Extractives of Three Southern African Medicinal Plants

by

Bronwen Ann Page

Submitted in fulfilment of the requirements for the degree of Master of Science, in the Department of Chemistry, University of Natal, Durban, 1998

The trees and plants in the forest listened to all that the animals said and planned, and resolved to do their utmost to bring their plans to nought. For man had done no harm to the vegetable creation....So they decided that for every disease that might fall upon man through the ill will of the beasts they would provide a remedy. Every single plant, from the huge trees down to the delicate moss was given a part in this curative scheme, and they became the medicine by means of which man is cured of all the ills that may afflict his body.

From "Myths of the North American Indians" **in** The Book of Myths by Amy Cruse (1972)

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Preface

The experimental work described in this thesis was carried out in the Department of Chemistry and Applied Chemistry, University of Natal, Durban, from February 1996 to December 1993, under the supervision of Prof. D.A. Mulholland and Dr N.R. Crouch.

This investigation represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

Signed: _____

Bronwen Ann Page, BSc(Hons)

Signed: _____ Signed: _____

Prof. D.A. Mulholland, PhD Dr N.R. Crouch, PhD

Abstract

In this investigation the chemistry of three southern African plants used for medicinal purposes was investigated. The plants were *Dioscorea dregeana, Avonia rhodesica* and *Equisetum ramossisimum.* Extracts of all three of these plants have been found to be active on the central nervous system. The structures of the compounds isolated were determined by using ^{1}H and ^{13}C n.m.r., i.r., UV-VIS, mass spectroscopy and chemical methods.

D. dregeana yielded two alkaloids and three aromatic compounds. *A. rhodesica* contained a large amount of wax which was analysed by GC-MS and its distribution and physical nature on the surface of the leaves was determined by SEM (scanning electron microscopy). This plant also contained two sterols. *Equisetum ramossisimum* extracts contained a carotenoid and several porphyrins, as well as large amounts of silica. A DTSA X-ray microanalysis system (which was a component of the scanning electron microscope) was used to determine the distribution of silica in the stem.

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List of Abbreviations

Ac	acetate group
bs	broad singlet
COSY	correlated nuclear magnetic resonance spectroscopy
d	doublet
dd	double doublet
ddd	double double doublet
dt	double triplet
DEPT	distortionless enhancement by polarisation transfer
GC-MS	gas liquid chromatography coupled with mass spectrometry
HETCOR	heteronuclear shift correlation nuclear magnetic resonance spectroscopy
HPLC	high performance liquid chromatography
Hz	Hz
i.r.	infrared spectroscopy
Me	methyl group
MS	mass spectrometry
NOE	nuclear overhauser effect
n.m.r.	nuclear magnetic resonance
q	quartet
s	singlet
t	triplet
t.l.c.	thin layer chromatography
UV-VIS	ultra-violet visible light spectroscopy

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Chapter 1: General introduction

For centuries medicinal plants have not only been the source of therapeutic compounds, but also the source of psychoactive ones. The use of psychoactive plants and their extracts has developed over such a long time that it has become an accepted part of the life of many ancient peoples (for example: khat – Ethiopia; fly agaric – Siberia; opium – South East Asia; cannabis – Middle East; cocaine – South America)(Brinkworth *et al.*, 1988). Two of the plants investigated in this work namely, *Dioscorea dregeana* (Kunth.) Dur. and Schinz (Dioscoreaceae) and *Avonia rhodesica* (NE. Br.) Rowl. (Portulacaceae), have been valued for their apparent narcotic properties, but unlike those listed above, the active components have not been isolated. The purpose of this chapter is to give an introduction to the mechanism of nerve function, as well as to give an overview of the South African plant species that are used for their effects on the central nervous system (CNS).

1.1. Introduction to the mechanism of nerve function

A change in the ion permeability of an axonal membrane resulting in an influx of sodium cations and efflux of potassium cations forms a wave of depolarisation known as the "transmission of the nerve impulse". When this impulse approaches the nerve-ending, calcium cations are actively transported into the cell through voltage regulated calcium channels, triggering the release of a neurotransmitter into the synapse between the two neurons (Brinkworth *et al.*, 1988).

According to Osborne (1983), neurotransmitters have been found to be chemicals that satisfy the following criteria:

1. They are present in the presynaptic ends of nerve cells.
2. Their precursors and biosynthetic enzymes are present in the nerve cell.
3. Stimulation of the neurons causes the release of the substance in physiologically significant amounts.
4. Application of the substance directly to the postsynaptic membrane results in a response identical to that caused by the presynaptic nerve cell.
5. Specific receptors that interact with the substance should be present in the postsynaptic membrane.
6. A specific inactivating mechanism should exist to ensure that interactions of the substance with its receptor are halted in a physiologically reasonable time.

Brinkworth *et al.* (1988) stated that neurotransmitters could be grouped into three main classes viz. (a) mono-amines (such as noradrenaline, adrenaline, dopamine, seratonin, histamine and acetylcholine), (b) amino acids (for example: glutamate and aspartate which are excitatory; and taurine and glycine which are inhibitory) and (c) peptides. Neuropeptides are divided into five categories (Palkovits, 1985):

1. hypothalmic neuropeptides: luteinizing hormone-releasing hormone, thyrotropin releasing hormone, corticotropin releasing hormone, growth hormone-releasing hormone, somatostatin, vasopressin, oxytocin.
2. brain-born 'pituitary' peptides: luteinizing hormone, growth hormone, thyrotropin, corticotropin, melanocyte stimulating hormones.
3. opioid peptides: β - endorphin, encephalins, dynorphins.
4. brain-born 'gastro-intestinal' peptides: pancreatic polypeptide, vasoactive intestinal polypeptide, cholycystokinin, bombesin, secretin, motilin, glucagon, insulin.
5. others: substance P, delta-sleep inducing peptide, angiotensin II, bradykinin, FMRF amide.

A receptor can be defined as a membrane-bound protein or protein complex which specifically binds a neurotransmitter, drug or hormone. According to Williams (1986), it should satisfy the following criteria:

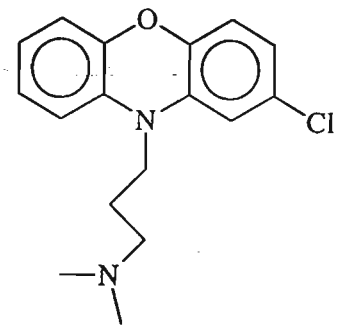
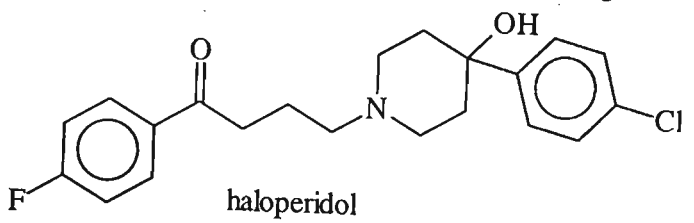
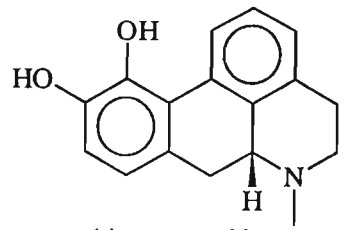
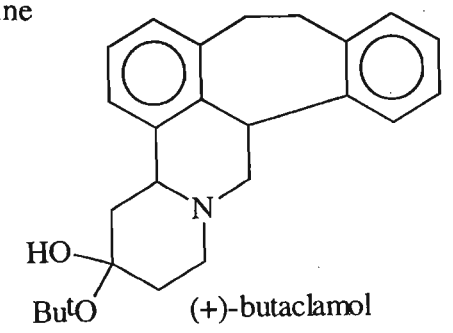
1. Binding of the ligand must be saturable, indicating that the number of receptor sites is finite.
2. Binding should be reversible.
3. There should be a correlation between the binding and the activity of agonists, as measured by dose-response curves.
4. Binding should be specific and selective.
5. There must be a correlation between the distribution of its binding in a tissue and the known localisation (or target site) of the ligand (neurotransmitter, drug or hormone that binds to the receptor).

These criteria led to the description of ligands as agonists and antagonists. An agonist binds to the receptor, triggering a physiological response while an antagonist binds to a receptor without triggering a response, thus blocking the action of the agonist.

Binding of an agonist to a receptor results in the production of a secondary messenger which, in turn, influences an effector system (usually an enzyme or an ion channel (Carman-Krzan, 1986). In the case of ion channels, the subsequent events occur within milliseconds whether the affect of the agonist is excitatory or inhibitory, whereas the enzyme linked response may take minutes or days to occur (Brinkworth *et al.*, 1988).

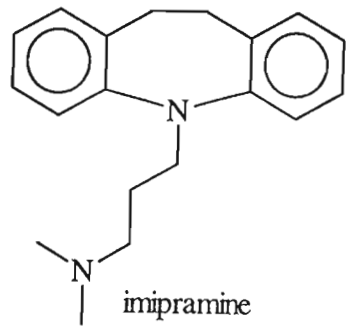
In order for a drug to act as a CNS receptor, it must possess both a strong affinity for the target receptor and specificity at that receptor relative to others (Table 1*a*)(Andrews and Lloyd, 1986).

Table 1*a*: Examples of drugs that act at neuroreceptors

Therapeutic Class	**Receptor Site**	**Compound**	**Action**
Antipsychotics	dopamine	 chlorpromazine	antagonist
	dopamine	 haloperidol	antagonist
	dopamine	 (-)-apomorphine	agonist
	dopamine	 (+)-butaclamol	antagonist

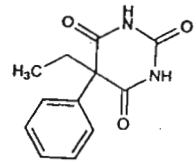
Antidepressants

catecholamine and serotonin transmitter sites in the CNS



imipramine

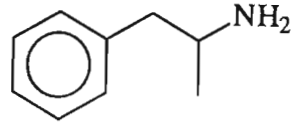
as above



phenobarbitone

Stimulants

action apparently not a receptor site.

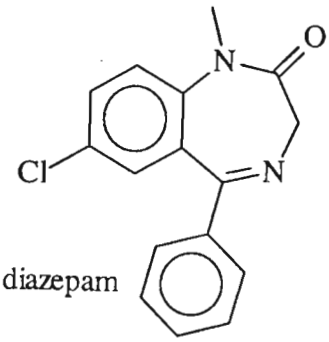


amphetamine

appear to act by enhancing the release and inhibiting the uptake of both noradrenaline and dopamine.

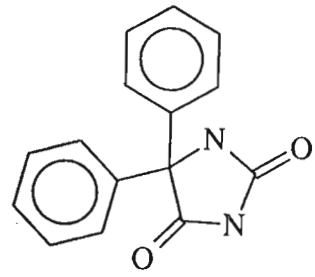
Anticonvulsants

GABA-benzodiazepine receptor-ionophore complex



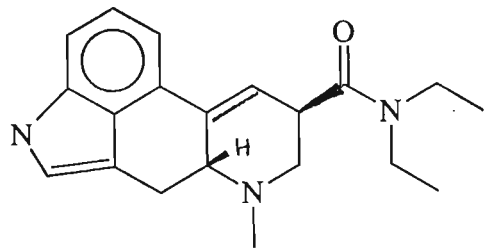
diazepam

as above



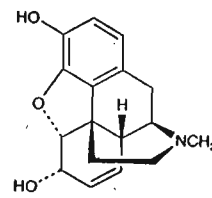
diphenylhydantoin

Hallucinogens — seratonin receptor



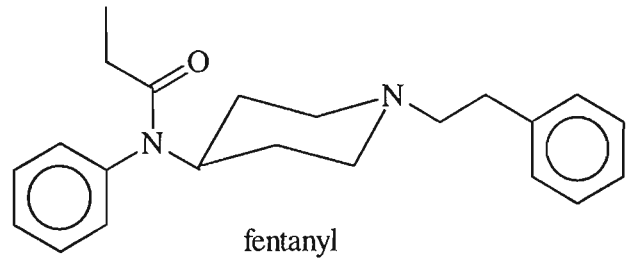
(+)-LSD

Opioid analgesics — μ-opioid binding site



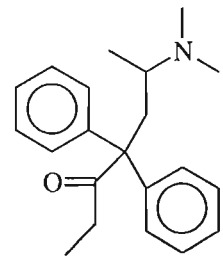
morphine

as above



fentanyl

as above



methadone

An aromatic group and a nitrogen atom are common features of the majority of CNS-active drugs, and are the primary binding groups whose topographical arrangement is fundamental to the activity of the drug classes. The nature and placement of secondary binding groups determines the different classes of CNS drug activity (Lloyd and Andrews, 1986).

According to Strassman (1995), hallucinogenic drugs have been used in psychiatric research and treatment. Hallucinogens have been shown to elicit a multifaceted clinical syndrome affecting many of the functions of the human mind, therefore investigations of these properties could enhance understanding of mind-brain relationships. Naturally occurring psychotic syndromes apparently share features with those elicited by these drugs, therefore understanding the effects and mechanisms of action of hallucinogens may provide novel insights into endogenous psychoses (Strassman, 1995).

Strassman (1995) also stated that "classical" hallucinogens could be divided into the following categories: phenethylamines (e.g. mescaline), indolealkylamines (e.g. psilocybin and N,N-dimethyltryptamine [DMT]) and lysergamides (e.g. LSD). 3,4-methylene-dioxymethamphetamine (MMDA) is a phenethylamine, and is reported to cause effects overlapping with those of classical compounds (Lister *et al.*, 1992).

1.2. South African plant species reported to have been used for their effects on the central nervous system (CNS)

Many South African plants are valued for their apparent action on the CNS (Table 1*b*)(Hutchings *et al.*, 1996). These can be divided under six headings: pain relief (PR), treatment of nervous conditions (N), causation of nervous conditions (NC), treatment of paralysis (P), induction of paralysis (IP) and induction of trances (T). Certain of the plants have been investigated previously and found to contain alkaloids (shown with an 'x' on the table) and others have either not been investigated or have not been found to contain alkaloids (shown with a '–' on the table). While many of the species had not been investigated, other species from the same genera had been found to contain alkaloids (shown with an 'o' on the table)(Southon and Buckingham, 1989).

Both *D. dregeana* and *A. rhodesica* are reportedly used as beer additives (Hutchings *et al.*, 1996; Steffens, 1995) and it was found that a number of South African plants have also been reported to have been used in the making of beer, some of which have been listed in the table below (Watt and Breyer-Brandwijk, 1932).

Table 1*b*: Table of plants used by Zulu traditional healers for conditions involving the CNS (Hutchings *et al.*, 1996; Southon and Buckingham, 1989)

Family	Species Name	PR	N	NC	P	IP	T	Alkaloids
Agavaceae	*Agave* spp.			x				-
Alliaceae	*Tulbaghia alliacea* L.f.		x					-
	T. violacea Harv.				x		x	-
Amaryllidaceae	*Crinum bulbispermum* (Burm. f.) Milne-Redh & Schweik.		x					x
	Scadoxus puniceus (L.) Friis & Nordal.		x					-
Araceae	*Acorus calamus* L.		x					-
Asphodelaceae	*Bulbine latifolia* (L.f.) Roem. & Schult.		x					-
Colchicaceae	*Gloriosa superba* L.			x				x
Commelinaceae	*Commelina africana* L.		x					-
Dioscoreaceae	*Dioscorea diversifolia* Griseb.		x					o
	D. dregeana		x			x		o
Hyacinthaceae	*Scilla nervosa* (Burch.) Jessop		x					-
Hypoxidaceae	*Hypoxis colchicifolia* Bak.		x					-
	H. hemerocallidea Fish & C.A. Mey		x					-
Iridaceae	*Moraea spathuluta* (L.f.) Klatt			x				-
	Dietes iridoides (L.) Sweet ex Klatt		x					-
	Ansellia africana Lindl.		x					-
Orchidaceae	*Cenchrus ciliaris* L.	x						-
Poaceae	*Crabbea hirsuta* Harv.	x						-
Acanthaceae	*Harpephyllum caffrum* Bernh. ex Krauss				x			-
Anacardiaceae	*Rhus chirindensis* Bak.f.		x					-
Annonaceae	*Annona senegalensis* Pers. Subsp. *senegalensis*	x	x					o
	Monanthotaxis caffra (Sond.) Verdc.	x						o
	Heteromorpha trifoliata (Wendl.) Eckl. & Zeyh.		x					-
Apiaceae	*Acokanthera oppositifolia* (Lam.) Codd	x	x					-
Apocynaceae	*Strophanthus gerrardii* Stapf		x					o
	S. luteolus Codd		x					o
	S. petersianus Klotzsh		x					o
	S. speciosus (Ward & Harv.) Reber		x					o
Asclepiadaceae	*Asclepias cucullata* (Schltr.) Schltr.	x						o
	Pachycarpus asperifolius Meisn.	x	x					-
	Stapelia gigantea N.E.Br.	x	x					-
	Xysmalobium undulatum (L.) Ait.f.	x	x					-
Boraginaceae	*Myosotis afropalustris* C.H.Wr.		x					o
Asteraceae	*Achyrocline stenoptera* (DC.) Hillard & Burtt						x	-
	Brachylaena discolor DC. subsp. *discolor* var. *discolor*	x					x	-
	B. elliptica (Thunb.) DC.	x	x					-
	Conyza scabrida DC.	x						-
	Ethulia conyzoides L.f. subsp. *conyzoides*	x						-
	Helichrysum aureonitens Sch. Bip.						x	-
	H. decorum DC.						x	-
	H. nudifolium (L.) Less.	x						-
	Printzia pyrifolia Less.		x					-
	Vernonia adoensis Sch. Bip. ex Walp		x					-
	Aster bakeranus Burtt Davy ex C.A.Sm.	x	x					-

Family	Species Name	PR	N	NC	P	IP	T	Alkaloids
Capparaceae	*Cadaba natalensis* Sond.		x					o
	Capparis tomentosa Lam.	x	x					x
Caryophyllaceae	*Dianthus crenatus* Thunb.						x	o
Celastraceae	*Catha edulis* (Vahl) Vorssk. ex. Endl.		x					x
Chenopodiaceae	*Chenopodium ambrosioides* L.		x					o
	Crassula alba Forssk. var. *alba*		x					-
Ebenaceae	*Euclea crispa* (Thunb.) Guerke subsp. *crispa*		x					-
Euphorbiaceae	*Monadenium lugardiae* N.E.Br.						x	-
	Phyllanthus reticulatus Poir.						x	o
Fabaceae	*Adenopodia spicata* (E.Mey.) Presl	x					x	-
	Argyrolobium tormentosum (Andr.) Druce						x	o
	Erythrophleum lasianthum Corbishley	x	x					o
	Millettia grandis (E.Mey.) Skeels		x					o
	Mundulea sericea (Willd.) A.Chev.	x						x
	Psoralea pinnata L.		x					-
	Tephrosia capensis (Jacq.) Pers. var. *augustifolia*		x					-
Lauraceae	*Cryptocarya latifolia* Sond.	x	x					o
	Ocotea bullata (Burch.) Baill.	x	x					o
Meliaceae	*Ekebergia capensis* Sparrm.	x						o
	Turraea floribunda Hochst.						x	-
Menispermaceae	*Cissampelos torulosa* E.Mey. ex Harv.	x					x	o
Myrsinaceae	*Rapanea melanophloeos* (L.) Mez	x						-
Oleaceae	*Olea woodiana* Knobl.	x						o
Pittosporaceae	*Pittosporum viridiflorum* Sims.	x					x	-
Portulacaceae	*Talinum caffrum* (Thunb.) Eckl. & Zeyh.	x						-
Ptaeroxylaceae	*Ptaeroxylon obliquum* (Thunb.) Radlk.	x	x					-
Ranunculaceae	*Anemone caffra* Eckl. & Zeyh.	x	x					-
Rhamnaceae	*Helinus integrifolius* (Lam.) Kuntze	x	x					-
	Rhamnus prinoides L'Herit	x	x					o
	Ziziphus mucronata Willd. subsp. *rhodesica* Drum	x						x
Rosaceae	*Prunus africana* (Hook.f.) Kalkm.	x						o
Rubiaceae	*Canthium ciliatum* (Klotzsch) Kuntze	x					x	o
	Rubia cordifolia L. subsp. *conotricha*	x					x	-
Rutaceae	*Clausena anisata* (Willd.) Hook.f. ex Benth.	x	x					x
	Xanthoxylon capense (Thunb.) Harv.				x			o
Sapindaceae	*Hippobromus pauciflorus* (L.f.) Radlk.		x				x	-
Sapotaceae	*Sideroxylon inerme* L. subsp. *inerme*				x			-
Scrophulariaceae	*Harveya speciosa* Bernh. ex Krauss		x					-
Solanaceae	*Datura metel* L.	x	x					x
	D. stramonium L.	x	x				x	x
	Solanum hermannii Dun.	x						o
	S. nigrum L.	x	x					x
Tiliaceae	*Corchorus asplenifolius* Burch.		x					o
Verbenaceae	*Vitex rehmanii* Guerke		x					o
	V. wilmsii Guerke var. *reflexa* (H. Pearson) Pieper	x	x					o

Table 1*c*: South African plants reported to have been used in the making of beer

Family	**Species**
Poaceae	*Pennisetum glaucum* (L.) R.Br.
Arecaceae	*Phoenix reclinata* Jacq *Hyphaene coriacae* Gaerth.
Aizoaceae	*Limium aethiopicum* Burm.
Anacardiaceae	*Sclerocarya birrea* (A.Rich.) subsp. *caffra*.
Celastraceae	*Catha edulis* (Vahl) Forsk. ex Endl.
Solanaceae	*Withania somnifera* (L.) Dun.

The three plants studied in this investigation were the monocotyledon *Dioscorea dregeana* (Dioscoreaceae), the dicotyledon *Avonia rhodesica* (Portulacaceae) and the pteridophyte *Equisetum ramosissimum* Desf. (Equisetaceae). Both the genera *Dioscorea* L. and *Equisetum* L. have been found to contain alkaloids (Dictionary of Natural Products on CD-ROM, 1997). Species of the genus *Anacampseros* have been investigated chemically and the isolation of betacyanins was reported (Hegnauer, 1962).

Chapter 2. Extractives from *Dioscorea dregeana*

Chapter 2.1. Introduction

Chapter 2.1.1. Taxonomy and distribution

Dioscorea L. is the largest of five genera of the monocotyledonous family Dioscoreaceae (yam family)(Table 2*a*). The family (which contains approximately 630 taxa [Mabberly, 1990]) was formerly placed in the order Liliales, but more recently the order Dioscoreales was established (Ayensu and Coursey, 1972).

Table 2*a*: Structure of the order Dioscoreales (Ayensu and Coursey, 1972)

Order	**Family**	**Genus**
Dioscoreales	Dioscoreaceae	*Avetra* H.Perrier
		Dioscorea
		Rajania L.
		Stenomeris Planch.
		Tamus L.
	Trichopodiaceae	*Trichopus* Gaertn.
	Stemonaceae	*Croomia* Torr.
		Stemona Lour.
		Stichoneuron Hook.f.

D. dregeana has stout, very twining stems with alternate leaves (Fig. 2.1*a*). The leaf blade is digitately trifoliate, each leaflet being triplinerved from base to apex. The flowers are dioecious: the male perianth being campanulate with a short tubule and six subequal oblanceolate segments, and the female perianth being smaller with a three-celled ovary containing two ovules per cell. The capsules formed are oblong and deflexed containing seeds which have an apical wing about the breadth of the nucleus (Baker, 1896).

The ancestors of the *Dioscorea* crop plants originated in areas of high rainfall such as tropical rainforests or in savanna areas where there were distincts wet and dry seasons. During the rainy season, the plants grow as vines, and in cultivation, they are usually trained on stakes, strings or wires. The vines produce separate male and female flowers, which usually occur in separate plants. The female flowers are followed by dehiscent capsules containing winged seeds, which are

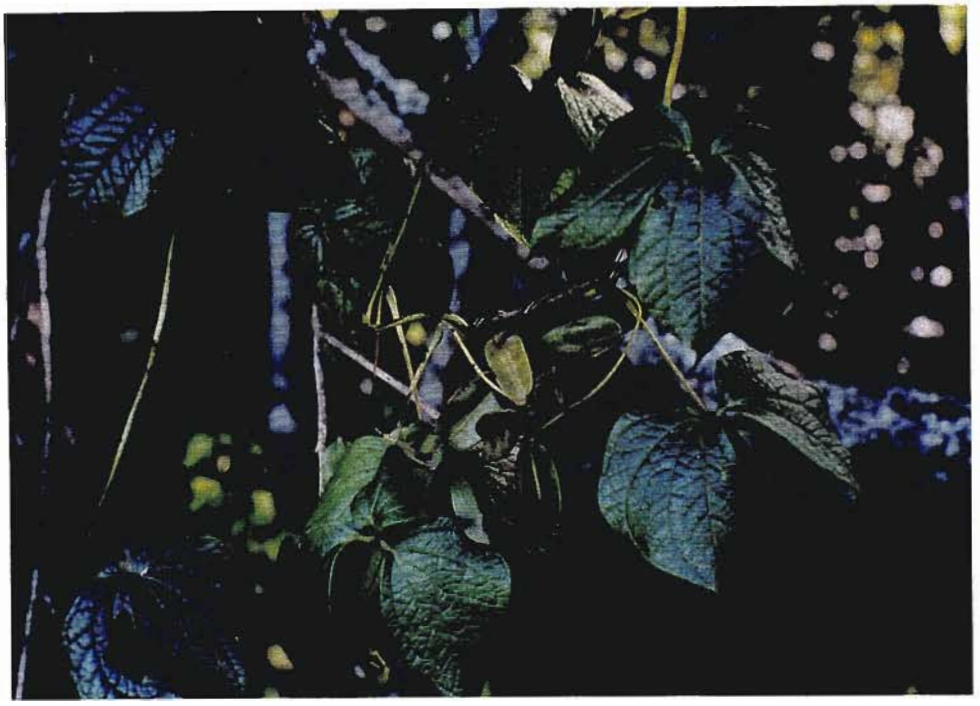


Fig. 2.1*a*: An aerial portion of a fruiting *D. dregeana*, Pietermaritzburg*

*Photograph taken by N.R. Crouch

Fig. 2.1*b*: A *D. dregeana* tuber sold at the Ezimbuzini market*

*Photograph taken by N.R. Crouch

wind-dispersed. During the dry season, the vine dies down and the dormant tubers persist. These natural storage organs are the economically useful parts of the plant (Fig. 2.1*b*)(Ayensu and Coursey, 1972).

According to Ayensu and Coursey (1972), the tuber can either be renewed annually, as in edible yams, or can be perennial, becoming larger and progressively more lignified. They can be propagated vegetatively by means of small tubers or pieces of tuber.

The yams of the Old and New Worlds are botanically distinct, those of the New World having chromosome numbers based on multiples of nine, and those of the Old World having multiples of ten. Also, the capacity to synthesize alkaloids appears to be confined to species of the Old World (Ayensu and Coursey, 1972).

Chapter 2.1.2. Ethnobotany

The Asian cultigen *D. alata* L. is a major food crop in many tropical countries, as the thick tubers are rich in starch (77-94%), protein (3.6-12.5%) and vitamins (B and C)(Neuwinger, 1996). While *Dioscorea* species share these characteristics and appear to be attractive food sources for both man and animals (Table 2*b*)(Ayensu and Coursey, 1972), certain species are not safe to eat.

Table 2*b*: *Dioscorea* species used as food crops (Ayensu and Coursey, 1972)

	Africa	**Asia**	**America**
Primary Species	*D. rotundata* Poir	*D. alata* L.	*D. trifida* L.f.
	D. cayensis Lam.	*D. esculenta* (Lour.) Burk.	
Secondary Species	*D. bulbifera* L.	*D. bulbifera* L.	*D. convulvulacea* Cham. *et* Schlecht
	D. preussi Pax.	*D. hispida* Dennst.	
	D. praehensilis Berth.	*D. pentaphylla* L.	
	D. sanziborensis Pax.	*D. nummularia* Lam.	
	D. dumetorum (Kunth) Pax.	*D. opposita* Thunb.	
		D. japonica Thunb.	

Many of the inedible species produce toxic compounds, which have been found in their tubers. Two types of toxic compounds characteristic of *Dioscorea* species are saponins and alkaloids (Hutchings *et al.*, 1996), both of which are usually water-soluble. In the case of *D. dregeana*, the tubers are only edible if they have been soaked in running water (Watt and Breyer-Brandwijk, 1962). If they are eaten after insufficient soaking, they can cause paralysis and if they are not soaked at all, consumption may result in narcosis (Watt and Breyer-Brandwijk, 1932).

Due to their high toxicity, many *Dioscorea* species are used as criminal and hunting poisons. Examples of species used for these purposes include: *D. dumetorum*, *D. bulbifera*, *D. quartiniana*, *D. sanziborensis*, *D. smilacifolia* Wildem. & Th.Dur., *D. trifida*, *D. cayensis*, *D. hirsuta* BI and *D. triphylla* L. (Neuwinger, 1996).

The water extracts of *D. dregeana*, however, have reportedly been used in the treatment of nervous conditions (hysterical fits and insanity [Watt and Breyer-Brandwijk, 1962] nervous spasms and cramp, [Pujol, 1990]), have sometimes been taken in mixtures during pregnancy to ensure easy childbirth (Varga and Veale, 1997), and used in the treatment of sores and wounds. The tubers have been used as a beer additive, and two teaspoonfuls of freshly macerated material are sufficient to make a person drunk (Gerstner, 1938).

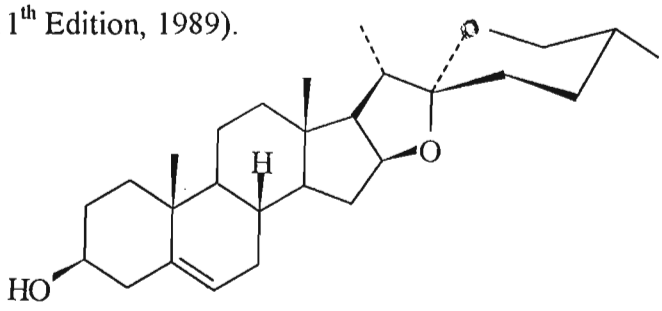
The Zulu people have been known to place maize (*Zea mays* L.) cobs boiled in a water extract of the tubers in their fields so that monkeys (or baboons) responsible for destroying their crops would, on eating these, become inebriated or suffer a short term paralysis, during which time they could be caught (Watt and Breyer-Brandwijk, 1932). A herbalist informant reported that a homeowner might soak some meat in an aqueous extract if his neighbour's dog is, for example, eating his chickens. The dog that eats the soaked meat apparently becomes paralysed for about twenty four hours and can be returned to its owner in this condition as proof of its trespass!

D. dumetorum extracts have been used to trap monkeys in the same way, as well as being used with *Strophanthus* DC. species as an arrow poison . The tubers are known to be poisonous to pigs, but are similarly edible after soaking in water (Watt and Breyer-Brandwijk, 1962).

2.1.3. Review of the previous chemical investigations of the genus Dioscorea

2.1.3.1. Compounds isolated from Dioscorea

Apart from the use of *Dioscorea* species as crop plants, certain species contain large amounts of the commercially useful saponin diosgenin (**1**). The conversion of this compound to progesterone was reported in 1947 by Marker *et al.* and now, several *Dioscorea* species are cultivated as a source of diosgenin, which is used in the synthesis of steroidal hormones (Merck Index, 11th Edition, 1989).



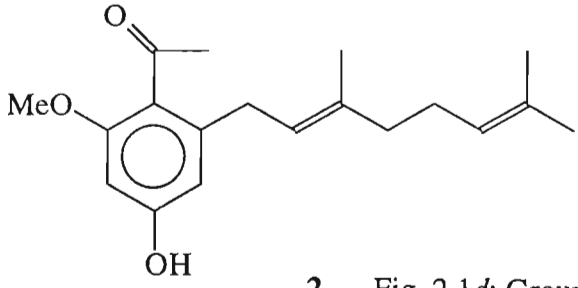
1

Fig. 2.1*c*: Diosgenin

As a result of its importance as a food crop, and because of the commercial use of diosgenin, the chemistry of the genus has been extensively investigated. Compounds isolated fall into four major groups: 1. aromatic compounds, 2. clerodadienes, 3. sterols and saponins, and 4. alkaloids.

2.1.3.1.1. Group 1: Aromatic compounds

Group 1a: 4-Hydroxy-2-[*trans*-3',7'-dimethyl-2',6'-dienoctenyl]-6-methoxyacetophenone (**2**) and 4,6-dihydroxy-2-O-[4'-hydroxybutyl]acetophenone were isolated from *D. bulbifera* (Gupta and Singh, 1989) and 6,7-dihydroxy-1,1-dimethylisochromane (**3**) was isolated from *D. cirrhosa* Lour. (Hsu and Chen, 1993).



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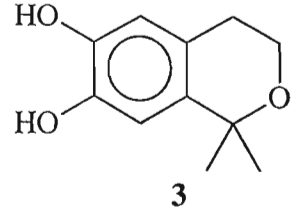

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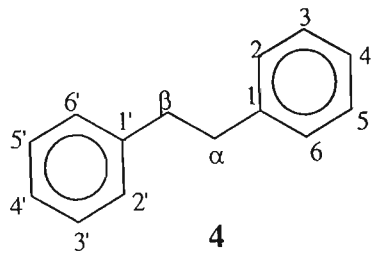
Fig. 2.1*d*: Group 1a compounds

Group 1b: Bibenzyl (**4**) derivatives have been isolated from *Dioscorea* species.



4

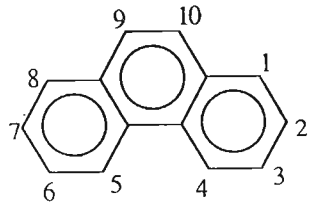
Fig. 2.1*e*: Bibenzyl skeleton

2,3-Dihydroxy-4,5-dimethoxybibenzyl (Batatasin II) was isolated from *D. batatas* Decne and *D. opposita*.

3,3′-Dihydroxy-5-methoxybibenzyl (Batatasin III), 3-hydroxy-3′,5-dimethoxybibenzyl, 3-hydroxy-5-methoxybibenzyl, 2′-hydroxy-3,5-dimethoxybibenzyl and 2′-hydroxy-3,4,5-trimethoxybibenzyl (Batatasin V) were isolated from *D. batatas* (Hashimoto *et al.*, 1972; Hashimoto and Hasegawa, 1974).

2,3′-Dihydroxy-5′-methoxybibenzyl (Batatasin IV) and 3,5-dihydroxybibenzyl were isolated from both *D. batatas* and *D. rotundata* (Hashimoto and Tajima, 1978; Fagboun *et al.*, 1987). *D. rotundata* infected with *Bothyodiplodia theobromae* also contained 2′,3,5-trihydroxybibenzyl (Fagboun *et al.*, 1987), and *D. bulbifera* infected with the same bacterium species contained 3,4',5-trihydroxybibenzyl (Adesanya *et al.*, 1989)

Group 1c: Phenanthrene (**5**) derivatives



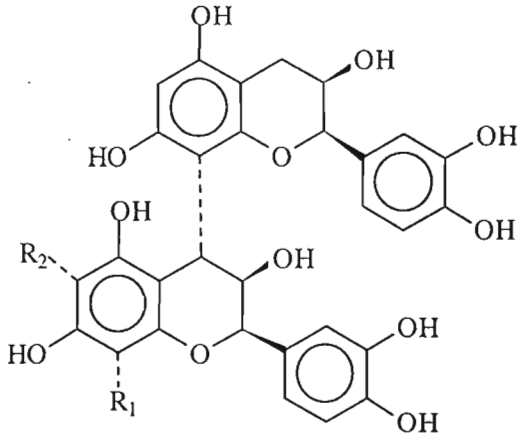
5

Fig. 2.1*f*: Phenanthrene skeleton

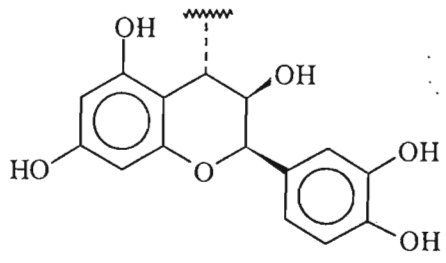
3-Hydroxy-2,5,7-trimethoxyphenanthrene (Batatasin I), and 1,2,5,7-tetrahydroxyphenanthrene were isolated from *D. batatas* (Takasugi *et al.*, 1987), 9,10-dihydro-2,7-dihydroxy-4,5,7-trimethoxyphenanthrene was isolated from *D. decipiens* Hook.f. (Sunder *et al.*, 1978) and 9,10-dihydro-2,4,5,6-tetrahydroxyphenanthrene was isolated from *D. bulbifera*. 1,3,5,6-Tetrahydroxyphenanthrene, 9,10-dihydro-5,6-dihydroxy-1,2,4-trimethoxyphenanthrene, 9,10-dihydro-5,6-dihydroxy-2,3,4-trimethoxyphenanthrene (prazerol), 9,10-dihydro-5,6-dihydroxy-2,4-

dimethoxyphenanthrene and 9,10-dihydro-5,6-dihydroxy-2,4-dimethoxyphenanthrene were isolated from *D.prazeri* Prain & Burkill (Wij *et al.*, 1978).

Group 1d: The last group of aromatic compounds contains flavans (**6-9**) from *D. cirrhosa* and anthocyanidins (**10**) isolated from *D. alata* (Shoyoma *et al.*, 1990).



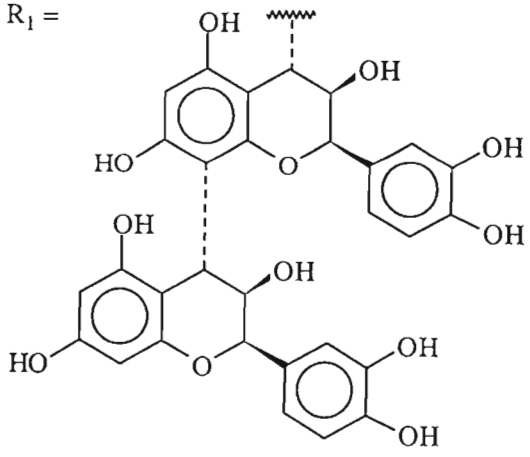
6 $R_1 = H$
$R_2 =$



= A *D. cirrhosa*

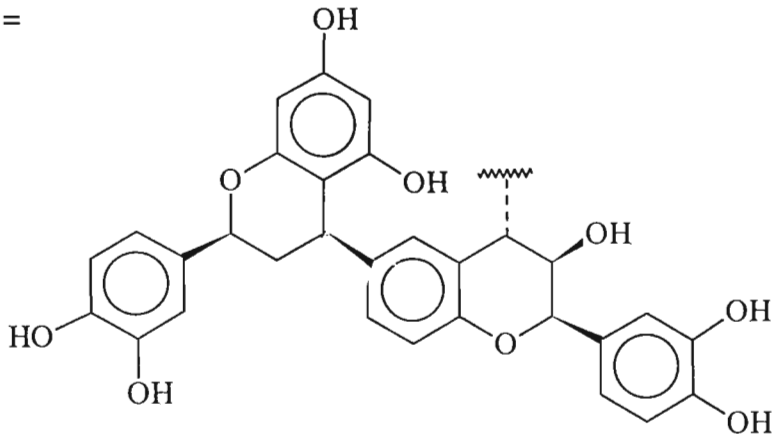
7 $R_1 = A, R_2 = H$ *D. cirrhosa*

8 $R_1 =$



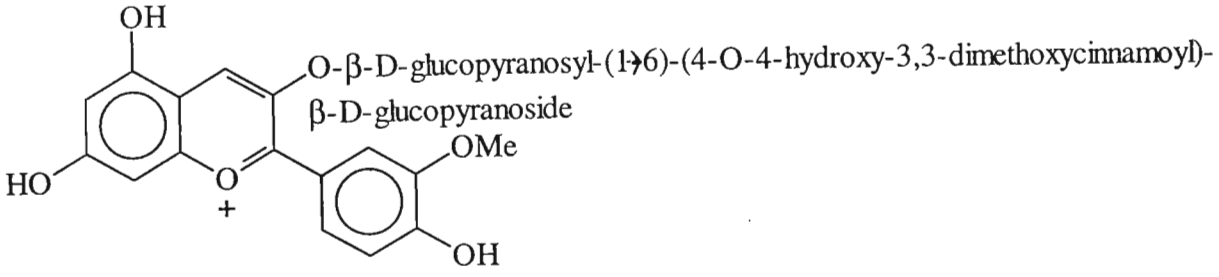
$R_2 = H$ *D. cirrhosa*

9 R_1 =



R_2 = H *D. cirrhosa*

10

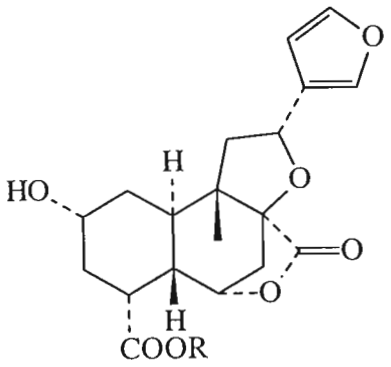


D. alata

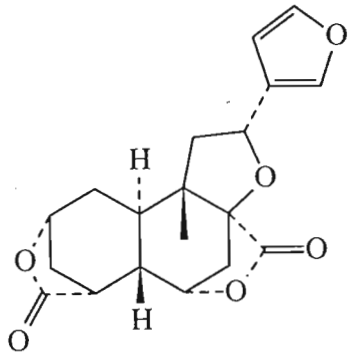
Fig. 2.1*g*: Group 1d compounds

2.1.3.1.2. Group 2: Clerodadienes

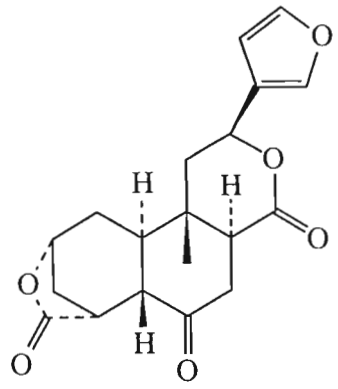
The following clerodadienes were isolated from *D. bulbifera* (Fig 2.1*h*)(Murray *et al.*, 1984)



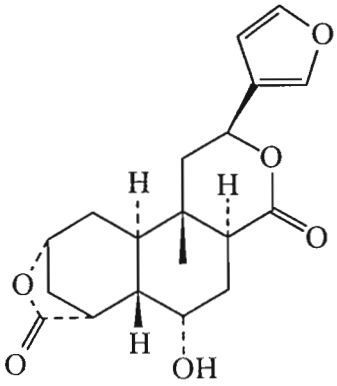
R= Me, diosbulbin A
R= H, diosbulbin C



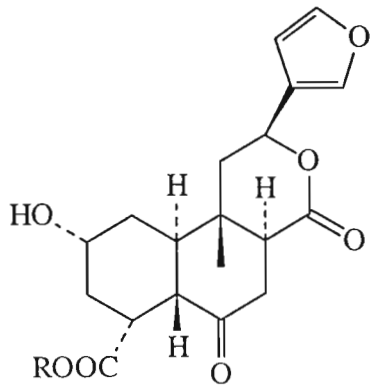
diosbulbin B



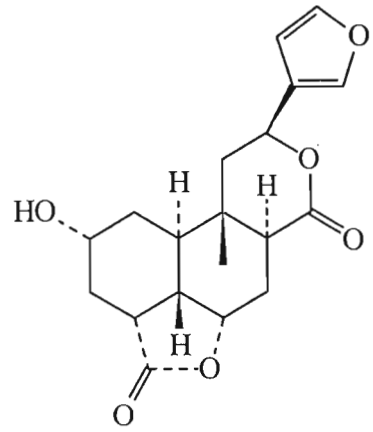
diosbulbin D



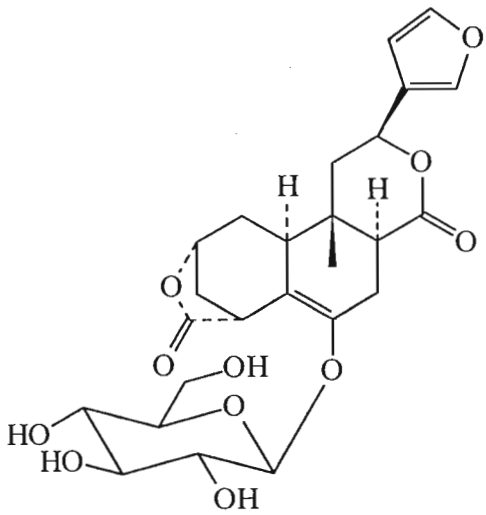
diosbulbin E



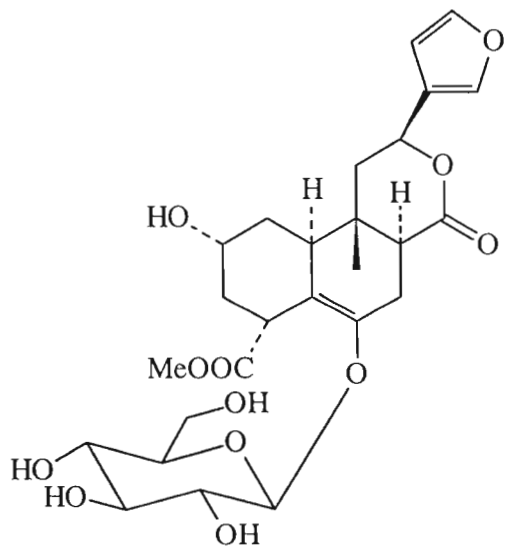
R= Me, diosbulbin F
R= $(CH_2)_3Me$, diosbulbin H



diosbulbin G



diosbulbinoside D



diosbulbinoside F

Fig. 2.1*h*: Clerodadienes from *D. bulbifera*

2.1.3.1.3. Group 3: Sterols and saponins

Group 3a: Sterols are widespread in the plant kingdom, so it is not surprising that many have been isolated from *Dioscorea* species. The sterols of a single species, *D. batatas* have been included as representatives of Group 3a: cholestanol (**11a**), 24*R*-methylcholestanol (**11b**), 24*S*-methylcholestanol (**11c**), 24*R*-ethylcholestanol (**11e**), cholesterol (**12a**), campesterol (**12b**), 24*S*-methylcholesterol (**12c**), 24-methylenecholesterol (**12d**), sitosterol (**12e**), stigmasterol (**12f**), isofucosterol (**12g**), clerosterol (**12h**), 24-methylene-25-methylcholesterol (**12i**), lathosterol (**13a**), cholest-8(14)-enol (**14a**), 24*R*-methylcholest-8(14)-enol (**14b**), 24*S*-methylcholest-8(14)-enol (**14c**) and 24*R*-ethylcholest-8(14)-enol (**14e**)(Fig. 2.1*i*)(Akihisa *et al.*, 1991).

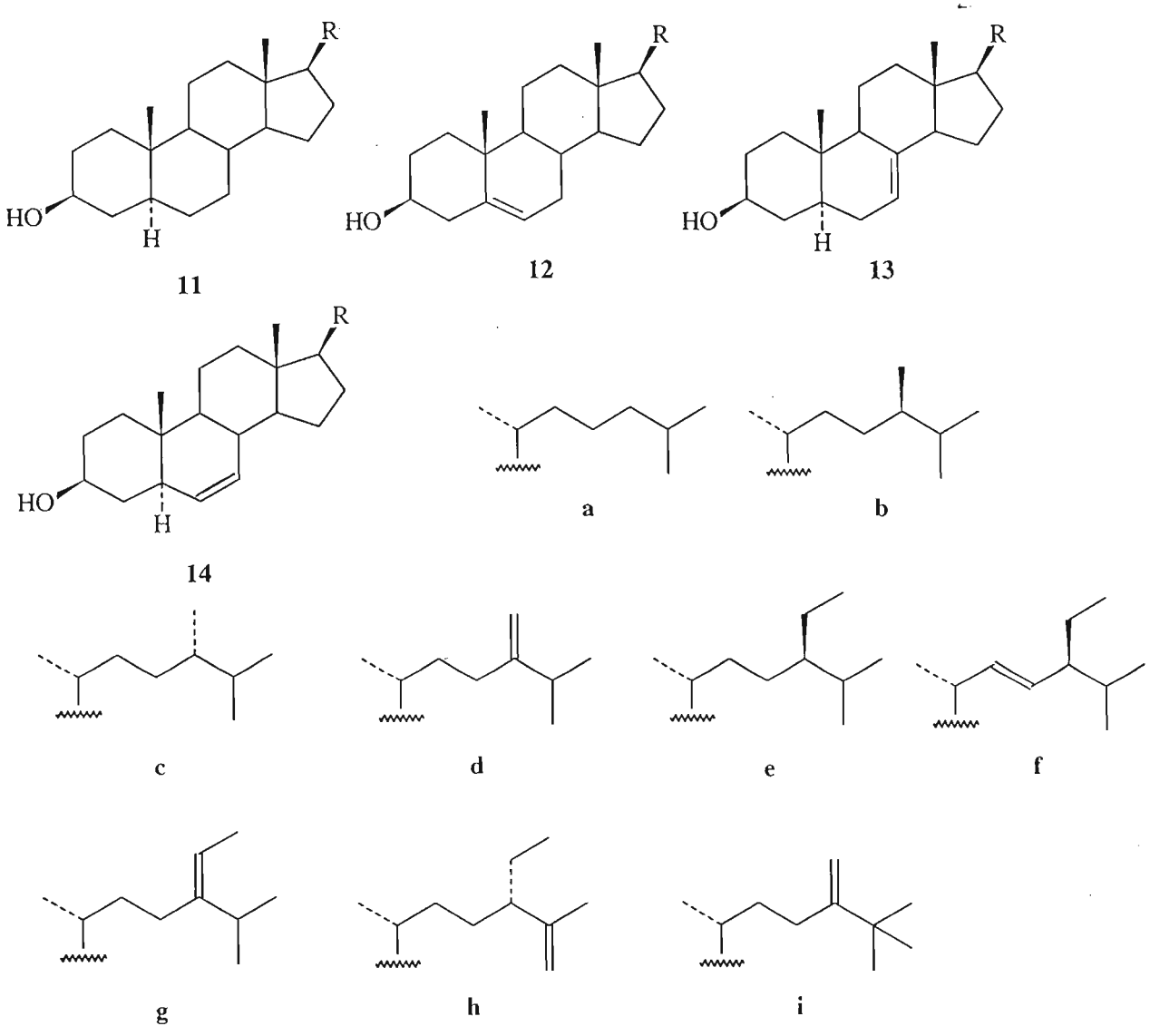
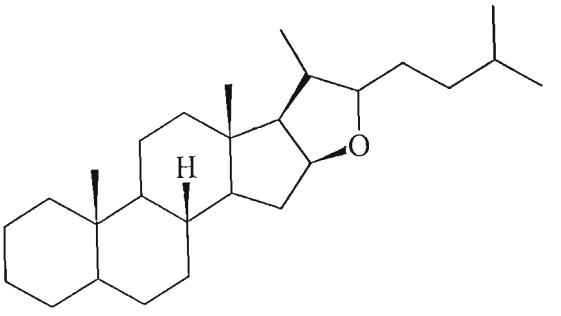


Fig. 2.1*i*: Sterols from *D. batatas*

Group 3b: All the sterols that belong to group 3b, have a furostane (**15**) skeleton.

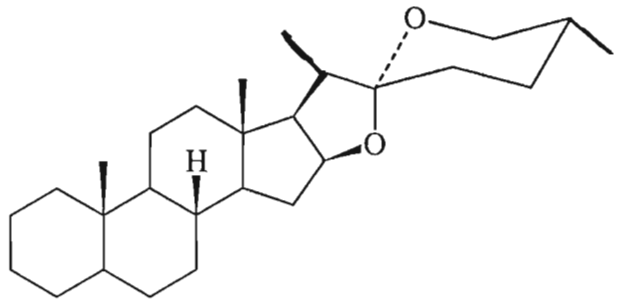


15

Fig 2.1*j*: Furostane skeleton

26-O-β-D-Glucopyranosyl-25*R*-furostane-2β,3α,22ζ-triol (protoyonogenin) and 26-O-β-D-glucopyranosyl-25*S*-5β-furostan-2β,3α,22ζ,-triol (protoneoyonogenin) were isolated from *D. tokoro* Makino (Tomita and Uomori, 1974; Uomori *et al.*, 1983) and a glucoside of 25ζ-furost-5-ene-3β,17α,20ζ,26-tetrol (nolonin) was isolated from *D. mexicana* Scheidw. (Marker and Lopez, 1947). 3-O-β-[α-L-Rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl]-26-O-β-D-glucopyranosyl-25*R*-furost-5-en-22*R*-ol (protodioscin) was isolated from *D. gracillima* Miq. (Kawasaki *et al.*, 1974) and 3-O-β-[α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl]-26-O-β-D-glucopyranosyl-25*R*-furost-5-en-22*R*-ol (protogracillin) was isolated from *D. septemloba* Thunb. (Kawasaki *et al.*, 1974). 3-O-β-[α-L-Rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl]-26-O-β-D-glucopyranosyl-22*R*-methoxy-25*R*-furost-5-ene (floribundasaponin E) and 3-O-β-[α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl]-26-O-β-D-glucopyranosyl-25*R*-furost-5-en-22*R*-ol (floribundasaponin F) were isolated from *D. floribunda* Mart. & Gal. (Mahato *et al.*, 1978).

Group 3c: Most of the compounds reported to have been isolated from *Dioscorea* species have a spirostane (**16**) skeleton and many of these are saponins. The list given in this introduction should not be considered to be exhaustive.



16

Fig. 2.1*k*: Spirostane skeleton

25*S*-Spirost-5-ene-3β,27-diol has been found to be present in many *Dioscorea* species (Minato and Shimaoka, 1963). 14-Hydroxy-(5β,25*R*)-spirostan-3-one (prazerigenin C), 14-hydroxy-4-en-3-one (prazerigenin B), 25*R*-spirost-5-ene-3β,14ζ-diol (prazerigenin A)(Rajaraman *et al.*, 1975), 3-O-β-[β-D-glucopyranosyl]-spirost-5-en-14ζ-ol, 3-O-β-[α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranosyl]-spirost-5-en-14ζ-ol and 25*S*-spirost-5-ene-3β,14ζ,27-triol (prazerigenin D)(Rajaraman *et al.*, 1982) were isolated from *D. prazeri*. 5β,25*R*-Spirostane-2β,3α-diol (yonogenin)(Uomori *et al.*, 1983), 2-O-α-L-arabinospiran-3β-ol (yononin), 25*R*-spirostane-1β,2β,3α,5β-tetrol (kokagenin), 1-O-α-[α-L-arabinopyranosyl)-5β,25*R*-spirostane-2β,3α-diol (tokoronin), 5β,25*R*-spirostane-2β,3α,4β-triol (isodiotigenin) and 5β,25*R*-spirostane-2β,3α,19-triol (19-hydroxyyonogenin) were found in *D. tokoro* (Miyahara *et al.*, 1975). The following two compounds were isolated from both *D. tokoro* and *D. temuipes*: 5β,25*R*-spirostane-1β,2β,3α-triol (tokorogenin)(Uomori *et al.*, 1983) and 5β,25*S*-spirostane-1β,2β,3α-triol (neotokorogenin)(Akahori *et al.*, 1973). *D. temuipes* Franch. & Sav. was also found by these authors to contain 1-O-β-[α-L-arabinopyranosyl]-5β,25*R*-spirostane-2β,3α-diol (neotokoronin), 5β,25*S*-spirostane-2β,3α,4β-triol (diotigenin) and 4β-acetyl-3α-methoxy-25*S*-spirostane-2β,3α,4β-triol (diotigenin) and 4β-acetyl-3α-methoxy-25*S*-spirostane-2β-ol (steroid G_4).

Diosgenin (**1**) and its 3-O-β-D-glucopyranoside and 3-O-α-L-rhamnopyranosyl-(1-2)-α-L-rhamnopyranosyl-(1-4)-β-D-glucopyranoside derivatives (trillin and dioscin respectively) are found in most *Dioscorea* species (Kawasaki *et al.*, 1962; Uomori *et al.*, 1983). Other derivatives of diosgenin that have been isolated include: diosgenin palmitate (*D. colletti* Hook.f.) and an unidentified diosgenin glycoside (*D. polystacha* Turcz.)(Rajaraman *et al.*, 1975).

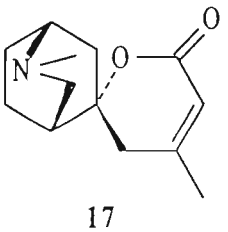
2.1.3.1.4. Group 4: Alkaloids

The aim of this investigation was to elucidate the structure of the compound(s) responsible for the effect of the plant extract on the CNS, therefore a more detailed discussion of the alkaloid composition of *Dioscorea* species is given.

Willaman *et al.* (1953) screened various plants for alkaloids, and found that *D. dregeana* was amongst those that did have an alkaloid component. However, subsequent investigation into the chemistry of this species has apparently not been undertaken.

In 1956, Bevan *et al.* reported the isolation of an alkaloid from *D. dumetorum* ($C_{13}H_{21}O_2N$), which acted as a convulsant poison when injected in mice. The LD_{50} was about 65 mg/kg. A concentration of 10^{-5} M reduced the response to acetylcholine, and enhanced the response to adrenaline on an isolated guinea pig ileum as well as on an isolated rabbit duodenum preparation. The structure of this compound was not determined. Dioscorine (**17**) apparently does not have the same effect (Bevan *et al.*, 1956).

Alkaloids that have been isolated from other *Dioscorea* species include: dioscorine (*D. hirsuta*, *D. hispida* [Pinder, 1952; Leete, 1977]), dihydrodioscorine (*D. dumetorum*, *D. sanziborensis* [Corley *et al.*, 1985]), dioscoretine (**18**)(*D. dumetorum* [Iwu *et al.*, 1990]) and dumetorine (**19**)(*D. dumetorum* [Corley *et al.*, 1985]).



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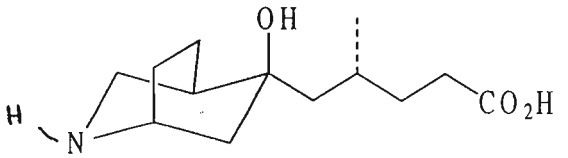

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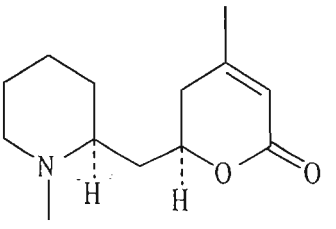

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Fig. 2.1*l*: Alkaloids of *Dioscorea*

Dihydrodioscorine isolated from *D. dumetorum* tubers produced convulsions in mice at doses of 12.5 mg/kg, and lasting hypotension was produced in a cat given intravenous doses of 100 mg/kg. Data obtained from experiments performed on cats and monkeys indicated that this compound had an excitant action on the cerebral cortex (Corley *et al.*, 1985). (It is therefore considered that the alkaloid isolated by Bevan *et al.* [1956] was dihydrodioscorine). These authors also stated that

the saponin dioscin, also isolated from these tubers produced similar convulsions, but was less toxic than the alkaloid.

In 1977, Leete published evidence supporting a scheme for the biosynthesis of dioscorine where nicotinic acid (**20**) was the proposed starting material (Fig. 2.1*m*).

2.1.3.2. Biosynthesis of dioscorine

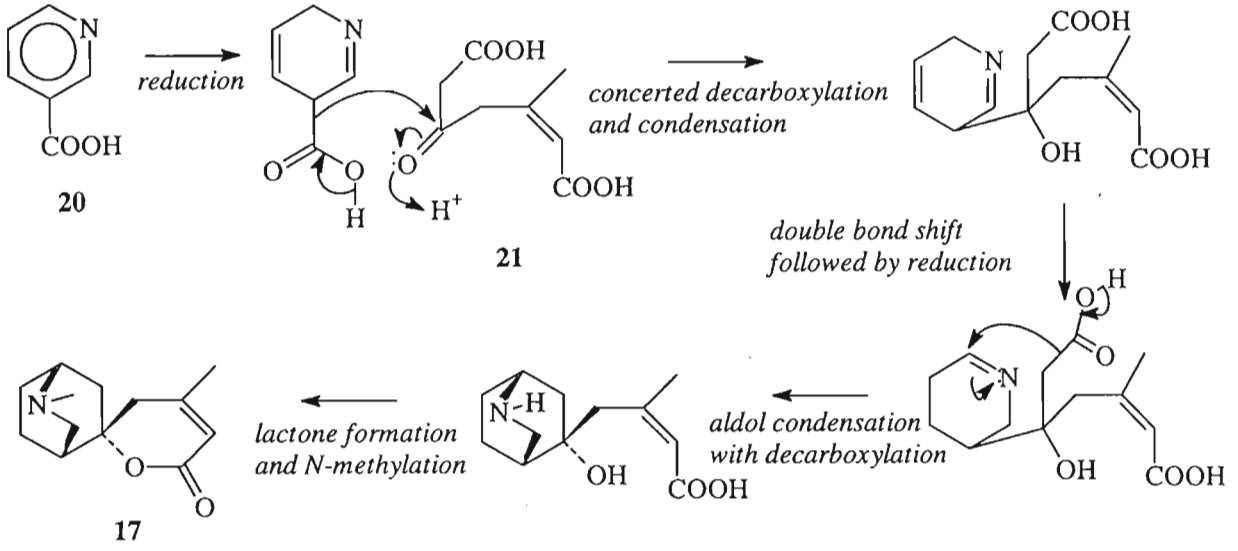

Fig 2.1*m*: Biosynthesis of dioscorine

The initial testing involved feeding nicotinic-[2-^{14}C] acid to *D. hispida* plants. Dioscorine (**17**) was isolated and found to be radiolabelled, therefore nicotinic acid (**20**) had been incorporated. According to Leete (1977), previous experiments had shown that administration of acetate-[1-^{14}C] resulted in radioactive dioscorine which was labelled on alternative carbons of the unsaturated lactone ring. This suggested that one of the initial condensation reagents was the branched 8-carbon unit (**21**) derived from four acetate units. In order to further support the proposed scheme it was necessary to show that all of the radioactivity was located at C-3 of dioscorine (**17**). This was done by a series of degradative reactions (Fig. 2.1*n*)(Leete, 1977).

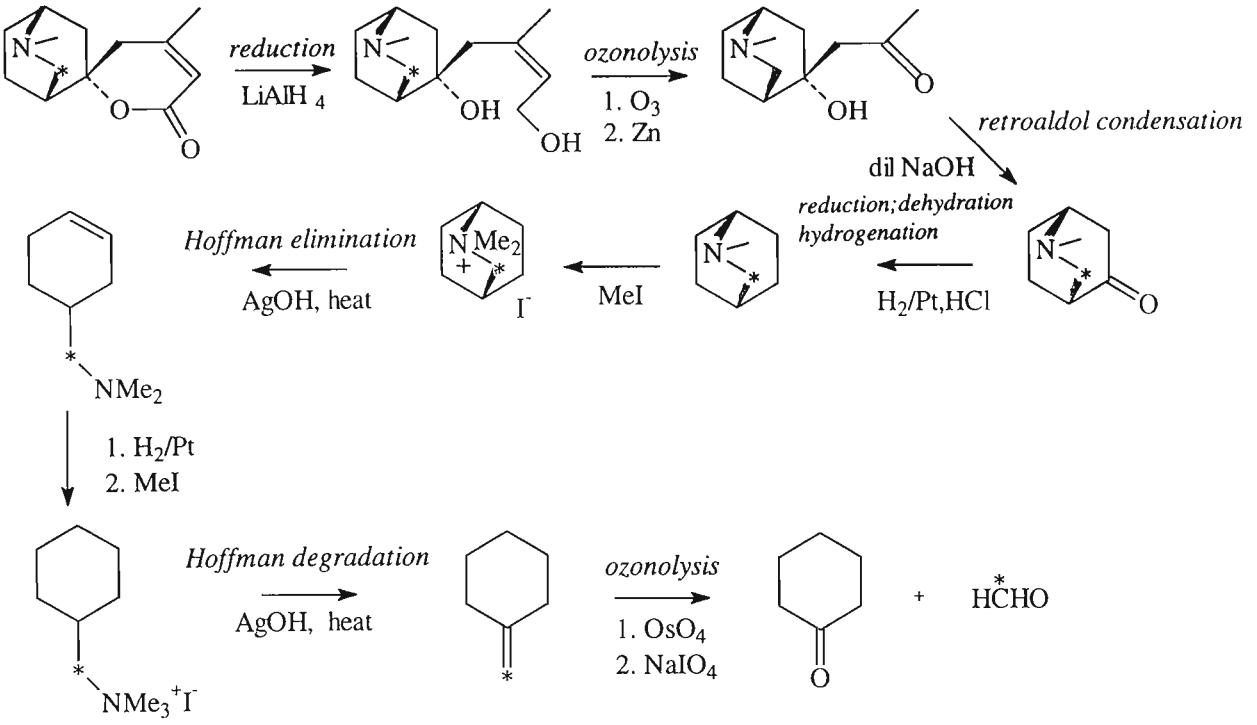

Fig. 2.1*n*: Degradative reactions of dioscorine

Another compound commonly found in *Dioscorea* species whose biosynthesis has been investigated (Seo *et al.*, 1981) is diosgenin.

2.1.3.3. Biosynthesis of diosgenin

Feeding experiments with sodium [1, 2–^{13}C] acetate on tissue cultures of *D. tokoro* yielded the following compounds (Fig. 2.1*o*)(Seo *et al.*, 1981). The bold straight lines represent the inclusion of two radiolabelled carbons from the same acetate group, and the asterisks represent the inclusion of a single radiolabelled carbon (Fig. 2.1*o*).

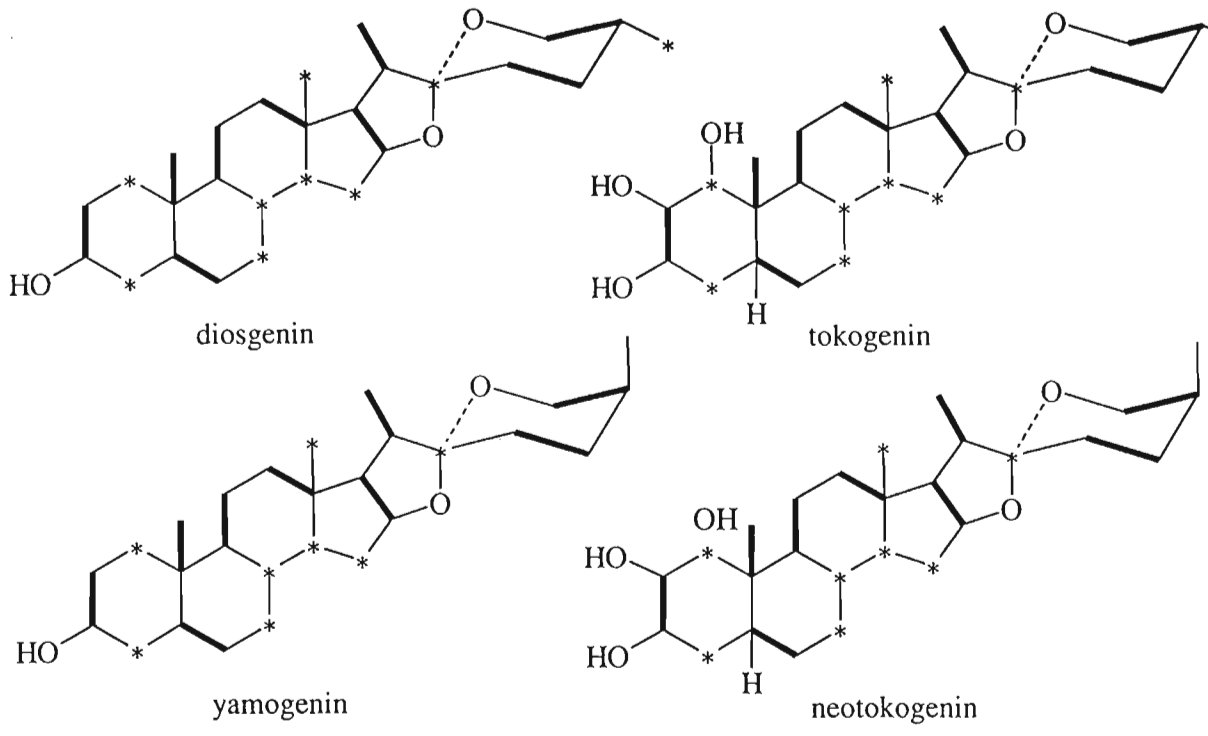

Fig. 2.1*o*: Radiolabelled sapogenins isolated in feeding experiments using [1,2-^{13}C] acetate

These results support the belief that steroidal sapogenins are synthesised in plants *via* the following route: cycloartenol to 26-hydroxycholesterol to sapogenin as shown below (Seo *et al.*, 1981).

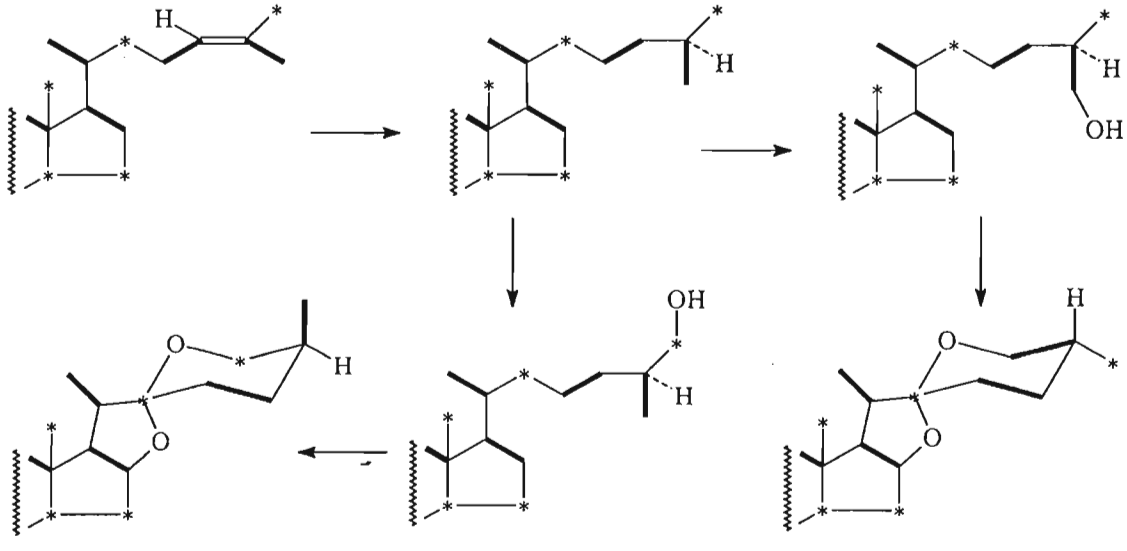

Fig. 2.1*p*: Biosynthesis of sapogenins from cycloartenol

2.1.4. Overview of other biosynthetic pathways pertinent to this chapter

2.1.4.1. Biosynthesis of natalensine

One of the compounds isolated in this investigation was an aromatic alkaloid similar in structure to natalensine. For this reason, the biosynthesis of natalensine has been included in this chapter.

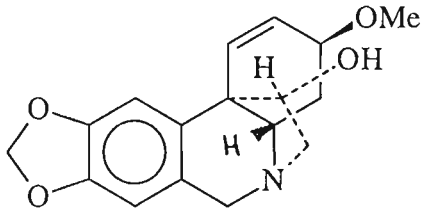

Fig. 2.1*q*: Natalensine

Natalensine is one of many Amaryllidaceae alkaloids, all of which have been hypothesised to form by the oxidative phenolic coupling of diphenyls such as O-methylnorbelladine (**22**)(Barton, 1963).

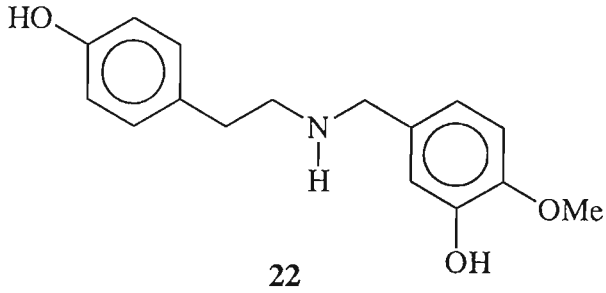

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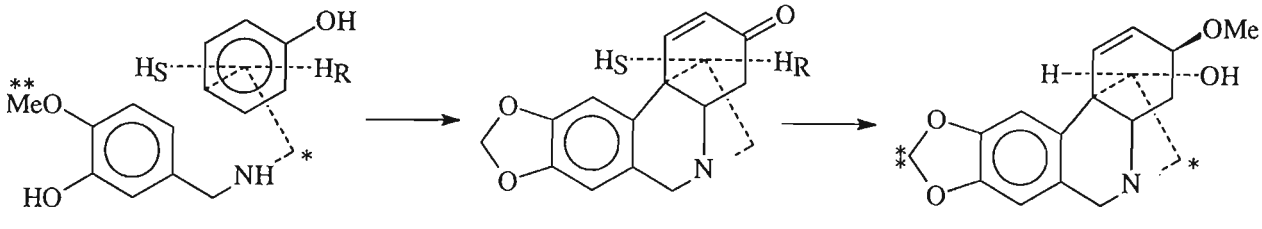
Fig. 2.1*r*: Precursor for the biosynthesis of natalensine

Feeding experiments performed by Barton (1963) using O-methylnorbelladine (**22**) radiolabelled with ^{14}C at position 1 and in the O-methyl group resulted in the formation of radiolabelled natalensine (Fig. 2.1*s*).



22 **23** Radiolabelled natalensine

Fig. 2.1*s*: Result of feeding experiment using radiolabelled O-methylnorbelladine (**22**)

From ths it could be seen that the methylenedioxy group was probably formed by oxidative cyclisation of the methoxy group. The mechanism proposed by the author (Barton, 1963) was that showm in Figure 2.1*t*.

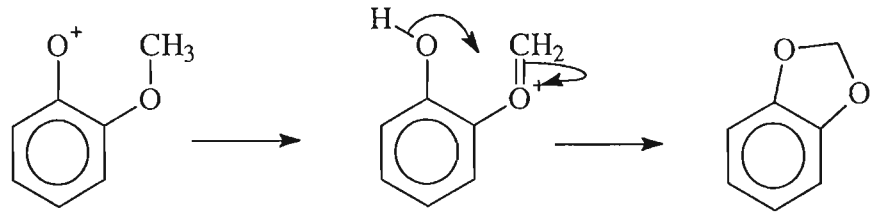


Fig. 2.1*t*: Mechanism for the formation of the methylenedioxy group

Compound **23** has been shown by Battersby *et al.* (1971) to be an intermediate in the biosynthesis of natalensine. It was not isolated in the abovementioned labelling experiment.

In their investigation of the biochemistry of galanthamine (**24**) Barton (1963) found that not only was radiolabelled [1-^{14}C]-norbelladine (**25**) incorporated, but so was (+/-)-[2-^{14}C]-tyrosine (**26**). They were also able to isolate a number of other metabolites that could be considered intermediates in the biosynthesis of (**25**)(Fig. 2.1*u*). It was not stated that these intermediates were definitely the same as those of the natalensine biosynthesis.

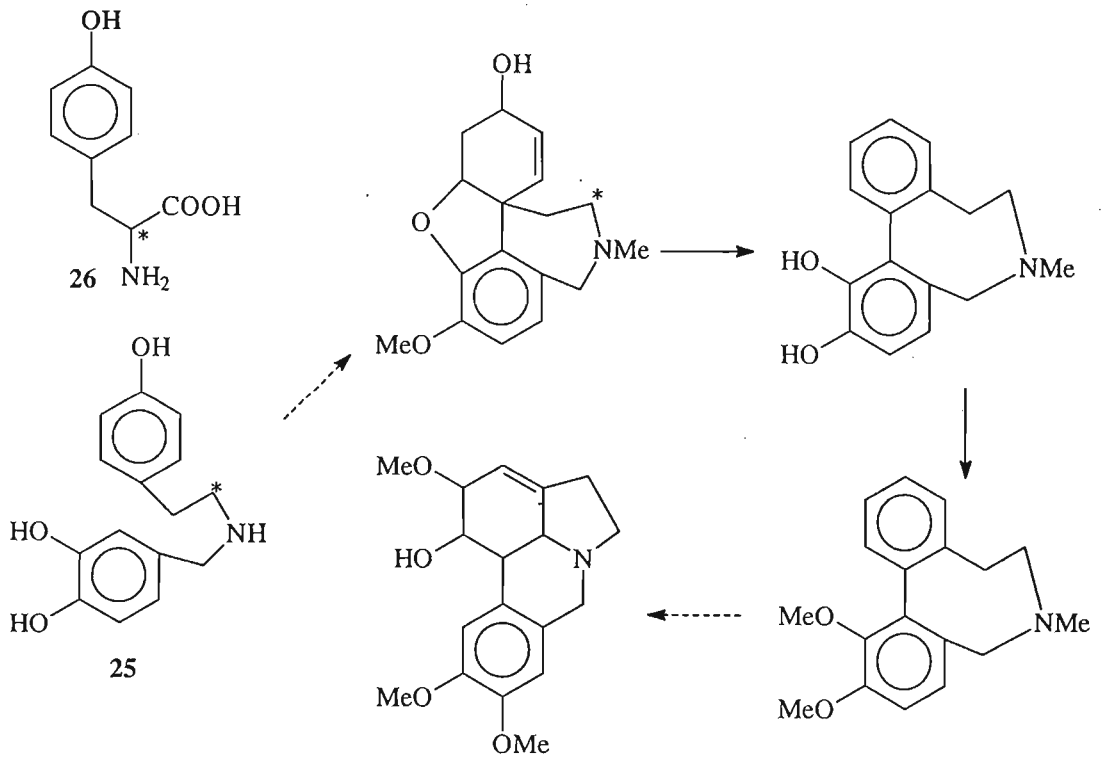


Fig. 2.1*u*: Biosynthesis of galanthamine

Tyrosine (**26**) is an aromatic amino acid and, like many aromatic compounds found in plants, it is a product of the shikimic acid pathway.

2.1.4.2. The shikimic acid pathway

The primary role of the shikimic acid pathway is the biosynthesis of aromatic amino acids. The first reaction is that of phosphoenolpyruvate with erythrose-4-phophate catalysed by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (Matthews and van Holde, 1991). The enzyme isolated from *Pseudomonas aeruginosa* has been resolved into two isozymes both of which are inhibited by tyrosine and tryptophan (both are aromatic amino-acid end-products)(Whitaker *et al.*, 1982). A series of reactions follows to form shikimic acid-3-phosphate (Fig. 2.1*v*)(Matthews and van Holde, 1991).

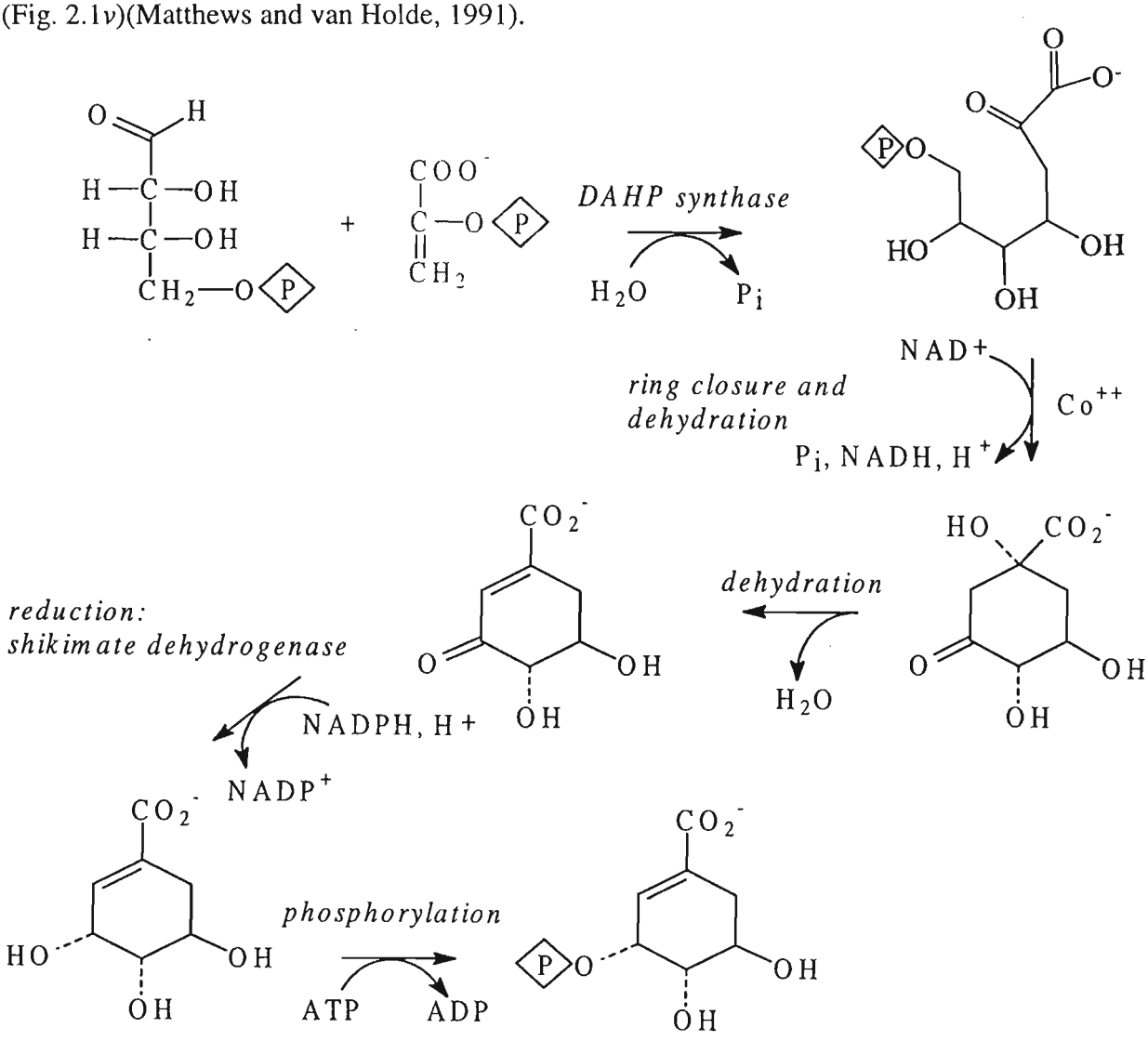


Fig. 2.1*v*: Formation of shikimic acid-3-phosphate

The formation of 5-enoylpyruvylshikimic acid-3-phosphate from shikimic acid-3-phosphate catalysed by 5-enoylpyruvyl shikimate-3-phosphate synthase is thought to proceed by an addition-elimination mechanism in which C-3 of phosphoenolpyruvate transiently becomes a methyl group in the enzyme bound intermediate (Fig. 2.1*w*)(Grimshaw *et al.*, 1982).

