, supporting its later position in the biosynthetic pathway.

Since the saturated salt, N-([4-^{14}C]-4-aminobutyl)pyrrolidinium dihydrochloride (112) was available, this was mixed with [2,3-^{3}H]-putrescine dihydrochloride to give a $^{3}H/^{14}C$ ratio of 1.5 and this mixture was fed to Senecio pleistocephalus. The isolated rosmarinine (20) had a ^{14}C specific incorporation‡ of 2.1% with a $^{3}H/^{14}C$ ratio of 2.5. This was somewhat unexpected because the precursor (112) was not postulated as an intermediate in the biosynthetic pathway to rosmarinine.

To investigate this point further, intermediate trapping experiments were carried out for the immonium ion (113), and the saturated compound (112) in S. pleistocephalus. [1,4-^{14}C]Putrescine dihydrochloride was fed to one S. pleistocephalus plant and after one day the plant was blended in methanol. The methanolic extract was divided into two. Inactive immonium salt was added to one of the methanolic portions. The mixture was then treated with sodium borohydride and the saturated products were converted into the N-phenylamino(thiocarbonyl) derivatives, employing the procedure of Golding and Nassereddin.[78]



(119)

PhNHCSNH$(CH_2)_4$NHCSNHPh

(120)

A radioscan of a chromatogram of the mixture indicated that most of the radioactivity (75%) was due to the derivative of putrescine (120). The remainder of the activity was attributed to the N-phenylamino(thiocarbonyl) derivative of the saturated salt (119), ca. 10%, and baseline material, ca. 10%. After separation from other species present in the mixture using preparative thin layer chromatography, the saturated salt derivative was shown to contain ca. 0.4% of the original radioactivity fed as [1,4-^{14}C]putrescine dihydrochloride. This indicates that the immonium ion is formed from putrescine in the Senecio pleistocephalus plant, and that it is a normal intermediate in rosmarinine biosynthesis.

Inactive N-(4-aminobutyl)pyrrolidinium dihydrochloride (112) was added to the second methanolic portion and the phenylisothiocyanate derivatives were formed as before. Autoradiography of a chromatogram of the mixture showed that most of the radioactivity was associated with the derivative of putrescine (ca. 90%) and baseline material (ca. 8%). The band on the chromatogram which corresponded to the N-phenylamino(thiocarbonyl) derivative of the saturated dihydrochloride (119) was extracted and crystallised. The derivative showed a total incorporation corresponding to $< 0.017\%$ ^{14}C. Thus, it can be concluded that N-(4-aminobutyl)pyrrolidinium dihydrochloride (112) is not a true intermediate in the biosynthetic pathway to rosmarinine in S. pleistocephalus. Additionally, since the saturated salt was incorporated into rosmarinine it may be assumed that the plant contains enzymes that can oxidise the saturated salt (112) to the corresponding immonium ion, which does lie on the pathway.

Attention was next directed towards the involvement of the immonium ion (108) in retronecine biosynthesis. Accordingly, some ^{14}C-labelled immonium ion (113) was mixed with [2,3-^{3}H]putrescine dihydrochloride to give a $^{3}H/^{14}C$ ratio of 12.3 and fed to one Senecio isatideus plant by the xylem pricking technique. One week after feeding was complete, retrorsine (12) was isolated and recrystallised to constant specific radioactivity. A ^{14}C specific incorporation‡ for retrorsine of 4.5%, with a $^{3}H/^{14}C$ ratio of 9.8 was measured. Alkaline hydrolysis of a sample of the retrorsine (12) gave retronecine (3) containing 95% of the specific radioactivity with a $^{3}H/^{14}C$ ratio of 9.9. Therefore, it is apparent that the immonium ion (113) is an efficient and specific precursor for retronecine biosynthesis. Furthermore, since the immonium ion is an efficient precursor for both rosmarinecine (21) and retronecine (3) biosynthesis, this narrows down the point at which the two pathways diverge. This might occur during the Mannich-type cyclisation of the immonium ion on an enzyme surface, or by epimerisation of the 1β-formyl-8α-pyrrolizidine (50a) leading to retronecine. (The 1α-aldehyde is not likely to epimerise to the 1β-isomer because of the retention of deuterium at this position in rosmarinecine biosynthesis, Chapter 4.3).

The saturated salt, N-([4-^{14}C]-4-aminobutyl)pyrrolidinium dihydrochloride (112), along with [2,3-^{3}H]putrescine dihydrochloride was also fed to S. isatideus ($^{3}H/^{14}C$ ratio of 22.3). A ^{14}C specific incorporation‡ for the isolated retrorsine of 5.4% with a $^{3}H/^{14}C$ ratio of 20.4 was obtained, showing that the saturated salt was incorporated efficiently into retrorsine.

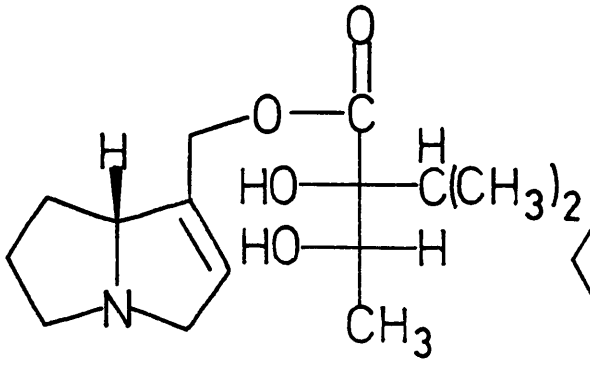
In order to provide evidence for the intermediacy of the immonium ion (108) in the biosynthesis of other pyrrolizidine alkaloids, the ^{14}C-labelled precursor (113) was fed, using the xylem pricking technique to two other plant species. The plants chosen were Cynoglossum australe [which contains cynaustine (121) and cynaustraline (122)] and Cynoglossum officinale [which contains echinatine (123)]. As before, the ^{14}C-labelled precursor (113) was mixed with [2,3-^{3}H]-putrescine dihydrochloride for each experiment to give an initial $^{3}H/^{14}C$ ratio. Table 6 shows the incorporation data for the ^{14}C-immonium ion into the alkaloids of the two plant species.‡ In each experiment, the ^{14}C-labelled immonium ion (113) was almost as efficient as putrescine in the biosynthesis of the respective alkaloids.

The ^{14}C feeding experiments discussed in this section, have provided good evidence for the participation of the immonium ion (108) in the biosynthetic pathways to a range of pyrrolizidine alkaloids.

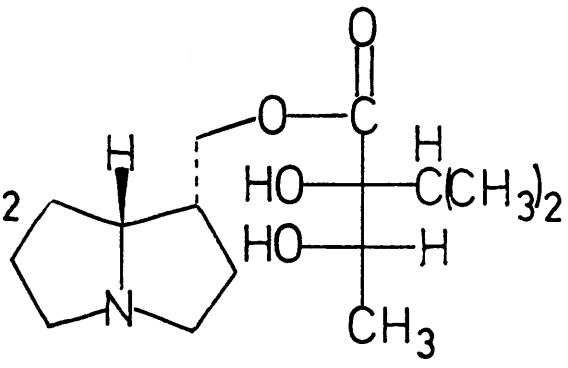
4.3 Pyrrolizidine Diols and Rosmarinecine as Intermediates in Rosmarinine Biosynthesis

(±)-Isoretronecanol (51b) had previously been shown to be an intermediate in the biosynthesis of rosmarinecine (21) (Chapter 2.1). We wished to establish the order of hydroxylation processes between (±)-isoretronecanol and rosmarinecine and to see if rosmarinecine is incorporated efficiently into rosmarinine (20). Therefore, rosmarinecine

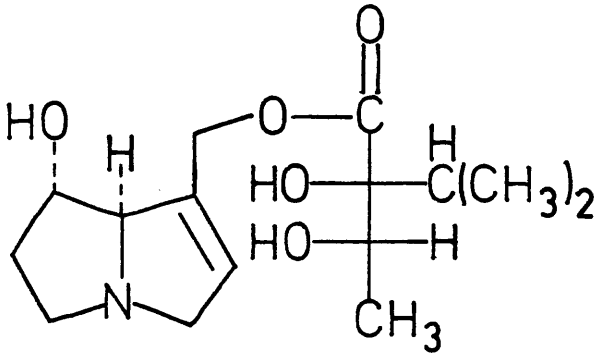
‡ Specific ^{14}C incorporation is calculated from {(molar activity of rosmarinine)/(molar activity of [^{14}C]immonium ion)} x 100%.



(121)



(122)



(123)

Table 6

Plant	Alkaloid	Length of Expt/d	Initial $^{3}H/_{14}C$	$^{3}H/_{14}C$ of alkaloid	% Total Incorpn	% Specific Incorpn
C. australe	Cynaustine	15	2.0	3.0	0.7	0.03
	Cynaustraline		2.0	2.9	1.0	0.05
C. officinale	Echinatine	15	2.4	3.9	1.3	0.8

(21) and platynecine (124) were prepared and fed to Senecio pleistocephalus. Both precursors were derived from ^{3}H-labelled rosmarinine, which was obtained by feeding one well-established plant with [2,3-^{3}H]putrescine dihydrochloride. Alkaline hydrolysis of a sample of the ^{3}H-rosmarinine furnished ^{3}H-rosmarinecine. Platynecine was prepared by mesylation of ^{3}H-rosmarinine, followed by reduction of the mesylate with sodium cyanoborohydride,[79] and hydrolysis of the product (Scheme 44).

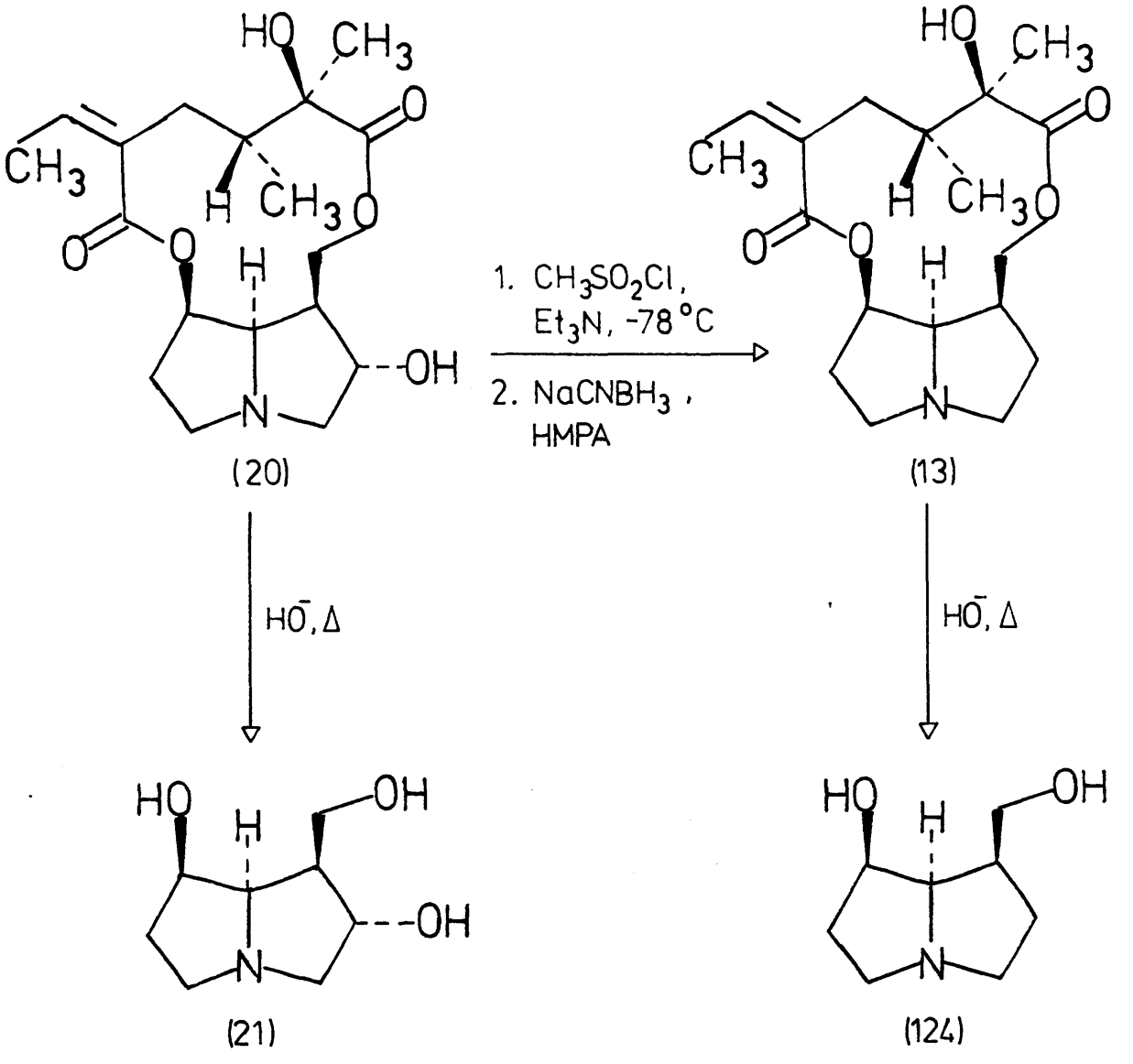
The samples of ^{3}H-rosmarinecine and ^{3}H-platynecine were both mixed with [1,4-^{14}C]putrescine dihydrochloride to give initial $^{3}H/^{14}C$ ratios of 16.1 and 12.3, respectively, and each mixture was fed to one Senecio pleistocephalus plant by the wick method.

The ^{3}H specific incorporation‡ for rosmarinine (20) was 4.0% with a $^{3}H/^{14}C$ ratio of 9.3 after feeding ^{3}H-rosmarinecine. The ^{3}H specific incorporation‡ for rosmarinine was 7.3% with a $^{3}H/^{14}C$ ratio of 10.2 after administering ^{3}H-platynecine. These results indicate that both rosmarinecine (21) and platynecine (124) are incorporated into rosmarinine with almost the same efficiency as putrescine (27).

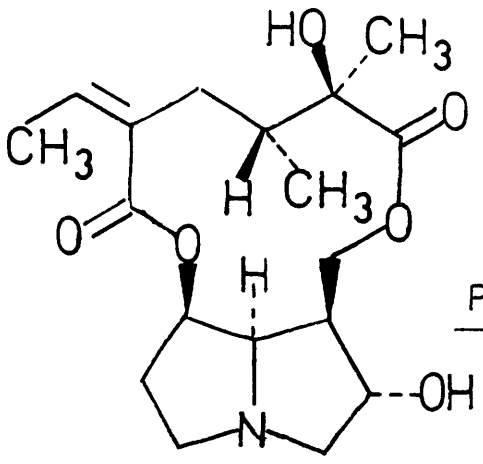
In addition to the two feeding experiments above, it had also been intended to feed the structural isomer (125) of platynecine. Unfortunately, a successful synthesis of the diol (125) could not be achieved. As shown in Scheme 45, initial efforts in the synthesis of the diol (125) concentrated on using rosmarinine (20) as starting material.

For the first step in the sequence, it was critical to choose a protecting group that was going to be stable towards basic conditions. Consequently, the acetal was selected. Three protecting group reactions

‡ see p.111 for explanation.

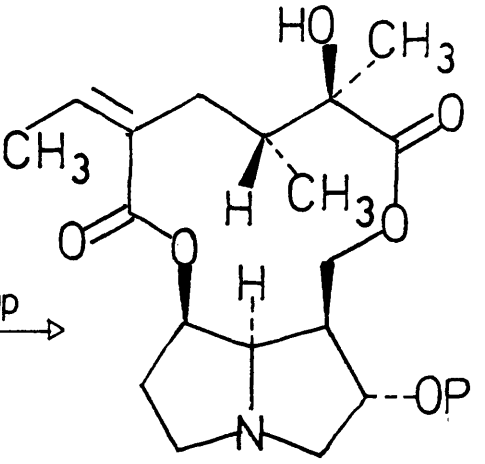

HO
CH3
O
CH3
H
CH3
O
O
O
H
OH
N
(20)
1. CH3SO2Cl,
Et3N, -78 °C
2. NaCNBH3,
HMPA
HO
CH3
O
CH3
H
CH3
O
O
O
H
N
(13)
HO⁻, Δ
HO⁻, Δ
HO
H
OH
OH
N
(21)
HO
H
OH
N
(124)

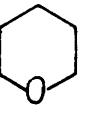
Scheme 44



(20)

Protecting Group

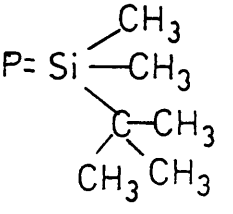


(126 a) P = 

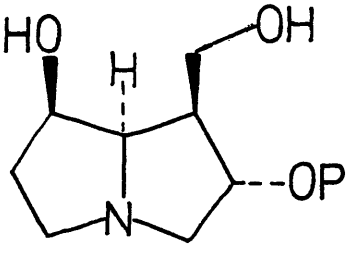
(126 b) P = $CH_2OCH_2CH_2OCH_3$

(126 c) P = CH_2OCH_3

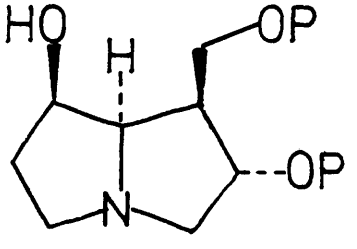
(126 d) P = CH_2Ph

(126 e) 

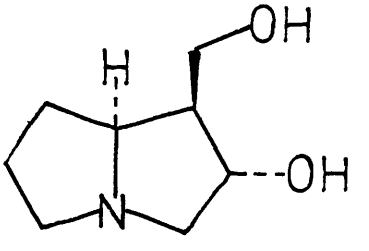
HO^-, Δ



Protecting Group



1. CH_3SO_2Cl, Et_3N -78°C
2. $NaCNBH_3$
3. Deprotection



(125)

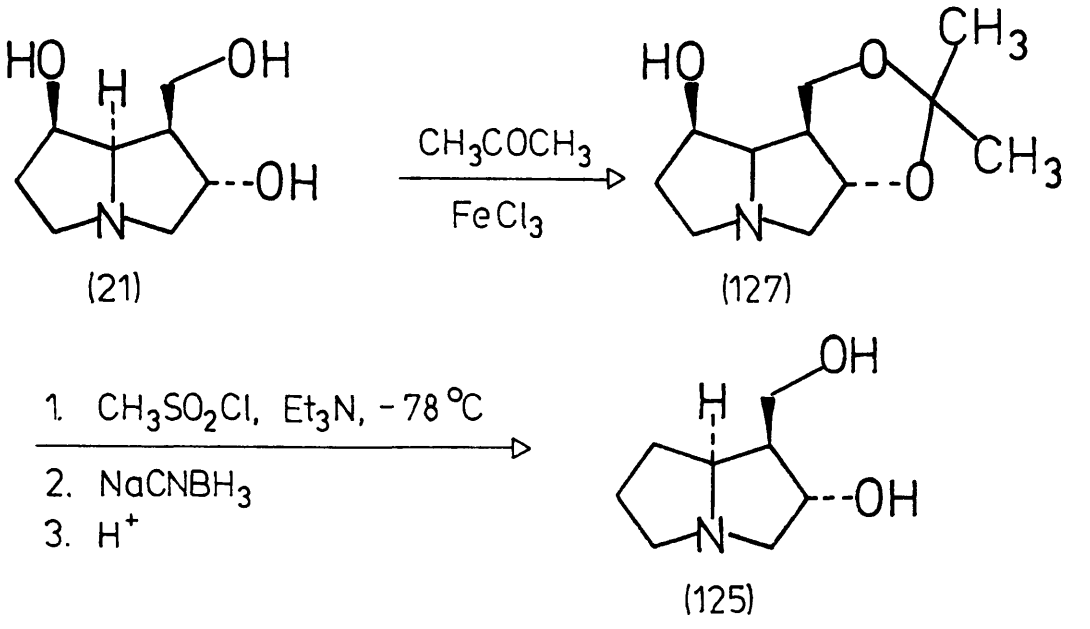
Scheme 45

involving the formation of an acetal at C-2 of rosmarinine were investigated. The formation of the tetrahydropyranyl (THP) ether of rosmarinine (126a) could not be accomplished under a variety of conditions. When rosmarinine was treated with methoxyethoxymethyl (MEM) chloride in order to produce the protected rosmarinine (126b), a mixture of products was obtained which was not easily separated. Finally, the preparation of the methoxymethyl (MOM) ether (126c) was attempted, but this also proved unsuccessful. It was therefore decided to consider an alternative type of protecting group. Accordingly, rosmarinine was treated with benzyl bromide and sodium hydride in the anticipation that 2-O-(benzyl)rosmarinine would be formed (126d). This reaction failed to give any of the desired product. Success was achieved when rosmarinine was stirred with tert.-butyldimethylsilyl chloride and imidazole in dry dimethyl formamide (DMF). This resulted in the formation of 2-O-tert.-(butyldimethylsilyl)rosmarinine (126e). The ^{1}H n.m.r. spectrum of (126e) in deuteriochloroform displayed two singlets at δ0.1 and 0.5 due to the two methyls attached to the silicon and a nine proton singlet at δ0.85 associated with the tertiary butyl group also attached to the silicon. Furthermore, high resolution mass spectrometry of the derivative established the molecular formula as $C_{23}H_{41}NO_6Si$. The success was short-lived because during the ensuing hydrolysis reaction, not only was the diester hydrolysed, but the tert.-butyldimethylsilyl protecting group was removed, resulting in rosmarinecine (21) as the product. At this point an alternative synthetic strategy, starting from rosmarinecine, was adopted (Scheme 46).

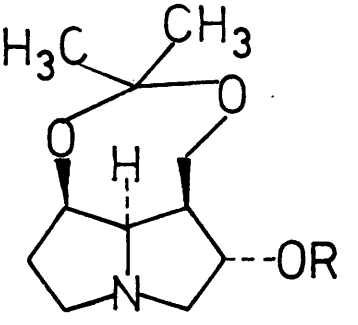
This procedure relied upon the expected preferential formation of a six-membered acetonide of rosmarinecine over a seven-membered system. Employing the method of Singh et al.,[80] rosmarinecine was dissolved in dry acetone and stirred with ferric chloride to give one product with molecular formula $C_{11}H_{19}NO_3$. A ^1H n.m.r. spectrum of the product exhibited two singlets at δ1.27 and 1.38, which was consistent with the formation of an acetonide. The ^1H n.m.r. spectrum however did not clearly reveal whether the 1,3-dioxane (127) or the 1,3-dioxepane (128) had been formed. Therefore it was decided to examine the ^{13}C n.m.r. spectrum because it has been shown that the chemical shift of an acetal carbon of an isopropylidine acetal is characteristic of ring-size.[81] In this case, it appeared from the large chemical shift value of the acetal carbon i.e., δ101.3, that a seven-membered ring had been formed. A typical ^{13}C chemical shift value for an acetal carbon which is part of a six-membered ring is ca. δ99. However, for complete verification, the acetonide was mesylated, reduced with sodium cyanoborohydride, and the product was stirred in aqueous acid to give platynecine (124). Thus, it became apparent that the 1,3-dioxepane (128) had formed. This was not totally unexpected since models had shown that the formation of both a six and seven-membered system was feasible.

Unfortunately, due to a lack of time, a different approach to the synthesis of 2α-hydroxy-1β-hydroxymethyl-8α-pyrrolizidine (125) could not be explored.

Since crystalline platynecine (124) became available during this work, this provided an opportunity to extend the limited range of



Scheme 46



(128) R = H

(129) R = SO_2CH_3

necine bases which have been studied by X-ray crystallography. Thus, an X-ray crystal structure analysis was carried out on platynecine, by Dr. A.A. Freer (Figure 14).[82]

Platynecine (124) has been shown to be an efficient precursor of rosmarinecine in S. pleistocephalus, but the status of the isomeric diol (125) in rosmarinine biosynthesis is still uncertain. Rosmarinecine is incorporated efficiently into rosmarinine.

‡ Specific ^{3}H incorporation is calculated from {(molar activity of rosmarinine (20))/(molar activity of [^{3}H] precursor)} x 100%.

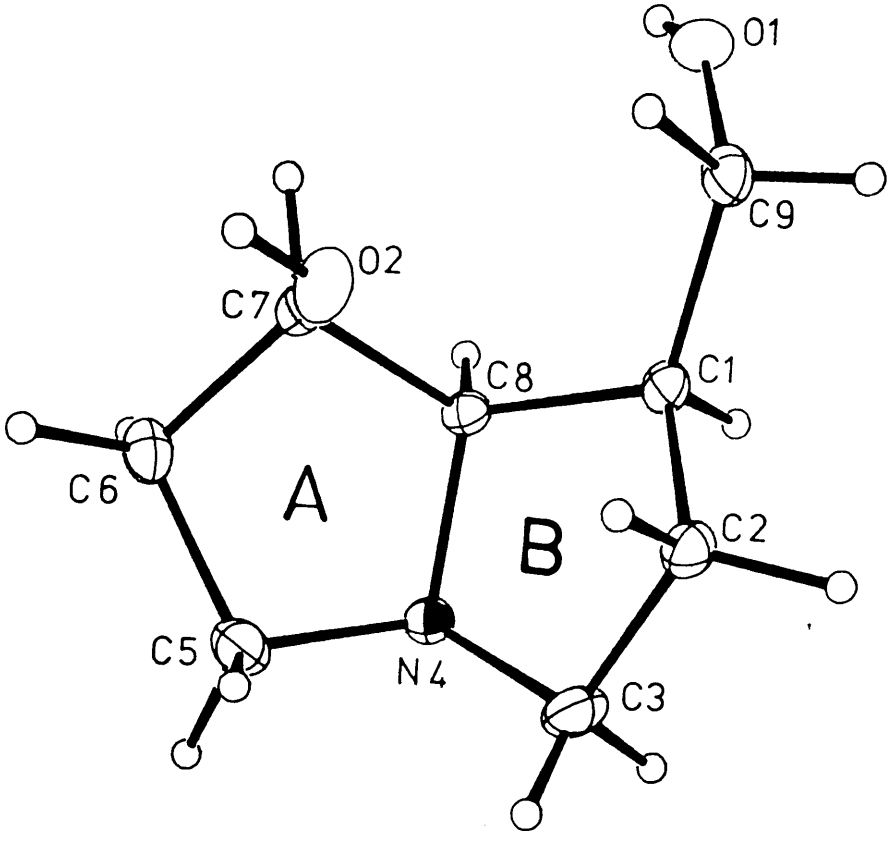
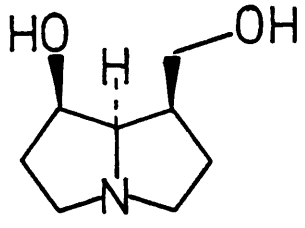


Figure 14

X-Ray Crystal Structure of Platynecine (124)[82].



(124)

Chapter 5

SYNTHESIS OF PYRROLIZIDINE ALKALOIDS AND ANALOGUES

5.1 Introduction

Pyrrolizidine alkaloids have attracted much attention because of their widespread distribution and the range of biological activities they exhibit, in particular hepatotoxicity.[1] The toxicity has been attributed to the pyrrolizidine nucleus in combination with an allylic ester function (Chapter 1.3). The most toxic alkaloids are the macrocyclic diesters formed by diesterification of a pyrrolizidine diol (usually retronecine) or triol with a necic diacid. Pyrrolizidine alkaloids with ring sizes of 11, 12, 13, and 14 have been isolated.[2]

In order to develop an understanding of structure-activity relationships in this area, synthetic routes to macrocyclic alkaloids and structurally related analogues have been devised. The following section describes such routes.

