Insecticidal Natural Products From Sri Lankan Plants

Thesis

INSECTICIDAL NATURAL PRODUCTS FROM SRI LANKAN PLANTS

by

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To my husband

Hemalal

ABSTRACT

This thesis describes investigations of the insecticidal compounds of three Sri Lankan plants, *Pleurostylia opposita* (Wall) Alston (Celastraceae), *Aegle marmelos* Correa (Rutaceae) and *Excoecaria agallocha* Linn. (Euphorbiaceae). After establishing the insecticidal activity of the extracts of three plants, separation of compounds was achieved by bio-assay directed chromatography, and the compounds were characterised by NMR especially 2D experiments, mass, UV and IR spectroscopy.

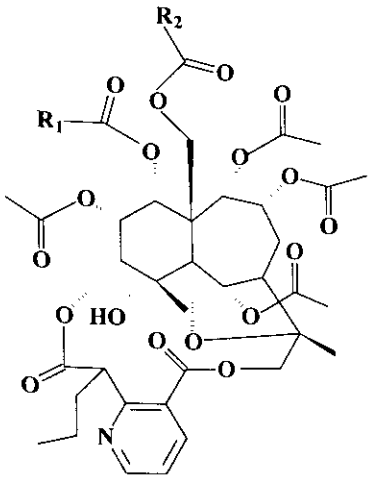
Three new macrocyclic sesquiterpene polyol ester alkaloids **1**, **2** and **3** containing a novel 6,7 ring skeleton have been identified from the petroleum ether-ethyl acetate extract of the stem bark of *Pleurostylia opposita*. They are homologues of known macrocyclic sesquiterpene alkaloids which contains a 6,6 ring system based on the β-dihydroagarofuran core. The extra methylene has been shown by NMR to be in the 8 position of the 6,7 ring. Observation of insecticidal activity in the *Pleurostylia* genus is novel. The previously reported 20-hydroxylupane-3-one has also been identified from the active extract and shown to be non-insecticidal.

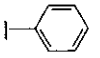
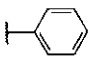
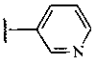
Two new insecticidal compounds **6** and **7** have been identified from the petroleum ether-ethyl acetate extract of the stem bark of *Aegle marmelos*. They are shown to be protolimonoids, and are senecioate ester analogues of the known isovalerate esters of C-21-β and C-21-α glabretal which were also isolated. Compounds **8** and **9** were also found to be insecticidal against mustard beetles

and houseflies. Separation and characterisation of epimeric mixtures were first achieved for the di p-nitrobenzoate derivative.

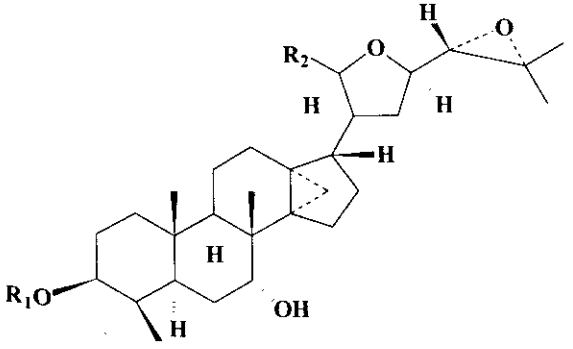
Previously recorded epoxyaurapten, marmesin, marmin and lupeol have been isolated from the active extract of *Aegle marmelos* and shown to be non-insecticidal.

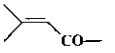
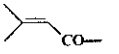
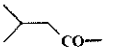
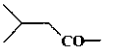
Five compounds **24-28** have been isolated from the ethyl acetate extract of the stem bark of *Excoecaria agallocha*. They all have the previously reported daphnane diterpenoid orthoester skeleton, and differ only in the orthoester alkyl side chain. Two of them **24** and **27** are novel. Insecticidal activity has not been recorded previously for extracts of *Excoecaria* species, nor for the daphnane orthoesters.



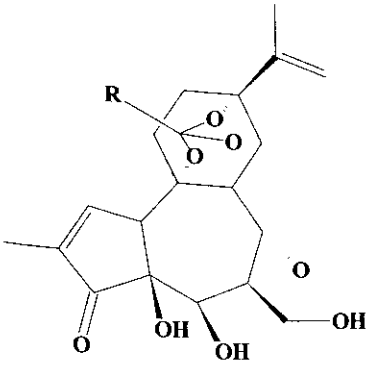
Compound	R_1	R_2
1		CH_3
2	CH_3	CH_3
3		

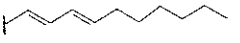
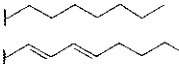
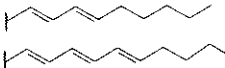
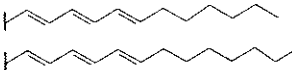
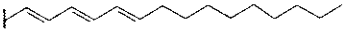
Compounds from *Pleurostylia opposita*



Compound	R_1	R_2
6		α-H, β-OH
7		β-H, α-OH
8		α-H, β-OH
9		β-H, α-OH

Compounds from *Aegle marmelos*



Compound	R
24	
25	
26	
27	
28	

Compounds from *Excoecaria agallocha*

Figure 1 : Insecticidal natural products

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ABBREVIATIONS

Ac	Acetyl
API	Atmospheric Pressure Ionisation
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
Bz	Benzoyl
br	Broad
^{13}C-^{1}H COSY	Carbon-Hydrogen Correlation Spectroscopy
DEAD	Diethyl azodicarboxylate
DEPT	Distortionless Enhancement by Polarisation Transfer
DDT	Dichloro Diphenyl Trichloroethane
DNA	Deoxyribonucleic Acid
DMF	N,N-Dimethylformamide
FPP	Farnesyl Pyrophosphate
FT	Fourier Transform
GPP	Geranyl Pyrophosphate
GC-MS	Gas Chromatography-Mass Spectroscopy
HMQC	^{1}H-Detected Heteronuclear Multiple-Quantum Coherence via Direct Coupling
HSQC	^{1}H-Detected Heteronuclear Simple-Quantum Coherence via Direct Coupling
HMBC	Heteronuclear Multiple Bond Connectivity by 2D Multiple Quantum NMR

^{1}H-^{1}H COSY	Hydrogen-Hydrogen Correlation Spectroscopy
HPLC	High Performance Liquid Chromatography
HPSE	High Pressure Soxhlet Extraction
HPTLC	High Performance Thin Layer Chromatography
kdr	Knock down resistance
IPP	Isopentenyl Pyrophosphate
IR	Infra Red
INADEQUATE	Incredible Natural Abundance Double Quantum Transfer Experiments
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectroscopy
LC-NMR	Liquid Chromatography- Nuclear Magnetic Resonance
LC-SFC	Liquid Chromatography- Supercritical Fluid Chromatography
LC-UV-MS	Liquid Chromatography- Ultra Violet-Mass Spectroscopy
MCPBA	m-Chloroperbenzoic acid
Me	Methyl
NT	Non Toxic
Nic	Nicotinyl
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement (and Exchange) Spectroscopy
NADH	Nicotinamide Adenine Dinucleotide, reduced form
PTLC	Preparative Thin Layer Chromatography
PCC	Pyridinium Chlorochromate

SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
SAR	Structure Activity Relationship
RI	Refractive Index
Rf	Retention factor
RNA	Ribonucleic Acid
RP	Reverse Phase
tlc	Thin Layer Chromatography
TMS	Tetramethylsilane
THF	Tetrahydrofuran
TOCSY	Total Correlation Spectroscopy
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Over the centuries people have struggled to protect crops against invasion by pests and microbial pathogens. They observed that some plants resisted pests better than others. Through trial and error they discovered the applications of these substances and developed procedures for their extraction. As a result, plant extracts have been widely used to protect crops both in the field and after harvest.

The origin of insecticides is lost in antiquity, but a thousand years ago, the Chinese used pyrethrum and derris species to control insects and the Romans used hellebore species as insecticides. The ancients also used certain spices to protect food, such as cinnamon, mustard, nutmeg and pepper, all of which are now known to contain insecticides and fungicides. Non-plant derived materials have also been used such as whitewash which was sprayed on grapevines to protect the foliage and fruit from insects in the 1880s.

As the years progressed, several plants were exploited more widely as a source of commercial insecticides. In the early 20 th century, botanical insecticides, such as pyrethrum, nicotine and rotenone were holding a prominent place among pest control products, competing with arsenicals such as paris green.

During the 1940s, synthetic chemicals were discovered including DDT for public health and plant protection.[1] Thereafter, the agricultural community has relied extensively on synthetic insecticides, such as organochlorines, organophosphates, carbamates and more recently, the pyrethroids.[1] Synthetic insecticides exhibit broad

spectrum and greater intrinsic activity as well as better physical properties. However, there are some drawbacks of synthetics over botanicals, such as being "environmentally nonfriendly". Some indiscriminately destroy beneficial species including the pest's natural enemies. The long degradation periods of some synthetics, especially chlorinated hydrocarbons, result in accumulation in food chains[2] and contamination of drinking water.[3] The most important as well as the most worrying drawback is the development of resistance to the synthetic insecticides.

Nevertheless, the success of synthetics in almost all facets of insect control has relegated botanicals to secondary markets, such as home, garden and veterinary uses and today, botanicals represent less than 5% of the commercial insecticide market (Figure 1.1).[4]

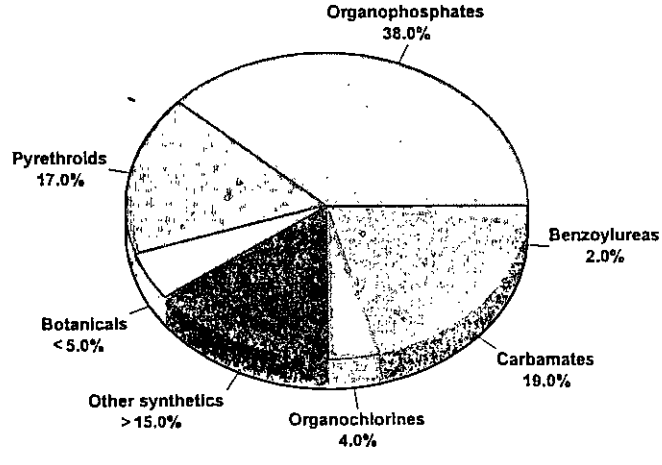

Figure 1.1 : World insecticide usage ($m)- 1996

Currently, five botanical insecticides, pyrethrum, rotenone, ryania, sabadilla and neem, are registered for use in the United States (Table 1.1).[5]

Table 1.1 : Current botanical insecticides

Insecticide	Country of Origin	Active Ingredients	% in TGAC[a]	US$/Kg[b]
Pyrethrum	Kenya	Pyrethrins, Cinerins	25	75
Rotenone	Peru	Rotenoids (isoflavones)	7.3	3
Ryania	Trinidad	Ryanodine alkaloids	0.1	3
Sabadilla	Venezuela	Veratrum alkaloids	0.8	8
Neem	India	Tetranortriterpenoids	25	37.5

[a] Technical grade active concentrations, [b] Approximate sale price in 1992

Currently, despite an enormous expenditure on agrochemicals world-wide, approximately one third of global food production is reportedly destroyed by different pest species (Table1.2).[6]

Table 1.2 : World Crop Losses -1996

	% Crop loss	
Insects and mites	12	
Plant pathogens	12	
Weeds	10	
Mammals and birds	1	
	35	= US $400 Thousand Million
Post Harvest Losses	20	
	55	= US $630 Thousand Million

Over 500 pest species have become resistant to the current classes of synthetic insecticides. Some present a major resistance management problem e.g. 10-20 species of arthropods, notably spider mites, mosquitoes, three lepidopterous cotton pests, and the housefly resist pyrethroids, organophosphates and carbamates.[7]

There are three main ways for resistance to develop against insecticides.[7] The most common involves detoxification of the insecticide before it reaches the target site; for instance the role of esterases in resistance to organophosphates as well as the hydrolases and oxidases to pyrethroids and benzoyl phenyl ureas.[8] Another resistance mechanism involves modifications in the binding sites[9, 10] which results in insensitivity of the target site to the pesticide, for example reduced sensitivity of acetylcholinesterase[11] to organophosphates and modified sodium channel proteins[12] to pyrethroids in houseflies. In addition, reduced penetration has also been recognised as having a role in the development of resistance.[7]

The major classes of insecticides have therefore become less effective to pests. The development of new pesticide products, especially those with alternative modes of action therefore continue to be a major priority for agrochemical industry. [13]

In the last two decades industry has concentrated on evaluating compounds from micro-organisms and synthetic screening programmes. However, the limited success of this approach has led to a revival of interest in plant metabolites which have played an important role in crop protection over thousands of years.

The plant kingdom contains at least 500 000 different species[14] and they produce a very diverse range of secondary metabolites. Only about 5-10% of these have been examined chemically for biologically active compounds and over 2 000 plant species have been reported to possess pest control properties.[15]

In the past, plants have provided important lead structures for the development of new classes of insecticides. For instance, the carbamates and pyrethroids, two of the three main classes of established insecticides are all analogues of natural compounds (Figure 1.2).

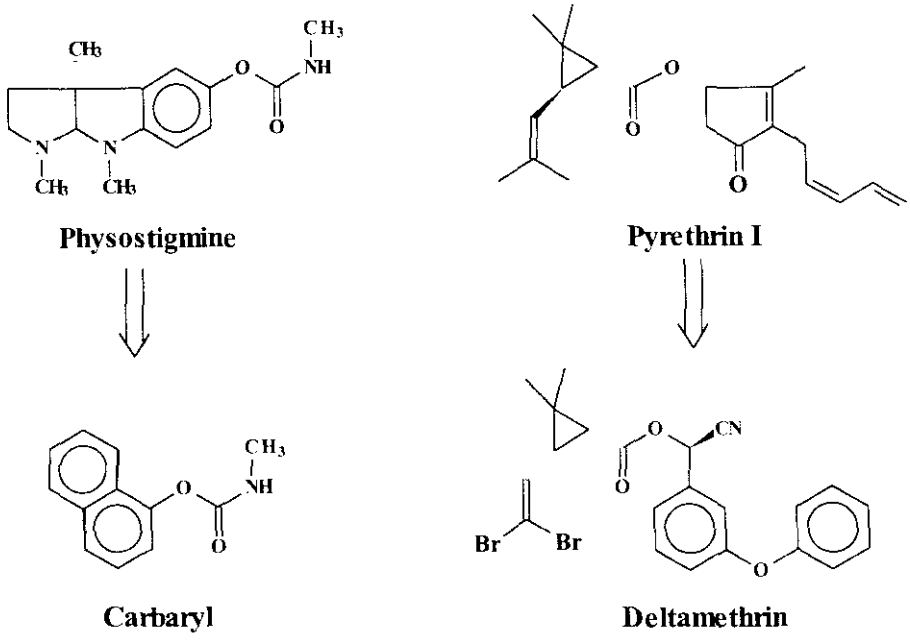

Figure 1.2 : Commercial insecticides from plant leads

Hence, the plant kingdom represents an enormous reservoir of new molecules either for direct development of botanical insecticides or as leads for the synthesis of insecticidal analogues with novel modes of action and possibly favourable mammalian toxicity.

1.2 SECONDARY METABOLISM

1.2.1 Introduction

Plant secondary metabolism produces a very diverse range of metabolites, such as terpenoids, alkaloids, polyacetylenes, flavonoids, phenols, mycotoxins, polyenes, amino acids and sugars offering a rich diversity of biologically active compounds. Many of these are believed to have evolved, to protect the plant from attack by animals, insects and microbial pathogens as well as weeds.

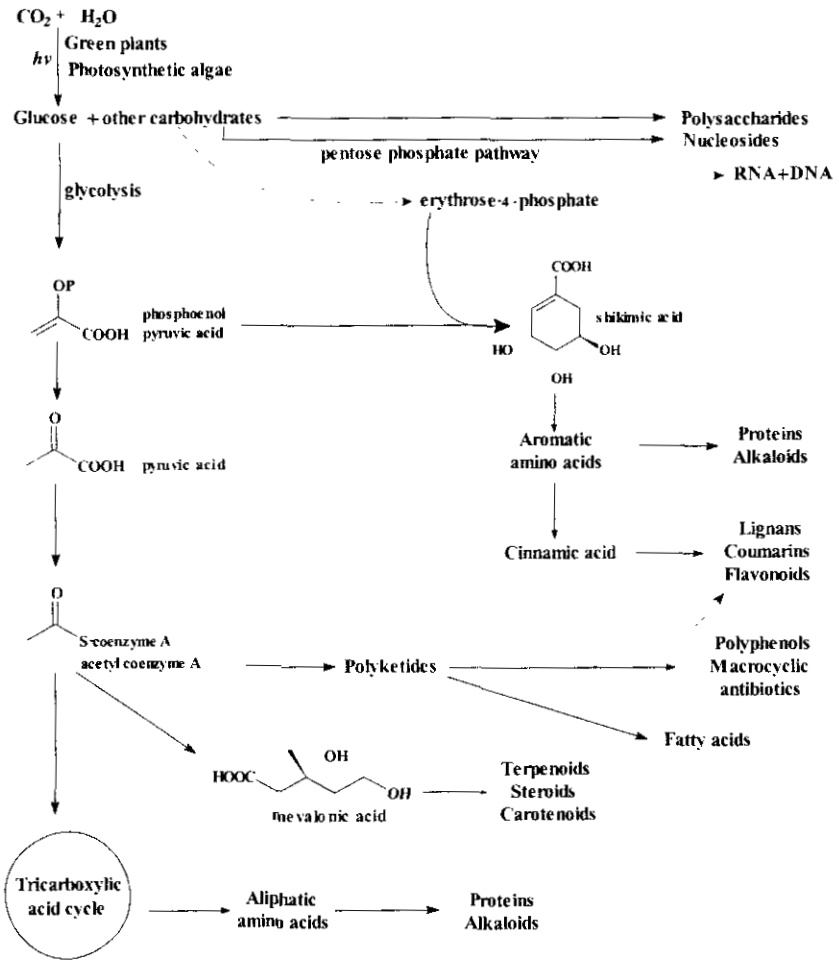

Figure 1.3 : Main streams of secondary metabolism (Adapted from J Mann, 1994)[20]

The secondary metabolites are derived principally from a few key intermediates of primary/intermediary metabolism, most often, phosphoenol pyruvate, acetyl co-enzyme A, malonyl co-enzyme A, α-amino acids and shikimate, by specific enzymes. Some enzymes appear to have a strong resemblance to certain enzymes of primary metabolism.[16] Secondary metabolites have a distribution restricted, mostly, to a single major taxon and are more characteristic of specific botanical sources.

Secondary metabolites are broadly divisible on the basis of their structures and biosynthetic origins as relating to one of several distinct biosynthetic pathways, namely, the mevalonic acid, the shikimic acid, the polyketide, the fatty acid, and the alkaloid pathways summarised in Figure 1.3.

1.2.2 **The mevalonic acid pathway**

Mevalonic acid, a C_6 acyclic compound, is the precursor of all terpenoids. The isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) which form from mevalonic acid condense together to form geranyl pyrophosphate (C_{10}-GPP). Sequential addition of further C_5 units (IPP), lead to farnesyl pyrophosphate (C_{15}-FPP) and geranylgeranyl pyrophosphate (C_{20}-GGPP) (Figure 1.4).[17] These regular acyclic terpenes, geranyl pyrophosphate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate can, by dimerisation, cyclisation and rearrangement, act as precursors to all of the various sub-classes of terpenes (Figure 1.4).[17, 18]

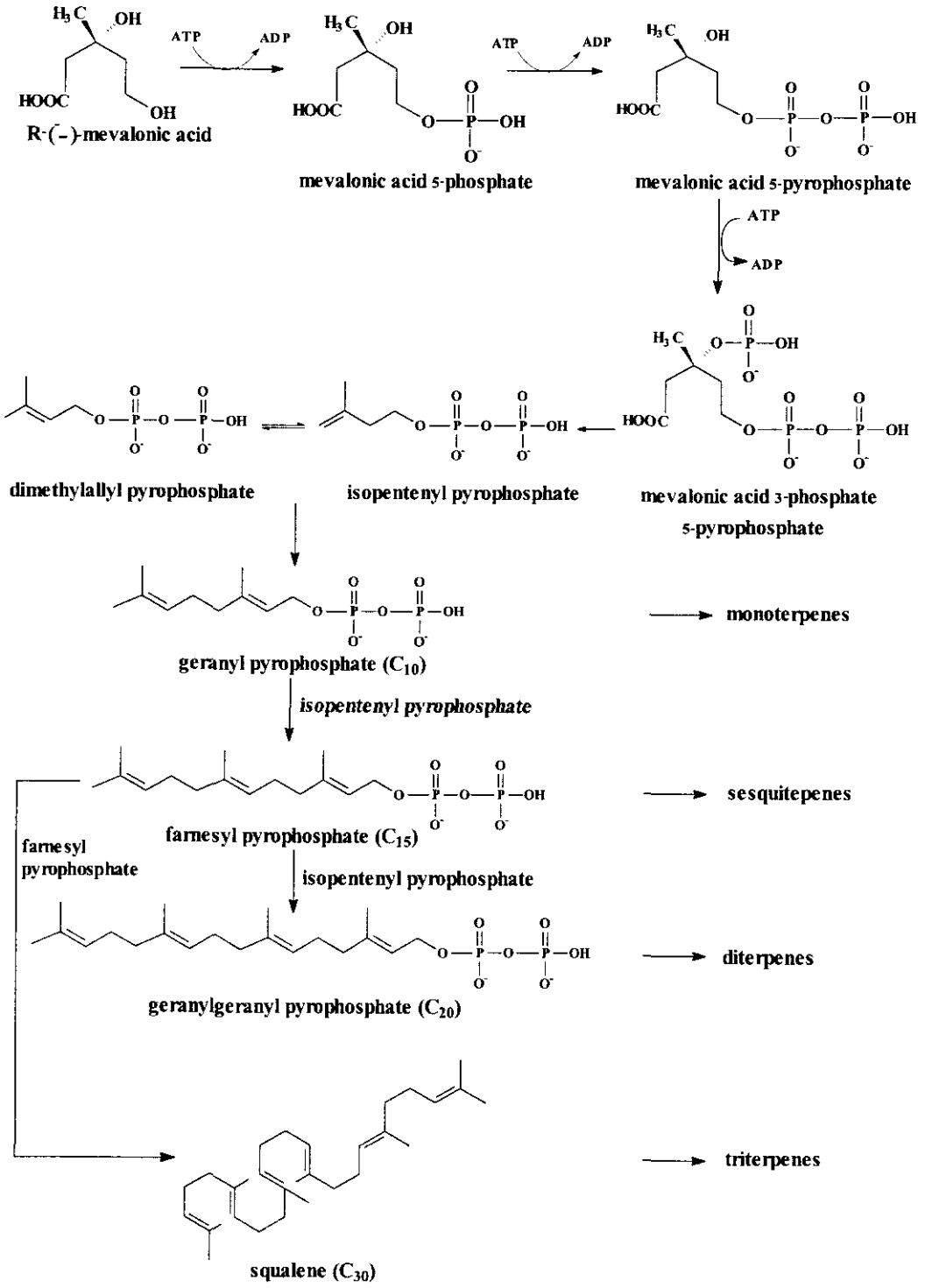

Figure 1.4 : The mevalonic acid pathway

1.2.3 **The shikimic acid pathway**

Shikimic acid is a key intermediate for several distinctive pathways to a range of *phenolic compounds* that exist in plants (Figure 1.5).[19]

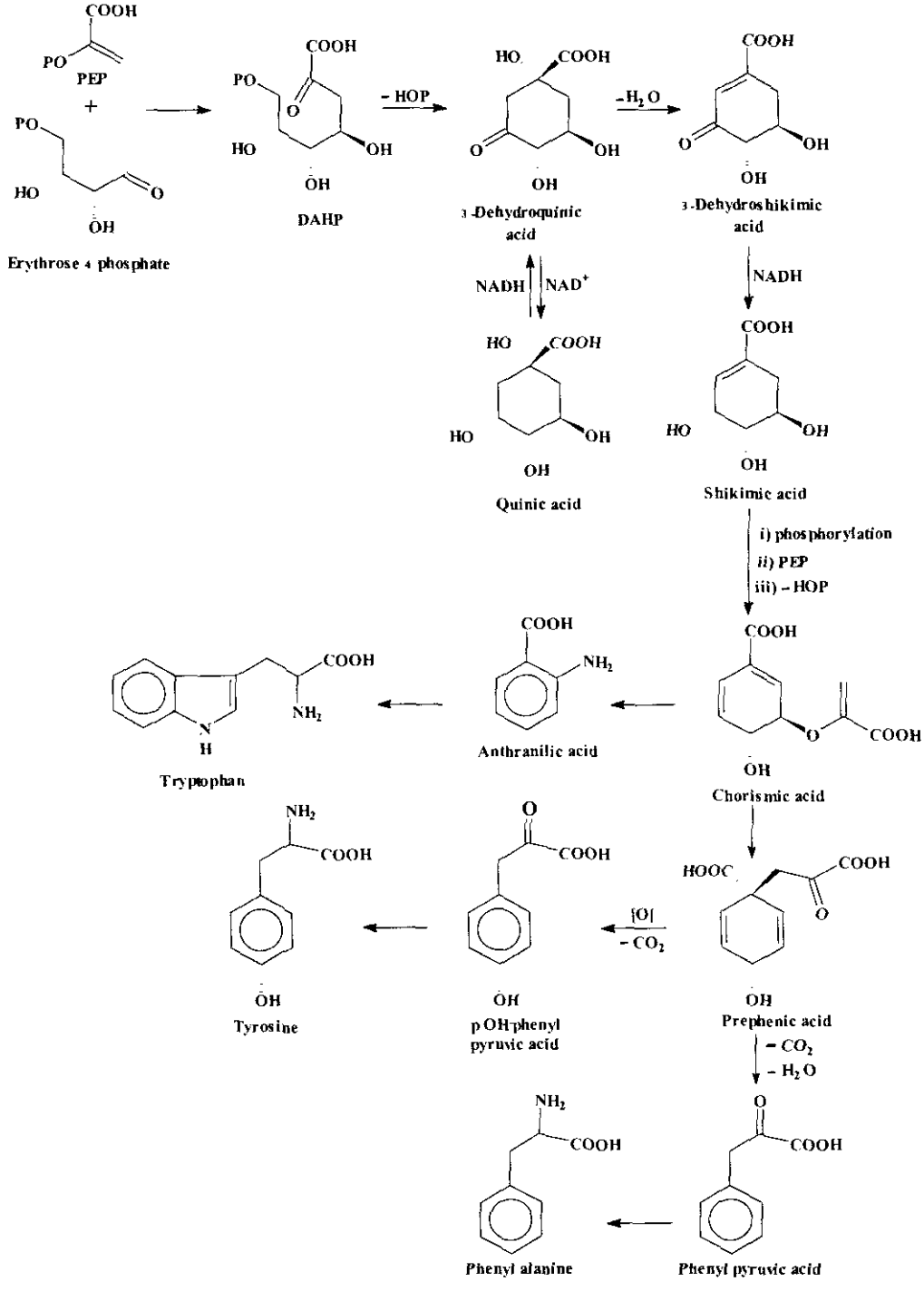

Figure 1.5 : The shikimic acid pathway

The shikimic pathway begins with the condensation of phosphoenol pyruvate (PEP) and D-erythrose-4-phosphate, yielding 3-deoxy-D-*arabino*-heptulosonate-7-phosphate [(DAHP) Figure 1.5]. Elimination of phosphoric acid gives the ketone, formally in its enol form which then cyclises to 3-dehydroquinic acid. Further elimination of water followed by reduction gives shikimic acid which is a precursor for several biosynthetic pathways, leading to aromatic amino acids, proteins, enzymes, alkaloids, phenyl propanoids, coumarins, flavonoids, lignins, cyanogenic glycosides, quinones and stilbenes.[19]

1.2.4 **The polyketide pathway**

Polyketide pathway gives rise to several important plant metabolites, such as fatty acids, olefins, acetylenes, some branched derivatives and aromatics. It starts with the linear Claisen condensation of acetyl co-enzyme A leading to β-keto esters, which either by reduction and repeated condensation give fatty acids. Further unsaturation of fatty acids gives acetylenes or by direct condensation gives polyketides.[17, 18] They can also cyclise to form a vast number of aromatic compounds such as acetophenone and pyrone derivatives and orsellinic acid.[17, 18] Other compounds of polyketide origin are derived by alteration of substituent by secondary processes, such as oxidation and attachment of non-acetate derived terminal units, as well as terminal changes which involve oxidative ring opening and reclosures.[17, 18]

1.2.5 **The alkaloids**

In plants, alkaloids show a great structural diversity and are biosynthesised from a restricted group of α-amino acids, ornithine, valine, leucine, isoleucine, phenylalanine, tyrosine, proline and tryptophan, many of which are hydrophobic in character. The carbon-nitrogen framework of the heterocyclic ring of many alkaloids is generated by the initial condensation, which may be either intramolecular or intermolecular, of an aldehyde and a primary amine[20]. These condensation products then give rise to a great variety of alkaloids.[17, 18]

1.3 BOTANICAL INSECTICIDES

1.3.1 Introduction

80% of the botanical insecticide market involves pyrethrins. Other important groups of botanical insecticides include the isoflavonoids (e.g. rotenone), the alkaloids (e.g. ryania, veratrum and nicotine) and the tetranortriterpenoids (e.g. azadiractin). Besides these, a multitude of insecticidal chemical constituents with different structural types have been identified from various plants. Among them N-butyl amides, physostigmine, toosendanin, citrus limonoids, coumarins (e.g. mammeins and furanocoumarins), benzofurans, acetogenins, quinones, pyrroles, chromenes, terthienyls and polyacetylenes were recognised as potent botanical insecticides.[13]

1.3.2 Pyrethrins

The most economically important and significant application of a plant natural product derives from the insecticidal properties of pyrethrins, which were isolated from the flower heads of the pyrethrum plant, *Tanacetum cinerariaefolium*, (Asteraceae). The powdered dry flower has been used as an insecticide from ancient times.[21] The commercial use originated in Persia in the early 1800s and by 1828, pyrethrum was being processed for commercial insect control. The majority of pyrethrum produced in the world comes from Kenya and Tanzania.

The more active constituents of pyrethrum, pyrethrin **I** and **II** were first identified partially by Staudinger and Ruzicka in the early 1920s, and fully by Schechter and co-workers in the period 1935-1949.[22] However, the complete structural elucidation

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also widely used for flea control on domestic pets and control of human headlice, although a significant proportion of humans are susceptible to pyrethrum-induced allergic dermatitis. All six of the esters show both kill and knockdown activities, but pyrethrin **I** is the most active for kill and pyrethrin **II** is the most effective as a knockdown agent for flying insects. They are also powerful insect repellents. Resistance has not developed to pyrethrins in insects as in many synthetic insecticides.

The rapid photodegradation of pyrethrins greatly limits their use in controlling agricultural pests. This eventually led to the development of synthetic ester pyrethroids and subsequently, nonester pyrethroids[22] with increased photostability, high insecticidal and broad spectrum of activity (e.g. mites) as well as low mammalian toxicity.

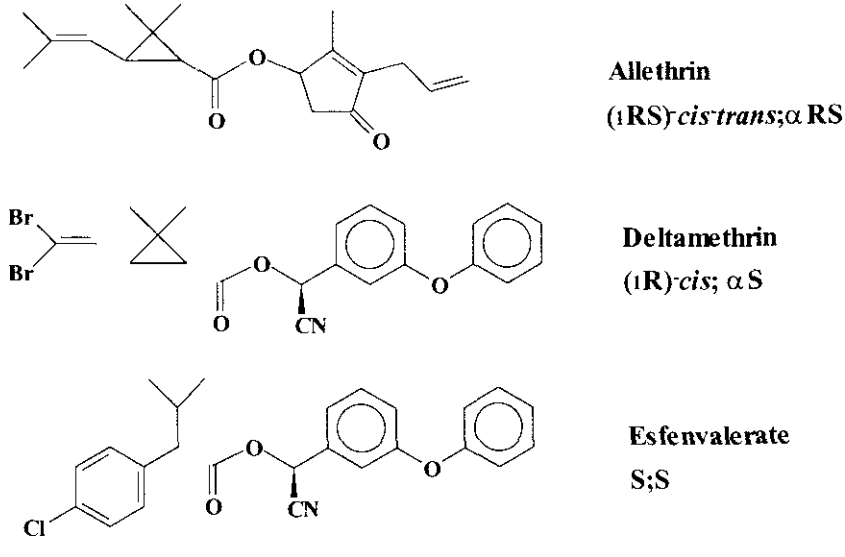


Figure 1.7 : Synthetic ester pyrethroids

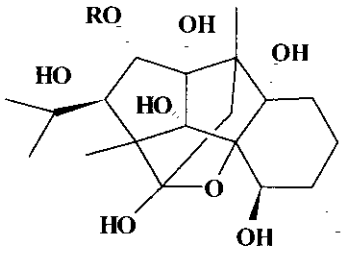
As a first example of analogue synthesis, allethrin (Figure 1.7) was developed as a commercial synthetic pyrethroid for domestic use.[22] After a number of structural modifications leading to improved household insecticides, pyrethroids, that are more suitable for use in the agricultural pest field were developed. Esfenvalerate and deltamethrin are the best known examples of their commercial success as agricultural insecticides (Figure 1.7).[22]

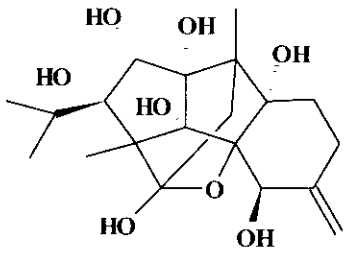
Even today, "pyrethrum" still remains one of the best lead structures from natural sources for agricultural applications. The properties of these analogues, such as excellent killing and paralysing activities, selective toxicity margins and controlled bio-degradability in the environment are still highly valued.

1.3.3 **Alkaloids**

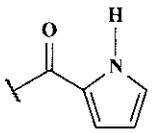
(a) **Ryania**

The neuromuscular poison ryanodine, a diterpenic alkaloid (Figure 1.8) is the active principle of ryania insecticides, obtained from *Ryania speciosa* Vahl, (Flacourtiaceae).[24] This represents the first example of a commercially successful natural insecticide discovered by rational screening of plant extracts. The leaves, stem and roots of this plant all contain the insecticidal ryanodine. *Ryania* species have been used in South America for euthanasia and as rat poisons, although there was no detailed knowledge of insecticidal properties.





R

Ryanodine 

Didehydroryanodol

Ryanodol H

Figure 1.8 : Ryanoids

Ryanodine poisons muscles by binding to calcium channels in the sarcoplasmic reticulum. This causes calcium ions to flow into the cell and death occurs rapidly.[25, 26] Ryanodine has a rat oral LD_{50} of 750 mg/kg but ryania can be considered as nontoxic at the concentrations recommended for use, as technical ryania contains less than 1% of ryanodine. The use of ryania is largely limited to control codling moths on apples and pears.

The derivatives of ryanodine and its decomposition products, ryanodol and didehydroryanodol were shown to have good knockdown activity against houseflies and cockroaches (Figure 1.8).[25]

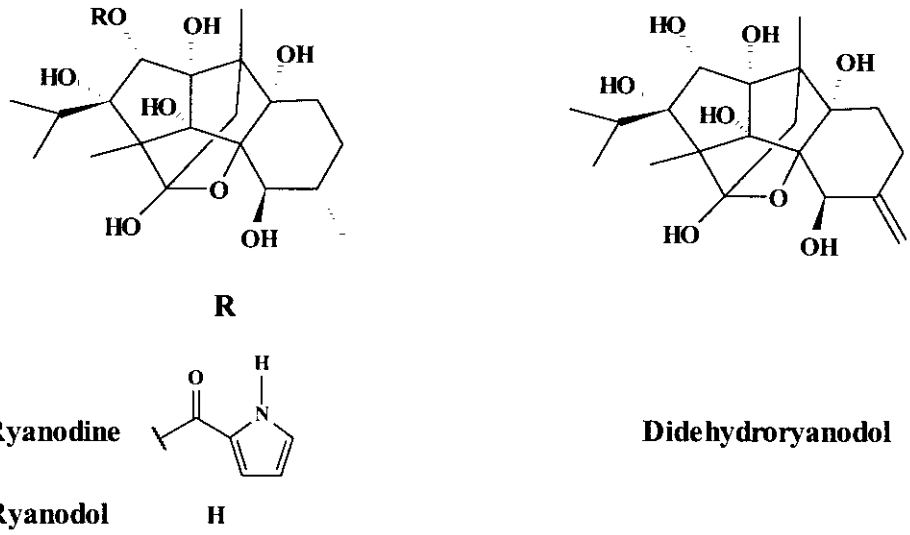


Figure 1.8 : Ryanoids

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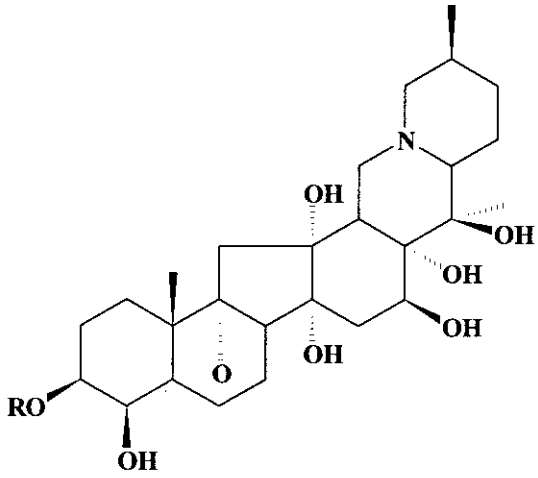
(b) **Sabadilla**

Sabadilla is the powdered ripe seeds of the lilly, *Schoenocaulon officinale* (Liliaceae), a plant native to South America. It was used for many years as a source of insecticide and is registered in the USA[5] for a wide range of pests in vegetable, fruit and berry crops. Its commercial application is now extremely limited.



	R
Veratridine	**3, 4 $(MeO)_2$ PhCO**
Cevadine	**Me**
Veracevine	**H**
3-(3,5-dimethoxy benzoyl) veracevine	**3, 5 $(MeO)_2$ PhCO**

Figure 1.9 : Veratrum alkaloids

The main active principles are the ceveratrum alkaloids, veratridine, cevadine and veracevine (Figure 1.9).[13] The use of sabadilla in the Americas dates back to the 1500s, and the product was used extensively in Europe and the USA from the late nineteenth to mid twentieth century.[27]

The ceveratrum alkaloids affect the sodium ion channel[28] and show very high levels of toxicity to mammals (LD_{50} ~ 12.5 mg/kg). However, commercial sabadilla contains only 0.8% alkaloids, making it relatively safe for use.

The rhizomes of *Veratrum album* (hellebore) contains closely related insecticidal veratrum alkaloids, protoveratrines (Figure 1.10). Only protoveratrine **A** shows high insecticidal activity against housefly and milkweed bug.

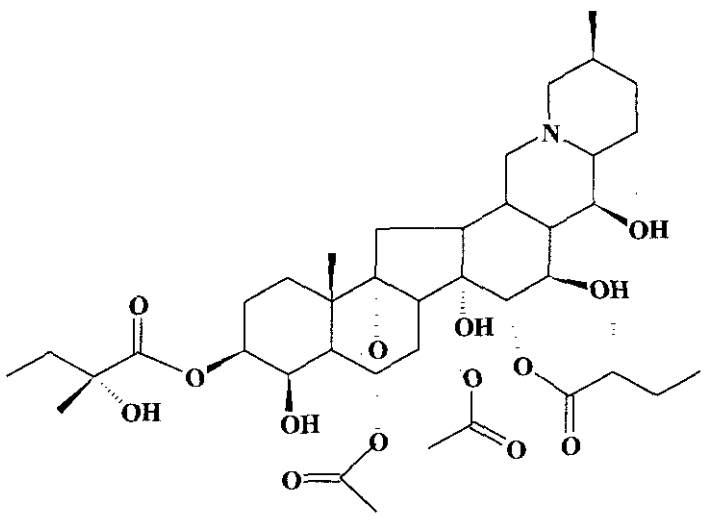


Figure 1.10 : Protoveratrine **A**

Recent SAR studies of veratrum alkaloids have been based on the variation of the 3-acyl group of veracevine.[29] The 3,5-dimethoxybenzoyl derivative was the most active compound, almost twice as insecticidal as the natural 3,4-dimethoxy derivative, but without an increase in mammalian toxicity (Figure 1.9).

(c) **Nicotine**

One of the most important natural alkaloids used in insect control is nicotine (Figure 1.11).[30] Nicotine is found in many species of *Nicotiana* (Solanaceae) but *N. rustica* L. is a much better source of this compound than the more familiar tobacco plant, *N.tabacum* L. The use of this plant as an insecticide dates back at least 200 years. Nicotine affects the nervous system by binding to the acetyl choline receptors[28]. It is little used today due to its high mammalian toxicity and low persistence.

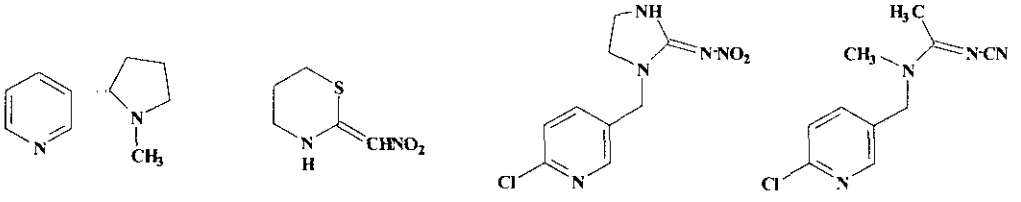


Figure 1.11 : Nicotinoids

The most significant advance in the development of nicotine based insecticides came with the discovery of imidacloprid (Figure 1.11).[31] Although its discovery was not based on the structure of nicotine, the structural similarity between the two has been recognised. The lead compound, tetrahydro-2-(nitromethylene)-2H-1,3-thiazine[32] (Figure 1.11) originated from random screening and was found to be highly active against a variety of insects, particularly lepidopterous larvae, and has acceptably low mammalian toxicity.[13] However, the major drawback is the extreme photochemical instability. Further development led to imidacloprid which has a

broad spectrum of activity and is particularly active as a systemic insecticide against sucking insects.[31] Its mammalian toxicity is low[31] and it has been shown to function as a nicotinic acetylcholine receptor agonist.[33] Further analogues of imidacloprid with increased spectrum of activity have now been developed, for example acetamiprid (Figure 1.11) which has high contact activity against lepidopterous species.[34]

(d) **Physostigmine**

Physostigmine is an alkaloidal carbamate (Figure 1.12), isolated from seeds of the African vine tree, *Physostigma venenosum* Balf. (Leguminosae). This was widely used by natives as an arrow poison to catch wild animals. The alkaloid was known as a neuro-toxic substance which inhibits Acetylcholine esterase (AchE)[28] and the toxicity is due to the presence of the carbamate group.

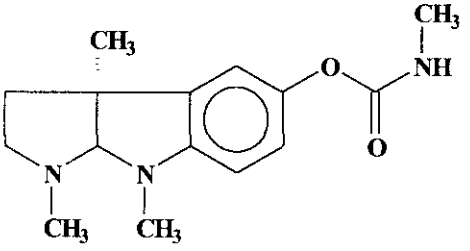


Figure 1.12 : Physostigmine

Recognition of the similarity of the poisoning symptoms of physostigmine and organophosphates, led to the development of synthetic carbamate insecticides in the 1950s. Early compounds developed include metolcarb (MTMC), carbofuran and methomyl (Figure 1.13). However, they had rather high mammalian toxicity.

Structural modifications involving additional metabolisible groups on the N-atom reduced toxicities against mammals, and also altered the spectrum of activity against pests. For instance, carbosulfan and benfuracarb (Figure 1.13) were specific insecticides against rice pests, water weevils and thrips. Further SAR studies led to the development of many other useful carbamate insecticides, including triazamate (Figure 1.13).[35, 36]

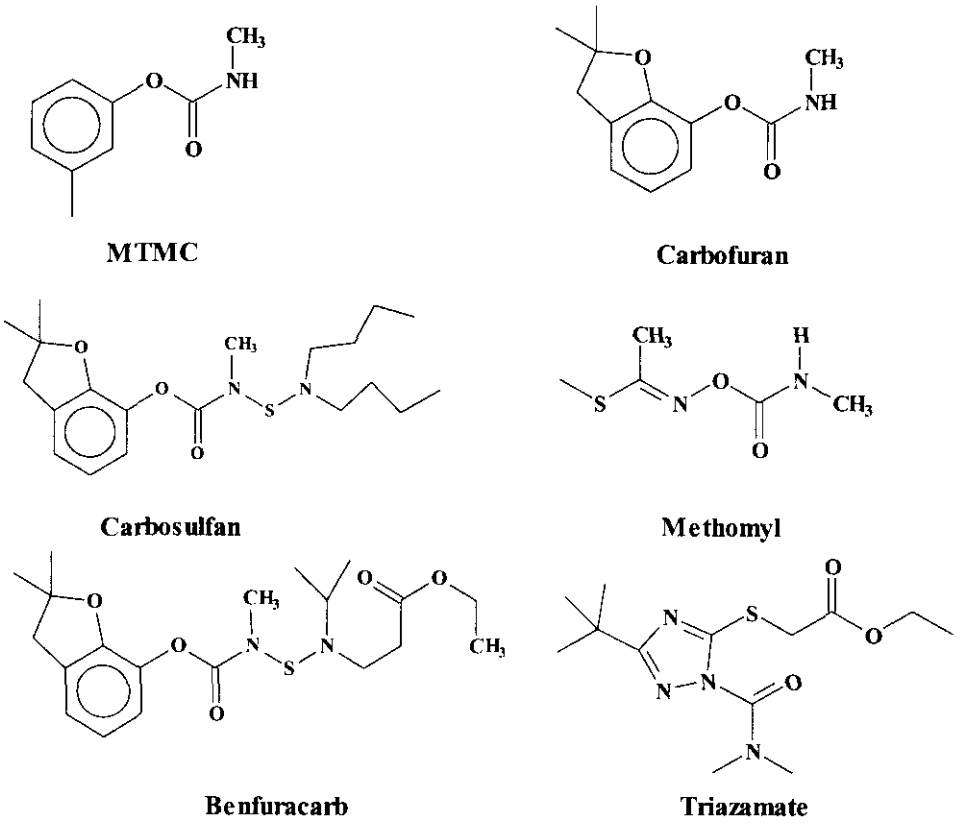


Figure 1.13 : Synthetic carbamates

1.3.4 **Isoflavonoids**

(a) **Rotenone**

Derris extract is a well-known botanical insecticide which still retains popularity with gardeners. It is obtained from the roots and tubers of *Derris elliptica* and similar compounds are obtained from *Lonchocarpus*, *Tephrosia* and *Mundulea* species [(Leguminosae) Figure 1.14]. The active principles are isoflavonoids that are specific inhibitors of cellular respiration, specifically blocking NADH oxidation.[37] They have moderate toxicity to vertebrates (rat oral LD_{50} ~ 132 mg/kg). However, rotenone is extremely toxic to fish and its principle use is as a commercial piscicide, following a traditional practice dating back at least 300 years. As an insecticide, rotenone is used primarily as a dust for home and garden use against horticultural pests.

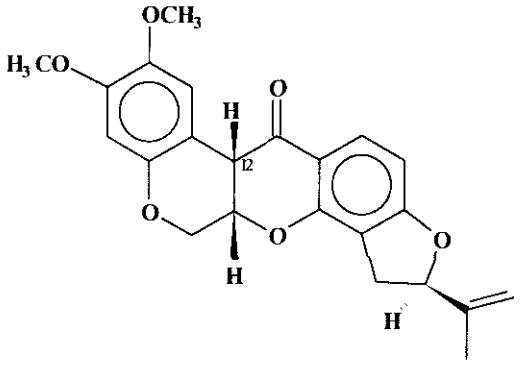


Figure 1.14 : Rotenone

SAR studies on synthetic analogues of rotenone have identified the key features necessary for activity.[38, 39] However, the analogue in which a methyl group is introduced at position 12 has been found to be more active than rotenone.

1.3.5 **Terpenoids**

(a) **Azadirachtin**

One of the few botanical insecticides attracting current interest as a natural product is neem oil, obtained from the seeds of the Indian neem tree, *Azadirachta indica* A. Juss (Meliaceae). Neem oil contains more than 70 triterpenoids but the most important active constituent is tetranortriterpenoid, azadirachtin (Figure 1.15).[40] This compound was first isolated in 1968 by Morgan owing to its outstanding antifeedant effect against the desert locust, *Schistocerca gregaria*[41] but the structure was not established fully until 1985.[42]

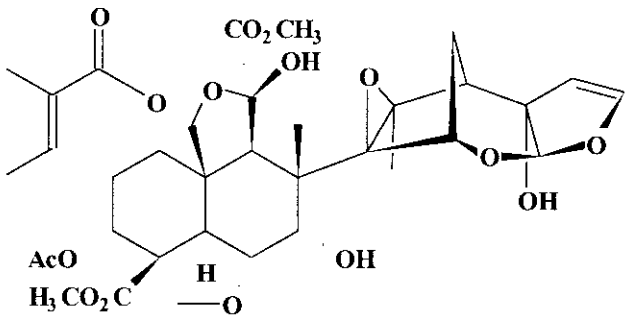


Figure 1.15 : Azadirachtin

Subsequent experiments demonstrated that azadirachtin also disrupted insect moulting at very low doses, and interfered with reproduction in adult insects. Therefore, neem differs markedly from other botanical insecticides in that it is not an acute toxin, but rather a natural insect growth regulator. The major physiological effects in insects are a consequence of reduced synthesis of ecdysteroids.[43] In addition, there is evidence for the direct action of azadirachtin on other organ

systems, including the midgut and the epidermis, as well as a well-characterised direct effect on the gustatory chemosensilla.[43]

Neem is a broad spectrum insecticide, particularly effective against lepidopteran, coleopteran and dipteran pests.[44] Neem has relatively poor contact toxicity, and for most pests it must be ingested to be effective which at least partly accounts for the selectivity favouring natural enemies in the field, making neem compatible with most integrated pest management programs.[45]

The mammalian toxicity of azadirachtin has not been published, however, products containing 3% azadirachtin are essentially nontoxic (rat oral LD_{50}> 5 g/kg). Azadirachtin is very susceptible to photodegradation, ensuring its environmental nonpersistence. Its combination of pest controlling effects (broad spectrum insect growth regulator and antifeedant, with some systemic action) and environmental attributes (nonpersistent, safe for pollinators, non-toxic to vertebrates) is unmatched among other natural insecticides.

The synthesis of azadirachtin has not yet been achieved although a great deal of work in this area is being undertaken.[40]

(b) **Toosendanin**

Toosendanin, a limonoid derived from the bark of the chinaberry trees (*Melia toosendan* and *Melia azadarach*; Meliaceae), was developed as a botanical insecticide in China over the past decade (Figure 1.16). It is an antifeedant, stomach poison and growth inhibitor, effective against lepidopteran pests[46] and least 100 times less active than azadirachtin. Toosendanin has been registered for use against a broad spectrum of orchard and vegetable pests in China such as, three-spotted plusia, cabbage worm, turnip sawfly, potato lady beetle and oriental tobacco budworm.

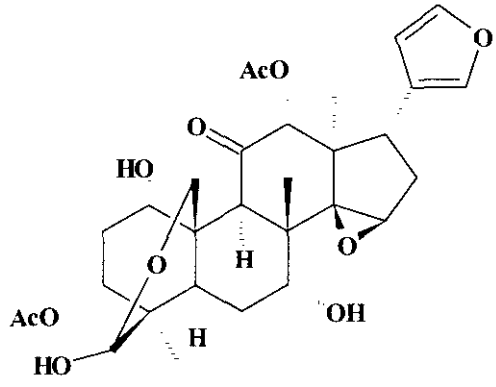
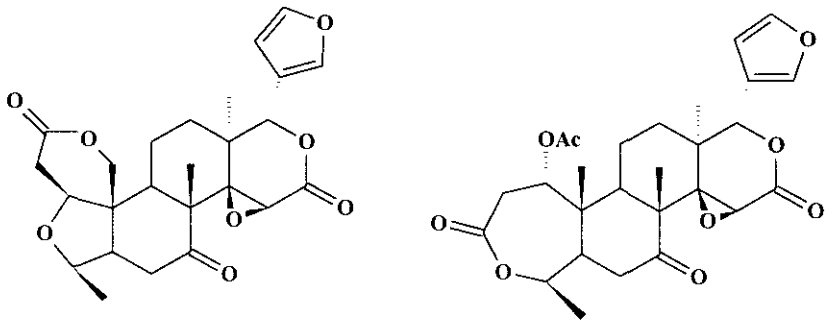


Figure 1.16 : Toosendanin

Vertebrate toxicity appears to be quite favourable (mouse oral LD_{50} >10 g/Kg). The technical grade active concentrate contains 60-80% of toosendanin as the principle active ingredient. However, the extracts are consistently more insecticidal than pure toosendanin itself, indicating the presence of more active constituents or synergistic effects.[47] The mode of action of toosendanin is unknown and seems to be a presynaptic blocking agent which acts on the neuromuscular system.

(c) **Citrus limonoids**

The major active limonoids isolated from the grapefruit seeds (Rutaceae) are modified triterpenes, limonin and nomilin (Figure 1.17).[48] Limonin is a potent antifeedant to the Colorado potato beetle (*Leptinotarsa decemlineata*), the most important pest of potatoes in North America and Europe.[49]



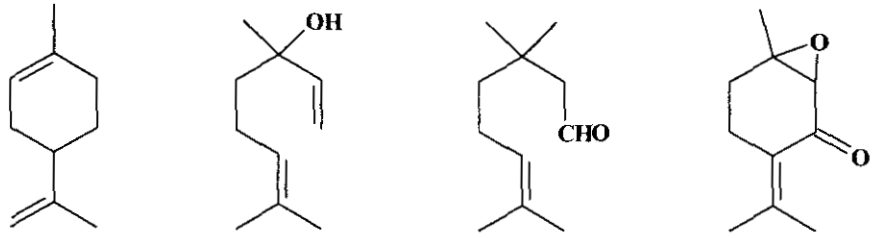
Limonin **Nomilin**

Figure 1.17 : Citrus limonoids

Nomilin is more active against lepidopterans as a larval growth inhibitor.[48] The vertebrate toxicity of these citrus limonoids is low.

(d) **Miscellaneous terpenoids**

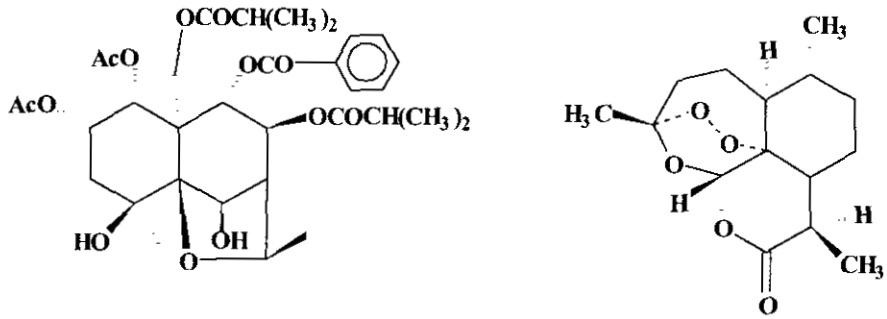
The insecticidal activity of monoterpenoids, especially from essential oils, has been evaluated against insect pests and some showed modest activities. These include limonene, linalool, citronellal and pulegone-1, 2-epoxide (Figure 1.18).[50, 51, 52, 53]



Limonene **Linalool** **Citronellal** **Pulegone-1, 2-epoxide**

Figure 1.18 : Monoterpenoids

The root bark of Chinese bitter sweet, *Celastrus angulatus* Max. (Celastraceae) has been used to control pests of vegetables for many years in China. The active constituent is a sesquiterpene polyol ester, angulatin **A** and the related analogues also possess strong antifeedant activities (Figure 1.19).[54]

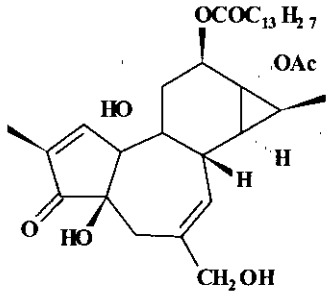


Angulatin A **Artemisinin**

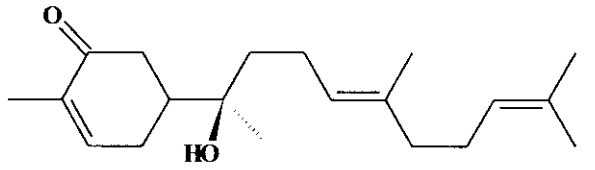
Figure 1.19 : Sesquiterpenoids

Members of the genus *Artemisia* (Asteraceae) produce a series of sesquiterpene lactone endoperoxides, many of which are active as insecticides and antifeedants and also fungicides, herbicides, antimicrobials and antimalarials (Figure 1.19).[55]

The tigliane diterpene ester, isolated from the seeds of *Croton tiglium* L. (Euphorbiaceae) and two other closely related esters exhibited both growth inhibitory and insecticidal activities (Figure 1.20).[56] The novel diterpene isolated from *Croton linearis* Jacq. showed activity to adult weevil, *Cylas formicarius elegantulus*, a serious pest on sweet potatoes (Figure 1.20).[57] Symptoms of poisoning were typical of neurotoxic insecticides.



