BIOSYNTHESIS AND SYNTHESIS

OF PYRROLIZIDINE ALKALOIDS

AND ANALOGUES

A thesis in part fulfilment of the

requirement for the Degree of

Doctor of Philosophy

by

Desmond Bernard Hagan

Department of Organic Chemistry

University of Glasgow

November 1987

ProQuest Number: 10997900

ProQuest 10997900

'What we call the beginning

is often the end

And to make an end is to make

a beginning

The end is where we start from'

                    T.S. Eliot

'The long and the short of the matter

is that I am running over the order

of a journey I have lately made'

              Lucien's Icaromenippus

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

Page

SUMMARY

This thesis deals with three aspects of research into pyrrolizidine alkaloids: (a) the synthesis of macrocyclic pyrrolizidine alkaloid analogues incorporating (+)-heliotridine (A); (b) the study of the biosynthesis of pyrrolizidine bases using specifically labelled molecules; and (c) the investigation of the pyrrolizidine alkaloid content of plants not previously studied.

(a). Synthesis of macrocyclic pyrrolizidine alkaloid analogues
     incorporating (+)-heliotridine

The synthesis of a series of novel macrocyclic diesters of (+)-heliotridine (A) has been achieved by the use of the Corey-Nicolaou double activation method of lactonisation. The base (A) was obtained by the alkaline hydrolysis of (+)-echinatine, a natural pyrrolizidine monoester isolated by methanolic extraction of Cynoglossum officinale. An alternative method of production of (+)-heliotridine utilised a short, known conversion of the pyrrolizidine base (+)-retronecine into (+)-heliotridine which was carried out with modifications.

The treatment of (+)-heliotridine (A) with glutaric anhydride derivatives selectively yielded 9-monoesters of (+)-heliotridine. Lactonisation of these monoesters was effected via the corresponding pyridine-2-thiolesters. The macrocyclic nature of these 11-membered pyrrolizidine alkaloid analogues was confirmed by spectroscopic studies. An X-ray analysis of 7,9-O,O'-(3,3-dimethylglutaryl)heliotridine (B) revealed the conformation of this bislactone in the solid state. The ester carbonyl

groups of (B) are anti-parallel. Attempted synthesis of 10-membered bislactones of (+)-heliotridine was unsuccessful.

(b). <u>Study of the biosynthesis of pyrrolizidine bases</u>

The biosynthetic portion of the project was to attempt to gain insight into the various processes taking place in the biosynthesis of different base portions of pyrrolizidine alkaloids by plants.

Previous work had shown that 1,4-diaminobutane (putrescine) is a good precursor of retronecine. A series of putrescines specifically labelled with ^{2}H or ^{13}C were synthesised by known routes. They were isolated as their dihydrochloride salts.

These salts were each mixed with a known activity of a radio-active tracer and were fed to <u>Cynoglossum officinale</u> by the xylem pricking method. The plants were later harvested and extracted to yield samples of (+)-echinatine, i.e. esters of (+)-heliotridine (A). Scintillation counting showed the specific incorporations to be below 0.5% per C_4-unit of the base portion and the ^{2}H and ^{13}C n.m.r. spectra of the (+)-echinatine samples, also showed evidence of the disappointingly low incorporations. An exception was noted with the use of [1-^{13}C]-putrescine dihydrochloride. A ^{13}C n.m.r. spectrum of (+)-echinatine was obtained in which the expected four (C-3,-5,-8 and -9) of the eight signals of the base portion, (+)-heliotridine (A), were enriched. The conditions of feeding were varied over the two subsequent summers but the same low incorporations always resulted. Only the base portion of (+)-echinatine was radioactive as shown by alkaline hydrolysis of each (+)-echinatine sample.

(A)

(B)

(C)

(D)
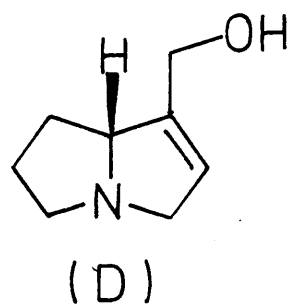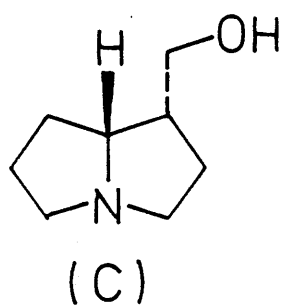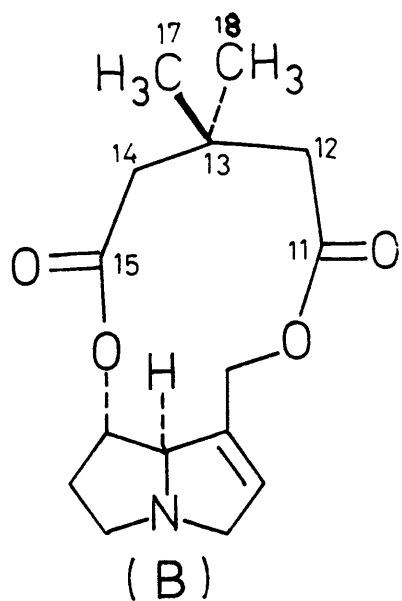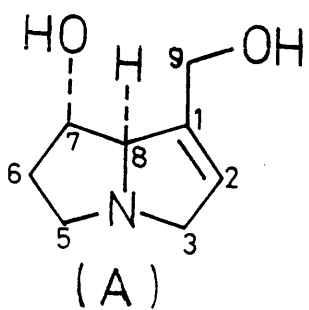
An investigation of <u>Cynoglossum australe</u> revealed the presence of a mixture of (+)-cynaustraline and (+)-cynaustine. Biosynthetic interest arises because of the presence of two rare 8β-bases, (+)-isoretronecanol (C) and (+)-supinidine (D) respectively. The specifically ^{2}H labelled compounds previously synthesised were fed to batches of young <u>Cynoglossum australe</u> plants. However, again the specific incorporations were low and inconclusive ^{2}H n.m.r. spectra were obtained. The experiments were repeated the following summer, but the same results were obtained. Alkaline hydrolysis of the alkaloid samples proved that the radioactivity present was located exclusively in the base portions, (C) and (D), of the alkaloids.

Putrescine with a radioactive ^{3}H label was fed as the dihydrochloride to several young <u>Cynoglossum australe</u> plants by the wick method. A good incorporation (> 1%) into each alkaloid was obtained, and radioactive samples of the two 1-hydroxymethyl pyrrolizidines (C) and (D) were obtained after separation of the alkaloids and subsequent hydrolysis. Each 8β-necine was then fed in turn to different batches of young <u>Cynoglossum australe</u> plants. Isoretronecanol (C) was a precursor for both alkaloids, but supinidine (D) was only incorporated into cynaustine. This finding is consistent with conversion of isoretronecanol into supinidine in the biosynthetic pathway.

Analogues of 1,4-diaminobutane have been prepared and fed to various plants which produce pyrrolizidine alkaloids. An element or group of similar size to hydrogen was used to produce an analogue. Racemic 2-methylputrescine and <u>R</u>-(+)-2-methylputrescine were synthesised by a literature method, and racemic 2-fluoroputrescine and the two

enantiomeric 2-fluoroputrescines were constructed by a new procedure. The 2-methylputrescines were both labelled with ^{3}H and fed to Cyno-glossum officinale, Senecio pleistocephalus, and Emilia flammea. Their fate in the plant extracts was followed by radioactive scintillation counting. N.m.r. spectroscopy and circular dichroism were employed in an attempt to study the absolute configuration of the two enantiomeric fluoro-putrescines, and n.m.r. spectroscopy was used to try to study their metabolism by the plants.

(c). Investigation of the pyrrolizidine alkaloid content of plants

H.p.l.c. was used to study the separation of alkaloid mixtures in Cynoglossum officinale, Cynoglossum australe, Cynoglossum nervosum, and Senecio glaberrimus. Analytical h.p.l.c. traces of the mixtures of pyrrolizidine alkaloids isolated from each of the plants, demonstrated the general applicability of this technique. Preparative h.p.l.c. of the alkaloid extract of Cynoglossum nervosum was used to separate three similar alkaloids, which were all esters of (+)-heliotridine (A).

ABBREVIATIONS

Ac — acetyl ($COCH_3$)

br — broad

^tBu — tertiary butyl (CMe_3)

c — concentration

c.d. — circular dichroism

CDI — N,N'-carbonyl diimidazole

COSY — correlation spectroscopy

C.S.I.R.O. — Commonwealth Scientific and Industrial Research Organisation

d — doublet

D — deuterium (^2H)

DBN — 1,8-diazabicyclo[4.3.0]non-5-ene

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

DCC — dicyclohexyl carbodiimide

DEAD — diethyl azodicarboxylate

dec. — decomposition

DEPT — distortionless enhancement by polarisation transfer

DIAD — diisopropyl azodicarboxylate

DIBAL — diisobutyl aluminium hydride

DMAP — 4-N,N-dimethylaminopyridine

DME — 1,2-dimethoxyethane

DMF — N,N-dimethylformamide

DMSO — dimethyl sulphoxide

DNA — deoxyribonucleic acid

e.e.	-	enantiomeric excess
Et	-	ethyl
h.p.l.c.	-	high pressure liquid chromatography
Hyd.	-	hydrolysis
i.r.	-	infra red
J	-	coupling constant
m	-	multiplet
max.	-	maximum
Me	-	methyl (CH_3)
MOM	-	methoxymethyl (CH_2OCH_3)
m.p.	-	melting point
m.s.	-	mass spectrometry
MSM	-	methane sulphonyl methyl (CH_2SOCH_3)
MTM	-	methane thio methyl (CH_2SCH_3)
n.m.r.	-	nuclear magnetic resonance
Nu	-	nucleophile
Ox.	-	oxidation
Ph	-	phenyl
p.l.c.	-	preparative layer chromatography
^{i}Pr	-	isopropyl ($CHMe_2$)
q	-	quartet
Red.	-	reduction
r.t.	-	room temperature
s	-	singlet
t	-	triplet
T	-	tritium (^3H)

TBDMS   –   tertiarybutyl dimethylsilyl

THF   –   tetrahydrofuran

t.l.c.   –   thin layer chromatography

TMS   –   tetramethylsilane

U.S.D.A. –   United States Department of Agriculture

U.S.S.R. –   Union of Soviet Socialist Republics

u.v.   –   ultra violet

PUBLICATIONS

Sections of this thesis have been accepted for publication, as shown below.

Pyrrolizidine Alkaloid Analogues. Synthesis of 11-Membered Macrocyclic Diesters of (+)-Heliotridine. Desmond B. Hagan and David J. Robins, J. Chem. Soc., Perkin Trans. 1, in press.

(+)-7,9-$\underline{O}$,$\underline{O}$'-(3,3-Dimethylglutaryl)heliotridine, A Pyrrolizidine Alkaloid Analogue. Andrew A. Freer, Desmond B. Hagan and David J. Robins, Acta Cryst., Sect. C, in press.

CHAPTER ONE

INTRODUCTION

1.1 Pyrrolizidine Alkaloids

Natural products lie at the heart of organic chemistry. Studies
of the substances which are present in living matter have not only given
a theme to the discipline but have continually enriched its development.

The pyrrolizidine group of alkaloids constitute a large class of
natural products.[1] Alkaloids of this type are important due to their
widespread occurrence and their varied and well documented biological
and medicinal properties. They contain the basic 1-azabicyclo[3.3.0]-
octane (pyrrolizidine) nucleus (1) or a related system (2). This nucleus
is commonly found as a 1-hydroxymethyl pyrrolizidine system, e.g.,
heliotridine (3).

The most frequently isolated pyrrolizidine alkaloids are esters.[2]
These may be monoesters [e.g. strigosine (4)], unsymmetrical diesters
[e.g. lasiocarpine (5)], or macrocyclic diesters. The macrocyclic
diester alkaloids have the highest hepatotoxicity.[3] The size of the
largest ring can vary from 11- [as in trichodesmine (6)] up to 14-
membered rings [such as parsonsine (7)]. Most macrocyclic diesters
contain retronecine as the base portion. It is curious that no macrocyclic
diesters of heliotridine have been isolated.

The base portions (necines) differ in the degree of hydroxyl-
ation of the amino alcohol, stereochemistry and the degree of unsaturation
of the rings. The acidic moiety of these alkaloids (necic acids) differ in

(1)

(2)

R^2O H $^9CH_2OR^1$

7 1

6 8 2

5 4 3

(3) $R^1=R^2=H$

(5) $R^1= COCOHCH(OCH_3)$

$(CH_3)_2COH$ CH_3

$R^2= (Z)-COC(CH_3)=CHCH_3$

H CH_2O O

H OH

CH_3 OH

CH_2CH_3

(4)

HO

HO CH_3 CH_3

$(CH_3)_2HC$

H

O

O

O

H

N

(6)

H_3C CH_3

CH H_3C OH

HO $CH(CH_3)_2$

CO_2

O

O H

N

(7)

H $NHCH_3$

O

N

(8)

H

N

CH_3 $(CH_2)_6CH_3$
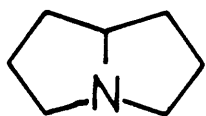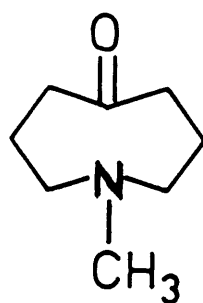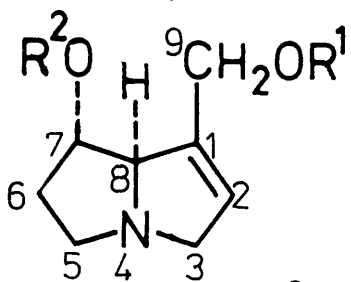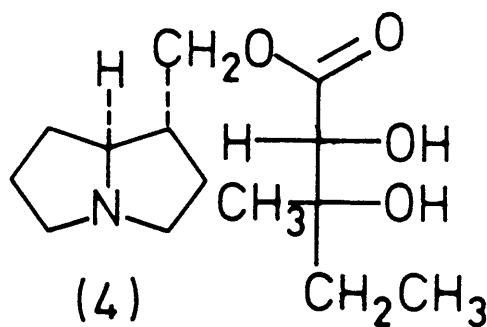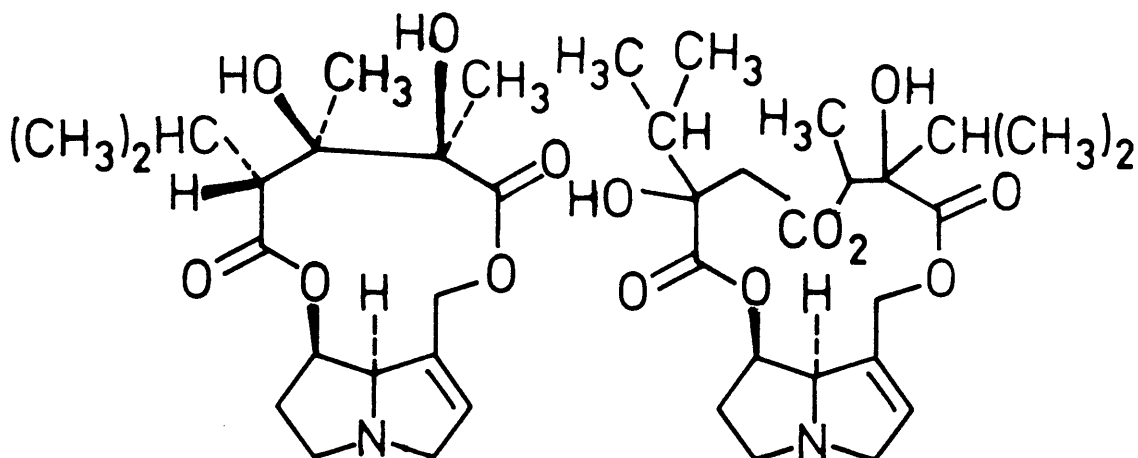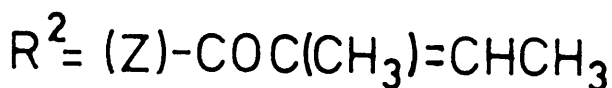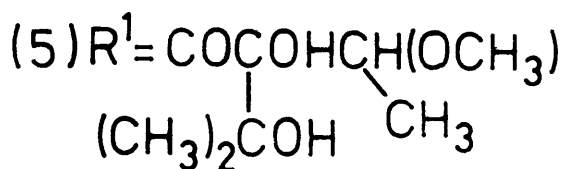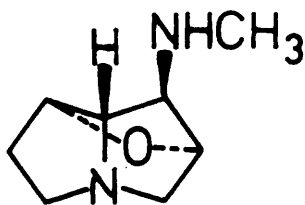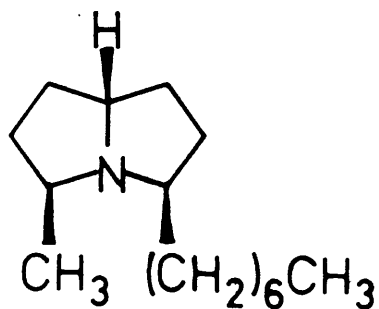
(9)

the degree of branching, hydroxylation and unsaturation. They normally

contain between five to ten carbon atoms and are mono- or dicarboxylic

acids. It is noteworthy that a significant number of alkaloids are non-

ester in nature, such as loline (8).

A comprehensive book by Bull et al.[1] and the annual reviews

presented in the Specialist Periodical Reports[4] and Natural Product

Reports[5] are to be recommended for additional information. A recent book

by Mattocks is also highly informative.[6]

1.2 Occurrence and Hazards of Pyrrolizidine Alkaloids

The pyrrolizidine alkaloids comprise a group of nitrogeneous

bases with a very wide distribution in the plant kingdom. They occur

mainly in genera of three plant families; the Compositae (Senecio), the

Leguminosae (Crotalaria), and the Boraginaceae (Cynoglossum, Helio-

tropium, Lindelofia, and Symphytum). Over 330 plant species have so

far been shown to contain pyrrolizidine alkaloids.[2] Culvenor has

estimated that 3% of the world's flowering plants may contain these

alkaloids.[7] It is known that plants are one of the most hazardous

materials ingested by humans of all ages.[8]

Molecules containing the pyrrolizidine nucleus have also been

found in the insect kingdom. For example, (3S,5S,8R)-3-heptyl-5-methyl

pyrrolizidine (9) is the presumed venomous constituent from the cryptic

thief ant Solenopsis sp. near tennesseensis.[9] Certain species of

Lepidoptera are known to store and metabolise pyrrolizidine alkaloids for

defence purposes. They also utilise them as a pheromone precursor with

additional effects on growth and sexual selection.[10]

The pyrrolizidine class are distinct from the main body of alkaloids in that they are hepatotoxic and thus resemble the mycotoxins, e.g. aflatoxin B_1 (10). They have received extensive chemical and biological study because of their considerable poisoning of livestock and humans.[1]

The hazard to health arises due to ingestion of the alkaloids over long periods of time in plants or foods in which they are present at low concentrations. Pyrrolizidine alkaloids occur mainly as the N-oxides, which are said to be free from the bitterness which causes mammals to reject other alkaloid-containing plants as feedstuffs. They may operate by permitting consumption in order to effect long-term reduction in the herbivore population. Samples of bread,[11] cereals,[12] milk and honey[13] have been found to be contaminated with these toxic substances. Deaths have frequently resulted. Numerous herbal teas, e.g. Symphytum officinale[14] (Russian comfrey), and many medicinal remedies[15] also contain appreciable quantities of pyrrolizidine alkaloids.

The disease recognises no boundaries and affects all climates and continents. Reported outbreaks of the disease, which has various historical, botanical and geographical names, have occurred for several centuries.[1]

The first pyrrolizidine alkaloids were isolated during the last century[16] and now over 200 alkaloids are known. Early this century, a Senecio species which was known to be responsible for livestock poisoning was shown to contain pyrrolizidine alkaloids.[17]

When tested upon animals the alkaloids were shown to produce liver lesions, which are the major characteristics of the disease. The

stricken animals were seen to succumb to "the staggers". Other well documented clinical signs follow, culminating in coma and death.[1] Other organs may also be affected, e.g. heart, lungs, kidneys. Pyrrolizidine alkaloid poisoning remains a serious health and economic hazard.

1.3   Toxicity and Metabolism of Pyrrolizidine Alkaloids

The alkaloids cause their effects due to reaction at the mito-chondrial membrane of the hepatocytes. The metabolism of the hepatocytes is damaged and a megalocytosis occurs where the cells continue to grow and enlarge, but cell division is inhibited. The liver eventually becomes composed of a small number of giant cells. This disruption of the metabolism leads to a shortening of the life-span of the hepatocytes. The liver ceases to function.

Not all pyrrolizidine alkaloids are toxic. This led Schoental to postulate that hepatotoxicity was related to 1,2-unsaturation in the basic moiety and to the intact ester structure.[18] Soon thereafter Culvenor suggested that the alkylation of biological nucleophiles in the liver was responsible for the toxicity.[19] The similarity between the pyrrolizidine alkaloids and other biological alkylating agents was noted as both gave rise to chromosome breakage, inhibition of cell division, mutagenesis and carcinogenesis. The branching of the acidic chain was also thought necessary for the hepatotoxic activity,[20] but this is now thought to be a secondary effect.[21]

Evidence for the mechanism of toxicity and the toxic species involved was provided by Mattocks, who demonstrated that dihydro-

(10)

(11) R = COR′
(12) R = H
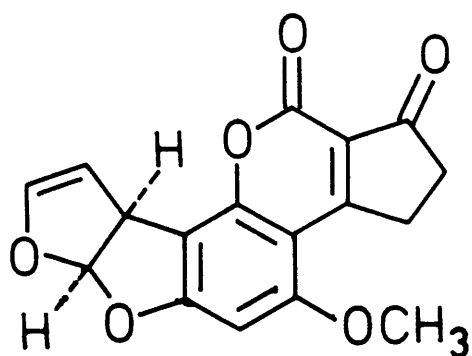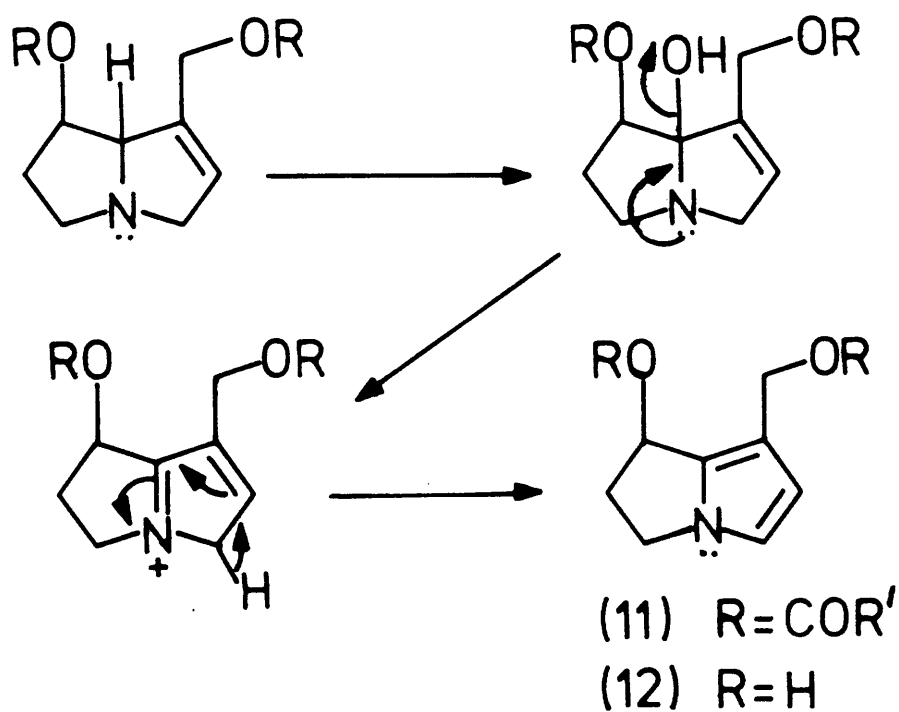
Scheme 1
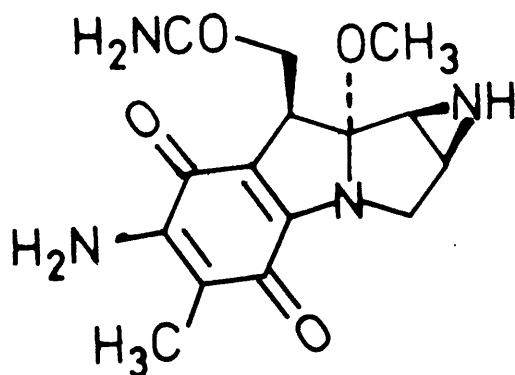
(13)

pyrrolizines (11) were more reactive than the parent alkaloids with respect to alkylation and that there was a good correlation between hepatotoxicity and the amount of pyrrole production.[22,23] Evidence for these metabolically produced pyrroles was detected primarily in the liver, but also in the heart, lung, kidneys, spleen, urine and several other tissues. Pyrroles were also produced from pyrrolizidine alkaloids in vitro using liver slices.

The metabolic pyrroles are thought to arise via hydroxylation and dehydration, as shown in Scheme 1. These reactions occur by the action of hepatic microsomal enzymes. The rate of pyrrole formation by microsomes in vitro is generally higher for macrocyclic than open diesters or monoesters. The initial hydroxylation step is subject to greater steric hinderance in open diesters and so this hydroxylation is thought to occur at C-8 rather than C-3.[24]

The dehydroalkaloids react rapidly with nucleophiles[25] or may be hydrolysed to the secondary toxic metabolite, the dehydronecine (12). Evidence for the former action has been detected in enzyme systems[26] and diseased animals.[27]

The pyrroles act as bifunctional alkylating agents and are released at the site of metabolism. The more acute lesions are consistent with the inactivation of certain coenzymes and component B vitamins, probably acting by alkylating sulphydryl groups.[28] The nucleic acids and proteins can also be alkylated in a manner similar to that of other toxins, e.g. mitomycin C (13).[29] Dehydroretronecine (14) is known to alkylate DNA to give stable adducts,[30] and to reproduce the pattern of liver lesions in vivo of its parent macrocycles.[31] Esters of heliotridine

$$R^1 \quad R^2 \qquad CH_2OH$$
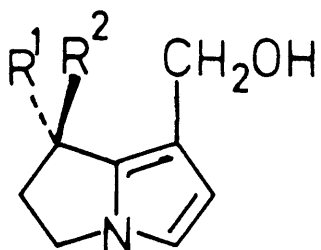
(14) $R^1 = H, R^2 = OH$

(15) $R^1 = OH, R^2 = H$

(11)
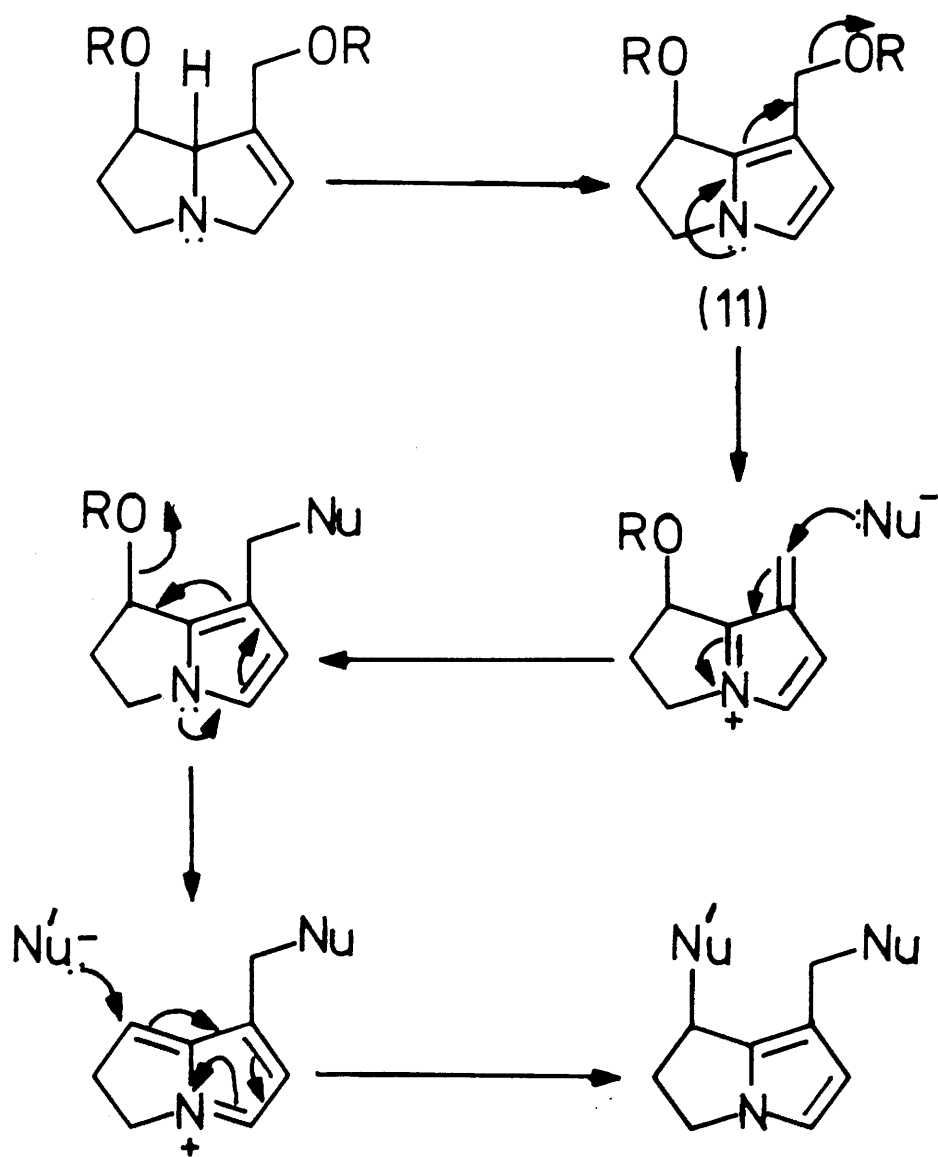
Scheme 2

are also converted into dehydroheliotridine (15).[32]   Electrophilic

alkylation can be related to the structural features required for hepato-

toxicity (Scheme 2).

The most toxic alkaloids are generally the macrocycles, because

of the steric hinderance around the ester groups.   This reduces

detoxification by esterase hydrolysis.   Water solubility, lipophilicity

and base strength also effect toxicity.

The three principal metabolic reactions involved are pyrrole

formation, N-oxidation and hydrolysis.   The first two require microsomal

oxidases.   Pyrrole formation is activating and the others detoxifying as

they produce water soluble excretable products.   This may happen via

urine or bile.   The primary metabolite may escape into the bloodstream

to reach and damage other tissues.   The conversion of N-oxides into the

tertiary amines may occur in the gut.[33]   The fates of the alkaloids are

summarised in Figure 1.   It is possible to see these alkaloids as the

outcome of some evolutionary drive towards a comparatively inert molecule

capable of rapid activation to an efficient cytocidal alkylating agent.

Several pyrrolizidine alkaloids are carcinogens and mutagens.[34,35]

In one of the studies, derivatives of heliotridine and otonecine (16),

including a pyrrolic compound (17), were found to be mutagenic.[35]

It should be noted that not all pyrrolizidine alkaloids are

harmful and many display very useful therapeutic properties.   For

example, sarracine (18) and platyphylline (19) are used in the U.S.S.R.

for the treatment of hypertension, gastrointestinal hypermobility and

peptic ulceration in humans.[1]   The relatively limited toxicity of indicine

N-oxide (20) from Heliotropium indicum allowed it to be chosen for

clinical trials.[36]   Indicine N-oxide is effective against gastrointestinal

Figure 1

Nu = nucleophile, ^{t}Nu = tissue-bound nucleophile

(16)

(17)

(18)

(19)

(20) R= CO
(CH₃)₂CH—OH
HO—H
CH₃

(21) R=CO
HO—C₆H₅
H—H
CH₃
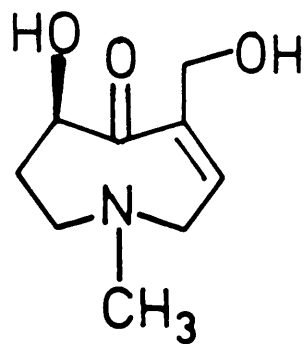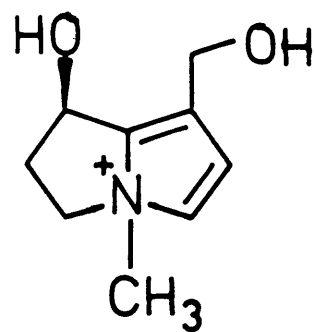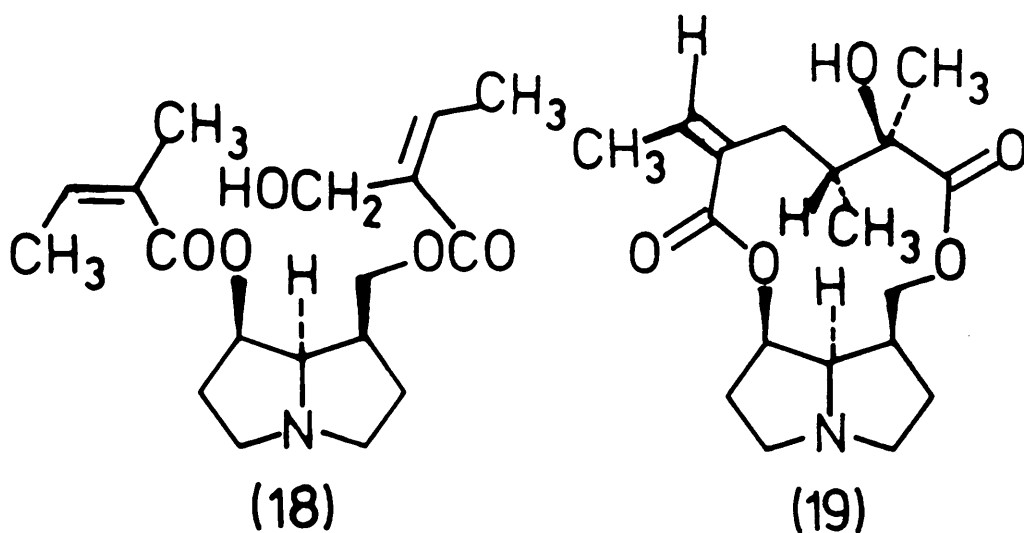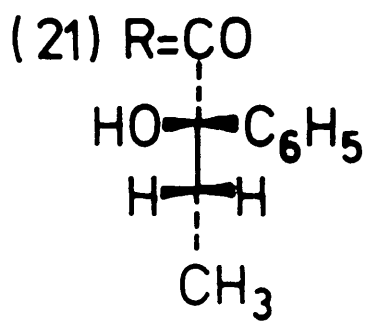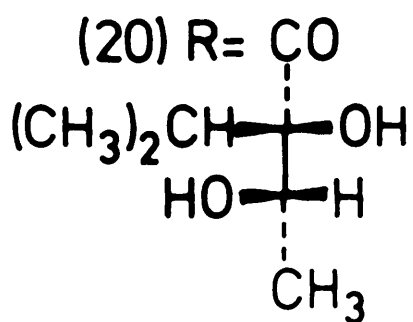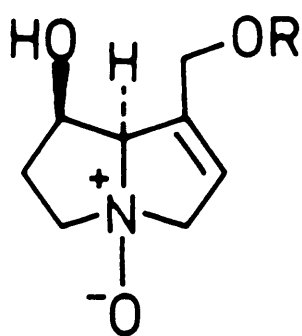
cancer and in cases of leukaemia and melanoma.[33,37] A semi-synthetic analogue (21) has been shown to be a more active anti-tumour agent.[38] This activity may be related to the alkylation of cancer cells.

A goal in this area is to develop a method for protecting animals which ingest pyrrolizidine alkaloids in their feed. This may be by metabolic detoxification, the use of additives, or some other method.

1.4   Outline of Project

This work is divided into several sections. The synthesis of pyrrolizidine alkaloids and analogues has received considerable attention. This area is reviewed in Chapter Two. Work on the synthesis of the first macrocyclic diesters incorporating heliotridine (3) is described in Chapter Three. The main intermediates in the biosynthetic pathway of retronecine have now been established but little is known about the route to other pyrrolizidine bases. Work on the biosynthesis of several pyrrolizidine bases is covered in Chapter Five. The use of structurally modified precursor analogues to gain information about biosynthetic pathways is increasing steadily. The preparation and biosynthetic studies involving putrescine analogues is also described in Chapter Five. Identification of pyrrolizidine alkaloids from plant species not previously investigated is discussed in Chapter Six.

CHAPTER TWO

SYNTHESIS OF PYRROLIZIDINE ALKALOIDS AND ANALOGUES

2.1   Introduction

A great many syntheses of necines and necic acids have been reported.   The combination of these moieties to prepare pyrrolizidine esters is also an area of immense chemical interest.   This can take the form of the synthesis of monoesters and simple open diesters, or the more challenging synthesis of macrocyclic dilactones.   A growth area is the preparation of semi-synthetic and synthetic analogues, which are required for studies of toxicology and structure-activity relationships.

2.2   Synthesis of Pyrrolizidine Bases

Considerable progress has been made by a number of workers in developing synthetic approaches to the less oxidized necines, and some headway has been made towards the total synthesis of the more complex bases.

The recent profusion of new routes to racemic pyrrolizidines has illustrated the interest in this field, e.g. the intramolecular alkyl-ations of activated cyclopropanes[39] (Scheme 3).   Few practical total syntheses of optically active pyrrolizidine derivatives have emerged in spite of the deceptive simplicity of these targets.   The first construction of a series of optically active 1-hydroxymethyl pyrrolizi-dines provided samples of both enantiomers of isoretronecanol (22), trachelanthamidine (23) and supinidine (24) from a readily available

Scheme 3

$(+)-(24)$

$(+)-(22)$ $R^1=H, R^2=CH_2OH$

$(+)-(23)$ $R^1=CH_2OH, R^2=H$

$(-)-(22)$ $R^1=CH_2OH, R^2=H$

$(-)-(23)$ $R^1=H, R^2=CH_2OH$

$(-)-(24)$
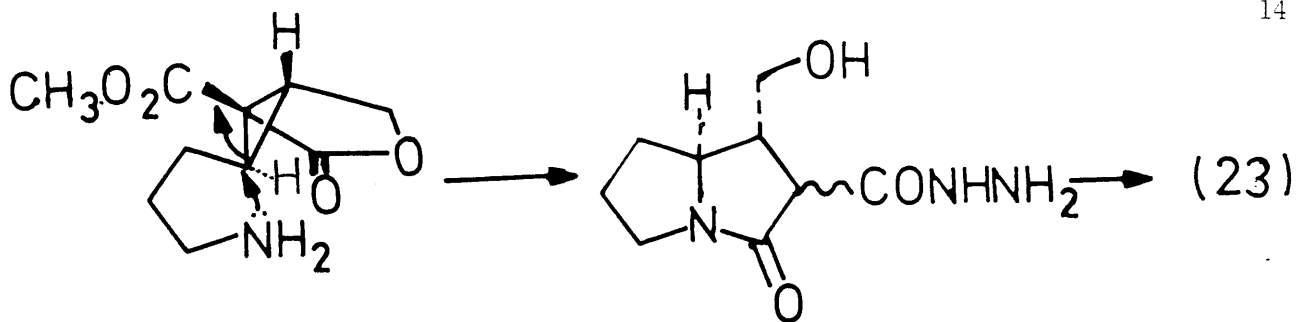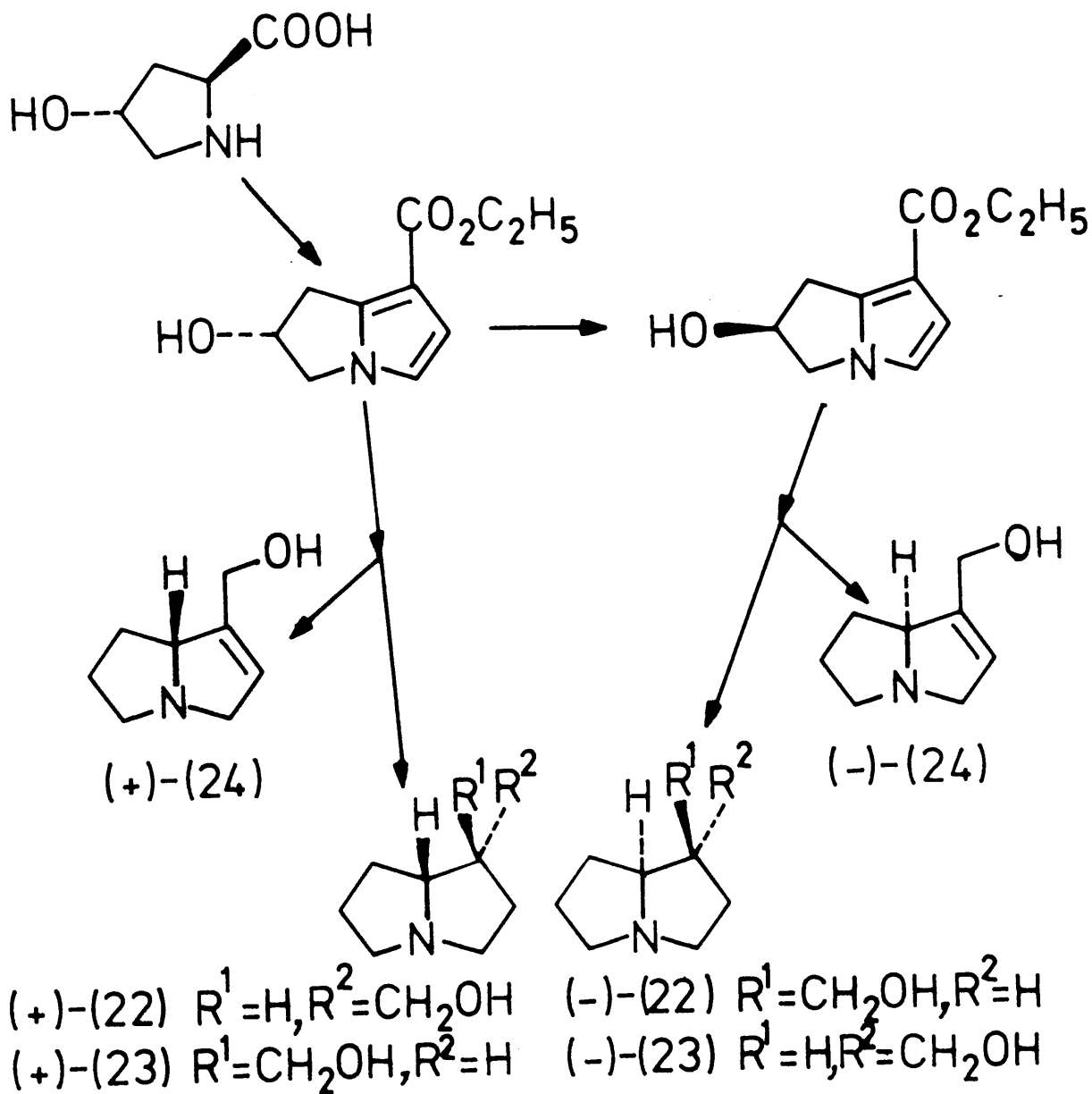
Scheme 4

starting material[40] (Scheme 4). Samples of (-)-rosmarinecine (25) have been produced from D-glucosamine[41] (Scheme 5).

The enantioselective synthesis of (+)-heliotridine (3) and seven other diols from a single intermediate (26) has been reported.[42] The opposite enantiomer of heliotridine has also been synthesised.[43] Both of these routes are noteworthy because of the simplicity of the starting materials (Scheme 6). The most common dihydroxynecine, retronecine (27), was first synthesised by Geissman and Waiss in racemic form via a lactone and the enantiomers were separated by resolution.[44] The same lactone, in chiral form, was used to synthesise (+)-croalbinecine (28) and (-)-platynecine (29).[45] Recently, (+)-retronecine was synthesised by the construction of an optically active tricyclic lactone (30)[46] (Scheme 7).

2.3 Synthesis of Acyclic Esters

Among the first pyrrolizidine monoesters to be synthesised were trachelanthamine (31) and viridiflorine (32) via a transesterification process.[47] This involved the treatment of the di-O-benzylether derivative of the methyl ester of an acid with the 1-hydroxymethyl pyrrolizidine.

The use of acid chlorides in the synthesis of pyrrolizidine alkaloids has been prevalent for a number of decades. The recombination of an amino alcohol and an acid to synthesise heliotrine (33) and supinine (34) by Culvenor et al.,[48] and the synthesis of benzoyl trachelanthamidine (35) by Men'shikov et al.[49] are two of the earliest examples.
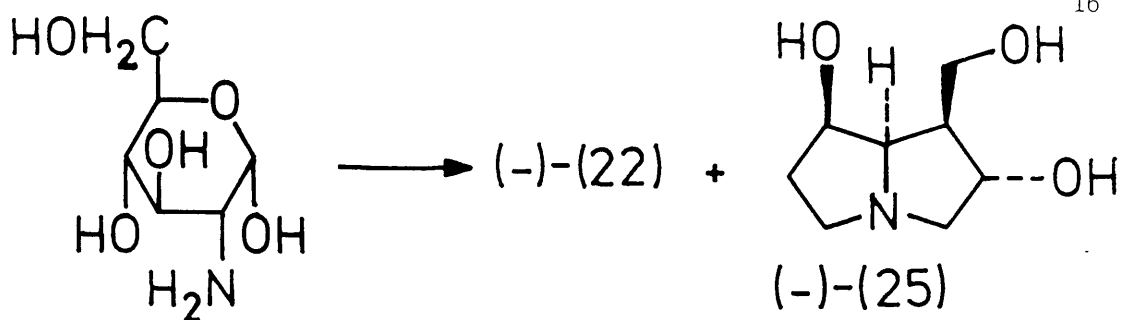
Scheme 5
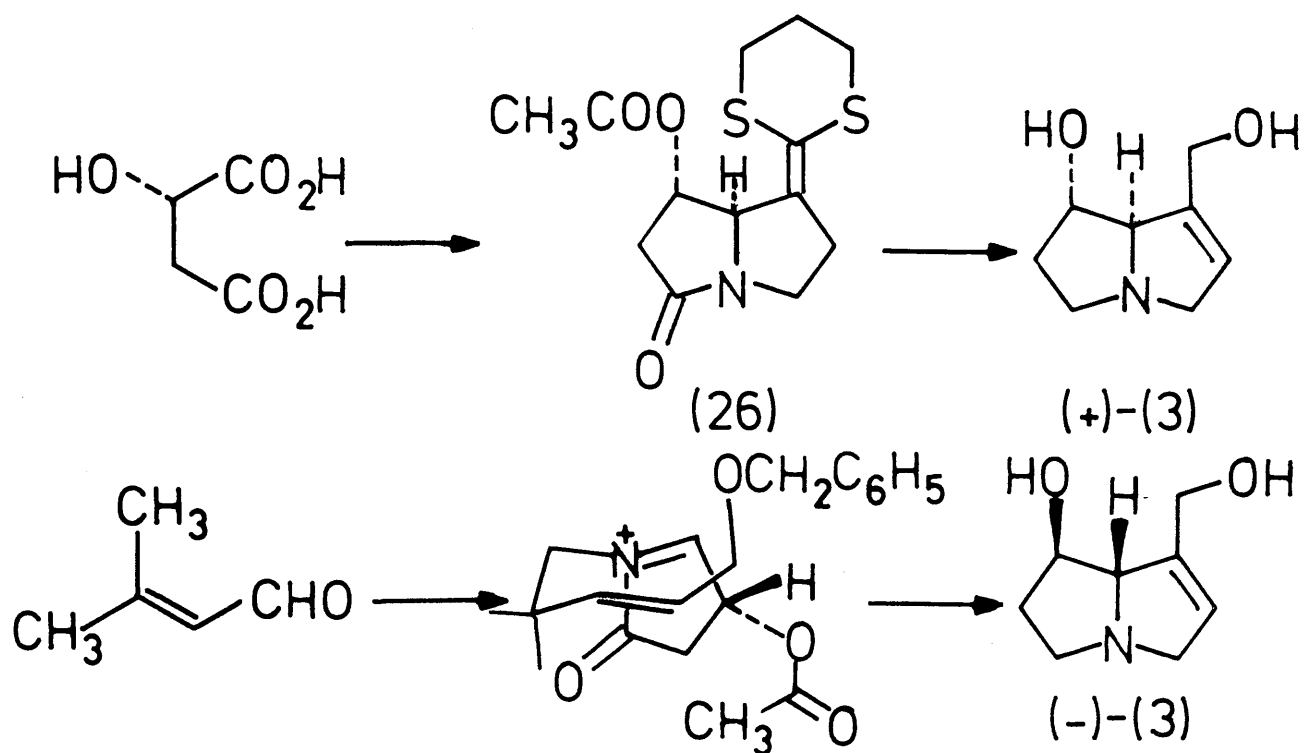
Scheme 6
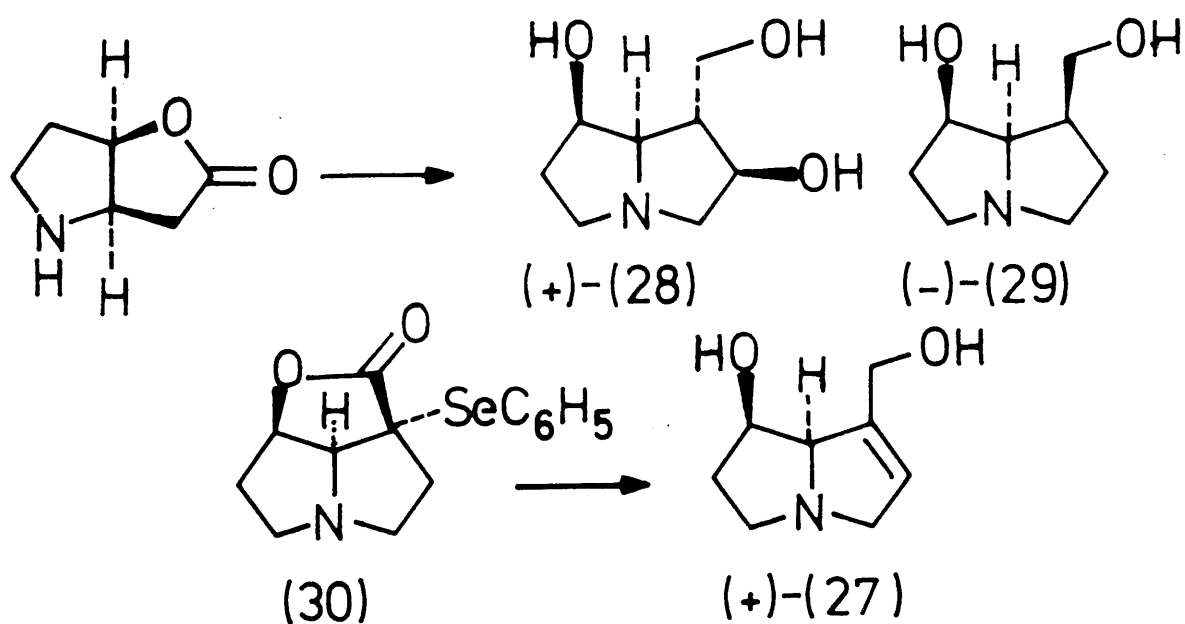
Scheme 7

Selective esterification of the allylic hydroxyl group has been achieved by the use of a coupling reagent, such as dicyclohexyl carbodiimide (DCC) or 1,1'-carbonyl diimidazole[50] (CDI). The esterifying acids were not always simple, but were sometimes α,β-unsaturated or hindered by α-hydroxyl functions (Scheme 8). In the case of 9-tiglylretronecine (36), the use of CDI was preferred over that of DCC as the reaction was cleaner in a useful yield and with less isomerised product. An unsymmetrical diester (37) was also prepared by the treatment of retronecine in turn with pivaloyl imidazole and an acid chloride. Other workers have employed CDI as a coupling agent in the synthesis of indicine N-oxide (19) and analogues, e.g. (20)[51] (Section 1.3). Indicine N-oxide has been prepared in a radioactivity labelled form with a view to studying the alkaloid's metabolism.[52]

2.4 Synthesis of Macrocyclic Diesters

The fact that a number of macrolide natural products have antibiotic and therapeutic properties has prompted interest in lactonisation procedures. A number of reviews have been published in this area.[53-56] The intramolecular esterification of a hydroxycarboxylic acid is dependent upon several specific structural features, e.g. unsaturation[57] and the length of the carbon chain.[58]

The synthesis of the macrocyclic diester alkaloids is one of the outstanding challenges in this field. The first synthesis of a bislactone containing retronecine was the construction of 13,13-dimethyl-1,2-didehydrocrotalanine (38).[59] The route made excellent use of the
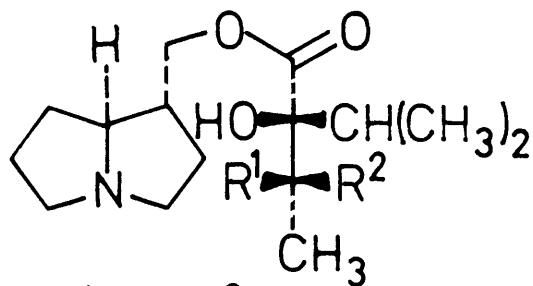
(31) R^1=H, R^2=OH
(32) R^1=OH, R^2=H

(33)

(34)

(35)

(27)

$\xrightarrow[\text{RCOOH}]{\text{DCC or CDI}}$

(36) R= CO

Scheme 8

$(CH_3)_2CHCOO$ $CH_2OCOC(CH_3)_3$
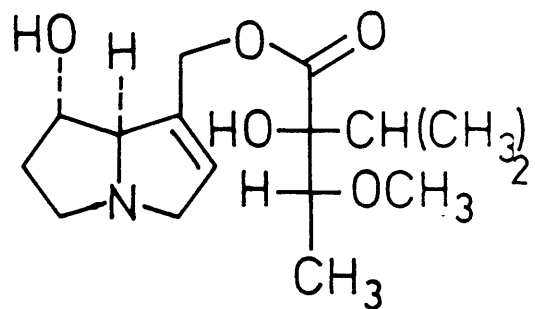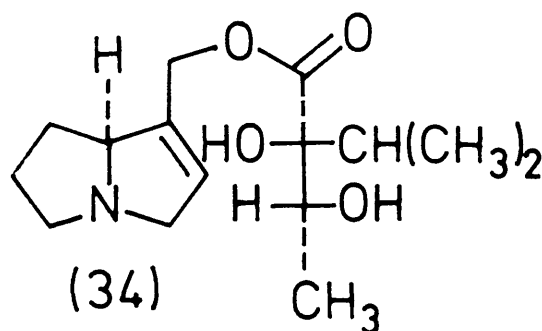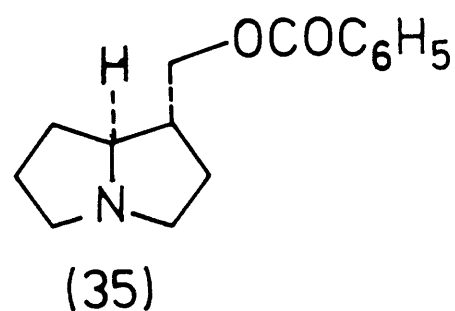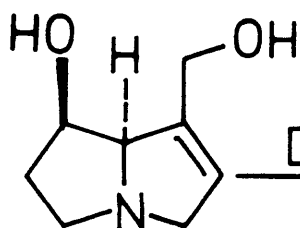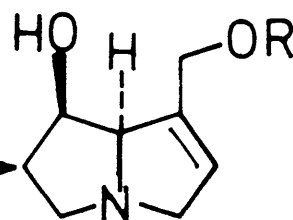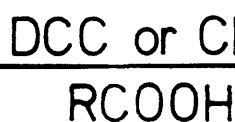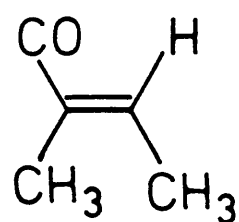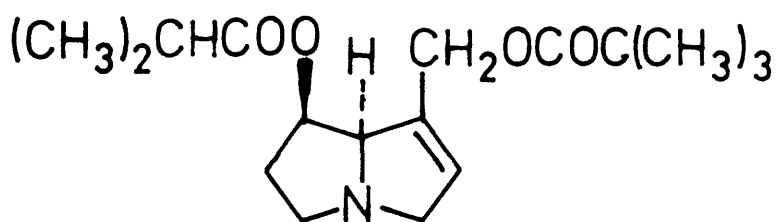
(37)

Corey-Nicolaou "double activation" method of lactonisation.[60] This highly efficient method is mild enough to be used in conjunction with complex and polyfunctional substrates and is discussed in greater detail in Chapter Four. The synthesis of a series of 11-membered macrocyclic diesters of (+)-retronecine,[61] including (+)-dicrotaline (39),[62] followed this initial success.

The first 10-membered macrocyclic pyrrolizidine diesters were also synthesised via this method,[63] e.g. 7,9-$\underline{O}$,$\underline{O}$'-(succinyl)retronecine (40). No pyrrolizidine alkaloids have been isolated with a ring of this size. A number of other natural products have been constructed by this procedure.[64] Another route to pyrrolizidine alkaloid analogues is via the displacement of an allylic chloride, such as is present in (-)-(7R,8R)-1-chloromethyl-1,2-didehydro-7-hydroxypyrrolizidine (41), by a carboxylate anion. This was used to provide further macrocyclic bislactones, e.g. (42) (Scheme 9).[65] The reaction of the appropriate anhydride probably formed the 7-monoester and intramolecular displacement by a carboxylate anion was effected by treatment with DBN, DBU or Hunig's base.

Relatively few total syntheses of natural pyrrolizidine alkaloids exist. As well as dicrotaline, mentioned previously, the only other 11-membered macrocycles constructed have been crispatine (43) and fulvine (44),[66] the $\underline{O}$-acetyl derivative of crobarbatine[67] and monocrotaline (45).[68]

The synthesis of crispatine and fulvine began with crispatic (46) and fulvinic (47) anhydrides respectively. Lactonisation was achieved by the displacement of a methanesulphonate ester with a
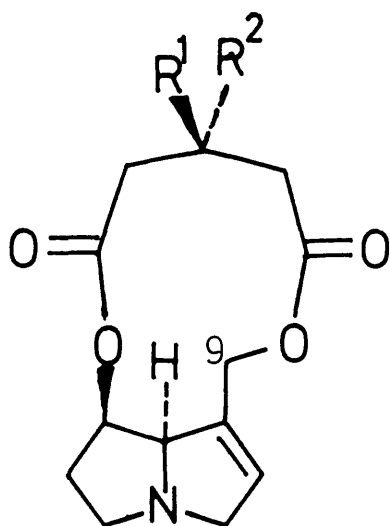
$(38)\ R^1=R^2=CH_3$

$(39)\ R^1=OH,\ R^2=CH_3$
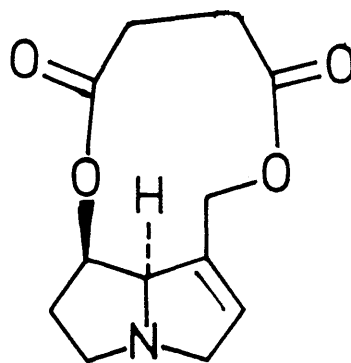
(40)
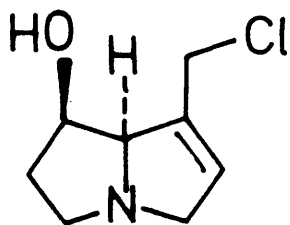
(41)
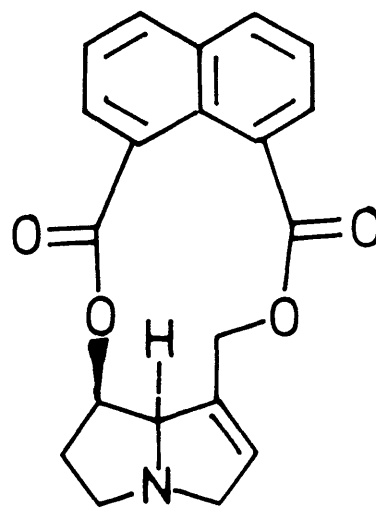
(42)

Scheme 9

carboxylate anion to form two separable diastereoisomers in each case (Scheme 10).

Crobarbatine acetate and a diastereoisomer were constructed via the use of a chiral lactone (48) (Scheme 11). The lactone was opened as its tertiary butyl thiolester and the free hydroxyl group was protected as the acetate ester. The remaining carboxylic acid grouping was activated as the imidazolide to form the thiolester (49). Treatment of (+)-retronecine with this thiolester and a catalytic amount of base was followed by intramolecular lactonisation to give two diastereoisomeric products, (50) and (51). Only one of the esters, (51), could be hydrolysed to the corresponding base (52). The authors could not conclusively prove that (52) corresponded to natural crobarbatine, or demonstrate which one of the esters was the acetate of this alkaloid.

Monocrotaline (45) was prepared by coupling of the protected acid (53) with a form of retronecine (54) and eventual fluoride induced cyclization to form acetal (55) (Scheme 12). Desilylation of the β-(trimethylsilyl)ethylester to generate a carboxylate anion in situ and displacement of a methanesulphonate group at C-9 of the necine moiety effected this transformation. Deprotection afforded the free base (45).