5.2 Synthesis of Macrocyclic Diesters

Most of the synthetic work on pyrrolizidine alkaloids has been directed towards the necines. Nevertheless, the outstanding challenge in this area has been the total synthesis of macrocyclic pyrrolizidine diesters. Indeed, it was not until the beginning of this decade that the first encouraging results were published. Robins and Sakdarat[83] reported the first synthesis of an eleven-membered dilactone (136) of (+)-retronecine (3).

The key reaction of this synthesis was the Corey-Nicolaou "double activation" method of lactonisation.[84] This method of internal esterification of hydroxy acids is highly efficient and can be used with complex and polyfunctional substrates. The carboxyl and hydroxyl groups are simultaneously activated to promote interaction. This is effected by the formation of a pyridine-2-thiolester,[85] which is doubly activated towards lactonisation by the internal proton transfer of a proton from the hydroxyl as shown in Figure 15. The transfer of this proton is facilitated by the basic nitrogen of the pyridine nucleus.

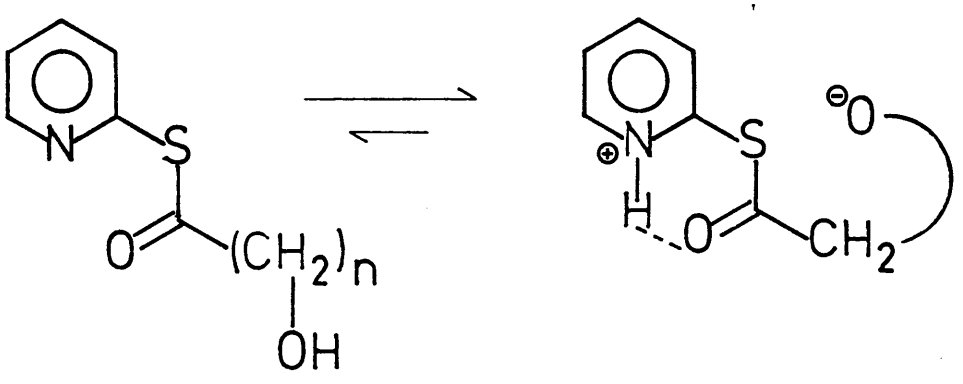


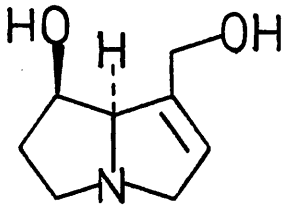
Figure 15

Full details of the synthesis of the dilactone (136) and a series of similar alkaloid analogues [(137) - (142)] were published soon after (Scheme 47).[86] Treatment of (+)-retronecine (3) with an equimolar amount of 3,3-dimethylglutaric anhydride in dry chloroform gave a quantitative mixture of the corresponding 7- (132) and 9- (133) monoesters of (+)-retronecine (3). There was no evidence of 7,9-diester formation probably because the initial zwitterionic monoesters were precipitated. The mixture of monoesters was converted into the corresponding pyridine-2-thiolesters (134) and (135), using 2,2'-dithiodipyridine (130) and triphenylphosphine (131). Lactonisation was then

effected by slow addition of the pyridine-2-thiolesters to refluxing chloroform and heating at reflux was continued for 12 hours. Isolation and purification of the dilactone by preparative thin layer chromatography afforded 13,13-dimethyl-1,2-didehydrocrotalanine (136) as an oil. The noteworthy feature in the ^{1}H n.m.r. spectrum of the dilactone (136) was the presence of an AB (J 12 Hz) due to the diastereotopic protons at C-9. The chemical shift difference between these protons was 1.24 p.p.m. The combination of spectral data was sufficiently convincing evidence for the formation of an eleven-membered macrocyclic diester.

Shortly, afterwards, Robins et al.[87] carried out the first synthesis of a macrocyclic pyrrolizidine alkaloid, dicrotaline (141). This was chosen as a synthetic target because it was the simplest of the eleven-membered macrocyclic diesters of (+)-retronecine. Using the procedure described above, 3-hydroxy-3-methylglutaric anhydride protected as its trimethylsilyl ether, reacted with (+)-retronecine (3) to give crude dicrotaline (141) and its C-13 epimer (142). No deprotection step was necessary as the protecting group was removed in the work-up. The two components were separated by preparative thin layer chromatography. The absolute configuration of each alkaloid was established by a specific degradation of each epimer to optically active mevalonolactone.

It is believed that steric hindrance at the α-positions of the diacid portions enhances toxicity by reducing the extent of detoxification by hydrolysis.[21] Therefore, Brown et al.[88] reacted (+)-retronecine (3) with meso-2,4-dimethylglutaric anhydride in dry dimethoxyethane



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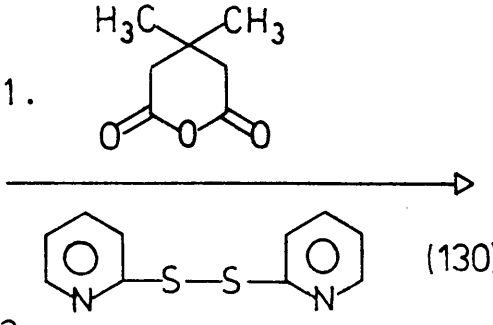
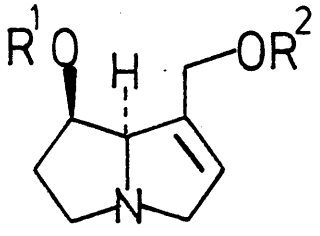


1. H_3C, CH_3

2. Ph_3P (131)

(130)

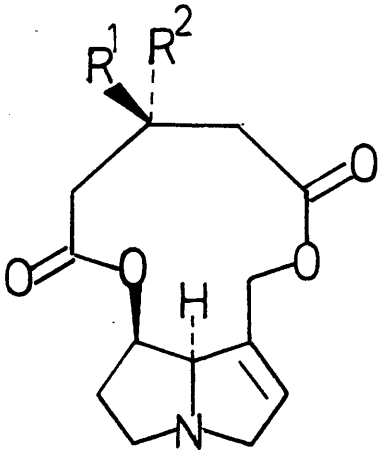


(132) R^1= $COCH_2CMe_2CH_2CO_2H$, R^2 = H

(133) R^1= H, R^2=$COCH_2CMe_2CH_2CO_2H$

(134) R^1= $COCH_2CMe_2CH_2COSPy$, R^2=H

(135) R^1= H, R^2=$COCH_2CMe_2CH_2COSPy$



	R^1	R^2
(136)	CH_3	CH_3
(137)	$(CH_2)_4$	
(138)	H	H
(139)	Ph	Ph
(140)	{ H / CH_3	CH_3 / H
(141)	OH	CH_3
(142)	CH_3	OH

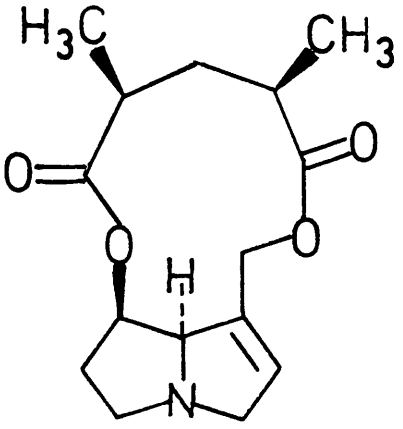
Scheme 47

(DME) to obtain a mixture of the corresponding 9- monoesters of (+)-retronecine. Lactonisation *via* the pyridine-2-thiolesters gave *ca.* 1.1 mixture of dilactones (143a) and (143b) which was separated by column chromatography. A sequence of chemoselective reactions was carried out on each diastereoisomer to establish the absolute configuration of the acid portions. In each case, hydrogenolysis and borohydride reduction of the dilactones afforded tetrahydro-3,5-dimethyl-2H-pyran-2-ones (144a) and (144b) of known configurations. *X*-Ray crystal structure analysis confirmed the structure of the (12*S*,14*R*)-isomer (144b), and established that the carbonyl bonds of the ester functions are synparallel.

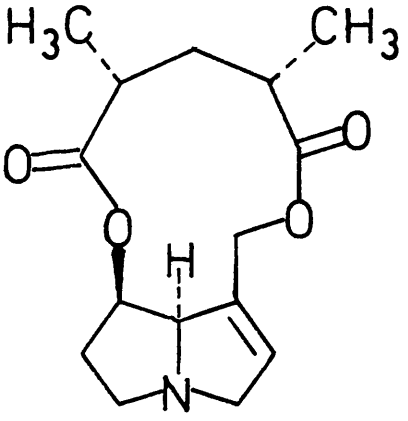
Macrocyclic pyrrolizidine diesters generally have 11- or 12-membered rings. No pyrrolizidine alkaloids containing ten-membered rings have so far been isolated although succinic acid derivatives are common plant constituents.

Burton and Robins[89] synthesised the first ten-membered macrocyclic diesters (145), (146), (147a) and (147b) of (+)-retronecine (3) by treating retronecine with succinic anhydride, phthalic anhydride and (±)-*trans*-cyclohexane-1,2-dicarboxylic anhydride, respectively, under Corey-Nicolaou conditions. The reaction of (±)-*trans*-cyclohexane-1,2-dicarboxylic anhydride with retronecine gave a 4:1 mixture of diastereoisomers (147). *X*-Ray crystal structure analysis on the succinate dilactone (145) showed that the ten-membered macrocycle adopts a conformation in which the carbonyl groups are antiparallel.

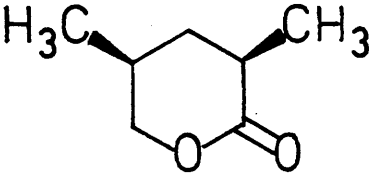
The low yield (16%) for analogue (146) prompted Burton and Robins to develop a different route to analogues containing aromatic



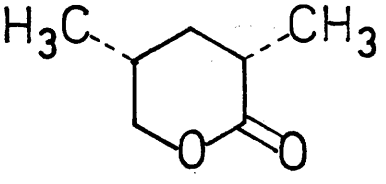
(143a)



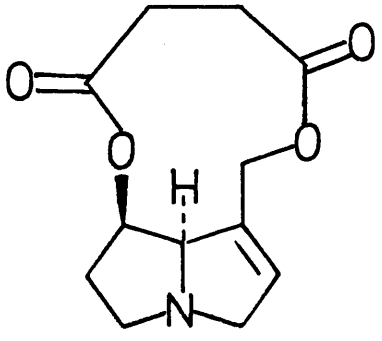
(143b)



(144a)



(144b)



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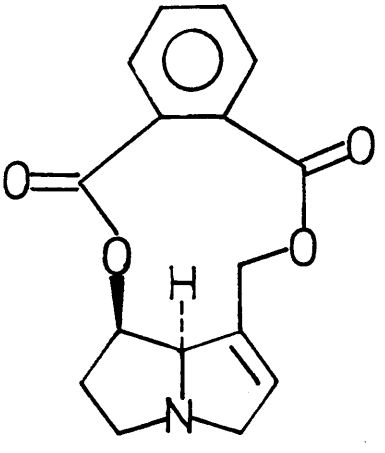
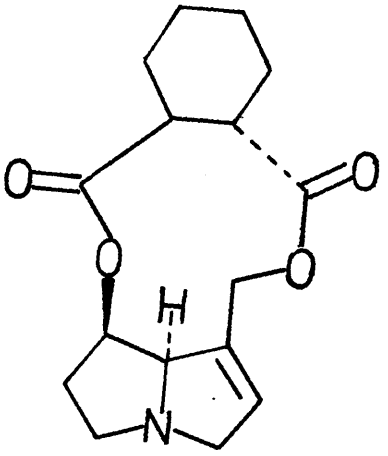
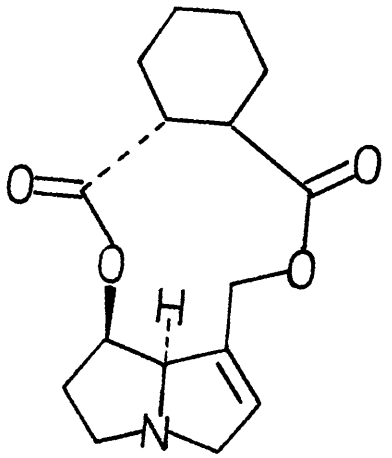


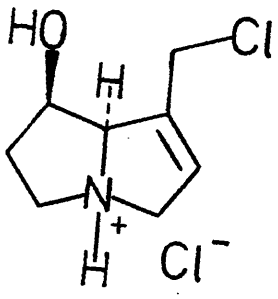
(146)



(147a)



(147b)



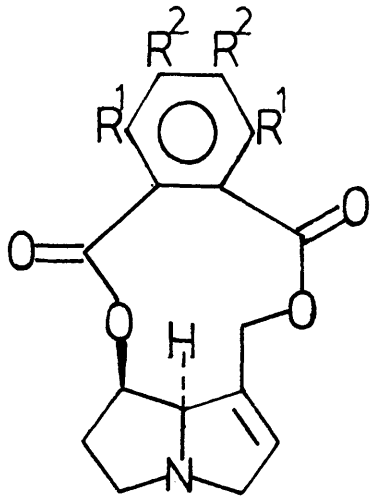
(148)

rings or unsaturation in the acid moiety.[90]

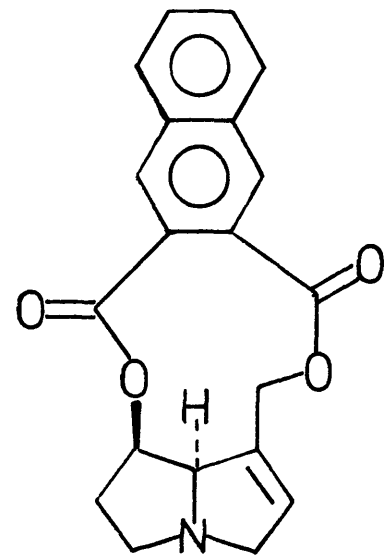
(+)-Retronecine was converted into (7R,8R)-1-chloromethyl-1,2-didehydro-7-hydroxypyrrolizidinium chloride (148) on treatment with thionyl chloride. Phthalic anhydride was then added to the allylic chloride (148) in DMF, along with two equivalents of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) forming the pyrrolizidine alkaloid analogue (146) in a yield of 57%. Similarly, other new pyrrolizidine alkaloids were prepared (149) - (156), by varying the aromatic and unsaturated anhydrides. Analogues (155) and (156) could not be synthesised using Corey-Nicolaou conditions. It is believed that each reaction probably involves the internal nucleophilic displacement of chloride by carboxylate anion. Using bases such as N,N-di-isopropylethylamine (Hunig's base) and potassium hydroxide in place of DBU resulted in products being formed in lower yield.

The new lactonisation procedure was tried with some saturated anhydrides. Cyclised products were obtained in lower yields, probably because the allylic chloride decomposed slightly and the rates of cyclisation were significantly slower. Thus, it was concluded that to prepare analogues using saturated anhydrides, the Corey-Nicolaou is the preferred method.

Masamune et al.[91] have developed a synthetic method for the construction of macrocyclic lactones. The procedure employs the tert.-butylthiolester of a hydroxy acid and mercuric trifluoroacetate or methanesulphonate to effect lactonisation, as shown in Scheme 48.



(146) $R^1 = R^2 = H$
(149) $R^1 = H,\ R^2 = Cl$
(150) $R^1 = R^2 = Br$
(151) $R^1 = R^2 = Cl$



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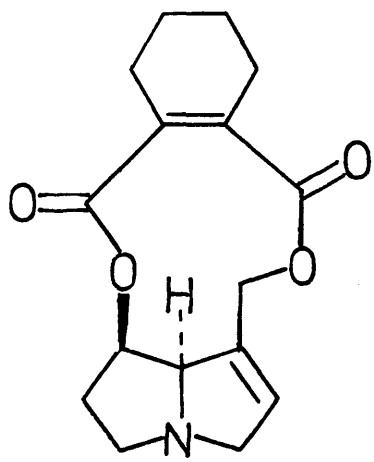

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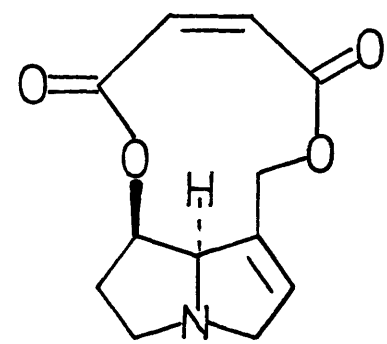

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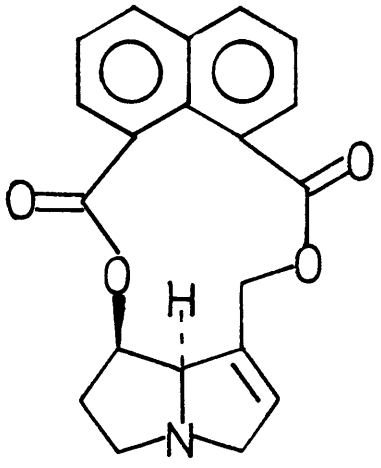

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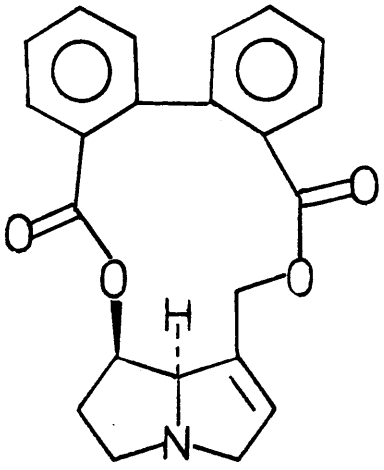

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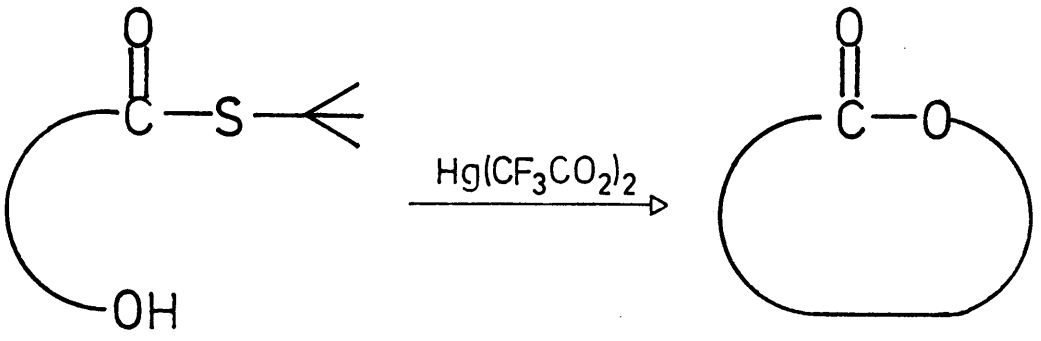


Scheme 48

It is believed that the cyclisation reaction proceeds through a metal complex as shown in Figure 16. Other thiophilic metal cations such as Cu(I), Cu(II) and Ag(I) can be used.

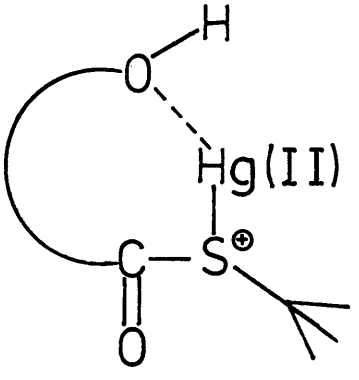


Figure 16

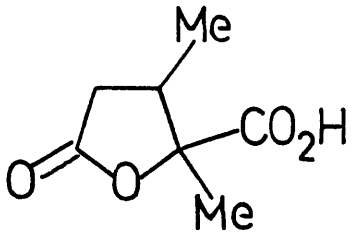
The 11-membered pyrrolizidine macrocyclic diesters, crobarbatine acetate (157a) and a diastereoisomer (157b) were synthesised by Huang and Meinwald[92] employing the above strategy (Scheme 49).

Crobarbatic acid lactone (158), shown to be the trans isomer, was converted into the methylene chloride soluble dimethylaluminium salt (159), which was subsequently reacted with dimethylaluminium

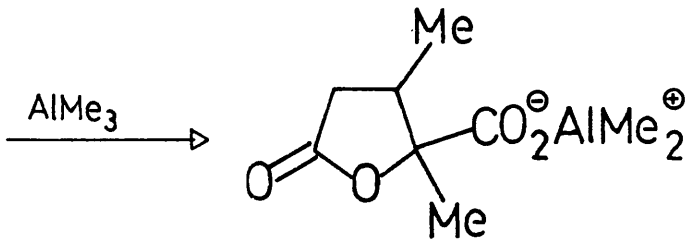
tert.-butylsulphide affording the desired thiolester (160). The tertiary hydroxyl was protected as its acetate before converting the free acid into the acylimidazolide (161) with N,N'-carbonyldi-imidazole (CDI). This activated the carbonyl group to attack from the primary allylic hydroxyl group of (+)-retronecine. The two components were coupled by adding the imidazolide (161) to a solution of retronecine in tetrahydrofuran (THF), containing a catalytic amount of sodium hydride. Various attempts to cyclise the allylic monoester failed. However, five equivalents of copper(I) trifluoromethanesulphonate-benzene complex were found to effect the crucial cyclisation in good yield. The two diastereoisomers (157) formed were separated using column chromatography.

The configurations of the acid portions of the dilactones were established by acid hydrolysis of each diastereoisomer and analysis of the Cotton effect exhibited by each lactonic acid (Figure 17). Attempts made to deacetylate both diastereoisomers under basic conditions to obtain the free alkaloids met with failure and produced retronecine. However, using acid catalysed conditions, the deacetylated product was produced in low yield, but the authors were unable to prove that this corresponded to natural crobarbatine.

The preparation of all the analogues discussed depended on a supply of (+)-retronecine (3) from natural sources, since the available synthetic routes to (+)-retronecine are lengthy and low yielding. This limited the amount of each analogue that was prepared, and since a reasonable amount is required for toxicological studies, a more accessible necine or necine analogue was necessary. Barbour and



(±) (158)



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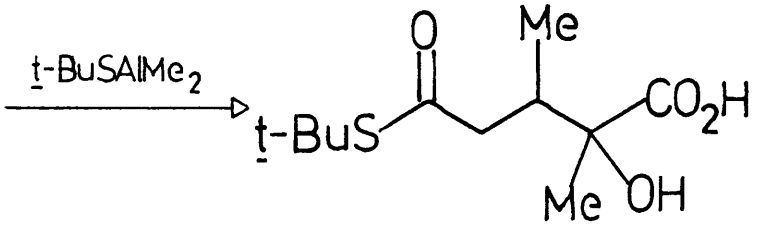

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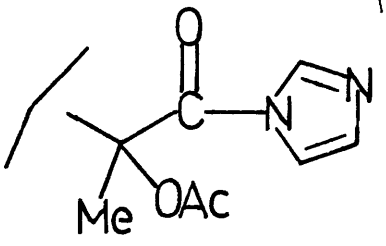

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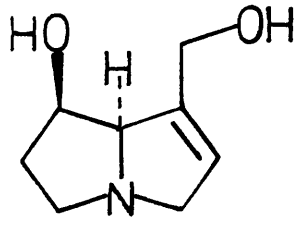
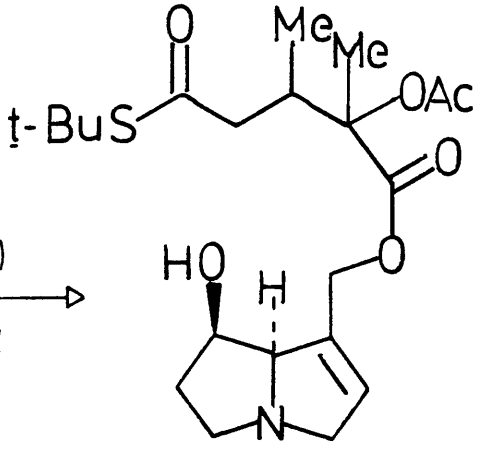
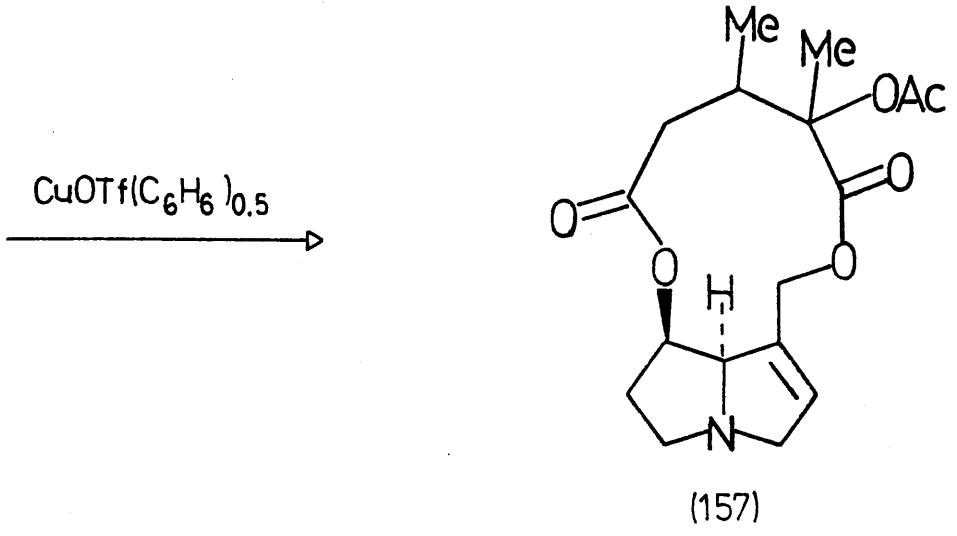


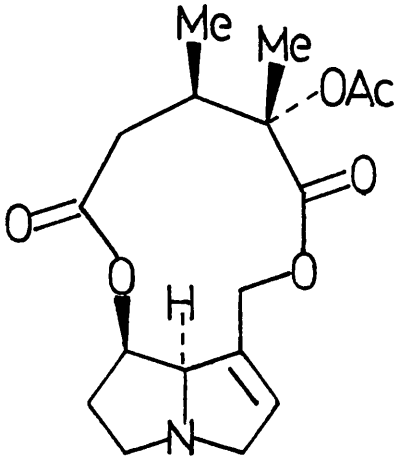
(3)



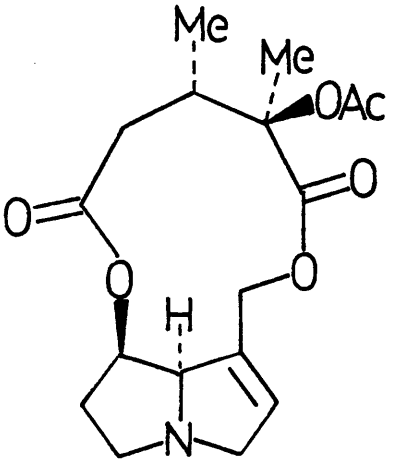


(157)

Scheme 49



(157a)