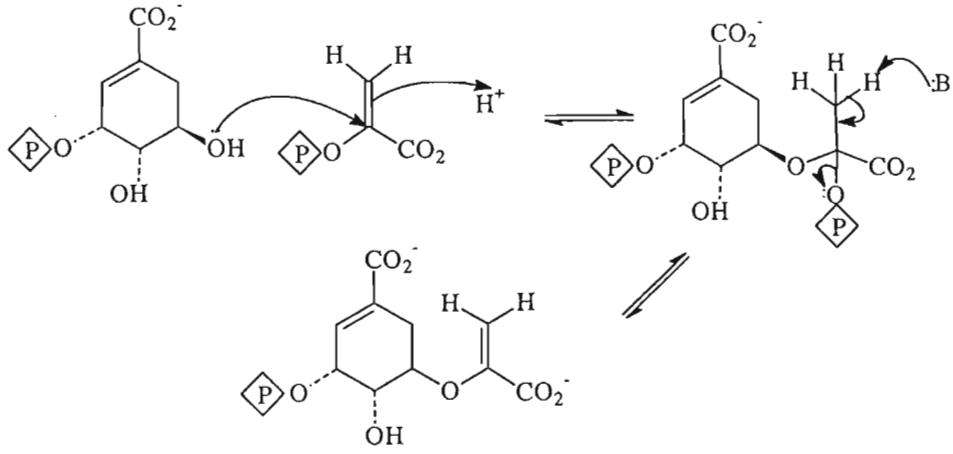


Fig. 2.1*w*: Formation of 5-enoylpyruvylshikimic acid-3-phosphate

Following this reaction is a dephosphorylation step to form chorismate, which subsequently undergoes the reaction catalysed by chorismate mutase shown below. Citrate has been found to be an inhibitor of this reaction (Fig. 2.1*x*)(Dewick, 1984).

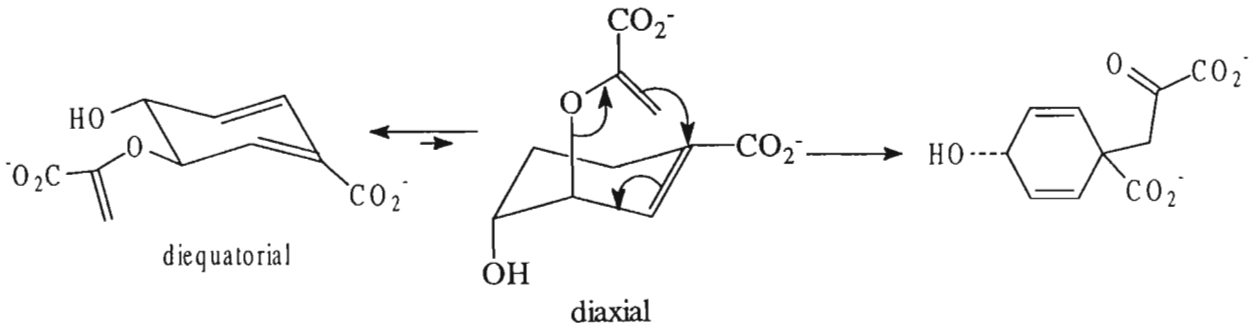


Fig. 2.1*x*: Formation of prephenate from chorismate

The subsequent reaction sequence that the prephenate undergoes depends on whether or not it remains bound to the enzyme (Fig. 2.1*y*)(Dewick, 1984).

One of the compounds isolated in this investigation was a cinnamic acid derivative. This class of compounds is biosynthesized from either phenylalanine or tyrosine. The enzyme phenylalanine ammonia-lyase catalyses the deamination of both of these amino acids (Fig. 2.1*z*).

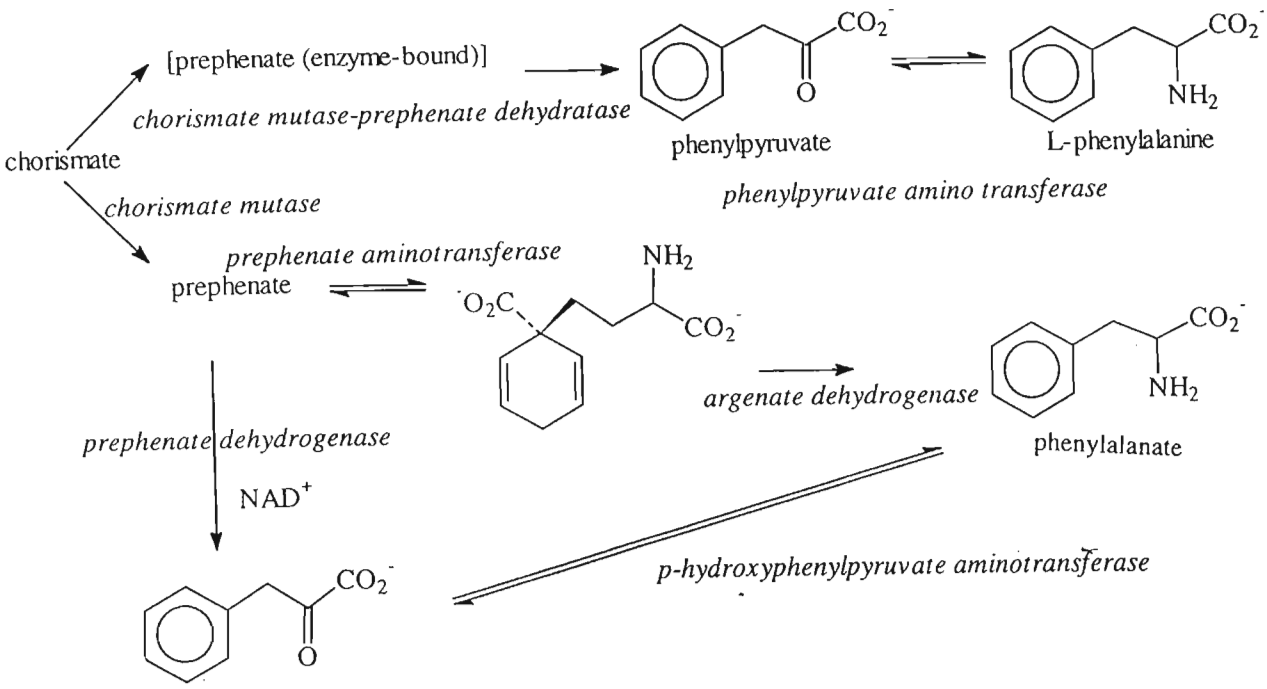


Fig. 2.1*y*: Reactions of chorismate

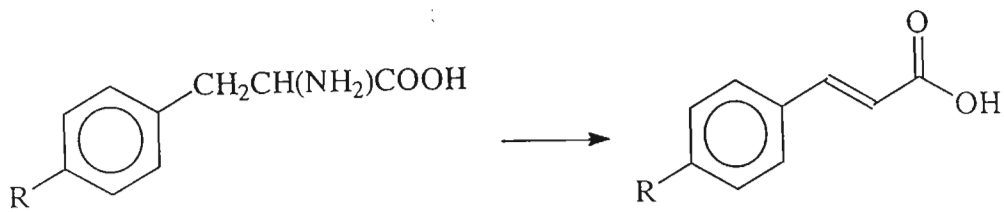


Fig. 2.1*z*: Deamination of aromatic amino acids*

*The enzyme responsible for this conversion is phenylalanine ammonia-lyase.

Hydroxylation of cinnamic acids has been found to be catalyzed by multifunctional phenolases which use molecular oxygen. In these reactions, the NIH shift is observed whereby migration of the hydrogen atom being replaced by a hydroxyl group occurs. This was shown using tritium labelled amino acids (Fig. 2.1a_1)(Vickery and Vickery, 1981a).

Both the phenanthrene and the bibenzyl compounds (*cf. 2.1.3.1.1*) are related to stilbenes. The following series of reactions is thought to occur in the biosynthesis of these compounds (Fig. 2.1b_1)(Dewick, 1984).

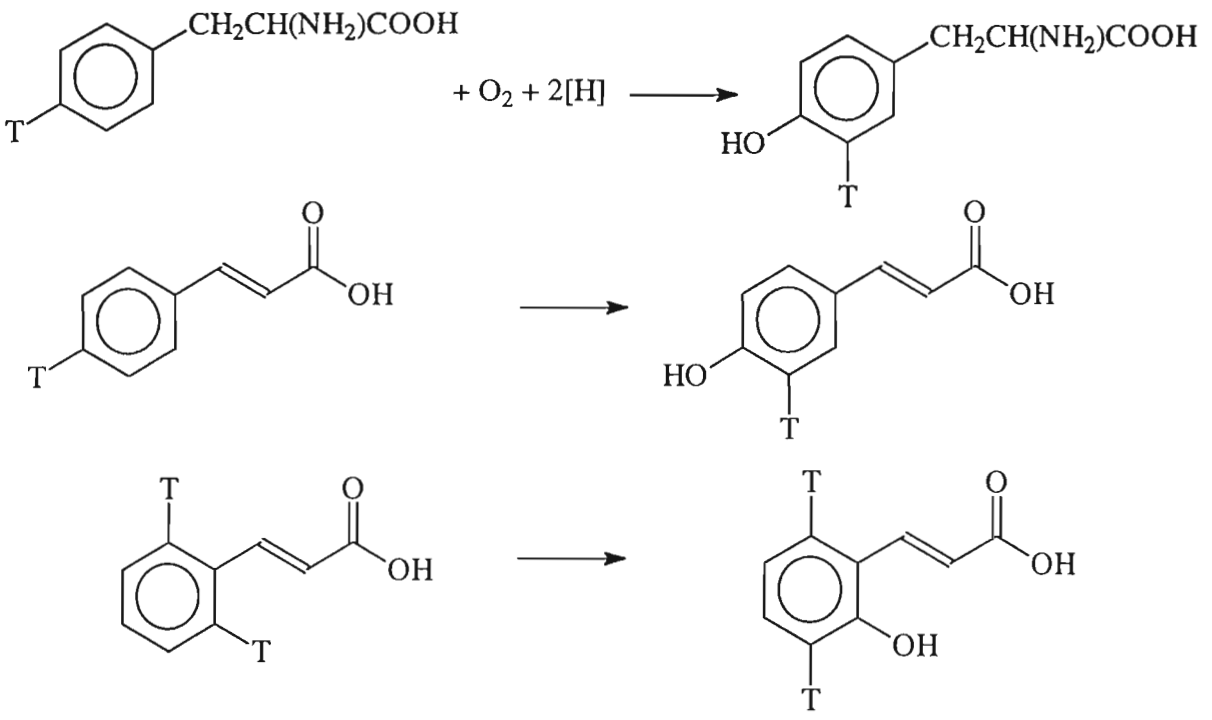


Fig. 2.1a_1: Examples of the NIH shift

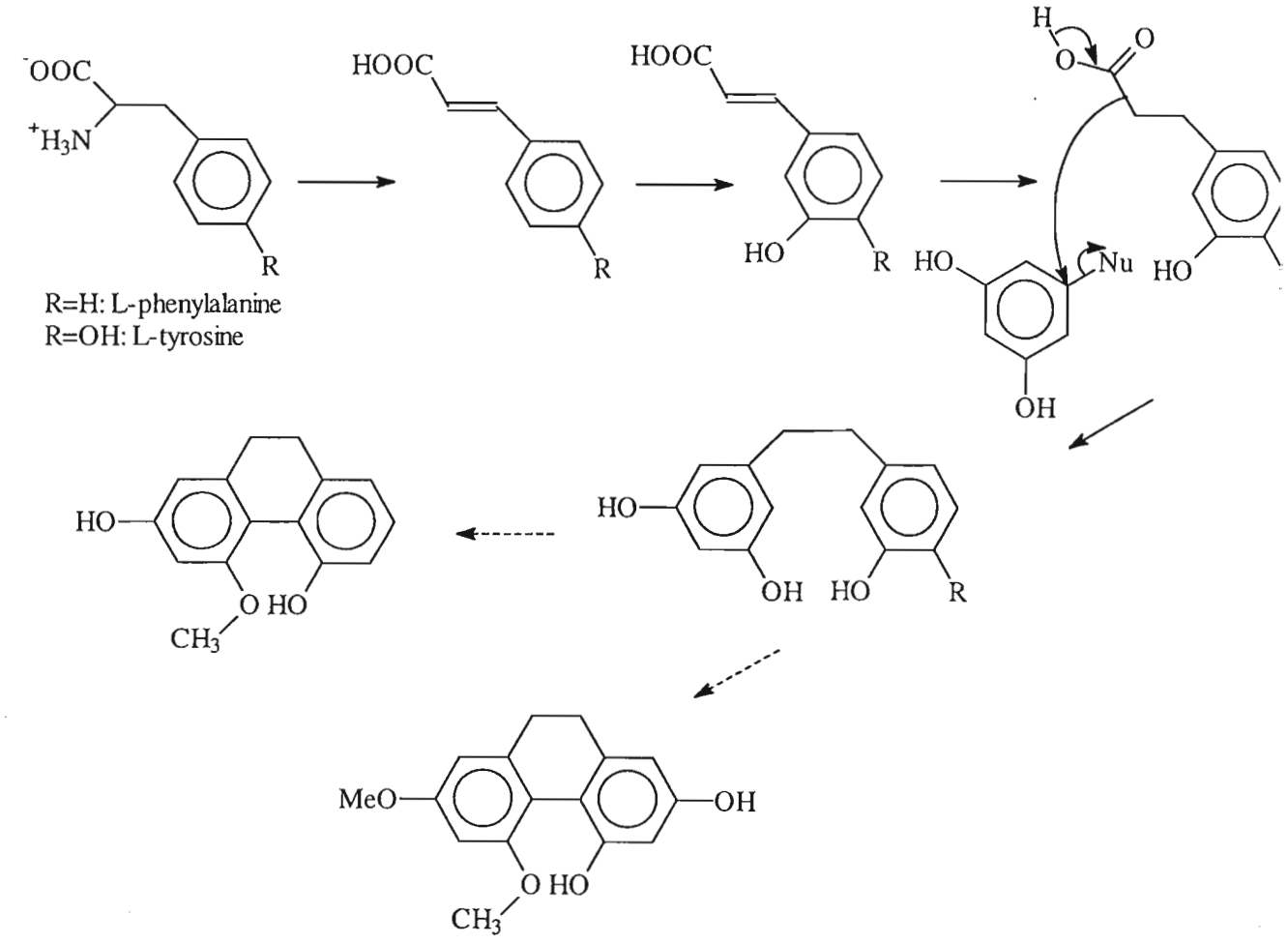


Fig. 2.1b_1: Biosynthesis of dihydrostilbenes

2.2. Results

The following compounds were isolated from *D. dregeana* in this investigation: (1) the alkaloid dioscorine (compound 1), (2) an alkaloid (compound 2), (3) the sterols sitosterol and stigmasterol, (4) a cinnamic acid derivative (compound 3), (5) a stilbene derivative (compound 4) and (6) a furan derivative (compound 5).

In order to elucidate the structure of compound 3, the following compounds were successfully synthesised: (1) the acyl derivative of compound 3, (2) 3-(3′-hydroxy-4′-methoxyphenyl)propenoic acid, (3) 3-(4′-hydroxy-3′-methoxyphenyl)propenoic acid, (4) butyl 3-(3′-hydroxy-4′-methoxyphenyl)propenoate and (5) butyl 3-(4′-hydroxy-3′-methoxyphenyl)propenoate.

In order to elucidate the structure of compound 2, the acetate derivative was made (compound 2A).

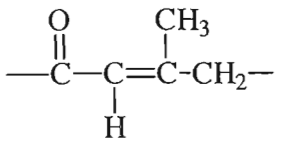
2.3. Discussion

2.3.1. Structure elucidation of compound 1

spectra: pages S1-S10

Compound 1 was isolated as an orange gum, which gave a positive reaction for alkaloids (Dragendorff's reagent). The carbon-13 n.m.r. spectrum showed the presence of two double bond carbons (δ 156.1 and 116.3) and one carbonyl group (δ 164.5). The resonances at δ 156.1 and 164.5 were not present on the DEPT spectrum so these represented non-protonated carbons. The resonance at δ 116.2, however, was shown to represent a =CH carbon atom (*S5*).

There was a sharp infrared absorption at 1707 cm^{-1} and two smaller ones at 1306 and 1243 cm^{-1} suggesting the presence of an α,β unsaturated carbonyl group; and there were two absorptions at 1637 and 1650 cm^{-1} indicating the presence of the double bond (*S10*). From the COSY spectrum (*S3*) it was possible to see that the alkene proton resonating at δ 5.78 (*dd*, J_1=1.29 Hz; J_2=2.60 Hz) was coupled to a proton represented by a broad singlet at δ 1.95 integrating to three protons. This indicated that one of the substituents on the non-protonated double bond carbon was a methyl group. The methyl group was long-ranged coupled to two protons resonating as a pair of doublets (δ 2.53 and 2.50) which were not further coupled. From this information, the following molecular fragment could be deduced:



A singlet at δ 79.2 in the carbon-13 n.m.r. spectrum indicated the presence of a fully substituted carbon alpha to either an oxygen or a nitrogen atom. The resonances at δ 53.0 and 52.5 were shown from the DEPT to represent a $-CH_2-$ and a –CH- group respectively. Their chemical shifts were very similar so they were considered to be in approximately electronically equivalent positions. In the region 0-45 ppm there were seven resonances: one methine carbon (δ 34.0), four methylene carbons (δ 39.4, 38.4, 19.1 and 18.5) and two methyl carbons (δ 41.8 and 23.3). Compound 1, therefore had thirteen carbon atoms.

All four alkaloids reported to have been constituents of *Dioscorea* species have thirteen carbon atoms (dioscorine $C_{13}H_{19}NO_2$; dihydrodioscorine $C_{13}H_{21}NO_2$; dumetorine $C_{13}H_{21}NO_2$ and dioscoretine $C_{13}H_{23}NO_3$). The only two to have α,β-unsaturated carbonyl groups were dioscorine and dumetorine.

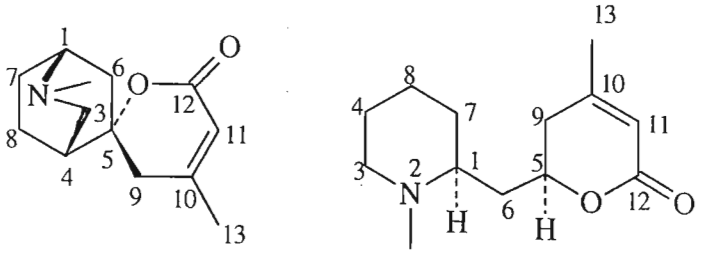


Fig. 2.3*a*: Dioscorine and dumetorine

Compound 1 could not have been dumetorine, because there are only two unprotonated carbons in dumetorine, whereas there are three in compound 1. It seemed reasonable, therefore that compound 1 should have a structure similar to that of dioscorine. Carbon-13 n.m.r. data for dioscorine (Leete, 1977) was compared with that of compound 1 (Table 2*c*).

The carbon-13 chemical shifts for the two compounds were not identical.

From the HETCOR spectrum (*S6, S7*) it could be seen that one of the carbons resonating at δ 18.7 was coupled to two protons, one of which resonated as a multiplet at δ 1.42 and the other

resonated as a multiplet at δ 2.12. This was repeated for all of the carbon resonances and the results are summarised in Table 2*d*.

Table 2*c*: Comparison between the ^{13}C n.m.r. chemical shifts of dioscorine and compound 1 (Leete, 1977)

Carbon Number	**Dioscorine in $CDCl_3$/ppm**	**Compound 1 in $CDCl_3$/ppm**
1	52.4 (CH)	52.5 (CH)
3	53.8 (CH_2)	53.0 (CH_2)
4	35.2 (CH)	33.9 (CH)
5	81.6	79.2
6	39.5 (CH_2)	39.1 (CH_2)
7	20.3 (CH_2)	18.7 (CH_2)
8	19.5 (CH_2)	18.7 (CH_2)
9	40.9 (CH_2)	38.3 (CH_2)
10	155.9	156.1
11	116.5 (CH)	116.3 (CH)
12	165.1	164.2
13	23.3 (CH_3)	23.4 (CH_3)
N-Me	42.6 (CH_3)	41.8 (CH_3)

Table 2*d*: The HETCOR results for compound 1*

Carbon resonance	**Resonance/ppm of the proton(s) to which the carbon is correlated**
52.5 (CH)	2.91 *p* (J=2.10 Hz)
53.0 (CH_2)	3.40 *dt* (J_1=2.79 Hz, J_2=11.97 Hz); 2.55 *dd* (J_1=2.47 Hz, J_2=9.75 Hz)
33.9 (CH)	1.77 *obscured multiplet*
38.3 (CH_2)	2.80 *d* (J=17.70 Hz), 2.50 *d* (J=17.70 Hz)
18.7a (CH_2)	1.50 *m*, 2.12 *m*
18.7b (CH_2)	1.70 *m*, 2.08 *obscured multiplet*
39.1 (CH_2)	2.21 *dt* (J_1=2.49 Hz; J_2=14.75 Hz) and 1.72 *dd* (J_1=2.37 Hz; J_2=14.91 Hz)
116.3 (CH)	5.78 *dd* (J_1=2.60 Hz; J_1=2.60 Hz)
23.4 (CH_3)	1.90 *s*
41.8 (CH_3)	2.45 *s*

*These chemical shifts were taken from the two dimensional spectra

Examination of the COSY and delayed HETCOR spectra of this compound led to the conclusion that this molecule was either dioscorine or the isomer where the stereochemistries at carbon 1 and carbon 4 are inverted. The latter has not previously been isolated, and has been suggested in order to explain the differences in the carbon-13 n.m.r. data between compound 1 and dioscorine. In the biosynthesis of dioscorine (*2.1.3.2*), the cyclisation reaction to form the carbon-1-carbon-6 bond would appear to be restricted to the stereochemistry exhibited by dioscorine. If, however, the reaction preceeding this was not stereospecific (or the enzyme-specified stereochemistry was inverted), the second isomer would result. Unfortunately, the proton n.m.r. data for dioscorine was not available from the literature. Although hydrogenation of compound 1 was successful, the product was lost due to contamination from the rotary evaporator, and because all of the sample was used for this reaction, it was not possible to obtain a mass spectrum.

From the COSY spectrum (*S3, S4*) the following connectivities could be deduced (Table 2*e*).

Table 2*e*: Connectivities deduced from the COSY spectrum of compound 1

Proton number (resonance/ppm)	**Protons to which this proton is correlated***
H-3a (3.4)	H-3b, H-8a (long range), H-4
H-1 (2.91)	H-6a, H-7a, H-6b
H-9a (2.80)	H-13, H-9b
H-3b (2.55)	H-4
H-9b (2.50)	H-13
H-6a (2.21)	H-6b
H-8a (2.12)	H-7a
H-7a (2.08)	H-8b
H-7b (1.70)	H-8b

*All redundant correlations have been omitted, for example: the correlation between H-3b and H-3a already shown by the correlation between H-3a and H-3b.

The delayed HETCOR (*S8, S9*) showed a number of connectivities many of which were over three bonds (Table 2*f*).

Table 2*f*: Correlations seen in the delayed HETCOR of compound 1

Carbon number	**Protons to which these carbons were correlated as seen in the delayed HETCOR**
1	H-1 (1 bond)
3	N-CH_3 (3 bonds)
4	H-3 (2 bonds)
5	H-6a (2 bonds), H-6b (2 bonds)
6	H-4 (3 bonds)
7	H-4 (3 bonds), H-6a (3 bonds)
8	H-3a (3 bonds)
9	H-13 (3 bonds)
10	H-9b (2 bonds), H-9a (2 bonds), H-13 (2 bonds)
11	H-11 (1 bond), H-9a (3 bonds), H-13 (3 bonds)
12	H-11 (2 bonds)
13	H-13 (1 bond)
N-CH_3	N-CH_3 (1 bond)

2.3.2. Structure elucidation of compound 2

spectra: S11-S22

Compound 2 was isolated as a colourless gum that decomposed on standing in pyridine shortly after a portion of a solution of the compound (in pyridine) had been removed for acetylation to form compound 2A. The molar mass of compound 2A was found to be 243.1432 g/mol corresponding to the molecular formula $C_{19}H_{21}NO_5$, therefore, compound 2 had the molecular formula $C_{17}H_{19}NO_4$.

From the carbon-13 n.m.r. spectrum of compound 2 (*S14*), there were nine carbons with chemical shifts greater than 100 ppm. Six corresponded to the carbons of an aromatic ring, two to double-bond carbons and one to a methylenedioxy carbon. There were six carbons in the region δ 56-δ 80 which corresponded to carbons α to either oxygen or nitrogen.

A literature survey of compounds with the required molecular formula was performed and it was proposed that this compound was a crinine-type alkaloid. Comparing the carbon-13 n.m.r. spectrum of this compound with that of many compounds of this type led to the conclusion that

this compound was similar to crinamine and natalensine (Table 2*d*)(the degree of saturation of the carbons was determined using a DEPT spectrum).

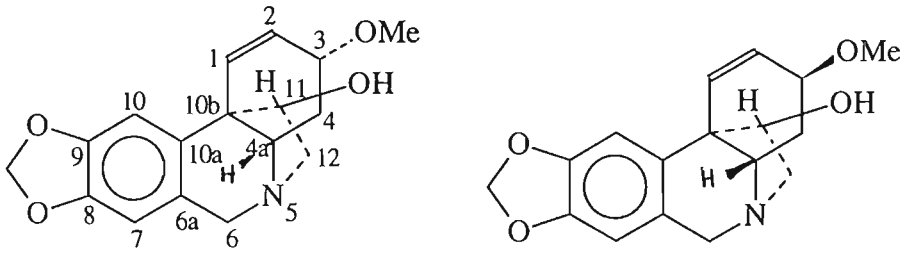


Fig 2.3*b*: Crinamine and natalensine

Table 2*g*: Comparison between the ^{13}C n.m.r. resonances for compound 2, crinamine and natalensine

Carbon Number	**Compound 2 in $CDCl_3$/ppm**	**Crinamine in $CDCl_3$/ppm (Vilidomat *et al.*, 1996)**	**#Natalensine in $CDCl_3$/ppm**
1	134.5 -CH-	136.8 -CH-	135.4
2	126.4 –CH-	123.6 -CH-	127.4
3	72.4 -CH-	76.0 -CH-	72.8
4	27.7 $-CH_2-$	30.1 $-CH_2-$	28.2
4a	63.0 -CH-	66.1 -CH-	63.6
6*	63.1 $-CH_2-$	63.5 $-CH_2-$	62.6
6a	125.5	126.6	126.7
7	106.9 -CH-	106.8 -CH-	106.8
8	146.5	146.1	146.1
9	146.9	146.4	146.5
10	103.5 -CH-	103.1 -CH-	103.3
10a	132.5	135.5	131.9
10b	50.2	50.2	50.1
11	79.5 -CH-	80.0 -CH-	80.0
12*	60.8 $-CH_2-$	61.2 $-CH_2-$	61.3
$-OCH_2O-$	101.0 $-CH_2-$	100.7 $-CH_2-$	100.8
-OMe	56.8 $-CH_3-$	55.6 $-CH_3-$	56.6

*Literature assignments should be interchanged, see discussion of the COSY

#Natalensine sample was kindly lent by K.H. Pegel

In the HETCOR spectrum (*S15*) it could be seen that the carbon atom resonating 134.5 ppm (C-1) in the carbon-13 spectrum was coupled with a proton resonating as a singlet in the proton n.m.r. spectrum at 6.38 ppm. By repeating this process for all the carbons it was possible to propose assignments for all the resonances in the proton n.m.r. spectrum.

Table 2*h*: The HETCOR results for compound 2

Carbon Number	**Coupled proton resonance/ppm**
1	6.38 *s* #
2	6.38 *d* #
3	3.85 *dd*
4	2.15 *dd*
4a	3.40 *m*
6*	3.45 *d*
7	6.47 *s*
10	6.82 *s*
11	4.02 *t*
12*	4.39 *d* and 3.76 *d*
$-OCH_2O-$	5.89 *d*
$-OCH_3$	3.34 *s*

*Values should be interchanged

#These resonances were nearly superimposed, but the presence of a doublet was clear on an expansion of the spectrum.

From the COSY spectrum (*S13*) it was found that the proton resonating at δ 4.39 was coupled to that at δ 3.76. Both of these protons were attached to the same carbon (HETCOR), so this coupling was geminal. The proton corresponding to the H-2 (δ 6.38 *d*) resonance was coupled to the proton resonating at δ 3.85 (H-3) which, in turn, was coupled to the proton resonating at δ 2.15 (H-4), which, in turn, was coupled to the proton resonating as a multiplet at δ 3.40 (H-4a) which was previously assigned to H-6 (extrapolated from Vilidomat *et al.*, 1996). This was not possible, because H-6 had no protonated neighbouring carbons. It was therefore proposed that the assignments of carbon resonances (and those of the protons) 6 and 12 given in the literature (Vilidomat *et al.*, 1996) should be interchanged. Confirming this further was the coupling between the triplet at δ 4.02 (H-11) and the δ 3.40 multiplet. There was long range (or *para*)

coupling between the two singlets at δ 6.82 (H-10) and 6.47 (H-11) as well as between the singlet at δ 6.47(H-7) and the pair of doublets at δ 4.39 and 3.76 (H-12).

On acetylation of this compound, a number of changes occurred, and these are summarised and compared with the corresponding chemical shifts of natalensine and natalensine acetate in Table 2*i*.

While all the chemical shifts changed on acetylation, those that were most notable were those of protons 1,2 and 11. This would strongly suggest that the –OH group was on carbon-11, and that the methoxy group was on carbon-3.

There were two factors that led to the conclusion that this molecule was in fact crinamine. (1) In the literature spectra for those compounds where the hydroxy group on C-11 is in the opposite orientation to that of natalensine and crinamine (i.e. *S* instead of *R*), the two protons on C-6 resonate at δ 4.70 and 4.54 (Pabuccuoglu *et al.*, 1989). This means that one of these protons is deshielded by the hydroxy group which is close to it in space. The stereochemistry at C-11 in compound 2 must therefore be *R*. (2) After acetylation, the proton on C-3 of natalensine was shielded relative to the unacetylated compound due to the presence of the acetate group in close proximity to it. This would not occur if the stereochemistry at C-3 was *R* and not *S*. After acetylation of compound 2 no significant change occurred in the chemical shift of H-3, therefore it is likely that the stereochemistry at C-3 of compound 2 was *R*. According to Dr J.J. Nair, who examined the spectra for this compound, compound 2 is crinamine, but comparative proton n.m.r. data was not available from the literature.*

*The proton n.m.r. data published by Haugwitz *et al.* (1965) was incomplete and certain of those chemical shifts that *were* published were calculated values.

2.3.3. Significance of the presence of the two alkaloids

Compound 1 was an alkaloid typical of the family Dioscoreaceae.

The second alkaloid isolated is more likely to be responsible for the paralytic effects of this plant. This compound was a β–phenethylamine, and as such would be very likely to have an effect on the central nervous system (Lloyd and Andrews, 1986). It is quite interesting to note the similarity between compound 2 and 3,4-methylene-dioxyamphetamine (MMDA or 'ecstacy')(Fig. 2.3*c*).

Table 2*i*: Changes in the ^{1}H n.m.r. chemical shift of compound 2 and of natalensine on acetylation

Proton number	**Resonances of compound 2/ppm**	**Resonances of compound 2A/ ppm**	**Resonances of natalensine/ppm**	**Resonances of natalensine-3-acetate/ppm**
1	6.38 *s*	6.32 *d* (J=10.08 Hz)	6.36 *d* (J=10.0 Hz)	6.28 *d* (J=10.07 Hz)
2	6.38 *d* (J=3.30 Hz)	6.14 *dd* (J_1=4.47 Hz; J_2=9.63 Hz)	6.25 *dd* (J_1=10.0 Hz; J_2=5.0 Hz)	6.17 *dd* (J_1=10.32 Hz; J_2=4.82 Hz)
3	3.85 *dd* (J_1=3.15 Hz; J_2=6.00 Hz)	3.83 *dt* (J_1=1.65 Hz; J_2=4.47 Hz)	3.82 *m*	3.83 *m* ($W_{1/2}$=8.19 Hz)*
4	2.15 *dd* (J_1=3.06 Hz; J_2=9.81 Hz)	1.89 *dd* (J_1=4.83 Hz; J_2=13.65 Hz)	2.11 *ddd* (J_1=13.7 Hz; J_2=4.2 Hz, J_3<1 Hz); 1.96 *dd* (J_1=13.6 Hz; J_2=4.6 Hz)	2.26 *m* ($W_{1/2}$=8.20 Hz); 2.00 *dd* (J_1=4.28 Hz; J_2=13.62 Hz)
4a	3.40 *m*	3.33 *dd* (J_1=13.29 Hz; J_2=4.08 Hz)	3.25 *dd* (J_1=13.6 Hz, J_2=4.2 Hz)	3.48 *m* ($W_{1/2}$=13.86 Hz)
6	4.39 *d* and 3.76 *d* (J=16.86 Hz)	4.33 *d* and 3.69 *d* (J=16.92 Hz)	4.25 *d* and 3.72 *d* (J=16.8 Hz)	3.83 *m* ($W_{1/2}$=8.19 Hz)* and 4.46 *d* (J=16.48 Hz)
7	6.47 *s*	6.44 *s*	6.41 *s*	6.44 *s*
10	6.82 *s*	6.88 *s*	6.74 *s*	6.89 *s*
11	4.02 *t* (J=4.38 Hz)	4.95 *dd*	3.30 *dd* (J_1=13.9 Hz; J_2=6.7 Hz) and 3.19 *dd* (J_1=13.9 Hz; J_2=3.3 Hz)	5.00 *dd* (J_1=3.78 Hz; J_2=6.59 Hz)
12	3.45 *d* (J=9.06 Hz)	3.33 *dd* (J_1=4.08 Hz; J_2=13.29 Hz)	3.96 *dd* (J_1=6.7 Hz; J_2=3.3 Hz)	3.48 *m* ($W_{1/2}$=13.86 Hz)

*Note that H-3 in natalensine acetate has become shielded such that the resonances for H-3 and H-6a are superimposed

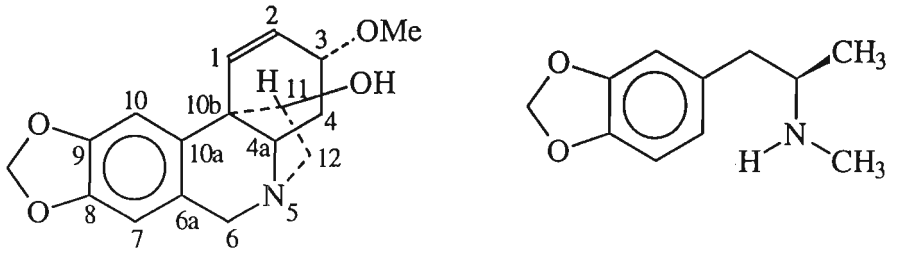


Fig. 2.3*c*: Compound 2 and 'ecstacy'

Crinamine has been shown to be a powerful hypertensive agent in dogs, and to also act as a respiratory depressant (Dictionary of Natural Products on CD-ROM, 1997). It was unusual to isolate this class of compound from a species outside of the Amaryllidaceae family, but from the biosynthesis of natalensine it is not unreasonable that such a biosynthetic pathway might exist in another family.

2.3.4. Structure elucidation of compound 3

spectra: S28-S46

Compound 3 was isolated from the methylene chloride extract as a white amorphous solid, which decomposed after the proton and carbon-13 n.m.r. spectra had been run. The proton n.m.r. spectrum (*S28*) showed resonances typical of a cinnamic acid–like structure namely two doublets at δ 6.27 and δ 7.59 both having a coupling constant of 15.93 Hz corresponding to the *trans*-oriented olefinic protons α and β to a carbonyl function.

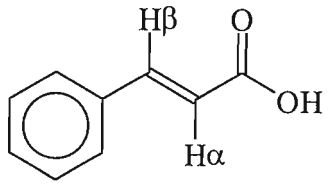


Fig. 2.3*d*: Cinnamic acid

There were three resonances in the aromatic region; each integrating to one proton so the benzene ring was trisubstituted. Examination of the coupling constants [7.06 (*dd*, J_o=8.10 Hz and J_m=1.9 Hz), 7.02 (*d*, J_m=1.83 Hz) and 6.90 (*d*, J_o=8.11 Hz)] suggested the following arrangement of substituents:

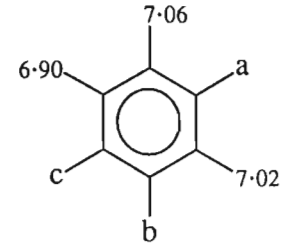


Fig. 2.3*e*: Arrangement of the substituents on the aromatic ring of compound 3*

*The numbers refer to the proton n.m.r. chemical shifts associated with the three aromatic protons

A sharp singlet at δ 3.91 integrating to three protons in the ^{1}H n.m.r spectrum indicated the presence of a methoxy group and a peak at δ 167.37 in the ^{13}C n.m.r. spectrum (*S30*) indicated the presence of an ester. The presence of at least ten carbon resonances in the alkyl region of the ^{13}C n.m.r spectrum suggested that the alcohol moiety of the ester was a long chain alkane.

An attempt was made to acetylate this compound. Even after addition of 4-dimethylaminopyridine (DMAP) and standing overnight, only a negligible amount of the compound had reacted (the reaction was monitored using thin layer chromatography). The compound was then treated with acetylchloride in pyridine and a highly exothermic reaction yielded compound 3A indicating the presence of a phenol group in compound 3. This compound did not decompose.

On acylation a singlet at δ 5.81 integrating to one proton disappeared (the phenolic proton) and a new singlet at δ 2.30 integrating to three protons was noted. A comparison between the proton n.m.r. data for compound 3 and compound 3A is shown (Table 2*j*).

Four NOE experiments (*S32-S35*) were performed on this compound. Irradiation of the methyl protons resonating at δ 3.84 resulted in a positive NOE for the proton resonating at δ 7.09. Irradiation of the doublet at δ 7.61 caused positive NOEs at δ 7.09 and 7.10 as did irradiation of the doublet at δ 6.36. The fact that protons *ortho* to the double bond should give positive NOE signals for both olefinic protons is consistent with literature findings (Atta-ur-Rahman *et al.*, 1997). Irradiation of the acyl methyl protons resulted in a positive NOE only at the methoxy group proton resonance.

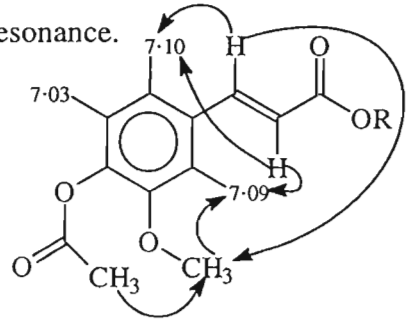


Fig. 2.3*f*: Results of the NOE experiments for compound 3A

Table 2*j*: Comparison between the proton n.m.r. data for compound 3 and compound 3A

Proton	**Compound 3 proton n.m.r. resonances/ppm (*S28*)**	**Compound 3A proton n.m.r. resonances/ppm (*S31*)**
Hα	6.27 *d* (*J*=15.93 Hz)	6.26 *d* (15.94 Hz)
Hβ	7.59 *d* (*J*=15.93 Hz)	7.61 *d* (16.00 Hz)
3 aromatic protons	6.90 *d* (J_o=8.11 Hz)	7.03 *d* (J_o=8.18 Hz)
	7.02 *d* (J_m=1.83 Hz)	7.09 broad singlet
	7.06 *dd* (J_o=8.10 Hz; J_m=1.90 Hz)	7.10 *dd* (J_o=7.63 Hz; J_m=1.71 Hz)
-OH	5.81 *s*	-
$-OCOCH_3$	-	2.30 *s*
$-OCH_3$	3.91 *s*	3.84 *s*
Alcohol moiety	4.17 *t* (*J*=6.71 Hz)	4.17 *t* (*J*=6.71 Hz)
	1.67 *p* (*J*=7.69 Hz)	1.68 *p* (*J*=7.2 Hz)
	0.86 *t* (*J*=6.48 Hz)	0.86 *t* (*J*=6.48 Hz)

In order to confirm the order of the substituents around the benzene ring, a synthesis of the two possible carboxylic acid derivatives was performed, as shown below:

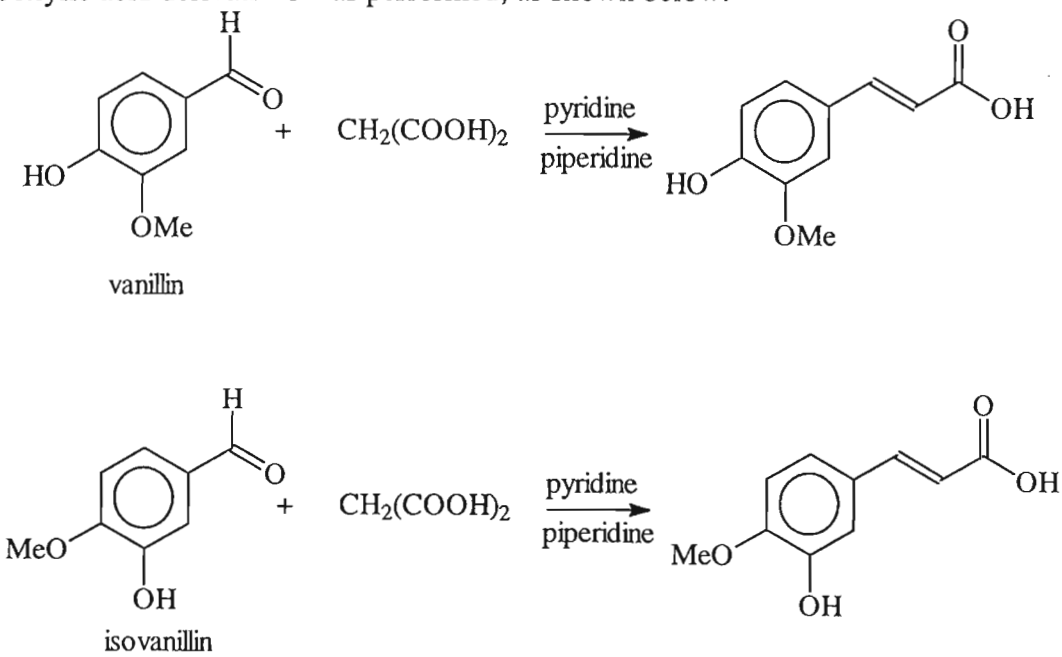


Fig. 2.3*g*: Synthesis of two possible carboxylic acid derivatives

This reaction is the Verley-Doebner modification of the Koevenagel condensation and involves the condensation of aldehydes with malonic acid in pyridine at 100° C with an accompanying decarboxylation. The proposed mechanism is as follows:

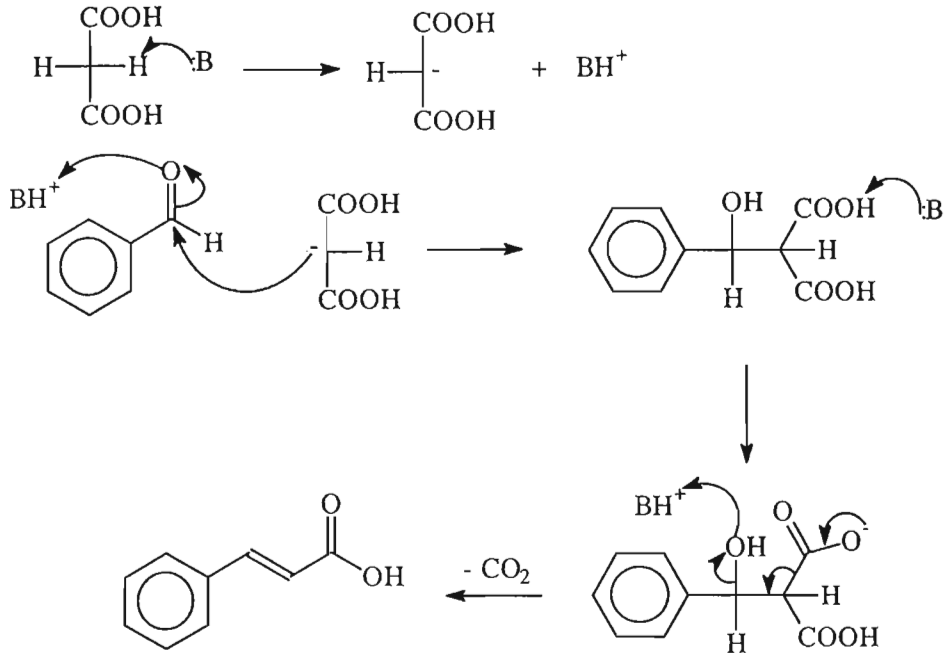


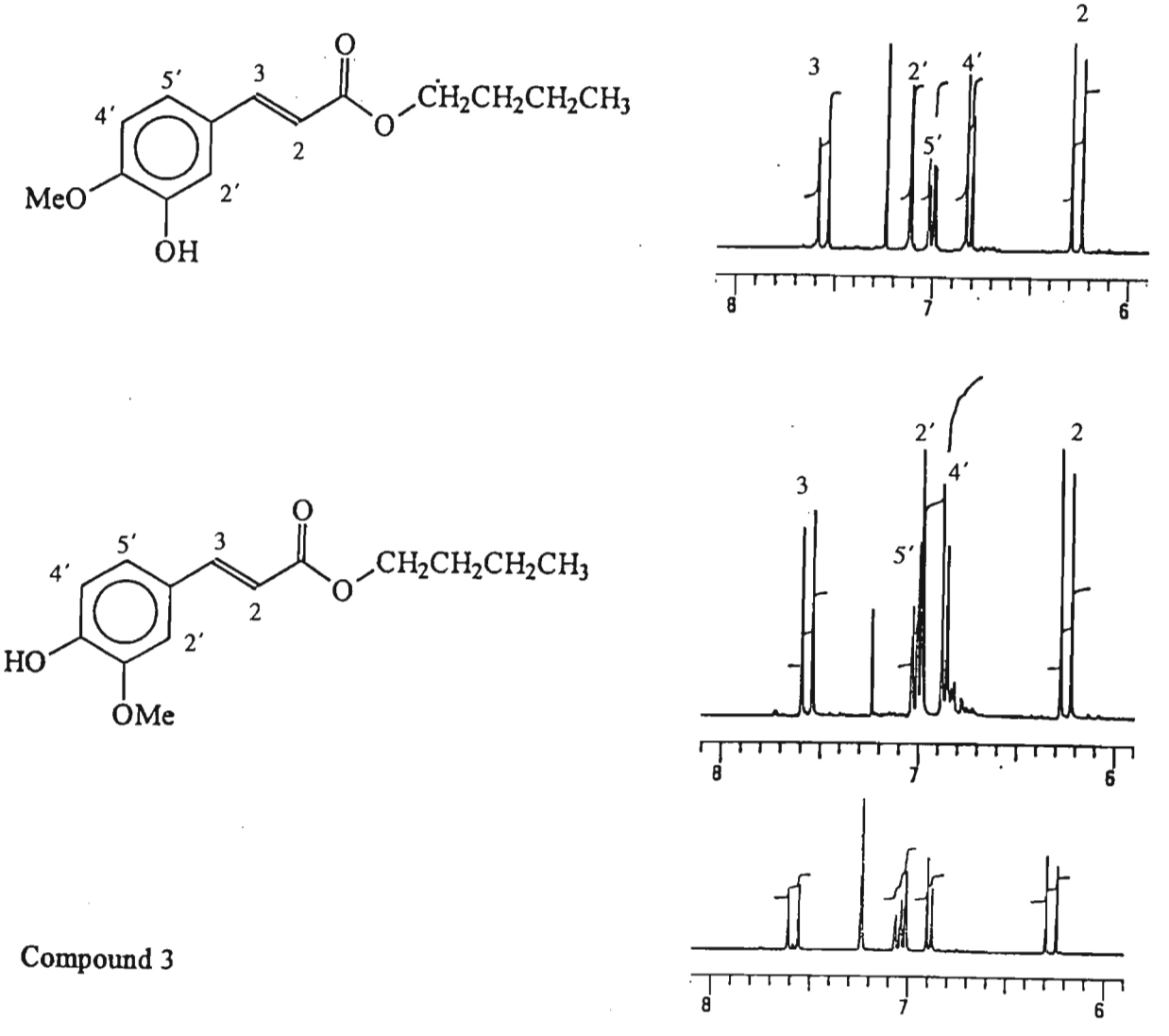
Fig. 2.3*h*: Mechanism for the Verley-Doebner modification of the Koevenagel condensation

Condensations of malonic acid and aromatic aldehydes give rise to the *trans*-cinnamic acid exclusively in all products whose configurations have been determined. In the formation of this isomer it is thought that the relief of steric strain in the intermediate malonic acid plays an important part (Jones, 1967).

The two carboxylic acids formed by this reaction were then butylated. From these results it was possible to see that that compound 3 was an ester of 3-(4'-hydroxy-3'-methoxyphenyl)propenoic acid (Fig. 2.3*i*). All that remained was to determine the length of the alkyl chain. Unfortunatly it was not possible to obtain a high resolution mass for the peak with the highest m/z value.

The highest major peak in the mass spectrum of compound 3A (*S38*) had an *m/z* value of 531. A smaller peak at *m/z* 560 was also evident and thought to be the molecular ion. The peak at *m/z* 531 would then represent the loss of the fragment of 29 ($-CH_2CH_3$). The most intense peak (*m/z* 503) would represent the loss of C_4H_9, a particularly stable ion due to its rearrangement from the straight chain form to the branched (tertiary) ion. The peaks at *m/z* 475 and 407 represented the loss of C_6H_{11} and C_8H_{15} respectively. If the molar mass of this compound was 560 g/mol then the

alkyl chain would have to be $-C_{22}H_{45}$. The absence of doublets in the alkyl region of the carbon-13 n.m.r. spectrum of this compound would suggest that the alkyl chain was not branched.



Compound 3

Fig. 2.3*i*: Comparison between the δ 8-δ 6 region of the proton n.m.r. spectra of the synthetic butyl esters and compound 3

2.3.5. Structure elucidation of compound 4

spectra: S47-S48

Compound 4 was isolated as a colourless gum from the ethyl acetate extract of *D. dregeana.* There were four aromatic resonances in the proton n.m.r. spectrum (*S47*)[δ 7.01 *dd* (2H), 6.70 *dd* (2H), 6.16 *d* (2H), 6.11 *t* (1H)] as well as a multiplet at δ 2.74 (4H). The carbon-13 n.m.r. spectrum (*S48*) showed eight aromatic resonances. A DEPT experiment was performed and these were found to represent four fully substituted carbon atoms [δ 159.2, 156.2, 145.7, 134.2] and four methine carbon atoms [δ 130.4, 116.1, 108.2, 101.2]. There were also two methylene carbon resonances at δ 39.7 and 38.0. After examination of the relative intensities, it was proposed that the peaks at δ 159.2, 130.4, 116.1 and 108.2 each represented two carbons. Calculations were then performed to predict the ^{13}C and ^{1}H n.m.r. spectra of 3,4′,5-trihydroxybibenzyl (Tables 2*k*-2*o*).

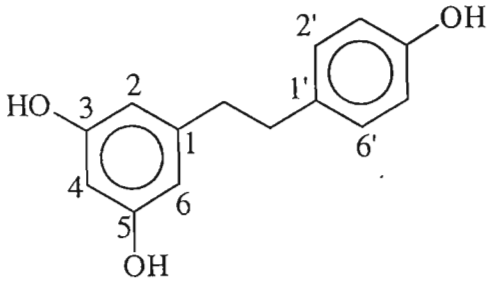


Fig. 2.3*j*: 3,4′,5-trihydroxybibenzyl

Table 2*k*: Influence of a functional group on the chemical shift positions of nearby carbons on a benzene ring (base value: benzene δ 128)(Kemp, 1991)

Substituent	***Ipso***	***Ortho***	***Meta***	***Para***
-OH	+27	-13	+1	-7
-R	+15	0	0	-2

Table 2*l*: Comparison between ^{13}C calculations, the ^{13}C spectrum for compound 4 and the values reported by Majumder and Pal (1993)

Carbon Number	**Calculated Value**	**Compound 4 resonances in CD_3OD/ppm**	**3,4′,5-trihydroxybibenzyl resonances in d_6-acetone/ppm (Majumder and Pal, 1993)**
1	145	145.7	145.0
2 and 6	108	108.2	107.8
3 and 5	156	159.2	157.6
4	100	101.2	100.6
1′	136	134.2	133.6
2′ and 6′	115	116.1	115.4
3′ and 5′	125	130.4	129.6
4′	153	156.3	154.6
α and β		39.7	38.2
		38.0	36.7

Table 2*m*: Shifts in position of benzene protons caused by substituents (base value: benzene δ 7.27)(Kemp, 1991)

Substituent	***Ortho***	***Meta***	***Para***
-R	-0.15	-0.1	-0.1
-OH	-0.4	-0.4	-0.4

Table 2*n*: Comparison between the calculated proton shifts and proton shifts of compound 4

Proton number	**Calculated Value**	**Compound 4/ppm**
H-2 and H-6	6.25	6.11
H-4	6.37	6.16
H-2′ and H-6′	6.72	6.70
H-3′ and H-5′	6.77	7.01

Table 2*o*: Detailed comparison between the ^1H n.m.r. values for compound 4 and 3,4′,5-trihydroxybibenzyl (Adesanya *et al.*, 1989)

Compound 4 in CD_3OD/ ppm	**3,4′,5-trihydroxybiphenyl in d_6-acetone/ppm**
7.01 (2H, *dd*, J_o=8.55 Hz, J_m=2.01 Hz)	6.94 (2H, *dd*, *J*=8.0 Hz and 1.8 Hz)
6.70 (2H, *dd*, J_o=8.37 Hz, J_m=2.20 Hz)	6.51 (2H, *dd*, *J*=8.0 Hz and 1.8 Hz)
6.16 (1H, *d*, J_m=2.14 Hz)	6.21 (3H, *br s*)
6.11 (2H, *t*, J_m=2.17 Hz)	
2.74 (4H, *m*)	2.71 (4H, *br s*)

From the comparison between the chemical shifts for the carbon-13 and proton n.m.r. spectra of compound 3 in $CDCl_3$ and 3,4′,5-trihydroxybiphenyl, it can be seen that chemical shifts are very dependant on the solvent in which the experiments are performed. In the carbon-13 spectra the most notable differences in the chemical shift values were between those carbons with attached hydroxy groups (carbons 3,4′ and 5). This is probably due to hydrogen bonding between the phenol hydrogens and the oxygen of acetone. The largest difference in the proton n.m.r. resonances exists between those of the 2′ and 6′ protons, but it is interesting to note that where coupling constants were reported by Adesanya *et al.* (1989) they were reasonably close to those reported in this investigation.

2.3.6. Significance of the Presence of the Aromatic Compounds

Zhao *et al.* (1994) reported the presence of the docosyl 3-(4-hydroxy-3-methoxyphenyl)propenoate from *Ligularia nelumbifolia* Hand.-Mazz a plant used in Chinese folk medicine for reducing inflammation, for the treatment of coughs and for curing apoplexy as well as tuberculosis. Glycoside esters of this class of compound are also commonly found linked to plant cell wall components and as such have been found to a play a role in plant tissue growth and biodegredation (Hosny and Rosazza, 1997).

Compound 4 belongs to Group 1(b) of compounds isolated from *Dioscorea*. It has been been isolated from *Cannabis sativa* L. (El-Feraly, 1984) and *Bulbophyllum triste* Reichb. f. (Majumder and Pal, 1993) as well as from *D. bulbifera* and *D. dumetorum*. The five other compounds already listed in this group were isolated from *D. batatas* innoculated with the bacterium *Pseudomonas cichorii*. Dihydropinosylvin, 3-hydroxy-5-methoxybibenzyl, 2′,3-dihydroxy-5-methoxybibenzyl (batatasin IV) and 2′-hydroxy-3,5-dimethoxybibenzyl have been shown to be antifungal compounds. Of these dihydropinosylvin (3,5-hydroxybibenzyl) qualified as a phytoalexin, because it was not found in the dormant bulbs (Takasugi *et al.*, 1987).

Both batatasin IV and batatasin V (2′-hydroxy-3,4,5-trimethoxybibenzyl) have been shown to be growth inhibitors (Hashimoto and Tajima, 1978).

The compound 3,4′-dihydroxy-5-methoxybibenzyl isolated from *Cannabis sativa* has been shown to produce effects similar to those produced by oestrogen on rat uteri, i.e. the compound caused an increase in size and mass of the rat uteri (Wirth *et al.*, 1981). These authors did not report the isolation or the testing of 3,4′,5-trihydroxybibenzyl.

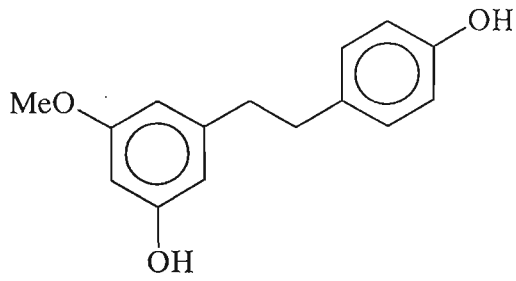


Fig. 2.3*k* :3,4′-dihydroxy-5-methoxybibenzyl

2.3.7. Structure Elucidation of Compound 5

spectra: S49-S55

Compound 5 was isolated as a clear colourless gum from the chloroform extract of the hydrolysis reaction (a portion of the methanol extract was hydrolysed with the aim of investigating the presence of sapogenins; no sapogenins were isolated). The proton n.m.r. spectrum (*S49*) revealed five peaks:

- A singlet at δ 9.55 which indicated the presence of either a carboxylic acid or an aldehyde proton. The peak integrated to one.
- A pair of doublets at δ 7.19 and 6.49 which had coupling constants of 3.6 and 3.9 Hz respectively. These peaks integrated to one proton each.
- A singlet at δ 4.69 which integrated to two protons; and
- A broad singlet at δ 1.22 which was thought to represent a hydroxy group proton.

The carbon-13 n.m.r. spectrum (*S54*) indicated the presence of six non-equivalent carbon atoms in the molecule:

- A doublet at δ 177.7 corresponding to an aldehyde carbon atom.
- Two singlets at δ 160.6 and 152.4 respectively, which indicated the presence of two unsaturated, fully substituted carbons.
- Two doublets at δ 122.8 and 110.0 respectively, which indicated the presence of two unsaturated, unsubstituted carbons.
- A triplet at δ 57.7 which corresponded to a $-CH_2O-$ carbon atom.

Three NOE experiments (*S51-S53*) were performed on this compound. Irradiation of the protons resonating at δ 4.69 resulted in a positive NOE at δ 6.49. Irradiation of the proton resonating at

δ 6.49 resulted in positive NOEs at δ 4.69 and 7.19. Irradiation of the proton resonating at δ 9.55 resulted in a positive NOE at δ 7.19.

On the basis of this information, the following structure was proposed:

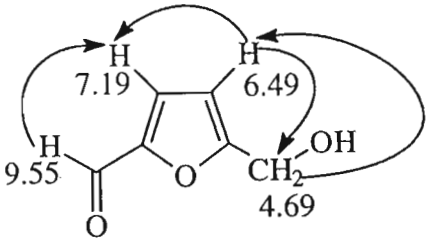


Fig. 2.3*l*: 5-hydroxymethyl-furan-2-carbaldehyde*

*The numbers refer to the ^1H n.m.r. resonances of the protons and the arrows refer to the results of the NOE experiments.

A comparison of the n.m.r. data for this compound (5-hydroxymethyl-furan-2-carbaldehyde) and the literature data are shown (Table 2*p*).

The expected molecular ion (obtained by high resolution mass spectroscopy) was *m/z* 126. While there was a peak at this value, there was also a peak at *m/z* 234 indicating the possible presence of the product of the condensation of two molecules of compound 5 shown below:

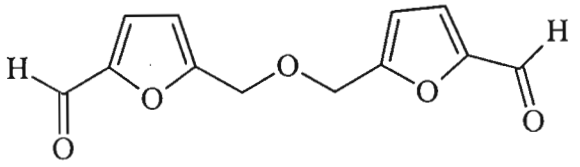


Fig 2.3*m*: The product of the condensation of two molecules of compound 5

The high resolution mass of the cation at *m/z* 126 (*S55*) was 126.0307 μ which supports the molecular formula $C_6H_6O_3$ (126.0317 μ). The presence of a peak at *m/z* 109 is consistant with the loss of a hydroxy group, and *m/z* 97 is consistant with the loss of an aldehyde group from this compound. The loss of hydrogen to form a cation at *m/z* 125 is also typical of the fragmentation of aldehydes.

The loss of an aldehyde group from the product of condensation was indicated by the cation at *m/z* 205. It is theorised that this compound formed in the mass spectrometer by the following reaction:

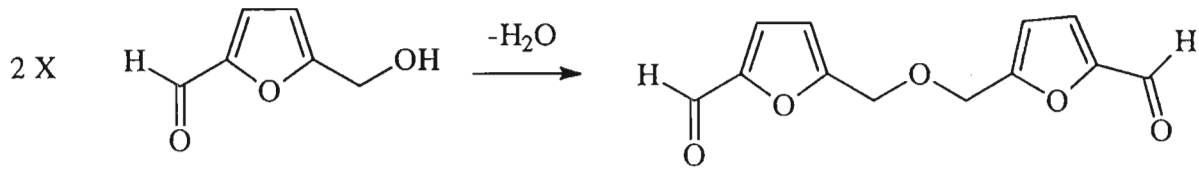


Fig. 2.3n: Dehydration reaction of compound 5

The fact that the reverse reaction would require water (water is unlikely to be present in the high vacuum of the mass spectrometer) and that the cation represented by the peak at *m/z* 126 was the one of highest concentration led to the conclusion that 5-hydroxymethylfuran-2-carbaldehyde was the isolated compound. The presence of a hydroxy stretch absorption in the infrared spectrum (*S50*) also confirmed this.

Table 2*p*: Comparison between the ^{1}H n.m.r and ^{13}C n.m.r. chemical shifts of compound 5 and 5-hydroxymethyl-furan-2-carbaldehyde

Hydrogen	**Compound 5 proton n.m.r resonances in $CDCl_3$/ppm**	**5-hydroxymethyl-furan-2-carbaldehyde proton n.m.r. resonances in $CDCl_3$/ppm (Timko *et al.*, 1977)**
$-CH_2-$	4.69 *s*	4.63 *s*
H-4	6.49 *d*, *J*=3.9 Hz	6.48 *d*, *J*=3.6 Hz
H-3	7.19 *d*, *J*=3.6 Hz	7.18 *d*, *J*=3.6 Hz
-COH	9.55 *s*	9.54 *s*
Carbon	**^{13}C n.mr. resonances in $CDCl_3$/ ppm**	**5-hydroxymethyl-furan-2-carbaldehyde ^{13}C n.m.r. resonances in $CDCl_3$/ppm (Hearn, 1976)**
2	152.4	152.1
3	122.8	124.9
4	110.0	110.4
5	161.6	160.6
2′	177.7	178.5
5′	57.7	57.0

Chapter 3: Extractives from *Avonia rhodesica*

3.1. Introduction

3.1.1. Taxonomy of Anacampserotae

Recently, the tribe Anacampserotae, of the family Portulacaceae was revised by Rowley (1994) who subdivided the genus *Anacampseros* L. into two genera: *Anacampseros* L. and *Avonia* (Mey. Ex Fenzl) Rowl. according to the criteria included in Table 3*a* recognising the taxa included in Table 3*b*.

Table 3a: The taxonomic criteria that led to the division of the genus *Anacampseros* into the two genera *Anacampseros* and *Avonia* (Rowley, 1994)

Classification	Tribe: Anacampserotae	
	Genus: *Anacampseros*	
	Section: *Anacampseros*	Section: *Avonia*
Habit	Rosettes without visible internodes	Overlapping scales without visible internodes
Stems	Thick and fleshy	
Leaves	Alternate	
	Terete to elliptical in section	
	Exposed	Minute and hidden
	With or without papillae	No papillae
Inflorescence	One or a few flowers on branched scapes with scattered bracts	Flowers solitary at stem tips
Anthesis	Pomeridian	
Stamen number	5-45	5-80
Fruits	Exocarp separating from endocarp and falling leaving seeds in a "basket"	
Seeds	Wingless or with low wings	Not winged
Distribution	South Africa	Africa: Cape, Zimbabwe and Somalia (or Yemen [Steffens, 1995])

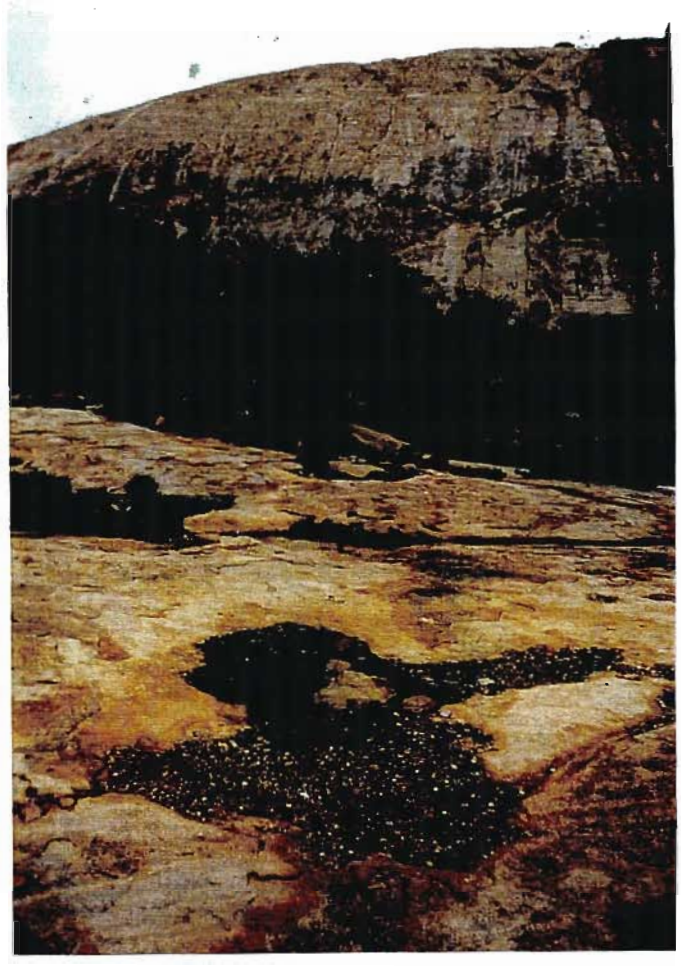


Fig. 3.1*a*: *above*: The natural habitat of *Avonia rhodesica* (University of the North, Pietersburg)
below: *Avonia rhodesica* present on a basolith behind the University of the North, Pietersburg*

*Photographs taken by N.R. Crouch

Table 3*b*: Recognised taxa in *Avonia* and *Anacampseros* (Tribe Anacampserotae)(Rowley, 1994; Steffens, 1995)

New Generic Classification	***Anacampseros***	***Avonia***
Recognised Species (Steffens, 1995)	*A. arachnoides* (Haw.) Sims *A. baeseckei* Dinter *A. bayeriana* Hammer *A. comptonii* Pillans *A. dielsiana* Dinter *A. filamentosa* (Haw.) Sims *A. lanceolata* (Haw.) Sweet *A. marlothii* V.Poelln. *A. subnuda* V.Poelln. *A. telephiastrum* DC	***A. albissima*** (Marl.) Rowley *A. dinteri* (Schinz) Rowley *A. herreana* (V.Poelln) Rowley ***A. papyracea*** (E.Meyer ex Fenzl.) Rowley ***A. quinaria*** (E.Meyer ex Fenzl.) Rowley *A. recurvata* (Schonl.) Rowley ***A. rhodesica*** (N.E.Br.)Rowl. *A. ruschii* (Dinter & V.Poelln.) Rowley ***A.ustulata*** (E. Meyer ex Fenzl.) Rowley

Those species given in bold type are those reportedly used as additives in "honeybeer" (Steffens, 1995). This usage is also indicated by the common (Afrikaans) names given to these plants, e.g. *A. albissima* is called "kareemoer": "karee" being the name of the honeybeer and "moer" meaning leaven or yeast (Steffens, 1995). Given that all additives belong the genus *Avonia*, it is possible that a chemical distinction exists between *Avonia* and *Anacampseros*. Characterisation of this compound (or class of compound) and subsequent chemotaxonomic assessment of *Anacampseros* (*sensu lato*) would substantiate or challenge the most recent revision of the Anacampserotae (Rowley, 1994). As indicated (Table 3b), *Avonia rhodesica* (N.E. Br.) Rowl. is one such taxon known to be used in beer making (Brown, 1914) and was investigated in the current study for its narcotic constituent(s) responsible for potentiating native brews.

3.1.2. Description of *Avonia rhodesica*

Avonia rhodesica (NE. Br.) Rowl. is a succulent which, according to Steffens (1995) and Gerbaulet (1992), occurs in Zimbabwe, South Africa north of the Vaal river and Yemen (or Somalia [Rowley, 1994]). Its aerial stems are so closely packed with leaves that the internodes are not exposed. The roots are not typically tuberous, although a basal caudex may be present. The green leaves which are less than twice as wide as they are thick, are only slightly exposed being hidden beneath imbricate papery scales or stipules which make the shoots appear white (Fig. 3.1*a*)(Rowley, 1994; Steffens, 1995). According to Marloth (1913), *Anacampseros* plants show a striking similarity to their surroundings and are therefore good examples of protective mimicry among plants. This characteristic of the genus was described in Burchell's (1824) account of his finding *Anacampseros filamentosa* (Haw.) Sims (*sensu Anacampseros lanigera* Burch.):

> *"....but I discovered almost accidentally, happening to sit down on the ground close by them, two small plants, the singularity of which consisted in their being so exactly of the colour of white limestone on which they grew, that scarcely any eye could have noticed them in walking by..."*
> *"Experience teaches, that many curious and minute plants will escape detection, unless sought out with more than ordinary attention."*
> Burchell (1824) Travels in the Interior of Southern Africa.

Marloth (1913) suggested that the stipules retard the transpiration of the delicate leaves and protect them from the rays of the sun by diffusing the light before it reaches the photosynthetic tissue, and stated that the stipules also absorb rain and dew. The flowers occur singly at the stem tip on short pedicels (Rowley, 1994).

3.1.3. Ethnobotany of *Avonia rhodesica*

It has been reported that *Avonia* species are readily eaten when found by grazing animals (Marloth, 1913), and that most *Anacampseros* and *Avonia* species can be consumed by humans (van Jaarsveld, 1987). The name given to *A. rhodesica* by the people native to Zimbabwe is qilika and its extensive use in the manufacture of intoxicating liquors was reported in the original description of the species by Brown (1914).

In August 1916, Ordinance no. 10 known as the 'Qilika Act' was issued following the debate in the Legislative Council of Rhodesia beginning on the first of May of that year (Qilika Ordinance, 1916). Evidence was put forward by Dr Mackenzie of Hartley who described how qilika was prepared from golden syrup, sugar, a little hops and qilika root which had been specially prepared (no information regarding this preparation was given). He said that fermentation took place rapidly and that in the course of a few hours the beer was ready for use. The beverage contained only a very small proportion of alcohol and its effects, which were attributed to the qilika root, were as follows:

> *"One to two cups resulted in great excitement and loss of reasoning power. As the dose was increased, incoordination became marked until a state of stupor intervened which could lead, in turn, to a comatose condition. After moderated quantities, the state of excitement was transient, but affected the individual's ability to work effectively, making him stupid for a considerable time as well as causing him to complain of headaches."*

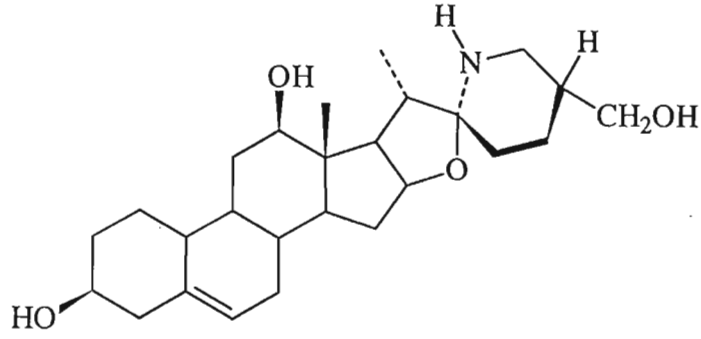
The following two passages are quoted directly from the report and give valuable information regarding the trade and the nature of this product.

> *"I consider that qilika is a most dangerous substance on account of the rapidity of its action and the violence of the excitement it produces. I do not think it should be regarded like kaffir beer, but rather as a noxious drug for the suppression of which special legislation is necessary."*

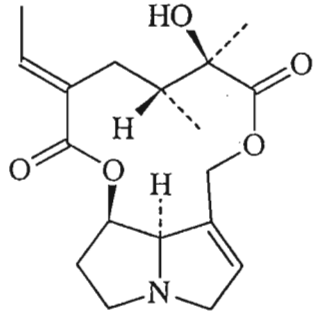
> *" ...different varieties of qilika are found in this country, but natives inform me that it is usually imported from the south already prepared. The root in the prepared state is an expensive commodity, and boys of the driver type, usually Xosas* (sic)*, are said to be the principal vendors..."*

The Act to prohibit the sale, possession or the use of qilika and other harmful substances defines qilika as the *"plant or shrub commonly called qilika or tirika under whatsoever designation known and shall include any part of such plant or shrub, any extract therefrom or any preparaion thereof, and any liquor or substance with which such plant, shrub, part, preparation or extract is mixed"* showing that at the time of writing, very little information about the plant used had been obtained.

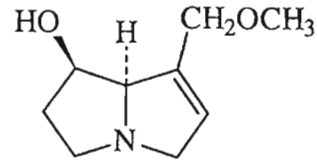
According to Watt and Breyer-Brandwiyk (1932) and Brown (1914), the natives of Rhodesia used *Anacampseros rhodesica* in the treatment of blackwater fever, malaria, blood poisoning, anthrax and dysentry; and the plant was reported to be poisonous. Other plants used in the treament of blackwater fever include: *Cannabis sativa* L., *Solanum nigrum* L., *Lippia asperifolia* Reichb., *Crotalaria burkeana* Benth., *Piliostigma reticulatum* [DC] Hochst, *Cassia fistula* L. and *Pterocarpus angolensis* (Dornan, 1916). This is of interest as *S. nigrum* is known to contain alkaloids (e.g. spirosol-5-ene-3,12,27-triol); and while *Crotalaria burkeana* and *Cassia fistula* have apparently not been chemically investigated, members of their respective genera have been shown to contain alkaloids (e.g. 15*E*-senecion and O^9-methylretronecine from *Crotalaria* Dill. ex Linn. species and chaksine and cassine from *Cassia absus*)(Southon and Buckingham, 1989).



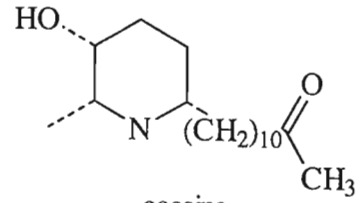
spirosol-5-ene-3,12,27-triol



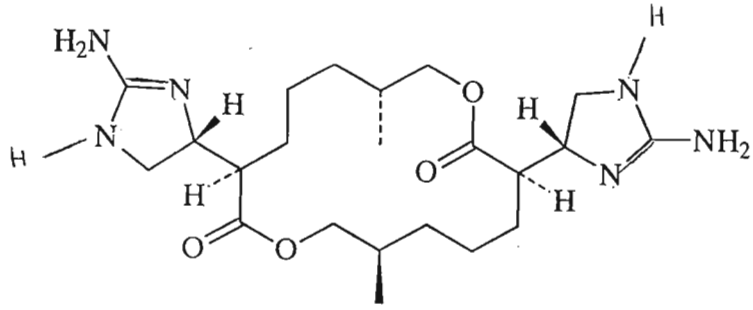
15*E*-senecion



O^9-methylretronecine



cassine



chaksine

Fig. 3.1*b*: Alkaloids of plants used in the treatment of black water fever

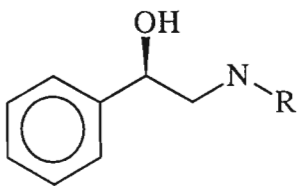
From the ethnobotanical information, it appeared likely that *Avonia* species and especially *A. rhodesica* should contain alkaloids (or other compounds) having an effect on the central nervous system.

Alkaloids have been isolated from other members of the Portulacaceae and these will be discussed in the overview of the chemical investigations of this family.

3.1.4. Brief overview of the previous chemical investigations of the family Portulacaceae

Of the genera of the Portulacaceae family, the one that has been most extensively investigated chemically is *Portulaca* Linn. (In fact none of the remaining genera are listed in the Dictionary of Natural Products [1994]). The compounds isolated from this genus could be divided into three main groups (1) β-phenethylamines, (2) betalaine pigment alkaloids and their precursors and (3) clerodane derivatives.

Group 1: β-phenethylamines: Noradrenaline (**1a**) and adrenaline (**1b**) have been isolated from *P. oleracea* Linn. and *P. grandiflora* Hook. respectively (Endress *et al.*, 1984; Smith *et al.*, 1977).



1a R=H
1b R=CH_3

Fig. 3.1*c*: β-phenethylamines

Group 2: Betalaine pigments and their precursors: Portulacaxanthins I (**2**), II (**3**) and III (**4**) have been isolated from *P. grandiflora*, as well as the compounds betalamic acid (**5**), betanidin (**6**), indicaxanthin (**7**), vulgaxanthin I (**8**) vulgaxanthin II (**9**) and humilixanthin (**10**)(Kimler *et al.*, 1971; Strack *et al.*, 1987). Dopaxanthin (**11**) has been found to be a constituent of a number of *Portulaca* species.

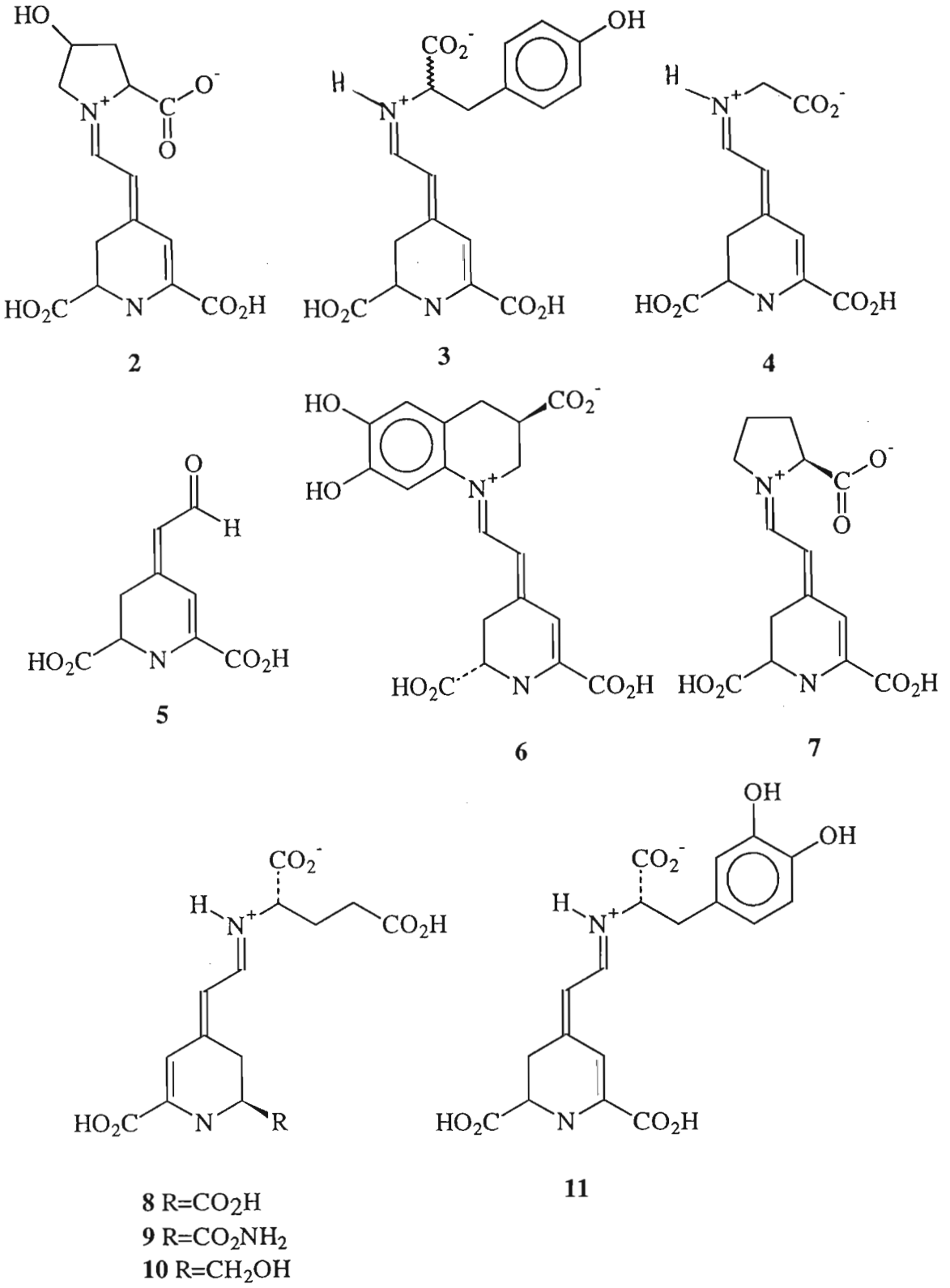


Fig. 3.1*d*: Betalaine pigments and their precursors

Anacampseros rufescens DC., *Calandrina grandiflora* Lindl. and various other *Calandrinia* H.B. & K.Nov. species apparently have been shown to contain betacyanins (Hegnauer, 1962).

