Activity of Triterpenoid Glycosides from the Root Bark of *Mussaenda macrophylla* against Two Oral Pathogens

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Four new triterpenoid glycosides were isolated from the root bark of *Mussaenda macrophylla*. Their structures were determined as 3-*O*-*β*-D-glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-16α-hydroxy-23-deoxyprotobassic acid (**1**), 28-*O*-*β*-D-glucopyranosyl-16α-hydroxy-23-deoxyprotobassic acid (**2**), 3-*O*-*β*-D-glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-16α-hydroxyprotobassic acid (**3**), and 3-*O*-{[*β*-D-glucopyranosyl-(1→6)]-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-*β*-D-glucopyranosyl-(1→2)}-*O*-*β*-D-glucopyranosyl-(1→3)-*O*-*β*-D-glucopyranosyl-cycloarta-22,24-dien-27-oic acid (mussaendoside W, **4**). Four known triterpenoids [3-*O*-acetyloleanolic acid (**5**), 3-*O*-acetyldaturadiol (**6**), rotundic acid (**7**), and 16α-hydroxyprotobassic acid (**8**)] were also isolated. The structures of **1**−**4** were determined by several spectroscopic techniques including 2D NMR methods. Compounds **1**−**6** showed inhibitory activity against a periodontopathic bacterium, *Porphyromonas gingivalis*, but were inactive against the cariogenic organism, *Streptococcus mutans*.

Mussaenda macrophylla Wall. (Rubiaceae) is a shrub with hairy branches that has attractive flowers and is called "dhobini" by local populations in Nepal.[1] Various parts of the plant have been used to treat persons with sore mouths, sore throats, and fevers.[2] The plant came to our attention because the fruits and root bark are reputed to have a sweetness-inducing effect when water is tasted. To date, there have been no reports in the literature on either the biological activity or phytochemistry of *M. macrophylla*. However, other species in this genus have afforded flavonoids;[3,4] phenylpropanoids;[3,4] and cycloartane-,[5−10] oleanane-,[9,11,12] and ursane-type triterpenoids.[10−12]

In the present investigation, the initial crude extracts and several purified constituents of the root bark of *M. macrophylla* have been evaluated against two oral pathogenic bacteria, *Streptococcus mutans* and *Porphyromonas gingivalis*, in response to the folkloric use of this plant for treating sore mouths.[2] Four new triterpenoid glycosides (**1**−**4**) were isolated and structurally determined from a 1-butanol-soluble extract of *M. macrophylla* root bark, and four triterpenoids of previously known structure (**5**−**8**) were obtained from petroleum ether- and EtOAc-soluble extracts of this same plant part (Chart 1). Compounds **1**−**6** were obtained in sufficient quantities to perform antimicrobial assays conducted as part of this study.

Results and Discussion

Both the FABMS and ESMS of **1** showed a pseudomolecular ion peak at *m*/*z* 809 $[M - H]^-$. From this information, together with an analysis of the ^{13}C NMR spectrum, it was apparent that this isolate was a triterpene glycoside containing two sugar units. The presence of an olefinic proton at δ_H 5.53 (H-12), which corresponded to the signal at δ_C 126.5 (C-12) in the HMBC NMR spectrum, and of seven methyl groups in the ^1H NMR spectrum of **1** were consistent with the aglycon being a substituted Δ^{12}-oleanane-type triterpene.[13] The ^{13}C NMR signals at C-2 (δ_C 70.8), C-6 (δ_C 67.2), and C-16 (δ_C 75.6), with respect to analogous data for oleanolic acid, suggested that these positions are substituted in **1**.[14] Two anomeric protons were observed at δ_H 6.17 and 4.90, which corresponded, in turn, to the signals at δ_C 95.7 and 106.6, and indicated the presence of two sugar units. Acid hydrolysis of **1** with 2 N HCl gave glucose and rhamnose, which were identified by GC and TLC comparison with authentic sugar samples. The anomeric proton resonance at δ_H 4.90 correlated with the glucosyl H-6′ signal at δ_H 4.39 in the TOCSY experiment,[15] indicating that this sugar unit was glucose. In the HMBC spectrum, the anomeric proton signal at δ_H 4.90 also correlated with the signal at δ_C 89.6 (C-3), which suggested that the glucose unit was connected to the C-3 hydroxyl group. The rhamnosyl anomeric proton (δ_H 6.18) exhibited a three-bond correlation with the C-28 carbonyl carbon resonance (δ_C 176.8). The rhamnosyl C-1″ signal at δ_C 95.7 indicated that this anomeric moiety was connected directly to the carboxylic acid substituent of the aglycon. The connectivity of the glucose unit and the stereochemistry at the C-3 position were confirmed by a ROESY experiment,[16] where a correlation was observed between the glucosyl anomeric proton (δ_H 4.90) and Hβ-3 at δ_H 3.39. Moreover, the ^1H NMR coupling constant of H-3 (J = 12.0 and 4.8 Hz) confirmed that the stereochemistry of C-3 was in the β position.[17] Thus, on analysis of all of the above information and by comparison of spectral data with similar compounds in the literature,[14] the structure of **1** was assigned as 3-*O*-*β*-D-glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-16α-hydroxy-23-deoxyprotobassic acid.