(157b)

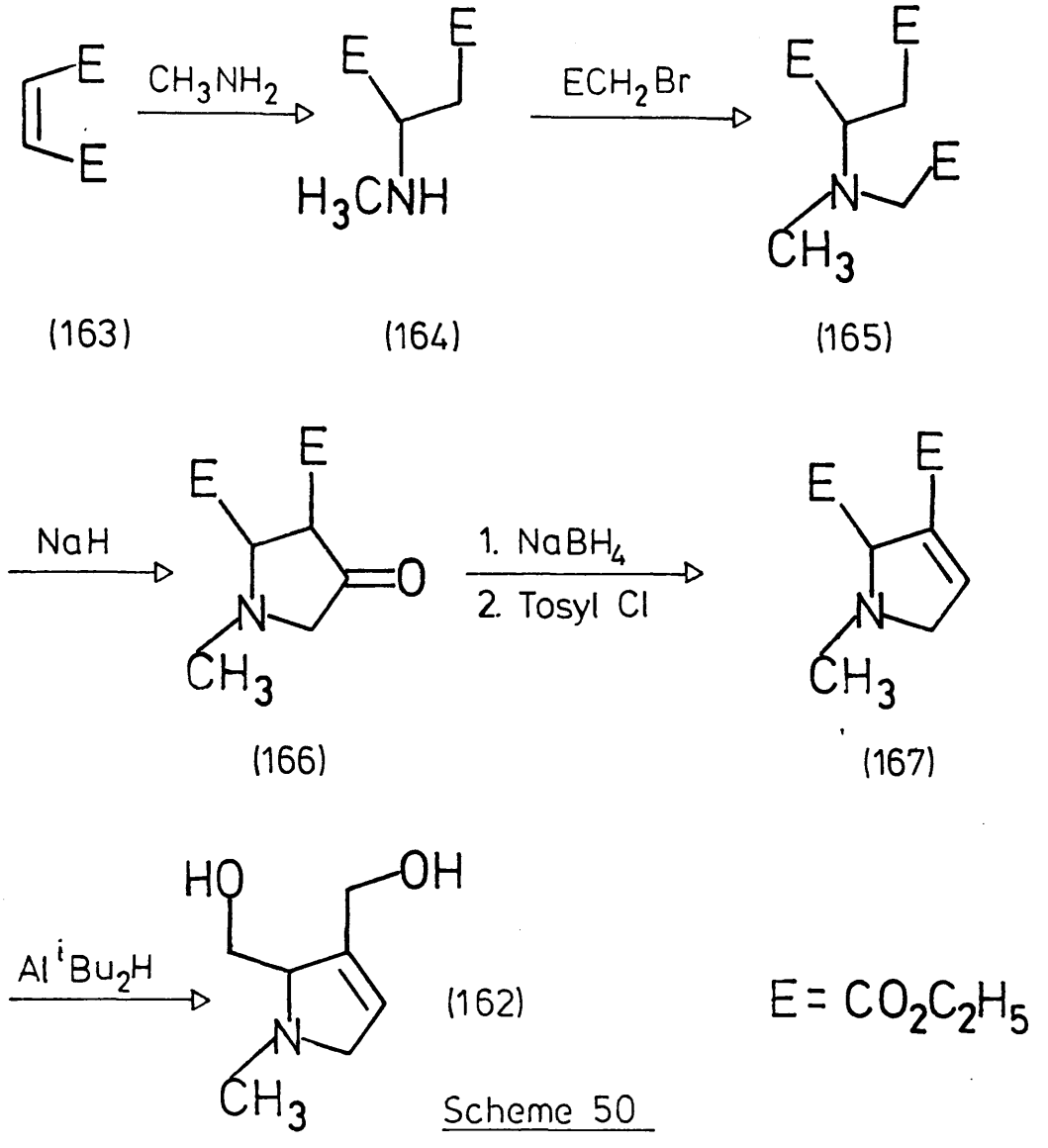
Figure 17

Robins[93] selected 2,3-bis(hydroxymethyl)-1-methyl-2,5-dihydropyrrole (162) (synthanecine A). This was chosen because it could be easily prepared and diester derivatives of synthanecine A were shown to produce damage to the livers of animals, similar to that caused by pyrrolizidine alkaloids.[94] In addition, Mattocks had demonstrated that the saturated ring in the pyrrolizidine nucleus system is not involved in the toxic process.[95]

(±)-Synthanecine A (162) was prepared by a slightly modified version of the route published by Mattocks (Scheme 50).[95]

Condensation of methylamine and diethyl maleate (163) afforded the diester (164), which was converted into the triester (165) with ethyl bromoacetate. Addition of sodium hydride effected a Dieckmann cyclisation, giving the cyclic ketone as product (166). Selective reduction of the ketone, followed by dehydration gave the desired unsaturation. Finally reduction of the diester (167), gave the diol, synthanecine A (162).

A series of 10- and 11-membered macrocyclic diesters of synthanecine A was constructed by Barbour and Robins,[93,96] using substituted succinic and glutaric anhydrides, respectively.



Scheme 50

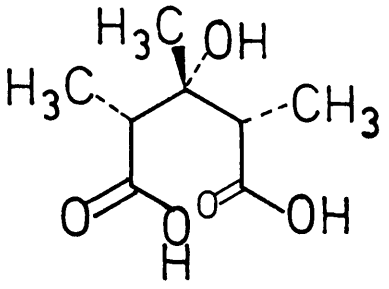
A preliminary investigation into the synthesis of (±)-crispatine (168) and (±)-fulvine (169) was conducted by Vedejs and Larsen[97] using the Corey-Nicolaou lactonisation method, without significant success. However, adoption of an alternative procedure afforded (168) and (169) in much improved yields. The key reaction was the displacement of a highly reactive mesylate by carboxylate anion.

Crispatic anhydride (171), prepared from crispatic acid (170) was protected as its methoxymethyl ether (172). Opening of the anhydride (172) using 2-trimethylsilylethoxydimethyl aluminium gave the

protected crispatic derivative (173), and the free acid was activated by forming the mixed phosphoric anhydride (174). Coupling of the anhydride with the lithium alkoxide of monosilyl(±)-retronecine (174) produced the 7-monoester (176a) and a diastereoisomer (176b) in equal quantities. Desilylation of the monoester liberated the primary hydroxyl group, which was then transformed into the mesylate. Without isolation, this was added to excess tetra-n-butylammonium fluoride hydrate in acetonitrile, causing spontaneous lactonisation, and forming two separable diastereoisomers (177a) and (177b). Quantitative deprotection of compounds (177a) and (177b) yielded (±)-crispatine (168) and (±)-fulvine (169), respectively (Scheme 51). The spectroscopic and chromatographic data of alkaloids (168) and (169) were identical to those of authentic samples.

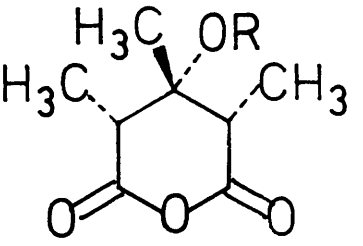
The only 12-membered macrocyclic pyrrolizidine alkaloid to be synthesised is integerrimine (178), first prepared by Narasaka et al.[98] The synthesis includes a novel means of lactonisation after using the methylthiomethyl ester ($CO_2CH_2SCH_3$) as a protecting group.

The methylthiomethyl (MTM) group is a well-known protecting group for alcohols and carboxylic acids. Deprotection is usually carried out by heavy metal salts or oxidising agents. The latter transforms the methylthiomethyl group into a methylsulphonylmethyl group which is readily hydrolysed by aqueous alkali (Scheme 52). The authors envisaged that an ester would be formed if the methylsulphonylmethyl ester was treated with a metal alkoxide instead of aqueous alkali.



(170)

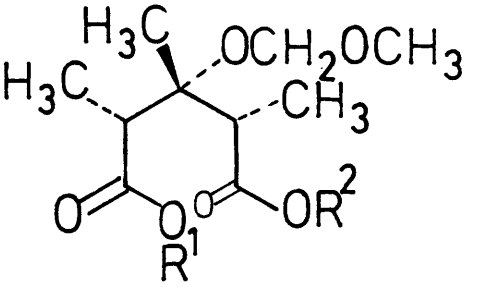
DCC,
P_2O_5, $(CH_3O)_2CH_2$



(171) R = H

(172) R = CH_2OCH_3

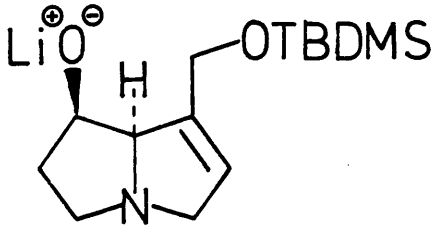
$(CH_3)_2AlOCH_2CH_2Si(CH_3)_3$



(173) R^1 = H, R^2 = $CH_2CH_2Si(CH_3)_3$

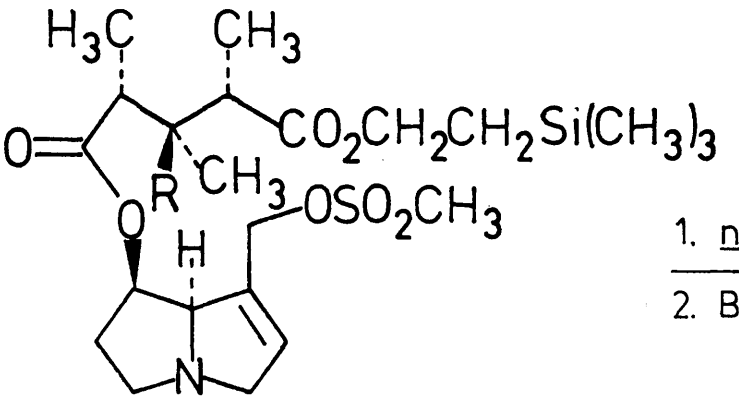
(174) R^1 = $PO(OEt)_2$,
R^2 = $CH_2CH_2Si(CH_3)_3$

(174) and



(175) + enantiomer

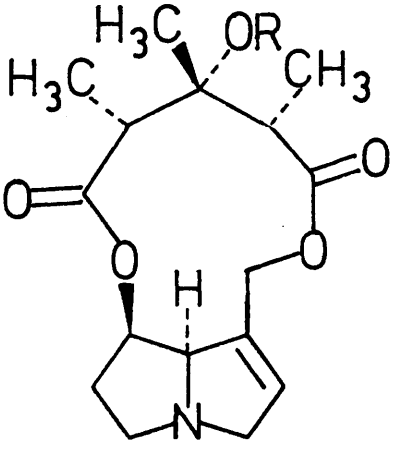
1. DMAP
2. H_2O-HF, THF
3. $MeSO_2Cl$, Et_3N, CH_2Cl_2



1. n - $Bu_4N^{\oplus}F^{\ominus}$
2. BF_3. Et_2O - EtSH

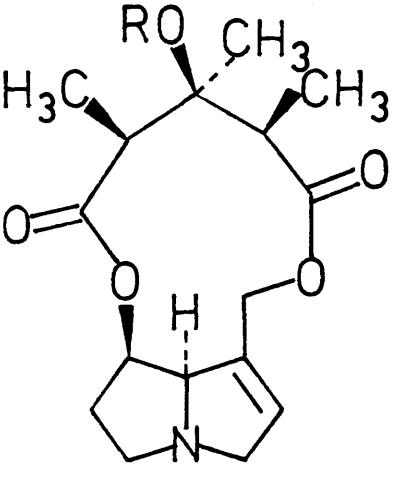
(176 a) R = OCH_2OCH_3

a diastereoisomer (176 b)



(177 a) R = CH_2OCH_3

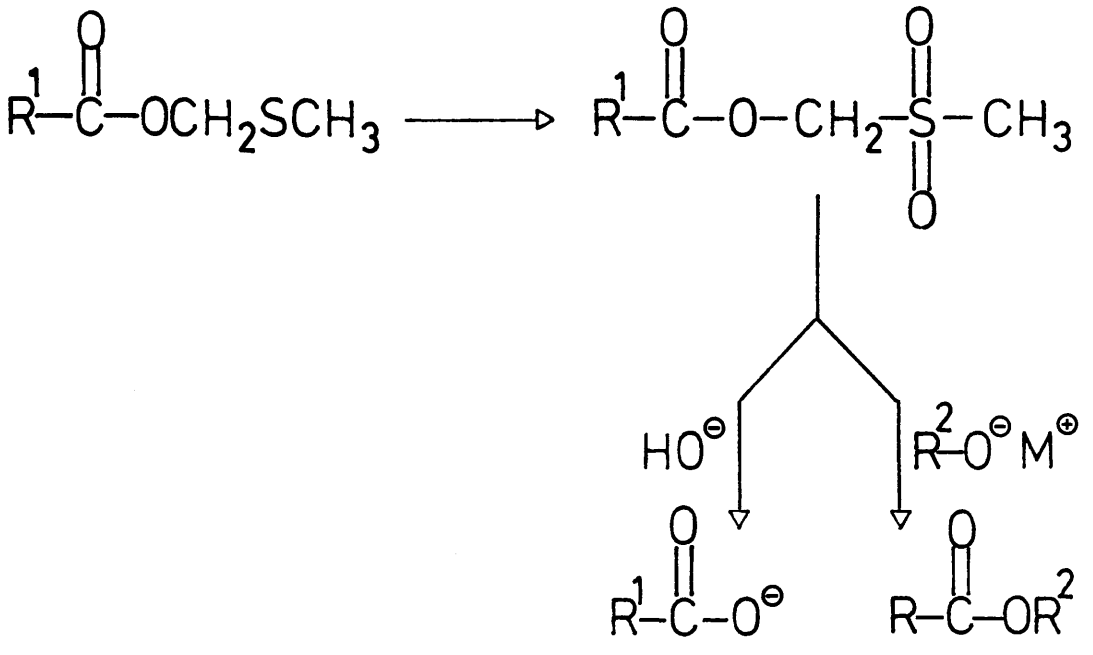
(168) R = H



(177 b) R = CH_2OCH_3

(169) R = H

Scheme 51



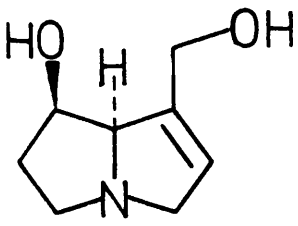
Scheme 52

In essence, the synthesis of integerrimine (178) involved the joining together of suitably protected retronecine and integerrinecic acid.

Retronecine (3) was prepared by modifying the synthetic procedure of Geissman.[99] Prior to coupling however, the allylic hydroxyl of retronecine was protected as the t-butyldimethylsilyl ether (179). Integerrinecic acid lactone (180) was prepared as reported[98] and protected to give derivatised integerrinecic acid (181). This was transformed into the acid anhydride (182) with 2-chloro-1-methyl-pyridinium iodide and triethylamine (Mukaiyama reagent). After formation of the lithium alkoxide of monosilyl(±)-retronecine (175) the two components (175) and (182) combined to give compound (183a) and its diastereoisomer (183b). The silyl groups were removed, and the sulphides were oxidised to the sulphones. Unfortunately, N-oxide

formation of the necines accompanied the latter reaction. Esterification of the activated acids was accomplished by the intramolecular nucleophilic displacement of the methylsulphonylmethyl groups to afford the dilactones (184). Reduction of the N-oxides (184) yielded a diastereoisomeric mixture of bases [(185a) and (185b)] which was separated. Removal of the methoxymethyl protecting group from compound (185a) furnished (±)-integerrimine (178), which was spectroscopically and chromatographically identical with a natural sample (Scheme 53). Two other syntheses of integerrimine have recently appeared.[98]

It is surprising that the synthesis of natural ,macrocyclic pyrrolizidine alkaloids has been restricted to a small number of examples. After the successful synthesis of dicrotaline (141) in 1981, it was anticipated that there would be an explosion of effort in this area. There are now a wide variety of techniques available for the construction of macrocyclic dilactones, and synthesis of different macrocyclic pyrrolizidine alkaloids is expected over the next few years.



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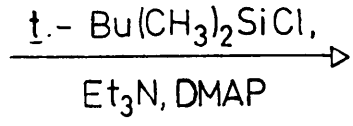

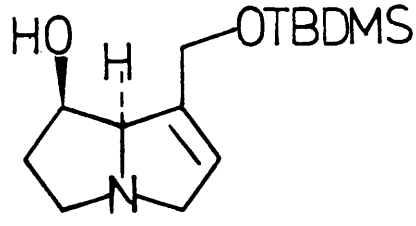

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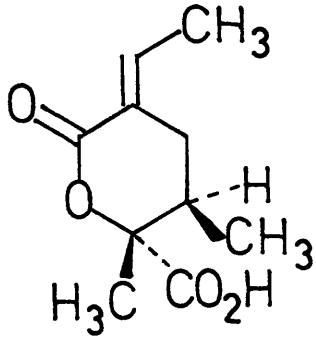

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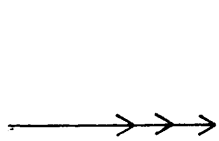
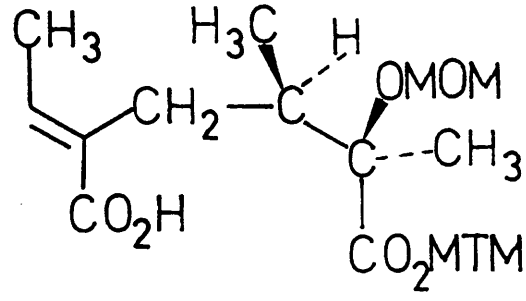
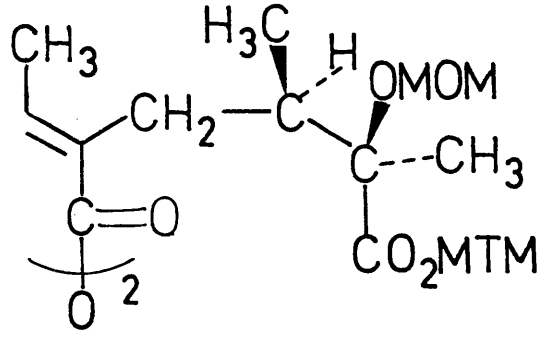
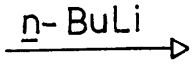


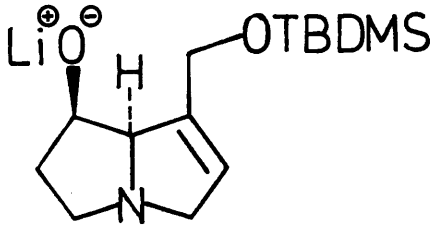


(181)



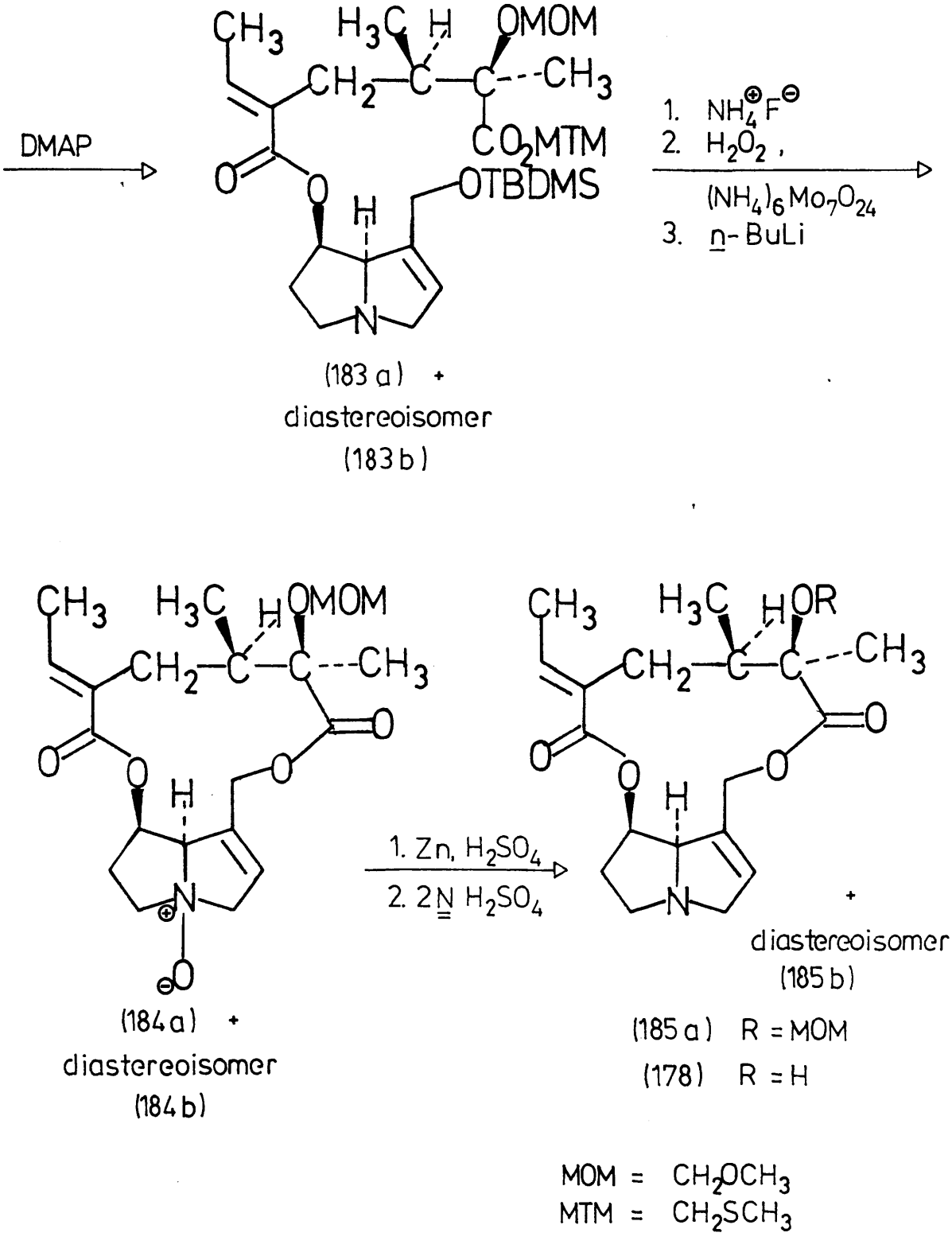
(182)

(179) 



(175)

+ (182)



(183 a) + diastereoisomer (183 b)

(184 a) + diastereoisomer (184 b)

(185 a) R = MOM

(178) R = H

+ diastereoisomer (185 b)

MOM = CH_2OCH_3

MTM = CH_2SCH_3

Scheme 53

Chapter 6

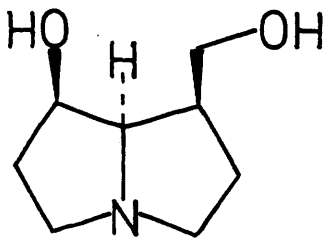
SYNTHESIS OF OPTICALLY ACTIVE MACROCYCLIC PYRROLIZIDINE ALKALOID ANALOGUES

6.1 Introduction

It has been reported that platyphylline (13) is widely used in the U.S.S.R. for the treatment of hypertension and internal ulcers.[1,100] Presumably this provides an important model for this type of pharmacological activity because platyphylline lacks the hepatotoxicity normally associated with pyrrolizidine alkaloids, since it has no 1,2-double bond. Thus, it was decided to prepare a saturated base, in optically active form, and to synthesise a range of macrocyclic diesters containing it. This would produce novel compounds which could be tested for useful biological activity by a pharmaceutical company. The structural features required for anti-ulcer and anti-hypertensive activities might then be established. This investigation had the additional incentive of achieving the first synthesis of macrocyclic diesters of a saturated pyrrolizidine base.

The base (22), (+)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine, a structural isomer of platynecine (124), was selected for these studies because it was readily available *via* a short and efficient route.[101]

The synthesis of a series of 12-membered and one 11-membered macrocyclic diesters of (22) was achieved employing the widely used Corey-Nicolaou "double activation" method of lactonisation[84] (Chapter 5).



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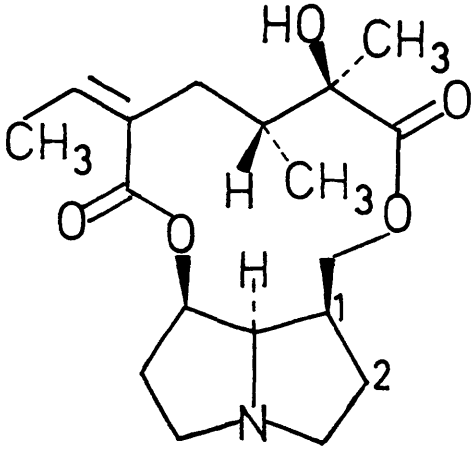
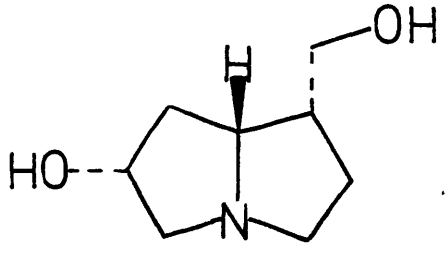


(13)



(22)

6.2 Synthesis of Optically Active Base (22)

The optically active base (22) was prepared according to the procedure of Robins and Sakdarat,[101] with one slight modification.

