

**THE PYRROLIZIDINE ALKALOIDS OF  
*SENECIO CHRYSOCOMA* AND *SENECIO PANICULATUS***

**THESIS**

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requirements for the Degree of  
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by

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*For a historian and a naturalist  
from a scientist*

*O! mickle is the powerful grace that lies  
In herbs, plants, stones, and their true qualities:  
For nought so vile that on earth doth live  
But to the earth some special good doth give,  
Within the infant rind of this weak flower  
Poison hath residence and medicine power:*

Romeo and Juliet, II, iii

## ABSTRACT

In order to compare the pyrrolizidine alkaloid content of two closely related species, *Senecio chrysocoma* and *S. paniculatus*, nine populations of plants, distributed between the two species, were examined. Three novel pyrrolizidine alkaloids, 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine, 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine and 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide, as well as eight known pyrrolizidine alkaloids, 7-angelylhastanecine, 9-angelylhastanecine, 7-angelylplatynecine, 9-angelylplatynecine, 9-angelylplatynecine-4-oxide, sarracine, neosarracine and retrorsine, were isolated and identified by NMR and GC-MS techniques. Traces of five tiglyl isomers, 9-tiglylplatynecine, 9-tiglylplatynecine-4-oxide, 7 $\beta$ -tiglyl-1-methylene-8 $\alpha$ -pyrrolizidine, sarranicine and neosarranicine, were also isolated and tentatively identified; however, these compounds could have been artefacts of the extraction and analytical procedures.

While both species of plant investigated, *S. chrysocoma* and *S. paniculatus*, were found to be morphologically different, their pyrrolizidine alkaloid content was, in fact, very similar. The presence of retrorsine in *S. paniculatus* plant extracts, but not in those from *S. chrysocoma* plants, was the only major chemical difference observed. It is perhaps significant that retrorsine was the only macrocyclic pyrrolizidine to be identified.

A comprehensive, computerised database of physical data for pyrrolizidine alkaloids has been compiled, which has facilitated the identification of new pyrrolizidines and the examination of trends in proton and carbon-13 NMR data for pyrrolizidine alkaloids.

A stereospecific synthesis of 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine was undertaken to confirm the absolute stereochemistry of the product isolated from *S. chrysocoma* and *S. paniculatus*. An inseparable 5:2 mixture of 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine and 7 $\beta$ -angelyl-1,2-didehydro-1-methyl-8 $\alpha$ -pyrrolizidine, together with a small amount of tiglyl isomer, was finally synthesised. The application of various chiral differentiating chromatographic and spectroscopic techniques confirmed that both the natural and synthetic products had the same stereochemistry, permitting the natural alkaloid to be identified as 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine.

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## LIST OF ABBREVIATIONS

Ac	acetyl
Bu	butyl
CAMELSPIN	cross-relaxation appropriate for minimolecules emulated by locked spins
CDI	<i>N,N'</i> -carbonyldiimidazole
CI	chemical ionisation
COLOC	carbon-detected correlation spectroscopy <i>via</i> long-range coupling
COSY	<sup>1</sup> H- <sup>1</sup> H correlation spectroscopy
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCCC	droplet counter current chromatography
DMAP	4,4-dimethylaminopyridine
EI	electron impact ionisation
Et	ethyl
GC-MS	gas chromatography-mass spectrometry
GI50	50% growth inhibition
GLC	gas liquid chromatography
HETCOR	heteroatom correlation
HIV	human immunodeficiency virus
HMBC	<sup>1</sup> H-detected heteronuclear multiple bond connectivity
HMQC	<sup>1</sup> H-detected heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
LC50	50% cytotoxicity
NBI	National Botanical Institute
NCI	National Cancer Institute
NICI	negative ion chemical ionisation
NMR	nuclear magnetic resonance
NOE	nuclear Överhauser effect
NOESY	nuclear Överhauser effect spectroscopy
Ph	phenyl
PICI	positive ion chemical ionisation
PLC	preparative layer chromatography
ROESY	rotating frame nuclear Överhauser effect spectroscopy
TGI	total growth inhibition
THF	tetrahydrofuran
TLC	thin layer chromatography

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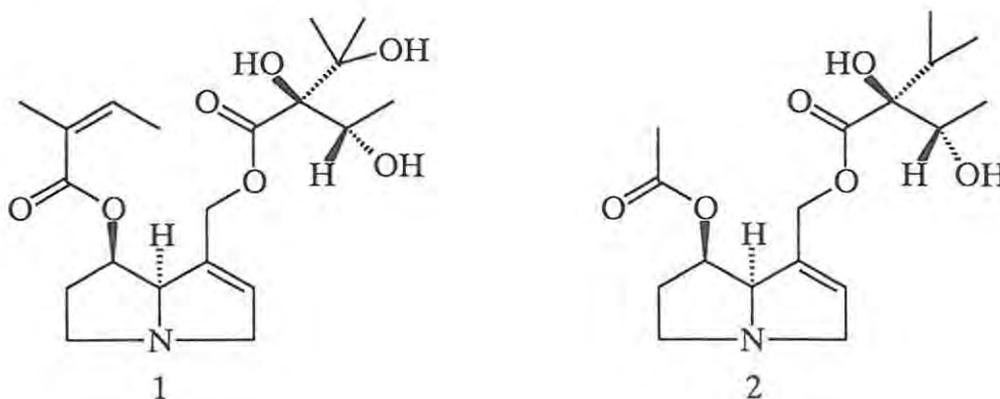
## 1. INTRODUCTION

*Senecio* species were recognised as toxic to livestock at the turn of the century.<sup>1-3</sup> The active metabolites, pyrrolizidine alkaloids, were only identified later. Since these earliest studies their toxicity has been of interest and pyrrolizidine alkaloids have been linked to a number of livestock diseases which are characterised by chronic hepatic lesions. Diseases, such as dunsiekte,<sup>4</sup> Molteno,<sup>4,5</sup> Pictou,<sup>5</sup> stomach staggers<sup>1</sup> and Winton,<sup>4,5</sup> are all forms of one disease, which is caused by a number of pyrrolizidine alkaloids.<sup>1,6</sup> The diseases have been reported in many countries and pyrrolizidine alkaloids are known to affect horses,<sup>7</sup> sheep,<sup>2,8</sup> goats,<sup>8</sup> cows,<sup>6,9,10</sup> pigs<sup>8</sup> and poultry.<sup>11</sup> Sheep, however, tend to be less susceptible to pyrrolizidine poisoning than cattle.<sup>10,12</sup> This is said to be due to different rumen microflora which, in sheep, can detoxify the pyrrolizidine alkaloids<sup>13</sup> and, possibly, to the presence of different hepatic pyrrolizidine alkaloid metabolising enzymes and to different absorption capabilities of the rumen wall.<sup>13</sup>

Pyrrolizidine alkaloid containing plants still account for large stock losses in many parts of the world, and it is estimated that stock loss, in the north western United States of America, as a result of such poisoning, costs millions of dollars annually.<sup>14</sup> The estimate for 1991 alone was \$20 million.<sup>9</sup> In Rio Grande do Sul *Senecio* species are the most important cause of premature death in adult cattle<sup>15</sup> and, in the Southern Australian autumn of 1993, *ca.* 150 thousand chickens and *ca.* 3000 pigs died in three months as a result of *Heliotropium europaeum* contaminated grain feed.<sup>11</sup> *H. europaeum* has been shown to contain pyrrolizidine alkaloids.<sup>16-20</sup> So great is the stock loss from pyrrolizidine alkaloids that, besides research into herbicides, biological control programmes are underway to control the growth of plants containing these alkaloids.<sup>21</sup>

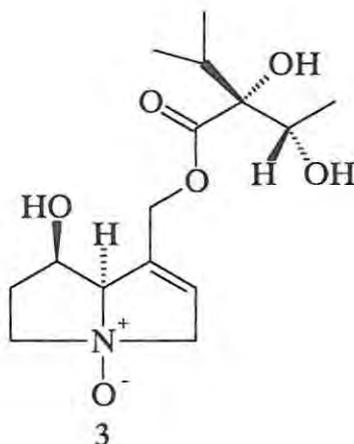
Hepatotoxic, teratogenic, mutagenic and carcinogenic effects of pyrrolizidine alkaloids have also been noted in humans. Large-scale human poisoning was first recognized in South Africa in the 1920's<sup>22</sup> and, subsequently, in Afghanistan,<sup>23</sup> India,<sup>24</sup> and the former

USSR.<sup>2</sup> Poisoning has usually resulted from the contamination of wheat, and thus bread, with pyrrolizidine alkaloid containing plant species.<sup>25</sup> However, there are now concerns about the presence of pyrrolizidine alkaloids in other food-stuffs and, in particular, in certain herbal remedies, bush teas, milk and honey.<sup>2</sup> People have died in Western Europe and the United States of America as a result of the consumption of medicinal herbs like *Senecio longilobus*.<sup>8</sup> Some countries have restricted the sale of Russian comfrey (*Symphytum X uplandicum*), which is used dried as a tea or fresh in salads, since the leaves contain 0.01-0.15% dry weight of hepatotoxic pyrrolizidine alkaloids (mainly echimidine 1 and lycopsamine-7-acetate 2).<sup>26</sup> Most of the Australian territories have introduced some form of noxious weed legislation to control the distribution of pyrrolizidine alkaloid containing plants.<sup>27</sup>



Although pyrrolizidine alkaloids are frequently toxic and carcinogenic, some have been shown to have antitumour properties. A number of pyrrolizidine alkaloids have been tested,<sup>28</sup> and are still being tested,<sup>29</sup> against a range of tumours. The only real success has been achieved with indicine-4-oxide 3 (see Section 1.4.2, p. 33).<sup>30</sup>

The presence of pyrrolizidine alkaloids in a wide diversity of plant families (see Section 1.1, p. 3) and their toxic effects on both man and livestock make them of considerable economic importance. Their range of biological activities encompassing their acute toxicity, on one hand, and their antitumour activity, on the other, have also stimulated research into their chemistry.



## 1.1 OCCURRENCE OF PYRROLIZIDINE ALKALOIDS

A considerable number of plant genera have been found to contain pyrrolizidine alkaloids (Table 1.1). The majority of these genera are in three families: Boraginaceae, Compositae, and Leguminosae, with *Heliotropium*, *Senecio*, and *Crotalaria* respectively being representative genera. In fact, pyrrolizidine alkaloids are most abundant within the huge *Senecio* genus, with over 150 species having been investigated.<sup>31</sup>

Pyrrolizidine alkaloids have also been found in insects which feed on pyrrolizidine containing plant species. The pheromones, danaidone **4**, danaidal **5**, and hydroxydanaidal **6**, of some male Lepidoptera are biosynthesised from ingested pyrrolizidine alkaloids.<sup>32</sup>

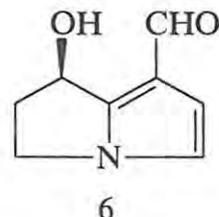
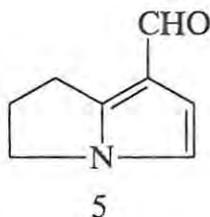
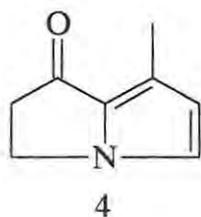
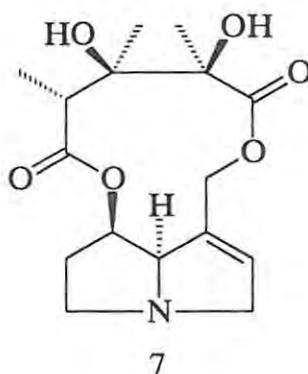


Table 1.1: Plant genera in which pyrrolizidine alkaloids have been found.<sup>31,33-55</sup>

Family	Genus
Apocynaceae	<i>Alafia, Anodendron, Parsonsia, Strophanthus, Tabernaemontana, Urechites</i>
Boraginaceae/ Ehretiaceae	<i>Alkanna, Amsinkia, Anchusa, Arnebia, Asperugo, Borago, Caccinia, Cerinthe, Cordia, Cryptantha, Cynoglossum, Echium, Ehretia, Hackelia, Heliotropium, Lappula, Lindelofia, Lithospermum, Macrotomia, Mertensia Messerschmidia, Moltikiopsis, Myosotis, Neatostema, Omphalodes, Onosma, Paracaryum, Paracynoglossum, Rindera, Solenanthus, Symphytum, Tournefortia, Trachelanthus, Trichodesma, Turneforcia, Ulegbekia</i>
Celastraceae	<i>Bhesa</i>
Compositae/ Asteraceae	<i>Adenostyles, Ageratum, Arnica, Brachyglottis, Cacalia, Chersodoma, Chromolaena, Cirsium, Conoclinium, Crassocephalum, Doronicum, Echinaceae, Emilia, Erechites, Eupatorium, Farfugium, Gynura, Homogyne, Jacmaia, Kleinia, Liatris, Ligularia, Nardosmia, Notonia, Packera, Petasites, Schismus, Senecio, Syneilesis, Tussilago, Werneria</i>
Convolvulaceae	<i>Ipomoea</i>
Elaeocarpaceae	<i>Aristotelia</i>
Euphorbiaceae	<i>Phyllanthus, Securinega</i>
Gramineae	<i>Festuca, Lolium, Thelepogon</i>
Leguminosae/ Fabaceae	<i>Adenocarpus, Alexa, Buchenroedera, Castanospermum, Crotalaria, Cytisus, Laburnum, Lotononis, Onosma</i>
Linaceae	<i>Hugonia</i>
Orchidaceae	<i>Chysis, Doritus, Hammarbya, Kingiella, Liparis, Malaxis, Phalaenopsis, Vanda, Vandopsis</i>
Papaveraceae	<i>Glaucium</i>
Ranunculaceae	<i>Caltha, Trollius</i>
Rhizophoraceae	<i>Cassiopourea</i>
Santalaceae	<i>Thesium</i>
Sapotaceae	<i>Mimusops, Planchonella</i>
Scrophulariaceae	<i>Castilleja, Melampyrum, Pedicularis</i>

Butterflies of the *Danaus* genus have been shown to sequester and store unmodified pyrrolizidine alkaloids from their food supply.<sup>32,56,57</sup> It is presumed that they use the pyrrolizidines for self-defence, as they have been shown to be distasteful to insect and arachnid predators.<sup>32,56,58</sup> The larval stage of the moth, *Utetheisa ornatrix*, obtains pyrrolizidine alkaloids, notably monocrotaline **7**, from the *Crotalaria* species on which it feeds;<sup>32,59</sup> the adult then converts monocrotaline **7** to hydroxydanaidal **6**. Another moth, *Gnophaela vermiculata*, modifies pyrrolizidines ingested from *Hackelia californica*.<sup>60</sup>



Chrysomelid beetles, which feed on pyrrolizidine alkaloid containing plants, also sequester the alkaloids and store them as *N*-oxides in their defence secretions.<sup>61-63</sup> They cannot oxidise pyrrolizidines, but must ingest them in the oxidised form if they are to be stored.<sup>62</sup> Those Chrysomelid beetles which do not feed on pyrrolizidine alkaloid containing plants reject such plants as a food supply.<sup>63</sup> Ants<sup>64</sup> and frogs<sup>65</sup> synthesise their own pyrrolizidine alkaloid derivatives as a defence mechanism. In ants the alkaloids are found in the venom, while in frogs they are found in the skin.

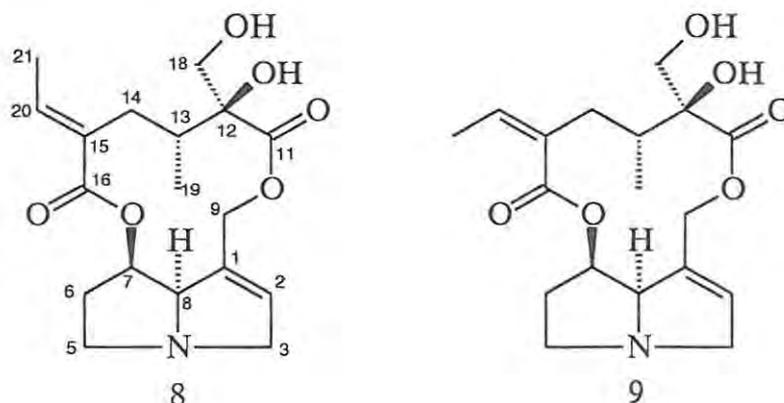
## 1.2 EXTRACTION AND PURIFICATION

Before the pyrrolizidine alkaloid components of a plant can be identified they have to be extracted and then purified to remove other chemical components. Then, as a plant generally contains more than one pyrrolizidine alkaloid, they have to be separated. Often the purification and separation can take place together. Although it is possible to identify

pyrrolizidines in a mixture using, for example, gas chromatography-mass spectrometry, it is necessary to have an absolutely pure pyrrolizidine alkaloid for biological testing. Very few pyrrolizidines are available commercially so they have to be isolated from natural sources or synthesised as required.

### 1.2.1 STABILITY OF PYRROLIZIDINE ALKALOIDS

If dried plant material is to be extracted, the stability of the pyrrolizidine components must be considered. During storage, the ratio of the pyrrolizidine alkaloids to their *N*-oxide derivatives can change.<sup>66</sup> Isomerisation can also take place, particularly between (*E*)- and (*Z*)-double bonds. Ultraviolet radiation has been shown to convert usaramine **8** to retrorsine **9**.<sup>67</sup> The only difference between the two being the stereochemistry of the C(15)-C(20) double bond. This being analogous to angelic acid esters, which over time have been shown to convert to tiglic acid esters;<sup>68</sup> a (*Z*)-double bond changing to an (*E*)-double bond. Plants must also be dried quickly to reduce the effects of enzyme action, without excessive heat or exposure to direct sunlight.<sup>69</sup> The alkaloids are susceptible to hydrolysis at high pH (> 9) and, therefore, basic solutions must be kept cool and must not be stored.<sup>69</sup> Moreover, pyrrolizidine alkaloids should not be stored in halogenated solvents due to the possibility of forming quaternary salts especially if exposed to sunlight.<sup>69</sup>



### 1.2.2 EXTRACTION OF PYRROLIZIDINE ALKALOIDS

A general approach is given by Mattocks.<sup>70</sup> Most often dried, shredded plant material is extracted using either hot or cold solvents.<sup>35,39,71-78</sup> The initial extraction involves two steps: one to remove fats from the plant, typically with hexane or petroleum ether,<sup>77,78</sup> and a second to extract the pyrrolizidine alkaloids with an alcohol.<sup>73,77,79,80</sup> The pyrrolizidine alkaloids are then separated from other alcohol soluble components by partition between an aqueous and an organic phase.<sup>37,79,81</sup> As pyrrolizidine alkaloids are often present in plants as *N*-oxides, which are not particularly soluble in organic solvents, they are reduced, while in acidic solution, with zinc dust<sup>81-85</sup> and then, finally, the acidic solution is basified<sup>39,73,80,81</sup> and the pyrrolizidine alkaloids are extracted into an organic solvent such as chloroform or ethyl acetate.<sup>37,81,86</sup> This is the most common approach to the extraction of pyrrolizidine alkaloids and was the one used in this investigation (Section 2.3, p. 51).

Other authors have used ion exchange resins to purify the alcoholic extract rather than using solvent partition.<sup>87</sup> The extraction system can be set up to be continuous with alcohol passing through the plant material and then through a cation exchange resin before returning to the plant material to repeat the cycle.<sup>88,89</sup> Huizing and Malingré<sup>90,91</sup> used a column containing a redox polymer, "Serdoxit", in conjunction with the ion exchange isolation. The redox column was to reduce any *N*-oxides present. One of the disadvantages of using a resin is that regeneration is difficult. Dowex-50, a commonly used resin, becomes coated with waxes and tar.<sup>92</sup> The regeneration is time-consuming and requires large volumes of hydrochloric acid. In addition, the beads tend to swell and crack.

A microextraction method has been developed to extract the pyrrolizidine alkaloids from herbarium specimens.<sup>93</sup> The finely shredded plant material is extracted with methanol using a sonifier for 10 minutes to solubilize the pyrrolizidines; the solution is then centrifuged and the supernatant collected. The solvent is removed with an air stream and then solutions of exact concentration are made up for comparison, with standards, using thin layer chromatography.

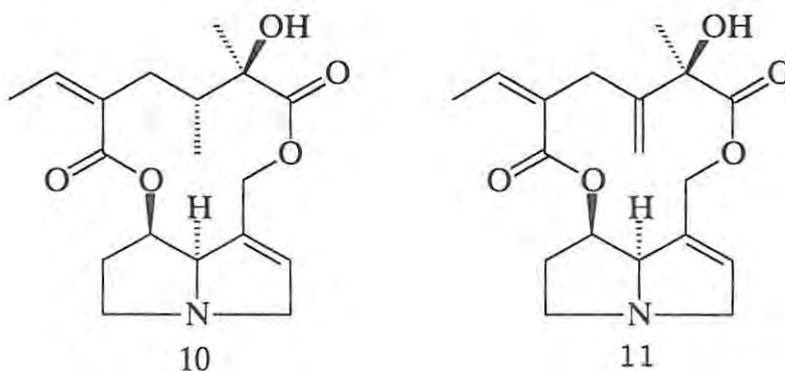
A methanol soxhlet extraction of plant material is time-consuming and requires large amounts of solvent and plant material. Off-line supercritical fluid extraction is often very rapid with high recovery from very small amounts of material.<sup>94</sup> An attempt to extract pyrrolizidine alkaloids with various densities of supercritical carbon dioxide was unsuccessful, probably due to an inappropriate polarity and solvent strength of the carbon dioxide. The polarity can be increased by adding methanol as a modifier. The most efficient extraction was achieved with a pressure of 15 MPa, a temperature of 55°C, a carbon dioxide density of 0.65 and using 800  $\mu\text{L}$  of methanol.<sup>94</sup> The success of the separation was measured using capillary gas chromatography.

### 1.2.3 SEPARATION AND PURIFICATION OF PYRROLIZIDINE ALKALOIDS

The mixture of pyrrolizidine alkaloids extracted can be separated into its individual components by a number of methods. Isomers are frequently extremely difficult to separate, although gas chromatography and high performance liquid chromatography are occasionally proving to be more successful.

#### 1.2.3.1 RECRYSTALLISATION

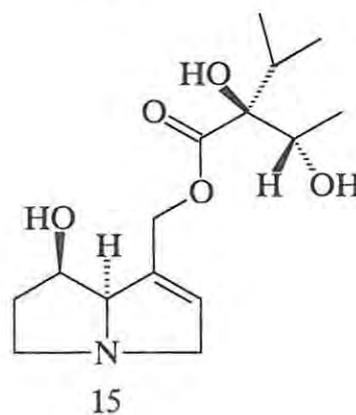
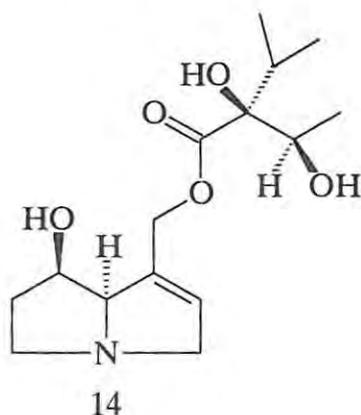
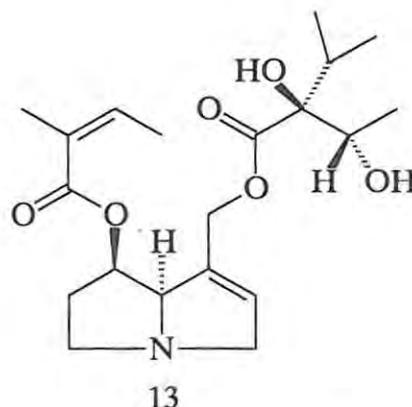
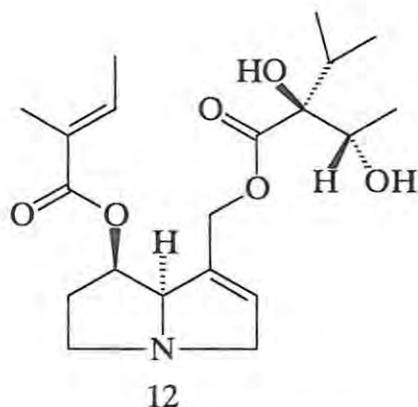
Crystalline pyrrolizidines can be purified by recrystallisation. Methanol,<sup>95,96</sup> ethanol,<sup>75,97-100</sup> ethyl acetate,<sup>101-106</sup> acetone,<sup>78,102,107-113</sup> water,<sup>114,115</sup> petroleum ether<sup>112,116-118</sup> and ether<sup>106</sup> have all been used for recrystallisation, either pure, or as mixtures.<sup>75,78,102,114,119-122</sup> Some pyrrolizidines, for example senecionine **10** and seneciophylline **11**, co-crystallise and hence can not be separated by recrystallisation.<sup>123-125</sup>



If the pyrrolizidine alkaloid is not crystalline a crystalline derivative, such as a perchlorate,<sup>78</sup> picrate<sup>100,106,126-129</sup> or picrolonate,<sup>129</sup> can be synthesised which can then be purified by recrystallisation. The wide variety of chromatographic techniques now available has reduced the need for recrystallisation.

### 1.2.3.2 THIN LAYER CHROMATOGRAPHY

The progress of the separation and purification can be followed using thin layer chromatography (TLC). A number of different solvent systems have been used for developing the plates, the choice being dependent on what stationary phase is used (commonly silica gel<sup>46-48,101,130-132</sup> and, more rarely, aluminium oxide<sup>46,47,132</sup>). The  $R_f$  values for individual pyrrolizidines vary depending on the solvent, temperature, degree of chamber saturation and amount of compound applied. A known alkaloid can be applied for comparison. Closely related isomers, like symphytine **12** and symlandine **13**, and intermedine **14** and lycopsamine **15**, are difficult if not impossible to separate by TLC.<sup>26,133</sup>



The pyrrolizidine alkaloids can be visualised on the plates using ultraviolet light or iodine vapour, but neither of these methods is specific for the alkaloids. For unsaturated pyrrolizidine alkaloids and their *N*-oxides, tests based on Ehrlich's reagent are the most sensitive. The plates are first sprayed with a reagent, *e.g.* *o*-chloranil (tetrachloro-*o*-benzoquinone), to convert the pyrrolizidines to pyrroles,<sup>134,135</sup> before being sprayed with Ehrlich's reagent.<sup>136</sup> Unfortunately, Ehrlich's reagent is very sensitive to water so is not easily stored for routine analyses. A modified iodobismuth reagent, Dragendorff's reagent, is very useful for detecting both saturated and unsaturated pyrrolizidines; however, it is not specific for pyrrolizidine alkaloids.<sup>137-139</sup>

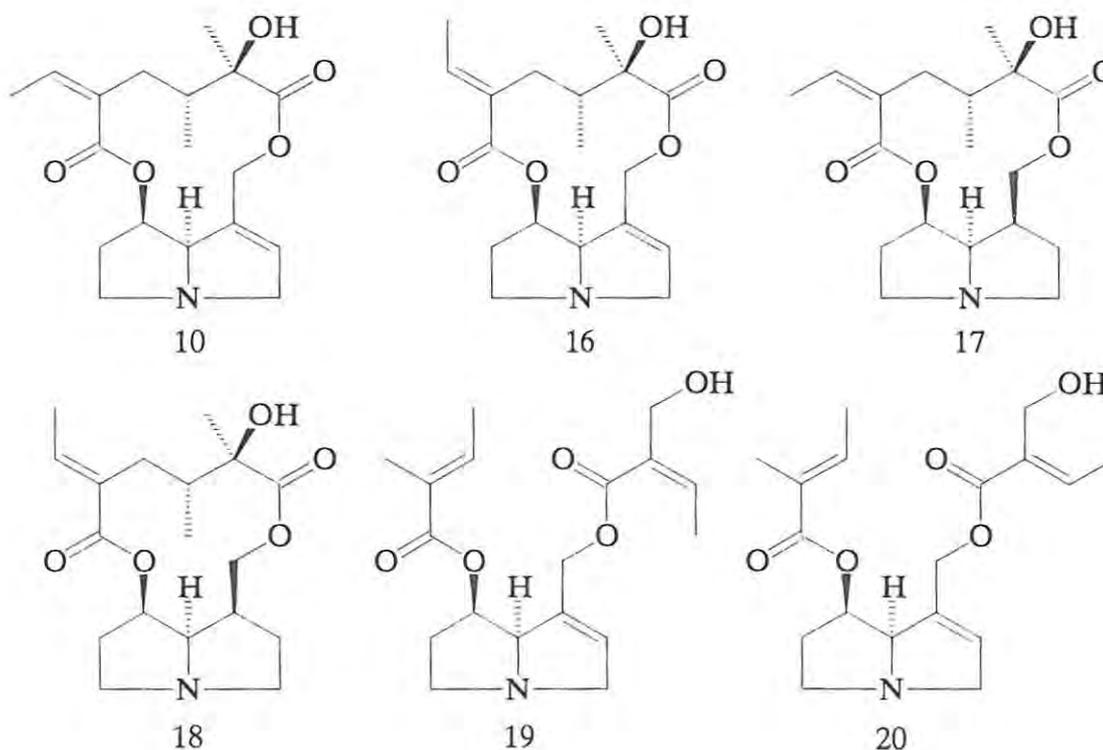
As TLC is used to follow the progress of the separation so preparative layer chromatography (PLC) is used to purify small amounts of crude pyrrolizidine alkaloid material for identification. Plates are normally coated with silica gel,<sup>46,47,76,132,140-144</sup> but alumina<sup>46,47</sup> has also been used. The solvents used are similar to those used for developing the analytical TLC plates.

### 1.2.3.3 COLUMN CHROMATOGRAPHY

Column chromatography has been a standard, preparative method of separating pyrrolizidine alkaloids. The solid stationary phases used have included silica gel,<sup>45,140,145-152</sup> Sephadex LH-20,<sup>153</sup> and aluminium oxide,<sup>47,141,154</sup> but silica gel is by far the most popular. Eluting solvents tend to be similar to those used for TLC, but often a gradient<sup>155</sup> is used, slowly increasing the polarity of the eluting solvent.<sup>60,145,154,156-159</sup> The disadvantage of column chromatography is the tendency of pyrrolizidine alkaloids to bind irreversibly to the solid stationary phase.<sup>67</sup> Column chromatography does not always give complete separation and is often followed by a series of preparative TLC separations. It is, however, relatively inexpensive and does not require complex apparatus. Flash column chromatography was used as the initial separation technique in this study.

## 1.2.3.4 GAS LIQUID CHROMATOGRAPHY

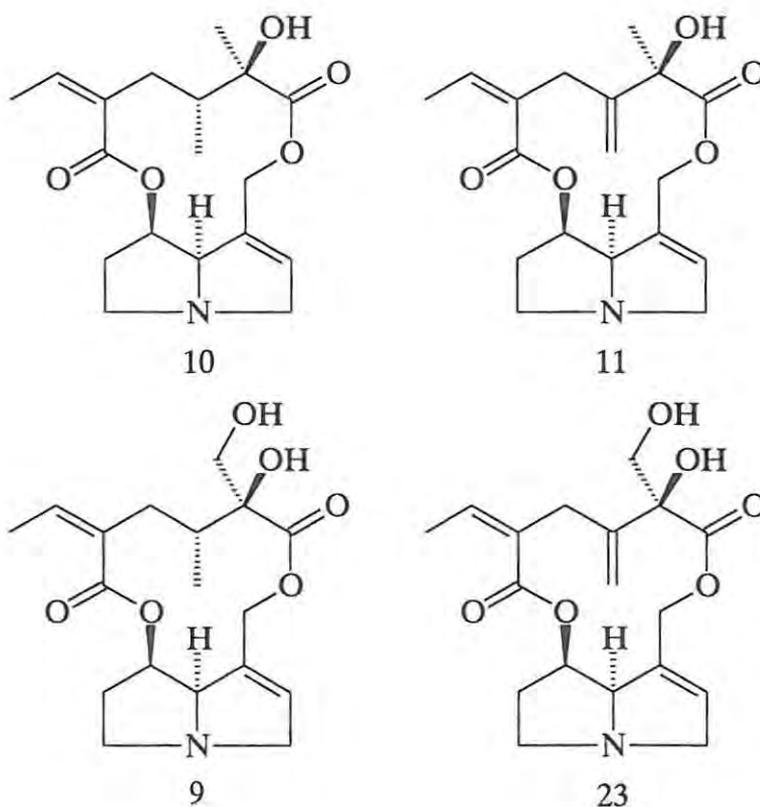
The first gas liquid chromatographic (GLC) characterisation of pyrrolizidine alkaloids was performed by Chalmers *et al.*<sup>160</sup> in 1965 and it is now a widely used technique.<sup>161</sup> Capillary columns have given better separation, but as a result GLC provides analytical rather than preparative separations. Coupled to mass spectrometry (Section 1.3.4, p. 18), GC-MS is a formidable identification technique. More recently GLC has been coupled to Fourier transform infra-red spectroscopy as well.<sup>162</sup> GLC is capable of resolving (*E*)- and (*Z*)-isomers, like senecionine **10** and integerrimine **16**, platyphylline **17** and neoplatyphylline **18**, and triagularicine **19** and neotriangularicine **20**,<sup>68</sup> but is less successful in separating diastereoisomers, such as lycopsamine **15** and intermedine **14**. The use of chiral stationary phases may improve this situation, but these systems do not yet appear to have been applied to pyrrolizidine alkaloids.





that allows for better ultraviolet detection. Pyrrolizidine alkaloids are best detected below 230 nm, and while THF shows strong absorption below this value, methanol does not. Alkaline conditions dissolve silica gel, so the slightly acidic conditions required for reverse phase HPLC increase the life of the column.<sup>75</sup> Preparative ion-pair adsorption HPLC has also produced good results as shown by the resolution of the pyrrolizidine alkaloids of comfrey.<sup>164,165</sup>

HPLC has been able to separate senecionine **10** from seneciphylline **11**<sup>166,167</sup> and retrorsine **9** from riddelline **23**.<sup>75</sup> More surprisingly, a resolution of C(15)-C(20) double bond isomers has been reported.<sup>168</sup> HPLC, unlike GLC, operates favourably at room temperature, thus preventing the formation of thermal degradation products.



### 1.2.3.6 DROPLET COUNTER CURRENT CHROMATOGRAPHY

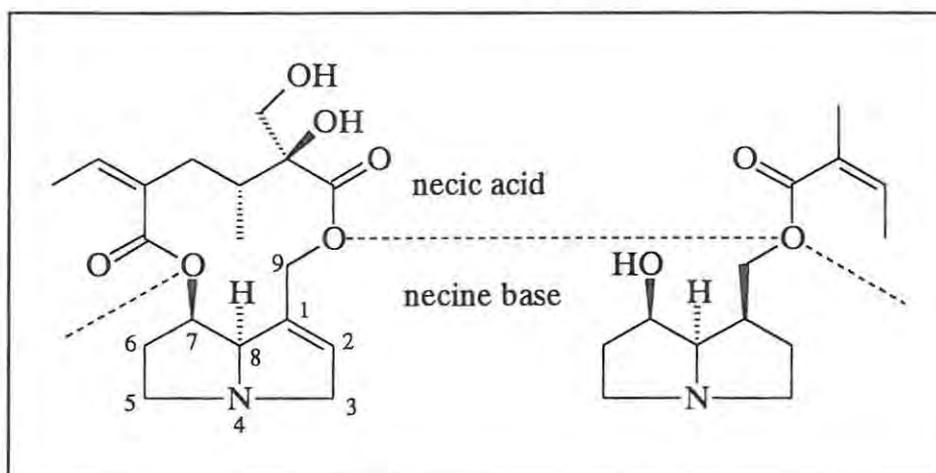
Pyrrrolizidine alkaloids tend to adsorb irreversibly to solid phases such as silica gel, alumina, and reverse phase HPLC stationary phases resulting in large losses of material.<sup>67,169</sup> Droplet counter current chromatography (DCCC) is a liquid-liquid extraction technique and involves the partition of the alkaloids between a steady stream of droplets and a surrounding stationary phase. The droplets can be ascending or descending. There is no loss of material due to adsorption. The method has been used for the efficient separation and purification of pyrrrolizidine alkaloids.<sup>35,67,72,169,170</sup> The solvents used for polar pyrrrolizidines include varying ratios of chloroform, methanol, and water,<sup>35,67</sup> in descending mode, while for moderately polar pyrrrolizidines chloroform-toluene-methanol-water (5:5:7:2),<sup>44,67,72,170</sup> has been used in ascending mode.

## 1.3 IDENTIFICATION

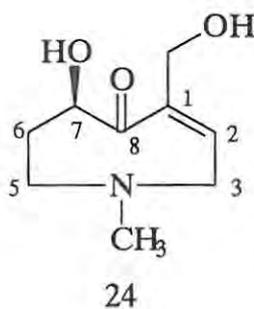
### 1.3.1 STRUCTURE OF PYRROLIZIDINE ALKALOIDS

Most pyrrrolizidine alkaloids consist of two parts: the necine base, a bicyclic ring system with a bridgehead nitrogen, and the necic acid(s) (Figure 1.1). The traditional, non-IUPAC, numbering system of the necine base is shown in Figure 1.1.

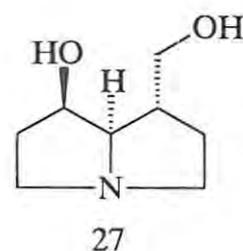
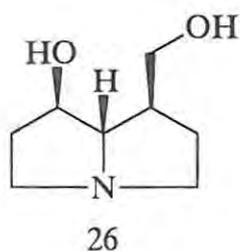
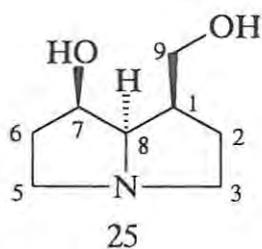
The ring is not planar but is actually "V"-shaped like a partly open book. Whether the "V" opens towards or away from the viewer depends on the stereochemistry at C-8. The base may be saturated or unsaturated; if unsaturated, the double bond is usually between carbons 1 and 2. Pyrrole types, with double bonds between carbons 1 and 8 and between carbons 2 and 3, are more rare. The base ring system may be oxygenated at C-9 and/or C-7 and, sometimes at carbons 1, 2 or 6. The otonecine base **24** has lost the C(8)-N bond and has, instead, a methyl group on the nitrogen and a ketone functionality at C-8.



**Figure 1.1:** The structure of pyrrolizidine alkaloids showing the traditional necine base numbering system.



Often the only difference between two necine bases is the stereochemistry at carbons 1, 7 or 8, *e.g.* platynecine **25**, hastanecine **26**, and turneforcidine **27**. Hydroxyl groups at either (or both) carbons 7 and 9 may be esterified to give a wide range of acyclic mono- and diesters and macrocyclic diesters. Any hydroxyl groups at carbons 2 and 6 may also be esterified.



There are a number of acids found as esters of necine bases, the simplest being acetic acid. Only acetate esters have been found at carbon-2, while for other positions a range of esters are known. Necic acids are mono- or dicarboxylic acids of branched carbon chains normally containing 5 to 10 carbon atoms, exceptions include acetic acid ( $C_2$ ), nervogenic acid ( $C_{17}$ ), malaxanic acid ( $C_{18}$ ), kurameric acid ( $C_{23}$ ) and nervosinic acid ( $C_{28}$ ).<sup>171</sup> The latter four are very rarely found. The majority of necine acids contain 10 carbon atoms. Monocarboxylic acids give acyclic esterification products, while dicarboxylic acids afford macrocycles. The macrocycle can be constructed of 11, 12, 13 or 14 atoms, as in crotalarine **28**, senecionine **10**, doronenine **29** and parsonsine **30**, respectively.

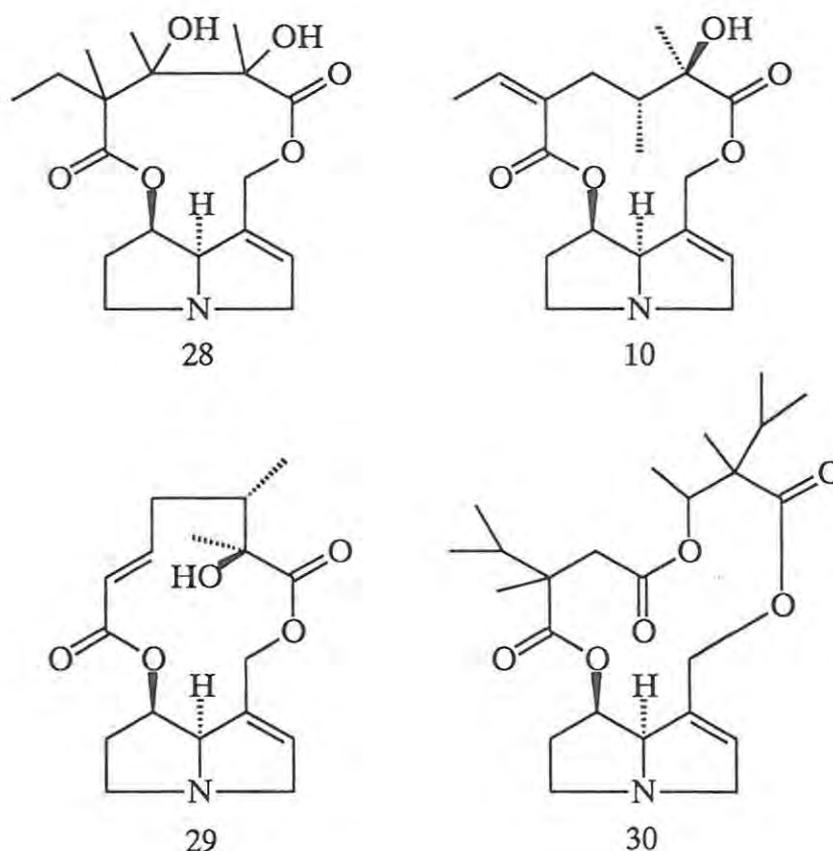
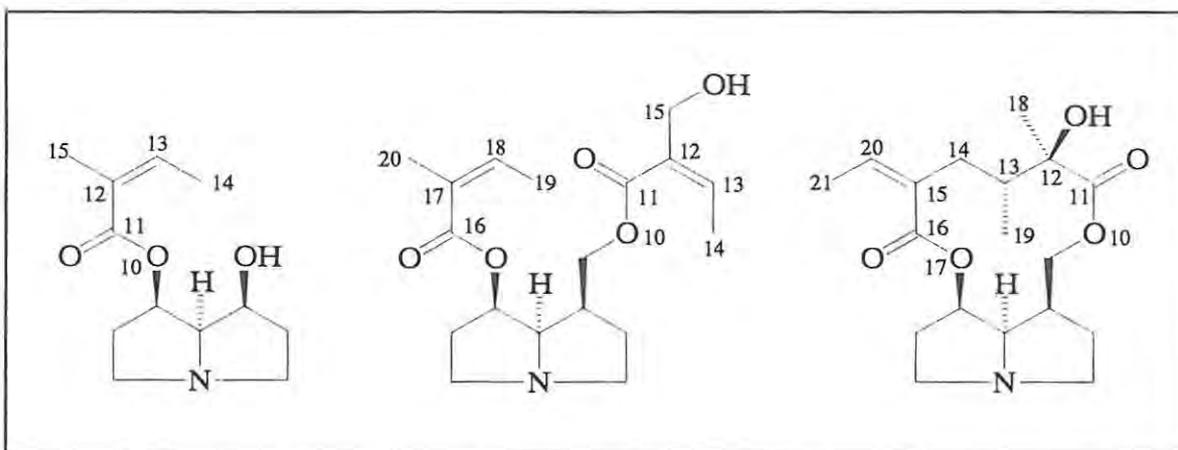


Figure 1.2 shows the traditional, non-IUPAC numbering system used for the necic acid portion of the pyrrolizidine alkaloid. For monoester and macrocyclic pyrrolizidines, the ester linkage oxygens are numbered. However, for acyclic diesters only one ester linkage oxygen is numbered and that is the one at C-9.



**Figure 1.2:** The traditional numbering of the necic acid portion of pyrrolizidine alkaloids.

### 1.3.2 MELTING POINTS AND X-RAY CRYSTAL STRUCTURES

Pure crystalline pyrrolizidine alkaloids have characteristic melting points, which can help to confirm an identification. More importantly, x-ray crystal structures can be obtained for pure crystalline alkaloids to provide an absolute identification. Crystal structures have been obtained for a number of pyrrolizidines as well as for a number of crystalline pyrrolizidine alkaloid derivatives.<sup>172</sup>

### 1.3.3 INFRA-RED SPECTROSCOPY

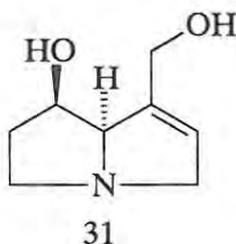
This technique is now seldom used in the identification of pyrrolizidine alkaloids. It does allow for the recognition of functional groups commonly found in these systems, *viz.*, carbon-carbon double bonds, hydroxyl groups, esters and terminal methylene groups.<sup>173,174</sup>  $\alpha,\beta$ -Unsaturated esters show a conjugated carbonyl stretch at 1710-1720  $\text{cm}^{-1}$  (a non-conjugated ester carbonyl band is at 1730-1740  $\text{cm}^{-1}$ ) and a C=C stretching band at 1640-1660  $\text{cm}^{-1}$ .<sup>174</sup> Unsaturated necine bases and esters also show a weak band at 3025-3090  $\text{cm}^{-1}$  due to vinyl hydrogen stretching.<sup>174</sup>

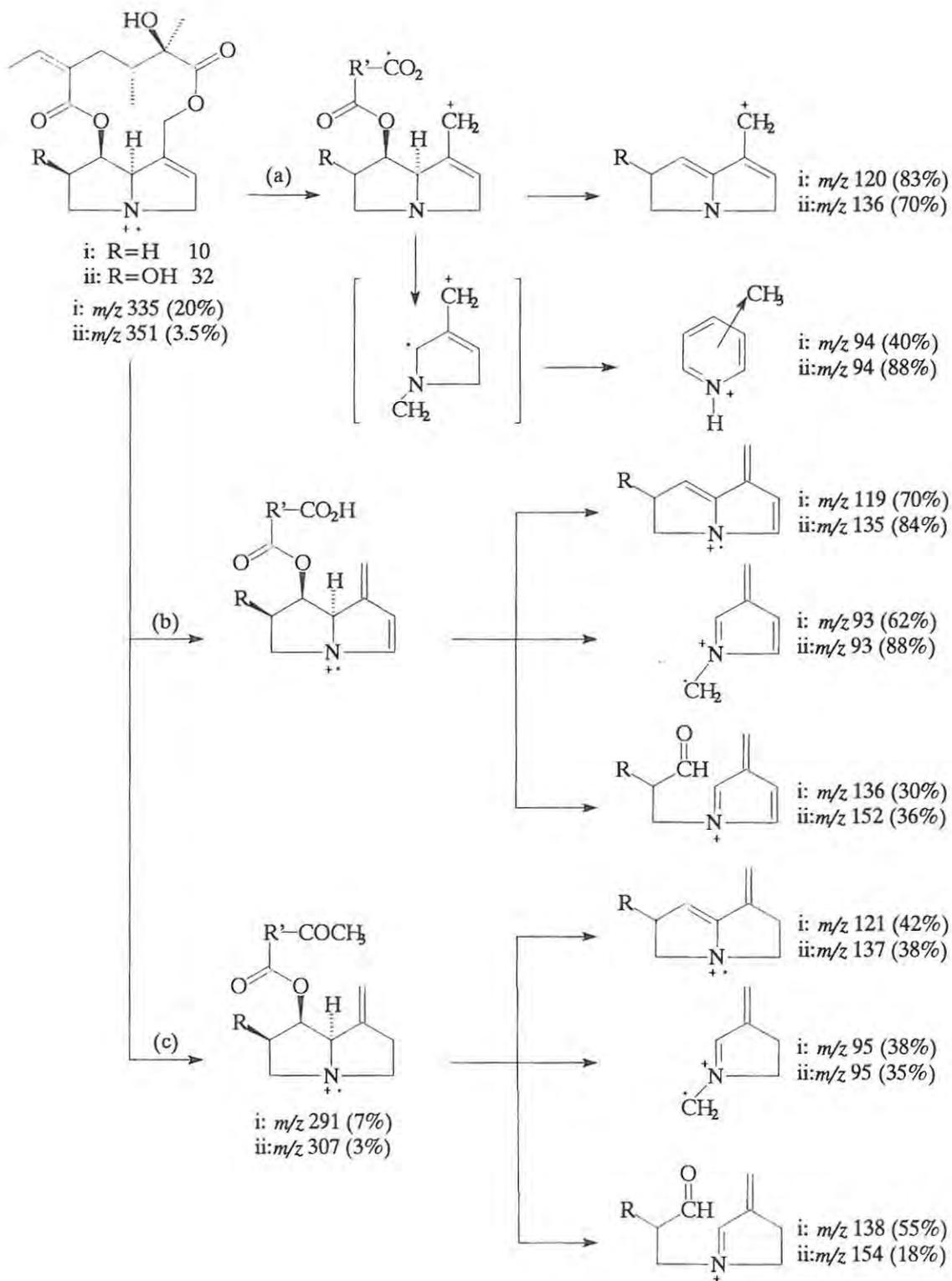
### 1.3.4 MASS SPECTROMETRY

This is a very useful technique for determining the structure of pyrrolizidine alkaloids, especially when coupled with gas chromatography. Both electron impact ionisation and chemical ionisation techniques have been employed, however, electron impact ionisation mass spectrometry of pyrrolizidine alkaloids tends to be more reproducible and gives a wide range of fragments. A review has been published on the mass spectrometry of pyrrolizidine alkaloids.<sup>175,176</sup> The main features of electron impact mass spectra are outlined below followed by a brief section on chemical ionisation mass spectra.

#### 1.3.4.1 PYRROLIZIDINE ALKALOIDS CONTAINING AN UNSATURATED NECINE BASE

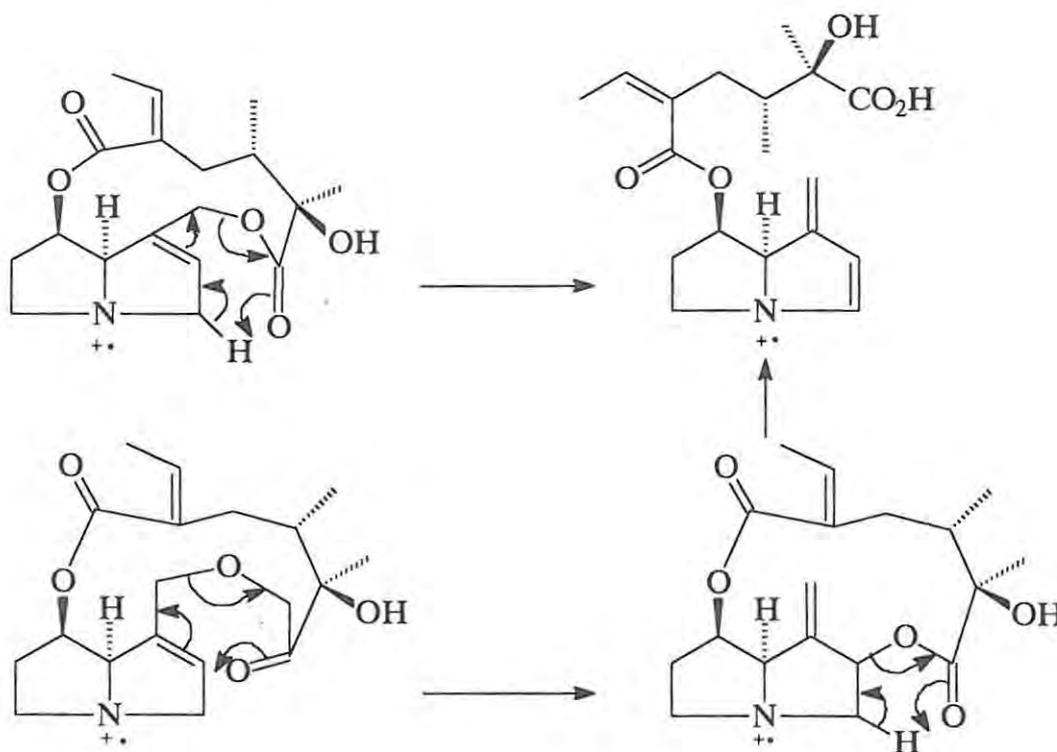
Retronecine **31**, a typical unsaturated necine base, is the most common of the necine bases and there is thus a great deal of information available on it. Macrocycles having a retronecine base typically show triads at  $m/z$  93, 94, and 95; 119, 120, and 121; and 136, 137, and 138.<sup>105,127,177-182</sup> As an example, the fragmentation of senecionine **10** and anacrotine **32** is shown in Scheme 1.1.<sup>177</sup> The only difference between anacrotine **32** and senecionine **10** is that the former has a hydroxyl group on carbon-6 and, consequently, until carbon-6 is lost the fragments of anacrotine will be 16 atomic mass units higher than the corresponding fragments of senecionine.



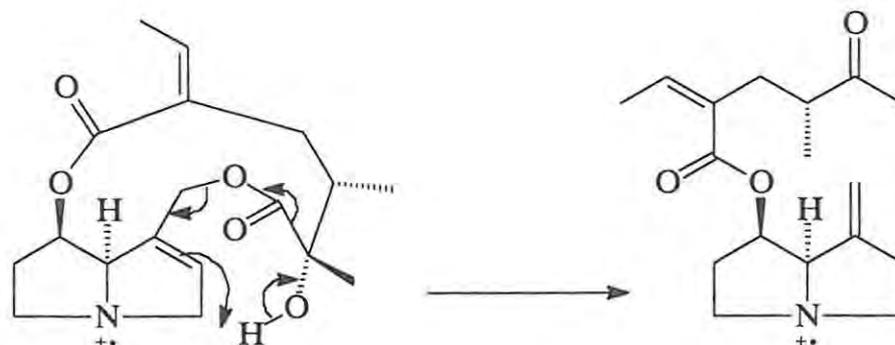


Scheme 1.1

Three possible fragmentation paths are illustrated in Scheme 1.1; path (a) is initiated by homolytic fission of the allylic ester moiety, path (b) by a 8- and/or 6-membered McLafferty rearrangements (Scheme 1.2), and path (c) by an 8-membered McLafferty rearrangement (Scheme 1.3).<sup>175</sup> Regardless of how the fragmentation was initiated, the triads around  $m/z$  94 and  $m/z$  137 result from eventual homolytic fission of the C(7)-(C)8 bond, followed by loss of the acid moiety, while acid loss by McLafferty rearrangement gives the triad  $m/z$  119, 120, and 121.<sup>175</sup>



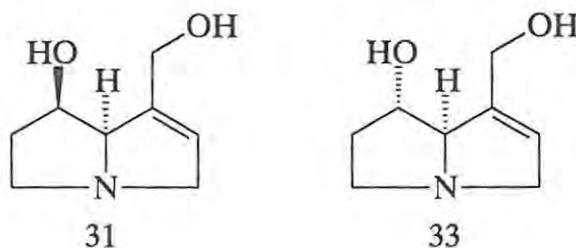
Scheme 1.2



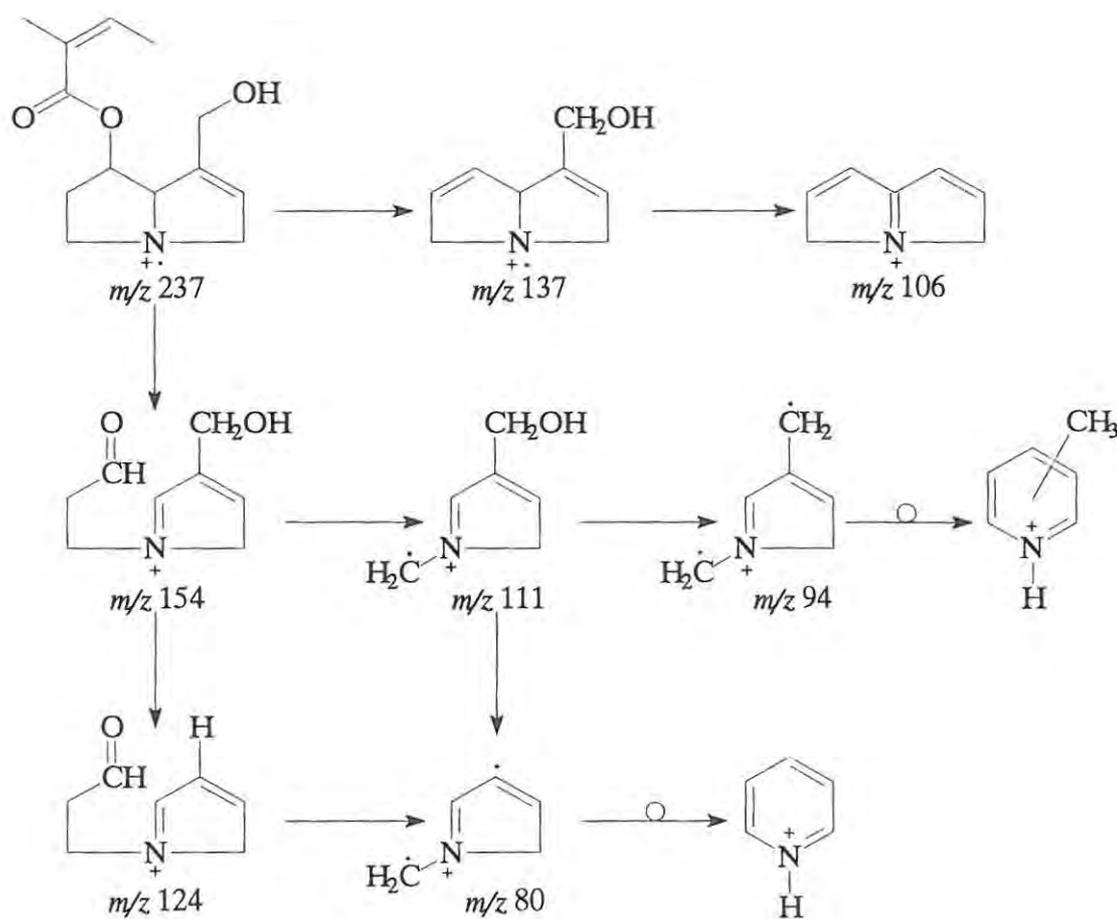
Scheme 1.3

Macrocycles can be distinguished from acycles by the presence of the  $M^+ - CO_2$  peak which is absent in the mass spectra of acycles.<sup>175</sup> In acyclic diesters the carboxylate moiety at C-9 is readily lost by McLafferty rearrangement and there are relatively few mass fragments found between the  $M^+$  peak and the peak for the loss of the acid. Of lesser importance is the loss of the ester group at C-7. In macrocycles, fission of the ester at C-9 does not result in the loss of the carboxylate group as it is still linked through to C-7; subsequent fragmentation of the acid, however, leads to numerous peaks.<sup>175</sup>

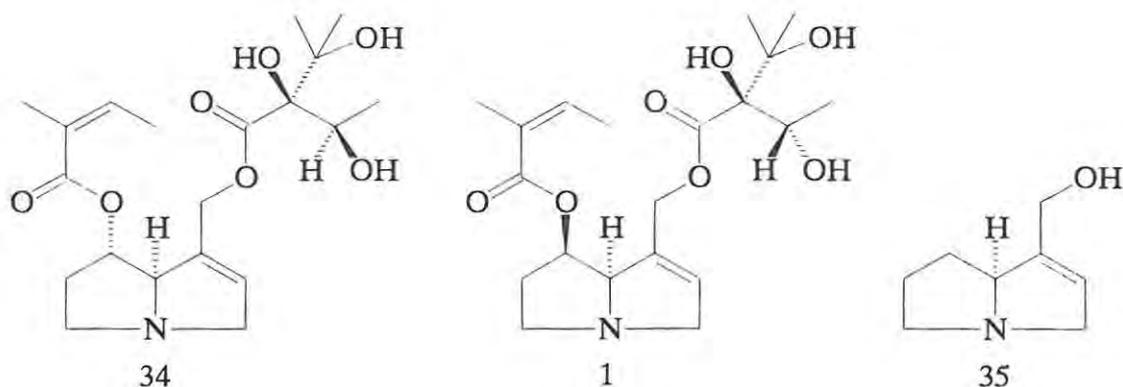
For the monoesters of retronecine **31** and heliotridine **33**, the ester at C-9 is particularly susceptible to cleavage. A triad at  $m/z$  137, 138, and 139 (one  $m/z$  unit higher than for macrocycles) is characteristic, and the peak at  $m/z$  138 is often the base peak. Usually the  $m/z$  119, 120, and 121 triad is insignificant, while the peak at  $m/z$  93 is strong and those at  $m/z$  94 and  $m/z$  95 have medium and low intensity, respectively.<sup>175</sup>



The diastereomers, retronecine **31** and heliotridine **33**, which differ only in the stereochemistry at carbon-7, exhibit identical fragmentation as do their 7-angelyl derivatives;<sup>183</sup> a proposed fragmentation pattern is shown in Scheme 1.4.<sup>183</sup> Heliosupine **34** and echimidine **1**, acyclic diesters of heliotridine and retronecine respectively, also exhibit identical fragmentation.<sup>183</sup> Supinidine **35**, in which the C-7 hydroxyl group is missing, has a similar mass spectrum to retronecine and heliotridine, but with additional peaks at  $m/z$  108, 110, 120, 122 and 139( $M^+$ ).<sup>184</sup>



Scheme 1.4

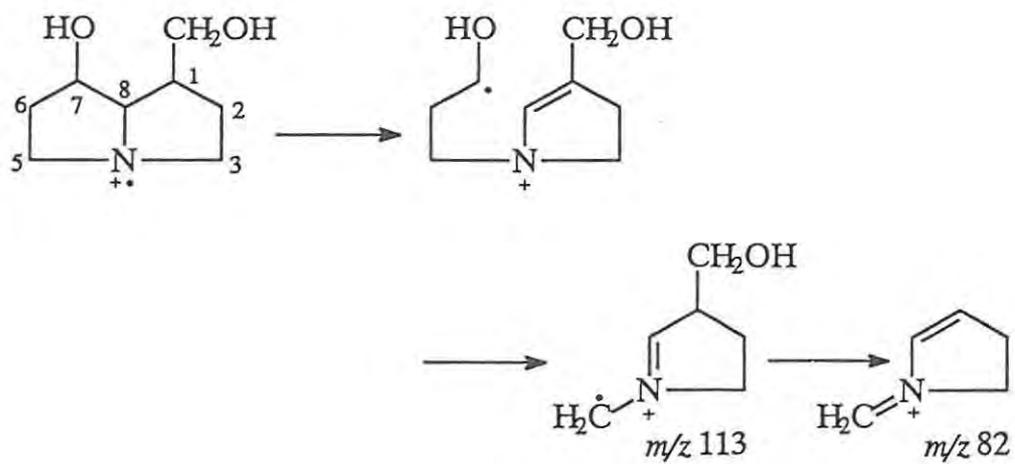
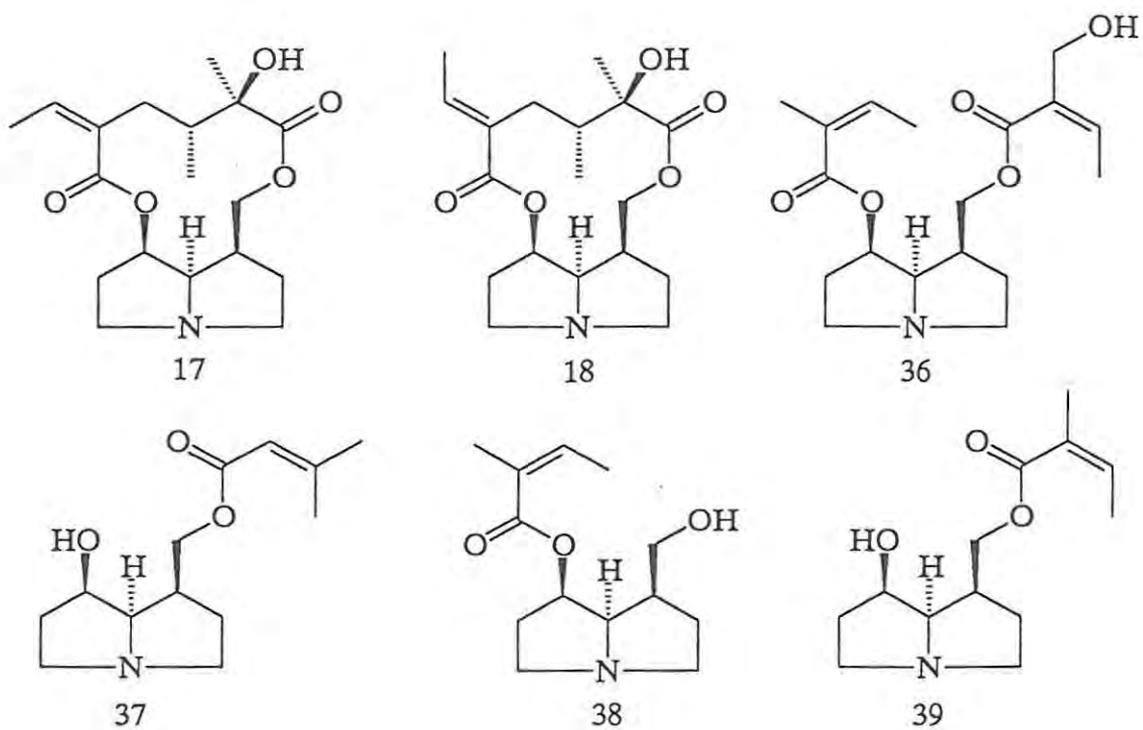


### 1.3.4.2 PYRROLIZIDINE ALKALOIDS CONTAINING A SATURATED NECINE BASE

The prominent peaks in the mass spectrum of a saturated necine pyrrolizidine alkaloid are typically two  $m/z$  units higher than those for their unsaturated analogues.<sup>185</sup> The base peak is often observed at  $m/z$  82,<sup>144,155,185-188</sup> and fragmentation is dominated by the loss of the ester at C-7 when this group is present.<sup>189,190</sup>

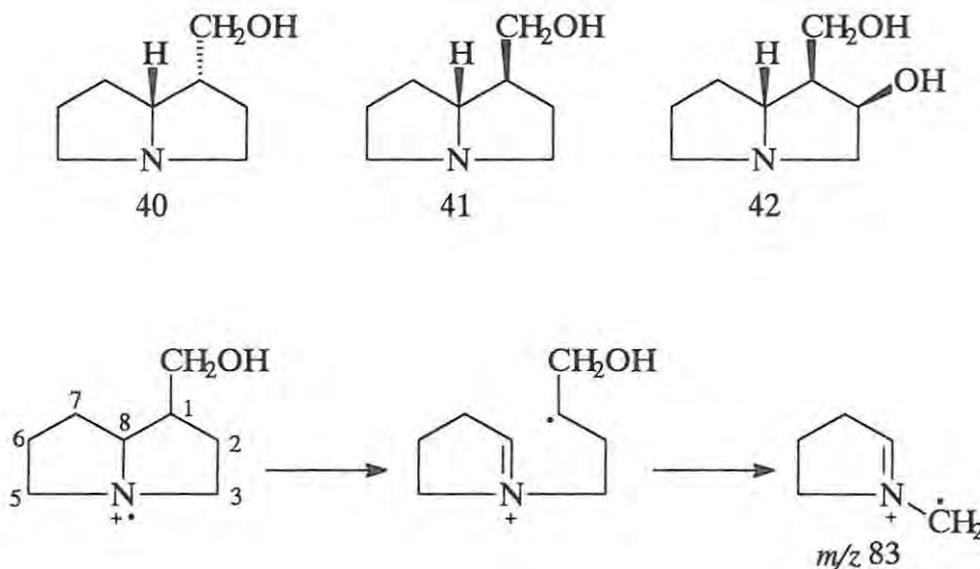
For macrocycles with a saturated base, *e.g.* platyphylline **17** and neoplatyphylline **18**, the base peak is observed at either  $m/z$  140 or  $m/z$  82.<sup>155,191</sup> The other important characteristic peaks are found at  $m/z$  95, 96, 122, 123 and 138.<sup>186,187,192</sup> For acyclic diesters, *e.g.* sarracine **36**, the base peak is often at  $m/z$  138.<sup>155</sup> Interestingly, the peak at  $m/z$  95 (66.7%) in fuchsisenecionine **37** is presumed to indicate esterification at C-9.<sup>193</sup> However, the mass spectra of 7- and 9-angelylplatynecine, **38** and **39**, are so similar<sup>155</sup> that no deductions about the location of the ester functions can be drawn from the intensity of the  $m/z$  95 peaks.

