A THESIS ENTITLED

THE STUDY OF NATURAL PRODUCTS

SUBMITTED TO

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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE FACULTY OF SCIENCE

BY

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CONTENTS

SUMMARY

This thesis consists of six chapters. A General Introduction is presented at the beginning, dealing briefly with the background of wood chemistry research, the nature of secondary metabolism and the objectives of the study. Furthermore, the biogenesis of diterpenoids, *nor*-lignans and phenylethylchromones are also discussed.

Chapter I concerns a discussion of the constituents of the normal and infected woods of ***Agathis borneensis***. Fourteen compounds from both woods were isolated, including four new compounds **(44, 46, 51, 53)**. Their structures were elucidated mainly by use of ^{1}H and ^{13}C n.m.r. and mass spectroscopy.

The occurrence of xanthones in ***Calophyllum*** species is reviewed in Chapter II. This is followed by a discussion of the extractives from the wood and bark of ***Calophyllum biflorum***. Five compounds were isolated, two of which are new compounds **(80, 82)**.

The petroleum ether and ethyl acetate extract constituents of the infected wood of ***Aquilaria malaccencis*** is discussed in Chapter III. Four phenylethylchromones were isolated, of which compound **(42)** is a new compound. In addition, a new sesquiterpenoid compound **(43)** was also isolated.

The results of a phytochemical screening of three ***Dipterocarpus*** species is described in Chapter IV. The wood extracts of ***D. costulatus***, ***D. cornutus*** and ***D. baudii*** were investigated. The timber of ***D. costulatus*** contains more extractives than the other two ***Dipterocarpus*** species. Dipterocarpol was found to be the major compound in all three species.

An investigation of the tetranortriterpenoid constituents of ***Azadirachta indica*** is discussed in Chapter V. Five tetranortriterpenoids were isolated and compound **(22)** was identified as a new compound. A detailed description of the preparation of 3-deacetyl-azadirachtin by mild alkaline hydrolysis and its reacetylation to azadirachtin is also given.

The final chapter deal with raffinose, a trisaccharide isolated from ***Delonix regia***. The structure was assigned by modern m.m.r. techniques including two-dimensional experiments. The ^{13}C n.m.r. resonances of raffinose peracetate are assigned for the first time.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

A knowledge of the chemistry of wood is very important in many areas of the timber industry, for example the manufacture of pulp and paper, counter action against insect and fungal attack, discolouration by light, acid, alkali, metal ions, glues and water etc.[1]. The chemistry of wood extractives has been widely studied in relation to most of the above problems and also in the growing areas of interest of organic chemistry, pharmacology and biochemical systematics and evolution. It is clear, therefore, that the investigation of the chemical constituents of timbers is an important area of research and may provide valuable results of both academic and industrial interest.

Natural products traditionally fall into two categories , primary and secondary metabolites. Polysaccharides, proteins, fats, lipids, amino acids nucleosides and nucleic acids are the fundamental building blocks of living matter and are considered to be the primary metabolites. The chemistry of the secondary metabolites, such as phenols, quinones, terpenes, alkaloids, and various pigments has been a productive area of research for the organic chemist.

Chemotaxonomy, the classification of species according to their chemistry, is based on the

occurrence in the trees and plants of compounds such as flavonoids, alkaloids and terpenoids. Such chemo-taxonomic knowledge of timber species may provide useful information about lesser known species which could be of commercial value. Wood chemistry has been an important element in this process.

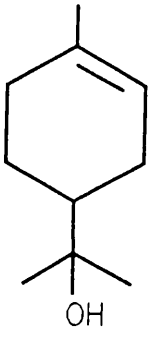
The study of natural products has long been a subject of fascination for the organic chemist. However, it was only in the eighteenth century that chemists began to investigate the extracts obtained from natural sources including timbers. Secondary metabolites, in particular those from plants, are considered to be an important factor in the co-evolution of plants, animals and insects. The impor-tance of chemical control is apparent in areas of survival e.g. defence chemicals, feedant, and anti-feedants, the pheromones and sex attractants.

The objectives of this thesis include:

1. To understand the behaviour of wood components.
2. The isolation, separation and structural elucidation of the chemical constituents of the wood, bark and leaf of some tropical wood species.
3. To gain insight into the sophiscated n.m.r. spectroscopic techniques currently used in natural product chemistry.
4. To investigate the chemistry of some of the compounds isolated.

In this chapter we shall refer only to a small number of natural products, in particular those related to the group of compounds isolated from ***Agathis borneensis*** and ***Aquilaria malaccencis***.

Terpenoids form one of the most important and diverse groups of secondary metabolites and they occur widely in resinous wood. Because of their fragrancy they have had an important role since ancient times in the manufacture of perfumes and cosmetics. Studies of terpenoids were instrumental in the early development of the synthesis of terpineol in 1901 by Perkin [2].



Terpineol

Monoterpenes often occur as mixtures of hydrocarbons ($C_{10}H_{16}$). The simpler members of the group were obtained by steam distillation of the parts of the plants to give oily mixtures known as essential oils. Later, compounds with higher a number of carbon atoms (C_{20} and C_{30}) were isolated from non-steam volatile saps, gums and resins of trees/plants. The biogenetic origin of these compound has been intensively studied. Early workers formulated the Isopropene Rule.

However, due to the increasing numbers of exceptions to the Isoprene Rule, this was restated by Ruzicka [3] as the Biogenetic Isoprene Rule which covered the possibility of rearrangement during biosynthesis.

The current classification of terpenoids is as follows:

No. of Carbon Atoms	Class
10	Monoterpenoid
15	Sesquiterpenoid
20	Diterpenoid
25	Sesterpenoid
30	Triterpenoid
40	Tetraterpenoid
>40	Polyterpenoid

Diterpenoids constitute a large portion of the resin of coniferous species. The amount of resin may be increased in response to wounding or heat. Some of the terpenoids play an important role in plant physiology and also in resistance to microorganisms. This was supported by the results of preliminary tests of some diterpenoids isolated from ***Agathis borneensis*** (Table 1).

Table 1. Bioassay of diterpenoids from

Agathis borneensis

(Carried out by ICI Plant Protection

Division, Jealott's Hill)

Compound No	Test Carried out	Result/Comments
(46)	F, I	Inactive
(6)	F, I	Complete control of ***Plasmopara viticola*** at 100 ppm
(50)	F, I	Inactive
(7)	F, I	Inactive

F= Fungicide/Bactericide,

I= Insecticide/Miticide/Nematicide

The results revealed that **3β, 16-dihydroxy-7-isopimarene-2, 15-dione (6)** was active against the vine downey pathogen *Plasmopara viticola*, giving a total control at 100 ppm as a combined protectant/systematic treatment. Unfortunately, it becomes inactive at lower doses.

The most studied biosynthesis in the diterpenoid area has been that of kaurene [4] which occurs in both the normal and enantio series. In ***A. borneensis*** only normal diterpenoids have been isolated. A scheme for the biosynthesis of diterpenoids from ***A. borneensis*** is

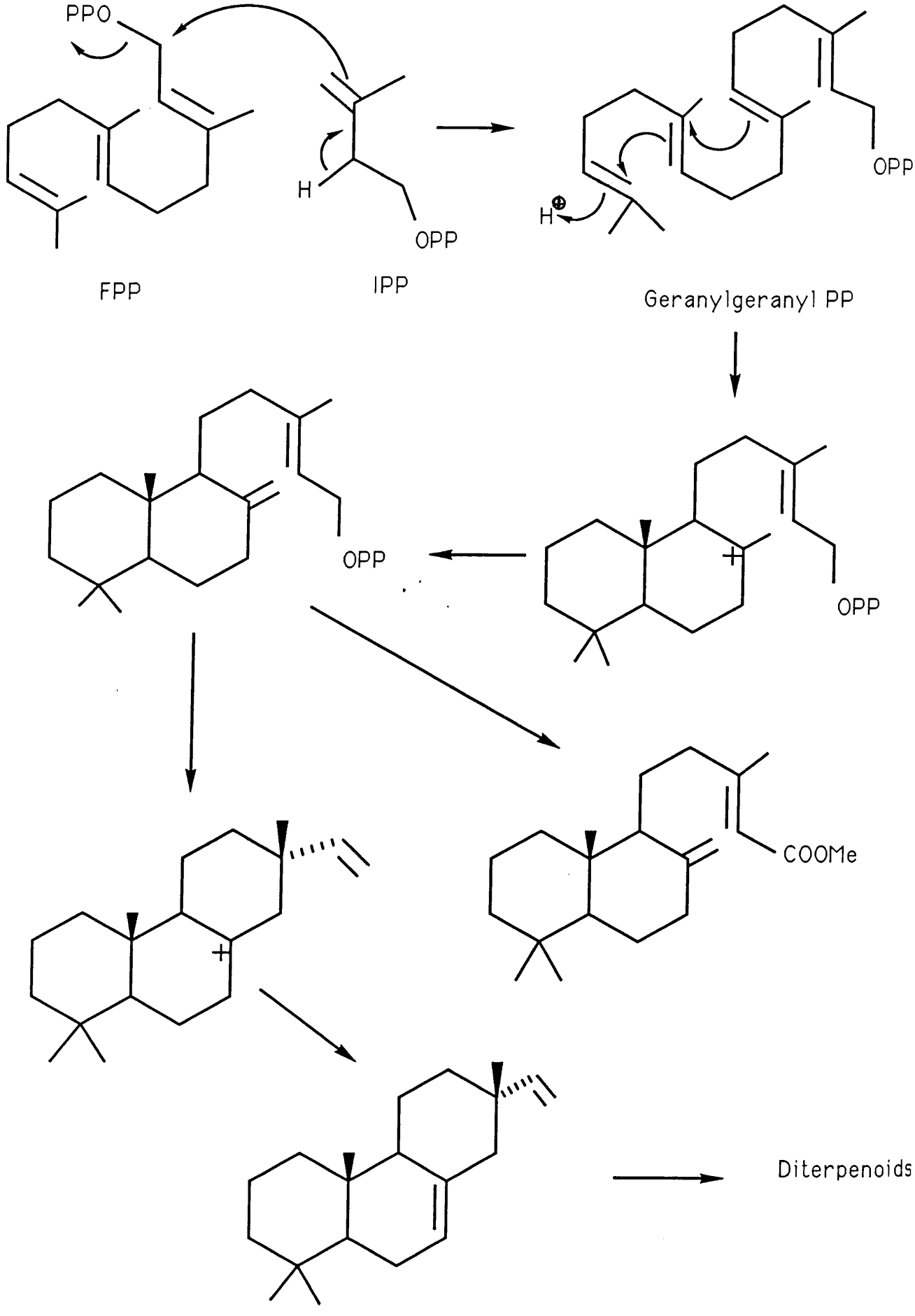


Fig. 1 Biosynthesis of diterpenoids

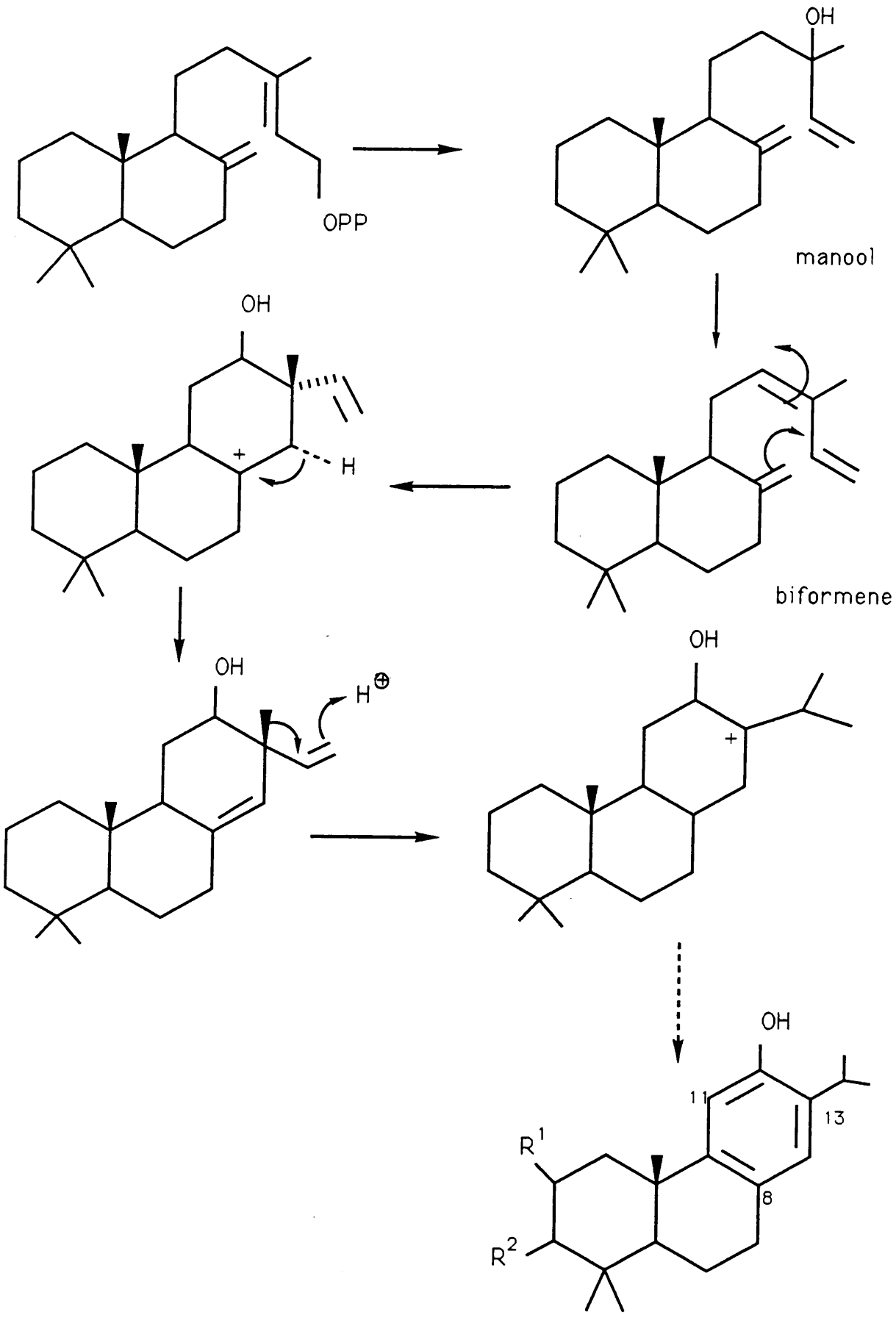
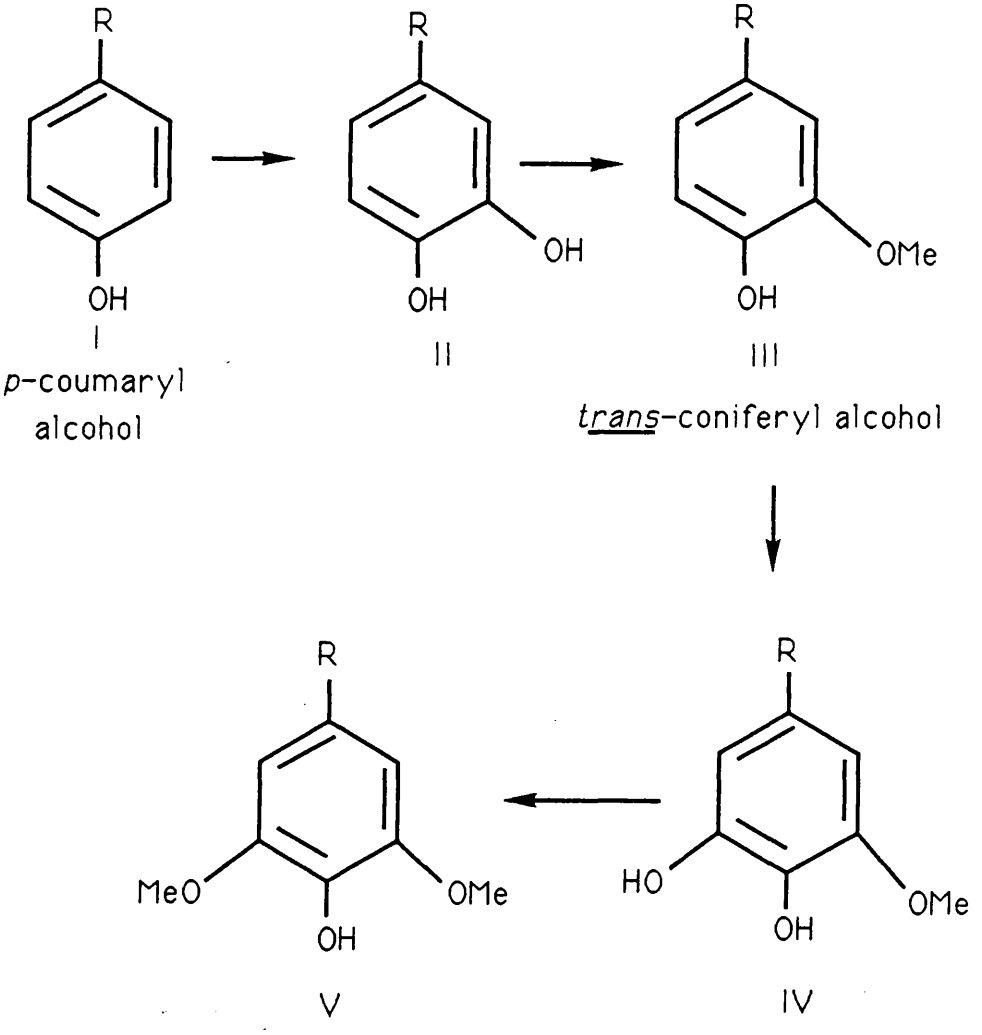


Fig. 2 Biosynthesis of 8,11,13- abietatriene


R
OH
I
p-coumaryl alcohol
R
OH
OH
II
R
OH
OMe
III
trans-coniferyl alcohol
R
HO
OH
OMe
IV
R
MeO
OH
OMe
V
trans-sinapyl alcohol

R = CH = CHCOOH

shown in Fig 1. It is generally accepted that the first step in the biosynthesis of diterpenoids is the proton initiated cyclization of a suitable conformation of geranylgeranyl pyrophosphate to labda-8(17),13-dien-15-yl pyrophosphate. Ring C is formed by *anti*-allylic displacement of pyrophosphate followed by proton loss to give a 7,15-pimaradiene. Subsequent hydroxylation, reduction and oxidation lead to a wide range of compounds.

A possible route to abietatriene diterpenoids is shown in Fig. 2. Allylic rearrangement to manool followed by dehydration affords the triene (biformene) [5] which can undergo oxygenation at position 12. Cyclisation of ring C, initiated by oxygen, would lead to a C-12 oxygenated compound. Rearrangement and aromatisation of ring C affords the abietatriene system. Later oxidation/hydroxylation may occur. The oxygenation at C-3 may be introduced at an early stage via 2,3-epoxygeranylgeranyl pyrophosphate.

Lignans are normally derived from two C_6-C_3 units and their presence been used as a basis of classification. The most common precursors of lignans are cinnamyl alcohol, particularly p-coumaryl alcohol, *trans*-coniferyl alcohol and *trans*-sinapyl alcohol. The formation of lignans involve the initial formation of radicals which then dimerise in various rational ways [6,7,8]. However, in the case of *nor*-lignan type

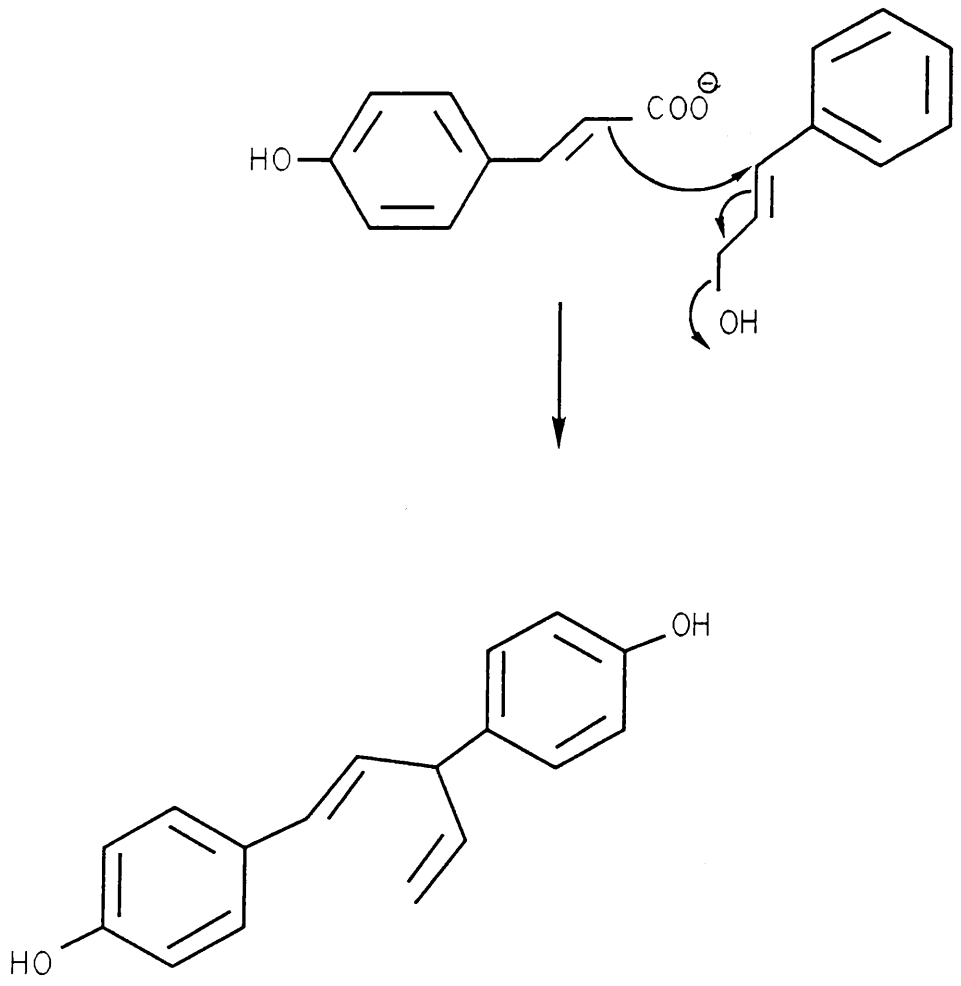


Fig. 3 Biosynthesis of *nor* - lignans

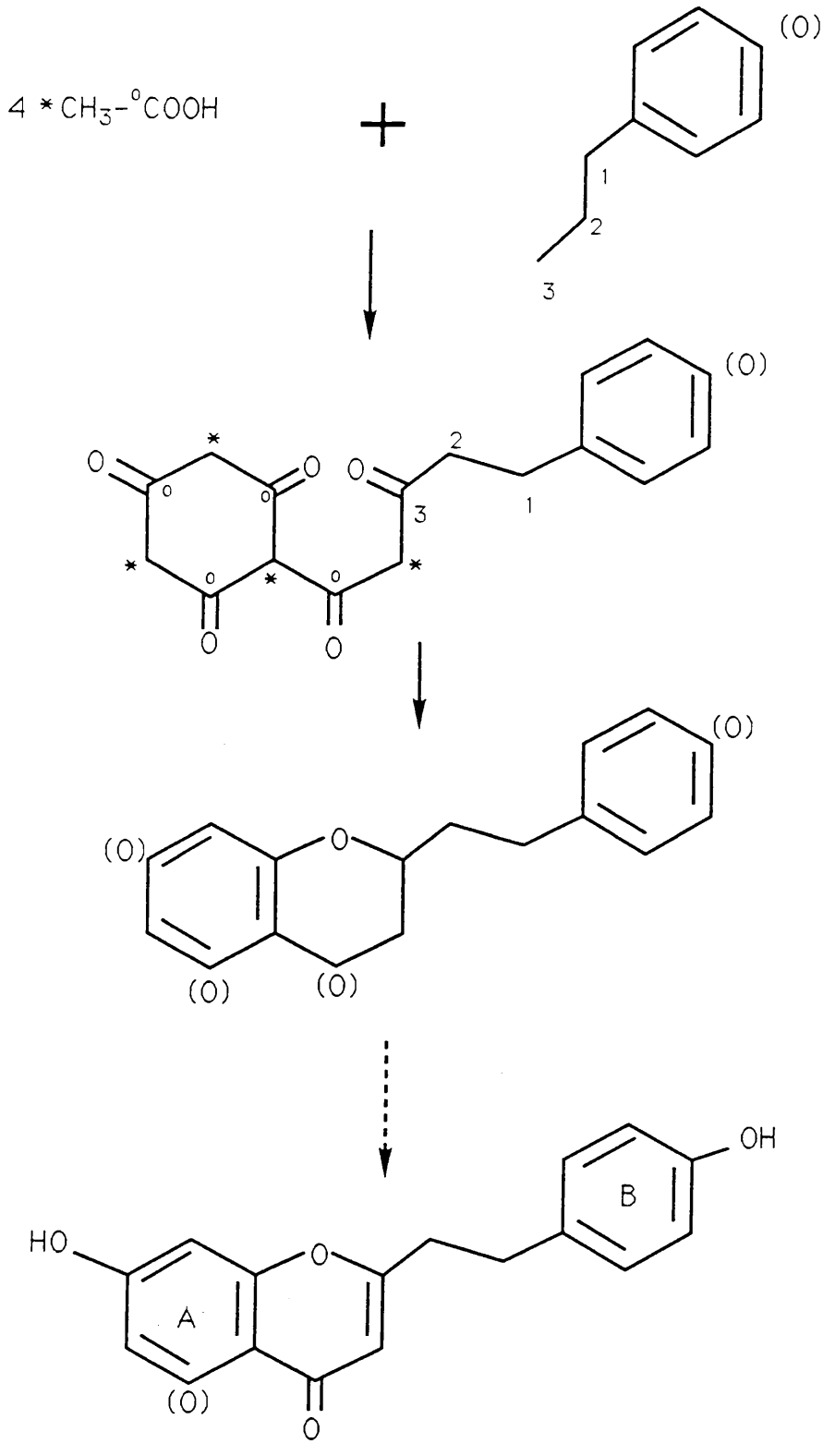


Fig. 4 Biosynthesis of phenylethylchromones

compounds, such as hinokiresinol **(36)** and agatharesinol **(11)** from ***A. borneensis***, it was suggested [9] that their formation may occur through alkylative decarboxylation. Such a route requires *p*-hydroxycinnamic acid and *p*-coumaryl alcohol which react as shown in Fig. 3.

Several phenylethylchromone compounds were isolated from ***Aquilaria malaccencis***. Such compounds are clearly flavonoid derivatives. It is generally accepted [10,11] that flavonoids are formed from three acetate units and a phenylpropanoid intermediate. derived from the shikimic acid pathway. The formation of phenylethylchromones may involve the condensation of four acetate units to form ring A while ring B arises from a phenylpropanoid precursor (Fig. 4). Cyclisation followed by appropriate elimination, enolisation, reduction etc. gives the phenylethyl chromones.

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CHAPTER I

DITERPENOIDS AND *nor*-LIGNANS FROM THE NORMAL AND INFECTED WOOD OF *Agathis borneensis*

INTRODUCTION

There are only two ***Agathis*** species [1] found in Malaysia namely ***Agathis borneensis*** (= ***A. damarra*** = ***A. alba***) and ***A. flavescens***. The former is important because of its use as a commercial timber. ***A. borneensis*** belong to a genus containing big trees of the family Araucariaceae which are found in Indo-China and through Malaysia to the Pacific and contain the Kauri pine of Australia and New Zealand. In Malaysia it is distributed through the mountain areas, normally above 350 m, from Serembam northwards and on the hills of Pahang.

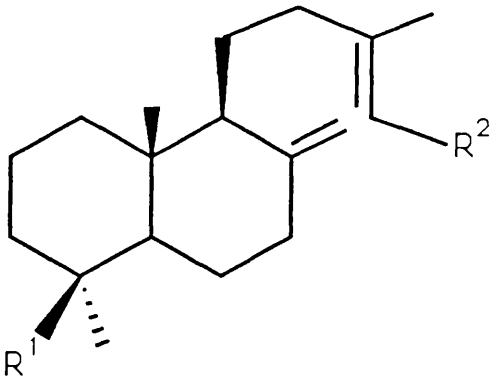
Foxworthy [2] reported that the resin is used in liniment by the Malay who sometimes apply small bits of fresh ***Agathis*** resin to the feet to prevent attack by leeches. The oleoresin has also been used in some spirit varnishes and lacquers and for preparation of linoleum. Usually the resin is deposited into a wound caused, for instance, by high winds cracking the wood in a fork and sometimes it drips to the ground if too much resin flows from the wound. It is the practice of the resin collectors to hack the trunk, cutting off strips of bark and exposing a wound to bleed, either at the base of the tree or at intervals up the trunk.

The timber is non-porous, whitish or yellowish to almost pale brown with a very slight difference

between sapwood and heartwood. Sometimes it is heavily impregnated with resin and is very hard and very dark in colour. The dark colour of the wood arises most probably from microbial infection of sapwood [3] resulting in the accumulation of induced secondary metabolites in the forms of gums. A previous study [4] showed a large increase in heptane-soluble compounds, usually, diterpenoids, of coniferous species due to mechanical wounding of the tree. Usually it is not sufficiently durable for outdoor use but is suitable for doors, window frames and musical instruments. It is regarded as a first class timber because of its excellent finish.

Heartwood components and the resin from conifers have been studied more systematically than those of tropical species, due to their outstanding importance. Minor constituents of the plant, especially those excreted into the dead heartwood or bark as metabolic end products, are much more valuable as taxonomic markers than constituents of highly specialized organs or intermediates in the general metabolism or components of purely mechanical structure such as cellulose or lignin.

The earliest recorded investigation [5] of ***Agathis*** resin was made in 1843 at about the time when export of the resin from New Zealand first began. Further chemical investigation [6] of the bled resin was

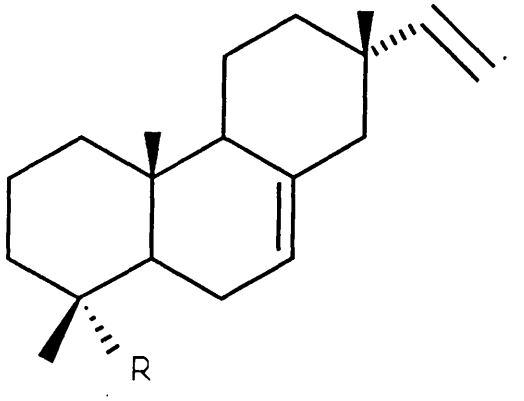


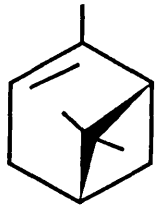
1. $R^1 = R^2 = COOH$ (agathic acid)

17. $R^1 = COOCH_3$; $R^2 = COOH$ (agathic acid monomethyl ester)

21. $R^1 = COOH$; $R^2 = COOMe$ (monomethyl agathate)

34. $R^1 = CH_2OH$; $R^2 = COOH$ (agatholic acid)

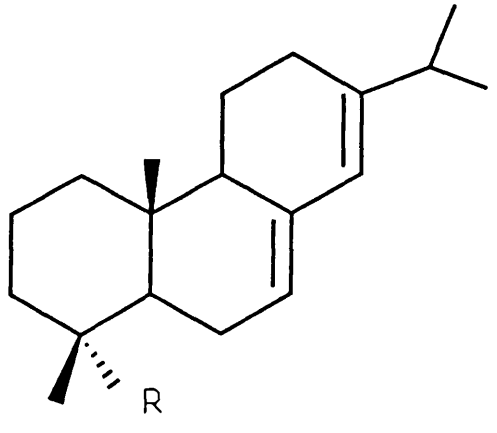




2. (+)-α-pinene

3. $R = CO_2H$

14. $R = CH_2OH$

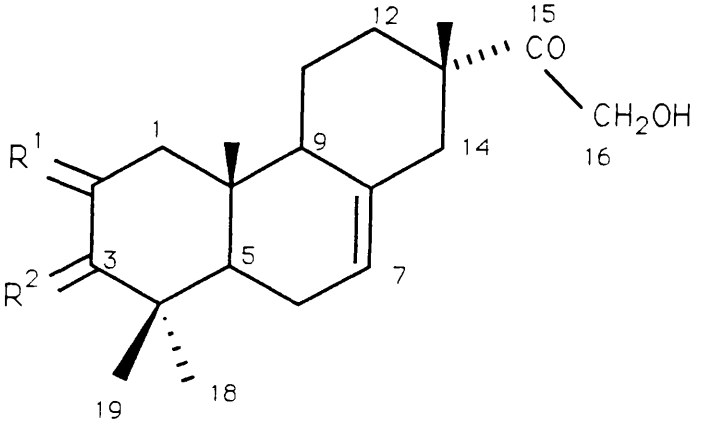


4. R = COOH (abietic acid)

18. R=COOMe (methyl abietate)

carried out by Tschirch and Niederstardt in 1901 who reported the presence of acidic (75 %) and neutral material (12 %) and oil (13 %). They also isolated agathic acid(**1**) for the first time in 10.5 % yield from the acidic fraction. Thirty years later (+)-α-pinene(**2**) was isolated by Hosking [7]. The resin derived from various species of the genus ***Agathis*** has been investigated by many workers. The earliest report on the chemical composition of Malaysian oleoresin was made in 1932 by Barry [8] who noted 3.5% volatile compounds. Isopimaric acid(**3**) and abietic acid(**4**) were found in 1964 by Gough [9] who suggested that the petroleum insoluble component of the resin consists predominantly of a copolymer of communic acid and communol. However, the resin still contains a fairly complex mixture of minor neutral, acid and phenolic constituents.

It was discovered later that agathic acid(**1**) is also present in other ***Agathis*** resin [10,11] including ***A. dammara*** (up to 15 %), ***A. palmerstonii*** (8.0 %), ***A. vitiensis*** (5%), ***A. robusta***, ***A. brownii*** (1%) and from ***A. microstachya*** [12]. Further chemical investigation of the resin from ***Agathis*** species was carried out by Carman and Dennis [13] who isolated communic acid and laevopimaric acid(**5**). Enzell and Thomas [14,15,16] investigated the heartwood resin of ***A. australis*** and isolated the closely related ketols

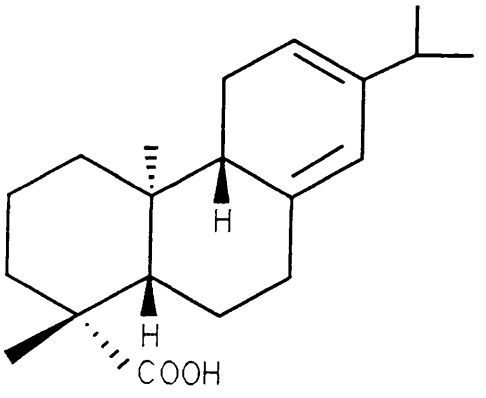


6. R^1 = O ; R^2 = β-OH,H

7. R^1 = H,H ; R^2 = O

8. R^1 = H,H ; R^2 = β-OH,H

46. R^1 = H,H ; R^2 = α-OH,H



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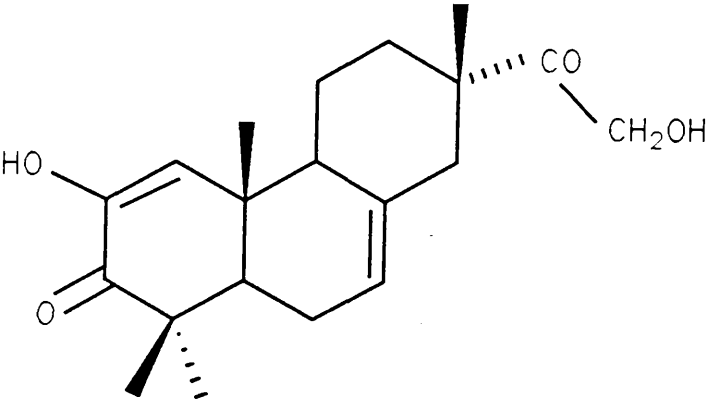

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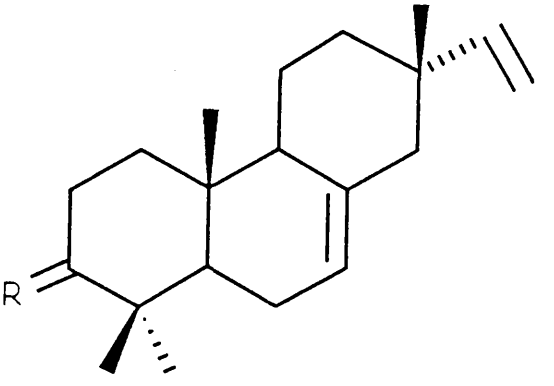
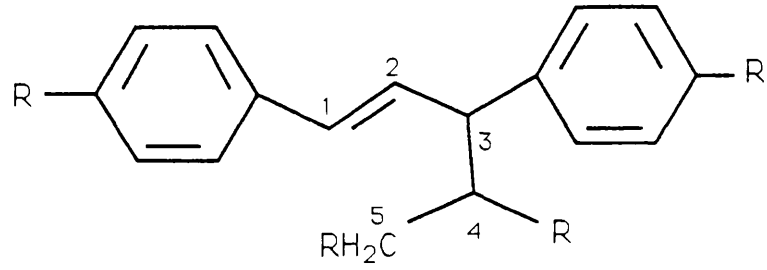


10. R = β-OH,H

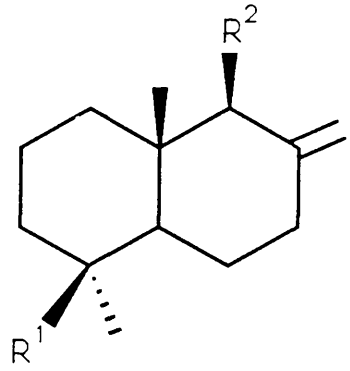
44. R = α-OH,H

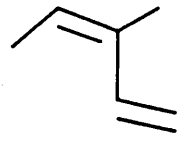
45. R = β-OAc,H

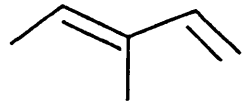


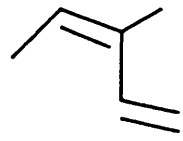
11. R = OH

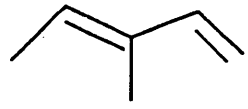
55. R= OAc

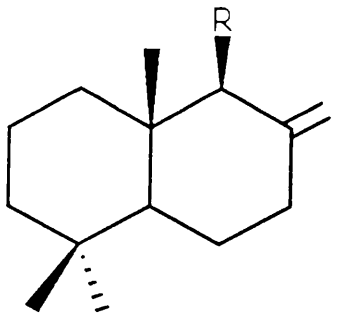


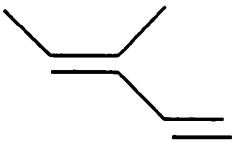
12. R^1 = COOH ; R^2 = 

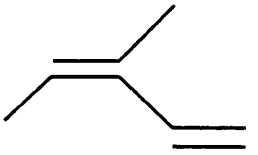
13. R^1 = COOH ; R^2 = 

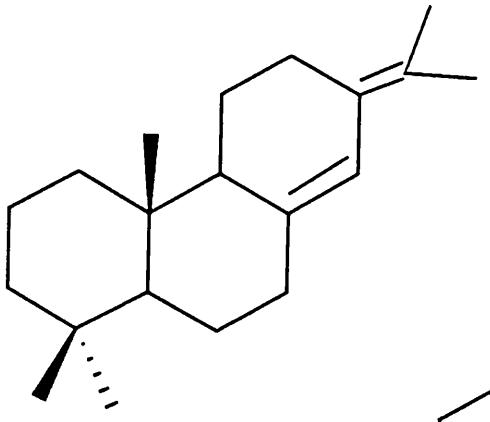
15. R^1 = CH_2OH; R^2 = 

16. R^1 = CH_2OH ; R^2 = 



19. R = 

20. R = 



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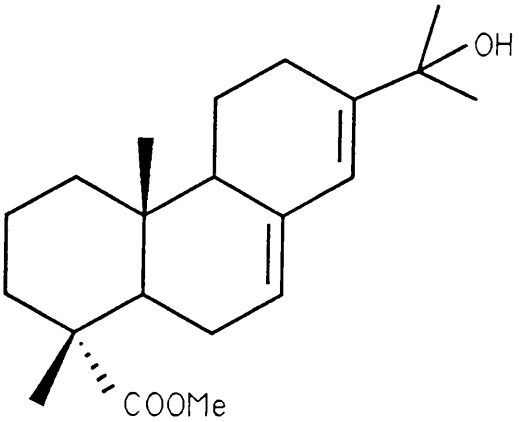

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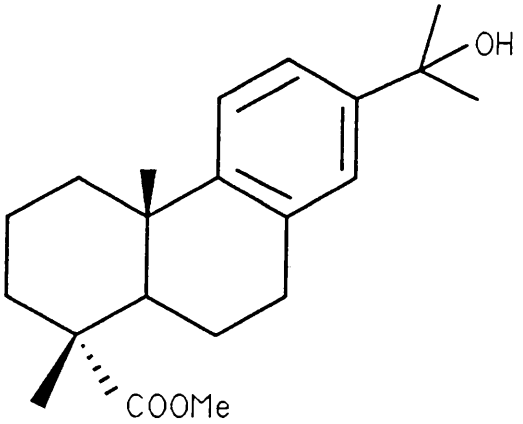
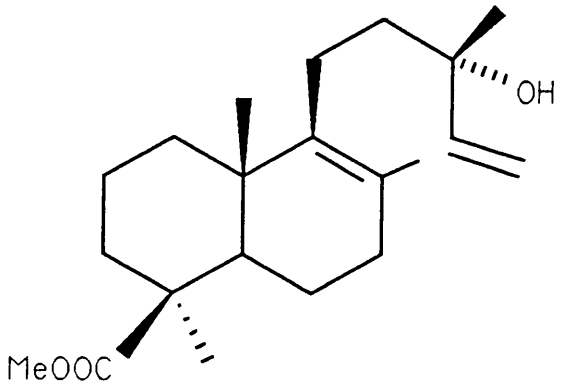


24.

araucarolone(6), araucarone(7), araucarol(8) and araucarenolone(9). Furthermore, they also found the known isopimaradien-3β-ol(10), β-sitosterol and number of minor constituents including agatharesinol (11). It is interesting to note that the wood resin, containing mainly araucarenolone (9), is quite difference in its constituents from the bled resin which contains isopimaric(2) and communic acid as the principle components along with agathic acid (1)and its monomethyl esters. A detailed chemical investigation of bled resin from ***A. australis*** by Thomas (1966)[17] revealed its principal components to be *cis*-communic(12), *trans*-communic(13), isopimaric(2) and abietic(3) acids, isopimaradienol(14), *cis*-communol(15), *trans*-communol(16) and agathic acid monomethyl ester(17). *Trans*-communic acid(13) is also present in ***A. palmerstonii*** and ***A. robusta*** [13].

Investigation of the oleoresin [18] from ***A. microstachya*** afforded methyl abietate(18), methyl *cis*-communate(19), methyl *trans*-communate(20), monomethyl agathate(21), agathic(4) and neoabietic (22) acids. A phytochemical survey [19] of the diterpenoid resins of North Queensland ***Agathis*** species by Carman and Marty afforded two new resin acids identified as the corresponding esters methyl 15-hydroxyabietate(23) and methyl 15-hydroxydehydro-abietate(24). Three new resin acid diterpenoids [20]



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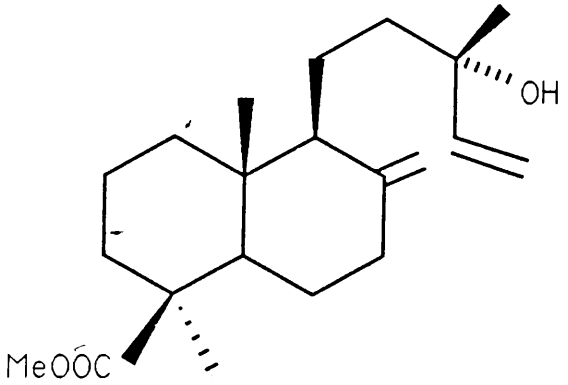

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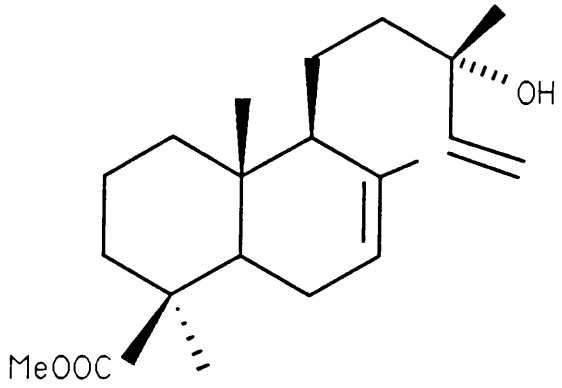

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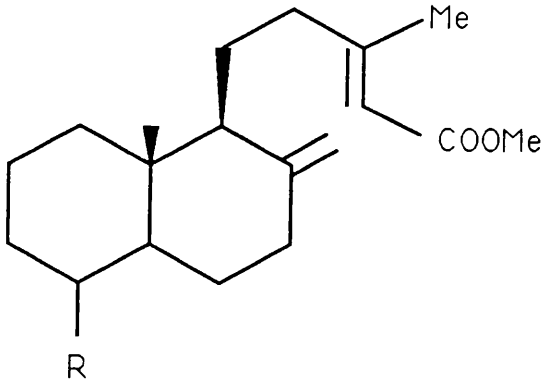
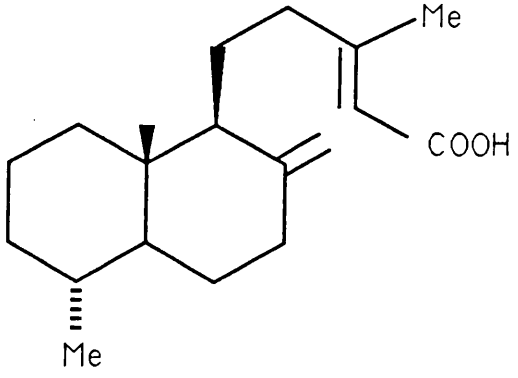


29. R = α-Me

30. R = β- Me

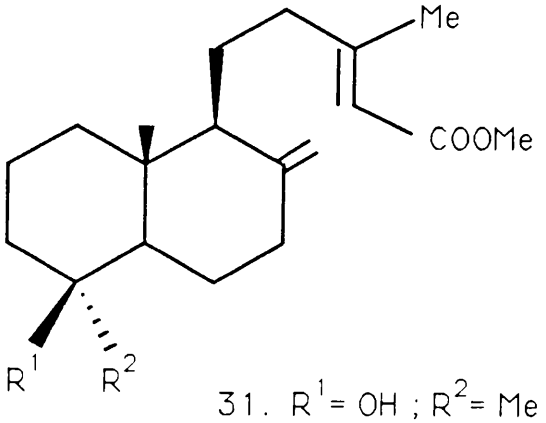


28.

were discovered in the resin of ***A. robusta*** and characterised as the esters, methyl 13β-hydroxylabda-8,14-dien-19-oate**(25)**, methyl 13β-hydroxylabda-7,14-dien-19-oate**(26)** and methyl 13β- hydroxylabda-8(17),14-dien-19-oate**(27)**.

Essential oil analysis [21] of ***A. australis*** was reported by Brigg, Kingsford and White who identified nineteen constituents and, furthermore, isolated isopimara-8,15-diene for the first time in nature. Kach Du Doc, Bastard and Fetizon [22] investigated the resin from ***A. lanceolata*** and isolated 19-noranticopalic acid**(28)** together with isopimaric acid**(3)**. A study of resin from [23] ***A. borneensis*** was reported by Sakami, Ohtani and Ichinohe in which three diterpenoids, isopimaric**(3)**, cis-communic**(12)** and agathic acids**(4)**, were identified.

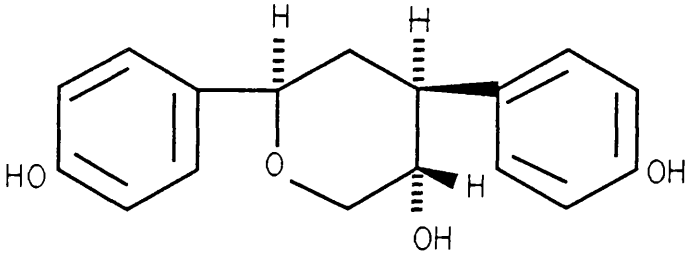
The bled resins [24] from ***A. vitiensis***, ***A. lancoelata*** and ***A. macrophylla*** were investigated by Smith, Marty and Peters by GC/MS analysis of the methylated fraction. The major components of ***A. vitiensis*** and ***A. macrophylla*** were virtually identical, thus supporting the hypothesis by Whitmore that ***A. vitiensis*** and ***A. robusta*** are synonyms of ***A. macrophylla*** [25]. ***A. lanceolata*** [26] resin, from New Caledonia, was investigated by Manh, Bastard and Fetizon. Five new diterpene acids were isolated and characterised as the methyl esters methyl 19-nor



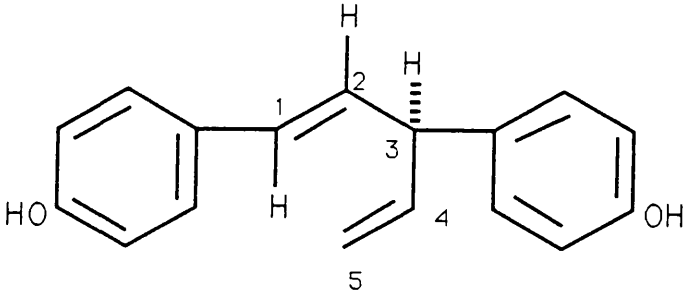
31. R^1 = OH ; R^2 = Me

32. R^1 = Me ; R^2 = OH

33. R^1 = CH_2OAc ; R^2 = H



35. Sugiresinol

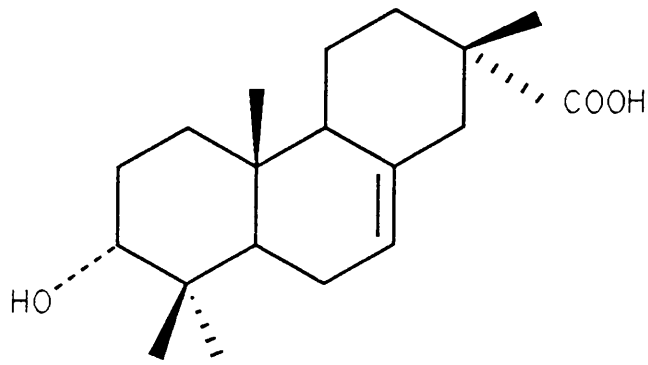


36. Hinokiresinol

anticopalate(29), methyl 18-noranticopalate(30), methyl 4β-hydroxy-19-noranticopalate(31), methyl 4α-hydroxy-18-noranticopalate(32) and methyl 19-acetoxyagatholate(33). Agathic acid monomethyl ester(21) and agatholic acid(34)were also isolated. A chemical investigation of the heartwood extract [27,28] of ***A. australis*** afforded a phenolic compound agatharesinol whose structure was established as (11). The studies showed that agatharesinol(11) is the most important constituent of the acidic fraction, representing about 5.0% and 0.3% of the total resin and heartwood respectively. A detailed study of the minor components of ***A. australis*** afforded further phenolic compounds, identified as sugiresinol(35) and hinokiresinol(36).

