A STUDY OF THE ALKALOID CONTENT OF THE SENECIO SPECIOSUS/MACROCEPHALUS COMPLEX

THESIS

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ABSTRACT

The isolation and identification of pyrrolizidine alkaloids from various plant species from 1988 to May 1991 are reviewed and the alkaloids of two indigenous plant species, ***Senecio speciosus*** **Willd** and ***Senecio macrocephalus*** **DC,** were investigated. A brief review of the methods used for isolation and identification of pyrrolizidines is also given.

S. speciosus was found to contain two new alkaloids, 7-senecioyl-9-sarracinylheliotridine and 7-isosarracinyl-9-sarracinyl-heliotridine, which were identified using high-field NMR techniques. A number of other alkaloids were tentatively identified using GC-MS. ***S. macrocephalus*** contains very little alkaloid, but a number of pyrrolizidine alkaloids were tentatively identified using GC-MS. Standard alkaloids for GC-MS work were obtained both by extraction from a number of plant species and by synthesis of simple monoester alkaloids. In this process the alkaloid neosarracine, previously described by GC-MS, was isolated and NMR data for this compound are reported for the first time.

S. speciosus and ***S. macrocephalus*** are morphologically very similar and their counterparts in the Grahamstown district exhibit features characteristic of both species. This could be due to hybridization, genetic mutation or simple variation within the species. The alkaloids of four local plant populations were examined in order to collect taxonomic markers whereby it was hoped that the Grahamstown plants could be satisfactorily classified. Three of the plant populations

were found to contain 7-senecioyl-9-sarracinylheliotridine and 7-angelyl-9-sarracinyl-heliotridine. One population was found to contain the known alkaloid retrorsine along with the new alkaloid 2-hydroxy-1,2-dihydrosenkirkine. The alkaloidal fractions of all four populations were compared using GC-MS and NMR techniques. Tentative taxonomic conclusions were drawn.

CHAPTER ONE

INTRODUCTION

1. REVIEW OF PYRROLIZIDINE ALKALOIDS

1988 to May 1991

1.1 Occurrence and distribution

The group of compounds known as pyrrolizidine alkaloids comprises over three hundred compounds identified to date, the majority of which occur naturally, although an increasing number are being derived synthetically or semisynthetically.

Naturally occurring pyrrolizidines are predominantly of plant origin and have been isolated from over four hundred and fifty species distributed among eighty-seven genera of fourteen plant families[1-40]. The largest number of pyrrolizidine alkaloids occur in the genus ***Senecio***. For this reason, and because the first pyrrolizidine alkaloid was isolated from this genus[41], these alkaloids are often referred to as the Senecio alkaloids. The distribution of species, genera and families containing pyrrolizidine alkaloids is shown (Table 1.1; updated from[1,42,43]).

Plants containing pyrrolizidine alkaloids are known to be toxic and have caused widespread livestock losses in many parts of the world[1]. Human poisoning has also been

Table 1.1. Pyrrolizidine containing genera

Family	Genera (with number of species investigated)
Apocynaceae	Alafia(1), Anodendron(1), Parsonsia(5), Urechtites(1).
Boraginaceae	Alkanna(1), Amsinckia(4), Anchusa(2), Asperugo(1), Borago(1), Buchenroedera(5), Caccinia(1), Cerinthe(1), Cordia(2), Cynoglossum(11), Echium(5),Ehretia(1), Hackelia(3), Heliotropium(38), Lappula(2), Lindelofia(9), Lithospermum(3), Lotononis(16), Macrotomia(1), Mertensia(3), Meerschmidia(1), Myosotis(2), Onosma(1), Paracarym(1), Paracynoglossum(1), Rindera(5), Solenanthus(4), Symphytum(7), Tournefortia(2), Trachelanthus(2), Trichodesmia(3), Ulugbekia(1).
Celastraceae	Bhesa(1).
Compositae	Adenostyles(3), Brachyglottis(1), Cacalia(5), Cirsium(1),Conoclinum(1), Crassocephalum(1), Doronicum(2), Echinaceae(2), Emilia(2), Erechtites(1), Eupatorium(6), Farfugium(1), Gynura(2), Jacmaia(1), Kleinia(1), Ligularia(5), Notonia(1), Odontocline(1), Petasites(4), Senecio(149), Syneilesis(1), Tussilago(1), Werneria(1).
Euphorbiaceae	Phyllanthus(1), Securinega(1).
Graminae	Festuca(1), Lolium(2), Schismus(1), Thelopogon(1).
Leguminosae	Adenocarpus(4), Alexia(1), Castanospermum(3), Crotalaria(64), Cytisus(1).
Linaceae	Hugonia(2).
Orchidaceae	Chrysis(1), Doritis(1), Hammarbya(1), Kingiella(1), Liparis(7), Malaxis(2), Phalaenopsis(14), Vanda(4), Vandopsis(2).
Ranunculaceae	Caltha(2).
Rhizoporaceae	Cassipourea(2).
Santalaceae	Thesium(1).
Sapotaceae	Mimusops(1), Planchonella(2).
Scrophulariaceae	Castilleja(3).

reported to occur through ingestion of pyrrolizidine alkaloids from contaminated foodstuffs and traditional herbal remedies and teas[43]. Some pyrrolizidine alkaloids have also been shown to exhibit anti-tumour activity[27,44] and as a result are of great interest in medical research fields.

The toxic and other biological effects of pyrrolizidines illustrate the importance of their study, both in continued screening of as yet uninvestigated plant sources for their presence and in synthetic studies of pyrrolizidine alkaloids and their analogues.

Pyrrolizidine alkaloids have also been used as chemotaxonomic markers in various botanical studies[36,45].

Pyrrolizidine alkaloids have also been isolated from some animal sources. Butterflies and moths of various genera feed on pyrrolizidine-containing plants and the ingested alkaloids are used for defence against predators and as pheromones[46,47]. For example, the black and white moth ***Gnophaela latipennis*** (Arctiidae) has recently been shown to contain pyrrolizidines[11], whilst other workers have demonstrated the uptake of pyrrolizidines from host plants by the parasitic genus ***Pedicularis***[48].

In all these instances the pyrrolizidines were obtained from plant sources in the feeding process.

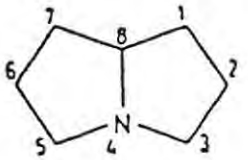
There is a group of naturally occurring pyrrolizidine alkaloids which are not of plant origin; a number of ant genera have been shown to produce pyrrolizidines as defence compounds[49].

1.2 Pyrrolizidine alkaloid Structure

All pyrrolizidine alkaloids contain the basic pyrrolizidine ring system (1). The diagram shows the traditional system for numbering the ring, which has been adopted throughout this work. The orientation of substituents is labelled as shown in (1). The fully saturated ring structure is non-planar and adopts an open 'V' shaped configuration[42]. Whether the V opens towards or away from the viewer depends on the orientation of the substituents on C_8.

Most pyrrolizidine alkaloids identified so far have the basic structure as shown in (1). A few alkaloids possess substituents such as methyl, methylene, carboxaldehyde, carboxylic acid and nitrogen groups at C_1, while some possess hydroxyl groups at other positions on the ring system, usually at C_2 or C_6. One or both of the hydroxyl groups (C_7 or C_9) may be esterified with a variety of acids (see Fig 1.3, pg 41 for numbering). The unesterified ring system is usually referred to as the "necine" and the acid portion as the "necic acid". A large number of pyrrolizidines possess a double bond between C_1 and C_2 and these alkaloids are usually toxic. The factors affecting the toxicity of pyrrolizidine alkaloids have been tabulated[1].

The alkaloids isolated and identified between March 1988 and May 1991 will now be reviewed. (The alkaloids identified between 1976 and March 1988 have already been reviewed elsewhere[42].)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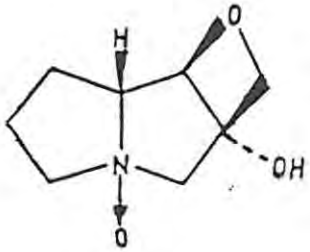
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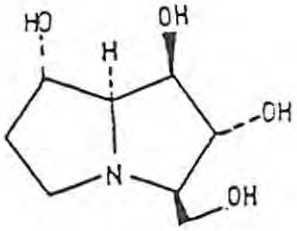
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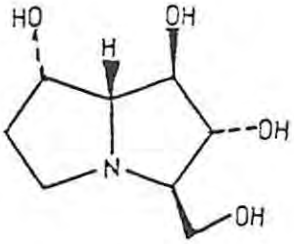
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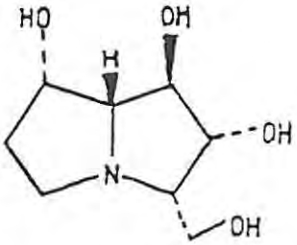
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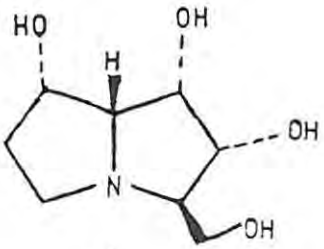
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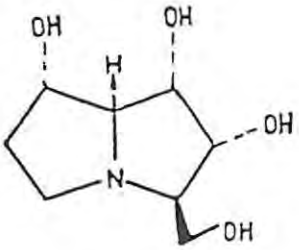
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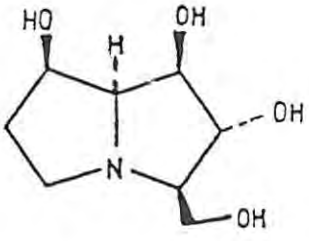
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1.2.1 The Necine Bases and Simple alkaloids

There are over forty different necine bases, but only about twenty of these occur commonly[1]. Unesterified bases are rarely extracted from plants, but are obtained by hydrolysis of the alkaloid extract. Simple pyrrolizidine alkaloids are not esterified and are composed of a modified necine base. The group of simple alkaloids is fairly large, and a number of new compounds have recently been identified.

Thus subulacine-4-oxide (**2**) was isolated from ***Heliotropium subulatum*** and its structure determined by NMR studies[6].

Alexine (**3**) is a new alkaloid from ***Alexia leiopetala*** and is the first alkaloid to exhibit a carbon substituent at C_3[15]. This is also the first time pyrrolizidines have been found in the genus ***Alexa***. A number of similar alkaloids have been isolated from ***Castanospermum australe***[51]. Australine (**4**) differs from alexine only in the orientation of the H at C_8. Australine is the first pyrrolizidine alkaloid to be identified as a glucosidase inhibitor.

3-Epiaustraline (**5**)[52], 1-epiaustraline (**6**)[18], 1,7α-diepialexine (**7**) and 7,7α-diepialexine (**8**)[17] were isolated from the same plant. The structures of all these compounds were elucidated using high-field and two-dimensional NMR studies. X-ray crystal structure analyses were performed on alexine, 1,7α-diepialexine and 7,7α-diepialexine.

Australine, 3-epiaustraline and 1-epiaustraline possess ß-stereochemistry, a property exhibited by a minority of necines[53].

Three new alkaloids of animal origin have been isolated. In a chemotaxonomic study of populations of ants of the genus ***Chelaner***, Jones et al[49] isolated the pyrrolizidines trans-2-butyl-5-(8-nonenyl)pyrrolidine (**9**), (5E, 8Z)-3,5-di-(5-hexenyl)-pyrrolizidine (**10**) and (5Z, 8E)-3-methyl-5-(8-nonenyl)pyrrolizidine (**11**). Structural identification of these compounds was performed by gas chromatography-mass spectrometry, using both synthetic and natural standards.

1.2.2 Monoester alkaloids

Monoester alkaloids are composed of a necine base esterified either at C_7 or C_9. A number of previously known monoester alkaloids have been isolated from new plant sources.
Heliotrine (**12**) and echinatine (**13**) were isolated from ***Lithospermum callosum*** **Vahl.** (***Moltikiopsis ciliata***)[12].
Lycopsamine (**14**) and intermedine (**15**) were isolated from ***Mertensia bakeri*** and ***Mertensia ciliata***, the first pyrrolizidines to be found in this genus[5]. ***Mertensia*** species are reported to be commonly consumed as an emergency ration by hikers.
Echinatine (**13**) and its N-oxide have been isolated from ***Lindelofia longiflora***[9].

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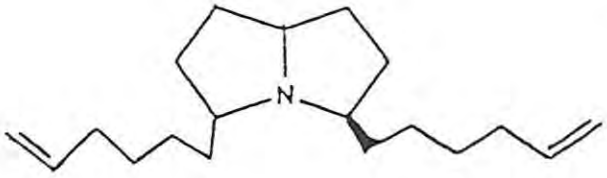

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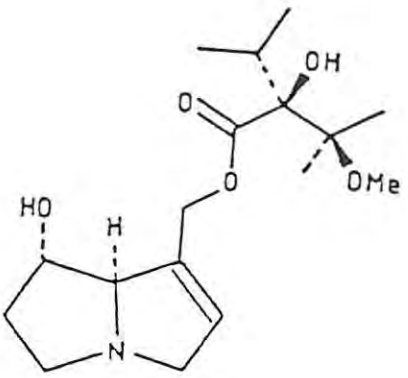

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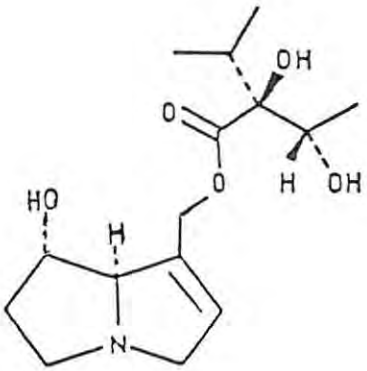

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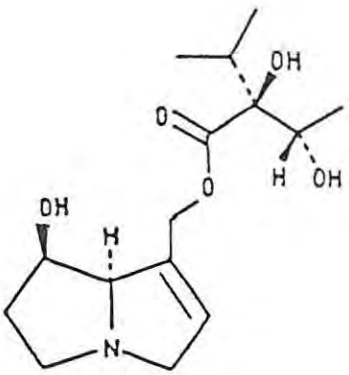

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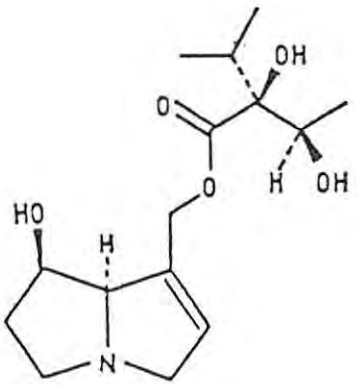

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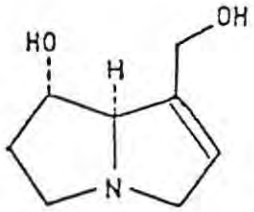
A further two genera have been added to the list of plants containing monoester pyrrolizidine alkaloids; intermedine (**15**) was isolated from ***Cerinthe minor***[33], while the presence of heliotridine (**16**) and the necine base 1-methylenepyrrolizidine (**17**) in the species ***Onosma heterophylla*** was demonstrated by gas chromatographic-mass spectrometric studies[32].

A number of ***Heliotropium*** species have been found to contain previously known alkaloids. 9-Angelylretronecine-4-oxide (**18**), supinine (**19**), heliotrine (**12**) and lasiocarpine (**20**) were isolated from ***Heliotropium bursiferum Wr ex Grisebach***[27]. The crude alkaloid extract from this plant showed antimicrobial activity.

Two-dimensional nuclear magnetic resonance studies were conducted on heliotrine and europine (**21**), isolated from ***Heliotropium bacciferum***[25]. Curassavine (**22**), echinatine (**13**), europine (**21**), heleurine (**23**), heliotrine (**12**) and lasiocarpine (**20**) were isolated from ***Heliotropium circinatum***[28]. Heliotrine, lasiocarpine, europine and supinine were obtained from ***Heliotropium hirsutissimum***[26].

Cynaustine (**24**) and cynaustraline (**25**), two of the minority of alkaloids possessing 8ß-stereochemistry[53], were isolated from ***Cynoglossum montanum***, together with echinatine (**13**) and heliosupine (**26**)[29]. Viridiflorine (**27**) and diacetyl-viridiflorine (**28**) have been isolated from ***Cynoglossum germanicum***, along with the alkaloid (**29**) and its acetate (**30**)[14]. The stereochemistry of alkaloid (**29**) has not yet been

established, although it appears probable that it is echinatine (13).



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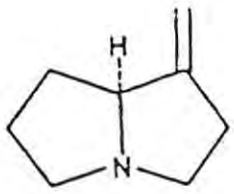

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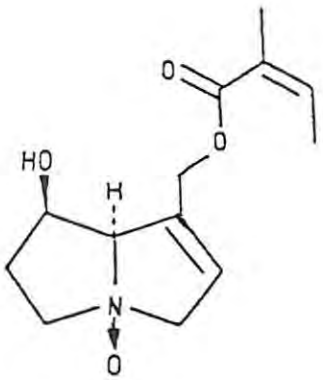

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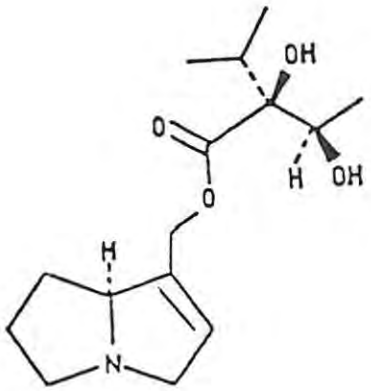

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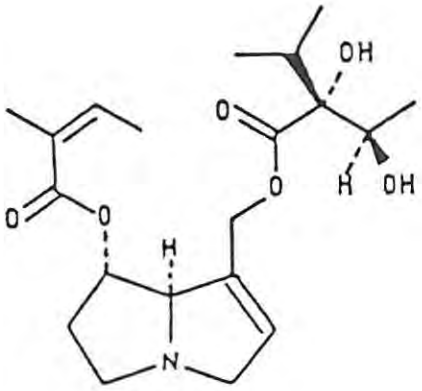

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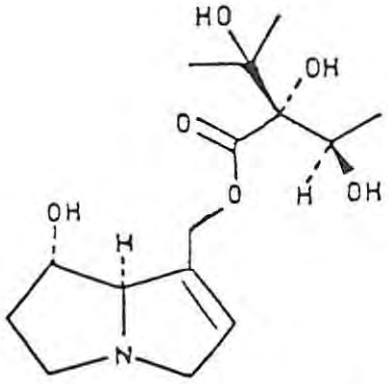

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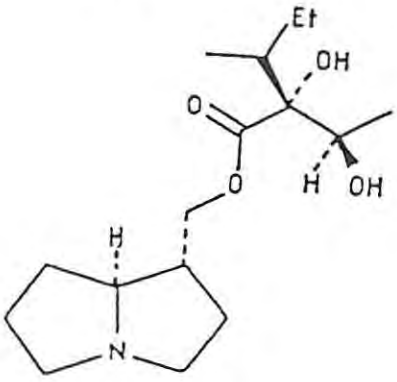

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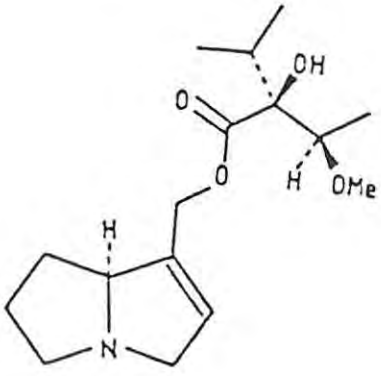

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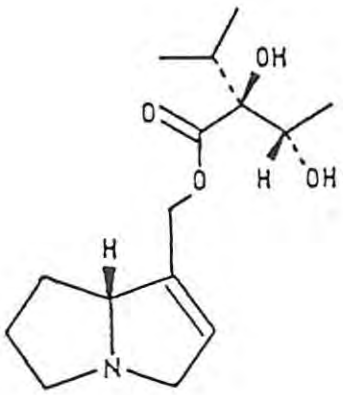

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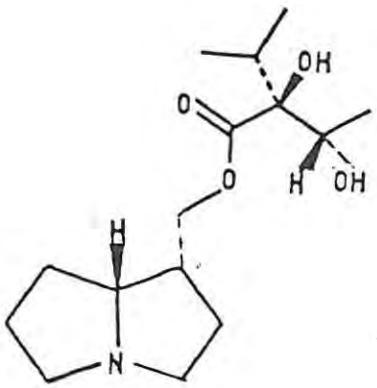

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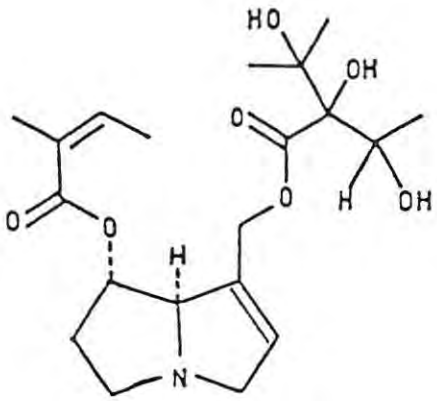

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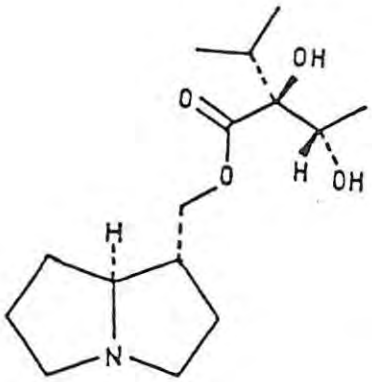

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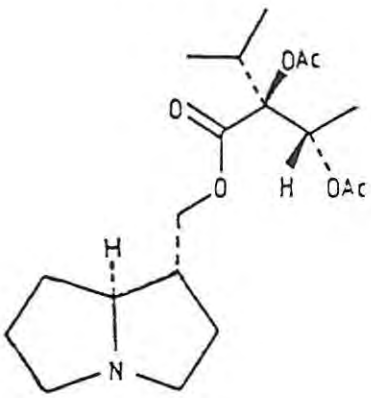

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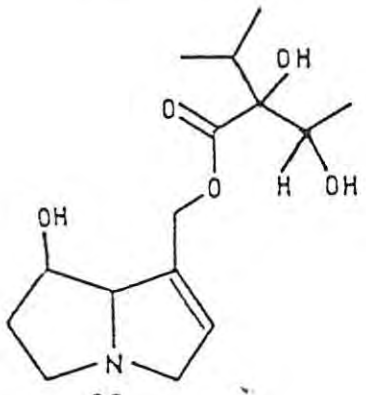

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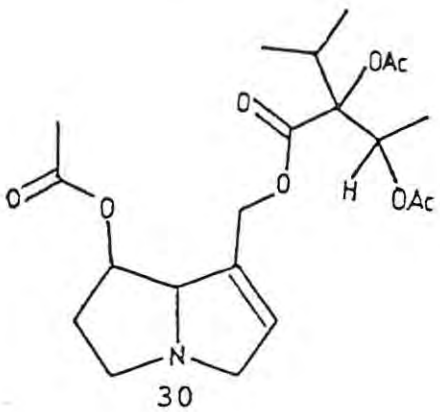

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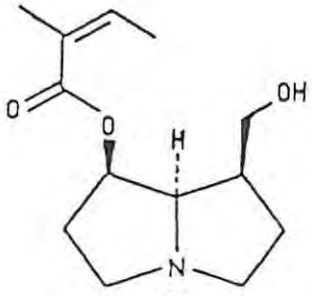
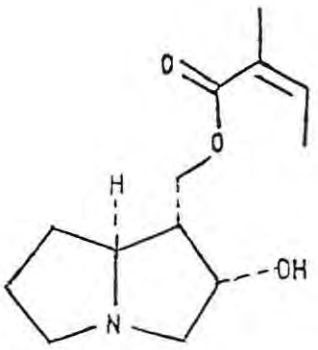


31

A number of new monoester alkaloids have been identified. The presence of 7-angelylplatynecine (**31**) and its N-oxide in ***Crotalaria scassellati*** was demonstrated by gas chromatography-mass spectrometry[54]. A new alkaloid, 2α-hydroxy-9-angelyloxy-(-)-trachelanthamidine-4-oxide (**32**) was isolated from ***Senecio deferens***[2]. A stereoisomer of 7-angelylplatynecine, 7-angelyl-turneforcidine (**33**), was isolated from ***Senecio integrifolius*** var.***Fauriri***, together with the known alkaloid 7-angelyl-heliotridine (**34**) and its N-oxide[39].

Further studies on ***Heliotropium*** species revealed a number of new monoester alkaloids. Davicino et al[55] isolated 9-(3'-isovaleryl)-viridiflorylretronecine (**35**) from both ***Heliotropium curassavicum*** var. ***curassavicum*** and ***Heliotropium curassavicum*** var. ***argentinum***, and 9-(3'-acetyl)-viridifloryl-retronecine (**36**) from the former. The latter alkaloid appears to be the same as the known alkaloid 3'-acetyllycopsamine, but since the original report on this compound does not include optical rotation data, this claim cannot be verified[55a]. Although retronecine (**37**) is the dominant necine base found in the ***Heliotropium*** genus, this is the first time that it has been isolated in the ***curassavicum*** species examined so far[56-58]. ***Heliotropium rotundifolium*** has been found to contain the previously isolated alkaloids europine (**21**), heliotrine (**12**), and lasiocarpine (**20**) as well as a new alkaloid identified as 5'-acetyleuropine (**38**)[7]. The alkaloids were separated by droplet counter-current chromatography, a technique which has been used increasingly in the last ten years[59].



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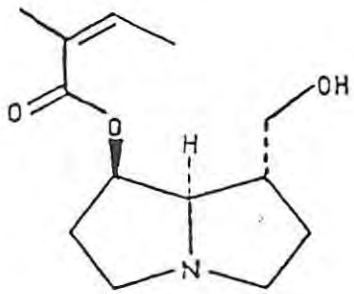

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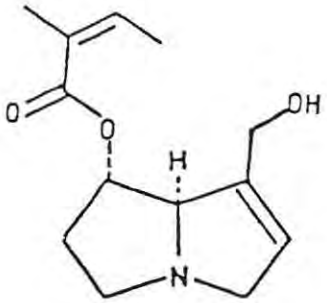

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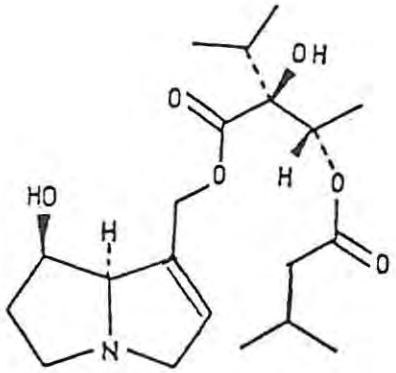

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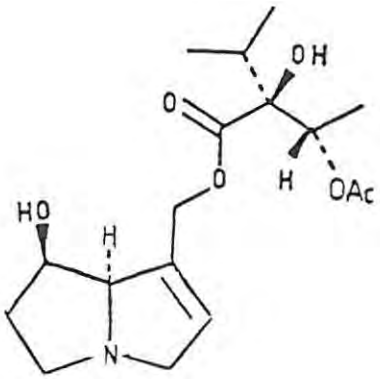

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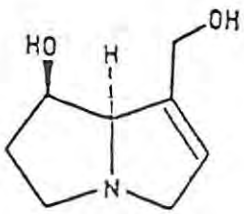

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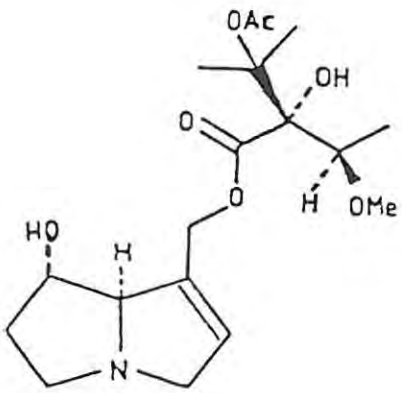

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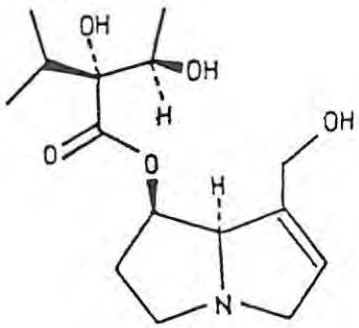

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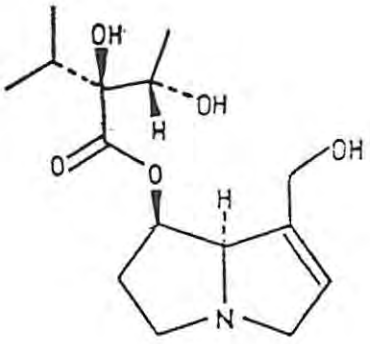
The new pyrrolizidine alkaloid iso-lycopsamine (**39**) was isolated from ***Heliotropium keralense***, along with intermedine (**15**) and retronecine(**37**)[8]. This plant has been used as a medicinal herb in Southern India.

Heliospathuline(**40**), heliospathine (**41**) and 7-curassavinyl-retronecine(**42**), also new monoester alkaloids, were isolated from ***Heliotropium spathulatum***[40].

A new alkaloid, hackelidine (**43**), has been isolated from ***Hackelia californica***[60].

Pyrrolizidine alkaloids have been found in the genus ***Hugonia*** for the first time[35]. This is the first reported occurrence of pyrrolizidines in the family Linaceae, to which this genus belongs. A number of ***Hugonia*** species were investigated and two novel alkaloids identified. Absouline (**44**) and isoabsouline (**45**) were isolated from the species ***Hugonia oroegena*** and ***Hugonia pencillanthemum***[35]. The two alkaloids differ only in the configuration of the double bond in the necic acid, as shown. It is interesting to note that the necine base in these two alkaloids is the unusual base 1-aminopyrrolizidine.

A new monoester of retronecine, named assamicadine (**46**), was isolated from ***Crotalaria assamica***[61]. The necic acid is interesting since it contains a γ-lactone, a feature found in only a few other pyrrolizidines. The relative stereochemistry of the lactone is not yet known.



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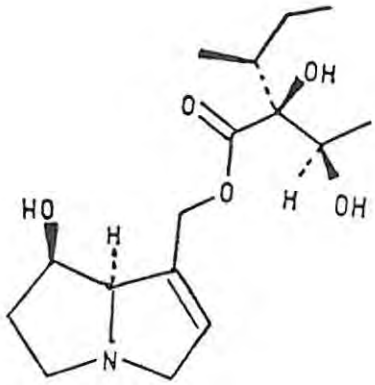

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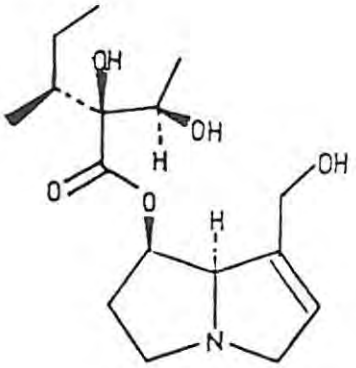

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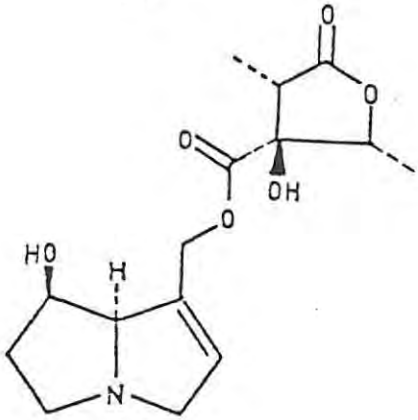

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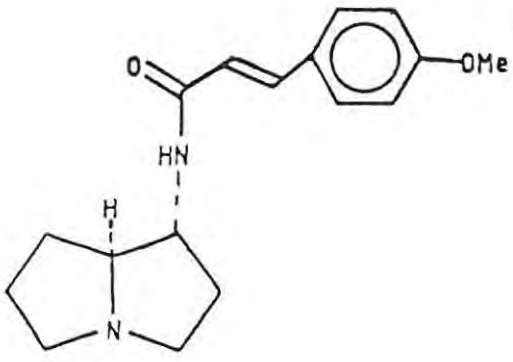

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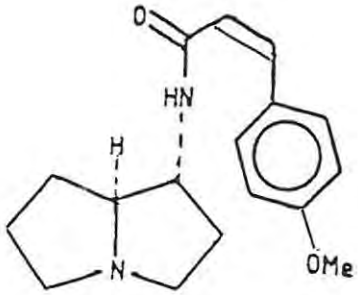

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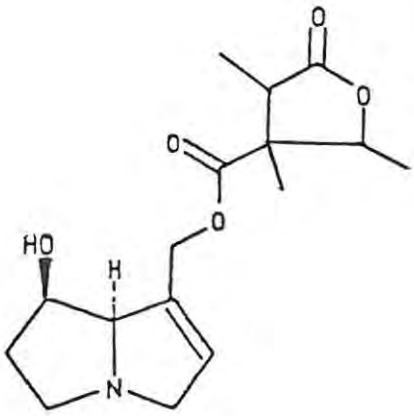


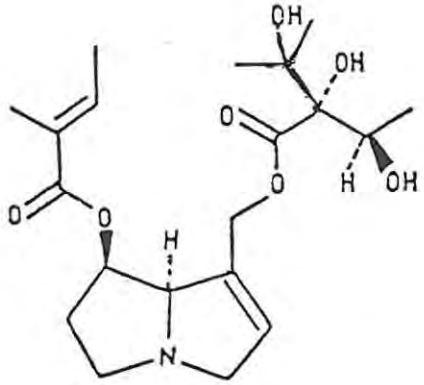
46

1.2.3. Acyclic diester alkaloids

Most new alkaloids of this class have retronecine as the necine base. Hydroxymyoscorpine (**47**) was isolated from ***Lithospermum erythrorhizon***, along with myoscorpine (**48**) and intermedine(**15**)[13]. Doriasenine (**49**) was isolated from ***Senecio doria***[62].

Two new pyrrolizidine alkaloids, longitubine (**50**) and neolatifoline (**51**) were isolated from ***Hackelia longituba*** along with the known alkaloid latifoline (**52**) and the known monoester alkaloids 9-angelylretronecine (**53**) and 7-angelylretronecine (**54**)[10].

Some confusion has arisen concerning the stereochemistry of latifolic acid. Latifoline was first discovered in 1973 and the stereochemistry assigned as shown in (**52**), based on chemical determination methods[63]. Roitman and Wong[64] performed a single-crystal X-ray crystallographic analysis on (+)latifolic acid. This study indicated that latifoline in fact had the stereochemistry (**55**). They speculated that samples may have been interchanged in the previous study. Stermitz and Hope resolved the problem by X-ray crystal studies of their own, the results of which confirmed the structure of latifoline as (**52**), in agreement with the original determination[63]. It seems that the reported data of Roitman and Wong do not meet the criteria for an unambiguous stereochemical determination. Consequently, the structure of longitubine should be represented by structure (**56**).



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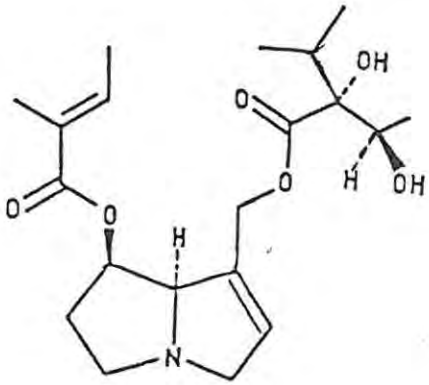

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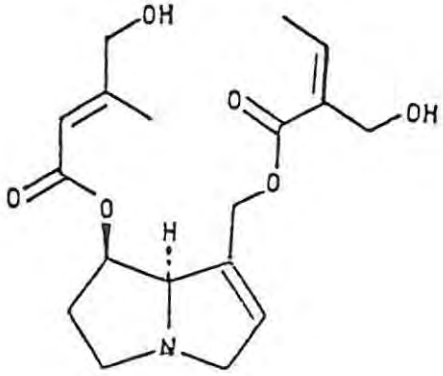

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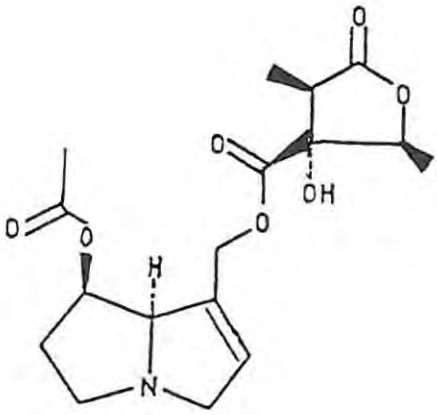

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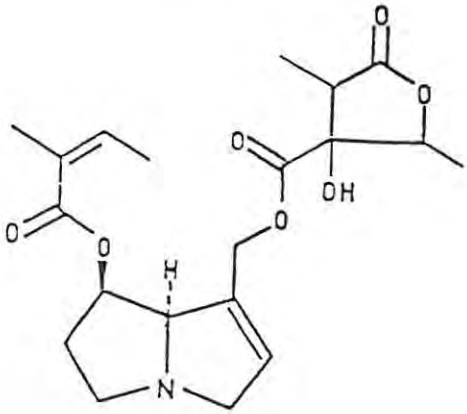

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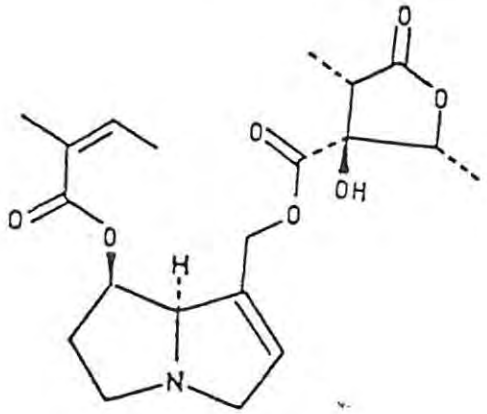

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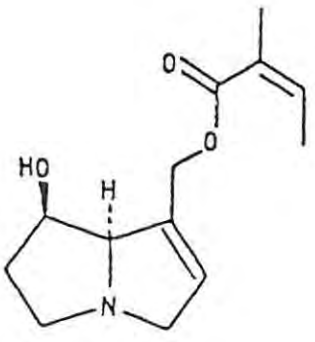

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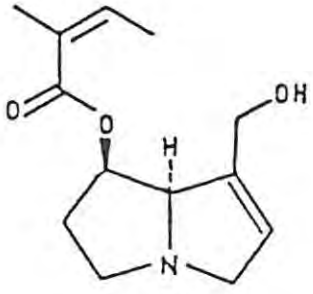

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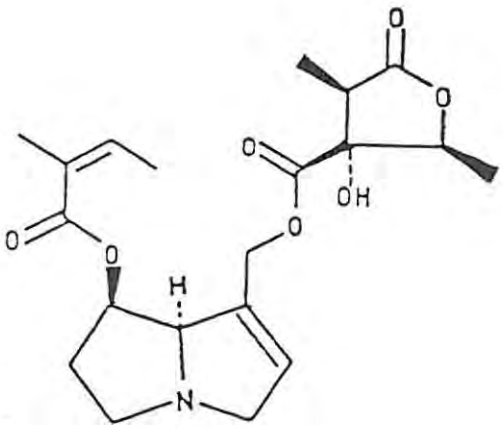
An independent study of ***Gnophaela latipennis***, an Arctiid moth, and its host plant ***Hackelia californica*** resulted in the discovery of two new pyrrolizidine alkaloids, 9-latifolyl-retronecine (**57**) and 7-acetyl-9-latifolylretronecine (**58**)[11]. With the evidence provided by Stermitz and Hope[63], it would seem that the latter alkaloid is the same as longitubine. Stermitz and Hope found that all data reported for this alkaloid were identical to those reported for longitubine[10], confirming identity of the two substances. 7-acetyl-9-latifolylretronecine should therefore also be represented by structure **56**. The new alkaloid 7-acetylhackelidine, isolated from ***Hackelia californica*** by Chinese workers, also seems to be identical to longitubine. Hackelidine (**43**) could thus be the same as 9-latifolylretronecine (**57**)[60]. No spectral data for hackelidine and its acetate were available for comparison.

A new water-soluble pyrrolizidine alkaloid quaternary salt, N-methyl-7,9-diangelyl-1-hydroxylplatynecium chloride (**59**) was isolated from ***Senecio integrifolius*** var. ***Fauriri***[39].
This is only the second example of this type of quaternary salt pyrrolizidine[42].



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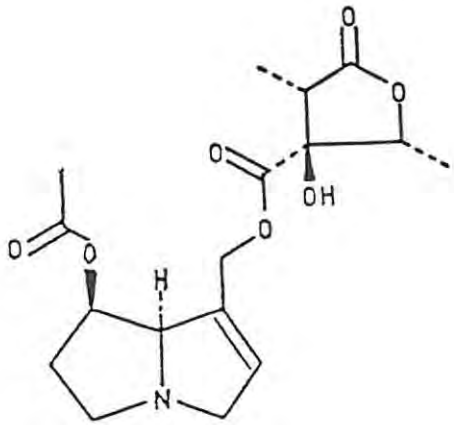

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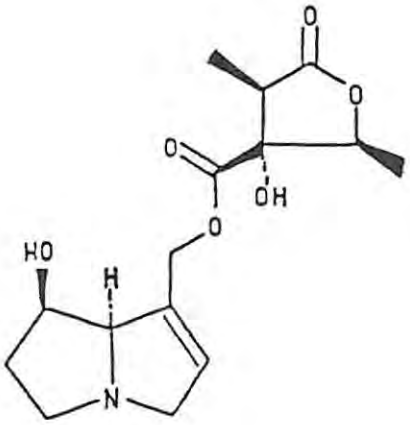

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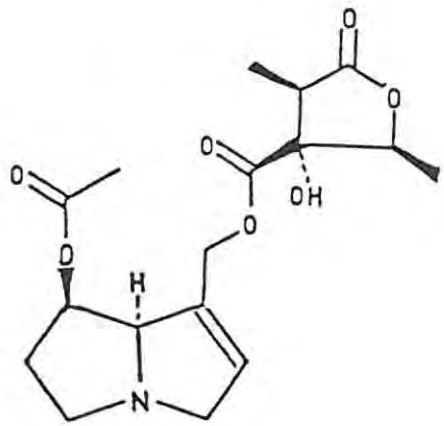

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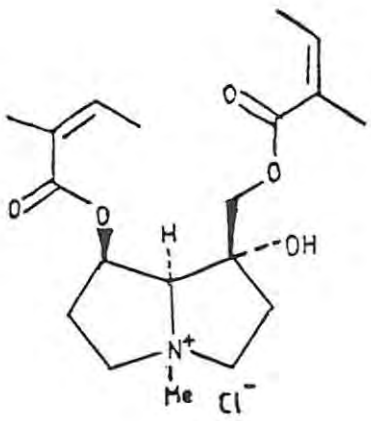

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1.2.4 Macrocyclic diester alkaloids

A macrocyclic diester alkaloid is composed of a necine base esterified to a dibasic acid, to produce a macrocyclic structure. Many such alkaloids are known.

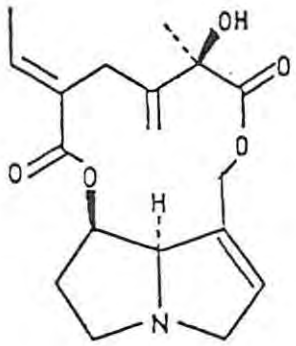
A re-investigation of ***Senecio vulgaris*** showed the presence of spartioidine (**60**) and usaramine (**61**) in addition to the previously reported alkaloids[65]. NMR data for usaramine are reported for the first time[65].

Senecio anonymus was investigated and ten twelve-membered macrocyclic pyrrolizidine alkaloids isolated[4]. The separation, carried out by droplet counter-current chromatography, afforded the known alkaloids senecionine (**62**), integerrimine (**63**), retrorsine (**64**), senkirkine (**65**), neosenkirkine (**66**), otosenine (**67**), hydroxysenkirkine (**68**), usaramine (**61**) and two new alkaloids, anonamine (**69**) and hydroxyneosenkirkine (**70**).

An investigation of ***Senecio gallicus***[66] afforded the known alkaloids ligularizine (**71**), senkirkine (**65**) and senecionine-4-oxide (**72**). The same workers isolated florosenine (**73**) and a new alkaloid from ***Senecio adonidifolius***[66], which was identified as 12,13,19-trihydroxy-15,20-epoxy-15,20-dihydro-(12S,15R,20R)senecionan-11,16-dione (**74**).

The co-occurrence of a pyrrolizidine alkaloid and its corresponding acylpyrrol has been reported for the first time[3]. Acetylsenecionine (**75**) and the acylpyrrol

desacetylsenaetnin (**76**) were isolated from ***Senecio magnificus***.



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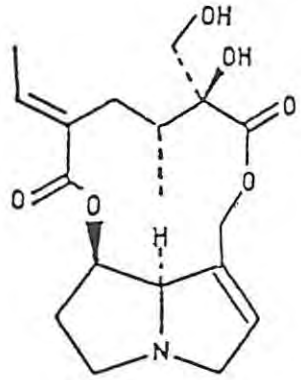

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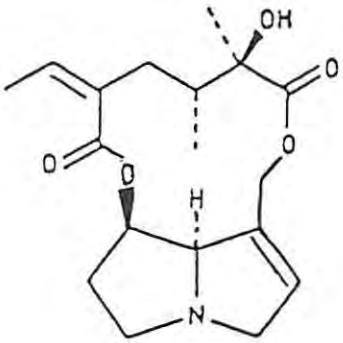

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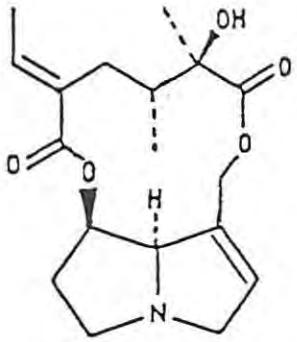

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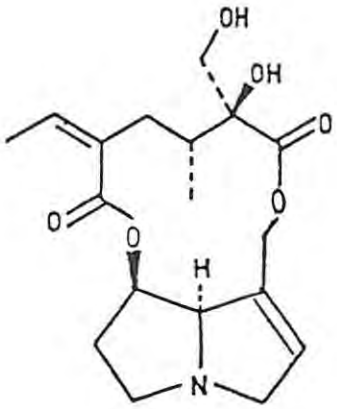

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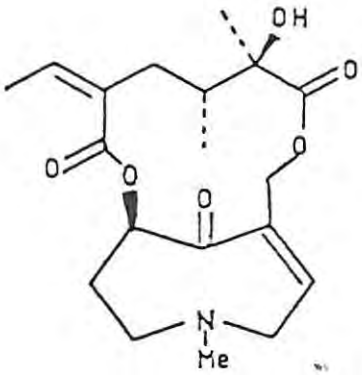

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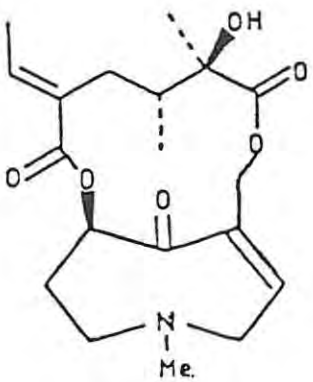

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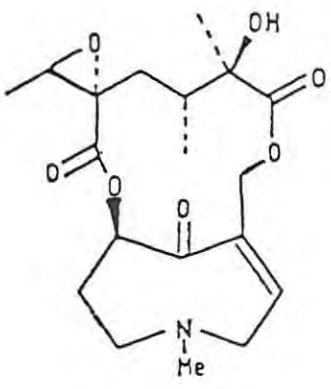

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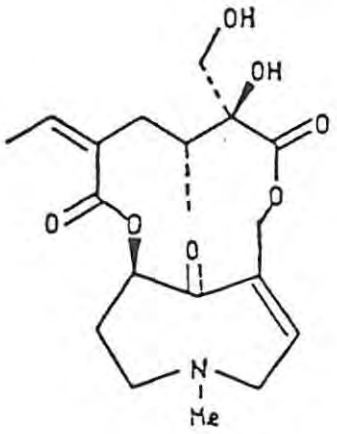

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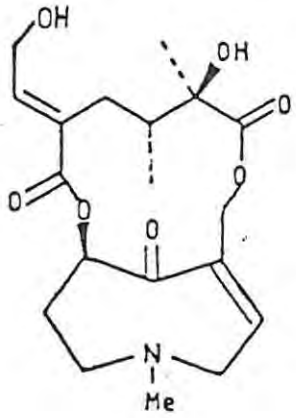

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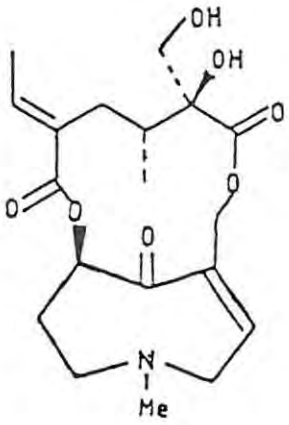

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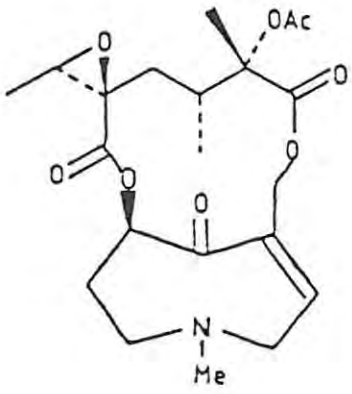

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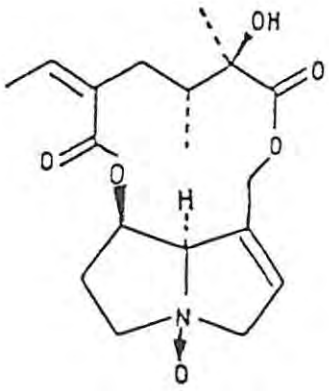

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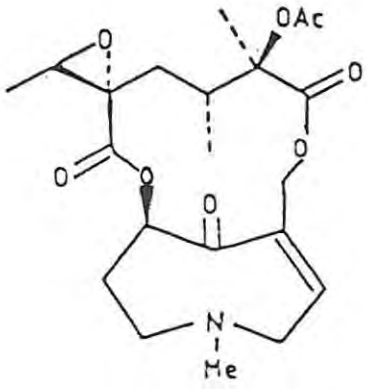

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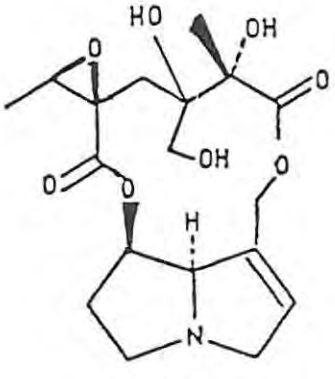

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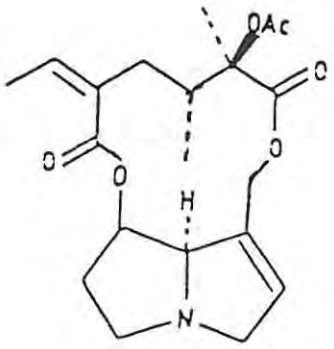
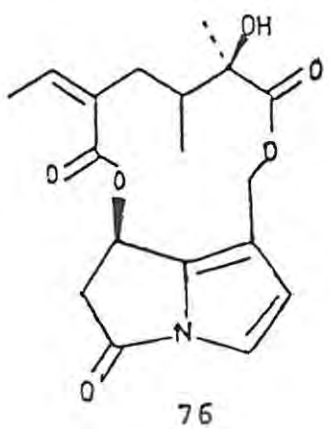


75

Other ***Senecio*** species also yielded macrocyclic diester alkaloids. Florosenine (**73**), senecionine (**62**), integerrimine (**63**), otosenine (**67**) and senkirkine (**65**) were isolated from ***Senecio glabellus***[67]. Senecionine, integerrimine and otosenine were isolated from ***Senecio sanguisorbae***[20]. Otosenine, senecionine and seneciphylline (**77**) were obtained from ***Senecio aquaticus Huds***[21]. Integerrimine and usaramine (**61**) were isolated from ***Senecio murorum***[22], while ***Senecio cilicius*** afforded integerrimine, senecionine and platyphylline (**78**)[23]. Bicchi *et al.* investigated the alkaloidal fraction of ***Senecio inaequidens*** by tandem gas chromatography-mass spectrometry (GC-MS) and gas chromatography-Fourier transform infra-red (GC-FTIR) studies[68,69]. Nineteen constituents were characterized and sixteen positively identified by these techniques. Alkaloids identified were senecivernine (**79**), senecionine, seneciphylline (**77**), spartioidine (**60**), integerrimine, retrorsine, usaramine, senkirkine, neosenkirkine(**66**), otosenine, acetylsenkirkine (**80**), florosenine, doronine (**81**), floridanine(**82**), floricaline (**83**) and the new alkaloid desacetyldoronine(**84**).

An investigation of ***Senecio argunensis***, a herb used in traditional Chinese medicine, yielded the known alkaloids senecionine, otosenine, erucifoline (**85**) and a new alkaloid, 21-hydroxyintegerrimine (**86**)[37].



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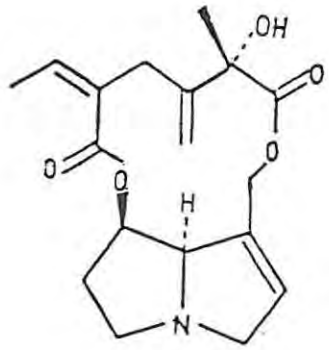

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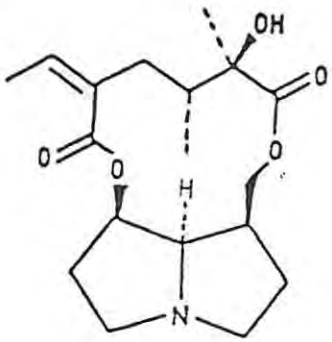

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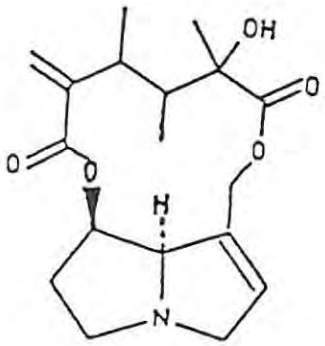

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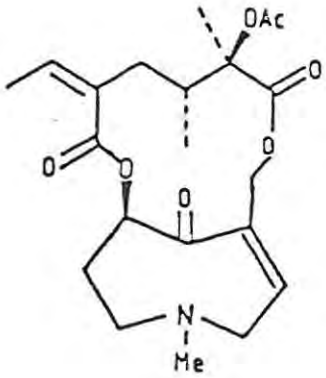

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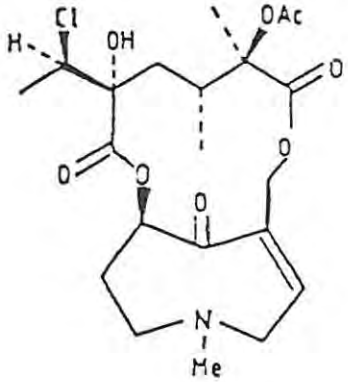

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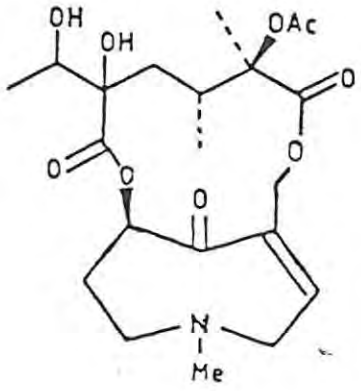

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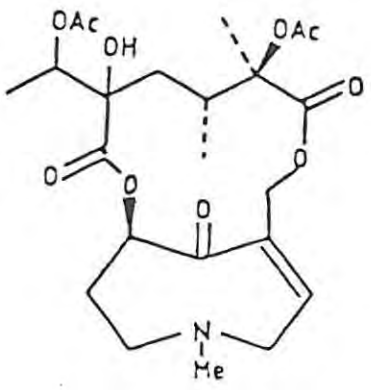

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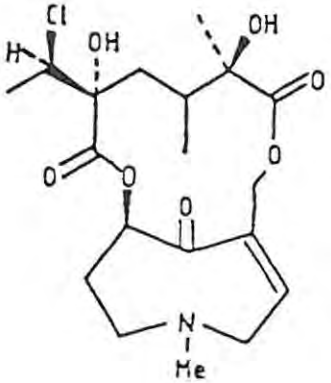

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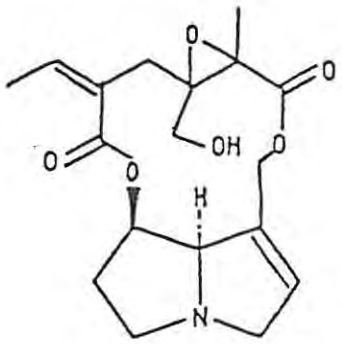

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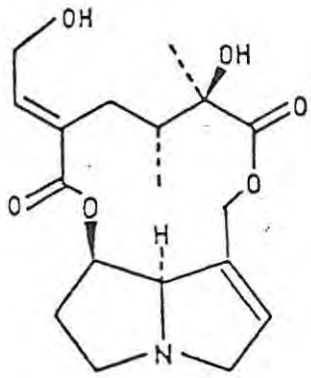

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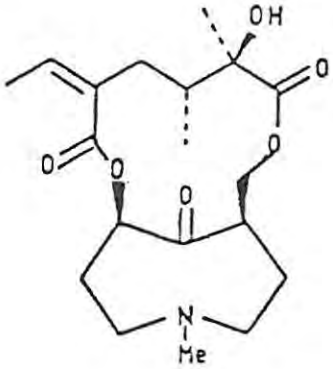

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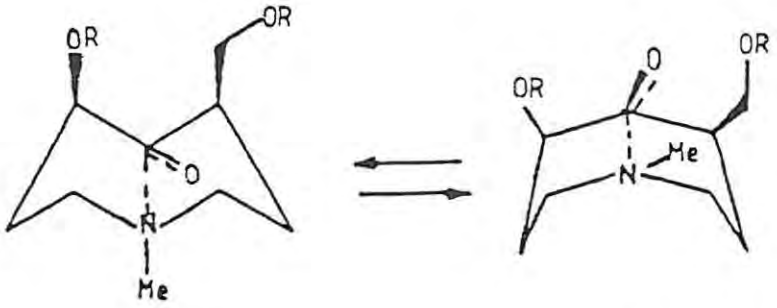
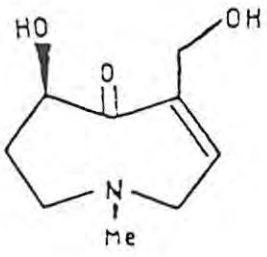


88a 88b

R = Senecic acid

Fig 1.1



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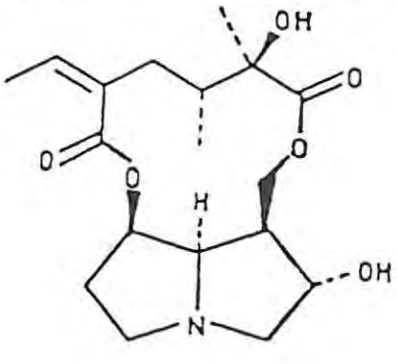


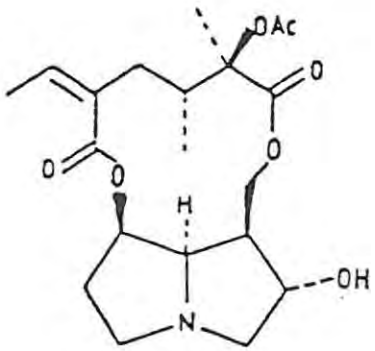
90

A new saturated otonecine pyrrolizidine, 1,2-dihydrosenkirkine (**87**), was isolated from ***Senecio integrifolius*** var. ***Fauriri***[39]. Two forms of this alkaloid, shown by structures (**88a**) and (**88b**), were in fact isolated by preparative thin layer chromatography, and were found to be interchangeable in solution. This phenomenon was also observed by 300 MHz NMR spectroscopy, since the two forms of the alkaloid exhibit very different chemical shift values at H_1, H_7, H_9, H_{20} and H_{21}[39]. Analysis of the compounds was completed by ^1H-^1H correlation spectroscopy (COSY) and ^1H-^{13}C heteroatomic correlation spectroscopy (HETCOR); the data thus obtained was in complete accordance with the assigned structures.

