Alkolides, coumarins, triterpenes and other biologically actine principals of some plant families of Sri Lanka

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FINAL REPORT

Grant No: RG/84/C/6

Grantees: Prof. G.P.Wannigama, Dr. B.M.R.Bandara, Dr. N.L.V.V. Karunaratne and Prof. S. Sotheeswaran

Title of Project: Alkaloids, coumarins, triterpenes and other biologically active principles of some plant families of Sri Lanka.

Date of award of the grant by NARESA: 1.1.1984

Abstract: The following plants have been studied during the period:

(a) (i) Cyclea burmanni (කැහි පිත්තන්) and (ii) Diploclisia glaucescens (ඇට තිත්ත වැල්) (Menispermaceae) and

(b) (i) Litsea gardneri (තලන්) and (ii) Actinodaphne speciosa (නික දවුල) (Lauraceae)

Both plants of the Menispermaceae are found in Sri Lanka as well as India while both plants of the Lauraceae are endemic to Sri Lanka.

The roots of C. burmanni, collected near Kandy gave the bisbenzyl-isoquinoline alkaloids, phaeanthine and limacine, while the same plant collected near Kalutara gave tetrandrine, the enantiomer of phaeanthine, indicating that the chemical constituents and hence bioactivity of a plant may depend on its site of collection. The 'non-phenolic' alkaloidal fraction of the plant, consisting of phaeanthine and limacine showed no hypotensive activity, no hemolytic activity and no anti-inflammatory activity. The mass spectral fragmentation of phaeanthine and limacine as well as the ^{13}C NMR spectrum of phaeanthine have been interpreted.

A quaternary alkaloid, palmatine has also been isolated from the roots of C. burmanni collected near Kandy.

The stem of Diploclisia glaucescens gave the arthropod moulting hormone, 20-hydroxyecdysone in the highest yield (3.2%) so far recorded from any natural source. This compound showed both fungicidal and insecticidal activity, but no molluscicidal activity. Other compounds isolated were (i) an unidentified sterol, (ii) two triterpenoid acids of the oleanane series namely, serjanic acid and phytolaccagenic acid, (ii) a bidesmosidic saponin tentatively identified as 3,28-di-O-β-D-glucopyranosylphytolaccagenic acid and (iv) vibo-quercitol. The bidesmodic saponin showed no molluscicidal activity. A mixture of compounds showing the hemolysis and froth tests for saponins was also obtained. This mixture showed molluscicidal activity.

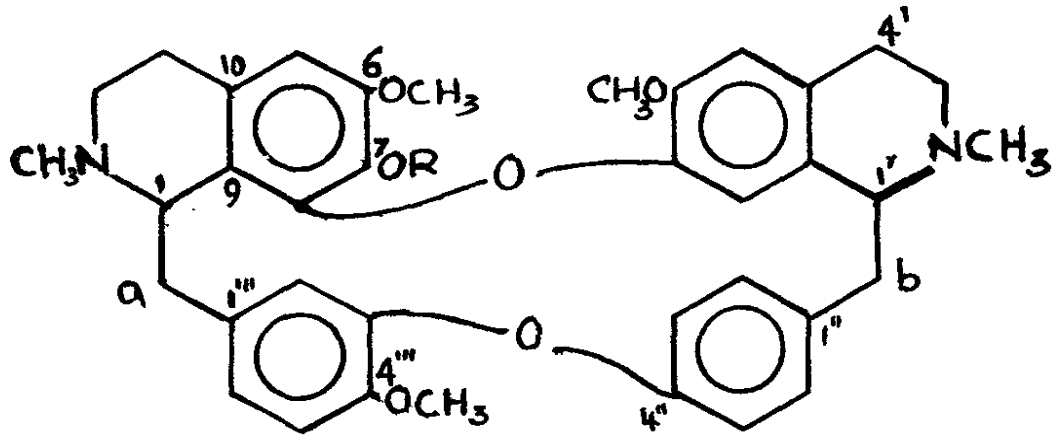
The fungicidal major component of the neutral fraction of the bark of Litsea gardneri has been isolated. The basic fraction gave the aporphine alkaloids, actinodaphnine and laurolitsine.

Three alkaloids have been isolated from the leaves of Actinodaphne speciosa. Two have been identified as the aporphine alkaloids, laurotetanine and N-methyllaurotetanine. The third is most probably the benzylisoquinoline alkaloid. N-methylcoclaurine.

Discussion

(a) (i) Cyclea burmanni is a creeper growing in South India as well as in Sri Lanka. The roots are used in the indigenous system of medicine for treatment of jaundice, stomachache, fever, leprosy and asthma. Indian chemists have isolated tetrandrine from the roots of the plant collected near Trivandrum (1).