In general, saturated 7,9-dihydroxy necine bases exhibit two significant peaks, one at  $m/z$  82 and the other at  $m/z$  113 (Scheme 1.5).<sup>194</sup> In saturated pyrrolizidine necine bases the initial fissions are of bonds  $\beta$  to the nitrogen.<sup>194</sup> Due to the 7-hydroxy group the C(7)-C(8) bond is  $\beta$  to both a nitrogen and an oxygen atom and is, therefore, most labile and breaks easily.



Scheme 1.5

When the 7-hydroxyl is absent, as in isoretronecanol **40** and trachelanthamidine **41**, C(1)-C(8) bond fission predominates, giving a base peak at  $m/z$  83 (Scheme 1.6).<sup>194</sup> Macronecine **42** has two hydroxyl groups: one at C-1 and the other at C-2, and the major peaks in the mass spectrum of this compound are the base peak at  $m/z$  83 and the peaks at  $m/z$  55 and 98.<sup>194</sup>



Scheme 1.6

### 1.3.4.3 OTONECINE-BASED PYRROLIZIDINE ALKALOIDS

In the otonecine base, as mentioned earlier, the N-C(8) bond is absent and there is a ketone functionality at C-8 and a methyl group on the nitrogen. Characteristically, these compounds show an  $M^+ - 15$  peak corresponding to the loss of the *N*-methyl group, and the base can generally be identified by strong peaks at  $m/z$  94, 96, 100, 110, 122, 123, 149, 150, 151 and 168.<sup>154,195,196</sup> The peaks at  $m/z$  100, 110, 151 and 168, however, are considered to be particularly diagnostic as they do not feature strongly in the mass spectra of other pyrrolizidine alkaloids.<sup>154</sup>

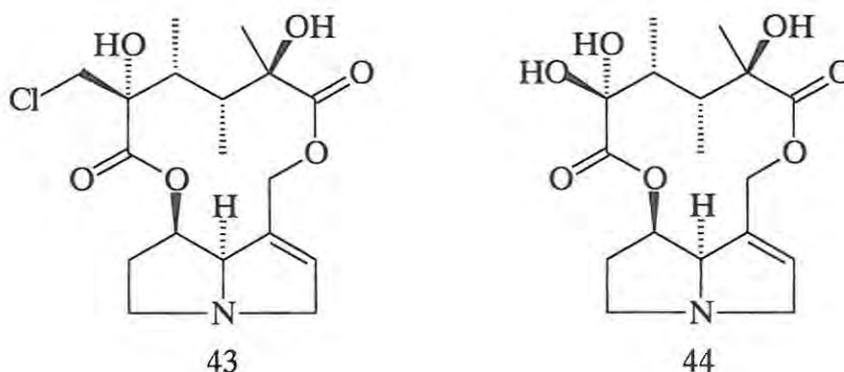
### 1.3.4.4 MASS SPECTRA OF PYRROLIZIDINE ALKALOID-4-OXIDES

Due to low volatility and thermal instability, pyrrolizidine alkaloid-4-oxides give very poor mass spectra.<sup>197</sup> *N*-oxides also elute poorly on some GLC columns used for pyrrolizidine

alkaloids and thus when GLC is coupled to mass spectrometry poor spectra generally result. In such cases the molecular ion peak is either very small or absent. The major fragments are associated with N-O cleavage<sup>197,198</sup> and the mass can, therefore, sometimes be determined by the presence of the peaks  $m/z$   $M^+-16$  and  $M^+-18$ , which represent the loss of O and H<sub>2</sub>O respectively.<sup>199</sup>

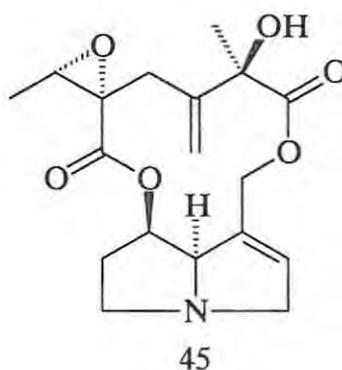
#### 1.3.4.5 USE OF MASS SPECTROMETRY TO DETERMINE THE MODE OF ESTER ATTACHMENT

Often one needs to establish which of two acid moieties is esterifying the 9-hydroxyl group, leaving the other to be bonded at position 7 or, in some cases, position 6. For 1,2-unsaturated pyrrolizidine alkaloids the allylic (or primary) ester at C-9 is more vulnerable to electron impact fission than the secondary ester (at C-7).<sup>154,189</sup> In an acyclic diester, therefore, the necic acid attached at carbon-9 is lost more readily than that attached to carbon-7. For macrocycles, cleavage of the C(9)-O bond will open the macrocycle and careful study of the fragmentation data may indicate the mode of ester attachment. Similar C(9)-O bond cleavage patterns apply to otonecine-based alkaloids. According to Breidenkamp and Wiechers,<sup>200</sup> however, this is an oversimplification of a complex fragmentation pattern. The necic acid component of merenskine **43**, scleratine **44**, and their derivatives is almost symmetrical so the mass spectra can accommodate both possible modes of ester attachment to the necine base. This is due to the transfer of the free radical centre within the acid portion of the pyrrolizidine alkaloid.<sup>200</sup>

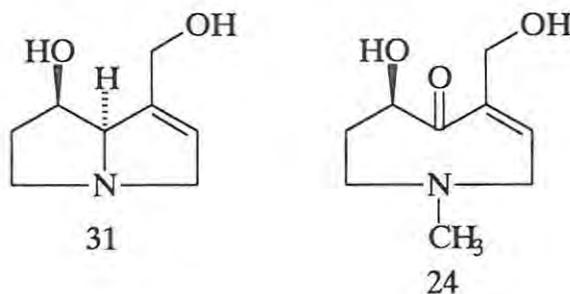


### 1.3.4.6 CHEMICAL IONISATION (CI)

The mass spectrometric patterns discussed above involve electron impact (EI) ionisation which is reproducible and so useful for identifying pyrrolizidine alkaloids. However, the high energy involved during EI ionisation means that the molecular ion peak is often not detected.<sup>176</sup> Chemical ionisation is a less energetic method for ionising a molecule and, therefore, the molecular ion peak is generally observed with little subsequent fragmentation occurring. Thus, the CI mass spectrum of jacozone **45** (using methane as the reagent gas) only exhibits three peaks,  $m/z$  120, 138 and 350.<sup>185</sup>



Proton transfer and hydride extraction reactions result in the formation of  $(M+1)^+$  and  $(M-1)^-$  ions in a sample. In nitrogen containing compounds, like pyrrolizidine alkaloids, proton transfer is more efficient than hydride extraction.<sup>176</sup> Chemical ionisation mass spectrometry is particularly useful for determining molecular weights of pyrrolizidine alkaloids but, surprisingly, only a relatively small number of pyrrolizidine alkaloids have been examined using this ionisation technique. Positive ion chemical ionisation (PICI) is achieved using methane or ammonia gas,<sup>93</sup> while hydroxide ions are used for negative ion chemical ionisation (NICI). The most striking feature of NICI mass spectra is the presence of relatively intense peaks, which are due to the necic acid portion of the pyrrolizidine alkaloid and which are not present in the PICI mass spectra.<sup>201,202</sup> Retronecine **31**- and otonecine **24**-based pyrrolizidines can be distinguished from each other by chemical ionisation mass spectrometry as only the retronecine-based systems give a  $(M-1)^-$  peak due to proton abstraction; otonecine-based alkaloids, on the other hand, give an  $M^-$  peak due to an electron capture process.<sup>201</sup>



### 1.3.5 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

With the advent of high field (400 MHz and higher) NMR instruments, NMR spectroscopy has become a very powerful means of determining the structure of pyrrolizidine alkaloids. Although the pyrrolizidine levels in plants are very low a proton NMR spectrum can be run on approximately 1 mg of material in a short time. A carbon-13 spectrum, however, takes considerably longer to acquire.

In view of the availability of extensive physical and spectroscopic (particularly proton and carbon-13 NMR) data for pyrrolizidine alkaloids, it became clear that a database would be an invaluable tool to facilitate identification of newly isolated pyrrolizidines. During the course of this project such a data base has been constructed, by remodelling and extensively enlarging a skeleton database begun earlier by co-workers. This database has helped tremendously in the identification of known pyrrolizidine alkaloids which were isolated in this study. In addition, other alkaloids, which were isolated, were rapidly shown to be new and hence needing additional investigation. A complete discussion of the development and application of this database is given in Section 3 (p. 97). A number of trends in the published proton and carbon-13 NMR data became apparent and these are discussed in Section 3.2 (p. 102).

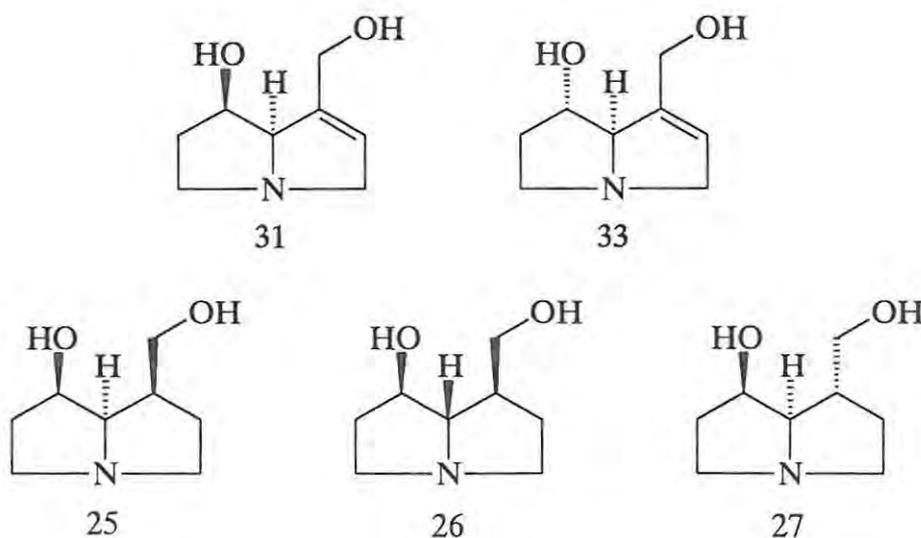
The signals in one dimensional spectra are not always readily distinguishable or easy to assign. Two dimensional spectra are increasingly being used to solve this problem. <sup>1</sup>H-<sup>1</sup>H

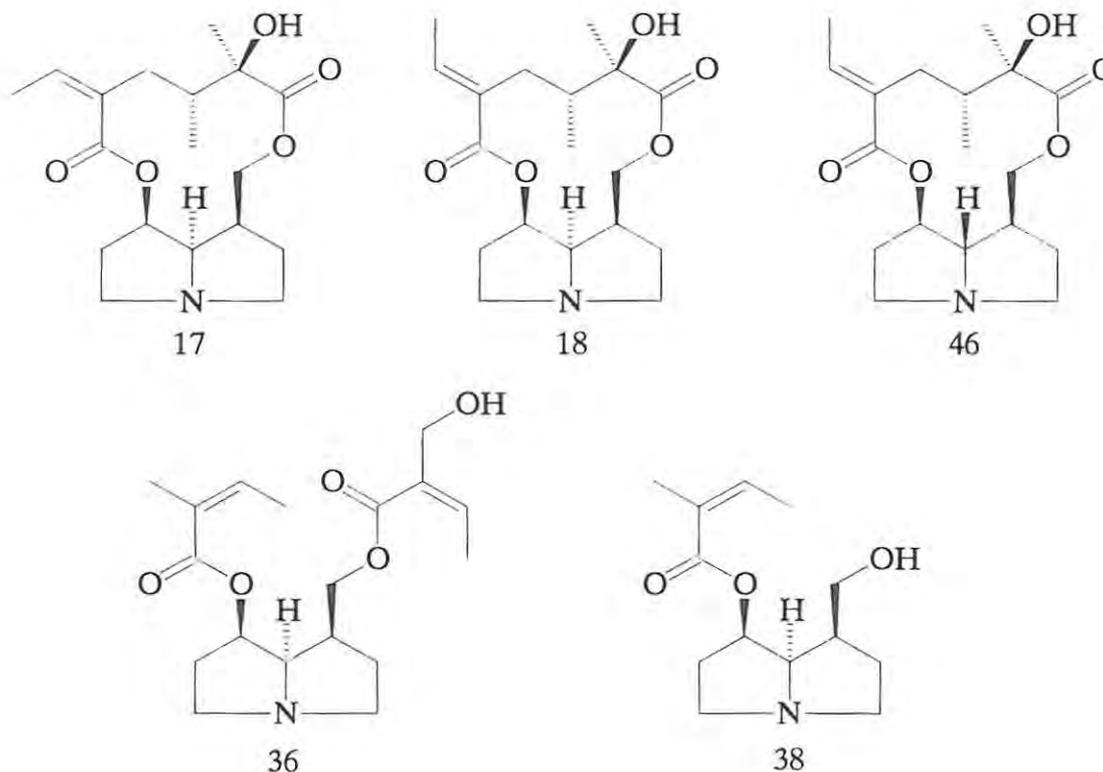
correlation spectroscopy (COSY) often helps with proton allocations.<sup>6,76,85,149,159,188,203-206</sup> The assigning of carbons is made easier by heteroatom correlation (HETCOR) or <sup>1</sup>H-detected heteronuclear multiple quantum coherence (HMQC) spectroscopy.<sup>6,67,76,113,149,159,188,203,205-209</sup> Both techniques detect <sup>13</sup>C-<sup>1</sup>H couplings but, for weak samples, HMQC spectroscopy is a better option as the coupling between the carbon nucleus and proton nucleus is detected from the side of the more sensitive proton.<sup>210-212</sup>

The problem of determining the mode of necic acid attachment to a necine base in diesters has also been solved by two dimensional NMR techniques. Both carbon-detected correlation spectroscopy *via* long-range coupling (COLOC) and proton-detected heteronuclear multiple bond connectivity (HMBC) spectroscopy have been used.<sup>76,85,149,159,209</sup> Both experiments detect coupling between a hydrogen and a carbon one or two atoms removed from the one to which the hydrogen is bonded. These techniques therefore show coupling between the carbonyl carbon of the necic acid and the protons on carbons 6, 7 or 9 of the necine base, hence linking the necic acid to the respective site. The proton-detected method is favored due to enhanced sensitivity.<sup>212,213</sup>

Relative stereochemistry of the necine base portion of pyrrolizidine alkaloids has been determined by two dimensional techniques which utilise the nuclear Överhauser effect (NOE), *viz.*, nuclear Överhauser effect spectroscopy (NOESY) and rotating frame nuclear Överhauser effect spectroscopy [ROESY - also know as CAMELSPIN (cross-relaxation appropriate for minimolecules emulated by locked spins)].<sup>149,204,206</sup> The nuclear Överhauser effect is also utilised in NOE-difference spectroscopy<sup>159,214,215</sup> which is a one dimensional technique with faster acquisition times than NOESY or ROESY as only the proton signals of importance are decoupled. Before these more advanced techniques were developed, the necine bases were distinguished, in a standard proton spectrum, by the width, at half-height, of the H-7 signal measured in Hertz<sup>216</sup> and the shift position of the H-6 signal.<sup>57,72,216,217</sup> For retronecine-based pyrrolizidines the H-6 multiplet is downfield of 2.3 ppm, while for heliotrine-based pyrrolizidines it is upfield of this value.

The width of the H-7 signal has been used to distinguish between retronecine **31**- and heliotridine **33**-based alkaloids and between platynecine **25**-, hastanecine **26**- and turneforcidine **27**-based pyrrolizidines.<sup>155,186,194,216,218</sup> For the unsaturated bases, retronecine and heliotridine, values for the width at half-height of 8.8 and 13.9 Hz, respectively, have been reported. For the esterified bases, however, there is an overlap. Culvenor and Woods<sup>216</sup> quote 11 Hz for both retronecine- and heliotridine-based pyrrolizidines and there are similar anomalies when looking at saturated bases. For unesterified platynecine a value of 8.2 Hz has been reported,<sup>216</sup> while for platynecine-based pyrrolizidines some published values include: platyphylline **17** (14.2 Hz),<sup>216</sup> neoplatyphylline **18** (17.4 Hz),<sup>194</sup> sarracine **36** (6.5 Hz),<sup>155</sup> sarracine-4-oxide (13 Hz)<sup>216</sup> and 7-angelylplatynecine **38** (6.4 Hz).<sup>155</sup> The big difference between the values for esterified and non-esterified necine bases is said to be due to the difference between *endo* and *exo* buckling.<sup>216</sup> The H-7 signal for hastanecine **26** has a width at half-height of 12.0 Hz,<sup>194,218</sup> while for hastacine **46**, a hastanecine-based pyrrolizidine, a value of 24 Hz has been reported.<sup>194</sup> With NMR data available for more pyrrolizidine alkaloids, it is now clear that the width of the H-7 signal at half-height is not definitive, and Aasen *et al.*<sup>194</sup> conclude that the half-height width does not assist in arriving at the stereochemistry at C-7 of hastanecine **26** and the amino alcohol from retusine (turneforcidine **27**). For turneforcidine **27** a value of 14.1 Hz has been reported.<sup>218</sup>





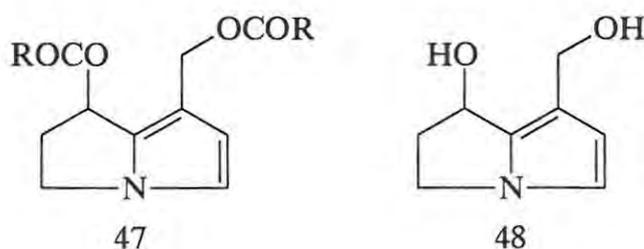
## 1.4 BIOLOGICAL ACTIVITY

### 1.4.1 TOXICITY

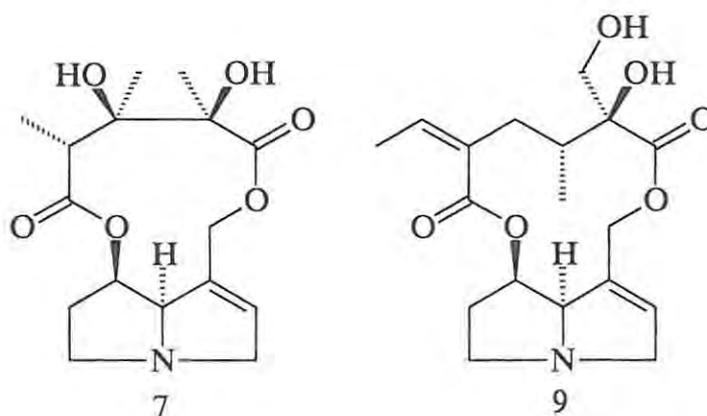
The toxic properties of pyrrolizidine alkaloids prompted the early investigations of plants containing them. Liver damage has been found to be the main characteristic of pyrrolizidine poisoning.<sup>219</sup> Their toxicity to other organs, *viz.*, lungs, heart, kidneys, pancreas, brain and intestines,<sup>220</sup> and their carcinogenicity were only recognised later.<sup>2</sup>

In the liver, the 1,2-unsaturated pyrrolizidine alkaloids are metabolised to cytotoxic pyrrolic compounds by microsomal enzymes. Pyrrolizidine alkaloids containing a saturated necine base, however, are not activated by these enzymes and thus are not

hepatotoxic.<sup>221</sup> The primary metabolites, pyrrolic esters **47**, are more reactive than the secondary metabolites, dehydroaminoalcohols **48**, as they can cause non-specific alkylation of cell constituents leading to necrosis of the liver and to a lesser extent of the lung endothelium.<sup>220,222</sup> Horses and cattle are more susceptible than sheep and goats to the toxic effects of pyrrolizidine alkaloids.<sup>2,28</sup>



Pyrrolizidine alkaloids have also been shown to cause pulmonary hypertension and arteritis which can lead to chronic heart damage.<sup>220</sup> Rats dosed with monocrotaline **7** have developed necrosis of the renal tubes, while monkeys dosed with retrorsine **9** have developed severe toxic nephritis with damaged glomeruli.<sup>220</sup> Other pyrrolizidines have destroyed pancreatic cells and caused the spongy degeneration of calf and sheep brains.<sup>220</sup> In northern Queensland, Australia, oesophageal disease in horses (or Chillagoe horse disease) has resulted from pyrrolizidine alkaloid ingestion, which causes ulceration of the oesophagus and squamous mucosa of the stomach.<sup>28</sup>

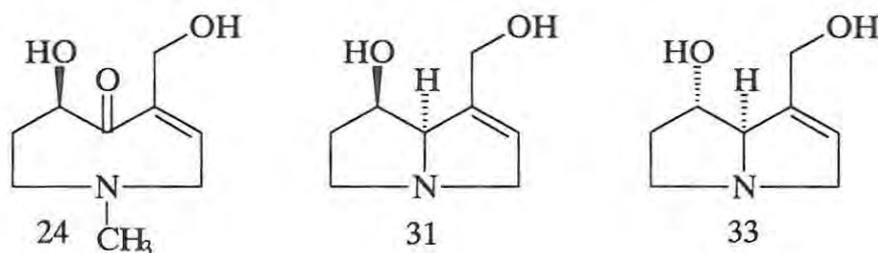


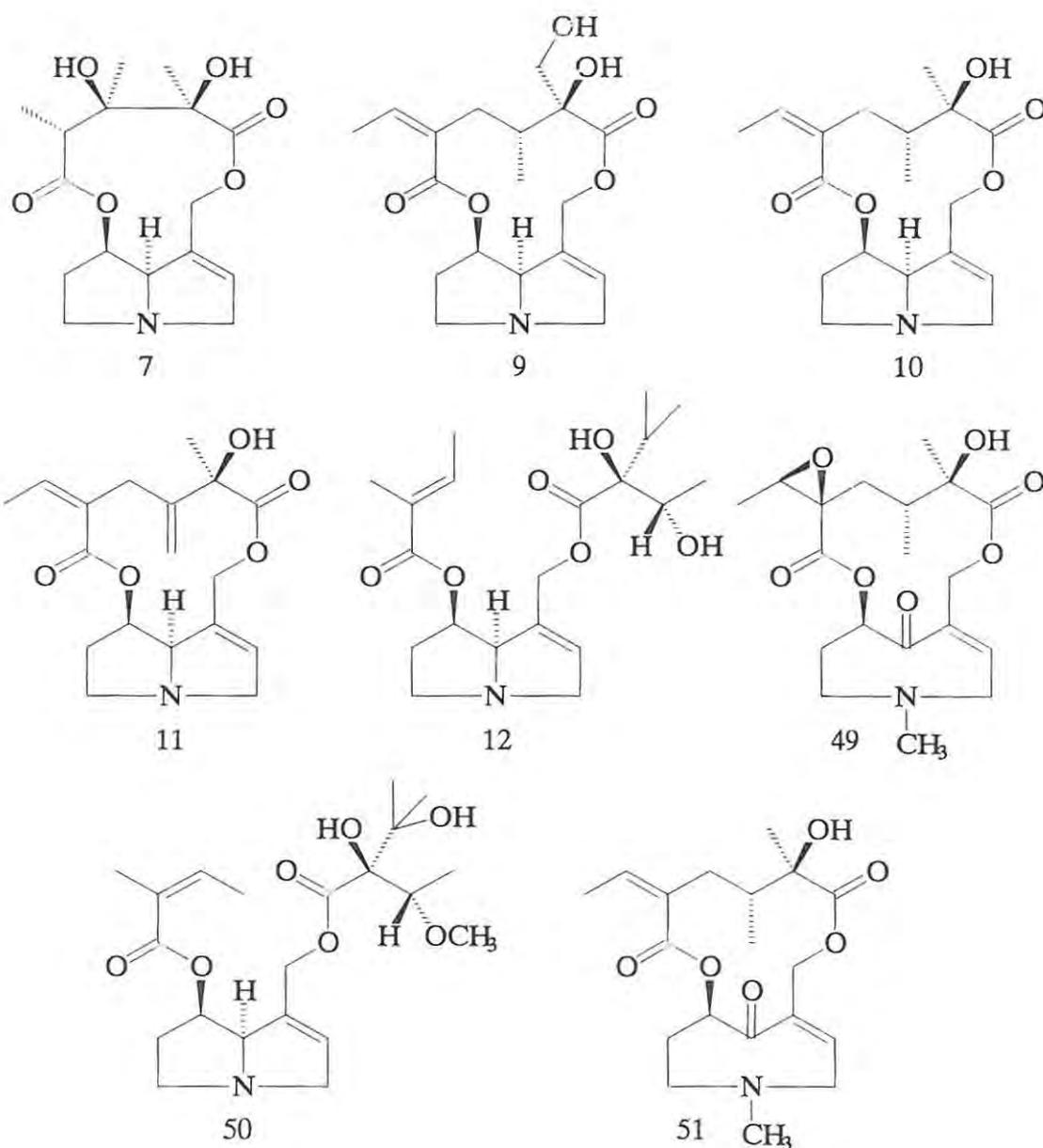
### 1.4.2 CARCINOGENICITY AND ANTITUMOUR PROPERTIES

In the early 1970's pyrrolizidine alkaloids were found to be carcinogenic.<sup>223</sup> As it was known that some foodstuffs, such as honey and milk,<sup>224-226</sup> contained pyrrolizidine alkaloids, a spate of investigations followed.

Hepatic enzymes convert pyrrolizidine alkaloids to cytotoxic metabolites, primarily, highly toxic pyrrolic esters. The tumours appear at the liver and not at the site of pyrrolizidine alkaloid application. The carcinogens are, therefore, the metabolites produced in the liver.<sup>227</sup>

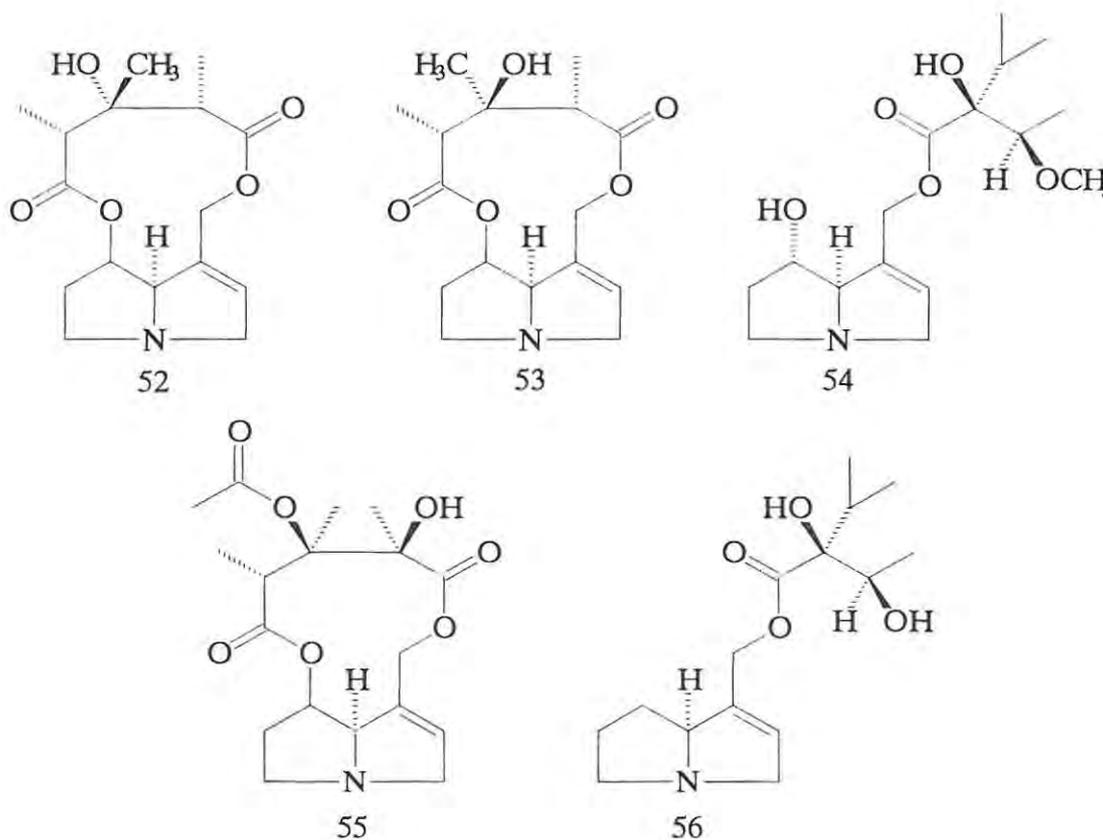
Macrocyclic or acyclic diester pyrrolizidine alkaloids containing otonecine **24**, retronecine **31** or heliotridine **33** are hepatocarcinogenic.<sup>228</sup> Thus, monocrotaline **7**, retrorsine **9** and its *N*-oxide, senecionine **10**, seneciophylline **11**, symphytine **12**, petasitenine **49**, lasiocarpine **50** and senkirkine **51** have caused liver tumours and carcinomas.<sup>229</sup> Some pyrrolizidine alkaloids are also known to cause tumours in the pancreatic tissue or in the central nervous system.<sup>230</sup>





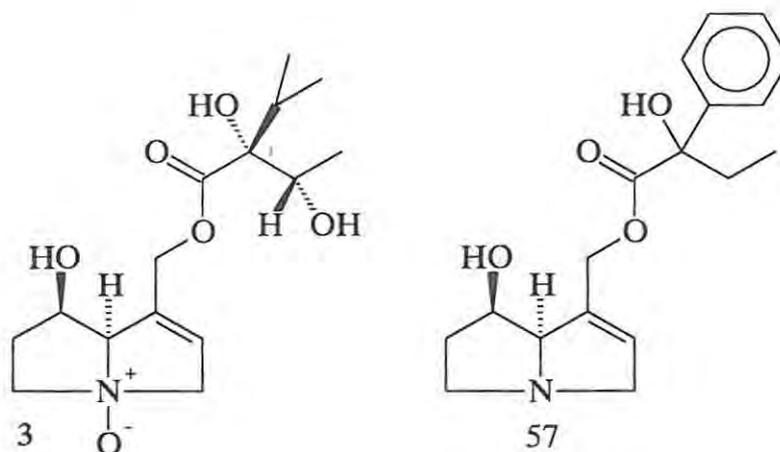
Interestingly, a number of pyrrolizidine alkaloids exhibit antitumour activity. In studies in which 18 pyrrolizidine alkaloids were tested, nine were active against one or more of the four tumour systems, adenocarcinoma 755, sarcoma 180, Walker 256 intramuscular and Walker 256 subcutaneous.<sup>28</sup> The nine alkaloids are monocrotaline **7**, senecionine **10**, lasiocarpine **50**, crispatine **52**, fulvine **53**, heliotrine **54** and its *N*-oxide, spectabiline **55**

and supinine **56**. Structure activity relationship studies have shown that the antitumour activity is not dependent on the allylic ester.<sup>231</sup>



Extracts of *Heliotropium indicum* were effective against Walker 256 tumours in rats and against leukaemia 1210 in mice.<sup>232</sup> The active component was found to be indicine-4-oxide **3**. To date, indicine-4-oxide is the only pyrrolizidine alkaloid to have undergone clinical tests.<sup>233</sup> Twenty nine human patients with advanced solid tumours were dosed with indicine-4-oxide, but showed no objective therapeutic response.<sup>234</sup> The major toxic effect was myelosuppression (bone marrow suppression). Indicine-4-oxide was also tested in ten people with advanced leukaemia, two of whom showed complete remission and one partial remission.<sup>235</sup> Once again the toxic effects were myelosuppression and jaundice. The mechanism of antitumor action of indicine-4-oxide is unknown.<sup>233</sup>

A semisynthetic pyrrolizidine, 9-(2-hydroxy-2-phenylbutyryl)-retronecine **57**, which is structurally similar to indicine-4-oxide **3**, is claimed to be more active against P388 lymphatic leukaemia.<sup>236</sup>



Other pyrrolizidine alkaloids are being tested for antitumour properties by the National Cancer Institute in the United States of America.<sup>29</sup>

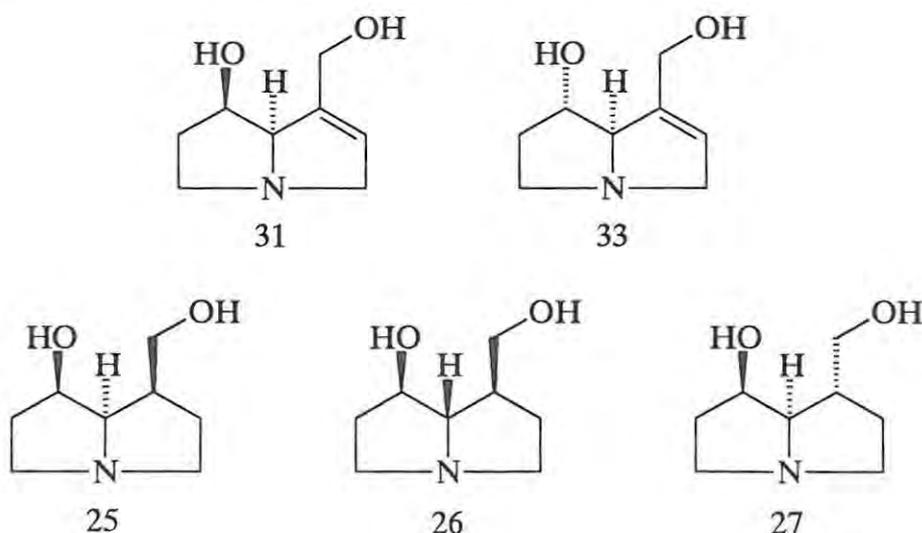
## 1.5 SYNTHESIS OF PYRROLIZIDINE ALKALOIDS

Pyrrolizidine alkaloids often need to be synthesised to confirm their stereochemistry. NMR techniques, for example NOE-difference spectroscopy, may permit the relative stereochemistry to be determined, but synthesis is often required for establishing absolute stereochemistry. Furthermore, only very small quantities of alkaloid are generally available from natural sources so, for further investigation of the metabolism and biological activity, synthesis of larger quantities may be required. The toxicity of natural pyrrolizidine alkaloids tends to outweigh their beneficial properties. Consequently, less toxic derivatives which keep the beneficial properties are being synthesised. Mattocks<sup>237</sup> reviewed the synthesis of pyrrolizidine alkaloids until 1985, while Robins' annual reviews<sup>238-252</sup> have reviewed the synthesis of pyrrolizidines to June 1994.

Pyrrolizidine alkaloids consist of a necine base and one or two necic acids. In the brief overview which follows, the synthesis of the two parts will be discussed separately followed by various coupling techniques.

### 1.5.1 SYNTHESIS OF NECINE BASES

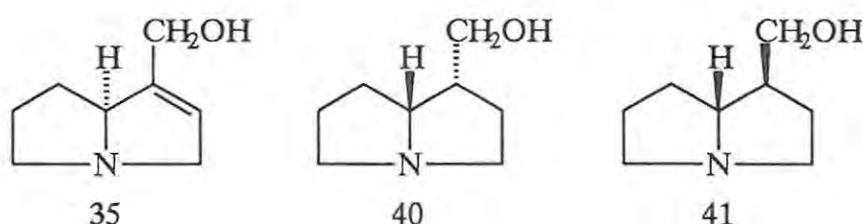
As can be expected the synthesis of a necine base can be very challenging, particularly if the stereochemistry is taken into account. Some bases differ only in stereochemistry (compare retronecine **31** with heliotridine **33**, and platynecine **25** with hastanecine **26** and turneforcidine **27**). There are two approaches to necine bases; an *ab initio* approach using acyclic or simple monocyclic precursors and a semi-synthetic approach utilising a readily available pyrrolizidine alkaloid.



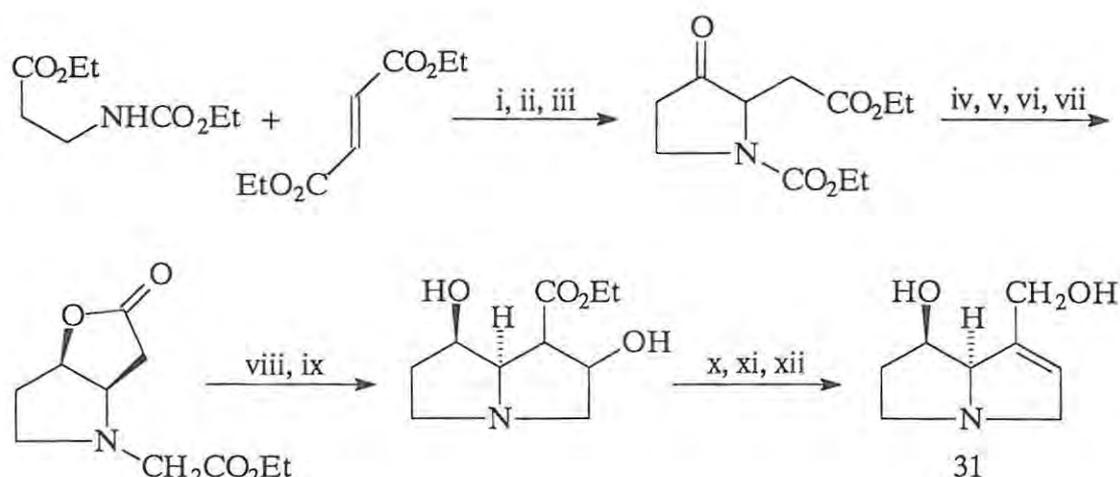
#### 1.5.1.1 AB INITIO SYNTHESIS

*Ab initio* syntheses often involve numerous steps and, over the years, a wide range of starting materials have been used. The stereochemistry, as mentioned earlier, is very important but many of the published syntheses produce both optical isomers of a necine base. As a result there is a continuing search for stereospecific procedures. The synthesis of optically active necine bases using *L*-proline derivatives, optically active malic acids, carbohydrates and chiral auxiliaries was reviewed by Dai *et al.*<sup>253</sup> in 1990. Many

syntheses for the simpler necine bases having only one hydroxyl group have been reported; such target molecules include supinidine **35**, isoretronecanol **40**, and trachelanthamide **41**. Those with more than one hydroxyl group are synthesised less frequently. Proline derivatives are often used to provide one of the five membered rings of the necine base.<sup>254-266</sup> The proline is then bonded to a carbon chain which cyclises to form the other half of the necine base.<sup>267-274</sup> Due to their low cost, availability, and precise stereochemistry, carbohydrates are being used increasingly as starting materials.<sup>275-277</sup>



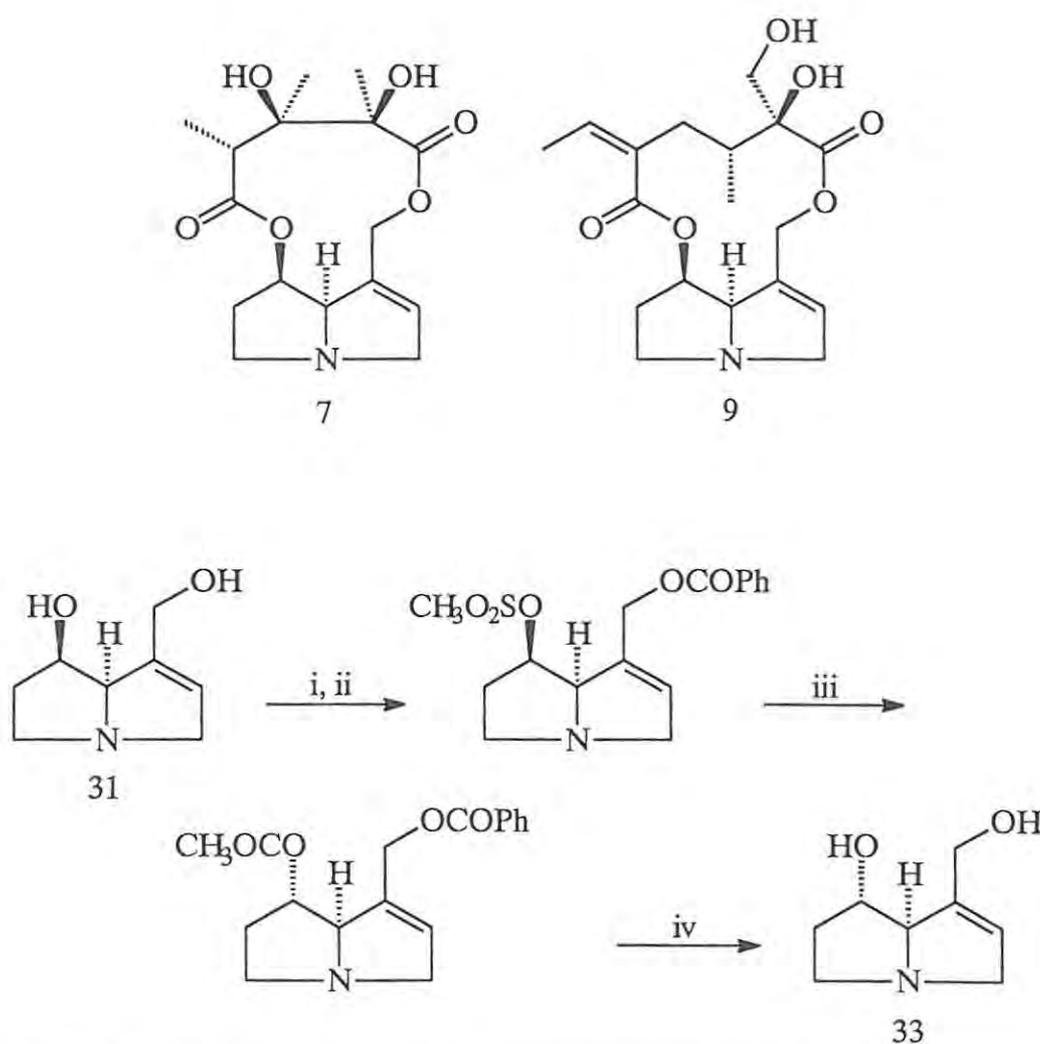
Another important starting material for the necine base is the Geissman-Waiss lactone, notable in its use in the very first synthesis of (+)-retronecine **31** (Scheme 1.7).<sup>278</sup> There are now many paths to the Geissman-Waiss lactone and its derivatives.<sup>245,247,256,259,263,279,280</sup>



**Scheme 1.7:** Reagents: i, Na; ii, HCl, H<sub>2</sub>O; iii, EtOH, HCl; iv, NaBH<sub>4</sub>; v, Ba(OH)<sub>2</sub>; vi, HCl; vii, BrCH<sub>2</sub>CO<sub>2</sub>Et; viii, KOEt; ix, H<sub>2</sub>, Pt, CH<sub>3</sub>CO<sub>2</sub>H; x, Ba(OH)<sub>2</sub>; xi, EtOH, HCl; xii, LiAlH<sub>4</sub>.

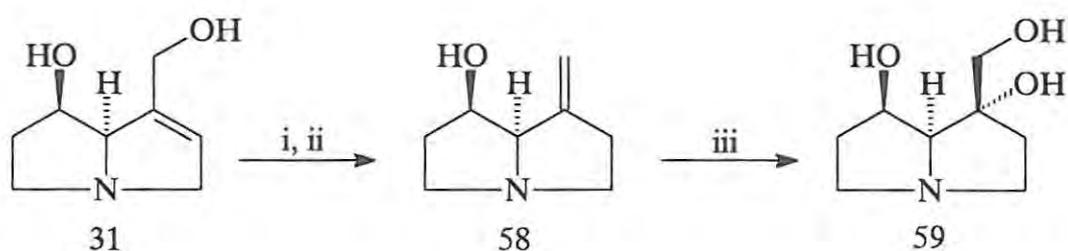
## 1.5.1.2 MODIFICATION OF AN EXISTING SYSTEM

Retronecine **31**, which is common to many pyrrolizidine alkaloids, has often been modified to produce other necine bases. It can be synthesised from simple precursors, but is regularly obtained by saponification of a macrocyclic, retronecine-containing pyrrolizidine alkaloid (*e.g.* monocrotaline **7** or retrorsine **9**) using a base such as barium hydroxide.<sup>202,208,236,281-284</sup> Once obtained, retronecine can then be altered to produce a variety of other necine bases. The stereochemistry at carbon-7 of retronecine **31** can be inverted as in the synthesis of heliotridine **33** (Scheme 1.8).<sup>285</sup>

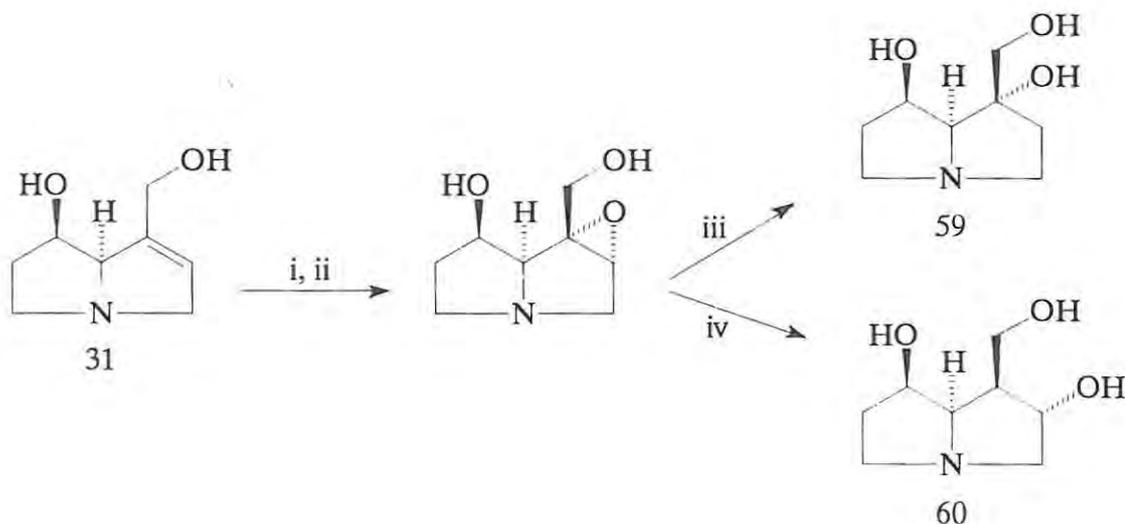


**Scheme 1.8:** Reagents: i,  $\text{PhCO}_2\text{H}$ ,  $N,N'$ -carbonyldiimidazole, THF, room temperature, 16 h; ii,  $\text{CH}_3\text{SO}_2\text{Cl}$ ,  $\text{Et}_3\text{N}$ ,  $\text{CH}_2\text{Cl}_2$ ,  $-2^\circ\text{C}$ , 1 h; iii,  $\text{CH}_3\text{CO}_2\text{Cs}$ , dimethylformamide; iv,  $\text{Ba}(\text{OH})_2$ , room temperature.

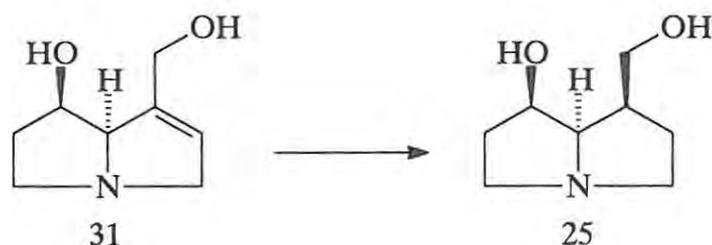
Rearrangement of the double bond in retronecine **31** from an endocyclic to an exocyclic position has recently been carried out by Hanselmann and Benn<sup>283</sup> to produce 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58**, which was then converted to hadinecine **59** (Scheme 1.9). The same authors have also synthesised hadinecine **59** and rosmarinecine **60** via the 1,2-epoxide of retronecine (Scheme 1.10).<sup>283</sup> Platynecine **25** (Scheme 1.11) has been synthesised from retronecine **31** by catalytic hydrogenation,<sup>286</sup> while free radical deoxygenation of retronecine **31** (Scheme 1.12) results in the production of supinidine **35**.<sup>287</sup>



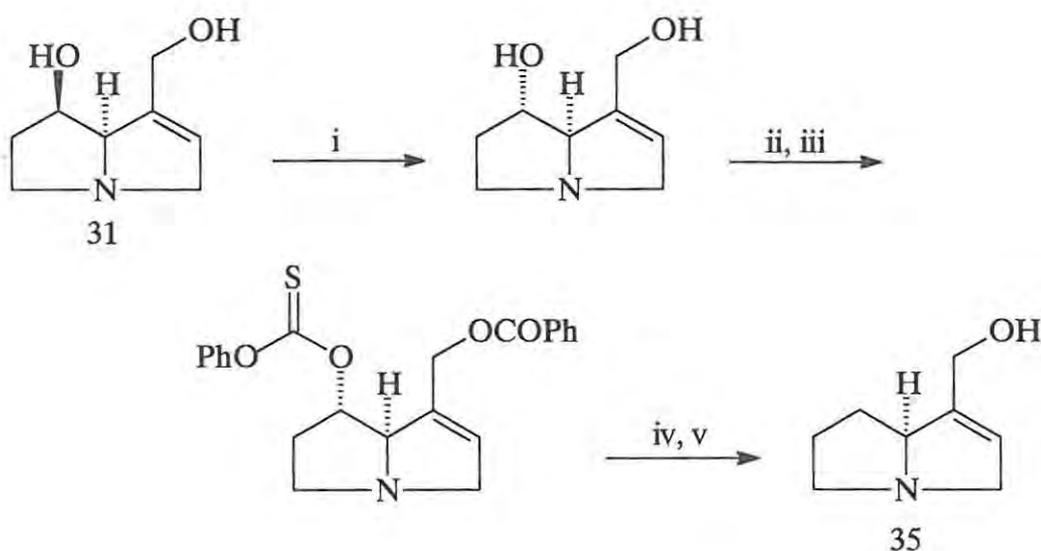
**Scheme 1.9:** Reagents: i,  $\text{SOCl}_2$ ; ii, Zn,  $1\text{M H}_2\text{SO}_4$ ; iii,  $\text{OsO}_4(\text{cat})$ , 4-methyl morpholine-*N*-oxide, acetone,  $\text{H}_2\text{O}$ .



**Scheme 1.10:** Reagents: i, *meta*-chloroperbenzoic acid; ii, Zn,  $\text{CH}_3\text{CO}_2\text{H}$ ; iii, catalytic hydrogenation; iv, diisobutylaluminium hydride.



**Scheme 1.11:** Reagents:  $H_2$ (1 atm), Pd-charcoal.

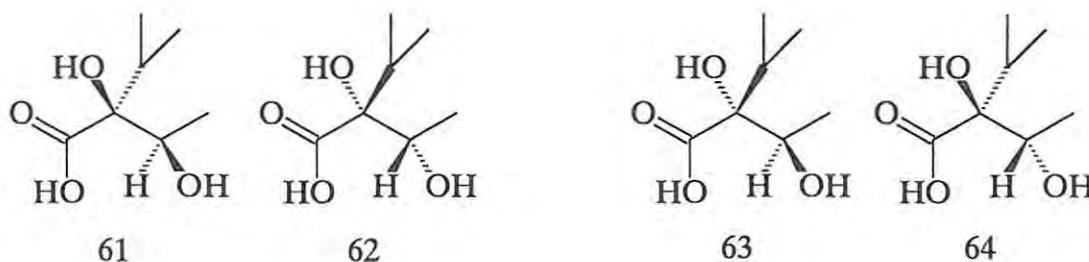


**Scheme 1.12:** Reagents: i, see Scheme 1.8; ii,  $PhCO_2H$ ,  $N,N'$ -carbonyldiimidazole, THF; iii,  $PhOCSCl$ , 4,4-dimethylaminopyridine,  $CH_3CN$ ; iv,  $n-Bu_3SnH$ , *azo-bis-isobutyronitrile*,  $PhCH_3$ ,  $75^\circ C$ , 3 h; v,  $NH_4F$   $CH_3OH$ ,  $60^\circ C$ , 18 h.

### 1.5.2 SYNTHESIS OF NECIC ACIDS

There are a wide variety of necic acids. They can be obtained directly from the parent pyrrolizidine alkaloids by hydrolysis or hydrogenolysis,<sup>288</sup> the latter approach, however, may be complicated by the formation of lactones and decomposition products. Necic acids are branched carbon chains with hydroxy, methoxy and acetoxy substituents. Since they may contain one or more chiral centres and/or double bonds, isomers are possible. One

of the main aims when synthesising necic acids is stereoselectivity, *e.g.* Kochetkov's<sup>289</sup> synthesis of all four isomers of 2-isopropyl-2,3-dihydroxybutyric acid, the [2*S*,3*R*] and [2*R*,3*S*] isomers of which are (+)- and (-)-trachelanthic acid (**61** and **62**), respectively, while the [2*R*,3*R*] and [2*S*,3*S*] isomers are (+)- and (-)-viridifloric acid (**63** and **64**). A number of pyrrolizidine alkaloids contain trachelanthic and viridifloric acid but until Kochetkov's synthesis their absolute stereochemistry was uncertain. Since then other shorter syntheses of one or more of the isomers have been realised.<sup>290-293</sup> As many of the acids are  $\gamma$ - or  $\delta$ -hydroxyacids they are isolated as the corresponding  $\gamma$ - or  $\delta$ -lactones, which are then used for coupling to the desired necine base.<sup>239,294,295</sup>



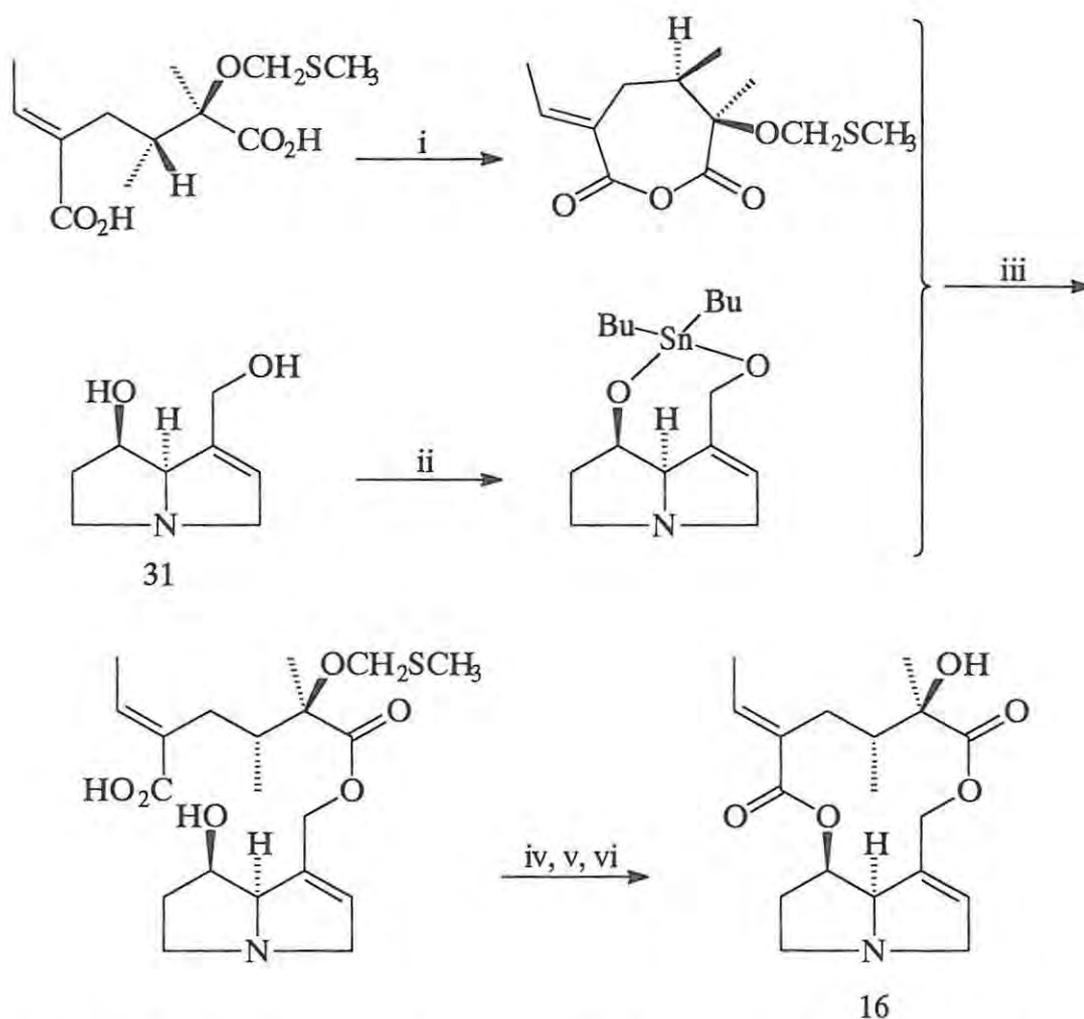
### 1.5.3 COUPLING REACTIONS

A variety of coupling reagents have been used in pyrrolizidine alkaloid syntheses to join a necine base to a necic acid. The 9-chloro necine base has been reacted with the sodium salt of the acid.<sup>281,296,297</sup> Diesters of retronecine have been synthesised by mixing retronecine with excess necic acid chloride.<sup>298</sup> Selective coupling to the 9-position of the necine base has been achieved using *N,N'*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP).<sup>202,292,293</sup> *N,N'*-carbonyldiimidazole (CDI) has been more successful as a selective coupling reagent for the synthesis of 9-monoesters.<sup>202,236,299</sup>

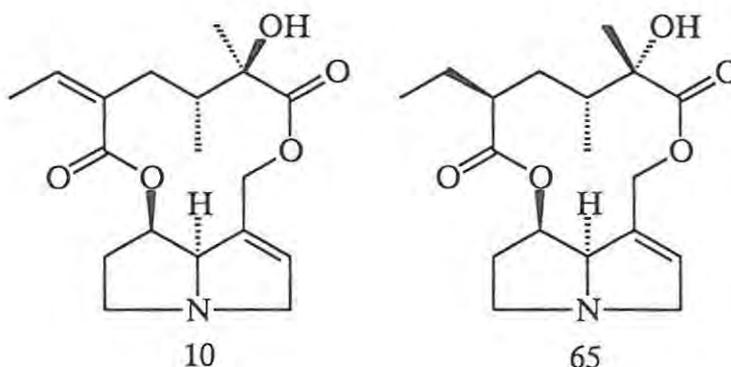
During coupling protection of the hydroxyl groups of the necic acid or selective protection of one or other of the hydroxyl groups of a dihydroxy necine base may be required.<sup>293,294,300</sup> The protection is to ensure regioselective coupling of an unsymmetrical diol to an unsymmetrical dicarboxylic acid and thus decrease the chance of having a

mixture of products. Such protection is not always essential as the allylic alcohol is more reactive than the secondary alcohol.

A modified form of the necic acid can be used instead of a protecting group to ensure regioselectivity. Niwa *et al.*<sup>300</sup> coupled a cyclic anhydride derivative of integerrinecic acid with a stannoxane obtained from retronecine to synthesise integerrimine **16** (Scheme 1.13). This method ensured regioselective coupling. A similar approach was used for the synthesis of senecionine **10**<sup>301</sup> and yamataimine **65**.<sup>302</sup>



**Scheme 1.13:** Reagents: i, *N,N'*-dicyclohexylcarbodiimide,  $\text{CH}_2\text{Cl}_2$ ; ii,  $\text{Bu}_2\text{SnO}$ , benzene, reflux; iii,  $5^\circ\text{C}$  to room temperature; iv, 2,4,6-trichlorobenzoylchloride,  $\text{Et}_3\text{N}$ , THF; v, 4,4-dimethylaminopyridine, reflux; vi,  $\text{Ph}_3\text{C}\cdot\text{BF}_4$ ,  $\text{CH}_2\text{Cl}_2$ .



## 1.6 AIMS OF THE PRESENT STUDY

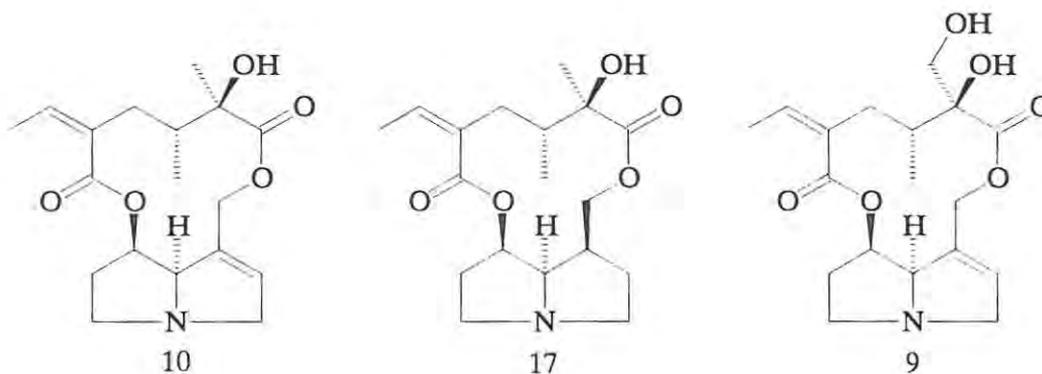
The present study aimed to isolate, identify, and compare the pyrrolizidine alkaloid content of *Senecio chrysocoma* and *S. paniculatus* as the two species have often been confused botanically. It was intended that the results would be of taxonomic assistance as pyrrolizidine alkaloids have previously been used as taxonomic markers in the *Symphytum*<sup>93</sup> and *Amsinkia*<sup>303</sup> species. During the course of the study the novel structured 7-angelyl-1-methylenepyrrolizidine alkaloids were identified and a synthesis of 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine was undertaken to confirm the stereochemistry.

An ancillary part of the project was the reconstruction, major updating, and completion of what was a skeleton database of published physical data on pyrrolizidine alkaloids.

## 2. SENECIO CHRYSOCOMA AND SENECIO PANICULATUS

Both *Senecio chrysocoma* and *S. paniculatus* grow on recently disturbed ground and are, therefore, found beside roads, in recently burnt areas, and on agricultural land. The pyrrolizidine alkaloid content of the plants can be used as an indication of their possible toxicity to livestock. There is also some discussion amongst botanists as to whether they are in fact separate species, and an investigation into the pyrrolizidine alkaloid content of both species should show the chemical differences and similarities between the two species.

*S. paniculatus* has been reported to contain the macrocyclic pyrrolizidine alkaloids, senecionine **10** and platyphylline **17**,<sup>31,33</sup> while *S. graminifolius*, synonymous with *S. chrysocoma* (see Section 2.1, p. 46), has been reported to contain retrorsine **9**.<sup>304</sup> *S. chrysocoma* had, therefore, initially been extracted as a potential source of macrocyclic pyrrolizidine alkaloid GC-MS standards.<sup>305</sup> However, no macrocyclic alkaloids had been found,<sup>152</sup> so a full investigation of the alkaloidal constituents was undertaken.



## 2.1 A BOTANICAL COMPARISON OF THE TWO SPECIES, *SENECIO CHRYSOCOMA* AND *SENECIO PANICULATUS*

The National Botanical Institute (NBI) of South Africa now views *Senecio chrysocoma* Meerb. and *Senecio paniculatus* Berg. as two different, yet closely related, species.<sup>306</sup> In 1894 *S. paniculatus* Berg. was described with three variants: *peucedanifolius*, *intermedius*, and *reclinatus*.<sup>307</sup> *S. paniculatus* Berg. var. *reclinatus* was also known as *S. chrysocoma* Meerb.<sup>307</sup> *S. chrysocoma* Meerb. was described as a separate species, in 1973, by Hilliard and Burt<sup>308</sup> although they gave *S. reclinatus* Linn. f., *S. graminifolius* Jacq., *S. paniculatus* Berg. var. *reclinatus* (Linn. f.), *S. paniculatus* auct. and *Jacobaea reclinata* (Linn. f.) Thunb. as synonymous names for the species. *S. chrysocoma* has been described as having entire linear to linear-lanceolate leaves and discoid flower heads (9-11 mm) with white, cream or yellow flowers (but mostly yellow).<sup>306,309</sup> *S. paniculatus*, on the other hand, has linear and entire to pinnatipartite leaves even on the same plant. The flower heads are discoid (6-8 mm) and also white, cream or yellow, although most of the NBI specimens are described as having white flowers.<sup>306</sup> The NBI records indicate that *S. chrysocoma* is found east of Uniondale, in the Western Cape, to southern Kwazulu-Natal, South Africa, while *S. paniculatus* is found to the west of Uniondale to the west coast of the Western Cape (See Figure 2.1).<sup>306,309</sup>

## 2.2 PLANT COLLECTIONS

In total, nine populations of plants were collected and extracted (see Figure 2.1): three east of Uniondale - at Grahamstown, at Trifolia near Humansdorp, and at the Port Elizabeth municipal water works' Van Stadens River dams; three west of Uniondale - on the farm Assegaaibos near Riversdale and at the summits of the Robinson and Tradouw Passes; and three from the east-west distribution borderline - near the Dieprivierhoogte picnic spot, beside the Wittedrif road, and alongside the Prince Alfred Pass. Specimen samples of the plants collected were identified by the staff of the Selmar Schönland Herbarium, Grahamstown, and Pauline Bohnen, a botanist in Riversdale.

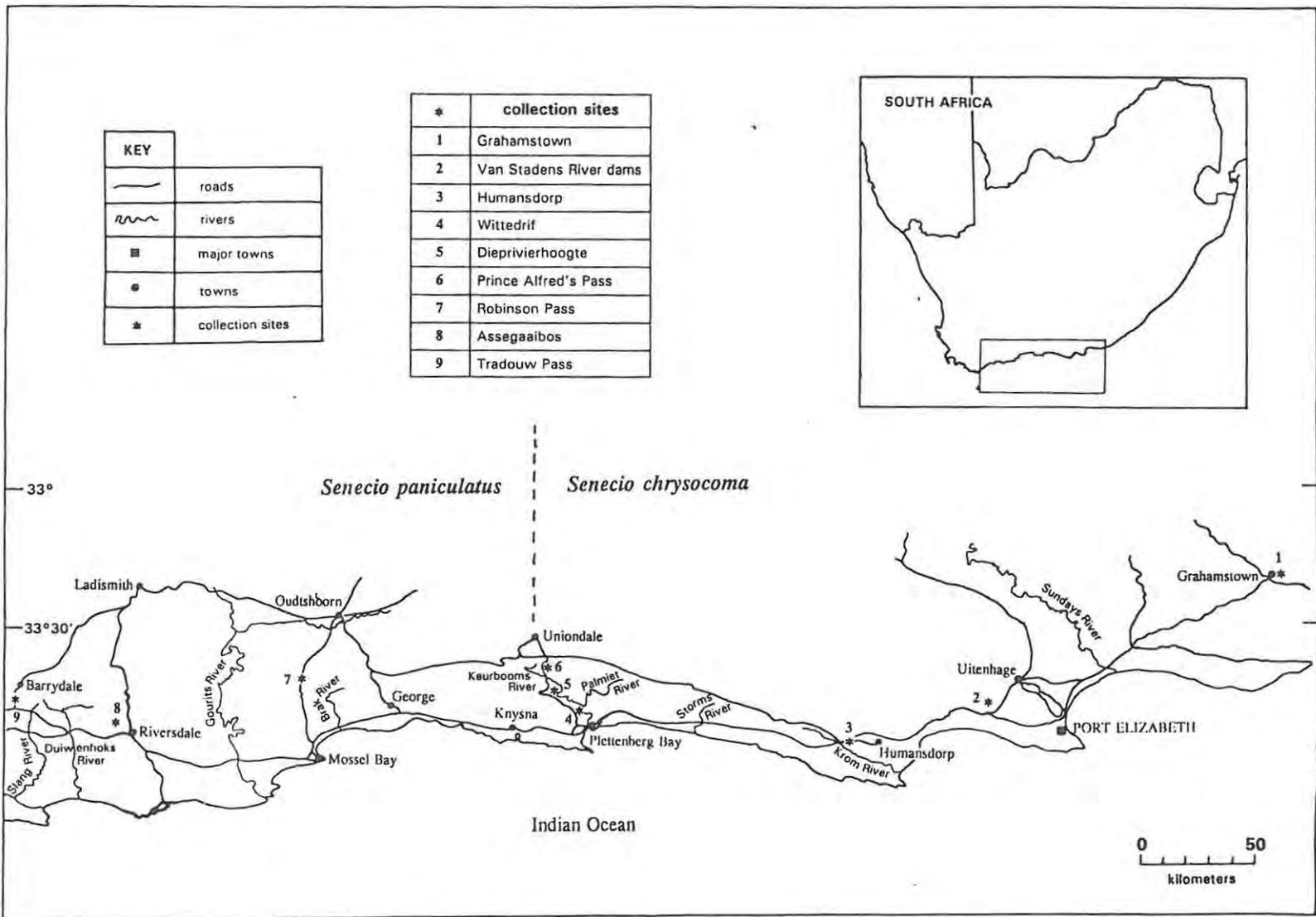
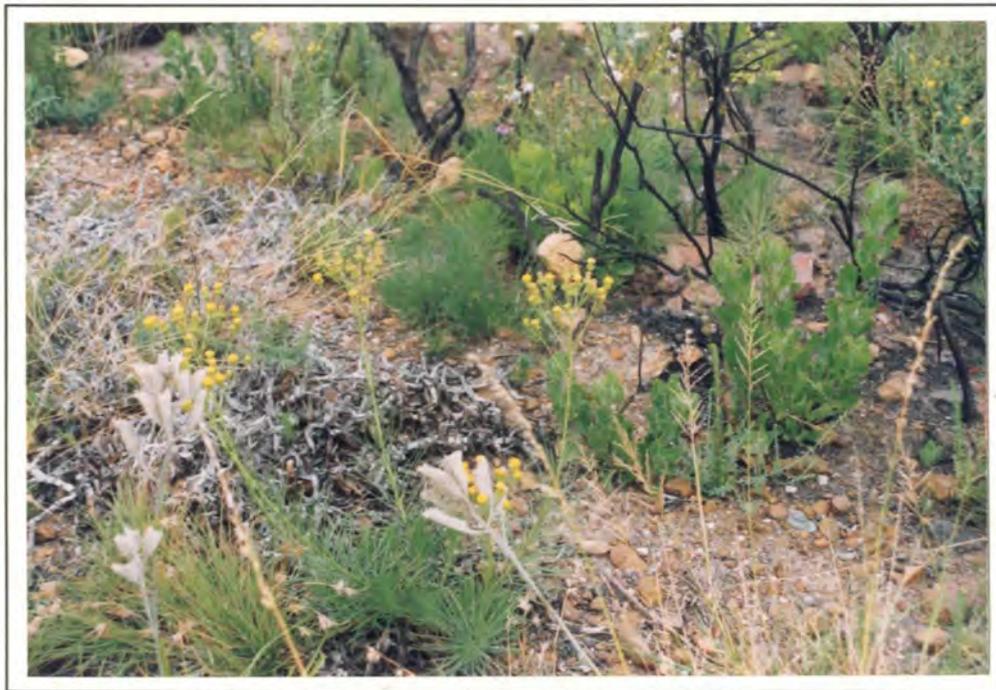


Figure 2.1: A map showing the distribution of *S. chrysocoma* and *S. paniculatus* and the sites at which plants were collected. (Drawn by B. C. Logie)

Those collected east of Uniondale and on the borderline were identified as *S. chrysocoma*. They had the entire linear-lanceolate leaves and those which were in flower had yellow disc florets (Figure 2.2). The three populations collected west of Uniondale were identified as *S. paniculatus*. The Assegaaibos population had finished flowering so the colour of the flowers is unknown, but the Robinson Pass population had yellow disc florets, while the Tradouw Pass population had white disc florets (Figure 2:3). All three had pinnatipartite leaves.