At the present time, only one 12-membered macrocyclic pyrrolizidine alkaloid, integerrimine (56), has been synthesised. In the first reported synthesis, the protected anhydride (57) was coupled with the lithium alkoxide of silylated retronecine (54) with p-dimethyl-aminopyridine (DMAP) as a catalyst[69] (Scheme 13). The allylic hydroxyl was desilylated and the methylthiomethyl group was converted into a methylsulphonylmethyl moiety. The activated acid was

Scheme 10

(46) $R^1=OH, R^2=CH_3$
(47) $R^1=CH_3, R^2=OH$

+ isomer

(43) $R^1=OH, R^2=CH_3$
(44) $R^1=CH_3, R^2=OH$
+
(12R,14S)-isomers

(48)

(27)

	R^1	R^2	R^3	R^4
(50)	CH_3	H	CH_3	OAc
(51)	H	CH_3	OAc	CH_3
(52)	H	CH_3	OH	CH_3

Scheme 11

(53)

(54)
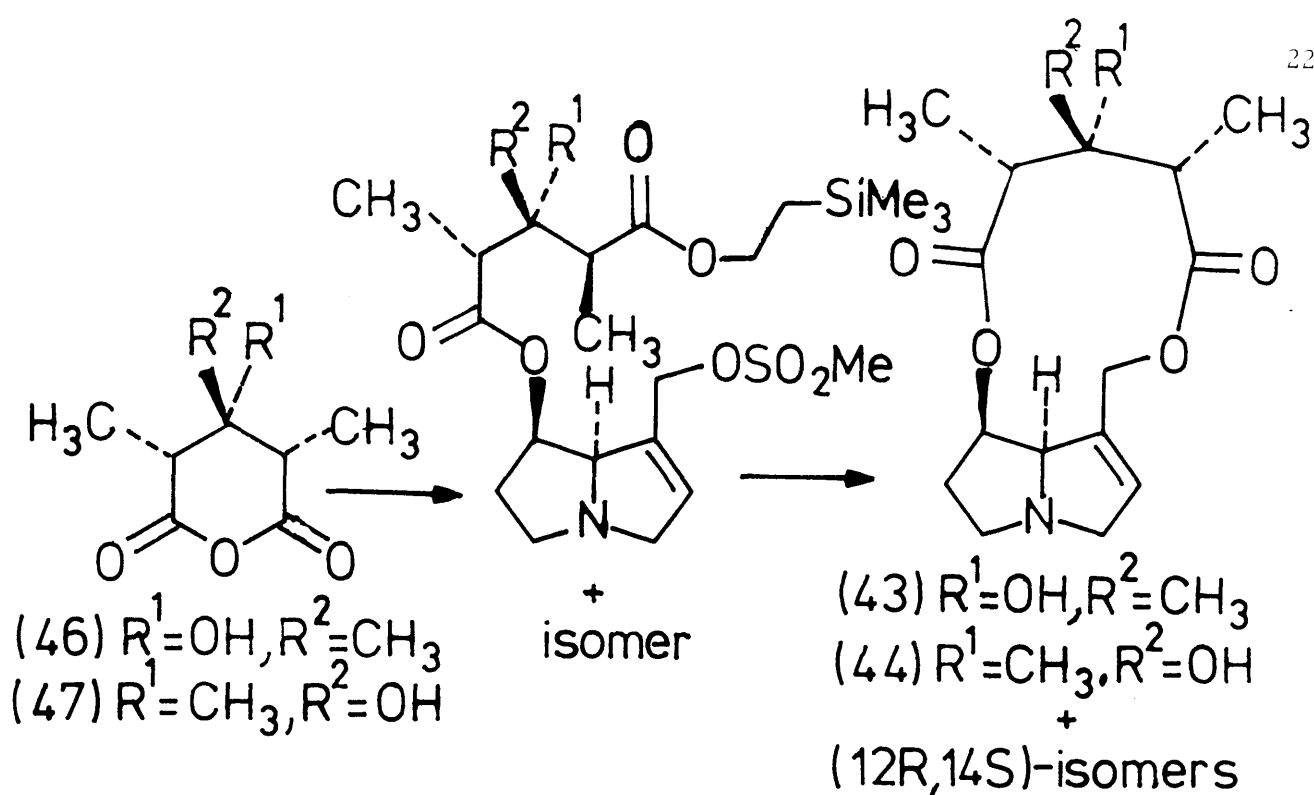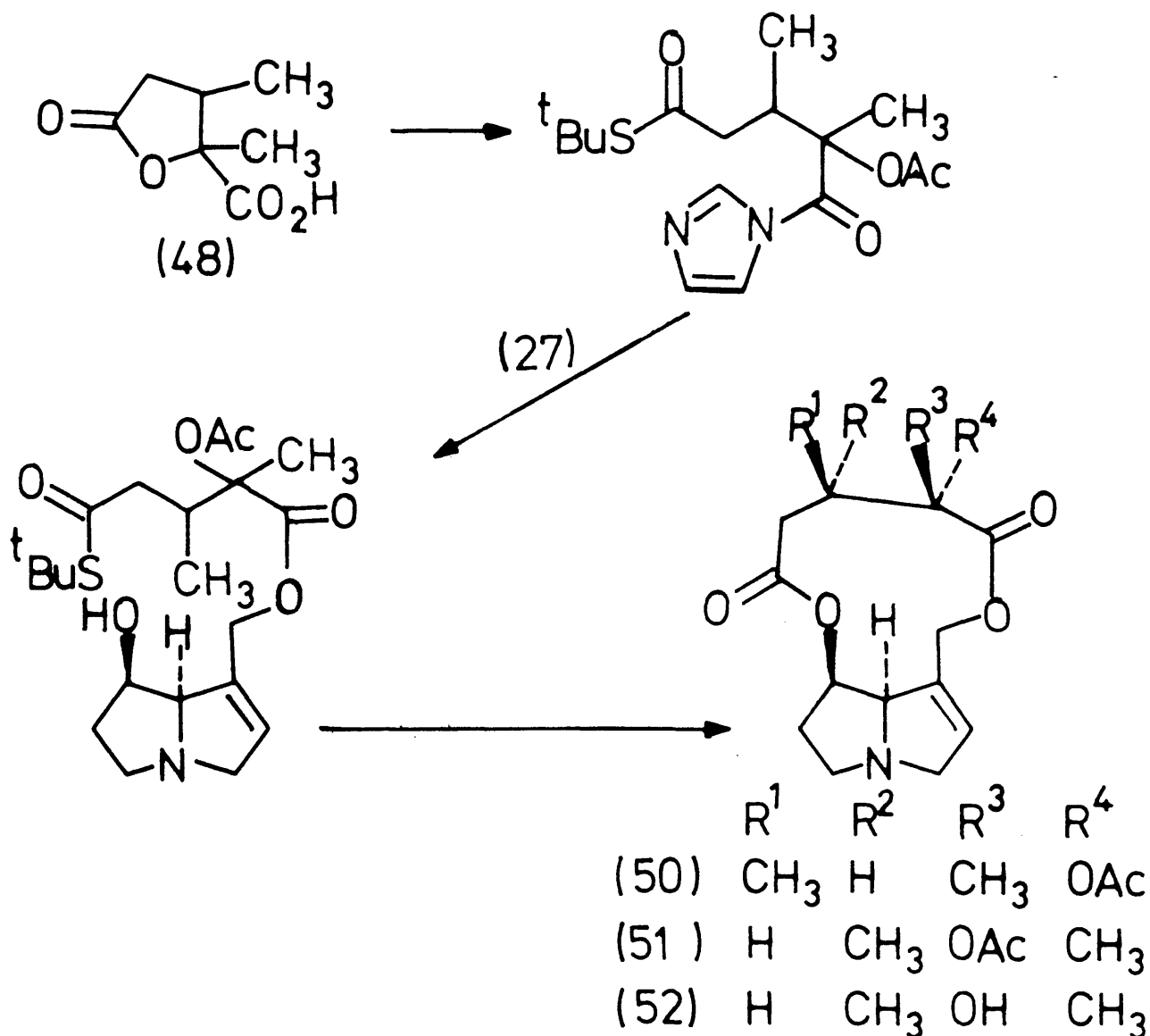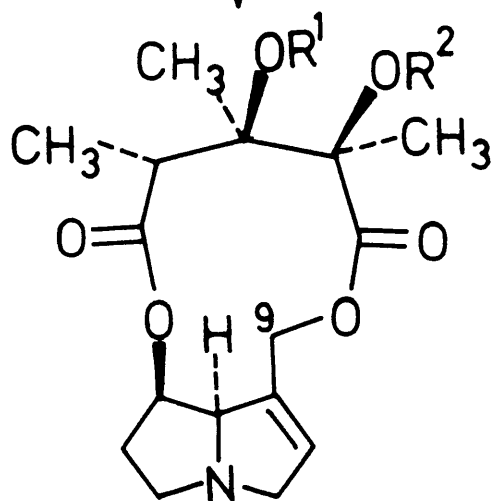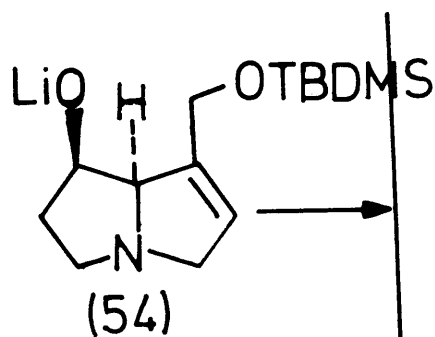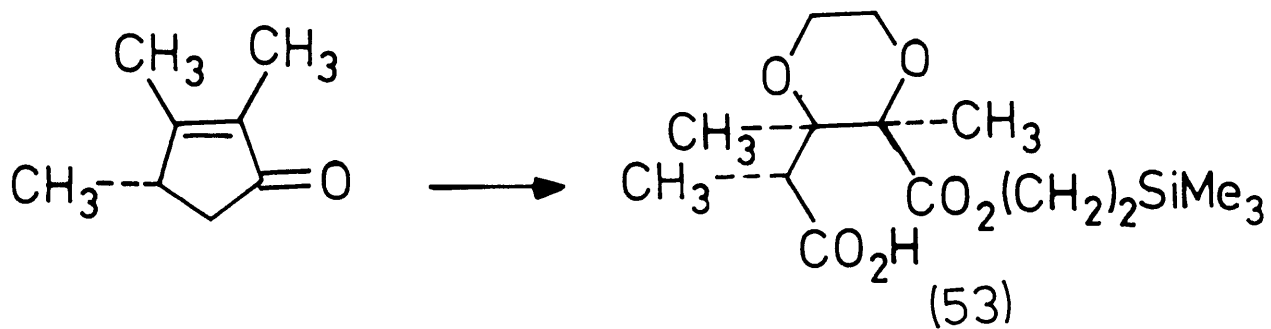
(45) $R^1 = R^2 = H$

(55) $R^1, R^2 = (CH_2)_2$

Scheme 12

Scheme 13

(54)

(57)

(56)

Scheme 14

(58)

(59)

(56)
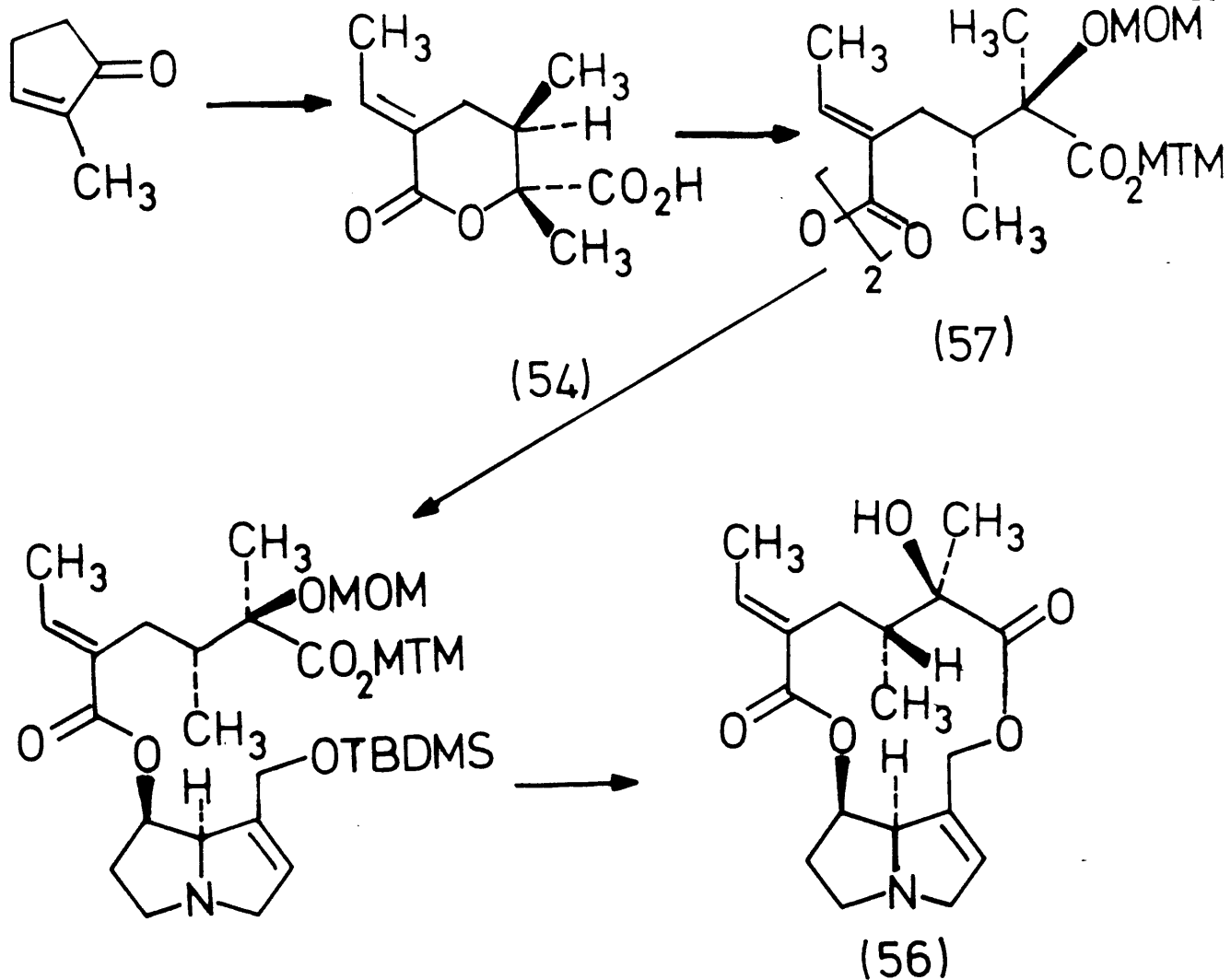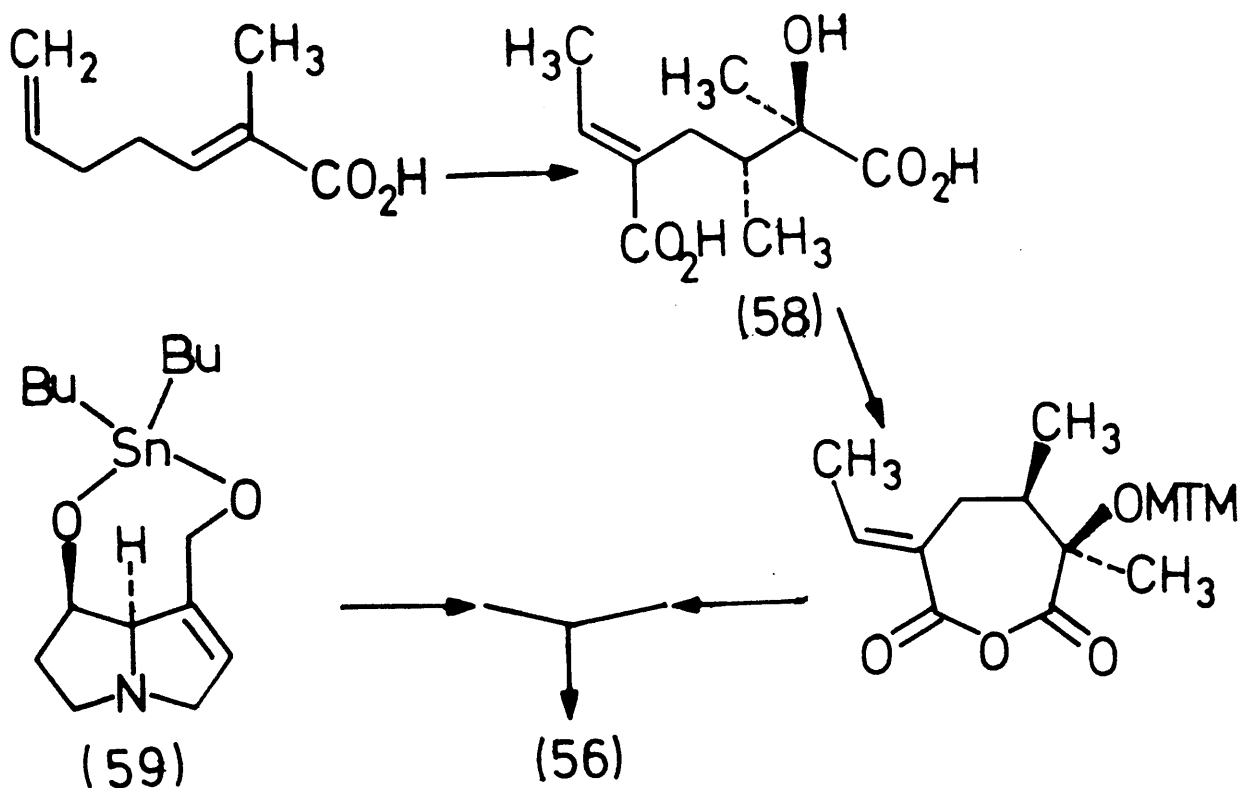
lactonised by intramolecular nucleophilic displacement. Reduction of the N-oxides, deprotection, and separation furnished samples of (±)-(56) and a diastereoisomer.

The same alkaloid was constructed by the regioselective reaction of a cyclic anhydride with a cyclic stannoxane (59)[70] (Scheme 14). This involved the synthesis of (-)-integerrinecic acid (58),[71] and eventually yielded (-)-(56).

Another synthesis of (-)-(56) utilised a silylated necine and a protected acid (60) to furnish a C-7 monoester[72] (Scheme 15). Modifications to the allylic alcohol group, followed by selective cleavage, spontaneous intramolecular displacement and final deprotection afforded integerrimine.

Analogues of macrocyclic bislactones based upon 2,3-bis-hydroxymethyl-1-methyl-3-pyrroline (synthanecine A)[73] (61) have been synthesised by the Corey-Nicolaou route,[74] and by the intramolecular nucleophilic substitution of an allylic chloride.[75] Using a combination of these two routes, macrocyclic diesters of synthanecine A containing 12 to 16-membered rings have been constructed.[76] These dilactones are metabolised in a manner similar to that of the natural alkaloids.[77]

OR

COOCH₃

$COOCH_3$

H

CH_3

R=$CH_2O(CH_2)_2SiMe_3$

CH_3 H_3C OTBDMS

$CO_2(CH_2)_2SiMe_3$

H

HO_2C

CH_3

(60)

HO H OTBDMS

CH_3 H_3C

OTBDMS

$CO_2(CH_2)_2SiMe_3$

CH_3

OR

H

N

(56)

R=TBDMS,H or SO_2Me

Scheme 15

OH OH

CH_3 N

(61)
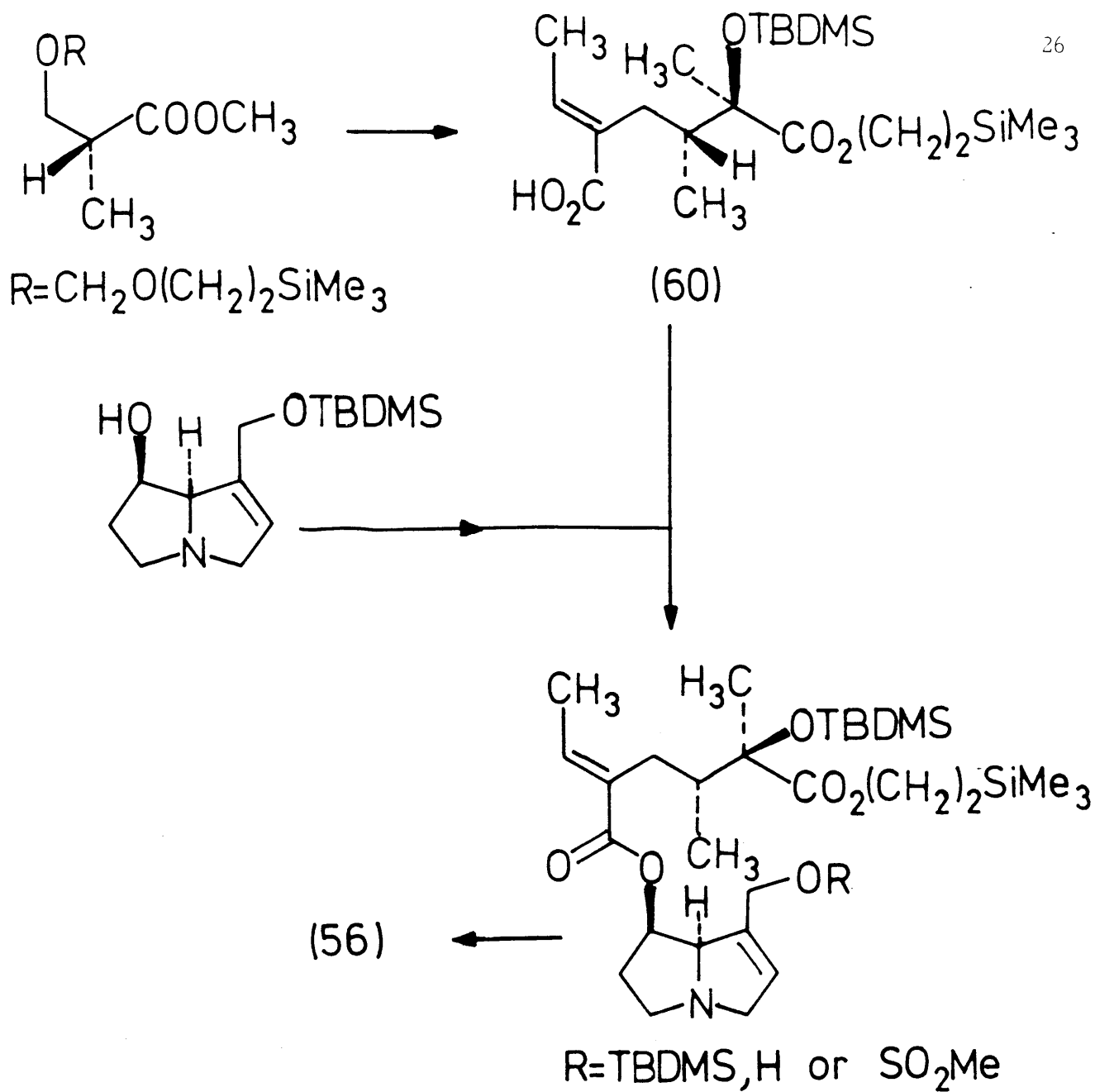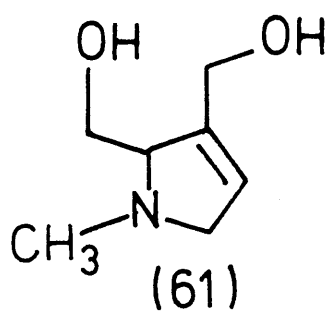
CHAPTER THREE

SYNTHESIS OF MACROCYCLIC DIESTERS OF HELIOTRIDINE

3.1   Introduction

Macrocyclic diester pyrrolizidine alkaloids have the highest hepatotoxicity.   Some typical LD_{50} values[1] are listed in Table 1.

A range of macrocyclic diesters of (+)-retronecine (27) was constructed by Robins et al. by use of the Corey-Nicolaou method (see Section 2.4).   In this work, the first synthesis of macrocyclic diesters containing (+)-heliotridine (3) was achieved via this route. A supply of (+)-heliotridine was obtained by hydrolysis of (+)-echinatine (62) and, to a smaller extent, by inversion of the configuration of the secondary hydroxyl of (+)-retronecine by a literature method.[78] An X-ray crystal structure analysis of one of these new pyrrolizidine alkaloid analogues was carried out.

3.2   Isolation of (+)-Echinatine

The first aim of the project was to locate a good source of heliotridine.   With this in mind, Cynoglossum officinale (family Boraginaceae) was investigated.   Previous workers had found this species contained a variety of alkaloids, with the exact pattern varying according to the location of the plants.   This is not unusual.

In the first study of this species, heliosupine (63) was isolated,[79] followed by heliosupine, echinatine and the corresponding N-oxides.[80]   Acetylheliosupine (64) and 7-angelylheliotridine (65) were

ALKALOID (Type)	LD_{50}^{a}
RETRORSINE (Macrocycle)	34
CYNAUSTINE (Monoester)	260
PLATYPHYLLINE (Satd. Diester)	252[b]
HELIOTRINE (Open Diester)	300
ECHINATINE (Monoester)	350
HELIOTRIDINE (Free Base)	1200
HELIOTRINE N-OXIDE	5000

Table 1

a. in male rats (mg/kg)

b. all deaths occurred within 1 hr.

(62) $R^1 = H, R^2 = CO-C(iPr)(OH)-CH(OH)-Me$

(63) $R^1 = (Z)-COCMe=CHMe,$
$R^2 = COC(OH)(CMe_2OH)CHOHCH_3$

(64) $R^1 = (Z)-COCMe=CHMe,$
$R^2 = COC(OH)(CMe_2OH)CH(OAc)CH_3$

(65) $R^1 = (Z)-COCMe=CHMe, R^2 = H$

(66)

(+)-(62)

$\xrightarrow{\text{i.}}$

(+)-(3)

+

(−)-(67)
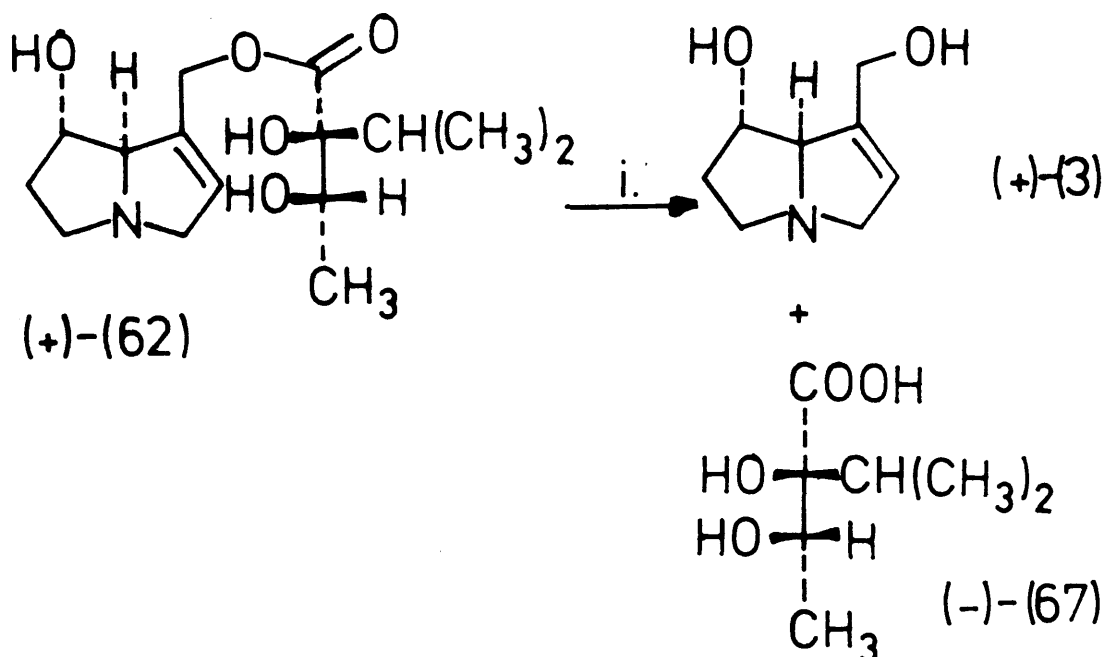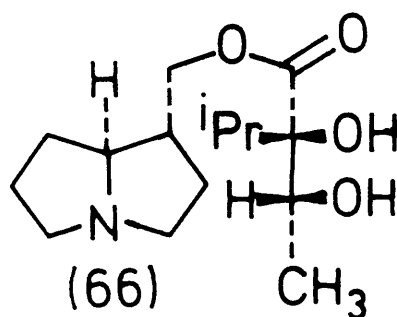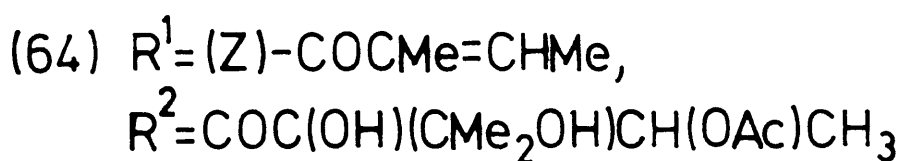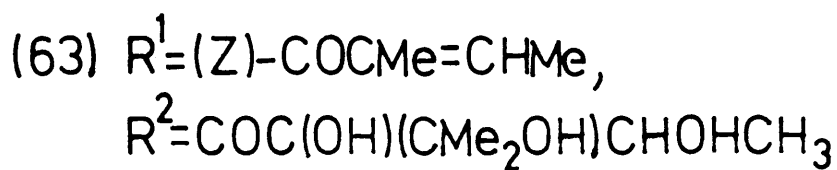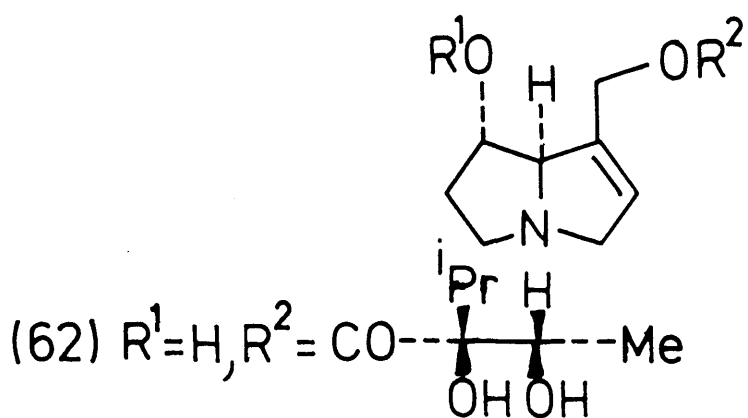
Scheme 16

Reagents: i. $Ba(OH)_2, H_2O, \Delta$.

also found.[81]   All these alkaloids are derivatives of heliotridine and

our choice of plant seemed to offer a good chance of achieving our aim.

More recently, the same species was shown to contain coromandalin (66).[82]

Our investigation of a batch of locally grown Cynoglossum

officinale revealed, after harvesting and methanolic extraction, a

mixture of pyrrolizidine alkaloids.   The major alkaloidal constituent was

(+)-echinatine (62).   The structure and stereochemistry was established

by spectroscopic analysis and alkaline hydrolysis to the free base, (+)-

heliotridine (3), and (-)-viridifloric acid (67)[83] (Scheme 16).   A 200

MHz ^{1}H n.m.r. spectrum (Figure 2) was carried out and a ^{1}H/^{13}C

correlation spectrum[84] (Figure 3) was used for the elucidation of the

structure.   For example, of particular interest is the assignment of

C-9/C-3 and C-7/C-8, which is of value in the biosynthetic work

(see Section 5.3).   This alkaloid was first isolated from Rindera

echinata (family Boraginaceae)[85] and the stereochemistry present in

heliotridine was originally deduced by chemical means.[86]

3.3   Synthesis of (+)-Heliotridine

Enantiomerically pure heliotridine was prepared by modificat-

ions to the method of Zalkow et al.,[78] which itself uses other well

known procedures (Scheme 17).

Although several procedures for the synthesis of retronecine

(27) have been developed (see Section 2.2), the base is more easily

obtained by the hydrolysis of pyrrolizidine alkaloids containing the

retronecine moiety.   This affords optically pure material.   For this

purpose, we made use of two readily available macrocycles.   Retrorsine

Figure 2

200MHz ^{1}Hn.m.r. spectrum of
(+)-Echinatine (62)
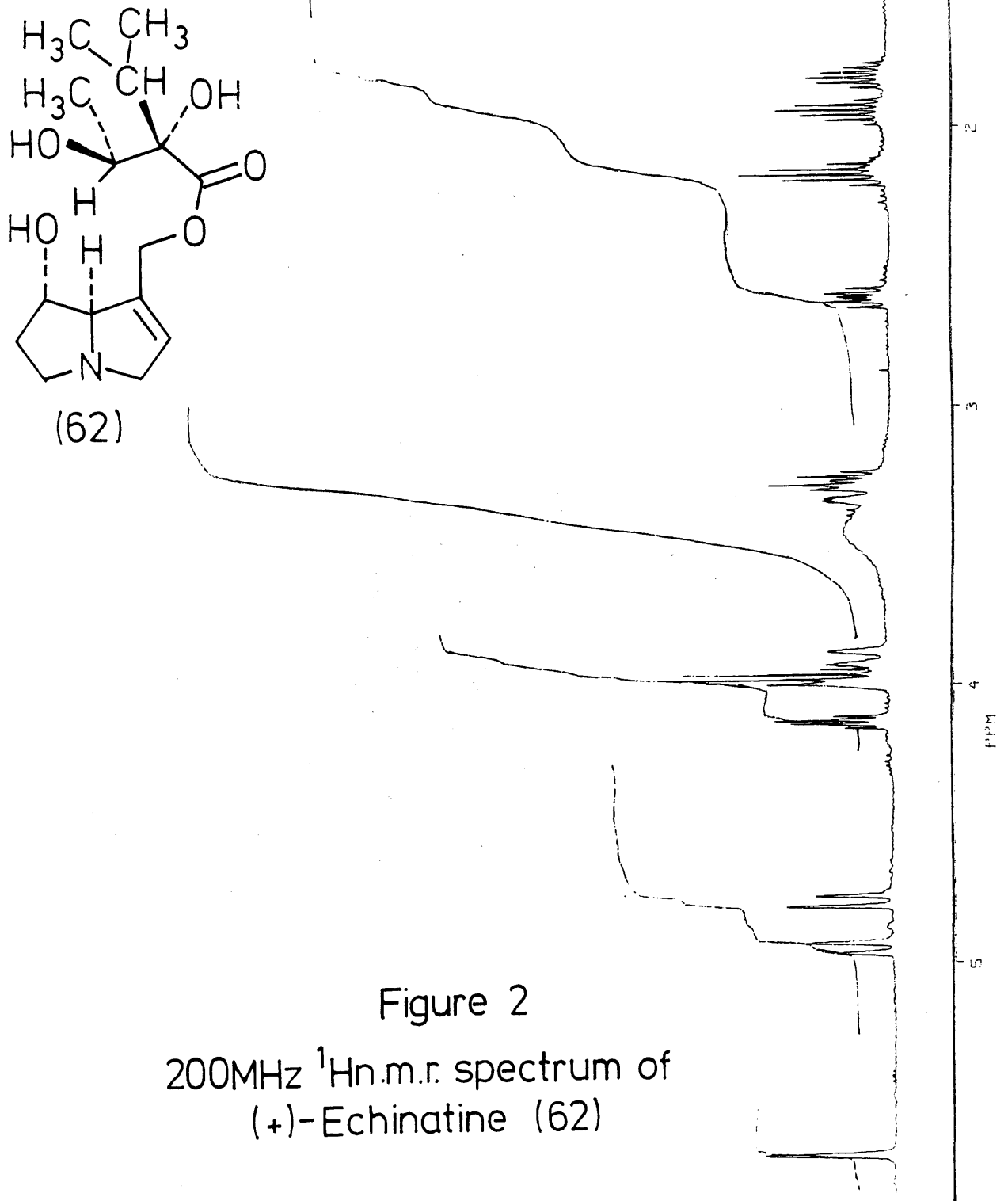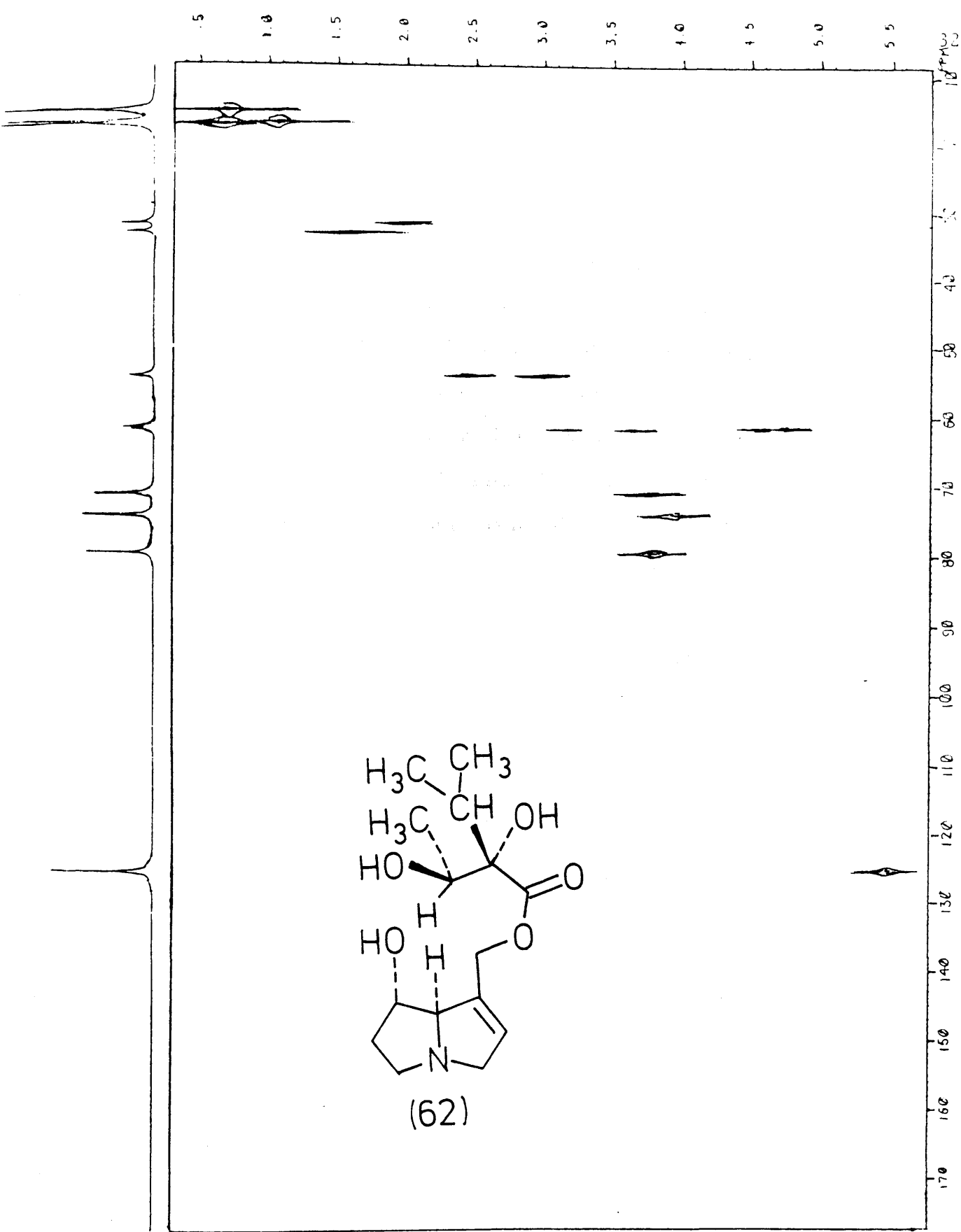
Figure 3

$^1H/^{13}C$ COSY n.m.r. spectrum of
(+)-Echinatine (62)

(68) was obtained by methanolic extraction of <u>Senecio isatideus</u> (family Compositae),[87] and riddelline (69) was produced by crystalliz- ation of the mother liquors of an extract of <u>Senecio riddellii</u> provided by Dr. R.J. Molyneux, U.S.D.A., Berkeley, California, U.S.A. Alkaline hydrolysis of these two diesters yielded respective acidic portions. The primary allylic hydroxyl of the free base was protected by coupling with benzoic acid using CDI in dry tetrahydrofuran (THF).[88] The reaction was selective and quantitative, affording a clean product.[50] No 7,9-dibenzoylretronecine was ever seen as evidenced by t.l.c. and n.m.r. spectroscopy. The product was the C-9 derivative (70) rather than the C-7 isomer as there was a discernible downfield acylation shift in the position of the C-9 protons in the [1]H n.m.r. spectrum. The reaction intermediate is expected to be an acylimidazole with esters resulting by the treatment with alcohol.

The 9-benzoylretronecine (70) was then converted into 7- methanesulphonyl-9-benzoylretronecine (71) by treatment with freshly distilled triethylamine and methanesulphonyl chloride under anhydrous conditions at a low temperature.[89] This key intermediate (71) was isolated, but not distilled, and used immediately in the next conversion. It has long been known that caesium carboxylates in dry $\underline{N}$,$\underline{N}$-dimethyl- formamide (DMF) are good nucleophiles and give clean SN^2 substitution.[90] Caesium benzoate and caesium propionate were prepared by the method of Kellogg[90] and each was used in turn to displace the good leaving group present in our key intermediate. In each case, no detectable amount of elimination occurred. 9-Benzoyl-7-propionyl heliotridine (72) and 7,9-dibenzoyl heliotridine (73) were obtained pure by the introduct-
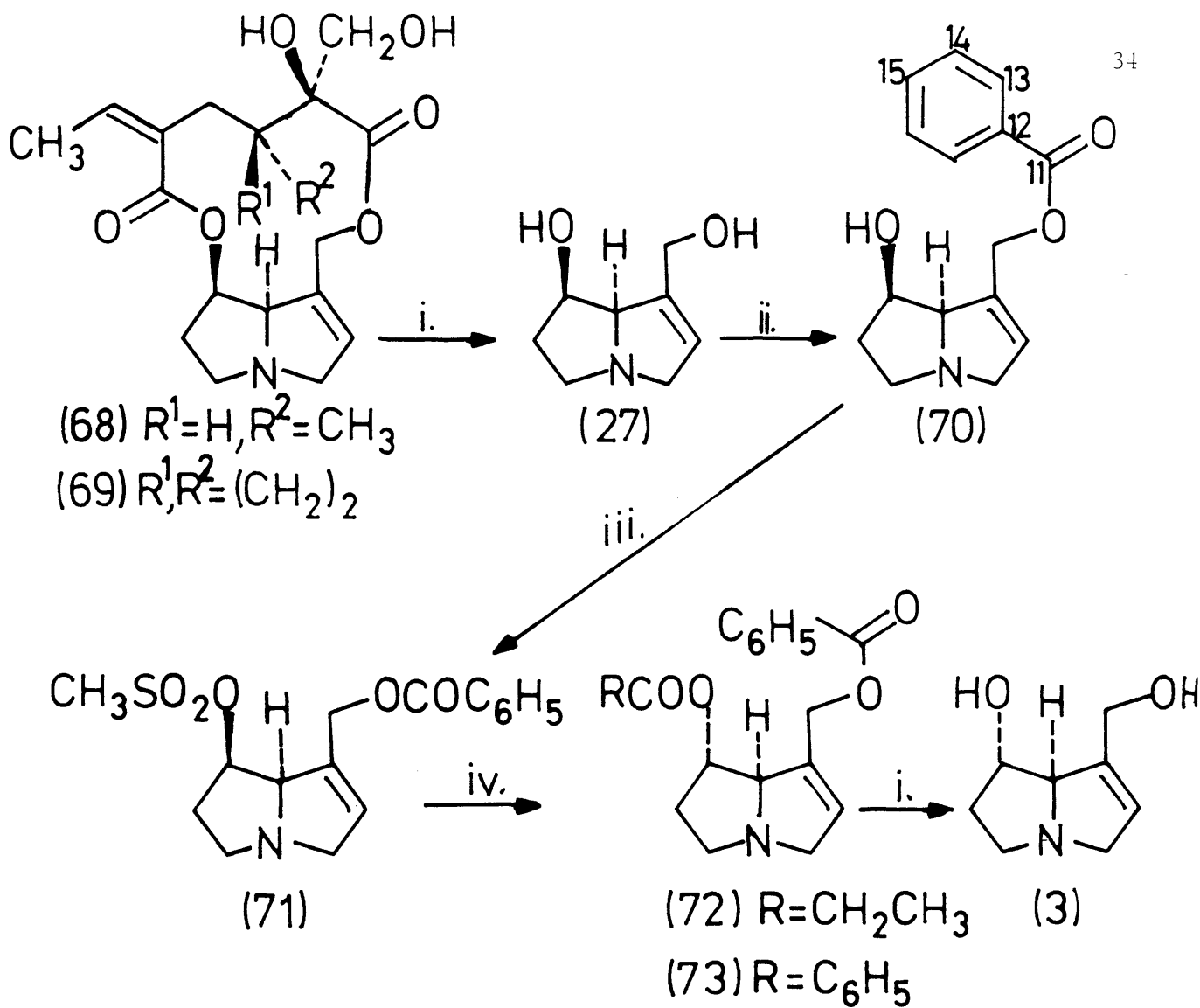
Scheme 17

Reagents: i. Ba(OH)$_2$, H$_2$O, Δ; ii. C$_6$H$_5$CO$_2$H, CDI, THF, r.t. ;
iii. NEt$_3$, CH$_3$SO$_2$Cl, -10°C, CH$_2$Cl$_2$; iv. RCO$_2$Cs,
DMF, Δ.

ion of an acid-base recycle into the work-up procedures. After full
characterization, these two open diesters were subjected in turn to
cleavage under basic conditions. The yields of (+)-heliotridine (3)
were useful and comparable, and the samples of the free base were
identical in all respects with that of the natural compound.

From the points of view of simplicity, speed and ease of
preparation, the best way to obtain (+)-heliotridine is by the hydrolysis
of a natural alkaloid, e.g. (+)-echinatine.

3.4 Synthesis of 11-membered Bislactones

A supply of (+)-heliotridine was obtained by the procedures
outlined in the previous two sections. The next aim was the synthesis
of the first macrocyclic diesters containing heliotridine. In order to
form the macrocyclic ring we chose to use glutaric anhydride and some
substituted derivatives.

After a series of trial reactions, the Corey-Nicolaou double
activation method of lactonisation was utilised to provide a series of
7,9-macrocyclic diesters of (+)-heliotridine. Biological study of a
series of 11-membered macrocyclic diesters of synthanecine A [e.g. (74)]
has confirmed that the diacid portion is important and that β,β-
disubstitution confers high resistance to esterase attack.[77] With this
knowledge, we used 3,3-dimethylglutaric anhydride to provide analogue
(75). The problem of diastereoisomerism is avoided by the use of this
symmetrically substituted glutaric anhydride. The route involves the
treatment of the base with the anhydride to form predominantly the C-9
monoester (76). This regioselective esterification was indicated by a

(74)

(3) + $\xrightarrow{i.}$ (76)

(76) $\xrightarrow{ii.}$ (77) $\xrightarrow{iii.}$ (75)
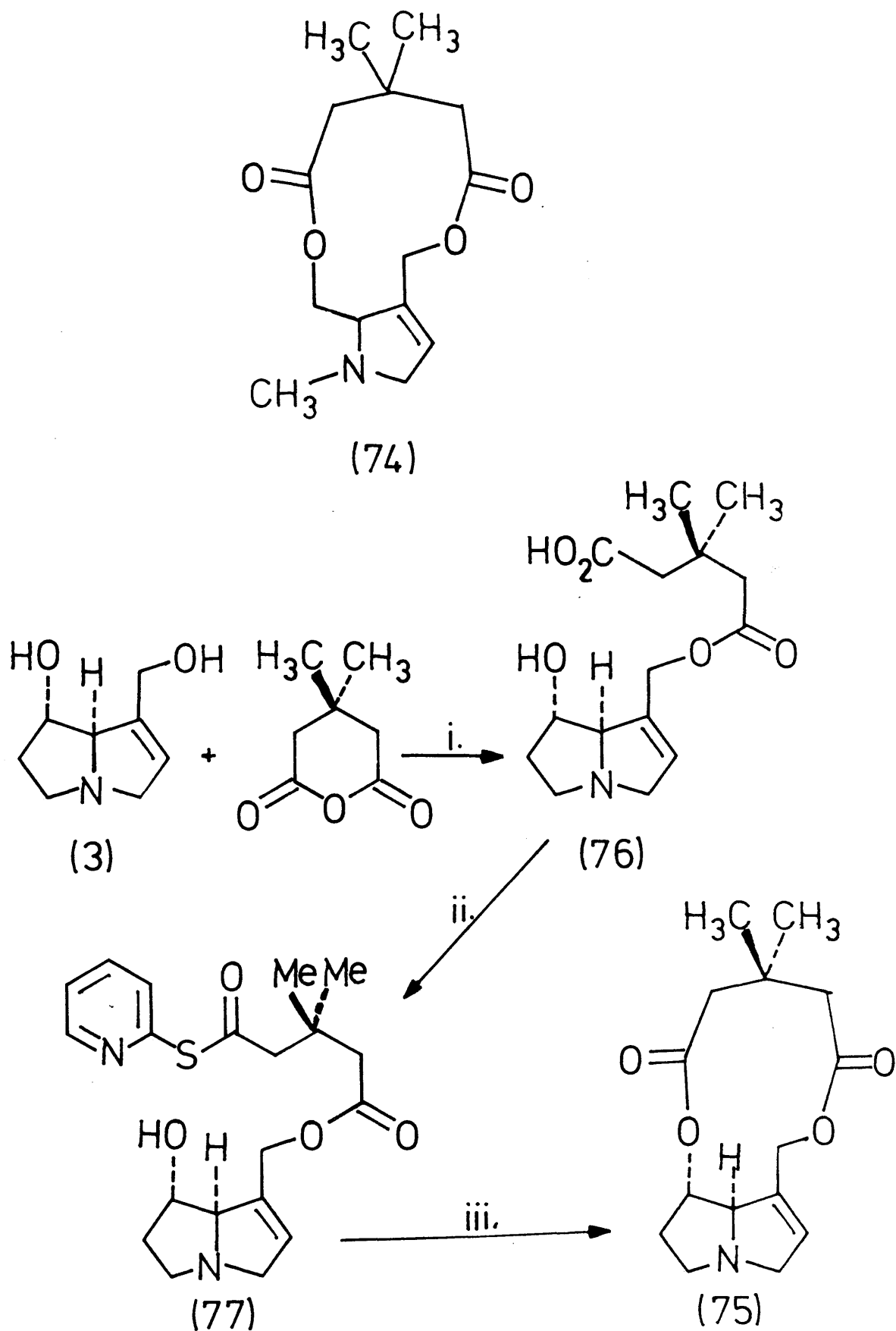
Scheme 18

Reagents: i. DME, Δ ; ii. PPh₃, $\left[\underset{N}{\bigcirc}\!S\right]_2$; iii. DME, Δ.

downfield shift for the C-9 protons in the ^{1}H n.m.r. spectra of the

monoesters as compared with the signal in the spectrum of heliotridine

(Figure 4) (the acylation shift).[91]   The 90 MHz ^{1}H n.m.r. spectrum of

a precipitate of the monoesters in deuteriomethanol showed signals for

the C-9 monoester (76) at δ_H 4.26 (7-H), 4.40 and 4.71 (2H, ABq,

9-H$_2$), and 5.53 p.p.m. (2-H) and for the C-7 monoester at δ_H 5.00

(7-H), 4.31 (2H, s, 9-H$_2$), and 5.50 p.p.m. (2-H). From the

appearance and integrations of these signals,[50] the ratio of 9- to

7-monoesters is 4:1.   Conversion of (76) into the C-9 thiolester (77)

was effected by the addition of 2,2'-dithiodipyridine and triphenyl-

phosphine to a suspension of the monoesters in dry 1,2-dimethoxy-

ethane (DME).   Rapid stirring was maintained until a homogeneous

solution was obtained and conversion was complete as seen by t.l.c.

Intramolecular lactonisation was carried out by heating the diluted

mixture at reflux in a further quantity of dry DME (Scheme 18).

Prolonged heating resulted in the formation of less polar pyrroles, as

evidenced by reaction with the Ehrlich's reagent (Section 3.6). Final

purification of the bislactone (75) was by an acid-base treatment to

remove by-products and by chromatography on a basic alumina column.

The acid-base recycle may be a reason for the moderate yield of

cyclised product and another procedure could result in an improvement

of this yield.

The macrocyclic nature of this new pyrrolizidine alkaloid

analogue was established by ^{1}H and ^{13}C n.m.r. spectroscopic studies.

One of the most important features in the ^{1}H n.m.r. spectrum of (75)

(Figure 5) is the appearance of the AB system due to the significant

Figure 4

200MHz ^{1}H n.m.r. spectrum of
(+)-Heliotridine (3)
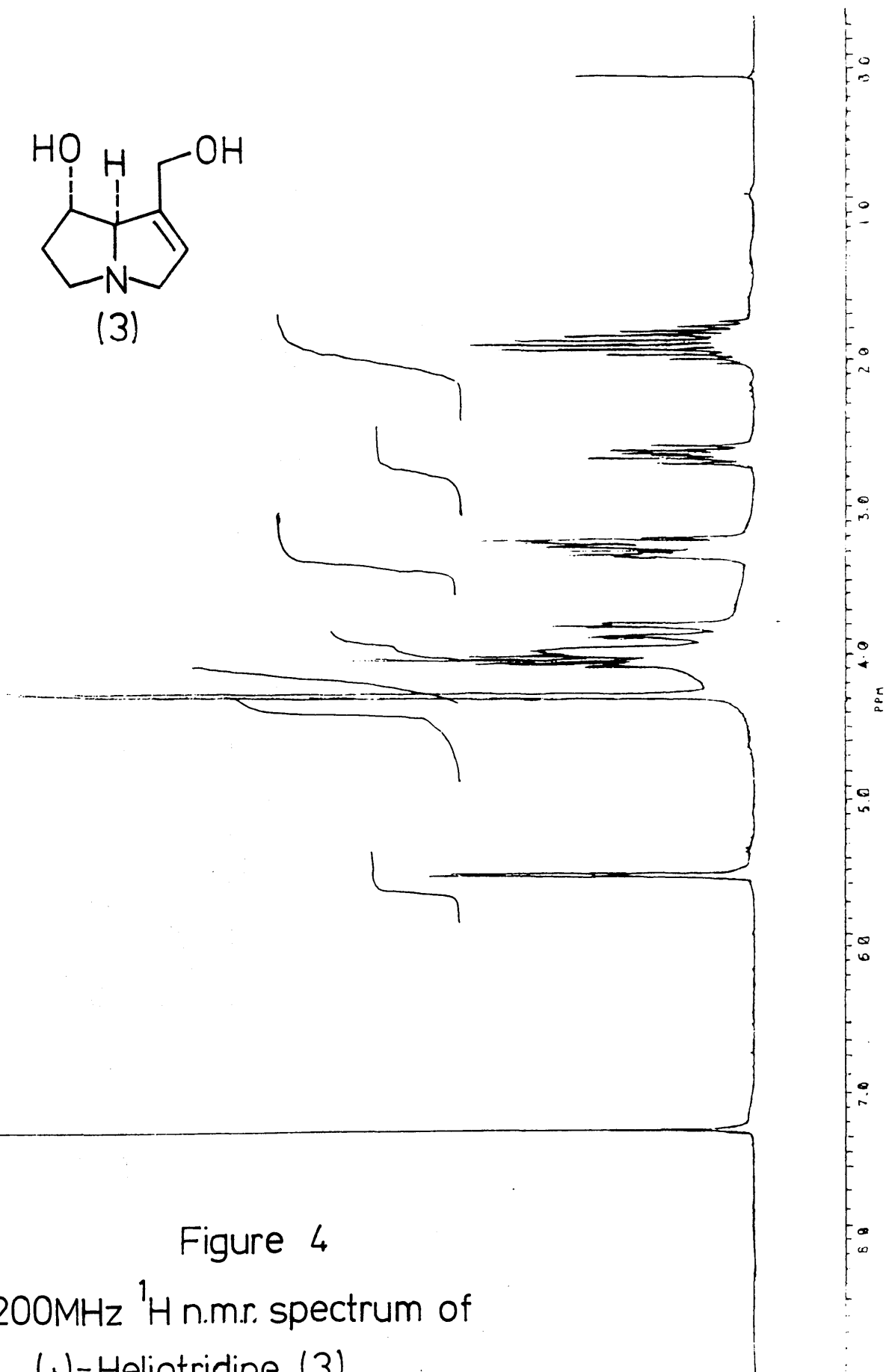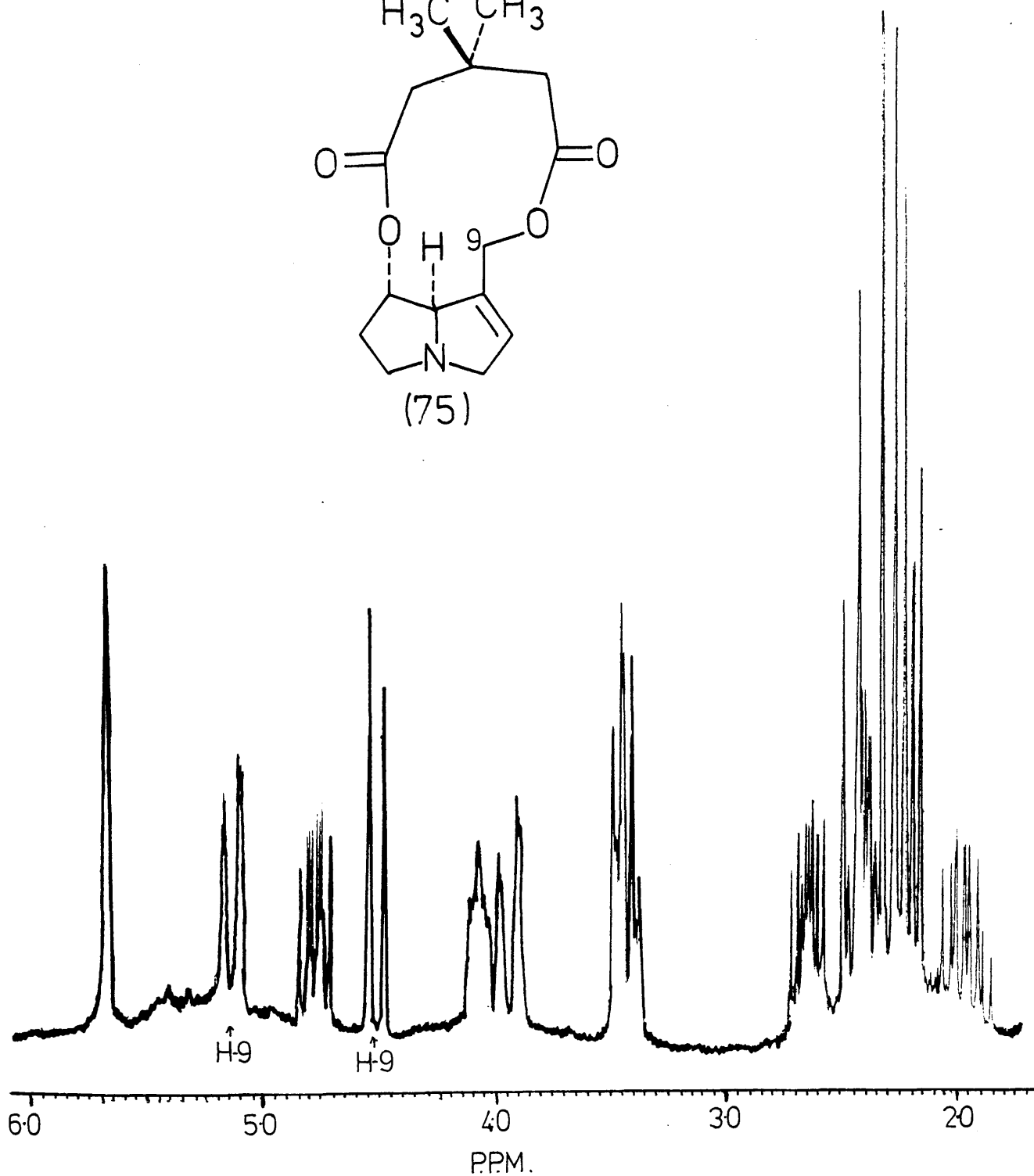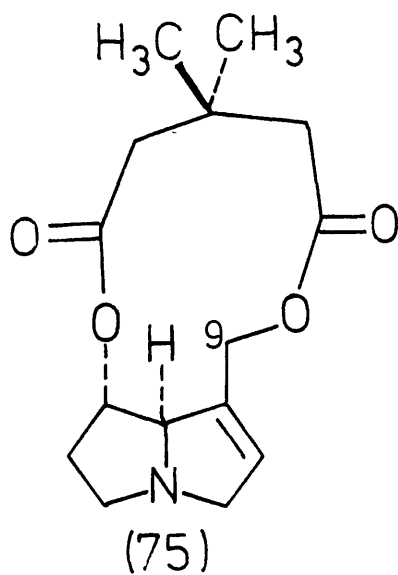
FIGURE 5

200MHz ^{1}H n.m.r. spectrum of
(+)-7,9-O̲,O̲-(3,3-Dimethyl glutaryl) heliotridine (75)

non-equivalence of the two diastereotopic protons at C-9. This
acylation shift can be seen when comparison is made between Figures
4 and 5. The chemical shift difference of 0.61 p.p.m. for the C-9
protons ($\Delta \delta$H-9) of (75) is within the typical range observed for 11-
membered macrocyclic diesters of (+)-retronecine.[1,2] The $\Delta \delta$H-9 value
can be used as a rough guide to the ring size of a retronecine macro-
cyclic diester. For 11-membered rings the difference in chemical
shifts is typically 0-0.75 p.p.m., whereas for 12-membered macrocycles,
the range is usually 1.10-1.55 p.p.m. (Table 2). The occurrence of
the significant $\Delta \delta$H-9 value is convincing evidence for the presence of
the macrocyclic diester system. Further AB systems are evident for
the two protons α with respect to the carbonyl carbon (Figure 5).

The mass spectral fragmentation pattern of (+)-7,9-$\underline{O}$,$\underline{O}$'-(3,3-di-
methylglutaryl)heliotridine (75) (Scheme 19) is characteristic of the
1-hydroxymethyl pyrrolizidine nucleus.[92] The formula $C_{15}H_{21}NO_4$
requires $\underline{M}$, 279.1470. An accurate mass measurement of the parent
ion revealed $\underline{M}^{+}$, 279.1469.

The absorption of compound (75) in the infra-red spectrum was
recorded with a peak at 1733 cm^{-1} for the absorption of the carbonyl
groups. This value is highly typical of diesters of heliotridine. The
solid (75) gave the correct microanalytical data.