The composition of ***Agathis*** wood was studied [29] by Fujita, Yoshimoto and Samajima who showed that the methanol extract comprised 80% of the total weight of the extract from three different solvents (hexane, ether, methanol). The major compounds were detected by g.c. analysis and the methanol extract was shown to contain the phenolic components hinokiresinol(36) and monomethylhinokiresinol. Furthermore, araucarol(8) and two other tricyclic diterpenoids were also identified.

Recently Cambie, Coddington, Stone, Tanaka, Hua and Arigayo [31] examined the heartwood of ***A. vitiensis***. They isolated three new diterpenoids, including



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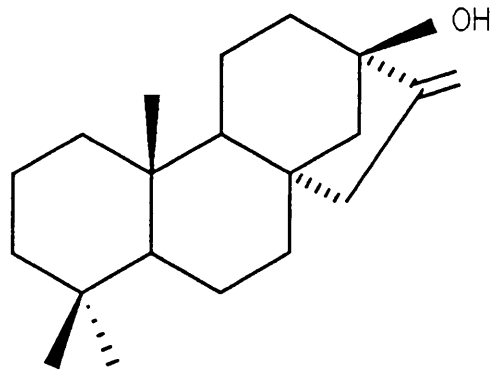

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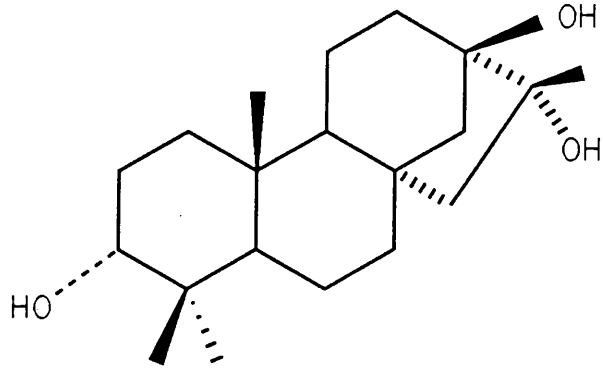

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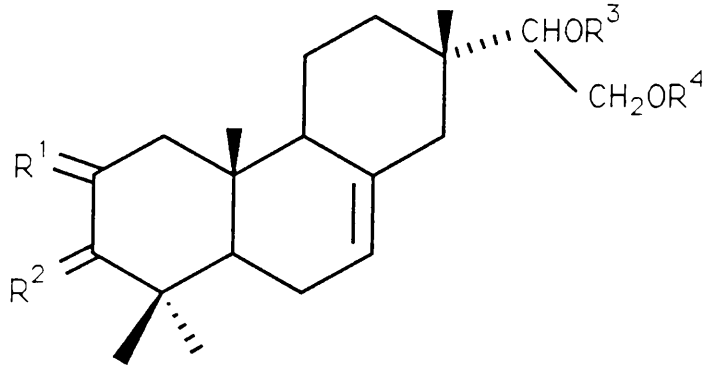


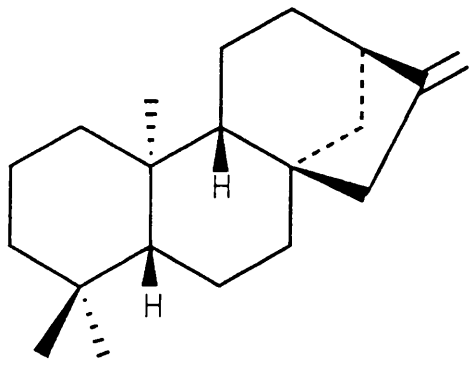
40 R^1 = H,H ; R^2 = α-OH,H ; R^3 = R^4 = H

47. R^1 = H,H ; R^2 = O ; R^3 = R^4 = H

48. R^1 = H,H ; R^2 = α- OAC,H ; R^3 = R^4 = Ac

53. R^1 = O ; R^2 = α-OH,H ; R^3 = R^4 = H

54. R^1 = O ; R^2 = β-OH,H ; R^3 = R^4 = H



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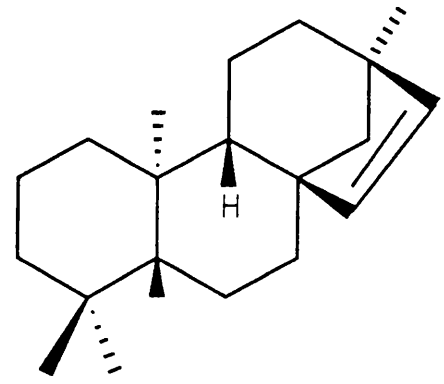

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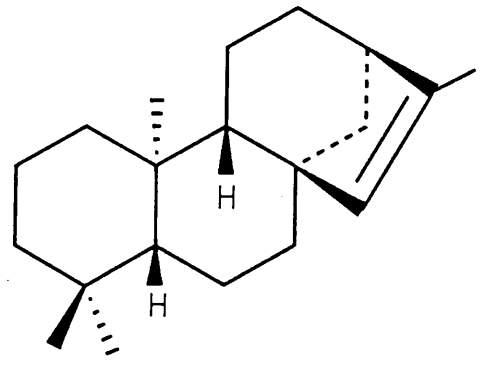
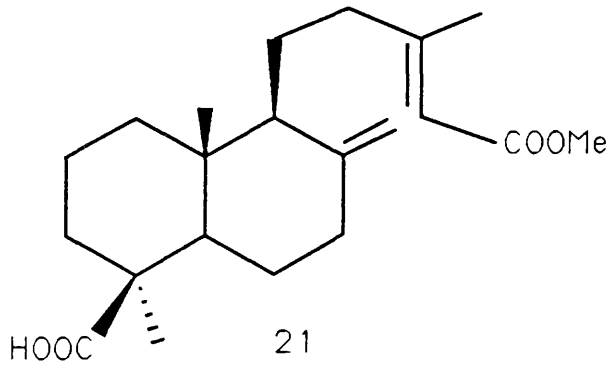


3α-hydroxy-16-nor-isopimar-7-en-15-oic acid**(37)**, kaur-16-ene-3α, 13-diol**(38)** and kaurane-3α, 13, 16α-triol**(39)**. They also isolated isopimar-7-ene-3α, 15, 16-triol **(40)** which had been reported earlier by Bohlmann and co-workers [31] from ***Palafoxia rosea***. Several known compounds were also found, including agatharesinol **(11)**, abietic acid**(3)**, agathic acid**(4)** and sitosterol.

The resin [32, 33, 34] from the leaf of ***Agathis*** species has also been investigated and several mono-, sesqui- and diterpenes including (-) kaurene**(41)**, (-) isokaurene**(42)** and cupressene**(43)** isolated.



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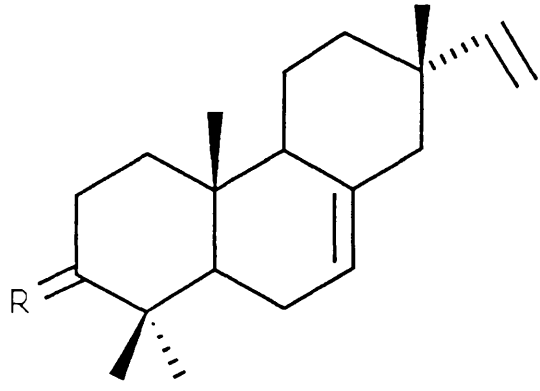
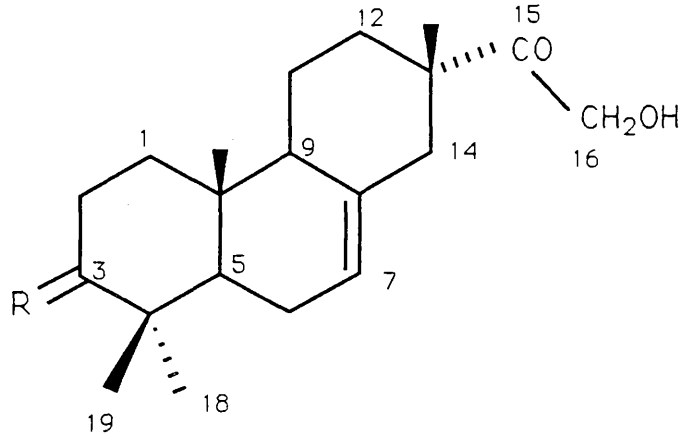


10. R =β-OH,H

44. R =α-OH,H

45. R = β-OAc,H



8. R = β-OH

46. R = α-OH

DISCUSSION

Both the normal and the infected wood of ***Agathis borneensis*** were investigated. The latter contained a higher proportion of organic metabolites, presumably in response to the infection. The structures of the compounds are discussed in order of increasing polarity. Fourteen compounds were isolated and characterised. Unfortunately several have already been described. The tricyclic diterpenoids all belong to the isopimarene series. This is easily established by the ^{13}C n.m.r. chemical shift of C-17. The absolute configuration of isolated compounds has not been established directly. Compounds are assumed to have the absolute configuration shown on the basis of comparison of the sign of the $[\alpha]_D$ (small and negative) with compounds of known absolute configuration.

The first two compounds, the epimers 7,15-isopimaradien-3α-ol **(44)** [35,36] and 7,15-isopimaradien-3β-ol **(10)** [15,37] had similar spectroscopic properties. HRMS confirmed the molecular formula as $C_{20}H_{32}O$ [m/z 288.2450 (21.5%) for **(44)**; 284.2453 (34.2 %) for **(10)**] for both compounds. The ^1H n.m.r. of both compounds showed signals for four tertiary methyls [δ_H 0.88 , 0.90, 0.97 (2) and 0.85, 0.88, 0.98 (2) respectively] [38] and a vinyl group attached to a

fully substituted sp^3 carbon [ABX system, δ_A 4.82, δ_B4.94 , δ_X5.82; J_{AB}=2.0 Hz, J_{AX}=10.0 Hz, J_{BX}=18.0 Hz respectively] characteristic of a pimarane/isopimarane skeleton. An additional isolated olefinic proton at δ_H 5.38 (m) suggested a 7,15-isopimaradiene structure. The i.r. spectrum of both compounds exhibited bands due to hydroxyl (3640 and 3620 cm^{-1} respectively) and monosubstituted double bond (992, 912 and 996, 912 cm^{-1} respectively). It seemed likely that the secondary hydroxyl groups were situated at C-3 in a 7,15-isopimaradiene skeleton. The carbinol proton of compound **(44)** appeared as δ_H 3.46 as a narrow multiplet indicating that it was equatorial (β) and hence the hydroxyl group was axial (α). In comparison, in **(10)** the corresponding proton was a doublet of doublets (J=10.0, 5.0 Hz) and hence the hydroxyl group was equatorial (β). H-3α (axial) normally appear at higher field than H-3β because of 1,3 diaxial interaction. Confirmation was obtained from the ^{13}C n.m.r. spectra of both compounds which showed they contained a 7,15-isopimaradiene skeleton, almost identical with reported values [39]. The stereochemistry of the hydroxyl was assigned on the basis of the ^{13}C n.m.r. chemical shifts of C-3 [δ_C 76.2 in **(44)** ; 79.3 in **(10)**]. The configuration of C-13 was confirmed by the chemical shift of Me-17 since it is known that axial methyls are usually more shielded than

equatorial ones [40]. Acetylation of 3β-hydroxy-7,15-isopimaradiene gave the 3β-acetate **(45)** which the ^{1}H n.m.r. showed of the acetate signal at δ_H 2.05 (s,3H).

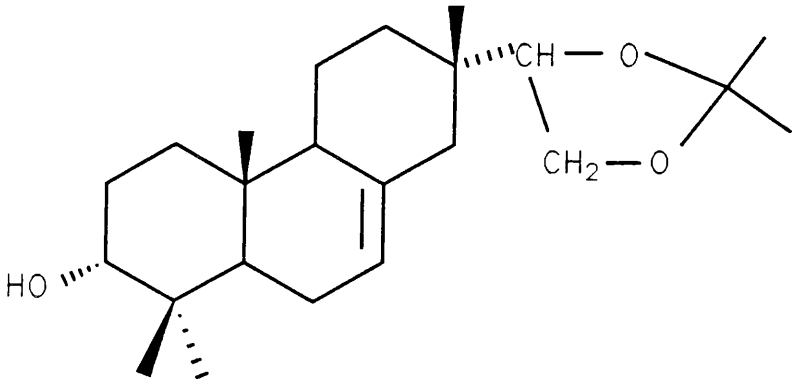
The ^{13}C n.m.r. spectrum of the next compound, monomethyl agathate**(21)**, showed signals characteristic for a labdane possessing a C-19 carboxyl group [ν_{COOH} 2940, 1710 cm^{-1}; δ_C 183.3 (s,C-19), δ 18.9 (q,C-18)] an αβ-unsaturated methyl ester in the side-chain [δ_C 167.3 (s,C-15), 50.8 (q,-OMe), 161.0 (s,C-13), 115.0 (d,C-14)] and an exomethylene group [δ_C 147.6 (s,C-8), 106.6 (t,C-17)]. The ^{1}H n.m.r. spectrum clearly showed a methyl ester [δ_H 3.76 (s)], two tertiary methyls [δ 0.58 and 1.22 (s)], a vinyl methyl [δ_H 2.13 (s)] and an exomethylene [δ_H 4.49 and 4.86 (both,s)]. Comparison of the ^{1}H n.m.r. chemical shifts of the isolated compound with literature values [18] for monomethyl agathate confirmed their identity. The parent ion was detected in the HRMS which had a mass at m/z 348.2301 (2.6 %) in agreement with the molecular formula $C_{21}H_{32}O_4$.

It was clear from the properties of the next compound, 3α,16-dihydroxy-7-isopimaren-15-one**(46)**, a new compound and the 3-epimer of araucarol**(8)**, that it possessed a 7-isopimarene skeleton with an α-hydroxyl substituent at C-3 [ν_{OH} 3460 cm^{-1}, δ_H 3.45 (s,br)]. A typical ketol side chain [16] consisting of a primary alcohol [ν_{max} 3480 cm^{-1}, δ_H 4.38 (s,2H-16), δ_C 64.0

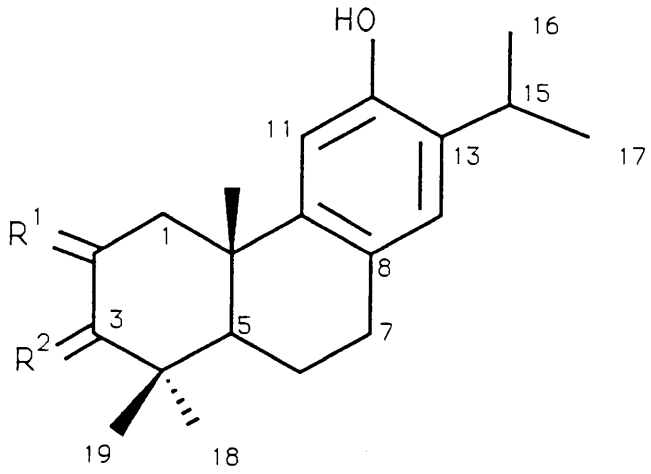
(t)], and a ketone [ν_{max} 1703 cm^{-1}, δ_C 215.0 (C-15)] was readily identified. The relative stereochemistry of the C-3 hydroxyl group as α was assigned from the chemical shift of C-3 [δ_C 76.0] and the lack of a large coupling of H-3. The HRMS also further confirmed the molecular formula as $C_{20}H_{32}O_3$ with a parent ion at m/z 320.2347 (12.8%)) and a base peak at m/z 243.2105 due to loss of H_2O and ketol side chain.

The ^1H n.m.r of the known 15,16-dihydroxy-7-isopimaren-3-one**(47)** which is also called 3-oxo-13-epipalarosan [31], showed signals for four tertiary methyls [δ_H 0.78, 1.05 (2), 1.11]. The olefinic proton H-7 appeared as an unresolved triplet centred at δ_H 5.45. The ^1H n.m.r. spectrum also showed a signal at δ_H 2.66 as a doublet of doublet of doublets (J=15.0, 10.0, 5.0 Hz) characteristic of an equatorial proton (H-2α) adjacent to ketone [41]. Comparison of the ^{13}C n.m.r. data with those of related compounds [30,42] suggested the placing of the carbonyl group [δ_C 217.0] at C-3. The ^1H n.m.r. signals of the diol side-chain appeared at δ_H 3.33 [dd, J=10.0, 2.5 Hz, H-16], δ_H 3.76 [dd, J=10.0, 2.5 Hz, H-16] and δ_H 3.37 [dd, J=10.0, 10.0 Hz, H-15] [30]. The presence of the diol side chain was further supported by the fragment ion at m/z 259.2059 [M^+-$CHOHCH_2OH$] in the HRMS.

The ^1H n.m.r. of the known 7-isopimarene-3α,15,16-triol**(40)** was similar to that of 15,16-



49.



50. R^1= H,H ; R^2= O

51. R^1= O ; R^2= β-OH,H

52. R^1=H,H ; R^2= β-OH,H

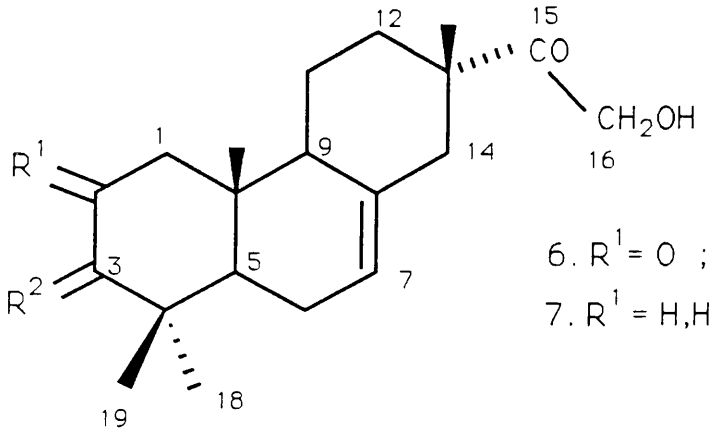
dihydroxy-7-isopimaren-3-one **(47)** apart from the signal at δ_H 3.44 (s, br, 1H) arising from the geminal proton of a 3α-hydroxyl group [30,31,34,35]. The ^{13}C n.m.r. chemical shift of C-3 (δ_C 76.0) also confirmed the stereochemistry of the hydroxyl group [30]. In addition the ^1H n.m.r. spectrum showed the presence of the primary [δ_H 3.34 (dd, J= 9.7 Hz, 2.9 Hz), 3.73 (dd, 1H, J=9.7 Hz, 2.9 Hz)] and secondary [δ_H3.52 (dd, 1H, J=9.7, 9.7 Hz)] hydroxyl groups of the diol side-chain. The HRMS showed a parent ion at m/z 322.2497 ($C_{20}H_{34}O_3$) and a base peak at m/z 261.2214 due to cleavage of the diol side chain.

Acetylation of the triol afforded the triacetate **(48)** whose ^1H and ^{13}C n.m.r. showed signals for three acetates [δ_H 2.10, 2.05 and δ 2.01; δ_C 20.9 (2), 21.3, 171.0 (2) and 170.7]. The triol was also converted to the acetonide **(49)** [δ_H 1.36, 1.41 (each 3H, s, acetonide methyls)].

The infected wood contained the known hinokione **(50)** [43]. The ^1H n.m.r. [44] revealed the presence of a hydroxyl proton [δ_H 5.20 (s, br)], two aromatic protons [δ_H 6.62 (s, H-11), 6.85 (s, H-14)], an isopropyl group [δ_H 3.13 (septet, J=8.0 , H-15), 1.22 and 1.23 (each d, J=6.9 Hz, 3H-16, 3H-17)] and three tertiary methyls [δ 1.14 , 1.18 and 1.27]. The position of the ketone [δ_C 217.4] was assigned by comparison of the ^{13}C n.m.r. chemical shifts with

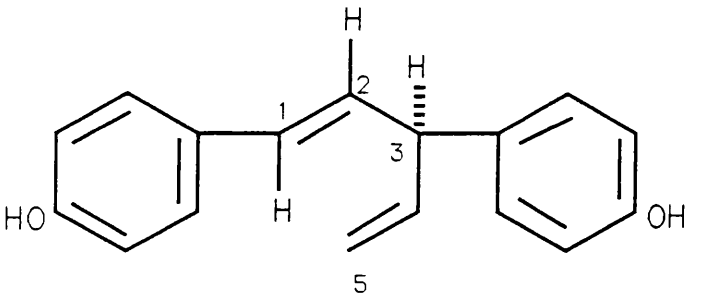
those of others aromatic diterpenoids [45,46]. The structure of the compound was further confirmed by its mass spectrum which showed a parent ion as the base peak at m/z 300.2079 [$C_{20}H_{28}O_2$].

The structure of 3β,12-dihydroxy -8,11,13-abietatrien-2-one**(51)**, a new compound, was established by comparison with the ^1H and ^{13}C n.m.r. spectra of related 8,11,13-abietatrienes [45,46]. The ^1H chemical shifts of the aromatic protons, isopropyl group and phenolic hydroxyl were almost identical with those of hinokione**(50)** and hinokiol **(52)** [44,45,46]. However the C-20 methyl resonance was shifted upfield (Δδ 0.4 ppm) due to shielding by the neighbouring carbonyl. Signals for an isolated methylene group [δ_H 2.63 (d, 1H, J=12.2 Hz), 3.11 (d, 1H, J=12.2 Hz)] [44] arising from the C-1 methylene, since the proton are deshielded by the aromatic ring with respect to araucarolone **(6)**, and a secondary carbinol group [δ_H 3.97 (s)] revealed the 3-hydroxy-2-keto system [47,48]. The ^{13}C n.m.r. of **(52)** was similar to that of hinokione**(50)** and hinokiol **(52)** [45,46] except for the signals of ring A which appeared at δ_C 210.9 (s, C-2), 82.8 (d, C-3) and 51.8 (t, C-1). Similar chemical shifts were observed for the ring A carbons of araucarolone **(6)**. This comparison supported the β (equatorial) configuration of the hydroxyl group at C-3. The mass spectrum of **(51)** revealed the parent ion as base peak at m/z 316.2049,

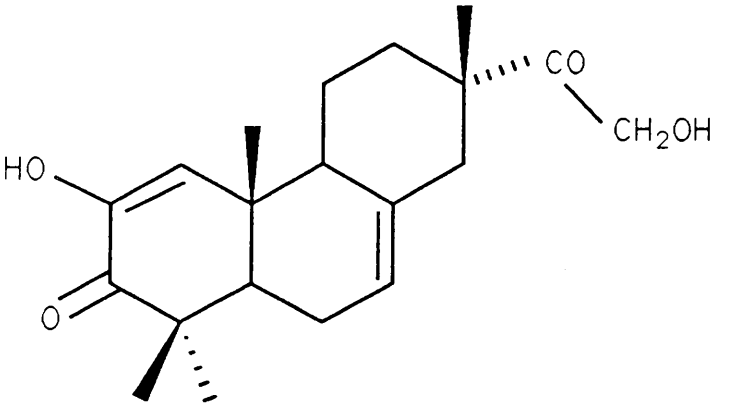


6. $R^1 = O$; $R^2 = \beta$-OH,H

7. R^1 = H,H ; $R^2 = O$



36. Hinokiresinol



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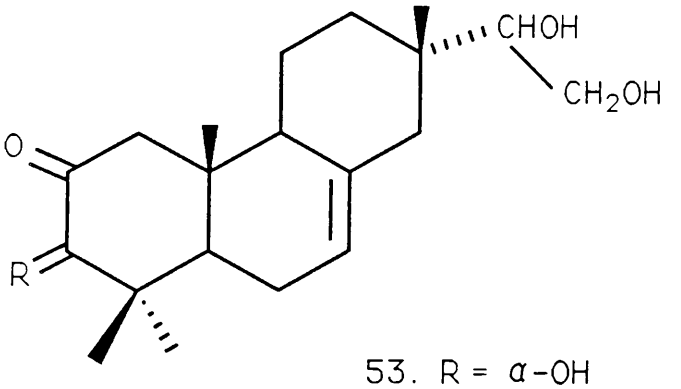

53. R = α-OH

54. R = β-OH

in agreement with the molecular formula $C_{20}H_{28}O_3$.

The ^1H n.m.r. spectrum of 3β,16-dihydroxy-7-isopimarene-2,15-dione (araucarolone) **(6)** afforded a signal at δ_H 4.36 (s, br, 2H) characteristic of a primary hydroxyl group in the ketol side chain of an 7-isopimarene [16]. Resonances for an isolated methylene group at δ_H 2.25 (d, J=12.0 Hz) and δ_H 2.62 (d, J=12.0 Hz) [49] and a secondary carbinol at δ_H 3.45 (s, br) indicated the presence of a ring A ketol. Comparison of the ^{13}C n.m.r. chemical shifts with those of araucarolone **(6)** confirmed the structure. Further evidence for the ketol side-chain was obtained from the HRMS. Loss of a $COCH_2OH$ fragment from the parent ion was observed (m/z 275.1990 [M^+-COCH_2OH], 78.3%).

The ^1H n.m.r. of the known hinokiresinol **(36)** first isolated from ***Chamaecyparis obtusa*** by Hirose and co-workers [49], showed resonances for a *trans*-disubstituted double bond [δ_H 6.25 (dd, J= 15.3, 6.4 Hz, H-2) and δ_H 6.23 (d, 1H, J=15.3 Hz, H-1)], a vinyl group [δ_H 5.15 (m, 2H-5), 6.06 (ddd, J=17.0, 10.3, 6.8 Hz)] and two AA'BB' systems [δ_H 7.24, 7.11, 6.76, 6.70, J_{obs}= 8.7 Hz)] arising from two *para*-disubstituted benzene rings. The AA' system at δ_H 7.11 showed a small coupling (0.5 Hz) with H-1. This indicated that the double bond is conjugated with that aromatic ring. The appearance of a benzylic methine proton [δ_H 4.12 (t, J=6.4 Hz, H-3)] coupled with two

olefinic protons in different double bonds was consistent with the structure (**36**) assigned to hinokiresinol. Confirmation of the structure was obtained from the ^{13}C n.m.r. spectrum recorded for the first time. The shifts and assignments are listed in the experimental section. The chemical shifts of C-1 and C-2 are very similar but these carbons are readily distinguished from C-3 and the vinyl group. No attempt was made to assign the aromatic carbons to specific rings.

The ketol side chain of the next compound, 16-hydroxy-7-isopimarene-3,15-dione (araucarone)(**7**), was readily identified by signals at δ_H 3.24 [OH] and δ_H 4.36 (s, br, 2H). The ^1H n.m.r. signal at δ_H 2.66 (ddd, 1H, J=16.0, 10.0, 3.0 Hz) for H-2α further suggested a ketone attached in ring A [16,41]. The olefinic proton H-7 [δ_H 5.48 (m)] had a similar pattern to the corresponding signals in other 7-isopimarenes. The ketol side-chain was also further supported by loss in the HRMS of a $COCH_2OH$ fragment [259.2071 (100%), M^+-COCH_2OH] from the parent ion. Hence, these spectroscopic data confirmed the structure of the isolated compound as 16-hydroxy-7-isopimarene-3,15 dione (araucarone) (**7**).

The next compound to be isolated was the diosphenol, 2,16-dihydroxy-1,7-isopimaradiene-3,15-dione (araucarenolone)(**9**), which showed the same

pattern as other 7-isopimarenes [16] for the ketol side-chain and the H-7 vinyl proton. The lack of coupling and the deshielded nature of a second vinyl proton [δ_H 6.19 (s)] were consistent with the presence of an enolised α-diketone system in ring A. The ^{13}C n.m.r. spectrum confirmed the siting of the double bond at C-1 [δ_C 123.1 (d)] and C-2 [δ_C 143.3 (s)] and the carbonyl at C-3 [δ_C 215.9 (s)]. The other carbon resonances were almost identical with those of related 7-isopimarenes with a ketol side-chain [16].

The ^{1}H n.m.r. spectrum of 3β,16-dihydroxy-7-isopimaren-15-one (araucarolone) **(6)** [16] was readily recognisable as arising from a 7-isopimarene with ketol side-chain. The geminal proton of a secondary, equatorial hydroxyl group gave a signal at δ_H 3.28 (m) and was assigned to C-3 by analogy with the other compounds in this series. The ^{13}C n.m.r chemical shift of C-3 [δ_C 79.2] confirmed this proposal. Further support for the ketol side-chain was obtained from the mass spectrum which showed loss of a $COCH_2OH$ fragment [M^+–$COCH_2OH$, 243.2102 (7.8 %)] from the parent ion due to α-cleavage.

The structure of another new compound from the extract was established as 3α,15,16-trihydroxy-7-isopimaren-2-one**(53)**, the C-3 epimer of fumotoshidin B, first found in ***Microlepia marginata*** [49]. The ^{1}H n.m.r. showed a geminal proton signal of a secondary

hydroxyl which appeared as a singlet at δ_H 4.38, indicating that a carbonyl group was attached to the C-2 position. Protons of an isolated methylene group, presumably C-1, were observed at δ_H 2.12 (d, J=13.8 Hz) and δ_H 2.21 (d, J=13.8 Hz), their geminal coupling again being consistent with the presence of a carbonyl at C-2 position [41]. Thus a 3-hydroxy-2-keto system was situated in ring A. The stereochemistry of the hydroxyl was assigned as α on the basis of ^{13}C n.m.r. chemical shift of C-3 [δ_C 71.1]. The olefinic proton as usual appeared at δ_H 5.46 (t, 1H, J=2.7 Hz) as in 7-isopimarenes. The diol side-chain was readily identified by comparison of the ^1H and ^{13}C n.m.r. chemical shifts with those of 7-isopimarene-3α, 15, 16-triol **(40)** and also by the loss of $CHOHCH_2OH$ from the parent ion in the mass spectrum. The HRMS also afforded a parent ion at m/z 336.2282 (7.3 %) ($C_{20}H_{32}O_4$).

Fumotoshidin B **(54)**[48], the 3-epimer of the previous compound, was also isolated. Its ^{13}C n.m.r. spectrum showed a signal at δ_C 82.4 (d) for C-3 bearing a 3β-hydroxy, a downfield shift of 11.3 ppm relative to the 3α-hydroxy compound **(53)**. As anticipated, there was great similarity of the proton shifts of both compounds apart for the resonance of H-3. In **(54)** H-3 appeared at δ_H 3.93 (s, 1H). In addition, comparison of the ^{13}C n.m.r. chemical shifts

of the ring A carbons with those of 3β,16-dihydroxy-7-isopimarene-2,15-dione (araucarolone)**(6)** showed close similarity . The HRMS showed a parent ion at m/z 336.2305 (5.6%) in agreement with the molecular formula $C_{20}H_{32}O_4$ α- Cleavage of the ketol side-chain again gave a fragment at m/z 275.2007 (18.3%). The possibility of equilibration of **(53)** and **(54)** during isolation and chromatography was not excluded.

The known lignan, agatharesinol **(11)** [27,28] was also isolated. Since it rapidly polymerised during purification on preparative t.l.c. it was characterised as its acetate **(55)**. The ^{1}H n.m.r. spectrum showed four acetates [δ_H 1.88 (s), 2.05 and δ 2.66 (2)] and eight aromatic protons as two overlapping AA'BB' systems [δ_H 7.31 (m, 4H) and 7.04 (m, 4H)], indicating again the presence of two <u>para</u>-disubstituted benzene rings. Two vinyl protons [δ_H 6.26 (dd, J=15.8, 8.3 Hz, H-2) and 6.48 (d, J=15.8 Hz, H-1)] revealed a <u>*trans*</u>-disubstituted double bond. The higherfield vinyl proton [δ_H 6.26, H-2] showed an additional coupling [J=8.3 Hz] with another proton [δ_H 3.08 (t, 1H, J=8.5 Hz, H-3)]. This proton in turn is coupled to a secondary acetate proton [δ_H 5.51 (ddd, J=8.3, 6.7, 3.1 Hz, H-4)] with a neighbouring primary acetate [δ_H 4.07 (dd, J=12.0, 6.7 Hz), 4.40 (dd, J=12.0, 3.1 Hz) , 2H-5]. The ^{13}C n.m.r., assigned for the first time, showed signals for two *p*-

disubstituted benzene rings (see experimental). The carbons of the disubstituted double bond [δ_c 129.1, 131.8] are slightly shifted relative to hinokiresinol **(36)**. The remaining carbons [δ_c 49.9 (d,C-3), 72.7 (d,C-4), 63.8 (t,C-5)] were readily assigned on the basis of their chemical shift and multiplicities. Further support for the structure of agatharesinol **(11)** was obtained from the HRMS. Loss of acetic acid from the parent ion gave a fragment at m/z 394.1416 (16.5%). Cleavage of C3-C4 led to a fragment at m/z 309.1106 (24.1 %).

GENERAL EXPERIMENTAL

All melting-points (m.p.) which are uncorrected, were determined on a Kofler hot-stage apparatus. Infrared (i.r.) spectra were recorded in CCl_4 solution unless otherwise stated on either Perkin Elmer 580 or 257 instruments. Ultraviolet absorption spectra were measured in ethanol or methanol solution using a Unicam S.P. 800 spectrometer. Mass spectra (m.s.) were recorded using an MS12 instrument (low resolution) and MS9025 instrument (high resolution). Unless otherwise stated nuclear magnetic resonance (n.m.r.) spectra were recorded for $CDCl_3$ solutions using Bruker WP200SY or AM 200SY instruments (^1H, 200 MHz, ^{13}C 50.32 MHz). Low field spectra were recorded at 90 MHz, on a Perkin-Elmer R32 instrument; or on a Varian XL 100 (^1H 100 MHz, ^{13}C 25.16 MHz) instrument. Chemical shifts are measured using the δ scale with tetramethylsilane as internal standard or relative to $CHCl_3$ at δ_H 7.25 or $CDCl_3$ at δ_C 77.0 unless otherwise stated. Kieselgel GF_{254} was used for preparative thin layer chromatography (t.l.c.) (0.5 mm adsorbent thicknes). Analytical t.l.c. plates were visualised using U.V. light (254 or 350 nm) or by heating for several minutes after spraying with ceric sulphate / H_2SO_4. All solvents and reagents used were of analytical grade except for column chromatography

where bulk solvents were used. Solvents were removed by using a Buchi rotary evaporator and water aspirator. Botanical specimens were identified by the Botanical staff of Forest Research Institute of Malaysia (FRIM) and the reference samples are deposited in the FRIM Herbarium in Kepong, Selangor.

EXPERIMENTAL

NORMAL WOOD

The wood of *A. borneensis* was obtained from the Tekam River Forest, Pahang, Malaysia. Sawdust (754 g) was extracted with hot petroleum ether (60-80) for three days and the solvent evaporated to yield viscous oily material (2.7 g). The brownish extract was adsorbed on Silica GF_{254} (5 g) and chromatographed over the same adsorbent (120 g). The extract was eluted with petroleum ether (60-80) containing increasing proportions of ethyl acetate and finally with increasing proportions of methanol. Fractions (100 ml) of eluate were collected as follows: fractions 1 (100% petroleum ether(60-80), 2-3 (petroleum ether (60-80)-ethyl acetate, 9:1), 4-8 (petroleum ether (60-80)-ethyl acetate, 4:1), 9-11 (petroleum ether (60-80)-ethyl acetate, 7:3), 12-14 (petroleum ether (60-80)-ethyl acetate, 3:2), 15-16 (petroleum ether(60-80)-ethyl acetate, 1:1), 17-18 (petroleum ether (60-80)-ethyl acetate, 2:3), 19-21 (petroleum ether (60-80)-ethyl acetate , 3;7), 22-23 (petroleum ether (60-80)-ethyl acetate, 1:4), 24-26 (petroleum ether (60-80)-ethyl acetate, 1:9), 27-28 (100% ethyl acetate), 29-31 (ethyl acetate-methanol, 4:1), 32 (ethyl acetate-methanol, 2:3) and 33 (100% methanol).

Fraction 4 (64.3 mg) was further separated by

preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 19:1) and afforded **7,15-isopimaradien-3α-ol(44)** (13 mg), m.p. 98-99°C (from petroleum ether (60-80)), $[\alpha]_D$-27° (c, 0.55 in $CHCl_3$), i.r. ν_{max} cm^{-1} 3640, 2958, 2925, 2865, 1637, 992, 912. ^1H n.m.r. [lit[15,35]: δ 0.88(s, 3H, Me-20), 0.90 (s, 3H, Me-17), 0.97 (s, 6H, Me-18, 19), 1.94 (br, 2H, 2H-14), 3.46(s, br, 1H, H-3eq), 4.82 (dd, 1H, J=10.0, 2.0 Hz, H-16*cis*), 4.94 (dd, 1H, J=18.0, 2.0 Hz, H-16*trans*), 5.38(m, 1H, H-7), 5.82 (dd, 1H, J=18.0, 10.0 Hz, H-15). ^{13}C n.m.r. : δ 14.8 (q, C-20), 20.1 (t, C-11), 21.5 (q, C-17), 22.7 (q, C-19), 23.1 (t, C-6)), 25.4 (t, C-2), 28.3 (q, C-18), 33.4 (t, C-1), 35.1 (s, C-10), 36.2 (t, C-12), 36.9 (s, C-4), 37.0 (s, C-13), 44.2 (d, C-5), 46.1 (t, C-14), 51.7 (d, C-9), 76.2 (d, C-3), 109.2 (t, C-16), 121.6 (d, C-7), 135.6 (s, C-8), 150.5 (d, C-15). [Found m/z (rel int.): 288.2452 (21.6). $C_{20}H_{32}O$ requires 288.2453. Other significant peaks in the HRMS were at m/z (rel. int.) : 273.2204(4.3) [M^+-CH_3], 255.2126 (35.2) [M^+-H_2O-CH_3]].

Fraction 5 (133.9 mg) was crystallised from petroleum ether (60 -80) to give **7,15-isopimaradien-3β-ol(10)**(24.6 mg), m.p. 145-146° C, $[\alpha]_D$-40° (c, 0,65 g in $CHCl_3$), [Lit [15] m.p. 146-147°C, $[\alpha]_D$ -36°], i.r. ν_{max} cm^{-1} 3620, 2968, 2929, 2855, 1638, 996, 912. ^1H n.m.r. [Lit [15,35]: δ 0.85 (s, 3H, Me-20), 0.88 (s, 3H, Me-17), 0.98 (s, 6H, Me-18, 19), 1.92 (br, 2H, 2H-

14), 3.24 (dd, 1H, J= 10.0, 5.0 Hz, H-3ax), 4.82 (dd, 1H, J=10.0, 2.0 Hz, H-16*cis*), 4.94 (dd, 1H, J=18.0, 2.0 Hz, H-16*trans*), 5.38 (1m, 1H, H-7), 5.82 (dd, 1H, J=18.0, 10.0 Hz, H-15).

^{13}C n.m.r : δ 14.9 (q, C-20), 15.6(q, C-19), 20.2(t, C-11), 21.5(q, C-17), 23.1(t, C-6), 27.5(t, C-2), 28.4(q, C-18), 35.4(s, C-10), 36.2(t, C-1), 36.8(C-13), 37.9(t, C-12), 38.6(s, C-4), 46.0(t, C-14), 50.1(d, C-5), 52.0(d, C-9), 79.3(d, C-3), 109.2(t, C-16), 121.5(d, C-7), 135.4 (s, C-8), 150.3(d, C-20). [Found m/z (rel. int.): 288.2453 (34.2). $C_{20}H_{32}O$ requires 288.2453. Other significant peaks in the HRMS were at m/z (rel. int.) : 270.2367(16.9) [M^+-H_2O], 255.2108(31.6) [$M^+-H_2O-CH_3$], 148.1248(41.1)].

The mother liquors of fraction 5 were acetylated with acetic anhydride (1ml) and pyridine (1ml) overnight. The product was purified by preparative t.l.c (petroleum ether (60-80)-ethyl acetate, 19:1) and yielded **3β-acetoxy-7, 15-isopimaradiene (45)** (51.1 mg) as a gum. ^{1}H n.m.r. : δ 0.86 (s, 3H, Me-17), 0.88 (s, 3H, Me-20), 0.98 (s, 6H, Me-18, 19), 2.05 (s, 3H, acetate), 4.59 (dd, 1H, J=3.0, 2.0 Hz, H-3ax), 4.82 (dd, 1H, J=10.0, 2.0 Hz, H-16*cis*), 4.94 (dd, 1H, J=18.0, 2.0 Hz, H-16*trans*), 5.38 (m, 1H, H-7), 5.82 (dd, 1H, J=18.0, 10.0 Hz, H-15).

Fraction 7 (67.9 mg) was purified by preparative t.l.c. (petroleum ether (60-80)- ethyl

acetate, 17:3) and afforded ***C-16* monomethyl agathate(21)** (10.2 mg), i.r. ν_{max} cm $^{-1}$ 2940, 1710, 1690, 980, 895, 810.

^{1}H n.m.r. [Lit [23]] : δ 0.85 (s, 3H, Me-20), 1.22 (s, 3H, Me-18), 2.13 (s, 3H, Me-17), 3.67 (s, 3H, -OMe), 4.49 (s, 1H, H-14), 4.86 (s, 1H, H-14), 5.63 (s, 1H, H-15).

^{13}C n.m.r. : δ 12.8 (q, C-20), 18.9 (q, C-18), 19.9 (t, C-2), 21.6 (t, C-6), 26.0 (t, C-11), 29.0 (q, C-9), 37.9 (t, C-7), 38.6 (t, C-12), 39.1 (t, C-1), 39.7 (t, C-3), 40.5 (s, C-10), 44.2 (s, C-4), 50.8 (q, C-21), 55.3 (d, C-5), 56.2 (d, C-9), 106.6 (t, C-17), 115.0 (d, C-14), 147.6 (s, C-8), 161.0 (s, C-13), 167.3 (s, C-15), 183.3 (s, C-19). [Found m/z (rel. int.): 348.2301 (2.6). $C_{21}H_{32}O_4$ requires 348.2301].

Fraction 14 (314.1 mg) was crystallised from chloroform and petroleum ether (60-80) to give **3α, 16-dihydroxy-7-isopimaren-15-one(46)** (86.1 mg), m.p. 116-117° C , $[\alpha]_D$ -27° (c, 1.12 in $CHCl_3$), i.r. ν_{max} cm^{-1} 3640, 3480, 2925, 2872, 1708, 1010, 925.

^{1}H n.m.r. : δ 0.89 (s, 3H, Me-20), 0.98 (s, 6H, Me-18, 19), 1.17 (s, 3H, Me-17), 3.45 (s, br, 1H, H-3eq), 4.38 (s, br, 2H-16), 5.48 (m, 1H, H-7).

^{13}C n.m.r. : δ 14.8 (q, C-20), 18.9 (q, C-17), 19.3 (t, C-11), 22.7 (q, C-19), 23.2 (t, C-6), 25.2 (t, C-2), 28.3 (q, C-18), 31.7 (t, C-12), 32.6 (t, C-1), 35.1 (s, C-10), 37.0 (s, C-4), 41.9 (t, C-14), 44.0 (d, C-5), 46.0 (s, C-13), 51.3 (d, C-9), 64.0 (t, C-16), 76.0 (d, C-3),

123.7 (d, C-7), 133.3 (s, C-8), 215.1 (s, C-15).
[Found m/z (rel. int.): 320.2351 (12.8). $C_{20}H_{32}O_3$ requires 320.2351. Other significant peaks in the HRMS were at m/z (rel. int.) : 302.223(29.0)[M^+-H_2O], 287.2009(57.1), 243.2105 (100) [M^+-H_2O, $COCH_2OH$]].

Fraction 18 (62.4 mg) was purified by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 1:1) to yield **15,16-dihydroxy-7-isopimaren-3-one(47)**(13.4 mg)(3-oxo-13-epipalarosan), m.p. 125-126°C (from petroleum ether (60-80)), $[\alpha]_D$-38° (c, 0.195 in $CHCl_3$ [Lit[40] m.p. 134°C, $[\alpha]_D$ -24°], i.r. ν_{max} cm^{-1} : 3400, 2930, 1710, 820.
^1H n.m.r. : δ 0.78 (s, 3H, Me-20), 1.05 (s, 6H, Me-19, 20), 1.11(s, 3H, Me-17), 2.67 (dd, 1H, J=15.0, 10.0, 5.0 Hz, H-2eq), 3.33 (dd, 1H, J=10.0 Hz, 2.5Hz, H-16a), 3.52 (dd, 1H, J=10.0, 10.0 Hz, H-15), 3.76 (dd, 1H, J=10.0 , 2.5 Hz, H-16b), 5.45 (m, 1H, H-7).
^{13}C n.m.r. : δ 14.7 (q, C-20), 17.2 (q, C-17), 19.8 (t, C-11), 22.6 (q, C-19), 23.8 (t, C-6), 25.5(q, C-18), 33.0 (C-12), 34.7 (t, C-2), 35.2 (s, C-10), 37.1 (s, C-13), 38.1 (t, C-1), 42.9 (t, C-14), 47.5 (s, C-4), 51.1 (d, C-5), 51.6 (d, C-9), 62.6 (t, C-16), 80.5 (d, C-15), 121.7 (d, C-7), 135.0 (s, C-8), 217.0 (s, C-3).
[Found m/z (rel. int.): 320.2352 (12.9). $C_{20}H_{32}O_3$ requires 320.2351. Other significant peaks in the HRMS were at m/z (rel. int.): 305.2080 (3.8) [M^+-CH_3], 287.2014 (15.1) [M^+-CH_3], 259.2059 (100), [M^+-

$CHOHCH_2OH$].

Combined fractions 21,22,23 (207 mg) were crystallised from petroleum ether (60-80) and a few drops of chloroform to afford **7-isopimarene-3α,15,16-triol(40)** (149.7 mg), m.p. 177-178°C, $[\alpha]_D$- 31° (c, 0.71 in $CHCl_3$), [Lit [33] m.p. 170-175°, $[\alpha]_D$ -22°], i.r. ν_{max} cm^{-1} 3610,3130,860.

^1H n.m.r. : δ 0.74 (s,3H,Me-13), 0.85 (s,3H,Me-20), 0.93 (s,3H,Me-19), 0.94 (s,3H,Me-18), 3.44 (s,br,1H,H-3eq), 3.34 (dd,1H,J=9.7 Hz, 2.6 Hz,H-16a), 3.52 (dd,1H,J=9.7 Hz, 9.7 Hz,H-15), 3.73 (dd, 1H, J=9.7 Hz, 2.6 Hz,H-16b), 5.38(m,1H,H-7).

^{13}C n.m.r. : δ 14.9 (q,C-20), 17.3 (q,C-17), 19.9 (t,C-11), 22.9 (q,C-19), 23.3 (t,C-6), 25.4 (t,C-2), 28.4(q,C-18), 32.0 (t,C-1), 33.4(t,C-12), 35.1 (s,C-10), 37.2 (s,C-13), 37.3 (s,C-4), 43.4 (t,C-14), 44.4 (d,C-5), 52.1 (d,C-9), 62.7 (t,C-16), 76.0 (d,C-3), 80.7 (d,C-15), 122.2 (d,C-7), 135.4 (s,C-8).

[Found m/z (rel. int.): 322.2507 (24.6). $C_{20}H_{34}O_3$ requires 322.2508. Other significant peaks in the HRMS were at m/z (rel. int.) : 307.2255 (24.5) [M^+-CH_3], 289.2170 (59.5) [M^+-H_2O-CH_3], 261.2214 (100) [M^+-$CHOHCH_2OH$]].

Part of the triol(40) (20.0 mg) was acetylated with acetic anhydride (1 ml)and pyridine (1ml) overnight. The product was purified by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 13:7)

to give **3α, 15, 16-triacetoxy-7-isopimarene (48)** (17.4 mg), i.r. ν_{max} cm^{-1} 2920, 1745, 1370, 1245.

^1H n.m.r. : δ 0.86 (s, 3H), 0.86 (s, 3H), 1.01 (s. 3H), 1.27 (s, 3H), 2.01 (s, 3H, acetate), 2.05 (s, 3H, acetate), 2.10 (s, 3H, acetate), 4.12 (dd, 1H, J=10.0 Hz, 3.0 Hz, H-16), 4.46 (dd, 1H, J=10.0, 3.0 Hz, H-16), 4.70 (s, br, 1H, H-3eq), 4.96 (dd, 1H, J=10.0, 10.0 Hz, H-15), 5.42 (m, 1H, H-7).

^{13}C n.m.r. : 14.8 (q, C-20), 17.8 (q, C-17), 19.5 (t, C-11), 22.4 (q, C-19), 22.7 (t, C-6), 22.9 (t, C-2), 27.9 (q, C-18), 32.5 (t, C-12), 33.1 (t, C-1), 35.0 (s, C-10), 36.2 (s, C-13), 37.0 (s, C-4), 42.6 (t, C-14), 45.1 (d, C-5), 51.6 (d, C-9), 63.2 (t, C-16), 78.3 (2) (d, C-3, 15), 122.5 (d, C-7), 134.3 (s, C-8), 171.0 (2) and 170.7 (s, acetate C=O), 20.9 (2) and 21.3 (q, acetate methyls). [Found m/z (rel. int.): 448.2825 (0.5). $C_{26}H_{40}O_6$ requires 448.2825. Other significant peaks in the HRMS were at m/z (rel.int.): 388.2616 (22.8) [M^+-CH_3COOH], 373.2394 (22.4) [M^+-CH_3, CH_3COOH]].

7-Isopimarene-3α, 15, 16-triol (40) (50.2 mg) was stirred in dry acetone in the presence of anhydrous copper sulphate. The product was purified by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 1:1) to yield the **acetonide (49)** (29.5 mg), i.r. ν_{max} cm^{-1} 3620, 1370, 860.