**Figure 2.2:** *Senecio chrysocoma*, with yellow disc florets and linear-lanceolate leaves. (Photograph: B.A. Logie)



**Figure 2.3:** *Senecio paniculatus*, with white disc florets and pinnatifid leaves. (Photograph: Caryl G. Logie)

The Grahamstown, Humansdorp and Van Stadens River dam populations were collected from recently burnt (within 18 months) areas. The Van Stadens River dam site was a felled pine tree forest and, at the time of collection, had horses grazing on it. The Assegaaibos population was gathered on land that supported cattle. The remaining populations were collected beside roads. All plants, therefore, were collected in regenerating environments, however, *S. paniculatus* plants were found in slightly more overgrown regions than the *S. chrysocoma* plants (Figure 2.4). Although some of the plants were collected where animals were grazing, there was little danger of intoxication since as will be shown later in this chapter, only traces of hepatotoxic pyrrolizidine alkaloids were found, and then only in *S. paniculatus* extracts.

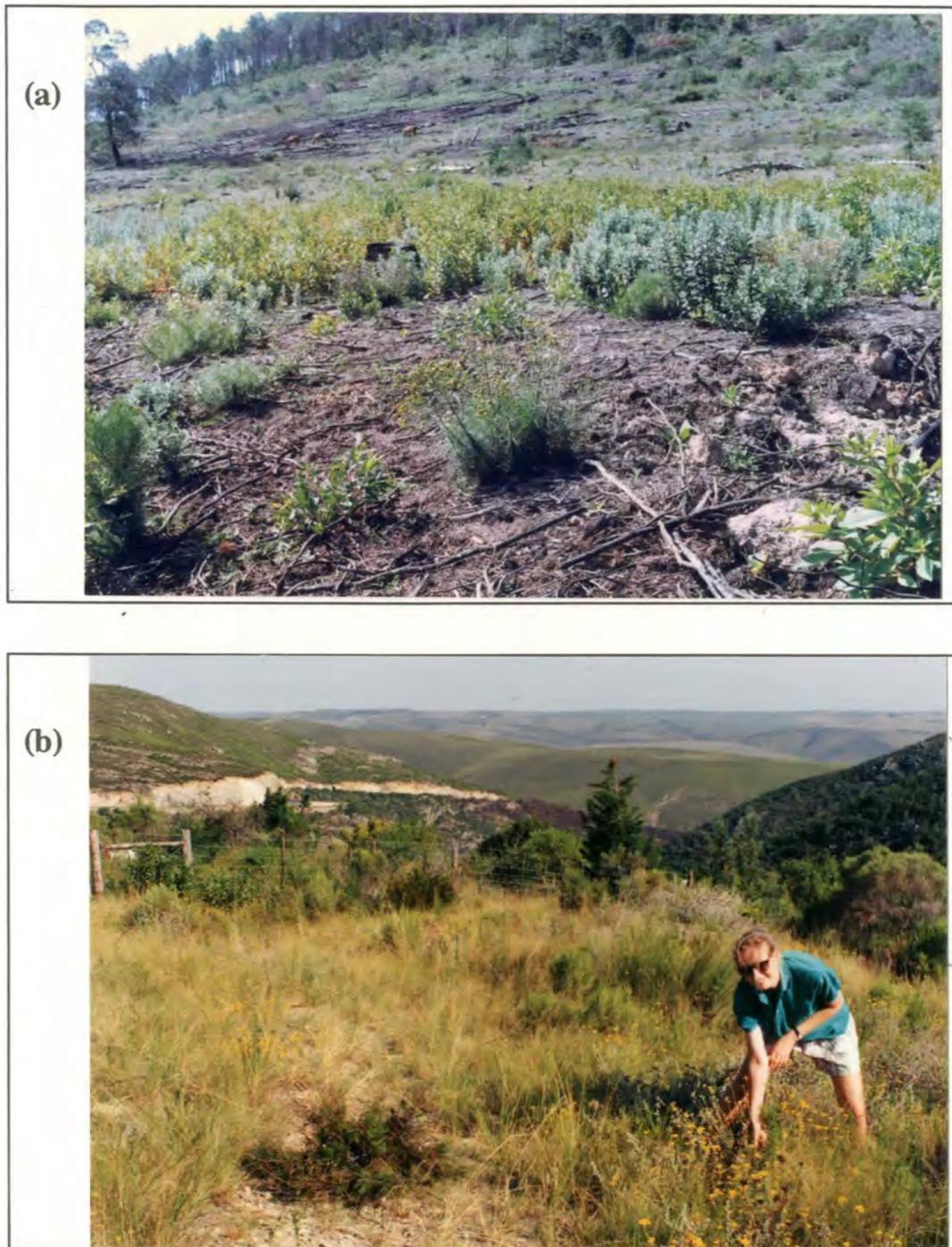


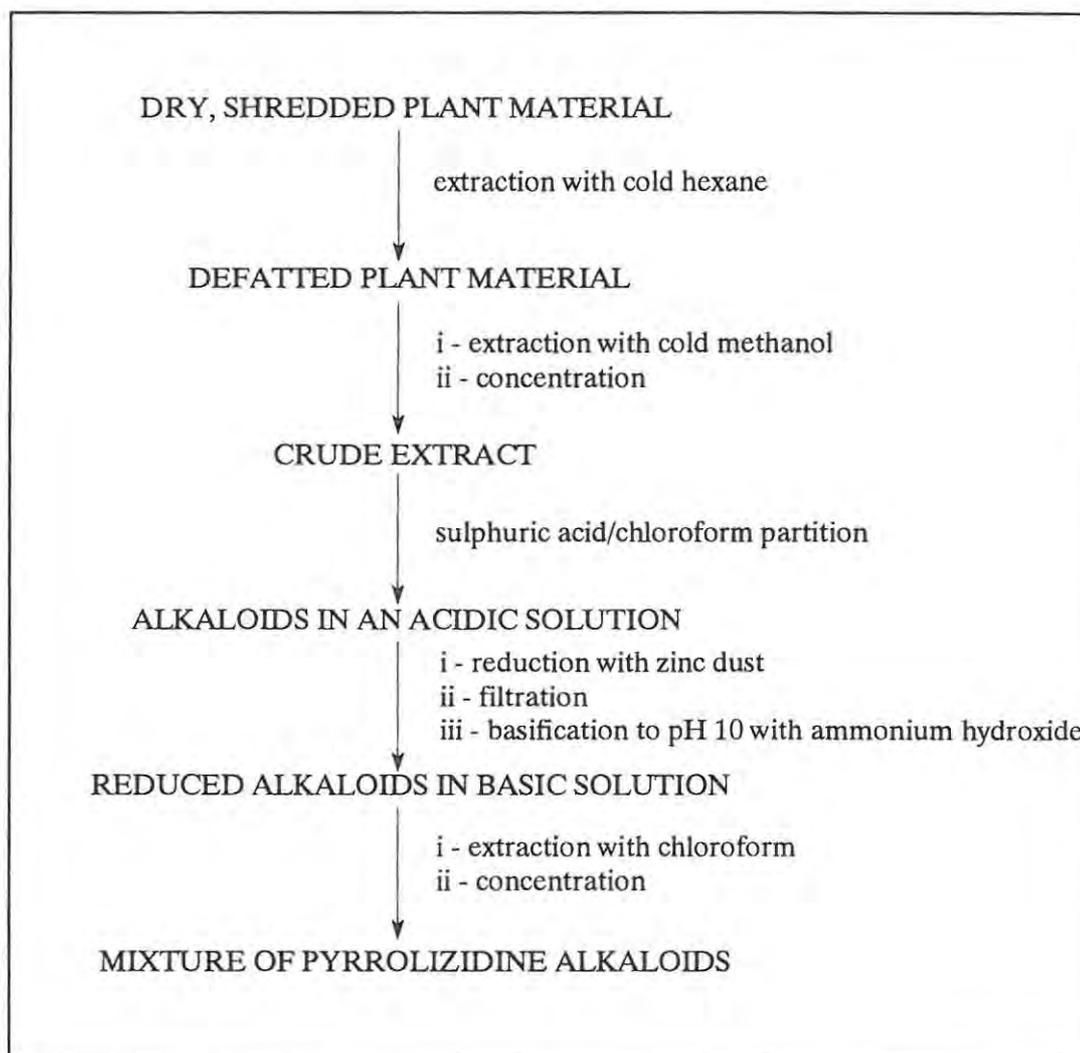
Figure 2.4: Examples of the typical environments in which (a) *S. chrysocoma* plants (Van Stadens River dams), and (b) *S. paniculatus* plants (Tradouw Pass) were found. (Photographs: Caryl G. Logie)

## 2.3 EXTRACTION OF THE PYRROLIZIDINE ALKALOIDS

The standard procedure (Figure 2.5), described in Section 5.3.1 (p. 137), was used for the extraction of the pyrrolizidine alkaloids from the nine plant populations. Soaking in cold solvents was chosen above continuous soxhlet extraction as the latter tended to cause charring. The extraction to remove waxes and chlorophylls can be delayed and the methanol extract washed with hexane or petroleum ether,<sup>71,75,185</sup> but it made more sense to remove as much non-alkaloidal material as possible before extracting the pyrrolizidines. The crude, tarry mixtures, obtained after concentrating the methanolic extract, are typically dissolved in either an acidic (sulphuric or hydrochloric acid) or an organic solvent (chloroform, dichloromethane or ethyl acetate) before continuing with the aqueous/organic separations.<sup>70</sup> In the present study, however, a large proportion of the crude mixture would not dissolve when only one solvent was used, so a 50:50 mixture of 2M-sulphuric acid and chloroform was used.

As pyrrolizidine alkaloid *N*-oxides are not particularly soluble in organic solvents, they were reduced with zinc dust prior to extraction of the alkaloids with chloroform. The most convenient way to do this was to add zinc dust to the acidic solution and stir overnight, but occasionally a shorter time sufficed. Despite the reduction step, two *N*-oxides were identified in this study. They were either not reduced, but still extracted, or they reformed after extraction. It is possible that they would be extracted even if they were not reduced as it has been shown that some *N*-oxides are soluble in organic solvents.<sup>70</sup> On the other hand, if the *N*-oxides reformed after extraction there should be a steady, noticeable increase in the *N*-oxide concentration with time, but this was not observed.

After the zinc reduction, the solution was filtered to remove excess zinc and the pH adjusted to 9 with concentrated ammonium hydroxide. In a few cases an emulsion developed at this pH which disappeared if the pH was increased to 10 or 11 by adding more ammonium hydroxide.



**Figure 2.5:** A general approach to the extraction of the pyrrolizidine alkaloids.

It was found that a re-extraction helped to clean the crude pyrrolizidine alkaloid mixture further before separation. The re-extraction involved dissolving the crude mixture in a small volume of chloroform, extracting with acid, washing the acidic solution with chloroform before increasing the pH to 10 and then extracting the pyrrolizidine alkaloids back into chloroform. This alkaloidal mixture was then separated to yield, as cleanly as possible, the individual pyrrolizidine alkaloids.

## 2.4 SEPARATION AND PURIFICATION OF THE PYRROLIZIDINE ALKALOIDS

Of the many techniques which have been used for the separation and purification of pyrrolizidine alkaloid extracts, the use of three was explored in this study and, ultimately, only two were used as droplet counter current chromatography proved unsuccessful. These two techniques, flash column and preparative layer chromatography (PLC) permitted adequate separation of the alkaloids. However, it was not possible to separate completely isomers that differed only in the geometry of a double bond in the necic acid portion of the pyrrolizidine. Angelic acid esters were always slightly contaminated by the tiglic acid equivalent, and sarracine and neosarracine could never be totally separated. Stelljes *et al.*<sup>68</sup> have noted the spontaneous conversion of angelates to tiglates during storage.

All the fractions obtained were analysed by TLC as a primary check of the degree of purification. Silica gel plates were used with a chloroform-methanol-25% ammonium hydroxide solution (85:14:1) solvent system. Developed plates were viewed under ultraviolet light and then generally sprayed with Dragendorff's reagent. Dragendorff's reagent was chosen in preference to Ehrlich's reagent for several reasons. Staining with Ehrlich's reagent was more time-consuming as the plates had to be sprayed first with an *o*-chloranil solution and then with Ehrlich's reagent, with warming in an oven after each application.<sup>135</sup> Saturated pyrrolizidine alkaloids do not stain with Ehrlich's reagent<sup>136</sup> (and most of the pyrrolizidine alkaloids identified in this investigation were saturated), while both unsaturated and saturated pyrrolizidines stain with Dragendorff's reagent. Moreover, Ehrlich's reagent is water sensitive making storage difficult; Dragendorff's reagent, on the other hand, can be stored for a longer time. The disadvantage of Dragendorff's reagent is its lack of selectivity; a positive response does not always indicate the presence of a pyrrolizidine.<sup>135,137,138,139</sup> The hexane extracts and the chloroform washings of the acidic fractions were tested for pyrrolizidines using Dragendorff's reagent and their presence was suggested by an orange spot. However, NMR spectroscopy indicated that the compound responsible was not a pyrrolizidine alkaloid.

Droplet counter current chromatography (DCCC) is a fairly new technique with which some authors have achieved very good separations of pyrrolizidine alkaloid mixtures.<sup>44,67,72,170</sup> In particular, *E/Z*-isomers were separated and, consequently, this was intended to be the primary method of separation. There is no solid support to irreversibly adsorb the alkaloids, the solvent consumption is low and a variable sample size (from milligram to multigram quantities) can be loaded.<sup>169</sup> However, the efficiency of the method depends entirely on droplet formation.<sup>169</sup> In the end, the only separation attempted was that of the pyrrolizidine alkaloids from the Grahamstown *S. chrysocoma* population. The solvent system chosen, chloroform-benzene-methanol-water (5:5:7:2), had been used previously by Zalkow *et al.*,<sup>67</sup> but a decent drop rate and form were never achieved. The drops tended to slide down the side of the tube and, towards the end of the "separation", stationary phase as well as mobile phase was being pumped off the columns. Zalkow *et al.*<sup>67</sup> also noticed that, towards the end of their separation, stationary phase was being pumped off the column. The tubes of the droplet counter current chromatograph were cleaned as described in the accompanying manual by flushing them sequentially with methanol, distilled water, a 20% Extran solution and then distilled water again before loading the stationary phase, but this did not improve the droplet formation. As a result, the separation obtained was extremely poor and extensive preparative layer chromatography (PLC) was required to separate the alkaloids of the Grahamstown population.

Flash column chromatography on silica gel was found to be a very good initial separation technique, but needed to be followed by comprehensive preparative layer chromatography; a few fractions were clean enough not to require PLC separation. The only crude mixtures not initially subjected to flash column chromatography were those from the Grahamstown and Dieprivierhoogte *S. chrysocoma* populations and the Assegaaibos *S. paniculatus* population. The Grahamstown population was initially separated by DCCC, as indicated above. As pyrrolizidine alkaloids tend to adhere to silica gel, a certain amount of methanol was required in the elution solvent. Since methanol does dissolve silica to some extent, a gradient elution was used where the percentage of methanol was slowly increased, with only the final elution, of approximately 200 mL, being neat

methanol. The silica that did dissolve was removed from the fractions by evaporating them to dryness and then dissolving the alkaloidal material in chloroform or dichloromethane and filtering.

Further separation of the fractions obtained from DCCC and flash column chromatography was achieved by preparative layer chromatography on silica plates using, generally, chloroform-methanol-25% ammonium hydroxide (85:14:1) as the mobile phase. Some of the fractions, particularly those collected off the droplet counter current chromatograph, were separated using a different chloroform-methanol-25% ammonium hydroxide mixture (80:19:1). In a few cases, the solvent system, chloroform-cyclohexane-diethylamine (5:4:1), gave better separation than the standard 85:14:1 (chloroform-methanol-25% ammonium hydroxide) mixture. The diethylamine helped to compact the bands which normally tended to streak. For mixtures of which there was very little material (< 15 mg) analytical glass-backed plates were used as "preparative" layer chromatography plates. As a 400 MHz nuclear magnetic resonance spectrometer was used for identifying the pyrrolizidine alkaloids, small samples of *ca.* 2 mg were not too small. Developed plates were viewed under ultraviolet light to distinguish the bands. On occasion, a thin strip on the side of the plate was sprayed with Dragendorff's reagent to aid in determining which bands could contain pyrrolizidine alkaloids. The bands of interest were removed from the plate and the compounds removed from the silica using a mixture of chloroform and methanol, and any dissolved silica was removed as described previously.

## 2.5 IDENTIFICATION OF THE PYRROLIZIDINE ALKALOIDS

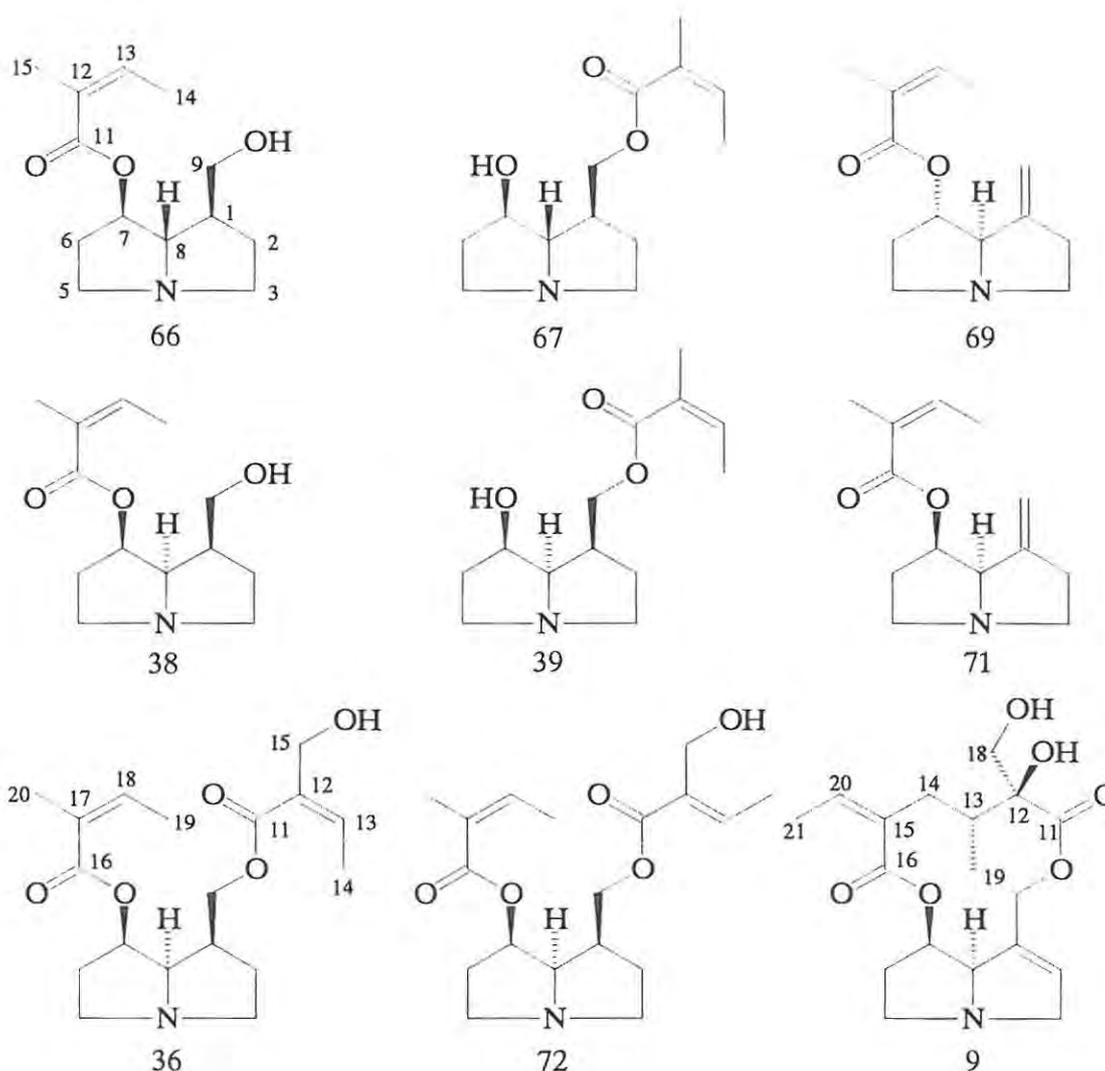
Table 2.i shows the pyrrolizidine alkaloids, identified by NMR techniques, in extracts from each population investigated. Additional alkaloids were identified in some populations' extracts by comparison of the individual gas chromatograms, but these are discussed in Section 2.6 (p. 79). The alkaloids identified by NMR spectroscopy can be divided into four groups:-

- i) the monoesters (7-angelylhastanecine **66**, 9-angelylhastanecine **67**, 7-angelylplatynecine **38**, 9-angelylplatynecine **39** and 9-angelylplatynecine-4-oxide **68**);
- ii) the 1-methylenepyrrolizidines (7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69**, 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide **70**, and 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**);
- iii) the acyclic diesters (sarracine **36** and neosarracine **72**); and
- iv) the macrocyclic diester alkaloid (retrorsine **9**).

The identity of the pyrrolizidines was determined almost exclusively by nuclear magnetic resonance (NMR) spectroscopy, although mass spectrometry was used to confirm the structures.

**Table 2.1:** Pyrrolizidine alkaloids, identified by NMR techniques, in the extracts from the various plant populations.

<b>Pyrrolizidine alkaloid</b>	<b>Population</b>
7-angelylhastanecine <b>66</b>	Grahamstown and Humansdorp
9-angelylhastanecine <b>67</b>	Van Stadens River Dams and Dieprivierhoogte
7-angelylplatynecine <b>38</b>	Wittedrif and Prince Alfred's Pass
9-angelylplatynecine <b>39</b>	Grahamstown, Humansdorp, Wittedrif, Dieprivierhoogte, Assegaaibos and Tradouw Pass
9-angelylplatynecine-4-oxide <b>68</b>	Wittedrif, Tradouw Pass, and Prince Alfred's Pass
7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine <b>69</b>	Van Stadens Dam
7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide <b>70</b>	Grahamstown
7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine <b>71</b>	all nine populations
sarracine <b>36</b>	all nine populations
neosarracine <b>72</b>	all nine populations
retrorsine <b>9</b>	Tradouw Pass



### 2.5.1 THE MONOESTER AND ACYCLIC DIESTER PYRROLIZIDINE ALKALOIDS

The identification of the five monoesters and two acyclic diesters is discussed together as the NMR spectra of these pyrrolizidines have very similar features due to their similar structures. Sarracine **36** and neosarracine **72**, the two acyclic diester pyrrolizidine alkaloids, could be named 7-angelyl-9-sarracinylplatynecine and 7-angelyl-9-isosarracinylplatynecine, respectively. The proton and carbon NMR spectra of

pyrrolizidine alkaloids show distinctive shifts (see Section 3.2, p. 102) and in these pyrrolizidine alkaloids, the chemical shifts for the protons on carbons 2, 7 and 9 were important.

To facilitate assignment of the  $^1\text{H}$  NMR signals of the pyrrolizidines examined (Table 2.2) COSY spectra [Figure 2.6 (9-angelylplatynecine 39) and Appendix] were required. The resonances for H-2 and H-6 overlap as do those for H-1, H-3 and H-5. The key to assigning the shifts was recognising the H-7, H-8 and H-9 resonances as they are in an uncluttered region of the spectrum (*ca.* 3.5-5.5 ppm).

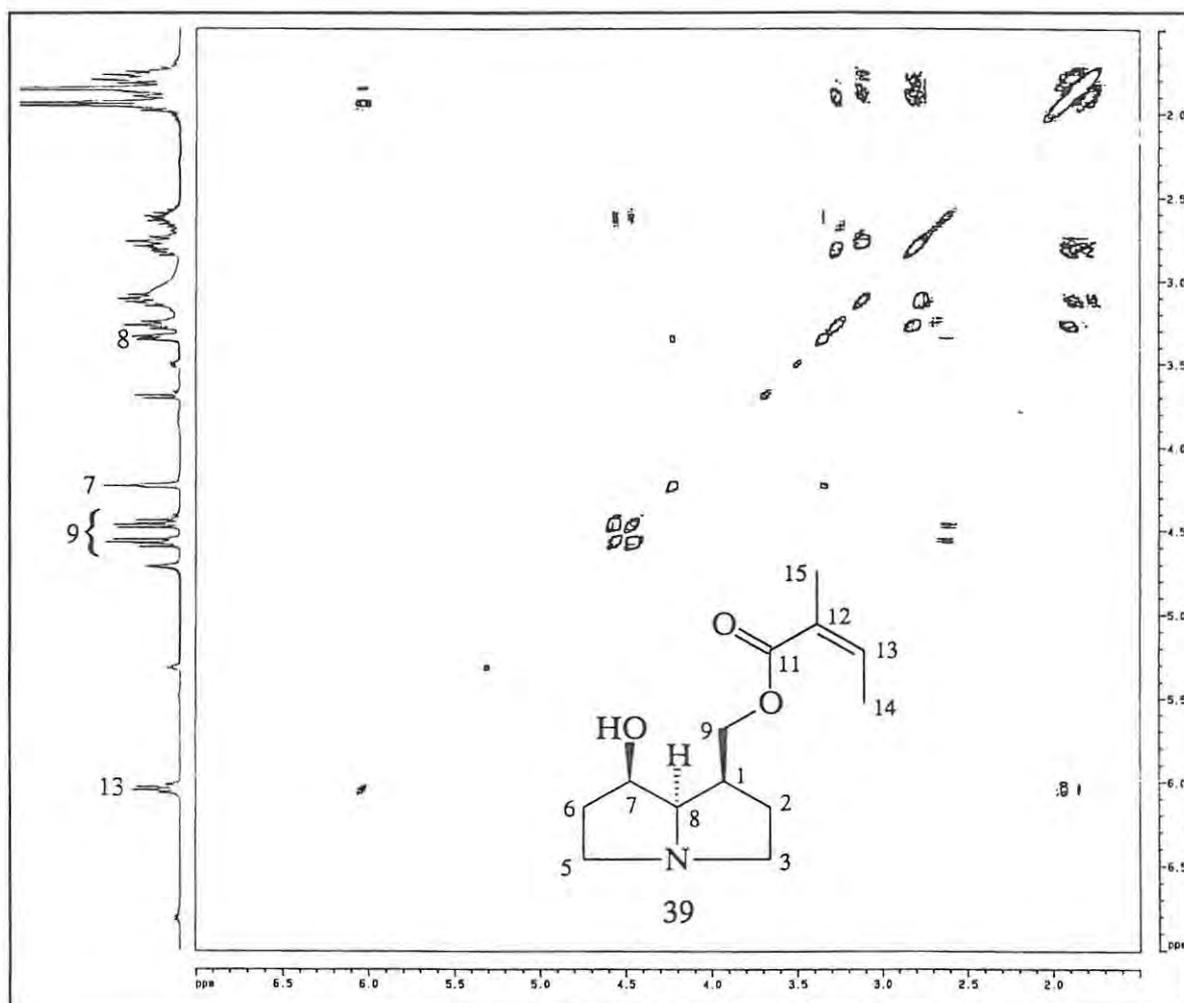


Figure 2.6: COSY spectrum of 9-angelylplatynecine 39.

**Table 2.2:** Proton NMR data (ppm) for 7-angelylhastanecine **66**, 9-angelylhastanecine **67**, 7-angelylplatynecine **38**, 9-angelylplatynecine **39**, 9-angelylplatynecine-4-oxide **68**, neosarracine **72** and sarracine **36**.\*

Atom†	66	67	38	39	68	72	36
1	2.42	2.83	2.31	2.64	3.19	2.68	2.83
2u	2.05	2.12	1.91	1.78	2.14	1.75	1.95
2d	2.08	2.26	2.14	1.90	2.32	1.81	
3u	2.84	3.19	2.68	2.75	3.70	2.74	2.90
3d	3.65	3.64	3.38	3.13	3.89-4.01	3.13	3.33
5u	2.88	3.21	2.77	2.81	3.83	2.64	2.79
5d	3.68	3.93	3.42	3.27	3.89-4.01	3.28	3.52
6u	2.22	2.17	2.17	1.81	2.20	1.94	2.05
6d	2.36		2.26	1.91	2.60	2.02	2.14
7	5.46	4.60	5.37	4.23	4.69	5.25	5.41
8	4.22	4.18	3.89	3.36	3.89-4.01	3.58	3.84
9u	3.60	4.49	3.57	4.47	4.47	4.14	4.24
9d	3.77	4.62	3.72	4.57	4.63	4.29	4.40
13	6.18	6.10	6.15	6.06	6.10	6.88	6.36
14	2.01	1.98	2.02	1.97	1.98	1.83	2.03
15	1.88	1.87	1.90	1.87	1.86	4.24	4.22
18						6.02	6.12
19						1.91	1.97
20						1.83	1.88

\* For solutions in CDCl<sub>3</sub>.

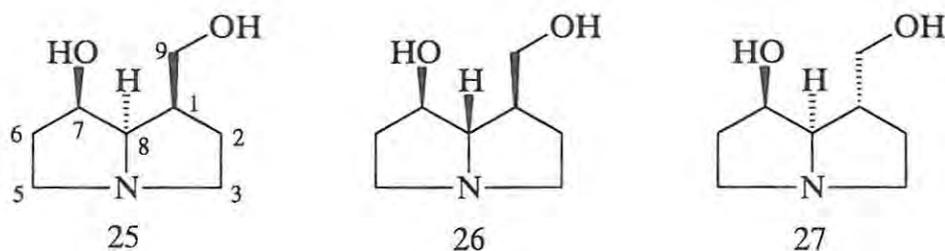
† Upfield (u) and downfield (d) shifts of non-equivalent methylene protons.

The site and extent of esterification was determined by examining the H-7 and H-9 chemical shifts. The non-equivalent 9-methylene protons were observed as a pair of signals (double doublets, when not obscured by other signals) *ca.* 0.2 ppm apart. Both the H-7 and H-9 resonances shift downfield by *ca.* 1 ppm on esterification of the adjacent hydroxyl groups. Thus, the H-7 signal for the 7-angelyl derivatives, 7-angelylhastanecine **66** and 7-angelylplatynecine **38**, resonates downfield of H-9 while the pattern is reversed for the 9-angelyl derivatives, 9-angelylhastanecine **67** and 9-angelylplatynecine **39**. For

the two 7,9-diesters, sarracine **36** and neosarracine **72**, both the H-7 and the H-9 signals are shifted downfield.

The H-7 and H-8 nuclei are coupled in all the pyrrolizidines examined, thus excluding the possibility of an otonecine base. The H-7 and H-6 nuclei are weakly coupled, and this coupling was not always observed on the COSY spectra without significantly increasing the signal intensity. The saturated character of the necine base portion of the alkaloids examined was indicated by the downfield position (between 1.9 and 2.5 ppm) of the H-2 signal. From careful consideration of the various coupling relationships unambiguous assignment of the other signals was possible.

There are three known saturated dihydroxypyrrolizidine bases, *viz.*, platynecine **25**, hastanecine **26**, and turneforcidine **27**. They have been distinguished by the width of the H-7 signal at half-height but this is not as well established for saturated necine bases as it is for the unsaturated bases. Although the width of the H-7 shift at half-height was determined for these pyrrolizidine alkaloids, nuclear Overhauser effect (NOE) difference spectroscopy was found to be more reliable.



The signals for the protons on carbons 1, 7, and 8 were irradiated individually in separate experiments. If the protons are on opposite faces of the necine base, irradiation of the signal for one will not cause an increase in intensity of the other. For the hastanecine-based alkaloids no through space interactions for the protons on carbons 1, 7 and 8 were apparent, as was the case for 7-angelylhastanecine **66** (see Figure 2.7) where irradiation of the H-8 nucleus causes no enhancement of the H-7 or H-1 resonances, indicating that H-8 is on a different face of the necine base from H-7 and H-1. The platynecine-based

alkaloids, on the other hand, showed interactions between H-7 and H-8, and between H-8 and H-1 (see Figure 2.8); the NOE-difference spectrum for 9-angelylplatynecine **39**, irradiated at H-8, shows enhancement of both the H-7 and H-1 signals, indicating that all three protons are on the same face. The width at half-height of the H-7 peak was 10.2 Hz for 7-angelylhastanecine **66** and 7.2 Hz for 9-angelylhastanecine **67**. For the platynecine based alkaloids, 7-angelylplatynecine **38**, 9-angelylplatynecine **39**, neosarracine **72** and sarracine **36**, however, the values ranged from 7.6 to 9.5 Hz; this range covers one of the hastanecine-based values, showing that this method is unreliable for saturated necine bases.

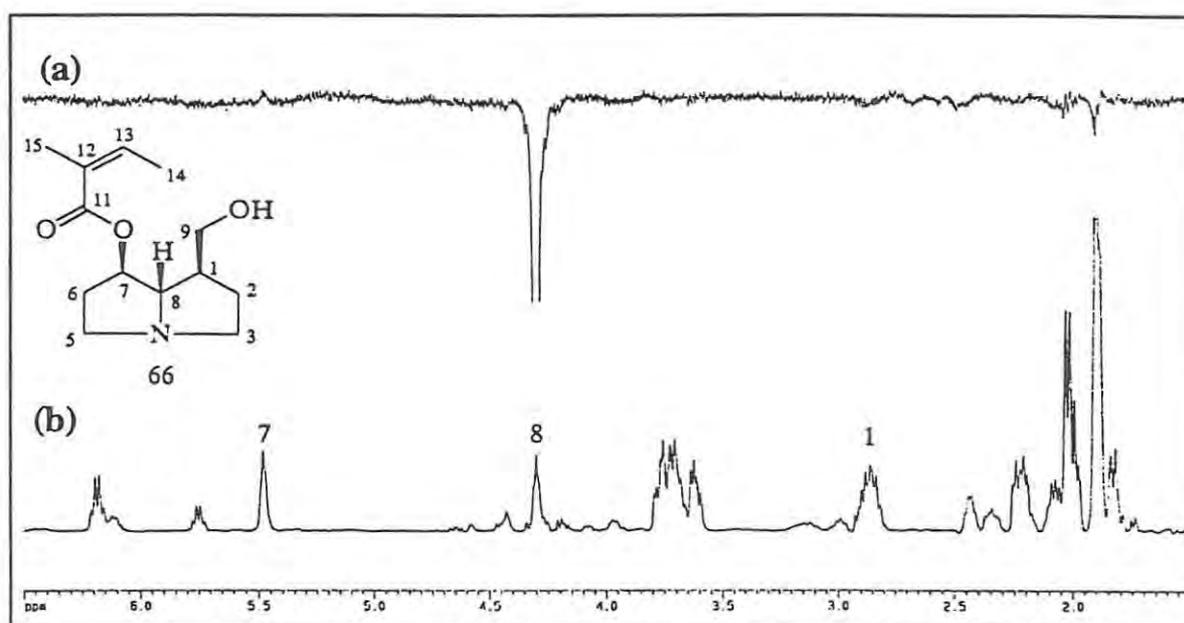
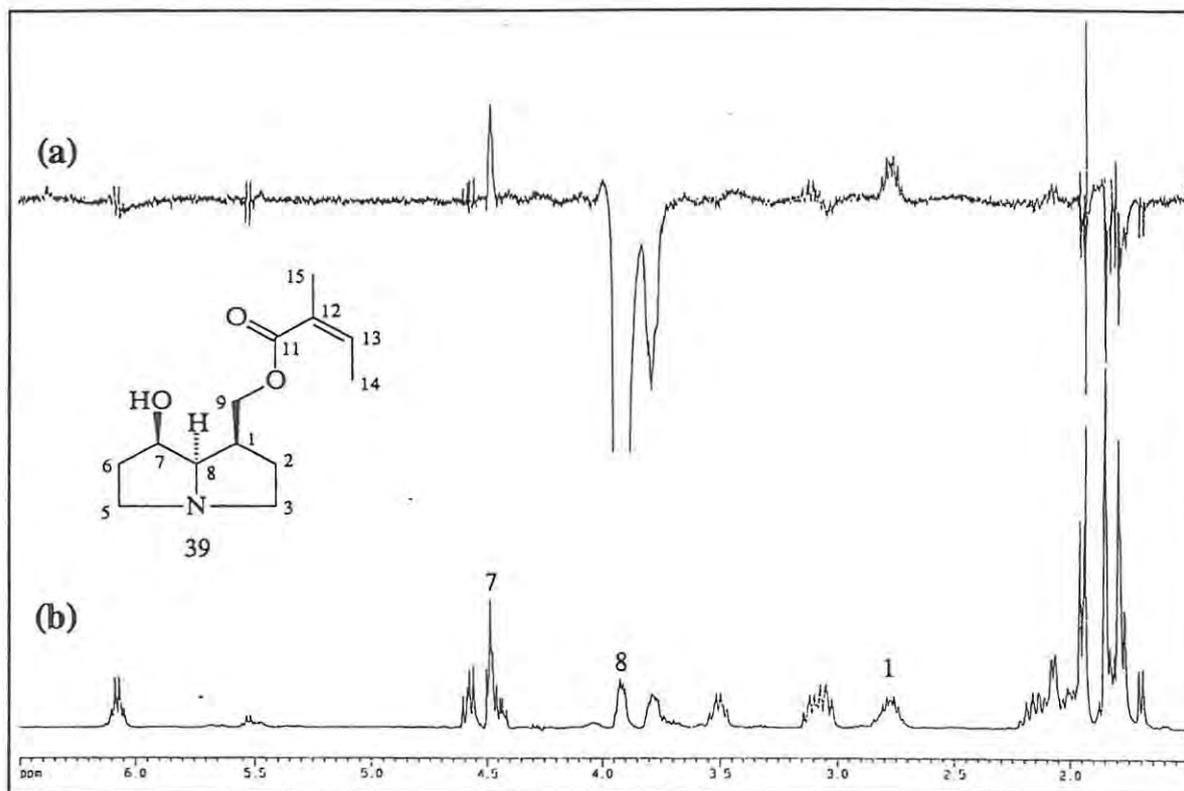


Figure 2.7: NOE-difference spectrum (a), irradiated at H-8, for 7-angelylhastanecine **66** compared to the <sup>1</sup>H NMR spectrum (b).



**Figure 2.8:** NOE-difference spectrum (a), irradiated at H-8, for 9-angelylplatynecine **39** compared to  $^1\text{H}$  NMR spectrum (b).

The necic acid portions of the pyrrolizidines were easily identified by their characteristic quartets for a vinylic proton between 6.0 and 7.0 ppm. The acids were angelic acid (*ca.* 6.1 ppm), present in the monoesters and the acyclic diesters, sarracinic acid (6.4 ppm) present in sarracine **36**, and its isomer isosarracinic acid (6.9 ppm) in neosarracine **72**. In addition, both sarracinic and isosarracinic acid have characteristic singlets at approximately 4.3 ppm due to their hydroxy methylene units. The quartet for the vinylic proton couples on the COSY spectrum to both methyl groups for angelic acid and a methyl and methylene group for sarracinic and isosarracinic acid. On the COSY spectra for mixtures of neosarracine and sarracine the different necic acid moieties are clearly visible, while the remainder of the proton resonances for the two pyrrolizidines cannot be distinguished from one another. Tiglic acid isomers were also present as indicated by the vinylic proton resonance at approximately 6.7 ppm, but they were present in such low

concentrations that not all shift values were visible for their compounds. In view of the similarity of the proton and carbon-13 NMR shifts for neosarracine and sarracine, the tiglate isomers probably have similar chemical shifts to their angelate counterparts. However, due to the known spontaneous conversion of angelates to tiglates, the possibility that tiglyl-containing alkaloids are an artefact of the isolation procedure cannot be ruled out. In fact, a small increase in the concentration of tiglic isomers was noticed with time even though the samples were stored at low temperature, *ca.* 5°C.

The carbon-13 shift assignments (Table 2.3) were made with the aid of HMQC spectra and by comparison with published data. There has been some conflict in the literature concerning the carbon-13 shifts for the angelate moiety of pyrrolizidine alkaloids. For example, in one publication<sup>155</sup> the carbon shifts of the angelate moiety in 7-angelylplatynecine **38** and 9-angelylplatynecine-4-oxide **68** have been differently assigned. The HMQC spectra [Figure 2.9 (9-angelylplatynecine **39**) and Appendix] of the angelate containing pyrrolizidines in this study showed clearly that the terminal methyl group of the necic acid moiety (C-14 in example **39**) has the upfield shift of *ca.* 15.6 ppm while C-15 resonates at *ca.* 20.5 ppm. The geometric isomer of angelic acid, tiglic acid, follows the same pattern but the terminal methyl group resonates slightly further upfield at *ca.* 14 ppm. Isosarracinic and sarracinic acid fit this pattern too.

The NMR data for 7-angelylhastanecine **66** and 9-angelylhastanecine **67** (Tables 2.2 and 2.3) have not previously been reported and the proton shift assignments, while atypically high compared to those of other monoester alkaloids, were confirmed by COSY and HMQC experiments. The signals for the necine base portion of pyrrolizidine alkaloids shift downfield when the alkaloid is oxidised. The observed shifts of the C-8 signals, however, clearly indicate that the nitrogen atoms of the hastanecine-based alkaloids examined (**66**, **67**) were not oxidised. On the other hand, the C-8 signal for 9-angelylplatynecine-4-oxide **68** moved from 70.30 ppm for the parent alkaloid **39** to 88.18 ppm for the oxidised form. The H-8 signal is also considerably further downfield.

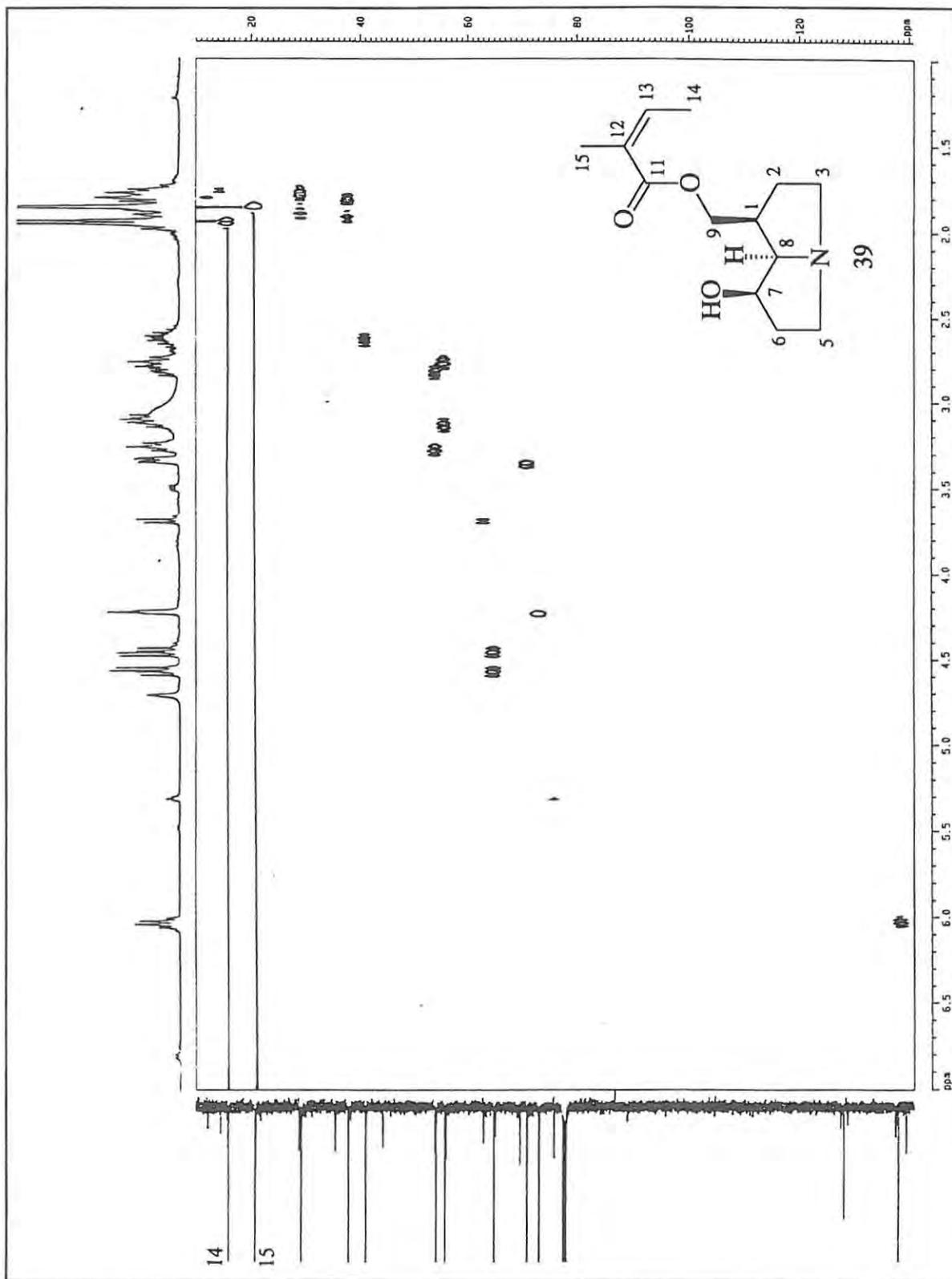


Figure 2.9: HMQC spectrum for 9-angelylplatynecine 39.

**Table 2.3:** Carbon-13 NMR data (ppm) for 7-angelylhastanecine **66**, 9-angelylhastanecine **67**, 7-angelylplatynecine **38**, 9-angelylplatynecine **39**, 9-angelylplatynecine-4-oxide **68**, neosarracine **72** and sarracine **36**.\*

Atom	<b>66</b>	<b>67</b>	<b>38</b>	<b>39</b>	<b>68</b>	<b>72</b>	<b>36</b>
<b>1</b>	40.22	39.98	40.13	40.70	37.98	39.94	40.01
<b>2</b>	30.26	28.69	30.98	28.91	28.94	28.49	28.64
<b>3</b>	54.88	54.32	55.13	55.23	70.41	54.68	54.62
<b>5</b>	52.36	53.85	52.33	53.69	68.24	53.27	53.44
<b>6</b>	34.48	36.73	34.85	37.41	34.81	34.69	34.76
<b>7</b>	73.29	71.55	73.93	72.72	71.51	74.91	74.69
<b>8</b>	71.69	71.48	71.82	70.30	88.18	68.43	68.87
<b>9</b>	63.46	62.28	64.75	64.33	62.58	63.90	63.23
<b>11</b>	166.29	167.65	166.83	168.10	167.78	166.89	166.68
<b>12</b>	126.59	127.20	126.96	127.77	127.35	131.93	131.74
<b>13</b>	140.83	139.20	140.23	137.83	138.98	140.93	140.98
<b>14</b>	15.87	15.47	15.88	15.65	15.86	14.04	15.67
<b>15</b>	20.51	20.54	20.58	20.68	20.56	56.10	68.89
<b>16</b>						166.46	166.36
<b>17</b>						126.79	126.62
<b>18</b>						139.55	140.55
<b>19</b>						15.51	15.78
<b>20</b>						20.51	20.21

\* For solutions in CDCl<sub>3</sub>.

The structures of the monoesters, 7-angelylhastanecine **66**, 9-angelylhastanecine **67**, 7-angelylplatynecine **38**, 9-angelylplatynecine **39** and 9-angelylplatynecine-4-oxide **68**, had now been determined. However, both the diesters, neosarracine **72** and sarracine **36**, had been discovered to contain an angelic acid moiety and one other, either isosarracinic or sarracinic acid, but the mode of attachment was unknown. This was determined by heteronuclear multiple bond correlation (HMBC) spectroscopy, which detects coupling between protons and carbons one or two removed from the one to which they are directly bonded. The HMBC spectrum of neosarracine **72** (Figure 2.10) shows coupling between H-7 and a carbonyl carbon and between that carbonyl carbon and the vinylic proton of angelic acid showing that the angelyl unit was attached at carbon 7 of the necine base

system. This leaves the isosarracinyll or sarracinyll unit to be attached at carbon 9. Moreover, the HMBC spectrum shows coupling between the vinylic proton of the isosarracinyll or sarracinyll moiety and the other carbonyl carbon and a coupling between this carbonyl carbon and the protons on carbon 9.

Mass spectrometry (Table 2.4) helped to confirm the results obtained from NMR techniques. The saturated bases were indicated by characteristic major peaks at  $m/z$  82, 95, 138, 139 and 140.<sup>185</sup> An intense peak at  $m/z$  95 (see Section 1.3.4.2, p. 23) is claimed to show esterification at carbon 9<sup>193</sup> and was absent from the spectra for 7-angelylhastanecine **66** and 7-angelylplatynecine **38**. On the other hand, the peak at  $m/z$  95 appeared as the base peak in the spectra for 9-angelylhastanecine **67** and 9-angelylplatynecine **39**. This observed difference in the mass spectra of 7- and 9-angelylplatynecine is, in fact, contrary to that which has been reported previously.<sup>155</sup> For sarracine **36** and neosarracine **72**, which are both esterified at position 9 as well as position 7, the peak at  $m/z$  95 is a major peak, but the base peak appears at  $m/z$  138, which corresponds to the loss of both necic acid moieties from the alkaloid. The mass spectrum of 9-angelylplatynecine-4-oxide **68** does not fit the pattern for saturated pyrrolizidines; this may be due to the typical low volatility and thermal instability of *N*-oxides.

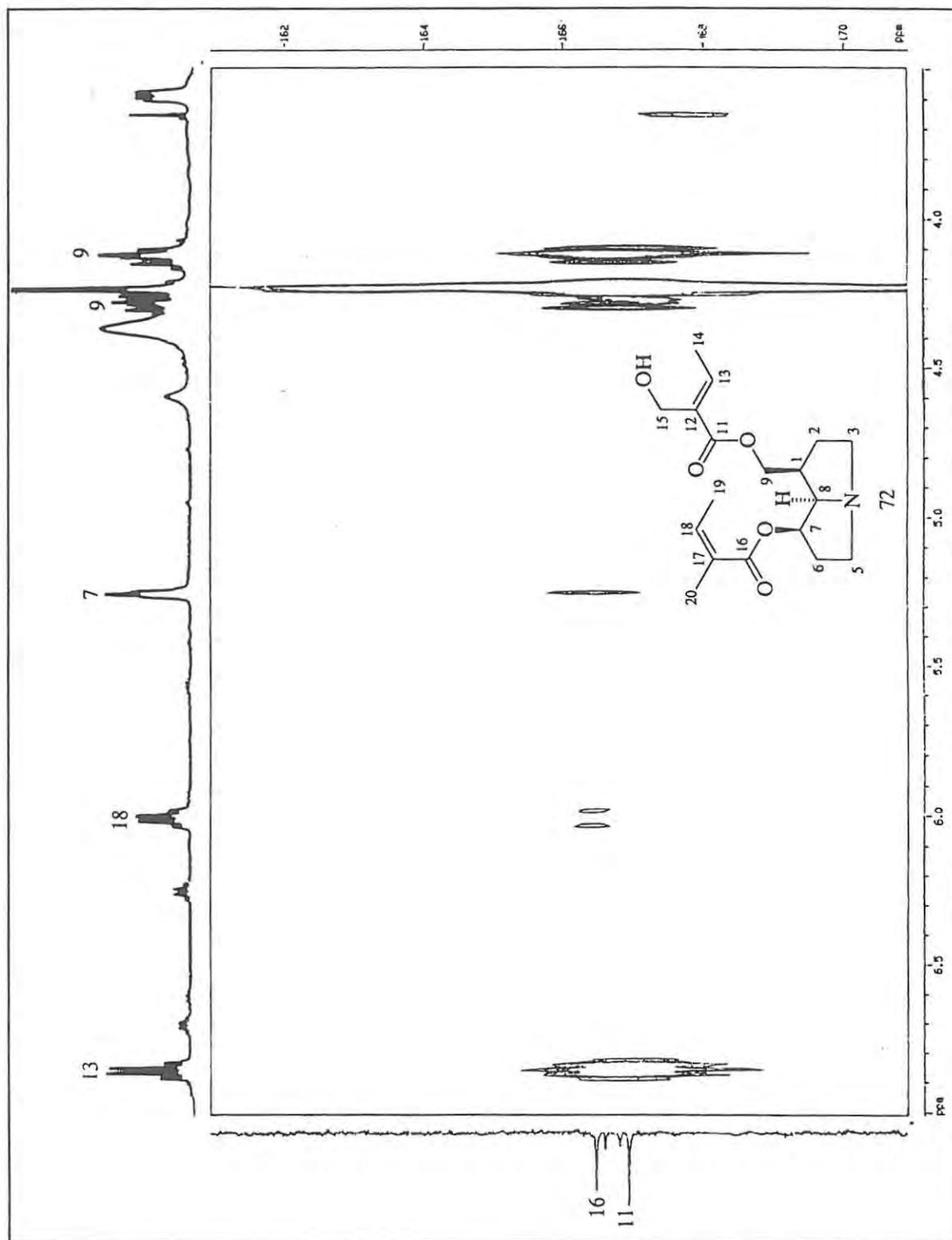


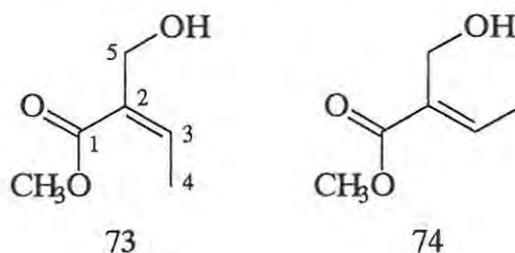
Figure 2.10: A section of the HMBC spectrum for neosarracine 72.

Table 2.4: Mass spectral data for the mono- and acyclic diester pyrrolizidine alkaloids.

Pyrrolizidine alkaloid	Mass Fragments*
7-angelylhastanecine <b>66</b>	239(M <sup>+</sup> ,0.3), 219(0.2), 156(47), 140(6), 139(64), 138(13), 114(12), 113(13), 83(13), 82(100), 55(9).
7-angelylplatynecine <b>38</b>	239(M <sup>+</sup> ,1), 208(1), 156(40), 140(21), 139(85), 138(29), 109(10), 108(41), 83(30), 82(100), 55(16).
9-angelylhastanecine <b>67</b>	239(M <sup>+</sup> ,6), 221(32), 195(6), 155(5), 140(29), 139(11), 138(16), 122(49), 121(7), 120(7), 96(91), 95(100), 94(19), 83(28), 82(96), 55(71).
9-angelylplatynecine <b>39</b>	239(M <sup>+</sup> ,2), 221(5), 195(1), 156(2), 140(6), 139(4), 138(5), 122(9), 121(2), 120(2), 96(36), 95(100), 94(5), 82(94), 55(19).
9-angelylplatynecine-4-oxide <b>68</b>	219(1), 120(14), 119(100), 118(7), 107(5), 106(59), 79(6), 77(4), 55(5).
sarracine <b>36</b>	237(8), 222(10), 140(37), 139(32), 138(100), 123(26), 122(57), 121(13), 96(46), 95(47), 83(35), 82(74), 55(35).
neosarracine <b>72</b>	237(9), 222(9), 140(29), 139(27), 138(100), 123(22), 122(38), 121(15), 96(30), 95(51), 83(24), 82(64), 55(27).

\* *M/z* values followed by % relative abundance in parentheses.

The extracts from the Grahamstown *S. chrysocoma* population contained sarracinic acid methyl ester **73** and isosarracinic acid methyl ester **74** but it is not known if these esters are artefacts of the extraction procedure or not. The proton NMR chemical shifts for compounds **73** and **74** (Table 2.5) correlate with those for the sarracinic and isosarracinic moieties of sarracine **36** and neosarracine **72**.

**Table 2.5:** Comparative proton NMR data (ppm)\* for sarracinic acid methyl ester **73**, isosarracinic acid methyl ester **74**, sarracine **36** and neosarracine **72**.†

Atom‡	73	36	74	72
3(13)	6.37(q)	6.36	6.99(q)	6.88
4(14)	2.06(d)	2.03	1.90(d)	1.83
5(15)	4.22(s)	4.22	4.35(s)	4.24
-OCH <sub>3</sub>	3.79(s)		3.77(s)	

\* Multiplicity in parentheses.

† For solutions in CDCl<sub>3</sub>.

‡ Number in parentheses is the equivalent atom number in sarracine and neosarracine.

## 2.5.2 THE 7-ANGELYL-1-METHYLENEPYRROLIZIDINE ALKALOIDS

1-Methylenepyrrolizidines have previously only been found in *Crotalaria* and *Onosma* species, which belong to the Legume family.<sup>41,310</sup> They had never previously been found esterified at carbon-7 or in *Senecio* species or in the Compositae family, and there are features of the NMR spectra of these compounds which are unusual for pyrrolizidine alkaloids.

The proton chemical shifts for these alkaloids are given in Table 2.6. Esterification at position 7 is indicated, in all three cases, by an H-7 chemical shift of *ca.* 5.6 ppm. What are assumed to be the H-9 signals are at an unusually low field, but indicate the possibility of esterification at position 9. Signals attributable to H-8 were also visible and indicate the presence of a bicyclic necine base (not an otonecine base) in each system. The upfield H-2 signals, once identified, indicated that the base was saturated. The H-1 signals were

not visible in the proton NMR spectra but can often be obscured by the signals for the protons on carbons 2, 3 or 5; unfortunately their presence could not be confirmed by integration as impurities in the fraction led to irregularities. However, as the H-2 shift was in an upfield position ("typical" in saturated necine bases) the H-1 signal was assumed to be present but obscured by overlapping signals. The esterifying acid in each spectrum was angelic acid as shown by a characteristic quartet at *ca.* 6.0 ppm. A far smaller quartet at *ca.* 6.7 ppm indicated the presence of very small amounts of the tiglic acid analogues, which could, again, be artefacts of the extraction procedure.

**Table 2.6:** Proton NMR data (ppm)\* for 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69**, 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide **70** and 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**.†

Atom‡	69	70	71
2u	2.66(t)	2.69(m)	2.55(m)
2d		3.12(m)	
3u	2.90	3.79(m)	2.76(m)
3d	3.41		3.16(m)
5u	2.92	3.84(m)	2.83(m)
5d	3.60(t)	3.91(m)	3.27(m)
6u	2.17(dd)	2.22(m)	2.08(m)
6d	2.32	3.01(m)	2.17(m)
7	5.62(t)	5.84(dt)	5.52(dt)
8	4.46(s)	4.66(d)	4.14(d)
9u	4.96(s)	5.04(d)	4.88(d)
9d	5.04(s)	5.24(d)	5.07(d)
13	6.07(q)	6.10(dq)	6.03(dq)
14	1.97(d)	1.98(dd)	1.99(dd)
15	1.83(s)	1.84(t)	1.84(t)

\* Multiplicity of shifts, where it could be determined, is in parentheses.

† For solutions in CDCl<sub>3</sub>.

‡ Upfield (u) and downfield (d) shifts of non-equivalent methylene protons.

The COSY spectra [Figure 2.11 (7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**) and Appendix] also did not reveal the presence of the H-1 signal. The H-2 signal couples to

H-3 but not H-1; neither does the H-8 signal couple to H-1. The two signals assumed to be the H-9 signals couple to H-8, as would be expected, but do not couple with each other, which is unusual. The rest of the couplings are typical for a pyrrolizidine alkaloid.

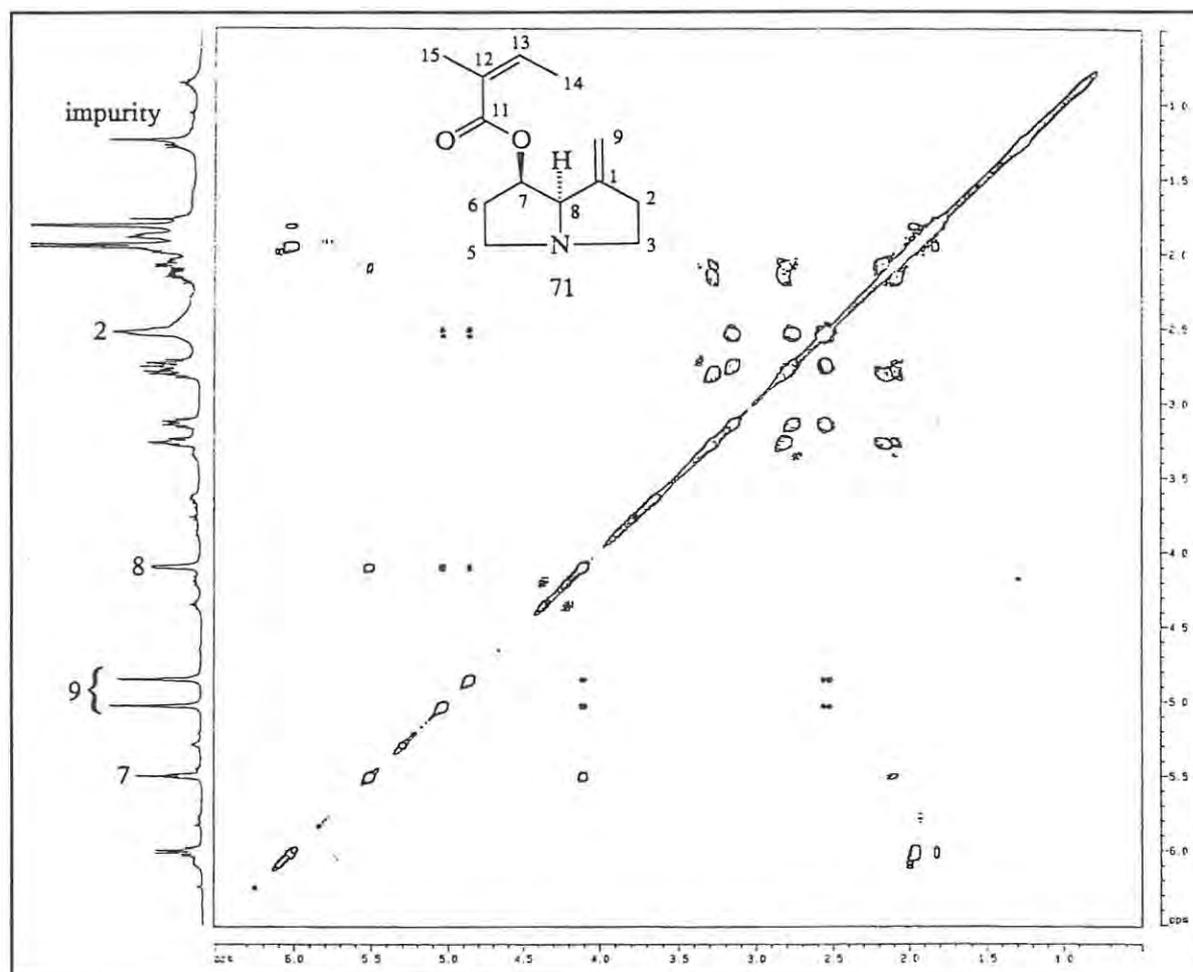


Figure 2.11: COSY spectrum for 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**.

The carbon-13 NMR spectra of 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**, 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69**, and 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide **70** (Table 2.7) have thirteen peaks which rule out the possibility of a diester, as eight signals are required for the necine base, leaving five, which are enough for a single angelate ester. There is one carbonyl peak as expected for a single angelate ester, but four vinylic carbons. Since only two are required for the angelate moiety, the base was, thus,

presumed to be unsaturated. If a necine base is unsaturated the double bond is usually between carbons 1 and 2 but the shift for H-2 and the COSY coupling patterns preclude this location. The proton shifts of the other ring protons (Table 2.6) show that there is no double bond within either of the base rings. This and the missing H-1 resonance lead to the conclusion that there is an exocyclic double bond between carbons 1 and 9 in the 7-angelyl-1-methylenepyrrolizidines.

**Table 2.7:** Carbon-13 NMR data (ppm) for 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69**, 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide **70**, and 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**.\*

Atom	69	70	71
1	†	139.74	147.09
2	33.45	32.63	33.92
3	53.70	68.47	54.46
5	52.60	67.36	52.81
6	34.21	32.07	34.35
7	73.91	72.41	74.95
8	71.68	89.63	71.94
9	109.89	112.49	107.68
11	166.28	165.83	166.87
12	127.16	126.63	127.86
13	139.21	140.18	137.84
14	15.69	15.73	15.61
15	20.46	20.32	20.55

\* For solutions in CDCl<sub>3</sub>.

† Too weak to be detected.

HMQC experiments [Figure 2.12 (7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**) and Appendix] confirmed the existence of the exocyclic double bond, as both H-9 nuclei couple to the same vinylic carbon. After assigning all the other carbons, carbon-12 (*ca.* 127 ppm) was assigned by comparison with other angelate esters, it was clear that the remaining quaternary carbon signal was due to carbon-1.