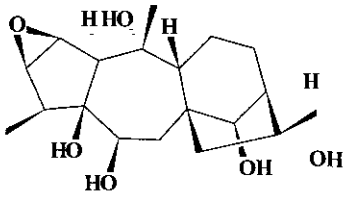
Tigliane ester



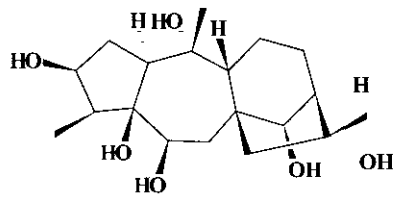
Diterpene from *C.linearis*

Figure 1.20 : Diterpenoids from *Croton* species

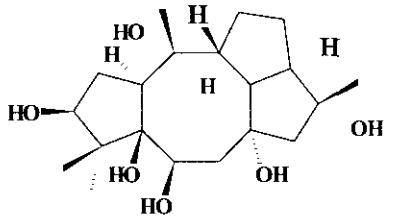
The grayanoid diterpenes, rhodojaponin III, grayanotoxin II and kalmanol, isolated from the dried flowers of the Chinese insecticidal plant, *Rhododendron molle* (Ericaceae) exhibited antifeedant, growth inhibitory and insecticidal activities against *Spodoptera frugiperda* and *Leptinotarsa decemlineata* (Figure 1.21).[58]



Rodojaponin III



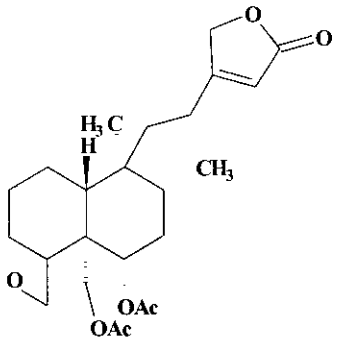
Grayanotoxin II



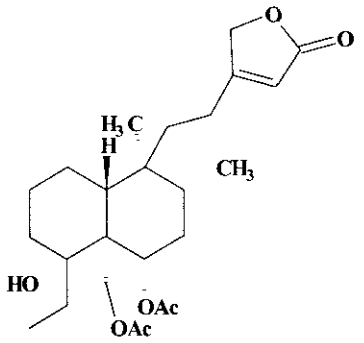
Kalmanol

Figure 1.21 : Grayanoid diterpenoids

A number of clerodane diterpenoids isolated from *Ajuga* plants (Labiatae)[59] showed potent insect antifeedant and modulating hormone activities, including feedant deterrent activity on the Mexican bean beetle, bollworm and *Pieris brassicae*[60] and juvenilisation of the beetworm.[61] The insecticidal principles are ajugarins, **I**, **II** (6-deacetylajugarin) and **III** (Figure 1.22).[59]



Ajugarin I



Ajugarin III

Figure 1.22 : Ajugarins

Conifers of the *Podocarpus* species (Podocarpaceae) are known to be resistant to many insects.[62, 63] The active principle is a norditerpene dilactone referred as nagilactone **D** (Figure 1.23).

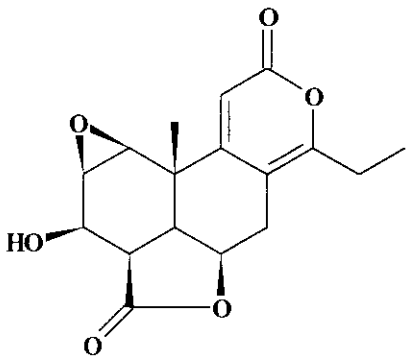


Figure 1.23 : Nagilactone **D**

1.3.6 **Unsaturated amides**

(a) **Isobutylamides**

Naturally occurring long-chain unsaturated isobutylamides from plants of the Piperaceae, Asteraceae and Rutaceae families have long been recognised to exhibit insecticidal properties.[64] One of the first structures of this type to be identified was pellitorine (Figure 1.24), isolated from *Anacyclus pyrethrum* (Asteraceae). The related compounds affinin, isolated from *Heliopsis longipes* Blake, pipercide, dihydropipercide and guineensine (Figure 1.24), isolated from the fruit of black pepper, *Piper nigrum* L. (Piperaceae), showed higher knockdown and lethal actions against adzuki bean weevil[65] and mosquito larvae.

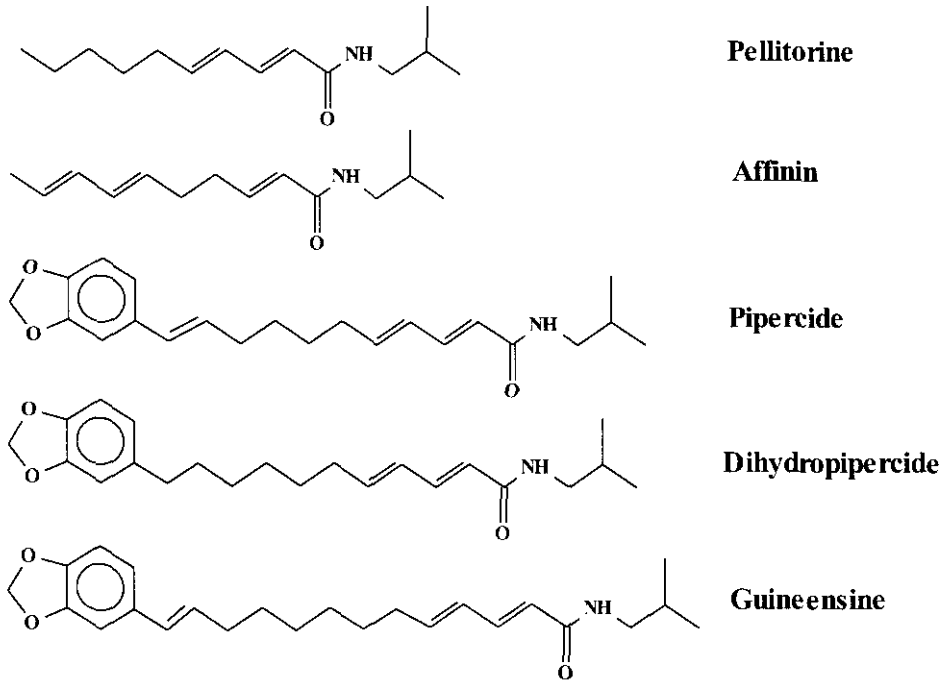


Figure 1.24 : Natural N-alkylamides

The derivatives which hold halogenated phenoxy rings substituted for the methylene-dioxy benzyl terminal group, such as the 3-bromophenyl ether analogue (Figure 1.25A), showed the greater insecticidal activities, about fifty times more than the natural parent molecule, dihydropipercide (Figure 1.24).[65] Structural modification by shortening the chain between phenyl and carbonyl and replacing terminal methylenedioxyphenyl by 6-chloro-2-napthyl or 3,5-difluorophenyl groups led to dienamide analogues **B** and **C** (Figure 1.25). These modified molecules exhibited potent activity against both houseflies and mustard beetles.[66]

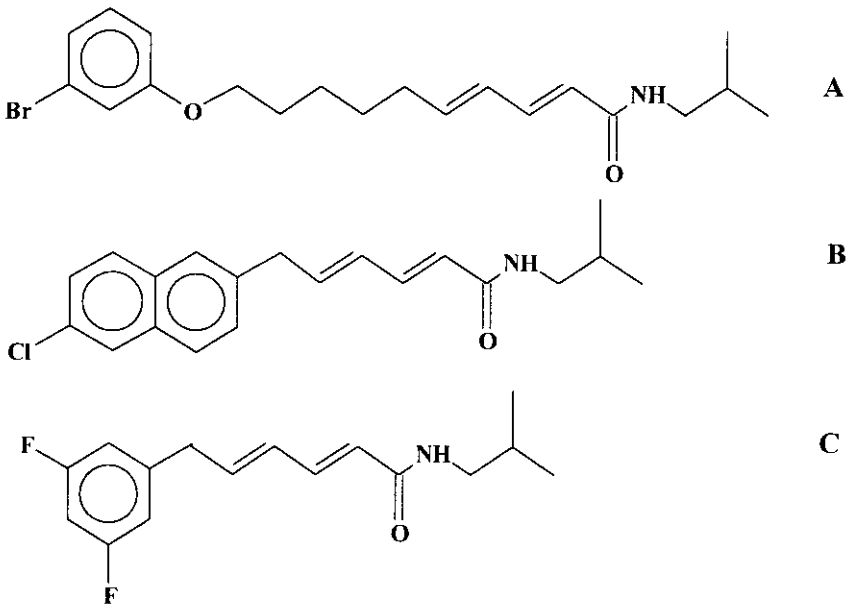


Figure 1.25 : Synthetic N-alkylamides

The isobutylamides cause rapid knockdown and kill of flying insects, in particularly pyrethroid resistant (kdr) strain of houseflies[67] but they are too unstable for use as agricultural products. They have been found to act by voltage-dependent blocking of the sodium channel.[68]

1.3.7 **Benzofurans**

(a) **Rocaglamides**

Rocaglamide, a highly substituted benzofuran (Figure 1.26) is the insecticidal constituent of *Aglaia odorata* (Meliaceae) which is an ornamental shrub, native to Indo-Malaysia.[69] This compound had previously been reported from the related species *A. elliptica* as an anti-leukemic drug. Rocaglamide inhibits larval growth and is insecticidal to both variegated cutworms and Asian armyworms.[70, 71] Related compounds isolated from the same plant also exhibited insecticidal activity.[72]

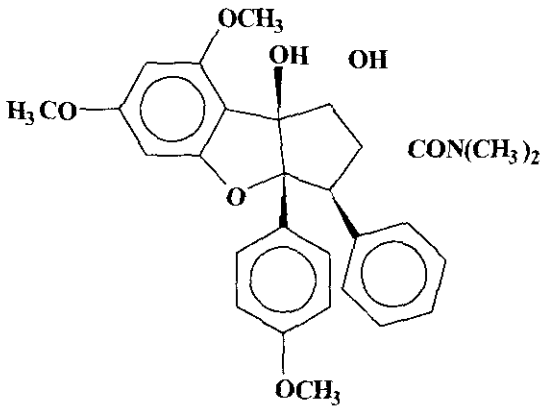
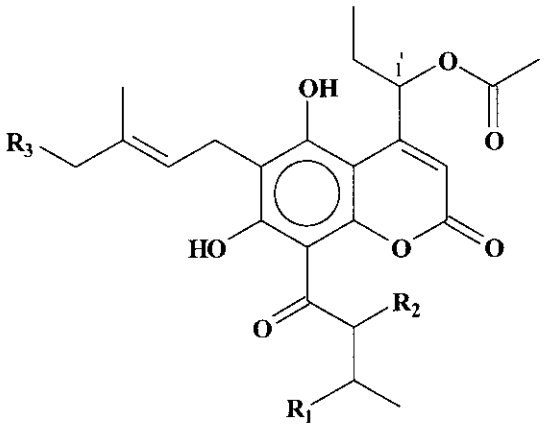


Figure 1.26 : Rocaglamide

Rocaglamide is a slow acting-toxin, but feeding is severely inhibited almost immediately in treated insects, suggesting that central inhibition of the feeding process is the proximate mode-of-action.[71]

1.3.8 **Coumarins**

The insecticidal principle of the genus *Mammea* (Guttiferaceae) is a coumarin which possess an acetyl functionality at the 1' position (Figure 1.27).[73] A series of insecticidally non-active related coumarins have been isolated from *Mammea* species and show that the acetoxy group at the 1' position is critical for activity. Mammalian toxicity has not been reported, but many natural coumarins are highly fish toxic. The powdered seeds are very toxic to common insect pests such as fall army worm, diamondback moth larvae, aphids and rice weevil as well as for larvae of mosquitoes, cockroaches, flies and ants.



$R_1 = CH_3$, $R_2 = R_3 = H$

$R_1 = H$, $R_2 = CH_3$, R_3 = Isopentenyl

Figure 1.27 : Mammea coumarins

1.3.9 **Acetogenins**

(a) **Annonins**

A series of insecticidal acetogenins have been isolated from seeds of *Annona* species (Annonaceae), sweetsop (*A. squamosa*) and soursop (*A. muriculata*). They have also been used as a traditional insecticide in many tropical countries. This species has long been known to possess several other types of biological activities including being cytotoxic, antitumor, antimalarial and antimicrobial. The insecticidal action of these plants was originally attributed to a series of benzylisoquinoline alkaloids known as annonaines (Fig 1.28).[74]

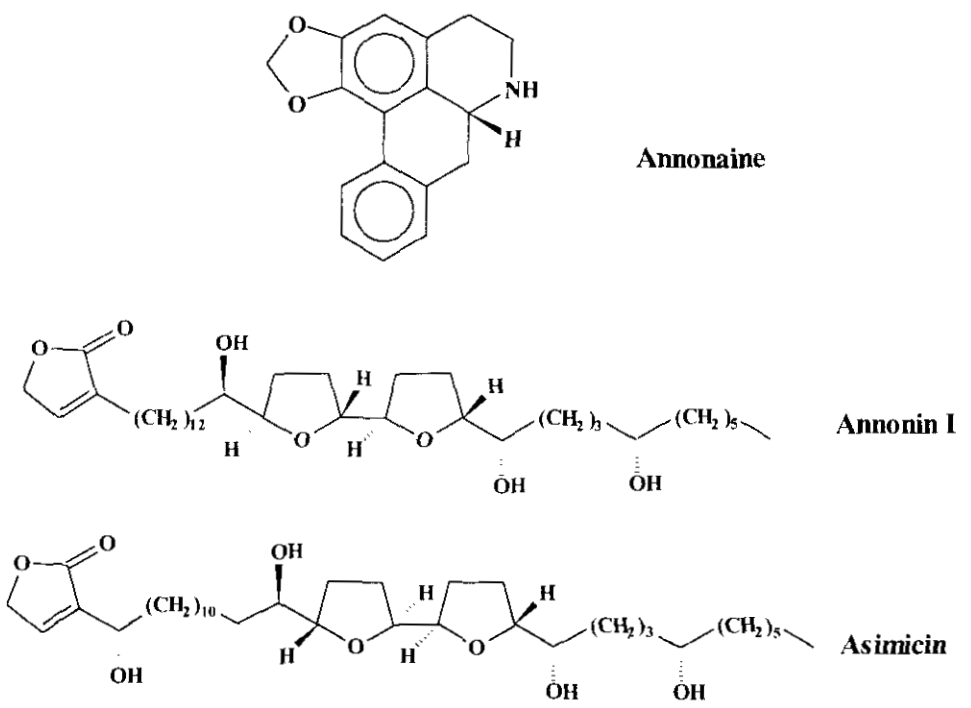
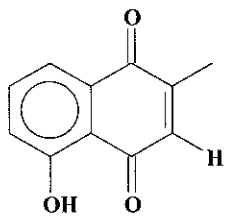


Figure 1.28 : Insecticidal compounds of *Annona* species

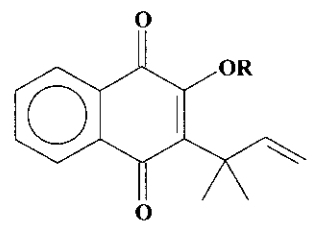
However, recent investigations have confirmed that the active principles are acetogenins and the most active principle is annonin **I** isolated from seeds of *A.squamosa* (Figure 1.28).[75] These acetogenins are slow acting toxins, particularly effective against lepidopterans such as diamondback moth, lady bird beetle and leafhoppers. They inhibit mitochondrial respiration via specific inhibition of NADH-cytochrome reductase, an action analogous to that of rotenone.[76, 77] Pure annonins exhibit significant vertebrate toxicity. [77] Another closely related insecticidal acetogenin, asimicin (Figure 1.28) was reported from the bark of the temperate pawpaw tree (*Asimina triloba*).[78]

1.3.10 Naphthoquinones

The first reported insecticidal naphthoquinone was plumbagin (Figure 1.29), isolated from *Plumbago euroepea* (Plumbaginaceae).[79] Recently, hydroxynaphthoquinones (Figure 1.29) have been isolated from a South American plant *Calceolaria andina* (Scrophulariaceae).[80]



Plumbagin



R = H, Ac

Hydroxy naphthoquinones

Figure 1.29 : Naphthoquinones

These compounds showed activity against houseflies, mustard beetles and especially against tobacco whitefly (*Bemisia tabaci*), two-spotted spider mite (*Tetranychus urticae*), and the peach-potato aphid (*Myzus persicae*), species that show resistance to many of the current commercial insecticides. The whitefly and mite activities are comparable to that of pyrethrins and rotenones and greater than those of several established synthetic insecticides.[81]

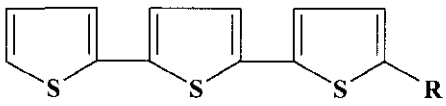
1.3.11 **Miscellaneous compounds**

(a) **Photoactivated insecticides**

Polyacetylenes, thiophenes and furanocoumarins are the most important classes of light-activated insecticidal compounds.[82, 83] They are only active in the presence of UV light and undergo photo-oxidation to generate activated oxygen species such as singlet oxygen or less commonly superoxide radicals. These activated oxygens cause damage to macromolecules by biological oxidations and disrupt biomembranes. In addition, a photosensitiser can intercalate with DNA molecules and under light activation, the excited state sensitiser undergoes cycloaddition reactions to form mono or difunctional adducts disrupting DNA molecules.[84] The major disadvantages of phototoxins are that they are general biocides and are rapidly photodegraded.

The thiophene, α-terthienyl, isolated from the marigold, *Tagetes erecta* (Asteraceae) is a potent photosensitising mosquito larvicide.[85] It showed excellent bioactivity comparable to synthetic insecticides against larvae of yellow fever

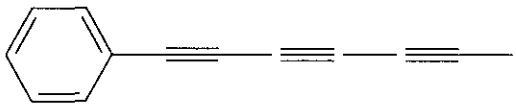
mosquito (*Aedes aegypti*) and malaria mosquito (*Anopheles stephensii*)[85] under artifical UV or natural sunlight.



Terthienyl R = H, CN, Me

Figure 1.30 : α-terthienyl

Pure α-terthienyl has appreciable toxicity to vertebrates (rat oral LD_{50} ~ 100 mg/Kg) but a formulation for mosquito control is significantly less toxic (LD_{50} >5 g/Kg).

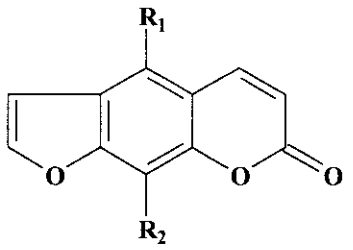


Phenyl heptatriyne

Figure 1.31 : Polyacetylenes

All photoactivated polyacetylenes have been isolated from plants of the family Asteraceae.[86] The photodynamic mode of action of polyacetylenes is less clear and a novel mode of action has been suggested.[83]

Furanocoumarins have been reported mainly from Apiaceae and Rutaceae. They react with pyrimidine bases in DNA and RNA on light activation.[87]

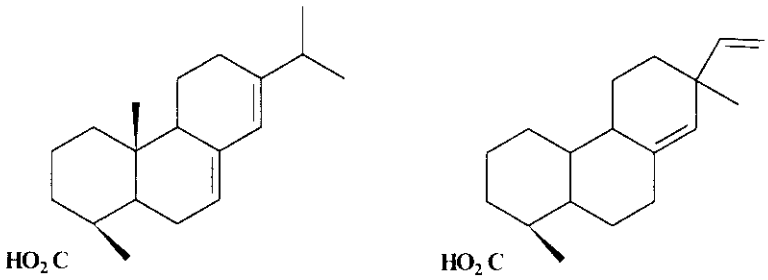


Xanthotoxin $R_1 = H$, $R_2 = OCH_3$, Psoralen $R_1 = R_2 = H$, Bergapten $R_1 = OCH_3$, $R_2 = H$

Figure 1.32 : Furanocoumarins

(b) Industrial by-products - Tall oil

Tall oil, a major by-product of the Kraft process for pulping softwood, has been shown to have insecticidal properties. The diterpene resin acids including abietic, dihydroabietic and pimaric acids are the major active principles in tall oil (Figure 1.33).[88]



Abietic acid **Pimaric acid**

Figure 1.33 : Insecticidal diterpene resin acids

They are antifeedants and growth inhibitors for agricultural insect pests such as variegated cutworm, cabbage looper and green peach aphid. Tall oil is currently being evaluated as an alternative to petroleum and mineral-based horticultural oils for control of soft-bodied insects and mites in vegetable and fruit crops.

1.4 IDENTIFICATION OF INSECTICIDAL NATURAL PRODUCTS

In the course of identification of novel insecticidal natural products, the collection of plants is the important starting point. There is no defined criterion for collection of plants. They can be collected at random or selected, using ethnobotanical and chemotaxonomic information. Chemotaxonomic studies of related species and families of the active plant can often provide an array of related compounds especially suitable for structure activity relationship studies. In addition, plants can be selected based on geographical locations, plant type and endangered species. Another alternative is the screening of plant materials available as industrial by products such as Grapefruit seeds[48] and wood extractives.[88] In plant selection, it is also important to consider plants with minimal pest damage and known biological activity as well as their ability to survive in harsh environments.

Different extraction techniques can be employed in sample preparation; e.g. the shake-filter method, sonication, soxhlet extraction, homogenisation, forced flow leaching, and dissolution[89] and as well as supercritical fluid (SFE)[90], accelerated solvent[91], microwave assisted solvent[92, 93], automated solvent[89] and high pressure soxhlet extractions (HPSE).[94]

The type and design of bioassay systems are also important in the identification of insecticidal natural products. Choice of life stages (e.g. adults, larvae and eggs), application routes (e.g. topical, residual, vapour and systemic), in *vivo* or in *vitro* bioassays are all important.

Once an activity has been found, the isolation of pure active compounds from the plant extracts is performed using bio-activity-guided fractionation procedures. For this combinations of chromatographic techniques are employed such as planar, column, including flash and vacuum techniques[95, 96, 97], low-pressure liquid (LPLC), medium-pressure liquid (MPLC), high-pressure liquid (HPLC), countercurrent and supercritical fluid (SFC)[90] chromatography.[98] Multistep chromatographic methods coupled with spectrometry can also be employed such as GC-MS, LC-MS[99, 100], LC-UV-MS[99], MAE-GC (Microwave-Assisted Extraction Coupled with Gas Chromatography)[101], LC-NMR[102, 103], and LC-SFC.[104]

The structure of compounds is established using spectroscopic techniques (NMR, MASS, IR, UV and X-ray crystallography). 2D NMR techniques such as COSY, HMQC, HMBC, NOESY, TOCSY and INADEQUATE[105, 106, 107] are exceptionally useful in structure elucidation.

1.5 **OBJECTIVE OF THE THESIS**

Over 400 plant species were collected from Sri Lanka and 300 species were previously unrecognised plants for insecticidal activity. Several active extracts were identified from preliminary bio-assay studies conducted at Rothamsted against a range of susceptible and resistant strains. In particular, three species, *Pleurostylia opposita* (Wall) Alston (Celastraceae), *Aegle marmelos* Correa (Rutaceae) and *Excoecaria agallocha* Linn. (Euphorbiaceae) were shown to have previously unrecognised insecticidal activity.

The object of the research described in this thesis was to identify novel insecticidal natural products from these Sri Lankan plants and to carry out preliminary structure activity relationship studies to find more promising active analogues.

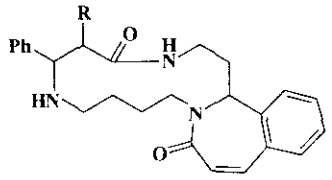
The following section reviews the existing knowledge about the three plants as relevant to the present study.

1.6 REVIEW OF PREVIOUS LITERATURE ON *PLEUROSTYLIA OPPOSITA*

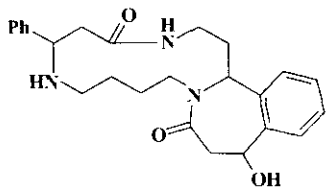
1.6.1 Introduction

Pleurostylia opposita (Wall) Alston (Celastraceae) is a moderately sized timber tree found in the dryzone of Sri Lanka. Timber is used for building construction and for furniture. The genus was considered to be a monotypic but more recently, six species[108] have been reported from Africa, Madagascar, India, Sri Lanka, Indo-Malaysia, Queensland and New Caledonia. No previous reports on the biological activity of extracts from the genus *Pleurostylia* are known.

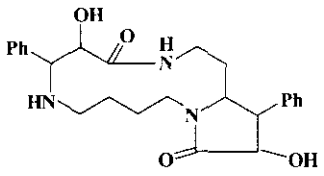
1.6.2 Chemistry of *Pleurostylia*



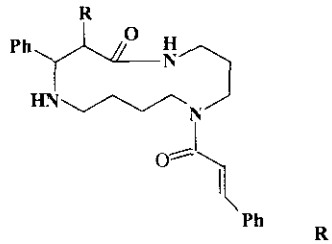
	R
7-hydroxypleurostyline	OH
Pleurostyline	H



7'-hydroxy-7', 8'-dihydropleurostyline



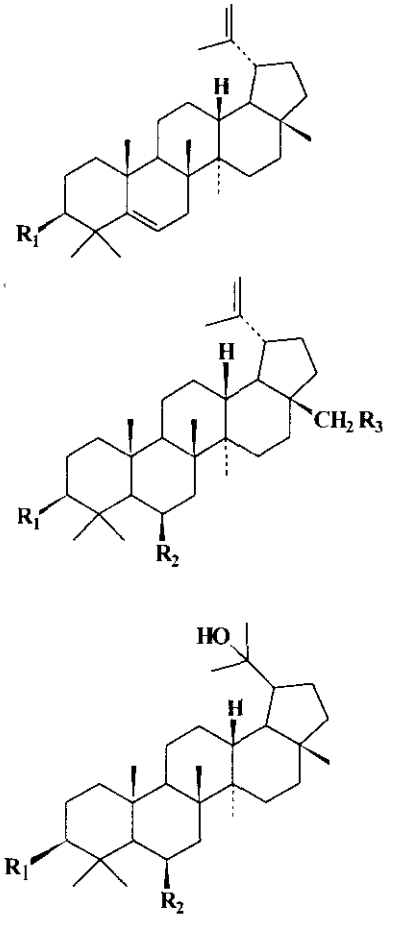
7-hydroxypleurocorine



	R
7-hydroxycelacinnine	OH
Celacinine	H

Figure 1.34 : Spermidine alkaloids

Pleurostylia has not been widely investigated for phytochemical constituents. Previous research has led to the isolation of macrocyclic spermidine alkaloids[109, 110, 111] from leaves of *P. opposita* and *P.affricana* (Figure 1.34). The related macrocyclic lactams derived from spermidine are known to possess several therapeutical activities.[110] In addition, several triterpenoids including oxygenated lupanes (Figure 1.35), friedelanes, α-amyrin, pristimerin and a sterol, β-sitosterol have also been reported from the stem bark and leaves of *P. opposita*.[112, 113, 114]



	R_1	R_2	R_3
5, 20(29)-Lupadien-3-ol	OH		
5, 20(29)-Lupadien-3-one	O		
6, 28-Dihydroxy 20(29)-lupen-3-one	O	OH	OH
20(29)-Lupene-3, 6-diol	OH	OH	H
6-Hydroxy-20(29)-lupen-3-one	O	OH	H
6, 20-Dihydroxy-3-lupanone	O	OH	
20-Hydroxy 3 lupanone	O	H	
3, 20-Dihydroxylupane	OH	H	

Figure 1.35 : Lupane triterpenoids

1.7 REVIEW OF PREVIOUS LITERATURE ON *AEGLE MARMELOS*

1.7.1 Introduction

Aegle marmelos Correa (Rutaceae) is a large deciduous tree, commonly known as "Bael", indigenous to India. *A. marmelos* is widely distributed in both dry and wet zones of Sri Lanka. The wood and the edible ripe fruit both have a strong aromatic scent.

A. marmelos is a well known medicinal plant used in an indigenous system of medicine. Practically every part of the tree has been used against various diseases especially for dysentery, asthmatic complaints, fever and various intestinal ailments. In addition, the root bark has been used as a fish poison.[115] The leaves of the plant were used in Bangladesh for fertility control.[116] The oil from seeds is used as a purgative and has shown antimicrobial and anthelmintic properties.[117] The water extract of leaves showed *in vitro* antifungal activity against rice blast and brown spot pathogens of rice plant.[118] The methanolic extract of roots of *A. marmelos* exhibited anti-lipid peroxidative activity.[119] More recently, insecticidal activity has been reported against cotton leaf worm, flour beetle and laboratory flies, but no further work has been done since then.[120]

1.7.2 Chemistry of *Aegle marmelos*

Aegle marmelos has been widely investigated for phytochemical constituents as well as for biologically active compounds. The first chemical investigation on root, seed, bark, leaves and fruits has been carried out by Dikshit and Dutt in 1930.[121]

From the fruit they isolated a furano coumarin, named "marmelosin" (imperatorin) which is an active laxative and diuretic along with reducing sugars and tannin (Figure 1.36).[122]

Since then, a wide range of coumarins, alkaloids, sterols, terpenes, flavonoids, anthraquinones, lignan-glucosides, tannins and volatile oils have been isolated from various parts of the plant. Among the coumarins, the furano coumarins, imperatorin (marmelosin)[122] and allo-imperatorin[123] were isolated from the fruit and xanthotoxin[124], xanthotoxol, marmesin[125], and psoralen[126] from the stem and root barks (Figure 1.36). The pyrano coumarin, aegelinol[127], was isolated from the root and stem barks (Figure 1.36). The hydroxy coumarins, umbelliferone[128] and scopoletin[126], and O-alkylated coumarins, aurapten[128], epoxyaurapten[129], marmin[130], 7-O-methylmarmin[129] and marminal[129], were isolated from the stem and root barks of *A. marmelos* (Figure 1.37).

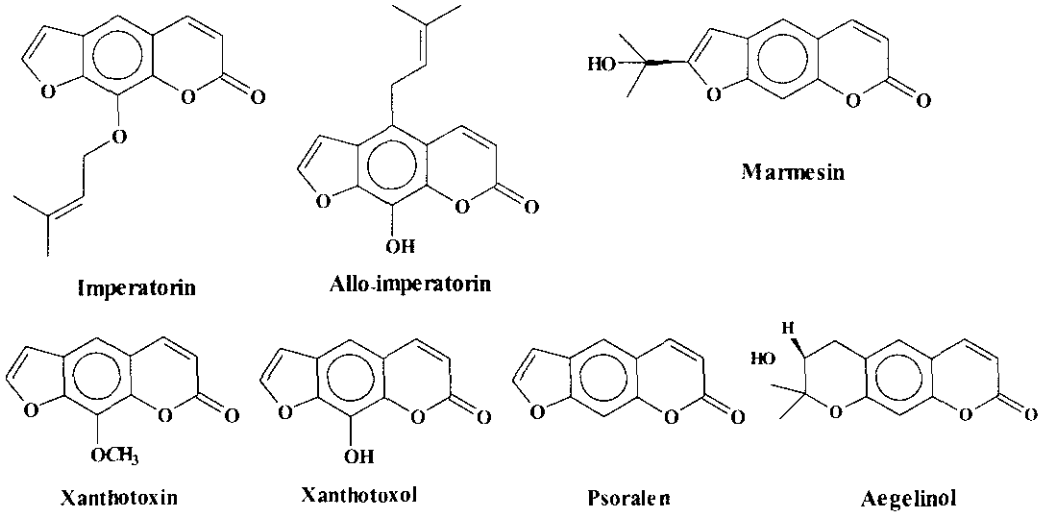


Figure 1.36 : Furano and pyrano coumarins

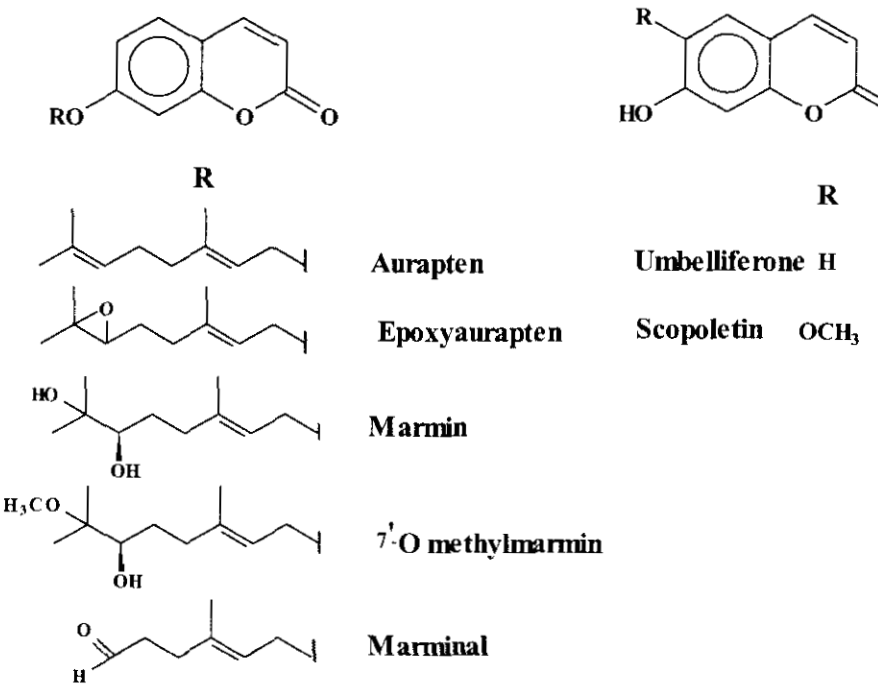


Figure 1.37 : Coumarins

Alkaloids have been widely reported to be present in *A. marmelos*. They include cinnamamide type alkaloidal amides, furoquinolines and halfordinol type alkaloids[131] from leaves, stem and root barks (Figure 1.38 and 1.39). The reported alkaloidal amides include aegeline[132], marmeline[133], tembamide and N-4-methoxystyrylcinnamide.[134]

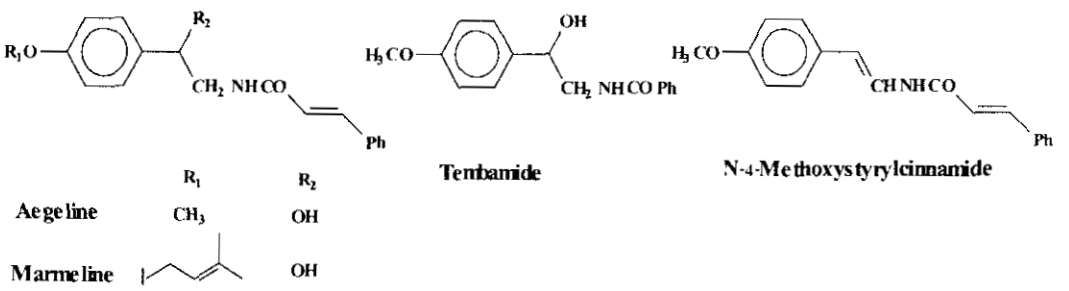


Figure 1.38 : Alkaloidal amides

The furoquinoline alkaloids include rutacin (skimmianine)[135], isoskimmianine[135], haplopine[115] and γ-fagarine [(aegelenine) Figure 1.39).[129]

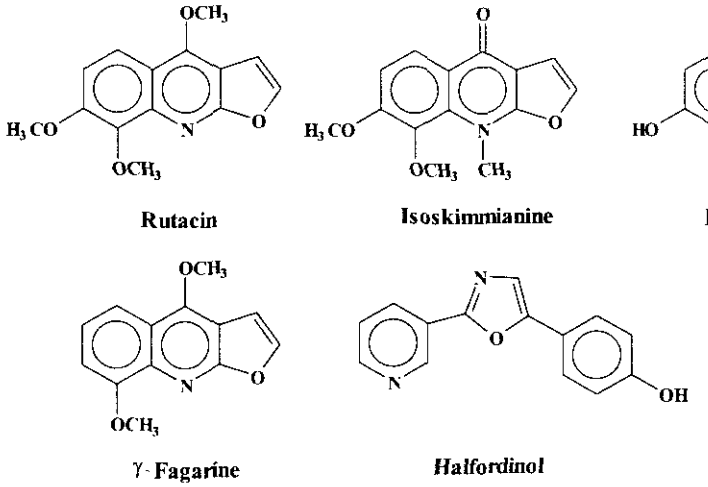


Figure 1.39 : Alkaloids

A number of flavonoids have been identified from *A. marmelos* including rutin and immunomodulatory C-glucosylated propelargonidins (Figure 1.40).[136]

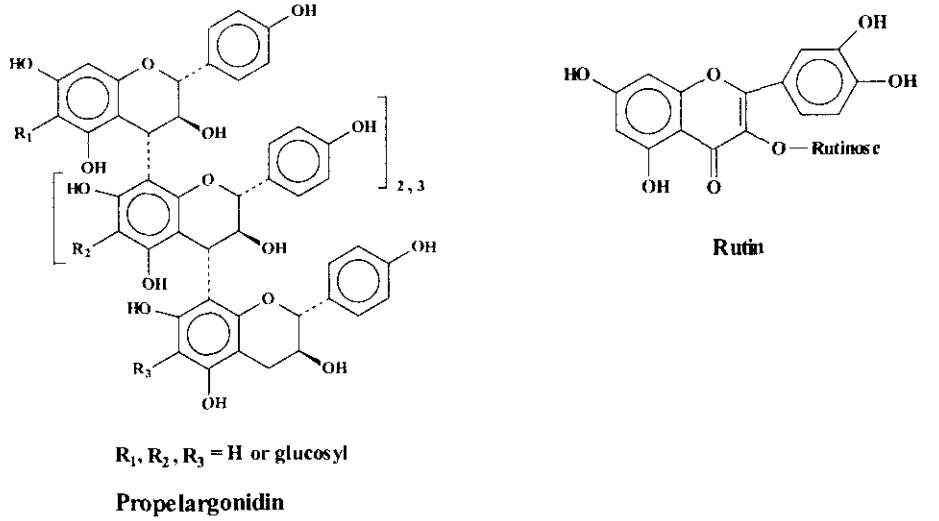
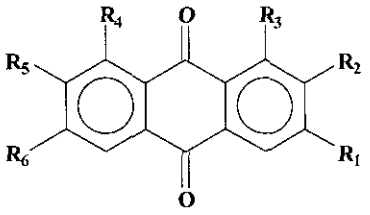


Figure 1.40 : Flavonoids

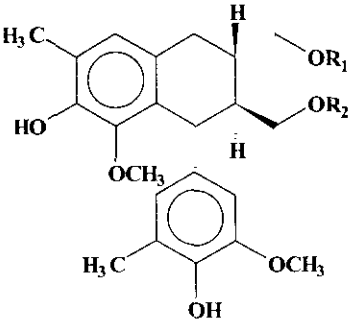
Anthraquinones[137] and several lyoniresinol lignan-glucosides[138] have also been isolated from the stem bark of *A.marmelos* (Figure 1.41).



$R_1 = R_6 = H$, $R_2 = CH_3$, $R_3 = OH$, $R_4 = R_5 = OCH_3$

$R_1 = CH_3$, $R_2 = R_4 = R_5 = H$, $R_3 = OCH_3$, $R_6 = OH$

Anthraquinones



$R_1 = H$, $R_2 =$ D-glucose

$R_1 = R_2 = H$

$R_1 =$ D-glucose, $R_2 = H$

Lignan glucosides

Figure 1.41 : Anthraquinones and lignans

In addition, the leaf oil contains monoterpene hydrocarbons, especially myrcene, oxygenated monoterpenes and sesquiterpenes.[139, 140] Volatile oils have also been reported from the fruit and *cis*-linalool oxide is the major aroma constituent.[141] The pulp and peel of the fruit have a terpene-like aroma due to terpene alcohols and β-ionones.[142] The injured trunk and the fruit exude gums which contain polysacharides.[143, 144]

1.8 REVIEW OF PREVIOUS LITERATURE ON *EXCOECARIA AGALLOCHA*

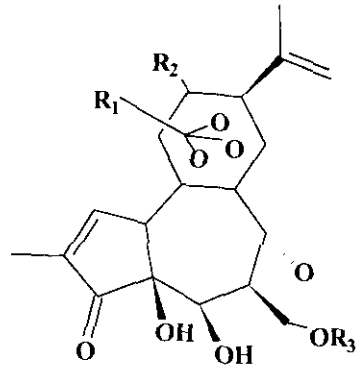
1.8.1 Introduction

Excoecaria agallocha Linn. (Euphorbiaceae) is a small tree known as "blinding tree" which grows extensively in mangrove ecosystems. The genus *Excoecaria* comprises forty species which are distributed throughout tropical Africa and Asia and are well known for the production of toxic metabolites.

The leaves and latex of *Excoecaria agallocha* have been reportedly used as a source of dart-arrow and fish poison in Sarawak[145], New Caledonia[146] and Goa[147]. The piscicidal constituent was identified as a daphnane diterpenoid orthoester.[146] In Thailand, the plant is used in herbal preparations[148] and in Pakistan, the latex has been used as a purgative and abortifacient and for treatment of ulcers, rheumatism, leprosy and paralysis.[149] *Excoecaria* species are very well known for extreme skin irritant and caustic properties, residing especially in their lattices. In addition, the latexes are known to possess biocidal effects on a variety of marine organisms and phytoplanktons as well as fungicidal activity. Surprisingly, no insecticidal activity has been reported from *Excoecaria*.

1.8.2 **Chemistry of *Excoecaria agallocha***

Previous phytochemical studies revealed that daphnane diterpenoid orthoesters, phorbol esters and related homologues occur widely in *Excoecaria* species. They are skin irritants (irritant *Excoecaria* factors) and some are tumour promoters. In *E. agallocha*, daphnane orthoesters have been reported from the latex and all possess orthoester aliphatic unsaturated alkyl side chains (Figure 1.42).[146, 148, 150]



R_1	R_2	R_3
$(CH_2)_8$	H	H
$(CH_2)_6$	H	H
$(CH_2)_4$	H	H
CHO	H	H
CHO	H	H
$(CH_2)_8$	H	H
$(CH_2)_{10}$	H	H
$(CH_2)_8OH$	OH	H
$(CH_2)_6OH$	OH	H

Figure 1.42 : Daphnane orthoesters

In *E. agallocha*, phorbol esters have been identified from the leaves and stem bark and they showed anti-HIV activity (Figure 1.43).[151]

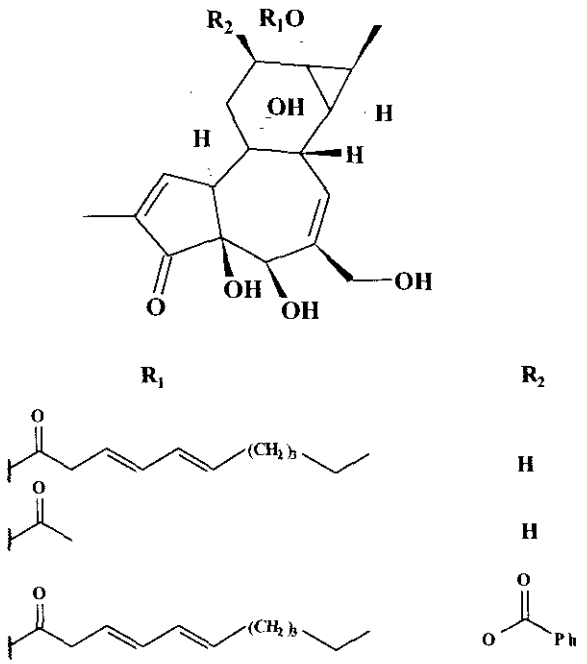
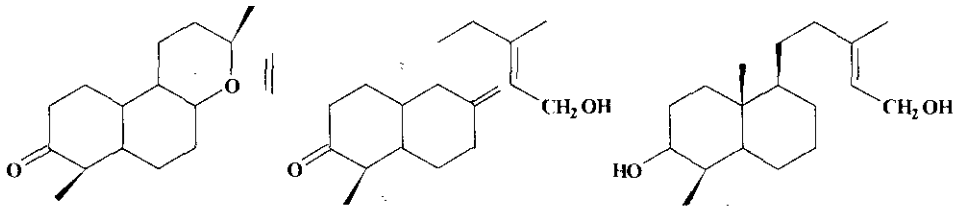
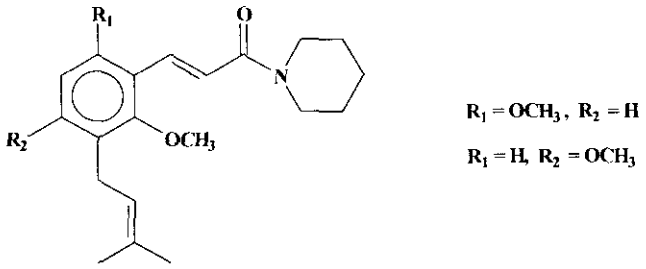


Figure 1.43 : Phorbol esters

Labdane-type diterpenoids[152], piperidine alkaloids[147] (Figure 1.44) and taraxerane triterpenoids[153] were also reported from the stem wood and leaves.



Labdane diterpenoids



Piperidine alkaloids

Figure 1.44 : Labdane diterpenoids and piperidine alkaloids

CHAPTER 2

RESULTS AND DISCUSSION

2.1 INTRODUCTION

Under an exploratory arrangement between IACR Rothamsted and University of Colombo, Sri lanka (1991-1993), over 400 plant species were collected from different geographical locations of Sri Lanka (tropical rain forests, wet, dry and arid parts, mangroves and marine) in a search for novel naturally occurring insecticides from plants. The plant extracts were examined for insecticidal activity against a range of species. Several plants exhibited promising activity (Table 2.1) and work in this thesis describes exploration of three species, *Pleurostylia opposita* (Wall) Alston (Celastraceae), *Aegle marmelos* Correa (Rutaceae) and *Excoecaria agallocha* Linn. (Euphorbiaceae) for their insecticidal constituents. Chromatographic techniques were used in fractionation to isolate the active compounds and the structure elucidation was achieved by spectroscopy, mainly NMR.

Table 2.1 : Promising insecticidal Sri Lankan plants

Plant species	Family	MB*	HF*	PX†	BT†	TU*
Pleurostylia opposita	Celastraceae	100	WT	WT	20	NT
Aegle marmelos	Rutaceae	100	79	20	NT	NT
Excoecaria agallocha	Euphorbiaceae	100	62	NT	NT	50
Acronychia pedunculata	Rutaceae	100	100	WT	NT	NT
Atalanta zeylanica	Rutaceae	90	NT	-	42	-
Feronia limonia	Rutaceae	60	NT	20	-	NT
Choroxylon swiotonia	Rutaceae	NT	50	NT	NT	NT
Walsura piscidia	Meliaceae	100	NT	NT	NT	-

table contd.

Annona squamosa	Annonaceae	100	NT	70	NT	-
Moringa oleifora	Moringaceae	80	NT	20	NT	-
Piper longum	Piperaceae	100	NT	NT	NT	-
Anacardium occidentale	Anacardiaceae	-	65	-	-	-
Acacia leucophloea	Leguminosae	55	NT	WT	-	-
Tephrosia purpurea	Leguminosae	100	NT	NT	-	-
Tagetus erecta	Compositae	90	-	-	-	-
Leucas zeylanica	Labiatae	70	NT	NT	-	-
Anamirta cocculus	Menispermacea	60	NT	NT	NT	-
Ardisia humilis	Myrsinaceae	85	100	30	-	WT
Hortonia angustifolia	Monimiaceae	NT	66	NT	NT	NT
Leea indica	Leeaceae	65	NT	NT	NT	-

Insecticidal activity is given as % mortality (* 20 µg/insect, † 10 µg/insect, ‡ 2000 ppm, ¶ 1000 ppm).

NT : non toxic, WT : weakly toxic (< 20%).

MB : mustard beetle (*Phaedon cochleariae*), HF : housefly (*Musca domestica*),

PX : larvae of diamondback moth (*Plutella xylostella*), BT : whitefly (*Bemisia tabaci*)

TU : mites (*Tetranychus urticae*).

2.2 ISOLATION AND CHARACTERISATION OF COMPOUNDS FROM *PLEUROSTYLIA OPPOSITA*

2.2.1 Introduction

Pleurostylia opposita (Wall) Alston (Celastraceae) is a tropical timber tree, and many such are known to possess insecticidal activity (Meliaceae, Olacaceae, Dipterocarpaceae).[154] The plant species of Celastraceae exhibit a range of biological activities[155], with some having been used to protect plants from insect damage[156, 157], in folk medicine[158], or as stimulants[159] from ancient times. The insecticidal principles of Celastraceae have been recognised as a range of alkaloids based on the β-dihydroagarofuran skeleton and mainly display antifeedant activity.[54, 160, 161]

The work reported here is concentrated on the insecticidal activity of *Pleurostylia opposita*, for which only a few phytochemical constituents have been reported for any member of the genus, and none of them are linked to insecticidal activity. Preliminary bio-assay studies of the insecticidal activity of the petroleum ether, ethyl acetate and ethanol extracts of the stem bark and leaves of *P. opposita* indicated that the stem bark is active against mustard beetles (Table 2.2).

Table 2.2 : Insecticidal activity of *Pleurostylia opposita*

Insect species	Stem bark			Leaves
	A	B	C	C
MB*	100	100	NT	NT
HF*	WT	NT	NT	NT
PX†	WT	NT	WT	NT
BT‡	20	NT	NT	-
TU¶	NT	NT	NT	-

*, †, ‡, ¶, NT, WT, MB, HF, PX, BT, TU - As table 2.1 (Page 57).

A : petroleum ether, B : ethyl acetate, C : ethanol

2.2.2 **Isolation of insecticidal compounds**

Sequential extraction of the stem bark of *P. opposita* showed that the activity was concentrated in the petroleum ether and ethyl acetate extracts (Table 2.2). Bio-assay guided fractionation of these combined active extracts in three steps (dry column flash chromatography, flash column chromatography and HPLC) allowed the isolation of two insecticidal compounds **1** and **2** and non-insecticidal compounds, **3** and **4** (Figure 2.1).

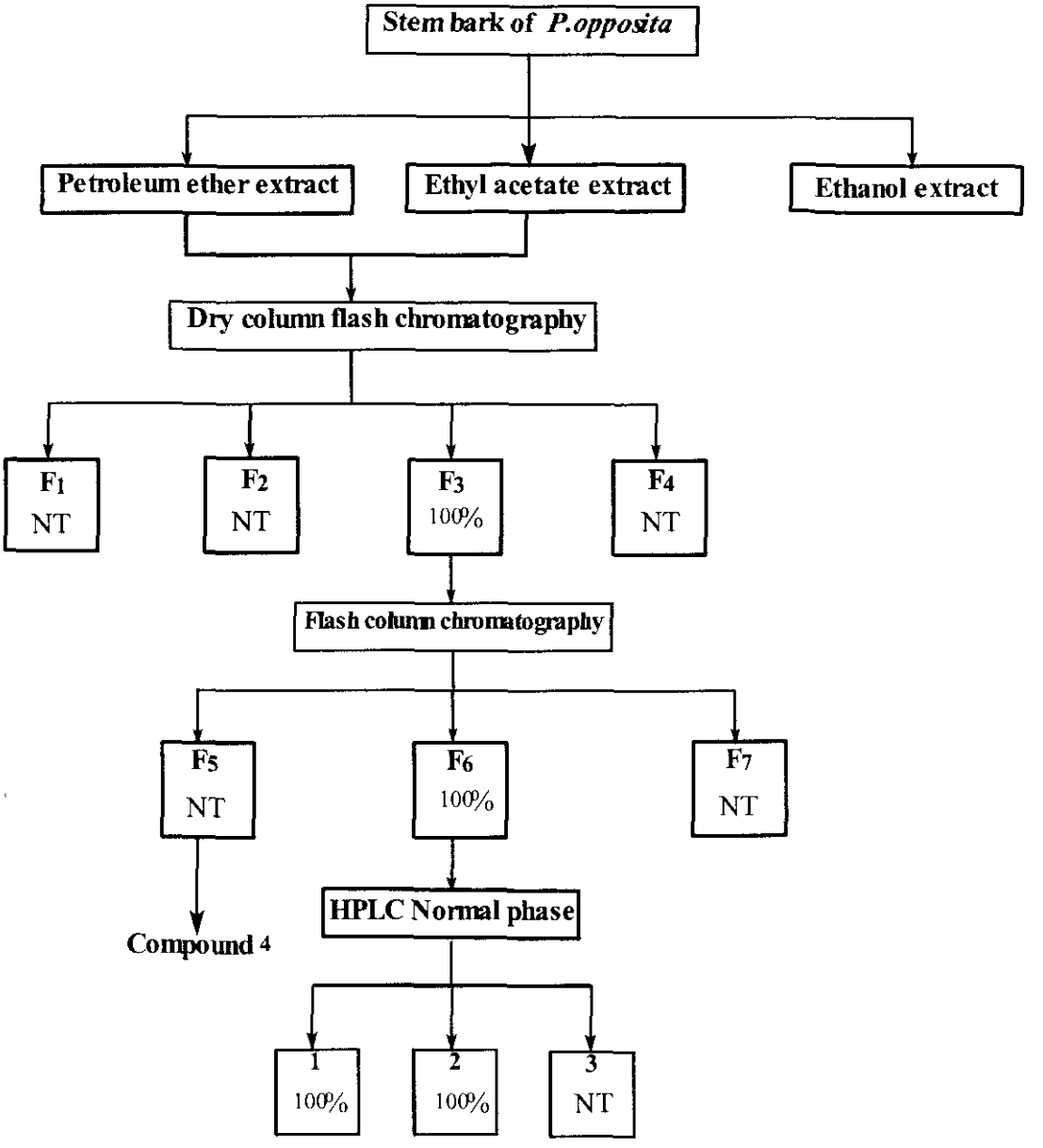


Figure 2.1 : Bio-assay guided fractionation of extracts of *Pleurostylia opposita*

2.2.3 Characterisation of compounds 1-4

(a) Characterisation of compound 1

Compound **1** was isolated from *Pleurostylia opposita* as a yellow oil in 0.03% yield. The compound gave a UV active spot (λ = 254 and 366 nm, 1:2:7, isopropanol:ethyl acetate:petroleum ether, Rf = 0.4) and a purple spot with anisaldehyde-H_2SO_4.[162] The alkaloid nature of the compound was suggested by its appearance as an orange spot with Dragendorff's-reagent.[162]

The IR spectrum revealed the characteristic absorptions of ester carbonyl (1738 cm^{-1}) and hydroxyl (3468 cm^{-1}) functionalities as well as the phenyl group (1467 and 1602 cm^{-1}). The UV spectrum showed absorptions at λ_{max} = 229, 203 and 269 nm.

The ^{13}C NMR spectrum showed the presence of forty four carbons in the molecule (Table 2.6, page 75). The multiplicities of the carbons were determined on the basis of DEPT spectrum, indicating eight methyl, five methylene, sixteen methine and fifteen quaternary carbons in the molecule. The ^{1}H NMR spectrum integrated for fifty one protons in thirty one different chemical environments in the molecule (Table 2.6, page 75). The direct connectivities of carbons and their respective protons were established from the analysis of the ^{13}C-^{1}H COSY and HSQC spectral data.

The high resolution electrospray (API) mass spectrum displayed the molecular ion at m/z 881.3192 which corresponds to a molecular formula of $C_{44}H_{51}O_{18}N$, and therefore a degree of unsaturation of twenty.

The ^{13}C spectrum showed the presence of nineteen sp^2 carbons and twenty five sp^3 carbons, of which eleven were oxygenated (δ 60.0, 68.8, 69.8, 69.9, 70.0, 71.4, 2×73.6, 75.7, 84.9 and 94.0)[163] of these, only one has a hydroxyl group (δ 4.90, br s), so the remainder are involved in C-O-C or C-O-CO groups. Such heavy oxygenation is reminiscent of the sesquiterpene polyol ester alkaloids found in other Celastraceae. Confirmation of this relationship came from comparison with published data, in particular for the 2,3-disubstituted pyridine fragment found in all these compounds.

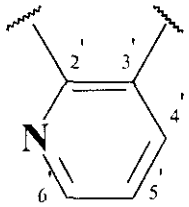


Figure 2.2 : Pyridyl fragment

The Table 2.3 lists the ^{13}C and ^{1}H shifts reported for a typical sesquiterpene polyol ester alkaloid, cangorinine W-II,[164] together with the observed peaks in the NMR spectrum of the compound **1**.

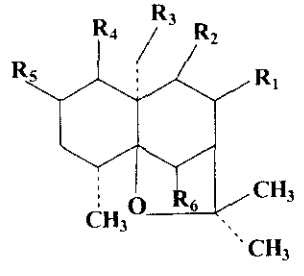
Table 2.3 : ^{13}C and ^{1}H NMR assignments of pyridine fragment

Position	Reported δ_C	Observed δ_C	Reported δ_H (J)	Observed δ_H(J)
2'	164.0	168.5	-	-
3'	124.6	129.4	-	-
4'	138.6	138.1	8.27, dd (8.0, 1.8)	8.40, dd (7.9, 1.8)
5'	121.2	120.7	7.27, dd (8.0, 4.7)	7.30, dd (7.9, 4.6)
6'	153.3	153.8	8.76, dd (4.8, 1.8)	8.83, dd (4.6, 1.8)

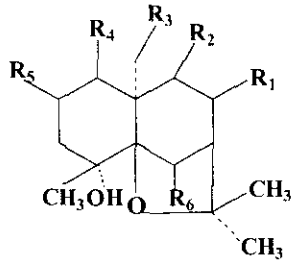
Coupling constants (J values) in parentheses are in Hz.

This clear correspondence justified closer inspection of published data on sesquiterpene polyol ester alkaloids, which is summarised in the next section.

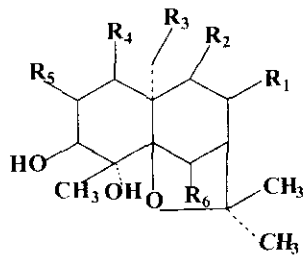
The sesquiterpene polyol ester alkaloids and sesquiterpene polyol esters reported in Celastraceae,[155] all possess a 6,6 ring system as part of a β-dihydroagarofuran skeleton with different ester functionalities (Figure 2.3).



R = OH, OBz, OAc, OCO-nC7H15, O-t-Cinn, OFu, OCO-nC9H19



R = OH, OAc, OBz, OFu

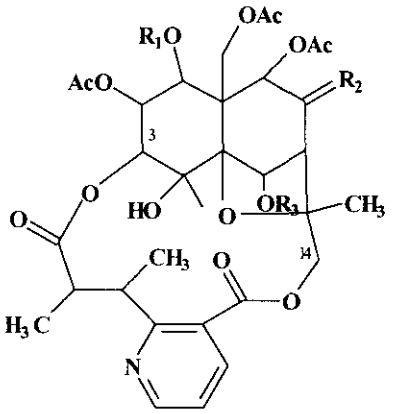


R = H, OH, OAc, OBz, OFu , ONic

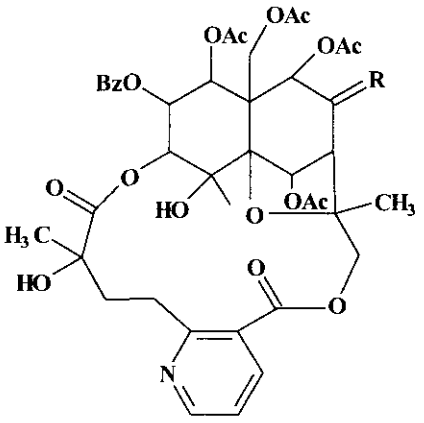
Figure 2.3 : β-dihydroagarofuran sesquiterpene polyol esters

In addition to the β-dihydroagarofuran skeleton, all the reported macrocyclic sesquiterpene polyol ester alkaloids contain a 2,3-disubstituted pyridine moiety which was derived from either evonic acid, wilfordic acid or other related acid

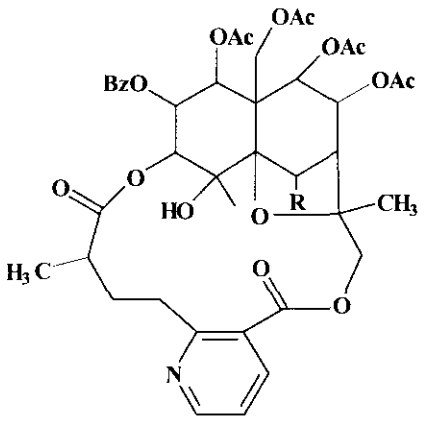
groups (Figure 2.4).[155] This macrocyclic structure was formed by ester linkages between one sesquiterpene molecule and the acid moiety at positions 3 and 14 (Figure 2.4).