The ring inversion shown in Fig 1.1 may occur in otonecine (**89**) and analogous compounds, but since the otonecine ring system is more rigid due to the presence of a double bond, the less stable form would probably never be observed[39].

1,2-dihydrosenkirkine (**87**) lacks this double bond and is more susceptible to ring-inversion; hence the isolation of both stereoisomers which exhibit different chromatographic and spectroscopic behaviour[39]. The saturated otonecine-type base has not been isolated before.

A group of closely related macrocyclic diesters was isolated from ***Senecio hadiensis*** **Forsk**, a plant used in folk medicines among the rural people of Kenya[70]. As well as the known alkaloid rosmarinine (**90**), the new alkaloids 12-acetyl-rosmarinine (**91**), neorosmarinine (**92**), hadiensine (**93**), 12-acetylhadiensine (**94**), 12-acetylneohadiensine (**95**) and



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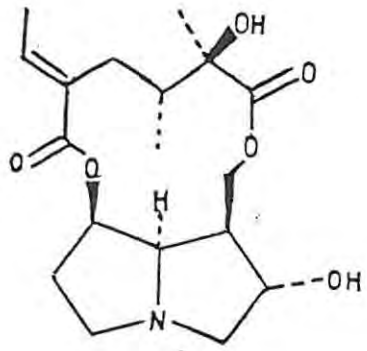

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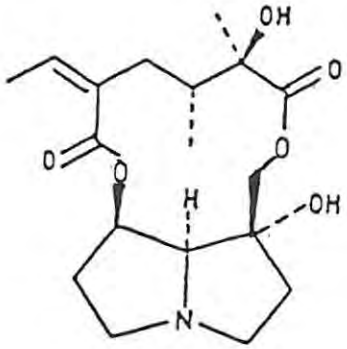

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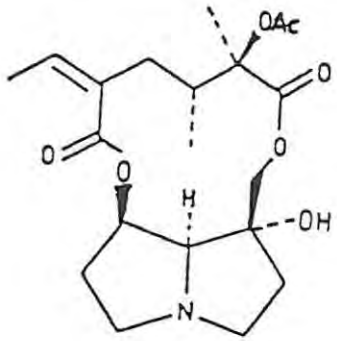

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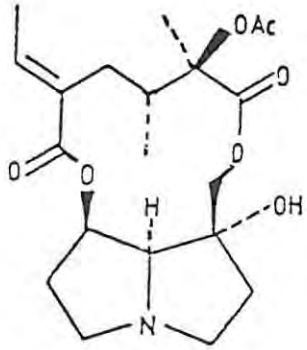

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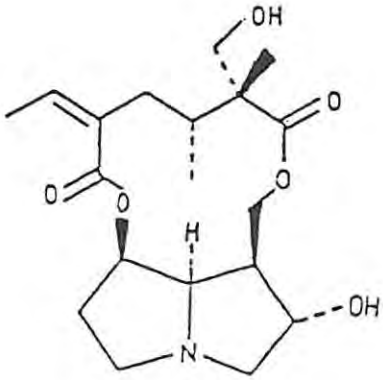

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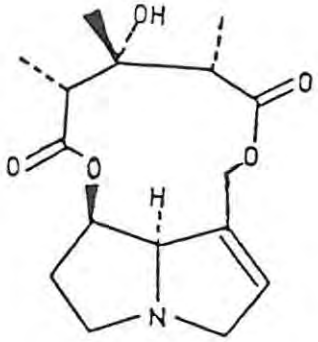

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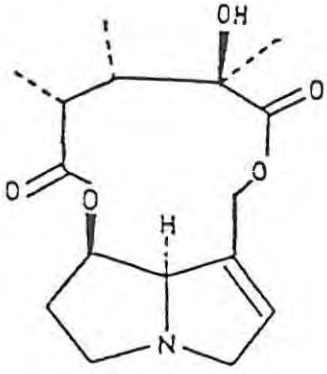


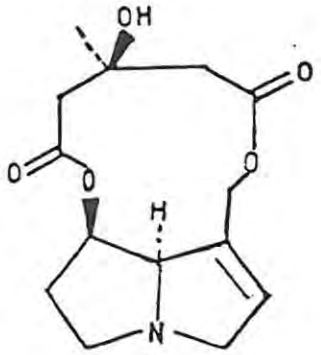
98

petitianine (**96**) were isolated and identified using NMR techniques. Hadiensine and its isomers are striking in that they possess a hydroxy group at C-1.

An investigation of the alkaloids crotaleschenine and crispatine, previously thought to be the same, showed that crispatine (**97**) is in fact an isomer of crotaleschenine (**98**)[50]. Crotaleschenine was isolated from ***Crotalaria leschenaultii*** and its structure elucidated by NMR, fast atom bombardment mass spectrometry (FAB) and X-ray crystallography[50].

Dicrotaline (**99**) and its acetyl derivative (**100**), the natural occurrence of which is reported for the first time, were isolated from ***Crotalaria lachnosema***[16]. ***Crotalaria nargutensis*** was also investigated and found to contain nilgirine (**101**), integerrimine (**63**), usaramine (**61**) and a new alkaloid, acetylintegerrimine (**102**), all identified by NMR spectroscopy, mass spectrometry and gas chromatography[16].

As part of a continuing investigation of ***Parsonsia laevigata***, Abe *et al.* isolated the new alkaloids parsonsianidine (**103**), 20-methylparsonsianidine (**104**) and parsonsianine (**105**) as minor components of the plant[38].



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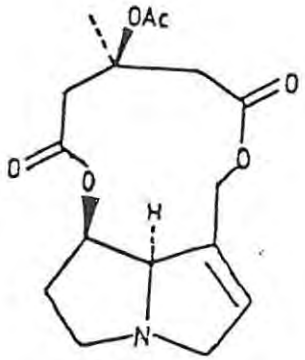

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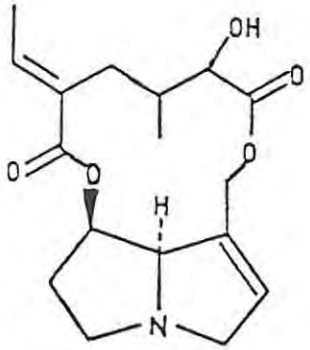

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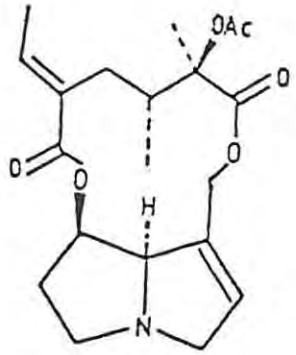

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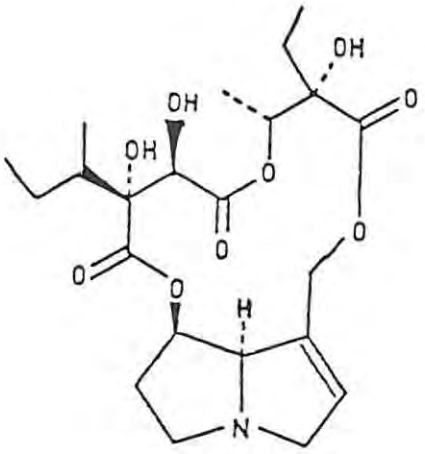

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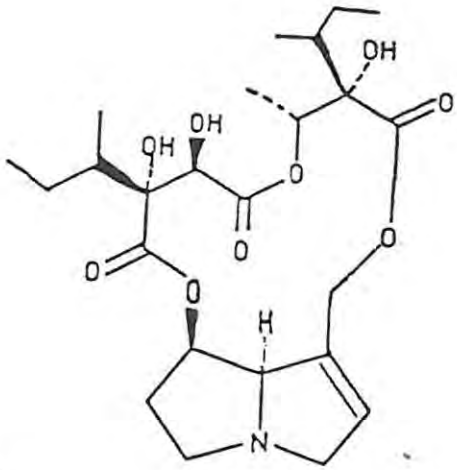

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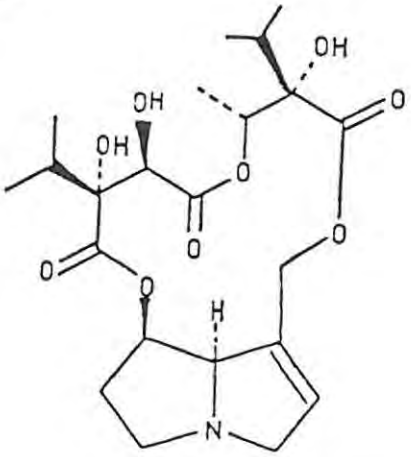


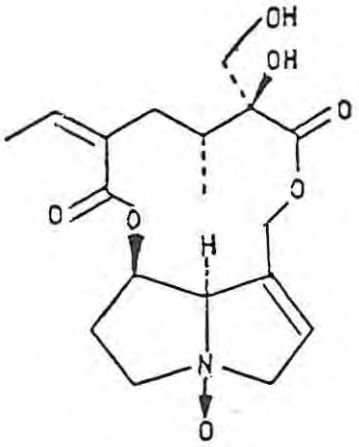
105

A number of new genera have been found to contain macrocyclic pyrrolizidines. ***Werneria decora*** was found to contain the known alkaloids isatidine (retrorsine-4-oxide) (**106**) and retronecine-4-oxide (**107**)[19]. Although other species belonging to this genus have been studied, this is the first species to contain pyrrolizidines.

Floridanine (**82**) has been isolated from ***Cordia sinensis*** and macrophylline (**108**) from ***Cordia mixa***, while ***Schismus barbatus*** was found to contain senecionine (**62**)[30].

A new alkaloid, 12-acetyljacoline (**109**), was isolated from ***Cirsium wallichii DC***[84].

A recent chemotaxonomic study has revealed the presence of pyrrolizidine alkaloids in the genera ***Lotononis*** and ***Buchenroedera*** for the first time[36]. Twenty-one species from the two genera were found to contain pyrrolizidines, the alkaloids isolated being senecionine (**62**), integerrimine (**63**), platyphylline (**78**) and neoplatyphylline (**110**).



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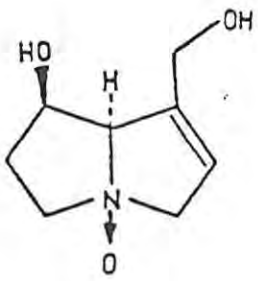

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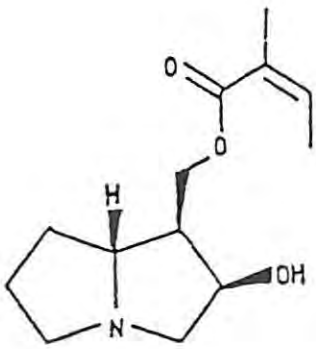

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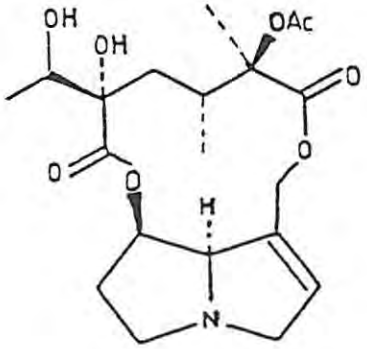

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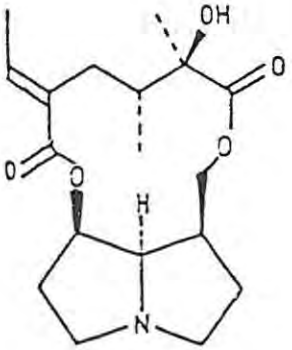

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1.3 Separation and identification of pyrrolizidine alkaloids

Pyrrolizidine alkaloids are usually extracted from dried, chopped plant material with hot or cold methanol. The methanol is evaporated to approximately one-tenth of its volume and the residue treated according to a standard method. Fig 1.2 shows a typical extraction procedure. The residue is treated with dilute aqueous acid, the solution washed with an organic solvent to remove all nonbasic organic-soluble material and treated with zinc dust to reduce N-oxides. The solution is then made alkaline with dilute NH_4OH and the free alkaloids extracted with an organic solvent. Alternatively, the methanolic solution may be passed through a cation exchange resin and the alkaloids eluted from this with dilute NH_4OH[18,71].

Traditional extraction methods involve large quantities of plant material, but the recent development of a micro-extraction method using sonification has made it possible to investigate the alkaloid content of small amounts of plant material (eg. herbarium specimens)[72].

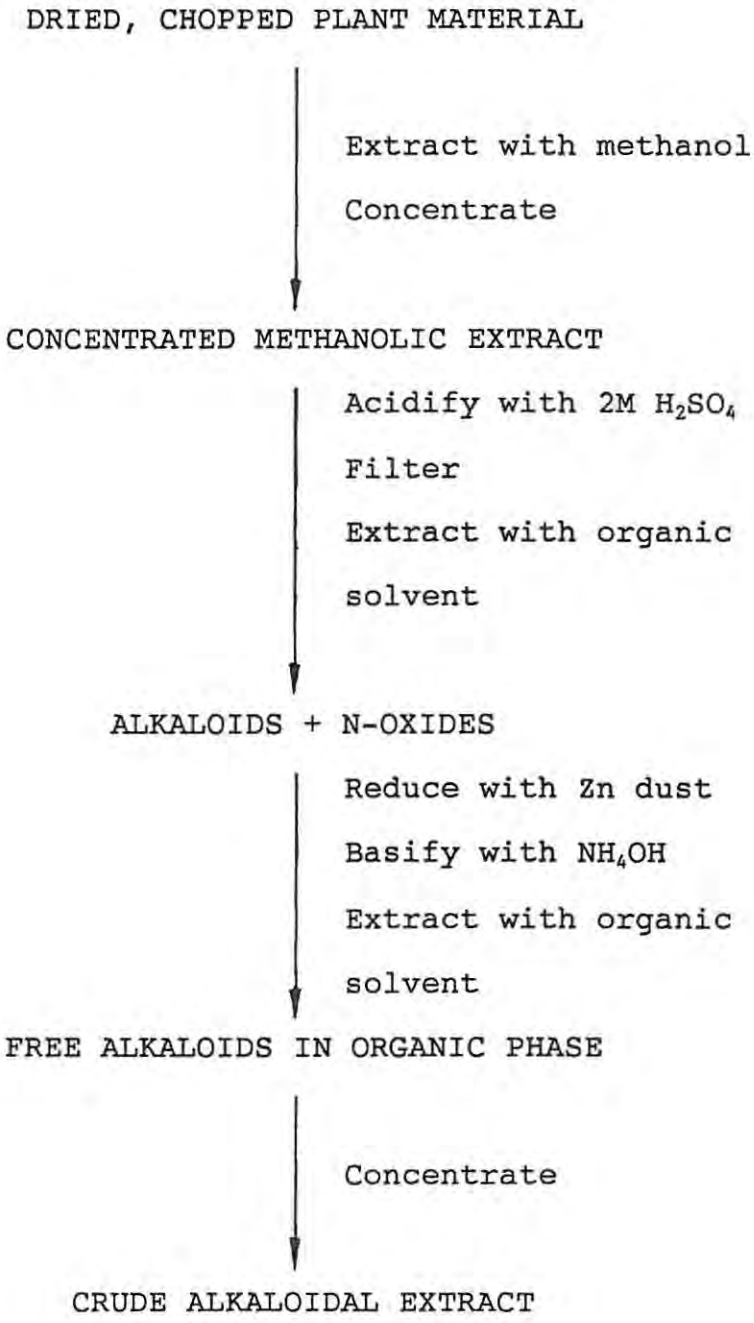


Fig 1.2 General extraction procedure

1.3.1 Preparative separation techniques

The crude alkaloidal extract thus obtained can be separated into individual components in a variety of ways. Crystalline components may be purified by crystallization, although co-crystallization of alkaloids often occurs. Suitable solvents are methanol, ethanol or acetone.

Column chromatography is the usual method of preparative separation. The solid support is usually silica or neutral alumina, although cellulose powder, powdered glass and celite have also been used[1]. A variety of solvent systems have been developed.

Column chromatography on a solid support has many disadvantages. The highly polar alkaloids tend to adsorb irreversibly onto the support, resulting in poor separation and large losses of material[4]. Repeated chromatography is often necessary in order to purify a sample, with resulting poor yields. The decomposition of alkaloids on silica has also been observed[73]. Column chromatography does have the advantage, however, of being relatively inexpensive and quick, especially if flash chromatography is used.

Preparative thin-layer chromatography is often used to purify small quantities of alkaloid. Again, the solid support is usually silica or neutral alumina, with solvent systems much the same as for column chromatography.

Vacuum-liquid chromatography (VLC) is essentially a preparative layer chromatographic separation run as a column,

the flow of which is activated by vacuum[74]. The technique involves step gradient elution and good separation of components may be achieved by careful selection of solvent gradients. Sample quantities ranging from 100mg to 10g may be separated. Since this technique was introduced by Pelletier[75], other workers have used it with success for pyrrolizidine alkaloids[11,39]. Once again, binding of the alkaloids to the silica gel is unavoidable.

Preparative high-performance liquid chromatographic separation of pyrrolizidines has been achieved using a reverse-phase column and ion-pair adsorption techniques, with good resolution[76,77]. The main disadvantage in the use of HPLC is its high cost and the technique is thus often used analytically rather than on a preparative scale[1]. Zalkow *et al.*, however, observed irreversible binding of pyrrolizidine alkaloids to the reverse-phase HPLC columns[4].

Droplet counter-current chromatography (DCCC) is a technique which has only recently come into use for alkaloid separation. DCCC is a liquid-liquid extraction technique based on the partitioning of solutes between a steady stream of droplets of mobile phase and a column of surrounding stationary phase[78]. It is particularly useful for the separation of polar natural products, since the absence of a solid support eliminates adsorption problems. In many cases, separation of complex mixtures of components has been achieved far more readily than with conventional chromatographic techniques. Zalkow *et al.*[4] separated ten macrocyclic pyrrolizidine

alkaloids from ***Senecio anonymus*** using DCCC, obtaining the pure components in sufficient quantity for NMR analysis. Other workers have used this technique with success for the separation of pyrrolizidine alkaloids[4,40,62,79]. Two excellent reviews on DCCC and related techniques have recently been published[59].

1.3.2 Analytical separation techniques

In many cases, plants contain complex mixtures of alkaloids which cannot easily be separated, and the total alkaloid content of the plant is very small. Accordingly, pure components cannot be obtained in sufficient quantity for conventional analysis and other methods have to be used to analyze such mixtures.

Analytical thin-layer chromatography is a very useful technique. Silica coated plates are used most frequently for pyrrolizidine alkaloid work. Alkaloids are detected on chromatograms by a variety of spray reagents. Dragendorff's reagent is commonly used, but has been found to react positively with non-alkaloidal compounds as well[80]. The iodoplatinate reagent is more specific[1], but also more expensive. The most convenient and sensitive method for visualising unsaturated pyrrolizidine alkaloids is to spray the chromatogram with a chloranil solution, heating briefly, then spraying with Ehrlich's reagent[81].

Thin-layer chromatography can thus give an indication of the

number and type of pyrrolizidines present in the crude alkaloid mixture. Tentative identification of alkaloids by comparison of R_f values is possible if standards are available, but many alkaloids have identical R_f values and components are often missed due to incomplete separation.

High-performance liquid chromatography (HPLC) may be used in conjunction with mass spectrometry for identification of the components of complex alkaloidal mixtures[1].

A more frequently used technique is tandem gas chromatography-mass spectrometry (GC-MS). The main drawback of gas chromatography is that underivatized alkaloids often decompose due to the high temperatures used for the separation. Many workers have analyzed alkaloid samples by derivitazation[82]. The recent development of specially inert columns for polar components has facilitated the GC study of underivatized pyrrolizidines and many workers now use GC-MS for analysis of complex alkaloid mixtures[69,83-87]. The alkaloid fraction of ***Senecio inaequidens*** was analyzed by gas chromatography and mass spectrometry in different ionization modes[68]. Nineteen pyrrolizidine alkaloids were characterized and sixteen identified by this method. Unfortunately, mass spectrometry is unable to distinguish between isomeric alkaloids, and results obtained can therefore be ambiguous. Reference standards are necessary for comparison of retention times and mass spectra in order to identify compounds unambiguously. A technique which is complementary to GC-MS is tandem gas

chromatography-Fourier transform infra-red (GC-FTIR). Infra-red spectroscopy can be used to characterize individual alkaloids by "fingerprinting". Useful information concerning unsaturation and functional groups may be obtained. It is possible using this technique to distinguish between isomers, although reference standards are necessary for complete identification. GC-FTIR is a powerful analytical tool when used in conjunction with GC-MS. In the study of the alkaloidal fraction of ***Senecio inaequidens***, mentioned previously, Bicchi *et al.* made use of both techniques[68], but unfortunately the cost of the GC-FTIR equipment is prohibitive.

1.3.3 Identification techniques

Alkaloids may be identified by comparison with authentic samples, but these are often difficult to obtain. They may be identified by comparison of their physical properties, such as melting point, optical rotation and electrophoretic mobility, with those of known compounds. Such physical properties are dependent on the degree of purity of the alkaloids and are therefore not definitive. Unknown alkaloids are usually identified using NMR spectroscopy. The use of high-field NMR spectroscopy is essential to obtain good resolution and two-dimensional NMR techniques provide unambiguous structural assignment. ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR are most commonly used. Nuclear Overhauser Enhancement

spectroscopy (NOESY) provides information about through-space interactions between protons in a molecule and can aid in determination of stereochemistry. Other techniques such as NMR-SPI may also be used. There is a vast amount of published NMR data and a review of the ^{13}C spectral data of over 130 pyrrolizidine alkaloids was published recently[88].

Infra-red analysis of the alkaloid can also provide a fair amount of information since individual alkaloids have characteristic IR bands. The IR spectrum provides a "fingerprint" of the alkaloid.

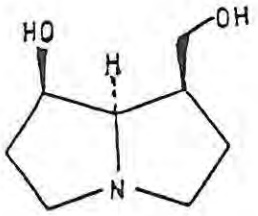
If insufficient material is available for NMR analysis, techniques such as GC-MS, GC-FTIR and HPLC are used to analyze an alkaloidal extract. These techniques are also used if separation of a mixture of alkaloids cannot be achieved in any other way.

1.3.4 Mass spectrometry of pyrrolizidine alkaloids

Pyrrolizidine alkaloids exhibit characteristic peaks in their mass spectra. It is possible to obtain a great deal of information about the structure of a pyrrolizidine alkaloid from electron impact mass spectrometry and both positive and negative ion mass spectrometry.

It is possible to identify the type of necine base present using diagnostic peaks. Saturated bases, such as platynecine (**111**) show intense ions at m/z 82 or 83, together with peaks at m/z 97 or 113[1]. The fragmentation pathway giving rise to these peaks is shown in Scheme 1.1. Unsaturated bases such as retronecine (**37**) show intense ions at m/z 80 or 81 and 95 or 111[1]. The fragmentation pathway is shown in Scheme 1.2. Otonecine (**89**) type bases exhibit characteristic peaks at m/z 94, 96 and 110[1] (Scheme 1.3).

The croalbinecine (**112**) type base is saturated and possesses a hydroxyl group at C_2. Dehydration occurs readily and the mass spectrum can thus exhibit peaks characteristic of both a saturated and unsaturated base[89]. It may also be difficult to identify the necine base of an alkaloid when it is esterified, since peak intensities are altered and the esterifying acid can give rise to peaks which are misleading; for example, the presence of angelic acid in heliosupine (**26**) gives rise to a peak at m/z 83, as shown in Scheme 1.4[89]. A peak at m/z 108 is usually taken to indicate a saturated necine base, but this peak is also prominent in the mass spectra of unsaturated monoester alkaloids such as isolycopsamine (**39**), heliospathuline (**40**) and 7-curassavinylretronecine (**42**)[40,90]. In esterified necines account must be taken of a range of diagnostic peaks.



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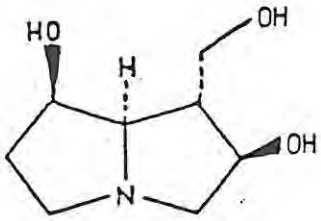

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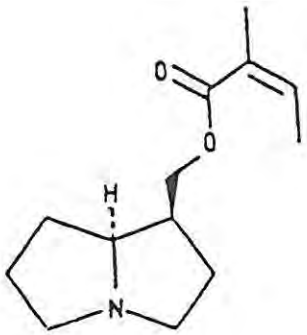

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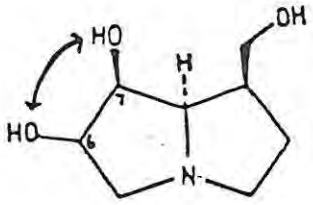

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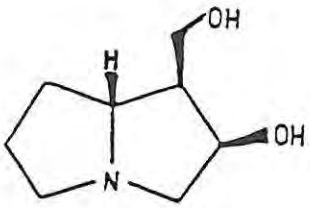

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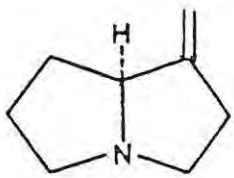

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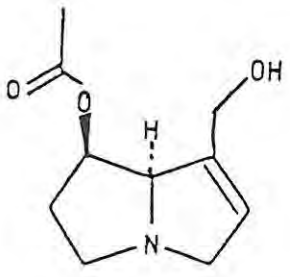

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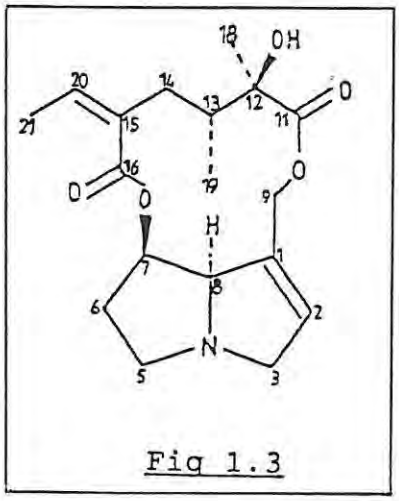
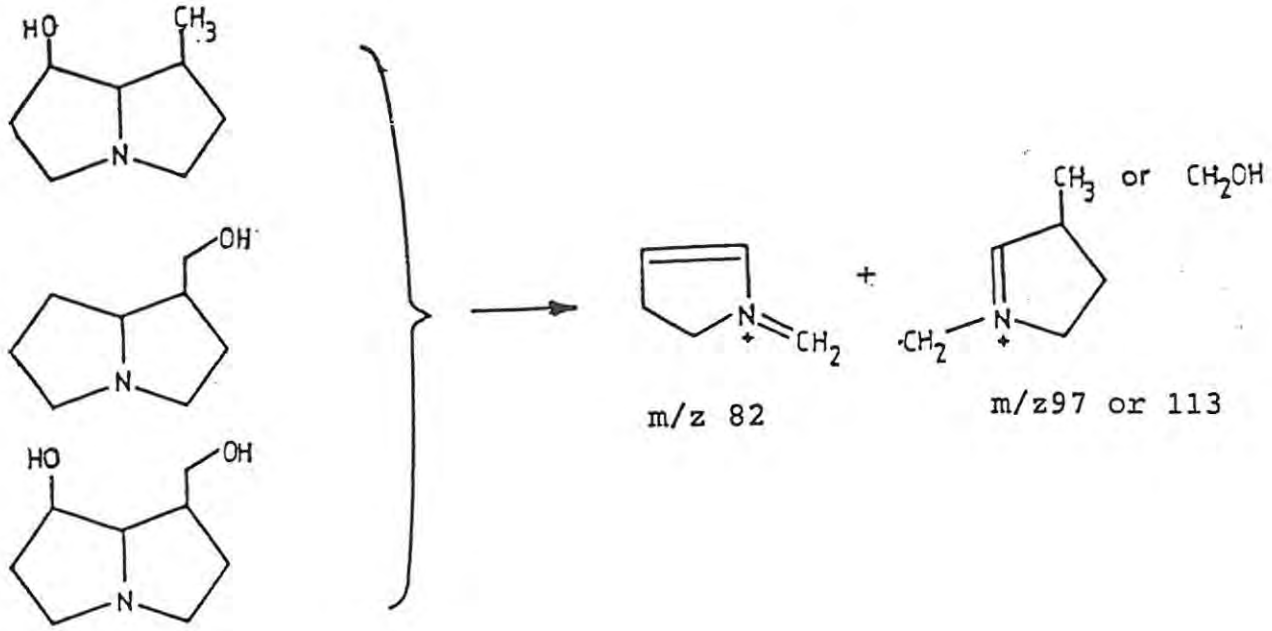
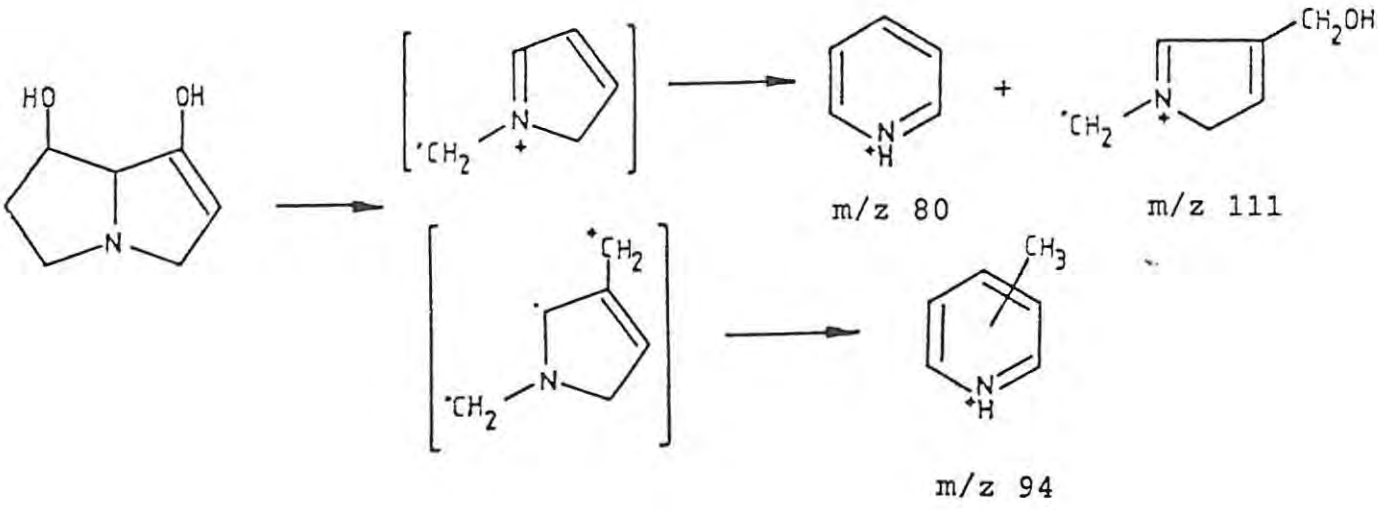


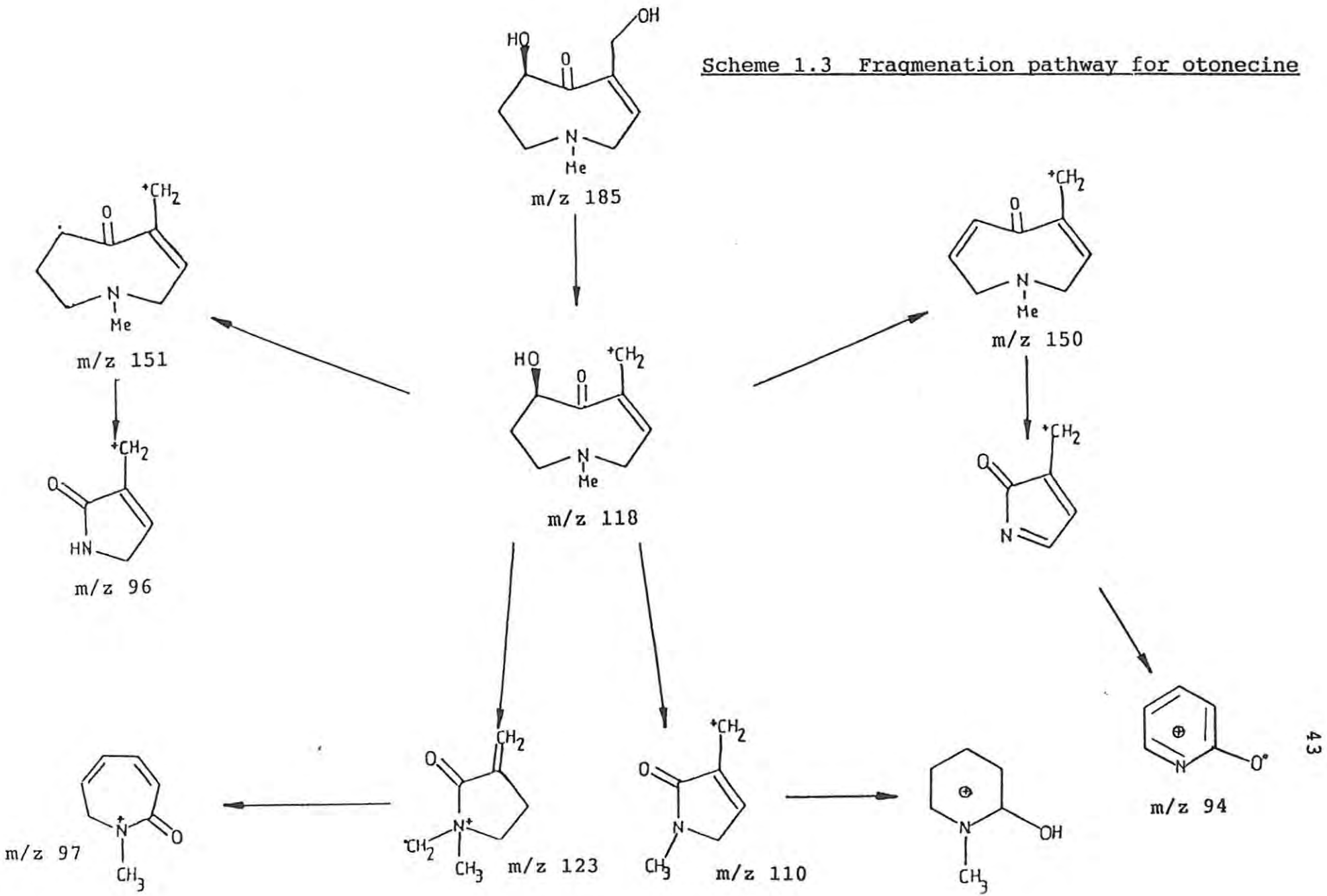
Fig 1.3



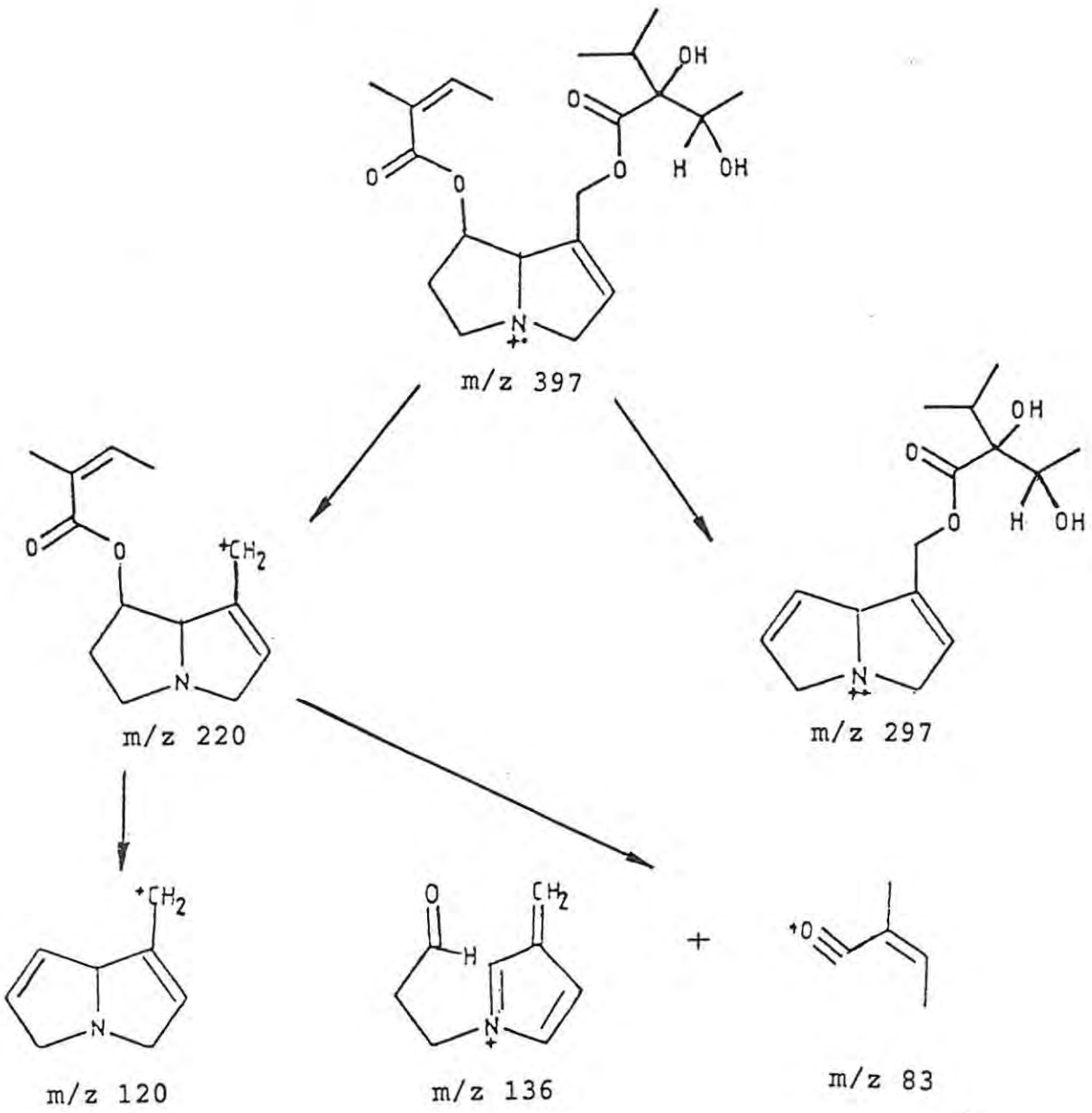
Scheme 1.1 A fragmentation pathway for platynecine



Scheme 1.2 A fragmentation pathway for retronecine



Scheme 1.3 Fragmenation pathway for otonecine

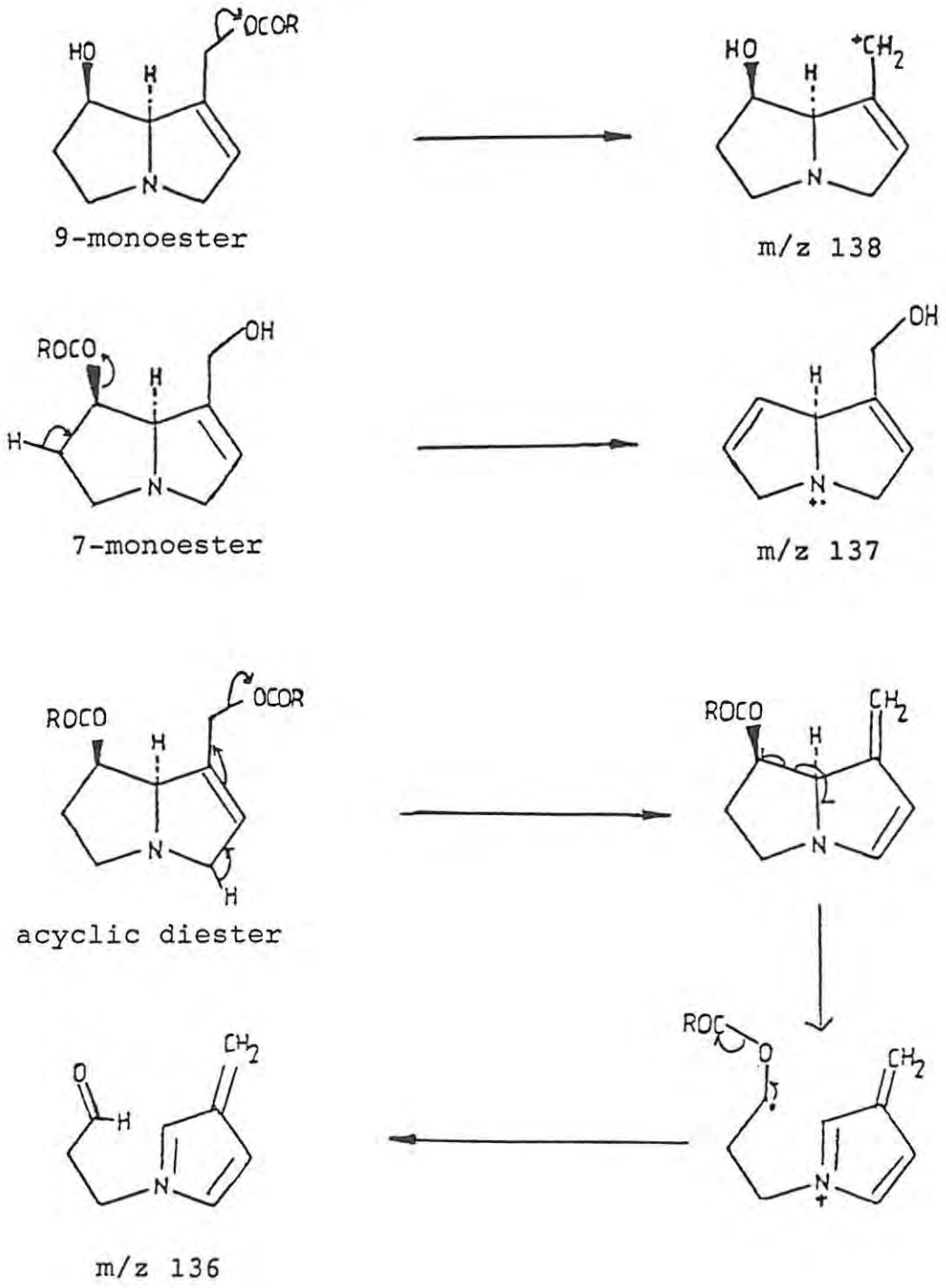


Scheme 1.4 Fragmentation pathway for heliosupine

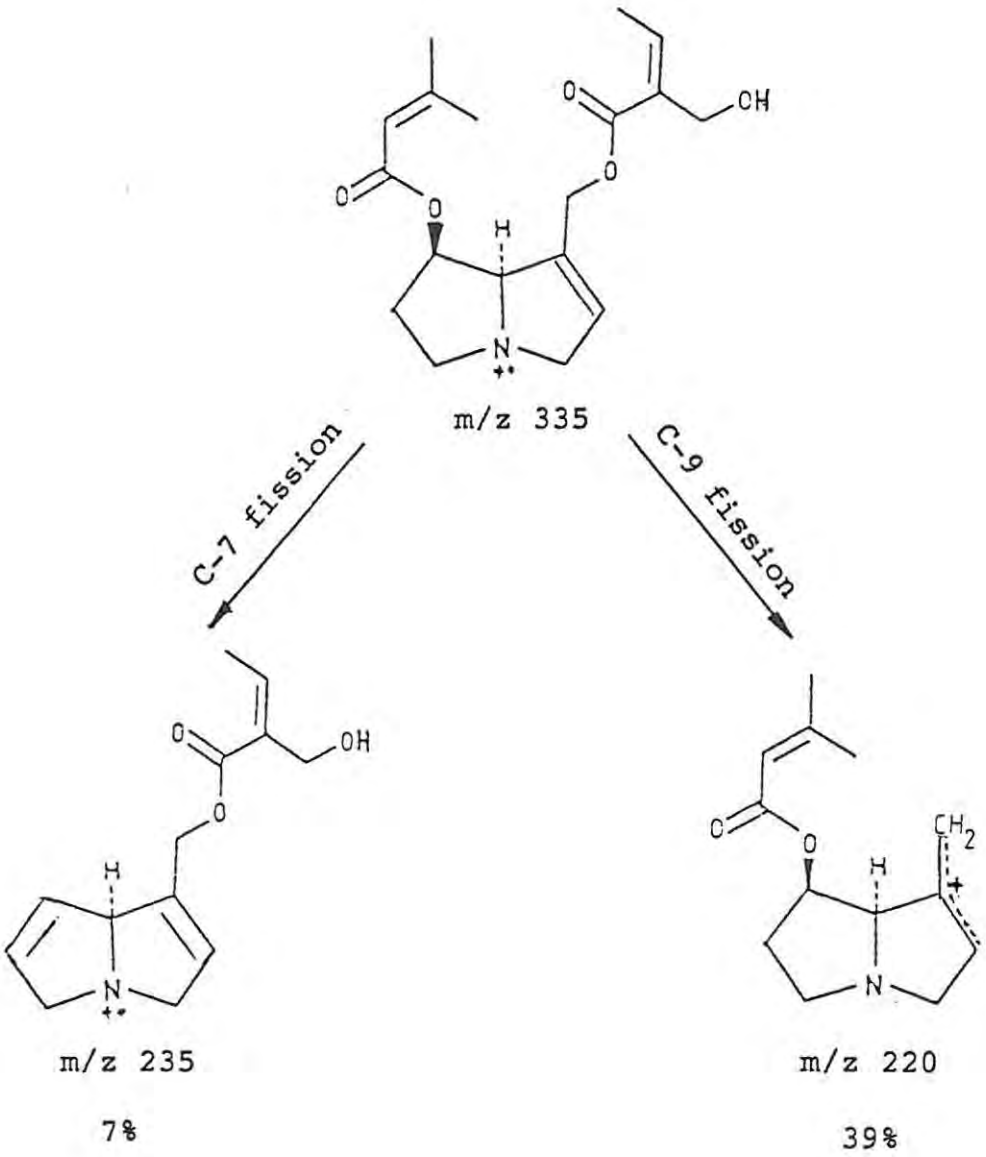
It is possible to ascertain whether the base is mono- or diesterified, and whether the diester is acyclic or macrocyclic. C9-monoesters of retronecine (**37**) exhibit an intense peak at m/z 138, C7-monoesters at m/z 137 and acyclic diesters at m/z 136 (Scheme 1.5)[7,55,91]. The corresponding monoesters of platynecine exhibit peaks at m/z two mass units higher; i.e at m/z 138, 139 or 140.

Macrocyclic diesters exhibit the characteristic "triads" of peaks; a group of peaks at m/z 80 and 81 with a second group at m/z 119, 120, 121 and a third at m/z 136, 137, 138 indicate an unsaturated base alkaloid[1]. "Triads" of peaks at m/z 82 and 83; m/z 121, 122, 123 and m/z 138, 139 and 140 are exhibited by saturated base alkaloids[1]. The triad at m/z 119, 120 and 121 (or two mass units higher) also appears in the spectra of acyclic diesters and monoester alkaloids.

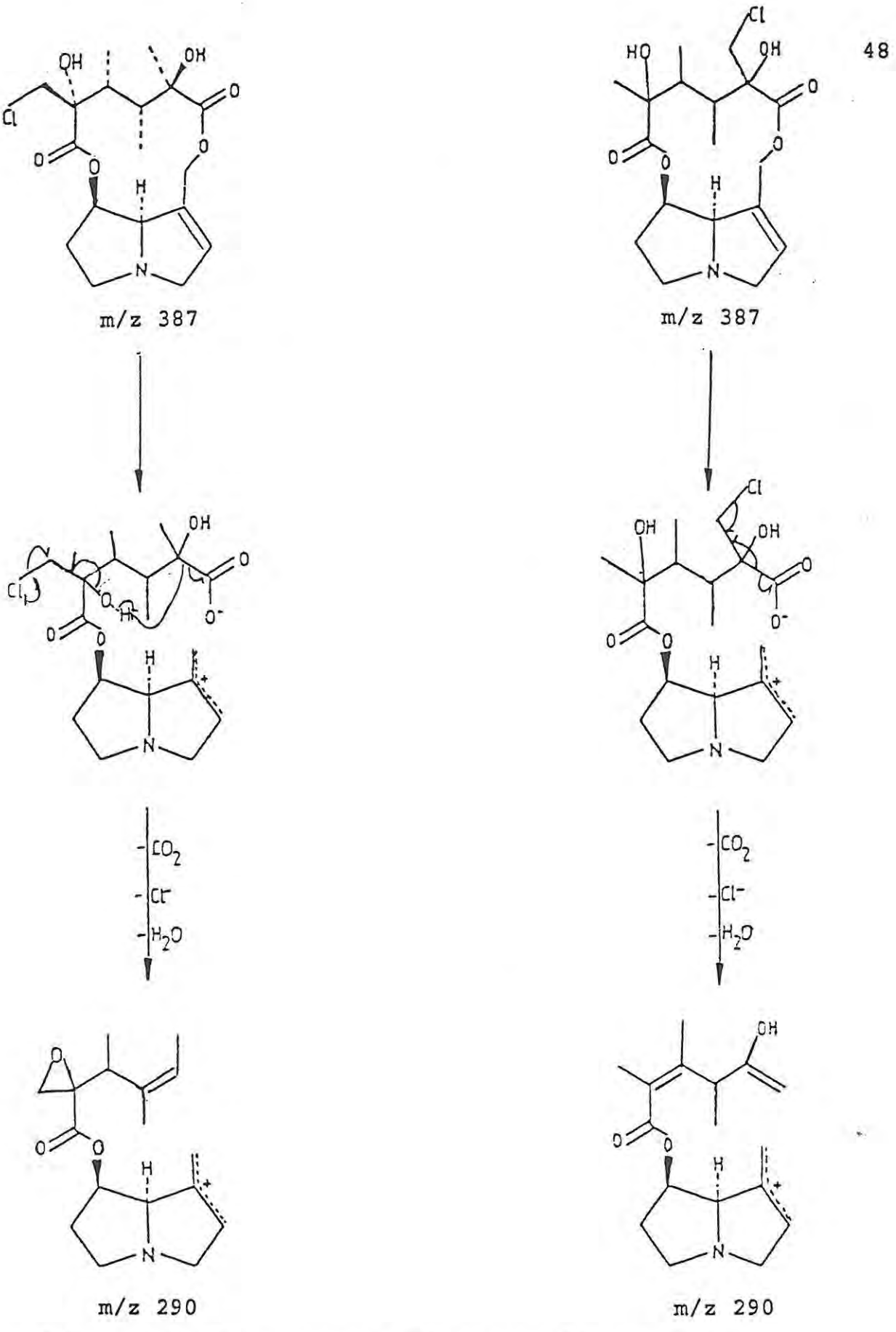
A great deal of information can be obtained from the relative intensities of peaks in the spectrum. The C_9 ester bond is weaker than the C_7 bond and will break more easily; hence ions corresponding to this cleavage will be more intense than ions corresponding to cleavage at the C_7 linkage (Scheme 1.6). In the case of acyclic diesters this enables one to ascertain the mode of ester attachment. In the case of macrocyclic diesters however, confusion can occur, since the ion corresponding to C_9 cleavage can also be formed by cleavage at C_7 with subsequent rearrangement[92]. This is shown in Scheme 1.7.



Scheme 1.5 Fragmentation of monoesters and acyclic diesters

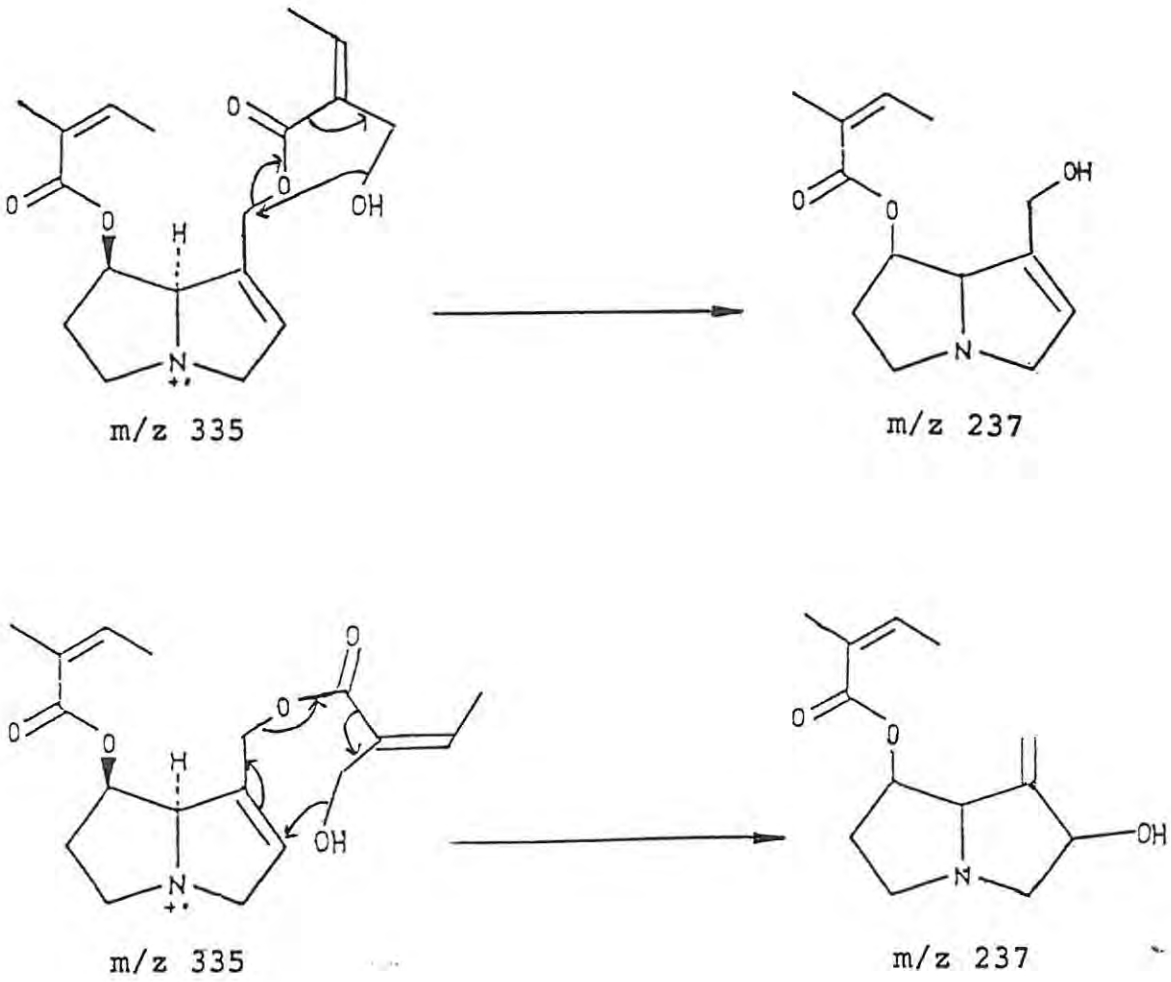


Scheme 1.6



Scheme 1.7 Rearrangement of macrocyclic diesters

Rearrangements play a large role in the fragmentation of acyclic diester and monoester alkaloids. Scheme 1.8 shows two possible mechanisms for such a rearrangement.