The roots of C.burmanni (275 g) collected near Peradeniya were extracted with hot methanol, the extract evaporated and the residue partitioned between 2N HCl and n-butanol. Basification of the aqueous layer with 25% NH_4OH followed by extraction with $CHCl_3$ gave the total alkaloids (10 g). Chromatographic separation of a portion (5 g) gave phaeanthine (262 mg) as colourless crystals, mp 150-152° and limacine (123 mg) as a light brown gummy solid (2).



Tetrandrine (R=Me): 1(S), 1'(S)

Phaeanthine (R=Me): 1(R), 1'(R)

Limacine (R=H): 1(R), 1'(R)

The melting point, specific rotation and ^1H NMR-spectrum of phaeanthine as well as the melting point and specific rotation of its picrate agreed well with recorded data for synthetic phaeanthine (3). The phaeanthine obtained was optically pure, since the O-methyl and N-methyl singlets in its ^1H NMR spectrum were only shifted downfield but not split by the chiral shift reagent, tris-(3-trifluoroacetyl-d-camphorato)-europium III, $Eu(tfc)_3$.

The fragmentation pattern in the mass spectrum of phaeanthine was interpreted on the basis of N-methyl fission, ether fission and benzylic fission.

The ^{13}C NMR spectrum of phaeanthine ($CDCl_3$) showed the presence of 38 carbon atoms. There were 37 signals one of which was due to two overlapping signals. DEPT analysis was used to differentiate $\underline{C}H_3$ and $\underline{C}H$ signals from $\underline{C}H_2$ signals.

Limacine was identified on the basis of its ^1H NMR spectrum, mass spectrum and specific rotation (4,5). The identity was confirmed by conversion to phaeanthine by reaction with diazomethane. The free phenolic hydroxyl group in limacine was placed at position 7, as the ^1H NMR spectrum of limacine showed only three O-methyl singlets, lacking the O-methyl singlet at the highest field in phaeanthine.

The fragmentation pattern in the mass spectrum of limacine was interpreted on the basis of N-methyl fission, benzylic fission, ether fission and retro-Diels-Alder reaction.

Limacine is insoluble in alkali as it has a hindered phenolic hydroxyl group. Together with phaeanthine, it was separated as the 'non-phenolic' alkaloidal fraction of the roots of C. burmanni collected near Kandy. This fraction did not have any influence on rat heart beat and blood pressure when administered intravenously at doses of 0.1-20 mg/kg. The fraction did not hemolyse sheep blood and did not show any toxicity in the rat carrageenan oedema bioassay. Oral feeding on female mice showed no toxicity even at a dose of 100 mg/kg.

The roots of C. burmanni (52 g) collected near Kalutara were extracted with hot methanol and the total alkaloids (1.9 g) isolated as for the Peradeniya sample. Separation was effected by column chromatography on silica gel followed by preparative layer chromatography

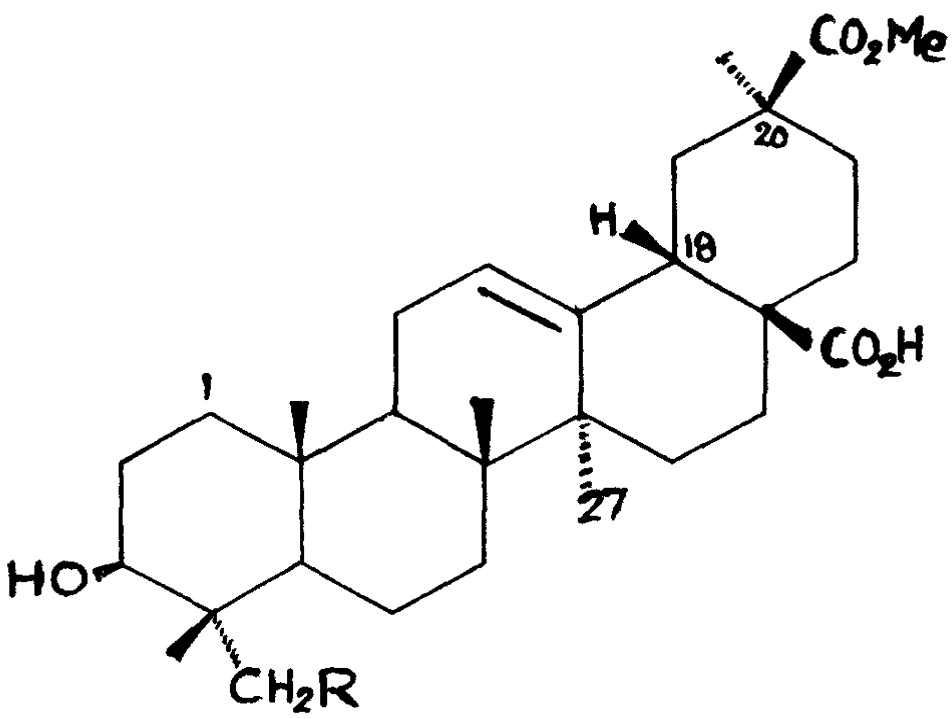
on silica gel. Tetrandrine (40 mg) was obtained as a light brown gummy solid. The ^{1}H NMR spectrum was identical with that of phaeanthine isolated earlier. The spectific rotation indicated the enantiomeric relationship with phaeanthine.