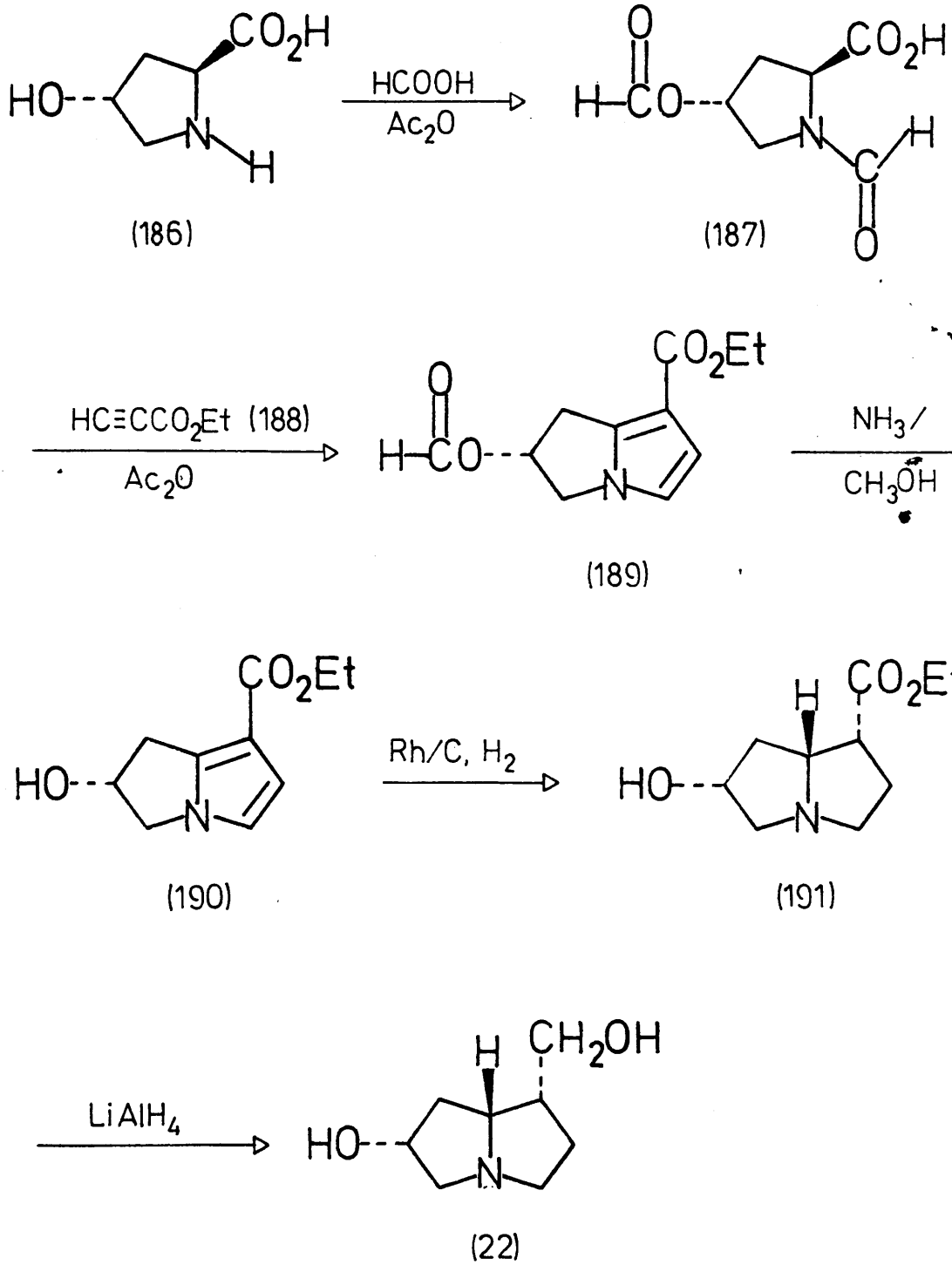
L-4-Hydroxyproline (186) was diformylated using formic acid and acetic anhydride. The N,O-diformyl derivative (187) then underwent a 1,3-dipolar cycloaddition reaction with ethyl propiolate (188) to produce one optically active product (189). A characteristic AB quartet for H-5 and H-6 of the dihydropyrrolizine appeared at δ6.6 (J 3 Hz) in the ^{1}H n.m.r. spectrum, and this along with the other signals and mass spectral data were consistent with the structure (189). Removal of the O-formyl group was achieved by stirring the ester (189) in conc.

ammonia/methanol affording the optically active alcohol (190). The next step involved the reduction of the pyrrole system. Unfortunately, use of a Pd/C catalyst, as suggested by Robins and Sakdarat did not furnish any of the desired product. However, when the hydrogenation was carried out with a Rh/C catalyst at 7 atm and 60°C for 24h, the crystalline ester (191) was formed in 47% yield. This hydrogenation resulted in the formation of two new chiral centres at C-1 and C-8 of the pyrrolizidine. The configurations at these two centres are controlled by the hydroxyl group on C-2 of the dihydropyrrolizine (190). This directs the cis addition of hydrogen from the less-hindered β-face, resulting in the formation of the thermodynamically less stable endo-ester. Finally, reduction of the ester using lithium aluminium hydride produced the optically active diol (22) (Scheme 54). The mechanism for the 1,3-dipolar cycloaddition reaction is discussed at the end of the chapter (6.5).

6.3 Synthesis of 12-Membered Dilactones

A range of 12-membered macrocyclic diesters of the pyrrolizidine diol (22), was constructed using substituted glutaric anhydrides. Initially, symmetrically substituted glutaric anhydrides were employed to provide the diacid component, thus preventing the formation of diastereoisomers.

The optically active diol (22) was dissolved in dry 1,2-dimethoxyethane (DME) and a series of 3,3-disubstituted glutaric anhydrides (192a-d) was added. In each experiment, a gum was


HO
CO2H
N
H
HCOOH
Ac2O
(186)
O
H–CO
CO2H
N
C
H
O
(187)
HC≡CCO2Et (188)
Ac2O
CO2Et
(189)
NH3 /
CH3OH
(190)
Rh/C, H2
(191)
LiAlH4
CH2OH
(22)

Scheme 54

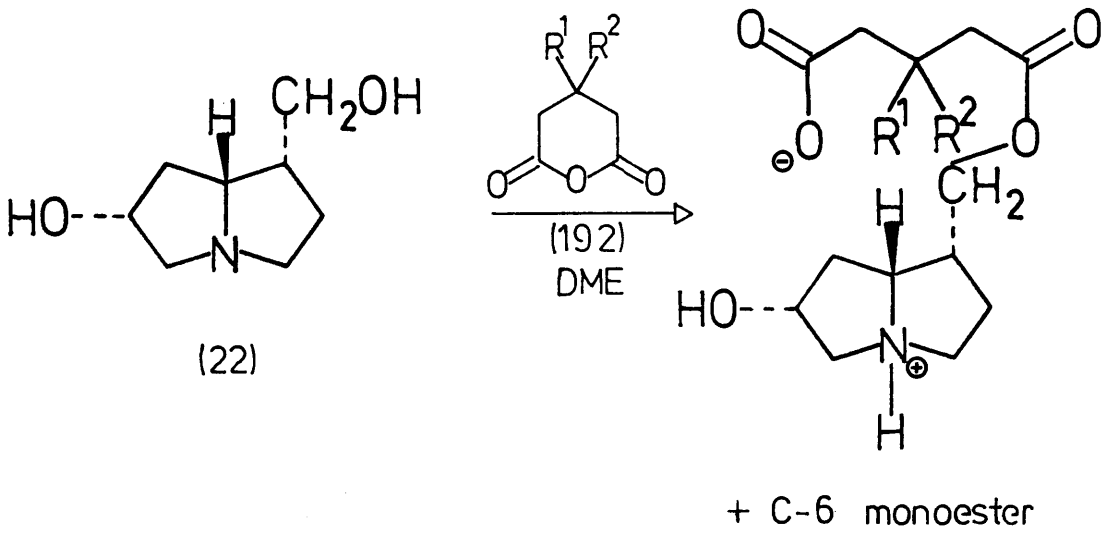
deposited on the walls of the reaction vessel, which was shown to contain a mixture of 6- and 9-monoesters. The disappearance of the base was monitored by thin layer chromatography. When a sample of this reaction mixture was applied to a silica gel plate eluted with the normal solvent system, (Chapter 8.1), the monoester mixture had an R_F of 0.1.

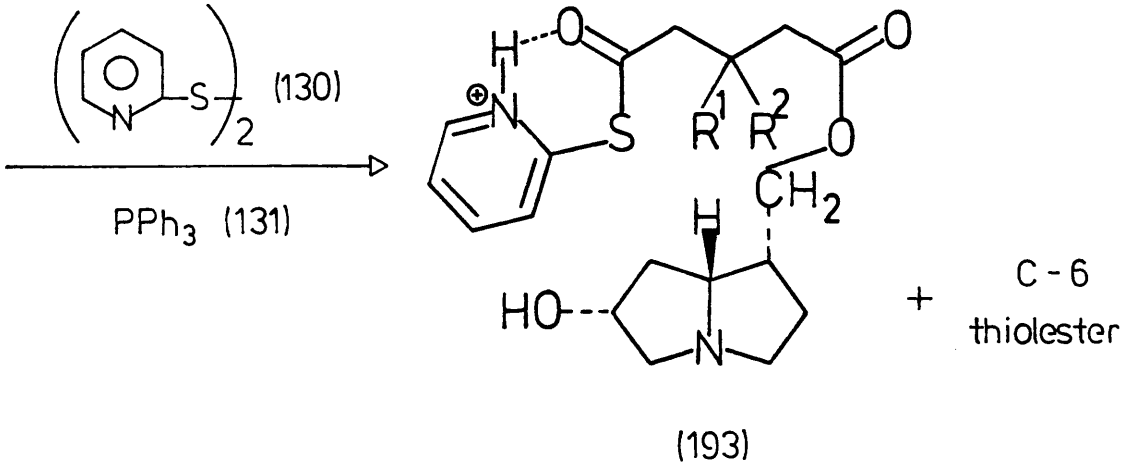
From the many lactonisation methods available, the Corey-Nicolaou "double-activation" method was chosen, as it had been successfully used in the preparation of other macrocyclic pyrrolizidine alkaloids (Chapter 5). Therefore, the pyridine-2-thiolesters (193a-d) were prepared by stirring each monoester with $1\frac{1}{2}$ equivalents of 2,2'-dithiodipyridine (130) and $1\frac{1}{2}$ equivalents of triphenylphosphine (131). Typically, the R_F value of the thiolesters was 0.65.

Lactonisation was finally effected under high dilution conditions. The solutions of thiolesters were added dropwise to refluxing DME and the resultant solution was heated at reflux for a further four days. A high dilution technique inhibited any intermolecular reactions, which may have led to the formation of dilides.

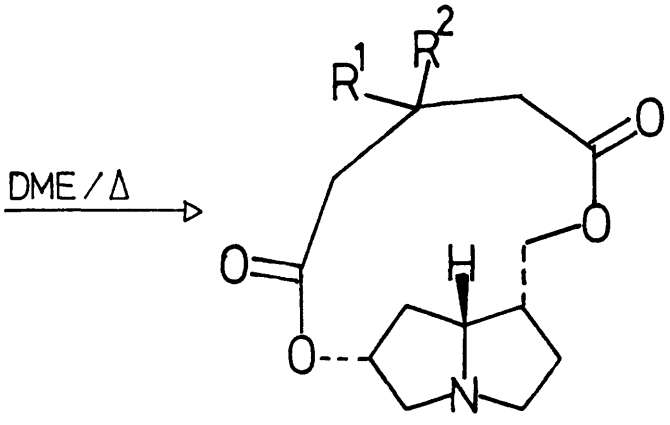
The product mixture was subjected to an acid/base recycle to remove most of the thiopyridone (196) and triphenylphosphine oxide (195). Finally, preparative thin layer chromatography was necessary to obtain pure samples of the optically active dilactones (194a-d) (Scheme 55). Table 7 shows the yields of macrocyclic diesters formed.

It was observed that dilactones (194b-d) were formed faster than diester (194a). The rates of such cyclisations depend upon two factors.[102] Firstly, the atoms at the end of the chain have to come within reacting distance (entropy factor). Secondly, steric and stereo-


HO
H
CH2OH
N
(22)
R1 R2
O
O
O
(192)
DME
O
O
R1 R2
O
CH2
H
HO
N
H
+ C-6 monoester


O
N
S
2
(130)
PPh3 (131)
H
O
O
N
S
R1 R2
O
CH2
H
HO
N
+
C-6
thiolester
(193)

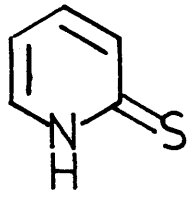
DME/Δ



	R^1	R^2
a	H	H
b	CH_3	CH_3
c	$(CH_2)_4$	
d	$(CH_2)_5$	

(194)

\+ $Ph_3P{=}O$ (195)

\+  (196)

Scheme 55

Table 7	% Yield of Macrocyclic Diester
(194a)	26
(194b)	30
(194c)	41
(194d)	37

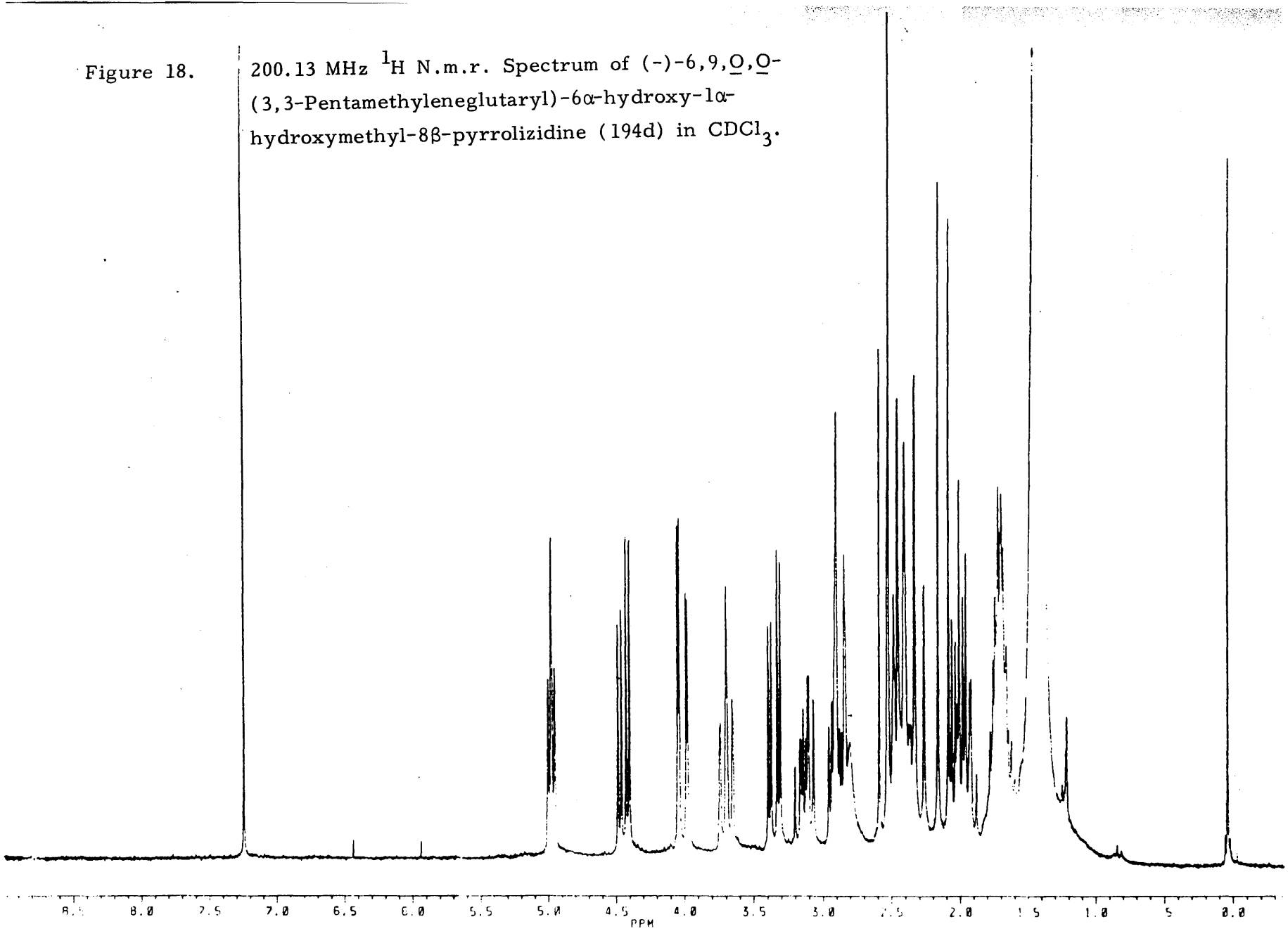
electronic interactions may hinder ring closure when the two ends meet (enthalpy factor). Thus, substituents at the 3-positions of the glutaric anhydride reduce the entropy effect because they restrict the number of rotational degrees of freedom of the thiolester chains, thereby promoting an efficient intramolecular process.

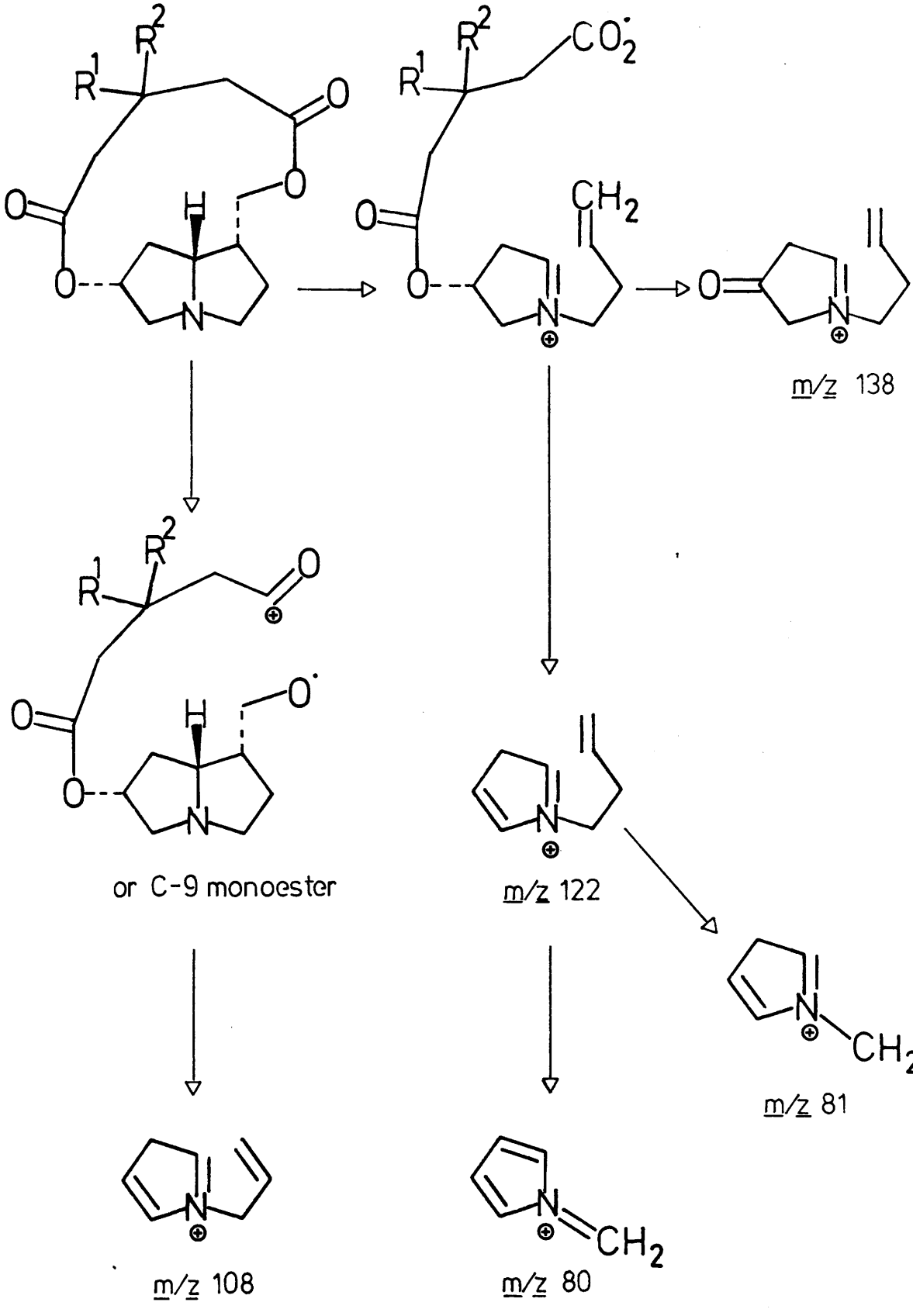
The ^{1}H n.m.r. spectra of the dilactones (194a-d) in deuteriochloroform contained some common diagnostic features. When the C-6 and C-9 hydroxyl groups of the base (22) were not esterified, the protons attached to those carbinol carbons gave rise to a quintet at δ4.33 and a doublet at δ3.65, respectively. On esterification,, the protons assigned to C-6 shifted downfield by ca. 0.7 p.p.m. and appeared as a triplet. Additionally, both of the C-9 protons of the base shifted downfield on esterification, and each appeared as a doublet of doublets since they were part of an ABX system. The difference in chemical shift of those two protons was 0.75 p.p.m. Furthermore, for dilactones (194b-d), an AB quartet was evident for the C-12 and C-14 protons. Figure 18 shows the ^{1}H n.m.r. spectrum of (194d).

The infra-red spectra of the macrocycles displayed absorption peaks at ca. 1735 cm^{-1} corresponding to the saturated ester carbonyls. There were no signals in the i.r. spectra due to carboxylic acids or hydroxyl groups. Correct accurate mass measurements were obtained for each macrocyclic product. Analysis of the mass spectra revealed that each showed a molecular ion and that each had similar fragmentation patterns with major fragments at m/z 138, 122, 108, 82, 81 and 80. Scheme 56 shows a possible fragmentation pattern.

Figure 18.

200.13 MHz ^{1}H N.m.r. Spectrum of (-)-6,9,O,O-(3,3-Pentamethyleneglutaryl)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (194d) in $CDCl_3$.





Scheme 56

The combination of mass spectral data, i.r. data and the distinctive signals in the ^{1}H n.m.r. spectra was excellent evidence for the formation of 12-membered macrocyclic diesters of the diol (22).

When the optically active base (22) was treated with 2,2-dimethylglutaric anhydride (197), subsequent lactonisation yielded one product as an oil, in low yield (10%). An accurate mass measurement on the oil gave the molecular formula $C_{15}H_{23}NO_4$, and analysis of its ^{1}H n.m.r. and mass spectra suggested that it had one of two structures, (198a) or (198b). Assuming that the initial step of this reaction is the attack of the primary alcohol of the base on the anhydride, steric considerations would suggest that C-5 of 2,2-dimethylglutaric anhydride is preferentially attacked. If the other carbonyl (C-1) is attacked, then the steric congestion developed in the transition state may be too severe. This would involve the formation of a bulky tetrahedral intermediate next to a carbon bearing two methyl groups. Thus, it is likely that the oil has structure (198b). An X-ray crystal structure analysis would have revealed the structure but attempts at crystallising the oil met with failure.

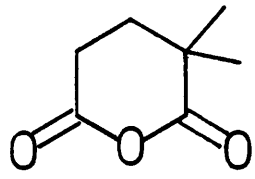
Finally, it was decided to prepare dilactones (199) with substituents on the α-positions of the diacid portions. This creates steric hindrance around the ester groups which is believed to reduce the likelihood of removal of the biological activity of these compounds by hydrolysis.[22] Therefore, the optically active diol (22) was treated with meso-2,4-dimethylglutaric anhydride (200) and the resultant monoesters were lactonised via the pyridine-2-thiolesters to give a 1:1 mixture of diastereoisomers, as seen by ^{13}C n.m.r. spectroscopy. The high

resolution mass spectrum of the mixture displayed the characteristic fragmentation pattern and a molecular ion corresponding to $C_{15}H_{23}NO_4$. The i.r. spectrum of the dilactones (199) contained carbonyl absorptions (ca. 1730 cm^{-1}) consistent with the presence of saturated esters. But the ^1H n.m.r. spectrum of the mixture was complicated owing to the doubling of peaks.

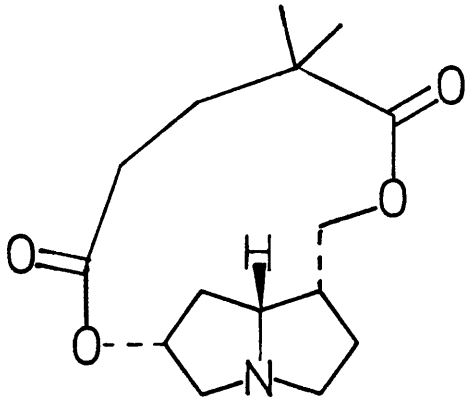
An attempt at producing the dilactone (194a) in a higher yield, was made using 2,2'-dithiobis(4-tert-butyl-1-isopropylimidazole) (201) instead of 2,2'-dithiodipyridine (130). It has been reported that imidazole thiolesters, such as (202), are more reactive (ca. 100 times) than the corresponding pyridine analogues,[103] and high yields of products are usually obtained. In this instance, it was found that the esterification did go faster with this modification (t.l.c. data), but the product yield did not significantly improve. Furthermore, purification of the final product was more difficult, because of the by-products of the reaction.

6.4 Synthesis of an 11-Membered Dilactone

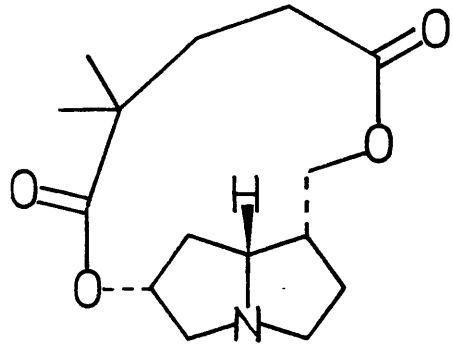
When (+)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (22) was reacted with succinic anhydride (203) in dry DME, the corresponding C-6 and C-9 zwitterionic monoesters precipitated. Lactonisation of this mixture of monoesters was carried out using the Corey-Nicolaou method with 2,2'-dithiodipyridine (130) and triphenylphosphine (131). Extraction of the basified reaction mixture with chloroform gave the succinate diester (204).



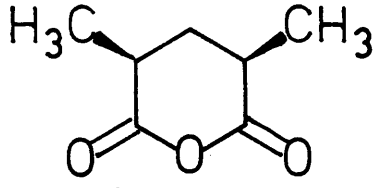
(197)



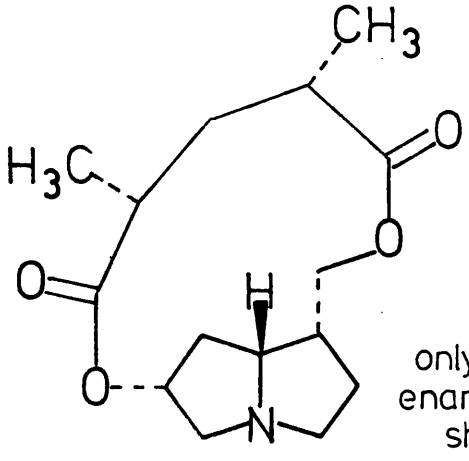
(198a)



(198b)

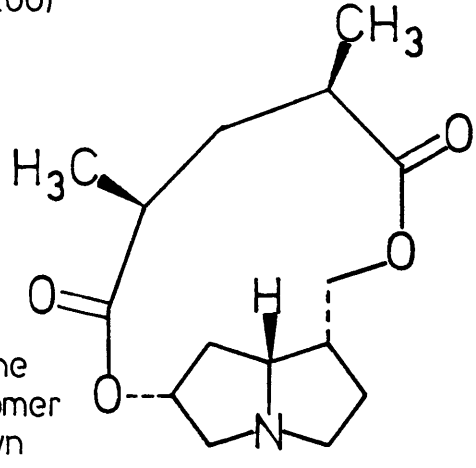


(200)

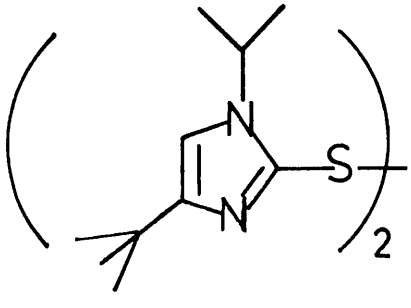


only one enantiomer shown

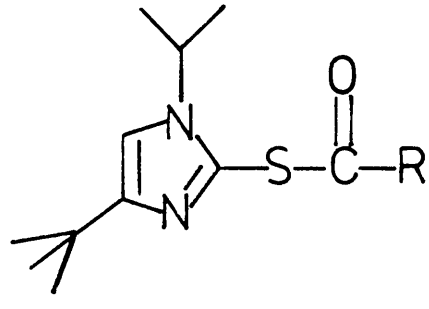
(199a)



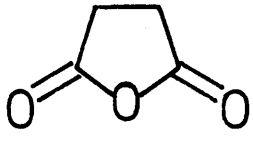
(199b)



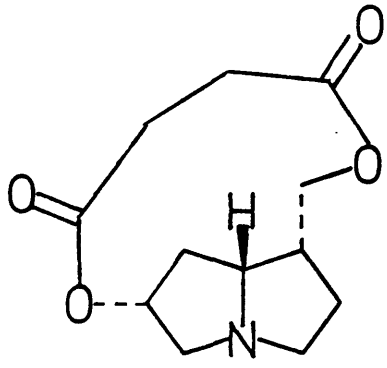
(201)



(202)



(203)



(204)

The i.r. spectrum of (204) showed the saturated ester carbonyls at 1745 cm^{-1}. High resolution mass spectrometry exhibited a fragmentation pattern similar to that obtained with the 12-membered diesters. The most important feature in the ^{1}H n.m.r. spectrum of (204) was the AB part of the ABX system at δ 3.90 and 4.72, due to the two diastereotopic protons at C-9 (Figure 19).