Scheme 1.8 Possible mechanisms for rearrangment in acyclic diesters

These fragmentation pathways apply mainly to the retronecine and platynecine type bases. The less common pyrrolizidine bases exhibit other characteristic peaks. A base peak at m/z 70 is considered by a number of authors to be indicative of a pyrrolizidine in which ring A is unsubstituted (**113**)[6,50], as is the combination of a base peak at m/z 83 and a prominent peak at m/z 98[93]. A base peak at m/z 82 occurring with a major peak at m/z 113 is characteristic of a 1-hydroxymethyl-pyrrolizidine with an OH at C_6 or C_7 (e.g.**114**)[93]. This combination is also considered characteristic of platynecine type pyrrolizidines[94]. A base peak at m/z 83 is typical of the macronecine base (**115**)[95]. The otonecine base (**89**) shows characteristic fragments at m/z 168, 150, 122, 110 and 96[96], while a base peak at m/z 124 is typical of 1-methylene-pyrrolizidines (**116**)[97].

Other typical diagnostic peaks occur. A peak at m/z M-44 (loss of CO_2) is typical of all macrocyclic diesters[98]. A peak at m/z M-17 is indicative of an OH group at C_{12}, since this peak is not observed in the spectra of alkaloids lacking this group[99-101] (see Fig 1.3 for numbering). A peak at m/z M-31 shows that a methyl ester is present[95]. A base peak at m/z M-31 is indicative of the loss of an exocyclic CH_2OH group[51]. A strong peak at m/z 180 is typical of a 7-acetylretronecine derivative (**117**)[102]. Acyclic diesters of retronecine in which the ester at C_7 is an acyl group show intense fragments at m/z 180, 136 and 120[103]. A peak at m/z M-15 indicates the presence of a CH_3 group on the nitrogen atom[101]. A peak at m/z M-18 indicates the loss of H_2O, which is considered to occur when

there is an OH at C_{15} (Fig 1.3 shows the numbering)[101]. Other workers have found that a peak at M-18 occurred when there was a CH_2OH group present at C_{12}[104]. Acyclic diesters of retronecine or platynecine which possess a C7-angeloyloxy (or tigloyloxy) group exhibit intense peaks at m/z 220, due to cleavage of the C_9 bond[105]. The same acid group at C_9 leads to an intense peak at m/z 237. A peak at m/z 101 is due to protonated angelic, tiglic or senecioic acid[105]. A peak at m/z 253 is indicative of a retronecine ester of hydroxysenecioic acid and one at m/z 235 indicates a retronecine ester of hydroxyangelic acid[62].

Although mass spectrometry can provide useful information concerning the overall structure of a pyrrolizidine alkaloid, relative stereochemistry cannot be determined, nor can geometric isomers always be distinguished. It is necessary to make use of reference standards for unambiguous structural assignment. Mass spectrometry is a powerful tool in its own right, but the information it provides can be greatly enhanced by the concurrent use of other spectroscopic techniques, where possible.

CHAPTER TWO

DISCUSSION

2. General

Senecio speciosus Willd. and ***Senecio macrocephalus*** DC. are closely related botanical species belonging to the genus ***Senecio***. Both plants bear purple daisy-like flowers and are often found growing in disturbed ground and pastures. Their physical appearance is very similar, but ***S. speciosus*** is a coastal plant while ***S. macrocephalus*** occurs at higher altitudes[106]. Neither of these plants has been investigated previously for alkaloid content. Counterparts of these plants found in the Grahamstown district are not easily classifiable as either ***Senecio speciosus*** or ***Senecio macrocephalus***, but exhibit characteristics intermediate to the two species. It is possible that, Grahamstown being an intermediate climatic and geographical zone, these plants represent hybrids of ***S. speciosus*** and ***S. macrocephalus***. Alternatively, the plants could represent genetic mutants or even an entirely different species. In order to examine the alkaloidal extracts of previously uninvestigated plant species and possibly to collect taxonomic markers whereby these plants could be satisfactorily classified, plants were collected from four separate locations in the Grahamstown district. These locations are shown on the map in Fig 2.1 and represent uniform populations of plants.

Fig 2.1 Map showing locations of plant populations

Authentic specimens of ***S. speciosus*** and ***S. macrocephalus*** were also collected.

These plants were extracted as described in Section 3.1. The alkaloid contents of the Grahamstown populations (named populations 1 to 4, respectively) and of ***S. macrocephalus*** were lower than that of ***S. speciosus***. Alkaloid contents expressed as percentage crude alkaloid mass per dry weight of plant are given in Table 2.1.

Table 2.1 Percentage crude alkaloid per dry weight of plant

Plant	% crude alkaloid
Senecio speciosus	0.32
Senecio macrocephalus	0.21
Population 1	0.10
Population 2	0.061
Population 3	0.05
Population 4	0.055

Initial examination of the crude extracts by analytical thin-layer chromatography (TLC) on silica gel in various solvent systems showed that the crude extracts from populations 1 to 4 were extremely complex. Attempts to isolate pure alkaloids using column chromatography, preparative TLC and vacuum-liquid chromatography[74,75] on silica gel were unsuccessful due to the irreversible adsorption of the components on to the solid support. This phenomenon has been noted by other workers[4].

Attempts to separate alkaloids from non-alkaloidal material using Dowex(H^+) cation exchange resin[18,71] were successful, but it proved difficult to elute all alkaloidal material from the resin subsequent to this separation. A resin column apparently stripped free of alkaloid, yielded further alkaloid when treated with fresh eluent after a few days.

Droplet counter current chromatography (DCCC) proved the only reliable method for separating the complex mixtures of alkaloids without appreciable loss of material[59,78]. Components were further purified by preparative TLC on silica gel.

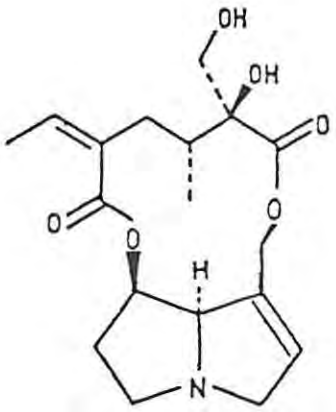
Owing to the small quantities of alkaloid present in the plants and the complexity of the alkaloid mixtures, analysis was largely dependent on gas chromatography-mass spectrometry (GC-MS). It was possible, using this technique, to separate mixtures of isomers which could not be separated by other methods. Since it is not possible to identify compounds conclusively by mass spectrometric data, standard alkaloids were required. These were obtained both by extraction from plants which have been investigated previously and by synthesis of simple monoester alkaloids.

The procedures whereby these standards were obtained will be discussed briefly before the analysis of the alkaloids present in ***Senecio speciosus***, ***S. macrocephalus*** and the local plant populations is described.

2.1 *Senecio othonnaeflorus* DC

This plant was investigated by Liddell *et al.*[107] and found to be exceptionally rich in retrorsine (**118**), with an alkaloid content as high as 1.5%. The plant was therefore extracted in order to obtain a supply of retrorsine, which is commonly found in ***Senecio*** species and is therefore a useful standard. The retrorsine could also be hydrolysed to provide the pyrrolizidine base retronecine (**119**), which was required for the synthesis of simple monoester alkaloids.

The retrorsine isolated was purified by recrystallization from acetone, and its structure confirmed by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. The NMR data obtained are in good agreement with those reported for retrorsine[88,108].



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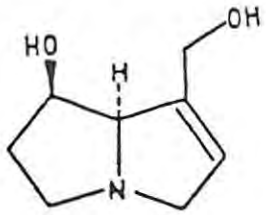

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2.2 *Senecio chrysocoma*

Senecio chrysocoma has often been confused with ***Senecio paniculatus***. Synonyms for ***Senecio chrysocoma*** include ***Senecio reclinatus***, ***S. graminifolius***, ***S. paniculatus*** Berg. ***var reclinatus*** and ***S. paniculatus auct.***, while ***S. paniculatus*** Berg. ***var paniculatus*** is considered to be the true ***S. paniculatus***[106]. ***Senecio paniculatus*** Berg. (variety unspecified) was investigated by Glonti in 1956[109], and reported to contain the alkaloids senecionine (**120≡62**) and platyphylline (**121≡78**). Since it was not certain whether the plant investigated by Glonti was the true ***S. paniculatus*** or not, the local ***S. chrysocoma*** was examined. The plant was found to contain 0.25% crude alkaloid. The crude extract exhibited two major components on GC, which were assumed to be the reported alkaloids. However, analysis by GC-MS revealed that the alkaloids present were not senecionine or platyphylline. Instead, the two major components appear to be monoester alkaloids of a saturated base. The first component exhibits the characteristic peak for a saturated base 7-monoester alkaloid at m/z 139, along with the diagnostic peaks at m/z 82, 94, 95, 122, 222 and 239 (M+)[7,55,91,110]. The molecular mass was confirmed by chemical ionization mass spectrometry (CIMS). The second major component exhibits the characteristic peak for a saturated base 9-monoester alkaloid at m/z 140, with a molecular mass of 239[7,55,91]. The analysis also showed the presence of isomeric 7- and 9-monoesters. Two 7-monoesters precede the

first major component and two 9-monoesters follow the second component. These alkaloids exhibit the characteristic fragment peaks corresponding to angelyl, tiglyl or senecioyl esters of platynecine[105]. The GC trace is shown in Fig 2.2.

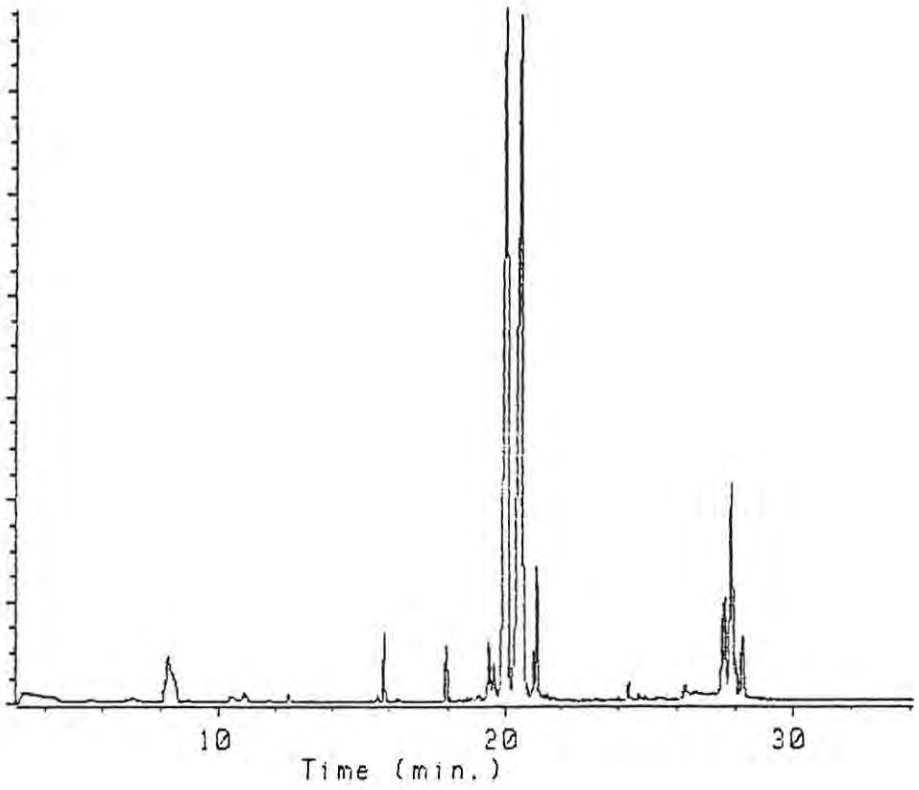


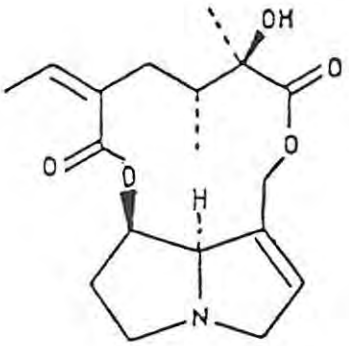
Fig 2.2 GC trace of crude extract from *Senecio chrysocoma*

A second smaller group of alkaloids emerges with a higher retention time. This group comprises at least three components (GC trace shown in Fig 2.2), all of which have a molecular mass of 337 (confirmed by CIMS) and exhibit similar fragment peaks. The large peak at m/z 138 identifies them as acyclic diesters of a saturated base[7,55,91]. Other diagnostic peaks appear at m/z 82, 94, 95, 120, 222 and 237. These fragment peaks are typical of sarracine (**122**) and its geometric isomers recently described by Stelljes *et al.*[111].

A discussion of the isolation of the major alkaloids of ***Senecio chrysocoma*** and of the structural analysis of these compounds using NMR techniques is relevant at this point, since analysis of the relatively simple isomeric mixtures obtained was extremely helpful in analyzing the more complex mixtures obtained from ***S. speciosus*** and ***S. macrocephalus***. In addition, one of the major alkaloids isolated has so far only been characterised by mass spectrometry.

The crude alkaloidal extract was separated by flash column chromatography on silica gel[112] to give the major components, pure by TLC, as yellow oils. NMR analysis of these compounds, labelled C1 and C2 respectively, indicated that they were not in fact senecionine and platyphylline. C1 appeared to be a mixture of 7-angelylplatynecine (**123**) and 9-angelyl-platynecine (**124**). Analysis of C1 by GC-MS confirmed its dual nature, the two components in fact being the two major components indicated in the analysis of the crude extract. C1 was further separated by VLC to give the pure monoesters.

The structures of these compounds were confirmed by ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H correlation spectroscopy (COSY)[113,114] and ^{1}H-^{13}C heteroatomic correlation spectroscopy (HETCOR)[113]. The NMR data for these compounds is given in Section 3.2 and agrees with reported data[88,110].



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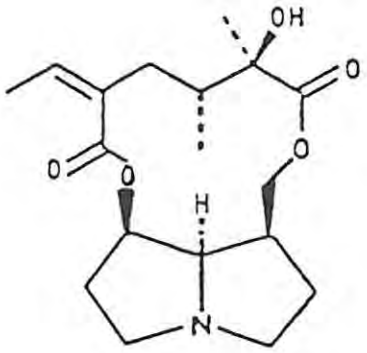

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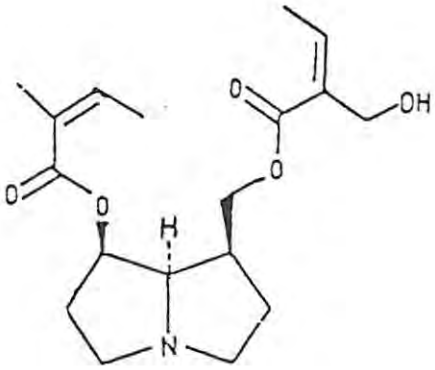

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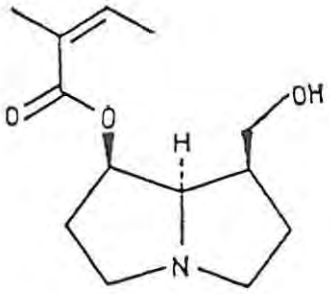

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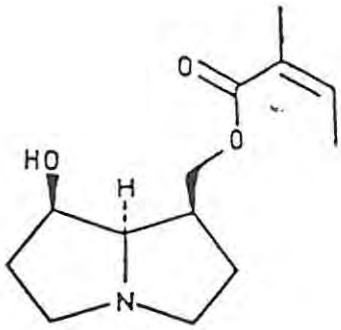
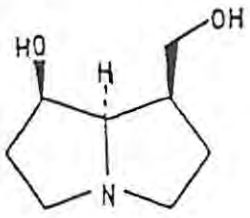


124

NMR analysis of C2 revealed that it was an acyclic diester of platynecine. The shift positions of the ring protons correspond to published data for platynecine (**125**) and its esters[110]. One of the esterifying acids is undoubtedly angelic acid, exhibiting a one-proton quartet at δ 6.11[90,105,110] which is coupled to two methyl groups. The other acid appears to be the geometric isomer of sarracinic acid. A quartet at δ 6.95, corresponding to the vinyl proton, establishes the *Z*-geometry of the double bond[115]. The shift positions of the ring protons are similar to those reported for sarracine (**122**)[110]. It is usually possible to ascertain the mode of ester attachment at the two linkage sites, since the C_9 linkage breaks more easily than the C_7 linkage and the corresponding peak in the mass spectrum is more intense[1,105]. In this case, however, the peaks at m/z 222 and m/z 237, corresponding to the cleavage of the two ester linkages[62,105], are not sufficiently different in intensity to allow such interpretation. Fortunately heteronuclear multiple bond correlation spectroscopy (HMBC)[113,116,117] could be used to determine the linkage mode. HMBC shows long-range through-bond connectivity in a molecule. Unlike HETCOR[113], which shows one-bond coupling between carbon and hydrogen atoms which are adjacent, the HMBC spectrum shows coupling across two and three bonds. Providing all the proton and carbon signals in the one-dimensional ^1H and ^{13}C NMR spectra have been correctly assigned (using COSY and HETCOR), the HMBC spectrum can provide very useful information concerning the overall structure of the molecule. A HETCOR spectrum is obtained by

detecting on ^{13}C and long run-times are often required, especially with small quantities of material[113]. The HMBC spectrum, however, is obtained by detecting on ^{1}H; the run-time is consequently much reduced and strong signals are obtained[113]. The HMBC spectrum of C2 showed strong coupling between the carbonyl at δ 168.2, the vinyl quartet at δ 6.95 (H-13) and the H-9 proton signals, confirming that neosarracinic acid is esterified at C-9.

It would therefore appear that the major diester present in this plant is the geometric isomer of sarracine, named neosarracine (**126**) by Stelljes *et al.*[111]. Neosarracine has been identified only by GC-MS and no NMR data has thus far been published for this compound. A fragmentation pathway for neosarracine is shown in Scheme 2.1.



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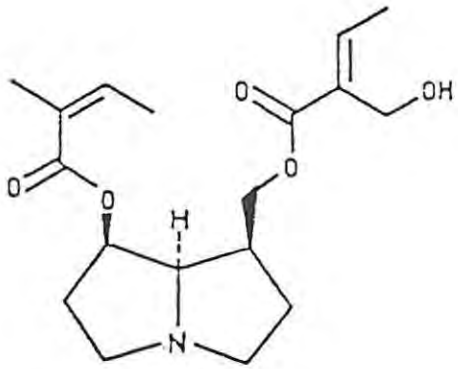
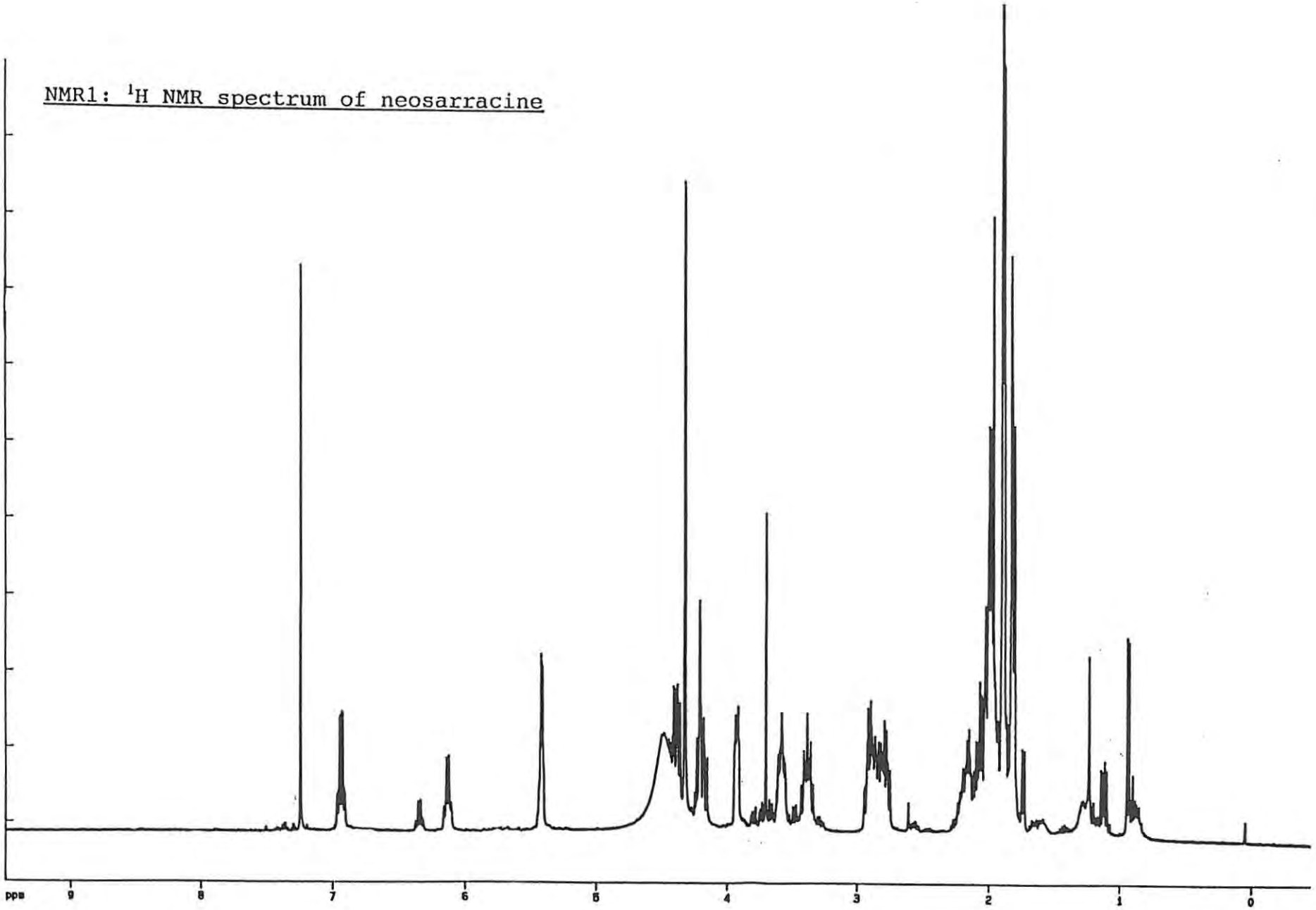
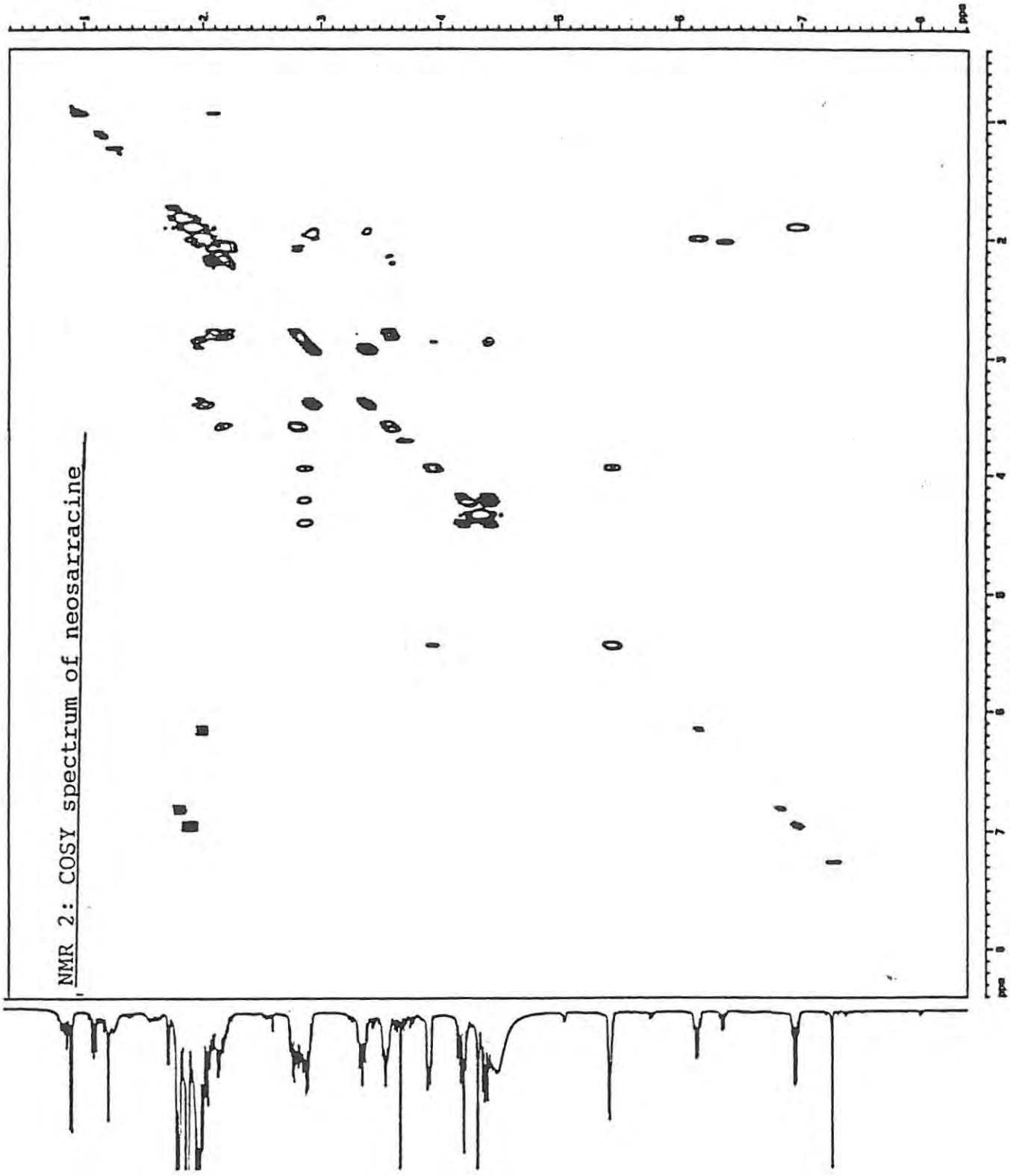
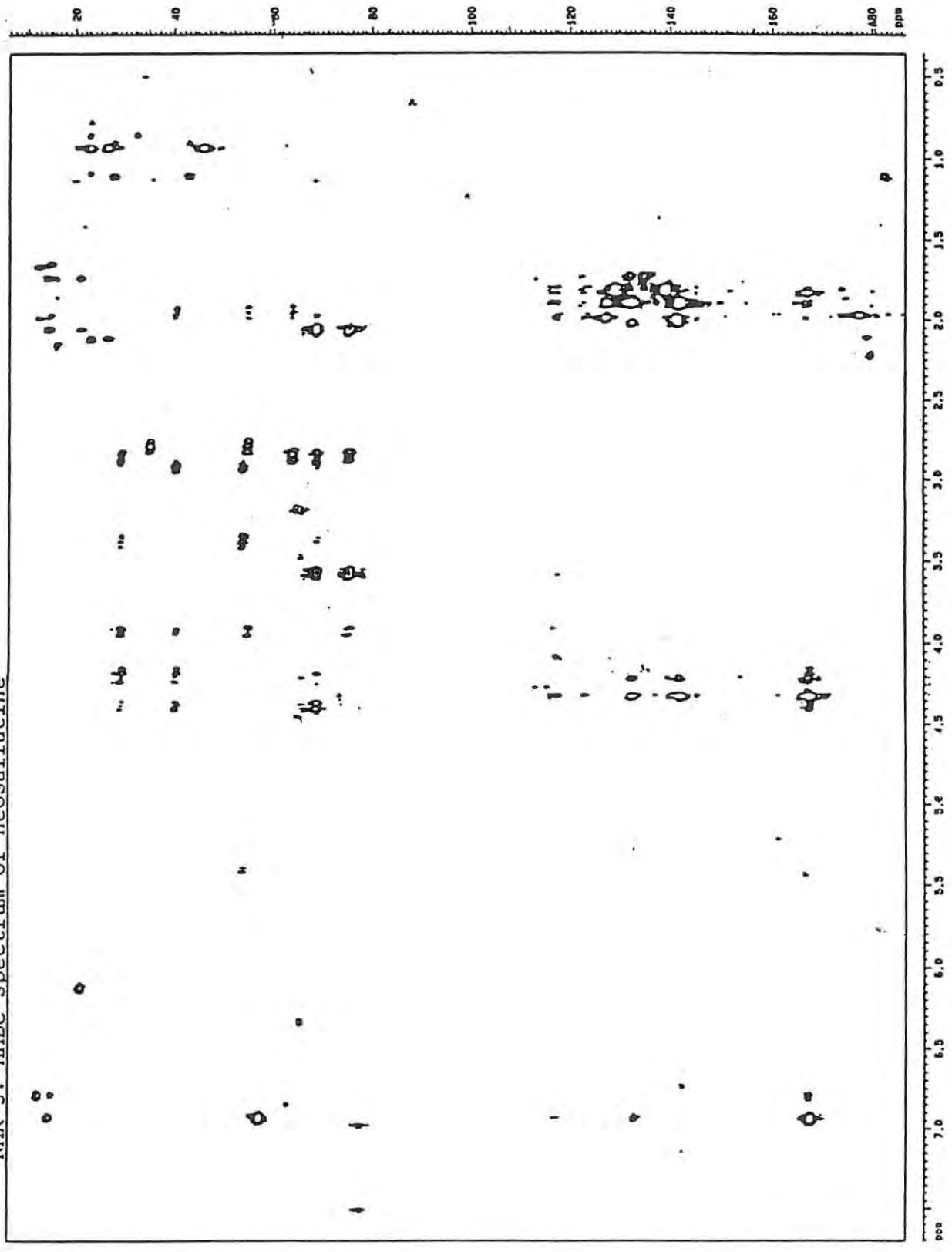


126

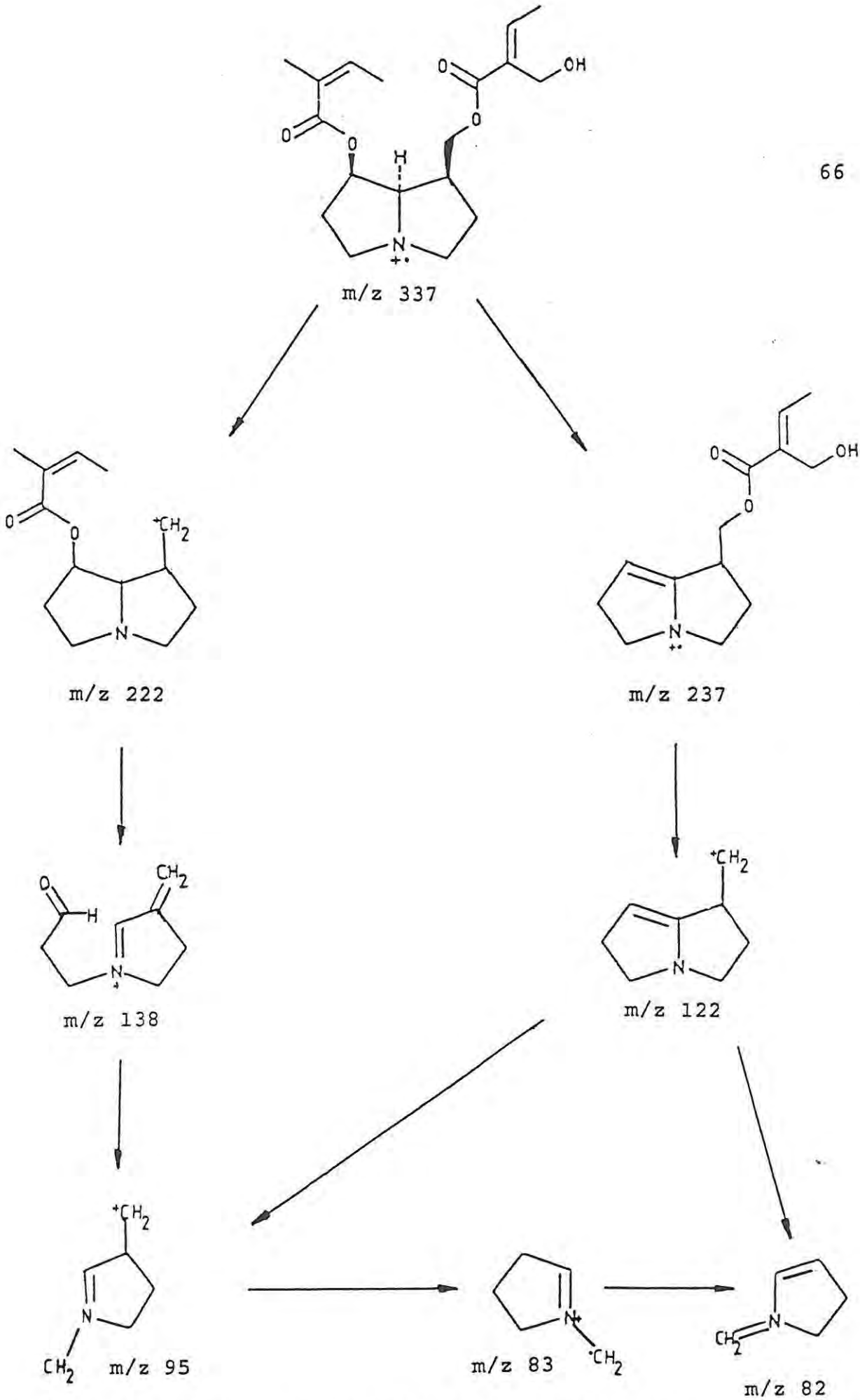
NMR1: ^{1}H NMR spectrum of neosarracine




NMR 2: COSY spectrum of neosarracine

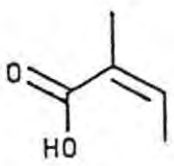


NMR 3: HMBC spectrum of neosarracine



Scheme 2.1 Fragmentation pathway for neosarracine

The isomerization of angelic acid (**127**) to tiglic acid (**128**) during esterification has been noted by Hoskins and Crout[115]. Stelljes *et al.* reported that this isomerization also occurs spontaneously over time in stored alkaloidal fractions[111]. The NMR spectrum of neosarracine (**126**) also exhibits a trace amount of sarracine (**122**), as indicated by a low intensity quartet at δ 6.38, which is the characteristic shift position for the vinyl proton in sarracinic acid[62,90,105,110]. The ratio of neosarracine to sarracine, calculated by comparing the ratio of the integrals of the H-13 protons, is 5:1. Since angelic acid appears to isomerize more readily than sarracinic acid (**129**), it would be logical to assume that, if the alkaloidal extract had isomerized, there would be no traces of angelic acid in the sample. In this case, however, there are no traces of tiglic acid; rather, it is the isomer of sarracinic acid which is present. It is therefore certain that neosarracine is the major diester alkaloid produced by the plant and is not an artefact. Angelic acid also does not seem to isomerize to tiglic acid in stored dried plant material.



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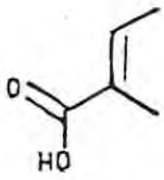

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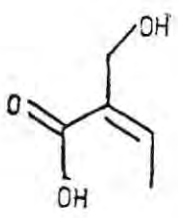
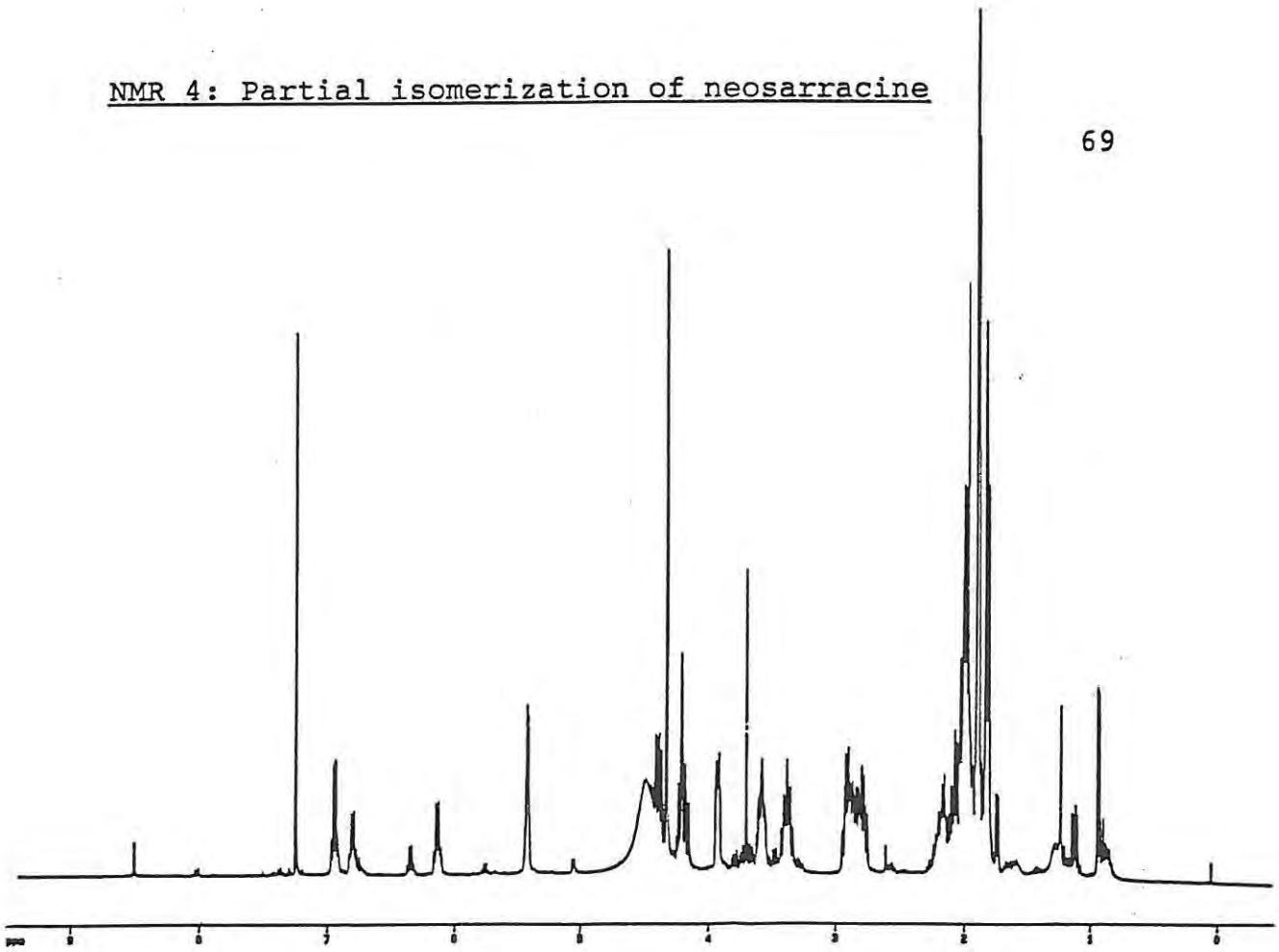


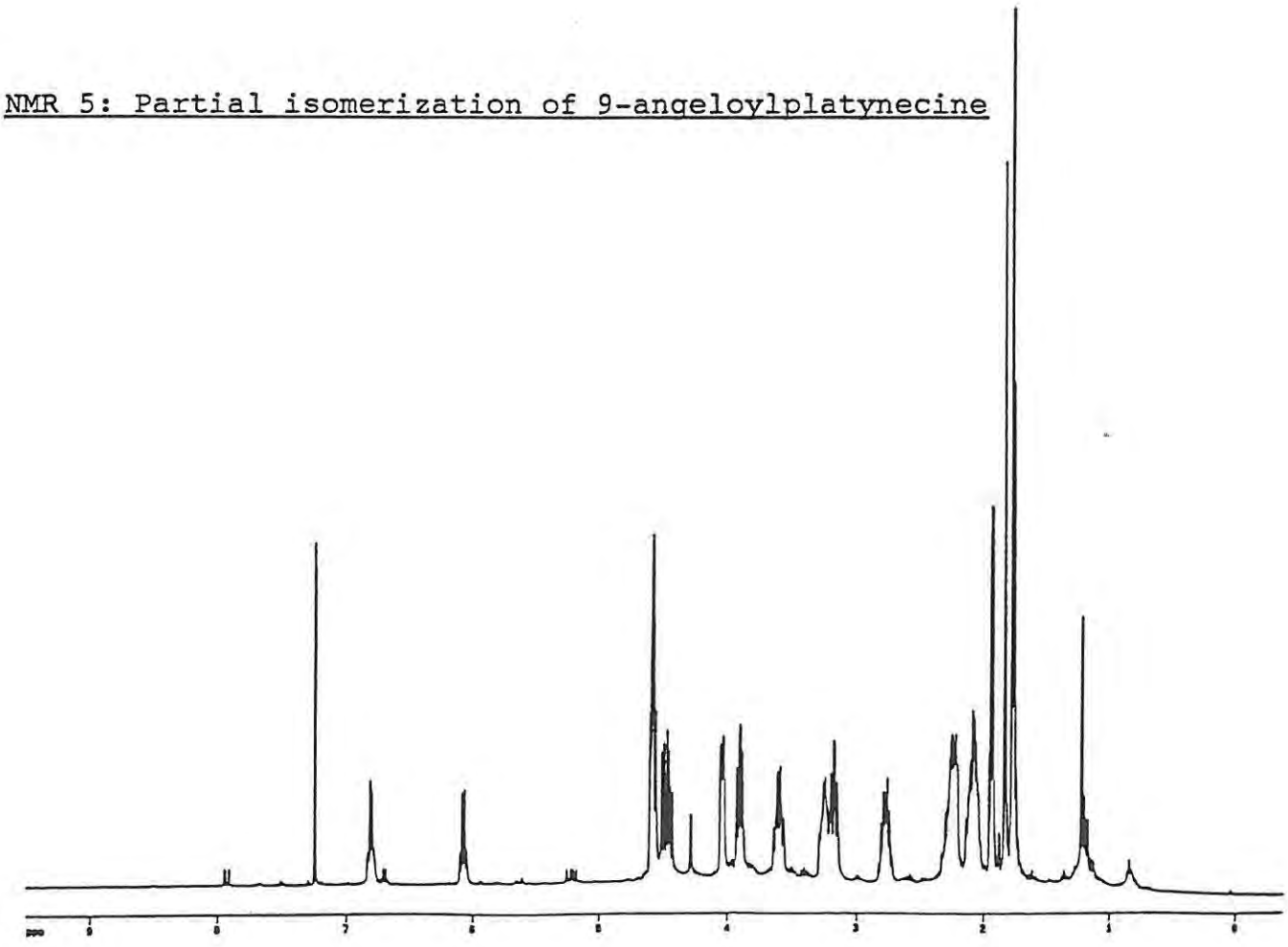
129

A second batch of ***Senecio chrysocoma*** was collected, dried, extracted and the major alkaloids isolated by DCCC and preparative TLC as described in Section 3.3. The extracts were stored at 5°C for six months and then examined by NMR spectroscopy. It was found that the monoesters had partially isomerized to 7-tiglylplatynecine (**130**) and 9-tiglyl-platynecine (**131**) respectively. The possible presence of small quantities of these compounds in the original crude plant extract was indicated by GC-MS analysis and has already been discussed. The fraction comprising mainly neosarracine with a trace of sarracine had also partially isomerized. A quartet, corresponding to the vinyl proton in a tiglyl group, appeared at δ 6.80, indicating the isomerization of the angelyl group[105,115]. The angelic vinyl quartet at δ 6.13 was still present. The quartet corresponding to the vinyl proton of neosarracinic acid at δ 6.95 appeared to have split, indicating the possible presence of a second compound containing this acid. The sarracinyl vinyl proton quartet at δ 6.38 had not disappeared. These results are consistent with the partial isomerization of neosarracine to neosarranicine (**132**) and of sarracine to sarranicine (**133**). Both these compounds were described recently for the first time by Stelljes *et al.*[111]. Once again, although these compounds are artefacts produced by spontaneous isomerization of the stored alkaloids, their possible presence as minor components in the original crude plant extract is indicated by the GC-MS analysis, as already discussed.

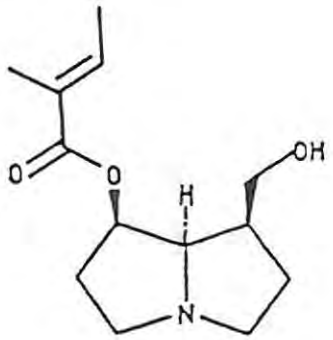
NMR 4: Partial isomerization of neosarracine



NMR 5: Partial isomerization of 9-angeloylplatynecine



From this analysis, one would deduce that the plant investigated by Glonti[109] was in fact the true ***Senecio paniculatus***. This preliminary investigation of ***Senecio chrysocoma*** is, therefore, the first examination of this species.



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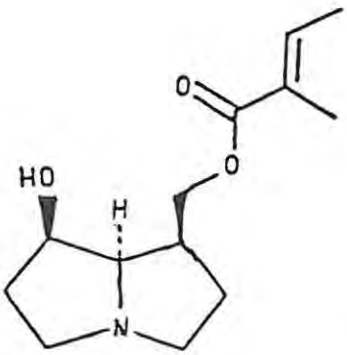

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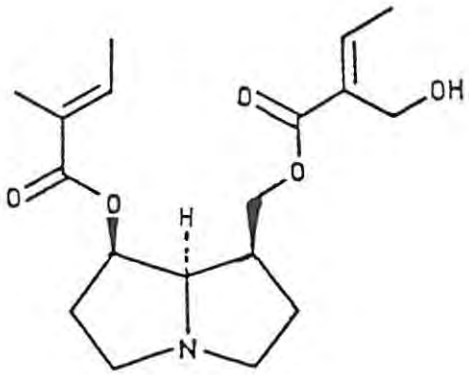

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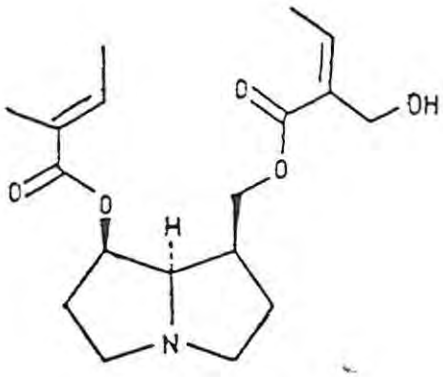


133

2.3 *Senecio pterephorus* DC

Senecio pterephorus **DC** has been investigated by a number of workers[118-122] and the alkaloids senecionine (**120**), seneciphylline (**134**), rosmarinine (**135**), retrorsine (**118**), pterophorine (**136**) and an uncharacterized alkaloid called acetylseneciphylline were identified. Since this plant occurs in abundance in the Albany district, it was collected and extracted to obtain these alkaloids for standards. The crude alkaloid content was found to be 0.06% and the extract appeared largely crystalline. Preliminary examination by analytical TLC on silica gel showed the presence of at least eight alkaloids, most of which exhibited a positive response to the chloranil - Ehrlich spray reagent, indicating that they were in fact unsaturated pyrrolizidines[1]. Analysis by GC-MS showed the presence of six diester alkaloids. The GC trace is shown in Fig 2.3.

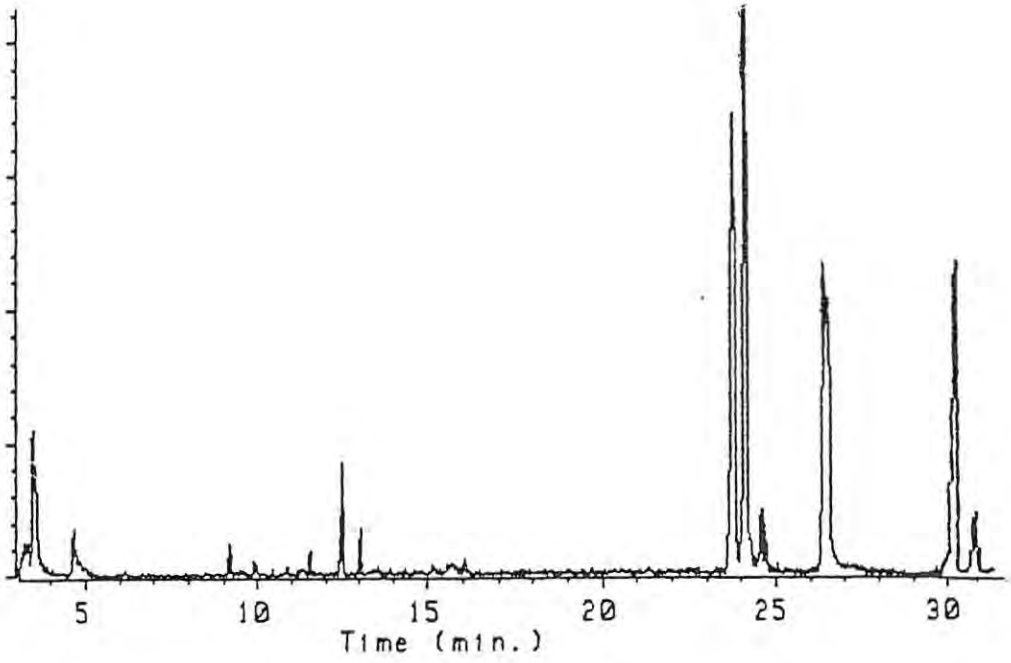
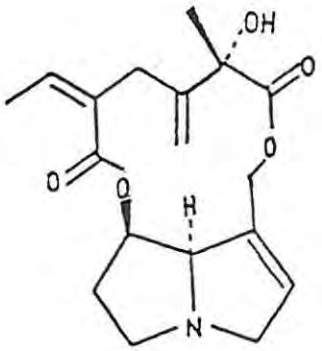


Fig 2.3 GC trace of crude extract from *Senecio pterophorus*



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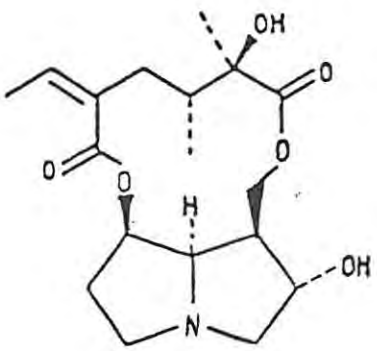

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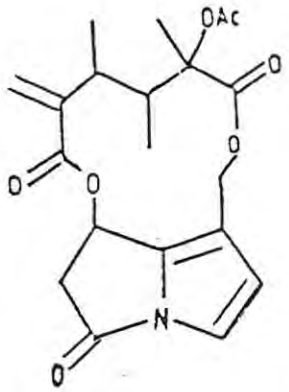

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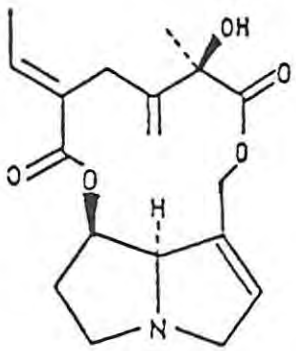

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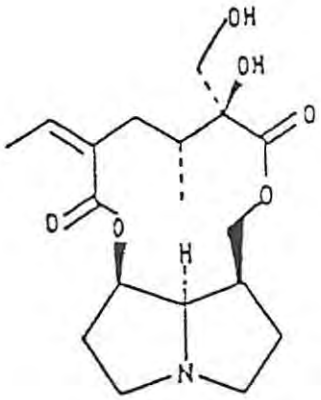

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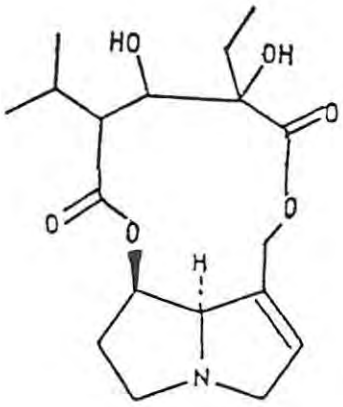

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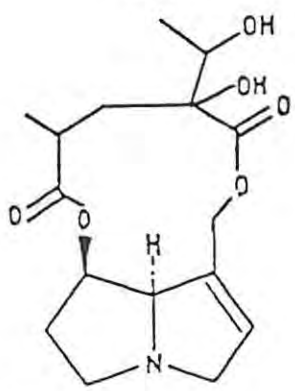

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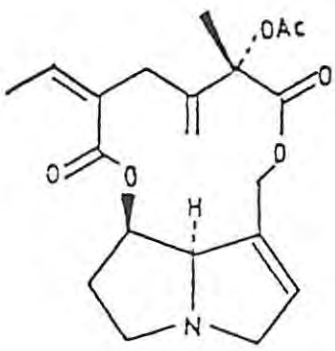


141

The first of the diester components (T_R 26.33 min) has a molecular mass of 335 and its fragmentation pattern fits that of senecionine (**120**)[123,124]. The fragmentation pathway for senecionine is given in Scheme 2.2. The second diester (T_R 26.81 min) appears to be the major component of the plant. It has a molecular mass of 333 and exhibits a fragmentation pattern characteristic of seneciphylline (**134**)[68,124]. A fragmentation pathway is shown in Scheme 2.3. The minor component following seneciphylline (T_R 27.28 min) also has a molecular mass of 333 and exhibits an identical fragmentation pattern. It would therefore appear that this compound is an isomer of seneciphylline, probably spartioidine (**137**)[65].

A diester with the surprising molecular mass of 375 is also present (T_R 29.02). Like the other three diesters, it exhibits the characteristic fragmentation pattern of a macrocyclic diester of an unsaturated pyrrolizidine base, *viz.* peaks at m/z 93, 119, 120, 136 and 137[1]. This alkaloid could be the uncharacterized acetylseneciphylline[120], which was also tentatively identified in ***Senecio alpinus*** by GC-MS[85].

The component at T_R 30.05 min has a molecular mass of 353 and exhibits the characteristic fragment peaks for a macrocyclic diester of a saturated base, *viz.* m/z 82, 120, 121, 122 and 138[1]. Peaks pertaining to the acid portion of the molecule, however, are low in intensity and the component could be one of several alkaloids, including dihydroretrorsine (**138**), axillaridine (**139**), desoxyaxillarine (**140**) and rosmarinine (**135**)[94,105,125]. Taking into consideration previous analysis of the plant, however, it would be reasonable to assume that the

compound is rosmarinine (**135**)[120]. Scheme 2.4 shows the fragmentation pathway. A very minor component at T_R 31.14 exhibits the same molecular mass and a similar fragmentation pattern and could be an isomer of rosmarinine. No traces of retrorsine (**118**), mass 351, were detectable; neither did the analysis show the presence of pterophorine (**136**).