Group 3: Clerodane derivatives: The following clerodienes were isolated from various *Portulaca* species (Fig. 3.1*e*)(Ohsaki et al., 1987; Ohsaki et al., 1988).

According to Hegnauer (1962) species in the Portulaceae have also been shown to contain flavanoids, saponins and aromatic acids. In *Avonia rhodesica*, however, the only compounds found to be present in significant quantities were waxes.

3.1.5. Waxes

A natural wax has, in the past, been defined as a long chain fatty acid esterified with a long chain alcohol, but this definition has now come to extend to any organic molecule which is solid at room temperature and is sufficiently hydrophobic to serve as a water repellant (Matthews and van Holde, 1991)(Table 3*c*).

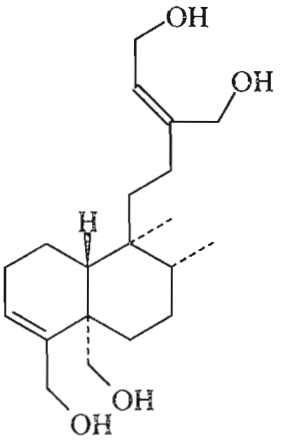
Table 3*c*: Compounds found to be present in cuticular waxes (Kolattukudy, 1970)

Compound Type	**Range**	**Most Common**	**Source**
Hydrocarbons			
n-alkanes	C_{25}-C_{35}	C_{29}, C_{31}	most plants
iso-alkanes	C_{25}-C_{35}	C_{27}, C_{29} ,C_{31} ,C_{33}	lavender blossom, poplar leaf
anteiso-alkanes	C_{24}-C_{36}	C_{28} ,C_{30} ,C_{32}	tobacco leaf
internally branched alkanes	C_{14}-C_{34}		rose petal, sugar cane
cyclic alkanes	C_{13}-C_{33}		tobacco, seed oils
n-alkenes	C_{17}-C_{33}	C_{27}, C_{29}, C_{31} ,C_{33}	cane sugar wax, rose petal wax, algae
branched alkenes	C_{17}-C_{33}		
terpenoid hydrocarbons	Pristane and cyclic hydrocarbons, phyllocladene, isophyllocladene, (+) kaurene, isokaurene and others		Podocarpaceae
aromatic hydrocarbon	anthracene and phenanthrene type with an attached alkane chain		banana leaf
Ketones and related compounds			
monoketones	C_{24}-C_{33}	C_{29}, C_{31}	cabbage, roses
β-diketones	(C_{33}) tritriacontan-16,17-dione,		*Eucalyptus* L'Herit., *Poa colenasoi*

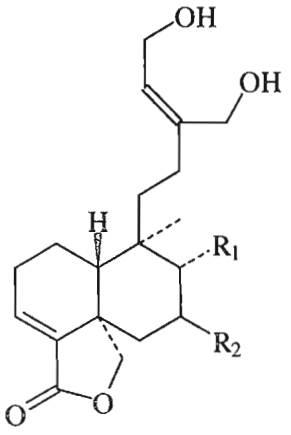
	(C_{31}) hentriacontan-14,16-dione		Hook.f., wheat
hydroxy β-diketones	(C_{31}) hentriacontan-9-ol-14,16-dione		wheat
Secondary alcohols	C_{20}-C_{33}	hentriacontan-16-ol hentriacontan-9-ol nonacosan-15-ol nonacosan-10-ol	pea leaves Rosaceae brussel sprouts apple and various trees
	C_9-C_{15}	C_9, C_{11}, C_{13}, C_{15}	*Eucalyptus risdoni* Hook.f.
Wax esters	C_{30}-C_{60}	C_{44}, C_{46}, C_{48}, C_{50}	most plants
Primary alcohols	C_{12}-C_{36}	C_{26}, C_{28}	most plants
Aldehydes	C_{14}-C_{34}	C_{26}, C_{28}, C_{30}	grapes, apple, pea, sugar cane
Acids			
alkanoic acids	C_{12}-C_{36}	C_{24}, C_{26}, C_{28}	most plants
alkenoic acids	C_{16}-C_{18}		apple
ω-hydroxy acids	C_{10}-C_{34}	C_{24}, C_{26}, C_{28}	carnauba, apple
α,β-Diols	C_{20}-C_{32}	C_{22}, C_{24}, C_{26}, C_{28}	carnauba, apple
Terpenes	ursolic and oleanolic acid		apple, grape
Flavones			*Eucalyptus*

Waxes are commonly found in the cuticle in a matrix with the biopolymer cutin. The cuticle is a protective layer associated with the external tissues of plants, without which, transpiration of most land plants would be so rapid that they would die. Although cuticular transpiration is usually much smaller than the flux of water passing through open stomata, it can exceed the loss of water through partially or completely closed stomata. Thus under conditions of water deficit, cuticular conductance of water vapour is an important determinant of water balance and drought reactivity (Premachandra *et al.*, 1994). This is especially true of plants living in arid, water stressed conditions such as those of the granite basoliths where *A. rhodesica* is typically found.

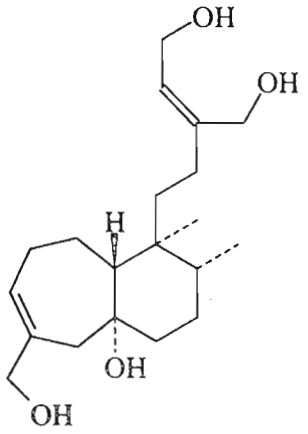
Wax that is present on the top the cutin matrix is epicuticular wax and that found within the cutin matrix is embedded wax (Bewick *et al.*, 1993). These waxes are usually multicomponent mixtures dominated by a particular compound or class of compounds (Meusel *et al.*, 1994).



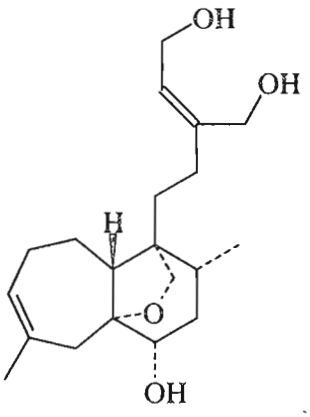
Jewenol-A



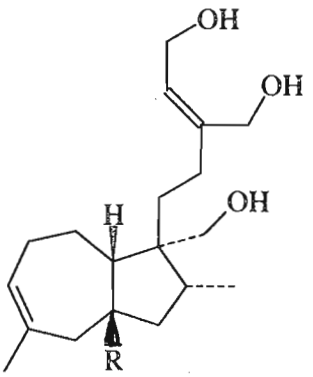
R_1= CH_2OH and R_2= H, Portulide A
R_1= CH_3 and R_2= H, Portulide B
R_1= CH_3 and R_2= OH, Portulide C
R_1= COH and R_2=H, Portulide D



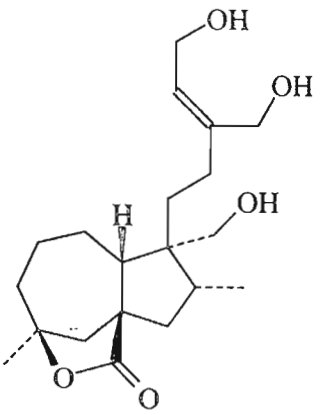
Jewenol-B



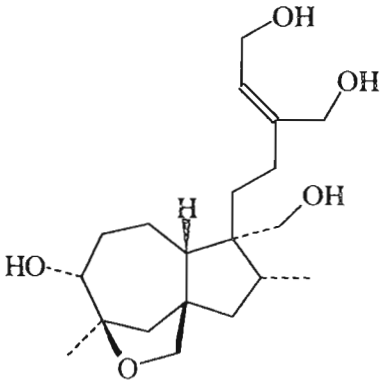
Portulen



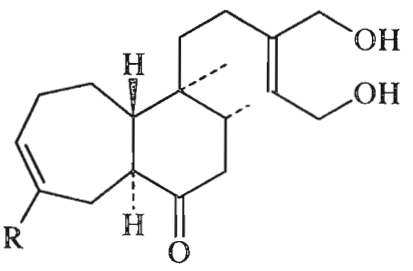
R=CH_2OH, Portulol
R=CHO, Portulal
R=COOH, Portulic acid



Portulolactone



3-hydroxyportulolether



R=Me, pilosanone A
R=CH_2OH, pilosanone B

Fig. 3.1*e*: Clerodanes from *Portulaca* species

Further functions of epicuticular wax suggested by Jetter and Riederer (1994) include: decreasing the wettability of the surface; additional resistance to stomatal transpiration; guarding the leaf-surfaces from accumulation of air-borne particles and spores; keeping the leaf surfaces dry and thus causing the germination conditions for spores to deteriorate; and protecting internal tissues and the photosynthetic system from excessive radiation.

The accumulation of epicuticular waxes has been shown to be associated with detrimental effects in long-lived columnar cactii in Argentina and Chile such as *Carnegia gigantea* Engelm. These accumulations obscured stomata leading to visible surficial damage such as scaling (appearance of tan to red-orange discolouration of surfaces) and barking (larger build-up of materials on the surfaces that appear brown to black) which resulted in premature death of the plant. It appeared that increased sunlight exposure could have been the causative agent, or at least have contributed to surface injuries (Evans *et al.*, 1994).

Epicuticular wax could be crystalline or amorphous. Straight tubules count among the most prominent and widespread forms of epicuticular waxes. The wax mixtures of these contain substantial amounts of either β-diketones (e.g. on the leaves of *Eucalyptus*) or asymetric secondary alcohols (e.g. on the needles of gymnosperms)(Jetter and Riederer, 1994).

Using scanning electron microscopy, Bacic *et al.* (1994) were able to show conclusively that recrystalisation of amorphous wax occurred on the needles of *Pinus sylvestris* L. This supported the hypothesis earlier proposed by Fox (1958) and Schulze *et al.* (1989) that epicuticular wax was inherently unstable and required continuous recrystallisation to maintain its three-dimensional structure.

Compound epicuticular rodlets of aliphatic wax lipids (mainly wax lipids) and triterpenols (and their acetates) have been shown to occur on the surface of *Strelitzia* Aiton and *Benincasa* Savi, respectively. The ultrastructure of the waxes could therefore be interpreted as a structural as opposed to a chemical convergency (Meusel *et al.*, 1994). While wax ultrastructure is not a useful taxonomic feature, the chemical composition of waxes or lipids can be (Vickery and Vickery, 1981).

3.1.6. Taxonomic classification on the basis of fatty acids

Waterman and Gray (1987) stated that the most successful use of fatty acid-profile analysis has been at the lower levels of classification, for example in *Carya* Nutt. (Juglandaceae), *Citrus* L.(Rutaceae) and in *Cornus* L. and *Nyssa* Gronov. Ex L. (Cornaceae). These authors also mention that the use of lipids as taxonomic markers in the family Brassicaceae has been explored. An examination of fatty acids in the Pittosporaceae, Araliaceae, Apiaceae, Simaroubaceae and Rutaceae revealed that petroselenic acid [18:1 (6*Z*)] was a significant component only in species of Araliaceae and Apiaceae, while tariric acid [18:1 (6*E*)] was found only in the Simaroubaceae. Alkanes have been used in the classification of *Citrus* (Rutaceae) and *Arbutus* L. (Ericaceae)(Waterman and Gray, 1987).

Groups of families can also be recognised on the basis of the major fatty acids released by hydrolysis of the lipids in their seeds (Vickery and Vickery, 1981b)

3.1.7. Overview of the biosynthetic pathways pertinent to this chapter

3.1.7.1. Biosynthesis of fatty acids

Except for the terpenes and the flavones, most of the components of both epicuticular and embedded wax are derived from long chain fatty acids, and as such are products (or by-products) of the acetate-malonate pathway (Vickery and Vickery, 1981b).

The synthesis of malonyl-coenzyme A (malonyl-CoA) from acetyl coenzyme A (acetyl-CoA) is the first step in the synthesis of fatty acids and is catalysed by acetyl-CoA carboxylase. This enzyme is a protein complex with three domains each of which performs a different task: one acts as a biotin carboxylase, the second as a biotin carboxyl carrier (BCCP) and the third as a transcarboxylase (Stumpf, 1980).

The next stage involves the condensation of a malonyl with an acetyl moeity, but before this can occur, the two moieties must be transferred from CoA to acyl carrier protein (ACP) molecules. The enzymes catalysing these two reactions are malonyl-CoA-ACP tranferase and acetyl-CoA-ACP transacylase respectively (Fig. 3.1*f* (Vickery and Vickery, 1981b).

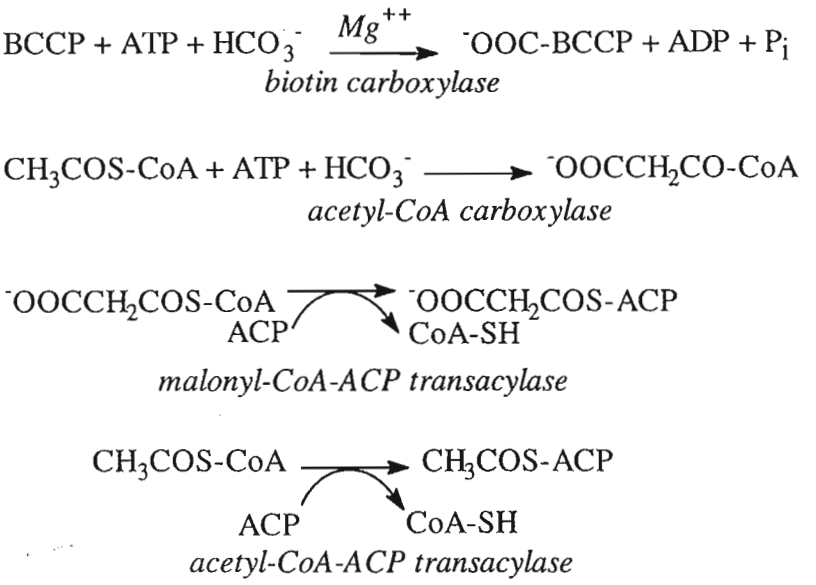


Fig. 3.1*f*: Formation of acetyl-ACP

β-Ketoacyl-ACP-synthase catalyses the condensation of acetyl-ACP and malonyl-ACP to form acetoacetyl-ACP and carbon dioxide. The reduction of acetoacetyl-ACP catalysed by β-ketoacyl-ACP reductase requires NADPH, and is followed by the dehydration of the product catalysed by 3-hydroxyacyl-ACP dehydrase. The final step in the sequence is the reduction of the *trans*-Δ^2-enoyl-ACP to butyryl-ACP catalysed by enoyl-CoA reductase. The four enzymes mentioned in this cycle are in a close association known as the fatty acid synthetase complex(Vickery and Vickery, 1981b).

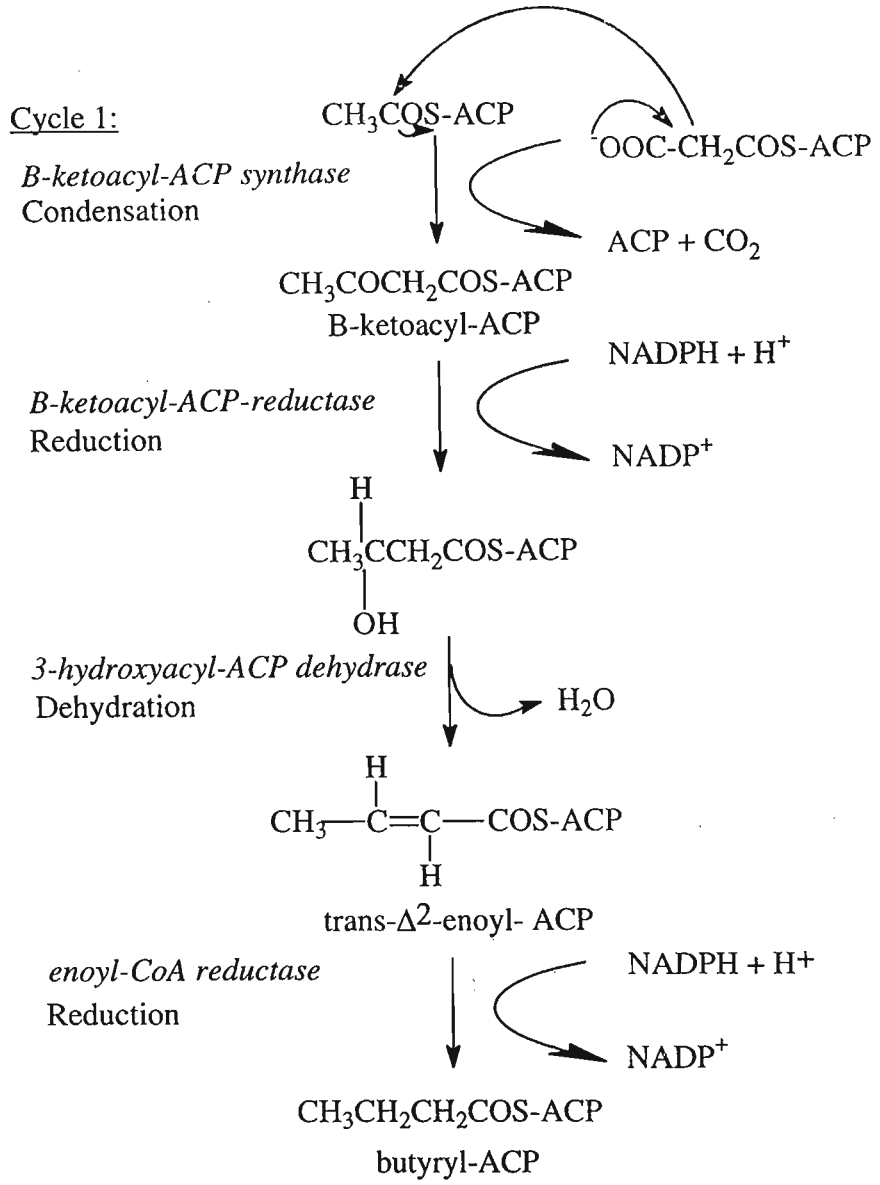


Fig. 3.1*g*: Formation of butyryl-ACP

This sequence is repeated six more times, the final product being palmityl-ACP (C_{16}).

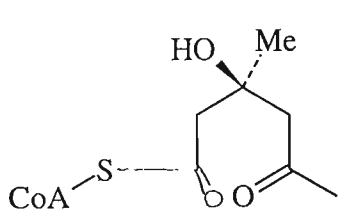
There are three possible termination reactions that this molecule could undergo viz. (1) transfer of the palmityl moeity to ACP which is not bound to the *de novo* fatty acid synthetase complex, (2) transfer to CoA, or (3) hydrolysis to the free acid (palmitic acid). Unbound palmityl-ACP can condense with a further malonyl-ACP to form stearyl-ACP. This reaction occurs in the "type one elongation system". Stearyl-ACP can either be transformed to stearyl-CoA, hydrolysed to form stearic acid or be elongated by further addition of malonyl units to the longer fatty acids found in waxes. These elongations occur in the "type two elongation sytem" (Vickery and Vickery, 1981b).

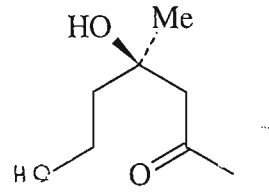
Two of the other compounds found to occur in *A. rhodesica* were triterpenols, and their biosynthesis will be discussed.

3.1.7.2. Biosynthesis of triterpenoids

As in the biosynthesis of fatty acids, the starting material for the synthesis of triterpenoids is acetyl CoA (**12**). Two molecules of acetyl CoA (**12**) combine to form acetoacetyl-CoA (**13**) which in turn is used to form (3*S*)-3-hydroxy-3-methylglutanyl-CoA (HMG-CoA) (**14**). NADPH then reduces this to mevalonic acid (**15**) in higher plants, the enzyme catalysing this reaction being hydroxymethylglutaryl-CoA reductase (Harrison, 1985).

12 $CH_3COS\ CoA$ **13** $CH_3COCH_2COS\ CoA$





14 **15**

Fig. 3.1*h*: Biosynthesis of mevalonic acid

The conversion of mevalonate into *R*-5-phospho-mevalonic acid (**16**) is catalysed by the enzyme mevalonate kinase (E.C.2.7.1.36) and is ATP-dependant. The enzyme has been shown to have both an essential amino-group and an essential thiol group (Soler *et al.*, 1979). The conversion of *R*-5-phospho-mevalonic acid (**16**) into *R* -5-diphosphomevalonic acid (**17**) is catalysed by phosphomevalonate kinase. This is a single polypeptide chain and, again, both thiol and amino functions are involved in the catalytic process (Harrison, 1985).

R-5-diphosphomevalonic acid (**17**) is then converted to isopentenyl diphosphate (**18**) by the enzyme diphosphomevalonate decarboxylase (E.C.4.1.1.33). This enzyme does not appear to require thiols for activation or for its stability, but contains an essential arginyl residue and is inhibited by phenyl and phenolic compounds (Shama Bhat and Ramasarma, 1979).

The interconversion of isopentenyl diphosphate (**18**) and dimethylallyldiphosphate (**19**) is an equilibrium reaction catalysed by the enzyme isopentyl-diphosphate-Δ-isomerase (E.C.5.3.3.2).

The following reactions are both catalysed by the enzyme dimethylallyl-*trans* transferase (E.C.2.5.1.1) [prenyl-transferase]: (a) condensation of isopentenyl diphosphate (**18**) with dimethylallyldiphosphate (**19**) to form geranyl diphosphate (**20**)(Laskovics and Poulter, 1981) which is the last reaction common to the synthesis of triterpenoids and carotenoids; (b) condensation of geranyl diphosphate (**20**) with isopentenyl diphosphate (**18**) to form farnesyl diphosphate (**21**) a possible mechanism for which is shown (Fig. 3.1*i*)(Poulter *et al.*, 1978).

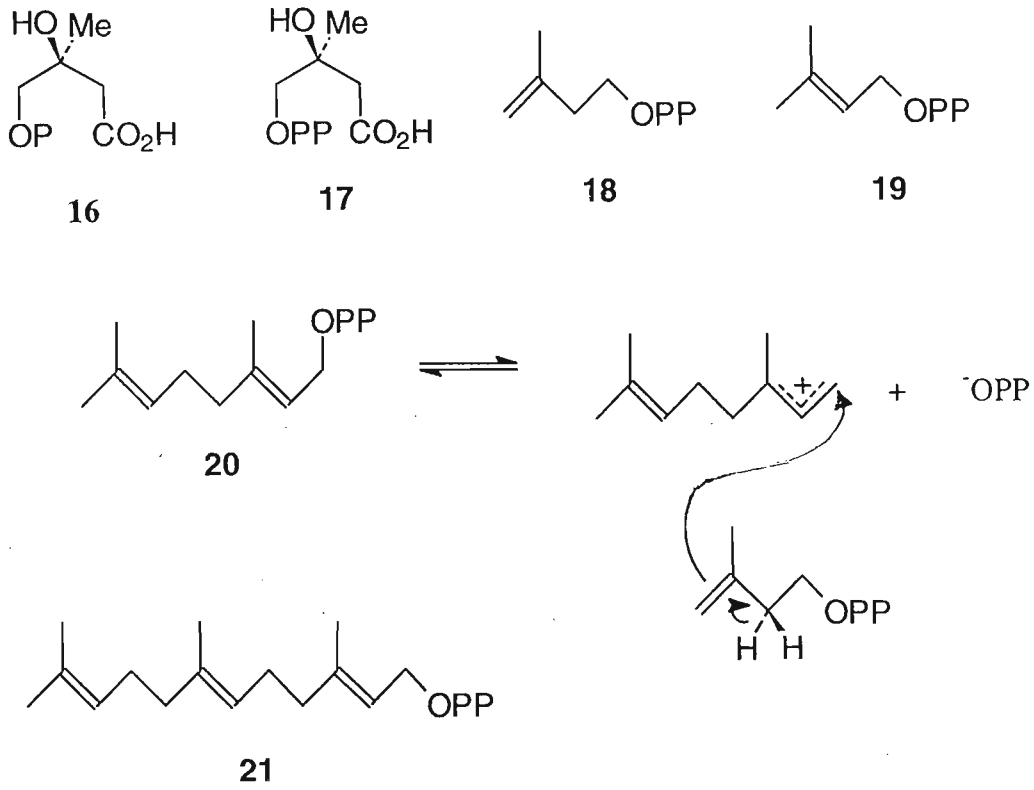


Fig. 3.1*i*: Formation of farnesyl diphosphate

Prenyltransferase has also been isolated from the higher plant species *Pisum sativum* L., *Gossypium hirsutum* L., and *Citrus sinensis* Osbeck. If it is assumed that the enzyme responsible for these reactions in higher plants is basically the same, then:

1. It exists in two forms, one of which can produce farnesyl diphosphate the other forming the 2*Z*, 6*E* isomer when provided with the appropriate starting materials.
2. It has a molecular weight of approximately 96000.
3. It has an essential thiol group and requires Mg^{++} for its activity (Laskovics and Poulter, 1981; Harrison, 1985).

The conversion of farnesyl diphosphate(**21**) to squalene (**23**) is catalysed by the enzyme squalene synthetase (E.C.2.5.1.21) with presqualene diphosphate (**24**) as an intermediate.

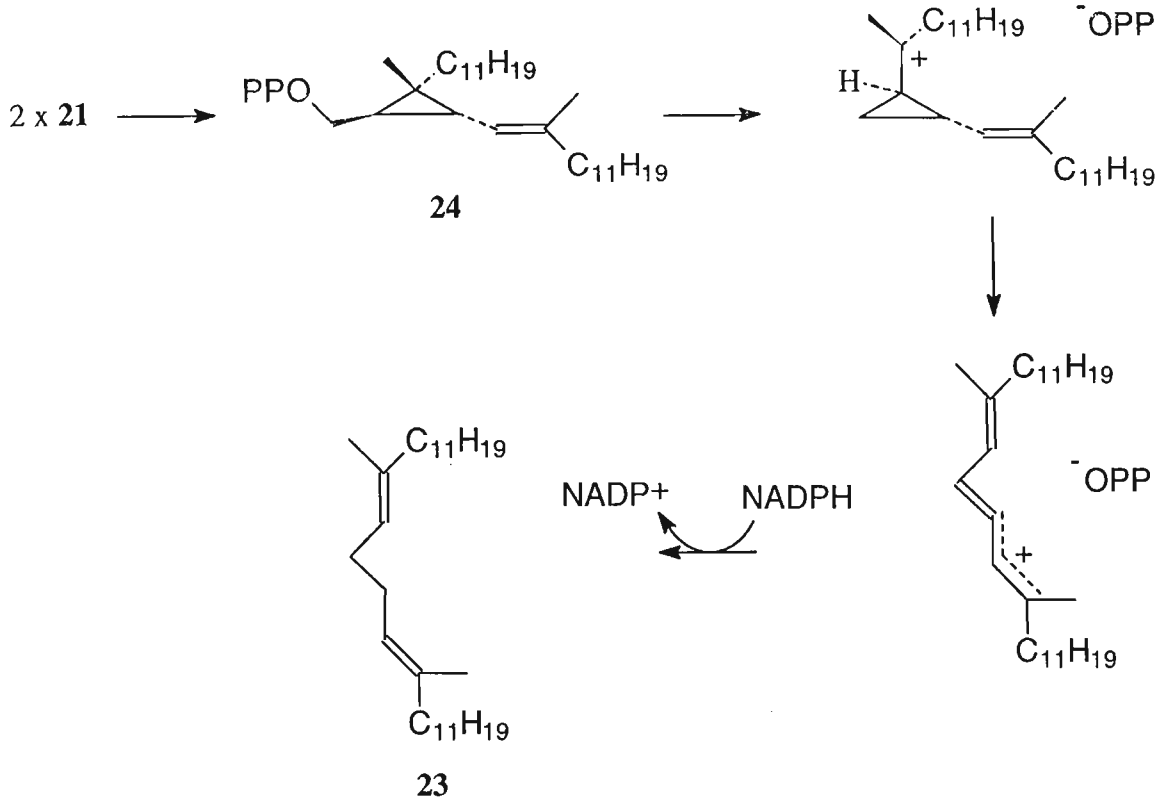
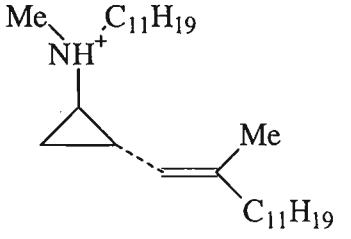


Fig. 3.1*j*: Biosynthesis of squalene

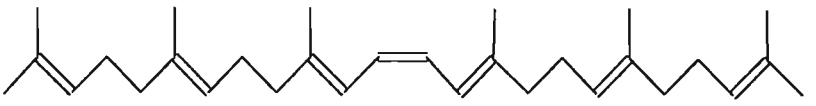
While the two ionic intermediates have not been isolated, their importance is supported by the inhibition of squalene synthetase by the ammonium diphosphate salt (**25**)(Harrison, 1985).



25

Fig. 3.1*k*: Squalene synthetase inhibitor

If NADPH is absent (and Mn^{2+} is present) 12Z-12,13-didehydrosqualene (**26**) is formed. This is a squalene synthetase-mediated reaction (Harrison, 1985).

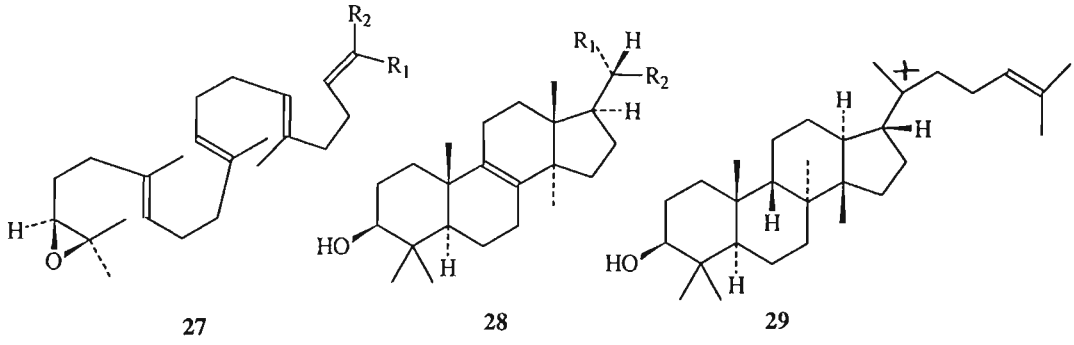


26

Fig. 3.1*l*: 12Z-12,13-didehydrosqualene

The next stage of the synthesis of triterpenoids is the oxidation of 12*Z*-12,13-didehydrosqualene (**26**) to form 3*S*-squalene-2,3-epoxide (**27**) in the presence of molecular oxygen and NADPH. The enzyme involved is squalene epoxidase (E.C.1.14.99.7)(Harrison, 1985).

The conversion of 3*S*-squalene-2,3-epoxide (**27**) to form lanosterol (**28**) is catalysed by the enzyme lanosterol synthase (E.C.5.4.99.7). If the derivatives **27a**, **b** and **c** are used, the corresponding products **28a**, **b** and **c** are formed. This demonstrates that the stereochemistry at C-20 is not determined by the relative size of the substituents at C-19, but by their stereochemical disposition about the Δ^{18} double bond. This is not consistant with the formation of the carbocation, (**29**), as an intermediate, but rather with a pathway involving the stereospecific participation of an enzyme-bound nucleophile in the cyclisation step (Harrison, 1985)(Fig. 3.1*m*).



27 28 29

27a and 28a; R_1= Me, R_2= $(CH_2)_2CH{=}CMe_2$
27b and 28b; R_1= Et, R_2= Me
27c and 28c; R_1= Me, R_2= Et
27d and 28d; R_1= Me, R_2= $(CH_2)_2CH$-CMe_2 (O)

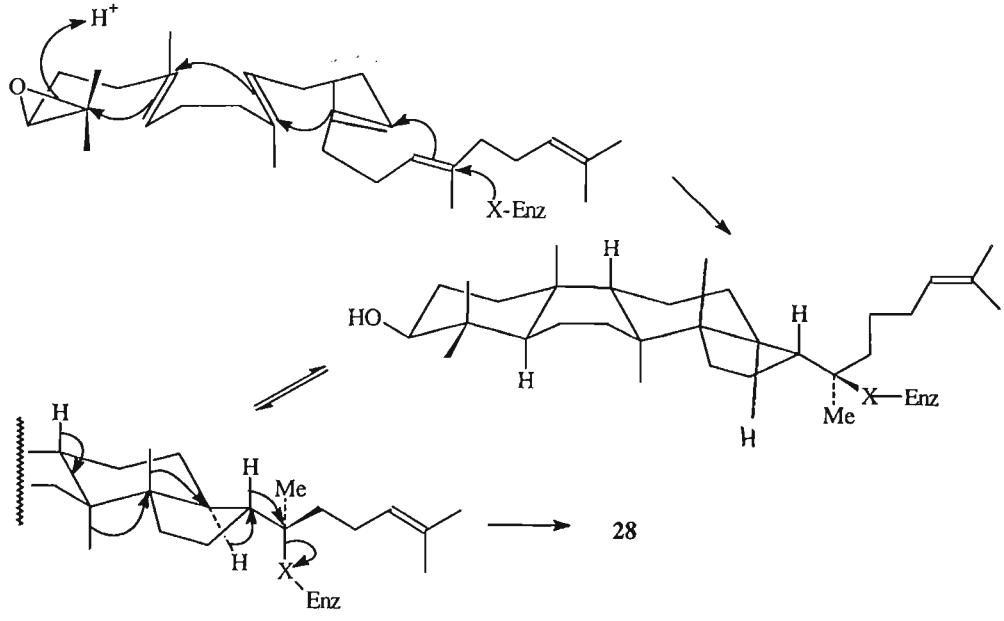


Fig. 3.1*m*: Biosynthesis of lanosterol

Labelling patterns for sterols biosynthesised from mevalonate [2-^{14}C(4R)-4-^{3}H$_1$] are shown below (Fig. 3.1*n*)(Lenton *et al.*, 1975).

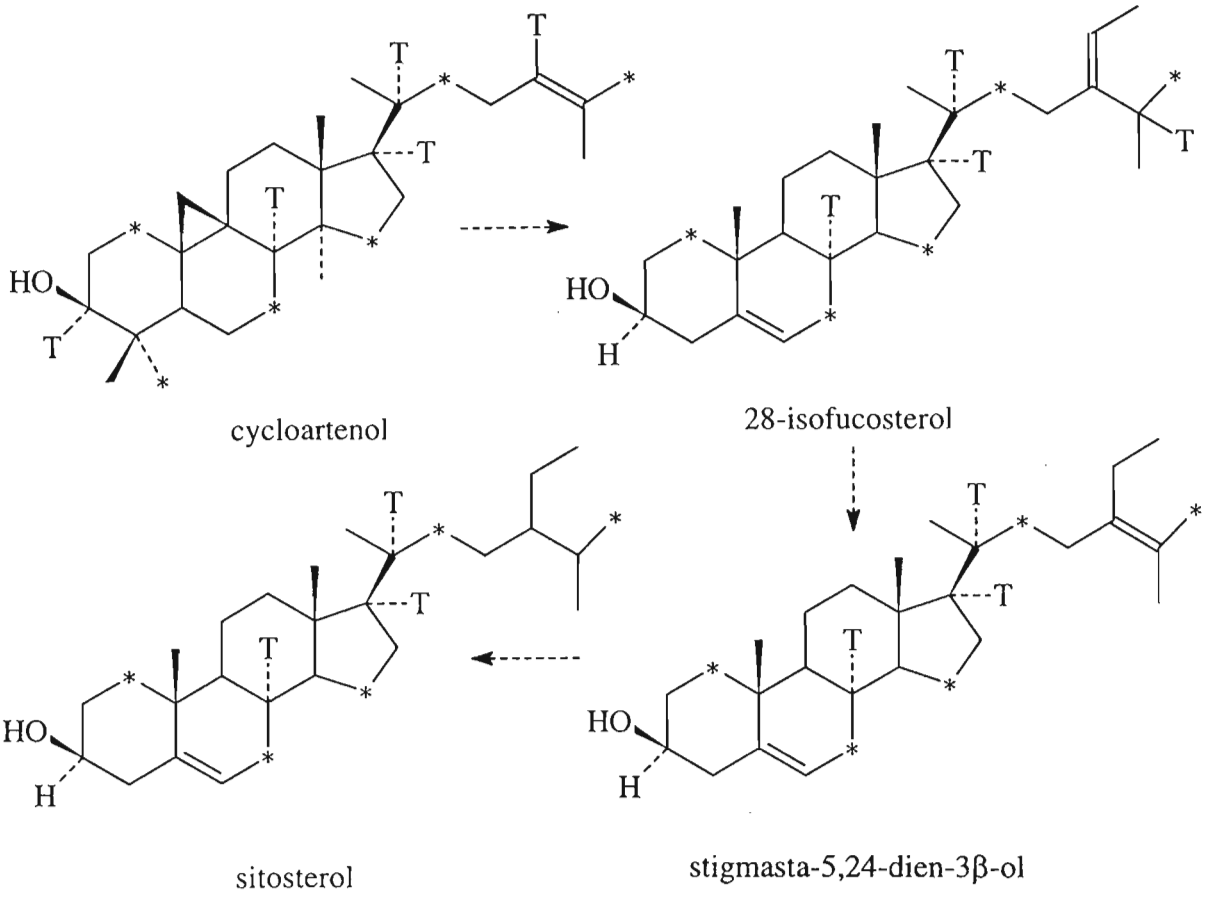


Fig. 3.1*n*: Labelling patterns for the biosynthesis of sterols from mevalonate [2-^{14}C(4*R*)-4-^{3}H$_1$]

A mechanism for the formation of the phytosterol side-chain proposed by Lenton *et al.* (1975) is also shown:

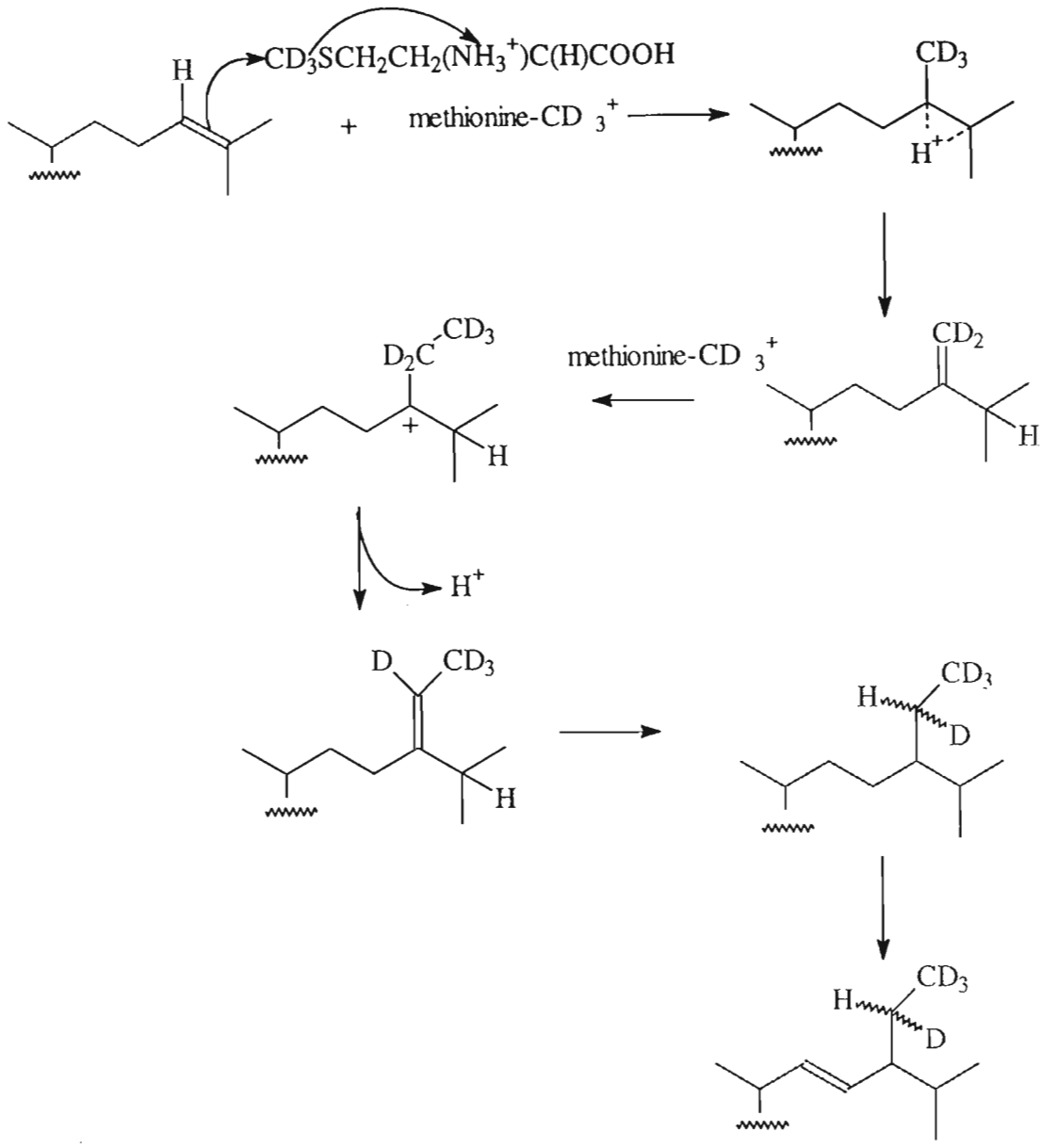


Fig. 3.1*o*: Mechanism for the formation of the phytosterol side-chain

Labelling experiments have been performed to determine the mechanism whereby the pentacyclic triterpenes ursolic acid (**29**), 2α-hydroursolic acid (**30**) and 3-*epi*-maslinic acid (**31**) are cyclised from **27** in tissue cultures of *Isodon japonicus* (Burman f.) H.Hara (a higher plant)(Fig. 3.1*p*).

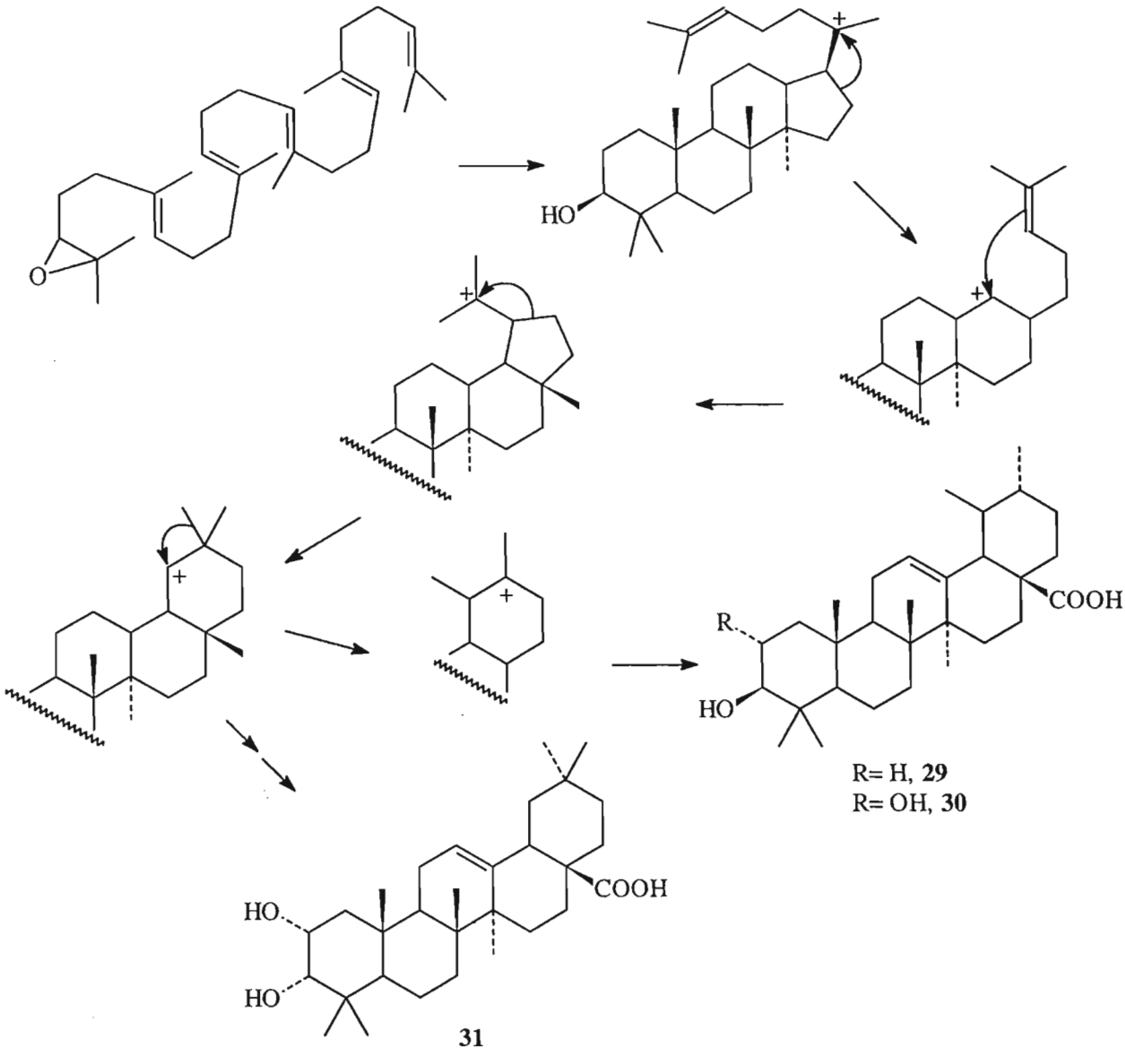


Fig. 3.1*p*: Mechanism for the formation of pentacyclic triterpenoids

3.2 Results

From the hexane and the ether extracts of *Avonia rhodesica,* white precipitates formed on cooling and were separated from the remainder of the extract by filtration. From their water insolubility, their appearance and their low melting points, they were thought to be waxes.

In this investigation of the waxes of *A. rhodesica*, methyl esters of the following fatty acids were shown to be the major constituents of the the hexane extract precipitate (characterised by GC-MS):

Behenic acid (Docosanoic acid) $C_{22}H_{44}O_2$ (9.4 %)

Lignoceric acid (Tetracosanoic acid) $C_{24}H_{48}O_2$ (9.76 %)

Palmitic acid (Hexadecanoic acid) $C_{16}H_{32}O_2$ (34.52 %)

Cerinic acid (Hexacosanoic acid) $C_{26}H_{52}O_2$ (6.48 %)

Compounds found in the ether extract precipitate included: nonacosane $C_{29}H_{60}$ (15.32 %), pentacosane $C_{25}H_{52}$ (26.68 %) and neophytidiene $C_{20}H_{38}$ (3.83 %)(characterised by GC-MS). Two compounds which gave mass spectra similar to (or the same as) those of dioctyl hexanedioate (14.04 %) and ethyl docosanoate (2.72 %) were also present.

The GC-MS had a spectrum library and use was made of this.

Scanning electron microscopy was used to examine the ultrastructure of the surface waxes.

Two triterpenols (β-amyrin [Compound 6] and a cycloartenol derivative [Compound 7]) were isolated as a mixture from the methylene chloride extract, and triacetylglycerol was isolated from the acetylated methanol extract. No nitrogenous compounds were found.

The triterpenol mixture was acetylated in an effort to separate the two compounds.

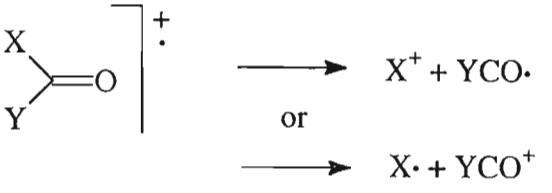
3.3. Discussion

3.3.1. Waxes

spectra: S56-S60

According to Tulloch (1987), the mass spectra of esters ($R\text{-}CO_2\text{-}R'$) show the presence of ions $[R'\text{-}1]^+$ and $[CO_2R']^+$ indicating the alcohol chain length and $[RCO]^+$ showing the acid chain length. The two types of cleavage important in the mass spectra of esters of fatty acids are α-cleavage and β-cleavage with McLafferty rearrangement (Kemp, 1991).

α-cleavage:



β-cleavage with McLafferty rearrangement:

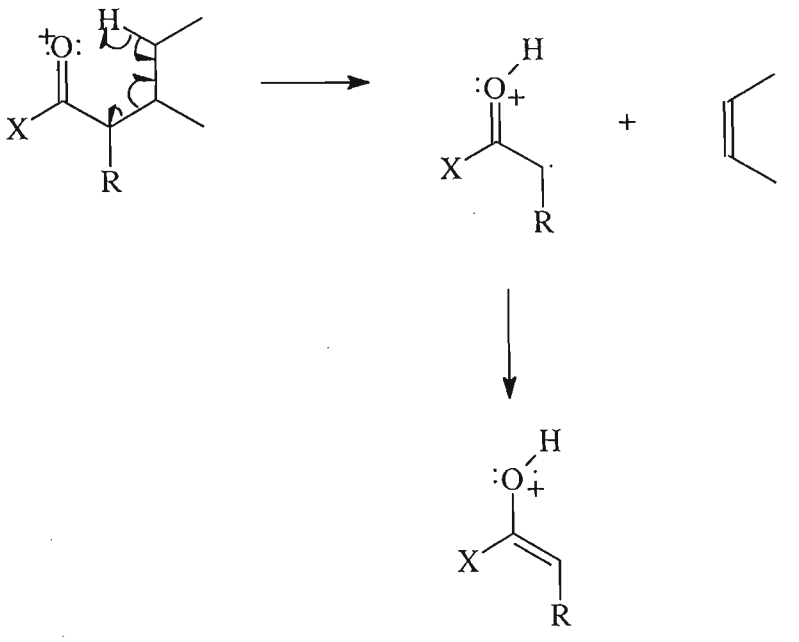
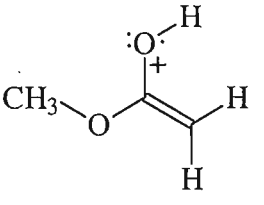


Fig. 3.3*a*: α and β-cleavage of fatty acids

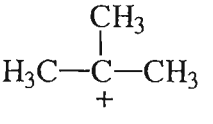
In the first four esters that were identified using GC-MS (the methyl esters), the peak of highest intensity was that at *m/z* 74 (Fig. 3.3*b*). This is consistant with the formation of :



This suggested the presence of methyl esters of fatty acids. X in all four cases was therefore OMe. Further confirmation of the identification as methyl esters was the fact that in all four compounds, there was a $[M - 31]^+$ (*m/z* 31 being the mass of OMe) representing the $Y\text{-}C{=}O^+$ of α-cleavage and a peak at *m/z* of 59 representing $CO_2CH_3^+$.

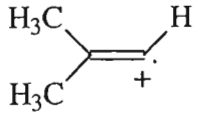
There were a number of other peaks common to all six esters:

1. $[M - 43]^+$. This was probably the loss of C_3H_7, a group typically lost in long chain alkanes (This peak was not seen in the spectrum of dioctyl ester).
2. Peak at *m/z* 57. This corresponded to the formation of $C_4H_9^+$. This cation is known to be very stable, as the unbranched form rearranges in the mass spectrometer to form the branched-chain form.



This rearrangement is analogous to the Wagner-Meerwein shift which occurs in S_N1 reactions involving cations, the more stable branched structures being preferred (Kemp, 1991).

3. Peak at *m/z* 55. This corresponded to the loss of two hydrogen atoms from $C_4H_9^+$.



3. Peaks at *m/z* 71 and 69. These corresponded to the analogous $C_5H_{11}^+$ and $C_5H_9^+$ peaks.
4. Peaks at *m/z* 85 and 83. These corresponded to the analogous $C_6H_{13}^+$ and $C_6H_{11}^+$ peaks.
5. Peak at *m/z* 87. This was thought to correspond to $[(CH_2)_3COOH]^+$.

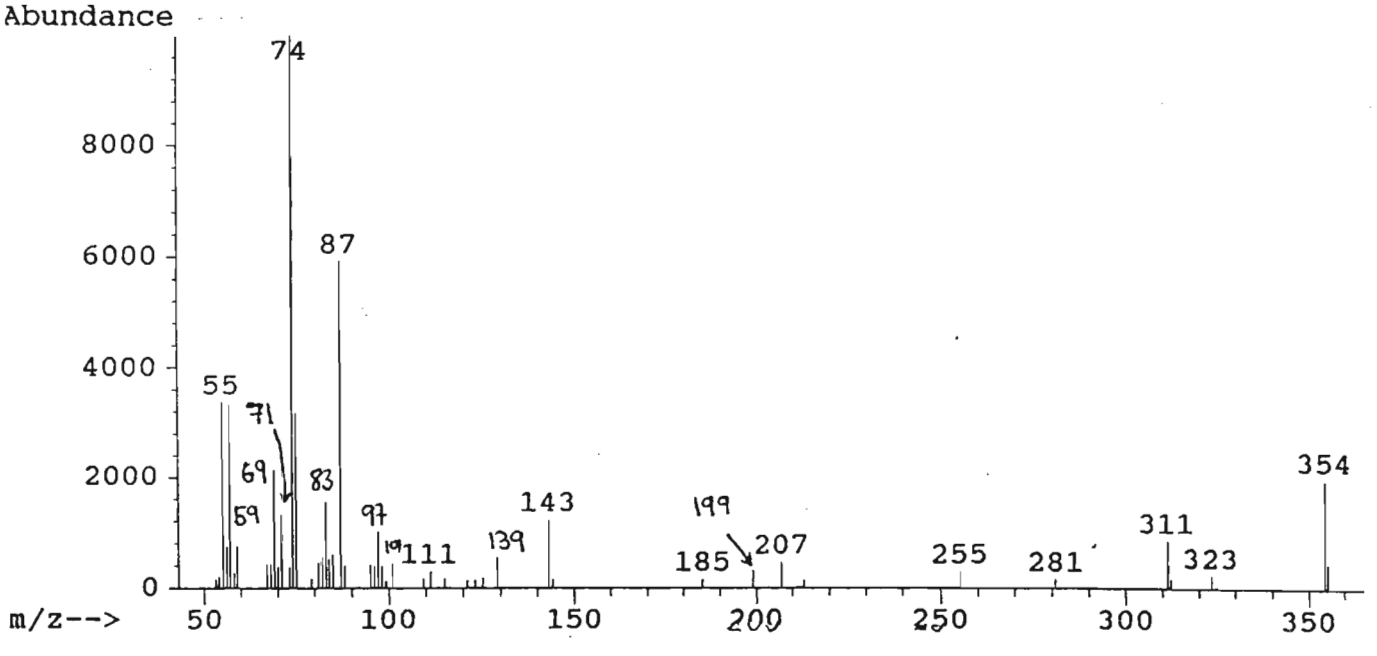
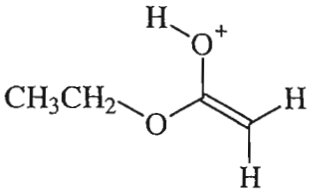


Fig. 3.3*b*: GC-MS spectrum of methyl docosanoate

The molecular ions of these four methyl esters had *m/z* values of 354, 382, 270 and 410 corresponding to the molar masses of methyl docosonoate (354.615 μ), methyl tetracosanoate (382.669 μ), methyl hexadecanoate (270.454 μ) and methyl hexacosanoate (410.722 μ) respectively. As the GC-MS instrument had a spectrum library, it was possible to confirm the identities of these compounds.

In the mass spectrum for ethyl docosanoate, the molecular ion had a *m/z* value of 368. A prominent peak at *m/z* 88 corresponded to the formation of the product of β-cleavage with McLafferty rearrangement shown below.



A peak at *m/z* 281 indicated the loss of 87 corresponding to the loss of the non-protonated form of this molecule. In all other respects, the mass spectrum of this compound was similar to those of the methyl esters.

In the mass spectrum for dioctyl hexadioate, the molecular ion was not present (Fig 3.3*c*).

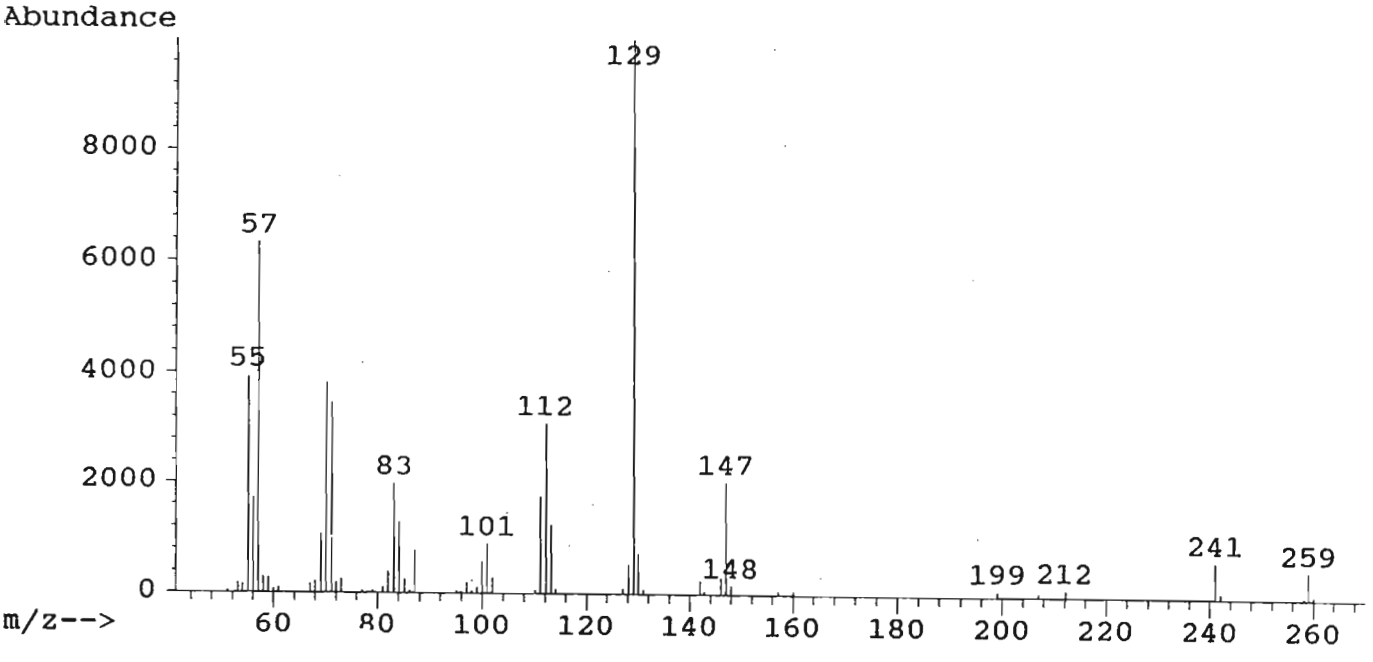


Fig. 3.3*c*: GC-MS spectrum of dioctyl hexadioate

The peak with the highest *m/z* value was at *m/z* 259, so the identification was tentative. Unlike all the other esters, products of β-cleavage with McLafferty rearrangement were absent from the spectrum. The fragmentation pattern was dominated by the cations formed by α-cleavage as shown below.

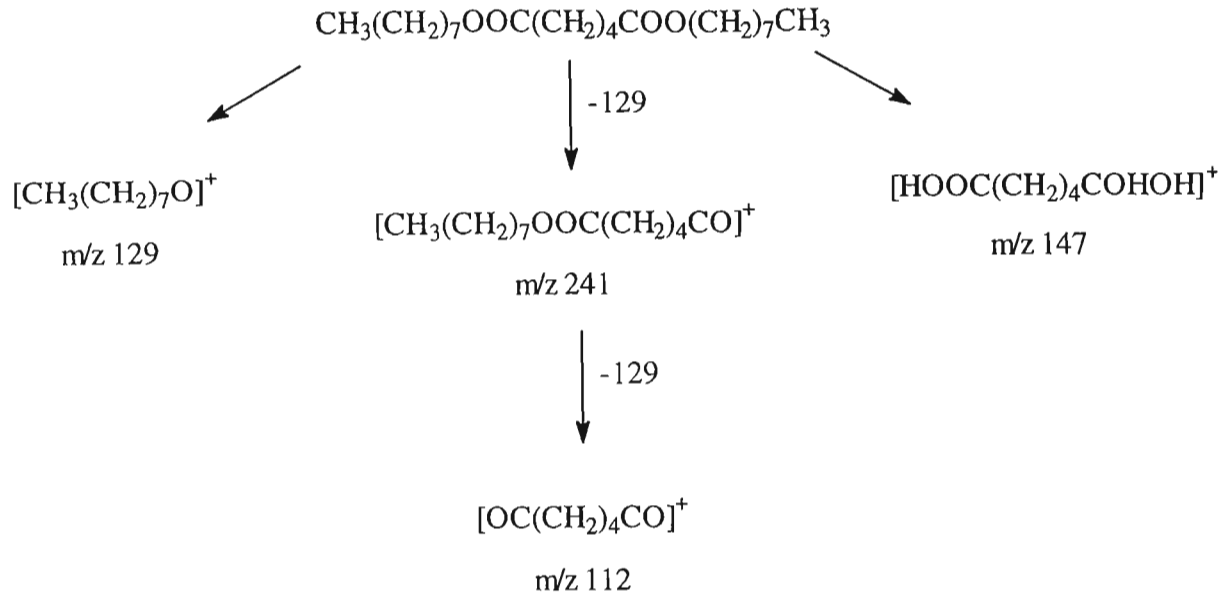


Fig. 3.3*d*: Fragmentation pattern for dioctyl hexanoate

Methyl esters of long acids have however been isolated from *Abies balsamae*. In this species methyl esters comprised 6-7% of the waxes, methyl triacontanoate being the major component (methyl esters being the only esters isolated). Before this, methyl esters had not been reported as components of epicuticular waxes of higher plants except in certain eragrostoid grasses in which methyl esters formed 0.25% of the ester fraction. It was suggested that methyl esters may be present in the waxes of conifers, but have been overlooked so far because only small amounts were present (Tulloch, 1987). Methyl hexadecanoate had however been isolated previously from the trunkwood of *Macherium kuhlmanii* (Ollis *et al.*, 1978) and methyl docosanoate had been isolated from *Cassia javanica* Gagnepain leaves (Chauduri and Chawla, 1985). Prior to performing the gas chromatography, attempts had been made to dissolve the waxes in hot methanol, thus the possibility that these were artefacts (formed by a transacylation reaction) cannot be overlooked. Efforts were made to repeat this exercise with smaller amounts of plant material, but difficulties were experienced in using the GC-MS. This work was not further pursued due to lack of time.

The other compounds that were characterised in the GC-MS data, are the long chain alkanes nonacosane and pentacosane, and the α,γ-diene neophytidiene. As earlier noted, long chain alkanes are an established constituent of the cuticular wax layer. They usually have between 19 and 37 carbons, and almost always have an odd number of carbon atoms, because they are formed by the decarboxylation of fatty acids.

$$CH_3(CH_2)_{28}COOH \longrightarrow CH_3(CH_2)_{27}CH_3 + CO_2$$

The GC-MS spectrum of nonacosane showed a molecular ion at *m/z* 408 consistant with the molecular formula $C_{29}H_{60}$ (408.793 μ) is shown (Fig. 3.3*e*).

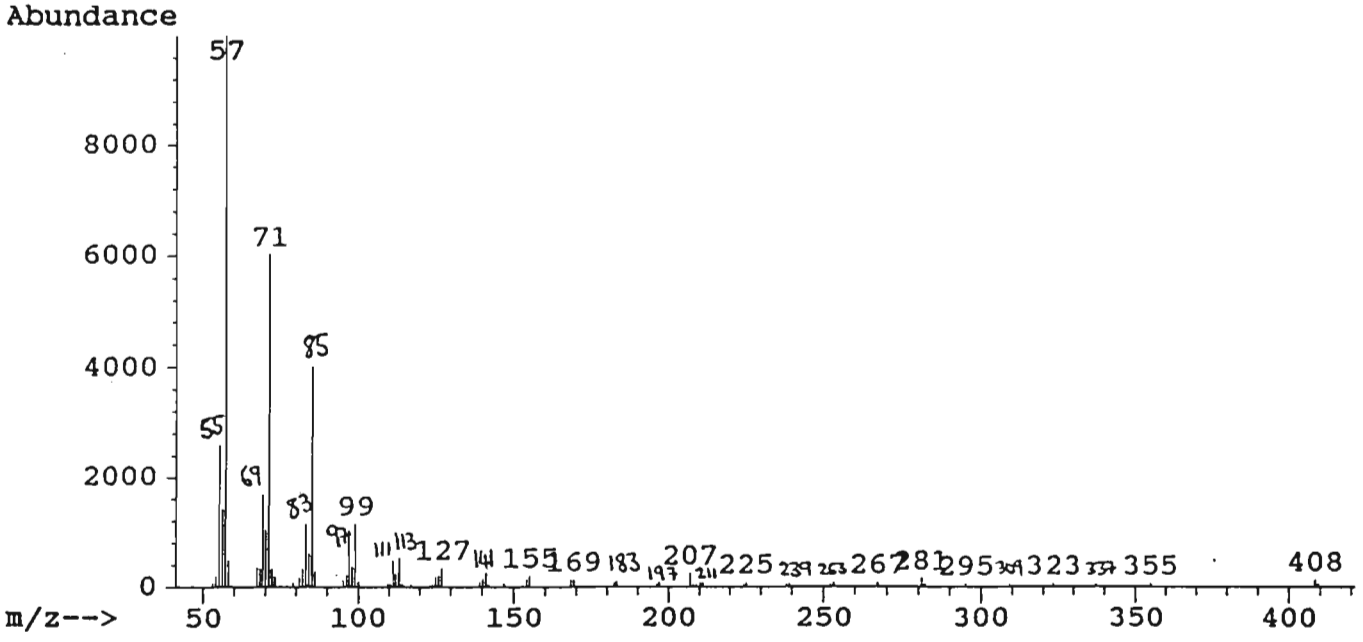


Fig. 3.3*e*: GC-MS spectrum of nonacosane

The fragmentation pattern was typical of that of long chain alkanes, that is, cations of the homologous series $C_nH_{2n-1}^+$. For n = 4,5,6,7,8,9 and 10, peaks corresponding to $C_nH_{2n-3}^+$ occured as well. These have already been discussed in the structure elucidation of the methyl esters. The spectrum of pentacosane was very similar, but fewer peaks were visible. The molecular ion occurred at *m/z* 352, which is consistant with the molecular formula $C_{25}H_{52}$ (352.686 μ). Nonacosane has been isolated from *Brassica oleracea* Linn. and *Artemisia annua* (Uluben and Halfon, 1976) and pentacosane has been isolated as a constituent of the natural waxes of many plant species (Karrer, 1981).

The structure of the last compound has been tentatively identified as that of neophytidiene (Fig. 3.3*f*).

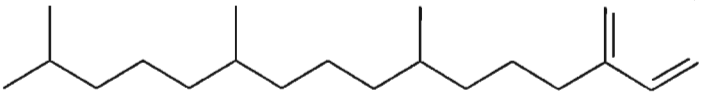


Fig. 3.3*f*: Neophytidiene

The GC-MS spectrum of this compound is shown below (Fig. 3.3*g*).

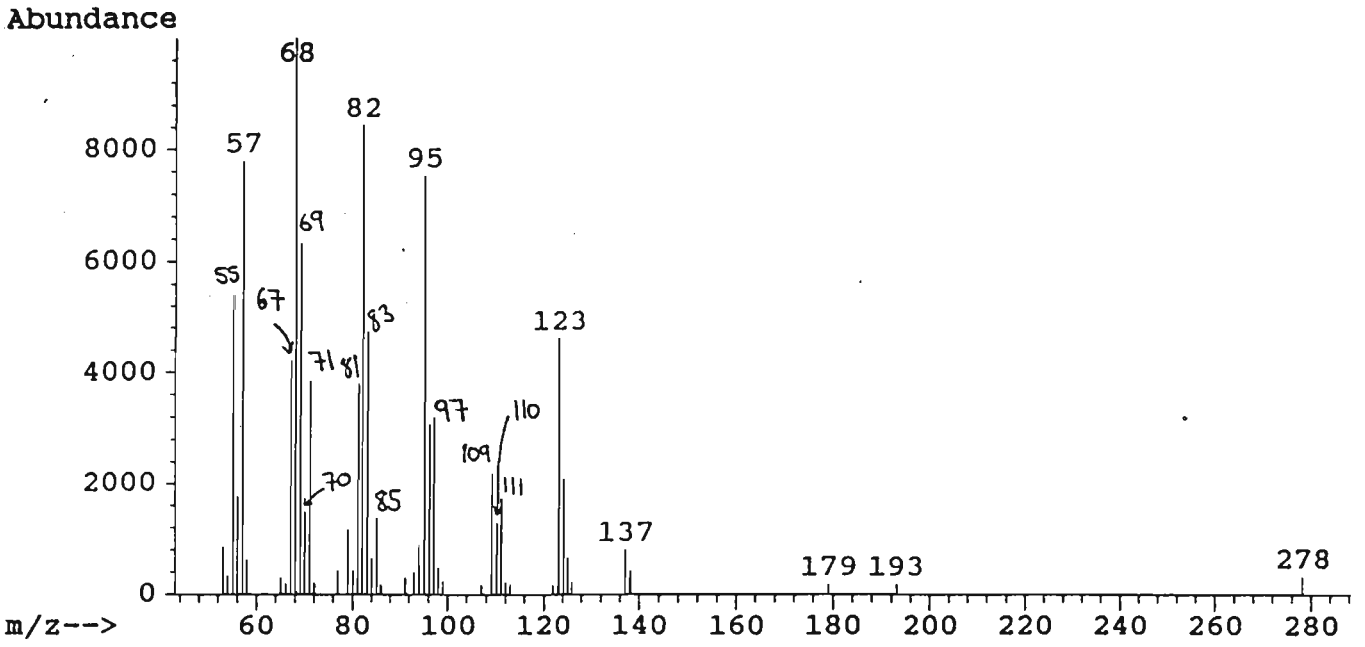
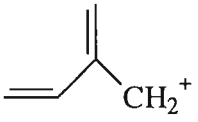


Fig. 3.3*g*: GC-MS spectrum of neophytidiene

A peak at *m/z* = 67 is consistant with the formation of:



The most common fragmentation in alkene groups involves the rupture of the allylic bond β to the double bond which gives rise to the resonance stabilised allylic cation (Fig. 3.3*h*)(Kemp, 1991).

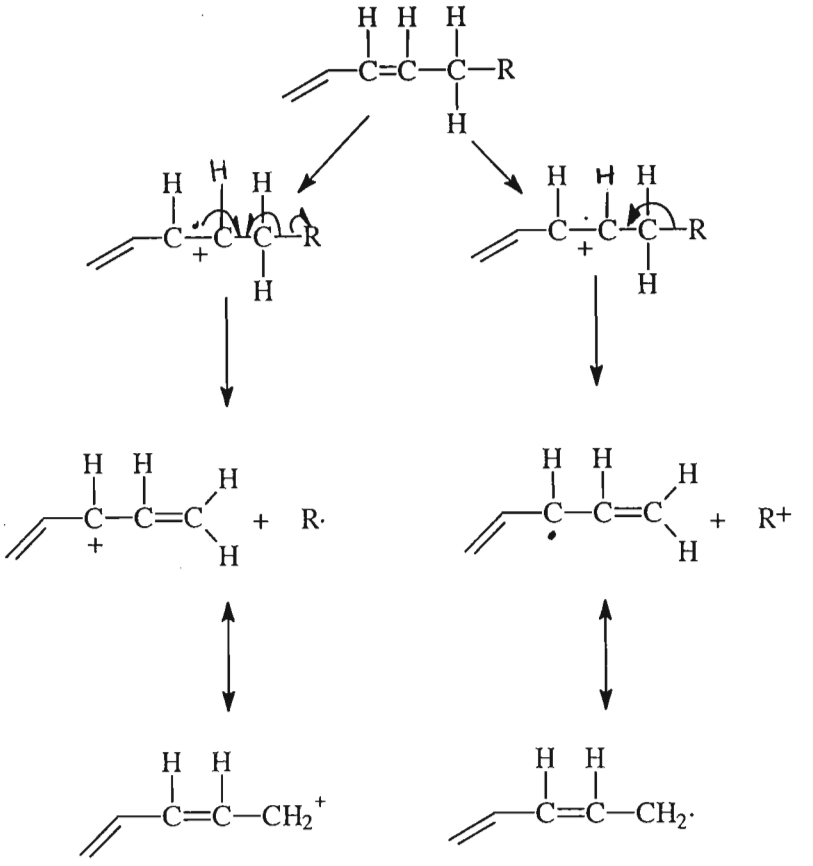


Fig. 3.3*h*: Common fragmentation patterns of alkene groups

McLafferty rearrangement my also occur in olefins provided that the γ carbon has a hydrogen on it (Fig. 3.3*i*).

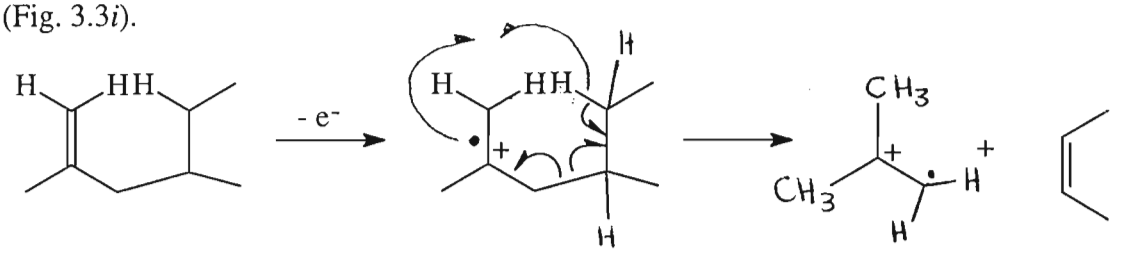
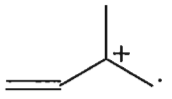
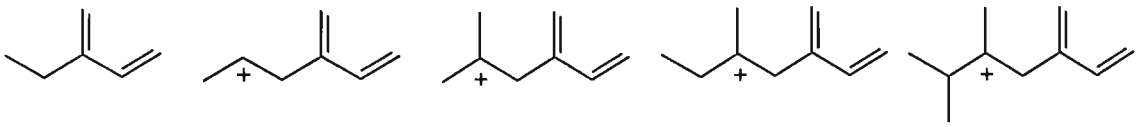


Fig. 3.3*i*: McLafferty rearrangement of alkenes

A peak at *m/z* = 68 is consistant with the formation of:



The homologous series $C_nH_{2n+1}^+$ discussed previously could also be seen (n = 4, 5, 6, 7, 8) as well as well as examples of the corresponding $C_nH_{2n-1}^+$ (n = 4, 5, 6, 7, 8). A further homologous series could be seen namely: $C_nH_{2n-3}^+$ (n = 6, 7, 8, 9, 10). This could be rationalised by the following fragment cations:



The only major peak that does not fit into any of these categories is m/z = 82 corresponding to $C_6H_{10}^+$. This could be rationalised by the following mechanism:

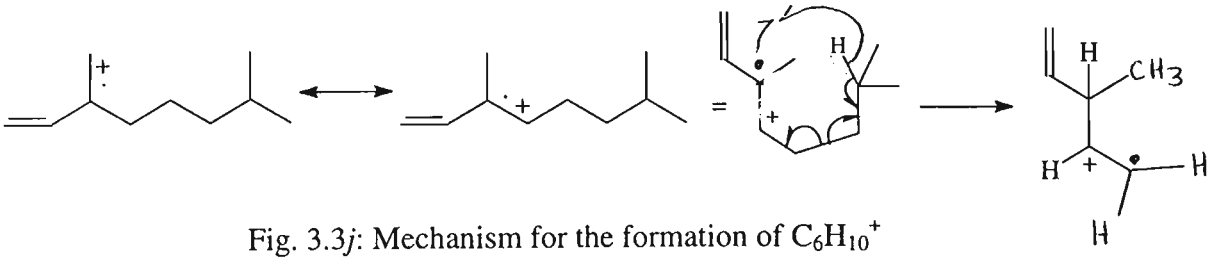


Fig. 3.3*j*: Mechanism for the formation of $C_6H_{10}^+$

The structure of neophytidiene was first determined by Rowland (1957) as part of his analyses of the ether soluble materials extracted from flue-cured tobacco. He suggested that it could be a result of the breakdown of phytol (ex chlorophyll) in the tobacco leaf.

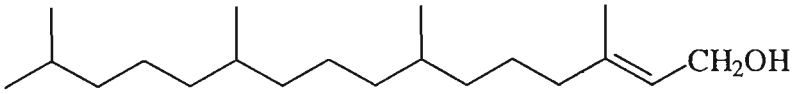


Fig. 3.3*k*: Phytol

These waxes were found in samples resulting from Soxhlet extraction (in hexane or methylene chloride) for longer than 24 hours therefore, they would consist of not only epicuticular waxes, but also of embedded waxes (Bewick *et al.*, 1993). The presence of surficial wax was visualised using scanning electron microscopy.

The presence of surficial wax crystals on the stipule surface (Figures 3.3*l* and *m*)(which is the surface directly exposed to the atmosphere) is consistent with the fact that the plant needs to be dessication tolerant. The wax crystals were plates that formed rosette-like structures on the surface. They were especially abundant on the tips of the stipules – the area closest to the small exposed leaf surface (Fig. 3.3*m*).

Given its habitat preferences, not only would *A. rhodesica* have to be dessication tolerant, it would also have to be adapted to high light intensities. With this in mind, it is interesting to note the morphology of the stipule surface (Figures 3.3*l* and *m*). In most plants, a pair of stipules is present at the base of the petiole (leaf stalk). They are usually small in comparison to the leaf size and

serve to protect the delicate bud at the leaf axil during its early development. In *Avonia* the stipules are much larger and have been modified to form scales which cover almost all the leaf surface (Rowley, 1995). According to Rowley (1995), the functions of these stipules include reflection and diffusion of light; limiting evaporation; and controlling aeration by curving in and out according to humidity.

Light microscopy was used to qualitatively determine the amount of light transmitted through and reflected from the stipule. It was interesting to note that the reflected light appeared to be more intense than the transmitted light, and that the light transmitted appeared to be mostly blue while the light reflected appeared to be mostly yellow (Fig. 3.3*n*). The u.v. spectrum of chlorophyll a showed two major absorptions, one at 403.35 nm (100%) and the other at 665.05 nm (43%) corresponding to the absorption of violet and red light respectively (*see chapter 4.3.3*). The relatively high absorption of blue light required for the light reactions of photosynthesis (Matthews and van Holde, 1991) would therefore account for the selective transmittance of blue light by the stipules.

It was noticed that the waxes, when floated on hot water, were molten. The melting points were found to be 52-60° C. In the molten state they were highly reflective. Attempts were made to visualise the melting of the waxes on the surface of stipules by attaching the hot-stage of the melting point apparatus to the light microscope (Biophot). Heating the stipule to 100° C resulted in no apparent change in the morphology of the stipule and the wax on the surface did not melt. There was also no change in the selectivity of transmittance of light by the stipule. The fact that the wax did not melt could mean that the stipule is an extremely poor conductor of heat and thus very effective in protecting the plant from excessive heat.