^{1}H N.m.r. spectroscopic data can be used to evaluate conformations of macrocyclic pyrrolizidine alkaloids in organic solution (see Chapter 1.4). In particular, the magnetic non-equivalence of the C-9 ($\Delta\delta$H-9) diastereotopic protons of the dilactones is indicative of the conformation adopted by the macrocycle in organic solution, as discussed in Chapter 1. Thus, it appears possible from Table 8, that the 12-membered macrocycles (194a-d) adopt different conformations in organic solution from the 11-membered dilactone (204). Unfortunately, this prediction could not be verified by X-ray crystallography.

Figure 19. 200.13 MHz ^{1}H N.m.r. Spectrum of (+)-6,9-O,O-(Succinyl)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (204) in $CDCl_3$.

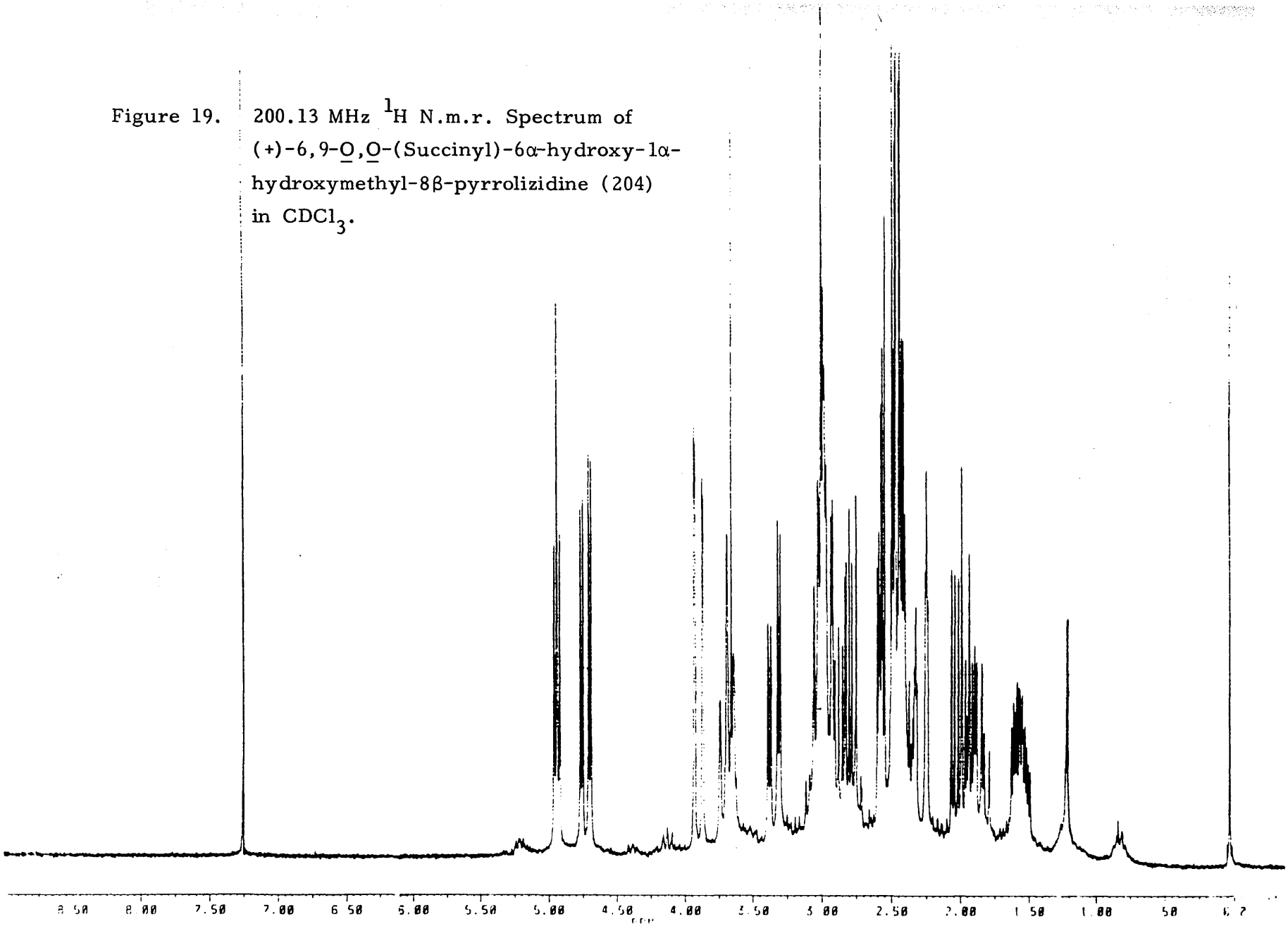


Table 8

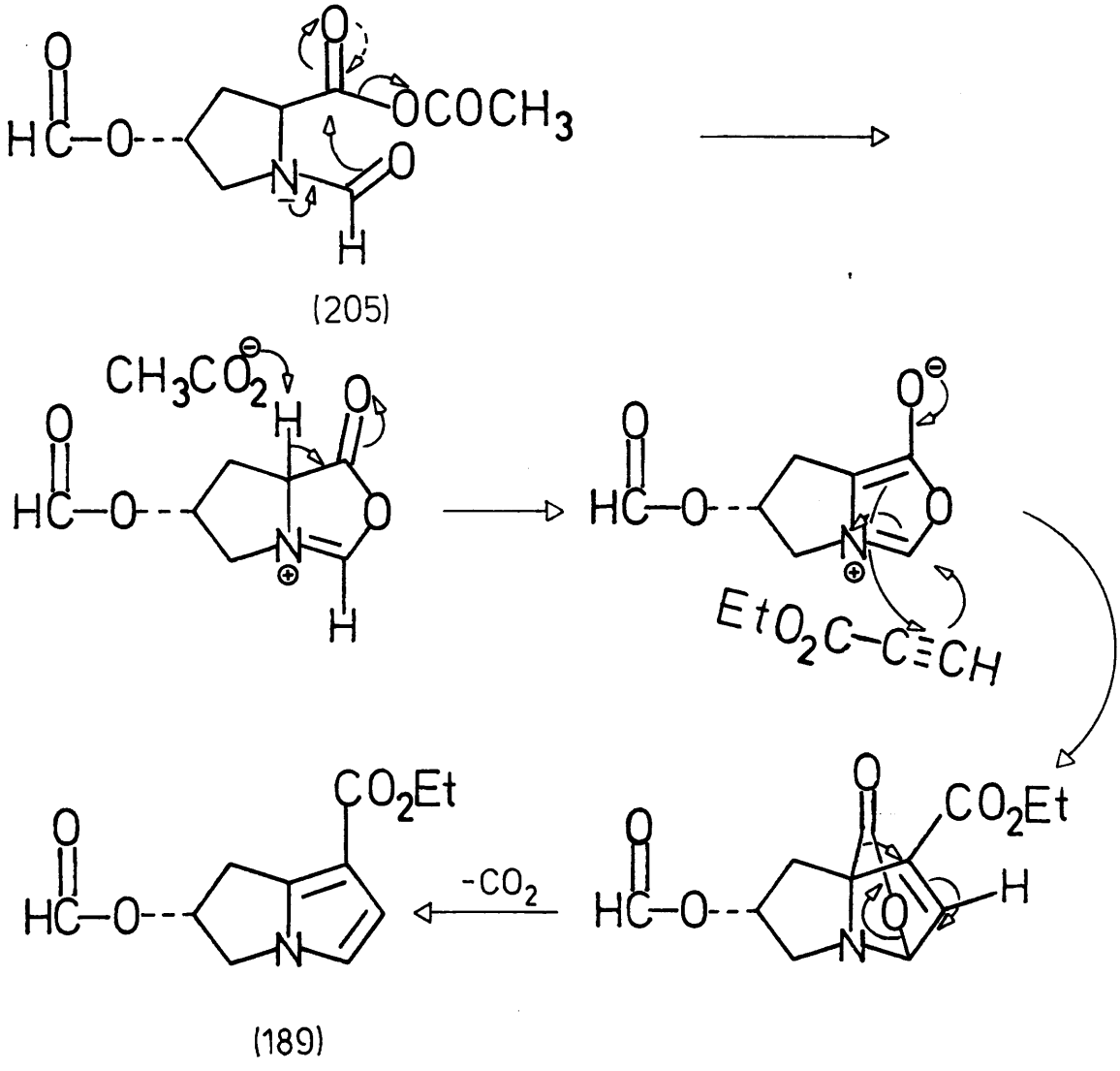
12-Membered Dilactone		Δδ H-9 (p.p.m.)
(194a)	glutarate	0.47
(194b)	3,3-dimethylglutarate	0.45
(194c)	3,3-tetramethyleneglutarate	0.40
(194d)	3,3-pentamethyleneglutarate	0.39
11-Membered Dilactone		
(204)	succinate	0.82

6.5 The 1,3-Dipolar Cycloaddition Reaction

The key reaction in the synthesis of the pyrrolizidine base (22) described in this chapter was the 1,3-dipolar cycloaddition. It is the great structural variety of both 1,3-dipoles and dipolarophiles that makes 1,3-dipolar cycloadditions very valuable and versatile in heterocyclic synthesis. Huisgen,[104] in 1961, was the first to recognise fully the general concept and scope of 1,3-dipolar cycloadditions. A 1,3-dipole is basically a system of three atoms amongst which are distributed four π-electrons as in an allyl anion system. The three atoms can be a wide variety of combinations of C, O, N, and other heteroatoms.

Huisgen and others have systematically studied the mechanism of 1,3-dipolar cycloadditions. In most cases, the evidence, like that for the Diels-Alder reaction, points to a concerted reaction in line

with orbital symmetry considerations.[105] Scheme 57 shows a postulated mechanism of the reaction between the mixed anhydride (205) and ethyl propiolate (188).



Scheme 57

Frontier Molecular Orbital (FMO) theory is used to explain regioselectivity in 1,3-dipolar cycloadditions. Essentially this attempts to predict the energy changes when two reagents approach, i.e., the interaction of the HOMO of one component with the LUMO of another, leading to bond formation. [HOMO and LUMO refer to highest occupied molecular orbital and lowest unoccupied molecular orbital, respectively]. Thus, it is necessary to know whether a particular reaction being looked at has a smaller separation between the HOMO (dipole) and the LUMO (dipolarophile) which will be common for electron-deficient dipolarophiles or, between the LUMO (dipole) and HOMO (dipolarophile), as will be common for electron-rich dipolarophiles. The former is called a dipole-HOMO controlled reaction and the latter a dipole-LUMO controlled reaction. If both frontier orbital interactions are large then this is classified as a HOMO-LUMO controlled reaction. Tables of data provide the energies of the HOMO's and LUMO's of dipoles and of dipolarophiles, making it possible to evaluate whether a reaction is Dipole-HOMO, HOMO-LUMO or Dipole-LUMO controlled.[106] But many reactions are not carried out on 'simple' unsubstituted dipoles and dipolarophiles, therefore the effects of substituents have also got to be considered.

Once the important interaction has been established, the coefficients of the relevant orbitals are assessed. Regioselectivity then follows by allowing large-large/small-small interactions to predominate.[107]

Now the reaction of N-formylproline (187) with ethyl propiolate will be discussed.

To begin with, the dominant frontier orbital interaction has to be classified. The dipole in this case is the azomethine ylide and the

dipolarophile, ethyl propiolate. Unfortunately, there is very little experimental data on orbital energies of substituted $H_2C{=}\overset{+}{N}H\overset{-}{C}H_2$ species. However, calculations have shown that electron-withdrawing groups such as the carbonyloxy substituent result in a lowering of the LUMO and a lesser lowering of the HOMO orbital energies. Figure 20 shows the approximate energies of the frontier molecular orbitals of the oxazolone (206).[108] Photo-electron spectroscopy has shown that the HOMO level of an alkyne is 0.4 to 0.9 eV lower than that of the corresponding alkene. On the other hand, the LUMO is not raised for alkynes relative to alkenes, as determined by u.v. spectroscopy.[107] The approximate energies of ethyl propiolate (the dipolarophile with an electron withdrawing substituent) are shown in Figure 20. Thus, it can be seen that this cycloaddition is a dipole-HOMO controlled reaction.

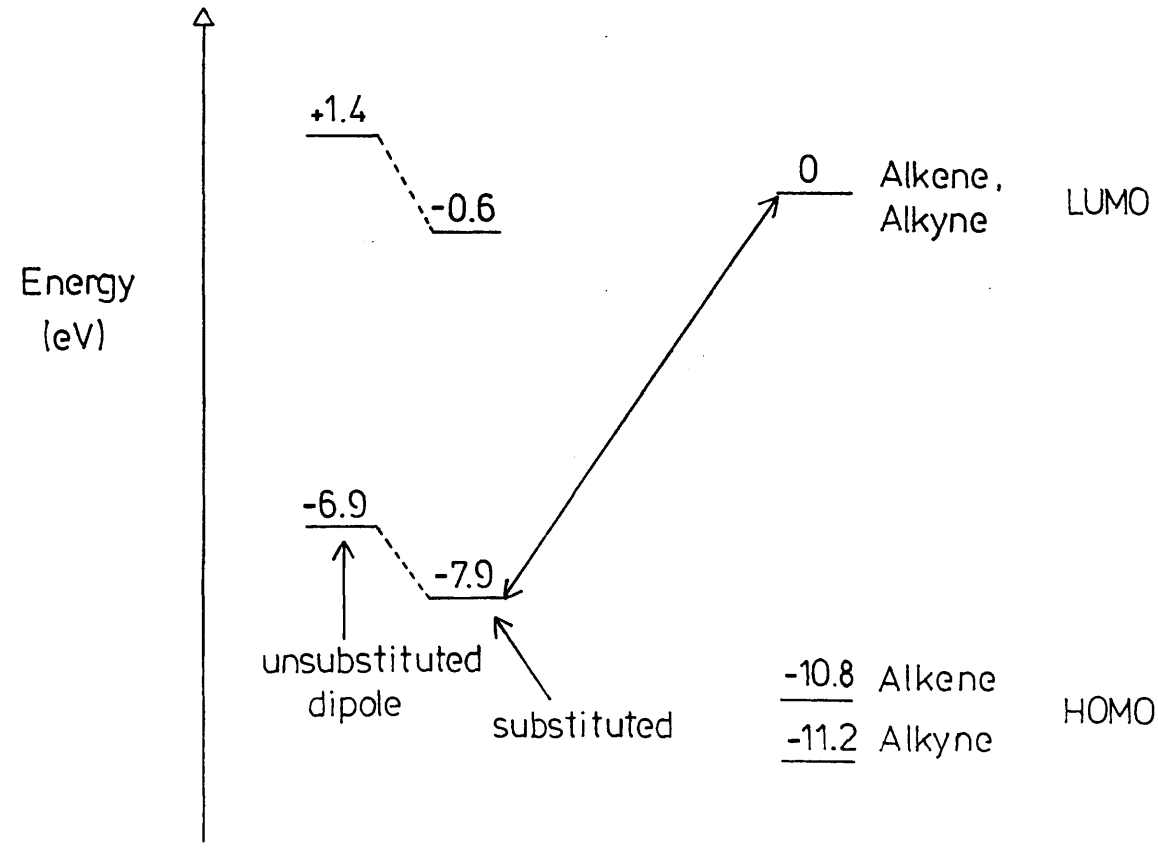


Figure 20

The coefficients of the atomic orbitals strongly influence regioselectivity in 1,3-dipolar cycloadditions. However, very few data are available in this respect, which can lead to some uncertainty in reasoning. Calculations have shown that the HOMO and LUMO of an alkene or alkyne with an electron-withdrawing substituent attached at one end have the largest coefficients at the opposite end (Figure 21)[107]. The HOMO in a 1,3-dipole has the largest coefficient on the anionic carbon, but when the dipole contains a central nitrogen atom, both ends of the dipole bear substantial negative charges, [-0.14 to -0.21 for the 'neutral' carbon atom, -0.20 to -0.40 for the anionic carbon atom and +0.23 to +0.47 eV for the central nitrogen atom].[108]

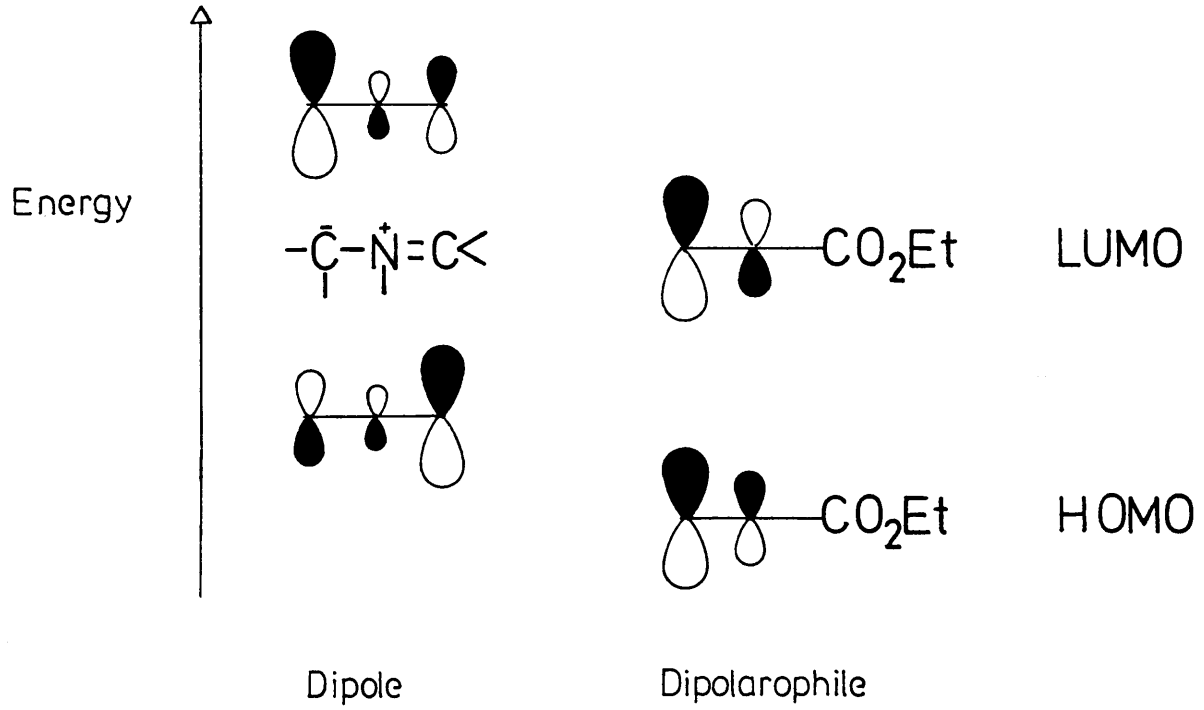
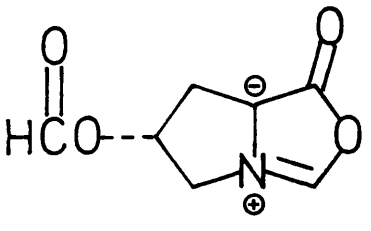


Figure 21



(206)

Thus, it is apparent that the normal interaction of such a 1,3-dipole with an electron-deficient alkene or alkyne in a dipole-HOMO controlled reaction would be for the anionic carbon orbital to overlap with the unsubstituted end of the alkene or alkyne (Figure 21). This reasoning suggests the opposite regiochemistry to that observed in the cycloaddition reaction. One possible explanation for this contradiction is that the carbonyloxy substituent has a crucial effect in determining the regioselectivity. The carbonyl must lower the coefficient at the anionic carbon end of the dipole, whereas the oxygen atom attached to the neutral carbon atom must increase the coefficient at that site in the HOMO. Consequently, the overlap occurs at the unsubstituted end of the alkyne with the neutral carbon of the oxazolone (206) to give the regioselectivity obtained.

The regioselectivity of this cycloaddition might have been easier to understand if sufficient data had been available on the atomic orbital coefficients of substituted 1,3-dipoles.

6.6 Conclusions

A series of 12-membered macrocyclic diesters of (+)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (22) has been synthesised, along with one 11-membered alkaloid analogue, employing the Corey-Nicolaou reaction. This method had two major drawbacks. Firstly, there was difficulty in removing all traces of the by-products, triphenylphosphine oxide (195) and thiopyridone (196). Secondly, and more importantly, the dilactones were formed in low to modest yields. This may be partly attributed to

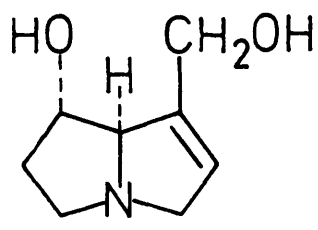
the instability of the cyclised products towards the conditions involved in the purification procedure. The acid/base work-up may have resulted in the partial hydrolysis of the diesters. Nevertheless, the work described here has laid the foundation for a more efficient and high yielding synthesis of the macrocycles, which will be necessary to study structure-activity relationships.

Chapter 7

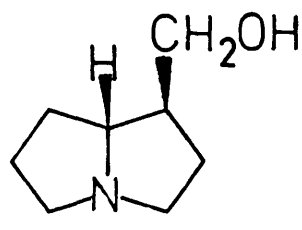
PYRROLIZIDINE ALKALOIDS FROM LINDELOFIA LONGIFLORA AND CYNOGLOSSUM MACROSTYLUM

7.1 Introduction

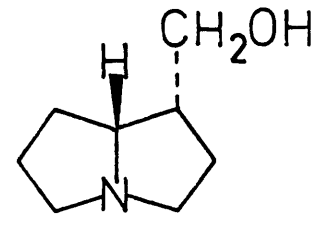
An investigation into the pyrrolizidine alkaloid content of two plants, Lindelofia longiflora and Cynoglossum macrostylum was conducted. Both species belong to the Boraginaceae family. Although this family contains the largest number of different genera shown to contain pyrrolizidine alkaloids, very few plant species of each genus have been studied. In fact, only ten species of the Lindelofia and Cynoglossum genera have been examined.[2] We therefore decided to investigate the alkaloidal content of species from these genera. As in many studies on the pyrrolizidine alkaloids present in plants, chemotaxonomic relationships were expected to be of value in arriving at the structures for the alkaloids. It is known that the majority of Lindelofia and Cynoglossum species which have been studied contain pyrrolizidine alkaloids which are monoesters of heliotridine (207), isoretronecanol (51b) or trachelanthamidine (51a). The esterifying acid is commonly viridifloric acid (208).



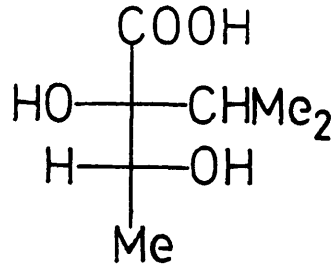
(207)



(51b)



(51a)



(208)

7.2 Extraction of Lindelofia longiflora

Lindelofia longiflora plants were obtained from the Edinburgh Botanic Garden. They were grown in the open ground and harvested when flowering. The fresh plant material was extracted with methanol, and the extract, after concentration, was taken up in dilute sulphuric acid. The acid solution, after being washed with dichloromethane, was stirred with zinc powder to reduce any N-oxides, filtered, and basified. The basic solution was extracted with dichloromethane to give one alkaloid in 0.1% yield, based on the dry weight of plant material.

7.3 Identification of Echinatine (123)

A chromatogram of the alkaloid gave a positive result (i.e. purple spot) when sprayed with o-chloranil followed by Ehrlich's reagent,[68] indicating the presence of 1,2-unsaturation. The i.r. spectrum exhibited an absorption at 1728 cm^{-1} due to an αβ-unsaturated ester system. High resolution mass spectrometry and analytical data established the molecular formula of the alkaloid as $C_{15}H_{25}NO_5$. Several pyrrolizidine alkaloids have this molecular formula, but after consideration of the ^1H and ^{13}C spectroscopic data of the alkaloid, it

was concluded that the most likely structure was echinatine (123).

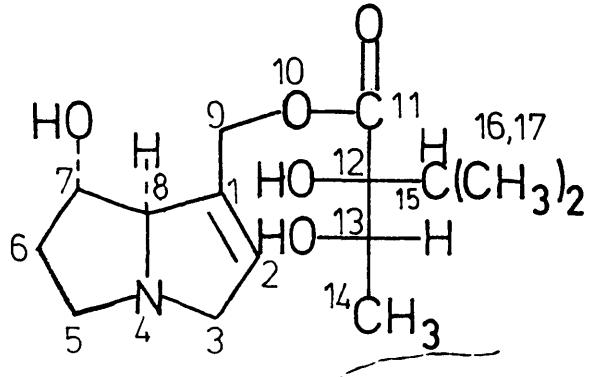
The ^{1}H n.m.r. spectrum of the alkaloid (Figure 22), run in deuteriochloroform, displays an AB quartet at δ4.79 and 4.96 (J 13.4 Hz) for the C-9 diastereotopic protons. The vinyl proton at C-2 appears as a broad singlet at δ5.70. The proton at C-7 is coupled to the two C-6 protons, giving rise to a triplet, and this in turn is split by the C-8 proton, thereby producing a doublet of triplets centred at δ4.15. The C-15 proton is coupled to the two non-equivalent methyl group protons at C-16 and C-17, generating a doublet of quartets at δ2.18 (J 6.8 Hz). The protons of the two methyl groups are coupled to the C-15 proton, producing two doublets at δ0.89 and 0.93 (J 6.8 Hz). Coupling of the C-13 proton to the C-14 methyl group protons results in a quartet at δ3.99 (J 6.6 Hz) and coupling of the C-14 methyl group protons with the C-13 proton gives rise to a doublet at δ1.27 (J 6.6 Hz).

The ^{13}C n.m.r. spectrum of the alkaloid was assigned on the basis of a DEPT experiment and good agreement with the data published for echinatine[109] was obtained.