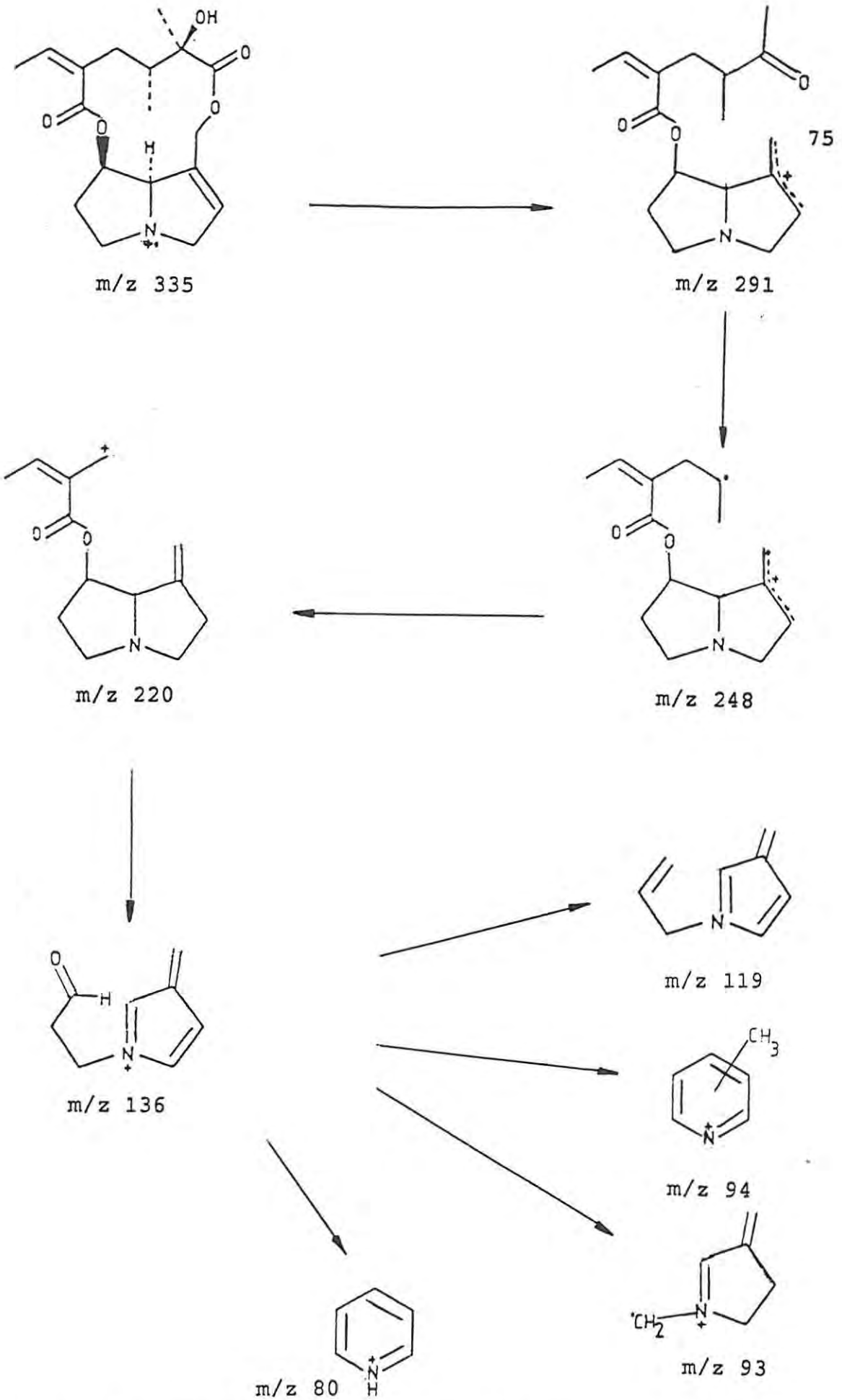
The GC-MS analysis therefore shows the presence of some of the reported alkaloids along with two unidentified compounds. A summary of these alkaloids is given in Table 2.2.

compound is rosmarinine (**135**)[120]. Scheme 2.4 shows the fragmentation pathway. A very minor component at T_R 31.14 exhibits the same molecular mass and a similar fragmentation pattern and could be an isomer of rosmarinine. No traces of retrorsine (**118**), mass 351, were detectable; neither did the analysis show the presence of pterephorine (**136**).

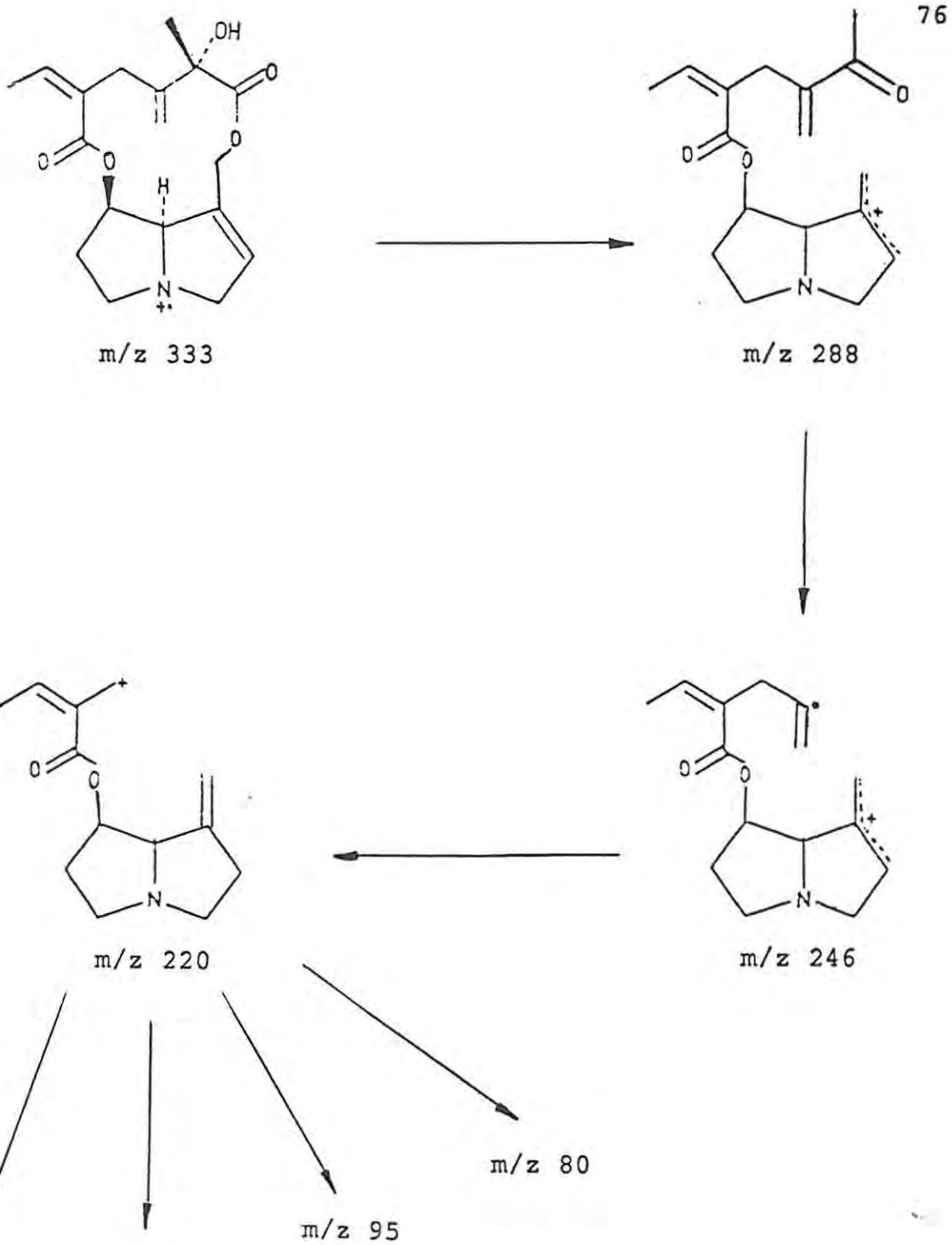
The GC-MS analysis therefore shows the presence of some of the reported alkaloids along with two unidentified compounds. A summary of these alkaloids is given in Table 2.2.

Table 2.2 Alkaloids identified in *Senecio pterephorus* by GC-MS

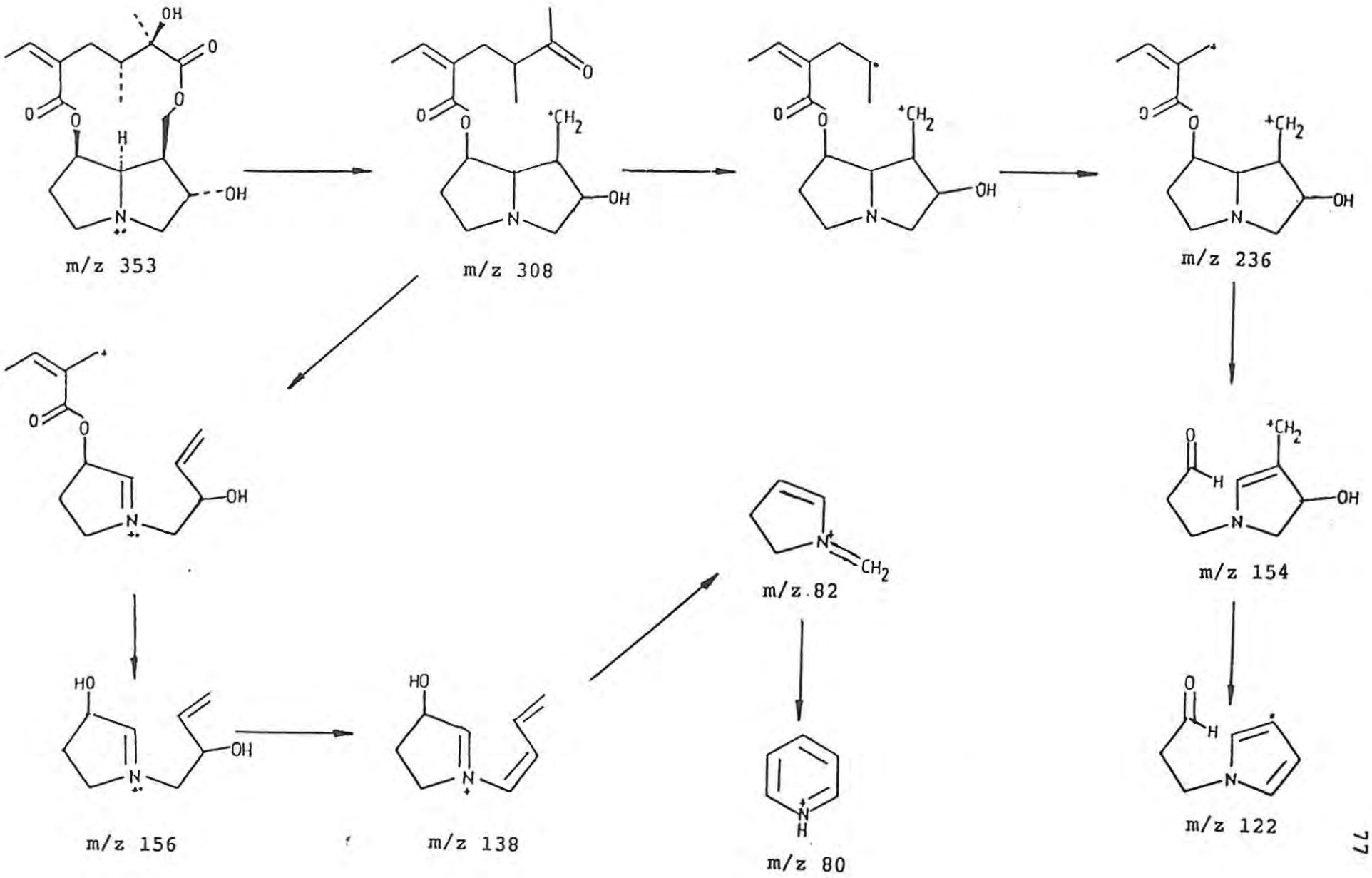
Component Number	T_R (min)	Important mass fragments	Possible Identity
1	26.33	335, 220, 153, 138, 137, 136, 121, 120, 119, 93	Senecionine
2	26.81	333, 246, 138, 137, 136, 121, 120, 119, 94, 93	Seneciphylline
3	27.28	333, 246, 138, 137, 136, 121, 120, 119, 94, 93	Spartioidine
4	29.02	375, 316, 220, 138, 137, 136, 121, 120, 119, 93	Acetyl-seneciphylline
5	30.05	353, 254, 156, 154, 138, 137, 136, 122, 121, 82	Rosmarinine
6	31.14	353, 252, 156, 154, 138, 137, 136, 122, 121, 82	Rosmarinine isomer



Scheme 2.2 Fragmentation pathway for senecionine



Scheme 2.3 Fragmentation pathway for seneciphylline



Scheme 2.4 Fragmentation pathway for rosmarinine

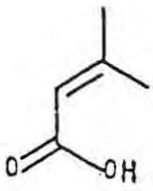
The plant was examined in more detail by a colleague, C G Logie, and the alkaloidal components isolated, purified and identified by NMR techniques[126]. Alkaloids found were seneciphylline (**134**) as the major component, spartioidine (**137**) (as a mixture with seneciphylline), rosmarinine (**135**), acetylseneciphylline (**141**) and a stereoisomer of rosmarinine[126]. I have used these alkaloids as GC standards.

2.4 Synthesis of standards

Retrorsine (**118**) was obtained from ***Senecio othonnaeflorus*** **DC** as previously discussed and hydrolysed to give retronecine (**119**). Hydrolysis using NaOH was only partially successful and examination of the reaction mixture by TLC showed that much retrorsine was still present even after refluxing for 72 hours. Hydrolysis with $Ba(OH)_2$ gave better results, although the yield of retronecine was lower than expected[79,115,127]. This was probably due to the high solubility of retronecine in the aqueous phase. The work-up procedure involved evaporation of the reaction solvent, dissolution of the residue in dilute aqueous acid and extraction with chloroform to remove the necic acid. The solution was then basified and extracted continuously with chloroform for seven days, but the yield of retronecine was still poor compared to published results[115,128]. Tiglic acid (**128**) was obtained from Sigma and isomerized to angelic acid (**127**) using the three-stage procedure described by Buckles *et al.*[129]. The reaction was successful, giving angelic acid in a 60% yield. Senecioic acid (**142**) was

synthesized from mesityl oxide (**143**) *via* 4-methyl-4-hydroxypentan-2-one (**144**)[130,131].

Various esterification procedures were attempted. Reaction of retronecine with the acid chloride has been used but is reported to give large quantities of diester rather than monoester[115,132]. A procedure involving the reaction of retronecine with NaH with subsequent coupling to the acid chloride was also attempted[79] but was unsuccessful; examination of the reaction mixture by TLC after 24 hours showed that it contained mostly unreacted retronecine, although a similar procedure was successfully used in the synthesis of 9-angelylturneforcidine (**145**) by Niwa *et al.*[133]. Selective esterification involving N N'-carbonyldi-imidazole (CDI)(**146**) gave the best results[115]. Thus the acid was reacted with CDI to form an acyl-imidazole complex which reacted with retronecine to produce the 9-monoester as the major product. Very little, if any, diester was detected. Products were purified by preparative TLC on silica gel. 9-Tiglyl-retronecine (**147**), 9-senecioyl-retronecine (**148**) and 9-angelylretronecine (**149**) were obtained in good yield. Physical and spectral data for these compounds are given in Section 3.5. Pure 9-angelylretronecine was unfortunately not obtained. A small proportion of the 9-tiglylretronecine isomer was present, as indicated by the appearance of a quartet at δ 6.88 in the ^{1}H NMR spectrum. This is the typical shift position of the vinyl proton in 9-tiglylretronecine[115]. The proportion of the two isomers was calculated by comparison of the integrals of the H-13 proton signals in the ^{1}H NMR



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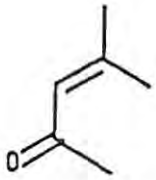

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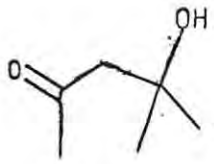

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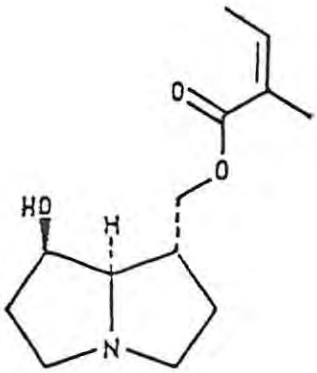

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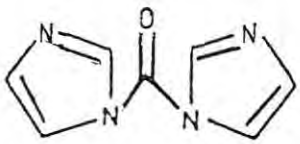

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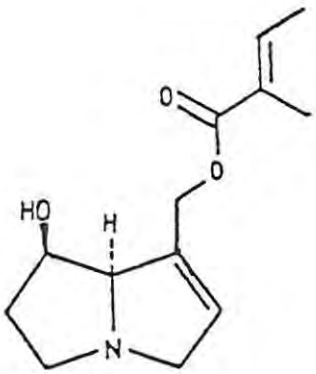

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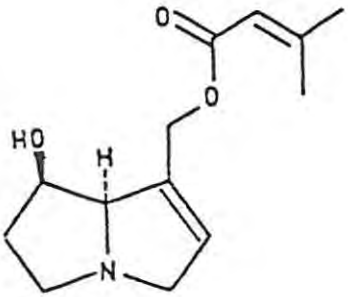

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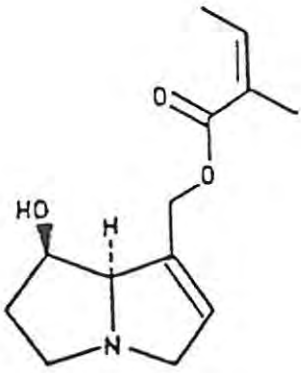

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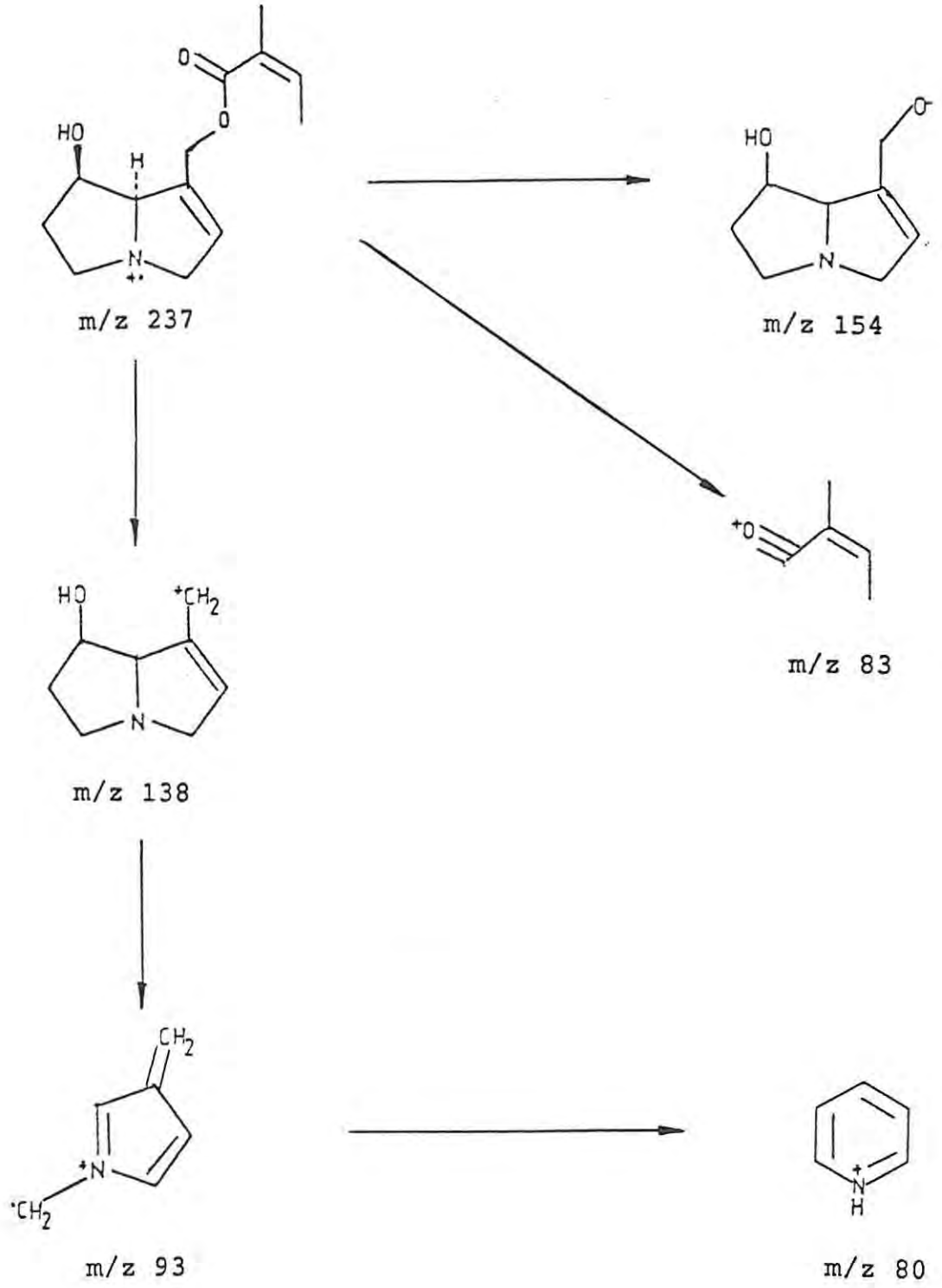
2.5 Alkaloid content of *Senecio speciosus* Willd

Senecio speciosus was collected, dried and extracted as described in Section 3.2. The crude alkaloidal content was found to be 0.32%. Analysis of the extract by GC-MS revealed that it contained a large portion of non-alkaloidal material despite the precaution of the double acid-base extraction procedure used. In addition, a certain amount of alkaloid degradation could be detected; many components were present in low concentration which showed fragmentation patterns characteristic of alkaloids. The molecular masses of these components (determined by CIMS) were characteristic of alkaloid fragments. Degradation is probably due to the high temperatures required to volatilize the alkaloids for analysis.

Purified extracts were also examined by GC-MS.

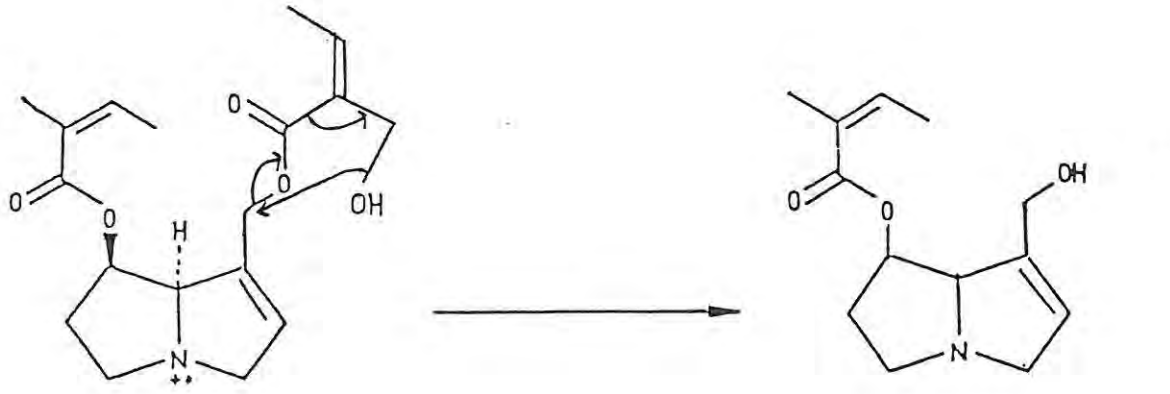
Senecio speciosus seems to contain relatively few alkaloids. Two alkaloids with similar mass spectra appear at T_R 20.3min and 20.7min respectively. Apparently both alkaloids have a molecular mass of 237 and exhibit the characteristic peaks for monoesters of an unsaturated base, *viz* at m/z 80, 93, 136, 136 and 138[1]. An intense peak at m/z 138 indicates that both compounds are 9-monoesters[7,55,91]. By comparison of retention times and mass spectra, these components were tentatively identified as 9-angelylretronecine (**149**) and 9-senecioyl-retronecine (**148**) respectively. Both are present in very low concentration. A fragmentation pathway for 9-angelyl-retronecine is shown in Scheme 2.5.

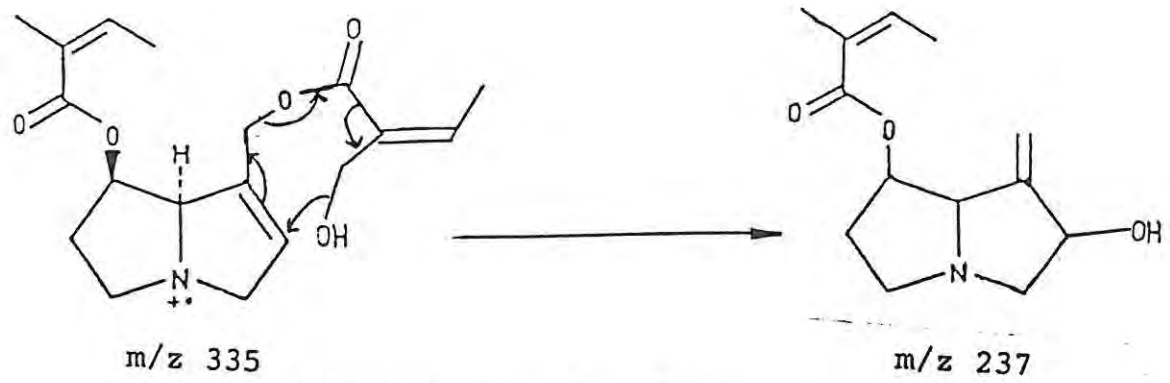


Scheme 2.5 Fragmentation pathway for 9-angelylretronecine

A number of acyclic diester alkaloids appear to be present; these are characterised by an intense peak at m/z 136[7,55,91]. All three discernible diesters have identical mass spectra; peaks at m/z 80, 93, 119, 120, 121 and 136 establish that they are all acyclic diesters of an unsaturated base[1]. Peaks at m/z 83 and 220 indicate the presence of an angelyl type group, while a peak at m/z 235 indicates a sarracinyl group[105]. The peak at m/z 220 is significantly more intense than that at m/z 235, suggesting that the sarracinyl group is attached at C-9 and the angelyl at C-7[105]. This group of diesters is thus isomeric, with angelyl, tiglyl or senecioyl groups at C-7 and sarracinyl, isosarracinyl or neosarracinyl groups at C-9. An intense peak at m/z 237 is characteristic of acyclic diesters of this type and arises *via* a rearrangement of the alkaloid[105]. A proposed mechanism for this rearrangement is shown in Scheme 2.6. The retention times of these diesters are 29.6 min, 29.8 min and 30.1 min respectively and their molecular mass is 335. Comparison of retention times and mass spectra with those of standard alkaloids indicate the absence of macrocyclic diester alkaloids and of saturated acyclic diesters.

A summary of the alkaloids tentatively identified by GC-MS is given in Table 2.4 and the GC trace is shown in Fig 2.4.





Scheme 2.6 Rearrangement for acyclic diesters

Table 2.4 Alkaloids identified in *Senecio speciosus* by GC-MS

Component	T_R (min)	Important mass fragments	Possible Identity
1	20.3	237, 138, 121, 120, 119, 93, 80	9-Angelyl-retronecine
2	20.7	237, 138, 121, 120, 119, 93, 80	9-Senecioyl-retronecine
3	29.6	335, 237, 220, 136, 121, 120, 119, 94, 93, 83, 80	Triangularine type
4	29.8	335, 237, 220, 136, 121, 120,119, 94, 93, 83, 80	Triangularine type
5	30.1	335, 237, 220, 136, 121, 120, 119, 94, 93, 83, 80	Triangularine type

The crude alkaloidal extract was subjected to droplet counter current chromatography (DCCC) followed by further purification using preparative TLC as described in Section 3.8. The alkaloidal fractions obtained are shown in the flow chart in Fig 2.5.

Owing to the small quantities obtained, it was not possible to obtain ^{13}C NMR spectra of the alkaloids and identification was based on ^{1}H NMR and ^{1}H-^{1}H correlation spectroscopy (COSY)[113,114].

Three alkaloidal components were obtained: S5(2mg), S6(5mg) and S8(2mg).

The ^{1}H NMR spectrum of S5 exhibits features typical of acyclic diester alkaloids. The chemical shift of the pyrrolizidine ring vinyl proton (H-2; δ 5.78) is characteristic of this type of alkaloid, the corresponding shift position for H-2 in macrocyclic diesters occurring slightly upfield of this value[105]. In addition, the difference in chemical shift between the geminal H-9 proton signals is small (0.14 ppm), whereas these signals are usually separated by up to 1.5 ppm for macrocyclic diester alkaloids[105]. Close examination of the two-dimensional COSY spectrum allowed assignment of the proton resonances, which agreed well with data already published for acyclic diester alkaloids[90,105,134]. The double bond between C-1 and C-2 in the pyrrolizidine ring allows allylic and homo-allylic coupling[135,136]. Thus H-8 couples to H-3d, H-3u, H-9d and H-9u while H-2 couples to the H-9 protons.

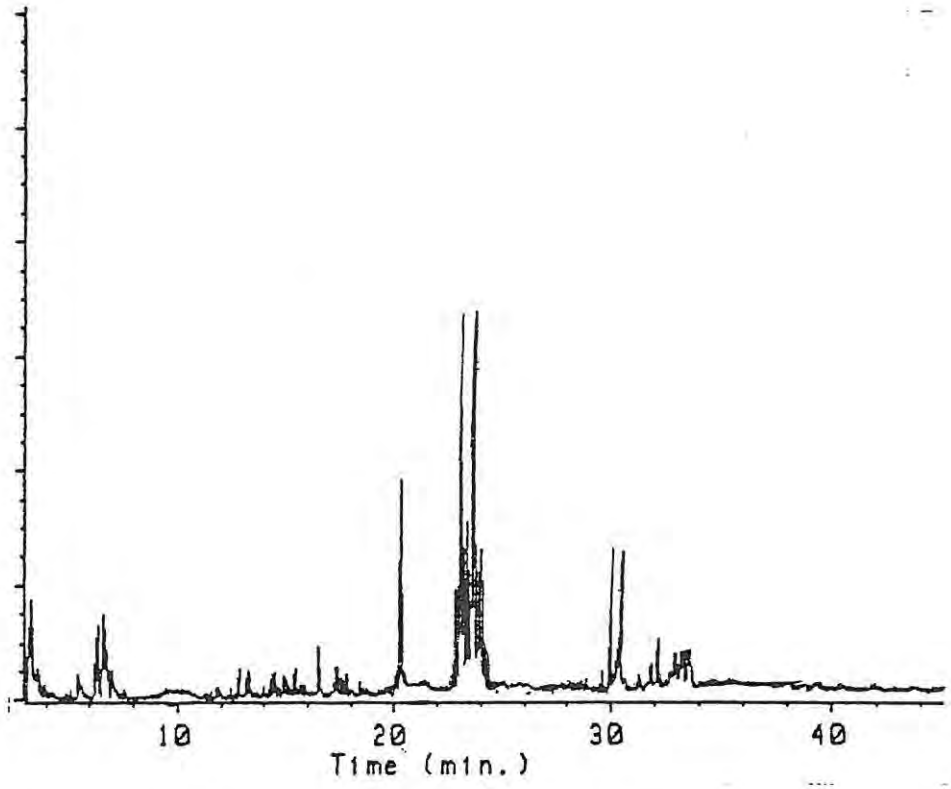


Fig 2.4 GC trace of crude extract from *S. speciosus* Willd

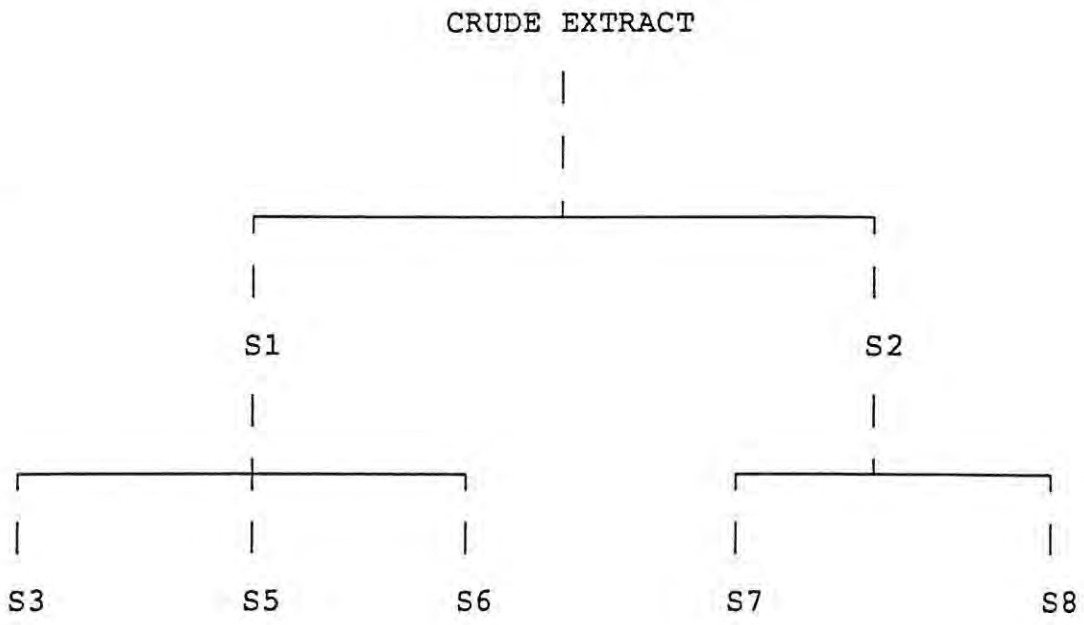
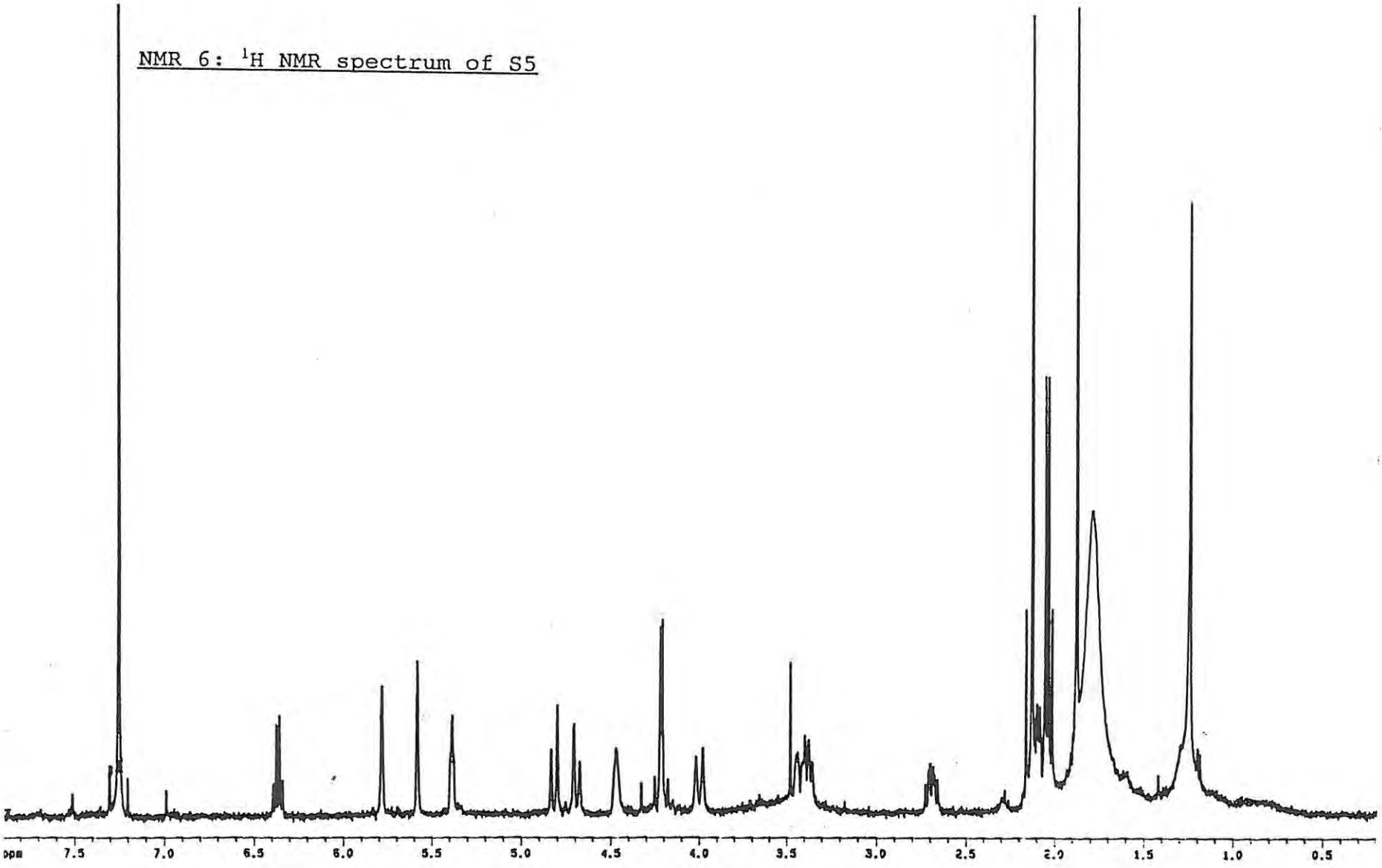
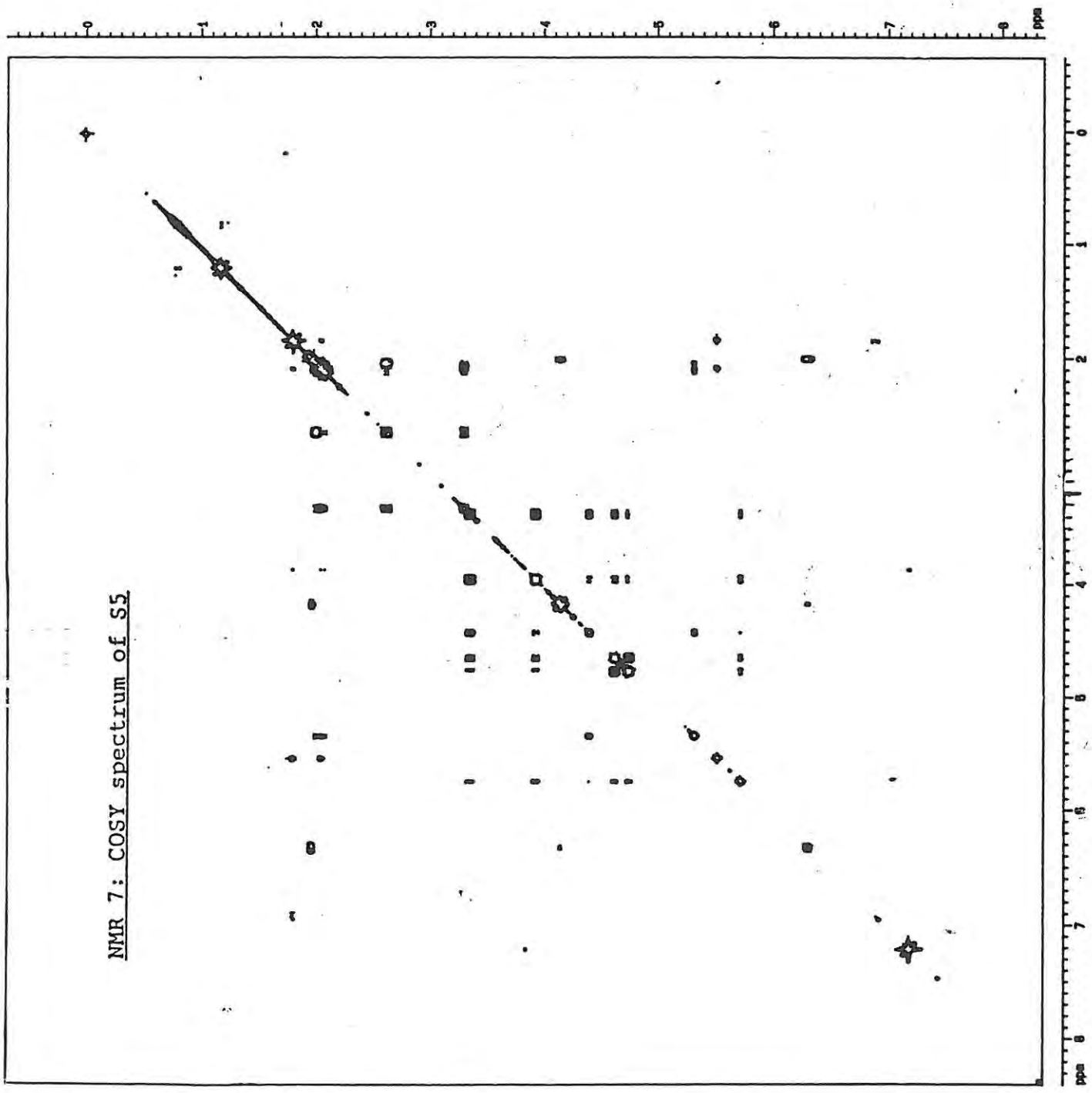


Fig. 2.5 Flow chart showing alkaloidal fractions isolated from *Senecio speciosus*.

NMR 6: ^{1}H NMR spectrum of S5





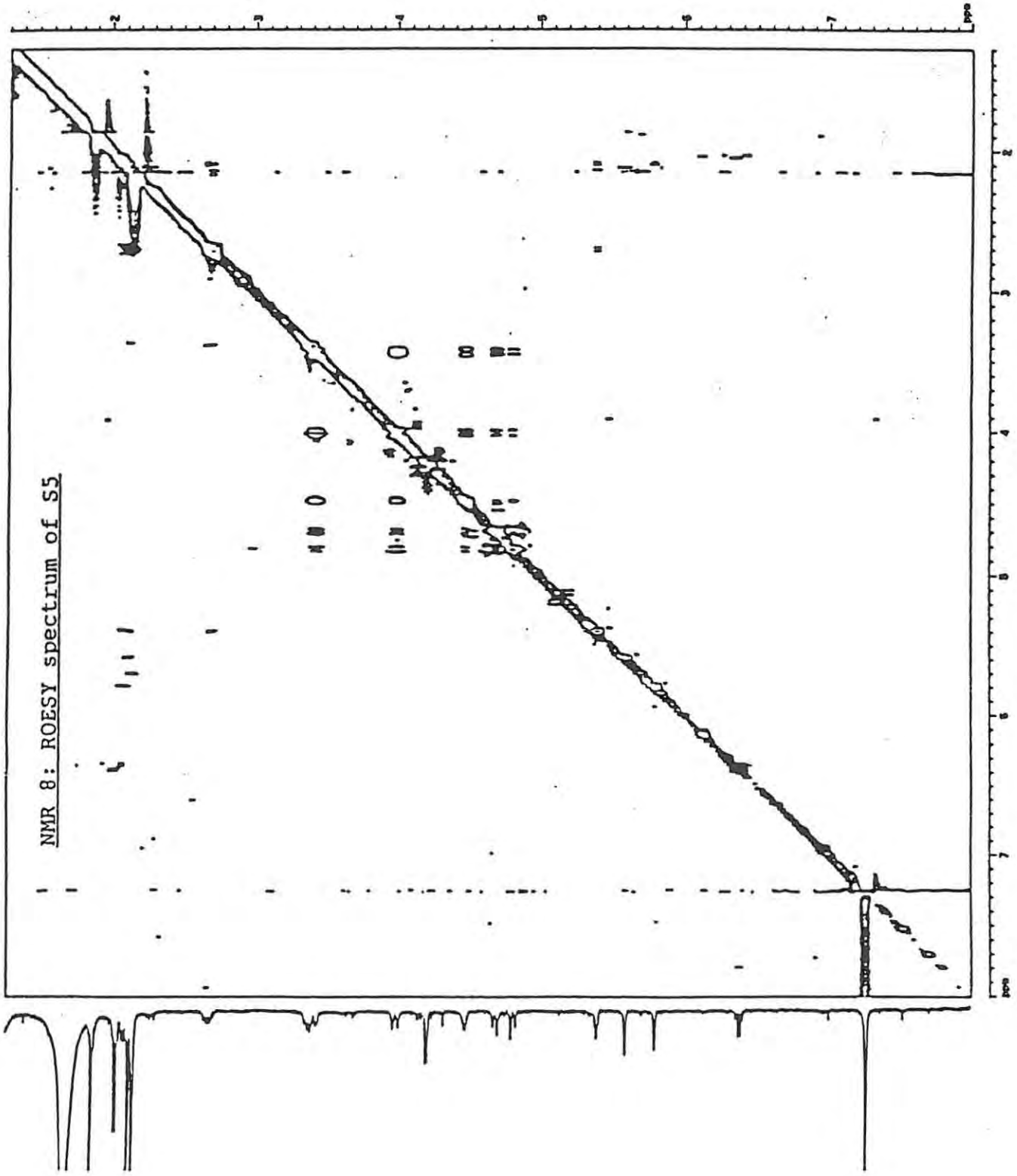
NMR 7: COSY spectrum of S5

A quartet appearing at δ 6.37 indicates a vinyl proton such as that in angelic acid[105]. The COSY spectrum shows that this signal couples to a doublet at δ 2.05 ppm, which can only be a methyl group. The vinyl quartet also shows long-range coupling to a doublet at 4.20 ppm, as does the methyl group. These shift values are typical for sarracinic acid[62,90,110] and the coupling patterns are consistent with this structure.

The other esterifying acid is clearly senecioic acid, typified by coupling in the COSY spectrum between a broad singlet at 5.59 ppm and two methyl groups at 2.10 ppm and 1.85 ppm respectively[90,134]. The coupling is again long-range through the double bond and coupling constants are therefore small.

S5 therefore appeared to be 7-senecioyl-9-sarracinyl-retronecine (**150**). This alkaloid has been isolated previously by Rüeger and Benn from ***Senecio triangularis*** **Hook**[90] and by Roeder *et al* from ***Senecio cacaliaster***[134]. There is, however, some discrepancy between the ^{1}H NMR data reported for the compound by the two authors, especially in the shift values for the ring protons. The data for S5 are in good agreement with the data reported by Rüeger and Benn[90], except for the chemical shift for H-8, which is 0.11 ppm downfield of the value reported by these workers and 0.04 ppm upfield of that reported by Roeder *et al.*[134]. If the NMR experiments were conducted at different temperatures the discrepancy is easily explained, but since the differences are only really evident in the shift values of the ring protons, the possibility that the alkaloids isolated from the two different plants are stereoisomers cannot be excluded.

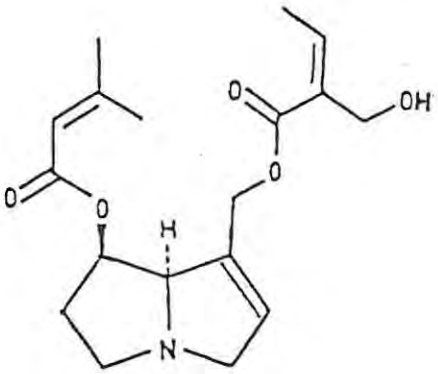
The relative stereochemistry of the pyrrolizidine ring in S5 was determined using Rotating-frame Overhauser enhancement spectroscopy (ROESY)[113,137]. Nuclear Overhauser enhancement spectroscopy (NOESY)[113,117] indicates through-space interactions between protons in a molecule and can aid in the determination of stereochemistry. This technique has been used successfully in the elucidation of pyrrolizidine alkaloid stereochemistry by a number of workers[18,70,138]. However, NOE effects are dependent on molecular tumbling rate and vary with molecular mass. It is often difficult to observe NOE effects for complex medium-sized molecules (e.g. oligosaccharides and alkaloids)[137]. This problem can be overcome by observing these transient NOE effects in the rotating frame, using an appropriate pulse sequence[137]. A ROESY spectrum therefore gives the same information as a NOESY spectrum. Both techniques were used, but ROESY proved more successful. Clear NOEs were observed between H-7 and H-5u, between H-9d, H-8, H-3d and H-3u, between H-9u, H-8, H-3d and H-5d, between H-8, H-9d, H9-u, H-3d and H-5d and between H-3u and H-5u. No interaction was observed between H-8 and H-7. This, together with the fact that H-7 and H-8 interact with different "sets" of H-3 and H-5 protons, indicates that H-7 and H-8 are on opposite faces of the ring system, i.e. the necine base in S5 is not retronecine. This proposal is supported by the one-dimensional ^{1}H spectrum of the compound. The shift value of the H-6 protons is considered to be indicative of the stereochemistry at H-7[62,135-6]. However, the H-6 proton signal for S5 occurs at 2.01-2.20ppm; not too high for heliotridine


NMR 8: ROESY spectrum of S5

but not too low for retronecine[135]. However, the shift position of the H-6 protons in retronecine-based alkaloids is generally downfield from this value[62,134-6]. The width of the H-7 proton signal at half-height (in Hz) can also give an indication of the stereochemistry at this positon[39,135]. The width of this signal in the ^1H spectrum of S5 was calculated to be 12.4Hz, a value typical of heliotridine-based alkaloids[39,135].

Two possibilities exist for the absolute stereochemistry of the ring, *viz*. H-8 is α to the ring and H-7 is ß (i.e. (+)-heliotridine-based) or H-8 is ß to the ring and H-7 is α (i.e. (-)-heliotridine based). The former arrangment is more common, 8-ß stereochemistry being exhibited by a minority of pyrrolizidines[53]. Unfortunately, the absolute stereochemistry cannot be obtained from a ROESY or a NOESY spectrum. The two possible structures (shown in Fig 2.6) are enantiomeric and would be expected to exhibit identical NMR spectra. A chiral probe could be used to distinguish between the enantiomers, as could optical rotation measurements, but this would only be of value if both were available for comparison. Hydrolysis of the ester linkages, followed by recovery, purification and identification of the necine base by physical measurements and spectroscopic techniques would establish which optical isomer is present; however insufficient material was available for this. The absolute stereochemistry of the base could therefore not be determined and will be represented as that of (+)-heliotridine (151), the known enantiomer, throughout this work. It must be borne in mind that the enantiomer (-)-

heliotridine (**152**) is also a possibility.



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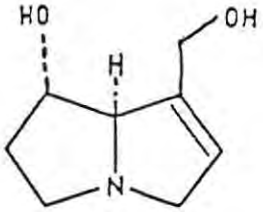

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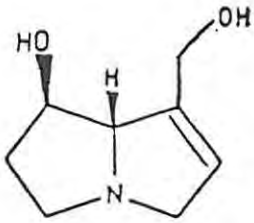

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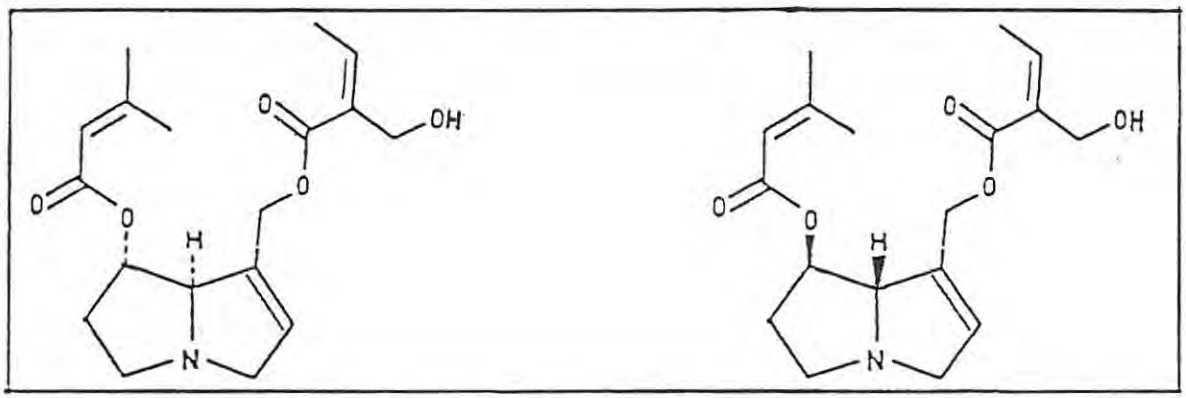
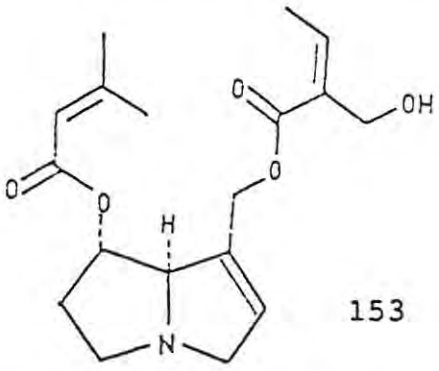


Fig 2.6 Possible structures for S5



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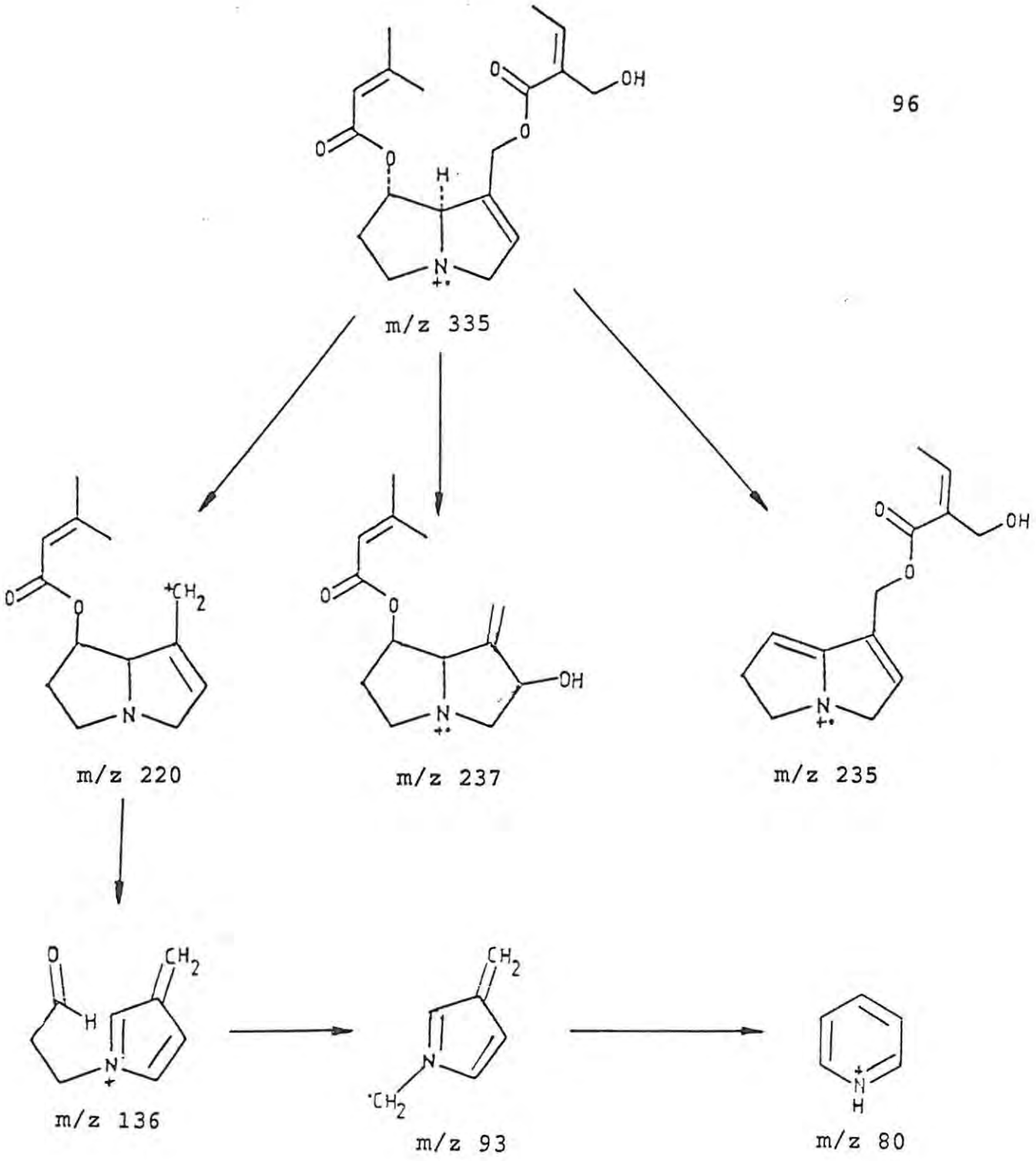
Analysis of S5 by GC-MS showed a major component (T_R 30.1 min) which comprises 86% of the total alkaloid content. This component has molecular mass 335 and exhibits the typical fragmentation pattern for an acyclic diester alkaloid of an unsaturated base[1,7,55,91], thus confirming the results obtained

by NMR analysis. The mode of esterification follows from the relative intensities of the peaks arising from cleavage of the two ester groups; the peak at m/z 220 (arising from the loss of sarracinic acid) is significantly more intense than that at m/z 235, which is due to the loss of senecioic acid[105]. In addition, an intense peak is observed at m/z 237. This peak arises *via* a rearrangement of the alkaloid[105], as shown in Scheme 2.6. The major component of S5 (86% of the total) is therefore 7-senecioyl-9-sarracinyl-heliotridine (**153**). Scheme 2.7 shows the fragmentation pathway for this compound, which is identical to that of its retronecine counterpart. The GC-MS analysis of S5 also shows the presence of two minor components at T_R 29.6 min and 29.8 min respectively. The mass spectra of these compounds are virtually identical to that of the main component, indicating that the three alkaloids are isomeric. Possibilities for the minor components include 7-angelyl-9-sarracinylheliotridine (**154**), 7-senecioyl-9-neosarracinylheliotridine (**155**), 7-angelyl-9-neosarracinyl-heliotridine (**156**) and 7-isosarracinyl-9-angelylheliotridine (**157**). No peaks other than those which pertain to 7-senecioyl-9-sarracinyl-heliotridine are visible in the ^1H NMR spectrum. The COSY spectrum, however, shows a faint coupling between a signal at δ 6.95 ppm, a signal at 2.05 ppm and one at 4.19 ppm, suggesting the presence of 7-senecioyl-9-neosarracinyl-heliotridine (**155**). However, since these signals are not visible in the one-dimensional spectrum, no conclusions can be drawn concerning the absolute identity of the minor alkaloids present.