(a) (ii) Diploclisia glaucescens is a creeper growing in the mid-country regions of India and Sri Lanka. The plant is reported to be used for the treatment of biliousness and venereal diseasess (6). Several phytoecdysteroids have been isolated from the seeds of the plant and their activity as insect control agents demonstrated (7). The principal compound,20-hydroxyecdysone, was isolated in a yield of 0.46%.

The presence of alkaloids in the leaves and twigs of the plant had been indicated in a preliminary survey (8). In this investigation, the mature stem of the plant was defatted with petroleum ether and then with methanol. The methanol extract (7 g) was partitioned between dichloromethane and 2N HCl. Basification of the acidic layer gave a mixture of alkaloids (140 mg). Separation of the alkaloids is in progress.

The non basic fraction (1.7 g) was separated by chromatography into six compounds. The least polar compound (65 mg), identified as serjanic acid was obtained as colourless crystals, mp 249-251°, $[\alpha]_D^{22}$ + 77° (c 0.20 in $CHCl_3$). The next more polar compound, the major compound (120 mg), identified as phytolaccagenic acid (10) was obtained as colourless crystals, mp 285-287°, $[\alpha]_D^{30}$ + 98.1° (c 0.13 in CH_3OH). Both serjanic acid and phytolaccagenic acid were shown to be present in the methanol extract and are not artefacts formed during the

isolation process. Both acids did not inhibit the growth of the fungus Cladosporium cladosporioides.



Serjanic acid: R = H

Phytolaccagenic acid : R = OH

Serjanic acid was first obtained as an amorphous solid by the acid hydrolysis of the saponins of a Serjania species (Sapindaceae). The acid has been characterised as its methyl ester (9). Identification of the acid in the present work was based on a spectral analysis of the acid, the methyl ester and its acetate, 3-O-acetylserjanic acid. The latter was obtained for the first time as colourless crystals, mp 196-198°, $[\alpha]_D^{22}$ + 27° (c 0.13 in $CHCl_3$).

Phytolaccagenic acid was first isolated from the acid hydrolysate of the saponin fraction of the pokeroot, Phytolacca americana (Phytolaccaceae) (10). Structure elucidation was based on chemical data and a spectroscopic analysis of the acid and derivatives (10,11,12). Identification of the acid in the present work was based on a spectral analysis of the acid and its diacetate, 3,23-di-O-acetylphytolaccagenic acid.

The present investigation provided unambiguous evidence for the structure and configuration of phytolaccagenic acid in the ring E. Thus the groups attached to C-20 were identified as methyl and carbomethoxyl from observation of $^{13}C-^{1}H$ coupling in heteronuclear scalar correlated spectra (between H-29 and C-30, C-30 and $CO_2\underline{CH}_3$, H-29 and C-20). The configuration at C-20 is established both from ^{13}C NMR studies as well as NOE studies in ^{1}H NMR. The chemical shifts (δ_c in $CDCl_3$ + CD_3OD) for C-20 and C-29 were 44.62 and 28.69 respectively. These values are very similar to recorded data for 18β-olean-12-ene (13), and indicate that the methyl and carbomethoxyl groups attached to C-20 are equatorial (α) and axial (β) respectively. In ^{1}H NMR, no NOE is observed between H-18 and H-27, whereas 1,3-diaxial interaction results in a strong NOE between H-18 and $CO_2\underline{CH}_3$.