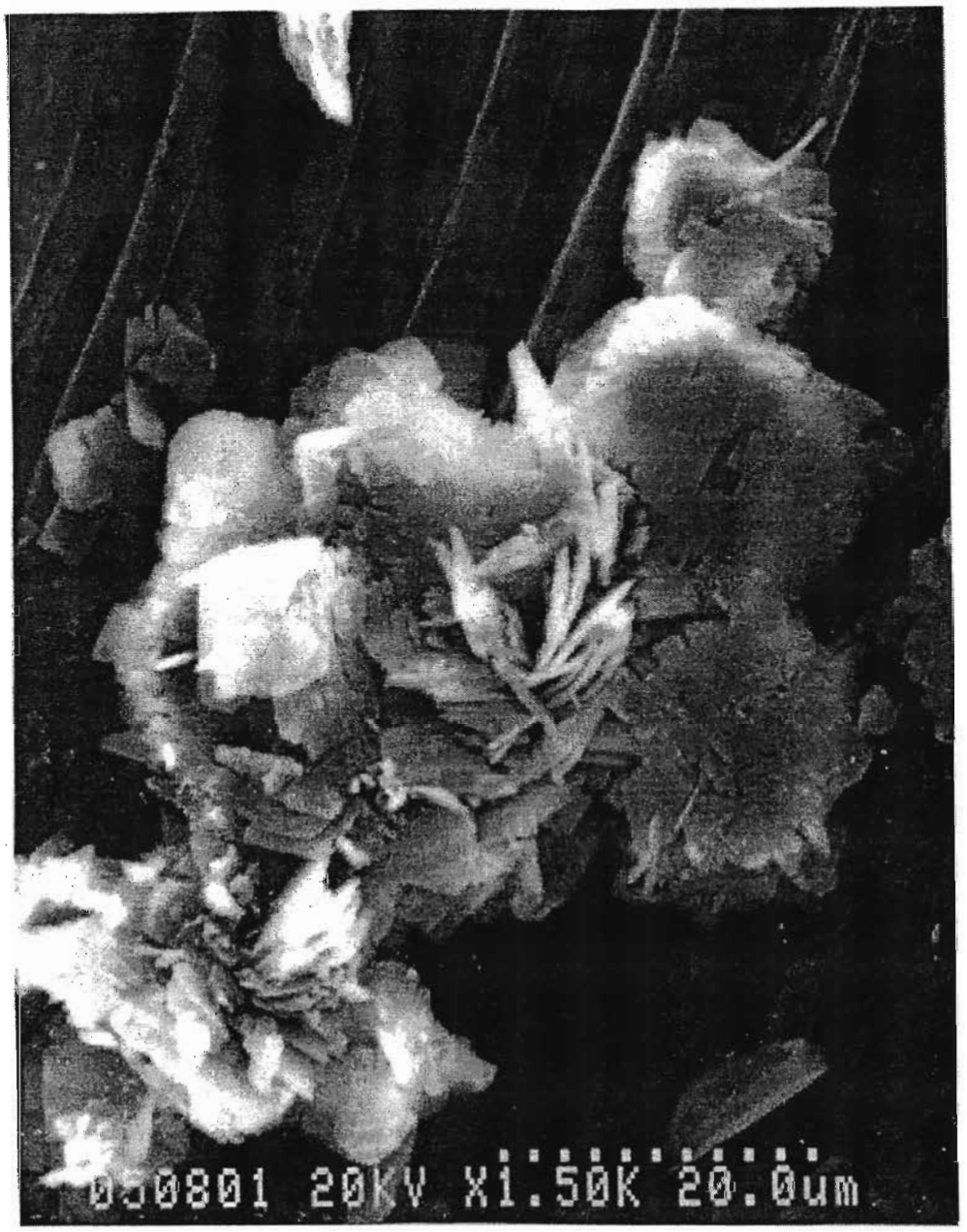


Fig. 3.3*l*: A rosette-like structure on the surface of *Avonia rhodesica* resulting from the arrangements of wax crystals

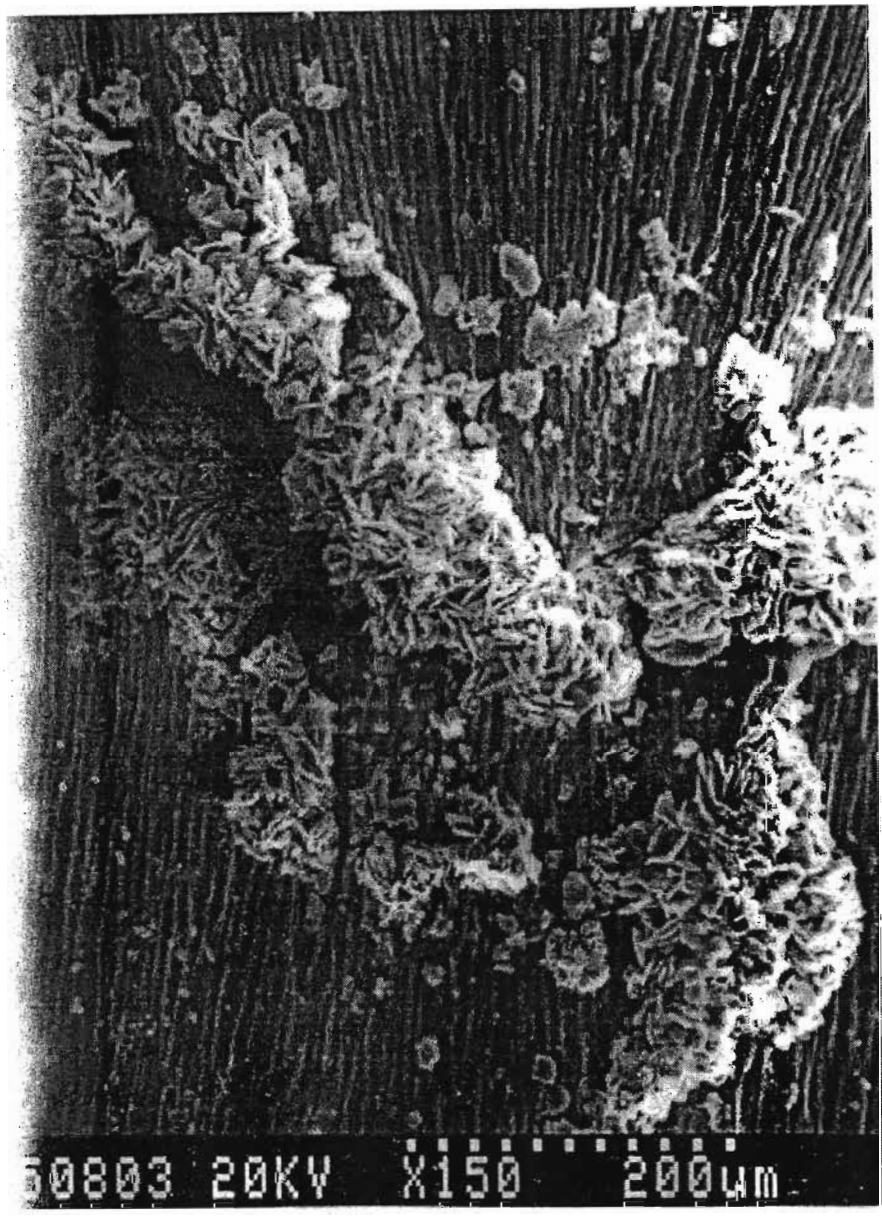


Fig. 3.3*m*: Proliferation of wax crystals on the tip of a stipule

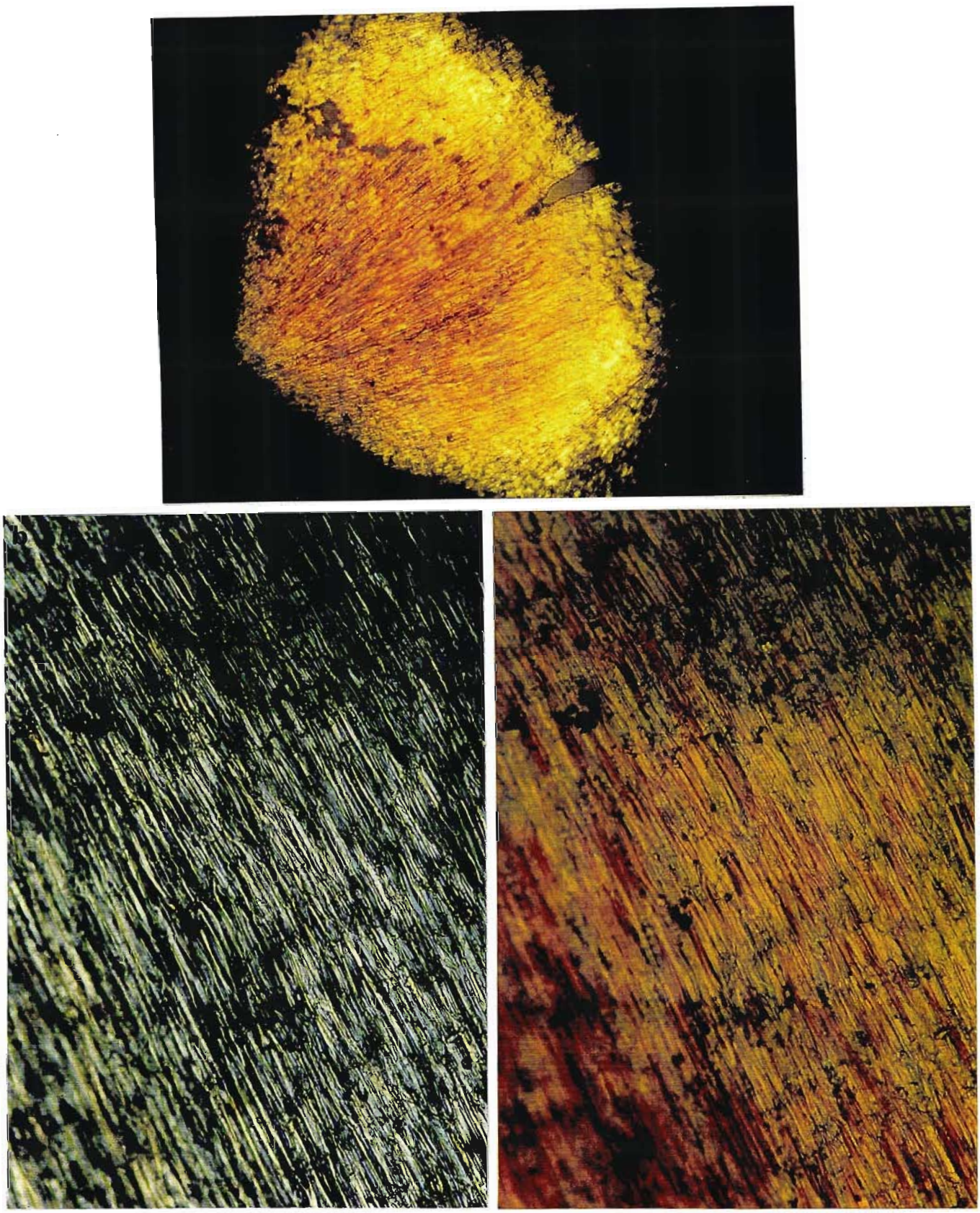


Fig. 3.3*n*: (a) Light reflected/scattered off an entire stipule of *Avonia rhodesica* (70x)
(b) Blue light transmitted through the stipule (180x)
(c) Yellow/orange light reflected/scattered off the surface of the stipule (180x)

3.3.2. Structure elucidation of compounds 6 and 7

spectra: S61-S67

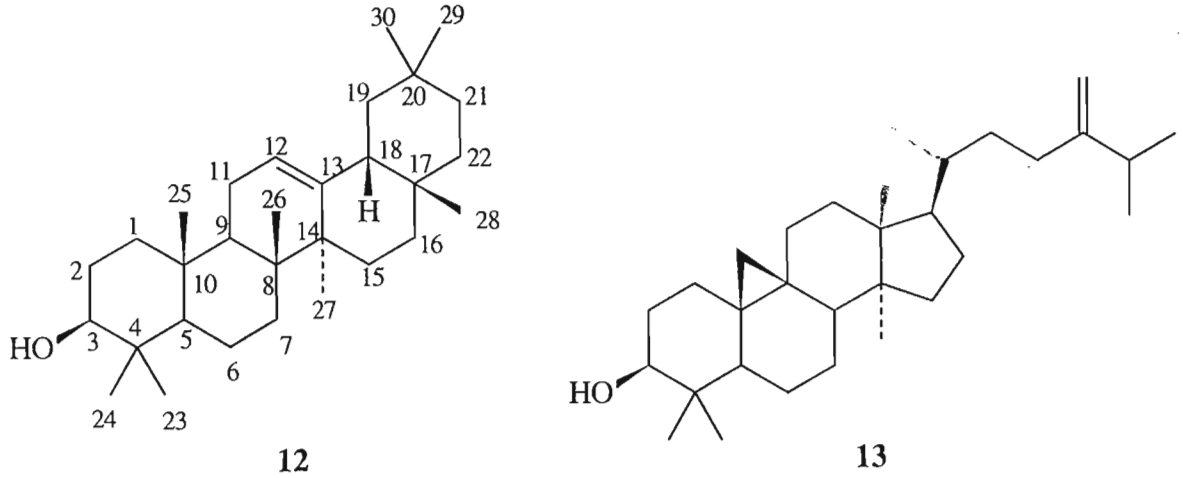


Fig. 3.3*o*: Compound 6 and compound 7

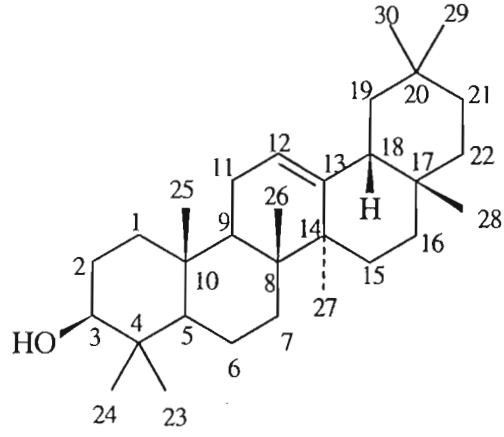
Compounds 6 and 7 were isolated as a mixture of triterpenols from the methylene chloride extract of *A. rhodesica*. Many attempts were made to separate this mixture using a number of solvent systems with silica gel, using LH-20 Sephadex, as well as normal phase HPLC (high performance liquid chromatography). From the carbon-13 n.m.r. spectrum (*S63, S65*) it was noted that certain peaks had higher intensities than others, and it was thought that those of similar intensity might represent the carbon atoms of one of the triterpenols, and those of lower intensity would represent the other.

The presence of two doublets at δ 0.53 (J=4.41) and 0.31 (J=4.14) on the proton n.m.r. spectrum (*S61*) suggested that one of the triterpenols had a cyclopropane ring. A multiplet at δ 3.22 integrating to two protons was thought to correspond to two protons on hydroxylated carbons. The presence of a triplet at δ 5.17 and a pair of doublets at δ 4.70 and 4.64 was also noted.

There were four carbon-carbon double bond resonances in the carbon-13 spectrum [δ 156.9, 145.2, 121.7 and 105.9] as well as two resonances at δ 79.0 and 78.0 indicating the presence of carbon atoms attached to oxygen atoms.

With this information, an extensive literature search was performed, and it was found that in 1975, Seo *et al.* published the carbon-13 spectra of a number of urs-12-enes and olean-12-enes isolated from *Isodon japonicus*. It was noteworthy that the chemical shifts for β-amyrin (**12**) were all

represented in the carbon-13 n.m.r. spectrum of the sample. Subsequent to the isolation of this mixture from *A. rhodesica*, another member of the research team isolated β-amyrin from *Cedrelopsis grevei* (Table 3*d*).



12

Fig. 3.3*p*: β-amyrin

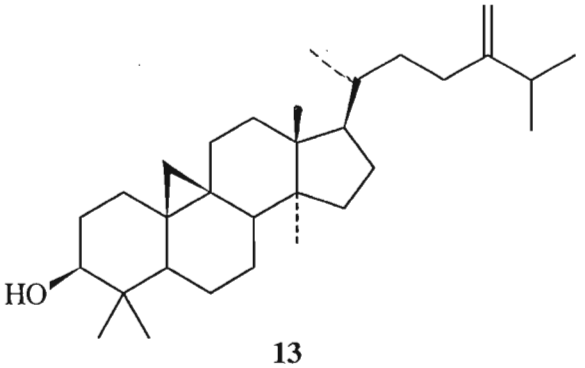
Table 3*d*: Comparison between the ^{13}C n.m.r. shifts for β-amyrin isolated from *Isodon japonicus*, *Cedrelopsis grevei* and *A. rhodesica*

Carbon Number	Chemical shifts/ppm for 12 in $CDCl_3$ from *I. japonicus* (*S63, S65*)	*Chemical shifts/ppm for 12 in $CDCl_3$ from *C. grevei* (*S64, S66*)	Chemical shifts/ppm for 12 in $CDCl_3$ from *A. rhodesica*
1	38.5	38.6 (CH_2)	38.6
2	27.0	26.9 (CH_2)	27.2
3	78.9	79.0 (CH)	78.8
4	38.7	38.8	38.8
5	55.1	55.2 (CH)	55.2
6	37.0	36.9 (CH_2)	37.2
7	32.6	32.6 (CH_2)	32.7
8	39.7	39.8	39.8
9	47.6	47.6 (CH)	47.6
10	18.3	18.4	18.3
11	23.4	23.5 (CH_2)	23.5
12	121.7	121.7 (CH)	121.7
13	145.0	145.2	145.2
14	41.7	41.7	41.7
15	28.3	28.4 (CH_3)	28.4

16	26.2	26.1 (CH_2)	26.2
17	32.5	32.5	32.5
18	47.2	47.2 (CH)	47.2
19	46.8	46.8 (CH_2)	46.8
20	31.1	31.1	31.1
21	34.8	34.7 (CH_2)	34.7
22	37.2	37.1 (CH_2)	37.2
23	28.1	28.1	28.1
24	15.5	15.6 (CH_3)	15.6
25	15.5	15.5 (CH_3)	15.5
26	16.8	16.8 (CH_3)	16.8
27	26.0	26.0 (CH_3)	26.1
28	27.3	27.2 (CH_2)	27.2
29	33.2	33.3 (CH_3)	33.3
30	23.6	23.7 (CH_3)	23.5

*β-amyrin spectra kindly provided by M. Kotsos

Compound 6 was therefore identified as β-amyrin. Examination of the remaining carbon-13 resonances led to the conclusion that compound 7 had a cycloartane skeleton with one double bond. The pair of doublets at δ 4.70 and 4.64 (each with a coupling constant J=1.5 Hz) were also associated with this compound, and because these are typical of terminal double bond protons that are non-equivalent, compounds having this functional group were examined in the literature search. It was found that in 1987, De Pascual Teresa *et al.* isolated 24-methylenecycloartenol (**13**) as one of the sterols in the latex of *Euphorbia broteri* Daveau.



13

Fig. 3.3*q*: 24-methylenecycloartenol

This compound was found to have carbon-13 n.m.r. shifts that compared favourably with the remaining signals of the triterpenol mixture (Table 3*e*). It was therefore proposed that compound 7 was 24-methylenecycloartenol.

Table 3*e*: Comparison between the ^{13}C chemical shifts of **13** and the remainder of those in the sample isolated from *A. rhodesica*

Carbon Number	**13 isolated from *E. broteri* in $CDCl_3$/ppm**	**13 isolated from *A. rhodesica* in $CDCl_3$/ppm**
1	30.1	29.9
2	30.5	30.4
3	78.8	79.0
4	40.5	40.5
5	47.2	47.1
6	21.2	21.1
7	28.2	28.2
8	48.0	48.0
9	20.1	20.0
10	26.2	26.0
11	26.1	25.8
12	35.7	35.6
13	45.4	45.3
14	48.9	48.8
15	33.0	32.8
16	26.6	26.5
17	52.4	52.3
18	18.1	18.0
19	29.9	29.9
20	36.2	36.1
21	18.4	18.4
22	35.2	35.0
23	31.4	31.3
24	156.8	156.9
25	33.9	33.8

26	21.9	21.9
27	22.1	22.0
28	19.4	19.3
29	14.1	14.1
30	25.5	25.4
31	106.1	105.9

3.4. The absence of alkaloids

It was disappointing to find that none of the extracts contained alkaloids. The methanol extract contained a complex mixture of sugars. A portion of this extract was acetylated and the only component isolated that was not an acetylated sugar was triacetylglycerol. A portion of the methanol extract was butylated to investigate the presence of nitrogen containing compounds that were also carboxylic acids, but no such compounds were obtained. Efforts to isolate alkaloids by acid/base solvent extractions of the methanol and water extracts were unsuccessful (Prof. Taylor suggested that this could be because small aromatic molecules are frequently not detected if in small amounts when this method is used due to their relatively poor solubility in solvents such as ether).

The fact that no alkaloids were isolated suggests that either the plant only produces alkaloids in a particular season, or that the plants role in the making of beer is that of a "yeast", or that the people who use this plant in the making of beer treat it in such a way that its chemical composition or properties changes. An example of the latter may be to expose it to a fungus or bacterium that produces alkaloids. It is also possible that the alkaloids are present in quantities too small to be detected.

The last plant that was studied in this investigation was *Equisetum ramosissimum*, and unlike both *Dioscorea dregeana* and *Avonia rhodesica*, the major constituents of this plant were pigments and silica.

Chapter 4. Chemical Investigation of *Equisetum ramosissimum*

4.1. Introduction

4.1.1. Taxonomy and distribution

Equisetum ramosissimum Desf. belongs to the class Sphenopsida. This class includes several living and many extinct representatives, the oldest fossils of which are considered to be of Devonian age. It appears that the period of widest distribution and greatest diversity of this group was that of the Carboniferous age. By the end of the Triassic, however, only a few relatively small, herbaceous representatives remained. The genus *Equisetum* L. (Order Equisetales, Family Equisetaceae) is the only remnant of this class, and, as it does not appear to have undergone any significant change in the course of time from the Carboniferous, it may be the oldest living genus of vascular plants in the world today (Foster and Gifford, 1974). Mabberly (1990) reported that there were 29 extant species in this genus.

An important characteristic of the class Sphenopsida is the subdivision of the shoot axis of the sporophyte into definite nodes and internodes (Foster and Gifford, 1974). At the nodes are whorls of relatively small leaves which are fused together except for their extreme tips to form leaf sheaths. The leaves are usually achlorophyllous, photosynthesis being carried out entirely by the green stems (Sporne, 1979). In *E. ramosissimum* well defined stem ribs are evident, each rib corresponding to a leaf in the node above, and the ridges in successive internodes alternating with one another. Together with the rough texture of *Equisetum*, these characteristics have led to the common names "horsetails" and "scouring rushes" (Foster and Gifford, 1974).

The shoot system of *Equisetum* consists of an aerial and an underground horizontal rhizome portion. The rhizome portion is perennial while the aerial portion may die back during autumn and winter. In *E. ramosissimum*, the black, root-bearing, subterranean stems are horizontal to erect and can be up to 8 mm in diameter. The aerial stems are up to 2 m high and their branches are formed in whorls at the nodes (Schelpe and Anthony, 1986).

Equisetum has a worldwide distribution (except for certain parts of Australasia), but the largest populations are found in the northern hemisphere. They typically grow in wet or damp places such as along the banks of streams or irrigation ditches (Foster and Gifford, 1974). In certain countries, e.g. Finland, these large populations have become a problem due to their extensive grazing by livestock (Borg, 1971).

4.1.2. Ethnobotany

In Finland, the large population of *E. palustre* L. plants has made it difficult to prevent the slight, but chronic poisoning of cattle due to the presence of the alkaloid palustrine. In acute cases, the reported symptoms include diarrhoea, refusal to eat and even coma, but for the most part, the symptoms are vague and poisoning often remains undiagnosed. It has however, become necessary in many cases to rather cultivate cereal than farm with cattle (Borg, 1971).

The early Voortrekkers in the Transvaal Highveld (*ca.* 1840) observed that their horses developed a form of shivers after having eaten the shoots of *E. ramosissimum* (Afr. "bewerasie") and could not be ridden for a time. These experiences led to the Afrikaans common name "bewerasiegras" for this plant (Smith, 1966).

Stockowners have stated that all classes of animals develop the following symptoms if driven shortly after eating horse-tail: staggering about in a drunken fashion, falling down, inability to rise, and when standing, shivering of the forequarters or whole bodies. It has been reported that it is rarely fatal if the animals are left undisturbed. Excitement, however, causes precipitation of the symptoms and exhaustion followed by death (Steyn, 1934). It was uncertain whether the toxic effects of the plant material diminished or disappeared on drying.

In the case of this southern African species, the toxicity has been attributed to the thiaminases it contains, and that, because of the rarity of the poisoning it is of academic interest only (Kellerman *et al.*, 1988).

Consumption of another plant which contains thiaminases, *Pteridium aquilinum* (L.) Kuhn, results in similar symptoms to those described above *viz.*, drowziness, dypsnoea, unsteady gait, pronounced staggers, tremors and an awkward stance (Kellerman *et al.*, 1988).

Like many Costa Rican species including *E. myriochaetum* Schlecht. *et* Cham, it is used medicinally as a treatment for human kidney trouble (Foster and Gifford, 1974). Watt and Breyer Brandwijk (1932) stated that a decoction was used as a diuretic, as a styptic especially in urinary trouble, and in the fomenting of septic inflammations. The sap from this plant is reportedly used to relieve toothache and is also applied to the wound after tooth extraction. Powdered stem and water or milk infusions have been reported to be administered to children with abdominal upsets. Rhizome decoctions have been used for infertility by the Sotho. The Dutch settlers used this plant for venereal diseases, diarrhoea, septic inflammations, glandular swelling earache, toothache and as diuretics and uterine styptics (Hutchings *et al.*, 1996).

Equisetum extracts are currently being used in:

1. An anticellulite cosmetic that apparently promotes transdermal transport and also has slimming effects (Voss *et al.*, 1995).
2. A vegetable juice health food rich in superoxide dismutase (Niwano *et al.*, 1993).
3. A liquid plant growth regulator which increased the yield of cucumber and paprika (Kalman *et al.*, 1993).
4. A hair preparation for the prevention of hair loss (Mathieu and Fangain Boudin, 1992).

4.1.3. Review of previous chemical investigations of the genus *Equisetum*

The majority of the compounds isolated form the genus are flavanoids. 3,4′,5,7-Hydroxyflavanone (**1**) has been isolated from a number of *Equisetum* species (Harbourne, 1971). Many flavone (**2**) derivatives have also been isolated.

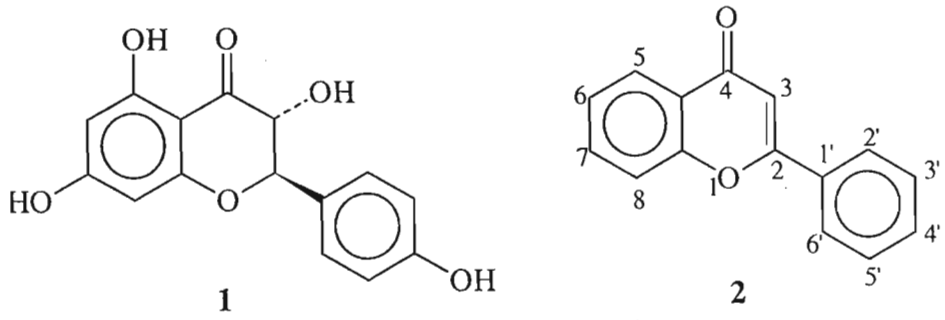


Fig. 4.1*a*: Flavones from *Equisetum* species

Four hydroxycinnamoyltransferases were isolated from *E. arvense* which were shown to be involved in the biosynthesis of some of the complex flavanoids from this species (Hohlfeld *et al.*, 1996). *E. telmateia* Ehrh. has been shown to contain 3-O-(6-O-acetyl-β-D-glucopyranosyl), 7-O-α-L-rhamnopyranosyloxy-4',5-dihydroxyflavone and 3-O-(6-O-acetyl-β-D-glucopyranosyl), 7-O-β-D-glucopyranosyloxy-4',5-dihydroxyflavone (Veit *et al.*, 1995). *E. silvaticum* contained 3-O-rutinosyl-7-O-α-L-rhamnopyranosyloxy-4',5-dihydroxyflavone and 7-O-α-L-rhamnopyranosylrutin (Ally and Geiger, 1975). 3-O-Rutinosyl-7-O-β-D-glucopyranosyloxy-4',5-dihydroxyflavone has been isolated from *E. palustre*, and *E. hyemale* L. contained 8-glucosyl-3-sophorosyl-4',5,7-trihydroxyflavone and 8-O-β-D-glucopyranosyl-3-O-sophorosyl-3',4',5,7-tetrahydroxyflavone (Geiger *et al.*, 1982).

E. arvense also contained two other compounds, namely, protogenkwanin (**3**) and onitin (**4**)(Hauteville *et al.*,1981).

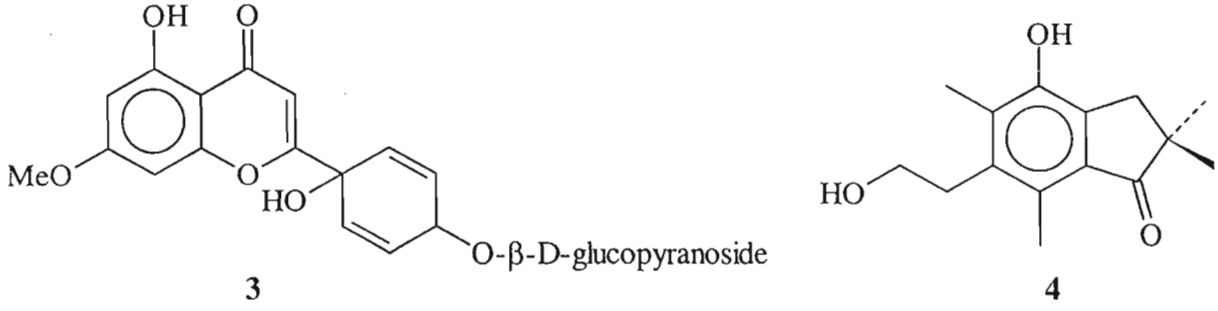
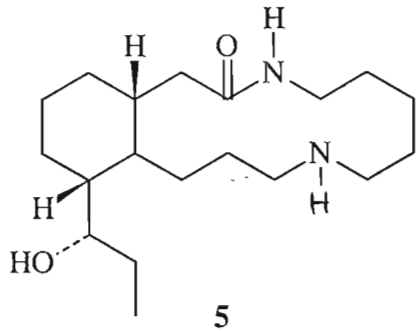


Fig. 4.1*b*: Protogenkwanin and onitin from *E. arvense*

The alkaloid palustrine (**5**) has been isolated from *E. palustre*, *E. arvense* and *E. sylvaticum* L. (Mayer *et al.*, 1978; Walchli *et al.*, 1978). A second alkaloid, 3-methoxypyridine (**6**) has been isolated from *E. arvense* (Manske, 1942). The total synthesis of palustrine was reported by Wasserman *et al.* in 1984.



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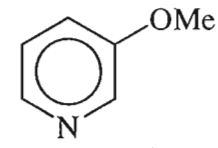

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Fig. 4.1*c*: Alkaloids from *Equisetum* species

Dimethyl sulfone has been found to be a constituent of *Equisetum* species (Langs, 1970).

Many *Equisetum* taxa accumulate minerals in their structures and can be used as indicators of the mineral content of the soil. The mineral content can be determined either by chemical or X-ray analysis of the plant tissues (Foster and Gifford, 1974).

The surface of all *Equisetum* species is irregular due to the deposition of silica (as opal)(Kaufman *et al.*, 1971). In *E. arvense* L., it forms either discrete knobs and rosettes on the epidermal surface and in *E. hyemale* L. it is deposited as a uniform pattern on and in the entire outer epidermis (Foster and Gifford, 1974). This observation was recently confirmed by electron probe microanalysis and electron spectroscopy by Sawatari *et al.* (1996). These workers found that the depth of the silica layer was 10 μm and observed that the worty portions also contained high silicon concentrations.

Possible functions of the silica include: (1) maintenence of the erectness of the plant; to compensate for the very low lignin content of the cell walls, (2) protection against pathogens and herbivores, and (3) prevention of excessive water loss (Kaufman *et al.*, 1971).

4.1.4. Silicon biochemistry

All soil growing plants contain silica, and in many plants (including rice, millet, barley, sunflower, beet, cucumber, corn, tomato and tobacco) it could be considered an essential element, because addition of silicon to culture solutions increases their growth rate and yield. In addition to this, these plants have been reported to exhibit silicon deficiency symptoms including: necrosis of the root and wilting of the leaves (beet) and necrotic spots on the leaves (barley, rice, cucumber). In rice, silicon-deficient shoots exhibit reduced fertility (Chen and Lewin, 1968).

According to Chen and Lewin (1968), the lower leaves of *Equisetum arvense* cultures grown in the absence of silicon, became brown or grey, an effect that later extended into the upper leaves. It was interesting that the increased weights of plants grown with higher silicon concentration was due to an increase in organic matter, not an increase in silica content (Chen and Lewin, 1968).

Chen and Lewin (1968) demonstrated that silicon supply reduces iron and manganese toxicity, and that the necrotic brown spots in silica-deficient plants was probably due to the presence of toxic levels of these elements.

These authors also showed that the rate of transpiration in silicon deficient rice plants was 30% higher than that of control plants. It is therefore probable that silicon is necessary to prevent wilting and death of certain plants including *Equisetum (*Chen and Lewin, 1968).

According to Birchall and Espie (1986), there is no evidence for Si-C bonds in biology, so organic bonding must involve either Si-O-C bonds or hydrogen bonds *via* silanol groups. These authors state that no success has been had in identifying stable Si-O-C species in aqueous solution at neutral pH, although complexes exist at high pH values with 1,2-dihydroxyphenols.

The silicon component of an unspecified *Equisetum* species was shown by Li *et al.* (1992) to have a protective action against experimental liver injury (using CCl_4 or pentobarbitol sodium) in rats and mice.

Carlisle (1986) stated that silicon was required in bone, cartilage and connective tissue formation. He also reported that *in vivo* studies showed that silicon affects the rate of bone mineralisation. Silicon deficiency is incompatible with normal growth and skeletal development in the chick and these abnormalities could be corrected by a silicon supplement. Silicon deficiency in the rat results in depressed growth and skull deformation (Carlisle, 1986).

In his review, Carlisle (1986) also reported that in human serum, silicon is present almost entirely as free soluble monosilicic acid; and that connective tissues such as aorta, trachea, tendon, bone and skin and its appendages are unusually rich in silicon. The high silicon content of connective tissue appears to arise mainly from its presence as an integral component of the glycosaminoglycans and their protein complexes which contribute to the structural framework of this tissue (Carlisle, 1986). It has been shown that there is an interaction between silicon and ascorbate (in cartilage) to give maximal production of hexosamines (Carlisle, 1986). Maximal prolyl hydroxylase (an enzyme involved in collagen biosynthesis) activity also depends on the presence of silicon. Silicon, therefore, has both a structural and a biochemical role in bone development. Connective tissue changes are prominent in aging, so it is not surprising to find a relationship between silicon and aging in certain tissue. The silicon content of the aorta, other arterial vessels and skin was found to decline with age (Carlisle, 1986).

According to Dobbie and Smith (1986), silicon is mainly derived from dietary intake and on a normal diet, humans excrete a large amount of silicon in their urine. With increasing renal functional impairment, urinary elimination of silicon decreases and serum silicon rises, therefore hypersilicaemia should be recognised as a further biochemical characteristic of the uraemic state (Dobbie and Smith, 1986). These authors also state that patients on silica antacids can produce pure silica kidney stones, which was explained by the fact that silicic acid has been shown to inhibit the action of the peptide inhibitors responsible for the mineral crystallization in urine.

Silica has in the past been used in the treatment of gallstones, asthma, coughs, tuberculosis of the lung and arteriosclerosis (Viehover and Prusky, 1938). These authors suggested that, because organic silica is a normal constituent of epithelial structure of human tissue, the water-soluble medication, if absorbed should lead to the formation of a network in the lung tissue giving it strength and greater

resistance against the attack of the organism. It was thought that a result of tuberculosis was the loss by the body of the ability to store silicic acid and therefore a loss in resistance to the destructive processes taking place in the disease (Viehoever and Prusky, 1938).

Apart from silica, the other compounds isolated in this investigation were pigments, one of which was a carotenoid. The other two were porphyrins.

4.1.5. Overview of the biosynthetic pathways pertinent to the chapter

4.1.5.1. Biosynthesis of carotenoids

The condensation of two molecules of geranyl diphosphate (**7**) to form prephytoene diphosphate (**8**) is the earliest step that is unique to the synthesis of carotenoids. Prephytoene diphosphate (**8**) is then converted to *cis*-phytoene (**9**) after which a series of dehydrogenations ensue with the formation of *trans*-phytofluene (**10**), all-*trans*-*Z*-carotene (**11**), neurosporene (**12**) and lycopene (**13**)(Harrison, 1986).

The *cis-trans* isomerism can occur at either the phytoene or the phytofluene stage. It would appear that this conversion is light-induced, because in experiments on a fungal species, *cis*-phytoene was found to accumulate in the dark (Harrison, 1986).

In the biosynthesis of carotenoids in *Capsicum annuum* L., the enzymes were found to be compartmentalised (Camara *et al.*, 1982a and 1982b). Phytoene synthesis occured in the stroma and the subsequent dehydrogenations and cyclisations occured in the membranous fraction (Camara *et al.* 1982a and 1982b). In turn, studies of the carotenoid biosynthesis in both an algal species and seedlings of radish (*Raphanus sativus* L.) show that there are two pools of β–carotene (**14**) *viz.* a small pool for the synthesis of other carotenoids and a large pool for the protection of photosynthetic apparatus in the chloroplast from damage by light (Harrison, 1986).

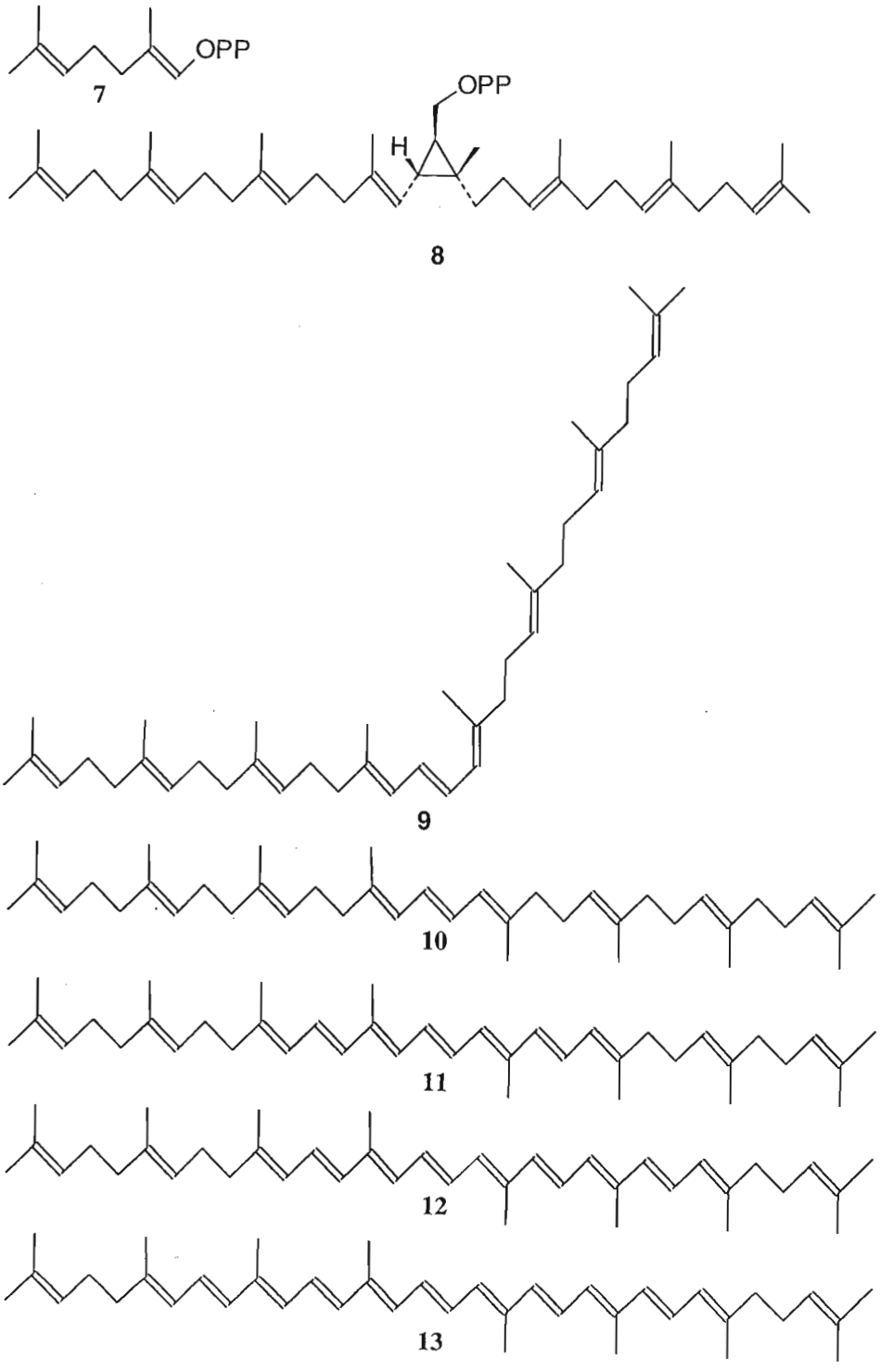


Fig. 4.1*d*: Formation of lycopene

A possible intermediate in the formation of lutein (**16**) is α-carotene (**15**). This reaction proceeds *via* two hydroxylation reactions, both of which occur with retention of configuration (Fig. 4.1*e*)(Milborrow *et al.*, 1982). [This was shown by using (3*RS*, 5*R*) and (3*RS*, 5*S*)[2-^{14}C, 5-^{3}H]mevalonic acid (**17**) as precursors in *Calendula officinalis* Linn.]

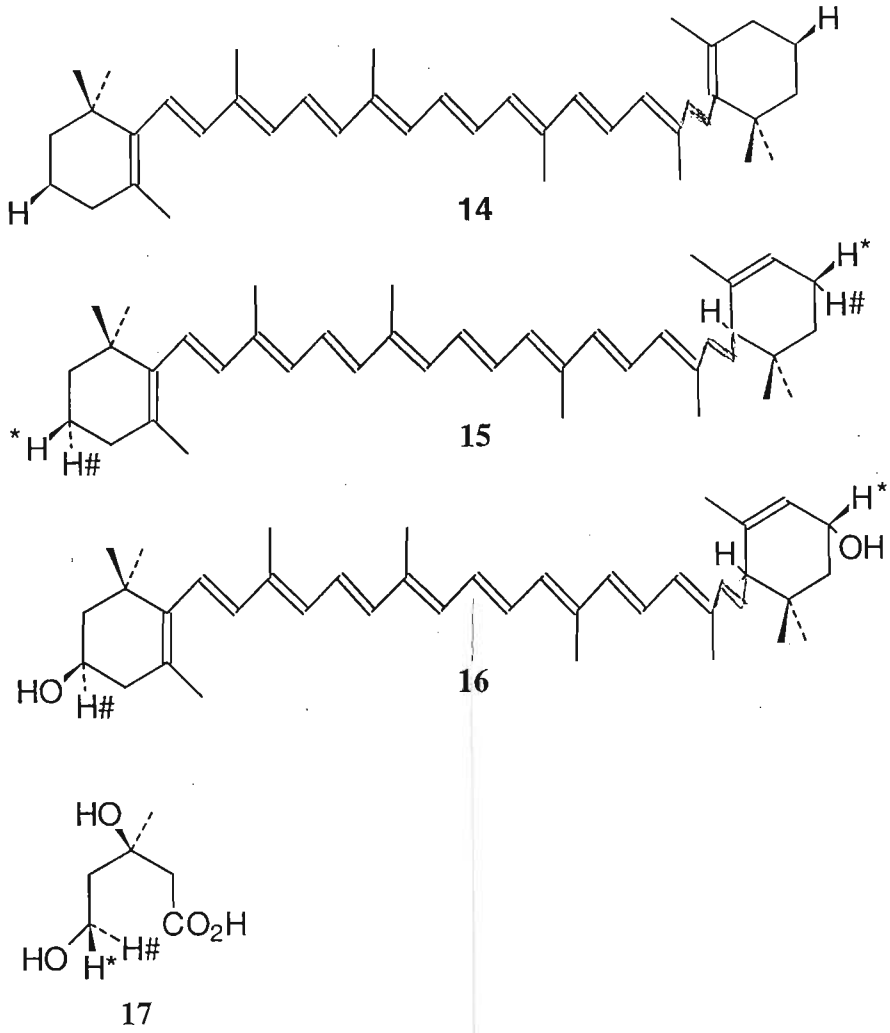


Figure 4.1*e*: Formation of lutein

The synthesis of zeaxanthin (**18**) from violaxanthin (**19**) has been shown to require NADPH and molecular oxygen (Costes *et al.*, 1979). Subsequent to illumination, the concentration of violaxanthin (**19**) decreased with a corresponding increase in the levels of antheraxanthin (**20**) and zeaxanthin (**18**). This reaction was found to be reversible, and in the dark the reverse transformations occurred. It has been shown that the de-epoxidation of violaxanthin (**19**) is linked to the oxidation of

plastohydroquinone-9. Pinacol-like rearrangements of violaxanthin (**19**) and antheraxanthin (**20**) are suspected to result in the formation of capsorubin (**21**) and capsanthin (**22**). It has been suggested that eschscholtzxanthin (**23**), a yellow pigment from *Eschscholtzia californica* Cham., is formed following the dehydration of a monoepoxide of zeaxanthin (**18**)(Harrison, 1986).

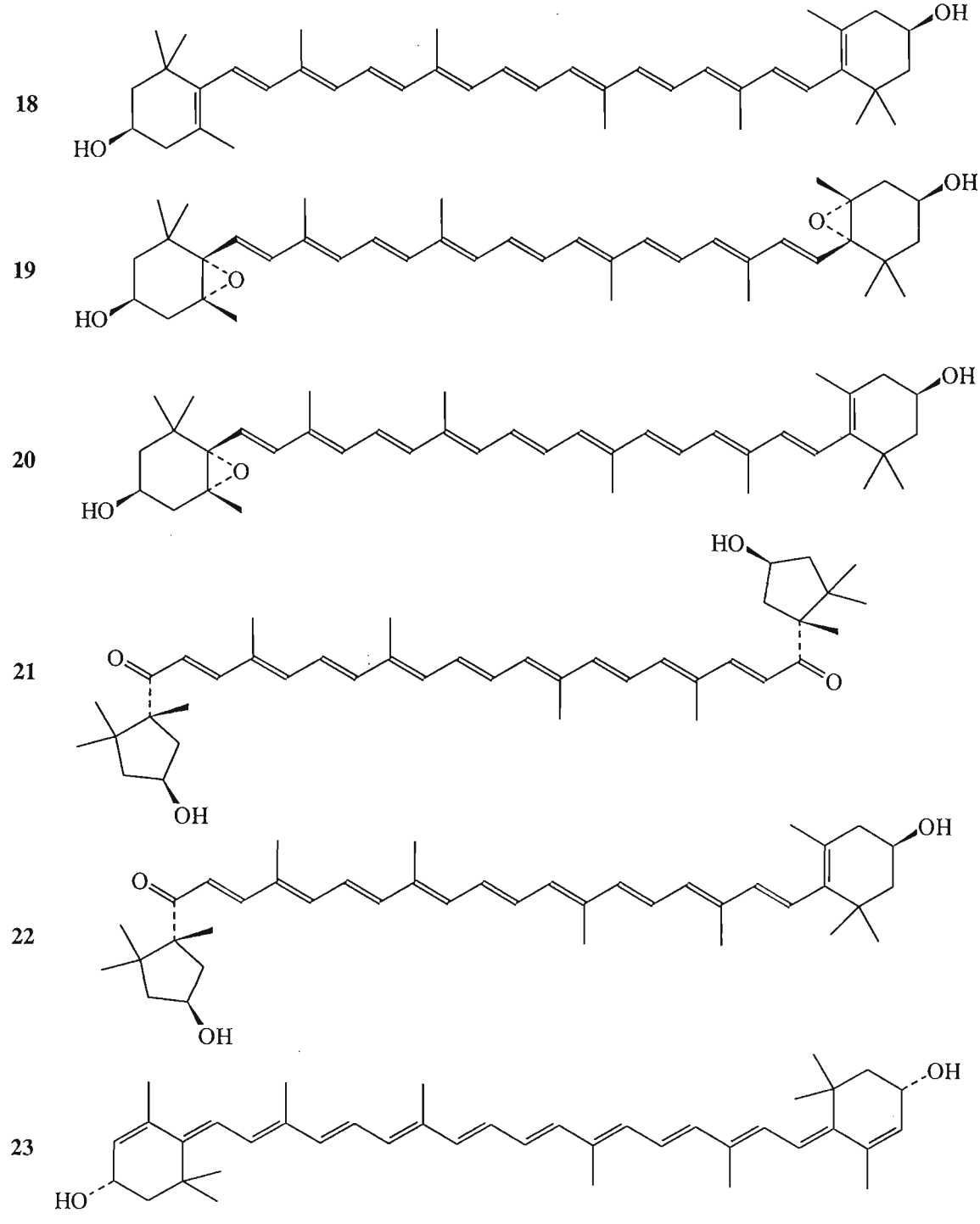


Fig. 4.1*f*: Products of carotenoid biosynthesis

The other two pigments that were isolated in this investigation were porphyrins and the biosynthesis of this class of compound will now be discussed.

4.1.5.2. Biosynthesis of porphyrins

In plants, the starting material for the synthesis of porphyrins is glutamate (24)(Weinstein and Beale, 1983). It is converted to δ-aminolaevulonic acid (**25**) according to the scheme shown in Fig. 4.1*g* (Meller *et al.*, 1975). δ-Aminolaevulonic acid dehydratase (E.C.4.2.1.24) catalyses the Knorr-type condensation of two molecules of δ-aminolaevulonic acid (**25**) to form porphobilinogen (**26**)(Matthews and van Holde, 1991). All known dehydratases are inhibited by laevulonic acid and the enzyme found in Rhodobacter sphaeroides [van Niel] Imhoff *et al.* requires either K^+ or Mg^{++} for activity (Leeper, 1985).

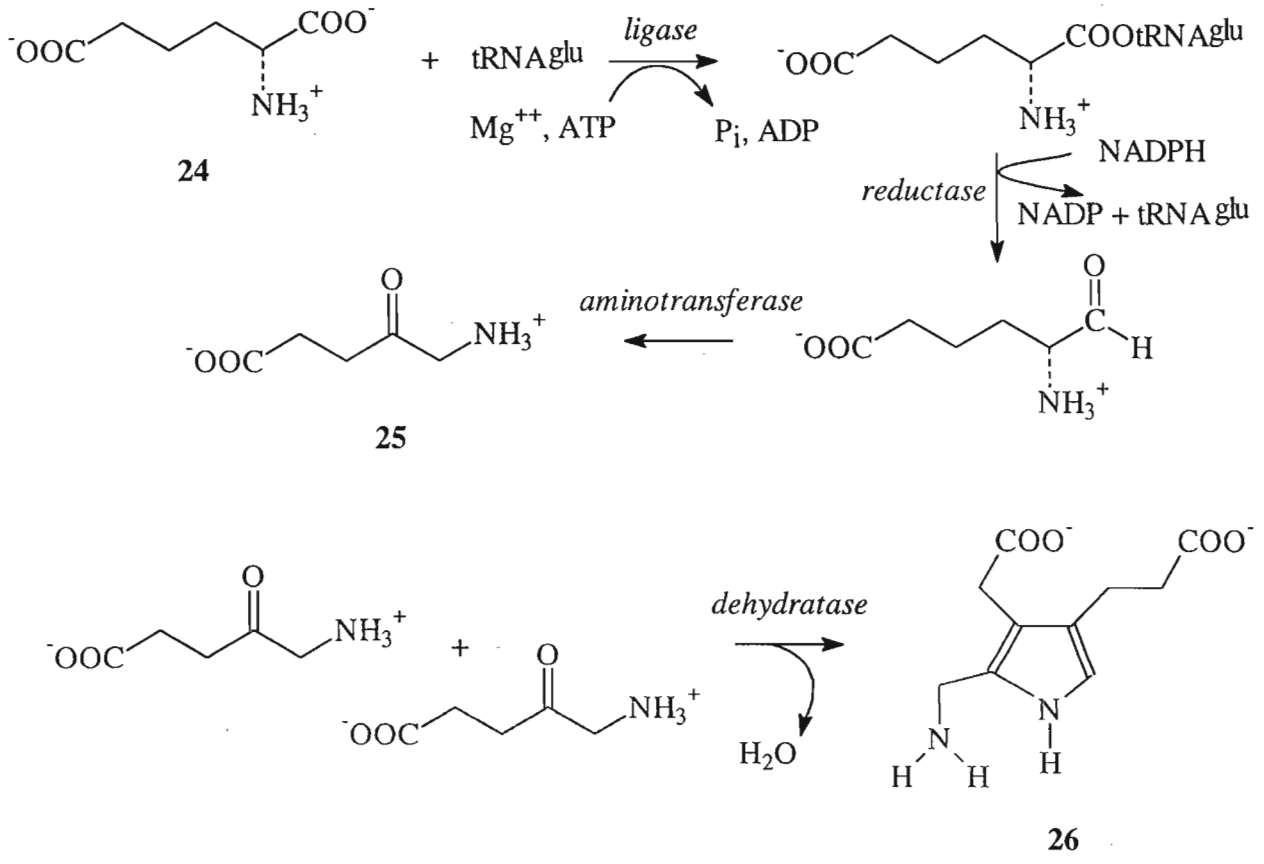


Figure 4.1*g*: Formation of porphobilinogen

Head to tail condensation of four molecules of δ-aminolaevulonic acid (**25**) to form hydroxymethylbilane (**26**) is catalysed by porphobilinogen deaminase (E.C.4.3.1.8). Kinetic experiments on the enzyme show that the first molecule of δ-aminolaevulonic acid (**25**) is covalently bound to the enzyme with the release of one molecule of NH_3. During the subsequent condensations, the di- and tri-pyrrolic intermediates remain bound to the enzyme. In the proposed mechanism (Fig. 4.1*h*), it is possible that the enzymic group X is the amino group of a lysine residue (Jordan and Berry, 1981). While no other substrate for the deaminase has been found, isoporphobilinogen, opsopyrroledicarboxylic acid and 2-aminomethylpyrrole-3,4-diacetic acid are competitive inhibitors of the enzyme (Leeper, 1985).

Cyclisation of hydroxymethylbilane (**26**) with rearrangement of ring D to form uroporphyrinogen III (**30**) is catalysed by cosynthetase (E.C.4.2.1.75). There are two plausible proposed mechanisms for this reaction shown in Figures 4.1*i* and *j*.

1. After the loss of water from hydroxymethylbilane (**26**) to give **27**, cyclisation onto the subsituted α-position of ring D to form spiro compound (**28**) occurs. Subsequent fragmentation could then lead to the formation of **29** with cyclisation to form uroporphyrinogen III (**30**).
2. An enzyme-bound electrophile holds ring D as it is detached for the other three pyrrole units, turned around and re-attached. In the scheme the electrophile is shown as an iminium ion.

The four-fold decarboxylation of uroporphyrinogen (**30**) to form coproporphyrinogen III (**32**) is catalysed by uroporphyrinogen decarboxylase (E.C.4.1.1.37)(Elder, *et al.*, 1983). The first decarboxylation is the fastest, and for this reason, it has been suggested that there are two or more catalytic sites on the enzyme *viz.* one for the first decarboxylation and the others for the remaining ones (De Verneuil *et al.*, 1980; Smith and Francis, 1981). This is supported by the following facts.

1. Decarboxylation of uroporphyrinogen (**30**) was inhibited by uroporphyrinogen I, but that of the intermediates was not (Straka and Kushner, 1983).
2. Decarboxylation of the intermediates, on the other hand, was inhibited by type I porphyrinogen (De Verneuil *et al.*, 1983).
3. Inhibition by polychlorinated biphenyls at low concentration, leads to accumulation of the heptacarboxylic acid intermediate (Kawanishi *et al.*, 1983).

   (The decarboxylase is also inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+} as well as by thiol directed reagents [De Verneuil *et al.*, 1983]).

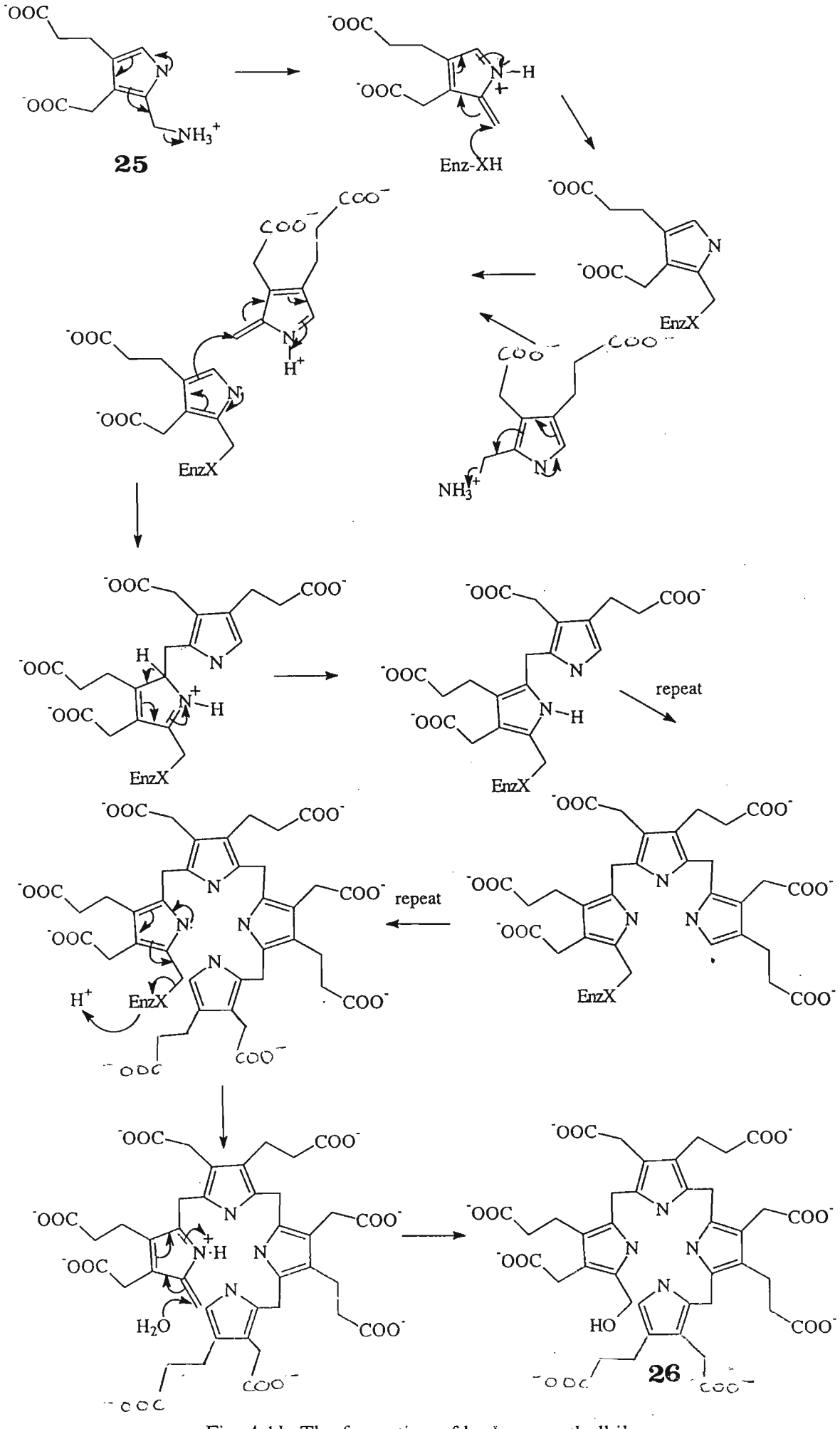


Fig. 4.1*h*: The formation of hydroxymethylbilane

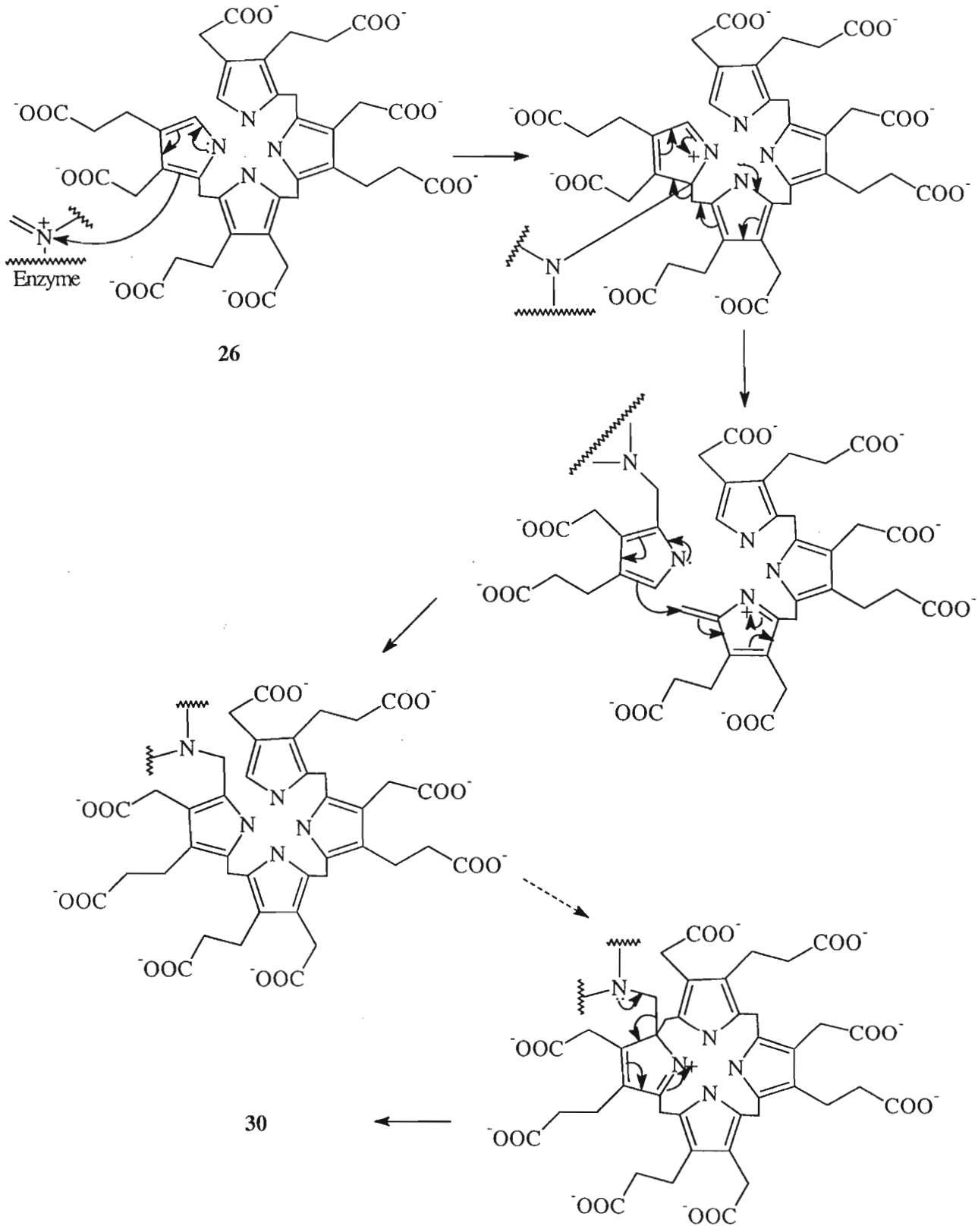


Fig. 4.1*i*: The first mechanism for the formation of uroporphyrinogen III

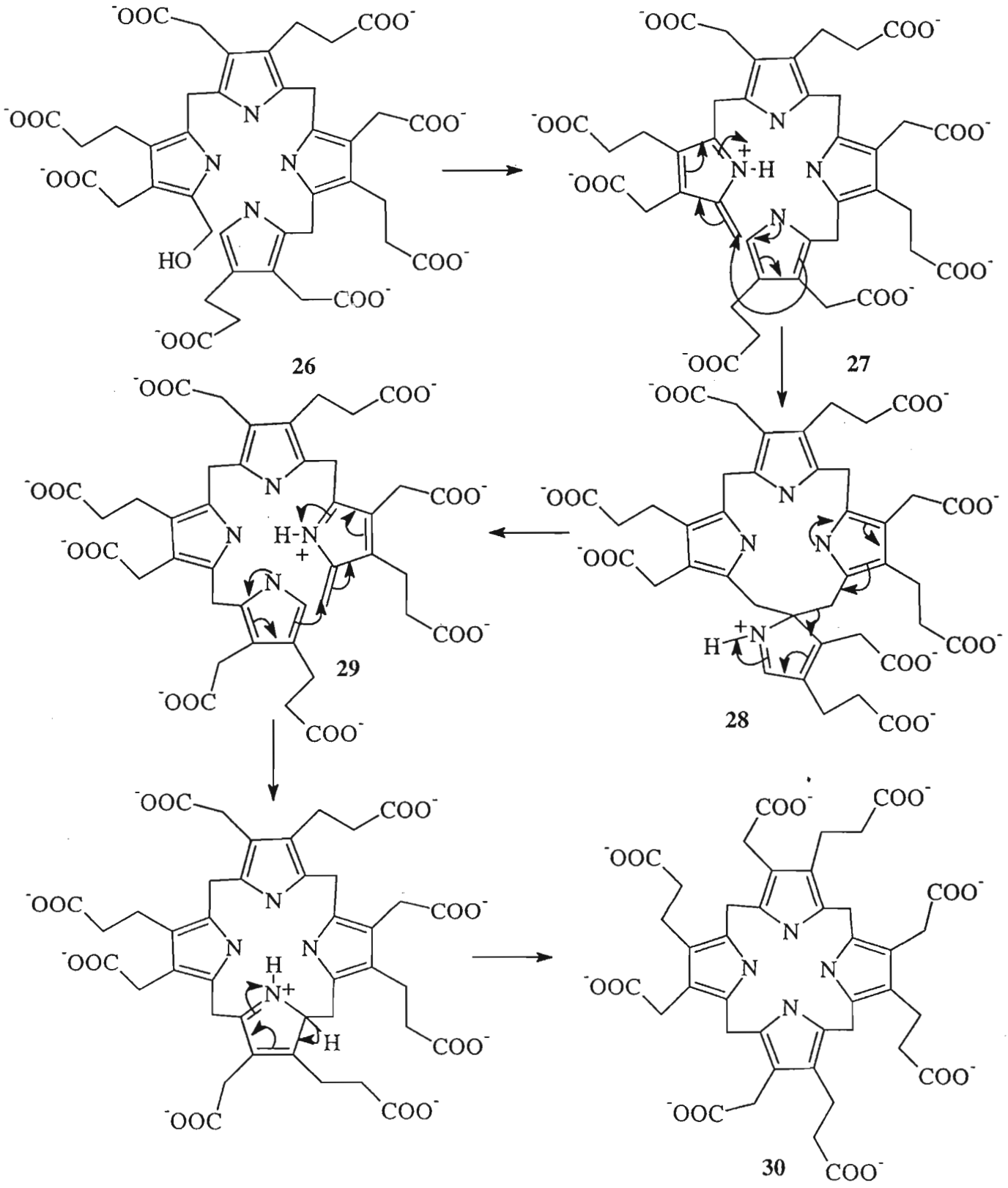


Fig. 4.1*j*: The second mechanism for the formation of uroporphyrinogen III

For uroporphyrinogen (**30**), the order of decarboxylations is not random. The first reaction gives **31** and the preferred subsequent order appears to be clockwise from ring D (*via* ring A and B) to ring C (De Verneuil *et al.*, 1983).

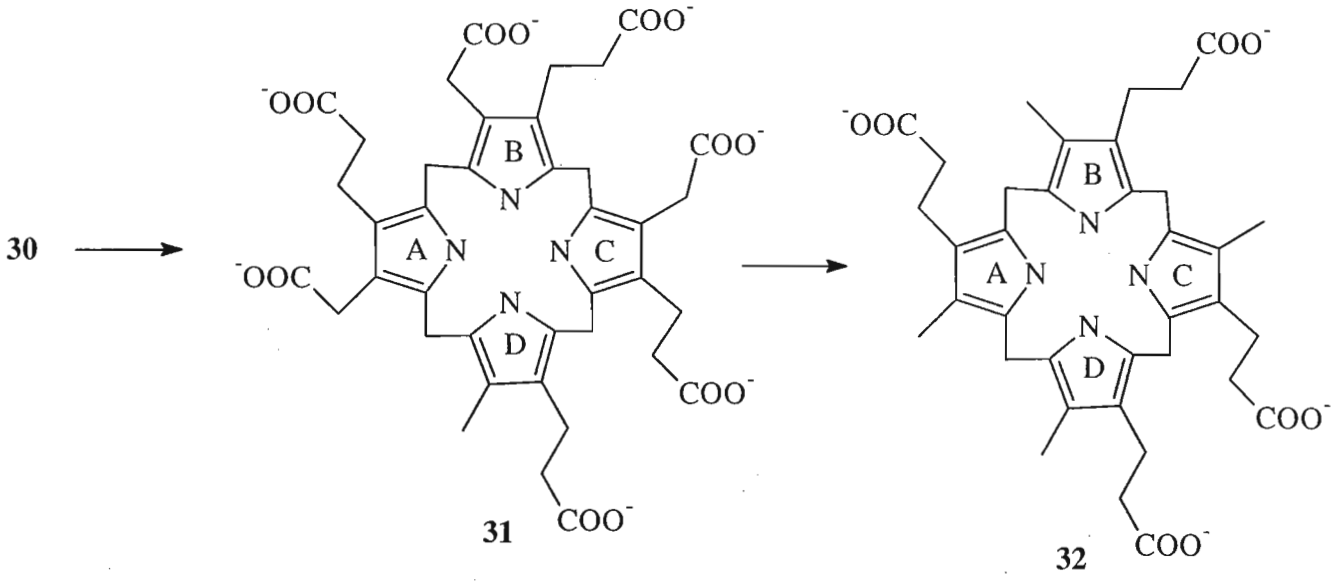


Fig. 4.1*k*: Formation of coproporphyrinogen III

A single enzyme, coproporphyrinogen oxidase (E.C.1.3.3.3), catalyses both of the oxidative decarboxylations of coproporphyrinogen III (**32**) to form proporphyrinogen IX (**34**)(Jackson *et al.*, 1980).

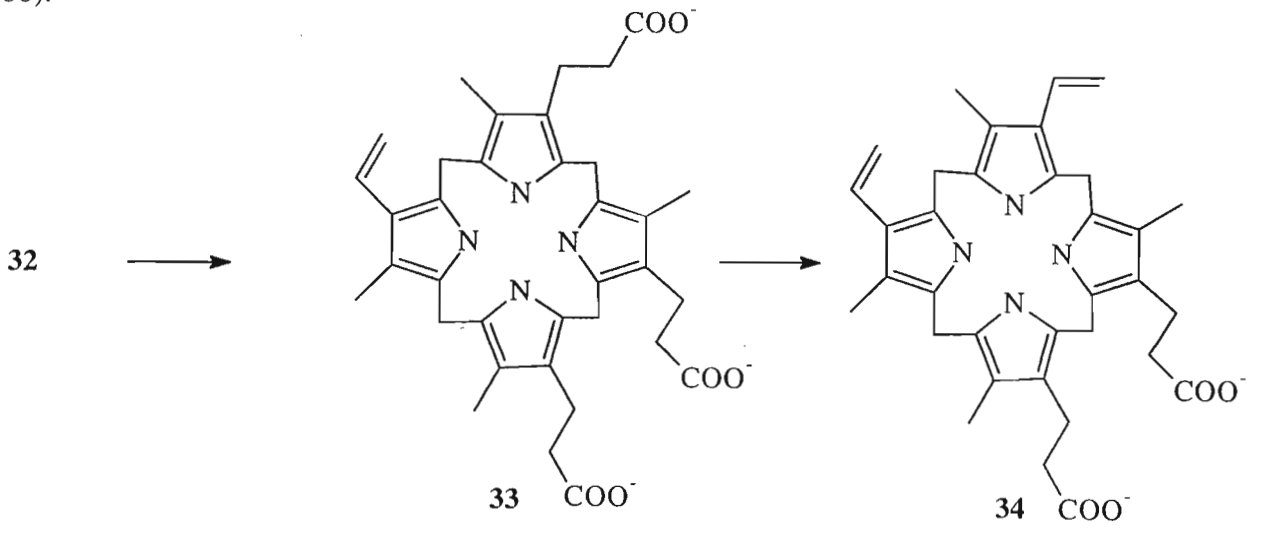


Fig. 4.1*l*: Formation of proporphyrinogen IX

Here, only one active site is involved (Elder, Evans, Jackson and Jackson, 1978). First, the propionate of ring A is converted to a vinyl group to form **33**, and then the corresponding reaction of ring B to form proporphyrinogen IX (**34**) occurs (Jackson *et al.*, 1978). In aerobic organisms, oxygen is the electron acceptor in the oxidation. The stereochemistry has been shown to involve the loss of the *pro-S*-hydrogen atom that is β to the carboxyl group in the propianoate side chain (Figure 4.1*k*). In eukaryotic cells the enzyme is mainly associated with the mitochondria and is thought to be located in the intermembrane space (Elder and Evans, 1978; Yoshinago and Sano, 1980). Two possible mechanisms for this reaction are shown below (Seehra *et al.*, 1983).

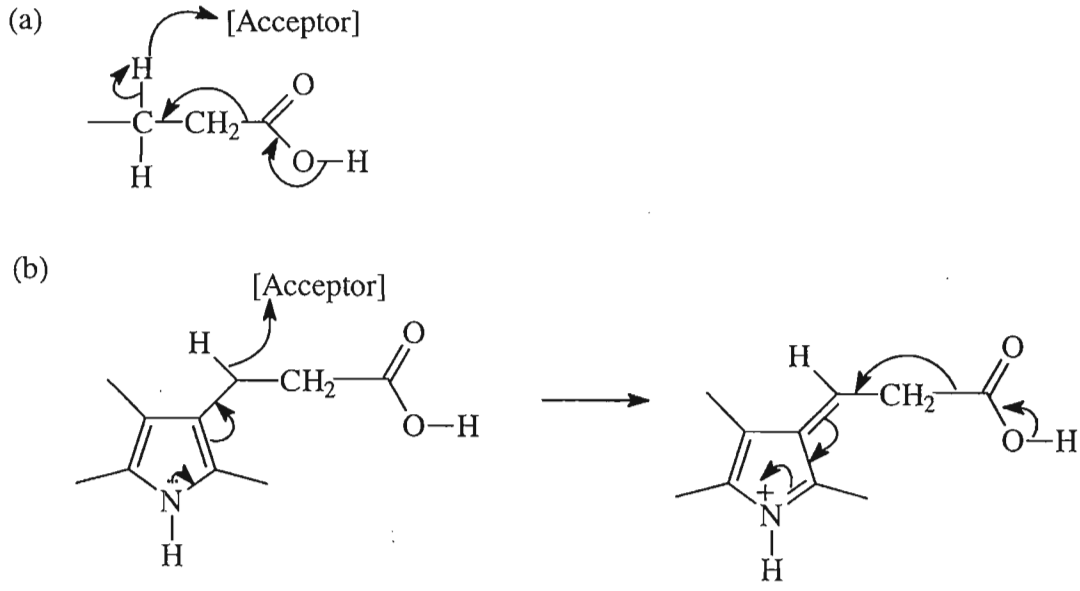


Fig. 4.1*m*: Possible mechanisms for the oxidative decarboxylations

Protoporphyrinogen oxidase (E.C.1.3.3.4) catalyses the oxidation of proporphyrinogen IX (**34**) to the corresponding prophytin (**35**) and uses oxygen as the oxidant. The oxidation involves the removal of four hydrogens in the meso position and two from the nitrogen atoms. The non-polar groups on rings A and B are an important factor. This step is followed by the ATP-dependant chelation of magnesium.

The methylation of the carboxyl group in the 13-propionate to give **36** is catalysed by *S*-adenosyl-L-methionine: magnesium protoporphyrin O-methyl transferase (E.C.2.1.1.11). It was found to require 5-adenosylmethionine (SAM)(Hinchigeri and Richards, 1982).

The reduction of the 8-vinyl group can either occur at this stage to form **37** or at the chlorophyllide stage (that is, reduction of **38** to **39**) depending on the plant species and whether it is day or night (Leeper, 1985).

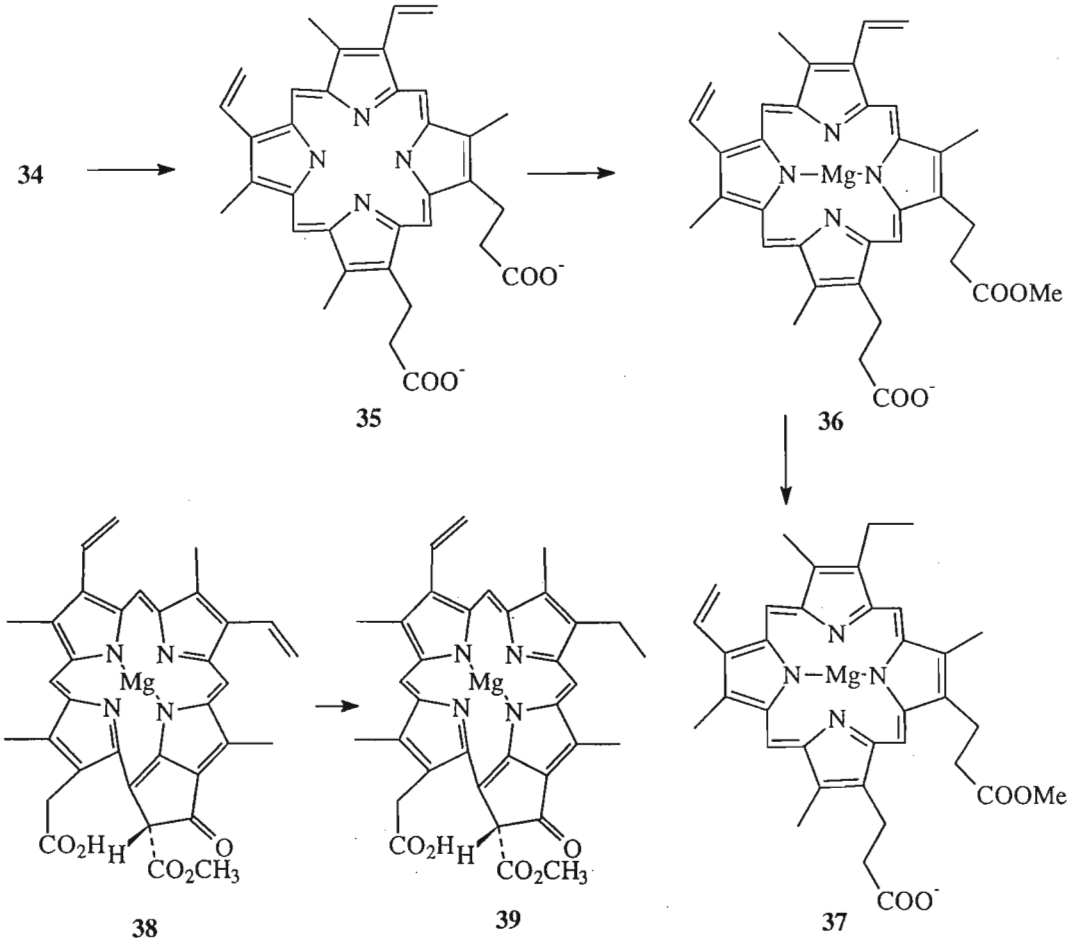


Fig. 4.1*n*: The reactions catalysed by protoporphyrinogen oxidase and protoporphyrin O-methyl transferase, and the reduction of the 8-vinyl group

The oxidation and cyclisation to form ring E requires oxygen, ATP and NAD(P)/NAD(P)H (Fig. 4.1*n*). For maximum efficiency, SAM is also required. This enzyme system is inhibited by both N-ethylmaleimide and dithiothreitol, which indicates the required presence of both free thiols and disulphide links (Leeper, 1985).

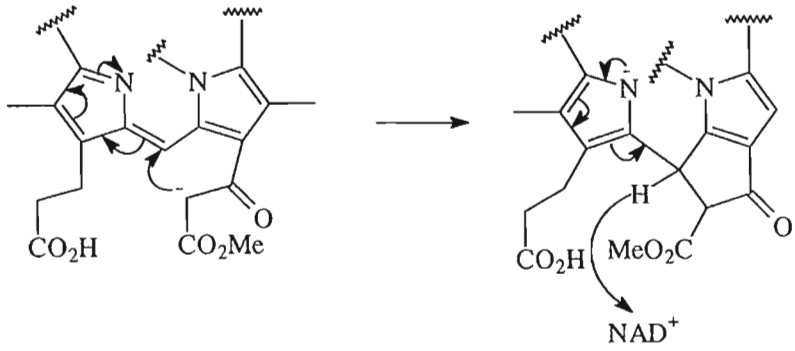
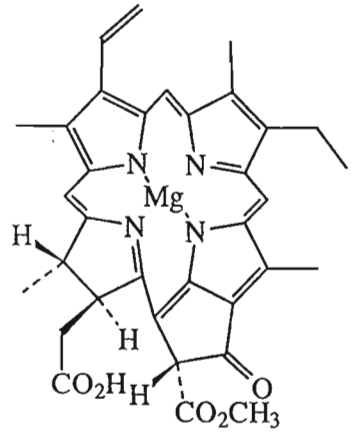


Fig. 4.1*o*: Oxidation and cyclisation to form ring E

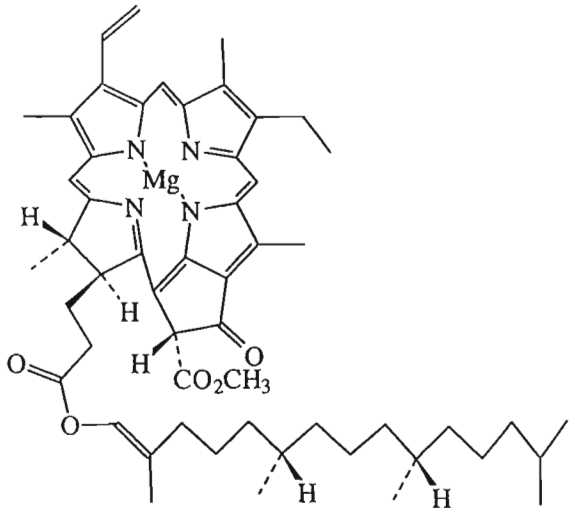
In higher plants, the reduction of **39** to chlorophyllide a (**40**) is catalysed by the light requiring enzyme NADPH:protochlorophyllide oxidoreductase (EC 1.6.99.1). **39**, which absorbs at 635 nm, binds to the enzyme in its reduced form. This chromophore absorbs at about 650 nm and is fluorescent. After absorption it is immediately converted into a non-fluorescent intermediate which absorbs at 690 nm. The intermediate then decays with a time constant of 3 μs to give a fluorescent chlorophyllide with and absorption maximum of about 678 nm (Ellsworth and Murphy, 1979).



40

Fig. 4.1*p*: Chlorophyllide a

The last stage of the synthesis of chlorophyll a (**41**) is the esterification of chlorophyllide a. There are two possible pathways that would lead to chlorophyll a (Fig. 4.1*r*)(Soll *et al.*, 1983).



41

Fig. 4.1*q*: Chlorophyll a

4.2. Results

4.2.1. Elemental analysis

An elemental X-ray analysis of the specimen obtained was performed, and the scanned material in Fig. 4.2*a*(i) contained the following elements (all percentages quoted are relative percentages of the element atoms): O (88.80%), Si (7.21%), Cl (1.56%), K (1.28%), Mg (0.76%) and Ca (0.40%) (Fig. 4.2*b*). The silica was found to be present as a thick layer on both the external and internal circumference of the stem [Fig. 4.2*a*(ii)]. The elements were not distributed evenly throughout the section and three point elemental analyses demonstrate this. In the outer cell wall of the external epidermis the five elements predominating are O (50.73%), Si (27.69%), Cl (9.67%), K (8.66%) and Ca (3.25%) (Fig. 4.2*c*). In the photosynthetic cortical region above the vascular tissue, the elemental composition is very different: O (54.10%), C (36.06%), Cl (2.81%), Mg (2.60%), K (2.03%), S (1.62%) and Ca (0.78%) (Fig. 4.2*d*). In the tissue immediately above the internal epidermis the abundant atoms were: K (37.25%), Cl (29.35%), Ca (18.32%) and S (15.09%)(Fig. 4.2*e*).

