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Report

Sesquiterpene Lactones and Other Constituents from a Cytotoxic Extract of *Michelia floribunda*[1]

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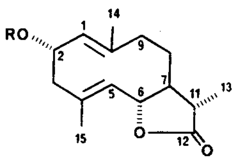
The pentane and $CHCl_3$ fractions of a crude extract of *Michelia floribunda* exhibited cytotoxic activity when tested in KB and P388 tumor cell cultures. Repeated chromatography led to the isolation of three cytotoxic sesquiterpene lactones (costunolide, parthenolide, and santamarine) and a cytotoxic isoquinoline alkaloid (liriodenine). Inactive sesquiterpene lactones obtained during the course of this study included dihydroparthenolide and two new glucosides of dihydrotamaulipin A and dihydroreynosin (1 and 2). The structures of these new compounds were determined through interpretation of their spectroscopic data including 2D-NMR spectroscopy. Syringin was also isolated from the extract.

KEY WORDS: *Michelia floribunda;* Magnoliaceae; cytotoxicity test; isolation; sesquiterpene lactones; dihydrotamaulipin A glucoside; dihydroreynosin glucoside.

INTRODUCTION

Continuing our search for potential antitumor agents from plant extracts, we have now examined *Michelia floribunda* Fin. & Gagnep. (Magnoliaceae), a small tree grown in the northern part of Thailand (1). A MeOH extract of the dried bark was fractionated by solvent partition; the pentane- and $CHCl_3$-soluble portions were found to be cytotoxic in both the KB nasopharyngeal carcinoma and the P388 murine leukemia cell lines. Chromatography of these active fractions afforded eight compounds, two of which were new sesquiterpene lactone glucosides (1 and 2) (Schemes I and II). To the best of our knowledge, there have been no previous biological or phytochemical investigations of *M. floribunda,* although some reports on other *Michelia* species are available in the literature. For example, crude extracts of *M. cathcartii* and *M. champaca* were found to have hypotensive effects (2–4), and those of *M. champaca, M. compressa,* and *M. kisopa* were reported to display cytotoxic activity (5–7). Chemically, *Michelia* species are known to contain sesquiterpene lactones and isoquinoline alkaloids (5,6,8–10). Essential oil compositions of some species have also been studied (11–13).



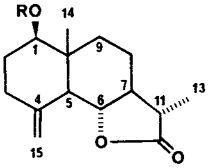
1 11β,13-Dihydrotamaulipin A β-D-glucoside
R = Glucose
3 11β,13-Dihydrotamaulipin A
R = H
4 Tamaulipin A
R = H, $\Delta^{11,13}$

Scheme I

MATERIALS AND METHODS

General Experimental Procedures

Melting points were determined using a Kofler hot-stage instrument and are uncorrected. The UV spectra were obtained with a Beckman DU-7 spectrophotometer, and IR spectra on a Nicolet MX-1 FT-IR spectrometer. NMR spectra (including 2D COSY and HETCOR spectra) were recorded on a Nicolet NMC-360 instrument, operating at 360 MHz for proton resonance and 90.8 MHz for carbon resonance. Low-resolution EI-MS was obtained with a Finnigan Model 4500 mass spectrometer, operating at 70 eV. FAB-MS was measured with a Finnigan MAT-90 mass spectrometer, using a glycerol matrix. Silica gel 60 (70-230 mesh, E. Merck) was used in all gravity chromatography, and silica gel (40-μm diameter, J. T. Baker) in flash chromatography. For



2 11β,13-Dihydroreynosin β-D-glucoside
R = Glucose
5 11β,13-Dihydroreynosin
R = H

Scheme II

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analytical and preparative TLC, Silica gel 60 F-254 precoated plates were obtained from E. Merck.

Plant Material

Stem bark of *M. floribunda* was collected in Thailand in 1985. Voucher specimens have been deposited at the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Solvent Extraction and Initial Chromatographic Separation

The powdered, air-dried stem bark (4.4 kg) was exhaustively extracted with 95% MeOH (ca. 20 liters) at room temperature. The MeOH extract was concentrated *in vacuo* to a syrup, taken up in MeOH–H_2O (1:2), and successively partitioned with pentane and $CHCl_3$. The $CHCl_3$ fraction (102 g; ED_{50}'s, 5.4 μg/ml in KB and 2.0 μg/ml in P388) was chromatographed on a column of silica gel (1.2 kg) eluting with $CHCl_3$–MeOH mixtures of increasing polarity. A total of 21 column fractions was subsequently obtained based on similar TLC profiles.