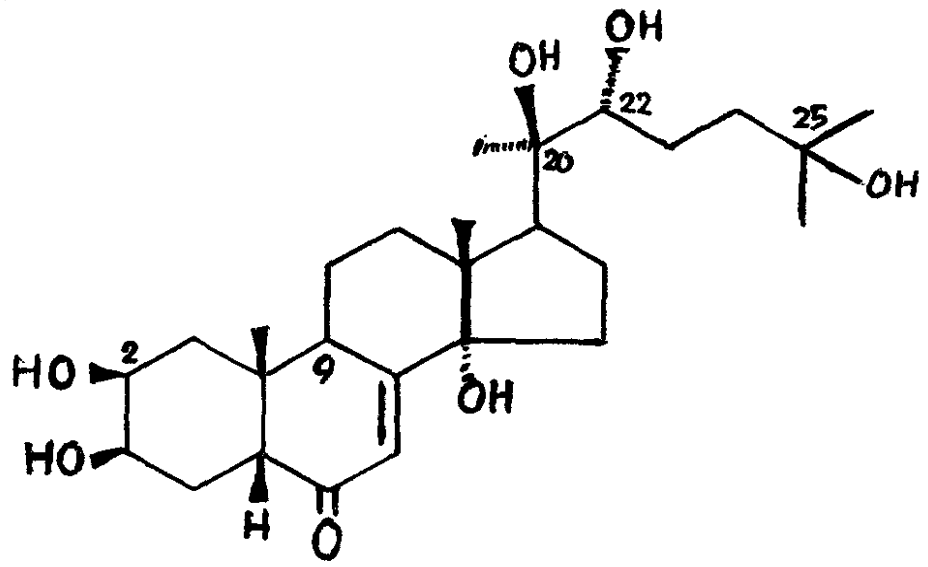
The presence of triterpenoid hydroxy acids in D. glaucescens indicated the probable presence of saponins in the plant. Indeed, a n-butanol extract of the methanol extract gave a positive response for saponins in both the froth and hemolysis tests. Further, the methanol extract showed fungicidal activity and reasonable molluscicidal activity. When Biomphalaria glabrata snails (molluscs), one of the intermediate hosts of the Schistosoma parasite, were exposed to the extract at a concentration of 200 ppm, the minimum concentration for activity, 100% mortality of the snails was observed.

In view of the interesting bioactivity shown by many saponins, a chemical fractionation of the methanol extract coupled with bioassay was undertaken. Chromatographic separation of the methanol extract (15 g) gave an unidentified compound 1 (30 mg) as colourless crystals, mp 156-158°, $[\alpha]_D^{22}$ -52.5° (c 0.40 in $CHCl_3$), 20-hydroxyecdysone (4 g) as colourless crystals, mp 242-244°, $[\alpha]_D^{22}$ +52.2° (c 0.23 in CH_3OH) and a new bidesmosidic saponin 2 (600 mg) as colourless crystals, mp 171-173°, $[\alpha]_D^{22}$ + 25° (c 0.12 in CH_3OH). Further separation could not be achieved. Elution of the unseparated mixture with methanol and concentration of the methanol extract gave vibo-quercitol (82 mg) as colourless crystals, mp 186°, $[\alpha]_D^{22}$ -50° (c 0.40 in H_2O). The methanol extract was evaporated and the residue (5.5 g) partitioned between n-butanol and water. The n-butanol extract (4.8 g) gave strongly positive froth and hemolysis tests and probably consists of three saponins.

The unidentified compound 1 was later obtained by chromatographic separation of the petroleum ether extract, (2 g of extract giving 0.37 g of 1). The compound shows a positive hemolysis test.

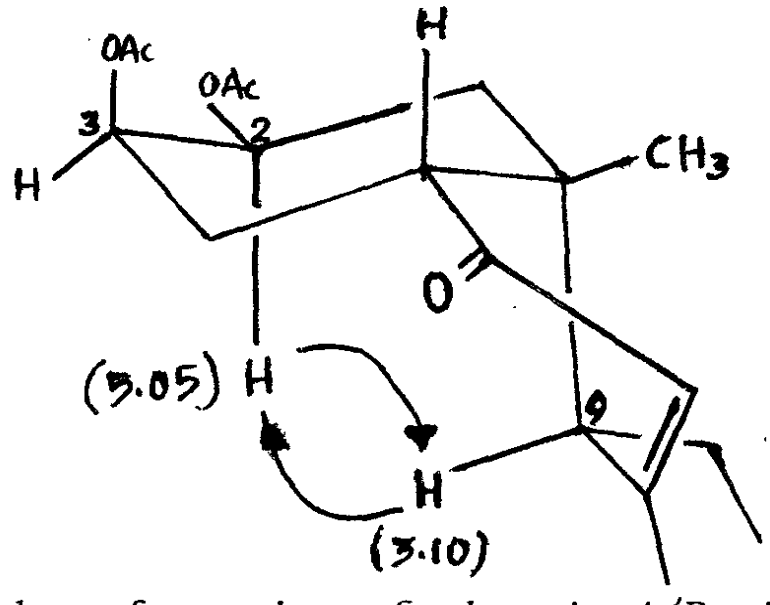
Spectroscopic data indicates that 1 is a hydroxylated steroid, with a conjugated system of double bonds.