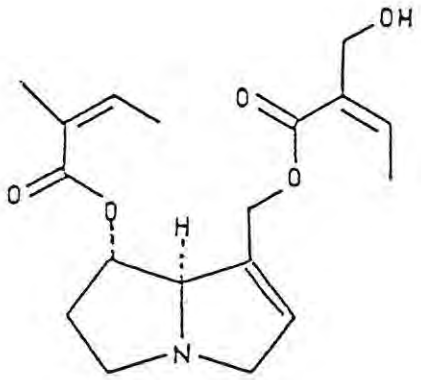


Scheme 2.7 Fragmentation pathway for 7-senecioyl-9-sarracinylheliotridine

The ^{1}H NMR spectrum of S8 is identical to that of S5, except that the signal for H-8 is 0.05ppm downfield of that for S5. The ROESY spectrum[113,137] of S8 shows the same through-space interactions, indicating that the stereochemistry is the same as that of S5. These spectra are included in Appendix 1.
Analysis of S8 by GC-MS showed a major component at T_R 30.1 min (82%) and one minor component at 29.6 min.
S8 therefore appears identical to S5, but with only one minor component present. No further information concerning the nature of the minor component could be obtained. 7-Senecioyl-9-sarracinylheliotridine (**153**) is therefore the major alkaloid present in ***Senecio speciosus***.

The analysis of S6 proved more difficult. The ^{1}H NMR of this compound again shows peaks characteristic of acyclic diester alkaloids, *viz.* the signal for H-2 is at δ 5.80 , while the signal for the H-9 protons occurs as a broad singlet at δ 4.75[90,105]. Although the signal for the H-9 protons is usually split, a multiplet or broad singlet is observed for monoester alkaloids and occasionally for acyclic diesters[39,40,62,123].
Shift values for the ring protons could easily be assigned from the two-dimensional COSY spectrum[113,114], which once again shows long-range coupling through the ring system *via* the double bond[135,136]. H-2 couples to H-9, which also couples to both H-3d and H-3u. H-8 couples to both the H-3 protons.
The identity of the esterifying acids could also be established from the COSY spectrum. A quartet at δ 6.38

couples to a doublet at δ 1.93 and a quartet at δ 4.20, which also couples to the signal at δ 1.93, indicating that one of the acids is sarracinic acid[105,110]. A broad singlet at δ 5.90 ppm coupling to a signal at δ 1.97 and to a signal at δ 4.12 shows that isosarracinic acid is also present[62,134]. This acid is a hydroxylated version of senecioic acid and occurs in the alkaloid sencalenine (**158**), isolated from ***Senecio cacaliaster*** by Roeder *et al.*[134].



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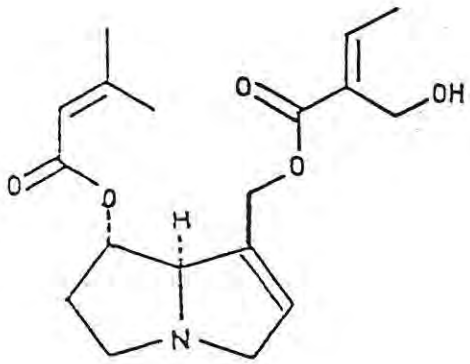

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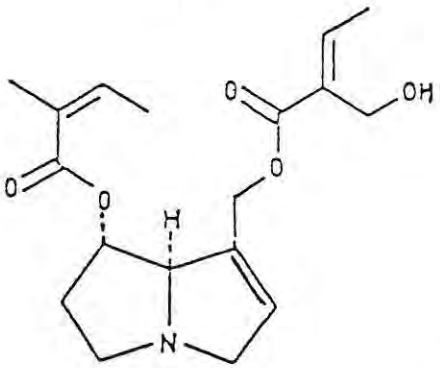

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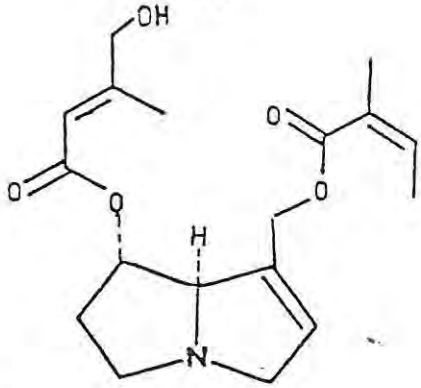

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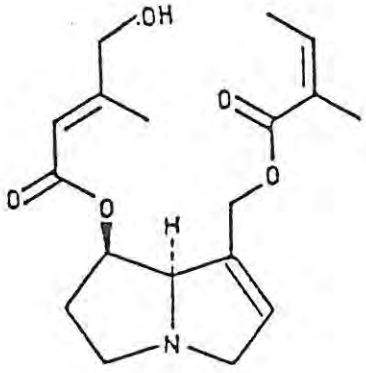
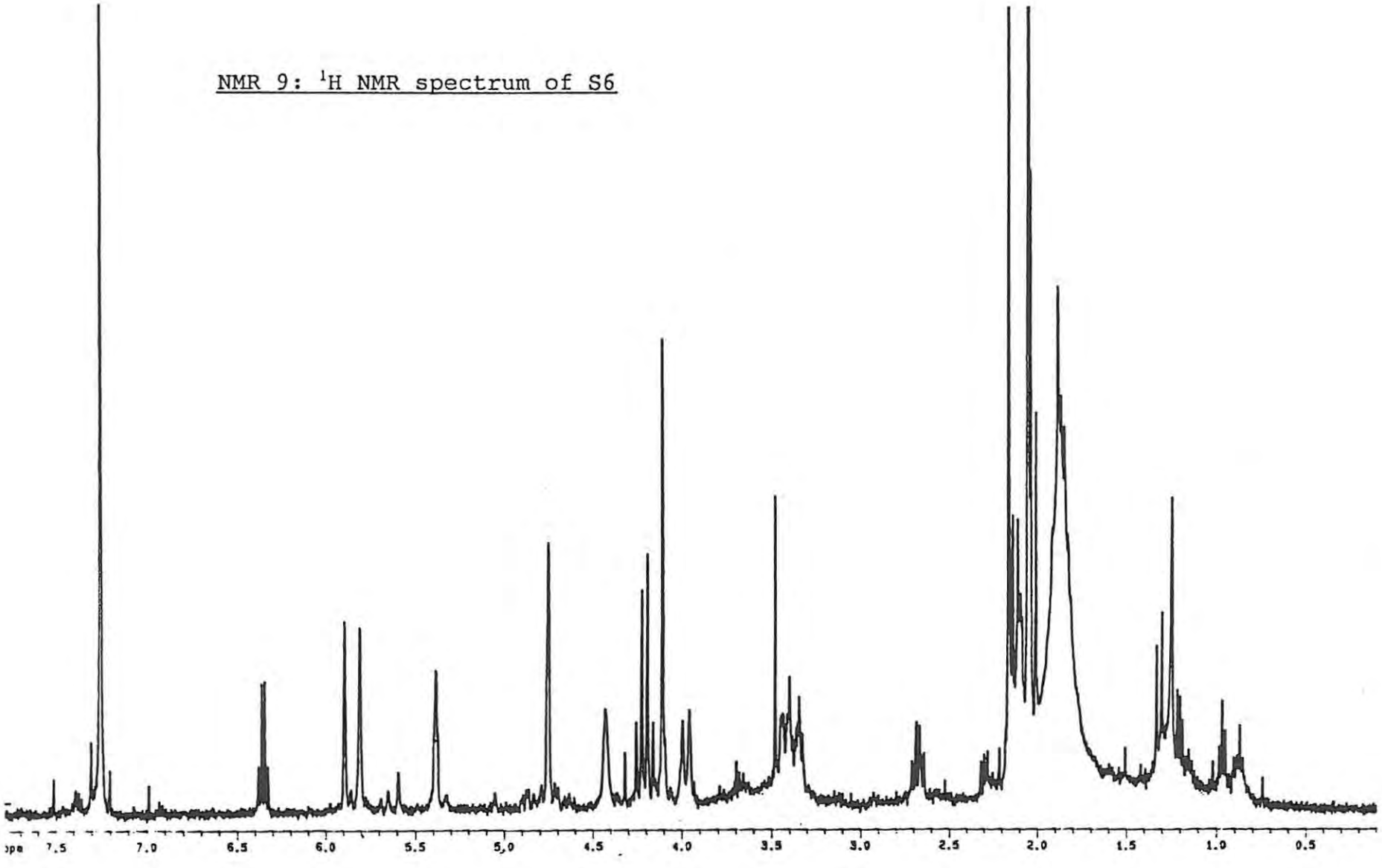
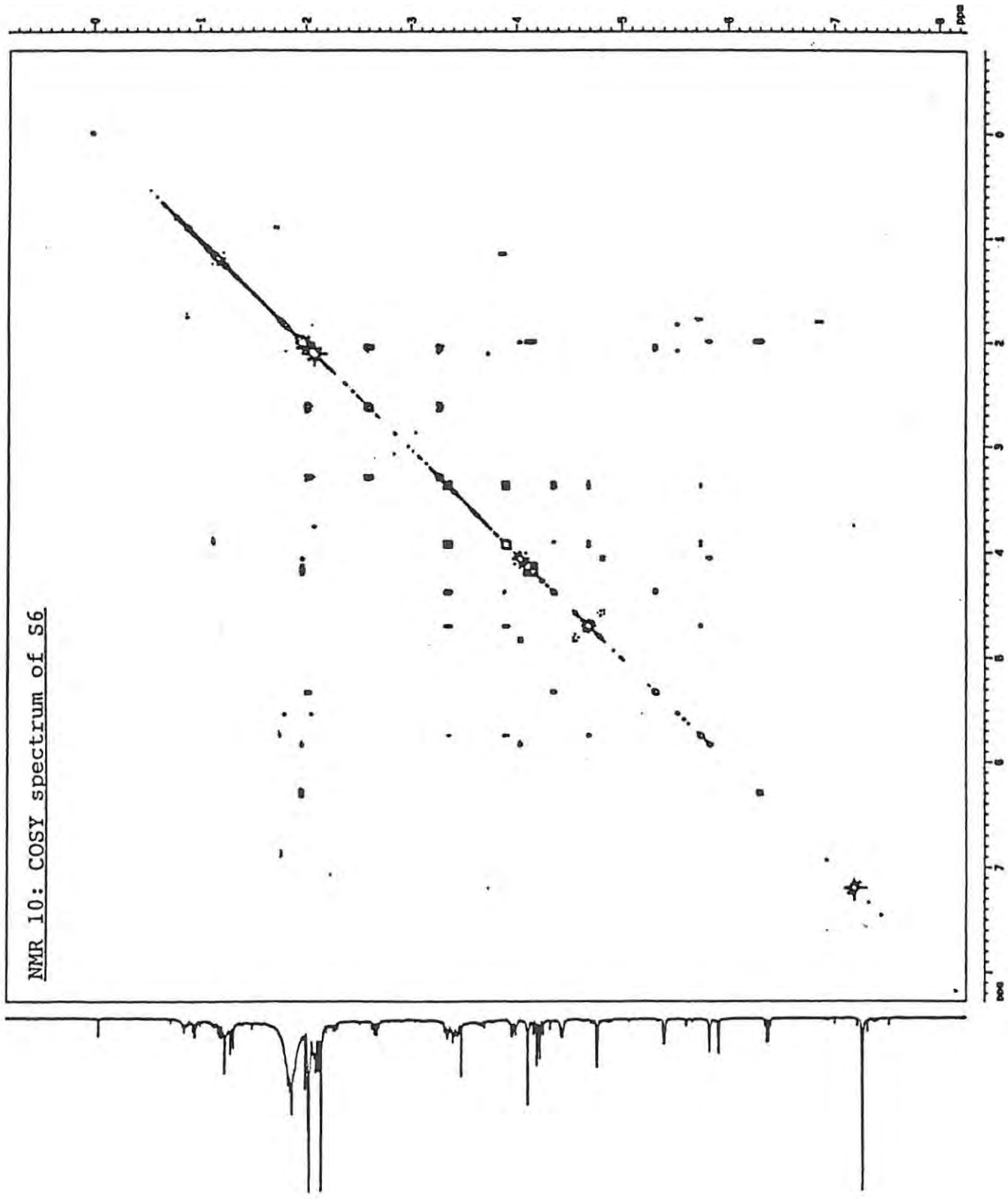


158

NMR 9: ^{1}H NMR spectrum of S6





NMR 10: COSY spectrum of S6

S6 therefore appears to be an acyclic diester alkaloid of an unsaturated base esterified with sarracinic and isosarracinic acids.

Analysis of S6 by GC-MS, however, gave unexpected results. Two major components appear to be present in approximately equal amounts with retention times of 20.3min and 20.6min and apparent molecular masses of 219 (or 238) and 237. Other peaks in the spectra indicate that an unsaturated base is present with fragmentation patterns characteristic of a C-9 and a C-7 monoester[1,7,55,91]. S6 could therefore possibly be a mixture of two monoesters with sarracinic and isosarracinic acids at the C-9 and C-7 positions respectively. However, if this were so, the ^{1}H NMR spectrum of S6 would be expected to exhibit two C-9 signals and two C-7 signals; one for each monoester. No such signals are apparent. If two 9-monoesters were present, the signal for the C-7 proton would shift upfield from between δ 5.4 and δ 5.35 to δ 4.3 in the ^{1}H NMR spectrum[110]. The H-7 signal for S6 is at 5.34 ppm. If S6 were a mixture of two C-7 monoesters, the collapsing of the C-9 proton signal to a broad singlet would be typical, but this signal would also shift upfield to between δ 3.7 and δ 4.2[39,110]. No such shift is observed. Moreover, if two compounds were present (one monoester with sarracinic acid and one with isosarracinic acid) the integration of the proton signals in the ^{1}H NMR spectrum would indicate the dual nature of the compound. The signals for the vinyl protons of the acids would integrate for a single proton each and the ring proton signals would correspondingly integrate for a double

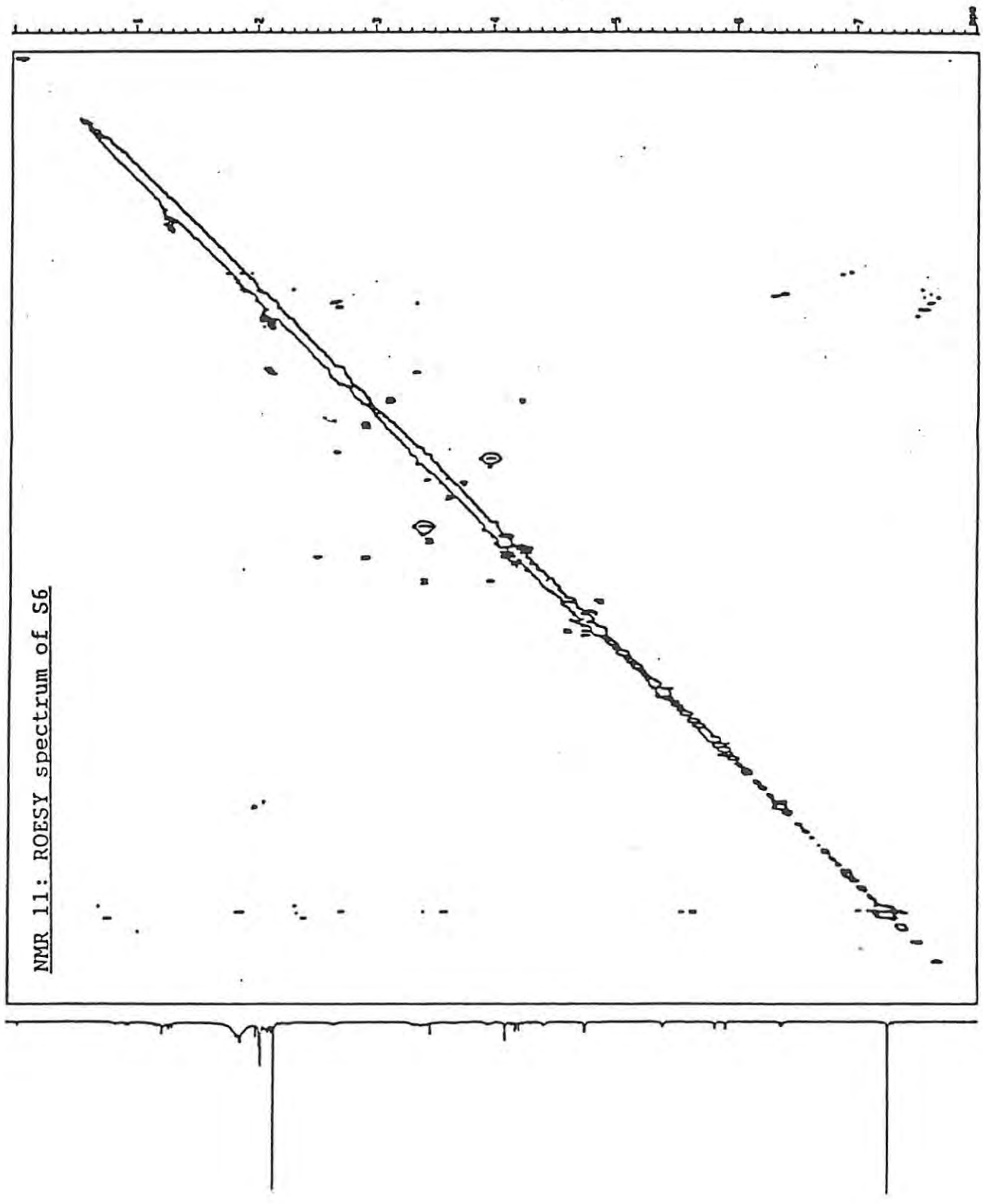
set of protons. Careful examination of the integrals for the two vinyl protons and for the ring protons indicate that only one compound is present. In addition, a molecular mass of 238 would not be possible since pyrrolizidine alkaloids always have odd masses[1]. It is possible that the high temperatures required to volatilise the alkaloids for GC analysis could cause degradation of the molecule. Peaks at m/z 219 and 237 are commonly encountered in the spectra of acyclic diester alkaloids[105,111,139] and the visible "components" would then be fragments of the alkaloid.

Confirmation of this proposal was obtained from the solid-probe mass spectrum of S6. The spectra of the two "fragments" show intense peaks at m/z 137 and 138 respectively, indicating that a 7-monoester and a 9-monoester of an unsaturated base are present[7,55,91]. If S6 were a mixture of two monoester alkaloids its mass spectrum would be expected to exhibit intense peaks at both these values. However, the solid probe mass spectrum of S6 shows an intense peak at m/z 136, a peak typical of acyclic diesters with an unsaturated base[7,55,91]. Only very low intensity peaks at m/z 137 and 138 are visible. S6 is therefore almost certainly a single alkaloid.

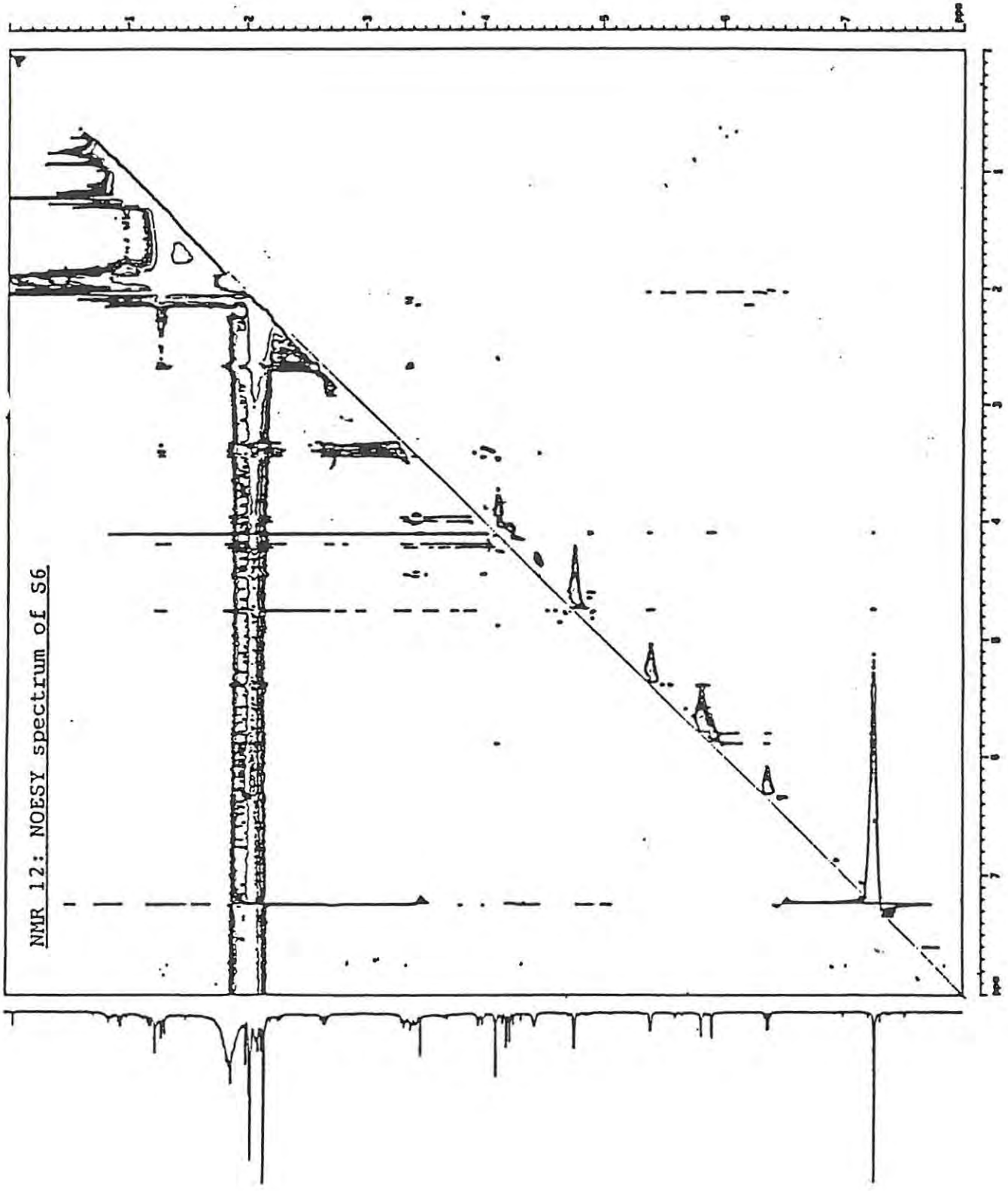
The mode of esterification in the diester was clarified by the fragmentation pattern of the molecule. A fragment peak at m/z 253 can only be formed by a rearrangement of the alkaloid if the sarracinyl group is at C-9. Isosarracinic acid at this position would exhibit an entirely different fragmentation[62]. A second rearrangement involving the isosarracinic acid at C-7 gives rise to a fragment peak at m/z 235. These

fragmentations are shown in Scheme 2.8. The alkaloid 7-isosarracinyl-9-sarracinylretronecine (**159**) has been isolated only once previously from ***Senecio doria*** by Röder *et al* and was given the trivial name doriasenine[62]. A comparison of the ^{1}H NMR data of S6 with that published for doriasenine revealed distinct differences, especially in the assignment of signals for H-3d, H-3u, H-5d, H-5u and H-6. The H-3 and H-5 proton signals reported by Röder are not split and the H-6 proton signal is downfield from the signal identified as H-6 for compound S6[62]. It is possible that the compounds are stereoisomers. NOESY[113,117,138] and ROESY[113,137] experiments were performed in an effort to obtain some information concerning the stereochemistry of S6. In both ROESY and NOESY spectra through-space interactions were observed between H-8, H-3d and H-3u, between H-8 and H-5u and between H-7 and H-9. No interactions were observed between H-8 and H-9 or between H-8 and H-7, suggesting that H-8 and H-7 are on opposite sides of the ring system[138].

The shift position of the H-6 protons is similar to that observed for S5 and S8, while the width of the H-7 signal at half-height is 12.5Hz, again indicating that the base is not retronecine but heliotridine (either the (+) or the (-) enantiomer)[39,62,135-6]. The alkaloid was named 7-isosarracinyl-9-sarracinylheliotridine (**160**).

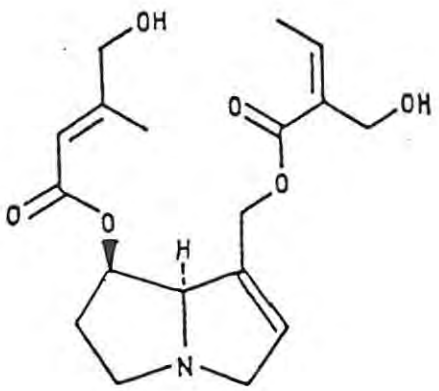


NMR 11: ROESY spectrum of S6



NMR 12: NOESY spectrum of S6

The diester alkaloids 7-senecioyl-9-sarracinylheliotridine (**153**) and 7-isosarracinyl-9-sarracinylheliotridine (**160**) were thus isolated from ***Senecio speciosus*** **Willd.** and the monoester alkaloids 9-angelylretronecine (**149**) and 9-senecioyl-retronecine (**148**) identified by GC-MS.



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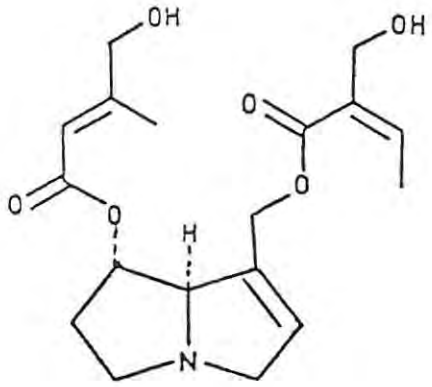

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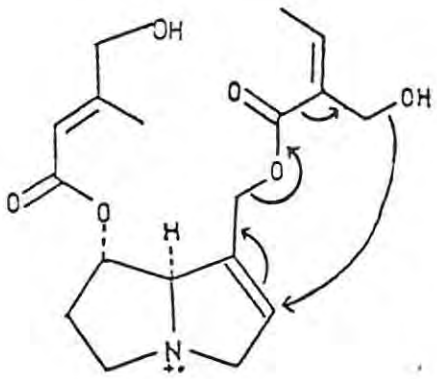
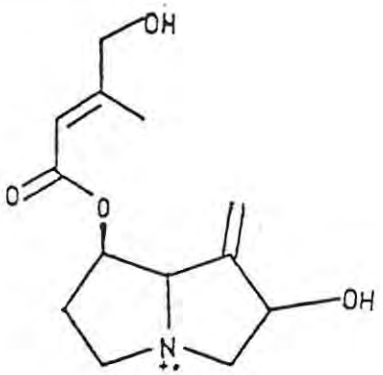
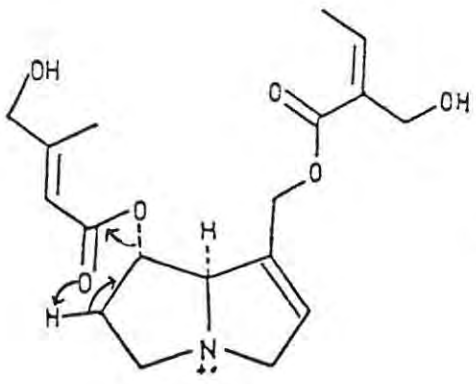


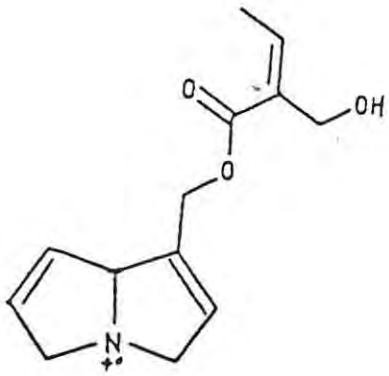
m/z 351



m/z 253



m/z 351



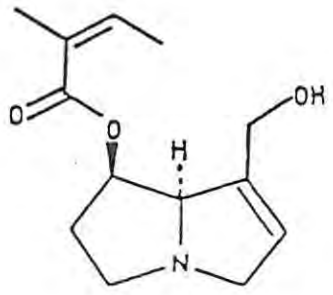
m/z 235

Scheme 2.8 Fragmentation pathway for 7-isosarracinyl-9-sarracinylheliotridine

2.6 Alkaloid content of *Senecio macrocephalus* DC

Senecio macrocephalus was collected, dried and extracted as described in Section 3.2. The crude alkaloid content was found to be 0.21%. Analysis by GC-MS showed that the crude extract contained a number of alkaloids. Some of these are, however, present in very low concentration, which makes their identification rather difficult since many of the fragment peaks in the spectra are of such low intensity that they are obscured by "background noise", ie. peaks produced as a result of column bleed and interference from the grease used in the instrument. Many of the components also appeared to be non-alkaloidal. Only those components which could be recognized as pyrrolizidine alkaloids from their mass spectra and whose spectra provided useful information will be discussed. All molecular masses were verified by CIMS.

The first alkaloidal component occurs at T_R 20.0min with molecular mass 237 and exhibits the characteristic fragmentation pattern for a C-7 monoester of an unsaturated base[1,7,55,91]. Peaks occur at m/z 137, 108, 93 and 80. This component could be 7-angelylretronecine (**161**), although it is present in such low concentration that it could conceivably be a degradation product. The fragmentation pathway is given in Scheme 2.9.



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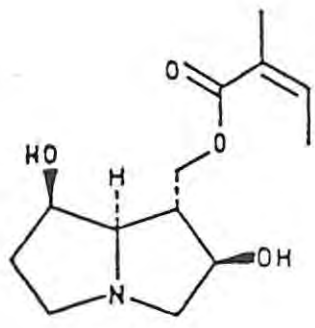

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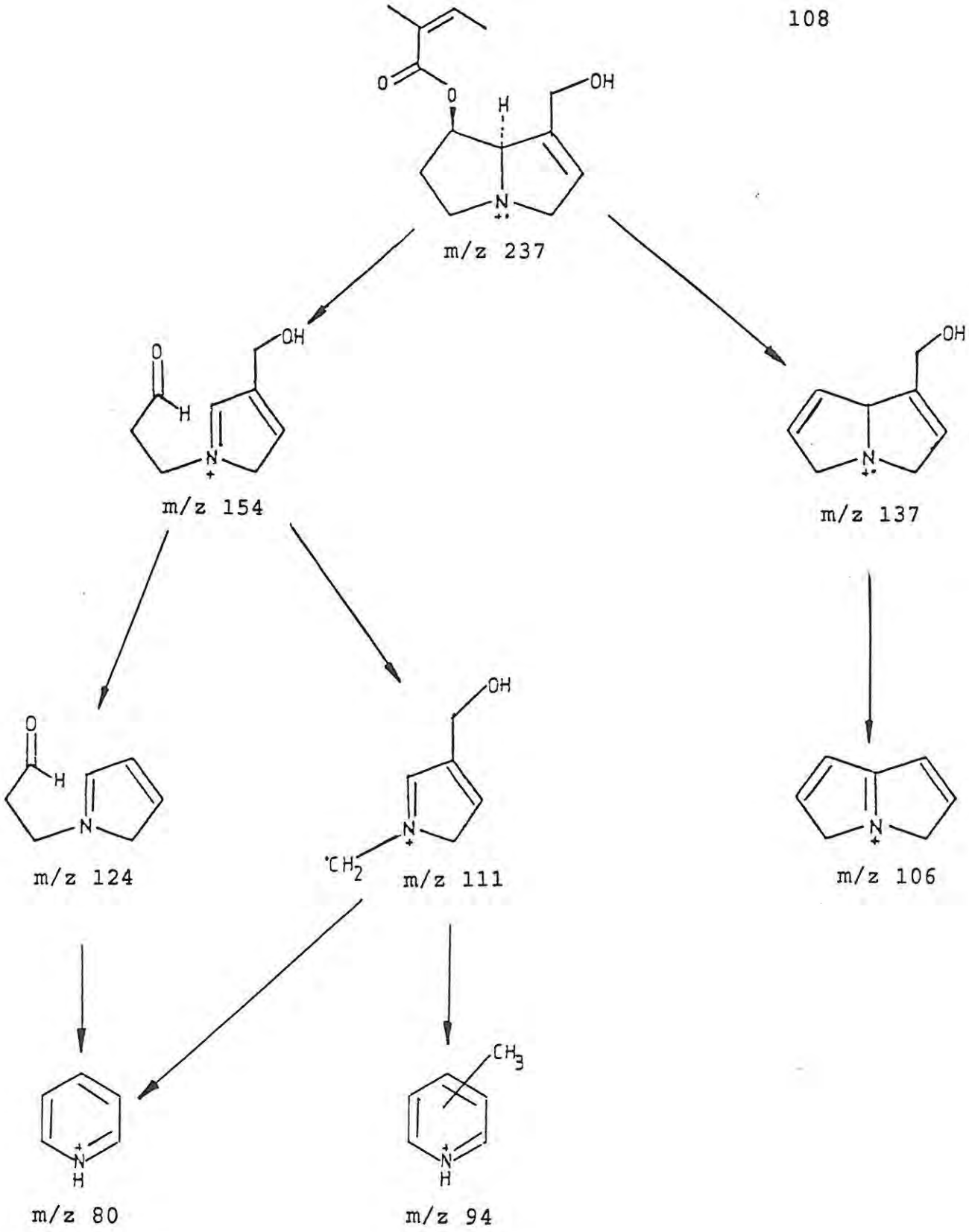
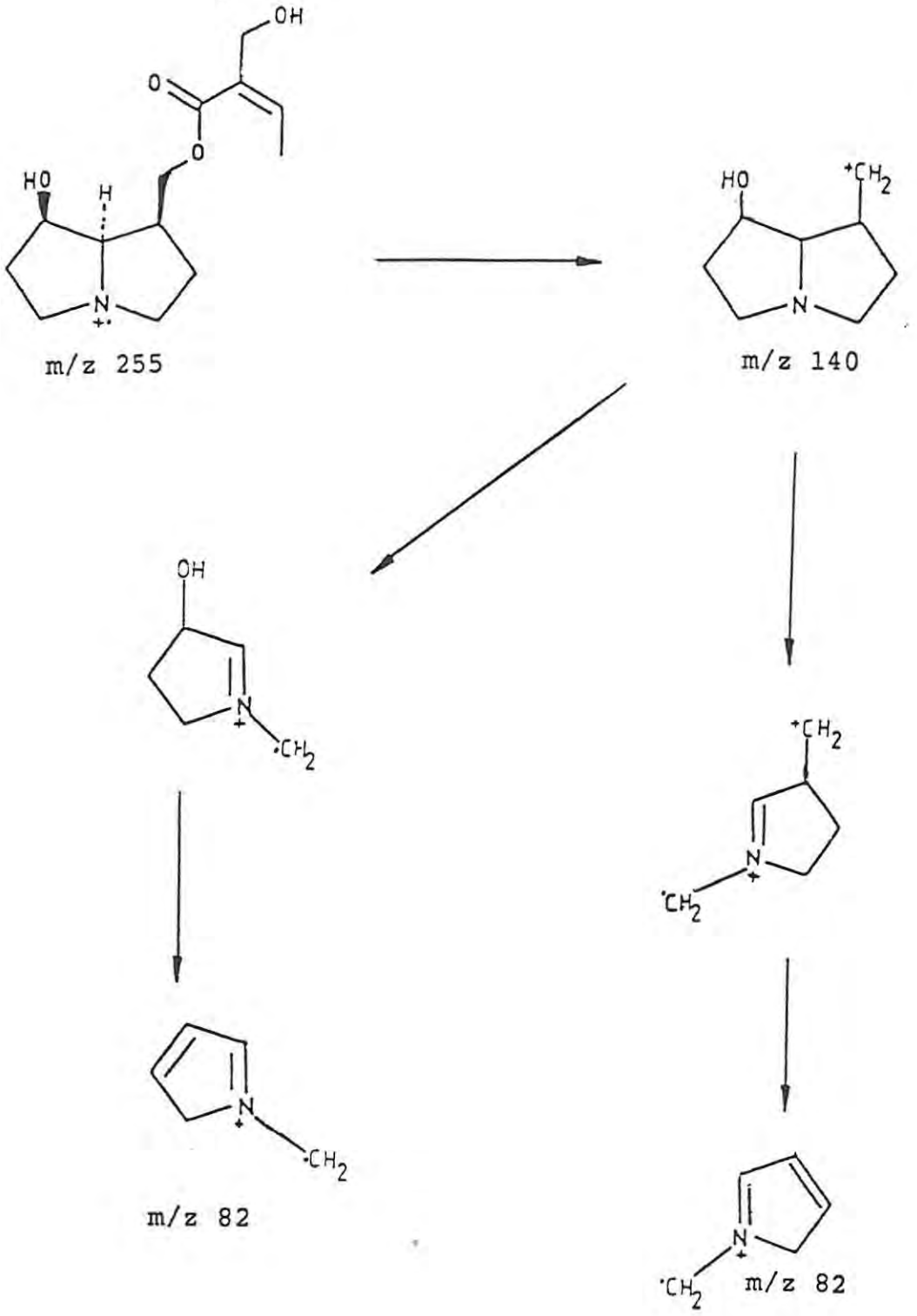


Scheme 2.9 Fragmentation pathway for 7-angelylretronecine

A component with molecular mass 255 occurs at T_R 24.5min and exhibits fragment peaks characteristic of a C-9 monoester of a saturated base alkaloid[1,7,55,91]. Characteristic fragment peaks occur at m/z 223, 140, 122, 121, 108 and 83. N-oxides of 9-angelylretronecine and its isomers have a molecular mass of 255, but since N-oxides are not mobile on the capillary GC column used this possibility may be ruled out. The fragmentation pattern also definitely indicates a saturated base alkaloid. The only known alkaloid with mass 255 is helifoline (**162**), in which the saturated base is hydroxylated at C-2[89]. However, the mass spectrum of component 2 does not resemble that reported for helifoline[89]. The fragmentation pattern for helifoline is similar to that of its unsaturated counterparts, since dehydration of the necine base occurs readily, followed by degradation identical to that of the retronecine monoesters[89].

An intense peak at m/z 238 shows that component 2 easily loses an OH group, suggesting a hydroxylated version of 9-angelyl-platynecine. The hydroxyl group would not be at C-2 since in that case the alkaloid would be an isomer of helifoline and would exhibit a different mass spectrum[89]. Peaks at m/z 238 and 223 are typical of sarracinic acid[105]. Possible structures are 9-sarracinyl-platynecine (**163**), 9-neosarracinylplatynecine (**164**) and 9-isosarracinyl-platynecine (**165**). A representative fragmentation pathway is shown in Scheme 2.10.

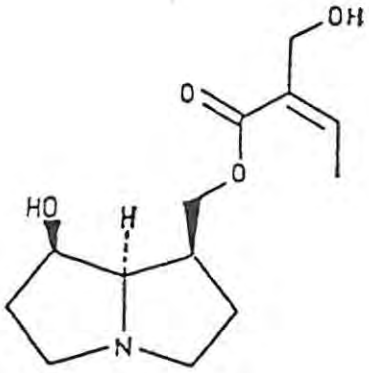


Scheme 2.10 Fragmentation pathway for 9-sarracinyl-platynecine

A component at T_R 26.0min appears to be C-9 monoester of a macronecine type base alkaloid with molecular mass 271. The only known alkaloid with this mass is procerine (**166**), which was characterized by mass spectrometry by Jovçeva *et al.* in 1978[140]. However, the mass spectrum of component 3 only exhibits fragment peaks which pertain to the base portion of the molecule and no conclusions can be drawn concerning the structure of the necic acid. The component could well be a C-9 version of procerine.

Component 4 (T_R 26.4min) has a molecular mass of 269 and exhibits characteristic fragment peaks for a C-9 monoester of a macronecine or rosmarinecine-based alkaloid[1,7,55,91]. No alkaloids with this mass are known. Fragment peaks occur at m/z 155, 123, 122, 109 and 83. A peak at m/z 252 (M-17) indicates that the compound has an hydroxyl group which is easily lost. The intense peak at m/z 155 is also reported to be present in the mass spectrum of procerine (**166**)[140], and it is possible that this component is a C-9 version of procerine with an double bond in the necic acid, as shown in structure (**167**).

Component 5 (T_R 31.9) has a molecular mass of 337 and exhibits the characteristic fragment peaks for a diester of a saturated base alkaloid[1,7,55,91]. Although the retention time for this component is close to that for neosarracine (**126**), its mass spectrum lacks the intense fragment at m/z 138 which would identify it as an acyclic diester of a saturated base[7,55,91]. In addition, other saturated base alkaloids of mass 337 exhibit a base peak at m/z 82[111], while the base peak for



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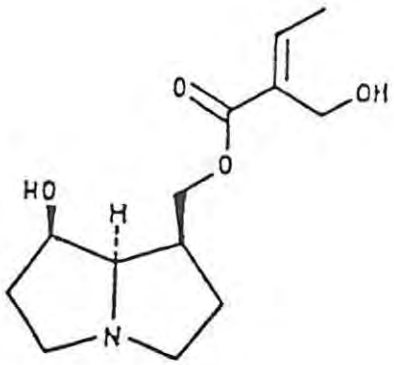

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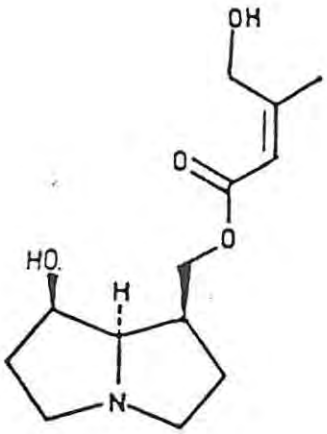

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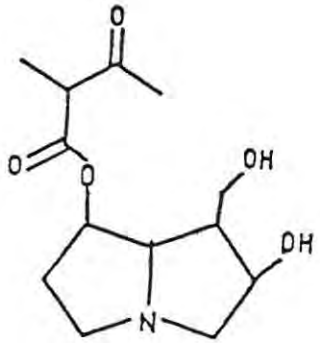

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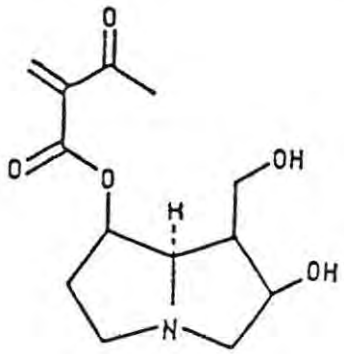

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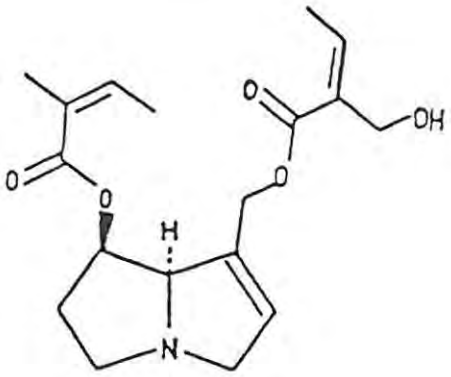

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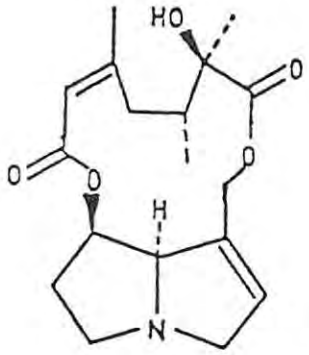

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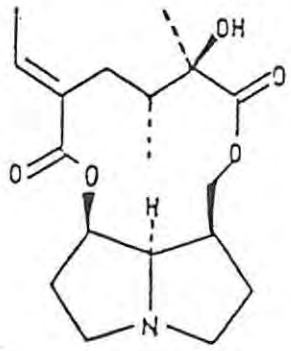

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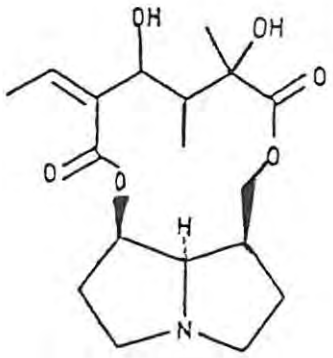


171

component 5 is at m/z 83. In this respect the spectrum resembles that of triangularine (**168**) and its isomers[111]; however, the fragmentation pattern is definitely not that of an unsaturated alkaloid. The spectrum is similar to that of bulgarsenine (**169**)[98], but as no reference material was available this tentative identification could not be verified. No mass spectrometric data for other saturated macrocyclic diester alkaloids such as platyphylline (**121**) and neoplatyphylline (**170**) was available for comparison.

Component 6 (T_R 33.3min) has a molecular mass of 353 and exhibits a fragmentation pattern characteristic of a macrocyclic diester (or a high molecular mass C-9 monoester) of an unsaturated base[1,7,55,91]. The mass spectrum is similar in some respects to that of rosmarinine (**135**), but the retention time is slightly lower. A number of possibilities exist, including axillaridine (**139**), desoxyaxillarine (**140**) and hygrophylline (**171**)[125,141]. Although the mass spectra recorded for axillaridine and desoxyaxillarine[125] are similar to that of component 6, no positive identification was possible. Unlike these two compounds, the mass spectrum of component 7 exhibits a base peak at m/z 83.

Due to the low alkaloid content of the plant, no pure alkaloids were isolated by chromatography, and the alkaloidal fractions obtained provided no further information when analyzed by GC-MS.

The GC-MS analysis of the crude alkaloid extract from ***Senecio macrocephalus*** clearly shows that it is very different from

that of ***Senecio speciosus***. Table 2.5 summarises the components present in ***S. macrocephalus*** and the GC trace is shown in Fig 2.7.

Table 2.5 Alkaloids identified in *Senecio macrocephalus* by GC-MS

Component	T_R (min)	Important mass fragments	Possible Identity
1	20.0	237, 137, 108, 93, 80, 55	7-Angelyl-retronecine
2	24.5	255, 238, 223, 140, 123, 122, 121, 108, 83, 82	9-Sarracinyl-platynecine type
3	26.0	271, 123, 122, 109, 83, 81	9-procerine type
4	26.4	269, 155, 123, 122, 109, 95, 83, 81, 55	Procerine type
5	31.9	337, 220, 178, 123, 122, 121, 108, 83, 55	Bulgarsenine, Platyphylline
6	33.3	353, 236, 138, 137, 136, 123, 123, 121, 83, 55	Axillaridine type

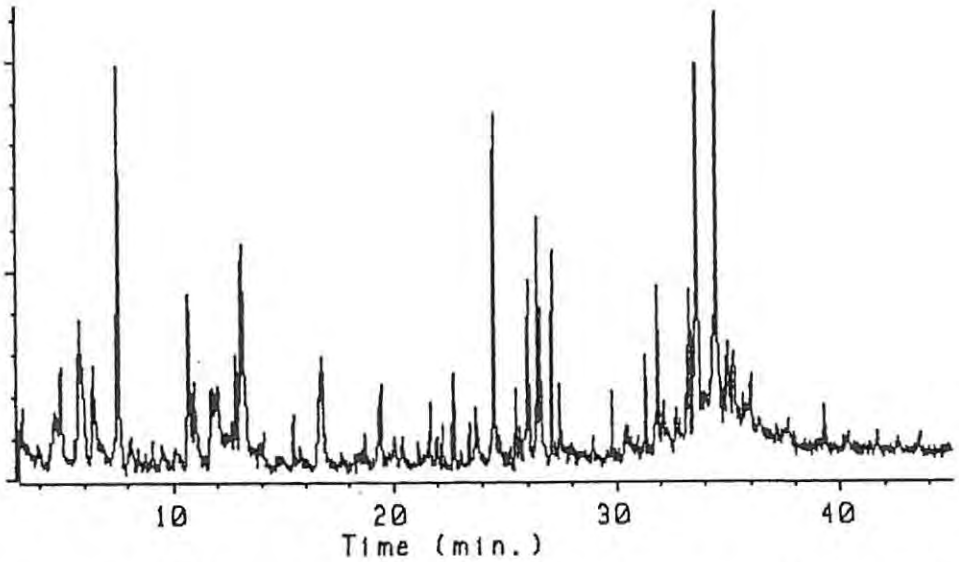


Fig 2.7 GC trace of crude extract from *S. macrocephalus* DC

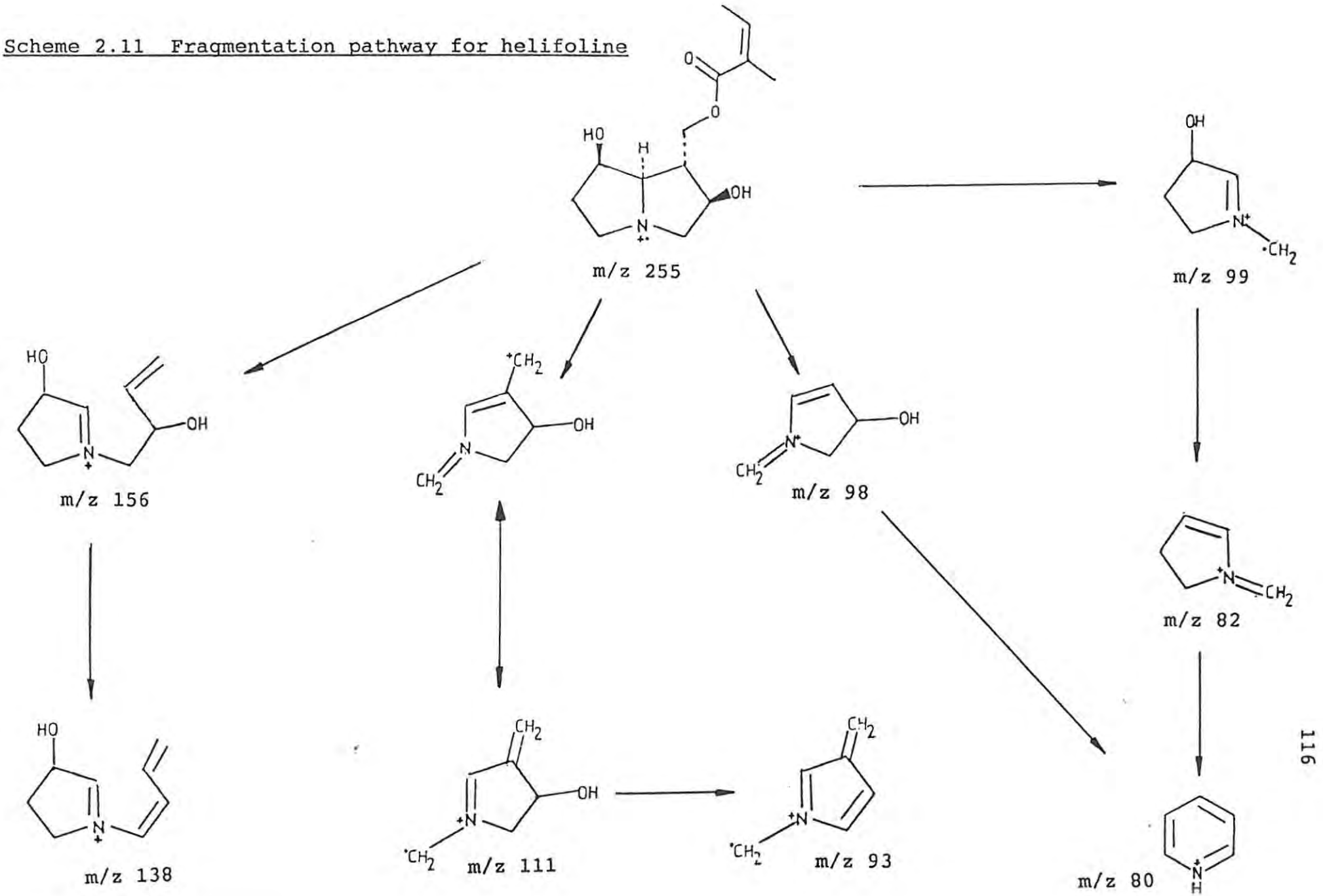
2.7 Alkaloid content of *Senecio speciosus/macrocephalus* Population 1

Population 1 of the local plants was collected, dried, chopped and extracted as described in Section 3.2. The crude alkaloid content was found to be 0.1%.

Analysis of the crude extract by GC-MS showed a number of alkaloidal components. The first three components have a molecular mass of 237 and exhibit the characteristic fragmentation patterns for monoesters of an unsaturated base[1,7,55,91]. The first component (T_R 20.0min) has an intense peak at m/z 137, identifying it as a C-7 monoester, while the other two components (T_R 20.3min and 20.7min) appear to be C-9 monoesters[7,55,91]. Comparison of retention times and MS data identified the latter components as 9-angelyl-retronecine (**149**) and 9-senecioylretronecine (**148**), while component 1 could be 7-angelylretronecine (**161**). Although no 7-monoester standards were available, tentative identification was possible based on the observed GC behaviour of pyrrolizidine alkaloids[111]. Fragmentation pathways are shown in Scheme 2.5 and Scheme 2.9.

Component 4 (T_R 21.4min) has molecular mass 255 and its mass spectrum is similar to that reported for helifoline (**162**)[89]. A fragmentation pathway is shown in Scheme 2.11.

Scheme 2.11 Fragmentation pathway for helifoline



A group of acyclic diester alkaloids, similar to that found in ***Senecio speciosus***, is also present. Four alkaloids with molecular mass 335 are discernible. Retention times are 29.3min, 29.6min, 29.8min and 30.1min. Mass spectra are similar to those of the corresponding components in ***S. speciosus***. Two more acyclic diesters are also discernible (T_R 32.8min and 33.2min). Both exhibit the intense fragment peak typical of this class of pyrrolizidines at m/z 136[7,55,91]. An intense peak at m/z 220 indicates the presence of an angelyl type group at C-7, while another peak at m/z 237 is typical of acyclic diesters with angelic acid at C-7[105]. A small fragment peak at m/z 235 is indicative of sarracinic acid[105]. It would therefore appear that these two alkaloids are isomers of the earlier group. Unfortunately, no molecular ion could be discerned even using CIMS, probably due to the low level of these alkaloids present. A summary of these alkaloidal components is given in Table 2.6 and the GC trace is shown in Fig 2.8.

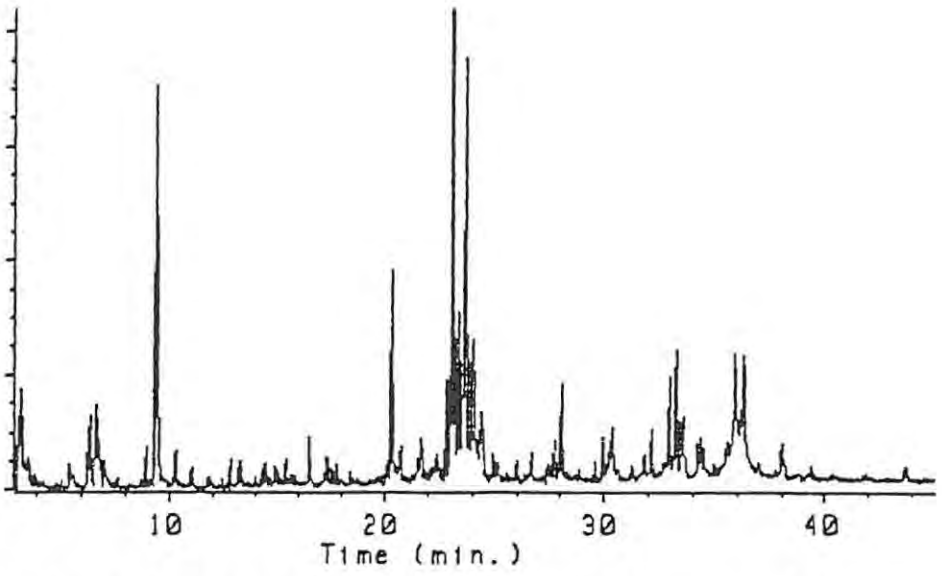


Fig 2.8 GC trace of crude extract from Population 1

Table 2.6 Alkaloids identified in Population 1 by GC-MS

Component	T_R (min)	Important mass fragments	Possible Identity
1	20.0	237, 137, 121, 120, 119, 93, 80	7-Angelyl-retronecine
2	20.3	237, 220, 138, 120, 119, 93, 80	9-Angelyl-retronecine
3	20.7	237, 138, 121, 120, 119, 93, 80	9-Senecioyl-retronecine
4	21.4	255, 138, 122, 121, 120, 119, 93, 83, 80	Helifoline type
5	29.3	335, 237, 220, 136, 121, 120, 119, 94, 93, 83, 80	Triangularine type
6	29.6	335, 237, 220, 136, 121, 120, 119, 94, 93, 83, 80	Triangularine type
7	29.8	335, 237, 220, 136, 121, 120, 119, 94, 93, 83, 80	Triangularine type
8	30.1	335, 237, 220, 136, 121, 120, 119, 94, 93, 83, 80	Triangularine type
9	32.8	237, 220, 136, 121, 120, 119, 94, 93, 83, 80	Triangularine type (Isomer of above?)
10	33.2	237, 220, 136, 121, 120, 119, 94, 93, 83, 80	Triangularine type (Isomer of above?)