The use of 3,3-tetramethylene and 3,3-pentamethylene glutaric
anhydrides led to the formation of macrocyclic bislactones (78) and (79)
respectively. These diesters have significant non-equivalence of the
C-9 protons with $\Delta \delta$H-9 values of 0.49 p.p.m. for (78) and 0.79 p.p.m.
for (79). The simplest macrocycle, (+)-7,9-$\underline{O}$,$\underline{O}$'-(glutaryl)heliotridine

MACROCYCLE	RING SIZE	$\Delta\delta H\text{-}9$ (p.p.m.)
MONOCROTALINE (45)	11	0·16
CRISPATINE (43)	11	0·32
FULVINE (44)	11	0·73
OTOSENINE (209)	12	1·14
INTEGERRIMINE (56)	12	1·25
SENECIONINE (205)	12	1·47
(75)	11	0·61
(78)	11	0·49
(79)	11	0·79
(80)	11	0·12
(81)/(82)	11	0·29 / 0·17

Table 2

m/e 279
(75)

m/e 137

m/e 119

m/e 220

m/e 220

m/e 120

m/e 93
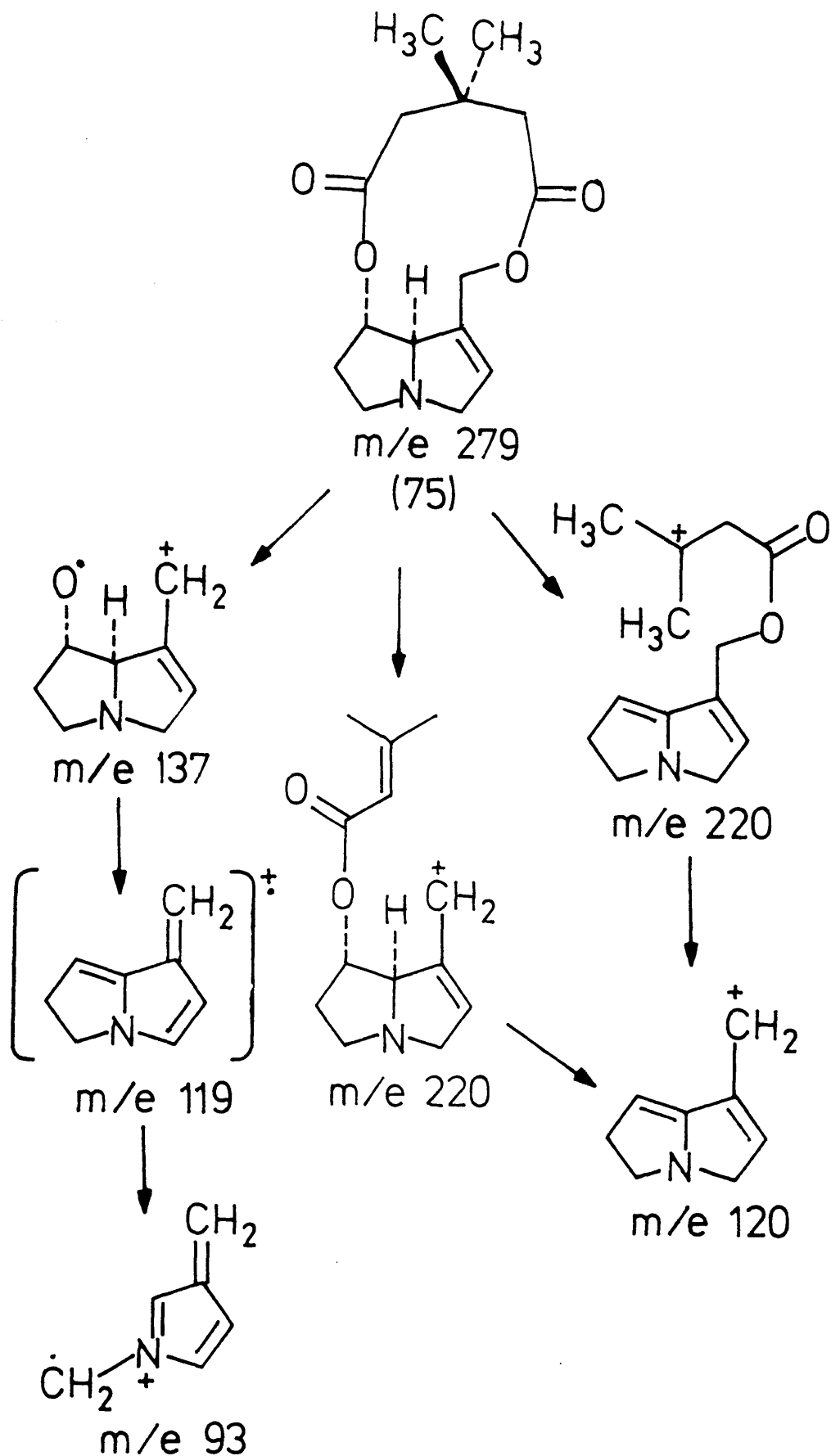
Scheme 19

Possible mass spectral fragmentation
pattern of (75)

43
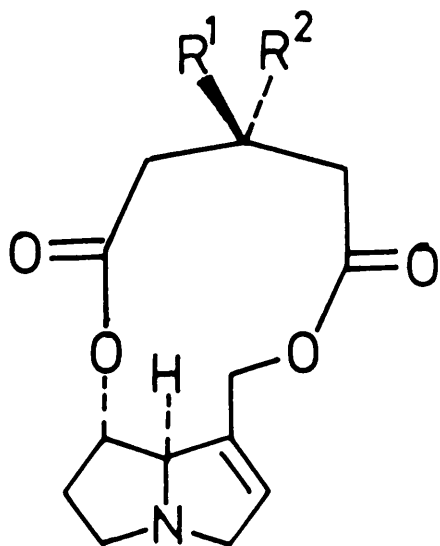
(78) $R^1, R^2 = (CH_2)_4$
(79) $R^1, R^2 = (CH_2)_5$
(80) $R^1 = R^2 = H$
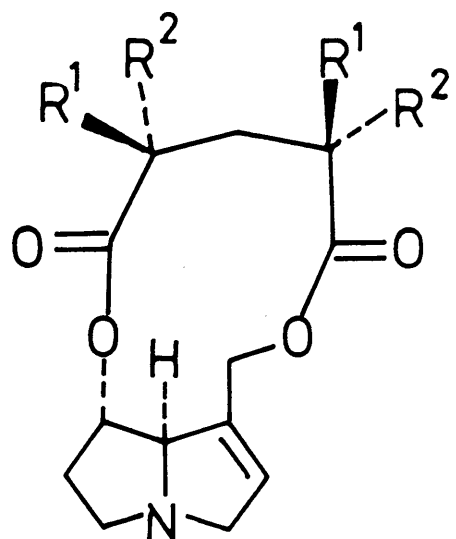
(81) $R^1 = CH_3, R^2 = H$
(82) $R^1 = H, R^2 = CH_3$
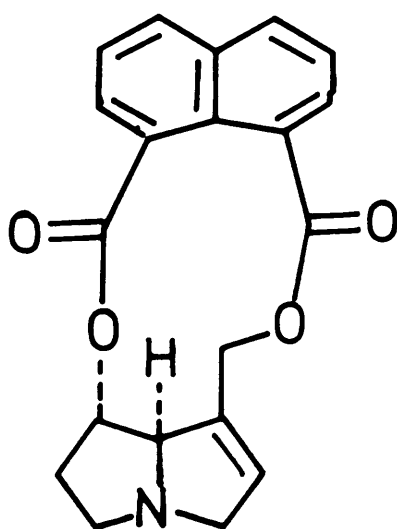
(83)
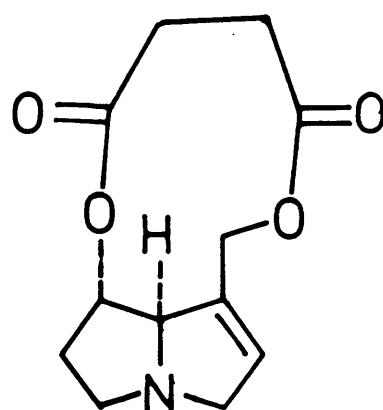
(84)

(80), was obtained as an oil and characterized as the picrolonate. This analogue has a relatively low $\Delta \delta$H-9 value of 0.12 p.p.m.

Compounds (81) and (82) were furnished by the use of meso-2,4-dimethylglutaric anhydride as an inseparable mixture of diastereo-isomers. Column chromatography, p.l.c. and h.p.l.c. were ineffectual in separating this mixture. Consideration of the ^{1}H n.m.r. spectrum of the mixture indicated that the isomers were present in a ratio of 2:1. The chemical shift differences for the C-9 protons are 0.17 p.p.m. for the major isomer and 0.29 p.p.m. for the minor component. Pyrrolizi-dine alkaloids with α-substituents are believed to enhance the toxicity by creating steric hinderance around the ester groups and thereby increasing the resistance to esterase hydrolysis.

When naphthalene-1,8-dicarboxylic anhydride was used, t.l.c. evidence indicated the formation of analogue (83), but this 11-membered macrocyclic diester could not be isolated. Treatment of (+)-heliotridine with 2,2-dimethylglutaric anhydride, resulted in no isolation of the desired cyclised products. Similarly, the reaction of the free base with homophthalic anhydride by the Corey-Nicolaou procedure failed to produce any cyclic diester.

3.5   Attempted Synthesis of 10-membered Bislactones

Endeavours to construct 10-membered macrocyclic diesters of (+)-heliotridine proved disappointing, although formation of the pyridine-2-thiolesters proceeded normally. Chromatographic evidence for the formation of 7,9-O,O'-(succinyl)heliotridine (84) was obtained. When phthalic anhydride was used in this procedure, no evidence was

obtained for the formation of a bislactone. Models of these 10-membered macrocycles indicated that there may be unfavourable steric interactions in the systems.

3.6 Chromatographic Analysis of Corey-Nicolaou Method

All of the macrocyclic diesters [(75), (78)-(82)] were prepared in a "one-pot" reaction as none of the intermediates were actually isolated and fully characterized. In order to optimise the yields at every stage of the route, careful monitoring by t.l.c. was required. This was helped by the fact that the intermediates have R_f values which fall within distinct ranges, and several of the compounds are u.v. active (Table 3).

The solvent system to use for the best separation of the free base and the resultant monoester on an analytical t.l.c. plate is 10% conc. ammonia in methanol. In this system the highly polar zwitter-ionic monoesters [e.g. (76)] have an average R_f value of 0.61, and (+)-heliotridine (3) has an R_f value of 0.37. In the normal t.l.c. solvent system[93] (1:14:85 = 0.89M NH_3:MeOH:$CHCl_3$), the monoester does not move a great deal from the baseline and (+)-heliotridine is essentially static (Table 3). Obviously the formation of the monoester was followed by the appearance of a compound of higher R_f and the disappearance of the heliotridine. The staining system that was used is specific for Δ^3-pyrrolines.[94] The pyrroline is oxidised to the corresponding pyrrole by o-chloranil, and the pyrrole is visualised by reaction with a modified Ehrlich's reagent[95] (Scheme 20).

COMPOUND	R_f values[1]	u.v. activity
Heliotridine (3)	0-0.05	X
Monoesters, e.g. (76)	0.06-0.09	X
Thiolesters, e.g. (77)	0.36-0.42	✓
Bislactones (75),(78)-(82)	0.51-0.69	X
Pyridine-2-thione (85)	0.62	✓
Triphenylphosphine (86)	0.75	✓

Table 3

1 = normal solvent system, 1:14:85 = 0.89M NH_3 : CH_3OH : $CHCl_3$

Scheme 20

(85)

$(C_6H_5)_3\overset{+}{P}-O^-$
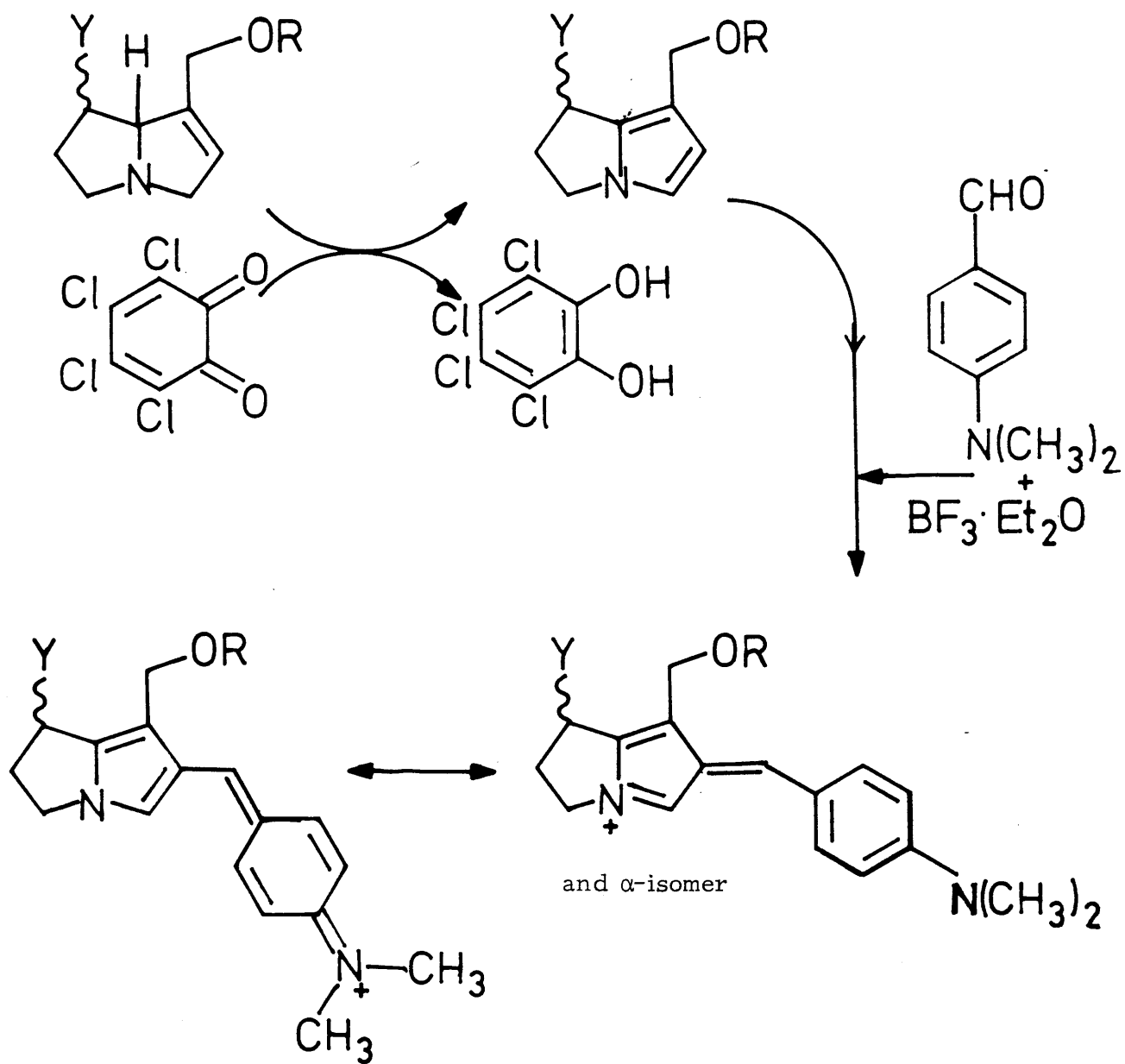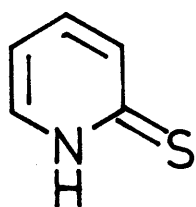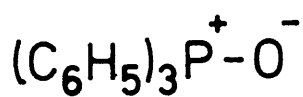
(86)

Conversion of the monoesters into the pyridine-2-thiolesters [e.g. (77)] was indicated by the appearance of a u.v. active compound and the concurrent loss of the monoester. This u.v. active compound stains purple with the o-chloranil/Ehrlich's reagent, as do all other didehydropyrrolizidines. Ring closure was brought about by adding the yellow DME solution of thiolesters to a larger volume of DME and heating at reflux under these high dilution conditions. The cyclic diesters [(75), (78)-(82)] are less polar than the thiolesters and stain purple. They have no appreciable u.v. activity. The two major by-products, pyridine-2-thione (85) and triphenylphosphine oxide (86), run close to the dilactones. Both by-products are visible under u.v. light and pyridine-2-thione stains a very vivid yellow colour with the visualising system. Thus it can be seen that the use of t.l.c. and the appropriate visualisation techniques can let us look at all of the reaction intermediates and end products.

3.7 X-Ray Analysis of Pyrrolizidine Alkaloids

The differing $\Delta \delta$H-9 values of these new pyrrolizidine alkaloid analogues [(75), (78)-(82)] are believed to reflect the different conformations of the diacid portions in the macrocyclic systems in organic solutions. The deshielding may be greater than 1 p.p.m. if the ester carbonyl group and the proton are almost coplanar. The double bond of the pyrrolizidine nucleus can also deshield the C-9 protons.

X-Ray data on 11-membered macrocyclic diesters of (+)-retronecine have shown that most have ester carbonyl groups that are

synperiplanar and directed downward from the large ring, while for
12-membered bislactones of (+)-retronecine the ester carbonyls are
antiperiplanar and directed outward from the macro-ring. The latter
case is also true for the toxic metabolite, dehydrosenecionine (87).
It was important to establish the conformations of these new alkaloid
analogues. For example, they may favour the oxidation of the 3-
pyrroline rings to the toxic pyrrole metabolites rather than the
detoxification processes of N-oxidation or lactone hydrolysis. It
should be noted however that the conformations adopted by the
alkaloids in the solid state and in organic solutions are not necessarily
the same as those present when the alkaloids are metabolised in vivo,
i.e. in dilute aqueous media.

The conformation of heliotridine (3) is thought to be composed
of a mixture of two rapidly interconverting forms in solution[96] and is
endo-puckered in the X-ray crystal structure.[97] Heliotrine (33)[98]
and lasiocarpine (5)[99] also exist with ring A in the endo-buckled form.
Colourless, orthorhombic, plate-shaped crystals of (+)-7,9-O,O'-(3,3-
dimethylglutaryl)heliotridine (75) were grown from cyclohexane and the
X-ray structure was elucidated. Ring A of the pyrrolizidine nucleus
is exo-buckled. The addition of the macrocycle has therefore induced
a conformational change to the exo-form which dominates other pyrroli-
zidine alkaloids and bases, e.g. (+)-retronecine (27).[97] The conform-
ation of the ester carbonyl groups of the 11-membered ring is anti-
parallel, and the ester carbonyl group at C-11 is oriented in the same
direction as H-8. The alternative antiparallel arrangement has been
found for trichodesmine (6), an 11-membered macrocyclic retronecine
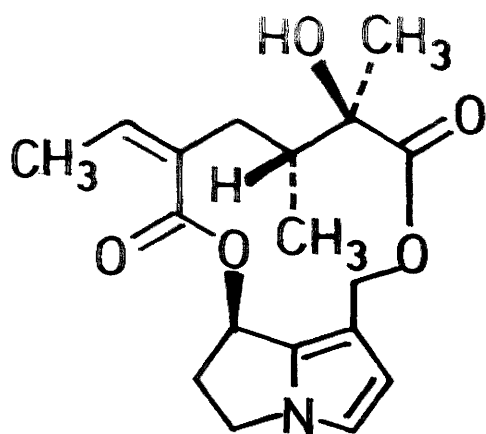
(87)
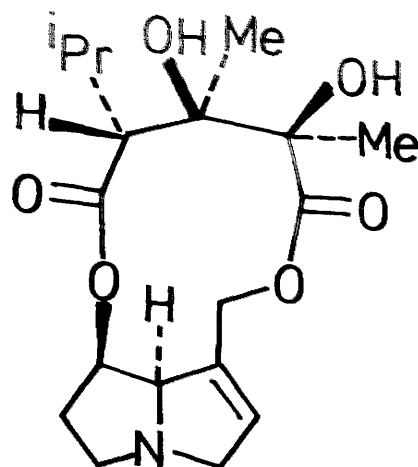
(6)
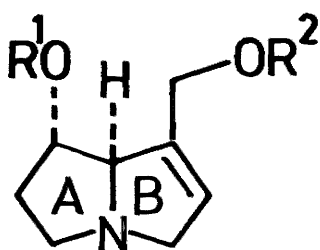
(3) R^1=H, R^2=H

(5) R^1= (Z)-COCMe=CHMe,
R^2=COC(OH)(CMe$_2$OH)CH(OMe)CH$_3$

(33) R^1=H, R^2=COC(OH)(CHMe$_2$)CH(OMe)CH$_3$
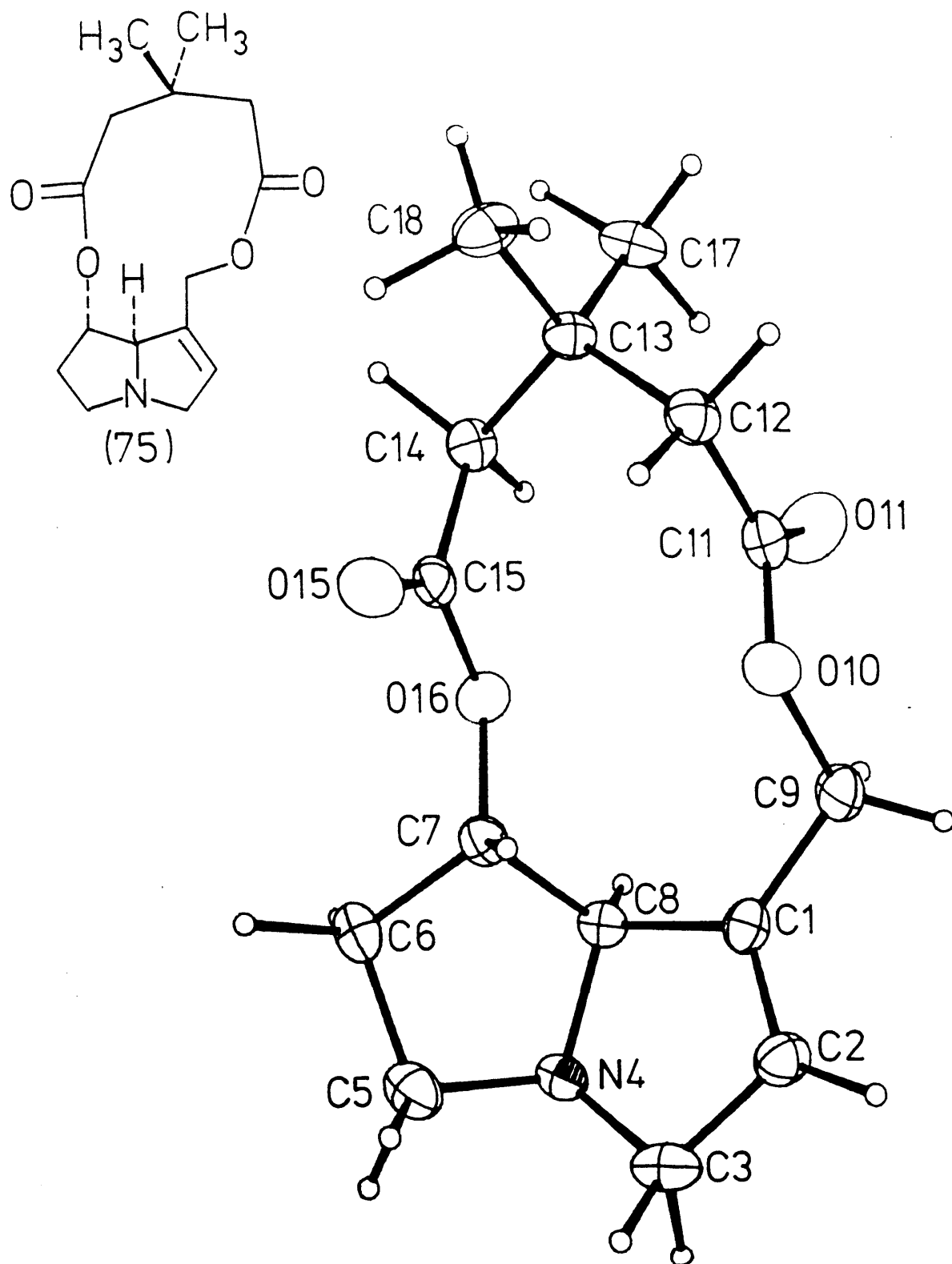
Figure 6

X-Ray Crystal Structure of
(+)-7,9-OO-(3,3-Dimethylglutaryl)heliotridine (75)

diester.[100] The macrocyclic diester (75) is shown in its conformation with the conventional numbering system[101] (Figure 6).

3.8   Summary and Conclusions

A series of 11-membered macrocyclic bislactones [(75), (78)-(82)] of (+)-heliotridine (3) have been prepared via the Corey-Nicolaou double activation method of lactonisation. Full structural elucidation was carried out. The base was isolated from natural sources and synthesised from (+)-retronecine (27). An X-ray crystal structure of one of these pyrrolizidine alkaloid analogues (75) revealed the conformation in the solid state.

3.9   Further Possible Studies

The diesters were synthesised in moderate yields only, probably in part due to the elaborate purification procedures required. The removal or replacement of the acid-base recycle should increase the final yields as the bislactones are undoubtedly unstable in these aqueous media. If the large amounts of coupling reagents, and hence by-products, could be reduced or even eliminated by trying different lactonisation procedures, the reaction efficiency might also improve. The hydrochloride salt of (7S, 8R)-1-chloromethyl-1,2-didehydro-7-hydroxy-pyrrolizidine (88) is a known compound[86b] and may be extremely useful in this respect. It is known that the corresponding allylic chloride (41) derived from retronecine can be successfully employed in the construction of macrocyclic bislactones (see Section 2.4).
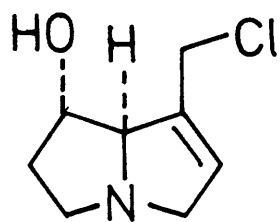
(88)
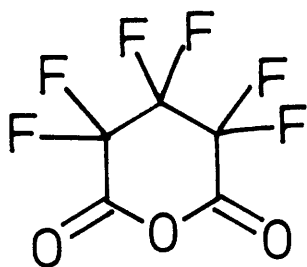
(89)
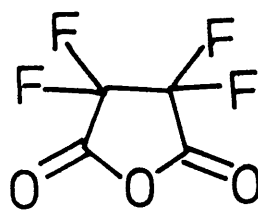
(90)

The testing of these new analogues containing (+)-heliotridine would be of interest to evaluate their biological properties, e.g. hepato-toxicity and carcinogenicity. Upon the completion of these studies, further modifications to the structure of the acidic and basic moieties may be desirable.

The production of other pyrrolizidine alkaloid analogues should also be an aim. The synthesis of macrocycles with varied ring sizes is a primary target. The use of novel cyclic anhydrides could eventually produce macrocyclic bislactones which might have interesting and possibly unusual biological and medicinal properties. For example, the substitution of a hydrogen atom by a fluorine atom or a trifluoromethyl group might affect the activity. Many fluorine-containing anhydrides and acids are readily available, e.g. perfluoroglutaric (89) and perfluoro-succinic (90) anhydrides.

CHAPTER FOUR

BIOSYNTHESIS OF THE PYRROLIZIDINE ALKALOIDS

4.1   Introduction

Investigations into the biosynthesis of natural products have led to the recognition of precursor-product relationships in many classes of compounds.   These studies were initially carried out using radio-isotopes (^{3}H, ^{14}C).   Originally, organic chemistry was the only tool available to establish labelling patterns in natural products by performing degradative reactions and making derivatives.   However, over the past few decades a number of non-degradative spectral methods have been introduced which have had a great impact upon the research into the biogenesis of naturally occurring compounds.   Application of such new methodology as ^{2}H and ^{13}C n.m.r. spectroscopy has resulted in a number of advances, several of which have occurred in the field of research into pyrrolizidine alkaloids.

4.2   Necine Bases

Almost all of the biosynthetic studies reported to date have been carried out upon the most frequently isolated necine, retronecine (27).   The initial prediction of Sir Robert Robinson that the pyrrolizidine nucleus originates by the condensation of two molecules has been substantiated.[102]   It is now widely accepted that ornithine (91) is used in the biosynthesis of the bicyclic necine structure.[103]   At some stage in the biosynthetic pathway, C-2 and 5 of ornithine become equivalent

suggesting that a symmetrical intermediate is involved, e.g. 1,4-diamino-butane (putrescine) (92).[104]   Arginine (93) is also known to be a precursor of retronecine.[105]   Generally, the radioactive alkaloids obtained after feeding were hydrolysed to retronecine and the retronecine was degraded to yield C-9 as formaldehyde[106] and C-5 to 7 as β-alanine.[107]   These results are summarised in Scheme 21.

The use of precursors specifically enriched with ^{13}C atoms has recently blossomed. With the aid of ^{13}C n.m.r. spectroscopy, the complete labelling patterns in retronecine have been established.   The feeding of [1-^{13}C]- and [1,4-$^{13}C_2$]putrescine, (94) and (95) respectively, resulted in the observation of four enhanced signals in the ^{13}C n.m.r. spectra.[108]   The use of [1,2-$^{13}C_2$]putrescine (96) led to the flanking of all eight ^{13}C n.m.r. signals in retronecine by doublets with the resultant observation of four pairs of ^{13}C-^{13}C coupling constants.[109] When [2,3-$^{13}C_2$]putrescine (97) was incorporated into retronecine, two pairs of these doublets with different coupling constants were seen.[108] Direct evidence for the involvement of a later symmetrical intermediate was obtained when [1-amino-^{15}N,1-^{13}C]putrescine (98) was used and the ^{13}C n.m.r. spectrum of the resultant alkaloid was analysed. As well as the expected enrichments, two doublets corresponding to C-3/N-4 and C-5/N-4 were seen.[108,110]   The enrichment at each site was approximately equal.   These ^{13}C-^{15}N species were also observed in the ^{15}N n.m.r. spectra.   The molecules of retronecine shown as being produced from (94)-(98) are representations of all the possible species involved in each case (Scheme 22).

(91) $R^1 = COOH, R^2 = H$

(92) $R^1 = R^2 = H$

(93) $R^1 = COOH$ $R^2 = C(=NH)NH_2$
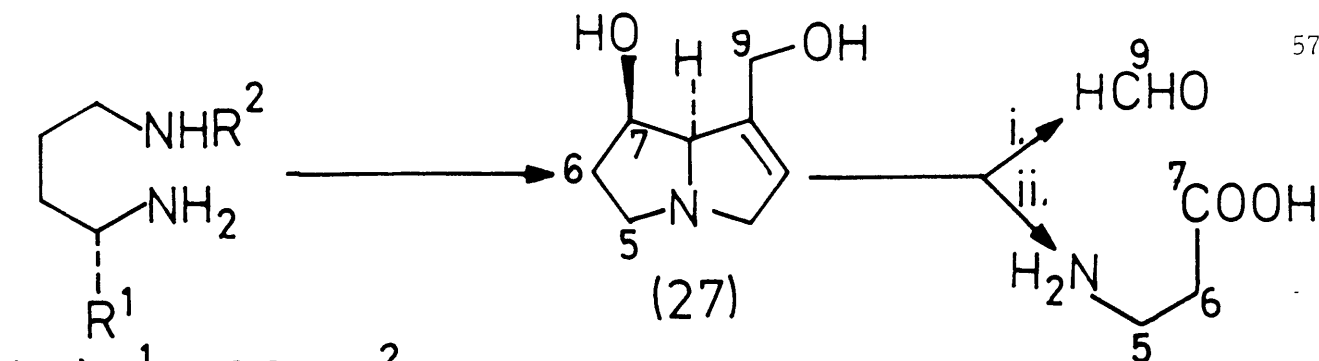
Scheme 21

Reagents: i. OsO_4, $NaIO_4$; ii. CrO_3, H_2SO_4.

(94)

(95)

(96)

(97)

(98)
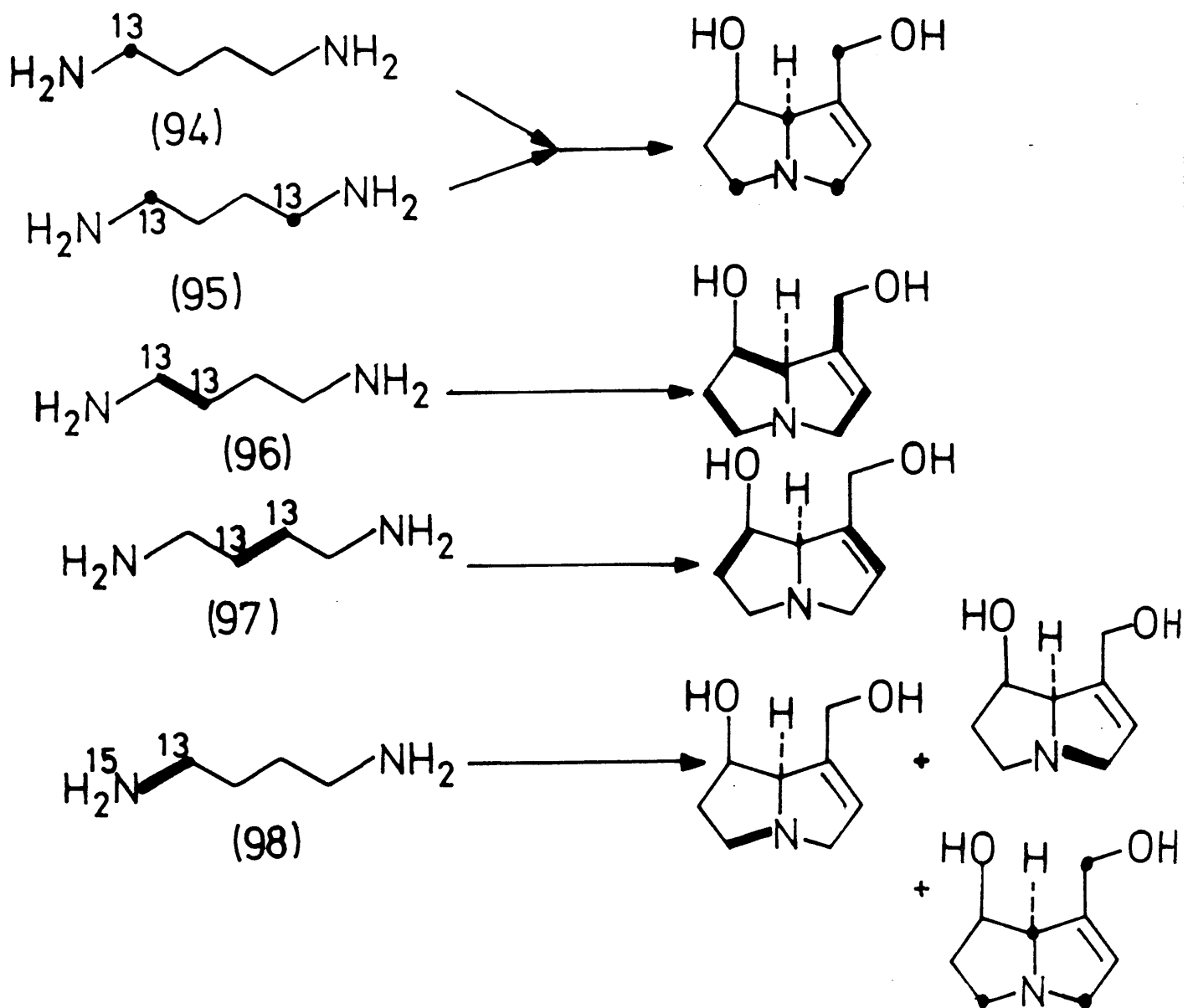
Scheme 22

● single label ▬ double label

The later symmetrical intermediate was shown to be $\underline{N}$-(4-aminobutyl)-1,4-diaminobutane (homospermidine)(99) by the efficient intact incorporation of $[1,9-^{14}C_2]$-[108] and $[1,9-^{13}C_2]$-homospermidine,[111] respectively (100) and (101). In the case of (101), two doublets were superimposed on the singlets corresponding to C-8 and 9 in the ^{13}C n.m.r. spectrum. Further support for homospermidine as an intermediate in retronecine biosynthesis has been obtained by its conversion into trachelanthamidine (23) using enzymes and physiological conditions.[112] The initial oxidation is believed to yield an aldehyde species which cyclizes intramolecularly to 1-formyl pyrrolizidine. The 1-hydroxymethyl pyrrolizidine (23) is afforded by reduction. Trachelanthamidine itself has been shown to be an intermediate along the biosynthetic pathway. The pulse-feeding of $^{14}CO_2$ to a plant which produces pyrrolizidine alkaloids resulted in the labelling of trachelanthamidine (23), supinidine (24) and retronecine (27),[113] with the saturated base exhibiting the highest specific radioactivity. The main sites of alkaloid biosynthesis appeared to be the leaves of this plant. Radioactive samples of trachelanthamidine and isoretronecanol (22) were independently synthesised by two groups and fed to different $\underline{Senecio}$ species.[114,115] In both cases, trachelanthamidine was incorporated into retronecine to a higher degree than putrescine, which itself was a much more efficient precursor than isoretronecanol. This result is consistent with putrescine being a more remote precursor than trachelanthamidine. By contrast, isoretronecanol is a very efficient precursor for rosmarinecine (25).[114] A pathway can be postulated to account for these findings (Scheme 23).

$$H_2N^XCH_2(CH_2)_3NH(CH_2)_3{}^XCH_2NH_2$$
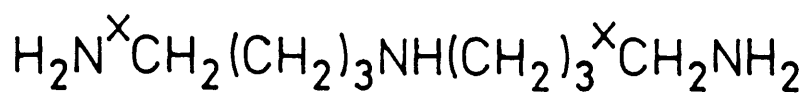
(99) x=12
(100) x=14
(101) x=13
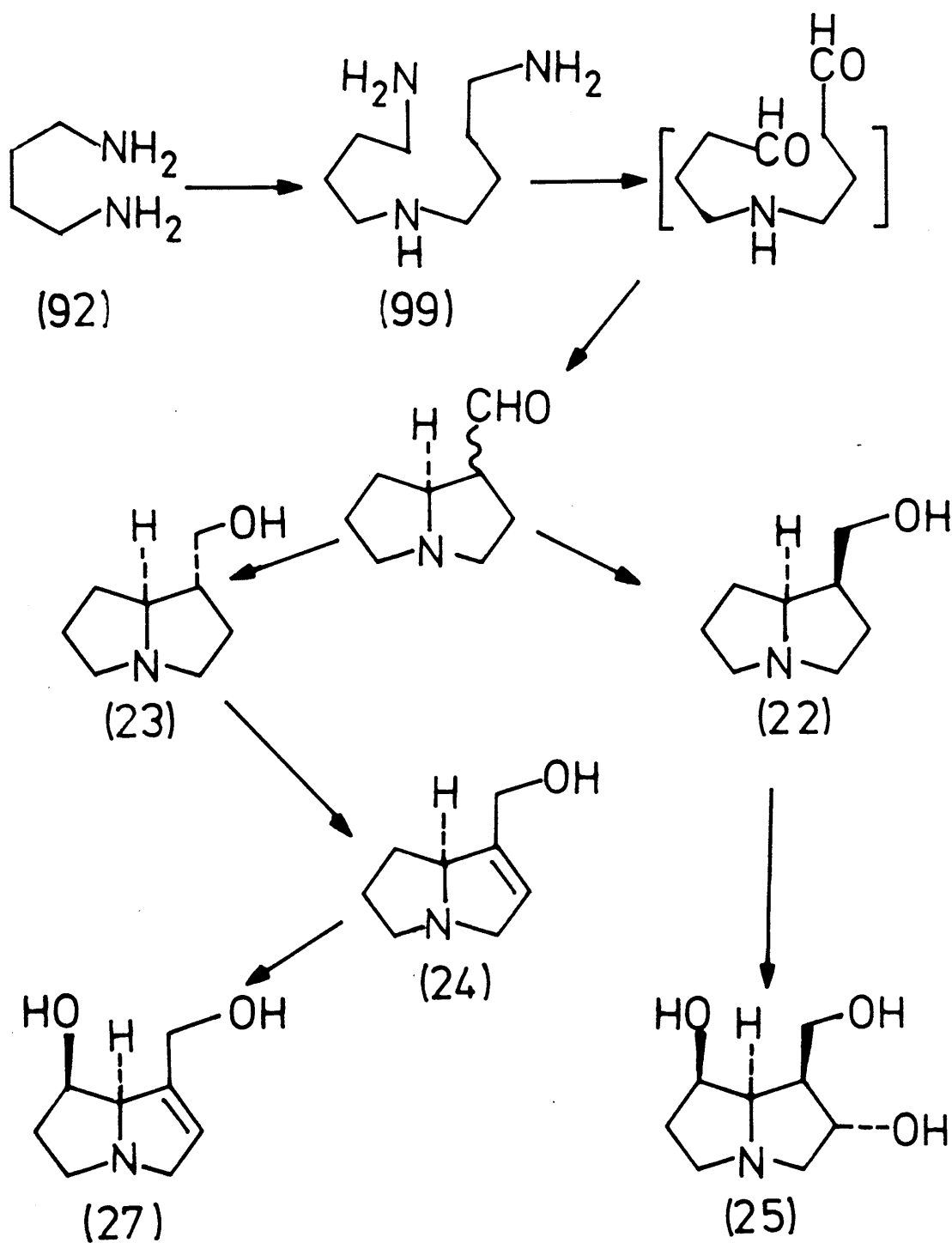
Scheme 23

In order to understand the stereochemistry inherent in the enzymic processes involved in the biosynthesis of pyrrolizidine alkaloids, specifically ^{2}H-labelled precursors were synthesised. The decarboxylation of basic α-amino acids and the oxidation of the resultant amines are the key steps in the biosynthesis of many alkaloids.[116] It is known that in the oxidative deaminations by amine oxidases (E.C.1.4.3.6) from pea seedlings, the stereospecific removal of the pro-S-hydrogen atom from the methylene group occurs. This may happen via an imine (Scheme 24).[117,118] There may be more than one type of reaction as some enzymes are known to use pyridoxal phosphate as cofactor and some do not. The detailed mechanism is not known for even one oxidase.

The labelling patterns in retrorsine derived biosynthetically from [1,1,4,4-^{2}H$_4$]- and [2,2,3,3-^{2}H$_4$]putrescine [(103) and (104) resp.] have been established by ^{2}H n.m.r. spectroscopy.[119] Incorporation of [1,1,4,4-^{2}H$_4$]putrescine led to the discovery that the hydride donor attacks the aldehyde precursor on the re-face of the carbonyl group as there was a ^{2}H n.m.r. signal at the C-9 pro-S position. The use of [2,2,3,3-^{2}H$_4$]putrescine showed a ^{2}H atom to be present at C-7α in the sample of retrorsine. This result indicates that the introduction of the 7β-hydroxyl group does not involve a keto or an enol intermediate.

When R-[1-^{2}H$_1$]- and S-[1-^{2}H$_1$]putrescine [(105) and (106) resp.] were used, the labelling patterns agreed with the previous results.[110,119] The use of R-[2-^{2}H$_1$]putrescine (107) led to the discovery that the pro-S hydrogen atom is lost at C-2 during the formation of the 1,2-double bond of retronecine.[120] This may be explained by hydroxylation of trachelanthamidine, followed by the elimination of

Scheme 24

$$H_2NCD_2CH_2CH_2CD_2NH_2 \qquad (103)$$

$$H_2NCH_2CD_2CD_2CH_2NH_2 \qquad (104)$$

(105) $R^1 = D, R^2 = H$
(106) $R^1 = H, R^2 = D$

(107) $R^1 = D, R^2 = H$
(108) $R^1 = H, R^2 = D$
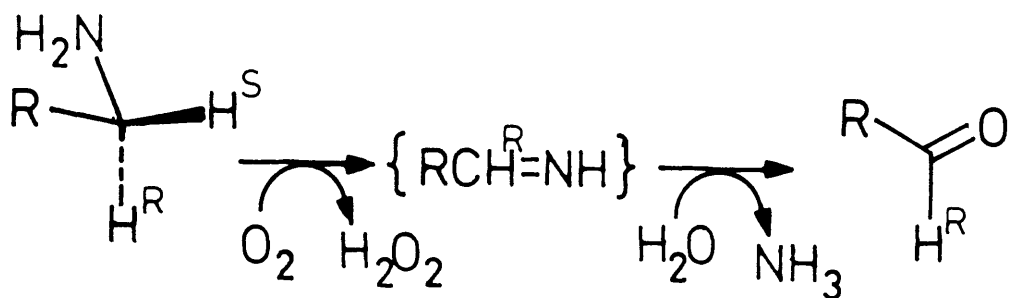
Scheme 25

Scheme 26
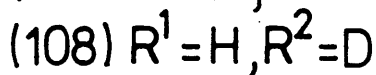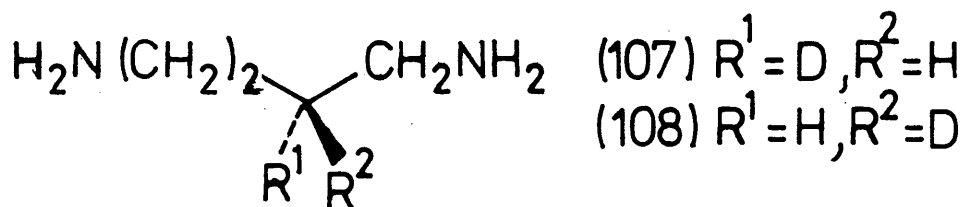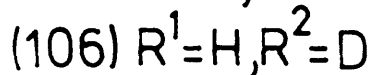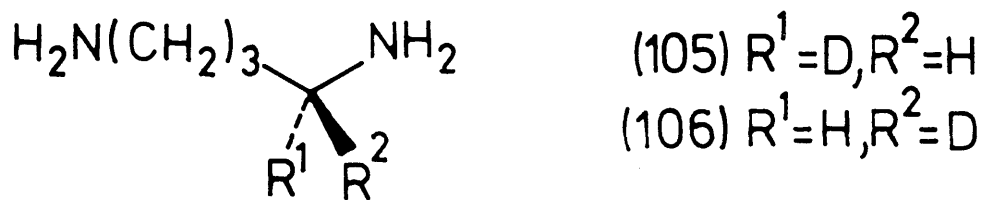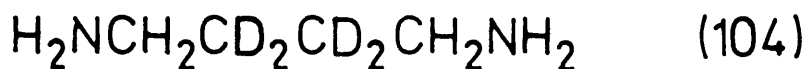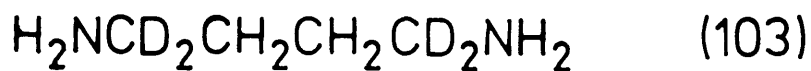
the elements of water. The pattern of incorporation of $\underline{S}$-[2-^{2}H$_1$]-putrescine (108) demonstrated that the hydroxylation step at C-7 proceeds with retention of configuration.[120]

The result of all of these experimental findings is the filling in of the stereochemical details of a number of the key steps in the biosynthetic pathway (Scheme 25). Oxidation of putrescine (92) to 4-aminobutanal (109) is followed by coupling with a further molecule of putrescine to form the imine (110). Reduction of this imine from the $\underline{si}$-face affords homospermidine (99). Further oxidation of homospermidine affords an aldehyde. Additional cyclisation and oxidation gives a pyrrolizidine aldehyde (111), which yields trachelanthamidine (23) on stereospecific reduction.

In the conversion of heliotrine (33) into hydroxydanaidal (112), a male moth pheromone, the configuration at C-7 is inverted (Scheme 26).[121]

4.3   Necic Acids

All of the necic acids thus far investigated have been shown to be derived from amino acid molecules. Heliosupine (63) was isolated from Cynoglossum officinale and contains two esterified acids, echimidinic (113) and angelic (114). Crout has demonstrated that the former is derived from valine by the incorporation of [4-^{14}C]valine (115).[122] Angelic acid is produced biosynthetically from isoleucine (116) as demonstrated by the incorporation of a universally ^{14}C enriched sample of this acid.[123] The use of [1-^{14}C]tiglic acid (117) and the specific incorporation of this geometrical isomer of angelic acid into heliosupine,

CH₃   CH₃ structure (117)

$(^{14}CH_3)_2CHCHCOOH$ with NH_2 (115)

H   CH₃ structure (114)

$(CH_3)_2C$—C—COOH with OH OH and CHOHCH₃ (113)

H   CH₃ structure (63)

$(CH_3)_2COH$
OCO—C—OH
$CHOHCH_3$

$CH_3CH_2CH(CH_3)CH(NH_2)COOH$ (116)
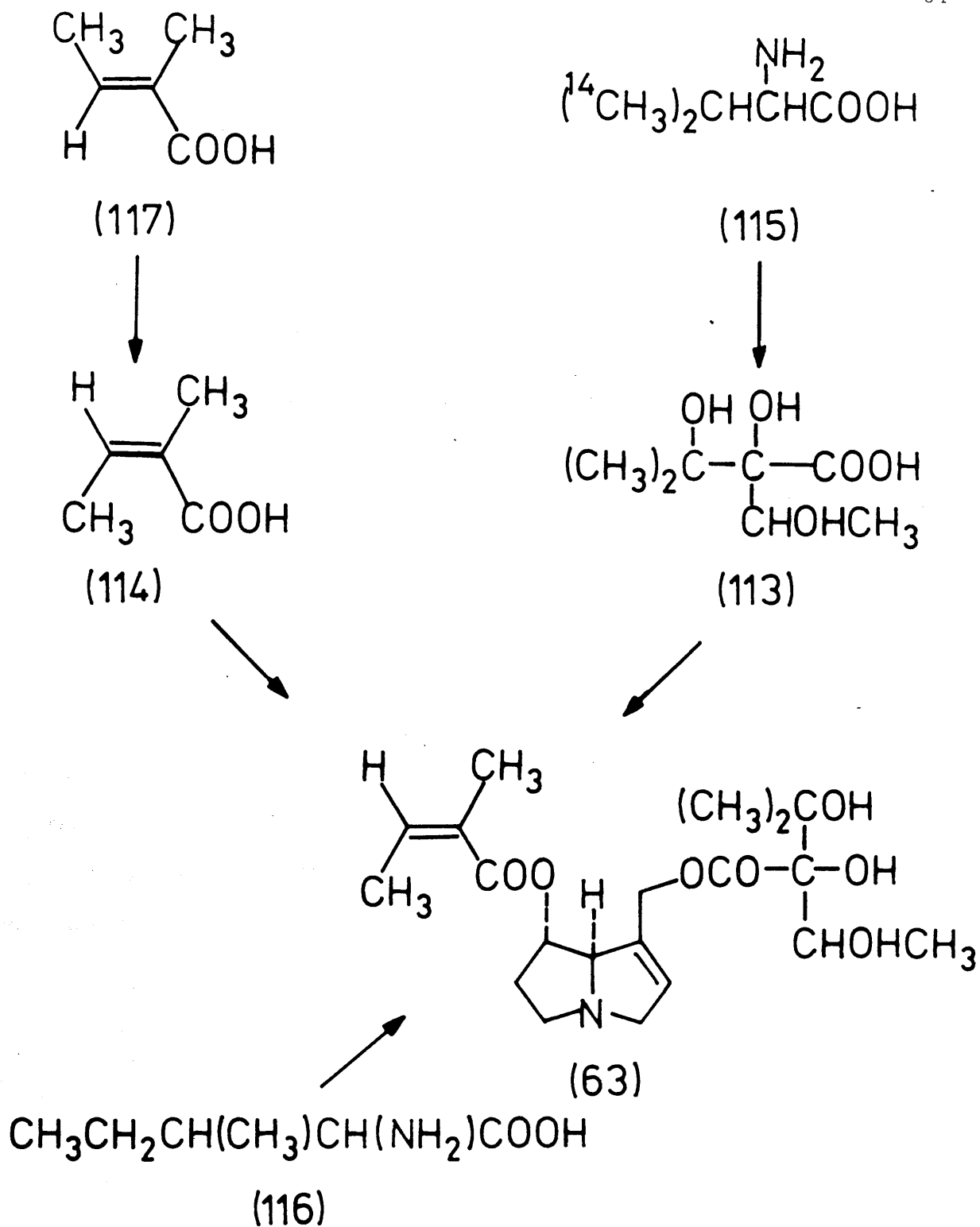
Scheme 27

suggests that isomerisation occurs during the production of the alkaloid.[124]

These results are interpreted diagrammatically in Scheme 27.

Many other studies upon the pathways used to construct the necic acids have taken place.[2] The majority of these involve the feeding of amino acid molecules. It is noteworthy that no macrocyclic diesters have 7α-hydrogen. This may be a consequence of biogenesis when two acid portions combine to form a macrocycle.[125]

4.4 The use of Analogues in Biosynthetic Studies

Analogues of known precursors can be of assistance in elucidating biosynthetic pathways. The ability or inability of living systems to convert chemically modified precursors into analogues of their normal metabolites can sometimes throw light upon the transformations occurring. The analogue substrates may be metabolized in an abnormal way, giving rise to new structural types. Indeed, the metabolism may be changed sufficiently so that growth is inhibited or the cells die.

The basis of the construction of a particular analogue of a known biosynthetic precursor is to replace an atom or group by a detectable label. One of the simplest cases is the replacement of a hydrogen by a fluorine atom. The properties of fluorine are ideally suited for this purpose (see Table 4). The element forms a strong bond to carbon and is not readily displaced. The van der Waals radius of fluorine is closely similar to that of hydrogen and the steric interactions with enzymes should be similar. Fluorine has even been called the fourth isotope of hydrogen. Fluorine is easy to observe in isolation by the use of ^{19}F n.m.r. spectroscopy and is relatively

Atom or Group	Van Der Waals Radii (Å)	C-X Bond Energy (kJ/mole)
H	1·20	416
F	1·35	485
Cl	1·80	327
Br	1·95	285
CH_3	2·00	356

Table 4

FCH_2COOH
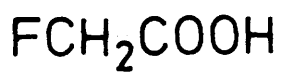
(118)
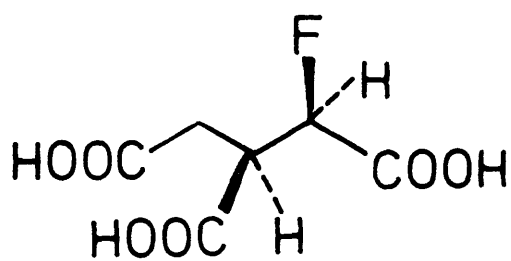
(119)

uncommon in living organisms. The attempted subterfuge at an enzyme receptor site is often detected by the system and may lead to pitfalls such as 'lethal synthesis'. This approach is used in cancer treatment. Balanced against the similarities of fluorine and hydrogen atoms are the drawbacks. The high electronegativity of the element frequently alters the chemical reactivity and electronic effects. Other atoms and groups have also attracted attention as a replacement for hydrogen and some of these are listed (Table 4).

Fluorinated analogues of natural substrates have been metabolized by a wide variety of biological systems. In most cases, the fluorinated precursor analogues were transformed into derivatives of the "normal" metabolites. The best results were obtained when the fluorine was remote from the sites of the transformations and the atom replaced, and thus the fluorine atom itself, was not normally lost in biological transformations.

The first discovery of C-F bonds in natural compounds was in the isolation of fluoroacetic acid (118) from Dichapetalum cymosum (Gifblaar).[126] Fluoroacetate is toxic and animals fed fluoroacetic acid were found to have a markedly increased concentration of citric acid in their organs, suggesting interference in the tricarboxylic acid pathway.[127] Administration of fluoroacetic acid to kidney homogenates resulted in the production of (+)-erythrofluorocitric acid (119). The site of action of fluorocitrate was shown to be at the mitochondrial membrane with potential inhibition of citrate transport across this barrier.[128] Fluorinated fatty acids and esters containing an even number of carbon atoms in the acid portion are known to be toxic,

68

possibly via degradation to acetate and fluoroacetate. The latter is the ultimate toxic principle.

A large number of studies with fluoroamino acids and micro-organisms have been carried out. Most bacteria are able to adapt to high levels of fluoroamino acids without being killed, and the acids can be incorporated into proteins by a variety of organisms.[129] For instance, [3-^{14}C]-p-fluorophenylalanine (120) was transformed by Scuttellaria galericulata into [2-^{14}C]-4'-fluorochrysin (121).[130] Tryptophan (122) was converted by Pseudomonas aurefaciens into the antibiotic pyrrolnitrin (123). The same species converted the unnatural substrates 6-fluorotryptophan (124) and 7-methyltryptophan (125) into 4'-fluoropyrrolnitrin (126) and 3'-methyl-3'-dechloropyrrolnitrin (127) respectively[131] (Scheme 28).

Metabolites isolated from the medium of resting cells of a Pseudomonas sp. incubated with p-fluorophenylacetic acid were R-(+)-monofluorosuccinic acid (128), trans-3-fluoro-3-hexenedioic acid (129), (-)-4-carboxymethyl-4-fluorobutanolide (130), 4-fluoro-2-hydroxy-phenylacetic acid (131) and 4-fluoro-3-hydroxyphenylacetic acid (132)[132] (Scheme 29). On the other hand, when the cells were growing, all the organic fluorine was released into the culture medium as fluoride ion.

The metabolism of the antitumour drug, 5-fluorouracil (133), has been studied in various systems. This compound is converted by Bacillus subtilis into 5-fluorouridine (134), by Brevibacterium ammoni-agenes to 5-fluorouridylic acid (135), and by Streptomyces cacaoi (var. asoensis) to 5-fluoropolyoxins M (136) and L (137)[133] (Scheme 30).

(120) → S.galericulata → (121)

(122) $R^1=R^2=H$ ------→ (123) $R^1=Cl, R^2=H$
(124) $R^1=H, R^2=F$ -----→ (126) $R^1=Cl, R^2=F$
(125) $R^1=CH_3, R^2=H$ -----→ (127) $R^1=CH_3, R^2=H$

P.aurefaciens

Scheme 28

(129)
+
(128)
(130)
+
(131) $R^1=H, R^2=OH$
(132) $R^1=OH, R^2=H$

Pseudomonas sp.
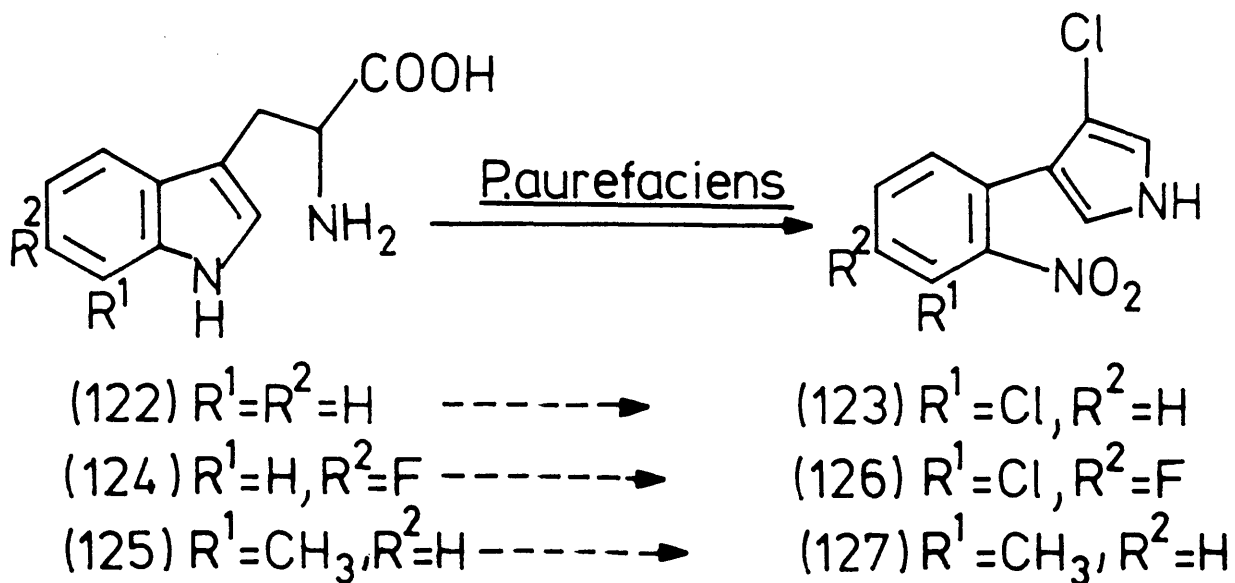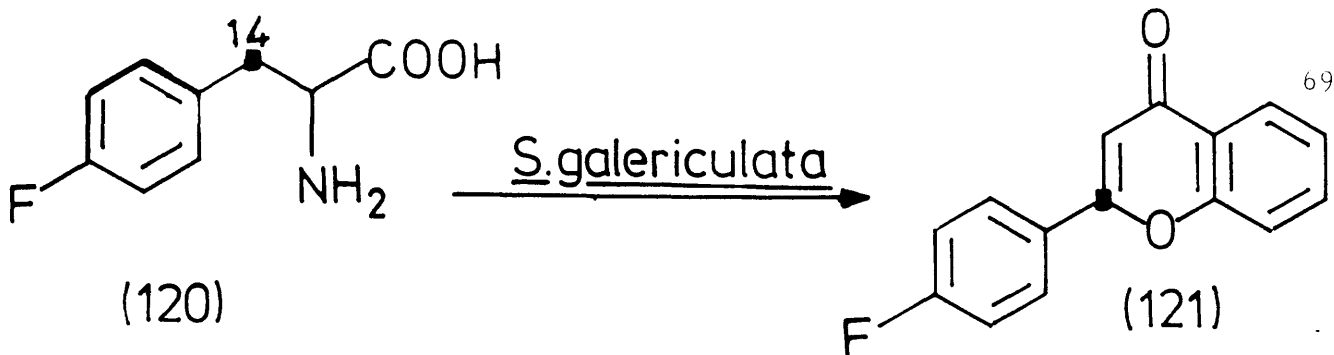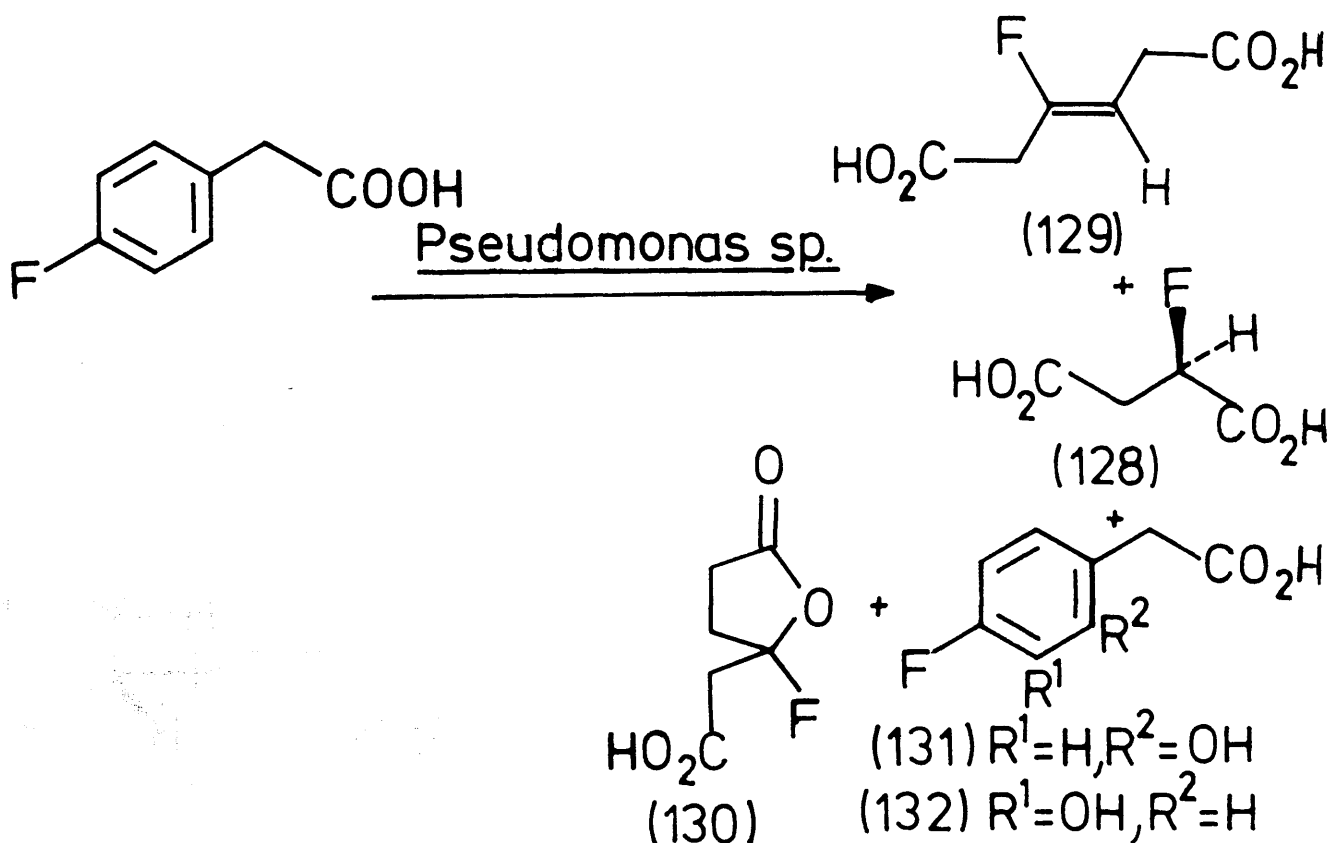
Scheme 29

Scheme 30

(133) R=F
(139) R=CH₃

(138) R=F
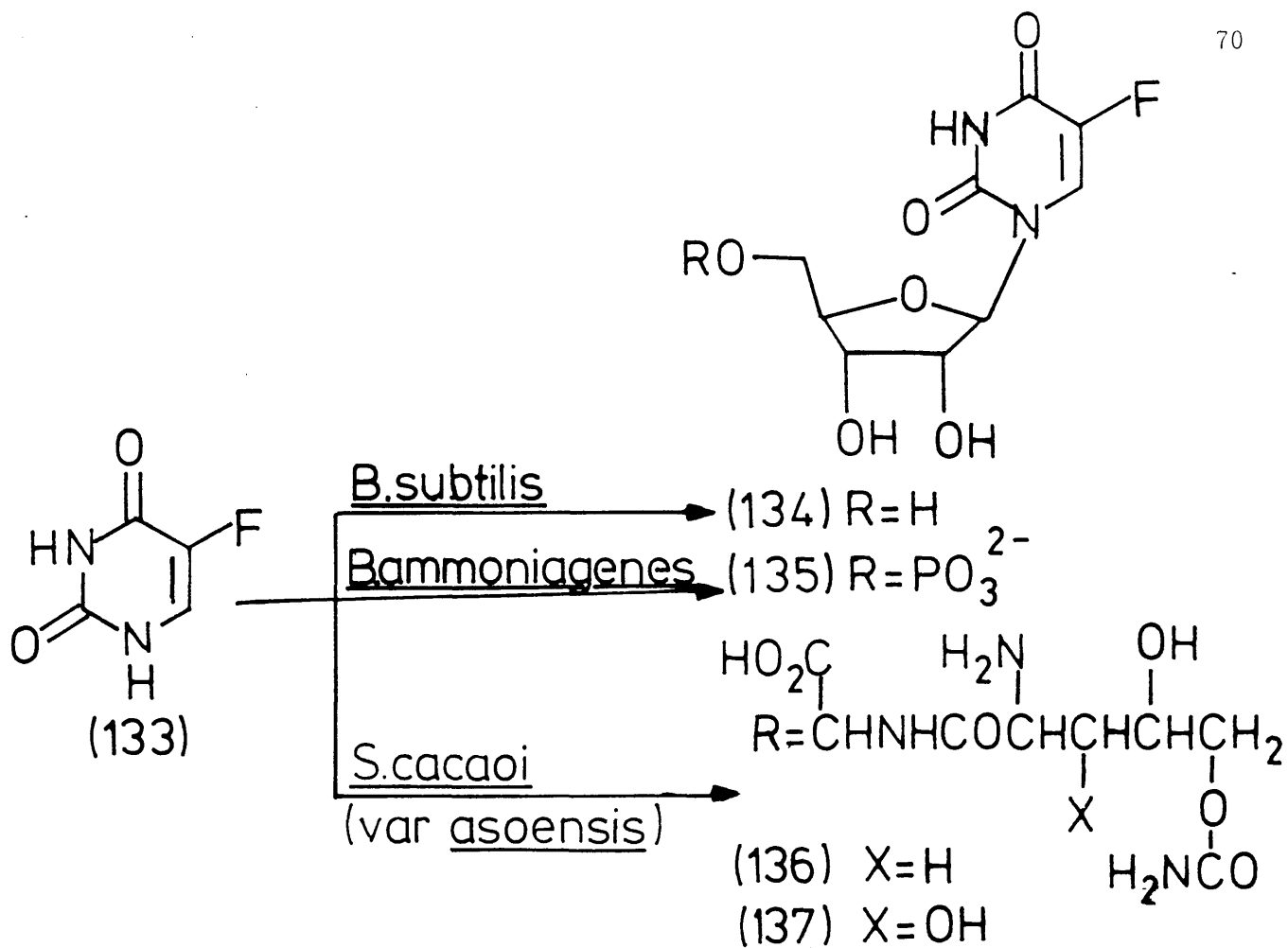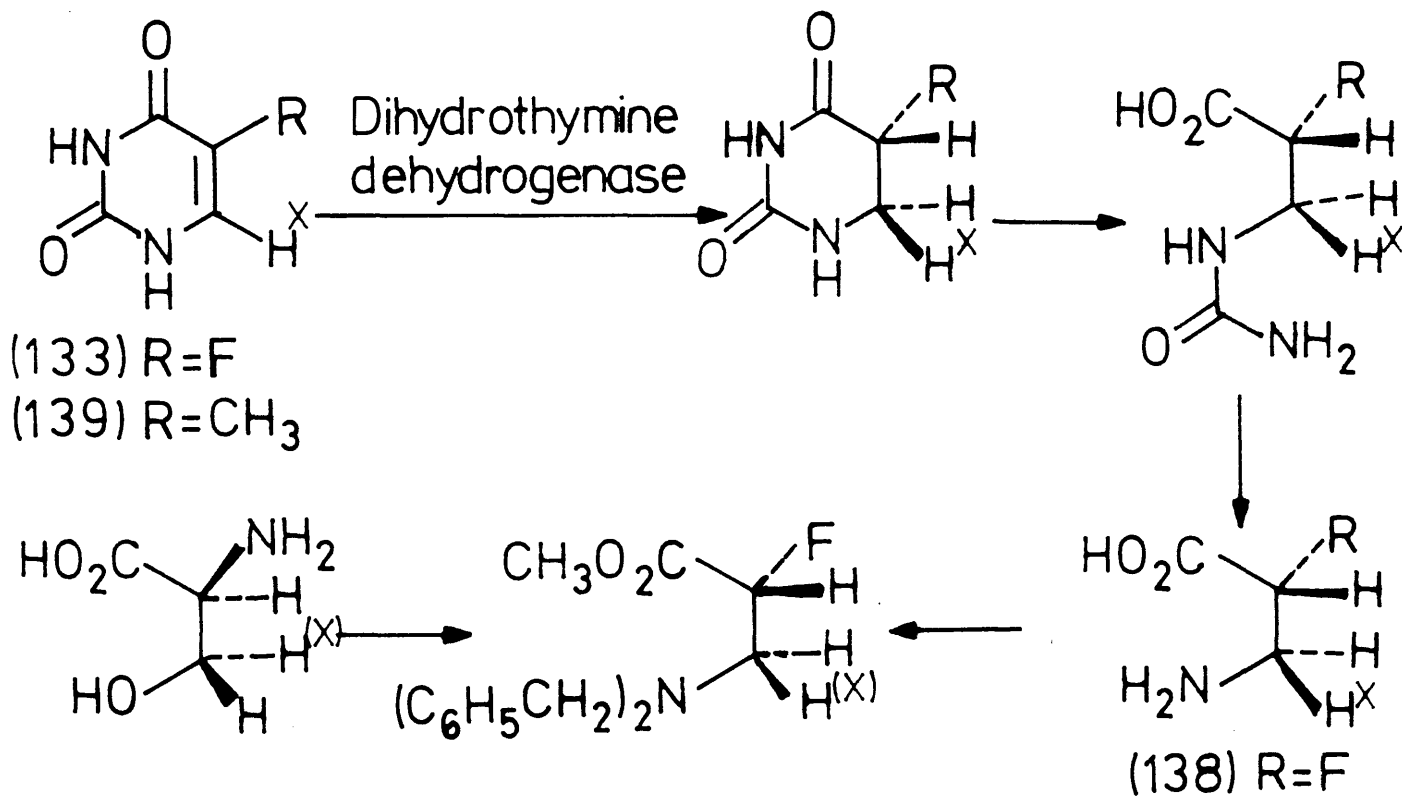
Scheme 31

Catabolism of 5-fluorouracil with a bovine liver enzyme system
produced R-3-amino-2-fluoropropanoic acid (138) via overall anti-addition
of hydrogen to the pyrimidine at the si-face of C-5 and 6.[134] This is
the same process as the catabolism of uracil and thymine (139), the
methyl analogue.[135] The structure and stereochemistry of (138) was
proved by the synthesis of a stable derivative from L-serine (Scheme 31).
The synthesis of thymidylic acid is blocked by 5-fluorouracil to cause
"thymineless death" of cells. This is an example of "lethal synthesis".