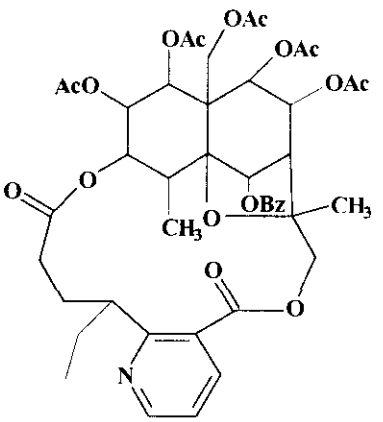
	R_1	R_2	R_3
Euonymine	**Ac**	**β-OAc, α-H**	**Ac**
Mayteine	**Bz**	**β-OAc, α-H**	**Ac**
Evonine	**Ac**	**O**	**Ac**
Cangorinine E-I	**Ac**	**β-OAc, α-H**	**Bz**



	R
Wilfordine	**β-OAc, α-H**
Alatamine	**β-OBz, α-H**



	R
Cangorinine W-I	**OBz**
Cangorinine W-II	**ONic**
Wilforine	**OAc**



Cassinin

Figure 2.4 : Sesquiterpene polyol ester alkaloids

The sesquiterpene polyol ester alkaloids all have up to seven esterifiable hydroxyls of which typically five or six are esterified with acetate, benzoate or nicotinate. Compound **1** contains one hydroxyl and six ester carbonyls (δ 170.4, 170.0, 169.9 168.9, 168.5 and 164.6), which are assigned to five acetates (δ_H 2.35, 2.30, 2.40, 2.25 and 1.40; δ_C 21.6, 21.4, 21.0, 20.9 and 19.9) and one benzoate (δ_H 2×7.83, 7.54, 2×7.42; δ_C 133.5, 2×129.5, 2×128.5 and 125.2).[54] The presence of the benzoyl ester moiety was further confirmed by the analysis of the COSY spectral data (Table 2.4, page 69) and the characteristic signals of benzene at δ 7.83 (2H, d, J = 7.0 Hz), 7.42 (2H, dd, J = 7.6 Hz) and 7.54 (1H, dd, J = 7.6 Hz).

The upfield shift of the acetate methyl at δ 1.40 is due to the presence of an anisotropic effect of the benzoyl ester group in its vicinity. The occurrence of such highly shielded acetate methyls has already been reported for sesquiterpene polyol ester alkaloids.[54]

The sesquiterpene polyol ester alkaloids differ most markedly in the fragment that links C-2' of the pyridine ring (Figure 2.2, page 62) to one of the macrocyclic lactone carbonyls. In the reported compounds variations observed include CH_2-CH_2-$CH(CH_3)$-; CH_2-CH_2-$C(OH)(CH_3)$-; $CH(CH_3)$-$CH(CH_3)$- and $CH(CH_2$-$CH_3)$-CH_2-CH_2- (Figure 2.4). In compound **1**, the $CH(CH_2$-CH_2-$CH_3)$- fragment was recognised by examination of the ^1H-^1H COSY spectra.

The presence of this novel four carbon skeleton (Figure 2.5) in **1** was supported by the vicinal and geminal couplings of the protons at δ 0.70, 1.75-2.00, 1.95-

2.20 and 4.40 of the COSY spectrum (Figure 2.13, page 74) and the HMBC coupling in the carbon at δ 42.1 with protons at δ 0.70 and 1.95-2.20 (Figure 2.14, page 76).

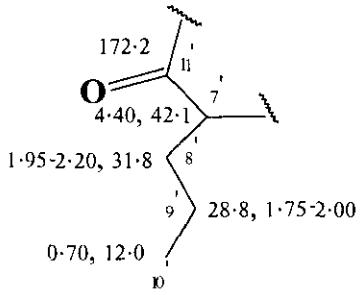


Figure 2.5 : Propyl fragment

The main carbon skeleton of compound **1** was assigned mainly on the basis of HMBC spectral data (Table 2.4, page 69). The oxygenated carbons resonating at δ 73.6 and 69.9 showed HMBC couplings with protons at δ 5.00, 5.20, 5.34, 5.38 and δ 4.60, 5.00, 5.85, respectively. Similarly, the carbon at δ 75.7 showed coupling with protons at δ 1.60 and 5.20. The ^{1}H NMR spectrum revealed two doublets at δ 5.00, 5.85 (1H, each, J = 3.9, 2.4 Hz, respectively) and a double doublet at 5.20 (1H, J = 2.4 and 3.9 Hz) which are attributed to vicinal protons. They were strongly correlated in the ^{1}H-^{1}H COSY spectrum as well (Figure 2.13, page 74).

Furthermore, the carbon at δ 69.8 which is an oxygenated quaternary, exhibited HMBC cross peaks with protons at δ 1.60, 5.00 and 5.20. A small cross-peak between the protons at δ 5.00 and δ 1.60 was also observed in the ^{1}H-^{1}H COSY spectrum (Table 2.4, page 69). The quaternary carbon at δ 172.2 showed HMBC

coupling with a proton at δ 5.00, indicating the connectivity of carbons δ 172.2 and 75.7 through an oxygen atom. The data analysed above, established the carbon connectivity as 73.6-69.9-75.7-69.8-22.8, for the fragment **A** of the molecule (Figure 2.6).

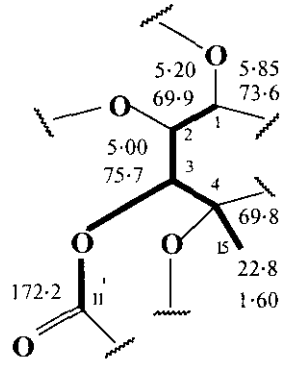


Figure 2.6 : Fragment **A**

The quaternary carbon at δ 94.0 showed HMBC coupling with protons resonating at δ 7.00, 5.00, 4.60, 2.12 and δ 1.60. Its deshielded chemical shift value was indirect evidence for the location, i.e. in a ring junction and attached to an oxygen function. Furthermore, oxygenated carbons at δ 73.6, 68.8 and 71.4, exhibited HMBC couplings with protons resonating at δ 2.12, 2.25-2.35; δ 2.25-2.35, 7.00 and δ 5.55, 5.85, 4.60, 2.25-2.35, respectively. Similarly, the carbons at δ 50.7 and 52.4 exhibited couplings with protons at δ 1.75, 3.60, 7.00 and 4.60, 5.20, 5.38, 5.55, 5.85, respectively (Table 2.4, page 69).

The ^{1}H NMR spectrum showed a doublet and a double doublet at δ 5.38 and 5.55 (1H, each, J = 5.8 Hz) indicating their close proximity. They were also strongly correlated in the ^{1}H-^{1}H COSY spectrum (Figure 2.13, page 74). The COSY

spectrum further showed cross-peaks between protons resonating at δ 2.25-2.35, 5.55 and 4.60 and 5.34. Thus, the carbons at δ 94.0, 73.6, 50.7, 31.2, 68.8, 71.4, 52.4 and 60.0 were closely located in the fragment **B** (Figure 2.7).

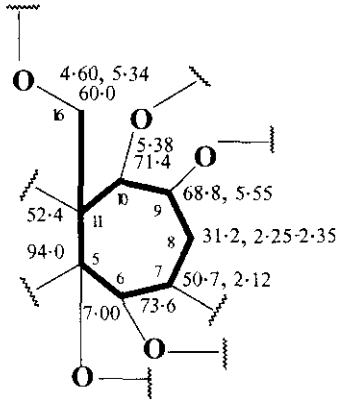


Figure 2.7 : Fragment **B**

The remaining oxygenated carbons, resonating at δ 84.9 and 69.9 showed HMBC couplings with protons at δ 7.00, 6.00, 3.60 and 1.75. The quaternary carbon at δ 166.4 exhibited similar couplings with protons at δ 3.60 and 6.00, establishing the structural fragment **C** shown in Figure 2.8.

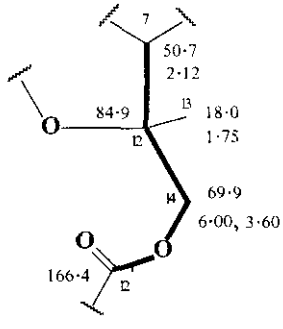


Figure 2.8 : Fragment **C**

Table 2.4 : ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and HMBC spectral data for compound **1** in $CDCl_3$

Position	^{1}H-^{1}H COSY	^{13}C-^{1}H COSY	HMBC
1	5.85 → 5.20	-	73.6 → 5.00, 5.20, 5.34 5.34, 5.38
2	5.20 → 5.00, 5.85	-	69.9 → 2.25, 4.60, 5.00, 5.85
3	5.00 → 1.60, 5.20	75.7 → 1.60	75.7 → 1.60, 5.20
4	-	69.8 → 1.60	69.8 → 1.60, 5.0, 5.2
5	-	94.0 → 1.60	94.0 → 1.60, 2.12, 4.60, 5.00, 7.00
6	-	-	73.6 → 2.12, 2.25-2.35
7	-	-	50.7 → 1.75, 3.60, 7.00
8	2.25-2.35 → 5.55	-	-
9	5.55 → 5.38, 2.25-2.35	-	68.8 → 2.25-2.35, 7.00
10	5.38 → 5.55	-	71.4 → 2.25-2.35, 4.60, 5.55, 5.85
11	-	-	52.4→ 4.60, 5.20, 5.38, 5.55, 5.85
12	-	84.9 → 1.75, 7.0	84.9 → 1.75,3.60, 6.00, 7.00
13	-	-	18.0 → 6.00
14	6.00 → 3.60	69.9 → 1.75	69.9 → 1.75
15	-	22.8 → 4.90	-
16	5.34 → 4.60	-	-
2'	-	-	168.5→ 8.40
3'	-	-	129.4 → 7.30
4'	8.40 → 7.30	138.1 → 8.83	-
5'	7.30 → 8.83, 8.40	-	-
6'	8.83 → 7.30	153.8 → 8.40	-
7'	4.40 → 1.95, 1.75	-	42.1→ 2.20, 1.95, 0.70
8'	1.95 → 2.2, 1.75, 4.40	-	-
9'	1.75-2.00 → 0.70, 4.40	28.8 → 0.70	28.8 → 2.20
10'	0.70 → 1.75, 2.00	-	-
11'	-	-	172.2 → 1.95, 5.00
12'	-	-	166.4 → 3.60, 6.00
1"	-	-	-
2"	7.83 → 7.42	129.5 → 7.42	-
3"	7.42 → 7.83, 7.54	-	-

table contd.

4"	7.54 → 7.42	-	-
5"	-	-	-
6"	-	-	-
7"	-	164.6 → 7.83	164.6 → 5.85, 7.83
1a	-	168.5 → 2.35	168.5 → 5.20, 2.35
1b	-	-	-
2a	-	170.0 → 2.25	170.0 → 7.00, 2.25
2b	-	-	-
3a	-	169.9 → 2.30	169.9 → 5.55, 2.30
3b	-	-	-
4a	-	168.9 → 1.40	168.9 → 5.38, 1.40
4b	-	-	-
5a	-	170.4 → 2.40	170.4 → 4.60, 5.34, 2.40
5b	-	-	-

Chemical shift values are in ppm.

The above HMBC spectral data clearly clarified the connectivity of each segment of one part of the molecule, thus establishing the fragment **1** (Figure 2.9).

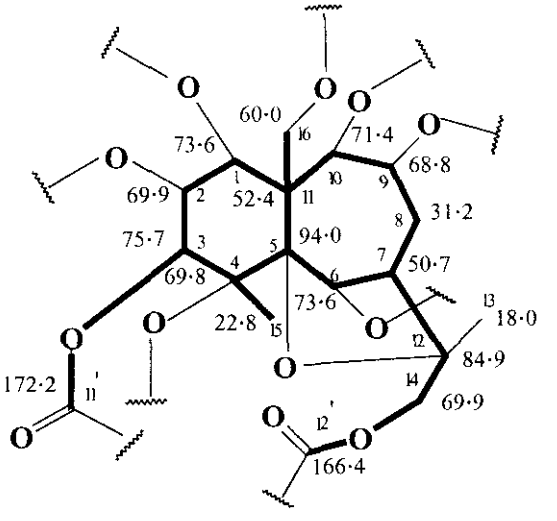


Figure 2.9 : Fragment 1

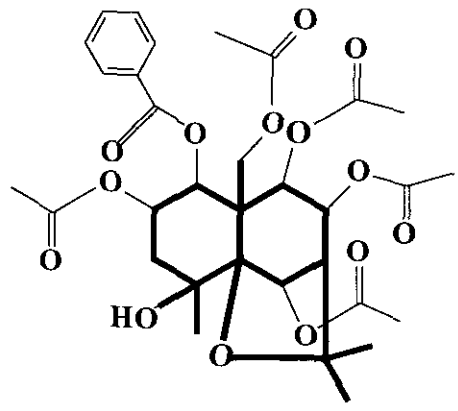


Figure 2.10 : β-dihydroagarofuran skeleton

Comparison of the reported values of the ^{1}H and ^{13}C NMR chemical shifts for the β- dihydroagarofuran skeleton (Figure 2.10) of the sesquiterpene polyol ester alkaloids[155, 164, 165] with those observed for fragment **1** (Figure 2.9) indicated a close similarity (Table 2.5, page 72). The only difference was the presence of an extra CH_2 group at δ 31.2, which suggests a 6,7 ring skeleton instead of the 6,6 ring in the reported products. This extra carbon atom was placed at position 8 of the ring on the basis of HMBC spectral data (Figure 2.11).

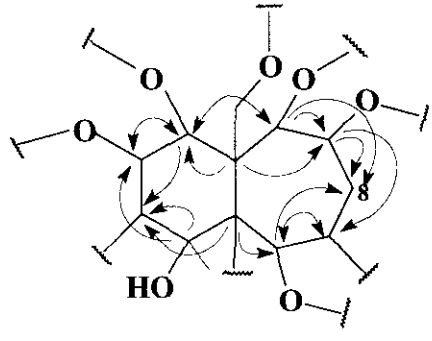


Figure 2.11 : Selected HMBC correlations for compound **1**

Table 2.5 : Comparison of ^{13}C and ^{1}H NMR spectral data of fragment **1** with those reported for cangorinine **W-II** in $CDCl_3$

Position	Reported δ_C	Observed δ_C	Reported δ_H (*J*)	Observed δ_H (*J*)
1	73.5	73.6	5.81, d (3.7)	5.85, d (4.0)
2	69.9	70.0	5.51, dd (3.7, 2.6)	5.20, dd (4.0, 2.4)
3	76.1	75.7	5.12, d (2.6)	5.00, d (2.4)
4	70.1	69.8	-	-
5	93.5	94.0	-	-
6	75.3	73.6	7.08, s	7.00, s
7	51.2	50.7	2.56, d (3.9)	2.12, m
8	-	31.2	-	2.25-2.35, m
9	69.3	68.8	5.60 dd (5.8, 3.9)	5.55, m
10	71.1	71.4	5.46, d (5.8)	5.38, d (5.8)
11	52.3	52.4	-	-
12	84.6	84.9	-	-
13	18.1	18.0	1.71, s	1.75, s
14	70.2	69.9	5.77, d, 3.71, d (11.8)	6.00, d, 3.60, d (11.9)
15	22.3	22.8	1.69, d (1.1)	1.60, s
16	60.8	60.0	5.54, d, 4.49, d (13.3)	5.34, d, 4.60, d (13.4)
11'	175.3	172.2	-	-
12'	167.1	166.4	-	-
4-OH	-	-	5.13 br s	4.90, br s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

The NMR spectral data of the fragment **1**, pyridine and propyl moieties were in agreement with those reported for similar skeletons from Celastraceae.[155, 160, 164, 166, 167, 168] They were connected to establish the main carbon framework of the molecule, on the basis of the application of homonuclear and heteronuclear correlation spectral data and data reported in literature of related skeleton types.

The molecular composition suggested the presence of one free hydroxyl group in the compound which was confirmed by the characteristic absorption of the hydroxyl functionality (3468 cm^{-1}) observed in the IR spectrum. This hydroxyl group was placed at C-4 on the basis of biogenesis and comparison of spectral data with the literature.[54]

The remaining assignment of **1** was the location of the six ester groups. This was assigned on the basis of the HMBC spectral data (Figure 2.14, page 76). The proton signals assignable to acetyl methyls at δ 2.35, 2.25, 2.30, 1.40 and 2.40 were correlated with the carbon signals at δ 168.5, 170.0, 169.9, 168.9 and 170.4, respectively, in the HMBC spectrum. Similarly, the proton signals at δ 5.20 (H-2), 7.00 (H-6), 5.55 (H-9), 5.38 (H-10) and 4.60, 5.34 (H-16) were correlated with the carbon signals at δ 168.5, 170.0, 169.9, 168.9 and 170.4, respectively. These clearly indicated that the five acetyl ester groups could be placed on C-2, C-6, C-9, C-10 and C-16. The remaining ester group is the benzoyl group and the ester linkage site is C-1. The benzoyl ester was therefore placed on C-1 which was further confirmed by the HMBC in which the carbon at δ 164.6 was correlated doubly with the proton at δ 5.85 (H-1) and the ortho proton of the benzoyl ester group at δ 7.83.

Thus, the structure of the compound, **1** was established as a novel sesquiterpene polyol ester alkaloid including one benzoate and five acetates. The upper part of the molecule contains a 6,7 ring system based on a β-dihydroagarofuran core and the lower part a fourteen membered bislactone ring (Figure 2.12). This

macrocyclic structure was formed by two ester linkages between one 6,7 ring molecule and the 2,3-disubstitued pyridine derived acid moiety, at positions 3 and 14.

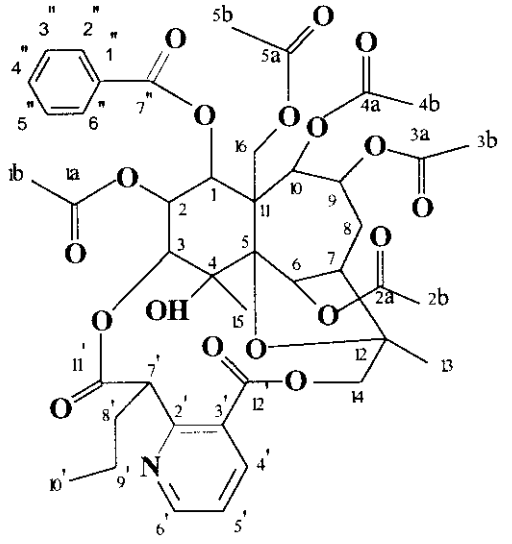


Figure 2.12 : Compound 1

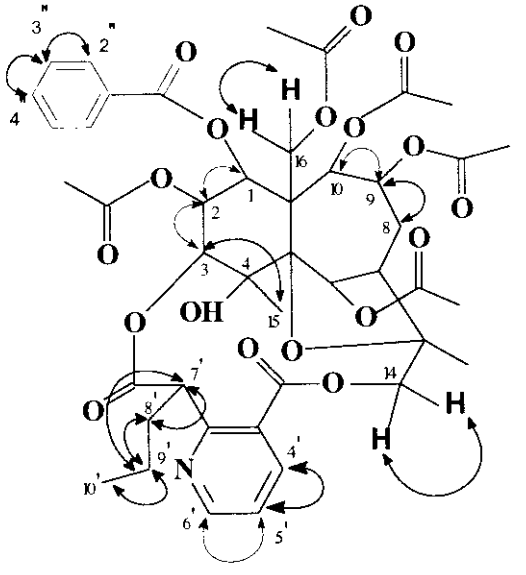


Figure 2.13 : ^{1}H-^{1}H COSY correlations for compound 1

Table 2.6 : ^{13}C and ^{1}H NMR spectral assignments of compound **1** in $CDCl_3$

Position	δ_C	DEPT	δ_H (*J*)
1	73.6	CH	5.85, d (4.0)
2	70.0	CH	5.20, dd (4.0, 2.4)
3	75.7	CH	5.00, d (2.4)
4	69.8	C_q	-
5	94.0	C_q	-
6	73.6	CH	7.00, s
7	50.7	CH	2.12, m
8	31.2	CH_2	2.25-2.35, m
9	68.8	CH	5.55, m
10	71.4	CH	5.38, d (5.8)
11	52.4	C_q	-
12	84.9	C_q	-
13	18.0	CH_3	1.75, s
14	69.9	CH_2	6.00, d, 3.60, d (11.9) (11.9)
15	22.8	CH_3	1.60, s
16	60.0	CH_2	5.34, d, 4.60, d (13.4)
2'	168.5	C_q	-
3'	129.4	C_q	-
4'	138.1	CH	8.40, dd (7.9, 1.8)
5'	120.7	CH	7.30, dd (7.9, 4.6)
6'	153.8	CH	8.83, dd (4.6, 1.8)
7'	42.1	CH	4.40, m
8'	31.8	CH_2	1.95-2.20, m
9'	28.8	CH_2	1.75-2.00, m
10'	12.0	CH_3	0.70, t (7.3)
11'	172.2	C_q	-
12'	166.4	C_q	-
1"	125.2	C_q	-
2", 6"	129.5	CH	7.83, d (7.0)
3", 5"	128.5	CH	7.42, dd (7.6)
4"	133.5	CH	7.54, dd (7.6)
7"	164.6	C_q	-

table contd.

1a	168.5	C_q	-
1b	20.9	CH_3	2.35, s
2a	170.0	C_q	-
2b	21.0	CH_3	2.25, s
3a	169.9	C_q	-
3b	21.6	CH_3	2.30, s
4a	168.9	C_q	-
4b	19.9	CH_3	1.40, s
5a	170.4	C_q	-
5b	21.4	CH_3	2.40, s
4-OH	-	-	4.90, br s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

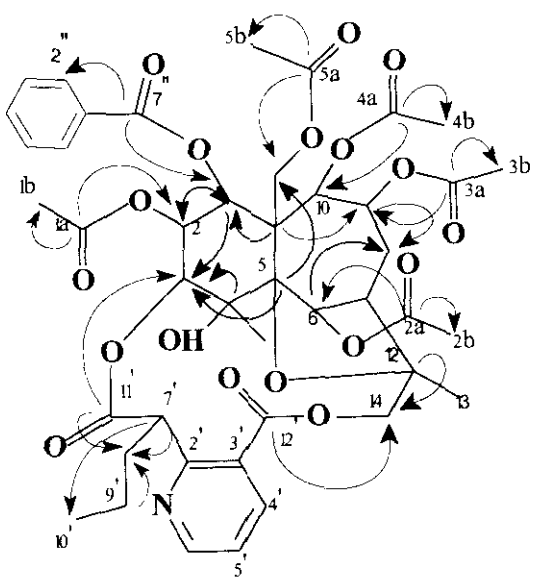


Figure 2.14 : HMBC correlations for compound 1

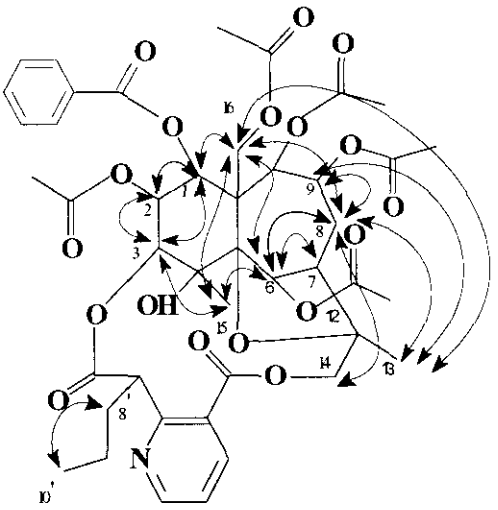


Figure 2.15 : NOESY correlations for compound **1**

The relative stereochemistry of the molecule was established by means of extensive NOESY experiments. H-1 and H-6 of the 6,6 ring system have been reported to have axial stereochemistry.[155, 54] In the NOESY spectrum, H-1 (J_{12} = 4.0 Hz) showed an interaction with H-2 suggesting the equatorial stereochemistry of H-2. This was confirmed by its coupling constants (Table 2.6, page 75). The observed NOESY interaction between H-2 and H-3 indicated the axial stereochemistry of H-3.

The H-6 proton exhibited interactions with protons, H-7, H-8, H-15 and H-16, indicating their close proximity in space. Since the dihedral angle between H-6_{ax} and H-7_{eq} is near to 90°, the proton H-6 appeared as a singlet.[155] The methyl protons at H-15 showed NOESY correlations with protons at H-3 and H-16 and the latter proton showed similar correlations with H-1, H-9 and H-13. In addition, the methyl protons at H-13 exhibited NOESY correlation signals with H-8 and H-

9 protons and the latter proton exhibited similar correlations with H-8 and H-10. The NOESY interactions between H_3-15 and H-16 confirmed the trans-ring fusion. The NOESY experiment also disclosed several other spatial correlations of the molecule as depicted in Figure 2.15 and Table 2.7 (page 79). These data clearly established the relative stereochemistry of the compound **1** as in Figure 2.16.

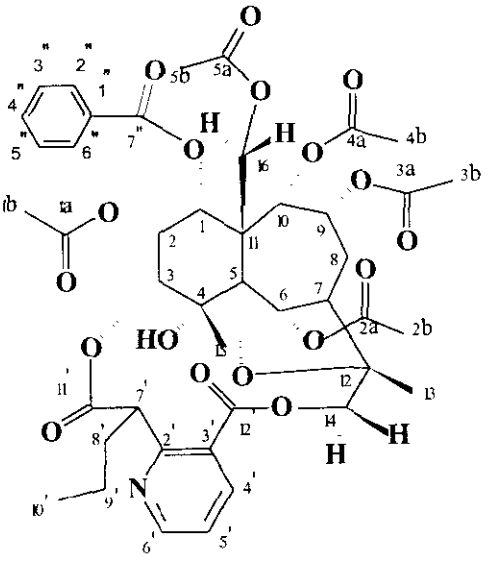


Figure 2.16 : The relative stereochemistry of compound 1

Table 2.7 : NOESY spectral data for compound **1** in $CDCl_3$

Position	δ_H	NOESY (^1H-^1H)
1	5.85, d	H-2, H-16
2	5.20, dd	H-1, H-3
3	5.00, d	H-1, H-2, H-15
6	7.00, s	H-7, H-8, H-15, H-16
7	2.12, m	
8	2.25-2.35, m	H-6, H-13, H-15, H-16
9	5.55, m	H-8, H-13
10	5.38, d	-
13	1.75, s	H-8, H-9, H-16
14	6.00, d, 3.60, d	H-8, H-14, H-16
15	1.60, s	H-3, H-6, H-16
16	5.34, d	H-1, H-6, H-9, 13, H-15
16	4.60, d	H-14, H-15
4'	8.40, dd	-
5'	7.30, dd	-
6'	8.83, dd	-
7'	4.40, m	-
8'	1.95-2.20, m	H-10'
9'	1.75-2.0, m	-
10'	0.70, t	H-8'
2"	7.83, d	-
3"	7.42, dd	-
4"	7.54, dd	-
5"	7.42, dd	-
6"	7.83,d	-

Chemical shift values are in ppm.

(b) **Characterisation of compound 2**

The compound **2** was isolated from *Pleurostylia opposita* as a yellow oil in 0.02% yield. The compound gave a UV active spot (λ = 254 and 366 nm, 1:2:7, isopropanol:ethyl acetate:petroleum ether, Rf = 0.38) and a purple spot with anisaldehyde-H_2SO_4.[162] The alkaloid nature of the compound was suggested since it appeared as a orange spot with Dragendorff' s-reagent.[162]

The IR spectrum showed the characteristic absorptions of ester carbonyl (1740 cm^{-1}) and hydroxyl (3462 cm^{-1}) functionalities. The UV spectrum also suggested the presence of a pyridine (λ_{max} = 269 nm) and ester carbonyl (λ_{max} = 206 nm) groups. The High resolution electrospray API mass spectrum showed the molecular ion at m/z 819.3035, suggesting the molecular formula to be $C_{39}H_{49}O_{18}N$. The molecular formula indicated a degree of unsaturation of sixteen.

The ^{13}C NMR spectrum showed the presence of thirty nine carbons in the molecule including nine methyls, five methylenes, eleven methines and fourteen quaternary carbons (Table 2.8, page 82). The ^{1}H NMR spectrum integrated for forty nine protons in the molecule. The NMR spectral data of the compound **2** (Table 2.8) showed a close spectral analogy to **1** (Table 2.6, page 75) but with six acetate esters (δ_H 1.30, 1.90, 2×2.15, 2.20 and 2.35; δ_C 25.3, 21.6, 21.3, 21.0, 20.8 and 20.5) instead of five acetates and a benzoate. Close comparison of the ^{1}H and ^{13}C NMR spectral data of **1** and **2** clearly revealed that the acetate at C-1 of **2** had replaced the benzoate of **1**. The H-1 proton at δ 5.85 of **1** has been shifted upfield to δ 5.55 in **2,** further indicating the location of the extra acetate on C-1. The

geminal protons at H-16 have therefore been slightly shifted upfield to δ 4.50 and 5.15 in the ^{1}H NMR spectrum.

All the remaining spectroscopic features of **2** were consistent with **1,** including the NOESY spectral data which suggested the same relative stereochemistry of both structures. The structure of the compound **2** (Figure 2.17) was therefore also established to be a novel macrocyclic sesquiterpene polyol ester alkaloid based on a β-dihydroagarofuran core. The macrocyclic structure contains a fourteen membered bislactone ring with a 2,3-disubstituted pyridine moiety.

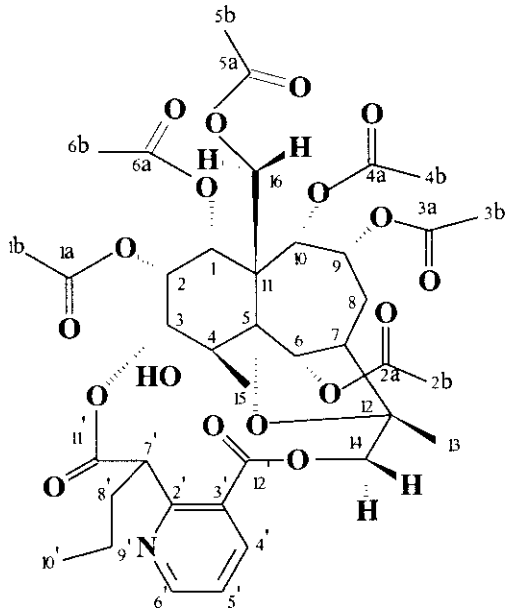


Figure 2.17 : Compound **2**

Table 2.8 : ^{13}C and ^{1}H NMR assignments for compound **2** in $CDCl_3$

Position	δ_C	DEPT	δ_H (J)
1	73.4	CH	5.55, d (3.9)
2	69.7	CH	5.20, dd (3.9, 2.4)
3	75.7	CH	4.97, d (2.4)
4	69.6	C_q	-
5	93.9	C_q	-
6	73.6	CH	7.00, s
7	50.8	CH	2.12, m
8	31.3	CH_2	2.25-2.35, m
9	68.9	CH	5.55, m
10	70.8	CH	5.48, d (5.8)
11	52.1	C_q	-
12	84.7	C_q	-
13	18.0	CH_3	1.73, s
14	69.9	CH_2	6.00, d, 3.60, d (11.7)
15	22.9	CH_3	1.68 s
16	60.0	CH_2	5.15, d, 4.50, d (13.5)
2'	168.0	C_q	-
3'	129.6	C_q	-
4'	138.2	CH	8.40, dd (7.9, 1.8)
5'	120.8	CH	7.13, dd (7.9, 4.6)
6'	153.8	CH	8.83, dd (4.6, 1.8)
7'	42.2	CH	4.40, m
8'	31.8	CH_2	1.95-2.20, m
9'	28.8	CH_2	1.75-2.00, m
10'	11.9	CH_3	0.70, t (7.3)
11'	172.3	C_q	-
12'	166.4	C_q	-
1a	168.0	C_q	-
1b	21.0	CH_3	2.15, s
2a	169.9	C_q	-
2b	20.8	CH_3	2.15, s
3a	170.1	C_q	-
3b	21.6	CH_3	2.20, s
4a	168.8	C_q	-
4b	20.5	CH_3	1.30, s
5a	170.3	C_q	-
5b	21.3	CH_3	2.35, s
6a	169.1	C_q	-
6b	25.3	CH_3	1.90, s
4-OH	-	-	4.90, br s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

(c) **Characterisation of compound 3**

The compound **3** was isolated from *Pleurostylia opposita* as a colourless oil in 0.01% yield. The compound gave a UV active spot (λ = 254 and 366 nm, 1:2:7, isopropanol:ethyl acetate:petroleum ether, Rf = 0.32) and a purple spot with anisaldehyde-H_2SO_4.[162] The alkaloid nature of the compound was suggested since it appeared as a orange spot with Dragendorff's-reagent.[162]

It showed IR absorptions characteristic of ester carbonyl (1751 cm^{-1}), hydroxyl (3448 cm^{-1}) and phenyl (1456 and 1603 cm^{-1}) functionalities. The UV spectrum also evidenced the presence of ester carbonyl (λ_{max} = 205 nm), pyridine (λ_{max} = 268 nm), benzoyl (λ_{max} = 228 nm) and nicotinyl (λ_{max} = 225 nm) groups. The High resolution electrospray API mass spectrum showed a molecular ion at m/z 944.3294, suggesting the molecular formula to be $C_{48}H_{52}O_{18}N_2$ which indicated a degree of unsaturation of twenty four.

The DEPT and ^{13}C NMR spectrum revealed the presence of forty eight carbons; seven methyls, five methylenes, twenty methines and sixteen quaternary carbons (Table 2.9, page 85). The ^{1}H NMR spectrum integrated for fifty two protons. Comparison of the NMR spectral data of the compound **3** (Table 2.9, page 85) with those of **1** (Table 2.6, page 75) indicated close similarity. The spectral data of **3** revealed the presence of four acetate esters (δ_H 1.35, 2.14, 2.23 and 2.34; δ_C 21.7, 21.3, 20.9 and 19.9), one benzoate ester (δ_H 2×7.75, 7.54, 2×7.30; δ_C 133.6, 2×129.5, 2×128.6 and 124.9) and one nicotinate ester[164] (δ_H 9.30, 8.90, 8.40 and 7.50; δ_C 153.5, 150.8, 138.6, 124.8 and 123.8). Close similarity of the ^{1}H and ^{13}C

NMR spectra of **1** and **3** indicated the nicotinate of **3** had replaced an acetate (at C-16) of **1**. The H-16 protons were slightly shifted to δ 5.45 and 4.55 as well as the H-1 and H-10 protons to δ 6.05 and 5.65, respectively, in **3**.

All the remaining spectroscopic features of **3** were consistent with **1**. Thus, the structure of **3** was established to be a novel sesquiterpene pyridine alkaloid which contain a fourteen membered bislactone ring, four acetates, one nicotinate and one benzoate ester groups (Figure 2.18).

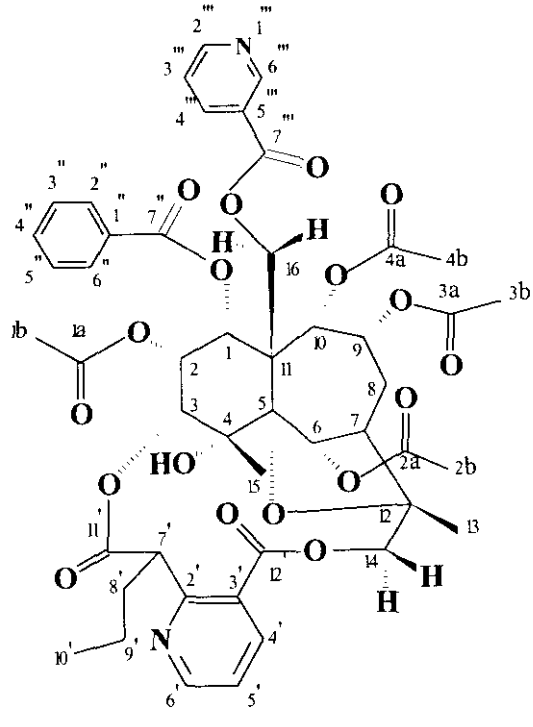


Figure 2.18 : Compound **3**

Table 2.9 : ^{13}C and ^{1}H NMR assignments for compound **3** in $CDCl_3$

Position	δ_C	DEPT	δ_H (*J*)
1	73.9	CH	6.05, d (4.0)
2	71.2	CH	5.20, dd (4.0, 2.4)
3	75.5	CH	5.15, d (2.4)
4	69.9	C_q	-
5	94.0	C_q	-
6	73.6	CH	7.06, s
7	50.8	CH	2.12, m
8	31.2	CH_2	2.25-2.35, m
9	68.9	CH	5.55, m
10	71.2	CH	5.65, d (5.8)
11	52.4	C_q	-
12	85.1	C_q	-
13	18.1	CH_3	1.75, s
14	70.3	CH_2	6.05, d, 3.40, d (11.9)
15	23.2	CH_3	1.65, s
16	60.3	CH_2	5.45, d, 4.55, d (13.4)
2'	167.9	C_q	-
3'	128.6	C_q	-
4'	137.6	CH	8.50, dd (7.9, 1.8)
5'	121.0	CH	7.40, dd (7.9, 4.6)
6'	153.9	CH	8.90, dd (4.6, 1.8)
7'	42.2	CH	4.40, m
8'	31.8	CH_2	1.95-2.20, m
9'	28.7	CH_2	1.75-2.00, m
10'	12.0	CH_3	0.72, t (7.3)
11'	172.2	C_q	-
12'	166.1	C_q	-
1"	124.9	C_q	-
2", 6"	129.5	CH	7.75, d (7.0)
3", 5"	128.6	CH	7.30, dd (7.6)
4"	133.6	CH	7.54, dd (7.6)
7"	164.6	C_q	-
2'''	153.5	CH	8.90, br s
3'''	124.8	CH	7.50, m
4'''	138.6	CH	8.40, dd
5'''	123.8	C_q	-
6'''	150.8	CH	9.30, br s
7'''	163.4	C_q	-

table contd.

1a	168.9	C_q	-
1b	21.3	CH_3	2.34, s
2a	170.0	C_q	-
2b	20.9	CH_3	2.14, s
3a	170.0	C_q	-
3b	21.7	CH_3	2.23 s
4a	168.4	C_q	-
4b	19.9	CH_3	1.35, s
4-OH	-	-	4.90, br s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

(d) **Characterisation of compound 4 as 20-hydroxylupan- 3-one**

Compound **4** was isolated from *Pleurostylia opposita* as a white crystalline solid (m.p. 220-224 °C) in 0.04% yield. The compound gave a UV active spot on a tlc plate (3:2, ethyl acetate:petroleum ether, Rf = 0.45).

The IR spectrum revealed the characteristic absorptions of hydroxyl (3611 cm^{-1}) and carbonyl (1699 cm^{-1}) functionalities. The UV spectrum also showed the presence of a carbonyl (λ_{max} = 206 nm) group. The low resolution electrospray API mass spectrum showed the molecular ion at m/z 442, suggesting the molecular formula to be $C_{30}H_{50}O_2$. The molecular formula indicated a degree of unsaturation of six. The ^{13}C NMR spectrum showed the presence of thirty carbons in the molecule including eight methyls, eleven methylenes, five methines and six quaternary carbons. The ^{1}H and ^{13}C NMR spectral data (Table 2.10) was indicative of a triterpene with thirty carbon atoms, most likely 20-hydroxylupan-3-one which had previously been reported from *Pleurostylia opposita*.[114] Comparison with reported data[114, 169] (Table 2.10) showed that compound **4** was indeed 20-hydroxylupan-3-one.

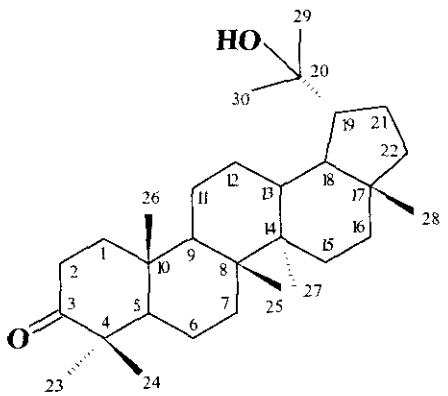


Figure 2.19 : Compound **4**

Table 2.10 : ^{13}C and ^{1}H NMR data for compound **4** and reported ^{13}C NMR data in $CDCl_3$

Position	δ_C	Reported δ_C	DEPT	δ_H
1	39.6	39.6	CH2	
2	34.2	34.6	CH2	2.49-2.41, m
3	218.3	218.0	C_q	
4	47.3	47.2	Cq	
5	54.8	54.9	CH	
6	19.7	19.7	CH2	
7	33.8	33.9	CH2	
8	41.3	41.4	Cq	
9	49.9	50.0	CH	
10	36.8	36.8	Cq	
11	21.9	22.0	CH2	
12	28.7	28.7	CH2	
13	37.6	37.7	CH	
14	43.6	43.6	Cq	
15	27.5	27.6	CH2	
16	35.5	35.6	CH2	
17	44.6	44.6	Cq	
18	48.2	48.3	CH	
19	49.6	49.7	CH	
20	73.5	73.4	Cq	
21	29.0	29.7	CH2	
22	40.3	40.2	CH2	
23	26.7	26.7	CH3	1.12, s
24	21.1	21.0	CH3	1.02, s
25	16.0	16.0	CH3	1.07, s
26	16.0	16.0	CH3	0.94, s
27	14.8	14.8	CH3	0.96, s
28	19.2	19.2	CH3	0.81, s
29	31.6	31.6	CH3	1.26, s
30	24.7	24.8	CH3	1.23, s

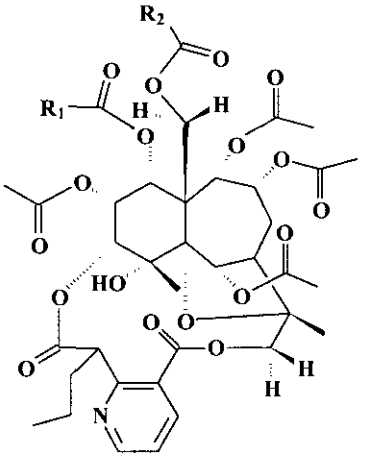
Chemical shift values are in ppm.

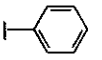
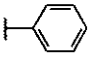
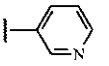
2.2.4 **Insecticidal activity**

The biological activity including insecticidal activity of the genus *Pleurostylia* (Celastraceae) has not been reported previously. Several Celastraceae plant species have been used as a natural insecticide for a long time in China[156, 157] The insecticidal principles of Celastraceae have been identified as β-dihydroagarofuran sesquiterpene polyester alkaloids.[155, 160, 161] However, non-alkaloid compounds have also been reported to possess insect antifeedant properties.[54]

In the screens at Rothamsted Experimental station, the stem bark of *Pleurostylia opposita* showed high insecticidal activity against mustard beetles (Table 2.2, page 59). The bio-assay results showed only compounds **1** and **2** exhibited high insecticidal activity. **1** and **2** have an LD_{50} of 0.027 and 0.10 μg/insect against mustard beetles, an activity comparable to pyrethrins and rotenones (Table 2.11). The activity of **1** is less than pyrethrins (0.01 μg/insect), much higher than nicotine (6 μg/insect) and comparable to rotenones (0.02 μg/insect). **1** and **2** showed no activity to houseflies and weak activity to larvae of the diamondback moth. The nicotinate derivative of **1** and **2** (compound **3**) is non insecticidal. The LD_{50} values of **1** and **2** suggest that the ester group is important in determining activity because while replacement of a C-1 benzoate of **1** by an acetate in **2** led to the retention of activity, replacement of the C-16 acetate of **2** by a nicotinate in **3** led to the complete loss of activity.

Table 2.11 : Insecticidal activity of compounds



	R_1	R_2
1		CH_3
2	CH_3	CH_3
3		

Compound	MB*	HF*	PX†
1	0.027	NT	WT
2	0.10	NT	WT
3	NT	NT	NT
Pyrethrines	0.01	0.30	-
Rotenones	0.02	0.60	>10
Nicotine	6	19	-

* Insecticidal activity is given in LD_{50} (μg/insect) or † as % mortality (10 μg/insect).

NT : non toxic, WT : weakly toxic (< 20%)

MB : mustard beetle (*Phaedon cochleariae*), HF : housefly (*Musca domestica*),

PX : larvae of diamondback moth (*Plutella xylostella*)

2.2.5 **Postulated biosynthesis of compounds 1, 2 and 3**

A proposal for the biosynthesis of the compounds **1**, **2** and **3** involves two component precursors, **A** and **B** (Figure 2.20).

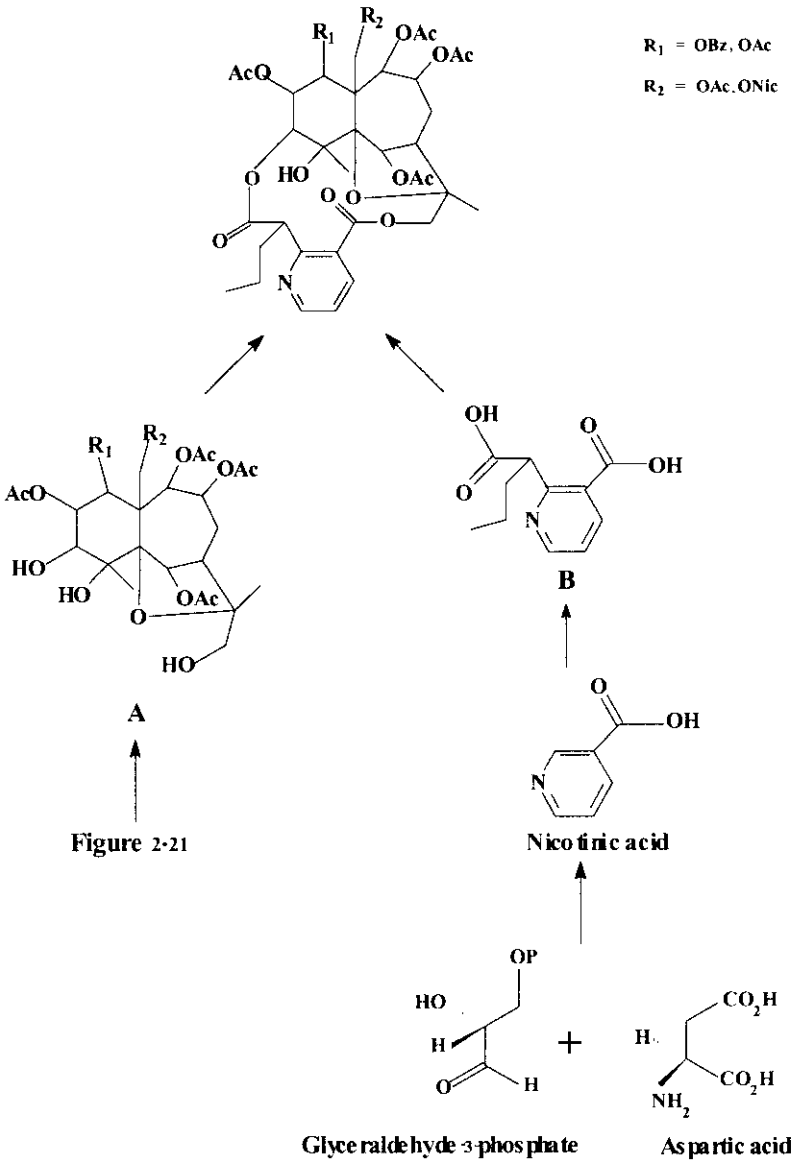


Figure 2.20: Postulated Biosynthetic pathway

The related 6,6 ring system of the precursor **A** is derived from the β-dihydroagarofuran skeleton and its biosynthetic pathway has been previously

reported[155] as proceeding via the sesquiterpene precursor, farnesyl pyrophosphate via the acetate-malonate pathway (Figure 2.21). The farnesyl pyrophosphate (FPP) undergoes direct ring closure with the terminal double bond in conjunction with the solvolysis of the allylic pyrophosphate. The germacrene cation formed is captured by water to yield the alcohol followed by a second ring closure to form the 6,6 ring system. A new cationic centre thus, regenerated by the 1,2-hydride shift, undergoes an internal cyclisation to form the β-dihydroagarofuran skeleton (Figure 2.21).[155]

The biosynthetic pathway to the 6,7 ring system with a furan ring is less likely to arise via the direct ring closure of the farnesyl pyrophosphate. A possibility is the insertion of the extra carbon unit into the 6,6 ring system during the biosynthesis of the β-dihydroagarofuran core. The biological precursor for the extra carbon unit could be S-Adenosyl-L-methionine since most biological one carbon additions involve this.[17]

The intermediate, **C** (Figure 2.21) formed during the biogenesis of the β-dihydroagarofuran skeleton instead of cyclising to the 6,6 system is thought to undergo a 1,4-hydride shift to form a new cationic centre. Subsequently, the elimination of a proton would result in a new double bond where S-Adenosylmethionine could react. Elimination of demethyl S-Adenosylmethionine would result in a cyclopropane intermediate. This cyclopropyl moiety could then open, thereby inserting an additional CH_2 unit to the 6,6 ring system to form the related 6,7 system. The cation formed by the ring opening of the cyclopropane

would undergo internal cyclisation to form the 6,7 furan ring system (Figure 2.21).

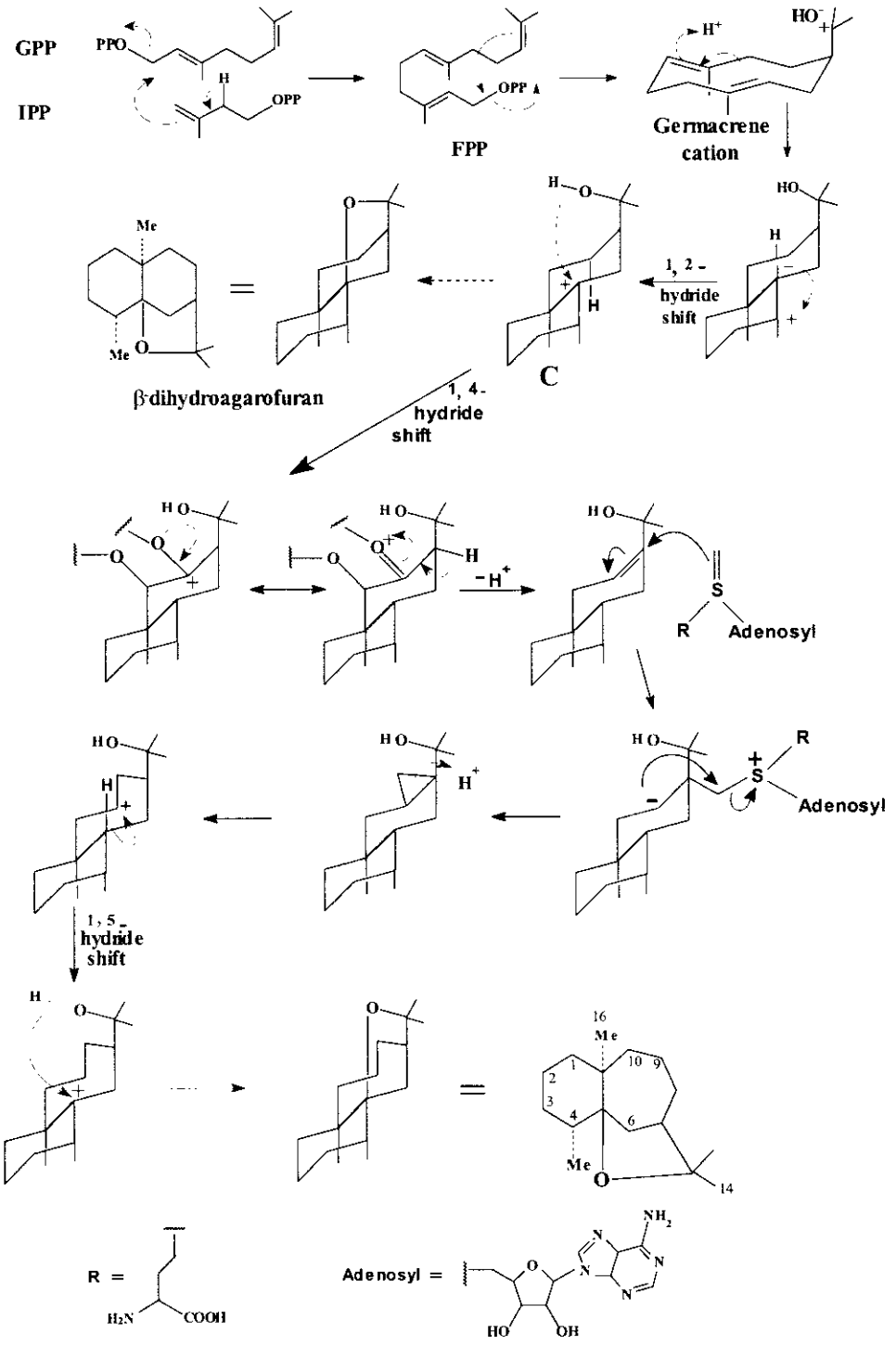


Figure 2.21 : Postulated Biosynthetic pathway of precursor **A**

Oxygenation at positions 1, 2, 3, 4, 6, 9, 10, 14 and 16 (Figure 2.21) results in a polyol, and esterification with the corresponding acids afford the trihydroxy polyester, **A** (Figure 2.20, page 91).

The precursor, **B** is formed from glyceraldehyde-3-phosphate and aspartic acid to form nicotinic acid,[20, 170] followed by the addition of the CH_3-$(CH_2)_3$-COOH moiety at the C-2 position (Figure 2.20, page 91).

Finally, combination of compounds **A** and **B** to form the fourteen membered bis-lactone ring results in compounds **1**, **2** and **3**.

2.3 ISOLATION, DERIVATISATION, CHARACTERISATION AND SYNTHESIS OF COMPOUNDS FROM *AEGLE MARMELOS*

2.3.1 Introduction

Preliminary bioassay studies of the petroleum ether, ethyl acetate and ethanol extracts of various parts of *Aegle marmelos* showed high insecticidal activity (Table 2.12) against mustard beetles (*Phaedon cochleariae*) and houseflies (*Musca domestica*) and prompted the present study.

Aegle marmelos Correa (Rutaceae) is a large deciduous tropical tree and is well known for its medicinal properties. The Rutaceae is a predominantly tropical family some of which produce edible fruits, volatile oils and a wide range of alkaloids having useful pharmacological activities.[171] Several Rutaceae plants, especially Citrus species, are known to possess insecticidal effects.[48]

A. marmelos has been widely investigated for bioactive phytochemical constituents since 1930[121] but insecticidal activity was not reported until 1996[120] after the preliminary studies which are described on page 56. This report indicated that the petroleum ether and alcohol extracts of *A. marmelos*, collected from Egypt, were active against flour beetles (*Tribolium confusum* Duv.), cotton leaf worm (*Spodoptera littoralis* Boisd.), and flies (*Drosophila melanogaster* Meig.).[120] However, the active constituents were not identified.

Table 2.12 : Insecticidal activity of *Aegle marmelos*

Insect	SB			RB			FR			LE		
species	A	B	C	A	B	C	A	B	C	A	B	C
MB*	100	100	NT	100	100	NT	35	60	NT	WT	NT	NT
HF*	79	WT	NT	NT	20	NT	WT	NT	NT	NT	NT	NT
PX†	WT	20	WT	25	20	WT	20	20	WT	NT	WT	WT
BT‡	NT	NT	WT	NT	NT	WT	WT	WT	NT	NT	NT	NT
TU¶	WT	NT	NT	NT	NT	NT	WT	NT	NT	NT	NT	NT

Insecticidal activity is given as % mortality (* 20 µg/insect, † 10 µg/insect, ‡ 2000 ppm, ¶ 1000 ppm)

NT : non toxic, WT : weakly toxic (< 20%). A : petroleum ether, B : ethyl acetate, C : ethanol

MB : mustard beetle (*Phaedon cochleariae*), HF : housefly (*Musca domestica*),

PX : larvae of diamondback moth (*Plutella xylostella*), BT: whitefly (*Bemisia tabaci*),

TU : mites (*Tetranychus urticae).* SB : stem bark, RB : root bark, FR : fruit, LE : leaves.

2.3.2 **Isolation of insecticidal compounds**

The sequential extraction of the stem bark revealed that the activity was concentrated in the petroleum ether and ethyl acetate extracts (Table 2.12). Dry column flash chromatography of the combined petroleum ether and ethyl acetate extracts on silica gel yielded an active fraction, $\mathbf{F_2}$ in 0.76% yield (Figure 2.22). TLC analysis of $\mathbf{F_2}$ (3:2, ethyl acetate:petroleum ether) showed a single spot (Rf = 0.37) under UV light (λ = 254 and 366 nm) but two spots with anisaldehyde-H_2SO_4.[162] The major spot was green (Rf = 0.37) beneath which there was a minor spot which was purple (Rf = 0.37-0.36).

Chromatography (column, PTLC and HPLC) failed to separate $\mathbf{F_2}$, but fractionation by crystallisation led to isolation of the major component (compound **5**, inactive) in the mother liquors, and an active minor component, $\mathbf{C_1}$ as a white crystalline solid in 0.11% yield (Figure 2.22). The formation of these crystals was highly dependent on the ambient temperature and solvent composition. Ethyl acetate:petroleum ether (1:1) and a temperature below 15 $^{\circ}$C were found to be the most appropriate conditions for recrystallisation.

The ^{1}H and ^{13}C NMR spectra, in particular the intensity of the signals in the ^{1}H NMR spectrum and the overlapping nature of the methylene and several methyl and methine carbon signals of the crystalline fraction, $\mathbf{C_1}$ indicated that it consisted mainly of a mixture of four related compounds, **6**, **7**, **8** and **9**.