The crude alkaloidal extract was subjected to DCCC and the alkaloids obtained were purified by preparative TLC as described in Section 3.10. The flow chart in Fig 2.9 shows the alkaloids isolated.

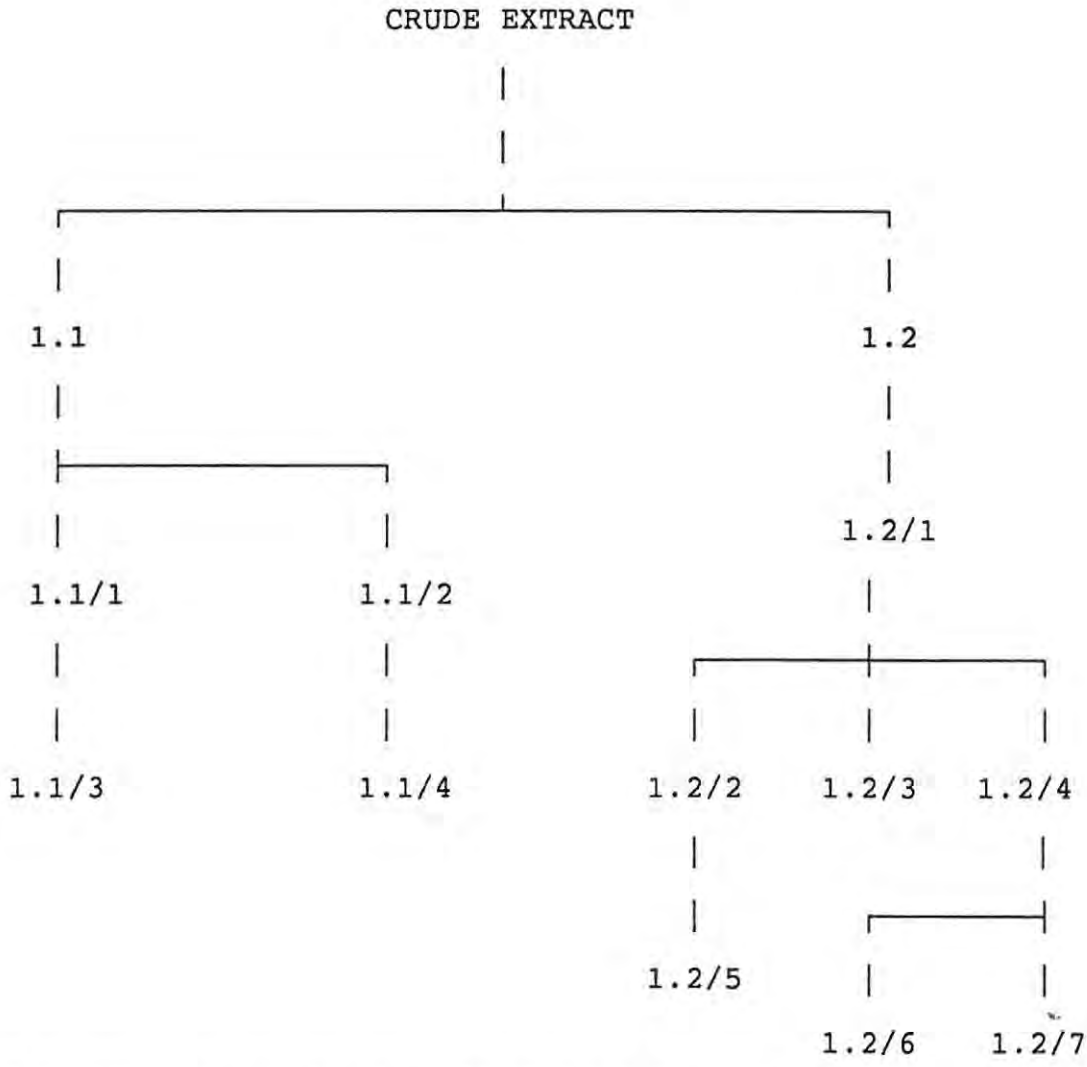
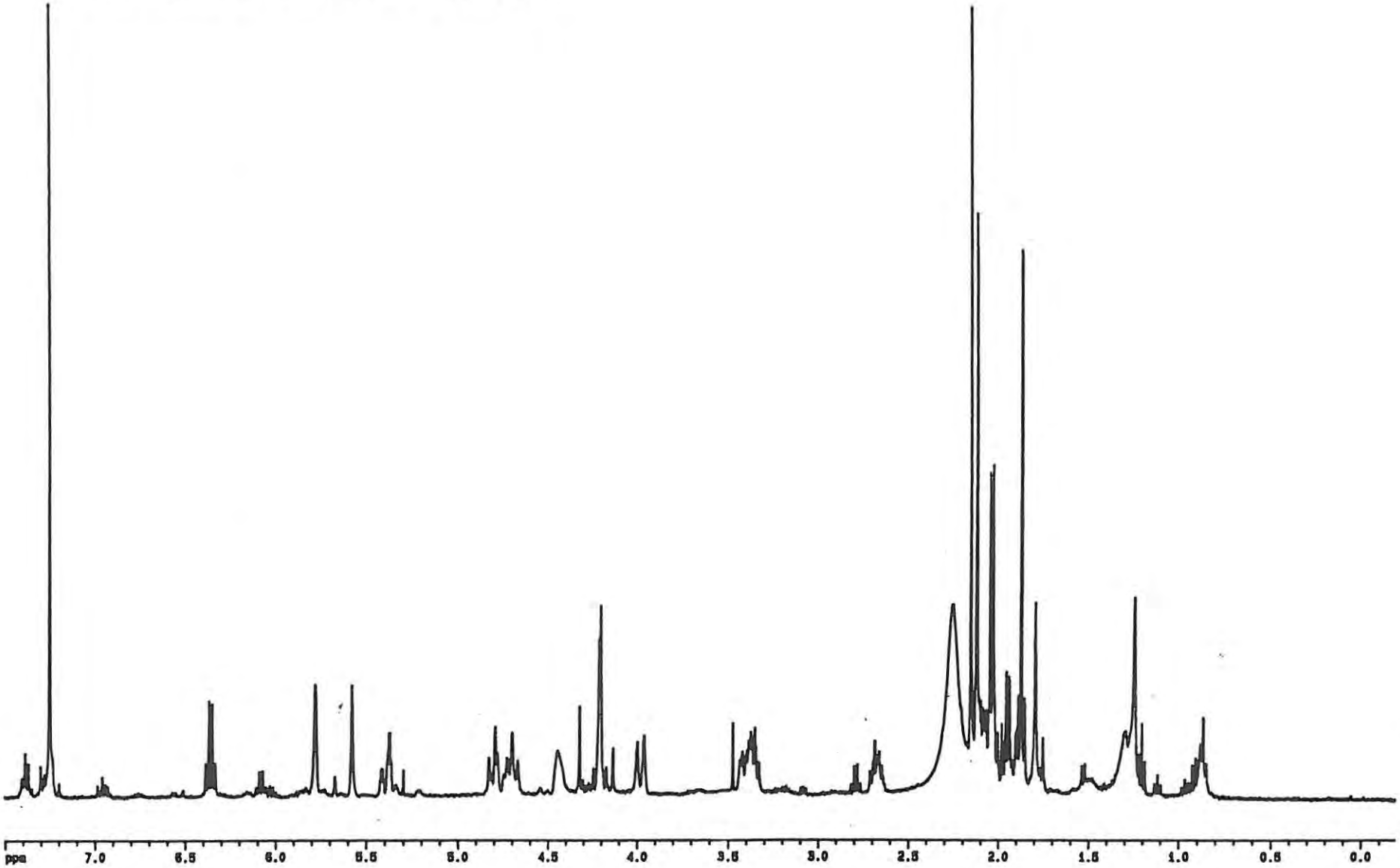


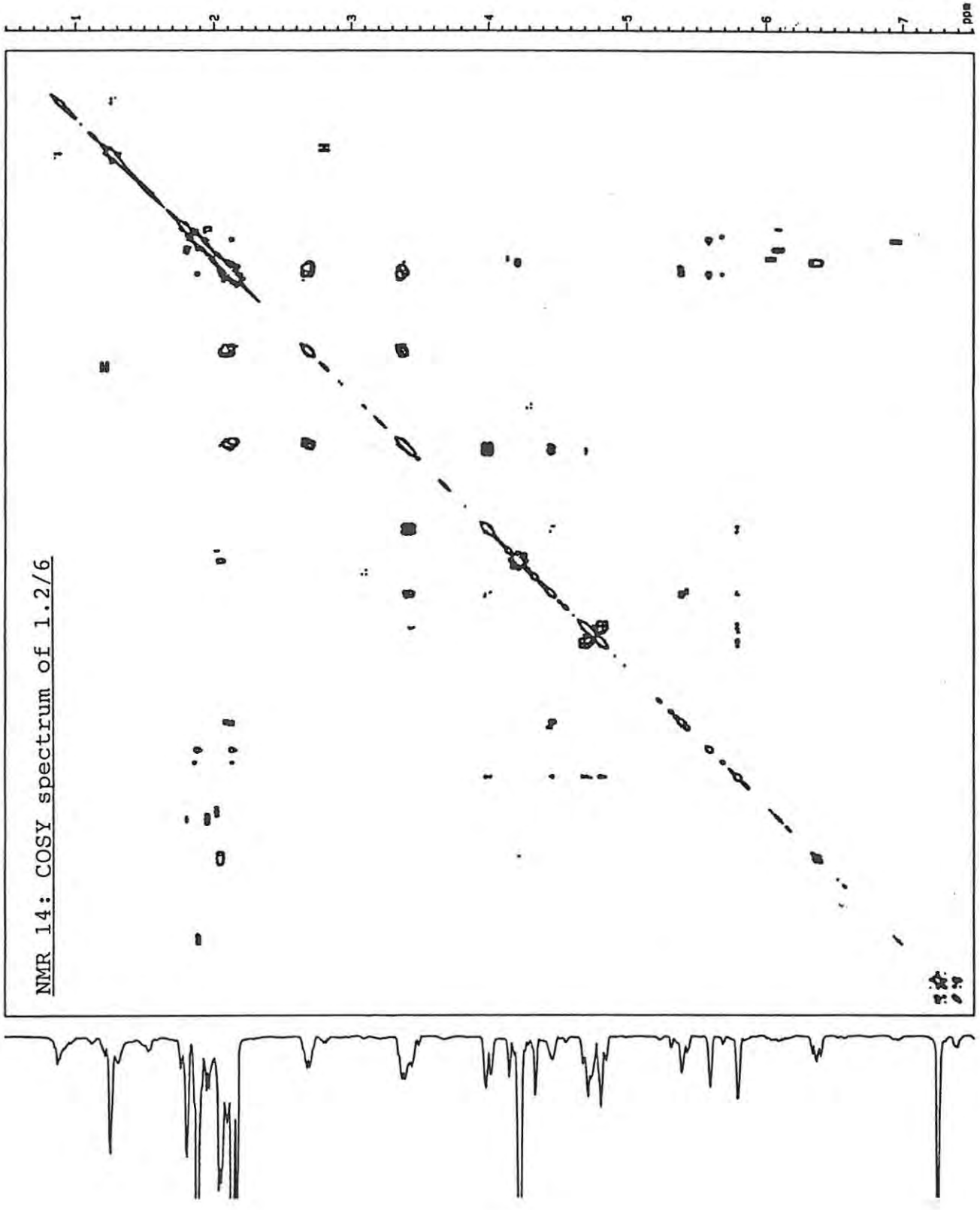
Fig 2.9 Flow chart showing alkaloidal fractions isolated from *S. speciosus/macrocephalus* Population 1

Fraction 1.2/3 was shown by GC-MS to be very impure, although the major component (T_R 21.4min) appeared to be a 9-monoester of a macronecine-type base[7,55,89,91]. This is almost certainly the alkaloid already identified by GC-MS of the crude extract and could well be helifoline (**162**).Fractions 1.2/5 and 1.2/6 have similar ^{1}H NMR spectra.

1.2/6 appears to consist mainly of 7-senecioyl-9-sarracinyl-heliotridine (**153**) and its spectrum is very similar to those of S5 and S8. The shift value for H-8 is δ 4.45 as for S5. However, the ring proton signals are not clearly defined; this is most obvious in the signals for the H-9 protons. In the ^{1}H NMR spectra of S5 and S8 these signals are a pair of well defined doublets, while in the spectrum of 1.2/6 these doublets are unresolved. The broad singlet at δ 5.38 (H-7) is partnered by a smaller signal at δ 5.41. Examination of the COSY spectrum[114] shows that both these signals couple to the signal for H-8 at δ 4.43. In addition to the signals for the vinyl protons of senecioic and sarracinic acids, a small quartet is visible at δ 6.10, which couples to two methyl group signals at δ 1.95 and δ 1.80. This is indicative of an angelyl group[105,134]. Analysis of 1.2/6 by GC-MS revealed the presence of two major and one minor component (T_R 29.6min, 29.8min and 30.1min respectively). All three alkaloids have a molecular mass of 335 and exhibit identical mass spectra. The component at 30.1min comprises 60% of the total and its mass spectrum is identical to that of S5.

NMR 13: ^{1}H NMR spectrum of 1.2/6

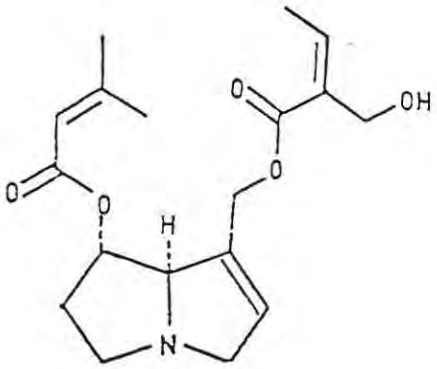



NMR 14: COSY spectrum of 1.2/6
ppm

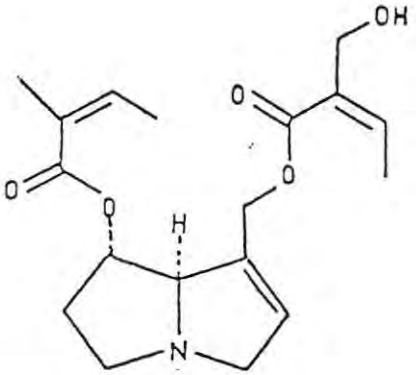
The major alkaloid of this fraction is therefore 7-senecioyl-9-sarracinylheliotridine (**153**).

The component at 29.8min comprises 35% of the total and that at 29.6min 5%. The mass spectra and retention times of these components are identical to those of the minor components present in S5 and S8. The component at 29.8min is almost certainly 7-angelyl-9-sarracinylheliotridine (**154**), based on the ^{1}H NMR spectrum. The corresponding retronecine-based alkaloid has been isolated from ***Senecio triangularis*** **Hook** by Roitman[105], who named it triangularine, and by Rueger and Benn[90], in independent studies. The ^{1}H NMR data for component 1.2/6-2 agree well with those reported by both authors, except for the shift value for H-8, which is downfield of the values reported[90,105]. A ROESY spectrum[113,137] of fraction 1.2/6 was obtained. Although 1.2/6 is a mixture, the ring proton signals for the two components overlap, except for the H-7 signals, and it would be expected that if the two alkaloids present have opposite stereochemistry this would be apparent. NOEs were observed between H-7 and H-5u, between H-9d, H-8, H-3d and H-3u and between H-9u, H-8, H-3d and H-3u. No interactions were observed between H-7 and H-8 for either alkaloid. The H-7 signal widths at half-height are 15Hz and 13.5Hz respectively, again indicating that the base is heliotridine[39,135-6].

The presence of a low intensity quartet at δ 6.95 indicates the strong possibility that the minor component of 1.2/6 is 7-angelyl-9-neosarracinylheliotridine (**156**)[105].



153



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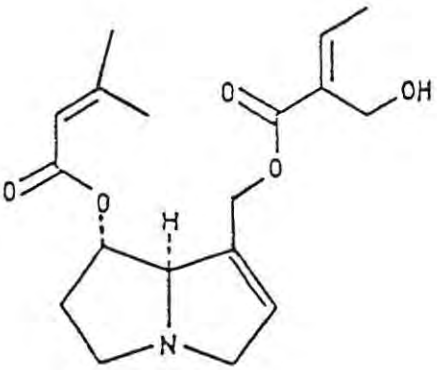
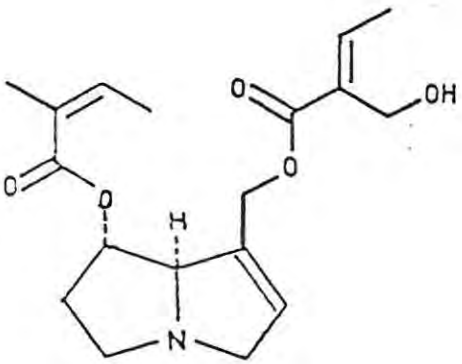
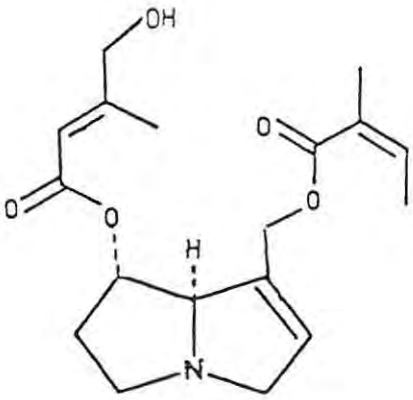


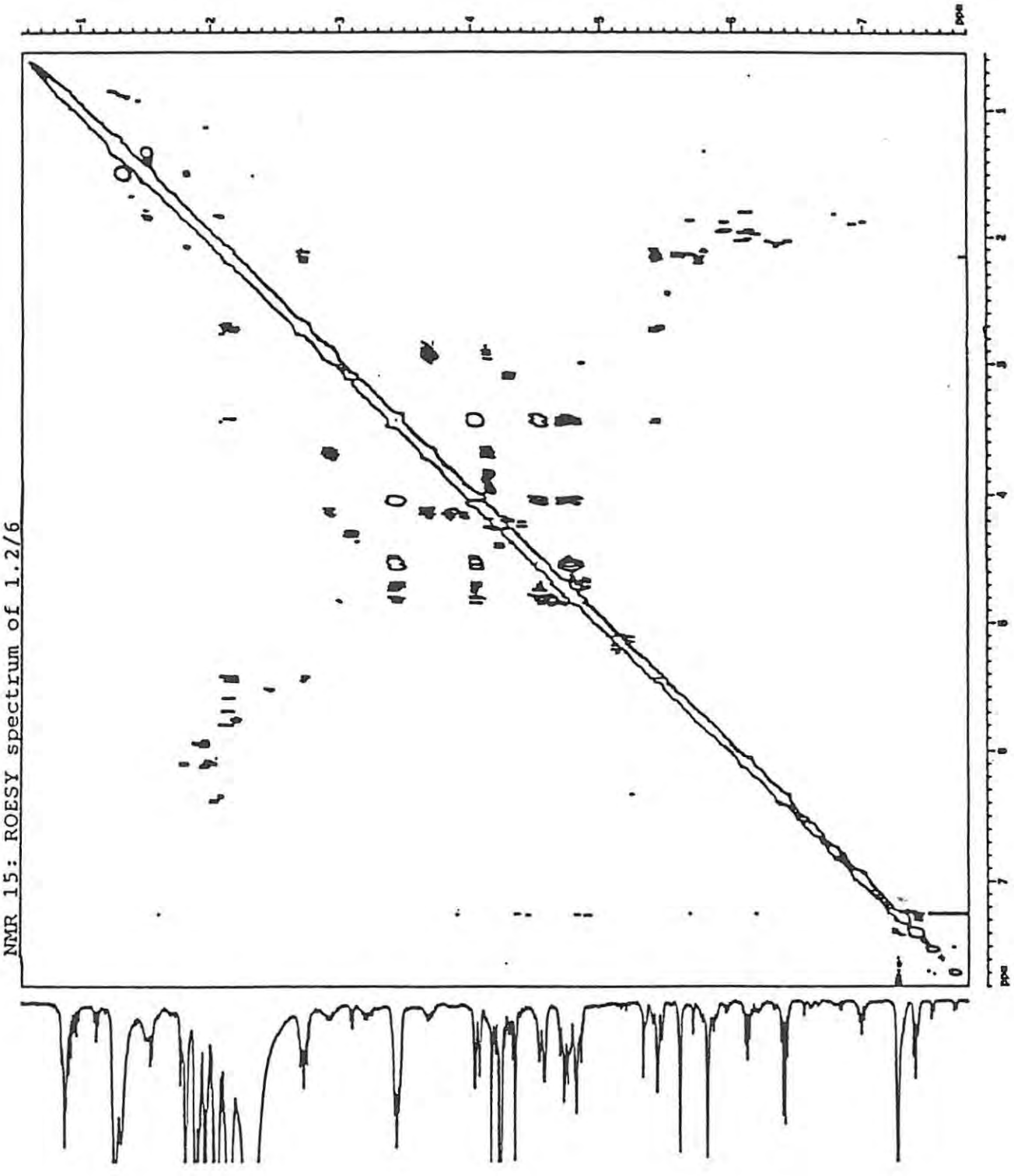
155



156



157



NMR 15: ROESY spectrum of 1.2/6

The retention times and mass spectra of components 1.2/6-2 and 1.2/6-3 are identical to those of the minor components of S5 and S8, indicating that 7-angelyl-9-sarracinylheliotridine and 7-angelyl-9-neosarracinylheliotridine are also present in ***Senecio speciosus***.

The ^{1}H NMR spectrum of 1.2/5 differs from that of 1.2/6 only in the ratio of the components. Integration of the signals for the respective H-7 protons indicates that the major components are present in approximately equal concentration, with slightly more 7-angelyl-9-sarracinylheliotridine present. This is confirmed by GC-MS analysis, which also shows a fourth isomeric alkaloid at T_R 29.3min. The identity of this alkaloid could not be established from the NMR spectra. Possibilities are 7-senecioyl-9-neosarracinylheliotridine (**155**) and 7-isosarracinyl-9-angelylheliotridine (**157**).
Several attempts were made to separate the two major components, but all were unsuccessful.
The diester alkaloids 7-senecioyl-9-sarracinylheliotridine (**153**) and 7-angelyl-9-sarracinylheliotridine (**154**) were thus obtained as a mixture from plant population 1 and ^{1}H NMR data for the two compounds are given in Table 2.7.
Comparison of results obtained by GC-MS and NMR spectroscopy show that these compounds could have been derived from ***Senecio speciosus***, while a monoester of mass 237 (T_R 20.0min) could have derived from ***Senecio macrocephalus***. If plant population 1 is indeed a hybrid of these two plants it seems to exhibit predominantly ***speciosus*** characteristics.

Table 2.7 ^{1}H NMR data for major alkaloids in fractions 1.2/5 and 1.2/6

#H	7-Senecioyl-9-sarracinyl-heliotridine	7-Angelyl-9-sarracinyl-heliotridine
2	5.78 (br s)	5.78
3d	3.98 (d)	3.99
3u	3.40 (m)	3.40
5d	3.38 (m)	3.36
5u	2.65 (m)	2.65
6	2.00-2.13 (m)	2.00-2.13
7	5.37 (br s)	5.41
8	4.43 (br s)	4.43
9d	4.81 (d)	4.82
9u	4.69 (d)	4.69
13	6.39 (q)	6.39
14	2.05 (d)	2.05
15	4.20 (d)	4.20
17	5.59 (s)	-
18	-	6.09
19	2.10 (s)	1.95
20	1.85 (s)	1.80

δ in ppm

Coupling constants were not measured due to signal overlap from the two alkaloids present.

2.8 Alkaloid content of *Senecio speciosus/macrocephalus* Population 2

Population 2 of the local plants was collected, dried, chopped and extracted as described in Section 3.2. The crude alkaloid content was found to be 0.061%.

The crude alkaloidal extract was examined by GC-MS and found to be much more complex than the extract from Population 1. The first alkaloidal component (T_R 20.0min) appears to be a C-7 monoester of an unsaturated base[7,55,91] (molecular mass 237) and has an identical mass spectrum to component 1 in both population 1 and ***S. macrocephalus***. This component could be 7-angelylretronecine (**161**).

Component 2 (T_R 24.5min) has a molecular mass of 255 and exhibits the characteristic fragment peaks for a C-9 monoester of a saturated base alkaloid[7,55,91]. The mass spectrum of this component is identical to that of component 2 in ***S. macrocephalus***. Prominent peaks at m/z 238 and 223 again suggest a hydroxylated version of angelic acid[105], while intense peaks at m/z 108 and 109 confirm the presence of a saturated platynecine-type base[90]. A fragmentation pathway is shown in Scheme 2.10.

Component 3 (T_R 26.0min) has molecular mass 271 and its mass spectrum is identical to that of component 4 in ***S. macrocephalus***. Once again, no peaks pertaining to the acid portion of the molecule are visible in the spectrum, but the component could well be a C-9 version of procerine[140].

A group of acyclic diester alkaloids similar to those found

in ***S. speciosus*** and population 1 is also present (T_R 29.6min, 29.8min and 30.1min). All three components have molecular mass 335 and their mass spectra are similar to those observed for 7-senecioyl-9-sarracinylheliotridine (**153**) and 7-angelyl-9-sarracinylheliotridine (**154**). Component 7 (T_R 32.07min) has molecular mass 351 and exhibits the fragmentation pattern characteristic of a macrocyclic diester of an unsaturated base[7,55,91]. Comparison of retention times and mass spectra allowed its tentative identification as retrorsine (**118**).
In addition, a second group of acyclic diesters similar to those found in population 1 was observed (T_R 32.8min and 33.2min). Fragment peaks at m/z 220, 235 and 237 again indicate the presence of angelic and sarracinic acids[105], while other peaks at m/z 136, 121, 120, 119 and 93 show that the alkaloids are acyclic diesters of an unsaturated base[1,7,55,91]. Once again no molecular ion could be detected. These alkaloids are summarized in Table 2.8 and the GC trace shown in Fig 2.10.

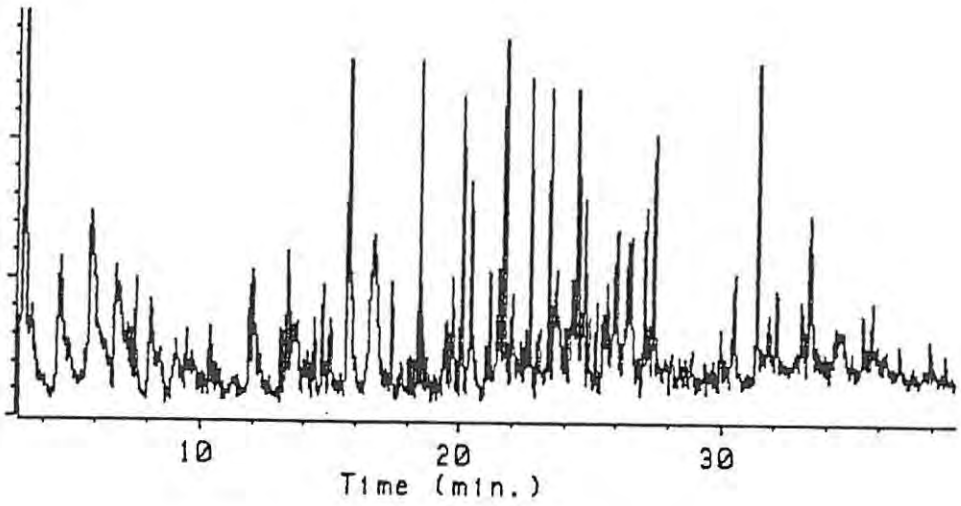


Fig 2.10 GC trace of crude extract from Population 2

Table 2.8 Alkaloids identified in Population 2 by GC-MS

Component	T_R (min)	Important mass fragments	Possible Identity
1	20.0	237, 220, 137, 108, 83, 80	7-Angelyl-retronecine
2	24.5	255, 238, 223, 140, 123, 122, 121, 108, 83, 82, 55	9-Sarracinyl-platynecine type
3	26.0	271, 123, 122, 109, 83, 81	9-Procerine type
4	29.6	335, 237, 219, 136, 121, 120, 119, 93, 83, 80, 55	Triangularine type
5	29.8	335, 237, 219, 136, 121, 120, 119, 93, 83, 80	Triangularine type
6	30.1	335, 237, 219, 136, 121, 120, 119, 93, 83, 80	Triangularine type
7	32.07	351, 281, 220, 138, 137, 136, 121, 120, 119, 93, 80	Retrorsine
8	32.8	237, 219, 136, 121, 120, 119, 93, 83, 80	Triangularine type (Isomer of above?)
9	33.2	237, 219, 136, 121, 120, 119, 93, 83, 80	Triangularine type (Isomer of above?)

The crude alkaloidal extract was subjected to DCCC and preparative TLC as described in Section 3.11. The flow chart in Fig 2.11. shows the components obtained.

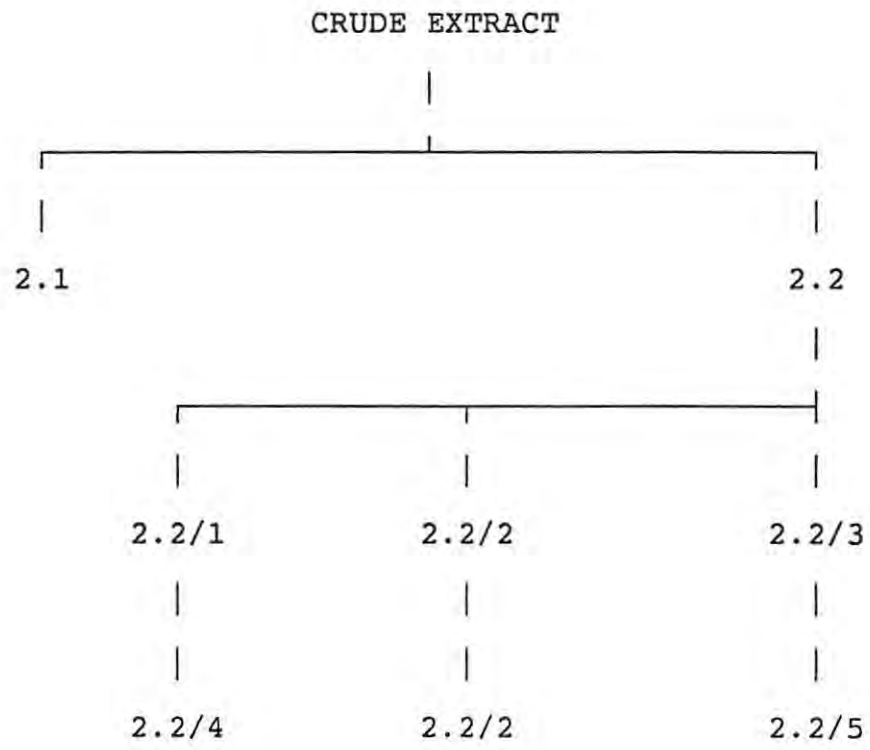
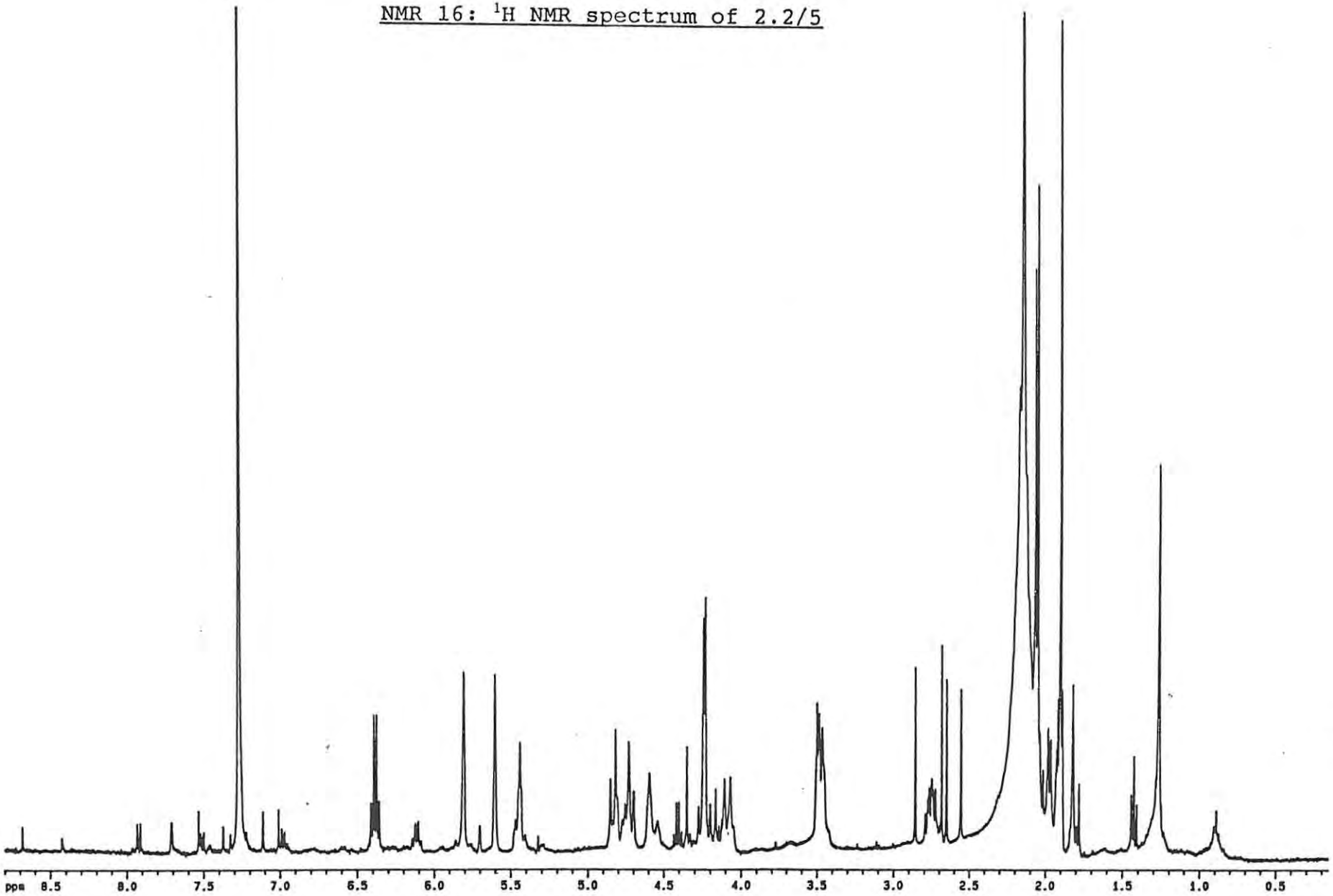
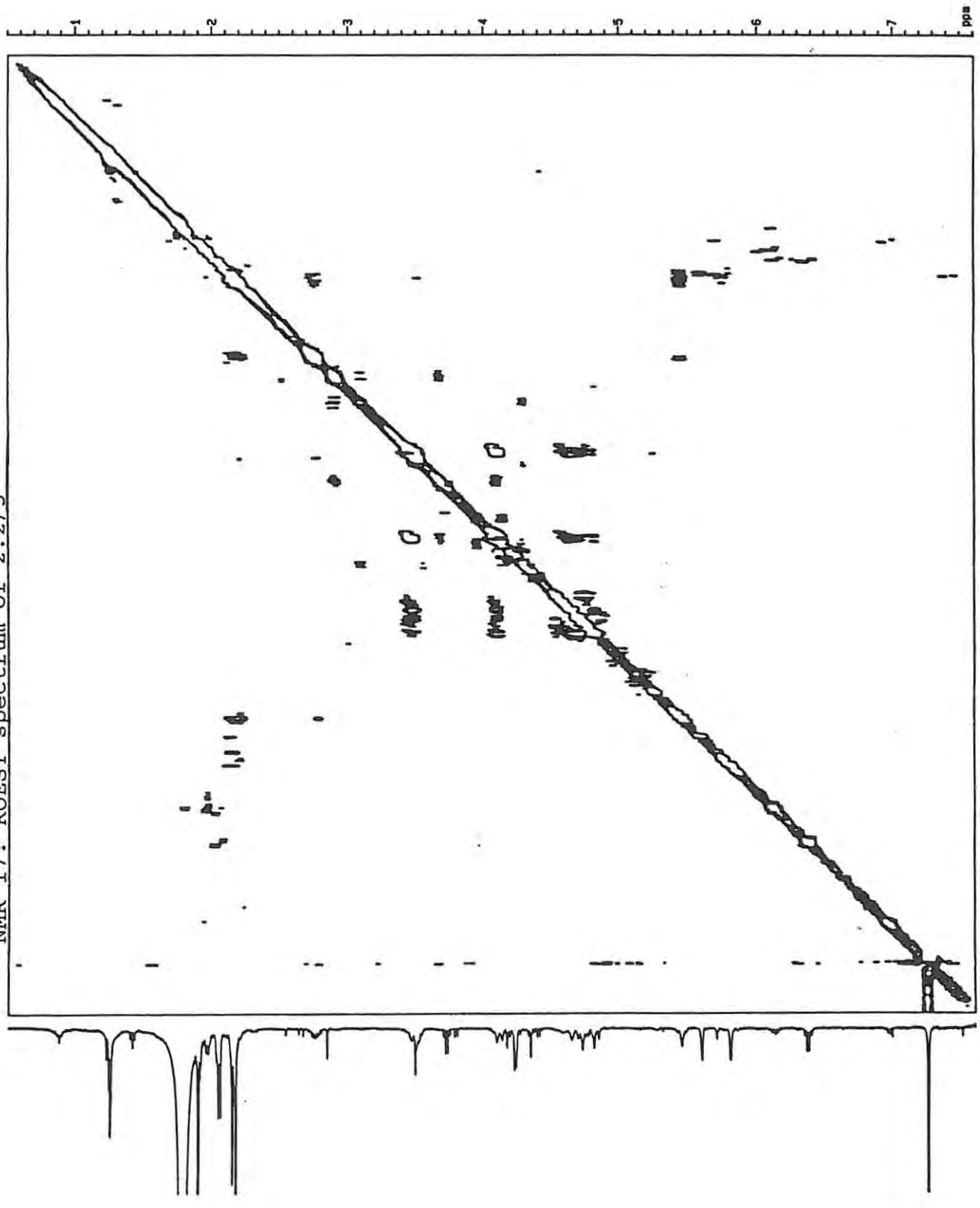


Fig 2.11 Flow chart showing alkaloidal fractions isolated from *S. speciosus/macrocephalus* Population 2

The major alkaloidal component of the plant was 2.2/5. Analysis of this fraction by GC-MS revealed that it was composed of two acyclic diester alkaloids of molecular mass 335 (T_R 29.8min and 30.1min). The mass spectra of these components are identical to those for the alkaloids isolated from Population 1.

NMR 16: ^{1}H NMR spectrum of 2.2/5





NMR 17: ROESY spectrum of 2.2/5

Table 2.9 ^{1}H NMR data for two alkaloids in Fraction 2.2/5

#H	7-Senecioyl-9-sarracinyl-heliotridine	7-Angelyl-9-sarracinyl-heliotridine
2	5.80 (s)	5.80
3d	4.09 (d)	4.08
3u	3.49 (m)	3.49
5d	3.45 (m)	3.45
5u	2.73 (m)	2.73
6	1.98-2.16 (m)	1.98-2.16
7	5.45 (br s)	5.48
8	4.59 (br s)	4.57
9d	4.83 (d)	4.82
9u	4.73 (d)	4.72
13	6.38 (q)	6.38
14	2.05 (d)	2.05
15	4.25 (d)	4.25
17	5.60 (s)	-
18		6.10
19	2.10 (s)	1.95
20	1.95 (s)	2.00

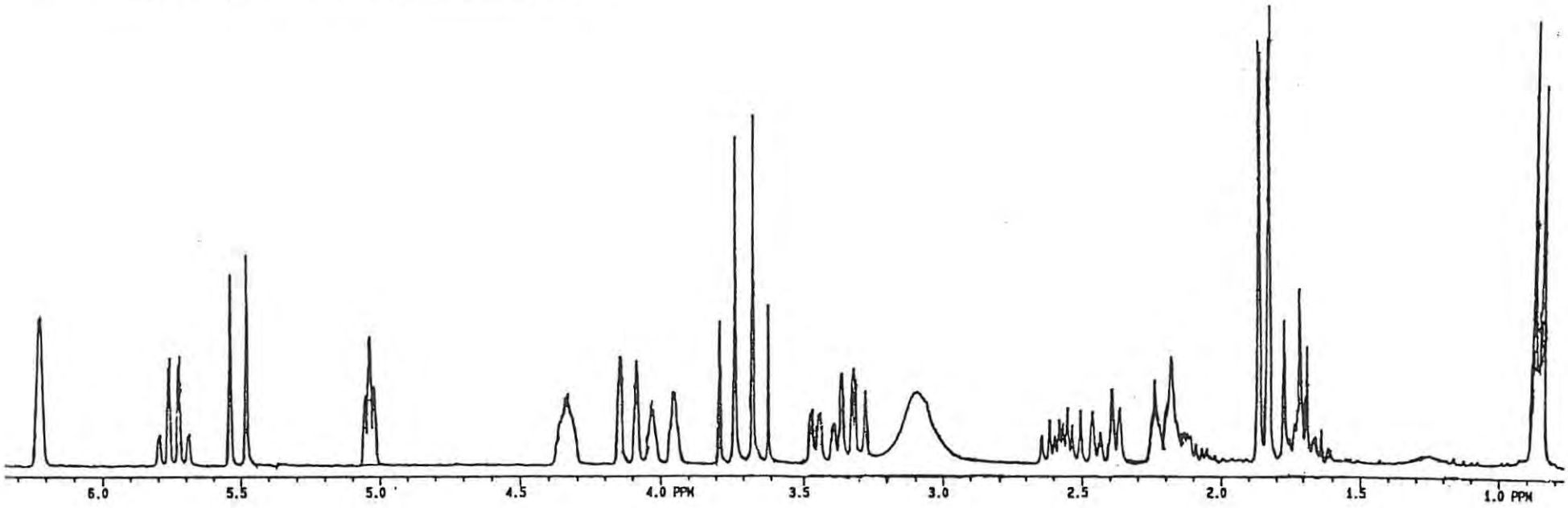
δ in ppm

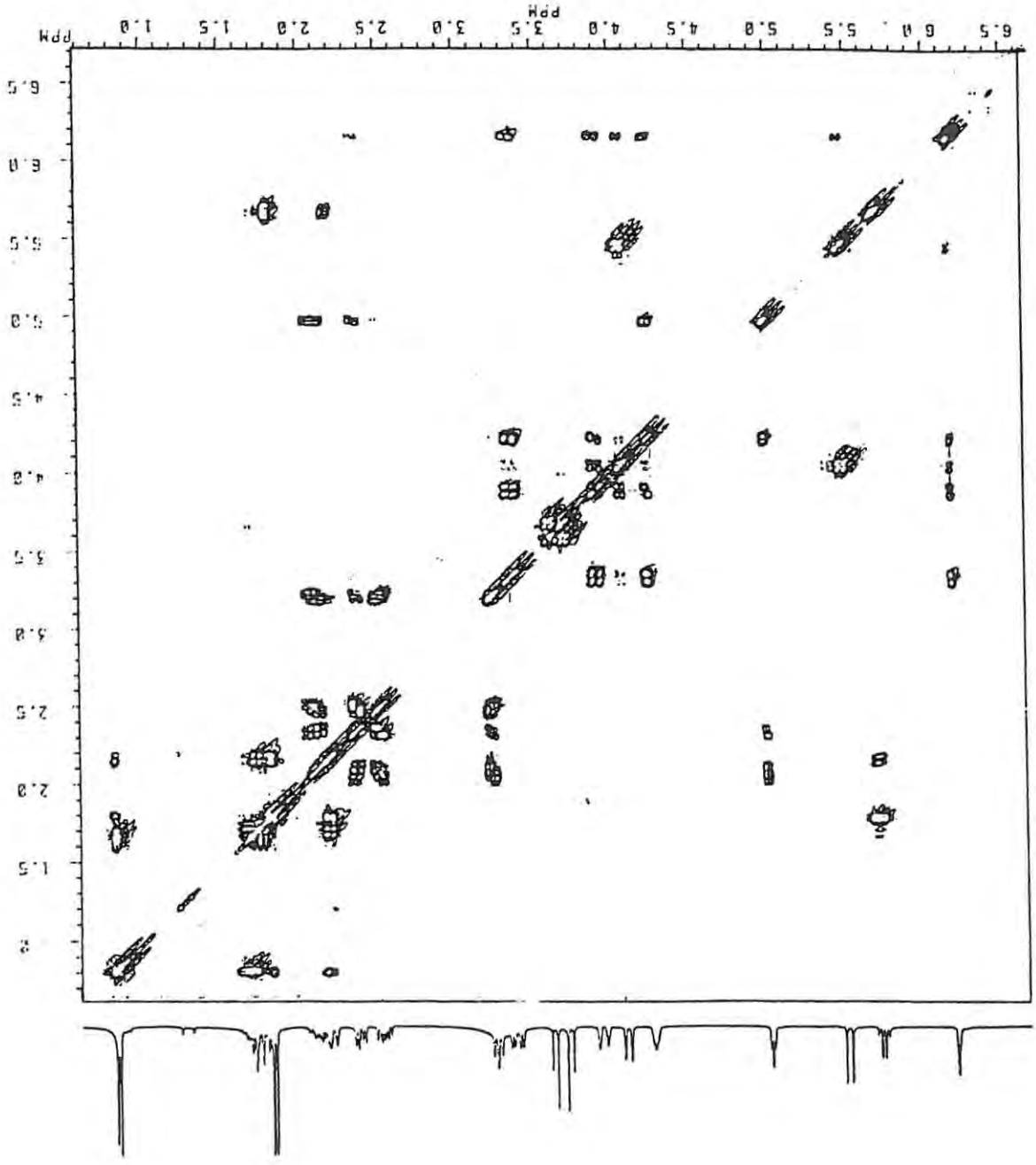
Coupling constants were not measured due to signal overlap in the two components present.

Examination of the ^{1}H NMR spectrum showed that the major component, which comprises 75% of the total, could be 7-senecioyl-9-sarracinylheliotridine (**153**). The minor component appears to be 7-angelyl-9-sarracinylheliotridine (**154**). The ^{1}H NMR data for the major component closely resembled those observed for S5 and S8. A ROESY spectrum[113,137] of 2.2/5 showed that the stereochemistry of the necine base was the same as for S5 and S8. The ^{1}H NMR data for the two alkaloids present in 2.2/5 are given in Table 2.9.

The second alkaloid isolated from Population 2 was 2.2/4, which was the only crystalline alkaloid obtained in the whole study. The ^{1}H NMR spectrum exhibits features typical of macrocyclic diester alkaloids of an unsaturated base; namely, the H-9 proton signals are 1.35 ppm apart, while the H-2 signal occurs at δ 6.17[65,108]. The structure of the compound followed logically from the COSY coupling patterns and the alkaloid was identified as retrorsine (**118**). The ^{1}H NMR spectrum was in good agreement with that observed for authentic retrorsine (see Sections 2.1 and 3.2). The shift positions of the H-6 protons are virtually identical to those reported for retrorsine[108] and the half-height width of the H-7 proton signal was calculated to be 8.7 Hz, confirming retronecine as the necine base for this compound[39,135].

NMR 18: ^{1}H NMR spectrum of 2.2/4





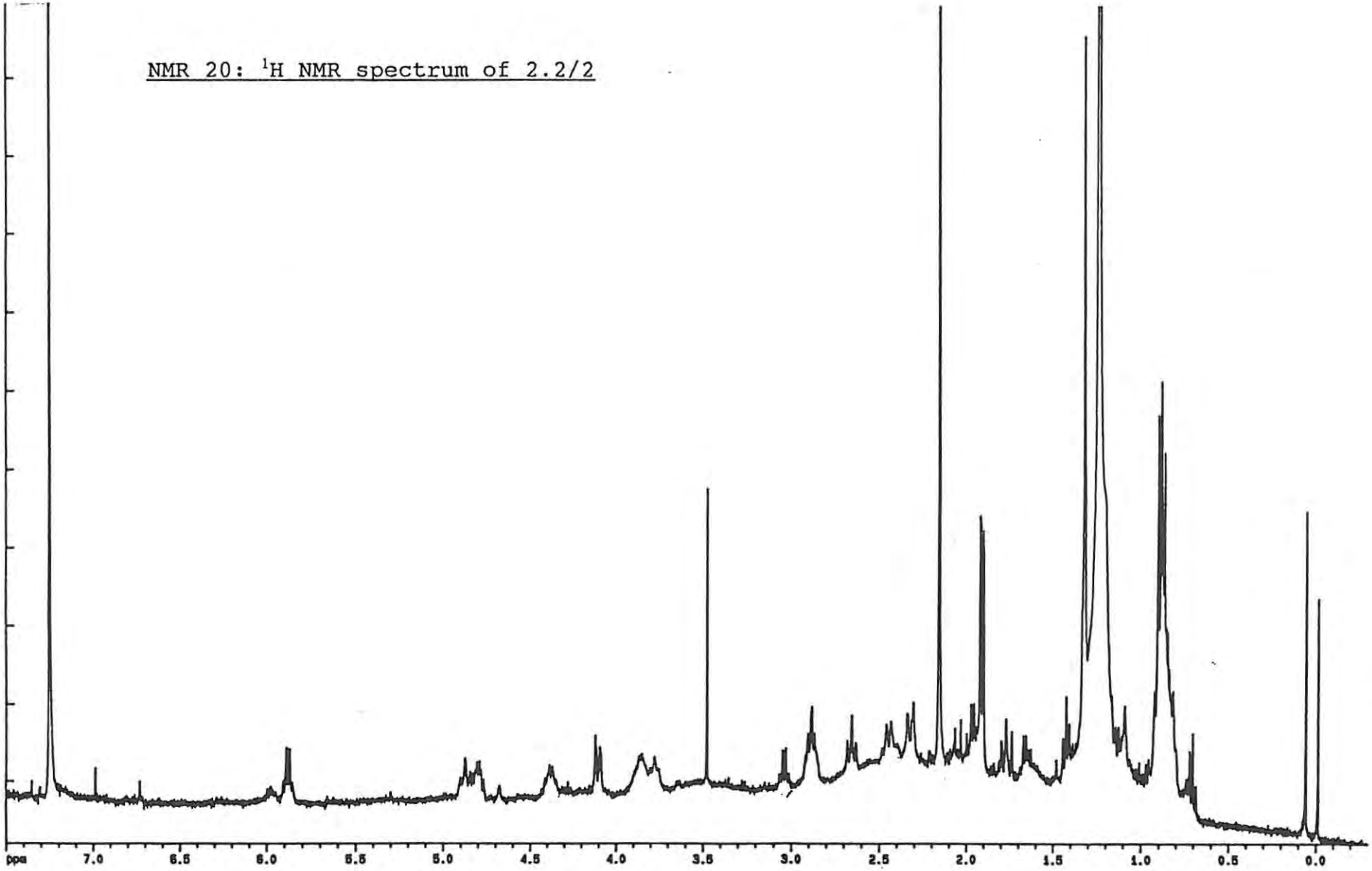
NMR 19: COSY spectrum of 2.2/4

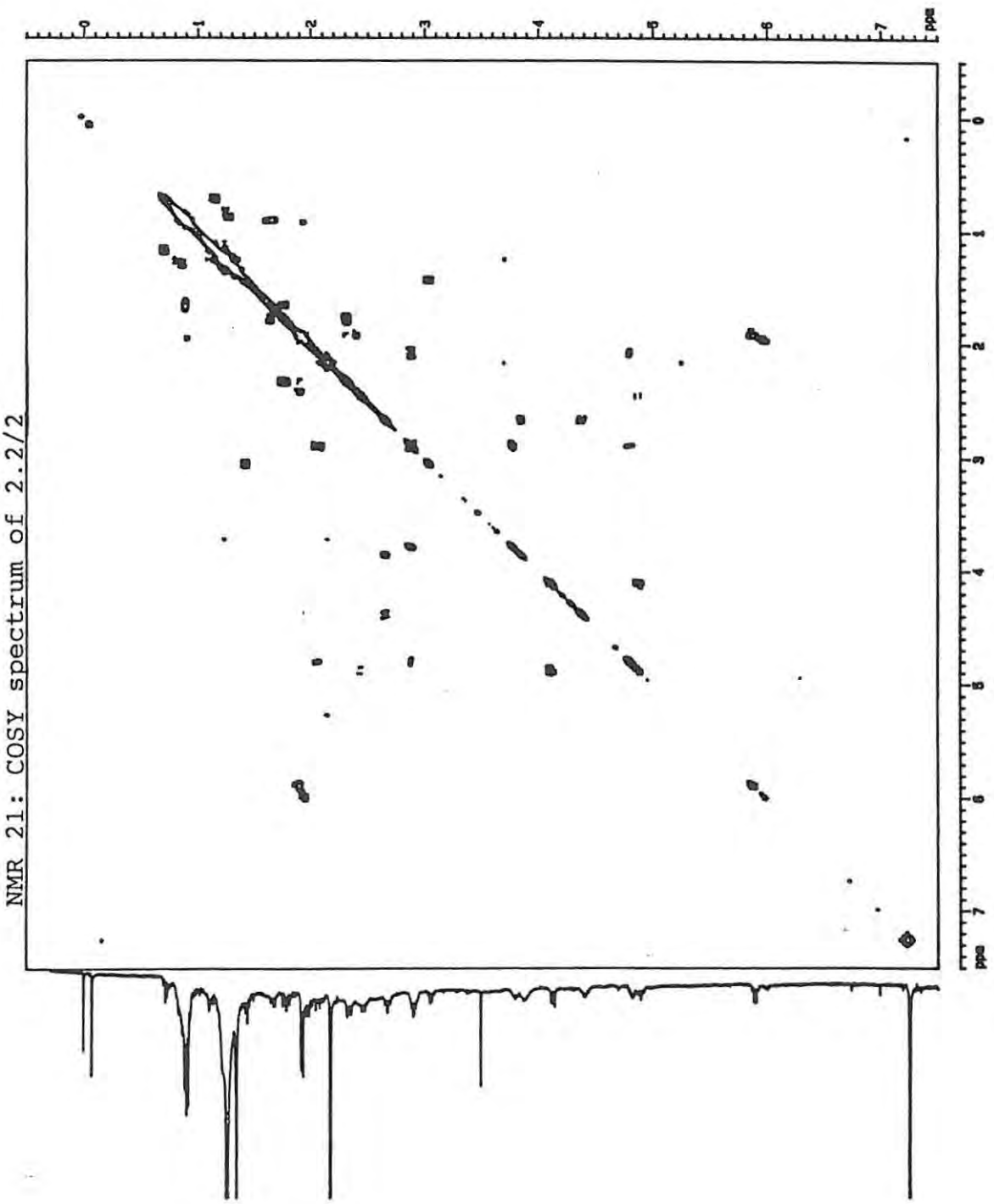
Analysis by GC-MS showed that a single major component was present. The retention time and mass spectrum were identical to those for retrorsine, thus confirming the presence of this alkaloid in the plant.

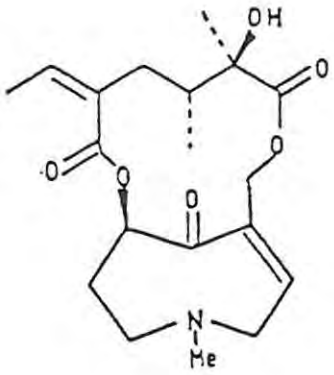
Component 2.2/2 was the minor alkaloid. The ^{1}H NMR spectrum suggested the presence of a saturated rosmarinecine-type base. The H-9 protons were once again separated by 0.8 ppm, a feature typical of twelve-membered macrocyclic diesters[65,141-2]. In the COSY spectrum[113,114], the H-9 proton signals couple to a peak at δ 2.42, which in turn is coupled to a peak at δ 4.38, identifying these protons as H-1 and H-2 respectively. The shift value for H-2 is typical of necines hydroxylated at C-2[89,105]. The rest of the ring protons can easily be assigned from the COSY coupling patterns. The signal for H-8 is missing, indicating an otonecine base, while the singlet at δ 2.13 is typical of the N-methyl group found in this class of compounds[143,144].