Isolation of Costunolide

From the pentane-soluble portion (85 g oil; ED_{50}'s, 22.5 μg/ml in KB and 19.4 μg/ml in P388), a colorless crystalline substance was obtained following partial removal of the solvent. The solid was collected by filtration and purified by preparative TLC ($CHCl_3$ as solvent) to afford costunolide (30 mg, 0.0007% yield from dried plant material) after repeated crystallization.

Isolation of Parthenolide, Dihydroparthenolide, and Santamarine

Column fraction 1 (5 g; eluted from $CHCl_3$; ED_{50}, 10.5 μg/ml in P388) was subjected to silica gel chromatography (100 g) eluting with petroleum ether–EtOAc (6:5). Subfractions 41–49 exhibited two major spots on TLC and were further purified by preparative TLC (toluene–ether, 7:3) to afford parthenolide (40 mg, 0.0009% yield) and dihydroparthenolide (15 mg, 0.0003% yield).

Another subfraction (89–106; ED_{50}, 7.8 μg/ml in P388) was also purified by preparative TLC ($CHCl_3$–MeOH, 98:2) to yield santamarine (35 mg, 0.0008% yield).

Isolation of Liriodenine

Column fraction 4 (1 g; eluted from $CHCl_3$–MeOH, 99:1; ED_{50}, 12.2 μg/ml in P388), following subsequent flash chromatography (40 g) eluted with $CHCl_3$–MeOH (98:2), afforded liriodenine (195 mg, 0.0044% yield).

Isolation of Syringin

Column fraction 14 (14 g; eluted from $CHCl_3$–MeOH, 85:15), although inactive in cytotoxicity assays, exhibited a major spot on TLC. It was therefore rechromatographed on a column (200 g) using $CHCl_3$–MeOH (85:15) as solvent. Syringin (198 mg, 0.0044% yield) was obtained following further purification by preparative TLC ($CHCl_3$–MeOH, 8:2).

Isolation of Dihydrotamaulipin A β-D-Glucoside (1) and Dihydroreynosin β-D-Glucoside (2)

Column fraction 13 (14 g; eluted from $CHCl_3$–MeOH, 95:5) was further separated on a silica gel column (250 g) eluted with $CHCl_3$–MeOH (85:15). Repeated chromatography of subfraction 14 (eluted from EtOAc–MeOH, 8:2) resulted in a semipurified substance. Final purification by another column (hexane–iPrOH, 4:6) afforded a pure solid, 1 (67 mg, 0.0015% yield); mp 213–215°C; UV (EtOH) λ_{max}210 nm end absorption; IR (KBr) ν_{max}1660, 1780, 3475 cm^{-1}; ^1H-NMR and ^{13}C-NMR, see Table I; FAB-MS (negative ion, glycerol matrix) *m/z* 823 $[2M\text{-}1]^-$, 411 $[M\text{-}1]^-$; EI-MS *m/z* 250 $[M^+\text{-}C_6H_{10}O_5]$, 232 $[M^+\text{-}C_6H_{12}O_6]$.

Subfraction 13, obtained following separation of the initial column fraction 13 on a column as described above, was shown by TLC to contain one major component. It was then purified on a short column (30 g) eluting with hexane–iPrOH (4:6) to yield compound 2 (20 mg, 0.0005% yield); mp 125–129°C; IR (KBr) ν_{max}890, 1760, 3465 cm^{-1}; ^1H-NMR and ^{13}C-NMR, see Table I; FAB-MS (negative ion, glycerol

Table I. ^1H and ^{13}C-NMR Data for Compounds 1 and 2

	Carbon chemical shift (δ, ppm)		Proton chemical shift (δ, ppm)	
Position	1[a]	2[a]	1[b]	2[b]
1	141.5	84.3	5.05, d (10)	3.7, dd (11, 5)
2	75.1	28.4	5.23, ddd (10, 10, 5.5)	1.5–2.1, m
3	47.5	34.5	2.68, dd (11, 5) 2.25, m	1.9–2.3, m —
4	130.6*,[c]	145.4	—	—
5	142.5	53.6*	4.87, d (10)	2.13, d (10)
6	83.0	81.0	4.57, t (10)	4.17, t (10)
7	56.2	54.0*	1.6, m	1.7, m
8	29.1	23.8	1.5–1.7, m	1.6–1.8 m
9	42.2	37.1	2.21, m 1.97, br t (11)	1.3, m 2.1, m
10	129.5*	43.4	—	—
11	43.2	42.1	2.35, dq (12, 7)	2.44, dq (12, 6)
12	180.1	182.2	—	—
13	13.3	12.7#	1.23, d (7)	1.16, d (6)
14	17.3	12.9#	1.53, s	0.88, s
15	18.5	109.9	1.42, s	4.77, br s 4.92, br s
1′	101.9	101.6	5.08, d (8)	4.32, d (6)
2′	75.3	75.0	4.1, m	3.13, dd (7, 6)
3′	78.1 ^	77.9 ^	4.3, m	3.3, m
4′	71.8	71.8	4.2, m	3.6, m
5′	78.2 ^	78.1 ^	3.9, m	3.2, m
6′	62.9	63.0	4.6, m 4.4, dd (12, 6)	3.6, m 3.8, m

[a] Solvents: pyridine-d_5 for 1 and CD_3OD for 2. Assignment was aided by single-frequency off-resonance and attached proton test experiments.