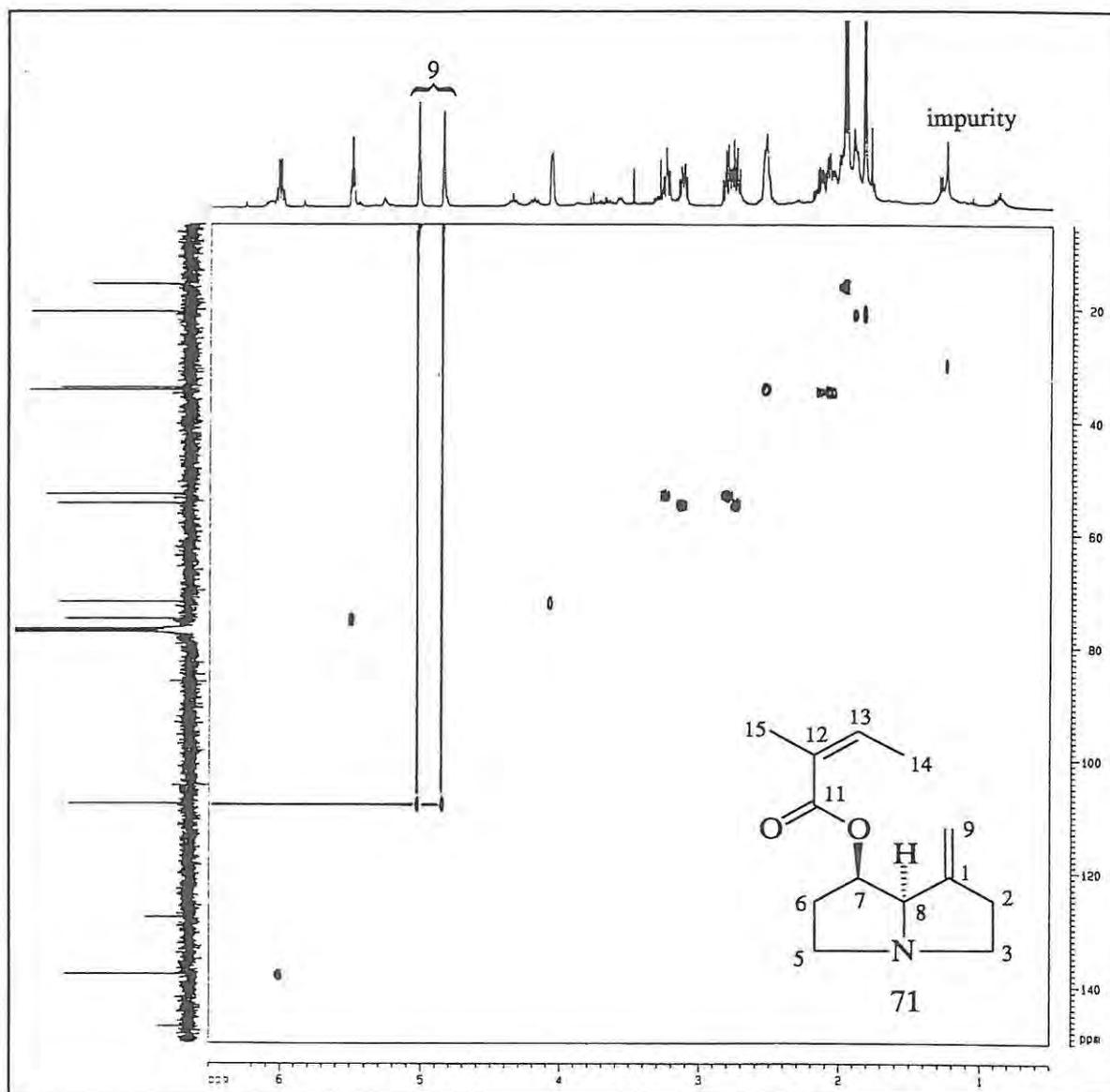


Figure 2.12: HMOC spectrum for 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**.

The basic skeleton was thus determined to be 7-angelyl-1-methylenepyrrolizidine but the stereochemistry at carbons 7 and 8 had not been established. To determine the *relative* stereochemistry NOE-difference spectroscopy was used. If H-8 is assumed to be in the  $\alpha$  position, which is most common, then the two possible diastereoisomers are 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** and 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69**. Irradiation of 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** at the H-8 resonance frequency (see Figure 2.13) produced an enhancement of the H-7 signal, while similar irradiation of

7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69** (see Figure 2.14) produced no enhancement of the H-7 signal. The *N*-oxide of 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine, easily recognisable by H-8 and C-8 chemical shifts of 4.66 and 89.63 ppm, respectively, was also identified. 7 $\beta$ -Angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** was extracted in far higher concentrations than 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69** and its *N*-oxide **70**, which were each only identified by NMR spectroscopy in one fraction.

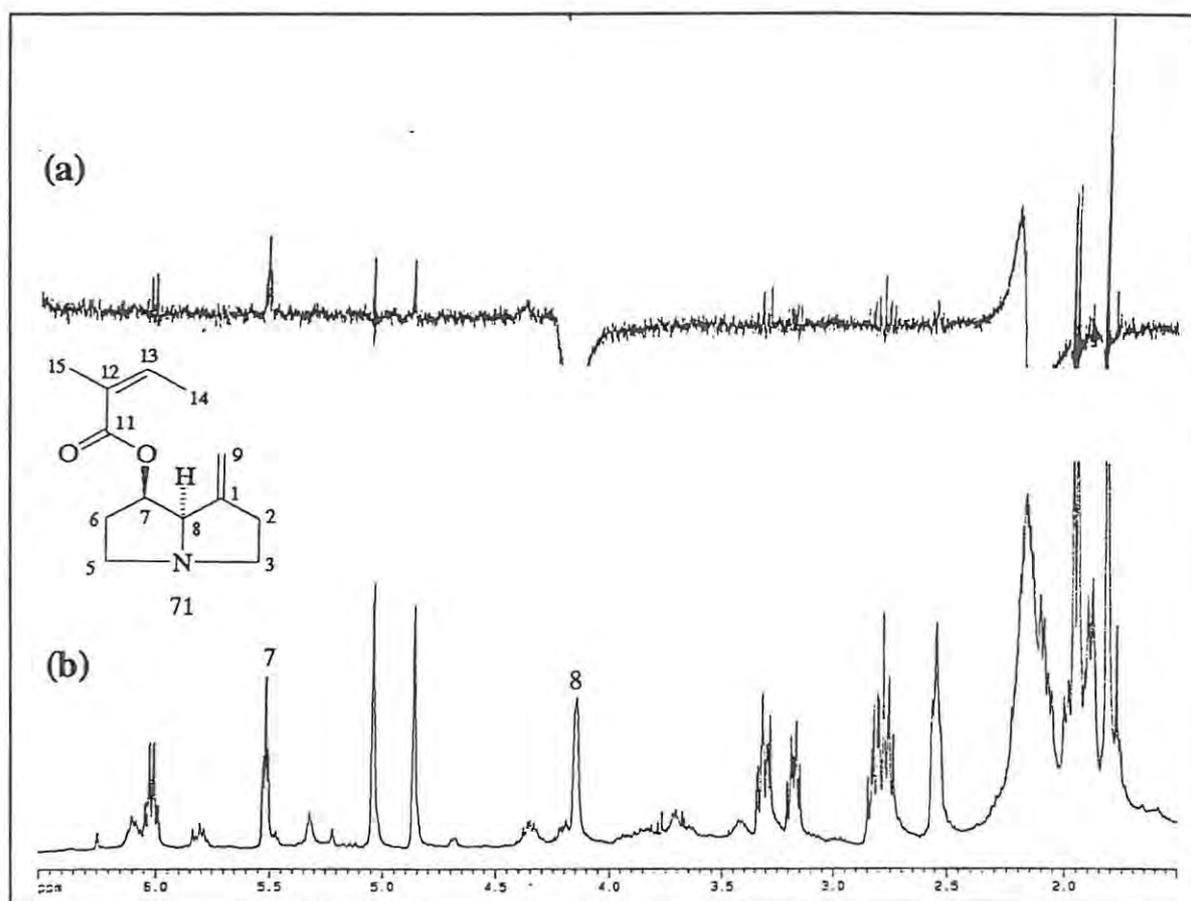
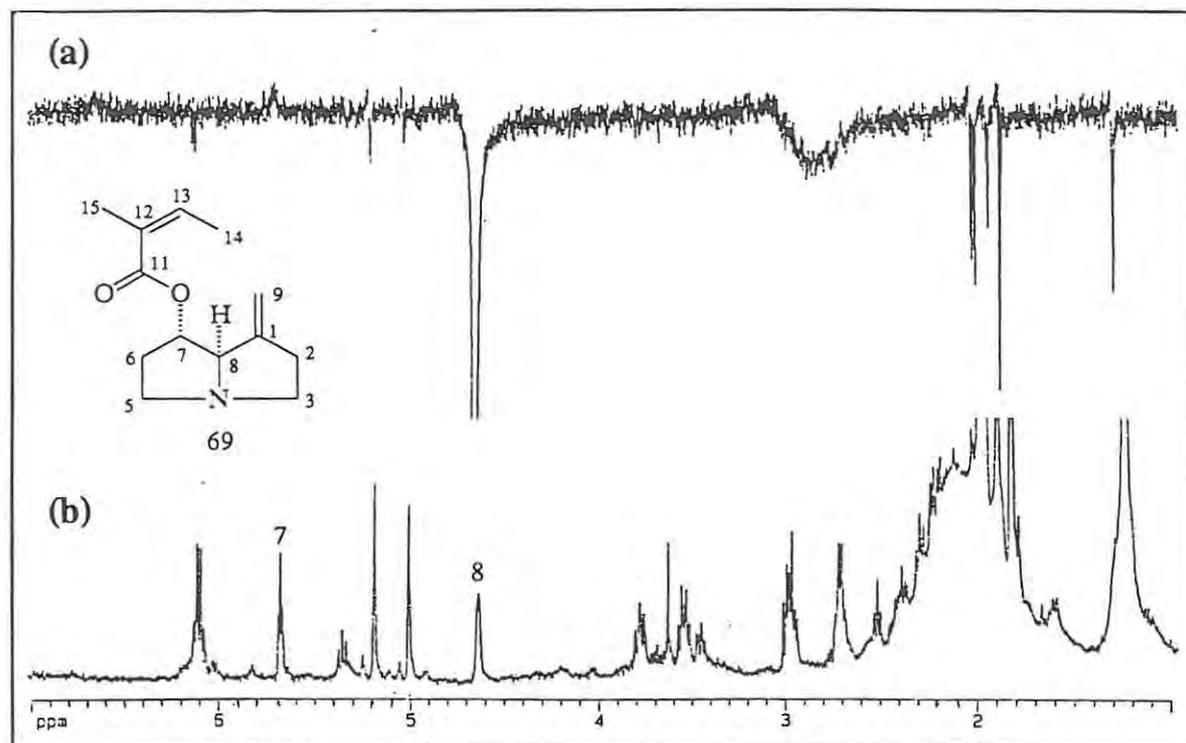


Figure 2.13: NOE difference spectrum (a), irradiated at H-8, for 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** compared to the <sup>1</sup>H NMR spectrum (b).



**Figure 2.14:** NOE-difference spectrum (a), irradiated at H-8, for  $7\alpha$ -angelyl-1-methylene- $8\alpha$ -pyrrolizidine **69** compared to the  $^1\text{H}$  NMR spectrum (b).

A stereoselective synthesis of  $7\beta$ -angelyl-1-methylene- $8\alpha$ -pyrrolizidine **71** was then undertaken to establish the *absolute* stereochemistry at positions 7 and 8 of the necine base portion of the alkaloid (see Section 4, p. 114). (Only a minute amount of  $7\alpha$ -angelyl-1-methylene- $8\alpha$ -pyrrolizidine **69** was present in the plant extracts effectively preventing comparisons from being made with any synthetic material.) The synthesis confirmed the stereochemistry of the natural alkaloid as  $7\beta$ -angelyl-1-methylene- $8\alpha$ -pyrrolizidine **71**. Since  $7\beta$ -angelyl-1-methylene- $8\alpha$ -pyrrolizidine **71** has the same stereochemistry at carbons 7 and 8 as platynecine **25**, it is possible that the  $7\alpha$ -angelyl analogue **69** has the same stereochemistry as hastanecine **26**, the other necine base present in the alkaloids identified in this study, and could thus be  $7\beta$ -angelyl-1-methylene- $8\beta$ -pyrrolizidine.

The EI mass spectra of  $7\beta$ -angelyl-1-methylene- $8\alpha$ -pyrrolizidine **71** and  $7\alpha$ -angelyl-1-methylene- $8\alpha$ -pyrrolizidine **69** did not show the molecular ion; the highest mass peak was

at  $m/z$  138, which represents the loss of the angelic acid moiety from the alkaloid. The base peak at  $m/z$  95 is contra-indicative of esterification at position 9. The CI spectra confirmed the mass of these novel compounds (**69**, **70**, and **71**) to be 221. The formula of 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** and 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69**, C<sub>13</sub>H<sub>19</sub>NO<sub>2</sub>, was also confirmed by high resolution mass spectrometry.

### 2.5.3 THE MACROCYCLIC DIESTER PYRROLIZIDINE ALKALOIDS

Retrorsine **9** was the only macrocyclic pyrrolizidine alkaloid identified and was also the only pyrrolizidine identified containing a "traditional" unsaturated necine base. Being a well-documented alkaloid, retrorsine was easily identified using the database (see Section 3.2.3, p. 110). The major features of the proton and carbon-13 NMR spectra (Table 2.8) that distinguish retrorsine **9** from the other pyrrolizidine alkaloids in these plants are:-

- i) the downfield positions of the H-2 (6.20 ppm) and corresponding C-2 signals (136.61 ppm), indicating an unsaturated necine base;
- ii) the large difference between the chemical shifts for the non-equivalent 9-methylene protons ( $\Delta H-9 = 1.41$  ppm) indicative of a 12-membered macrocycle (see Section 3.2.1, p. 102); and
- iii) characteristic resonances of the necic acid portion of the alkaloid, most notably the signal at 5.74 ppm for the H-20 vinylic proton and the signals at 3.62 and 3.74 ppm for the 18-methylene protons. The necic acid portion of retrorsine, isatinecic acid consists of ten carbons not five as in angelic, sarracinic and isosarracinic acids.

Table 2.8: Proton and carbon-13 NMR data (ppm) for retrorsine 9.\*

Atom†	<sup>1</sup> H	<sup>13</sup> C
1		132.38
2	6.20	136.61
3u	3.40	62.59
3d	3.97	
5u	2.55	52.94
5d	3.29	
6u	2.08	34.73
6d	2.40	
7	5.02	74.98
8	4.31	77.47
9u	4.10	61.06
9d	5.51	
11		175.65
12		81.29
13	1.68	35.69
14u	1.72	37.89
14d	2.12	
15		131.18
16		167.31
18u	3.62	66.86
18d	3.74	
19	0.85	11.68
20	5.74	134.85
21	1.83	15.05

\* For solutions in CDCl<sub>3</sub>.

† Upfield (u) and downfield (d) shifts of non-equivalent methylene protons

## 2.6 A CHEMICAL COMPARISON OF THE TWO SPECIES, *SENECIO CHRYSOCOMA* AND *S. PANICULATUS*

Although the pyrrolizidine alkaloids associated with each species were not identified by NMR spectroscopy in all the populations of that species, comparison of GLC chromatograms of the crude extracts revealed that the three populations of each group, viz., *S. chrysocoma*, borderline *S. chrysocoma*, and *S. paniculatus*, contained similar pyrrolizidine alkaloids and only slight differences between the groups were noted.

A representative GLC chromatogram for each group is shown in Figure 2.15 (the other populations' chromatograms are in the Appendix). It is necessary to note that the GLC column used, the HP-ultra 2, was cleaned during the course of the study and a section of the column was removed, so the retention times for the alkaloids do not match precisely from one group to the next; the pattern of the peaks, however, is the same, and the chromatograms can be divided into several sections:-

- i) impurities with a retention time of less than 9.5 minutes;
- ii) 7-angelyl-1-methylenepyrrolizidines with retention times between 9.5 and 11.5 minutes;
- iii) monoesters with retention times between 11.5 and 16 minutes;
- iv) acyclic diesters eluting between 19 and 21 minutes; and
- v) macrocyclic diesters eluting at *ca.* 21 minutes (observed in one set of chromatograms only).

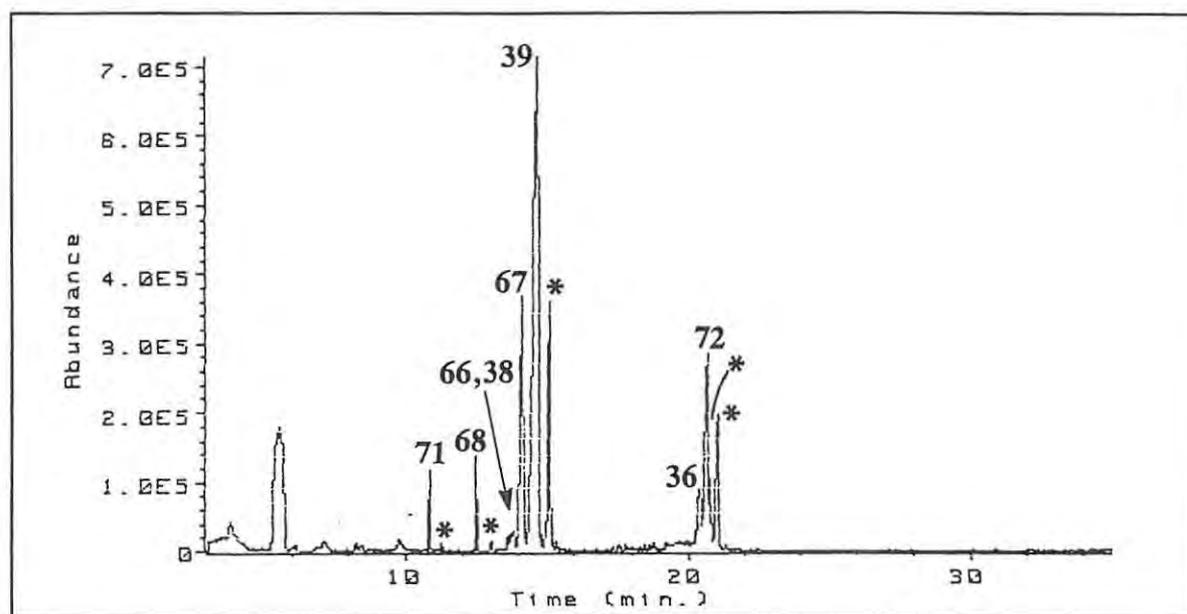


Figure 2.15a: The GLC chromatogram of the crude extract from the Humansdorp *S. chrysocoma* population.

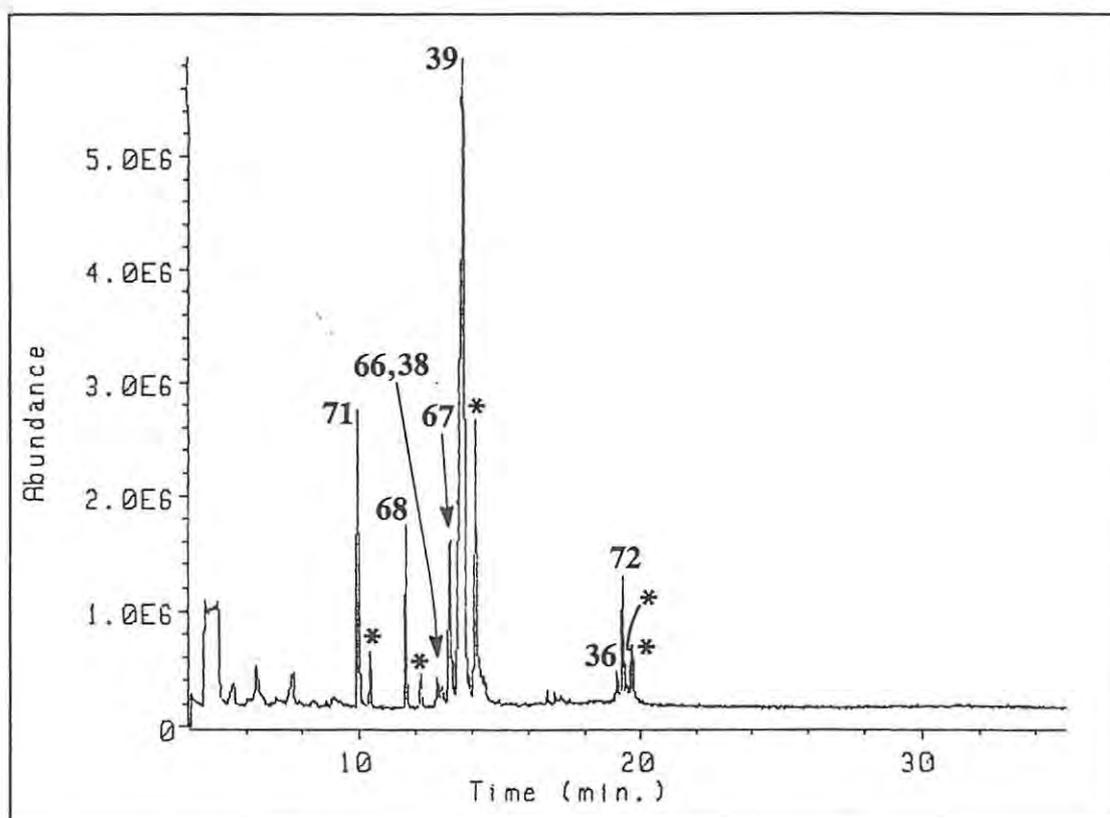
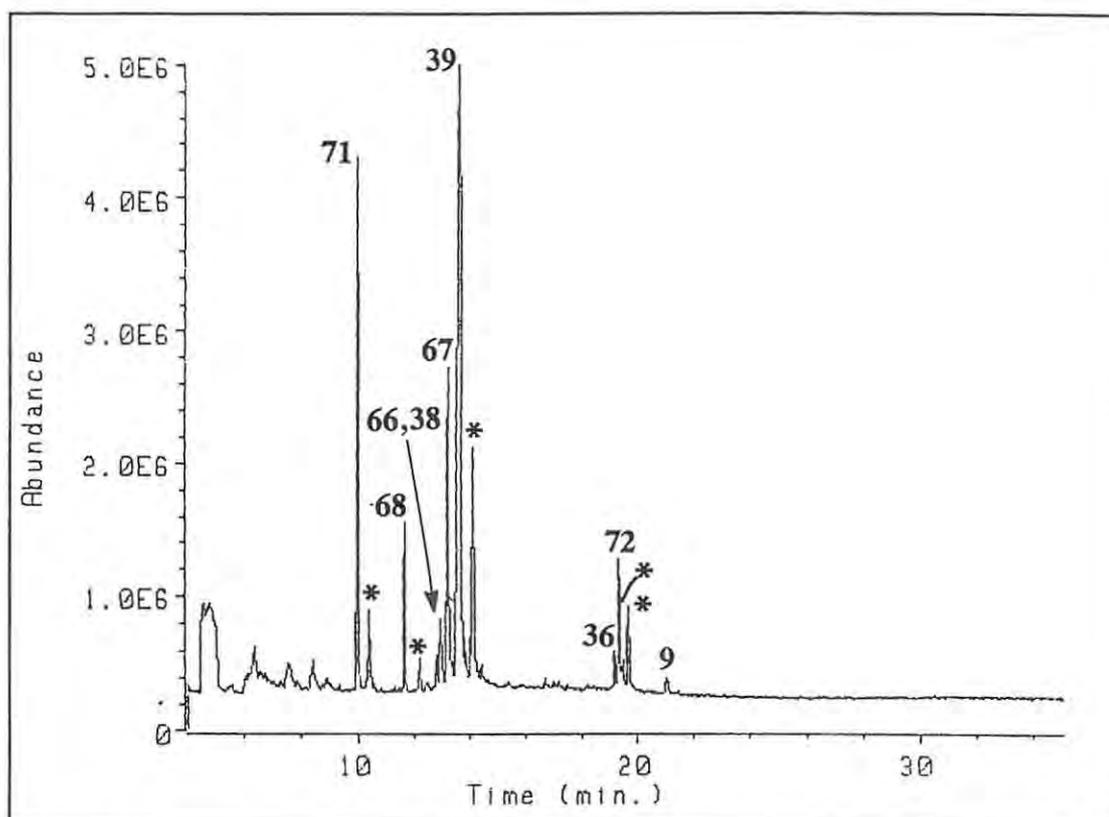


Figure 2.15b: The GLC chromatogram of the crude extract from the Dieprivierhoogte *S. chrysocoma* population.



**Figure 2.15c:** The GLC chromatogram of the crude extract from the Assegaaibos *S. paniculatus* population.

Most of the pyrrolizidine alkaloids responsible for peaks on the GLC chromatograms (Figure 2.15) have been identified by NMR spectroscopy and these peaks are labelled with the structure number of the alkaloid. From these chromatograms it can be seen that 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**, 9-angelylplatynecine-4-oxide **68**, 7-angelylhastanecine **66**, 7-angelylplatynecine **38**, 9-angelylhastanecine **67**, 9-angelylplatynecine **39**, sarracine **36** and neosarracine **72** are present in the extracts from all three groups.

The borderline *S. chrysocoma* populations (Dieprivierhoogte, Prince Alfred's Pass, and Wittedrif) have the same pyrrolizidine alkaloid content as the *S. chrysocoma* populations (Grahamstown, Humansdorp, and Van Stadens River dams), except that no 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69** was identified in extracts from the borderline populations.

7 $\alpha$ -Angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69** has a slightly shorter retention time than the diastereomeric 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**, but it and its *N*-oxide **70** are not visible on the GLC chromatograms of the crude extracts as they are present in such low concentrations. In fact, 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69** was only identified, by NMR spectroscopy, in extracts from the Van Stadens River dams population, while the *N*-oxide was only identified in extracts from the Grahamstown population. However, a peak for the unoxidised form was present in the chromatogram of the fractions obtained from crude extracts of the Grahamstown, Humansdorp, and Van Stadens River dams.

The pyrrolizidine alkaloid content of the *S. paniculatus* populations (Assegaaibos, Robinson Pass, and Tradouw Pass) differs from that of the *S. chrysocoma* populations (Grahamstown, Humansdorp, and Van Stadens River dams) by the absence of 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69** and the presence of a peak at *ca.* 21 minutes, which was later identified as retrorsine **9**. This was the only macrocycle present and also the only "typical" unsaturated pyrrolizidine alkaloid. Retrorsine **9** was identified by NMR spectroscopy in the Tradouw Pass *S. paniculatus* population's extract, but a peak for the alkaloid appears on the GLC chromatograms of all three *S. paniculatus* populations.

The hastanecine-based pyrrolizidines, 7-angelylhastanecine **66** and 9-angelylhastanecine **67**, were identified by NMR techniques in the *S. chrysocoma* populations but not in the borderline *S. chrysocoma* or *S. paniculatus* populations; however, there are corresponding signals for these alkaloids in the chromatograms for the *S. chrysocoma* borderline and *S. paniculatus* populations.

Although the populations contain similar alkaloids they are present in different concentrations (Table 2.9a,b,c). In all the groups, 9-angelylplatynecine **39** has the highest concentration, while there is no clear pattern for the remaining pyrrolizidine alkaloids. Different locations and collection during different seasons are thought to be responsible for differences in pyrrolizidine alkaloid concentrations within a single species.<sup>311,312</sup> The reproductive organs and young leaves of plants have been shown to contain more

pyrrolizidine alkaloids than the mature vegetative parts,<sup>184,313-317</sup> therefore, for maximum alkaloid yields pre-flower and budding plants should be extracted. The plants extracted in this study, however, were not all collected during the same phase of their development; in particular, the *S. chrysocoma* borderline (Dieprivierhoogte, Prince Alfred's Pass, and Wittedrif) and *S. paniculatus* (Assegaibos, Robinson Pass and Tradouw Pass) populations were collected at the very end of, or just after, their flowering season, while the *S. chrysocoma* populations (Grahamstown, Humansdorp, and Van Stadens River dams) were collected at the beginning of, or during, their flowering season. Even though the *S. chrysocoma* borderline populations were collected at the end of their flowering season they generally contained more pyrrolizidines than the other *S. chrysocoma* populations, which is contrary to what was expected. The *S. paniculatus* populations, on the other hand, contained fewer pyrrolizidines than all the *S. chrysocoma* populations.

**Table 2.9a:** Concentrations of identified pyrrolizidine alkaloids in plant extracts from the *S. chrysocoma* populations (A-Grahamstown, B-Humansdorp, and C-Van Stadens River dams) as a percentage of dry plant weight.

Pyrrolizidine alkaloid	A %/10 <sup>-2</sup>	B %/10 <sup>-2</sup>	C %/10 <sup>-2</sup>
7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine <b>69</b>	0.27	0.16	0.14
7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine <b>71</b>	6.6	2.1	2.9
9-angelylplatynecine-4-oxide <b>68</b>	0.95	0.97	0.89
7-angelylhastanecine <b>66</b>	0.52	0.39	0.42
7-angelylplatynecine <b>38</b>	0.20	0.44	0.42
9-angelylhastanecine <b>67</b>	5.1	7.1	4.5
9-angelylplatynecine <b>39</b>	28.8	22.1	11.0
sarracine <b>36</b>	3.9	1.2	1.9
neosarracine <b>72</b>	14.0	4.2	3.0
<b>Total concentration</b>	<b>60.3</b>	<b>38.7</b>	<b>25.2</b>

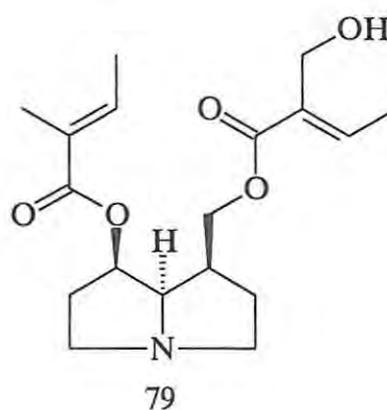
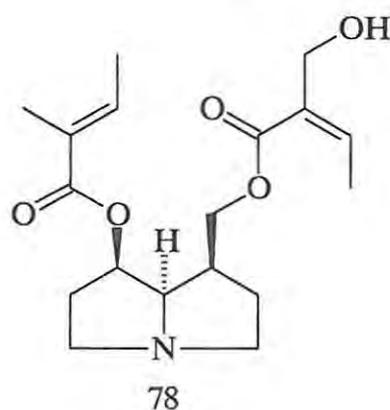
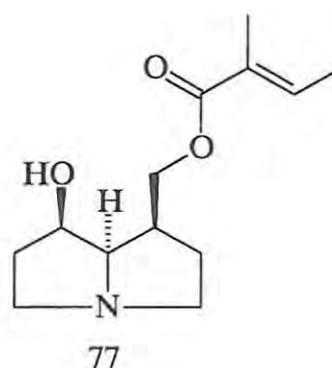
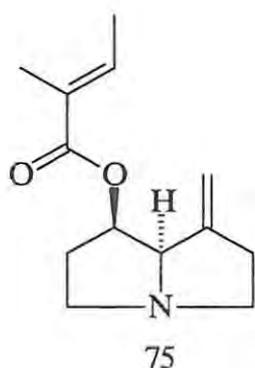
**Table 2.9b:** Concentrations of identified pyrrolizidine alkaloids in plant extracts from the *S. chrysocoma* border-line populations (D-Dieprivierhoogte, E-Prince Alfred's Pass, and F-Wittedrif) as a percentage of dry plant weight.

<b>Pyrrolizidine alkaloid</b>	<b>D</b> %/10 <sup>-2</sup>	<b>E</b> %/10 <sup>-2</sup>	<b>F</b> %/10 <sup>-2</sup>
7β-angelyl-1-methylene-8α-pyrrolizidine <b>71</b>	0.90	3.2	3.4
9-angelylplatynecine-4-oxide <b>68</b>	0.30	0.44	0.41
7-angelylhastanecine <b>66</b>	0.13	0.44	1.2
7-angelylplatynecine <b>38</b>	0.052	0.63	0.48
9-angelylhastanecine <b>67</b>	1.2	12.0	12.6
9-angelylplatynecine <b>39</b>	11.8	49.0	35.1
sarracine <b>36</b>	0.41	0.27	0.80
neosarracine <b>72</b>	1.4	0.88	2.7
<b>Total concentration</b>	16.2	66.9	56.7

**Table 2.9c:** Concentrations of identified pyrrolizidine alkaloids in plant extracts from the *S. paniculatus* populations (G-Assegaibos, H-Robinson Pass, and I-Tradouw Pass) as a percentage of dry plant weight.

<b>Pyrrolizidine alkaloid</b>	<b>G</b> %/10 <sup>-2</sup>	<b>H</b> %/10 <sup>-2</sup>	<b>I</b> %/10 <sup>-2</sup>
7β-angelyl-1-methylene-8α-pyrrolizidine <b>71</b>	2.5	0.92	0.84
9-angelylplatynecine-4-oxide <b>68</b>	0.30	0.23	0.36
7-angelylhastanecine <b>66</b>	0.25	0.12	0.25
7-angelylplatynecine <b>38</b>	0.71	0.48	0.34
9-angelylhastanecine <b>67</b>	4.7	3.8	2.7
9-angelylplatynecine <b>39</b>	11.3	12.5	7.5
sarracine <b>36</b>	0.32	0.47	1.1
neosarracine <b>72</b>	1.1	1.5	2.4
retrorsine <b>9</b>	0.11	0.32	0.21
<b>Total concentration</b>	21.3	20.3	15.7

In the representative GLC chromatograms of the crude extracts (see Figure 2.15) there are five unidentified peaks on each chromatogram (labelled \*) with retention times slightly longer than 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**, 9-angelylplatynecine-4-oxide **68**, 9-angelylplatynecine **39**, sarracine **36** and neosarracine **72**, respectively. It is possible that these represent the corresponding tiglyl isomers, namely, 7 $\beta$ -tiglyl-1-methylene-8 $\alpha$ -pyrrolizidine **75**, 9-tiglylplatynecine-4-oxide **76**, 9-tiglylplatynecine **77**, sarranicine **78** and neosarranicine **79**. The mass spectra of these compounds correlate well with their corresponding angelyl isomers (Table 2.10); as the necic acid components are geometric isomers and necine bases are identical, similar mass fragmentation patterns are expected.



**Table 2.10:** Mass spectral data of the proposed tiglyl derivatives and their angelyl isomers.

Pyrrolizidine alkaloid	Mass fragment†
7β-angelyl-1-methylene-8α-pyrrolizidine <b>71</b>	138(42), 121(42), 95(100), 80(7), 67(7), 55(13).
7β-tiglyl-1-methylene-8α-pyrrolizidine <b>75</b>	138(38), 121(45), 95(100), 67(7), 55(11).
9-angelylplatynecine <b>39</b>	221(5), 195(1), 156(2), 140(6), 139(4), 138(5), 122(9), 96(36), 95(100), 82(94), 55(19).
9-tiglylplatynecine <b>77</b>	221(7), 195(2), 156(1), 138(4), 122(5), 96(13), 95(100), 82(72), 55(17).
sarracine <b>36</b>	237(8), 222(10), 140(37), 139(32), 138(100), 123(26), 122(57), 121(13), 96(46), 95(47), 83(35), 82(74), 55(35).
sarranicine <b>78</b>	237(11), 222(12), 140(38), 139(29), 138(100), 122(78), 96(38), 95(49), 83(36), 82(76), 55(32).
neosarracine <b>72</b>	237(9), 222(9), 140(29), 139(27), 138(100), 123(22), 122(38), 121(15), 96(30), 95(51), 83(24), 82(64), 55(27).
neosarranicine <b>79</b>	237(11), 222(12), 140(51), 139(37), 138(100), 122(53), 96(47), 95(79), 83(42), 82(90), 55(42).

† *M/z* values followed by % relative abundance in parentheses.

Proton NMR spectra of fractions which, by GC-MS, contained a supposed tiglyl compound, revealed a quartet at *ca.* 6.7 ppm, which is characteristic of the vinylic proton of the tiglic acid moiety. The tiglyl compounds were never present in large amounts and no single tiglyl compound was completely identified by NMR techniques. The temperatures involved in GLC analyses could easily have caused an increase in the percentage of tiglyl isomers. Stelljes *et al.*<sup>68</sup> have noted that *trans* isomers always elute after their *cis* isomers. They were looking, in particular, at sarracine **36** (7-angelyl-9-sarracinyplatynecine) and neosarracine **72** (7-angelyl-9-isosarracinyplatynecine), and the corresponding tiglyl isomers, sarranicine **78** (7-tiglyl-9-sarracinyplatynecine) and neosarranicine **79** (7-tiglyl-9-isosarracinyplatynecine). Isosarracinic acid is the *trans* isomer of sarracinic acid, while tiglic acid is the *trans* isomer of angelic acid. When the sarracine/neosarracine region of the GLC chromatograms in Figure 2.15 is expanded

(e.g. Figure 2.16), four peaks can be distinguished. The first two components have been identified by NMR spectroscopy as sarracine **36** and neosarracine **72**; in view of the observation by Stelljes *et al.*,<sup>68</sup> the other two could be the corresponding tiglic isomers (**78** and **79**). These deductions are consistent with the expectation that sarracine **36** (in which both necic acids, angelic and sarracinic, are the *cis* isomers) should have the shortest retention time of the four isomers, while neosarracinic **79** (in which both necic acids are the *trans* isomers) would have the longest retention time.

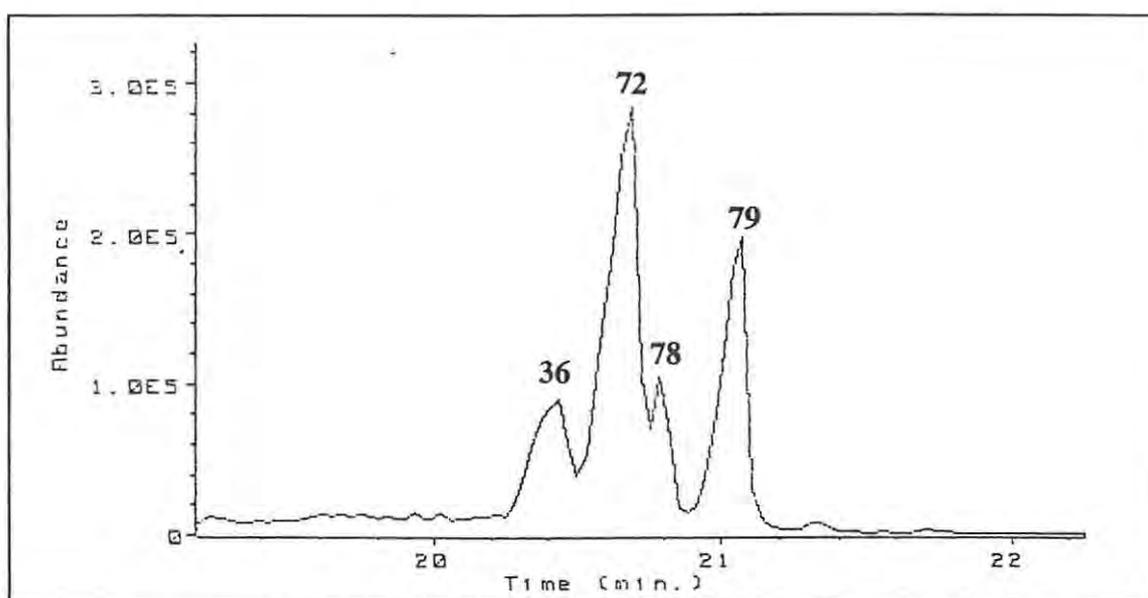


Figure 2.16: The expanded sarracine/neosarracine region of the GLC chromatogram.

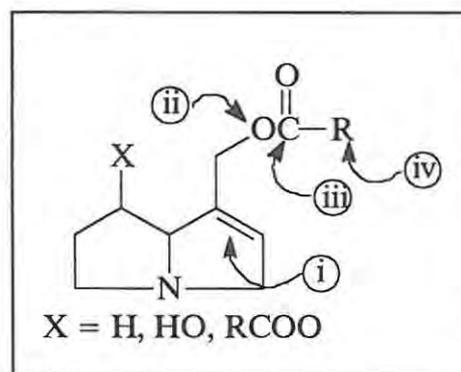
As indicated previously (p. 45), *S. paniculatus* has been recorded as containing senecionine **10** and platyphylline **17**,<sup>31,33</sup> but as neither of these was found to be present the original publication<sup>318</sup> was consulted and it was discovered that *S. grandifolius* and not *S. paniculatus* had actually been extracted. Nowhere has *S. grandifolius* been found as a synonym for *S. paniculatus*. On the other hand, *S. graminifolius*, which is given as a synonym for *S. chrysocoma*, has been found to contain the macrocyclic pyrrolizidine retrorsine **9**. *S. graminifolius* could, therefore, be the genuine *S. paniculatus*.

## 2.7 BIOLOGICAL ACTIVITY

The cytotoxicity of pyrrolizidine alkaloids is caused by pyrrolic metabolites which are formed in the liver.<sup>319</sup> This, in turn, depends on the alkaloid having a structure that has the potential to be converted to toxic metabolites and the ability of an animal's enzymes to effect this conversion.<sup>319</sup> The minimum structural requirements for hepatotoxicity of naturally occurring pyrrolizidine alkaloids include (see Figure 2.17):-

- i) an unsaturated necine base portion;
- ii) a hydroxyl group on carbon 9 and, preferably, on carbon 7 too;
- iii) esterification of at least one of the hydroxyl groups; and
- iv) a branched necic acid moiety.<sup>319</sup>

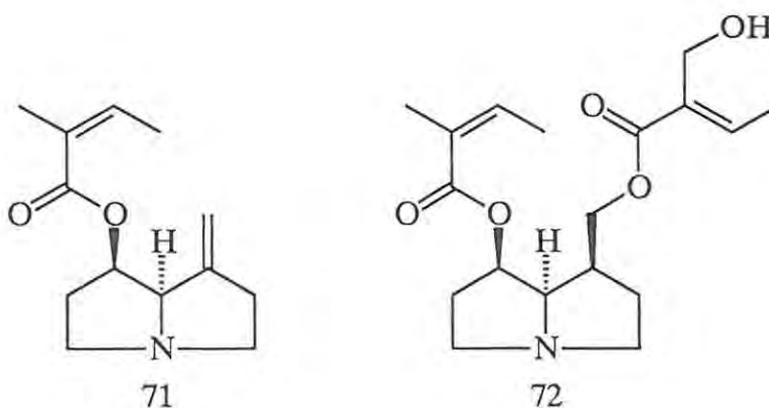
**Figure 2.17:** The minimum structural requirements for hepatotoxicity of naturally occurring pyrrolizidine alkaloids.<sup>319</sup>



The alkaloids identified in this study, with the exception of retrorsine **9**, do not fit the first requirement, which is essential for pyrrole formation. Retrorsine **9** is present in *Senecio paniculatus*, but in very low concentrations, and is absent in *S. chrysocoma*, so neither species should be exceedingly toxic to stock. The 7-angelyl-1-methylenepyrrolizidines (**69**, **70**, **71**) have an exocyclic double bond on the necine base moiety but, as far as has been determined, this will not migrate to an endocyclic position to allow for pyrrole formation.<sup>320</sup> It is interesting to note that Erhlich's spray reagent detects unsaturated pyrrolizidine alkaloids as their pyrrole derivatives.<sup>136,321</sup> For TLC identification a 1% solution *o*-chloranil in toluene is used as a spray to convert the alkaloids to their pyrrole derivatives,<sup>134-136</sup> which then stain purple with Erhlich's reagent. Retrorsine **9** readily undergoes this conversion and is seen as a purple spot on TLC plates, while the

platynecine-based alkaloids did not give a positive response. The 7-angelyl-1-methylenepyrrolizidines did not stain purple under the same conditions and were therefore assumed not to form the pyrrole derivatives essential for cytotoxicity. However, this does not rule out the possibility that enzymes can catalyse the conversion.

The National Cancer Institute (NCI), in the United States of America, tested neosarracine **72** and the 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** for antitumour activity. A sample of neosarracine was requested by the NCI and as 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine was a novel pyrrolizidine and, therefore, an untested structural arrangement it was sent for testing. The NCI tests provide an initial evaluation of a potential antitumour agent on as little as 25 mg.<sup>322</sup> In these tests, the cell panel consists of 60 human cell lines from nine cancer types (lung, colon, melanoma, renal, ovarian, central nervous system, leukaemia, prostate and breast) against which the agents are tested at a minimum of five different concentrations. Cell viability or growth is estimated by a sulphorhodamine B (SRB) protein assay.<sup>322</sup> Neosarracine **72** showed mild activity against a few cell lines and was thus tested twice, but 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** exhibited no antitumour activity.



Dose response curves (in which percentage growth is plotted against the  $\log_{10}$  of the concentration of test compound) for 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** are shown in Figure 2.18. Horizontal lines representing a percentage growth of +50, 0, and -50 are given. The concentrations corresponding to where the curves cross these lines, if they do,

are the 50% growth inhibition (GI50), total growth inhibition (TGI), and 50% cytotoxicity (LC50) concentrations, respectively. From these curves it can be seen that for 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine 50% growth inhibition concentration is only achieved once, for a colon cancer, over the tested range.

For neosarracine **72** the mean graphs (Figure 2.19) are shown instead of the dose response curves. These graphs summarise the data obtained from the dose response curves in a manner which readily shows any above average activity against any particular cell line. Bars extending to the right show sensitivity of that cell line to the test compound compared to the average of all the tested cell lines. The bar scale is logarithmic, hence, a bar two units to the right implies that the compound achieved the response parameter at a concentration one hundredth of the mean concentration required over all cell lines and thus, that cell line is particularly sensitive to the test compound. Bars to the left indicate sensitivity less than the mean. If a response parameter could not be determined by interpolation, on the dose response curves, the bar length shown is the highest or lowest concentration tested and the response parameter is preceded by > or <, respectively. Neosarracine **72** showed activity against leukaemia, colon cancer and melanoma cell lines and less activity against renal and breast cancer cell lines, but at no time was the activity exceptional and worthy of further investigation. It is interesting to note the activity shown against all the forms of leukaemia tested as it was against leukaemia that indicine-4-oxide **3**, the only pyrrolizidine alkaloid to undergo clinical trials, was marginally successful;<sup>30</sup> the toxicity of the alkaloid being a major side effect.<sup>234</sup>

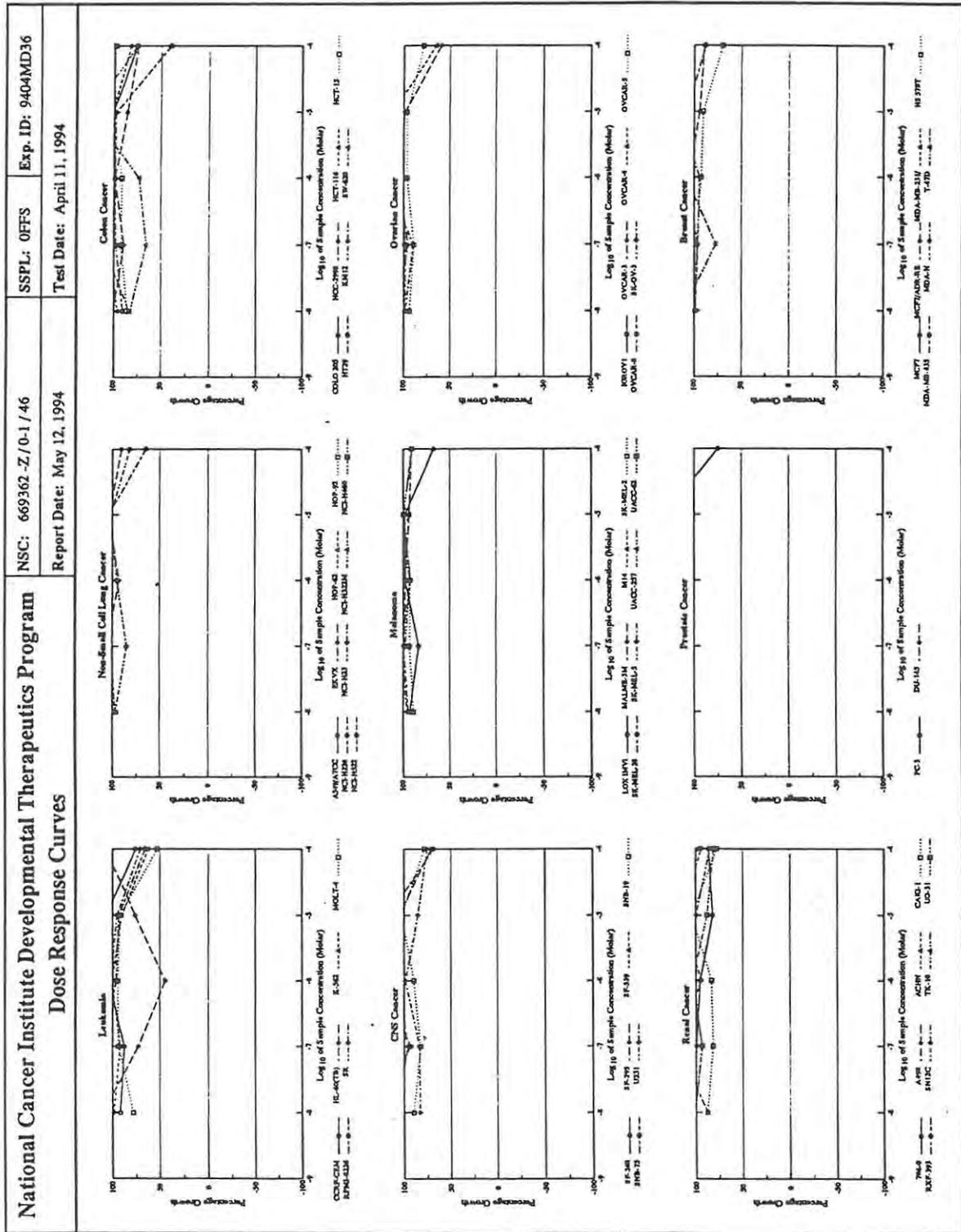


Figure 2.18: Dose response curves for 7β-angelyl-1-methylene-8α-pyrrolizidine's 71 antitumour tests.

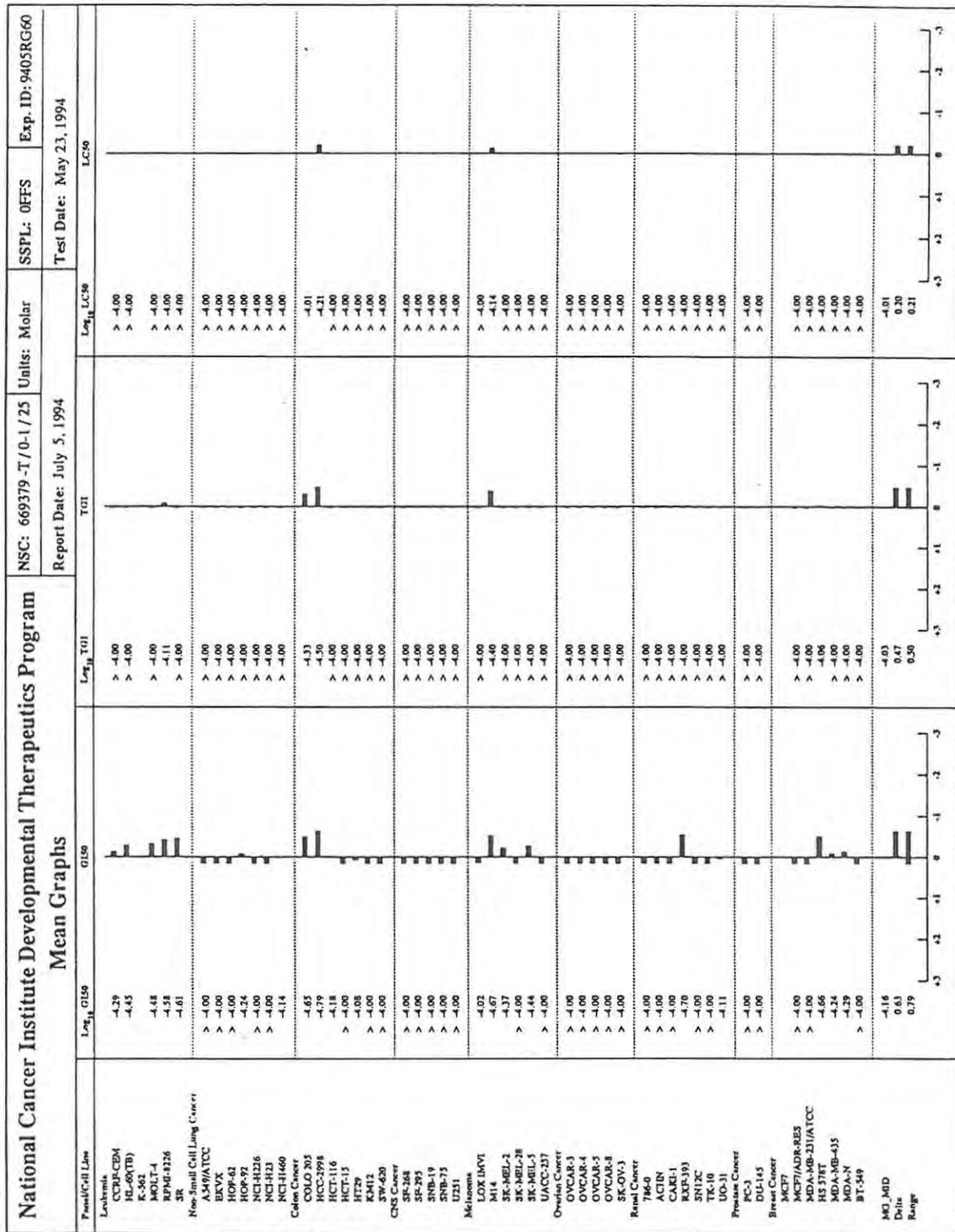


Figure 2.19: Mean graphs of results of neosarracine's 72 antitumour tests.

Neosarracine **72** was also tested by the NCI as a potential anti-human immunodeficiency virus (HIV) agent. The test used by the NCI<sup>323</sup> is designed to detect agents acting at any stage of the viral reproductive cycle and involves the killing of T4 lymphocytes by the HIV. The results of the test are shown in Figure 2.20. The solid line is the percentage surviving HIV-infected cells treated with neosarracine relative to uninfected, untreated controls and the line, therefore, expresses the *in vitro* anti-HIV activity of neosarracine. The dashed line is the percentage surviving uninfected cells treated with neosarracine relative to the same uninfected, untreated controls and represents the *in vitro* growth inhibitory properties of the alkaloid. The viral cytopathic effect is represented by the dotted line and shows the extent of cell destruction by the virus in absence of treatment. This property is used as a quality control parameter. As can be deduced from the graph, neosarracine **72** was inactive against HIV.

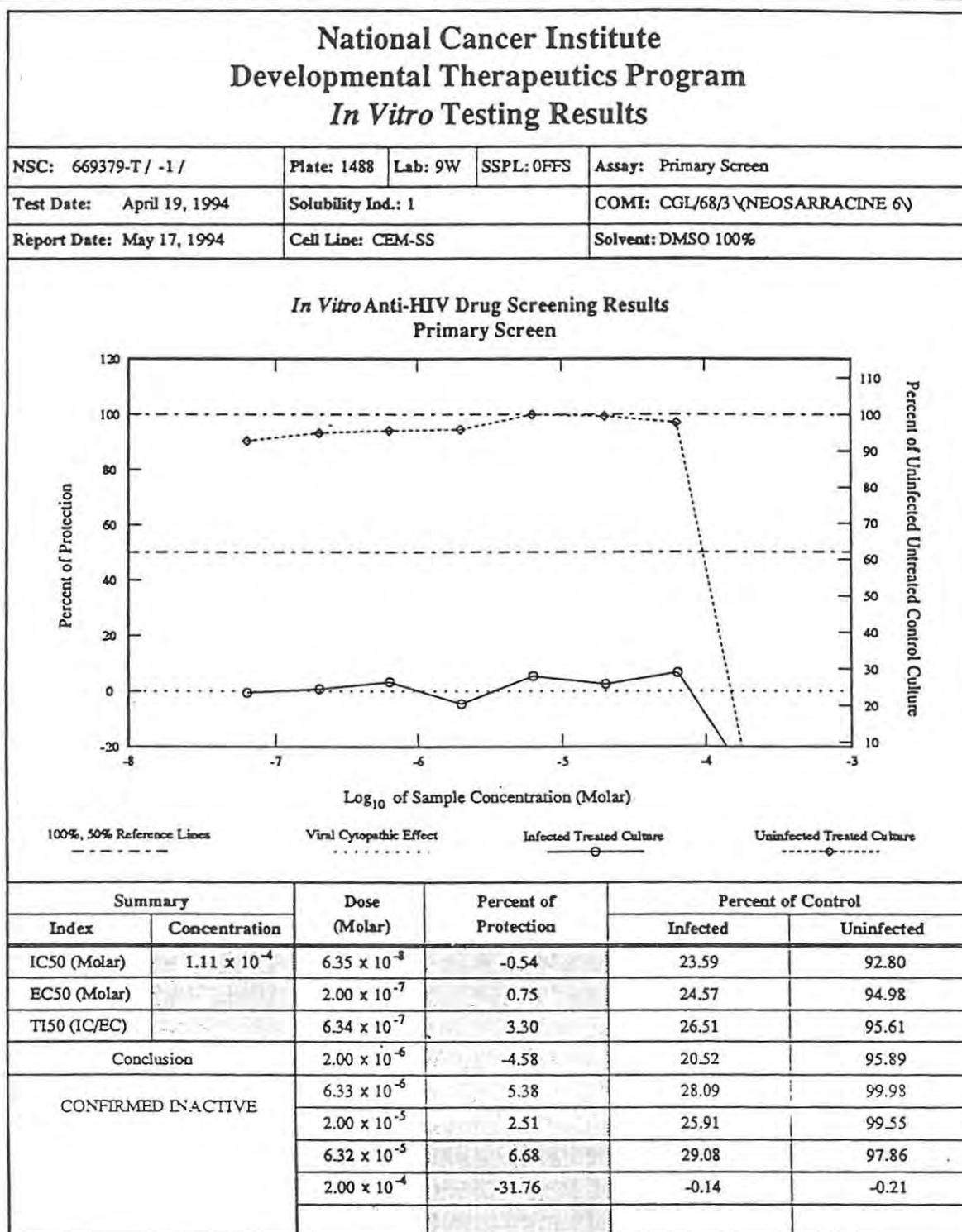


Figure 2.20: The results of the anti-HIV tests performed with neosarracine 72.

## 2.8 CONCLUSION

In total, eight known pyrrolizidine alkaloids, 7-angelylhastanecine **66**, 7-angelylplatynecine **38**, 9-angelylhastanecine **67**, 9-angelylplatynecine **39** and its *N*-oxide **68**, sarracine **36**, neosarracine **72** and retrorsine **9**, were extracted from the plant material and identified by NMR spectroscopy. The NMR data for the known 7-angelylhastanecine **66** and 9-angelylhastanecine **67** has been reported for the first time. The novel structured 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69** and its *N*-oxide **70**, 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**, were also identified in the plant extracts by NMR techniques. With the exception of 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69** and its *N*-oxide **70**, present in *S. chrysocoma*, and retrorsine **9**, present in *S. paniculatus*, all the remaining alkaloids were found in both *S. chrysocoma* and *S. paniculatus*. The presence, in both species, of a further five alkaloids, 7 $\beta$ -tiglyl-1-methylene-8 $\alpha$ -pyrrolizidine **75**, 9-tiglylplatynecine **77** and its *N*-oxide **76**, sarranicine **36** and neosarranicine **72**, has been proposed on the strength of the GC-MS data, but these compounds could be artefacts of the isolation and identification processes.

A morphological difference between the two species, *S. chrysocoma* and *S. paniculatus*, was definitely apparent in the plants collected for this investigation; those plants collected west of Uniondale had pinnatipartite leaves, while those east of Uniondale had linear-lanceolate leaves. Despite the morphological difference between the two species, it was clear that there is very little difference in their pyrrolizidine alkaloid content. However, the presence of retrorsine **9** in *S. paniculatus* is one distinguishing feature, particularly as it has a different basic structure from the other pyrrolizidines extracted from these plants.

None of the alkaloids which have been identified, with the exception of retrorsine **9**, have the requisite structure for hepatotoxicity. Moreover, retrorsine was present in such low concentrations (0.0011-0.0032% of dry plant weight) that the toxicity of *S. paniculatus* to stock is probably negligible. Neosarracine **72** and 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** were tested for antitumour activity, and neosarracine **72** for anti-HIV activity.

Neosarracine exhibited limited antitumour activity but no anti-HIV activity, while 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** showed no antitumour activity.

### 3. THE PYRROLIZIDINE ALKALOID DATABASE

The physical data for many pyrrolizidine alkaloids are now known and are published in a wide selection of journals. When investigating a new plant this information can be used to identify the pyrrolizidines present, but the variety of ways in which the information is reported makes access difficult. Consequently, a database of the physical data of pyrrolizidine alkaloids was compiled during the course of this study. Combining all the known data into a single database has made the data more readily available and easier to use. Moreover, not only are the data in the same format, making comparisons decidedly simpler, but trends can also be determined.

This database originally consisted of the physical data for about 100 pyrrolizidine alkaloids and was created using dBase® III. It has now been upgraded to hold the data for 490 pyrrolizidines and has been transferred to a more user friendly relational database programme system, Paradox® 3.5. Since the original compilation of the database, Borland has released Paradox® 5.0 for Windows. The added advantage of the improved system is that figures can now be included alongside the text and, as it is a Windows-based programme, structures of the pyrrolizidine alkaloids can easily be imported from ChemWindow®, a powerful chemical structure drawing programme. It is pertinent to note that files in Paradox® 5.0 and dBase® are now interconvertible.

#### 3.1 THE DATA INCLUDED IN THE DATABASE

As much published information as possible has been included (from 1940 to 1995), however, since Russian, Chinese and Japanese papers are not readily accessible or easily translated, most of the information has been gleaned from papers written in English, German, and French. The data held in the database can either be viewed on the screen as a table or as a printed report, the form of which can be designed by the user - a useful feature of Paradox®. Usually, the most suitable report would be one that presented all the

data for a single pyrrolizidine alkaloid on a single page; such a report has been designed and is illustrated in Figure 3.1.

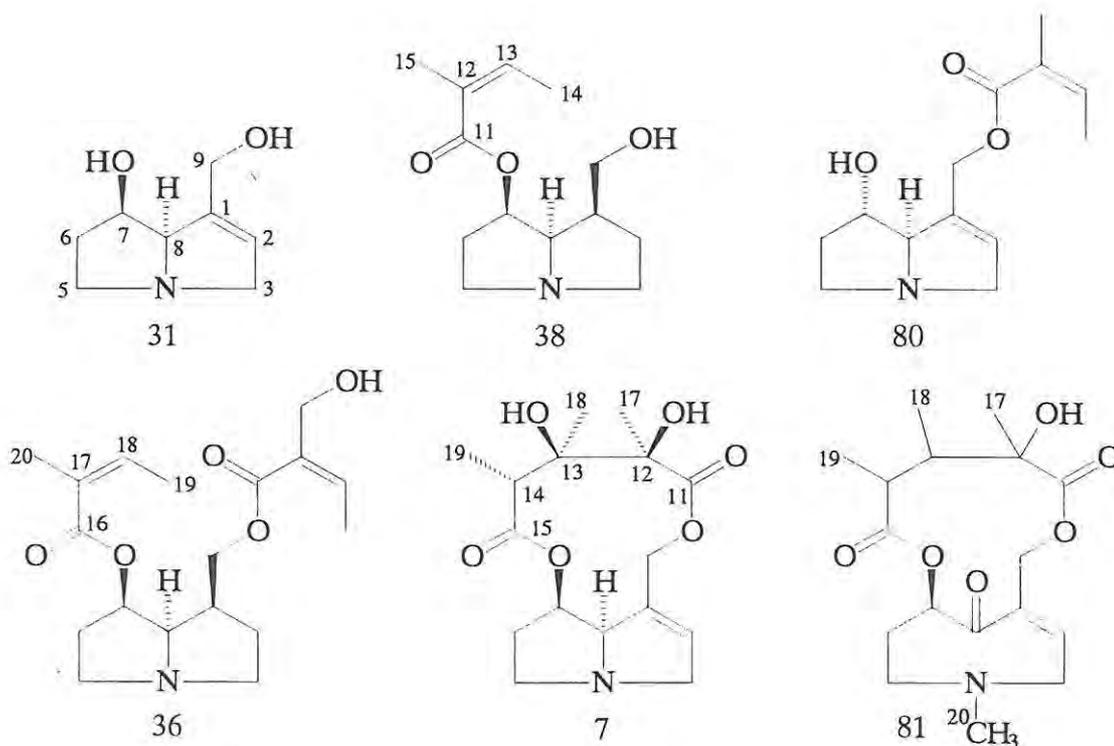
In such reports, the pyrrolizidine is referred to by its common name (A), although any known synonyms, which may include other common names or a systematic/IUPAC name (B) are also recorded. In addition, the alkaloid is identified by its formula (C), written in the same format for all the pyrrolizidines, and its molecular mass (D). The Chemical Abstracts Service (CAS) registry number (E) is present for those alkaloids for which it is readily known. If the pyrrolizidine is a crystalline solid the melting point (F) is given in °C. If it is not a solid, "gum" or "oil" is recorded in the melting point column, depending on what is reported. The specific rotation (G), where it is known, is given together with the solvent in which it was measured. Although the data are only taken from a few papers, as many reports of the alkaloid as possible are noted (H), as they may give additional, helpful information, including reports of the occurrence of the alkaloid in different botanical, or in some cases zoological, species; the references from which each set of physical data were taken, are also quoted (I). All the pyrrolizidine alkaloids have been assigned a type depending on their basic structure; this is the system followed by Roeder<sup>324</sup> in his carbon-13 NMR spectroscopy review. For ease of correlation each type has been given a number (J) as shown in Table 3.1.

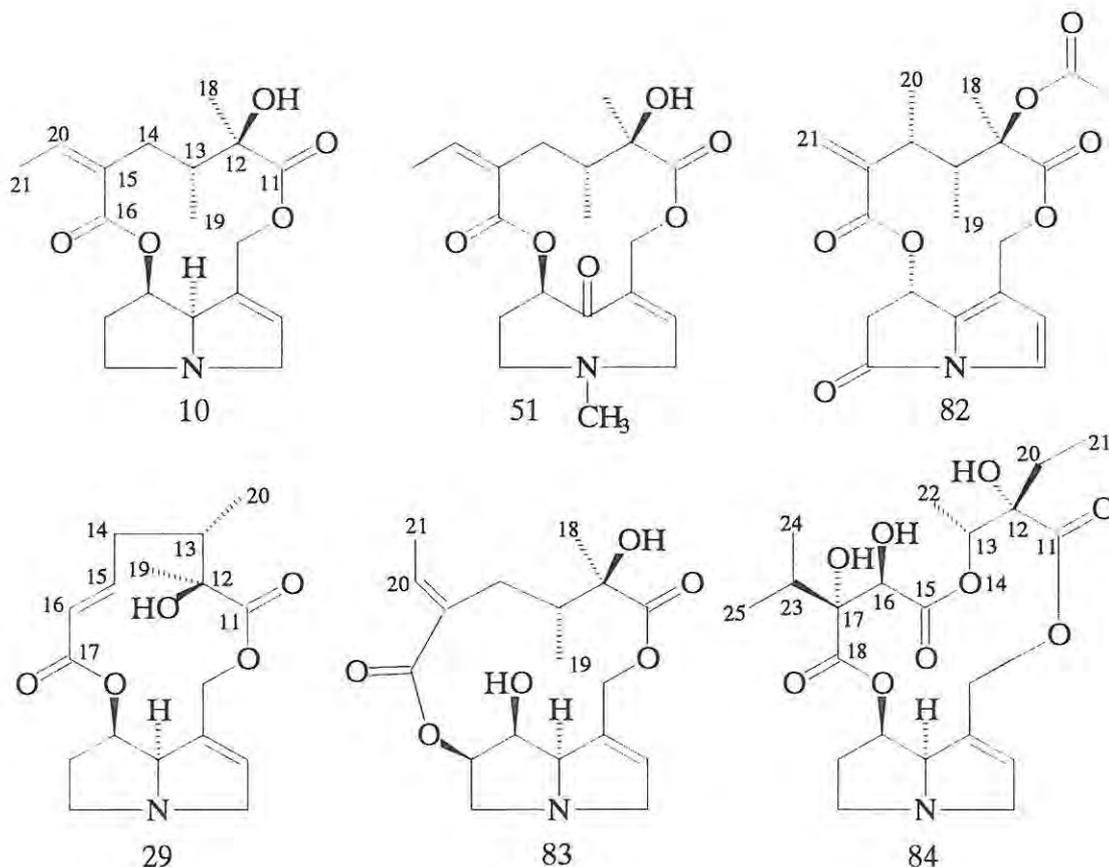
Common name: Retrorsine <b>A</b>				
Synonyms: $\beta$ -Longilobine <b>B</b>				
Formula : C <sub>18</sub> H <sub>25</sub> N.O <sub>6</sub> <b>C</b>		CAS registry no: 480-54-6 <b>E</b>		
Molecular mass : 351 <b>D</b>		Melting pt: 216-7 <b>F</b>		Specific rotation: -23.2 EtOH <b>G</b>
References : 6,125,150,325-328 <b>H</b>				
Alkaloid type : 7 <b>J</b>				
-----				
H-NMR data. <b>K</b>		Solvent: CDCl <sub>3</sub> <b>L</b>		Reference: 329 <b>I</b>
-----				
1 :	7a :	14a :	19a :	26 :
2a : 6.20	7b :	14b : 2.19	19b :	27 :
2b :	8 : 4.27	15 :	20a : 5.71	28 :
3a : 3.39	9a : 4.09	16a :	20b :	29 :
3b : 3.94	9b : 5.49	16b :	21 : 1.83	30 :
5a : 2.53	12a :	17a :	22 :	:
5b : 3.26	12b :	17b :	23 :	OH :
6a : 2.15	13a : 1.64	18a : 3.62	24 :	:
6b : 2.38	13b :	18b : 3.74	25 :	:
-----				
C-13 NMR data. <b>K</b>		Solvent: CDCl <sub>3</sub> <b>L</b>		Reference: 330 <b>I</b>
-----				
1 : 132.5	9 : 61.1	17 :	24 :	
2 : 136.7	11 : 175.6	18 : 66.9	25 :	
3 : 62.8	12 : 81.4	19 : 11.7	26 :	
5 : 53.0	13 : 35.7	20 : 134.6	27 :	
6 : 34.7	14 : 37.9	21 : 15.0	28 :	
7 : 75.0	15 : 131.3	22 :	29 :	
8 : 77.5	16 : 167.3	23 :	30 :	
-----				
EI-MS reference: 142 <b>I</b>				
EI-MS : 351 307 246 220 136 120B 117 95 93 80 <b>M</b>				
NICI-MS reference: 331 <b>I</b>				
NICI-MS : 352 351B 333 319 231 114 <b>M</b>				
PICI-MS reference: 332 <b>I</b>				
PICI-MS : 352 320 292 246 220 138 136 120B 95 <b>M</b>				
IR reference: 146 <b>I</b>				
Infra-red data: 3580 3430 1738 1710 <b>N</b>				
X-ray data reference: 98 <b>O</b>				

Figure 3.1: The report sheet for retrorsine, an example of a typical report sheet generated by Paradox®.