The yield recorded in the isolation of 20 -**hydroxyecdysone** (3.2%) is the highest yield so far recorded for this moulting hormone from any natural source.



20 - Hydroxyecdysone

Identification of 20-hydroxyecdysone was based on its UV, IR, ^{1}H NMR and ^{13}C NMR and mass spectral data. Further confirmation was available from a study of the spectral data of its 2,3,22-triacetate and 2,3,22,25-tetraacetate (14,15). NOE studies confirm the assignments of the signals of the protons at positions 2,3,9 and 22. These studies indicate that the cis- A/B ring system in the triacetate adopts the same conformation in solution as in 20-hydroxyecdysone in the solid state (16). The alternative conformation for the cis-A/B ring system is of higher energy due to a 1,3-diaxial interaction between the acetoxyl group at C-2 and the methyl group at C-10. In the preferred conformation, H-2 and H-9 are situated close to each other, whereas they are much further apart in the conformation of higher energy. The observation of a positive NOE between H-2 (δ 5.05) and H-9 (δ 3.10) in the triacetate is evidence for its preferred conformation.

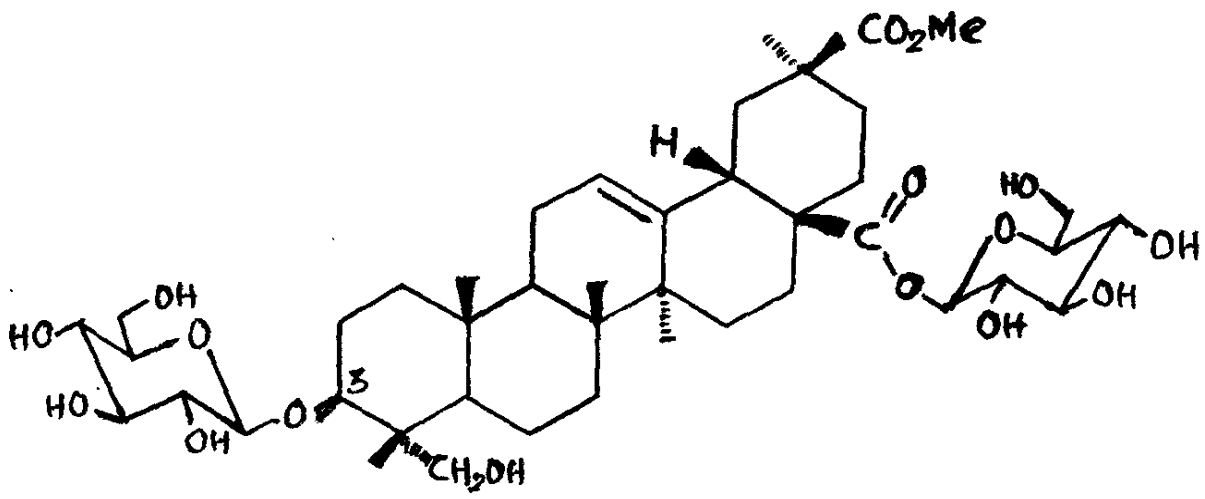


Preferred conformation of the cis-A/B ring system in the triacetate of 20-hydroxyecdysone.

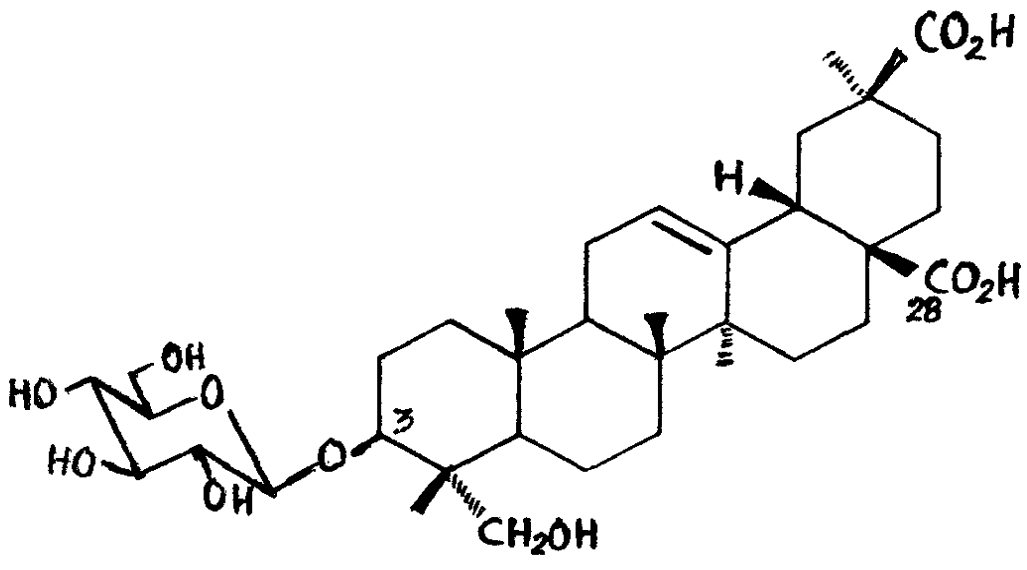
The presence of hydrophilic and hydrophobic parts in 20-hydroxyecdysone constitutes a structural similarity with saponins. Strongly positive froth and hemolysis tests are shown by 20-hydroxyecdysone as for saponins. However the compound shows no activity against B.glabrata up to a concentration of 50 ppm. Activity against C. cladosporioides is also shown by the compound. This activity is enhanced in both the triacetate and tetraacetate. Preliminary obsevations indicate moderate insecticidal activity (against Aphis craccivora).