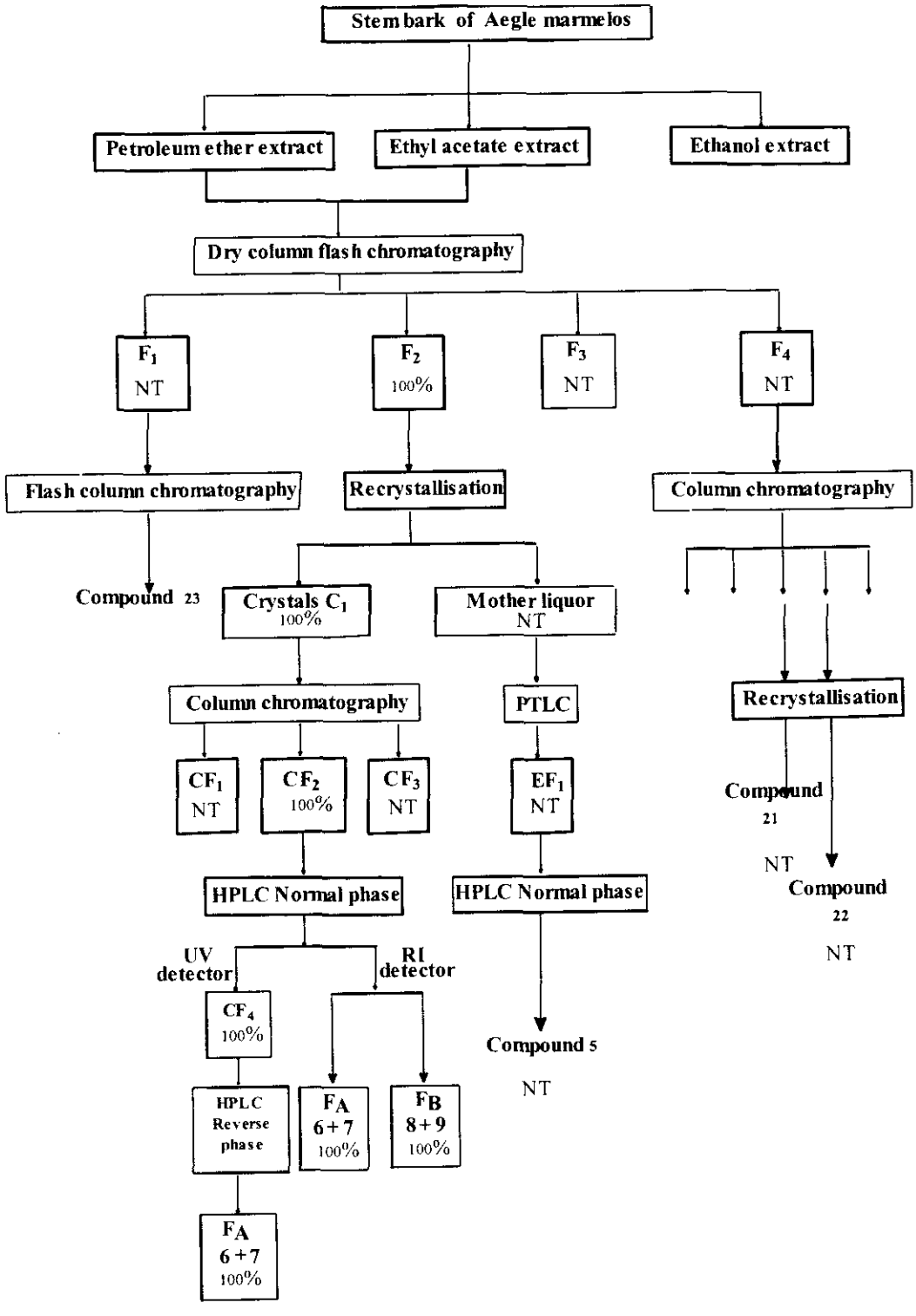


Figure 2.22 : Bio-assay guided fractionation extracts of *Aegle marmelos*

Bio-assay guided sequential chromatography of $\mathbf{C_I}$ (Figure 2.22) by silica gel column, normal phase and reverse phase HPLC (Table 2.13) allowed the isolation of the major insecticidal constituent, $\mathbf{F_A}$ as a mixture of two compounds, **6** and **7** (2:1) in 0.006% yield.

Table 2.13 : HPLC conditions

Column	Solvent	Flow rate (ml/min)
Silica	Isopropanol:tetrahydrofuran:hexane (2:25:73)	2
Silica	Isopropanol:diethyl ether:hexane (1:35:64)	2
C_8	Water:acetonitrile (1:9)	2
C_{18}	Water:acetonitrile (1:9)	2

Due to the poor UV signal a difficulty arose during the HPLC separation of the natural products, suggesting only poor chromophores in the compounds at λ = 254 and 366 nm. The UV diode array detector indicated that the most appropriate wavelength for detection was 220 nm and subsequent HPLC separations were monitored at this wave length. Consequently solvents with a UV cut-off point > 220 nm were not appropriate even though they gave a good separation on high performance analytical tlc. Isopropanol:tetrahydrofuran:hexane (2:25:73) and isopropanol:diethyl ether:hexane (1:35:64) were the most appropriate solvent systems for normal phase and water:acetonitrile (1:9) for reverse phase separations at λ = 220 nm.

An alternative experiment, using the Refractive Index detector (RI) for HPLC detection showed that, in addition to fraction $\mathbf{F_A}$, a second insecticidal fraction $\mathbf{F_B}$

was also present. The ^{1}H and ^{13}C NMR spectra indicated that $\mathbf{F_B}$ also was a mixture of two compounds (**8** and **9**). Further separation of the two mixtures by normal and reverse phase HPLC with RI detector was unsuccessful. The other minor compounds in the fraction $\mathbf{C_1}$ could not be isolated using the RI detector due to its lower sensitivity.

Further attempts to separate the mixture of **6** and **7** by HPLC using three columns, C4, C8 and nitrile, eluted with water:tetrahydrofuran, water:methanol and water:acetonitrile were unsuccessful. A series of Supercritical Fluid Chromatographic (SFC) experiments was carried out with a silica column using supercritical carbon dioxide as a mobile phase with organic modifiers (*acetonitrile* and *methanol*) applying a range of temperatures (32, 45 and 55 $^{\circ}C$) and pressures (3.95 and 4.35 Kpsi). However, these failed to separate the compounds.

Further purification of the non-active fraction $\mathbf{F_1}$ by flash column chromatography (Figure 2.22, page 98) led to the isolation of non-active compound **23**. Fractionation of $\mathbf{F_4}$ (Figure 2.22, page 98) by column chromatography followed by crystallisation also led to the isolation of two non-active compounds **21** and **22**.

2.3.3 **Characterisation of compound 5**

The major component of active fraction $\mathbf{F_2}$ is compound **5**, which was isolated from the mother liquors left on crystallisation. The compound gave a fluorescing UV active spot (3:2, ethyl acetate:petroleum ether, Rf = 0.37) and a green spot with anisaldehyde-H_2SO_4.[162]

The presence of a carbonyl function was evident from the strong band at 1736 cm^{-1} in the IR spectrum. It also showed the absorptions characteristic of an aromatic ring (1605 cm-1) and a ester (1285 and 1226 cm^{-1}) functionality. The UV spectrum showed absorptions at λ_{max} = 323 and 206 nm. The low resolution electrospray API mass spectrum displayed the molecular ion at m/z 314, suggesting the molecular formula to be $C_{19}H_{22}O_4$ which indicated a degree of unsaturation of nine.

The analysis of ^{13}C and ^{1}H NMR spectra indicated the presence of the 7-substituted coumarin skeleton (δ_H 6.80, d, J = 2.5 Hz; 6.83, dd, J = 2.5, 8.8 Hz; 7.34, d, J = 8.8; 6.24, d, J = 9.5 Hz and 7.62, d, J = 9.5 Hz) and a geranyloxy derived side chain (methylene carbons at δ 27.1, 36.3, 65.3; olefinic carbon at δ 119.1; olefinic quaternary carbon at δ 141.3 and two oxygenated carbons at δ 58.3, 63.7) in the molecule.[172]

These data indicated the structure of compound **5** to be epoxyaurapten [7-(6',7'-epoxy-3',7'-dimethyl-2-octenyloxy) coumarin] which is known to occur in *Aegle marmelos* and several other Rutaceous plants (Figure 2.23).[129, 172] By way of

confirmation the NMR spectral data of **5** is in agreement with that previously reported for epoxyaurapten (Table 2.14).[172]

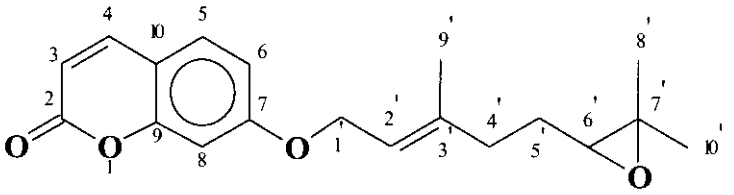


Figure 2.23 : Compound **5**

Table 2.14 : ^{13}C and ^{1}H NMR data of compound **5** and reported ^{13}C NMR data in $CDCl_3$

Position	δ_C	Reported δ_C	DEPT	δ_H (J)
2	161.0	161.3	C_q	-
3	113.1	113.1	CH	6.24, d (9.5)
4	143.3	143.5	CH	7.62, d (9.5)
5	128.7	128.8	CH	7.34, d (8.8)
6	113.2	113.2	CH	6.83, dd (8.8, 2.5)
7	162.0	162.1	C_q	-
8	101.5	101.7	CH	6.80, d (2.5)
9	155.9	155.9	C_q	-
10	112.5	112.6	C_q	-
1'	65.3	65.4	CH_2	4.60, d (6.5)
2'	119.1	119.1	CH	5.52, t (6.5)
3'	141.3	141.5	C_q	-
4'	36.3	36.3	CH_2	2.25, m
5'	27.1	27.2	CH_2	1.68, m
6'	63.7	63.9	CH	2.69, t (6.3)
7'	58.3	58.4	C_q	-
8'	24.8	24.9	CH_3	1.30, s
9'	16.8	16.8	CH_3	1.78, s
10'	18.8	18.8	CH_3	1.27, s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

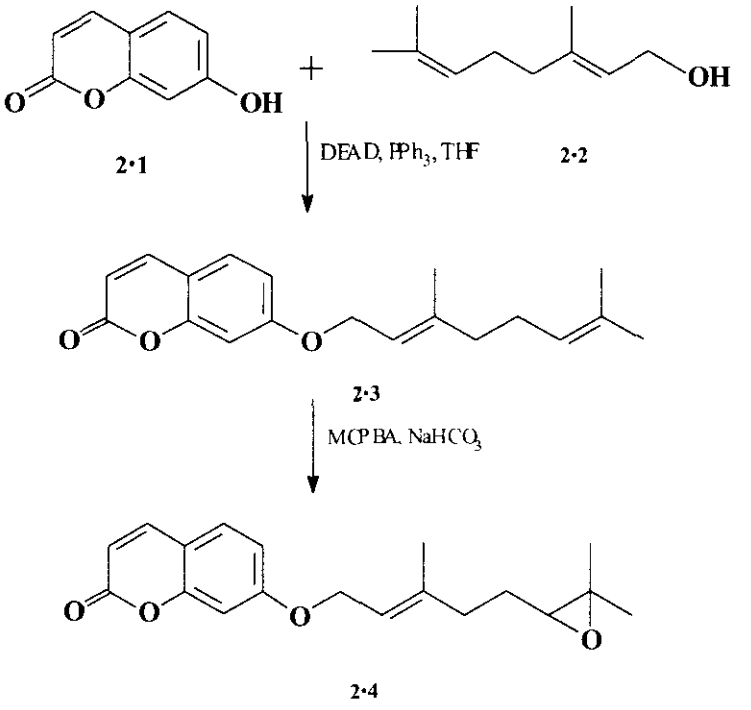
2.3.4 **Synthesis of Epoxyaurapten**

The total synthesis of epoxyaurapten (**2.4**) was achieved in two steps, via O-alkylation and epoxidation as outlined in Scheme 2.1. The key intermediate is aurapten (**2.3**) which was reported from *Aegle marmelos*.[128] Among different types of O-alkylation of coumarins that have been reported in the literature,[173] geranyl ethers were synthesised in varying yields for instance 7-geranyloxy coumarin was prepared in 63% yield by the reaction of the sodium salt of 7-hydroxy coumarin with geranyl bromide in dimethyl formamide at room temperature.[174] However, analogous alkylations to give 7-substituted geranyl and related derivatives under virtually identical conditions, proceeded in very low yields.[173]

In the present study, the O-alkylation of 7-hydroxy coumarin (**2.1**) was achieved using the Mitsunobu protocol.[175] The O-alkylation of different phenolic substrates has been successfully achieved using Mitsunobu conditions[176] but the O-alkylation of coumarins has not been reported in the literature.

The 7-hydroxy coumarin was treated with geraniol (**2.2**) in the presence of triphenylphosphine and Diethyl azodicarboxylate (DEAD) at 0 $^{\circ}$C. The reaction mixture was purified by column chromatography on silica gel which gave aurapten (**2.3**) as a white crystalline solid. The NMR spectral data indicated that product was in agreement with the reported data of aurapten.[172]

The epoxidation of aurapten was carried out using m-chloroperbenzoic acid (MCPBA) in dichloromethane with $NaHCO_3$.[177] The reaction was completed within 20 min and the tlc showed two spots, as expected, (3:2, ethyl acetate:petroleum ether, Rf = 0.37 and 0.21) with anisaldehyde-H_2SO_4.[162] The chromatography of the resultant mixture, afforded, epoxyaurapten (**2.4**) as a colourless oil in 71% yield. The NMR spectral data of the product was in agreement with the reported data of epoxyaurapten.[172, 177] The minor compound of the reaction mixture was identified as a diepoxyaurapten. The same reaction procedure has been used in the literature, but without $NaHCO_3$. The reaction had to be left for 15 h to obtain the epoxyaurapten in 87% yield.[177]



Scheme 2.1 : Synthesis of Epoxyaurapten

However, a synthetic sample of epoxyaurapten was nontoxic, indicating that the active constituent was the minor component of the active fraction ($\mathbf{F_2}$).

2.3.5 **Characterisation of fraction F_A and F_B**

Fractions $\mathbf{F_A}$ and $\mathbf{F_B}$ both were isolated from *Aegle marmelos* as colourless oils in 0.006% and 0.004% yields. They were visualised as purple spots (2:3, ethyl acetate:petroleum ether, Rf = 0.37-0.36) with anisaldehyde-H_2SO_4.[162]

The IR spectrum of $\mathbf{F_A}$ and $\mathbf{F_B}$ each showed absorptions characteristic of hydroxyl (3587 cm^{-1}) functionality and $\mathbf{F_A}$ only showed absorptions characteristic of an α, β-unsaturated ester (1704 cm^{-1}). The UV spectrum of $\mathbf{F_A}$ also evidenced the presence of an α, β-unsaturated ester at λ_{max} = 216 nm. The low resolution electrospray API mass spectrum of $\mathbf{C_1}$ gave molecular ions at m/z 570 and 572 for $\mathbf{F_A}$ and $\mathbf{F_B}$.

The ^{13}C NMR spectrum of $\mathbf{F_A}$ and $\mathbf{F_B}$ each displayed resonances for thirty five carbons for the major compound. They included signals due to one hemiacetal carbon at δ 98.4 for $\mathbf{F_A}$ and δ 98.2 for $\mathbf{F_B}$ and five sp^3 oxygenated carbons at δ 78.5, 76.7, 74.3, 67.6 and 58.1 and the remaining sp^3 carbons include eight methyls, nine methylenes, four methines and five quaternaries. In addition, three sp^2 carbons were observed in $\mathbf{F_A}$ corresponding to one carbonyl (δ 166.5) and one double bond (δ 156.0 and 116.9). In $\mathbf{F_B}$, only one sp^2 carbon was observed at δ 173.0.

The ^1H NMR spectrum of $\mathbf{F_A}$ exhibited the presence of two hydroxyl groups at δ 2.55 and 2.49. It also showed five oxymethines proton signals from the major compound at δ 5.43, 4.68, 4.00-3.85, 3.76 and 2.84. In $\mathbf{F_B}$, two hydroxyl groups

were observed at δ 2.44 and 2.38 and five oxymethine protons observed at δ 5.40, 4.63, 4.00-3.82, 3.74 and 2.80.

Extensive signal overlapping was observed in the aliphatic region (δ 2.17-0.80) of the ^{1}H NMR spectrum of both fractions. Because of this, H-H correlations and long-range C-H correlations were complex, and in some instances could not be observed in the COSY spectrum. In the ^{13}C NMR spectrum, signal overlapping was observed for all methylenes and several methyl and methine carbons of both fractions $\mathbf{F_A}$ and $\mathbf{F_B}$.

Because of the complexity of the spectra observed for the crystalline solid, $\mathbf{C_1}$, and the fractions obtained from it by direct chromatographic methods, interpretation "from scratch" could not be achieved. In an attempt to obtain a single pure compound other approaches were examined. These involved derivatisation combined with chromatography.

2.3.6 **Derivatisation of active compounds**

The rationale behind the derivatisation was to achieve better chromatographic separation by making physico-chemical differences (mainly polarity) between the inseparable compounds. Since the parent compounds lack UV absorbing chromophores at λ = 254 nm, one aim of derivatisation was to increase the absorptivity by introducing chromophores, and thus allow more sensitive detection.

(a) **p-Nitrobenzoyl derivatives**

p-Nitrobenzoyl chloride was used as a benzoylation reagent for hydroxyl functional groups which greatly enhanced the detectability at λ = 254 nm. Benzoylation was carried out with the crystalline solid, $\mathbf{C_1}$ using a large excess of p-nitrobenzoyl chloride in pyridine in the presence of catalytic amount of 4-N,N dimethylamino pyridine at room temperature.[178] The reaction was completed within three hours. After the workup, the presence of one major spot was indicated by tlc. Repeated chromatography of the reaction mixture (PTLC-HPLC), afforded two novel crystalline fractions. The combined Mass and NMR spectral data, suggested that they were each single pure compounds (**10** and **11**), and that they corresponded to major components in the insecticidal mixture $\mathbf{C_1}$.

(b) **Acetyl derivatives**

The crystalline solid, $\mathbf{C_1}$ was subjected to acetylation using a large excess of acetic anhydride in pyridine at room temperature.[179] After workup, the reaction mixture was purified by bio-assay guided repeated chromatography (silica gel column-HPLC) to obtain three novel insecticidal compounds **12**, **13**, **14** and a non- insecticidal fraction containing a mixture of three compounds, **15-17**.

(c) **Buffered PCC oxidation**

The buffered PCC oxidation[180] of the crystalline solid, $\mathbf{C_1}$ with pyridinium chlorochromate, anhydrous potassium acetate in dichloromethane at room temperature, afforded the insecticidal mixture containing compounds **18** and **19**. Further separation of this by both normal and reverse phase HPLC was unsuccessful. The NMR spectral data revealed that this is a mixture of two diketo compounds.

(d) **Hydrolysis**

Acid hydrolysis was carried out using methanolic HCl under reflux.[181] Repeated chromatography of the reaction mixture (silica gel column-reverse phase HPLC), afforded a non-insecticidal novel compound, **20** (4.67 mg).

The base hydrolysis of $\mathbf{C_1}$ with aqueous methanolic potassium hydroxide at room temperature was unsuccessful.[182] The presence of the starting material was indicated by the tlc with several other minor compounds after 24 hours. After

workup, repeated chromatography (dry column-HPLC) of the mixture, allowed isolation of the minor component as a mixture in a very low yield (6.70 mg). The spectral data suggested the presence of the senecioic acid ester moiety and no other major spectral changes were indicated. The senecioic acid ester group is less reactive, sterically encumbered at C-3 and therefore, resistant to both acid and base hydrolysis.

(e) **Methyl ether derivatives**

Methylation was carried out with a large excess of dimethyl sulphate and anhydrous potassium carbonate in dry acetone and longer refluxing hours.[183] After seventy two hours, the reaction mixture was purified by column chromatography, yielding mainly the unreacted starting material.

Harsh conditions are needed for the methylation of secondary hydroxyl groups, providing an earlier indication of the unreactivity of this functional group. The use of harsh conditions might be disadvantageous due to the other reactive functionalities in the molecule.

Therefore, the methylation was attempted using a large excess of freshly prepared silver oxide (I) and methyl iodide in the dark and the reaction was accelerated using periodic sonication.[184] The reaction had to be kept longer than expected for the starting material to disappear. After prolonged stirring over three days, unreacted starting material was recovered.

The unsuccessful results suggest that the hydroxyl groups are resistant towards methylation.

(f) **Silyl ether derivatives**

The silylation with excess of t-butyldimethylsilyl chloride (TBDMS) and imidazole, as a basic activator in N,N-Dimethylformamide (DMF) at room temperature[185] was unsuccessful. The reaction mixture was left for a longer period than normal, but even after 48 hours, mainly the starting material was recovered with traces of unidentified artefacts. The negative results suggest that the hydroxyl groups were also resistant to silylation.

(g) **Reduction**

Reduction by lithium aluminium hydride in dry diethyl ether at room temperature[186], afforded mainly the unreacted starting material with several other minor compounds. The mixture was purified by chromatography, however, the spectral data of the minor component provided little information.

2.3.7 **Characterisation of p-nitrobenzoyl derivatives 10 and 11**

(a) **Characterisation of compound 10**

Compound **10** was a white crystalline solid (m.p. 169-170 °C). It gave a UV active spot (λ = 254 and 366 nm, in 2:3, ethyl acetate:petroleum ether, Rf = 0.41) and a purple spot with anisaldehyde-H_2SO_4.[162]

It showed IR absorptions characteristic of α, β-unsaturated ester (1722, 1607 cm^{-1}) and epoxide (988 cm^{-1}) functionalities. The UV spectrum also evidenced the presence of α, β-unsaturated (λ_{max} = 216 nm) and p-nitrobenzoyl (λ_{max} = 259 nm) ester moieties.

The presence of two p-nitrobenzoyl ester groups in compound **10** was confirmed by the characteristic NMR chemical shifts of the p-nitrobenzoate.[178]

The DEPT and ^{13}C NMR spectra led to the identity of forty nine carbons in the molecule. The multiplicity studies indicated the presence of eight methyl, nine methylene, eighteen methine and fourteen quaternary carbons in the molecule. These included signals due to one hemiacetal carbon at δ 102.9, and five sp^3 oxygenated carbons at δ 79.8, 78.1, 76.7, 64.7 and 57.2. Three sp^2 carbons were observed for one carbonyl (δ 165.8) and one double bond (δ 115.9 and 157.0) in addition, to the sp^2 carbons of the p-nitrobenzoyl ester group.

The low resolution electrospray API mass spectrum displayed the molecular ion at m/z 868, suggesting the molecular formula to be $C_{49}H_{60}O_{12}N_2$. The molecular formula indicated a degree of unsaturation of twenty one.

The ^{1}H NMR spectrum revealed a pair of doublets, each integrating for one proton, at δ 0.25 and 0.67 (J = 5.5 Hz), suggesting two geminal protons on a cyclopropane ring.[187] The cyclopropyl methylene carbon was observed at δ 15.1 in the ^{13}C NMR spectrum. They also showed correlations in the ^{1}H-^{1}H COSY spectrum (Figure 2.30, page 120).

The ^{1}H and ^{13}C NMR spectra both revealed the presence of a trisubstituted epoxide at δ 2.77 (d, J = 7.3 Hz; δ_C 64.7 and 57.1) in the compound **10**.[188] The ^{1}H NMR spectrum also showed signals due to six tertiary methyl groups at δ 0.67, 0.90, 0.98, 1.20, 1.29 and 1.32 (3H, each, s) and four oxymethine protons at δ 4.01-3.95 (qd, J = 9.8, 7.3, 2.7 Hz), 4.68 (s), 5.31 (br s) and 6.55 (d, J = 2.5 Hz).

Extensive signal overlapping was observed in the aliphatic region (δ 0.89-2.18) of the ^{1}H NMR spectrum. These signals accounted for eight methylene and three methine protons and several of them were overlapped with the methyl signals.

The characteristic olefinic proton at δ 5.60 (H-2') which was coupled with protons at δ 2.15 (H-4') and 1.95 (H-5'), indicated the presence of a senecioic acid ester moiety (Figure 2.24) in **10**.[189] This was further confirmed by the olefinic carbon signal at δ 115.9 which showed HMBC couplings with the H-4' and H-5' methyl

protons. In addition, the quaternary carbon at δ 157.0 showed similar couplings with the same methyl protons (Table 2.16, page 119).

The coupling network of the molecule was assigned mainly on the basis of HMBC and TOCSY spectral data (Table 2.16, 2.17, page, 119, 122). The senecioate carbonyl at δ 165.8 showed a three-bond correlation with the oxymethine proton H-3 which was coupled to the carbon at δ 76.7, indicating the senecioate was attached to the oxygen at C-3. The C-1 and C-5 carbons showed HMBC couplings with the H-3 proton at δ 4.68. The C-3 and C-5 carbons both exhibited similar couplings with H-28 and H-29 methyls at δ 0.90 and 0.67, respectively. These methyl protons showed ^{1}H-^{1}H correlations in the TOCSY spectrum (Figure 2.32, page 121), establishing their geminal disposition and the attachment to C-4. The tertiary methyls at δ 0.98 (H-19) and 1.20 (H-30) were placed on C-10 and C-8, respectively, on the basis of HMBC spectral data (Figure 2.31, page 121).

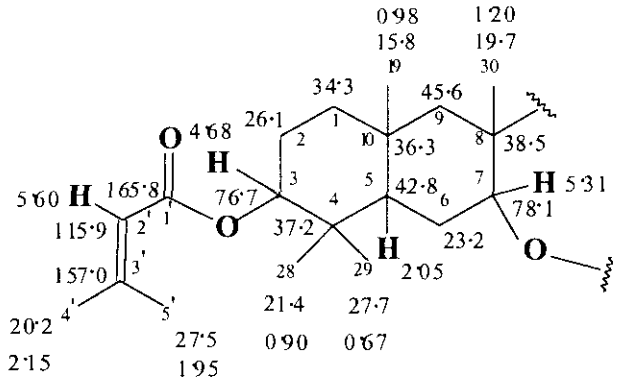


Figure 2.24 : Fragment 1

In addition, carbons at C-5, C-8 and C-9 showed HMBC correlations with the H-7 proton at δ 5.31 and the C-7 carbon also showed similar correlations with the H-30 proton (Figure 2.31, page 121). All these spectral data account for the assignment of fragment **1** in the molecule (Figure 2.24).

The location of the cyclopropane ring in the molecule (Figure 2.25) was assigned on the basis of HMBC spectral data, in which the carbons at C-13, C-14, C-16 and C-17, showed couplings (Figure 2.31, page 121) with the cyclopropyl methylene protons at H-18 (δ 0.25 and 0.67), thus establishing the fragment **2** of the molecule (Figure 2.25).

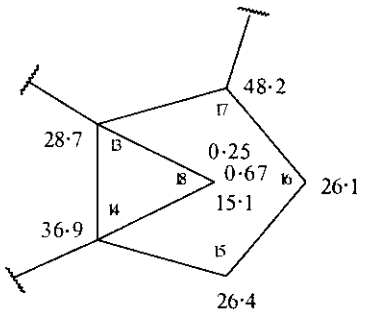


Figure 2.25 : Fragment **2**

The presence of a trisubstituted epoxide was further confirmed by the HMBC spectral data in which correlation between an oxygenated quaternary carbon at 57.2 (C-25) and H-24, H-26 and H-27 protons was observed. The C-24 showed correlations with H-22, H-23, H-26 and H-27 protons and C-23 showed similar correlations with protons at H-21, H-22 and H-24 in the HMBC spectrum. The ^{1}H-^{1}H COSY spectrum exhibited correlations between the protons at H-20 to H-

24 (Figure 2.30, page 120). The C-20 and C-21 carbons showed HMBC correlations with the proton at H-17 which in-turn showed TOCSY correlations with protons at H-20 to H-24 (Figure 2.32, page 121), thus assigning the fragment **3** of the molecule (Figure 2.26) and the location at C-17.

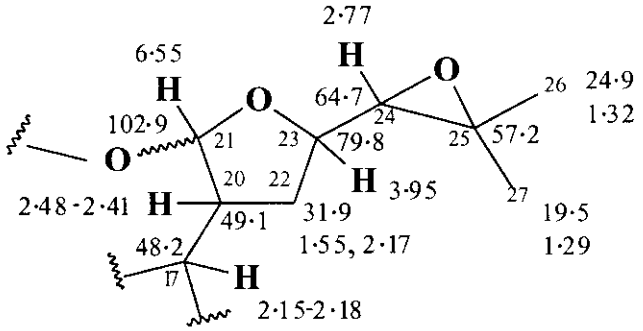


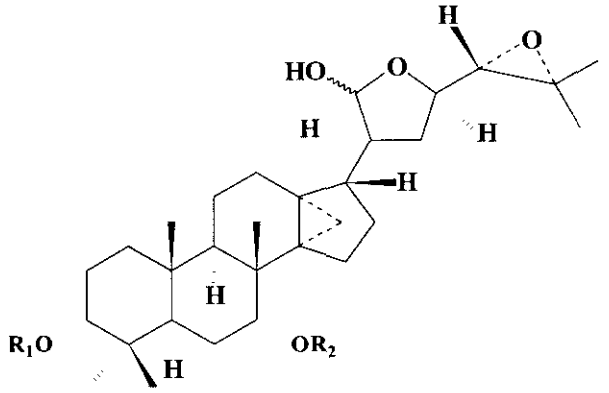
Figure 2.26 : Fragment **3**

The location of the two p-nitrobenzoyl ester groups was assigned on the basis of HMBC spectral data (Figure 2.31, page 121) in which the proton signals at δ 5.31 (H-7) and 6.55 (H-21) were correlated with the carbon signals at δ 163.4 and 164.0, respectively. These clearly indicated that two p-nitrobenzoates could be placed on C-7 and C-21.

Comparison of the ^{1}H NMR spectrum of both di p-nitrobenzoate and nonbenzoate clearly indicated that the protons resonating at δ 3.70 and 5.43 were shifted to δ 5.31 and 6.55, respectively, thus indicating the location of two p-nitrobenzoates on C-7 and C-21.

The characteristic signals identified for melianone-type side chain in fragment **3** (Figure 2.26) and cyclopropane ring (Figure 2.25) suggested the main carbon

skeleton of **10** is analogous to those of glabretal (e.g. Skimmiarepin **A** and **B**) and ailanthol triterpenes (Figure 2.27 and 2.28).[190, 191] Comparison of the reported ^{13}C NMR shifts for the 3-isovaleryl glabretal, Skimmiarepin **A**[188] with those observed for **10** (Table 2.15, page 117), identified the similar parent skeleton in both compounds but different ester functionality at C-3.



	R_1	R_2
Skimmiarepin A	$COCH_2CH(CH_3)_2$	**H**
Skimmiarepin B	$COCH{=}CHCH{=}CHCH{=}CHCH_2CH_2CH_3$	**H**
Glabretal	**H**	**Ac**

Figure 2.27 : Glabretal triterpenes

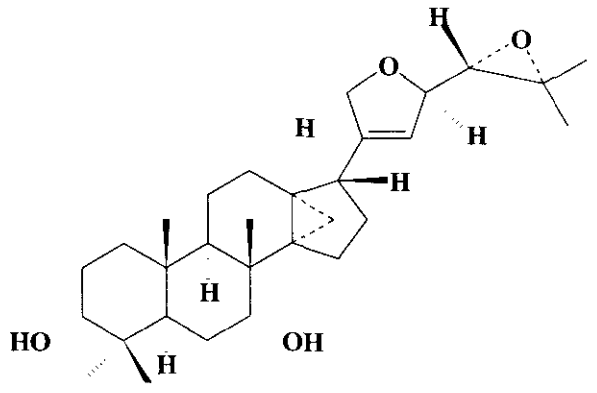


Figure 2.28 : Ailanthol

Table 2.15 : ^{13}C and ^{1}H NMR data of compound **10** and reported ^{13}C NMR data in $CDCl_3$

Position	Reported δ_C	Observed δ_C	DEPT	δ_H (*J*)
1	**34.1**	34.3	CH_2	1.41*, 1.21*, m
2	**26.6**	26.2	CH_2	1.90*, 1.62, m
3	**77.8**	76.7	CH	4.68, s
4	**37. 5**, 37.2	37.3	C_q	-
5	**41.5**, 45.5	42.8	CH	2.05-2.01, dd (8.9, 2.4)
6	**24.5**	23.3	CH_2	1.70, m
7	**74.3**	78.1	CH	5.31, br s
8	**39.2**, 39.3	38.5	C_q	-
9	**44.2**, 44.0	45.6	CH	1.49, m
10	**36.4**	36.3	C_q	-
11	**16.5**, 16.3	16.6	CH_2	1.32, m
12	**23.2**	22.7	CH_2	1.56, 0.89, m
13	**29.3**, 28.9	28.7	C_q	-
14	**37.2**	36.9	C_q	-
15	**25.9**, 26.2	26.4	CH_2	1.90*, 1.80, m
16	**27.8**, 26.5	26.0	CH_2	1.69, 1.52, m
17	**45.0**, 48.5	48.2	CH	2.15-2.18*, m
18	**14.1**, 13.9	15.1	CH_2	0.67, d, 0.25, d (5.5)
19	**16.0**, 15.9	15.8	CH_3	0.98, s
20	**49.6**, 51.0	49.1	CH	2.48-2.41, qd (6.1, 5.4, 2.5)
21	**98.1**, 102.0	102.9	CH	6.55, d (2.5)
22	**31.1**, 33.3	31.9	CH_2	2.17*, 1.55, m
23	**78.4**, 77.8	79.8	CH	4.01-3.95, qd (9.8, 7.3, 2.7)
24	**67.7**, 65.4	64.7	CH	2.77, d (7.3)
25	**57.2**, 57.4	57.2	C_q	-
26	**25.3**, 25.2	24.9	CH_3	1.32, s
27	**19.7**, 19.8	19.5	CH_3	1.29, s
28	**22.2**	21.5	CH_3	0.90, s
29	**28.3**	27.7	CH_3	0.67, s
30	**19.5**, 19.8	19.7	CH_3	1.20, s
	Isovalerate	**Senecioate**		
1'	172.4	165.8	C_q	-
2'	44.0	115.9	CH	5.60, s
3'	26.0	157.0	C_q	-
4'	22.7	20.2	CH_3	2.15, s
5'	22.7	27.5	CH_3	1.95, s

table contd.

		Nitrobenzoate		
1"	-	163.4	C_q	-
2"	-	150.6	C_q	-
3", 7"	-	130.8	CH	8.38-8.17, m
4", 6"	-	123.6	CH	8.38-8.17, m
5"	-	135.5	C_q	-
1"'	-	164.0	C_q	-
2"'	-	150.4	C_q	-
3"', 7"'	-	130.6	CH	8.21-8.17, m
4"', 6"'	-	123.6	CH	8.21-8.17, m
5"'	-	136.7	C_q	-

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

Bold numbers denote major epimer. * - Peaks obscured

The structure of **10** was established as a 3-senecioyl glabretal triterpene belonging to the class of protolimonoids (Figure 2.29). Compound **10** is novel and consists of a senecioate ester, two p-nitrobenzoate esters, a cyclopropane ring and a side chain at C-17 with a hemiacetal ring and a trisubstituted epoxide.

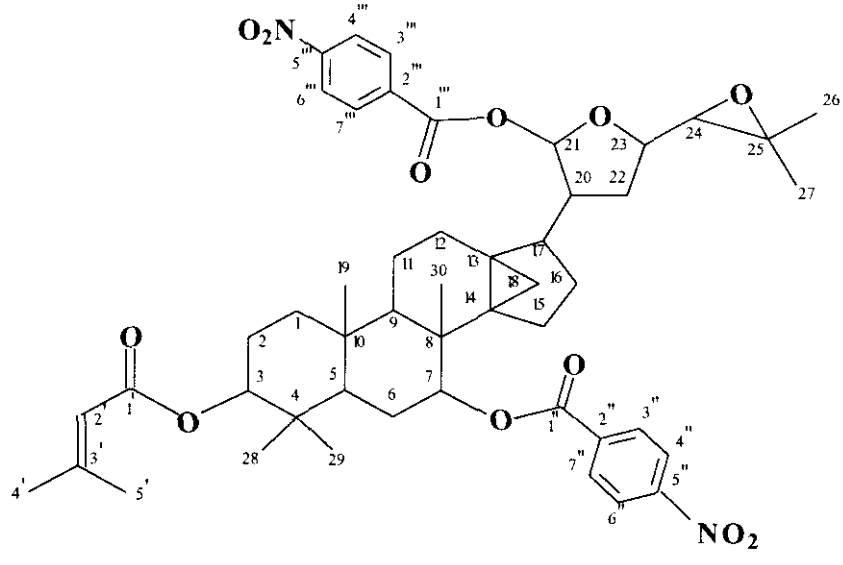


Figure 2.29 : Compound **10**

Table 2.16 : ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and HMBC spectral data for compound **10** in $CDCl_3$

Position	^{1}H-^{1}H COSY	^{13}C-^{1}H COSY	HMBC
1	1.21 → 1.90	34.3 → 0.98	4.68, 0.98
2	1.90 → 4.68, 1.62, 1.21	-	-
3	4.68 → 1.90, 1.62	76.7 → 0.90, 0.67	0.9, 0.67
4	-	-	-
5	-	42.8 → 0.90, 0.67	5.31, 4.68, 0.90, 0.98, 0.67, 1.90
6	1.70 → 5.31	-	2.05, 0.67, 0.90
7	5.31 → 1.70	78.1 → 1.20	2.05, 1.20
8	-	38.5 → 1.20	5.31
9	-	45.6 → 0.98	5.31, 1.32, 1.20, 0.98, 0.89
10	-	36.3 → 0.98, 0.67	4.68, 2.05, 1.7, 1.49 1.20, 0.67, 0.98
11	1.32 → 1.56, 0.89	-	1.49, 0.89
12	1.56 → 1.32, 0.89	-	-
13	-	-	1.56, 1.52, 0.25
14	-	-	1.80, 1.52, 1.49, 1.20, 0.25
15	1.90 → 1.69, 1.52	-	-
16	1.69, 1.52 → 2.18-2.15, 1.90	-	0.25
17	2.15-2.18 → 1.69, 1.52	48.2 → 1.69-1.52	6.55, 2.48-2.41,1.55 1.52, 1.69, 0.25
18	0.67 → 0.25	-	-
19	-	-	2.05, 0.9
20	2.48-2.41 → 6.55, 2.17, 1.55	49.2 → 1.55	2.15-2.18
21	6.55 → 2.48-2.41	102.9 → 2.48-2.41	2.48-2.41, 2.15-2.18
22	2.17 → 4.01-3.95, 2.48, 1.55	-	6.55
23	4.01-3.95 → 2.77, 2.17, 1.55	79.8 → 6.55, 1.55	6.55, 2.77, 1.55,1.32
24	2.77 → 4.01-3.95	64.7 → 1.29	4.01-3.95,1.55, 1.32, 1.29
25	-	57.2 →1.29	2.77, 1.32, 1.29
26	-	24.9 → 1.29	1.29
27	-	19.5 → 1.32	1.32
28	-	21.5 → 0.90	2.05
29	-	27.7 → 0.67	-
30	-	-	1.49, 0.98

table contd.

1'	-	165.8 → 4.68, 2.15	4.68, 5.6
2'	5.60 → 2.15, 1.95	115.9 → 2.15, 1.95	2.15, 1.95
3'	-	157.0 → 2.15, 1.95	2.15, 1.95
4'	2.15 → 5.60	20.2 → 5.60, 1.95	5.60, 1.95
5'	1.95 → 5.60	27.5 → 5.60, 2.15	5.60, 2.15
1''	-	163.4 → 5.31	5.31, 8.38-8.17
2''	-	150.6 → 8.38-8.17	8.38-8.17
3'', 7''	-	-	8.38-8.21
4'', 6''	-	-	8.38-8.17
5''	-	135.5 → 8.38-8.17	8.38
1'''	-	164.0 → 8.21-8.17	6.55, 8.21-8.17
2'''	-	150.4 → 8.21-8.17	8.21-8.17
3''', 7'''	-	-	8.17
4''', 6'''	-	-	8.21-8.17
5'''	-	136.7 → 8.21-8.17	8.21

Chemical shift values are in ppm.

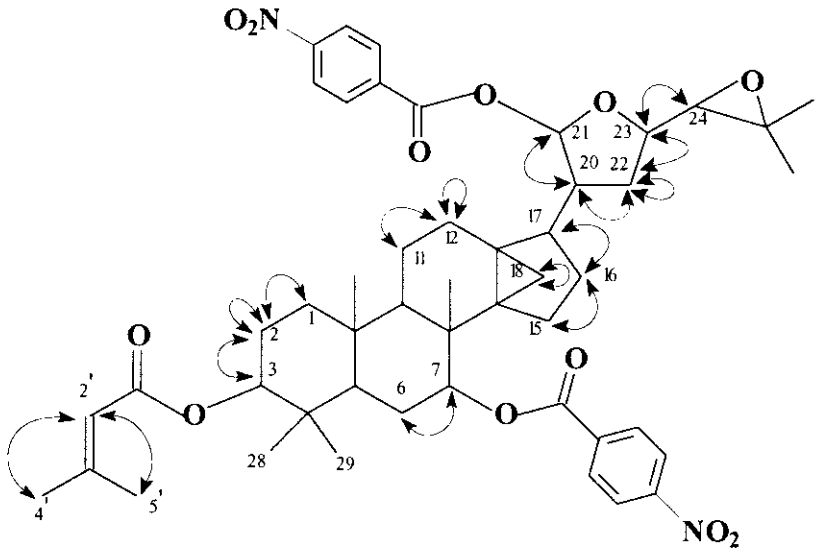


Figure 2.30 : ^{1}H-^{1}H COSY correlations for compound **10**

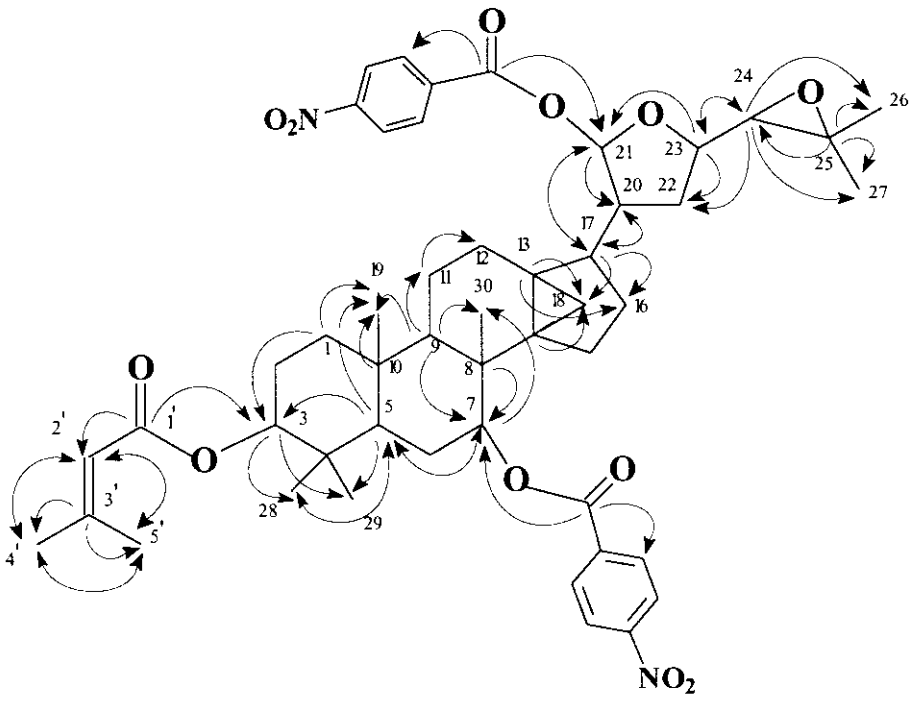


Figure 2.31 : HMBC correlations for compound **10**

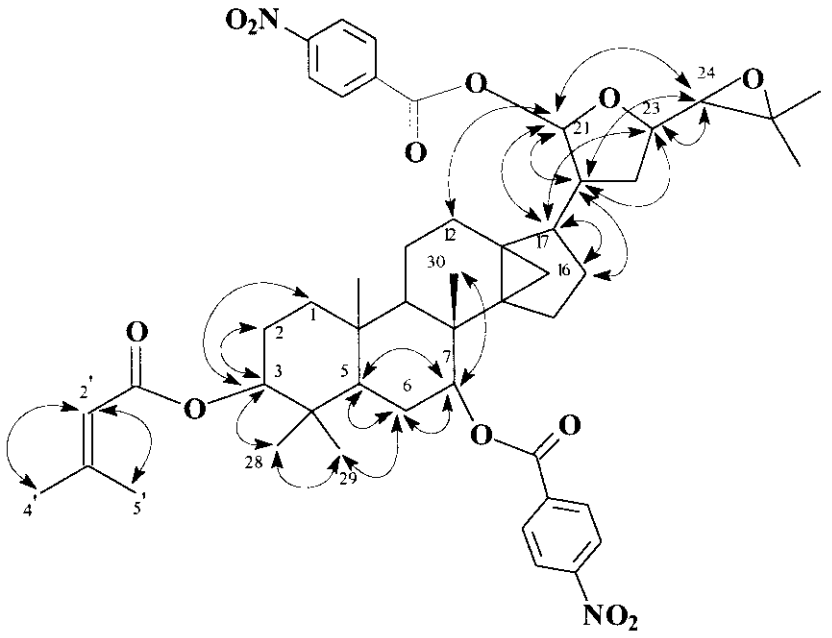


Figure 2.32 : Selected TOCSY correlations for compound **10**

Table 2.17 : TOCSY spectral data for compound **10** in $CDCl_3$

Position	δ_H	50 ms	80 ms	150 ms
1	1.41, 1.21	-	4.68	4.68, 1.90, 1.62, 0.98
2	1.90 , 1.62	4.68	4.68	4.68, 1.62, 1.41, 1.21 0.67
3	4.68	1.90, 1.62, 1.21	1.90, 1.62, 1.41, 1.21, 0.90	1.90, 1.62, 1.41, 1.21, 0.90
5	2.05-2.01	1.70	5.31, 1.70	5.31, 1.70
6	1.70	5.31, 2.05,	5.31, 2.05-2.01	5.31, 2.05-2.01, 0.67
7	5.31	2.05-2.01, 1.70	2.05, 1.70, 1.20	2.05, 1.70, 1.20
11	1.32	-	-	1.20, 0.89
12	1.56, 0.89	-	1.90, 1.80	2.15-2.18, 1.32, 1.20
15	1.90, 1.80	-	0.89	1.69, 1.52, 1.32, 0.67
16	1.69, 1.52	-	-	2.48-2.41, 2.15-2.18 1.90, 1.80
17	2.15-2.18	1.90, 1.69, 1.55, 1.52	6.55, 3.95, 2.77, 1.55, 1.20, 0.89	6.55, 2.77, 2.48, 1.52, 1.20, 0.89
18	0.67, 0.25	-	1.90, 1.69, 1.32	1.90, 1.80
19	0.98	-	-	1.41, 1.21
20	2.48-2.41	6.55, 2.77	6.55, 3.95, 2.77, 1.55	2.77, 2.15-2.18, 1.62 1.55, 1.52
21	6.55	2.48-2.41	2.77, 2.48, 2.15, 1.55, 1.90, 0.89	2.77, 2.48, 2.15-2.18, 1.55, 1.52, 0.89
22	2.17, 1.55	-	6.55, 2.77, 2.48, 2.15, 1.55	6.55, 2.48
23	4.01-3.95	2.77, 2.15 , 1.55	2.77, 2.48, 2.15, 1.55	2.77, 2.48-2.41
24	2.77	3.95, 2.48, 1.55	6.55, 3.95, 2.48, 2.15, 1.55, 1.29	3.95, 2.48, 2.18-2.15, 1.55, 1.29
27	1.29	-	2.77	2.77
28	0.90	-	-	4.68
29	0.67	0.90	1.90, 1.70	1.90, 1.70, 1.62
30	1.20	-	5.31	5.31, 2.15-2.18, 1.32, 0.89
2'	5.60	2.15, 1.95	2.15, 1.95	2.15, 1.95
4'	2.15	5.60	5.60	5.60
5'	1.95	5.60	5.60	5.60

Chemical shift values are in ppm.

(b) **Characterisation of compound 11**

Compound **11** was a white crystalline solid (m.p 166-167 °C) which produced a UV active spot (λ = 254 and 366 nm, in 2:3, ethyl acetate:petroleum ether, Rf = 0.41) and a purple spot with anisaldehyde-H_2SO_4.[162]

The IR spectrum showed the absorptions characteristic of ester carbonyl (1720 cm^{-1}) and epoxide (987 cm^{-1}) functionalities. The UV spectrum further confirmed the presence of an ester carbonyl (λ_{max} = 204 nm) and a p-nitrobenzoyl ester (λ_{max} = 260 nm).

The DEPT and ^{13}C NMR spectra led to the recognition of forty nine carbons in the molecule, including eight methyls, ten methylenes, eighteen methines and thirteen quaternaries.

The ^{1}H and ^{13}C NMR spectral data of the compound **11** (Table 2.18) were quite similar to those of **10** (Table 2.15, page 117), except the absence of NMR shifts of the senecioate ester. Instead, several new peaks were observed at δ_H 2.20-2.10, 2.10-2.00, 0.92, 0.91 and δ_C 172.2 (C_q), 44.1 (CH_2), 25.7 (CH), 22.6 (CH_3), 22.5 (CH3) which were identical to those reported for the isovaleryl acid ester moiety in glabretals.[188] The ^{1}H-^{1}H and long-range ^{13}C-^{1}H COSY spectra clearly indicated the H-H and C-H correlations within the isovalerate group (Figure 2.34 and 2.35, page 127). **11** has therefore a similar parent skeleton to **10**, except for having two extra hydrogens, that is, the senecioate is replaced by an isovalerate at the C-3 position (Figure 2.33).

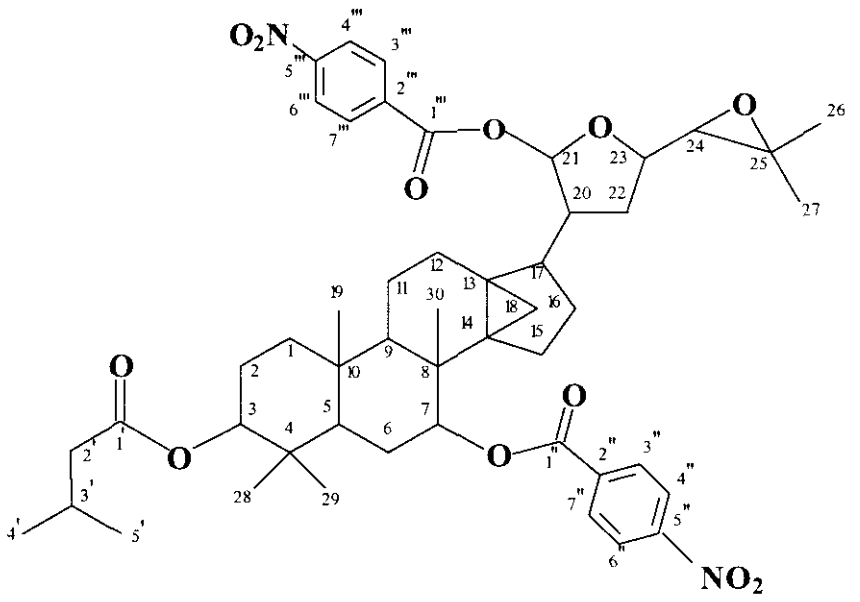


Figure 2.33 : Compound **11**

Table 2.18 : ^{13}C and ^{1}H NMR spectra assignments of compound **11** in $CDCl_3$

Position	δ_C	DEPT	δ_H (*J*)
1	34.2	CH_2	1.50-1.41, m
2	26.0	CH_2	1.95-1.52, m
3	77.5	CH	4.67, s
4	37.3	C_q	-
5	42.9	CH	2.00-1.95, m
6	23.2	CH_2	1.75-1.70, m
7	78.1	CH	5.30, br s
8	38.5	C_q	-
9	45.6	CH	1.50-1.49, m
10	36.2	C_q	-
11	16.6	CH_2	1.48-1.36*, m
12	22.7	CH_2	1.62, 1.58, m
13	28.7	C_q	-
14	36.8	C_q	-
15	26.3	CH_2	1.90-1.80, m
16	26.0	CH_2	1.89, 1.52, m

table contd.

17	48.1	CH	2.20-2.10, m
18	15.2	CH_2	0.70 d, 0.26, d (5.7)
19	15.7	CH_3	0.98, s
20	49.1	CH	2.48-2.40, qd (6.3)
21	103.0	CH	6.56, d (2.5)
22	31.8	CH_2	2.12, 1.50, m
23	79.8	CH	4.02-3.97, qd (13.1, 9.3, 3.9)
24	64.7	CH	2.76, d (7.7)
25	57.2	C_q	-
26	24.9	CH_3	1.32, s
27	19.5	CH_3	1.30, s
28	21.5	CH_3	0.90, s
29	27.9	CH_3	0.67, s
30	19.7	CH_3	1.20, s
$1'$	172.2	C_q	-
$2'$	44.1	CH_2	2.20-2.12, m
$3'$	25.7	CH	2.10-2.00, m
$4'$	22.6	CH_3	0.92, d
$5'$	22.5	CH_3	0.91, d
$1''$	163.4	C_q	-
$2''$	150.6	C_q	-
$3''$, $7''$	130.8	CH	8.33-8.16, m
$4''$, $6''$	123.6	CH	8.33-8.16 m
$5''$	135.5	C_q	-
$1'''$	164.0	C_q	-
$2'''$	150.4	C_q	-
$3'''$, $7'''$	130.5	CH	8.33-8.16, m
$4'''$, $6'''$	123.6	CH	8.33-8.16, m
$5'''$	136.5	C_q	-

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

* - Peaks obscured.

Table 2.19 : ^{1}H-^{1}H and ^{13}C-^{1}H COSY spectral data for compound **11** in $CDCl_3$

Position	^{1}H-^{1}H COSY	^{13}C-^{1}H COSY
1	1.50-1.41→ 1.95-2.00	-
2	2.00-1.95 → 4.67, 1.50, 1.41	
3	4.67 → 2.00-1.95	77.6 → 0.90
6	1.75-1.70 → 5.30	-
7	5.30 → 1.75-1.70	78.1 → 1.20
9	1.50-1.49 → 1.20	45.6 → 0.98
10	-	36.2 → 0.90, 0.67
11	1.48-1.36 →0.87	-
12	1.58 → 0.87	-
13	-	28.7 → 0.87
14	-	36.8 →1.20
15	1.90-1.80 → 1.89, 1.52	-
16	1.89, 1.52 → 1.90-1.80	-
17	2.20-2.10 → 2.48-2.40, 1.89	-
18	0.70 → 0.26	-
20	2.48-2.40 → 6.56, 2.20-2.10, 2.12, 1.50	-
21	6.56 → 2.48-2.40	103.0 → 2.76, 2.48-2.40
22	2.12, 1.50 → 4.02-3.95, 2.48-2.40	-
23	4.02-3.97 → 2.76, 2.20-2.10, 2.12, 1.50	79.8 → 1.30
24	2.76 → 4.02-3.97	64.7 → 1.32, 1.30
25	-	57.2 → 1.32, 1.30
26	-	24.9 → 1.30
27	-	19.5 → 1.32
28	-	21.5 → 0.67
29	-	27.9 → 0.90
30	1.20 → 1.50-1.49	19.7 → 1.48-1.36
1'	-	172.2 → 2.20-2.12
2'	-	44.1 → 0.92, 0.91
3'	2.10-2.00 → 0.92, 0.91	-
4'	0.92 → 2.10-2.00	22.6 → 0.91
5'	0.91 → 2.10-2.00	22.5 → 0.92
1''	-	163.4 → 8.33-8.16
2''	-	150.6 → 8.33-8.16
3'', 7''	-	130.8 → 8.33-8.16

table contd.

$4^{''}, 6^{''}$	-	123.6 → 8.33-8.16
$5^{''}$	-	135.5 → 8.33-8.16
$1^{'''}$	-	164.1 → 8.33-8.16
$2^{'''}$	-	150.4→ 8.33-8.16
$3^{'''}, 7^{'''}$	-	130.5 → 8.33-8.16
$4^{'''}, 6^{'''}$	-	123.6 → 8.33-8.16
$5^{'''}$	-	136.5 → 8.33-8.16

Chemical shift values are in ppm.

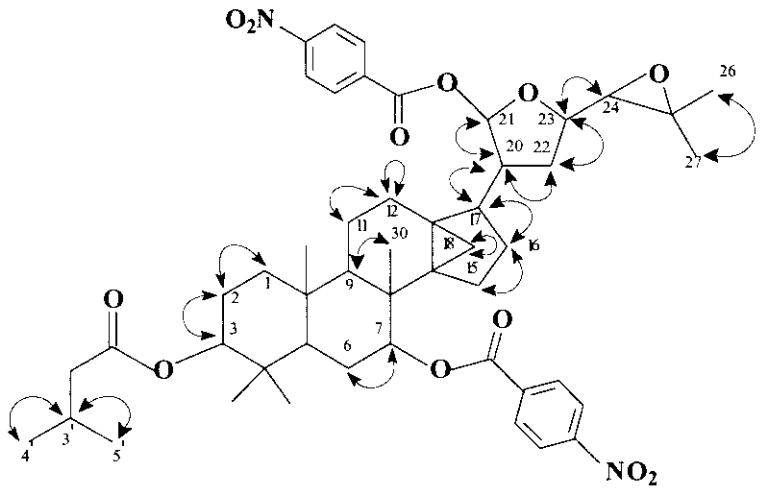


Figure 2.34 : ^{1}H-^{1}H COSY correlations for compound **11**

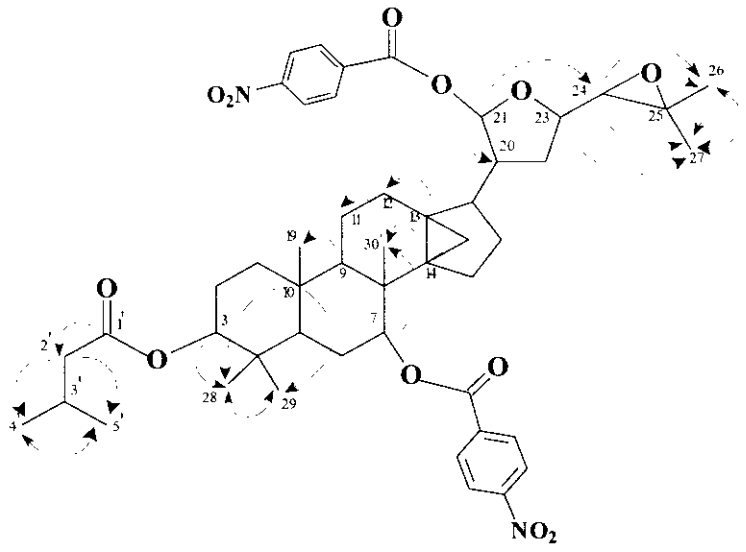


Figure 2.35: Long-range ^{13}C-^{1}H COSY correlations for compound **11**

2.3.8 **The structure of compounds 6-9**

With this structural information, it was possible to interpret the spectra of the underivatised insecticidal mixture $\mathbf{F_A}$ and $\mathbf{F_B}$.