Table 3.1: The number assigned to each of the pyrrolizidine alkaloid types.

No.	Alkaloid Type	Example
1	necine bases	retronecine <b>31</b>
2	7-monoesters	7-angelylplatynecine <b>38</b>
3	9-monoesters	9-angelylheliotridine <b>80</b>
4	7,9-acyclic diesters	sarracine <b>36</b>
5	11-membered crotalananes	monocrotaline <b>7</b>
6	11-membered with otonecine base	croaegyptine <b>81</b>
7	12-membered senecionanes	senecionine <b>10</b>
8	12-membered otonecanes	senkirrine <b>51</b>
9	12-membered pterophoranes	pterophorine <b>82</b>
10	13-membered dorononanes	doronanine <b>29</b>
11	13-membered madurensanes	madurensine <b>83</b>
12	14-membered parsonanes	parsonsianidine <b>84</b>
13	miscellaneous types	





The NMR data (K) were chosen from the indicated publication (I); the recorded shifts are not an average of many reports, but are the most complete and/or most up-to-date set. Preference was also given to data which were obtained in deuterated chloroform to facilitate standardisation. Some pyrrolizidine alkaloids, such as necine bases and *N*-oxides, however, are not soluble in chloroform so the solvent used is also recorded (L). For the mass spectrometry data (M) the ten strongest signals were chosen (if more than ten were published) and the base peak is indicated by a capital B after the mass value. This method does not necessarily show up the trends, some of which rely on the presence of triads (see Section 1.3.4, p. 18), but the reference (I) permits the original report to be consulted. Only the first six absorption frequencies are quoted for the infra-red spectroscopy data (N), while only a reference (O) is given if the X-ray crystal structure has been determined. It

should be noted that for some pyrrolizidine alkaloids very little information has been reported and, therefore, there are a number of blank spaces.

A complementary database of references accompanies the database of pyrrolizidine alkaloids and the Paradox<sup>®</sup> system allows for on-screen linking between the two. The usual facts are listed in the reference database including author(s), journal, title and contents key words. The reference number is the link between the reference database and the pyrrolizidine alkaloid database.

## 3.2 UTILISING THE DATABASE

Although the database contains a variety of data, the NMR data have proved to be the most useful in identifying pyrrolizidine alkaloids. The only previously published major collection of pyrrolizidine data comprised carbon-13 NMR shifts for 136 pyrrolizidine alkaloids,<sup>324</sup> which was updated in 1991 to include the data for 26 more pyrrolizidines.<sup>333</sup> However, using the database developed in this project a paper containing the proton NMR shifts for 372 pyrrolizidine alkaloids has been published,<sup>334</sup> and a paper reporting the carbon-13 NMR data of *ca.* 300 pyrrolizidine alkaloids is to be submitted for publication.<sup>335</sup> As the data are now all in the same format, various trends in the proton and carbon-13 NMR shifts have become apparent. Comparison of the data for pyrrolizidine alkaloids with similar structures will be discussed below, and will be followed by an application of the database to a particular example.

### 3.2.1 TRENDS IN THE PROTON NMR DATA FOR PYRROLIZIDINE ALKALOIDS

As already noted pyrrolizidine alkaloids consist of two parts, the necine base and the necic acid(s). The protons of the necine base have very distinctive shifts, the degree of saturation and the oxygenation pattern being easily recognisable. The important signals are for the protons on carbons 2, 6, 7, 8 and 9.

The type of alkaloid, be it an acyclic mono- or diester or a macrocyclic diester, can often be determined from the chemical shifts of the 7- and 9-proton signals. When the necine base is hydroxylated at C-7 and C-9, the protons on carbons 7 and 9 resonate at *ca.*  $\delta$ 4.5 and  $\delta$ 3.5, respectively. In both cases, if the hydroxyl group is esterified, the proton signal shifts *ca.* 1 ppm downfield. Should there be no oxygen on C-7, then the 7-methylene protons are usually chemically non-equivalent, being diastereotopic, and their shifts are in the region  $\delta$ 1.5-2.2. The 9-methylene protons are also normally chemically non-equivalent and the difference in the two chemical shifts,  $\Delta$ H-9, was shown by Culvenor and Woods to reflect the size of a macrocycle.<sup>216</sup> These authors give a  $\Delta$ H-9 range of 1.0-1.5 ppm for 12-membered macrocycles (*e.g.* senecionine **10**) and  $\Delta$ H-9 less than 0.9 ppm for 11-membered macrocycles (*e.g.* monocrotaline **7**). With all the NMR data now available for pyrrolizidine alkaloids, it has become clear that the  $\Delta$ H-9 value (Table 3.2) is only unambiguously diagnostic for 12-membered macrocyclic rings between narrow limits (*viz.* 1.25-1.54 ppm). It is also clear that there is no characteristic  $\Delta$ H-9 value for 11-membered macrocycles. Very few 13- and 14-membered macrocycles are known. For the 13-membered 7,9-diester macrocycles (*e.g.* doronenine **29**) there is no trend for  $\Delta$ H-9, but for the 6,9-diester (*e.g.* madurensine **83**) the  $\Delta$ H-9 value (1.58-1.65 ppm) is diagnostic. The 14-membered macrocycles (*e.g.* parsonsianidine **84**) have a very small  $\Delta$ H-9 value (0.75-0.95) and their *N*-oxides have an even smaller value (0.06-0.20), but they are not distinctive as they fall within the wider ranges for the 11- and 12-membered macrocycles.

**Table 3.2:**  $\Delta$ H-9 values for macrocyclic pyrrolizidine alkaloids.

macrocycle size	$\Delta$ H-9/ppm
11-membered	0.15-1.24 (majority < 1.0)
12-membered	0.08-1.54
13-membered (6,9-diester)	1.58-1.65
13-membered (7,9-diester)	no trend
14-membered	0.75-0.95

The H-8 signal shifts downfield for *N*-oxides, but, obviously, is absent for the otonecine-based pyrrolizidine alkaloids. The signals of all the remaining protons of the necine base portion of pyrrolizidine alkaloids are also shifted downfield for the *N*-oxide relative to the unoxidised form. Saturation of the base is indicated by the position of the H-2 signal. Thus, for saturated bases the signals are at approximately  $\delta$ 2.0-2.5, and the two methylene protons can be non-equivalent, while for typical unsaturated bases, the signal is at approximately  $\delta$ 5.8, and for 2-hydroxy bases at approximately  $\delta$ 4.0. The 6-methylene protons may be observed to be non-equivalent but their signals tend to be close together and appear at approximately 2.3 ppm. If C-6 is hydroxylated the H-6 signal is observed at approximately  $\delta$ 4.0; when the hydroxyl group at either C-2 or C-6 is esterified the methine proton resonates downfield at  $\delta$ 5.0-5.5.

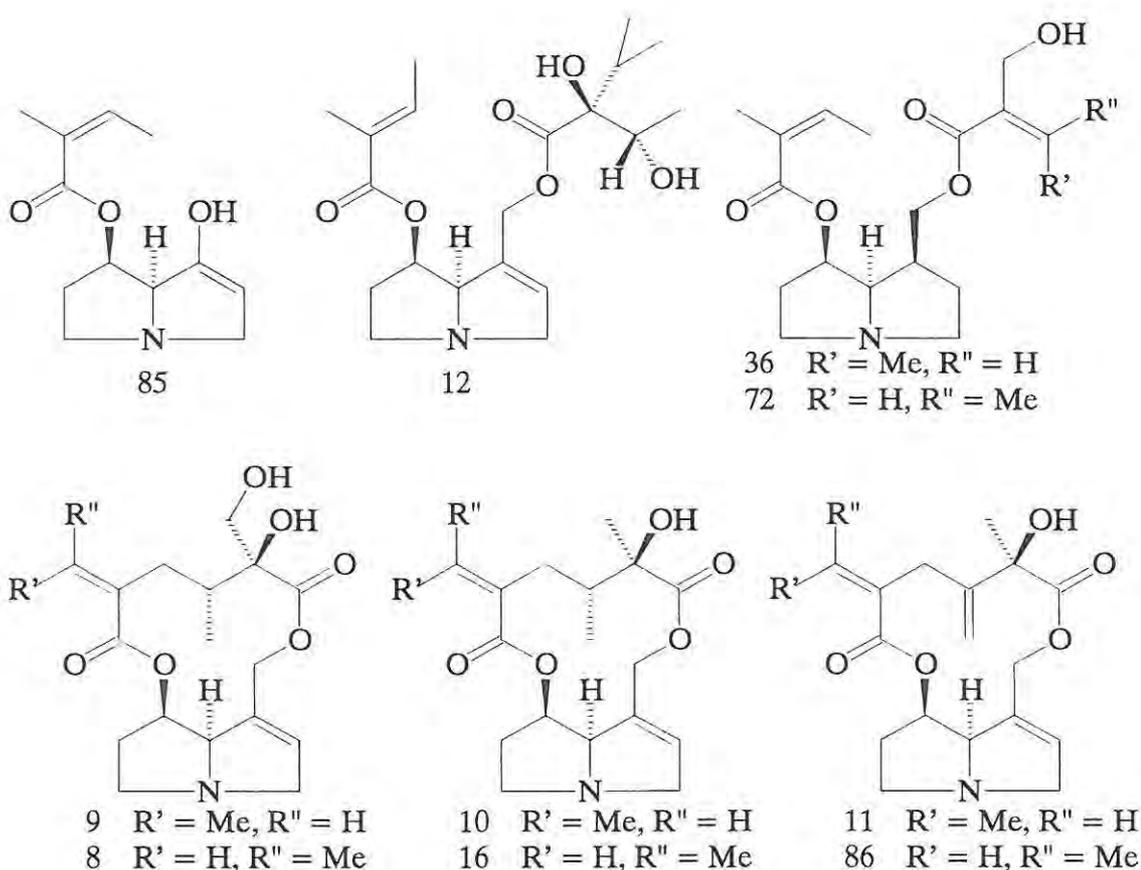
The chemical shift values for protons on C-1 vary between 2 and 4 ppm, with the larger values representing *N*-oxides. The signal is, of course, absent in the spectra of the common,  $\Delta^2$ -unsaturated pyrrolizidines, as well as the rare 1-hydroxy or 1-methylene bases; it is also absent for the pyrrole types. Pyrroles will be distinguished by signals in the aromatic region, for example, the H-2 signal at  $\delta$ 6.4-6.6.

The chemical shifts of the necic acid protons are not as distinctive as those for the bases, since many of the acids have very similar structures. The acids themselves can be divided into four groups:-

- i) the angelic/tiglic acid types;
- ii) the trachelanthic/viridifloric types;
- iii) the dicarboxylic acids which form macrocycles; and
- iv) a miscellaneous group containing aromatic rings, other ring systems and sulphur groups.

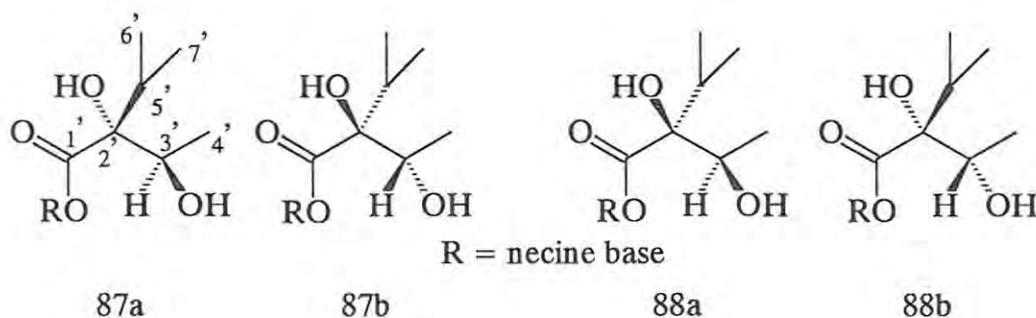
(*E*)- and (*Z*)-double bonds in unsaturated necic acids can often be distinguished by the shifts of the vinylic protons. For the more common (*Z*)-configuration the vinylic proton resonates close to  $\delta$ 6.0 - a pattern illustrated by the angelic acid moiety in 7-angelylretronecine **85** ( $\delta$ 6.11),<sup>336</sup> sarracinic acid in sarracine **36** ( $\delta$ 6.38),<sup>155</sup> isatinecic acid

in retrorsine **9** ( $\delta 5.71$ ),<sup>329</sup> senecic acid in senecionine **10** ( $\delta 5.71$ )<sup>329</sup> and seneciphyllic acid in seneciphylline **11** ( $\delta 5.83$ ).<sup>329</sup> For the (*E*)-configuration the corresponding signal is typically further downfield; for example, the tiglic acid moiety in symphytine **12** ( $\delta 6.70$ ),<sup>337</sup> isosarracinic acid in neosarracine **72** ( $\delta 6.97$ ),<sup>147</sup> integerrinecic acid in integerrimine **16** ( $\delta 6.52$ ),<sup>109</sup> the isomer of seneciphyllic acid in spartioidine **86** ( $\delta 6.70$ )<sup>338</sup> and retronecic acid in usaramine **8** ( $\delta 6.50$ ).<sup>338</sup> The peak multiplicity, viz. a quartet, distinguishes the vinyl signal in these acids from the H-2 signal in unsaturated pyrrolizidines which resonates as a broad singlet.



Certain trends may also be observed in the <sup>1</sup>H NMR data of the saturated viridifloric and trachelanthic type necic acids. These compounds are stereoisomers of 2,3-dihydroxy-2-isopropylbutyric acid and all four isomers have been synthesised and characterised.<sup>289</sup> (+)-Viridifloric acid is the (2*R*,3*R*) isomer (**87a**, R=H) and, hence, the (2*S*,3*S*) isomer

(**87b**, R=H) is (-)-viridifloric acid, while the (2*S*,3*R*) and (2*R*,3*S*) (**88a** and **88b**, R=H) isomers are the (+)- and (-)-trachelanthic acids, respectively. It is possible to distinguish between the viridiflorate and trachelanthate systems using the proton signals, but the differences are small and some of the signals are obscured by the signals for the necine base part of the pyrrolizidine. The viridifloryl moiety **87** in pyrrolizidine alkaloids is characterised by the following signals: H-5' greater than  $\delta 2.10$ , H-4' usually greater than  $\delta 1.24$  and H-3' less than  $\delta 4.0$ . When an ester function is present at C-3', H-3' is shifted downfield to *ca.* 5 ppm and is no longer diagnostic. The equivalent shifts for the trachelanthic moiety **88** are: H-5' less than  $\delta 2.10$ , H-4' usually less than  $\delta 1.20$  and H-3' greater than  $\delta 4.0$ . In addition, the chemical shift difference between the H-6' and H-7' signals is normally greater than 0.4 ppm for the viridifloric group and less than 0.4 ppm for the trachelanthic group.



Replacement of the hydrogen on carbon-5' of viridifloric and trachelanthic acids with a hydroxyl group provides lasiocarpic and echimidinic acids, respectively. There are far fewer reports on these acids, but they do appear to follow the same trends as viridifloric and trachelanthic acids and can be distinguished similarly.

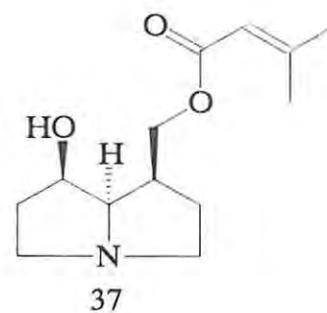
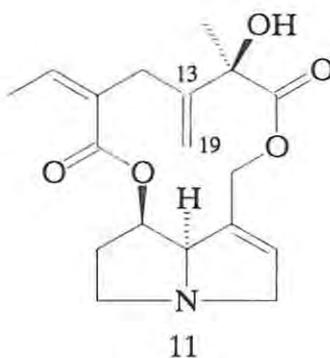
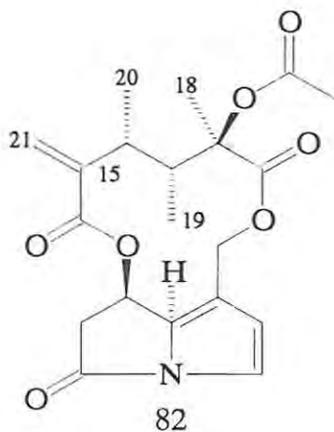
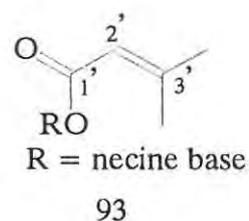
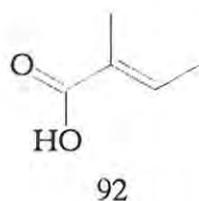
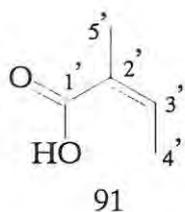
(-)-Heleuric acid (2-hydroxy-2-isopropyl-3-methoxybutyric acid) **89** is stereochemically analogous to (+)-trachelanthic acid and H-4' and H-5' follow the trends observed for trachelanthic acid. However, H-3' resonates below  $\delta 3.60$ , which is upfield of the equivalent proton in trachelanthic acid. Similarly, curassavic acid (2,3-dihydroxy-2-isobutylbutyric acid) **90** is analogous to viridifloric acid, and the H-3' and H-4' shifts of



between 79 and 85 ppm. Carbon-2, for saturated necine bases, and carbon-6, for all necine bases, resonate at 24-40 ppm but, when they are hydroxylated, the signals are found downfield between 68 and 79 ppm.

The  $^{13}\text{C}$  signals for carbons 3 and 5 are typically found between 50 and 80 ppm. Carbon-3 usually resonates further downfield than C-5, while both signals move further downfield, by approximately 15 ppm, if the nitrogen is oxidised. Carbon-7, when not hydroxylated, has a value of  $\delta$ 25-31 ppm, and when hydroxylated the shift is 68-80 ppm. The shift values for C-9 usually range from 56 to 68 ppm.

There are, however, fewer common trends for the carbons of the necic acid portion of the pyrrolizidine than there are for the necic acid protons. For example, the chemical shift differences for the carbons of (*E*)- and (*Z*)-double bonds are not as noticeable as those for the attached protons. In the case of the geometric isomers angelic acid **91** and tiglic acid **92**, however, there is a notable difference in the carbon shifts. For angelate systems the shift for C-4' of the angelic acid moiety is between 15.4 and 16.2 ppm, while for tiglate systems it is approximately 11.9 ppm. The  $^{13}\text{C}$  NMR data for far fewer tiglic acid-containing pyrrolizidines have been reported. The carbon-5' nucleus in angelate systems resonates between 19.8 and 21 ppm, compared to 14.5 ppm for tiglate systems. It is interesting to note that some authors have reversed the values for carbons 4' and 5', even varying the assignment within one report.<sup>155</sup> Increasing use is being made of two-dimensional NMR techniques, and these have shown that carbon-5' resonates downfield of carbon-4'. Double bonds that only have substituents at one end, like the  $\Delta^{15}$ -bond in pterophorine **82** and the  $\Delta^{13}$ -bond in seneciophylline **11**, have characteristic shifts, *i.e.* the substituted vinyl carbons resonate downfield (at 143-149 ppm) of the vinyl methylene carbons (at 114-123 ppm). The vinylic carbons (C-2' and C-3') in senecioate **93** systems are typically close to 115 and 159 ppm, respectively. An exception is fuchsisenecionine **37** with the chemical shifts for the two carbons being 135.9 and 131.9 ppm, respectively.<sup>324</sup> The carbons of the terminal methyl groups of the senecioic moiety resonate at two different values between 20 and 28 ppm.



Many pyrrolizidine alkaloids are acetylated at various sites; the acetyl carbonyl carbon resonates at approximately 170 ppm and the methyl carbon at approximately 21 ppm.

## 3.2.3 AN ILLUSTRATIVE APPLICATION OF THE DATABASE

The information held on the database helped to identify the pyrrolizidine alkaloid component of a fraction obtained from the extraction of the Tradouw Pass population of *S. paniculatus*. The proton spectrum (Figure 3.2) showed a mixture of compounds that did not correspond to any compounds previously isolated from the extracts. As it was unclear which peaks belonged to the major component a COSY spectrum (Figure 3.3) was also acquired for this fraction.

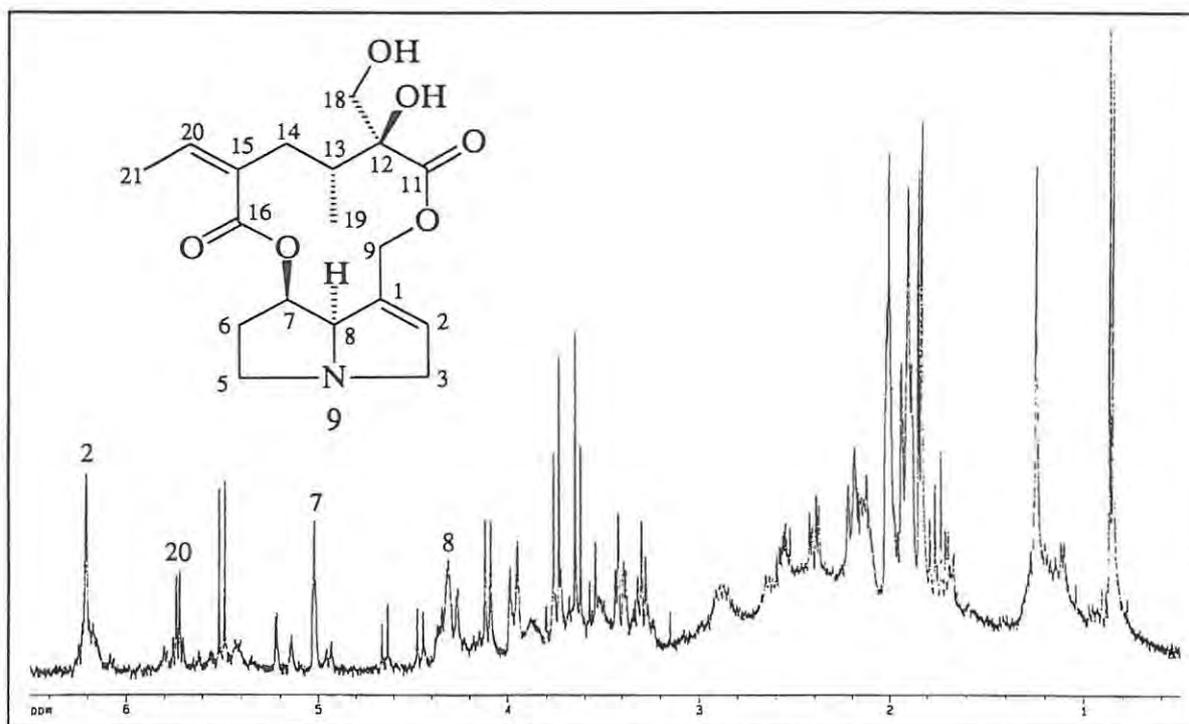
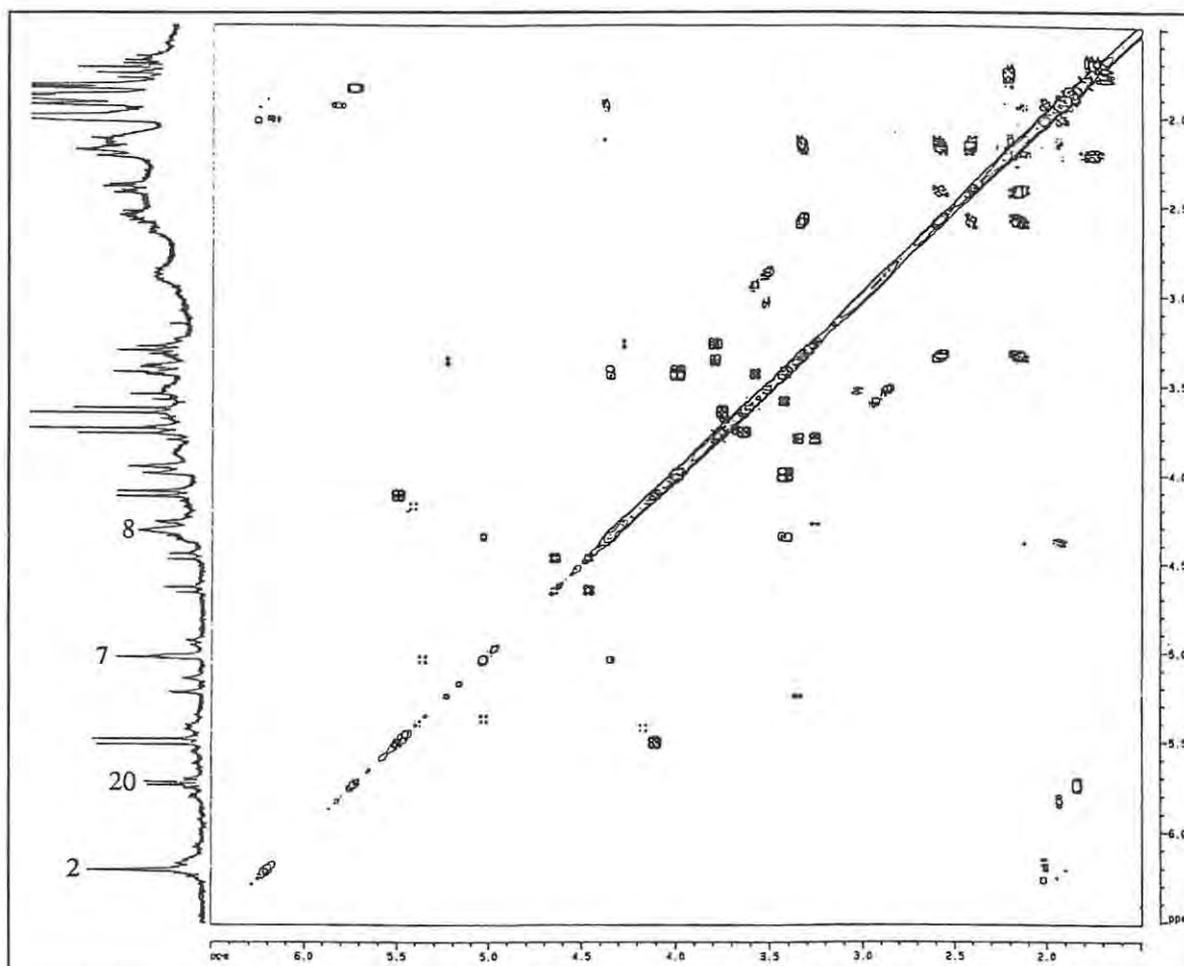


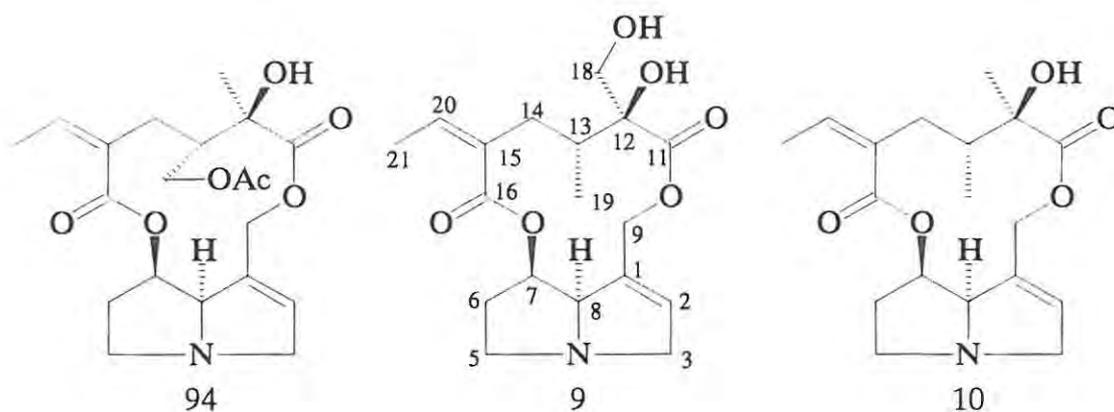
Figure 3.2: <sup>1</sup>H NMR spectrum of a fraction obtained from the extraction of the Tradouw Pass *S. paniculatus* population.



**Figure 3.3:** The COSY spectrum of the same fraction as depicted in Figure 3.2 to enable the signals of the major compound to be identified.

With the help of known trends and the COSY coupling patterns, the alkaloid was identified as possibly having an unsaturated necine base with an H-2 signal at 6.2 ppm. A search of the data held for H-2 between 6.1 and 6.3 ppm narrowed the choice from 490 to 71 possibilities - still too many for individual comparison with the acquired spectrum. Searching for H-7 chemical shifts greater than 4.9 ppm but less than 5.1 ppm and H-8 shifts between 4.15 and 4.35 ppm reduced the options to a more manageable 15. The designated alkaloid type (see Table 3.1), for the majority of the 15 pyrrolizidines, was 7, which represents 12-membered senecionanes. This observation suggested that the signal at 5.74 ppm could belong to H-20 on the necic acid portion of the alkaloid. Entering this option into the database narrowed the choice to one of three pyrrolizidine alkaloids, *viz.*,

gynuramine-19-acetate **94**, retrorsine **9** or senecionine **10**. Comparison of the proton data available (*e.g.* Figure 3.1)<sup>329</sup> for these three alkaloids with the acquired proton spectrum confirmed identification of the alkaloid as retrorsine **9**. The carbon-13 assignments, made with the aid of an HMQC spectrum (Figure 3.4), also matched the published <sup>13</sup>C NMR data for retrorsine.<sup>330</sup>



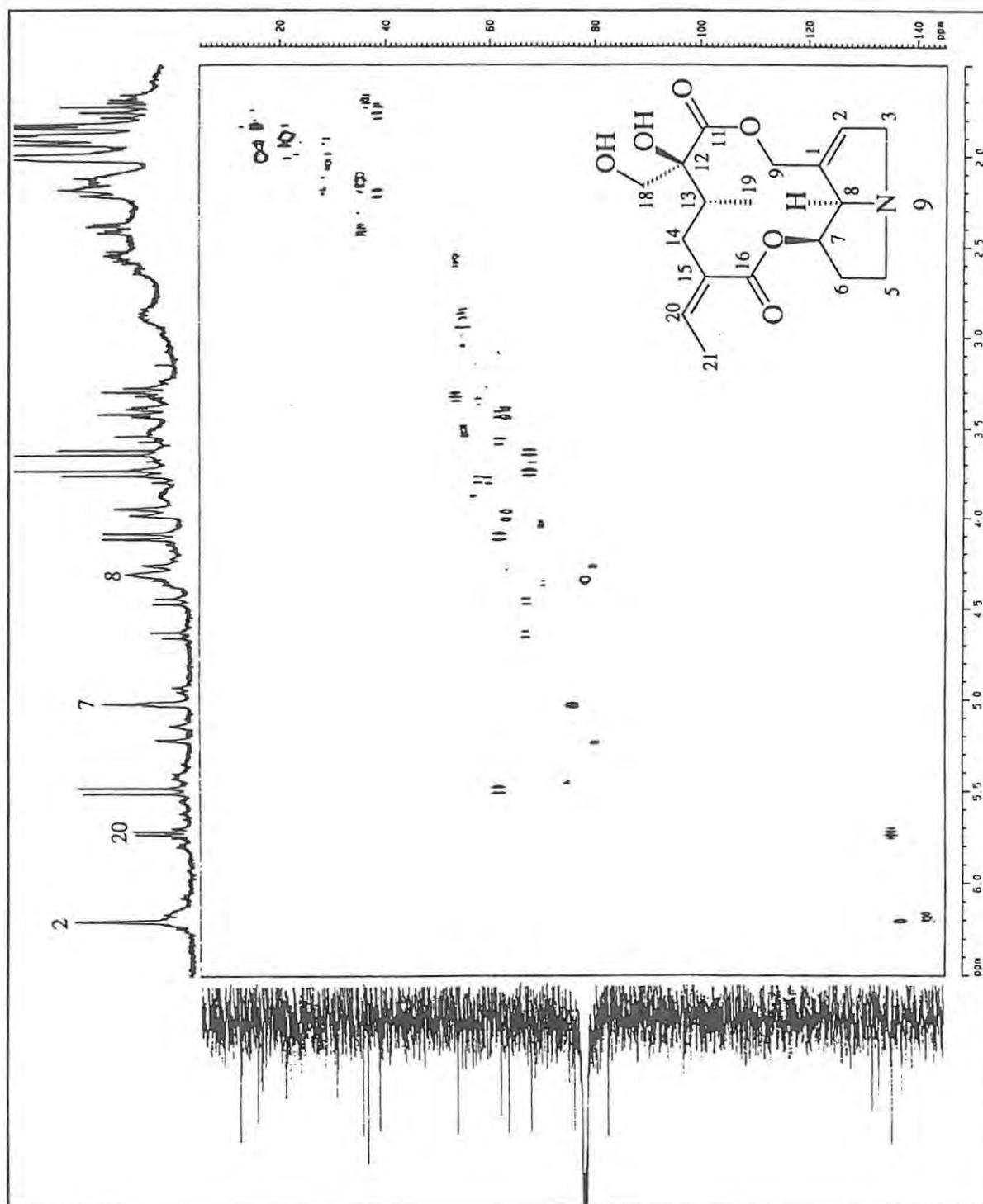
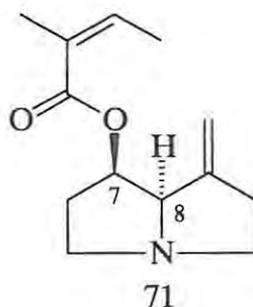


Figure 3.4: The HMBC spectrum of the same fraction as depicted in Figure 3.2 to enable the carbon-13 shifts to be assigned.

#### 4. THE SYNTHESIS OF 7 $\beta$ -ANGELYL-1-METHYLENE-8 $\alpha$ -PYRROLIZIDINE

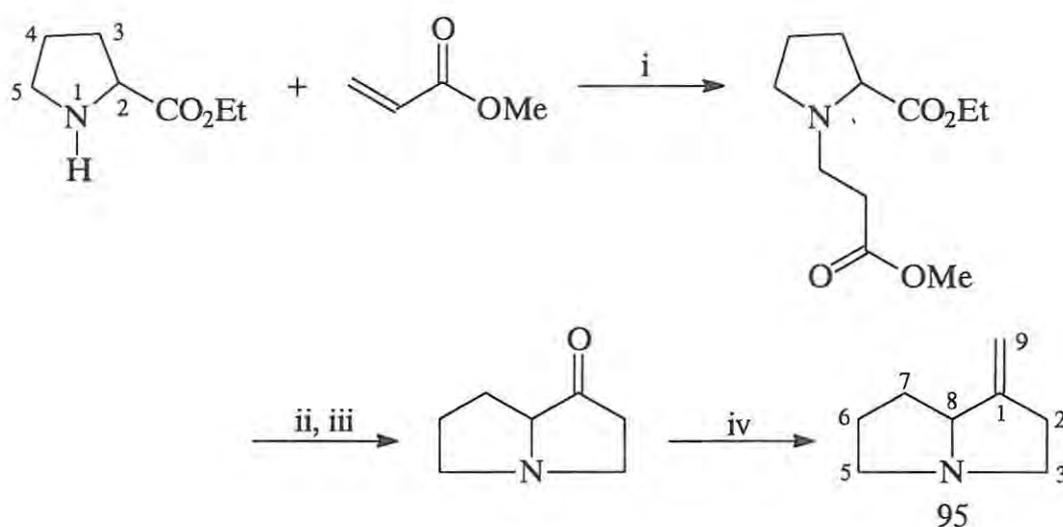
7 $\beta$ -Angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** was extracted from both *Senecio chrysocoma* and *S. paniculatus*. The pyrrolizidine structure is novel in that the unsaturation is exocyclic and there is no oxygen attached to carbon 9 (*cf.* Section 1.3.1, p. 14). The basic structure and the relative stereochemistry were determined by NMR techniques (Section 2.5.2, p. 70), but a synthesis was undertaken to determine the absolute stereochemistry at positions 7 and 8. There are two general strategies for the synthesis of pyrrolizidine alkaloids:-

- i) an *ab initio* approach starting with an acyclic or monocyclic precursor; and
- ii) the modification of an existing pyrrolizidine alkaloid system.



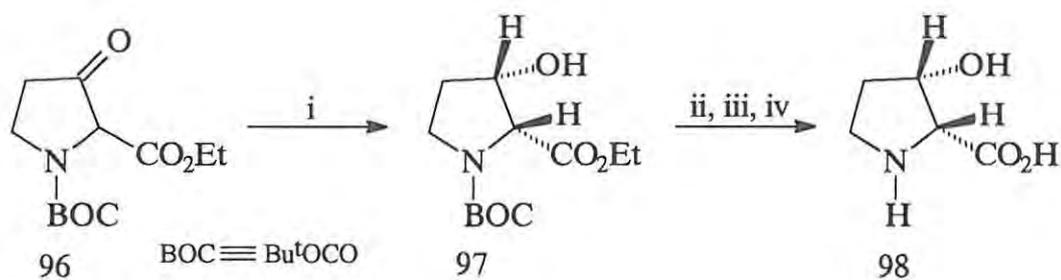
##### 4.1 THE *AB INITIO* APPROACH

In 1961, Kochetkov *et al.*<sup>339</sup> published a synthesis of 1-methylenepyrrolizidine **95** (Scheme 4.1), starting with ethyl prolinolate and methyl acrylate. This synthetic procedure indicated a possible approach to 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** but, as a hydroxy group was required at position 7 of the 1-methylenepyrrolizidine **95**, the starting material needed to be a 3-hydroxyprolinolate.

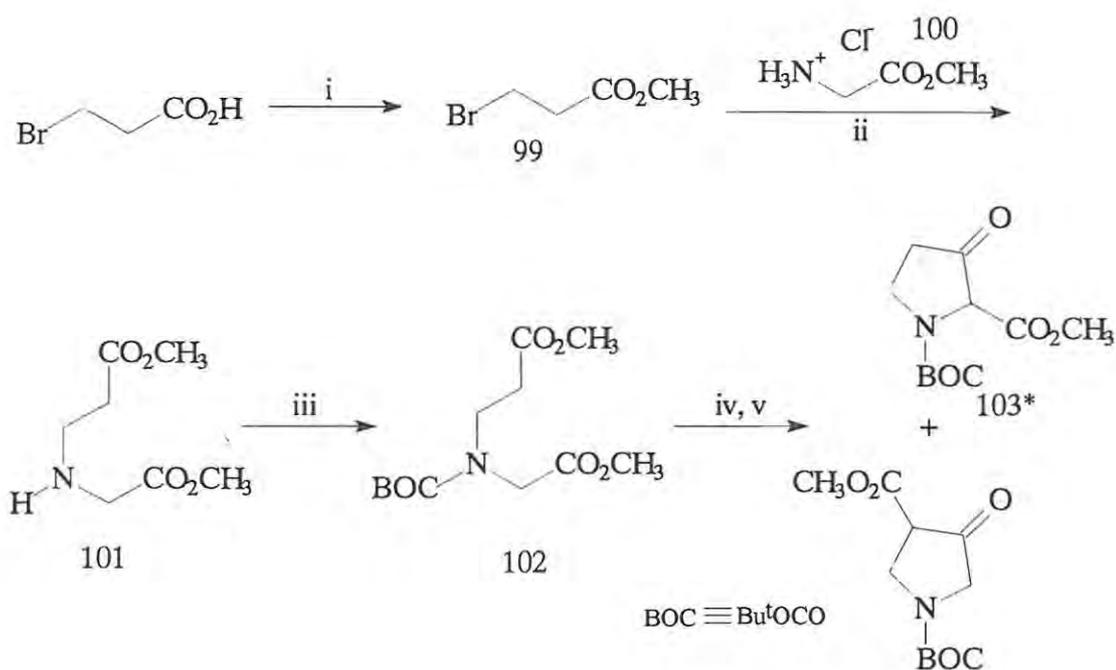


**Scheme 4.1:** Reagents: i, reflux, 24 h; ii, EtONa, xylene, 150-160 °C, 1.5 h; iii, 10% HCl, reflux, 3 h; iv, triphenylphosphinomethylene, Et<sub>2</sub>O, reflux, 6 h, then room temperature, 48 h.

Routes to non-racemic 3-hydroxyproline are rare, but Cooper *et al.*<sup>263</sup> (Scheme 4.2) have synthesised (+)-*cis*-(2*R*,3*S*)-3-hydroxyproline **98** by bakers' yeast reduction of a  $\beta$ -oxo proline derivative **96**, which in turn was synthesised from acyclic precursors (Scheme 4.3).<sup>340</sup> In the present study, an attempt was made to repeat this synthesis, but difficulties were encountered at the critical yeast reduction step. NMR analysis of the isolated product showed no signs of the desired product. This approach was abandoned when the modification of an existing system successfully gave the desired product with defined stereochemistry. In any event, the stereochemistry of the product from the *ab initio* approach may well have been questionable. Using (+)-*cis*-(2*R*,3*S*)-3-hydroxyproline **96** as the starting material would most likely have resulted in 7 $\alpha$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine instead of the desired 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine. Kochetkov *et al.*'s synthesis<sup>339</sup> (Scheme 4.1) afforded racemic 1-methylenepyrrolizidine **95** (resolved by crystallisation as the tartrate derivatives) but it is unclear if optically pure proline was used as the starting material.



**Scheme 4.2:** Reagents: i, bakers' yeast, sucrose, H<sub>2</sub>O, 30°C, 24 h; ii, CH<sub>2</sub>Cl<sub>2</sub>, trifluoroacetic acid, room temperature, 2 h; iii, KOH, CH<sub>3</sub>OH, H<sub>2</sub>O, room temperature, 16 h; iv, H<sup>+</sup>.

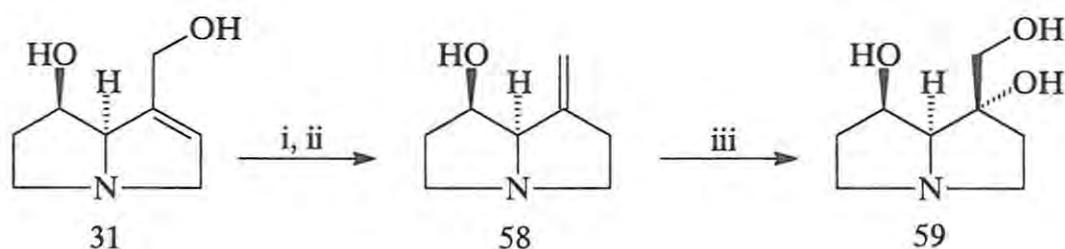


**Scheme 4.3:** Reagents: i, CH<sub>3</sub>COCl, CH<sub>3</sub>OH, 20°C, 18 h; ii, NaOH, H<sub>2</sub>O; iii, di-*tert*-butyl dicarbonate (BOC<sub>2</sub>O), CH<sub>2</sub>Cl<sub>2</sub>, Et<sub>3</sub>N; iv, KO<sup>t</sup>Bu, toluene; v, CH<sub>3</sub>COOH.

\* 2-methyl analogue of compound 96.

## 4.2 MODIFICATION OF AN EXISTING PYRROLIZIDINE SYSTEM

This approach to 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** was prompted by a recent synthesis published by Hanselmann and Benn,<sup>283</sup> in which hadinecine **59** was synthesised from retronecine **31** with 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** as an intermediate (Scheme 4.4).



**Scheme 4.4:** Reagents: i,  $\text{SOCl}_2$ ; ii, Zn, 1M  $\text{H}_2\text{SO}_4$ ; iii,  $\text{OsO}_4(\text{cat})$ , 4-methyl morpholine *N*-oxide, acetone,  $\text{H}_2\text{O}$ .

The disadvantage of using this route to 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** is that the yield is limited by the amount of natural product that can be extracted and purified (or synthesised) for use as the starting material. The advantage, however, is that as the stereochemistry of retronecine is known, the stereochemistry of the product is predetermined.

## 4.2.1 ISOLATION OF RETRONECINE

Retronecine **31** can be enantioselectively synthesised by a variety of procedures, but is readily available by saponification of retronecine-based macrocycles, e.g. monocrotaline **7** or retrorsine **9**.<sup>202,208,236,282-284</sup> *Senecio orthonnaeflorus*, a widely spread, local *Senecio* species, has been shown to contain large quantities of retrorsine **9** (1.2% of dry plant weight) and no other pyrrolizidines.<sup>341</sup> Consequently, dry, *S. orthonnaeflorus* plant material was extracted by conventional methods as described in Section 5.4.2 (p. 156). Retrorsine **9** was obtained as cream coloured crystals with a melting point of 214-216°C

(literature value 216°C<sup>142,328</sup>). The <sup>1</sup>H NMR spectrum (Figure 4.1) confirmed the absence of impurities so no recrystallisation was attempted and the retrorsine was hydrolysed by refluxing with barium hydroxide in aqueous medium.

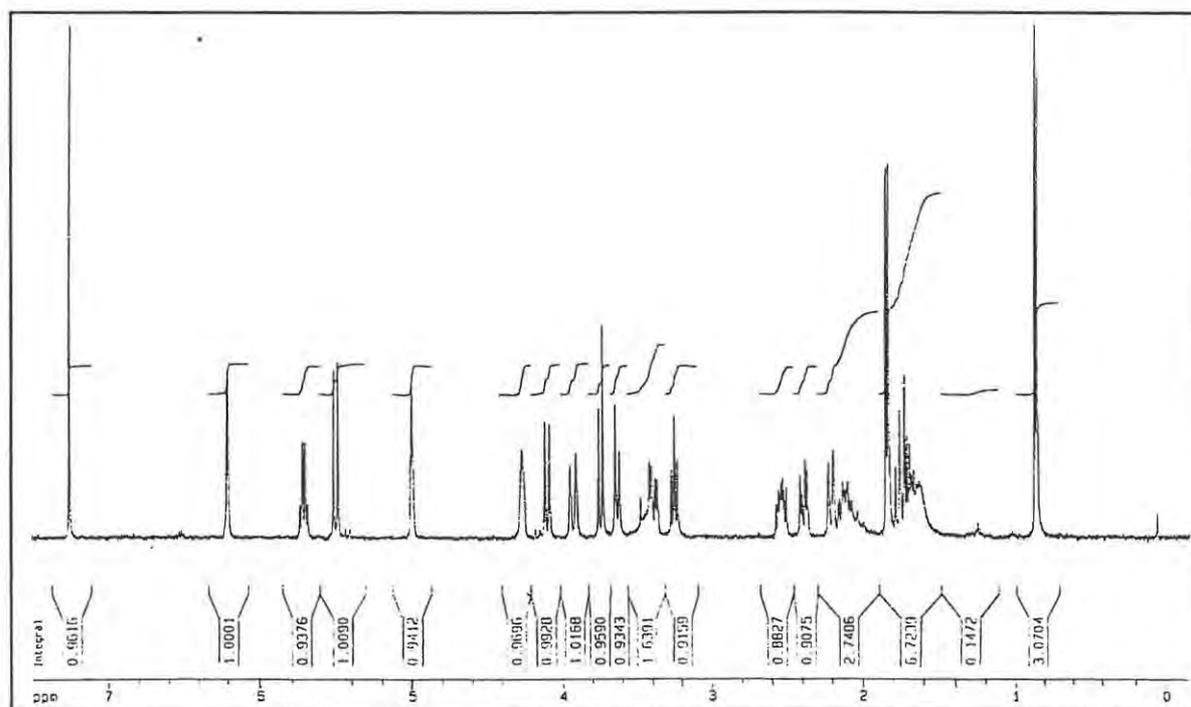
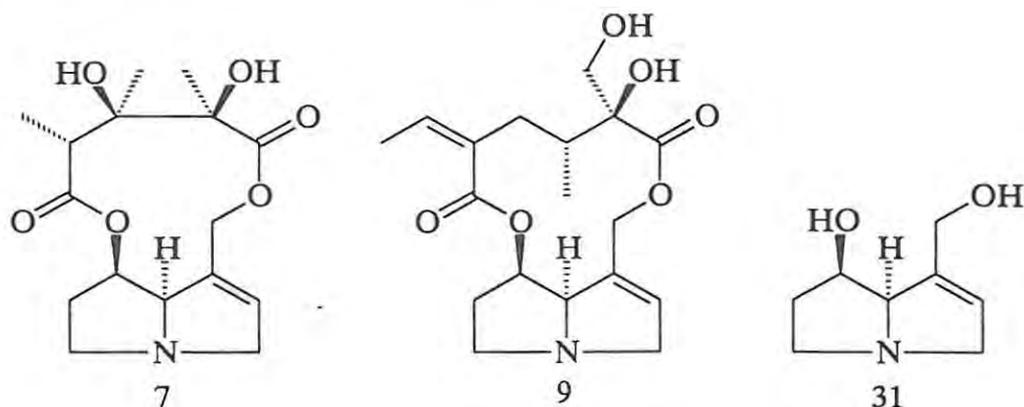
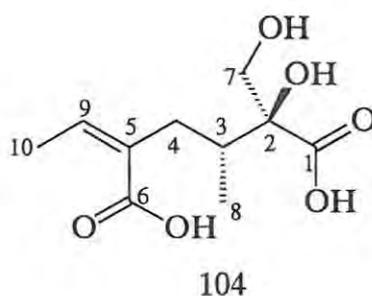


Figure 4.1: <sup>1</sup>H NMR spectrum of retrorsine 9 extracted from *S. orthonnaeflorus*.

The hydrolysis took longer than expected; refluxing for one to five hours<sup>202,284,342,343</sup> failed to hydrolyse all the retrorsine 9, and a reflux period of approximately 10 hours was required. After the barium had been removed from solution as barium carbonate, the pH

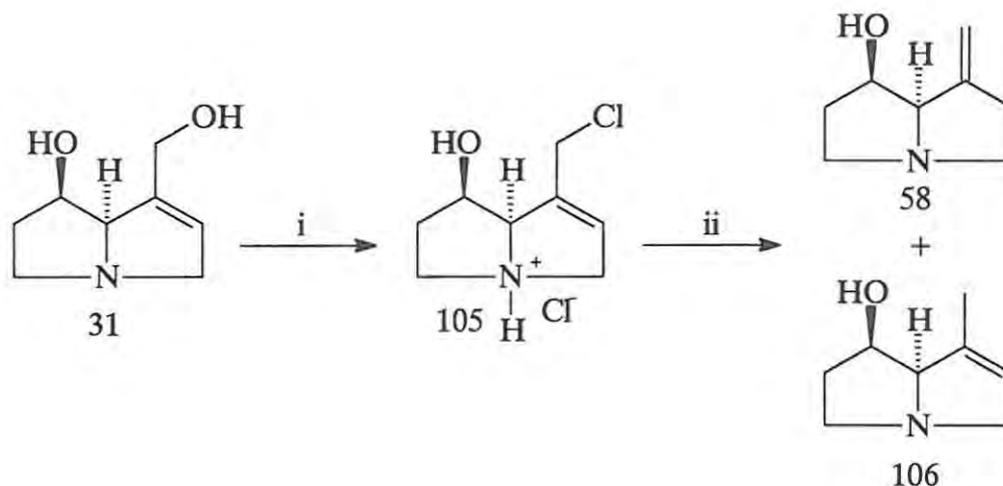
was increased to 9 or 10 and the retronecine isolated by continuous extraction. The yield of retronecine was lower than expected (26%). (Heliotridine had been extracted in 85% yields by this method.<sup>343</sup>) The solubility of retronecine in aqueous medium could have been the problem and the yield of retronecine **31** was improved when the hydrolysis product was evaporated to dryness and the gummy residue refluxed with chloroform to extract the retronecine. When this procedure was repeated a precipitate formed during the removal of the solvent, the amount of which increased when chloroform was added. The precipitate was removed by filtration and identified as isatinecic acid **104**, while the filtrate subsequently afforded cream coloured crystals of retronecine **31** in a 37.7% yield. Although the yield was still not as high as had been reported for other hydrolyses the product was clean and crystalline. Acetone has been the solvent of choice for crystallising retronecine,<sup>80,202,278,284,344</sup> but no success was obtained with this solvent. Other mixtures of solvents were tried with equally little success and, eventually, the only method to give crystals involved dissolving the gummy retronecine in chloroform, then removing the chloroform *in vacuo* and leaving the sample under vacuum for many hours. Consequently, it was very pleasing to obtain clean crystalline retronecine **31** directly from work-up of the hydrolysis mixture.



#### 4.2.2 MODIFICATION OF RETRONECINE TO AFFORD 7 $\beta$ -HYDROXY-1-METHYLENE-8 $\alpha$ -PYRROLIZIDINE

7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** was synthesised in two steps from retronecine **31** following the route outlined in Scheme 4.5. 1-Chloromethyl-1,2-didehydro-7 $\beta$ -hydroxy-8 $\alpha$ -pyrrolizidine hydrochloride **105** was prepared according to Adams and van Duuren's

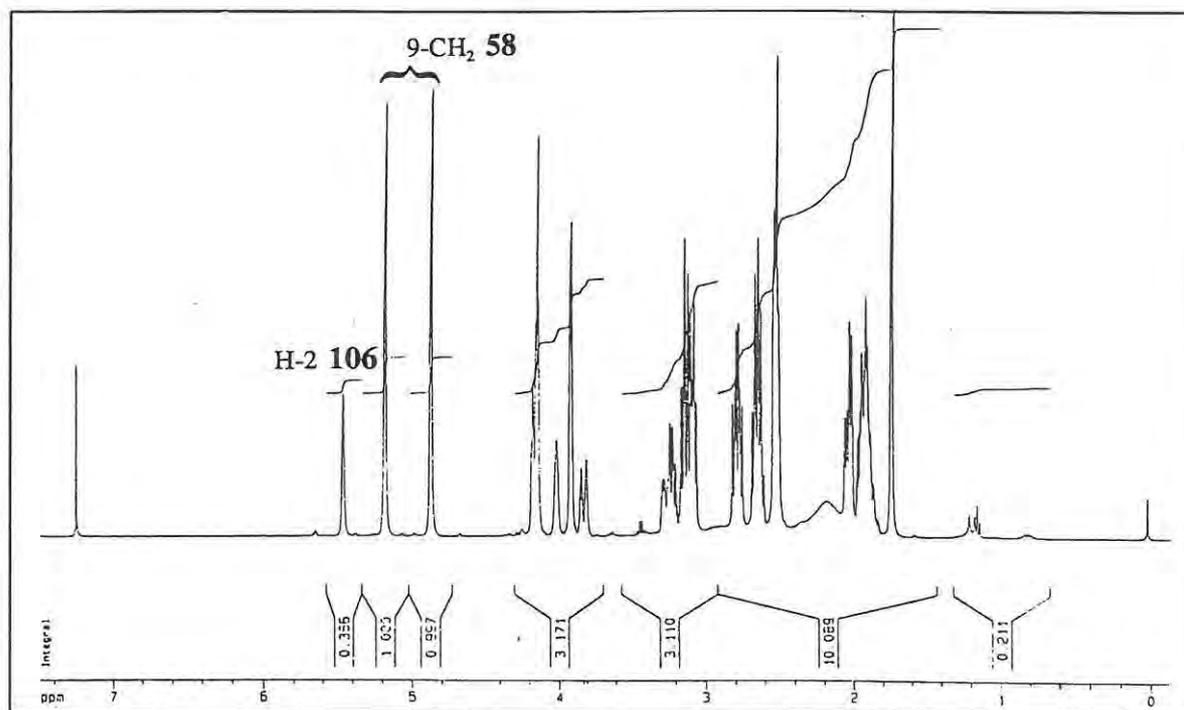
procedure,<sup>345</sup> which involved slowly adding crystalline retronecine to thionyl chloride. To facilitate the addition of gummy retronecine **31** to thionyl chloride, the necine base was pre-dissolved in dichloromethane, however the yield obtained was significantly lower (13%) than that obtained from the solvent-free method (64%).



**Scheme 4.5:** Reagents: i, SOCl<sub>2</sub>, 0°C; ii, Zn, 1M-H<sub>2</sub>SO<sub>4</sub>.

The hydrochloride **105**, once formed, was reduced with zinc dust and acid, as described by Culvenor and Smith,<sup>346</sup> to yield a mixture of the desired product, 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58**, together with 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106**. Both compounds are distinctly indicated by the vinyl signals in the <sup>1</sup>H NMR spectrum of the product [Figure 4.2 (COSY and HMQC spectra in the Appendix)] and the impurities are clearly minimal. From the integration of the signals for the vinylic protons the ratio of 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** to 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106** was determined to be 5 to 2. The two compounds were not separated at this stage as they were only formed in small amounts and, due to the known irreversible binding of pyrrolizidines to silica, considerable loss of product could have occurred. An attempt to obtain separation on TLC was unsuccessful as with all systems tried the two compounds had the same R<sub>f</sub> value. It was hoped that once the two compounds, 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-

methyl-8 $\alpha$ -pyrrolizidine **106**, had been esterified with angelic acid **91** separation would be possible.

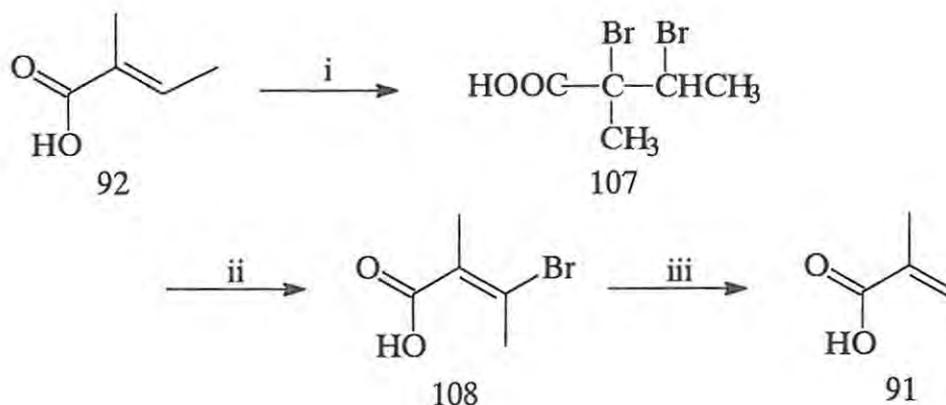


**Figure 4.2:**  $^1\text{H}$  NMR spectrum of the mixture of 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106**.

### 4.2.3 SYNTHESIS OF ANGELIC ACID

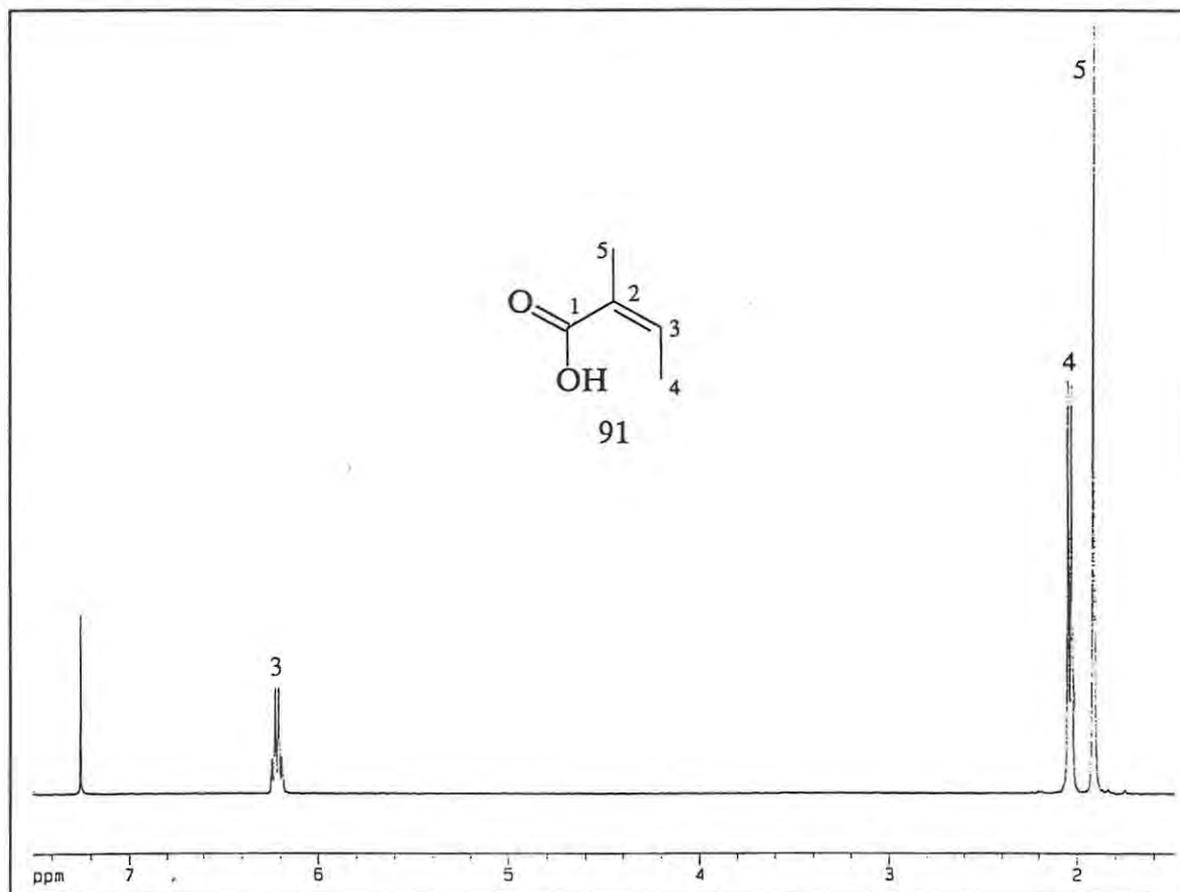
Angelic acid **91** is not as stable as its *trans* isomer, tiglic acid **92**; isomerisation has been observed to take place under acidic or basic conditions especially at high temperatures.<sup>347,348</sup> As a result, care must be taken during the synthesis and subsequent reactions of angelic acid **91**. It is, however, possible to handle the angelic acid **91** under mild conditions without marked isomerisation,<sup>347</sup> and crystalline angelic acid can be kept in the laboratory for years without any isomerisation taking place.<sup>347</sup> There are many syntheses for tiglic acid,<sup>347,349</sup> while for angelic acid the most effective synthesis, which

affords the required product uncontaminated by tiglic acid, is the one reported by Buckles and Mock (Scheme 4.6).<sup>349</sup>



**Scheme 4.6:** Reagents: i, Br<sub>2</sub>; ii, 25% KOH in CH<sub>3</sub>OH, K<sub>2</sub>CO<sub>3</sub>, 55°C; iii, Na-Hg amalgam, H<sub>2</sub>O, room temperature.

In the present investigation, commercially available tiglic acid **92** was used for the synthesis. The only change from Buckles and Mock's procedure<sup>349</sup> was that  $\beta$ -bromoangelic acid **108** was stirred with the sodium-mercury amalgam for longer than 24 hours, *i.e.* until the mercury had been restored. As expected, angelic acid **91** was obtained without contamination by tiglic acid **92** - the quartet at *ca.* 6.7 ppm for the vinylic proton of tiglic acid clearly being absent from the proton NMR spectrum of the product (Figure 4.3).



**Figure 4.3:** <sup>1</sup>H NMR spectrum of angelic acid **91** with no quartet at *ca.* 6.7 ppm for tiglic acid **92** contamination.

Having now synthesised both the necine base, 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58**, and the necic acid, angelic acid **91**, all that remained was to couple the two together.

#### 4.2.4 SYNTHESIS OF 7 $\beta$ -ANGELYL-1-METHYLENE-8 $\alpha$ -PYRROLIZIDINE

The coupling of angelic acid **91** with 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106** was not as simple as had originally been anticipated.

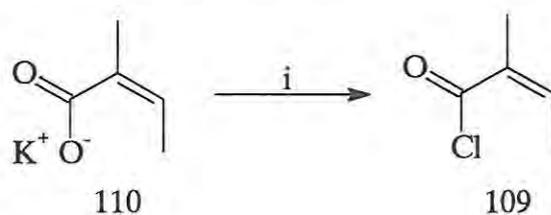
*N,N'*-Carbonyldiimidazole (CDI) has been used for the coupling of necic acids and necine bases<sup>202,236,299,350</sup> and, in particular, for the selective synthesis of 9-monoesters of

retronecine **31**. However, diesters of retronecine **31** and heliotridine **33** and 7-monoesters of heliotridine have also been synthesised using CDI.<sup>202,299,351</sup> Consequently, it was hoped that in the absence of the 9-hydroxyl group, as in 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106**, esterification would occur at the 7-hydroxyl group. Hoskins and Crout's procedure,<sup>202</sup> involving stirring a mixture of CDI and angelic acid in chloroform until carbon dioxide production had stopped and then adding retronecine and stirring overnight, was followed but, after stirring for 24 hours at room temperature, TLC analysis showed that no product had formed. There was obvious reluctance to heat the mixture in case the angelic acid isomerised and, therefore, the reaction mixture was stirred at room temperature for 72 hours, by which time some product had formed. Heating the reaction mixture initially to 35°C and then to reflux, appeared to increase the amount of product but, after work-up, none of the desired product could, in fact, be identified by <sup>1</sup>H NMR spectroscopy. This technique provides a quick method of checking if esterification has taken place as the signal for the H-7 nucleus shifts downfield from *ca.* 4.5 ppm for the unesterified necine base to *ca.* 5.5 ppm for the esterified base.

Coupling of retronecine **31** with various acids has also been achieved by using *N,N'*-dicyclohexylcarbodiimide (DCC), with or without 4,4-dimethylaminopyridine (DMAP). However, application of this method of coupling angelic acid to an alcohol has been observed to afford a large proportion of the tiglyl isomer, particularly when heat was involved.<sup>202,352</sup> Despite this drawback, an attempt was made to synthesise 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** using these reagents and the procedure given in Section 5.4.2 (p. 156). The reaction was attempted in deuterated dichloromethane to facilitate rapid NMR analysis, but no esterification was observed; not even a tiglyl isomer was obtained, despite stirring the reaction mixture for 24 hours. The <sup>1</sup>H NMR spectrum did show that a large portion of the angelic acid had been isomerised to tiglic acid (indicated by the appearance of a quartet at 6.7 ppm), so heating of the mixture was not attempted.

The alkaloid, 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71**, was finally obtained by coupling angelic acid chloride **109** with the lithium alkoxide of the necine base, which was

generated using butyllithium. Angelyl chloride **109** has previously been synthesised by first treating angelic acid **91** with one equivalent butyllithium to produce the lithium carboxylate, before reacting with oxalyl chloride,<sup>353</sup> or by the reaction of the sodium salt of angelic acid with phosphorus oxychloride.<sup>347</sup> In both cases the angelyl chloride **109** was used immediately without isolation or purification as angelyl chloride **109** rapidly decomposes to angelic acid on exposure to moisture. Beeby<sup>354</sup> reacted potassium angelate **110** with oxalyl chloride and a drop of dimethyl formamide at 0°C to give the acid chloride **109** (Scheme 4.7) and this was the method used in the present study.