Compound 2 showed strong absorption in its IR spectrum at 3350 (broad), 1730 and 1080 cm^{-1} (broad) characteristic of hydroxyl, ester and glycosidic units respectively. Hydrolysis of 2 with 4N HCl gave phytolaccagenic acid and D-glucose, as the only sugar. Compound 2 failed to react with diazomethane, showing the absence of a free carboxyl group and hence the attachment of D-glucose as a C-28 ester. Reaction of 2 with 0.5N KOH hydrolysed the C-28 ester as well as the C-30 ester giving the prosapogenin 3 as colourless crystals, mp 262°, $[\alpha]_D^{22}$ + 33° (c 0.15 in CH_3OH). The IR spectrum of 3 did

not indicate the presence of ester groups while the strong absorption at 1700 cm^{-1} indicated carboxyl. The prosapogenin 3 was evidently a dicarboxylic acid, since it reacted with diazomethane giving a product showing absorption for two carbomethoxyl groups (δ 3.62 and 3.66) in its ^1H NMR spectrum (C_5D_5N). Five hydroxyl groups were present in compound 3 since its acetylation product showed the presence of five acetate groups (δ 2.01, 2.02, 2.10, 2.11 and 2.24) in its ^1H NMR spectrum (C_5D_5N). Compound 2 showed two anomeric doublets (δ 5.12 and 6.29, J = 7.8 Hz) whereas compound 3 showed only one anomeric doublet (δ 5.17, J=7.6 Hz) in their ^1H NMR spectra (C_5D_5N). The coupling constants of the doublets indicated β-D-glucopyranosyl residues. Smaller coupling constants are observed for the anomeric protons of α-D-glucopyranosyl, α- and β-D-glucofuranosyl residues (17). Hence compound 2 is the bidesmosidic saponin, 3,28-di-O-β-D-glucopyranosyl-phytolaccagenic acid, while the prosapogenin 3 is 3-O-β-D-glucopyranosyl-esculentic acid. The dicarboxylic acid, esculentic acid (18) is the product of alkaline hydrolysis of phytolaccagenic acid.



3,28-di-O-β-D-glucopyranosylphytolaccagenic acid, 2



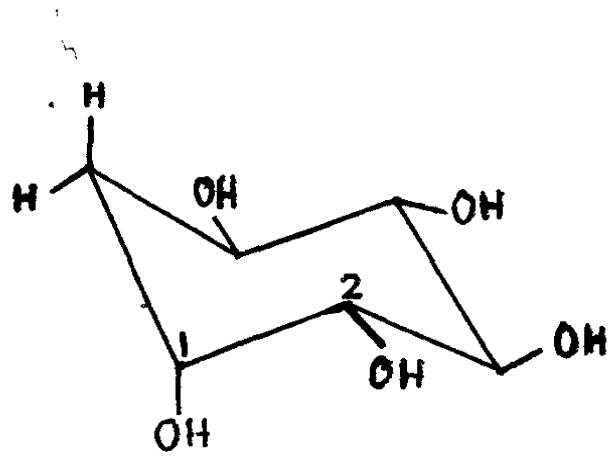
3-O-β-D-glucopyranosylesculentic acid, 3

The ^{1}H NMR and ^{13}C NMR spectra of the compound 2 and its nonaacetate provided further evidence for the structure assigned to 2. The ^{1}H NMR spectrum ($CDCl_3$) of the nonaacetate showed two anomeric doublets (δ 4.51 and 5.55, J = 7.8 Hz) as well as a broad multiplet (δ 5.0 to 5.25) integrating for six axial protons of the six CHOAc groups of two acetylated β-D-glucopyranosyl moieties.