A molecular ion peak of **2** at *m*/*z* 666 in the negative-ion ESMS and ^{13}C NMR data enabled an elemental formula of $C_{36}H_{58}O_{11}$ to be deduced and reflected the fact that only one sugar unit was attached to the aglycon when compared with **1**. This turned out to be glucose after acid hydrolysis on a TLC plate. The ^1H and ^{13}C NMR spectra of **2** were supportive of this glycoside having the same aglycon as found in **1**. The location of the glucose unit was determined from the ^{13}C NMR spectrum and by a HMBC experiment.

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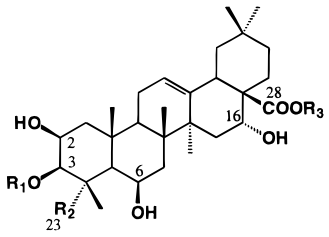
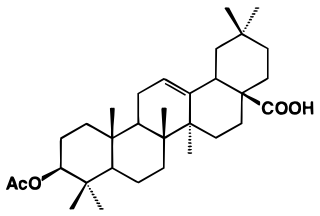
Chart 1



	R_1	R_2	R_3
1	β-glc	CH_3	α-rha
2	H	CH_3	β-glc
3	β-glc	CH_2OH	α-rha



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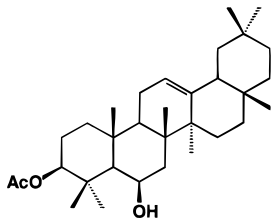

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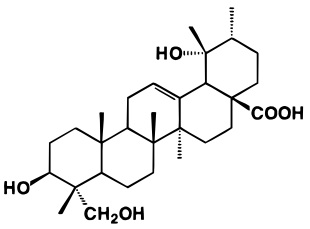

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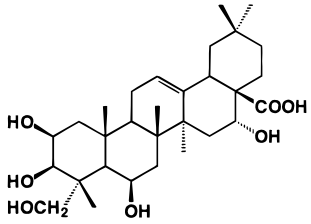

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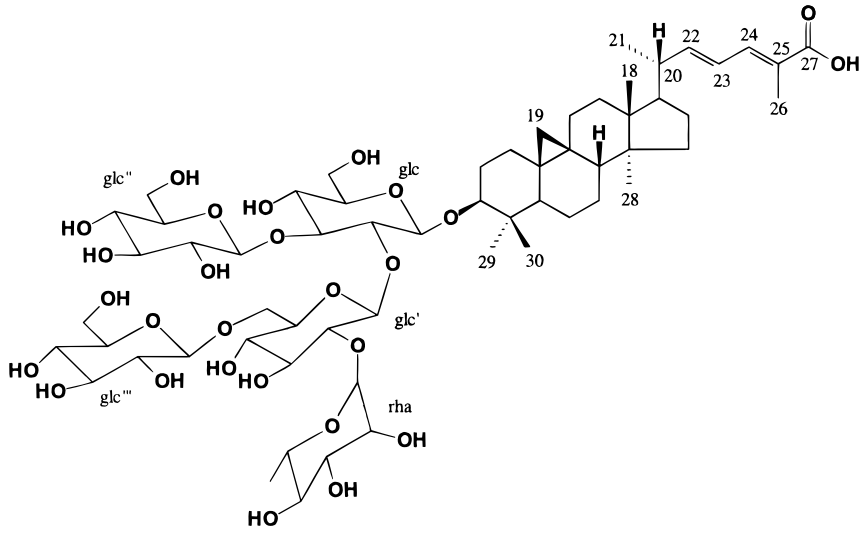

4

In the ^{13}C NMR spectrum, only one anomeric carbon signal with a chemical shift at δ_C 95.8 was observed, which suggested the attachment of the sugar unit to the carboxylic acid unit (C-28). The HMBC spectrum showed a correlation between the glucosyl anomeric proton at δ_H 6.31 and the carbonyl carbon of C-28 at δ_C 176.4, while there was no correlation with C-3 as found in **1**. Thus, the structure of **2** was assigned as 28-*O*-*β*-D-glucopyranosyl-16α-hydroxy-23-deoxyprotobassic acid.