[b] Solvents: pyridine-d_5 for 1 and CD_3OD for 2. Assignment was aided by $^1H,^1H$-COSY and $^1H,^{13}C$-HETCOR (for 1 only) experiments. Coupling constants (*J* values in Hz) are given in parentheses.

[c] (*,#, ^) Assignments may be interchanged.

matrix) *m/z* 823 [2M-1]$^-$, 411 [M-1]$^-$; EI-MS *m/z* 233 [M$^+$-$C_6H_{11}O_6$].

Enzymatic Hydrolysis of 1 and 2

To an aqueous acetate buffer solution (pH 5) of 1 or 2 (5 mg), β-glucosidase (2–3 mg) was added, and the mixture kept at 37°C overnight and then extracted with EtOAc. Purification of the crude product by chromatography on silica gel ($CHCl_3$–MeOH) afforded 3 (from 1) and 5 (from 2). The water layer was analyzed on a cellulose TLC plate developed in pyridine–EtOAc–HOAc–H_2O (36:36:7:21), to compare with standard sugars. Glucose was detected in both mixtures.

Bioassays for Cytotoxic Activity

In vitro cytotoxicity assays were performed using KB and P388 tumor cell lines as described in the literature (14–16).

RESULTS AND DISCUSSION

Fractionation of a MeOH extract of the stem bark of *M. floribunda* was performed by extracting successively with pentane and $CHCl_3$. Biological evaluation of these fractions for *in vitro* cytotoxicity in both the KB and P388 tumor cell lines indicated that the strongest activity was located in the $CHCl_3$-soluble portion, while the pentane fraction was weakly active.

Costunolide was obtained from the pentane fraction by crystallization. Chromatography of the $CHCl_3$ fraction on silica gel led to the isolation of seven metabolites, including five known compounds, (parthenolide, dihydroparthenolide, santamarine, liriodenine, and syringin) and two new sesquiterpene lactone glucosides, 1 and 2. All of the known compounds except syringin have been reported from *Michelia* species including *M. compressa* (6), *M. lanuginosa* (10), and *M. champaca* (5). They were identical in all respects (co-TLC, NMR, MS) to authentic samples available in our laboratories. Syringin was obtained for the first time from the genus *Michelia*. Its identity was established by comparing its UV, IR, NMR, and MS spectra with those published in the literature (17).

The new compound 1 was obtained as an amorphous powder, mp 213–215°C. The ^{13}C-NMR spectrum (Table I) displayed 21 carbon signals, 6 of which were readily assignable to a glucose moiety. The FAB-MS spectrum gave a [M-1]$^-$ signal at *m/z* 411, analyzing for a molecular formula of $C_{21}H_{32}O_8$(M^+ 412) for the compound. The presence of a glucose moiety was substantiated by two prominent mass fragment peaks in the EI-MS spectrum at *m/z* 250 (M^+-$C_6H_{10}O_5$) and 232 (M^+-$C_6H_{12}O_6$). The compound was therefore a sesquiterpene glucoside.

A standard APT pulse sequence was employed to delineate the carbon resonances. The aglycone portion of the molecule composed of three methyl, three methylene, six methine (two of which were oxygen-bearing and two were olefinic), two olefinic quaternary carbons, and one carbonyl carbon. An IR signal at 1780 cm^{-1} indicated the presence of a gamma-lactone. These features were consistent with a dihydrogermacranolide structure.

In the ^{1}H-NMR spectrum (Table I), three methyl signals were observed. One of them, a doublet (J = 7 Hz) at 1.23 ppm, is typical of the 13-Me on the lactone ring. It was clearly coupled to a doublet of quartet at 2.35 ppm (H-11) in the ^{1}H,^{1}H-COSY spectrum. The other two methyl protons (14- and 15-Me), when examined in the COSY spectrum, were found to display long-range correlation signals with olefinic protons at 5.05 and 4.87 ppm, respectively. The latter signals were therefore H-1 and H-5. The doublet at 4.87 ppm was subsequently assigned to H-5 through careful examination of the COSY spectrum. Thus, this signal was found to couple with H-6 (4.57 ppm), which in turn was coupled to an upfield aliphatic signal at 1.6 ppm (H-7). The H-7 multiplet was also coupled to H-11 and two methylene protons (8-CH_2). It followed that the signal at 5.05 ppm must be H-1.