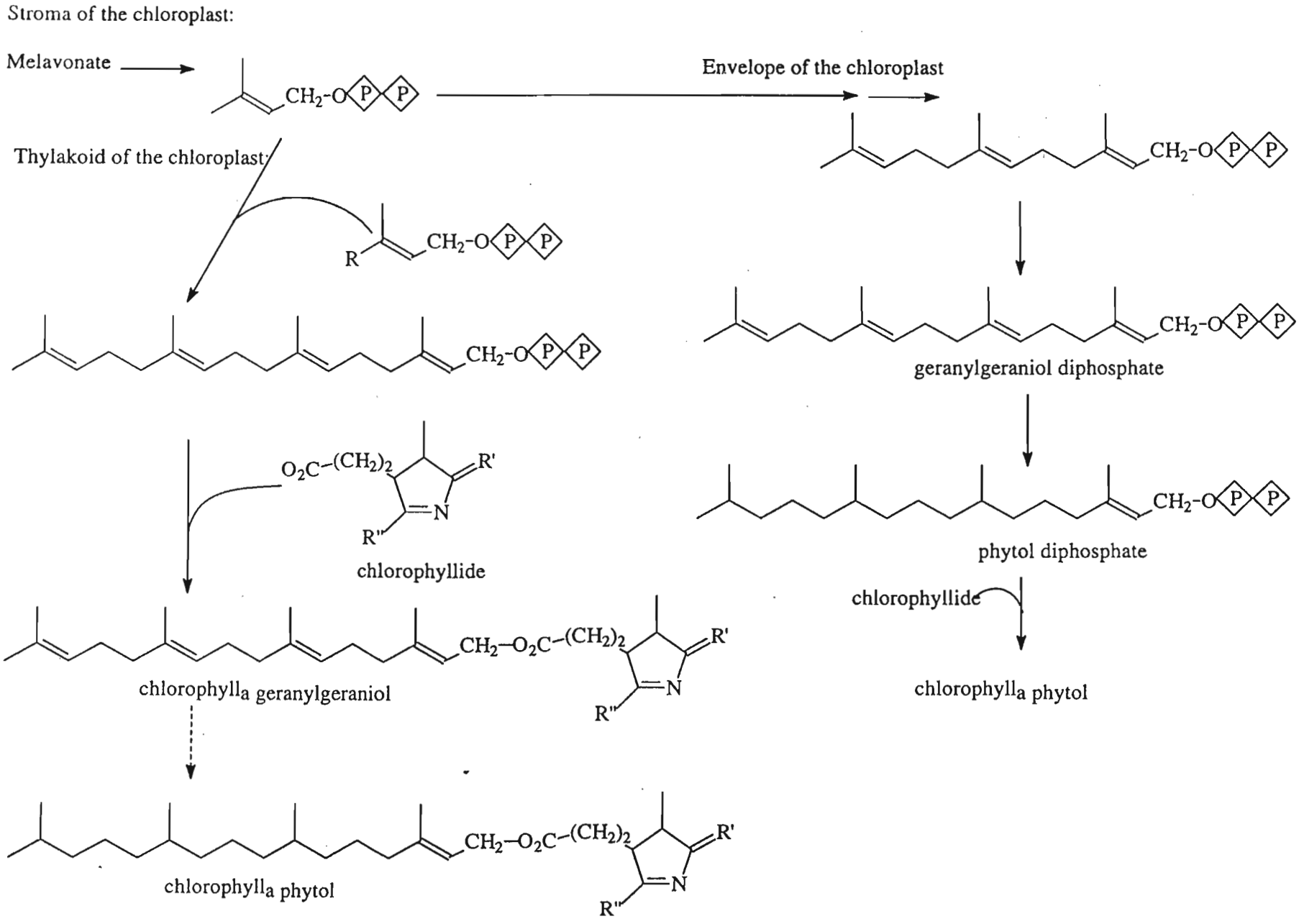


Fig. 4.1*r*: The formation of chlorophyll a in the chloroplast

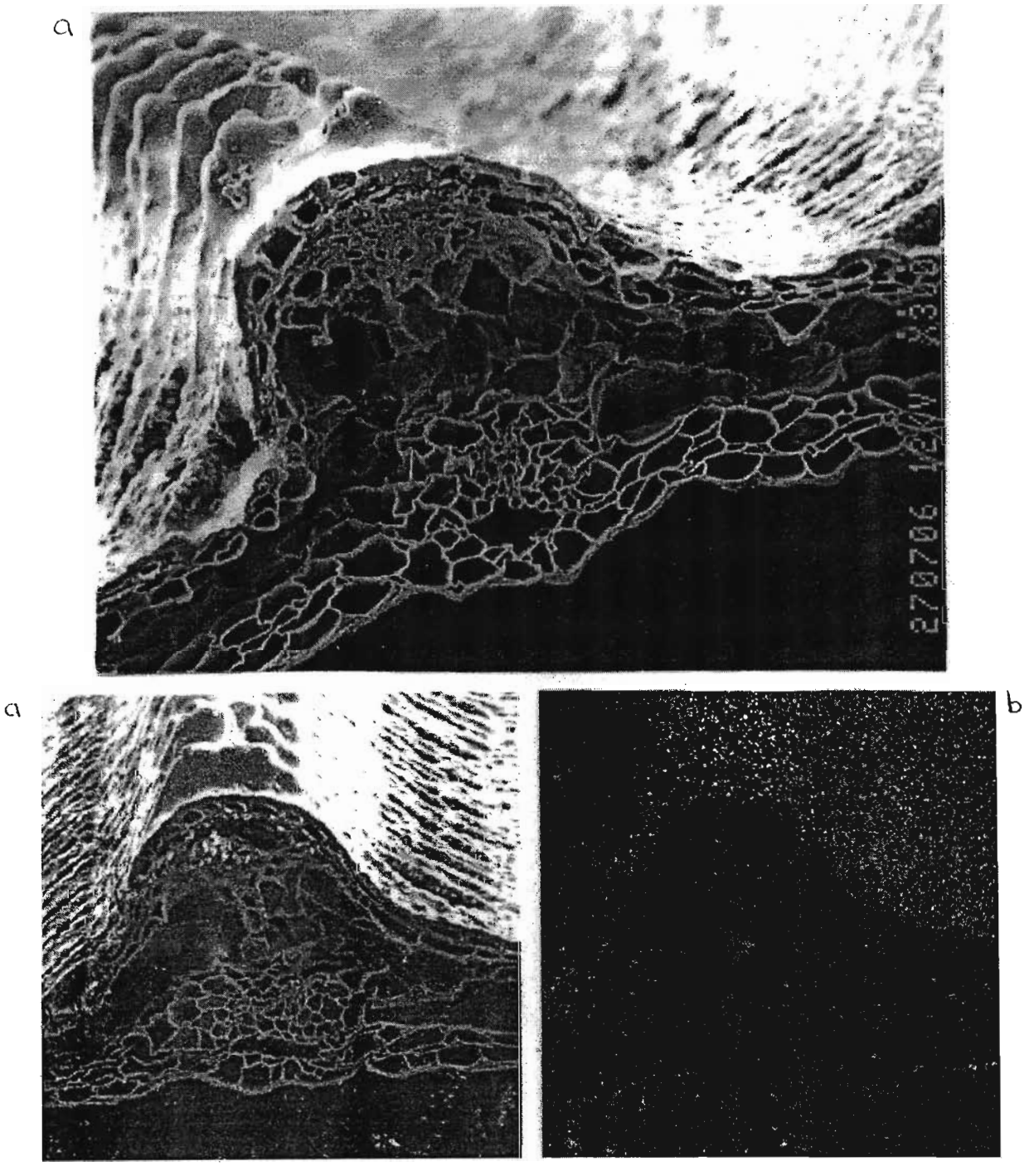
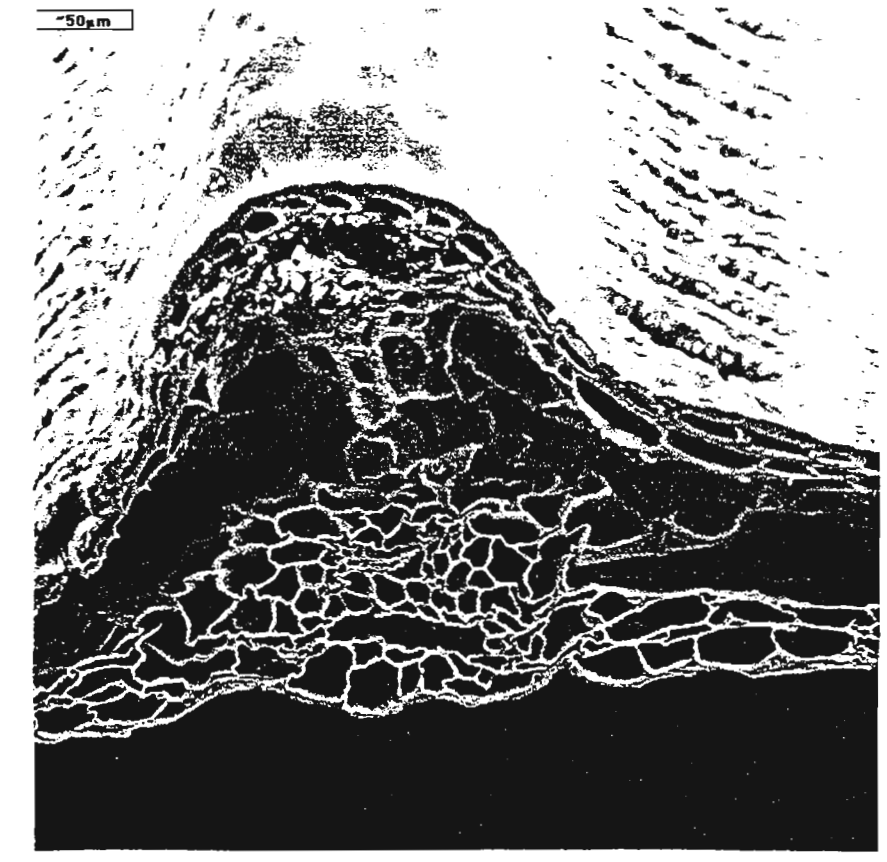


Fig 4.2*a* (a): Scanning electron micrograph of the freeze fractured transverse section of *Equisetum ramosissimum*

(b): X-ray emissions map showing the distribution of silicon in the tissue scanned.



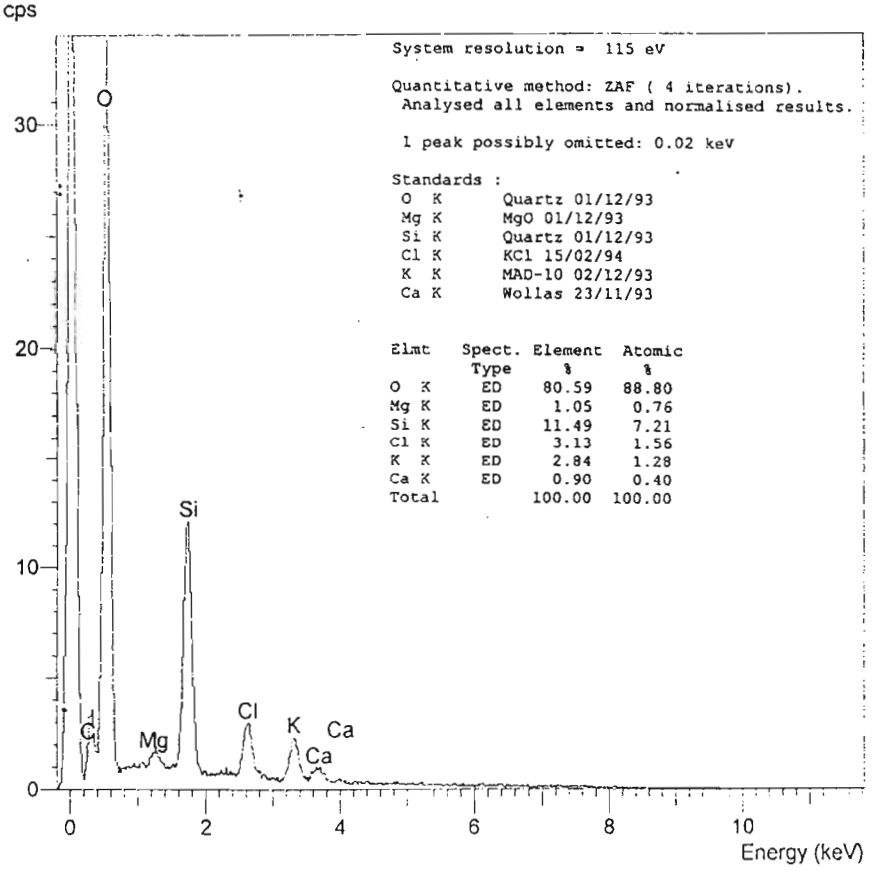


Fig. 4.2*b*: Elemental analysis for the entire transverse section

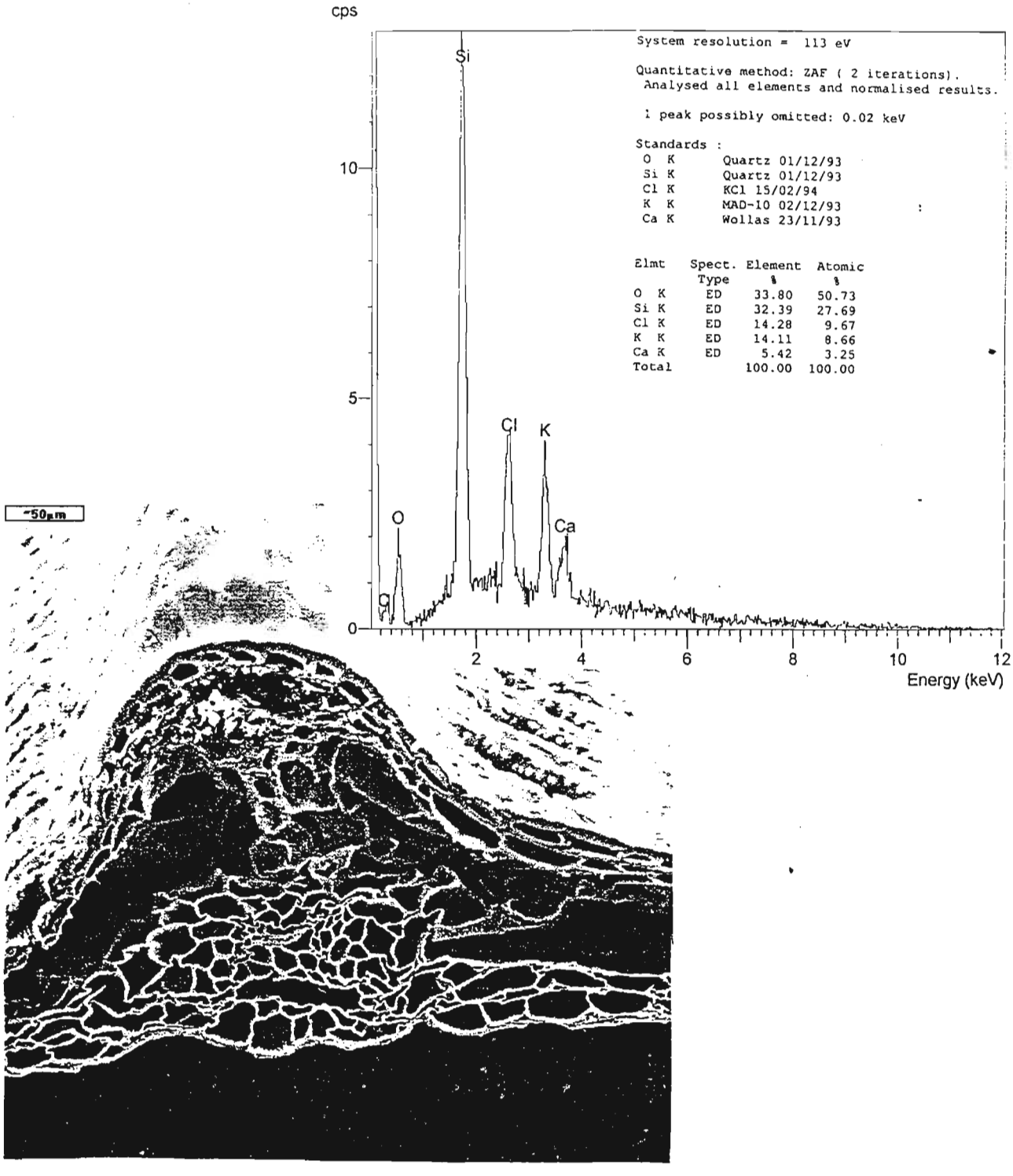


Fig. 4.2*c*: Point elemental analysis on the outer cell wall of the external epidermis

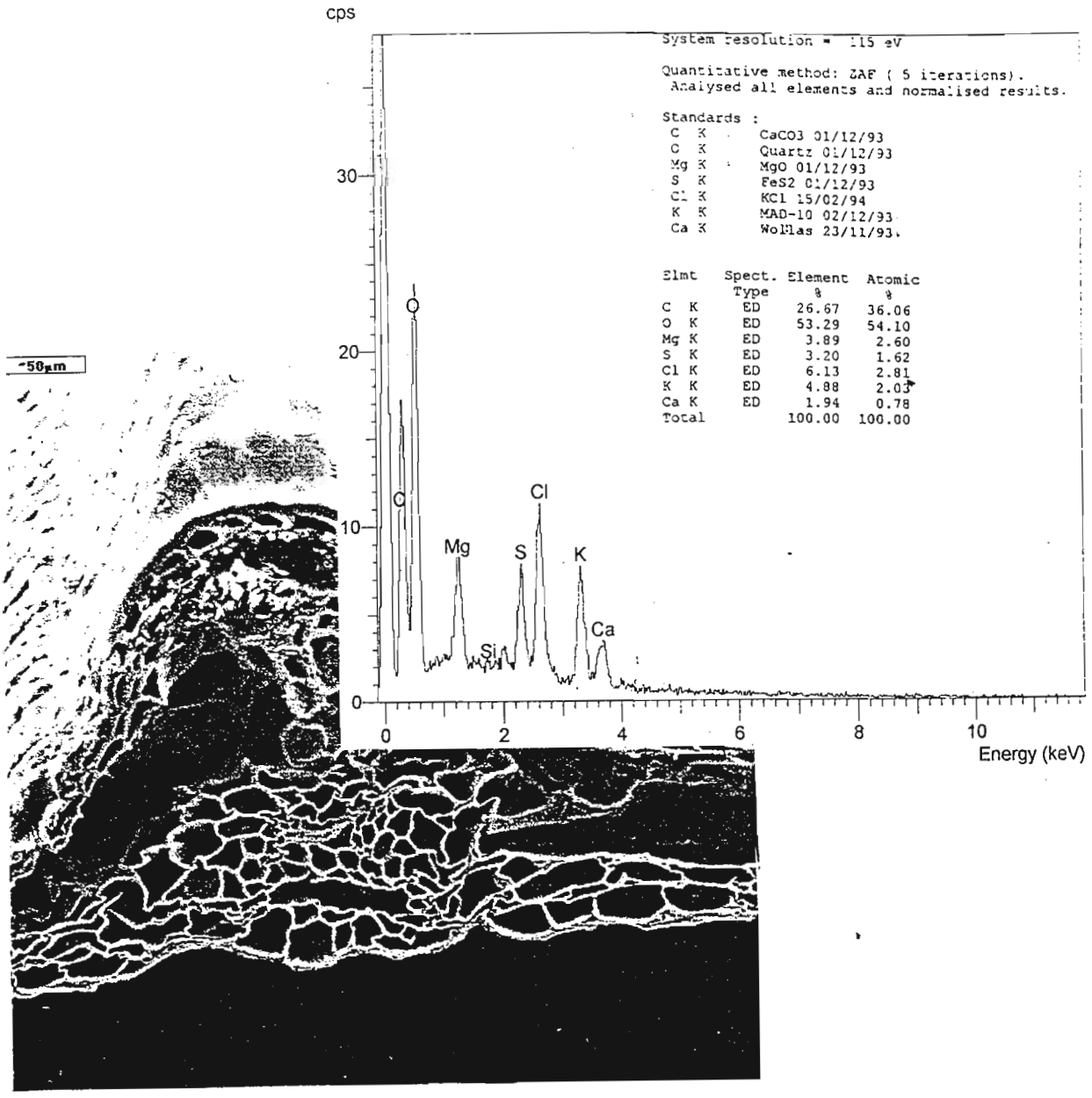


Fig. 4.2*d*: Point elemental analysis in the photosynthetic cortical region above the vascular tissue

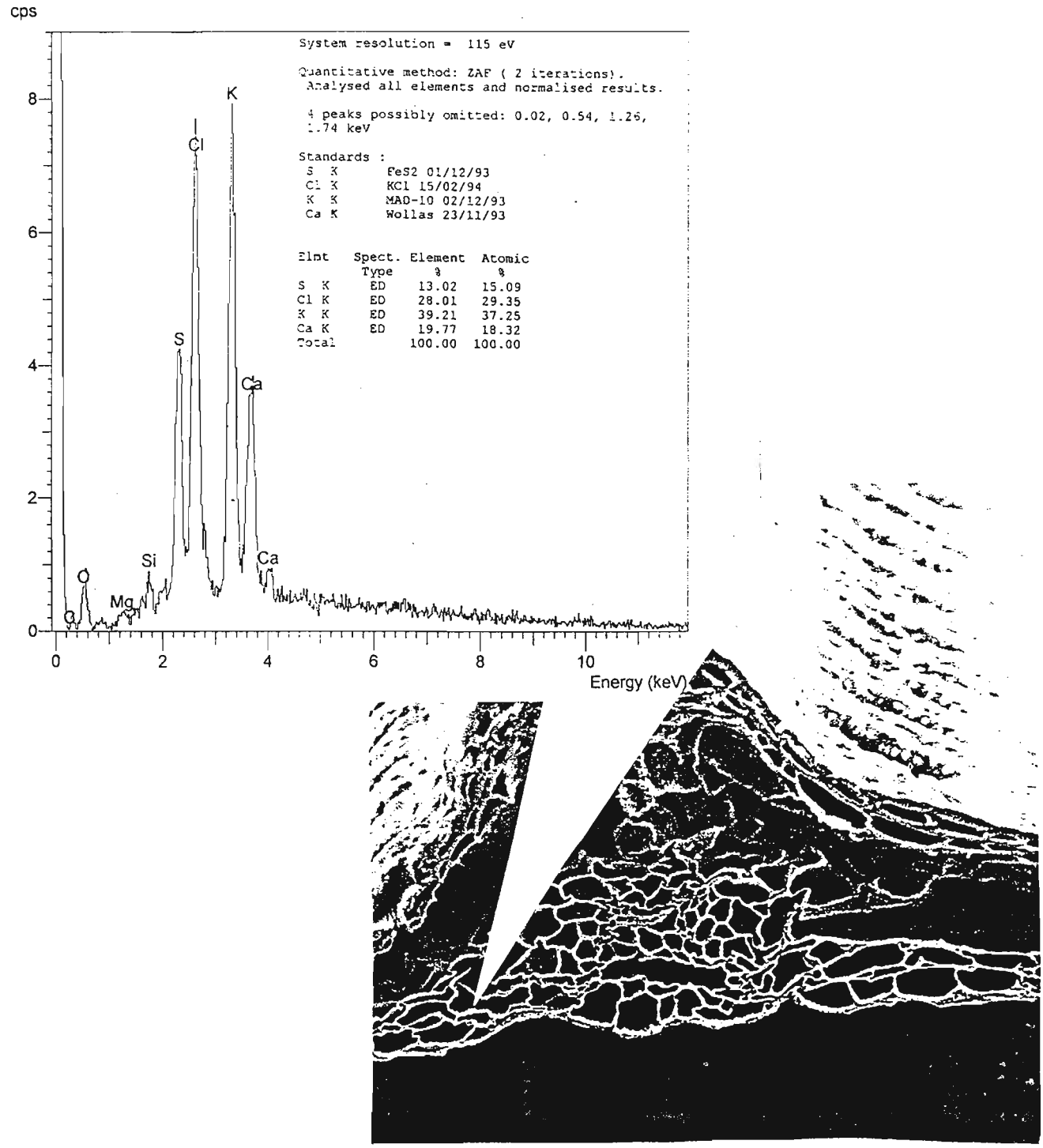


Fig. 4.2*e*: Point elemental analysis in the region immediately above the internal epidermis

4.2.2. Surface and transverse section morphology

It can be seen that the surface pattern of *E. ramosissimum* is irregular, accounting for its rough texture (Fig. 4.2*f*)(Foster and Gifford, 1974). It is also clear that the silica deposit is amorphous.
The stomata are arranged in pairs in two longitudinal rows in the furrows between the ribs and are slightly sunken [Fig. 4.2*f*(i)]. The guard cells also have a fine coating of silica particles as can be seen on the closed stoma [Fig. 4.2*f*(ii)]. In transverse section [Figure 4.2*a*(i)], it can be seen that the stem is cylindrical with a large central cavity. The cell walls of the outer epidermis are thick and have a generous deposition of silaceous material. The cell walls of the internal epidermis are less thick, but nevertheless contain a fair concentration of silica, as can be seen the associated X-ray map. Beneath this epidermis is the cortex. The transverse section of *E. ramosissimum* appears very much like that of *E. hyemale* (*cf.* Foster and Gifford, 1974). Even though the freeze fracture surface is slightly distorted, the following anatomical features can be seen: (a) schlerenchymatous tissue which is extensively developed opposite the ridge, (b) a "vallecular canal" situated beneath one of the "valleys", (c) a protoxylem lacuna called a "carinal canal" associated with a vascular bundle (the latter is not clearly visible), and *d* the region of photosynthetic tissue between adjacent ribs.

4.2.3. Organic compounds isolated

The carotenoid all-*trans*-3*R*,3'*R*,6'*R*-lutein; the two porphyrins pheophytin a and 13^2-*S*-hydroxypheophytin a and sitosterol were isolated from the methylene chloride extract of this plant.

4.3. Discussion

4.3.1. Significance of the elemental analysis

From the surface photographs (Figures 4.2*a* and 4.2*f*) and the cross sectional X-ray elemental analysis map for silicon, it is proposed that *E. ramosissimum* exhibits deposition types described by Foster and Gifford (1974) namely the discrete knobs and rosettes characteristic of *E. arvense* and the uniform pattern on and in the entire epidermis as seen in *E. hyemale*. This may provide additional taxonomic insight into its position within the genus (Hauke, 1978).

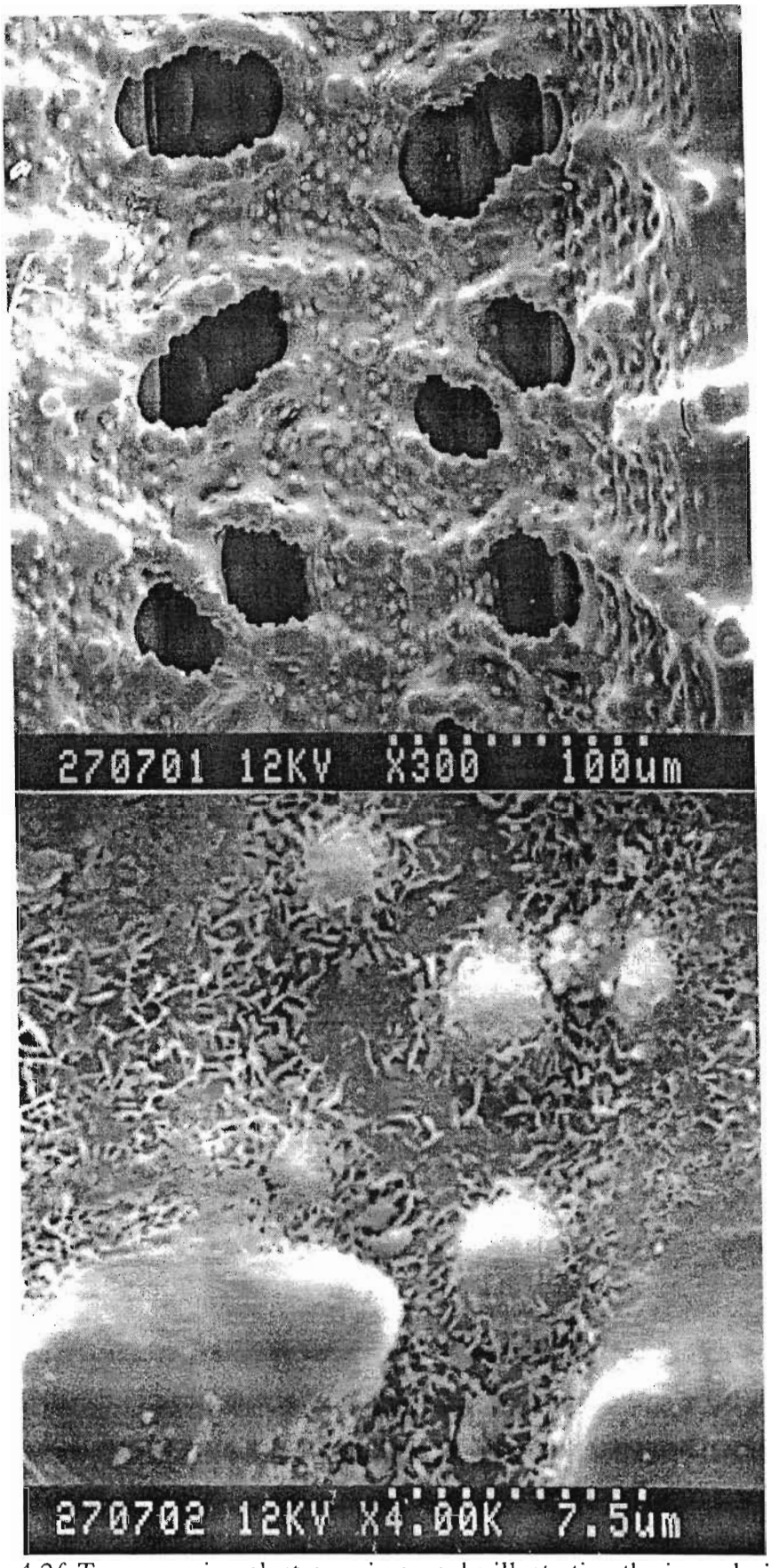
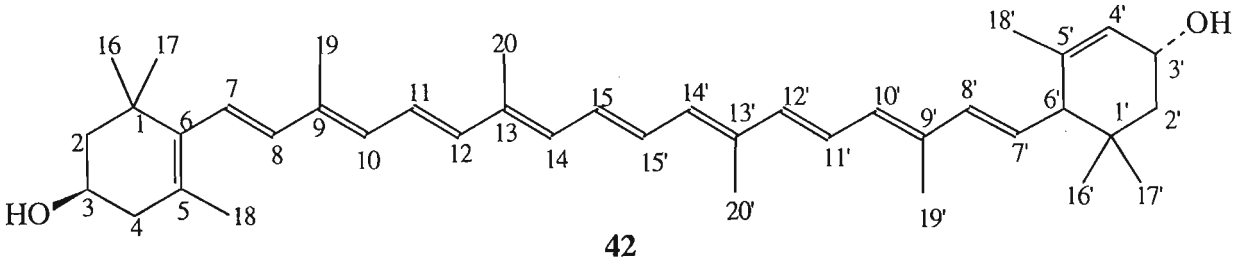


Fig. 4.2*f*: Two scanning electron micrographs illustrating the irregularity of the surface of *Equisetum ramosissimum*

4.3.2. Structure elucidation of compound 8

spectrum: S68



42

Fig. 4.3*a*: All-*trans*-3*R*, 3′*R*,6′*R*-lutein

There were three facts about compound 8 that led to the conclusion that this compound was a carotenoid: (i) the compound was a red gum which discoloured on standing in the pure form (i.e. when it was exposed to air and light), (ii) the absence of aromatic protons as seen in the ^{1}H n.m.r. spectrum (*S68*)(many plant pigments are aromatic) and (iii) the ratio of double bond to alkane hydrogens (15:39) was consistant with the carotenoid skeleton shown above.

From the integration of the proton n.m.r. spectrum, the number of protons excluding those associated with two hydroxy groups was fifty-four, suggesting a molecular formula of $C_{40}H_{56}O_2$. (mass spectrum was not obtained due to decomposition of the sample). The number of double bond equivalents was therefore thirteen. Most carotenoids have nine conjugated double bonds and two six-membered rings. The remaining two double bond equivalents could be accounted for by a double bond in each of the two rings.

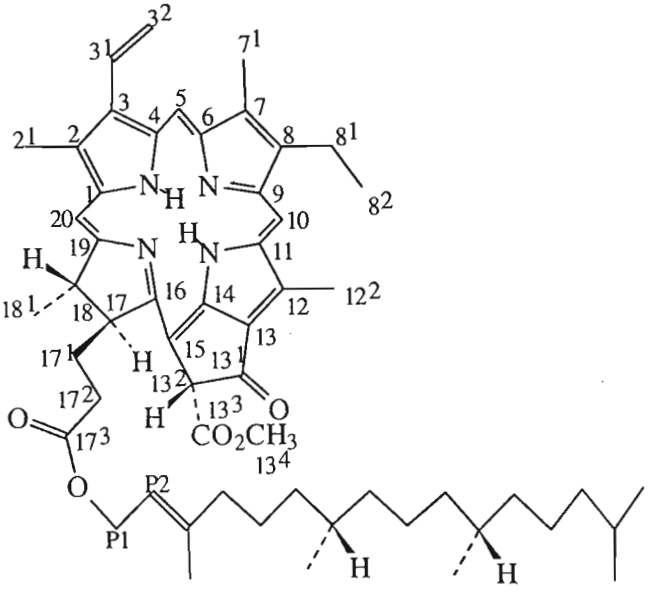
The two multiplets at δ 3.97 and 4.21 were found to be typical of carotenoids with hydroxy groups on the 3 and 3′ carbons and were therefore assignable to H-3 and H-3′ respectively. The two multiplets were not equivalent, so it seemed reasonable that the molecule was not symmetrical about the 15-15′ bond. This was confirmed by the fact that there were fifteen double bond protons, fourteen of which could be accounted for by the conjugated system between the two rings, therefore only one of the ring double bond carbons had a proton attached to it.

Steric considerations aside, there is only one possible situation where this could occur within the biological constraints on the positions of the methyl groups on the two rings, that is, the situation where the double bonds are between carbons 5 and 6, and 5′ and 4′.

A naturally occurring compound with this basic structure is all-*trans*-(3R, 3′R, 6′R)-lutein (**42**). The proton n.m.r. spectrum of compound 8 was compared to that of all-*trans*-3R, 3′R, 6′R-lutein (**42**) (Mayer and Rüttimann, 1980)(Table 4*a*). The chemical shifts and coupling constants were found to agree with those of all-*trans*-3R, 3′R, 6′R-lutein (**42**)(Fig 4.3*b*).

5.3.3. Structure elucidation of compound 9

spectra: S69-S71



43

Fig. 4.3*c*: Compound 9

Compound 9 was isolated as a black gum, which was recrystallised from 50 % MeOH: 50 % CH_2Cl_2. In solution it was dark green, and it emitted an orange fluorescence under ultraviolet. light. At first the proton n.m.r. spectrum (*S69*) seemed strange as there appeared to be three aldehyde peaks (δ 9.46, 9.30 and 8.54) as well as a peak at δ –1.74. Comparison of the proton spectrum with that of other pheophytins (Matsuo *et al.*, 1996) revealed that the three high field resonances corresponded to protons on the outside of the porphyrin ring (10-H, 5-H and 20-H respectively), and two peaks at δ –1.74 and δ 0.50 represented the two N-H protons in the centre of the ring (This was established by addition of D_2O).

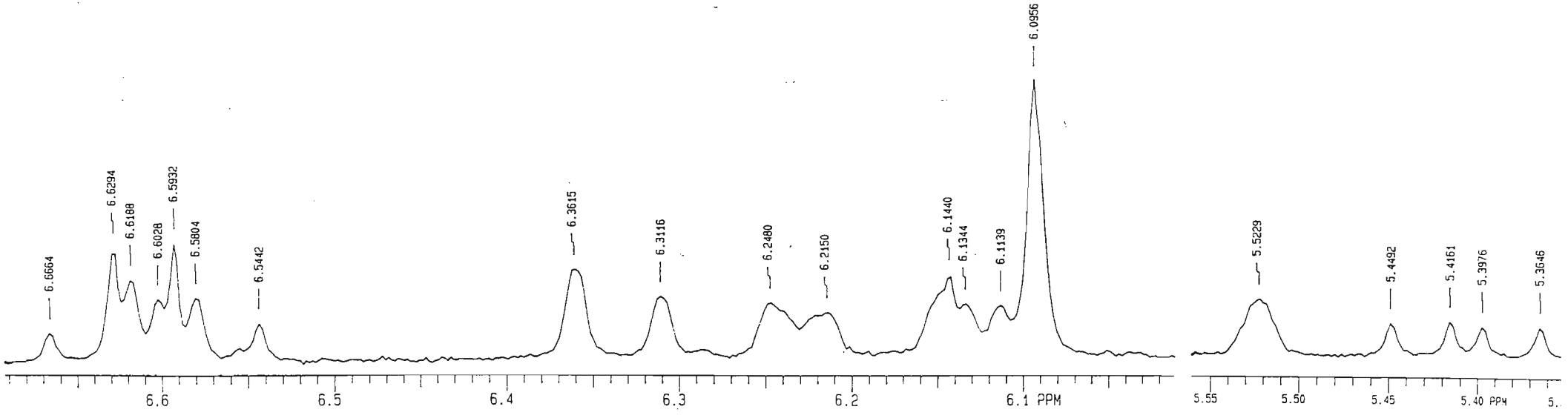


Fig. 4.3*b*: Expansion of the ^{1}H n.m.r. spectrum of compound 8 in $CDCl_3$

Table 4*a*: Comparison between the ^{1}H-n.m.r chemical shifts of compound 8 and lutein

Compound 8 in $CDCl_3$/ppm	**Lutein, $CDCl_3$/ppm, 270 MHz (Mayer and Ruttimann, 1980)**
0.83, 0.98 (2*s*, 6H)	0.85, 1.00 (2*s*, 6H, CH_3 [16′and 17′])
1.05 (*s*, 6H)	1.07 (*s*, 6H, CH_3 [16 and 17])
1.34 (*dd*, *J*=6.9 Hz, *J*=13.2 Hz, 1H)	1.37 (*dd*, $J_{2',3}$=7 Hz, J_{gem}=12 Hz, 1H, H_{ax}-C[2])
1.46 (*t*, *J*=11.79 Hz)	1.48 (*t*, $J_{2,3}$ =12 Hz, J_{gem}=12 Hz, 1H, H_{ax}-C[2′])
1.60 (*s*, 3H)	1.63 (*s*, 3H, CH_3 [18′])
1.71 (*s*, 3H)	1.74 (*s*, 3H, CH_3 [18])
1.76 (*m*, $W_{1/2}$=10.20 Hz, 1H)*	
1.82 (*dd*, *J*=5.91 Hz, J = 13.2 Hz)	1.84 (*dd*, $J_{2',3}$=6 Hz, J_{gem}=13 Hz, 1H, H_{eq}-C[2′])
1.89 (*s*, 3H)	1.91 (*s*, 3H, CH_3 [19′])
1.95 (*s*, 9H)	1.97 (*s*, 9H, CH_3 [19], CH_3 [20], CH_3 [20′])
2.04 (*bm*, 1H)	2.04 (*dd*, $J_{3,4}$=10 Hz, J_{gem}=17 Hz, 1H, H_{ax}-C[3])
2.33-2.40 (*m*, 2H)	2.33-2.45 (*m*, 2H, H-C[6′] and H-C[4])
3.97 (*m*, 1H)	4.0 (*m*, 1H, H_{ax}-C[3])
4.21 (*b*, 1H)	4.25 (*b*, H_{eq}-C[3′])
5.41 (*dd*, *J*=8.4 Hz, *J*=17.07 Hz, 1H)	5.43 (*dd*, $J_{6',7}$=10 Hz, $J_{7',8}$=17.55 Hz, 1H, H-C[7′]
5.52 (*bs*, 1H)	5.55 (*s*, 1H, H-C[4′])
6.10 (*s*, 2H)	6.12 (*s*, 2H, H–C[7] and H-C[8])
6.13 (*m*, 3H)	6.15 (*m*, 3H, H-C[8′], H-C[10], H-C[10′])
6.23 (*bd*, *J*=9.9 Hz, 2H)	6.26 (*m*, 2H, H-C[14] and H-C[14′]
6.34 (*d*, *J*=14.97 Hz, 2H)	6.36 (*d*, $J_{11,12}$=15 Hz, 2H, H-C[12] and H-C[12′])
6.54 - 6.67 (*m*, 4H)	6.55-6.71 (*m*, 4H, H-C[11], H-C[11′], H-C[15] and H-C[15′]

*Mayer and Rüttimann (1980) do not assign a resonance to H_{eq}-C[2]

There are twenty-two conjugated electrons in the porphyrin ring, therefore, according to the 4n+2 rule, the compound is aromatic. The π electrons are delocalised over the aromatic system and can be induced to circulate in the presence of an applied field producing an electric current called the ring current. This electrical field has associated with it a magnetic field which is diamagnetic (opposing B_o) in the centre of the ring and paramagnetic (augmenting B_o) on the outside of the ring, thus the protons on the inside and on the periphery resonate at lower and higher δ values respectively (Kemp, 1991). The values of the peripheral protons in pheophytins are shifted so far downfield, that it follows that the ring-induced magnetic field is quite large and that the compounds have substantial aromatic character.

A double doublet at δ 7.98 (J_1=11.60 Hz, J_2=17.71 Hz) in the proton-n.m.r. spectrum was assigned to H-3^1. The two protons on C-3^2 were not equivalent and had different chemical shifts: H-3^2[*Z*] was associated with a double doublet at δ 6.16 (J_1=1.44 Hz, J_2=11.47 Hz) and H-3^2[*E*] with a double doublet at δ 6.29 (J_1=1.44 Hz, J_2=17.09 Hz).

Four singlets (δ 3.86, 3.67, 3.38 and 3.22) each integrating to three protons were assigned to 13^3-OCH_3, 12-CH_3, 2-CH_3 and 7-CH_3. None of these methyl groups had neighbouring carbons that were protonated, so it was not possible to assign these with any certainty on the basis of the proton-n.m.r. spectrum.

A quartet at δ 3.67 (*J*=7.69 Hz) integrating to two protons was assigned to the two protons at C-8^1 and a triplet (*J*=7.63 Hz) integrating to three protons at δ 1.68 was assigned to the three protons at C-8^2.

The broad doublet at δ 4.19 (1H, *J*=8.13 Hz) was thought to correspond to H-17, while the double triplet at δ 5.11 integrating to one proton corresponded to P2-H. A multiplet at δ 4.27-4.51 integrating to three protons was thought to be assignable to P1-H and 18-H. The assignment of proton peaks of porphyrin ^{1}H n.m.r. spectra will be discussed further under the structure elucidation of compound 10.

The ^{13}C n.m.r. spectra of pheophytins was found to be characterised by a large number of resonances

corresponding to fully substituted carbons at chemical shifts greater than 100 ppm. In compound 9 (*S70*) there were eighteen such peaks, corresponding to fifteen double bonds in the porphyrin ring and three carbonyl groups *viz.* C-13^1 (δ 189.6), C-13^3 (δ 172.2), C-17^3 (δ 172.9). Most notable among the double bond resonances were those at δ 156.0 (C-6), 150.9 (C-9), 163.3 (C-16) and 169.6 (C-19) all of which were associated with carbons bonded to a non-protonated nitrogen atom.

The carbon-13 n.m.r. shifts of compound 9 compared favourably with those of pheophytin a (**43**) isolated from the liverwort *Plagiochila ovalifolia* Mitt (Matsuo *et al*, 1996)(Table 3*b*).

Table 4*b*: Comparison between the ^{13}C n.m.r. shifts of pheophytin a and compound 9

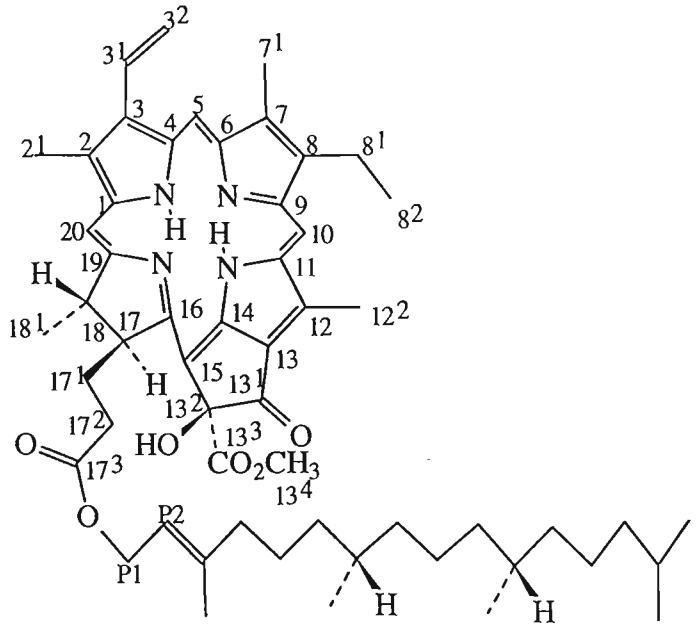
Carbon Number	**Compound 9 in $CDCl_3$/ppm**	**Pheophytin a in $CDCl_3$/ppm (Matsuo *et al.*, 1996)**
1	142.8	142.9
2	131.8	131.8
2^1	12.1 (CH_3)	12.1 (CH_3)
3	136.5	136.5
3^1	129.1 (CH)	129.0 (CH)
3^2	122.8 (CH_2)	122.8 (CH)
4	136.3	136.2
5	97.5 (CH)	97.5 (CH)
6	156.0	155.5
7	136.2	136.1
7^1	11.3 (CH_3)	11.2 (CH_3)
8	145.2	145.2
8^1	19.6 (CH_2)	19.7 (CH_2)
8^2	16.3 (CH_3)	16.3 (CH_3)
9	150.9	151. 0
10	104.4 (CH)	104.4 (CH)
11	137.9	137.9
12	129.0	129.1
12^1	12.1 (CH_3)	12.2 (CH_3)

13	129.0	129.0
13^1	189.6	189.6
13^2	64.7 (CH)	64.7 (CH)
13^3	172.2	173.0
13^4	52.9 (CH_3)	53.0 (CH_3)
14	149.7	150.0
15	105.2	105.2
16	161.3	161.1
17	51.1 (CH)	51.1 (CH)
17^1	29.8 (CH_2)	29.8 (CH_2)
17^2	31.2 (CH_2)	31.2 (CH_2)
17^3	172.9	173.0
18	50.1 (CH)	50.1 (CH)
18^1	22.7 (CH_3)	22.7 (CH_3)
19	169.6	172.2
20	93.1 (CH)	93.1 (CH)

The u.v. spectrum showed two major absorptions, one at 403.35 nm (100 %) and the other at 665.05 nm (43 %). These correspond to absorption of violet and red light respectively. Pheophytins are photosynthetic chromophores, and their function in the cell is to absorb light of specific energy (Matthews and van Holde, 1991).

4.3.4. Structure elucidation of compound 10

spectra: S72-S75



44

Fig. 4.3*d*: Compound 10

Compound 10 was also isolated as a black gum which was recrystallised from methylene chloride/methanol. It was dark green in solution and fluoresced orange under u.v. light. Three singlets (δ 9.61, 9.47, 8.64) and one double doublet (8.00 [J_1=11.53 and J_2=17.89 Hz]) were visible in the proton n.m.r. spectrum (*S72*). These are characteristic of porphyrins and correspond to H-10, H-5, H-20 and H-3 respectively. Two sets of double doublets at δ 6.29 and 6.18 (J_1=1.49 Hz, J_2=16.42 Hz; J_1=1.40 Hz, J_2=10.20 Hz) corresponded to the *E* and *Z* proton on C-3^1. The proton n.m.r. spectrum also showed four singlet peaks (δ 3.72, 3.60, 3.41 and 3.24) each integrating to three protons. These corresponded to the methyl group protons (12-CH_3, 2-CH_3 and 7-CH_3) and the methoxy group protons (13^3-OCH_3). The protons associated with C-18 and C-17 were represented by double doublets at δ 4.16 (J_1=6.72 Hz and J_2=2.49 Hz) and δ 4.47 (J_1=2.43 Hz and J_2=7.26 Hz). The protons of carbons P1 and P2 resonated at δ 4.54 (*multiplet*, 2H) and 5.20 (*t*, *J*=7.30, 1H) respectively.

From the COSY spectrum (*S73*) it could be seen that the triplet at δ 5.20 was coupled to the double doublet at δ 4.54 consistant with the proposal that these are P1-H and P2-H respectively. The double

doublet at δ 4.16 (17-H) was coupled to a multiplet integrating to two protons at δ 2.27 (2H-17^1). This in turn was coupled to two multiplets, δ 2.91 and 2.53 which represented the two protons attached to C-17^2. It is interesting that these two protons are not equivalent. This is probably due to restricted rotation at this bond caused by the length and bulk of the phytol chain as well as the presence of both an ester group α and a double bond γ to carbon-17^2. A quartet at δ 3.69 was coupled to a triplet at δ 1.68 and these peaks were assigned to the protons on carbons 8^1 and 8^2 respectively. The triplet at δ 4.47 was coupled with a broad singlet integrating to three protons at δ 1.60 and these two peaks were assigned to 18-H and the 18-CH_3 group protons respectively.

Instead of a peak at δ 6.26 corresponding to 13^2-H (as was seen in the spectrum of compound 9 – pheophytin a), there was a resonance at δ 5.51 which collapsed on addition of D_2O and was therefore considered to be an hydroxy group proton resonance at the 13^2 position. There were two stereochemical possiblities namely, *R* and *S*, both of which have been isolated previously from excreta of the silkworm *Bombix mori* L. and shown to have cytostatic and cytotoxic effects (Nakatini *et al.*, 1981)(Table 4*c*).

Table 4*c*: Comparison between the reported ^{1}H n.m.r. chemical shifts for 13*R*- and 13*S*-pheophytin a. and pheophytin a (Nakatini *et al.*, 1981); and the pheophytins isolated in this investigation (compounds 9 and 10)

Proton Resonance	**Pheophytin a in $CDCl_3$/ppm**	**13*S*-hydroxy-pheophytin a in $CDCl_3$/ppm**	**13*R*-hydroxy-pheophytin a in $CDCl_3$/ppm**	**Compound 9 in $CDCl_3$/ppm**	**Compound 10 in $CDCl_3$/ppm**
10-H	9.51	9.61	9.59	9.51	9.61
5-H	9.37	9.47	9.45	9.37	9.47
20-H	8.55	8.63	8.61	8.54	8.64
3-CH	7.99	8.04	8.04	7.98	8.00
3^1 -CH_2	~6.25	~6.25	~6.25	6.16[Z], 6.27 [*E*]	6.18[Z], 6.29 [*E*]

13^2 –H	6.26	-	-	6.25	-
13^2 –OH	-	5.53	5.34	-	5.51
P-1-H	4.46	4.47	4.47	4.54	4.47
P-2-H	5.12	5.18	5.18	5.11	5.19
18-H	4.45	4.56	4.56	4.47	4.54
17-H	4.16	4.15	4.69	4.19	4.16
13^3 –OCH_3	3.88	3.73	3.72	3.86	3.72
12-CH_3	3.68	3.61	3.63	3.67	3.60
2-CH_3	3.40	3.43	3.42	3.38	3.41
7-CH_3	3.22	3.26	3.25	3.22	3.24

The configuration at C-13^2 of 13^2-hydroxypheophorbides can be correlated with the ^{1}H n.m.r. shift of 17-H (which is *R*). The hydrogen at carbon-17 is distinctly deshielded by the 13^2-hydroxy group when C-13^2 has the *R* configuration, i.e. when the two groups are located on the same side of the molecular plane. The observed shift is between +0.45 and +0.53. When C-13^2 has the opposite configuration, the 17-H signal is not significantly different, the change in shift being between –0.05 and +0.01 (Nakatini *et al.*, 1981). From the 17-H resonance at δ 4.16 it was possible to determine that the compound was 13^2-*S*-hydroxypheophytin.

The carbon-13 n.m.r. spectrum (*S74*) for compound 10 was compared with those of the 13*S*- and 13*R*-hydroxypheophytins isolated from the liverwort *Plagiochila ovalifolia* (Matsuo *et al.*, 1996), but the carbon shifts of the two isomers were too similar to make any conclusions.

While both compounds can be formed by the auto-oxidation of chlorophyll a, this process results in both epimers in the ratio of 4:5 (Nakatini *et al.*, 1981) therefore it is unlikely that this compound is an artefact.

The porphyrin moiety of both compound 9 and compound 10 is esterified with a phytol chain. Comparison of the two compounds' carbon-13 n.m.r. data with phytol isolated from *Caulerpa filiformis* (Suhr) Hering is shown (Table 4*d*).

Table 4*d*: Comparison of the ^{13}C n.m.r. shifts of the phytyl chains of the two porphyrins isolated with those of phytol

Phytol	**Compound 9**	**Compound 10**
140.3	142.0	142.1
123.1 (CH)	122.8	123.0
59.4 (CH_2)	61.5	61.6
39.9 (CH_2)	39.8	39.8
39.4 (CH_2)	39.3	39.4
37.4 (CH_2)	37.4	37.4
37.4 (CH_2)	37.3	37.3
37.3 (CH_2)	37.2	37.3
36.7 (CH_2)	36.6	36.7
32.8 (CH)	32.8	32.8
32.7 (CH)	32.6	32.6
29.7 (CH_2)	29.7	29.7
28.0 (CH)	27.9	28.0
25.1 (CH_2)	25.0	25.0
24.8 (CH_2)	24.8	24.8
24.5 (CH_2)	24.4	24.4
22.7 (CH_3)	22.7	22.7
22.6 (CH_3)	22.6	22.6
19.8 (CH_3)	19.7	19.7
19.7 (CH_3)	19.6, 19.5	19.7, 19.6
16.2 (CH_3)	16.3	16.3

4.3.5. Significance of the presence of these pigments

Photodynamic cytotoxicity has been observed with certain porphyrins and used in the diagnosis and treatment of malignant tumours. The mechanism of action for methyl esters of porphyrins is different to that of phytyl esters of porphyrins. The mechanism of action of the methyl esters is postulated to

involve penetration and probably fixation of the compounds in the membrane, followed by photosensitized formation of singlet oxygen as shown below (Nakatini *et al.*, 1981).

$$P + h\upsilon \longrightarrow P^{*3}$$

$$P^{*3} + O_2{}^3 \longrightarrow P + O_2{}^1$$

$P^{*3} + O_2{}^3 \longrightarrow P^{+\cdot} + O_2{}^{-\cdot}$ can also occur, therefore the participation of the superoxide radical in the cytotoxic affect is also possible. In the case of phytyl esters such as compound 10, the cytostatic activity also occurs in the dark, suggesting another mode of action. Also, while cell division is inhibited, no cell death is observed in the presence if this type of compound. It has been suggested that the long chain hinders the molecule form entering the membrane, limiting its action to the level of membrane-bound extrinsic enzymes (Nakatini *et al.*, 1981).

Carotenoids, like pheophytins, are involved in the light harvesting reactions of photosynthesis, and they protect plant organelles against singlet-oxygen-induced damage. According to Martin *et al.*, 1996) studies have shown that there is an inverse relationship between the increased intake of carotenoid-rich fruits and vegetables and the incidence of degenerative diseases such as cancer. *In vitro* studies have also demonstrated that carotenoids can:

1. Inhibit chemically induced neoplastic transformation.
2. Induce remission of oral leukoplakia.
3. Quench free radicals such as singlet oxygen.
4. Modulate immune activity.

Direct evidence for the role of carotenoids in antioxidant activity was described by Martin *et al.* (1996) in their investigations using the human liver cell line HepG2. They showed that carotenoid (either β-carotene or lutein) loaded cells were protected against changes in lipid peroxidation, lactate dehydrogenase release and amino acid and deoxyglucose transport induced by the oxidant *tert*-butylhydroperoxide.

Chapter 5: General conclusion

Three plants used for medicinal purposes by people native to southern Africa were chemically investigated. The aim was to isolate the compound or compounds from each of these indigenous species that was responsible for the plants' effects on the central nervous sytem.

The first, *Dioscorea dregeana*, was a monocotyledonous plant belonging to the family Dioscoreaceae; the second, *Avonia rhodesica* was a dicotyledonous plant belonging to the family Portulacaceae and the third, *Equisetum ramosissimum* was a pteridophyte belonging to the family Equisetaceae.

In the case of *D. dregeana*, two alkaloids were isolated. One of these was thought to be crinamine – a β-phenethylamine - which is reported to have an effect on the central nervous system being a transient hypotensive in dogs (Dictionary of Natural Products on CD-ROM, 1997). This fact is reminiscent of the story reported at the Ezimbuzini market about the use of the aqueous extracts of the tubers of *D. dregeana* to paralyse trespassing dogs. According to Strassman (1995) certain phenethylamines (such as mescaline) are hallucinogenic, which could account for the use of these tubers as beer additives.

Dihydrodioscorine, a compound closely related in structure to dioscorine (the probable identity of the other alkaloid isolated) has also been reported to have an effect on the central nervous system. It has been shown to be a convulsant poison which reduces the response to acetylcholine and enhances the response to adrenaline (Bevan *et al.*, 1956) as well as having an excitant action on the cerebral cortex (Corley *et al.*, 1985).

A number of other compounds were also isolated from this plant. These were dodecanosyl 3-(4'-hydroxy-3'-methoxyphenyl)propenoate, 3,4',5-trihydroxybibenzyl, sitosterol, stigmasterol and 4-hydroxymethylfuran-2-carbaldehyde.

The investigation of *A. rhodesica* was less successful as no alkaloids were isolated from this plant. However, a number of esters and wax components were characterised by GC-MS and two triterpenols (β-amyrin and 24-methylenecylcoartenol) were found to be present. Epicuticular wax crystals were visualised using scanning electron microscopy and the physical nature of the stipule

was investigated. These results confirmed the fact that this plant is well adapted to high temperatures and high light intensities.

E. ramosissimum yielded three pigments: lutein, pheophytin a and 13^2-*S*-hydroxypheophytin a. Consumption of carotenoids like lutein has been found to be beneficial as they inhibit chemically induced neoplastic transformation, induce remission of oral leukoplakia, quench free radicals such as singlet oxygen and modulate immune activity (Martin *et al.*, 1996). Porphyrins have been found to be useful in the diagnosis and treatment of malignant tumours by radiation therapy (Nakatini *et al.*, 1981).

This plant was also found to contain a large amount of silica and the distribution of the element silicon was investigated. It was found to be present as a layer on the external and the internal epidermis. The beneficial effects of the consumption of silica include a protective action against liver injury (Li *et al.*, 1992) and greater connective tissue strength (Viehover and Prusky, 1938).

Chapter 6: Experimental

6.1. General

6.1.1. Nuclear Magnetic Resonance Spectroscopy

Proton and carbon-13 n.m.r. spectra were recorded at room temperature on a Varian Gemini 300 MHz spectrometer using either deuteriochloroform ($CDCl_3$) or deuteriomethanol (CD_3OD) as the solvent. The chemical shifts of the proton spectra were recorded relative to the chloroform singlet at δ 7.24 or the methanol singlet at δ 4.90 and those of the carbon-13 spectra were assigned relative to the central line of the $CDCl_3$ triplet at δ 77.09 or the CD_3OD peak at δ 49.6.

6.1.2. Infrared Spectroscopy

Fourier transform infraréd spectra were recorded on a Nicolet Impact 400 FT-IR spectrometer. The samples were dissolved in chloroform and dropped onto NaCl discs. The solvent was evaporated and the spectra were obtained.

6.1.3. Melting Point Determination

Melting points were determined on a Kofler micro hotstage melting point apparatus and are uncorrected. Compounds for melting point determination were either crystallised or precipitated from appropriate solvents.

6.1.4. Optical Rotations

Optical rotations were recorded at room temperature in chloroform on an Optical Activity Ltd Type AA-5 polarimeter.

6.1.5. UV-VIS spectroscopy

UV-visible spectoscopy was performed at room temperature on a Varian Cary IE UV-visible spectrophotometer. The solvents used were methylene chloride and acetonitrile.

6.1.6. Mass Spectroscopy

High resolution masses and mass spectra were recorded by Dr. P. Boshoff at the Cape Technikon on a Finnigan 1020 GC-MS spectrophotometer using the injection and solid probe methods.

6.1.7. Chromatography

Analytical thin layer chromatography (t.l.c.) was performed using 0.2 mm thick aluminium-backed silica gel 60 sheets (Merck Art. 5553), employing solvent systems in the appropriate ratios. The solvents used were carbon tetrachloride, hexane, benzene, methylene chloride, chloroform, ethyl acetate, ethanol and methanol.

The spots on the t.l.c. plates were visualised either using a UV lamp or by the compounds' reactions with anisaldehyde spray reagent. This reagent was prepared by adding anisaldehyde (1.25 ml) to cold methanol (50 ml) to which was added concentrated sulphuric acid (2.5 ml) and more methanol to make up 100 ml. The reagent was refrigerated.

Separation of the extracts was achieved using column chromatography. In most cases, the packing material was silica gel 60 (0.040-0.053 mm particle size, 230-400 mesh ASTM, Merck Art. 9385) or its recycled product. The different column diameters used were 8,6, 2.5 and 1.5 cm. For final purification of some of the compounds columns were made of 0.75 cm diameter pasteur pipettes packed with silica gel. The solvents used for elution were carbon tetrachloride (only used for the final purification of compound 8), hexane, methylene chloride, ethyl acetate and methanol in appropriate ratios.

6.1.8. Alkaloid Tests

6.1.8.1. Dragendorff reagent according to Munier for alkaloids and other nitrogen – containing compounds (Merck, 1978)

Bismuth (III) nitrate (1.7 g) and tartaric acid (20 g) were dissolved in water (80 ml) to form solution A. Potassium iodide (16 g) was dissolved in water (40 ml) to form solution B. Equal portions of solution A and B were mixed to form a stock solution which was stored in the freezer. Shortly before use, a solution of tartaric acid (10 g) in water (50 ml) was added to 10 ml of the stock

solution. To test, the sample was spotted onto a silica plate and a drop of the reagent was added. A dark orange colour formed if an alkaloid was present.

6.1.8.2. Iodine-potassium iodide acidic for alkaloids (Merck, 1978)

Glacial acetic acid (2 ml) was added to a solution of iodine (1 g) and potassium iodide (10 g) in water (50 ml). This solution was made up to 100 ml with water. To test, the sample was spotted onto a silica plate and a drop of the reagent was added. A dark brown colour formed if an alkaloid was present. It was not as selective as the Dragendorff reagent as it gave weak false positive results for compounds like unsaturated fatty acids and aromatic aldehydes.

6.2. Extractives of *Dioscorea dregeana* (Kunth.) Dur.& Schinz.

Two tubers were collected in February, 1996 from the Ezimbuzini Muthi Market (*Crouch and Page 751*, NH). The total wet mass of plant material was 1.7 kg. These were sliced and air dried on top of the oven. The dry mass of the plant material was 0.5 kg. The material was then ground in a liquidiser to a fine powder and extracted with hexane, methylene chloride, ethyl acetate and methanol using a soxhlet apparatus for 24 hours each.

Compounds from the hexane (a mixture of sitosterol and stigmasterol), methylene chloride (compound 3) and ethyl acetate extracts (compound 4) were isolated and purified using column chromatography. The methanol extract was divided into three. The first portion was acidified with dilute HCl and extracted with ether. The acid fraction was then made basic by the careful addition of NaOH. This was then extracted three times with ether to yield compound 2. The second portion was acetylated using pyridine and acetic anhydride. Separation of this mixture yielded compound 1. In an effort to isolate sapogenins, the remainder of the extract was dissolved in 1 % sulphuric acid and refluxed for 1 hour. This was then neutralised using a saturated solution of sodium bicarbonate and extracted with chloroform. This yielded compound 5.

6.2.1. Physical data of compound 1

Dioscorine

Description: orange gum

Yield: 150 mg

Melting Point: n/a

u.v.: E_{max}=236.57 nm

i.r./cm^{-1}: 2933.1 (C-H *str*), 2873.9 (C-H *str*), 1707.3 (C=O *str*), 1650.5 (C=C *str*), 1637.3 (C=C *str*), 1442.4 (C-H *def*), 1382.7 (C-H *def*), 1305.7 (C-O or C-N *str*), 1242.5 (C-O or C-N *str*), 1163.4, 1130.5, 1071.3, 1014.6, 854.1.

Optical rotation was not obtained.

^{1}H n.m.r. in $CDCl_3$/ppm: 5.78 (*q*, *J*=1.29 Hz), 3.30 (*dt*. J_1=11.97 Hz, J_2=2.79 Hz), 2.91 (p, *J*=2.10 Hz), 2.65 (*t*, *J*=17.70 Hz), 2.56 (*dd*, J_1=2.49 Hz, J_2=9.75 Hz), 2.48 (*s*), 2.21 (*tt*, J_1=14.88 Hz, J_2=3.12 Hz), 1.95 (*s*), 1.85 (*dd* J_1=2.37 Hz, J_2=14.91 Hz), 1.65 (*ddt*, J_1=2.01 Hz, J_2=5.07 Hz, J_3=12.93 Hz), 1.50 (*ddt*, J_1=2.88 Hz, J_2=4.95 Hz, J_3=16.92 Hz), 1.20 (*s*)

^{13}C n.m.r. in $CDCl_3$/ppm: 164.48 (*s*), 156.06 (*s*), 116.25 (*d*), 79.78 (*s*), 52.48 (*d*), 52.34 (*t*), 41.79 (*q*), 39.39 (*q*), 38.44 (*t*), 34.05 (*d*), 23.35 (*q*), 19.05 (*t*), 18.52 (*t*)

6.2.2. Physical data for compound 2

Crinamine

Description: colourless gum

Yield: 100 mg

Melting point: n/a

u.v. not performed due to decomposition of sample

i.r. not performed due to decomposition of sample

Optical Rotation: not performed due to decomposition of sample

^{1}H n.m.r. in $CDCl_3$/ppm: 6.82 (*s*), 6.47 (*s*), 6.38 (*s*), 6.38 (*d*, *J*=3.30 Hz), 5.89 (*d*, *J*=1.25 Hz), 4.39 (*d*, *J*=16.98 Hz), 4.02 (*t*, *J*=4.38 Hz), 3.85 (*q*, *J*=3.15 Hz), 3.76 (*d*, *J*=16.86 Hz), 3.45 (*d*, *J*=9.06 Hz), 3.40 (*m*, $W_{1/2}$=4.10 Hz), 3.35 (*s*), 2.15 (*dd*, *J*1=3.06, J_2=9.81 Hz)

^{13}C n.m.r. in $CDCl_3$/ppm: 146.86 (*s*), 146.52 (*s*), 134.52 (*s*), 132.52 (*d*), 126.4 (*d*), 125.5 (*s*), 106.90 (*d*), 103.46 (*d*), 101.03 (*t*), 79.54 (*d*), 72.40 (*d*), 63.07 (*d*), 63.00 (*t*), 60.80 (*t*), 56.76 (*q*), 50.23 (*s*), 27.66 (*t*)

6.2.2.1. Acetylation of compound 2

Acetic anhydride (3 ml) was added to a small portion of compound 2 dissolved in pyridine (3 ml) and allowed to stand overnight. Methanol was added to remove the acetic anhydride and the solvent was removed under reduced pressure. Toluene was added (5 ml at a time) until evaporation under reduced pressure resulted in complete removal of all the pyridine (detected by smell). Methanol was added to remove the traces of toluene from the mixture. Thin layer chromatography confirmed that the compound had been acetylated, and column chromatography was performed to purify the product (Compound 2A).

6.2.2.2. Physical data for compound 2A

Description: White amorphous solid

^{1}H n.m.r. in $CDCl_3$/ppm: 6.88 (*s*), 6.44 (*s*), 6.32 (*d*, J=10.08 Hz), 6.14 (*ddd*, J_1~1 Hz, J_2=4.47 Hz, J_3=9.63 Hz), 5.87 (*s*), 4.95 (*q*, J=3.72 Hz), 4.33 (*d*, J=16.92 Hz), 3.83 (*dt*, J_1=1.65 Hz, $J2$=4.47 Hz), 3.69 (*d*, J=16.92 Hz), 3.33 (*dd*, J_1=13.29 Hz, J_2=4.08 Hz), 1.89 (*dd*, J_1=4.38 Hz, J_2=13.65 Hz)

^{13}C n.m.r in $CDCl_3$/ppm: protonated carbons: 129.5, 127.7, 127.0, 106.6, 104.0, 100.9, 80.5, 72.6, 62.9, 61.3, 60.7, 56.5, 29.7, 28.4, 21.2*

unprotonated carbons: 175, 146, 134, 50**

*values obtained from a proton decoupled spectrum performed on a 500 MHz n.m.r. instrument at the University of Pretoria

**values obtained by examination of the long range HETCOR performed on the abovementioned instrument

MS: Molar mass 243.1432 g/mol; M^+ *m/z*=343 (100), M^++1 *m/z*=344(22)The remainder of the peaks *m/z*(%): 342(2), 328(2), 312(2), 301(4), 300(4), 385(4), 284(13), 283(11), 282(2), 272(2), 271(2), 270(7), 269(11), 268(11), 257(4), 256(2), 254(2), 253(4), 252(16), 246(4), 242(9), 241(9), 240(13), 239(1), 238(4), 229(4), 228(11), 227(9), 226(9),

225(20), 224(18), 223(13), 222 (2), 214(2), 213(4), 212(11), 211(16), 210(16), 209(20), 201(2), 200(2), 199(4), 198(2), 188(16), 187(2), 186(2), 185(4), 183(4), 182(2), 181(18), 173(4), 166(2), 165(2), 160(2), 159(2), 155(2), 154(2), 153(9), 152(9), 149(9), 141(4), 139(4), 128(4), 127(4), 116(2), 115(9), 114(2), 102(2), 97(2), 87(9), 77(4), 71(7), 70(2), 69(4), 57(9), 56(4), 55(7), 51(2), 45(11), 44(4), 43(31), 42(7), 41(9), 39(4), 29(2), 28(10), 27(4).

Optical rotation, melting point, i.r. and u.v. data were not obtained due to decomposition of the sample.

6.2.3. Physical data for compound 3

Dodecanonosyl 3-[4' hydroxy, 3'-methoxyphenyl]propenoate

Description: white amorphous solid, which decomposed in $CDCl_3$.

Yield: 120 mg

Melting Point: n/a

u.v. not performed due to decomposition of the compound

i.r. not performed due to decomposition of the compound

Optical Rotation: not performed due to decomposition of the compound

^{1}H n.m.r. in $CDCl_3$/ppm: 7.59 (*d*, J = 15.93 Hz), 7.06 (*dd*, J_o = 8.10 Hz and J_m = 1.9 Hz), 7.02 (*d*, J = 1.83 Hz), 6.90 (i, J = 8.11 Hz), 6.27 (*d*, J = 15.93 Hz), 5.81 (*s*), 4.17 (*t*, J = 6.71 Hz), 3.91 (*s*), 1.67 (*p*, J = 7.69 Hz), 0.86 (*t*, J = 6.48 Hz).

^{13}C n.m.r. in $CDCl_3$/ppm: 167.37, 147.86, 144.59, 127.06, 123.03, 115.70, 114.67, 109.26, 64.61, 55.93, 31.92, 29.70, 29.60, 29.55, 29.36, 29.31, 28.77, 26.00, 22.68, 14.12. (A DEPT spectrum was not performed)

6.2.3.1. Acylation of compound 3

A small amount of compound 3 was dissolved in pyridine (1 ml). To this, acetyl chloride was added dropwise (2 ml). The resultant mixture was left overnight and worked up in the same way described in *6.2.2.1.* T.l.c. confirmed that the acylation to form compound 3A had taken place.

6.2.3.2. Physical data of compound 3A

Description: White amorphous solid.

Melting point: 68° C

Yield: 100 mg

u.v.E_{max}/nm: 319.35 (53), 310.85 (64), 228.85 (100)

i.r./cm^{-1}:2917.9 (C-H *str*), 2850.5 (C-H *str*), 1762.6, 1717.0, 1637.3 (alkene C=C *str*), 1601.3 (aromatic C=C *str*), 1508.9 (aromatic C=C *str*), 1472.3 (aromatic C=C *str*or C-H *def*), 1259.3 (C-O *str* or O-H *def* coupled), 1202.2 (C-O *str*), 1162.9, 1122.6, 1033.2, 758.1 (out of plane C-H *def*)

Optical rotation in methylene chloride: $[\alpha]_D = 0$

^{1}H n.m.r. in $CDCl_3$/ppm: 7.61 (*d*, *J*=16.00 Hz), 7.10 (*dd*, J_o=7.63 Hz, J_m=1.71 Hz), 7.09 (*br s*), 7.03 (*d*, J_o=8.18 Hz) 6.26 (*d*, *J*=15.94 Hz), 4.17 (*t*, *J*=6.71 Hz), 3.84 (*s*), 2.30 (*s*), 1.68 (*p*, *J*=7.2 Hz), 0.86 (*t*, *J*=6.48 Hz)

^{13}C n.m.r. in $CDCl_3$/ppm: 168.8 (*s*), 166.9 (*s*), 151.4 (*s*), 143.8 (*d*), 141.4 (*s*), 133.5 (*s*), 123.2 (*d*), 121.2 (*d*), 118.5 (*d*), 111.2 (*d*), 64.8 (*t*), 55.9 (*q*), 34.1 (*t*), 31.9 (*t*), 29.7 (*t*), 29.6 (*t*), 29.5 (*t*), 29.4 (*t*), 29.3 (*t*), 29.1 (*t*), 28.7 (*t*), 26.0 (*t*), 24.9 (*t*), 22.7 (*t*), 22.6 (*q*), 14.1 (*q*)

6.2.3.3. Synthesis of 3-(3'-hydroxy-4'-methoxyphenyl)propenoic acid and 3-(4'-hydroxy-3'-methoxyphenyl)propenoic acid

Malonic acid (5.2 g) and isovanillin (vanillin)(3.8 g) were dissolved in pyridine (10 ml) in a round bottomed flask. A catalytic amount of piperidine (0.38 ml) was added to this mixture which was then refluxed for 1.5 hours and allowed to stand overnight. The solution was then transferred to a beaker which was placed in an ice-water bath. Concentrated HCl (100 ml) was carefully added resulting in a cream (salmon pink for vanillin reaction) precipitate. The products of both reactions filtered and recrystallised from water to form clear needles and a salmon pink powder respectively.

6.2.3.4. Physical data of 3-(3'-hydroxy-4'-methoxyphenyl)propenoic acid

Description: White needles

Melting point: 203° C

Yield: 3.0 g

u.v.E_{max}/nm: 321.35 (100), 291.80 (92.06), 240.05 (74.31), 217.55 (91.34)

i.r./cm^{-1}: 3369.3 (-OH *str* carboxylic acid or phenol), 2942.2 (aromatic or double bond C-H *str*), 2852.9, 1669.8 (C=O *str* α,β- unsaturated), 1630.6, 1609.7 (aromatic C=C *str*), 1583.6 (aromatic C=C *str*), 1515.7 (aromatic C=C *str*), 1460.8, 1442.5, 1421.6, 1327.6, 1267.5, 1210.0 (C-O *str* ????unsaturated), 1163.0, 1136.9, 1022.0, 982.8 (trans out-of-plane C-H *def*), 961.4, 860.0 (out of plane C-H *def*), 818.2 (out of plane C-H *def*), 763.3 (out of plane C-H *def*), 690.2 (out of plane C-H *def*), 572.6

^{1}H n.m.r. in CD_3OD/ppm: 7.59 (*d*, J = 15.93 Hz), 7.11 (*broad singlet*), 7.08 (*dd*, J_m =1.96 Hz, J_o = 8.30 Hz), 6.97 (*d*, J_o = 8.12 Hz), 6.30 (*d*, J = 15.94 Hz), 3.92 (*s*).

6.2.3.5. Physical data of 3-(4'-hydroxy-3'-methoxyphenyl)propenoic acid

Description: Salmon pink powder

Melting point: 174° C

Yield: 4.1 g

^{1}H n.m.r. in CD_3OD/ppm: 7.63 (*d*, J = 15.87 Hz), 7.21 (i, J_m = 1.89 Hz), 7.10 (*dd*, J_m = 1.89 Hz, J_o = 8.18 Hz), 6.85 (*d*, J_o = 8.18 Hz), 6.35 (*d*, J = 15.87 Hz), 3.93 (*s*).

6.2.3.6. Butylation of 3-(3'-hydroxy-4'-methoxyphenyl)propenoic acid and 3-(4'-hydroxy-3'-methoxyphenyl)propenoic acid

Both butylations were performed using the Dean-Stark Apparatus. The acid (200 mg) was dissolved into a mixture of butanol (50 ml) and benzene (50 ml). A drop of concentrated sulphuric acid was added to the mixture which was then then refluxed for three hours. After cooling, of a saturated solution of sodium bicarbonate was added (2x50 ml) to remove the acid. The resulting benzene-butanol fraction was then evaporated under reduced pressure to yield the butyl ester. In both cases it was necessary to perform column chromatography to purify the compounds.

6.2.3.7. Physical data of butyl 3-(3'-hydroxy-4'-methoxyphenyl)propenoate

Description: White powder

Melting point: 189° C

Yield: 150 mg

u.v.E_{max}/nm: 320.51 (100%), 293.06 (90.78), 239.51 (72.95), 216.06 (95.96)

i.r./cm^{-1}: 3409.6 (-OH *str*), 2960.7 (C-H *str*), 2936.7 (C-H *str*), 2875.2 (C-H *str*), 2843.2 (C-H *str*), 1705.5, 1633.7 (alkene C=C *str*), 1608.8 (aromatic C=C *str*), 1512.2 (aromatic C=C *str*), 1443.1 (aromatic C=C *str*), 1462.4 (aromatic C=C *str* or C-H *def*), 1443.1 (aromatic C=C *str* or C-H *def*), 1400.0 (C-H *def*), 1305.0 (C-O *str* or OH *def* coupled), 1266.4 (C-O *str* or OH *def* coupled), 1208.4 (C-O *str*), 1158.7, 1131.1, 1067.5, 1028.9, 984.7 (trans out-of-plane C-H *def*), 879.8, 860.4 (out of plane C-H *def*), 816.2 (out of plane C-H *def*), 766.5 (out of plane C-H *def*), 595.3 (out of plane C-H *def*)

^{1}H n.m.r. in $CDCl_3$/ppm: 7.56 (*d*, *J*=15.94 Hz), 7.12 (*d*, J_m=2.01 Hz), 7.01 (*dd*, J_m=2.07 Hz, J_o=8.31 Hz), 6.82 (*d*, *J*o=8.31 Hz), 6.27 (*d*, *J*=15.93 Hz), 4.17 (*t*, *J*=6.59 Hz), 3.90 (*s*), 1.66 (*p*, *J*=6.72 Hz), 1.41 (*sestet*, *J*=7.63 Hz), 0.94 (*t*, *J*=7.27 Hz)

6.2.3.8. Physical data of butyl 3-(4'-hydroxy-3'-methoxyphenyl)propenoate

Description: White powder

Melting point: 234° C

Yield: 100 mg

u.v. E_{max}/nm: 319.00 (100), 293.60 (79), 233.6 (72), 227.25 (68)

i.r./cm^{-1}: 3400 (OH *str*), 2958.9 (aromatic/alkene C-H *str*), 2929.4 (C-H *str*), 1729.4 (C=C *str*), 1637.3 (C=C *str*), 1597.8 (C=O *str*), 1513.7 (aromatic C=C *str*), 1466.2 (C-H *def*), 1387.2 (C-H *def*), 1269.1 (C-O *str*), 1160.0 (C-O *str*), 1977.9, 1031.8, 992.3 (out of plane alkene C-H *def*), 854.1, 821.2 (out of plane para substituted aromatic C-H *str*)

^{1}H n.m.r. in $CDCl_3$/ppm: 7.57 (*d*, *J*=15.88 Hz), 7.02 (*dd*, J_1=1.89 Hz, J_2=10.01 Hz), 6.99 (*s*), 6.88 (*d*, *J*=8.12 Hz), 6.26 (*d*, *J*=15.87 Hz), 4.17 (*t*, *J*=6.96 Hz), 3.87 (*s*), 1.65 (*pentet*, *J*=6.96 Hz), 1.40 (*sestet*, *J*=7.26 Hz), 0.93 (*t*, *J*=7.33 Hz)

6.2.4. Physical data of compound 4

3,4',5-Trihydroxybibenzyl

Description: Clear Gum.

Yield: 80 mg

Melting Point: n/a

i.r. and u.v. spectra were not obtained

Optical rotation in methanol: $[\alpha]_D = 0$

^{1}H n.m.r. in CD_3OD/ppm: 7.00 (*dd*, J_o = 8.55 Hz, J_p = 2.01 Hz), 6.70 (*dd*, J_o = 8.37 Hz, J_m = 2.20 Hz), 6.20 (*d*, J_m = 2.14 Hz), 6.10 (*t*, J_m = 2.17 Hz), 2.70 (*m*)

^{13}C n.m.r. in CD_3OD/ppm: 159.2 (*s*), 156.3 (*s*), 145.7 (*s*), 134.2 (*d*), 130.4 (*d*), 116.1 (*d*), 108.2 (*d*), 101.2 (*d*), 39.7 (*t*), 38.0 (*t*)

6.2.5. Physical data of compound 5

5-Hydroxymethylfuran-2-carbaldehyde

Description: Clear colourless gum.