The replacement of hydrogen or hydroxyl moieties by a fluorine
atom very often alters the biological activity of the molecule. Studies
upon 3β-fluorovitamin D_3 (140), synthesised from cholesta-5,7-diene-3β-
ol, illustrate this.[136] The fluoroanalogue was less active than the parent
vitamin D_3 with regard to intestinal calcium transport and bone calcium
mobilization. The hydrogen analogue on the other hand was totally
inactive.

Several simple fluoroaromatic compounds have been incorporated
to give analogues of natural products. This is exemplified by the use
of 4-fluorophenylthioacetic acid as the side chain for a penicillin (141).[137]

As a final example of aberrant biosynthesis using fluorine, the
fine work carried out upon the alkaloid nicotine is worthy of description.
Aberrant biosynthesis is a term used to describe the conversion of an
unnatural "precursor" into an unnatural compound, presumably using
the same enzymes as the normal biosynthetic pathway. The feeding of
5-fluoronicotinic acid (142) to Nicotiana tabacum yielded 5-fluoronicotine
(143).[138] The tobacco plants were apparently able to build up a
resistance to the fluoroacid, but the dosage was critical. The use of a

(140)

(141)

(142)

N.tabacum → (143) R =

N.glauca → (144) R =
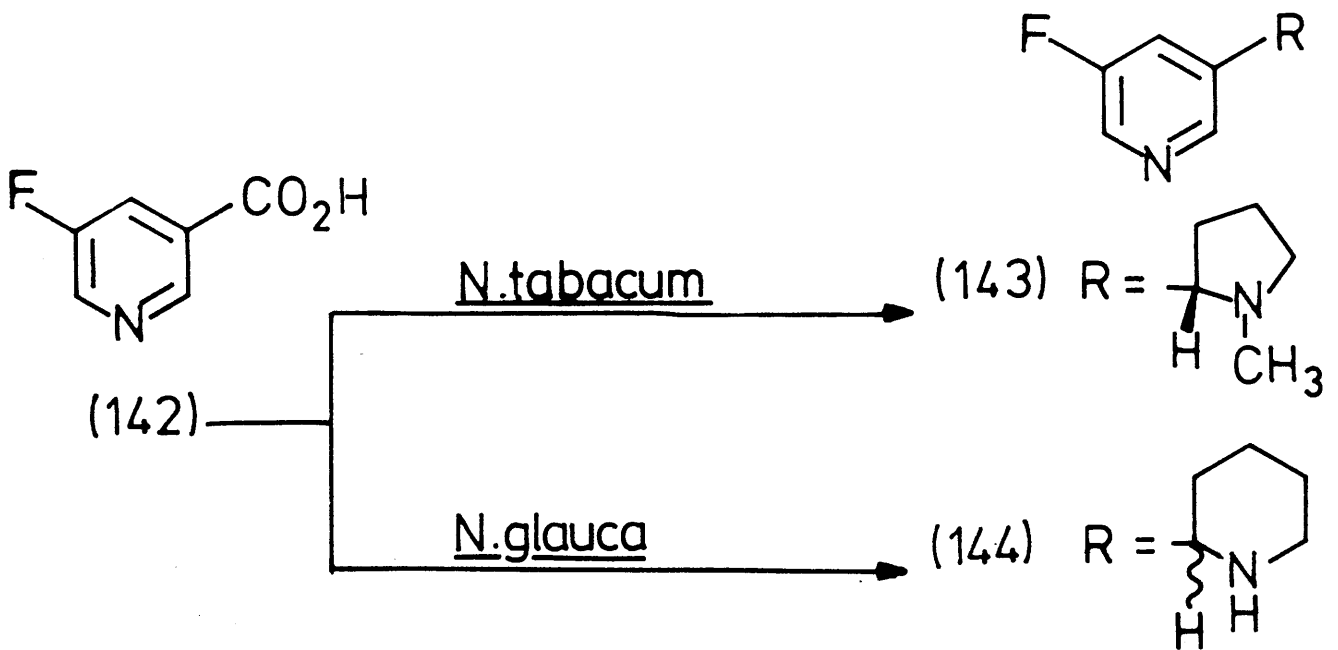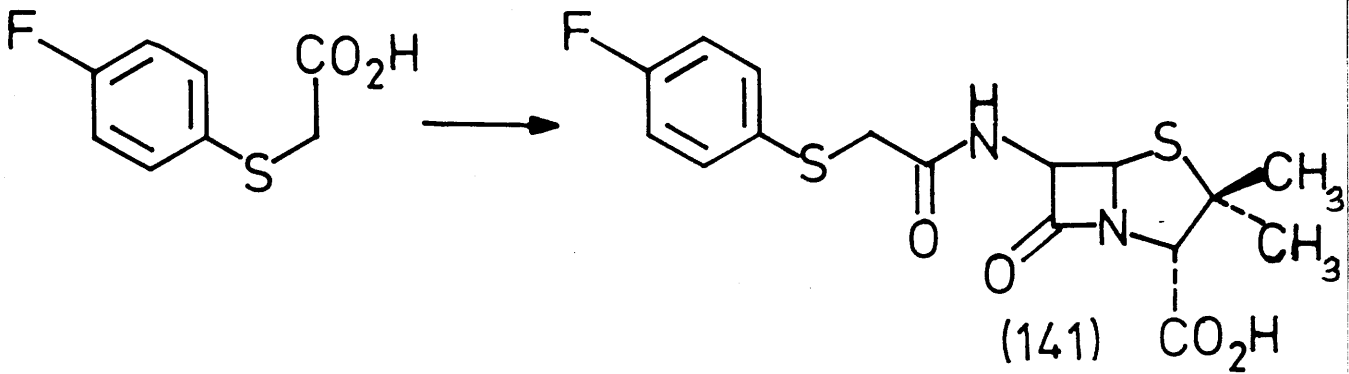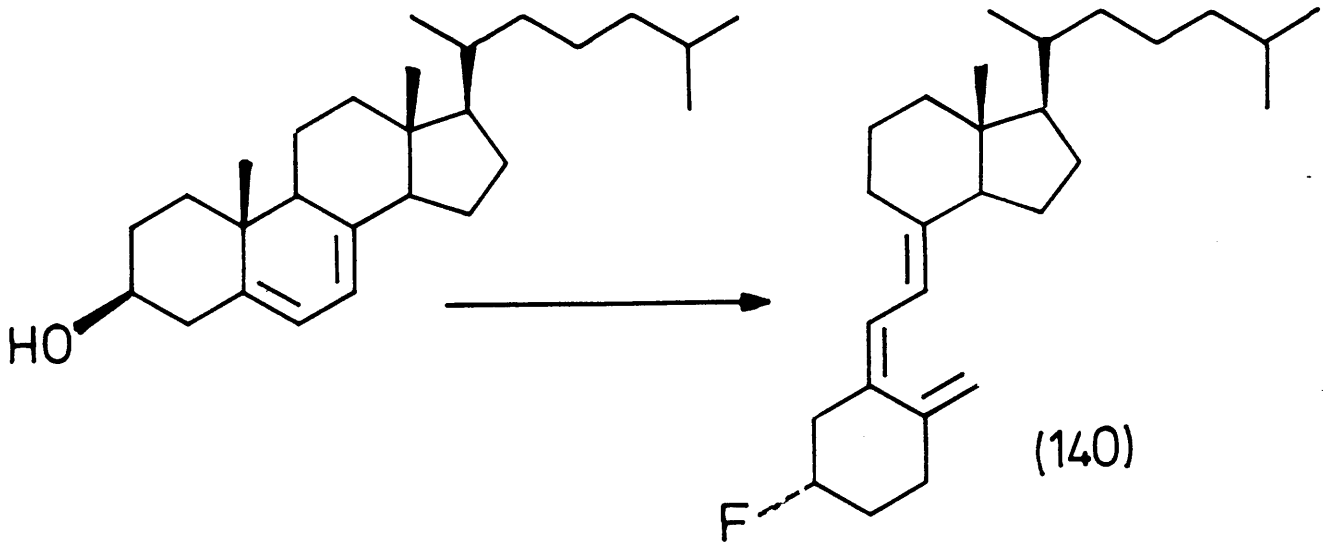
^{14}C label at C-5 and 6, and the unambiguous synthesis of 5-fluoro-
nicotine confirmed this result. Later, the same acid (142) was
incorporated by Nicotiana glauca into 5-fluoroanabasine (144), and
again labelling of C-5 and 6 was useful to confirm the finding.[139]
The 5-position was an excellent position to substitute as the hydrogen
at this position is incorporated intact and is not apparently involved in
any modifications.

As mentioned earlier, other atoms and groups are suitable for
analogue work. The feeding of codeine derivatives to Papaver somni-
ferum (the opium poppy) led to the isolation of a whole range of
morphine analogues.[140] The synthesis of 1-bromomorphine (145) and
morphine methyl ether (146) by this poppy exemplify these experiments.
Administration of 1,3-dimethyl-1-pyrrolinium chloride to Nicotiana
glutinosa led to the production of an unnatural alkaloid, 3'-methylnicotine
(147).[141]

All of the previous examples have involved the use of a
structurally modified form (A') of a well defined precursor (A) to
construct an analogue (Z') of the final product (Z) (Scheme 31). The
use of an analogue can lead to the retardation of a step, e.g. formation
of D'. This retardation may lead to the accumulation of C' or the use
of an alternative pathway to produce an intermediate E', which will throw
light on the structure of the compounds in the biosynthetic pathway.

Evidence for the discovery of an alternative pathway if a
blockage arises, was found in the oxidation of phytanic acid (148).[142]
The normal course of events is β-oxidation, but the β-position is now
blocked. The reading frame now shifts by a one-carbon unit and

P.somniferum

(145) R¹=Br, R²=H
(146) R¹=H, R²=CH₃

N.glutinosa

(147)

Scheme 31

(148)

α-Ox. → CO₂

successive
β-Ox.

CH₃CH(CH₃)CO₂H + 3CH₃CO₂H

+ 3 CH₃CH₂CO₂H
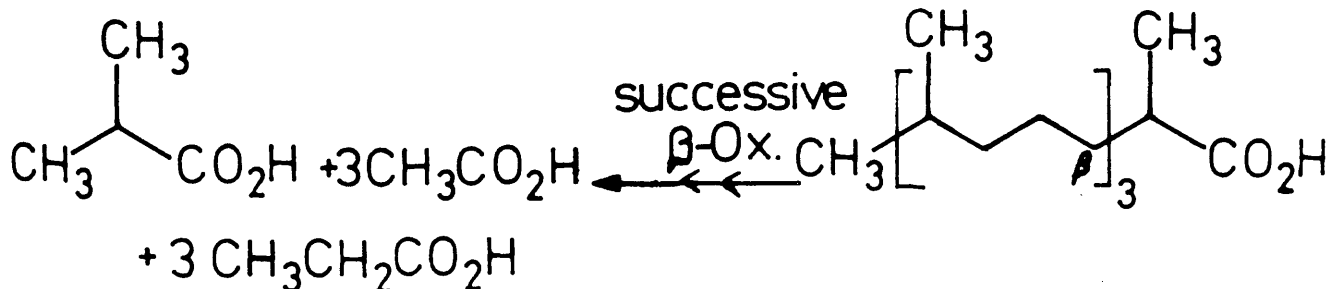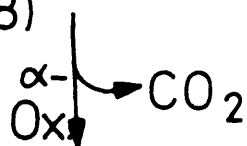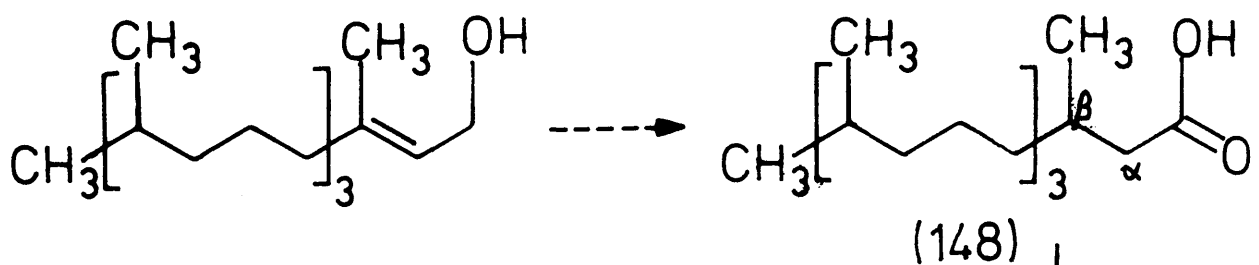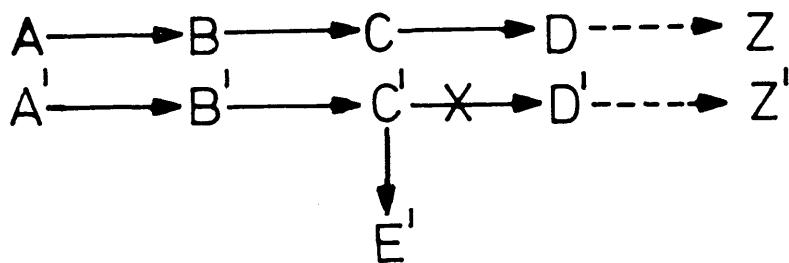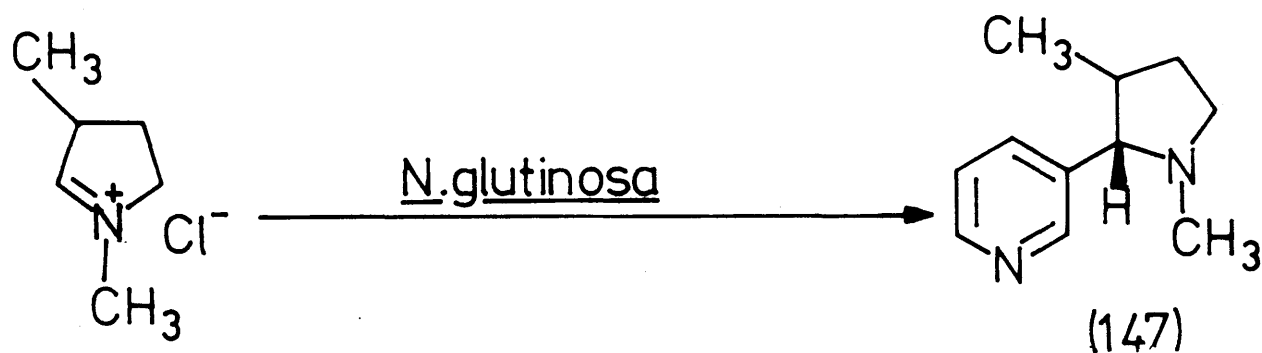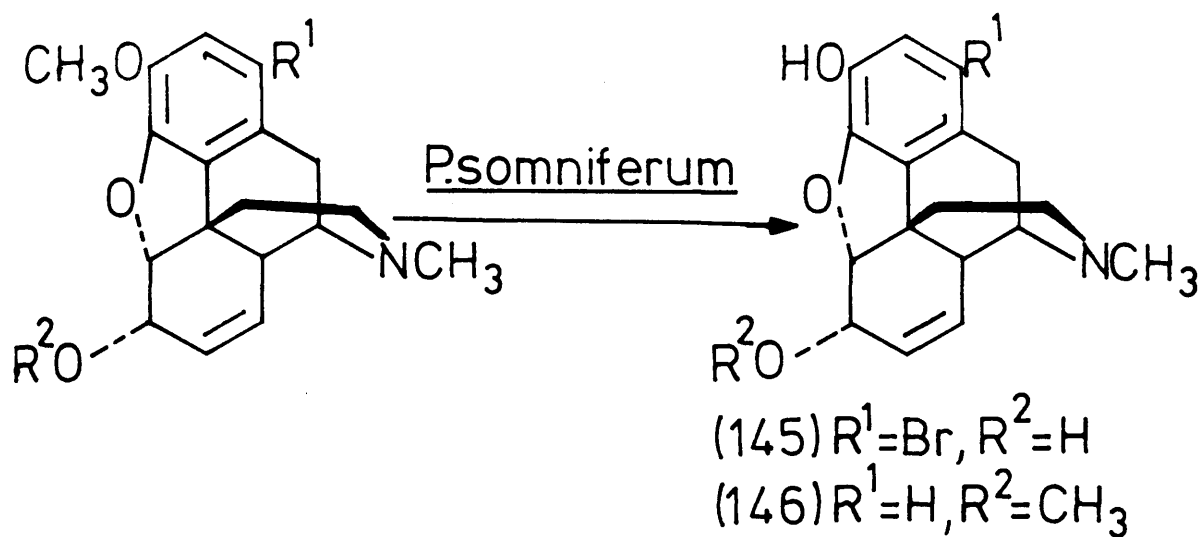
Scheme 32

α-oxidation converted phytanic acid into 2,6,10,14-tetramethylpentadeca-

noic acid (pristanic acid) which can readily undergo normal β-oxidation[143]

(Scheme 32). The bond energy released in the cleavage of a carbon-

carbon bond is far more effectively trapped by the β-oxidation pathway

than by the α-oxidation system. In Refsum's disease of the nervous

system, the capacity for α-oxidation is missing and phytanic acid cannot

be rapidly metabolized and accumulates in the tissue.

The formation of unnatural products in vivo should be useful

in the preparation of analogues and the study of the metabolism and

interrelationships of biologically active natural products.

The use of specially labelled molecules to attempt to study the

biosynthesis of pyrrolizidine bases, which have not been previously

investigated, is detailed in the following chapter. The synthetic

formation of analogues of a known precursor molecule and their use upon

plants which produce pyrrolizidine alkaloids is also described in Chapter

Five.

CHAPTER FIVE

INVESTIGATIONS INTO THE BIOSYNTHESIS OF THE NECINE BASES

5.1   Introduction

The majority of the biosynthetic studies upon the necine bases reported to date, have been concerned with (+)-retronecine (27). Previous work[2] had suggested that 1,4-diaminobutane (putrescine) would be a good precursor for (+)-heliotridine (3). A series of specifically labelled putrescines was therefore synthesised by known routes. These molecules were fed to plants which produce monoesters of (+)-heliotridine (3), (+)-isoretronecanol (22) and (+)-supinidine (24). An investigation into possible later intermediates in the biosynthesis of the latter two bases was undertaken by experiments with 1-hydroxymethylpyrrolizidines. Analogues of putrescine which contain a methyl group or a fluorine atom in place of a hydrogen atom have been prepared. These compounds were fed to various plants producing pyrrolizidine alkaloids and a study was made of their metabolism by the plants.

5.2   The Use of ^{2}H-Labelled Molecules with Cynoglossum officinale

5.2.1   Synthesis of ^{2}H-labelled putrescines

The biosynthesis of the pyrrolizidine alkaloid retrorsine (68) has been studied in Senecio isatideus plants using ^{2}H-labelled putrescines.[119,120] Convincing evidence for the fate of hydrogen atoms in retronecine biosynthesis was provided by the use of these precursors in conjunction with ^{2}H n.m.r. spectroscopy on the retronecine samples

produced. This avoids the need to degrade the retronecine moiety
(see Section 4.2). Several of the putrescines used in the above experiments were synthesised.

The dihydrochloride of $[1,1,4,4-{}^2H_4]$putrescine (103) was prepared by catalytic reduction of succinonitrile under an atmosphere of deuterium gas.[119] The solvent used was deuterioacetic acid and was generated by the deuteriolysis of acetic anhydride.[144] The atmosphere of deuterium gas was formed in situ by judicious addition of deuterium oxide to a suspension of lithium in paraffin oil, and the gas was dried by passage through silica gel and paraffin. The salt of (103) was furnished by acidification of the product with hydrochloric acid (Scheme 33). Inspection of the ${}^1H$ n.m.r. and the mass spectra of this material indicated that the putrescine (103) produced by this method contained over 95% of a ${}^2H_4$ species.

$[2,2,3,3-{}^2H_4]$Putrescine (104) was synthesised as the dihydrochloride salt after exchanging the hydrogens of succinonitrile for deuterium by heating in deuterium oxide.[119] This process was repeated thrice to ensure a high incorporation of deuterium. Catalytic hydrogenation of the $[2,2,3,3-{}^2H_4]$succinonitrile (149) with the Adams' catalyst gave (104), and acidification with hydrochloric acid produced the salt (Scheme 34). The ${}^2H$ content of both (104) and (149) was over 99% of a ${}^2H_4$ species.

The dihydrochloride salt of $[1,1-{}^2H_2]$putrescine (150), which was not used in the investigation of retronecine, was synthesised via 4-phthalimidobutanenitrile (151). Reaction of N-(3-bromopropyl)phthalimide with sodium cyanide in dry dimethylsulphoxide (DMSO) provided

$$R^1, R^2, H, \text{—OH}$$

(3) $R^1=H, R^2=OH$

(27) $R^1=OH, R^2=H$

$$NC(CH_2)_2CN \xrightarrow{\text{i.}} H_2NCD_2CH_2CH_2CD_2NH_2$$

(103)
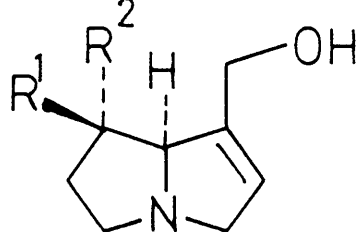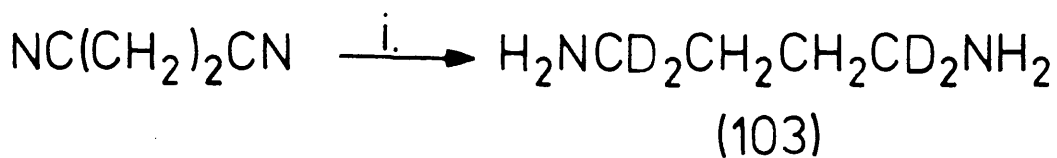
Scheme 33

Reagents: i. PtO_2, CH_3CO_2D, D_2.

$$NC(CH_2)_2CN \xrightarrow{\text{i.}} NC(CD_2)_2CN$$

(149) $\xrightarrow{\text{ii.}}$

$$H_2NCH_2CD_2CD_2CH_2NH_2$$

(104)

Scheme 34

Reagents: i. D_2O, Δ; ii. PtO_2, CH_3CO_2H, H_2.

(151)

$$\xrightarrow{\text{ii.–iii.}} H_2NCD_2(CH_2)_3NH_2$$

(150)
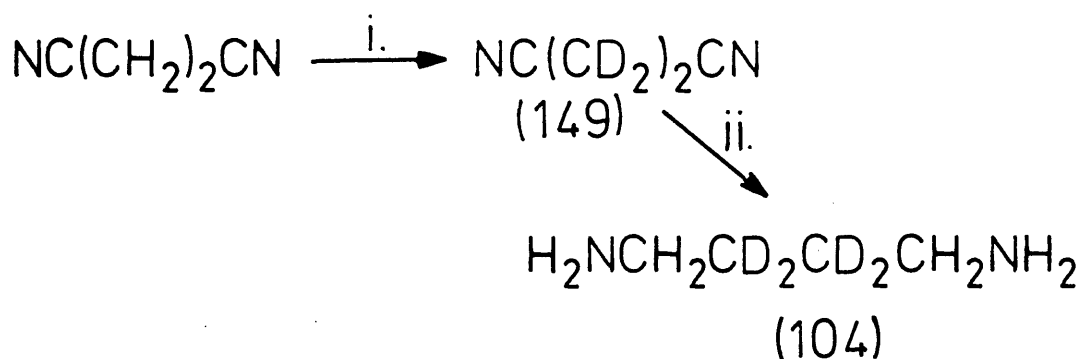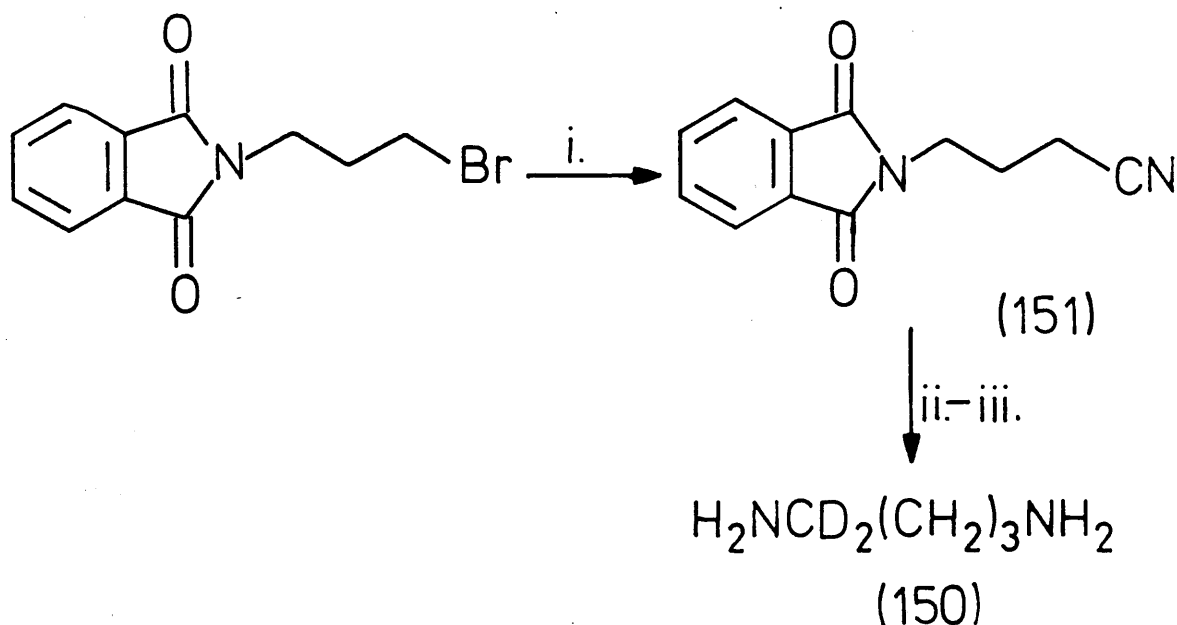
Scheme 35

Reagents: i. $NaCN, DMSO, \Delta$; ii. PtO_2, CH_3CO_2D, D_2;
iii. HCl, H_2O, Δ.

(151). Catalytic reduction of nitrile (151) under an atmosphere of deuterium in deuterioacetic acid was followed by acid hydrolysis to give putrescine (150) as the dihydrochloride salt. The salt was estimated to contain over 90% $^2H_2$ species. This compound (150) has been prepared previously by Callery et al.[145]

With a view to establishing the stereochemistry of some of the enzymic processes involved in the biosynthesis, four stereospecifically monodeuteriated putrescines [(105) - (108)] were synthesized. R-[1-$^2H_1$]- and S-[1-$^2H_1$]putrescines [(105) and (106) respectively] were made by known routes.[146]

Putrescine (105) was prepared by decarboxylation of L-ornithine in ^2H_2O using L-ornithine decarboxylase (E.C.4.1.1.17). Similar decarboxylation of DL-[2-$^2H_1$]ornithine in H_2O yielded (105) and unchanged D-[2-$^2H_1$]ornithine, which were separated. The $^2H_1$ content of each putrescine sample was estimated to be over 90%. The more challenging synthesis of R-[2-$^2H_1$]- and S-[2-$^2H_1$]putrescines [(107) and (108) respectively] were carried out by modifications to a known route[120] and full characterisation of the isolable intermediates. The route does not involve any enzymic reactions and seems to be highly enantioselective. The chiral starting material for the synthesis of (107) was S-aspartic acid, and was converted into S-chlorobutanedioic acid (152) (Scheme 36). The transformation occurred with retention of configuration, and required concentrated hydrochloric and nitric acids in the presence of urea.[147] The acid (152) was converted into dimethyl S-chlorobutanedioate (153), and reduction with diisobutylaluminium hydride (DIBAL) in toluene afforded S-2-chlorobutane-1,4-diol (154). Reduction of the chlorodiol

$H_2N(CH_2)_3$ NH_2

(105) $R^1 = D, R^2 = H$

(106) $R^1 = H, R^2 = D$

R^1 R^2

COOH

H_2N———H $\xrightarrow{i.}$ Cl———H $\xrightarrow{ii.}$ Cl———H

CH_2COOH CH_2COOH CH_2COOCH_3

(152)   (153)

COOH   COOH   COOCH_3

$\downarrow$ iii.

CH_2Br CH_2OH CH_2OH

H———D $\xleftarrow{v.}$ H———D $\xleftarrow{iv.}$ Cl———H

CH_2CH_2Br CH_2CH_2OH CH_2CH_2OH

(158)   (155)   (154)

$\downarrow$ vi.-vii. $\downarrow$ viii.

CH_2NH_2 COOH

H———D   H———D $\xrightarrow{ix.}$

$CH_2CH_2NH_2$ CH_2COOH

(107)   (156)   (157)
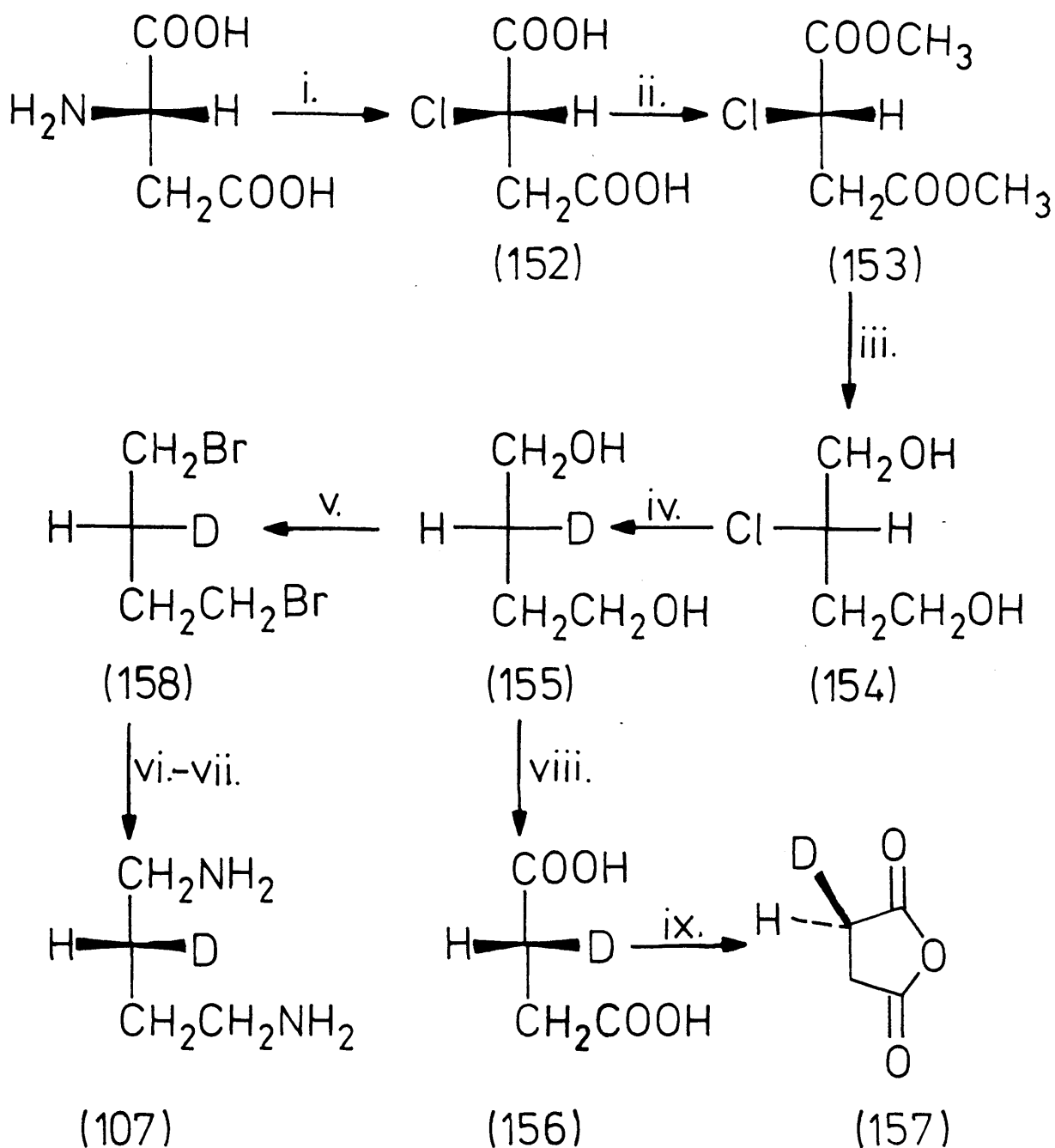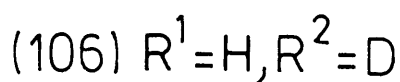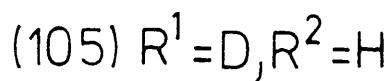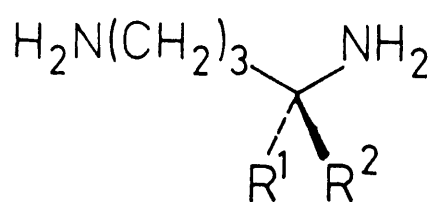
Scheme 36

Reagents: i. c.HCl, c. HNO_3, H_2NCONH_2, Δ; ii. CH_3OH, $SOCl_2$, -10°C; iii. DIBAL, $C_6H_5CH_3$, N_2, -30°C; iv. $LiAlH_4$, THF, 0°C, Ar; v. c.H_2SO_4, c.HBr, -10°C $\rightarrow$ +100°C; vi. NaN_3, DMSO, -5°C; vii. $LiAlH_4$, THF, N_2, r.t.; viii. $Na_2Cr_2O_7$, 2$\underline{M}$ H_2SO_4, -10°C; ix. Ac_2O, Δ.

(154) with lithium aluminium deuteride introduced one deuterium atom and

resulted in inversion of configuration to provide $\underline{R}$-[2-^{2}H$_1$]butane-1,4-diol

(155). The ^{2}H$_1$ content of this deuteriodiol (155) was checked by oxidation

to $\underline{R}$-[2-^{2}H$_1$]butanedioic (succinic) acid (156) with sodium dichromate and

formation of $\underline{R}$-[2-^{2}H$_1$]butanedioic anhydride (157). The mass spectrum of

(157) was checked against that of undeuteriated succinic anhydride and

this revealed a ^{2}H$_1$ content of greater than 95%. Formation of $\underline{R}$-[2-^{2}H$_1$]-

1,4-dibromobutane (158) was brought about by treatment of deuterio-

diol (155) with concentrated hydrobromic and sulphuric acids, and

reaction with sodium azide in DMSO formed $\underline{R}$-[2-^{2}H$_1$]-1,4-diazobutane.

This intermediate was not isolated, but was immediately reduced with

lithium aluminium hydride to furnish $\underline{R}$-[2-^{2}H$_1$]putrescine (107). The

salt was formed by passing dry hydrogen chloride gas through the

solution of the diamine (107) in THF. Synthesis of the dihydro-

chloride salt of $\underline{S}$-[2-^{2}H$_1$]putrescine (108) was effected by the

analogous series of reactions performed upon $\underline{R}$-aspartic acid and

subsequent intermediates [(159) - (162) and (165)] (Scheme 37). In

the original work,[120] the sample of (108) was contaminated with some

[1,1,2-^{2}H$_3$]putrescine dihydrochloride due to incomplete reduction of

dimethyl $\underline{R}$-chlorobutanedioate (160). Reduction of the unreacted

chlorodiester (160) with lithium aluminium deuteride led to the product-

ion of [1,1,2-^{2}H$_3$]butane-1,4-diol. This over-deuteriated diol was

eventually converted into [1,1,2-^{2}H$_3$]putrescine dihydrochloride. The

problem was overcome by a modification to the work-up procedure which

led to a 96% yield of (154) and a 95% yield of (161), as compared to

45% in the original paper.[120] Several other minor changes led to
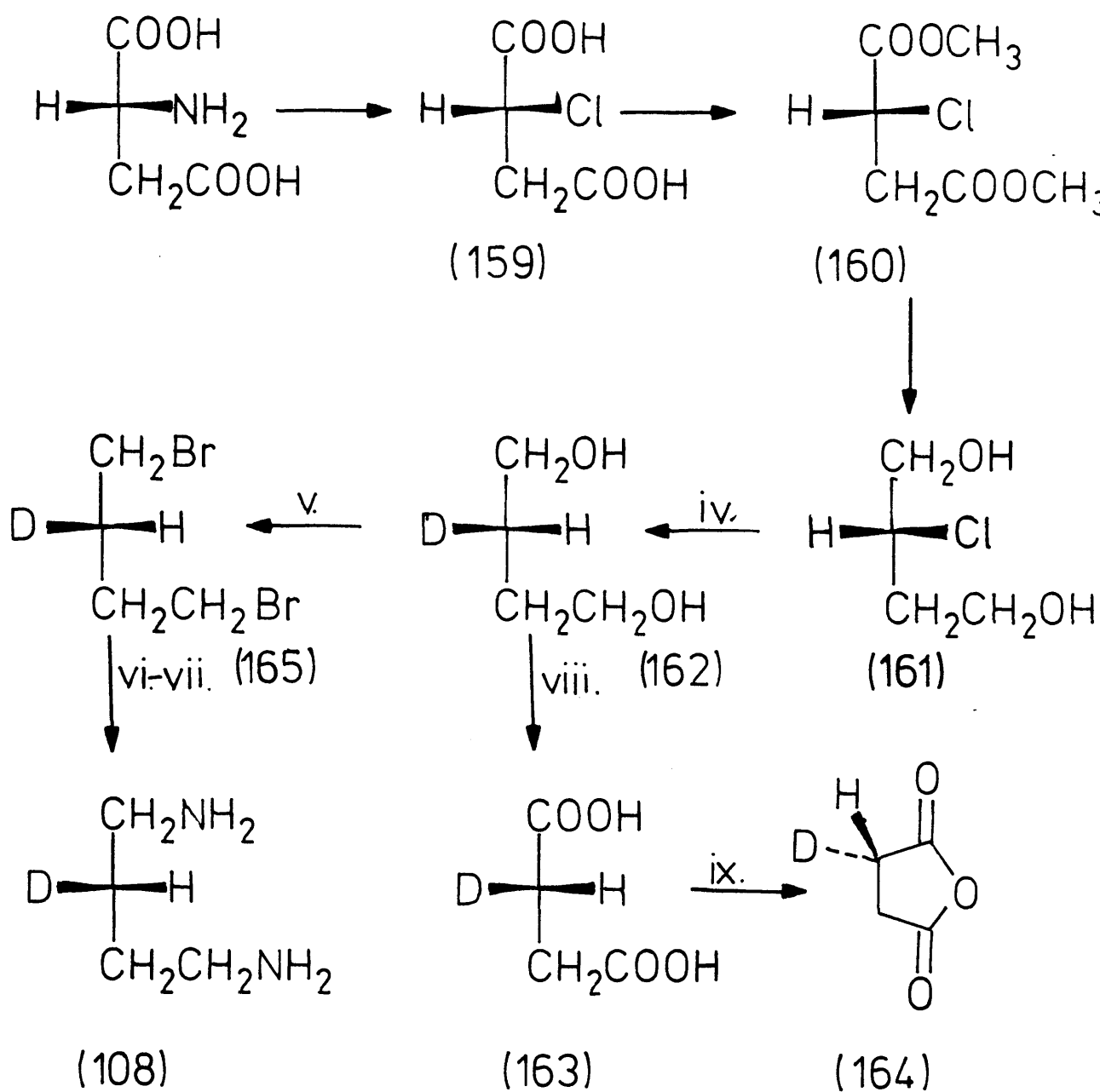
Scheme 37

Reagents: as in Scheme 36.

$$H_2N^{14}CH_2CH_2CH_2^{14}CH_2NH_2$$

(166)
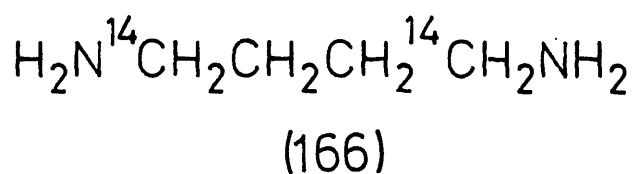
improvements in the reported yields of several of the steps.

Conversion of deuteriodiol (162) into $\underline{S}$-[2-$^2H_1$]butanedioic acid (163)

and $\underline{S}$-[2-$^2H_1$]butanedioic anhydride (164), revealed a $^2H_1$ content of

over 95%.

5.2.2   Feeding of ^2H-labelled putrescines

As mentioned earlier (Section 3.2), an investigation of

locally grown Cynoglossum officinale revealed the presence of

(+)-echinatine (62). It was intended to study the biosynthesis of

(+)-heliotridine (3) by feeding the specifically ^2H-labelled compounds

[(103) - (108) and (150)] to this species and analysing the samples of

(+)-echinatine produced in each experiment.

Accordingly, a measured quantity of each of these putresc-

ines, as their dihydrochloride salts, was mixed with a known quantity

of a radioactive tracer, [1,4-^{14}C]putrescine (166) dihydrochloride,

and this mixture was fed to a batch of Cynoglossum officinale by the

xylem pricking method.   This method involves the absorption of a

sterile, aqueous solution of the salt through a puncture made in the

stem of the plant over a period of five alternate days.   After two

more weeks, the plants were harvested and extracted to yield samples

of (+)-echinatine.

Scintillation counting showed the specific incorporations of

putrescine to be below 0.5% per C_4-unit of the base portion in every

case.   Specific ^{14}C incorporation   per C_4-unit for a putrescine

molecule is calculated from [(molar activity of echinatine x 1/2)/

(molar activity of precursor)] x 100%.   The ^2H n.m.r. spectra also

showed evidence of the disappointingly low incorporations.   Usually

no signals were evident, and when some were obtained there was

generally no correspondence with any signals in the ^{1}H n.m.r.

spectrum of (+)-echinatine (Figure 2).   The incorporations were as

low as 0.1% per C_4-unit in some experiments.   These findings might

be attributed to a wide variety of factors - the poor summer, feeding

technique, and time of feeding.

The conditions of feeding were varied over the two

subsequent summers, but the same low magnitude of specific incorpor-

ations per C_4-unit always resulted.   The same species of plant had

been used in previous experiments by other workers, who also

reported low incorporations.   Crout observed incorporations of 0.25%

and 0.21% for valine[122] and isoleucine[123] feedings to Cynoglossum

officinale, whilst McGaw and Woolley obtained an incorporation of 0.86%

for tiglic acid.[124]   All three experiments used radioactively labelled

acids.

Each of our feeding experiments showed that putrescine was

a specific precursor for (+)-heliotridine (3), as in each case the

alkaloid was hydrolysed as previously (Section 3.2), and the base

portion (3) contained most of the radioactive label (> 95%).

5.3   The Use of ^{13}C-Labelled Molecules with Cynoglossum officinale

5.3.1   Synthesis of ^{13}C-labelled putrescines

The feeding of molecules labelled with ^{13}C has the same advantages as the use of ^{2}H-labelled compounds. The number of chemical manipulations required to complete the analysis after the isolation of the metabolite is reduced as the need for degradation to isolate specific parts of the molecule is eliminated. This is not the case with radioactive precursors, where degradations are time consuming and may produce errors.

Accordingly, two ^{13}C-labelled putrescines were synthesised by known methods[108] and fed to Cynoglossum officinale. The dihydrochloride of [1-^{13}C]putrescine (167) was prepared by treatment of N-(3-bromopropyl)phthalimide with sodium [1-^{13}C]cyanide to provide 4-phthalimidobutane nitrile, which was reduced under an atmosphere of hydrogen and the product was hydrolysed with aqueous hydrochloric acid (Scheme 38). [2,3-^{13}C$_2$]Putrescine (168) dihydro-chloride was synthesised from 1,2-dibromo-[1,2-^{13}C$_2$]-ethane by treatment with potassium cyanide, reduction of the resultant [2,3-^{13}C$_2$]-succinonitrile, and conversion into the dihydrochloride salt (Scheme 39).

5.3.2   Feeding of ^{13}C-labelled putrescines

A known amount of [1-^{13}C]putrescine (167) dihydrochloride was 'spiked' by the addition of [1,4-^{14}C]putrescine (166) dihydro-chloride. Pulsed feeding of a sterile aqueous solution of this mixture
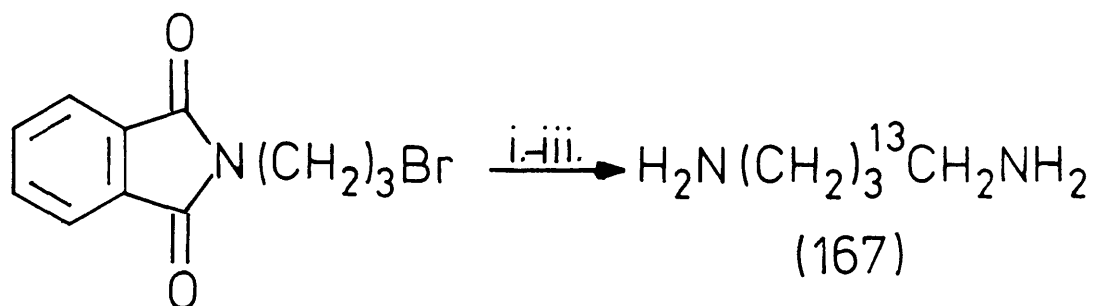
Scheme 38

Reagents:i.Na^{13}CN,DMSO,Δ;ii.H$_2$,PtO$_2$,CH$_3$CO$_2$H;
iii.dil.HCl(aq.).

Br(^{13}CH$_2$)$_2$Br $\xrightarrow{\text{i.-ii.}}$ H$_2$NCH$_2$(^{13}CH$_2$)$_2$CH$_2$NH$_2$

(168)
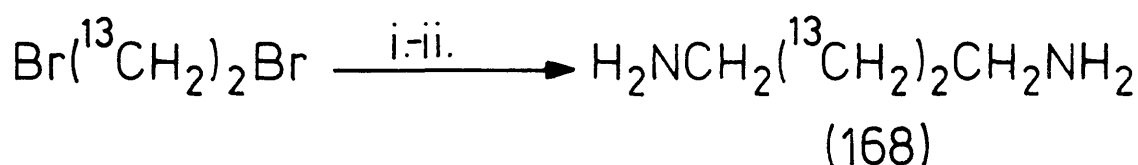
Scheme 39

Reagents:i.KCN,DMSO,Δ;ii.H$_2$,PtO$_2$,CH$_3$CO$_2$H.

into the xylems of young Cynoglossum officinale plants, followed by harvesting and methanolic extraction led to the isolation of a sample of (+)-echinatine (62). The 50 MHz ^{13}C-{^1H} n.m.r. spectrum of this alkaloid (Figure 7) was compared with that of the natural material run under the same conditions (Figure 8). The four enhanced signals observable corresponded to C-3, C-5, C-8 and C-9 of (+)-echinatine,[*], with enrichment factors for each position of 1.18, 0.78, 0.76 and 0.78% ($\pm$ 0.12%) ^{13}C respectively. The enrichment factor for each labelled site in (+)-echinatine (62) is the excess of ^{13}C above natural abundance and is calculated as [(integral of labelled site-natural abundance integral)/(natural abundance integral)] x 1.1%. The average enrichment factor for each labelled site was 0.88% ^{13}C, and the estimated ^{13}C specific incorporation was 1.93% per C_4-unit of putrescine [1.93% = 0.88 x 2/91 x 100%, where 91/2 atom % ^{13}C was the average enrichment at each labelled position of putrescine (167)].

The dihydrochloride of [2,3-$^{13}C_2$]putrescine (168) was fed to young Cynoglossum officinale plants. A relatively low ^{14}C specific incorporation of 0.27% per C_4-unit was obtained in (+)-echinatine. The ^{13}C-{^1H} n.m.r. spectrum of the alkaloid in deuteriochloroform (CDCℓ_3) was disappointing. The identification by observation of ^{13}C-^{13}C doublets was hampered by the noise present on the baseline, and the expected doublets were not evident. In both this experiment and in the previous case, alkaline hydrolysis of the alkaloid to produce (+)-heliotridine (3) confirmed that the radioactive ^{14}C label was almost exclusively located in the base portion (> 94%).

Figure 7

50MHz ^{13}C-$\{^1$H$\}$ n.m.r. spectrum of (+)-Echinatine
derived from [1-^{13}C]putrescine (167)
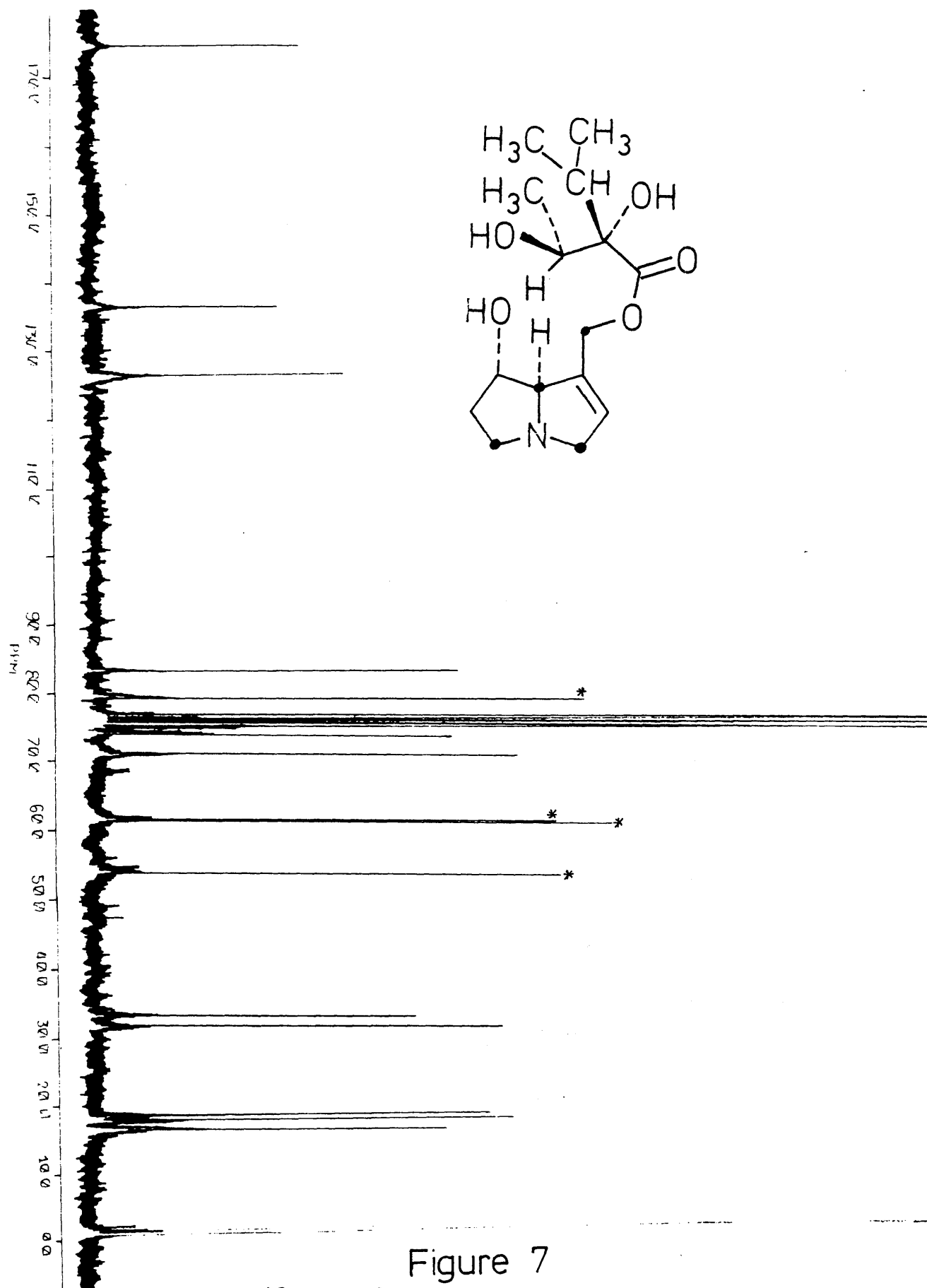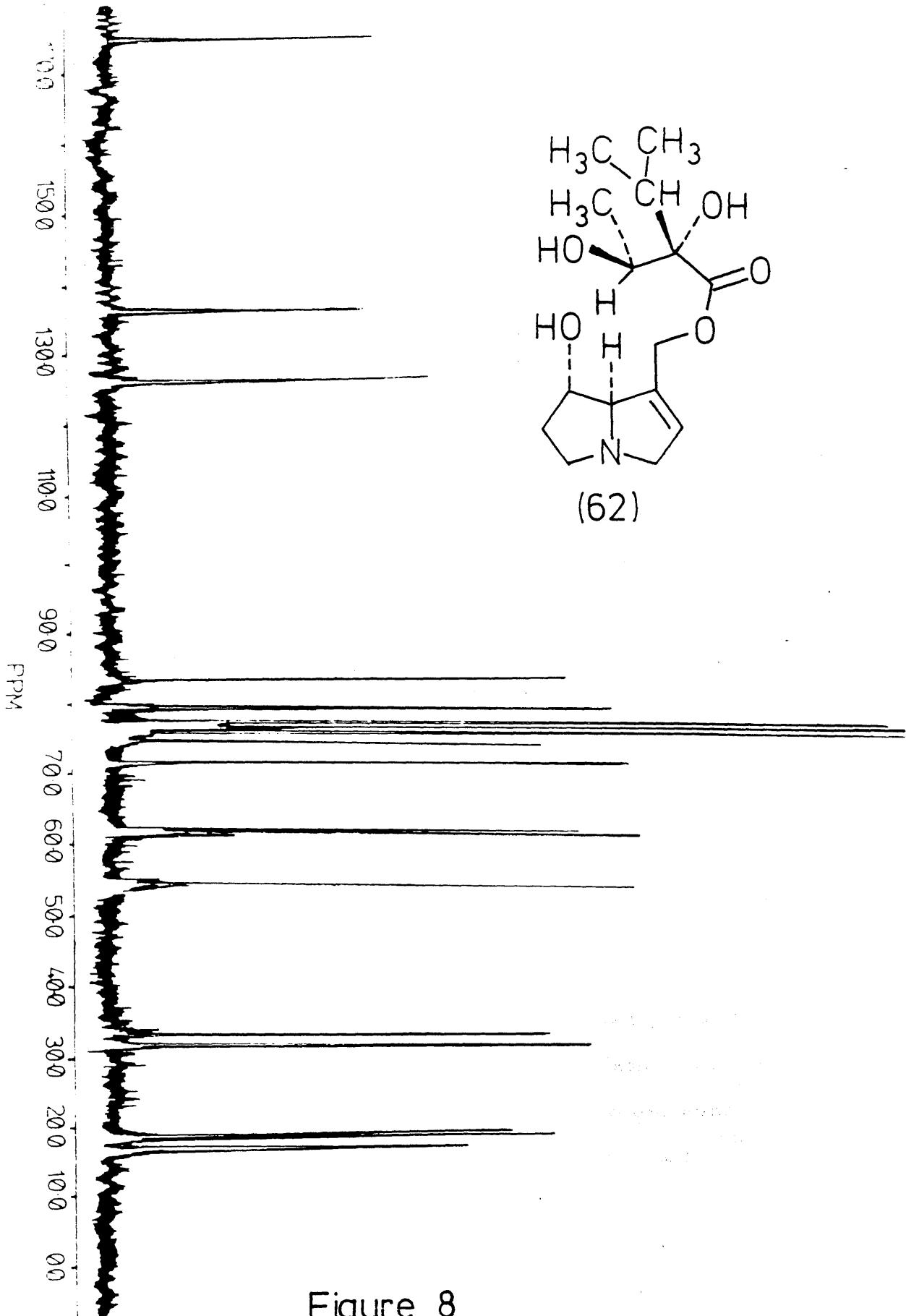
Figure 8

50MHz ^{13}C-{^{1}H} n.m.r. spectrum of (+)-Echinatine (62)

5.4   Investigations into the Biosynthesis of 8β-Necine Bases

5.4.1   Introduction

The occurrence of a wide variety of pyrrolizidine alkaloids is well documented.[1,2] Over 200 structures are known, but of these, only about 25 have the hydrogen atom at C-8 in the β-configuration. Furthermore, only one of these 8β-alkaloids is a macrocyclic diester, hastacine (169),[148] while the remainder are C-9 monoesters, such as macrophylline (170)[148] and planchonelline (171).[149] At the present time, no work upon the biosynthesis of any 8β-bases has been published.   The investigation of Cynoglossum australe (R.Br.) by Culvenor and Smith,[150] led to the discovery of two monoesters of 8β-bases.   This species was chosen for our investigation.

5.4.2   Isolation of (+)-Cynaustraline and (+)-Cynaustine

Cynoglossum australe were grown locally from seeds kindly provided by Dr. C.C.J. Culvenor of the C.S.I.R.O., Melbourne, Victoria, Australia.   Harvesting of the plants and methanolic extraction revealed the presence of two alkaloids.   The alkaloid with higher R_f (0.35) stained in the manner of a 1,2-unsaturated pyrrolizidine with o-chloranil and Ehrlich's reagent,[94] but the alkaloid with lower R_f (0.32) gave a different colour when treated with o-chloranil. This was expected from the findings of Culvenor and Smith,[150] who reported the isolation of (+)-cynaustraline (172) and (+)-cynaustine (173), the C-9 (-)-viridifloryl esters of (+)-isoretronecanol (22) and (+)-supinidine (24), respectively.   The number of known pyrrolizi-

(169)

(170) $R^1 = OH$, $R^2 = CO$ — CH_3, CH_3, H

(171) $R^1 = H$, $R^2 = CO$ — H, H, SCH_3
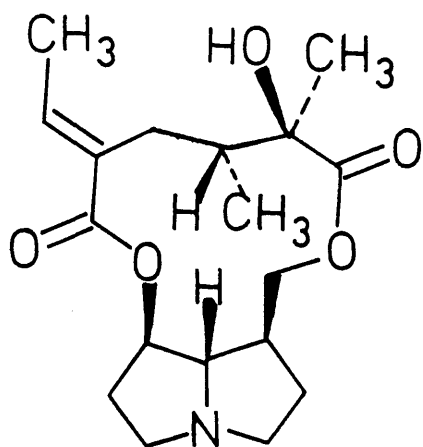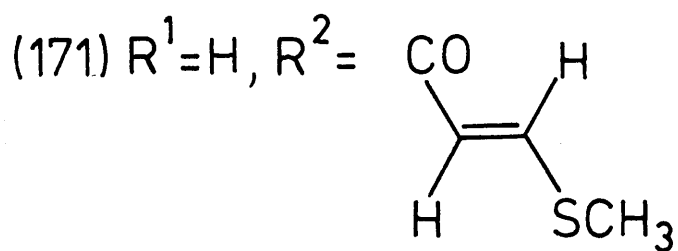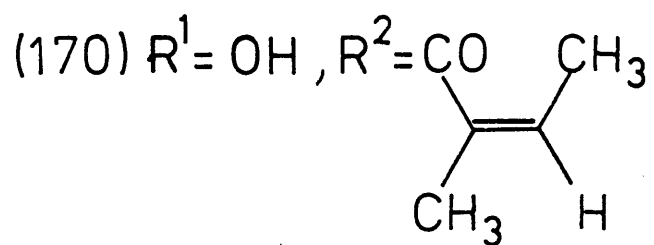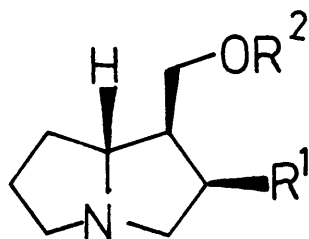
dine alkaloids containing (+)-isoretronecanol is in single figures and (+)-cynaustine (173) is the only alkaloid known to contain (+)-supinidine.