In the region of 3.5–5.5 ppm of the ^{1}H-NMR spectrum, several other signals were readily assignable to protons belonging to a glucose moiety by an examination of the ^{1}H,^{13}C-HETCOR spectrum. The only proton signal which remained unassigned in this region was a multiplet at 5.25 ppm. This must be the methine proton attached to the carbon bearing the glucose. Careful examination of this proton signal in the COSY spectrum indicated that it gave rise to three correlation signals. Two of these peaks were related to two methylene protons (at 2.25 and 2.68 ppm). The third correlation signal was found to be related to H-1 (5.05 ppm), suggesting that the sugar portion was attached on C-2, and the methylene signals at 2.25 and 2.68 ppm belonged to 3-CH_2.

The stereochemistry at C-2, C-6, and C-11 was inferred from the coupling information. Thus, a large coupling constant between H-2 and H-1 (J = 10 Hz) is consistent with a β orientation of H-2 in 2-substituted germacranolides (18–20). The H-6 lactone proton, coupled with both H-5 and H-7 with J values of 10 Hz, was typical for a *trans*-lactone. On the other hand, a large J value of 12 Hz between H-7 and H-11 also indicated a diaxial relationship. The 13-Me was therefore assigned to an equatorial position.

The structure of compound 1 was thus determined to be 11β,13-dihydrotamaulipin A β-D-glucoside. Enzymatic hydrolysis of 1 afforded glucose and dihydrotamaulipin A (**3**), whose ^{1}HNMR spectrum was almost identical to that of 1, with the exception that the glucose protons were absent and the H-2 was shifted upfield (4.65 ppm). Such spectral data were also comparable to the published spectrum (obtained at 60 MHz) of dihydrotamaulipin A (21). Tamaulipin A (**4**) has been previously reported from *Ambrosia confertiflora* (21) and was found to be cytotoxic (22).

Compound 2, mp 125–129°C, had the same molecular formula as 1 ($C_{21}H_{32}O_8$) as suggested by both the FAB-MS and the ^{13}C-NMR spectra. The ^{13}C-NMR spectrum revealed six carbon signals of a glucose moiety. The rest of the spectrum indicated that the aglycone part contained, contrary to 1, only two methyl groups and no olefinic carbons besides an exocyclic methylene (which was also revealed from an IR peak at 890 cm^{-1}). The compound was, therefore, concluded to be a tricyclic sesquiterpene lactone.

In the ^{1}H-NMR spectrum, a prominent feature in the upfield region was two methyl signals, one of which being a doublet at 1.16 ppm (J = 6 Hz) coupled to a doublet of quartet at 2.44 ppm. This was characteristic for the 13-Me and H-11 of a sesquiterpene lactone. Most other signals in this region were overlapped and unresolved. The downfield region (3.0–5.5 ppm), however, revealed several well-defined signals. The exocyclic methylene protons could be readily identified at 4.77 and 4.92 ppm. All signals due to the glucose moiety were also easily assigned through a COSY spectrum. A methine signal appearing as a triplet at 4.17 ppm ($J = 10$ Hz) was typical for H-6 of a sesquiterpene *trans*-lactone. It was coupled to two upfield signals at 1.7 and 2.13 ppm. The multiplet at 1.7 ppm could be ascribed to H-7 on the basis of its coupling to the H-11 doublet of quartet. It followed that the signal at 2.13 ppm was due to H-5. Since correlation signals were observed clearly between H-5 and the two exocyclic methylene protons, the latter could be assigned to H_2-15. All this information strongly suggested that the compound is a glucoside of simple dihydroeudesmanolide type.

Enzymatic hydrolysis of **2** afforded glucose and dihydroreynosin (**5**), whose identity was established by a direct comparison with an authentic sample (co-TLC, MS). Compound **2** was therefore determined to be 11β,13-dihydroreynosin β-D-glucoside. Spectroscopic data of the aglycone portion were agreeable with those published for 11β,13-dihydroreynosin (**5**) (23). The couplings of H-1 clearly indicated that the proton is in an axial orientation.

All isolates were evaluated for cytotoxic activity in both KB and P388 systems. The cytotoxic properties of sesquiterpene lactones such as costunolide, parthenolide, santamarine and liriodenine have been well documented (5,6,22, 24–26), and they were all active in both cell lines (ED_{50}'s, <2.5 μg/ml). As expected, the dihydro derivatives (including **1** and **2**) and syringin were devoid of any significant cytotoxicity.

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