The structure of the necic acid follows logically from the COSY spectrum. A quartet at δ 5.85 coupled to a methyl group at δ 1.89 is typical of the vinyl proton H-20 found in alkaloids such as senkirkine (**172**) and rosmarinine (**135**)[4,105]. The shift values of the remaining signals and their connectivity are in good agreement with the necic acid found in both these alkaloids[4,105].

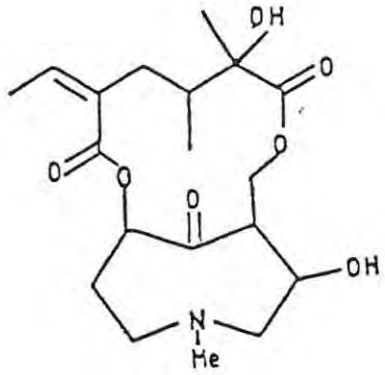
NMR 20: ^{1}H NMR spectrum of 2.2/2



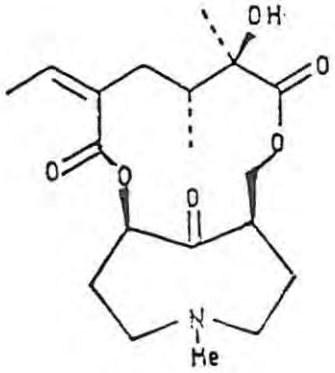

NMR 21: COSY spectrum of 2.2/2
ppm
0
1
2
3
4
5
6
7



172



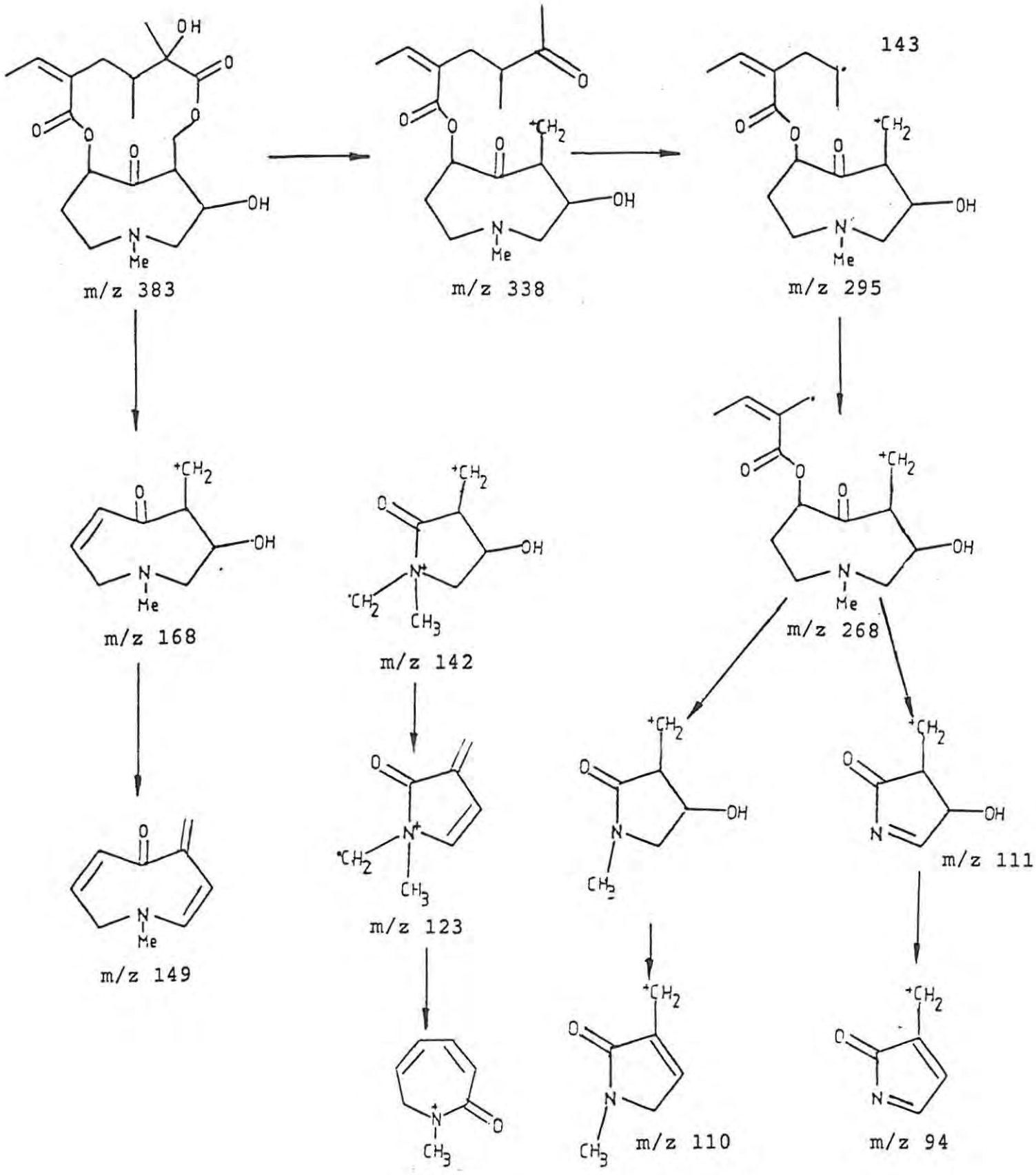
173



174

2.2/2 therefore appears to be 2-hydroxy-1,2-dihydrosenkirkine (**173**). Until recently only unsaturated otonecine-based alkaloids were known. The first saturated otonecine-based alkaloid, 1,2-dihydrosenkirkine (**174**), was isolated from ***Senecio integrifolius*** **var.Fauriri** early this year by Roeder and Liu[39]. No otonecine-based alkaloids with ring hydroxyl groups are known.

The ^{1}H NMR data observed for 2.2/2 agreed well with that reported for 1,2-dihydrosenkirkine[39] and for rosmarinine[105], indicating a similar stereochemistry to these two compounds. Unfortunately no useful information could be obtained from NOESY[113,117] or ROESY[113,137] experiments, probably due to the small quantity of material available. The mass spectrum of 2.2/2 exhibits fragment peaks characteristic of otonecine-based alkaloids[1,96], further supporting the proposed structure. Unlike 1,2-dihydrosenkirkine (**174**), 2.2/2 readily undergoes dehydration to form the corresponding unsaturated alkaloid which then fragments in a similar fashion to senkirkine[39,144]. This behaviour is typical of macronecine or rosmarinecine-based alkaloids[89,105]. A proposed fragmentation pathway is shown in Scheme 2.12. The IR spectrum of the compound showed a ketone carbonyl at ν 1705 cm^{-1} in addition to the usual ester carbonyl, which is typical of otonecine-based alkaloids[138,144]. A peak between 1550 and 1600 cm^{-1} is considered to be indicative of an interaction between the keto group at C-8 and the nitrogen of the base[140], although there are a number of other strong vibrations occuring in this region (e.g. nitro N=O and N-H bend).



Scheme 2.12 Proposed fragmentation for 2-hydroxy-1,2-dihydrosenkirkine

A medium intensity band at 1560 cm^{-1}, which could be due to the nitrogen-keto group interaction, is present[140]. Further confirmation of the structure should be obtainable from a ^{13}C spectrum, but unfortunately insufficient material was available. The data obtained, however, is consistent with the structure as proposed. 2-Hydroxy-1,2-dihydrosenkirkine (**173**) is a new alkaloid.

The new acyclic diester alkaloids 7-senecioyl-9-sarracinyl-heliotridine (**153**) and 7-angelyl-9-sarracinylheliotridine (**154**), the known macrocyclic diester retrorsine and the new alkaloid 2-hydroxy-1,2-dihydrosenkirkine were isolated from Population 2. The acyclic diesters could have been derived from ***S. speciosus***, while a number of the other alkaloids identified by GC-MS are identical to those observed in ***S. macrocephalus***. However, the two minor alkaloids isolated cannot be explained in terms of hybridization.

2.9 Alkaloid content of *Senecio speciosus/macrocephalus* Population 3

Plant population 3 was collected, dried and extracted as described in Section 3.2. The crude alkaloid content was found to be 0.05%. Analysis of the crude extract by GC-MS showed that very little alkaloid was actually present.

The first two components are monoesters of an unsaturated base alkaloid of molecular mass 237, exhibiting fragment peaks at m/z 121, 120, 119, 95 and 80[1,7,55,91]. The first component (T_R 20.0min) is a C-7 monoester and the second (T_R 20.3min) a C-9 monoester, as indicated by intense fragment peaks at m/z 138 and 137 respectively[55]. From their retention times and mass spectra, component 2 was identified as 9-angelylretronecine (**149**) while component 1 could be 7-angelylretronecine (**161**). Component 3 occurs at T_R 21.4min and has a molecular mass of 255. The mass spectrum of this component is identical to that of component 4 in Population 1 and could thus be helifoline (**162**)[89]. A fragmentation pathway is shown in Scheme 2.11. Component 4 (T_R 24.5min) has a molecular mass of 255 and exhibits a mass spectrum characteristic of a C-9 monoester of a saturated base alkaloid[1,7,55,91]. The mass spectrum is identical to that of component 3 from ***S. macrocephalus*** and component 2 from Population 2. A hydroxylated version of 9-angelyl-platynecine, such as 9-sarracinylplatynecine (**163**), is again suggested.

Component 5 (T_R 26.0min) has a molecular mass of 271 and its mass spectrum is identical to that of component 4 from

S. macrocephalus. This component is also found in Populations 1 and 2.

No diester alkaloids were observed in this plant.

The crude alkaloidal extract was subjected to DCCC and preparative TLC as described in Section 3.12, but no pure alkaloids could be isolated. Alkaloidal fractions obtained confirmed the results of GC-MS. The alkaloids tentatively identified in this way are shown in Table 2.10 and the GC trace is shown in Fig 2.12.

Table 2.10 Alkaloids identified in Population 3 by GC-MS

Component	T_R (min)	Important mass fragments	Possible Identity
1	20.0	237, 137, 108, 93, 83, 80	7-Angelyl-retronecine
2	20.3	237, 138, 121, 120, 119, 93, 83, 80	9-Angelyl-retronecine
3	21.4	255, 138, 121, 120, 119, 83, 80	Helifoline type
4	24.5	255, 238, 223, 140, 123, 122, 121, 108, 83, 80	9-Sarracinyl-platynecine type
5	26.0	271, 122, 121, 120, 109, 83, 82	9-Procerine type

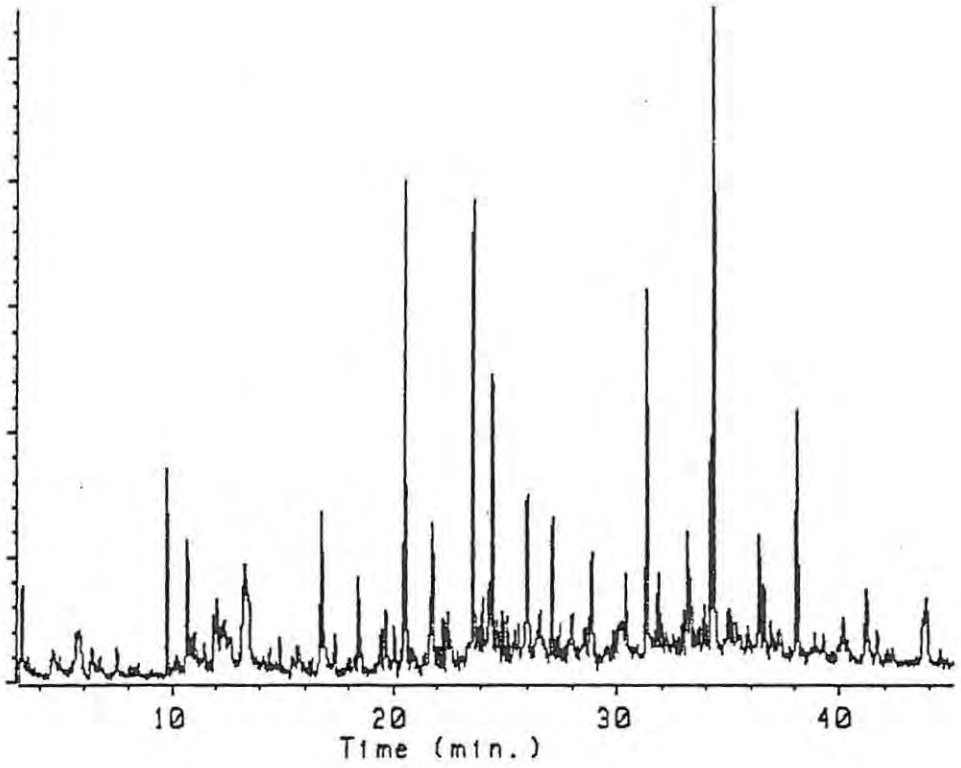


Fig 2.12 GC trace of crude extract from Population 3

2.10 Alkaloid content of *Senecio speciosus/macrocephalus* Population 4.

Population 4 was collected, dried and extracted as described in Section 3.2. The crude alkaloid content was found to be 0.055%. Analysis of the crude extract by GC-MS showed that the alkaloid fraction of this plant is very similar to that of population 2, except that no retrorsine appears to be present. Component 1 (T_R 20.0min) exhibits a fragmentation pattern typical of a C-7 monoester of an unsaturated base[1,7,55,91] and has molecular mass 237. This component is probably 7-angelylretronecine (**161**).

A second component at T_R 24.5min with mass 255 has a mass spectrum identical to that of component 3 from ***S. macrocephalus***. The fragmentation pattern is again consistent with the structure 9-sarracinylplatynecine (**163**) or an isomer.

Component 3 (T_R 26.0min, mass 271) once again appears identical to component 4 from ***S. macrocephalus***. Both components 2 and 3 are present in the crude extracts of populations 2 and 3.

A group of acyclic diesters similar to those observed in ***S. speciosus*** and populations 1 and 2 is also present. Retention times are 29.6min, 29.8min and 30.1min respectively and the mass spectra are identical to those of the corresponding diesters from the other plants. Also present are the two acyclic diesters (T_R 32.8min and 33.2min respectively) observed in the crude extracts of populations 1 and 2. No

molecular ions could be discerned, even using CIMS. The mass spectra once again exhibit fragment peaks at m/z 220, 237 and 235, indicating the presence of angelic and sarracinic acids[105]. A summary of these alkaloids appears in Table 2.11 and the GC trace is shown in Fig 2.13.

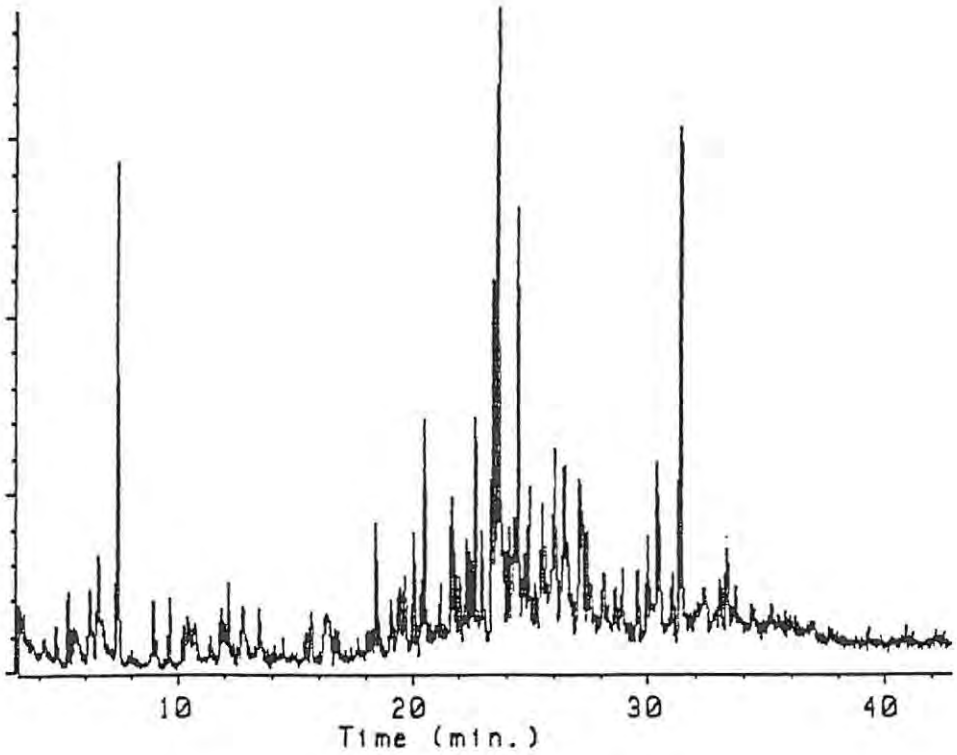


Fig 2.13 GC trace of crude extract from Population 4

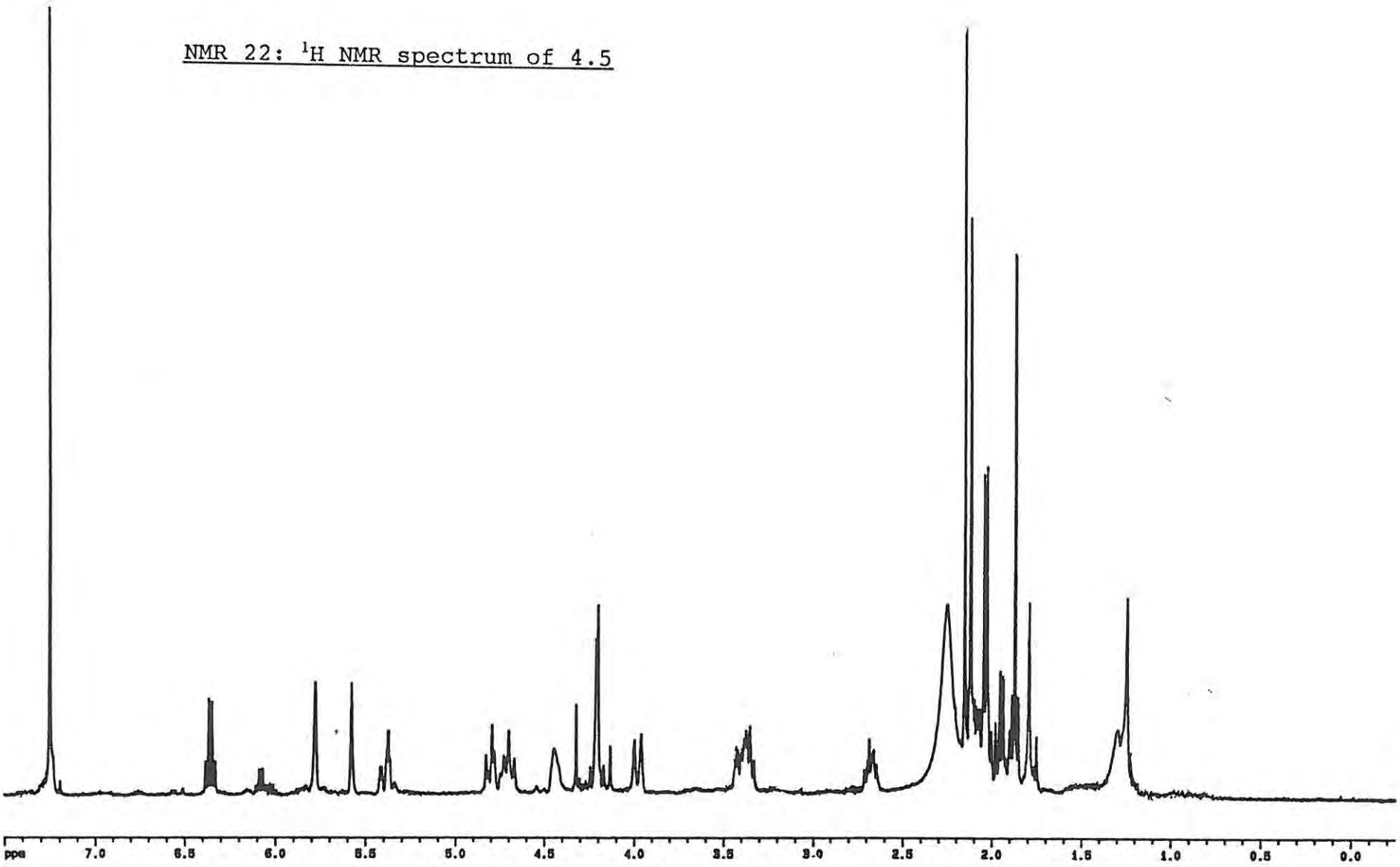
Table 2.11 Alkaloids identified in Population 4 by GC-MS

Component	T_R (min)	Important mass fragments	Possible Identity
1	20.0	237, 137, 93, 93, 80	7-Angelyl-retronecine
2	24.5	255, 238, 223, 140, 108, 83, 80, 55	9-Sarracinyl-platynecine type
3	26.0	271, 122, 121, 120, 119, 109, 95, 82, 80, 55	9-Procerine type
4	29.6	335, 237, 219, 136, 121, 120, 119, 93, 83, 80, 55	Triangularine type
5	29.8	335, 237, 219, 136, 121, 120, 119, 93, 83, 80, 55	Triangularine type
6	30.1	335, 237, 219, 136, 121, 120, 119, 93, 83, 80, 55	Triangularine type
7	32.8	237, 219, 136, 121, 120, 119, 83, 80	Triangularine type (Isomer of above?)
8	33.2	237, 219, 136, 121, 120, 119, 83, 80	Triangularine type (Isomer of above?)

The crude alkaloidal extract was subjected to DCCC and a single alkaloidal fraction (4.5) isolated. This appeared pure by TLC. The ^{1}H NMR spectrum of 4.5 is almost identical to that of 1.2/6. The COSY[113,114] coupling patterns indicate that the major component is again 7-senecioyl-9-sarracinyl-heliotridine (**153**) and the minor component 7-angelyl-9-sarracinylheliotridine (**154**). A ROESY spectrum[113,137] confirmed that the stereochemistry of the two alkaloids is the same at that of S5 and S8.

Analysis of 4.5 by GC-MS indicated the presence of two components (T_R 29.8min and 30.1min respectively), both of which exhibit the characteristic fragmentation pattern for an acyclic diester of an unsaturated base[7,55,91]. The mass spectra are identical to those observed for 7-senecioyl-9-sarracinyl-heliotridine and 7-angelyl-9-sarracinyl-heliotridine, isolated from ***Senecio speciosus***, Population 1 and Population 2.

NMR 22: ^{1}H NMR spectrum of 4.5



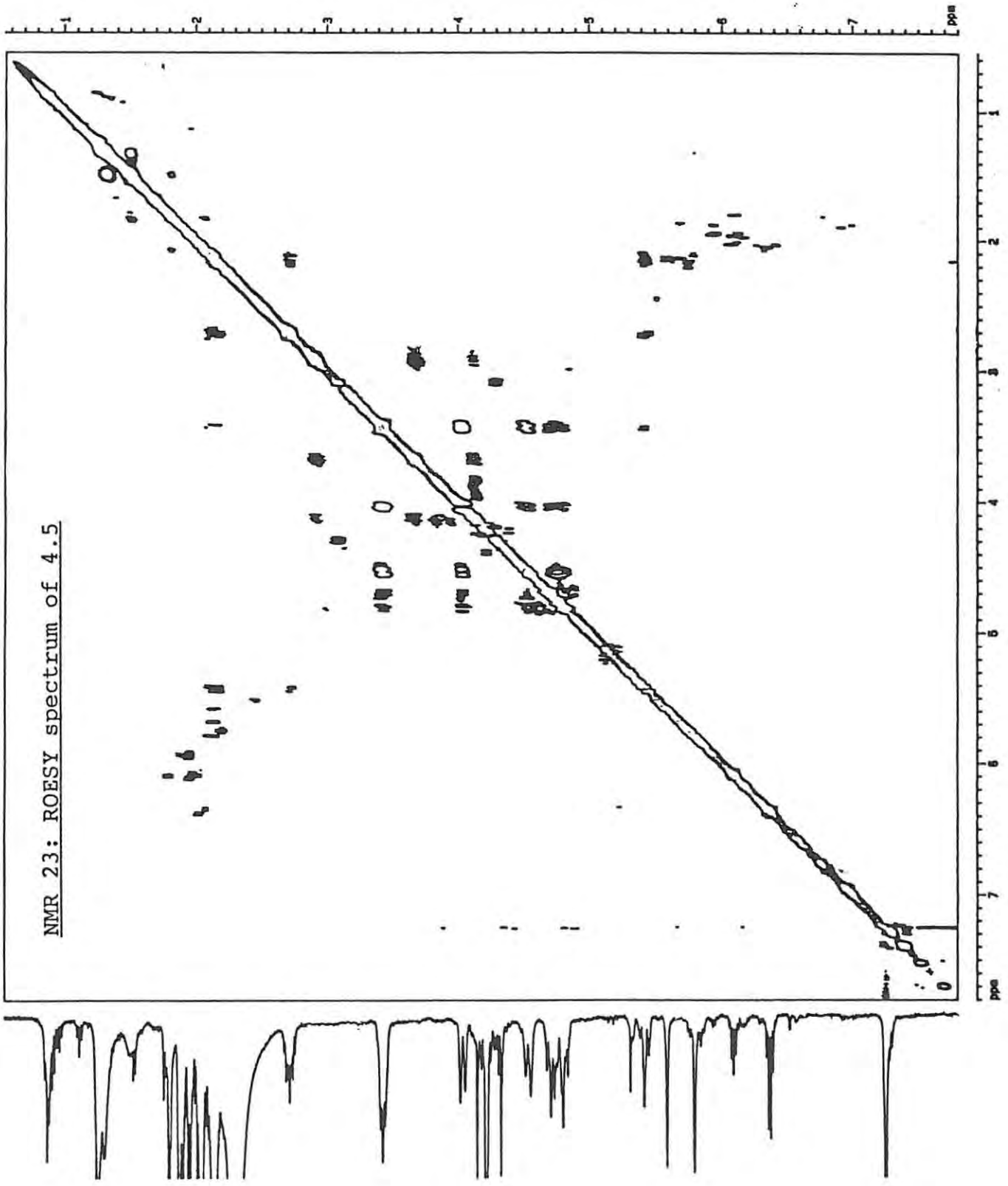

NMR 23: ROESY spectrum of 4.5
ppm
ppm

Table 2.12 ^{1}H NMR data for two alkaloids in Fraction 4.5

#H	7-Senecioyl-9-sarracinyl-heliotridine	7-Angelyl-9-sarracinyl-heliotridine
2	5.80 (s)	5.80
3d	3.98 (d)	3.98
3u	3.40 (m)	3.40
5d	3.38 (m)	3.38
5u	2.65 (m)	2.65
6	1.98-2.12 (m)	1.98-2.12
7	5.42 (br s)	5.39
8	4.43 (br s)	4.43
9d	4.82 (d)	4.81
9u	4.72 (d)	4.70
13	6.38 (q)	6.38
14	2.05 (d)	2.05
15	4.20 (d)	4.20
17	5.58 (s)	-
18	-	6.09
19	2.05 (s)	1.80
20	1.85 (s)	1.95

δ in ppm

Coupling constants were not measured due to signal overlap from the two alkaloids present.

2.11 Conclusions

The primary aim of this research was to study the alkaloid content of two previously uninvestigated plant species belonging to the genus ***Senecio***. ***Senecio speciosus*** **Willd** was found to contain pyrrolizidine alkaloids in appreciable quantity and two new compounds were isolated, *viz.* 7-senecioyl-9-sarracinylheliotridine (**153**) and 7-isosarracinyl-9-sarracinylheliotridine (**160**). A number of other pyrrolizidines were tentatively identified by GC-MS. The alkaloid content of ***Senecio macrocephalus*** **DC** was very low and no pure compounds were isolated. Investigation of the crude alkaloid extract by GC-MS, however, revealed the presence of a number of pyrrolizidines. Most of the alkaloids identified from the two plants exhibit structural features considered to impart cytotoxicity in a pyrrolizidine; *viz.* an unsaturated base ring, esterification of hydroxyl groups attached to the ring and branching of the acid moiety[1]. The alkaloids of ***Senecio speciosus*** are less toxic than those of ***Senecio macrocephalus***, being largely acyclic diesters with α,ß-unsaturated acid moieties. Macrocyclic diesters are considered to be the most toxic of the pyrrolizidines[1]. It is highly unlikely, however, that these plants would pose a threat to livestock since their total alkaloid content is very low.

Also studied were four populations of plants from the Grahamstown area which exhibit physical characteristics intermediate to those of ***S. speciosus*** and ***S. macrocephalus***.

Three of these plants yielded 7-senecioyl-9-sarracinyl-heliotridine (**153**) and 7-angelyl-9-sarracinylheliotridine (**154**) as major alkaloids, indicating their close relationship to ***S. speciosus***. Other alkaloids, identified by GC-MS, could easily have been derived from ***S. macrocephalus***. Table 2.13 compares the alkaloids identified in the six plants by GC-MS, while a comparison of major alkaloids present (identified by NMR techniques) is given in Table 2.14.

It is important to note that no conclusions can be drawn at this stage concerning the possible taxonomic implications of these results, since such a small sample can hardly be considered statistically significant. In order to determine conclusively whether the Grahamstown plants are indeed hybrids of ***S. speciosus*** and ***S. macrocephalus***, it would be necessary to sample a far greater number of plant populations over a much wider geographical area. It is also difficult to establish whether the differences in alkaloid content exhibited by plant populations are in fact due to genetic influence or the influence of the environment on the plant[145,146]. Since alkaloids are considered to be produced as part of the plant's defence system, it is quite conceivable that a plant would produce more alkaloids if threatened by fire, grazing pressures, etc than if these dangers were absent[46,47,146,147].

This is a major problem which faces taxonomists; a hybrid plant population, once formed, does not remain static. At first, the plant exhibits characteristics of both parents, but gradually certain characters are selected in preference

Table 2.13. Comparison of the alkaloids identified by GC-MS in the six plant populations

Component T_R	Mass	S	1	2	3	4	M
20.0	237		+	+	+	+	+
20.3	237	+	+		+		
20.7	237	+	+				
21.4	255		+		+		
24.5	255			+	+	+	+
26.0	271			+	+	+	+
26.4	269						+
29.3	335	+	+	+		+	
29.6	335	+	+	+		+	
29.8	335	+	+	+		+	
30.1	335	+	+	+		+	
31.9	337						+
32.1	351			+			
32.8	?		+	+		+	
33.2	?		+	+		+	
33.3	353						+

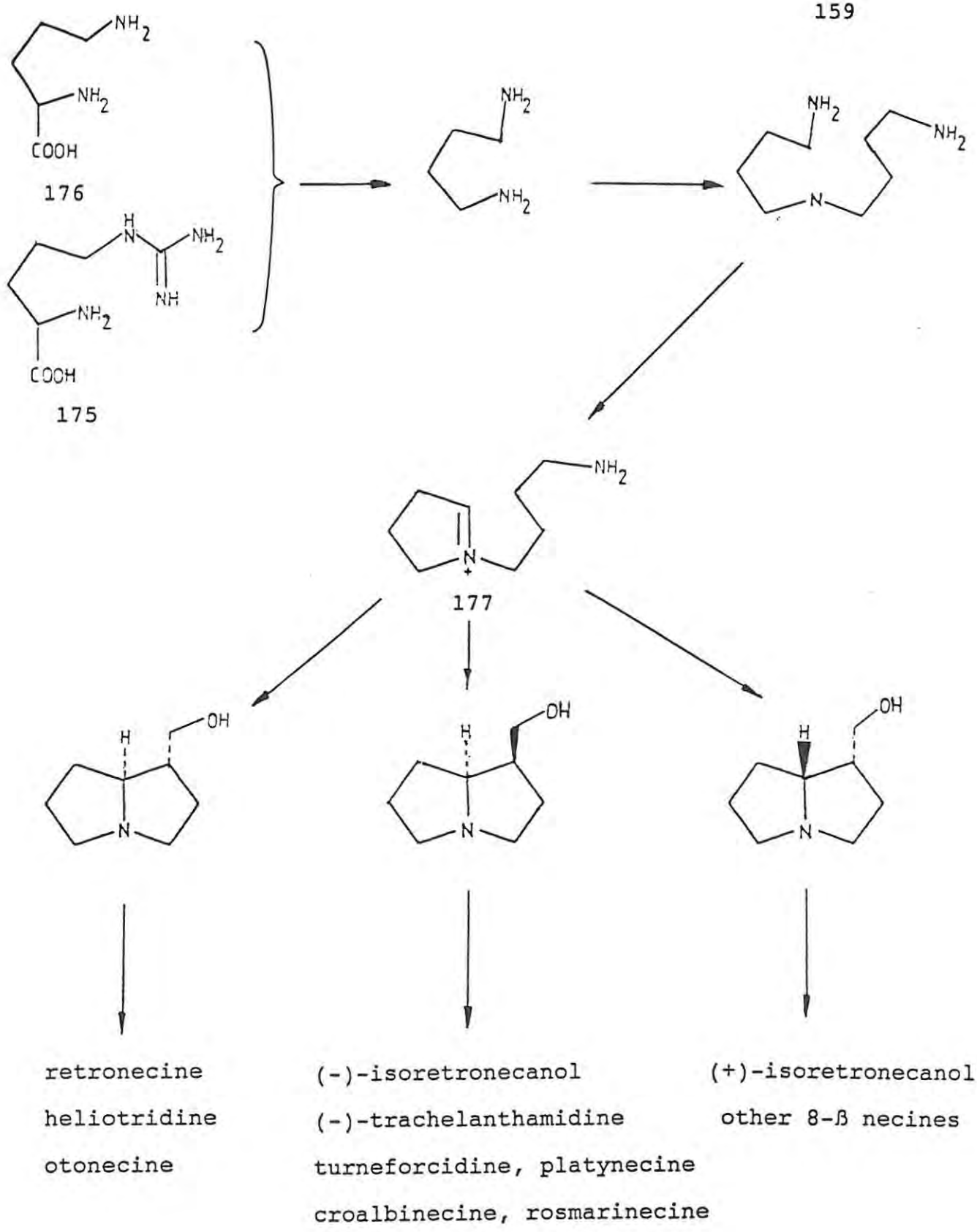
Table 2.14. Comparison of the major alkaloids identified in the six plant populations by NMR

Alkaloid	S	1	2	3	4	M
7-Senecioyl-9-sarracinyl-heliotridine	+	+	+		+	
7-Angelyl-9-sarracinyl-heliotridine	+	+	+		+	
7-Isosarracinyl-9-sarracinylheliotridine	+					
Retrorsine			+			
2-Hydroxy-1,2-dihydro-senkirkine			+			

S = *S. speciosus*
1 = Population 1
2 = Population 2
3 = Population 3
4 = Population 4
M = *S. macrocephalus*

to others[146]. The plant may find itself in a different environment to its parents, and may develop certain characteristics in order to cope[145,146]. Unless a study of genetic material can be made, there is no way of knowing whether the genetic capability to produce such characteristics was inherited from one, or both, of the parents, or whether genetic mutation has taken place. From the point of view of the botanist, therefore, plants should be grown from seed and raised in identical environments before any conclusive taxonomic evidence can be obtained. This process is both tedious and time-consuming and is out of the question in most chemical studies such as this one, where taxonomic concerns are usually secondary. Despite these difficulties, pyrrolizidine alkaloids have been used successfully as taxonomic markers in a number of studies[36,45,72,147-50].

This study can be regarded as a preliminary taxonomic survey and a few tentative conclusions can be drawn. From the comparisons shown in Tables 2.13 and 2.14, it would appear that the theory of hybridization is upheld. Most of the alkaloids identified could be derived from either ***S. speciosus*** or ***S. macrocephalus***. It is generally considered that the biosynthetic pathway whereby a compound is formed is of greater taxonomic importance than its mere presence in a plant[145,146]. The biosynthesis of necine bases has been studied by a number of authors and it is generally believed that all necines are produced from either L-ornithine (**175**) or L-arginine (**176**) *via* the pathway shown in Scheme 2.13[151-6]. After the formation of the iminium ion (**177**), as shown[157],



retronecine
heliotridine
otonecine

(-)-isoretronecanol
(-)-trachelanthamidine
turneforcidine, platynecine
croalbinecine, rosmarinecine

(+)-isoretronecanol
other 8-ß necines

Scheme 2.13 Biosynthesis of the necines

the biosynthesis diverges into three separate pathways[53,158]. On this basis, the appearance of the isomeric group of diester alkaloids identified in Populations 1, 2 and 4 by GC-MS (T_R 32.8min and 33.2min; shown in Table 2.13), which are not apparent in either parent plant, is not surprising. Likewise, the saturated monoester alkaloid (T_R 21.4min) occuring in Populations 1 and 3, could have been derived from ***S. macrocephalus***, which produces other saturated base alkaloids. It is the presence of the two minor alkaloids in Population 2 which is surprising. Otonecine (**89**) has recently been shown to be derived from retronecine (**119**)[159] and the co-occurrence of these two alkaloidal types is not uncommon[39,45,66,69,85,148,160]. However, this is only applicable to the traditional unsaturated otonecine. The biosynthesis of the newly discovered saturated otonecine-based alkaloid 1,2-dihydrosenkirkine (**174**)[39] is not yet understood. In addition, since the absolute stereochemistry of the major alkaloids of Population 2 is not known, it is quite possible that retrorsine (**118**), 2-hydroxy-1,2-dihydrosenkirkine (**173**) and the major acyclic diester alkaloids are produced *via* three separate biosynthetic pathways in this plant. Whether the capability to exercise these additional biosynthetic routes is inherited or has developed in response to the environment of the plant cannot be determined. The reason for the sudden production of retrorsine can be surmised by taking into account the environment of the plant[146]. Plant population 2 was collected on the grassy slopes of the commonage around Jameson Dam and is subject to grazing pressures. The other

plants were collected from reserves or sheltered areas. Retrorsine, as a macrocyclic diester, is potentially far more toxic than the acyclic diester alkaloids[1] and it is possibly produced in the Population 2 plants as a defence chemical[46,47,147]. Necine bases hydroxlated at C-2 are generally considered to be non-hepatotoxic[1], so that the presence of 2-hydroxy-1,2-dihydrosenkirkine remains a mystery. Unlike the more familiar unsaturated otonecine-based alkaloids, this alkaloid could not have formed from the unsaturated alkaloids in the plant[53,158,159]. In any event, the ***Senecio speciosus/macrocephalus*** plant complex appears to be an interesting one from both the chemical and botanical viewpoints and is worthy of further study.

CHAPTER THREE

EXPERIMENTAL

3. General methods

Plants were collected while flowering, air-dried and chopped in a hammer-mill before extraction. Extraction was carried out as described in each case. Chromatography of plant extracts was carried out on a droplet counter current chromatograph (Buchi 670) in the solvent systems indicated. Fine (230-400 mesh ASTM, Merck No. 9385) silica gel 60 and neutral alumina (Merck No.9660624) were also used for chromatography of alkaloids. Preparative TLC plates were prepared by coating glass plates (20 x 20 cm) with silica gel PF_{254} (Merck No.7747) to an approximate thickness of 2 mm. For sample quantities of less than 50 mg glass plates (20 x 5 cm) pre-coated with silica gel 60 F_{254}(Merck No. 5714) were used.

All chromatography and syntheses were monitored on 0.2 mm thick plastic backed silica gel 60 F_{254} plates (Merck No. 5735). Spray reagents used for visualization of alkaloids were Dragendorff's reagent, prepared according to the variation by Munier and Machebouef[161], and the chloranil-Ehrlich's spray reagent system developed by Mattocks[81,162-3]. Melting points (uncorrected) were determined on a Kofler block. Infra-red spectra were determined as KBr discs or in $CHCl_3$ solution on a Perkin Elmer 180 infra-red spectrophotometer.

Proton and ^{13}C NMR and two-dimensional NMR experiments were

determined on a Bruker 400 MHz instrument by Dr J R Liddell, Dr L A S Parolis and Prof P T Kaye at Rhodes University. $CDCl_3$ was used as the internal standard (δ=7.25ppm) and all spectra were recorded in $CDCl_3$ at 300K.

Gas chromatography experiments were performed on a HP 5890A gas chromatograph using nitrogen carrier gas and a flame ionisation detector under the conditions indicated. The column used was the HP Ultra 2 fused silica capillary column (0.2mm x 25m) packed with cross-linked 5% phenyl methyl silicone (film thickness 0.33μ). This capillary column was also used in the GC-MS experiments. These experiments involved the coupling of the capillary column in a HP 5890A gas chromatograph to a HP 5988A mass spectrometer. The carrier gas was helium (flow rate 30ml/min). Both 70 eV electron impact mass spectra and positive ion chemical ionization mass spectra (using methane as reactor gas; source pressure 0.85 Torr; source temperature 200°C) were determined.

3.1 Extraction procedure used for all plants

Air-dried plant material was chopped and soaked in methanol at room temperature for seven days. The methanol was run off and concentrated to approximately one-tenth of its volume. The solution was acidified with H_2SO_4 (2M), filtered and extracted three times with $CHCl_3$. The $CHCl_3$ extracts were discarded. The acidic aqueous phase was stirred with Zn dust for 6 hours, the Zn filtered off and the solution basified with concentrated NH_4OH to pH 8.5. The basic solution was

extracted six times with ethyl acetate, the ethyl acetate extracts combined and the solvent removed *in vacuo*. The residue obtained was acidified with aqueous H_2SO_4 (2M), extracted three times with $CHCl_3$, basified with concentrated NH_4OH and extracted six times with ethyl acetate. The ethyl acetate extracts were combined and the solvent removed *in vacuo* to give the crude alkaloid extract. The second acidification, extraction and basification step was necessary to ensure the complete removal of resins from the sample preliminary to GC analysis.

3.2 Extraction of *Senecio othonnaeflorus*

Senecio othonnaeflorus **DC** was collected by C G Logie on Mountain Drive near Grahamstown in January 1991 and identified at the Department of Botany, Rhodes University. Dried, chopped plant material (2.5kg) was extracted by the general procedure already described and a creamy crystalline residue (25g; 1%) was obtained. This was recrystallized from acetone to give retrorsine (**118**) as white crystals, mp 215°C (lit. mp 215 - 216°C[1]).

IR spectrum :3600 - 3550, 1750, 1725, 1650, 1350

^{1}H NMR spectrum :δ 6.20(1H;H-2;d; J=1.3Hz), 3.39(1H;H-3d;m; J=16Hz), 3.95(1H;H-3u;m), 2.54(1H;H-5d;m), 3.26(1H;H-5u;m), 2.14(1H;H-6d;m), 2.39(1H;H-6u;m), 5.01(1H;H-7;t; J=2.5Hz), 4.28(1H;H-8;s), 4.09(1H;H-9d;d; J=11.3Hz), 5.49(1H;H-9u;d; J=11.3Hz), 1.64(3H;H-13;m), 1.74(1H;H-14d;m),

2.20(1H;H-14u;m), 3.63(1H;H18d;d; J=11.3Hz), 3.75(1H;H-18u;d; J=11.2Hz), 0.85(3H;H-19;d; J=6.8Hz), 5.72(1H;H-20;q; J=7.5Hz), 1.85(3H;H-21;dd; J=7.2Hz; J=1.5Hz).

EIMS m/z (rel. int.): 351(M^+,3.5), 320(7), 246(19), 220(37), 138(45), 137(26), 136(100), 121(44), 120(97), 119(76), 95(43), 93(52), 80(10).

3.3 Extraction of *Senecio chrysocoma*

Senecio chrysocoma was collected in November 1987 by Dr J R Liddell on Mountain Drive near Grahamstown and a voucher specimen deposited at the Albany Museum (Voucher No. JRL 12). Dried chopped plant material (200g) was extracted by the general procedure already described and the crude alkaloidal extract was obtained as a yellow oil (600 mg; 0.3%). This extract was subjected to flash column chromatography[112] on silica gel with the solvent system $CHCl_3$: MeOH : NH_3 (85:13:2). Two fractions were obtained: C1 (260mg) and C2 (60mg).

C1 was found by GC-MS analysis to consist of two components and was subjected to vacuum-liquid chromatography (VLC)[74,75] on silica gel. Gradient elution was used, beginning with chloroform and increasing the percentage of methanol from 1% through to 10%. Two fractions were obtained: C3 (100mg) and C4 (145mg).

C3: yellow oil; 100mg (9-angelylplatynecine (**124**))

IR spectrum :3350, 1715, 1650, 1450, 1150

^{1}H NMR spectrum :δ 2.73(1H;H-1;m), 1.69-2.20(2H;H-2;m), 3.26(1H;H-3d;m), 2.85-2.95(1H;H-3u;m), 3.44(1H;H-5d;m), 2.85-2.95(1H;H-5u;m), 1.70-2.20(2H;H-6;m), 4.34(1H;H-7;br s), 3.52(1H;H-8;dd; J=12.8Hz; J=3.2Hz), 4.62(1H;H-9d;d; J=7.6Hz), 4.48(1H;H9-u;d; J=7.7Hz), 6.09(1H;H-13;dq; J=11,4Hz; J=2.1Hz), 1.99(3H;H-14;d; J=11.4Hz), 1.89(3H;H-15;d; J=2.1Hz).

^{13}C NMR spectrum :δ 40.9(C1), 29.1(C2), 55.4(C3), 53.9(C5), 37.5(C6), 72.2(C7), 70.3(C8), 64.6(C9), 168.2(C11), 127.9(C12), 137.9(C13), 20.6(C14), 15.8(C15).

EIMS: m/z (rel. int) :239(M^+,3), 221(5), 156(4), 140(6), 139(7), 138(4), 122(5), 96(27), 95(76), 83(12), 82(100), 55(26).

CIMS :240(100), 238(21), 221(6), 140(42).

C4: yellow oil; 145mg (7-angelylplatynecine (**123**))

IR spectrum :3350, 1710, 1650, 1430, 1150

^{1}H NMR spectrum :δ 2.63(1H;H-1;m), 1.86(1H;H-2d;m), 1.76(1H;H-2u;m), 3.18(1H;H-3d;m), 2.78(1H;H-3u;m), 3.29(1H;H-5d;m), 2.72(1H;H-5d;m), 2.06(2H;H-6;m), 5.34(1H;H-7;br s), 3.45(1H;H-8;dd; J=12Hz; J=3.3Hz), 3.64(2H;H-9;d; J=8.3Hz),

6.10(1H;H-13;dq; J=10.5Hz; J=1.9Hz
2.02(3H;H-14;d; J=10.2Hz), 1.89(3H;H-15;d;
J=1.9Hz).

^{13}C NMR :δ 44.1(C1), 29.0(C2), 55.7(C3), 53.7(C5),
spectrum 35.1(C6), 75.3(C7), 69.1(C8), 62.2(C9),
167.0(C11), 127.4(C12), 139.3(C13),
20.8(C14), 15.8(C15).

EIMS :239(M^+,1), 157(4), 156(14), 139(51),
m/z (rel. int.) 122(4), 120(1.5), 113(12), 96(3), 95(2),
83(23), 82(100), 55(20).

CIMS :240(100), 238(20), 221(7), 140(45).

C2: yellow oil, 60mg

IR spectrum :3600-3200, 1720, 1650, 1360, 1230

^{1}H NMR spectrum :δ 2.72(1H;H-1;m), 1.89(1H;H-2d;m),
1.82(1H;H-2u;m), 3.18(1H;H-3d;m),
2.82(1H;H-3u;m), 3.35(1H;H-5d;m),
2.72(1H;H-5u;m), 1.95-2.02(2H;H-6;m),
5.34(1H;H-7;br s), 3.64(1H;H-8;dd;
J=11.5Hz; J=3.5Hz), 4.38(1H;H-9d;d),
4.22(1H;H-9u;d; J=8.7Hz), 6.95(1H;H-13;q;
J=7.2Hz), 1.89(3H;H-14;d; J=7.1Hz),
4.34(1H;H-15;s), 6.12(1H;H-18;dq; J=7.2Hz;
J=1.4Hz), 1.98(3H;H-19;d; J=7.2Hz),
1.90(3H;H-20;d; J=1.5Hz).

^{13}C NMR :δ 40.2(C1), 28.8(C2), 55.1(C3), 53.6(C5),
spectrum 35.0(C6), 75.2(C7), 68.8(C8), 64.3(C9),
166.8(C11), 132.0(C12), 141.0(C13),

14.3(C14), 56.7(C15), 167.2(C16), 127.1(C17), 139.8(C18), 15.8(C19), 20.8(C20).

EIMS m/z (rel. int.) :337(M^+,0.2), 237(11), 222(9), 138(100), 123(15), 122(32), 96(26), 95(47), 83(28), 82(75), 55(43)

CIMS :338(100), 238(26), 237(17), 222(33), 140(10), 122(39)

GC-MS analysis was carried out using the following GC conditions: injector temperature 275°C; detector temperature 300°C; initial temperature 100°C (3 min); heating rate 8°C per minute; final temperature 275°C (25 min).

A second batch of ***Senecio chrysocoma*** was collected on Mountain Drive near Grahamstown in January 1991 and a specimen identified at the Albany Museum. Dried, chopped plant material (2.5kg) was extracted according to the general procedure and the crude alkaloid obtained as a brown gum (6.5 g; 0.26%).

1g of crude alkaloid was subjected to DCCC in the descending mode using the solvent system $CHCl_3$: C_6H_6 : MeOH : H_2O (5:5:7:2), flow rate 15 ml/hr. 15 ml fractions were collected and monitored by TLC on silica using the solvent system $CHCl_3$: MeOH : NH_3 (85:14:1). Fractions 12 - 32 were combined to give C5 (350mg) and fractions 45 - 63 were combined to give C6 (500mg).

C5 was subjected to VLC as for C1 and two fractions obtained: C7 (150mg) and C8 (175mg).

C6 was purified by repeated preparative TLC on silica gel using the solvent system $CHCl_3$: MeOH : NH_3 (85:14:1) to obtain a single fraction, C9 (35mg) as a yellow oil.

These fractions were stored for six months at 5°C. Their NMR analysis is discussed in Section 2.2.

3.4 Extraction of *Senecio pterephorus* DC

Senecio pterephorus **DC** was collected on Mountain Drive near Grahamstown in November 1987 by Dr J R Liddell and a voucher specimen deposited at the Albany Museum (Voucher No. JRL13). Dried, chopped plant material (200g) was extracted by the general procedure and 500 mg (0.25%) crude alkaloidal extract obtained. This extract appeared crystalline. The extract was examined by analytical TLC using the solvent system $CHCl_3$: MeOH: NH_3 (85:14:1).

GC-MS analysis was carried out as for the ***Senecio chrysocoma*** extract.

3.5 Synthesis of standards

3.5.1 Hydrolysis of retrorsine (**118**)[128]

Retrorsine (13g, 0.038mol) and $Ba(OH)_2.8H_2O$ (36g, 0.11mol) in 250ml water was refluxed for 15h. The solution was cooled, saturated with CO_2 by addition of dry ice and the $BaCO_3$ filtered off. The filtrate was concentrated to 50ml, basified with Na_2CO_3, saturated with NaCl and extracted continuously with $CHCl_3$ for seven days. The $CHCl_3$ extract was concentrated

in vacuo to give a solid residue which was recrystallized from acetone to give retronecine (**119**) (2.51 g; 43%), mp 120°C (lit. mp 120 - 121°C[1]).

IR spectrum :3600 - 3200, 2950, 1650, 1285

^{1}H NMR spectrum :δ 5.61(1H;H-2;s), 3.74(1H;H-3d;dd; J=13.8Hz, J=1.7Hz), 3.33(1H;H-3u;dd; J=10.4Hz, J=5.2Hz), 3.14(1H;H-5d;t; J=7.7Hz), 2.68(1H;H-5u;m), 1.79-1.93(2H;H-6;m), 4.07(1H;H-7;br s), 4.00(1H;H-8;d; J=11.3Hz), 4.22(2H;H-9;s).

EIMS :155(M^+,1), 111(23), 94(12), 93(10), 80(100)

m/z (rel.int.)

3.5.2 Synthesis of angelic acid (**127**)[129]

Tiglic acid (**128**) (Sigma No. T3131)(10g, 0.1mol) in 20ml anhydrous CCl_4 was added to Br_2 (16g, 0.1mol) and allowed to stand overnight. The solution was heated to reflux until the solution turned light orange (*ca* 2 hours). The solvent was removed *in vacuo* to leave a creamy crystalline residue which was recrystallized from hexane to give α,ß-dibromo-α-methylbutyric acid (18.52g; 72%) as creamy needles. Mp 82-83°C (lit. mp 82-88°C[129]). α,ß-dibromo-α-methylbutyric acid (16g, 0.062mol) was dissolved in 20ml methanol and *ca* 100g of a 25% solution of KOH in methanol added slowly with stirring. 5g anhydrous K_2CO_3 was added to suppress carboxylation. The temperature was increased to 55°C and maintained for 2 hours. CO_2 was bubbled through the solution to remove excess KOH. The solution was filtered and the precipitate washed with warm

methanol. The methanolic solutions were combined and the methanol removed *in vacuo*. The residue was dissolved in 20ml water and acidified to pH 3 with HCl (5M). Upon cooling the acidic solution in ice a white precipitate formed. This was filtered, dried and recrystallized from hexane to give long white needles of ß-bromoangelic acid (5.4g; 49%). Mp 90-91°C (lit mp 92-94°C[129]).

ß-bromoangelic acid (3g, 0.016mol) was dissolved in 25ml water and cooled to 5°C. A finely ground Na-Hg amalgam (66g of a 9% amalgam; 0.26 gram-atoms of Na) was added slowly in small pieces. The solution was stirred slowly at room temperature for 72 hours. The aqueous layer was separated from the Hg, which was washed with 10ml water. The combined aqueous solutions were acidified to pH 3 with concentrated HCl and the solution cooled in ice. A white precipitate formed. This was filtered, dried and recrystallized from hexane to give large colourless plates of angelic acid (**127**) (0.915g; 57%), mp 44°C (lit mp 44-46°C[129]).

The structure of the product was confirmed by IR and NMR spectroscopy.

IR spectrum :3300 - 2800, 1700, 1680, 1630, 1280

^{1}H NMR spectrum :δ 6.10(1H;alkene CH;dq; J=8Hz; J=1.2Hz), 1.98(3H;CH_3;d; J=8Hz), 1.75(3H;CH_3;d; J=1.2Hz).

3.5.3 9% Na-Hg amalgam[164]

Sodium (6.0g, 0.26mol) was placed in a 250ml round-bottomed three-necked flask which was flushed with N_2 and fitted with a dropping funnel. The funnel was charged with Hg (60g, 4.5ml). 2ml of Hg was added to the Na and the flask warmed with a free flame until the reaction started. The rest of the Hg was added slowly with heating and shaking. The molten amalgam was poured into a mortar, where it solidified immediately. The amalgam (66g) was ground finely in a N_2 atmosphere.

3.5.4 Synthesis of senecioic acid(**142**)[130,131,165]

375 ml of commercial acetone was shaken over anhydrous K_2CO_3 to dry it and then placed in a round-bottomed flask fitted with a Soxhlet and a double-surfaced condenser. A soxhlet thimble was charged with anhydrous $Ba(OH)_2$ and placed in the soxhlet. The acetone was distilled through the $Ba(OH)_2$ for 96 hours to give crude 4-methyl-4-hydroxypentan-2-one (**144**). The crude product (150ml) was distilled slowly over I_2 (0.05g) and three fractions collected:

1. Bp 56 - 80°C. Mainly acetone (30ml)
2. Bp 80 - 126°C. Mesityl oxide and water (55ml)
3. Bp 126 -131°C. Mesityl oxide (40ml)

Fraction 2 was separated, the mesityl oxide dried over anyhdrous K_2CO_3 and added to fraction 3 to give mesityl oxide (**143**) (85ml, 67.5g; 63%).

375g commercial $Ca(OCl)_2$ (HTH; 70% hypochlorite) was dissolved in 4l warm water. A warm solution of K_2CO_3 (262.5g) and KOH

(75g) in 750ml water was added. This mixture was shaken vigorously until the gel formed initially became liquid. The suspended solid was filtered off, washed with 300ml water and sucked dry. The solution, containing 300g (3.45mol) KOCl, was cooled to 10°C.

Mesityl oxide (75g, 0.77mol) in dioxane (150ml) was added to the cooled solution of KOCl and stirred. The mixture began to reflux within 5 minutes. Stirring was continued for 2 hours, with cooling when necessary (the reaction became quite vigorous at times). Excess hypochlorite was decomposed by the addition of sodium metabisulphite (*ca* 3g). 50% H_2SO_4 (*ca* 150ml) was added dropwise with stirring and cooling until pH 3. The cooled solution was then extracted with diethyl ether (6 x 500ml), the combined ethereal extracts combined, dried over anhydrous K_2CO_3 and the ether removed *in vacuo*. A dark liquid residue, from which white needles separated on cooling, was obtained. The residue was recrystallized from light petroleum (bp 40-60°C) to give long white needles of senecioic acid (**142**) (26.7g; 35%), mp 64-68°C (lit mp 68°C[130,131]).