$\mathbf{F_A}$ is a mixture of compounds **6** and **7** epimeric at C-21 due to the presence of a hemiacetal ring in the molecule. The hemiacetal carbons were observed at δ 98.4 and 102.3 in the ^{13}C NMR spectrum. The hemiacetal ring opens and closes in solution; thus, a mixture of epimers is always present, resulting in a complex ^{13}C NMR spectrum. However, since one epimer was present in a much higher concentration than the other, the characterisation of $\mathbf{F_A}$ focused on the major epimer.

A trisubstituted epoxide (δ_H 2.84, d, J = 7.4 Hz; δ_C 67.6, 58.1), a cyclopropyl methylene group (δ_H 0.75, 0.48, d, J = 4.4 Hz; δ_C 13.7), six tertiary methyl groups (δ_H 1.32, 1.31, 1.02, 2×0.89, 0.85) and five oxymethine protons (δ_H 5.43, 4.68, 4.00-3.85, 3.76 and 2.84) were recognised in the spectra of $\mathbf{F_A}$ which were already identified for di p-nitrobenzoates (**10** and **11**). The epoxide functionality is located in the melianone-type side chain which was evident by the resonances at δ 5.43 (d), 4.00-3.85 (m) and a pair of doublets at δ 2.84 and 2.70 (J = 7.4 Hz) in the two epimers.[192]

The presence of a senecioic acid ester moiety was proved since the signals identified for this group in the di p-nitrobenzoate (δ_H 5.77, br s, 2.18, s, 1.90, s; δ_C 166.5, 116.9, 156.0, 20.3, 27.4) were still present.

Comparison of the ^{1}H NMR spectrum of $\mathbf{F_A}$ with the di p-nitrobenzoate (**10**) indicated that protons resonated at δ 3.70 and 5.43 were shifted to δ 5.31 and 6.55, respectively, in **10**, thus identifying the location of two OH groups in $\mathbf{F_A}$ as on C-7 and C-21.

The spectra of $\mathbf{F_A}$ were compared (Table 2.20) with those reported for the α- and β-epimers of glabretal isovalerate, which occurs in *Skimmia* japonica (Rutaceae) [188] and *Zanthoxylum petiolare* (Rutaceae)[193] and with this information, the structures of compound **6** and **7** were assigned as 3-senecioyl glabretal triterpenes (Figure 2.36). These two senecioate esters **6** and **7** are novel and occur naturally as a mixture of α- and β-epimers at C-21.

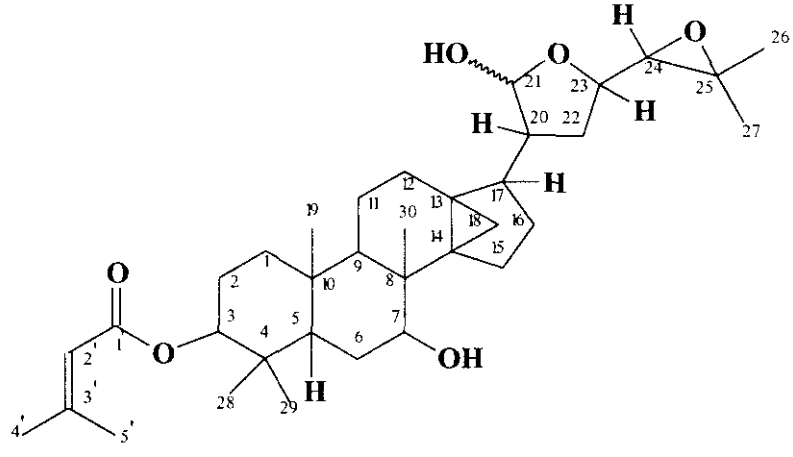


Figure 2.36 : Compound 6 and 7

Table 2.20 : ^{13}C and ^{1}H NMR data of $\mathbf{F_A}$ and reported ^{13}C NMR data in $CDCl_3$

Position	δ_C	Reported δ_C	DEPT	δ_H (*J*)
1	**33.8**, 33.7	**34.1**	CH_2	1.50*, 1.21, m
2	**27.5**	**26.6**	CH_2	1.92, 1.60*, m
3	**76.7**	**77.7**	CH	4.68, dd
4	**37.2**, 37.3	**37.5**, 37.2	C_q	-
5	**41.2**, 41.3	**41.5**, 45.5	CH	2.15-2.00, m
6	**24.1**	**24.5**	CH_2	1.70, m
7	**74.3**	**74.3**	CH	3.76, br s
8	**38.9**, 39.0	**39.2**, 39.3	C_q	-
9	**44.0**, 43.8	**44.2**, 44.0	CH	1.50-1.40*, m
10	**36.2**, 36.1	**36.4**	C_q	-
11	**16.1**, 16.0	**16.5**, 16.3	CH_2	1.32**, m
12	**22.8**	**23.2**	CH_2	1.60-1.50*, m, 0.80**
13	**28.9**	**29.3**, 28.9	C_q	-
14	**36.9**	**37.2**	C_q	-
15	**25.8**, 25.6	**25.9**, 26.2	CH_2	1.90-1.80**, m
16	**26.3**, 26.2	**27.8**, 26.5	CH_2	1.65-1.50*, m
17	**44.7**, 48.5	**44.9**, 48.5	CH	2.20, m
18	**13.7**, 13.5	**14.2**, 13.9	CH_2	0.75, d, 0.48, d (4.4)
19	**15.7**, 15.6	**16.0**, 15.9	CH_3	0.89, s
20	**49.3**, 51.0	**49.6**, 51.0	CH	2.24, m
21	**98.4**, 102.3	**98.1**, 102.0	CH	5.43, dd (4.1)
22	**30.8**, 30.3	**31.1**, 33.3	CH_2	2.15, 1.50*, m
23	**78.5**, 77.8	**78.4**, 77.8	CH	4.00-3.85, m
24	**67.6**, 65.0	**67.7**, 65.4	CH	2.84, d, (7.4), 2.7, d (7.7)
25	**58.1**, 57.3	**57.2**, 57.4	C_q	-
26	**25.0**, 24.9	**25.3**, 25.2	CH_3	1.32, s
27	**19.5**, 19.4	**19.7**, 19.8	CH_3	1.31, s
28	**21.8**	**22.2**	CH_3	0.89, s
29	**27.7**, 28.6	**28.0**	CH_3	0.85, s
30	**19.1**	**19.5**, 19.8	CH_3	1.02, s
7-OH	-	-	-	2.49, s
21-OH	-	-	-	2.55, s

table contd.

	Senecioate	Isovalerate		
1'	166.5	172.4	C_q	-
2'	116.9	44.0	CH	5.77, br s
3'	156.0	26.0	C_q	-
4'	20.3	22.7	CH_3	2.18, s
5'	27.4	22.7	CH_3	1.90, s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

Bold numbers denote major epimer. * - Assignments may be interchanged. ** - Peaks obscured.

Comparison of the spectra of $\mathbf{F_B}$ with those of glabretal isovalerate (Table 2.21) showed that they are identical to those previously isolated from *Skimmia japonica* (Rutaceae)[188] and subsequently from *Zanthoxylum petiolare* (Rutaceae).[193] The structure of **8** and **9** were assigned as 3-isovaleryl glabretal triterpenes which occur naturally as a mixture of α- and β-epimers (Figure 2.37).

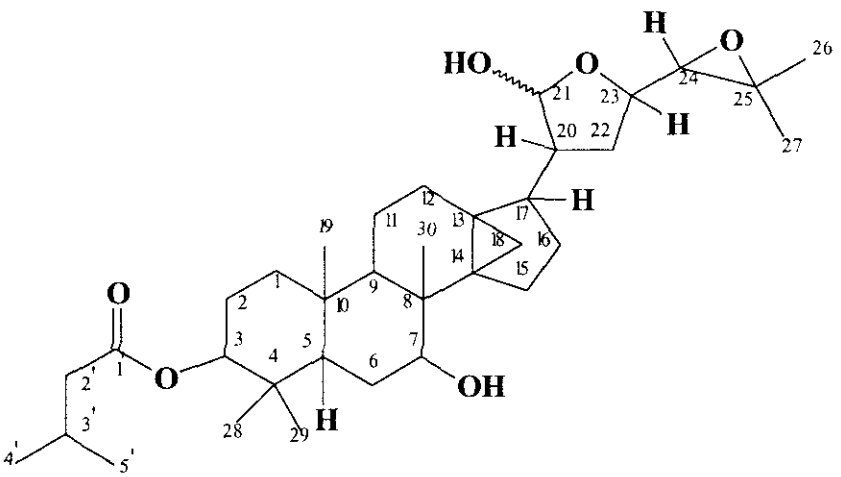


Figure 2.37 : Compound **8** and **9**

Table 2.21: ^{13}C and ^{1}H NMR data of $\mathbf{F_B}$ and reported ^{13}C NMR data in $CDCl_3$

Position	δ_C	Reported δ_C	DEPT	δ_H (J)
1	**33.8**, 33.7	**34.1**	CH_2	1.51*, 1.21*, m
2	**27.5**	**26.6**	CH_2	1.94, 1.60*, m
3	**76.7**	**77.7**	CH	4.63, dd, 4.64 dd
4	**37.2**	**37.5**, 37.2	C_q	-
5	**41.2**, 41.3	**41.5**, 45.5	CH	2.15-2.00*, m
6	**24.2**	**24.5**	CH_2	1.68, m
7	**74.4**	**74.3**	CH	3.74, br s
8	**38.9**, 39.0	**39.2**, 39.3	C_q	-
9	**44.0**, 43.8	**44.2**, 44.0	CH	1.51-1.48*, m
10	**36.2**, 36.1	**36.4**	C_q	-
11	**16.1**	**16.5**, 16.3	CH_2	1.32**, m
12	**22.9**	**23.2**	CH_2	1.60-1.51*, m, 0.80**
13	**29.0**	**29.3**, 28.9	C_q	-
14	**37.0**	**37.2**	C_q	-
15	**25.8**, 25.7	**25.9**, 26.2	CH_2	1.89-1.80**, m
16	**26.3**	**27.8**, 26.5	CH_2	1.65-1.50*, m
17	**44.8**, 48.3	**44.9**, 48.5	CH	2.20*, m
18	**13.7**	**14.2**, 13.9	CH_2	0.75, d, 0.40, d (4.4)
19	**15.7**, 15.7	**16.0**, 15.9	CH_3	0.89, s
20	**49.4**	**49.6**, 51.0	CH	2.25, m
21	**98.2**, 103.0	**98.1**, 102.0	CH	5.40, dd
22	**30.8**	**31.1**, 33.3	CH_2	2.14, 1.51*, m
23	**78.5**, 77.5	**78.4**, 77.8	CH	4.00-3.82, m
24	**67.6**, 65.2	**67.7**, 65.4	CH	2.80, d, 2.7, d
25	**58.1**	**57.2**, 57.4	C_q	-
26	**25.6**, 25.0	**25.3**, 25.2	CH_3	1.32, s
27	**19.5**, 19.4	**19.7**, 19.8	CH_3	1.31, s
28	**20.2**	**22.2**	CH_3	0.89, s
29	**27.7**, 27.8	**28.0**	CH_3	0.85, s
30	**19.2**	**19.5**, 19.8	CH_3	1.03, s
7-OH	-	-	-	2.38, s
21-OH	-	-	-	2.44, s, 2.43, s
1'	173.0	172.4	C_q	-
2'	44.0	44.0	CH_2	2.20-2.14, m
3'	27.4	26.0	CH	2.00-1.80, m

table contd.

4'	22.4	22.7	CH_3	0.97, d
5'	21.9	22.7	CH_3	0.96, d

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

Bold numbers denote major epimer. * - Assignments may be interchanged. ** - Peaks obscured

2.3.9 **Stereochemistry of compounds 6-9**

Previous work on glabretal triterpenes reported that the hydroxyl group at C-21 (δ 98.1) for the major epimer is β-oriented and the minor (δ 102.0) is α-oriented.[192, 194] With this information the senecioate ester **6** can be assigned as the β-epimer (δ 98.4) and **7** as the α-epimer (δ 102.3) at C-21. The isovalerate esters **8** and **9** are the β- and α-epimers at C-21, respectively (δ 98.2 and 103.0).

2.3.10 **Stereochemistry of p-nitrobenzoates**

Compound **10** was assumed to be the α-epimer at C-21 [(δ 102.9) Figure 2.39, page 134] based on the literature reported values for α (δ 102.3) and β (δ 98.1) epimers of glabretal isovalerates.[192] Biogenetically, H-19, H-29 and H-30 tertiary methyls are β-oriented and H-28 is α-oriented in glabretal triterpenes.[192]

The NOESY correlation, in particular, the anticipated enhancement between H-21 and H-30; H-21 and H-17 confirmed their close proximity in space and thus that compound **10** is the α-epimer. Figure 2.40 and 2.41 (page 136 and 137) show computer generated (ALCHEMY III, TRIPOS) 3D molecular representations of lowest energy confirmations of α- and β-epimers, respectively. According to this, such NOESY correlations can only occur for the α-epimer (Figure 2.40, page

136) and not for the β-epimer (Figure 2.41, page 137). This confirms that the α and β stereochemistry were correctly assigned in the previously published work. [192, 194] The NOESY experiment also indicated other spatial correlations of the molecule (Table 2.22). Consequently the relative stereochemistry of compound **10** was established as shown in Figure 2.39.

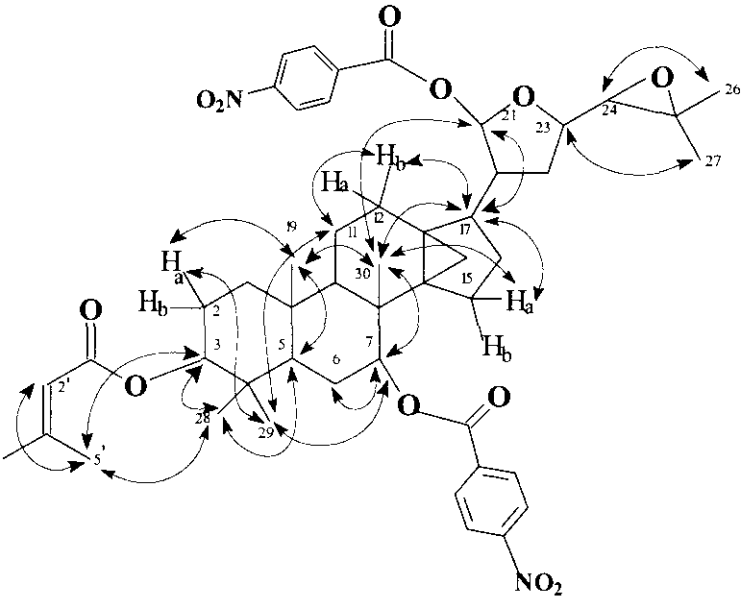


Figure 2.38 : NOESY correlations for compound **10**

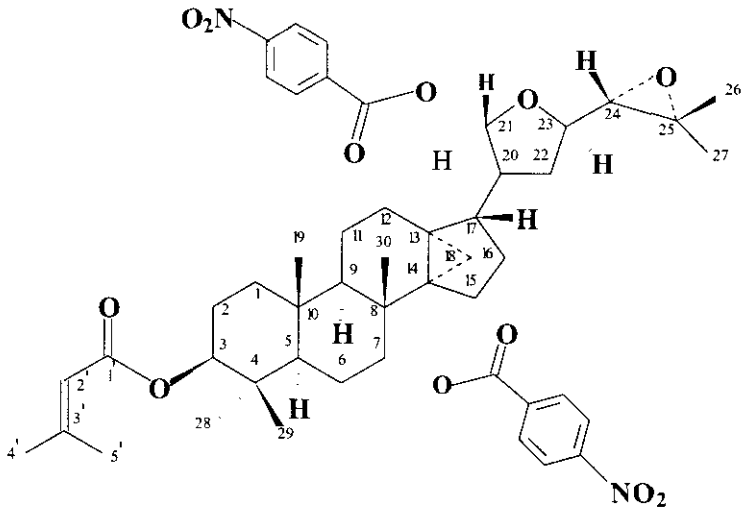


Figure 2.39 : The relative stereochemistry of compound **10**

Table 2.22 : NOESY spectral data for compound **10** in $CDCl_3$

Position	δ_H	NOESY (^1H-^1H)
2a	1.90	H-19, H-29, H-30
2b	1.62	-
3	4.68	H-18b, H-28, H-2'
5	2.05-2.01	H-28
7	5.31	H-6, H-18a, H-29, H-30
11	1.32	H-12b, H-29
12a	1.56	-
12b	0.89	H-11, H-17
15a	1.90	H-17, H-30
15b	1.80	-
17	2.15-2.18	H-12b, H-21, H-29,H-30
18a	0.25	H-7
18b	0.67	H-3
19	0.98	H-2a, H-29, H-30
21	6.55	H-17, H-29, H-30, H-5'
23	4.01-3.95	H-27
24	2.77	H-26
26	1.32	H-24
27	1.29	H-23
28	0.90	H-3, H-5
29	0.67	H-2a, H-7, H-11, H-17, H-18a, H-19,
30	1.20	H-2a, H-7, H-15a, H-17, H-19, H-21,
2'	5.60	H-5'
5'	1.95	H-3, H-28, H-2'

Chemical shift values are in ppm.

Compound 10 - α- epimer

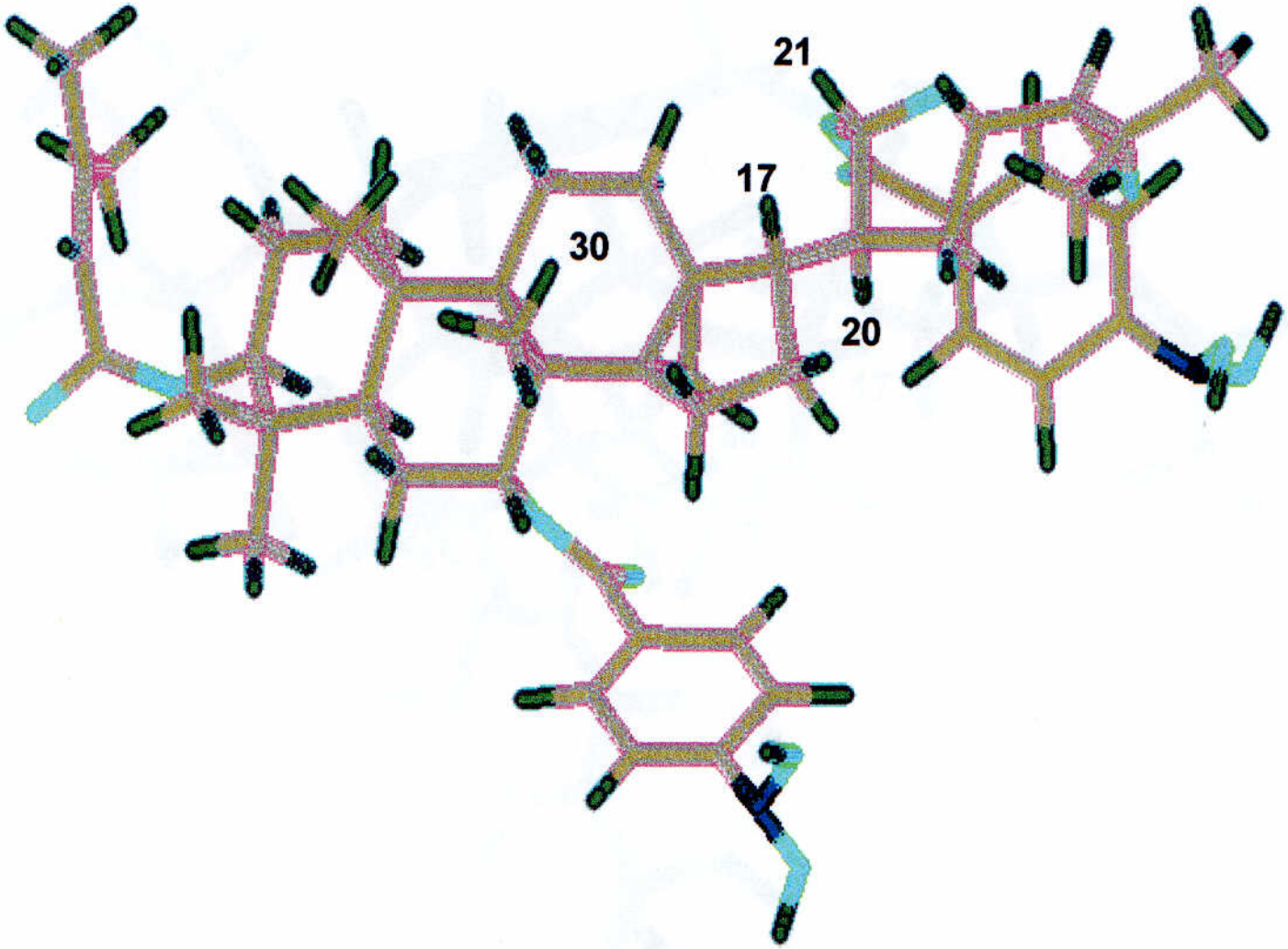


Figure 2.40 : 3D- Representation of compound 10

β- epimer

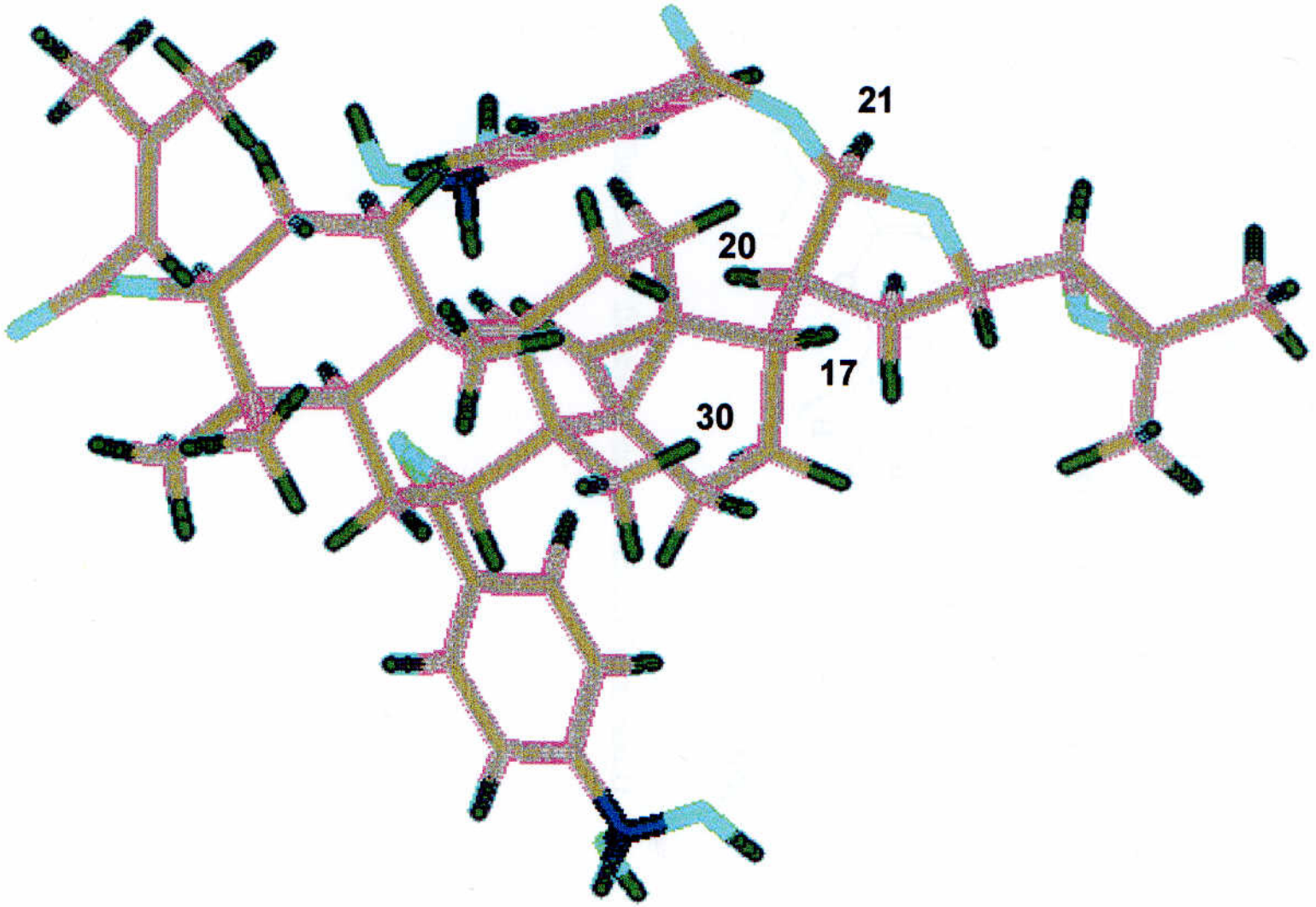


Figure 2.41 : 3D- Representation of β-epimer

The NOESY spectral data of compound **11** (Table 2.23) was similar to those observed for **10**, thus indicating the same relative stereochemistry for **11** (Figure 2.42).

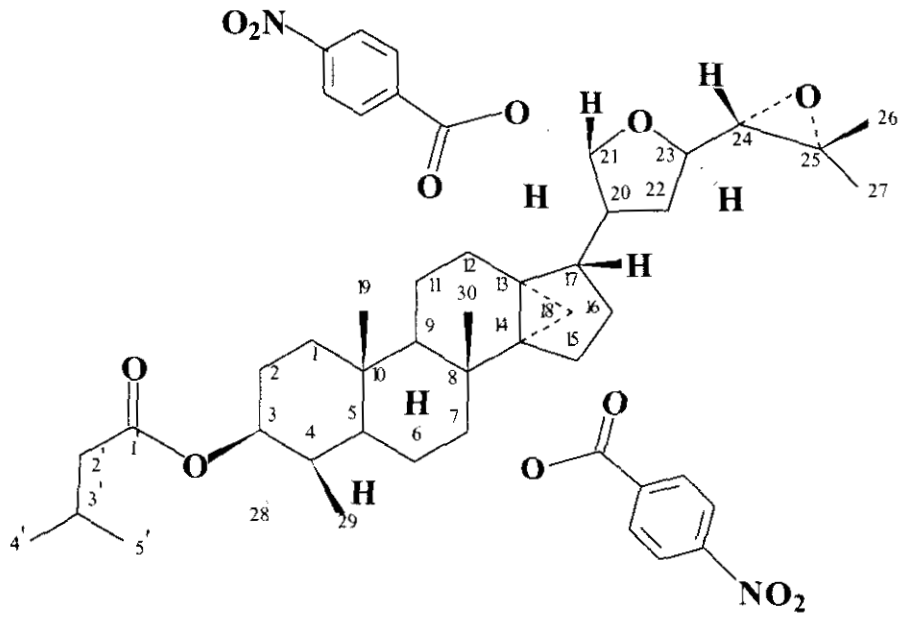


Figure 2.42 : The relative stereochemistry of compound 11

Table 2.23 : NOESY spectral data for compound **11** in $CDCl_3$

Position	δ_H	NOESY (^1H-^1H)
1	1.50, 1.41	H-3
2a	1.95	H-19, H-29, H-30
2b	1.52	-
3	4.67	H-1, H-18b, H-28
5	2.00-1.95	H-28
6	1.75-1.70	H-7, H-29
7	5.30	H-6, H-18a, H-29, H-30
9	1.50-1.49	H-28
11	1.48-1.36	H-29
12a	1.58	-
12b	0.87	H-17
15a	1.90-1.80	H-30
15b	1.80	-
17	2.20-2.10	H-12b, H-19, H-21, H-26, H-29,
18a	0.26	H-7, H-29, H-30
18b	0.70	H-3, H-28
19	0.98	H-2a, H-17, H-29, H-30
20	2.48-2.40	H-23, H-27
21	6.56	H-17, H-29, H-30
23	4.02-3.97	H-27, H-20
24	2.76	H-26
26	1.32	H-17, H-24, H-30
27	1.30	H-20, H-23
28	0.90	H-3, H-5, H-9, H-18b
29	0.67	H-2a, H-6, H-7, H-11, H-17, H-
30	1.20	H-2a, H-7, H-15a, H-17, H-18a,

Chemical shift values are in ppm.

2.3.11 **Characterisation of acetyl derivatives 12-17**

(a) **Characterisation of compound 12**

Compound **12** was a colourless oil and visualised as a purple spot (2:3, ethyl acetate-petroleum ether, Rf = 0.35) with anisaldehyde-H_2SO_4.[162]

The IR spectrum showed the absorptions characteristic of α, β-unsaturated ester (1741, 1646 cm^{-1}), hydroxyl (3007 cm^{-1}), and epoxide (949 cm^{-1}) functionalities. The UV spectrum also evidenced the presence of an α, β-unsaturated ester (λ_{max} = 216 nm).

The high resolution electrospray API mass spectrum showed the molecular ion at m/z 612.3905, suggesting the molecular formula to be $C_{37}H_{56}O_7$. The molecular formula indicated a degree of unsaturation of ten.

The presence of one acetyl ester group in **12** was shown by the methyl singlet at δ 2.08 in the ^1H NMR spectrum. This was further confirmed by the characteristic acetylation shift of 0.87 ppm, observed for the -CH-O proton resonating at δ 5.43. The nonacetylated hydroxyl group at C-7 was observed as a broad singlet at δ 2.40 in the ^1H NMR spectrum which was further confirmed by the characteristic IR absorption for hydroxyl group. In addition, the proton at δ 3.70 remained unshifted during the acetylation, indicating its attachment to the carbon bearing the hydroxyl group.

The compound, **12** exhibited a close spectral analogy to **10** (Table 2.24 and 2.15, page 117) and the reported isovaleryl glabretals[188], thus the structure was established by comparing mainly the NMR spectral data. The parent skeleton of **12** is therefore, an intact glabretal with a senecioate ester[189] at C-3, hydroxyl at C-7 and a melianone-type side chain[192] at C-17 (Figure 2.43). The HMBC and TOCSY spectral data of **12** clarified the connectivity of each segment of the molecule (Figure 2.45 and 2.46, page 145, 147) which helped to confirm the structure as shown in Figure 2.43. The relative stereochemistry of the molecule (Figure 2.43) was established on the basis of the NOESY experimental data (Table 2.27, page 148) and the value of the epimeric carbon at C-21 (δ 97.4), thus the molecule is the β-epimer.

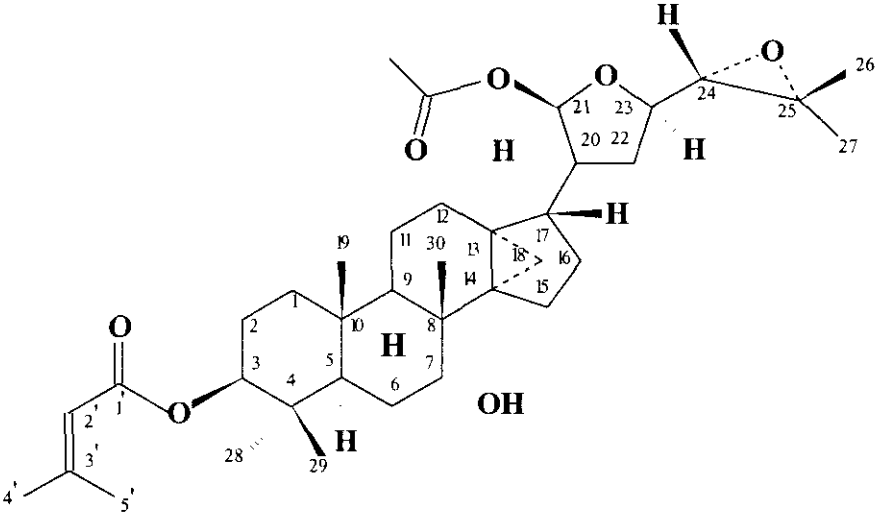


Figure 2.43 : Compound **12**

Table 2.24 : ^{13}C and ^{1}H NMR spectra assignments of compound **12**

in $CDCl_3$

Position	δ_C	DEPT	δ_H (*J*)
1	33.7	CH_2	1.22-1.21*, 1.19, m
2	26.2	CH_2	1.60, 1.02*, m
3	76.9	CH	4.70, dd (5.2, 2.6)
4	37.3	C_q	-
5	41.4	CH	2.00-1.95, dd (7.5, 5.0)
6	25.1	CH_2	1.65-1.55, m
7	74.1	CH	3.70, br s
8	39.1	C_q	-
9	43.7	CH	1.60, m
10	36.2	C_q	-
11	16.0	CH_2	1.30*, m
12	22.8	CH_2	1.60, m, 0.87*
13	29.9	C_q	-
14	36.7	C_q	-
15	24.2	CH_2	0.95, 1.60, m
16	27.5	CH_2	0.97, 0.87*, m
17	44.8	CH	2.07*, m
18	13.2	CH_2	0.74, d, 0.40, d
19	15.6	CH_3	0.90, s
20	48.0	CH	2.11-2.00, m
21	97.4	CH	6.30, d (3.3)
22	30.7	CH_2	2.07-1.92, 1.60, m
23	79.8	CH	3.90, qd (7.7, 6.6, 3.3)
24	66.7	CH	2.60, d (7.7)
25	57.1	C_q	-
26	24.9	CH_3	1.32, s
27	19.3	CH_3	1.27, s
28	21.8	CH_3	0.90, s
29	27.7	CH_3	0.84, s
30	19.4	CH_3	1.02, s

table contd.

1'	165.5	C_q	-
2'	116.9	CH	5.70, s
3'	155.8	C_q	-
4'	20.3	CH_3	2.17, s
5'	27.4	CH_3	1.90, s
7-OH	-	-	2.40, s
$COCH_3$	170.0	C_q	-
$OCOCH_3$	21.6	CH_3	2.08, s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

* - Peaks obscured.

Table 2.25 : ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and HMBC spectral data for compound **12** in $CDCl_3$

Position	^{1}H-^{1}H COSY	^{13}C-^{1}H COSY	HMBC
1	1.21-1.22 → 1.60, 1.02	33.7 → 0.90	4.70 , 0.90
2	1.60 → 4.70, 1.02	-	-
3	4.70 → 1.60, 1.02	76.9 → 0.90	0.84, 0.90
4	-	37.3 → 0.90	2.00-1.95
5	2.00-1.95 → 0.90, 0.84	41.4 → 0.90	4.70, 3.70, 0.9, 0.84
6	1.65-1.55 → 3.70	-	-
7	3.70 → 1.65-1.55	74.1 → 1.02	1.02
8	-	39.1 → 1.02	1.02
9	1.60 →1.30	43.7 → 1.02, 0.90	3.70, 1.02
10	-	36.2 → 0.90	2.0-1.95, 1.65-1.55, 1.02, 0.90
11	1.30 → 1.60, 0.87	-	2.0-1.95, 1.60, 1.02
12	1.60, 0.87 → 1.30	-	2.00-1.95, 1.02
14	-	36.7 → 1.02	1.02, 0.97, 0.95
15	0.95 → 0.97	-	0.97
16	0.97 → 0.95	-	1.60, 0.95
17	2.07 → 0.97, 0.87	-	1.60, 1.30, 1.02, 0.97, 0.95, 0.90, 0.40
18	0.74 → 0.40	-	-
19	0.90 → 2.00-1.95	-	2.00-1.95, 1.30

table contd.

20	2.11-2.00 → 6.30	-	-
21	6.30 → 2.11-2.00	-	-
22	2.07-1.92, 1.60 → 3.90	30.7 → 2.11-2.00, 2.07	-
23	3.90 → 2.60, 2.07-1.92, 1.60	79.8 → 6.30	6.30
24	2.60 → 3.90	66.7 → 1.32	1.32, 1.27
25	-	57.1 → 1.32	1.32, 1.27
26	-	24.9 → 1.27	1.27
27	-	19.3 → 1.32	-
28	0.90 → 2.00-1.95	21.8 → 0.84	-
29	0.84 → 2.00-1.95	27.7 → 0.90	1.65-1.55, 0.90
30	-	19.4 → 1.30	1.30, 0.90
1'	-	165.8 → 4.70, 2.17	2.17
2'	5.70 → 2.17, 1.90	116.9 → 1.90	2.17, 1.90
3'	-	155.8 → 2.17, 1.90	2.17, 1.90
4'	2.17 → 5.70	20.3 → 1.90	5.70, 1.90
5'	1.90 → 5.70	27.4 → 2.15	5.70 , 2.17
$COCH_3$	-	170.0 → 2.08	2.08
$OCOCH_3$	-	21.6 → 2.11-2.00	-

Chemical shift values are in ppm.

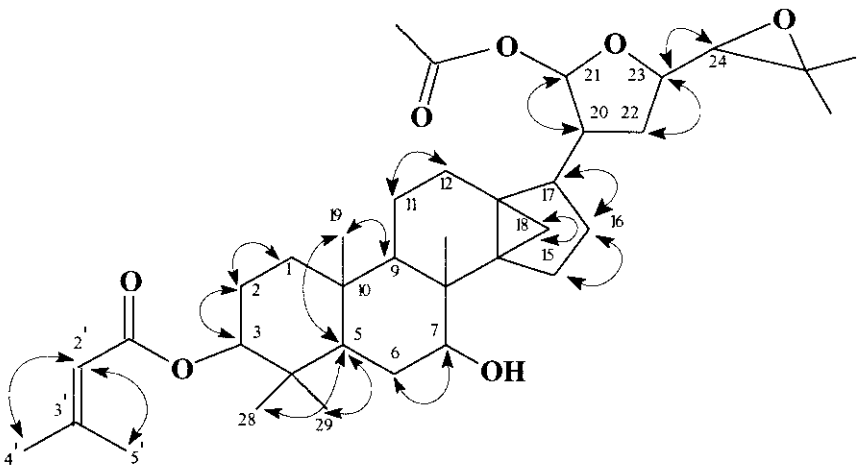


Figure 2.44 : ^{1}H-^{1}H COSY correlations for compound **12**

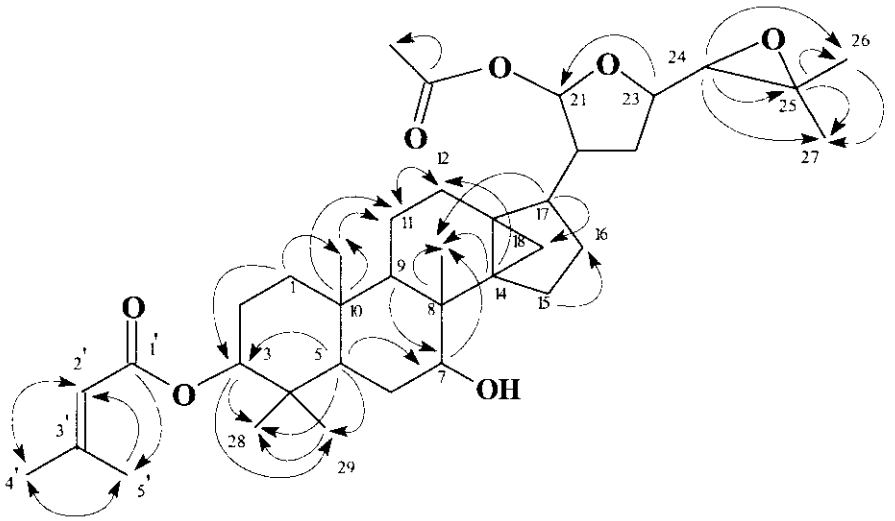


Figure 2.45 : HMBC correlations for compound **12**

Table 2.26 : TOCSY spectral data for compound **12** in $CDCl_3$

Position	δ_H	150 ms
1	1.21-1.22*, 1.19, m	4.70, 2.00-1.95, 1.60, 1.02, 0.90
2	1.60, m, 1.02*	4.70, 2.00-1.95, 1.22-1.21, 1.19
3	4.70, dd	2.00-1.95, 1.60, 1.22-1.21, 1.19
5	2.00-1.95, m	3.70, 1.22-1.21, 0.90, 0.84
6	1.65-1.55, m	3.70, 0.90
7	3.70, br s	2.00-1.95, 1.65-1.55, 1.02, 0.90
9	1.60, m	1.30, 1.22-1.21
11	1.30*	1.60
12	1.60, m, 0.87*	1.30
15	1.60, 0.95, m	2.07, 0.97, 0.87
16	0.97, m, 0.87*	2.07, 1.60
17	2.07*	6.30, 2.11-2.00, 2.07-1.92, 1.60, 1.02
18	0.74, d, 0.40, d	2.07, 2.11-2.00, 1.60
19	0.90, s	3.70, 2.00-1.95, 1.65-1.55, 1.60
20	2.11-2.00, m	6.30, 3.90, 2.60, 1.60, 1.32
21	6.30, d	3.90, 2.60, 2.08, 2.07, 2.11-2.00, 1.60
22	2.07-1.92, 1.60, m	2.60, 2.07, 2.11-2.00, 1.32
23	3.90, qd	6.30, 2.60, 2.08, 2.07, 2.11-2.00, 1.60
24	2.60, d	6.30, 3.90, 2.08, 2.07, 1.60, 1.32
26	1.32, s	2.60, 2.11-2.00, 2.07-1.92, 1.60
27	1.27, s	1.60, 1.32
28	0.90, s	2.00-1.95, 1.65-1.55, 1.60
29	0.84, s	2.00-1.95, 1.65-1.55
30	1.02, s	3.70, 2.07
2'	5.70, s	2.17, 1.90
4'	2.17, s	5.70
5'	1.90, s	5.70
$OCOCH_3$	2.08, s	6.30, 3.90, 2.60

Chemical shift values are in ppm. * - Peaks obscured

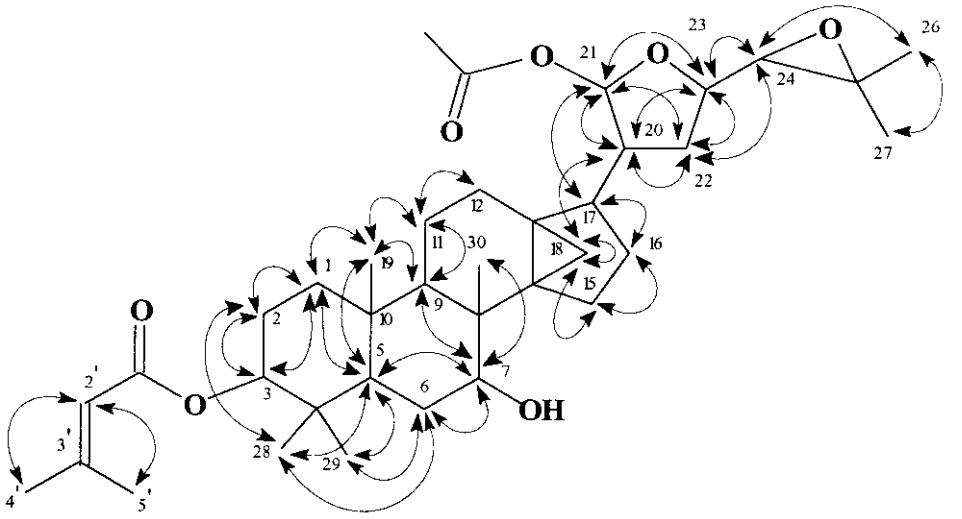


Figure 2.46 : TOCSY correlations for compound **12**

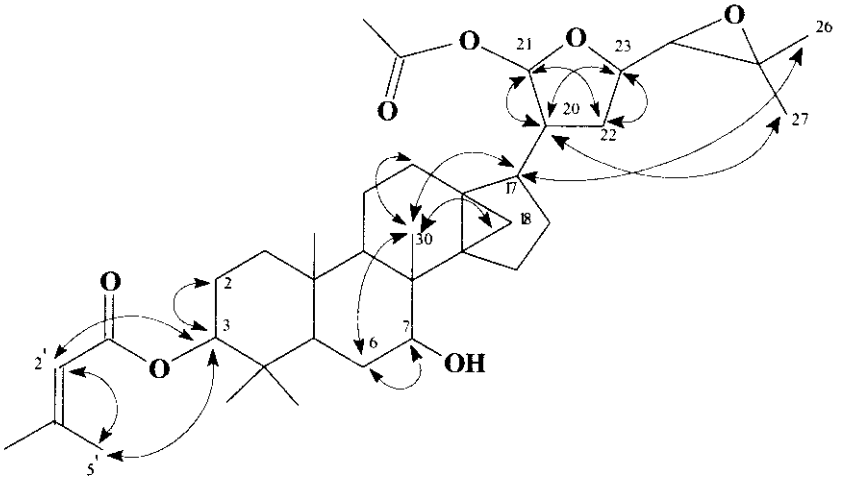


Figure 2.47 : NOESY correlations for compound **12**

Table 2.27 : NOESY spectral data for compound **12** in $CDCl_3$

Position	δ_H	NOESY (^1H-^1H)
2a	1.60, m	H-3
2b	1.02*	-
3	4.70, dd	H-2a, H-5'
6	1.65-1.55, m	H-7, H-30
7	3.70, br s	H-6
12a	1.60, m	H-30
12b	0.87*	-
17	2.07*	H-21, H-26, H-30
18a	0.74, d	H-19
18b	0.40, d	-
19	0.90, s	H-18a
20	2.11-2.00, m	H-23, H-27
21	6.30, d	H-17, H-22b
22a	1.60, m	H-23
22b	2.07-1.92, m	H-21, H-30
23	3.90, qd	H-20, H-22a
26	1.32, s	H-17
27	1.27, s	H-20
30	1.02, s	H-6, H-12a, H-17, H-22b
2'	5.70, s	H-3, H-5'
5'	1.90, s	H-3, H-2'

Chemical shift values are in ppm. * - Peaks obscured

(b) **Characterisation of compound 13**

Compound **13** was a colourless oil and visualised as a purple spot (2:3, ethyl acetate:petroleum ether, Rf = 0.35) with anisaldehyde-H_2SO_4.[162]

The IR spectrum showed the absorptions characteristic of α, β-unsaturated ester (1731, 1656 cm^{-1}), hydroxyl (3031 cm^{-1}), and epoxide (910 cm^{-1}) functionalities. The UV spectrum also evidenced the presence of an α, β-unsaturated ester (λ_{max} = 215 nm). The high resolution electrospray API mass spectrum showed the molecular ion at m/z 612.3889, suggesting the molecular formula to be $C_{37}H_{56}O_7$ which indicated a degree of unsaturation of ten.

The presence of one acetyl ester group in **13** was shown by the methyl singlet at δ 2.06 in the ^1H NMR spectrum. The acetate bearing the epimeric carbon (C-21) was observed at δ 101.1, instead of δ 97.4 as in the compound **12**. The characteristic acetylation shift of 0.83 ppm was observed for the proton at δ 5.43 (H-21) in **13**. The ^1H NMR spectrum exhibited a broad singlet at δ 2.44, indicating a nonacetylated hydroxyl group in the molecule which was further confirmed by the IR spectrum.

Compound, **13** exhibited the same molecular ion in the mass spectrum and identical NMR spectral data to those of **12** (Table 2.28 and 2.24, page 151 and 142), except for the ^{13}C chemical shift for the epimeric carbon at C-21 (δ 101.1), indicating that **13** is the α-epimer of **12** (Figure 2.48).

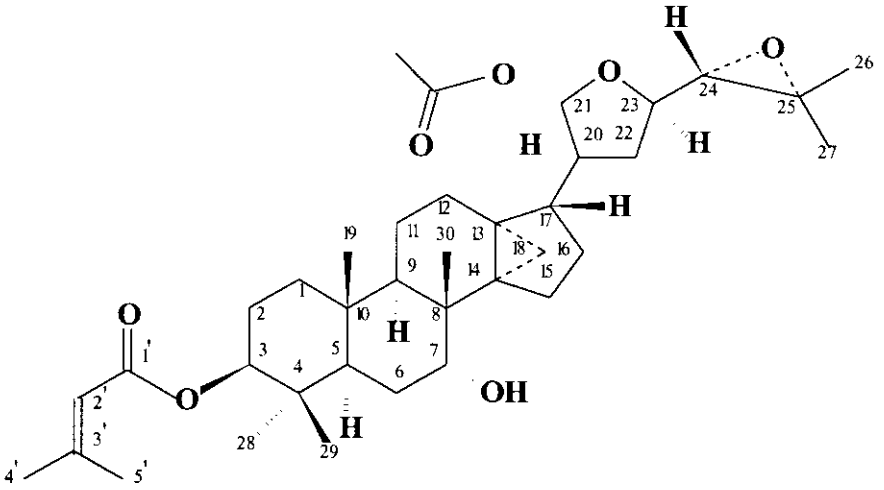


Figure 2.48 : Compound **13**

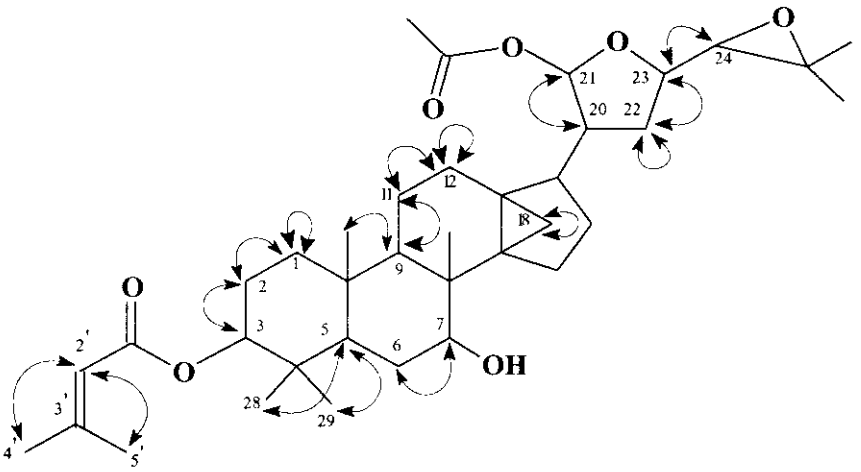


Figure 2.49 : ^{1}H-^{1}H COSY correlations for compound **13**

Table 2.28 : ^{13}C and ^{1}H NMR spectra assignments of compound **13** in $CDCl_3$

Position	δ_C	DEPT	δ_H (J)	^{1}H-^{1}H COSY
1	33.7	CH_2	1.27-1.21*, 1.19*, m	1.60, 1.02
2	25.8	CH_2	1.60, 1.02*, m	4.68, 1.27-1.21
3	76.8	CH	4.68, dd	1.60, 1.02
4	37.2	C_q	-	-
5	41.1	CH	2.00-1.92, m	0.85, 0.89
6	25.7	CH_2	1.60, m	3.76
7	74.2	CH	3.76, br s	1.60
8	38.9	C_q	-	-
9	43.7	CH	1.60-1.55*, m	0.90
10	36.2	C_q	-	-
11	16.0	CH_2	1.32*, 1.27*, m	1.60
12	22.6	CH_2	1.60, 0.90*, m	1.32
13	28.3	C_q	-	-
14	36.4	C_q	-	-
15	24.1	CH_2	0.95*, 1.66*, m	-
16	25.8	CH_2	0.97*, 0.87*, m	-
17	48.0	CH	2.07-1.92, m	-
18	13.8	CH_2	0.77, d, 0.49, d (4.9)	-
19	15.6	CH_3	0.90, s	1.60
20	48.5	CH	2.33, qd	6.26
21	101.1	CH	6.26, d (3.0)	2.33
22	32.1	CH_2	2.07-1.92, 1.60, m	3.90
23	79.1	CH	3.90, qd	2.75
24	64.7	CH	2.75, d (7.4)	3.90, 2.07-1.92, 1.60
25	57.0	C_q	-	-
26	24.8	CH_3	1.33, s	-
27	19.4	CH_3	1.31, s	-
28	21.7	CH_3	0.89, s	2.00-1.92
29	27.6	CH_3	0.85, s	2.00-1.92
30	19.4	CH_3	1.04, s	-
1'	166.4	C_q	-	-
2'	116.9	CH	5.76, dd (1.4, 1.1)	2.18, 1.90
3'	155.8	C_q	-	-
4'	20.1	CH_3	2.18, d (1.1)	5.76
5'	27.2	CH_3	1.90, d (1.1)	5.76

table contd.

7-OH	-	-	2.44, br s	-
$COCH_3$	170.5	C_q	-	-
$OCOCH_3$	21.4	CH_3	2.06, s	-

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

* - Peaks obscured.

(c) **Characterisation of compound 14**

Compound **14** was a colourless oil and visualised as a purple spot (2:3, ethyl acetate:petroleum ether, Rf = 0.35) with anisaldehyde-H_2SO_4.[162]

The IR spectrum showed the absorptions characteristic of α, β-unsaturated ester (1744 cm^{-1}) and epoxide (951 cm^{-1}) functionalities. The UV spectrum also evidenced the presence of an α, β-unsaturated ester (λ_{max} = 216 nm). The high resolution electrospray API mass spectrum showed the molecular ion at m/z 654.4009, suggesting the molecular formula to be $C_{39}H_{58}O_8$ which indicated a degree of unsaturation of eleven.

The presence of two acetyl ester groups in **14** was shown by the methyl singlets at δ 2.04 and 2.09 in the ^{1}H NMR spectrum. The protons at δ 3.70 and 5.43 of the non acetylated compound were shifted downfield to δ 4.98 and 6.26, respectively in **14**.

The compound, **14** exhibited a close spectral analogy to **12** and **13**, except for the presence of two acetates at C-7 and C-21. The value of the shift for C-21 (δ 97.5) shows that the substituent is β-oriented. Compound **14** is therefore, the diacetate of compound **6** (Figure 2.50).

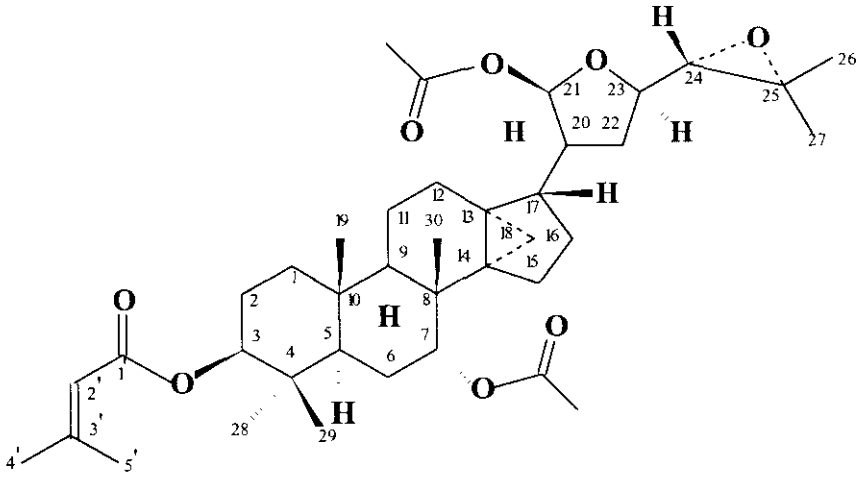


Figure 2.50 : Compound **14**

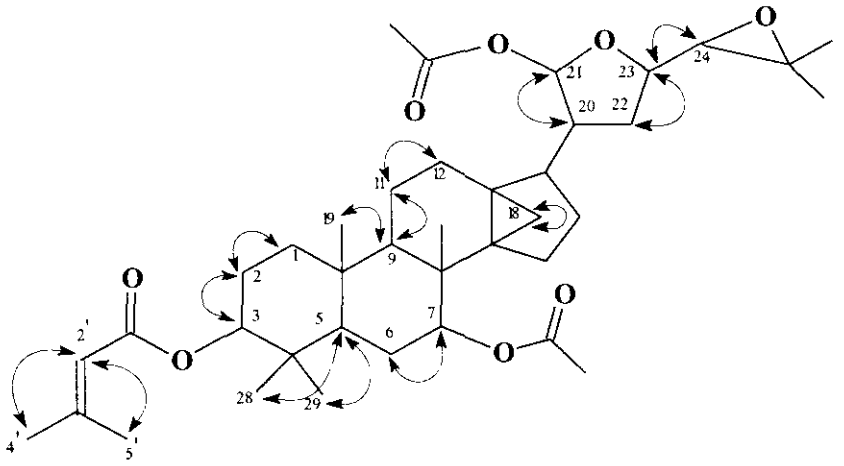


Figure 2.51 : ^{1}H-^{1}H COSY correlations for compound **14**

Table 2.29 : ^{13}C and ^{1}H NMR spectra assignments of compound **14** in $CDCl_3$

Position	δ_C	DEPT	δ_H (J)	^{1}H-^{1}H COSY
1	33.8	CH_2	1.27-1.21*, 1.19, m	1.60
2	26.5	CH_2	1.79-1.64*, 1.60, m	4.70, 1.27
3	76.9	CH	4.70, s	1.79-1.64, 1.60
4	37.0	C_q	-	-
5	42.4	CH	2.00-1.80, m	0.87, 0.75
6	25.6	CH_2	1.55, m	4.98
7	75.9	CH	4.98, s	1.55
8	38.2	C_q	-	-
9	44.9	CH	1.60, m	1.32, 0.90
10	36.1	C_q	-	-
11	16.5	CH_2	1.32*, m	1.60
12	22.6	CH_2	1.60, m,	1.32
13	29.0	C_q	-	-
14	37.0	C_q	-	-
15	23.1	CH_2	0.95, 1.60, m	-
16	27.6	CH_2	0.97, 0.87*, m	-
17	44.6	CH	2.08*, m	-
18	14.4	CH_2	0.71, d, 0.31, d (5.5)	-
19	15.7	CH_3	0.90, s	1.60
20	48.0	CH	2.10-2.00, m	6.26
21	97.5	CH	6.26, d (3.3)	2.10-2.00
22	30.6	CH_2	2.10*, 1.60, m	3.87
23	79.8	CH	3.87, qd (9.6, 7.7)	2.67, 2.10, 1.60
24	66.6	CH	2.67, d (7.7)	3.87
25	57.1	C_q	-	-
26	24.9	CH_3	1.32, s	-
27	19.3	CH_3	1.27, s	-
28	21.5	CH_3	0.87, s	2.00-1.80
29	27.6	CH_3	0.75, s	2.00-1.80
30	19.5	CH_3	1.08, s	-
1'	166.2	C_q	-	-
2'	116.9	CH	5.70, s	2.20, 1.93
3'	155.6	C_q	-	-
4'	20.4	CH_3	2.20, s	5.70
5'	27.6	CH_3	1.93, s	5.70

table contd.