Column chromatography on basic alumina eventually separated these two similar pyrrolizidine monoesters. Analysis of the more polar alkaloid (R_f 0.32) showed that it was a saturated pyrrolizidine monoester and spectroscopic data were in agreement with literature values[148,150] for (172). The less polar alkaloid (R_f 0.35) was identical with (+)-cynaustine (173).[150] Final conformation of the structures and stereochemistry of (172) and (173) was provided by alkaline hydrolysis of samples of the two alkaloids to (+)-isoretronecanol (22) from (172), and to (+)-supinidine (24) from (173). Analysis of the bases agreed with that of previous workers.[150,151] Having confirmed Cynoglossum australe as a source of these two unusual 8β-bases, an investigation into their biosynthesis was undertaken.

5.4.3.   Feeding of [2]H-labelled putrescines

The molecules chosen for an investigation of the biosynthesis of (+)-isoretronecanol (22) and (+)-supinidine (24), were the [2]H-labelled putrescines [(103) - (105), (107), (108), and (150)] synthesised earlier (Section 5.2.1). These specifically labelled compounds were fed to batches of young Cynoglossum australe plants as a mixture of their dihydrochloride salts and a known quantity of a radioactive tracer (166). The compounds were fed by pulse-feeding on five alternate days into the xylems of the plants. After a further

(172) R= CO   (22) R=H

$$HO-C-CH(CH_3)_2$$
$$HO-C-H$$
$$CH_3$$

(173) R=CO   (24) R=H

$$HO-C-CH(CH_3)_2$$
$$HO-C-H$$
$$CH_3$$

$$H_2NC^3H_2CH_2CH_2C^3H_2NH_2$$
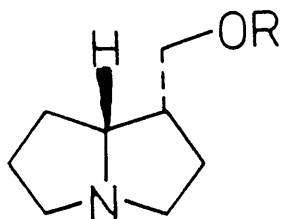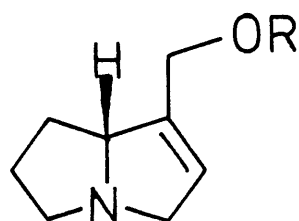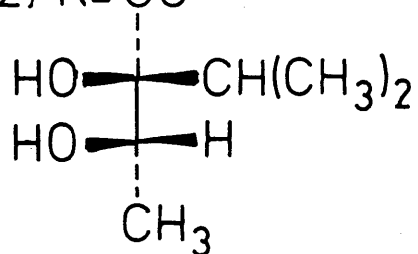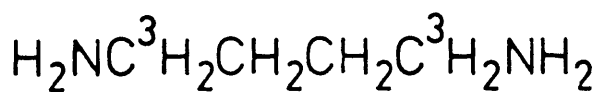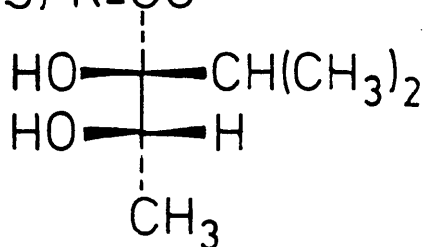
(174)
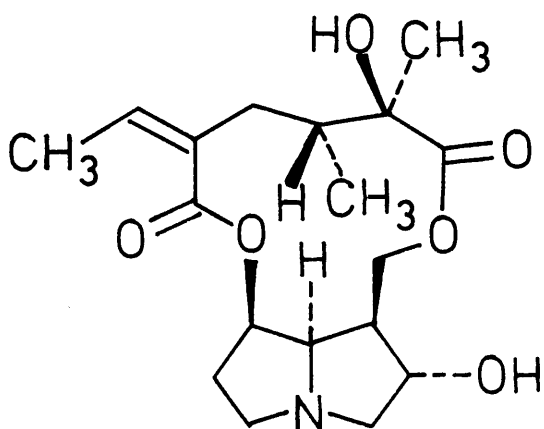
(175)

period of two weeks, the plants were harvested and the alkaloid mixture was isolated by methanolic extraction. Column chromatography on basic alumina with increasing proportions of methanol in dichloromethane of each of the mixtures, provided samples of (+)-cynaustraline (172) and (+)-cynaustine (173). As with the experiments upon Cynoglossum officinale (Section 5.2.2.), the incorporations of ^{14}C as detected by scintillation counting of radioactive solutions, were disappointingly low (generally ca. 0.1% per C_4-unit). The 31 MHz ^{2}H n.m.r. spectra obtained were inconclusive. The experiments were repeated the following summer, but the same results were obtained. Alkaline hydrolysis of the alkaloids proved that the radioactivity present was located predominantly in the 8β-base portion (> 95%).

5.4.4 Feeding of ^{3}H-labelled molecules

Alongside the use of ^{2}H-labelled precursors detailed previously, it was decided to use radioactive ^{3}H labels to investigate the biosynthesis of the two 8β-bases present in Cynoglossum australe. Putrescine labelled with tritium (^{3}H, T) (174) was fed as its dihydrochloride to a batch of young plants by the wick method. This method involves the threading of the stem of the plant and immersion of the ends of the thread in a vial containing a sterile aqueous solution of [1,4-^{3}H]putrescine (174) dihydrochloride. A good incorporation (> 1%) into each alkaloid was obtained, and after separation and subsequent hydrolysis, radioactive samples of the two 1-hydroxymethyl pyrrolizidines were obtained (Scheme 40). Each of these 8β-bases was fed in turn to different batches of young Cynoglossum australe
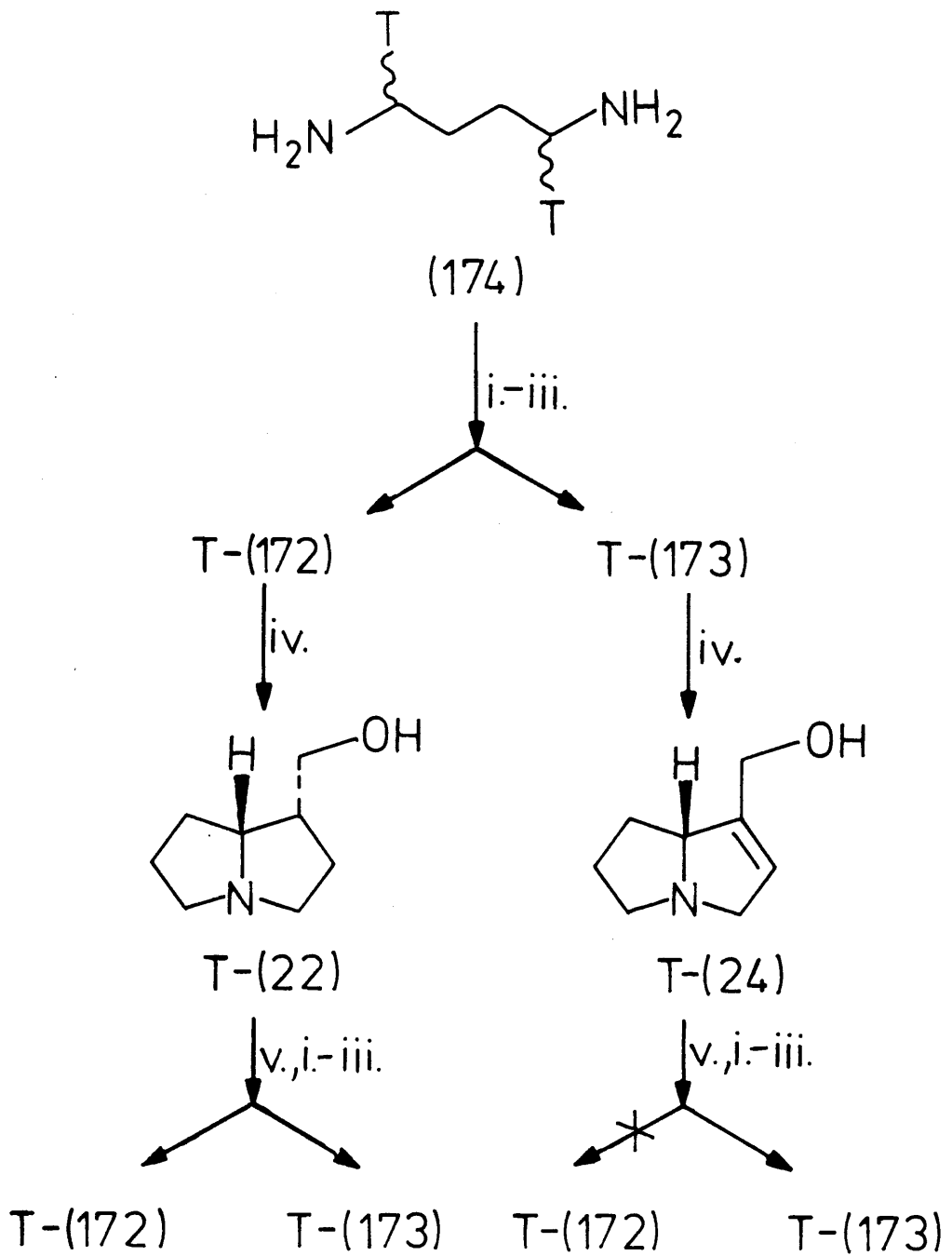
Scheme 40

Procedures: i. Feed to C.australe ; ii. Methanolic extraction; iii. Separation; iv. $Ba(OH)_2$, H_2O, Δ; v. Add (166) and measure $^3H{:}^{14}C$ ratio.

Expt.	Compound Isolated	Activity (µCi)	Expt.	Compound Isolated	%Incorp. ^{3}H	%Incorp. ^{14}C	^{3}H:^{14}C Ratio Fed	^{3}H:^{14}C Ratio Isolated
A	T-(172)	5·2	B	T-(172)	1·3	1·2	11·7	12·5
	T-(173)	2·1		T-(173)	1·2	1·4	11·7	11·0
	T-(22)	3·4		T-(175)	<0·1	2·5	11·7	<1
	T-(24)	1·2	C	T-(172)	<0·1	1·2	7·6	<1
				T-(173)	1·1	1·0	7·6	7·8

Expt. A = Feeding of ^{3}H-putrescine (174)

Expt. B = Feeding of ^{3}H-isoretronecanol (22)/(166)

Expt. C = Feeding of ^{3}H-supinidine (24)/(166)

Table 5

plants as an aqueous solution mixed with a known quantity of
[1,4-^{14}C]putrescine (166) dihydrochloride. The ratios of ^{3}H:^{14}C
were measured before feeding of the mixtures, and after isolation and
separation of the alkaloids. These ratios and the incorporations are
summarised in Table 5. The results suggest that (+)-isoretronecanol
(22) is a precursor for both alkaloids (172) and (173), but (+)-supin-
idine (24) is only incorporated into (+)-cynaustine (173). This is
consistent with the formation of supinidine via isoretronecanol, which
agrees with earlier conclusions (Section 4.2). A mixture of tritiated
(+)-isoretronecanol (22) and [1,4-^{14}C]putrescine (166) dihydrochloride
was fed to Senecio pleistocephalus, which produces (-)-rosmarinine
(175).[152] The putrescine (166) was incorporated efficiently into the
saturated macrocycle, but no incorporation of the 8β-base (22) into
the 8α-alkaloid (175) was detected by scintillation counting. This
showed that the 8β-base cannot be epimerized to the 8α-configuration
in Senecio pleistocephalus and incorporated into rosmarinine, possibly
via an immonium ion.

5.5   Synthesis and Feeding of 2-Methylputrescines

5.5.1   Introduction

In an effort to study further the biosynthesis of pyrrolizi-
dine alkaloids, substituted putrescines were chosen as targets for
feeding to plants producing these alkaloids. The van der Waals
radius of a methyl group (2.0Å) is not too dissimilar from that of a
hydrogen atom (1.2Å) (Table 4), and it was our hope that the enzymes
involved would not be able to differentiate between normal putrescine

and 2-methyl analogues in most steps of the biosynthetic pathway. To this end two 2-methylputrescines were synthesised.

5.5.2 Synthesis of 2-methylputrescines

The synthesis of R-(+)-2-methylputrescine (176) was first carried out by von Braun and Jostes[153] many years ago, and this route was chosen as the basis for our synthesis of the analogues. Readily available R-(+)-3-methyladipic(3-methylhexan-1,6-dioic)acid (177) was converted quantitatively into R-(+)-3-methyladipic diamide (178) by treatment with gaseous ammonia at low temperature. The diamide was reacted under Hofmann rearrangement conditions[154] with sodium hypobromite generated in situ to form R-(+)-2-methylputrescine (176). This diamine was isolated as the dibenzoyl derivative (179), and then obtained as a dihydrochloride salt by treatment with concentrated hydrochloric acid under sealed tube conditions at a high temperature and pressure (Scheme 41).

The Hofmann rearrangement is an intramolecular rearrangement which occurs with retention of configuration.[155] The reactive species is thought to be an isocyanate, which is hydrolysed to the primary amine (Scheme 42).

The same series of reactions used in the synthesis of (176), was used to convert racemic 3-methyladipic acid (180) into the racemic diamide (181), then into the dibenzoyl derivative (182), and eventually into the racemic 2-methylputrescine (183) dihydrochloride (Scheme 41). None of the reactions are low yielding, and the route proved synthetically useful for our purposes.

HOOC—CH₂CH₂—C(R¹R²)—CH₂—COOH

(177) R¹=CH₃, R²=H
(180) R¹,R²=CH₃,H

i. →

H₂NCO—CH₂CH₂—C(R¹R²)—CH₂—CONH₂

(178) R¹=CH₃, R²=H
(181) R¹,R²=CH₃,H

↓ ii.-iii.

H₂N—CH₂CH₂—C(R¹R²)—CH₂—NH₂

(176) R¹=CH₃, R²=H
(183) R¹,R²=CH₃,H

(both as their
dihydrochloride salts)

← iv.

C₆H₅C(O)—NH—CH₂CH₂—C(R¹R²)—CH₂—NC(O)C₆H₅

(179) R¹=CH₃, R²=H
(182) R¹,R²=CH₃,H
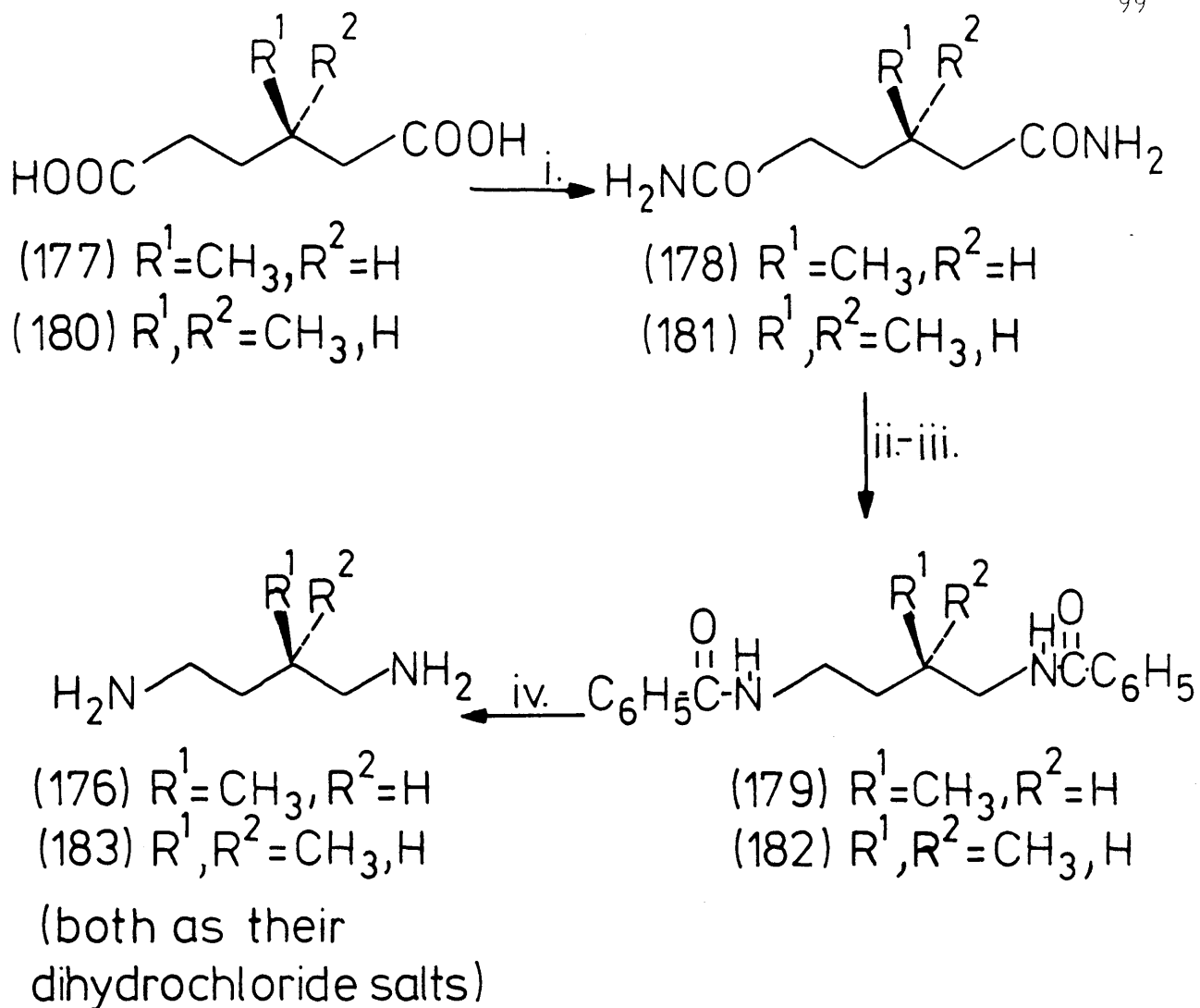
Scheme 41

Reagents: i. NH₃, C₂H₅OH, -40°C; ii. NaOH, Br₂, H₂O, ice, Δ;
iii. C₆H₅COCl, Δ; iv. c.HCl, Δ, pressure.

R—C(O)—NH₂ →[NaOBr]→ R—C(O)—NHBr →[OH⁻]→ R—N=C=O →[OH⁻]→ R—NH₂
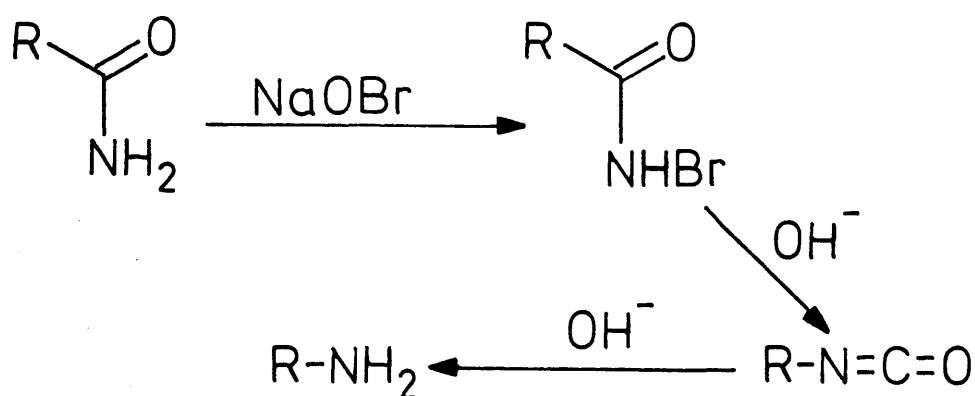
Scheme 42

After the successful syntheses of 2-methylputrescines (176) and (183), it was decided to introduce a radioactive ^{3}H label at some point with a view to the feeding of the radioactive 2-methylputrescines. Our initial attempts to exchange the protons were performed upon (±)-3-methyladipic acid (180). Reaction of acid (180) with strong base in deuterium oxide at reflux temperature produced no exchange as evidenced by ^{1}H n.m.r. spectroscopy. This method of analysis also showed the failure of concentrated sulphuric acid in deuterium oxide at reflux to effect exchange. The integrations of the ^{1}H n.m.r. signals for the hydrocarbon chain were unchanged after both these reactions. Efficient exchange was eventually brought about by heating the (±)-3-methyladipic acid (180) with deuterium oxide at high temperature and pressure under sealed tube reaction conditions (Scheme 43). This exchange was about 25% efficient as evidenced by integration of the ^{1}H n.m.r. signals of the acid (180). This mode of exchange was used for the successful introduction of a tritium label into both (176) and (180) to produce tritiated 3-methyladipic acids. These two radioactive acids were converted into two radioactive samples of 2-methylputrescines (173) and (180).

5.5.3   Feeding of 2-methylputrescines

Having obtained the desired radioactive putrescine analogues, it was decided to feed (176) and (183) to a range of plants which produce pyrrolizidine alkaloids. As mentioned earlier, Cynoglossum officinale and Senecio pleistocephalus produce (+)-echinatine (62) and (-)-rosmarinine (175) respectively. Emilia flammea Cass. (Compositae)

$$R \diagdown COOH \xrightarrow{\text{i.}} R \diagdown COOH$$
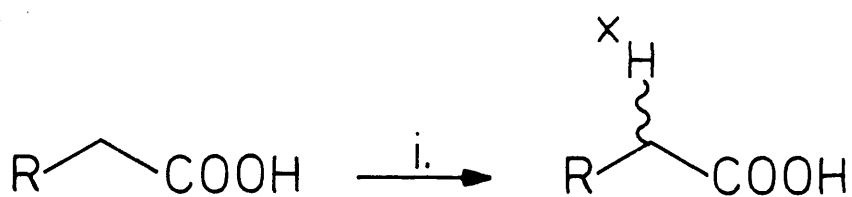
Scheme 43

Reagents: i. $^X H_2O$, Δ, pressure.

x = 2 or 3
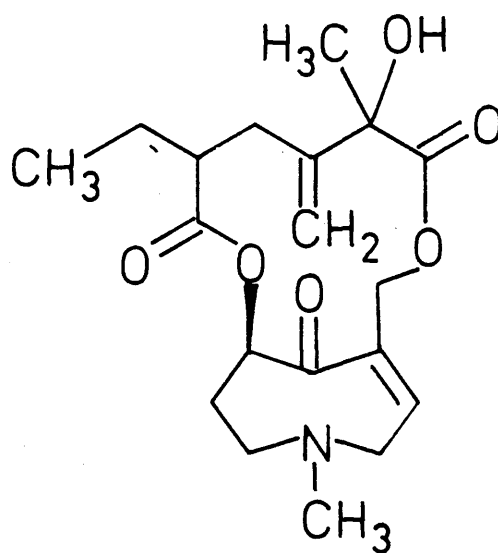
(184)

produces (-)-emiline (184),[156] and samples of all three plants were
available. A known activity of tritiated $\underline{R}$-(+)-2-methylputrescine
(176) dihydrochloride was mixed with a small quantity of [1,4-^{14}C]-
putrescine (166) dihydrochloride to produce a mixture with a ^{3}H:^{14}C
ratio measured by scintillation counting. The same procedure was
followed with (±)-2-methylputrescine (183) dihydrochloride and the
^{3}H:^{14}C ratio was measured.

The radioactive mixtures were fed separately in turn to
Cynoglossum officinale, Senecio pleistocephalus, and Emilia flammea by
the wick method to well-established plants. The compounds were fed
over two successive days, and the plants were allowed to grow for one
week and then harvested. The alkaloids were isolated by methanolic
extractions, and the fate of the radioactive labels was followed by
radioactive scintillation counting. The results are summarised in
Table 6. Encouragingly, both 2-methylputrescines were incorporated
into basic material in all three species about 1/4 to 2/5 as well as
ordinary putrescine. The two products isolated from Senecio
pleistocephalus both showed a radioactive band at R_f 0.30 when t.l.c.
radioscans were run. The incorporations of the samples produced by
Cynoglossum officinale and Emilia flammea were too low to be able to
detect any definite radioactive bands (Table 6). The radioactive bands
from both t.l.c.'s of the Senecio pleistocephalus feeds were scraped
off, extracted, and the ^{3}H:^{14}C ratios were measured by scintillation
counting. The ratios in both cases were close to the ratios in the
alkaloid extract, at 1.03 for the feeding of (176) and 3.39 for the use
of (183). The n.m.r. spectroscopic and t.l.c. data of all these

Alkaloid isolated	³H-methyl putrescine fed	³H:¹⁴C ratio		Specific Incorp. of	
		fed	isolated	³H	¹⁴C (%)
Echinatine (62)	(176)	3·78	1·06	0·26	0·93
	(183)	11·65	3·06	0·21	0·81
Rosmarinine (175)	(176)	3·99	1·15	0·70	2·40
	(183)	11·65	4·58	0·83	2·11
Emiline (184)	(176)	3·38	1·12	0·44	1·33
	(183)	8·92	2·18	0·34	1·40

Table 6

products showed no detectable differences from those of the natural

alkaloids each plant produces.

5.6   Synthesis and Feeding of Fluoroputrescines

5.6.1   Introduction

The use of fluorine to 'tag' a molecule throughout its

metabolism along a biosynthetic pathway has been expanded upon

earlier (Section 4.4).   The aims of this section of work were to

develop a short and efficient route to an analogue of putrescine with

fluorine present, and then to attempt to study the metabolism of this

fluorine containing molecule by plants which produce pyrrolizidine

alkaloids.

5.6.2   Introduction of fluorine

New reagents and improved techniques for the selective

introduction of fluorine have contributed to advances in the chemistry

of mental health, cancer chemotherapy, anti-inflammatory and anti-

parasitic agents, and antibiotics, e.g. fluoroprostaglandins.[157]   The

use of ^{18}F labelled biochemical tracers for medical purposes has

recently blossomed, and this isotope also has commercial applications

in fields such as agrochemicals and pharmaceuticals.

A wide variety of methods can be used to fluorinate

molecules.[158]   The cheapest source of fluorine is hydrogen fluoride,

but its use is restricted due to some unwelcome properties.

Elemental fluorine has become increasingly important as a selective

fluorinating agent.   The critical factor is to avoid radical fluorination.

This is achieved by the dilution of fluorine with an inert gas in the presence of a radical abstractor, with a suitable solvent at a low temperature. For example, trans-4-tertiary butyl cyclohexyl p-nitro-benzoate (185) can be fluorinated in a selective electrophilic manner at the remote tertiary carbon atom (Scheme 44).[159] Electrophilic fluorinations of this type appear to occur preferentially at the tertiary carbon atom, probably due to the high affinity of fluorine towards this high electron density region (C_4-H). Fluorinations with elemental fluorine in polar solvents, e.g. acetic acid, are also known, with the reactive species considered to be an organic hypofluorite (RCO_2F). Inorganic hypofluorites react particularly well with aromatic and unsaturated systems, e.g. OF_2, OHF, $CsSO_4F$.

Among the well-known fluorinating agents, sulphur tetra-fluoride and its derivatives are dominant. Sulphur tetrafluoride is selective and effective, and can work on a preparative scale or be catalytically further activated on addition of a Lewis acid. If we look at the fluorination of a carbonyl group, two alternative ideas arise. The Lewis acid can serve as a polarizing agent for the carbonyl and the nucleophilic attack of SF_4 is facilitated.[160] The effect may also be a mutual effect between the Lewis acid (LX_n) and SF_4, with SF_3^+ or $[F_3S^{\delta+}\cdots F\cdots {}^{\delta-}LX_n]$ as the fluorinating agent.[161] Foremost amongst the derivatives of SF_4 is N,N-diethylaminosulphur-trifluoride (DAST).[162] This is an extremely good fluorinating agent, as it is more convenient than SF_4, and the generally mild reactions proceed with lower temperatures and pressures, faster reaction times and higher yields. Glass vessels can be used, and rearranged

(185)

$$R = CO\text{—}\langle \rangle\text{—}NO_2$$

Scheme 44

(186)
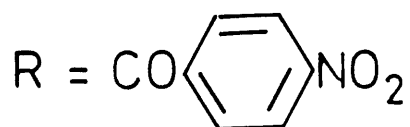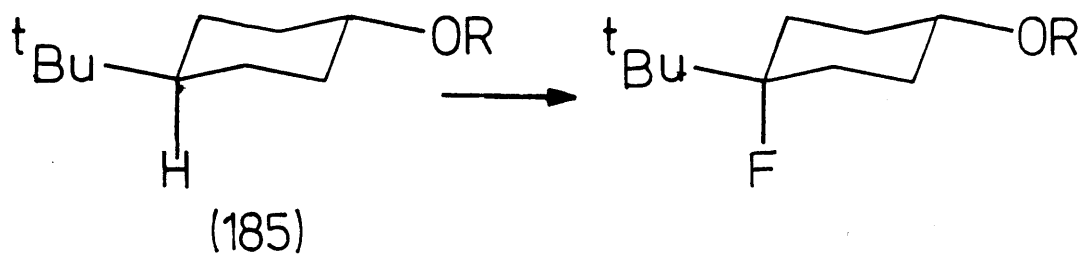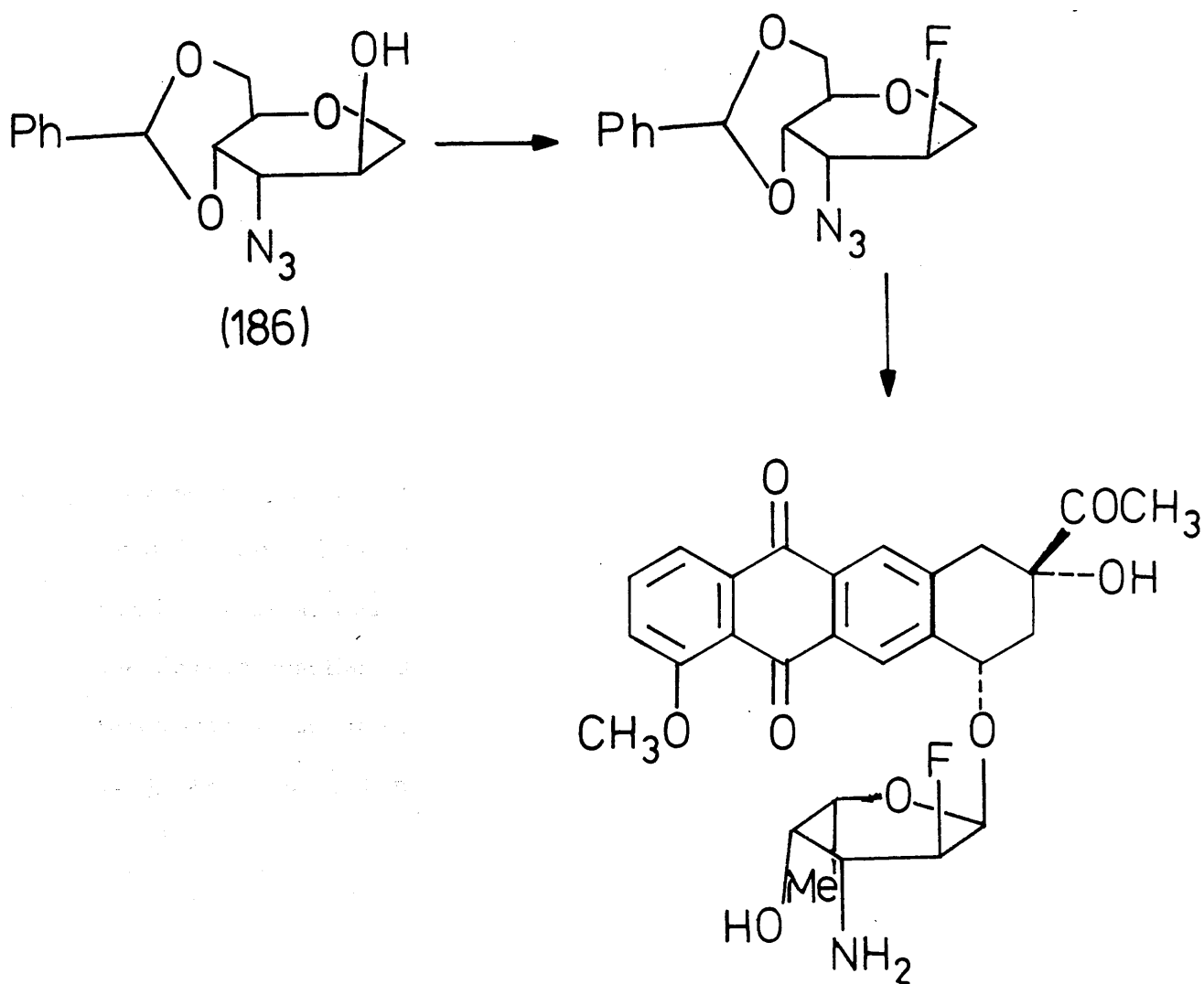
Scheme 45

products, dehydration and other serious side reactions are generally not encountered.[163] For example, DAST replaces the free hydroxyl in the carbohydrate derivative (186) with retention of configuration (Scheme 45).[164] The reagent can also replace a carbonyl oxygen with two fluorine atoms.[162] The yields with DAST are generally higher than those resulting from use of Ishikawa's reagent (187).[165] Other nitrogen-containing fluorinating agents are available, e.g. N-fluoro-2-pyridone (188).

The most mentioned inorganic fluorinating agent in recent years is xenon difluoride. This reagent has diverse effects, e.g. it can replace a carboxylic acid grouping with a fluorine atom,[166] or add across a double bond[167] (Scheme 46).

5.6.3   Synthesis of 2-fluoroputrescine

A recent synthesis of 2-fluoroputrescine (189) proceeded in a very low overall yield, but made use of DAST in the fluorination step (Scheme 47).[168] The production of 2-fluoro-1,4-dibromobutane from 2-hydroxy-1,4-dibromobutane, was followed by conversion of this fluorobutane into the corresponding diphthalimide. The protected putrescine was resistant to hydrolysis, and eventually deprotection was brought about only under extremely forcing conditions in a very low yield. A great deal of starting material was still present in spite of the drastic reaction conditions. A study of alternative routes only led to decomposition of the fluoro-compounds. The tetrafluoroputrescine (190) was also synthesised (Scheme (48).[168]

R_2N—C(F)=C(F)—CF_3 / $R_2NCF_2CHFCF_3$

(187)

(188)

$$R\text{-COOH} \xrightarrow[\text{CH}_2\text{Cl}_2,\,20^\circ\text{C}]{\text{XeF}_2 : \text{HF} (1:1)} R\text{-COOXeF}$$

$$RF \longleftarrow R^\bullet \longleftarrow R\text{-COO}^\bullet$$

Scheme 46a

$$\xrightarrow[\text{CH}_3\text{CN}]{\text{XeF}_2,\, h\nu}$$
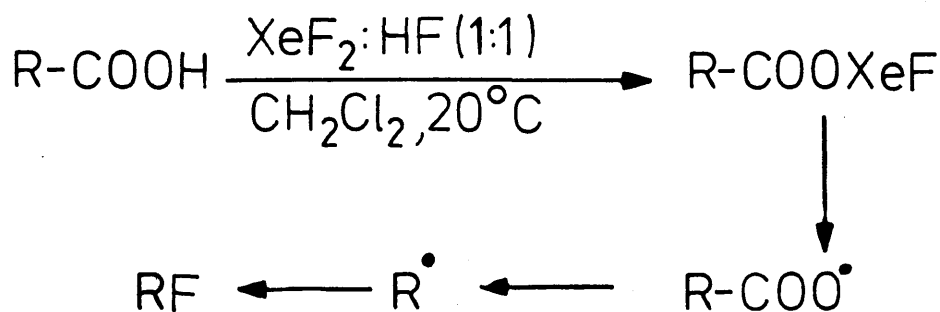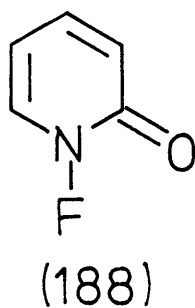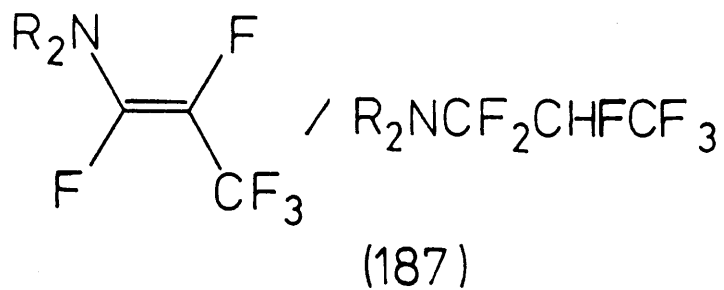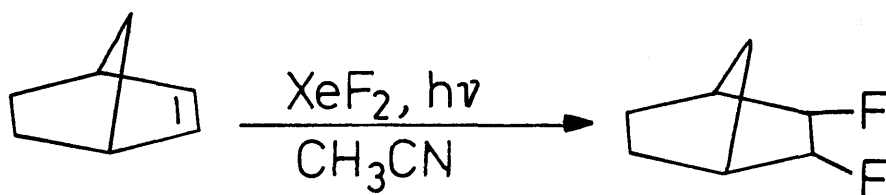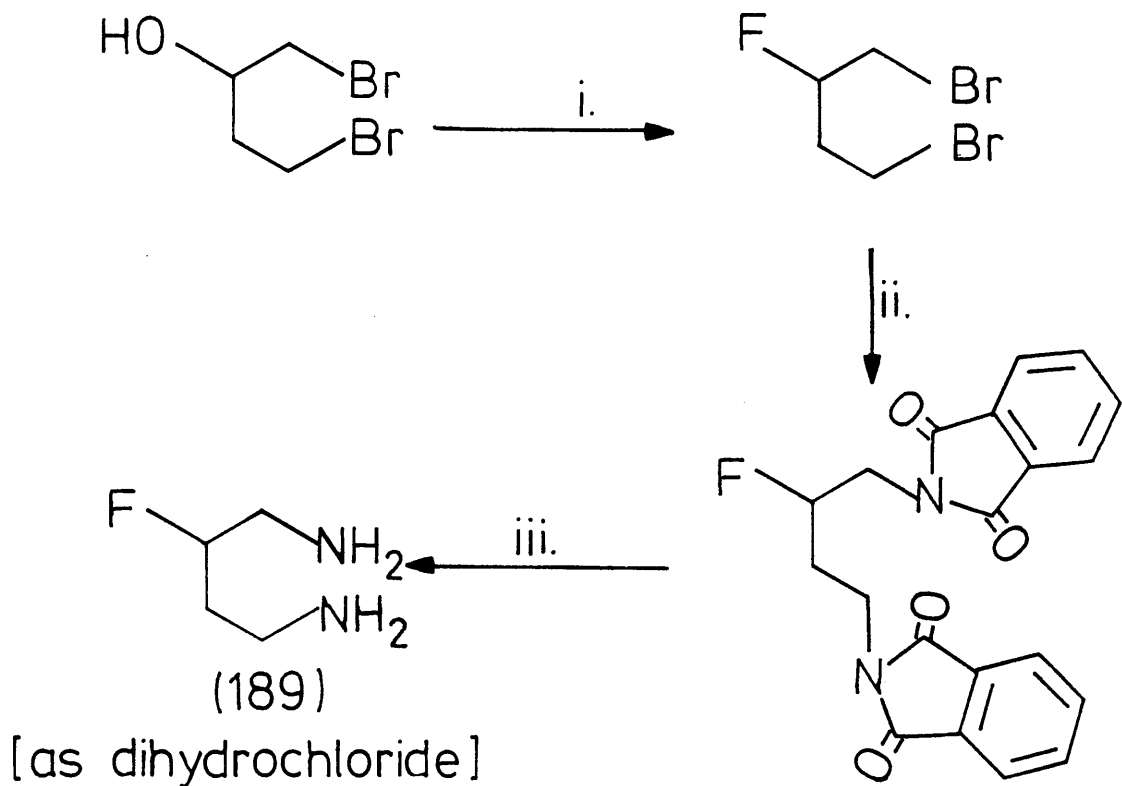
Scheme 46b

Scheme 47

Reagents: i. DAST, CH_2Cl_2, $-78°C$; ii. KN(phthalimide), DMF, 12h, 100°C;

iii. c.HCl, 72h, 120°C.

$$H_2NCO(CF_2)_2CONH_2 \xrightarrow{\ i.\ } H_2NCH_2CF_2CF_2CH_2NH_2$$

(190)
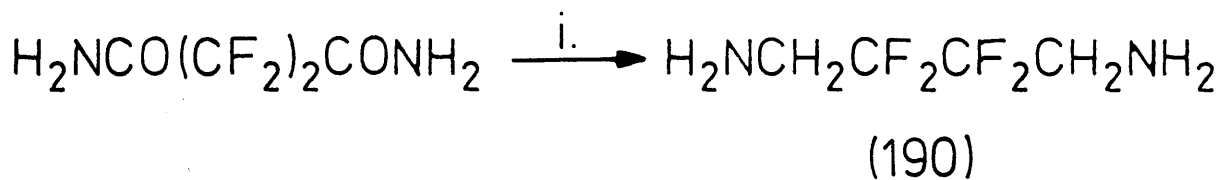
Scheme 48

Reagents: i. BH_3, THF, 3h, 70°C.

For our synthesis of racemic 2-fluoroputrescine (189), we chose readily available racemic malic acid. The acid was converted quantitatively into diethylmalate (191).[169] Fluorination with DAST gave one clean product, diethyl 2-fluorosuccinate (192), which was distilled under high vacuum (Scheme 49). The 90 MHz ^{1}H n.m.r. spectrum of this diester (Figure 9) shows the large geminal J_{HCF} coupling of 47.2 Hz. A coupling of this magnitude is characteristic of the CHF group and appears throughout the analysis of the 2-fluorocompounds. Diester (192) was reduced with diisobutyl aluminium hydride (DIBAL) or lithium aluminium hydride to provide samples of 2-fluoro-1,4-butanediol (193). This diol was converted into 2-fluoroputrescine (189) by treatment with a solution of hydrazoic acid in benzene, diethylazodicarboxylate, and excess triphenylphosphine in tetrahydrofuran.[170] The dihydrochloride salt of (189) was isolated by the addition of aqueous hydrochloric acid. The alcohol was converted into an azide by the Mitsunobu reaction,[171] and an in situ Staudinger reaction[172] of the azide with triphenylphosphine produced an iminophosphorane intermediate. Hydrolysis was effected by the treatment with acid.

The overall yields of all the reactions were good and very little purification of the products was required.

5.6.4   Investigations into the stereoselectivity of the DAST reaction

In the vast majority of the cases where the DAST reaction was used, the replacement of hydroxyl by fluorine generally proceeded with retention of configuration.[158]

HO$_2$C—CH(OH)—CH$_2$—CO$_2$H

$\xrightarrow{\text{i}}$

C$_2$H$_5$O$_2$C—CH(OH)—CH$_2$—CO$_2$C$_2$H$_5$

(191)

↓ ii.

C$_2$H$_5$O$_2$C—CHF—CH$_2$—CO$_2$C$_2$H$_5$

(192)

$\xleftarrow{\text{iii.}}$

HOH$_2$C—CHF—CH$_2$—CH$_2$OH

(193)

↓ iv.–v.

H$_2$N—CH$_2$—CHF—CH$_2$—CH$_2$—NH$_2$

(189)
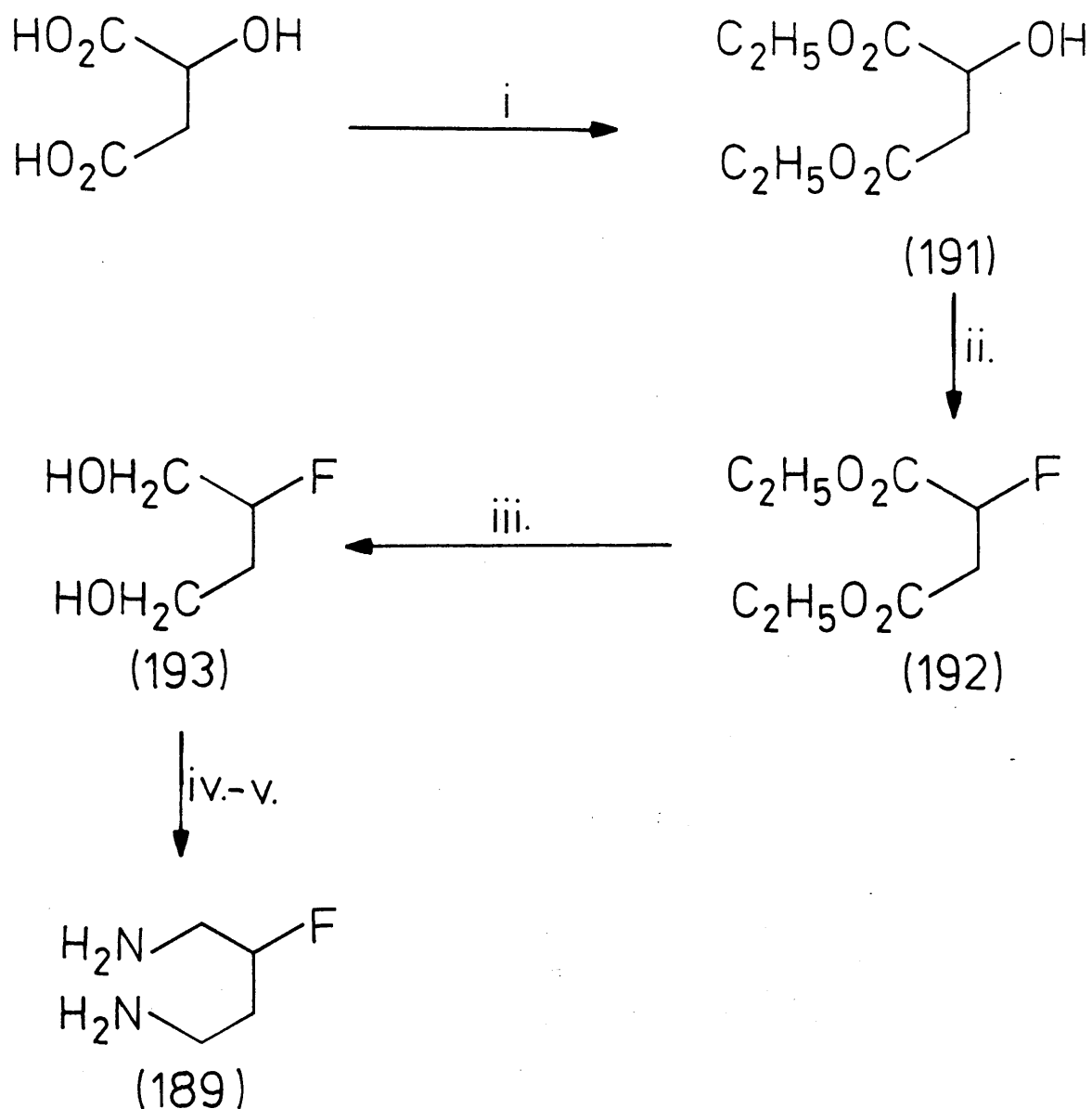
Scheme 49

Reagents: i. C$_2$H$_5$OH, C$_6$H$_5$CH$_3$, c.HCl, Δ; ii. DAST, CH$_2$Cl$_2$, −70°C; iii. DIBAL, C$_6$H$_5$CH$_3$, −40°C or LiAlH$_4$, Et$_2$O, 0°C; iv. HN$_3$, C$_6$H$_6$, PPh$_3$, EtO$_2$C-N=N-CO$_2$Et, Δ; v. HCl (aq.), Δ.

$C_2H_5O_2C$ — — F

$C_2H_5O_2C$

(192)

x

y

1 p.p.m. = 90Hz

2Hz = 1mm

0 p.p.m.
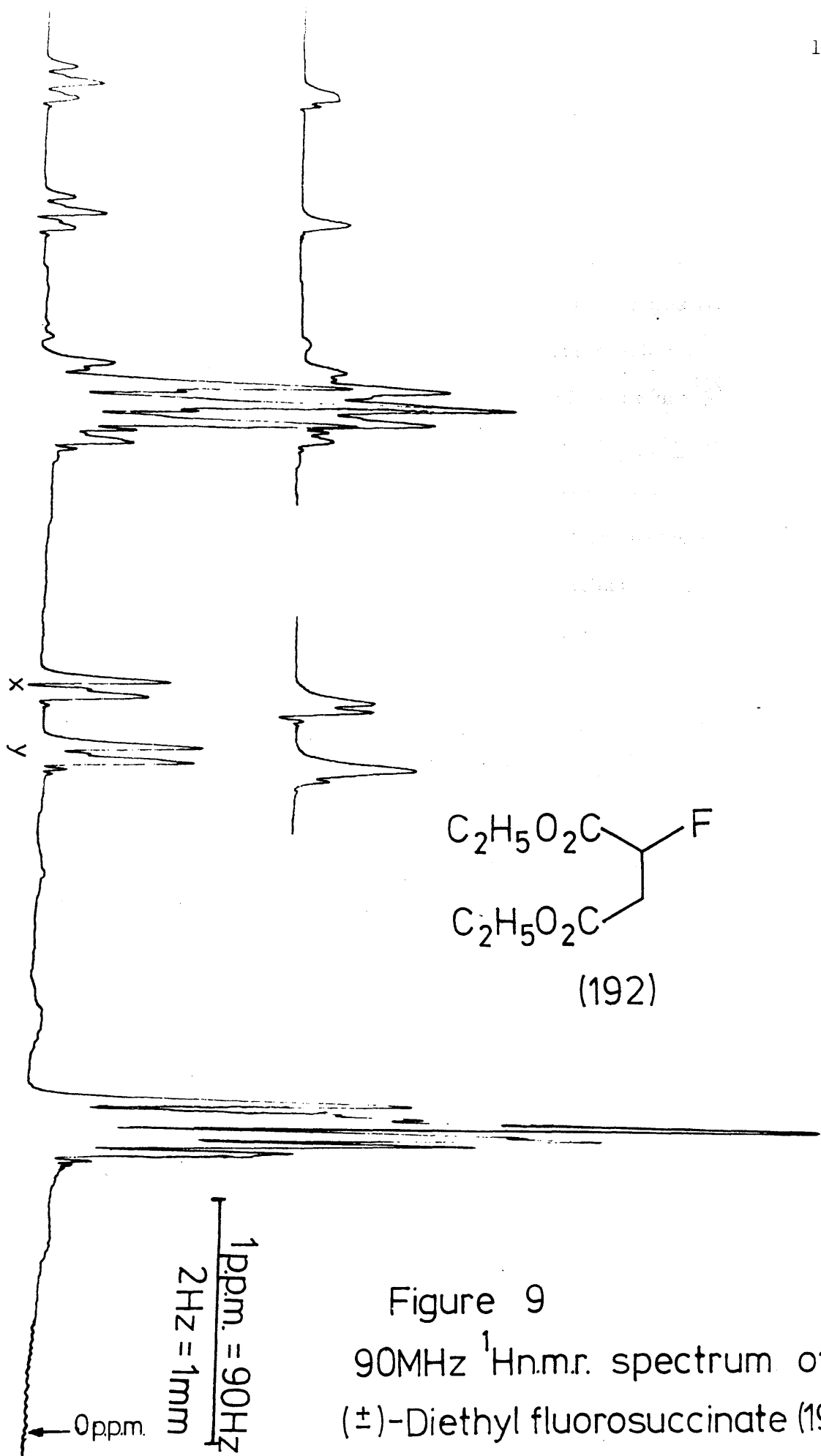
Figure 9

90MHz ^{1}Hn.m.r. spectrum of

(±)-Diethyl fluorosuccinate (192)

If the conversion of diethyl malate into diethyl fluorosuccinate occurred with retention of configuration, then the route detailed above could be used to produce two enantiomeric 2-fluoroputrescines by starting with the readily available optically active malic acids. Accordingly, $\underline{R}$-diethyl malate (194) was synthesised as before[169] and converted into the diethylfluorosuccinate (195). Similarly, $\underline{S}$-diethyl malate (196) was converted into diethylfluorosuccinate (197).

Chemists have long appreciated that a chiral environment might dissimilarly perturb the properties of enantiomeric molecules.[173] We decided to use a chiral solvating agent (CSA) to perturb the diethyl fluorosuccinates (195) and (197), and to view the differing extents of perturbation from the ^{1}H n.m.r. spectra. The CSA decided upon was $\underline{R}$-(+)-phenylethylamine (198). Nearly all CSAs contain a group of high diamagnetic anisotropy near the asymmetric centre.

The liquid CSA was added to all three diethyl fluorosuccinates [(192), (195) and (197)], along with a few drops of $CDCl_3$ containing 2% tetramethylsilane as internal standard, and the 90 MHz ^{1}H n.m.r. spectra were recorded (Figure 10a.-c.). There are easily seen characteristics of CSA-induced non-equivalence for the 2-H and 3-H_2 of the diesters. The doublet of doublets due to 3-H_2 is shifted in all three cases, but in the two 'enantiomers', (195) and (197), the signals are shifted to different degrees. In the case of (195)/(198), the left hand portion ($\underline{x}$) of the signal is shifted upfield by 32 Hz (Figure 10b), and the upfield shift in the case of (197)/(198) is 34 Hz (Figure 10c). The midpoint of the right hand doublet ($\underline{y}$) is shifted by 30 Hz for pairing (195)/(198) (Figure 10b), and by 42 Hz in the case of (197)/
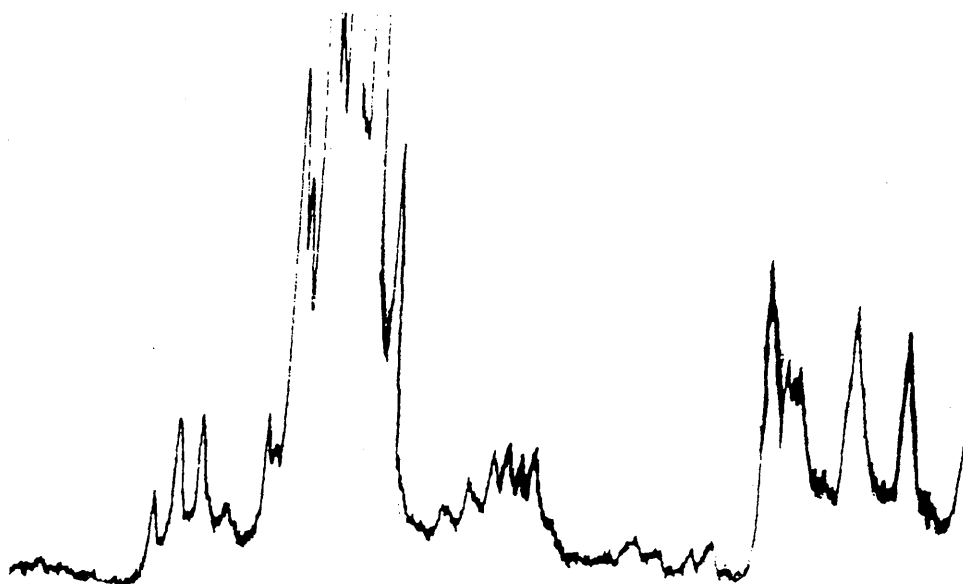
Figure 10a. (192) / (198)
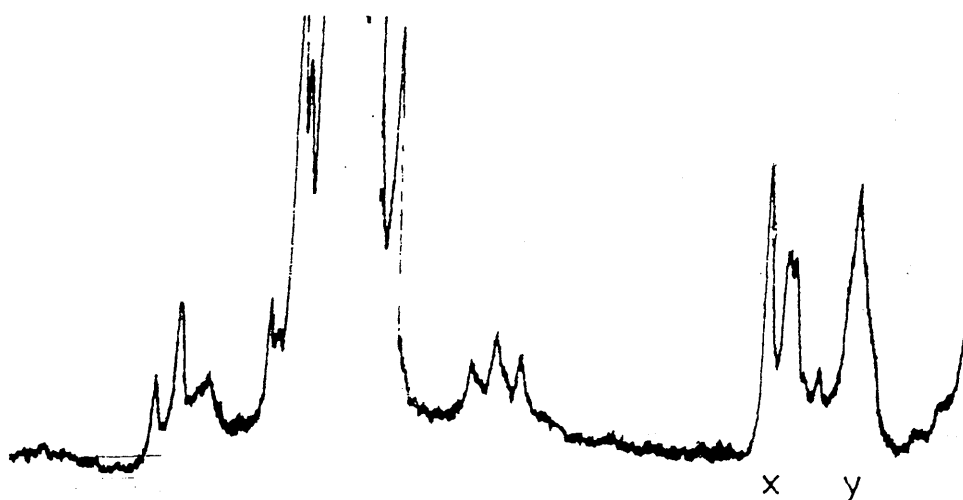
Figure 10b. (195) / (198)

Figure 10c. (197) / (198)

Chiral solvating agent (CSA) induced non-equivalence in the 90 MHz
[1]H n.m.r. spectra of the diethylfluorosuccinates [(192),(195),(197)]
with R-(+)-phenylethylamine (198).

(198) (Figure 10c). The ^{1}H n.m.r. spectrum of the racemate (192) with (198) (Figure 10a) is almost identical to the combined spectra of the two esters with CSA. Upfield shifts are seen for the respective H-2 signals, when compared to the ^{1}H n.m.r. spectrum of (192)[173] (Figure 9).

From this evidence, the reaction of the samples of diethyl malate [(191), (194) and (196)] with DAST seems to be very stereo-selective. The two 'enantiomers', (195) and (197), gave optical activity measurements which are roughly equal in magnitude, but opposite in sign. If the reaction of diethyl malate with DAST has occurred with retention,[158] then the R-configuration can be tentatively assigned to (195) and the S-configuration to (197). These two diethyl fluorosuccinates, (195) and (197), were reduced as for the racemate (192), to diols (199) and (200) respectively, which were subsequently converted in turn into the 2-fluoroputrescines, (201) and (202) (Scheme 50). Both pairs of compounds gave approximately equal and opposite rotations, and all samples gave correct analytical data. A portion of both 'enantiomeric' diethylfluorosuccinates were hydrolysed[174] to produce two fluorosuccinic acids, (128) obtained from diester (195) and diacid (203) from diester (197).

A point of note is the instability of many of these fluorinated molecules. Upon standing, they are prone to decomposition into unfluorinated molecules. An illustration of this was seen in the 94 and 188 MHz ^{19}F n.m.r. spectra. After some samples had stood for a while, very little fluorine was left in the product. The samples of fluoro-succinic acid, (128) and (203), were submitted for circular dichroism

$C_2H_5O_2C$—R^1—R^2
$C_2H_5O_2C$

$\longrightarrow$

$C_2H_5O_2C$—R^1—R^2
$C_2H_5O_2C$

(194) $R^1=H, R^2=OH$ (195) $R^1=H, R^2=F$

(196) $R^1=OH, R^2=H$ (197) $R^1=F, R^2=H$

H_2NH_2C—R^1—R^2
H_2NH_2C

$\longleftarrow$

HOH_2C—R^1—R^2
HOH_2C

(201) $R^1=H, R^2=F$ (199) $R^1=H, R^2=F$

(202) $R^1=F, R^2=H$ (200) $R^1=F, R^2=H$

Scheme 50

CH_3—NH_2—H
C_6H_5

(198)

HO_2C—R^1—R^2
HO_2C

(128) $R^1=H, R^2=F$

(203) $R^1=F, R^2=H$
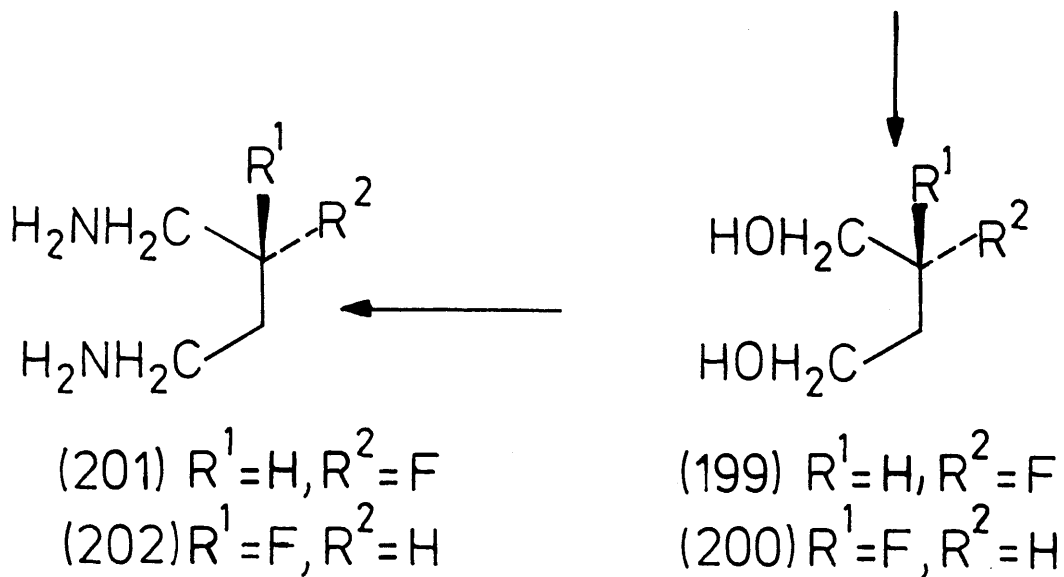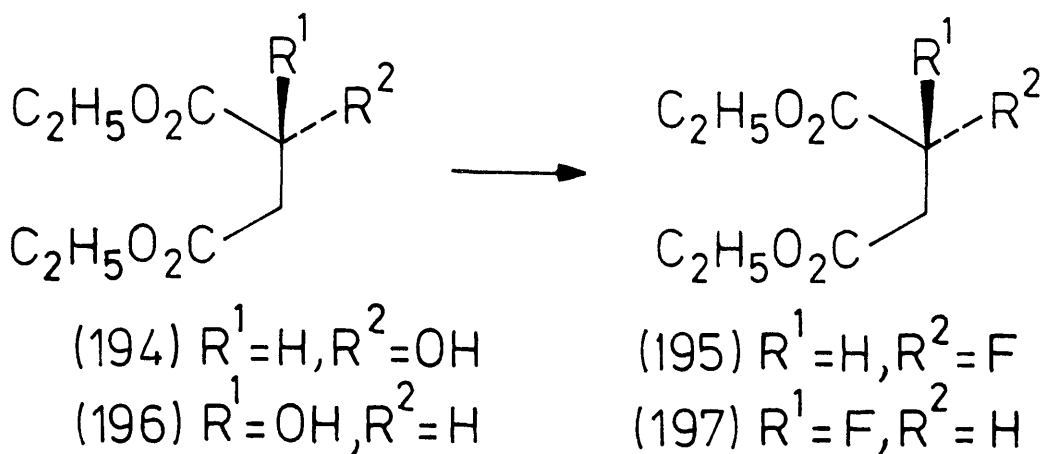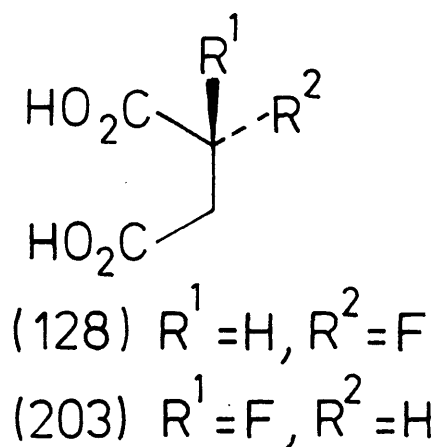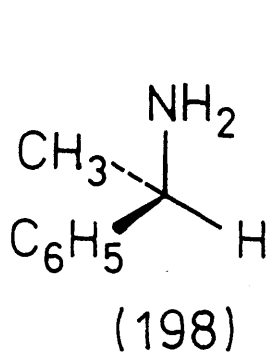
(c.d.) spectra, but the curves were unsatisfactory and inconclusive due to the presence of a chemical impurity. In order to obtain the vital missing data (^{19}F n.m.r. spectra and absolute stereochemistry), fresh samples of the compounds need to be prepared. Absorption c.d. data on the fluorosuccinic acids can hopefully be related to those of the chlorosuccinic acids.[132] Another possibility may be to use vibrational infra red circular dichroism (i.r.c.d.), with the samples of diethyl fluorosuccinate, which appear to be relatively stable. The i.r. c.d. technique was recently used to investigate the stereochemistry of a biological process.[175]

Once the successful synthesis of the desired compounds was complete, a range of feeding experiments was attempted.