The ^1H and ^{13}C NMR spectra of **3** were similar to analogous data for **1** and **2**. However, six methyl groups could be assigned to the aglycon, and the chemical shifts at δ_H 4.69 and 3.99 (H-23) in the ^1H NMR spectrum and at δ_C 68.6 (CH_2) in the ^{13}C NMR spectrum indicated that the C-23 position was hydroxylated. A HMBC correlation was observed between one of the hydroxylated methylene protons (δ_H 4.69, H-23) and the C-24 methyl carbon (δ_C 16.6). The FABMS pseudomolecular ion peak at *m*/*z* 825, $[M - H]^-$, when compared with that of **1**, also suggested the presence of one additional hydroxyl group in the molecule of **3** relative to **1**. Acid hydrolysis on the TLC plate of **3** confirmed the presence of glucose and rhamnose as in **1**. HMBC correlations between the glucosyl anomeric proton (δ_H 4.94) with C-3 of the aglycon (δ_C 89.0) and between the rhamnosyl anomeric proton (δ_H 6.22) and the C-28 carboxylic acid functionality of the aglycon (δ_C 176.2) indicated that the sugar linkages were the same as in **1**. Thus, the structure of **3** was identified as 3-*O*-*β*-D-glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-16α-hydroxyprotobassic acid.

An elemental formula of $C_{60}H_{96}O_{27}$ was suggested for **4** from its FABMS (both positive- and negative-ion) and APT ^{13}C NMR data. In the ^1H NMR spectrum, two characteristic doublets appearing at δ_H 0.31 and 0.53 indicated that the aglycon included a cyclopropyl ring and hence might be a cycloartane-type triterpene.[18] In the ^{13}C NMR spectrum, four olefinic carbons at δ_C 147.9, 134.6, 128.9, and 123.4 were observed and gave evidence for the presence of two double bonds. A characteristic CH_3-21 doublet proton at δ_H 1.08 (J = 6.2 Hz), which is common in the side chain of cycloartane-type triterpenes, was observed.[10] This proton was long-range correlated with one of the olefinic carbons

Table 1. ^{13}C and ^{1}H NMR Data and HMBC Correlations for Mussaendoside W (**4**) (125 and 500 MHz, C_5D_5N)

position	δ_C[a]	δ_H, mult. (*J* in Hz)	HMBC (H→C)
1	32.3 t	1.54, 1.20 m	
2	29.7 t	2.37 m	
3	90.4 d	3.43 dd (12.2, 4.1)	
4	41.4 s		
5	48.0 d	1.53 m	
6	21.2 t	1.55 m	
7	26.3 t		
8	47.7 d	1.31 m	
9	19.9 s		
10	26.2 s		
11	26.6 t		
12	33.0 t	1.60 m	
13	45.6 s		
14	49.2 s		
15	35.0 t		
16	28.8 t		
17	52.0 d	1.70 m	
18	18.5 q	1.04 s	12
19	29.9 t	0.31, 0.53 br s	
20	41.3 d	2.19 m	24, 25
21	20.0 q	1.08 d (6.2)	17, 22
22	147.9 d	5.93 dd (14.4, 9.0)	24
23	123.4 d	6.39 dd (14.3, 11.4)	4
24	134.6 d	7.46 d (10.9)	
25	128.9 s		
26	13.1 q	2.19 s	24, 25, 27
27	168.7 s		
28	19.5 q	0.96 s	13, 15
29	26.0 q	1.31 s	
30	15.5 q	1.20 s	3, 8, 29
Glc-1	105.1 d	4.91 d (7.6)	3
Glc-2	77.7 d	4.62 m	
Glc-3	79.0 d	4.14 m	
Glc-4	71.1 d	3.75 m	
Glc-5	75.0 d	4.01 m	
Glc-6	62.0 t	4.55 m	
Glc-1′	101.4 d	5.58 d (7.7)	Glc-2
Glc-2′	80.5 d	4.38 m	
Glc-3′	77.9 d	4.30 m	
Glc-4′	77.6 d	4.25 m	
Glc-5′	74.8 d	3.99 m	
Glc-6′	72.0 t	4.62, 4.73 m	
Glc-1″	104.6 d	5.27 d (7.8)	Glc-3
Glc-2″	75.9 d	4.03 m	
Glc-3″	77.7 d	4.51 m	
Glc-4″	71.2 d	4.15 m	
Glc-5″	78.6 d	4.04 m	
Glc-6″	62.3 t	4.39 m	
Glc-1‴	104.3 d	5.14 d (7.8)	Glc-6′
Glc-2‴	76.9 d	4.00 m	
Glc-3‴	78.1 d	4.22 m	
Glc-4‴	78.0 d	4.32 m	
Glc-5‴	77.6 d	4.03 m	
Glc-6‴	62.5 t	4.25 m	
Rha-1	101.9 d	6.16 br s	Glc-2′
Rha-2	72.2 d	4.77 m	
Rha-3	72.3 d	4.72 m	
Rha-4	74.0 d	4.35 m	
Rha-5	69.4 d	4.78 m	
Rha-6	18.9 q	1.84 d (6.0)	