IR spectrum :3600 - 2800, 1710, 1690, 1250, 1180

^{1}H NMR spectrum :δ 5.45(1H;alkene CH;br s), 1.92(3H;CH_3;s), 1.65(3H;CH_3;s).

3.5.5 Synthesis of monoesters of retronecine using N,N'-carbonyldi-imidazole. General procedure[115].

N,N'-carbonyldi-imidazole (**146**) (440mg, 2.72mmol) and the carboxylic acid (256mg, 2.56mmol) in 10ml dry, ethanol-free chloroform were stirred until CO_2 evolution stopped (about 30

minutes). Retronecine (400mg, 2.56mmol) was added and the reaction stirred under N_2 at room temperature for 48 hours. The solvent was removed *in vacuo* and the reaction products purified by preparative TLC on silica gel.

3.5.6 Reaction of tiglic acid with retronecine

Tiglic acid (**128**), CDI and retronecine (**119**) were reacted according to the general procedure. A brown gum was obtained which was subjected to preparative TLC on silica, first with the solvent system C_6H_6 : $CHCl_3$: $HN(Et)_2$ (7:2:1) and then with the solvent system $CHCl_3$: MeOH : NH_3 (85:14:1) to give an oil. This was dissolved in benzene and applied to a column of neutral alumina (d 5cm, h 4cm). Elution with benzene removed minor impurities. Elution with $CHCl_3$ gave a pale yellow oil which was identified as 9-tigloylretronecine (**147**) by ^1H NMR. Yield 235mg (39%).

IR spectrum :3600-3000, 1710, 1650, 1430, 1260

^1H NMR spectrum :δ 5.70(1H;H-2;s), 3.85(1H;H-3d;t; J=4.4Hz), 3.20(1H;H-3u;m), 3.65(1H;H-5d;d; J=17.8Hz), 3.20(1H;H-5u;m), 2.1-2.25(2H;H-6;m), 4.52(1H;H-7;br s), 4.38(1H;H-8;d; J=18.6Hz), 4.75(2H;H-9;d; J=13.3Hz), 6.88(1H;H-13;dq; J=9.2Hz; J=1.4Hz), 1.70(3H;H-15;d; J=1.5Hz), 1.85(3H;H-14;d; J=9.2Hz).

EIMS m/z (rel int) :237(M^+;0.1), 193(0.4), 154(6), 138(9), 137(15), 136(6), 94(45), 93(100), 80(33)

3.5.7 Reaction of senecioic acid with retronecine

Senecioic acid (**142**), CDI and retronecine were reacted according to the general procedure. A brown gum was obtained which was subjected to preparative TLC on silica, first with the solvent system C_6H_6 : $CHCl_3$: $HN(Et)_2$ (7:2:1) and then with the solvent system $CHCl_3$: MeOH : NH_3 (80:18:2) to give an oil. This was dissolved in benzene and applied to a column of neutral alumina (d 5cm, h 4cm). Elution with benzene gave minor impurities. Elution with $CHCl_3$ gave a pale yellow oil which was identified as 9-senecioylretronecine (**148**) by ^1H NMR spectroscopy. Yield 415mg (68%).

IR spectrum :3600-3000, 1720, 1640, 1250

^1H NMR spectrum :δ 5.65(1H;H-2;s), 3.55(1H;H-3d;s), 2.92(1H;H-3u;m), 3.43(1H;H-5d;s), 2.75(1H;H-5u;m), 2.03(2H;H-6;m), 4.12(1H;H-7;d; J=17.8Hz), 4.47(1H;H-8;d; J=27.6Hz), 4.59(2H;H-9;s), 5.62(1H;H-12;br s), 1.63(3H;H-14;s), 1.80(3H;H-15;s).

EIMS :237(M^+,0.1), 193(0.4), 154(8), 138(9),

m/z (rel int) 137(12), 136(6), 94(44), 93(100), 80(31).

3.5.8 Reaction of angelic acid with retronecine

Angelic acid (**127**), CDI and retronecine were reacted according to the general procedure. A brown gum was obtained which was subjected to preparative TLC on silica gel first with the solvent system C_6H_6 : $CHCl_3$: $HN(Et)_2$ (7:2:1) and then with the solvent system $CHCl_3$: MeOH : NH_3 (85:14:1) to give a yellow

oil. This was dissolved in benzene and applied to a column of neutral alumina (d 5cm, h 4cm). Elution with benzene removed minor impurities. Elution with $CHCl_3$ gave a pale yellow oil (223mg ;37%). ^1H NMR showed that this was predominantly 9-angelylretronecine (**149**). The presence of a small proportion of 9-tiglylretronecine (**147**) was indicated by the appearance of a quartet at δ 6.88. The ratio of the two isomers, calculated by comparison of the integrals of the H-13 quartets in the ^1H NMR spectrum, was 5:1, the major component being 9-angelylretronecine. The dual nature of the product was confirmed by GC, the ratio of components being calculated by comparison of the peak intensities in the GC trace as 5:1.

IR spectrum :3550-3300, 1715, 1660

^1H NMR spectrum :δ 5.80(1H;H-2;s), 3.85(1H;H-3d;d; J=13.3Hz), 3.35(1H;H-3u;dd; J=17.8Hz; J=2.7Hz), 3.30(1H;H-5d;m), 2.75(1H;H-5u;m), 1.90-2.01(2H;H-6;m), 4.28(1H;H-7;br s), 4.19(1H;H-8;br s), 4.76(2H;H-9;s), 6.10(1H;H-13;dq; 8Hz; J=2Hz), 1.85(3H;H-15;d; J=1.9Hz), 1.95(3H;H-14;d; J= 8.1Hz).

EIMS :237(M^+,0.1), 193(0.5), 154(9), 138(11),

m/z (rel int) 137(15), 136(7), 94(42), 93(100), 80(35).

3.6 GC-MS of alkaloid standards

The alkaloid monoesters synthesized, retrorsine (**118**) obtained from ***Senecio othonnaeflorus*** **DC**, retronecine (**119**) and the alkaloids isolated from ***Senecio pterephorus*** **DC** by C G Logie[126]

were used as GC standards. All alkaloids were dissolved in spectroscopic grade methanol for analysis. The following GC conditions were used: injector temperature 275°C; detector temperature 300°C; initial temperature 120°C (3min); heating rate 6°C per minute; final temperature 275°C (25min). These conditions were also used for GC-MS analysis of crude alkaloid extracts and alkaloids isolated from the plants.
Retention times of these standards were calculated relative to methanol and are given in Table 2.3.

3.7 General chromatographic procedures used for *Senecio speciosus/macrocephalus* populations

All plants were collected as described in each case and extracted according to the general procedure. The crude alkaloidal extract was in each case first subjected to DCCC, mode as indicated. The solvent system used for DCCC was C_6H_6 : $CHCl_3$: MeOH : NH_3 (5:5:7:2) (Solvent 1), flow rate 15 ml/hr. 15 ml fractions were collected and monitored by TLC on silica using the solvent system $CHCl_3$: MeOH : NH_3 (85:14:1) (Solvent 2). Alkaloids were visualized using Dragendorff's spray reagent[161].
Fractions thus obtained were purified by preparative TLC on silica gel using solvent systems as follows:
Solvent 2 $CHCl_3$: MeOH : NH_3 (85:14:1)
Solvent 3 $CHCl_3$: C_6H_{12} : $HNEt_2$ (5:4:1)
Solvent 4 $CHCl_3$: $HNEt_2$ (9:1)

3.8 Extraction of *Senecio speciosus* Willd

***Senecio speciosus* Willd** was collected at the Gonubie Nature Reserve in May 1991 and a voucher specimen deposited at the Herbarium at Rhodes University Botany Department (Voucher No. MRG5). Dried, chopped plant material (1kg) was extracted by the general procedure already described and the crude alkaloidal extract obtained as a brown gum (3.2g; 0.32%). 0.2g of the crude extract was reserved for analysis by GC-MS. These results are discussed in Section 2.5.

3g of the crude extract was subjected to DCCC in the ascending mode in Solvent 1. Appropriate fractions were combined. Two fractions containing alkaloids were obtained: S1 (350mg) and S2 (92mg) respectively.

S1 was subjected to preparative TLC on silica gel, first in Solvent 2 and then with Solvent 3. Three alkaloidal fractions were obtained: S3 (2mg), S5 (2mg) and S6 (5mg). All gave a single spot on TLC in a variety of solvent systems.

S2 was subjected to preparative TLC on silica gel in Solvent 1 and two fractions were obtained: S7 (5mg) and S8 (5mg). The response of S7 to Dragendorff's reagent was very poor. S8 showed faint residual impurities on TLC but due to the small quantity of material could not be purified further.

All purified alkaloids were analysed by GC-MS, and ^{1}H NMR spectroscopy. These results are discussed in Section 2.5.

S5: yellow oil, 2mg.

GC-MS showed that S5 contains a major component with two minor components, all with a mass of 335. Retention times are 29.7min (86%), 29.9min (7%) and 30.1min (7%). S5 is thus

essentially pure and was identified as 7-senecioyl-9-sarracinylheliotridine (**153**) by ^{1}H NMR spectroscopy and COSY experiments (See Section 2.5).

IR spectrum :3600-3450, 1720, 1660

^{1}H NMR spectrum :δ 5.78(1H;H-2;s), 3.98(1H;H-3d;d; J=15.4Hz), 3.42(1H;H-3u;m), 3.37(1H;H-5d;m), 2.68(1H;H-5u;m), 2.01-2.20(2H;H-6;m), 5.38(1H;H-7;t; J=0.9Hz), 4.47(1H;H-8;br s), 4.82(1H;H-9d;d; 13.3Hz), 4.68(1H;H-9u;d; J=13.4Hz), 6.37(1H;H-13;q; 7.3Hz), 2.05(3H;H-14;d; J=7.3Hz), 4.20(2H;H-15;d; J=4.5Hz), 5.59(1H;H-17;s), 2.10(3H;H-19;s), 1.85(3H;H-20;s).

EIMS :335(M^+,0.8), 237(15), 235(1), 220(8),

m/z (rel int) 219(4), 136(56), 120(34), 119(30), 94(100), 93(75), 83(91), 80(43).

S8: yellow oil, 2mg.

GC-MS showed that S8 contains one major and one minor component, both with mass 335. Retention times are 29.7min (82%) and 30.1min (18%).

^{1}H NMR and COSY studies indicate that the major component in S8 is in fact 7-senecioyl-9-sarracinylheliotridine (**153**), the ^{1}H NMR spectrum being identical to that of S5 (See Section 2.5).

IR spectrum :3600-3400, 1720, 1660

^{1}H NMR spectrum :δ 5.78(1H;H-2;s), 4.00(1H;H-3d;d); J=15.4Hz), 3.42(1H;H-3u;m), 3.39(1H;H-5d;m), 2.69(1H;H-5u;m), 2.02-2.15(2H;H-6;m), 5.38(1H;H-7;t J=0.9Hz), 4.52(1H;H-8;br s), 4.82(1H;H-9d;d; 13.5Hz), 4.68(1H;H-9u;d; J=13.3Hz), 6.38(1H;H-13;q; J=7.3Hz), 2.05(3H;H-14;d; J=7.3Hz), 4.20(2H;H-15;d; J=5Hz), 5.59(1H;H-17;s), 2.10(3H;H-19;s), 1.85(3H;H-20;s).

EIMS :335(M^+,0.7), 237(16), 235(2), 220(9),

m/z (rel. int.) 219(3), 136(57), 120(37), 119(32), 94(100), 93(76), 83(90), 80(41).

S6: yellow oil, 5mg.

IR spectrum :3600-3100, 1715, 1650, 1255

^{1}H NMR spectra :δ 5.80(1H;H-2;s), 3.98(1H;H-3d;d; J=16Hz), 3.45(1H;H-3u;m), 3.37(1H;H-5d;m), 2.68(1H;H-5u;m), 1.95-2.10(2H;H-6;m), 5.38(1H;H-7;t; J=0.9Hz), 4.43(1H;H-8;br s), 4.75(2H;H-9;s), 6.38(1H;H-13;q; J=7.1Hz), 1.93(3H;H-14;d; J=7.1Hz), 4.20(2H;H-15;q; J=8.9Hz), 5.90(1H;H-18;s), 1.97(3H;H-20;s), 4.12(2H;H-21;s).

EIMS :351(M^+), 254(0.7), 253(0.1), 236(0.6), 235(0.1), 154(3), 136(48), 121(12), 120(27), 119(24), 94(77), 93(100), 80(29).

3.9 Extraction of *Senecio macrocephalus* DC

***Senecio macrocephalus* DC** was collected at Hogsback in January 1991 and a voucher specimen deposited at the Herbarium at Rhodes University (Voucher No. MRG4). 300g dried, chopped plant material was extracted according to the general procedure and 0.62g crude alkaloid (0.21%) obtained as a brown gum. A small amount was reserved for analysis by GC-MS.

The remaining crude extract was subjected to DCCC in the ascending mode in Solvent 1. Appropriate fractions were combined. A large amount of non-alkaloidal material was discarded; two fractions containing alkaloid were obtained: M1 (200mg) and M2 (100mg).

M1 was subjected to DCCC in the descending mode using Solvent 1. Appropriate fractions were combined. A single fraction containing alkaloid was obtained: M3 (30mg). The response of the fraction to Dragendorff's reagent was very faint. The fraction was also not pure by TLC but attempts to resolve it into individual components by preparative TLC in various solvent systems were unsuccessful.

M2 was subjected to preparative TLC on silica Solvent 2. Two fractions were obtained: M4 (28mg) and M5 (1mg). M5 appeared pure by TLC.

M4 was further purified by preparative TLC using Solvent 4. A single fraction was obtained: M6 (1mg).

Analysis of both M5 and M6 by GC-MS indicated that the level of alkaloid present was in fact very low and that neither component was pure. No useful information could be obtained from NMR spectroscopy.

3.10 Extraction of Senecio speciosus/macrocephalus

Population 1

The locations of local populations are indicated on the map in Fig 2.1 (page 53). Population 1 was collected below the Settlers' Monument near Grahamstown in December 1990 and a voucher specimen deposited at the Albany Museum (Voucher No. JRL 18). Dried, chopped plant material (2kg) was extracted according to the general procedure and 2.0g (0.1%) crude alkaloid obtained as a brown gum. A small amount of this was reserved for analysis by GC-MS.

The remaining crude extract was subjected to DCCC in the ascending mode using Solvent 1. Appropriate fractions were combined. Approximately 0.4g non-alkaloidal material was discarded. Two alkaloidal fractions were obtained: 1.1 (300mg) and 1.2 (220mg).

Fraction 1.1 was subjected to DCCC in the descending mode using Solvent 1. Appropriate fractions were combined. A single alkaloidal fraction was obtained (255mg), which was subjected to DCCC as for 1.1. Two alkaloidal fractions were obtained: 1.1/1 (100mg) and 1.1/2 (55mg).

1.1/1 was subjected to preparative TLC on silica in Solvent 2. A single alkaloidal fraction was obtained: 1.1/3 (21mg). This appeared to contain two alkaloids but could not be purified further.

1.1/2 was subjected to preparative TLC on silica as for 1.1/1. A single alkaloidal fraction was obtained: 1.1/4 (1mg). The response of this fraction to Dragendorff's reagent was poor.

Analysis of 1.1/3 by GC-MS indicated that the level of alkaloid was very low.

Fraction 1.2 was subjected to DCCC as for fraction 1.1. A single alkaloidal fraction was obtained: 1.2/1 (100mg), which was subjected to preparative TLC on silica using Solvent 2. Three fractions were obtained: 1.2/2 (18mg), 1.2/3 (21mg) and 1.2/4 (19mg). 1.2/3 could not be purified further.

1.2/2 was subjected to preparative TLC on silica using Solvent 3 to give a single alkaloidal component: 1.2/5 (1mg).

1.2/4 was subjected to preparative TLC as for 1.2/2 to give two alkaloidal components: 1.2/6 (1mg) and 1.2/7 (1mg). 1.2/5 and 1.2/6 appeared pure by TLC, while 1.2/7 appeared to contain an impurity, but due the low quantity of material could not be purified further.

1.2/3 (21mg); yellow oil.

Analysis of this component by GC-MS showed that it was very impure; however the major component (T_R 21.4min) appears to be a 9-monoester of a saturated base alkaloid.

EIMS :255(M^+), 239(0.3), 238(15), 223(22), 178(8), 122(12), 108(95), 95(12), 83(9), 82(10), 55(100).

1.2/5 (1mg); yellow oil

Analysis of this fraction by GC-MS showed that it contained two major and two minor components, all with molecular mass 335, with similar mass spectra. The retention times are 29.3min(3%), 29.6min(5%), 29.8(51%) and 30.1min(41%).

Analysis by NMR spectroscopy indicates the possibility that the two major components are 7-senecioyl-9-sarracinyl-heliotridine (**153**) and 7-angelyl-9-sarracinylheliotridine (**154**), while the minor components could be 7-angelyl-9-neosarracinylheliotridine (**156**) and 7-senecioyl-9-neosarracinylheliotridine (**155**). Analysis of this fraction is discussed in detail in Section 2.7 and ^{1}H NMR data for the two major components are given in Table 2.7.

The mass spectra of the components are as follows:

EIMS (29.3min) :335(M^+,1.6), 237(15), 236(4), 235(1.9),
m/z (rel. int.) 220(25), 219(11), 136(65), 121(24),
120(67), 119(32), 94(100), 93(70), 83(43),
80(37), 55(73).

EIMS (29.6min) :335(M^+,2), 237(17), 236(3), 235(3),
m/z (rel. int.) 220(16), 219(9), 136(68), 121(21),
120(62), 119(29), 94(100), 93(72), 83(39),
80(40), 55(74).

EIMS (29.8min) :335(M^+,1.3), 237(16), 236(2), 235(2.6),
m/z (rel. int.) 220(12), 219(8), 136(54), 121(20),
120(49), 119(29), 94(100), 93(69), 83(43),
80(32), 55(57).

EIMS (30.1min) :335(M^+,2), 237(22), 236(2.5), 235(3),
m/z (rel. int.) 220(11), 219(6), 136(66), 121(16),
120(40), 119(35), 94(100), 93(69), 83(72),
80(39), 55(50).

1.2/6 (1mg); yellow oil

Analysis of this fraction by GC-MS showed that it contained two major and one minor component, all with a mass of 335, with similar mass spectra. The retention times are 29.6min(5%), 29.8min(35%) and 30.1(60%).

Analysis by NMR spectroscopy indicates the possibility that the two major components are 7-senecioyl-9-sarracinyl-heliotridine (**153**) and 7-angelyl-9-sarracinylheliotridine (**154**), while the minor component could be 7-angelyl-9-neosarracinylheliotridine. Analysis of this fraction is discussed in detail in Section 2.7 and ^{1}H NMR data for the two major components given in Table 2.7.

Mass spectra of the components are as follows:

EIMS (29.6min) :335(M^+,0.7), 237(0.5), 236(1.5), 235(1.4),
m/z (rel. int.) 220(11), 219(6), 136(36), 121(15),
120(31), 119(14), 94(100), 93(37), 83(27),
80(22), 55(38).

EIMS (29.8min) :335(M^+,1.7), 237(17), 236(2), 235(1.9),
m/z (rel. int.) 220(12), 219(4), 136(47), 121(17),
120(29), 119(21), 94(100), 93(48), 83(50),
80(26), 55(40).

EIMS (30.1min) :335(M^+,1.8), 237(23), 236(2), 235(3),
m/z (rel. int.) 220(10), 219(5), 136(55), 121(14),
120(33), 119(31), 94(100), 93(56), 83(57),
80(30), 55(38).

1.2/7 (1mg); yellow oil

Analysis of this component by GC-MS showed that it was a mixture of a number of compounds with very little alkaloid present.

3.11 Extraction of Senecio speciosus/macrocephalus Population 2

Population 2 was collected at Jameson Dam near Grahamstown in December 1989 and a voucher specimen deposited at the Herbarium, Rhodes University (Voucher No. MRG2). Dried, chopped plant material (1.5kg) was extracted according to the standard procedure to give the crude alkaloid extract as a brown gum (0.915g; 0.061%). A small quantity was reserved for analysis by GC-MS.

The remaining crude alkaloid was subjected to DCCC in the ascending mode using Solvent 1. Appropriate fractions were combined. Two apparently alkaloidal fractions were obtained: 2.1 (180mg) and 2.2 (200mg), although fraction 2.1 showed poor response to Dragendorff's reagent and chloranil - Ehrlich's reagent.

Fraction 2.2 was subjected to DCCC in the descending mode using Solvent 1. Three alkaloidal fractions were obtained: 2.2/1 (61mg), 2.2/2 (40mg) and 2.2/3 (30mg).

2.2/1 was purified by preparative TLC on silica gel Solvent 2 to give a single alkaloid 2.2/4 (1mg).

2.2/2 was subjected to preparative TLC on silica as for 2.2/1. Several fractions were obtained, all less than 1mg in quantity, but only one responsed to Dragendorff's reagent.

This was named 2.2/2.

2.2/3 was subjected to preparative TLC on silica as for 2.2/1. A single fraction was obtained (15mg) and was further purified by preparative TLC on silica using Solvent 3. A single alkaloid was obtained: 2.2/5 (2mg). This compound showed a single spot on TLC. The analysis of these fractions is discussed in Section 2.8.

2.2/2 (0.5mg), yellow oil

Analysis of this compound by GC-MS showed the presence of a single major component together with a small group of minor components. Analyis by NMR techniques gave surprising results; the compound appears to be 2-hydroxy-1,2-dihydrosenkirkine (**173**). This analysis is discussed in detail in Section 2.8.

IR spectrum :3600-3300, 1705, 1710, 1560

^{1}H NMR spectrum :δ 2.42(1H;H-1;m), 4.38(1H;H-2;m),
3.89(1H;H-3d;m), 2.65(1H;H-3u;t; J=9.5Hz),
3.78(1H;H-5d;m), 2.86(1H;H-5u;m),
2.86(1H;H-6d;m), 1.95(1H;H-6u;m),
4.79(1H;H-7;m), 4.89(1H;H-9d;m),
4.10(1H;H-9u;d; J=10.9Hz),
1.63(1H;H-13;m), 2.31(1H;H-14d;d; J=14Hz),
1.76(1H;H-14u;d; J= 13.8Hz),
1.33(3H;H-18;s), 0.88(3H;H-19;m),
5.85(1H;H-20;q; 6.8Hz), 1.89(3H;H-21;d;
J=7.1Hz), 2.13(3H;H-22;s).

EIMS :168(3), 156(4), 150(14), 149(14) 140(8),
136(29), 111(29), 109(23), 97(38), 95(37),

94(42), 83(100), 80(27), 58(60), 55(91).

2.2/4 (1mg), creamy crystals, mp 212-214 °C.

GC-MS showed that this component was largely pure, with a major component at T_R 32.07 min.

IR spectrum :3600-3400, 1750, 1725, 1650

^{1}H NMR spectrum :δ 6.20(1H;H-2;s), 3.94(1H;H-3d;d; J=16Hz), 3.43(1H;H-3u;m), 3.31(1H;H-5d;m), 2.54(1H;H-5u;m), 2.36(1H;H-6d;m), 2.15(1H;H-6u;m), 5.01(1H;H-7;t; 6Hz), 4.28(1H;H-8;m), 5.50(1H;H-9d;d; J=19.2Hz), 4.11(1H;H-9u;d; J=19.2Hz), 1.65 (1H;H-13;m), 2.21(1H;H-14d;m), 1.72(1H;H-14u;m), 3.74(1H;H-18d;d; J=11.3Hz), 3.62(1H;H-18u;d; J=11.3Hz), 0.91(3H;H-19;d; J=15Hz), 5.66(1H;H-20;dq; J=9Hz; J=1.5Hz), 1.80(3H;H-21;dd; J=9Hz; J=1.6Hz).

EIMS m/z (rel. int.) :351(M^+,3.5), 281(2.3), 220(10.7), 138(35), 137(12), 136(67), 121(41), 120(85), 119(57), 94(100), 93(76), 80(74).

2.2/5 (2mg), yellow oil

Analysis by GC-MS showed the presence of two components; T_R 29.8min(25%) and T_R 30.1min(75%), both with mass 335. Analysis by NMR spectroscopy indicates that these components are possibly 7-senecioyl-9-sarracinylheliotridine (**153**) and 7-angelyl-9-sarracinylheliotridine (**154**). Analysis is

discussed in detail in Section 2.8 and ^{1}H NMR data for the two components given in Table 2.9.

Mass spectra are as follows:

EIMS (29.8min) :335(M^+,1), 237(2), 236(16), 235(2),
m/z (rel. int.) 220(3), 219(13), 136(10), 119(20),
92(100), 91(77), 81(69).

EIMS (30.1min) :335(M^+,1.5), 237(3), 236(18), 235(2.5),
m/z (rel. int.) 220(2), 219(9), 136(10), 121(2), 120(18),
119(36), 92(100), 91(74), 81(75).

3.12 Extraction of Senecio speciosus/macrocephalus Population 3

Population 3 was collected at Faraway Farm near Grahamstown in January 1990 and a voucher specimen deposited at the Herbarium, Rhodes University (Voucher No. MRG3).

Dried, chopped plant material (1kg) was extracted according to the general procedure to give the crude alkaloidal extract as a brown gum (0.5g; 0.05%). A small quantity was reserved for analysis by GC-MS.

The remaining crude alkaloid was subjected to DCCC using Solvent 1. Appropriate fractions were combined. The response of the fractions to Dragendorff's reagent was extremely uncertain. A single fraction was obtained: 3.1 (100mg). This fraction was subjected to preparative TLC on silica gel using Solvent 4. One alkaloidal fraction was obtained: 3.2 (12mg), which was further purified by preparative TLC on silica using

Solvent 3. Two alkaloidal components were obtained: 3.3(2mg) and 3.4(1mg). Both components showed a faint response to Dragendorff's reagent but no useful information could be obtained using NMR spectroscopy.

3.13 Extraction of Senecio speciosus/ macrocephalus Population 4

Population 4 was collected on Mountain Drive near Grahamstown in March 1990 and a voucher specimen deposited at the Herbarium, Rhodes University (Voucher No. MRG1). Dried, chopped plant material (2kg) was extracted according to the general procedure and the crude alkaloid obtained as a brown gum (1.1g; 0.055%). A small quantity was reserved for analysis by GC-MS.

The remaining crude alkaloid was subjected to DCCC in the ascending mode using Solvent 1. Appropriate fractions were combined. Two apparently alkaloidal fractions were obtained: 4.1 (170mg) and 4.2 (105mg).

4.1 was subjected to preparative TLC on silica gel, first in Solvent 2 and then in Solvent 3. Two fractions which gave a positive response to Dragendorff's reagent were obtained; 4.3 (2mg) and 4.4 (1mg). An analysis by GC-MS, however, indicated that these fractions contained little or no alkaloid.

4.2 was subjected to DCCC in the descending mode using Solvent 1. A single alkaloidal component was obtained, which was further purified by preparative TLC on silica using Solvent 3 to give a single alkaloid: 4.5 (1mg). This component

appeared pure by TLC.

4.5 (1mg); yellow oil

Analysis of this fraction by GC-MS indicated that two components were present, in approximately equal quantity: T_R 29.8min(56%) and 30.1min(44%).

Analysis by NMR spectroscopy showed that these components are possibly 7-senecioyl-9-sarracinylheliotridine (**153**) and 7-angelyl-9-sarracinylheliotridine (**154**). ^{1}H NMR data for the two components are given in Table 2.12.

Mass spectra were as follows:

EIMS (29.8min) :335(M^+,2), 237(23), 236(2), 235(3),
m/z (rel. int.) 220(10), 219(5), 136(55), 121(14),
120(33), 119(31), 94(100), 93(56),
83(57), 80(30), 55(38).

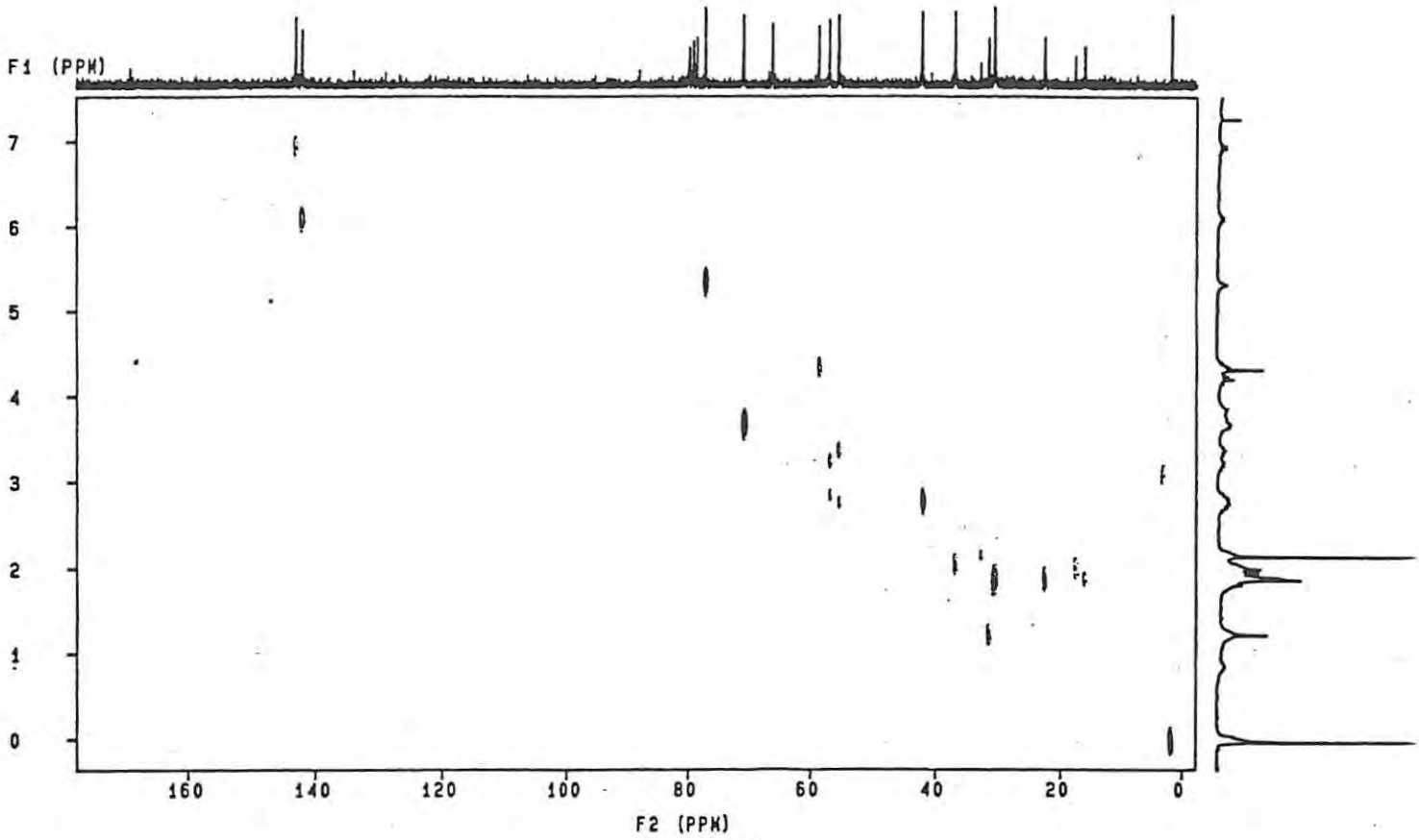
EIMS (30.1min) :335(M^+,1.6), 237(20), 236(2), 235(2),
m/z (rel. int.) 220(11), 219(4), 136(62), 121(15),
120(35), 119(29), 94(100), 93(62),
83(49), 80(35), 55(62).

APPENDIX I

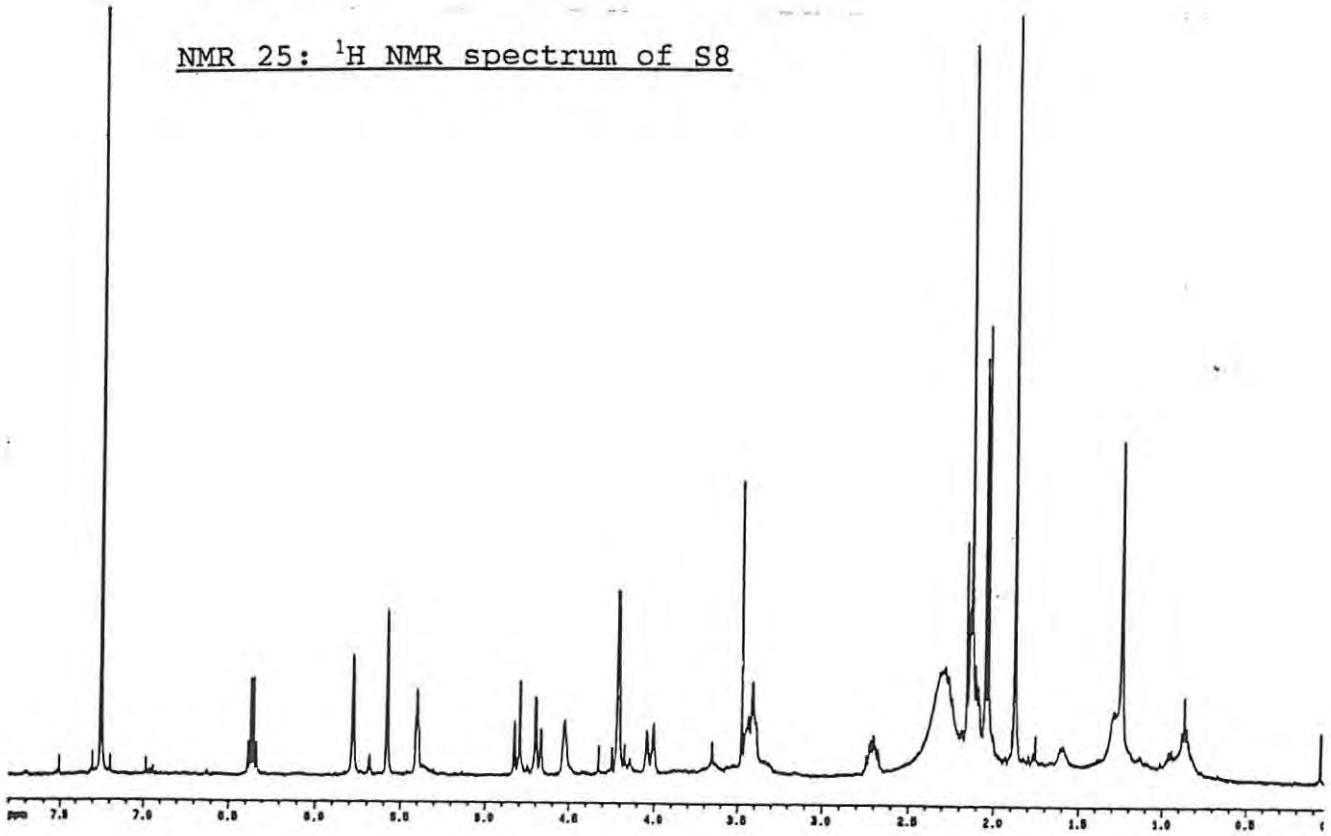
NMR SPECTRA

NOT INCLUDED IN TEXT

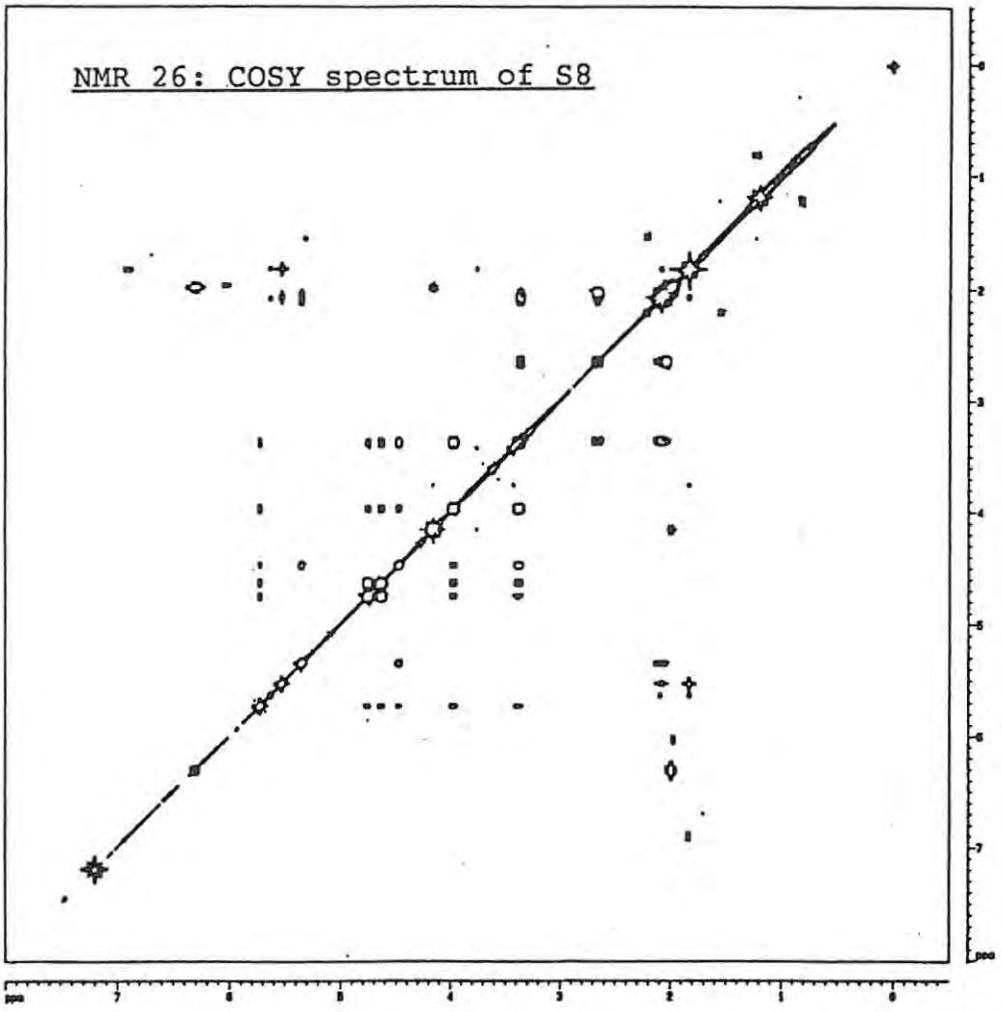
NMR 24: HETCOR spectrum of neosarracine



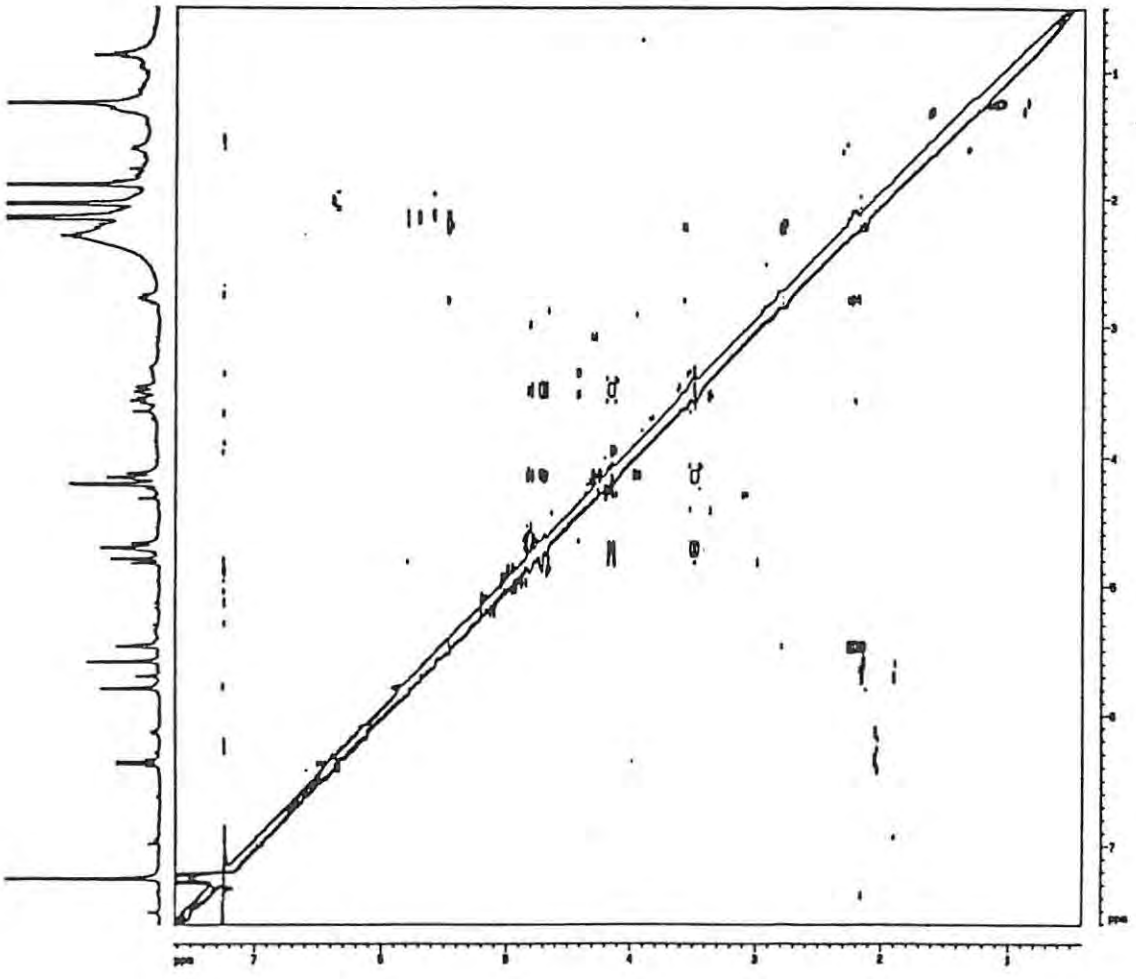
NMR 25: ^{1}H NMR spectrum of S8



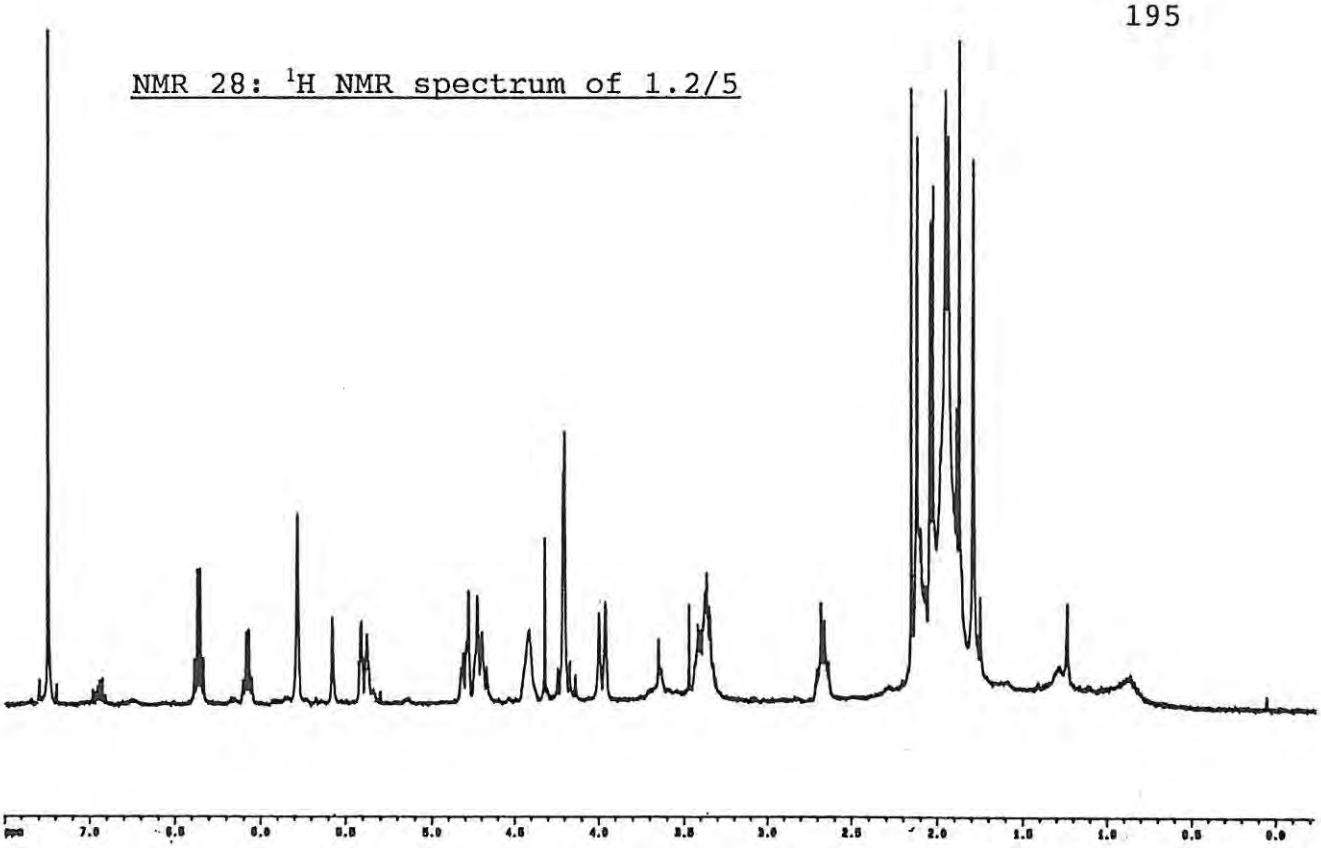
NMR 26: COSY spectrum of S8



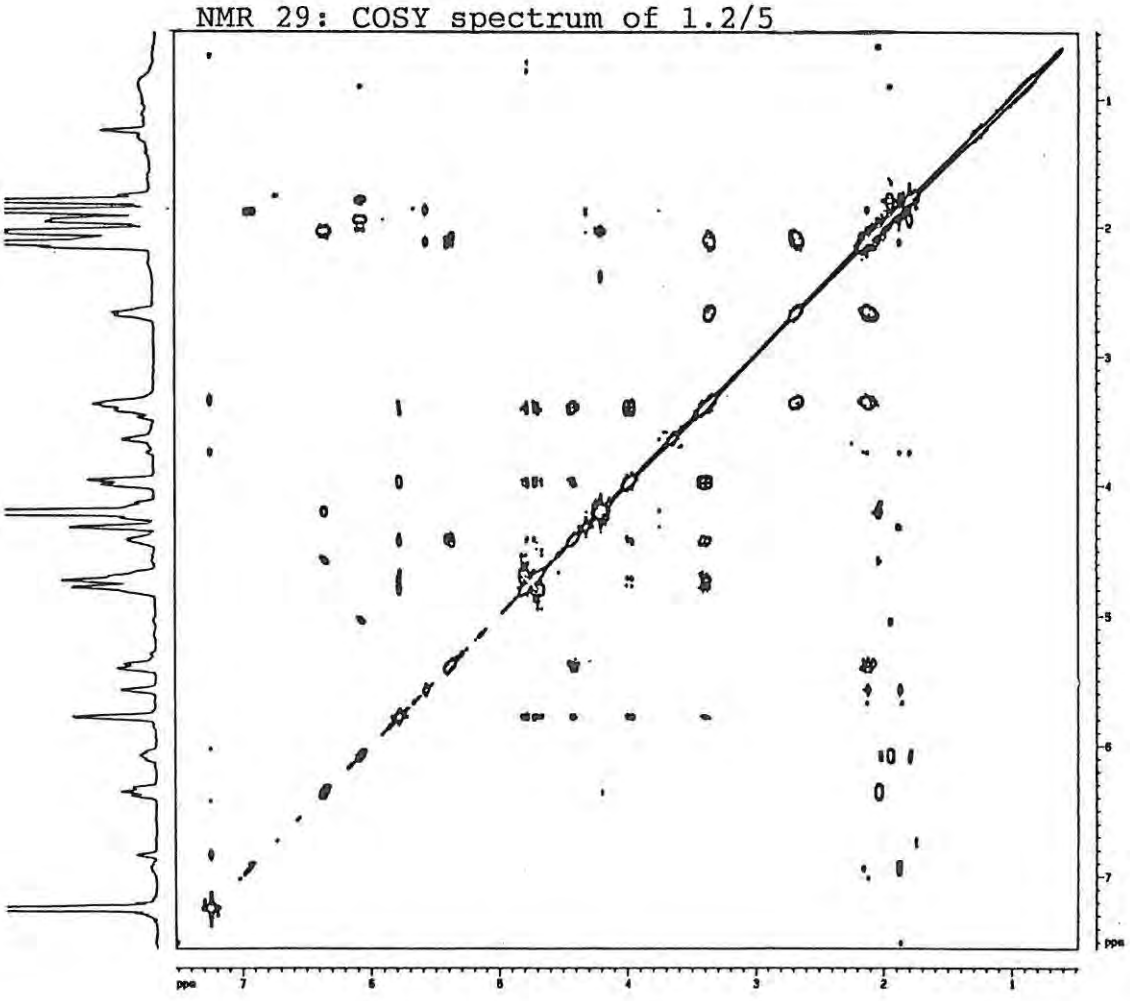
NMR 27: ROESY spectrum of S8



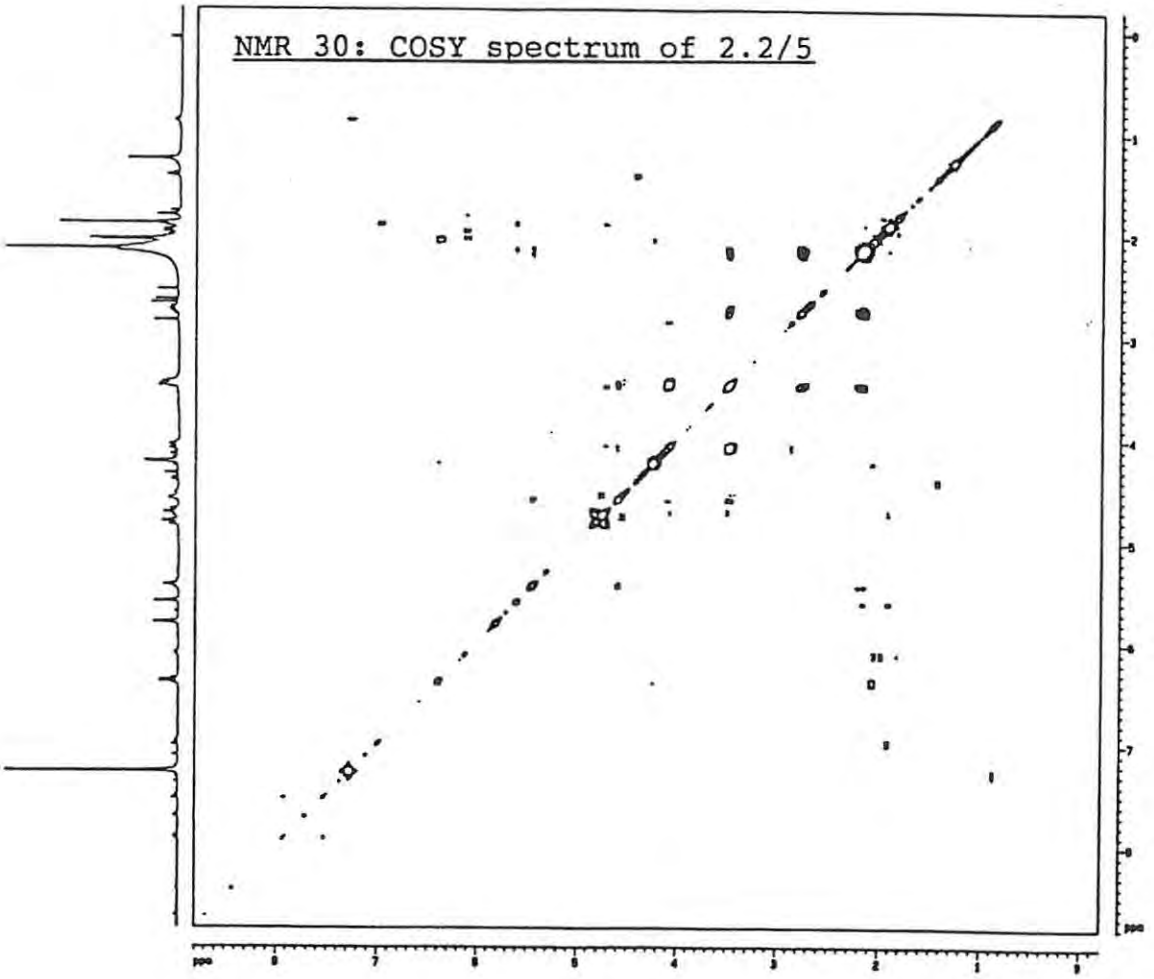
NMR 28: ^{1}H NMR spectrum of 1.2/5



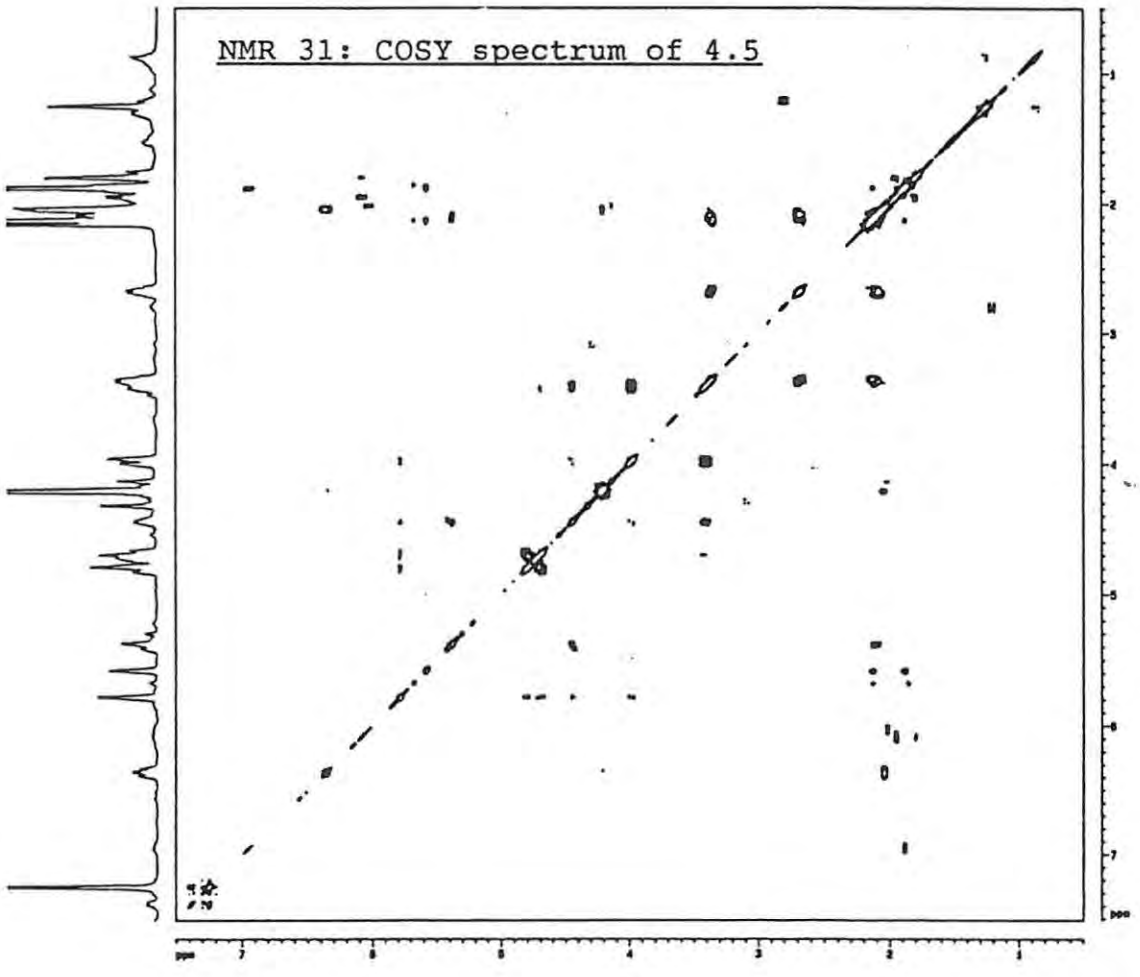
NMR 29: COSY spectrum of 1.2/5



NMR 30: COSY spectrum of 2.2/5



NMR 31: COSY spectrum of 4.5



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