^1H n.m.r. : δ 0.78 (s, 3H), 0.89 (s, 3H), 0.98 (s, br, 6H), 1.06 (s, 3H), 1.14 (s, 3H), 1.36 and 1.41

(s, each 3H, acetonide), 3.47 (s, br, 1H, H-3eq), 3.76-3.92 (m, 3H, H-15, 2H-16), 5.41 (m, 1H, H-7).

^{13}C n.m.r. : δ 14.8 (q, C-20), 17.3 (q, C-17), 19.5 (t, C-11), 22.7 (q, C-19), 23.0 (t, C-6), 25.1 (t, C-2), 28.3 (q, C-18), 31.8 (t, C-8), 33.1 (t, C-1), 35.0 (s, C-10), 36.0 (s, C-13), 37.0 (s, C-4), 42.6 (t, C-14), 44.1 (d, C-5), 51.9 (d, C-9), 64.8 (t, C-16), 76.1 (d, C-3), 84.3 (d, C-15), 121.9 (d, C-7), 135.0 (s, C-8), {acetonide: 108.6 (s, O-C-O), 26.3 and 25.3 (both q, methyls)}. [Low resolution MS m/z (rel. int.) : 362.0 (1.9). $C_{23}H_{38}O_3$ requires 362.0. Other significant peaks m/z (rel. int.) : 347.0 (1.9)[M^+-CH_3], 261.1 (1.9) [$M^+-C_5H_9O_2$]].

The **acetonide (49)** was further subjected to Collins' oxidation. Chromium trioxide (48 mg) was added to a stirred solution of pyridine (78 mg) in dry methylene chloride (3 ml) and the mixture stirred for 15 minutes. The **acetonide (49)** (15.2 mg) in dry methylene chloride was added to the reaction flask and stirring continued for one hour. The product was purified by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 7:3) to yield the keto-acetonide (8.8 mg) . ^{1}H n.m.r. : δ 0.78 (s, 3H), 1.05 (s, br, 6H), 1.01 (s, 3H), 1.42 and 1.48 (each s, 3H, acetonide), 3.71-3.95 (3H, m), 5.45 (m, 1H).

The keto-acetonide was dissolved in aqueous acetic acid (5ml) (water-acetic acid- 1:19) and the

solution heated under reflux for two hours. Separation by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 7:3) afforded **15,16,-dihydroxy-7-isopimaren-3-one(47)** (2.2 mg) identical with an authentic specimen.

INFECTED WOOD

The powdered infected wood (1239.3 gm) was extracted with hot petroleum ether for two days and the extract was concentrated to yield a brownish viscous material (17.7 g). Part of the extract (4.5 g) was adsorbed on Silica GF_{254} (10 g) and chromatographed over the same adsorbent (120 g). The extract was eluted with petroleum ether (60-80) and petroleum ether (60-80) containing increasing proportion of ethyl acetate and finally with ethyl acetate containing increasing proportions of methanol. About 100ml fraction of the eluate being collected as follow: 1 (petroleum ether (60-80)-ethyl acetate, 9:1), 2-5 (petroleum ether- (60-80)-ethyl acetate, 4:1), 6-10 (petroleum ether (60-80)-ethyl acetate, 7:3), 11-18 (petroleum ether (60-80)-ethyl acetate, 3:2), 19-25 (petroleum ether (60-80)-ethyl acetate, 1:1), 26-29 (petroleum ether (60-80)-ethyl acetate, 2:3), 30-32 (petroleum ether (60-80)-ethyl acetate, 3:7), 33-36 (petroleum ether (60-80-ethyl acetate, 1:4), 37 (petroleum ether (60-80)-ethyl acetate, 1:9),

38 (100% ethyl acetate), 39-40 (ethyl acetate-methanol, 4:10), 41 (ethyl acetate-methanol, 3:2), 43 (ethyl acetate-methanol, 1:4) and 44 (100% methanol).

The crude fraction 6 (65.9 mg) was crystallised from petroleum ether (60 -80) and afforded **hinokione (50)** (38.6 mg), m.p. 194-195°C, $[\alpha]_D$+128° (c, 0.33 in $CHCl_3$), [Lit [45] m.p. 191-192°C, $[\alpha]_D$ + 111.9°], i.r. ν_{max} cm^{-1} 3620, 2970, 1710, 1510, 1390, 1370, 1170, 1120.

^1H n.m.r. : δ 1.11 (s, 3H, Me-18), 1.14 (s, 3H, Me-19), 1.18 (s, 3H, Me-20), 1.22 and 1.23 (each d, 3H, J= 6.9 Hz, Me-16, 17), 3.13 (septet, 1H, J=8.0 Hz, H-15), 6.62 (s, 1H, H-11), 6.85(s, 1H, H-12).

^{13}C n.m.r. : δ 20.5 (t, C-6), 21.1 (q, C-19), 21.7 (q, C-16), 22.6 (q, C-17), 24.5 (q, C-20), 27.0 (d, C-15), 27.0 (q, C-18), 30.2 (t, C-7), 34.7 (t, C-2), 37.2 (s, C-10), 37.7 (t, C-1), 47.5 (s, C-4), 50.9 (d, C-5), 112.0 (d, C-11), 126.8 (s, C-8, 14), 231.6 (s, C-13), 145.8 (s, c-9), 151.4 (s, C-12), 217.4(s, C-3). [Found m/z (rel. int.): 300.2089 (100). $C_{20}H_{28}O_2$ requires 300.2089. Other significant peaks in the HRMS were at m/z (rel. int.): 285.1852(38.67)[M^+-CH_3], 243.1389 (23.47) [$M^+-CH_3-CH_2CO$]].

Fraction 10 (16.0 mg) was purified on preparative t.l.c. (petroleum ether (60-80)-chloroform-ethyl acetate, 30:70:7). The main band was crystallised from petroleum ether (60-80) and gave

3β, 12-dihydroxy-8, 11, 13-abietatrien-2-one(51) (3.6 mg), m.p. 197-198°C, $[\alpha]_D$+32 (c, 0.195 in $CHCl_3$), i.r. ν_{max} cm^{-1}: 3630, 3510, 2980, 1725.

^1H n.m.r. : δ 0.78 (s, 3H, Me-20), 1.15 (d, 3H, J_4= 0.6 Hz, Me-18), 1.22 and 1.23 (each d, 3H, J=6.9 Hz, Me-16, 17), 1.26 (s, 3H, Me-19), 2.87 (m, 2H), 2.63 (d, 1H, J=12.3 Hz, H-1ax), 3.11 (d, 1H, J=12.3 Hz, H-1eq), 3.12 (septet, 1H, J=6.9 Hz, H-15), 3.50 (s, OH, D_2O exchangeable), 3.97 (s, 1H, H-3), 4.94 (br, OH, D_2O exchangeable), 6.49 (s, 1H, H-11), 6.68 (s, 1H, H-14).

^{13}C n.m.r. : δ 16.3 (q, C-20), 19.2 (t, C-6), 22.6 (q, C-16), 22.7 (q, C-17), 25.8 (q, C-19), 26.9 (q, C-16), 29.2 (q, C-18), 29.8 (t, C-7), 43.5 (s, C-10), 45.0 (s, C-4), 49.2 (d, C-5), 51.8 (t, C-1), 82.8 (d, C-3), 110.8 (d, C-11), 126.9 (s, C-8), 127.0 (d, C-14), 133.0 (s, C-13), 145.3 (s, C-9), 151.4 (s, C-12), 210.9 (s, C-2).

[Found m/z (rel. int.): 316.2039 (100). $C_{20}H_{28}O_3$ requires 316.2039. Other significant peaks in the HRMS were at m/z (rel. int.): 301.1824 (16.9) $[M^+-CH_3]$, 243.1756 (63.7) $[M^+-C_3H_5O]$, 201.1287 (63.7) $[M^+-115]$].

Fraction 20 (495.5 mg) was crystallised from petroleum ether 60-80 and afforded **3β, 16-dihydroxy-7-isopimarene-2, 15-dione (Araucarolone)(6)** (217.0 mg), m.p. 153-154°C, $[\alpha]_D$-44° (c, 1.635 in $CHCl_3$), [Lit [15] m.p. 157-159°C, $[\alpha]_D$ -42°], i.r. ν_{max} cm^{-1} : 3590, 2960, 1705, 995, 830.

^1H n.m.r. : δ 0.78 (s, 3H, Me-20), 0.88 (s, 3H, Me-19),

1.07 (s, 3H, Me-18), 1.17 (s, 3H, Me-17), 2.25 (d, 1H, J=12.0 Hz, H-1), 2.62 (d, 1H, J=12.0 Hz, H-1ax), 3.45 (br, OH, D_2O exchangeable), 3.97 (br, 1H, H-3), 4.36 (br, 2H, 2H-16), 4.42 (br, OH, D_2O exchangeable), 5.55 (br, 1H, H-17)

^{13}C n.m.r. : δ 15.5 (q, C-20), 16.4 (q, C-19), 19.0 (q, C-17), 19.6 (t, C-11), 19.6.6 (t, C-6), 28.7 (q, C-18), 32.4 (t, C-12), 41.6 (t, C-14), 42.3 (t, C-10), 45.1 (s, C-4), 45.8 (s, C-13), 49.2 (d, C-5), 51.6 (d, C-9), 60.0 (t, C-1), 64.0 (t, C-16), 82.4 (d, C-3), 123.6 (d, C-7), 133.1 (s, C-8), 210.3 (s, C-2), 214.5 (s, C-15).

[Found m/z (rel. int.) : 334.2144 (68.4). $C_{20}H_{30}O_4$ requires 334.2144. Other significant peaks in the HRMS were at m/z (rel. int.): 319.1903 (26.4) [M^+-CH_3], 303.1966 (100) [M^+-CH_2OH], 275.1997 (78.3) [M^+-$COCH_2OH$]].

Fraction 21 (69.0 mg) was purified by preparative t.l.c (petroleum ether (60-80)-chloroform-ethyl acetate, 30:70:7) and gave **hinokiresinol(36)** (25 mg) which polymerised within several hours.

^1H n.m.r. : δ 4.12 (t, 1H, J=6.8 Hz, H-3), 5.15 (m, 2H-5), 6.06(ddd, J=17.0, 10.3, 6.8 Hz, H-4), 6.20 (dd, J=16.0, 6.8 Hz, H-2), 6.32 (br, d, 1H, J=16.0 Hz, H-1), 6.76 (d, 2H, J=8.7 Hz), 6.70 (d, 2H, J=8.7 Hz), 7.11 (d, 2H, J=8.7 Hz), 7.24 (d, 2H, J=8.7 Hz),

^{13}C n.m.r. :

C-Atom	Aromatic-C	Aromatic-C
1 129.7 (d)	154.8 (s)	154.0 (s)
2 129.9 (d)	115.4 (d)-2C	115.3 (d)-2C
3 51.4 (d)	129.2 (d)-2C	127.5 (d)-2C
4 140.4 (d)	135.0 (s)	130.3 (s)
5 115.2 (t)		

Fraction 26 (78.0 mg) was separated into four bands by preparative t.l.c. (petroleum ether (60-80)-chloroform-ethyl acetate, 50:50;7). Band 1 (the least polar band) was crystallised from chloroform and gave **16-hydroxy-7-isopimarene-3,15-dione (araucarone) (7)**(23.0 mg), m.p. 104-105°C , $[\alpha]_D$-37° (c, 0.13 in $CHCl_3$), [Lit [16] 115-116°C, $[\alpha]_D$ -51°], i.r. ν_{max} cm^{-1} : 3500, 2980, 1710, 850.

^1H n.m.r. : δ 1.07 (s, 3H, Me-20), 1.08 (s, 6H, Me-18, 19), 1.12 (s, 3H, Me-17), 2.66 (ddd, 1H, J=16.0, 10.0, 3.0 Hz, H-2eq), 3.24 (br, OH, D_2O exchangeable), 4.36 (s, br, 2H-16), 5.48 (m, 1H, H-7).

^{13}C n.m.r. : δ 14.8(q, C-20), 18.9 (q, C-17), 19.5 (t, C-11), 22.6 (q, C-19), 23.9 (t, C-6), 25.6 (q, C-18), 32.5 (t, C-12), 34.6 (t, C-2), 35.2 (s, C-10), 37.9 (t, C-1), 41.7 (t, C-14), 45.9 (s, C-13), 47.4 (s, C-4), 50.6 (d, C-5), 51.5 (d, C-9), 64.0 (t, C-16), 123.2 (d, C-7), 133.3 (s, C-8), 214.8 (s, C-15), 216.4 (s, C-3).

[Found m/z (rel. int.): 318.2195 (45.9). $C_{20}H_{30}O_3$ requires 318.2193. Other significant peak in the HRMS were at m/z (rel. int.) : 287.2010 (81.4) [M^+-CH_2OH], 259.2071 (100) [M^+-COCH_2OH]].

Band 2 (9.0 mg) was identified as **2,16-dihydroxy-1,7-isopimaradiene-3,15-dione (Araucarenolone) (9)**(9.0 mg) which polymerised within several hours of its isolation. ^1H n.m.r. : δ 1.08 (s,3H,H-17), 1.20 (s,3H,H-18), 1.24 (s,3H,H-19), 1.58 (s,3H,H-20), 3.24 (br,OH,D_2O exchangeable), 4.38 (s,br,,2H-16), 5.56 (br,1H-H7), 6.02 (br,OH,D_2O exchangeable), 6.19(s,1H-H1).

^{13}C n.m.r. : δ 15.5 (q,C-20), 18.6 (q,C-17), 18.9 (t,C-11), 22.1 (q,C-19), 22.4 (t,C-6), 24.9 (q,C-18), 31.9 (t,C-12), 36.0 (s,C-10), 41.5 (t,C-14), 43.0 (s,C-4), 45.5 (s,C-13), 47.6 (d,C-5), 48.1 (d,C-9), 63.6 (t,C-16), 123.1 (d,C-1), 123.3 (d,C-7), 132.7 (s,C-8), 143.3 (s,C-2), 213.8 (s,C-15), 215.9 (s,C-3).

Band 3 was crystallised from petroleum ether (60-80) and gave **3β,16-hydroxy-7-isopimaren-15-one (Araucarol)(8)** (8.0 mg), m.p. 117-119°C, $[\alpha]_D$ + 14.4 (c, 0.09 in $CHCl_3$), [Lit [16] m.p. 135-136°C, $[\alpha]_D$-24°], i.r. ν_{max} cm $^{-1}$ 3630, 3480, 2925, 2850, 1705, 1020, 995, 840.

^1H n.m.r. : δ 0.86 (s,3H,Me-20), 0.94 (s,6H,Me-18,19), 1.05 (s,3H,Me-17), 3.28(dd,J=10.0,5.0 Hz, H-3ax), 4.36 (br,2H,2H-16), 4.39 (s,-OH,D_2O

exchangeable), 5.48 (br, 1H, H-7).

^{13}C n.m.r. : δ 14.9 (q, C-20), 15.6 (q, C-19), 18.9 (q, C-17), 19.3 9t, C-11), 23.9 9t, C-6), 27.4 (t, C-2), 28.3 (q, C-18), 32.6 (t, C-12), 35.4 (s, C-10), 37.8 (t, C-1), 38.6 (s, C-4), 41.7 (t, C-14), 45.9 (s, C-13), 49.8 (d, C-5), 51.5 (d, C-9), 64.0 (t, C-16), 79.2 (d, C-3), 123.5 (d, C-7), 133.1 (t, C-8), 215.1 (t, C-15).

[Found m/z (rel.int.) : 320.2352 (3.2). $C_{20}H_{32}O_3$ requires 320.2351. Other significant peaks in the HRMS were at m/z (rel. int.): 305.2135 (2.4) $[M^+-CH_3]$, 302.2248 (3.0) $[M^+-H_2O]$, 287.2016 (4.6) $[M^+-CH_3-H_2O]$, 243.2102 (7.9) $[M^+-COCH_2OH]$].

Crystallisation of band 4, the most polar band, from petroleum ether (60-80) afforded **3α, 16-dihydroxy-7-isopimaren-15-one(46)**(25 mg) (see page 35).

Fraction 37 (51.9 mg) was purified by preparative t.l.c. (chloroform-ethyl acetate, 1:1, three developments) and afforded **3α, 15, 16-trihydroxy-7-isopimaren-2-one(53)** (7.0 mg) and **3ß, 15, 16-trihydroxy-7-isopimaren-2-one(54)**(20.7 mg).

(1) 3α, 15, 16-trihydroxy-7-isopimaren-2-one(53)

i.r. ν_{max} cm^{-1} : 3620, 3490, 2960, 2920, 1695, 1255.

^1H n.m.r.: δ 1.00 (s, 3H, Me-20), 1.06 (s, 3H, Me-18), 1.07 (s, 3H, Me-19), 1.10(s, 3H, Me-17), 2.12 (d, 1H, J=13.8 Hz, H-1), 2.21 (d, 1H, J=13.8 Hz, H-1), 3.22 (br, OH),

3.40-3.80 (br, 3H, H-15, 2H-16), 4.00 (s, br, OH), 4.38 (s, 1H, H-3), 5.46 (t, 1H, J=2.7 Hz, H-7).

^{13}C n.m.r : 16.7 (q, C-20), 17.4 (q, C-17), 19.3 (t, C-11), 19.9 (q, C-19), 23.1 (t, C-6), 30.0 (q, C-18), 32.5 (t, C-12), 34.5 (s, C-10), 37.8 (s, C-13), 41.7 (t, C-1), 43.4 (t, C-140, 45.9 (s, C-4), 49.7 (d, C-9), 52.4 (d, C-5), 64.0 (t, C-16), 71.1 (d, C-3), 78.2 (d, C-15), 123.8 (d, C-7), 132.7 (s, C-8), (C-2 not detected).

[Found m/z (rel. int.): 336.2301 (7.4). $C_{20}H_{32}O_4$ requires 336.2301. Other significant peaks in the HRMS were at m/z (rel. int.) : 321.2084 (5.8) [M^+-CH_3], 319.2262 (3.1) [M^+-H_2O], 275.2006 (21%) [M^+-$CHOHCH_2OH$], 105.0698 (68 %), 55.0548 (100)].

(ii) 3β, 15, 16-trihydroxy-7-isopimaren-2-one(54)

i.r. ν_{max} cm^{-1} 36400, 3500, 2980, 2960, 1715, 1260.

^{1}H n.m.r. : δ 0.75 (s, 6H, Me-18, 19), 0.85 (s, 3H, Me-20), 1.15 (s, 3H, Me-17), 2.27 (d, 1H, J=12.5 Hz), 2.59 (d, 1H, J=12.5 Hz), 3.34 (dd, 1H, J=9.4 Hz, 2.6 Hz), 3.76 (dd, 1H, J=10.5, 2.6 Hz), 3.48 (dd, 1H, J=10.5, 9.4 Hz), 3.93 (s, 1H, H-3ax), 5.43 (t, 1H, J=2.6 Hz).

^{13}C n.m.r. : δ 15.5 (q, C-20), 16.4 (q, C-19), 17.2 (q, C-17), 19.8 (t, C-11), 23.3 (t, C-6), 28.7 (q, C-18), 32.8 (t, C-12), 42.4 (s, C-10), 42.6 (t, C-14), 45.2 (s, C-4), 49.0 (d, C-9), 51.6 (t, C-1), 52.3 (d, C-5), 62.6 (t, C-16), 80.4 (d, C-15), 82.4 (d, C-3), 122.0 (d, C-7), 134.6 (s, C-8), 210.9 (s, C-2), C-13 not

detected. [Found m/z (rel. int.): 336.2301 (5.6). $C_{20}H_{32}O_4$ requires 336.2301. Other significant peaks in the HRMS were at m/z (rel. int.) : 321.2065 (6.2)[M^+-CH_3], 319.1919 (3.7) [M^+-H_2O], 275.2007 (57.2) [M^+-$CHOHCH_2OH$], 105.0609 (100)].

The infected wood was further extracted with methanol and a viscous brownish material was obtained after evaporating the solvent. The extract (17 g) was chromatographed on silica gel GF_{254} (120 g). Mainly polymerised material was obtained and further purification by preparative t.l.c. was unsuccessful. However, a fraction (258.9 mg), from 40% ethyl acetate in petroleum ether (60-80), was acetylated with acetic anhydride (1ml) and pyridine (1ml) overnight. The acetylated product was purified by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 7:3, three developments) and yielded **agatharesinol tetraacetate (55)** as an oil.

$[\alpha]_D$ -3.7° (c, 5.16 in $CHCl_3$), [Lit [15] -19° (acetone)], i.r. ν_{max} cm^{-1} 1762, 1743, 1500, 1365, 1190, 900.

^1H n.m.r. : δ 1.88 (s, 3H, acetate), 2.05 (s, 3H, acetate), 2.66 (s, 6H, acetate), 3.80 (t, 1H, J=8.3 Hz, H-3), 4.07 (dd, 1H, J=12.0, 6.7 Hz, H-5), 4.40 (dd, 1H, J=12.0, 3.1 Hz, H-5), 5.51 (ddd, 1H, J=8.3, 6.7, 3.1 Hz, H-4), 6.26 (dd, 1H, J=15.8, 8.3 Hz, H-2), 6.48 (d, 1H, J=15.8 Hz, H-1), 7.04 (m. 4H), 7.31(m, 4H),

^{13}C n.m.r :

C-Atom	Aromatic-C	Aromatic-C
1 129.1 (d)	149.5 (s)	150.0 (s)
2 131.8 (d)	121.6 (d)-2C	122.0 (d)-2C
3 49.9 (d)	127.2 (d)-2C	127.9 (d)-2C
4 72.7 (d)	136.9 (s)	134.2 (s)
5 63.8 (t)		

Acetate : 169.2(2)(s), 170,5(s), 170.0(s),
20.9(2)(q), 20.6(2) (q)

[Cal. for $C_{25}H_{26}O_8$: 454.1628. No parent ion detected. Significant peaks in the HRMS were at m/z (rel. int.) : 394.1416 (16.5)[$M^{+}-CH_3COOH$], 309.1106 (24.1) [$M^{+}-C_6H_9O_4$], 267.1010 (45.8) [$M^{+}-C_8H_{11}O_5$], 121.0291 (100)[$C_7H_5O_2$]].

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CHAPTER II

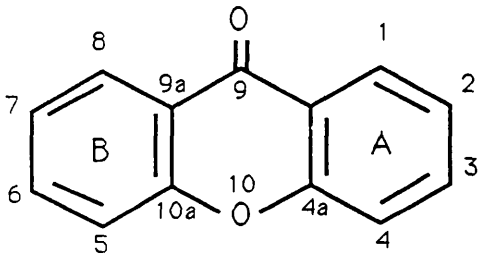
REVIEW OF *Calophyllum* PRODUCTS.

THE EXTRACTIVES OF *Calophyllum biflorum*

REVIEW OF THE CHEMISTRY OF CALOPHYLLUM PRODUCTS

INTRODUCTION

Calophyllum species the sub-family **Calophylloideae** a large genus of the family Guttiferae and are normally found in the warm humid tropical conditions. There are 49 ***Calophyllum*** species known to occur in the Malay Peninsula [1]. ***Calophyllum inophyllum*** is the most widely distributed of the ***Calophyllum*** species and is found mainly in the lowland area, particularly in coastal forest. The timber, which is locally known as "bintangor", varies in quality between species and is generally strong and durable and has been widely used for railway sleepers, machinery and cabinet work [2]. The trees grow very slowly and are not suitable for reforestation but they can be used as roadside shade trees since they are strong and not readily broken. Occasionally the sapwood and heartwood are distinct. The colour of the heartwood varies from pink to light mahagony and it is commercially agreed that the darker the colour the better the quality of the wood. Close examination of the heartwood shows the presence of the minute white or bright yellow sticky deposits within the cavities. Initial chemical investigations of ***Calophyllum*** species were mainly concerned with the constituents of the seed oil. Recently, reports have appeared on the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1
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B
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3
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10a
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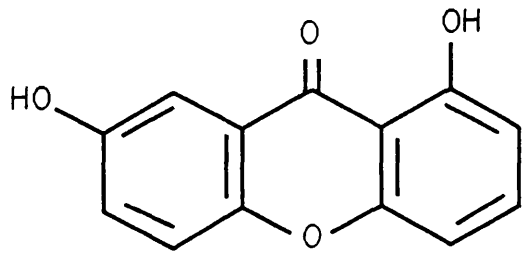
4

1.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HO
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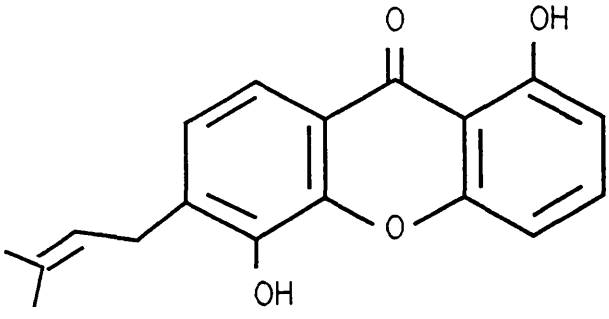
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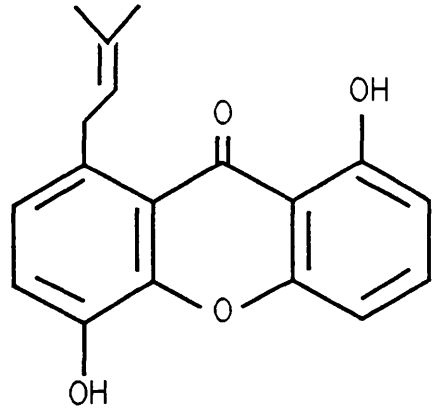
3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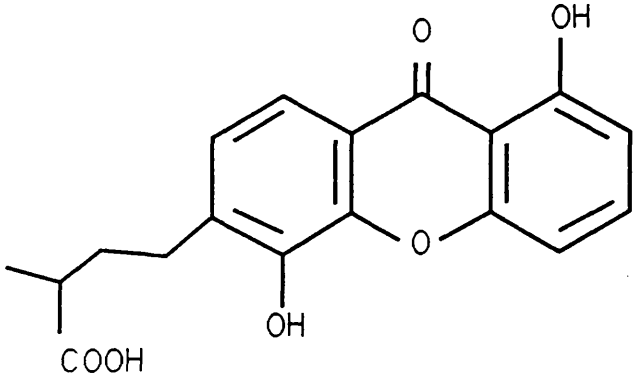
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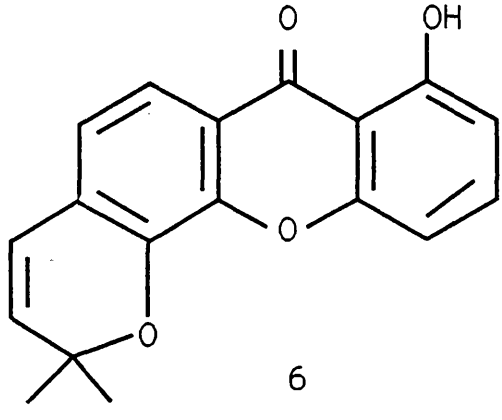
5.


O
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6

constituents of the bark, leaves and heartwood. Several reviews [3-5] of the constituents of ***Guttifereae*** family have been reported which discuss in detail the chemistry of ***Calophyllum*** The recent review by Bennet and Lee [5] reported that over eighty new compounds have been isolated since the last review in 1980. Approximately 110 ***Calophyllum*** species have been identified so far and about 32 species have been chemically investigated. The results of these investigations are reviewed below.

XANTHONES

Xanthones, prenylated xanthones and related benzophenones have been isolated from 21 species and can be classified according to oxygenation pattern. To take account of their mixed biogenetic origin xanthones are numbered 1-4 in the acetate derived ring A, normally characterised by 1,3-dioxygenation, and 5-8 in the shikimate derived ring B(1). However, if only ring B is oxygenated the lowest numbers are used except for biosynthetic discussions.

The structure of simple oxygenated xanthones is normally elucidated by analysis of UV, i.r. and ^1H n.m.r. data. The UV spectrum varies in a characteristic manner depending on the oxygenation pattern. Additional information on the location of hydroxyl groups may be obtained from the changes of the UV

spectra on addition of $AlCl_3$, a shift reagent for chelated hydroxyls, NaOAc, NaOH and boric acid. These data are useful for preliminary assignment which can then be substantiated by n.m.r. chemical shift data. Recently ^{13}C n.m.r. recently has been widely used for the determination of xanthone structures [10].

Further confirmation of the structure may be obtained by synthesis. The conventional method of xanthone synthesis is condensation of an *ortho*-oxygenated benzoic acid and a reactive phenol in the presence of phosphorous oxychloride and zinc chloride [11].

Mono-oxygenated xanthones

Only two mono-oxygenated xanthones have been isolated from ***Calophyllum*** species.

1. **2-hydroxyxanthone**

   C. cordato-oblongum [12], *C. trapezifolium* [13]

   C. zeylanicum [14]

2. **4-hydroxyxanthone**

   C. brasilliense [15,16], *C. cordato-oblongum* [12]

Di-oxygenated xanthones

Four types of dioxygenated xanthone are found and 1,5 -and 1,7 xanthone substitution is common.

1. **1,5-dihydroxyxanthone**

   C. bracteatum [17], *C. pulcherrimum* [16]

   C. tomentosum [13], *C. trapezifolium* [19]

   C. walkeri [20], *C. zeylanicum* [14]

2. **1,7-dihydroxyxanthone(2)**

   C. bracteatum [17], *C. calaba* [17], *C. cuneifolium* [21]

   C. fragrans [22], *C. inophyllum* [23]

   C. pulcherrimum [18], *C. ramiflorum* [24]

   C. sclerophyllum [25], *C. soulttri* [21]

   C. thwaitesii [20], *C. tomentosum* [13]

   C. trapezifolium [19], *C. walkeri* [20]

   C. zeylanicum [14]

3. **1-hydroxy-5-methoxyxanthone**

   C. soulattri [21]

4. **3-hydroxy-4-methoxyxanthone**

   C. cordato-oblongum [12]

Tri-oxygenated xanthones

Only 1,5,6-trihydroxyxanthone has been found but methyl ethers occur widely, particularly 1,6-dihydroxy-5-methoxyxanthone (Buchanoxanthone).

1. **1,6-dihydroxy-5-methoxyxanthone**

   C. calaba [17], *C. cordato-oblongum* [12]

   C. cuneifolium [21], *C. fragrans* [22], *C. inophyllum* [23]

   C. soulattri [21], *C. tomentosum* [13]

   C. trapezifolium [13], *C. walkeri* [20]

2. **1,7-dihydroxy-3-methoxyxanthone**

   C. brasilliense [16]

3. **2,8-dihydroxy-1-methoxyxanthone**

   C. calaba [17]

4. **8-hydoxy-1,2-dimethoxyxanthone**

   C. fragrans [22]

5. **2-hydroxy-1,8-dimethoxyxanthone**

   C. calaba [17]

6. **1-hydroxy-3,7-dimethoxyxanthone**

   C. brasilliense [15,16]

7. **1-hydroxy-6,7-dimethoxyxanthone**

   C. ramiflorum [24]

8. **1,5,6-trihydroxyxanthone**

   C. calaba [17], *C. cordato-oblongum* [12], *C. fragrans* [22]

   C. inophyllum [23], *C. scriblitifolium* [26]

Tetraoxygenated xanthones

Very few examples of this type of xanthone have been reported. Only one has four free hydroxyl groups. The others are methyl ethers.

1. **1,5-dihydroxy-2,3-dimethoxyxanthone**

   C. walkeri [20]

2. **1,7-dihydroxy-3,6-dimethoxyxanthone**

   C. inophyllum [27]

3. **3,8-dihydroxy-1,2-dimethoxyxanthone**

   C. trapizefolium [19]

4. **1,3,5-trihydroxy-2-methoxyxanthone**

   C. bracteatum [17]

5. **1,3,5,6-tetrahydroxy xanthone**

   C. sclerophyllum [25]

6. **3,5,6-trihydroxy-1-methoxyxanthone**

   C. Sclerophyllum[25]

Pentaoxygenated xanthones

The only xanthone of this type to be discovered is 1,8-dihydroxy-2,3,7-trimethoxyxanthone from ***C. bracteatum*** [17].

Alkylated xanthones

The mono and di-C5 substituted alkylated xanthone are predominantly found in the Guttiferae family. The C5 substituent usually occurs as 3-methyl-but-2-propenyl or less often 1,1-dimethylprop-2-enyl. The presence of prenyl and geranyl groups on the xanthone nucleus can be of chemotaxonomic value. In many cases the alkylated group has undergone oxidative cyclization with a hydroxyl group giving 2,2-dimethyl-pyrano (chromene ring), dihydropyrano, 2,2,3-tri-methylfurano (possible artefacts) and occasionally 2-isopropenyldihydrofurano compounds. In some cases hydroxylation or dehydration of the side chain may occur. Alkylated xanthones are limited largely to 1,3,5-, 1,3,7-, 1,3,5,6-, 1,3,6,7- oxygenation. Di-

and penta-oxygenated compounds are rare and no mono-oxygenated alkylated xanthone has been reported so far.

Mono-C5-Dioxygenated xanthones

In this group, two types of structure are found with the prenyl group intact or in a modified form such as a chromene ring or a carboxylic acid group.

1. **Guanandin**(3)

   C. bracteatum [17], *C. brasilliense* [15,28]

   C. calaba [17], *C. cuneifolium* [21], *C. inophyllum* [27]

   C. scribitifolium [27], *C. walkeri* [20]

2. **Isoguanandin**(4)

   C. brasilliense [15,28]

3. **Scribilitifolic acid**(5)

   C. calaba [17], *C. cordato-oblongum* [12],

   C. cuneifolium [21], *C. scribitofolium* [27]

4. **Dehydrocycloguanandin**(6)

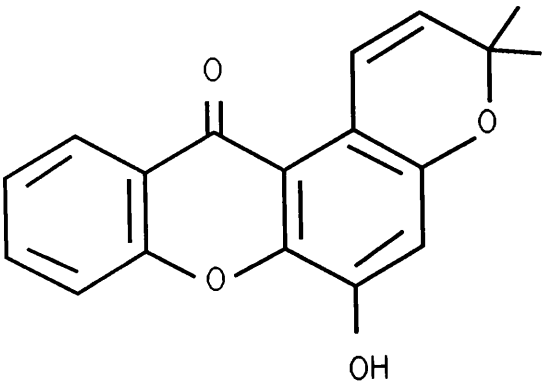
   C. brasilliense [28]

5. **Cordato-oblonguxanthone**(7)

   C. cordato-oblongum [12]

Mono-C5-trioxygenated xanthones

Examples of 2-or 4-prenylated xanthones from have been found in several species. Cyclization of the prenyl group is common, with 6-deoxyjacareubin(8) occurring widely.



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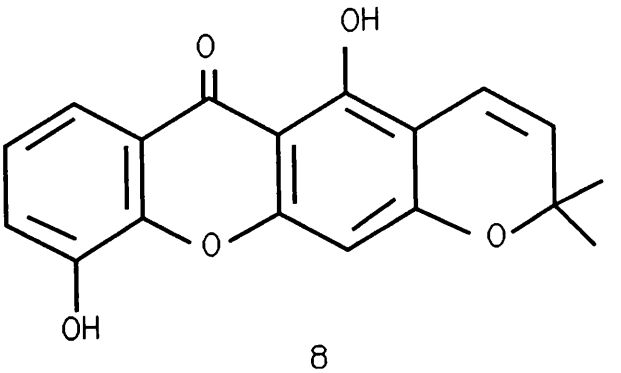

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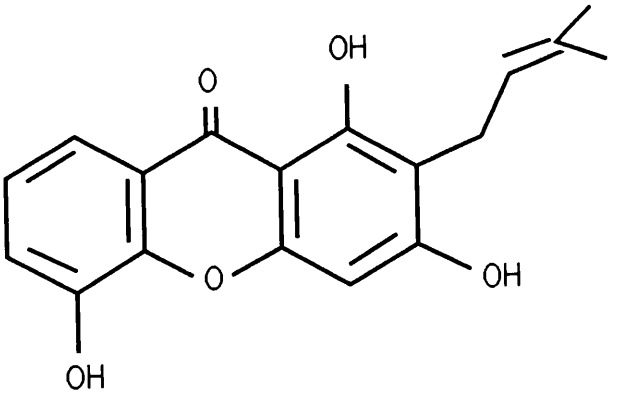

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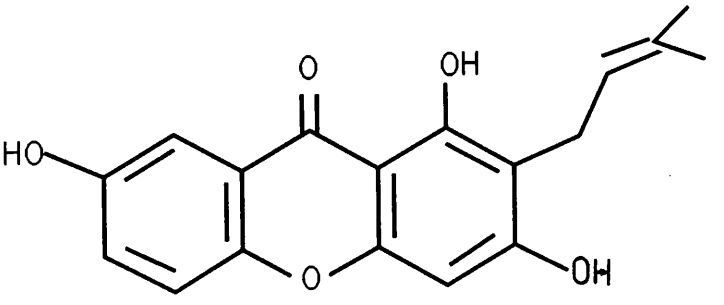

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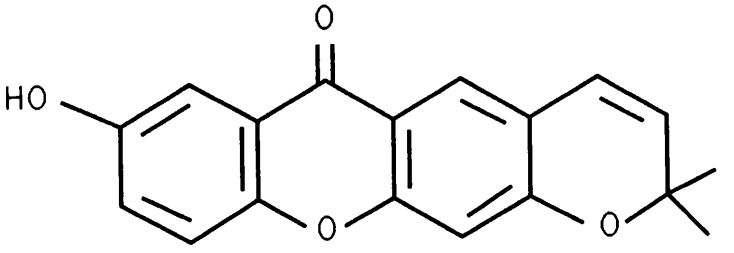


11

1. **6-Deoxyjacareubin(8)**

   C. bracteatum [17], *C. brasilliense* [28], *C. calaba* [17]

   C. cuneifolium [21], *C. inophyllum* [23,27]

   C. neo-ebudicum [29], *C. scribilitifolium* [26]

   C. soulattri [21], *C. tomentosum* [13]

   C. trapezifolium [13], *C. zeylanicum* [14]

2. **1,3,5-trihydroxy-2-(3-methylbut-2-enyl)xanthone(9)**

   C. cuneifolium [21], *C. scribitifolium* [26]

   C. soulattri [21], *C. tomentosum* [13], *C. walkeri* [20]

3. **1,3,7-trihydroxy-2-(3-methylbut-2-enyl)xanthone(10)**

   C. canum [30], *C. neo-ebudicum* [29], *C. scriblitifolium* [26]

4. **Osajaxanthone(11)**

   C. brasilliense [28], *C. canum* [30]

   C. scribitifolium [26]

Di-C5-Trioxygenated xanthones

In this group calabaxanthone is the most widespread. Trapezifolixanthone **(13)** and thwaitesixanthone **(15)** are found in several species while the remaining compounds are less common.

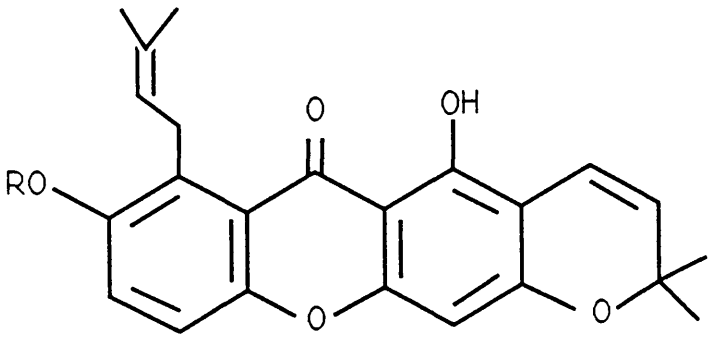
1. **Calabaxanthone(12)**

   C. bracteatum [17], *C. calaba* [17,31]

   C. cuneifolium [21], *C. tomentosum* [13]

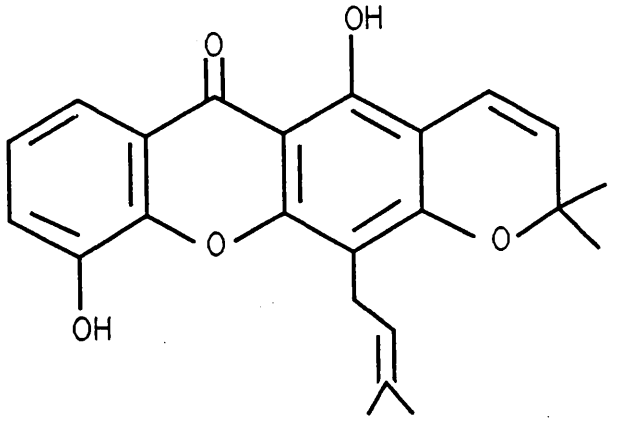
   C. trapezifolium [32], *C. walkeri* [20]

   C. zeylanicum [14]

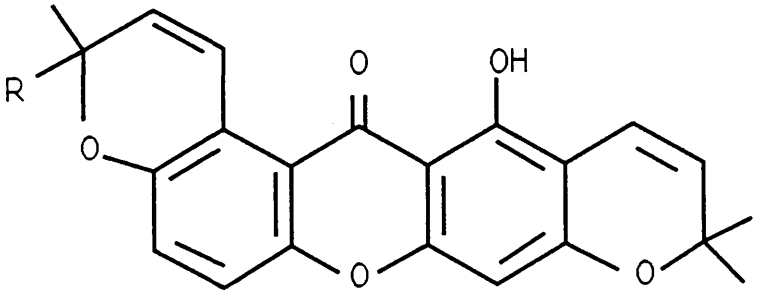


(12) R = Me

(14) R= H

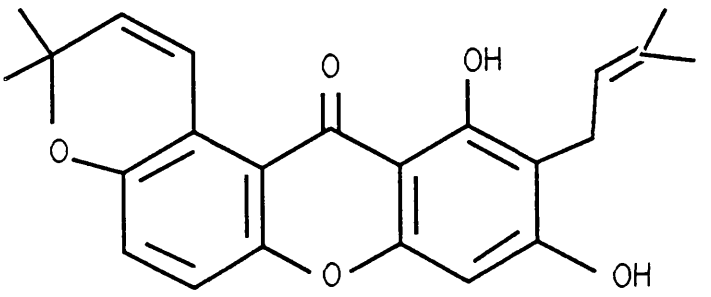


(13)

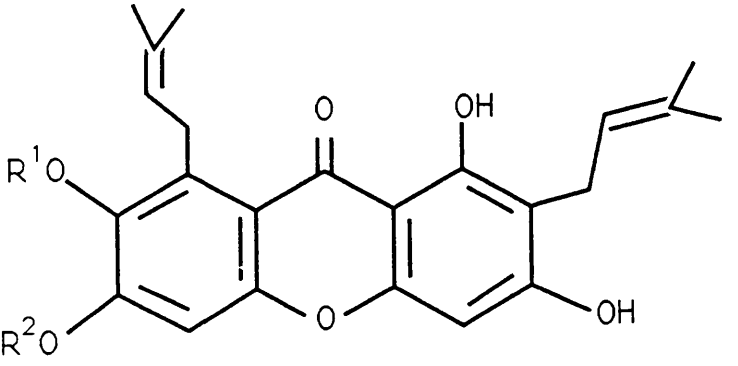


15. R= Me

16. R= CH_2OH

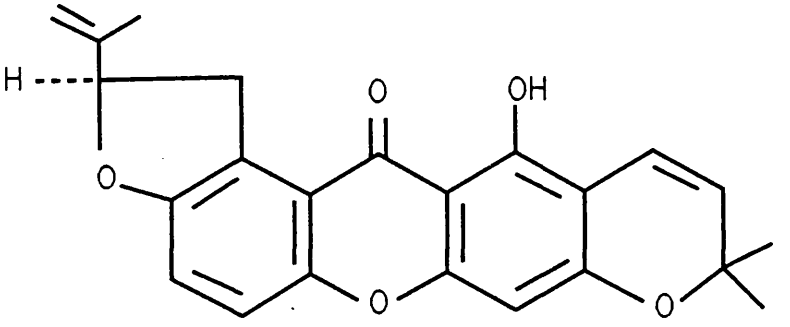


(17)



18. $R^1 = R^2 = H$

19. $R^1 = H$; $R^2 = Me$



(20)

2. **Trapezifolixanthone(13)**

   C. cuneifolium [21], *C. trapezifolium* [32], *C. calaba* [33]

3. **Demethylcalabaxanthone(14)**

   C. walkeri [34]

4. **Thwaitesixanthone(15)**

   C. cuneifolium [21], *C. thwaitesii* [20], *C. walkeri* [35]

5. **Thwaitesixanthonol(16)**

   C. walkeri [35]

6. **Calothwaitesixanthone(17)**

   C. thwaitesii [33]

7. **6-Deoxy-γ-mangostin(18)**

   C. thawaitesii [33]

8. **6-deoxymangostin(19)**

   C. bracteatum [34], *C. calaba* [31,33]

9. **Compound (20)**

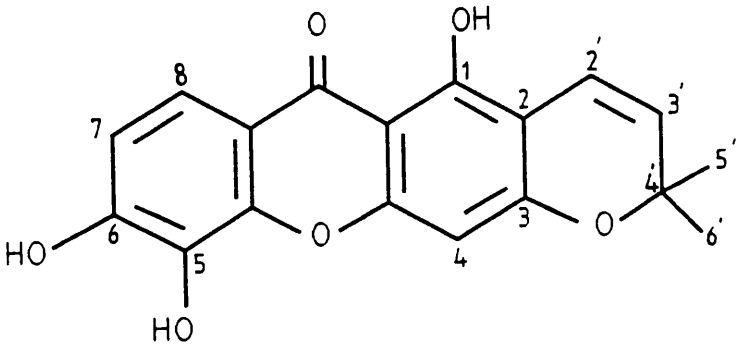
   C. walkeri [35]

Mono-C5-tetraoxygenated xanthones

Only two simple monoprenylated tetraoxygenated xanthone have discovered. However, jacareubin (21) which has a chromene ring, is found in a large numbers of ***Calophyllum*** species and is considered as a chemotaxonomic marker for this genus.

1. **Jacareubin(21)**

   C. bracteatum [17], *C. brasilliense* [36], *C. calaba* [17] *C. canum* [29], *C. cordato-oblongum* [12]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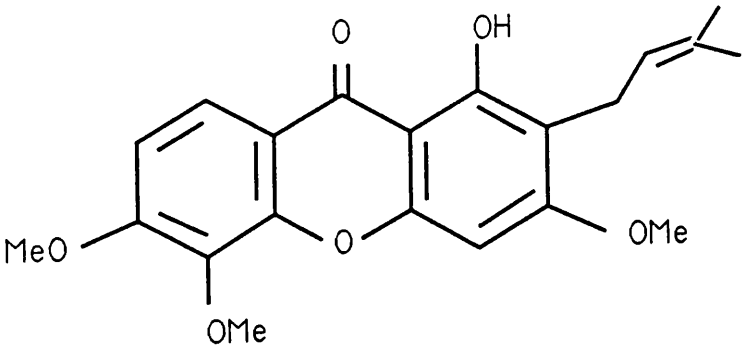
(21)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MeO
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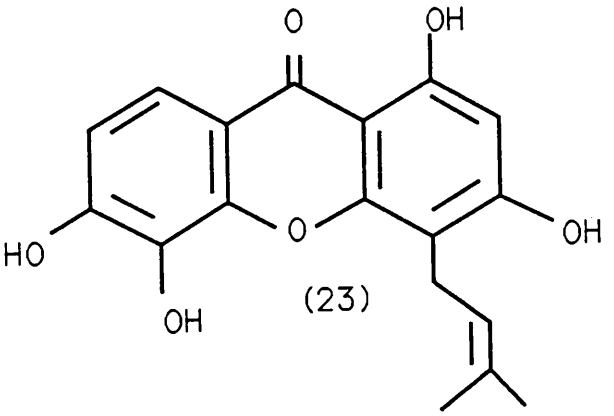
OMe
OMe

(22)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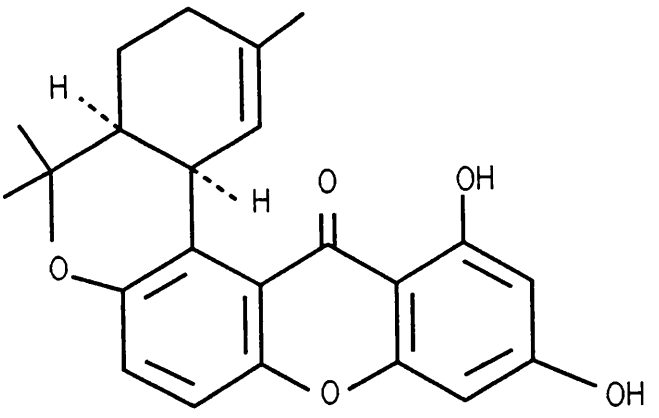
OH
HO
O
OH
OH

(23)


H
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H
O
O
OH

(24)

C. cuneifolium [21], *C. fragrans* [22]

C. inophyllum [23], *C. neo-ebudicum* [30]

C. ramiflorum [13], *C. sclerophyllum* [25]

C. scriblifolium [26], *C. thwaitesii* [20]

C. tomentosum [13], *C. trapezifolium* [32]

C. walkeri [20], *C. zeylanicum* [14]

2. **1-hydroxy-3,5,6-trimethoxy-2-(3-methylbut-2-enyl)xanthone (22)**

   C. ramiflorum [24]

3. **1,3,5,6-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone (23)**

   C. canum [30], *C. fragrans* [22] *C. inophyllum* [30]

   C. neo-ebudicum [29], *C. sclerophyllum* [25]

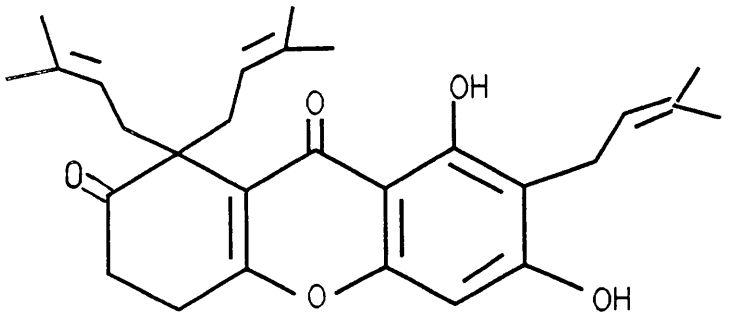
   C. scriblitifolium [26]

More Complex Xanthones

Calozeyloxanthone **(24)** from ***C. zeylanicum*** [14] has an unusual cyclised C_{10} unit while zeyloxanthonone **(25)** [37,38] has the novel feature of two prenyl groups attached to the C-8 of a necessarily non-aromatic ring A. The latter **(25)** is the first example of a natural triprenylated tetrahydroxanthone and is of considerable biogenetic and chemotaxonomic interest.