[a] Multiplicities determined from the APT ^{13}C NMR spectrum.

of the side chain at δ_C 147.9 (C-22) from the HMBC spectrum (Table 1). The H-22 signal at δ_H 5.93 showed a long-range correlation with another olefinic carbon at δ_C 134.6 (C-24), and the CH_3-26 signal at δ_H 2.19 correlated with two olefinic carbons at δ_C 134.6 (C-24) and 128.9 (C-25). The position of the carbonyl carbon of the carboxylic acid was also determined from the HMBC spectrum, from a cross-peak resonance observed between the methyl protons at CH_3-26 (δ_H 2.19) and the C-27 carbonyl carbon (δ_H 168.7). From these correlations, the side chain structure was determined as having a conjugated double bond and one terminal carboxylic acid group. As a result of these observations and by comparison of spectral data with a model compound,[10] the aglycon structure of **4** was assigned as cycloarta-22,24-dien-27-oic acid. The saccharide composition of **4** was identified by GC and TLC, and the sugars were identified as glucose and rhamnose. In the ^{1}H NMR spectrum of **4**, five anomeric proton signals at δ_H 4.91, 5.58, 5.27, 5.14, and 6.16 were observed, corresponding to signals at δ_C 105.1, 101.4, 104.6, 104.3, and 101.9, respectively, and indicating that **4** possesses five sugar units. Four anomeric protons (δ_H 4.91, 5.58, 5.27, and 5.14) showed β-glycosidic linkages according to the coupling constants of their anomeric protons (J = 7.6−7.8 Hz).[17] A characteristic rhamnosyl CH_3-6 proton doublet signal (δ_H 1.86, J = 6.0 Hz) was observed.[17] From the $^{1}H-^{1}H$ COSY and TOCSY spectra, all proton signals belonging to each sugar moiety in **4** were identified, starting from the anomeric protons. All sugar connectivities were established using NOESY and HMBC experiments. In the NOESY spectrum, cross-peak signals were observed between H_{Glc-1} and H-3, $H_{Glc-1'}$ and H_{Glc-2}, $H_{Glc-1''}$ and H_{Glc-3}, H_{Rha-1} and $H_{Glc-2'}$, and $H_{Glc-1'''}$ and $H_{Glc-6'}$. The HMBC experiment showed long-range correlations between H_{Glc-1} and C-3, $H_{Glc-1'}$ and C_{Glc-2}, $H_{Glc-1''}$ and C_{Glc-3}, H_{Rha-1} and $C_{Glc-2'}$, and $H_{Glc-1'''}$ and $C_{Glc-6'}$ (Table 1). Thus, the structure of **4** was assigned as 3-*O*-{[β-D-glucopyranosyl-(1→6)]-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)}-*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-cycloarta-22,24-dien-27-oic acid. In accordance with a previous convention in the naming of cycloartane glycosides from *Mussaenda* species,[5−10] compound **4** has been assigned the trivial name, mussaendoside W.

The known compounds **5**−**8** were identified as 3-*O*-acetyloleanolic acid,[19,20] 3-*O*-acetyldaturadiol,[21] rotundic acid,[22] and 16α-hydroxyprotobassic acid,[11,14] by data comparison with literature values.

In an antimicrobial screen against two oral pathogens, compounds **1**−**6** were found to inhibit the growth of *P. gingivalis* (Table 2), the Gram-negative anaerobic oral bacterium most commonly associated with human gum disease. Among these, compounds **3**, **5**, and **6** were either equally potent to or more potent than their respective crude extract of origin. The EtOAc and 1-butanol extracts were more active than the petroleum ether and aqueous extracts. None of the plant extracts or pure isolates was active against *S. mutans* (all exhibited MIC values of >1250 μg/mL), a Gram-positive facultative anaerobic coccus that is the etiologic agent of human dental caries. The differential antimicrobial activity observed against *P. gingivalis* suggests that compounds **3**, **5**, and **6** may benefit periodontal health. Because the fruits and roots of *M. macrophylla* have been used traditionally to treat sore mouths in Nepal,[2] the

Table 2. Antimicrobial Activity (MIC values) of Extracts of *M. macrophylla* and Compounds **1**−**6** against *P. gingivalis*[a]

extract/compound	MIC (μg/mL)
petroleum ether extract	312
EtOAc extract	156
1-BuOH extract	156
aqueous extract	312
1	312
2	312
3	78
4	156
5	39
6	78
chlorhexidine[b]	0.312

[a] For assay protocols, see Experimental Section. [b] Positive-control substance.

observed antimicrobial activity of **1**−**6** adds some validity to this ethnomedical use. However, whether these active compounds exert any antiviral affects awaits further testing. Previously, several compounds of plant origin have shown activity against oral pathogens.[23,24] A triterpenoid, oleanolic acid, and some flavonoids, including kaempferol, myricetin, and rhamnocitrin, inhibited the growth of *P. gingivalis*, with the MIC value of oleanolic acid isolated from *Syzygium aromaticum* being 625 μg/mL. In the present investigation, the activity of 3-*O*-acetyloleanolic acid (**5**) against *P. gingivalis* was about 20 times more potent (39 μg/mL) than oleanolic acid, thereby demonstrating an enhancement of activity as a result of the substitution of a more nonpolar functionality in **5** when compared with the parent triterpene acid.