$COCH_3$	170.0	C_q	-	-
$OCOCH_3$	21.5	CH_3	2.09, s	-
$COCH_3$	170.2	C_q	-	-
$OCOCH_3$	21.50	CH_3	2.04, s	-

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

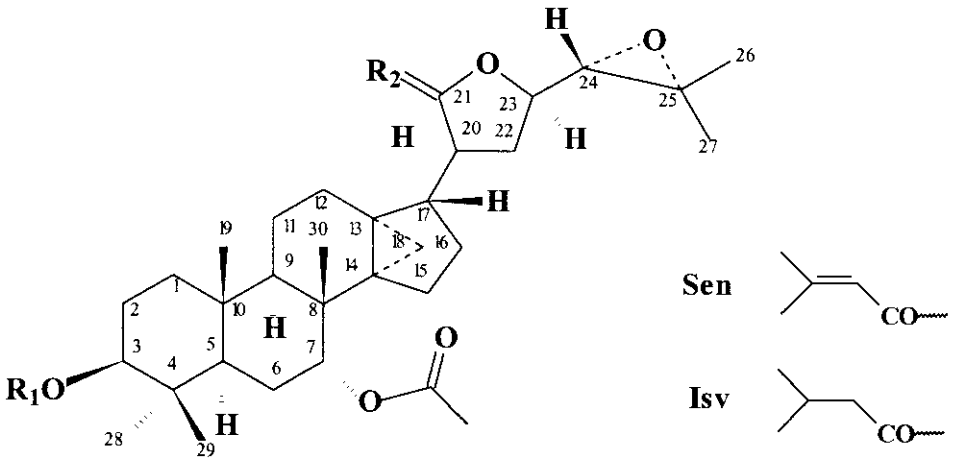
* - Peaks obscured.

(d) **Characterisation of compounds 15-17**

Compounds **15-17** were isolated as a mixture of C-7 and C-21 diacetates. This was a white crystalline solid and visualised as a purple spot (2:3, ethyl acetate-petroleum ether, Rf = 0.35) with anisaldehyde-H_2SO_4.[162]

The presence of both the senecioate (δ_C 166.2, 155.6, 116.8, 20.5, 27.6; δ_H 5.77, br s, 2.18, s, 1.90, s) and isovalerate esters (δ_C 172.5, 44.1, 27.6, 22.5, 21.6; δ_H 2.20-2.17, m, 2.07-1.92, m, 0.98, d, 0.96, d) was suggested by the ^1H and ^{13}C NMR spectral data of the mixture. In addition, the presence of a pair of ^{13}C signals at δ 97.5 and 101.1, suggested that this is also an epimeric mixture of α- and β-isomers.

The presence of six acetyl ester groups were shown by the integration and chemical shifts of methyl singlets at δ 2×2.04, 2.05, 2.06 and 2×2.08 in the ^1H NMR spectrum, suggesting a mixture of three diacetates. The intensity of ^1H and the overlapping nature of carbon signals of the main carbon frame work indicated a common parent skeleton of all three compounds. This mixture contained C-7 and C-21 diacetates of related senecioate and isovalerate esters of **7**, **8** and **9** (Figure 2.52).



Compound	R_1	R_2
15	**Sen**	**β-H, α-OAc**
16	**Isv**	**α-H, β-OAc**
17	**Isv**	**β-H, α-OAc**

Figure 2.52 : Compounds 15-17

2.3.12 **Characterisation of oxidised derivatives 18, 19**

Compounds **18** and **19** were isolated as a mixture. This was a colourless liquid which gave a UV active spot (λ = 254 and 366 nm, in 1:4 ethyl acetate-petroleum ether, Rf = 0.57) and a purple spot with anisaldehyde-H_2SO_4.[162]

The IR spectrum showed the presence of the γ-lactone (1774 cm^{-1}), α, β-unsaturated ester (1708, 1655 cm^{-1}) and epoxide (995 cm^{-1}) functionalities.

Comparison of the ^{1}H and ^{13}C NMR spectral data of $\mathbf{F_A}$ (Table 2.20, page 130) with the oxidised mixture (Table 2.30) indicated the absence of epimeric carbons at δ 98.4 and 74.3, their associated protons (δ 5.43 and 3.76), and the two hydroxyl groups in the mixture. Instead, two new peaks were observed at δ 215.1 (C-7) and 178.1 (C-21) for carbonyl functionality in the ^{13}C NMR spectrum. Due to the introduced γ-lactone ring at C-17, the protons, H-17 (δ 2.20), H-20 (δ 2.24) and H-23 (δ 3.85-4.00) were shifted downfield to δ 2.47, 2.90 and 4.15, respectively, in the ^{1}H NMR spectrum. In addition, H-6 protons (δ 1.70) were also shifted to δ 2.56 and 2.16-2.17 due to the newly introduced carbonyl at C-7. With all this spectral information, the structure of the compounds in the mixture were tentatively assigned as the senecioate and isovalerate esters of the C-7, C-21 di-oxo analogues of α- and β-glabretal (Figure 2.53).

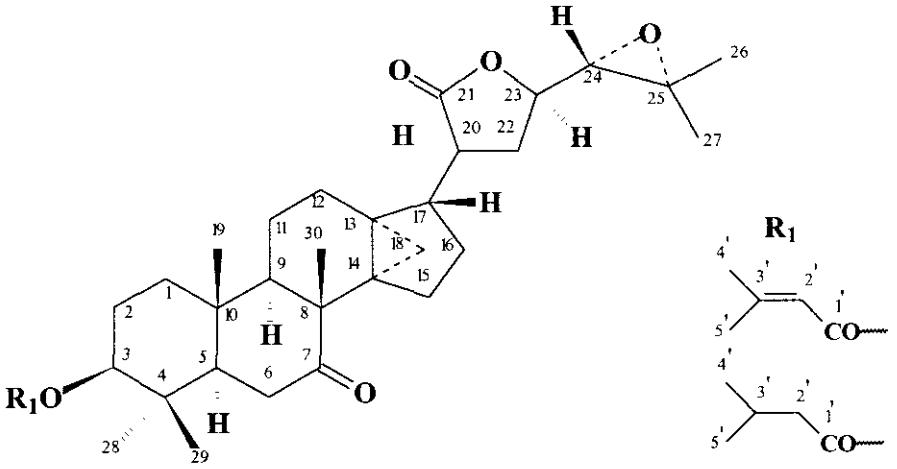


Figure 2.53 : Compounds **18** and **19**

Table 2.30 : ^{13}C and ^{1}H NMR spectra assignments of compound **18, 19**

in $CDCl_3$

Position	δ_C	DEPT	δ_H (*J*)	^1H-^1H COSY
1	**34.6**	CH_2	1.60, 1.12*, m	1.60
2	**27.0**	CH_2	1.92, 1.60, m	4.71, 1.60
3	**77.2** (76.4)	CH	4.71, d (9.6)	1.92, 1.60
4	**37.0** (37.3)	C_q	-	
5	**50.4**	CH	1.65, m	2.56, 2.17-2.16, 1.08, 0.97
6	**35.6**	CH_2	2.56, 2.17-2.16, m	1.65
7	**215.1**	C_q	-	-
8	**50.2**	C_q	-	-
9	**51.8**	CH	1.08**, m	1.32
10	**37.2**	C_q	-	-
11	**16.8**	CH_2	1.32*, m	2.00-1.90, 1.60, 1.08
12	**22.7** (22.6)	CH_2	2.00-1.90, 1.60*, m	1.32
13	**28.7**	C_q	-	-
14	**33.8**	C_q	-	-
15	**28.3**	CH_2	2.00-1.90, m	1.60
16	**21.4** (21.4)	CH_2	1.60*, 0.84, m	2.47, 2.00-1.90
17	**44.7**	CH	2.47, m	2.90, 1.60, 0.84
18	**15.1**	CH_2	0.58, 0.44, d (5.5)	-

table contd.

19	**16.0**	CH_3	1.08, s	1.65, 1.32
20	**40.8**	CH	2.90, sep	2.47, 2.17-2.00
21	**178.1**	C_q	-	-
22	**26.5**	CH_2	2.17-2.00*, 1.90, m	4.15, 2.90
23	**78.5**	CH	4.15, sep	2.82, 2.17-2.00, 1.90
24	**64.3**	CH	2.82, d (7.4)	4.15
25	**57.3**	C_q	-	-
26	**24.8**	CH_3	1.32, s	-
27	**19.5**	CH_3	1.35, s	-
28	**21.1**	CH_3	0.97, s	1.65
29	**27.4**	CH_3	0.84, s	-
30	**18.8**	CH_3	1.38, s	-
Senecioate				
1'	166.0	C_q	-	-
2'	116.3	CH	5.66, s	2.17, 1.90
3'	156.7	C_q	-	-
4'	20.3	CH_3	2.17, s	5.66, 1.90
5'	27.5	CH_3	1.90, s	5.66, 2.17
Isovalerate				
1'	172.5	C_q	-	-
2'	43.8	CH_2	2.20-2.12, m	-
3'	25.8	CH	2.10-2.00, m	0.95, 0.93
4'	22.5	CH_3	0.93, d	2.10-2.00
5'	22.4	CH_3	0.95, d	2.10-2.00

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

* - Peaks interchangeable. ** - Peaks obscured. **Bold** numbers correspond both isovalerate and senecioate analogues. ( ) - Corresponds only isovalerate analogue

2.3.13 **Characterisation of hydrolysis derivative 20**

The compound, **20** was a colourless oil which was visualised as a purple spot (1:9, water:acetonitrile, Rf = 0.42) with anisaldehyde-H_2SO_4.[162]

The IR spectrum showed the presence of α, β-unsaturated ester (1709 cm^{-1}) and hydroxyl (3618 cm^{-1}) functionalities. The UV spectrum also indicated the presence of an α, β-unsaturated ester (λ_{max} = 216 nm).

Comparison of the NMR spectral data of **20** with $\mathbf{F_A}$ (Table 2.20, page 130) indicated the absence of signals for a trisubstituted epoxide (δ_H 2.84; δ_C 67.6, 58.1) and a cyclopropane ring (δ_H 0.48, 0.75; δ_C 13.7). Instead, three new peaks at δ 4.30 (H-24), 3.50 (24-OH) and 3.36 (25-OH) were shown and the proton at δ 4.00-3.85 (H-23) was shifted to δ 4.12 in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum revealed two new resonances of oxygenated carbons, one secondary (δ 76.9) and one quaternary (δ 74.0) confirming the presence of a 24, 25-dihydroxy side chain at C-17.[195] The ^{13}C spectrum also revealed two additional sp^2 carbons at δ 162.4 and 119.3 assigned for a Δ^{14-15} double bond and a methyl carbon at δ 22.4 assigned as C-18 (Figure 2.54).

The structure of the compound, **20** was tentatively assigned as an analogue of glabretal with the 3-senecioyl ester group, a Δ^{14-15} double bond, two hydroxyl groups in the main carbon skeleton and a C-17 side chain with a hemiacetal ring and two hydroxyl groups, one secondary and one tertiary (Figure 2.54).

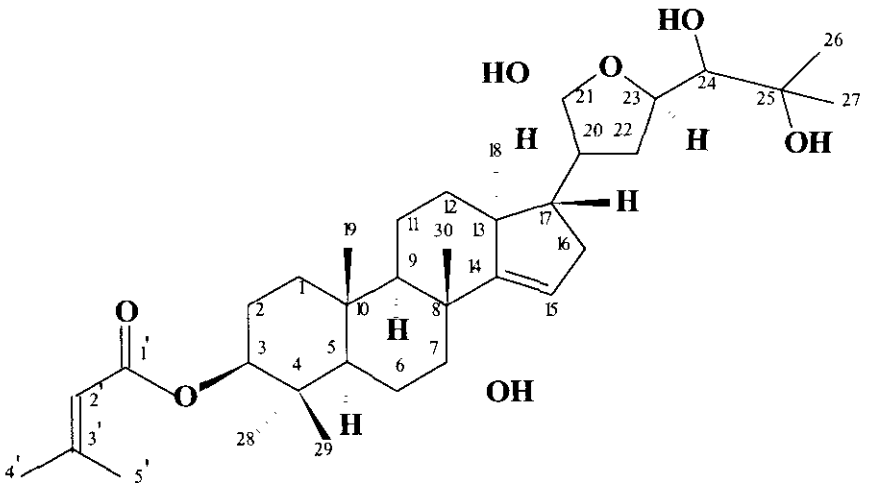


Figure 2.54 : Compound **20**

Table 2.31 : ^{13}C and ^{1}H NMR spectra assignments of compound **20** in $CDCl_3$

Position	δ_C	DEPT	δ_H (*J*)
1	34.7	CH_2	1.50, 1.25**, m
2	26.5	CH_2	1.92, 1.65, m
3	76.5	CH	4.70, dd
4	37.5	C_q	-
5	41.6	CH	2.10-2.00, m
6	23.6	CH_2	1.70, m
7	72.2	CH	3.92, s
8	44.4	C_q	-
9	43.8	CH	1.30**, m
10	36.2	C_q	-
11	16.2	CH_2	1.40-1.30**, m
12	33.3	CH_2	1.55, 0.80**, m
13	45.9	C_q	-
14	162.4	C_q	-

table contd.

15	119.3	CH	4.82, m
16	33.7	CH_2	1.60-1.40*, m
17	55.7	CH	2.20-2.15, m
18	22.4	CH_3	1.08, s
19	15.2	CH_3	0.92, s
20	47.0	CH	2.40-2.30, m
21	109.6	CH	5.46 dd
22	29.7	CH_2	1.95*, 1.60*, m
23	77.8	CH	4.12*, m
24	76.9	CH	4.30*, m
25	74.0	C_q	-
26	27.8	CH_3	1.28, s
27	26.4	CH_3	1.26, s
28	21.8	CH_3	0.91, s
29	27.7	CH_3	0.85, s
30	19.4	CH_3	1.06, s
1'	166.6	C_q	-
2'	117.0	CH	5.76, s
3'	155.8	C_q	-
4'	20.3	CH_3	2.05, s
5'	27.4	CH_3	1.89, s
7-OH	-	-	2.30*, s
21-OH	-	-	3.22*, s
24-OH	-	-	3.50, s
25-OH	-	-	3.36, s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

* - Peaks interchangeable **- Peaks obscured.

2.3.14 **Postulated biosynthesis of compounds 6-9**

The biosynthesis of related protolimonoids of **6-9** has been proposed previously.[196, 197] They are thought to arise from a Δ^7 tirucallol precursor (Figure 2.55) which is characteristic only in plant families of Rutales (Rutaceae, Meliaceae, Cneoraceae, Simaroubaceae and Burseraceae).

Compounds **6-9** could be derived from the same precursor, tirucallol which is derived from the triterpene precursor squalene via the acetate-mevalonate pathway.[20] According to literature, the Δ^7-bond of tirucallol is epoxidised to a 7-epoxide, which is then opened inducing a Wagner-Meerwein shift of Me-14 to C-8, formation of the OH-7, and introduction of a double bond at C-14/C-15 (Figure 2.55).[196] This could account for both the presence of hydroxyl at C-7 and the correct stereochemistry of the C-30 methyl group in the compounds **6-9**. The C-18 methyl could be involved in the formation of the cyclopropane ring at C-13, C-14.

Subsequently, the side chain at C-17 can be cyclised to form the 17-tetrahydrofuran ring with 21-OH and 23 isobutenyl side chain which would then undergo oxygenation to form a diol at C-24 and C-25 and subsequently an epoxide (Figure 2.55).

Finally, dihydroxy compound can undergo esterification at C-3 with senecioyl and isovaleryl acids to form the corresponding esters in compounds **6-9** (Figure 2.55).

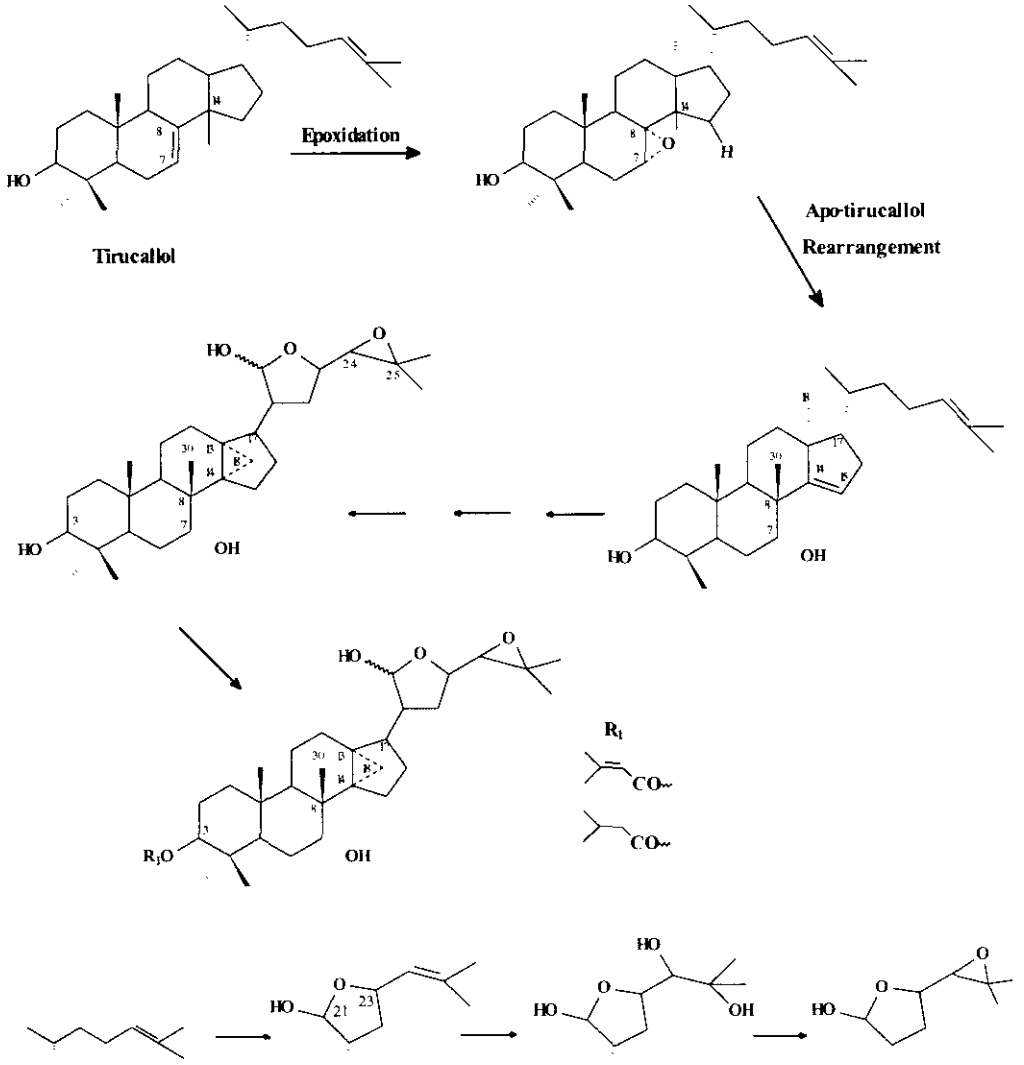


Figure 2.55 : Postulated biosynthetic pathway

2.3.15 **Characterisation of compound 21**

Compound **21** was isolated from *Aegle marmelos* as a white crystalline solid (m.p. 189-190 °C) in 0.004% yield. It was visualised as a fluorescing UV active spot (3:2, ethyl acetate:petroleum ether, Rf = 0.23) and a pink spot with anisaldehyde-H_2SO_4.[162]

The presence of a carbonyl function was evident from the strong band at 1725 cm^{-1} in the IR spectrum. It also showed absorptions characteristic of hydroxyl (3474 cm^{-1}), aromatic (1626 cm^{-1}) and furan (1130 cm^{-1}) functionalities. The UV spectrum showed absorptions at λ_{max} = 335 and 206 nm. The low resolution electrospray API mass spectrum displayed the molecular ion at m/z 246, suggesting the molecular formula to be $C_{14}H_{14}O_2$ which indicated a degree of unsaturation of eight.

The ^1H NMR spectrum indicated two olefinic proton signals corresponding to a cis double bond[198] (δ 6.21 and 7.59, J = 9.5 Hz) and two aromatic proton singlets (δ 6.73 and 7.22) attributable to a 1,2,4,5-tetra-substituted benzene ring. The ^1H NMR spectrum also showed the signals for two methyls (δ 1.38 and 1.24, s), two benzylic protons (δ 3.22, ddd) and a dihydrofuran α-proton (δ 4.74, dd J = 9.2, 8.6).

Comparison of these data with known metabolites from *A. marmelos* established the structure of compound **21** to be marmesin (Figure 2.56) which was in

agreement with the reported NMR spectral data (Table 2.32).[198] Marmesin has also been previously reported from other Rutaceous plants.[125, 198]

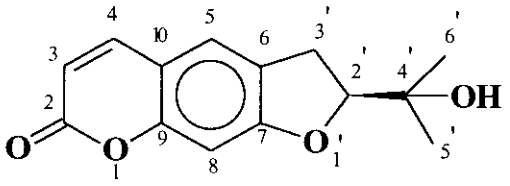


Figure 2.56 : Compound **21**

Table 2.32 : ^{13}C and ^{1}H NMR data of compound **21** and reported ^{13}C NMR data in $CDCl_3$

Position	δ_C	Reported δ_C	DEPT	δ_H (*J*)
2	161.5	160.5	C_q	-
3	112.3	111.2	CH	6.21, d (9.5)
4	143.7	144.6	CH	7.59, d (9.5)
5	123.4	123.8	CH	7.22, s
6	125.1	125.5	C_q	-
7	163.2	163.3	C_q	-
8	97.9	96.7	CH	6.73, s
9	155.6	155.1	C_q	-
10	112.8	121.1	C_q	-
2'	91.1	91.0	CH	4.74, dd (9.2, 8.6)
3'	29.5	28.7	CH_2	3.22, ddd
4'	71.6	70.0	C_q	-
5'	26.1	25.8	CH_3	1.38, s
6'	24.3	24.8	CH_3	1.24, s
4'-OH	-	-	OH	1.86, s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

2.3.16 **Characterisation of compound 22**

Compound, **22** was isolated from *Aegle marmelos* as a white crystalline solid in 0.003% yield. It was detected as a fluorescing UV active spot (3:2, ethyl acetate:petroleum ether, Rf = 0.18) and a green spot with anisaldehyde-H_2SO_4.[162]

The IR spectrum revealed the presence of carbonyl (1731 cm^{-1}), hydroxyl (3589 and 3013 cm^{-1}), aromatic (1610 cm^{-1}) and ester (1729 cm^{-1}) functionalities. The UV spectrum showed absorptions at λ_{max} = 324 and 207 nm. The low resolution electrospray API mass spectrum displayed the molecular ion at m/z 332, suggesting the molecular formula to be $C_{19}H_{24}O_5$ which indicated a degree of unsaturation of eight.

The ^{13}C NMR spectrum indicated the presence of nineteen carbons in the molecule. The presence of a 7-substituted coumarin ring was suggested by the comparison of ^{1}H and ^{13}C NMR spectral data with those reported for 7-substituted coumarins (Table 2.33)[172] especially epoxyaurapten **5**. The carbon signals of the C-10 side chain were identical to those of **5** apart from the carbon signals at δ 73.0 and 77.9. These carbon shifts indicated a cleavage of the epoxide to a diol in **22** (Figure 2.57). In the ^{1}H-NMR spectrum, a new double doublet was observed at δ 3.34 accompanied by the disappearance of the triplet at δ 2.69. These data established the structure of the compound, **22** to be marmin [7-(6',7'-dihydroxygeranyloxy) coumarin] which was in agreement with the reported spectral data (Table 2.33 and Figure 2.57).[172] Marmin has been previously reported from *Aegle marmelos*.[130]

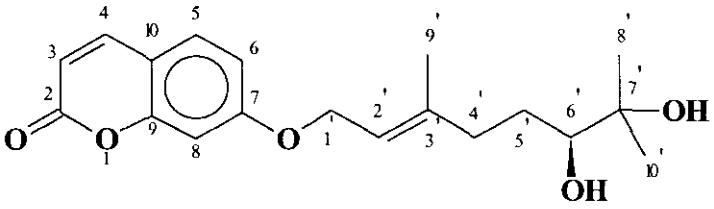


Figure 2.57 : Compound **22**

Table 2.33 : ^{13}C and ^{1}H NMR data of compound **22** and reported ^{13}C NMR values in $CDCl_3$

Position	δ_C	Reported δ_C	DEPT	δ_H (*J*)
2	161.9	160.9	C_q	-
3	113.3	113.1	CH	6.25, d (9.3)
4	143.5	143.9	CH	7.64, d (9.3)
5	128.7	129.5	CH	7.35, d (9.5)
6	113.3	113.2	CH	6.80, m
7	162.0	162.6	C_q	-
8	101.5	101.8	CH	6.80, m
9	155.6	156.4	C_q	-
10	112.0	112.9	C_q	-
1'	65.4	65.9	CH_2	4.61, d (6.6)
2'	118.9	119.1	CH	5.50, m
3'	142.2	142.7	C_q	-
4'	36.5	37.3	CH_2	2.41-2.34, m
5'	29.4	30.3	CH_2	2.41-2.34, m
6'	77.9	78.2	CH	3.34, dd
7'	73.0	72.6	C_q	-
8'	23.2	25.9	CH_3	1.17, s
9'	16.8	16.8	CH_3	1.78, s
10'	26.5	26.0	CH_3	1.21, s
7'-OH	-	-	OH	1 .89, s
6'-OH	=	=	OH	2.09, s

Chemical shift values are in ppm. Coupling constants (*J* values) in parentheses are in Hz.

2.3.17 **Characterisation of compound 23**

Compound, **23** was isolated from *Aegle marmelos* as a white crystalline solid (m.p. 196-197 °C) in 0.002 % yield. It was non UV active and visualised as a blue spot (3:2, ethylacetate:petroleum ether, Rf = 0.73) with anisaldehyde-H_2SO_4.[162]

The IR spectrum showed hydroxyl absorptions at 3462 cm^{-1}. The low resolution electrospray API mass spectrum displayed the molecular ion at m/z 426, suggesting the molecular formula to be $C_{30}H_{50}O$ which indicated a degree of unsaturation of six.

The ^1H and ^{13}C NMR spectral data (Table 2.34) was indicative of a triterpene with thirty carbon atoms. Compound **23** was assigned to be lup-20(29)-en-3-ol (Figure 2.58) previously reported from *Aegle marmelos*[128] by comparison with NMR data (Table 2.34).[199, 200]

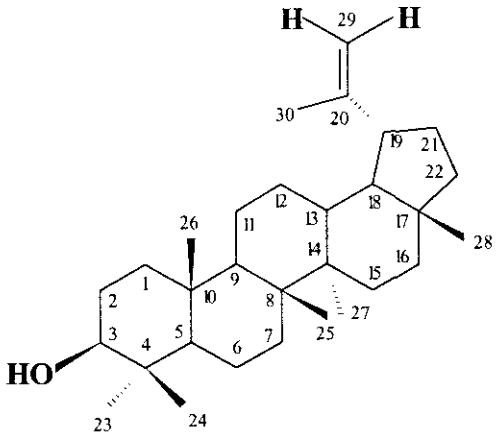


Figure 2.58 : Compound **23**

Table 2.34: ^{13}C and ^{1}H NMR data of compound **23** and reported ^{13}C NMR data in $CDCl_3$

Position	δ_C	Reported δ_C	DEPT	δ_H (*J*)
1	38.7	38.7	CH_2	
2	27.4	27.4	CH_2	2.40, t d
3	79.0	78.8	CH	
4	38.9	38.8	C_q	
5	55.3	55.2	CH	
6	18.3	18.3	CH_2	
7	34.3	34.2	CH_2	
8	40.8	40.8	C_q	
9	50.4	50.4	CH	
10	37.2	37.1	C_q	
11	20.9	20.9	CH_2	
12	25.1	25.1	CH_2	
13	38.0	38.0	CH	
14	42.8	42.8	C_q	
15	27.4	27.4	CH_2	
16	35.6	35.5	CH_2	
17	43.0	42.9	C_q	
18	48.3	48.3	CH	
19	48.0	47.9	CH	
20	151.0	150.6	C_q	
21	29.9	29.8	CH_2	
22	40.0	39.9	CH_2	
23	28.0	28.0	CH_3	0.97, s
24	15.4	15.4	CH_3	0.76, s
25	16.1	16.1	CH_3	0.83, s
26	16.0	15.9	CH_3	1.03, s
27	14.6	14.5	CH_3	0.94, s
28	18.0	18.0	CH_3	0.79, s
29	109.3	109.2	CH_2	4.68, 4.57, d
30	19.3	19.3	CH_3	1.68, s
-	-	-	OH	3.20, q

Chemical shift values are in ppm.

2.3.18 **Insecticidal Activity**

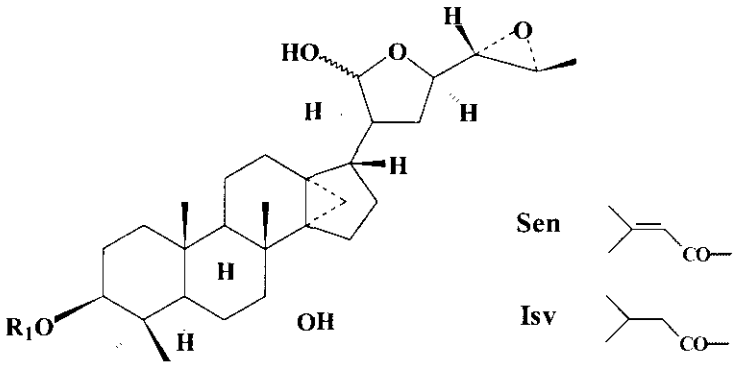
The insecticidal activity of *Aegle marmelos* (Rutaceae) was reported (after our preliminary survey in 1993) against cotton leaf worm (*Spodoptera littoralis* Boisd), confused flour beetles (*Tribolium confusum* Duv) and laboratory flies (*Drosophilla melanogaster* Meig)[120] but the constituents responsible were not identified. In the screens at Rothamsted Experimental station the plant showed activity against the mustard beetles and houseflies (Table 2.12, page 96). The two major active compounds were identified as 3-senecioyl and 3-isovaleryl glabretal triterpenoids, belonging to the class of protolimonoids. The related compounds, Skimmiarepin **A** and **B**, isolated from the fruits and leaves of *Skimmia japonica* (Rutaceae) showed insect growth inhibitory activity against the silk worm (*Bombyx mori* L.).[188] Other protolimonoids affect insect growth and feeding.[196]

The bio-assay results showed that the natural products are active against the mustard beetles and house flies (Table 2.35). The epimeric mixture of senecioate and isovalerate, fraction $\mathbf{C_1}$ has an LD_{50} of 0.028 μg/insect against mustard beetles and 71% activity at 20μg/insect for houseflies. The epimeric mixture of senecioate ($\mathbf{F_A}$) has an LD_{50} of 0.02 μg/insect, an activity comparable to the pyrethrins and rotenones and the housefly activity was 68% at 20μg/insect. Similarly, the epimeric mixture of isovalerate ($\mathbf{F_B}$) has mustard beetle activity of 100% and housefly activity of 70% at 20 μg/insect. The mustard beetle activities of the natural products are much higher than nicotines (6 μg/insect) and comparable with rotenones (0.02 μg/insect) but less than pyrethrins (0.01

μg/insect). The natural products showed no activity to mites and larvae of the diamondback moth.

At this stage it is not possible to conclude whether the activity is due to one or both epimers, α and β as only α- and β-mixtures were available for bio-assays.

Table 2.35 : The insecticidal Activity of Natural Products



Fraction	R_1	MB*	HF**	PX†	TU‡	BT¶
C_1	Sen, Isv	0.028	71	15	NT	NT
F_A	Sen	0.02	68	NT	NT	-
F_B	Isv	100**	70	-	-	-

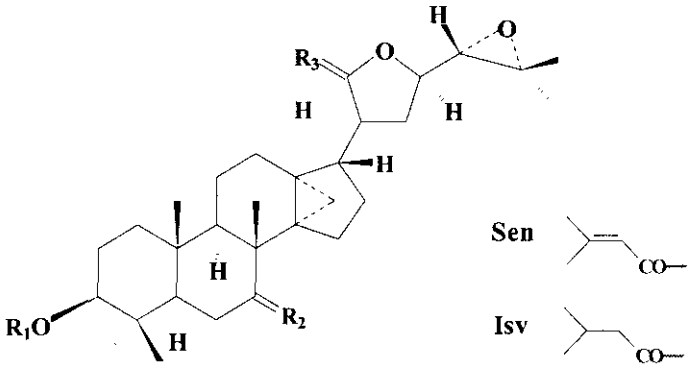
* Insecticidal activity is given in LD_{50} (μg/insect) or ** as % mortality (20 μg/insect) or

† as % mortality (10 μg/insect) or ‡ 1000 ppm or ¶ 2000 ppm.

NT : non toxic. MB : mustard beetle (*Phaedon cochleariae*), HF : housefly (*Musca domestica*), TU : mites (*Tetranychus urticae*), PX : larvae of diamondback moth (*Plutella xylostella*), BT : white fly (*Bemisia tabaci*)

Derivatives are less active than natural products (Table 2.36). Among the acylated derivatives the most active are the acetates with the diacetate (**14**) most active against both mustard beetles and houseflies. The α-epimer of the monoacetate (**13**) appears to be more active than the β-epimer (**12**). The mixture of senecioate and isovalerate diacetates showed no activity (**15-17**). All the acetates are nontoxic to mites.

Table 2.36 : The insecticidal Activity of Synthetic Derivatives



Compound	R_1	R_2	R_3	MB*	HF†	TU¶
12	Sen	β-H, α-OH	α-H, β-OAc	0.2	28	NT
13	Sen	β-H, α-OH	β-H, α-OAc	0.15	65	NT
14	Sen	β-H, α-OAc	α-H, β-OAc	0.1	78	NT
15-17	Sen, Isv	β-H, α-OAc	α, β-OAc	NT	NT	10
10	Sen	β-H, α-O-p-NO_2Bz	β-H, α-O-p-NO_2Bz	NT	NT	-
11	Isv	β-H, α-O-p-NO_2Bz	β-H, α-O-p-NO_2Bz	NT	NT	-
18, 19	Sen, Isv	O	O	100†	70	-

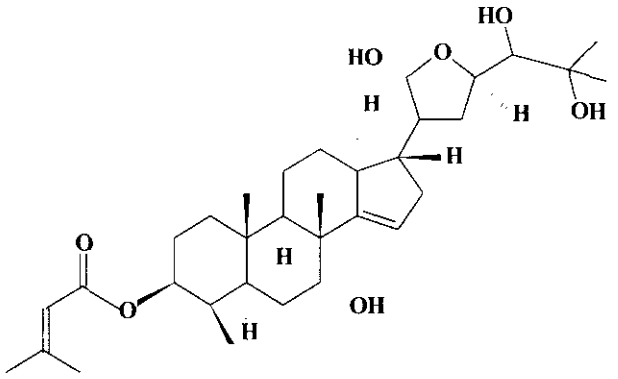
* Insecticidal activity is given in LD_{50} (μg/insect) or † as % mortality (5 μg/insect) or ¶ 1000 ppm.

NT, MB, HF, TU - As Table 2.35 (page, 172)

The di p-nitrobenzoyl derivatives (**10** and **11**) are nontoxic to both mustard beetles and houseflies (Table 2.36). Mustard beetle and housefly activities are retained in the oxidised derivatives (**18**, **19**).

Acid hydrolysis dramatically reduces the mustard beetle activity and completely destroy the housefly activity of the molecule (Table 2.37). This demonstrates that the epoxide and cyclopropane functionalities are required for the pronounced activity.

Table 2.37 : The Insecticidal Activity of Hydrolysed Derivative (**20**)



MB	HF	TU
37	NT	NT

Insecticidal activity is given as % mortality (5 μg/insect).

NT, MB, HF, TU - As Table 2.35 (page, 172).

In summary, the insecticidal activity has highlighted that the site of action is sensitive to the different ester groups at C-7 and C-21 as well as to the different functionalities in the molecule, such as hydroxyl, cyclopropyl and epoxide.

2.4 ISOLATION AND CHARACTERISATION OF COMPOUNDS FROM *EXCOECARIA AGALLOCHA*

2.4.1 Introduction

Preliminary bio-assay studies of the extracts of the stem bark and leaves of *Excoecaria agallocha* indicated good insecticidal activity against mustard beetles, houseflies and mites (Table 2.38).

Excoecaria agallocha Linn. (Euphorbiaceae) is distributed extensively in mangrove ecosystems. Plants in the Euphorbiaceae are known to produce a range of toxic metabolites with pro-inflammatory, tumour-promoting, skin irritant and co-carcinogenic activities.[201] In addition, tigliane esters reported from *Croton* species of Euphorbiaceae have been known to possess insecticidal effects.[56]

E. agallocha exhibits several biological activities including piscicidal[146], skin irritant[150] and fungicidal, and is used in herbal preparations.[148] However, no insecticidal activity for the genus *Excoecaria* has been reported.

The work reported here describes the characterisation of the insecticidal constituents of *E. agallocha*.

Table 2.38 : Insecticidal activity of *Excoecaria agallocha*

Insect species	Stem bark			Leaves		
	A	B	C	A	B	C
MB*	WT	100	80	NT	100	50
HF*	NT	28	NT	NT	62	NT
TU†	NT	NT	NT	NT	50	NT
BT‡	NT	NT	NT	NT	NT	NT

Insecticidal activity is given as % mortality (* 20 µg/insect, † 1000 ppm, ‡ 2000 ppm).

NT : non toxic, WT : weakly toxic (< 20%). A : petroleum ether, B : ethyl acetate, C : ethanol

MB : mustard beetle (*Phaedon cochleariae*), HF : housefly (*Musca domestica*),

TU : mites (*Tetranychus urticae)*, BT: whitefly (*Bemisia tabaci*),).

2.4.2 **Isolation of insecticidal compounds**

Sequential extraction of the stem bark and leaves with petroleum ether, ethyl acetate and ethanol, revealed that the activity was concentrated in the ethyl acetate and ethanol extracts (Table 2.38). The combined active extract was fractionated by dry column, flash column and finally, by reverse phase HPLC to isolate the compounds **24**-**28** (Figure 2.59).

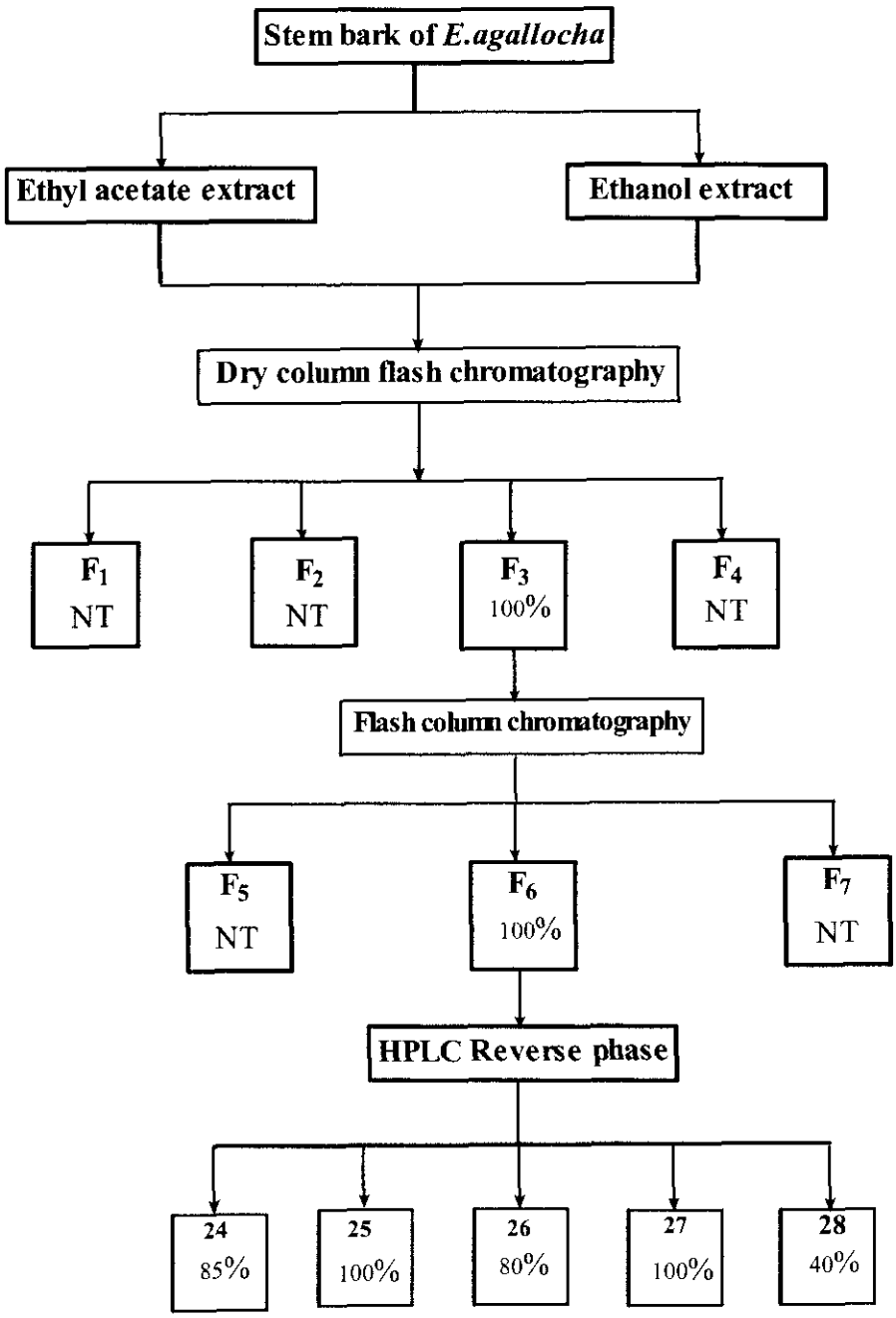


Figure 2.59 : Bio-assay guided fractionation of extracts of *Excoecaria agallocha*

2.4.3 **Characterisation of compounds 24-28**

(a) **Characterisation of compound 24**

The compound **24** was isolated from *Excoecaria agallocha* as a colourless oil in 0.003% yield. The compound was visualised as a UV active spot (λ = 254 and 366 nm, 1:9, water:methanol, Rf = 0.55) and a purple spot with anisaldehyde-H_2SO_4.[162]

The IR spectrum showed the characteristic absorptions of conjugated carbonyl (1690 cm^{-1}), a cyclopentenone (1797 cm^{-1}), double bond (1670 cm^{-1}), conjugated double bond (1630 cm^{-1}) and hydroxyl (3513 and 3021 cm^{-1}) functionalities. The UV spectrum also revealed the presence of a conjugated diene and an enone system (λ_{max} = 233 nm).

The DEPT and ^{13}C NMR spectra led to the recognition of thirty one carbons in the molecule including four methyls, eight methylenes, eleven methines and eight quaternary carbons (Table 2.39, page 180). The low resolution electrospray API mass spectrum displayed the molecular ion at m/z 542, suggesting the molecular formula to be $C_{31}H_{42}O_8$ which indicated a degree of unsaturation of eleven.

The ^{13}C and ^{1}H NMR spectra exhibited a characteristic signals for a 9,13,14-orthoester function (δ 118.0), an isopropylene side chain (δ_H 5.02, s, 4.91, s, 1.80, br s), a 6(7)-epoxy group (δ_H 3.49; δ_C 64.1, 60.3) and an α-β unsaturated carbonyl system (δ 210.0, 161.3, 137.0).[202]

Comparison of the ^{1}H and ^{13}C NMR chemical shifts for daphnane ortho esters reported from *E. agallocha*[148] and Euphorbiaceae[202, 203] with those observed for the compound **24** (Table 2.39), showed strong similarities leaving only the nature of the esterified acid moiety at C-1" to be established.

The ^{13}C, ^{1}H and ^{1}H-^{1}H COSY spectral data indicated the alkyl moiety at C-1" contained four contiguous vinyl carbons and six aliphatic carbons (Figure 2.60). These were readily assigned by the correlations from C-1' through C-10' in the COSY spectrum and with the support of the literature reported data.[202] Coupling constants of the ^{1}H NMR spectrum (Table 2.39) indicated ***E***-geometries of both 1',2'- and 3',4'-double bonds of the alkene side chain. With all this information the structure of the compound **24** was established as 12-deoxy daphnane, 9,13,14-orthoester diterpene with a C-10 alkyl side chain (Figure 2.61). This orthoester alkyl moiety in **24** has not been reported from the "daphnetoxine type" diterpenes of *E. agallocha* or any plant of Euphorbiaceae.

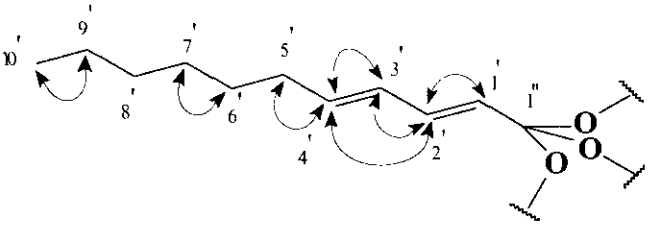


Figure 2.60 : ^{1}H-^{1}H COSY correlations for alkyl side chain

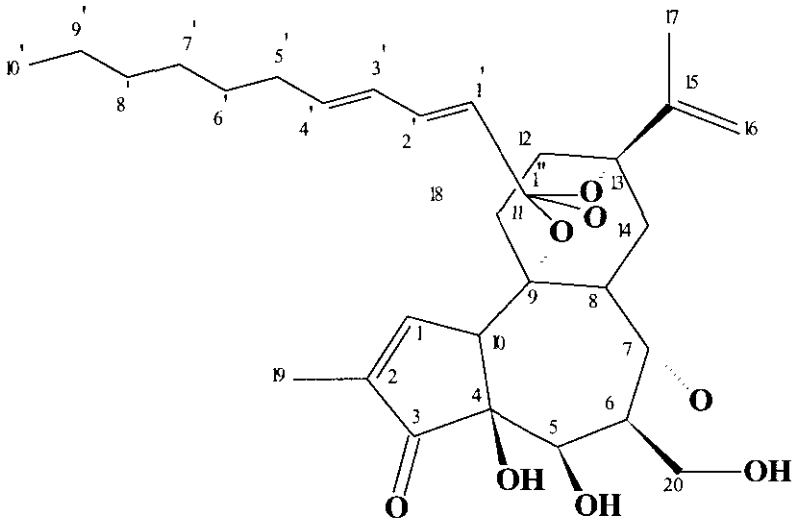


Figure 2.61: Compound **24**

Table 2.39 : ^{13}C and ^{1}H NMR spectra assignments of compound **24** and reported ^{13}C NMR data in $CDCl_3$

Position	δ_C	Reported δ_C	DEPT	δ_H (*J*)	^{1}H-^{1}H COSY
1	161.3	161.0	CH	7.64, s	7.64 → 3.84, 1.80
2	137.0	136.6	C_q	-	-
3	210.0	209.6	C_q	-	-
4	83.0	84.4	C_q	-	-
5	84.4	82.0	CH	4.26, s	4.26 → 3.49
6	60.3	60.5	C_q	-	-
7	64.1	64.1	CH	3.49, s	3.49 → 4.26
8	36.6	36.7	CH	2.95, d	2.95 → 4.43
9	72.2	72.4	C_q	-	-
10	48.1	48.2	CH	3.84, m	3.84 → 7.64, 1.80
11	34.9	34.9	CH	2.48, q	2.48 → 1.18
12	36.4	36.5	CH_2	3.46, s	-
13	81.9	79.6	C_q	-	-
14	72.0	71.8	CH	4.43, d	4.43 → 2.95
15	146.0	146.2	C_q	-	-
16	113.0	111.2	CH_2	5.02, 4.91, s	5.02, 4.91 → 1.80
17	20.3	20.3	CH_3	1.80, s	1.80 → 5.02, 4.91
18	9.9	9.8	CH_3	1.18, d	1.18 → 2.48

table contd.

19	19.0	18.9	CH_3	1.80, s	1.80 → 7.64
20	65.0	65.1	CH_2	3.80-3.70, m	-
1'	122.7	122.9	CH	5.71, d, (15.4)	5.71 → 6.70
2'	138.9	138.7	CH	6.70, q, (15.4, 9.5)	6.70 → 6.06, 5.71, 5.60
3'	134.8	134.6	CH	6.06, q (15.4, 9.5)	6.06 → 6.70, 5.85
4'	128.8	128.8	CH	5.85, m (15.4, 4.7)	5.85 → 6.06, 2.24
5'	32.6	32.6	CH_2	2.24, m	2.24 → 5.85
6'	30.0	28.7	CH_2	2.09, q	2.09 → 1.36
7'	28.7	31.3	CH_2	1.36, m	1.36 → 2.09
8'	31.3	22.4	CH_2	1.27, br s	-
9'	22.5	-	CH_2	1.27, br s	1.27 → 0.91
10'	14.1	13.9	CH_3	0.91, dd	0.91 → 1.27
1''	118.0	116.5	C_q	-	-
4-OH	-	-	-	2.24, s	-
5-OH	-	-	-	4.00, s	-
20-OH	-	-	-	3.60, s	-

Chemical shift values are in ppm.

(b) **Characterisation of compound 25**

The compound **25** was isolated from *E. agallocha* as a colourless oil in 0.004% yield. The compound was visualised as a UV active spot (λ = 254 and 366 nm, 1:9, water:methanol, Rf = 0.55), and a purple spot with anisaldehyde-H_2SO_4.[162]

Comparison of the ^1H chemical shifts for **24** with those observed for **25** indicated a close structural analogy (Table 2.39 and 2.40). The 5,7,6 tricyclic ring skeleton is identical for both compounds with a 6,7 epoxide group, hydroxyls at C-4, C-5 and C-20, an isopropenyl side chain at C-13 and an orthoester alkyl moiety which bridges C-9, C-13 and C-14 of the six membered ring.

The only significant difference of **25** was the absence of signals corresponding to the vinyl carbons in the the alkyl side chain of **24**. Integration of the proton signals and characteristic chemical shift values of **25**, tentatively assigned the alkyl side chain at C-1" as n-heptyl (Figure 2.62) which has been reported from Euphorbiaceae.[202]

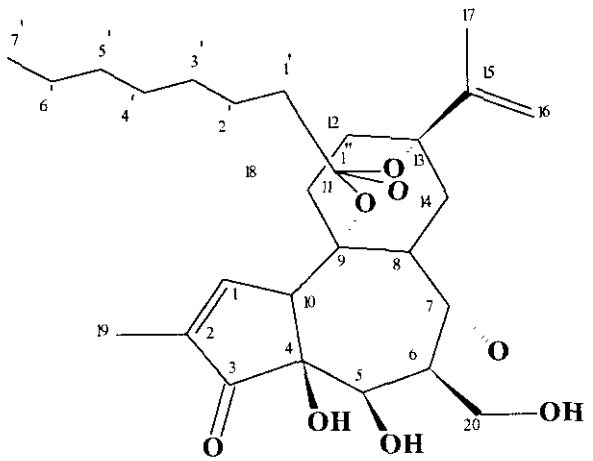


Figure 2.62 : Compounds **25**

Table 2.40 : ^{1}H NMR spectral data of compound **25** in $CDCl_3$

Position	δ_H (J)
1	7.64, br s
2	-
3	-
4	-
5	4.26, s
6	-
7	3.49, s
8	2.95, d
9	-
10	3.84, m
11	2.48, q
12	3.46, s
13	-
14	4.43, d
15	-
16	5.02, s, 4.91, s
17	1.80, s
18	1.18, d
19	1.80, s
20	3.70-3.80, m
1'	2.24, m
2'	2.09, q
3'	1.36, m
4'	1.29, s
5'	1.29, s
6'	1.29, s
7'	0.88, dd
1"	-
4-OH	2.24, s
5-OH	4.00, s
20-OH	3.60, s

Chemical shift values are in ppm.

(c) Characterisation of compounds 26-28

The compounds **26-28** were isolated from *E. agallocha* as colourless oils in 0.008, 0.006 and 0.006% yields, respectively. They were visualised as UV active spots (λ = 254 and 366 nm, 1:9, water:methanol, Rf = 0.55 and 0.45) and purple spots with anisaldehyde-H_2SO_4.[162]

The IR spectrum of compounds **26-28** showed the characteristic absorptions of hydroxyl (3528, 3500 cm^{-1}), conjugated carbonyl (1698, 1694 cm^{-1}), double bond (1670, 1672 cm^{-1}) and conjugated double bond (1638, 1631 cm^{-1}) functionalities. Their UV spectrum also indicated the presence of a conjugated double bond and an enone system (λ_{max} = 239 nm).

The low resolution electrospray API mass spectrum of compounds **26-28** showed the molecular ions at m/z 528, 568 and 610 suggesting the molecular formulae to be $C_{30}H_{40}O_8$, $C_{33}H_{44}O_8$ and $C_{36}H_{50}O_8$, respectively. Molecular formulae indicated a degree of unsaturation of eleven and twelve in compounds **26-28**.

Comparison of the ^1H and ^{13}C NMR spectral data of **26-28** with those of **24** indicated that they were analogues of **24** (Table 2.39, page 180 and 2.41) but having different alkyl side chains at C-1". The orthoester alkyl side chain of the compound **24** was reduced by one CH_2 unit in **26** which was assigned as 2,4-decadienoate. In **27** the alkyl side chain was further extended by one olefinic double bond and in **28** by one olefinic double bond and three CH_2 units which

were assigned as 2,4,6-tridecatetraenoate and 2,4,6-hexadecatetraenoate, respectively (Figure 2.63).

The 5,7,6 tricyclic parent skeleton is common to all three compounds. Additional carbon units in **27** and **28** were supported by their ^{1}H and ^{13}C NMR spectral data (Table 2.41) and the size of the alkyl side chain was confirmed by their mass spectrum. The compounds **26** and **28** were previously isolated from the latex of *E. agallocha* as Excoecariatoxins, **A3** and **A1**, respectively.[150] The alkyl moiety in **27** has not been reported from the "daphnetoxin type" diterpenes of *E. agallocha* or any other plant of the Euphorbiaceae.

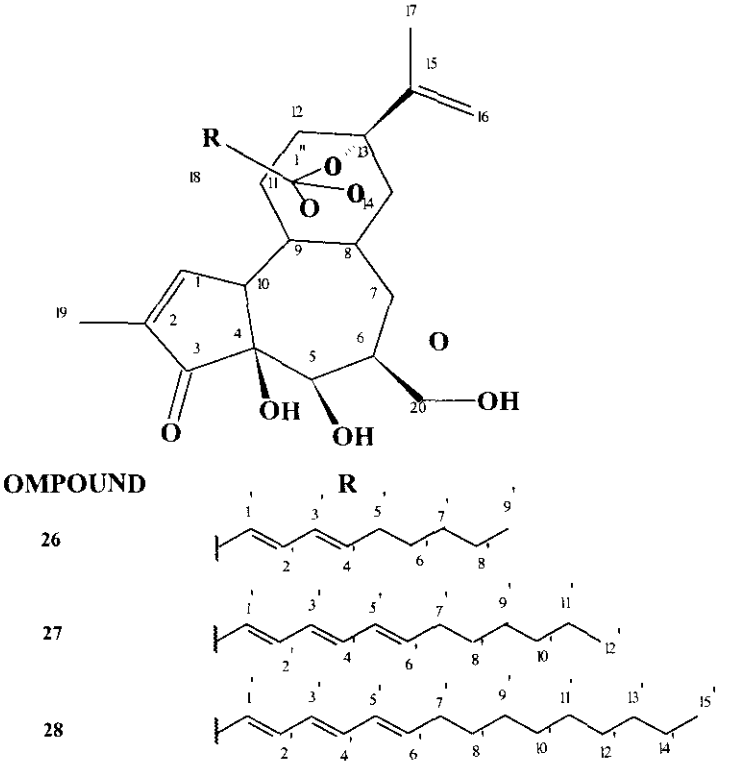


Figure 2.63: Compounds **26-28**

Table 2.41 : ^{13}C and ^{1}H NMR spectra assignments of compounds **26-28**

in $CDCl_3$

Position	26-δ_C	27-δ_C	28-δ_C	DEPT	26-δ_H	27-δ_H	28-δ_H
1	161.3	161.2	161.2	CH	7.64, s	7.64, s	7.64, s
2	137.0	136.6	136.6	C_q	-	-	-
3	210.0	210.0	209.7	C_q	-	-	-
4	83.0	83.0	83.0	C_q	-	-	-
5	84.4	84.4	84.4	CH	4.26, s	4.25, s	4.30, s
6	60.3	60.3	60.7	C_q	-	-	-
7	64.1	64.1	64.1	CH	3.70, s	3.70, s	3.80, s
8	36.7	36.7	36.6	CH	2.95, d	2.95, d	2.95, d
9	72.2	72.1	71.5	C_q	-	-	-
10	48.1	48.1	48.1	CH	3.84, m	3.88, m	3.80, m
11	34.8	34.8	34.8	CH	2.49, q	2.50, q	2.40, m
12	36.4	36.4	36.4	CH_2	3.45, s	3.46, s	3.50, s
13	81.9	81.9	81.9	C_q	-	-	-
14	72.0	72.0	72.5	CH	4.43, d	4.45, d	4.45, d
15	146.0	145.0	146.1	C_q	-	-	-
16	111.3	111.4	111.3	CH_2	5.02, 4.9,s	5.02, 4.9,s	5.02, 4.9, s
17	20.4	20.4	20.4	CH_3	1.80, br s	1.80, s	1.80, s
18	9.9	9.9	9.9	CH_3	1.18, d	1.18, d	1.18, d
19	19.0	18.9	18.9	CH_3	1.80, br s	1.80, s	1.80, s
20	65.0	65.0	65.1	CH_2	3.75, m	3.75, m	3.75, m
1'	122.7	123.9	124.1	CH	5.77, m	5.77, m	5.77, d
2'	138.9	137.4	137.3	CH	6.75, q	6.75q	6.70, q
3'	134.8	134.6	134.5	CH	6.10, m	6.10, q	6.10, m
4'	128.8	128.6	128.6	CH	5.77, m	5.77, m	5.85 ,m
5'	32.7	136.5	136.5	CH_2/CH	2.24, m	6.31, m	6.35, m
6'	29.7	130.0	130.0	CH_2/CH	2.10, q	6.10, q	6.10, m
7'	31.3	32.9	32.9	CH_2	1.25, s	2.25, m	2.24, m
8'	22.5	29.7	29.2	CH_2	1.25, s	2.10, q	1.36, m
9'	14.1	29.1	29.3	CH_3/CH_2	0.87, dd	1.37, m	1.25, s
10'	-	31.8	29.4	CH_2	-	1.27 br s	1.25, s

table contd.