5.6.5 Feeding of 2-fluoroputrescines

As with the 2-methylputrescines (Section 5.5), samples of the two 2-fluoroputrescines (201) and (202), were pulse-fed as their dihydrochloride salts by the wick method to Cynoglossum officinale, Senecio pleistocephalus, and Emilia flammea. They were fed as a sterile aqueous solution over 2 days. The plants were allowed to grow for a further week and then harvested, extracted, and the alkaloids were isolated as previously described. The plants appeared to suffer some damage as a result of feeding, particularly around the area of feeding on the stems.

The detection of any incorporation of fluorine into the alkaloids was attempted by 188 MHz ^{19}F n.m.r. The spectra of rosmarinine (175) and emiline (184) fed these fluoroputrescines showed very little

evidence of any significant incorporations. Signals at $\underline{ca}$. δ_F -186

p.p.m. were obtained as expected due to CH$\underline{F}$ bonds, but they were

far too weak to be taken as a definite result.

The fluoroputrescines were fed roughly 24h after final

crystallisation and may have decomposed to a degree in this short time.

The amounts fed were relatively small ($\sim$ 100 mg) as there was the

danger of toxicity if the solution was too concentrated. The amount

fed should probably be increased, and the pulse-feeding continued for

longer than 2 days. The synthesis of molecules of 2-fluoroputrescine

which contain a radioactive label (e.g. ^{3}H, ^{14}C, or ^{18}F) is needed in

order to measure the incorporation of the compound more easily.

Hopefully these modifications would result in a measured definite

incorporation of fluorine.

5.7   Further Work

On the basis of the work reported, further experiments can be

devised.

The synthesis and feeding of 2-methylputrescines with a ^{13}C

label might enable the analogues formed to be seen in the ^{13}C n.m.r.

spectra of the alkaloid extracts. In addition, the synthesis of 2-fluoro-

putrescines with a radioactive ^{18}F label present, would enable the

incorporation to be measured simply by scintillation counting.

The use of deuteriated putrescines upon Cynoglossum officinale

by feeding intact plants seems to be fruitless, and another plant

species should be sought out as a good source of (+)-heliotridine (3).

Different feeding methods might also be attempted. The feeding of

later intermediates, such as isoretronecanol (22), is a worthwhile area
of biosynthetic research to pursue further.   Labelled analogues of
later intermediates in the biosynthetic pathway could also be prepared
and fed to plants that produce pyrrolizidine alkaloids.   The later
intermediates are generally incorporated better than molecules such as
putrescine.

The use of h.p l.c. as a method for the rapid investigation
and separation of alkaloids and their analogues is also worthy of more
study.

CHAPTER SIX

INVESTIGATIONS INTO THE PYRROLIZIDINE ALKALOID CONTENT
OF PLANTS

6.1   Introduction

        Pyrrolizidine alkaloids are found in a variety of plant species
which are widely distributed throughout the world (Section 1.2).
The alkaloids have also been identified in a variety of human food
sources.   The difficulty in studying pyrrolizidine alkaloids has often
been the isolation of the alkaloids as a complex mixture of similar
compounds.   Various methods of separation have been used, and this
section will describe the use of high pressure liquid chromatography
(h.p.l.c.) to isolate pure alkaloids from a complex mixture.

        The first h.p.l.c. method to separate successfully pyrrolizi-
dine alkaloids was used for the isolation of retrorsine (68), seneciphyl-
line (204), and senecionine (205) from Senecio vulgaris (common
groundsel).[176]   This procedure used a THF/0.01 M ammonium carbonate
(pH 7.8) solvent system, and was also applied to Senecio longilobus
(threadleaf groundsel)[177] and Senecio jacobaea (tansy ragwort).[178]
Senecio longilobus contains the same macrocyclic diesters as Senecio
vulgaris plus an additional alkaloid, riddelline (69).   A quantitative
h.p.l.c. method was used to separate symphytine-N-oxide (206) and
echimidine-N-oxide (207) from Symphytum radix.[179]   Tittel el al. used
a methanol/water solvent system for elution of the N-oxides and monitored
their u.v. absorption at 220 nm.   The mixture of two N-oxides was

(68) $R^1 = H, R^2 = CH_3, R^3 = CH_2OH$

(69) $R^1, R^2 = CH_2, R^3 = CH_2OH$

(204) $R^1, R^2 = CH_2, R^3 = CH_3$

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

(206) $R^1 = (E)-COCMe=CHMe,$

$R^2 = CO$--$\overset{^iPr}{\underset{HO}{|}}$--$\overset{H}{\underset{OH}{|}}$--$Me$

(207) $R^1 = (Z)-COCMe=CHMe,$

$R^2 = COC(OH)(CMe_2OH)CHOHCH_3$
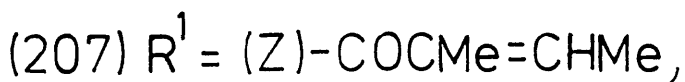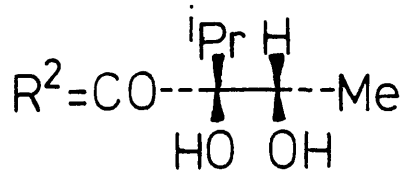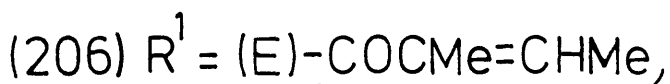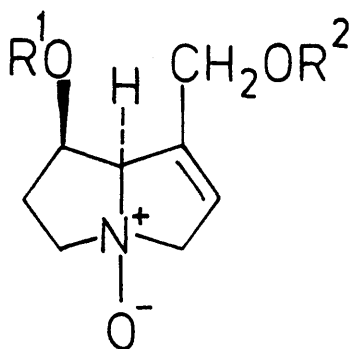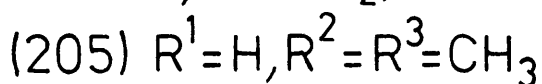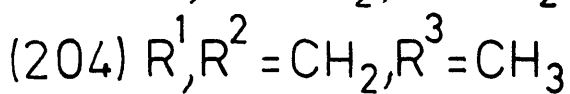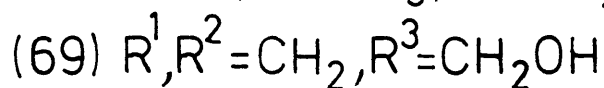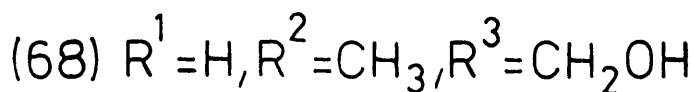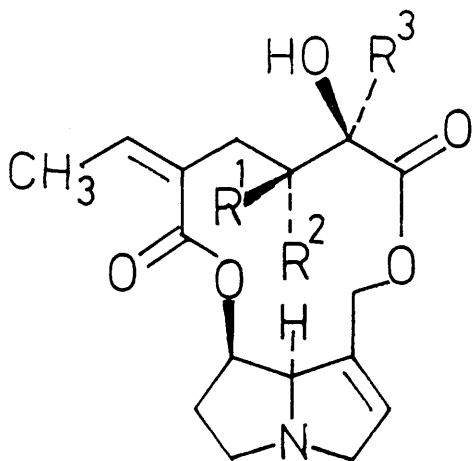
reduced and the alkaloids were separated with dichloromethane/propanol

as the solvent system with monitoring at 238 nm. An h.p.l.c. reversed

phase system was used to separate macrocyclic pyrrolizidine alkaloids

containing otonecine (208) from Petasites japonicus.[180] The previous

methods were found to be not applicable to this mixture but elution of

the column with methanol/0.02 M ammonium carbonate (pH 8.2) provided

samples of pure otosenine (209), petasitenine (210), neopetasitenine (211),

and senkirkine (212). The advantages of the h.p.l.c. method of

chromatography are the low solvent costs, the speed of separation and

the fact that no derivatives have to be made. Many of the pyrrolizidine

alkaloids were initially separated using time-consuming silica gel and

alumina columns in conjunction with t.l.c. techniques, and this method is

obviously still applicable to the separation of pyrrolizidine alkaloids.

H.p.l.c. has been used to isolate two dihydropyrrolizidine alkaloid

metabolites from an in vitro mouse hepatic microsomal mix.[181] This is a

good example of the extension of this separation method to biological

problems. The newer techniques of isolation and purification have

encouraged the increased activity in the search for new alkaloids. With

this objective in mind, we used an h.p.l.c. technique upon the alkaloid

mixtures isolated from Cynoglossum officinale and Cynoglossum australe,

and applied the experience to the separation of the alkaloid extracts of

Cynoglossum nervosum and Senecio glaberrimus.
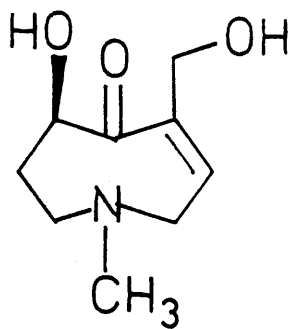
(208)
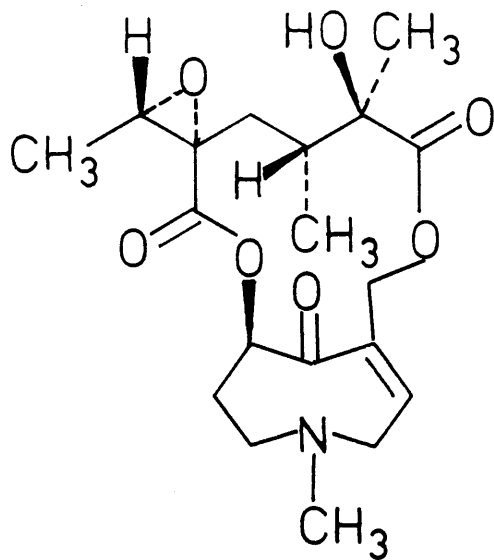
(209)
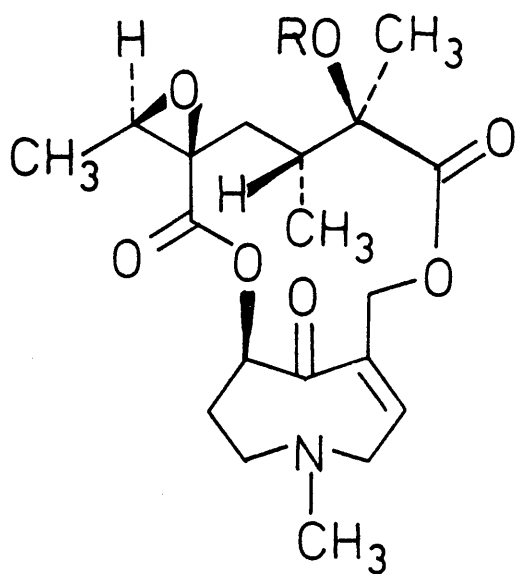
(210) R=H

(211) R=COCH₃
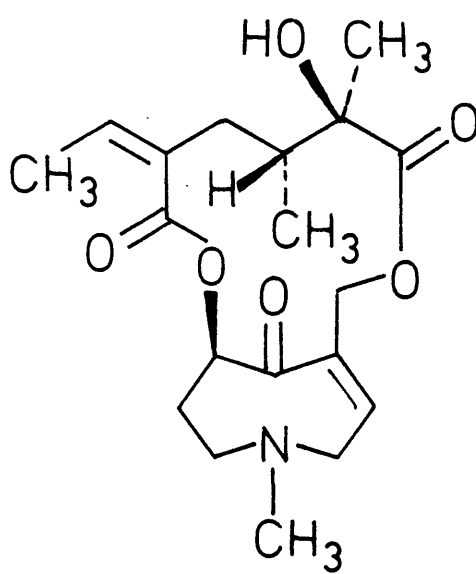
(212)

6.2   Analytical H.P.L.C.

The first plant species we decided to apply h.p.l.c. techniques to was Cynoglossum officinale.   This was known to contain mainly (+)-echinatine (62) (Section 3.2), but the samples isolated from the plants always contained a minor pyrrolizidine alkaloid which could never be isolated pure by column chromatography.   When an aliquot of the alkaloid mixture was applied to a Perkin Elmer 3 x 3 silica column and eluted with the solvent system,[182] diethylether: [5% v./v. (2.5% NH_3/ H_2O) in methanol] (75:25), an analytical h.p.l.c. trace (Figure 11) was obtained.   The main peak is (+)-echinatine, retention time (R_T, min) 3.78 (92.9%).   Other peaks were evident at R_T 5.78 (3.6%) and 8.02 (3.5%).   The latter was assigned to (+)-heliotridine (3) by comparison with an authentic sample, and the peak with R_T 5.78 was assigned to the minor alkaloid.   The initial peaks (R_T < 2.00) are due to the solvents in the injection of the samples.   When a t.l.c. of the alkaloid mixture was run, (+)-echinatine and the minor alkaloid had R_f values of 0.30 and 0.28 respectively.   Therefore, a mixture which was very difficult to separate by traditional column chromatography, was rapidly separated by h.p.l.c.

When a sample of the alkaloid mixture isolated from Cynoglossum australe was applied to the h.p.l.c. column and eluted as before, the analytical h.p.l.c. trace (Figure 12) again showed evidence of a good separation, although the peaks were broader than before.   The R_T values were 7.71 (71.4%) and 4.21 (28.6%), which were assigned to (+)-cynaustraline (172) and (+)-cynaustine (173) respectively.   This
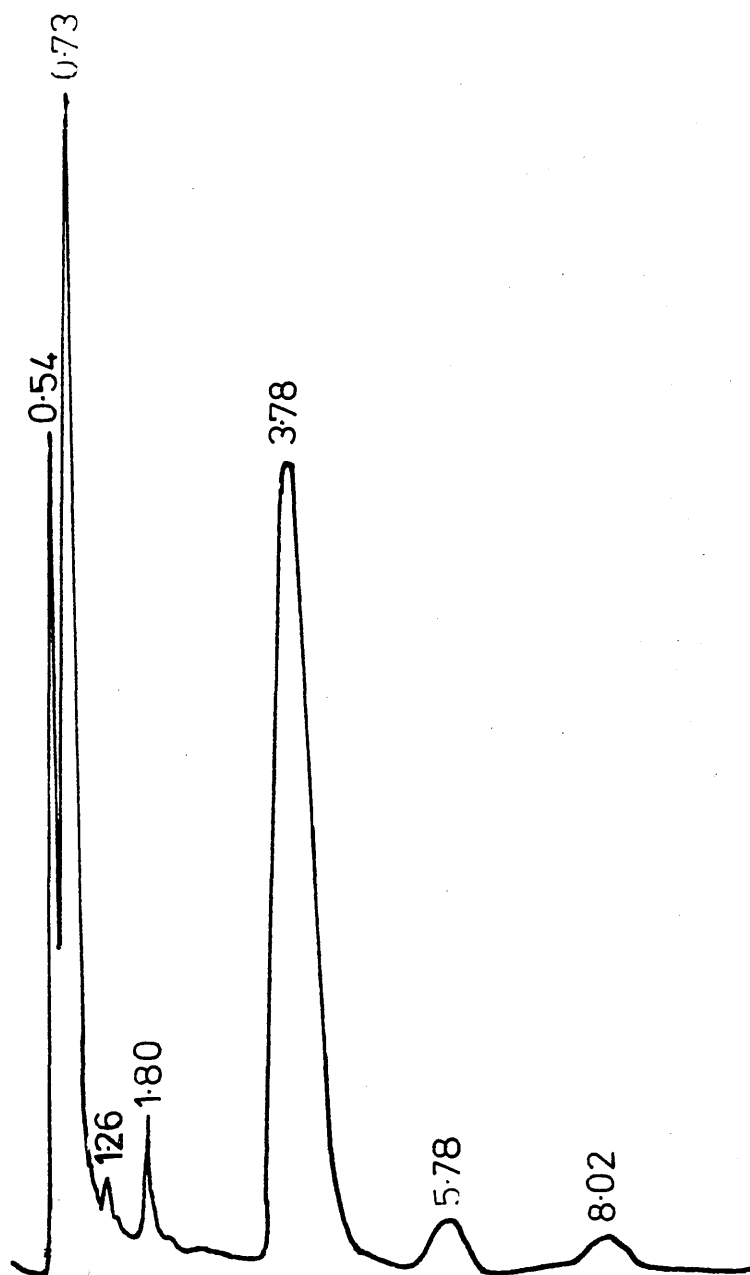
Figure 11

Analytical h.p.l.c. trace of the alkaloid mixture

isolated from Cynoglossum officinale
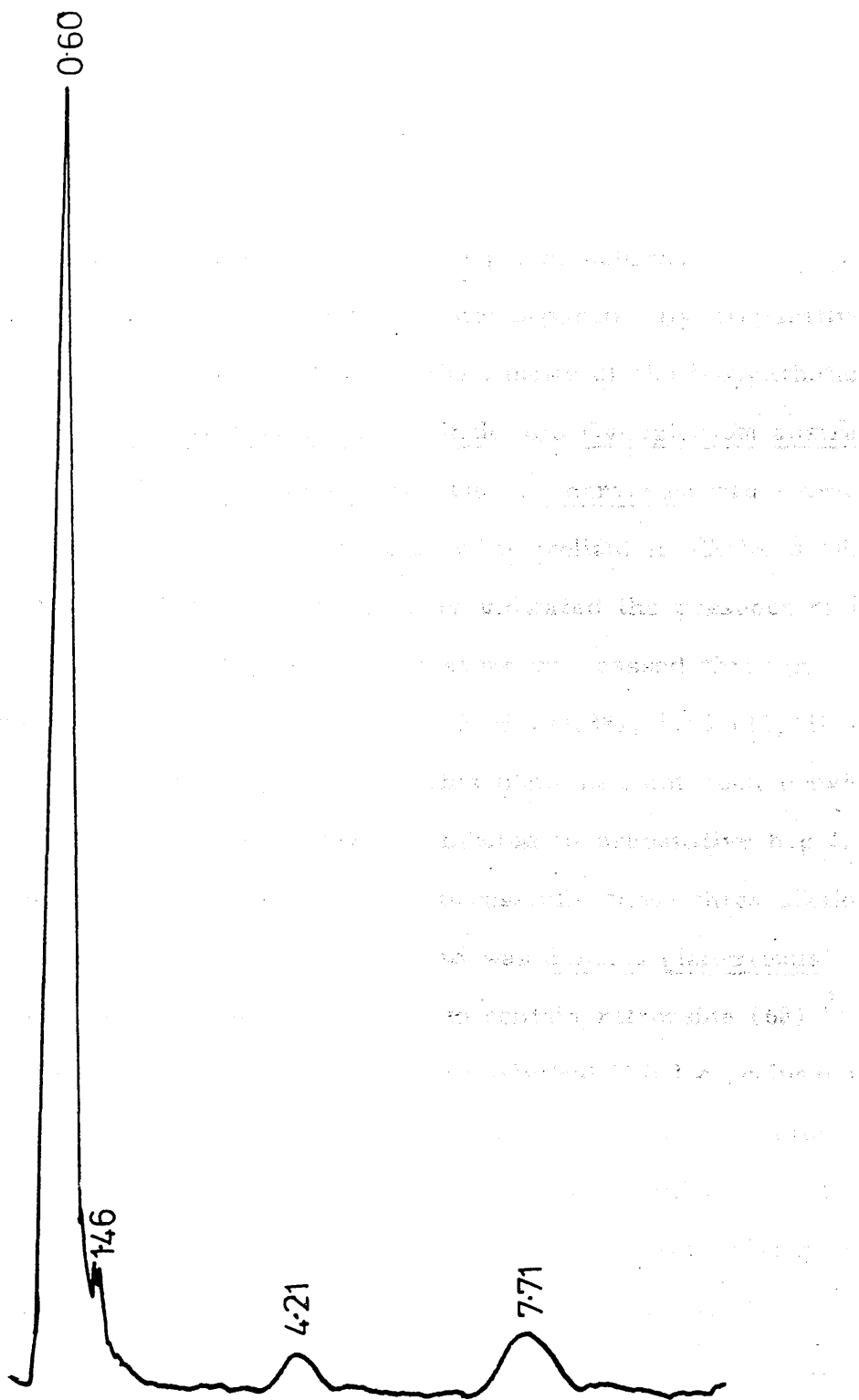
Figure 12

Analytical h.p.l.c. trace of the alkaloid mixture isolated

from Cynoglossum australe

mixture was extremely difficult to separate by column chromatography on

basic alumina, but was easier on an h.p.l.c. column.

The alkaloid samples were not separated by preparative h.p.l.c.,

but this may be a way to improve the results of the biosynthetic feeding

experiments upon Cynoglossum officinale and Cynoglossum australe.

Another Cynoglossum species, C. nervosum was extracted in

the usual manner to yield a mixture of pyrrolizidine alkaloids (R_f 0.30

and 0.26). Although the t.l.c. only indicated the presence of two

alkaloids, when an aliquot of the mixture was passed through the h.p.l.c.

separation system, three peaks (R_T 3.98 (58.3%), 5.03 (15.2%) and 6.36

(26.5%)) were seen (Figure 13). This plant had not been previously

investigated and the extract was subjected to preparative h.p.l.c.

analysis (Section 6.3) to separate successfully these three alkaloids.

The final plant investigated was Senecio glaberrimus.

Previously, this species was shown to contain retrorsine (68).[2,183]

When this extract was applied to the analytical h.p.l.c. column and

eluted with diethylether: [5% v./v. (2.5% NH_3/H_2O) in methanol],

(75:25), an h.p.l.c. trace with 4 peaks of very similar R_T values

resulted (Figure 14a). It was decided to reduce the polarity of the

eluting solvent and the relative proportions were changed to diethylether:

[5% v./v. (2.5% NH_3/H_2O) in methanol], (90:10). The h.p.l.c. trace

(Figure 14b) was then more evenly spread with R_T values of 2.71, 4.34,

5.47, and 6.84. When a pure sample of retrorsine was applied to the

column, it was observed at R_T 2.74. This suggests that the most polar

alkaloid from Senecio glaberrimus on the h.p.l.c. system is retrorsine,

but a preparative h.p.l.c. column would need to be run to obtain pure
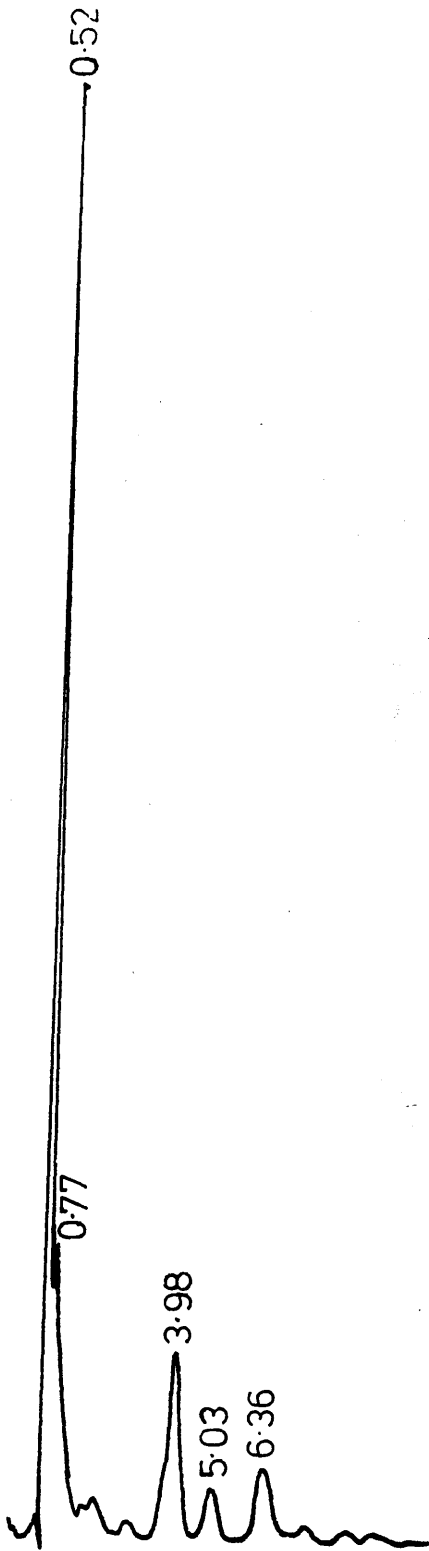
Figure 13

Analytical h.p.l.c. trace of the alkaloid

mixture isolated from Cynoglossum nervosum
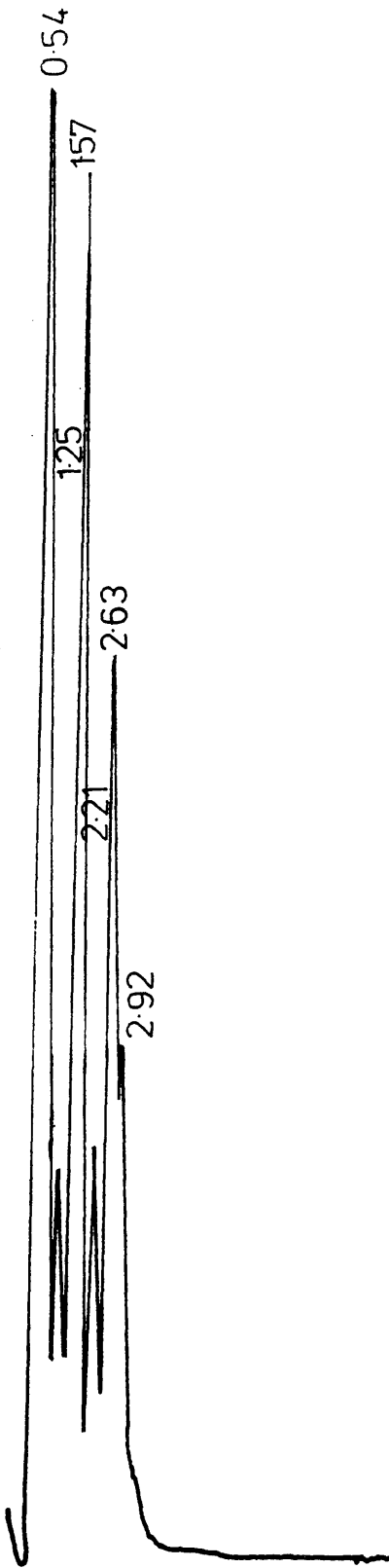
Figure 14a
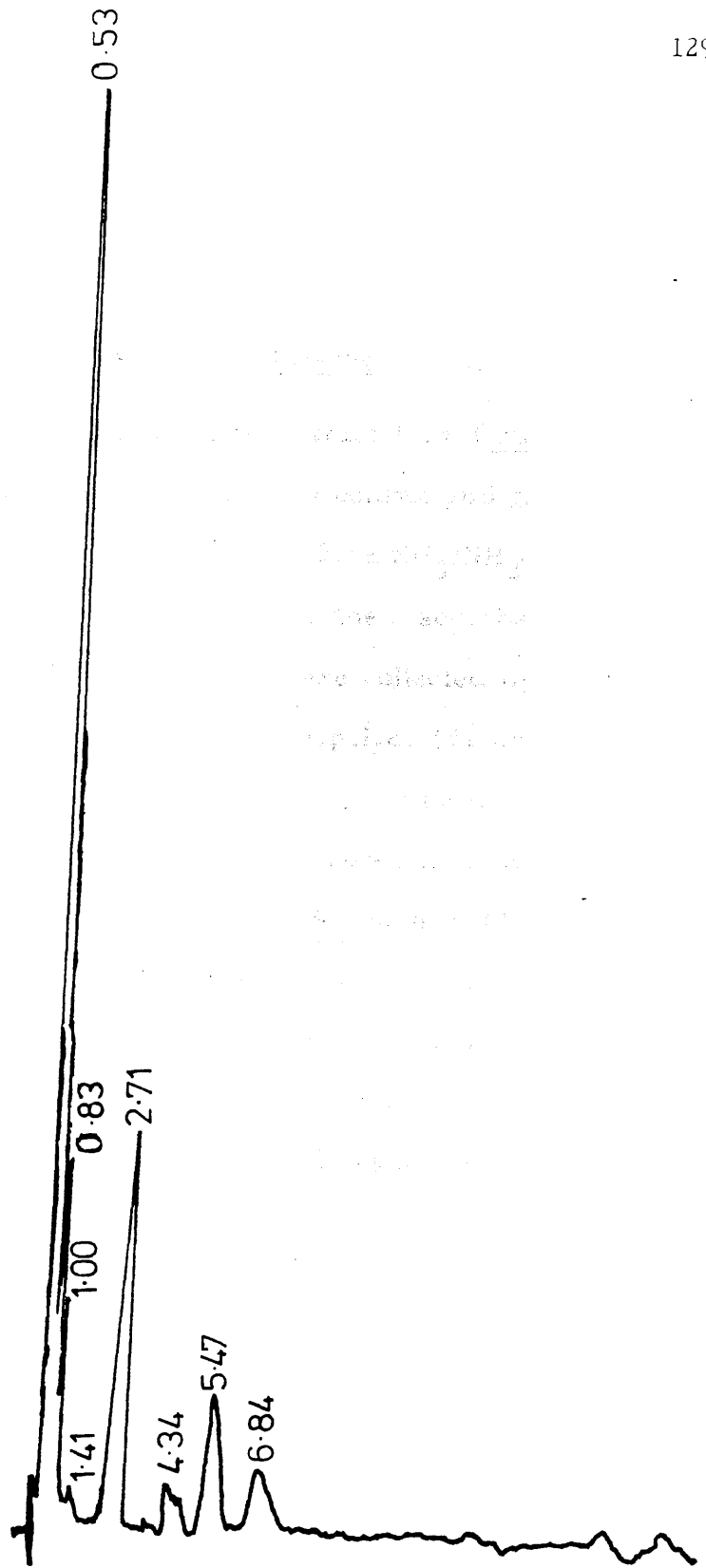
Figure 14b

Analytical h.p.l.c. traces of the alkaloid

mixture isolated from Senecio glaberrimus

samples for full characterisation and identification of the alkaloids.

6.3   Preparative H.P.L.C. of Cynoglossum nervosum

A concentrated sample of the alkaloid extract from Cynoglossum nervosum was applied to a Knauer Li Chrosol Si 60 column and the mixture was eluted with diethylether: [5% v./v. (2.5% NH_3/NH_2O) in methanol], 75:25. The alkaloids were detected by their absorbance in the u.v. spectrum at 220 nm, and the fractions were collected by hand. The separation was not as good as the analytical h.p.l.c. (Figure 13) suggested, and the polarity of the solvent system was reduced to 90:10 to effect a better separation. The retention times were all raised as follows: R_T 3.98 → 13.23, R_T 5.03 → 16.68, and R_T 6.36 → 19.51, on an analytical h.p.l.c. trace (Figure 15). This solvent system was used as the eluant for a preparative h.p.l.c. and the alkaloids were detected and collected as previously. In this case, a good separation of the alkaloids was achieved and pure samples of all three alkaloids were obtained. The alkaloid with R_T 13.23 was identical in all respects to a sample of (+)-echinatine (62) obtained from Cynoglossum officinale. The alkaloids with R_T 16.68 and 19.51 were thought to be heliotrine (33) and rinderine (213) by comparison with known data.[183-186] The small quantity of these alkaloids meant that the respective hydrolyses of the samples of (62), (33), and (213) to the base (3) and the corresponding acids was impractical. These reactions are required for final conformation of the structures of the alkaloids extracted.
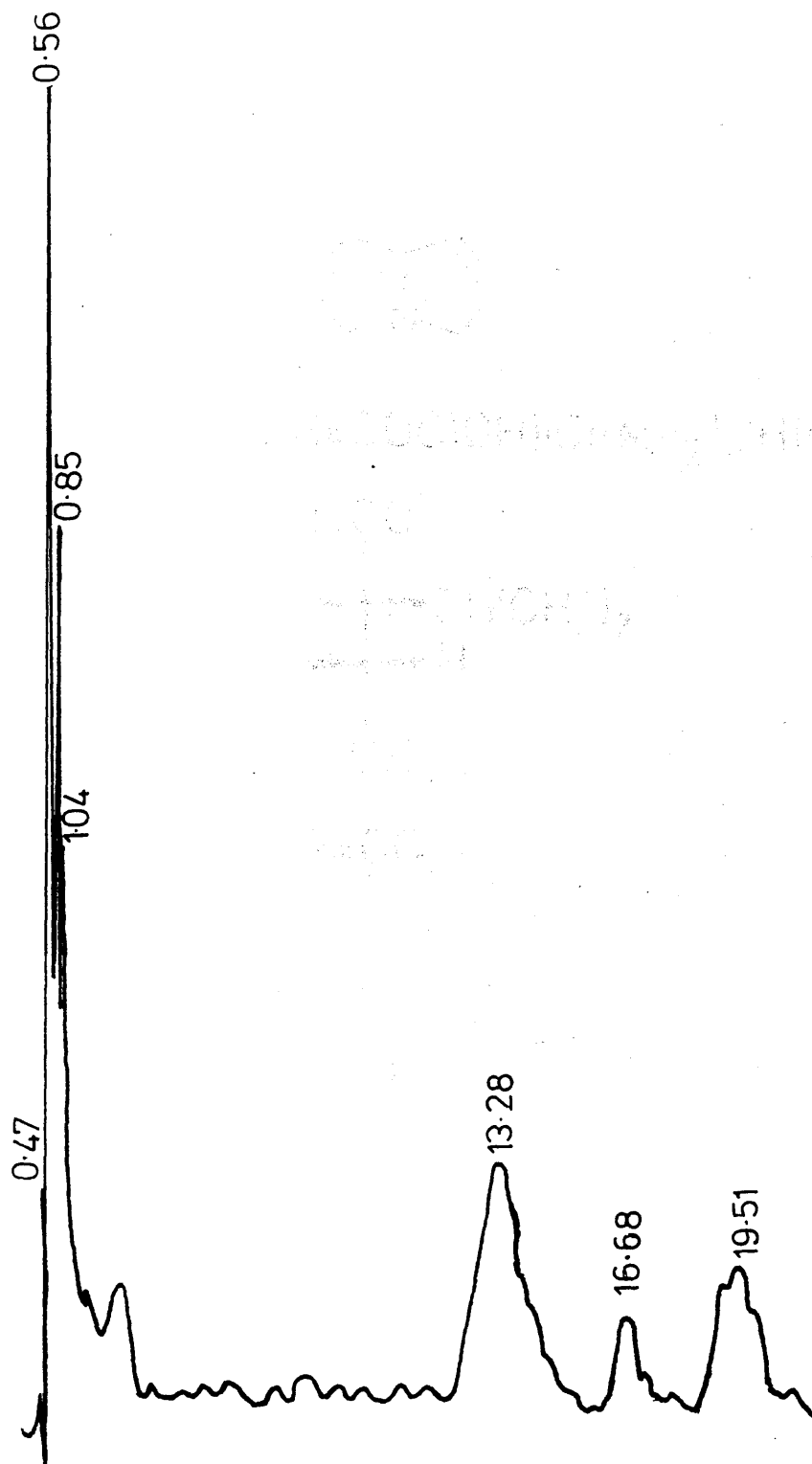
Figure 15

Preparative h.p.l.c. trace of the alkaloid mixture

isolated from Cynoglossum nervosum

(33) R=COC(OH)(CHMe$_2$)CH(OMe)CH$_3$

(62) R=CO

HO—|—CH(CH$_3$)$_2$
HO—|—H

CH$_3$

(213) R=CO

HO—|—CH(CH$_3$)$_2$
H—|—OH

CH$_3$
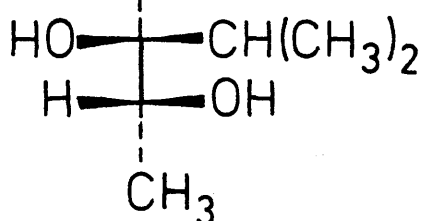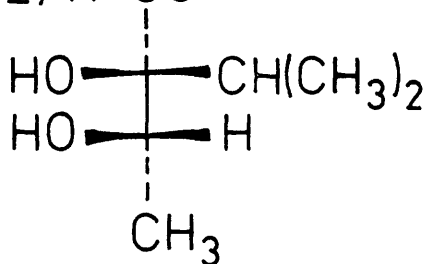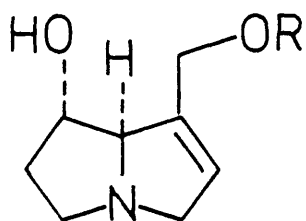
CHAPTER SEVEN

EXPERIMENTAL

7.1 General

Melting points (m.p.) were measured with a Kofler hot-stage apparatus. 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 94 MHz (δ_F) or 25 MHz (δ_C), a Bruker WP-200 SY spectrometer operating at 200 MHz (δ_H), 188 MHz (δ_F), 50 MHz (δ_C) or 31 MHz (δ_D) or with a Bruker WH-360 spectrometer operating at 360 MHz (δ_H) or 55 MHz (δ_D). N.m.r. spectra were recorded for solutions in deuteriochloroform with tetramethylsilane as internal standard unless otherwise stated. Infra red spectra were obtained on a Perkin Elmer 580 spectrophotometer. Optical rotations were measured with an Optical Activity Ltd. AA-10 Polarimeter. Mass spectra were determined with A.E.I. MS 12 or 902 spectrometers.

T.l.c. of the bases was carried out on Kieselgel G plates of 0.25 mm thickness developed with chloroform-methanol-conc. ammonia (85:14:1) unless otherwise stated. The location of the bases was carried out by oxidation with o-chloranil, followed by treatment with Ehrlich's reagent.[95]

1,2-Dimethoxyethane (DME) and tetrahydrofuran (THF) were dried by distillation from potassium hydroxide and then from sodium and benzophenone under argon immediately prior to use. Dry dichloromethane, methane sulphonyl chloride, methanol, thionyl chloride, and triethylamine

were furnished as described by Perrin et al.[187] N,N-Dimethylformamide

(DMF) was dried utilising 3Å molecular sieves as detailed by Burfield

and Smithers.[188] Organic solutions were dried with anhydrous sodium

sulphate, and solvents were evaporated off under reduced pressure

below 50°C.

7.2 Experimental to Chapter Three

7.2.1. Isolation and Hydrolysis of (+)-Echinatine

(+)-Echinatine (62).- Freshly harvested young Cynoglossum

officinale (5 kg) were soaked overnight in methanol and extracted

repeatedly with methanol until the extracts were colourless. The

combined methanol extracts were concentrated under reduced pressure.

The residue was taken up in methylene chloride (100 ml) and extracted

with 2M sulphuric acid (2 x 100 ml). The combined acidic layers were

washed with methylene chloride (4 x 100 ml) and stirred with powdered

zinc metal (10g) for 4h.[189] After filtration through Celite 535, the

solution was made alkaline with conc. ammonia and extracted with chloro-

form (4 x 100 ml). The aqueous solution was basified more strongly by

the addition of potassium hydroxide and extracted with chloroform

(4 x 100 ml). The combined chloroform extracts were dried, filtered,

and concentrated to a light brown foam, which contained one major

component, R_f 0.30. Purification by chromatography on basic alumina

and elution with 25% v/v chloroform in dichloromethane gave (+)-echina-

tine (62) as a gum, 5.06g (0.1%), $[\alpha]_D^{20}$ + 12.3° (c 0.94, CHCl$_3$)

(lit.[190] $[\alpha]_D^{22}$ + 15.0°, c 2.6, EtOH); $\nu_{max.}$ (thin film) 3400, 2973,

2936, 2885, 1728 and 1230 cm^{-1}; δ_H (200 MHz) 0.89 and 0.93 (6H, both

d, $\underline{J}$ 6.8 Hz, 16- and 17-H_3), 1.27 (3H, d, $\underline{J}$ 6.6 Hz, 14-H_3), 1.86 (1H, m, 6-H), 1.96 (1H, m, 6-H), 2.18 (1H, dq, $\underline{J}$ 6.8 Hz, 15-H), 2.62 (1H, ddd, $\underline{J}$ 10.7, 7.0 and 6.1 Hz, 5-H), 3.27 (1H, dd, $\underline{J}$ 10.8 and 6.5 Hz, 5-H), 3.37 (1H, dd, $\underline{J}$ 3.0 and 1.5 Hz, 3-H), 3.60 (1H, br s, O$\underline{H}$), 3.88 (1H, dd, $\underline{J}$ 3.1 and 1.5 Hz, 3-H), 3.97 (1H, m, 8-H), 3.99 (1H, q, $\underline{J}$ 6.6 Hz, 13-H), 4.01 (2H, br s, 2 x O$\underline{H}$), 4.15 (1H, dt, $\underline{J}$ 6.0 Hz, 7-H), 4.79 and 4.96 (2H, ABq, $\underline{J}$ 13.4 Hz, 9-H_2), and 5.70 p.p.m. (1H, br s, 2-H); δ_C (50 MHz) 15.7 and 17.8 (C-16 and -17), 17.2 (C-14), 32.2 (C-15), 33.5 (C-6), 54.2 (C-5), 61.7 (C-3), 62.0 (C-9), 71.6 (C-13), 74.2 (C-7), 79.7 (C-8), 84.1 (C-12), 125.6 (C-2), 136.1 (C-1), and 173.9 p.p.m. (C-11); $\underline{m/z}$ 299 ($\underline{M}^+$) (4%), 156, 139, 138 (100%), 137, 136, 120 and 95 (Found: $\underline{M}^+$, 299.1735;   C, 60.60;   H, 8.21;   N, 4.35. $C_{15}H_{25}NO_5$ requires $\underline{M}$, 299.1732;   C, 60.18;   H, 8.42;   N, 4.68%). The picrolonate had m.p. 210-212°C (lit.[190] 214°C) (Found: C, 53.4; H, 6.1;   N, 12.6. $C_{25}H_{33}N_5O_{10}$ requires C, 53.3;   H, 5.9;   N, 12.4%).

(+)-Heliotridine (3).-   Echinatine (62) (1.02g, 3.39 mmol) was heated at reflux with barium hydroxide (2.00g, 11.67 mmol) in water (25 ml) for 4h.   Solid carbon dioxide was added to the cooled solution which was then filtered.   The filtrate was basified to > pH 10 with potassium hydroxide and continuously extracted with chloroform for 48h to yield (+)-heliotridine (3) (450 mg, 85%) m.p. 116-117°C (acetone) (lit.[190] m.p. 115-116°C); $[\alpha]_D^{20}$ + 26.6° ($\underline{c}$ 1.2, MeOH) (lit.[190] $[\alpha]_D^{20}$ + 30.0°, $\underline{c}$ 1.6 MeOH); $\nu_{max.}$ (KBr) 3340, 2880, 2620, and 2480 cm^{-1}; δ_H (200 MHz) 1.91 (2H, m, 6-H_2), 2.64 (1H, dt, $\underline{J}$ 10.8 and 6.5 Hz, 5-H), 3.24 (1H, dt, $\underline{J}$ 10.7 and 6.2 Hz, 5-H), 3.36 (1H, m, 3-H), 3.80-

4.15 (2H, br s, 2 x O$\underline{\text{H}}$), 3.85 (1H, dd, $\underline{J}$ 15.5 and 1.9 Hz, 3-H), 3.99 (1H, m, 8-H), 4.05 (1H, dt, $\underline{J}$ 5.7 and 4.5 Hz, 7-H), 4.28 (2H, s, 9-H$_2$), and 5.50 p.p.m. (1H, d, $\underline{J}$ 1.5 Hz, 2-H); δ_{C} (50 MHz) 33.0 (C-6), 53.5 (C-5), 58.6 (C-9), 61.4 (C-3), 74.2 (C-7), 79.3 (C-8), 121.8 (C-2), and 140.9 p.p.m. (C-1); $\underline{m}/\underline{z}$ 155 ($\underline{\text{M}}^+$) (13%), 111 and 80 (100%) (Found: $\underline{\text{M}}^+$, 155.0951; C, 62.18; H, 8.51; N, 9.05. $C_8H_{13}NO_2$ requires $\underline{\text{M}}$, 155.0946; C, 61.91; H, 8.44; N, 9.03%).

(-)-Viridifloric acid (67).- The solution was acidified with 4$\underline{\text{M}}$ hydrochloric acid solution and continuously extracted with diethyl-ether for 48h to yield (-)-viridifloric acid (67) (440 mg, 80%), m.p. 120-121°C (ethyl acetate:petroleum ether b.p. 40-60°C, 1:1) (lit.[191] m.p. 119-120°C); $[\alpha]_D^{20}$ - 2.1° ($\underline{c}$, 3.0, H$_2$O) (lit.[191] $[\alpha]_D^{20}$ - 2.2°, $\underline{c}$ 1.1, H$_2$O); $\nu_{\text{max.}}$ (CHCℓ_3) 2966, 2566, and 1705 cm^{-1}; δ_{H} (90 MHz) 0.93 and 0.95 (6H, both d, $\underline{J}$ 6.6 Hz, 6- and 7-H$_3$), 1.21 (3H, d, $\underline{J}$ 7.0 Hz, 4-H$_3$), 2.14 (1H, m, 5-H), 4.08 (1H, q, $\underline{J}$ 7.0 Hz, 3-H), 5.37 (2H, br s, 2 x O$\underline{\text{H}}$), and 8.70 p.p.m. (1H, br s, COO$\underline{\text{H}}$); δ_{C} (25 MHz, D$_2$O) 18.0 and 19.5 (C-6 and -7), 19.2 (C-4), 34.7 (C-5), 72.7 (C-3), 86.3 (C-2), and 179.4 p.p.m. (C-1); $\underline{m}/\underline{z}$ 118 ($\underline{\text{M}}^+$ -44) (8%), 103, 85, 57, 56, 45 and 43 (100%) (Found: $\underline{\text{M}}^+$, 118.0998. $C_6H_{14}O_2$ requires $\underline{\text{M}}$, 118.0995. Found: C, 51.73; H, 8.87. $C_7H_{14}O_4$ requires C, 51.82; H, 8.70%).

7.2.2   Synthesis of (+)-Heliotridine

(+)-Retronecine (27).-   Retrorsine (68) was obtained by

methanolic extraction of Senecio isatideus grown in a greenhouse.

Riddelline (69) was furnished by crystallization of the mother liquors

provided by Dr. R.J. Molyneux as mentioned previously (see Section 3.3).

The alkaloid was originally provided by methanolic extraction of

S. riddellii.   Alkaline hydrolysis[192] of both (68) and (69) provided

samples of (+)-retronecine (27) (90-95% yield), m.p. 119-120°C (acetone)

(lit.[104] m.p. 120-121°C); $[\alpha]_D^{26}$ + 45.1° (c 1.2, MeOH) (lit.[97] $[\alpha]_D^{26}$

+ 55.0°, c 1.0 EtOH); $\nu_{max.}$ (KBr) 3330, 2865, and 1315 cm^{-1}; δ_H

(90 MHz, D_2O) 2.00 (2H, m, 6-H_2), 2.66 (1H, dt, J 12.0 and 6.0 Hz,

5-H), 3.17 (1H, dt, J 8.8 and 7.2 Hz, 5-H), 3.36 (1H, dd, J 14.9 and

2.0 Hz, 3-H), 3.80 (1H, dd, J 15.1 and 2.0 Hz, 3-H), 4.14 (1H, br m,

8-H), 4.24 (2H, s, 9-H_2), 4.39 (1H, m, 7-H), and 5.75 p.p.m. (1H, d,

J 2.0 Hz, 2-H); δ_C (25 MHz, D_2O) 37.8 (C-6), 55.1 (C-5), 60.8 (C-3),

63.7 (C-9), 72.9 (C-7), 78.8 (C-8), 127.3 (C-2), and 139.7 (C-1);

m/z 155 (M^+) (21%), 138, 111, 94, 81, and 80 (100%) (Found: M^+,

155.0947, C, 61.86;   H, 8.52;   N, 9.11. $C_8H_{13}NO_2$ requires M,

155.0947;   C, 61.94;   H, 8.39;   N, 9.03%).

9-Benzoylretronecine (70).-   Benzoic acid (0.80g, 6.55 mmol)

and CDI (1.15g, 7.09 mmol) were added to dry THF (80 ml) and stirred

under an argon atmosphere at 20°C for 30 min.[49,50]   Retronecine

(0.50g, 6.45 mmol) was added and the reaction mixture was stirred at

this temperature for a further 18h.   The organic solution was washed

with saturated sodium bicarbonate (3 x 20 ml), saturated brine solution

(1 x 20 ml) and dried over anhydrous Na_2SO_4. The solution was filtered and evaporated to dryness under reduced pressure to yield (70) as a light yellow oil, R_f 0.34 (1.55g, 5.98 mmol, 93%); $[\alpha]_D^{21}$ + 10.0° (c 2.2, CHCl$_3$); $\nu_{max.}$ (CCl$_4$) 2855, 1725 and 1266 cm^{-1}; δ_H (90 MHz) 1.90 (2H, m, 6-H$_2$), 2.64 (1H, m, 5-H), 3.40 (2H, m, 3- and 5-H), 3.73 (1H, m, 3-H), 3.99 (1H, m, 8-H), 4.21 (1H, m, 7-H), 4.50 (1H, br s, OH), 4.62 and 4.80 (2H, ABq, J 13.2 Hz, 9-H$_2$), 5.52 (1H, s, 2-H), and 7.05-7.81 p.p.m. (5H, m, Ar-H); δ_C (25 MHz) 34.4 (C-6), 53.7 (C-5), 61.3 and 62.9 (C-3 and -9), 74.6 (C-7), 79.2 (C-8), 121.7 (C-2), 127.8 (C-12), 128.5 and 129.5 (C-13 and -14), 133.2 (C-1), 134.4 (C-15), and 166.1 p.p.m. (C-11); m/z 259 (M$^+$) (2%), 154, 137, 122, 105, 93, and 77 (100%) (Found: M$^+$, 259.1217; C, 69,59; H, 6.34; N, 5.61. $C_{15}H_{17}NO_3$ requires M, 259.1209; C, 69.47; H, 6.61; N, 5.40%).

9-Benzoyl-7-methanesulphonyl retronecine (71).- 9-Benzoyl-retronecine (70) (1.55g, 5.98 mmol) was dissolved in dry dichloromethane (50 ml) at -10°C under an atmosphere of argon. Triethylamine (0.91g, 1.25 ml, 9.01 mmol) and methanesulphonylchloride (0.89g, 0.60 ml, 7.77 mmol) were added sequentially via syringe, and the reaction was then stirred at -10°C for 90 min.[89] The reaction mixture was then washed in turn with equal volumes (50 ml) of ice-water, saturated sodium bicarbonate solution, and brine. The organic solution was dried over anhydrous Na_2SO_4 and the solvent removed in vacuo to leave (71) as a light amber oil, R_f 0.50 (1.20g, 3.55 mmol, 60%); $[\alpha]_D^{26}$ - 7.3° (c 6.3, CHCl$_3$); $\nu_{max.}$ (CHCl$_3$) 3005, 1723, 1673 and 1272 cm^{-1}; δ_H (90 MHz)

1.91 (2H, m, 6-H$_2$), 2.72 (1H, m, 5-H), 3.05 (3H, s, C$\underline{H}_3$), 3.40 (1H,

m, 5-H), 3.52 (1H, m, 3-H), 3.87 (1H, m, 3-H), 4.25 (1H, m, 8-H),

4.90 and 4.99 (2H, ABq, $\underline{J}$ 13.9 Hz, 9-H$_2$), 5.20 (1H, m, 7-H), 5.72 (1H, s,

2-H), and 7.40-8.05 p.p.m. (5H, m, Ar-H); δ_C (25 MHz) 34.6 (C-6),

39.4 ($\underline{C}$H$_3$), 54.0 (C-5), 60.4 and 61.1 (C-3 and -9), 76.7 (C-7), 78.9

(C-8), 124.2 (C-2), 128.3 and 128.7 (C-13 and -14), 129.8 (C-12),

132.3 (C-15), 133.6 (C-1), and 166.2 p.p.m. (C-11); $\underline{m}/\underline{z}$ 337 ($\underline{M}^+$)

(2%), 215, 120, and 105 (100%) (Found: $\underline{M}^+$, 337.0987; C, 57.43;

H, 5.73; N, 4.01. C$_{16}$H$_{19}$NO$_5$S requires $\underline{M}$, 337.0984; C, 57.26, H,

5.67; N, 4.17%).

<u>Caesium propionate</u> .- Caesium carbonate dihydrate (29.0g,

80.1 mmol) was dissolved in Analar methanol (150 ml) with stirring at

30°C over 15 min.[90] Propionic acid was dried and a portion (13.0g,

176.2 mmol) in methanol (50 ml) was added dropwise over 30 min.   The

reaction mixture was then stirred for a further 90 min, before the

solvent was removed under reduced pressure to leave a white powder.

The powder was washed with diethyl ether (100 ml) and dried over

pastillated paraffin and phosphorus pentoxide for 24h under reduced

pressure to leave pure caesium propionate (28.0g, 136.2 mmol, 85%),

m.p. 184-186°C (dec.); $\nu_{max.}$ (KBr) 2975, 2920, and 1560 cm^{-1};

δ_H (90 MHz, D$_2$O) 0.96 (3H, t, $\underline{J}$ 6.1 Hz, C$\underline{H}_3$) and 2.18 p.p.m. (2H,

q, $\underline{J}$ 5.8 Hz, C$\underline{H}_2$) (Found: C, 17.43; H, 2.58. C$_3$H$_5$O$_2$Cs requires

C, 17.48; H, 2.43%).

Caesium benzoate.- Caesium carbonate dihydrate (27.5g, 76.0 mmol) in methanol (150 ml) and benzoic acid (20.4g, 167.2 mmol) in methanol (75 ml) were reacted in an analogous manner to the previous preparation,[90] to provide caesium benzoate as white crystals (27.2g, 107.2 mmol, 70.5%), m.p. > 230°C (dec.); $\nu_{max.}$ (KBr) 3060, 1632, and 1340 cm^{-1}; δ_H (90 MHz, D_2O) 7.56 (3H, m, 4-H_2 and 5-H) and 7.99 p.p.m. (2H, m, 3-H_2) (Found: C, 33.21; H, 2.12. $C_7H_5O_2Cs$ requires C, 33.08; H, 1.96%).

9-Benzoyl-7-propionyl heliotridine (72).- Caesium propionate (1.22g, 5.94 mmol) was added to a rapidly stirred solution of dry DMF (60 ml) under a nitrogen atmosphere. The key intermediate 9-benzoyl-7-methanesulphonyl retronecine (72) (0.50g, 1.48 mmol), in dry DMF (2 ml) was added dropwise over a period of 10 min, and the reaction solution was then heated at 85°C for 18h.[90] After cooling to 20°C over a further 12h, the solvent was removed to leave a dark brown residue. This residue was taken up in chloroform (10 ml) and then extracted with 4M hydrochloric acid solution (2 x 10 ml). The combined acidic layers were washed with chloroform (5 x 10 ml), and the pH of the aqueous phase was raised to > pH 9 with conc. ammonia solution. The alkaline solution was extracted with chloroform (2 x 50 ml), and the pH was further increased by the addition of sodium hydroxide. Extraction with chloroform (2 x 50 ml) and drying of the combined organic layers over anhydrous Na_2SO_4, followed by filtration and removal of the solvent in vacuo, left the title compound (72) as an amber oil, R_f 0.54 (0.35g, 1.11 mmol, 75.1%); $[\alpha]_D^{23}$ - 14.2° (c 2.0, $CHCl_3$); $\nu_{max.}$ ($CHCl_3$)

3020, 2950, 1720,1672, and 1273 cm^{-1}; δ_H (90 MHz) 1.07 (3H, t, J 6.0

Hz, C$\underline{H}_3$), 1.94 (2H, m, 6-H$_2$), 2.25 (2H, q, J 5.7 Hz, C$\underline{H}_2$), 2.67 (1H,

m, 5-H), 3.20 (1H, m, 5-H), 3.32 (1H, m, 3-H), 3.90 (1H, m, 3-H),

4.09 (1H, m, 8-H), 4.99 and 5.04 (2H, ABq, J 14.5 Hz, 9-H$_2$), 5.09

(1H, m, 7-H), 5.75 (1H, d, J 2.1 Hz, 2-H), and 7.43-8.04 p.p.m. (5H,

m, Ar-H); δ_C (25 MHz) 20.5 ($\underline{C}H_3$), 34.7 (C-6), 39.0 ($\underline{C}H_2$), 54.6

(C-5), 60.1 and 60.7 (C-3 and -9), 76.5 and 78.5 (C-7 and -8), 124.6

(C-2), 128.3 and 128.6 (C-13 and -14), 129.8 (C-12), 132.9 (C-15),

133.6 (C-1), 166.7 (C-11), and 174.8 p.p.m. (C-17); m/z 315 ($\underline{M}^+$)

(5%), 210, 194, 136, 119, and 105 (100%) (Found: $\underline{M}^+$, 315.1470;

C, 68.79; H, 6.67; N, 4.07. $C_{18}H_{21}NO_4$ requires $\underline{M}$, 315.1485;

C, 68.57; H, 6.67; N, 4.44%).

<u>7,9-Dibenzoylheliotridine (73)</u>.- Caesium benzoate (1.51g,

5.94 mmol) in dry DMF (60 ml) and 9-benzoyl-7-methanesulphonyl

retronecine (71) in dry DMF (2 ml) were reacted as above[90] to provide

the diester (73) as a light brown foam, R_f 0.59 (0.36g, 0.99 mmol,

67.0%); $[\alpha]_D^{20}$ - 8.4° ($\underline{c}$ 2.8, CHCℓ_3); $\nu_{max.}$ (CHCℓ_3) 3015, 2935,

and 1673 cm^{-1}; δ_H (90 MHz) 1.87 (2H, m, 6-H$_2$), 2.69 (1H, m, 5-H),

3.36 (2H, m, 3- and 5-H), 3.68 (1H, m, 3-H), 3.97 (1H, m, 8-H), 4.95

and 5.04 (2H, ABq, J 15.1 Hz, 9-H$_2$), 5.14 (1H, m, 7-H), 5.75 (1H, d,

J 1.9 Hz, 2-H), and 7.40-8.05 p.p.m. (10H, m, Ar-H); δ_C (25 MHz)

34.6 (C-6), 54.3 (C-5), 60.2 and 61.0 (C-3 and -9), 76.6 and 78.7

(C-7 and -8), 124.5 (C-2), 128.3, 128.5, 128.6 and 128.7 (C-13, -14,

-19 and -20), 129.8 and 129.9 (C-12 and -18), 132.0 and 132.4 (C-15

and -21), 133.9 (C-1), 166.5 and 166.9 p.p.m. (C-11 and -17); m/z

363 ($\underline{M}^+$) (3%), 259, 136, 122, 120, 119, and 105 (100%) (Found: $\underline{M}^+$,

363.1475; C, 72.89; H, 5.66; N, 4.07. $C_{22}H_{21}NO_4$ requires $\underline{M}$,

363.1470; C, 72.73; H, 5.79; N, 3.86%).

(+)-Heliotridine (3).- The two pyrrolizidine diesters,(72) and

(73), were hydrolysed[192] with alkali to yield samples of (+)-heliotridine

[72% from (72) and 67% from (73)]. The analysis of the free base (3)

agreed in all respects with that of natural (+)-heliotridine (see Section

7.2.1).

7.2.3   Synthesis of Macrocyclic Bislactones

General procedure.- The anhydride (0.35 mmol) was added to

a solution of (+)-heliotridine (3) (0.30 mmol) in dry DME (10 ml) under

a nitrogen atmosphere. After 24h, triphenylphosphine (0.60 mmol) and

2,2'-dithiodipyridine (0.60 mmol) were added, and the mixture was

stirred vigorously for 48h. The homogeneous solution was transferred

by syringe over 15 min to dry DME (150 ml) heated at reflux under

nitrogen. Heating at reflux was continued for 14h. The solution was

cooled and concentrated to an oil which was dissolved in chloroform

(10 ml). The chloroform solution was extracted with $\underline{1M}$ citric acid

(4 x 8 ml). The acidic solution was washed with chloroform (4 x 10 ml)

and made alkaline with conc. ammonia. The basic solution was

extracted with chloroform (4 x 15 ml). The chloroform extracts were

dried, filtered, and concentrated to an oil, which was purified by

chromatography on basic alumina and elution with dichloromethane [(75)

and (79)], or with increasing proportions (5-20%) of chloroform in

dichloromethane [(78) and (80)-(82)].

(+)-7,9-$\underline{O}$,$\underline{O}$'-(3,3-Dimethylglutaryl)heliotridine (75) was
obtained as needles, m.p. 90-92°C (cyclohexane) (55 mg, 0.197 mmol,
61%), R_f 0.53; $[\alpha]_D^{20}$ + 9.6° ($\underline{c}$ 2.7, CHCℓ_3); $\nu_{max.}$ (CHCℓ_3) 2955,
2923, 1733, 1415, and 1258 cm^{-1}; δ_H (200 MHz) 1.20 (6H, s, 17- and
18-H$_3$), 1.89 (1H, m, 6-H), 2.14 and 2.39 (2H, ABq, $\underline{J}$ 13.3 Hz, 12-
or 14-H$_2$), 2.18 and 2.29 (2H, ABq, $\underline{J}$ 14.9 Hz, 14- or 12-H$_2$), 2.34
(1H, m, 6-H), 2.59 (1H, ddd, $\underline{J}$ 12.3, 9.7 and 5.5 Hz, 5-H), 3.37 (1H,
d, $\underline{J}$ 15.3 Hz, 3-H), 3.39 (1H, dd, $\underline{J}$ 9.4 and 7.5 Hz, 5-H), 3.87 (1H,
dd, $\underline{J}$ 15.5 and 1.4 Hz, 3-H), 4.00 (1H, m, 8-H), 4.44 and 5.05 (2H,
ABq, $\underline{J}$ 13.0 Hz , 9-H$_2$), 4.71 (1H, ddd, $\underline{J}$ 12.7, 8.3 and 8.2 Hz, 7-H),
and 5.62 p.p.m. (1H, s, 2-H); δ_C (50 MHz) 29.5 and 30.4 (C-17 and
-18), 32.9 (C-6), 33.6 (C-13), 44.1 and 44.9 (C-12 and -14), 54.1
(C-5), 59.8 and 62.4 (C-3 and -9), 74.2 (C-7), 79.7 (C-8), 125.9
(C-2), 136.5 (C-1), 170.9 and 171.7 p.p.m. (C-11 and -15); $\underline{m}/\underline{z}$ 279
($\underline{M}^+$) (18%), 220, 137, 136, 120, 119 (100%), 118, 117 and 93
(Found: $\underline{M}^+$, 279.1469; C, 64.66; H, 7.78; N, 4.52. C$_{15}$H$_{21}$NO$_4$
requires $\underline{M}$, 279.1470; C, 64.49; H, 7.58; N, 5.01%).

(+)-7,9-$\underline{O}$,$\underline{O}$'-(3,3-Tetramethyleneglutaryl)heliotridine (78) was
obtained as prisms, m.p. 94-96°C (cyclohexane) (56 mg, 0.184 mmol, 57%),
R_f 0.75; $[\alpha]_D^{20}$ + 7.88° ($\underline{c}$ 3.3, CHCℓ_3); $\nu_{max.}$ (CHCℓ_3) 2965, 2915, 1745, 1438,
and 1178 cm^{-1}; δ_H (200 MHz) 1.15-1.82 (8H, m, 17-, 18-, 19- and
20-H$_2$), 1.80-2.08 (1H, m, 6-H), 2.30-2.45 (4H, m, 12- and 14-H$_2$),
2.48-2.61 (1H, m, 6-H), 2.85-3.00 (1H, m, 5-H), 3.42 (1H, d, $\underline{J}$ 15.0
Hz, 3-H), 3.57 (1H, m, 5-H), 4.01 (1H, d, $\underline{J}$ 14.8 Hz, 3-H), 4.08 (1H,
m, 8-H), 4.56 and 5.05 (2H, ABq, $\underline{J}$ 13.5 Hz, 9-H$_2$), 4.80 (1H, ddd, $\underline{J}$

12.9, 8.1 and 7.5 Hz, 7-H), and 5.62 p.p.m. (1H, s, 2-H); δ_C (50 MHz).
23.2 and 23.9 (C-18 and -19), 31.7 (C-6), 34.2 and 35.2 (C-17 and -20),
44.1 and 44.4 (C-12 and -14), 45.0 (C-13), 53.9 (C-5), 59.8 and 61.7
(C-3 and -9), 69.1 (C-7), 76.2 (C-8), 124.1 (C-2), 136.3 (C-1), 171.2
and 171.9 p.p.m. (C-11 and -15); $\underline{m/z}$ 305 ($\underline{M}^+$) (5%), 278, 277 (100%),
199, 185, 136, 120 and 119 (Found: $\underline{M}^+$, 305.1631; C, 66.66; H, 7.70;
N, 4.86. $C_{17}H_{23}NO_4$ requires $\underline{M}$, 305.1627; C, 66.86; H, 7.59; N,
4.59%).