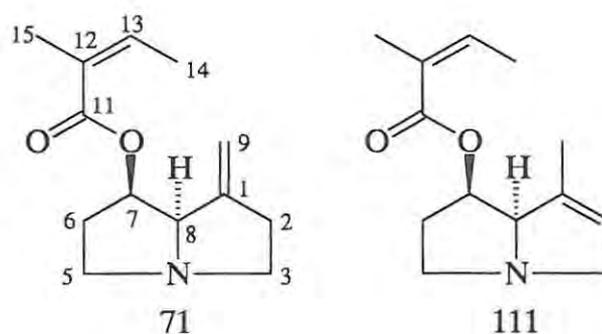


**Scheme 4.7:** Reagents: i, (COCl)<sub>2</sub>, dimethylformamide (1 drop), Et<sub>2</sub>O, 0°C.

The potassium angelate **110** was prepared by neutralising a solution of angelic acid **91** in ethanol with an equimolar amount of 30% ethanolic potassium hydroxide. The original procedure cites an 85% solution<sup>354</sup> but this could not be achieved. After addition of acetone a salt precipitated which was dried under a high vacuum before being converted to the acid chloride. After stirring potassium angelate with oxalyl chloride for two hours under dry nitrogen, the excess oxalyl chloride was removed under a slight vacuum without heating, while carefully avoiding exposure to moisture.

Angelyl chloride **109** has been coupled to secondary alcohols by simply stirring it in solution with the alcohol at room temperature.<sup>354,355</sup> However, after several unsuccessful attempts, it was decided to add the angelyl chloride **109** to the lithium alkoxide derivative of the necine base (a 5:2 mixture of 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-methylpyrrolizidine **106**) to ensure coupling - a method which has been reported to inhibit tiglactate formation.<sup>352,356</sup> The reaction mixture was easily purified, after work-up, by preparative layer chromatography on silica gel to give a 5:2 mixture of

the required 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** together with 7 $\beta$ -angelyl-1,2-didehydro-1-methyl-8 $\alpha$ -pyrrolizidine **111** (Figures 4.4 and 4.5); a small amount of the tiglata isomer was also formed as indicated by a quartet at 6.75 ppm on the proton spectrum. Separation of the two major components could not be achieved by various preparative chromatographic techniques. A wide range of solvents with differing polarities were used with normal and reverse phase silica, alumina and cellulose. Even if the 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** and 7 $\beta$ -angelyl-1,2-didehydro-1-methyl-8 $\alpha$ -pyrrolizidine **111** could have been separated from each other, the residual tiglata isomer would still have interfered if optical rotations were used to distinguish between the synthetic and natural products. Chiral differentiating techniques were therefore used to determine if the extracted alkaloid was the enantiomer of the synthesised alkaloid or not.



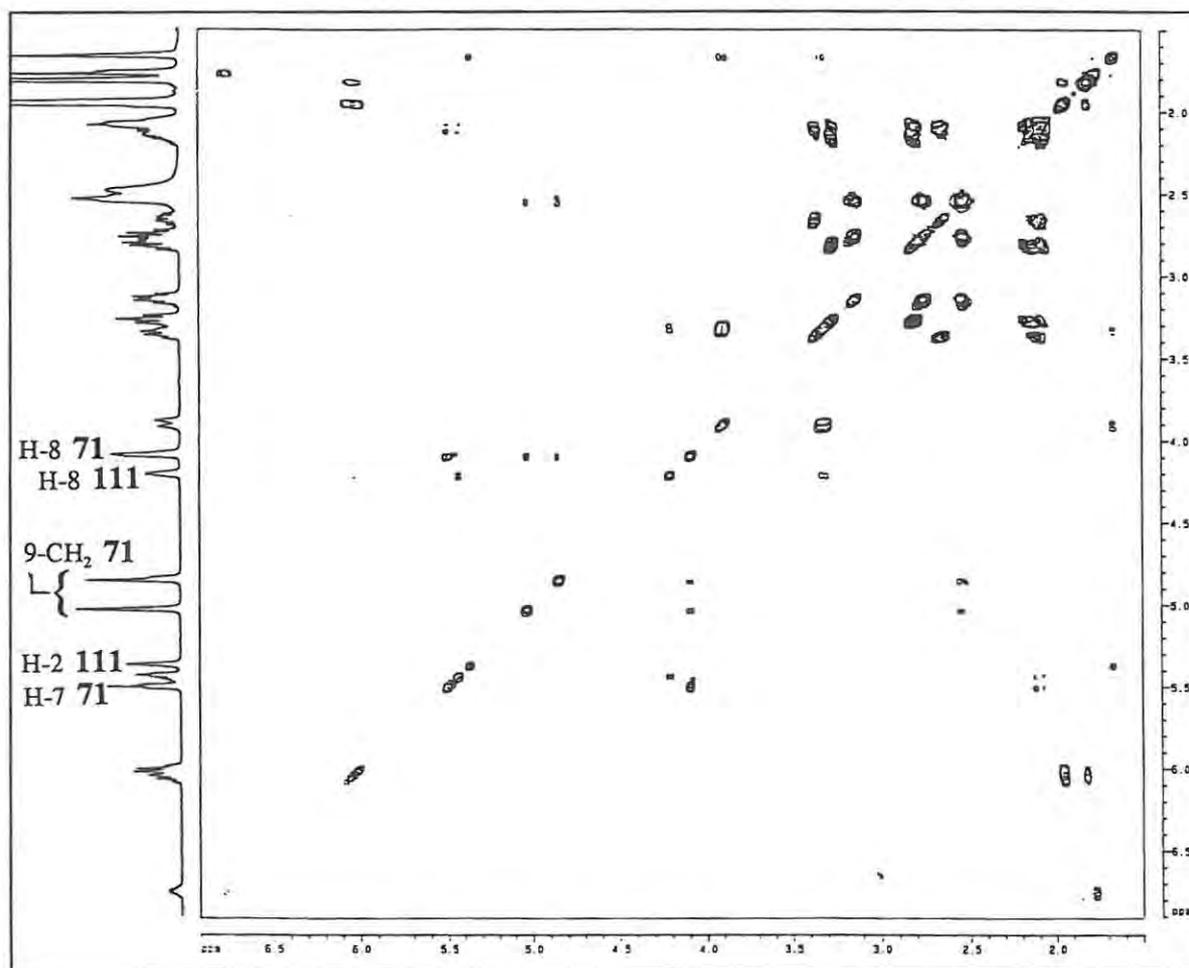
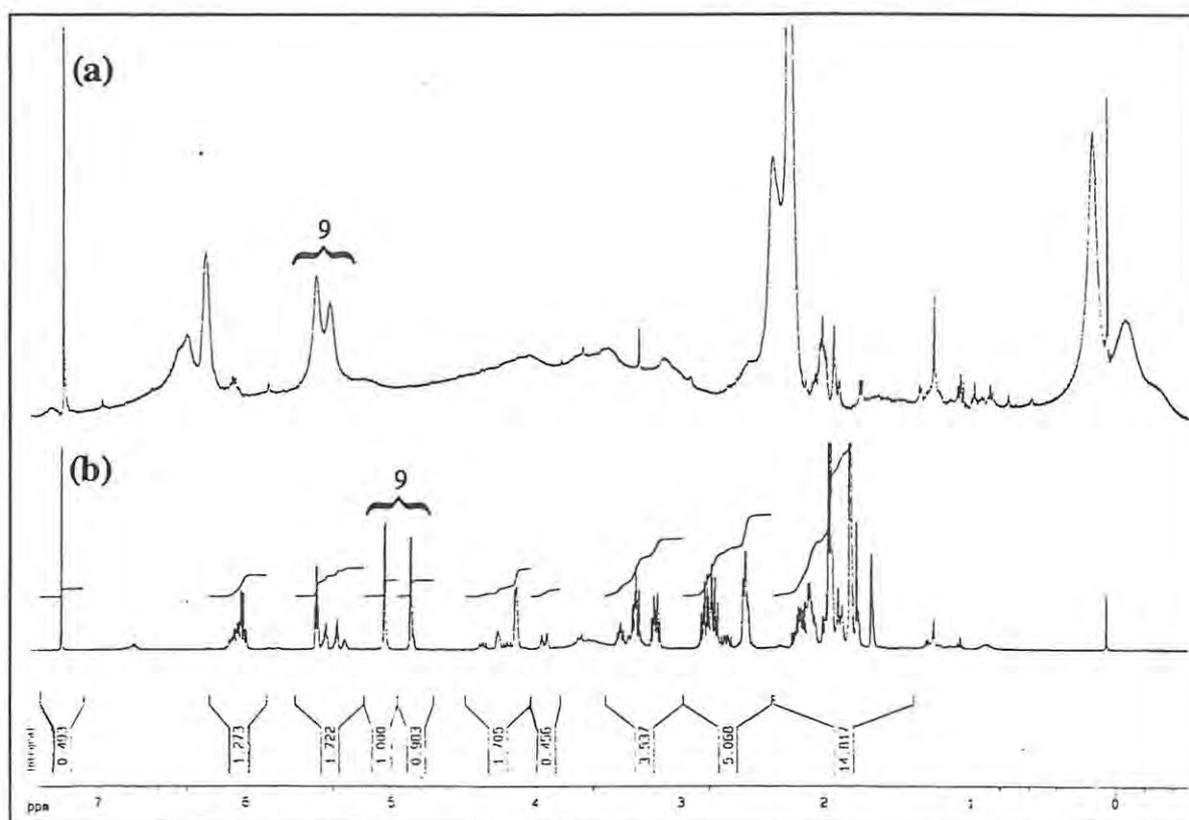


Figure 4.4: COSY spectrum of the mixture of 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine 71 and 7 $\beta$ -angelyl-1,2-didehydro-1-methyl-8 $\alpha$ -pyrrolizidine 111 with a quartet at  $\delta$ 6.75 indicating the presence of tiglyl isomers.



An NMR chiral shift reagent, tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]europium, was added separately to the synthesised product, the natural product and a mixture of the two. Chiral shift reagents tend to cause peak broadening which is more pronounced at high frequency (400 MHz).<sup>357</sup> The shifts that were easiest to recognise were those for the 9-methylene protons and the mixture of the synthesised and natural products exhibited only one set of these peaks in the <sup>1</sup>H NMR spectrum suggesting the same absolute stereochemistry. Figure 4.6 shows the <sup>1</sup>H NMR spectrum of the mixture of the synthesised and natural products compared to the same sample after the addition of chiral shift reagent. The H-9 signals have shifted downfield but have not split.

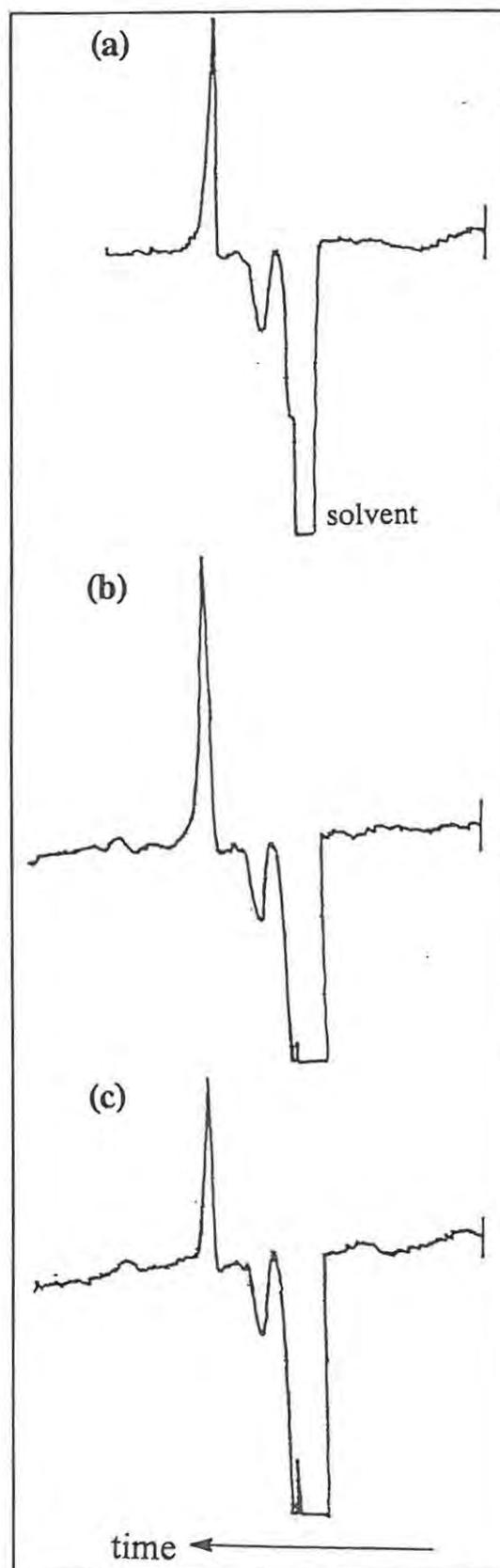


**Figure 4.6:** <sup>1</sup>H NMR spectra of a mixture of synthesised and natural 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** before (b) and after (a) the addition of a chiral shift reagent.

Both analytical HPLC and analytical GLC, using chiral columns, were then used to examine the synthesised product, the natural product, and a mixture of the two. No splitting of the peaks occurred when the mixture was injected supporting the stereochemical identity of the products. Two different solvent systems were used with a reverse phase, chiral HPLC column and, in neither case, was any peak splitting observed (see Figure 4.7).

Three different temperatures, 90, 100, and 110°C, were used for the GLC analyses. At 110°C two peaks were visible on the chromatogram of the synthesised product, presumably corresponding to the two products, 7 $\beta$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **71** and 7 $\beta$ -angelyl-1,2-didehydro-1-methyl-8 $\alpha$ -pyrrolizidine **111**. Decreasing the temperature to 100°C, caused the peak with the higher retention time to split, possibly due to the presence of both angelyl and tiglyl isomers. A further decrease to 90°C did not change the peak pattern, but only increased the retention times. Consequently, all three samples were run at 100°C. Figure 4.8 shows the chromatograms at 100°C for the synthesised product, the natural product and the mixture of the two and it is clear that the major constituents have identical retention times.

**Figure 4.7:** HPLC chromatograms, using a 6:4 ratio of methanol (with 0.2% TFA) and water as the solvent system on a chiral HPLC column, for (a) the synthesised product, (b) the mixture of synthesised and natural products, and (c) the natural product.



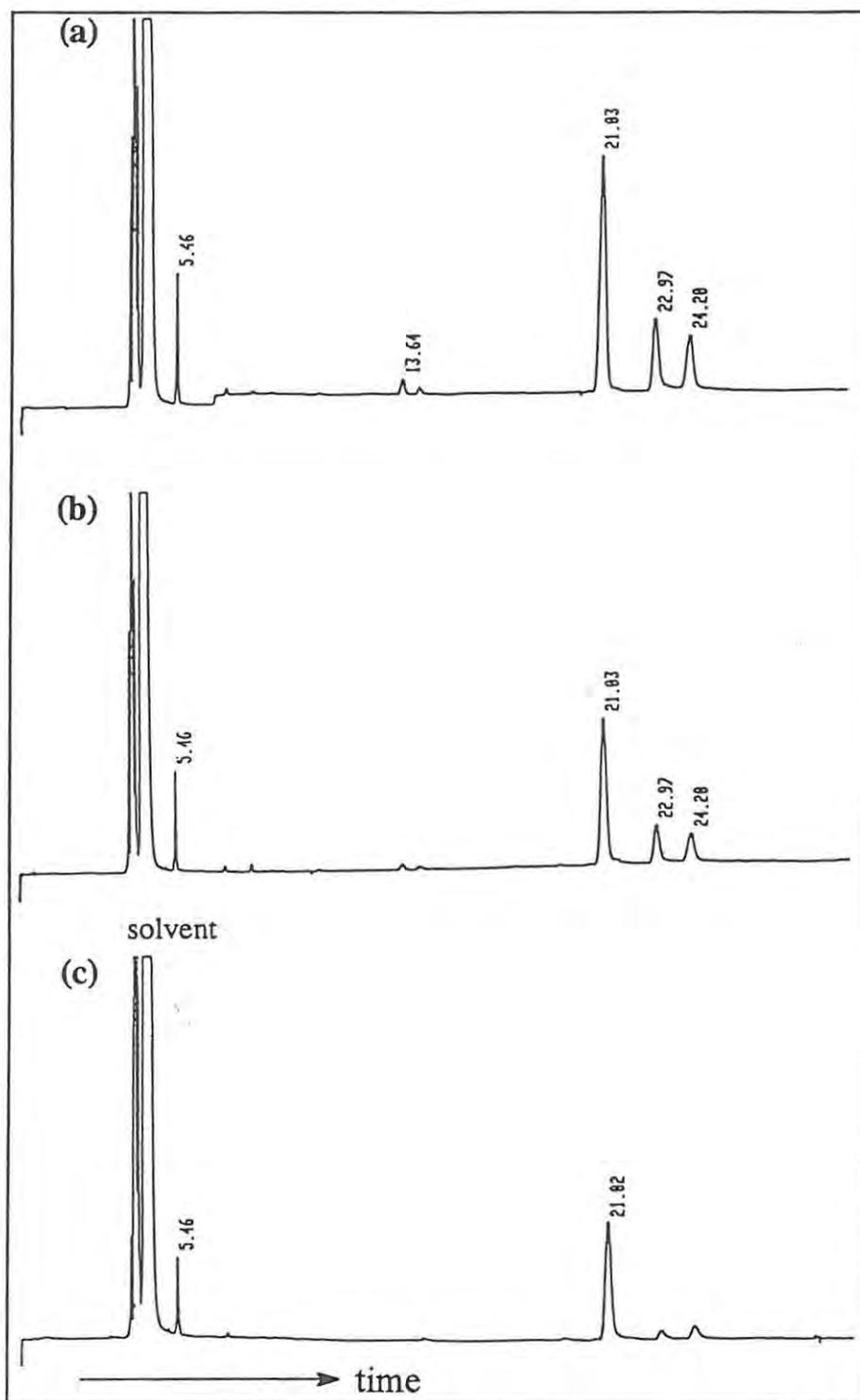
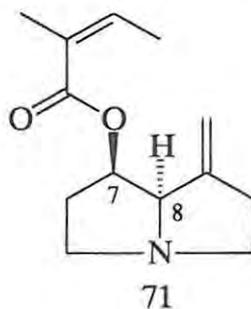


Figure 4.8: GLC chromatograms obtained using a chiral GLC column at 100°C for (a) the synthesised product, (b) a mixture of synthesised and natural products, and (c) the natural product.

The 7 $R$ ,8 $R$ -stereochemistry is, in fact, expected for the natural alkaloid as the platynecine-based alkaloids, which predominate in these plants, have that stereochemistry and the GLC, HPLC and NMR evidence all point towards the synthesised and natural product having the same stereochemistry. It is therefore concluded that the alkaloid isolated from *Senecio chrysocoma* and *S. paniculatus* was 7 $R$ -angelyl-1-methylene-8 $R$ -pyrrolizidine 71.



## 5. EXPERIMENTAL

### 5.1 GENERAL METHODS

The separation of pyrrolizidine alkaloids and the progress of reactions were monitored by analytical thin layer chromatography (TLC) on plastic backed, 0.2 mm thick silica gel 60 F<sub>254</sub> plates (Merck Art. no. 1.05735). Two different spray reagents were used for the visualisation of pyrrolizidine alkaloids, *viz.*, Dragendorff's reagent (see below) and *ortho*-chloranil/Erhlich's reagent (see below). For flash column chromatography<sup>358</sup> silica gel 60 (230-400 mesh ASTM; Merck Art. no. 1.09385) was used. Preparative layer chromatography plates (10x20 cm and 20x20 cm) were prepared from silica gel 60 PF<sub>254</sub> (Merck Art. no. 7747) according to the supplier's instructions. For small samples (< 15 mg) pre-coated (with a layer thickness of 0.25 mm), glass backed 5x20 cm silica gel 60 F<sub>254</sub> plates were used (Merck Art. no. 5714). A Büchi B-670 chromatograph was used, in descending mode, for droplet counter current chromatography (DCCC).

Standard analytical gas liquid chromatography (GLC) was performed on an HP 5890A chromatograph fitted with an HP ultra-2 fused silica capillary column (0.2 mm x 25 m) packed with cross-linked 5% phenyl methyl silicone (film thickness 0.33  $\mu$ m). An initial temperature of 120°C was maintained for 1 minute; the temperature was then increased at 10°C per minute to a final temperature of 275°C, which was maintained for 15-25 minutes. The injector and detector temperatures were 275 and 300°C, respectively, and the carrier gas was hydrogen.

Chiral GLC was performed on an HP 5890 chromatograph fitted with an FS-Lipodex A fused silica chiral column (0.25 mm x 50 m). The injector and detector were set at 250°C and the carrier gas was helium. Isothermal run conditions were used at 90, 100 or 110°C.

A Spherisorb S5-chiral 1 reverse phase column was used for HPLC analyses with two different solvent systems:-

- i) methanol with 0.2% trifluoroacetic acid at 0.5 mL per minute or
- ii) a 6:4 ratio of methanol (with 0.2% trifluoroacetic acid) and water at 1 mL per minute.

The detector was a Waters R401 differential refractometer.

For gas chromatography-mass spectrometry determinations an HP 5890A gas chromatograph was coupled to an HP 5988A mass spectrometer. The same GLC column, an HP ultra-2, was used but the operating conditions were slightly different from those used for analytical GLC. An initial temperature of 110°C was maintained for 1 minute before the temperature was increased by 10°C per minute to a final temperature of 275°C which was maintained for 20 minutes. The injector and detector temperatures were 275 and 300°C, respectively. The carrier gas was helium. Both electron impact (EI) and chemical ionisation (CI; using either methane or ammonia as the ionising gas) mass spectra were obtained.

High resolution mass spectral data was obtained on a Kratos MS 80RF mass spectrometer at the Cape Technikon Mass Spectrometry Unit.

All NMR spectra were acquired on a Brüker AMX400 spectrometer at 400 MHz for protons and 100 MHz for carbon-13 and at a temperature of 303K unless otherwise stated. The solvent used, unless otherwise stated, was deuterated chloroform and chemical shifts are quoted relative to the solvent peaks [ $\delta_{\text{H}}$  7.25 (for  $^1\text{H}$ ) and  $\delta_{\text{C}}$  77.0 (for  $^{13}\text{C}$ )]. Solvent peaks were also used to reference spectra run in deuterated dimethylsulphoxide [ $\text{DMSO-}d_6$  ( $\delta_{\text{H}}$  2.50;  $\delta_{\text{C}}$  39.43)]. For proton spectra determined in deuterium oxide ( $\text{D}_2\text{O}$ ) the signals are quoted relevant to the solvent peak ( $\delta_{\text{H}}$  4.82) and for carbon-13 spectra a drop of deuterated methanol was added for calibration ( $\delta_{\text{C}}$  49.05).  $J$  values are given in Hertz.

Melting points are uncorrected and were determined on a Kofler micro heating stage.

Antitumour and anti-human immunodeficiency virus tests were performed by the National Cancer Institute in the United States of America.

*Dragendorff's reagent.*<sup>138-</sup>

The stock solution was prepared by mixing a solution of bismuth (III) nitrate (0.85 g), glacial acetic acid (10 mL), and water (40 mL) with a solution of potassium iodide (20 g) and water (50 mL). Stock solution (1 mL), glacial acetic acid (2 mL), and water (10 mL) were mixed together to make the spray solution, which stained pyrrolizidine alkaloids bright orange.

*ortho-Chloranil/Erhlich's reagent.*<sup>136-</sup>

For the identification of unsaturated pyrrolizidine alkaloids, developed TLC plates were initially sprayed with a 1% solution of *ortho*-chloranil in toluene and warmed in the oven (80°C) for several minutes before being sprayed with Erhlich's reagent.

Erhlich's reagent was prepared in small volumes as it is highly moisture sensitive. *para*-Dimethylaminobenzaldehyde (0.5 g) was dissolved in dried absolute ethanol (25 mL) containing boron trifluoride etherate (0.5 mL). After spraying with Erhlich's reagent the plate was again warmed for several minutes at 80°C. Unsaturated pyrrolizidine alkaloids stained purple.

## 5.2 DRY SOLVENTS

Ethanol was dried by reaction with magnesium ethoxide.<sup>359</sup> Clean dry magnesium turnings (5.0 g), iodine (0.5 g), and absolute ethanol (75 mL) were heated in a 2 L round bottom flask, under dry nitrogen, until a vigorous reaction occurred. After the reaction had subsided, heating was continued until all the magnesium had been converted to the white ethoxide. Absolute ethanol (1 L) was added and the mixture boiled under reflux for 1 hour before the dry solvent was distilled off. Methanol was dried by the same procedure.

Dichloromethane and toluene were stored over anhydrous calcium chloride before being boiled under reflux and then distilled from calcium hydride. Carbon tetrachloride was dried by boiling under reflux with calcium chloride.

Ethanol-free, dry chloroform was prepared by washing the chloroform five times with an equal volume of water, drying over calcium chloride in the dark for 24 hours, boiling under reflux and then distilling.<sup>202</sup>

Diethyl ether and tetrahydrofuran were dried by boiling under reflux, in an atmosphere of dry nitrogen, with sodium wire and benzophenone as an indicator.

### 5.3 EXTRACTION, PURIFICATION, AND IDENTIFICATION OF THE PYRROLIZIDINE ALKALOIDS

Plants from 6 populations of *Senecio chrysocoma* and 3 populations of *S. paniculatus* were collected. The *S. chrysocoma* populations were collected from Mountain Drive, Grahamstown; Trifolia, Humansdorp; Van Stadens River dams; Wittedrif road; Prince Alfred's Pass; and close to the Dieprivierhoogte picnic spot. The former three were collected in November 1991, January 1992, and February 1992, respectively, while the latter three were all collected in February 1994. The *S. paniculatus* populations were collected in February 1994 on the farm Assegaaibos near Riversdale, beside the Robinson Pass 40 km from Oudtshoorn, and at the summit of the Tradouw Pass. Voucher specimens for all populations are held by the Selmar Schönland Herbarium, Albany Museum, Grahamstown.

#### 5.3.1 EXTRACTION PROCEDURE (see Table 5.1a,b for data for each population)

Air-dried, milled plant material (roots, stems, leaves and flowerheads) (column A, Table 5.1a,b) was initially covered with cold hexane and soaked for 2 days to remove fats. The hexane was decanted and replaced with cold methanol for 4 days to extract the

pyrrolizidines. The methanol extract was concentrated *in vacuo* and the resulting gummy residue dissolved in equal volumes of 2M-sulphuric acid and chloroform (column B, Table 5.1a,b). The aqueous and organic phases were separated, and the organic phase was further extracted with 2M-sulphuric acid (three times using the volume quoted in column C; Table 5.1a,b). The combined aqueous phases were washed with chloroform (three times using the volume quoted in column C; Table 5.1a,b) and then zinc dust was added and the mixture stirred overnight to reduce the *N*-oxides. The solution was filtered to remove excess zinc, basified to approximately pH 10 with concentrated ammonium hydroxide, and immediately extracted with chloroform. The chloroform was evaporated to leave the crude alkaloidal mixture. Before attempting any chromatographic separations, this mixture was cleaned further by redissolving in a smaller volume of chloroform and extracting with 2M-sulphuric acid. The acid extracts were basified to pH 10 with ammonium hydroxide, as before, and extracted with chloroform. The chloroform was removed *in vacuo* to leave the mixture of pyrrolizidine alkaloids (column D, Table 5.1a,b).

**Table 5.1a:** Data for the extraction of *Senecio chrysocoma* populations.

<i>S. chrysocoma</i> populations	A: dry mass (g)	B: volume of 2M-H <sub>2</sub> SO <sub>4</sub> (mL)*	C: volume of solvent (mL)	D: Yield of crude alkaloids (g),(%)†
Grahamstown	1204	500	200	8.803,(0.73)
Humansdorp	864	500	200	5.530,(0.64)
Van Stadens River Dams	728	250	200	3.067,(0.42)
Dieprivierhoogte	139	50	60	0.326,(0.23)
Prince Alfred's Pass	357	100	100	3.015,(0.84)
Wittedrif	280	100	100	1.820,(0.64)

\* Together with an equal volume of CHCl<sub>3</sub>.

† The crude pyrrolizidine alkaloids as a percentage of dry plant weight.

**Table 5.1b:** Data for the extraction of *Senecio paniculatus* populations.

<i>S. paniculatus</i> populations	A: dry mass (g)	B: volume of 2M-H <sub>2</sub> SO <sub>4</sub> (mL)*	C: volume of solvent (mL)	D: Yield of crude alkaloids (g),(%)†
Assegaibos	859	150	100	2.203,(0.26)
Robinson Pass	819	100	100	2.184,(0.27)
Tradouw Pass	497	150	100	0.992,(0.20)

\* Together with an equal volume of CHCl<sub>3</sub>.

† The crude pyrrolizidine alkaloids as a percentage of dry plant weight.

### 5.3.2 SEPARATION AND PURIFICATION OF THE PLANT EXTRACTS

The separation of the extracts of the nine populations is shown on flow charts (Figures 5.1-5.9); several techniques (designated: A; B1-3; and C) were used for separating and purifying the crude extracts.

**A:** Flash column chromatography performed on columns with a diameter of 50 mm and a silica bed height of 350 mm. A gradient elution was employed using varying ratios of chloroform and methanol (Table 5.2) and 50 mL fractions were collected.

**B:** Preparative thin layer chromatography using 20x20 cm plates for *ca.* 50 mg samples, 10x20 cm plates for 20-25 mg samples and 5x20 cm plates for samples smaller than 15 mg. Samples larger than 50 mg were separated on more than one 20x20 cm plate. Three different solvent systems were used:-

1: chloroform-methanol-25% ammonium hydroxide (85:14:1)

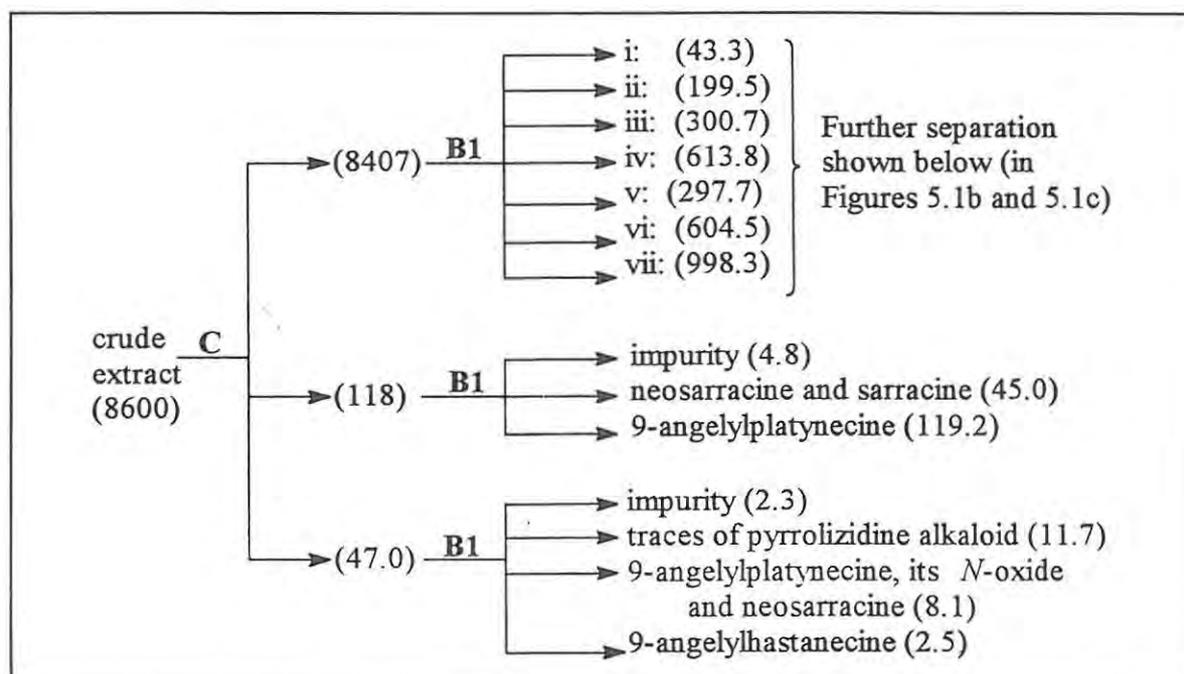
2: chloroform-cyclohexane-diethylamine (5:4:1)

3: chloroform-methanol-25% ammonium hydroxide (80:19:1)

**C:** Droplet counter current chromatography with chloroform-benzene-methanol-water (5:5:7:2) as the solvent system and a drop rate of 15 mL per hour.

**Table 5.2:** Chloroform:methanol volume ratios used for gradient elution flash column chromatography, with the volume of each mixture used in parentheses.

Population	Chloroform:Methanol(volume/L)
Humansdorp	85:15(1); 3:2(1); 0:1(0.5)
Van Stadens River Dams	4:1(1); 3:1(1); 3:2(0.5); 0:1(0.25)
Wittedrif	4:1(1); 7:3(1); 3:2(1); 0:1(0.3)
Prince Alfred's Pass	4:1(1); 7:3(1); 3:2(1); 0:1(0.25)
Robinson Pass	4:1(1); 7:3(1); 13:7(1); 0:1(0.25)
Tradouw Pass	4:1(2); 7:3(1); 1:1(1)



**Figure 5.1a:** Flow diagram for the separation of the crude extract from the Grahamstown *S. chrysocoma* population, the mass of each fraction (in mg) being indicated by the figure in parentheses.

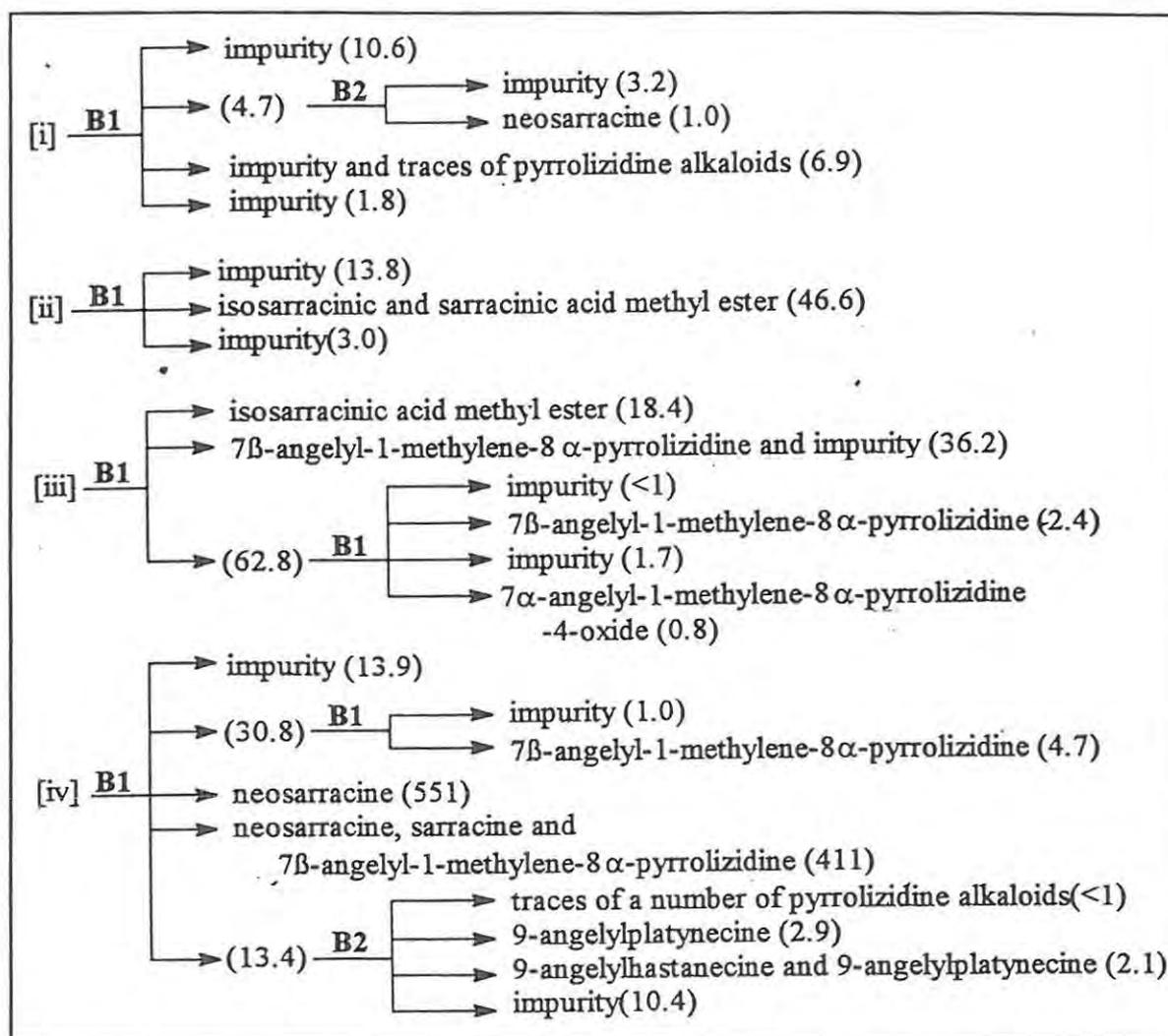


Figure 5.1b: Flow diagram (contd.) for the separation of the crude extract from the Grahamstown *S. chrysocoma* population, the mass of each fraction (in mg) being indicated by the figure in parentheses.

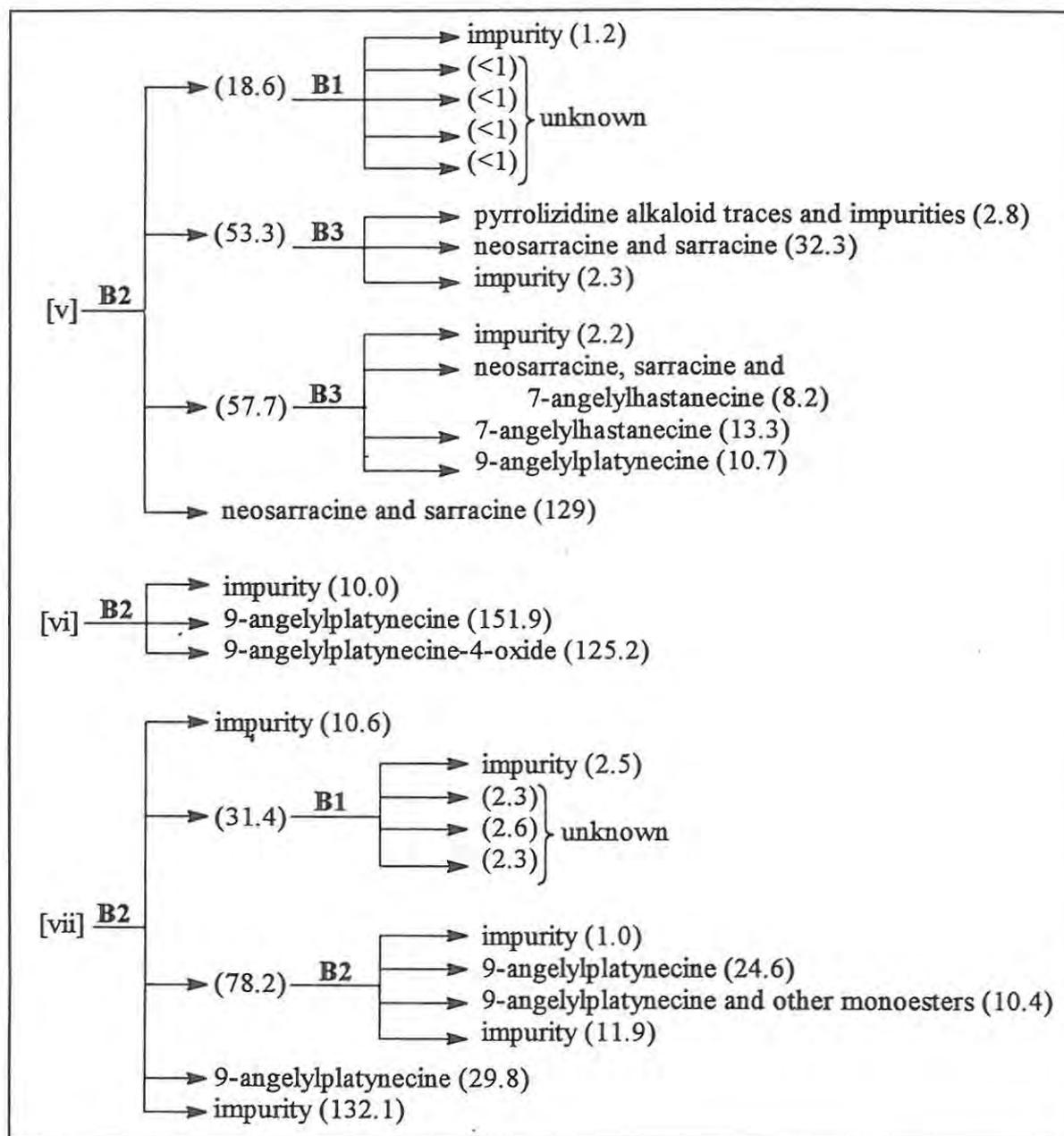
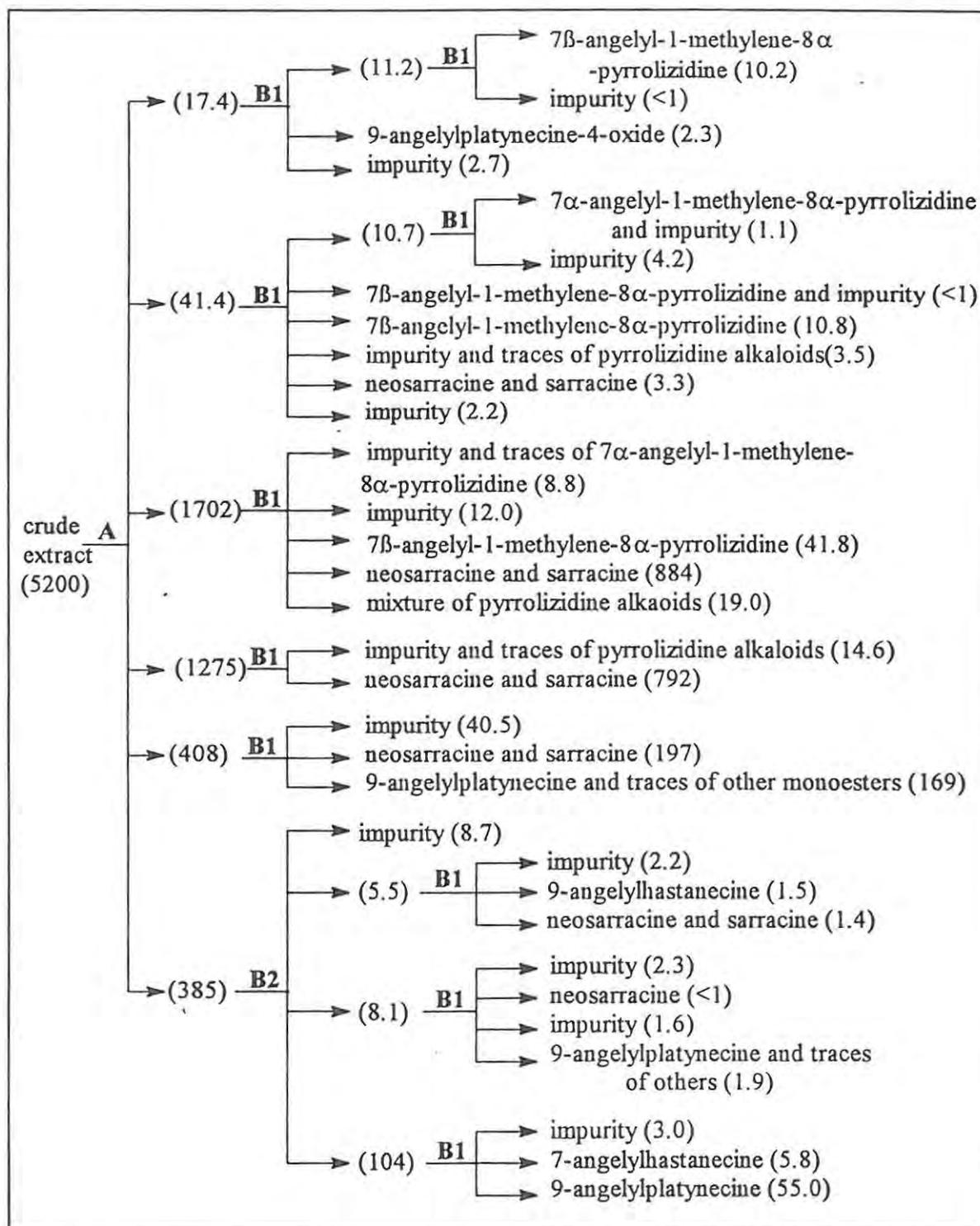
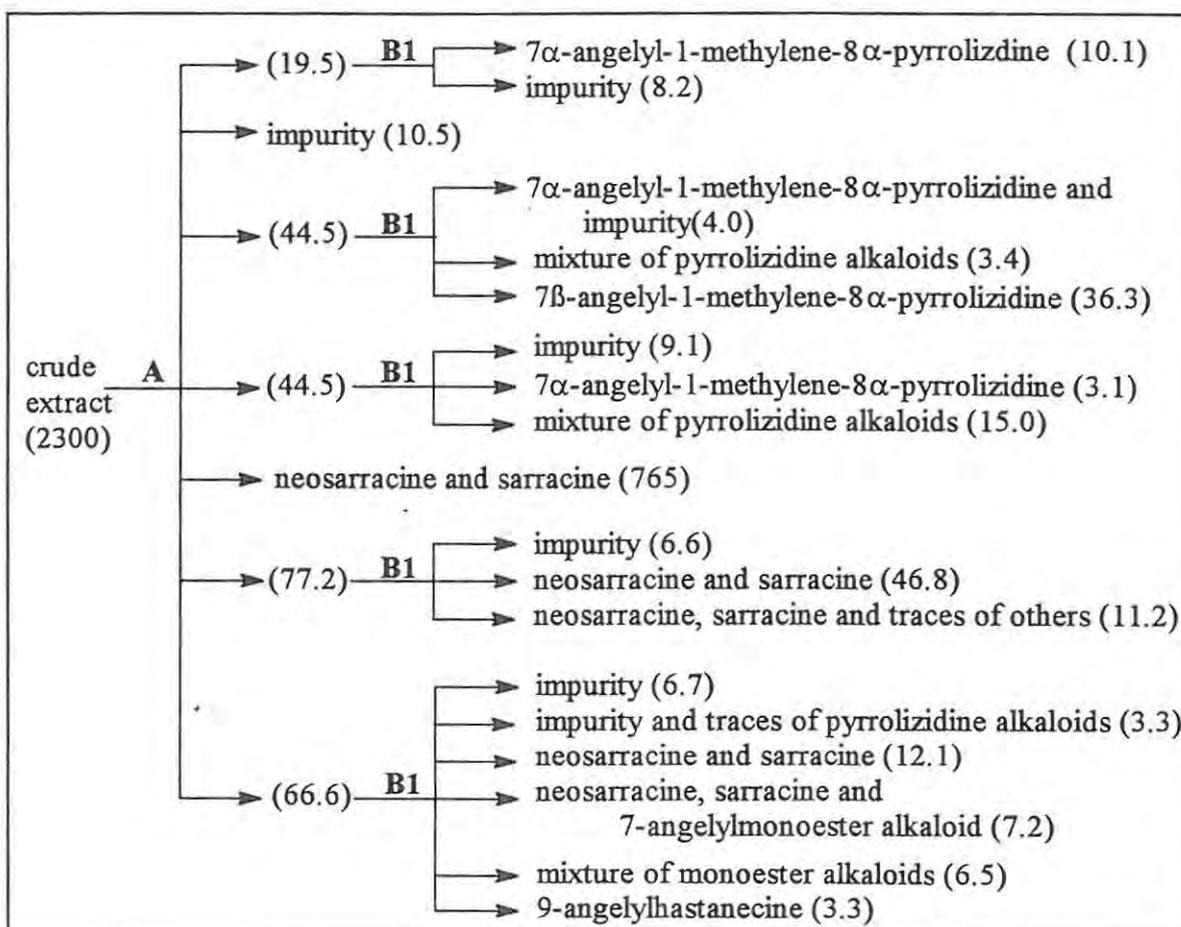


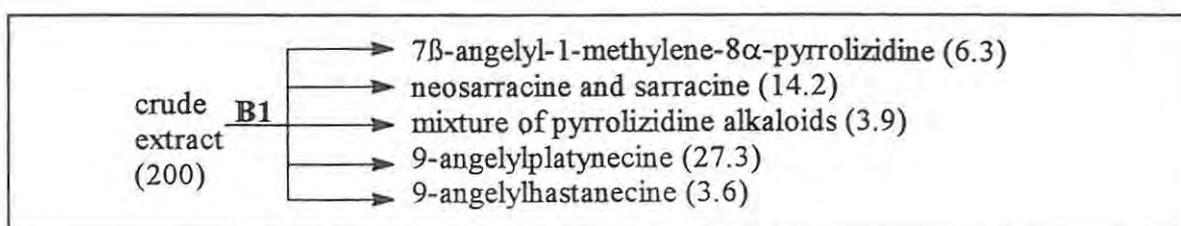
Figure 5.1c: Flow diagram (contd.) for the separation of the crude extract from the Grahamstown *S. chrysocoma* population, the mass of each fraction (in mg) being indicated by the figure in parentheses.



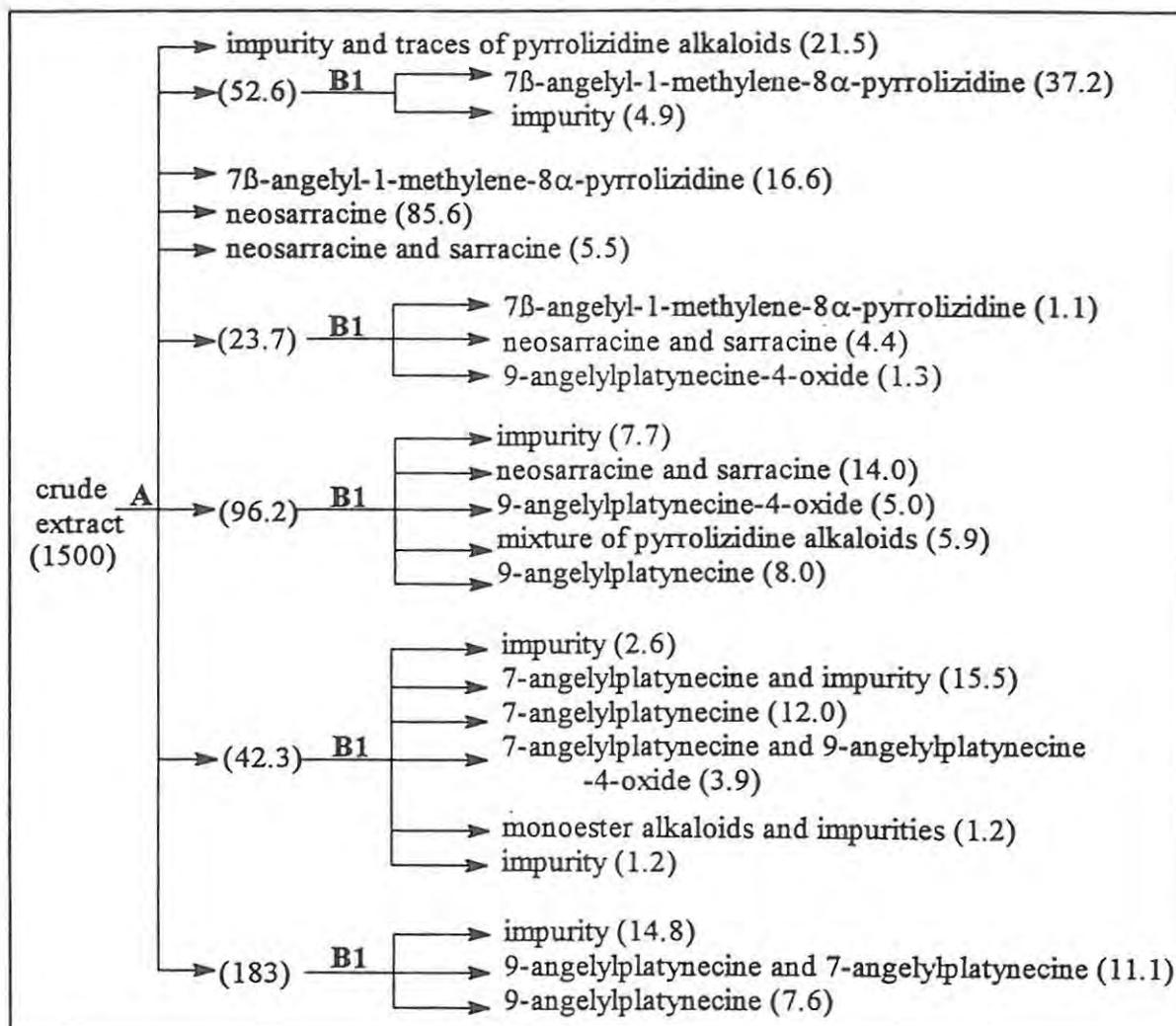
**Figure 5.2:** Flow diagram for the separation of the crude extract from the Humansdorp *S. chrysocoma* population, the mass of each fraction (in mg) being indicated by the figure in parentheses.



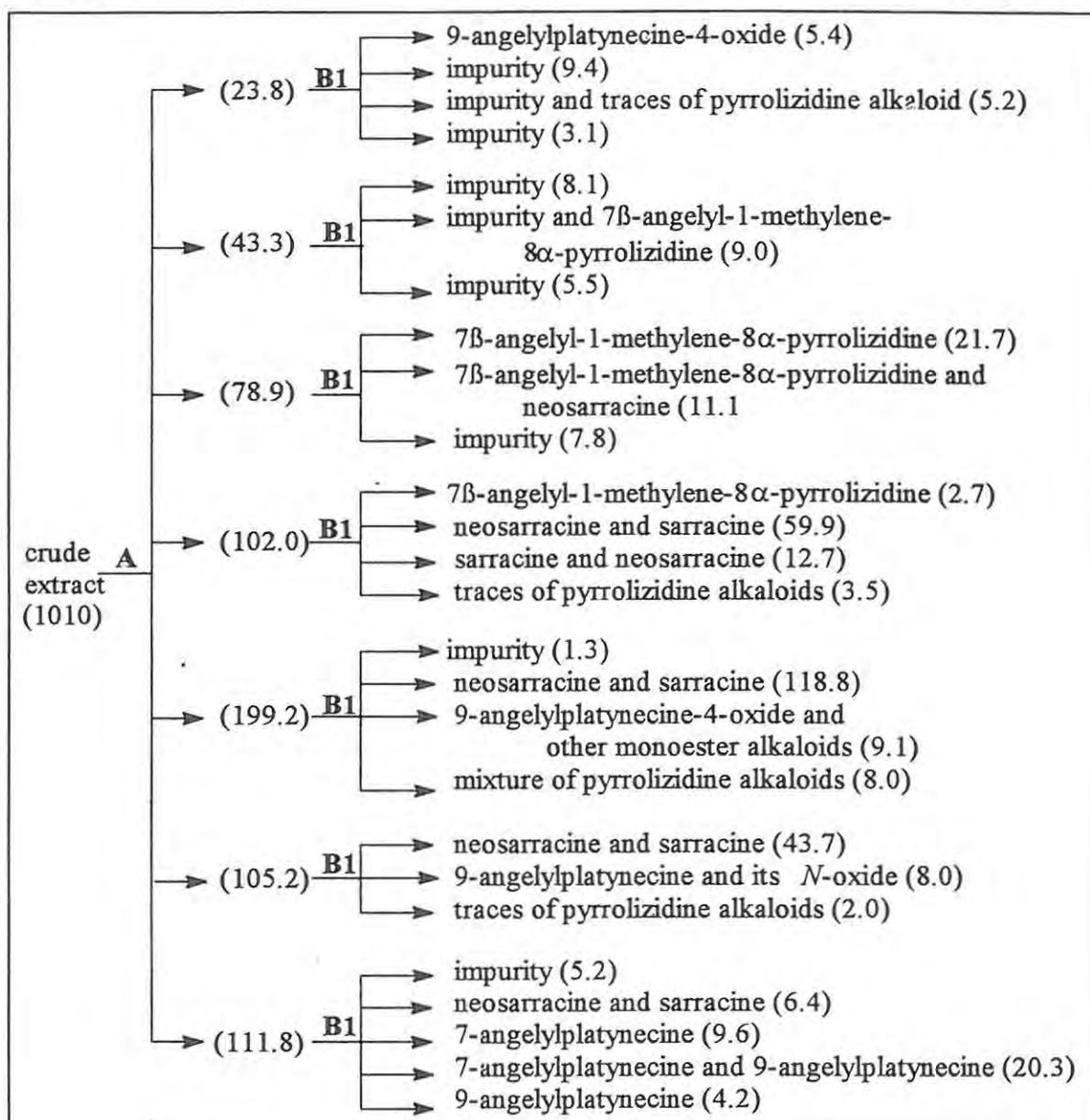
**Figure 5.3:** Flow diagram for the separation of the crude extract from the Van Stadens River dams *S. chrysocoma* population, the mass of each fraction (in mg) being indicated by the figure in parentheses.



**Figure 5.4:** Flow diagram for the separation of the crude extract from the Dieprivierhoogte *S. chrysocoma* border-line population, the mass of each fraction (in mg) being indicated by the figure in parentheses.



**Figure 5.5:** Flow diagram for the separation of the crude extract from the Prince Alfred's Pass *S. chrysocoma* border-line population, the mass of each fraction (in mg) being indicated by the figure in parentheses.



**Figure 5.6:** Flow chart for the separation of the crude extract from the Wittedrif *S. chrysocoma* border-line population, the mass of each fraction (in mg) being indicated by the figure in parentheses.

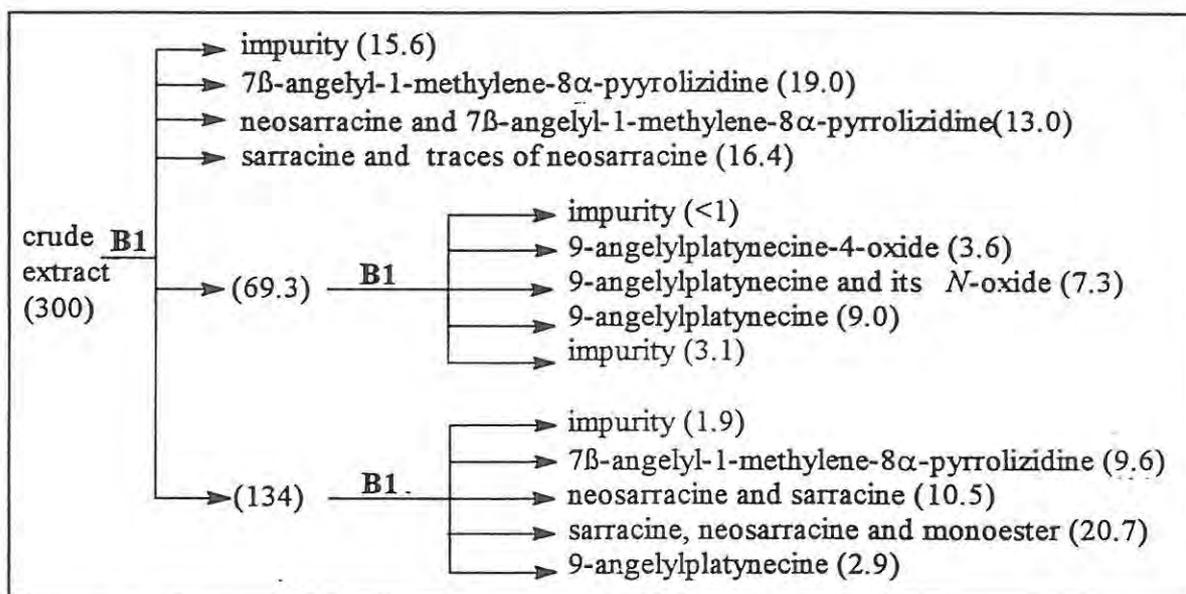
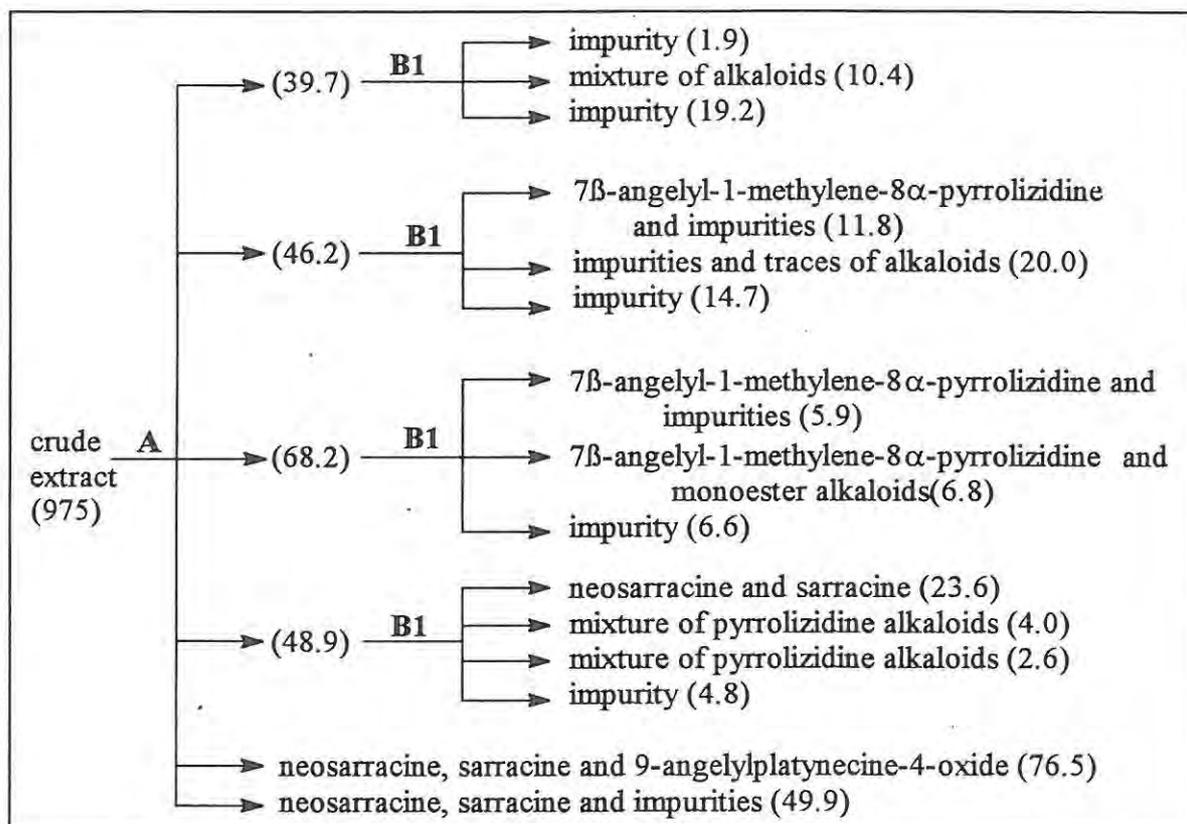
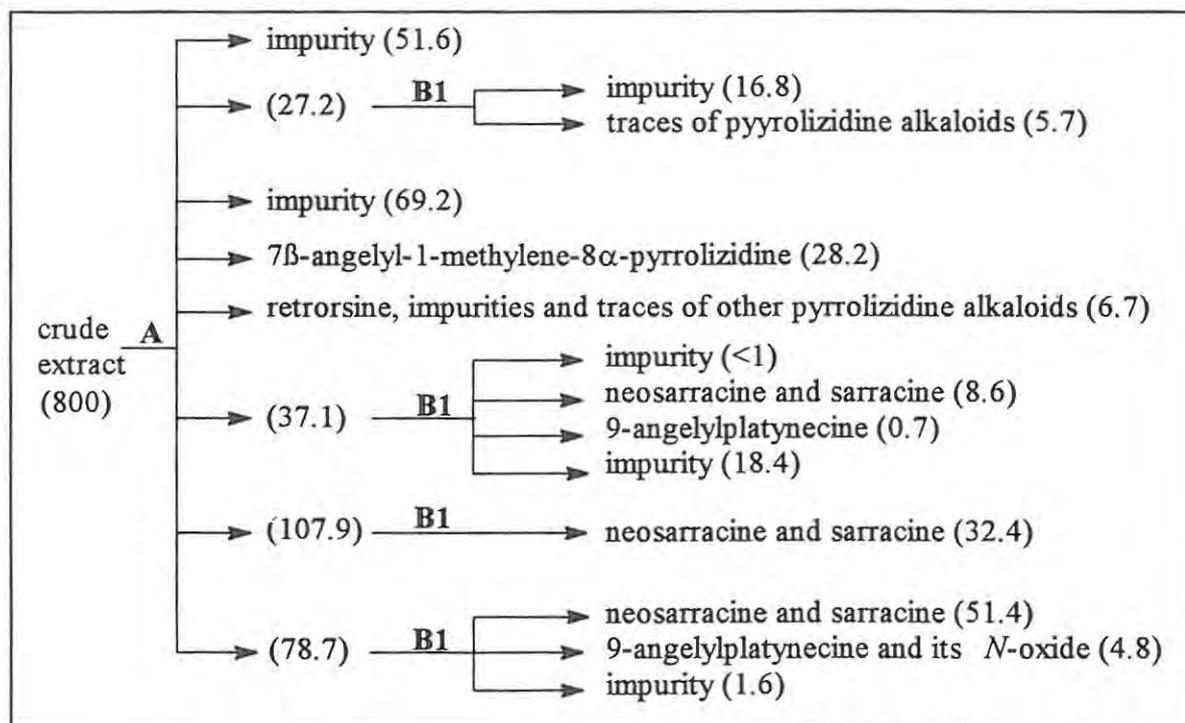


Figure 5.7: Flow chart for the separation of the crude extract from the Assegaaiibos *S. paniculatus* population, the mass of each fraction (in mg) being indicated by the figure in parentheses.



**Figure 5.8:** Flow chart for the separation of the crude extract from the Robinson Pass *S. paniculatus* population, the mass of each fraction (in mg) being indicated by the figure in parentheses.



**Figure 5.9:** Flow chart for the separation of the crude extract from the Tradouw Pass *S. paniculatus* population, the mass of each fraction (in mg) being indicated by the figure in parentheses.

### 5.3.3 ANALYTICAL DATA FOR THE ISOLATED ALKALOIDS

*Retrorsine 9*;  $\delta_{\text{H}}$  see Section 5.4.2 (p. 156);  $\delta_{\text{C}}$  see Section 5.4.2 (p. 156); EI  $m/z$  246(12%), 220(22), 139(11), 138(45), 137(24), 136(95), 122(21), 121(45), 120(100), 119(77), 118(18), 109(16), 108(19), 95(40), 94(63), 93(76), 80(29), 53(22) and 44(38); CI( $\text{CH}_4$ )  $m/z$  350[(M-1)<sup>+</sup>, 100%], 349(6), 348(7), 336(2), 151(2), 136(3), 119(3), 118(19), 117(14) and 116(4).

*Sarracine 36*; gum;  $\delta_{\text{H}}$  1.88(3H, 20- $\text{CH}_3$ ), 1.95(2H, 2- $\text{CH}_2$ ), 1.97(3H, 19- $\text{CH}_3$ ), 2.03(3H, 14- $\text{CH}_3$ ), 2.05 and 2.14(2H, 6- $\text{CH}_2$ ), 2.79 and 3.52(2H, 5- $\text{CH}_2$ ), 2.83(1H, 1-CH), 2.90 and 3.33(2H, 3- $\text{CH}_2$ ), 3.84(1H, 8-CH), 4.22(2H, 15- $\text{CH}_2$ ), 4.24 and 4.40(2H, 9- $\text{CH}_2$ ), 5.41(1H, 7-CH), 6.12(1H, 18-CH) and 6.36(1H, 13-CH);  $\delta_{\text{C}}$  15.67(C-14), 15.78(C-19), 20.21(C-20), 28.64(C-2), 34.76(C-6), 40.01(C-1), 53.44(C-5), 54.62(C-3), 63.23(C-9),

68.87(C-8), 68.89(C-15), 74.69(C-7), 126.62 (C-17), 131.74(C-12), 140.55(C-18), 140.98(C-13), 166.36(C-16) and 166.68(C-11); EI  $m/z$  237(8%), 222(10), 140(37), 139(32), 138(100), 123(26), 122(57), 121(13), 96(46), 95(47), 83(35), 82(74) and 55(35); CI (CH<sub>4</sub>)  $m/z$  336[(M-1)<sup>+</sup>, 100%], 335(6), 318(7), 237(4), 236(21), 235(8), 220(17), 138(3), 120(6), 118(3) and 117(3).