NEOFLAVONOIDS

Neoflavonoids with a C6-C3-C6 skeleton including other 4-substituted coumarins, chromones, chromans and coumarinic acid occur in many ***Calophyllum*** species. In fact, the first neoflavonoid, calophyllolide **(26)** was



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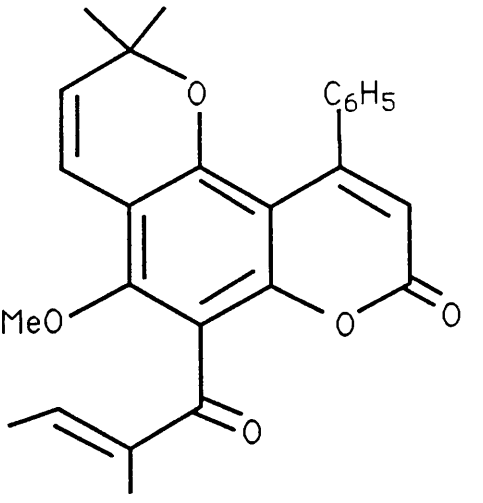

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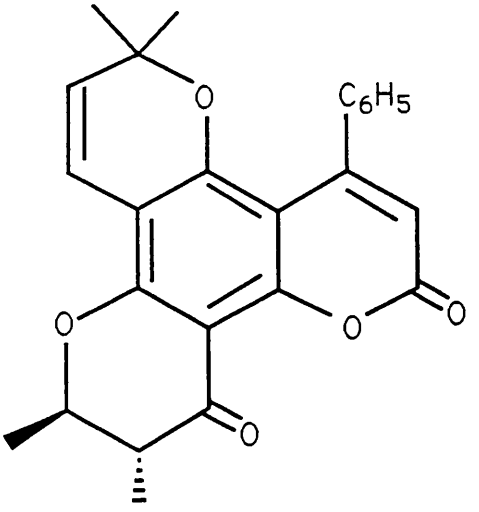
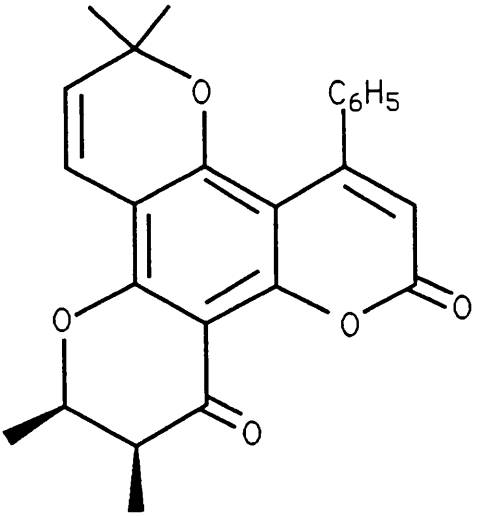
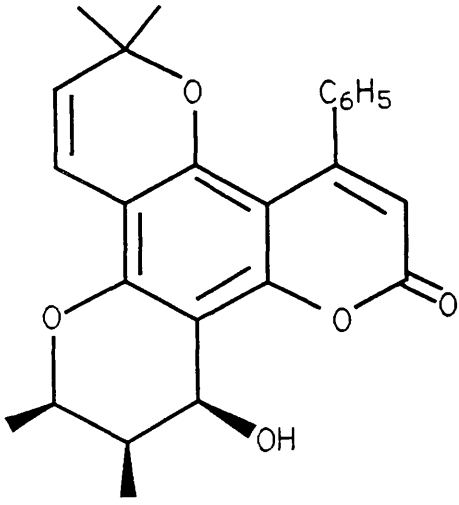


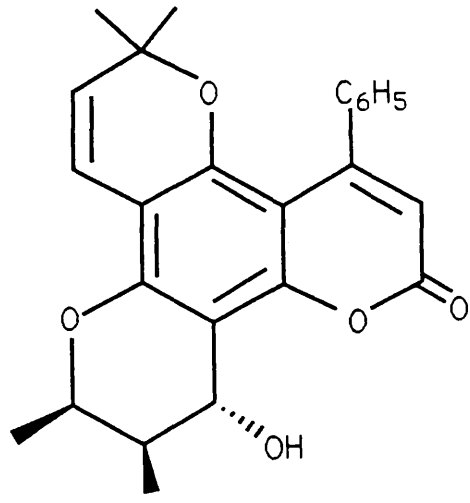
27. (+)-*trans*-inophyllolide



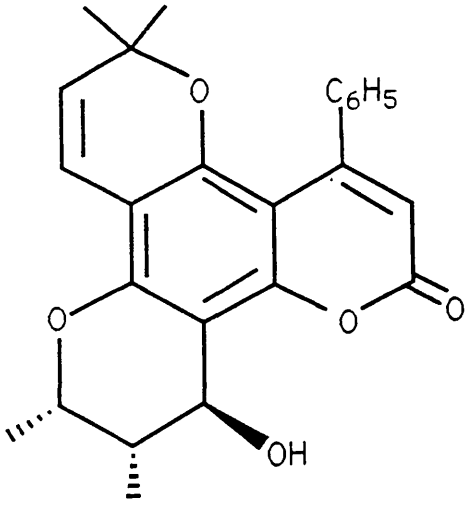
28. (+) - *cis* -inophyllolide



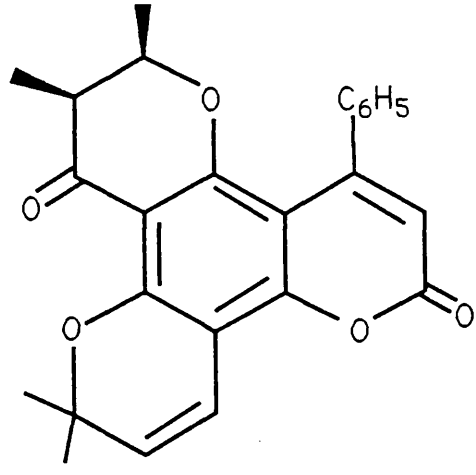
29. Inophyllum A



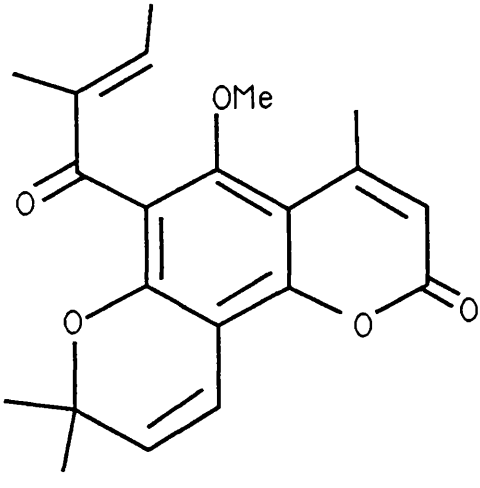
30. Inophyllum B



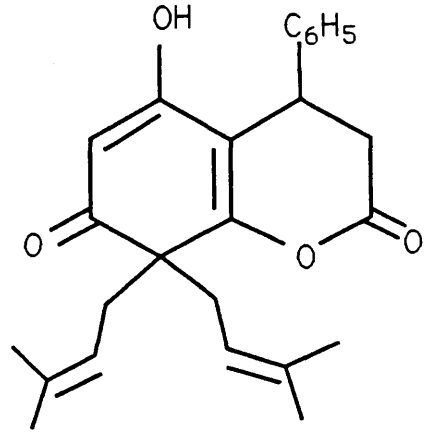
31. Inophyllum D



32. Tomentolide A



33. Apetatolide

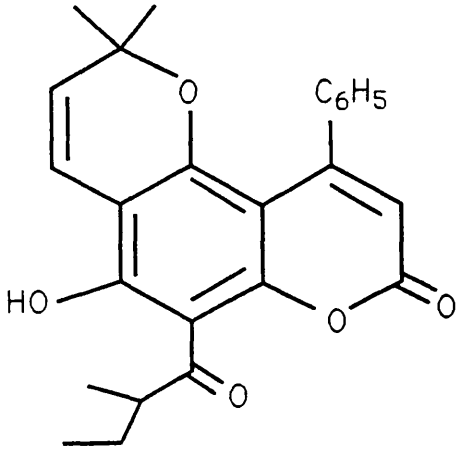


34. Calofloride

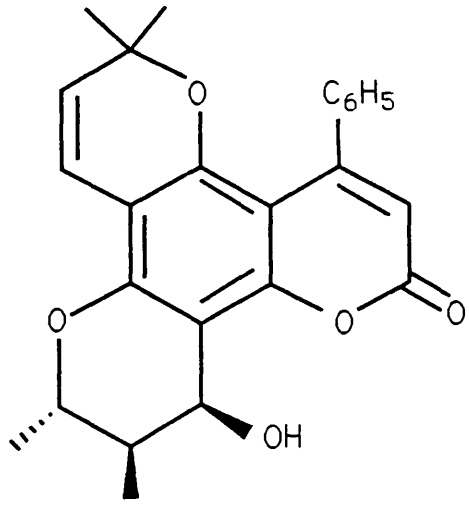
isolated from *C. inophyllum* [39] although the structure was not known at that time. The elucidation of the 4-phenylcoumarin structure of calophyllolide(26) and inophyllolide was first achieved by Polonsky [40,41,42]. Table 1 shows a representative group of the coumarin compounds which have been isolated from *Calophyllum* species [43,44].

Table 1. Neoflavonoids in *Calophyllum* species

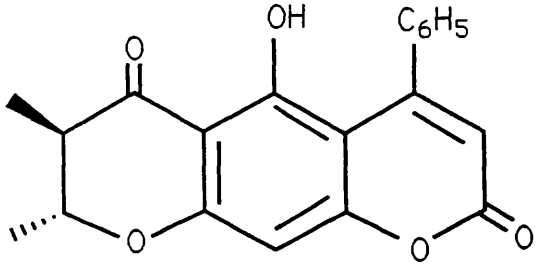
Compound	Species	Part Examined
4-Phenyl coumarin		
Calophyllolide(26)	*C. inophyllum* [42]	nut
	C. bracteatum [45]	nut
(±), (+)-trans-inophyllolide(27)	*C. inophyllum* [46]	nut, leaves
(+)-cis-inophyllolide(28)	*C. inophyllum* [46]	nut, leaves
Calophyllum A (29)	*C. moonii* [47]	leaves
	C. inophyllum [46,48]	leaves
Inophyllum B (30)	*C. inophyllum* [46,48]	leaves
Inophyllum D (31)	*C. inophyllum* [46,48]	leaves
Tomentolide A(32)	*C. tomentosum* [46]	nut
Apetatolide(33)	*C. apetalum* [49]	nut
Calofloride(34)	*C. verticilattum* [50]	nut
Ponnalide(35)	*C. inophyllum* [51,52]	nut



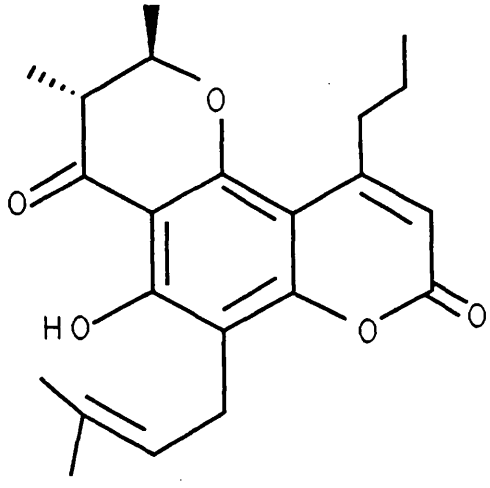
35. Ponnalide



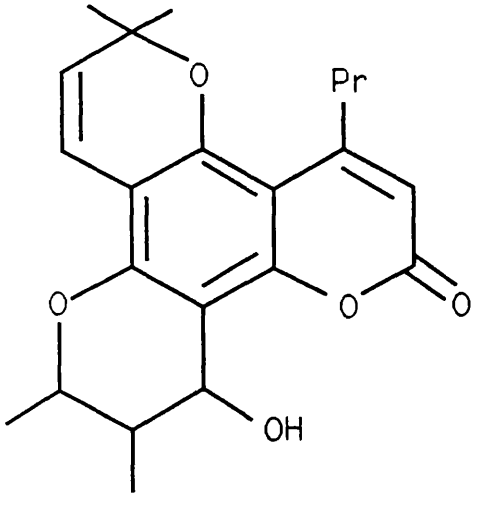
36. Soulattrolide



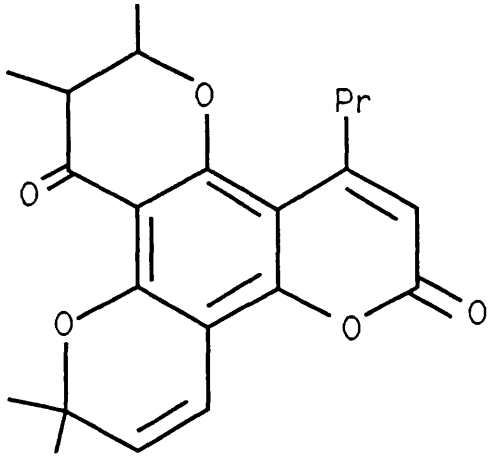
37. Calaustralin



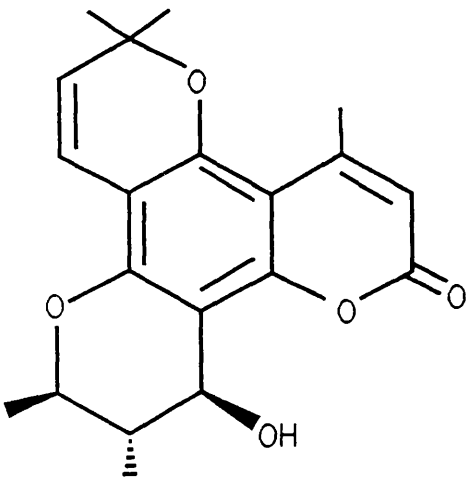
38. Recedensolide



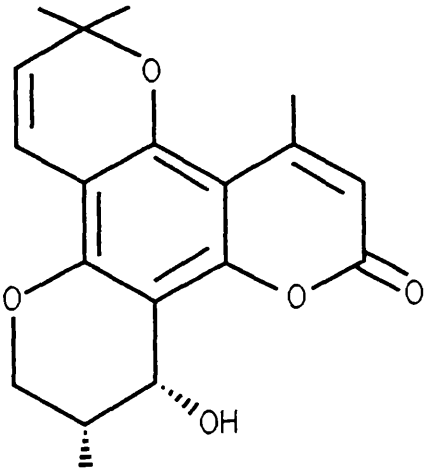
39. Costatolide



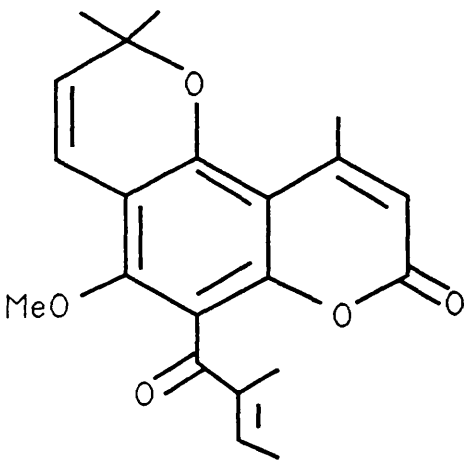
40. Tomentolide B



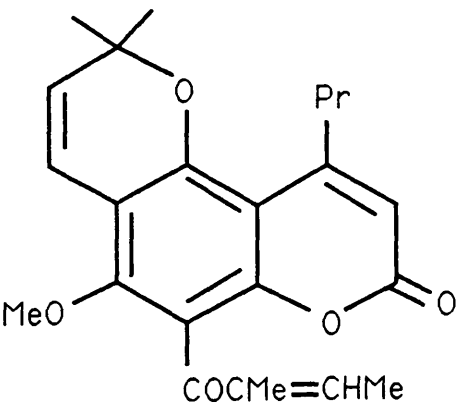
41. Cordatolide A



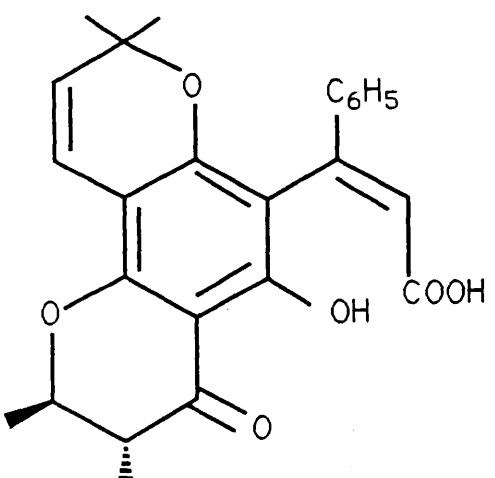
42. Cordatolide B



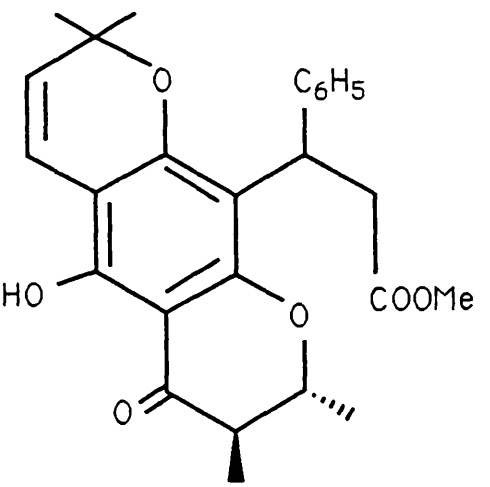
43. Oblongulide



Compound 44



45. Calophyllic acid



46. Chaplieric acid methyl ester

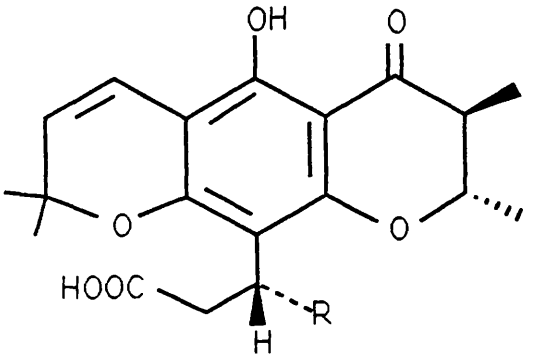
Calaustralin (36)	*C. australianum* [53]	bark, resin
	C. inophyllum [54]	nut
Soulattrolide (37)	*C. soulattri* [21]	bark
	C. moonii [47]	leaves
	C. cuneifolium [21]	bark

4-Alkyl coumarin

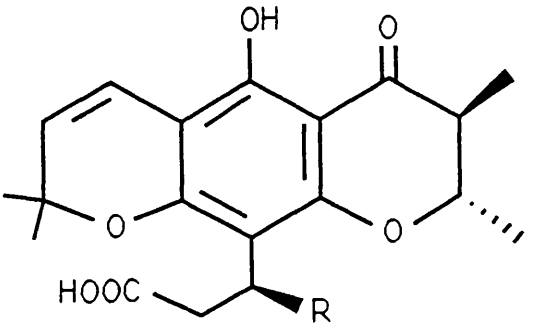
Recedensolide (38)	*C. recedens* [55]	bark
Costatolide (39)	*C. costatum* [56]	resin
Tomentolide B (40)	*C. tomentosum* [44, 49]	nut
Cordatolide A (41)	*C. cordato-oblongum* [57]	leaves
Cordatolide B (42)	*C. cordato-oblongum* [57]	leaves
Oblongulide (43)	*C. cordato-oblongum* [57]	leaves
Compound (44)	*C. inophyllum* [58]	-

Chromanone acid

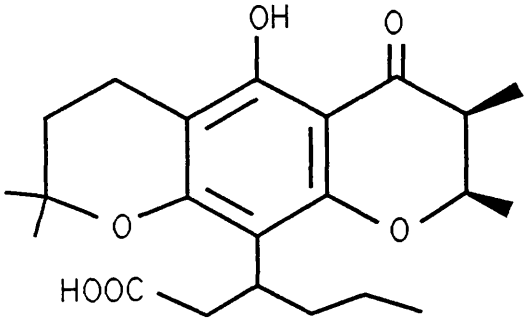
Calophyllic acid (45)	*C. inophyllum* [42]	nut
Chapelieric acid-methyl ester (46)	*C. chapelieri* [59]	nut
Blancoic acid (47)	*C. blancoi* [60]	bark
	C. brasiliense [61]	nut
Apetalic acid (48) and Isoapetalic acid (49)	*C. apetalum* [62]	bark
	C. brasiliense [61]	nut
	C. calaba [63]	bark
	C. bracteatum [63]	bark
	C. moonii [63]	bark



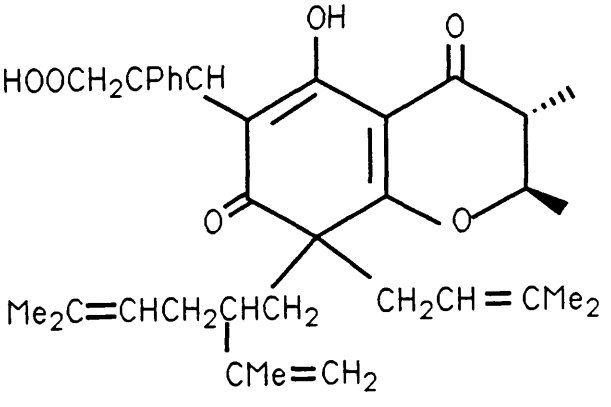
47. R=C_5H_{11} Blancoic acid
49. R= C_3H_7 Isoapetalic acid
51. R= C_4H_9



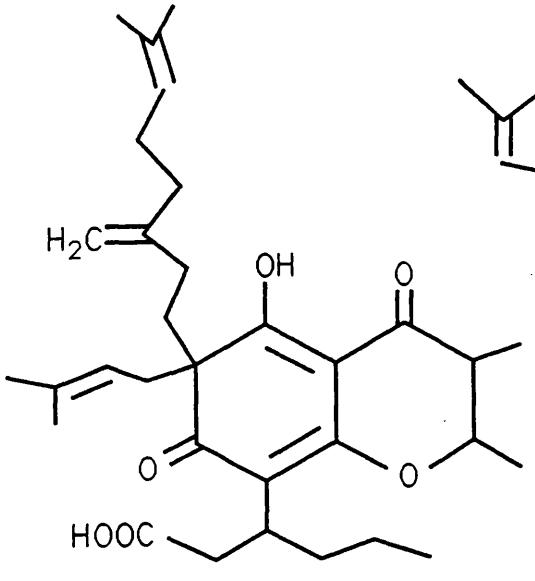
48. R=C_3H_7 Apetalic acid
52. R = C_4H_9
53. R= C_5H_{11}



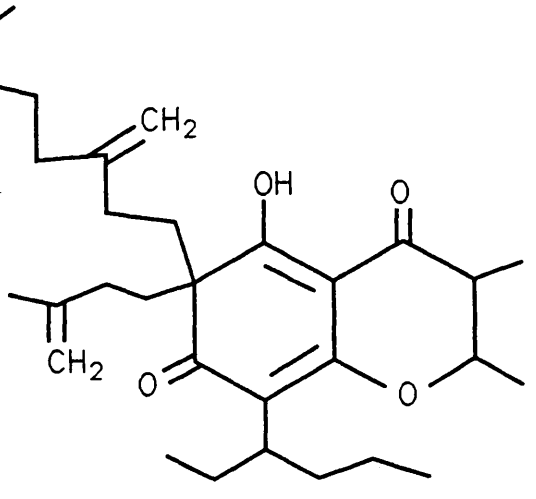
50. Dihydroapetalic acid



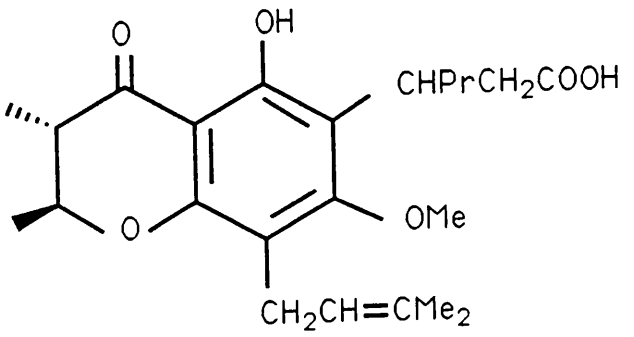
54.Calophynic acid



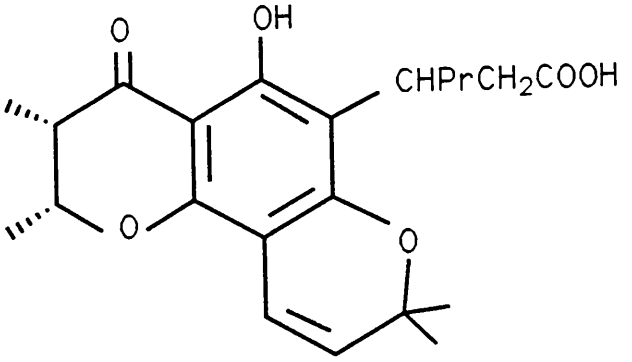
56.Brasiliensic acid



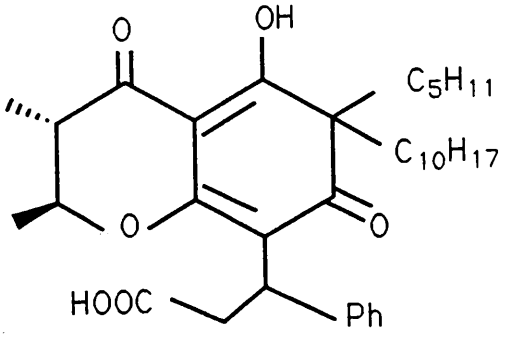
55. Inophyllolidic acid



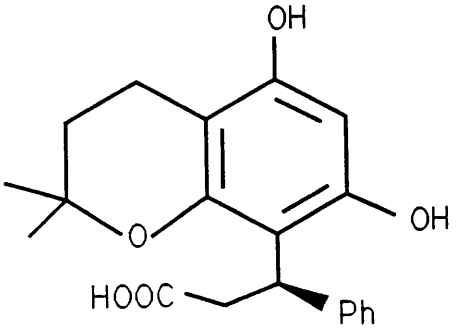
57. Recedensic acid



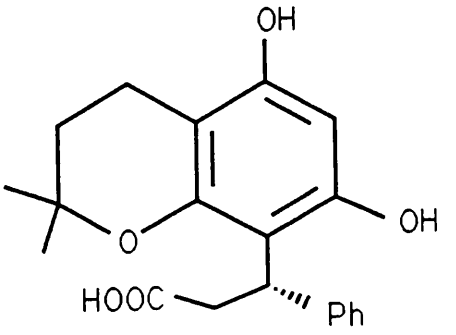
58. Isocalolongic acid



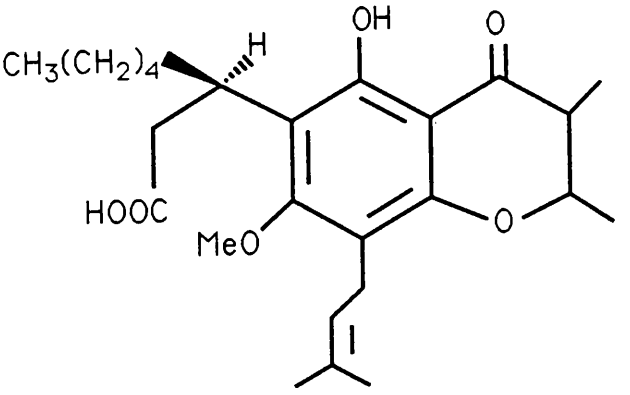
59. Calozeylanic acid



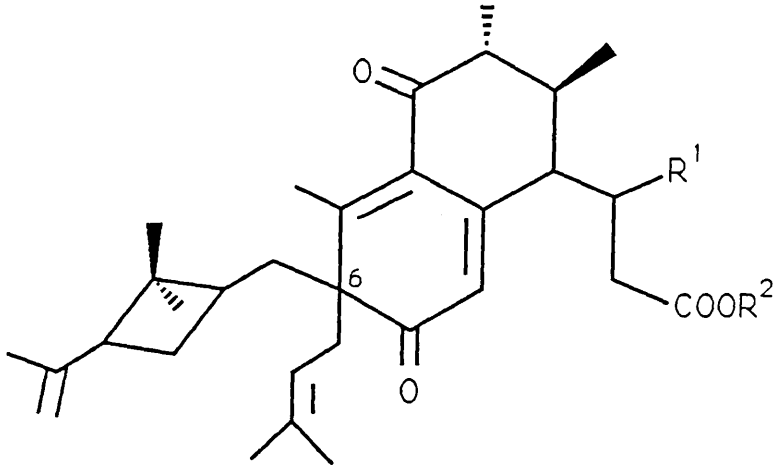
60. Thwaitesic acid



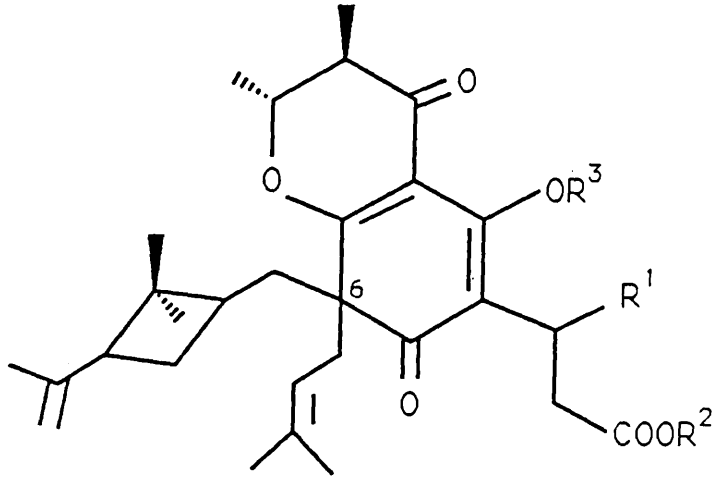
61. Isothwaitesic acid



62. Papuanic acid



63, 64 . R^1 = n-pentyl, R^2 = Me , R^3 = Me
(Different in Configuration at C-6)

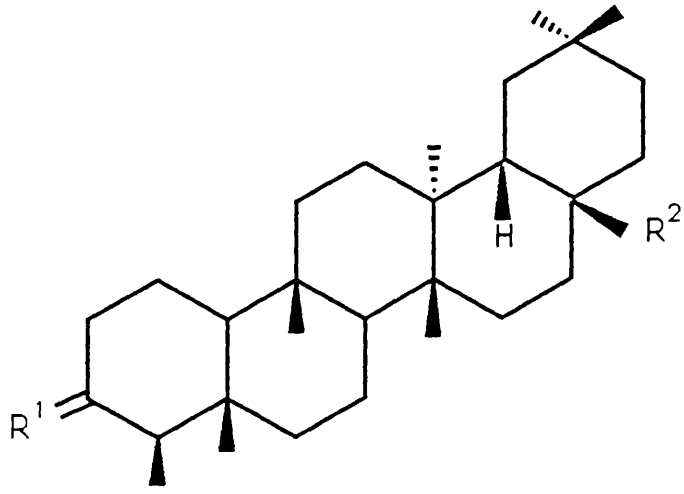


65. R^1 = n-Pentyl , R^2 = Me, R^3 = Me

	}	*C. trapezifolium* [63]	bark
	}	*C. macrocarpum* [35]	bark
	}	*C. walkeri* [35,63]	bark
	}	*C. chapelieric* [59]	nut
Dihydroapetalic acid (50)		*C. chapelieric* [59]	nut
Compound (51)		*C. brasiliense* [61]	nut
Compound (52)		*C. brasiliense* [61]	nut
Compound (53)		*C. brasiliense* [61]	nut
Calophynic acid(54)		*C. inophyllum* [64]	nut
Brasiliensic acid(55)		*C. brasiliense* [65]	resin
Inophyllolidic acid (56)		*C. brasiliensi* [65]	resin
Recedensic (57)		*C. recedens* [55]	bark
Isocalolongic acid (58)		*C. recedens* [55]	bark
Calozeylanic acid (59)	}	*C. thwaitesii* [63]	bark, nut
	}	*C. zeylanicum* [63,]	bark
	}	*C. walkeri* [63,47]	bark, leaves
Thwaitesic acid(60)		*C. thwaitesii* [66]	nut
Isothwaitesic acid(61)		*C. thwaitesii* [66]	nut
Papuanic acid (62)		*C. papuanum* [67]	bark
Caloverticillic acid A(63)		*C. verticillatum* [68]	bark
Caloverticillic acid B(64)		*C. verticillatum* [68]	bark
Caloverticillic acid C(65)		*C. verticilattum* [68]	bark

TERPENOIDS

Some ***Calophyllum*** species contain terpenoid compounds. Triterpenoids with a friedo-oleanane skeleton are the most abundant and occur mainly in the



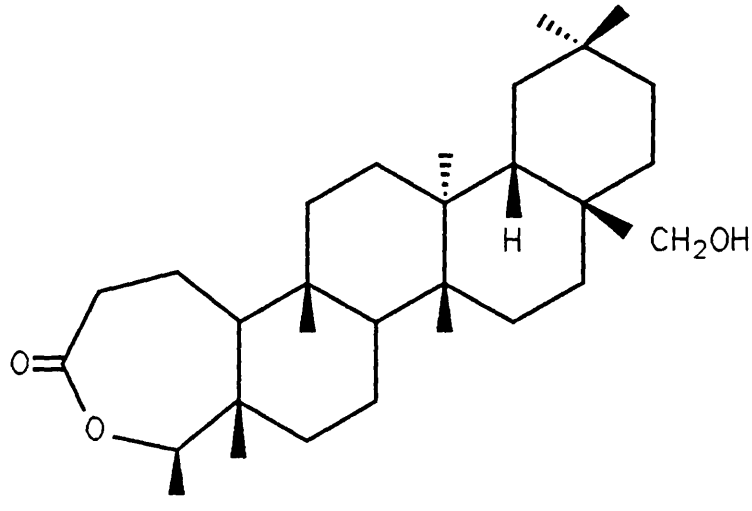
66 . $R^1 = O$; $R^2 = Me$

67. $R^1 = \beta$-OH, H ; $R^2 = Me$

68. $R^1 = O$; $R^2 = CH_2OH$

69. $R^1 = O$; $R^2 = CHO$

70. $R^1 = \beta$-OH,H ; $R^2 = CH_2OH$



71. Apetalactone

leaves and bark. No triterpenoids have been found in the nut.

Table 2. Triterpenoids in ***Calophyllum*** species

1. **Friedelin (66)**

   C. calaba [69], *C. cordato-oblongum* [57]

   C. moonii [47], *C. thwaitesii* [66], *C. walkeri* [47]

   C. apetalum [62,70], *C. inophyllum* [71],

   C. cuneifolium [54], *C. zeylanicum* [72]

   C. trapezifolium [47], *C. amoenum* [73]

   C. tomentosum [68], *C. lankaensis* [66]

2. **Friedelan-3β-ol (67)**

   C. calaba [69], *C. tomentosum* [74], *C. amoenum* [73]

   C. zeylanicum [72], *C. apetalum* [70], *C. inophyllum* [71]

3. **Canophyllol (68)**

   C. calaba [69], *C. cordato-oblongum* [57]

   C. lankaensis [66], *C. thwaitesii* [66]

   C. trapezifoilum [47], *C. walkeri* [47]

   C. inophyllum [75]

4. **Canophyllal (69)**

   C. calaba [69], *C. lankaensi* [66], *C. inophyllum* [75]

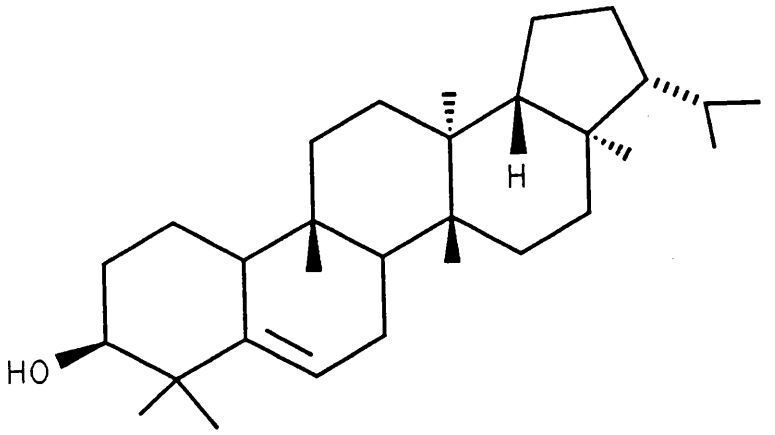
   C. thwaitesii [66]

5. **Friedelan-3β,28-diol (70)**

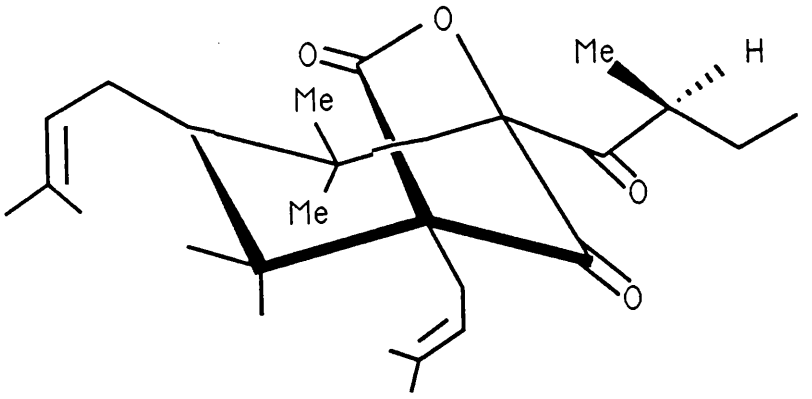
   C. calaba [66]

6. **Apetalactone (71)**

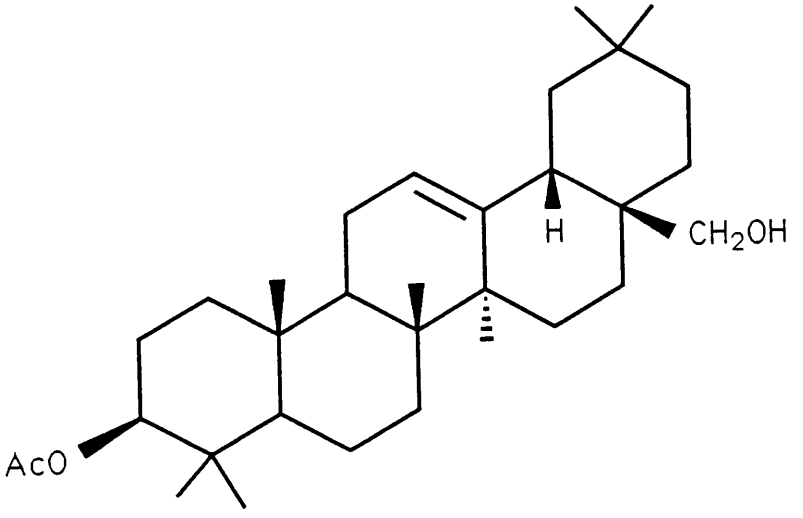
   C. moonii [47], *C. thwaitesii* [66], *C. apetalum* [76]



72. Simiarenol



73. Soulattrone



74. Erythrodiol-3-acetate

C. tomemtosum [75], *C. lankaensis* [66]

7. **β-Simiarenol (72)**

   C. cuneifolium [21], *C. trapezifolium* [32]

8. **Soulattrone A (73)**

   C. soulattri [77]

9. **Erythrodiol-3-acetate (74)**

   C. inophyllum [78]

CHEMOTAXONOMIC CONSIDERATIONS

Secondary metabolites of various classes play an important role in the principles of chemotaxonomy [79,80,81]. Xanthones, which occur widely in the Guttiferae and have almost forty different oxygenation patterns, should be of systematic significance in this family. There are about 180 ***Calophyllum*** species distributed from India to New Guinea and they form a homogenous group based on the xanthone content [82]. ***Calophyllum*** species have many xanthones in common which occur throughout the genus while the rare compounds occur only in a few species. Almost all the 21 xanthone-containing species have both simple and prenylated xanthones. Jacareubin **(21)** occurs in 17 of these species and 6-deoxyjacareubin **(8)** in 11 , suggesting these compounds as a taxonomic markers for the genus [71]. This uniformity of the occurrence of xanthones may be due to the rather narrow geographical range and the close relationship of the species

investigated. The xanthone content of ***Calophyllum*** species often varies greatly within the plant and this can cause difficulties in reaching valid taxonomic conclusion when confirming species. A possible geographical variation in the xanthone content has been suggested in ***C. calaba*** [31,45,83].

Neoflavonoid and other 4-substituted coumarins which co-occur in many ***Calophyllum*** species have similar patterns of substitution (cf Table 1). The only taxonomic significance of the neoflavonoid content concerns the different substitution from those of the Leguminosae [43]. The acyl and the isoprenoid substituents are present in the phloroglucinol-type A ring of all Guttiferae 4-phenylcoumarins with the exception of ponnalide(35).

Most triterpenoids from ***Calophyllum*** species were found in the leaf extract. Almost all species contain friedelin (66) except for ***C. soulattari*** (cf Table 2).

Constituents of Malaysian *Calophyllum* species

There are 49 ***Calophyllum*** species[1] known to occur in Malay Peninsula where ***C. inophyllum*** is the most dominant and widely distributed. Its seed, bark and leaves have been investigated extensively. The constituents of the leaves [46] include 10,11-*cis* and *trans*-isomers (27), (28) of (+)inophyllolide. In

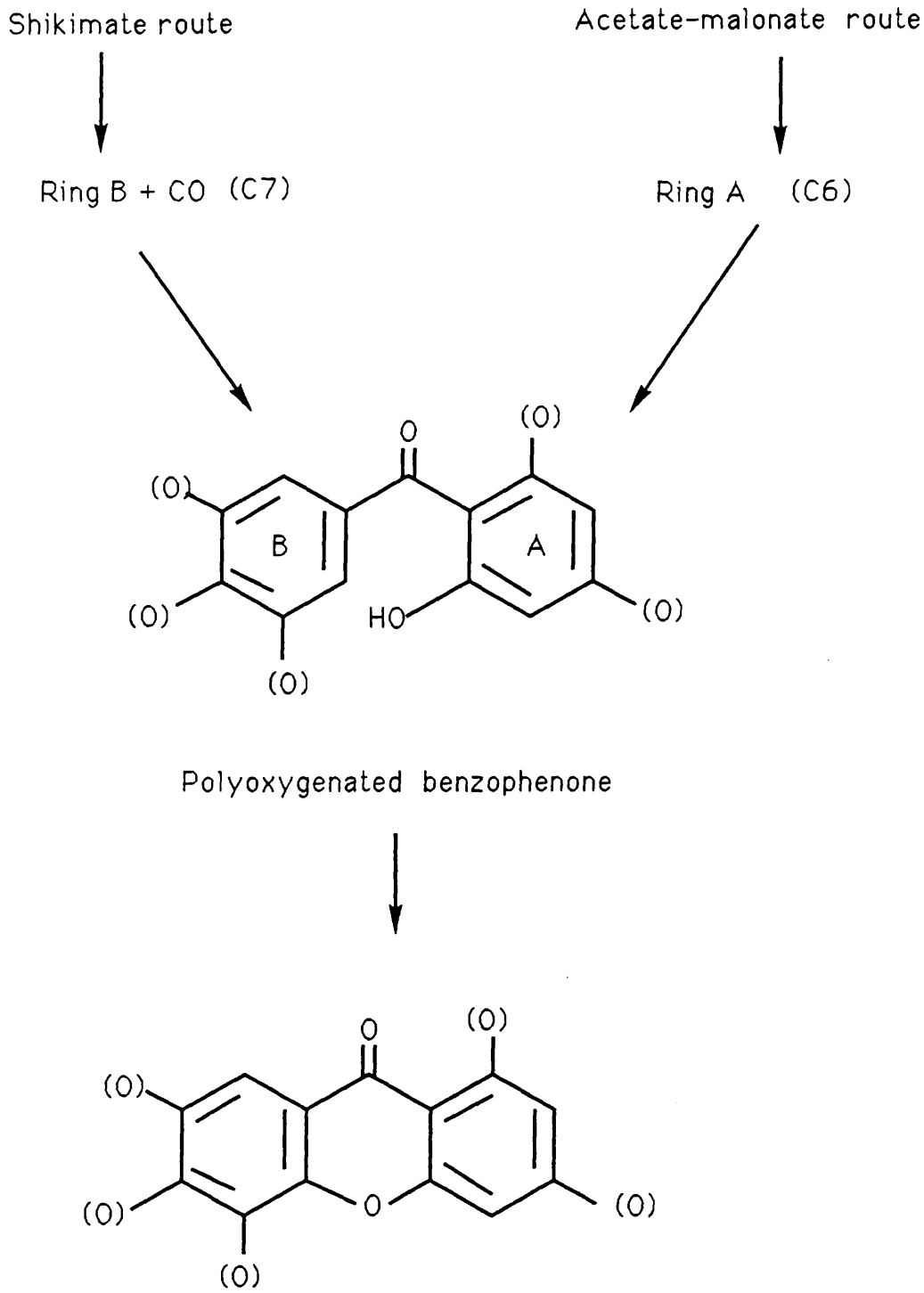
addition, inophyllum A (29) is the first natural 4-phenylcoumarin possessing a 2,3-dimethylchromanol ring isolated from this species.

The heartwood of ***C. sclerophyllum*** from Sarawak, Malaysia has been investigated by Jackson *et al.* [25]. Jacareubin(21), 1,7-dihydroxyxanthone and three related 1,3,5,6-tetraoxygenated xanthones i.e 2-(3,3-dimethylallyl)-1,3,5,6-tetrahydroxyxanthone(23), 3,5,6-trihydroxy-1-methoxyxanthone and 1,3,5,6 tetrahydroxyxanthone were isolated.

Studies on the heartwood of ***C. scriblitifolium***[26] obtained from Serawak afforded seven xanthones include 1,5,6-trihydroxyxanthone, 6-desoxyjacareubin (8), 1,3,5-trihydroxy-2-(3-methylbut-2-enyl)xanthone (9), 1,3,7-trihydroxy-2-(3-methylbut-2-enyl)xanthone (10), osajaxanthone (11), jacareubin(21) and 1,3,5-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone (23).

Further investigation of the heartwood of ***C. fragrans*** [22], also from Serawak, afforded six xanthones namely 1,7-dihydroxyxanthone, 1,6-dihydroxy-5-methoxyxanthone, 1,5,6-trihydroxyxanthone, 6-deoxyjacareubin(8), jacareubin(21) and 2-(3,3-dimethylalllyl)-1,3,5,6-tetrahydroxyxanthone(23).

More recently, the stem bark of ***C. macrocarpum*** collected in Kuala Lompat, Malaysia has been investigated [35]. The petrol extract yielded apetalic acid(48), a known chromanone acid neoflavonoid.



Scheme 1. Biosynthesis of xanthone
(*Gentiana lutea*)

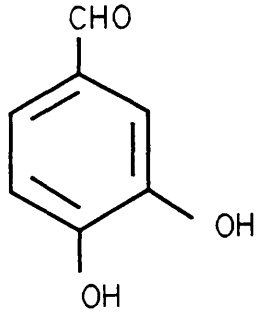
Furthermore, sitosterol, stigmasterol and protocatechualdehyde(75) were also isolated.

The species *C. biflorum* is normally found along the banks of rocky rivers and is distributed throughout Kelantan, Terengganu and Pahang. No chemical investigation of this species has been reported. We have investigated the bark and wood of this species. The results are presented below.