(+)-7,9-$\underline{O}$,$\underline{O}$'-(3,3-Pentamethyleneglutaryl)heliotridine (79) was
obtained as needles, m.p. 101-102°C (cyclohexane) (49.1 mg, 0.154
mmol, 41%), R_f 0.69; $[\alpha]_D^{20}$ + 6.0° ($\underline{c}$ 5.0, CHCℓ_3); $\nu_{max.}$ (CHCℓ_3^-)
2934, 1732, 1454, 1231, and 1158 cm^{-1}; δ_H (200 MHz) 1.33-1.72 (10H,
m, 17-, 18-, 19-, 20- and 21-H$_2$), 1.80-2.05 (2H, m, 6-H$_2$), 2.27 and
2.38 (2H, ABq, $\underline{J}$ 15.1 Hz, 14- or 12-H$_2$), 2.30 and 2.43 (2H, ABq, $\underline{J}$
13.5 Hz, 12- or 14-H$_2$), 2.62 (1H, ddd, $\underline{J}$ 12.5, 9.7 and 5.5 Hz, 5-H),
3.40 (1H, d, $\underline{J}$ 14.5 Hz, 3-H), 3.41 (1H, dd, $\underline{J}$ 9.7 and 7.5 Hz, 5-H),
3.85 (1H, m, 3-H), 4.01 (1H, m, 8-H), 4.41 and 5.15 (2H, ABq, $\underline{J}$
12.9 Hz, 9-H$_2$), 4.72 (1H, ddd, $\underline{J}$ 10.8, 8.3 and 6.3 Hz, 7-H), and
5.66 p.p.m. (1H, s, 2-H); δ_C (50 MHz) 21.5 and 21.6 (C-18 and -20),
25.8 (C-19), 32.2 (C-6), 36.4 (C-13), 37.0 and 37.9 (C-17 and -21),
41.9 and 42.4 (C-12 and -14), 54.2 (C-5), 59.8 and 62.6 (C-3 and -9),
75.8 (C-7), 77.5 (C-8), 126.3 (C-2), 136.7 (C-1), 171.1 (C-15) and
177.1 p.p.m. (C-11); $\underline{m/z}$ 319 ($\underline{M}^+$) (6%), 277, 149, 136, 120 and 119
(100%) (Found: $\underline{M}^+$, 319.1784; C, 67.49; H, 7.58; N, 4.57.
$C_{18}H_{25}NO_4$ requires $\underline{M}$, 319.1783; C, 67.69; H, 7.89; N, 4.39%).

(+)-7,9-O,O'-(Glutaryl)heliotridine (80) was obtained as a pale yellow oil which could not be crystallised, (20 mg, 0.080 mmol, 41%), R_f 0.51; $[\alpha]_D^{20}$ + 3.3° (c 1.2, CHCl$_3$); $\nu_{max.}$ (CHCl$_3$) 2980, 2935, 1735, 1278, and 1183 cm^{-1}; δ_H (200 MHz) 1.12-1.34 (2H, m, 13-H$_2$), 1.43-1.60 (1H, m, 6-H), 2.00-2.11 (2H, m, 12- and 14-H), 2.24-2.64 (4H, m, 5-, 6-, 12- and 14-H), 3.41 (1H, d, J 10.0 Hz, 3-H), 3.56 (1H, m, 5-H), 4.05 (1H, dd, J 10.1 and 1.5 Hz, 3-H), 4.18 (1H, m, 8-H), 4.82 and 4.94 (2H, ABq, J 15.0 Hz, 9-H$_2$), 5.01 (1H, ddd, J 9.7, 7.7 and 7.5 Hz, 7-H), and 5.53 p.p.m. (1H, s, 2-H); δ_C (50 MHz) 20.9 (C-13), 31.9 (C-6), 33.6 and 34.7 (C-12 and -14), 53.8 (C-5), 61.0 and 61.8 (C-3 and -9), 75.5 and 75.6 (C-7 and -8), 122.1 (C-2), 137.1 (C-1), 172.6 and 172.7 p.p.m. (C-11 and -15); m/z 251 (M$^+$) (13%), 136, 120, 119, 93 and 59 (100%) (Found: M$^+$, 251.1158; C, 61.91; H, 6.60; N, 5.83. C$_{13}$H$_{17}$NO$_4$ requires M, 251.1157; C, 62.14; H, 6.82; N, 5.57%).

The picrolonate had m.p. 192-194°C (Found: C, 53.51; H, 4.58; N, 13.65. C$_{23}$H$_{25}$N$_5$O$_9$ requires C, 53.59; H, 4.85; N, 13.59%).

7,9-O,O'-[(2R,4S)-Dimethylglutaryl]heliotridine (81) and 7,9-O,O'-[(2S,4R)-dimethylglutaryl]heliotridine (82) were obtained as a mixture of diastereoisomers which could not be separated, (15.3 mg, 0.055 mmol, 40%), R_f 0.55; $\nu_{max.}$ (CHCl$_3$) 2963, 2930, 1734, 1456, 1260, and 1184 cm^{-1}; δ_H (200 MHz) 1.09-1.42 (6H, m, 17- and 18-H$_3$), 1.46 (1/3H, m, 13H), 1.51 (2/3H, m, 13-H), 1.95-2.63 (6H, complex, 5-,

12-, 13-, 14-H and 6-H$_2$), 3.30-3.45 (2H, m, 3- and 5-H), 3.86 (2/3H, m, 3-H), 3.92 (1/3H, m, 3-H), 4.15 (2/3H, m, 8-H), 4.30 (1/3H, m, 8-H), 4.61 and 4.90 (2/3H, ABq, J 14.0 Hz, 9-H$_2$), 4.73 and 4.90 (4/3 H, ABq, J 14.1 Hz, 9-H$_2$), 4.86 (1/3H, m, 7-H), 5.08 (2/3H, ddd, J 13.0, 8.9 and 6.3 Hz, 7-H), 5.45 (1/3H, s, 2-H), and 5.55 p.p.m. (2/3H, s, 2-H); δ_C (50 MHz) 17.9, 18.2, 19.4 and 19.6 (C-17 and -18), 31.9 and 32.0 (C-6), 38.8 and 39.7 (C-13), 39.2, 39.4, 39.6 and 39.7 (C-12 and -14), 53.8 and 53.9 (C-5), 60.5, 61.6, 61.7 and 62.1 (C-3 and -9), 75.1, 75.2, 75.5 and 75.8 (C-7 and -8), 120.6 and 122.9 (C-2), 137.3 and 137.7 (C-1), 174.8, 175.2, 175.6 and 175.7 p.p.m. (C-11 and -15); m/z 279 (M$^+$) (11%), 206, 136, 120, 119 (100%) and 117 (Found: M$^+$, 279.1474;   C, 64.66;   H, 7.70;   N, 4.86. C$_{15}$H$_{21}$NO$_4$ requires M, 279.1470;   C, 64.49;   H, 7.58;   N, 5.01%).

An X-ray crystal structure analysis of (+)-7,9-O,O'-(3,3-dimethylglutaryl)heliotridine (75) was carried out by Dr. A.A. Freer.[193]

7.3 Experimental to Chapter Five

7.3.1 Synthesis of ^{2}H-labelled Putrescines

[1,1,4,4-^{2}H$_4$]Putrescine (103) Dihydrochloride.- 1,2-Dicyano-ethane (succinonitrile) (2.50g, 31.3 mmol) was added to a suspension of platinum(IV) oxide (Adam's catalyst) (375 mg, 15% by weight) in mono-deuterioacetic acid (60 ml, > 99 atom % ^{2}H$_1$ species).[119] The reaction mixture was stirred under an atmosphere of deuterium for 3 days until the catalyst precipitated out of solution. The reaction mixture was filtered through a pad of celite 535 and then evaporated to dryness under reduced pressure. The residue was dissolved in 1.2M hydro-chloric acid and evaporated to dryness again. The solid obtained was crystallised from 90% aqueous ethanol to furnish white needles of [1,1,4,4-^{2}H$_4$]putrescine (103) dihydrochloride (2.20g, 13.3 mmol, 42.7%), m.p. > 290°C; $\nu_{max.}$ (KBr) 3130, 3050, 2815, and 1408 cm^{-1}; δ_H (90 MHz, D$_2$O) 1.98 p.p.m. (2- and 3-H$_2$); δ_D (31 MHz, H$_2$O) 2.86 p.p.m. (1- and 4-^{2}H$_2$, > 95 atom % ^{2}H$_4$); δ_C (25 MHz, D$_2$O with 1,4-dioxan as standard at 69.1 p.p.m.) 26.2 (C-2 and -3) and 42.1 p.p.m. (C-1 and -4); m/z 93 (MH$^+$) (0.3%), 75, 45, 44, 38 and 36 (100%) (Found: MH$^+$, 93.1331. C$_4$H$_9$^{2}H$_4$N$_2$ requires MH, 93.1330). (Found: C, 29,34; N, 16.90. C$_4$H$_{10}$^{2}H$_4$N$_2$Cl$_2$ requires C, 29.09, N, 16.97%).

Monodeuterioacetic acid was generated by heating acetic anhydride (97.4g, 90.0 ml, 0.95 mol) and deuterium oxide (18.8g, 17.0 ml, 0.94 mol) under an argon atmosphere for 2 days at 75°C. Deuteriolysis was completed by the addition of concentrated sulphuric

acid (18$\underline{M}$, 0.2 ml) and heating at reflux for 2h.[144]   The monodeuterio-

acetic acid (103 ml, 109.2g, 1.79 mol, 95%) was distilled over 116-117°C

at atmospheric pressure (lit.[144] b.p. 50°C/57 mm) as a clear liquid;

$\nu_{max.}$ (neat) 3000, 2300 and 1728 cm^{-1}; δ_H (90 MHz) 2.05 p.p.m.;

δ_C (25 MHz) 20.8 (C-2) and 177.8 p.p.m. (C-1).

Deuterium gas was generated $\underline{in}$ $\underline{situ}$ by dropping deuterium

oxide onto lithium metal in rapidly stirred paraffin.   The gas was

passed immediately into the reaction vessel after drying by passing

through paraffin and silica gel in turn.   Prior to reaction and after

completion, the reaction apparatus was thoroughly flushed with argon.

[2,2,3,3-^{2}H$_4$]Succinonitrile (149).- Deuterium oxide (35 ml,

99.8 atom % ^{2}H) was added to succinonitrile (3.65g, 45.6 mmol) and

heated at reflux with stirring for 24h.[119]   The mixture was evaporated

to dryness under reduced pressure, and the procedure was repeated

with fresh aliquots of deuterium oxide (3 x 35 ml).   [2,2,3,3-^{2}H$_4$]-

Succinonitrile (149) solidified to a clear waxy solid and was recrystall-

ised from benzene (3.45g, 41.1 mmol, 90.1%), m.p. 54-56°C (lit.[194]

m.p. 57°C); $\nu_{max.}$ (CHCℓ_3) 3020, 2260, and 1689 cm^{-1}; δ_H (90 MHz)

no signals detected; δ_D (31 MHz) 2.67 p.p.m. (2- and 3-^{2}H$_2$, > 99

atom % ^{2}H$_4$); δ_C (25 MHz) 14.0 (C-2 and -3) and 117.8 p.p.m. (C-1

and -4); $\underline{m/z}$ 84 ($\underline{M}^+$) (76%), 82, 58 and 56 (100%) (Found: $\underline{M}^+$,

84.0629; C, 57.32; N, 33.49. C$_4$^{2}H$_4$N$_2$ requires $\underline{M}$, 84.0625; C,

57.14; N, 33.33%).

[2,2,3,3-^{2}H$_4$]Putrescine (104) Dihydrochloride.- [2,2,3,3-^{2}H$_4$]Succinonitrile (149) (2.2g, 26.2 mmol) was added to a suspension of platinum(IV) oxide (330 mg, 15% by weight) in glacial acetic acid (90 ml).[119] The mixture was stirred under an atmosphere of hydrogen at 20°C for 35h, by which time no more hydrogen was being taken up and the catalyst had fallen out of solution. The reaction mixture was then filtered through a pad of celite 535, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 1.2$\underline{M}$ hydrochloric acid and the solution was again evaporated to dryness under reduced pressure. The resulting light brown solid was crystallised from aqueous ethanol to yield white needles of [2,2,3,3-^{2}H$_4$]putrescine (104) dihydrochloride (1.95g, 11.8 mmol, 45.0%), m.p. > 290°C; $\nu_{max.}$ (KBr) 3070, 3010, 2940, and 1452 cm^{-1}; δ_H (90 MHz, D$_2$O) 3.18 p.p.m. (1- and 4-H$_2$); δ_D (31 MHz, D$_2$O) 1.56 p.p.m. (2- and 3-^{2}H$_2$, > 99 atom % ^{2}H$_4$); δ_C (25 MHz, D$_2$O with 1,4-dioxan as standard at 69.1 p.p.m.) 26.5 (C-2 and -3) and 42.0 p.p.m. (C-1 and -4); $\underline{m}/\underline{z}$ 93 ($\underline{M}$H$^+$) (0.2%), 75, 45, 43, 38 and 36 (100%) (Found: $\underline{M}$H$^+$, 93.1326; C$_4$H$_9$^{2}H$_4$N$_2$ requires $\underline{M}$H, 93.1330) (Found: C, 29.40; N, 16.64. C$_4$H$_{10}$^{2}H$_4$N$_2$Cl$_2$ requires C, 29.09; N, 16.97%).

4-Phthalimidobutane nitrile (151).- $\underline{N}$-(3-Bromopropyl)-phthalimide (9.00g, 33.6 mmol) was dissolved in dry DMSO (100 ml) with stirring. Sodium cyanide (2.00g, 40.8 mmol) was added and the reaction mixture was heated at 60°C for 14h, and then allowed to cool. Portions (100 ml) of water and diethyl ether were added; the layers were separated, and the aqueous phase was washed with further

diethyl ether (100 ml). The combined ethereal extracts were dried over anhydrous magnesium sulphate, filtered and evaporated to dryness under reduced pressure. The residue was crystallised from chloroform to yield 4-phthalimidobutane nitrile (151) as white plates (5.01g, 23.4 mmol, 69.7%), m.p. 82-83°C; $\nu_{max.}$ (CHCℓ_3) 3020, 1720, 1398, and 719 cm^{-1}; δ_H (100 MHz) 2.11 (2H, dt, $\underline{J}$ 7.0 and 6.5 Hz, 3-H$_2$), 2.51 (2H, t, $\underline{J}$ 7.0 Hz, 2-H$_2$), 3.79 (2H, t, $\underline{J}$ 6.5 Hz, 4-H$_2$) and 7.75 p.p.m. (4H, br s, Ar-H); δ_C (25 MHz) 14.9 (C-2), 24.7 (C-3), 36.6 (C-4), 119.2 (C-1), 123.2 (C-8), 131.8 (C-7), 134.1 (C-9) and 168.0 p.p.m. (C-6); $\underline{m}/\underline{z}$ 214 ($\underline{M}^+$) (18%), 161, 160 (100%) and 105 (Found: $\underline{M}^+$, 214.0742; C, 67.40; H, 4.53; N, 12.86. $C_{12}H_{10}N_2O_2$ requires $\underline{M}$, 214.0742; C, 67.29; H, 4.67; N, 13.08%).

$\underline{[1,1-{}^2H_2]Putrescine\ (150)\ Dihydrochloride}$.- 4-Phthalimido-butane nitrile (151) (500 mg, 2.34 mmol) was added to a suspension of platinum(IV) oxide (75 mg, 15% by weight) in monodeuterioacetic acid (40 ml) and the mixture was stirred under an atmosphere of deuterium until no more gas was taken up (60h), and the catalyst precipitated. After filtration through a pad of celite 535, the solution was evaporated to dryness $\underline{in\ vacuo}$. The residue was taken up in $\underline{4M}$ hydrochloric acid (25 ml), then stirred for 12h at 20°C and heated at reflux for 4h. After cooling to -10°C to precipitate out the phthalic acid, the reaction mixture was filtered, and evaporated to dryness. The residue was recrystallised from 80% aqueous ethanol to yield, after drying over pastillated paraffin and phosphorus pentoxide for 24h $\underline{in\ vacuo}$, [1,1-${}^2H_2$]putrescine (150) dihydrochloride (321 mg, 1.97 mmol, 84.2%),

m.p. > 295°C; $\nu_{max.}$ (KBr) 3080, 3020, 1452, and 1444 cm^{-1}; δ_H (90

MHz, D$_2$O) 1.56 (4H, m, 2- and 3-H$_2$) and 2.89 p.p.m. (2H, m, 4-H$_2$);

δ_D (31 MHz, H$_2$O) 2.88 p.p.m. (1-^{2}H$_2$, > 90 atom % ^{2}H$_2$); δ_C (25 MHz,

D$_2$O with 1,4-dioxan as standard at 69.1 p.p.m.) 26.3 (C-2 and -3),

41.3 (C-1) and 41.5 p.p.m. (C-4); m/z 91 (MH$^+$) (3%), 73, 45, 43, 38

and 36 (100%) (Found: MH$^+$, 91.1196. C$_4$H$_{11}$^{2}H$_2$N$_2$ requires MH,

91.1204) (Found: C, 29.31; N, 16.77. C$_4$H$_{12}$^{2}H$_2$N$_2$Cℓ$_2$ requires

C, 29.45; N, 17.18%).

R-[1-^{2}H]Putrescine (105) Dihydrochloride and S-[1-^{2}H]-

Putrescine (106) Dihydrochloride.- These salts were prepared as

described earlier,[146] with a ^{2}H$_1$ content of > 95% in both samples.

Preparation of R-[2-^{2}H]Putrescine (107) Dihydrochloride.-

S-2-Chlorobutanedioic acid (152). S-Aspartic acid (100g, 0.75 moles)

and urea (10g, 0.17 moles) were dissolved in conc. hydrochloric acid

(10M, 150 ml) with stirring at room temperature (20°C). Concentrated

nitric acid (11M, 150 ml) was added to this rapidly stirred mixture and

the reaction was heated at 80°C for 12h. A brown gas was evolved

from the reaction vessel. After cooling to 0°C, the white crystals of

S-2-chlorobutanedioic acid (152) were filtered, washed with ice-cold

water, and dried for 48h in vacuo over pastillated paraffin and phos-

phorus pentoxide (102g, 0.67 mol, 89%) m.p. 180-181°C (lit.[147] m.p.

176°C); $[\alpha]_D^{20}$ - 18.7° (c 1.7, H$_2$O) (lit.[147] $[\alpha]_D^{20}$ - 20.1°, H$_2$O);

$\nu_{max.}$ (KBr) 3000, 1712, 1421, and 1280 cm^{-1}; δ_H (100 MHz, CDCℓ$_3$

with d$_6$-DMSO) 2.95 (2H, ABX system, J 17.1 and 7.0 Hz, 3-H$_2$), 4.59

(1H, t, J 7.0 Hz, 2-H) and 10.00 p.p.m. (2H, br s, 2 x COOH);

δ_C (25 MHz, CDCℓ_3 with d_6-DMSO) 39.8 (C-3), 52.9 (C-2), 172.9 and 174.0 p.p.m. (C-1 and -4); m/z 136 (10%) and 134 (32%) (both $\underline{M}^+$-18), 108, 106, 73, 71, 64, 62 and 45 (100%) (Found: $\underline{M}^+$-18, 135.9734 and 133.9764. $C_4H_3O_3{}^{37}C\ell$ requires $\underline{M}$-18, 135.9741 and $C_4H_3O_3{}^{35}C\ell$ requires $\underline{M}$-18, 133.9771) (Found: C, 31.53; H, 3.37; Cℓ, 23.32. $C_4H_5O_4C\ell$ requires C, 31.50; H, 3.30; Cℓ, 23.24%).

Dimethyl S-2-chlorobutanedioate (153).- S-2-Chlorobutanedioic acid (150) (91.1g, 0.60 mol) was dissolved in dry methanol (1ℓ) with vigorous stirring at -10°C. Dry thionyl chloride (100 ml, 163.1g, 1.37 mol) was added over a period of 30 min, and the reaction mixture was allowed to warm up to 20°C. Excess reactant and solvent were removed in vacuo, and the resulting oil was partitioned between diethyl ether (100 ml) and saturated sodium hydrogen carbonate (100 ml). The aqueous phase was washed with a further portion of diethyl ether (100 ml), and the combined organic layers were dried over anhydrous sodium sulphate. After filtration, the solution was concentrated under reduced pressure to an amber oil which was distilled (b.p. 90-91°C/5.0 mm Hg) to provide dimethyl $\underline{S}$-2-chlorobutanedioate (153) as a clear oil (81.1g, 0.45 mol, 75%); $[\alpha]_D^{20}$ - 43.2° ($\underline{c}$ 4.0, CHCℓ_3) (lit.[195] $[\alpha]_D$ - 42.2°, CHCℓ_3); $\nu_{max.}$ (CHCℓ_3) 3020, 1745, and 1442 cm^{-1}; δ_H (100 MHz) 3.06 (2H, ABX system, $\underline{J}$ 17.0 and 7.0 Hz, 3-H$_2$), 3.71 and 3.80 (6H, both s, 2 x OC$\underline{H}_3$), and 4.68 p.p.m. (1H, t, $\underline{J}$ 7.0 Hz, 2-H). δ_C (25 MHz) 39.5 (C-3), 51.7 and 52.2 (OC$\underline{H}_3$), 53.3 (C-2), 169.3 and 169.9 p.p.m. (C-1 and -4); m/z 182 (1%) and 180 (3%) (both $\underline{M}^+$), 151, 150, 149, 148, and 113 (100%) (Found: $\underline{M}^+$, 182.0146 and 180.0199.

$C_6H_9O_4{}^{37}Cl$ requires $\underline{M}$, 182.0161 and $C_6H_9O_4{}^{35}Cl$ requires $\underline{M}$, 180.0191) ·
(Found: C, 39.87; H, 4.99; Cl, 19.64. $C_6H_9O_4Cl$ requires C, 39.89;
H, 4.99; Cl, 19.67%).

$\underline{S}$-2-Chlorobutane-1,4-diol (154).- Dimethyl $\underline{S}$-2-chlorobutane-
dioate (153) (12g, 66.5 mmol) was dissolved in dry toluene (sodium dried)
(50 ml) and added to dry toluene (25 ml) at -30°C under nitrogen.
Diisobutylaluminium hydride (DIBAL) in toluene (1.5$\underline{M}$, 200 ml, 0.30 moles)
was added dropwise into the reaction mixture over a period of 30 min,
then the solution was stirred at -30°C for a further 4h. After warming
up to room temperature, ethyl acetate (30 ml) was added, followed by
acetone (100 ml). The reaction mixture was then poured into a slurry
of celite 535 (80g) in acetone (100 ml). This slurry was stirred vigor-
ously until a gel was formed. The gel was broken up by the addition
of methanol (200 ml) and water (200 ml), and stirring was continued
throughout. When the evolution of gas had ceased, the solution was
filtered and the solid was washed with a further amount of methanol
(4 x 200 ml). The filtrate was concentrated in vacuo to yield a light
brown oil which was distilled (b.p. 200°C/0.5 mm Hg) to furnish a clear
liquid, $\underline{S}$-2-chlorobutane-1,4-diol (154) (8g, 64 mmol, 96%); $[\alpha]_D^{20}$
- 44.1° ($\underline{c}$ 7.4, MeOH) (lit.[120] $[\alpha]_D^{19}$ - 45.0°, $\underline{c}$ 1.2, MeOH); $\nu_{max.}$
(thin film) 3480, 2975, and 1052 cm^{-1}; δ_H (100 MHz) 2.03 (2H, m,
3-H$_2$), 3.80 (4H, m, 1- and 4-H$_2$), 4.15 (2H, br s, 2 x O$\underline{H}$) and 4.32
p.p.m. (1H, m, 2-H); δ_C (25 MHz) 37.1 (C-3), 58.8 (C-4), 60.5
(C-2), and 66.6 p.p.m. (C-1); $\underline{m/z}$ 126 (0.1%) and 124 (0.3%) (both $\underline{M}^+$)
78, 76, 58, 57 and 43 (100%) (Found: $\underline{M}^+$, 126.0240 and 124.0315;

C, 38.45; H, 7.23; Cℓ, 28.61. $C_4H_9O_2{}^{37}Cℓ$ requires $\underline{M}$, 126.0262, $C_4H_9O_2{}^{35}Cℓ$ requires $\underline{M}$, 124.0292, $C_4H_9O_2Cℓ$ requires C, 38.57; H, 7.28; Cℓ, 28.46%).

R-[2-^{2}H$_1$]Butane-1,4-diol (155).- S-2-Chlorobutane-1,4-diol (154) (6g, 48.2 mmol) was dissolved in dry THF (20 ml) and was added dropwise over 15 min to a suspension of lithium aluminium deuteride (98 atom %, 2.4g, 57.1 mmol) in dry THF (50 ml) at 0°C. After allowing the reaction temperature to rise gradually to 20°C, the solution was heated at reflux for 2h. When the solution was cool, saturated aqueous sodium sulphate (2 ml) was added and the mixture was stirred vigorously for 10 min. then filtered. The clear solution was concentrated under reduced pressure and the residue was distilled (b.p. 200°C/0.6 mm Hg) to yield $\underline{R}$-[2-^{2}H$_1$]butane,1,4-diol (155) as a clear oil (4g, 44.0 mmol, 91%); $\nu_{max.}$ thin film) 3450, 2940, 2880, and 1050 cm^{-1}; δ_H (90 MHz) 1.52 (3H, m, 2-H and 3-H$_2$), 3.47 (4H, m, 1- and 4-H$_2$), and 3.55 p.p.m. (2H, br s, 2 x O$\underline{H}$); δ_D (31 MHz, CHCℓ$_3$) 1.51 p.p.m. (2-^{2}H$_1$, 94 atom % ^{2}H$_1$); δ_C (25 MHz) 29.8 (C-2), 30.0 (C-3), and 62.6 p.p.m. (C-1 and -4); $\underline{m}/\underline{z}$ 91 ($\underline{M}^+$) (2%), 73, 72, 58, 57 and 43 (100%) (Found: $\underline{M}^+$, 91.0719; C, 53.01. $C_4H_9{}^2H_1O_2$ requires $\underline{M}$, 91.0743; C, 52.75%).

R-[2-^{2}H$_1$]Butanedioic acid (156).- $\underline{R}$-[2-^{2}H$_1$]Butane-1,4-diol (155) (500 mg, 5.5 mmol) was added to a solution of sodium dichromate dihydrate (5g, 16.8 mmol) in dilute sulphuric acid (2$\underline{M}$, 25 ml) at -10°C. The reaction mixture was heated to 60°C for 2h, cooled, extracted with diethyl ether (3 x 50 ml) and the combined ethereal extracts were dried. After filtration, the solvent was removed in vacuo to leave a solid, which

was recrystallised from acetone to yield R-[2-^{2}H$_1$]butanedioic acid (156)

(455 mg, 3.8 mmol, 70%), m.p. 187-189°C; δ_H (90 MHz, CDCℓ_3 with

d$_6$-DMSO) 2.45 (3H, m, 2-H and 3-H$_2$), and 12.92 p.p.m. (2H, br s,

2 x COO$\underline{H}$); $\underline{m}/\underline{z}$ 119 ($\underline{M}^+$) (2%), 102, 101, 75, 74, 56 and 45 (100%).

<u>R-[2-^{2}H$_1$]Butanedioic anhydride (157)</u>.- R-[2-^{2}H$_1$]Butanedioic

acid (156) (230 mg, 1.9 mmol) was heated with acetic anhydride (1.0 ml,

10.5 mmol) for 2h at 50°C. Excess reagent was removed under reduced

pressure below 40°C, and the residue was crystallised from chloroform

to yield R-[2-^{2}H$_1$]butanedioic anhydride (157) as needles (125 mg, 1.3

mmol, 65%), m.p. 119-120°C; δ_H (90 MHz, CDCℓ_3 with d$_6$-DMSO) 2.98

p.p.m. (m); $\underline{m}/\underline{z}$ 101 ($\underline{M}^+$) (21%), 74, 73, 56, 55 and 45 (100%).

Comparison with a mass spectrum of an unlabelled sample of butanedioic

(succinic) anhydride indicated a ^{2}H$_1$ content of > 95%.

<u>R-[2-^{2}H$_1$]-1,4-Dibromobutane (158)</u>.- A mixture of conc.

hydrobromic acid (48%, 6$\underline{M}$, 100 ml) and conc. sulphuric acid (10$\underline{M}$, 75

ml) were stirred at -10°C for 15 min.[196] R-[2-^{2}H$_1$]Butane-1,4-diol

(155) (4g, 44.0 mmol) was added over 5 min, and the solution was then

heated at reflux temperature for 4h. Upon cooling, the aqueous phase

was extracted twice with diethyl ether (2 x 250 ml), and the combined

organic layers were washed in turn with water (100 ml) and saturated

aqueous sodium hydrogen carbonate (100 ml) and dried. Filtration

followed by evaporation to dryness <u>in vacuo</u> yielded a brown oil, and

R-[2-^{2}H$_1$]-1,4-dibromobutane (158) (6.7g, 30.8 mmol, 70%) was afforded

by distillation (b.p. 66-67°C/1.4 mm Hg); $\nu_{max.}$ (CHCℓ_3) 2970, 1440,

1250, 700 and 560 cm^{-1}; δ_H (90 MHz) 2.05 (3H, m, 2-H and 3-H$_2$), and

3.45 p.p.m. (4H, m, 1- and 4-H$_2$); δ_D (31 MHz, CHCℓ_3) 2.02 p.p.m.

(2-^{2}H$_1$, 93 atom % ^{2}H$_1$); δ_C (25 MHz) 30.3 (C-2), 32.6 (C-3), and

41.8 p.p.m. (C-1 and -4); m/z 219 (1%), 217 (2%) and 215 (1%) (all M$^+$),

138, 136 and 56 (100%) (Found: M$^+$, 218.9017, 216.9038 and 214.9054;

C, 22.38; ^{1}H/^{2}H, 4.18. C$_4$H$_7$^{2}H$_1$^{81}Br$_2$ requires M, 218.9014;

C$_4$H$_7$^{2}H$_1$^{81}Br$_1$^{79}Br$_1$ requires M, 216.9035; C$_4$H$_7$^{2}H$_1$^{79}Br$_2$ requires M,

214.9055; C$_4$H$_7$^{2}H$_1$Br$_2$ requires C, 22.15; ^{1}H/^{2}H, 4.18%).

R-[2-^{2}H$_1$]Putrescine (107) Dihydrochloride.- Sodium azide

(2.4g, 36.9 mmol) was dissolved in dry DMSO (25 ml) at -5°C with

gentle stirring.[119] R-[2-^{2}H]-1,4-Dibromobutane (158) (3.5g, 16.1

mmol) was added dropwise and the solution was stirred for 14h. After

pouring the reaction mixture into water (500 ml), the solution was

extracted with diethyl ether (2 x 200 ml). The combined ethereal

layers were washed with water (4 x 100 ml), dried, filtered and concen-

trated to an amber oil, R-[2-^{2}H$_1$]-1,4-diazobutane (1.8g, 12.8 mmol,

79.5%). The diazide was not purified, but taken up in dry THF (50 ml).

Lithium aluminium hydride (960 mg, 28.2 mmol) was added and the

reaction mixture was stirred at 20°C for 18h under a nitrogen atmos-

phere,[197] then the reaction was quenched by the addition of saturated

aqueous sodium sulphate (2 ml). The organic solution was filtered,

dried, and refiltered, before dry hydrogen chloride gas was generated

in situ and passed through the organic solution to precipitate the salt.

Hydrogen chloride gas was produced by adding conc. sulphuric acid to

solid sodium chloride in a carefully controlled dropwise manner, and it

was dried by passage through paraffin and silica gel. The salt was

collected by filtration, dried over pastillated paraffin and phosphorus

pentoxide for 24h, and recrystallised from 95% aqueous ethanol to provide

R-[2-^{2}H$_1$]putrescine (107) dihydrochloride as white needles (5.4g, 33.1

mmol, 85%), m.p. > 300°C; $\nu_{max.}$ (KBr) 3080, 3015, 1470, and 1452 cm^{-1};

δ_H (90 MHz, D$_2$O) 1.59 (3H, m, 2-H and 3-H$_2$), and 2.87 p.p.m. (4H,

m, 1- and 4-H$_2$); δ_D (31 MHz, H$_2$O) 1.58 p.p.m. (2-^{2}H$_1$, > 90 atom %

^{2}H$_1$); δ_C (25 MHz, D$_2$O) 24.4 (C-2), 24.7 (C-3), and 39.8 p.p.m.

(C-1 and -4); m/z 90 (MH$^+$) (0.5%), 73, 45, 38 and 36 (100%) (Found:

MH$^+$, 90.1149; C, 29.84; N, 17.01; Cℓ, 44.25. C$_4$H$_{13}$^{2}HN$_2$Cℓ$_2$

requires MH, 90.1141; C, 29.63; N, 17.28; Cℓ, 43.83%).

Preparation of S-[2-^{2}H]Putrescine (108) Dihydrochloride.-

This compound was prepared, in an analogous manner to (107) from

R-aspartic acid. The analysis of this synthesis is detailed below.

R-2-Chlorobutanedioic acid (159) (26g, 0.039 moles, 92%), m.p.

179-180°C; $[\alpha]_D^{20}$ + 18.9° (c 1.6, H$_2$O); $\nu_{max.}$ (KBr) 3000, 1715, 1420

and 1275 cm^{-1}; δ_H (100 MHz, CDCℓ$_3$ with d$_6$-DMSO) 2.98 (2H, ABX

system, J 17.0 and 6.9 Hz, 3-H$_2$), 4.61 (1H, t, J 7.0 Hz, 2-H), and

10.05 (2H, br s, 2 x COOH); δ_C (25 MHz, CDCℓ$_3$ with d$_6$-DMSO) 39.5

(C-3), 52.3 (C-2), 169.9 and 170.8 p.p.m. (C-1 and -4); m/z 136 (11%)

and 134 (33%) (both M$^+$-18) (Found: M$^+$-18, 135.9740 and 133.9773;

C, 31.45; H, 3.52; Cℓ, 23.07. C$_4$H$_3$O$_3$^{37}Cℓ requires M-18, 135.9741

and C$_4$H$_3$O$_3$^{35}Cℓ requires M-18, 133.9771; C$_4$H$_5$O$_4$Cℓ requires C, 31.50;

H, 3.30; Cℓ, 23.24%).

Dimethyl R-2-chlorobutanedioate (160) (24g, 133 mmol, 92%),

b.p. 67-69°C/1.5 mm Hg; $[\alpha]_D^{20}$ + 42.7° ($\underline{c}$ 5.0, CHCl$_3$) (lit.[195] + 42.0°,

CHCl$_3$); $\nu_{max.}$ (CHCl$_3$) 3015, 1750, and 1440 cm^{-1}; δ_H (100 MHz)

3.05 (2H, ABX system, $\underline{J}$ 17.0 and 7.0 Hz, 3-H$_2$), 3.65 and 3.75 (6H,

both s, 2 x OC$\underline{H}_3$), and 4.68 p.p.m. (1H, t, $\underline{J}$ 7.0 Hz, 2-H); δ_C

(25 MHz) 39.8 (C-3), 51.9 and 52.5 (OC$\underline{H}_3$), 53.9 (C-2), 169.4 and

170.2 p.p.m. (C-1 and -4); $\underline{m/z}$ 182 (2%) and 180 (6%) (both $\underline{M}^+$)

(Found: $\underline{M}^+$, 182.0170 and 180.0191; C, 39.69; H, 5.07; Cl, 19.59.

C$_6$H$_9$O$_4{}^{37}$Cl requires 182.0161; C$_6$H$_9$O$_4{}^{35}$Cl requires 180.0188;

C$_6$H$_9$O$_4$Cl requires C, 39.89; H, 4.99; Cl, 19.64%).

R-2-Chlorobutane-1,4-diol (161) (3.9g, 31.5 mmol, 95%), b.p.

74-75°C/0.05 mm Hg; $[\alpha]_D^{20}$ + 43.5° ($\underline{c}$ 15.1, MeOH) [lit.[120] $[\alpha]_D^{21}$

+ 36.7° ($\underline{c}$ 1.2, MeOH)]; $\nu_{max.}$ (thin film) 3350, 2960, and 1050 cm^{-1};

δ_H (100 MHz) 2.02 (2H, m, 3-H$_2$), 3.80 (4H, m, 1- and 4-H$_2$), 4.20

(2H, br s, 2 x O$\underline{H}$) and 4.35 p.p.m. (1H, m, 2-H); δ_C (25 MHz)

37.0 (C-3), 58.5 (C-4), 60.6 (C-2), and 66.4 p.p.m. (C-1); $\underline{m/z}$ 126

(0.2%) and 124 (0.6%) (both $\underline{M}^+$), 78, 76, 58, 57 and 43 (100%) (Found:

$\underline{M}^+$, 126.0241 and 124.0299; C, 38.54; H, 7.38; Cl, 28.64.

C$_4$H$_9$O$_2{}^{37}$Cl requires $\underline{M}$, 126.0262; C$_4$H$_9$O$_2{}^{35}$Cl requires $\underline{M}$, 124.0292;

C$_4$H$_9$O$_2$Cl requires C, 38.57; H, 7.28; Cl, 28.46%).

S-[2-^{2}H$_1$]Butane-1,4-diol (162) (1.1g, 12.1 mmol, 75.1%),

b.p. 84-86°C/0.04 mm Hg; $\nu_{max.}$ (thin film) 3420, 3020, and 1048 cm^{-1};

δ_H (90 MHz, CDCl$_3$ with d$_6$-DMSO) 1.66 (3H, m, 2-H and 3-H$_2$), 3.55

(4H, m, 1- and 4-H$_2$), and 3.67 p.p.m. (2H, br s, 2 x O$\underline{H}$); δ_D

(31 MHz, CHCl$_3$) 1.65 p.p.m. (2-^{2}H$_1$, 95 atom % ^{2}H$_1$); δ_C (25 MHz)

29.9 (C-2), 30.0 (C-3), and 62.6 p.p.m. (C-1 and -4); $\underline{m}/\underline{z}$ 91 ($\underline{M}^+$)

(1%), 73, 72, 58, 57, and 43 (100%) (Found: $\underline{M}^+$, 91.0752; C, 52.56.

$C_4H_9{}^2H_1O_2$ requires $\underline{M}$, 91.0743; C, 52.75%).

$\underline{S\text{-}[2\text{-}{}^2H_1]Butanedioic\ acid}$ (163) (240 mg, 2.02 mmol, 73%)

m.p. 188-189°C (acetone); δ_H (90 MHz, d_6-DMSO) 2.40 (3H, m, 2-H

and 3-H_2), and 12.95 p.p.m. (2H, br s, 2 x $CO_2\underline{H}$); $\underline{m}/\underline{z}$ 119 ($\underline{M}^+$)

(0.1%), 102, 101, 75, 56 and 45 (100%).

$\underline{S\text{-}[2\text{-}{}^2H_1]Butanedioic\ anhydride}$ (164) (51 mg, 0.51 mmol, 61%),

m.p. 118-120°C (chloroform); δ_H (90 MHz, $CDCl_3$ with d_6-DMSO) 2.99

p.p.m. (m); $\underline{m}/\underline{z}$ 101 ($\underline{M}^+$) (13%), 74, 56, 55 and 45 (100%). ${}^2H_1$

content of > 95% was indicated by comparison with the mass spectrum of

undeuteriated butanedioic anhydride.

$\underline{S\text{-}[2\text{-}{}^2H_1]\text{-}1,4\text{-}Dibromobutane}$ (165) (1.9g, 8.7 mmol, 79%),

b.p. 100°C/5 mm Hg; $\nu_{max.}$ ($CHCl_3$) 2950, 1436, 1260, 698, and 565

cm^{-1}; δ_H (90 MHz) 1.96 (3H, m, 2-H and 3-H_2), and 3.50 p.p.m.

(4H, m, 1- and 4-H_2); δ_D (31 MHz, $CHCl_3$) 1.97 p.p.m. (2-${}^2H_1$, 95

atom % ${}^2H_1$); δ_C (25 MHz) 30.5 (C-2), 32.3 (C-3), and 41.4 p.p.m.

(C-1 and -4); $\underline{m}/\underline{z}$ 219 (2%), 217 (4%) and 215 (2%) (all $\underline{M}^+$), 138, 136

and 56 (100%) (Found: $\underline{M}^+$, 218.9019, 216.9034, and 214.9058; C, 22.40;

${}^1H/{}^2H$, 3.93. $C_4H_7{}^2H_1{}^{81}Br_2$ requires $\underline{M}$, 218.9014; $C_4H_7{}^2H_1{}^{81}Br_1{}^{79}Br_1$

requires $\underline{M}$, 216.9035; $C_4H_7{}^2H_1{}^{79}Br_2$ requires $\underline{M}$, 214.9055;

$C_4H_7{}^2H_1Br_2$ requires C, 22.15; ${}^1H/{}^2H$, 4.18%).

$\underline{S-[2-^{2}H_{1}]Putrescine}$ (108) $\underline{dihydrochloride}$ (1.14g, 7.0 mmol,

80.1%), m.p. $>$ 300°C (dec.) (95% aqueous ethanol); $\nu_{max.}$ (KBr)

3077, 3010, 1462, and 1450 cm^{-1}; δ_H (90 MHz, D_2O) 1.61 (3H, m, 2-H

and 3-H$_2$), and 2.88 p.p.m. (4H, m, 1- and 4-H$_2$); δ_D (31 MHz, H_2O)

1.60 (2-$^{2}H_1$, $>$ 90 atom % $^{2}H_1$); δ_C (25 MHz, D_2O) 24.7 (C-2), 24.9

(C-3), and 40.0 p.p.m. (C-1 and -4); $\underline{m/z}$ 90 ($\underline{MH}^+$) (0.3%), 72, 45,

43, 38 and 36 (100%) (Found: $\underline{MH}^+$, 90.1153; C, 29.53; N, 16.94;

Cℓ, 43.57. $C_4H_{13}{}^{2}H_1N_2C\ell_2$ requires $\underline{MH}$, 90.1141; C, 29.63; N, 17.28;

Cℓ, 43.83%).

7.3.2 Feeding of ^{2}H-labelled Putrescines

$\underline{Cynoglossum}$ $\underline{officinale}$ plants were grown from seeds

(Suttons Seeds Ltd.) in a standard compost. In the first series of

experiments, eight well-established plants were used. [1,4-^{14}C]-

Putrescine (166) dihydrochloride (5 µCi) was added to each ^{2}H-labelled

putrescine (200 mg). Sterile aqueous solutions of the precursor mixtures

were introduced into the xylems of the plants through stem punctures

made with a sterile needle. The solutions were applied as droplets

centred over the stem puncture. Feeding was carried out on five

alternate days, and the plants were allowed to grow for a further two

weeks before harvesting. (+)-Echinatine (62) was isolated as described

previously (Section 7.2.1). Scintillation counting of an aliquot of the

alkaloids in methanol was carried out to determine the specific incorpor-

ations per C$_4$-unit of the ^{14}C-labelled putrescine (166). The incorpor-

ations were all below 0.5% per C$_4$-unit. The 31 MHz ^{2}H n.m.r. spectra

revealed the very low incorporations of the ^{2}H-labelled putrescines

[(103)-(108), (150)].   In most cases, no definite signals were present.
When a trace of a signal was detected, there was never a correspondence
with signals due to the base moiety in the ^{1}H n.m.r. spectrum of
(+)-echinatine (Figure 2).

The second series of feeding experiments used the same method,
but the plants were fed earlier in the summer (June) in comparison to
the previous experiments (August-September).   The same series of ^{2}H-
labelled putrescines, again gave the same disappointingly low results.
The alkaloids were hydrolysed in turn to give radioactive samples of
(+)-heliotridine (3), crystallised from acetone.   The 55 MHz ^{2}H n.m.r.
spectra of these necine bases gave no ^{2}H signals of note.   The specific
incorporation per C_4-unit were yet again 0.5% or below, but the bases
contained > 95% of the specific radioactivity.

The final feeding experiments with ^{2}H-labelled putrescines
[(103)-(105), (107), (108), (150)], were performed with eight young
plants early in their growing season.   The amounts used were as before,
the method was basically the same, and the ^{14}C incorporations were as
low as previously.   The 55 MHz ^{2}H n.m.r. spectra of the alkaloids
were proof of the low incorporations of ^{2}H into (+)-echinatine (62).
The only ^{2}H signals which corresponded with any in the ^{1}H n.m.r. of
(+)-echinatine, were those due to the natural abundance of ^{2}H in the
methyl groups of the acidic portion.

Radioscans of silica gel G t.l.c. plates of 0.25 mm thickness
developed with chloroform-methanol-concentrated ammonia (85:14:1) did
not detect any appreciable radioactivity in any of the natural products.

7.3.3   Synthesis and Feeding of ^{13}C-labelled Putrescines

[1-^{13}C]Putrescine (167) and [2,3-^{13}C$_2$]putrescine (168) were synthesised as their dihydrochloride salts by Dr. J. Rana by literature methods.[108]   A quantity (30 mg) of each was mixed with [1-^{14}C]-putrescine (166) dihydrochloride (2.5 µCi), and the mixture was fed to two young Cynoglossum officinale plants by the xylem pricking method over two successive days, and the plants were allowed to grow for a further two weeks prior to harvesting.

The 50 MHz ^{13}C-{^{1}H} n.m.r. spectrum of the alkaloid resulting from the feeding of (167) showed enhanced signals at 54.2, 61.7, 62.0 and 79.7 p.p.m.   The specific incorporation of ^{13}C was 1.93% per C$_4$-unit, and that of ^{14}C was 0.35% per C$_4$-unit.

The 50 MHz ^{13}C-{^{1}H} n.m.r. spectrum of (+)-echinatine obtained from the use of (168) was inconclusive as any doublets were obscured by noise on the baseline.   The specific incorporation per C$_4$-unit for ^{14}C-putrescine (166) was 0.27% as measured by scintillation counting.

Both alkaloids from the above experiments were hydrolysed in alkaline solution to provide samples of (+)-heliotridine which were both radioactive (94-96% of the specific ^{14}C radioactivity), albeit with low incorporations (0.25-0.30% per C$_4$-unit).

7.3.4   Experiments upon 8β-Necine Bases

7.3.4.1   Extraction

Cynoglossum australe plants were grown locally from seeds provided by Dr. C.C.J. Culvenor of the C.S.I.R.O., Australia.   Several young plants (total weight 400g) were harvested, washed with water, and repeatedly extracted with methanol until the extracts were colourless. The combined methanol extracts were treated in the same manner as those of Cynoglossum officinale (Section 7.2.1) to provide a mixture (640 mg, 0.16%) of two pyrrolizidine alkaloids.   After several attempts, successful separation was achieved by column chromatography on basic alumina and elution with gradually increasing proportions of methanol (0-1.0%) in dichloromethane.   The major alkaloid isolated was (+)-cynaustraline (172) as a gum, 345 mg, R_f 0.32, $[\alpha]_D^{20}$ + 43.2° (c 2.0, MeOH) (lit.[150] $[\alpha]_D^{20}$ + 48.0°, c 1.1, EtOH); $\nu_{max.}$ (CHCl$_3$) 3369, 2966, 2931, 1729 and 1232 cm^{-1}: δ_H (200 MHz) 0.87 and 0.91 (6H, both d, J 6.9 Hz, 16- and 17-H$_3$), 1.22 (3H, d, J 6.6 Hz, 14-H$_3$), 1.69 (1H, m, 7-H), 1.94 (2H, m, 6-H$_2$), 2.01 (1H, m, 2-H), 2.10 (1H, m, 7-H), 2.15 (1H, m, 2-H), 2.19 (1H, dq, J 7.0 Hz, 15-H), 2.21 (1H, m, 1-H), 2.66 (1H, dt, J 6.5 and 3.5 Hz, 3-H), 2.74 (1H, dt, J 11.0 and 6.5 Hz, 5-H), 3.26 (1H, dt, J 9.9 and 6.4 Hz, 5-H), 3.48 (1H, dt, J 7.1 and 3.3 Hz, 3-H), 3.76 (1H, m, 8-H), 3.99 (1H, q, J 6.6 Hz, 13-H), 4.20 and 4.31 p.p.m. (2H, dABq, J 11.9 and 7.1 Hz, 9-H$_2$); δ_C (50 MHz) 15.7 and 16.8 (C-16 and -17), 17.4 (C-14), 25.7 (C-7), 30.3 (C-6), 31.8 and 32.0 (C-2 and -15), 45.2 (C-1), 55.5 and 55.7 (C-3 and -5), 65.3 and 67.3 (C-8 and -9), 71.6 (C-13), 83.9 (C-12), and

172.3 p.p.m. (C-11); $\underline{m}/\underline{z}$ 285 ($\underline{M}^+$) (2%), 181, 141, 124, 83, 57 and 43

(100%) (Found: $\underline{M}^+$, 285.3837. $C_{15}H_{27}NO_4$ requires $\underline{M}$, 285.3826).

The picrolonate had m.p. 147-148°C (lit.[150] m.p. 149-150°C) (Found:

C, 54.45; H, 6.59; N, 12.93. $C_{25}H_{35}N_5O_9$ requires C, 54.59; H,

6.37; N, 12.73%).

The minor alkaloid isolated was (+)-cynaustine (173), 90 mg,

R_f 0.35, $[\alpha]_D^{21}$ + 15.2° ($\underline{c}$ 1.7, MeOH) (lit.[150] $[\alpha]_D^{20}$ + 13.2°, $\underline{c}$ 1.6,

EtOH); $\nu_{max.}$ (CHCℓ_3) 3200, 2965, 2933, 1726, and 1218 cm^{-1}; δ_H

(200 MHz) 0.89 and 0.96 (6H, both d, $\underline{J}$ 6.8 Hz, 16- and 17-H$_3$), 1.24

(3H, d, $\underline{J}$ 6.0 Hz, 14-H$_3$), 1.59 (1H, m, 7-H), 1.76 (2H, m, 6-H$_2$),

1.99 (1H, m, 7-H), 2.17 (1H, dq, $\underline{J}$ 6.9 Hz, 15-H), 2.50 (1H, m, 5-H),

3.15 (1H, m, 5-H), 3.38 (1H, dd, $\underline{J}$ 3.7 and 1.2 Hz, 3-H), 3.83 (1H,

dd, $\underline{J}$ 3.5 and 1.5 Hz, 3-H), 4.02 (1H, q, $\underline{J}$ 6.2 Hz, 13-H), 4.38 (1H,

m, 8-H), 4.73 and 4.90 (2H, ABq, $\underline{J}$ 11.0 Hz, 9-H$_2$), and 5.70 p.p.m.

(1H, br s, 2-H); δ_C (50 MHz) 15.9 and 17.1 (C-16 and -17), 17.5

(C-14), 25.6 (C-6), 29.9 (C-7), 32.3 (C-15), 54.2 (C-5), 60.4 (C-3),

63.6 (C-9), 67.9 (C-8), 71.1 (C-13), 83.8 (C-12), 121.3 (C-2), 134.7

(C-1), and 173.0 p.p.m. (C-11); $\underline{m}/\underline{z}$ 283 ($\underline{M}^+$) (4%), 239, 179, 151,

139, 122, 93, 80, 55 and 43 (100%) (Found: $\underline{M}^+$, 283.3641. $C_{15}H_{25}NO_4$

requires $\underline{M}$, 283.3668). The picrate had m.p. 134-135°C (lit.[150] m.p.

135-136°C) (Found: C, 49.41; H, 5.24; N, 11.29. $C_{21}H_{28}N_4O_{11}$

requires C, 49.17; H, 5.46; N, 10.93%).

Each of these alkaloids was hydrolysed in turn with barium

hydroxide to provide samples of (-)-viridifloric acid (67)[191] from both

(172) and (173), (+)-isoretronecanol (22) from (172), and (+)-supinidine

(24) from (173). All characterization data agreed with literature

values.[150,151,191]

7.3.4.2   Feeding of ^{2}H-labelled putrescines

A sample of each ^{2}H-labelled putrescine [(103)-(108), (150)], (200 mg) was mixed with a known quantity (5 μCi) of [1,4-^{14}C]-putrescine (166), as their dihydrochlorides, and fed as sterile aqueous solutions to batches of young Cynoglossum australe on five alternate days by the xylem pricking method.   After a further two weeks growing time, the plants were harvested and the alkaloid mixture was extracted as before.   The specific incorporations per C_4-unit, as seen by radioactive scintillation counting of an aliquot in methanol, were all ~ 0.1% in magnitude.   The 31 MHz ^{2}H n.m.r. spectra showed signals due to ^{2}H present, but there was never any correspondence with the signals present in the ^{1}H n.m.r. spectra.

The following summer, ^{2}H-labelled putrescines [(105)-(107)] were again fed to batches of Cynoglossum australe, but earlier in their growing season, and well before they were due to flower.   The same low magnitude of incorporations (0.3-0.6% per C_4-unit) were seen after harvesting, extraction and separation.   The 55 MHz ^{2}H n.m.r. spectra gave practically no signals as evidence of any ^{2}H incorporation. Alkaline hydrolysis and scintillation counting as before gave radioactive samples of both 8β-necines, containing > 95% of the specific radioactivity.

7.3.4.3   Feeding of ^{3}H-labelled compounds

[1,4-^{3}H]Putrescine (174) dihydrochloride (1 mCi, 37 MBq) was fed by the wick method which involved threading the stem of a young plant and immersion of the ends of the thread in a vial containing a sterile aqueous solution of (174).   Five young Cynoglossum australe

plants were used, and the radioactive sample was diluted to 1 ml and

divided. After two successive days of feeding, the plants were

allowed to grow for two further weeks and then harvested (wet weight

3 kg) and extracted with methanol as before. An aliquot of the

mixture of pyrrolizidine alkaloids was subjected to radioactive scintill-

ation counting to reveal an average ^{3}H incorporation of 1.2% per C_4-

unit. This mixture (500 mg) was separated as before to give tritiated

samples of (+)-cynaustraline (172) (300 mg, 1.05 mmol, 5.9 μCi) and

(+)-cynaustine (173) (85 mg, 0.30 mmol, 2.1 μCi). These alkaloids

were hydrolysed as described previously. Hydrolysis of (172)

provided (+)-isoretronecanol (22) (95 mg, 0.67 mmol, 3.8 μCi), m.p.

39-41°C (lit.[151] m.p. 40-41°C); $[\alpha]_D^{24}$ + 65.1° (c 2.6, MeOH) (lit.[151]

$[\alpha]_D^{20}$ + 71.7°, c 1.0, EtOH); $\nu_{max.}$ (CHCl$_3$) 3350 cm^{-1}; δ_H (90 MHz)

1.70 (2H, m, 7- and 2-H), 1.95 (2H, m, 2- and 6-H), 2.01 (1H, m,

7-H), 2.05 (1H, m, 1-H), 2.60 (2H, m, 3- and 5-H), 3.04 (1H, m, 5-H),

3.22 (1H, dt, J 9.0 and 4.5 Hz, 3-H), 3.33 (1H, m, 8-H), 3.63 (2H,

dd, J 6.0 and 1.5 Hz, 9-H$_2$), and 4.32 (2H, br s, OH). Monoester

(173) furnished, upon alkaline cleavage, (+)-supinidine (24) (33 mg,

0.24 mmol, 1.2 μCi), $[\alpha]_D^{24}$ + 8.5° (c 2.6, MeOH) (lit.[150] $[\alpha]_D^{20}$ + 9.2°,

c 2.1, EtOH); $\nu_{max.}$ (CHCl$_3$) 3330 cm^{-1}; δ_H (90 MHz) 1.56 (1H, m,

7-H), 1.76 (2H, m, 6-H$_2$), 1.99 (1H, m, 7-H), 2.50 (1H, m, 5-H), 3.06

(1H, m, 5-H), 3.21 (1H, m, 3-H), 3.75 (1H, m, 3-H), 4.13 (1H, m,

8-H), 4.21 (2H, dd, J 13.1 and 2.0 Hz, 9-H$_2$), 4.31 (2H, br s, OH),

and 5.50 p.p.m. (1H, br s, 2-H).

A sample of the radioactive (+)-isoretronecanol (22) (20 mg

~ 0.9 μCi) was mixed with a quantity of [1,4-^{14}C]putrescine (166)

dihydrochloride ($\sim$ 0.075 μCi), and the ^{3}H:^{14}C ratio was measured by scintillation counting (11.7). The mixture was diluted (total volume 1 ml) and fed to three young Cynoglossum australe plants by the wick method over two successive days. After one week, the plants (175g) were harvested, extracted with methanol, and the alkaloid mixture (150 mg, 0.09%) was isolated and separated. The ^{3}H:^{14}C ratios present in the samples of (+)-cynaustraline (172) (70 mg) and (+)-cynaustine (173) (25 mg) resulting from this experiment are detailed in Table 5.

The same amount of precursors, with the same ratio (11.7) of radioactive labels, was fed to one Senecio pleistocephalus plant over two days by the wick method. The plant was allowed to grow for a week prior to harvesting (55g), and the alkaloid (175) (25 mg, 0.06%) was extracted in the usual way. The radioactive ratio was measured by scintillation counting (Table 5).

A sample of radioactive (+)-supinidine (24) (15 mg, $\sim$ 0.6 μCi) was mixed with an aliquot of ^{14}C-putrescine (166) dihydrochloride (0.075 μCi) and the ^{3}H:^{14}C ratio was measured by scintillation counting (7.6). The mixture was diluted (total volume 1 ml) and fed to two young Cynoglossum australe plants by the wick method over two successive days. After one week, the plants were harvested (130g), extracted with methanol, and the alkaloid mixture (100 mg, 0.08%) was isolated and separated to furnish (+)-cynaustraline (172) (45 mg) and (+)-cynaustine (173) (16 mg). The ^{3}H:^{14}C ratios in each of the monoesters are detailed in Table 5. The alkaloids all gave correct analytical data.

7.3.5   Synthesis and Feeding of 2-Methylputrescines

7.3.5.1   Synthesis of 2-methylputrescines

R-(+)-3-Methyladipic diamide (178).- R-(+)-3-Methyladipic

acid (177) (9.22g, 57.6 mmol) was dissolved in ethanol at -40°C with

stirring.   Dry ammonia gas (600 ml) was distilled from sodium and

bubbled through the reaction mixture over 5h at -40°C.   A white

precipitate formed immediately.   The reaction temperature was allowed

to warm up to room temperature (20°C) over 14h, then the solvent was

removed in vacuo.   The resultant white solid was recrystallised from

water to give the title compound (178) as a dihydrate (9.52g, 49.1

mmol, 85.2%), m.p. 188-189°C (H_2O) (lit.[153] m.p. 191°C); $[\alpha]_D^{20}$

+ 15.8° (c 2.0, H_2O) (lit.[153] $[\alpha]_D^{22}$ + 14.7°, H_2O); $\nu_{max.}$ (KBr) 2950,

2940, 2850, 1569, and 1545 cm^{-1}; δ_H (90 MHz, D_2O) 0.85 (3H, d, J

7.0 Hz, 7-H_3), 1.15-1.68 (3H, m, 3-H and 4-H_2), and 2.07-2.35 p.p.m.

(4H, m, 2- and 5-H_2); δ_C (25 MHz, D_2O) 19.4 (C-7), 31.4 (C-4),

33.6 (C-3), 36.9 (C-5), 43.8 (C-2), 183.6 and 184.4 p.p.m. (C-1 and

-6); m/z 142 (M^+-16) (4%), 114, 101, 83, 69, and 55 (100%) (Found:

M^+-16, 142.1761. $C_7H_{12}NO_2$ requires M-16, 142.1773) (Found: C,

43.31;   H, 9.04;   N, 14.21. $C_7H_{14}N_2O_2 \cdot 2H_2O$ requires C, 43.29;   H,

9.28;   N, 14.43%).

R-(+)-2-Methylputrescine (176) dihydrochloride.- Bromine

(10.2g, 63.8 mmol) was added dropwise to a stirring solution of sodium

hydroxide (14.2g, 335.0 mmol) in water (28 ml) and ice (40g).

R-(+)-3-Methyladipic diamide (178) dihydrate (5.0g, 25.8 mmol) was

added cautiously in small portions to the reaction mixture, which was

then warmed at 40°C until the mixture became clear (1h).[153] Heating

was continued for a further 4h, before the reaction solution was cooled,

filtered and shaken gently with benzoyl chloride (12g, 85.4 mmol).

The dibenzoyl putrescine derivative (179) was filtered off and recrystall-

ised from 95% aqueous ethanol (6.88g, 22.2 mmol, 86.0%), m.p. 150-151°C

(lit.[153] m.p. 154°C); $[\alpha]_D^{20}$ + 1.0° ($\underline{c}$ 2.0, MeOH) (lit.[153] $[\alpha]_D^{15}$ + 1.2°,

py); $\nu_{max.}$ (CHCℓ_3) 1694 cm^{-1}; δ_H (90 MHz, D$_2$O) 0.89 p.p.m.

(3H, d, $\underline{J}$ 6.6 Hz, 7-H$_3$), 1.31-1.63 (3H, m, 3-H and 4-H$_2$), 3.83 (4H,

m, 2- and 5-H$_2$), 7.46 (6H, m, $\underline{m}$- and $\underline{p}$-Ar-H), and 8.10 p.p.m. (4H,

dd, $\underline{J}$ 7.0 and 1.8 Hz, $\underline{o}$-Ar-H); $\underline{m}/\underline{z}$ 310 ($\underline{M}^+$) (0.3%) (Found: C, 73.61;

H, 7.04; N, 8.74. C$_{19}$H$_{22}$N$_2$O$_2$ requires C, 73.55; H, 7.10; N, 9.03%).

This $\underline{R}$-(+)-$\underline{N}$,$\underline{N}$'-dibenzoyl-2-methylputrescine (179) was

dissolved in concentrated hydrochloric acid (10$\underline{M}$, 25 ml) in a tube.

The vessel was sealed and heated at 130°C with stirring for 13h behind

a safety shield. Upon cooling, the precipitate was filtered off, and

evaporation of the filtrate to dryness under reduced pressure afforded

$\underline{R}$-(+)-2-methylputrescine (176) dihydrochloride (3.40g, 19.4 mmol, 75.2%

from diamide), m.p. 145-147°C (lit.[153] m.p. 143°C); $[\alpha]_D^{21}$ + 6.0°

($\underline{c}$ 2.0, H$_2$O) (lit.[153] $[\alpha]_D^{22}$ + 5.6°, H$_2$O); $\nu_{max.}$ (KBr) 3080, 3023,

1473 and 1451 cm^{-1}; δ_H (90 MHz, D$_2$O) 1.18 (3H, d, $\underline{J}$ 7.1 Hz, 5-H$_3$),

1.80 (3H, m, 2-H and 3-H$_2$) and 3.11 p.p.m. (4H, m, 1- and 4-H$_2$);

δ_C (25 MHz, D$_2$O with 1,4-dioxan as standard at 69.1 p.p.m.) 19.4 (C-5),

31.0 (C-3), 34.6 (C-2), 42.5 (C-4) and 48.2 p.p.m. (C-1); $\underline{m}/\underline{z}$ 103

($\underline{M}H^+$) (2%), 91, 69, 57 and 36 (100%) (Found: $\underline{M}H^+$, 103.1885.