Yield: 170 mg

Melting Point: n/a

i.r./cm^{-1}: 3367.5 (-OH *str*), 2922.2 (C-H *str*), 2854.1 (aldehyde C-H *str*), 1734.5 (aldehyde C=O *str*), 1676.5 (C=C *str*), 1522.0, 1461.2 (C-H *def*), 1385.3 (C-H *def*), 1274.0 (C-O *str*), 1193.0 (C-O *str*), 1021.0 (C-O *str*), 816.4, 777.9.

u.v.: spectrum was not obtained

Optical rotation in methylene chloride: $[\alpha]_D = 0$

^{1}H n.m.r. in $CDCl_3$/ppm: 9.55 (*s*), 7.19 (*d*, *J* = 3.6 Hz), 6.49 (*d*, *J* = 3,9 Hz), 4.69 (*s*)

^{13}C n.m.r. in $CDCl_3$/ppm: 177.68 (*d*), 160.56 (*s*), 152.39 (*s*), 122.77 (*s*), 110.00 (*d*), 57.65 (*t*)

MS: *m/z*(%): 234(4), 233(3), 205(7), 167(4), 153(4), 151(6), 149(7), 137(6), 126(28), 125(15), 110(42), 109(100), 97(31), 85(10), 83(10), 81(9), 71(13), 69(13), 57(21), 55(13), 53(13), 51(6), 43(16), 41(28)

6.3. Extractives of *Avonia rhodesica* (NE.Br.)Rowl.

The fresh mass of the Avonia rhodesica plant material collected (Page 1, NH) was 830 g, and after air drying on top of the oven the mass was reduced to 240 g. The plant material was then ground in a coffee grinder and extracted using a soxhlet apparatus in the same way as described for *Dioscorea dregeana*. In all of the extracts, a white solid mass precipitated on cooling which was filtered. These solids were found to be wax mixtures. GC-MS of these mixtures was performed. A great deal of difficulty was experienced separating any other compounds from these extracts as the wax concentrations were so high. A mixture of two triterpenols was, however, found in the methylene chloride extract (compound 6 and compound 7).

6.3.1. GC-MS

The instrument used was a Hewlett-Packard 5890 Series 2 gas chromatograph attached to a Hewlett-Packard 5971 mass spectrometer fitted with a 30 m x 0.25 mm (internal diameter) column. The stationary phase was cross-linked 5 % phenyl methyl silicone with a film thickness of 0.25 μm. The mobile phase was helium. The initial temperature was 100° C and the final temperature was 260° C.

6.3.1.1. Methyl hexacosanoate

MS: *m/z*(%): M^+ = 410 (19); [M + 1]+ = 411(6); 367(4), 311(2), 281(4.5), 253(1), 209(2), 208(2), 207(15), 143(20), 129(6), 111(6), 101(5), 97(7), 87(44), 85(7), 83(13), 75(32), 74(100), 73(8), 71(19), 69(24), 67(6), 59(6), 57(35), 55(38), 56(8)

6.3.1.2. Methyl tetraconsanoate

MS: *m/z*(%): M^+ = 382(24); [M + 1]+ = 383(6); 351(1), 339(6), 283(3), 241(1), 207(6), 199(2), 143(14), 129(6), 97(11), 87(69), 83(11), 75(26), 71(15), 69(22), 59(6), 57(38), 55(34)

6.3.1.3. Methyl docosonoate

MS: *m/z*(%): M^+ = 354(20); [M + 1]+ = 355(4); 323(2), 311(8), 281(1), 255(3), 207(6), 199(3), 185(1), 143(13), 139(6), 111(3), 101(4), 97(10), 87(60), 83(15), 75(32), 74(100), 71(13), 69(21), 59(8), 57(34), 55(34)

6.3.1.4. Methyl hexadecanoate

MS: *m/z*(%): M^+ = 270(6); 239(3), 227(6), 199(2), 185(2), 143(8), 129(4), 101(3), 87(62), 75(17), 71(6), 69(13), 59(10), 57(22), 55(25)

6.3.1.5. Nonacosane

MS: *m/z*(%): M+ = 408(1); 355(1), 337(1), 323(1), 309(1), 295(1), 281(2), 267(1), 253(1), 239(1), 225(1), 211(1), 207(2), 197(1), 183(1), 169(1), 155(1), 141(2), 127(3), 113(5), 111(5), 99(11), 97(10), 85(39), 83(11), 71(60), 69(17), 57(100), 55(26)

6.3.1.6. Neophytidiene

MS: *m/z*(%): M^+ = 278(2); 193(1), 179(1), 137(8), 124(21), 123(46), 111(17), 110(13), 109(22), 97(31), 96(30), 85(14), 83(47), 82(84), 81(38), 79(11), 71(38), 70(15), 69(63), 68(100), 67(42), 57(78), 55(53)

6.3.1.7. Pentacosane

MS: *m/z*(%): 352(1), 281(1), 207(3), 169(1), 168(1), 155(1), 154(1), 141(1), 140(1), 113(4), 112(3), 111(4), 99(10), 98(3), 97(7), 96(1), 86(3), 85(38), 84(6), 83(8), 82(3), 81(1), 73(1), 72(3), 71(54), 70(14), 69(14), 68(3), 67(3), 58(6), 57(100), 56(13), 55(25), 54(3), 53(1)

6.3.1.8. Ethyl docosanoate

MS: *m/z*(%): 369(6), 268(15), 325(3), 281(4), 213(3), 208(3), 207(10), 157(8), 149(3), 143(4), 135(3), 125(3), 115(7), 112(3), 111(7), 109(4), 102(6), 101(49), 99(3), 98(6), 97(13), 96(6), 95(4), 89(21), 88(100), 87(4), 86(3), 85(17), 84(7), 83(17), 82(4),

81(6), 79(4), 73(21), 72(3), 71(25), 70(17), 69(28), 68(3), 67(8), 61(8), 60(11), 58(4), 57(45), 56(14), 55(39), 54(4)

6.3.1.9. Dioctyl hexanedioate

MS: *m/z*(%): 259(4), 241(6), 212(1), 199(1), 148(1), 147(21), 146(3), 130(7), 129(100), 128(6), 113(13), 112(31), 111(17), 102(3), 101(8), 100(6), 97(1), 87(7), 85(1), 84(13), 83(20), 82(3), 73(1), 72(1), 71(35), 70(38), 69(10), 68(1), 59(3), 58(3), 57(63), 56(17), 55(39), 54(1), 53(1)

6.3.1.10. Physical data of the mixture of compound 6 and compound 7

β-Amyrin and 24-methylenecycloartenol

i.r./cm^{-1}: 3434.0 (O-H *str*), 2919.1 (C-H *str*), 2849.5 (C-H *str*), 1709.7 (C=C *str*), 1466.2 (C-H *def*), 1387.2 (C-O *str* or O-H *def* coupled), 1264.1 (C-O *str* or O-H *def* coupled), 1025.2 (=CH_2, out of plane C-H *str*), 998.9 (=CH_2 out of plane C-H *str*)

^{13}C n.m.r in $CDCl_3$/ppm: 156.9, 145.2, 121.7, 105.9, 79.0, 78.8, 55.18, 52.3, 48.8, 48.0, 47.6, 47.2, 47.1, 46.8, 45.3, 41.7, 40.5, 39.8, 38.8, 38.6, 37.2, 37.2, 36.1, 35.6, 35.0, 34.7, 33.8, 33.3, 32.8, 32.7, 32.5, 31.3, 31.1, 30.4, 29.9, 29.9, 28.4, 28.2, 28.1, 27.2, 27.2, 26.5, 26.2, 26.1, 26.0, 25.8, 25.5, 25.4, 23.5, 23.5, 22.0, 21.9, 21.1, 20.0, 19.3, 18.4, 18.4, 18.0, 16.8, 15.6, 14.1

6.3.2. Scanning Electron Microscopy

Fresh plant material was fixed in liquid nitrogen and gold-coated using a Polaron E 5100 Sputter Coater. The material was then placed in the JEOL JSM 35 Scanning Electron Microscope and photographs were taken.

6.4. Compounds Isolated from *Equisetum ramosissimum* Desf.

Equisetum ramosissimum plant material was collected (Crouch 745, NH), air dried on top of the oven, and then ground into a fine powder (100 g) using a liquidiser. The material was then extracted using a soxhlet apparatus in the same way as the other two species. The hexane and methylene

chloride extracts were found to contain the same compounds (t.l.c.) and were combined. This combined extract yielded compound 8, compound 9 and compound 10.

In all of the extracts, a white precipitate formed which was found to be silica (i.r.). SEM with XDT was performed to determine the location of the silica in the tissue as well as to determine the approximate concentration of silicon (and other elements) in the tissue.

6.4.1. X-ray maps

The fresh plant specimens were fixed in liquid nitrogen and placed in the scanning electron microscope. The DTSA X-ray microanalysis system was used to acquire the elemental analysis and to obtain the images mapping the distribution of the silica. In order to obtain the micrographs included in the text, the specimens were gold-coated as described in *6.2.2.2.*

6.4.2. Physical data of compound 8

All-trans-3R, 3'R, 6'R-lutein

^{1}H n.m.r. in $CDCl_3$/ppm: 6.54-6.67 (*m*), 6.34 (*d*, J=14.97 Hz), 6.23 (*br d*, J=9.9 Hz), 6.13 (*m*), 6.10 (*s*), 5.52 (*s*), 5.41 (*dd*, J_1=8.4 Hz, J_2=17.07 Hz), 4.21 (*s*), 3.97 (*m*), 2.33-2.40 (*m*), 2.04 (*s*), 1.95 (*s*), 1.89 (*s*), 1.82 (*dd*, J_1=5.91 Hz, J_2=13.2 Hz), 1.76 (*m*, $W_{1/2}$=10.20 Hz), 1.71 (*s*), 1.60 (*s*), 1.46 (*t*, J=11.79 Hz), 1.34 (*dd*, J_1=6.90 Hz, J_2=13.2 Hz), 1.05 (*s*), 0.98 (*s*), 0.83 (*s*)

6.4.3. Physical data of compound 9

Pheophytin a

Description: Black microcrystalline powder. Precipitated from MeOH/$MeCl_2$

Yield: 230 mg

Melting Point: 123° C (Lit., grease from petroleum ether, melting point: 120°C)

i.r./cm^{-1}: 2966.2, 2928.6, 2860.1, 1728.9, 1460.9, 1380.0, 1272.4, 1122.3, 1071.9, 1038.9, 742.1

u.v.: E_{max} = 403.35 nm (100%); 505.10 nm (12.62 %), 535.45 nm (10.97 %), 607.90 nm (9.78 %), 665.05 nm (43.93 %)

^{1}H n.m.r./ppm: 9.51 (*s*), 9.37 (*s*), 8.54 (*dd*, J_1=11.60 Hz, J_2=17.71 Hz), 6.27 (*dd*, J_1=1.44 Hz, J_2=17.09 Hz), 6.16 (*dd*, J_1=1.44 Hz, J_2=11.47 Hz), 5.11 (*dt*, J_1=1.23 Hz, J_2=8.16 Hz), 4.46 (*m*, $W_{1/2}$= 31.95 Hz), 3.86 (*s*), 3.67 (*s*), 3.38 (*s*), 3.22 (*s*), 2.60 (*m*, $W_{1/2}$=12.9 Hz), 2.46 (*m*, $W_{1/2}$=17.3 Hz), 2.32 (*m*, $W_{1/2}$=18.03 Hz), 2.17 (*m*,

$W_{1/2}$=18.60 Hz), 1.86 (*t*, *J*=7.62 Hz), 1.54 (*d*, *J*=6.90 Hz), 0.82 (*q*, *J*=6.60 Hz), 0.77 (*t*, *J*=6.15 Hz)

^{13}C n.m.r./ppm: 189.6, 172.9, 172.2, 169.6, 161.3, 150.9, c156, 149.7, 145.2, 142.8, 142,0, 137.9, 136.5, 136.3, 136.2, 131.8, 129.1, 129.0, 122.8, 117.7, 105.2, 104.4, 97.5, 93.1, 76.3, 64.7, 61.5, 52.9, 52.8, 51.1, 50.1, 39.8, 39.3, 37.4, 37.3, 37.2, 36.7, 32.8, 32.7, 31.2, 29.8, 29.7, 27.9, 25.0, 24.8, 24.4, 24.4, 23.1, 22.7, 22.6, 19.7, 19.6, 19.5, 17.4, 16.3, 12.1, 11.3

MS: not performed

6.4.4. Physical data of compound 10

13^2-S-Hydroxypheophytin a

Description: Black microcrystalline powder. Precipitated from MeOH/$MeCl_2$

Yield: 120 mg

Melting Point: 183-187° C

i.r./cm^{-1}: 2958.1, 2926.5, 2859.0, 1730.6, 1618.8, 1460.6, 1379.2, 1270.8, 1158.4, 1122.5, 1072.4, 1041.1, 742.4

u.v.: E_{max} = 406.30 nm (100 %); 503.70 nm (13.70 %), 523.10 nm (9.48 %), 532.90 nm (11.07 %), 608.15 nm (9.21 %), 627.55 nm (7.05 %), 664.85 nm (38.22 %)

^{1}H n.m.r./ppm: 9.61 (*s*), 9.47 (*s*), 8.64 (*s*), 8.00 (*dd*, J_1=11.53 Hz, J_2= 17.89 Hz), 6.29 (*dd*, J_1=1.47 Hz, J_2=16.42 Hz), 6.18 (*dd*, J_1=1.40 Hz, J_2=10.20 Hz), 5.51 (*s*), 5.19 (*m*), 4.47 (*t*, *J*=6.48 Hz), 4.54 (*dd*, J_1=2.43 Hz, J_2=7.26 Hz), 4.16 (*dd*, J_1=6.72 Hz, J_2=2.49 Hz), 3.72 (*s*), 3.60 (*s*), 3.41 (*s*), 3.24 (*s*), 3.69 (*q*, *J*=8.55 Hz), 2.91 (*m*, $W_{1/2}$=18.0 Hz), 2.53 (*m*, $W_{1/2}$=18.0 Hz), 1.68 (*t*, *J*=7.59 Hz), 1.59 (*J*=7.51 Hz), 1.49 (*p*, *J*=6.60 Hz).

^{13}C n.m.r./ppm: 192.0, 173.6, 173.0, 172.8, 172.5, 145.2, 142.8, 142.1, 137.9, 136.4, 136.4, 136.3, 131.9, 129.1, 123.0, 117.9, 117.7, 104.3, 98.0, 93.9, 89.0, 76.9, 76.6, 76.4, 61.6, 61.4, 53.4, 51.9, 50.4, 50.3, 39.8, 39.8, 39.4, 37.4, 37.3, 37.3, 36.7, 32.8, 32.6, 31.6, 31.2, 29.7, 29.6, 28.0, 25.0, 24.8, 24.4, 22.7, 22.6, 19.7, 19.7, 19.6, 19.5, 17.4, 16.3, 12.4, 12.1, 11.3

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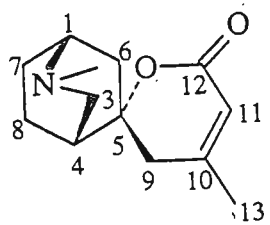
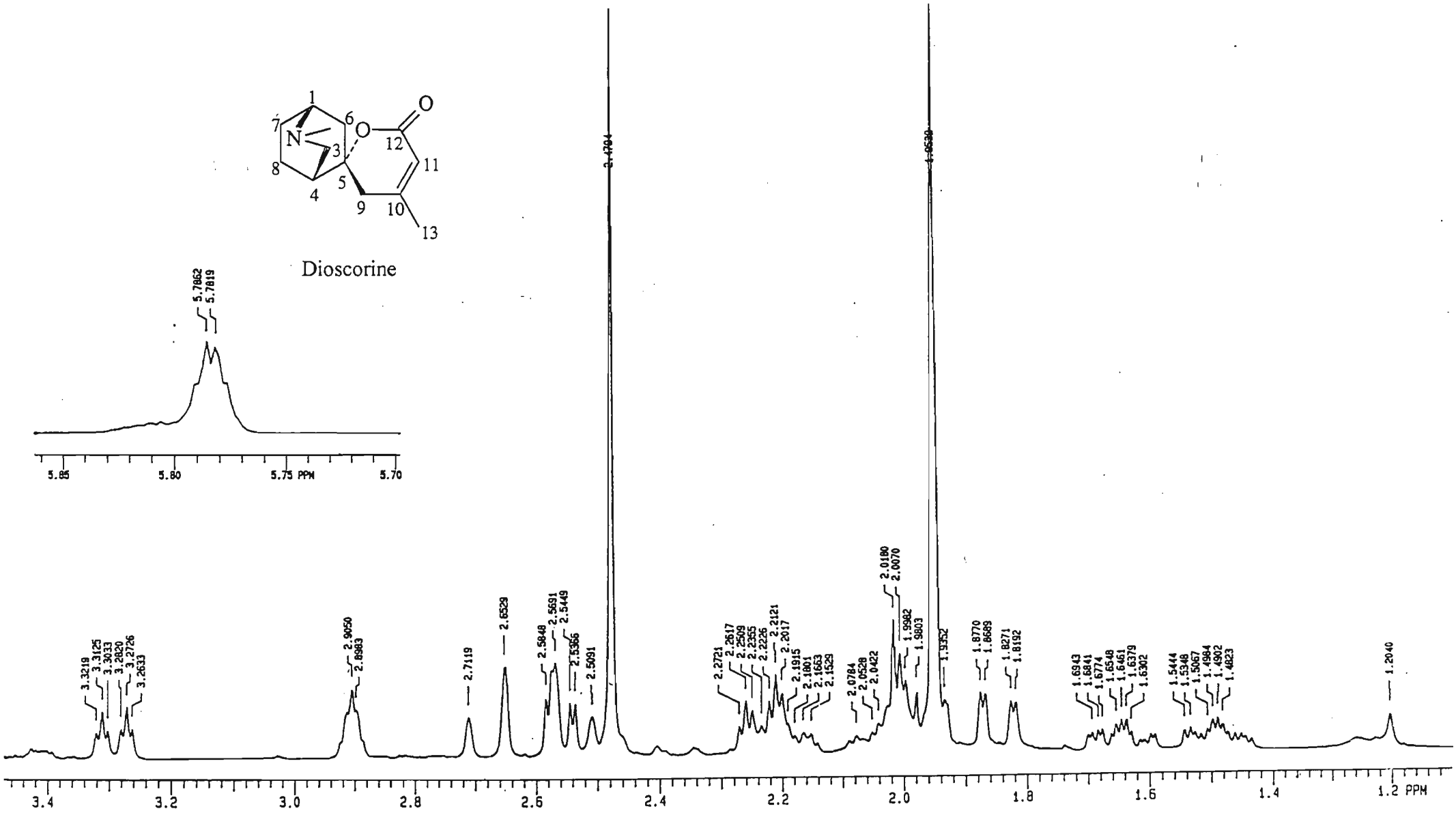
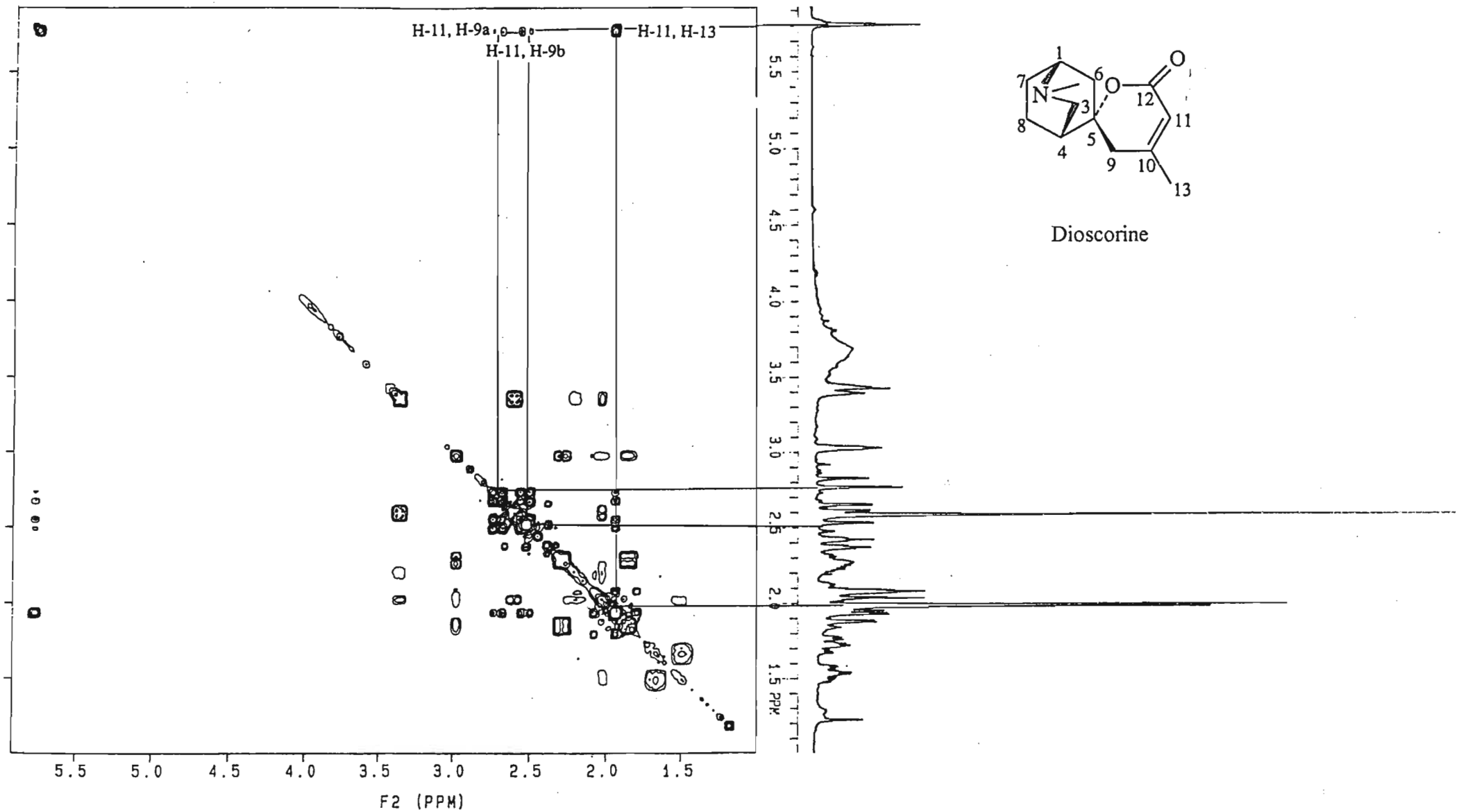


Dioscorine

Spectrum 1 ^{1}H n.m.r. spectrum of Compound 1 in $CDCl_3$



Spectrum 2 Expansion of the ^1H n.m.r. spectrum of Compound 1 in $CDCl_3$



Dioscorine

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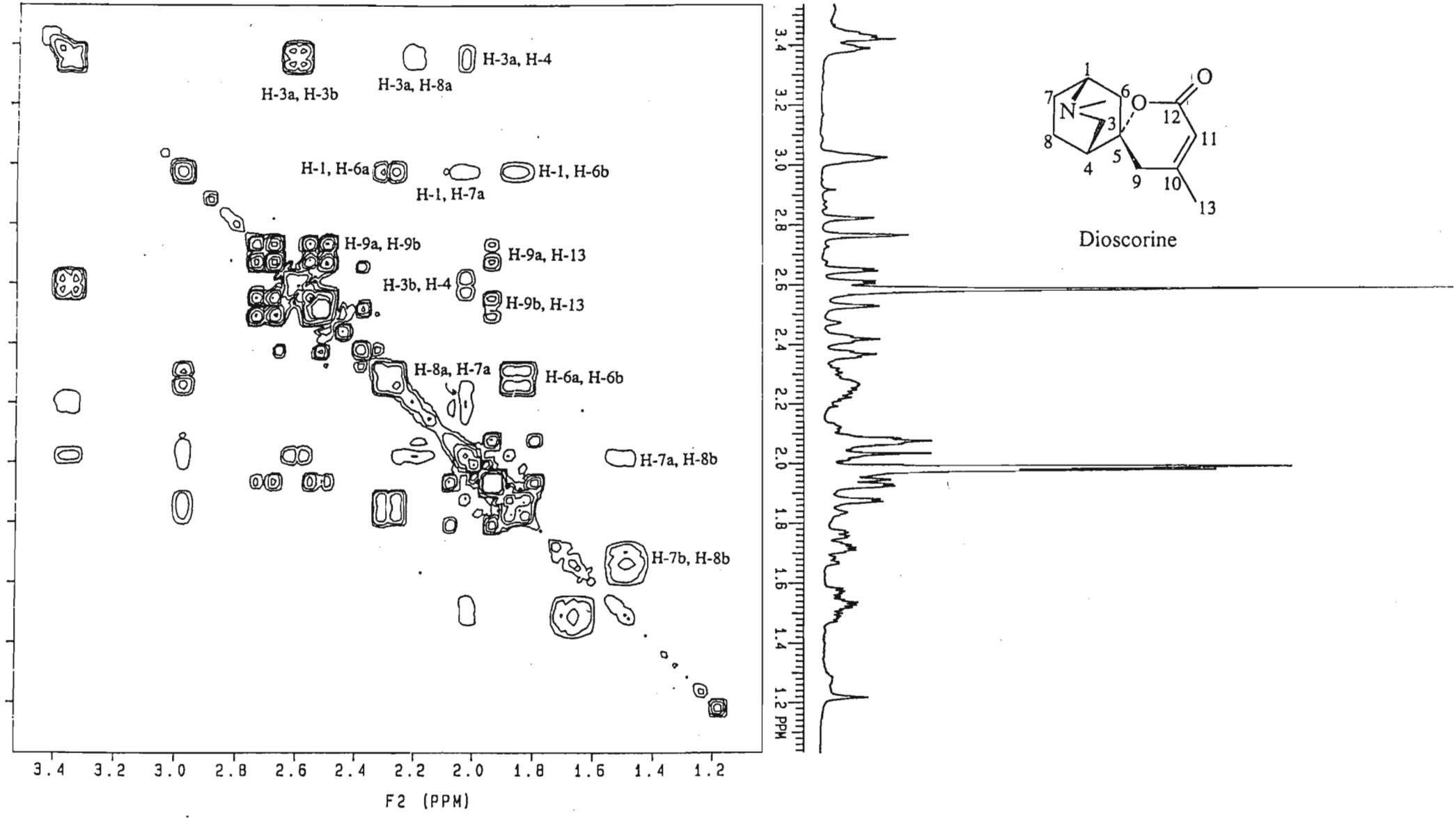

Dioscorine

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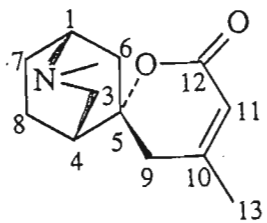

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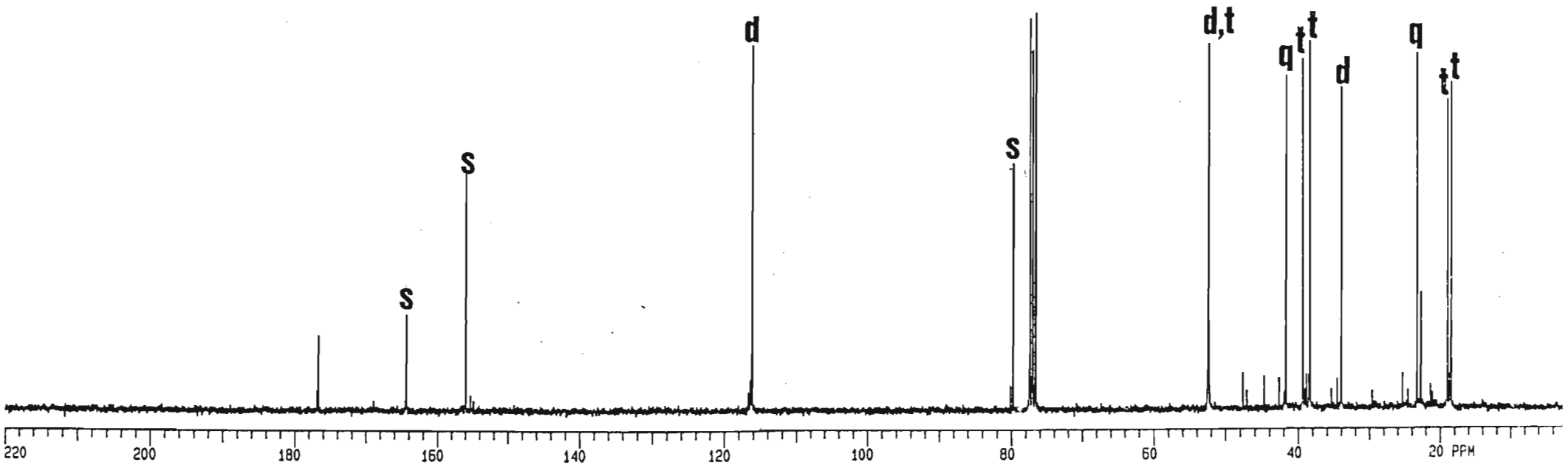
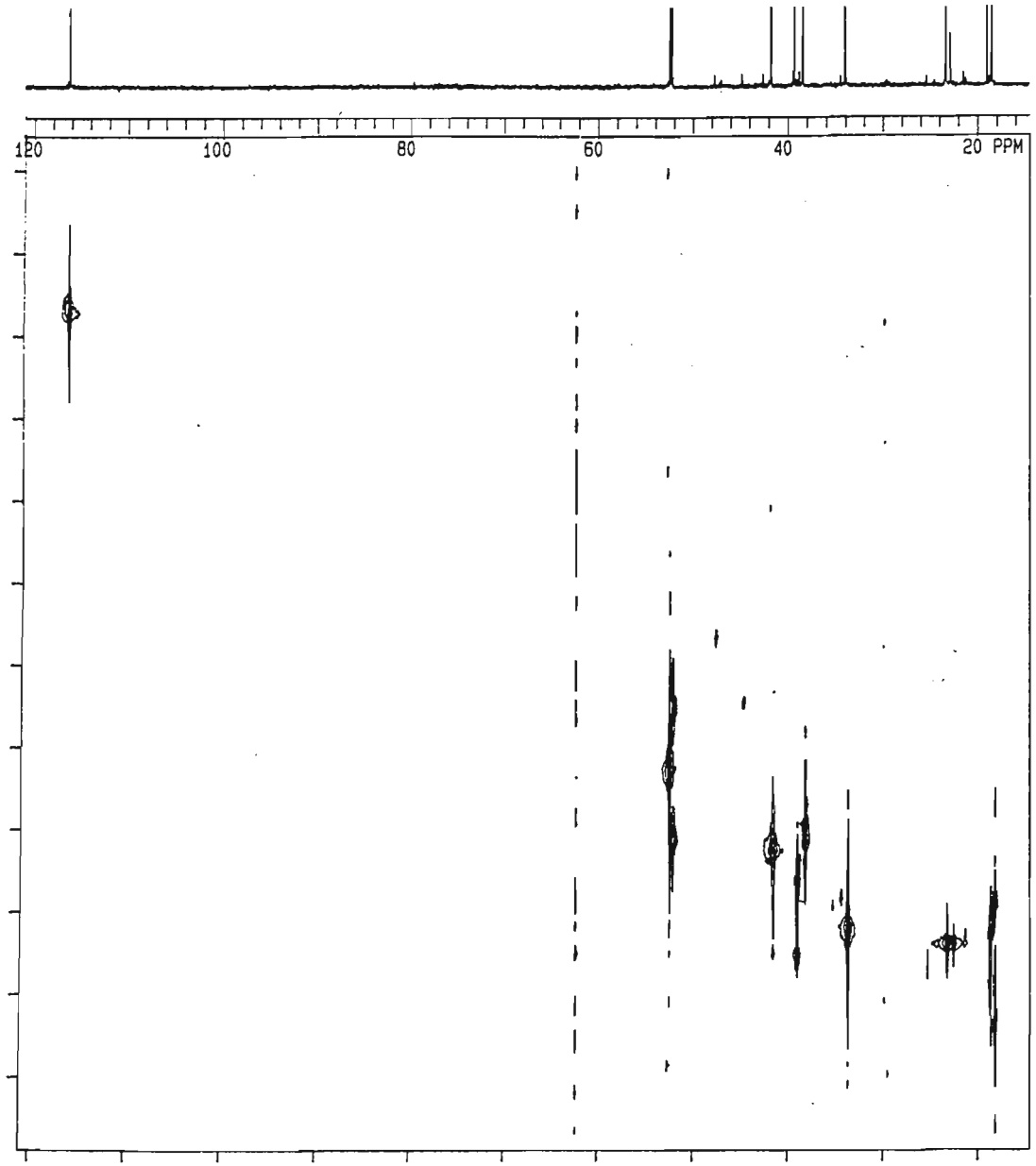
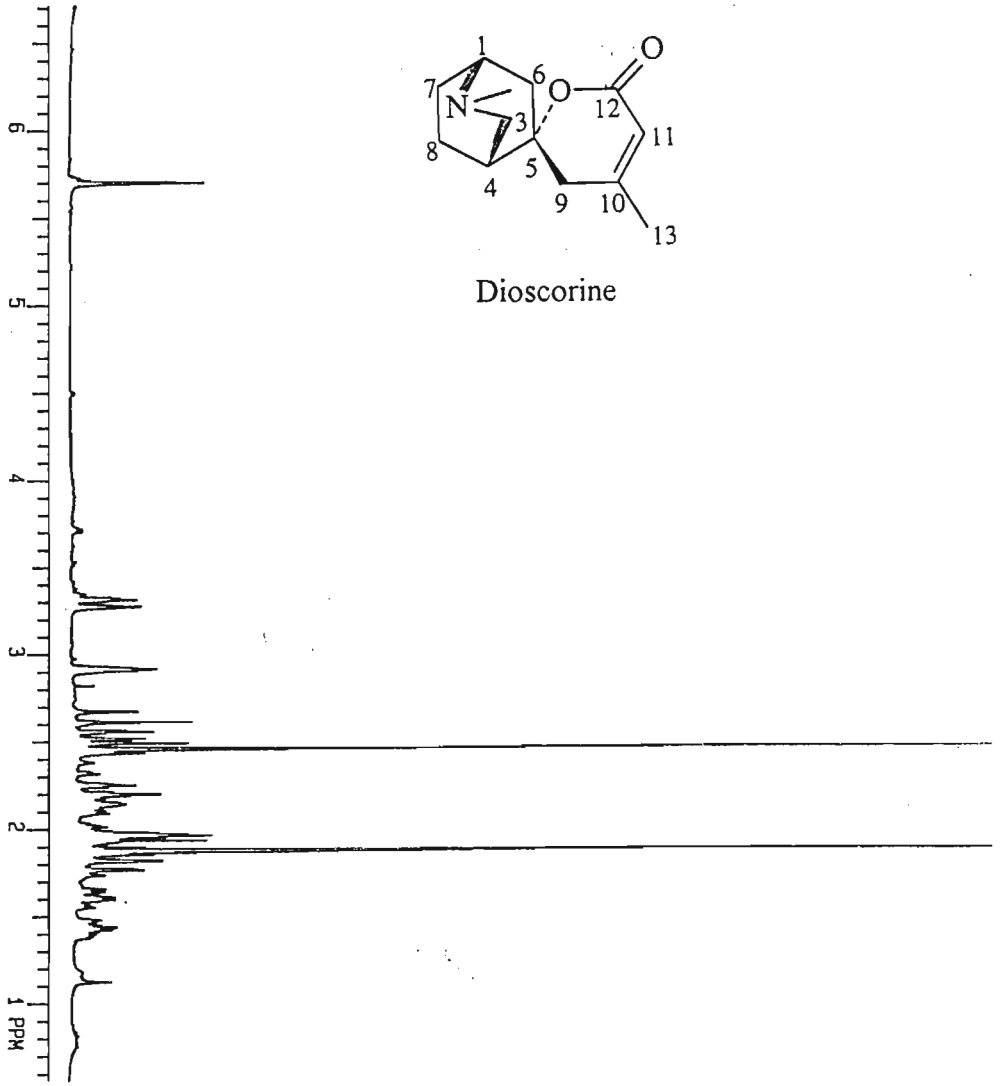


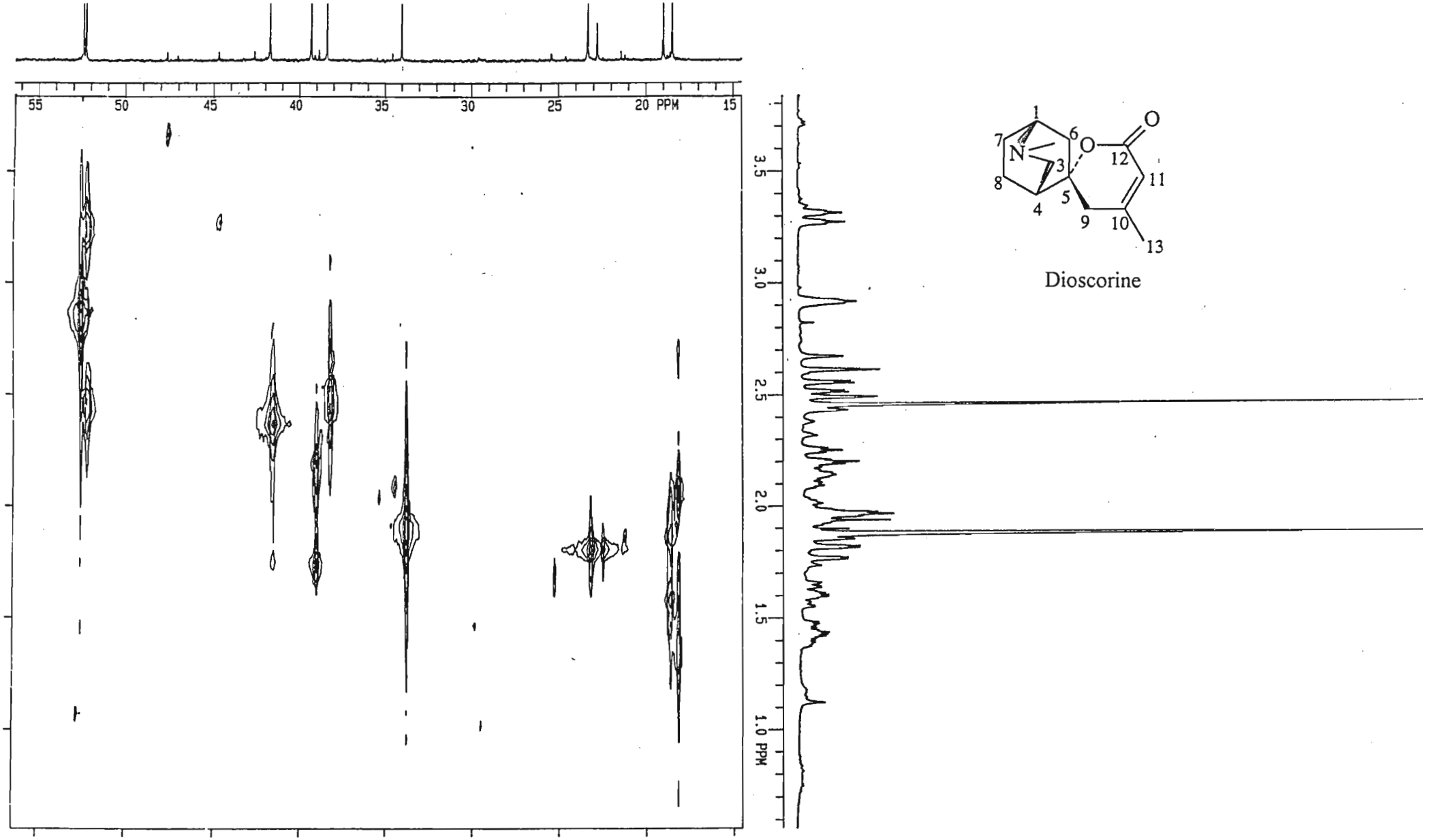
Spectrum : ^{13}C n.m.r. spectrum of Compound 1 in $CDCl_3$





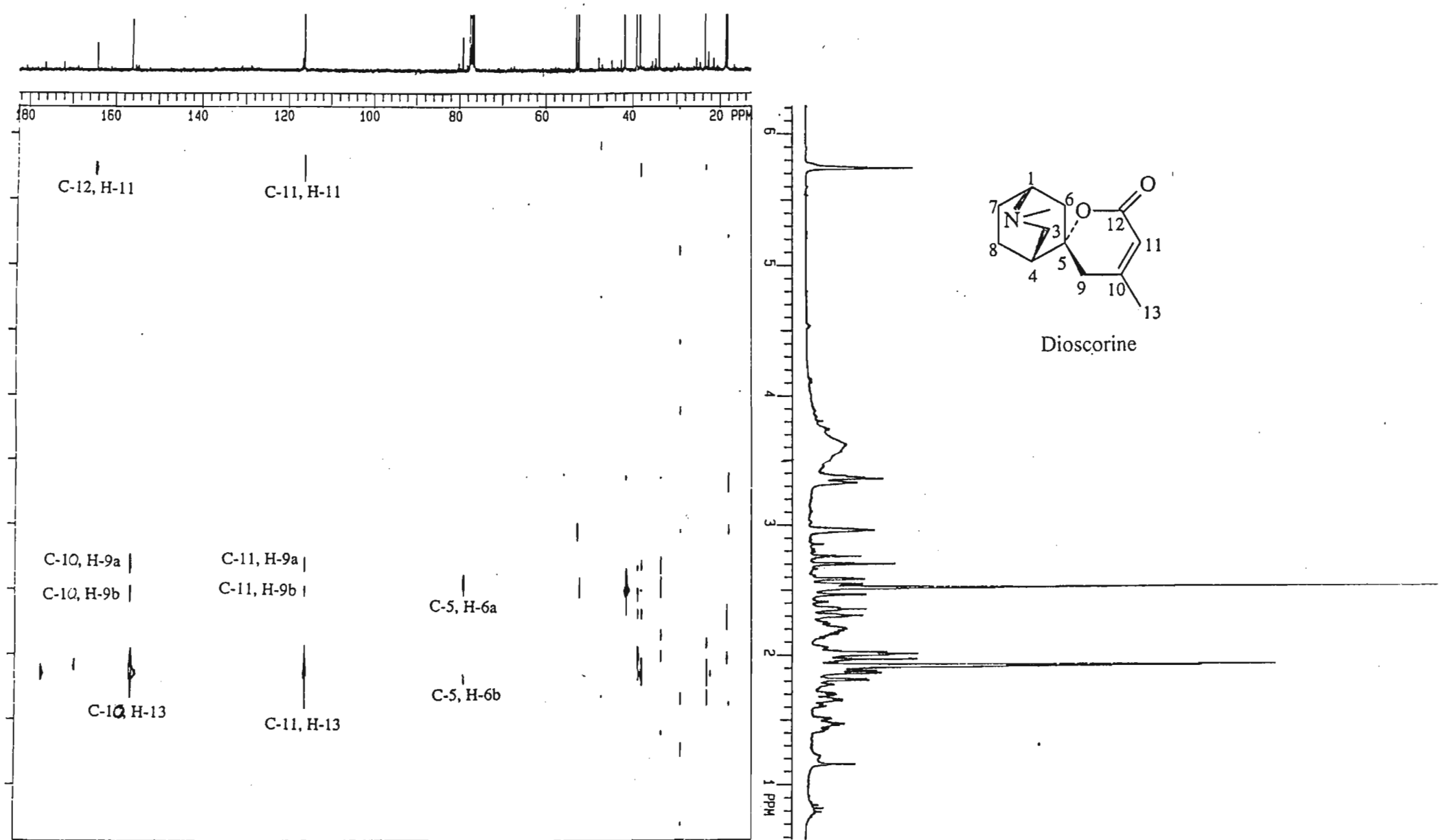
Dioscorine

Spectrum : HETCOR spectrum of compound 1 in $CDCl_3$



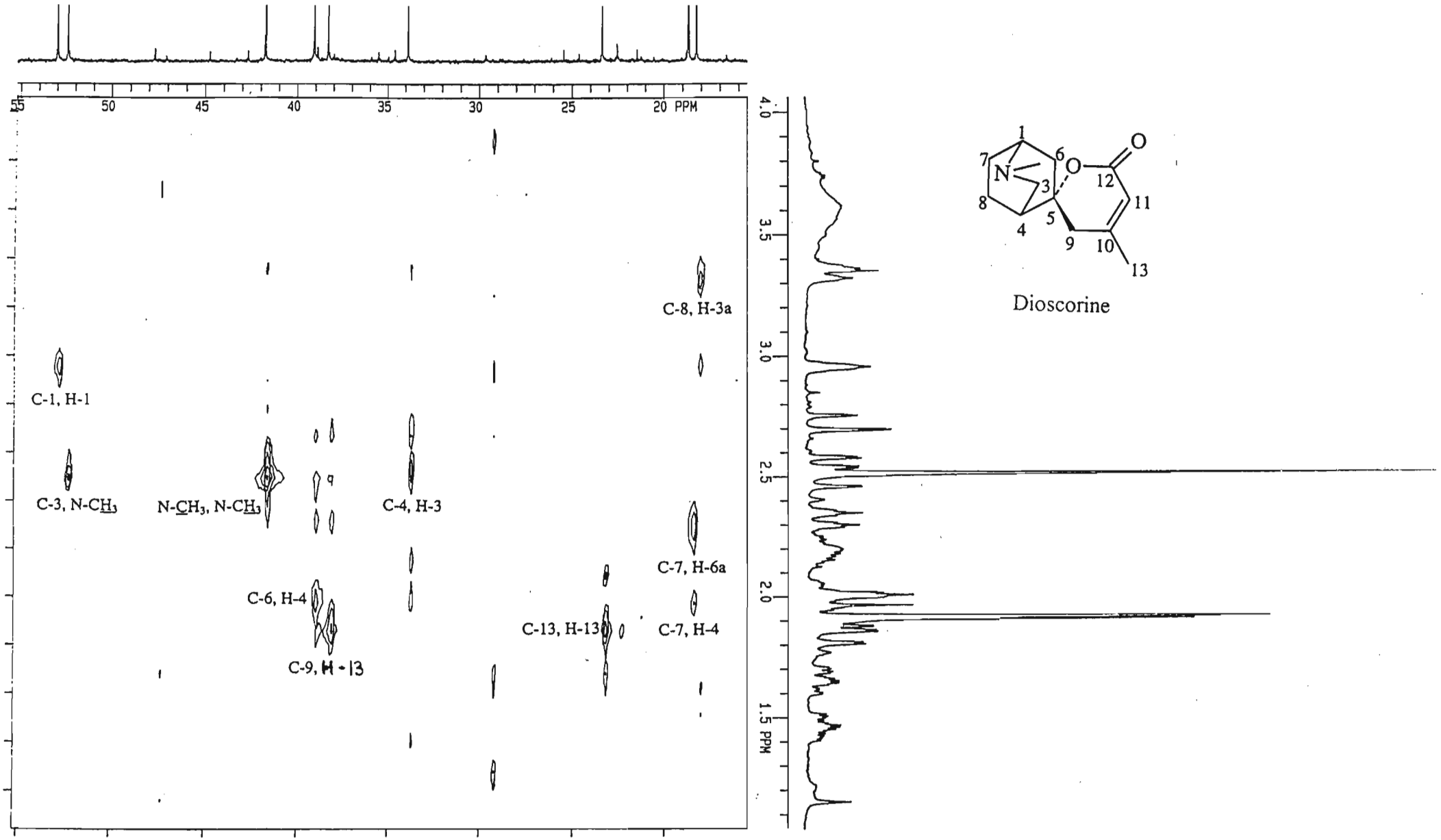
Dioscorine

Spectrum : Expansion of the HETCOR spectrum of compound 1 in $CDCl_3$

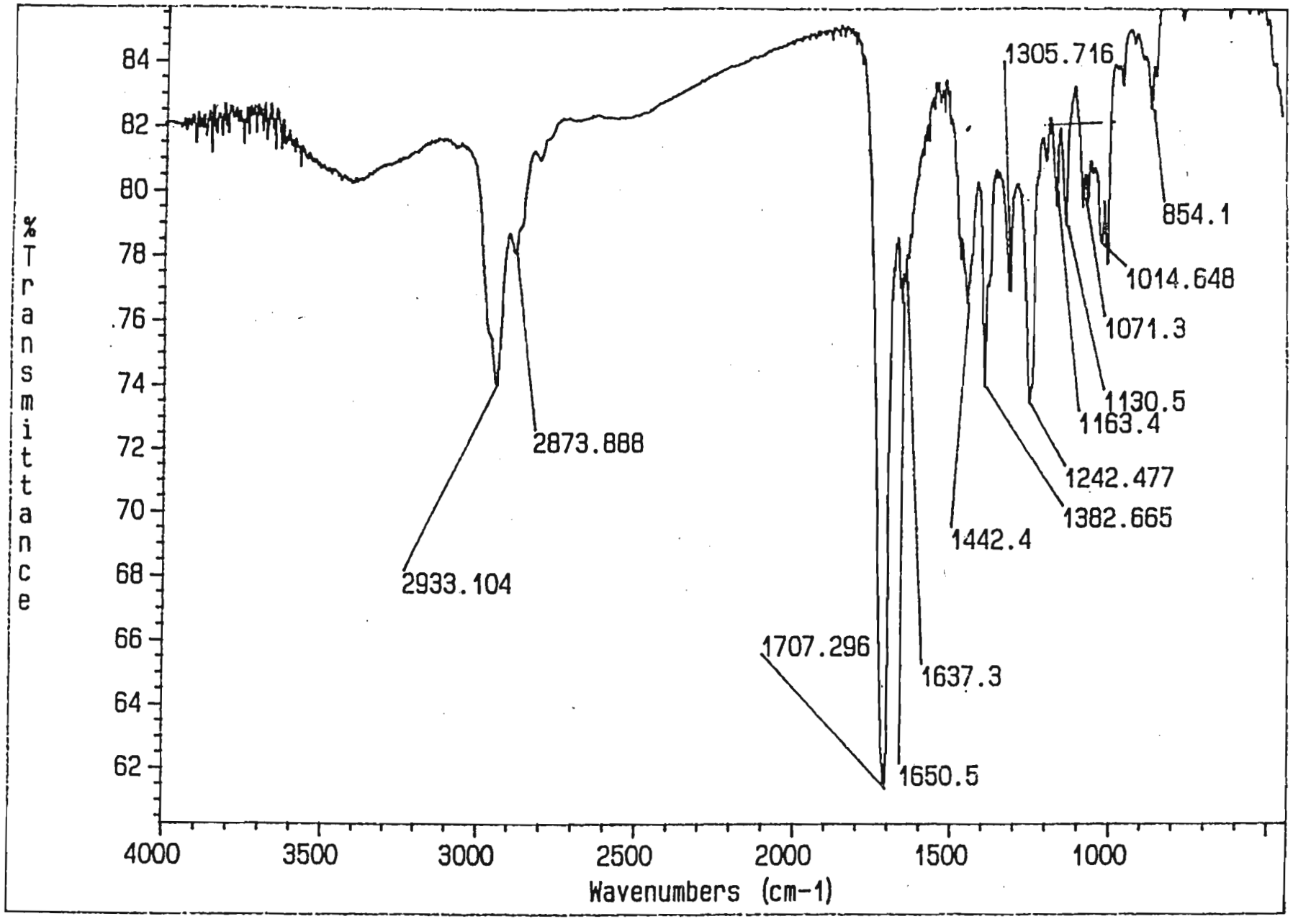


Dioscorine

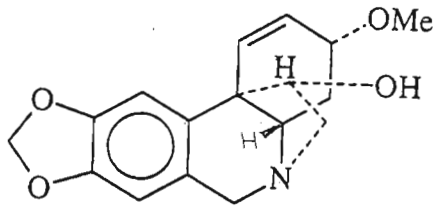
Spectrum : Delayed HETCOR spectrum of compound 1 in $CDCl_3$



Spectrum : Expansion of the delayed HETCOR spectrum of compound 1 in $CDCl_3$

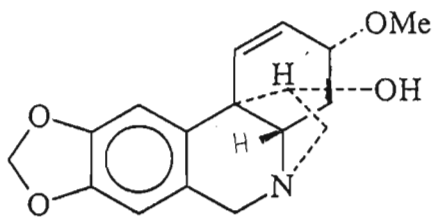


Spectrum : Infrared spectrum of compound 1 (dioscorine)

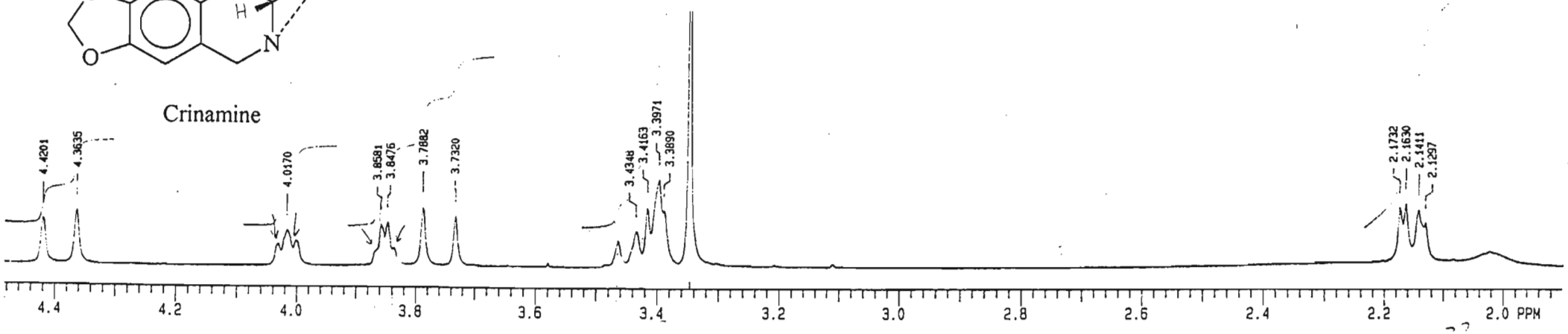


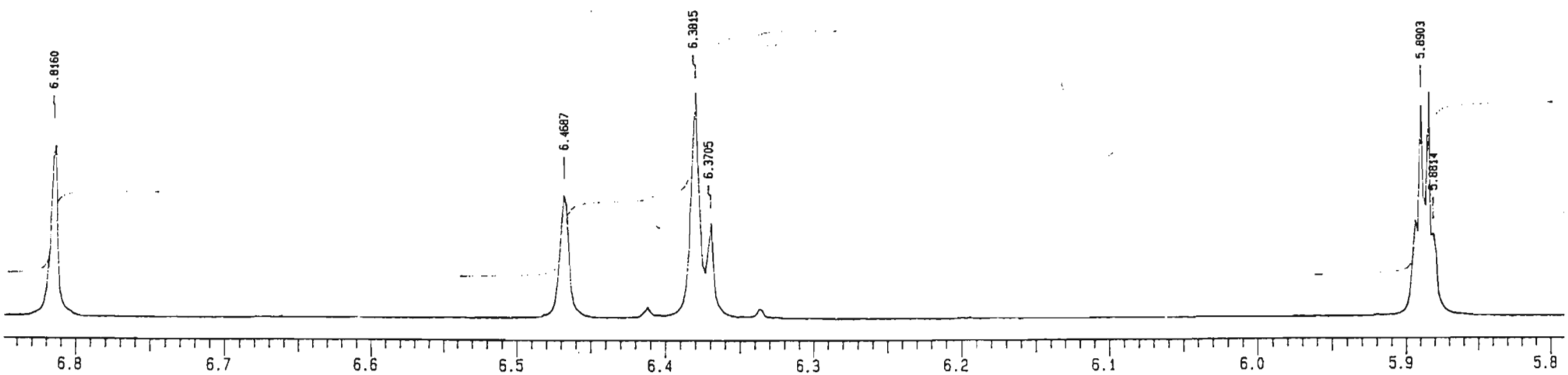
Crinamine

Spectrum : ^{1}H n.m.r. spectrum of Compound 2 in $CDCl_3$

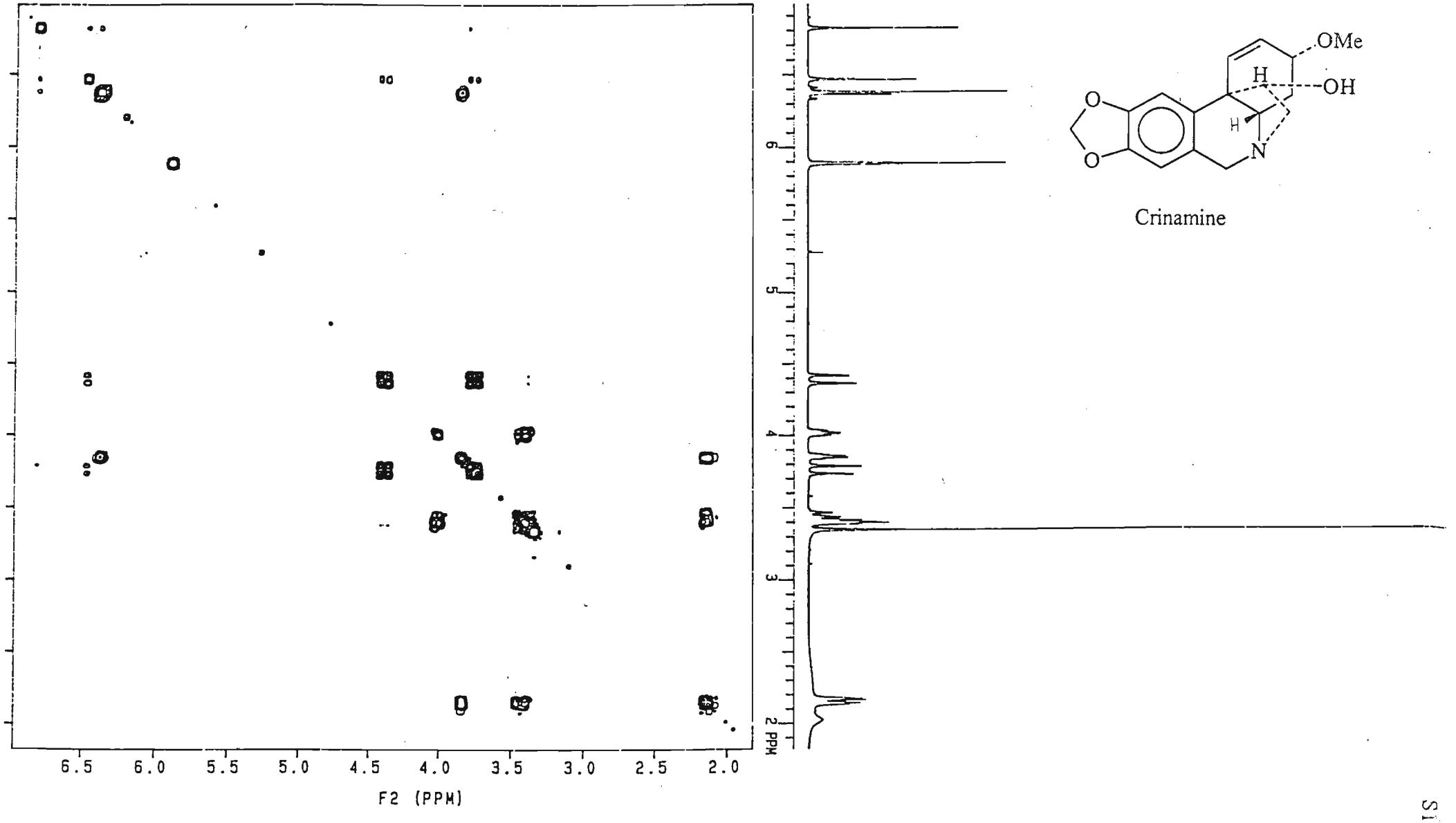


Crinamine



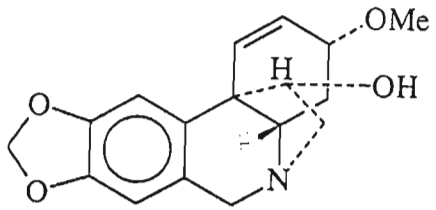


Spectrum : Expansion of the ^{1}H n.m.r. spectrum of compound 2 in $CDCl_3$



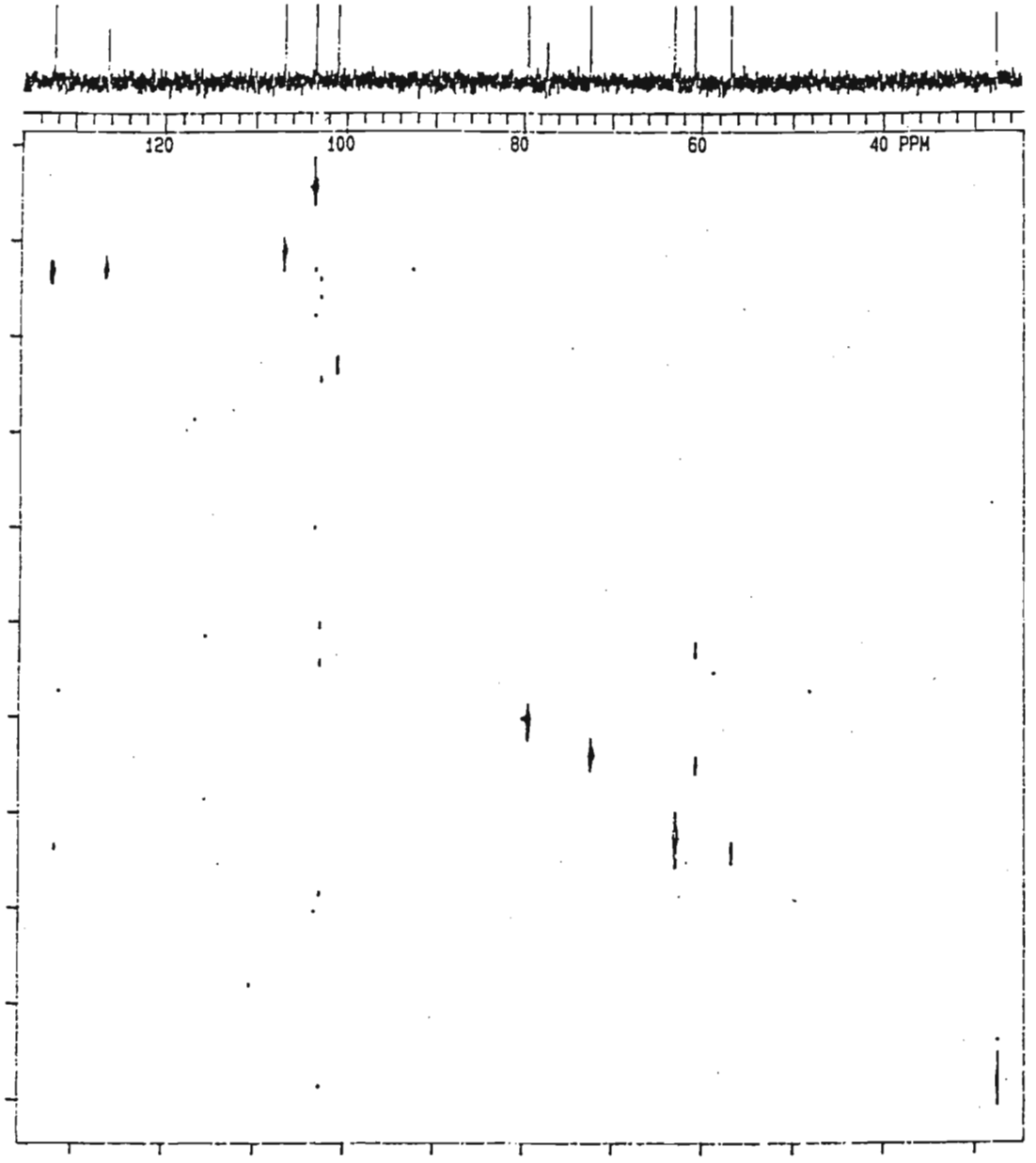
Crinamine

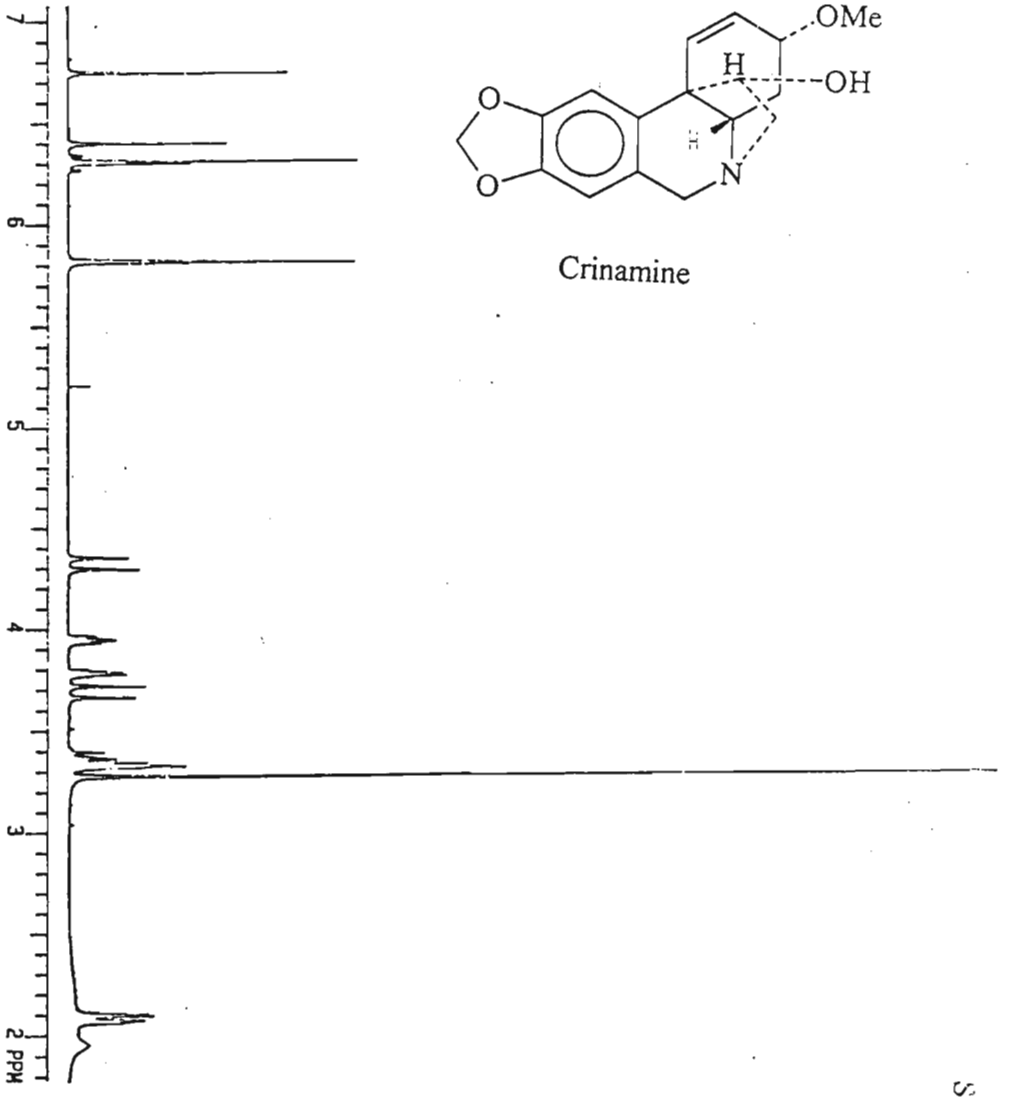
Spectrum : COSY spectrum of Compound 2 in $CDCl_3$



Crinamine

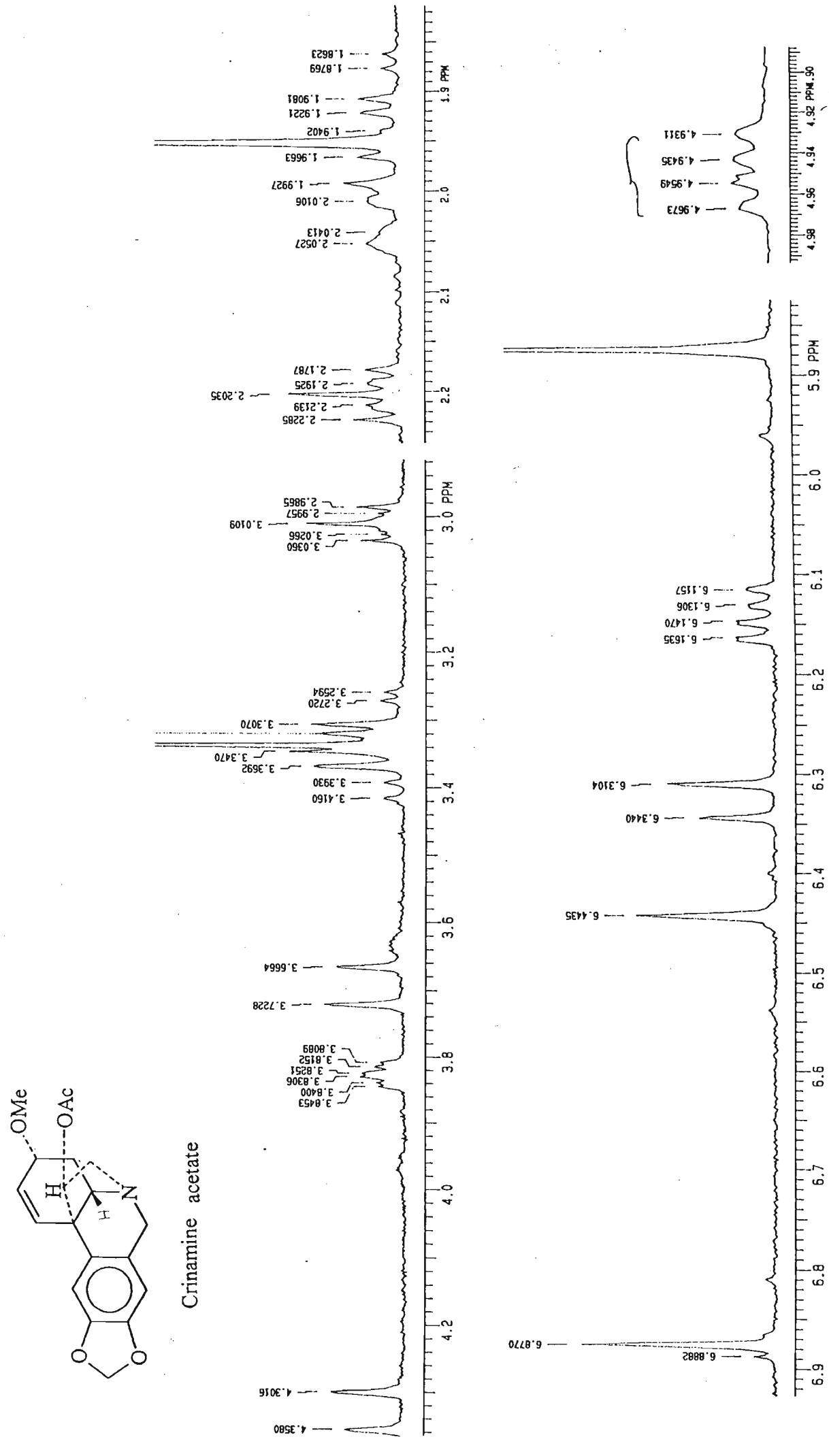
Spectrum : ^{13}C n.m.r. spectrum of Compound 2 in $CDCl_3$



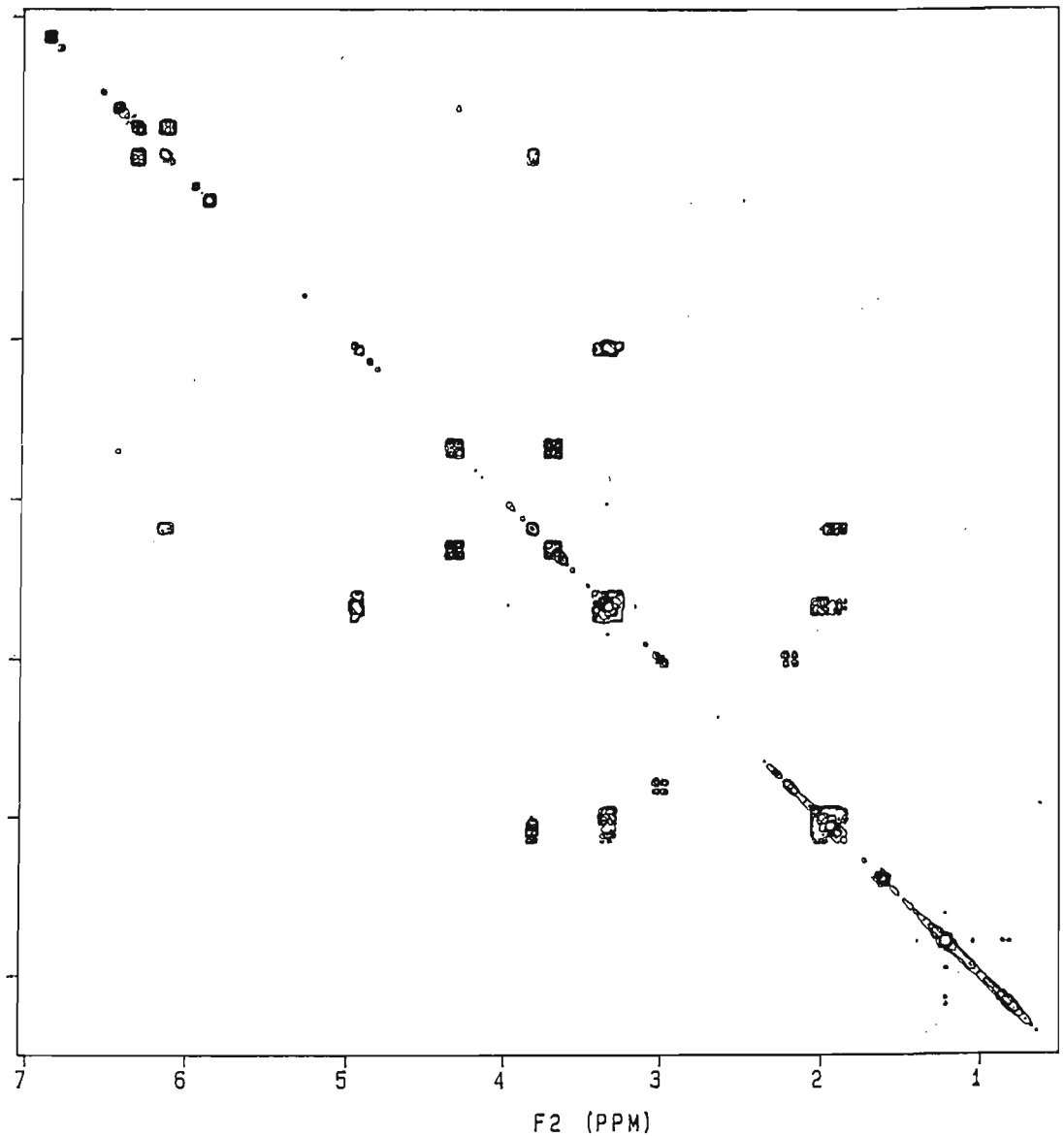


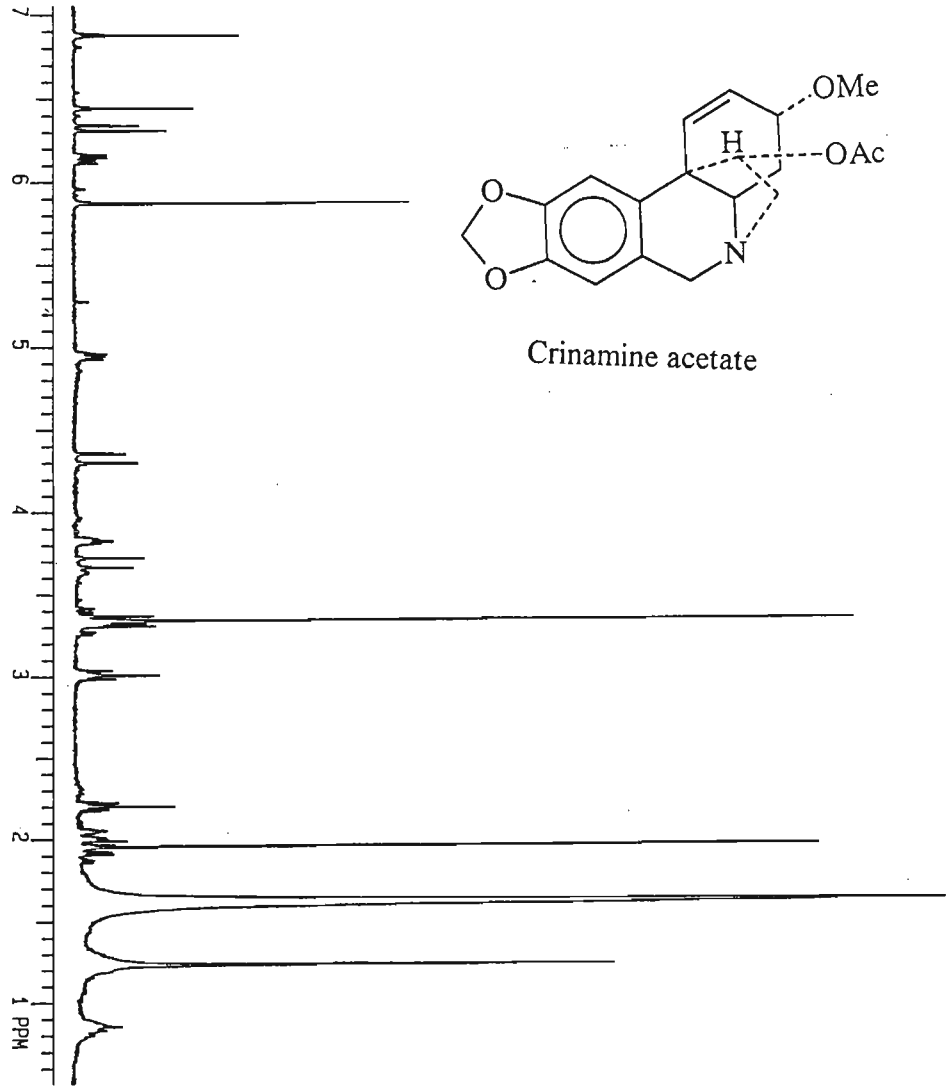
Crinamine

Spectrum : HETCOR spectrum of Compound 2 in $CDCl_3$



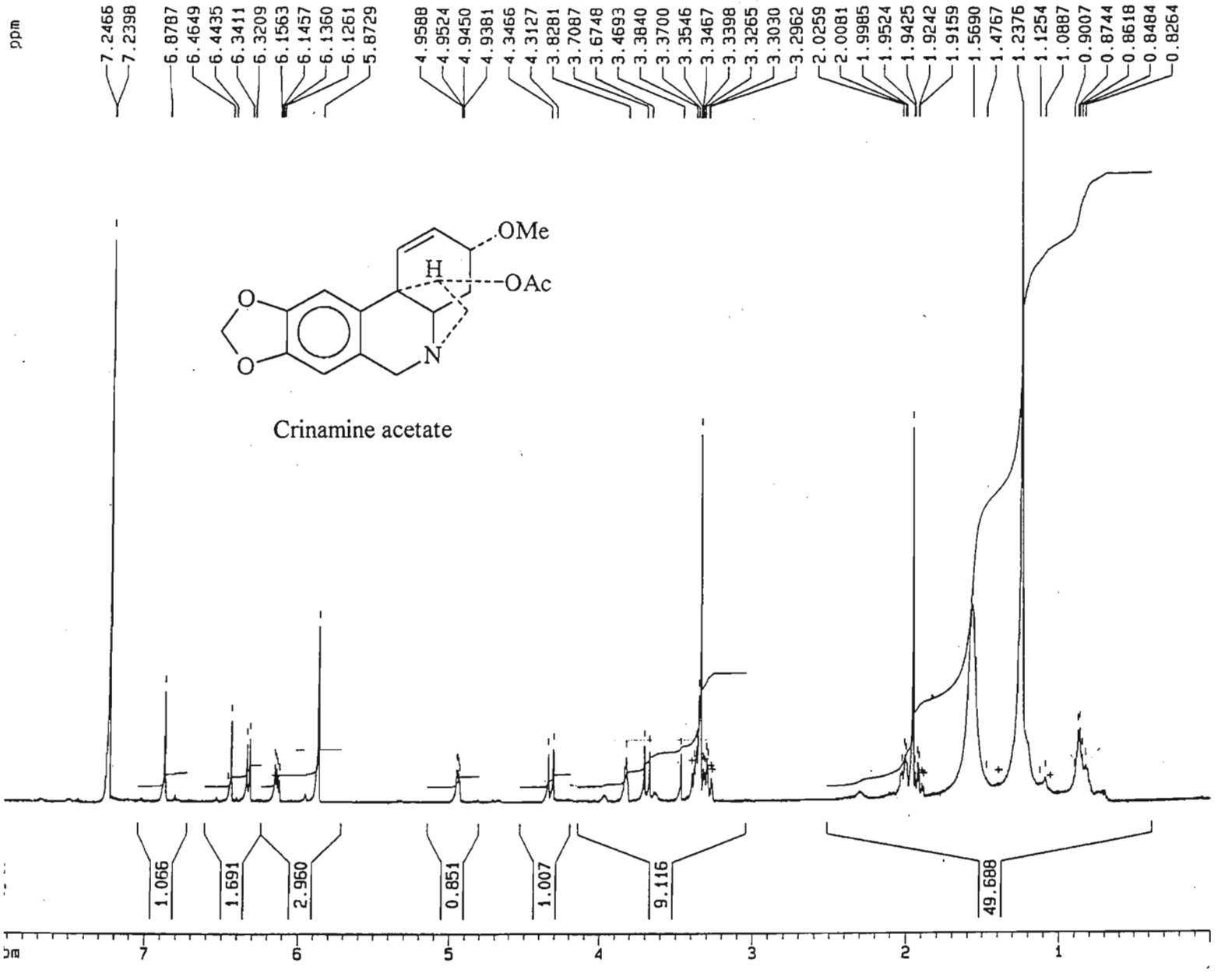
Spectrum : Expansion of the ^1H n.m.r. spectrum of compound 2A in $CDCl_3$