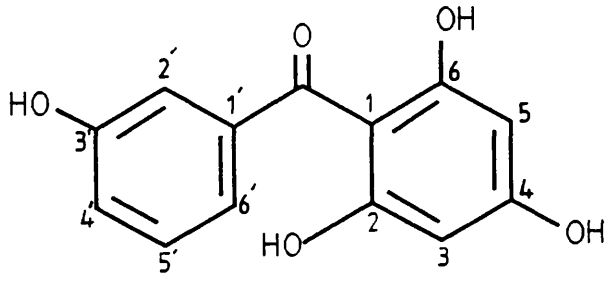
BIOSYNTHESIS

XANTHONES

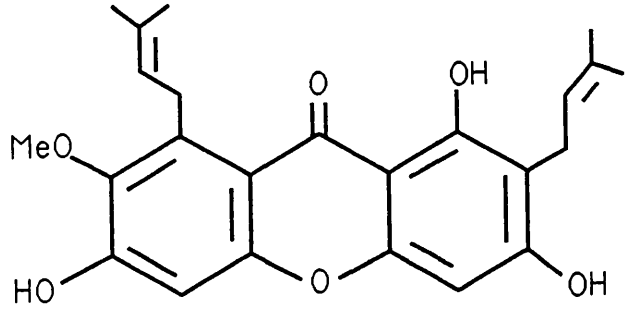
Early biosynthetic studies suggested that ring B and the attached carbonyl group (C7) are formed from the shikimic acid pathway whereas ring A (C6-unit) arises via the acetate-malonate polyketide route. It was concluded that a polyhydroxy benzophenone or its biogenetic equivalent is an intermediate in xanthone formation. However, initial studies were limited to 1,3,7-trioxygenated xanthones from *Gentiana lutea* (Gentianaceae) and the results [84,85] showed that the xanthone nucleus was formed from acetate (ring A) and C6-C1 unit derived from phenylalanine (ring B) (Scheme 1). The role of a benzophenone as intermediate has been supported by the incorporation of tritiated 2,3',4,6-tetrahydroxybenzophenone (76) [86]. Recently the first biosynthetic study of xanthone formation in



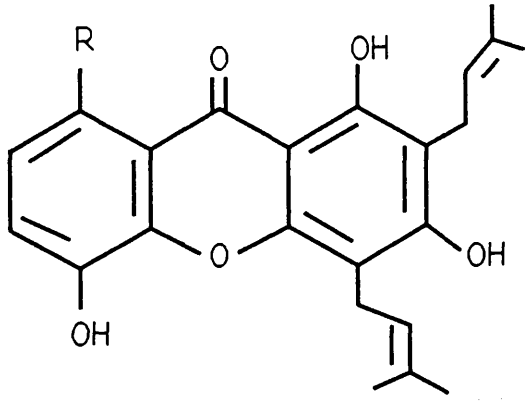
75. Protocatechualdehyde



76. Benzophenone

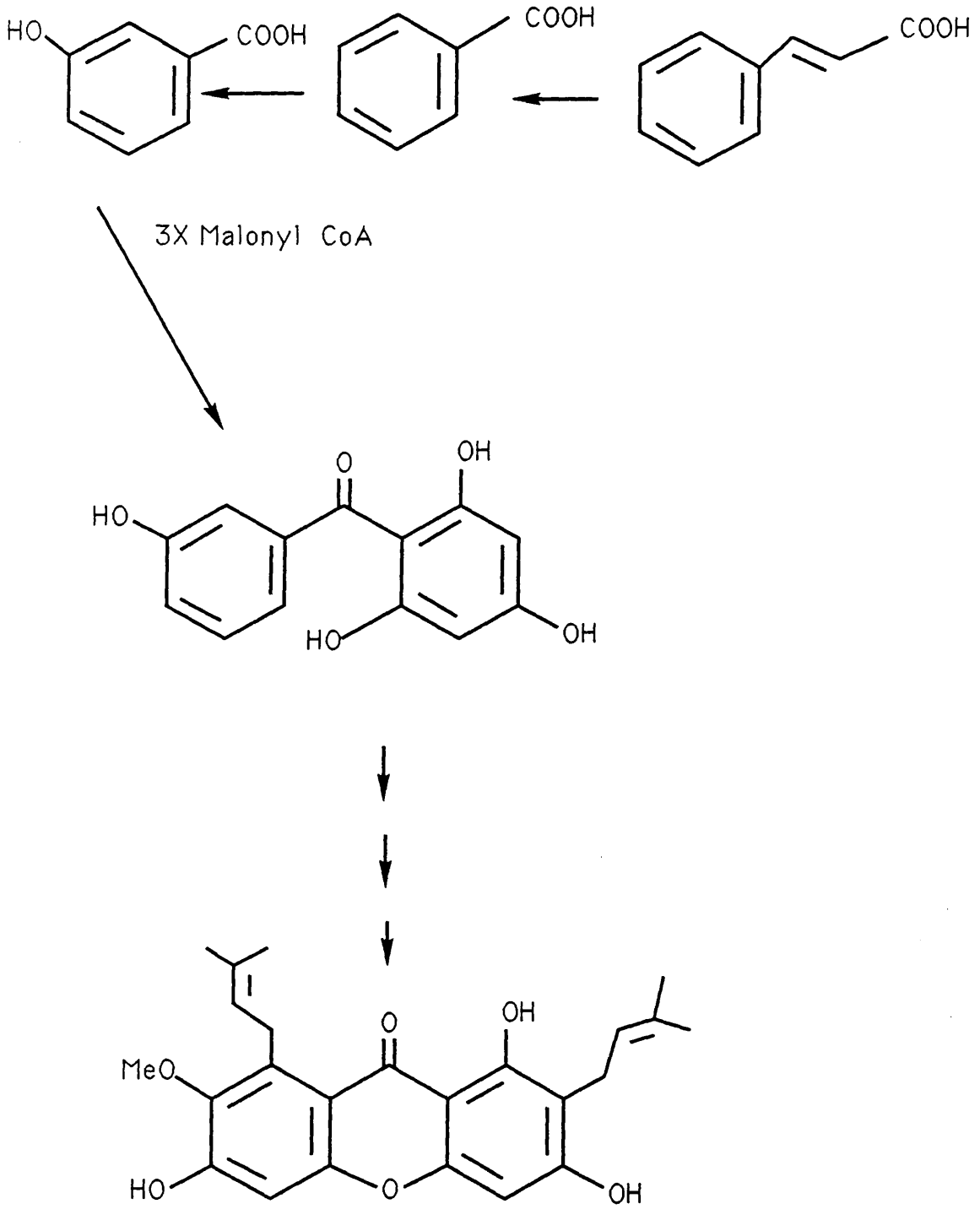


77. Mangostin



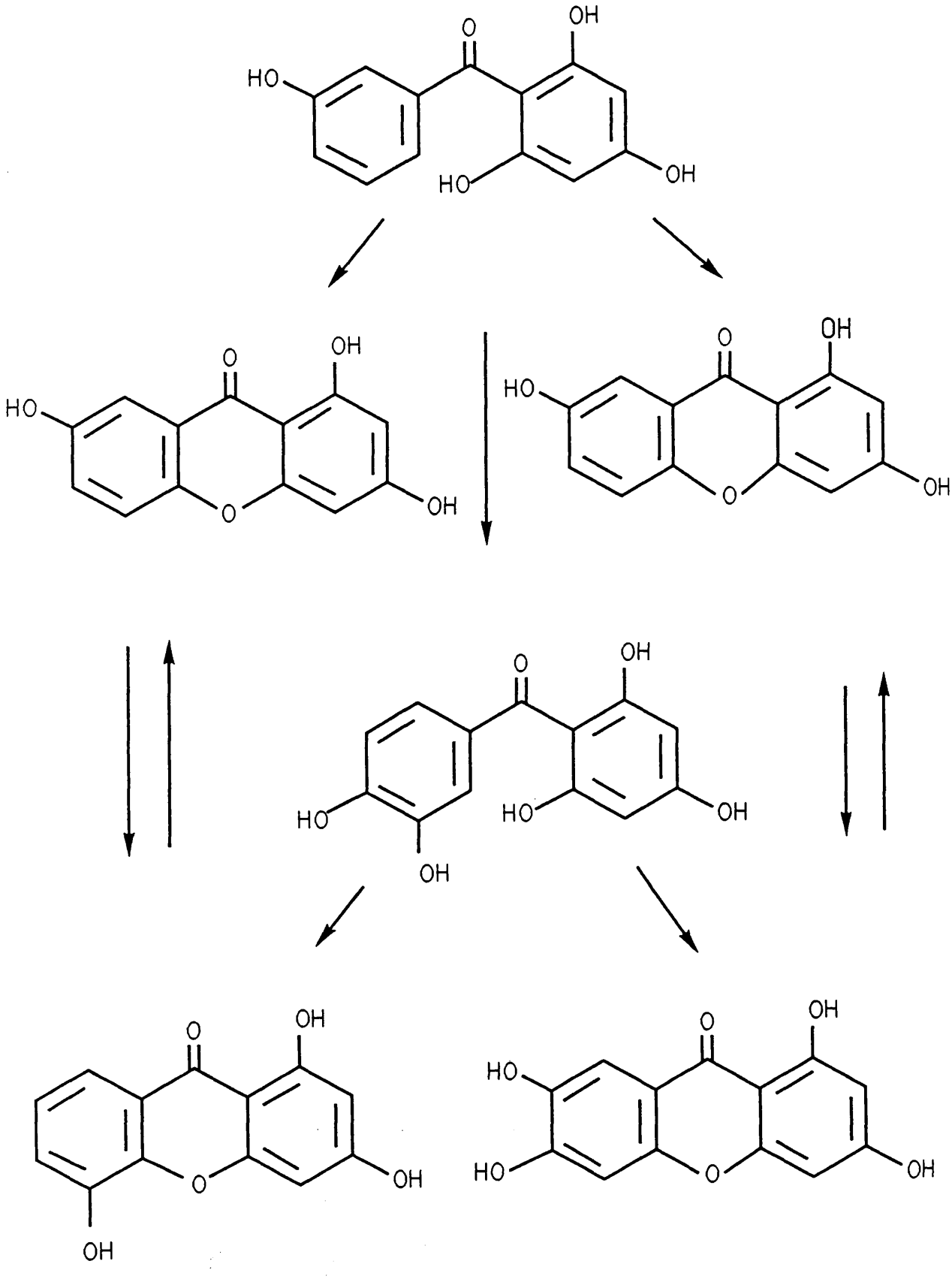
78. R = H

79. R = OH



Scheme 2. Biosynthesis of xanthone

(*Garcinia mangostana*)

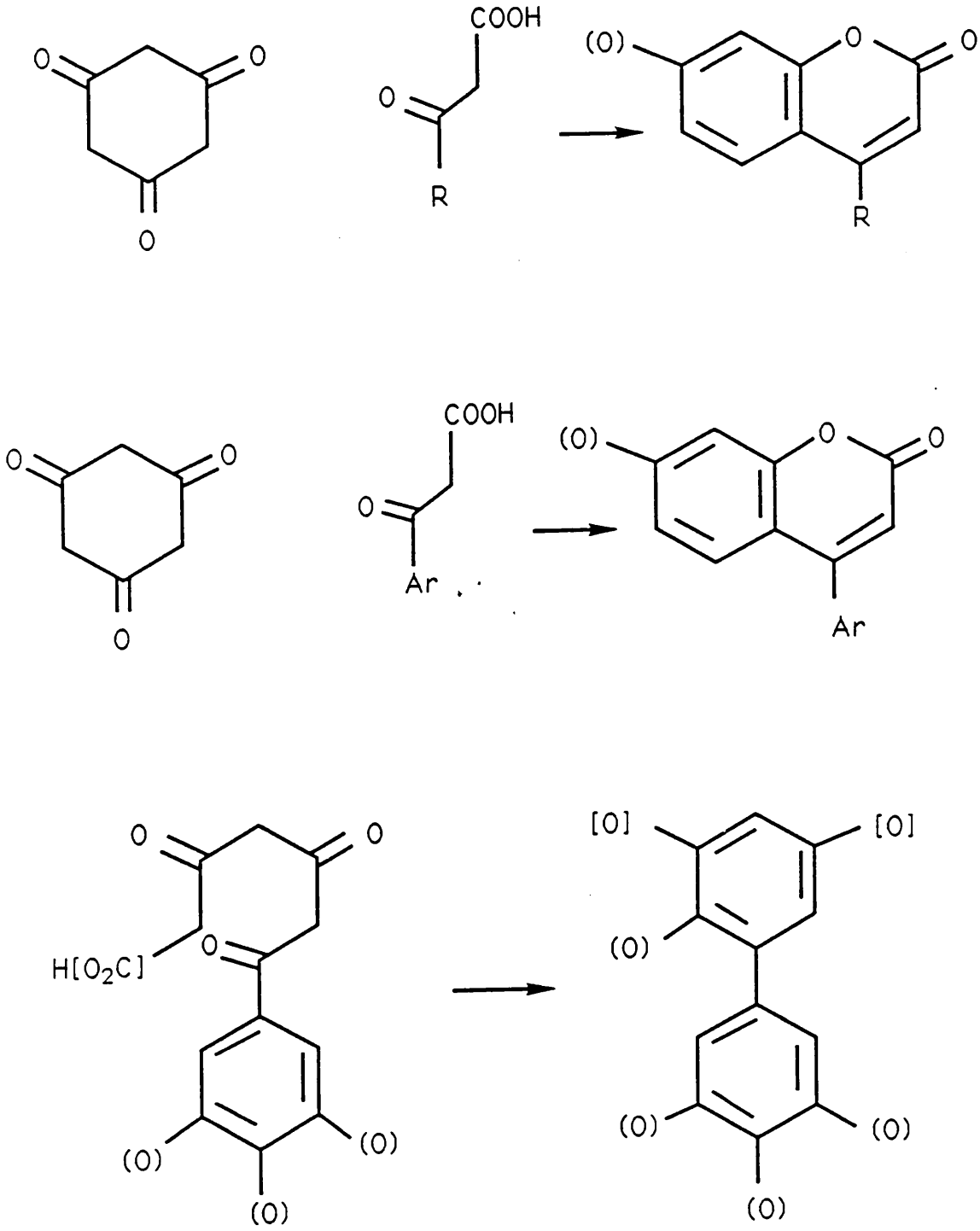


Scheme 3.

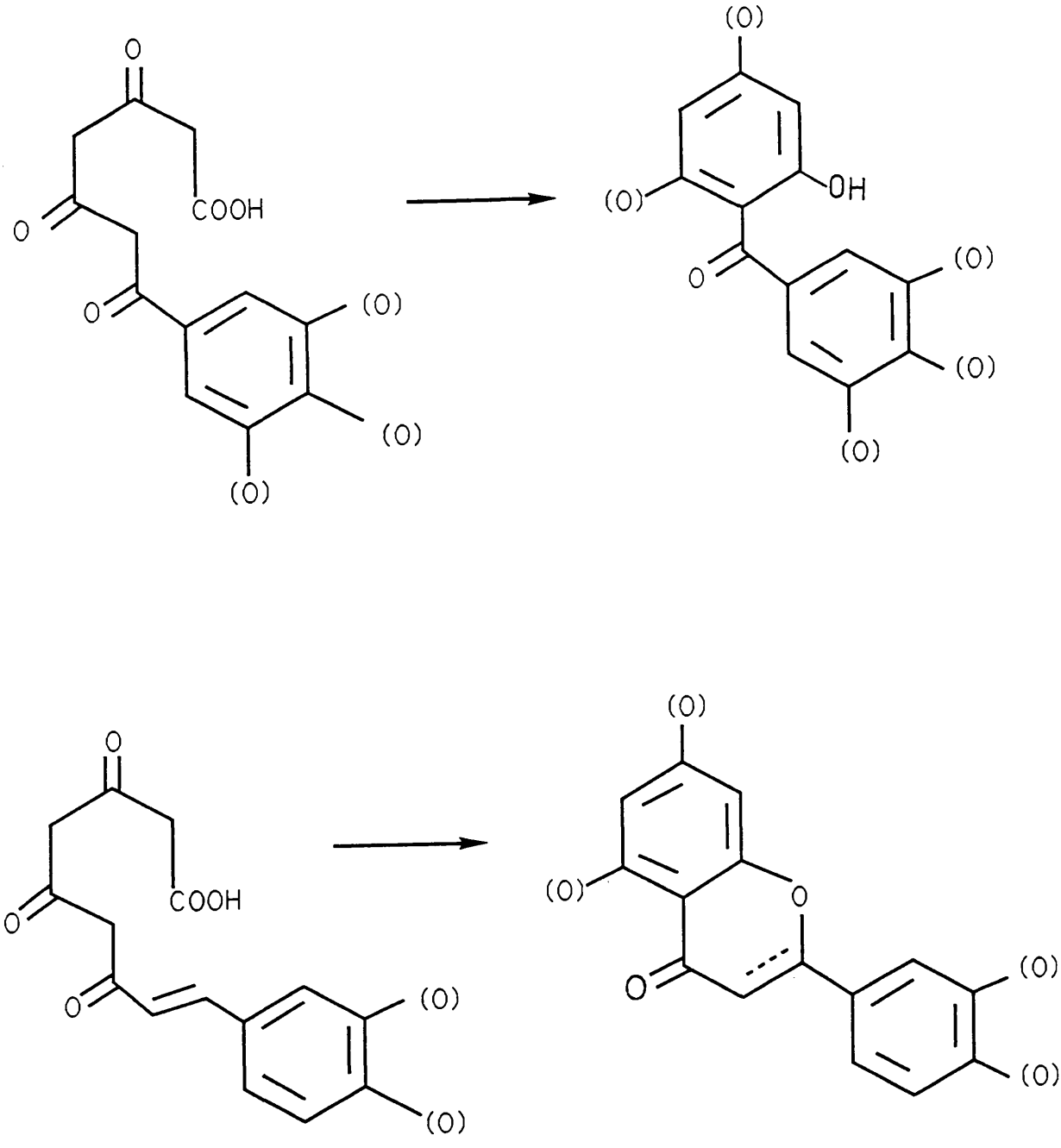
the Guttiferae showed that cinnamic acid, benzoic acid, m-hydroxybenzoic acid and the benzophenone (76) as well as malonic acid are efficient precursors of mangostin (77) in ***Garcinia mangostana***. The biosynthetic pathway is shown in the Scheme 2. Studies [67] with labelled benzophenone (76) showed that it is significantly incorporated into 8-desoxygartinin (78) and gartinin (79) in the same plant. The results suggest the involvement of benzophenones in the biosynthesis of xanthones in most higher plants. Phenol oxidative coupling can be considered the most likely mode of xanthone formation. Carpenter and co-workers [6] concluded that oxidative coupling of a series of suitably hydroxylated benzophenones can account for the formation of all major xanthone oxygenation patterns. This suggestion is compatible with the latest biosynthetic results since the benzophenones all require ***meta-*** or 3'-oxygenation for oxidation coupling and may be derived from benzophenone (76)(Scheme 3). It is clear that shikimate-derived intermediates with p-hydroxylation are unsuitable for direct phenolic oxidation to xanthone. Initially, explanations involving spirodiene intermediates which undergo rearrangement or nuclear oxidation/reduction processes were invoked [6]. Labelling studies, however, revealed that labelled *p*-hydroxybenzoic and coumaric acids are very poor

precursors of xanthones. Direct *meta*-hydroxylation of benzoic acid has now been suggested. A re-evaluation of the involvement of shikimate derivatives is certainly required.

No studies on the biosynthesis of polyisoprenylated xanthones have appeared. However, prenylation normally occurs *ortho*- to an oxygen function and 2-prenylation is most common in the 1,3-dioxygenated xanthone probably because of the prenyl group inhibits reduction of the 3-hydroxyl group. Very few examples occur of mono-prenylation in the 4,8- or 7-position. A second prenyl group normally occurs at the 4- or 8-position. 2,4-Diprenylation is usually found in 1,3,5-, 1,3,5,6- or 1,3,5,8- oxygenated xanthones and not with 1,3,7- or 1,3,6,7-oxygenated. Interestingly 2,8-diprenylation occur with 1,3,7- and 1,3,6,7-oxygenated. There is very little evidence to suggest whether prenylation occurs at the benzophenone or xanthone stage although the latter tend to be favoured based on previous studies [88]. 2-Prenyl-1,3,5-trihydroxyxanthone has been postulated as a precursor to 6-deoxyjacareubin **(8)** and jacareubin **(21)** in which a route involving 6-oxygenation at the xanthone stage. This suggestion was supported by the co-occurence of these three compounds in four ***Calophyllum*** species. Similarly 2-prenyl-1,3,7-trihydroxyxanthone can lead to mangostin **(77)** via 6-deoxy-γ-mangostin **(18)**.



Scheme 4a

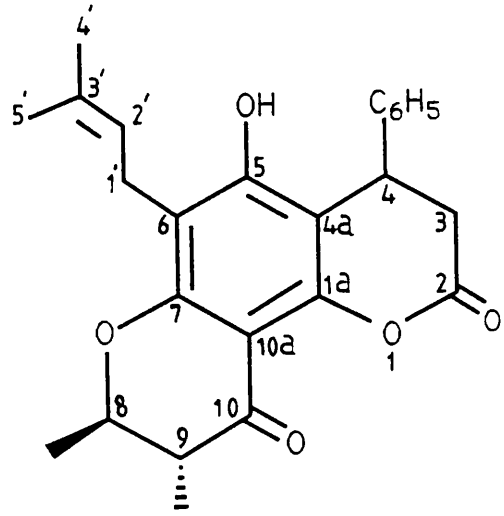


Scheme 4b.

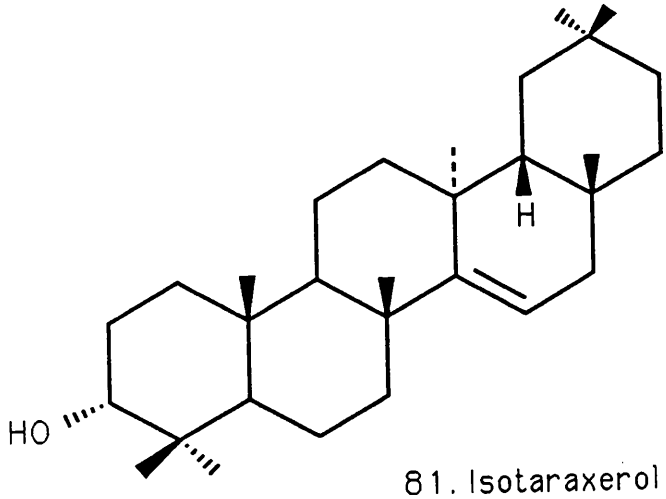
Various modifications (hydroxylation, further prenylation, cyclisation etc.) of these two basic 2-prenylxanthones can lead to at least half of the known prenylated xanthones.

NEOFLAVONOIDS

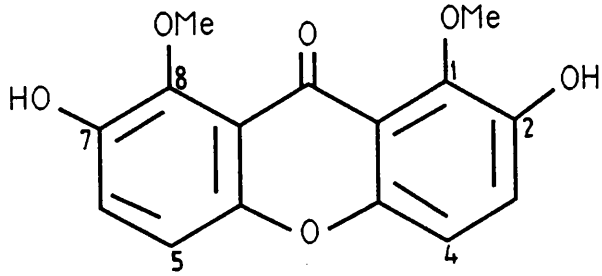
The co-occurrence of 4-arylcoumarins and with xanthones in ***Calophyllum*** suggests that they may be formed by analogous biosynthetic pathways [7] involving an aldol type condensation of a polyketide with a β-keto acid (Scheme 4a and 4b). This hypothesis can be extended to incorporate the formation of various classes of phenolic compounds-xanthones, benzophenones, aucuparins, 4-alkyl and 4-aryl-coumarins and flavonoids.



80. Dihydrocoumarin



81. Isotaraxerol



82. 2,7-dihydroxy-1,8-dimethoxyxanthone

DISCUSSION

The main compound isolated from the stem bark of *C. biflorum* was the unusual dihydrocoumarin $C_{25}H_{26}O_5$ whose structure was shown to be **(80)** on the following evidence. Its ^1H n.m.r. spectrum showed signals at δ_H 1.22 (d, 3H, J=6.9 Hz, C-9Me), δ_H 1.53 (d, 3H, J=6.3 Hz, C-8Me), δ_H 2.56 (dq, 1H, J=11.3, 6.9 Hz, H-9) and δ_H 4.22 (dq, 1H, J=11.3, 6.9 Hz, H-8) which revealed the typical *trans*-2,3-dimethylchromanone ring which is a common element in *Calophyllum* products [42,46,49,53,60]. The proton at C-8 is downfield with respect to the proton at C-9 due to deshielding effect of the neighbouring oxygen. The ^1H n.m.r also showed the presence of an isopentenyl chain [77,89] which gives rise to signals at δ_H 1.68 (s, 3H), δ_H 1.79 (s, 3H), δ_H 3.34 (d, 2H, J=7.4 Hz) and δ_H 5.19 (t septets, 1H, J=7.4, 1.4 Hz). The presence of an unsubstituted phenyl group [46], presumably attached to the dihydrocoumarin ring, was apparent from the appearance of a 5H multiplet at δ_H 7.26 and a band in the i.r. at 760 cm^{-1}. The lack of a vinyl resonance for H-3 normally observed in 4-substituted coumarin [22,49,53] and the presence of an ABX system [δ_H 2.98 (dd, J=15.9, 6.1 Hz), 3.08 (dd, J=15.9, 2.8 Hz), 4.61 (dd, J=6.1, 2.8 Hz)] provided strong evidence for a 4-substituted dihydrocoumarin system. I.r. bands at 3620, 1780 and 1690 cm^{-1}

indicated free hydroxyl and lactone ring functionality. It was clear that there was no chelated hydroxyl presence. This conclusion was also supported by the absence of a bathochromic shift of the UV maxima on addition of 5% ethanolic aluminium chloride [90,91].

The evidence thus far suggests a 4-phenyl-3,4-dihydrocoumarin with a free phenolic hydroxyl, a prenyl group and a 2,3-dimethylchromanone ring attached to ring A. A decision on the placing of these substituents was arrived at by comparing the ^1H chemical shift values of the dimethylchromanone ring with those of the compounds tomentolide A **(32)** [49] and *trans*-inophyllolide **(27)** [46]. In **(32)** both the methyl groups and the geminal protons are strongly shielded by the 4-phenyl group. The values observed for compound **(80)** accord well with those of **(27)** and are quite different from those of **(32)** (see table 3).

Table 3. ^1H n.m.r. of Compound 27, 32 and 80

Compound	Selected ^1H signals			
	H-9	H-8	-8Me	-9Me
Tomentolide A(32)	2.18	3.78	0.71	1.00
Inophyllolide(27)	2.46	4.32	1.58	1.21
Compound (80)	2.56	4.22	1.58	1.22

Thus the 2,3-dimethylchromanone ring must be attached to C-7 and C-10a of the coumarin nucleus. The free nature of the phenolic hydroxyl group indicates that it is attached to C-5 remote from any of the other oxygens. The prenyl group is therefore attached at C-6 . Thus structure **(80)** is assigned to the new dihydrocoumarin.

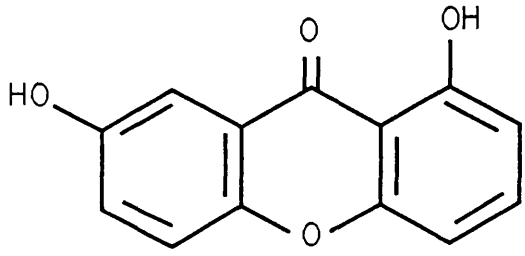
The ^{13}C n.m.r. spectra of the dihydrocoumarin **(80)** was assigned by comparing with model systems and clearly showed signals at δ_C 21.6 (t), 121.6 (d), 132.2 (s), 25.7 (q) and 17.8 (q) for the isopentenyl chain [8,77]. Resonances for the aromatic carbons of the 4-phenyl substituent were observed at δ_C 129.0 (2 X C,d), 127.7 (2 X C,d), 127.3 (d) and 140.9(s). The *trans*-2,3-dimethylchromanone ring was established [57] by signals at δ_C 10.0 (q), 19.7 (q), 79.0 (d) , 46.0 (d), 200.0 (s), 105.3 (s) and 157.6 (s). C-2 and C-3 of the dihydrocoumarin moiety appeared at δ_C 36.5 (t) and 34.1(d) respectively while the carbonyl resonance [57] was found at δ_C 166.5 ppm. Assignments of the remaining carbons of the coumarin nucleus (see experimental) are tentative and must await confirmation by 2D long-range δ_C/δ_H correlation.

The structure of compound **(80)** was also further supported by HRMS analysis which showed, as base peak, the parent ion at m/z 406.1770 $C_{25}H_{26}O_5$. The expected loss of methyl radical [m/z 391.1589 (21.95)]

and loss of CO from the pyrone ring [m/z 378.1817 (7.8%)] from the molecular ion were also observed.

The dihydrocoumarin **(80)** is the first example of its type to be isolated and is therefore of biosynthetic interest. It may be a precursor of *trans*-inophyllolide **(27)** to which it is related by oxidation, cyclisation and dehydrogenation.

A pentacyclic triterpenoid was present in the same fraction as the dihydrocoumarin **(80)**. Separation of the triterpenoid by preparative t.l.c. was unsuccessful, the two compounds have the same R_f values. However, on g.l.c., two peaks (R_t 10.1 and 22.3 mins) were observed, the minor (R_t 22.3 mins) of which may be attributed to the triterpenoid. The ^1H n.m.r. signals of the triterpenoid could be observed in the spectrum of the dihydrocoumarin **(80)**. The spectrum showed eight methyl singlets at δ_H 0.84, 0.95, 0.98, 1.00, 1.04, 1.09, 1.14 and 1.19. Two one-proton triplets at δ_H 3.42 (1H, J=2.7 Hz) and δ_H 5.62 (1H, J=5.6 Hz) could be assigned respectively to the equatorial proton of a 3α-hydroxy group in ring A as in isotaraxerol **(81)** [92] and to an olefinic proton. The mass spectrum of the trimethylsilyl derivatives revealed the parent ion at m/z 498 and hence the triterpenoid has the molecular formula $C_{30}H_{50}O$. Other high mass fragmentation included m/z 483 (M^+-CH_3), 408 (M^+-Me_3SiOH) and 393 ($M^+-Me_3SiOH-CH_3$). The most



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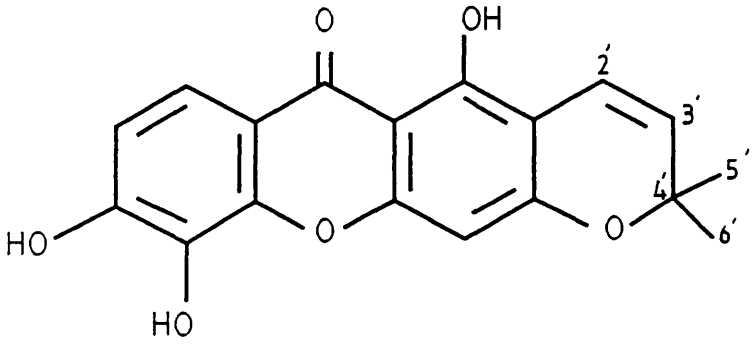
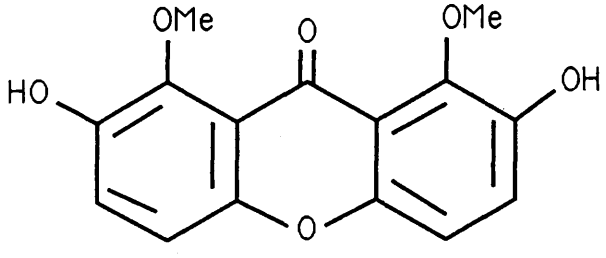


(21)



82. 2,7-dihydroxy-1,8-dimethoxyxanthone

significant cleavage of the molecule involved loss m/z 134 ($C_{10}H_{14}$) to give fragments at m/z 274 and 259. The formation of the fragment at m/z 134 must involve cleavage of ring B or ring D of a pentacyclic skeleton and cannot be rationalised in term of expected Diel-Alder fragmentation. Comparison of the ^1H n.m.r. methyl group shifts of isotaraxerol (**81**) with the unknown compound showed that they are different [92]. The triterpenoid remains unidentified at present.

The widespread 1,5 -dihydroxyxanthone(**2**) was found in the 30 % ethyl acetate fraction. The HRMS analysis revealed the base peak/the parent ion at m/z 228.0422 (100 %) [$C_{13}H_8O_4$]. Further loss of CO as expected produced the usual dibenzofuran fragment ion [93] at m/z 172.0512 (4.2%). The i.r. spectrum showed a phenolic hydroxyl (ν 3415 cm^{-1}) and a carbonyl (ν 1649 cm^{-1}). The UV spectrum (λ_{max} 233, 248, 315, 368 nm) [27] gave the first indication that the xanthone had a 1,5-oxygenation pattern. The ^1H n.m.r. spectrum showed a chelated hydroxyl which gave signal at δ_H 12.63 (s, 1H). The presence of the chelated hydroxyl group was further supported by a bathochromic shift in the UV maxima on addition of 5% ethanolic aluminium chloride [90,91]. This indicated that the hydroxy must be attached to C-1. It has been shown previously that the aromatic proton attached to C-8 is deshielded by the *ortho* ring carbonyl group [94].

Thus a signal at δ_H 7.59 (dd, 1H, J=7.6, 1.9 Hz) must be attributed to H-8. The chemical shift and coupling constants of the other aromatic protons in ring A were almost identical with reported values [94]. However, the coupling constants of protons in ring B differed slightly from reported values. Comparison of the ^{13}C n.m.r spectra of **(2)** with the reported values[10] revealed virtual identity and confirmed the structure of the 1,5,-dihydroxy-xanthone.

Flash chromatography of the ethyl acetate extract yield a yellow crystalline constituent whose the structure was established as 2,7-dihydroxy-1,8-dimethoxyxanthone **(82)**. HRMS analysis showed the parent ion/base peak [$C_{15}H_{12}O_6$] at m/z 288.0639. The other significant fragmentation gave M^+-CH_3 (26.3 %) and M^+-H_2O (26.3) fragments. The i.r. spectrum of the compound showed typical bands for phenolic hydroxyl (ν 3400 cm^{-1}) and xanthone carbonyl (ν 1650 cm^{-1}) absorption. The ^1H n.m.r. spectrum showed only two *ortho*-coupled aromatic proton signals at δ_H 7.15 (d, 2X1H, J=9.1 Hz) and δ_H 7.32 (d, 2X1H, J=9.1 Hz). The chemical shifts of these protons clearly excluded attachment to C-1 and C-8 since the deshielding effect of the carbonyl is not apparent. It is clear that the substitution of both rings must be identical and that the molecule is symmetrical. The only other protons observed were attributed to hydroxyl [δ_H 5.79

(s, br, 2X OH)] and methoxyl[δ_H 4.04 (s, 2XOMe)] substituents. In the absence of chelation, the hydroxyl must be attached to C-2 and C-7 and hence the methoxyl at C-1 and C-8. The UV spectrum (λ_{max} 241, 264, 362 nm) also supported the oxygenation pattern of **(82)** and is similar to that of 2,8-dihydroxy-1-methoxyxanthone. The absence of the chelated hydroxyl was further supported by the absence of shifts on the UV maxima on addition of 5% ethanolic aluminium chloride. Support for the structure **(82)** was obtained from the ^{13}C n.m.r. spectrum despite failure to see all the signals. The symmetry of the molecule was apparent since only five resonances were clearly observed. Thus the aromatic doublets appeared at δ_C 113.7 (C-4, C-5) and 122.1 (C-3, C-6), the methoxyl carbon at δ_C 62.6 [supporting the lack of an *ortho*-proton], and the oxygenated carbons at δ_C 150.3 (C-4a, C-10a) [cf 1,2,3-trihydroxyxanthone, C-4a, δ_C 150.3], 145.1 [C-1, C-8, C-2, C-7]. A weak signal at δ_C 174.9 could arise from the carbonyl group C-9 [cf δ_C 174.9 for 1,5-dimethoxy-xanthone]. The resonance for C-8a and C-9a should appear at approximately δ_C 112.0. It was not seen but could be concealed under the signal for C-4 and C-5. Thus the new compound is 2,8-dihydroxy-1,7-dimethoxy-xanthone **(82)**.

The known xanthone jacareubin **(21)** was found in the petroleum ether extract of the wood. Its HRMS

spectrum showed a parent ion at m/z 326.07851 [$C_{18}H_{14}O_6$] and a base peak at m/z 311.5590 which arose by loss of methyl from the 2,2-dimethylpyran ring to give a stable benzopyrylium ion, a common fragment in this species [95]. The i.r. spectrum of jacareubin (21) showed typical hydroxyl (ν 3420 cm^{-1}) and carbonyl (ν 1650 cm^{-1}) signals. The ^1H n.m.r. spectrum showed typical signal resonances for a 2,2-dimethylpyran unit [96] at δ_H 1.45 (s, 2X Me), δ_H 5.58 (d, 1H, J=10.00 Hz) and δ_H 6.58 (d, 1H, J=10.0 Hz). Two proton doublets at δ_H 6.82 (d, 1H, J=8.0) and δ_H 7.50 (d, 1H, J=8.0 Hz) were assigned to the aromatic protons at C-7 and C-8, the latter being deshielded by the carbonyl group [93]. A positive Gibbs' test indicated the presence of a chelated hydroxyl attached to C-1. The remaining aromatic proton singlet at δ_H 6.34 was assigned to H-4.

The ^{13}C n.m.r. spectrum of jacareubin (21) had resonances for a carbonyl group [δ_C 180.0] and several oxygenated aromatic carbons at [δ_C 132.6 (s) (C-5); δ_C 152.1 (C-6); δ_C 159.6 (C-1)]. The olefinic carbons of the 2,2-dimethylpyran ring gave rise to signals at δ_C 128.1 (d) and 114.9 (d) while the methyl groups appeared at δ_C 27.9 (2, XMe). The other shifts are listed in the Experimental section. Comparison of the ^{13}C n.m.r. signals with the reported values [10] confirmed identity.

EXPERIMENTAL

The bark and wood of ***C. biflorum*** were obtained from the Tekam River Forest, Pahang, Malaysia. Finely ground bark (572.4 g) of ***C. biflorum*** was extracted with hot petroleum ether (60-80) for one day and the extract was concentrated to give a yellow solid extract(44.2 g). Part (35.0 g) of the extract was adsorbed on 60.0 gm silica gel GF_{254} and chromatographed over 120.0gm of the same adsorbent. The extract was eluted with petroleum ether containing increasing proportion of ethyl acetate. About 100 ml fractions of the eluate were collected as follows: Fraction 1 (100% petroleum ether (60-80)), 2-5 (petroleum ether (60-80)-ethyl acetate, 4:1), 6-10 (petroleum ether (60-80)-ethyl acetate , 7:3), 11-13 (petroleum ether (60-80)-ethyl acetate, 3:2), 14-15 (petroleum ether (60-80)-ethyl acetate, 1:1), 16 (petroleum ether (60-80)-ethyl acetate, 2:3), 17 (petroleum ether (60-80)-ethyl acetate, 3:7), 18 (petroleum ether (60-80)-ethyl acetate, 1:4) and 19 (100 % ethyl acetate).

The crude fraction 3 (752.3 mg) was purified by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 19:1) and gave the **dihydrocoumarin (80)** $C_{25}H_{26}O_5$, (111.9 mg), m.p. 97-98°C, $[\alpha]_D$-38.8° (c, 0.66 in $CHCl_3$). UV λ_{max} nm: 282, 345 (ε resp. :

14600, 34600). Gibbs' test : λ_{max} no alteration in the presence of $AlCl_3$. I.r. ν_{max} cm^{-1} 3620, 2940, 1780, 1690, 760.

^1H n.m.r. : δ 1.22 (d, 3H, J= 6.9 Hz, C-9Me), 1.53 (d, 3H, J=6.3 Hz, C-8Me), 1.68 (s, 3H), 1.79 (s, 3H), 2.56 (m, 1H, J=6.9 , 11.3 Hz, H-9) , 3.34 (dd, 2H, J=7.4 Hz), 4.22 (m, 1H, J=6.3, 11.3 Hz, H-8), 4.61 (dd, 1H, J=6.1 , 2.8 Hz), 5.19 (t, br, 1H, J=7.4, 1.4 Hz), 7.24 (s, -OH), 7.26 (complex, m, 5H).

^{13}C n.m.r. : δ 10.0 (q, C-8Me), 17.8 (q, C-5'), 19.7 (q, C-9Me), 21.6 (t, C-1'), 25.7 (q, C-4'), 34.1 (d, C-4), 36.5 (t, C-3), 46.0 (d, C-9), 79.0 (d, C-8), 104.3 (s, C-10a), 105.3 (s, C-4a), 108.9 (s, C-6), 121.6 (d, C-2'), 127.3 (d, C-4"), 127.7 (d, C-3", 5"), 129.0 (d, C-2", 6"), 132.2 (s, C-3'), 140.9 (s, C-1"), 156.4 (s, C-5), 157.6 (s, C-7), 158.8 (s, C-1a), 166.5 (s, C-2), 200.0 (s, C-10). [Found m/z (rel. int.): 406.1770 (100). $C_{25}H_{26}O_5$ requires 406.1780. Other significant peaks in the HRMS were at m/z (rel. int.) : 407.1811 (23.0) [M^++1], 391.1589 (21.9), [M^+-CH_3], 378.1817 (7.8) [M^+-CO], 55.0536 (25.0 %)].

A minor component which accompanied the **dihydrocoumarin (80)** could not be separated by t.l.c.. A TMS derivative was prepared and subjected to GC/MS analysis using a DB-1, 60 m capillary column at oven temperature 265°C . The minor component afforded a peak at R_t 22.3 mins which gave a parent ion at m/z

498, corresponding to the TMS ether of a pentacyclic triterpenoid $C_{30}H_{50}O$.

^1H n.m.r. : δ 0.85 (s, 3H), 0.95 (s, 3H), 0.98 (s, 3H), 1.00 (s, 3H), 1.04 (s, 3H), 1.09 (s, 6H), 1.14 (s, 3H), 1.16 (s, 3H) 3.42 (t, 1H, J=2.7 Hz), 5.62 (t, 1H, 5.6 Hz).

Fraction 12 (16.5 mg) was crystallised from methanol to give **1,5-dihydroxyxanthone(2)** (11 mg), m.p. 265-267°C, [lit [20] 264-265 °C], UV λ_{max} (nm) : 248 (ε , 11610). Gibbs' test λ_{max} (EtOH+$AlCl_3$) nm : 263, 338, 410 (ε resp. : 12900, 22580, 19350). I.r. ν_{max} cm^{-1}: 3415, 1649, 1580, 780, 730.

^1H n.m.r. (d_6-DMSO) : δ 6.80 (dd, 1H, J=7.6, 0.8 Hz, H-2), 7.05 (dd, 1H, J=7.7, 0.8 Hz, H-4), 7.28 (t, 1H, J=7.6 Hz, H-7), 7.37 (dd, 1H, J=7.9 , 1.9 Hz, H-6), 7.59 (dd, 1H, J=7.6 , 1.9 Hz, H-8), 7.73 (t, 1H, J=8.3 Hz, H-3), 7.94 (s, 1H, OH), 12.63 (s, 1H, OH).

^{13}C n.m.r. (d_6-DMSO): δ 107.4 (d, C-4), 108.2 (s, C-9a), 110.2 (d, C-2), 114.6 (d, C-8), 120.9 (s, C-8a), 121.1 (d, C-6), 124.4 (d, C-7), 137.4 (d, C-3), 145.2 (s, C-10a), 146.5 (s, C-5), 155.6 (s, C-4a), 161.0 (s, C-1), 182.1 (s, C-9). [Found m/z (rel. int.) : 228.0415 (100). $C_{13}H_8O_4$ requires 228.0422. Other significant peak in the HRMS were at (m/z) (rel. int.)200.0453 (9.33 %) [M^+-CO], 172.0512 (4.2%) , 171.0445 (6.3)].

The bark was further extracted with ethyl acetate for eight hours and afforded a dark yellow solid (37.3 g). The extract (3.5 g) was adsorbed on

silica gel GF_{254} (6.5 g) and chromatographed over the same adsorbent (120.0 g). The extract was eluted with petroleum ether (60-80) containing increasing proportion of ethyl acetate. About 100 ml fractions of the eluate were collected as follows: Fraction 1-2 (petroleum ether (60-80)-ethyl acetate, 4:1), 3 (petroleum ether (60-80)-ethyl acetate, 3:2), 4 (petroleum ether (60-80)-ethyl acetate, 2:3), 5 (petroleum ether (60-80)-ethyl acetate, 1:4), 6 (100 % ethyl acetate).

Fraction 5 (17.5 mg) was crystallised from petroleum ether (60-80) to give **2,7-dihydroxy-1,8-dimethoxyxanthone(82)** (10.2 mg) $C_{15}H_{12}O_6$, m.p. 235-237°C, UV λ_{max} nm : 241, 264, 362 (ε resp. : 82058, 73230, 9470). Gibbs' test : λ_{max} no alteration in the presence of $AlCl_3$. I.r. (KBr Disc) ν_{max} cm^{-1} 3400, 2920, 1650, 1620, 1475, 1425, 1110.

^1H n.m.r. (CD_3OD) : δ 4.04 (s, 2 X OMe), 5.79 (b, OH), 7.32 (d, 2 X 1H, J= 9.1), 7.15 (d, 2 X 1H, J= 9.1 Hz).

^{13}C n.m.r. (CD_3OD): 62.6 (q, 2XOMe), 113.7 (d, C-4, 5), 122.1 (d, C-3, 6), 145.1 (s, C-1, 2, 7, 8), 150.3 (s, C-4a, 10a), 174.9 (s, C-9), C-8a, 9a are not detected.

[Found m/z (rel. int.): 288.0639 (100). $C_{15}H_{12}O_6$ requires 288.0634. Other significant peaks in the HRMS were at m/z (rel. int.) : 273.0396 (26.3 %) [M^+-CH_3], 270.0525 (26.32 %) [M^+-H_2O]. 250.042 (26.2 %) , 245.044 (58.3%) [M^+-CO], 230.0213 (45.3 %) , 202.0271

(31.5%)] .

The ground wood (587.78 g) of ***C. biflorum*** was extracted with petroleum ether (60-80) for one day and the extract was concentrated to yield a yellow solid (1.33 g). Trituration with petroleum ether (60-80) yielded a solid yellow residue (180 mg) which was crystallised from chloroform to give **jacareubin(21)** $C_{16}H_{14}O_6$(38.6 mg), m.p. 252-254°C [lit [36] m.p.256°C], UV λ_{max} nm : 295, 358 (ε resp. : 27630, 12230). Gibbs' test UV λ_{max} (EtOH + $AlCl_3$) nm : 301, 390 (ε resp. : 27630, 13240). I.r. (KBr Disc) max cm^{-1} 3420, 2980, 1650, 1610, 1580, 1420, 770, 730. ^1H n.m.r. (CD_3OD) : δ 1.45 (s, 2XMe), 5.58 (d, 1H, J=10.0 Hz), 6.34 (s, 1H), 6.58 (d, 1H, J= 10.0 Hz), 6.82 (d, 1H, J= 8.0 Hz), 7.50 (d, 1H, 8.0 Hz). ^{13}C n.m.r. (D_6MSO) : 27.9 (q, C-5',6'), 78.1 (s, C-4'), 94.6 (d, C-4), 102.3 (s, C-9a), 103.8 (d, C-2), 113.0 (s, C-8a), 113.2 (d, C-7), 115.9 (d, C-8), 128.1 (d, C-2'), 132.6 (s, C-5), 146.1 (s, C-10a), 152.1 (s, C-6), 156.6 (2)(s, C-3 and C-4a), 157.0 (s, C-3a), 159.6 (s, C-1), 180.0 (s, C-9). [Found m/z (rel. int.): 326.0785 (11.43 %). $C_{16}H_{14}O_6$ requires 326.0790. Other significant peaks in the HRMS were at (m/z) (rel.int.) : 311.1559 (100.0) [M^+-CH_3].

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CHAPTER III

THE PHENYLETHYLCHROMONES AND SESQUITERPENOIDS

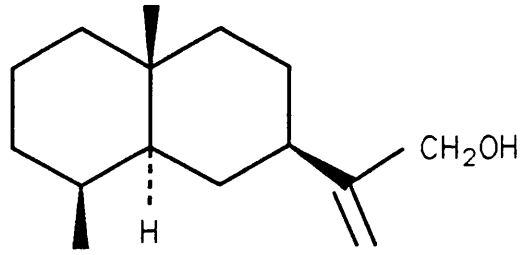
FROM THE INFECTED WOOD EXTRACT OF

Aquilaria malaccencis

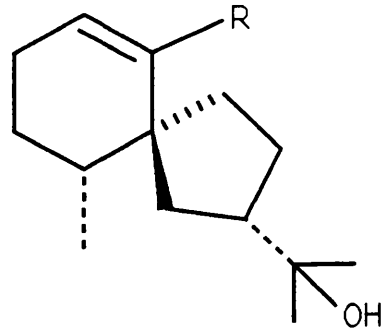
INTRODUCTION

Aquilaria malaccencis from the family Thymelaeacea [Aquilariodeae] is a tree with a smooth bark, usually whitish. There are five species of ***Aquilaria*** [1] found in West Malaysia namely ***A. hirta***, ***A. beccariana***, ***A. rostrata***, ***A. malaccencis*** and ***A. microcarpa***. However only one species, ***A. malaccencis***, is common and widespread. The timber is light, soft and very perishable and so of little commercial use. Sometimes, however, attack by a fungal pathogen, entering either through a wound or by encroachment, will cause a dense dark brown resin stained heartwood known as "gaharu" [2]. It is still unclear what happens to normal heartwood on microbial infection. It is assumed that phytoalexins are produced in response to the infection. The infected heartwood is highly valued and yields an oil, agarwood oil, used for incense. The wood is utilised in native medicine in many ways including the production of smoke for treating asthma. There has been extensive trading throughout the East from early times from the countries where the tree grows. Agarwood oil, exotic in character and of high fixture value, makes the wood greatly prized for incense.

Studies on the steam distillation of the wood of ***A. agallocha*** [3], infected with an ***Aspergillus*** species, revealed that the oil yield varied from 0.31 % to 1.5 %,

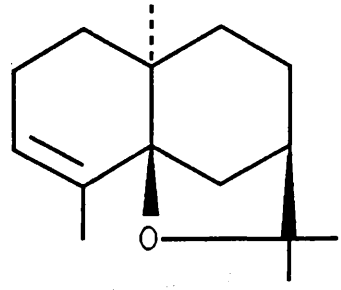


1. Agarol

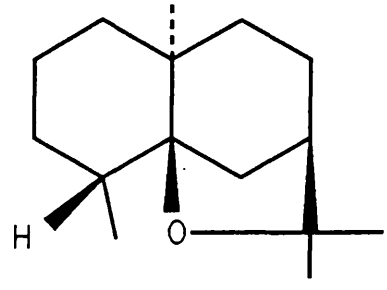


2. R= Me (agarospirol)

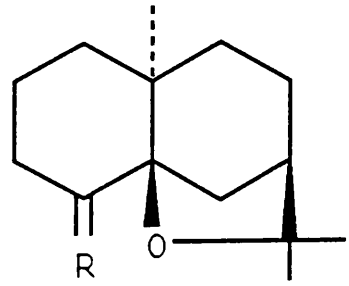
16. R= CHO (oxoagarospirol)



3. α-agarofuran

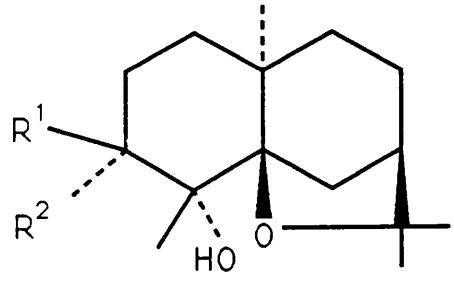


4. Dihydroagarofuran



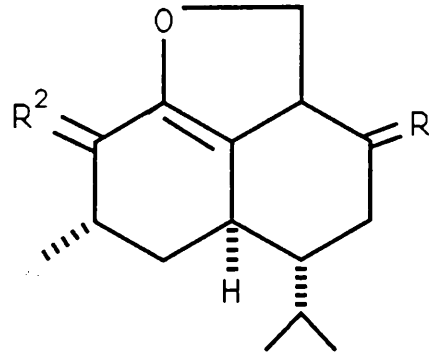
5. R = CH_2 (β- agarofuran)

6. R= O (norketoagarofuran)



7. $R^1 = R^2 = H$

8. R^1= OH ; R^2 = H



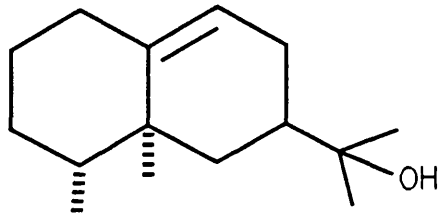
9. $R^1 = R^2 = O$

10. R^1 = O ; R^2 = α-OH,H

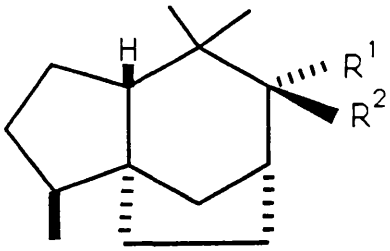
mainly depending on the quality of the raw material. Exploratory studies [4] on the change of essential oil formation and oil constituents with the state of growth of agarwood failed to reach any definitive conclusions.