C$_5$H$_{15}$N$_2$ requires $\underline{M}H$, 103.1869) (Found: C, 34.35; H, 9.29; N, 15.66.

C$_5$H$_{16}$N$_2$Cℓ_2 requires C, 34.28; H, 9.14; N, 16.00%).

(±)-3-Methyladipic diamide (181).- Racemic 3-methyladipic acid (180) (5g, 31.3 mmol) was converted as before into racemic 3-methyladipic diamide (181) dihydrate (4.7g, 24.2 mmol, 77.2%), m.p. 184-5°C; $\nu_{max.}$ (KBr) 2955, 2942, 2850, 1570 and 1540 cm^{-1}; δ_H (90 MHz, D_2O) 0.89 (3H, d, $\underline{J}$ 7.0 Hz, 7-H_3), 1.20-1.66 (3H, m, 3-H and 4-H_2), and 2.05-2.33 p.p.m. (4H, m, 2- and 5-H_2); δ_C (25 MHz, D_2O) 19.5 (C-7), 31.6 (C-4), 33.9 (C-3), 38.1 (C-5), 44.0 (C-2), 184.6 and 184.9 p.p.m. (C-1 and -6); $\underline{m}/\underline{z}$ 142 ($\underline{M}^+$-16) (5%), 114, 101, 69 and 55 (100%) (Found: $\underline{M}^+$-16, 142.1781. $C_7H_{12}NO_2$ requires $\underline{M}$-16, 142.1773) (Found: C, 43.21; H, 8.99; N, 14.49. $C_7H_{14}N_2O_2 \cdot 2H_2O$ requires C, 43.29; H, 9.28; N, 14.43%).

(±)-2-Methylputrescine (183).- Racemic 3-methyladipic diamide (181) dihydrate (3.9g, 20.1 mmol) was reacted in the manner of (178) to form the dibenzoyl derivative (182) (Found: C, 73.54; H, 6.87; N, 9.16. $C_{19}H_{22}N_2O_2$ requires C, 73.55; H, 7.10; N, 9.03%). This (±)-$\underline{N}$,$\underline{N}'$-dibenzoyl-2-methylputrescine (179) was hydrolysed with concentrated hydrochloric acid under sealed tube conditions to provide the title compound (183) as the dihydrochloride salt (2.9g, 16.4 mmol, 81.6%), m.p. 142-3°C; $\nu_{max.}$ (KBr) 3081, 3025, 1475 and 1452 cm^{-1}; δ_H (90 MHz, D_2O) 1.15 (3H, d, $\underline{J}$ 7.0 Hz, 5-H_3), 1.83 (3H, m, 2-H and 3-H_2), and 3.10 p.p.m. (4H, m, 1- and 4-H_2); δ_C (25 MHz, D_2O with 1,4-dioxan as internal standard at 69.1 p.p.m.) 19.4 (C-5), 31.1 (C-3), 34.5 (C-2), 42.8 (C-4) and 47.9 p.p.m. (C-1); $\underline{m}/\underline{z}$ 103 ($\underline{M}H^+$) (1%), 91, 67, 57 and 36 (100%) (Found: $\underline{M}H^+$, 103.1864. $C_5H_{15}N_2$ requires $\underline{M}H$, 103.1869) (Found: C, 34.46; H, 9.21; N, 16.29. $C_5H_{16}N_2C\ell_2$ requires C, 34.28; H, 9.14; N, 16.00%).

(±)-[^{2}H]-3-Methyladipic acid.- Sodium hydroxide (0.53g, 13.3 mmol) was dissolved in deuterium oxide (50 ml) and racemic 3-methyladipic acid (180) (1g, 6.25 mmol) was added. The reaction was heated at reflux temperature for 14h, then was allowed to cool, acidified and the acid was extracted with diethyl ether (4 x 50 ml). The organic layers were dried over anhydrous sodium sulphate and evaporated to dryness to leave a white solid. The 90 MHz ^{1}H n.m.r. spectrum of this solid in D_2O was identical with that of the starting acid (180). No exchange seemed to have taken place as evidenced by integration.

Racemic 3-methyladipic acid (180) (1g, 6.25 mmol) was dissolved in dilute hydrochloric acid solution (4M) in D_2O (50 ml), then heated at reflux temperature for 18h, and allowed to cool. Extraction of the substituted adipic acid with diethyl ether (4 x 50 ml), was followed by removal of the solvent in vacuo. The 90 MHz ^{1}H n.m.r. spectrum of the resultant white solid was also identical in all respects to that of the original acid (180), including the integration of the peaks.

Racemic 3-methyladipic acid (180) (500 mg, 3.12 mmol) was dissolved in deuterium oxide (25 ml) in a tube. The vessel was sealed and stirred at 180°C for 24h behind a safety screen. The reaction was allowed to cool over 6h, after which time the solvent was removed under reduced pressure, and a white solid was obtained. The 90 MHz ^{1}H n.m.r. spectrum of the product was identical in most respects with that of the starting material (180), but the integration of the signals due to 2-H_2 and 5-H_2 was only ~ 75% of the magnitude of the signals of the unlabelled material.

[^{3}H]-3-Methyladipic acids.- Racemic 3-methyladipic acid (180) (500 mg, 3.12 mmol) was dissolved in tritiated water (T$_2$O) (4 ml, ~ 15 mCi) and a radioactive tritium (^{3}H) label was inserted by the above experimental procedure to produce a sample of [^{3}H]-(180) (448 mg, 2.80 mmol, 89.7%) with an activity of 25.7 μCi (9.2 μCi mmol).

Similar treatment of R-(+)-methyladipic acid (177) (500 mg, 3.12 mmol) provided [^{3}H]-(177) (477 mg, 2.98 mmol, 95.4%) with an activity of 8.8 μCi (2.9 μCi mmol^{-1}).

The racemic [^{3}H]-3-methyladipic acid (180) was reacted as described previously to provide [^{3}H]-(181) (420 mg, 2.16 mmol, 77.3%; 19.2 μCi, 8.8 μCi mmol^{-1}), and Hofmann conditions[193] furnished [^{3}H]-(183) (285 mg, 1.63 mmol, 75.3%; 13.5 μCi, 8.3 μCi mmol^{-1}).

The sample of [^{3}H]-R-(+)-3-methyladipic acid (177) was used to furnish [^{3}H]-(178) (447 mg, 2.30 mmol, 77.3%; 7.2 μCi, 3.0 μCi mmol^{-1}), and Hofmann rearrangement[193] eventually provided [^{3}H]-(176) (340 mg, 1.93 mmol, 84.1%, 5.0 μCi, 2.6 μCi mmol^{-1}).

7.3.5.2   Feeding of [^{3}H]-2-methylputrescines

Cynoglossum officinale and Emilia flammea were grown on open ground by the Botany Department, Glasgow University, and Senecio pleistocephalus was grown in a greenhouse.   All radioactive mixtures were fed as sterile aqueous solutions (1 ml) by the wick method, over 2 successive days.   The plants were allowed to grow for one week from the last day of feeding, prior to harvesting, methanolic extraction and isolation of the respective alkaloids.   All putrescines were used as their dihydrochloride salts, and were fed as detailed in Table 7.   The radio-

Plant	^{3}H-METHYL PUTRESCINE FED	AMOUNT FED	ACTIVITY FED (µCi)	UNLABELLED PUTRES. ADDED	RATIO FED ^{3}H:^{14}C	ALKALOID ISOLATED	RATIO FOUND ^{3}H:^{14}C	% INCORP. PER C$_4$-UNIT ^{3}H	^{14}C
CYNOGLOSSUM	(176)	50mg 0·29mmol	0·20	25mg	3·78	(62)	1·06	0·26	0·93
OFFICINALE	(183)	40mg 0·23mmol	0·15	20mg	11·65	(62)	3·06	0·21	0·81
SENECIO	(176)	54mg 0·31mmol	0·20	28mg	3·99	(175)	1·15	0·69	2·40
PLEISTOCEPHALUS	(183)	40mg 0·23mmol	0·15	20mg	11·65	(175)	4·58	0·83	2·11
EMILIA	(176)	45mg 0·26mmol	0·20	25mg	3·38	(184)	1·12	0·44	1·33
FLAMMEA	(183)	35mg 0·20mmol	0·15	20mg	8·92	(184)	2·18	0·34	1·40

Table 7

active mixtures were diluted with unlabelled methylputrescine before feeding.

Radioactive scintillation counting was used to measure the ^{3}H:^{14}C ratios present in the mixture prior to feeding and in the alkaloids isolated after extraction. Radioscans of the two samples of (175) isolated on silica gel G plates of 0.25 mm thickness developed with chloroform-methanol-conc. ammonia (85:14:1) showed only one radioactive band (R$_f$ 0.30) in both cases. This was coincident with a non-radioactive sample of rosmarinine (175).

7.3.6 Synthesis and Feeding of 2-Fluoroputrescines

7.3.6.1 Synthesis of (±)-2-fluoroputrescine

(±)-Diethylmalate (191).- Racemic malic acid (40g, 0.298 mole) was dissolved in a rapidly stirred solution of ethanol (125 ml). Toluene (80 ml), conc. hydrochloric acid (1 ml), and a further amount of ethanol (50 ml) were added to the reaction mixture, which was then heated at 40°C for 8h.[169] The azeotropic mixture of ethanol, toluene and water was distilled off under reduced pressure below 50°C. Toluene (80 ml) and ethanol (125 ml) were added to the residue, and the solution heated at 40°C for 8h again. The solvent was removed in vacuo as before, and the residue was distilled (b.p. 0.30 mm Hg/120°C) (lit.[195] 27 mm Hg/150-2°C) to yield (±)-diethylmalate (191) as a clear oil (50.9g, 0.268 moles, 89.9%); $\nu_{max.}$ (thin film) 3485, 2982, 2941, and 1737 cm^{-1}; δ_H (90 MHz) 1.28 and 1.30 (6H, both t, J 7.1 Hz, 2 x CH$_3$), 2.82 (2H, d, J 5.2 Hz, 3-H$_2$), 3.76 (1H, br s, OH), 4.13

and 4.29 (4H, both q, $\underline{J}$ 7.0 Hz, 2 x C$\underline{H}_2$), and 4.53 p.p.m. (1H, t, $\underline{J}$

5.0 Hz, 2-H); δ_C (25 MHz) 14.2 (C$\underline{H}_3$), 39.0 (C-3), 61.0 and 61.8

(C$\underline{H}_2$), 67.6 (C-2), 170.8 (C-4), and 173.6 p.p.m. (C-1); $\underline{m/z}$ 190

($\underline{M}^+$) (0.2%), 145, 117, 89, 71, and 43 (100%) (Found: $\underline{M}^+$, 190.0875;

C, 50.27; H, 7.29. $C_4H_{14}O_5$ requires $\underline{M}$, 190.0858; C, 50.52; H,

7.42%).

<u>(±)-Diethylfluorosuccinate (192)</u>.- Diethylaminosulphur

trifluoride (4.0 ml, 4.88g, 30.3 mmol) was added <u>via</u> syringe to dry

dichloromethane (100 ml) at -75°C with stirring under N$_2$. Racemic

diethylmalate (191) (4.5g, 23.7 mmol) in dichloromethane (40 ml) was

added dropwise over 40 min. <u>via</u> a syringe, and the reaction temperature

allowed to warm from -75°C up to 0°C over 14h.[134] The reaction was

quenched with ice-cold water (100 ml) and ethyl acetate (350 ml) and

the solution was stirred vigorously for 4h. The aqueous layer was

separated and extracted with ethyl acetate (100 ml). The combined

organic layers were washed with water (100 ml), dried over anhydrous

sodium sulphate, and filtered. The solvent was removed <u>in</u> <u>vacuo</u> to

leave a light brown oil, which was distilled (b.p. 100°C/1.1 mm Hg)

(lit.[174] b.p. 72°C/1.0 mm Hg) to furnish (±)-diethylfluorosuccinate

(192) as a colourless liquid (4.44g, 23.1 mmol, 97.4%); $\nu_{max.}$ (CHCℓ_3)

3024, 2987, 1737, 1185, and 1100 cm^{-1}; δ_H (90 MHz) 1.27 and 1.31

(6H, both t, $\underline{J}$ 7.0 Hz, 2 x C$\underline{H}_3$), 2.82 (2H, dd, $\underline{J}$ 23.3 and 5.9 Hz,

3-H$_2$), 4.16 and 4.22 (4H, both q, $\underline{J}$ 7.0 Hz, 2 x C$\underline{H}_2$), and 5.15 p.p.m.

(1H, dt, $\underline{J}$ 47.6 and 5.9 Hz, 2-H); δ_C (25 MHz) 13.7 (C$\underline{H}_3$), 36.9

(C-3), 60.9 and 61.5 (C$\underline{H}_2$), 85.1 (C-2), 164.6 (C-4) and 168.7 p.p.m.

(C-1); $\underline{m}/\underline{z}$ 191 ($\underline{M}^+$-1) (1%), 173 (100%), 145, 128, 127, and 117

(Found: $\underline{M}^+$-1, 191.0827. $C_4H_{12}O_4F$ requires $\underline{M}$-1, 191.0819)

(Found: C, 49.97; H, 6.92; F, 9.99. $C_4H_{13}O_4F$ requires C, 50.00;

H, 6.82; F, 9.84%).

($\pm$)-2-Fluorobutane-1,4-diol (193)

<u>Run 1</u>: Lithium aluminium hydride (450 mg, 11.8 mmol) was added to
dry diethyl ether (20 ml) at -20°C under a N_2 atmosphere. Racemic
diethylfluorosuccinate (192) (500 mg, 2.6 mmol) was dissolved in diethyl
ether (5 ml) and added dropwise <u>via</u> syringe over 5 min to the reaction
mixture. The temperature of the solution was allowed to warm up to
room temperature (20°C) over 12h, and the reaction quenched by the
addition of saturated aqueous sodium sulphate (1 ml). The solution was
filtered, ethyl acetate (50 ml) and methanol (50 ml) were added, and
the mixture was filtered again. The filtrate was concentrated under
reduced pressure to leave an amber oil, which was distilled (b.p. 0.55
mm Hg/200°C) to leave ($\pm$)-2-fluorobutane-1,4-diol (193) as a clear
liquid (220 mg, 2.1 mmol, 79.1%); $\nu_{max.}$ (thin film) 3360, 2940, 2878,
1296, 1160, and 1075 cm^{-1}; δ_H (90 MHz, D_2O) 2.08 (2H, m, 3-H_2),
3.69 (4H, m, 1- and 4-H_2), and 4.52 (1H, dm, $\underline{J}$ 48.2 Hz, 2-H); δ_C
(25 MHz, D_2O) 34.1 (C-3), 64.0 (C-4), 66.2 (C-1), and 68.9 p.p.m.
(C-2); $\underline{m}/\underline{z}$ 108 ($\underline{M}^+$) (0.1%), 89, 63, and 45 (100%) (Found: $\underline{M}^+$,
108.0565; C, 44.56; H, 8.24; F, 17.82. $C_4H_9O_2F$ requires $\underline{M}$,
108.0586; C, 44.44; H, 8.39; F, 17.57%).

Run 2:   Racemic diethylfluorosuccinate (192) (500 mg, 2.6 mmol) in dry

toluene (5 ml) was added to rapidly stirred toluene (5 ml) at -30°C

under a N_2 atmosphere.   DIBAL (1.5$\underline{M}$, 20 ml) in toluene was dropped

into the reaction mixture over 10 min, and the stirred solution was

allowed to warm up to 20°C (r.t.) over 14h.   Ethyl acetate (10 ml) and

acetone (20 ml) were added, and the reaction mixture was then poured

onto a slurry of celite 535 (20g) in acetone (25 ml).   This slurry was

stirred vigorously until it formed a gel, which was broken by the

addition of methanol (35 ml) and water (35 ml).   Throughout the

addition, the solution was stirred and a gas was given off.   When the

gas evolution was complete, the slurry was filtered and the solid was

washed with methanol (4 x 50 ml).   The combined filtrates were

concentrated in vacuo to leave an amber oil, which was distilled (b.p.

180°C/0.46 mm Hg) to leave (±)-2-fluorobutane-1,4-diol (193) as a clear

liquid (240 mg, 2.2 mmol, 85.1%) which was analysed as described

previously.

(±)-2-Fluoroputrescine (189).- Racemic 2-fluorobutane-1,4-

diol (193) (0.90g, 8.3 mmol) was taken up in dry THF (10 ml) under

an inert argon atmosphere, and to this was added a solution of

hydrazoic acid (1.05$\underline{M}$, 21 ml) in benzene over 5 min with cooling (0°C)

and stirring.[171]   Diethylazodicarboxylate (DEAD) (4.44g, 21.9 mmol)

in dry THF (10 ml) was added cautiously, and the mixture was stirred

for 30 min.[172]   Triphenylphosphine (11.54g, 44.0 mmol) in dry THF

(50 ml) was added dropwise at such a rate that the reaction temperature

did not exceed 40°C, and the reaction mixture was then stirred at 50°C

for 3h.[170]   After cooling to room temperature, water (2 ml) was added

and the solution was heated at 50°C for a further 3h. The solvents
were removed in vacuo, and the residue was partitioned between
hydrochloric acid solution (1M, 80 ml) and dichloromethane (80 ml).
After separation, the acidic layer was washed with a further volume
(80 ml) of dichloromethane, and the layers were again separated. The
aqueous phase was then evaporated to dryness under reduced pressure
to furnish a white solid.   The title compound (189) was crystallised
from acetone:95% aqueous ethanol (1:1) (0.84g, 4.7 mmol, 56.2%), m.p.
> 225°C (dec.); $\nu_{max.}$ (KBr) 3080, 3020, 1472, 1451, and 1205 cm^{-1};
δ_H (90 MHz, D$_2$O) 1.65 (2H, m, 3-H$_2$), 3.63 (4H, m, 1- and 4-H$_2$),
and 4.44 p.p.m. (1H, dm, J 50.0 Hz, 2-H); δ_C (25 MHz, D$_2$O with
1,4-dioxan as standard at 69.1 p.p.m.) 32.5 (C-3), 44.4 (C-4), 44.9
(C-1), and 60.5 (C-2); δ_F (188 MHz, D$_2$O) - 188.2 p.p.m. (relative
to CFCl$_3$, δ_F = 0 p.p.m.); m/z 107 (MH$^+$) (0.1%), 88, 73, 45, 38, and
36 (100%) (Found: MH$^+$, 107.0967. C$_4$H$_{12}$N$_2$F requires MH, 107.0984)
(Found: C, 26.98; H, 7.60; F, 10.14. C$_4$H$_{13}$N$_2$FCl$_2$ requires C,
26.83; H, 7.32; F, 10.61%).

7.3.6.2   Synthesis of 'enantiomeric' 2-fluoroputrescines

R-(+)-Diethylmalate (194).- The title compound was prepared[169]
in the manner of the racemate (191), and distilled (b.p. 140°C/0.44 mm
Hg) as a clear oil (1.41g, 7.40 mmol, 99.2%); $[\alpha]_D^{20}$ + 5.75° (c 14.0,
CHCl$_3$); $\nu_{max.}$ (thin film) 3490, 2980, 2940, and 1738 cm^{-1}; δ_H (90
MHz) 1.29 and 1.30 (6H, both t, J 7.1 Hz, 2 x CH$_3$), 2.80 (2H, d, J
5.1 Hz, 3-H$_2$), 3.75 (1H, br s, OH), 4.20 and 4.34 (4H, both q, J
7.1 Hz, 2 x CH$_2$), and 4.50 p.p.m. (1H, t, J 5.1 Hz, 2-H); δ_C (25

MHz) 14.3 ($\underline{C}H_3$), 39.0 (C-3), 61.0 and 61.8 ($\underline{C}H_2$), 67.3 (C-2), 170.8 (C-4) and 173.5 p.p.m. (C-1); $\underline{m}/\underline{z}$ 190 ($\underline{M}^+$) (0.1%), 145, 117 (100%), 89 and 71 (Found: $\underline{M}^+$, 190.0841; C, 50.44; H, 7.49. $C_8H_{14}O_5$ requires $\underline{M}$, 190.0858; C, 50.52; H, 7.42%).

S-(-)-Diethylmalate (196).- The title compound was synthesised as described previously and distilled (b.p. 128°C/0.36 mm Hg) as a clear oil (1.42g, 7.41 mmol, 99.3%); $[\alpha]_D^{20}$ - 5.90° ($\underline{c}$ 14.0, CHCℓ_3). All other analytical data were as above.

(-)-Diethylfluorosuccinate (195).- $\underline{R}$-(+)-Diethylmalate (194) (1.41g, 7.40 mmol) was converted into the diethylfluorosuccinate (195) by treatment[134] with DAST as described earlier, to furnish the title compound (195) as a clear oil (0.90g, 4.69 mmol, 63.2%) (b.p. 120°C/ 0.95 mm Hg); $[\alpha]_D^{22}$ - 4.0° ($\underline{c}$ 4.5, CHCℓ_3); $\nu_{max.}$ (CHCℓ_3) 3025, 2990, 1770, 1746, 1280, 1181, and 1100 cm^{-1}; δ_H (90 MHz) 1.28 and 1.33 (6H, both t, $\underline{J}$ 7.4 Hz, 2 x C$\underline{H}_3$), 2.95 (2H, dd, $\underline{J}$ 24.0 and 6.1 Hz, 3-H$_2$), 4.19 and 4.28 (4H, both q, $\underline{J}$ 7.0 Hz, 2 x C$\underline{H}_2$), and 5.35 p.p.m. (1H, dt, $\underline{J}$ 47.2 and 5.5 Hz, 2-H); δ_C (25 MHz) 14.0 ($\underline{C}H_3$), 37.1 (C-3), 60.9 and 61.3 ($\underline{C}H_2$), 85.1 (C-2), 164.6 (C-4), and 168.6 p.p.m. (C-1); $\underline{m}/\underline{z}$ 191 ($\underline{M}^+$-1) (0.8%), 173, 145, 128, 127, and 117 (100%) (Found: $\underline{M}^+$-1, 191.0806. $C_4H_{12}O_4F$ requires $\underline{M}$-1, 191.0819) (Found: C, 50.25; H, 6.75; F, 10.01. $C_4H_{13}O_4F$ requires C, 50.00; H, 6.82; F, 9.84%).

(+)-Diethylfluorosuccinate (197).- The title compound (197) was prepared from $\underline{S}$-(-)-diethyl malate (196) and distilled (b.p. 105°C/ 0.90 mm Hg) as a clear oil (0.91g, 4.74 mmol, 63.4%); $[\alpha]_D^{22}$ + 3.9° ($\underline{c}$ 9.1, CHCℓ_3). The other data were identical to those for the previous compound (195).

(-)-Diethylfluorosuccinate (195) (100 mg, 0.52 mmol) was dissolved in CDCℓ_3 (1 ml) and $\underline{R}$-(+)-phenylethylamine (198) (126 mg, 1.04 mmol) was added. The 90 MHz ^{1}H n.m.r. spectrum was partially analysed as follows; δ_H 2.49 and 2.76 (2H, d, $\underline{J}$ 24.3 Hz, 3-H$_2$), 3.61 and 4.51 p.p.m. (1H, dt, $\underline{J}$ 80.5 and 6.0 Hz, 2-H) (Figure 10b).

(+)-Diethylfluorosuccinate (197) (100 mg, 0.52 mmol) was dissolved in CDCℓ_3 (1 ml) and $\underline{R}$-(+)-phenylethylamine (198) (126 mg, 1.04 mmol) was added. The 90 MHz ^{1}H n.m.r. spectrum was partially analysed as follows; δ_H 2.36 and 2.74 (2H, d, $\underline{J}$ 34.2 Hz, 3-H$_2$), 3.56 and 4.42 p.p.m. (1H, dt, $\underline{J}$ 77.4 and 6.0 Hz, 2-H) (Figure 10c).

When racemic diethylfluorosuccinate (192) (100 mg, 0.52 mmol) was dissolved in CDCℓ_3 (1 ml) and $\underline{R}$-(+)-phenylethylamine (198) (126 mg, 1.04 mmol), the 90 ^{1}H n.m.r. spectrum was almost a direct addition of the spectra arising from the above two experiments (Figure 10a); δ_H 2.40 and 2.73 (2H, dm, 3-H$_2$), 3.56 and 4.49 p.p.m. (1H, dm, 2-H). The ^{1}H n.m.r. spectra, and in particular the integrations of the signals due to 3-H$_2$, suggested that the enantiomeric excess (e.e.) of (195) and (197) is greater than 95% in both cases.

(+)-2-Fluorobutane-1,4-diol (199).- The title compound was prepared in the same way (Run 2) as was racemic 2-fluorobutane-1,4-diol (193), and distilled (b.p. 0.35 mm Hg/135°C) as a clear oil (0.38g, 3.52 mmol, 75.1%); $[\alpha]_D^{23}$ + 4.3° (c 1.2, MeOH).

(-)-2-Fluorobutane-1,4-diol (200).- The diol was furnished as previously, by the use of DIBAL, as a clear oil (0.40g, 3.70 mmol) (b.p. 0.40 mm Hg/150°C); $[\alpha]_D^{23}$ - 3.0° (c 1.0, MeOH). Both this compound (200) and the previous diol (199) gave similar data to those of the racemic 2-fluorobutane-1,4-diol (193).

(+)-2-Fluoroputrescine (201).- Conversion of (+)-2-fluorobutane-1,4-diol (199) into the diamine (201) proceeded as for the racemic compound (193), and provided an off-white solid (201) (1.13g, 6.30 mmol, 80.1%); $[\alpha]_D^{23}$ + 4.5° (c 1.8, H_2O).

(-)-2-Fluoroputrescine (202).- The title compound was synthesised from (-)-2-fluorobutane-1,4-diol (200) by the use of the described method, as an off-white solid (202) (1.06g, 5.92 mmol, 79.9%); $[\alpha]_D^{23}$ - 4.9° (c 2.2, H_2O). Both the diamines were obtained and analysed as their dihydrochloride salts.

(±)-Fluorosuccinic acid.- Racemic diethyl 2-fluorosuccinate (192) was hydrolysed[174] with aqueous sulphuric acid (2M, 10 ml), to furnish (±)-fluorosuccinic acid, m.p. 142-144°C (ethyl acetate) (lit.[174] 144-145°C); $\nu_{max.}$ (KBr) 3000, 1738, and 1230 cm^{-1}; δ_H (90 MHz, d_6-DMSO) 2.84 (2H, ddd, J 26.1, 17.2, and 4.9 Hz, 3-H_2), 5.20 (1H, dd, J 47.2 and 5.0 Hz, 2-H), and 12.05 p.p.m. (2H, br s, 2 x CO_2H);

$\underline{m}/\underline{z}$ 136 ($\underline{M}^+$) (0.1%), 118, 117, 98, 91, 89, 71, 54, and 44 (100%) (Found: $\underline{M}^+$, 136.0147. $C_4H_5O_4F$ requires $\underline{M}$, 136.0172).

When (-)-diethylfluorosuccinate (195) and (+)-diethylfluoro-succinate (197) were hydrolysed as in the literature method,[174] both the samples of fluorosuccinic acid, (128) and (203), could not be obtained crystalline.

The c.d. spectra of (128) and (203) were run by Dr. Alex. F. Drake, University of London. The compounds were dissolved in methanol and checked over the 185-300 nm range. The masking of the expected peaks [lit.[132] (+)-fluorosuccinic acid, ($\underline{c}$ 0.15, MeOH), positive Cotton effect, max. at 232.5 nm, $(\phi)^{20°} = + 1114°$] was ascribed to the impurities present in the samples.

7.3.6.3   Feeding of 2-Fluoroputrescines

Cynoglossum officinale and Emilia flammea were grown from seeds on open ground, and Senecio pleistocephalus was propagated from stem cuttings and grown in a greenhouse. All 2-fluoroputrescines were fed as sterile aqueous solutions (1 ml) by the wick method, over two successive days. The plants were allowed to grow for one week from the last day of feeding, prior to harvesting, methanolic extraction and isolation of the alkaloids. All bases were fed as their dihydrochloride salts.

A sample of (+)-2-fluoroputrescine (201) dihydrochloride (100 mg) was fed to one C. officinale plant. The same amount of (201) was fed to one E. flammea and S. pleistocephalus plant. The same quantity of (-)-2-fluoroputrescine (202) dihydrochloride was fed to

one each of the three plant species.

The 188 MHz ^{19}F n.m.r. spectra of the alkaloid samples

isolated were run in CHCℓ_3/CFCℓ_3, and only one small signal at ca.

186.7 p.p.m. was observed.

7.4 Experimental to Chapter Six

7.4.1 General

The alkaloids were extracted by the procedure followed for

the isolation of (+)-echinatine (62) from Cynoglossum officinale (Section

7.2.1). The h.p.l.c. solvent system used was diethyl ether: [5%

(2.5% conc. ammonia in water) in methanol],[182] in a measured ratio

(x:y). The eluting solvents were degassed with helium for 5 min before

use. The columns were flushed with the particular solvent system used

in the experiment prior to loading of the column, and the injecting

needles were flushed prior to use.

The solvents were pumped through by a Perkin Elmer Series

400, and the samples were injected by a Perkin Elmer ISS 100 using a

150 μℓ loop for analytical h.p.l.c. and a 2 ml loop for preparative

h.p.l.c. Detection was by using a Perkin Elmer LC 90 set at a wave-

length of 220 nm, and the fractions were collected in test tubes by hand.

The column used for analytical h.p.l.c. was a Perkin Elmer 3 x 3 silica

column and the column used in preparative h.p.l.c. was a Knauer Li-

Chrosol Si 60 (length 250 mm, ID 16 mm, packing 7 μm).

7.4.2   Analytical h.p.l.c.

An aliquot (100 mg) of the alkaloid mixture isolated from

Cynoglossum officinale was taken up in the h.p.l.c. solvent system

(75:25) (0.1 ml), and was then filtered through a pad of glass filter

paper.   A small sample (1 μℓ) was injected onto the analytical column

and then elution was carried out with the solvent system (75:25).   The

h.p.l.c. trace (Figure 11) was recorded over a period of 10 min, with

the solvent pumped through the column at a rate of 1 ml min^{-1}.   The

retention times (R_T, min) and percentage composition of the mixture,

were as follows: 3.78 (92.9%), 5.78 (3.6%), and 8.02 (3.5%).   The R_T

(3.78) alkaloid was (+)-echinatine (62), the R_T (5.78) compound was

the minor alkaloid, and R_T (8.02) was due to the presence of a small

quantity of (+)-heliotridine (3), as seen by t.l.c. of the fractions.

By the same procedure as above, a portion (100 mg) of the

extract from Cynoglossum australe was dissolved in the h.p.l.c. solvent

system (75:25) (1.0 ml), and filtered.   An injection (10 μℓ) onto the

analytical column was followed by elution with the solvent system

(75:25).   The h.p.l.c. trace (Figure 12) was recorded over a period

of 10 min, with a pumping rate of 1 ml min^{-1}.   The R_T values (min)

were as follows: 7.71 (71.4%) and 4.21 (28.6%).   The alkaloid eluted

first was (+)-cynaustine (173), followed by (+)-cynaustraline (172) as

checked by t.l.c. of the fractions.

Cynoglossum nervosum (280g) was grown by the Botanical

Gardens, Edinburgh, and was extracted to yield a mixture (250 mg,

0.09%) of pyrrolizidine alkaloids.   T.l.c. of the mixture, showed two

spots (R_f 0.30 and 0.26) upon staining with o-chloranil and Ehrlich's

reagent.[95]   Column chromatography upon basic alumina with a variety

of solvent systems (dichloromethane and/or chloroform with methanol;

diethyl ether, toluene and methanol) could not separate this mixture.

The mixture (200 mg) was dissolved in the h.p.l.c. solvent system

(75:25), filtered and an aliquot (1 $\mu\ell$) was injected onto the analytical

h.p.l.c. column.   The column was eluted by pumping the solvent

system (75:25) through at a rate of 1 ml min^{-1} and the h.p.l.c. trace

(Figure 13) was recorded over a period of 10 min.   Three peaks due to

alkaloids were clearly visible; R_T: 3.98 (58.3%), 5.03 (15.2%), and

6.36 min (26.5%).   The alkaloid mixture was separated by preparative

h.p.l.c. (Section 7.4.3).

Senecio glaberrimus was grown in South Africa, and there

extracted (by Dr. D.J. Robins).   The crude extract was partitioned

between chloroform (5 ml) and saturated aqueous sodium bicarbonate

(5 ml).   The layers were separated, and the aqueous phase washed

with chloroform (3 x 5 ml).   The combined organic layers were dried

over anhydrous magnesium sulphate, filtered and evaporated to dryness

under reduced pressure to provide a mixture (300 mg) of pyrrolizidine

alkaloids as an oil.   Column chromatography on basic alumina could not

effect separation.   T.l.c. of the mixture gave rise to three alkaloid

stains with R_f 0.34, 0.38, and 0.56.   The application of a sample (20

mg) dissolved in the solvent system (75:25) (50 $\mu\ell$) to the analytical

h.p.l.c. column, gave rise to the h.p.l.c. trace in Figure 14a when the

pumping rate was 1 ml min^{-1} for 10 min.   If the peaks due to the

presence of solvents are deducted from this analytical h.p.l.c. trace,

then four peaks due to alkaloids are evident; R_T: 1.57, 2.21, 2.63,

and 2.92 min. In order to attempt a better separation, the polarity of the solvent system was reduced. Accordingly a sample (20 mg) of the mixture was dissolved in the solvent system (90:10) and applied onto the analytical h.p.l.c. column. The elution with the solvent system (90:10) was carried out at the rate of 1 ml min^{-1} for 20 min, and the spectrum (Figure 14b) was recorded. The new R_T values were as follows: 2.71, 4.34, 5.47, and 6.84 min.

7.4.3 Preparative h.p.l.c.

The alkaloid mixture (180 mg) isolated from Cynoglossum nervosum (Section 7.4.2) was dissolved in the h.p.l.c. solvent system (75:25) (1.0 ml). A portion (0.34 ml) was applied onto the Knauer LiChrosol Si60 column and eluted with the solvent system (75:25) at a rate of 5 ml min^{-1} for 20 min. The h.p.l.c. trace was recorded and the fractions collected every 30s. The h.p.l.c. trace revealed only a partial separation as the peaks were overlapping. T.l.c. of the fractions confirmed this. The relevant fractions were combined, added to the original mixture and evaporated to dryness.

This alkaloid mixture (170 mg) was dissolved in the h.p.l.c. solvent system (90:10) (1.0 ml), and a small sample (1 μl) was injected onto the analytical h.p.l.c. column. Elution was carried out with the solvent system (90:10) flowing at 1 ml min^{-1} for 20 min, and the trace was recorded. A good separation was again achieved. A larger volume (0.4 ml) of the mixture was applied onto the preparative h.p.l.c. column, and eluted with the solvent system (90:10) at 5 ml min^{-1} for 30 min, with fractions collected ever 30s. The trace was recorded

(Figure 15), and revealed the efficient separation of the three alkaloids.

R_T: 13.28, 16.68, and 19.51 min. T.l.c. of the fractions confirmed

this separation, and the pure alkaloids were isolated and characterized

by combining the relevant fractions and evaporating to dryness in vacuo.

The first alkaloid eluted (25 mg), R_T: 3.98 (75:25) and

13.28 min (90:10), was identical in all characterization data to an

authentic sample of (+)-echinatine (62).

The second alkaloid isolated (14 mg), R_T: 5.03 (75:25) and

16.68 min (90:10), analysed for (-)-heliotrine (33), m.p. 124-125°C

(acetone) (lit.[195] m.p. 125-126°C); $[\alpha]_D^{20}$ - 4.1° (c 14, CHCl$_3$)

(lit.[195] $[\alpha]_D^{20}$ - 3.75°, EtOH); $\nu_{max.}$ (CHCl$_3$) 3200, 2980, 2840, 1726,

1265, and 1100 cm^{-1}; δ_H (200 MHz) 1.20 and 1.24 (6H, both d, J 6.9

Hz, 16- and 17-H$_3$), 1.29 (3H, d, J 6.5 Hz, 14-H$_3$), 2.19 (2H, m, 6-H$_2$),

2.21 (1H, dq, J 6.9 Hz, 15-H), 2.68 (1H, m, 5-H), 3.23 (3H, s, 18-H$_3$),

3.28 (1H, m, 5-H), 3.38 (1H, dd, J 3.0 and 1.6 Hz, 3-H), 3.89 (1H, dd,

J 3.0 and 1.5 Hz, 3-H), 3.98 (1H, q, J 6.5 Hz, 13-H), 4.10 (2H, br s,

2 x OH), 4.19 (1H, m, 8-H), 4.31 (1H, m, 7-H), 4.72 and 5.08 (2H,

ABq, J 14.1 Hz, 9-H$_2$), and 5.93 p.p.m. (1H, br s, 2-H); δ_C (50 MHz)

13.1 (C-14), 16.4 and 16.9 (C-16 and -17), 33.0 (C-15), 33.9 (C-6),

54.2 (C-5), 57.1 (C-18), 62.0 and 62.8 (C-3 and -9), 75.6 (C-7), 78.5

(C-8), 80.0 (C-13), 82.5 (C-12), 127.3 (C-2), 136.3 (C-1), and 176.0

p.p.m. (C-11); m/z 313 (M$^+$) (1%), 254, 211, 181, 139, 138, 119, 111,

and 94 (100%) (Found: M$^+$, 313.1874. C$_{16}$H$_{27}$NO$_5$ requires M, 313.1889).

The final compound (7 mg) R_T: 6.36 (75:25) and 19.51 min

(90:10), analysed for (+)-rinderine (213), m.p. 98-99°C (acetone)

(lit.[195] 100-101°C); $[\alpha]_D^{20}$ + 22.9° (c 7, CHCl$_3$) (lit.[195] + 24.6°, EtOH);

$\nu_{max.}$ (CHCℓ_3) 3400, 2976, 1725, and 1230 cm^{-1}; δ_H (200 MHz) 0.99 and 1.05 (6H, both d, $\underline{J}$ 6.9 Hz, 16- and 17-H$_3$), 1.24 (3H, d, $\underline{J}$ 6.6 Hz, 14-H$_3$), 1.99 (2H, m, 6-H$_2$), 2.25 (1H, dq, $\underline{J}$ 6.8 Hz, 15-H), 2.60 (1H, m, 5-H), 3.23 (1H, m, 5-H), 3.38 (1H, dd, $\underline{J}$ 2.9 and 1.4 Hz, 3-H), 3.81 (1H, br s, O$\underline{H}$), 3.83 (1H, dd, $\underline{J}$ 2.9 and 1.5 Hz, 3-H), 3.90 (2H, br s, 2 x O$\underline{H}$), 3.95 (1H, m, 8-H), 4.00 (1H, q, $\underline{J}$ 6.6 Hz, 13-H), 4.14 (1H, m, 7-H), 4.70 and 4.89 (2H, ABq, $\underline{J}$ 13.7 Hz, 9-H$_2$), and 5.53 p.p.m. (1H, br s, 2-H); δ_C (50 MHz) 17.0 and 17.2 (C-16 and -17), 17.9 (C-14), 33.0 (C-15), 34.1 (C-6), 54.3 (C-5), 61.9 and 62.2 (C-3 and -9), 69.5 (C-13), 74.9 (C-7), 80.1 (C-8), 83.4 (C-12), 126.8 (C-2), 133.9 (C-1), and 177.2 p.p.m. (C-11); $\underline{m/z}$ 299 ($\underline{M}^+$) (2%), 157, 156, 139, 138, 137, 120, and 119 (100%) (Found: $\underline{M}^+$, 299.1719. C$_{15}$H$_{25}$NO$_5$ requires $\underline{M}$, 299.1733).

REFERENCES

1. L.B. Bull, C.C.J. Culvenor, and A.T. Dick, "The Pyrrolizidine Alkaloids", North-Holland, Amsterdam, 1968.

2. D.J. Robins, Fortschr. Chem. Org. Naturstoffe, 1982, 41, 115.

3. A.R. Mattocks, "Phytochemical Ecology", ed. J.B. Harborne, Academic Press, London, 1980.

4. J.E. Saxton, "The Alkaloids", ed. J.E. Saxton, Specialist Periodical Reports, The Chemical Society, London, 1971-75, Vols. 1-5; D.H.G. Crout, "The Alkaloids", ed. M.F. Grundon, Specialist Periodical Reports, The Chemical Society, London, 1976-77, Vols. 6-7; D.J. Robins, "The Alkaloids", ed. M.F. Grundon, Specialist Periodical Reports, The Chemical Society, London, 1978-83, Vols. 8-13.

5. D.J. Robins, Nat. Prod. Rep., 1984, 1, 235; 1985, 2, 294; 1986, 3, 297.

6. A.R. Mattocks, "Chemistry and Toxicology of Pyrrolizidine Alkaloids", Academic Press, London, 1986.

7. C.C.J. Culvenor, "Toxicology in the Tropics", ed. R.L. Smith and E.A. Bababunmi, Taylor and Francis, London, 1980.

8. J.O. Dickson and R.R. King, "Effects of Poisonous Plants on Livestock", ed. R.F. Keeler, K.R. Van Kempen, and L.F. James, Academic Press, London, 1978, p.201.

9. M.S. Blum, H.M. Fales, T.H. Jones, and C.R. Thompson, J. Org. Chem., 1980, 45, 4778.

10. M. Boppré, Naturwissenschaften, 1986, 73, 17 (Chem. Abs., 1986, 104, 85628z).

11. G.N. Aram, A.A. Merzad, O. Mohabbat, G.G. Sediq, R.N. Srivastava, and M.S. Younos, Lancet, 1976, 2, 269.

12. Y.K. Joshi, M. Narndronathan, B.N. Tandon, H.D. Tandon, and R.K. Tandon, Lancet, 1976, 2, 271.

13. M.E. Benson, A.E. Johnson, R.J. Molyneux, and J.N. Roitman, J. Agric. Food Chem., 1979, 27, 494.

14. M. Haga, I. Hirono, and H. Mori, J. Nat. Cancer Inst., 1978, 61, 865.

15. A.E. Johnson, G.B. Merrill, and R.J. Molyneux, J. Agric. Food Chem., 1985, 33, 50.

16. A. Grandval and H. Lajoux, Compte Rendus L'Acad. Sci., 1895, 120, 1120.

17. H.E. Watt, J. Chem. Soc., 1909, 95, 463.

18. R. Schoental, Nature, 1957, 179, 361.

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

20. A.R. Mattocks and R. Schoental, Nature, 1960, 185, 842.

21. A.R. Mattocks, Nature, 1970, 228, 174.

22. A.R. Mattocks, Nature, 1968, 217, 723.

23. A.R. Mattocks and I.N.H. White, Chem.-Biol. Interact., 1971, 3, 383.

24. C.C.J. Culvenor, Trends Pharmacol. Sci., 1985, 6, 18.

25. C.C.J. Culvenor, J.A. Edgar, L.W. Smith, and H.J. Tweeddale, Aust. J. Chem., 1970, 23, 1853.

26. D.R. Buhler and B. Kedzierski, <u>Anal. Biochem.</u>, 1986, <u>152</u>, 59.

27. J.A. Burguera, G.T. Edds, and O. Osuna, <u>Orlando Rev. Fac. Farm</u>, Univ. Los Andes, 1983, <u>23</u>, 65 (<u>Chem. Abs.</u>, 1985, <u>102</u>, 60915m); J.A. Burguera and G.T. Edds, <u>Acta Cient, Venez.</u>, 1984, <u>35</u>, 67 (<u>Chem. Abs.</u>, 1985, <u>102</u>, 73826h).

28. R. Schoental, <u>F.E.B.S. Letters</u>, 1976, <u>61</u>, 111.

29. V.N. Iyer and W. Szybalski, <u>Science</u>, 1964, <u>145</u>, 55.

30. K.A. Robertson, <u>Cancer Res.</u>, 1982, <u>42</u>, 8.

31. J.R. Allen, C.F. Cheeney, and I.C. Hau, <u>C.F. Proc. Soc. Exp. Biol. Med.</u>, 1973, <u>144</u>, 834.

32. C.C.J. Culvenor, J.A. Edger, M.V. Jago, and L.W. Smith, <u>Mol. Pharmacol.</u>, 1970, <u>6</u>, 402.

33. M.M. Ames, J.S. Kovach, and G. Powis, <u>Cancer Res.</u>, 1979, <u>39</u>, 3564.

34. R. Schoental, <u>Cancer Res.</u>, 1968, <u>28</u>, 2237.

35. T. Furuya, T. Matsushima, M. Nagao, A. Shirai, T. Sugimura, and H. Yamanaka, <u>Mutation Res.</u>, 1979, <u>68</u>, 211.

36. M. Axelrod, M. Kugelman, W.C. Liu, T.J. McBride, and K.V. Rao, <u>Lloydia</u>, 1976, <u>39</u>, 125.

37. M.M. Ames, E.T. Creagan, R.G. Hahn, J.S. Kovach, C.G. Moertel, and G. Powis, <u>Cancer Res.</u>, 1979, <u>39</u>, 4540.

38. L.T. Gelbaum, M.M. Gordon, M. Miles, and L.H. Zalkow, <u>J. Org. Chem.</u>, 1982, <u>47</u>, 2501.

39. S. Danishefsky, <u>Acc. Chem. Res.</u>, 1979, <u>12</u>, 66 and references cited therein.

40. D.J. Robins and S. Sakdarat, J. Chem. Soc., Chem. Commun.,
     1979, 1181; J. Chem. Soc., Perkin Trans. 1, 1981, 909.

41. Y. Amemiya, M. Kinoshita, K. Takahashi, and K. Tatsuda,
     J. Am. Chem. Soc., 1983, 105, 4096.

42. A.R. Chamberlin and J.Y.L. Chung, J. Am. Chem. Soc., 1983,
     105, 3653; J. Org. Chem., 1985, 50, 4425.

43. D.J. Hart and J.Y.L. Chung, J. Chem. Soc., Chem. Commun.,
     1985, 135; J. Org. Chem., 1985, 50, 235.

44. T.A. Geissman and A.C. Waiss, J. Org. Chem., 1962, 27, 139.

45. M. Benn and H. Rüeger, Heterocycles, 1983, 20, 1331.

46. Y. Miyachi, H. Niwa, O. Okamoto, Y. Uosaki, and K. Yamada,
     Tetrahedron Lett., 1986, 27, 4605 and references cited therein.

47. N.K. Kochetkov, V.N. Kulakov, and A.M. Likhosherstov,
     Tetrahedron, 1969, 25, 2313.

48. C.C.J. Culvenor, A.T. Dann, and L.W. Smith, Chem. Ind., 1959,
     20.

49. E.L. Gurevich and I.V. Men'shikov, Zh. Obsh. Khim., 1947, 17,
     1714 (Chem. Abs., 1948, 42, 25981).

50. D.H.G. Crout and W.M. Hoskins, J. Chem. Soc., Perkin Trans. 1,
     1977, 538.

51. T.J. Fleischmann, L.T. Gelbaum, J.A. Glinski, M.M. Gordon,
     L.S. McGowan, and L.H. Zalkow, J. Med. Chem., 1985, 28, 687.

52. P. Kari, J.R. Piper, and Y.F. Shealy, J. Lab. Com. Radiopharm.,
     1981, 18, 1579.

53. K.C. Nicolaou, Tetrahedron, 1977, 33, 683.

54.   T.G. Back, Tetrahedron, 1977, 33, 3041.

55.   E. Haslam, Tetrahedron, 1980, 36, 2409.

56.   S. Masamune, Aldrich. Acta, 1978, 11, 23.

57.   P. Deslongchamps, S. Lamothe, and H. Lin, Can. J. Chem., 1984,
      62, 2395.

58.   C. Galli, G. Illuminati, L. Mandolini, and P. Tamborra, J. Am.
      Chem. Soc., 1977, 99, 2591.

59.   D.J. Robins and S. Sakdarat, J. Chem. Soc., Chem. Commun.,
      1980, 282.

60.   E.J. Corey and K.C. Nicolaou, J. Am. Chem. Soc., 1974, 96, 5614.

61.   J.A. Devlin, D.J. Robins, and S. Sakdarat, J. Chem. Soc.,
      Perkin Trans. 1, 1982, 1117.

62.   J.A. Devlin and D.J. Robins, J. Chem. Soc., Chem. Commun.,
      1981, 1272; K. Brown, J.A. Devlin, and D.J. Robins, J. Chem. Soc.,
      Perkin Trans. 1, 1983, 1819.

63.   M. Burton and D.J. Robins, J. Chem. Soc., Perkin Trans. 1.,
      1985, 611.

64.   K.C. Nicolaou, Tetrahedron, 1977, 33, 683.

65.   M. Burton and D.J. Robins, J. Chem. Soc., Perkin Trans. 1,
      1986, 585.

66.   S.D. Larsen and E. Vedejs, J. Am. Chem. Soc., 1984, 106, 3030.

67.   J. Huang and J.J. Meinwald, J. Am. Chem. Soc., 1981, 103, 861.

68.   S. Ahmad, S.D. Larsen, E. Vedejs, and S. Westwood, J. Org. Chem.,
      1987, 52, 3937.

69.   D. Guédin-Vuong, K. Narasaka, T. Sakakura, and T. Uchimaru,
      J. Am. Chem. Soc., 1984, 106, 2954.

70. H. Ishiwata, A. Kuroda, Y. Miyachi, H. Niwa, Y. Uosaki, and
     K. Yamada, Tetrahedron Lett., 1986, 27, 4609.

71. Y. Miyachi, H. Niwa, Y. Uosaki, and K. Yamada, Tetrahedron
     Lett., 1986, 27, 4601.

72. S.J. Ohira and J.D. White, J. Org. Chem., 1986, 51, 5492.

73. A.R. Mattocks, J. Chem. Soc., Perkin Trans. 1, 1974, 707;
     A.R. Mattocks, ibid, 1978, 896.

74. R.H. Barbour and D.J. Robins, J. Chem. Soc., Perkin Trans. 1,
     1985, 2475.

75. R.H. Barbour and D.J. Robins, J. Chem. Soc., Perkin Trans. 1,
     in press.

76. R.H. Barbour and D.J. Robins, unpublished results.

77. R.H. Barbour, H.E. Driver, A.R. Mattocks, and D.J. Robins,
     Chem.-Biol. Interact., 1986, 58, 95.

78. J.A. Glinski and L.H. Zalkow, Tetrahedron Lett., 1985, 26, 2857.

79. Y.G. Borisyuk and I.V. Man'ko, Ukr. Khim. Zhur., 1957, 23, 362
     (Chem Abs., 1958, 52, 2187i); I.V. Man'ko, Ukr. Khim. Zhur.,
     1959, 25, 627 (Chem. Abs., 1960, 54, 12494c).

80. Z. Sykulska, Acta Pol. Pharm., 1962, 19, 183 (Chem. Abs., 1963,
     59, 2876e); Z. Jerzmanowska and Z. Sykulska, Diss. Pharm.,
     1964, 16, 71, 81 (Chem. Abs., 1964, 61, 16438b).

81. E. Pederson, Dansk. Tid. Farm., 1970, 44, 287 (Chem. Abs., 1971,
     74, 72780e).

82. P.A. Cockrum, C.C.J. Culvenor, J.A. Edgar, J.L. Frahn,
     S. Mohanraj, L.W. Smith, and P.S. Subramanian, J. Chem. Soc.,
     Chem. Commun., 1978, 423.

83. G.P. Men'shikov, Zhur. Obsh. Khim., 1948, 18, 1736 (Chem. Abs, 1949, 43, 2625).

84. R. Benn and H. Günther, Angew. Chem., Int. Ed. (Eng.), 1983, 22, 350.

85. S.O. Denisova and G.P. Men'shkiov, Sbor. Stat. Obsh. Khim., 1953, 2, 1458 (Chem. Abs., 1955, 49, 5496f).

86a. A.D. Kuzovokov and G.P. Men'shikov, Zhur. Obsh. Khim., 1949, 19, 1702 (Chem. Abs., 1950, 44, 113).

86b. R. Adams and B.L. Van Duuren, J. Am. Chem. Soc., 1954, 76, 6379.

87. H.A. Khan and D.J. Robins, J. Chem. Soc., Chem. Commun., 1981, 146.

88. L.T. Gelbaum, M.M. Gordon, M. Miles, and L.H. Zalkow, J. Org. Chem., 1982, 47, 2501.

89. R.K. Crossland and K.L. Servis, J. Org. Chem., 1970, 35, 3195.

90. R.M. Kellogg, W.H. Kruizinga, and B. Strijtveen, J. Org. Chem., 1981, 46, 4321.

91. C.C.J. Culvenor, Tetrahedron Lett., 1966, 10, 1091.

92. H. Nesvadba, N. Neuner-Jehle, and G. Spiteller, Monats. Chem., 1965, 96, 321.

93. C.K. Atal, G.S. Khajuria, and R.K. Sharma, J. Chrom., 1965, 19, 433.

94. F. De Boer, H.J. Huizing, and T.M. Malingré, J. Chrom., 1980, 195, 407; R.J. Molyneux and J.N. Roitman, ibid, 412.

95. A.R. Mattocks, Anal. Chem., 1967, 39, 443.

96. C.C.J. Culvenor, M.L. Heffernan, and W.G. Woods, Aust. J. Chem., 1965, 18, 1605.

97. L.T. Gelbaum, J.A. Glinski, D. Van Der Veer, and L.H. Zalkow, Acta Cryst., Sect. C., 1985, 41, 1342.

98. S.J. Wodak, Acta Cryst., Sect. B., 1975, 31, 569.

99. C.C.J. Culvenor, D.G. Hay, and M.F. Mackay, Acta Cryst., Sect. B, 1982, 38, 155.

100. B. Tashkhodzhaev, M.R. Yagudaev, and S.Y. Yunosov, Khim. Prir. Soed., 1979, 3, 368 (Chem. Abs., 1980, 92, 111194k).

101. D.H.G. Crout, C.C.J. Culvenor, W. Klyne, W.P. Mose, J.D. Renwick, and P.M. Scopes, J. Chem. Soc. (C), 1971, 3653.

102. R. Robinson, "The Structural Relations of Natural Products", Oxford University Press, London, 1955, p.72.

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

104. W. Bottomley and T. Geissman, Phytochemistry, 1964, 3, 357.

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

106. C.A. Hughes, R. Letcher, and F.L. Warren, J. Chem. Soc., 1964, 4974.

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

108. H.A. Khan and D.J. Robins, J. Chem. Soc., Chem. Commun., 1981, 146, 554; J. Chem. Soc., Perkin Trans. 1, 1985, 101.

109. J. Rana and D.J. Robins, J. Chem. Res. (S), 1983, 326.

110. I.D. Spenser, Pure Appl. Chem., 1985, 57, 453.

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

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

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

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

115. E. Leete and J. Rana, *J. Chem. Soc., Chem. Commun.*, 1985, 1742.

116. R.B. Herbert, "Alkaloid Biosynthesis" in "Comprehensive Organic Chemistry", ed. D.H.R. Barton and W.D. Ollis, Pergamon Press, Oxford, 1979, Vol. 5, p.1072.

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

118. A.R. Battersby, J. Staunton, and J. Tippett, *J. Chem. Soc., Perkin Trans. 1*, 1982, 455.

119. J. Rana and D.J. Robins, *J. Chem. Soc., Chem. Commun.*, 1983, 1222 and 1984, 517; *J. Chem. Soc., Perkin Trans. 1*, 1986, 983.

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

121. T.W. Bell, M. Boppré, and D. Schneider, *Experientia*, 1984, 40, 713.

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

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

124. B.A. McGaw and J.G. Woolley, *Phytochemistry*, 1979, 18, 1647.

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

126. J.C.S. Marais, *J. Vet. Sci. Anim. Ind.*, 1944, 20, 67 (*Chem. Abs.*, 1945, 39, 4166[5]).

127. R.A. Peters, Adv. Enzymology, 1957, 18, 113.

128. E. Kirsten, E. Kun, and M.L. Sharma, Mol. Pharmacol., 1978, 14, 172.

129. R.E. Marquis, "Handbook of Experimental Pharmacology", Springer-Verlag, Berlin, 1970, Vol. 20, Part 2, Ch.5, p.166.

130. D.S. Magrill and J.E. Watkin, Fourth International Symposium on the Chemistry of Natural Products, Stockholm, June 26th-July 2nd, 1966, Abstracts, p.146.

131. R.P. Elander, M. Gorman, R.L. Hamill, and J. Mabe, Biochem. Biophys. Res. Commun., 1968, 31, 294.

132. E.R. Blakeley and D.B. Harper, Can. J. Microbiol., 1971, 17, 635.

133. K. Kieslich, "Microbial Transformations of Non-Steroid Cyclic Compounds", Georg Thiéme, Stuttgart, 1976, p.233.

134. D. Gani, P.B. Hitchcock, and D.W. Young, J. Chem. Soc., Perkin Trans. 1, 1985, 1363.

135. D. Dani and D.W. Young, J. Chem. Soc., Chem. Commun., 1983, 576.

136. B.A. Corradino, S.B. Kost, R. Kumar, J.M. Londowski, and L.K. Revelle, J. Steroid Biochem., 1985, 22, 469.

137. H.D. Hollingworth, Brit. Pat., 916488, 1959 (Chem. Abs., 1963, 58, 9600a).

138. G.B. Boden, E. Leete, and M.F. Manuel, Phytochemistry, 1971, 10, 2687.

139. E. Leete, J. Org. Chem., 1979, 44, 165.

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

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

142. J. Avigan, J.H. Herndon, G.W.A. Milne, E. Mize, D. Steinberg,
     and B.W. Uhlendorf, Science, 1967, 156, 1740.

143. P.K. Stumpf, "Lipid Metabolism", ed. S.J. Wakil, Academic Press,
     London, 1970, p.85.

144. C.R. Hauser, D.G. Hill, S.M. Luck, and A.T. Stewart Jr.,
     J. Am. Chem. Soc., 1959, 81 2784.

145. P.S. Callery, E.M. Jakubowski, M.S.B. Nayar, and M. Stogniew,
     Experientia, 1982, 38, 431.

146. J.C. Richards and I.D. Spenser, Can. J. Chem., 1982, 60, 2810;
     D.J. Robins, Phytochemistry, 1983, 22, 1133.

147. P. Karrer, W. Kasse, and H. Reschofsky, Helv. Chim. Acta,
     1947, 30, 271.

148. A.J. Aasen, C.C.J. Culvenor, and L.W. Smith, J. Org. Chem.,
     1969, 34, 4137.

149. N.K. Hart and J.A. Lamberton, Aust. J. Chem., 1966, 19, 1259.

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

151. A.P. Arendaruk, R.V. Konovalova, and N.F. Proskurnina,
     Zh. Obsh. Khim., 1960, 30, 670 (Chem. Abs., 1960, 54, 24835c).

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

153. F. Jostes and J. Von Braun, Berichte, 1926, 59, 1091 (Chem.
     Abs., 1926, 20, 2989).

154. A.W. Hofmann, Berichte, 1881, 14, 2725; E.S. Wallis and J.F. Lane,
     Org. Reactions, 1946, 3, 267.

155. C.L. Arais and J. Kenyon, J. Chem. Soc., 1939, 916.

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

157. "Biomedicinal Aspects of Fluorine Chemistry", ed. R. Filler and
      Y. Kobayashi, Elsevier Biomedical Press, New York, 1982.

158. A. Haas and M. Lieb, Chimia, 1985, 39, 134.

159. Y. Faust, C. Gal, and S. Rozen, J. Am. Chem. Soc., 1980, 102,
      6860.

160. W.C. Smith, Angew. Chem., 1962, 74, 742; Angew. Chem. Int.
      Ed. Engl., 1962, 1, 467.

161. E.F. Kagan and D.G. Martin, J. Org. Chem., 1962, 27, 3164.

162. A.V. Kirsanov, L.N. Markovskij, and V.E. Pashinnik, Synthesis,
      1973, 787.

163. W.J. Middleton, J. Org. Chem., 1975, 40, 574.

164. S. Castillon, A. Dessinges, R. Faghih, G. Lukacs, A. Olesker,
      and T.T. Ton, J. Org. Chem., 1985, 50, 4913.

165. N. Ishikawa, H. Iwakiri, and A. Takaoka, Bull. Chem. Soc. Japan,
      1979, 52, 3377.

166. K.K. Jokri, T.B. Patrick, and D.H. White, J. Org. Chem., 1983,
      48, 4159.

167. M.L. Druelinger, R.A. Hildreth, and S.A. Shackleford,
      Tetrahedron Lett., 1982, 23, 1059.

168. J. Chaney, G.A. Digenis, A.A. Hawi, and K. Saito, J. Fluorine
      Chem., 1987, 35, 663.

169. F. Elghozy and R. Locquin, Bull. Soc. Chim., 1927, 41, 445.

170. E. Fabiano, B.T. Golding, and M.M. Sadeghi, Synthesis, 1987,
      190.

171. O. Mitsunobu, Synthesis, 1981, 1.

172. Y.G. Gololobov, L.F. Kasukhin, and I.N. Zhmurova, Tetrahedron, 1981, 37, 437.

173. J. Drabowicz and M. Mikolajczyk, "N.M.R. Chiral Solvating Agents", in "Topics in Stereochemistry", ed. N.L. Allinger, E.L. Eliel, and S.H. Wilen, Wiley-Interscience, 1982, Vol. 13, Ch.4, p.263.

174. F.H. Dean and F.L.M. Pattison, Can. J. Chem., 1963, 41, 1833.

175. D.H.G. Crout, A.F. Drake, D.L. Rathbone, and G. Siligardi, J. Chem. Soc., Chem. Commun., 1987, 1834.

176. C.W. Qualls and H.J. Segall, J. Chrom., 1978, 150, 202.

177. R.J. Molyneux and H.J. Segall, Res. Commun. Chem. Pathol. Pharm., 1978, 19, 545.

178. H.J. Segall, Toxicol. Lett., 1978, 1, 279.

179. H. Hinz, G. Tittel, and H. Wagner, Planta Medica, 1979, 37, 1.

180. H. Ishiwata, H. Niwa, and K. Yamada, J. Chrom., 1983, 257, 146.

181. J.L. Dallas, W.F. Haddon, and H.J. Segall, Drug Metab. Dispos., 1984, 12, 68.

182. Y. Mino and N. Ota, Shoyakugaku Zasshi, 1979, 33, 140 (Chem. Abs., 1980, 92, 169114a).

183. F.L. Warren, Fortschr. Chem. Org. Naturstoffe, 1955, 12, 198.

184. C.C.J. Culvenor, A.J. Jones, and L.W. Smith, Aust. J. Chem., 1982, 35, 1173.

185. S.T. Akramov, F. Kiyamitdinova, and S.Y. Yunusov, Dokl. Akad. Nauk Uz.S.S.R., 1962, 19, 29 (Chem. Abs., 1964, 61, 11005).

186. J.L. Beal, R.W. Doskotch, and R.A. Locock, Lloydia, 1966, 29, 201.

187. D.D. Perrin, W.L.F. Armarego, and D.R. Perrin, "Purification of laboratory chemicals", Pergamon Press, Oxford, 1966.

188. D.R. Burfield and R.H. Smithers, J. Org. Chem., 1978, 43, 3966.

189. C.C.J. Culvenor, L.J. Drummond, and J.R. Price, Aust. J. Chem., 1954, 7, 277.

190. H.C. Crowley and C.C.J. Culvenor, Aust. J. Chem., 1959, 12, 694.

191. L.J. Dry and F.L. Warren, J. Chem. Soc., 1952, 3445.

192. D.H.G. Crout, N.M. Davies, E.H. Smith, and D. Whitehouse, J. Chem. Soc., Perkin Trans. 1, 1972, 671.

193. A.A. Freer, D.B. Hagan, and D.J. Robins, Acta Cryst., Sect. C, in press.

194. C.R. Witschonke, Anal. Chem., 1954, 26, 562.

195. "Dictionary of Organic Compounds", ed. J. Buckingham, Chapman and Hall, New York, 5th ed., 1982.

196. R. Alul, M. Bausch, and J.S. Chickos, J. Org. Chem., 1981, 46, 3559.

197. J.H. Boyler, J. Am. Chem. Soc., 1951, 73, 5865.