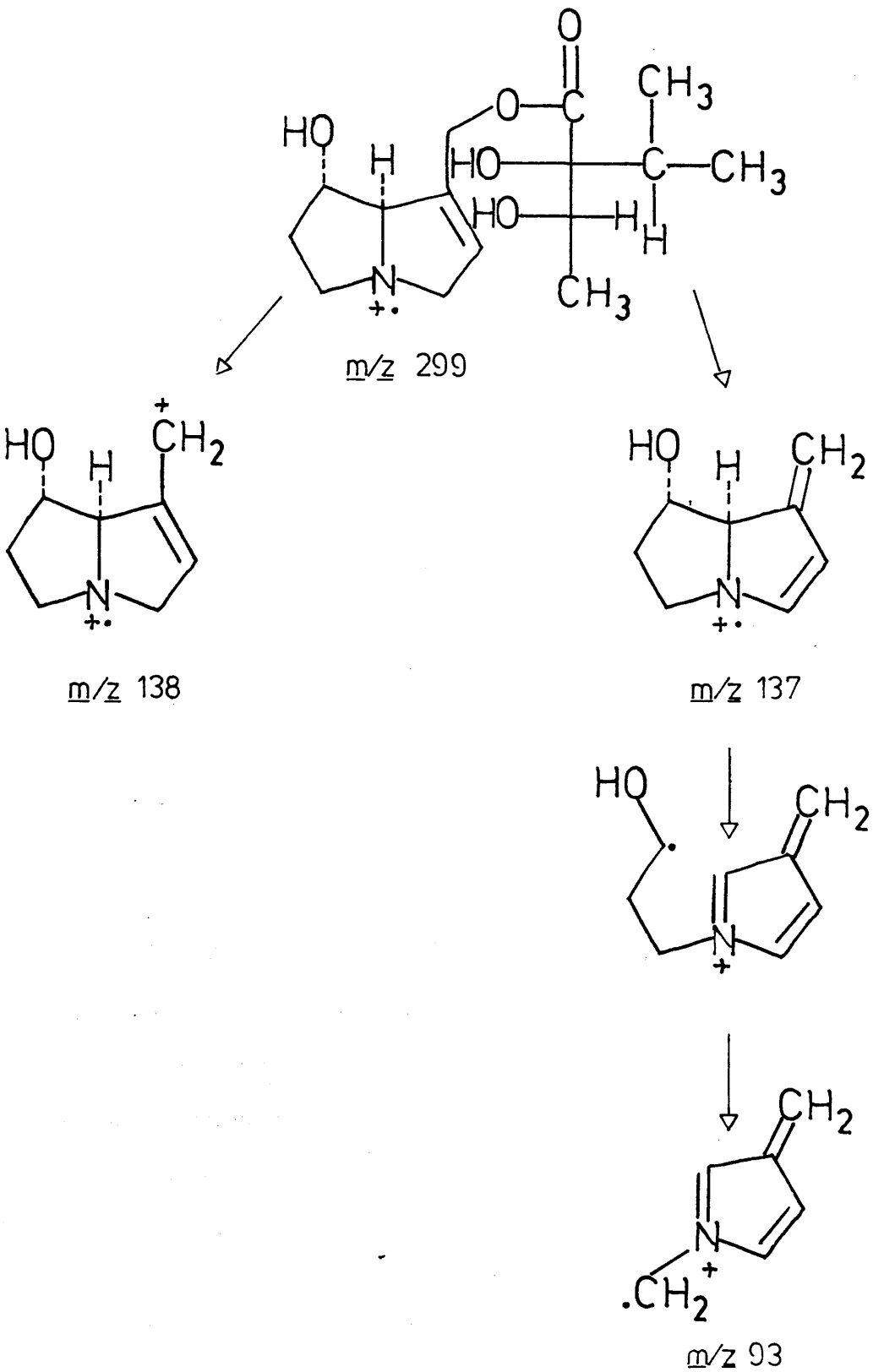
The mass spectrum of the alkaloid contained a molecular ion at m/z 299. The base peak was at m/z 138, resulting from fission of the allylic ester of the alkaloid. Fragment ions at m/z 137 and 93 were also intense. The former was produced after a McLafferty rearrangement and loss of the viridifloric acid (208) portion that had esterified C-9. Scheme 58 shows a probable fragmentation pathway.

Finally, the structure of the alkaloid was confirmed by comparison of the i.r., ^{1}H and ^{13}C n.m.r. spectra and mass spectrum

Figure 22. 200.13 MHz ^{1}H N.m.r. spectrum of echinatine (123) in $CDCl_3$.



Probable Fragmentation Pattern for Echinatine (123)



Scheme 58

with an authentic sample of echinatine, isolated by D.B. Hagan from Cynoglossum officinale.

The first isolation of echinatine (123) was from Rindera echinata Regel in 1953, by Men'shikov and Denisova.[110] Its structure was established by the same group of workers.

7.4 Extraction of Cynoglossum macrostylum

Cynoglossum macrostylum was grown from seeds supplied by the Edinburgh Botanic Garden, in the open ground, and the plants were harvested when red flowers first appeared. Fresh plant material was extracted employing the method detailed in 7.2. A crude alkaloid mixture was obtained in 0.1% yield, based on the dry weight of plant material. An examination of the mixture by t.l.c. showed three bands. The component of lowest R_F was present in the greatest amount. Separation of these alkaloids was accomplished by column chromatography on basic alumina.

7.5 Identification of Heliosupine (84)

The alkaloid which was obtained from the middle band on chromatography, was shown to contain 1,2-unsaturation when treated with o-chloranil followed by Ehrlich's reagent.[68] The i.r. spectrum indicated an αβ-unsaturated ester system at 1730 cm^{-1}. The absorption due to the carbon-carbon double bond of the αβ-unsaturated ester system was at 1650 cm^{-1}. After examination of the ^{1}H and ^{13}C n.m.r. spectroscopic data of the alkaloid, it was deduced that this component of the

mixture was heliosupine (84).

The ^{1}H n.m.r. spectrum of the alkaloid run in deuteriochloroform is similar to that published for heliosupine[111] (Figure 23). Thus, an easily recognisable AB system at δ4.89 and 5.05 (J 13.1 Hz) is due to the non-equivalent C-9 protons. Since the chemical shift difference between these two protons does not exceed 0.4 p.p.m., this is good evidence that the alkaloid does not contain a macrocyclic system.[1] A distorted triplet centred at δ5.23 is due to acylation of the hydroxy group at C-7. The broad singlet centred at δ5.90 is assigned to the vinyl proton at C-2. The protons belonging to the two methyl groups at C-16 and C-17 give rise to a six proton singlet at δ1.24. A quartet at δ4.18, associated with the proton at C-13, arises from coupling with the adjacent protons of the methyl group at C-14. Conversely, the methyl group protons at C-14 couple with the single proton at C-13 and produce a doublet centred at δ1.28. A three proton singlet at δ1.85 is due to the methyl group at C-23. A doublet at δ1.95 is a consequence of the coupling of the C-22 methyl group protons to the olefinic proton at C-21. Finally, the doublet of quartets at δ6.12 arises from the olefinic proton at C-21 coupling to the C-22 and C-23 methyl group protons. The complicated signals at δ2.90-4.35 are due to the methylene protons at C-3, C-5 and C-6, and to the methine proton at C-8.

The ^{13}C n.m.r. spectrum of the alkaloid is also similar to that reported for heliosupine (84).[111] A DEPT pulse sequence confirmed the number of each type of carbon atom in the alkaloid.

The mass spectrum of heliosupine (84) did not show a molecular ion at m/z 397, but displayed peaks at m/z 297 and m/z 220 due to M^{+}-

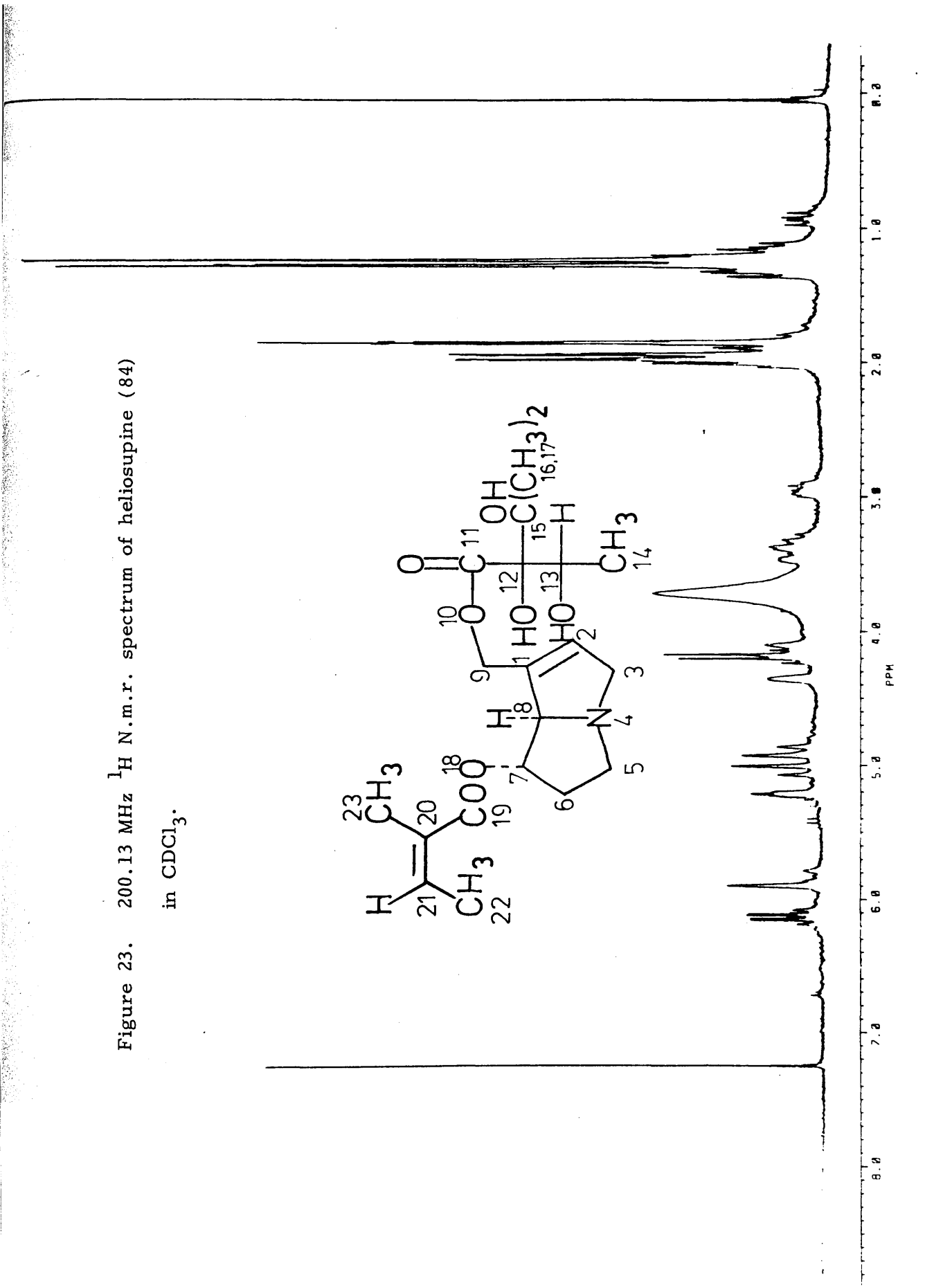


Figure 23. 200.13 MHz ^1H N.m.r. spectrum of heliosupine (84) in $CDCl_3$.

angelic acid and $\underline{M}^{+}$-echimidinic acid, respectively. McLafferty rearrangements and rearrangements involving loss and gain of hydrogen atoms from the pyrrolizidine ring accounted for peaks of lower intensity in the spectrum. Scheme 59 shows a possible fragmentation of heliosupine (84).

Final confirmation of the identity was provided by a non-depression of a mixed melting point of the picrate of heliosupine, with an authentic sample supplied by Prof. D.H.G. Crout.

Heliosupine (84) was first isolated from Heliotropium supinum L., (family Boraginaceae) by Denisova et al., and its structure was elucidated by the same group of workers.[112]

7.6 Identification of Echinatine (123)

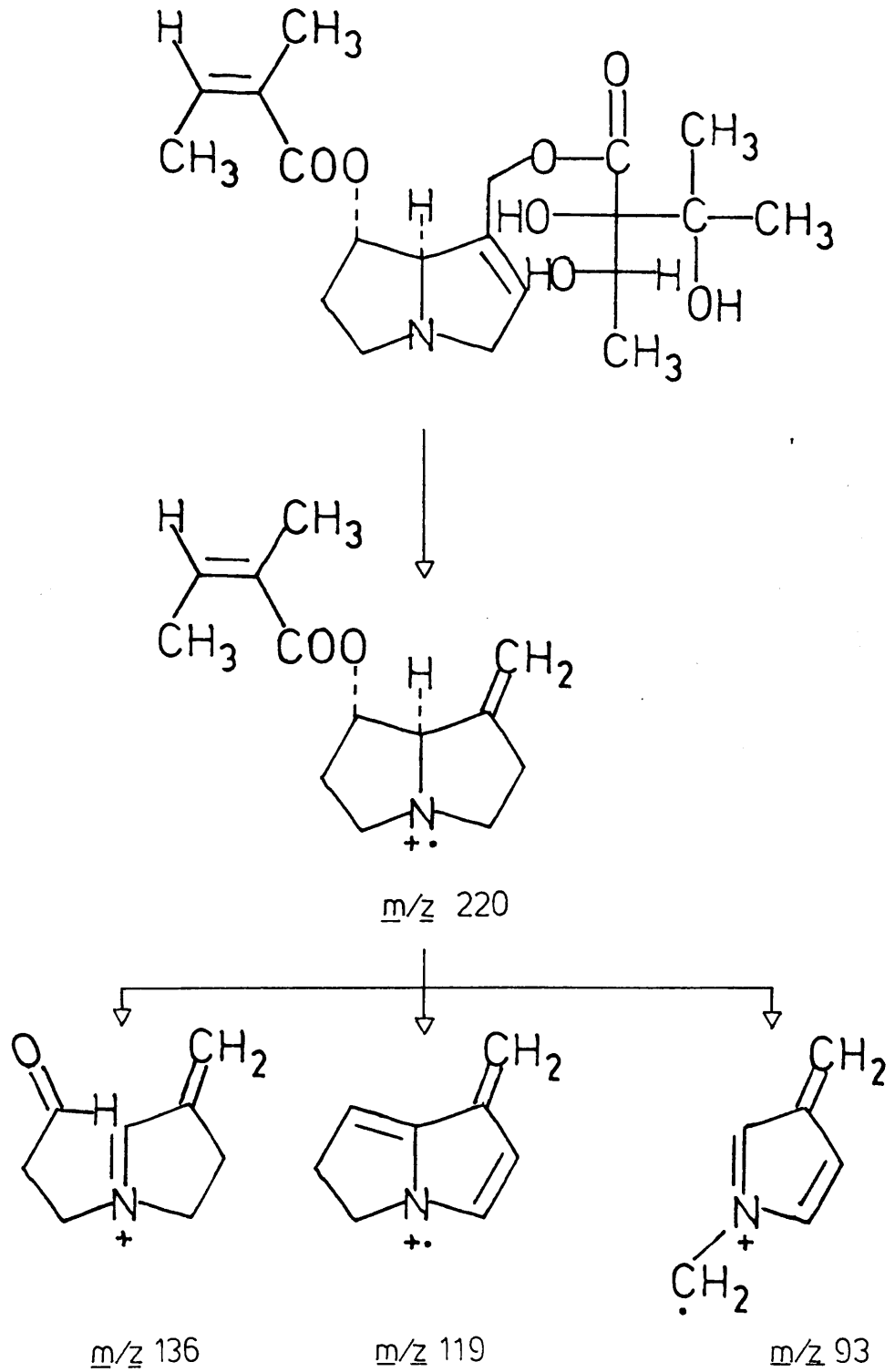
The most polar component of the alkaloid mixture was echinatine (123). This was the major component of the mixture of alkaloids (75%) and all the spectroscopic data were identical to those reported in 7.3.

The least polar alkaloid (gave a positive result with Ehrlich's reagent) could not be identified because of insufficient material.

7.7 Conclusions

Lindelofia longiflora was shown to contain one pyrrolizidine alkaloid, echinatine (123). Cynoglossum macrostylum was shown to produce three pyrrolizidine alkaloids. two of which were identified as echinatine (123) and heliosupine (84). The former was the most abundant.

Probable Fragmentation Pattern for Heliosupine (84)



Scheme 59

Chapter 8

EXPERIMENTAL

8.1 General

All melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with an Optical Activity Ltd. AA 10 Polarimeter. Infra red spectra were obtained on a Perkin Elmer 580 spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Perkin Elmer R32 spectrometer operating at 90 MHz (δ_H), a Varian XL-100 spectrometer operating at 25 MHz (δ_C), a Bruker WP200-SY spectrometer operating at 200 MHz (δ_H), 50 MHz (δ_C) and 30.72 MJz (δ_D). Spectra were recorded for solutions in deuteriochloroform unless otherwise stated, with tetramethylsilane as internal standard. Mass spectra were obtained with A.E.I. MS 12 or 902 spectrometers.

T.l.c. was carried out on Kieselgel G plates of 0.25 mm thickness and developed with chloroform-methanol-conc. ammonia (85:14:1) unless otherwise stated. The alkaloids were detected by the modified Dragendorff reagent,[113] or by oxidation with o-chloranil, followed by treatment with Ehrlich's reagent.[68]

All ^{13}C-labelled compounds were purchased from B.O.C. Prochem Ltd., London. Radiochemicals were purchased from Amersham International. Radioactivity was measured with a Philips PW 4700 Liquid Scintillation Counter using toluene-methanol solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each

determination. Radioactive samples were recrystallised to constant specific radioactivity and they were counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for radioscanning of t.l.c. plates.

1,2-Dimethoxyethane (DME) and tetrahydrofuran (THF) were dried by distillation from potassium hydroxide and then from sodium-benzophenone under argon prior to use. N,N-Dimethylformamide (DMF) and acetone were dried utilising 3A° molecular sieves.[114] Dichloromethane and chloroform were pre-dried with calcium chloride, and then distilled from phosphorus pentoxide.[115] Organic solutions were dried with anhydrous magnesium sulphate and solvents were evaporated off under reduced pressure below 50°C.

8.2 Experimental to Chapter 3

Standard Procedure for Extracting Rosmarinine (20) from Senecio pleistocephalus

Fresh leaves and stems (200g) of Senecio pleistocephalus S. Moore plants (family Compositae), were finely chopped and blended with methanol in portions. The blended extracts were filtered and the methanolic filtrates were concentrated under reduced pressure. The resulting green residue was dissolved in dichloromethane (100 ml) and extracted with 2M sulphuric acid (3 x 100 ml). The acidic extracts were combined and washed with dichloromethane (6 x 150 ml). Powdered zinc metal (5g) was added and the mixture was stirred at room temperature for 2h. After filtering through Celite, the filtrate was made basic by adding concentrated ammonia solution (pH 9) and the resultant alkaline solution was extracted with dichloromethane (4 x 300 ml). The combined organic extracts were dried, filtered, and concentrated under vacuum to afford crude rosmarinine (20), which was recrystallised from dichloromethane/acetone (1:1), to give fine white crystals (290 mg), m.p. 204°C (decomp.) (lit.,[69] 202-204°C); $[\alpha]_D^{14}$ -85.6° (c 1, MeOH), (lit.,[69] $[\alpha]_D^{24}$ -85.3°); $\nu_{max.}$ (KBr disc) 3420, 2970, 2920, 1740, 1720, and 1160 cm^{-1}; δ_H (200 MHz) 0.94 (3H, d, J 6.5 Hz, 19-H), 1.32 (3H, s, 18-H), 1.75 (1H, m, 13-H), 1.82 (3H, dd, J 7.2 Hz and 1.5 Hz, 21-H_3), 1.98 (1H, m, 6α-H), 2.04 (1H, m, 6β-H), 2.21 (2H, m, 14-H_2), 2.48 (1H, m, 1-H), 2.59 (1H, m, 5β-H), 2.90 (1H, dd, J 11.1 Hz and 8.0 Hz, 3β-H), 3.03 (1H, dd, J 11.1 Hz and 7.5 Hz, 3α-H), 3.24 (1H, ddd, J 9 Hz, 7 Hz and 2 Hz, 5α-H), 3.54 (1H, dd, J 7.7 Hz and 3.2 Hz, 8-H),

4.08 (1H, dd, J 12.5 Hz and 1.0 Hz, 9-Hpro-R), 4.20 (1H, ddd, J 9.5 Hz, 8.0 Hz and 7.3 Hz, 2-H), 4.87 (1H, dd, J 12.6 Hz and 5.2 Hz, 9-Hpro-S), 5.00 (1H, ddd, J 3.5 Hz, 3.3 Hz and 1.2 Hz, 7-H), and 5.76 p.p.m. (1H, dq, J 7.2 Hz and 1 Hz, 20-H); δ_C (50 MHz) 11.6 (C-19), 15.1 (C-21), 25.6 (C-18), 34.4 (C-6), 37.8 (C-13), 39.5 (C-14), 49.1 (C-1), 53.5 (C-5), 61.3 (C-3), 62.2 (C-9), 69.1 (C-2), 69.3 (C-8), 75.3 (C-7), 77.5 (C-12), 132.7 (C-15), 134.4 (C-20), 167.5 (C-16), and 180.6 p.p.m. (C-11); m/z 353 (M^+, 3%), 227, 156, 155, 154, 139, 138 (100%), 137, 122, 112, 111, and 82 (Found: M^+, 353.1847. $C_{18}H_{27}NO_6$ requires M, 353.1839). (Found: C, 60.98; H, 7.40; N, 3.87. $C_{18}H_{27}NO_6$ requires C, 61.17; H, 7.70; N, 3.96%).

1,4,-Diamino-[1-^{13}C]butane dihydrochloride (89).- Dry, powdered sodium [^{13}C]cyanide (1g, 20 mmol, 91 atom % ^{13}C) was added to a solution of N-(3-bromopropyl)phthalimide (39) (4.47g, 16.6 mmol) in dry dimethylsulphoxide (50 ml). The mixture was stirred at 90°C for 2h, and then left overnight at room temperature. Diethyl ether (300 ml) was added, and the mixture was washed with water (6 x 50 ml), and brine (3 x 50 ml). The organic layer was dried (Na_2SO_4), filtered and concentrated to yield 4-phthalimido-[1-^{13}C]butanenitrile as an oil (1.75g, 49%); $\nu_{max.}$ (film) 2200, 1775, and 1715 cm^{-1}; δ_H (90 MHz), 2.09 (2H, t, J 7.2 Hz), 2.4 (2H, m), 3.82 (2H, t, J 7.2 Hz), and 7.75 p.p.m. (4H, m); $\delta_C\{^1H\}$ (25 MHz) 118.6 p.p.m. (s); m/z 216 (M^+), 160, 133, 105, 104, and 76.

4-Phthalimido-[1-^{13}C]butanenitrile (0.5g, 2.3 mmol) was added to a suspension of platinum(IV) oxide (75 mg) in glacial acetic acid (12 ml),

and the mixture was hydrogenated at atmospheric pressure for 4h. The mixture was filtered, and the filtrate was concentrated to give crude 4-phthalimido-1-amino-[1-^{13}C]butane (450 mg, 88%). The crude product was hydrolysed by heating it at reflux in conc. HCl (15 ml) for 20h. Precipitated phthalic acid was removed by filtration after cooling the reaction product to 0°C. The filtrate was evaporated to dryness, and the residue was recrystallised from aqueous ethanol to yield 1,4-diamino-[1-^{13}C]butane dihydrochloride (89) (276 mg, 83%); δ_H (90 MHz) (D_2O) 1.79 (4H, br s) and 3.08 p.p.m. (2.2H, br s + 1.8H, d, $\underline{J}$ (^{13}C-^{1}H) 150 Hz); $\delta_C\{^1H\}$ (25 MHz) (D_2O) 39.6 p.p.m. (s).

1,4-Diamino-[2,3-$^{13}C_2$]butane dihydrochloride (37).- This was prepared by the method of Khan and Robins[41] with a modification to the reduction step.

A solution of sodium cyanide (1.5g, 31 mmol) in water (5 ml) was added to 1,2-dibromo[1,2-$^{13}C_2$]ethane (81%, $^{13}C_2$, 18% ^{13}C ^{12}C, and 1% $^{12}C_2$ species) (1g, 5.1 mmol) in ethanol (100 ml), and the mixture was heated at reflux for 4h. The solution was cooled and concentrated. The residue was taken up in water (50 ml) and extracted continuously with chloroform for 24h. The chloroform extracts were dried, filtered, and evaporated under reduced pressure to give [2,3-$^{13}C_2$]succinonitrile, (253 mg, 59%); m.p. 53-54°C [lit.,[41] (for unlabelled material) 57°C]; $\nu_{max.}$ (thin film) 2258 cm^{-1}. The dinitrile (230 mg, 2.8 mmol) was added to a suspension of platinum(IV) oxide (Johnson Matthey) (35 mg, 15% by weight) in glacial acetic acid (10 ml). The mixture was stirred under a

hydrogen atmosphere until no more hydrogen was taken up (ca. 30h). The suspension was filtered through Celite, and the filtrate was concentrated to give an oil. The oil was dissolved in 4M hydrochloric acid (10 ml) and concentrated to afford crude [2,3-$^{13}C_2$]putrescine dihydrochloride (37), which was recrystallised from aqueous ethanol (95%), (360 mg, 79%); δ_H (90 MHz) (D_2O) 2.3 (0.4H, s + 3.6H, d, J (^{13}C-^{1}H) 125 Hz), and 3.7 p.p.m. (4H, br s); $\delta_C\{^1H\}$ (25 MHz) (D_2O) 26.3 p.p.m. (s).

1,4-Diamino-[1-amino-^{15}N, 1-^{13}C]butane dihydrochloride (38).- The title compound (38) was prepared according to the method of Khan and Robins,[41] in an overall yield of 61%; δ_H (90 MHz) (D_2O) 1.8 (4H, br s), and 3.1 p.p.m. (2.2H, br s + 1.8H, d, J (^{13}C-^{1}H) 135 Hz); $\delta_C\{^1H\}$ (25 MHz) (D_2O) 41.2 p.p.m. (d, J 4.5 Hz).

[1,9-$^{13}C_2$]Homospermidine trihydrochloride (47).- Preparation of the trihydrochloride (47) was carried out by Dr. H.A. Khan.[41] The salt contained ca. 96% $^{13}C_2$ and 4% $^{13}C_1$ species; $\delta_C\{^1H\}$ (25 MHz) (D_2O) 39.6 p.p.m.

1,4-Diamino-[1,1-$^{2}H_2$]butane dihydrochloride (92)† [cf. method of Callery et al.[73]].- 4-Phthalimidobutanenitrile (2.36g, 11 mmol) was added to a suspension of platinum(IV) oxide (0.32g) in monodeuterioacetic acid. The mixture was stirred under a deuterium atmosphere at atmospheric pressure for 60h. The mixture was filtered through Celite, and the

† see p.175 for explanation

filtrate was concentrated to give crude 4-phthalimido-[1-$^2H_2$]-1-amino-butane. The crude product was heated at reflux in 4M hydrochloric acid (70 ml) for 6h. The reaction mixture was cooled to 0°C, and the precipitated phthalic acid was filtered off. The filtrate was evaporated to dryness, and the residue was recrystallised from aqueous ethanol to yield [1,1-$^2H_2$]butane-1,4-diamine (92) dihydrochloride (1.51g, 84.2%); δ_H (90 MHz) (D_2O) 1.77 (4H, br s) and 3.06 p.p.m. (2H, br s); δ_D (25 MHz) (H_2O) 3.05 p.p.m. (s). The diphenylamino(thiocarbonyl) derivative had m.p. 177-178°C [lit.,[116] (for unlabelled material), 177-179°C]; δ_H (90 MHz) [$(CD_3)_2SO$; 80°C] 1.58 (s, 4H), 3.48 (s, 2H), 7.25 (m, 10H), 7.75 (s, 2 x NH), 9.35 (s, 2 x NH); m/z 360 (unlabelled material m/z 358).