The first chemical investigation of agarwood was reported by Kafuku and Ichikawa [5] in 1935. In their investigation benzylacetone and dihydrocinnamic acid were isolated following saponification of the crude extract. Agarol(1) [6] was the first sesquiterpenoid to be isolated from the solvent-extracted essential oil of the fungus infected ***A. agallocha***. Its absolute configuration was established as in (1). Agarospirol(2) [7], a spiro-terpenoid, was later identified as one of the major components after agarol(1). The structure of agarospirol(2) was established by degradative studies and spectroscopic measurements and was further supported by synthesis of the derived ketone.

Further analysis of the agarwood oil [8,9] from ***A. agallocha*** afforded six minor sesquiterpenoids, the selinane tetrahydrofurans α-agarofuran(3), dihydroagarofuran(4), β-agarofuran(5), nor-ketoagarofuran(6), 4-hydroxyagarofuran(7) and 3,4-dihydroxydihydroagarofuran(8). In contrast, investigation of the ethanol extract [10] of ***A. agallocha*** resulted in the isolation of two cadinane sesquiterpenoids whose structures were established as gmelofuran(9) and dihydrogmelofuran(10).

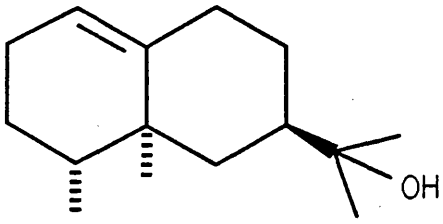


11. Jinkoh - eremol

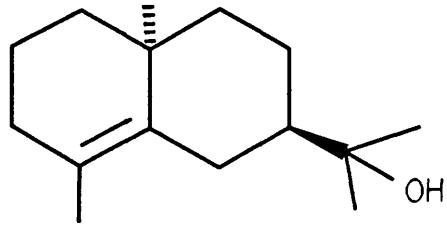


12. R^1 = H ; R^2 = CH_2OH (Jinkohol II)

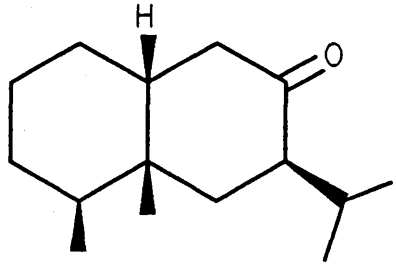
14. R^1 = Me ; R^2 = OH (Jinkohol)



13. Kusunol



15. (-) 10-epi-δ- eudesmol

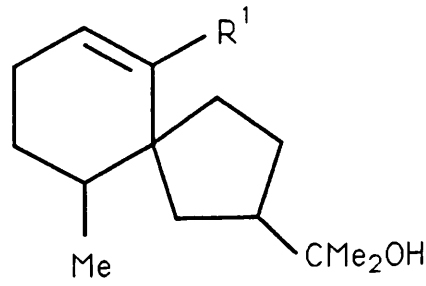


17. (+) dihydrokaranone

The constituents of the essential oil from ***A. malaccencis*** [11], a different kind of agarwood, include two new sesquiterpenoid alcohols, jinkoh-eremol(11) and jinkohol II(12), together with the known agarospirol(2), kusunol(13) [12] and jinkohol(14) [13] as the major constituents. Further investigation [14] of the benzene extract of ***A. malaccencis*** by HPLC identified three known fragrant sesquiterpenoids, α-agarofuran(3), (-)-10-epi-γ-eudesmol(15) and oxo-agarospirol(16).

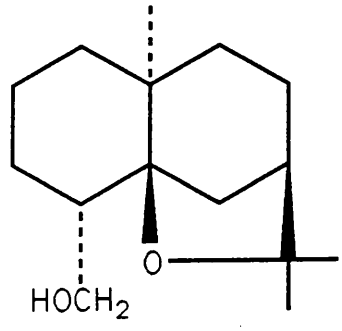
A detailed study [15] of the sesquiterpenoid content of the two different kinds of agarwood discussed above by a combination of GLC and GC/MS suggested that agarospirol(2), jinkoh-eremol(11), kusunol(15) and oxo-agarospirol(16) occurred abundantly, while, ***A. malaccencis*** , and not ***A. agallocha***, contained a large amount of (-)-10-epi-γ-eudesmol(15), jinkohol(14) and jinkohol II(12). In addition, nor-ketoagarofuran(6) and (+)dihydrokaranone(17) were found only in ***A. agallocha***.

Thus, the agarwood from ***A. malaccencis*** and ***A. agallocha*** can be easily distinguished on the basis of morphological studies [15] and sesquiterpenoid content. However, the agarwood called kanankoh [15] does not show any strong similarity in chemical composition to the agarwoods mentioned above. More detailed analytical results would be useful in placing the identification of agarwoods on a firmer basis. A study of the chemical constituents of ***A. sinensis*** [16,17] from China afforded



18. R^1= COOH

19. R^1= CHO



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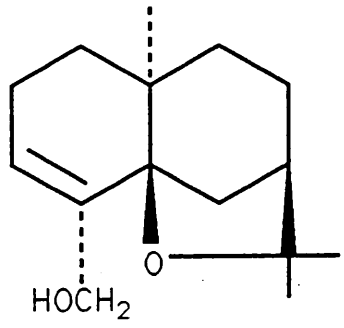
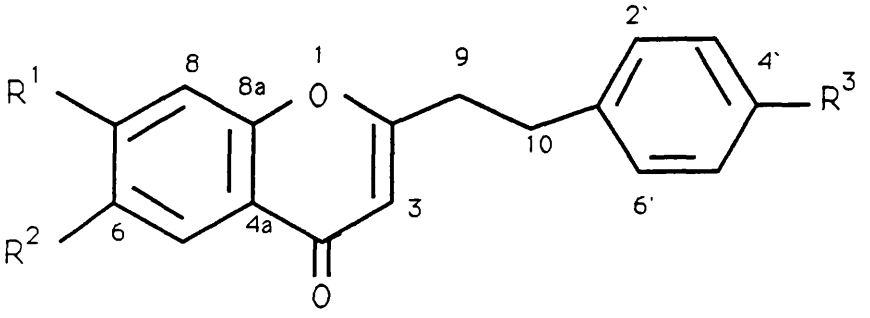


21.



22. $R^1 = R^2 = H$; $R^3 = OMe$

23. $R^1 = R^2 = R^3 = H$

29. $R^1 = R^3 = H$; $R^2 = OH$

30. $R^1 = R^3 = H$; $R^2 = OMe$

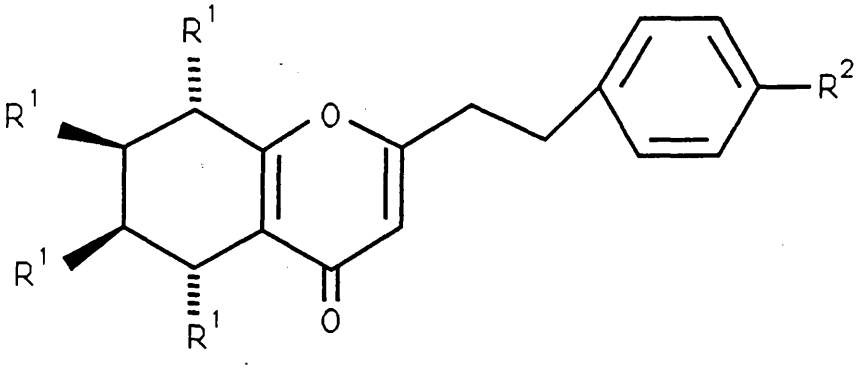
31. $R^1 = R^2 = OMe$; $R^3 = H$

32. $R^1 = H$; $R^2 = R^3 = OMe$

37. $R^1 = H$; $R^2 = R^3 = OMe$

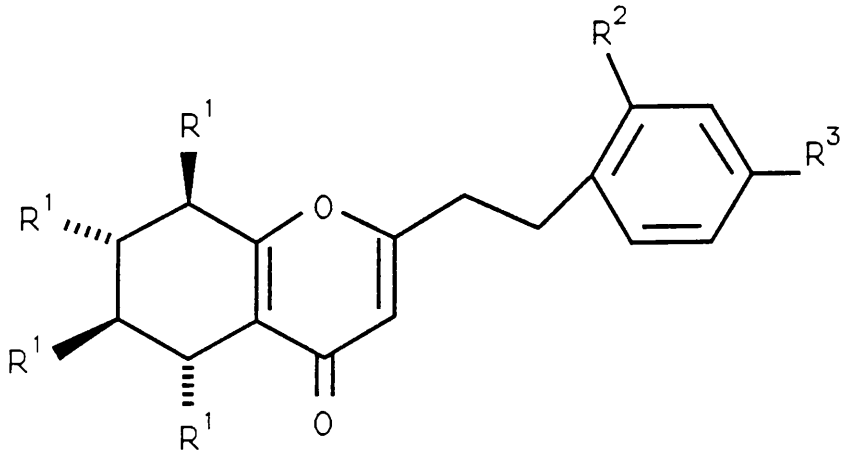
42. $R^1 = R^2 = R^3 = OMe$

44. $R^1 = R^3 = H$; $R^2 = OAc$



24. $R^1 = OH$; $R^2 = H$

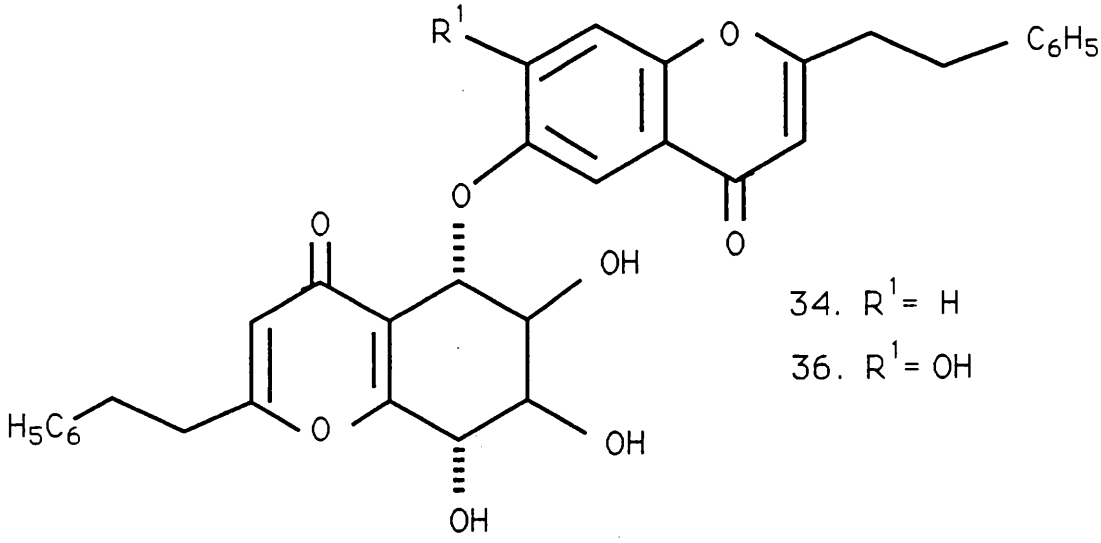
26. $R^1 = OAc$; $R^2 = OMe$



25. R^1 = OH ; R^2 = R^3 = OMe

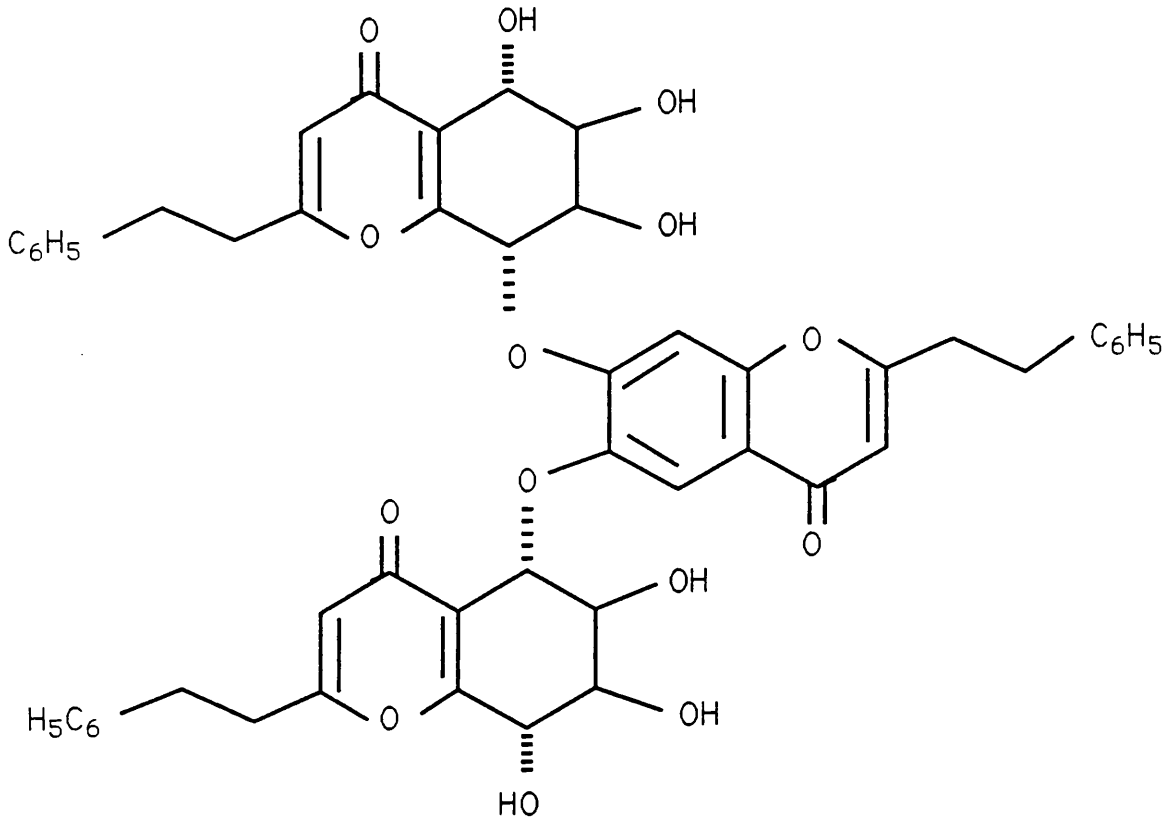
27. R^1 = OH ; R^2 = H ; R^3 = OMe

28. R^1 = OH ; R^2 = H ; R^3 = OH



34. R^1 = H

36. R^1 = OH



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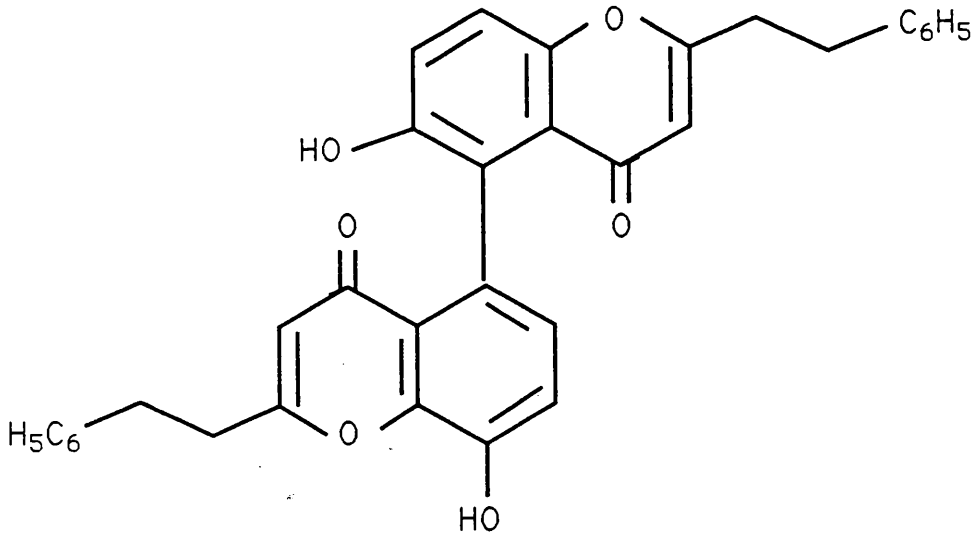
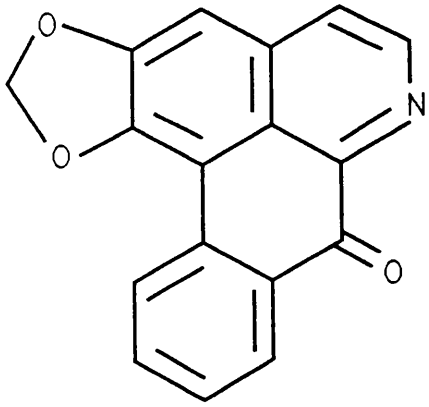


35.

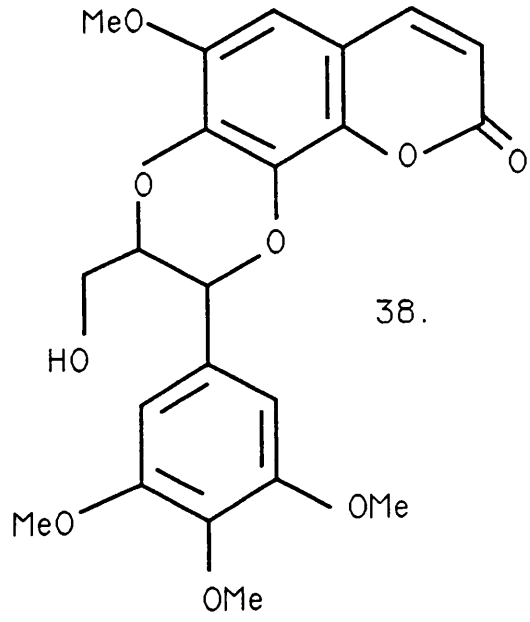
four new sesquiterpenoids, baimuxinic acid(18), baimuxinal (19), baimuxinol(20) and dehydrobaimuxinol(21).

The principal non-terpenoids constituents of agarwood are dervatives of phenylethylchromone . A common example is the biogenetically interesting, highly-oxygenated metabolite agarotetrol(22), from ***A. agallocha*** [18] whose absolute configuration has been established. Investigation of the acetone extract ***Aquilaria*** [19,20,21,22] species (Jinko) resulted the isolation of a range of phenylethylchromone derivatives including 2-[2-(4'-methoxyphenylethyl)]chromone(23), 2-(2-phenylethyl)chromone(24), agarotetrol(22), isoagarotetrol(25), 2-[2-(4 methoxyphenylethyl)-5α, 6β, 7β, 8α-tetracetoxy-5, 6, 7, 8-tetrahydrochromone(26), 2-[2-(4-methoxyphenylethyl)]-5α, 6β, 7α, 8β-tetrahydroxy-5, 6, 7, 8-tetrahydrochromone(27), 2-[2-(2-hydroxyphenylethyl)]-5α, 6β, 7α, 8β-tetrahydroxy-5, 6, 7, 8-tetrahydrochromone(28), 6-hydroxy-(29), 6-methoxy-(30) and 6, 7-dimethoxy-2-(2-phenylethyl)chromone(31), 6-methoxy-2-[2-(3-methoxyphenylethyl]chromone(32). In addition, trimers(33) and dimers (34), (35) and (36) have also been isolated from the acetone extract of agarwood [23,24]. A further chromone derivative, 2-[2-(4-methoxyphenyl-ethyl)]-6-methoxychromone(37), has been obtained from the benzene extract of ***A. agallocha*** [25].

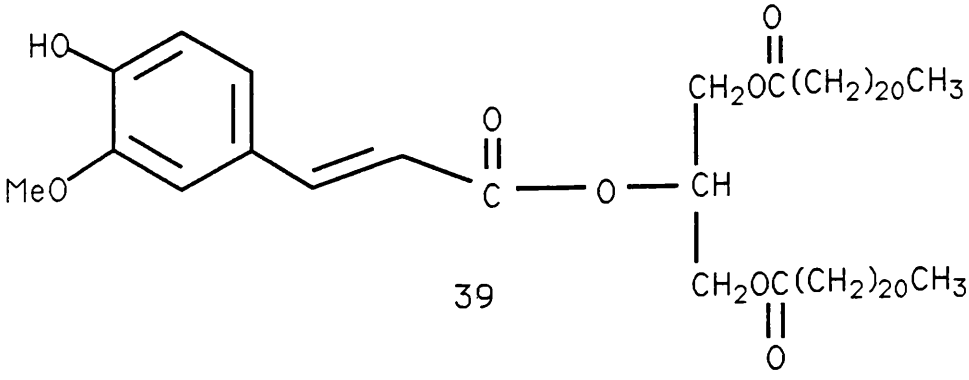
Pyrolysis of 2-[2-(4-methoxyphenylethyl)]



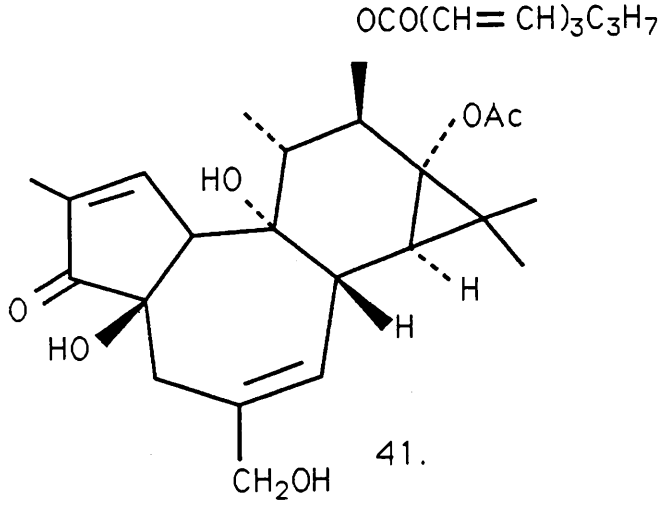
40. Liriodenine



38.



39



41.

chromone (23) and 2-(2-phenylethyl)chromone(24) at 150°C produced 4-methoxybenzaldehyde and benzaldehyde respectively. This result suggested that the chromones which are odourless at room temperature contribute to the pleasant lasting odour when agarwood is burnt.

Other compounds from ***Aquilaria*** species include, 1, 3-dibehenyl-2-ferulyl glyceride(38), the known 12-O-*n*-deca-2, 4, 6-trienoylphorbol-13-acetate(39) from the stem bark of ***A. malaccenceis***, the 7-oxo-aporphine alkaloid liriodenine(40) which was isolated [26] from the chloroform extract of the heartwood ***A. agallocha***, and aquillochin(41), a coumarinolignan compound from ***A. agallocha*** [27]. Studies [28] have revealed that both (38) and (39) display *in vitro* cytotoxic activity against the P-388 lymphophytic leukemia system.

Effort to stimulate [29] the agarwood oils were made using herbicides such as 2, 4-D, 2, 4, T and MCPA. The experiments failed to induce the formation of infected ***A. agallocha*** or to increase the oil yield. Further study of the formation of oleoresin [30] was attempted using ***A. sinensis*** which normally does not contain oleoresin or secretory tissue. The trees were notched in an effort to stimulate oleoresin formation. There was no indication of increased oleoresin content as a result of the tree notching although some fungal activity was observed in the notches.

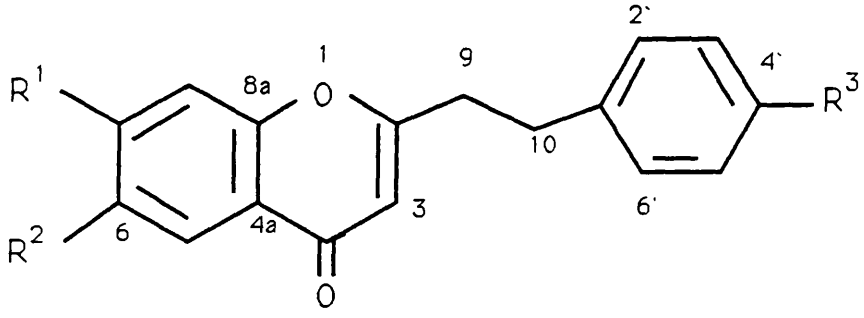
A study [31] of the correlation of quality of agarwood with the presence of chromones, using a dual thin layer chromatography scanner and standard samples of the 2-(2-phenylethyl)chromones agarotetrol(**22**) and isoagarotetrol(**25**) has been reported. These chromones are normally present in large amount in agarwood. The results revealed that, in general their presence is associated with high quality of agarwood, with some limitations. The good properties of agarwood named "kanankoh" were shown by the high intergrated value obtained by scanning at the 270 and 400 nm suggesting that constituents other than chromone derivatives may also influence the quality of agarwood.

DISCUSSION

Extraction of the infected wood with petroleum ether (60-80) and ethyl acetate followed by chromatography afforded several phenylethylchromones and sesquiterpenoids. The spectroscopic properties of these compounds are discussed below.

The first compound, the known 6,7-dimethoxy-2-(2-phenylethyl)chromone(31) [19] appeared to be a chromone derivative from its i.r. spectrum ν 1645 cm^{-1} (γ-pyrone) and ν 1420 cm^{-1} (aromatic ring) [19,32] which showed no hydroxyl adsorption. The HRMS analysis of (28) showed the parent ion at m/z 310.1206 (56.3 %) consistent with the molecular formula $C_{19}H_{19}O_4$. The base peak at m/z 91.0532 $[C_7H_7]^+$ was formed through the bond fission between C-9 and C-10 [24].

Its ^1H n.m.r. spectrum showed signals for two methoxyl groups at δ_H 3.95 (s, 3H) and δ_H 3.97 (s, 3H) and one phenylethyl group at δ_H 7.22 (m, 5H) and δ_H 2.96 (m, 4H) lacking substitution on its aromatic ring. Three singlet protons observed at δ_H 6.09, δ_H 6.85 and δ_H 7.49 were attributed to the protons attached to C-3, C-8 and C-5 respectively in the chromone moiety. The lack of coupling indicated that the two methoxyl groups were attached to C-6 and C-7. The ^{13}C n.m.r. spectrum further confirmed the suggested structure as it showed two signals at

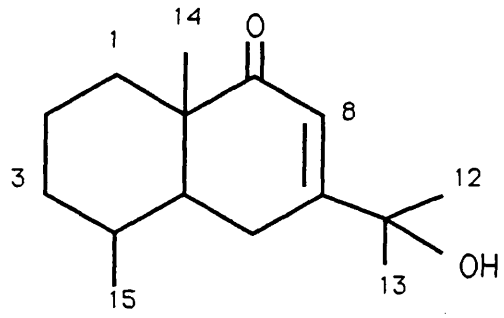


29. $R^1 = R^3 = H$; $R^2 = OH$

31. $R^1 = R^2 = OMe$; $R^3 = H$

42. $R^1 = R^2 = R^3 = OMe$

44. $R^1 = R^3 = H$; $R^2 = OAc$



43.

δ_C 152.4(s) and 154.2(s) for the neighbouring aromatic carbons with methoxyl substituents. These values are in agreement with the calculated values [32,33]. Two methoxyl group signals were observed at δ_C 56.3 (q) and 56.4 (q), values which indicate the presence of an *ortho* proton in each case. The carbon resonances of the $-CH_2CH_2-$ unit appeared at δ_C 33.0 (t) and 36.0(t).

The next compound isolated was a new chromone whose the structure was established as 6,7-dimethoxy-2[2-(4-methoxyphenylethyl)]chromone **(42)**. The compound exhibited i.r. bands arising from a phenylethyl group (ν 1422 cm^{-1}) and a substituted γ-pyrone ring (ν 1650, 1610 cm^{-1}) [32] and lacked hydroxyl absorption. HRMS analysis gave the parent ion at m/z 340.1311 (9.8 %) consistent with the molecular formula $C_{20}H_{20}O_5$. The base peak was found at m/z 121.0654 $[C_8H_9O]^+$ arising from expected bond fission between C-9 and C-10 [25].

The ^1H n.m.r. spectrum showed three methoxyl groups at δ_H 3.97 (s, 3H), δ_H 3.95 (s, 3H) and δ_H 3.77 (s, 3H), two of which were placed at C-6 and C-7 because the presence of two aromatic proton singlets at δ_H 7.49 (H-5) and 6.85 (H-8) and two *ortho* oxygenated carbons [δ_C 152.4 (C-6) and 154.2 (C-7)]. These shifts are in agreement with the corresponding shifts for 6,7-dimethoxy-2(2-phenylethyl)chromone **(31)**. H-3 appeared as expected at δ_H 6.08 (s). The remaining aromatic protons constituted an AA'BB'

system at δ_H 6.82 (d, 2X1H, J=8.7 Hz, H-3',5') and δ_H 7.08 (d, 2X1H, J=8.7 Hz, H-2',6') [25] indicating a *para*-disubstituted benzene ring. The chemical shifts of H-3' and H-5' are consistent with **(42)**, in particular C-4' at δ_C 158.2 [25,27]. Comparison with related compounds **(21)** and **(35)** provided convincing support for the structural proposal.

The next new compound from the extract was a sesquiterpenoid which was assigned the tentative structure 11-hydroxy-7-eudesmen-9-one **(43)** on the basis of its spectroscopic properties. The UV absorption maximum at 234 nm and the i.r. spectrum (ν 3660, 1670 cm^{-1}) showed that **(43)** contained both conjugated enone and hydroxyl functionality [33]. The High Resolution Mass Spectrometry did not give a parent ion. Facile loss of H_2O led to the highest mass fragment ion at m/z 218.1672 (27.66 %) $[C_{15}H_{22}O]$. The molecule is thus $C_{15}H_{24}O_2$ and is bicarbocyclic. The base peak at m/z 59.0485 supported the presence of a hydroxyisopropyl group.

The ^1H n.m.r spectrum showed the α-hydrogen of an enone system at δ_H 5.73 (t, 1H, J=1.3 Hz) coupled with two allylic protons. The ^{13}C n.m.r. signals at δ_C 199.7 (s), 124.4 (d) and 170.9 (s) were in accord with a trisubstituted conjugated enone system with the β-carbon fully substituted. In addition, the ^1H n.m.r. spectrum showed signals for one secondary [δ_H 0.95

(d, J=6.7 Hz)] and three tertiary methyl groups [δ_H 1.06, 1.16, 1.18], two of which were deshielded and part of a hydroxyisopropyl group. The carbon singlet at δ_C 72.4 (s) and the mass spectrum supported this suggestion. The structural units observed spectroscopically can be ready occommodated in a eudesmane skeleton, leading to structure **(43)** as a possible, but not unique solution. Effort to obtain confirmatory evidence from NOE experiments were singularly unsuccessful.

The ethyl acetate extract of ***A. malaccencis*** afforded the known 6-hydroxy-2-(2-phenylethyl) chromone**(29)** [19]. The i.r. spectrum showed that the compound had hydroxyl (ν 3285 cm^{-1}), carbonyl (ν 1630 cm^{-1}) and aromatic (ν 1455 cm^{-1}) absorption. Characteristic UV maxima appeared at λ_{max} 225, 236, 268, 326 cm^{-1} [19,34]. Signals in the ^1H n.m.r. spectrum at δ_H 2.96 (4H) and δ_H 7.21 (5H) showed the presence of phenylethyl group. The other aromatic protons overlapped partially with the phenyl protons, except for H-5 (δ_H 7.48 br,s). The structure was further supported by the ^{13}C n.m.r. spectrum, especially the signal at δ_C 154.6 for C-6 . The Low Resolution Mass Spectrometry revealed a parent ion at m/z 266, confirming the molecular formula $C_{17}H_{14}O_3$. Furthermore, the expected bond fission at C-9 and C-10 [25] gave rise to the fragment m/z 91 $[C_7H_7]^+$ as

the base peak.

The monoacetate derivative of the chromone **(44)** showed acetate signals at δ_H 2.29 (s, 3H) and δ_C 169.3 (C=O) and δ_C 21.0 (q, CH_3) . The monoacetate also showed a molecular ion m/z 308 [$C_{19}H_{16}O_5$] and a major fragment ion at m/z 266 due to loss of ketene from the parent ion. Again bond fission between C-9 and C-10[25] was observed leading to the fragment ion at m/z 91 which was also the base peak.

EXPERIMENTAL

The infected wood of ***A. malaccencis*** was collected in Terenganu, Malaysia. The wood (599.7 g) was powdered in a mill and extracted with hot petroleum ether (60-80) for eight hours. After filtration the solvent was evaporated to give a brownish oily solid extract (3.1 g). The extract was adsorbed on silica gel GF_{254} (6.0 g) and chromatographed over the same adsorbent (120.0 g), eluting first with petroleum ether (60-80) and then with petroleum ether containing increasing proportion of ethyl acetate. Fractions of eluate (100 ml) were collected as follows: Fraction 1-2 (100% petroleum ether), 3-4 (petroleum ether (60-80)-ethyl acetate, 9:1), 5 (petroleum ether (60-80)-ethyl acetate, 4:1), 6-8 (petroleum ether (60-80)-ethyl acetate, 7:3), 9-11 (petroleum ether (60-80)-ethyl acetate, 3:2), 12-15 (petroleum ether (60-80)-ethyl acetate, 1:1), 16 (petroleum ether(60-80)-ethyl acetate, 2:3), 17 (100% ethyl acetate).

Fraction 9 (82.0 mg) was separated by preparative thin layer chromatography (t.l.c.) (petroleum ether (60-80)-ethyl acetate , 9:1) into two bands. The less polar band (51.9 mg) was crystallised from petroleum ether to give **6,7-dimethoxy-2-(2-phenylethyl)chromone(31)**,

m.p. 135-136°C [Lit [18] m.p. 142-143°C], UV λ_{max} nm : 203 , 230, 247, 275, 310 (ε resp. 57200, 39910, 21280, 70300, 15290). I.r. ν_{max} cm^{-1} 2960, 2920, 1645, 1610, 1520, 1420, 700.

^1H n.m.r. : δ 2.96 (m, 4H), 3.95 (s, 3H), 3.97 (s, 3H), 6.09 (s, H-3), 6.85 (s, H-8), 7.22 (m, 5H), 7.49 (s, H-5).

^{13}C n.m.r. : δ 33.1 (t, C-10), 36.0 (t, C-9), 56.3 (q, OCH_3), 56.4 (q, OCH_3), 99.5 (d, C-8), 104.4 (d, C-5), 109.6 (d, C-3), 117.0 (s, C-4a), 126.5 (d, C-4'), 128.2 (d, C-2', 6'), 128.7 (d, C-3', 5'), 139.8 (s, C-1'), 147.4 (s, C-8a), 152.4 (s, C-7), 154.2 (s, C-6), 167.4 (s, C-2), 177.4 (s, C-4). [Found m/z (rel.int.): 310.1206 (56.3). $C_{19}H_{18}O_4$ requires 310.1205. Other significant peaks in the HRMS were at m/z (rel.int.): 295.0971 (1.8) [M^+-OCH_3], 279.0991 (1.2) [M^+-OCH_3, CH_3], 91.0532 $[C_7H_7]^+$(100).

The second band (15.6 mg) was further purified by preparative t.l.c. using the same solvent system (two developments) to give a pure unknown compound. $C_{15}H_{22}O_4$(9.0. mg). I.r. ν_{max} cm^{-1} : 3500, 2930, 2960, 1700, 1630, 1450, 1385, 1165, 1040, 1000.
[Found m/z (rel. int.): 266.1529 (23.5). $C_{15}H_{22}O_4$ requires 266.1518. Other significant peaks were at m/z (rel int.) : 251.1294 (3.0) [M^+-CH_3]. 248.1422 (32.5) [M^+-H_2O], 208.1105 (37.3)[M^+-58].

Fraction 12 (25.0 mg) was purified by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 9:1, four developments) and yielded a new compound **6,7-dimethyoxy-2[2-(4-methoxyphenylethyl)] chromone(42)** (14.2 mg) $C_{20}H_{20}O_5$, m.p. 129-130°C (petroleum ether (60-80)). UV λ_{max} nm : 203, 227, 247, 275, 313 (ε resp. 68000, 40000, 17040, 16200, 11070). I.r. ν_{max} cm^{-1} : 3010, 2960, 2940, 1650, 1610, 1510, 1420.

^1H n.m.r. : δ 2.94 (m, 4H), 3.77 (s, 3H), 3.95 (s, 3H), 3.97 (s, 3H), 6.08 (s, H-3), 6.82 and 7.08 (AA'BB', J=8.7 Hz), 6.84 (s, H-8), 7.49 (s, H-5).

^{13}C n.m.r : δ 32.2 (t, C-10), 36.3 (t, C-9), 55.2 (q, OCH_3), 56.4 (q, $2XOCH_3$), 99.5 (d, C-8), 104.4 (d, C-5), 109.6 (d, C-3), 117.0 (s, C-4a), 128.2 (d, C-2',6'), 128.7 (d, C-3',5'), 139.8 (s, C-1'), 147.4 (s, C-8a), 152.4 (s, C-7), 154.2 (s, C-6), 158.2 (s, C-4'), 167.4 (s, C-2), 177.4 (s, C-4). [Found m/z (rel. int.): 340.1311 (9.8). $C_{20}H_{20}O_5$ requires 340.1311. Other significant peaks in the HRMS were at m/z (rel. int.) : 121.0654 (100) $[C_8H_9O]^+$.

Fraction 13 (28.0 mg) was subjected to further purification by preparative t.l.c (100% chloroform) and afforded an oily new compound, tentatively **11-hydroxy-7-eudesmen-9-one (43)** (14.6 mg) $C_{15}H_{24}O_2$. $[\alpha]_D$ + 85° (c, 0.695 in $CHCl_3$). UV λ_{max} nm : 233 (ε, 16080). I.r. ν_{max} cm^{-1} : 3660, 2960, 1670.

^{1}H n.m.r. : δ 0.95 (d, 3H, J=6.7 Hz, H-15), 1.06 (s, 3H), 1.16 (s, 3H), 1.18 (s, 3H), 2.21 (br, 1H), 5.73 (t, 1H, J=1.3 Hz, H-8).

^{13}C n.m.r. : δ 14.9 (q), 16.9 (q), 26.9 (q), 27.3 (q), 27.7 (t), 32.9 (t), 39.1 (s, C-10), 39.6 (t), 40.5 (d, C-4), 42.0 (t, C-6), 43.8 (d, C-5), 72.4 (s, C-11), 124.4 (d, C-8), 170.9 (s, C-7), 199.7 (C-9). [No parent ion was detected. Other significant peaks in the HRMS were at m/z (rel. int.) : 221.1545 (4.3 %) [M^+-CH_3], 218.1672 (22.7 %) [M^+-H_2O], 203.1441 (2.2 %) [M^+-CH_3, H_2O], 178.1364 (27.0) [M^+-58], 177.1241 (3.4%) [$M^+-C_3H_7O$], 59.0485 (100 %)].

The infected wood sawdust was further extracted with ethyl acetate for a day and the solvent was evaporated to give a brownish oily material (22.0 g). Part of the extract (7.1 g) was adsorbed on silica gel GF_{254} (15.0 g) and chromatographed over of the same adsorbent (120.0 g). The extract was eluted with petroleum ether (60-80) and petroleum ether (60-80) containing increasing proportions of ethyl acetate. Fractions of eluate (100 ml) were collected as follows: Fraction 1 (petroleum ether (60-80)-ethyl acetate, 1:4), 2 (petroleum ether (60-80)-ethyl acetate, 3:2) , 3 (petroleum ether (60-80)-ethyl acetate, 1:1), 4 (petroleum ether (60-80)-ethyl acetate, 2 :3), 5 (petroleum ether (60-80)-ethyl acetate, 1:4) and 6 (100 % ethyl acetate).

Fraction 5 (71.8 mg) was purified by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 3:7, five developments) and gave **6-hydroxy-2-(2-phenylethyl)chromone(29)**(43.2 mg) which, on crystallised from chloroform-methanol afforded white crystals(22.4 mg), m.p. 210-211°C [lit.[18] 224-225°C]. UV λ_{max} nm : 225, 236, 268, 326 (ε resp. , 6670, 8480, 2160, 2340). I.r. ν_{max} cm^{-1} 3285, 2940, 1630, 1600, 1455, 700 .

^1H n.m.r. (CD_3OD) : δ 2.96 (m, 4H), 6.12 (s, H-3), 7.21 (m, 5H), 7.36 (m, 2H, H-7, 8), 7.48 (br, s, H-5).

^{13}C n.m.r. (D_6MSO) : δ 32.1 (t, C-10), 34.8 (t, C-9), 107.5 (d, C-5), 108.6 (d, C-3), 119.3 (d, C-8), 122.7 (d, C-7), 124.0 (s, C-4a), 126.1 (d, C-4'), 128.3 (d, C-2', 3', 5', 6'), 140.0 (s, C-1'), 149.6 (s, C-8a), 154.6 (s, C-6), 168.2 (s, C-2), 176.6 (s, C-4). [Found m/z (Low resolution MS)(rel.int.): 266 (77.2). $C_{17}H_{14}O_3$ requires 266.0. Other significant peak in the LRMS was at m/z (rel int.): 91 (100) $[C_7H_7]^+$].

The chromone(29) was further acetylated with acetic anhydride (1 ml) and pyridine (1 ml) at ambient temperature overnight. The usual work-up yielded **6-acetoxy-2-(2-phenylethyl)chromone (44)**, m.p. 78-79°C (from petroleum ether (60-80)). I.r. ν_{max} cm^{-1} : 1780, 1657, 1475, 1445, 1200, 1172.

^1H n.m.r. : δ 2.29 (m, 3H, OAc), 2.98 (m, 4H), 6.11 (s, H-3), 7.24 (m, 5H), 7.41 (m, 2H, H-7, H-8), 7.86 (m, H-5).

^{13}C n.m.r. : δ 21.0 (q, acetate), 33.0 (t, C-10), 36.1 (t, C-9), 109.9 (d, C-3), 117.8 (d, C-5), 119.4 (d, C-8), 124.5 (d, C-4a), 126.6 (d, C-4'), 127.6 (d, C-7), 128.3 (d, C-3', 5'), 128.7 (d, C-2', 6'), 139.6 (s, C-1'), 147.4 (s, C-8a), 153.9 (s, C-6), 168.6 (s, C-2), 169.3 (s, C-O, acetate), 177.4 (s, C-4). [Found m/z (Low resolution)(rel. int.): 308 (4.5). $C_{19}H_{16}O_5$ requires 308.0. Other significant peaks in the LRMS were at m/z (rel. int.): 266 (45.0%) [$M^{+\cdot}-CH_2CO^{+\cdot}$], 91 (100%) $[C_7H_7]^+$].

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CHAPTER IV

PHYTOCHEMICAL SCREENING OF

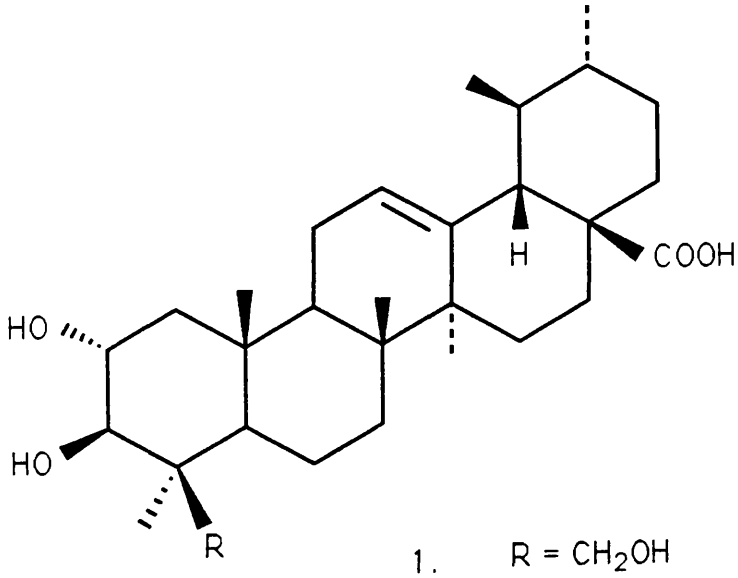
Dipterocarpus SPECIES

INTRODUCTION

The Dipterocarpaceae family (order Theales) contains about 550 plant species, generally tall, wood trees, widely distributed in the rain forests of South-East Asian countries. They are characterized by the abundant secretion of resin such as dammar. ***Dipterocarpus*** species are the most abundant tree species in the lowland zone forest and are important for their durable timber, edible fruit and useful resin. The resin is used in microscopy to preserve specimens and has also been used as a component of experimental insecticide formulations[1]. ***Dipterocarpus*** resin, known locally as **"minyak keruing"**, is often seen in large quantities in the freshly cut logs, exuding and covering the exposed sapwood at the end of the logs. Commercial dammar resin is soft and has a clear to yellow colour while the dammar resin freshly collected from the trunk of trees is a dark brown colour and is viscous.

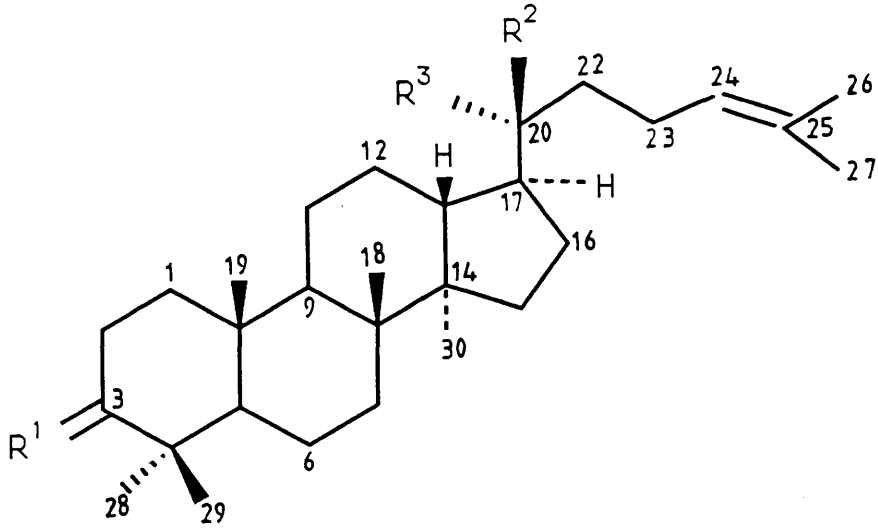
The earliest systematic investigation of dammar resin was reported by Tshirch and Glimann [2] who described a compound called dammarolic acid. Mladenovic and Barkovic [3] isolated the so-called dammarolic acid which later Brewis and Halsall [4] showed to be asiatic acid(1).

A triterpenoid, hydroxydammarenone-II(20S)(2)



1. R = CH_2OH

11. R = Me

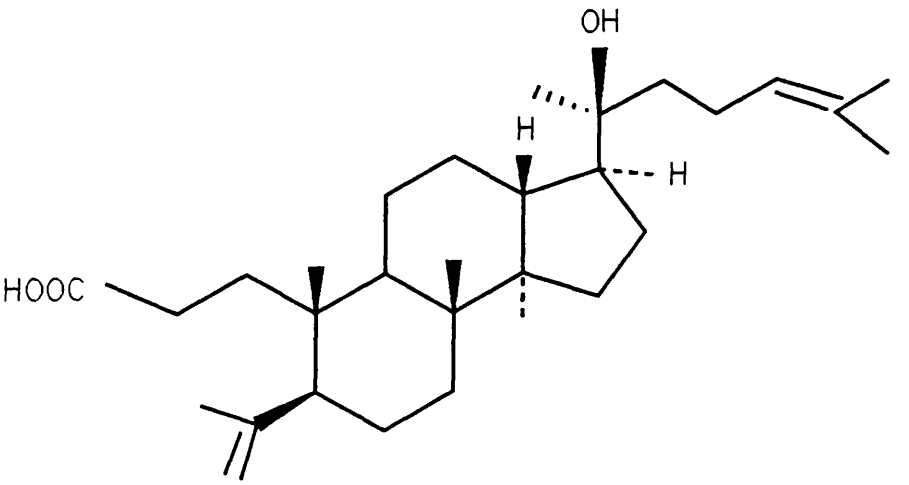


2. R^1 = O ; R^2 = OH ; R^3 = Me

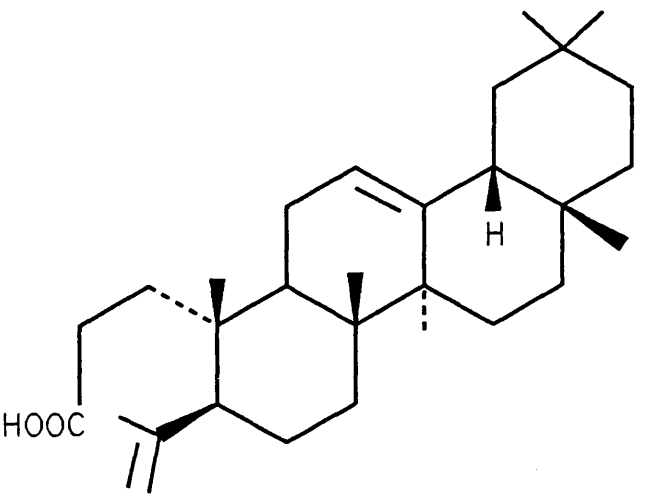
3. R^1 = β-OH, H ; R^2 = Me ; R^3 = OH

4. R^1 = β-OH,H ; R^2 = OH ; R^3 = Me

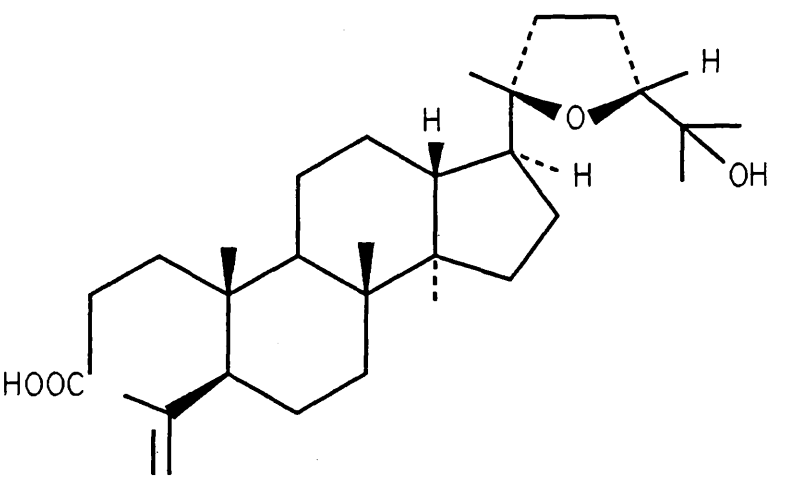
9. R^1 = O ; R^2 = Me ; R^3 = OH



5. Dammarenolic acid



6. Nyctanthic acid



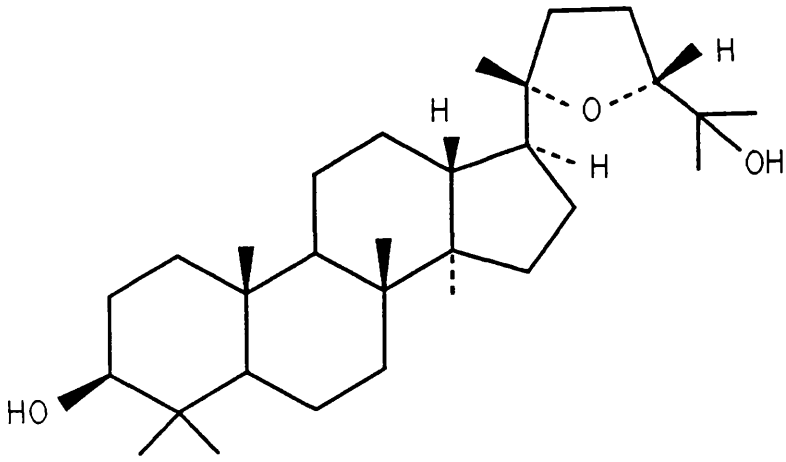
7. Shoreic acid

was first isolated by van Itallie [5] from ***D. hasseltii*** and ***D. trinervis***. Later Godson *et al.* [6] isolated dipterocarpol(2) from **D. lowrii Hook** and confirmed its identity with hydroxydammarenone-II(2), also isolated by Cosserat *et al.* [7] from ***D. dyeri***, ***D. alatus***, ***D. intricatus*** and ***D. atrocarpifollius***.