Although chlorhexidine has a considerably lower MIC than the compounds identified in this study (0.312 μg/mL for *P. gingivalis*, Table 2), its side effects, such as teeth staining and taste alteration, have been well documented. We believe that plant-derived antimicrobial compounds may serve as alternatives to the commonly used chemicals for dental plaque and disease control.

Experimental Section

General Experimental Procedures. Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin−Elmer model 241 polarimeter at 25 °C. UV spectra were recorded on a Beckman DU-7 spectrometer. IR spectra were measured on ATI Mattson Genesis series FT−IR spectrometer. ^{1}H, ^{13}C NMR, APT, ^{1}H−^{1}H COSY, HMQC, HMBC, NOESY, ROESY, and TOCSY spectra were recorded on either a Bruker DPX 300 NMR spectrometer or a Bruker DRX 500 NMR spectrometer with TMS used as internal standard. FABMS and EIMS were recorded on a Finnigan MAT-90 instrument. ESMS were recorded on Hewlett−Packard 5989B mass spectrometer with a Hewlett−Packard 59987A electrospray interface. Semipreparative HPLC was carried out on a Waters 600 controller attached to a YMC ODS-AQ Pack (250 × 20 mm i.d., S-5 μm, 120 Å; YMC, Inc., Wilmington, NC) column, and a YMC ODS-AQ Guard Pack (50 × 20 mm i.d., S-5 μm, 120 Å) guard column was used. The peaks were detected at 210 nm using a Waters 996 photodiode array detector. Gas chromatography was carried out on a Varian model 3400 gas chromatograph using a DB-1 fused silica capillary column (30 m × 0.32 mm i.d.; 0.25 μm film thickness; J&W Scientific, Folsom, CA). The column was held at the ion source temperature of 120 °C for 1 min then programmed at 10 °C/min to 270 °C. The injector and detector temperatures were maintained at 220 and 280 °C, respectively. Silylated sugar derivatives were prepared from trimethylsilyl chloride in pyridine using Sigma-Sil-A (Sigma Chemical Co., St. Louis, MO).

Plant Material. The root bark of *Mussaenda macrophylla* was collected near the village of Chamdilla, Lamjung District, Nepal, in July 1997. The plant was identified by one of us (A.E.D.), and a voucher specimen has been deposited at the Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL.

Test Organisms. Two organisms, *S. mutans* IB and *P. gingivalis* (ATTC33277), representative of a cariogenic and a periodontopathic oral pathogen, respectively, were chosen for this investigation. They were maintained at the culture library collection at the College of Dentistry, University of Illinois at Chicago.

Determination of Minimum Inhibitory Concentration (MIC) Values. The growth media employed were brain−heart infusion broth (BBL Microbiology System, Cockkeyville, MO) for *S. mutans* and trypticase soy broth−yeast extract medium supplemented with cysteine hydrochloride (0.05%), menadione (0.02 μg/mL), hemin (5 μg/mL), and potassium nitrate (0.02%) for *P. gingivalis*. All cultures were incubated at 37 °C. Cultures of *P. gingivalis* were incubated in an anaerobic growth chamber (Forma Scientific Inc., Marietta, OH) in 10% H_2, 5% CO_2, and 85% N_2.

Sterile 96-well microtiter plates were used. Each well contained colony forming units (CFU)/mL of test bacteria (5 × 10^6 for *S. mutans*; 5 × 10^5 for *P. gingivalis*), a serially diluted test compound, and the respective growth medium. Triplicate samples were performed for each test concentration. The controls included inoculated growth medium without test compounds. Sample blanks contained uninoculated growth medium only. All plates were incubated at 37 °C under appropriate atmospheric conditions with growth estimated spectrophotometrically (650 nm) after 48 h using a microtiter plate reader (Power Wave 200 Microplate Scanning Spectrophotometer, Bio-Tech Instruments Inc., Winooski, VT). The MIC value for each test organism was defined as the minimum concentration of test compound limiting turbidity to <0.05 absorbance at 650 nm. As a positive control, chlorhexidine was used (Sigma) and exhibited MIC values of 0.312 μg/mL against both *S. mutans* and *P. gingivalis*.