1,4-Diamino-[1,4-$^2H_4$]butane dihydrochloride (91)†.- The ^2H-labelled putrescine (91) was made from succinonitrile as described by Rana and Robins.[49] The ^1H n.m.r. spectrum indicated that the dihydrochloride contained ca. 96% $^2H_4$ species; δ_D (30.7 MHz) (H_2O) 2.86 p.p.m. (s).

1,4-Diamino-[2,3-$^2H_4$]butane dihydrochloride (104)†.- Preparation of the dihydrochloride (104) was accomplished using the method of Rana and Robins.[49] The salt contained > 99% $^2H_4$ species; δ_D (30.7 MHz) (H_2O) 1.56 p.p.m. (s).

(R)-1,4-Diamino-[1-^2H]butane dihydrochloride (52).- Enzymatic decarboxylation of L-ornithine in D_2O using L-ornithine decarboxylase, gave the title compound (52), according to the method of Spenser and Richards.[50] From ^1H n.m.r. spectroscopic data, the deuterium content

was estimated to be 97% $^2H_1$; δ_D (30.7 MHz) (H_2O) 2.87 p.p.m. (s).

(S)-1,4-Diamino-[1-^{2}H]butane dihydrochloride (53).- The enantiomer was synthesised by enzymatically decarboxylating [2-^{2}H]-DL-ornithine (55) in water using L-ornithine decarboxylase.[50] The ^{1}H n.m.r. spectrum (H_2O) was identical to that of (R)-[1-^{2}H]putrescine dihydrochloride. The deuterium content was estimated to be ca. 90% $^2H_1$ species; δ_D (30.7 MHz) (H_2O) 2.75 p.p.m. (s).

(R)-1,4-Diamino-[2-^{2}H]butane dihydrochloride (65)† and (S)-1,4-Diamino-[2-^{2}H]butane dihydrochloride (66)†.- The enantiomerically ^{2}H-labelled putrescines (65) and (66) were prepared according to the procedure of Kunec and Robins.[54] Mass spectral and n.m.r. data on (2R)-[2-^{2}H]putrescine dihydrochloride (65) indicated ca. 98% $^2H_1$ species; δ_D (30.7 MHz) (H_2O) 1.55 p.p.m. (s). Similar data showed that the enantiomer (66) was ca. 83% $^2H_1$ species; δ_D (30.7 MHz) (H_2O) 1.58 p.p.m. (s).

† I should like to express my gratitude to my colleagues, Desmond B. Hagan who kindly provided precursors (91), (92) and (104) and to Dr. Ellen K. Kunec who provided samples (65) and (66).

Feeding Methods.- Senecio pleistocephalus S. Moore plants were obtained from the Royal Botanic Garden, Edinburgh, and were propagated from stem cuttings and grown in pots in a standard compost in a greenhouse. One well-rooted plant was used for each experiment. A sample of [1,4-^{14}C]putrescine dihydrochloride (5 or 10 μCi) was added to each ^{13}C- and ^{2}H-labelled putrescine, and [1,9-^{14}C]homospermidine trihydrochloride (20 μCi) was added to [1,9-$^{13}C_2$]homospermidine (47) trihydro-

chloride. Each precursor was divided into equal portions, which were dissolved in sterile water and fed by the wick method, on successive days for 7-10 days. One week after administration of each precursor, the plants were harvested. The labelled rosmarinine was isolated according to the standard procedure (p.170), and recrystallised to constant specific radioactivity from dichloromethane-acetone. In each experiment radioscans of t.l.c. plates showed one radioactive band coincident with authentic unlabelled rosmarinine at R_F 0.30. Incorporation figures for each experiment are provided in Table 5, p.94.

8.3 Experimental to Chapter 4

8.3.1 [1-^{14}C]4-Chlorobutanenitrile (110).- Sodium cyanide (588 mg, 12.0 mmol) and sodium [^{14}C]cyanide (500 μCi) were dissolved in dry dimethylsulphoxide (DMSO) (40 ml) at 90°C under a dry argon atmosphere. 3-Chloro-1-propanol methanesulphonate (109) (2.0g, 111.6 mmol) was added with stirring, and heating was continued at 85-90°C for 2h, then the mixture was allowed to cool to room temperature overnight. The mixture was diluted with diethyl ether (100 ml) and water was added (50 ml). The layers were separated and the aqueous fraction was extracted with further diethyl ether (75 ml). The organic extracts were combined, washed with water (4 x 100 ml) and brine (2 x 100 ml), filtered, and concentrated to give a yellow oil. To remove traces of DMSO and starting material, the oil was dissolved in diethyl ether (20 ml) and passed through a short silica column (10g). The column was eluted with diethyl ether (50 ml) and the combined organic solutions were concentrated to give the chloronitrile (110) as a pale yellow oil (725 mg, 58%, 28.3 μCi mmol^{-1}). All physical properties were identical to authentic, unlabelled material e.g., δ_H (90 MHz) 2.15 (2H, m, $C\underline{H}_2$-CH_2CN), 2.55 (2H, t, $\underline{J}$ 7 Hz, $C\underline{H}_2$-CN), and 3.65 p.p.m. (2H, t, $\underline{J}$ 7 Hz, $C\underline{H}_2$-Cl).

N-([4-^{14}C]-3-Cyanopropyl)pyrrolidine (111).- A solution of the 4-chlorobutanenitrile (110) (688 mg, 6.65 mmol) in dry butanol (3 ml) was added to a stirred mixture of pyrrolidine (667 μl, 8.0 mmol), anhydrous sodium carbonate (707 mg, 6.65 mmol) and potassium iodide (184 mg, 1.11 mmol), and was stirred for 18h. The solution was cooled, and the solid

which was filtered off, was washed well with diethyl ether (50 ml). The washings and filtrate were combined and extracted with 4M hydrochloric acid (3 x 50 ml). The aqueous extracts were washed with diethyl ether (2 x 100 ml), basified with sodium carbonate and extracted with diethyl ether (3 x 100 ml). The ethereal extracts were dried, filtered, and concentrated to give an oil which was distilled; yield 403 mg, 44%, 30.8 μCi $mmol^{-1}$; b.p. 55°C (0.2 mm Hg). All physical properties were identical to authentic unlabelled material, e.g. $\nu_{max.}$ ($CHCl_3$) 2260 cm^{-1} (CN); δ_H (90 MHz) 1.76 (6H, m), and 2.49 p.p.m. (8H, m).

N-([4-^{14}C]4-Aminobutyl)pyrrolidinium dihydrochloride (112).- Adam's catalyst [Platinum(IV) oxide] (30 mg, 15% w/w) was added to the ^{14}C-labelled nitrile (111) (200 mg, 1.45 mmol) in glacial acetic acid (5 ml), and the mixture was hydrogenated at atmospheric pressure for 18h. The catalyst was removed by filtering through Celite and concentrated hydrochloric acid (8 ml) was added. The solution was concentrated under reduced pressure to give a yellow solid. The dihydrochloride (112) was obtained by slow recrystallisation from aqueous ethanol (95%); (280 mg, 90%, 55.4 μCi $mmol^{-1}$), m.p. 256-258°C; $\nu_{max.}$ (KBr disc) 3000 cm^{-1} ($-\overset{+}{N}H_3$); δ_H (90 MHz) (D_2O) 1.62 (4H, m, 7- and 8-H_2), 1.90 (4H, m, 3- and 4-H_2), 2.95 (4H, m, 2- and 5-H_2), 3.05 (2H, m, 9-H_2), and 3.55 p.p.m. (2H, m, 6-H_2); δ_C (50.3 MHz) (D_2O) 23.6, 23.7, 25.0 (C-3, -4, -7, and -8), 47.9 (C-9), 55.1 (C-6), and 55.2 p.p.m. (C-2 and -5). The phenylamino(thiocarbonyl) (119) derivative had m.p. 189°C; m/z 277 (M^+, 7%) (Found: M^+, 277.1609. $C_{15}H_{23}N_3S$

requires M, 277.1612).

N-([4-^{14}C]-4-Aminobutyl)-1,2-didehydropyrrolidinium chloride hydrochloride (113).- To mercury(II) acetate (1.18g, 3.7 mmol) in 5% aqueous acetic acid (6 ml) was added the dihydrochloride (112) (200 mg, 0.9 mmol), and the mixture was heated at reflux for 4h. The solution was cooled, and the mercury(I) acetate was filtered off. The filtrate was saturated with hydrogen sulphide to remove excess mercury(II) ions as black mercury(II) sulphide. This was filtered off through Celite and the process was repeated. A pale yellow filtrate was obtained which was made alkaline by the addition of 40% aqueous sodium hydroxide (4 ml), and then extracted with chloroform (3 x 20 ml). The chloroform extracts were dried and filtered. Addition of dry HCl gas gave the immonium salt (113), (102 mg, 51%, 64.6 μCi mmol^{-1}), m.p. 215-216°C; $\nu_{max.}$ (nujol) 3000 ($-\overset{+}{N}H_3$), 2025, and 1688 cm^{-1} ($>C=\overset{+}{N}<$); δ_H (90 MHz) (D_2O) 1.60-2.05 (6H, m, 4-, 7- and 8-H_2), 2.21 (2H, m, 3-H_2), 2.90-3.20 (4H, complex, 5- and 9-H_2), 3.95 (2H, m, 6-H_2), and 8.65 p.p.m. (1H, br s, 2-H); δ_C (25 MHz) (D_2O) 20.0, 24.1 and 24.4 (C-4, -7 and -8), 39.5 (C-3), 47.5 (C-9), 53.9 and 59.6 (C-5 and -6), and 182.2 p.p.m. (C-2); (Found: C, 44.90; H, 8.49; N, 12.93. $C_8H_8N_2Cl_2$ requires C, 45.06; H, 8.51; N, 13.18%).

A sample of the product (24 mg, 0.11 mmol) was dissolved in deuteriomethanol (2 ml) and stirred with sodium cyanoborodeuteride (15 mg, 0.22 mmol) for 4h at room temperature. Excess cyanoborodeuteride was destroyed by slow addition of 10% sodium hydroxide (ca. 2 ml). The resulting solution was extracted with chloroform (4 x 5 ml).

The chloroform extracts were dried, filtered, and concentrated to give an oil. The oil was taken up in concentrated hydrochloric acid and then evaporated to dryness to give [2-^{2}H]-N-(4-aminobutyl)pyrrolidinium dihydrochloride (118) (17 mg, 71%); δ_H (200 MHz) (D_2O) 1.65 (4H, m, 7- and 8-H_2), 1.95 (4H, m, 3- and 4-H_2), 2.90 (3H, m, 2-H and 5-H_2), 3.15 (2H, m, 9-H_2), and 3.50 p.p.m. (2H, m, 6-H_2).

Feeding Methods - (1). The ^{14}C-labelled immonium salt (113), along with [2,3-^{3}H]putrescine dihydrochloride were administered to four plants which contain pyrrolizidine alkaloids viz., Senecio pleistocephalus, S. isatideus, Cynoglossum australe and C. officinale. The wick procedure of feeding was employed for the S. pleistocephalus plant, while the xylem pricking technique was used for the others. The feeding periods lasted five days, and after a further ten days, the alkaloids from each plant species were extracted and recrystallised to constant specific radioactivity. In each experiment a radioscan of a chromatogram of each extracted alkaloid, developed in the usual solvent system, showed one radioactive band coincident with each authentic unlabelled alkaloid. Rosmarinine was hydrolysed to rosmarinecine with barium hydroxide (see p.182). Retrorsine was hydrolysed in a similar fashion to give retronecine. The $^{3}H/^{14}C$ ratios were measured for each base.

(2) N-([4-^{14}C]-4-Aminobutyl)pyrrolidinium dihydrochloride (112) was fed to one Senecio pleistocephalus and to one Senecio isatideus plant, as described above. Autoradiography of a chromatogram of each of the

extracted alkaloids, displayed one radioactive band coincident with each authentic unlabelled alkaloid.

Intermediate Trapping Experiments.- A solution of [1,4-^{14}C]putrescine dihydrochloride (50 μCi) was fed to one well-established S. pleistocephalus plant by the wick method. After 24h, the plant was blended in methanol (200 ml) and the filtered extract was used for the following two experiments.

Experiment 1 - Inactive immonium salt (113) (40 mg) and sodium borohydride (71 mg, 10 equivalents) were added to the methanolic extract (100 ml) and the mixture was stirred for 24h. Isothiocyanatobenzene (1 ml) was added to the solution, which was stirred for a further 3d at room temperature. Brine (100 ml) was added and the resultant solution was extracted with dichloromethane (4 x 100 ml). The organic extracts were dried, filtered, and concentrated to give a mixture of derivatised polyamines. A radioscan of a chromatogram of the mixture developed in CH_2Cl_2-MeCN (9:1) indicated two major bands at R_F 0.30 (75%) and 0.65 (10%). These bands correspond to the phenylamino(thiocarbonyl) derivatives (120) and (119) of putrescine and N-(4-aminobutyl)pyrrolidine, respectively. Some radioactivity was also observed at R_F 0.0 (10%). The band with R_F 0.65 was removed, and the compound was crystallised and counted.

Experiment 2 - N-(4-Aminobutyl)pyrrolidinium dihydrochloride (112) (25 mg) and isothiocyanatobenzene (1 ml) were added to the methanolic extract (100 ml), which was stirred for 3d at room temperature. The reaction

was worked up as described above to yield a crystalline residue. Autoradiography of a chromatogram of the crystalline material developed in CH_2Cl_2-MeCN (9:1), displayed one major band (90%), R_F 0.31 corresponding to the phenylamino(thiocarbonyl) derivative (120) of putrescine. A weaker band (10%) was also visible at R_F 0.0. The band which corresponded to the N-phenylamino(thiocarbonyl) derivative of the saturated dihydrochloride (119), was extracted and crystallised.

8.3.2 (1β,2α,7β,8α)-1-Hydroxymethyl-2,7-pyrrolizidinediol (Rosmarinecine) (21) - Rosmarinine (20) (500 mg, 1.42 mmol) was dissolved in water (25 ml) and the solution was heated under reflux with barium hydroxide octahydrate (1.78g, 5.65 mmol) for 4h. Solid carbon dioxide was added to the cooled solution and the precipitated barium carbonate was filtered off. The filtrate was saturated with sodium carbonate and continuously extracted with chloroform for 48h. Concentration of the chloroform extract under reduced pressure gave rosmarinecine (21) (160 mg, 65%), m.p. 170-171°C (lit.,[117a] 171-172°C); $[\alpha]_D^{18}$ -11.6° (c 2 in MeOH) (lit.,[117a] -11.8°); $\nu_{max.}$ (KBr disc) 3350, 2980, 2940, 2495, 2480, and 1480 cm^{-1}; δ_H (90 MHz) (CD_3OD) 1.90 (2H, m, 6-H_2), 2.32 (1H, m, 1-H), 2.85-3.35 (4H, m, 3- and 5-H_2), 3.48 (1H, dd, J 4 Hz and 3 Hz, 8-H), 3.80 (1H, dd, J 11 Hz and 5 Hz, 9-H), 4.05 (1H, dd J 11 Hz and 6 Hz, 9-H), 4.30 (1H, m, 7-H), and 4.41 p.p.m. (1H, ddd, J 9Hz, 9Hz and 5 Hz, 2-H); δ_C (25 MHz) (CD_3OD) 36.0 (C-6), 51.3 (C-1), 54.9 (C-5), 59.4 (C-3), 60.1 (C-9), 70.9 (C-8), and 72.0 and 72.5 p.p.m. (C-2 and -7); m/z 173 (M^+, 9%), 129, 113, 100, 99,

98 (100%), and 82 (Found: $\underline{M}^+$, 173.1053. $C_8H_{15}NO_3$ requires $\underline{M}$, 173.1052).

Acidification of the remaining aqueous solution with $2\underline{M}$ sulphuric acid, and continuous extraction with ether gave the lactone of senecic acid, m.p. 129°C (lit.,[117b] 129-130°C).

(1β,7β,8α)-7-Hydroxy-1-hydroxymethylpyrrolizidine (Platynecine) (124) - A solution of retronecine (3) (200 mg), 1.3 mmol) in absolute ethanol (10 ml), to which was added 10% Pd/C catalyst (30 mg, 15% by weight), was hydrogenated at 1 atm. After 4h, reduction was complete. The solution was filtered through Celite and the ethanol was removed under vacuum. The syrupy product was taken up in boiling acetone (2 ml), and platynecine (124) crystallised from the cooled solution (182 mg, 90%), m.p. 148-148.5°C (lit.,[118] 148-149°C); $[\alpha]_D^{20}$ -55.1° ($\underline{c}$ 1 in $CHCl_3$) (lit.,[118] -56.8°); $\nu_{max.}$ (KBr disc) 3350, 2940, 2880, 2480, and 1480 cm^{-1}; δ_H (200 MHz) 1.60-2.20 (4H, complex, 2- and 6-Hz), 2.39 (1H, m, 1-H), 2.70-3.15 (4H, complex, 3- and 5-Hz), 3.18 (1H, m, 8-H), 3.85 (1H, dd, $\underline{J}$ 11.3 Hz and 4.3 Hz, 9-H), 3.95 (1H, dd, $\underline{J}$ 11.3 Hz and 2.4 Hz, 9-H), and 4.20 p.p.m. (1H, dt, $\underline{J}$ 4.6 Hz and 1.7 Hz, 7-H); δ_C (25 MHz) 28.6 (C-6), 36.5 (C-2), 43.9 (C-1), 53.6 and 55.3 (C-3 and -5), 61.6 (C-9), 71.1 (C-8), and 73.1 p.p.m. (C-7); $\underline{m}/\underline{z}$ 157 ($\underline{M}^+$, 7%) 113, 83, 82 (100%), and 81 (Found: $\underline{M}^+$, 157.1108. $C_8H_{15}NO_2$ requires $\underline{M}$, 157.1103). The structure was also confirmed by Dr. A.A. Freer by $\underline{X}$-ray crystal structure analysis.[82]

[^{3}H]Rosmarinine - A sample of [2,3-^{3}H]putrescine dihydrochloride (1.0 mCi) was fed to one well-established Senecio pleistocephalus plant over four d. Ten d later, the plant was harvested and the [^{3}H]rosmarinine (240 mg, 0.15% yield based on wet weight of plant material) was isolated by the standard procedure (p.170). A radioactive count indicated a total incorporation of ca. 11% (Activity 107 μCi; 157.8 μCi mmol^{-1}). A t.l.c. of the tritiated rosmarinine showed one spot coincident with authentic unlabelled rosmarinine at R_F 0.3. All spectroscopic data were identical to those reported on p.170.

[^{3}H]Rosmarinecine - Tritiated rosmarinine (40 mg, 0.11 mmol; 157.8 μCi mmol^{-1}) in water (5 ml) was heated under reflux with barium hydroxide octahydrate (142 mg, 0.45 mmol), for 4h. The solution was cooled, treated with solid carbon dioxide, and the precipitated barium carbonate was filtered off. The filtrate was basified with sodium carbonate and continuously extracted with chloroform for 48h. The chloroform extract was dried, filtered, and concentrated under vacuum to give [^{3}H]rosmarinecine (12 mg, 60%; 128.7 μCi mmol^{-1}). A t.l.c. of the [^{3}H]rosmarinecine on a silica gel plate developed with methanol-conc. ammonia (9:1) showed one spot, R_F 0.3, coincident with authentic unlabelled rosmarinecine. The spectroscopic data were identical to those reported on p.182.

[^{3}H]Platynecine - Methanesulphonyl chloride (72 μl, 0.9 mmol, 1.1 equiv.), [^{3}H]rosmarinine (80 mg, 0.2 mmol; 157.3 μCi mmol^{-1}), and unlabelled rosmarinine (220 mg, 0.6 mmol) were dissolved in dry dichloromethane (8 ml) and the mixture was cooled to -78°C. Triethylamine (129 μl,

0.9 mmol, 1.1 equiv.), was added *via* syringe and the mixture was stirred at -78°C for 1/2h. The solution turned pale yellow and this colour deepened slightly as the reaction was allowed to reach room temperature. The mixture was poured into water (60 ml) and extracted with dichloromethane (3 x 60 ml). The combined organic extracts were washed with brine (50 ml), dried, and concentrated to give 2-*O*-methanesulphonyl-[^{3}H]rosmarinine (340 mg, 93%); $\nu_{max.}$ ($CHCl_3$), 1720, 1355, and 1175 cm^{-1}; δ_H (90 MHz) 3.1 p.p.m. (3H, s). The [^{3}H]mesylate (300 mg, 0.7 mmol) was dissolved in dry hexamethylphosphoric triamide (HMPTA) (4 ml) and added to a two-necked round bottom flask containing sodium cyanoborohydride (219 mg, 3.5 mmol, 5 equiv.) *via* a syringe. The mixture was stirred at 95-100°C for 18h, then cooled. The solution was diluted with brine (20 ml) and extracted with diethyl ether (3 x 25 ml). The combined ethereal extracts were washed with brine (3 x 25 ml). [Vigorous shaking with brine was required to remove all traces of HMPTA]. The ether solution was dried, filtered, and concentrated to yield [^{3}H]platyphylline, (119 mg, 51%). The ^{1}H n.m.r. spectrum showed no singlet at 3.1 p.p.m. due to the mesylate. Alkaline hydrolysis of the tritiated platyphylline, carried out as for [^{3}H]rosmarinine above, afforded [^{3}H]platynecine (38 mg, 68%; 19.4 μCi $mmol^{-1}$). A t.l.c. of the [^{3}H]-platynecine on a silica gel plate developed with methanol-conc. ammonium (9:1) showed one spot, R_F 0.2, coincident with authentic unlabelled platynecine.

Feeding Methods - The ^{3}H-labelled necines, rosmarinecine and platynecine, were both mixed with [1,4-^{14}C]putrescine dihydrochloride to give initial $^{3}H/^{14}C$ ratios of 16.1 and 12.3, respectively. Unlabelled putrescine dihydrochloride was added to each sample to ensure that equal molar quantities of each compound were administered. The two mixtures were each fed to one Senecio pleistocephalus plant by the wick method. Details of each experiment are given in Table 9. Rosmarinine was isolated and recrystallised to constant specific radioactivity. In each experiment radioscans of t.l.c. plates showed one band coincident with authentic unlabelled rosmarinine at R_F 0.3.

Table 9. Incorporation of ^{3}H-necines into rosmarinine in Senecio pleistocephalus plants

Base	Quantity fed/mg	$^{3}H/^{14}C$ of precursors	$^{3}H/^{14}C$ of rosmarinine	^{3}H Specific Incorporation in rosmarinine
Rosmarinecine	5	16.1	9.3	4.0
Platynecine	16	12.3	10.2	7.3

(-)-7,9-O-C-(Isopropylidenyl)rosmarinecine (128). - Rosmarinecine (200 mg, 1.16 mmol) was dissolved in dry acetone (2 ml) and ferric chloride (60 mg) was added. The mixture was stirred at 40°C for 36h under an inert atmosphere. The reaction was terminated by adding 10% potassium carbonate solution (5 ml), which resulted in the formation of a dark brown

syrup. This was extracted with chloroform (3 x 10 ml), and the combined chloroform extracts were washed with brine (4 x 10 ml). Concentration of the dried organic extract gave a crude sample of the title compound (128). Recrystallisation from dichloromethane afforded a pure sample of the acetonide (128), (115 mg, 47%), m.p. 176-177°C; R_F 0.65; $[\alpha]_D^{20}$ -131° (c 2 in $CHCl_3$); $\nu_{max.}$ ($CHCl_3$) 3350, 3050, 2960, 2925, 2400, 1230, and 750 cm^{-1}; δ_H (90 MHz) 1.27 and 1.38 (6H, 2 x s, 13- and 14-H_3), 2.05 (2H, m, 6-H_2), 2.40-3.05 (5H, complex, 1-H, 3- and 5-H_2), 3.80 (2H, m, 9-H_2), 3.90 (1H, dd, J 3.8 Hz and 3.0 Hz, 8-H), and 4.20 and 4.45 p.p.m. (2H, 2 x br s, 2- and 7-H_2); δ_C (25 MHz) 23.5 and 25.6 (C-13 and -14), 37.0 (C-6), 49.6 (C-1), 52.3 (C-5), 61.0 (C-3 and -9), 70.4 (C-8), 72.0 (C-2), 76.6 (C-7), and 101.3 p.p.m. (C-11); m/z 213 (M^+, 11%), 155, 149, 138, 112, 99, and 43 (100%) (Found: M^+, 213.1364. $C_{11}H_{19}NO_3$ requires M, 213.1364).

Attempted Synthesis of 2α-Hydroxy-1β-hydroxymethyl-8α-pyrrolizidine (125). - The acetonide (128) (100 mg, 0.5 mmol) and methanesulphonyl chloride (40 μl, 0.5 mmol) were dissolved in dry dichloromethane (2 ml) and cooled to -78°C. Triethylamine (196 μl, 0.5 mmol) was added and the mixture was stirred at -78°C for 1/2h. The solution turned pale yellow and the colour deepened as the reaction was allowed to reach room temperature. The solution was poured onto water (2 ml) and extracted with dichloromethane (3 x 5 ml). The organic extracts were washed with brine (1 x 15 ml), dried, filtered, and concentrated under vacuum to give the mesylate (129), as a crystalline solid, (100 mg, 73%), m.p. 186-187°C; δ_H (90 MHz) 3.10 p.p.m. (3H, s); m/z 291 (M^+, 4%)

(Found: $\underline{M}^+$, 291.1143. $C_{12}H_{21}NO_5S$ requires $\underline{M}$, 291.1141). The mesylate (129) (90 mg, 0.3 mmol) was treated with sodium cyanoborohydride (78 mg, 1.2 mmol) in dry HMPTA (2 ml) at 95-100°C for 18h under argon. Brine (4 ml) was then added to the cooled solution and the resultant milky solution was extracted with diethyl ether (3 x 6 ml). The combined ether extracts were washed with brine (2 x 10 ml) and then concentrated to give an oil (31 mg, 51%). Without purification, the oil (25 mg, 0.1 mmol) was dissolved in 3M hydrochloric acid (2 ml) and it was stirred at 70°C for 4h. The cooled solution was basified with 10% potassium carbonate solution (ca. 10 ml) and extracted with ethyl acetate (3 x 15 ml). The total organic extract was dried, filtered, and concentrated to give platynecine (124) (12 mg, 65%). The spectroscopic data were identical to those reported earlier (p.183).

Attempted Synthesis of 2,O-(Tetrahydropyranyl)rosmarinine (126a).- To rosmarinine (20) (250 mg, 0.7 mmol) and freshly distilled dihydropyran (64 µl, 0.7 mmol) in dry dichloromethane (6 ml) at 0°C under argon, was added p-toluenesulphonic acid monohydrate (1 mg). After the mixture was stirred for 10 min at 0°C, it was stirred for 18h at ambient temperature. The mixture was then partitioned between dichloromethane (20 ml) and a solution of brine (10 ml) and saturated sodium bicarbonate (10 ml). The organic layer was washed with brine (2 x 10 ml), dried, filtered, and concentrated to yield starting material.