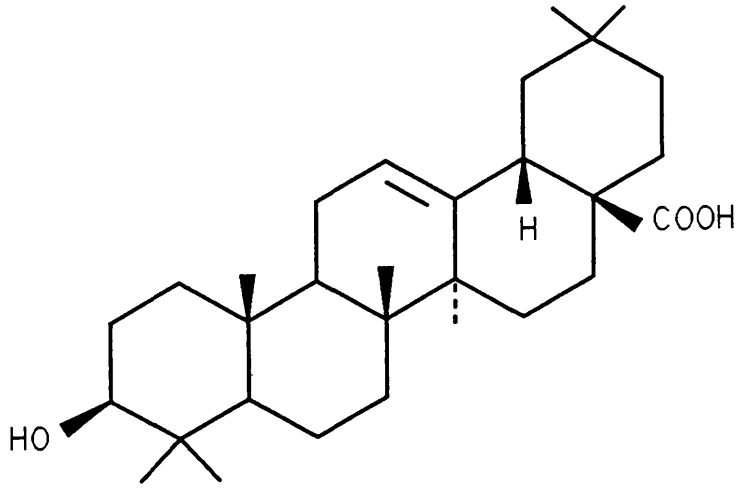
A further deatailed study of dammar resin carried out by Mills and Werner, resulted in the identification of ten dammarane triterpenoids [8]. The neutral and acidic triterpenoid constituents of dammar resin were also investigated and dammarenediol-I(3), -II(4), dammarenolic(5) and nyctanthic(6) acids isolated [9,10].

An early study [11] of the leaf of twenty-eight ***Dipterocarpus*** species showed that the phenolic constituents have three interesting characteristics related to the presence or absence of: (i) leuco-anthocyanins (ii) 3,4,5-vicinal trihydroxy groups in the phenolic constituents (iii) polyhydroxy and hydroxy methoxy aromatic acids. These characteristics are valuable from a taxonomic point of view. Bisset *et al.* [12] reported an interesting study in which the resins of 35 ***Shorea*** species from Malaysia were investigated. Shoreic acid(7) as well as oxygenated sesquiterpenoids were proposed as taxonomic markers of the genus ***Shorea***.

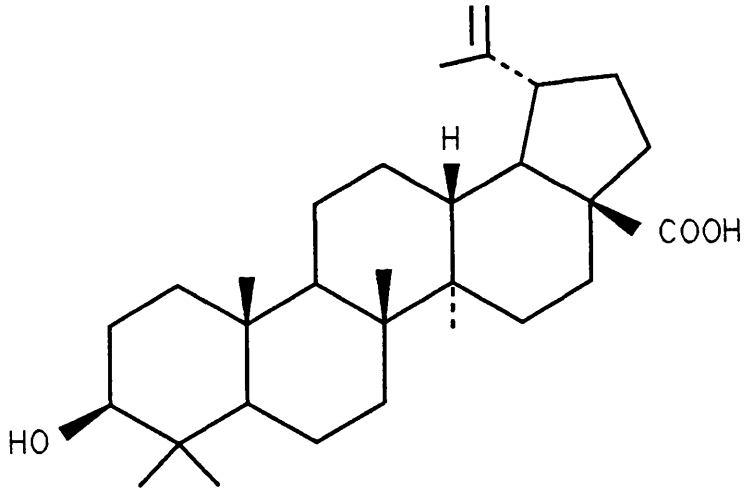
Bisset *et al.* [13] analysed seventy eight samples



8. Ocotillol



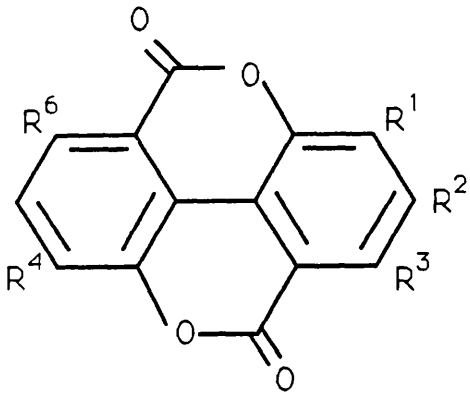
10. Oleanolic acid

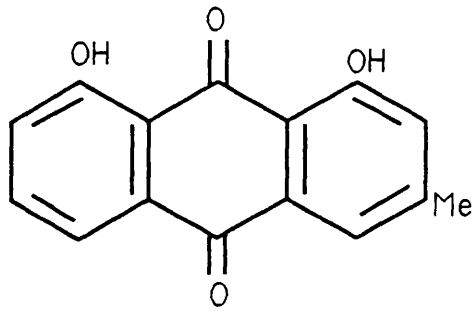


12. Betulinic acid

of resin from different species of ***Dipterocarpus*** and identified several sesquiterpenoids and triterpenoids, including ocotillol(**8**) and its C-20 epimer. An investigation of resin from ***Hopea pubescers*** [14] from Malaysia (Dipterocarpaceae) afforded three triterpenoids identified as hydroxydammarenone-I(20R)(**9**), dammarenediol-I(**3**) and oleanolic acid(**10**). Further study [15] on the resin of ***D. hispidus*** and ***D. zeylanicus*** afforded asiatic acid(**1**) and 2α,3β-dihydroxyurs-12-en-28-oic acid(**11**). The bark of ***D. hispidus*** contains betulinic acid (**12**) , dipterocarpol(**2**) and 3β,20β-dihydroxydammar-23-ene (dammarenediol-II(20S)(**4**) whilst the timber contains dipterocarpol(**2**) and asiatic acid(**1**).

The benzene soluble extractives of the heartwood of ***D. grandiflorus*** have been investigated [16] and contain 3.21% saponifiables, 0.27% combined acid, 0.16% fatty acid, 0.05% phenolic, 0.03% resin acids, 0.03% other acid and 0.06% of diethyl ether insoluble. The distribution of some triterpenoids and phenolic compounds in the extractives of endemic ***Dipterocarpus*** species of Sri Lanka have been studied by Sultanbawa *et al.* [17]. In this study, the extract of bark and/or timber of 11 species belonging to the genera ***Cotybolium, Hopea, Shorea, Vateria*** and ***Vatica*** yielded several triterpenes, hexamethylcoruleoellagic acid(**13**), pentamethylflavellagic acid(**14**),





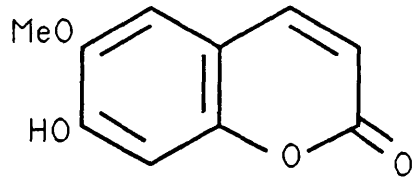
16. Chrysophanol

13. $R^1, R^2, R^3, R^4, R^5, R^6$ = OMe

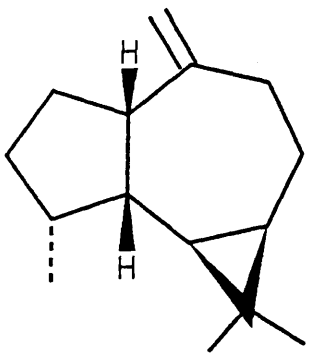
14. R^1, R^2, R^3, R^4, R^5 = OMe ; R^6 = H

15. R^1, R^2, R^4, R^5 = OMe ; R^3, R^6 = H

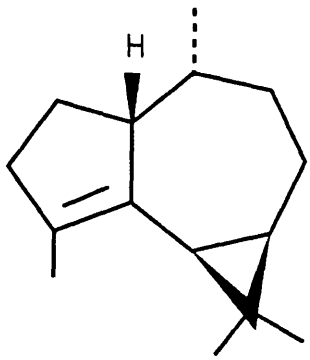
24. R^1, R^2, R^4, R^5 = OH ; R^3, R^6 = H



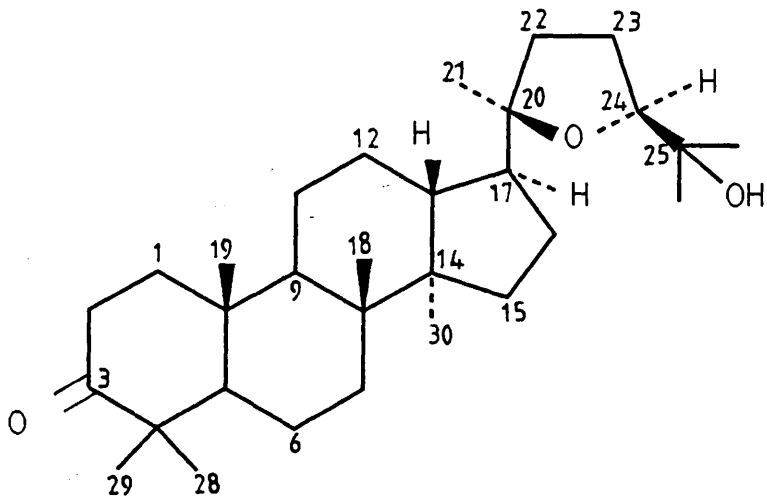
17. Scopoletin



19. Alloaromadendrene



18. α-Gurjunene

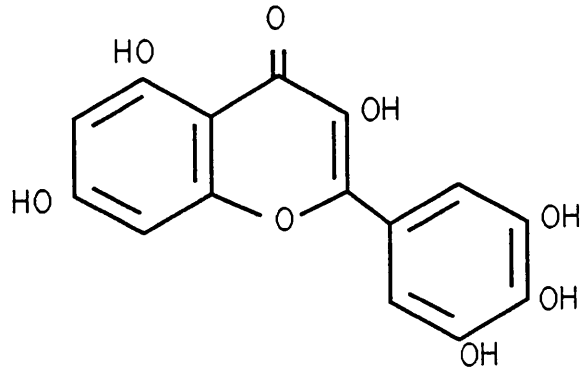


20. Ocotillone II

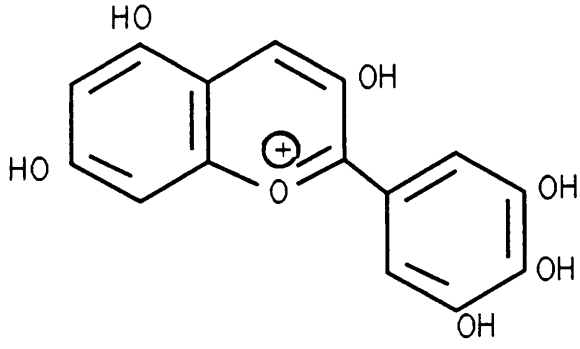
tetramethylellagic acid(15), chrysophanol(16) and scopoletin(17). The distribution of these compounds in 18 other species, including four ***Dipterocarpus*** species, was examined by t.l.c screening.

A bioassay study [18] of nine triterpenoids from dammar resin for antiviral activity against *Herpes simplex* virus type I *in vitro* showed a significant reduction in viral cytophatic effect when Vero cells were exposed continously to 1-10 µg/ml of compound for 48 hours after the viral challenge.

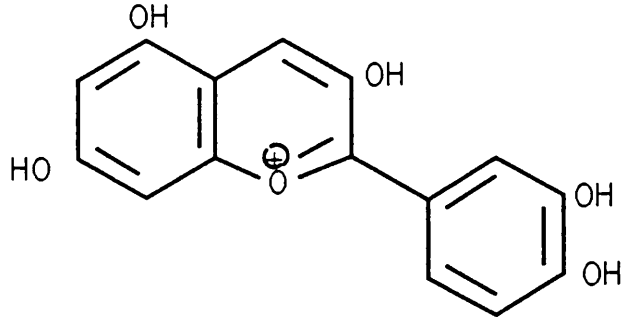
Recently a study of resin [19] from ***Dipterocarpus kerii*** resulted the isolation of sesquiterpenoids.



21. Myrcetin



22. Delphinidin



23. Cyanidin

DISCUSSION

The resin of ***D. baudii*** and ***D. cornutus*** has been studied by Bisset et al [13]. They isolated sesquiterpenoids and triterpenoids including α-gurjunene (18), alloaromadendrene (19) and dipterocarpol (2). Calarene and ocotillone-II (20) were also isolated from ***D. baudii*** but not from ***D. cornutus***. No work has been reported on ***D. costulatus*** resin. The leaves of ***D. costulatus*** contain several phenolic compounds including myrcetin (21), delphenedin (22) and quercitin while the leaves of ***D. baudii*** contain cyanidin (23) and ellagic acid (24) but no quercitin. The above studies showed that leaves of ***Dipterocapus*** species do not contain dipterocarpol (20S) (2)[11]. However, the timber of ***D. baudii***, ***D. costulatus*** and ***D. cornutus*** has not been investigated so far. In this study, the yield of the timber extract of the above species is shown in Table 1.

Table 1. Timber extract

Species	pet. ether (%)	Ethyl acetate (%)
D. costulatus	4.87	0.79
D. cornutus	0.91	0.27
D. baudii	1.61	1.62

It can been from Table 1. that ***D. costulatus*** contains a higher amount of petroleum ether(60-80) soluble extract compared to the other two ***Dipterocarpus*** species. Analysis of the flash chromatography fractions showed that all three species contain dipterocarpol(2) as a major component. The structure of dipterocarpol(2) was established from its ^{1}H n.m.r. spectrum which showed seven methyls signals at δ_H 0.73. 0.80, 0.87, 0.91, 0.97, 1.45 and 1.52 and olefinic proton at δ_H 4.95 (t, 1H, J=7.0 Hz). The chemical shifts were identical with literature values [14,20]. The m.p. and $[\alpha]_D$ were also identical with reported values [14,20].

Further purification of the flash chromatography fraction of the extract of ***D. cornutus*** and ***baudii*** yielded only fatty materials. Since it was more abundant the timber extract of ***D. costulatus*** was examined in more detail.

From fraction 4 of the flash column, ocotillone-II (20) was isolated and crystallised from petrolem ether (60-80). The ^{1}H n.m.r. was identical with the reported values [22,23]. The structure was further supported by HRMS. The fragment ions at m/z 399.326 (M^+-59) and 205.1593 are in agreement with the suggested pattern of fragmentation from ocotillone II(20) [21]. Furthermore, the expected base peak [15,21] was observed at m/z 143.1073. The m.p. and $[\alpha]_D$ were

also identical with the earlier findings [15,21].

The structure of dammarenediol-II(4) from fraction 5 was established by comparison of its ^1H n.m.r. spectrum with reported data [14,18,23]. The mass spectroscopic data also supported the proposed structure. The fragment ion at m/z 426 [M^+-H_2O] and also m/z 207 and 109 are typical for dammarenediol compounds [15,20].

The more polar fraction (7,8 and 10) of the flash chromatography were purified by preparative t.l.c. but only mixtures of triterpenoids were obtained. Clearly the timber extract of ***D. costulatus*** contains more constituents than ***D. cornutus*** and ***D. baudii***. It may be significant that ocotillone II (20) was found only in ***D. costulatus***. Dipterocarpol(2) appears to be present in almost all the ***Dipterocarpus*** species and as a major constituent.

EXPERIMENTAL

The timber of the ***Dipterocarpus*** species was obtained from Tekam River Forest, Pahang, Malaysia. The dried timber was chipped and powdered in a mill. The powdered timber was then extracted with hot petroleum ether (60-80) and ethyl acetate successively. The solvents were removed by rotary evaporator and viscous yellow extracts were obtained.

Timber of *D. costulatus*

The petroleum ether extract (11.04 g) was adsorbed on silica gel GF_{254} and was chromatographed over 120.0 g of the same adsorbent. The extract was eluted with petroleum ether (60-80) and increasing proportion of ethyl acetate. About 100 ml fractions of the eluate were collected as follows: Fraction 1 (petroleum ether (60-80)-ethyl acetate, 9:1), 2 (petroleum ether (60-80)-ethyl acetate, 4:1), 3 (petroleum ether (60-80)-ethyl acetate, 7:3), 4 (petroleum ether (60-80)-ethyl acetate, 3:2), 6-7 (petroleum ether (60-80)-ethyl acetate, 3:7), 8-9 (petroleum ether (60-80)-ethyl acetate, 1:4) and 10-11 (100 % ethyl acetate).

From fraction 3 and 4 a white crystalline solid was obtained. Recrystallisation from petroleum ether (60-80) gave **dipterocarpol (2)** (1.9 g) as white shiny

flakes. M.p. 134-135°C, $[\alpha]_D$ +67° (c, 2.37 in $CHCl_3$), [lit [14,20] m.p. 135-136°C , $[\alpha]_D$ + 66°], i.r. ν_{max} cm^{-1} : 3620, 1705, 1455, 1385

^1H n.m.r. : δ 0.73 (s, 14-Me), 0.80 (s, 8-Me), 0.87 (s, 4β-Me), 0.91 (s, 4α-Me), 0.97 (s, 20-Me), 1.45 (s, 26-Me), 1.52 (s, 27-Me), 4.95 (t, 1H, J=7.0 Hz, 24-H). [High Resolution Mass Spectroscopy m/z (rel. int.): 424.3690 **$C_{30}H_{50}O$** [M^+-H_2O] (51.0%), 205.1947 (18.4%), 109.1018 **C_8H_{13}** (100%)].

Fraction 4 was crystallised from petroleum ether (60-80) to give **ocotillone-II(20)** (439.0 mg). M.p. 163-164°C, $[\alpha]_D$ +54° (c, 1.01 in $CHCl_3$), [Lit [22,23] m.p. 164-165°C $[\alpha]_D$ +59], i.r. $\nu_{max}$$cm^{-1}$: 3583, 1705, 1455, 1385

^1H n.m.r. : δ 0.88 (s, 14-Me), 0.95 (s, 10-Me), 1.00 (s, 18-Me), 1.04 (s, 4β-Me), 1.09 (s, 4α-Me), 1.11 (s, 20-Me), 1.15 (s, 26-Me), 1.20 (s, 27-Me), 2.45 (m, 2H), 3.78 (triplet like, 1H). [High Resolution Mass Spectroscopy m/z (rel int.): 443.3527 **$C_{29}H_{47}O_3$** [M^+-CH_3] (17%), 399.3260 [M^+-59] (12.4%), 205.1593 (7.6%), 143.1073 [$C_8H_{15}O_2$] (100%)].

Fraction 5 was purified by preparative t.l.c (petroleum ether(60-80)-ethyl acetate-4:1), four developments) and precipitated from aqueous MeOH to afford a white material (10.6 mg) which by t.l.c. appeared to be a mixture of two compounds the major of which was identified as **dammarenediol-II(4)** from the

^{1}H n.m.r. and mass spectra.

^{1}H n.m.r. : δ 0.80 (s, Me), 0.87 (s, Me), 0.89 (s, Me), 0.98 (s, Me), 1.15 (s, Me), 1.63 (s. Me), 1.71 (s, Me), 2.10 (m, 2H), 3.20 (m, 1H), 5.15 (t, 1H, J=7.0 Hz). Low Resolution Mass Spectroscopy m/z (rel. int.): 426 $C_{30}H_{50}O$ [M^+-H_2O] (20.2%), 207 (24.5%), 127 (19.0%), 109 [C_8H_{13}] (100%)].

The crude fraction 7 was subjected to preparative t.l.c. (petroleum ether (60-80)-ethyl acetate, 7:3) but unfortunately gave only a mixtures (90.5 mg).

Fraction 8 was thrice purified by preparative t.l.c. using the same solvent system but again afforded only a mixture (7.0 mg).

Purification of fraction 10 by preparative t.l.c. (petroleum ether (60-80)-ethyl acetate , 3:2, three developments) and gave another mixture (11.1 mg) of dammarene-type compounds.

Timber of *D. cornutus*

The petroleum ether (60-80) extract (7.01 g) was chromatographed on a column of silica gel GF_{254} (120.0 gm). Elution with petroleum ether(60-80) -ethyl acetate (7:3) gave dipterocarpol **(2)** (1.134 g). Elution with other proportion of solvents gave only fatty materials.

Timber of *D. baudii*

The petroleum ether extract (13.8 g) was chromatographed on a column of silica GF_{254} (120.0 g). Elution with 30% ethyl acetate in petroleum ether (941.0 mg) gave **dipterocarpol (2)** (455.0 mg). Further elution of the extract gave only fatty materials.

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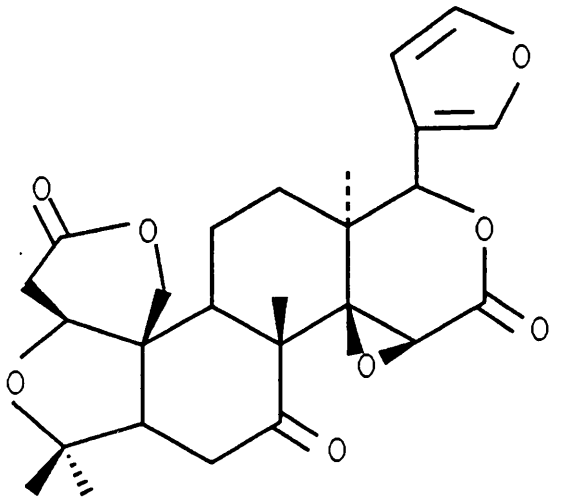
CHAPTER V

TETRANORTRITERPENOIDS FROM THE SEED OF *Azadirachta indica* AND THE PREPARATION OF 3-DEACETYLAZADIRACHTIN



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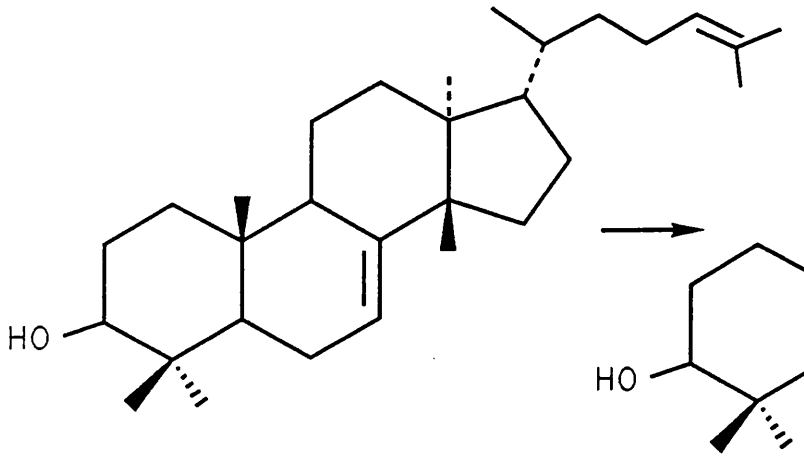

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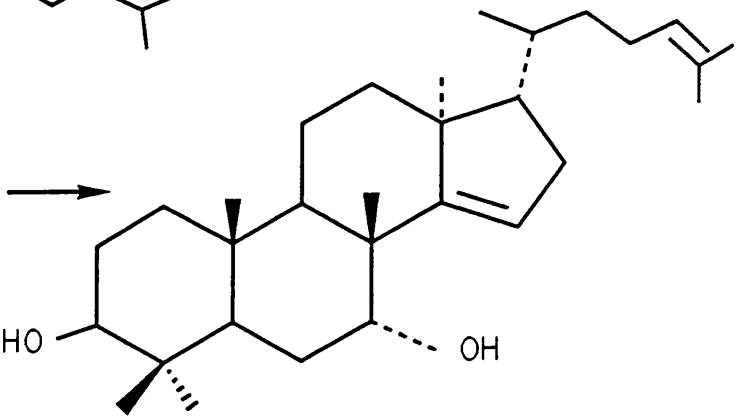

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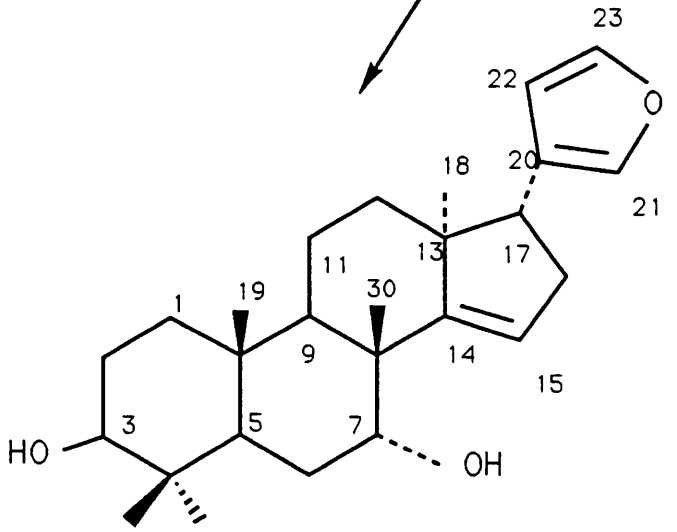
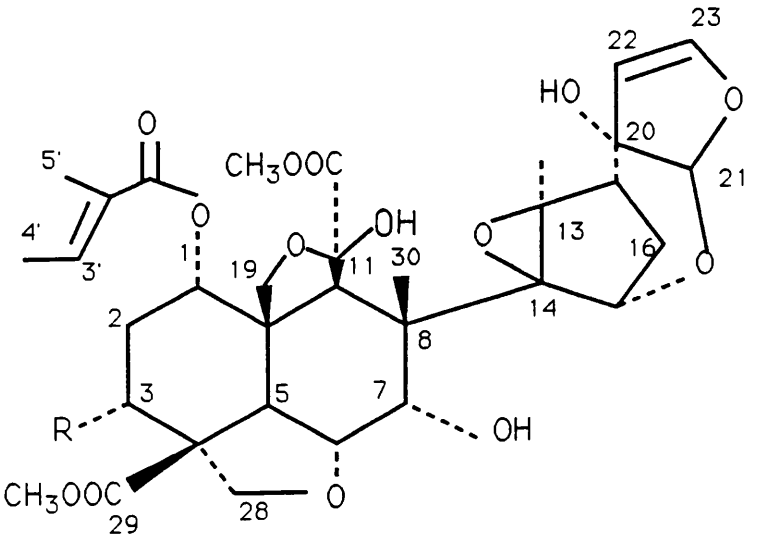


4

INTRODUCTION

Tetranortriterpenoids or limonoids form a large group of modified triterpenoids found mainly in the Meliaceae and Rutaceae families. The term limonoid is derived from limonin (1) [1,2], a bitter principle from *Citrus* species, whose structural elucidation stimulated interest in this type of compound. Tetranortriterpenoids are thought to arise in nature from simple tirucallol (euphol) derivatives eg. (2) which undergo an *apo*-rearrangement, probably via a 7α,8α-epoxide, to give an *apo*-tirucallol derivative (3) with an oxygen at 7α, a methyl migrated to C-8 from C-14 and a Δ^{14} double bond. Loss of the four terminal carbons of the side chain followed by furan ring formation affords the simplest limonoid (4). Other limonoids are formed by ring cleavages, oxidations and rearrangements.

Several reviews [3-8] of the limonoids in the Meliaceae family have appeared. More recent reviews on the limonoids of *Azadirachta indica* [9,10] and *Melia azedarach* [9] have been published. The most interesting and most complex compound from *Azadirachta indica* is azadirachtin (5), a strong locust antifeedant which was first isolated by Morgan [11]. Azadirachtin (5) is one of the most powerful antifeedant and ecdysis inhibitory compounds ever isolated from a botanical



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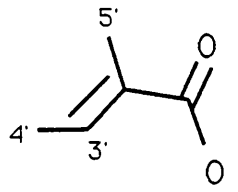
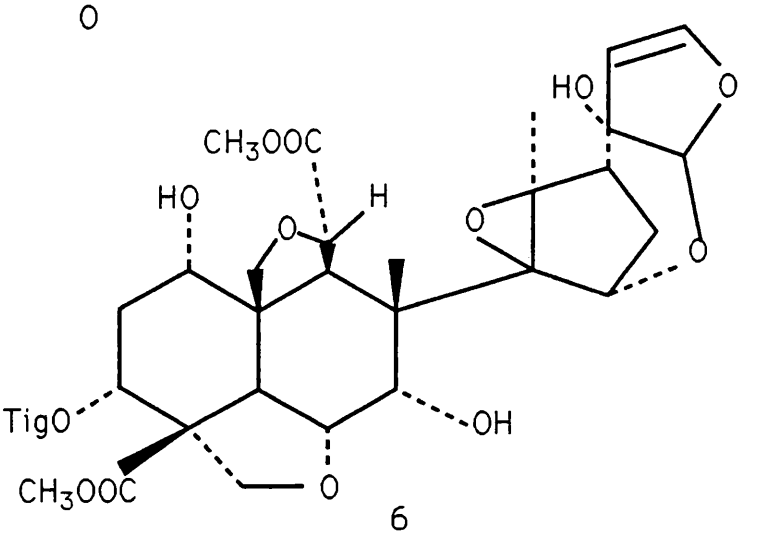
18. R = OH

OTig =





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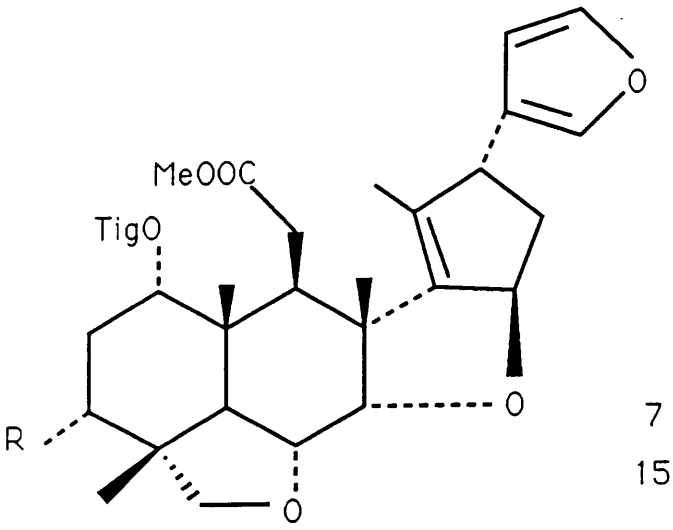
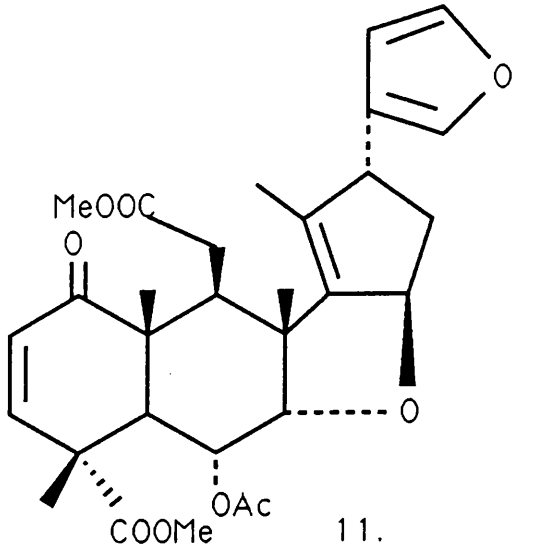


7 R = OAc

15. R = OH



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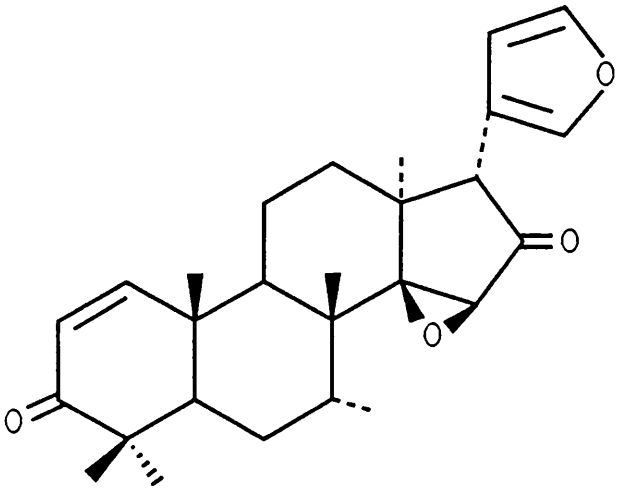

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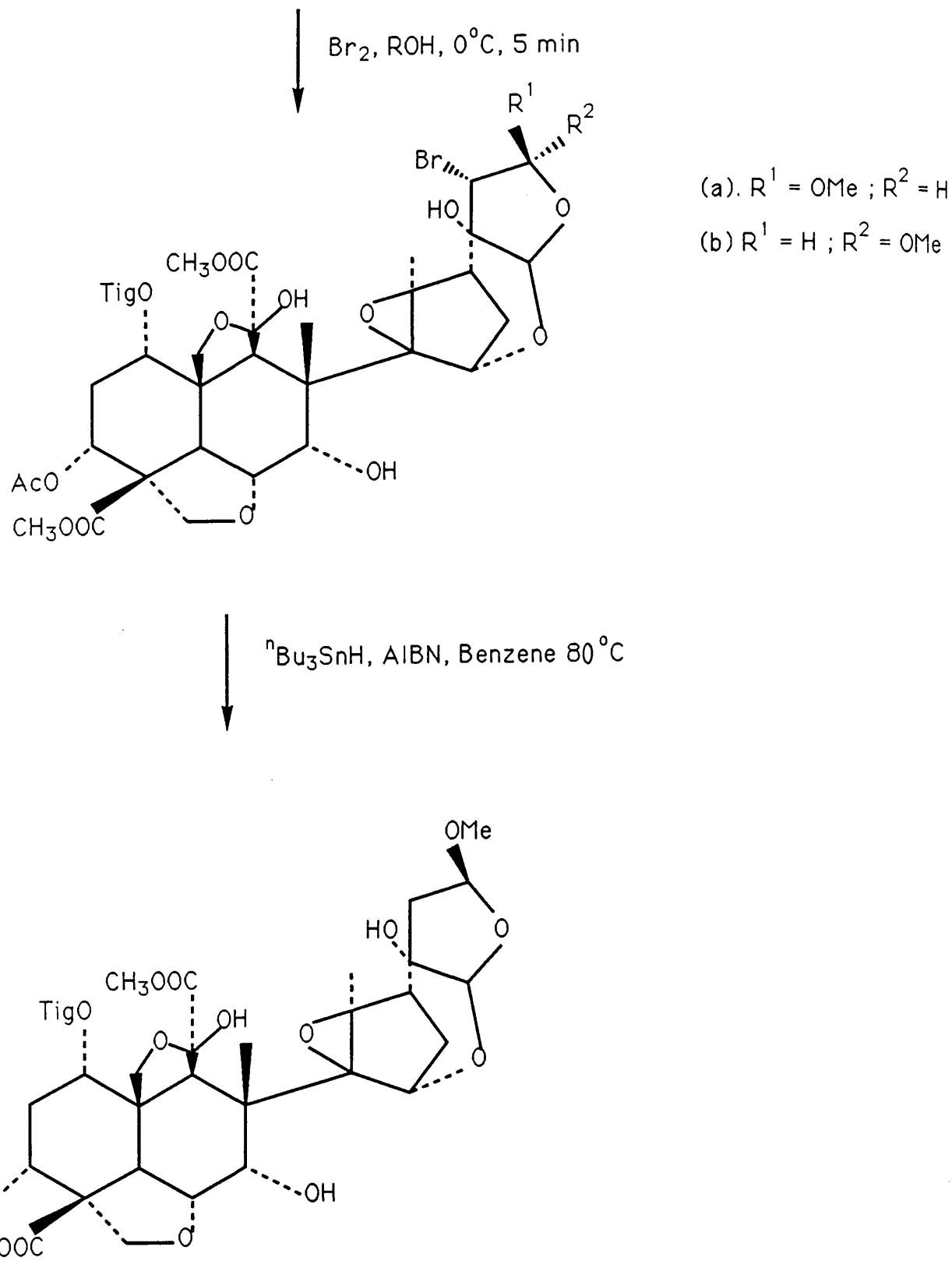
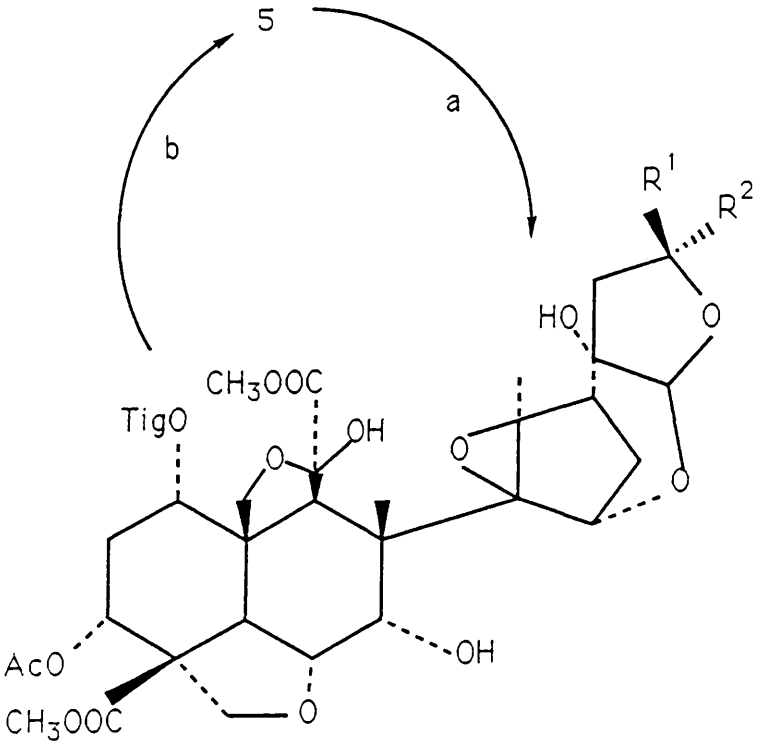


(a). R^1 = OMe ; R^2 = H

(b) R^1 = H ; R^2 = OMe

8. (Major)

Scheme 1.



9. R^1 = H, R^2 = OAC

10. R^1 = OAc, R^2 = H

Scheme 2. a) AcOH, RT, 72 h

b) 1.7 X 10^{-3} mm Hg, 175^{0}C, 5 mins

source [12]. The structural elucidation of azadirachtin (5) has been discussed in detail by Taylor [13], Ley [14], Turner [15] and Kraus [16]. The structure was recently established correctly, first by Kraus [16], and confirmed by X-ray crystallographic analysis [17,18].

Initial synthetic approaches to azadirachtin (5) have been reported [19]. Chemical modification of azadirachtin (5) and the related 3-tigloylazadirachtol (6) and salannin (7) have also been carried out [20]. The biological activity of azadirachtin derivatives has also been further investigated [20]. Hydrogenation of the C-22,23 enol ether double bond does not significantly diminish the activity of either azadirachtin (5) or the deoxy series. Bulky substituents at C-22 and C-23 cause a considerable drop in antifeedant potency. However, salannin (7) and related compounds are apparently poor anti-feedants [20].

Azadirachtin (5) also can be converted to the natural product 22,23-dihydro-23β-methoxyazadirachtin (8) via selective bromomethoxylation of the 22,23 enol ether double bond and tri-n-butyltin hydride reduction [21] (Scheme 1). Pyrolysis of the corresponding acetic acid adducts (9) and (10) afforded azadirachtin (5) in high yield (Scheme 2).

Pharmacology of Limonoids

Very little systematic work has been done in this field. The only notable work, a structure activity study of selected limonoids from two families (Meliaceae and Rutaceae) against the marine P-388 lymphocytic leukemia system (PS system) was done by Pettit *et al.* [22]. The presence of both a 19,29-hemiketal and a 14β,15β-epoxide is important for pronounced inhibition of the PS *in vitro* cell line. Substitution of an A-ring α,β- unsaturated ketone (3-oxo-1-ene) for the ketol led to diminished activity, while reduction of the olefin caused complete loss of activity. At the dose levels employed, even very good PS *in vitro* inhibition was not translated into PS *in vivo* antineoplastic effects.

Azadirachta indica

Azadirachta indica (syn. ***Melia azadirachta***, ***Melia indica***, ***Margosa***), known in the vernacular as "neem" and "nimba", belongs to the Meliaceae family and is widely distributed in Asia, Africa and other tropical parts of the world. Almost every part of the tree including its roots, leaves and fruits have been used in folkloric and traditional systems of medicine, in agricultural, and in industrial and commercial exploitation [23]. The tree has been used in the treatment of diseases of bacterial and fungal origin[24]

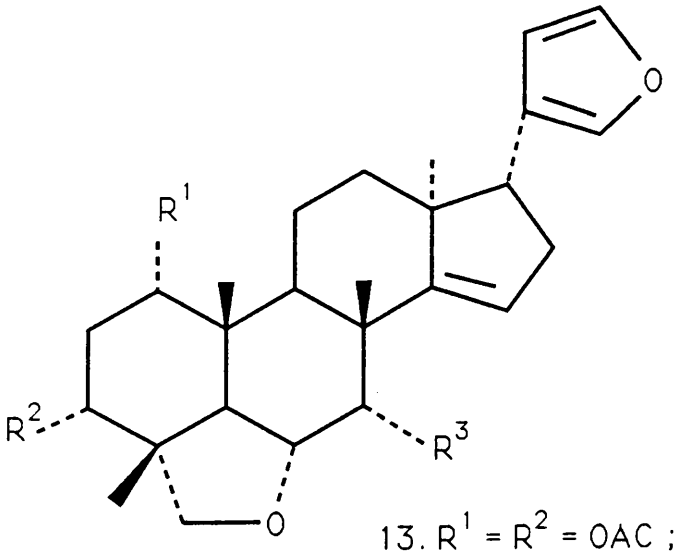
while the seed oil has commercial possibilities for lighting and heating and in the manufacture of lubricant and soap.

Of primary importance are its potent antifeedant and ecdysis (moulting) inhibitory activities against pests and insects [25]. However, it is still too early predict its significance for utilization on a commercial scale. Nimbin **(11)** and nimbinin **(12)** were the first two crystalline compounds isolated from the seed oil [26]. About 100 constituents have so far been isolated from different parts of the tree and their structures elucidated. These include protolimonoids, limonoids, pentanortriterpenoids and hexanortriterpenoids. Since our interest lies in azadirachtin **(5)** is not our intention to discuss other extractives in detail.

DISCUSSION

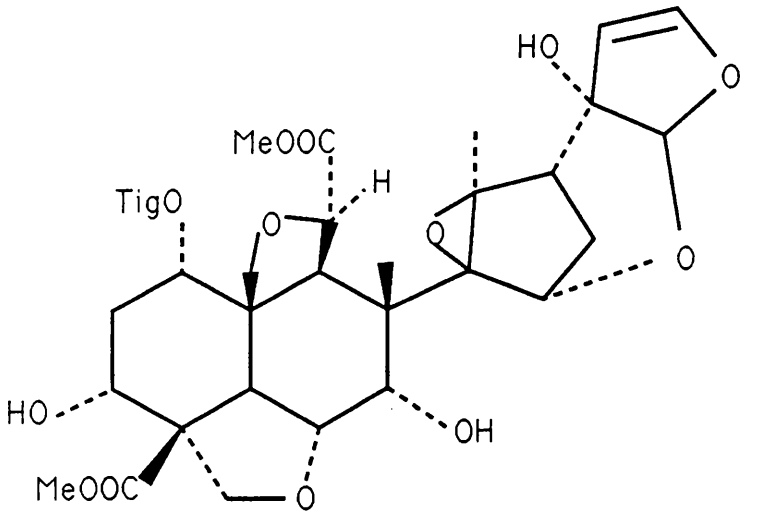
The seed of ***Azadirachta indica*** has been investigated by many workers. The main interest of these investigations has been the techniques of isolation and purification and the structural elucidation and biological activity of the isolated compounds. Since the correct structure of azadirachtin (5) was established recently there has been a growing interest in the investigation its mode of action. As part of a collaborative effort with Dr. Strang , Department of Biochemistry , University of Glasgow, we undertook a reinvestigation of the constituents of the seed, primarily to provide a supply of pure azadirachtin (5) for insect work.

The extraction with methanol was followed by the method suggested by Schroeder [27], with some modification (see experimental). It is interesting to note that prior removal of fat, sugar and other polymeric material by solvent partition increases the efficiency of the isolation and purification of tetranortriterpenoids from the flash chromatography fractions. Thus, the method suggested by Schroeder [27] proved to be much more efficient than the normal method of extraction and purification. Our approach, using flash chromatography, gave quite a high yield of azadirachtin (5). However, the isolation and



13. $R^1 = R^2 = OAC$; $R^3 = OTig$

16. $R^1 = R^2 = OAc$; $R^3 = OH$



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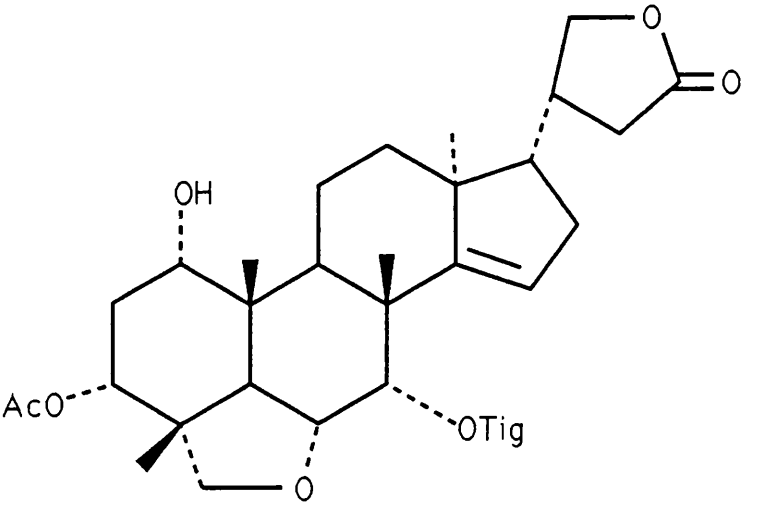

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purification became more difficult the lower the concentration of azadirachtin (**5**) in the extract. Thus, only the more abundant compounds were isolated in a pure state using flash chromatography and preparative t.l.c. techniques. After several lots of preparative t.l.c. a new compound, tentatively identified as 1,3-diacetyl-7-tigloylvilasinin (**13**), was isolated along with several known compounds namely salannin (**7**), azadirachtin (**5**), 3-deacetyl-11-desoxyazadirachtin (**14**) and 3-deacetylsalannin (**15**).

Azadirachtin (**5**), a microcrystalline solid, was the most abundant material in the seed extract. Considerable discussion of the high field n.m.r. features of azadirachtin (**5**) and derivatives has appeared in the literature [13→18]. It is not our intention to discuss in these assignments in detail. Comparison showed that most ^1H n.m.r. signals are in accord with the published data [13→16]. Other physical properties are almost identical with the reported values.

Salannin (**7**) and 3-deacetylsalannin (**15**) are two closely related compounds. The only difference in the ^1H n.m.r. spectra of these compounds are the chemical shifts of H-3 (salannin (**7**) δ_H 4.82, 3-deacetyl salannin (**15**) δ_H 3.80). The other signals are almost the same and are in accord with the reported values [28→30].

The structure of the new compound, 1,3-diacetyl-7-tigloylvilasinin **(13)**, was assigned on the basis of its spectroscopic properties (Table 3). The ^{1}H n.m.r. spectrum revealed the typical features of a vilasinin type skeleton [31]. Comparison of the ^{1}H n.m.r. with 1,3-diacetylvilasinin **(16)** revealed the identical chemical shifts for H-1 (δ_H 4.76) and H-3 (δ_H 4.95) suggesting that the acetates are attached to C-1 and C-3. Comparison of the H-7 signal (δ_H 5.71, d, J=3.0 Hz) with that of 3-acetoxy-7-tigloylvilasinin lactone **(17)** [32] showed close similarity. Likewise H-6 in the two cases has virtually the same chemical shift. It seems likely, therefore, that the tiglate is located at C-7. Such arguments, based solely on similarity of chemical shifts, cannot be regarded as definitive and hence structure **(13)** must be tentative. A long range correlation experiment is necessary to provide conclusive evidence for the siting of the esters. Lack of material has prevented further study.

Repeated purification by preparative t.l.c gave 3-deacetyl-11-desoxyazadirachtin **(14)** as a pure compound. It is interesting that this known compound was previously isolated by reverse phase HPLC. The ^{1}H n.m.r. spectral data (see Table 4) were in agreement with those reported by Ley [32]. The lack of an acetate signal at C-3 was apparent from the chemical shift of H-3 (δ_H 3.52). The appearance of a signal at δ_H 4.46

for H-11 and the differences in chemical shift of H-19 and H-9 relative to azadrachtin **(5)** provided further indication of structure **(14)**. There was insufficient material to crystallise and thus no physical comparison was possible.