Extraction and Isolation. Dried ground plant material (800 g) was extracted with 100% MeOH (2 L × 3) using a percolator. The MeOH solution was evaporated under reduced pressure to give 35 g of a residue. A portion of this residue (34 g) was dissolved in MeOH (100 mL) and suspended in H_2O (900 mL). The aqueous solution was partitioned between 1 L of petroleum ether, EtOAc, and 1-BuOH, respectively (three times each), to afford petroleum ether (12.7 g), EtOAc (2.5 g), and 1-BuOH (5.5 g) fractions. A portion of the petroleum-ether extract (10 g) was subjected to Si gel column chromatography using petroleum ether−EtOAc−MeOH (19:1:0→3:1:1, gradient mixture) as solvents. A precipitate was formed in EtOAc from the fractions eluted with petroleum ether−EtOAc (4:1) elution to give **5** (5.2 mg). A portion of the EtOAc extract (2 g) was subjected to Si gel column chromatography eluted with petroleum ether−EtOAc−MeOH (2:3:0→0:1:1, gradient mixtures). The combined fractions eluted with petroleum ether−EtOAc (2:3) were subjected to low-pressure reversed-phase (C_{18}) Si gel column chromatography using a gradient of 80%→95% MeOH in H_2O to give **6** (6.8 mg), **7** (3.4 mg), and semipurified **8**. The final purification of **8** (2.5 mg) was accomplished by semipreparative HPLC using 90% MeOH in H_2O as mobile phase with a flow rate of 5 mL/min. A portion of the 1-BuOH fraction (5 g) was chromatographed over Si gel by column chromatography using EtOAc−MeOH (9:1→1:5, gradient mixtures). Fractions eluted with EtOAc−MeOH (1:2) were combined and subjected to column chromatography over Diaion HP-20 (Mitsubishi Kasei, Tokyo, Japan) using 0, 20, 50, 80, and 100% MeOH in H_2O as eluents. A fraction eluted with 50% MeOH in H_2O was then subjected to Si gel column chromatography using $CHCl_3$−MeOH (3:1→1:2, gradient mixtures). The fractions eluted with $CHCl_3$−MeOH (1:2) were combined and subjected to low-pressure reversed-phase (C_{18}) Si gel column chromatography using 60% MeOH in H_2O as eluent, and yielded **1** (15 mg), **2** (6 mg), and semi-purified **3** and **4**. Further low-pressure reversed-phase (C_{18}) Si gel column chromatography using 55% MeOH in H_2O afforded **3** (4 mg). Further column chromatography with Sephadex LH-20 using 100% MeOH as an eluent afforded **4** (7.4 mg).

3-*O*-β-D-Glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-16α-hydroxy-23-deoxyprotobassic acid (1): white amorphous powder (15 mg); mp 233−235 °C (dec); $[\alpha]_D$ −8.0° (*c* 0.1, pyridine); UV (MeOH) λ_{max} (log ϵ) 215 (3.26), 245 (3.31), 256 (3.38), 261 (3.29) nm; IR (film) ν_{max} 3399, 2928, 2872, 1735, 1607, 1422, 1245, 1064 cm^{-1}; ^{1}H NMR (300 MHz, C_5D_5N) δ 6.18 (1H, d, J = 7.8 Hz, H-1″), 5.53 (1H, br s, H-12), 4.90 (1H, br s, H-1′), 4.78 (1H, br s, H-6), 4.52 (1H, br s, H-2), 4.43 (1H, br s, H-16), 4.41−4.20 (5H, overlapping signals, H-2′, 4′, 6′, 2″, and 3″), 4.16 (1H, m, H-3′), 4.08 (1H, dd, J = 14.0, 7.1 Hz, H-4″), 3.39 (1H, dd, J = 12.0, 4.8 Hz, H-3), 3.20 (1H, d, J = 14.0 Hz, H-18), 1.68 (3H, br s, CH_3-25), 1.64 (3H, s, CH_3-23), 1.61 (3H, br s, CH_3-6″), 1.50 (3H, s, CH_3-26), 1.30 (3H, s, CH_3-27), 1.14 (3H, s, CH_3-24), 0.95 (3H, s, CH_3-29), 0.90 (3H, s,

CH_3-30); ^{13}C NMR (75 MHz, C_5D_5N) δ 176.8 (s, C-28), 143.5 (s, C-13), 126.5 (d, C-12), 106.6 (d, C-1′), 95.7 (d, C-1″), 89.6 (d, C-3), 78.9 (d, C-3′), 78.1 (d, C-5′), 75.6 (d, C-2′), 75.2 (d, C-4″), 75.1 (d, C-16), 73.7 (d, C-4′), 73.7 (d, C-2″), 70.8 (d, C-2), 70.8 (d, C-3″), 68.0 (d, C-5″), 67.2 (d, C-6), 61.9 (t, C-6′), 56.4 (d, C-5), 48.5 (d, C-9), 47.5 (t, C-1), 47.1 (t, C-17), 46.9 (t, C-19), 42.7 (s, C-4), 42.6 (s, C-14), 41.7 (d, C-18), 40.6 (t, C-7), 39.4 (s, C-10), 39.1 (s, C-8), 36.6 (t, C-15), 34.0 (t, C-21), 33.5 (t, C-22), 33.2 (q, C-29), 30.7 (s, C-20), 28.0 (q, C-23), 26.2 (q, C-27), 23.7 (t, C-11), 23.6 (q, C-30), 18.6 (q, C-25), 18.6 (q, C-6″), 14.2 (q, C-24); FABMS (negative-ion mode) *m/z* 809 $[M - H]^-$ (100), 647 $[M - Glc - H_2O]^-$ (38); ESMS (negative-ion mode) *m/z* 809 $[M - H]^-$ (100), 477 (23), 404 (10), 323 (51).