*7-Angelylplatynecine 38*; gum;  $\delta_H$  1.90(3H, 15-CH<sub>3</sub>), 1.91 and 2.14(2H, 2-CH<sub>2</sub>), 2.02 (3H, 14-CH<sub>3</sub>), 2.17 and 2.26(2H, 6-CH<sub>2</sub>), 2.31(1H, 1-CH), 2.68 and 3.38(2H, 3-CH<sub>2</sub>), 2.77 and 3.42(2H, 5-CH<sub>2</sub>), 3.57 and 3.72(2H, 9-CH<sub>2</sub>), 3.89(1H, 8-CH), 5.37(1H, 7-CH) and 6.15(1H, 13-CH);  $\delta_C$  15.88(C-14), 20.58(C-15), 30.98(C-2), 34.85(C-6), 40.13(C-1), 52.33(C-5), 55.13(C-3), 64.75(C-9), 71.82(C-8), 73.93(C-7), 126.96(C-12), 140.23(C-13) and 166.83(C-11); EI  $m/z$  239(M<sup>+</sup>, 1%), 208(1), 156(40), 140(21), 139(85), 138(29), 109(10), 108(41), 83(30), 82(100) and 55(16); CI(NH<sub>3</sub>)  $m/z$  240[(M+1)<sup>+</sup>, 100%], 238(2), 156(1), 149(2), 139(2), 138(3), 122(1) and 99(1).

*9-Angelylplatynecine 39*; gum;  $\delta_H$  1.78 and 1.90(2H, 2-CH<sub>2</sub>), 1.81 and 1.91(2H, 6-CH<sub>2</sub>), 1.87(3H, 15-CH<sub>3</sub>), 1.97 (3H, 14-CH<sub>3</sub>), 2.64(1H, 1-CH), 2.75 and 3.13(2H, 3-CH<sub>2</sub>), 2.81 and 3.27(2H, 5-CH<sub>2</sub>), 3.36(1H, 8-CH), 4.23(1H, 7-CH), 4.47 and 4.57(2H, 9-CH<sub>2</sub>) and 6.06(1H, 13-CH);  $\delta_C$  15.65(C-14), 20.68(C-15), 28.91(C-2), 37.41(C-6), 40.70(C-1), 53.69(C-5), 55.23(C-3), 64.33(C-9), 70.30(C-8), 72.72(C-7), 127.77(C-12), 137.83(C-13) and 168.10(C-11); EI  $m/z$  239(M<sup>+</sup>, 2%), 221(5), 195(1), 156(2), 140(6), 139(4), 138(5), 122(9), 121(2), 120(2), 96(36), 95(100), 94(5), 83(28), 82(94) and 55(19); CI(NH<sub>3</sub>)  $m/z$  240[(M+1)<sup>+</sup>, 100%], 140(2), 138(1) 99(1) and 95(1).

*7-Angelylhastanecine 66*; gum;  $\delta_H$  1.88(3H, 15-CH<sub>3</sub>), 2.01(3H, 14-CH<sub>3</sub>), 2.05 and 2.08(2H, 2-CH<sub>2</sub>), 2.22 and 2.36(2H, 6-CH<sub>2</sub>), 2.42(1H, 1-CH), 2.84 and 3.65(2H, 3-CH<sub>2</sub>), 2.88 and 3.68(2H, 5-CH<sub>2</sub>), 3.60 and 3.77(2H, 9-CH<sub>2</sub>), 4.22(1H, 8-CH), 5.46(1H, 7-CH) and 6.18(1H, 13-CH);  $\delta_C$  15.87(C-14), 20.51(C-15), 30.26(C-2), 34.48(C-6), 40.22(C-1), 52.36(C-5), 54.88(C-3), 63.46(C-9), 71.69(C-8), 73.29(C-7), 126.59(C-12), 140.83(C-13) and 166.29(C-11); EI  $m/z$  239(M<sup>+</sup>, 0.3%), 219(0.2), 156(47), 140(6), 139(64), 138(13),

114(12), 113(13), 83(13), 82(100) and 55(9); CI(NH<sub>3</sub>) *m/z* 240[(M+1)<sup>+</sup>, 100%], 239(2), 238(11), 139(4), 138(8), 136(2), 134(1), 122(2), 120(3), 118(2), 114(1), 99(1) and 96(1).

*9-Angelylhastanecine 67*; gum;  $\delta_{\text{H}}$  1.87(3H, 15-CH<sub>3</sub>), 1.98 (3H, 14-CH<sub>3</sub>), 2.12 and 2.26(2H, 2-CH<sub>2</sub>), 2.17(2H, 6-CH<sub>2</sub>), 2.83(1H, 1-CH), 3.19 and 3.64(2H, 3-CH<sub>2</sub>), 3.21 and 3.93(2H, 5-CH<sub>2</sub>), 4.18(1H, 8-CH), 4.49 and 4.62(2H, 9-CH<sub>2</sub>), 4.60(1H, 7-CH) and 6.10(1H, 13-CH);  $\delta_{\text{C}}$  15.47(C-14), 20.54(C-15), 28.69(C-2), 36.73(C-6), 39.98(C-1), 53.85(C-5), 54.32(C-3), 62.28(C-9), 71.48(C-8), 71.55(C-7), 127.20(C-12), 139.20(C-13) and 167.65(C-11); EI *m/z* 239(M<sup>+</sup>, 6%), 221(32), 195(6), 155(5), 140(29), 139(11), 138(16), 122(49), 121(7), 120(7), 96(91), 95(100), 94(19), 83(28), 82(96) and 55(71); CI(NH<sub>3</sub>) *m/z* 240[(M+1)<sup>+</sup>, 100%], 238(3), 156(2), 140(3), 139(3), 138(3), 122(2) and 113(1).

*9-Angelylplatynecine-4-oxide 68*; gum;  $\delta_{\text{H}}$  1.86(3H, 15-CH<sub>3</sub>), 1.98 (3H, 14-CH<sub>3</sub>), 2.14 and 2.32(2H, 2-CH<sub>2</sub>), 2.20 and 2.60(2H, 6-CH<sub>2</sub>), 3.19(1H, 1-CH), 3.70-4.01(5H, 3-CH<sub>2</sub>, 5-CH<sub>2</sub>, 8-CH), 4.47 and 4.63(2H, 9-CH<sub>2</sub>), 4.69(1H, 7-CH) and 6.10(1H, 13-CH);  $\delta_{\text{C}}$  15.86(C-14), 20.56(C-15), 28.94(C-2), 34.81(C-6), 37.98(C-1), 62.58(C-9), 68.24(C-5), 70.41(C-3), 71.51(C-7), 88.18(C-8), 127.35(C-12), 138.98(C-13) and 167.78(C-11); EI *m/z* 219(1), 120(14), 119(100), 118(7), 107(5), 106(59), 79(6), 77(4) and 55(5).

*7 $\alpha$ -Angelyl-1-methylene-8 $\alpha$ -pyrrolizidine 69*; gum (Found: M<sup>+</sup>, 221.1415. C<sub>13</sub>H<sub>19</sub>NO<sub>2</sub> requires *M*, 221.1416);  $\delta_{\text{H}}$  1.83(3H, 15-CH<sub>3</sub>), 1.97(3H, 14-CH<sub>3</sub>), 2.17 and 2.32(2H, 6-CH<sub>2</sub>), 2.66(2H, 2-CH<sub>2</sub>), 2.90 and 3.41(2H, 3-CH<sub>2</sub>), 2.92 and 3.60(2H, 5-CH<sub>2</sub>), 4.46(1H, 8-CH), 4.96 and 5.04(2H, 9-CH<sub>2</sub>), 5.62(1H, 7-CH) and 6.07(1H, 13-CH);  $\delta_{\text{C}}$  15.69(C-14), 20.46(C-15), 33.45(C-2), 34.21(C-6), 52.60(C-5), 53.70(C-3), 71.68(C-8), 73.91(C-7), 109.89(C-9), 127.16(C-12), 139.21(C-13) and 166.28(C-11); EI *m/z* 138(41%), 121(38), 95(100), 80(6), 67(7) and 55(14); CI(NH<sub>3</sub>) *m/z* 222[(M+1)<sup>+</sup>, 100%], 158(1), 138(2), 121(2) and 95(3).

*7 $\alpha$ -Angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide 70*; gum;  $\delta_{\text{H}}$  1.84(3H, 15-CH<sub>3</sub>), 1.98(3H, 14-CH<sub>3</sub>), 2.22 and 3.01(2H, 6-CH<sub>2</sub>), 2.69 and 3.12(2H, 2-CH<sub>2</sub>), 3.79(2H, 3-CH<sub>2</sub>), 3.84 and 3.91(2H, 5-CH<sub>2</sub>), 4.66(1H, 8-CH), 5.04 and 5.24(2H, 9-CH<sub>2</sub>), 5.84(1H, 7-CH) and 6.10(1H, 13-CH);  $\delta_{\text{C}}$  15.73(C-14), 20.32(C-15), 32.07(C-6), 32.63(C-2), 67.36(C-5), 68.47(C-3), 89.63(C-8), 72.41(C-7), 112.49(C-9), 126.63(C-12), 139.74(C-1), 140.18(C-13) and 165.83(C-11).

*7 $\beta$ -Angelyl-1-methylene-8 $\alpha$ -pyrrolizidine 71*; gum (Found: M<sup>+</sup>, 221.1419. C<sub>13</sub>H<sub>19</sub>NO<sub>2</sub> requires M, 221.1416);  $\delta_{\text{H}}$  1.84(3H, 15-CH<sub>3</sub>), 1.99(3H, 14-CH<sub>3</sub>), 2.08 and 2.17(2H, 6-CH<sub>2</sub>), 2.55(2H, 2-CH<sub>2</sub>), 2.76 and 3.16(2H, 3-CH<sub>2</sub>), 2.83 and 3.27(2H, 5-CH<sub>2</sub>), 4.14(1H, 8-CH), 4.88 and 5.07(2H, 9-CH<sub>2</sub>), 5.52(1H, 7-CH) and 6.03(1H, 13-CH);  $\delta_{\text{C}}$  15.61(C-14), 20.55(C-15), 33.92(C-2), 34.35(C-6), 52.81(C-5), 54.46(C-3), 71.94(C-8), 74.95(C-7), 107.68(C-9), 127.86(C-12), 137.84(C-13), 147.09(C-1) and 166.87(C-11); EI *m/z* 138(42%), 121(42), 95(100), 80(7), 67(7) and 55(13); CI(NH<sub>3</sub>) *m/z* 222[(M+1)<sup>+</sup>, 100%], 158(1), 138(2), 121(3) and 95(4).

*Neosarracine 72*; gum;  $\delta_{\text{H}}$  1.75 and 1.81(2H, 2-CH<sub>2</sub>), 1.83(6H, 14-CH<sub>3</sub>, 20-CH<sub>3</sub>), 1.91(3H, 19-CH<sub>3</sub>), 1.94 and 2.02(2H, 6-CH<sub>2</sub>), 2.68(1H, 1-CH), 2.74 and 3.13(2H, 3-CH<sub>2</sub>), 3.28 and 3.64(2H, 5-CH<sub>2</sub>), 3.58(1H, 8-CH), 4.14 and 4.29(2H, 9-CH<sub>2</sub>), 4.24(2H, 15-CH<sub>2</sub>), 5.25(1H, 7-CH), 6.02(1H, 18-CH) and 6.88(1H, 13-CH);  $\delta_{\text{C}}$  14.04(C-14), 15.51(C-19), 20.51(C-20), 28.49(C-2), 34.69(C-6), 39.94(C-1), 53.27(C-5), 54.68(C-3), 56.10(C-15), 63.90(C-9), 68.43(C-8), 74.91(C-7), 126.79 (C-17), 131.93(C-12), 139.55(C-18), 140.93(C-13), 166.46(C-16) and 166.89(C-11); EI *m/z* 237(9%), 222(9), 140(29), 139(27), 138(100), 123(22), 122(38), 121(15), 96(30), 95(51), 83(24), 82(64) and 55(27); CI(NH<sub>3</sub>) *m/z* 338[(M+1)<sup>+</sup>, 100%], 322(1), 236(3), 220(2), 140(1), 139(1), 137(3), 134(5), 133(1), 123(1), 122(7), 118(2), 95(2) and 94(1).

## 5.4 THE SYNTHESIS OF 7 $\beta$ -ANGELYL-1-METHYLENE-8 $\alpha$ -PYRROLIZIDINE 71

### 5.4.1 *AB INITIO* APPROACH

#### *Methyl 3-bromopropanoate 99.*<sup>340</sup>-

Acetyl chloride (2.0 mL, 28 mmol) was added dropwise to a stirred solution of 3-bromopropanoic acid (100 g, 654 mmol) in dry methanol (400 mL). Stirring was continued at room temperature for 21 hours and the solvent was then removed *in vacuo* keeping the temperature below 30°C. The residue was dissolved in dichloromethane (250 mL), and washed sequentially with saturated aqueous sodium hydrogen carbonate (50 mL), water (2x50 mL) and saturated brine (50 mL), before being dried with anhydrous magnesium sulphate. The solvent was evaporated to give methyl 3-bromopropanoate **99** as a yellow oil (104 g, 95.2%) sufficiently pure to be used directly in the next step;  $\delta_{\text{H}}$  2.87(2H, t,  $J_{2,3}$  6.8, 2-CH<sub>2</sub>), 3.52(2H, t,  $J_{2,3}$  6.8, 3-CH<sub>2</sub>) and 3.67(3H, s, CH<sub>3</sub>O);  $\delta_{\text{C}}$  25.74(C-3), 37.46(C-2), 51.89(CH<sub>3</sub>O) and 170.83(C-1).

#### *Methyl glycinate hydrochloride 100.-*

Thionyl chloride (73 mL, 1.0 mol) was added dropwise to a stirred solution of glycine (75 g, 1.0 mol) in dry methanol (750 mL). Stirring was continued at room temperature overnight. The solvent was removed *in vacuo* to afford crystalline methyl glycinate hydrochloride **100** as white needles (124 g, 98.8%); m.p. 174-176°C (lit.,<sup>360</sup> 175°C) (from methanol and chloroform);  $\delta_{\text{H}}$ (D<sub>2</sub>O) 3.95(3H, s, CH<sub>3</sub>O) and 4.04(2H, s, 2-CH<sub>2</sub>);  $\delta_{\text{C}}$ (D<sub>2</sub>O) 42.73(C-2), 56.03(CH<sub>3</sub>O) and 171.30(C-1).

#### *Dimethyl 3-azahexane-1,6-dicarboxylate 101.*<sup>340</sup>-

10M-Sodium hydroxide (11.9 mL) was added in one portion to a cooled, stirred solution of methyl glycinate hydrochloride **100** (15.0 g, 119 mmol) in water (35 mL). When the temperature had returned to 0°C, methyl 3-bromopropanoate **99** (19.9 g, 119 mmol) in methanol (70 mL) was added dropwise over 35 minutes, followed by a solution of

potassium carbonate (7.9 g, 57 mmol) in water (88 mL). The resulting cloudy mixture was stirred at room temperature overnight. The solvents were removed *in vacuo* at 40°C until a precipitate began to form. Just enough water was added to dissolve the precipitate and the resulting mixture was extracted with dichloromethane (5x45 mL). The combined extracts were dried with anhydrous magnesium sulphate and the solvent evaporated to give dimethyl 3-azahexane-1,6-dicarboxylate **101** (12.04 g, 57.6%);  $\delta_{\text{H}}$  1.77(1H, s, NH), 2.47(2H, t,  $J_{4,5}$  6.6, 5-CH<sub>2</sub>), 2.86(2H, t,  $J_{4,5}$  6.6, 4-CH<sub>2</sub>), 3.38(2H, s, 2-CH<sub>2</sub>), and 3.65 and 3.68(6H, s and s, 2xCH<sub>3</sub>O);  $\delta_{\text{C}}$  34.58(C-5), 44.69(C-4), 50.60(C-2), 51.55 and 51.69(2xCH<sub>3</sub>O), and 172.60 and 172.76(C-1, C-6).

*3-tert-Butyl 1,6-dimethyl 3-azahexane-1,3,6-tricarboxylate 102.*<sup>340-</sup>

Di-*tert*-butyl dicarbonate (20.6 g, 94.2 mmol) in dry dichloromethane (60 mL) was added dropwise over 30 minutes to an ice-cold, stirred solution of dimethyl 3-azahexane-1,6-dicarboxylate **101** (15.0 g, 85.6 mmol) and triethylamine (12.3 mL, 8.8 mmol) in dry dichloromethane (60 mL). The resulting solution was stirred at room temperature overnight before being diluted with dichloromethane (80 mL) and washed sequentially with 2M-hydrochloric acid (190 mL), water (2x190 mL), and saturated brine (95 mL). The organic layer was then dried with anhydrous magnesium sulphate and the solvent was evaporated. The resulting oil was vacuum distilled (*ca.* 0.6 mmHg) to give two fractions: one boiling at 88-92°C which was *tert*-butyl alcohol and the other at 124-129°C which was the desired 3-*tert*-butyl 1,6-dimethyl 3-azahexane-1,3,6-tricarboxylate **102** (21.7 g, 92.1%);  $\delta_{\text{H}}$ (DMSO-*d*<sub>6</sub>, 353K) 1.39[9H, s, (CH<sub>3</sub>)<sub>3</sub>C], 2.57(2H, t,  $J_{4,5}$  6.9, 5-CH<sub>2</sub>), 3.48(2H, t,  $J_{4,5}$  6.9, 4-CH<sub>2</sub>), 3.61 and 3.66(6H, s and s, 2xCH<sub>3</sub>O) and 3.96(2H, s, 2-CH<sub>2</sub>).  $\delta_{\text{C}}$ (DMSO-*d*<sub>6</sub>, 353K) 27.39[(CH<sub>3</sub>)<sub>3</sub>C], 32.51(C-5), 43.76(C-4), 48.93(C-2), 50.59 and 50.91(2xCH<sub>3</sub>O), 78.79[(CH<sub>3</sub>)<sub>3</sub>C], 154.02(NCO<sub>2</sub>), and 169.72 and 171.15(C-1, C-6).

*1-tert-Butyl 2-methyl 3-oxopyrrolidine-1,2-dicarboxylate 103.*<sup>263-</sup>

A solution of 3-*tert*-butyl 1,6-dimethyl 3-azahexane-1,3,6-tricarboxylate **102** (6.30 g, 22.9 mmol) in dry toluene (11.5 mL) was added dropwise to an ice-cold, stirred solution of potassium *tert*-butoxide (3.60 g, 32.1 mmol) in dry toluene (65 mL) under dry nitrogen. Stirring was continued at 0°C for 15 minutes. Glacial acetic acid (2.5 mL) was added in

one portion followed immediately by an ice-cold solution of sodium dihydrogen phosphate (92 mL of a 10% aqueous solution of  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ). The mixture was shaken, left to separate, and the organic layer removed. The aqueous phase was extracted with dichloromethane (4x50 mL). The combined organic extracts were washed with pH 7 phosphate buffer (3x75 mL), dried with anhydrous magnesium sulphate and the solvent evaporated. The crude residue was dissolved in toluene (150 mL), washed with pH 9.5 carbonate buffer (10x50 mL) and water (20 mL), and then dried with anhydrous magnesium sulphate. The solvent was evaporated to give the desired regioisomer 1-*tert*-butyl 2-methyl 3-oxopyrrolidine-1,2-dicarboxylate **103** (3.48 g, 62.4 %);  $\delta_{\text{H}}$  1.39-1.50[9H, m,  $(\text{CH}_3)_3\text{C}$ ], 2.63(2H, t,  $J$  2.6, 4- $\text{CH}_2$ ), 3.65-4.01(2H, m, 5- $\text{CH}_2$ ), 3.78(3H, s,  $\text{CH}_3\text{O}$ ) and 4.52(1H, m, 2-CH);  $\delta_{\text{C}}$  28.20[ $(\text{CH}_3)_3\text{C}$ ], 36.32(C-4), 41.52(C-5), 65.59[ $(\text{CH}_3)_3\text{C}$ ], 81.05(C-2), 153.95(N-CO<sub>2</sub>) and 170.71(CO<sub>2</sub>CH<sub>3</sub>).

*pH 7 Phosphate Buffer.-*

Sodium chloride (8.0 g), potassium chloride (0.2 g), potassium dihydrogen phosphate (0.2 g) and sodium phosphate-2H<sub>2</sub>O (0.15 g) were dissolved in deionised water (1000 mL) and carefully brought to pH 7 with a few drops of concentrated sodium hydroxide.

*pH 9.5 Carbonate Buffer.-*

Sodium hydrogen carbonate (11.76 g) and sodium carbonate (6.36 g) were dissolved in deionised water (1000 mL).

*Attempted preparation of 1-tert-butyl 2-methyl 3-hydroxypyrrolidine-1,2-dicarboxylate **112\***.*<sup>263</sup>

A mixture of 1-*tert*-butyl 2-methyl 3-oxopyrrolidine-1,2-dicarboxylate **103** (1.0 g, 3.9 mmol), tap water (80 mL), sucrose (15 g) and dried baker's yeast (10 g) was shaken at 30°C for 24 hours. Celite was added to the mixture, which was filtered and the solids were washed with water (20 mL). The combined filtrates were saturated with sodium chloride and extracted with diethyl ether (5x50 mL). The combined extracts were washed with water (2x25 mL), dried with anhydrous magnesium sulphate and the solvent was

\* 2-methyl analogue of compound **97** (Scheme 4.2, p. 116).

evaporated *in vacuo*, but the desired product, 1-*tert*-butyl 2-methyl 3-hydroxypyrrolidine-1,2-dicarboxylate **112** could not be identified in the residue.

#### 5.4.2 MODIFICATION OF THE NATURAL PYRROLIZIDINE SYSTEM, RETRONECINE **31**, TO AFFORD 7 $\beta$ -ANGELYL-1-METHYLENE-8 $\alpha$ -PYRROLIZIDINE **71**

##### *Retrorsine 9*.-

Dried *Senecio orthonnaeflorus* plant material (1.226 kg) was extracted by the same general procedure as described for *S. chrysocoma* and *S. paniculatus*. The methanol extract was concentrated and dissolved in a mixture of 2M-sulphuric acid (500 mL) and chloroform (500mL). The organic and aqueous phases were separated and the organic layer was extracted with 2M-sulphuric acid (3x200 mL). The combined aqueous layers were washed with chloroform (3x200 mL) and then stirred overnight with zinc dust. The solution was filtered to remove excess zinc, basified to pH 10 with concentrated ammonium hydroxide and extracted with chloroform (5x200 mL). The chloroform extracts were dried with anhydrous magnesium sulphate and the solvent evaporated to give retrorsine **9** as a crystalline solid (20.55 g, 1.7% of dry plant weight); m.p. 214-216°C (lit.,<sup>142,328</sup> 216°C);  $\delta_{\text{H}}$  0.86(3H, d, *J* 6.4, 19-CH<sub>3</sub>), 1.68(1H, m, 13-CH), 1.74 and 2.12(2H, m and m, 14-CH<sub>2</sub>), 1.83(3H, dd, *J* 1.4 and 7.0, 21-CH<sub>3</sub>), 2.21 and 2.39(2H, d and dd, *J* 12.8 and *J* 5.9, 14.0, 6-CH<sub>2</sub>), 2.53 and 3.25(2H, m and t, *J* 8.4, 5-CH<sub>2</sub>), 3.39 and 3.93(2H, dd and d, *J* 7.1, 15.4 and *J* 14.3, 3-CH<sub>2</sub>), 3.63 and 3.74(2H, d and d, *J* 11.2 and *J* 11.2, 18-CH<sub>2</sub>), 4.10 and 5.50(2H, d and d, *J* 11.5 and *J* 11.8, 9-CH<sub>2</sub>), 4.26(1H, d, *J* 2.0, 8-CH), 5.00(1H, t, *J* 3.2, 7-CH), 5.72(1H, q, *J* 7.2, 20-CH) and 6.21(1H, s, 2-CH);  $\delta_{\text{C}}$  11.65(C-19), 14.99(C-21), 34.73(C-6), 35.64(C-13), 37.91(C-14), 52.96(C-5), 61.12(C-3), 62.85(C-9), 66.89(C-18), 75.11(C-8), 77.49(C-7), 81.36(C-12), 131.29(C-15), 132.519(C-1), 134.54(C-2), 136.86(C-20), 167.41(C-16) and 175.63(C-11).

*Retronecine 31.*-

Method 1: A mixture of retrorsine (20.0 g, 56.9 mmol) and barium hydroxide octahydrate (16.0g, 50.7 mmol) in water (250 ml) was boiled under reflux for four hours. After cooling the mixture, it was saturated with carbon dioxide using dry ice and filtered to remove the precipitated barium carbonate. The filtrate was concentrated *in vacuo* to approximately 100 mL, brought to pH 9 with sodium carbonate, then saturated with sodium chloride and continuously extracted with chloroform for 17 hours. The chloroform extract was concentrated *in vacuo* to yield a gummy residue which was shown, by NMR analysis, to contain some retronecine. Hydrolysis was deemed to be incomplete and the chloroform insoluble material (7.02 g) was boiled under reflux with barium hydroxide octahydrate (15.0g, 47.5 mmol) in water (200 mL) for 20 hours. The same workup procedure as above was followed, except that the continuous extraction took 72 hours, yielding retronecine 31 as a gum (2.35 g, 26.5%).

Method 2: A mixture of retrorsine (7.5 g, 21 mmol), barium hydroxide octahydrate (17.4 g, 55.2 mmol) and water (150 ml) was boiled under reflux for 9.5 hours. After cooling the solution was saturated with carbon dioxide using dry ice and filtered to remove precipitated barium carbonate. The filtrate was evaporated *in vacuo* and the residue dissolved in methanol. The methanolic solution was filtered and evaporated to leave a brown gum which was boiled under reflux with chloroform for 5.5 days. The chloroform was changed twice during this time. The chloroform extracts were combined, dried with magnesium sulphate and the solvent evaporated *in vacuo* to give crude retronecine (2.13g, 65.4%). Attempted recrystallisation from acetone was unsuccessful.

Method 3: A mixture of retrorsine (21.0 g, 60.0 mmol), barium hydroxide octahydrate (48.9 g, 155 mmol) and water (450 ml) was boiled under reflux for 16 hours. The cooled solution was saturated with carbon dioxide using dry ice and then filtered to remove precipitated barium carbonate. The filtrate was evaporated *in vacuo* until a precipitate began to form. Chloroform was added and the resulting mixture was stirred vigorously overnight at room temperature before filtering to remove the precipitate, which was

identified as isatinecic acid **104**,  $\delta_{\text{H}}(\text{D}_2\text{O})$  0.94(3H, d,  $J_{3,8}$  6.0, 8-CH<sub>3</sub>), 1.74(3H, d,  $J_{9,10}$  6.8, 10-CH<sub>3</sub>), 2.05(2H, m, 4-CH<sub>2</sub>), 2.31(1H, m, 3-CH), 3.75 and 3.86(2H, d and d,  $J_{7,7}$  11.6 and  $J_{7,7}$  11.6, 7-CH<sub>2</sub>), and 5.43(1H, q,  $J_{9,10}$  6.8, 9-CH);  $\delta_{\text{C}}(\text{D}_2\text{O})$  13.00(C-8), 15.42(C-10), 37.38(C-3), 38.00(C-4), 67.17(C-7), 83.38(C-2), 124.49(C-9), 139.97(C-5), 180.54(C-6) and 181.16(C-1).

The organic and aqueous layers of the filtrate were separated. The organic layer was dried with anhydrous magnesium sulphate and the solvent evaporated to yield a brown gum, which contained a small amount retronecine **31**. The aqueous layer was evaporated to dryness *in vacuo* and was stirred with additional chloroform, at room temperature for six days, after which the chloroform was removed, dried with magnesium sulphate and the solvent evaporated *in vacuo* to yield retronecine **31** as cream crystals (3.52 g, 37.7%); m.p. 119-121°C (lit.,<sup>202</sup> 120-121°C);  $\delta_{\text{H}}$  1.99(2H, m, 6-CH<sub>2</sub>), 2.78 and 3.32(2H, m and t,  $J$  7.2, 5-CH<sub>2</sub>), 3.46 and 3.93(2H, dd and dd,  $J$  5.4, 15.5 and  $J$  2.0, 15.6, 3-CH<sub>2</sub>), 4.17 and 4.38(2H, d and d,  $J_{9,9}$  11.6 and  $J_{9,9}$  12.0, 9-CH<sub>2</sub>), 4.27(1H, s, 8-CH), 4.34(1H, t,  $J$  3.0, 7-CH) and 5.74(1H, s, 2-CH);  $\delta_{\text{C}}$  35.43(C-6), 54.09(C-5), 59.10(C-3), 61.93(C-9), 71.19(C-7), 79.47(C-8), 127.46(C-2) and 137.32(C-1).

*1-Chloromethyl-1,2-didehydro-7 $\beta$ -hydroxy-8 $\alpha$ -pyrrolizidine hydrochloride 105.*<sup>345</sup>

Retronecine **31** (0.186 g, 1.20 mmol) was added slowly, under dry nitrogen, to ice-cold, stirred thionyl chloride (0.28 mL, 3.9 mmol). The mixture was stirred at 0°C for 30 minutes before the excess thionyl chloride was removed under vacuum without heating. The black, gummy residue was dissolved in absolute ethanol, decolourised with activated carbon and filtered. Diethyl ether was added to give 1-chloromethyl-1,2-didehydro-7 $\beta$ -hydroxy-8 $\alpha$ -pyrrolizidine hydrochloride **105** as crystals (0.161 g, 63.9%); m.p. 150-153°C (from absolute ethanol and diethyl ether) (lit.,<sup>345</sup> 152-153°C);  $\delta_{\text{H}}(\text{D}_2\text{O})$  2.32(2H, m, 6-CH<sub>2</sub>), 3.43 and 4.06(2H, m and m, 5-CH<sub>2</sub>), 4.06 and 4.59(2H, m and d,  $J$  16.0, 3-CH<sub>2</sub>), 4.38 and 4.50(2H, d and d,  $J_{9,9}$  13.1 and  $J_{9,9}$  13.0, 9-CH<sub>2</sub>), 4.84(1H, s, 7-CH), 5.16 (1H, s, 8-CH) and 6.08(1H, s, 2-CH);  $\delta_{\text{C}}(\text{D}_2\text{O})$  36.19(C-6), 40.21(C-9), 55.19(C-5), 62.40(C-3), 70.19(C-7), 79.25(C-8), 125.74(C-2) and 134.37(C-1).

*7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine 58 and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine 106.*<sup>346</sup>-

A mixture of 1-chloromethyl-1,2-didehydro-7 $\beta$ -hydroxy-8 $\alpha$ -pyrrolizidine hydrochloride **105** (230 mg, 1.09 mmol), 1M-sulphuric acid (7 mL) and zinc dust was stirred for 2 hours. The solution was filtered to remove excess zinc, basified to pH 10 with dilute sodium hydroxide and extracted with chloroform (5x20 mL). The extracts were dried with anhydrous magnesium sulphate and evaporated to give a 5:2 mixture of 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** [ $\delta_{\text{H}}$  1.93 and 2.04(2H, m and dd,  $J$  6.4 and 13.2, 6-CH<sub>2</sub>), 2.53(2H, t,  $J$  6.7, 2-CH<sub>2</sub>), 2.65 and 3.11(2H, m and m, 3-CH<sub>2</sub>), 2.79 and 3.11(2H, m and m, 5-CH<sub>2</sub>), 3.92(1H, s, 8-CH), 4.15(1H, t,  $J$  3.8, 7-CH), and 4.88 and 5.18(2H, s and s, 9-CH<sub>2</sub>);  $\delta_{\text{C}}$  34.72(C-2), 35.58(C-6), 52.85(C-5), 54.98(C-3), 72.26(C-7), 73.79(C-8), 107.15(C-9) and 149.08(C-1)] and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106** [ $\delta_{\text{H}}$  1.75(3H, s, 9-CH<sub>3</sub>), 1.85-1.93(2H, m, 6-CH<sub>2</sub>), 2.65 and 3.20(2H, m and m, 5-CH<sub>2</sub>), 3.26 and 3.82(2H, m and d,  $J$  8.0, 3-CH<sub>2</sub>), 4.02(1H, s, 8-CH), 4.18 (1H, t,  $J$  3.1, 7-CH) and 5.46(1H, s, 2-CH);  $\delta_{\text{C}}$  14.16(C-9), 36.53(C-6), 53.95(C-5), 63.15(C-3), 70.68(C-7), 80.25(C-8), 124.71(C-2) and 134.14(C-1)].

*$\alpha,\beta$ -Dibromo- $\alpha$ -methylbutyric acid (2,3-dibromo-2-methylbutanoic acid) 107.*<sup>349</sup>-

Bromine (12.9 mL, 0.250 mol) was added dropwise to an ice-cold, stirred solution of tiglic acid **92** (25.0 g, 0.250 mol) in dry carbon tetrachloride (50 mL). After completion of the addition the reaction mixture was allowed to warm to room temperature, left to stand overnight and then boiled under reflux until the solution was light orange in colour. The solvent was removed *in vacuo* to give crystals of  $\alpha,\beta$ -dibromo- $\alpha$ -methylbutyric acid **107** (55.24 g, 85.0%); m.p. 81-83°C (from hexane) (lit.,<sup>349</sup> 82-88°C);  $\delta_{\text{H}}$  1.91(3H, d,  $J_{3,4}$  6.8, 4-CH<sub>3</sub>), 1.99(3H, s, 5-CH<sub>3</sub>), 4.84(1H, q,  $J_{3,4}$  6.8, 3-CH) and 9.86(1H, br s, OH);  $\delta_{\text{C}}$  20.93(C-4), 21.12(C-5), 51.06(C-3), 61.45(C-2) and 174.89(C-1).

*$\beta$ -Bromoangelic acid [(E)-3-bromo-2-methyl-2-butenoic acid] 108.*<sup>349</sup>-

To a stirred solution of  $\alpha,\beta$ -dibromo- $\alpha$ -methylbutyric acid **107** (40.0 g, 0.154 mol) in methanol (25 mL) was added, dropwise, a 25% methanolic potassium hydroxide solution (226 mL). Potassium carbonate (4.0 g) was added to prevent decarboxylation and the

reaction mixture was heated for 2 hours at *ca.* 55°C. Excess potassium hydroxide was removed by saturating the hot mixture with carbon dioxide, using dry ice and filtering while warm. The filter cake was washed with warm methanol and the combined methanol fractions were evaporated to dryness. Water (30 mL) was added to the residue which was then acidified to pH 3 with 3M-hydrochloric acid to precipitate  $\beta$ -bromoangelic acid **108** (8.35g, 30.4%); m.p. 90-93°C (from hexane) (lit.,<sup>349</sup> 92-94.5°C);  $\delta_{\text{H}}$  2.11(3H, d,  $J_{4,5}$  1.5, 5-CH<sub>3</sub>), 2.75(3H, d,  $J_{4,5}$  1.5, 4-CH<sub>3</sub>) and 8.53(1H, br s, OH);  $\delta_{\text{C}}$  20.94(C-5), 28.53(C-4), 127.39(C-2), 140.41(C-3) and 171.42(C-1).

*Angelic acid [(Z)-2-methyl-2-butenoic acid] 91.*<sup>349</sup>-

$\beta$ -Bromoangelic acid **108** (1.00 g, 5.59 mmol) was stirred in water (9 mL) at 5°C and small pieces of a 9% sodium-mercury amalgam were added. The resulting reaction mixture was stirred slowly at room temperature for 64 hours. The solution was separated from the mercury, which was rinsed with water (10 mL). The combined aqueous fractions were acidified to pH 3 with concentrated hydrochloric acid. Angelic acid **91** precipitated on cooling (0.337 g, 60.3%); m.p. 42-44°C (from hexane) (lit.,<sup>349</sup> 44-46°C);  $\delta_{\text{H}}$  1.91(3H, t,  $J$  1.5, 5-CH<sub>3</sub>), 2.03(3H, dd,  $J$  1.5 and 6.6, 4-CH<sub>3</sub>) and 6.22(1H, dq,  $J$  1.4 and 7.3, 3-CH);  $\delta_{\text{C}}$  16.00(C-4), 20.31(C-5), 127.23(C-2), 141.03(C-3) and 173.72(C-1).

*9% Sodium-mercury amalgam.*<sup>361</sup>-

Sodium (6.0 g, 0.26 mol), cut into small pieces, was placed in a 250 mL round bottom flask fitted with a dry nitrogen inlet, a nitrogen outlet and a dropping funnel. The dropping funnel was charged with mercury (60 g, 0.30 mol). About one third of the mercury was added to the sodium and the flask was warmed cautiously with a bunsen flame until the reaction started. The remainder of the mercury was added slowly with shaking and heating. The molten amalgam was poured into a mortar where it solidified immediately. The amalgam was crushed, in the mortar, under a stream of dry nitrogen.

*7β-angelyl-1-methylene-8α-pyrrolizidine 71 and 7β-angelyl-1,2-didehydro-1-methyl-8α-pyrrolizidine 111.-*

A solution of 7β-hydroxy-1-methylene-8α-pyrrolizidine **58** and 1,2-didehydro-7β-hydroxy-1-methyl-8α-pyrrolizidine **106** (0.345 g, 5:2 mixture, 2.48 mmol) in dry tetrahydrofuran (10 mL) was added, *via* cannula, to a flame-dried, 4-necked flask flushed with nitrogen. The mixture was cooled to 0°C and 1.6 M-butyllithium in hexane (1.6 mL, 2.48 mmol BuLi) was added dropwise. The mixture was stirred at 0°C for 30 minutes before angelyl chloride **109** (0.586 g, 4.94 mmol) was added. The reaction mixture was then allowed to warm to room temperature and was stirred overnight. The pH was adjusted to 9 with aqueous sodium bicarbonate and the mixture was extracted with chloroform (5x15 mL). The combined extracts were dried with anhydrous magnesium sulphate and the solvent evaporated *in vacuo* to give a brown oil. Preparative layer chromatography on silica gel with chloroform-methanol-25% ammonia (85:14:1) as eluent yielded a 5:2 mixture (145 mg, 26.4%) of 7β-angelyl-1-methylene-8α-pyrrolizidine **71** [ $\delta_{\text{H}}$  1.81(3H, s, 15-CH<sub>3</sub>), 1.95(3H, d, *J* 6.8, 14-CH<sub>3</sub>), 2.09 and 2.15(2H, m and m, 6-CH<sub>2</sub>), 2.53(2H, br s, 2-CH<sub>2</sub>), 2.78 and 3.13(2H, m and m, 3-CH<sub>2</sub>), 2.81 and 3.26(2H, m and t, *J* 8.0, 5-CH<sub>2</sub>), 4.08(1H, s, 8-CH), 4.85 and 5.02(2H, s and s, 9-CH<sub>2</sub>), 5.50(1H, t, *J* 4.2, 7-CH) and 6.04(1H, m, 13-CH);  $\delta_{\text{C}}$  15.64(C-14), 20.53(C-15), 33.89(C-2), 34.33(C-6), 52.81(C-5), 54.43(C-3), 71.96(C-8), 74.88(C-7), 107.83(C-9), 127.80(C-12), 137.98(C-13), 146.87(C-1) and 166.83(C-11); EI *m/z* 221(M<sup>+</sup>, 1%), 139(8), 138(84), 122(15), 121(89), 120(23), 96(59), 95(100), 94(22), 83(8), 82(8), 80(14), 67(13) and 55(16)] and 7β-angelyl-1,2-didehydro-1-methyl-8α-pyrrolizidine **111** [ $\delta_{\text{H}}$  1.67(3H, s, 9-CH<sub>3</sub>), 1.81(3H, s, 15-CH<sub>3</sub>), 1.95(3H, d, *J* 6.8, 14-CH<sub>3</sub>), 2.09(2H, m, 6-CH<sub>2</sub>), 2.66 and 3.35(2H, m and m, 5-CH<sub>2</sub>), 3.32 and 3.89(2H, m and d, *J* 14.0, 3-CH<sub>2</sub>), 4.20(1H, s, 8-CH), 5.36(1H, s, 2-CH), 5.43(1H, t, *J* 3.6, 7-CH) and 6.06(1H, m, 13-CH);  $\delta_{\text{C}}$  13.96(C-9), 15.60(C-14), 20.45(C-15), 34.60(C-6), 54.19(C-5), 62.83(C-3), 73.32(C-7), 78.64(C-8), 123.02(C-2), 134.36 and 136.97(C-1 and C-12), 138.45(C-13) and 166.97(C-11); EI *m/z* 139(4%), 138(49), 122(8), 121(57), 120(15), 96(35), 95(100), 94(17), 83(5), 82(6), 80(7), 67(9) and 55(15)] together with traces of corresponding tiglyl isomers.

Attempted preparations

Method 1: CDI coupling.<sup>202</sup>-

Angelic acid **91** (72 mg, 0.72 mmol) and *N,N'*-carbonyldiimidazole (124 mg, 0.763 mmol) were stirred together in dry, ethanol-free chloroform (2 mL) under a dry nitrogen atmosphere for 1 hour. A 5:2 mixture of 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106** (100 mg, 0.718 mmol) was added in dry, ethanol-free chloroform (2 mL) and the reaction was left to stir at room temperature for 24 hours. Analytical TLC showed that no reaction had taken place so reaction was stirred at room temperature for another 3 days after which time TLC showed that a product had begun to form. The mixture was heated at 35°C for 1.5 hours and then under reflux for 2 hours. The reaction mixture was diluted with chloroform (5 mL) and extracted with 2M-sulphuric acid (5x5 mL). The combined acid extractions were washed with chloroform (3x5 mL), basified to pH 10 with 25% ammonium hydroxide and extracted with chloroform (5x10 mL). The combined chloroform layers were dried with anhydrous magnesium sulphate and the solvent was evaporated. None of the desired product was found to be present in the residue (as determined by NMR analysis).

Method 2: DCC-DMAP coupling.-

Two solutions were prepared in flame dried, 3 mL, stoppered test tubes:-

- a) 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106** (5 mg, 5:2 mixture, 35  $\mu$ mol) and 4-dimethylaminopyridine (DMAP) (15mg) in deuterated dichloromethane (0.7 mL); and
- b) angelic acid **91** (14 mg, 140  $\mu$ mol) and dicyclohexylcarbodiimide (DCC) (70 mg) dissolved in deuterated dichloromethane (0.3 mL) by shaking and warming.

When the bubbling in (b) had stopped, the DMAP/alcohol solution (a) was added to the angelic acid/DCC solution (b) and the mixture shaken well. The mixture was left to stand with occasional shaking for 0.5 hour. NMR analysis of the mixture showed that no coupling had taken place; the reaction mixture was then left to stir overnight, but still no coupling resulted.

*Angelyl chloride 109.*<sup>354</sup>-

A suspension of potassium angelate **110** (0.872 g, 6.31 mmol) was stirred in dry diethyl ether (20 mL) at 0°C under a dry nitrogen atmosphere. Oxalyl chloride (3.5 mL) and a drop of dry dimethylformamide were added and the stirring was continued at 0°C for 2 hours. The excess oxalyl chloride was removed under a slight vacuum at room temperature. Dry carbon tetrachloride was added to aid the removal of oxalyl chloride. Angelyl chloride **109** (0.586 g, 78.3%) was obtained as a pale yellow oil, which was used immediately without further purification.

*Potassium angelate 110.*<sup>354</sup>-

Angelic acid **91** in ethanol was neutralised by the dropwise addition of an equimolar quantity potassium hydroxide in ethanol. The solvent was removed *in vacuo* without heating to afford potassium angelate **110** as a white crystalline product.

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## 7. APPENDIX

### 7.1 TWO DIMENSIONAL NMR SPECTRA NOT INCLUDED IN THE TEXT

**Figure 7.1** COSY spectrum of 7-angelylhastanecine **66**.

**Figure 7.2** COSY spectrum of 9-angelylhastanecine **67**.

**Figure 7.3** COSY spectrum of 7-angelylplatynecine **38**.

**Figure 7.4** COSY spectrum of 9-angelylplatynecine-4-oxide **68**.

**Figure 7.5** COSY spectrum of sarracine **36**.

**Figure 7.6** COSY spectrum of neosarracine **72**.

**Figure 7.7** HMQC spectrum of 7-angelylhastanecine **66**.

**Figure 7.8** HMQC spectrum of 9-angelylhastanecine **67**.

**Figure 7.9** HMQC spectrum of 7-angelylplatynecine **38**.

**Figure 7.10** HMQC spectrum of 9-angelylplatynecine-4-oxide **68**.

**Figure 7.11** HMQC spectrum of sarracine **36**.

**Figure 7.12** HMQC spectrum of neosarracine **72**.

**Figure 7.13** COSY spectrum of 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69**.

**Figure 7.14** COSY spectrum of 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide **70**.

**Figure 7.15** HMQC spectrum of 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine **69**.

**Figure 7.16** HMQC spectrum of 7 $\alpha$ -angelyl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide **70**.

**Figure 7.17** COSY spectrum of the mixture of 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106**.

**Figure 7.18** HMQC spectrum of the mixture of 7 $\beta$ -hydroxy-1-methylene-8 $\alpha$ -pyrrolizidine **58** and 1,2-didehydro-7 $\beta$ -hydroxy-1-methyl-8 $\alpha$ -pyrrolizidine **106**.

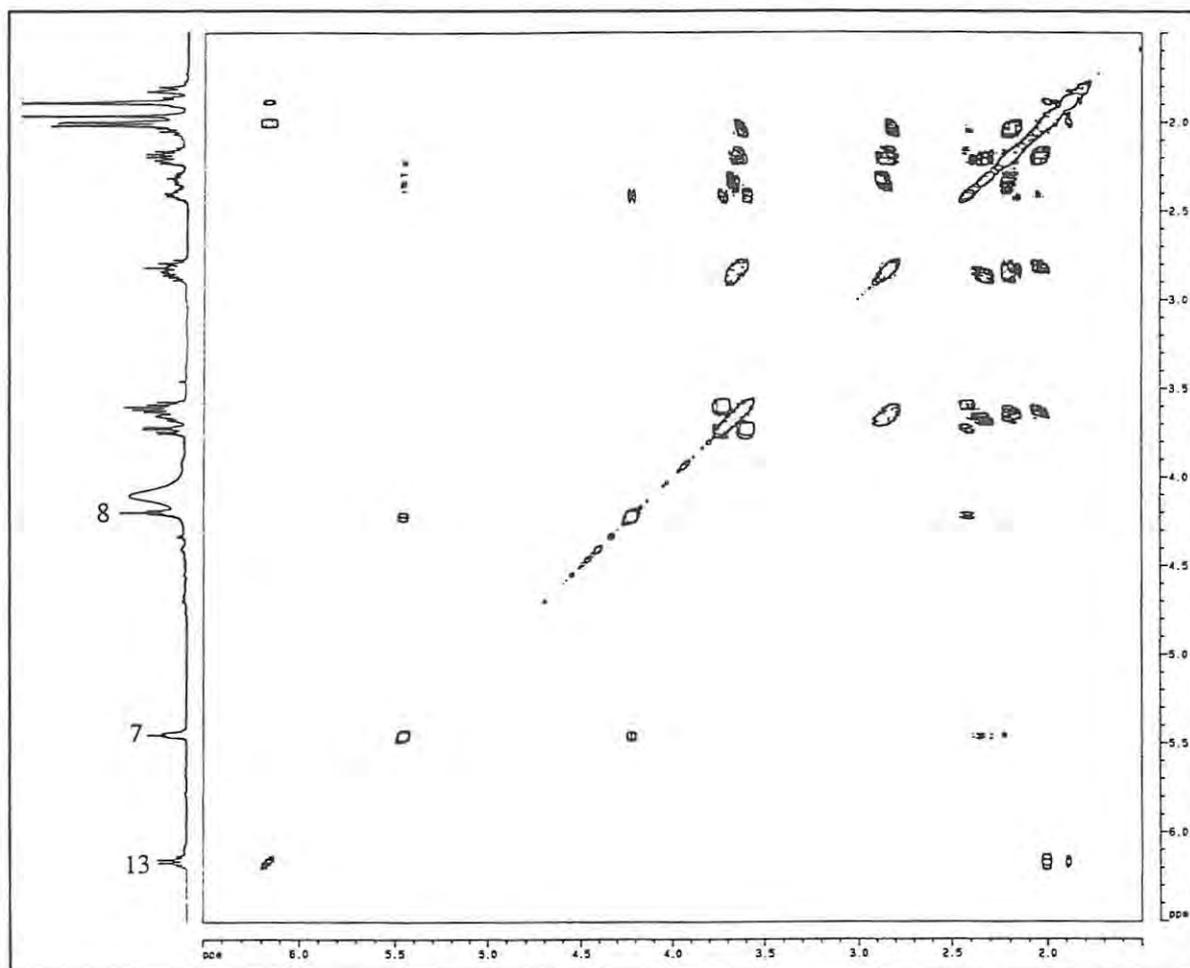
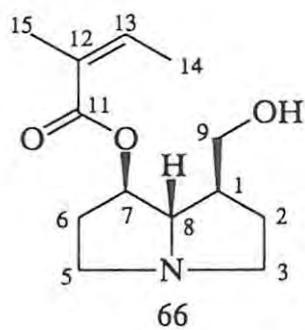


Figure 7.1: COSY spectrum of 7-angelylhastanecine 66.

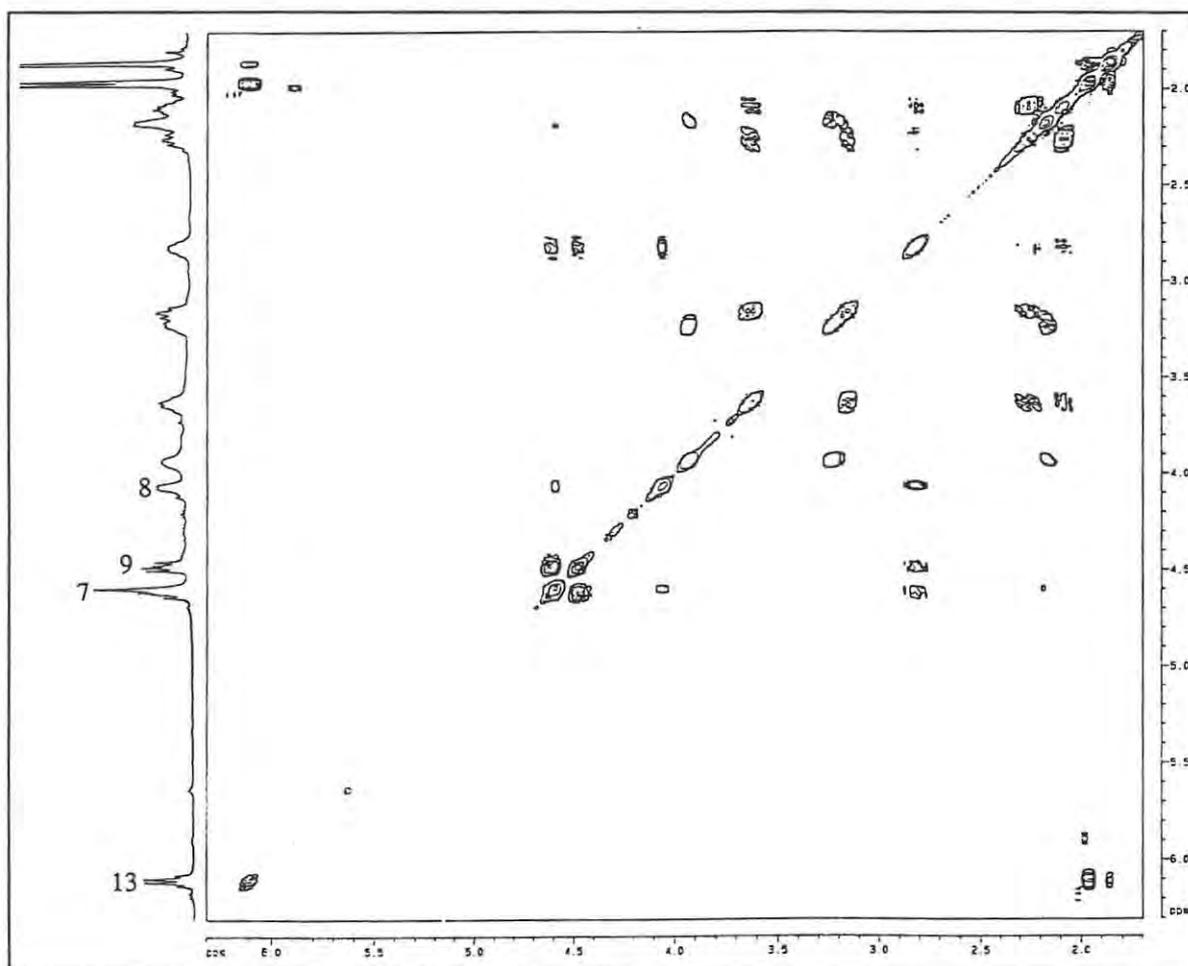
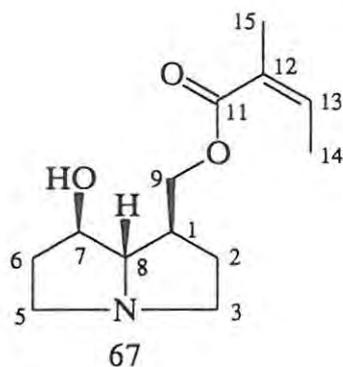


Figure 7.2: COSY spectrum of 9-angelylhastanecine 67.

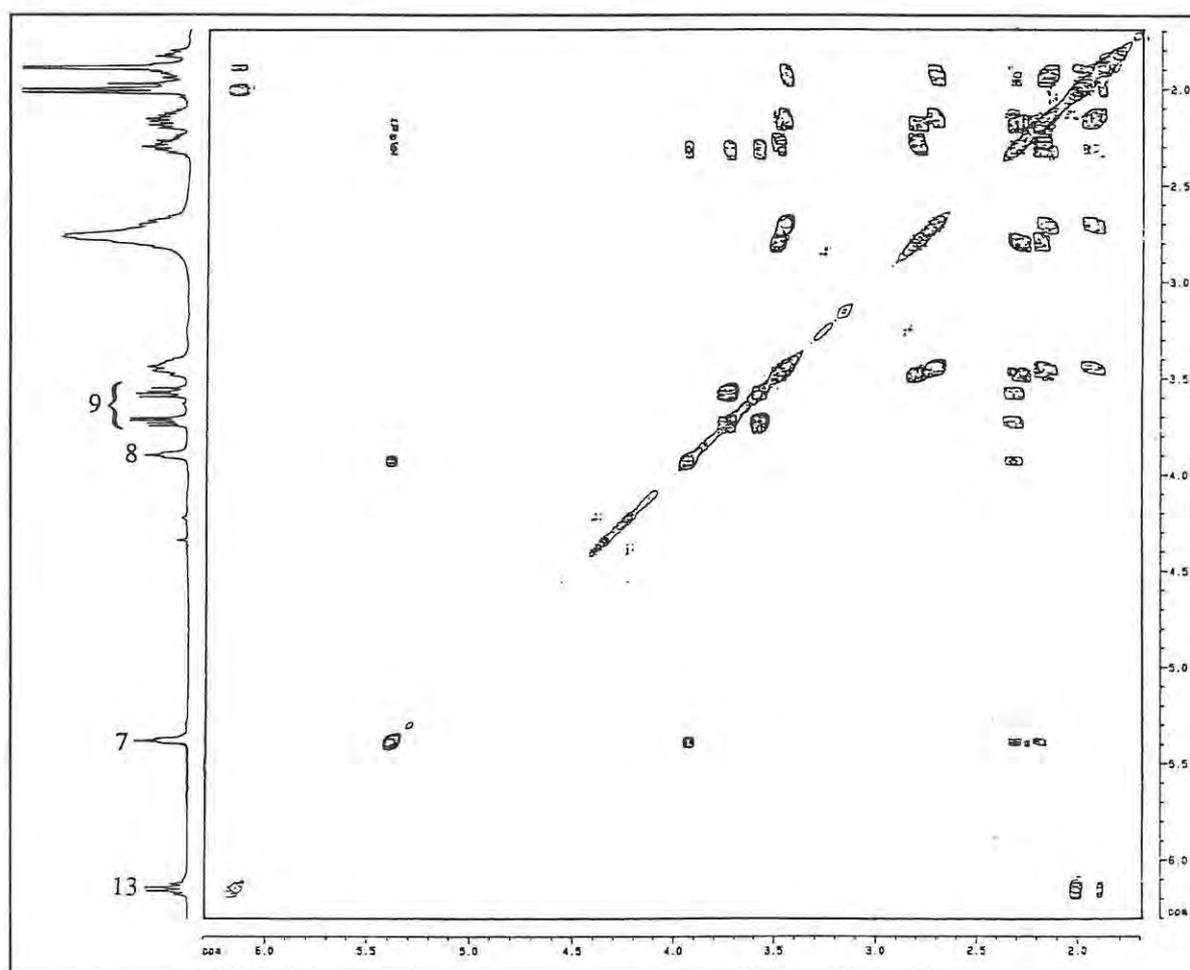
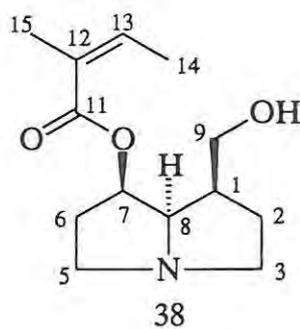


Figure 7.3: COSY spectrum of 7-angelylplatynecine 38.

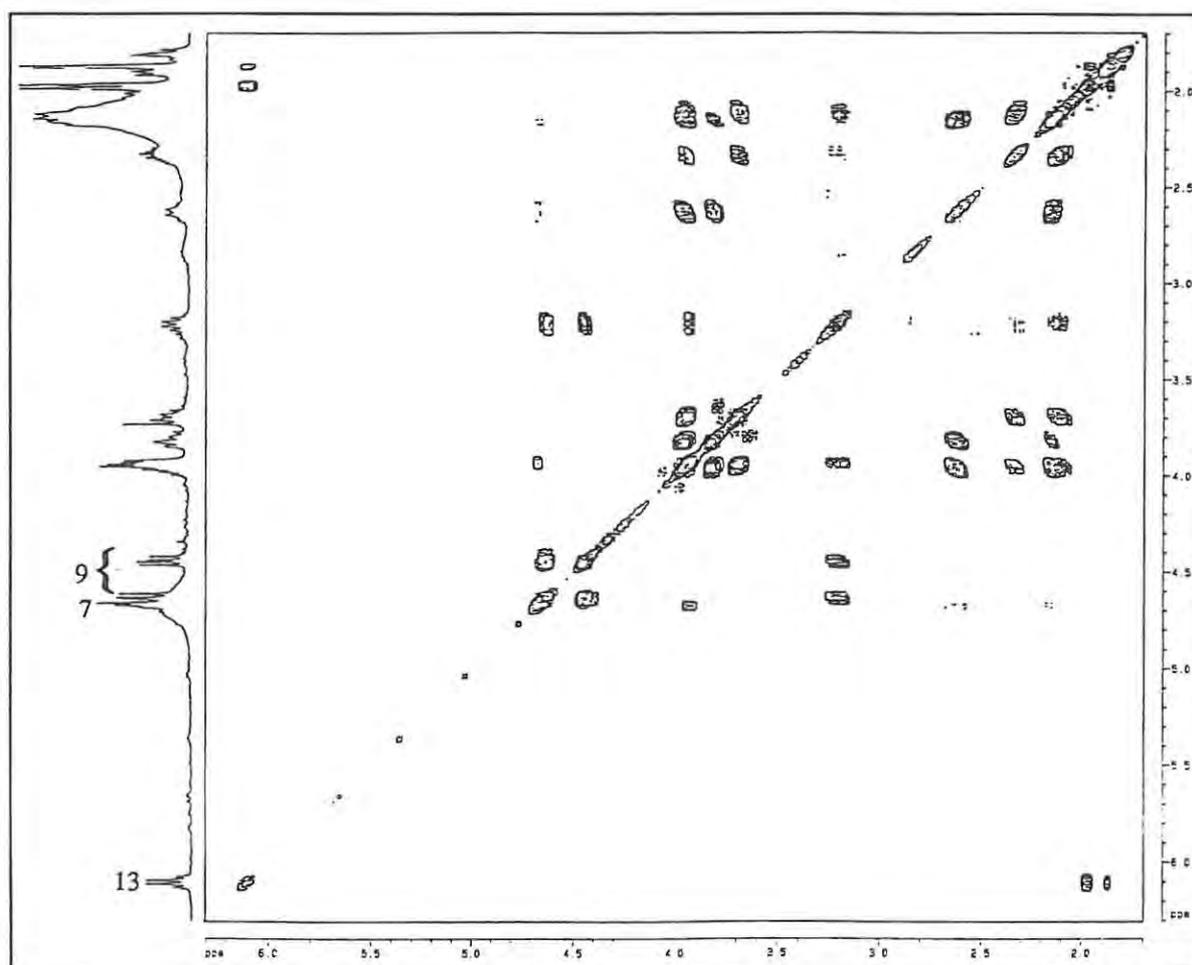
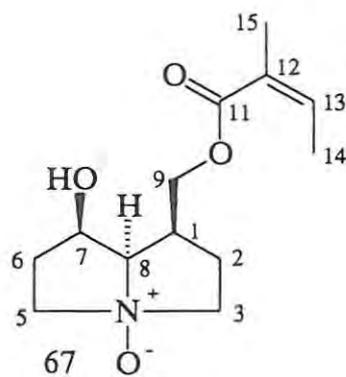


Figure 7.4: COSY spectrum of 9-angelylplatynecine-4-oxide **68**.

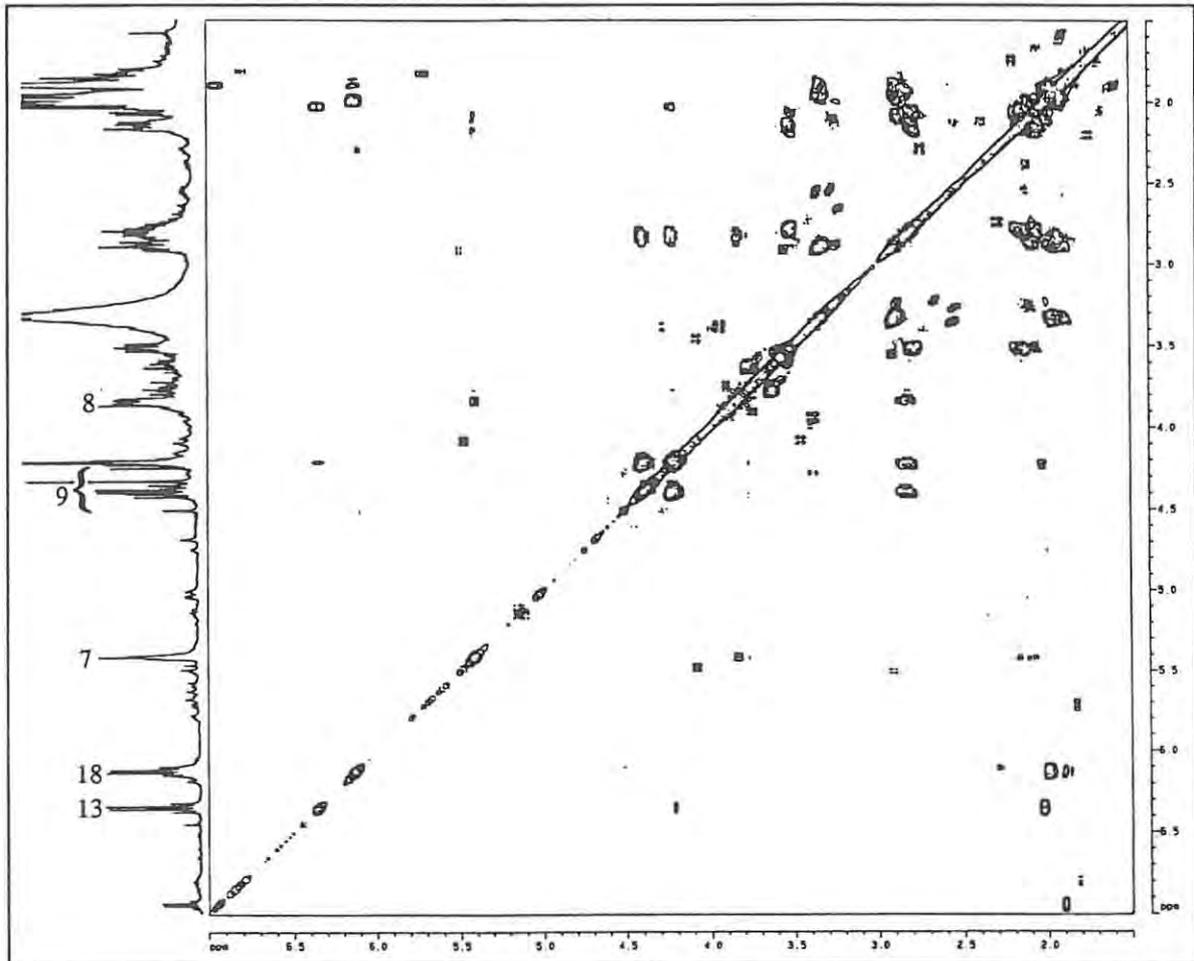
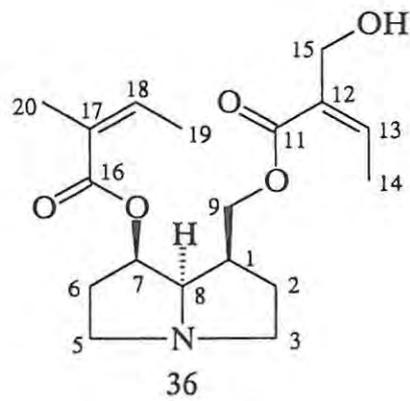


Figure 7.5: COSY spectrum of sarracine 36.