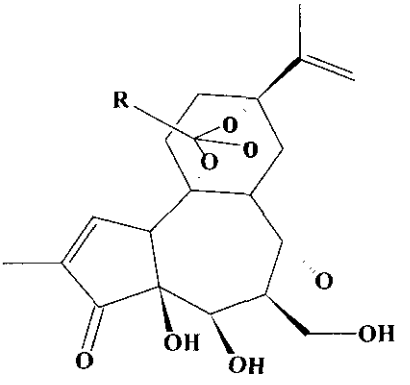
11'	-	22.7	29.5	CH_2	-	1.27 br s	1.25, s
12'	-	14.1	29.5	CH_3/CH_2	-	0.90, dd	1.25, s
13'	-	-	31.9	CH_2	-	-	2.10, m
14'	-	-	22.7	CH_2	-	-	1.25, s
15'	-	-	14.2	CH_3	-	-	0.88, t
1''	118.0	118.0	116.4	C_q	-	-	-
4-OH	-	-		-	2.24, s	2.24, s	2.50, s
5-OH	-	-		-	4.02, s	4.00, s	4.20, s
20-OH	-	-		-	3.63, s	3.60, s	3.80, s

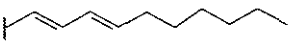
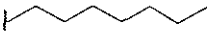
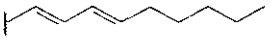
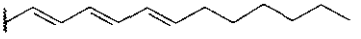
Chemical shift values are in ppm.

2.4.4 **Insecticidal activity**

The insecticidal activity of the genus *Excoecaria* (Euphorbiaceae) has not been reported. The insecticidal constituents were identified as daphnane orthoester diterpenoids **24-28**.

Table 2.42 : Insecticidal activity of compounds



OMPOUND	R
24	
25	
26	
27	
28	

Compound	Mustard beetle (*Phaedon cochleariae*)
24	85
25	100
26	80
27	100
28	40

Insecticidal activity is given as % mortality (5 µg/insect).

The bio-assay results show that compounds **25** and **27** have the highest activity against mustard beetles (Table 2.42). Compounds **24** and **26** are less active and **28** shows only weak activity. The size of the orthoester alkyl side chain is clearly important in determining activity.

2.4.5 **Postulated Biosynthesis of compounds 24-28**

No detailed studies have been reported on the biosynthesis of daphnane diterpenoids nor on related hydrocarbon skeletons. Adolf and Hecker[204] reported a hypothetical discussion of possible biosynthetic pathways of various diterpenoid compounds of the family Euphorbiaceae and Thymelaeaceae including daphnane type diterpenoids. Based on these reports[204, 205] the biosynthetic pathway leading to the compounds **24-28** can be postulated as illustrated in Figure 2.64.

According to this scheme, compounds have been derived from the diterpene precursor geranyl-geranyl pyrophosphate (GGPP) via the acetate-mevalonate pathway. Casbene has been considered to be the biogenetic precursor which cyclises to form lathyranes, tiglianes and subsequently daphnanes, which then lead to formation of an orthoester.

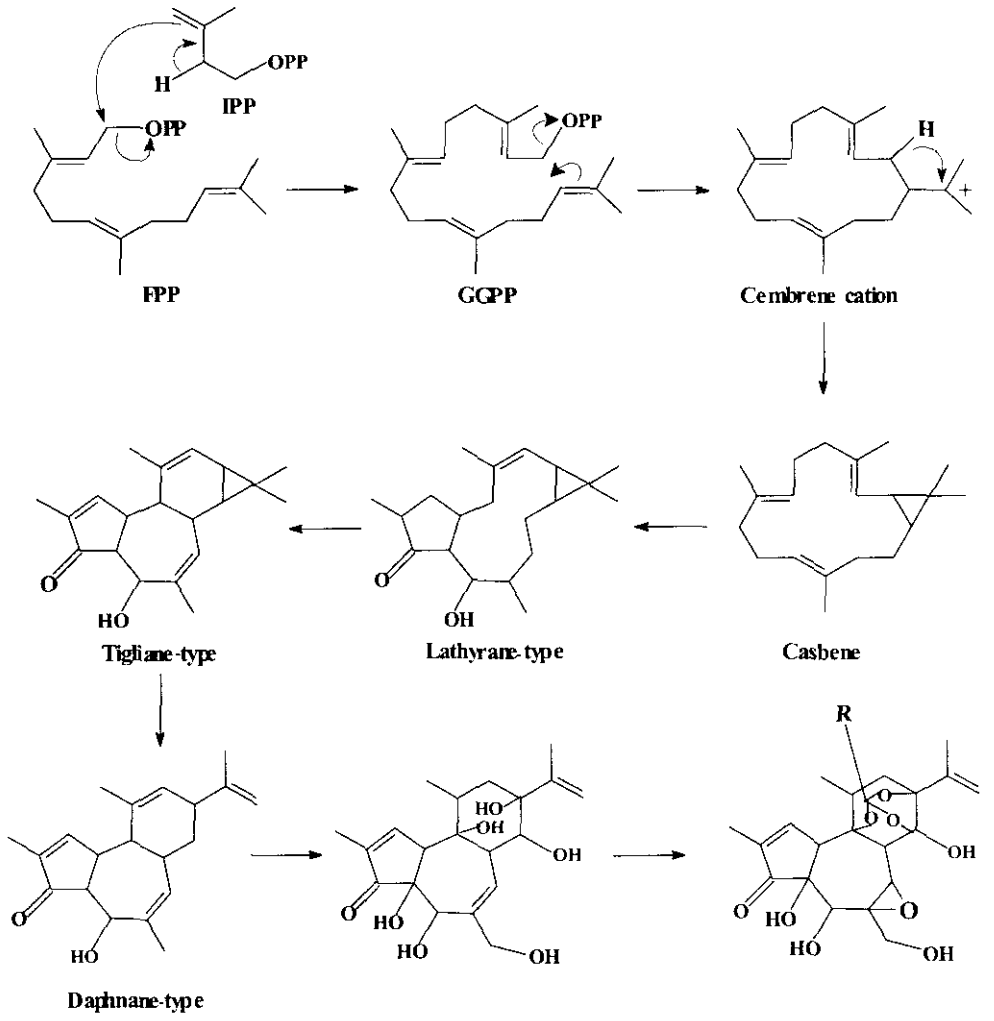


Figure 2.64: Postulated Biosynthetic pathway to compounds **24-28**

2.5 SUMMARY

2.5.1 *Pleurostylia opposita*

Three novel macrocyclic sesquiterpene polyol ester alkaloids, compounds **1**, **2** and **3** were identified from *Pleurostylia opposita* as the insecticidal principles in the present study. They possess a 6,7 ring skeleton, analogous to the β-dihydroagarofuran core which contains a 6,6 ring skeleton and a fourteen membered bislactone ring containing a 2,3-disubstituted pyridine moiety. They differ only in the nature of the six ester groups. Compound **1** contains five acetates and one benzoate, while compound **2** contains six acetates, and **3** four acetates, one nicotinate and one benzoate.

This is the first time that sequiterpene polyol ester alkaloids have been identified and insecticidal activity detected in the genus *Pleurostylia*. The compounds **1**, **2** and **3** are homologues of the known sesquiterpene polyol ester alkaloids from Celastraceae.[155] The molecular framework for **1**, **2** and **3** contains an extra CH_2 group compared to all the previously reported compounds in this series. This additional carbon atom, indicated by the NMR spectral data, is present in the bicyclo [5,4,0] undecane ring system at position 8. The 6,7 ring system analogues of the β-dihydroagarofuran core is previously unknown in any form, but sesquiterpenoids based on other 6,7 ring systems such as himachalenes and the widdrols, have been reported from several plant species.[206]

Sesquiterpene polyol ester alkaloids have low potential as leads to develop as a new class of commercial insecticides due to their structural complexity, poor spectrum and low activity when compared to commercial standards. However, further knowledge of the toxophore for this class of compounds will help with the overall assessment of commercial possibilities. Further testing of the crude extract against other commercially important pest species is required in order to ascertain whether the plant extract has potential as a botanical pesticide.

2.5.2 *Aegle marmelos*

The major insecticidal component of *Aegle marmelos* (Rutaceae) is a novel glabretane pentacyclic triterpene, belonging to the class of protolimonoids exemplified by glabretal. The isolation of the active principle was difficult as there were four closely related compounds, accompanied by a large amount of the non-active compound epoxyaurapten. Although many phytochemical studies were reported from *A. marmelos*, there are no reported active structures.

The presence of three coumarins epoxyaurapten, marmesin and marmin and a triterpene, lupeol in *A. marmelos* has been already reported, but they were found to be non-active in the present bio-assays.

Bio-assay guided chromatographic separations showed that minor components were responsible for the insecticidal activity. A Combination of derivatisation and HPLC allowed the isolation of the two pure di p-nitrobenzoates, which were characterised fully, especially by NMR as derivatives of 3-senecioyl and 3-

isovaleryl glabretals. Re-examination of the parent spectra and 2-D experiments including inverse experiments (HMBC and TOCSY) led to the conclusion that the major components of the insecticidal mixture were the related senecioate and isovalerate esters **6**, **7**, **8** and **9**.

As well as the above C-21 α-di p-nitrobenzoates, other derivatives isolated included a β-diacetate and C-21 α- and β-monoacetates, a hydrolysis product with a changed skeleton, and a diketo oxidation product. None of these derivatives, except the oxidation product of isovalerate, has been reported previously.

Glabretal compounds may provide leads for SAR based programmes to develop new classes of commercial insecticide. However, such possibilities cannot be assessed without testing on a broader range of insect species, especially commercially important insects. Due to the low yields and the complexity of isolation of the active constituents the most likely possibility of developing the plant extract is as a botanical insecticide.

2.5.3 ***Excoecaria agallocha***

Excoecaria agallocha (Euphorbiaceae) is known for the production of toxic metabolites, but no insecticidal activity has been reported for any member of the genus *Excoecaria*. In the screens at Rothamsted the plant showed activity against mustard beetles, houseflies and mites.

The insecticidal principles of *E. agallocha* have now been identified as the daphnane polyol orthoester diterpenoids, already identified in *E. agallocha*, as well as other Euphorbiaceae and Thymelaeaceae.[202] These toxic diterpenoids have been reported to possess pro-inflammatory, tumour-promoting, skin irritant, co-carcinogenic and piscicidal activities[150] but, the discovery of their insecticidal activity is novel.

Of the five compounds isolated, **24** and **27** with 2,4-decaenoate and 2,4,6-tridecatetraenoate side chains are novel among the "daphnetoxin type" diterpenes of Euphorbiaceae and Thymelaeaceae.

Daphnane orthoester compounds have low commercial potential to develop new classes of insecticide because they are skin irritants and the related phorbol esters are co-carcinogenic.

CHAPTER 3

EXPERIMENTAL

3.1 GENERAL

High and low resolution mass spectra were recorded on a Micromass Autospec double focusing mass spectrometer equipped with an electrospray API interface. The samples were introduced in a solution of methanol and 1% (v/v) acetic acid.

The ^{1}H NMR spectra were recorded on Jeol JNM-GX 400 FT NMR, λ-400 and α-500 spectrometers, operating at 400 and 500 MHz, respectively. The ^{13}C NMR spectra were recorded on the same instruments operating at 100 and 125 MHz, respectively. The DEPT, COSY and NOESY spectra were recorded at 400 MHz on a Jeol JNM-GX 400 FT NMR spectrometer. DEPT experiments were recorded at 90° and 135° and NOESY spectra were recorded with mixing time of 500 ms. The HMBC, HSQC and TOCSY spectra were recorded on Jeol λ-400 and α-500 NMR spectrometers using pulse field gradient. TOCSY spectra were performed with mixing times 50 ms, 80 ms and 150 ms.

Structural elucidation of the compounds was based mainly on these NMR experiments. The ^{1}H, ^{13}C and DEPT NMR techniques[187, 207, 208] were extensively used in the structure elucidation of the simple and known compounds. 2D NMR correlation spectroscopy,[105, 187] including ^{1}H-^{1}H, ^{13}C-^{1}H and long-range ^{13}C-^{1}H COSY techniques, was used widely to assign complex, new compounds. Where ever possible the stereochemistry of the molecules was assigned from the NOESY spectrum.[187] Inverse 2D NMR experiments including HSQC[107], HMBC and TOCSY[106] were extensively used to establish the entire molecular frame work of

compounds. Unless otherwise stated, deuteriochloroform ($CDCl_3$) was used as solvent for NMR. Chemical shift values (δ) were reported in ppm downfield from tetramethylsilane (TMS).

Infrared spectra were recorded on a Nicolet Impact 410 IR and FT IR spectrometer. UV spectra in ethanol were obtained on a Shimadzu UV-160A UV-visible recording spectrophotometer. Melting points were determined on a Gallenkamp electrothermal capillary tube melting point apparatus.

All solvents were distilled prior to use. Petroleum ether refers to the fraction boiling at 60-80 °C. HPLC grade solvents supplied by Rathburn were used in HPLC and SFC separations. All HPLC solvents were degassed using an ultrasonic bath for about 30 min before use.

A Panasonic 800 WD type (800W) microwave oven was used in microwave extraction of plant materials. Acid non-washed diatomaceous silica [(celite), 90% SiO_2], supplied by Sigma chemicals was used in most of the routine filtrations.

Analytical thin layer chromatography (tlc) was carried out using either plastic-backed plates supporting a 0.25 mm layer of silica gel $60F_{254}$ (No.5735), or pre-coated glass plates supporting a 0.25 mm layer of RP-$18F_{254}$ (No. 15685) and silica gel $60F_{254}$ (No.5719), or pre-coated HPTLC glass plates supporting a 0.10 mm layer of silica gel $60F_{254}$ (No. 5628), all supplied by Merck.

For visualisation, UV light (λ = 254 and 366 nm) and spray reagents (anisaldehyde-H_2SO_4, Dragendorff's-reagent[162] and 5% phosphomolybdic acid-ethanol) were used and colour was observed by heating.

Preparative tlc was carried out using 20×20 cm glass plates supporting a 1 mm layer of silica gel 60GF, supplied by Anachem (No. 2013). Products were best visualised by UV light (λ = 254 and 366 nm) or by treatment of a small portion on both side edges of the plate with an appropriate spray reagent.

Silica gel supplied by Merck [(No. 7734) 70-230 mesh ASTM, particle size: 63-200 μm] was used for gravity column chromatography. Flash column chromatography was carried out using 230-400 mesh silica gel (particle size: 40-63 μm), supplied by Merck (No. 9385). Silica gel 60H supplied by Merck (No. 64271) was used for dry column chromatography. Reverse phase gravity column chromatography and flash column chromatography was carried out using silica gel 60 RP-18 (particle size: 40-63μm), supplied by Merck (No. 10167). Sephadex LH-20 (particle size: 25-100 μm) was used in gel filtration chromatography, supplied by Fluka (No. 84952). Products of all column chromatographic separations were described in the order of their elution.

HPLC was performed with 10 mm ID×250 mm L Dynamax, 60 Å, 8 μ silica, nitrile, C_8, C_{18} and 300 Å, 5 μ C_4 semi-preparative columns coupled to a Gilson Model 305 and 306 LC pumps and an Applied Biosystems 1000s Diode array detector, monitored at four different wave lengths simultaneously (λ = 220, 254, 280 and 366 nm). The Flow rate of the mobile phase was 2.0 or 3.0 ml min^{-1}.

Preparative HPLC separations were performed with 21.44 mm ID×250 mm L Dynamax, 60 Å, 8 μm silica and 300 Å, 5 μm C_{18} preparative columns coupled to a Gilson Model 305 and 306 LC pumps and a Dynamax, UV-1, variable wave length UV/visible absorbance detector.

Supercritical Fluid Extractions (SFE) and Supercritical Fluid Chromatography (SFC) were performed on Gilson SF_3 SFC system coupled to a Gilson Model 305 LC pump which was used to pressurise the carbon dioxide above its critical points and an Applied Biosystems 1000s Diode array detector. A Gilson Model 306 LC pump was used to deliver modifier e.g. acetonitrile to a Gilson 811B dynamic mixer. SFC separations were carried out with a 10 mm ID×250 mm L Dynamax, 60 Å 8 μ silica semi-preparative column.

The yields of the fractions and compounds were expressed as a percentage of the weight of dry plant material used for extraction.

3.2 COLLECTION AND PREPARATION OF PLANT MATERIAL

All plant materials including mangrove and marine plants and fruiting bodies were collected from Sri Lanka by the Natural Product Group of Department of Chemistry, University of Colombo during the period 1992-1996.

Stem bark, root bark, leaves and fruits of *Aegle marmelos* were collected from Western Province, Colombo. Stem bark and leaves of *Pleurostylia opposita* were collected from Central Province, Anuradhapura. Stem bark and leaves of *Excoecaria agallocha* were collected from the lagoon at Negambo, Western

Province of Sri Lanka. *A. marmelos*, *P. opposita* and *E. agallocha* were identified by late Professor S Balasubramanium of the Department of Botany, University of Peradeniya, Sri Lanka. The voucher specimens of plants were preserved in the Department of Botany, University of Colombo, Sri Lanka. All plant materials were air dried and finely powdered.

3.3 EXTRACTION

Air dried, powdered plant materials were extracted sequentially into three solvents using either normal, microwave, high pressure Soxhlet or supercritical fluid extraction techniques.

3.3.1 Normal extraction

Air dried, powdered plant material (20 g) was sequentially extracted with petroleum ether, ethyl acetate and ethanol (2×100 ml of each) using a mechanical stirrer for 20 min at room temperature. Each extract was filtered through a celite bed and concentrated in *vacuo*.

3.3.2 Microwave extraction

A modified commercial microwave oven was used with a reflux condenser which connect to a culture vessel through a glass column.[92, 209]

The powdered plant material (20 g) was suspended with petroleum ether (100 ml) in a culture vessel and placed in a microwave oven. The plant material was irradiated until the contents in the vessel started to reflux (2.8 min). The extract was

filtered through a celite bed and concentrated in *vacuo*. The extraction procedure was repeated with another 100 ml of petroleum ether. The irradiation step was then sequentially repeated with the residual plant material using ethyl acetate and ethanol (2×100 ml of each). The irradiation time was 3-4 min depending on the solvent used.

3.3.3 **Liquid carbon dioxide extraction - High Pressure Soxhlet extraction**

A J & W high pressure Soxhlet extractor[94] was used for small scale extraction of plant material. The powdered plant material (6 g) was placed in the glass thimble in the Soxhlet assembly and the entire extraction unit was placed inside the pressure chamber. Dry ice (180 g) was added into the outside shell of the glass assembly and the tightly closed extraction unit was placed in a water bath (45 °C) to a depth of approximately 6 cm. Ice water (0-10 °C) was circulated through the cold finger condenser using a peristaltic pump.

The pressure of the system was around 800 psi before starting the condensation and fell to 600-700 psi as condensation began. Condensed carbon dioxide was delivered to the Soxhlet assembly, and extraction of plant material started. Several hours of operation (7 h or more) were necessary to complete the extraction. Before opening the system, the assembly was cooled to approximately -78 °C using acetone-dry ice bath to bring down the pressure to zero.

3.3.4 **Supercritical Fluid Extraction (SFE)**

The finely ground plant material (0.5-1 g) was placed in the extraction vessel (2.5 ml) containing a small amount of celite at the bottom. The extraction vessel was placed in the oven and connected to the SFE.[90] The pressure (3.98 Kpsi), flow rate (4 ml min^{-1}) and oven temperature (32 °C) were set. The choice of SFE conditions, temperature, pressure, flow rate and modifier depended on the specific material to be extracted and the polarity of the product being studied.[90] When the system reached equilibrium, the injection valve was switched to the inject position to start the extraction. Supercritical carbon dioxide was forced through the plant material to extract the desired compounds. The extraction profile was monitored with an on-line UV detector set at 254 nm until the absorbance returned to the base line. The extract was collected in an ice-cooled round bottom flask containing a small amount of trapping medium (ethyl acetate). The same extraction procedure was sequentially performed with the residual plant material at an oven temperature of 40, 50 and 60 °C.

3.4 ISOLATION OF COMPOUNDS

Bio-assay guided chromatographic techniques were used in the isolation of the insecticidal compounds from the plant extracts. Whenever possible the isolated compounds were crystallised as a method of final purification.

3.4.1 Chromatographic techniques

TLC systems were extensively used for preliminary screening of active extracts, to monitor the progress of all chromatographic separations and to check the purity of isolated fractions and compounds.

Dry column flash chromatography (Vacuum liquid chromatography-VLC) [95, 96] and flash column chromatography[97] were used widely in the initial separation of crude extracts. Preparative tlc[210] was carried out to separate the compounds especially in the small scale separations (sample size ≤ 100 mg). Column chromatography[210] was used mostly in further purification of the fractions and on several occasions to isolate the pure compounds. Sephadex LH-20 gel filtration chromatography[211] was used to separate the active compounds from high molecular weight pigments and bulky non-active compounds.

Normal and reverse phase semipreparative and preparative HPLC systems[98, 210] were extensively used in the final purification of active compounds. In addition, semi-preparative HPLC was routinely used to check the purity of isolated compounds. SFC[90] was used for further purification of mixtures which failed to separate by normal and reverse phase HPLC.

3.5 INSECTICIDAL BIO-ASSAY

Insecticidal activity of plant extracts, fractions and compounds was assessed against mustard beetles, houseflies, whitefly, diamond-back moth larvae and mites by colleagues in the Resistance group within the Biological and Ecological Chemistry Department at IACR-Rothamsted. A 2% (w/v) solution of the sample was prepared by using either an acetone or acetone:ethanol (1:1) solution and this was used as a stock solution for bio-assay. Plant extracts were tested at much larger doses in preliminary assays, 20 μg/insect for mustard beetle and housefly, 10 μg/insect for diamondback moth, 2000 ppm/insect for whitefly and 1000 ppm/insect for mites. For line tests a closely spaced range of concentrations was used in comparative tests with the standard bioresmethrin. LD_{50} and LC_{50} values were calculated from the several mortalities, using a probit analysis software package (POLO-PC).[212]

3.5.1 Housefly (*Musca domestica*)

The houseflies were treated by the topical application method. Female flies (30 per concentration) were dosed on the thorax with 1 μl of the test solution. The treated flies were maintained at 20 °±1 °C and fed on a cotton wool plug soaked in a sucrose solution. Line tests used 6 dose rates per compound, two replicates of 15 flies for each. The percentage of mortality was assessed 24 and 48 h after treatment. The LD_{50} values were calculated in micrograms of test compound per fly.[213, 214]

3.5.2 **Mustard beetle (*Phaedon cochleariae* Fab)**

The mustard beetles were treated by the topical application method. A 1 µl of the test solution was dosed ventrally to adult mustard beetles (20 per concentration) using a micro drop applicator. The treated beetles were stored in petri dishes at 20 °C. Line tests used 5 dose levels per compound and two replicates of 20 beetles for each. Beetles were assessed for percentage mortality after 48 h. The LD_{50} values were calculated as for house flies.

3.5.3 **Diamondback moth (*Plutella xylostella*)**

The diamondback moth larvae were treated by topical application method. Fifth instar larvae (30 per concentration) were dosed with 0.5 µl of the test compound in acetone. Treated larvae were maintained at 22 °C and mortality was assessed as failure to pupate 5 days later. Line tests used 5 dose rates per compound, three replicates of 10 larvae for each. The LD_{50} values were calculated as for houseflies.

3.5.4 **Whitefly (*Bemisia tabaci*)**

White flies were treated using a vial confinement method. The test was carried out using three different strains of whitefly, one susceptible and two resistant to pyrethroid insecticides. The susceptible strain (SUD-S) was collected in Sudan in 1978 from cotton. The resistant strains, Ned 3 and USA-B, were collected from gerbera, Netherlands in 1992 and from poinsettia, Southern USA in 1985, respectively.

A solution of test compound in acetone (100 µl) was placed in 10 ml glass vials and evaporated with rotation to deposit a film of the compound. Adult whiteflies (30 per replicate, 3 replicates per dose, 5, 7 dose levels) were confined inside the vial for 1 h and then transferred onto untreated cotton leaf discs which were kept moist on a bed of agar gel. The temperature was maintained at 25 °C and mortality assessed after 48 h. LC_{50} values (in ppm) were calculated as above.

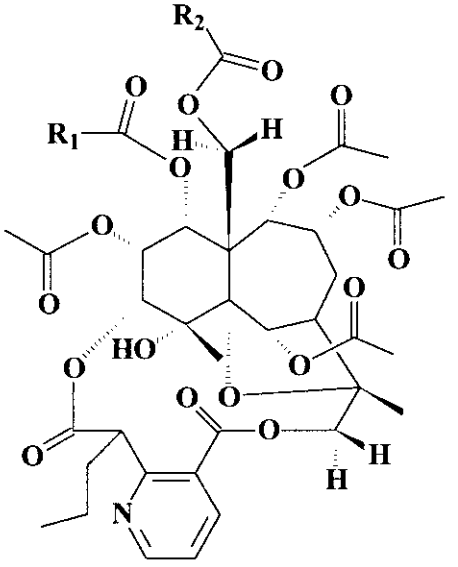
3.5.5 **Mites (*Tetranychus urticae*)**

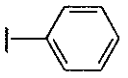
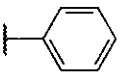
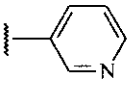
The mites were treated by microimmersion of topical application method[215] using three different strains of mites, one susceptible and two resistant to bifenthrin and carbaryl. The susceptible strain (GSS) was supplied by Schering, AG, Berlin. The resistant strain, NYR-Bif-1000 was provided by the department of Entomology, Cornell University, New York, having subjected a field strain to selection with bifenthrin. The resistant strain, UK-S Carb-600 was obtained by applying selection with carbaryl to the UK-S strain provided by Shell Research Limited, Sittingbourne, UK.

Adult female mites (25 per replicate, 3 replicates, 5, 6 dose rates) were immersed in 35 µl of an acetone:water (1:4) solution of the test compound for 30 seconds. The treated mites were maintained at 21 °±2 °C and mortality was assessed 72 h after treatment. Mites exhibiting repetitive (non-reflex) movement of more than one locomotory appendage after this period were recorded as alive. The LC_{50} values (in ppm) were calculated as above.

3.6 BIO-ASSAY GUIDED EXTRACTION AND ISOLATION OF COMPOUNDS FROM *PLEUROSTYLIA OPPOSITA*

3.6.1 Isolation of compounds 1, 2, and 3



COMPOUND	R_1	R_2
1		CH_3
2	CH_3	CH_3
3		

Air-dried, finely powdered stem bark (300 g) of *Pleurostylia opposita* was sequentially extracted with petroleum ether (2×1.5 l), ethyl acetate (2×1.5 l) and ethanol (2×1.5 l) using the microwave extraction procedure. The petroleum ether and ethyl acetate extracts were found to be insecticidal.

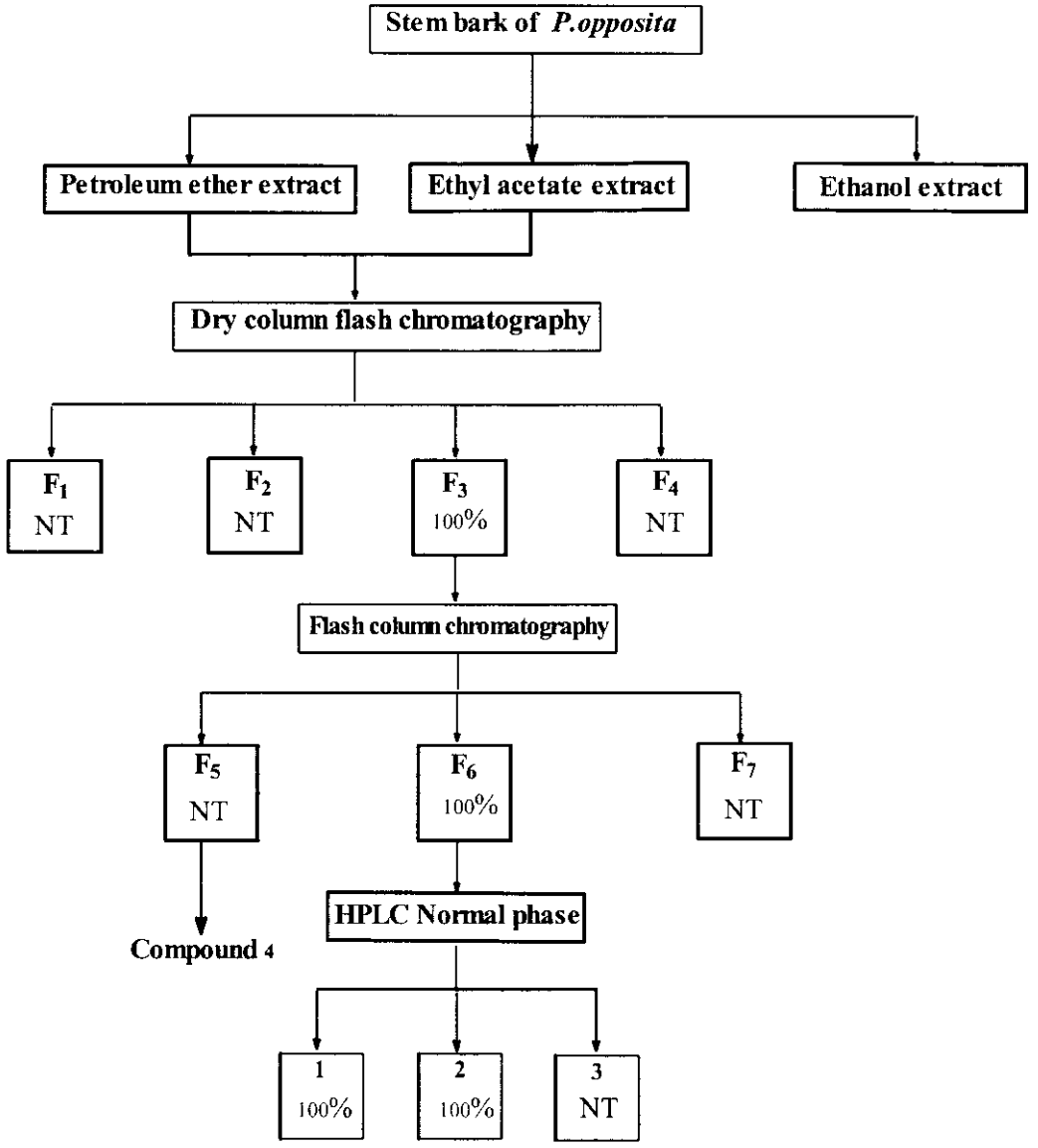


Figure 3.1 : Bio-assay guided fractionation of extracts of *Pleurostylia opposita*

These two active extracts were combined (10.4 g) and subjected to dry column flash chromatography (Figure 3.1) on silica gel eluting with a gradient solvent system of petroleum ether:ethyl acetate (2:3, 3:7, 1:4, 1:9, 2×75 ml, each) and finally ethyl acetate (3×75 ml). The combined insecticidal fractions (954.6 mg) were further purified by silica gel flash column chromatography (Figure 3.1) eluting with petroleum ether:ethyl acetate:isopropanol (6:3:1 to 5:4:1; 200 ml, 400 ml,

respectively) and finally ethyl acetate:isopropanol (9:1, 200 ml), to afford the active fraction, $\mathbf{F_6}$ (331 mg).

Fraction $\mathbf{F_6}$ (92.3 mg) was subjected to preparative HPLC (silica column, 10 ml min^{-1}, UV 254 nm) eluting with hexane:ethyl acetate:isopropanol (5:4:1), to afford, insecticidal compounds, **1** (26.0 mg, 0.03%, retention time 6.0 min) and **2** (15.0 mg, 0.02%, retention time 7.2 min) as yellow oily liquids and non-insecticidal compound **3** (7.4 mg, 0.01%, retention time 13.9 min) as a colourless oily liquid.

Compound (1)

^{1}H-NMR : δ 8.40 (1H, dd, J = 7.9, 1.8 Hz), 8.83 (1H, dd, J = 4.6, 1.8 Hz), 7.83 (2H, d, J = 7.0 Hz), 7.54 (1H, dd, $J_1 = J_2$ = 7.6 Hz), 7.42 (2H, dd, $J_1 = J_2$ = 7.6 Hz), 7.30 (1H, dd, J = 7.9, 4.6 Hz), 7.00 (1H, s), 6.00 (1H, d, J = 11.9 Hz), 5.85 (1H, d, J = 4.0 Hz), 5.55 (1H, m), 5.38 (1H, d, J = 5.8 Hz), 5.34 (1H, d, J = 13.4 Hz), 5.20 (1H, dd, J = 4.0, 2.4 Hz), 5.00 (1H, d, J = 2.4 Hz), 4.90 (1H, br s), 4.60 (1H, d, J = 13.4 Hz), 4.40 (1H, m), 3.60 (1H, d, J = 11.9 Hz), 2.40 (3H, s), 2.35-2.25 (2H, m), 2.35 (3H, s), 2.30 (3H, s), 2.25 (3H, s), 2.12 (1H, m), 2.20-1.95 (2H, m), 2.00-1.75 (2H, m), 1.75 (3H, s), 1.60 (3H, s), 1.40 (3H, s), 0.70 (3H, t, J = 7.3 Hz).

^{13}C-NMR : δ 172.1 (C_q), 170.4 (C_q), 170.0 (2×C_q), 168.9 (C_q), 168.5 (2×C_q), 166.4 (C_q), 164.6 (C_q), 153.8 (CH), 138.1 (CH), 133.5 (CH), 129.5 (CH), 129.4 (CH), 129.4 (C_q), 128.5 (2×CH), 125.2 (C_q), 120.7 (CH), 94.0 (C_q), 84.9 (C_q), 75.7 (CH), 73.6 (2×CH), 71.4 (CH), 70.0 (CH), 69.9 (CH_2), 69.8 (C_q), 68.8 (CH), 60.0 (CH_2), 52.4 (C_q), 50.7 (CH), 42.1 (CH), 31.8 (CH_2), 31.2 (CH_2), 28.8 (CH_2), 22.8

(CH_3), 21.4 (CH_3), 21.6 (CH_3), 21.0 (CH_3), 20.9 (CH_3), 19.9 (CH_3), 18.0 (CH_3), 12.0 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3689, 3468, 3436, 3020, 2985, 1738, 1602, 1467, 1378, 1247, 1044, 737, 733. λ_{max} (EtOH, nm) 269, 229, 203. **Mass Spec**. (ESI) m/z 882.3192 $[M+H]^+$, 904.3019 $[M+Na]^+$, 920.2772 $[M+K]^+$ (Calcd. for $C_{44}H_{52}O_{18}N$ is 882.3184).

Compound (2)

^{1}H-NMR : δ 8.83 (1H, dd, *J* = 4.6, 1.8 Hz) 8.40 (1H, dd, *J* = 7.9, 1.8 Hz), 7.13 (1H, dd, *J* = 7.9, 4.6 Hz), 7.00 (1H, s), 6.00 (1H, d, *J* = 11.7 Hz), 5.15 (1H, d, *J* = 13.5 Hz), 5.55 (1H, d, *J* = 3.9 Hz), 5.55 (1H, m), 5.48 (1H, d, *J* = 5.8 Hz), 5.20 (1H, dd, *J* = 3.9, 2.4 Hz), 4.97 (1H, d, *J* = 2.4 Hz), 4.90 (1H, br s), 4.50 (1H, d, *J* = 13.5 Hz), 4.40 (1H, m), 3.61 (1H, d, *J* = 11.7 Hz), 2.35-2.25 (2H, m), 2.35 (3H, s), 2.12 (1H, m), 2.20-1.95 (2H, m), 2.20 (3H, s), 2.15 (3H, s), 2.15 (3H, s), 1.75-2.00 (2H, m), 1.90 (3H, s), 1.73 (3H, s), 1.68 (3H, s), 1.30 (3H, s), 0.70 (3H, t, *J* = 7.3 Hz).

^{13}C-NMR : δ 172.3 (C_q), 170.3 (C_q), 170.1 (C_q), 170.0 (C_q), 169.1 (C_q), 168.8 (C_q), 168.0 (2×C_q), 166.4 (C_q), 153.8 (CH), 138.2 (CH), 129.6 (C_q), 120.8 (CH), 93.9 (C_q), 84.7 (C_q), 75.7 (CH), 73.6 (CH), 73.4 (CH), 70.8 (CH), 69.9 (CH_2), 69.7 (CH), 69.6 (C_q), 69.0 (CH), 60.0 (CH), 52.1 (C_q), 50.8 (CH), 42.2 (CH), 31.8 (CH_2), 31.3 (CH_2), 28.8 (CH_2), 25.3 (CH_3), 22.9 (CH_3), 21.6 (CH_3), 21.3 (CH_3), 21.0 (CH_3), 20.8 (CH_3), 20.5 (CH_3), 18.0 (CH_3), 11.9 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3617, 3542, 3462, 3029, 2973, 2869, 2257, 2092, 1885, 1740, 1565, 1377, 1033,

920, 845. λ_{max} (EtOH, nm) 269, 206. **Mass Spec.** ((ESI) m/z 820.3035 $[M+H]^+$, 842.2867 $[M+Na]^+$, 858.2614 $[M+K]^+$ (Calcd. for $C_{39}H_{50}O_{18}N$ is 820.3027).

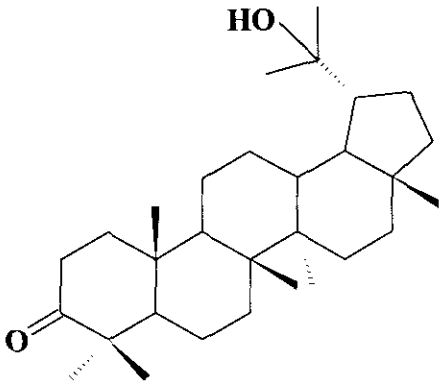
Compound (3)

^{1}H-NMR : δ 9.30 (1H, br s), 8.90 (1H, dd, J = 4.6, 1.8 Hz), 8.90 (1H, br s), 8.50 (1H, dd, J = 7.9, 1.8 Hz), 8.40 (1H, dd), 7.75 (2H, d, J = 7.0 Hz), 7.54 (1H, dd, J_1 = J_2 = 7.6 Hz), 7.50 (1H, m), 7.40 (1H, dd, J = 7.9, 4.6 Hz), 7.30 (2H, dd, J_1 = J_2 = 7.6 Hz), 7.06 (1H, s), 6.05 (1H, d, J = 11.9 Hz), 6.05 (1H, d, J = 4.0 Hz), 5.65 (1H, d, J = 5.8 Hz), 5.55 (1H, m), 5.45 (1H, d, J = 13.4 Hz), 5.20 (1H, dd, J = 4.0, 2.4 Hz), 5.15 (1H, d, J = 2.4 Hz), 4.90 (1H, br s), 4.55 (1H, d, J = 13.4 Hz), 4.40 (1H, m), 3.40 (1H, d, J = 11.9 Hz), 2.35-2.25 (2H, m), 2.34 (3H, s), 2.23 (3H, s), 2.20-1.95 (2H, m), 2.14 (3H, s), 2.12 (1H, m), 2.00-1.75 (2H, m), 1.75 (3H, s), 1.65 (3H, s), 1.35 (3H, s), 0.72 (3H, t, J = 7.3 Hz).

^{13}C-NMR : δ 172.2 (C_q), 170.0 (2×C_q), 168.9 (C_q), 168.4 (C_q), 168.0 (C_q), 166.1 (C_q), 164.6 (C_q), 163.4 (C_q), 153.9 (CH), 153.5 (CH), 150.8 (CH), 138.6 (CH), 137.6 (CH), 133.6 (CH), 129.5 (2×CH), 128.6 (2×CH), 128.6 (C_q), 124.9 (C_q), 124.8 (CH), 123.8 (C_q), 121.0 (CH), 94.0 (C_q), 85.1 (C_q), 75.5 (CH), 73.9 (CH), 73.6 (CH), 71.2 (2×CH), 70.3 (CH_2), 69.9 (C_q), 68.9 (CH), 60.3 (CH_2), 52.4 (C_q), 50.8 (CH), 42.2 (CH), 31.8 (CH_2), 31.2 (CH_2), 28.7 (CH_2), 23.2 (CH_3), 21.7 (CH_3), 21.3 (CH_3), 20.9 (CH_3), 19.9 (CH_3), 18.1 (CH_3), 12.0 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3728, 3448, 3021, 2962, 2927, 1751, 1603, 1567, 1456, 1372, 1235, 1098, 1039, 870, 740. λ_{max} (EtOH, nm) 268, 228, 225, 205. **Mass Spec.** (ESI) m/z 945.3294

$[M+H]^+$, 967.3111 $[M+Na]^+$, 983.2877 $[M+K]^+$ (Calcd. for $C_{48}H_{53}O_{18}N_2$ is 945.3293).

3.6.2 **Isolation of compound 4**



Fraction, $\mathbf{F_5}$ obtained from silica gel flash column chromatography (Figure 3.1, page 208) was eluted with petroleum ether:ethyl acetate:isopropanol (6:3:1) to afford the non-active compound **4** (120.6 mg, 0.04%), a white crystalline solid, m.p. 220-224 °C.

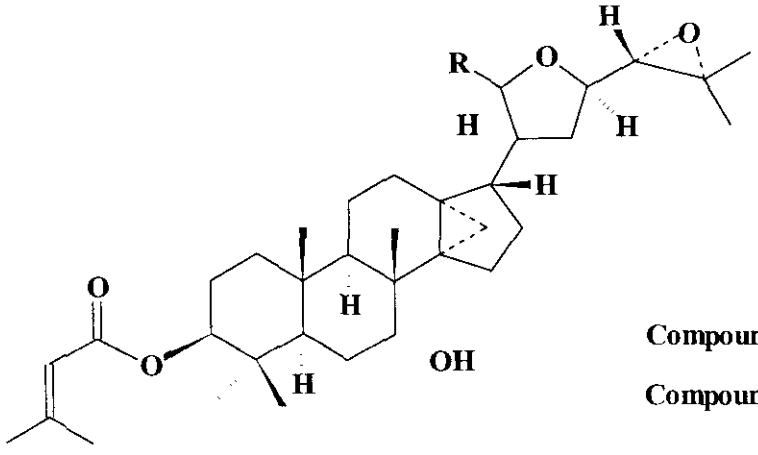
^{13}C-NMR : δ 218.3 (C_q), 73.5 (C_q), 54.8 (CH) 49.9 (CH), 49.6 (CH), 48.2 (CH), 47.3 (C_q), 44.6 (C_q), 43.6 (C_q), 41.3 (C_q), 40.3 (CH_2), 39.6 (CH_2), 37.6 (CH), 36.8 (C_q), 35.5 (CH_2), 34.2 (CH_2), 33.8 (CH_2), 31.6 (CH_3), 29.0 (CH_2), 28.7 (CH_2), 27.5 (CH_2), 26.7 (CH_3), 24.7 (CH_3), 21.9 (CH_2), 19.7 (CH_2), 19.2 (CH_3), 21.1 (CH_3), 16.0 (2×CH_3), 14.8 (CH_3).

m.p. 220-224 °C. ν_{max} ($CHCl_3$, cm^{-1}) 3611, 2929, 2856, 1699, 1468, 1382.

λ_{max} (EtOH, nm) 205. **Mass Spec**. m/z 443 $[M+H]^+$.

3.7 BIO-ASSAY GUIDED EXTRACTION, ISOLATION, SYNTHESIS AND DERIVATISATION OF COMPOUNDS FROM *AEGLE MARMELOS*

3.7.1 Isolation of compounds 6, 7



	R
Compound 6	α-H, β-OH
Compound 7	β-H, α-OH

Air-dried, finely powdered stem bark (365 g) of *Aegle marmelos* was sequentially extracted with petroleum ether (2×1.5 l), ethyl acetate (2×1.5 l) and ethanol (2×1.5 l) using microwave extraction procedure. The combined petroleum ether and ethyl acetate extracts gave a dark yellow insecticidal residue (6.7 g) on evaporation in *vacuo*.

The combined extract (6.7 g) was subjected to dry column flash chromatography (Figure 3.2, page 214) on silica gel by gradient elution with petroleum ether:ethyl acetate (3:2, 1:1, 2:3, 3:7; 2×75 ml, each) and finally ethyl acetate (3×75 ml). The recrystallisation of the active fraction, $\mathbf{F_2}$ (2.79 g, 0.76%) with petroleum ether:ethyl acetate (1:1), yielded the active fraction, $\mathbf{C_1}$, as a white crystalline solid, containing a mixture of compounds (393 mg, 0.11%, m.p. 167-168 °C) in a 2:1 ratio

(Figure 3.2). This insecticidal fraction, C_1 (253.4 mg) was chromatographed on silica gel column (Figure 3.2) eluting with chloroform:isopropanol (49:1, 400 ml).

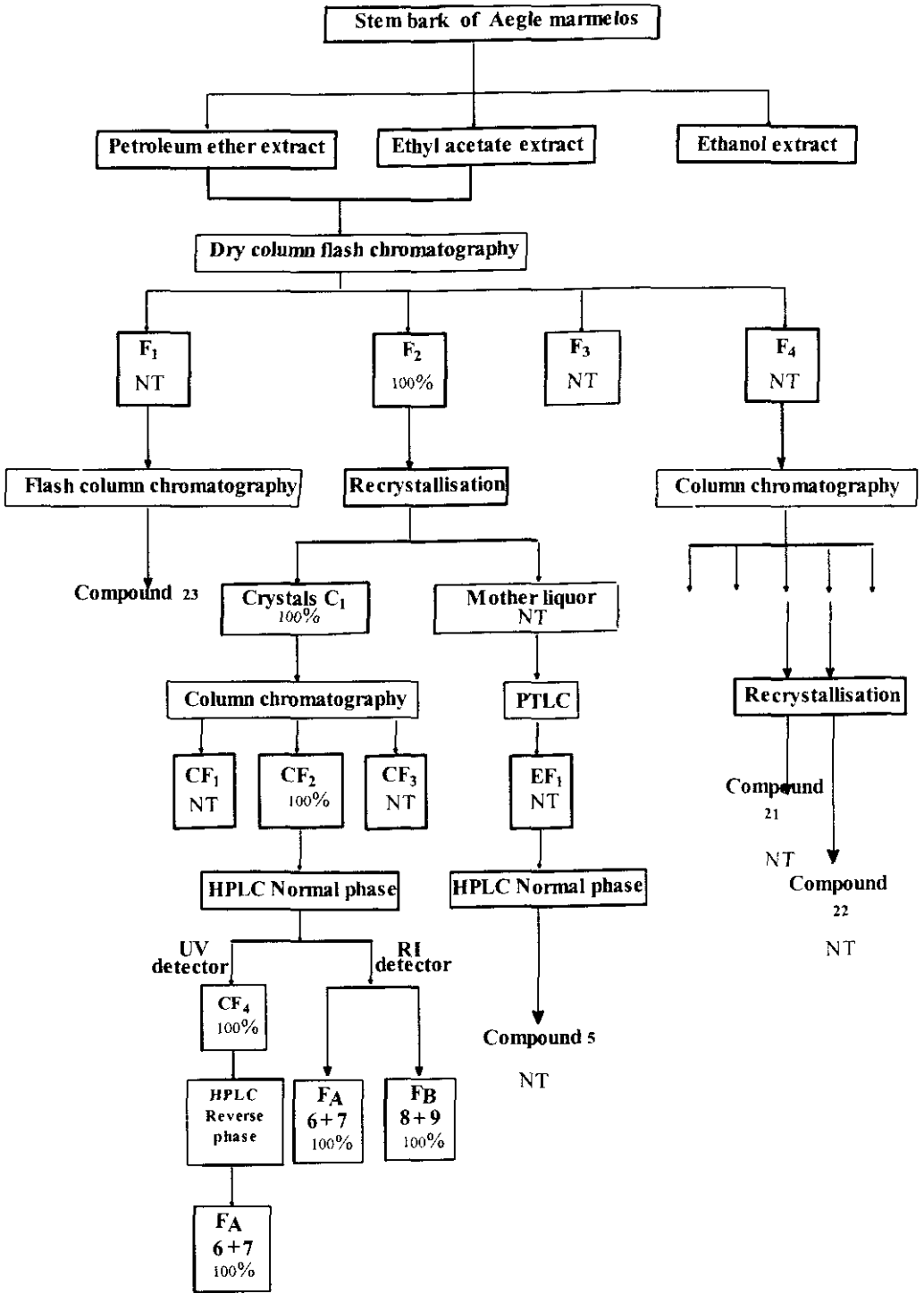


Figure 3.2 : Bio-assay guided fractionation of extracts of *Aegle marmelos*

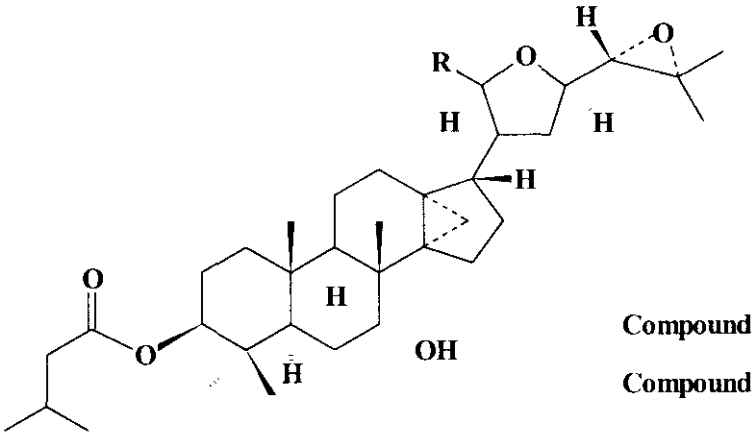
The active fraction, **CF_2** (125.8 mg) was further purified by semi-preparative HPLC (silica column, 2 ml min^{-1}, UV 220 nm) in hexane:THF:isopropanol (73:25:2) to afford the active fraction, **CF_4** (64.5 mg, retention time 12 min) which was further purified by semi-preparative HPLC (C_8 column, 2 ml min^{-1}, UV 220 nm) in acetonitrile:water (9:1) to afford the insecticidal fraction (20.3 mg, 0.006%, retention time 16.5 min), containing compounds, **6** and **7** as a 2:1 mixture.

^{1}H-NMR : δ 5.77 (1H, dd), 5.43 (1H, dd, J = 4.1 Hz), 4.68 (1H, dd), 4.65 (1H, dd, minor), 4.00-3.85 (1H, m), 3.76 (1H, br s), 2.84 (1H, d, J = 7.4 Hz), 2.70 (1H, d, J = 7.7 Hz, minor), 2.55 (1H, s), 2.49 (1H, s), 2.24 (1H, m), 2.20 (1H, m), 2.18 (3H, s), 2.15 (1H, m), 2.15-2.00 (1H, m), 1.92 (1H, m), 1.90 (3H, s), 1.90-1.80 hid (2H, m), 1.70 (2H, m), 1.60 obs (1H, m), 1.65-1.50 obs (2H, m), 1.60-1.50 obs (1H, m), 1.50 obs (2H, m), 1.50-1.40 obs (1H, m), 1.32 hid (2H, m), 1.32 (3H, s), 1.31 (3H, s), 1.21 (1H, m), 1.02 (3H, s), 0.89 (6H, s, H-19), 0.85 (3H, s), 0.80 hid (1H, m), 0.75 (1H, d, J = 4.4 Hz), 0.48 (1H, d, J = 4.4 Hz).

^{13}C-NMR : δ 166.5 (C_q, both), 156.0 (C_q, both), 116.9 (CH, both), 102.3 (CH, minor), 98.4 (CH, major), 78.5 (CH, major), 77.8 (CH, minor), 76.7 (CH, both), 74.3 (CH, both), 67.6 (CH, major), 65.0 (CH, minor), 58.1 (C_q, major), 57.3 (C_q, minor), 51.0 (CH, minor), 49.3 (CH, major), 48.5 (CH, minor), 44.7 (CH, major), 44.0 (CH, major), 43.8 (CH, minor), 41.3 (CH, minor), 41.2 (CH, major), 39.0 (C_q, minor), 38.9 (C_q, major), 37.3 (C_q, minor), 37.2 (C_q, major), 36.9 (C_q, both), 36.2 (C_q, major), 36.1 (C_q, minor), 33.8 (CH_2, major), 33.7 (CH_2, minor), 30.8 (CH_2, major), 30.3 (CH_2, minor), 28.9 (C_q, both), 28.6 (CH_3, minor), 27.7 (CH_3, major),

27.5 (CH_2, both), 27.4 (CH_3, both), 26.3 (CH_2, major), 26.2 (CH_2, minor), 25.8 (CH_2, major), 25.6 (CH_2, minor), 25.0 (CH_3, major), 24.9 (CH_3, minor), 24.1 (CH_2, both), 22.8 (CH_2, both), 21.8 (CH_3, both), 20.3 (CH_3, both), 19.5 (CH_3, major), 19.4 (CH_3, minor), 19.1 (CH_3, both), 16.1 (CH_2, major), 16.0 (CH_2, minor), 15.7 (CH_3, major), 15.6 (CH_3, minor), 13.7 (CH_2, major), 13.5 (CH_2, minor). ν_{max} ($CHCl_3$, cm^{-1}) 3587, 2998, 2925, 2863, 1704, 1456, 1390, 1151, 1071, 1025, 989, 877, 853. λ_{max} (EtOH, nm) 204, 216. **Mass Spec.** (ESI) m/z 592 $[M+Na]^+$.

3.7.2 **Isolation of compounds 8, 9**



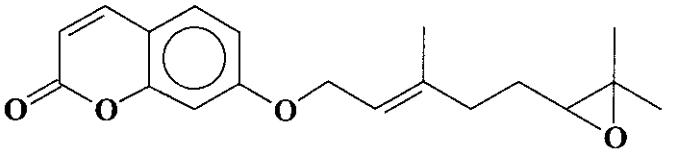
	R
Compound 8	α-H, β-OH
Compound 9	β-H, α-OH

The active fraction, **CF_2** (20 mg) obtained from silica gel column chromatography (Figure 3.2, page 214) was also purified by semipreparative HPLC (silica column, 1 ml min^{-1}) in chloroform:isopropanol (97:3) using an RI detector to afford two insecticidal fractions, first (3.5 mg, 0.006%, retention time 10 min) and second (2.3 mg, 0.004%, retention time 13 min). The former is a mixture of compounds, **6** and **7** and the latter is a mixture of **8** and **9** both in 2:1 ratio.

^{1}H-NMR : δ 5.40 (1H, dd), 4.64 (1H, dd, minor), 4.63 (1H, dd), 4.00-3.82 (1H, m), 3.74 (1H, br s), 2.80 (1H, d), 2.70 (1H, d, minor), 2.44 (1H, s), 2.43 (1H, s, minor), 2.38 (1H, s), 2.25 (1H, m), 2.20 (1H, m), 2.20-2.14 (2H, m), 2.14 (1H, m), 2.15-2.00 (1H, m), 2.00-1.80 (1H, m), 1.94 (1H, m), 1.89-1.80 hid (2H, m), 1.68 (2H, m), 1.60 obs (1H, m), 1.65-1.50 obs (2H, m), 1.60-1.51 obs (1H, m), 1.51 obs (2H, m), 1.51-1.48 obs (1H, m), 1.32 hid (2H, m), 1.32 (3H, s), 1.31 (3H, s), 1.21 obs (1H, m), 1.03 (3H, s), 0.89 (6H, s), 0.85 (3H, s), 0.80 hid (1H, m), 0.75 (1H, d, J = 4.4 Hz), 0.40 (1H, d, J = 4.4 Hz).

^{13}C-NMR : δ 173.0 (C_q, both), 103.0 (CH, minor), 98.2 (CH, major), 78.5 (CH, major), 77.5 (CH, minor), 76.7 (CH, both), 74.4 (CH, both), 67.6 (CH, major), 65.2 (CH, minor), 58.1 (C_q, both), 49.4 (CH, both), 48.3 (CH, minor), 44.8 (CH, major), 44.0 (CH_2, both), 44.0 (CH, major), 43.8 (CH, minor), 41.2 (CH, major), 41.3 (CH, minor), 39.0 (C_q, minor), 38.9 (C_q, major), 37.3 (C_q, minor), 37.2 (C_q, major), 37.0 (C_q, both), 36.2 (C_q, major), 36.1 (C_q, minor), 33.8 (CH_2, major), 33.7 (CH_2, minor), 30.8 (CH_2, both), 29.0 (C_q, both), 27.8 (CH_3, minor), 27.7 (CH_3, major), 27.5 (CH_2, both), 27.4 (CH, both), 26.3 (CH_2, both), 25.9 (CH_2, major), 25.7 (CH_2, minor), 25.6 (CH_3, major), 25.0 (CH_3, minor), 24.2 (CH_2, both), 22.9 (CH_2, both), 22.4 (CH_3, both), 21.9 (CH_3, both), 20.2 (CH_3, both), 19.5 (CH_3, major), 19.4 (CH_3, minor), 19.2 (CH_3, both), 16.1 (CH_2, both), 15.7 (CH_3, major), 15.6 (CH_3, minor), 13.7 (CH_2, both). ν_{max} ($CHCl_3$, cm^{-1}) 3587, 3321, 2860, 1270, 1025, 985, 875. λ_{max} (EtOH, nm) 204. **Mass Spec**. (ESI) m/z 594 $[M+Na]^+$.

3.7.3 **Isolation of compound 5**

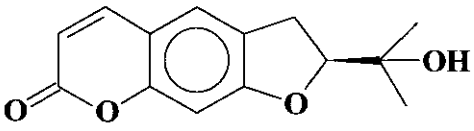


The oily liquid (122 mg) obtained from the mother liquor after recrystallisation of $\mathbf{F_2}$ (Figure 3.2, page 214) was subjected to preparative thin layer chromatography in petroleum ether:ethyl acetate (3:2). The resulted non-active fraction, $\mathbf{EF_1}$ (52.9 mg) was then subjected to semi-preparative HPLC (silica column, 3 ml min^{-1}, UV 254 nm) in hexane:ethyl acetate (2:3), to afforded, the non-active compound **5** as a colourless oil (13 mg, 0.28%, retention time 8 min).