28-*O*-β-D-Glucopyranosyl-16α-hydroxy-23-deoxyprotobassic acid (2): white amorphous powder (6 mg); mp 225−228 °C (dec); $[\alpha]_D$ −8.9° (*c* 0.1, pyridine); UV (MeOH) λ_{max} (log ϵ) 217 (3.21), 256 (3.17), 268 (3.11) nm; IR (film) ν_{max} 3380, 2930, 2853, 1744, 1609, 1425, 1366, 1069, 1031 cm^{-1}; ^1H NMR (300 MHz, C_5D_5N) δ 6.31 (1H, d, *J* = 7.6 Hz, H-1′), 5.51 (1H, m, H-12), 4.79 (1H, m, H-6), 4.37−4.22 (4H, overlapping signals, H-2, H-4′, H-5′, and H-6′), 4.01 (1H, m, H-3), 3.32 (1H, m, H-18), 1.69 (3H, s, CH_3-26), 1.68 (3H, s, CH_3-25), 1.55 (3H, s, CH_3-24), 1.50 (3H, s, CH_3-27), 1.34 (3H, s, CH_3-23), 0.95 (3H, s, CH_3-29), 0.91 (3H, s, CH_3-30); ^{13}C NMR (75 MHz, C_5D_5N) δ 176.4 (s, C-28), 143.5 (s, C-13), 124.1 (d, C-12), 95.8 (d, C-1′), 79.2 (d, C-3), 78.5 (d, C-3′), 75.3 (d, C-2′), 74.1 (d, C-16), 73.8 (d, C-4′), 72.7 (d, C-5′), 71.1 (d, C-2), 67.4 (d, C-6), 62.2 (t, C-6′), 56.4 (d, C-5), 48.6 (d, C-9), 47.1 (t, C-1), 47.0 (t, C-19), 46.9 (s, C-17), 42.8 (s, C-14), 42.6 (s, C-4), 41.8 (d, C-18), 40.6 (t, C-7), 39.4 (s, C-8), 39.3 (t, C-21), 36.8 (s, C-10), 34.1 (t, C-15), 33.2 (q, C-29), 30.8 (t, C-22), 28.2 (q, C-27), 26.3 (q, C-23), 23.8 (t, C-11), 23.7 (q, C-30), 18.8 (q, C-25), 18.7 (q, C-26), 17.1 (q, C-24); FABMS (negative-ion mode) *m/z* 647 $[M - H_2O - H]^-$ (37); ESMS (negative-ion mode) *m/z* 666 $[M]^-$ (3), 223 (29), 185 (86), 131 (47), 115 (100).