The ^{13}C NMR spectrum (C_5D_5N) of compound 2 showed signals for C-23(δ_c 64.77) and the two C-6' atoms of the β-D-glucopyranosyl moieties (δ_c 61.63 and 61.97). The positions of the C-23 signals in compound 2 as well as its nonaacetate excluded the possibility of glycosylation through C-23. In such cases, the C-23 signals are known to appear at lower fields (ca. δ_c 80) (19). Glycosylation through C-3 and C-28 in compound 3 is evident from the signals at δ_c 82.19 and 175.97 respectively, in agreement with recorded data (20). The corresponding signals in the nonaacetate ($CDCl_3$) appear at δ_c 83.55 and 175.07 respectively. The anomeric carbon atoms attached to the genin through C-3 and C-28 appear, in agreement with recorded data, at δ_c 105.83 and 95.75 respectively (21). The corresponding signals in the nonaacetate appear at δ_c 102.59 and 91.53 respectively. Signals for C-2', C-3', C-4' and C-5' in each β-D-glucopyranosyl moiety of compound 2 agree well with recorded data (21).

The bidesmosidic saponin structure assigned to compound 2 is consistent with the observed lack of activity against B. glabrata up to a concentration of 50 ppm (22). Compound 2 gave a weakly positive hemolysis test. It showed no activity against the fungus C. cladosporioides.

Identification of vibo-quercitol (1,2,4/3,5-cyclohexanepentol) was based on a study of its physical properties as well as ^{1}H NMR and ^{13}C NMR spectra (23). Two dimensional ^{1}H NMR studies established the connectivities between the seven protons attached to carbon.



Vibo- quercitol (1,2,4/3,5-cyclohexanepentol)

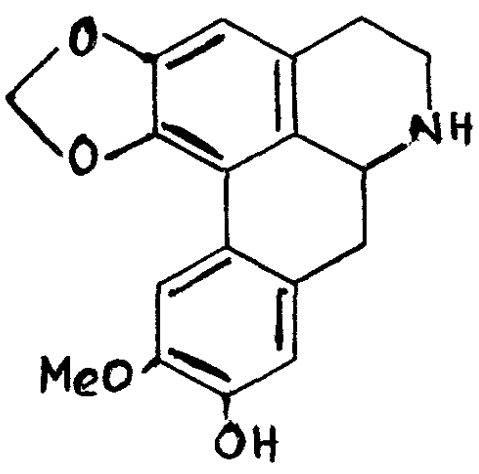
(b) (i) Litsea gardneri, endemic to Sri Lanka, is a tree of moderate size, growing in the wet lowland forests of the island. The plant has not been reported as yet for any medicinal activity. A preliminary survey has indicated the presence of trace quantitites of alkaloids in the leaves and twigs of the plant (8).

In this investigation, the bark of the plant (400 g) was defatted with petroleum ether. Evaporation of the extract gave a brown solid (4 g). The major component of this extract, inhibiting the growth of C. cladosporioides, has been isolated.

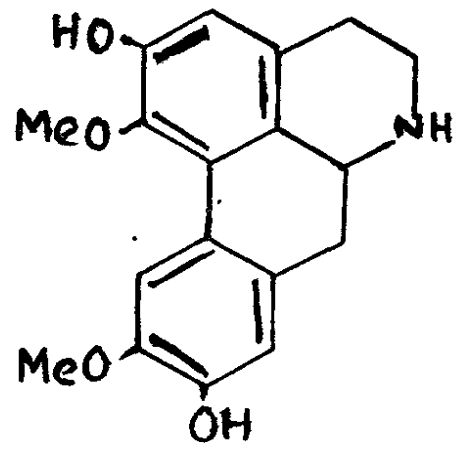
The bark residue from the petroleum ether extraction was basified with 5% NH_4OH and the alkaloids extracted with CH_2Cl_2.

Evaporation of the dichloromethane extract gave a brown solid (15 g) which was partitioned between 2N HCl and CH_2Cl_2. Basification of the acid extract with 20% NH_4OH, extraction with CH_2Cl_2 and evaporation gave a brown solid (700 mg).

Chromatographic separation of the bases (650 mg) gave actinodaphnine (30 mg) as a light brown amorphous solid, $[\alpha]_D^{22}$ + 8.7° (c 0.23 in EtOH) and laurolitsine (80 mg), also as a light brown amorphous solid, $[\alpha]_D^{22}$ + 75° (c 0.20 in EtOH).