Crinamine acetate

Spectrum : COSY spectrum of Compound 2A



Crinamine acetate

Current Data Parameters

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PROCNO	1

F2 - Acquisition Parameters

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Time	11.48
PULPROG	zg
SOLVENT	CDCl3
AQ	3.6372681 sec
FIDRES	0.137467 Hz
DW	111.0 usec
RG	2860
NUCLEUS	1H
D1	1.5000000 sec
P1	9.1 usec
DE	138.8 usec
SFO1	500.1374301 MHz
SWH	4504.50 Hz
TD	32768
NS	256
DS	2

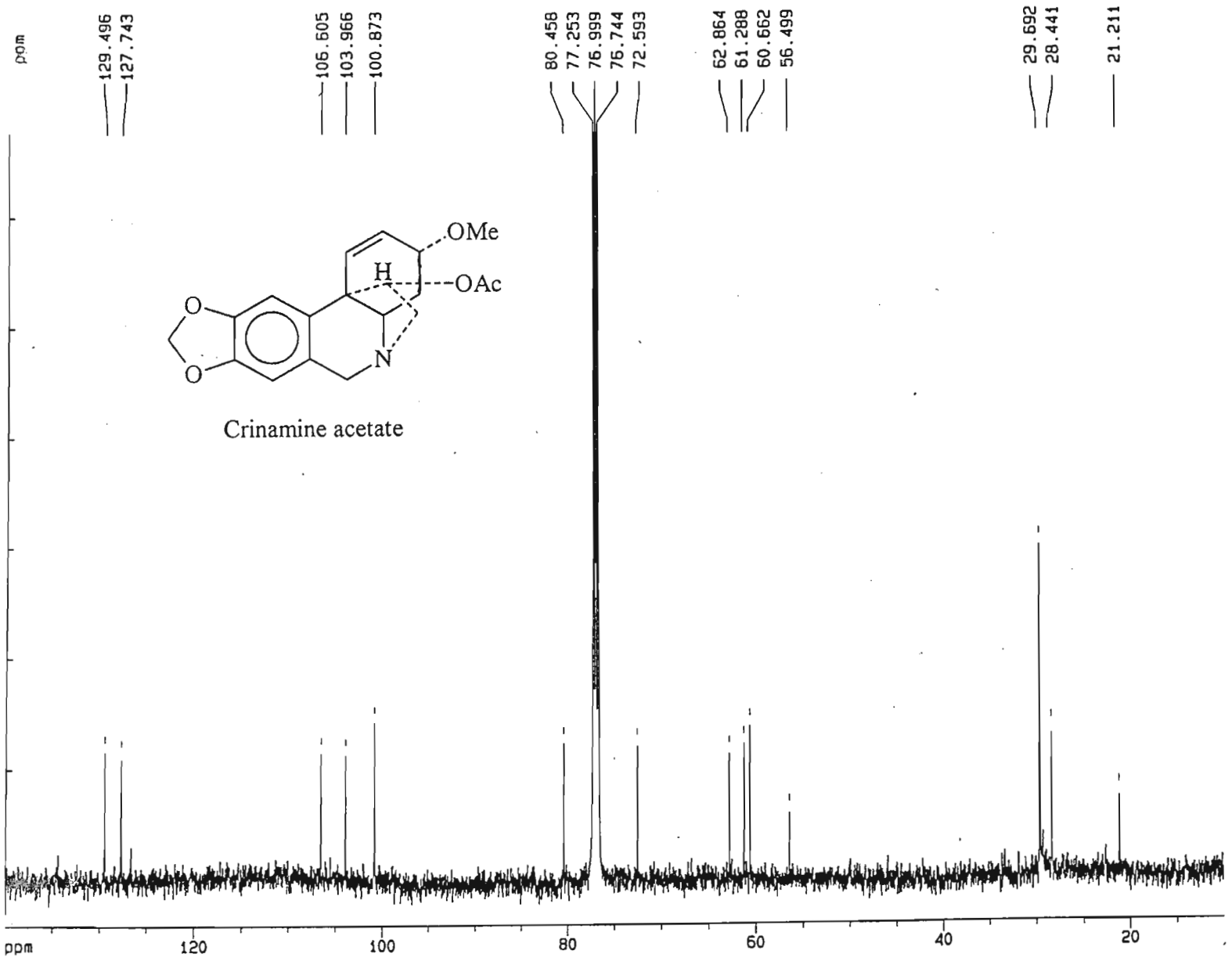
F2 - Processing parameters

SI	16384
SF	500.1354296 MHz
WDW	GM
SSB	0
LB	-0.50 Hz
GB	0.5
PC	1.00

1D NMR plot parameters

CX	22.00 cm
F1P	8.000 ppm
F1	4001.08 Hz
F2P	0.000 ppm
F2	0.00 Hz
PPMCM	0.36364 ppm/cr
HZCM	181.86743 Hz/cm

pectrum : ^{1}H n.m.r. spectrum of compound 2A (500 MHz n.m.r. instrument)



Current Data Parameters

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PROCNO	1

F2 - Acquisition Parameters

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FIDRES	0.635783 Hz
DW	12.0 use
RG	45500
NUCLEUS	13C
D12	0.0000200 sec
DL6	26.00 dB
D1	1.5000000 sec
P31	100.0 use
D11	0.0300000 sec
DL5	25.00 dB
P1	11.8 use
DE	17.1 use
SFO1	125.7703757 MHz
SWH	41666.67 Hz
TD	65536
NS	40960
DS	2

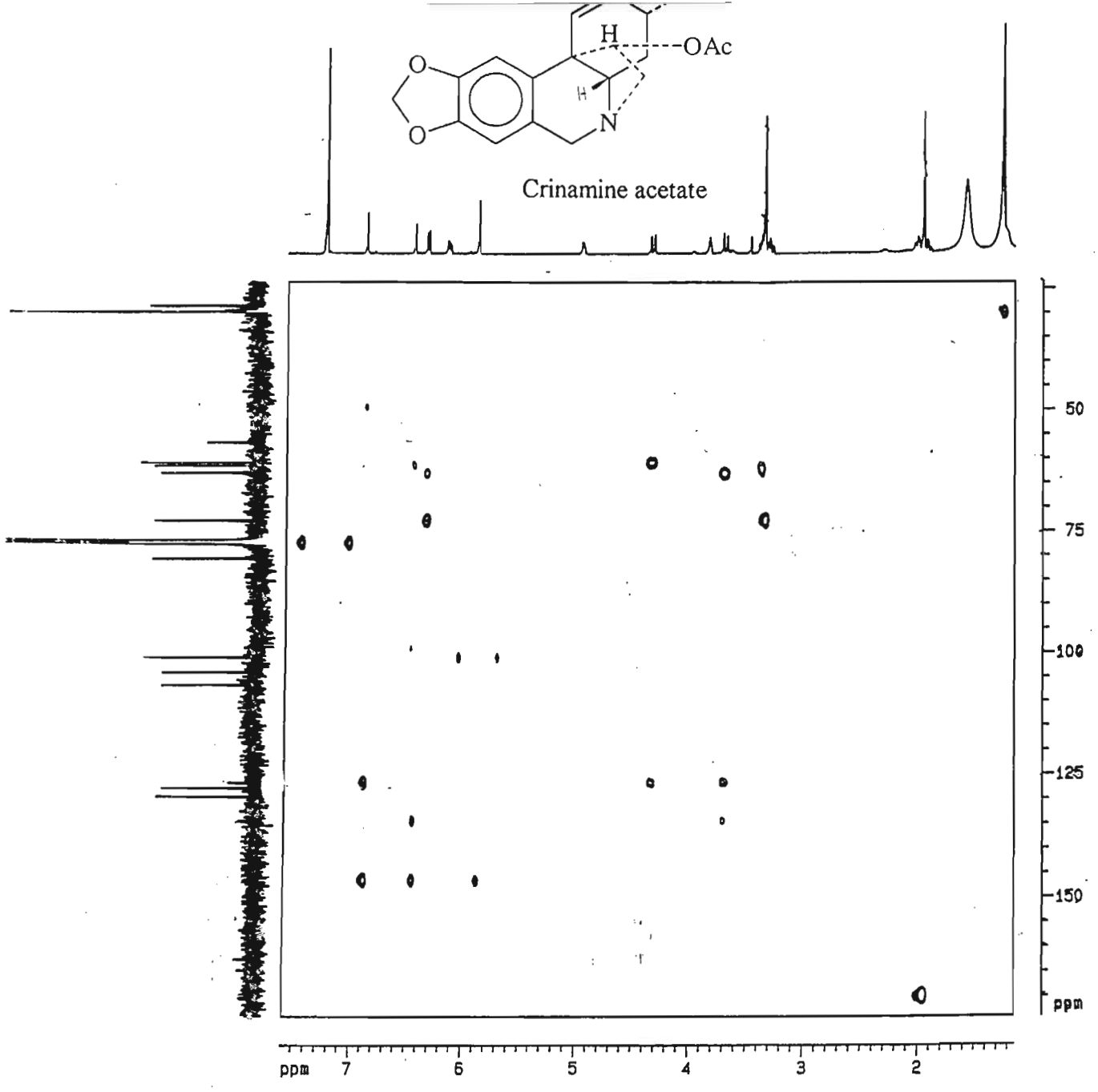
F2 - Processing parameters

SI	32768
SF	125.7591486 MHz
WDW	EM
SSB	0
LB	2.00 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	22.00 cm
F1P	140.000 ppm
F1	17606.28 Hz
F2P	10.000 ppm
F2	1257.59 Hz
PPMCM	5.90909 ppm
HZCM	743.12225 Hz/

Spectrum : Proton decoupled ^{13}C n.m.r. spectrum of compound 2A in $CDCl_3$ (500 MHz n.m.r. instrument)