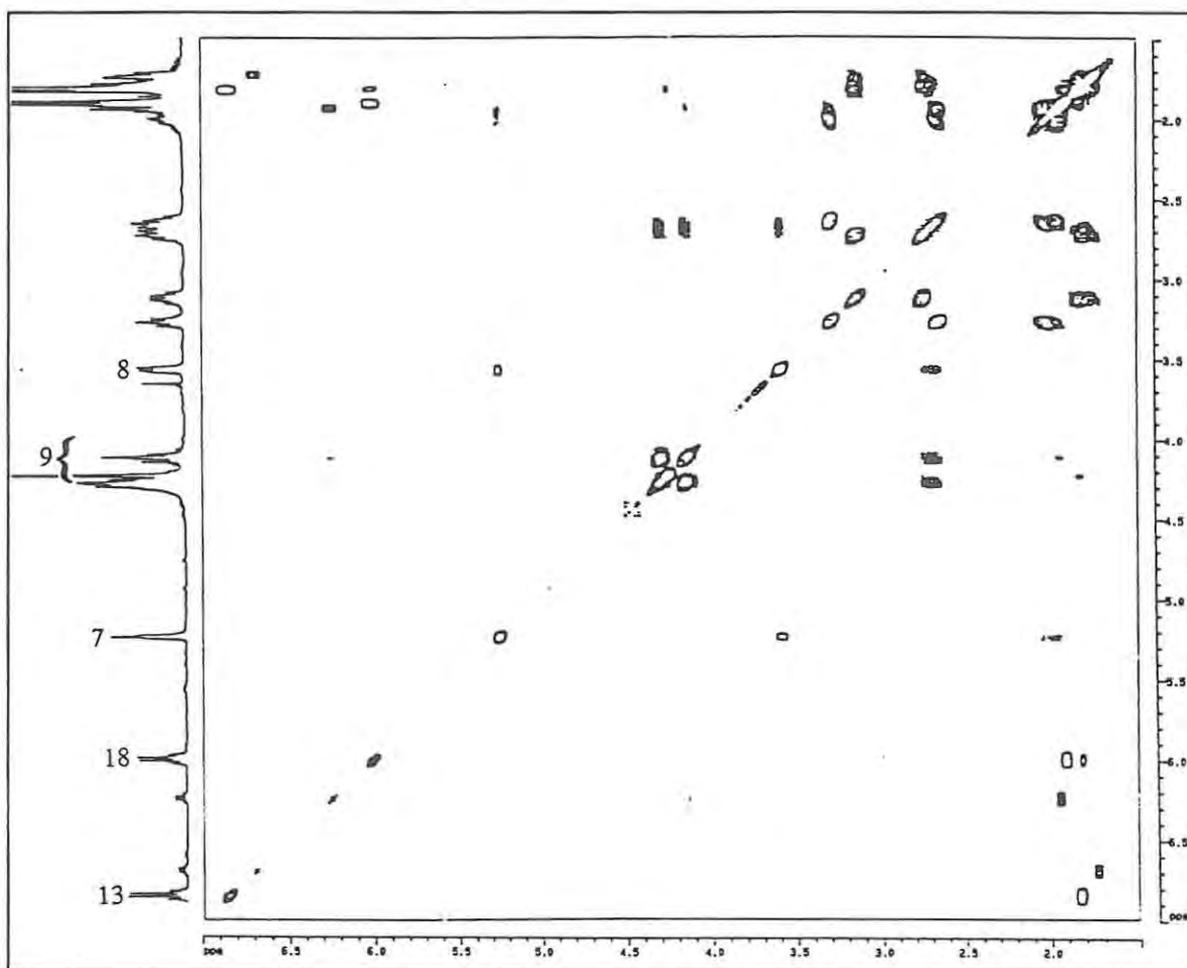
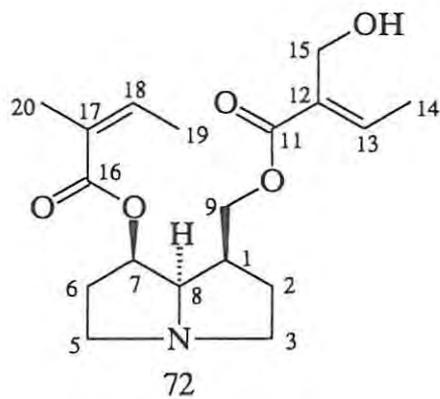


Figure 7.6: COSY spectrum of neosarracine 72.

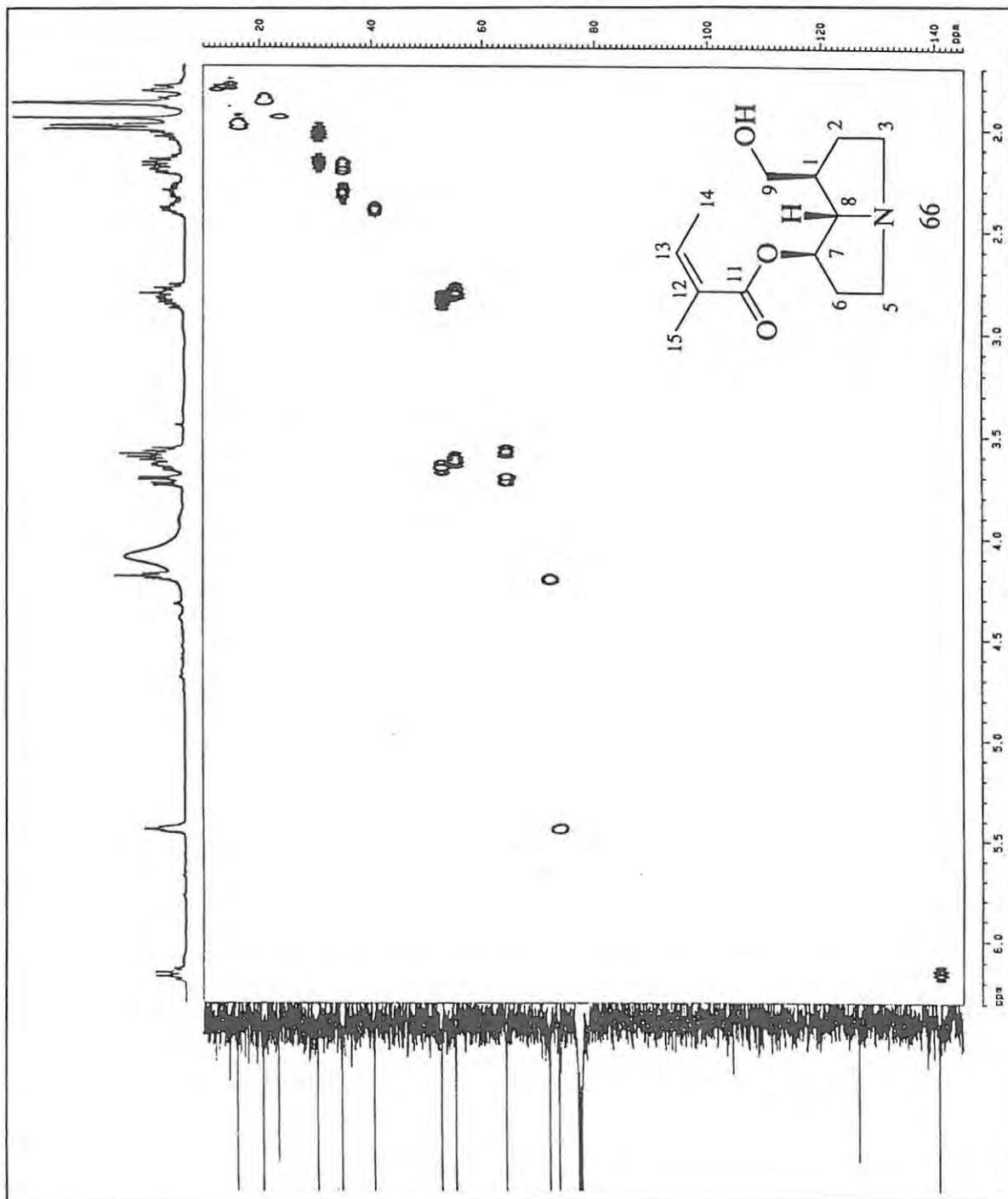


Figure 7.7: HMQC spectrum of 7-angelylhastanecine **66**.

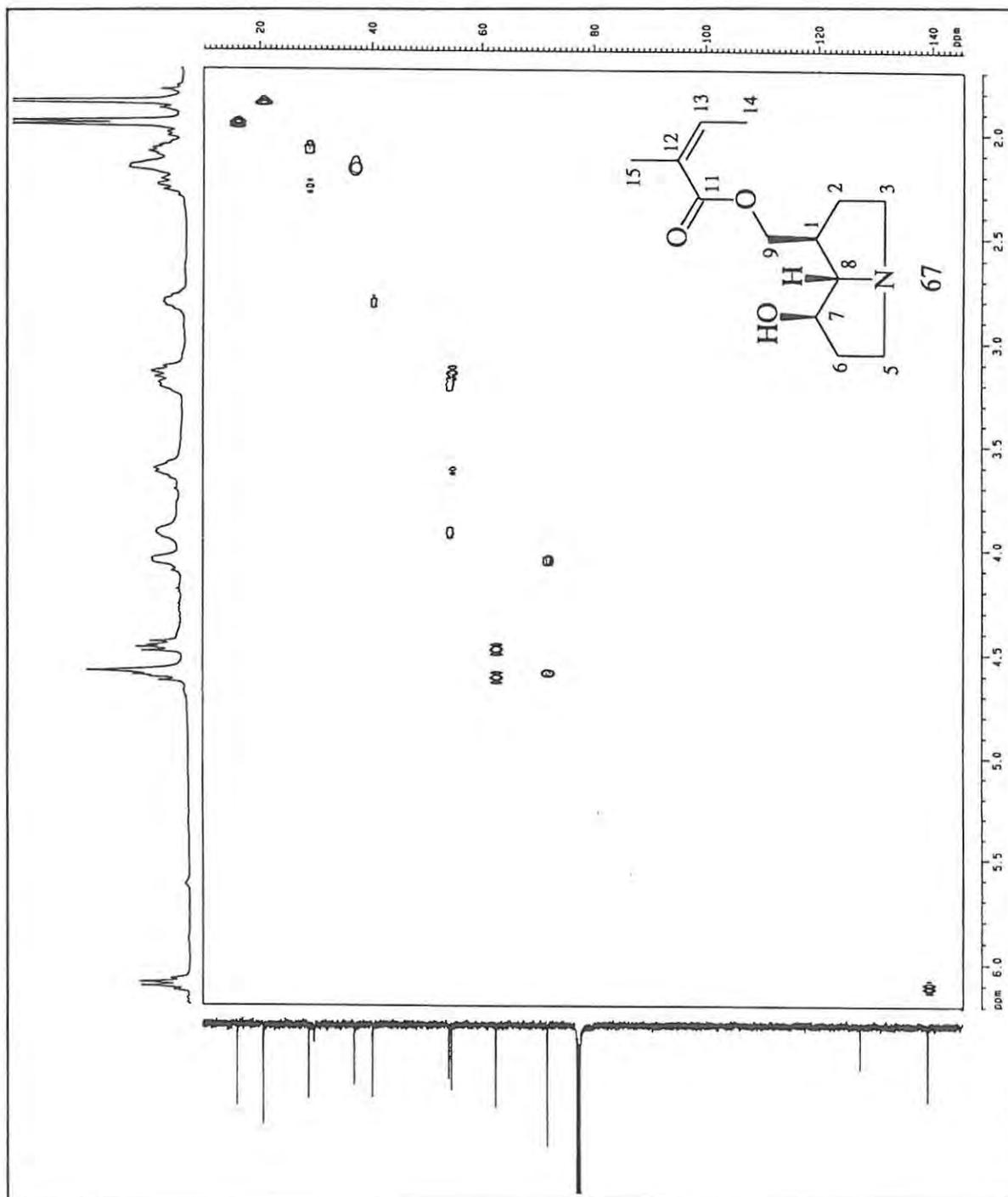


Figure 7.8: HMQC spectrum of 9-angelylhastanecine 67.

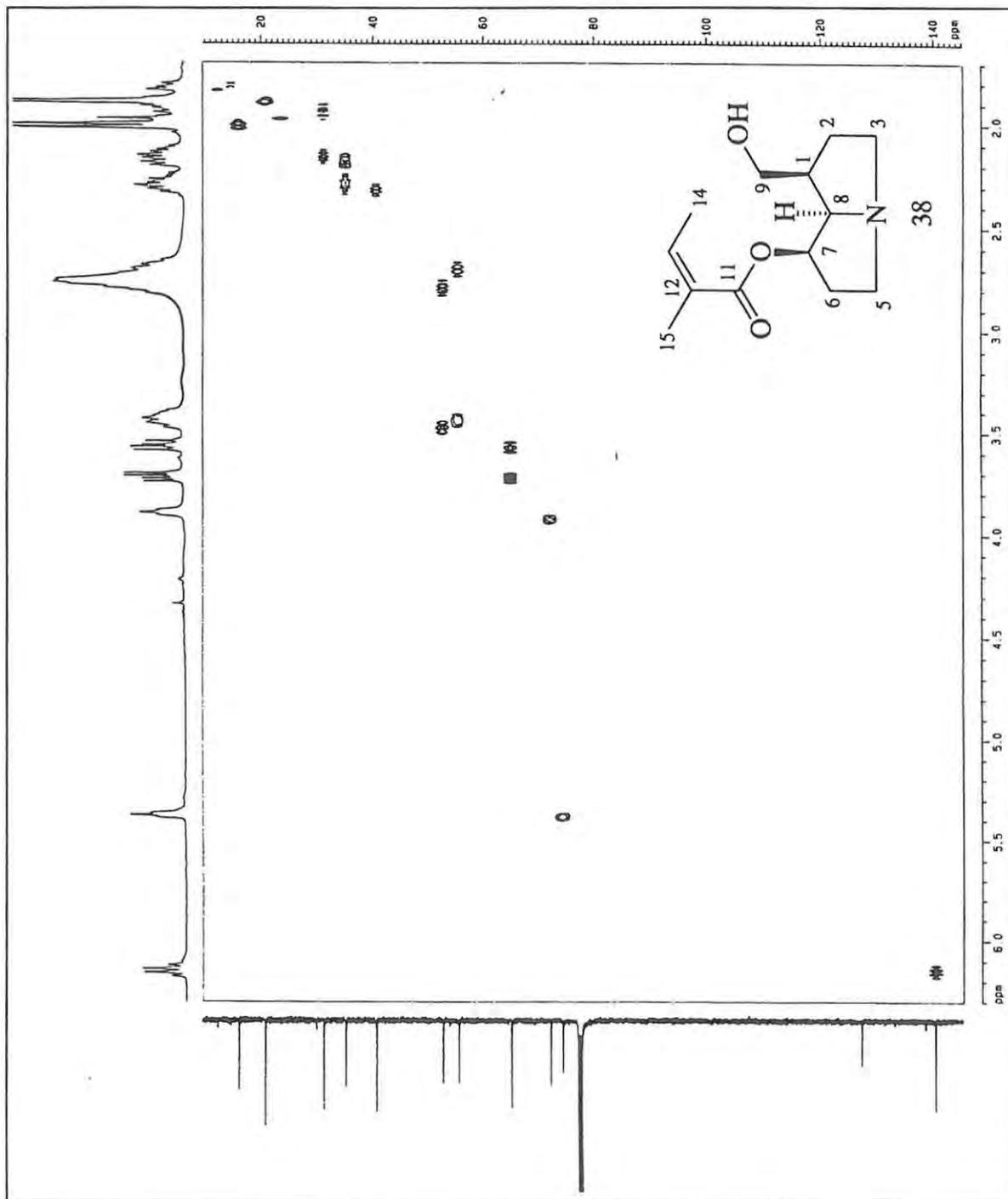


Figure 7.9: HMQC spectrum of 7-angelylplatynecine 38.

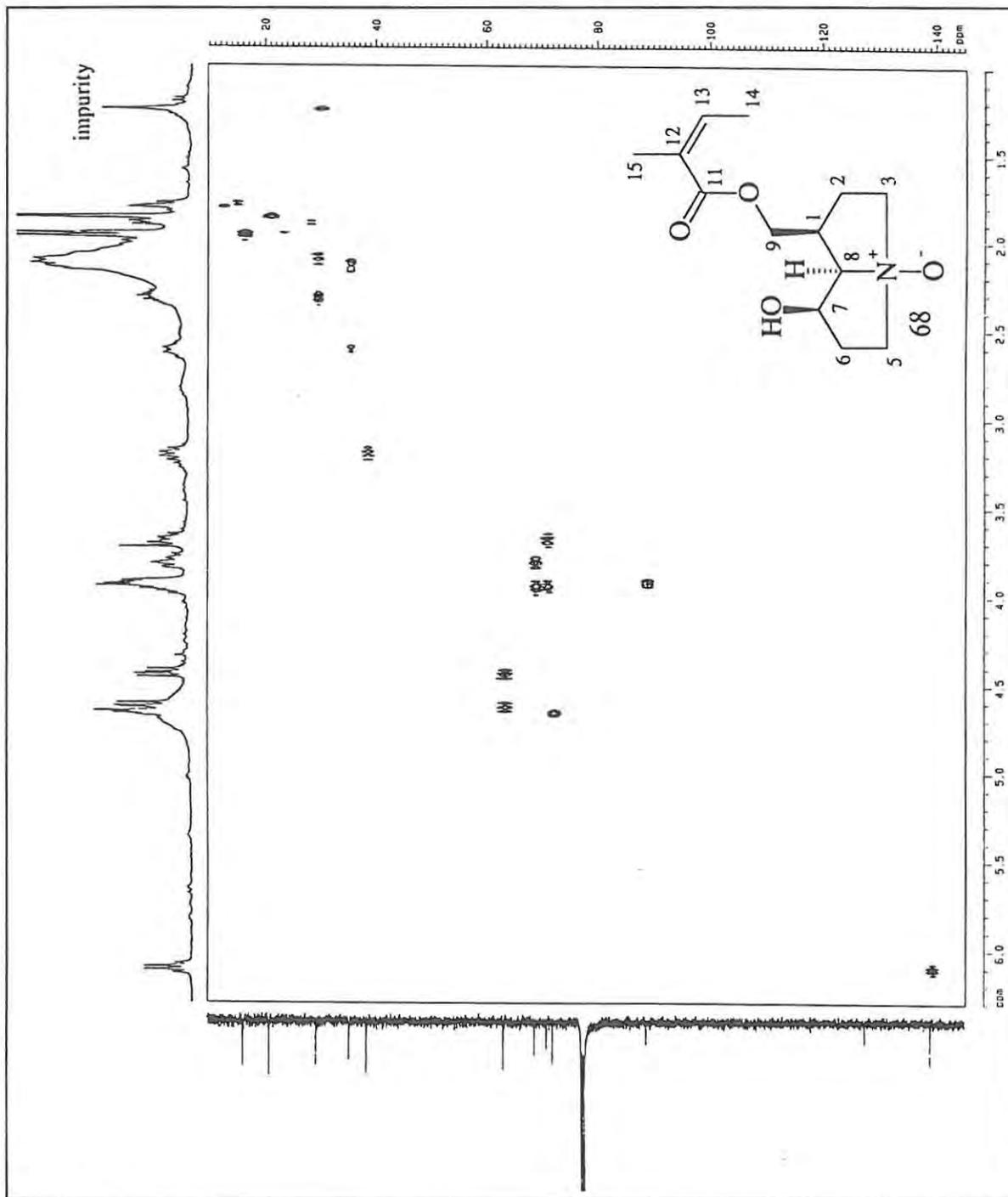


Figure 7.10: HMQC spectrum of 9-angelylplatynecine-4-oxide 68.

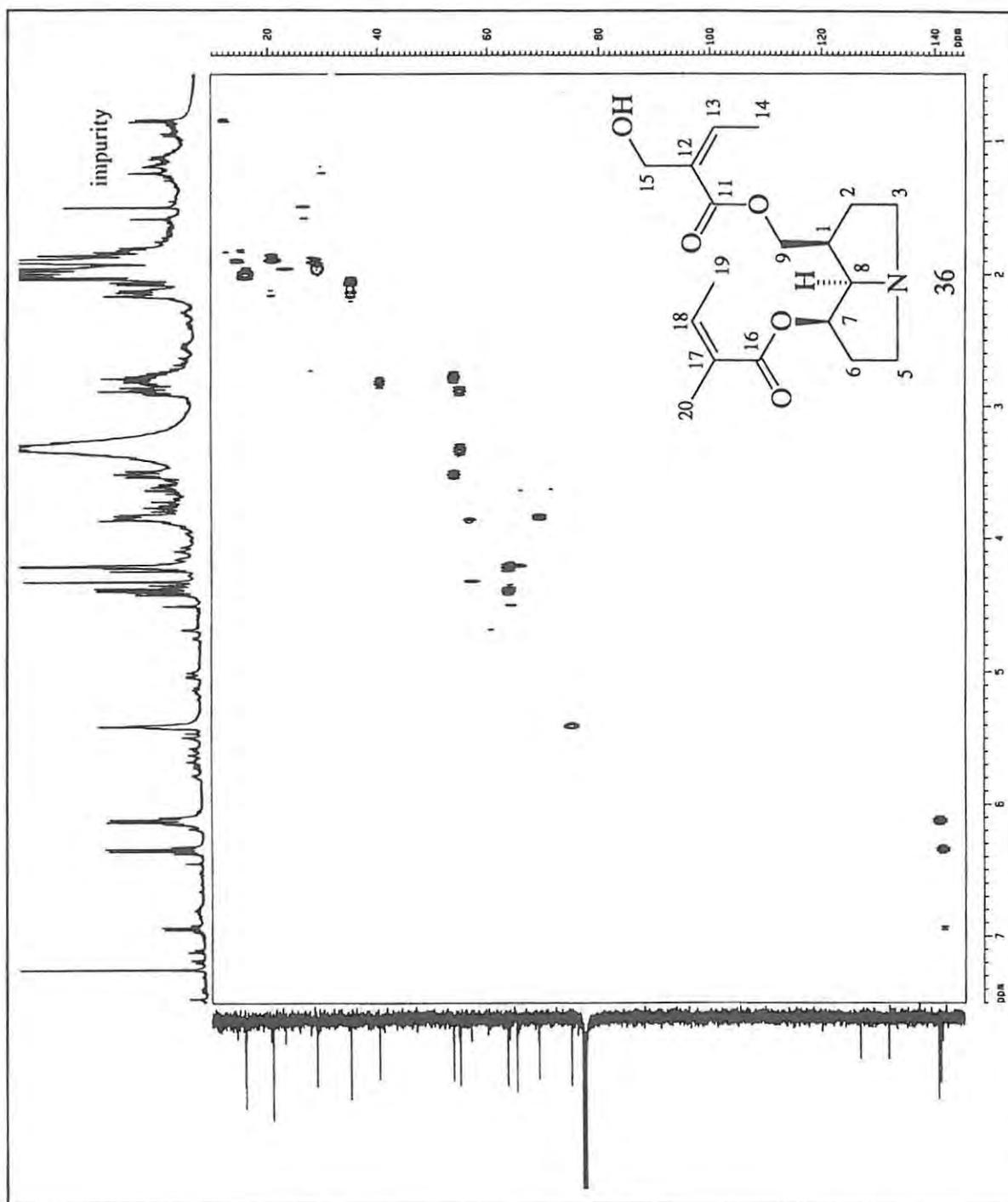


Figure 7.11: HMQC spectrum of sarracine 36.

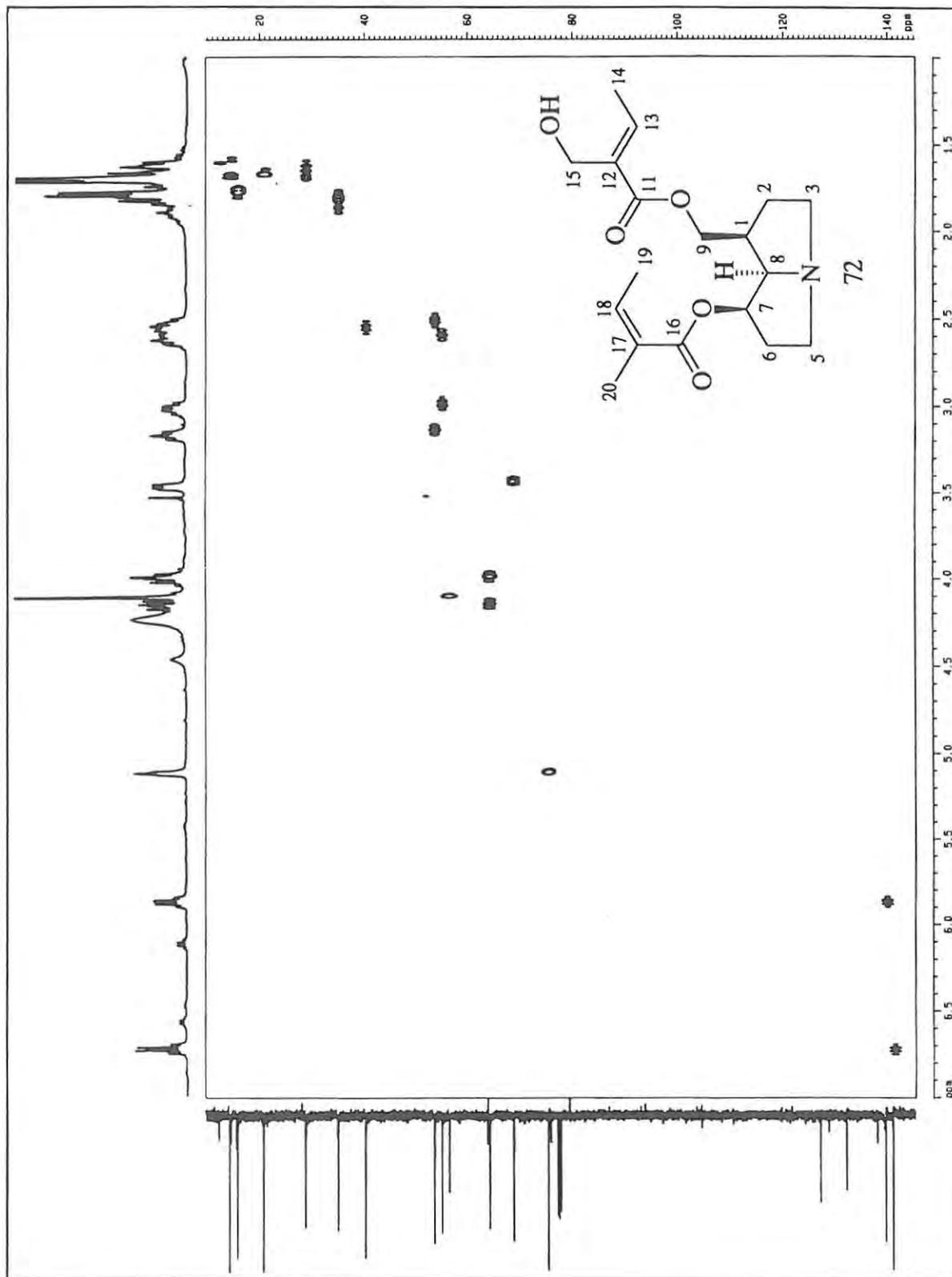


Figure 7.12: HMQC spectrum of neosarracine 72.

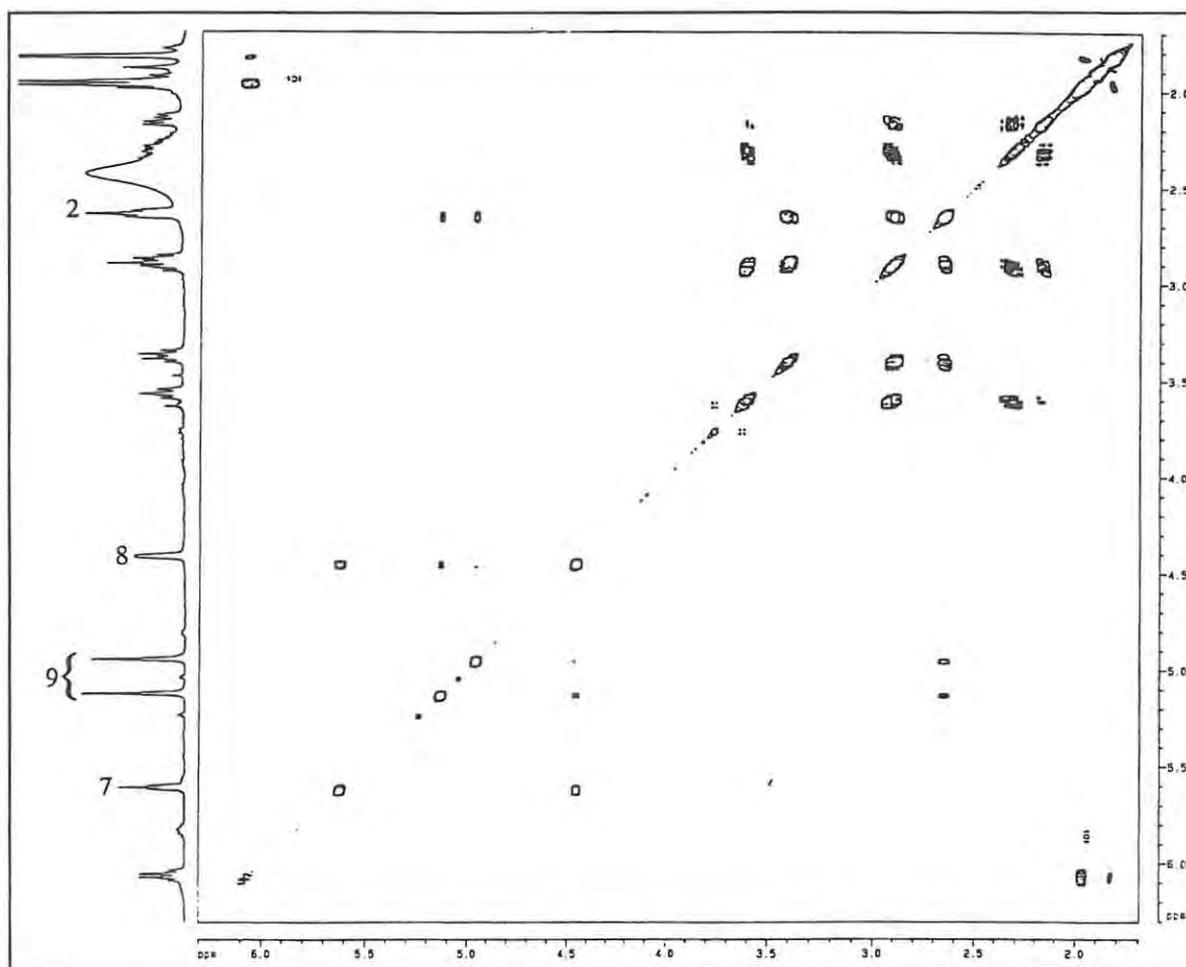
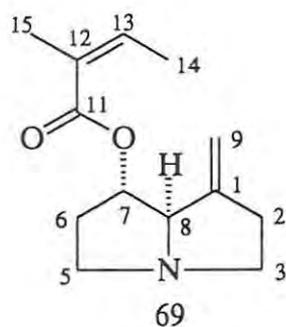


Figure 7.13: COSY spectrum of 7 $\alpha$ -angeryl-1-methylene-8 $\alpha$ -pyrrolizidine **69**.

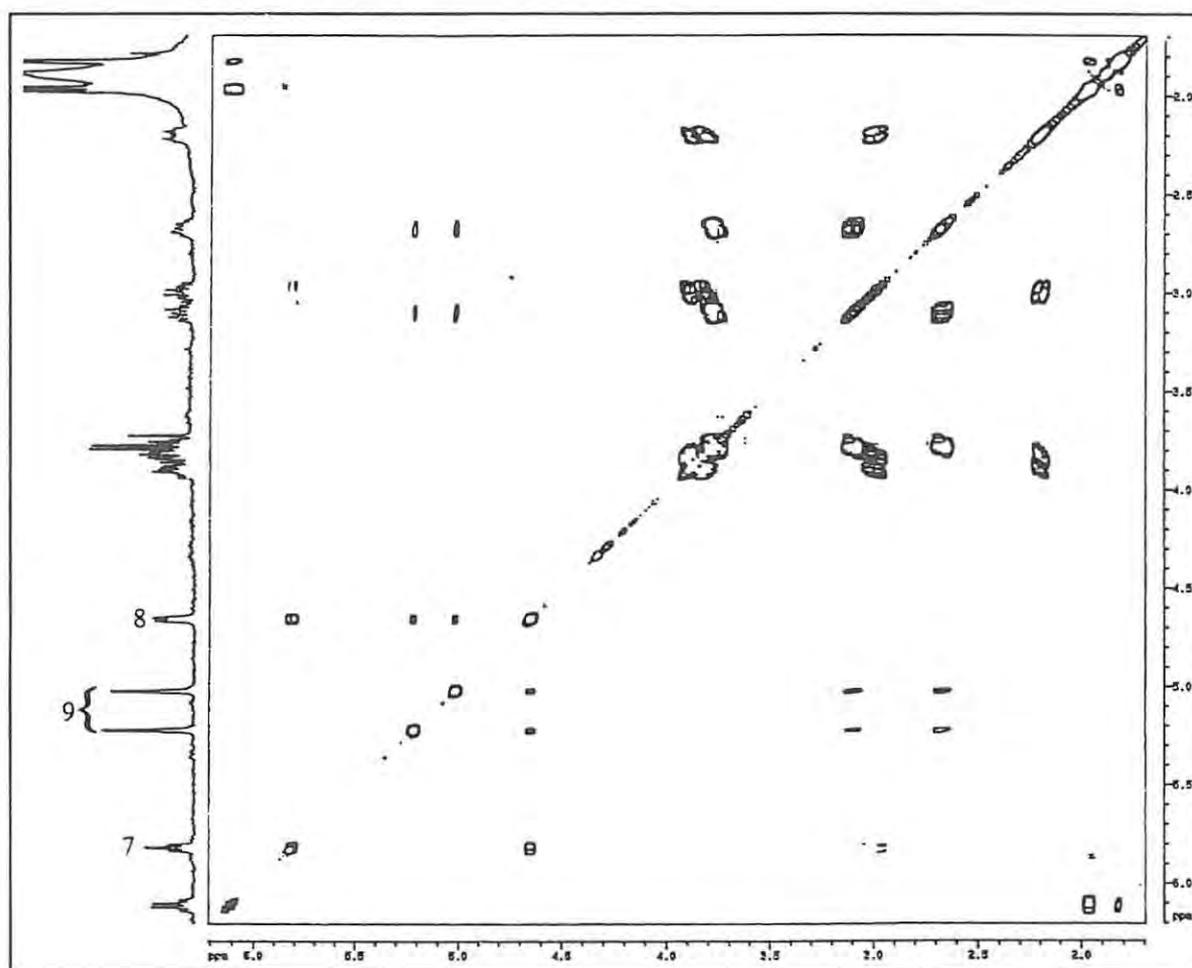
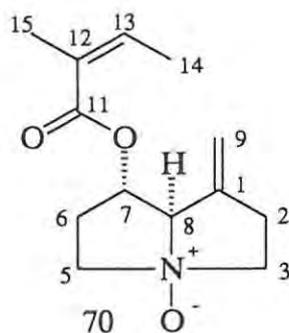
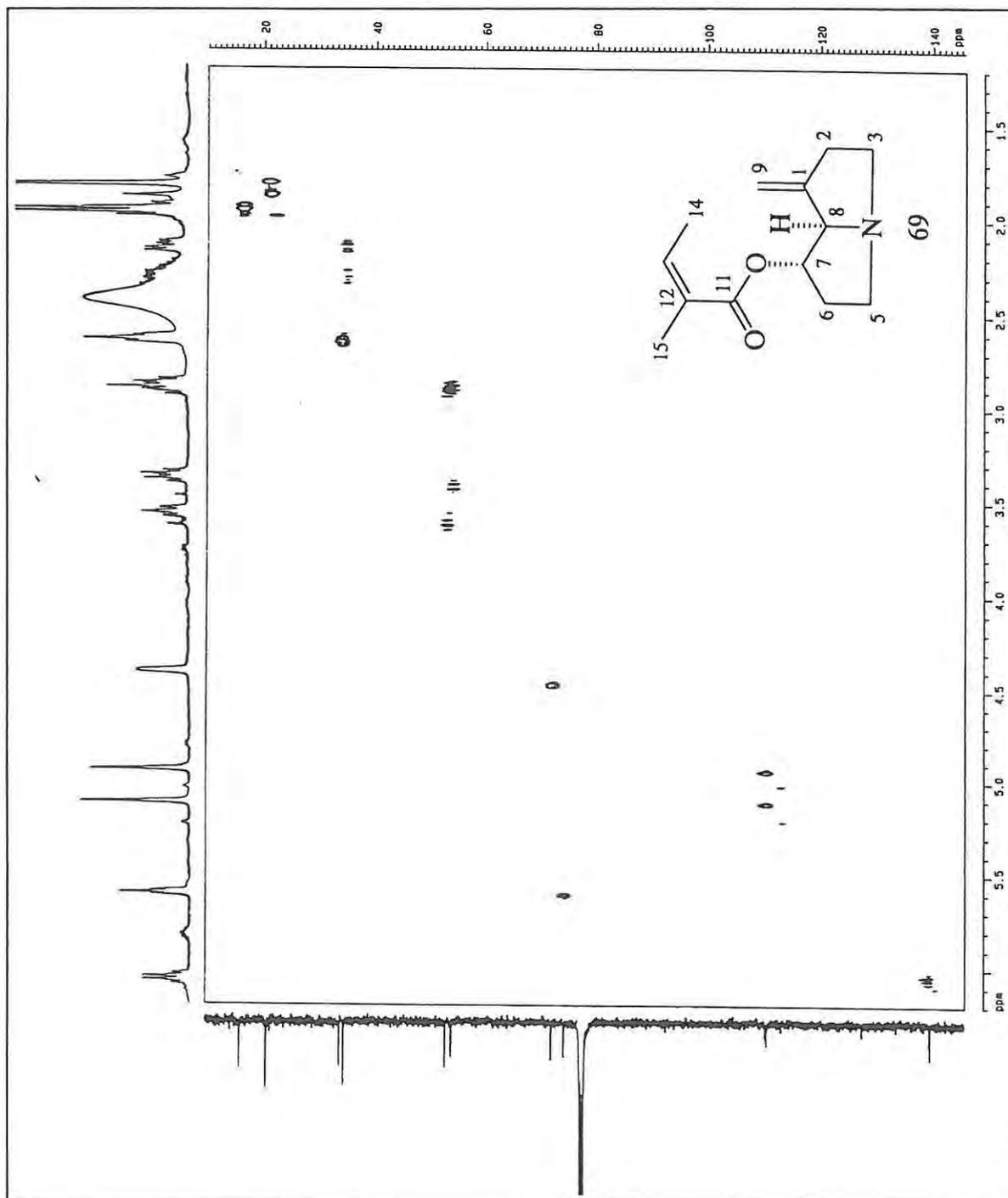


Figure 7.14: COSY spectrum of 7 $\alpha$ -angeryl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide 70.



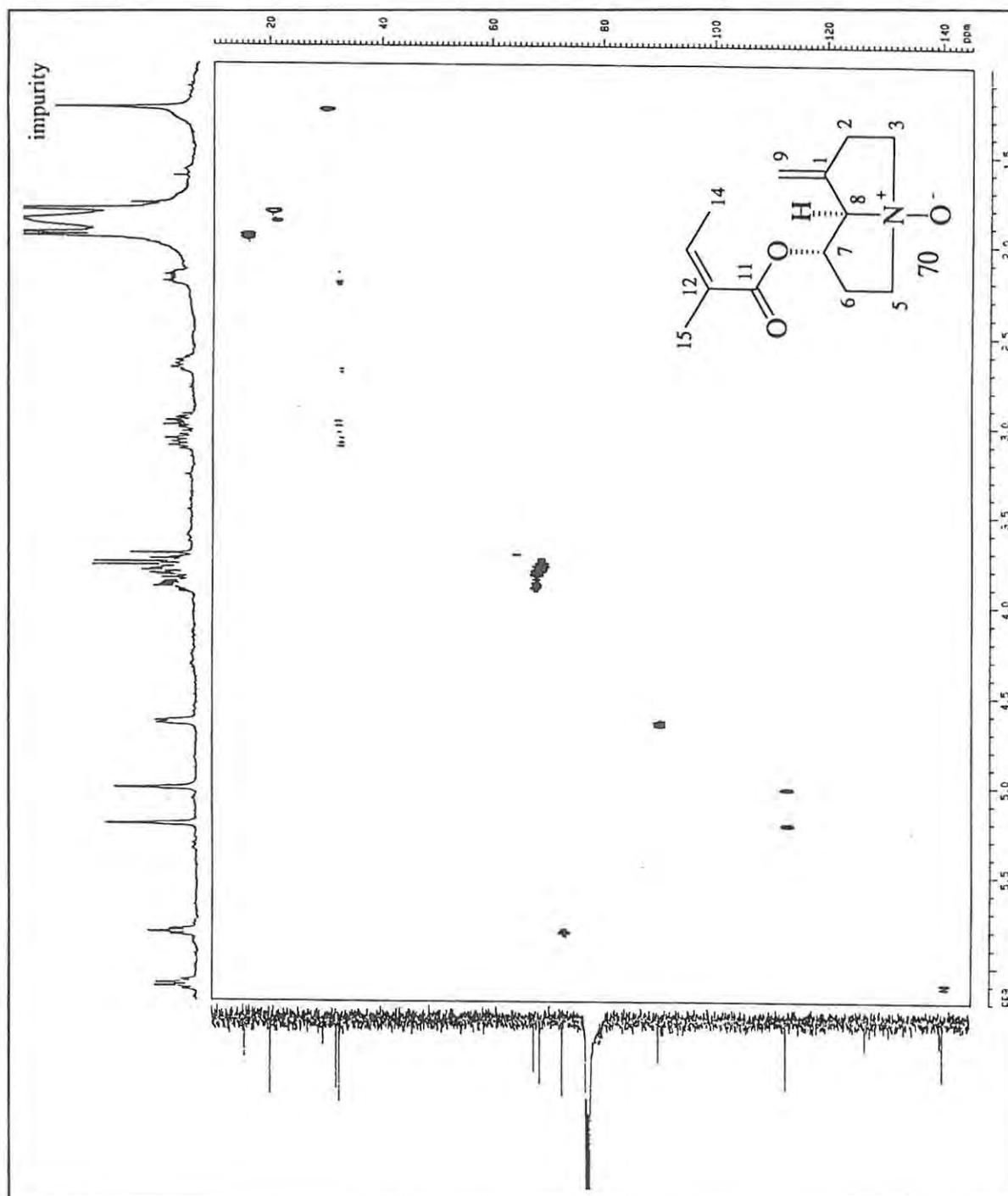


Figure 7.16: HMQC spectrum of 7 $\alpha$ -angeryl-1-methylene-8 $\alpha$ -pyrrolizidine-4-oxide 70.

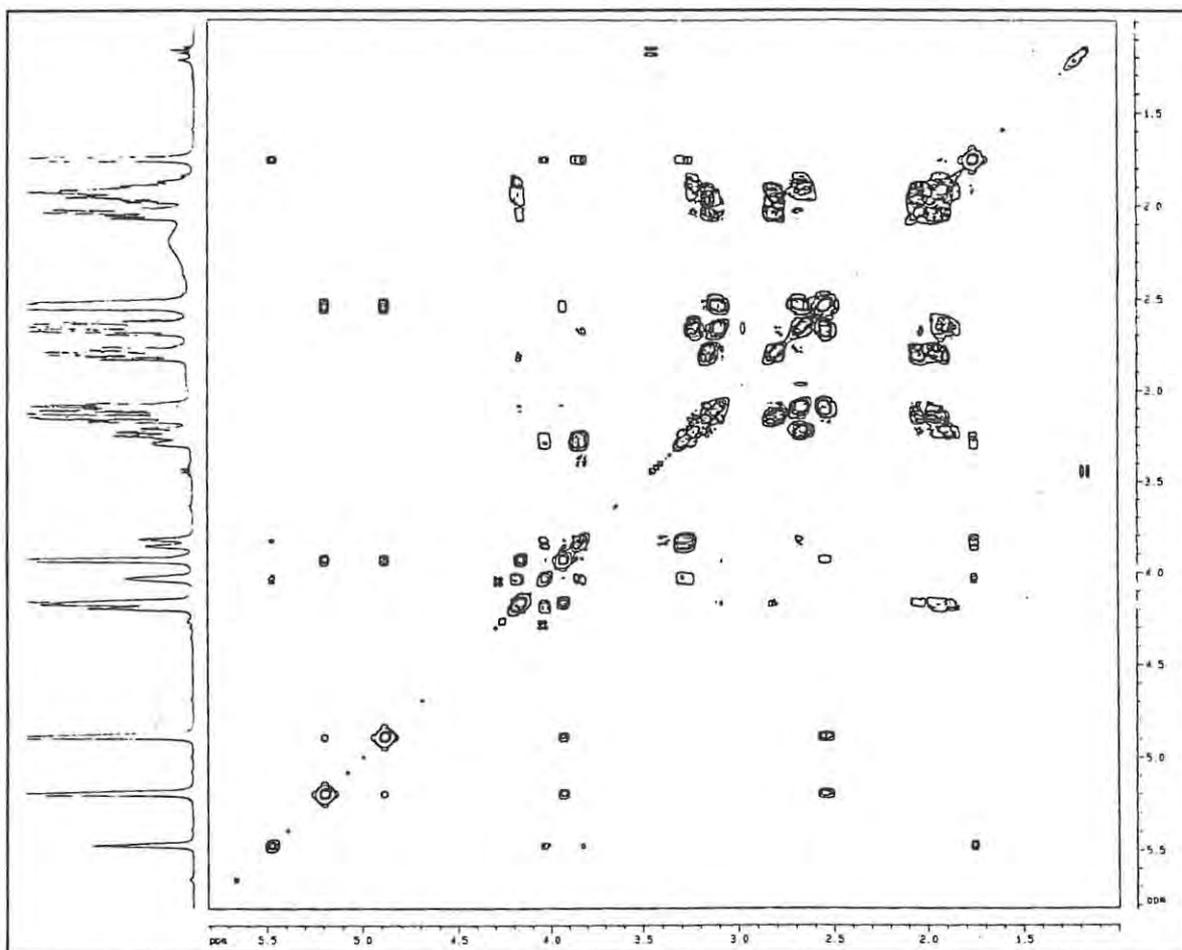
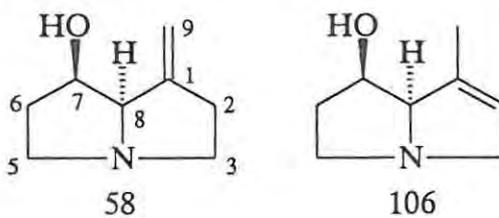


Figure 7.17: COSY spectrum of the mixture of 7β-hydroxy-1-methylene-8α-pyrrolizidine **58** and 1,2-didehydro-7β-hydroxy-1-methyl-8α-pyrrolizidine **106**.

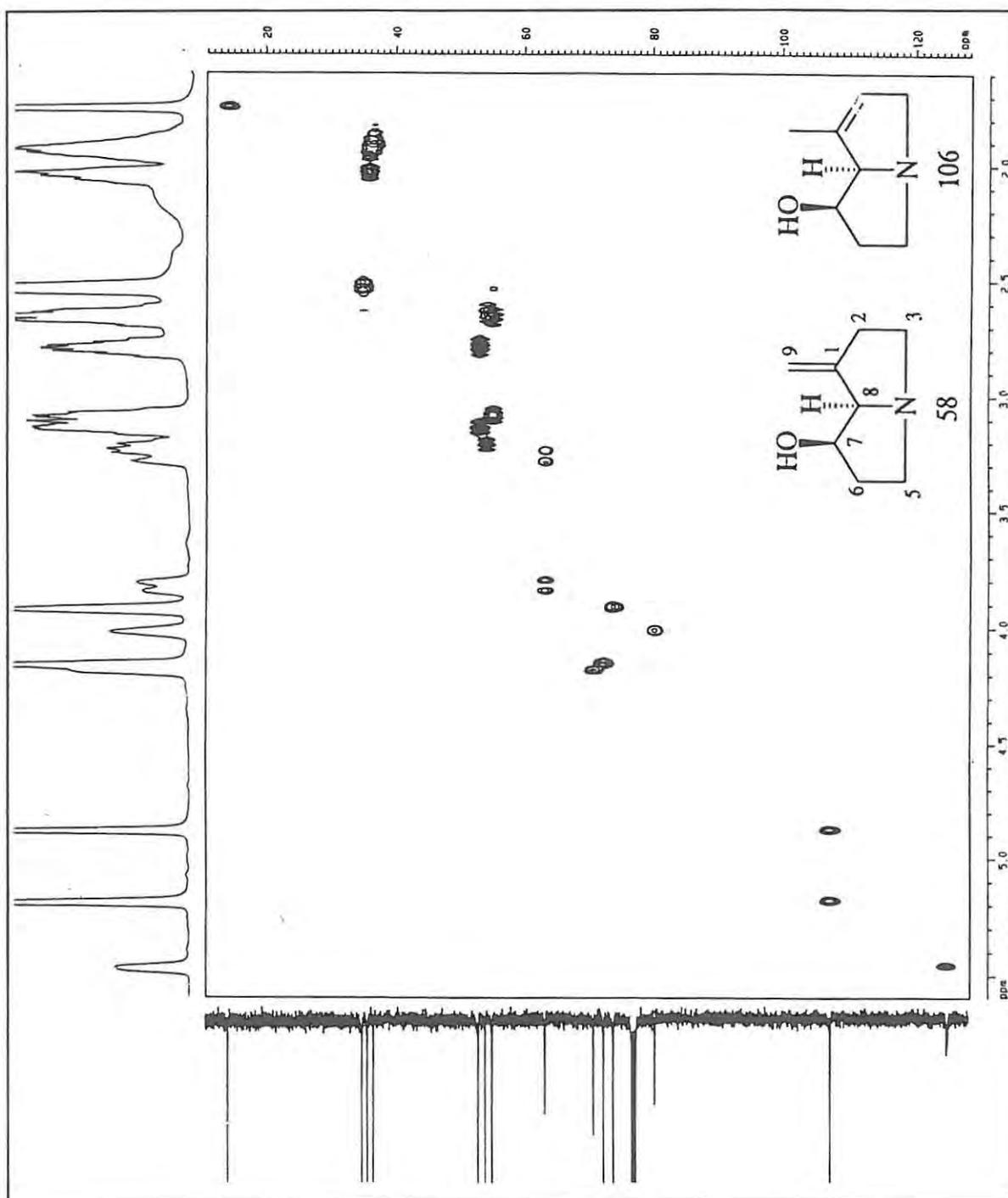


Figure 7.18: HMQC spectrum of the mixture of 7β-hydroxy-1-methylene-8α-pyrrolizidine **58** and 1,2-didehydro-7β-hydroxy-1-methyl-8α-pyrrolizidine **106**.

## 7.2 GLC CHROMATOGRAMS OF CRUDE EXTRACTS NOT INCLUDED IN THE TEXT

**Figure 7.19** GLC chromatogram of the crude extract from the Grahamstown *S. chrysocoma* population.

**Figure 7.20** GLC chromatogram of the crude extract from the Van Stadens River dams *S. chrysocoma* population.

**Figure 7.21** GLC chromatogram of the crude extract from the Prince Alfred's Pass *S. chrysocoma* border-line population.

**Figure 7.22** GLC chromatogram of the crude extract from the Wittedrif *S. chrysocoma* border-line population.

**Figure 7.23** GLC chromatogram of the crude extract from the Robinson Pass *S. paniculatus* population.

**Figure 7.24** GLC chromatogram of the crude extract from the Tradouw Pass *S. paniculatus* population.

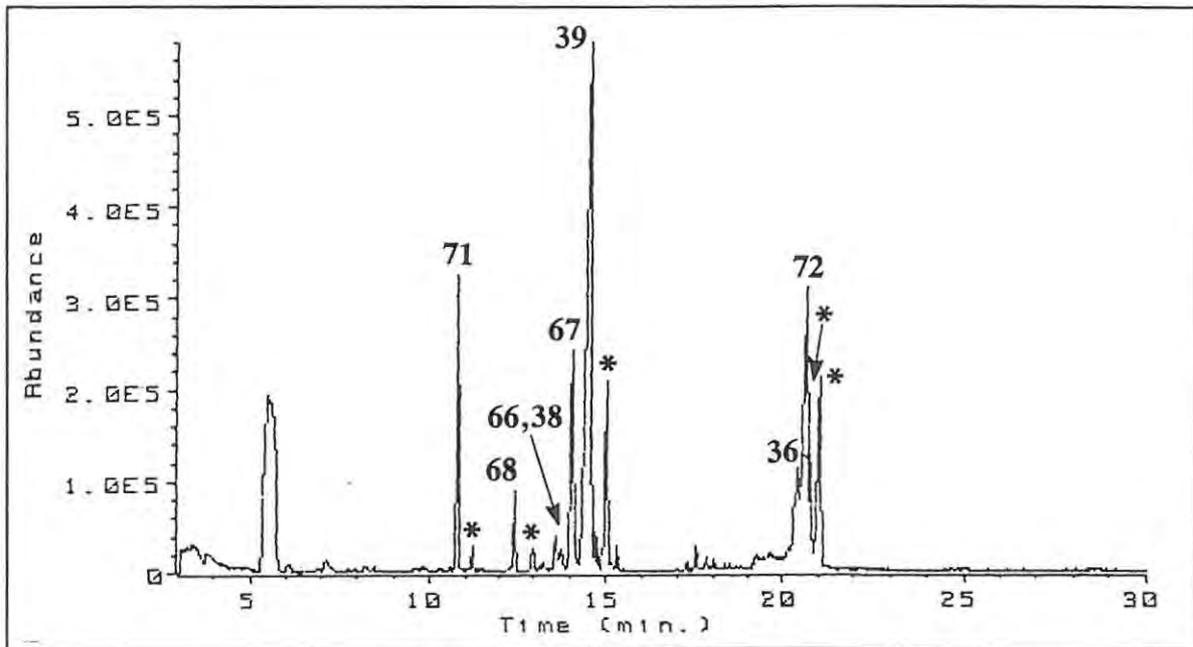


Figure 7.19: GLC chromatogram of the crude extract from the Grahamstown *S. chrysocoma* population.

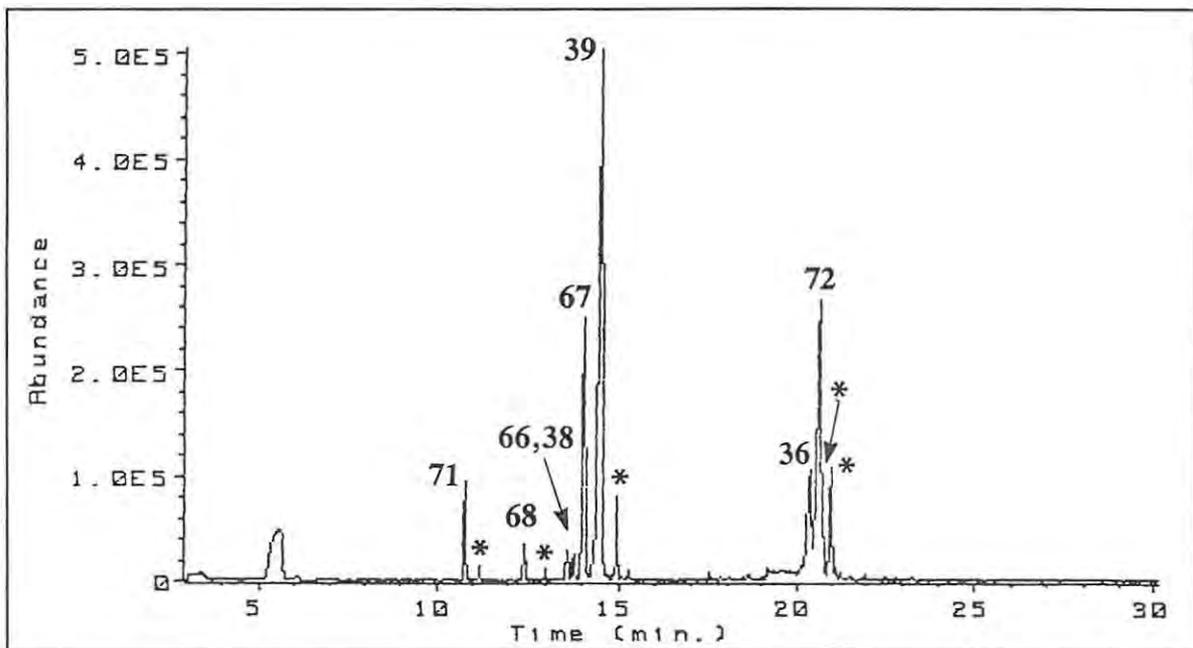


Figure 7.20: GLC chromatogram of the crude extract from the Van Stadens River dams *S. chrysocoma* population.

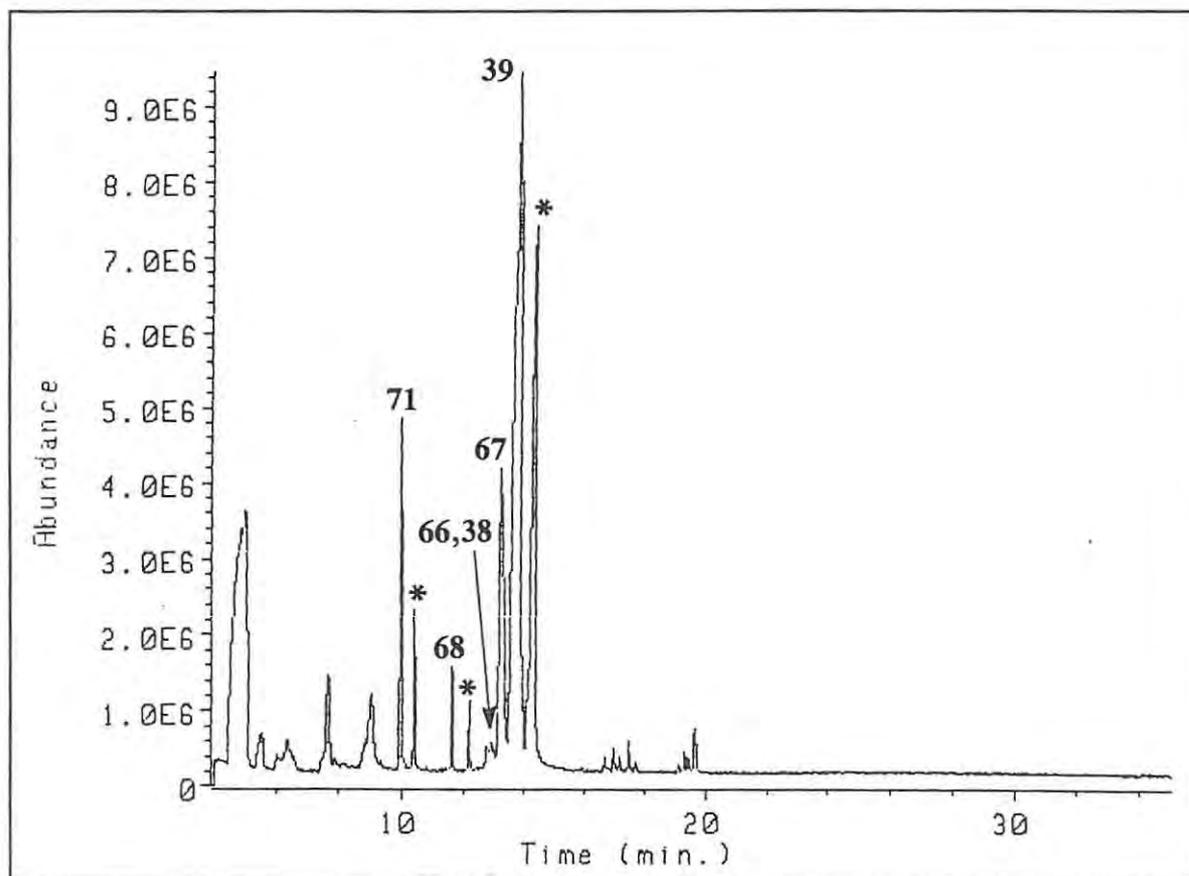


Figure 7.21: GLC chromatogram of the crude extract from the Prince Alfred's Pass *S. chrysocoma* border-line population.

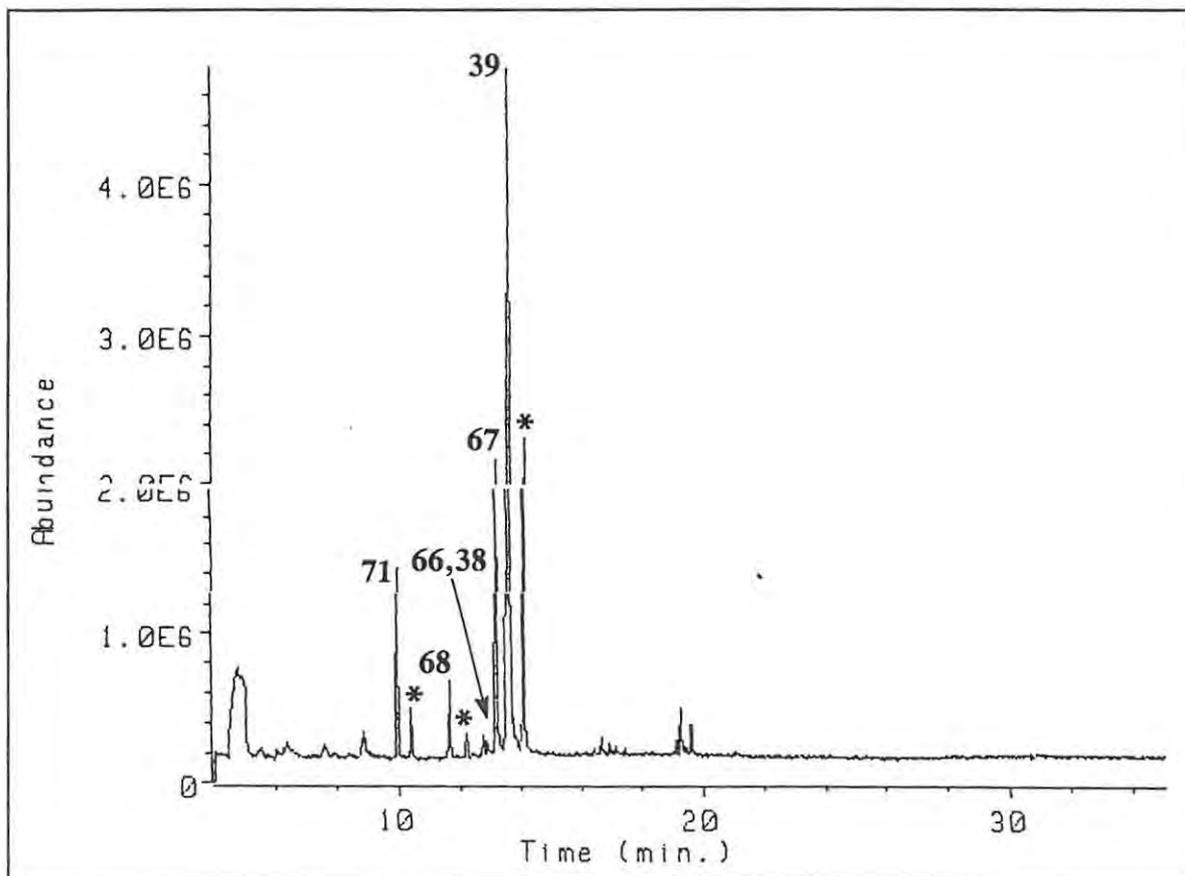


Figure 7.22: GLC chromatogram of the crude extract from the Wittedrif *S. chrysocoma* population.

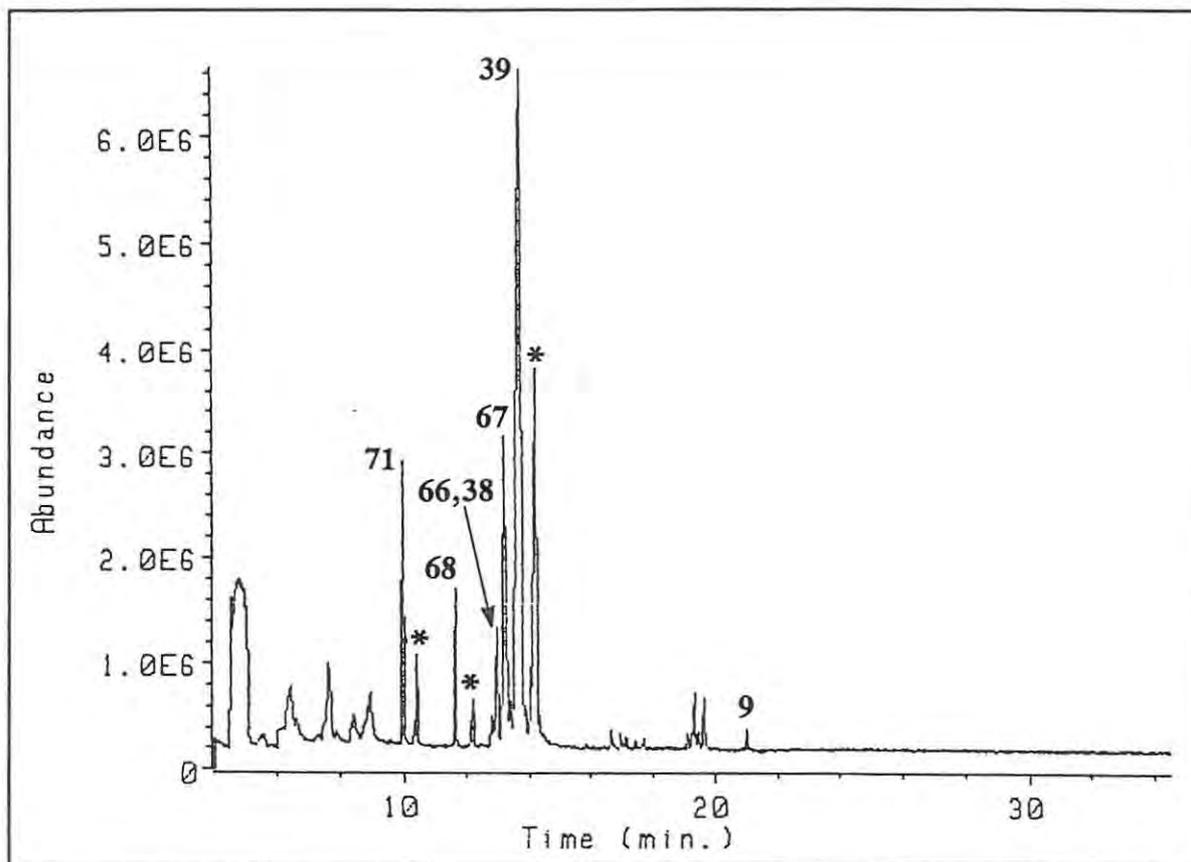


Figure 7.23: GLC chromatogram of the crude extract from the Robinson Pass *S. paniculatus* population.

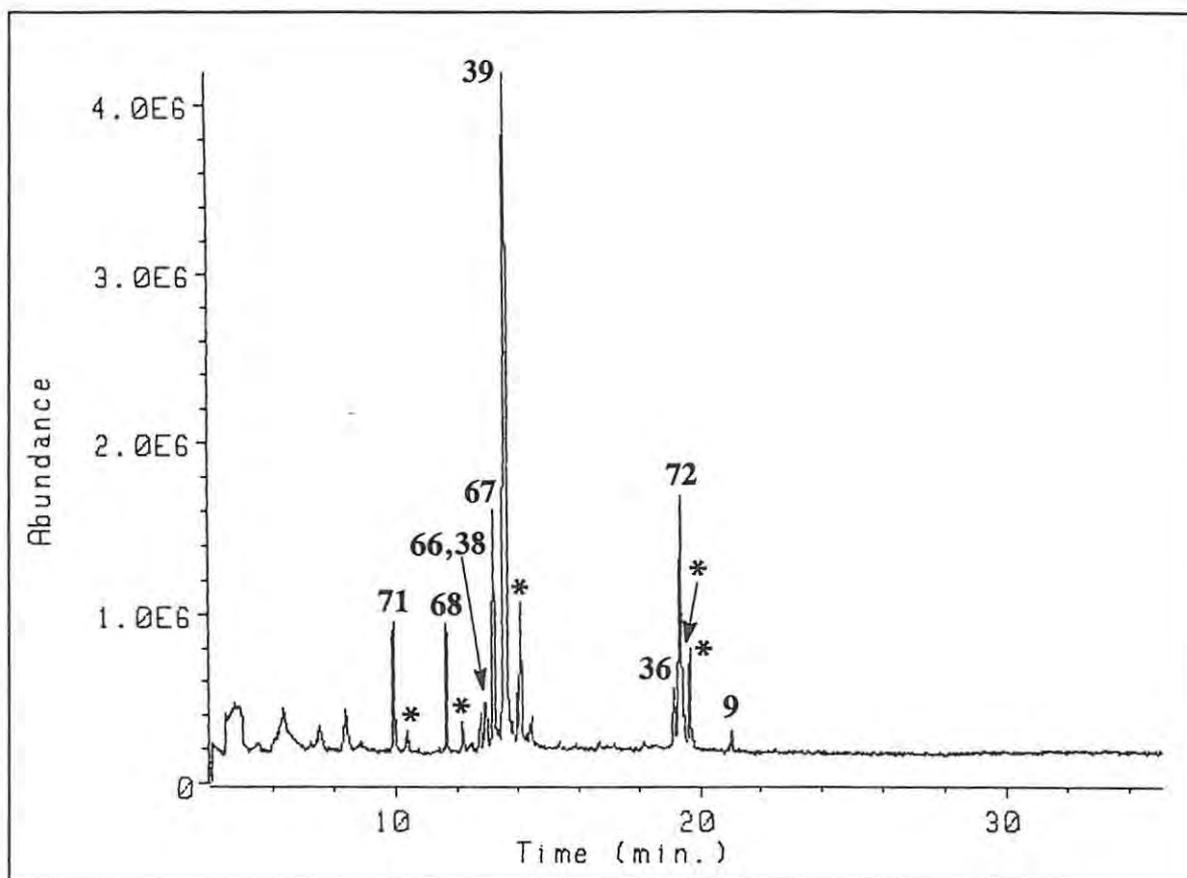


Figure 7.24: GLC chromatogram of the crude extract from the Tradouw Pass *S. paniculatus* population.