Actinodaphnine



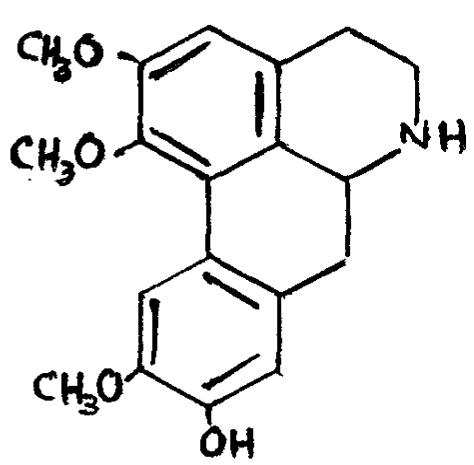
Laurolitsine

The identity of these bases was established on the basis of their specific rotations, UV, ^1H NMR and mass spectra (24). Further confirmation was available from a study of the specific rotations, UV, ^1H NMR and mass spectra of their acetyl derivatives, N,O-diacetylactinodaphnine (25) and N,O,O-triacetyllaurolitsine (26).

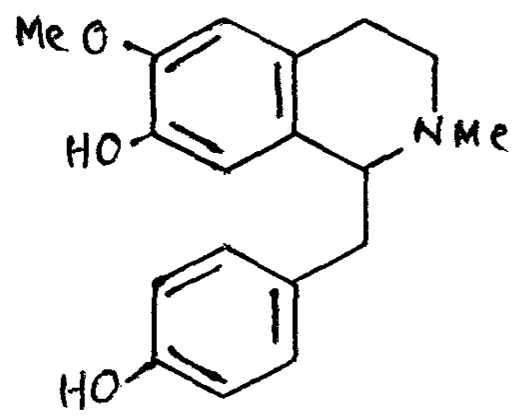
(b) (ii) Actinodaphne speciosa is endemic to Sri Lanka. It is a tree of moderate size growing in the forests of the upper montane zone of the island. This plant has not been reported as yet for any medicinal activity. A preliminary survey had indicated the presence of alkaloids in the leaves and twigs of the plant (8).

In this investigation, the leaves of the plant (800 g) were extracted with hot light petroleum and the residue extracted with methanol. Evaporation of the methanol yielded a dark green solid (104 g). This solid (100 g) was partitioned between 2N HCl and dichloromethane. The aqueous fraction was washed with dichloromethane and basified with 20% NH_4OH, and the bases extracted with dichloromethane. Evaporation of the solvent gave a light brown solid (478 mg).

Chromatographic separation of the bases (470 mg) gave the following compounds in order of increasing polarity: N-methyllaurotetanine (20 mg) as colourless crystals, mp 101°, $[\alpha]_D^{22}$ + 47° (c 0.13 in $CHCl_3$); a white amorphous solid (12 mg), tentatively identified as N-methylcoclaurine; laurotetanine (75 mg) as light brown crystals, mp 125°, $[\alpha]_D^{22}$ + 100° (c 0.12 in EtOH).



Laurotetanine



N-Methylcoclaurine

The identity of laurotetanine and N-methyllaurotetanine was established on the basis of their specific rotations, UV, ^1H NMR and mass spectra (27,28). The identity of laurotetanine was further confirmed by a spectral analysis of its N,O-diacetyl derivative (29). The identity of N-methyllaurotetanine was further confirmed by its synthesis from laurotetanine by N-methylation (30).

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Remarks:

1. Professor S.Sotheeswaran continued to collaborate on the project, even after his resignation from the University of Peradeniya, at the end of 1985.
2. Professor W. Kraus of the University of Hohenheim, West Germany has collaborated in the work described. He has provided considerable spectral data.
3. Dr. Diego Cortes of the University of Valencia ,Spain has also provided spectral data and collaborated in the work.
4. Dr. K. Hostettmann of the University of Lausanne, Switzerland and Dr. N.K.B.Adikaram are specially thanked for bioactivity determinations.
5. The C.I.S.I.R. has provided a few mass spectra.
6. Dr. B.M.R.Bandara left on sabbatical leave in February 1988.

The number of communications of the results of this work are as follow

(a) SLAAS 1984 (01)
1986 (01)
1987 (02)

(b) Institute of Chemistry : 1987 (01)

(c) 5th ASOMPS, Seoul, Korea, 1984 (01)

(d) Princess Congress, Bangkok, Thailand, 1987(01)

Signature of Grantees: Ramnigama Ekanayake

Signature of Head of Department: R. P. Gunawardana

HEAD
Department of Chemistry
University of Peradeniya.

Signature of Dean: 03 JUN 1988

Dean/Faculty of Science
University of Peradeniya

Signature of Vice-Chancellor: An excellent report 6/6/88

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