Spectrum : Long range HETCOR of compound 2A in $CDCl_3$ (500 MHz n.m.r. instrument)

```
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NAME                    pagedde
EXPNO                         3
PROCNO                        1

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Time                      19.55
PULPROG          inv4gplplrnd
SOLVENT                   CDCl3
AQ                0.1135840 sec
FIDRES            4.398930 Hz
DW                    111.0 use
RG                       2800
NUCLEUS                    1H
P16                  1000.0 use
L21                       200
P1                        9.1 use
D1                1.5000000 sec
D2                0.0034500 sec
P3                       11.8 use
SFO2             125.7703757 MHz
D6                0.0500000 sec
D0                0.0000030 sec
D27               0.0000030 sec
D16               0.0004000 sec
P2                       18.2 use
D13               0.0000040 sec
DE                      138.6 use
SFO1             500.1374301 MHz
SWH                 4504.50 Hz
TD                        1024
NS                          96
DS                          16
IN0               0.0000120 sec

                F1 - Acquisition para
MCO                          2
TD                         256
SFO1             125.7704 MHz
FIDRES           152.769422 Hz
SW               331.292 ppm

                F2 - Processing parame
SI                        1024
SF               500.1354298 MHz
WDW                       SINE
SSB                          0
LB                        0.00 Hz
GB                           0
PC                        1.00

                F1 - Processing para
SI                        1024
MC2                         QF
SF               125.7691486 MHz
WDW                       SINE
SSB                          0
LB                        0.00 Hz
GB                           0

                2D NMR plot paramet
CX2                      13.00 cm
CX1                      13.00 cm
F2PLO                    7.380 ppm
F2LO                  3790.89 Hz
F2PHI                    1.141 ppm
F2HI                   570.87 Hz
F1PLO                  174.045 ppm
F1LO                  21867.81 Hz
F1PHI                   23.583 ppm
F1HI                   2957.01 Hz
F2PPMCM                0.49525 ppm
F2HZCM               247.86380 Hz/
F1PPMCM                11.57334 ppm
F1HZCM              1455.45374 Hz/
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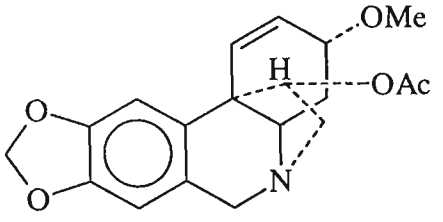
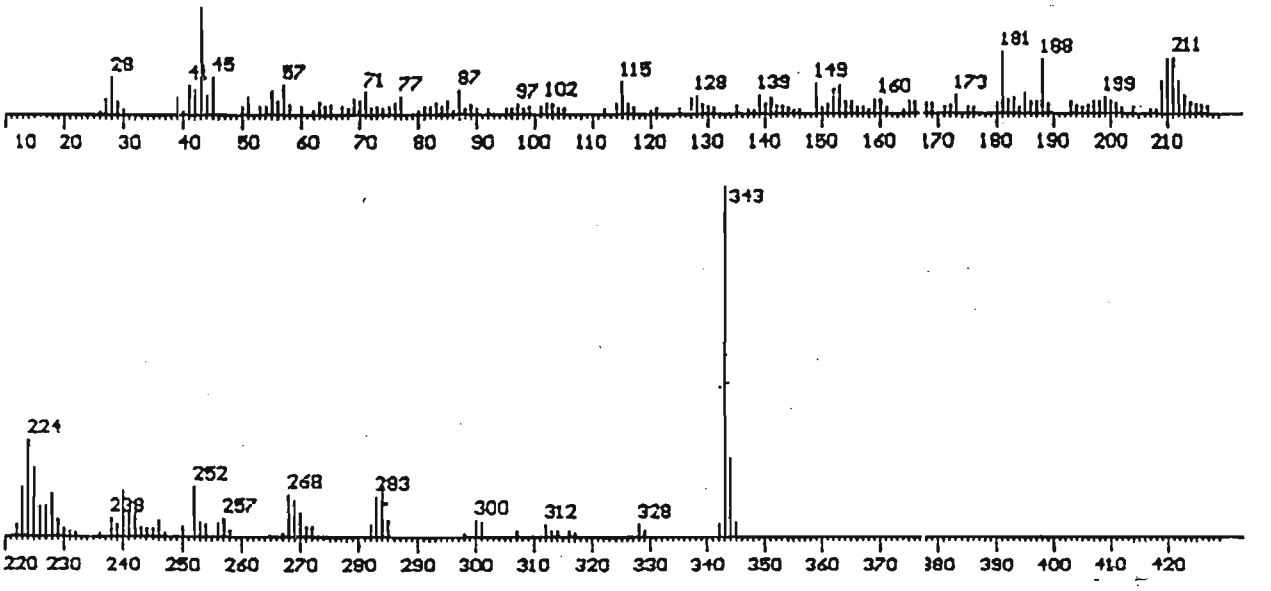
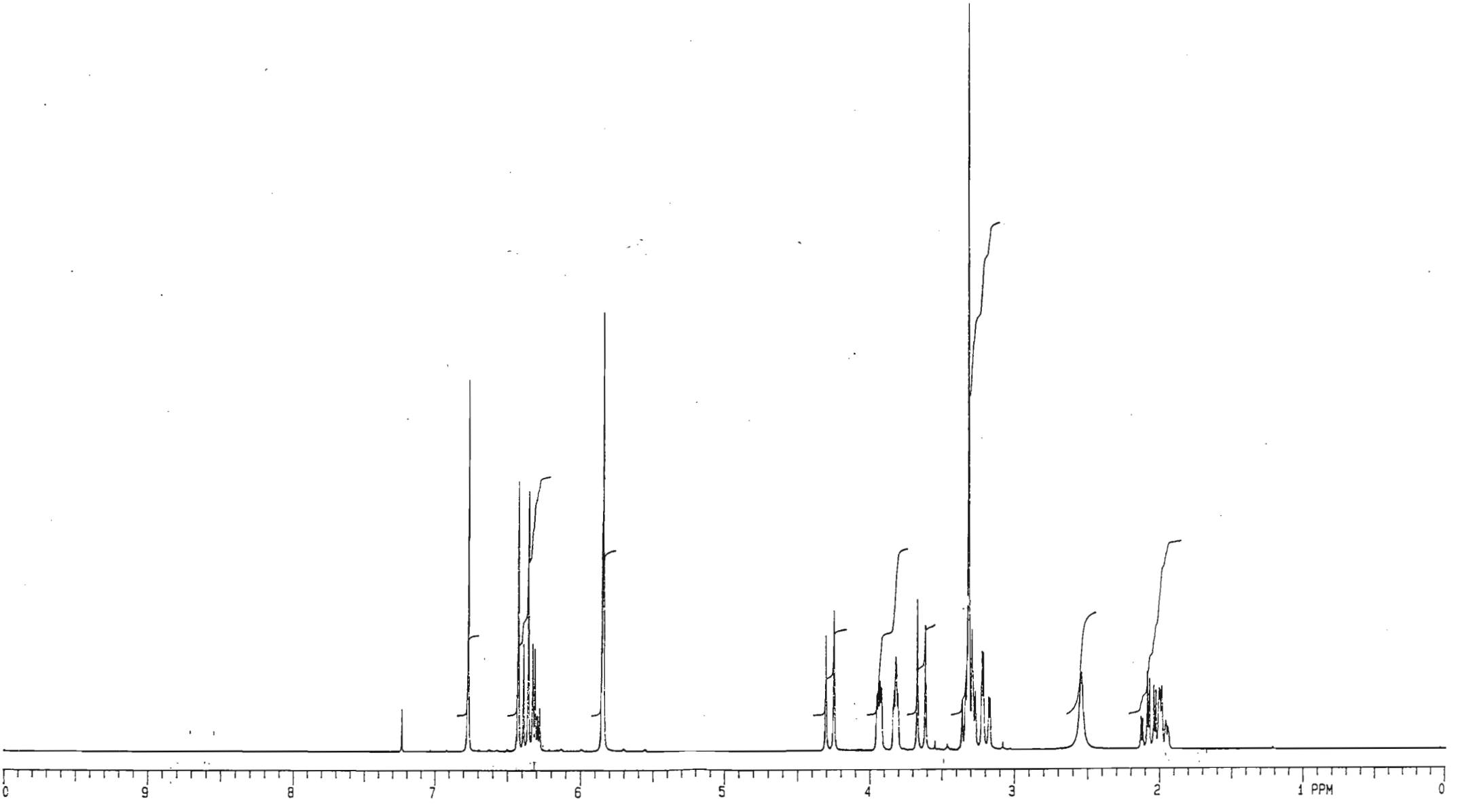


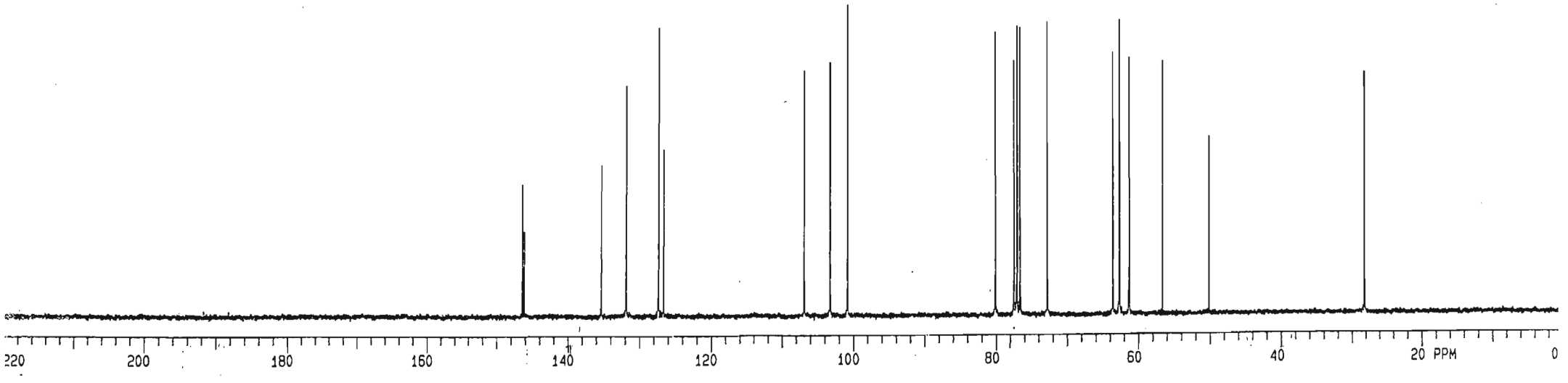
Crinamine acetate



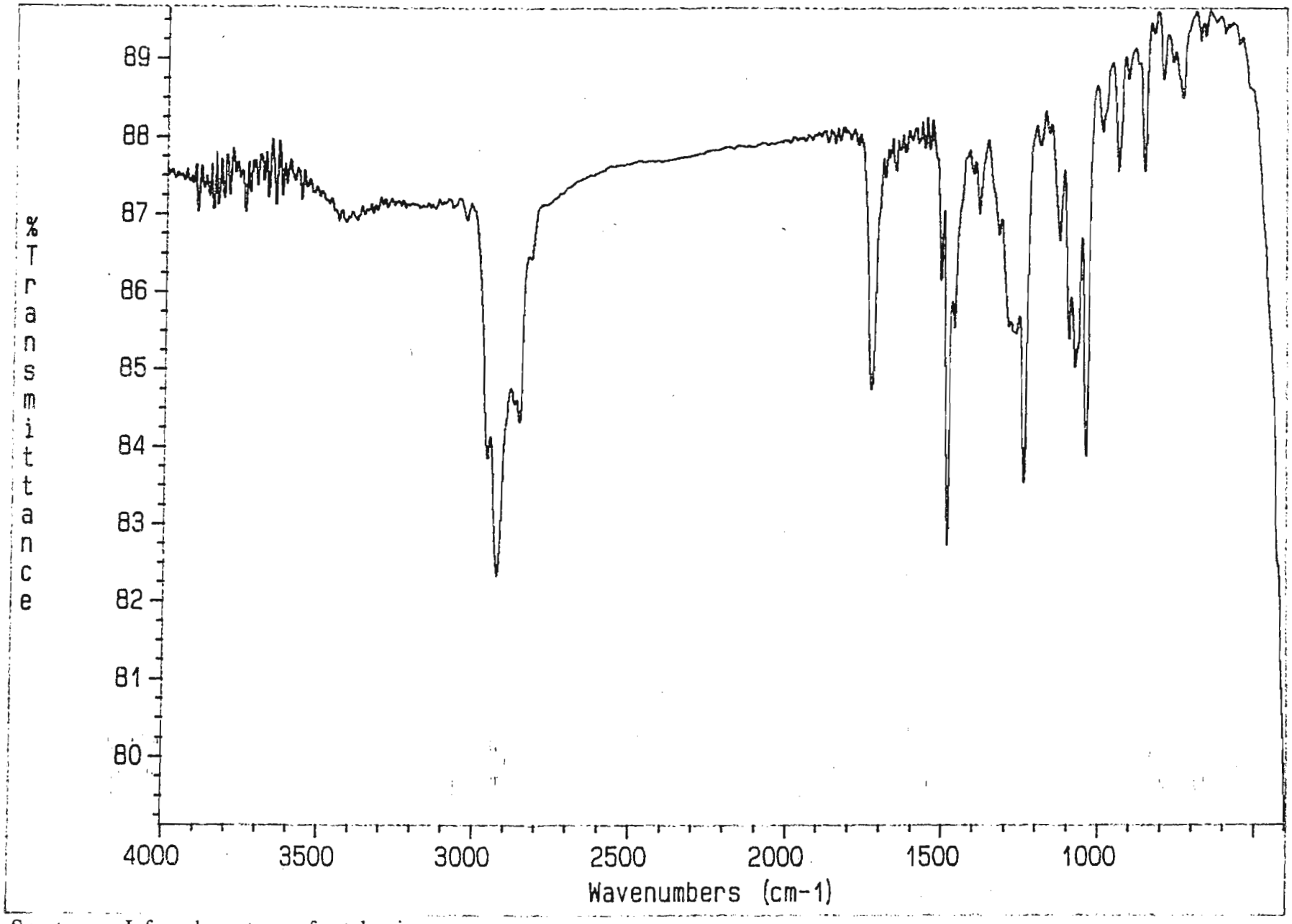
Spectrum : Mass spectrum of Compound 2A



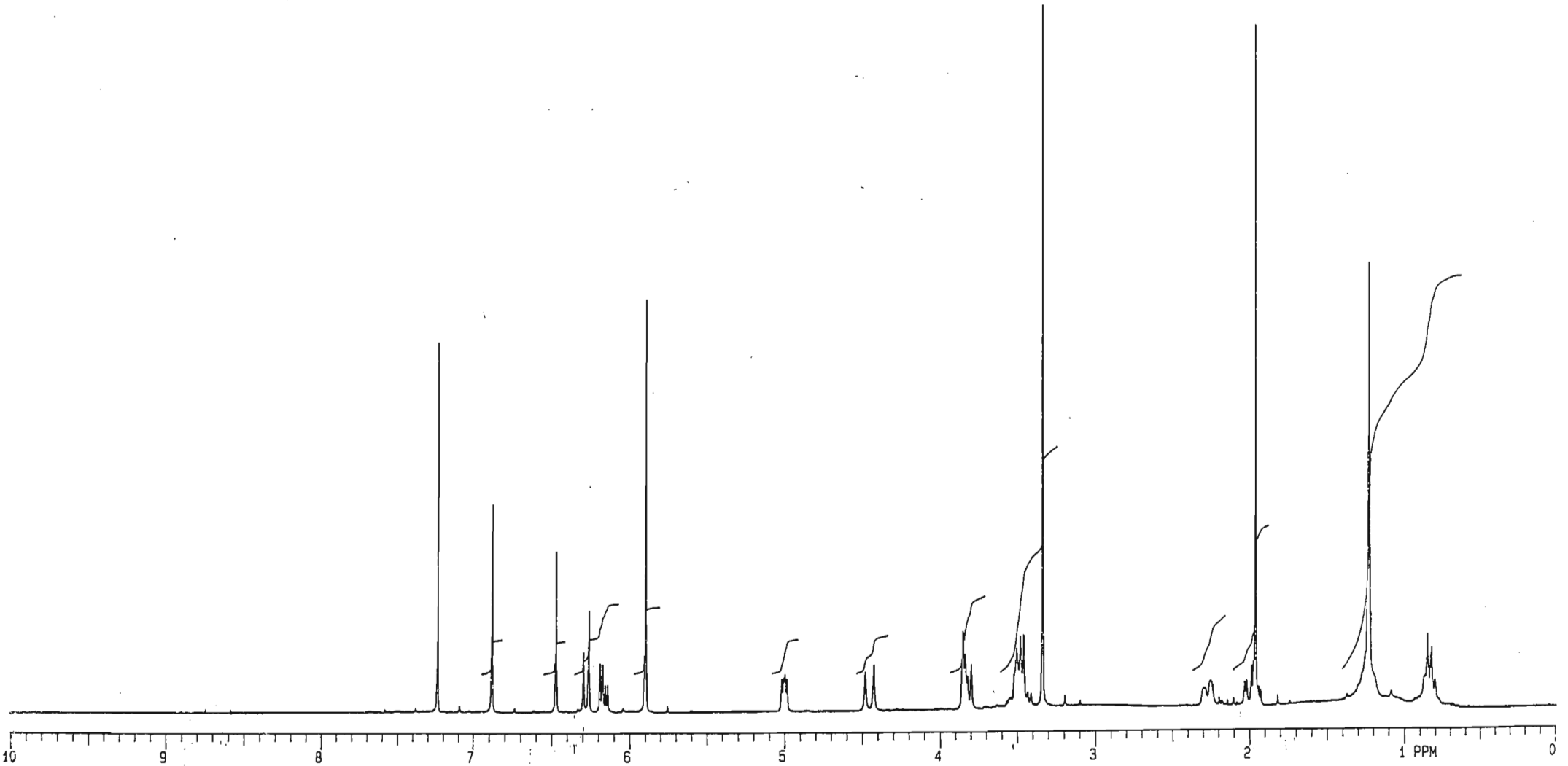
Spectrum : ^{1}H n.m.r. spectrum of natalensine



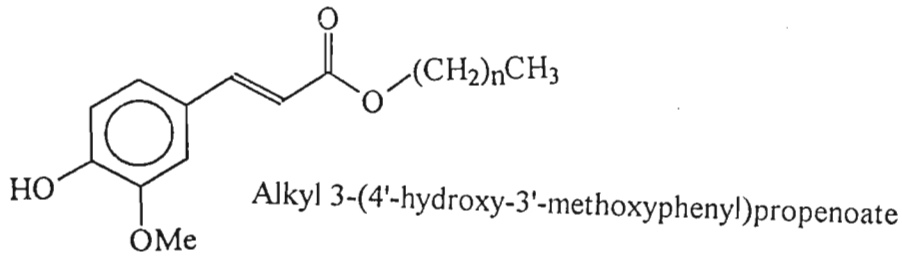
Spectrum : ^{13}C n.m.r. spectrum of natalensine



Spectrum : Infrared spectrum of natalensine

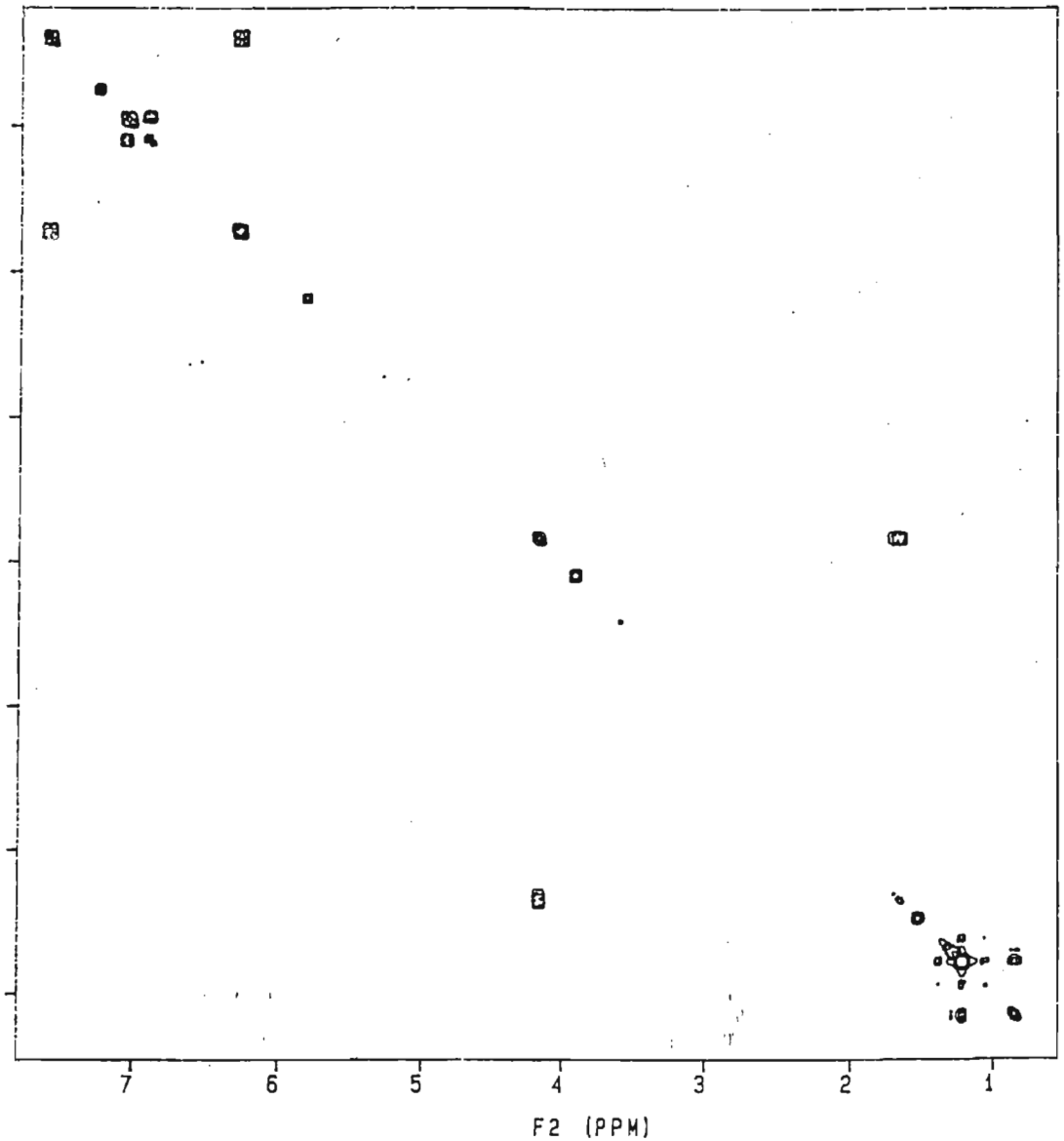


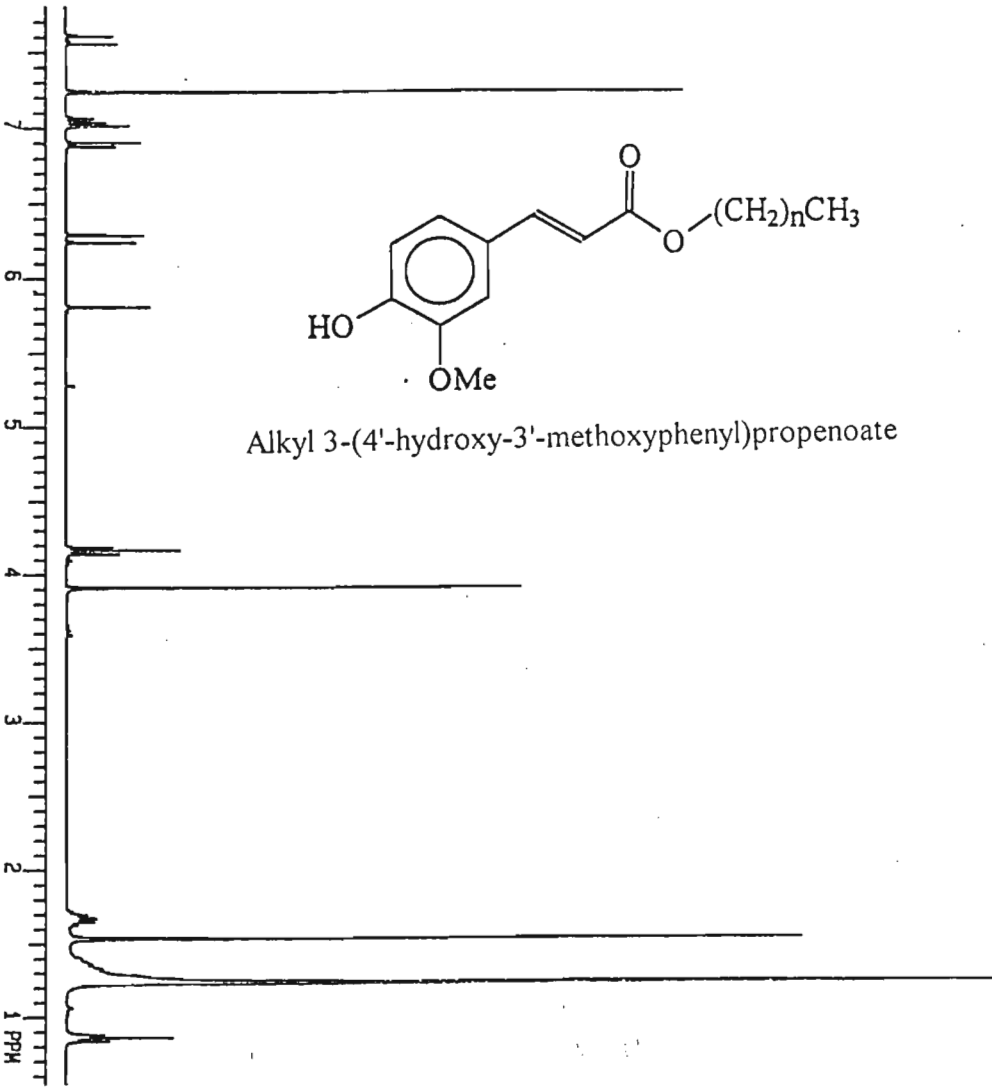
Spectrum : ^{1}H n.m.r. spectrum of natalensine acetate



Alkyl 3-(4'-hydroxy-3'-methoxyphenyl)propenoate

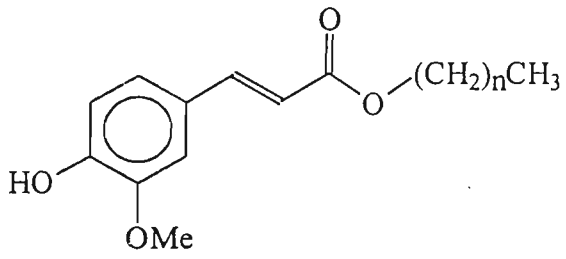
Spectrum : ^{1}H n.m.r. spectrum of compound 3 in $CDCl_3$





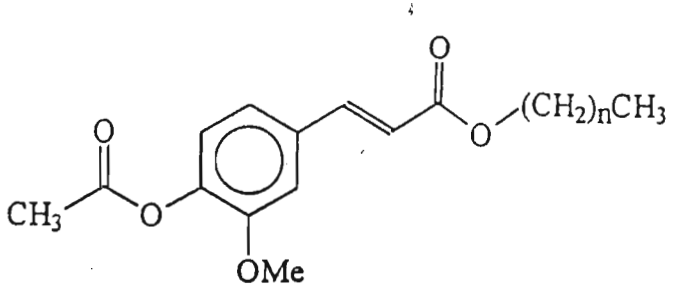
Alkyl 3-(4'-hydroxy-3'-methoxyphenyl)propenoate

Spectrum : COSY spectrum of Compound 3



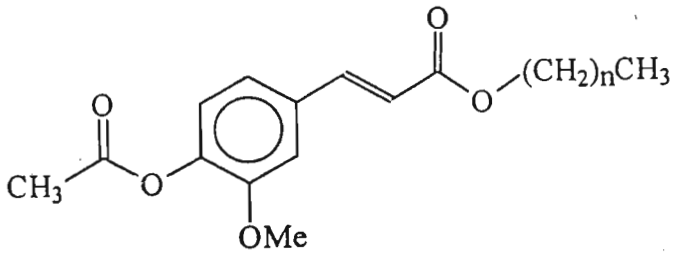
Alkyl 3-(4'-hydroxy-3'-methoxyphenyl)propenoate

Spectrum : ^{13}C n.m.r. spectrum of compound 3 in $CDCl_3$



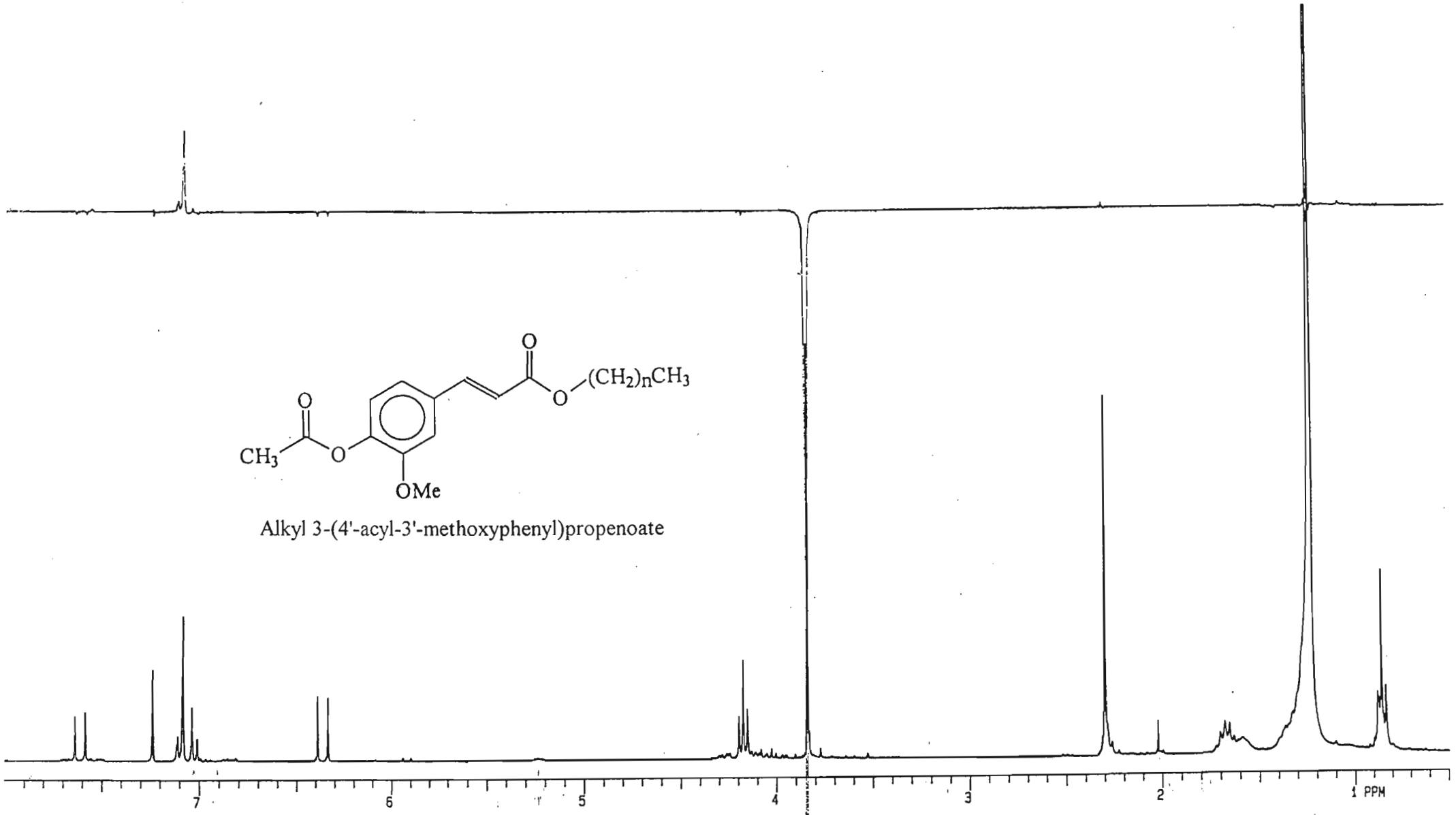
Alkyl 3-(4'-acyl-3'-methoxyphenyl)propenoate

Spectrum : ^{1}H n.m.r. spectrum of Compound 3A



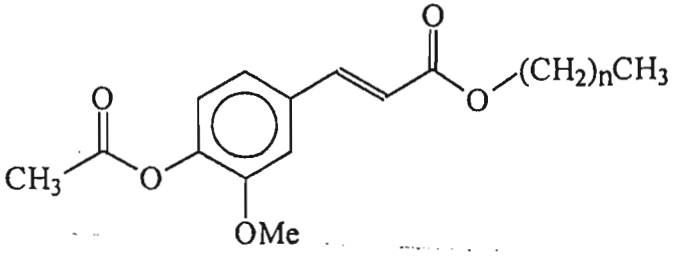
Alkyl 3-(4'-acyl-3'-methoxyphenyl)propenoate

ɔectrum : NOE spectrum resulting from the irradiation of the protons resonating at δ 2.30 (compound 3A)



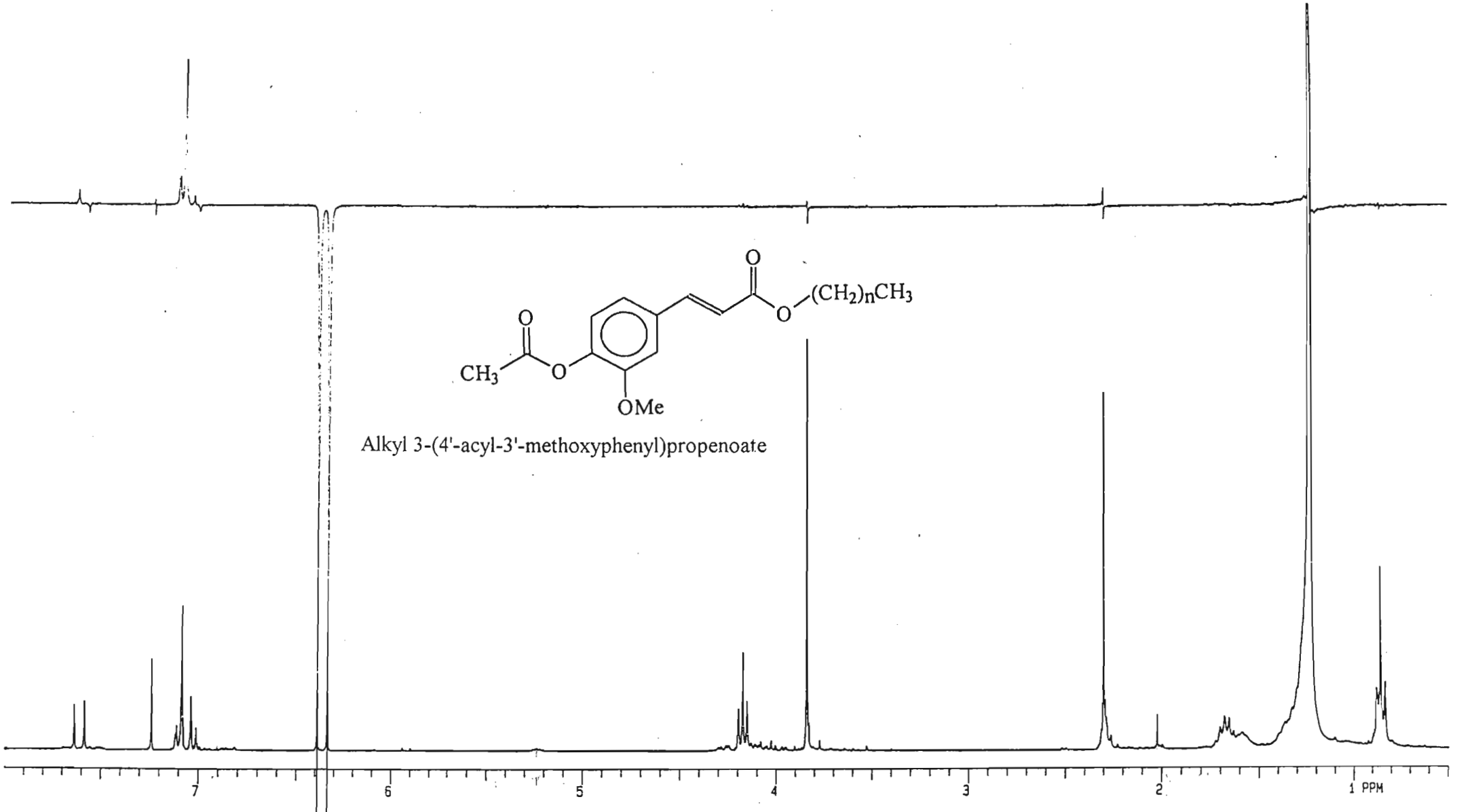
Alkyl 3-(4'-acyl-3'-methoxyphenyl)propenoate

Spectrum : NOE spectrum resulting from the irradiation of the protons resonating at δ 3.84 (compound 3A)



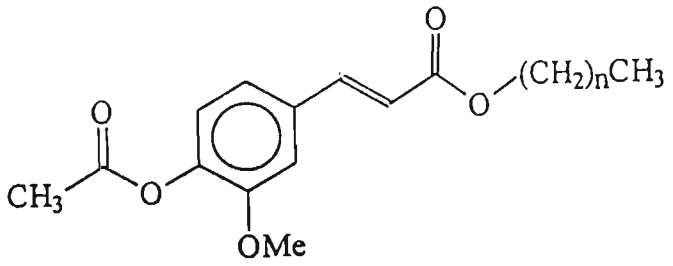
Alkyl 3-(4'-acyl-3'-methoxyphenyl)propenoate

Spectrum : NOE spectrum resulting from the irradiation of the proton resonating at δ 7.59 (compound 3A)



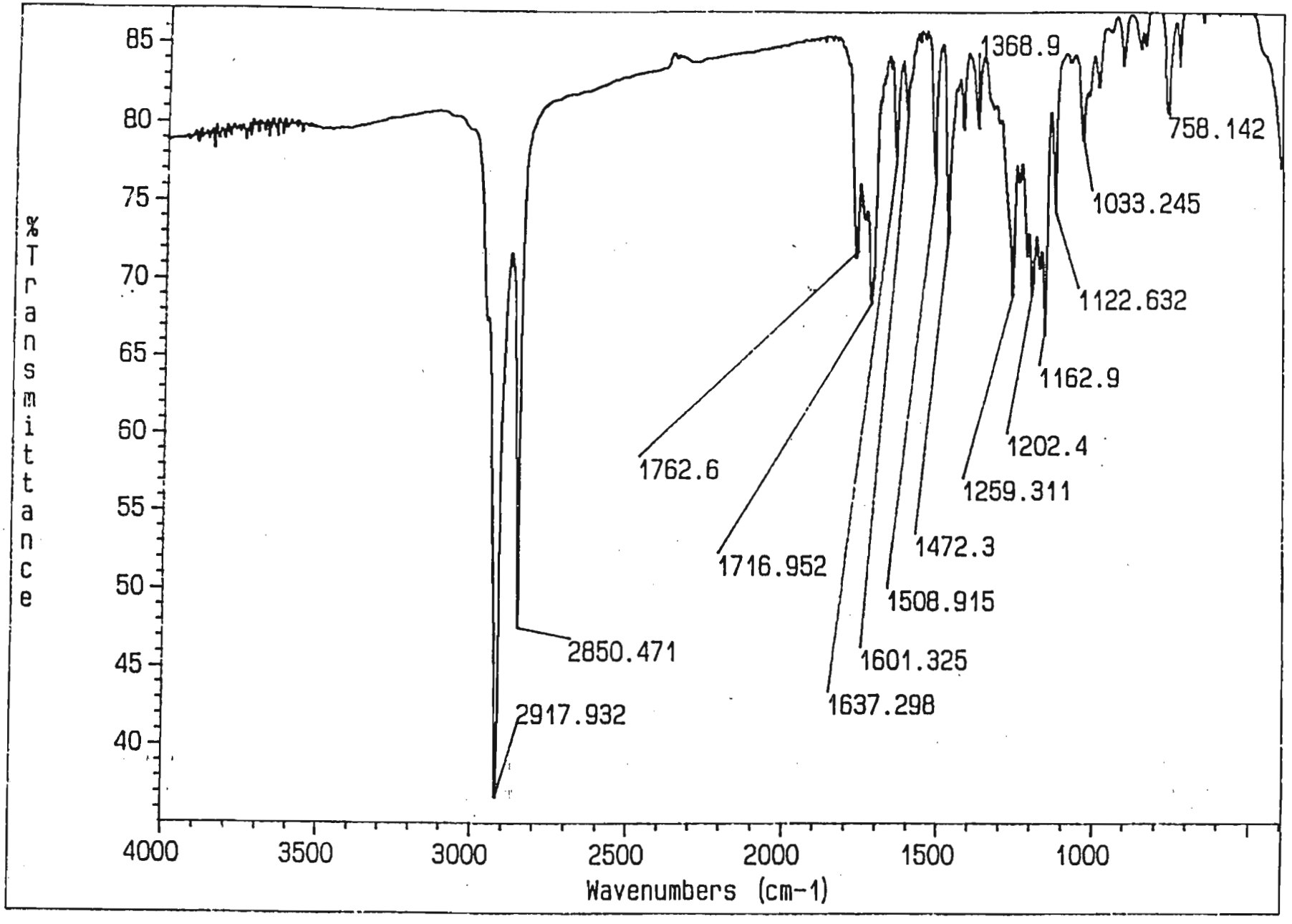
Alkyl 3-(4'-acyl-3'-methoxyphenyl)propenoate

Spectrum : NOE spectrum resulting from the irradiation of the proton resonating at δ 6.26 (compound 3A)

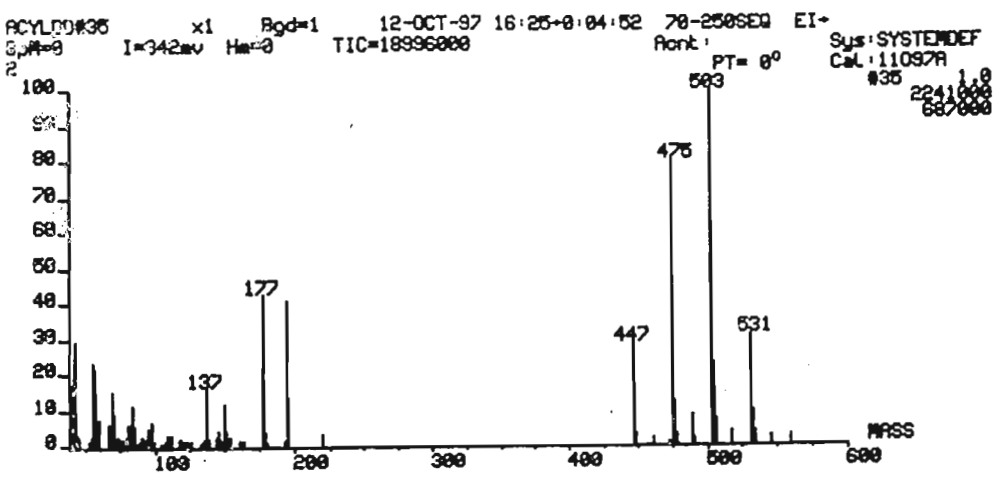


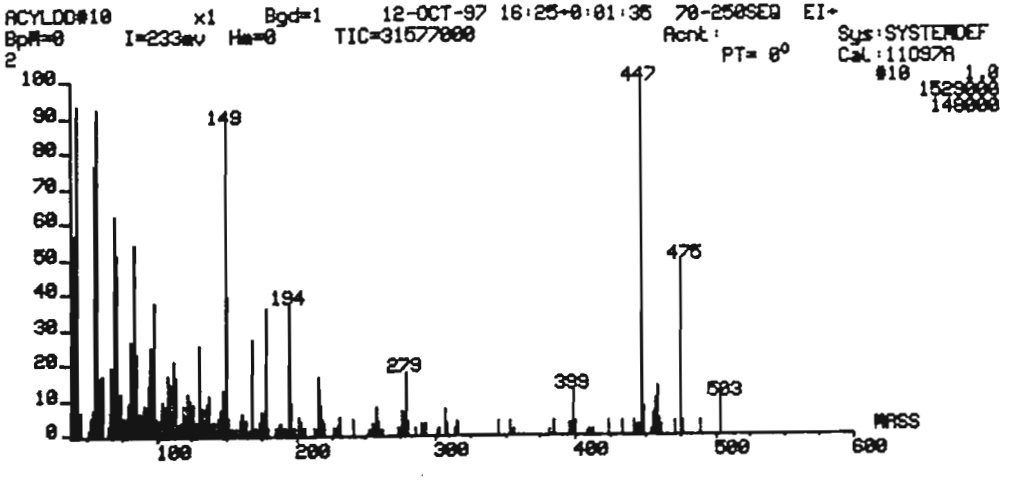
Alkyl 3-(4'-acyl-3'-methoxyphenyl)propenoate

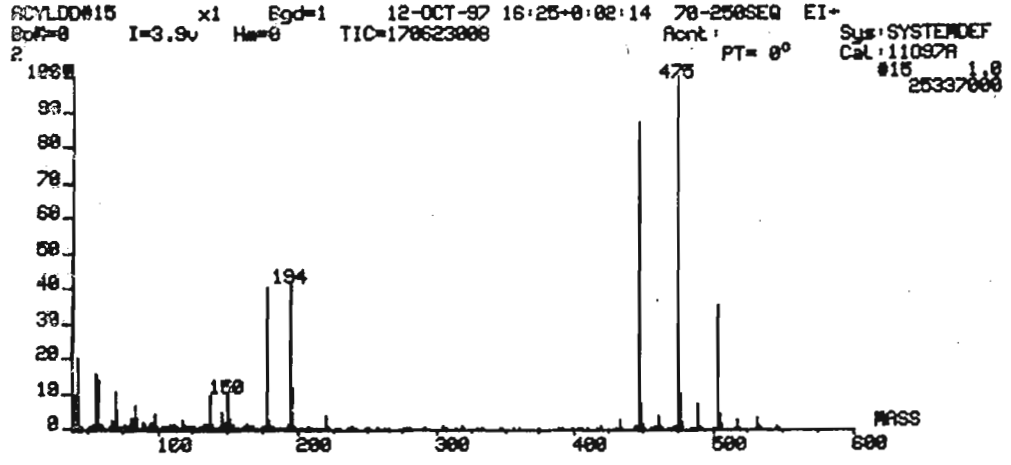
Spectrum : ^{13}C spectrum of Compound 3A

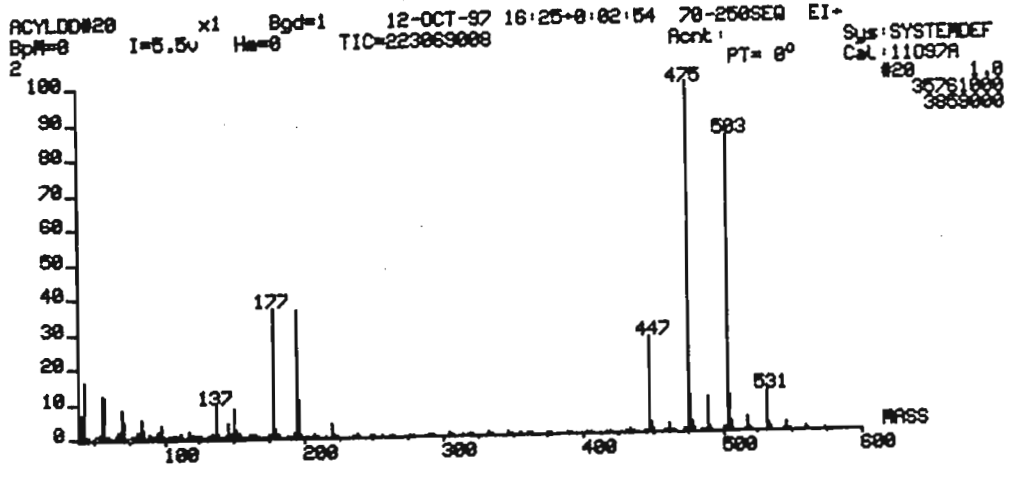


Spectrum : Infrared spectrum of compound 3A (alkyl 3-(4'-acyl-3'-methoxyphenyl)propenoate)

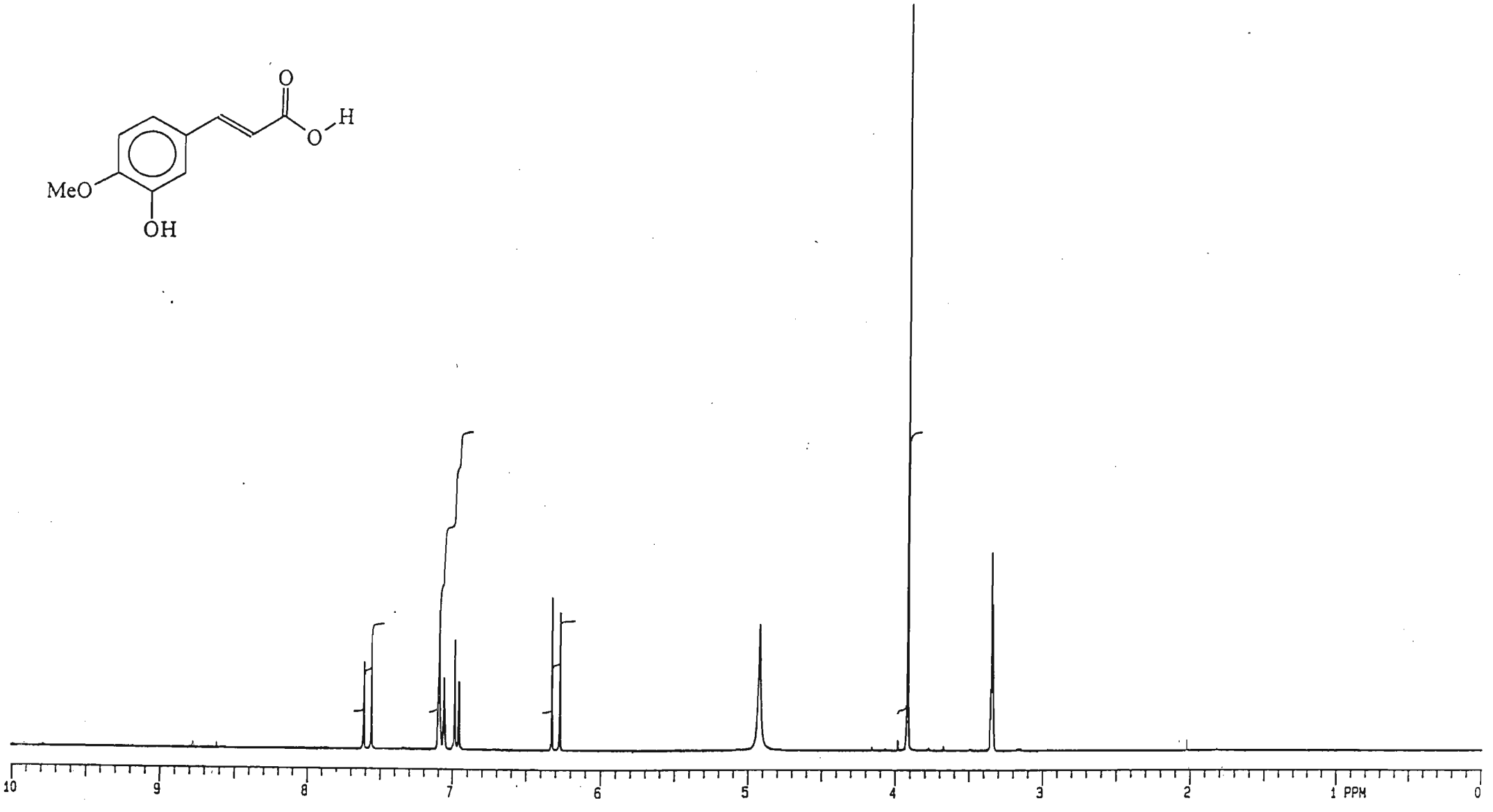




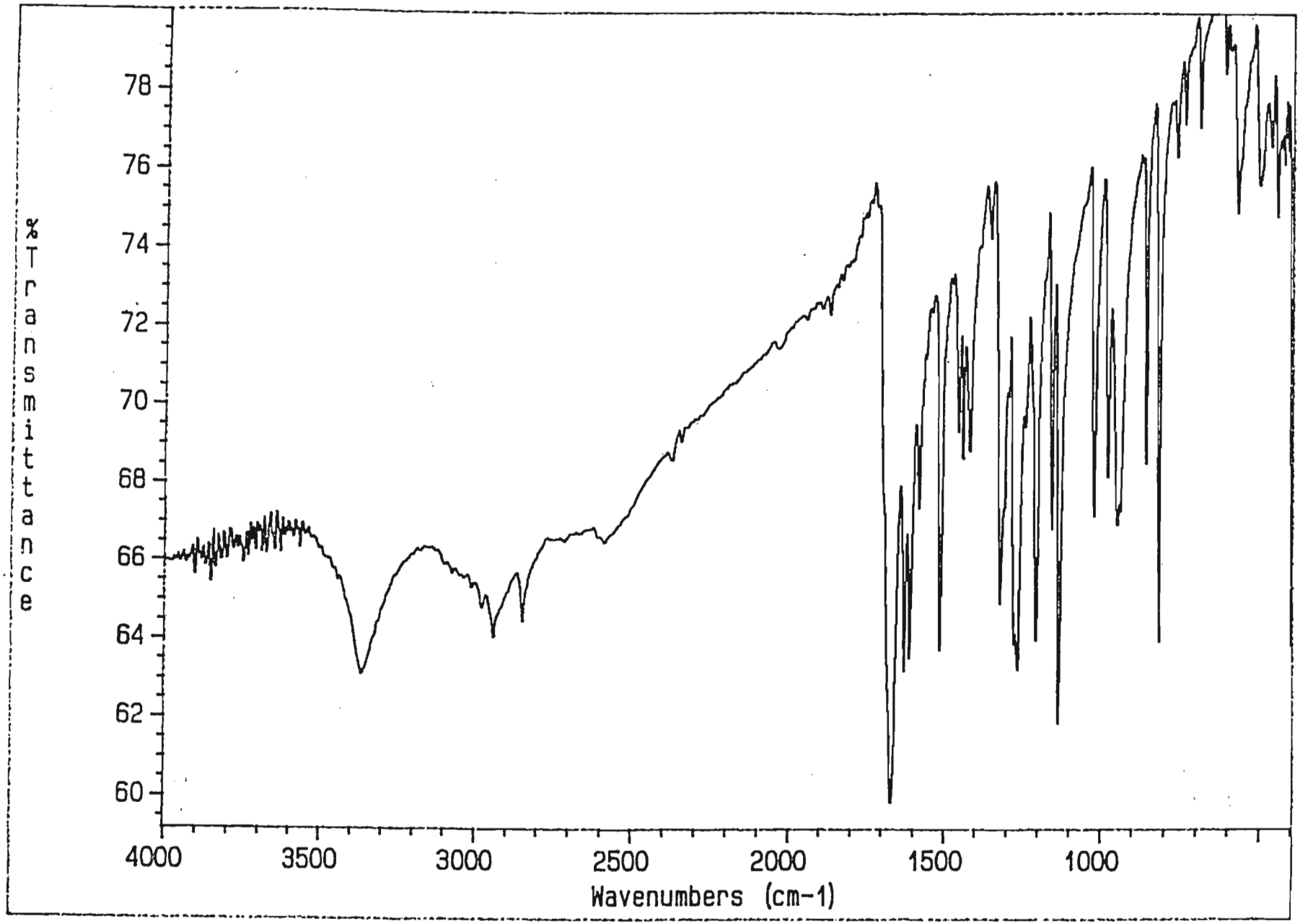




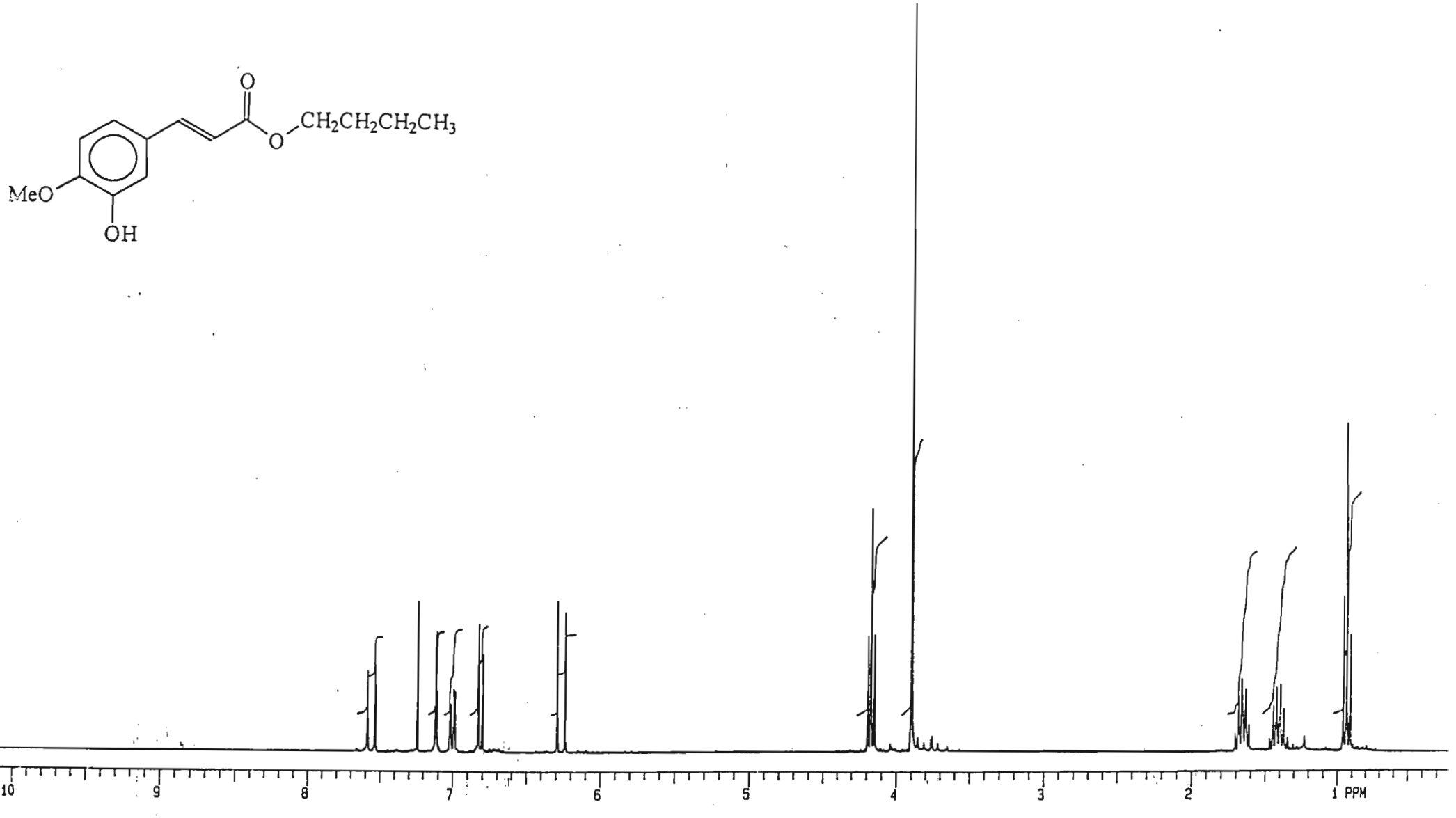
Spectrum : Mass spectrum of compound 3A (alkyl 3-(4'-acyl-3'-methoxyphenyl)propenoate)



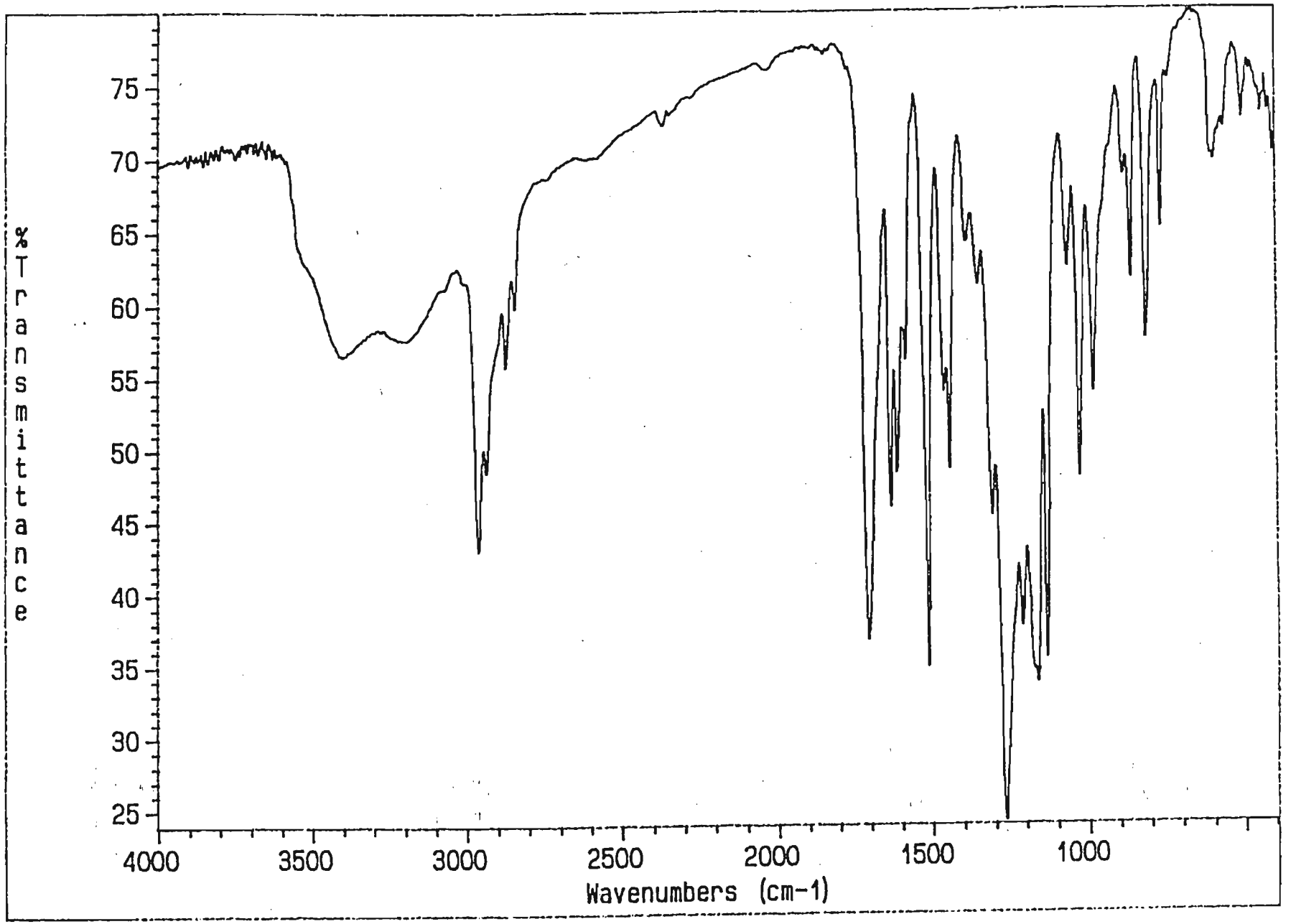
Spectrum : ^{1}H n.m.r. spectrum of 3-(3'-hydroxy-4'-methoxyphenyl)propenoic acid



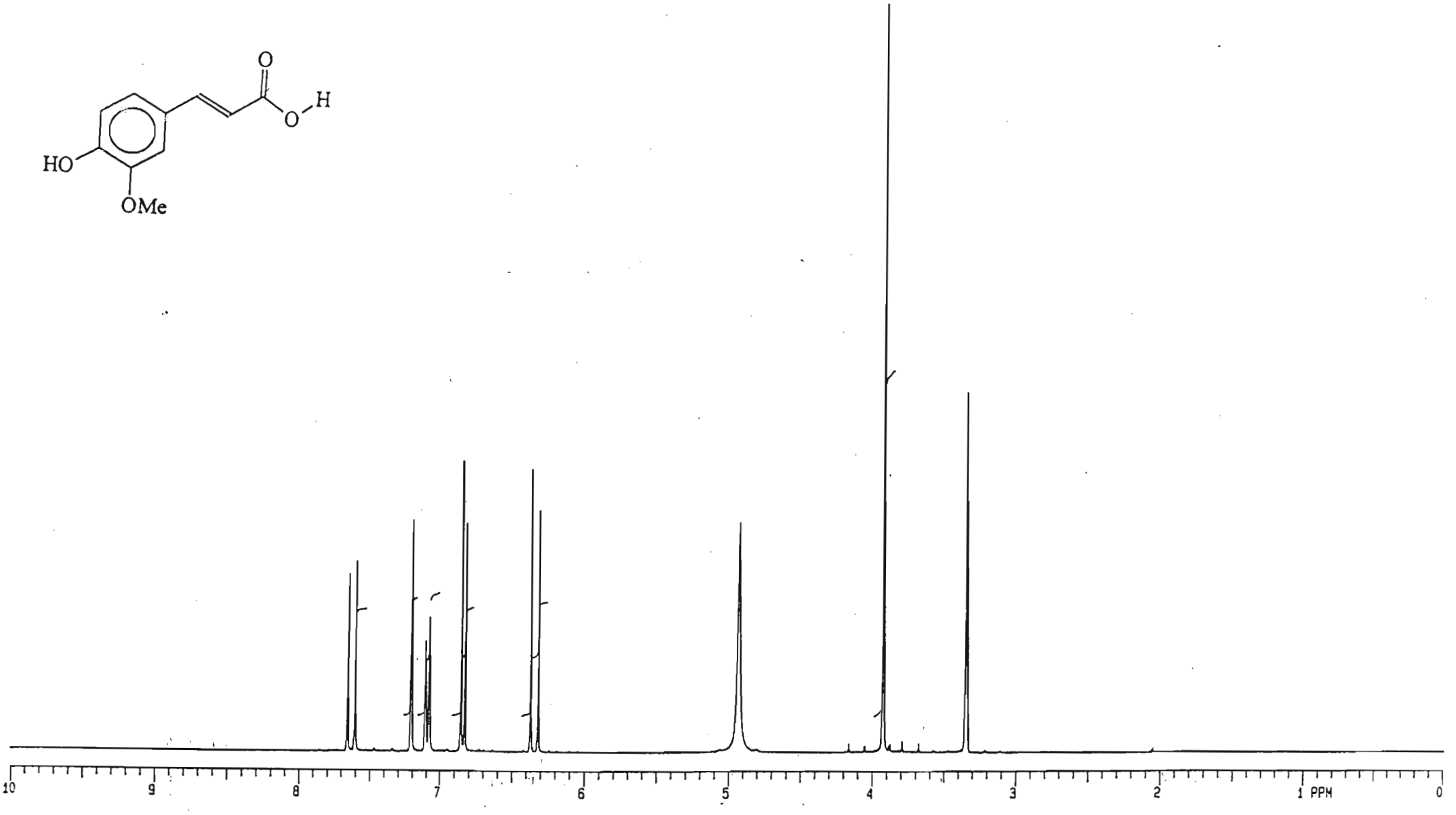
Spectrum : Infrared spectrum of 3-(3'-hydroxy-4'-methoxyphenyl)propenoic acid



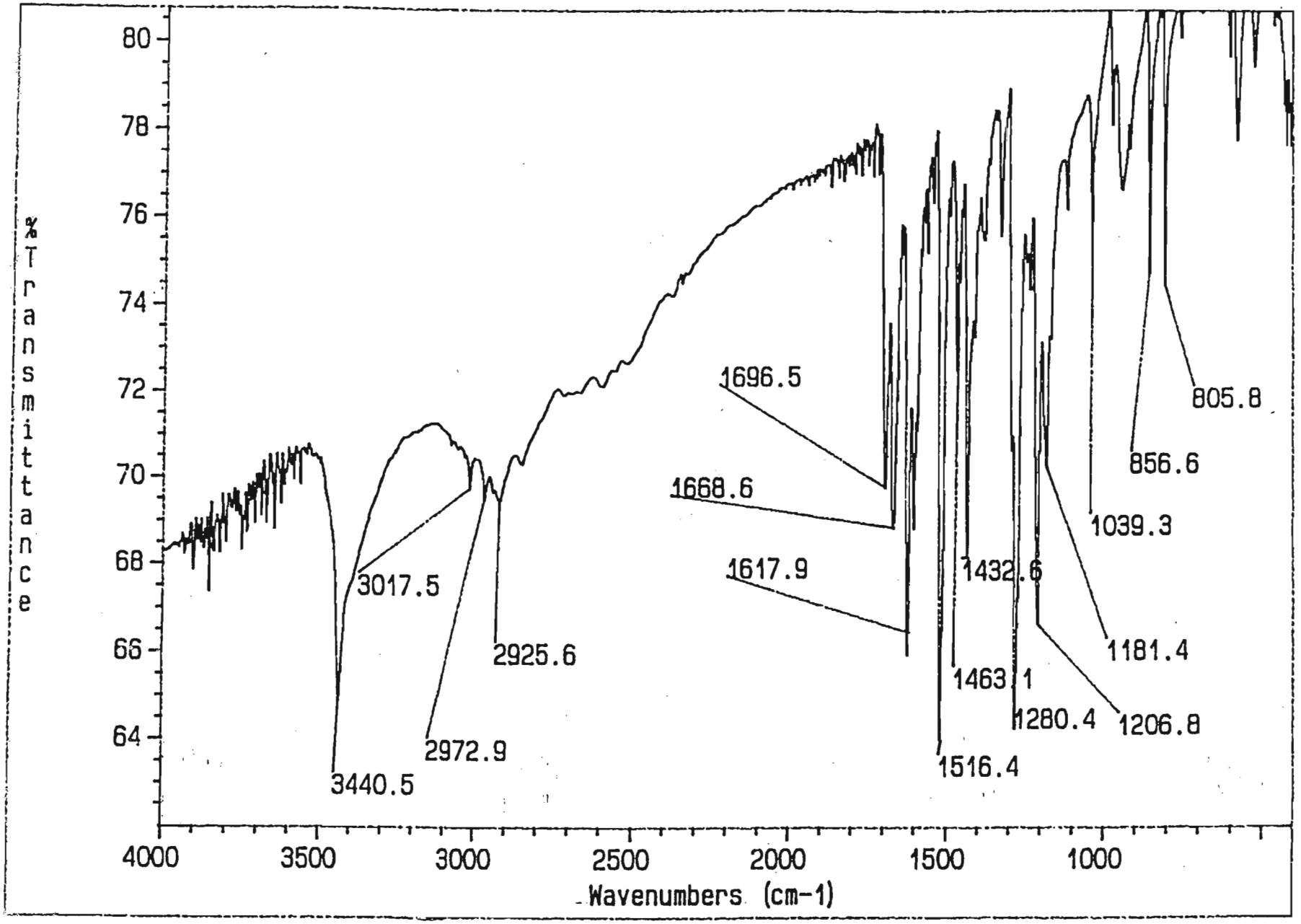
Spectrum : ^{1}H n.m.r. spectrum of butyl 3-(3'-hydroxy-4'-methoxyphenyl)propenoate



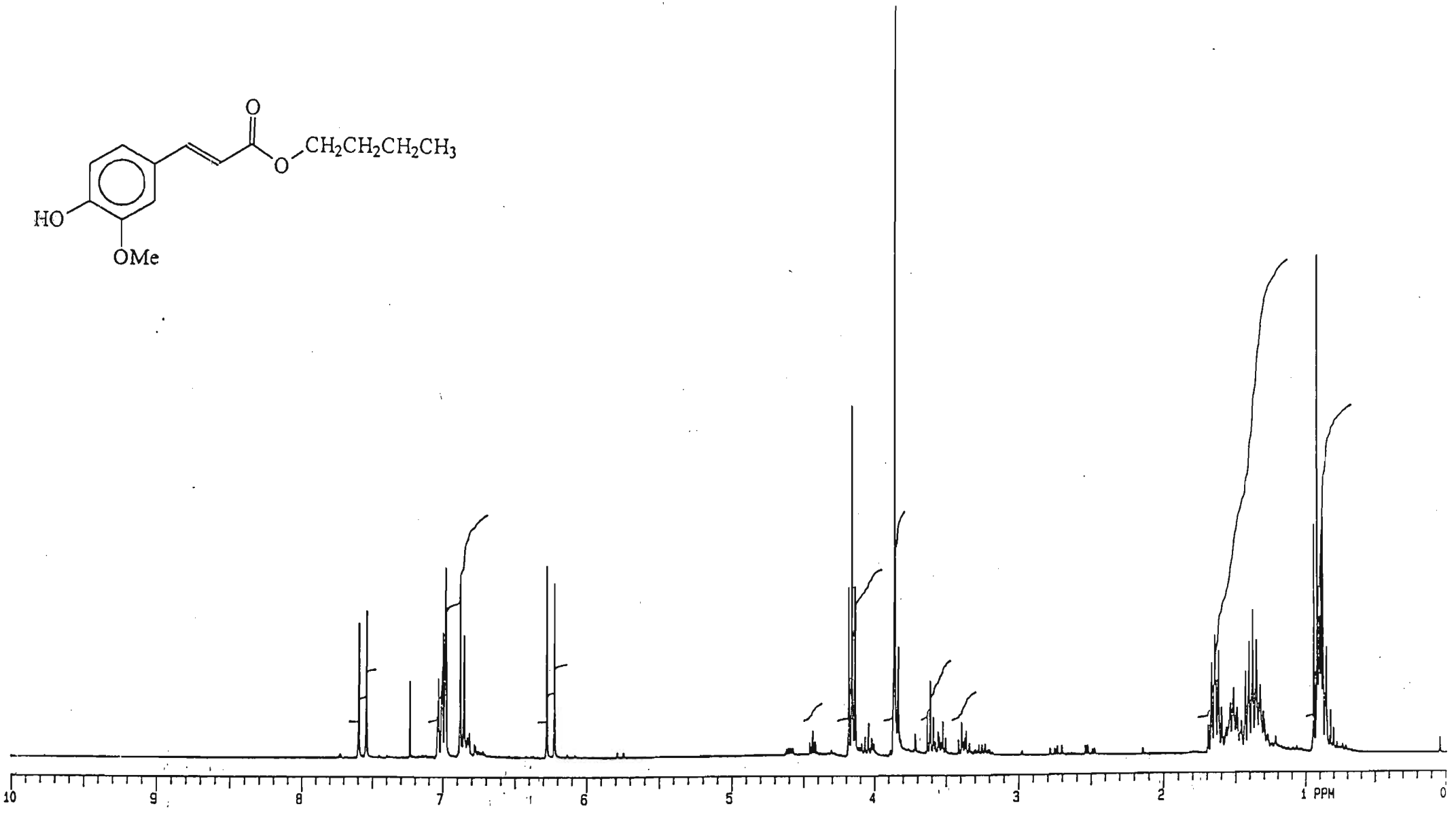
Spectrum : Infrared spectrum of butyl 3-(3'-hydroxy-4'-methoxyphenyl)propenoate



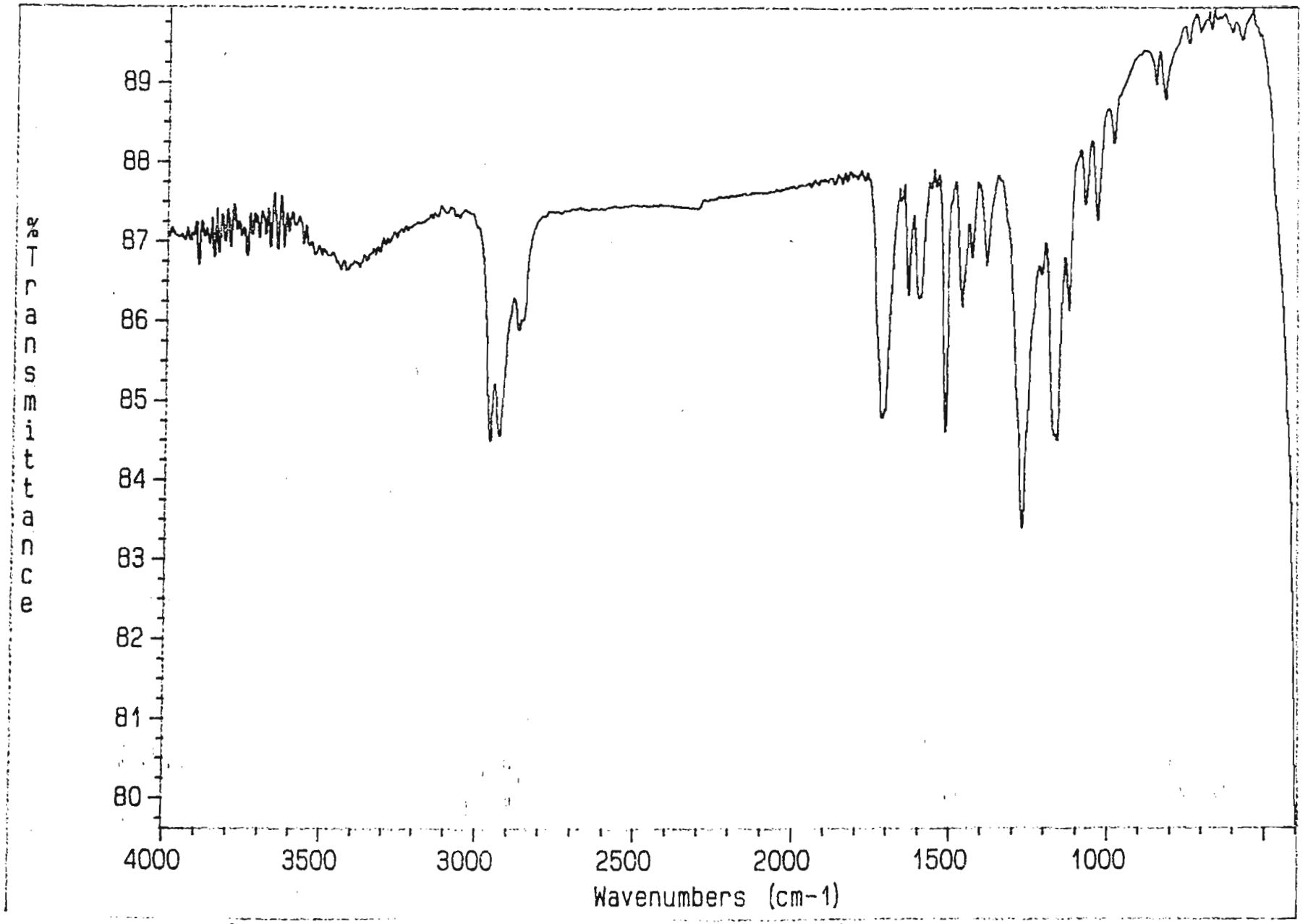
Spectrum : ^{1}H n.m.r. spectrum of 3-(4'-hydroxy-3'-methoxyphenyl)propenoic acid



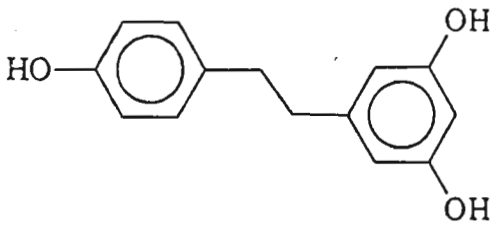
Spectrum : Infrared spectrum of 3-(4'-hydroxy-3'-methoxyphenyl)propenoic acid



Spectrum : ^{1}H n.m.r. spectrum of butyl 3-(4'-hydroxy-3'-methoxyphenyl)propenoate

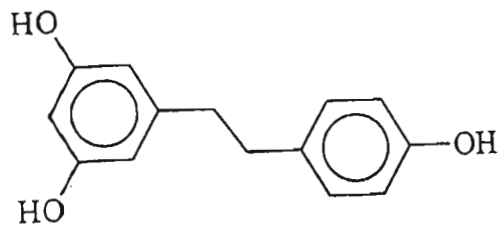


Spectrum : Infrared spectrum of butyl 3-(3'-hydroxy-4'-methoxyphenyl)propenoate



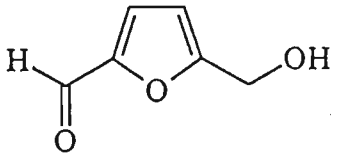
3,4',5-Trihydroxybibenzyl

Spectrum : ^{1}H n.m.r. spectrum of Compound 4



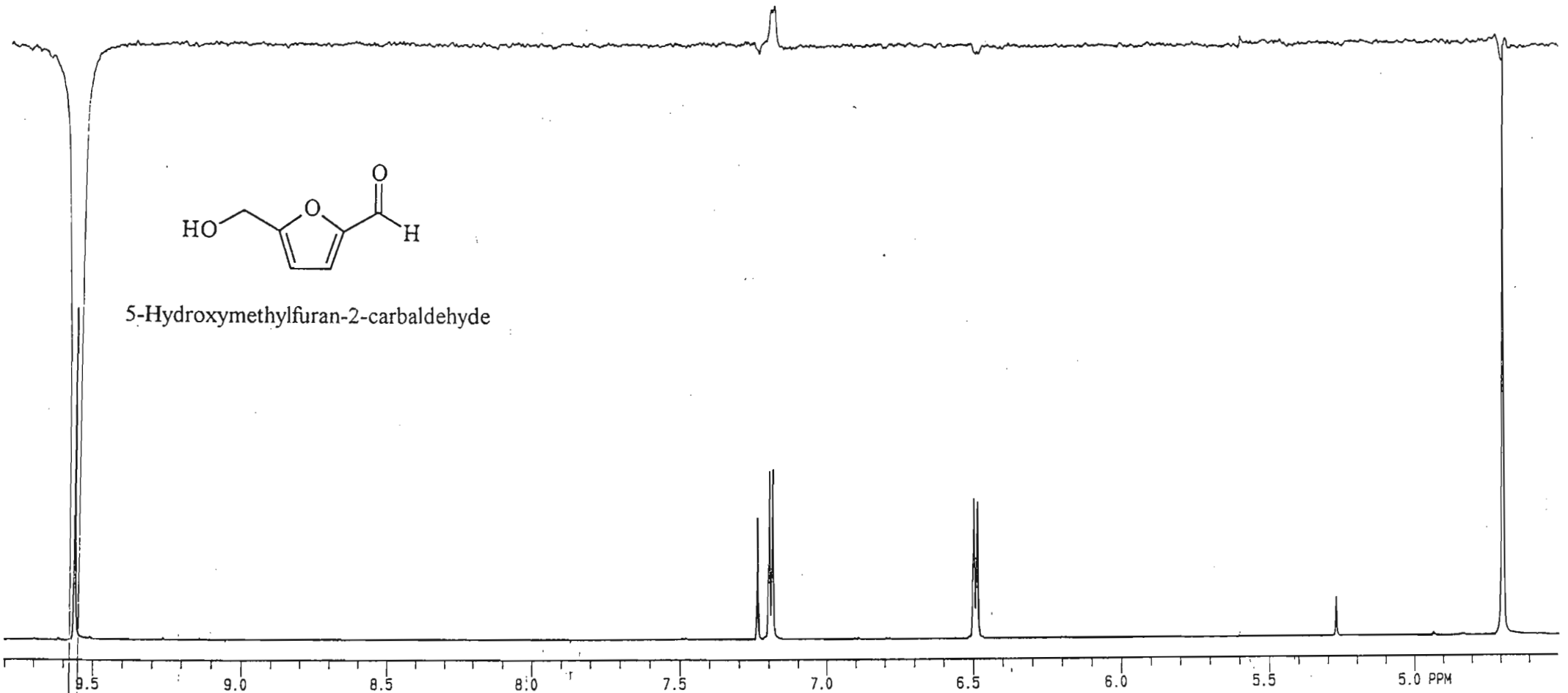
3,4',5-Trihydroxybibenzyl

Spectrum : ^{13}C n.m.r. spectrum of compound 4 in CD_3OD



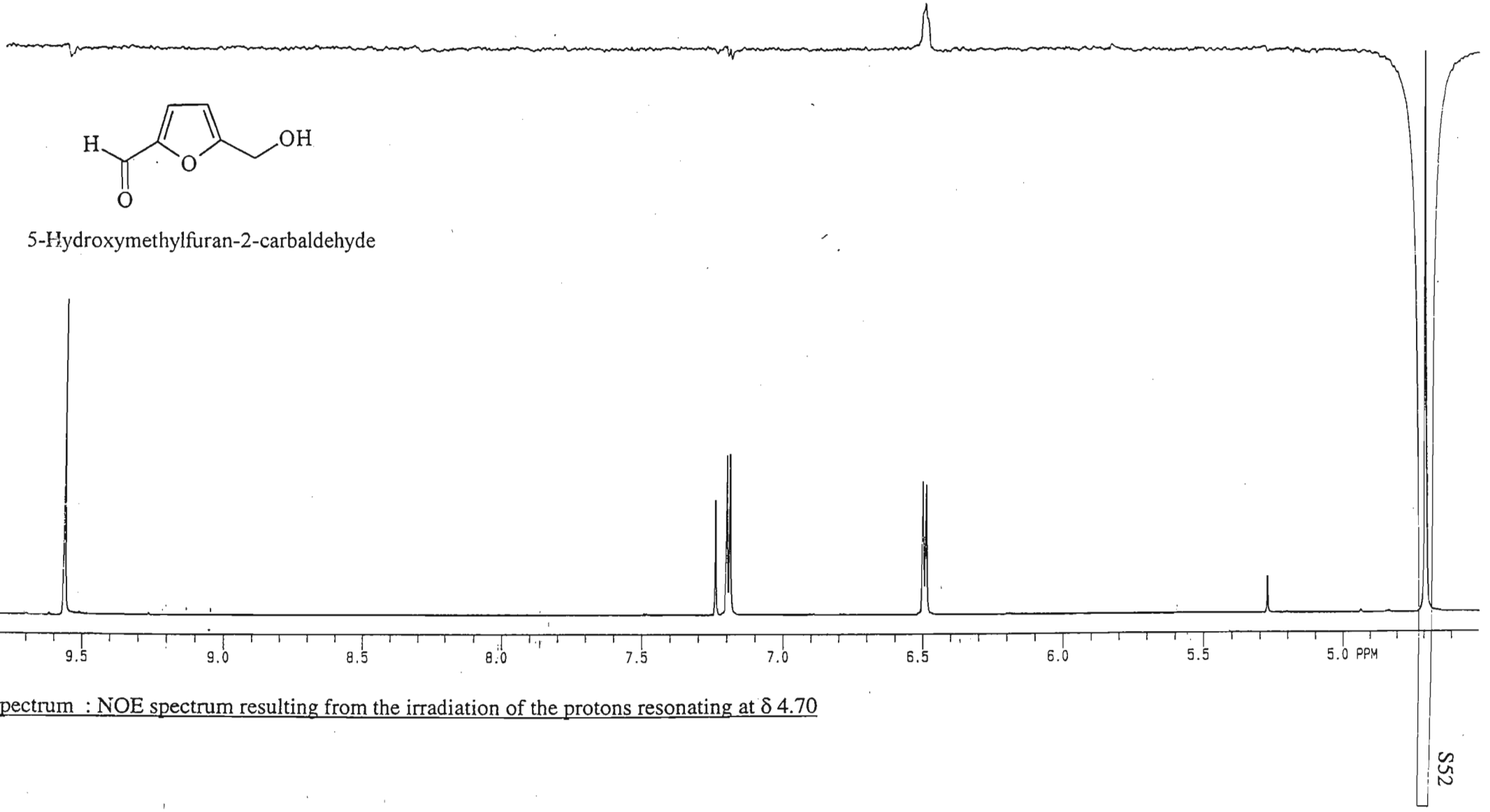
5-Hydroxymethylfuran-2-carbaldehyde

Spectrum : ^{1}H n.m.r spectrum of compound 5 in $CDCl_3$



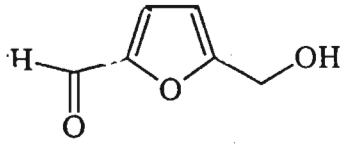
5-Hydroxymethylfuran-2-carbaldehyde

Spectrum : NOE spectrum resulting from the irradiation of the proton resonating at δ 9.55



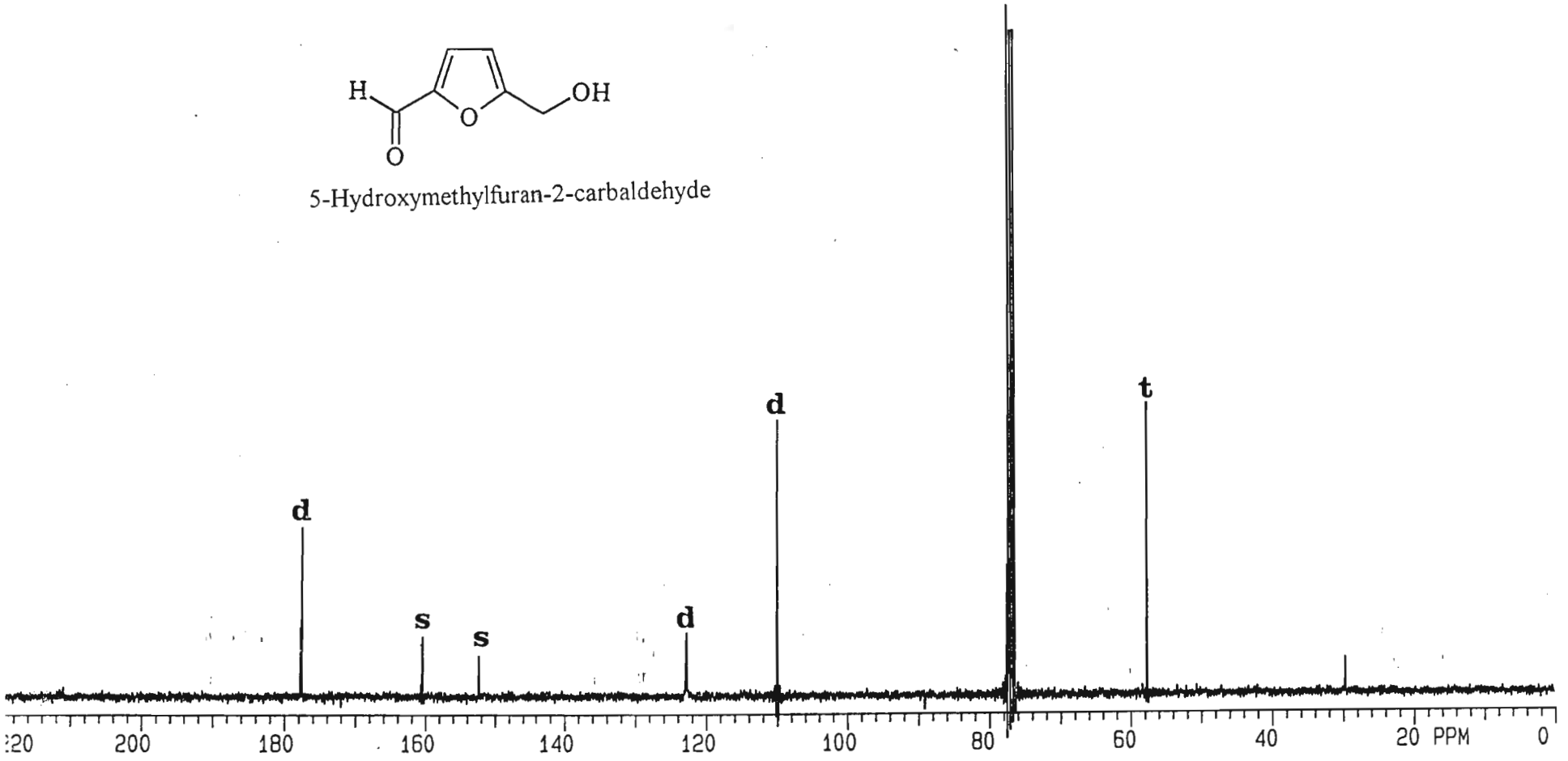
5-Hydroxymethylfuran-2-carbaldehyde

Spectrum : NOE spectrum resulting from the irradiation of the protons resonating at δ 4.70

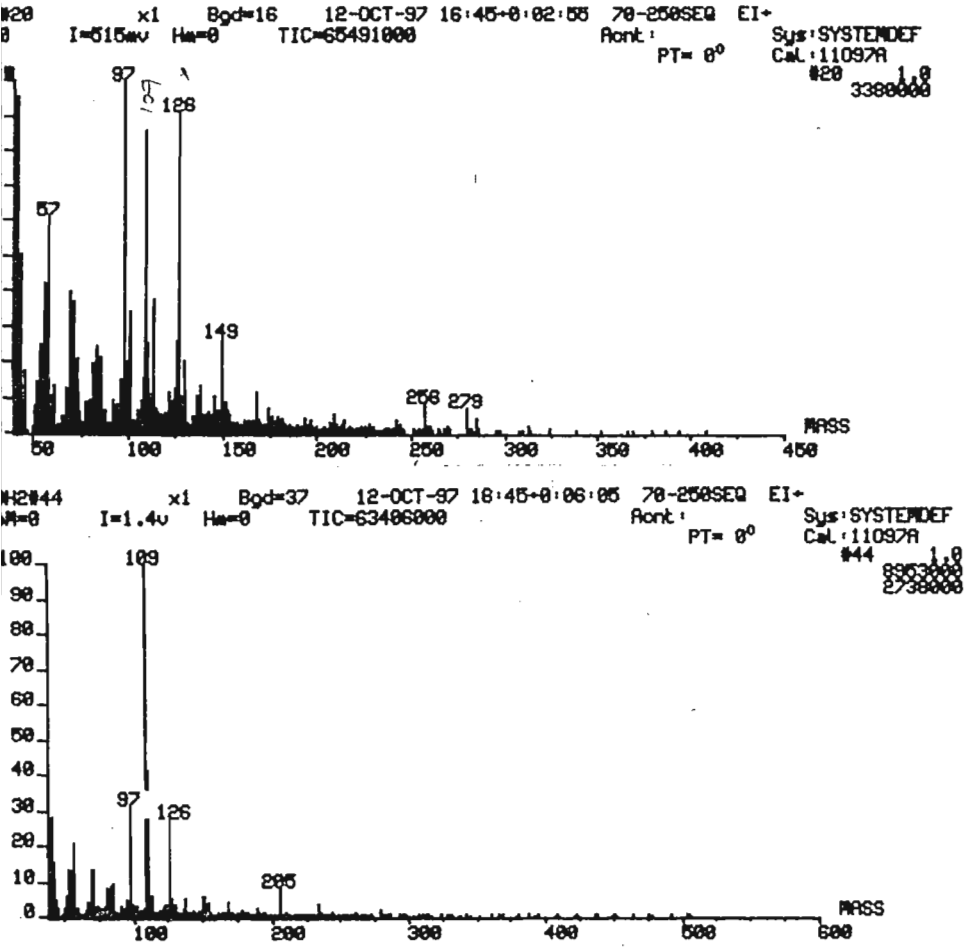


5- Hydroxymethylfuran-2-carbaldehyde

Spectrum : NOE spectrum resulting from the irradiation of the proton resonating at δ 6.50



pectrum : ^{13}C n.m.r. spectrum of compound 5 in $CDCl_3$



Spectrum: Mass spectrum of compound 5

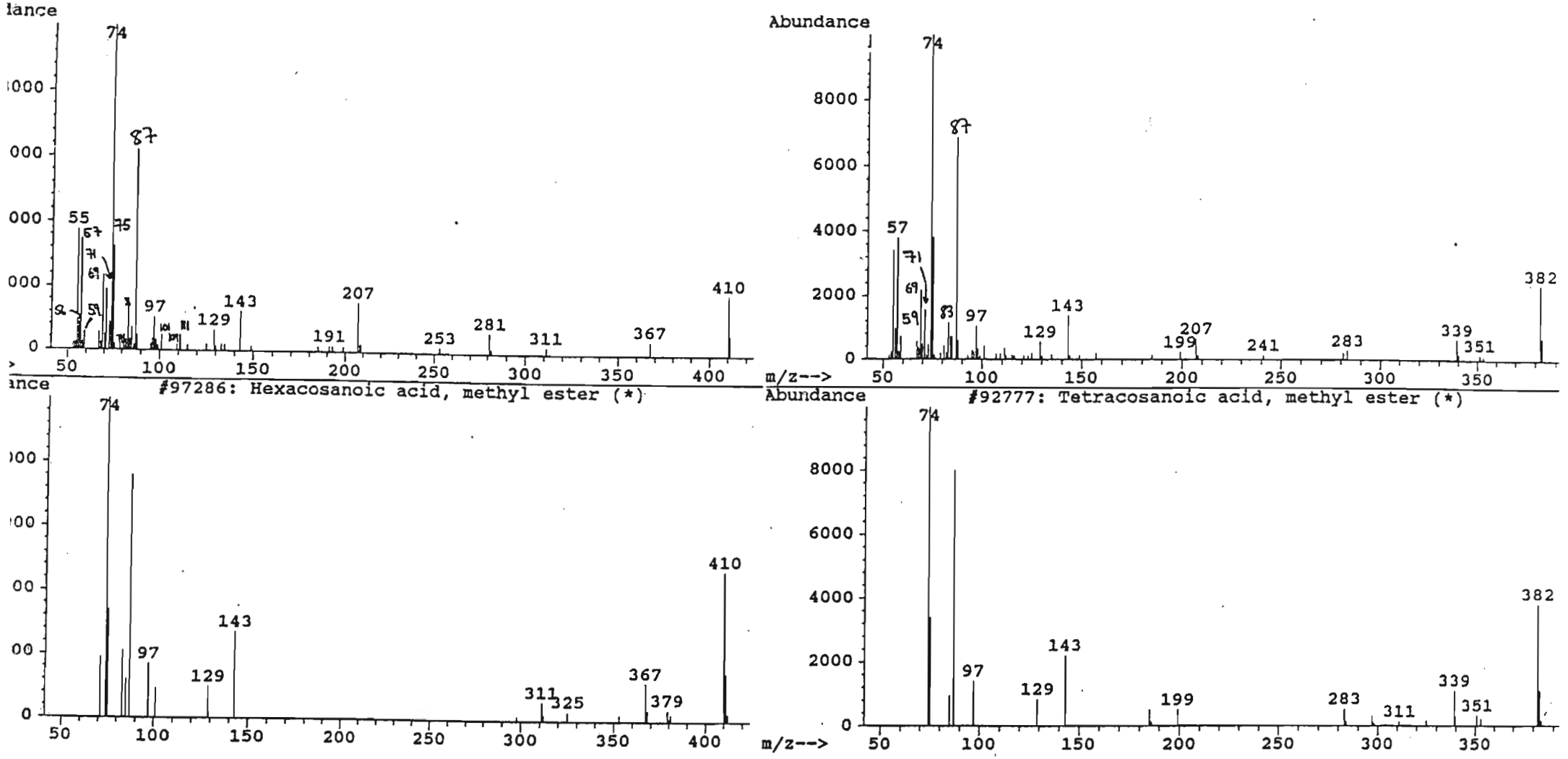


Figure S1 GC-MS spectra of methyl hexadecanoate and methyl tetracosanoate from *Avonia rhodesica* with the comparative library spectra

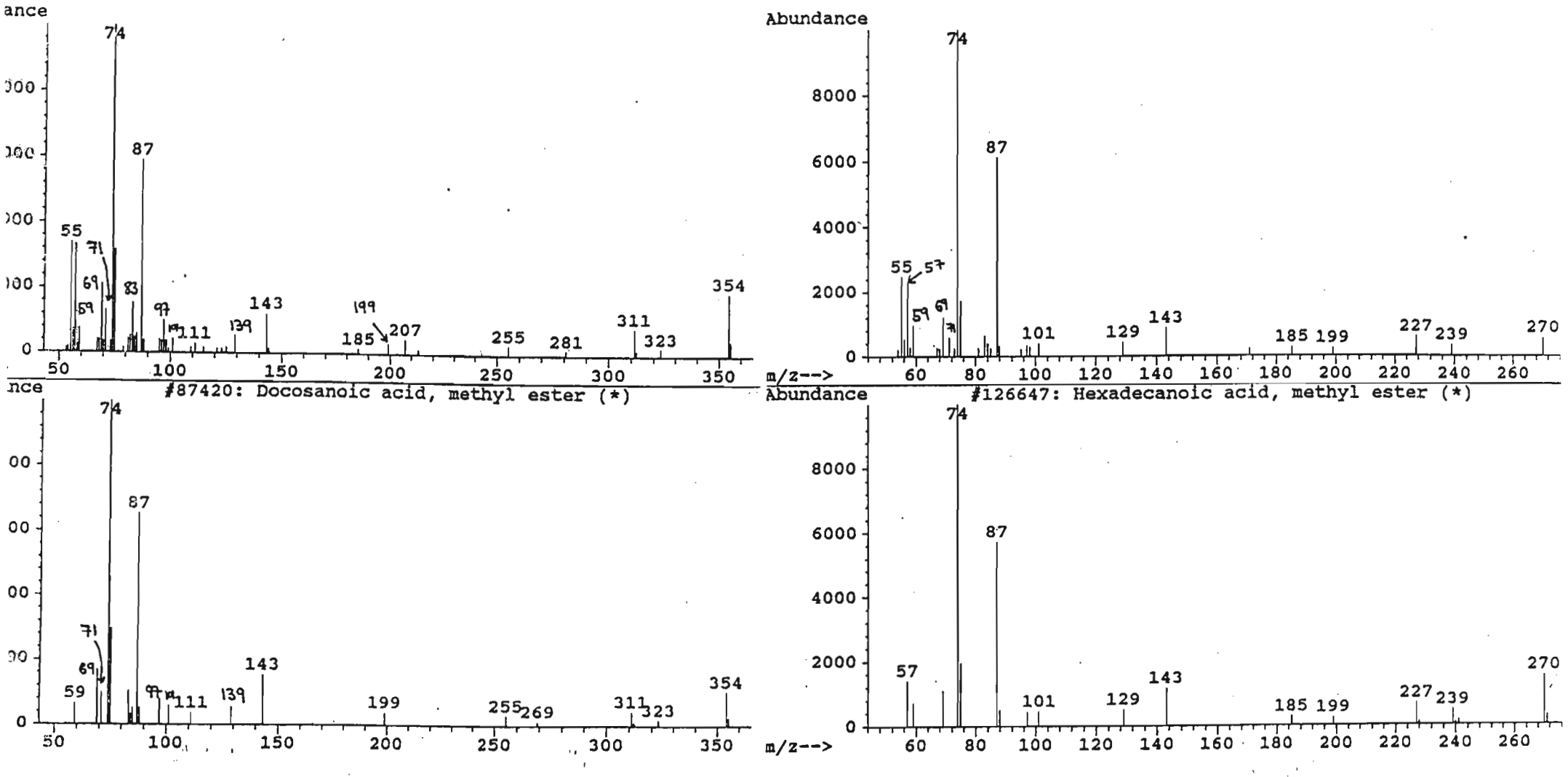


Figure S2 GC-MS spectra of methyl docosanoate and methyl hexadecanoate from *Avonia rhodesica* with the comparative library spectra

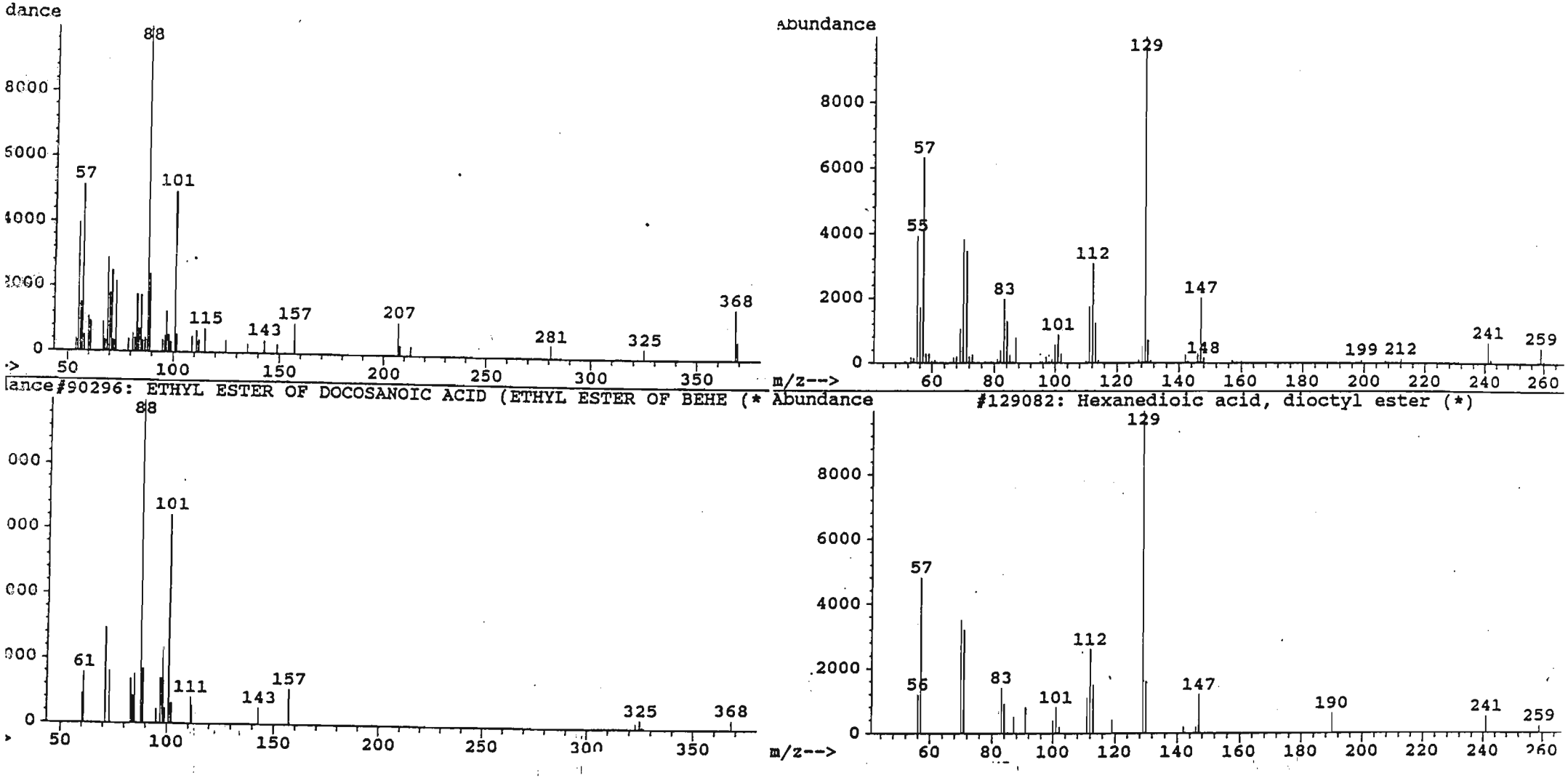


Figure S3 GC-MS spectra of ethyl docosanoate and dioctyl hexadecanoate from *Avonia rhodesica* with the comparative library spectra

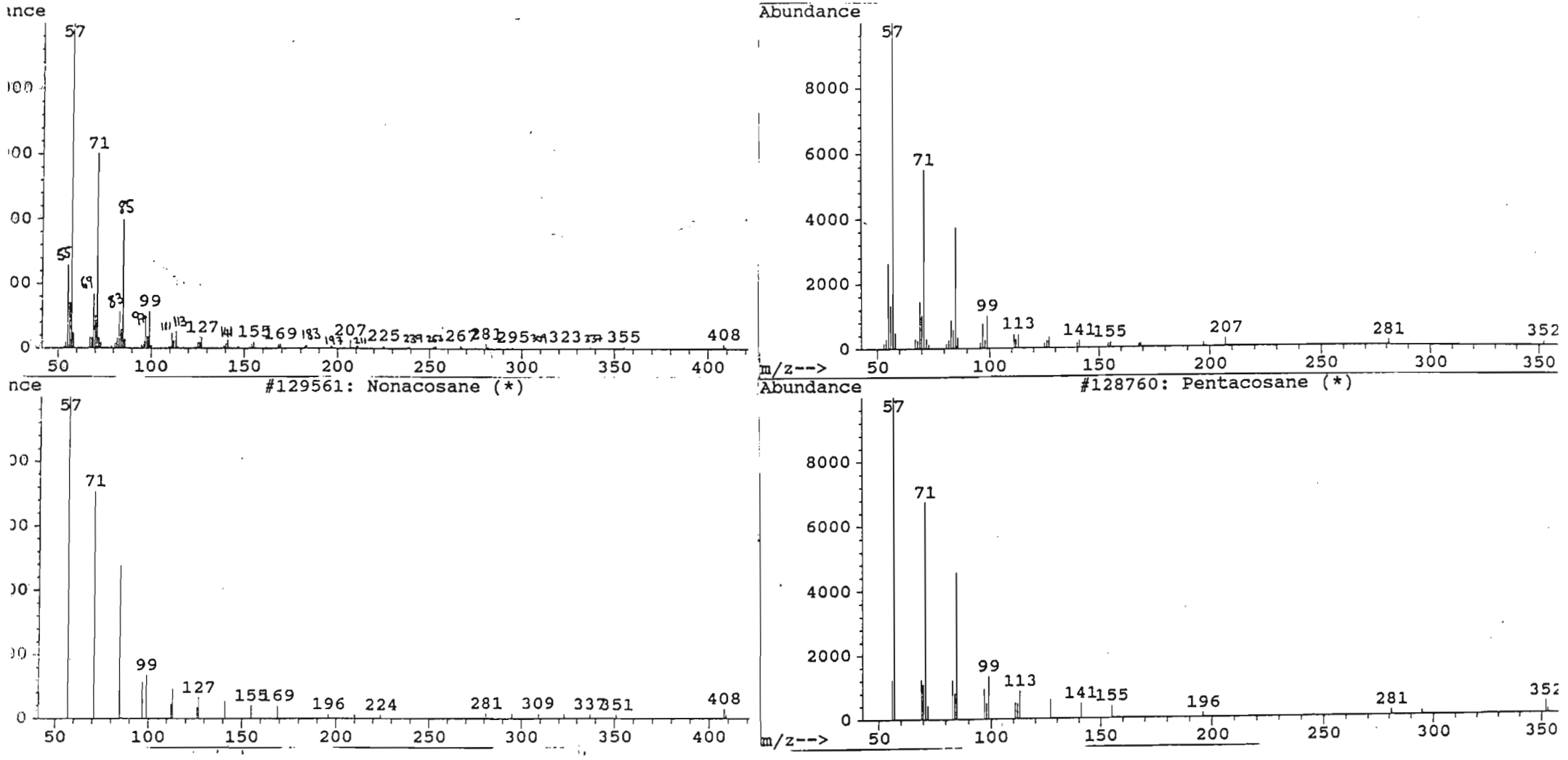


Figure S4 GC-MS spectra of nonacosane and pentacosane from *Avonia rhodesica* with the comparative library spectra

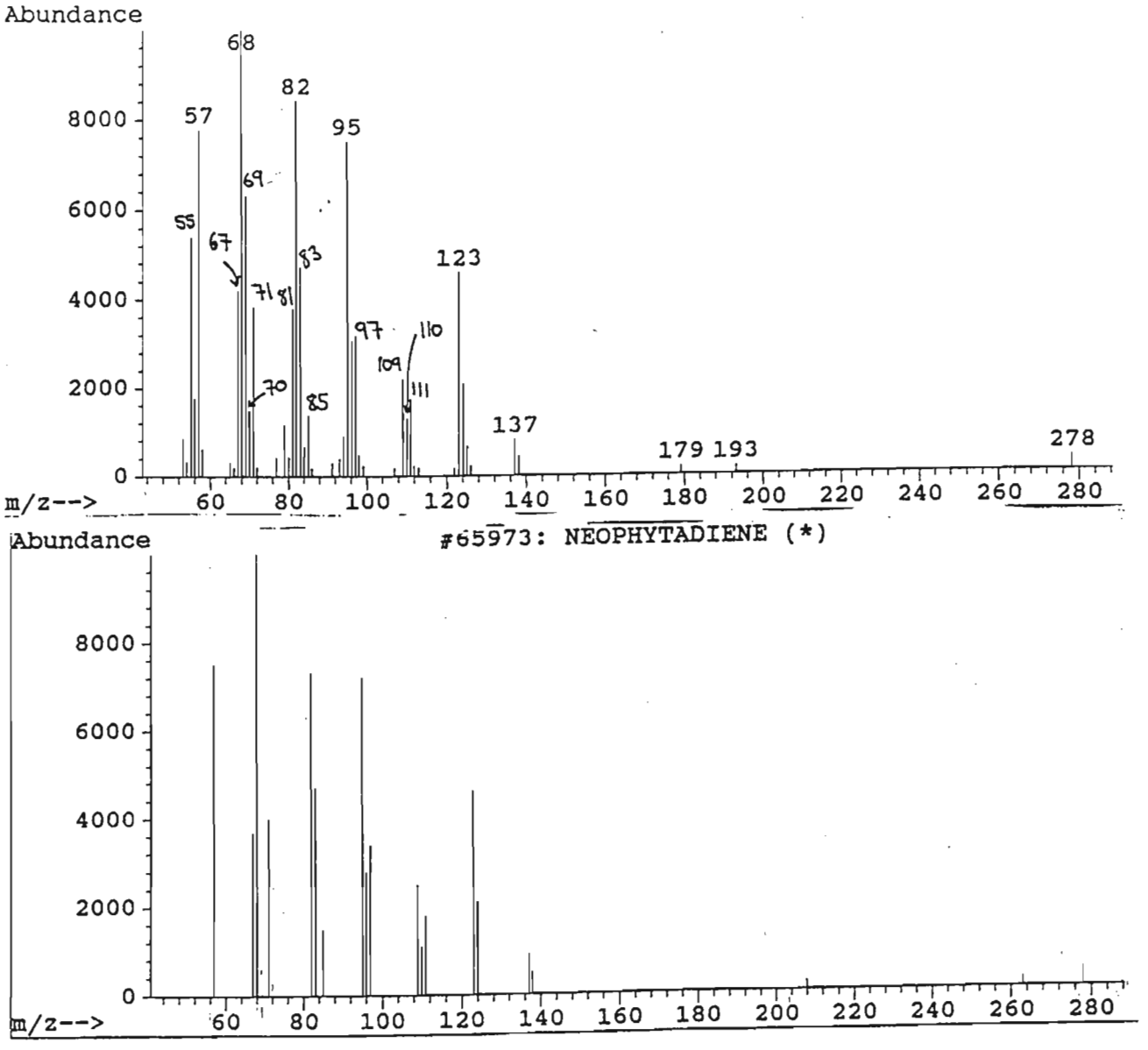
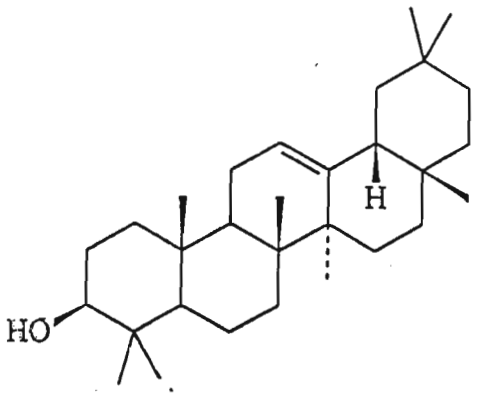
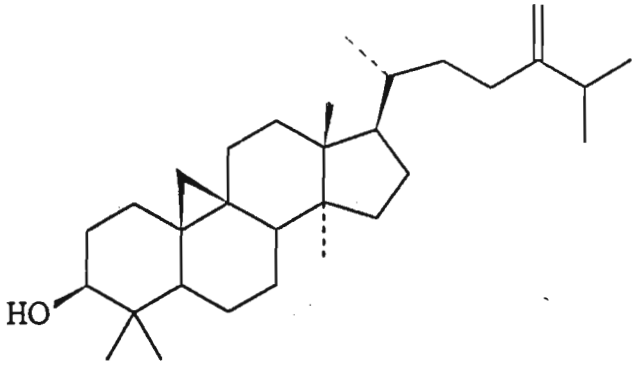


Figure S5 GC-MS spectrum of neophytidiene from *Avonia rhodesica* with the comparative library spectrum

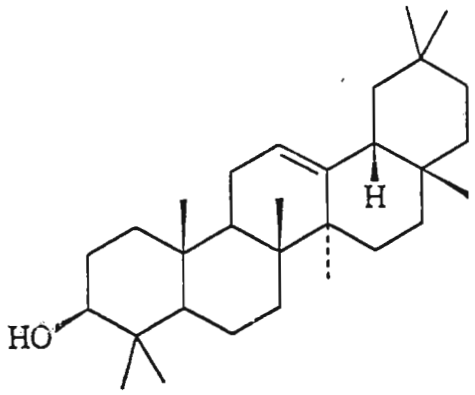


β-Amyrin

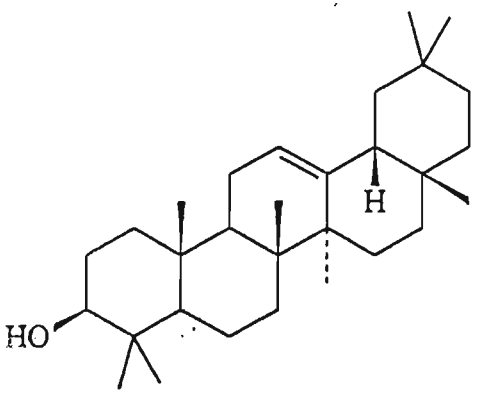


24-Methylenecycloartenol

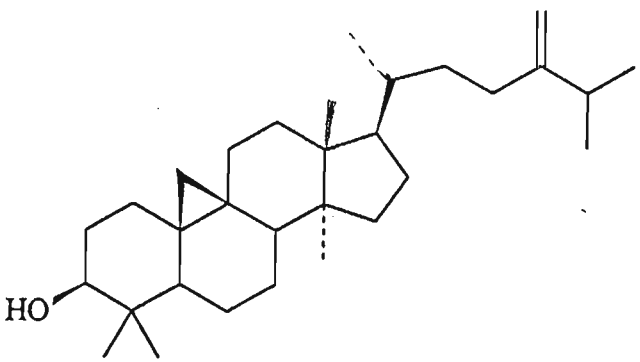
Spectrum : ^{1}H n.m.r. spectrum of the mixture of Compound 6 and Compound 7



Spectrum : ^{1}H n.m.r. spectrum of β-amyrin

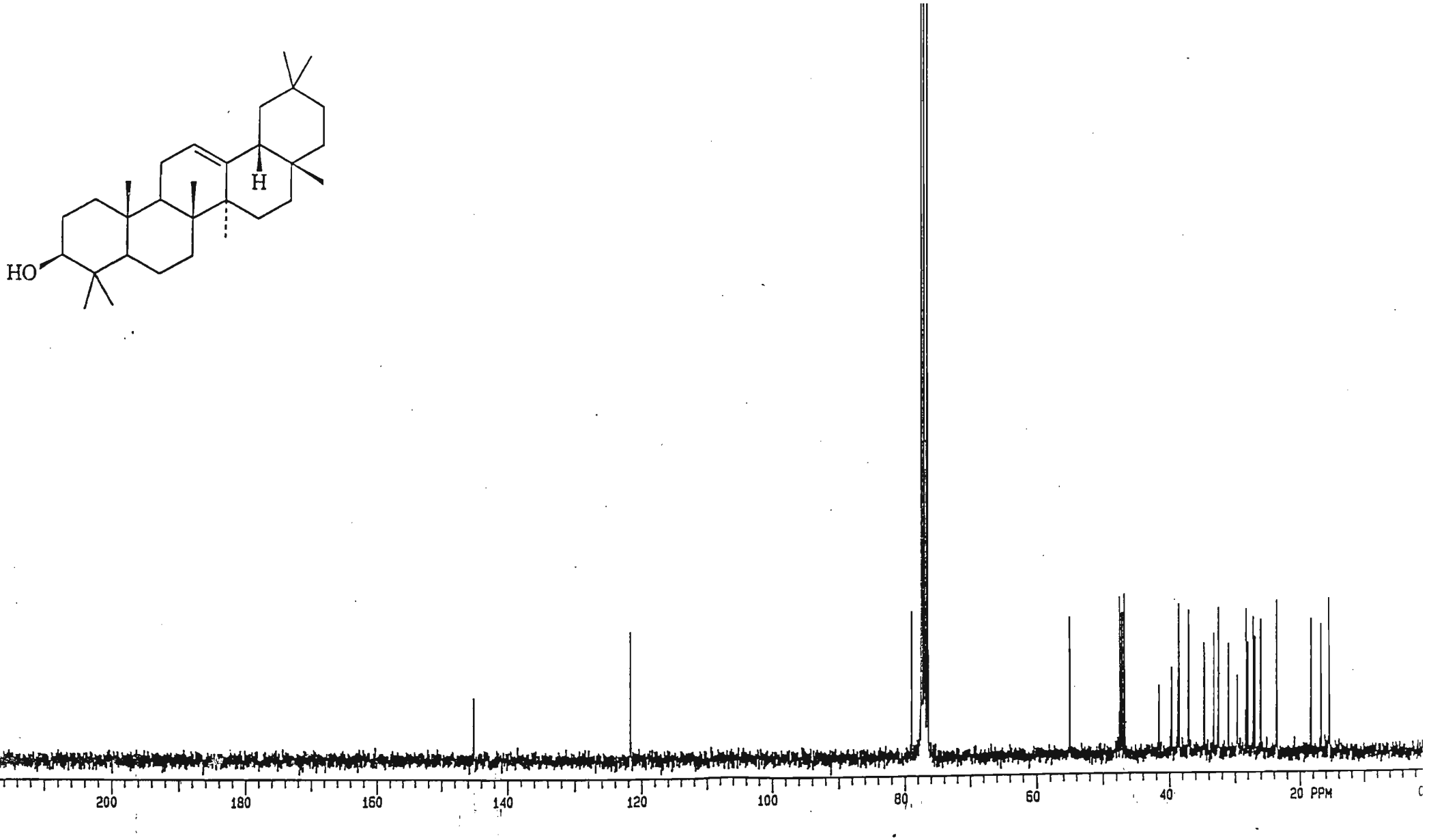


β-Amyrin

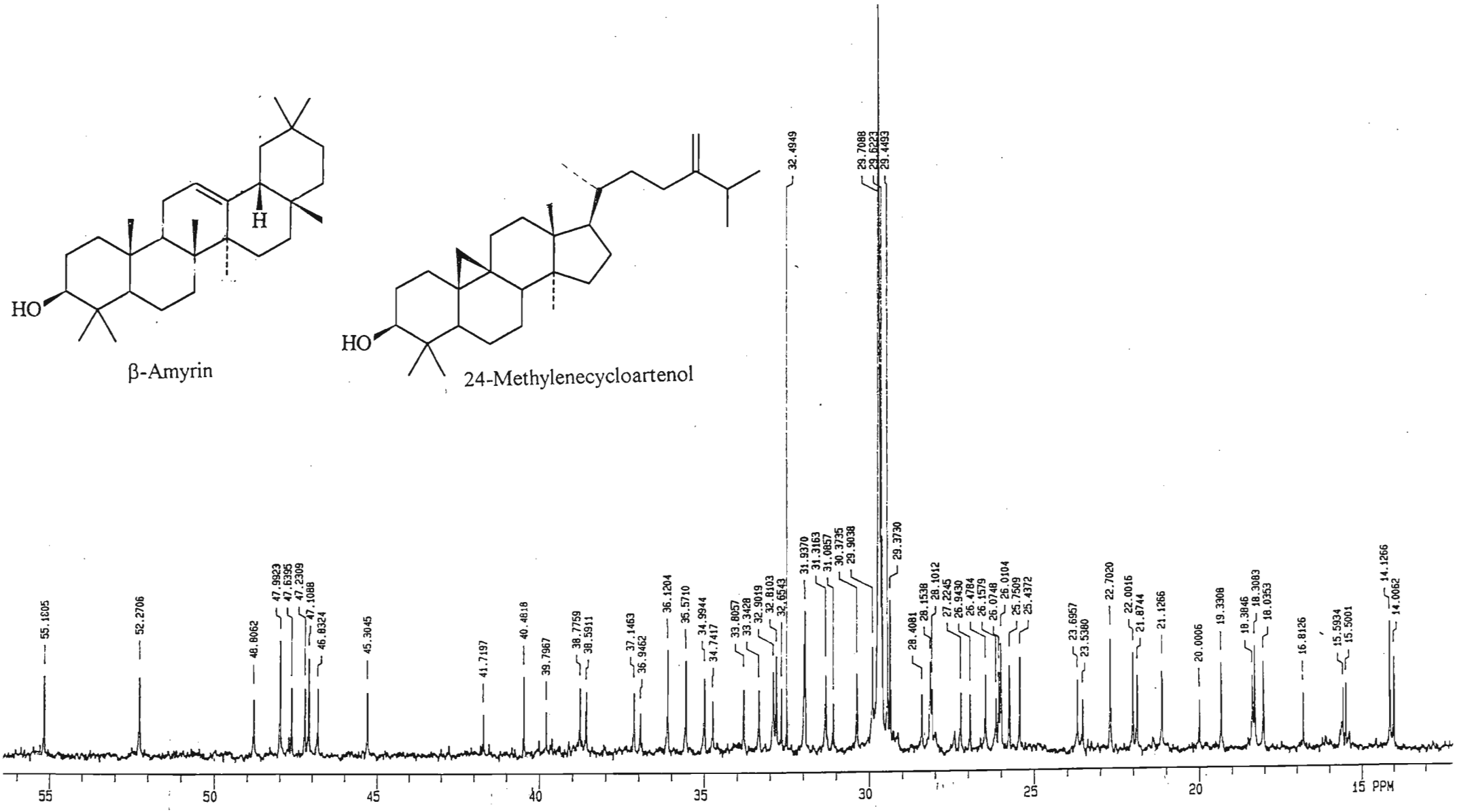


24-Methylenecycloartenol

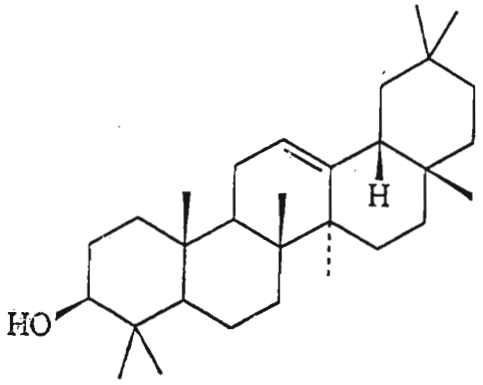
Spectrum : ^{13}C spectrum of the mixture of Compound 6 and Compound 7

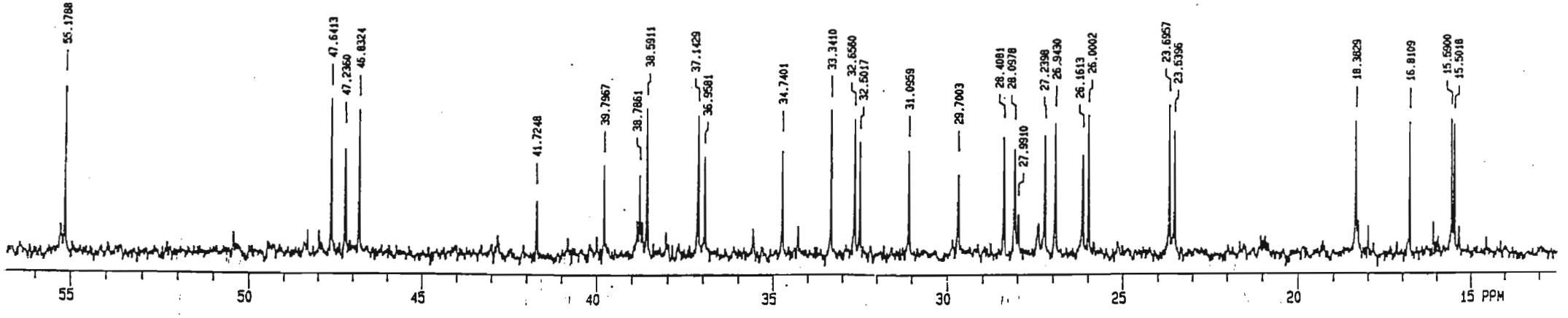


Spectrum : ^{13}C spectrum of β-amyrin

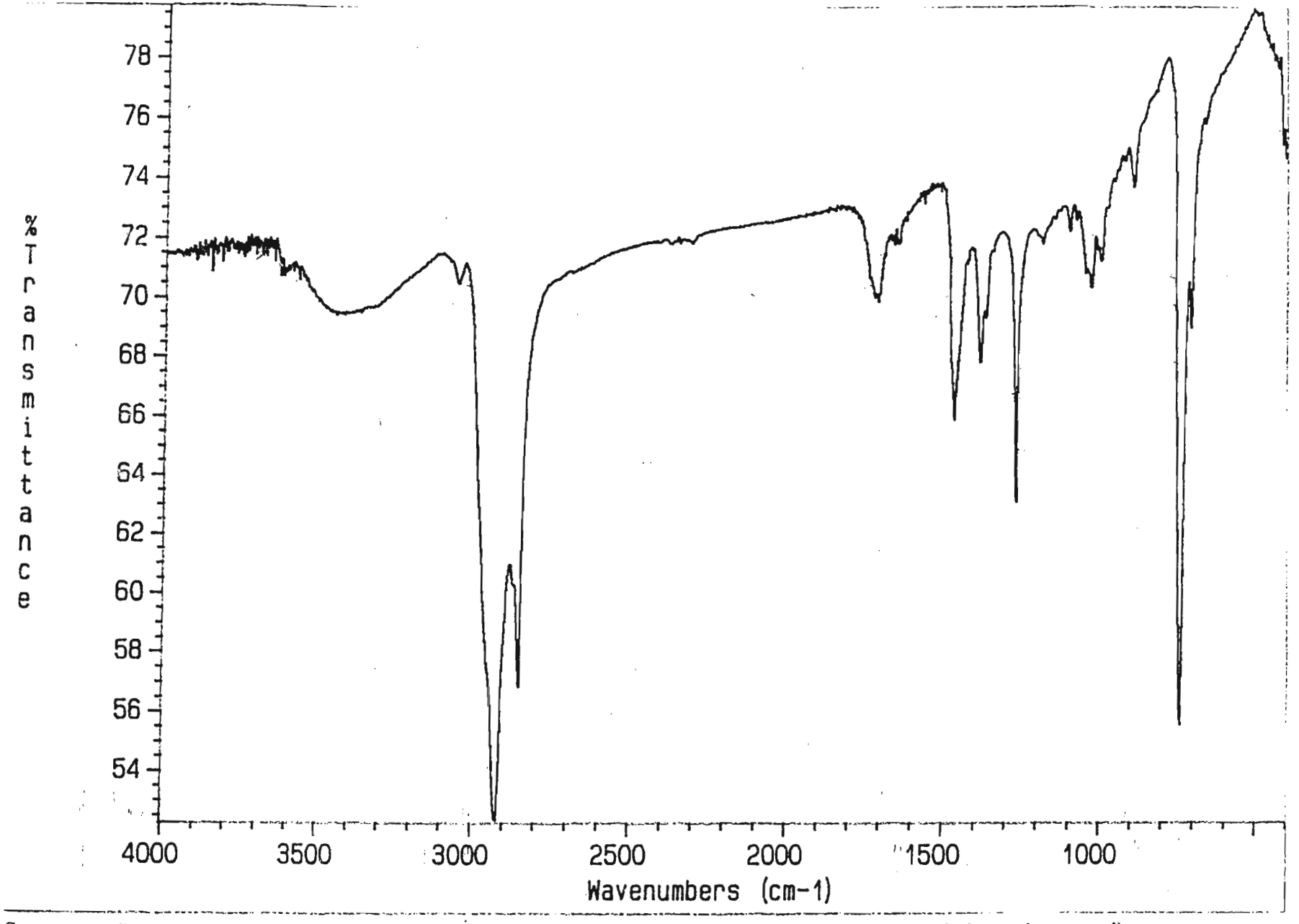


Spectrum : Expansion of the ^{13}C spectrum of the mixture of compound 6 and compound 7 in $CDCl_3$

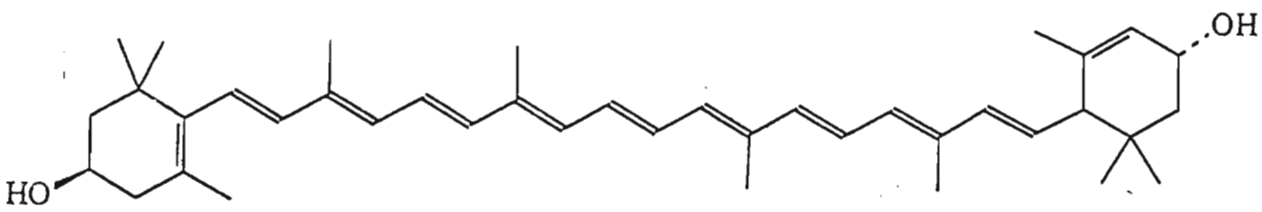




Spectrum : Expansion of the ^{13}C spectrum of β-amyrin

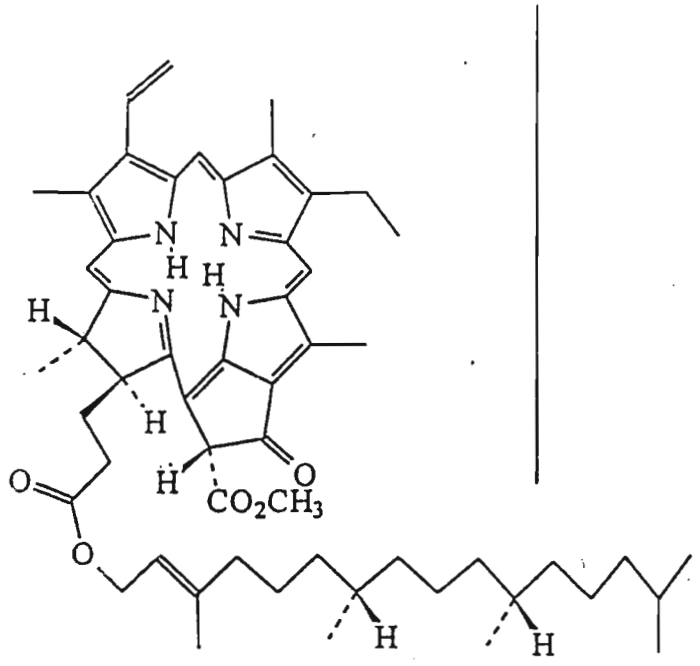


Spectrum : Infrared spectrum of the mixture of compound 6 and compound 7 (β-amyrin and 24-methylenecylcoartenol)



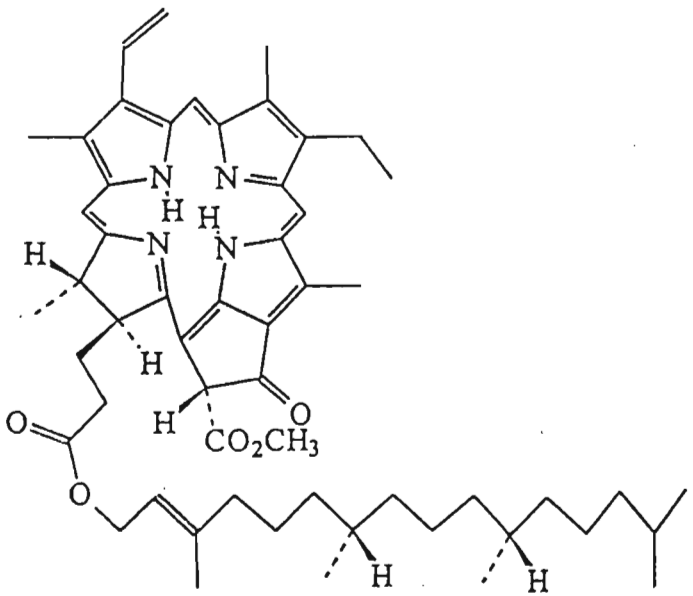
Lutein

Spectrum : ^{1}H n.m.r. spectrum of Compound 8



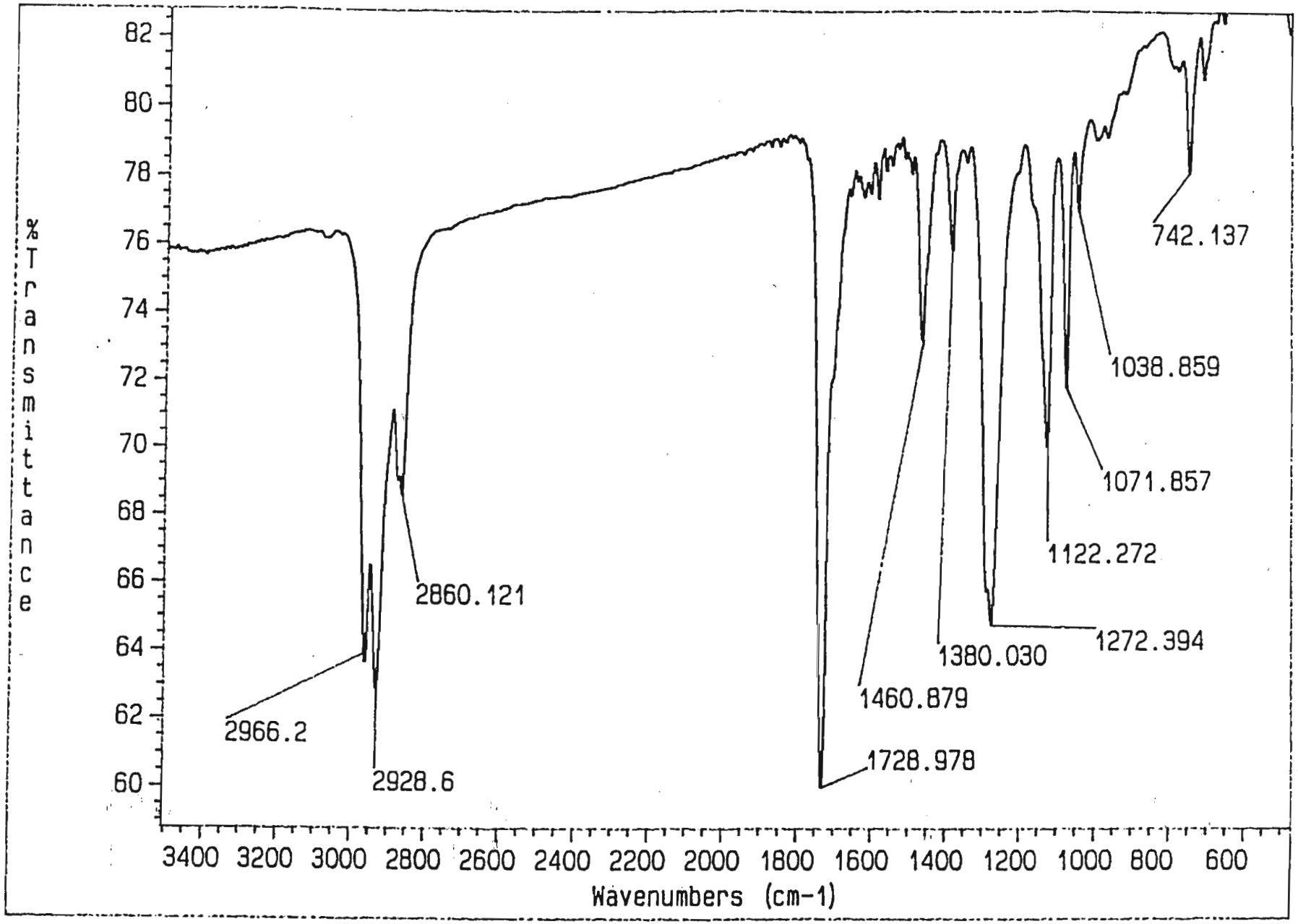
Pheophytin a

Spectrum : ^{1}H n.m.r. spectrum of Compound 9

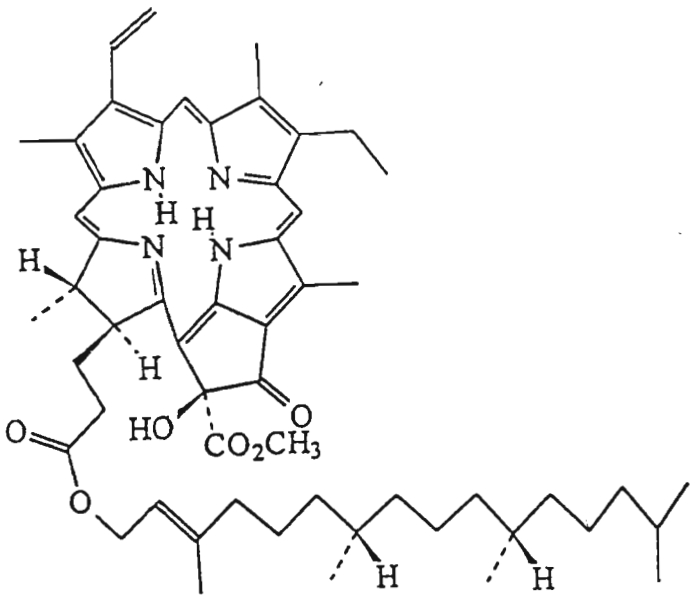


Pheophytin a

spectrum : ^{13}C n.m.r. spectrum of compound 9 in $CDCl_3$

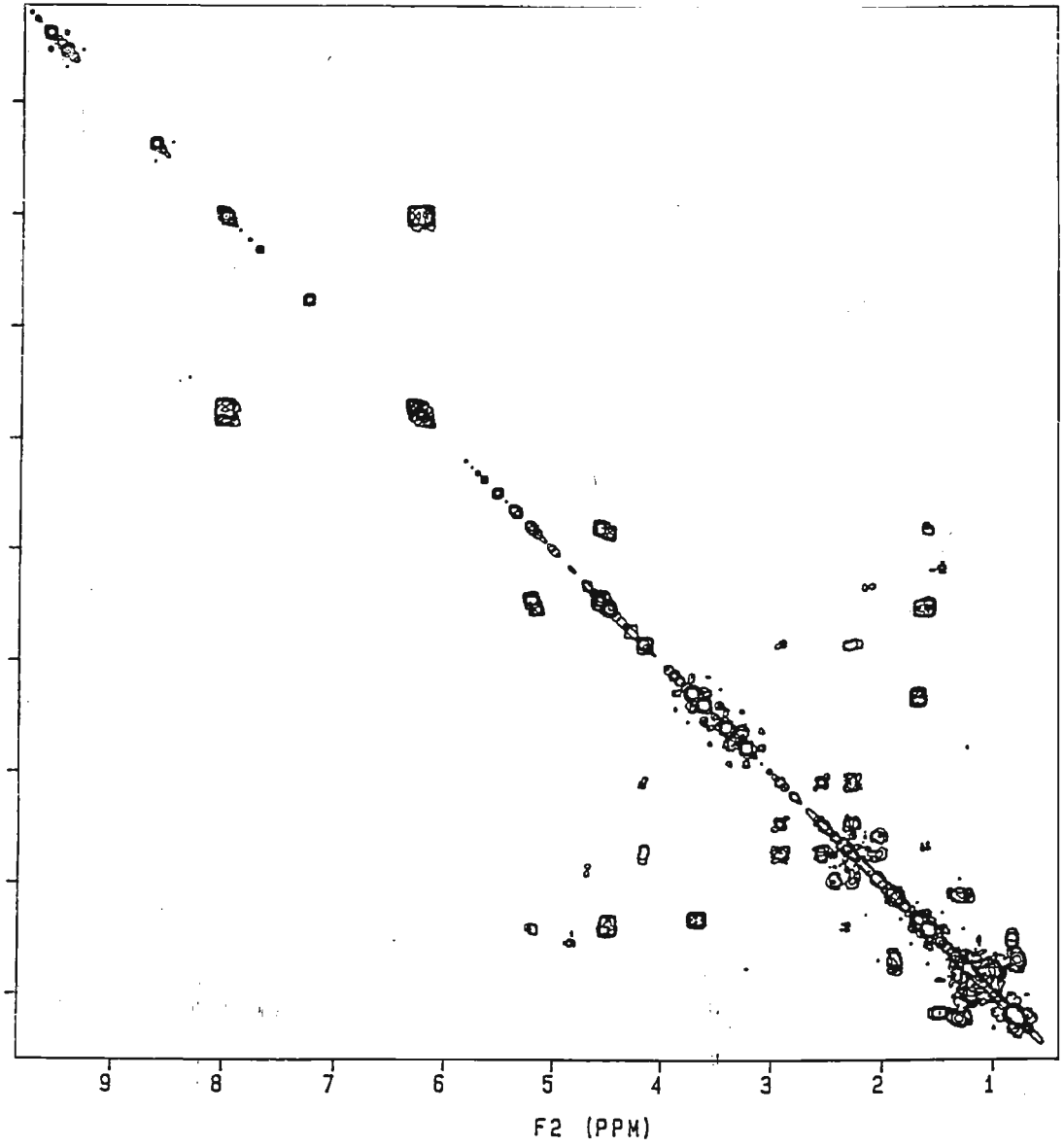


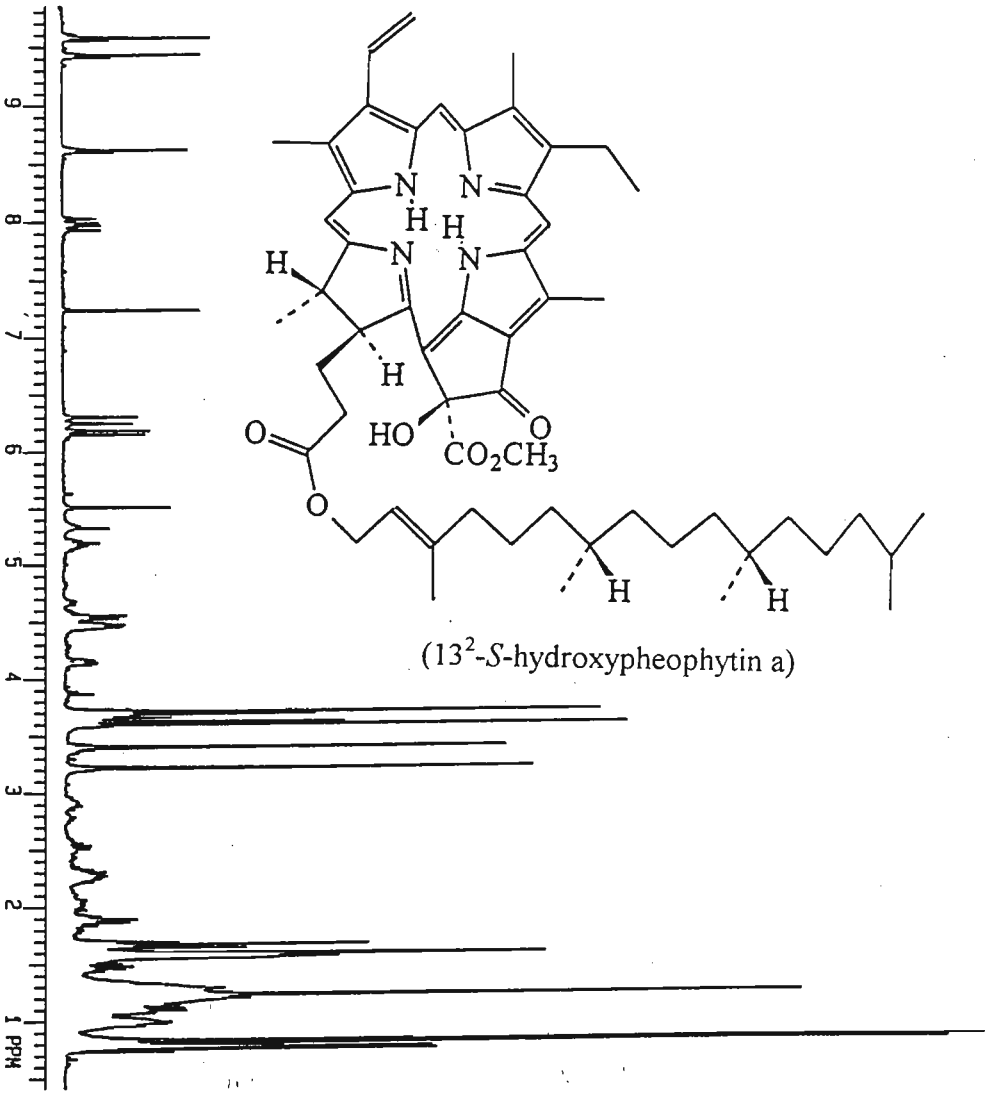
Spectrum : Infrared spectrum of compound 9 (pheophytin a)



13^2-*S*-Hydroxypheophytin a

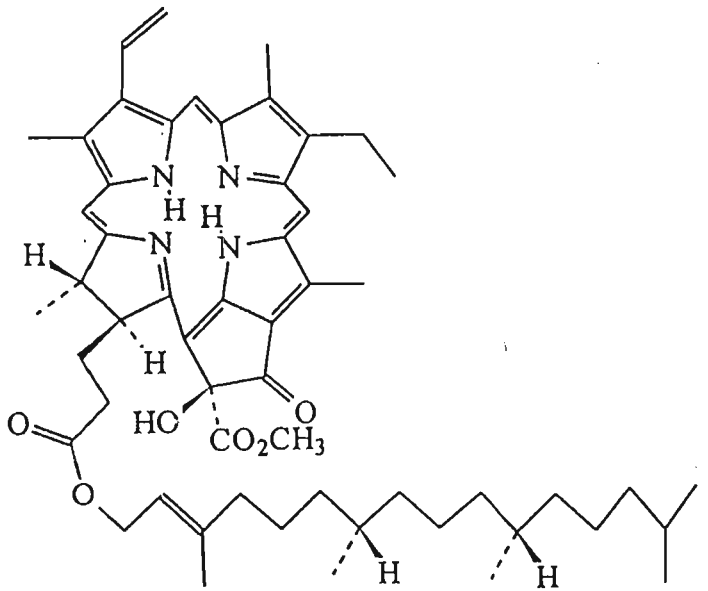
Spectrum : ^{1}H n.m.r. spectrum of Compound 10





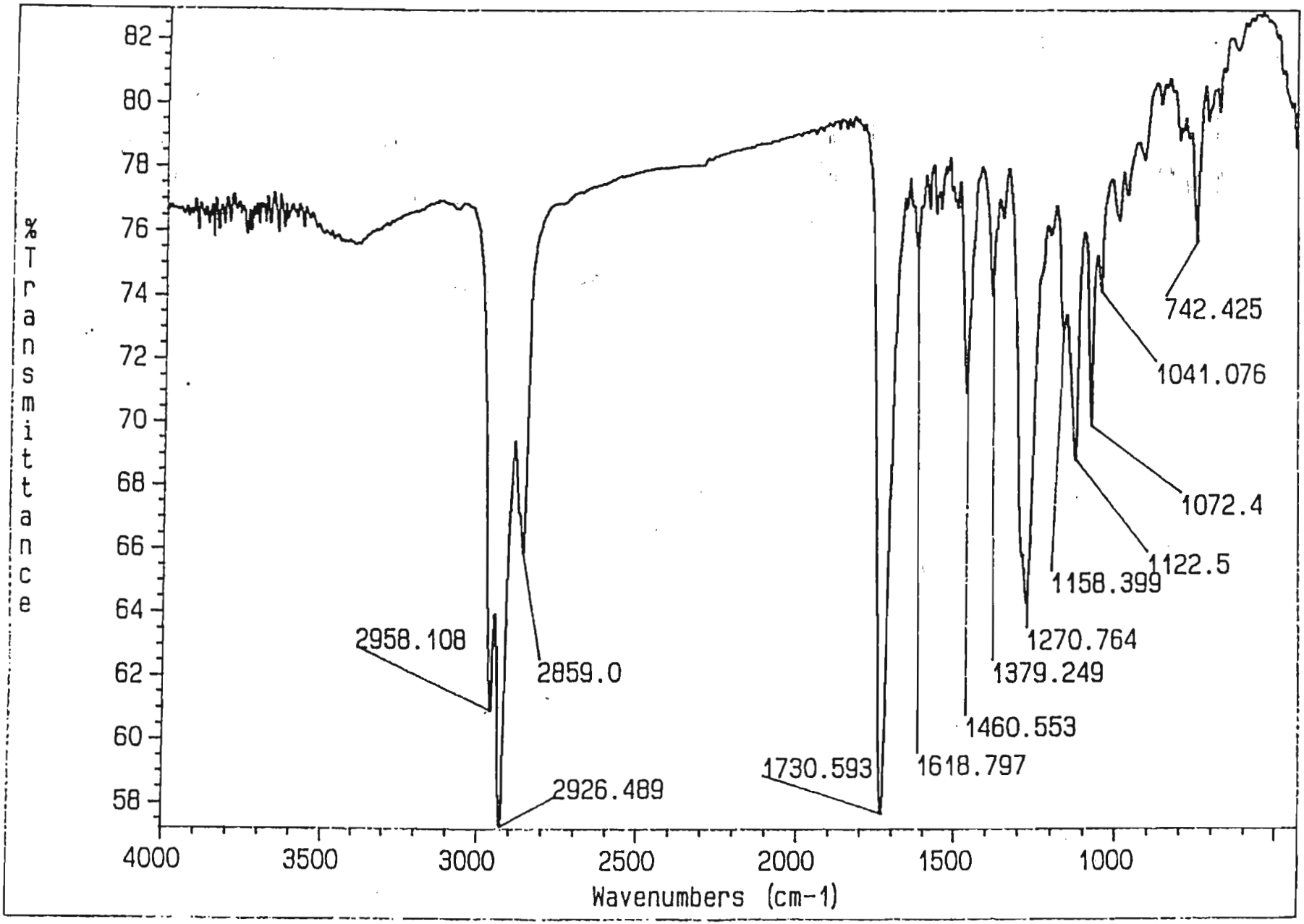
(13^2-*S*-hydroxypheophytin a)

Spectrum : COSY spectrum of Compound 10



13^2-*S*-Hydroxypheophytin a

pectrum : ^{13}C n.m.r. spectrum of compound 10 in $CDCl_3$



Spectrum : Infrared spectrum of compound 10 (13^2-*S*-hydroxypheophytin a)