^{1}H-NMR : δ 7.62 (1H, d, *J* = 9.5 Hz), 7.34 (1H, d, *J* = 8.8 Hz), 6.83 (1H, dd, *J* = 8.8, 2.5 Hz), 6.80 (1H, d, *J* = 2.5 Hz), 6.24 (1H, d, *J* = 9.5 Hz), 5.52 (1H, t, *J* = 6.5 Hz), 4.60 (2H, d, *J* = 6.5 Hz), 2.69 (1H, t, *J* = 6.3 Hz), 2.25 (2H, m), 1.78 (3H, s), 1.68 (2H, m), 1.30 (3H, s), 1.27 (3H, s).

^{13}C-NMR : δ 162.0 (C_q), 161.0 (C_q), 155.9 (C_q), 143.3 (CH), 141.3 (C_q), 128.7 (CH), 119.1 (CH), 113.2 (CH), 113.1 (CH), 112.5 (C_q), 101.5 (CH), 65.3 (CH_2), 63.7 (CH), 58.3 (C_q), 36.3 (CH_2), 27.1 (CH_2), 24.8 (CH_3), 18.8 (CH_3) 16.8 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3009, 2981, 2930, 1736, 1505, 1404, 1285, 1226, 1128, 1004, 893. λ_{max} (EtOH, nm) 323, 206. **Mass Spec.** (ESI) m/z 337 $[M+Na]^+$.

3.7.4 **Isolation of compound 21**

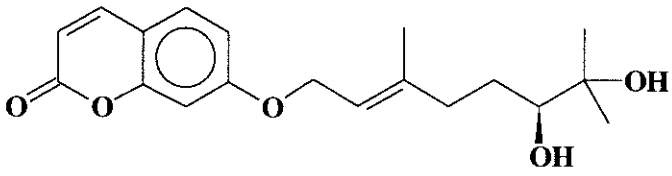


The non-active fraction, $\mathbf{F_4}$ (798.5 mg), obtained from dry column flash chromatography (Figure 3.2, page 214) was further purified on silica gel column (Figure 3.2, page 214) by gradient elution with petroleum ether:ethyl acetate (3:2 and 2:3, 500 and 200 ml, respectively) and finally ethyl acetate (200 ml). The 7th fraction (72.1 mg) which was eluted with petroleum ether:ethyl acetate (3:2) was recrystallised with the same solvent mixture, to afford the non-active compound **21** as a white crystalline solid (14.4 mg, 0.004%, m.p. 189-190 °C).

^{1}H-NMR : δ 7.59 (1H, d, J = 9.5 Hz), 7.22 (1H, s), 6.73 (1H, s), 6.21 (1H, d, J = 9.5 Hz), 4.74 (1H, dd, J = 9.2, 8.6 Hz), 3.22 (2H, ddd), 1.86 (1H, br s), 1.38 (3H, s), 1.24 (3H, s).

^{13}C-NMR : δ 163.2 (C_q), 161.5 (C_q), 155.6 (C_q), 143.7 (CH), 125.1 (C_q), 123.4 (CH), 112.8 (C_q), 112.3(CH), 97.9 (CH), 91.1 (CH), 71.6 (C_q), 29.5 (CH_2), 26.1 (CH_3), 24.3 (CH_3). **m.p.** 189-190 °C. ν_{max} ($CHCl_3$, cm^{-1}) 3618, 3474, 3019, 2939, 2835, 1725, 1626, 1566, 1492, 1393, 1130, 1017. λ_{max} (EtOH, nm) 335, 206. **Mass Spec**. (ESI) m/z 247 $[M+H]^+$, 515 $[2M+Na]^+$, 761 $[3M+Na]^+$.

3.7.5 **Isolation of compound 22**

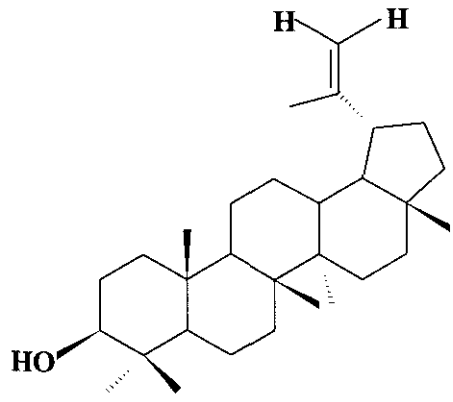


The eighth fraction which was eluted with petroleum ether:ethyl acetate (2:3) from silica gel column (Figure 3.2, page 214) in Section 3.7.4 (page 219) was recrystallised with petroleum ether:ethyl acetate (3:2) and afforded the non-active compound **22** as a white crystalline solid (13.4 mg, 0.004%, m.p. 123-124 °C).

^{1}H-NMR : δ 7.64 (1H, d, J = 9.3 Hz), 7.35 (1H, d, J = 9.5 Hz), 6.80 (1H, m), 6.80 (1H, m), 6.25 (1H, d, J = 9.3 Hz), 5.50 (1H, m), 4.61 (2H, d, J = 6.6 Hz), 3.34 (1H, dd), 2.41-2.34 (4H, m), 2.09 (1H, s), 1.89 (1H, s), 1.78 (3H, s), 1.21 (3H, s), 1.17 (3H, s).

^{13}C-NMR : δ 162.0 (C_q), 161.9 (C_q), 155.6 (C_q), 143.5 (CH), 142.2 (C_q), 128.7 (CH), 118.9 (CH), 113.3 (2×CH), 112.0 (C_q), 101.5 (CH), 77.9 (CH), 73.0 (C_q), 65.4 (CH_2), 36.5 (CH_2), 29.4 (CH_2), 26.5 (CH_3), 23.2 (CH_3), 16.8 (CH_3). **m.p.** 123-124 °C. ν_{max} ($CHCl_3$, cm^{-1}) 3589, 3013, 2934, 2855, 1731, 1610, 1511, 1407, 1279, 1122, 1001, 897, 838. λ_{max} (EtOH, nm) 324, 207. **Mass Spec.** (ESI) m/z 355 $[M+Na]^+$, 687 $[2M+Na]^+$.

3.7.6 **Isolation of compound 23**

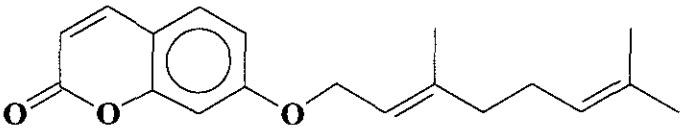


The non-active fraction, $\mathbf{F_1}$ (1.37 g) obtained from dry column flash chromatography (Figure 3.2, page 214) was subjected to flash column chromatography on silica gel in petroleum ether:ethyl acetate (3:2, 400 ml). The 2nd fraction, afforded the non-active compound **23** as a white crystalline solid (7.7 mg, 0.002%, m.p. 196-197 °C).

^{13}C-NMR : δ 151.0 (C_q), 109.3 (CH_2), 79.0 (CH), 55.3 (CH), 50.4 (CH), 48.3 (CH), 48.0 (CH), 43.0 (C_q), 42.8 (C_q), 40.8 (C_q), 40.0 (CH_2), 38.9 (C_q), 38.7 (CH_2), 38.0 (CH), 37.2 (C_q), 35.6 (CH_2), 34.3 (CH_2), 29.9 (CH_2), 28.0 (CH_3), 27.4 (2×CH_2), 25.1 (CH_2), 20.9 (CH_2), 19.3 (CH_3), 18.3 (CH_2), 18.0 (CH_3), 16.1 (CH_3), 16.0 (CH_3), 15.4 (CH_3), 14.6 (CH_3). **m.p.** 196-197 °C. ν_{max} ($CHCl_3$, cm^{-1}) 3631, 3462, 3019, 2944, 2838, 2360, 2342, 1602, 1464, 1333, 1222, 1016. λ_{max} (EtOH, nm) 205. **Mass Spec.** (ESI) m/z 875 $[2M+Na]^+$.

3.7.7 Synthesis of epoxyaurapten

(a) Synthesis of aurapten



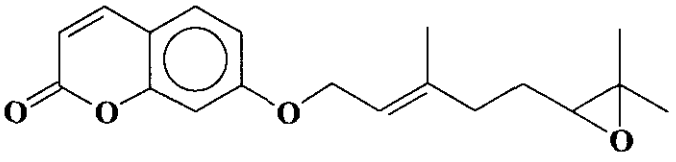
To a stirred solution of 7-hydroxycoumarin (0.53 g, 3.3 mmol), geraniol (0.5 g, 3.2 mmol) and triphenylphosphine (1.7 g, 6.5 mmol) in THF (10 ml) at 0 °C was added Diethyl azodicarboxylate [(DEAD) 0.57 g, 3.2 mmol] dropwise and mixture was warmed to room temperature over 2 h.[175] Evaporation of the solvent in *vacuo*, yielded crude 7-geranyloxycoumarin (2.9 g) which was purified by column chromatography eluting with petroleum ether:ethyl acetate (9:1, 400 ml) to afford 7-geranyloxycoumarin (181.0 mg, m.p. 68-69 °C, Lit. 68 °C) as a white crystalline solid.

^{1}H-NMR : δ 7.64 (1H, d, J = 9.6 Hz), 7.36 (1H, d, J = 8.5 Hz), 6.86 (1H, d, J = 8.5 Hz), 6.82 (1H, s), 6.24 (1H, d, J = 9.6 Hz), 5.47 (1H, t, J = 6.6, 1.1 Hz), 5.08 (1H, sep), 4.60 (2H, d, J = 6.6 Hz), 2.28-2.02 (4H, m),), 1.76 (3H, s), 1.67 (3H, s), 1.60 (3H, s)

^{13}C-NMR : δ 162.1 (C_q), 161.3 (C_q), 155.8 (C_q), 143.5 (CH), 142.4 (C_q), 132.0 (C_q), 128.7 (CH), 123.6 (CH), 118.4 (CH), 113.2 (CH), 112.9 (CH), 112.4 (C_q), 101.6 (CH), 65.5 (CH_2), 39.5 (CH_2), 26.2 (CH_2), 25.7 (CH_3), 17.7 (CH_3), 16.8

(CH_3). **m.p.** 68-69 °C. ν_{max} ($CHCl_3$, cm^{-1}) 3017, 2974, 2929, 2857, 1724, 1614, 1546, 1412, 1229, 1128, 998, 898, 835. λ_{max} (EtOH, nm) 323, 206.

(*b*) **Synthesis of epoxyaurapten**



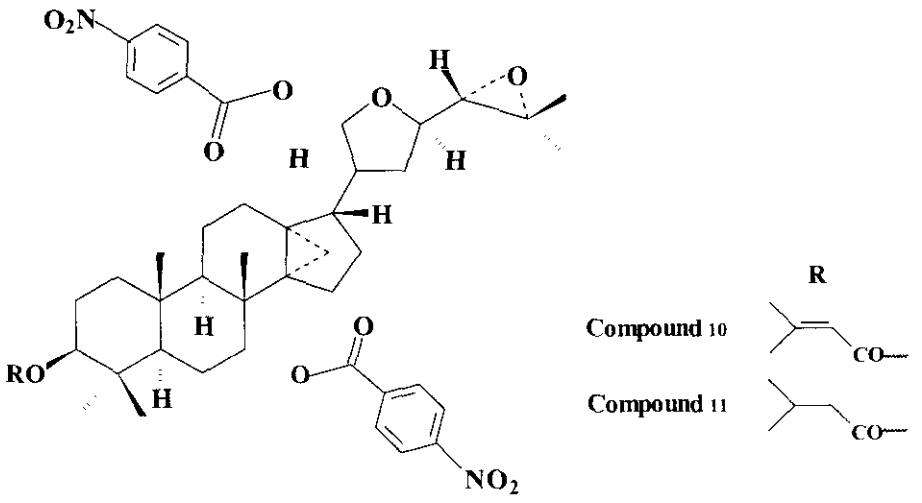
Aurapten (96 mg, 321 mmol) and m-chloroperbenzoic acid [(MCPBA) 111 mg, 641 mmol] was stirred in dichloromethane (5 ml) with sodium bicarbonate (27 mg, 320 mmol) for 20 min at room temperature, then an aqueous solution of sodium sulphate was added, and stirring continued for another 20 min.[177] The organic phase was washed with sodium bicarbonate solution (2×10 ml), saturated sodium chloride (2×10 ml), water (2×10 ml), and dried over anhydrous magnesium sulphate. Evaporation of the solvent in *vacuo* yielded crude epoxyaurapten (92 mg), which was purified by column chromatography on silica gel eluting with petroleum ether:ethyl acetate (7:3, 300 ml) to afford epoxyaurapten (72 mg, 71%) as a colourless oil.

^{1}H-NMR : δ 7.62 (1H, d, J = 9.5 Hz), 7.34 (1H, d, J = 8.8 Hz), 6.83 (1H, dd, J = 8.8, 2.5 Hz), 6.80 (1H, d, J = 2.5 Hz), 6.24 (1H, d, J = 9.5 Hz), 5.52 (1H, t, J = 6.5 Hz), 4.60 (2H, d, J = 6.5 Hz), 2.69 (1H, t, J = 6.3 Hz), 2.25 (2H, m), 1.78 (3H, s), 1.68 (2H, m), 1.30 (3H, s), 1.27 (3H, s).

^{13}C-NMR : δ 162.0 (C_q), 161.0 (C_q), 155.7 (C_q), 143.3 (CH), 141.3 (C_q), 128.7 (CH), 119.1 (CH), 113.2 (CH), 113.1 (CH), 112.5 (C_q), 101.5 (CH), 65.3 (CH_2), 63.7 (CH), 58.3 (C_q), 36.3 (CH_2), 27.1 (CH_2), 24.8 (CH_3), 18.8 (CH_3), 16.8 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3009, 2981, 2930, 1736, 1605, 1404, 1285, 1226, 1128, 1004, 893. λ_{max} (EtOH, nm) 323, 206. **Mass Spec.** (ESI) m/z 337 $[M+Na]^+$.

3.7.8 **Derivatisation studies**

(a) **p-Nitrobenzoylation**



To a solution of fraction $\mathbf{C_1}$ [(264 mg) Figure 3.2, page 214] in pyridine (10 ml) was added p-nitrobenzoyl chloride (775 mg) and 4-N, N dimethyl aminopyridine (100 mg) and stirred for 3 h at room temperature.[178] The reaction mixture was diluted with dichloromethane (30 ml) and washed with 5% sodium carbonate solution (3×30 ml). The combined dichloromethane extract was washed with 2M hydrochloric acid (3×30 ml), saturated sodium chloride (3×30 ml), water (3×30 ml), and dried over anhydrous sodium sulphate. Filtration and evaporation of the solvent in *vacuo* gave a colourless semi crystalline solid (388 mg).

The crystalline solid (388 mg) was purified by column chromatography eluting with petroleum ether:ethyl acetate (4:1 and 1:1, 300 and 200 ml, respectively) to afford a non-insecticidal crystalline solid (208 mg). This (127 mg) was subjected to preparative HPLC (silica column, 8 ml min^{-1}, UV 254 nm) eluting with

hexane:ethyl acetate:isopropanol (79:20:1), afforded non-insecticidal compounds **10** (46.8 mg, retention time 16.5 min) and **11** [(25 mg, retention time 18 min).

Compound (10)

^{1}H-NMR : δ 8.38-8.17 (4H, m), 8.21-8.17 (4H, m), 6.55 (1H, d, J = 2.5 Hz), 5.60 (1H, s), 5.31 (1H, br s), 4.68 (1H, s), 4.01-3.95 (1H, qd, J = 9.8, 7.3, 2.7 Hz), 2.77 (1H, d, J = 7.3 Hz), 2.48-2.41 (1H, qd, J = 6.1, 5.4, 2.5 Hz), 2.17 hid (1H, m), 2.15 (3H, s), 2.15-2.18 hid (1H, m), 2.05-2.01 (1H, dq, J = 8.9, 2.4 Hz), 1.95 (3H, s), 1.90 hid (2H, m), 1.80 (1H, m), 1.70 (2H, m), 1.69 (1H, m), 1.62 (1H, m), 1.56 (1H, m), 1.55 (1H, m), 1.52 (1H, m), 1.49 (1H, m), 1.41 hid (1H, m), 1.32 (2H, m), 1.32 (3H, s), 1.29 (3H, s), 1.20 (3H, s), 1.21 hid (1H, m), 0.98 (3H, s), 0.90 (3H, s), 0.89 (1H, m), 0.67 (3H, s), 0.67 (1H, d, J = 5.5 Hz), 0.25 (1H, d, J = 5.5 Hz).

^{13}C-NMR : δ 165.8 (C_q), 164.0 (C_q), 163.4 (C_q), 157.0 (C_q), 150.6 (C_q), 150.4 (C_q), 136.7 (C_q), 135.5 (C_q), 130.8 (2×CH), 130.6 (2×CH), 123.6 (4×CH), 116.0 (CH), 102.9 (CH), 79.8 (CH), 78.1 (CH), 76.7 (CH), 64.7 (CH), 49.2 (CH), 57.2 (C_q), 48.2 (CH), 45.6 (CH), 42.8 (CH), 38.5 (C_q), 37.3 (C_q), 36.9 (C_q), 36.3 (C_q), 34.3 (CH_2), 32.0 (CH_2), 28.7 (C_q), 27.5 (CH_3), 27.7 (CH_3), 26.4 (CH_2), 26.2 (CH_2), 26.1 (CH_2), 24.9 (CH_3), 23.3 (CH_2), 22.7 (CH_2), 21.5 (CH_3), 20.2 (CH_3), 19.7 (CH_3), 19.5 (CH_3), 16.6 (CH_2), 15.8 (CH_3), 15.1 (CH_2). **m.p.** 169-170 °C.

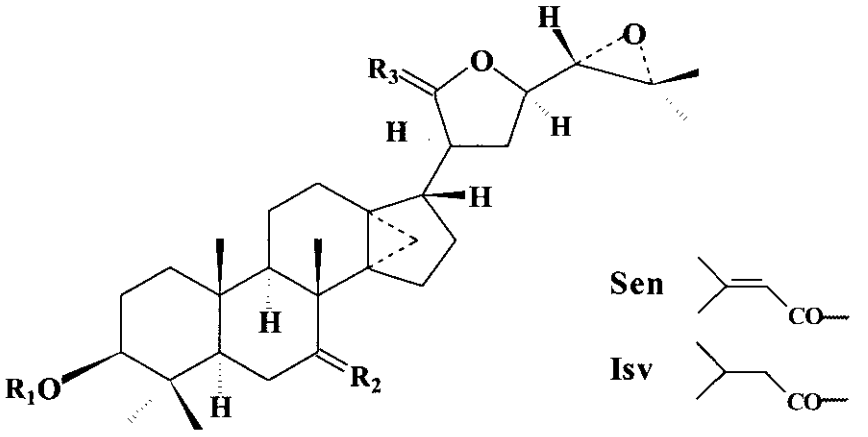
ν_{max} ($CHCl_3$, cm^{-1}) 2958, 2929, 2854, 1722, 1607, 1529, 1459, 1379, 1349, 1281, 1232, 1150, 1119, 1102, 1074, 1014, 988, 907, 875, 858, 794. λ_{max} (EtOH, nm) 259, 216, 204. **Mass Spec.** (ESI) m/z 907 $[M+K]^+$.

Compound (11)

^{1}H-NMR : δ 8.33-8.16 (8H, m), 6.56 (1H, d, J = 2.5 Hz), 5.30 (1H, br s), 4.67 (1H, s), 4.02-3.97 (1H, qd, J = 13.1, 9.3, 3.9 Hz), 2.76 (1H, d, J = 7.7 Hz), 2.48-2.40 (1H, qd, J = 6.3 Hz), 2.20-2.12 (2H, m), 2.20-2.10 (1H, m), 2.12 (1H, m), 2.10-2.00 (1H, m), 2.00-1.95 (1H, m), 1.90-1.80 (2H, m), 1.95-1.52 (2H, m), 1.75-1.70 (2H, m), 1.89 (1H, m), 1.62 (1H, m), 1.58 (1H, m), 1.52 (1H, m), 1.50 (1H, m), 1.50-1.49 (1H, m), 1.50-1.41 (2H, m), 1.48-1.36 hid (2H, m), 1.32 (3H, s), 1.30 (3H, s), 1.20 (3H, s), 0.98 (3H, s), 0.92 (3H, d), 0.91 (3H, d), 0.90 (3H, s), 0.67 (3H, s), 0.70 (1H, d, J = 5.7 Hz), 0.26 (1H, d, J = 5.7 Hz).

^{13}C-NMR : δ 172.2 (C_q), 164.0 (C_q), 163.4 (C_q), 150.6 (C_q), 150.4 (C_q), 136.5 (C_q), 135.5 (C_q), 130.8 (2×CH), 130.5 (2×CH), 123.6 (4×CH), 103.0 (CH), 79.8 (CH), 78.1 (CH), 77.6 (CH), 64.7 (CH), 57.2 (C_q), 49.1 (CH), 48.1 (CH), 45.6 (CH), 44.1 (CH_2), 42.9 (CH), 38.5 (C_q), 37.3 (C_q), 36.8 (C_q), 36.2 (C_q), 34.2 (CH_2), 31.8 (CH_2), 28.7 (C_q), 27.9 (CH_3), 26.3 (CH_2), 26.0 (2×CH_2), 25.7 (CH), 24.9 (CH_3), 23.2 (CH_2), 22.7 (CH_2), 22.6 (CH_3), 22.5 (CH_3), 21.5 (CH_3), 19.7 (CH_3), 19.5 (CH_3), 16.6 (CH_2), 15.7 (CH_3), 15.1 (CH_2). **m.p.** 166-167. ν_{max} ($CHCl_3$, cm^{-1}) 3019, 2963, 2872, 2356, 2336, 1720, 1534, 1353, 1284, 1122, 1102, 1015, 987, 911, 875. λ_{max} (EtOH, nm) 260, 204.

(b) **Acetylation**



Compound	R_1	R_2	R_3
12	Sen	β-H, α-OH	α-H, β-OAc
13	Sen	β-H, α-OH	β-H, α-OAc
14	Sen	β-H, α-OAc	β-H, β-OAc
15	Sen	β-H, α-OAc	α-H, α-OAc
16	Isv	β-H, α-OAc	α-H, β-OAc
17	Isv	β-H, α-OAc	β-H, α-OAc

The fraction, $\mathbf{C_1}$ [(50 mg) Figure 3.2, page 214] in pyridine (1 ml) was stirred with acetic anhydride (3 ml) for 18 h at room temperature.[179] The reaction mixture was diluted with water (10 ml) and extracted with diethyl ether (3×10 ml). The combined ethereal extracts were washed with hydrochloric acid (2M; 3×30 ml), saturated sodium chloride (3×30 ml), water (3×30 ml), and dried over anhydrous sodium sulphate. Filtration and evaporation of the solvent in *vacuo* gave a colourless insecticidal oily liquid (130.5 mg) which was purified by column chromatography eluting with petroleum ether:diethyl ether (1:1, 300 ml) to afford the active fraction as a crystalline solid (83 mg). This (83 mg) was subjected to

semi-preparative HPLC (silica column, 2ml min^{-1}, UV 220 nm) eluting with hexane:diethyl ether:isopropanol (64:35:1), to afford the insecticidally active compounds, **14** (12.4 mg, retention time 23 min), **13** (3.8 mg, retention time 27 min), **12** (11.0 mg, retention time 36 min) as colourless oily liquids and a non-active fraction (34.9 mg, retention time 20 min) containing a mixture of compounds **15**, **16** and **17** as a white crystalline solid.

Compound (12)

^{1}H-NMR : δ 6.30 (1H, d, *J* = 3.3 Hz), 5.70 (1H, s), 4.70 (1H, dd, *J* = 5.2, 2.6 Hz), 3.90 (1H, qd, *J* = 7.7, 6.6 Hz), 3.70 (1H, br s), 2.60 (1H, d, *J* = 7.7 Hz), 2.40 (1H, s), 2.17 (3H, s), 2.11-2.00 (1H, m), 2.08 (3H, s), 2.07-1.92 (1H, m), 2.07 hid (1H, m), 2.00-1.95 (1H, dd, *J* = 7.5 Hz), 1.90 (3H, s), 1.65-1.55 (2H, m), 1.60 (4H, m), 1.30 hid (2H, m), 1.32 (3H, s), 1.27 (3H, s), 1.22-1.21 hid (1H, m), 1.19 (1H, m) 1.02 (3H, s), 1.02 hid (1H, m), 0.95 (1H, m), 0.97 (1H, m), 0.90 (6H, s), 0.87 hid (2H, m), 0.84 (3H, s), 0.74 (1H, d, *J* = 4.9 Hz), 0.40 (1H, d, *J* = 4.9 Hz).

^{13}C-NMR : δ 170.0 (C_q), 165.5 (C_q), 155.8 (C_q), 116.9 (CH), 97.4 (CH), 79.8 (CH), 76.9 (CH), 74.1 (CH), 66.7 (CH), 57.1 (C_q), 48.0 (CH), 44.8 (CH), 43.7 (CH), 41.4 (CH), 39.1 (C_q), 37.3 (C_q), 36.7 (C_q), 36.2 (C_q), 33.7 (CH_2), 30.7 (CH_2), 29.0 (C_q), 27.7 (CH_3), 27.5 (CH_2), 27.4 (CH_3), 26.2 (CH_2), 25.1 (CH_2), 24.9 (CH_3), 24.2 (CH_2), 22.8 (CH_2), 21.8 (CH_3), 21.6 (CH_3), 20.3 (CH_3), 19.4 (CH_3), 19.3 (CH_3), 16.0 (CH_2), 15.6 (CH_3), 13.2 (CH_2). ν_{max} ($CHCl_3$, cm^{-1}) 3007, 2950, 2872, 2362, 2339, 1741, 1702, 1646, 1463, 1388, 1152, 1091, 1027, 1002, 949, 884. λ_{max}

(EtOH, nm) 206, 216. **Mass Spec.** (ESI) m/z 635.3905 $[M+Na]^+$, 651.3624 $[M+K]^+$ (Calcd. for $C_{37}H_{56}O_7Na$ is 635.3924).

Compound (13)

^{1}H-NMR : δ 6.26 (1H, d, *J* = 3.0 Hz), 5.76 (1H, dd, *J* = 1.4, 1.1 Hz), 4.68 (1H, dd), 3.90 (1H, qd), 3.76 (1H, br s), 2.75 (1H, d, *J* = 7.4 Hz), 2.44 (1H, s), 2.33 (1H, qd), 2.18 (3H, d, *J* = 1.1 Hz), 2.07-1.92 (1H, m), 2.06 (3H, s), 2.07-1.92 (1H, m), 2.00-1.92 (1H, m), 1.90 (3H, d, *J* = 1.1 Hz), 1.66 hid (1H, m), 1.60-1.55 hid (1H, m), 1.60 (5H, m), 1.33 (3H, s), 1.32 hid (1H, m), 1.31 (3H, s), 1.27 hid (1H, m), 1.27-1.21 hid (1H, m), 1.19 hid (1H, m), 1.04 (3H, s), 1.02 (1H, m), 0.95 hid (1H, m), 0.97 hid (1H, m), 0.90 (3H, s), 0.90 hid (1H, m), 0.89 (3H, s), 0.87 hid (1H, m), 0.85 (3H, s), 0.77 (1H, d, *J* = 4.9 Hz), 0.49 (1H, d, *J* = 4.9 Hz).

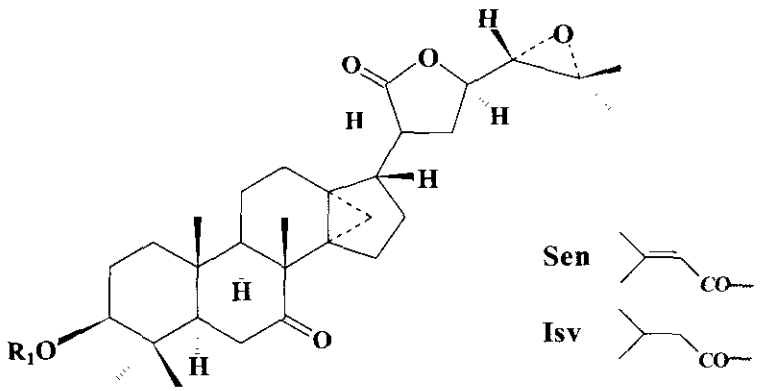
^{13}C-NMR : δ 170.5 (C_q), 166.4 (C_q), 155.8 (C_q), 116.9 (CH), 101.1 (CH), 79.1 (CH), 76.8 (CH), 74.2 (CH), 64.7 (CH), 57.0 (C_q), 48.5 (CH), 48.0 (CH), 43.7 (CH), 41.1 (CH), 38.9 (C_q), 37.2 (C_q), 36.4 (C_q), 36.2 (C_q), 33.7 (CH_2), 32.1 (CH_2), 28.3 (C_q), 27.6 (CH_3), 27.4 (CH_3), 25.8 ($2\times CH_2$), 25.7 (CH_2), 24.8 (CH_3), 24.1 (CH_2), 22.6 (CH_2), 21.7 (CH_3), 21.4 (CH_3), 20.1 (CH_3), 19.4 ($2\times CH_3$), 16.0 (CH_2), 15.6 (CH_3), 13.8 (CH_2). ν_{max} ($CHCl_3$, cm^{-1}) 3031, 2927, 2850, 2360, 1731, 1556, 1459, 1253, 1043, 910. λ_{max} (EtOH, nm) 215. **Mass Spec.** (ESI) m/z 635.3889 $[M+Na]^+$, 651.3608 $[M+K]^+$ (Calcd. for $C_{37}H_{56}O_7Na$ is 635.3884).

Compound (14)

^{1}H-NMR : δ 6.26 (1H, d, J = 3.3 Hz), 5.70 (1H, s), 4.98 (1H, s), 4.70 (1H, s), 3.87 (1H, qd, J = 9.6, 7.7 Hz), 2.67 (1H, d, J = 7.7 Hz), 2.10 hid (1H, m), 2.09 (3H, s), 2.08 hid (1H, m), 2.20 (3H, s), 2.10-2.00 (1H, m), 2.00-1.80 (1H, m), 2.04 (3H, s), 1.93 (3H, s), 1.79-1.64 hid (1H, m), 1.60 (5H, m), 1.55 (2H, m), 1.32 hid (2H, m), 1.32 (3H, s), 1.27-1.21 hid (1H, m), 1.27 (3H, s), 1.19 (1H, m), 1.08 (3H, s), 0.97 (1H, m), 0.95 (1H, m), 0.90 (3H, s), 0.87 hid (3H, s), 0.87 (1H, m), 0.75 (3H, s), 0.71 (1H, d, J = 5.5 Hz), 0.31 (1H, d, J = 5.5 Hz).

^{13}C-NMR : δ 170.2 (C_q), 170.0 (C_q), 166.2 (C_q), 155.6 (C_q), 116.9 (CH), 97.5 (CH), 79.8 (CH), 76.9 (CH), 75.9 (CH), 66.6 (CH), 57.1 (C_q), 48.0 (CH), 44.9 (CH), 44.6 (CH), 42.4 (CH), 38.2 (C_q), 37.0 (C_q), 37.0 (C_q), 36.1 (C_q), 33.8 (CH_2), 30.6 (CH_2), 29.0 (C_q), 27.6 (CH_2, 2×CH_3), 26.5 (CH_2), 25.6 (CH_2), 24.9 (CH_3), 23.1 (CH_2), 22.6 (CH_2), 21.5 (2×CH_3), 21.4 (CH_3), 20.4 (CH_3), 19.5 (CH_3), 19.3 (CH_3), 16.5 (CH_2), 15.7 (CH_3), 14.4 (CH_2). ν_{max} ($CHCl_3$, cm^{-1}) 3019, 2960, 2874, 1744, 1447, 1374, 1238, 1150, 1027, 951, 907, 880, 813. λ_{max} (EtOH, nm) 216. **Mass Spec.** (ESI) m/z 677.4009 $[M+Na]^+$, 693.3741 $[M+K]^+$ (Calcd. for $C_{39}H_{58}O_8Na$ is 677.4029).

(c) **Buffered PCC oxidation**



Compound	R_1
18	Sen
19	Isv

To a slurry of *pyridinium chlorochromate* (133 mg, 0.61 mmol), anhydrous potassium acetate (2 mg) and powdered 4 Å molecular sieves (50 mg) stirred in dry dichloromethane (10 ml) under nitrogen at room temperature, was slowly added a solution of fraction, $\mathbf{C_1}$ [(50 mg) Figure 3.2, page 214] in dichloromethane (2 ml). [180] The mixture was stirred for 2.5 h before being filtered through silica gel, eluting with diethyl ether. Evaporation of the solvents in *vacuo* yielded a colourless insecticidal oil (174 mg).

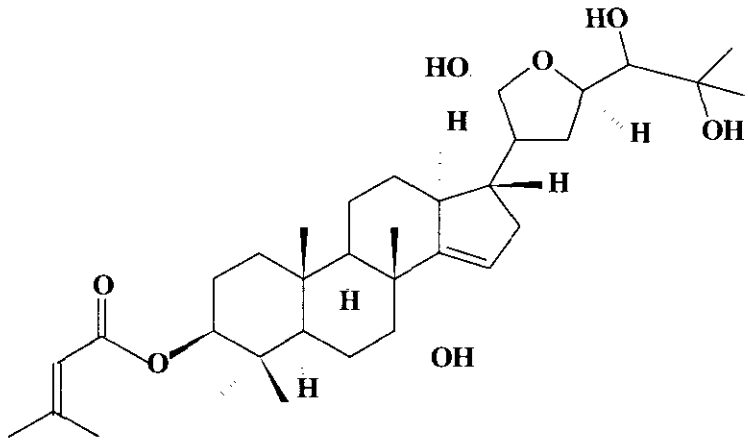
This was purified by silica gel column chromatography in petroleum ether:ethyl acetate (4:1 and 1:1, 300 ml and 200 ml, respectively). The active fraction (59.1 mg) was subjected to preparative tlc (petroleum ether:ethyl acetate, 3:2) and the active fraction (25.3 mg) was further purified by preparative HPLC (silica column, 10 ml min^{-1}, UV 220 nm) in hexane:diethyl ether:isopropanol (64:35:1), to afford,

insecticidal fraction (12.8 mg, retention time 30 min), as a colourless liquid containing compounds, **18** and **19** as a 2:1 mixture.

^{1}H-NMR : δ 5.66 (1H, s), 4.71 (1H, d, *J* = 9.6 Hz), 4.15 (1H, sep), 2.90 (1H, sep), 2.82 (1H, d, J = 7.4 Hz), 2.56 (1H, m), 2.47 (1H, m), 2.20-2.12 (2H, m, minor), 2.10-2.00 (1H, m, minor), 2.17 (3H, s), 2.17-2.00 obs (1H, m), 2.17-2.16 (1H, m), 2.00-1.90 (3H, m), 1.92 (1H, m), 1.90 (1H, m), 1.90 (3H, s), 1.65 (1H, m), 1.60 (2H, m), 1.60 obs (2H, m), 1.38 (3H, s), 1.35 (3H, s), 1.32 obs (2H, m), 1.32 (3H, s), 1.12 obs (1H, m), 1.08 hid (1H, m), 1.08 (3H, s), 0.97 (3H, s), 0.95 (3H, d, minor), 0.93 (3H, d, minor), 0.84 (1H, m), 0.84 (3H, s), 0.58 (H, d, J = 5.5 Hz), 0.44 (H, d, J = 5.5 Hz).

^{13}C-NMR : δ 215.1 (C_q, both), 178.1 (C_q, both), 172.5 (C_q, minor), 166.0 (C_q, major), 156.7 (C_q, major), 116.3 (CH, major), 78.5 (C-23, both), 77.2 (CH, major), 76.4 (CH, minor), 64.3 (CH, both), 57.3 (C_q, both), 51.8 (CH, both), 50.4 (CH, both), 50.2 (C_q, both), 44.7 (CH, both), 43.8 (CH_2, minor), 40.8 (CH, both), 37.3 (C_q, minor), 37.2 (C_q, both), 37.0 (C_q, major), 35.6 (CH_2, both), 34.6 (CH_2, both), 33.8 (C_q, both), 28.7 (C_q, both), 28.3 (CH_2, both), 27.5 (CH_3, major), 27.4 (CH_3, both), 27.0 (CH_2, both), 26.5 (CH_2, both), 25.8 (CH, minor), 24.8 (CH_3, both), 22.7 (CH_2, major), 22.6 (CH_2, minor), 22.5 (CH_3, minor), 22.4 (CH_3, minor), 21.4 (CH_2, major), 21.3 (CH_2, minor), 21.1 (CH_3, both), 20.3 (CH_3, major), 19.5 (CH_3 ,both), 18.8 (CH_3, both), 16.8 (CH_2, both), 16.0 (CH_3, both), 15.1 (CH_2, both). ν_{max} ($CHCl_3$, cm^{-1}) 3023, 3006, 2964, 2881, 1774, 1708, 1655, 1460, 1388, 1279, 1150, 1026, 995, 979, 907, 852, 814. λ_{max} (EtOH, nm) 216.

(d) **Acid hydrolysis**



The fraction, $\mathbf{C_1}$ [(100 mg) Figure 3.2, page 214] in methanolic hydrochloric acid (40 ml MeOH, 20 ml HCl, 10%) was refluxed for 6 h.[181] The reaction mixture was concentrated in *vacuo*, diluted with water (20 ml) and extracted with chloroform (3×20 ml). The combined chloroform extracts were evaporated in *vacuo*, yielding a yellow crystalline solid (95.5 mg). The solid was purified by silica column chromatography eluting with hexane:diethyl ether:isopropanol (64:35:1, 400 ml). The major fraction (19.9 mg) was subjected to semi-preparative HPLC (C_{18} column, 2 ml min^{-1}, UV 220 nm) eluting with acetonitrile:water (9:1), to afford mainly the compound **20** as a colourless oil (4.7 mg, 0.004%, retention time 15 min).

Compound (20)

^{1}H-NMR : δ 5.76 (1H, s), 5.46 (1H, dd), 4.82 (1H, m), 4.70 (1H, dd), 4.30 obs (1H, m), 4.12 (1H, m), 3.92 (1H, s), 3.50 (1H, s), 3.36 (1H, s), 3.22 obs (1H, s), 2.40-2.30 (1H, m), 2.30 obs (1H, s), 2.12-2.15 (1H, m), 2.10-2.00 (1H, m), 2.05

(3H, s), 1.95 obs (1H, m), 1.89 (3H, s), 1.92 (1H, m), 1.70 (2H, m), 1.65 (1H, m), 1.60-1.40 obs (2H, m), 1.60 obs (1H, m), 1.55 (1H, m), 1.50 (1H, m), 1.40-1.30 hid (2H, m), 1.30 hid (1H, m), 1.28 (3H, s), 1.26 (3H, s), 1.25 hid (1H, m), 1.08 (3H, s), 1.06 (3H, s), 0.92 (3H, s), 0.91 (3H, s), 0.85 (3H, s), 0.80 hid (1H, m).

^{13}C-NMR : δ 166.6 (C_q), 162.4 (C_q), 155.8 (C_q), 119.3 (CH), 117.0 (CH), 109.6 (CH), 77.8 (CH) 76.5 (CH), 72.2 (CH), 60.4 (CH), 57.5 (C_q), 55.7 (CH), 47.0 (CH), 45.9 (C_q), 44.4 (C_q), 43.8 (CH), 41.6 (CH), 37.5 (C_q), 36.2 (C_q), 34.7 (CH_2), 33.7 (CH_2), 33.3 (CH_2), 29.7 (CH_2), 27.8 (CH_3), 27.7 (CH_3), 27.4 (CH_3), 26.5 (CH_2), 26.4 (CH_3), 23.6 (CH_2), 22.4 (CH_3), 21.8 (CH_3), 20.3 (CH_3), 19.4 (CH_3), 16.2 (CH_2), 15.2 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3618, 2971, 2931, 1709, 1452, 1388, 1153, 1086, 1032, 950, 879, 818. λ_{max} (EtOH, nm) 205, 216.

(e) **Base hydrolysis**

To a stirred solution of the fraction, $\mathbf{C_1}$ [(70 mg) Figure 3.2, page 214], in methanol:THF (10 ml, 1:1) at room temperature was added a solution of potassium hydroxide (630 mg) in water, during 20 min.[182] The reaction mixture was stirred overnight at room temperature and poured into a mixture of diethyl ether (15 ml) and water (35 ml). After acidification to pH 4-5 with hydrochloric acid (2M; 12 ml) the aqueous phase was separated and extracted further with diethyl ether (2×30 ml). The combined organic phase was washed with saturated sodium chloride (2×30 ml), water (2×30 ml), and dried over anhydrous sodium sulphate. Filtration and evaporation of the solvent in *vacuo*, afforded a colourless liquid (68 mg).

This (68 mg) was subjected to dry column flash chromatography eluting with a gradient solvent system of petroleum ether:ethyl acetate (1:1 and 2:3, 4×15 ml, each) and finally ethyl acetate (3×15 ml) to afford mainly the starting material. The nonstarting-material fraction (12 mg) was further purified by semi-preparative HPLC (silica column, 3 ml min^{-1}, UV 220 nm) eluting with hexane:diethyl ether:isopropanol (24:25:1) to afford a mixture (6.7 mg, retention time 11 min) which could not be characterised.

(f) **Methylation**

Method 1

The fraction, $\mathbf{C_1}$ [(50 mg) Figure 3.2, page 214], a large excess of dimethyl sulphate (6.82 μl, 500 mg) and dry potassium carbonate (200 mg) were refluxed in dry acetone (4.5 ml) for 72 h.[183] The reaction mixture was diluted with water (5 ml) and extracted with diethyl ether (3×10 ml). The ether extract was washed with saturated sodium chloride (2×10 ml), water (2×10 ml), dried over anhydrous sodium sulphate and evaporated *in vacuo* to give a oily liquid (62.0 mg). The oily liquid was purified by column chromatography eluting with petroleum ether:ethyl acetate (3:2, 200 ml), to afford mainly unreacted starting material which showed identical ^{1}H and ^{13}C NMR spectra with those of $\mathbf{C_1}$ and the tlc analysis indicated similar Rf values of both.

Method 2

The fraction,$\mathbf{C_1}$ [(50 mg) Figure 3.2, page 214], and freshly prepared silver (I) oxide (200 mg) were dissolved in methyl iodide (25 ml). The reaction mixture was stirred at room temperature in the dark for 72 h with periodic sonication.[184] The mixture was filtered through celite using dichloromethane and evaporated in *vacuo* to afford mainly unreacted starting material. The NMR and tlc analysis showed that the product was identical with $\mathbf{C_1}$.

(g) ***Silylation***

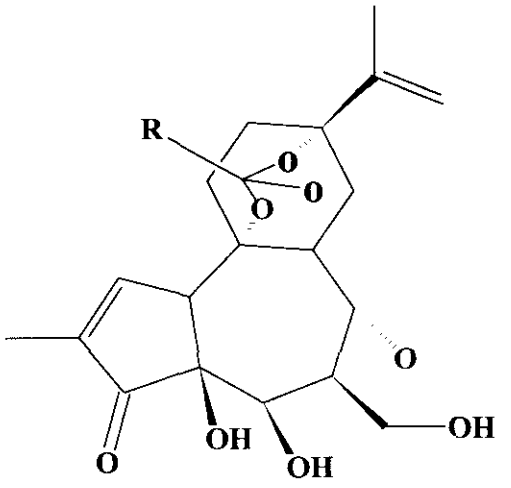
To a stirred solution of t-butyldimethylsilyl chloride (74 mg, 6 mmol), and imidazole (44.64 mg, 8 mmol) in dry DMF (4 ml) at room temperature under nitrogen was added fraction $\mathbf{C_1}$ [(50 mg) Figure 3.2, page 214], dissolved in DMF (1 ml).[185] The resulting solution was stirred at room temperature for 48 h. The reaction mixture was diluted with water (30 ml) and extracted with diethyl ether (3×30 ml). The combined ether extract was washed successively with hydrochloric acid (2M; 3×30 ml), saturated sodium chloride (3×30 ml), water (3×30 ml), and dried over anhydrous sodium sulphate. Filtration and evaporation of the solvent in *vacuo* gave a colourless oil. The oil was purified by silica gel column chromatography eluting with petroleum ether:ethyl acetate (3:2, 200 ml), to afford, mainly unreacted starting material which showed similar NMR spectra to those of $\mathbf{C_1}$.

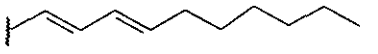
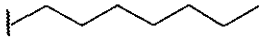
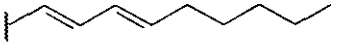
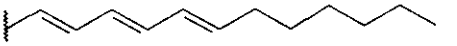
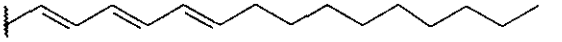
(h) **Reduction**

To a stirred solution of lithium aluminium hydride (20 mg) in dry diethyl ether (10 ml) was added, dropwise, the fraction $\mathbf{C_1}$ [(29 mg) Figure 3.2, page 214] in diethyl ether (5 ml) at 0 °C under nitrogen.[186] The reaction mixture was stirred at room temperature for 5 h with frequent tlc monitoring. The mixture was treated by successive dropwise addition of water (0.02 ml), 15% sodium hydroxide solution (0.02 ml) followed by water (0.06 ml). The white precipitate which formed was filtered under vacuum and washed with diethyl ether. The filtrate was concentrated in *vacuo* affording the oily liquid (27 mg) and this was purified by dry column flash chromatography eluting with a gradient solvent system of petroleum ether:ethyl acetate (1:1, 2:3, 3:7, 1:4; 4×5 ml, 2×5 ml, 2×5 ml, 2×5 ml, respectively) and finally ethyl acetate (4×5 ml), to afford a mixture containing mostly the unreacted starting material. The ^{1}H NMR showed identical peaks to those of $\mathbf{C_1}$.

3.8 BIO-ASSAY GUIDED EXTRACTION AND ISOLATION OF COMPOUNDS FROM *EXCOECARIA AGALLOCHA*

3.8.1 Isolation of compounds



OMPOUND	R
24	
25	
26	
27	
28	

Air-dried, finely powdered stem bark (200 g) of *Excoecaria agallocha* was extracted sequentially with ethyl acetate (3×1 l) and ethanol (3×1 l) at room temperature using the microwave extraction procedure. The ethyl acetate and ethanol extracts were found to be insecticidal.

The combined active extracts (7.1 g) were subjected to dry column flash chromatography (Figure 3.3) on silica gel by gradient elution with petroleum

ether:ethyl acetate (4:1, 3:2, 2:3, 3×75 ml each) and finally ethyl acetate (3×75 ml). The active fraction $\mathbf{F_3}$ (764 mg) was further purified by flash column chromatography (Figure 3.3) on silica gel in petroleum ether:ethyl acetate (7:3, 600 ml) to afford a yellow oily insecticidal liquid, $\mathbf{F_6}$ (156 mg).

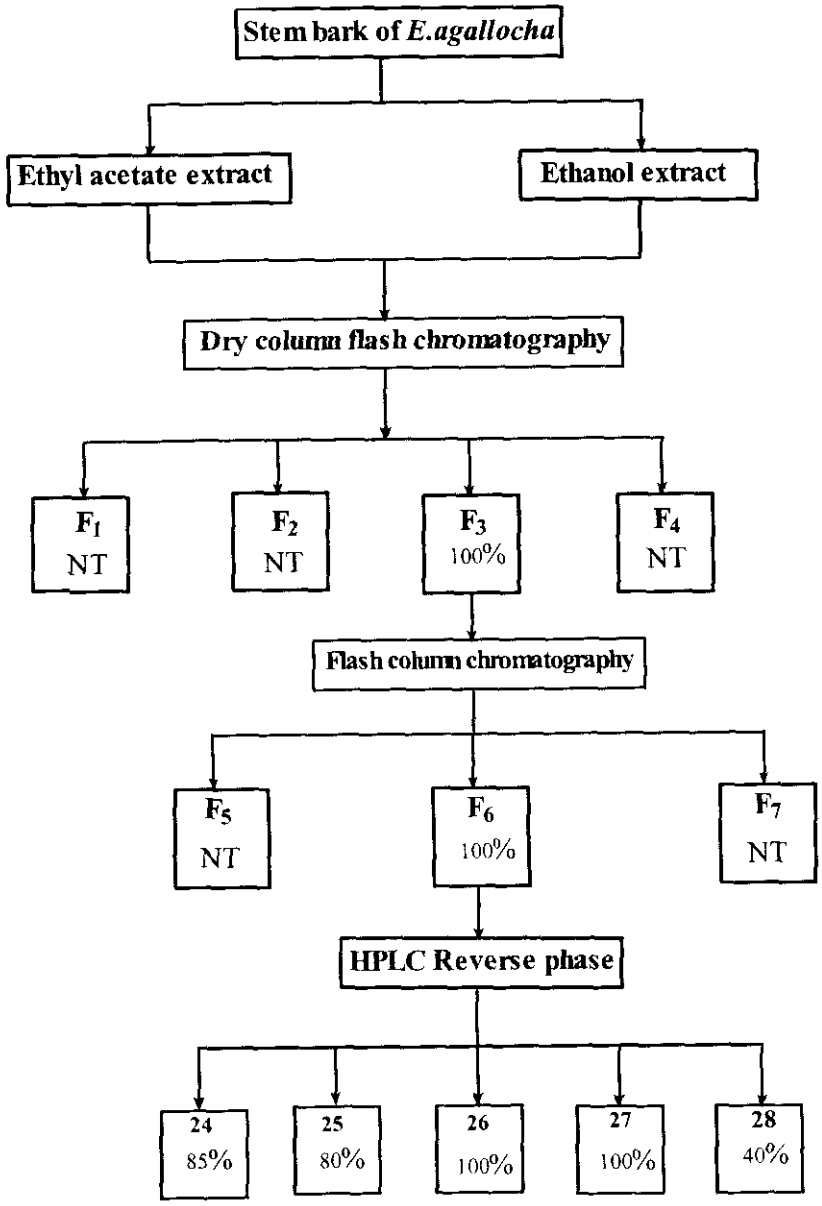


Figure 3.3 : Bio-assay guided fractionation of extracts of *Excoecaria agallocha*

The oily liquid (56 mg) was subjected to preparative HPLC (C_{18} column, 10 ml min^{-1}, UV 254 nm) using acetonitrile:water (9:1), to afford the insecticidal compounds **24** (19.5 mg, 0.003%, retention time 11min), **25** (2.7 mg, 0.004%, retention time 22 min), **26** (5.7 mg, 0.008%, retention time 15.5 min), **27** (4.4 mg, 0.006%, retention time 23 min) and **28** (4.3 mg, 0.006%, retention time 25 min).

Compound (24)

^{1}H-NMR : δ 7.64 (1H, s), 6.70 (1H, q), 6.06 (1H, m), 5.85 (1H, m), 5.71 (1H, d), 5.02 (1H, s), 4.91 (1H, s), 4.43 (1H, d), 4.26 (1H, s), 4.00 (1H, s), 3.84 (1H, m), 3.80-3.70 (2H, m), 3.60 (1H, s), 3.49 (1H, s), 3.46 (2H, s), 2.95 (1H, d), 2.48 (1H, q), 2.24 (2H, m), 2.24 (1H, s), 2.09 (2H, q), 1.80 (3H, s), 1.80 (3H, s), 1.36 (2H, m), 1.27 (4H, br s), 1.18 (3H, d), 0.91 (3H, dd).

^{13}C-NMR : δ 210.0 (C_q), 161.3 (CH), 146.0 (C_q), 139.0 (CH), 137.0 (C_q), 134.8 (CH), 128.8 (CH), 122.7 (CH), 118.0 (C_q), 113.0 (CH_2), 84.4 (CH), 83.0 (C_q), 81.9 (C_q), 72.2 (C_q), 72.0 (CH), 65.0 (CH_2), 64.1 (CH), 60.3 (C_q), 48.1 (CH), 36.6 (CH), 36.4 (CH_2), 34.9 (CH), 32.6 (CH_2), 31.3 (CH_2), 30.0 (CH_2), 28.7 (CH_2), 22.5 (CH_2), 20.3 (CH_3), 19.0 (CH_3), 14.1 (CH_3), 9.9 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3513, 3021, 2922, 2856, 2259, 1797, 1690, 1670, 1630, 1464, 1389, 1285, 1093, 1033, 917, 903. λ_{max} (EtOH, nm) 233, 204. **Mass Spec.** (ESI) m/z 565 $[M+Na]^+$, 581 $[M+K]^+$.

Compound (25)

^{1}H-NMR : δ 7.64 (1H, br s), 5.02 (1H, s), 4.91 (1H, s), 4.43 (1H, d), 4.26 (1H, s), 4.00 (1H, s), 3.80-3.70 (2H, m), 3.84 (1H, m), 3.60 (1H, s), 3.49 (1H, s), 3.46 (2H, d), 2.95 (1H, d), 2.48 (1H, q), 2.24 (2H, m), 2.24 (1H, s), 2.09 (2H, q), 1.80 (6H, s), 1.18 (3H, d), 1.36 (2H, m), 1.29 (6H, s), 0.88 (3H, dd). λ_{max} (EtOH, nm) 203.

Compound (26)

^{1}H-NMR : δ 7.64 (1H, s), 6.75 (1H, q), 6.10 (1H, m), 5.77 (2H, m), 5.02 (1H, s), 4.90 (1H, s), 4.43 (1H, d), 4.26 (1H, s), 4.02 (1H, s), 3.84 (1H, m), 3.75 (2H, m), 3.70 (1H, s), 3.63 (1H, s), 3.45 (2H, s), 2.95 (1H, d), 2.49 (1H, q), 2.24 (2H, m), 2.24 (1H, s), 2.10 (2H, q), 1.80 (6H, br s), 1.25 (4H, s), 1.18 (3H, d), 0.87 (3H, dd).

^{13}C-NMR : δ 210.0 (C_q), 161.3 (CH), 146.0 (C_q), 139.0 (CH), 137.0 (C_q), 134.8 (CH), 128.8 (CH), 122.7 (CH), 118.0 (C_q), 111.3 (CH_2), 84.4 (CH), 83.0 (C_q), 81.9 (C_q), 72.2 (C_q), 72.0 (CH), 65.0 (CH_2), 64.1 (CH), 60.3 (C_q), 48.1 (CH), 36.7 (CH), 36.4 (CH_2), 34.8 (CH), 32.7 (CH_2), 31.3 (CH_2), 29.7 (CH_2), 22.5 (CH_2), 20.4 (CH_3), 19.0 (CH_3), 14.1 (CH_3), 9.9 (CH_3). **Mass Spec.** (ESI) m/z 555 $[M+Na]^+$, 567 $[M+K]^+$.

Compound (27)

^{1}H-NMR : δ 7.64 (1H, s), 6.75 (1H, q), 6.31 (1H, m), 6.10 (1H, q), 6.10 (1H, q), 5.77 (2H, m), 5.02 (1H, s), 4.91 (1H, s), 4.45 (1H, d), 4.25 (1H, s), 4.00 (1H, s), 3.88 (1H, m), 3.75 (2H, m), 3.70 (1H, s), 3.60 (H, s), 3.46 (2H, s), 2.95 (1H, d), 2.50 (1H, q), 2.25 (2H, m), 2.24 (1H, s), 2.10 (2H, q), 1.80 (3H, s), 1.80 (3H, s), 1.18 (3H, d), 1.37 (2H, m), 1.27 (4H, br s), 0.90 (3H, dd).

^{13}C-NMR : δ 210.0 (C_q), 161.2 (CH), 145.0 (C_q), 137.4 (CH), 136.6 (C_q), 136.5 (CH), 134.6 (CH), 130.0 (CH), 128.6 (CH), 124.0 (CH), 118.0 (C_q), 111.4 (CH_2), 84.4 (CH), 83.0 (C_q), 81.9 (C_q), 72.1 (C_q), 72.0 (CH), 65.0 (CH_2), 64.1 (CH), 60.3 (C_q), 48.1 (CH), 36.7 (CH), 36.4 (CH_2), 34.8 (CH), 32.9 (CH_2), 31.8 (CH_2), 29.7 (CH_2), 29.1 (CH_2), 22.7 (CH_2), 20.4 (CH_3), 19.0 (CH_3), 14.1 (CH_3), 9.9 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3528, 3021, 2922, 2855, 1698, 1670, 1638, 1458, 1382, 1254, 1042, 997, 939, 856. λ_{max} (EtOH, nm) 269, 239, 203. **Mass Spec.** (ESI) m/z 591 $[M+Na]^+$.

Compound (28)

^{1}H-NMR : δ 7.64 (1H, s), 6.70 (1H, q), 6.35 (1H, m), 6.10 (2H, m), 5.85 (1H, m), 5.77 (1H, d), 5.02 (1H, s), 4.90 (1H, s), 4.45 (1H, d), 4.30(1H, s), 4.20 (1H, s), 3.80 (1H, m), 3.80 (1H, s), 3.80 (1H, s), 3.75 (2H, m), 3.50 (2H, s), 2.95 (1H, d), 2.50 (1H, s), 2.40 (1H, m), 2.24 (2H, m), 2.10 (2H, m), 1.80 (3H, s), 1.80 (3H, s), 1.36 (2H, m), 1.25 (10H, s) 1.18 (3H, d), 0.88 (3H, t).

^{13}C-NMR : δ 209.7 (C_q), 161.1 (CH), 146.1 (C_q), 137.3 (CH), 136.6 (C_q), 136.5 (CH), 134.5 (CH), 130.0 (CH), 128.6 (CH), 124.1 (CH), 116.4 (C_q), 111.3 (CH_2), 84.4 (CH), 83.0 (C_q), 81.9 (C_q), 71.5 (C_q), 72.5 (CH), 65.1 (CH_2), 64.1 (CH), 60.7 (C_q), 48.1 (CH), 36.6 (CH), 34.8 (CH), 36.4 (CH_2), 32.9 (CH_2), 31.9 (CH_2), 29.5 (2×CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 22.7 (CH_2), 20.4 (CH_3), 19.0 (CH_3), 14.1 (CH_3), 9.9 (CH_3). ν_{max} ($CHCl_3$, cm^{-1}) 3500, 2925, 2849, 1694, 1672, 1631, 1457, 1380, 1039, 1001, 912. λ_{max} (EtOH, nm) 270, 203. **Mass Spec.** (ESI) m/z 633 $[M+Na]^+$, 1243 $[2M+Na]^+$.

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