One of the objectives in this investigation was the preparation of a radiolabelled azadirachtin **(5)** for the bioassay work. With this end in view we undertook the preparation of 3-deacetylazadirachtin **(18)**. It was reported [30] earlier that 3-deacetyl-azadirachtin **(18)** could be prepared using 2% KOH. Several attempts at the preparation of 3-deacetyl-azadirachtin **(18)** using small amounts of azadirachtin **(5)** and 2% KOH failed to yield any hydrolysed product after esterification with diazomethane. The reaction clearly needs higher amounts of azadirachtin. However, hydrolysis using 1 % KOH for 3½ hours afforded 16.7% of 3-deacetylazadirachtin **(18)** after esterification of the crude acidic product. This yield is much higher than that obtained with 2 equivalents of sodium carbonate. The structure of 3-deacetyl azadirachtin **(18)** was established by comparison of its ^{1}H n.m.r. spectrum with that of azadirachtin **(5)**. The most obvious differences are the lack of an acetate signal and the upfield shift of H-3 (δ_H 4.36) . In the ^{13}C n.m.r. spectrum of **(18)** C-3 (δ_C 71.6) moves downfield compared to C-3 of azadirachtin **(5)**.

Detailed comparison with the ^{1}H and ^{13}C n.m.r. spectra of azadirachtin **(5)** showed that most other signals remained unchanged.

Reacetylation of 3-deacetylazadirachtin **(18)** with acetic anhydride and pyridine proceeded smoothly to afford azadirachtin **(5)** (56%), identified by ^{1}H n.m.r. and analytical t.l.c. comparison with standard azadirachtin **(5)**. This opens the way for the preparation of labelled azadirachtin by reacetylation of 3-deacetylazadirachtin **(18)** using ^{14}C-acetic anhydride and pyridine. Preparation of the ^{14}C labelled azadirachtin now in progress.

EXPERIMENTAL

Two methods of extraction and purification were carried out.

Method 1

The ***Azadirachta indica*** "neem" seed, collected in Pakistan, was ground with a Wiley mill. The ground neem seed (874.4 g) was soaked in petroleum ether overnight. The defatted seed was then filtered , dried and extracted with methanol for one day. The solvent was filtered and evaporated to dryness. The dried extract was subjected to partitioning with ethyl acetate-water(1:1). The ethyl acetate fraction was collected and the solvent was removed by rotary evaporator. A dried extract (10.9 g) was obtained and subjected to a quick flash chromatography (7.0 cm dia. X 10.0 cm height) using silica gel GF_{254} as adsorbent. Four fractions were colected as follows: Fraction 1 (ethyl acetate-methanol, 4:1) (4.3 g), fraction 2 (ethyl acetate-methanol, 7:3) (1.1 g), fraction 3 (ethyl acetate-methanol, 1:1) (2.9 g) and fraction 4 (methanol) (2.3g).

Fraction 3 was subjected to crystallisation in CCl_4 and yielded crude azadirachtin **(5)**(503.5 mg) confirmed by ^1H n.m.r.. The mother liquor (2.3 g) was further fractionated using more efficient flash

chromatography and eluting with petroleum ether and increasing proportions of ethyl acetate, and finally with methanol. A total of 16 fractions (100 ml each) were collected as follows: Fraction 1 (petroleum ether-ethyl acetate, 3:2), 2-3 (petroleum ether-ethyl acetate, 1:1), 4-5 (petroleum ether-ethyl acetate, 2:3), 6-8 (petroleum ether-ethyl acetate, 3:7), 9-10 (petroleum ether-ethyl acetate, 1:4), 11-13 (ethyl acetate), 14-15 (ethyl acetate-methanol, 9:1) and 16 (ethyl acetate-methanol, 4:1). Crystallisation of fractions 6-12 from CCl_4 yielded more azadirachtin **(5)** (322.4 mg).

Method II

A more efficient method of extraction was followed as suggested by Schroeder [27] with some modification. Powdered ***Azadirachta indica*** seed (1763.5 g) was extracted with petroleum ether (60-80) (2 X 4 litres) and left to stand for overnight in petroleum ether (60-80) (2 litres). The solvent with the fatty material was discarded. The extracted ground seed was then extracted again by soaking in methanol for 2-3 hours (3 X 2 litres) and finally allowed to stand overnight in methanol (2 litres). All the methanol fractions (8 litres) were combined and the solvent was evaporated to give a viscous dark green material (79.4 g). The extract then subjected to

solvent partition with petroleum ether (60-80) and 95% methanol in water to remove any remaining fatty material. The 95% methanol-water fraction was evaporated to give a crude extract (76.2 g) which was dissolved in ethyl acetate and washed with water to remove any sugar and protein. Evaporation of the solvent yielded 25.4 g of dried material. The dried extract was dissolved again in ethyl acetate and filtered through silica gel GF_{254} column (7.0 cm diameter X 10.0 cm height) and after removing of the solvent afforded 17.1 g of filtered extract. The extract then subjected to more efficient flash chromatography (7.0 cm diameter X 14.0 cm height) and about 100 ml of eluant was collected for every fraction. A total of 14 fractions were collected as follows.

Fraction 1-10 (ethyl acetate-petroleum ether (60-80), 3:1), 11-12 (methanol-ethyl acetate, 1:4), 13 (methanol-ethyl acetate, 2:3) and 14 (methanol). Analytical t.l.c comparison showed that fractions 2-5 contained mainly compounds less polar than azadirachtin (**5**) and only a relatively small amount of azadirachtin(**5**). However, fractions 6 to 11 contained relatively high concentrations of azadirachtin **(5)** together with a small amount of more polar compounds.

Fractions 12 to 14 contained mainly more polar compounds. Fractions 6 to 9 were crystallised from

CCl_4 four times and to give pure azadirachtin **(5)** (1.9 g) as a white powder.

Isolation of salannin (7), 1,3-diacetyl-7-tigloylvilasinin (13), 3-deactyl-11-desoxyazadirachtin (14) and 3-deacetylsalannin (15) from flash chromatography fractions of Method 1

The mother liquor of fraction 7 was purified by preparative t.l.c. (60% ethyl acetate in petroleum ether (60-80)- two runs) and two major bands 1 and 2 were collected. Band 1 which was less polar than band 2, was purified again by preparative t.l.c. (40 % ethyl acetate in petroleum ether (60-80)-four runs) and a further two bands were colllected. The upper band was crystallised from carbon tetrachloride to afford salannin **(7)**(4.0 mg). The second band contained, as a major component, the new compound 1,3-diacetyl-7-tigloylvilasinin **(13)** (13.9 mg).

Band 2 was purified again by preparative t.l.c. (60 % ethyl acetate in petroleum ether -four runs) and afforded 3-deacetyl-11-desoxyazadirachtin **(14)** (13.6 mg). The mother liquor of fraction 2 was purifed further by preparative t.l.c. (60% ethyl acetate in petroleum ether-four runs) and yielded 3-deacetylsalannin **(15)** (84.2 mg). Many minor

compounds still remain in the flash chromatography fractions.

Azadirachtin(5)

m.p. 158-160°C , $[\alpha]_D$ -38° (c, 3.44 in $CHCl_3$),

[Lit [30] m.p. 156°C , $[\alpha]_D$ -46°], i.r. ν_{max} cm^{-1} 3450 (br), 1730, 1720, 1648, 1620.

^1H n.m.r See Table 1.

Salannin (7)

i.r. ν_{max} cm^{-1} 1730, 1705, 1648

^1H n.m.r. see Table 2

1,3-Deacetyl-7-tigloylvilasinin (13)

m.p. 174-176°C, $[\alpha]_D$ -33° (c, 0.19 in $CHCl_3$),

i.r. ν_{max} cm^{-1} 3440, 1732, 1648

^1H n.m.r. see Table 3

3-Deacetyl-11-desoxyazadirachtin (14)

i.r. ν_{max} cm^{-1} 3480, 1730, 1650, 1620.

^1H n.m.r. see Table 4

3-Deacetylsalannin (15)

m.p. 180-181°C, $[\alpha]_D$ + 30°(c, 0.9 in $CHCl_3$), [Lit [29] 214-215° (ethyl acetate), $[\alpha]_D$ +134°], i.r. ν_{max} cm^{-1} 3450, 1730, 1648

^1H n.m.r. see Table 5

3-Deacetylazadirachtin (18)

m.p. 143-145°C, $[\alpha]_D$ -32°(c, 0.49 in $CHCl_3$)

i.r. ν_{max} cm^{-1} 3480, 1645

^1H n.m.r. see Table 6., ^{13}C n.m.r. see Table 7

Preparation of 3-deacetylazadirachtin (18)

Various concentrations of aqueous potassium hydroxide were tried for the hydrolysis of azadirachtin **(5)**. It was concluded that 2.0 % potassium hydroxide was too strong for the hydrolysis since no products were detected. The following two methods of hydrolysis were more successful :-

1. **Hydrolysis of azadirachtin (5) with 1 % potassium hydroxide**

Azadirachtin **(5)** (128.5 mg) in methanol (7.0 ml) was treated with aqueous 1 % potassium hydroxide solution (4.0 ml) for 3½ hours at room temperature. The mixture was made just acidic with dilute hydrochloric acid and extracted with ethyl acetate (4 X 10.0 ml). Evaporation of the solvent gave a crude acidic product (88.5 mg).

2. **Hydrolysis of azadirachtin(5) with sodium carbonate**

A portion (68.2 mg) of azadirachtin **(5)** in methanol (3.0 ml) was added to sodium carbonate (23.5 mg) in water (3.0 ml). The reaction was allowed to stand for three days. The reaction mixture was made just acidic with dilute hydrochloric acid and extracted with ethyl acetate (4 X 10.0 ml).

Evaporation of the solvent gave crude acidic product (55.1 mg)

Esterification of the acidic products

Chloroform solution of both crude acidic products were esterified by addition of excess diazomethane. The esterified product was then purified by preparative t.l.c.(80 % ethyl acetate in petroleum ether (60-80) - three runs) and afforded 16.7 % and 8.8 % of 3-deacetylazadirachtin **(18)** by method I and II respectively. The 3-deacetylazadirachtin **(18)** was crystallised from CCl_4 as a microcrystalline powder.

Reacetylation of the 3-deacetylazadirachtin(18)

The 3-deacetylazadirachtin **(18)** (8.9 mg) was acetylated with acetic anhydride (0.5 ml) and pyridine (0.4 ml) . The reaction was allowed to stand overnight at room temperature. After removing of the excess acetic anhydride and pyridine, the crude product was purified by thin layer chromatography (petroleum ether(60-80)-ethyl acetate, 2:3) and afforded azadirachtin **(5)** (5.0 mg) identical with the authentic material by ^1H n.m.r and analytical t.l.c (R_f 0.5 in ethyl acetate).

Table 1. ^{1}H n.m.r of azadirachtin (5)

H-atom	
1-H	4.75 (dd, J=2.8, 3.0 Hz)
2-H_α	2.27 (ddd, J=16.5, 3.0, 2.7 Hz)
2-H_β	2.10 (ddd, J=16.5, 2.9, 2.9 Hz)
3-H	5.47 (dd, J= 2.5, 2.7 Hz)
5-H	3.38 (d, J=12.5 Hz)
6-H	4.60 (dd, J=12.5, 2.6 Hz)
7-H	4.75 (d, J=2.6 Hz)
9-H	3.30 (s)
11-H	-
15-H	4.65 (d, J=3.0 Hz)
16-H	1.70 (ddd, J=13.5, 5.0, 4.0 Hz)
16-H	1.22 (d, J=13.5 Hz)
17-H	2.35 (d, J=5.0 Hz)
18-H	1.98 (s)
19-H	3.62 (d, J=9.5 Hz)
19-H	4.16 (d, J=9.5 Hz)
21-H	5.65 (s)
22-H	5.05 (d, J=3.0 Hz)
23-H	6.43 (d, J=3.0 Hz)
28-H_α	4.05 (d, J=9.0 Hz)
28-H_β	3.74 (d, J=9.0 Hz)
30-H	1.72 (s)
7-OH	3.05 (s)
11-OH	5.07 (s)
20-OH	3.20 (s)
OMe	3.65 (s)
29-OMe	3.76 (s)
OAc	1.95 (s)
3'-H	6.93 (qq, J=7.5, 1.5 Hz)
4'-H	1.75 (dq, J=7.5, 1.5 Hz)
5'-H	1.81 (dq, J=1.5, 1.1 Hz)

Table 2. ^{1}H n.m.r. of Salannin (7)

H-Atom	
1-H	4.93 (t, J=3.0 Hz)
2-$H_{\alpha,\beta}$	2.05-2.25 (m)
3-H	4.82 (t, J=3.0 Hz)
5-H	2.79 (d, J=12.5 Hz)
6-H	3.92 (dd, J=12.5, 3.0 Hz)
7-H	4.18 (d, J=3.0 Hz)
11-H_{α}	n.d
11-H_{β}	2.29 (dd, J=15.0 , 9.5 Hz)
15-H	5.41 (ddq, J=7.5 , 1.0, 1.5 Hz)
16-H_{α}	2.20 (dd, J=12.5, 5.0 Hz)
16-H_{β}	2.11 (dd, J=12.5, 7.5, 5.0 Hz)
17-H	3.60 (dd, J=7.5, 1.0 Hz)
18-H	1.65 (d, J=1.0 Hz)
19-H	0.96 (s)
21-H	7.32 (m)
22-H	6.27 (m)
23-H	7.23 (m)
28-H_{α}	4.15 (d, J=8.0 Hz)
28-H_{β}	3.62 (d, J=8.0 Hz)
29-H	1.20 (s)
30-H	1.28 (s)
3'-H	6.96 (qq, J-7.0 , 1.0 Hz)
4'-H	1.81 (dq, J=7.0 , 1.0 Hz)
5'-H	1.91 (dq, J=1.5, 1.0 Hz)
OMe	3.22 (s)
OAc	1.92 (s)

Table 3. ^{1}H n.m.r. of 1,3-diacetyl-7-tigloyl vilasinin (13).

H-atom	
1-H	4.76 (t, J=3.0 Hz)
2-H$_{\alpha,\beta}$	2.15-2.20 (m)
3-H	4.96 (t. J=3.0 Hz)
5-H	2.80 (d, J=12.5 Hz)
6-H	4.06 (dd, J=12.5, 3.0 Hz)
7-H	5.71 (d, J=3.0 Hz)
9-H	2.84 (m)
11-H	1.85-1.45 (m)
12-H	
15-H	5.61 (m)
16$_{\alpha,\beta}$	2.50 (m)
17-H	3.20 (dd, J=12.5, 5.0 Hz)
18-H	0.87 (s)
19-H	1.04 (s)
21-H	7.29 (m)
22-H	6.28 (m)
23-H	7.23 (m)
28-H$_\alpha$	3.46 (br, d, J=8.5 Hz)
28-H$_\beta$	3.56 (d, J=8.5 Hz)
29-H	1.13 (s)
30-H	1.18 (s)
3'-H	6.92 (qq, J=7.0 , 1.0 Hz)
4'-H	1.75 (dq, J=7.0 , 1.0 Hz)
5'-H	1.85 (m)
OAc	2.03 (s)
OAc	2.11 (s)

Table 4. ^{1}H n.m.r. of 3-deacetyl-11-desoxyazdirachtin (**14**)

H-atom	
1-H	5.52 (t, J=2.9 Hz)
2-H$_\alpha$	2.27 (dt, J=15.2 , 2.5 Hz)
2-H$_\beta$	2.10 (m)
3-H	3.52 (m)
5-H	3.32 (d, J=12.7 Hz)
6-H	4.53 (dd, J=12.7, 2.8Hz)
7-H	4.71 (d, J=2.8)
9-H	3.19 (s)
11-H	4.46 (s)
15-H	4.58 (d, J=2.9 Hz)
16-H	1.64 (ddd, J= 13.0, 5.0, 4.0 Hz)
16-H	1.33 (d, J=13.0 Hz)
17-H	2.36 (d, J=4.7 Hz)
18-H	2.04 (s)
19-H	3.94 (d, J=9.4 Hz)
19-H	4.49 (d, J=9.4 Hz)
21-H	5.65 (s)
22-H	5.03 (d, J=2.9 Hz)
23-H	6.43 (d, J=2.9 Hz)
28-H$_\alpha$	4.04 (d, J=9.0 Hz)
28-H$_\beta$	3.83 (d, J=9.0 Hz)
30-H	1.43 (s)
7-OH	2.78 (s)
11-OH	-
20-OH	-
OMe	3.75 (s)
29-OMe	3.75 (s)
OAc	-
3'-H	6.49 (qq, J=7.1, 1.3 Hz)
4'-H	1.79 (dq, J=7.1, 1.3 Hz)
5'-H	1.84 (m)

Table 5. ^{1}H n.m.r. of 3-deacetylsalannin (15)

H-Atom		
1-H	5.00	(t, J=3.0 Hz)
2-H_α	2.26	(dt, J=3.0, 12.0 Hz)
2-H_β	2.05	(dt, J=3.0 , 15.0 Hz)
3-H	3.80	(t, J=3.0 Hz)
5-H	2.70	(d, J=13.0 Hz)
6-H	3.99	(dd, J=3.0, 13.0 Hz)
7-H	4.17	(d, J=3.0 Hz)
11-H_α	2.14	(dd, J=3.0, 15.0 Hz)
11-H_β	2.30	(dd, J=10.0, 15.0 Hz)
15-H	5.37	(m)
16-H_α	2.25	(dd, J=5.0, 12.0 Hz)
16-H_β	2.11	(ddd, J=12.0, 7.5, 5.0 Hz)
17-H	3.60	(dd, J=7.5, 1.5 Hz)
18-H	1.63	(d, J=1.5 Hz)
19-H	0.93	(s)
21-H	7.28	(m)
22-H	6.27	(m)
23-H	7.23	(m)
28-H_α	4.12	(d, J=8.0 Hz)
28-H_β	3.63	(d, J=8.0 Hz)
29-H	1.15	(s)
30-H	1.29	(s)
3'-H	6.88	(qq, J=7.0 , 1.0 Hz)
4'-H	1.84	(dq, J=7.0, 1.0 Hz)
5'-H	1.90	(d, J=1.0 Hz)
OMe	3.15	(s)
OH	2.42	(s)

Table 6. ^{1}H n.m.r. of 3-deacetylazadirachtin (18)

H-Atom	
1-H	4.84 (t, J=2.7 Hz)
2-H	2.25 (dt, J=16.5 , 2.7, Hz)
2-H	2.67 (m)
3-H	4.36 (dt, J=7.3, 3.1 Hz)
5-H	3.21 (d, J=12.5 Hz)
7-H	4.62 (dd, J=12.5, 2.8 Hz)
9-H	3.32 (s)
15-H	4.65 (d, J=3.5 Hz)
16-H	1.66 (ddd, J=13.3, 5.4, 3.9 Hz)
16-H	1.29 (d, J=13.3 Hz)
17-H	2.38 (d, J=5.2 Hz)
18-H	2.01 (s)
19-H	3.58 (d, J=9.8 Hz)
19-H	4.22 (d, J=9.8 Hz)
21-H	5.65 (s)
22-H	5.04 (d, J=2.9 Hz)
23-H	6.44 (d, J=2.9 Hz)
28-H_α	4.06 (d, J=8.6 Hz)
28-H_β	3.61 (d, J=8.6 Hz)
30-Me	1.75 (s)
17-OMe	3.76 (s)
29-OMe	3.88 (s)
3'-H	6.83 (qq, J=7.1, 1.4 Hz)
4'-H	1.78 (dq, J=7.1, 1.1 Hz)
5'-H	1.84 (dq, J=1.2, 1.3 Hz)
7-OH	2.75 (s, br)
11-OH	5.02 (s)
20-OH	2.93 (s, br)
3-OH	n.d

Table 7. ^{13}C n.m.r. of 3-deacetylazadirachtin (18)

C-Atom	
C-1	66.0 (d)
C-2	32.0 (t)
C-3	71.6 (d)
C-4	53.3 (s)
C-5	35.3 (d)
C-6	73.7 (d)
C-7	75.8 (d)
C-8	52.4 (s)
C-9	44.7 (d)
C-10	52.5 (s)
C-11	104.2 (s)
C-12	n.d
C-13	69.8 (s)
C-14	69.4 (s)
C-15	77.0 (d)
C-16	25.1 (t)
C-17	48.7 (d)
C-18	18.3 (q)
C-19	71.8 (t)
C-20	83.5 (s)
C-21	108.7 (d)
C-22	107.4 (d)
C-23	147.0 (d)
C-28	73.0 (t)
C-29	173.1 (s)
C-30	21.2 (q)
12-OMe	53.2 (q)
29-OMe	52.5 (q)
C-1'	n.d
C-2'	128.1 (s)
C-3'	138.3 (d)
C-4' Me	14.5 (q)
C-5' Me	12.1 (q)

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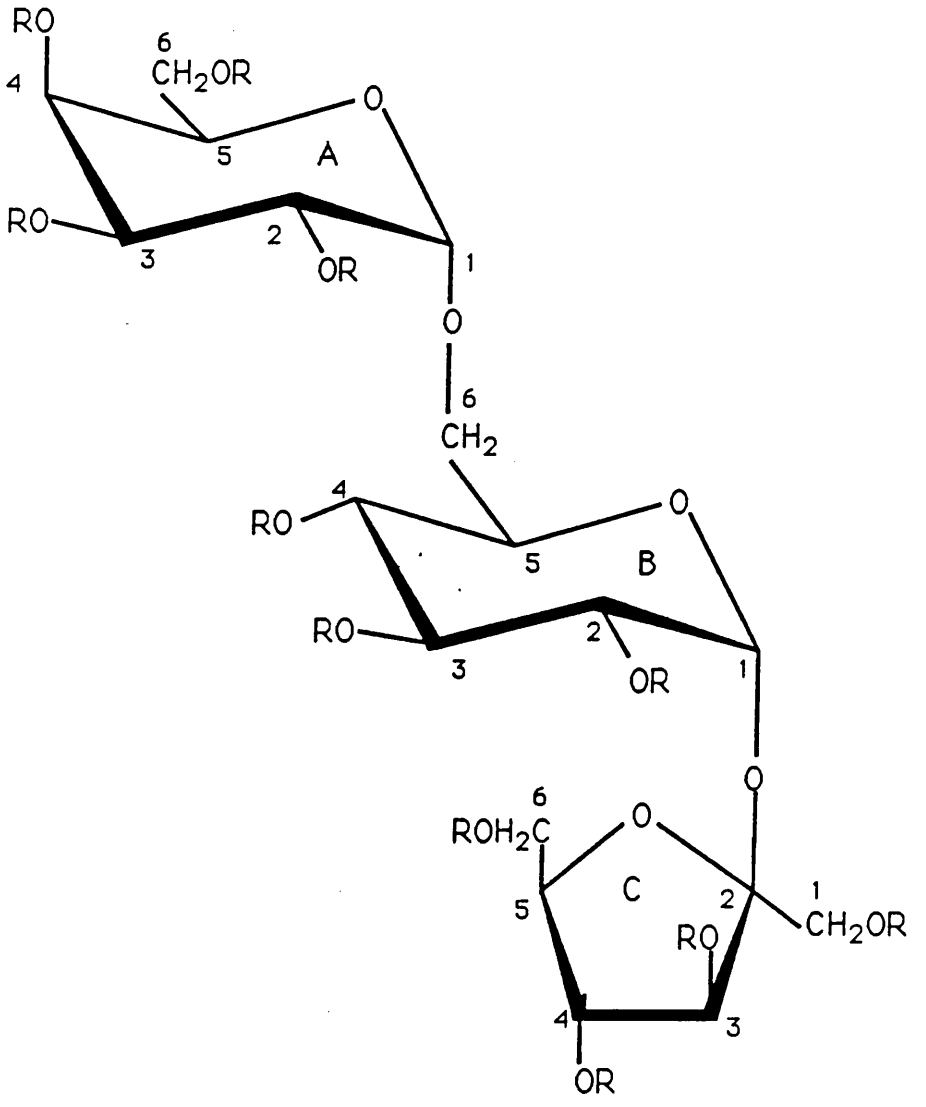
CHAPTER VI

STRUCTURE ELUCIDATION OF RAFFINOSE FROM

Delonix regia.

A TWO-DIMENSIONAL N. M. R. SPECTROSCOPY APPROACH

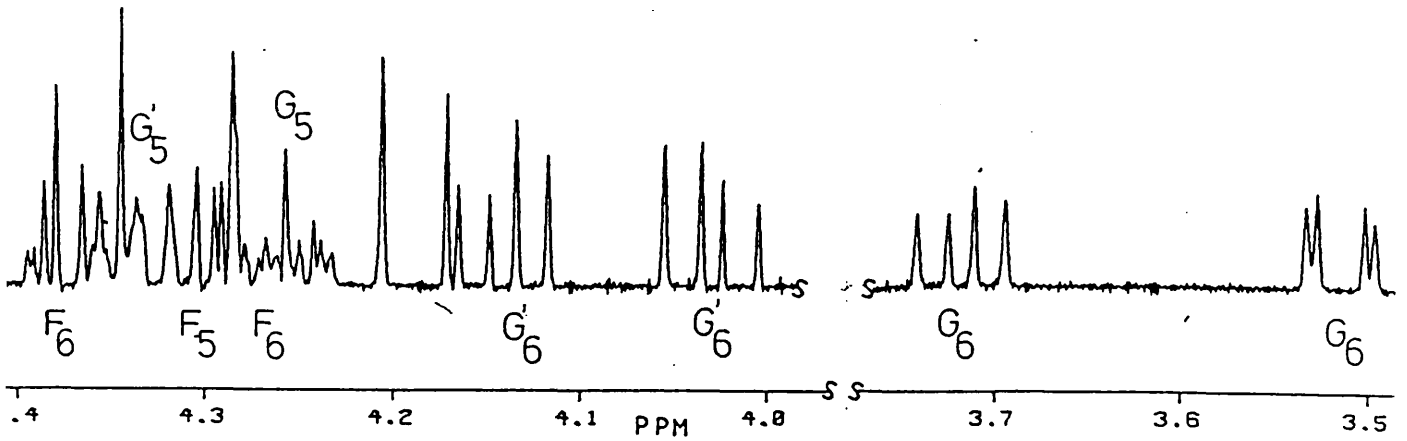


1. R = H (Raffinose)
2. R = Ac (Raffinose peracetate)

DISCUSSION

This chapter is concerned with the structural elucidation and the assignment of the ^{1}H and ^{13}C resonances of the peracetate of a trisaccharide, subsequently identified as raffinose **(1)**, from the plant ***Delonix regia*** (Leguminosae) from Cameroon. It is clear from the ^{1}H n.m.r. spectrum of the compound that it is a sugar derivative though the considerable overlap of resonances at 200 MHz makes it impossible to distinguish most of the individual signals. The ^{13}C n.m.r. spectrum reveals its trisaccharide nature (Table 2), showing three anomeric carbons [δ_C 100.5 (d), 105.2 (s) and 93.4 (d)]. No attempt was made to assign the ^{1}H and ^{13}C resonances of the free trisaccharide. The compound was converted into its peracetate **(2)**. This has the advantage of spreading out the ^{1}H resonances since those attached to carbons bearing acetyl groups will be deshielded. At 200 MHz there are still several regions of the proton spectrum of **(2)** which are not amenable to interpretation because of overlap. The 360 MHz ^{1}H n.m.r. spectrum of **(2)** reveals most of the individual protons and their coupling constants. The appropriate regions of the spectrum are shown in Figure 1. Interpretation was facilitated by use of a COSY spectrum of **(2)** also run at 360 MHz (Figure 2).

Fig. 1. The ^1H n.m.r. of raffinose peracetate (2) at 360 MHz (in $CDCl_3$ / C_6D_6)



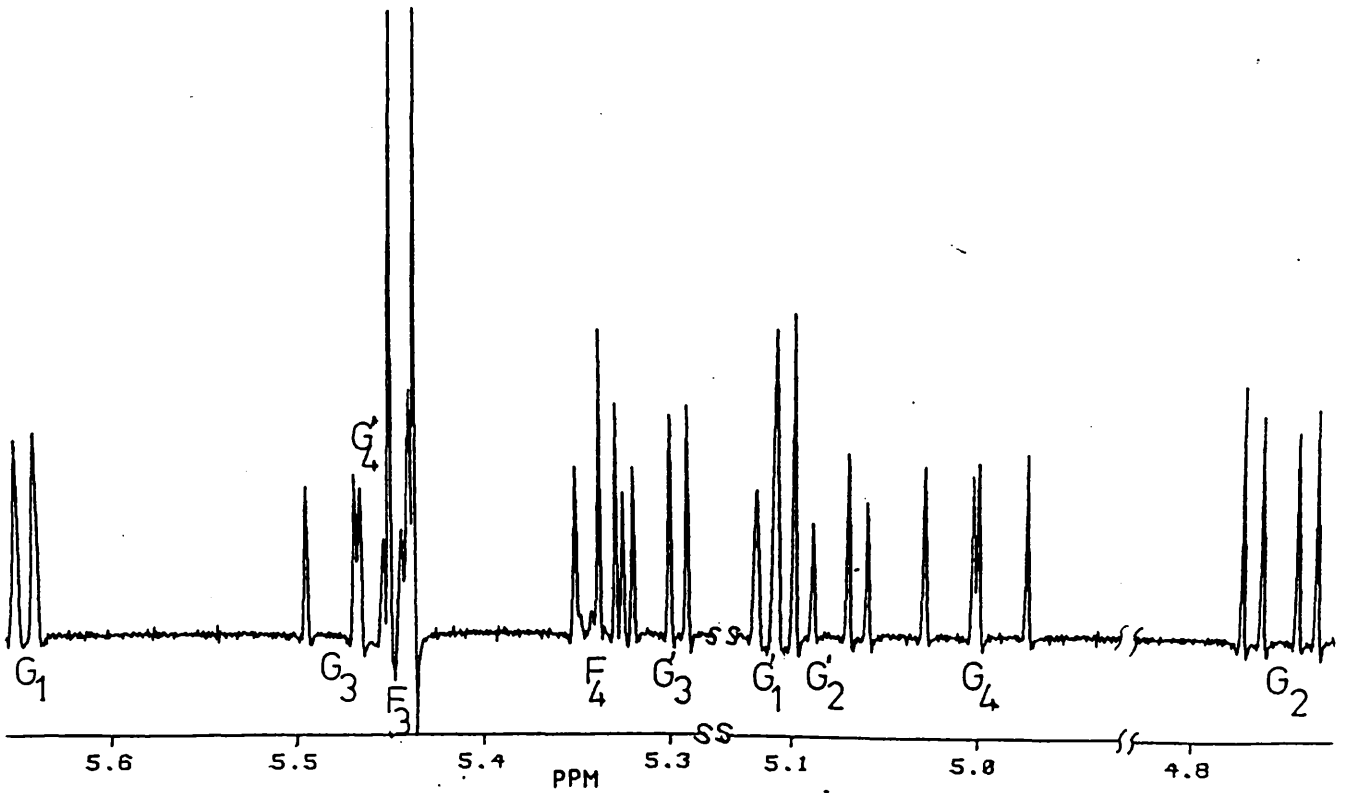


Fig. 2. COSY spectrum of raffinose peracetate (2) at 360 MHz

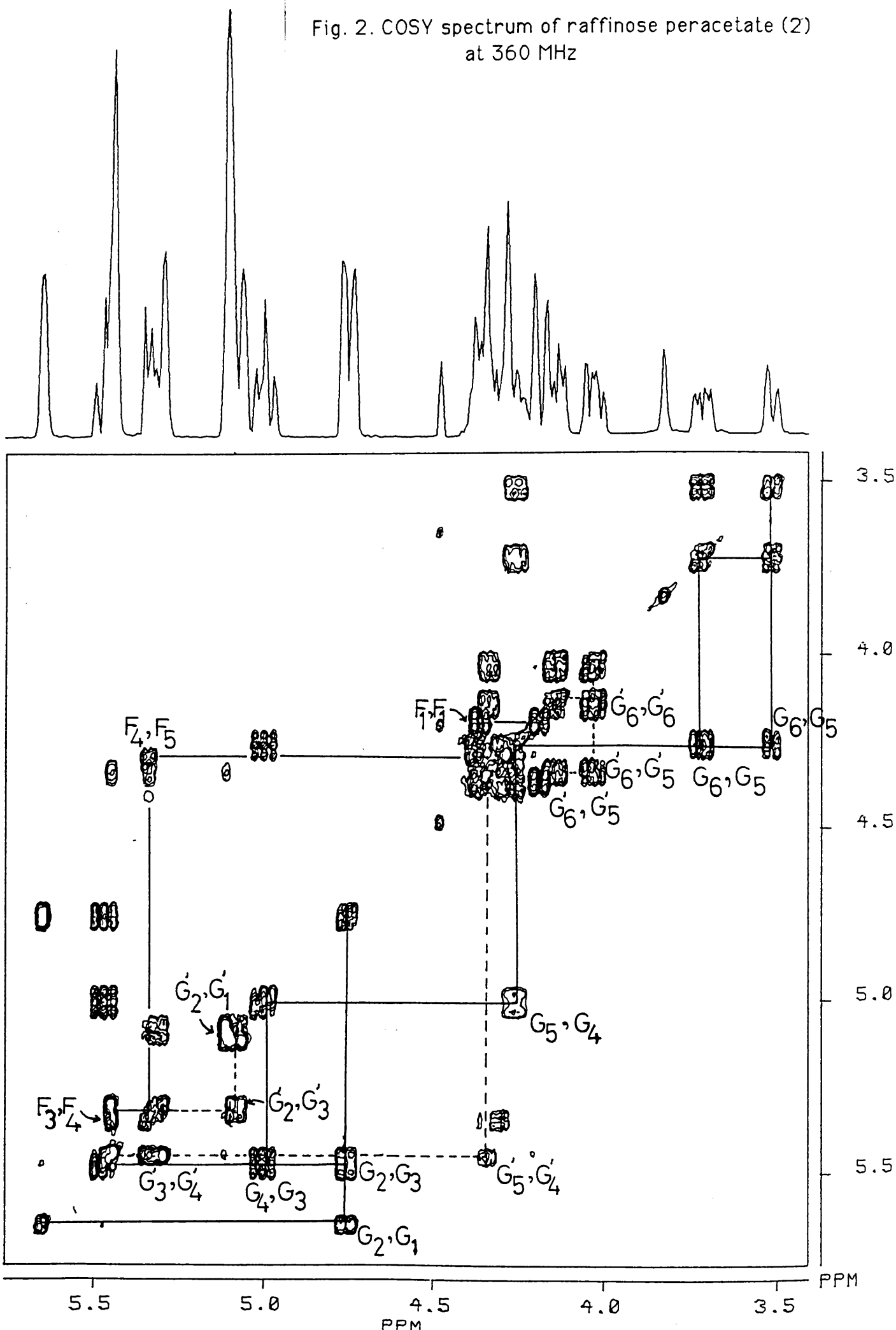
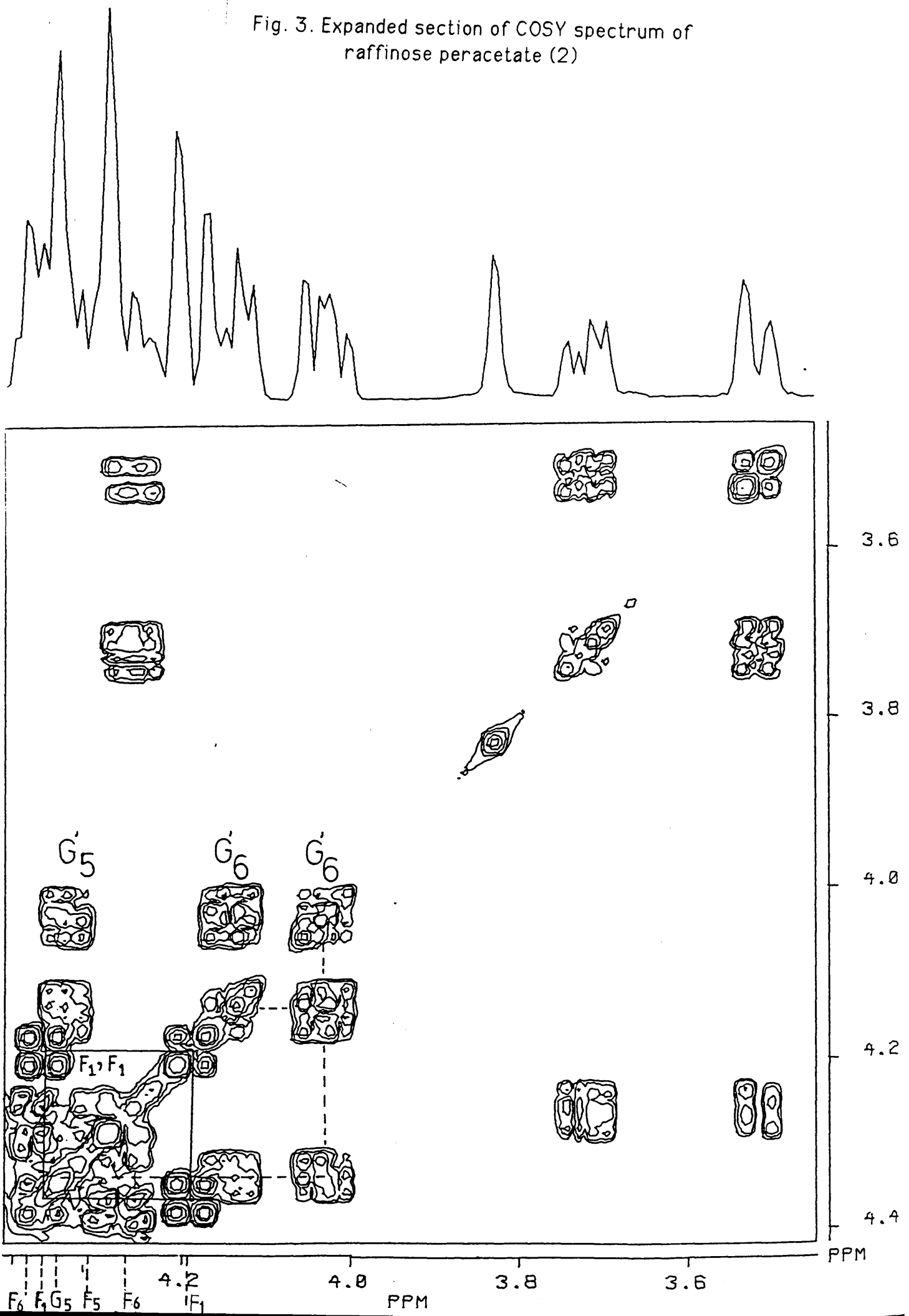


Fig. 3. Expanded section of COSY spectrum of raffinose peracetate (2)



The easiest sugar to identify is glucose. All the protons except H-5 are readily apparent and the connectivity sequence is easily followed in the COSY spectrum. The ^1H chemical shifts and coupling constants are listed in Table 1. The chemical shift of 2H-6 indicates the involvement of C-6 in a glycosidic link. The small coupling constant of H-1 (J=3.7 Hz) reveals the α configuration at C-1.

The second sugar is clearly a ketose since it has two $-CH_2OR$ groups and a singlet anomeric carbon (δ_C 105.1). Two protons are readily identified, H-3 [δ_H 5.44 (d, J=4.6 Hz)] and H-4 [δ_H 5.34 (t, J=4.6 Hz)]. These are consistent with the presence of fructose. The positions of the remaining protons H-5 and 2H-6 are obtainable from the expansion of the COSY spectrum (Figure 3). The 2H-1 resonances appear as an isolated AB system. It seems likely that the fructose and glucose units are linked as in sucrose. Comparison with the ^1H and ^{13}C n.m.r. resonances of sucrose peracetate [1] confirm this suggestion. Thus the 'unknown' glycoside consists of sucrose linked via C-6 of glucose to another monosaccharide unit.

The proton shifts and coupling constants of the third unit, obtained from the 360 MHz ^1H n.m.r. and COSY spectra as above, are listed in Table 1. The nature of H-4 [δ_H 5.45 (dd, J=3.4, 1.3 Hz)] indicates that the sugar is galactose. The anomeric

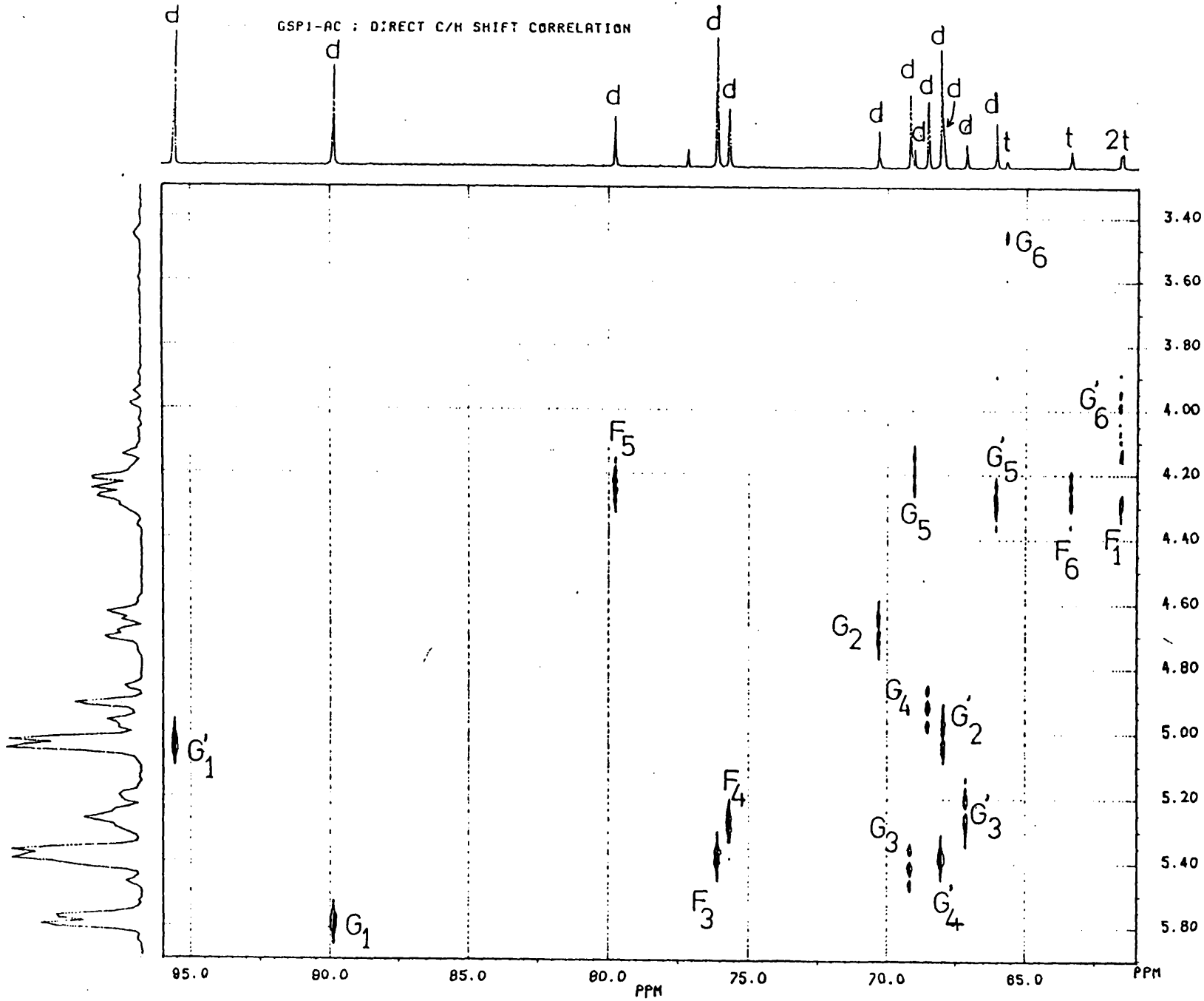


Fig. 4. Heteronuclear C-H chemical shift correlation of raffinose peracetate (2)

configuration is clearly α (J= 3.7 Hz) . The structure of the gycoside is therefore α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (1). Consultation of the literature showed that this is a known compound raffinose (1). The ^{1}H [2] and ^{13}C [3,4,5] n.m.r. assignments of raffinose (1) have been published. The details of the peracetate (2) have not been published.

The assignments of the ^{13}C resonances of the acetates of carbohydrates on the basis of acetylation shifts and comparison with the free sugar is fraught with difficulty because of H-bonding effects. Definitive assignments require a 2D δ_C/δ_H correlation experiment. The 2D δ_H/δ_H correlation spectrum of raffinose peracetate (2) is shown in Figure 4. Since the ^{1}H chemical shifts of all the protons are already known it is a relatively simple matter to make the asignments listed in Table 2. Potential confusion between H-3 of fructose and H-4 of galactose which have the same chemical shift is easily avoided by comparison with the known ^{13}C chemical shifts of sucrose peracetate [1].

The above discussion illustrates the advantages of the application of modern n.m.r. techniques to carbohydrate structure, especially to small oligosaccharides. The subject has been reviewed recently by Bock and Thøgersen [6]. The potential for

the use of delayed COSY experiments is illustrated by the structure elucidation of the oligorhamnosides from ***Cleistopholis glauca*** [7].

EXPERIMENTAL

The seed of ***Delonix regia*** (Leguminosae) was collected from the highland grass area of Ngaoundere, Cameroon. The seed was ground after removing the hard pericarps. The ground seed (2.2 kg) was extracted successively with hexane, ethyl acetate and methanol. The methanol extract (3.5 litres) was concentrated and left to stand for one week at room temperature. A white precipitate was formed and was filtered off to yield a white solid (9.1 g). Part of the solid was washed with a mixture of methanol and acetone several times and crystallised from methanol and a few drops of water to yield pure crystalline **raffinose (1)**, m.p. 133-134°C, $[\alpha]_D$ +76° (c, 0.35 in MeOH), [Lit [9] m.p. 118°C (from H_2O), $[\alpha]_D$+123°(in H_2O)], i.r. ν_{max} (KBr Disc) cm^{-1}: 3300-3480 (br, OH)

^{13}C n.m.r. see Table 2.

Acetylation of raffinose (1)

Raffinose **(1)**(17.0 mg) was acetylated under the usual condition using acetic anhydride (1 ml) and dry pyridine (1 ml) overnight at room temperature. The crude product was purified by preparative t.l.c. (ethyl acetate: petroleum ether(60-80), 1:1 , three developments) to afford, after crystallisation from methanol, raffinose peracetate**(2)** (13.7 mg),

m.p. 90-92°C, ^{1}H n.m.r. See Table 1, ^{13}C n.m.r. See Table 2.

N.M.R. Experimental

The 360 MHz ^{1}H COSY spectrum was acquired using Jeener's [9] two pulse sequence P1 - t_1 - P2 - acquire with a 16 step phase cycle for anti-echo selection; P1 was 90° and P2 was 60°, and the recycle delay was 3 sec. 80 transients (and 2 dummy scans) were collected for each of 256 t_1 values. Sine-bell squared weighting functions in both dimensions were used and magnitude spectra were calculated. Zero-filling in f_1 gave a digital resolution of 2.3 Hz/data point in both dimensions. Both unsymmetrised and symmetrised spectral matrices were examined.

The 50 MHz ^{13}C/^{1}H 2D chemical shift correlation spectrum was obtained using the standard sequence [10] 90^0_H-½t_1-180^0_C-½t_1-Δ_1-90^0_H, 90^0_C-Δ_2-*decouple*, *acquire*, with a 32 step phase cycle arranged for 'quadrature detection' in f_1[11]. The defocussing delay Δ_1 = 3.4 ms and the refocussing delay Δ_2=1.7 ms; the recycle delay was 1s. 160 transients (and 4 dummy scans) were acquired for each of 256 values of t_1. Sine-bell weighting functions shifted by π/6 were used in both dimensions, and power spectra were calculated. The digital resolution was 2.0 Hz/data point in f_2 and (after zero-filling) 1.8 Hz/data point in f_1.

Table 1. ^1H n.m.r. of raffinose peracetate (2) (in a mixture of $CDCl_3$ and C_6D_6) at 360 MHz

Signal	δ_H
Ring A (Galactose)	
G'1	5.11 (d, J=3.7 Hz)
G'2	5.08 (dd, J=10.5, 3.6 Hz)
G'3	5.32 (dd, J=10.5, 3.4 Hz)
G'4	5.45 (dd, J=3.4, 1.3 Hz)
G'5	4.34 (dd, J=7.1, 6.1, 1.3 Hz)
G'6A	4.03 (dd, J=11.2, 7.1 Hz)
G'6B	4.14 (dd, J=11.2, 6.1 Hz)
Ring B (Glucose)	
G1	5.65 (d, J=3.7 Hz)
G2	4.75 (dd, J=10.4, 3.7 Hz)
G3	5.47 (dd, J=10.5, 9.4 Hz)
G4	5.00 (dd, J=10.5, 9.4 Hz)
G5	4.26 (ddd, J=10.5, 6.1, 2.0 Hz)
G6A	3.72 (dd, J=11.1, 6.1 Hz)
G6B	3.53 (dd, J=11.1, 2.0 Hz)
Ring C (Fructose)	
F1A	4.36 (d, J=12.3 Hz)
F1B	4.19 (d, J=12.3 Hz)
F2	-
F3	5.44 (d, J=4.6 Hz)
F4	5.34 (t, J=4.6 Hz)
F5	4.31 (m)
F6	4.38 (m)
FG	4.27 (m)
OAc	1.67, 1.93, 1.97, 1.98, 2.01, 2.02, 2.03, 2.04, 2.05, 2.06, 2.09 (all singlets)

Table 2. ^{13}C n.m.r. of raffinose (1) and its peracetate(2)

Signal	Raffinose(1) (CD_3OD)	Peracetate(2) ($CDCl_3$)
Ring A (Galactose)		
G'1	100.5	95.8 (d)
G'2	71.4	68.2 (d)
G'3	71.0	67.4 (d)
G'4	71.9	68.3 (d)
G'5	72.4	66.3 (d)
G'6	62.7	61.8 (t)
Ring B (Glucose)		
G1	93.4	90.0 (d)
G2	74.4	70.5 (d)
G3	73.0	69.4 (d)
G4	70.5	68.7 (d)
G5	73.0	69.2 (d)
G6	65.9	65.9 (t)
Ring C (Fructose)		
F1	63.1	61.8 (t)
F2	105.2	104.9 (s)
F3	79.0	76.3 (d)
F4	75.2	75.9 (d)
F5	83.4	79.9 (d)
F6	63.8	63.6 (t)
OAc	-	170.5 (s)(2), 170.5 (s), 170.2 (s), 170.1 (s)(3), 170.0 (s), 169.7 (s), 169.6(s), 169.5 (s) 20.73 (q), 20.66 (q), 20.62 (q), 20.53 (q)

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