The above procedure was repeated, using elevated temperatures, but starting material was returned.

Attempted Synthesis of 2-O-(2-Methoxyethoxymethyl)rosmarinine (126b).- Rosmarinine (20) (200 mg, 0.57 mmol) was dissolved in dry dichloromethane (5 ml), under an inert atmosphere. MEM chloride (65 µl, 0.57 mmol) and then N,N'-diisopropylethylamine (Hunig's base) (99 µl, 0.57 mmol) were added and the solution was stirred at room temperature for 24h. Water (1 ml) was added, and the organic layer was separated. The aqueous layer was extracted with dichloromethane (3 x 2 ml). The combined organic extracts were dried, filtered, and concentrated to give an oil, which contained an unidentifiable mixture of products.

Attempted Synthesis of 2-O-(Benzyl)rosmarinine (126d).- Rosmarinine (20) (200 mg, 0.6 mmol) was dissolved in dry DMF (3 ml) and added slowly with stirring to a suspension of sodium hydride (23 mg, 0.6 mmol, 60% dispersion in oil) in DMF (1 ml) under argon. After addition was complete, tetrabutylammonium iodide (21 mg, 0.06 mmol) and benzyl bromide (71 µl, 0.6 mmol) were added, and the mixture was stirred for 18h at room temperature. The solution, to which brine had been added (1 ml), was passed through a small column of florosil. Removal of the solvent by distillation gave a solid which was shown to be rosmarinine by ^{1}H n.m.r. spectroscopy.

The above procedure was repeated at 50°C and 90°C, and in both cases, starting material was recovered.

Attempted Synthesis of 2-O-(Methoxymethyl)rosmarinine (126c).- To a stirred solution of rosmarinine (20) (250 mg, 0.7 mmol) in dry chloroform (4 ml) were added DME (626 µl, 0.7 mmol) and phosphorus pentoxide (2g). After 24h, the slurry was poured onto ice-cooled sodium carbonate

solution (10 ml). The mixture was extracted with chloroform (3 x 10 ml). The combined chloroform extracts were washed with brine, dried, filtered, and evaporated to give mostly rosmarinine. T.l.c. indicated another compound, R_F 0.8, which may have been the title compound. However, it was present in an insufficient quantity for it to be further analysed.

2-O-tert.-(Butyldimethylsilyl)rosmarinine (126e).- Rosmarinine (20) (140 mg, 0.4 mmol) was dissolved in dry DMF (0.75 ml), under an inert atmosphere. tert-Butyldimethylsilyl chloride (90 mg, 0.6 mmol) and imidazole (41 mg, 0.6 mmol) were added and the reaction mixture was stirred for 48h at room temperature. The reaction mixture was taken up in chloroform (10 ml) and washed with brine (2 x 10 ml). The aqueous layers were combined and extracted with chloroform (1 x 20 ml). The total chloroform extracts were dried, filtered, and evaporated under vacuum to give an oil, which slowly crystallised to give the ether (126e) (160 mg, 86%); m.p. 94-96°C; R_F 0.8; $[\alpha]_D^{24}$ -66.5° (c 8 in $CHCl_3$); $\nu_{max.}$ (KBr disc) 2960, 1740, 1720, 1260, and 1120 cm^{-1}; δ_H (90 MHz) 0.1 and 0.15 (6H, 2 x s, 2 x CH_3-Si), and 0.85 p.p.m. (9H, s, 3 x CH_3-C) and the remaining signals were the same as for rosmarinine; m/z 467 (M^+, 7%), 410, 270, 268, 122, 120, and 82 (100%) (Found: M^+, 467.2710. $C_{23}H_{41}NO_6Si$ requires M, 267.2703).

Attempted Synthesis of 2-O-tert.-(Butyldimethylsilyl)rosmarinecine.- The TBDMS ether of rosmarinine (126e) (152 mg, 0.32 mmol) in water (8 ml) was heated under reflux with barium hydroxide octahydrate (113 mg, 0.36 mmol, 1.1 equiv.) for 2h. The solution was allowed to cool and

treated with solid carbon dioxide. The resultant slurry was filtered, and the filtrate was continuously extracted with chloroform for 48h, after further basification with sodium carbonate. The organic extract was dried, filtered, and concentrated to give rosmarinecine (21).

8.4 Experimental to Chapter 6

8.4.1 Preparation of (+)-6α-Hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (22).-

(-)-(2S,4R)-N-Formyl-4-formyloxyproline (187), ethyl (+)-(2R)-2-formyloxy-2,3-dihydro-1H-pyrrolizine-7-carboxylate (189) and ethyl (+)-(2R)-2-hydroxy-2,3-dihydro-1H-pyrrolizine-7-carboxylate (190) were prepared as detailed by Robins and Sakdarat.[101]

Ethyl (+)-6α-hydroxy-8β-pyrrolizidine-1α-carboxylate (191).- A solution of ethyl (+)-(2R)-2-hydroxy-2,3-dihydro-1H-pyrrolizine-7-carboxylate (190) (5g, 25 mmol) in acetic acid (75 ml) was hydrogenated at 7 atm for 24 h at 60°C using 10% Rh-C (1g) as catalyst. The catalyst was removed by filtering through Celite, and the filtrate was concentrated to give an oil. The oil was taken up in 1M HCl (16 ml) and washed with chloroform (2 x 25 ml). The aqueous solution was basified with conc. ammonia solution and extracted with chloroform (3 x 30 ml). The chloroform extracts were dried, filtered, and concentrated. The product was obtained as white crystals from ethyl acetate, (2.1g, 47%). All physical data were identical to those reported in the literature,[101] e.g., m.p. 109°C (lit., 109-110°C).

(+)-6α-Hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (22).- This compound was prepared as before[101] and had δ_C (25 MHz) (CD_3OD) 27.6 (C-2), 33.7 (C-7), 45.6 (C-1), 55.2 (C-3), 62.9 and 63.4 (C-5 and -9), 65.8 (C-8), and 73.1 p.p.m. (C-6). The picrolonate had m.p. 171-172° (decomp.) (Found: C, 51.2, H; H, 5.54; N, 16.35. $C_{18}H_{23}N_5O_7$ requires C, 51.3; H, 5.50; N, 16.61%).

8.4.2 Synthesis of 12-Membered Macrocyclic Diesters of (+)-6α-Hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (22)

General Procedure.- Glutaric anhydride or a derivative (1.1 mmol) was added to a solution of the optically active diol (22) (1 mmol) in dry DME (30 ml) under argon. The reaction mixture was then stirred at 40°C for 1h and the homogeneous solution was stirred for 18h at room temperature, to ensure that all the base had reacted to form a zwitterionic monoester (t.l.c., R_F 0.05-0.10). 2,2'-Dithiodipyridine (130) (1.5 mmol) and triphenylphosphine (131) (1.5 mmol) were added and the mixture was stirred vigorously until thiolester formation was complete (t.l.c., R_F ca. 0.65, 24h). The resultant clear yellow solution was diluted with DME (120 ml) and heated at reflux under argon until lactonisation was complete (t.l.c., R_F 0.35, 4d). The reaction mixture was cooled and then evaporated under pressure to afford a yellow oil. This oil was dissolved in chloroform (10 ml) and extracted with 1M citric acid (4 x 4 ml). The acidic extracts were combined and washed with chloroform (6 x 20 ml), then basified with concentrated ammonia solution and extracted with chloroform (4 x 25 ml). The chloroform extracts were dried, filtered, and concentrated to give crude cyclised products as yellow oils. Purification was achieved by preparative thin layer chromatography, eluting with chloroform-methanol-conc. ammonia (85:14:1).

(-)-6,9-O,O-(Glutaryl)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (194a).- Using glutaric anhydride (192a), the glutarate dilactone (194a) was obtained as a powder (26% yield), m.p. 79-80°C [from benzene-hexane]; R_F 0.35; $[\alpha]_D^{21}$ -4.1° (c 3 in MeOH); $\nu_{max.}$ (CCl_4) 2960, 2930, 2860, 1740, 1330, 1245, 1150, and 1010 cm^{-1}; δ_H (200 MHz) 1.70 (2H, m, 2-H_2), 2.00 (2H, m, 13-H_2), 2.12 (2H, m, 7-H_2), 2.40 (1H, m, 1-H), 2.45 (4H, m, 12- and 14-H_2), 3.00-3.40 (4H, m, 3- and 5-H_2), 3.75 (1H, m, 8-Hβ), 4.05 (1H, dd, J 12.2 Hz and 4.3 Hz, 9-H), 4.52 (1H, dd, J 12.2 Hz and 1.2 Hz, 9-H), and 5.11 p.p.m. (1H, m, 6-Hβ); δ_C (50 MHz) 20.0 (C-13), 28.9 (C-2), 33.1 (C-7), 34.5 and 35.1 (C-12 and -14), 40.1 (C-1), 54.3 (C-3), 58.6 and 61.1 (C-5 and -9), 65.0 (C-8), 76.4 (C-6), and 172.2 and 172.5 p.p.m. (C-11 and -15); m/z (M^+, 19%), 140, 138, 122 (100%), 121, 120, 110, 109, 108, 82, 81, 80, 69, and 55 (Found: M^+, 253.1315; C, 61.44; H, 7.57; N, 5.34. $C_{13}H_{19}NO_4$ requires M, 253.1314; C, 61.64; H, 7.56; N, 5.53%).

(-)-6,9-O,O-(3,3-Dimethylglutaryl)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (194b).- The above procedure was repeated using 3,3-dimethylglutaric anhydride (192b) to give the title compound (194b) (30% yield) as a non-crystalline solid, m.p. 84°C [from benzene-hexane]; R_F 0.35; $[\alpha]_D^{15}$ -11° (c 3 in MeOH); $\nu_{max.}$ (CCl_4) 2955, 2920, 2870, 2850, 1735, 1325, 1170, and 1145 cm^{-1}; δ_H (200 MHz) 1.27 (6H, s, 17- and 18-H_3), 1.72 (2H, m, 2-H_2), 1.98 (2H, m, 7-H_2), 2.13 and 2.61 (2H, ABq, J 13.6 Hz, 12-H_2 or 14-H_2); 2.24 and 2.27 (2H, ABq, J 15.3 Hz, 12-H_2 or 14-H_2), 2.42 (1H, m, 1-H), 2.90 (2H, complex, 3- and 5-H), 3.15 (1H, m, 3-H), 3.35 (1H, dd, J 13.5 Hz and 4.5 Hz, 5-Hβ),

3.73 (1H, m, 8-Hβ), 4.03 (1H, dd, J 12.1 Hz and 2.2 Hz, 9-H), 4.48 (1H, dd, J 12.1 Hz and 4.9 Hz, 9-H), and 4.98 p.p.m. (1H, t, J 4.8 Hz, 6-Hβ); δ_C (50 MHz) 28.3 (C-2), 29.4 and 30.5 (C-17 and -18), 33.3 (C-13), 33.7 (C-7), 41.6 (C-1), 44.7 and 44.8 (C12 and -14), 56.2 (C-3), 62.2 and 62.8 (C-5 and -9), 65.4 (C-8), 75.8 (C-6), and 171.3 and 171.8 p.p.m. (C-11) and -15); m/z 281 (M^+, 20%), 140, 138, 122 (100%), 121, 120, 82, 81, 80, and 55 (Found: M^+, 281.1634; C, 64.00; H, 8.31; N, 4.73. $C_{15}H_{23}NO_4$ requires M, 281.1627; C, 64.03; H, 8.24; N, 4.98%).

(-)-6,9-O,O-(3,3-Tetramethyleneglutaryl)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (194c).- The title compound (194c) was prepared (41% yield) as a powdered solid when 3,3-tetramethyleneglutaric anhydride (192c) and the pyrrolizidine diol (22) were treated as described in the general procedure; m.p. 90-91°C [from benzene-hexane]; R_F 0.35; $[\alpha]_D^{21}$ -26° (c 3 in MeOH); $\nu_{max.}$ (CCl_4) 2960, 2920, 2870, 2850, 1730, 1155, 1135, and 1120 cm^{-1}; δ_H (200 MHz) 1.66 (8H, m, 17-, 18-, 19-, and 20-H_2), 1.72 (2H, m, 2-H_2), 2.00 (2H, m, 7-H_2), 2.22 and 2.59 (2H, ABq, J 13.6 Hz, 12-H_2 or 14-H_2); 2.29 and 3.35 (2H, ABq, J 15.2 Hz, 12-H_2 or 14-H_2), 2.42 (1H, m, 1-H), 2.91 (2H, complex, 3- and 5-H), 3.15 (1H, m, 3-H), 3.35 (1H, dd, J 13.5 Hz and 4.8 Hz, 5-Hβ), 3.73 (1H, m, 8-Hβ), 4.05 (1H, dd, J 12.1 Hz and 2.4 Hz, 9-H), 4.45 (1H, dd, J 12.1 Hz and 5.2 Hz, 9-H), and 5.10 p.p.m. (1H, t, J 5 Hz, 6-Hβ); δ_C (50 MHz) 23.2 and 24.0 (C-18 and -19), 28.6 (C-2), 33.6 (C-7), 38.9 and 39.4 (C-17 and -20), 41.7 (C-1), 42.8 and 43.2 (C-12 and -14), 44.1 (C-13), 56.1 (C-3), 62.1 and 62.8 (C-5 and -9),

65.8 (C-8), 76.0 (C-6), and 171.6 and 172.1 p.p.m. (C-11 and -15); m/z 307 (M^+, 8%), 122 (100%), 121, 120, 108, 82, 81, 80, and 55 (Found: M^+, 307.1787; C, 66.6; H, 8.27; N, 4.40. $C_{17}H_{25}NO_4$ requires M, 307.1783; C, 66.4; H, 8.20; N, 4.56%).

(-)-6,9-O,O-(3,3-Pentamethyleneglutaryl)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (194d).- When the base (22) was treated with 3,3-pentamethyleneglutaric anhydride (192d) by the standard procedure, the title compound (194d) was obtained (37% yield); m.p. 97-98°C [from benzene-hexane]; R_F 0.35; $[\alpha]_D^{18}$ -15° (c 3 in MeOH); $\nu_{max.}$ (CCl_4) 2930, 2855, 1730, 1450, 1330, and 1165 cm^{-1}; δ_H (200 MHz), 1.44 (10H, m, 17-, 18-, 19-, 20- and 21-H_2), 1.73 (2H, m, 2-H_2), 2.00 (2H, m, 7-H_2), 2.12 and 2.44 (2H, ABq, J 15.3 Hz, 12-H_2 or 14-H_2); 2.30 and 2.50 (2H, ABq, J 13.6 Hz, 12-H_2 or 14-H_2), 2.45 (1H, m, 1-H), 2.87 (1H, m, 5-H), 2.89 (1H, m, 3-H), 3.12 (1H, m, 3-H), 3.35 (1H, dd, J 13.4 Hz and 4.5 Hz, 5-Hβ), 3.70 (1H, m, 8-Hβ), 4.05 (1H, dd, J 12.1 Hz and 2.5 Hz, 9-H), 4.44 (1H, dd, J 12.1 Hz and 5.1 Hz, 9-H), and 4.98 p.p.m. (1H, t, J 4.8 Hz, 6-Hβ); δ_C (50 MHz) 21.3 and 21.4 (C-18 and -20), 25.7 (C-19), 28.4 (C-2), 33.7 (C-7), 36.0 (C-13), 36.6 and 38.0 (C-17 and -21), 41.8 (C-1), 42.1 and 42.5 (C-12) and -14), 56.2 (C-3), 62.3 and 62.8 (C-5 and -9), 65.5 (C-8), 75.7 (C-6), and 171.5 and 171.9 p.p.m. (C-11 and -15); m/z 321 (M^+ 19%), 122 (100%) 121, 120, 95, 82, 81, 80, 68, 67, and 55 (Found: M^+, 321.1925; C, 67.30; H, 8.42; N, 4.21. $C_{18}H_{27}NO_4$ requires M, 321.1935; C, 67.26; H, 8.47; N, 4.36%).

(-)-6,9-O,O-(2,2-Dimethylglutaryl)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (198).- Treatment of 2,2-dimethylglutaric anhydride (197) as described in the general procedure afforded the title compound (198) (10% yield), as an oil, R_F 0.35; $[\alpha]_D^{20}$ -19° (c 3 in MeOH); $\nu_{max.}$ (CCl_4) 2960, 2920, 2890, 2870, 1735, 1295, 1270, 1140, and 1120 cm^{-1}; δ_H (200 MHz) 1.24 and 1.27 (3H, s, 17-H_3 or 18-H_3), 1.78 (2H, m, 2-H_2), 1.90-2.45 (6H, m, 7-, 13- and 14-H_2), 2.57 (1H, m, 1-H), 2.95-3.13 (3H, m, 3-H_2 and 5-H), 3.20 (1H, dd, J 13.4 Hz and 4.8 Hz, 5-Hβ), 3.77 (1H, m, 8-Hβ), 3.87 (1H, dd, J 12.1 Hz and 3.9 Hz, 9-H), 4.61 (1H, dd, J 12.1 Hz and 1.1 Hz, 9-H), and 5.05 p.p.m. (1H, m, 6-H); δ_C (50 MHz) 24.4 and 27.1 (C-17 and -18), 28.6 (C-2), 30.8 (C-13), 33.2 (C-12 or -14), 34.9 (C-7), 40.1 (C-1), 41.6 (C-12 or -14), 53.8 (C-3), 58.3 and 63.5 (C-5 and -9), 64.7 (C-8), 78.1 (C-6), and 172.7 and 176.5 p.p.m. (C-11 and -15); m/z 281 (M^+, 11%), 122 (100%), 121, 120, 108, 82, 81, 80, 69, 68, and 55 (Found: M^+, 281.1632. $C_{15}H_{23}NO_4$ requires M, 281.1627).

(-)-6,9-O,O-(Meso-2,4-Dimethylglutaryl)-6α-hydroxy-1α-hydroxymethyl 8β-pyrrolizidine [(199a) + (199b)].- A mixture of two diastereoisomers [(199a) + (199b)] (R_F 0.30-0.35) was obtained when the optically active pyrrolizidine diol (22) was treated with meso-2,4-dimethylglutaric anhydride (200) as described in the standard procedure (21% yield); $\nu_{max.}$ (CCl_4) 2960, 2930, 2870, 1730, 1260, and 1165 cm^{-1}; δ_H (200 MHz) 1.10-1.20 (6H, 2d, J 7.1 Hz, 17- and 18-H_3), 1.31 and 1.38 (2H, t, J 2.7 Hz, 13-H_2), 1.72-2.20 (6H, complex, 2-H_2, 7-H_2, 12-H and 14-H), 2.45 (1H, m, 1-H), 2.95 - 3.30 (4H, m, 3- and 5-H_2), 3.75 (1H, m, 8-Hβ), 4.05 - 4.45 (2H, m, 9-H_2) and 5.00 p.p.m. (1H, m,

6-H); δ_C (50 MHz) 17.5, 18.0, 19.5 and 19.7 (C-17 and -18), 28.7 and 29.0 (C-2), 33.6 and 33.8 (C-7), 36.6 and 37.5 (C-12 and -14), 37.9 and 38.0 (C-13), 40.0 and 40.2 (C-1), 55.0 and 55.8 (C-3), 61.4, 63.3, 63.4 and 63.6 (C-5 and -9), 64.7 and 65.7 (C-8), 76.4 and 76.7 (C-6), and 174.9, 175.2, 175.5 and 176.1 p.p.m. (C-11 and -15); m/z 281 (M^+, 11%), 122 (100%), 121, 120, 108, 82, 81, 80, 68, and 55 (Found: M^+, 281.1630. $C_{15}H_{23}NO_4$ requires M, 281.1627).

Modification of the General Procedure

Use of 2,2'-Dithiobis(4-tert.-butyl-1-isopropylimidazole)[103] (201).- (+)-6α-Hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (22) (100 mg, 6.37 mmol) was treated with glutaric anhydride (80 mg, 7.02 mmol) as described in the general procedure, except that 2,2'-dithiobis(4-tert.-butyl-1-isopropylimidazole) (201) was added instead of 2,2'-dithiodipyridine (130). The solution of the intermediate thiolester in DME was heated at reflux for 24h only. The cyclised product obtained after the work-up (45 mg, 28%), was identical in all respects to (-)-6,9-O,O-glutaryl-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (194a) obtained previously (p.194).

(+)-6,9-O,O-(Succinyl)-6α-hydroxy-1α-hydroxymethyl-8β-pyrrolizidine (204).- The title compound (204) was prepared (20% yield) as an oil when succinic anhydride (202) was reacted with the optically active diol (22) as described in the general procedure. R_F 0.30; $[\alpha]_D^{20}$ +8° (c 3 in MeOH); $\nu_{max.}$ (CCl_4) 2965, 2930, 1745, 1440, 1260, and 1170 cm^{-1}; δ_H (200 MHz) 1.59 (2H, m, 2-H_2), 1.92 (2H, m, 7-H_2), 2.35-

2.50 (4H, m, 12- and 13-H_2), 2.56 (1H, m, 1-H), 2.75-3.10 (3H, m, 3-H_2 and 5-H), 3.35 (1H, dd, $\underline{J}$ 13.9 Hz and 4 Hz, 5-Hβ), 3.69 (1H, m, 8-Hβ), 3.90 (1H, dd, $\underline{J}$ 12.1 Hz and 1.2 Hz, 9-H), 4.72 (1H, dd, $\underline{J}$ 12.1 Hz and 3.9 Hz, 9-H), and 4.94 p.p.m. (1H, t, $\underline{J}$ 4.3 Hz, 6-Hβ); δ_C (50 MHz) 27.1 (C-2), 31.4 and 32.4 (C-12 and -13), 34.5 (C-7), 43.1 (C-1), 56.6 (C-3), 61.8 and 62.3 (C-5 and -9), 64.5 (C-8), 76.7 (C-6), and 171.2 and 173.1 p.p.m. (C-11 and -14); $\underline{m}/\underline{z}$ 239 ($\underline{M}^+$, 25%), 154, 122, (100%), 121, 120, 110, 108, 107, 106, 95, 94, 82, 81, 80, and 55 (Found: $\underline{M}^+$ 239.1157. $C_{12}H_{17}NO_4$ requires $\underline{M}$, 239.1158).

8.5 Experimental to Chapter 7

8.5.1 - The alkaloidal content of Lindelofia longiflora (family Boraginaceae) was investigated. The procedure which was used for extracting rosmarinine (20) from Senecio pleistocephalus plants (p.170) was employed. Freshly harvested plant material (1 Kg) gave a viscous gum (950 mg). This was identified as echinatine (123), R_F 0.3; $[\alpha]_D^{20}$ +12.3° (c 0.9 in EtOH) (lit.,[1] [α] +15.0°); $\nu_{max.}$ (thin film) 3400, 2973, 2936, 1728, and 1230 cm^{-1}; δ_H (200 MHz) 0.89 and 0.93 (3H, both d, J 6.8 Hz, 16- and 17-H_3), 1.27 (3H, d, J 6.6 Hz, 14-H_3), 1.86-1.96 (2H, m, 6-H_2), 2.18 (1H, dq, J 6.8 Hz, 15-H), 2.62 (1H, ddd, J 10.7 Hz, 7.0 Hz and 6.1 Hz, 5-H), 3.27 (1H, dd, J 10.8 Hz and 6.5 Hz, 5-H), 3.37 (1H, dd, J 3.0 Hz and 1.5 Hz, 3-H), 3.60 (1H, br s, OH), 3.88 (1H, dd, J 3.1 Hz and 1.5 Hz, 3-H), 3.97 (1H, m, 8-H), 3.99 (1H, q, J 6.6 Hz, 13-H), 4.01 (2H, br s, 2 x OH), 4.15 (1H, dt, J 6.0 Hz, 7-H), 4.85 (2H, ABq, J 13.4 Hz, 9-H_2), and 5.70 p.p.m. (1H, br s, 2-H); δ_C (50 MHz) 15.8 and 17.9 (C-16 and -17), 17.4 (C-14), 32.3 (C-15), 33.6 (C-6), 54.3 (C-5), 61.7 (C-3), 62.0 (C-9), 71.5 (C-13), 74.2 (C-7), 80.0 (C-8), 84.1 (C-12), 125.7 (C-2), 136.1 (C-1), and 174.0 p.p.m. (C-11); m/z 299 (M^+, 2%), 156, 139, 138 (100%), 137, 136, 120, and 93 (Found: M^+ 299.1732; C, 60.06; H, 8.24; N, 4.35. $C_{15}H_{25}NO_5$ requires M, 299.1733; C, 60.18; H, 8.42; N, 4.68%).

8.5.2 - Cynoglossum macrostylum (family Boraginaceae) was grown and cropped when flowering. Methanolic extracts of the plant gave a mixture of three alkaloids, shown by thin layer chromatography. Purification of these alkaloids was accomplished by column chromatography on basic alumina, eluting with increasing proportions of methanol in dichloromethane. The major alkaloid present (ca. 75%) was echinatine (123). The physical data were identical to that reported in 8.5.1. The other alkaloid identified was heliosupine (84), R_F 0.5; $[\alpha]_D^{21}$ -4.8° (c 1 in EtOH) (lit.,[1] [α] -4.3°); $\nu_{max.}$ ($CHCl_3$) 3500, 2980, 1730, and 1710 cm^{-1}; δ_H (200 MHz) 1.24 (6H, s, 16- and 17-H_3), 1.28 (3H, d, J 6.5 Hz, 14-H_3), 1.85 (3H, s, 23-H_3), 1.90 (2H, m, 6-H_2), 1.95 (3H, d, J 7.0 Hz, 22-H_3), 2.90-3.03 (1H, m, 5-H), 3.20-3.45 (2H, m, 3- and 5-H), 3.70 (3H, br s, 3 x OH), 4.08 (1H, m, 3-H), 4.18 (1H, q, J 6.4 Hz, 13-H), 4.35 (1H, m, 8-H), 4.95 (2H, ABq, J 13.1 Hz, 9-H_2), 5.23 (1H, br t, 7-H), 5.90 (1H, br s, 2-H), and 6.12 p.p.m. (1H, dq, J 7.2 Hz and 1.4 Hz, 21-H); δ_C (50 MHz) 15.9 (C-22), 18.5 (C-14), 20.4 (C-23), 24.7 and 26.0 (C-16 and -17), 30.1 (C-6), 54.0 (C-5), 61.7 and 61.9 (C-3 and -9), 69.7 (C-13), 73.7 (C-15), 77.6 (C-8), 78.8 (C-7), 82.9 (C-12), 127.2 (C-20), 128.8 (C-2), 133.9 (C-1), 139.7 (C-21), 168.0 (C-19), and 174.2 p.p.m. (C-11); m/z 297 (M^+, 2%), 221, 220, 139, 138, 137, 136, 120 (100%), 119, 106, 95, 94, and 93 (Found: M^+, 297.1566; $C_{15}H_{23}NO_5$ requires M, 297.1576). The picrate had m.p. 104-105°C (lit.,[1] 103-106°C), (from ethanol), (Found: C, 49.80; H, 5.50; N, 8.98. $C_{26}H_{34}O_{14}N_4$ requires C, 49.84; H, 5.45; N, 8.94%).

There was insufficient material for identification of the third alkaloid.

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