3-*O*-β-D-Glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-16α-hydroxyprotobassic acid (3): white amorphous powder (4 mg); mp 238−242 °C (dec); $[\alpha]_D$ −5.2° (*c* 0.1, pyridine); UV (MeOH) λ_{max} (log ϵ) 219 (3.30), 271 (2.92) nm; IR (film) ν_{max} 3376, 2925, 2862, 1735, 1606, 1418, 1062, 1031 cm^{-1}; ^1H NMR (300 MHz, C_5D_5N) δ 6.22 (1H, d, *J* = 8.1 Hz, H-1″), 4.94 (1H, d, *J* = 7.7 Hz, H-1′), 4.79 (1H, br s, H-6), 4.77 (1H, br s, H-6), 4.69 (1H, d, *J* = 10.1 Hz, H-23a), 4.48 (1H, br s, H-16), 4.43 (1H, br s, H-2), 4.34−4.10 (5H, overlapping signals, H-2′, H-4′, H-6′, H-2″, and H-3″), 3.99 (1H, m, H-23b), 3.45 (1H, m, H-3), 3.21 (1H, m, H-18), 1.71 (6H, s, CH_3-25, CH_3-6″), 1.64 (3H, s, CH_3-26), 1.52 (3H, s, CH_3-24), 1.30 (3H, s, CH_3-27), 0.94 (3H, s, CH_3-29), 0.90 (3H, s, CH_3-30); ^{13}C NMR (75 MHz, C_5D_5N) δ 176.2 (s, C-28), 143.1 (s, C-13), 129.0 (d, C-12), 106.2 (d, C-1′), 95.2 (d, C-1″), 88.9 (d, C-3), 78.5 (d, C-3′), 77.8 (d, C-5′), 74.9 (d, C-4″), 74.8 (d, C-16), 74.7 (d, C-2′), 73.4 (d, C-4′), 73.2 (d, C-2″), 70.5 (d, C-3″), 70.1 (d, C-2), 68.6 (t, C-23), 67.8 (d, C-5″), 66.8 (d, C-6), 61.5 (t, C-6′), 55.9 (d, C-5), 48.0 (d, C-9), 46.8 (t, C-19), 46.6 (t, C-1), 46.6 (s, C-17), 42.2 (s, C-4), 42.2 (s, C-14), 41.2 (d, C-18), 40.1 (t, C-7), 38.7 (s, C-8), 36.2 (s, C-10), 36.2 (t, C-15), 33.8 (t, C-21), 32.7 (q, C-29), 30.6 (t, C-22), 30.3 (s, C-20), 25.7 (q, C-27), 24.1 (t, C-11), 23.1 (q, C-30), 20.8 (q, C-25), 18.1 (q, C-26), 18.1 (q, C-6″), 16.6 (q, C-24); FABMS (negative-ion mode) *m/z* 825 $[M - H]^-$ (62), 647 $[M - rha - H_2O]^-$ (81); FABMS (positive-ion mode) *m/z* 871 $[M + 2Na - H]^+$ (10), 848 $[M + Na]^+$, (10), 802 (14), 693 (16), 594 (22), 356 (100).

3-*O*-{[β-D-Glucopyranosyl-(1→6)]-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)}-*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosylcycloarta-22,24-dien-27-oic acid (mussaendoside W) (4): white amorphous powder (7.4 mg); mp 175−178 °C; $[\alpha]_D$ −4.3° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 218 (3.11), 240 (3.19), 280 (3.18) nm; IR (film) ν_{max} 3600, 3285, 2795, 1687, 1504, 1213, 1034 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; FABMS (positive-ion mode) *m/z* 1247 $[M - 1]^+$ (3), 584 (64), 566 (66), 482 (100), 370 (56); ESMS (negative-ion mode) *m/z* 1248 $[M]^-$ (3), 686 (100), 338 (22), 255 (61).

3-*O*-Acetyloleanolic acid (5): white amorphous powder (5.2 mg); mp 258−260 °C [lit.[19] 263−265 °C]; $[\alpha]_D$ +28.0° (*c* 0.05, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 247 (2.03) nm; IR (film) ν_{max} 3634, 3423, 2942, 1727, 1369, 1242, 1019 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.29 (1H, m, H-12), 4.51 (1H, dd, *J* = 11.2, 5.2 Hz, H-3), 2.84 (1H, dd, *J* = 14.0, 3.9 Hz, H-18), 2.06 (3H, s, $OCOCH_3$), 1.14 (3H, s, H-27), 0.96 (3H, s, CH_3-25), 0.94 (3H, s, CH_3-30), 0.87 (3H, s, CH_3-24), 0.76 (3H, s, CH_3-26); ^{13}C NMR data consistent with literature values;[20] EIMS (70 eV) *m/z* 498 $[M]^+$ (3), 438 (15), 248 (100), 203 (86), 190 (58).

3-*O*-Acetyldaturadiol (6): white amorphous powder (6.8 mg); mp 214−217 °C [lit.[21] 227−236 °C]; $[\alpha]_D$ +27.6° (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 274 (2.53) nm; IR (film) ν_{max} 3510, 2937, 1725, 1561, 1452, 1370, 1251, 1031 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.30 (1H, br s, H-12), 4.50 (1H, br s, H-6), 4.44 (1H, m, H-3), 2.06 (3H, s, $OCOCH_3$), 1.31 (3H, s, CH_3-25), 1.24 (3H, s, CH_3-26), 1.08 (3H, s, CH_3-24), 1.04 (3H, s, CH_3-27), 0.94 (3H, s, CH_3-23), 0.91 (6H, s, CH_3-29,30), 0.89 (3H, s, CH_3-28); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.7 (s, $OCOCH_3$), 144.2 (s, C-13), 123.8 (d, C-12), 81.2 (d, C-3), 67.9 (d, C-6), 55.5 (d, C-5), 49.6 (d, C-9), 46.7 (s, C-17), 46.6 (t, C-19), 42.7 (s, C-14), 42.1 (d, C-18), 41.1 (t, C-7), 40.4 (s, C-4), 38.6 (t, C-1), 38.4 (s, C-10), 38.1 (s, C-8), 36.2 (t, C-21), 33.8 (t, C-22), 32.9 (q, C-29), 30.5 (s, C-20), 27.4 (q, C-23), 25.6 (q, C-27), 23.5 (t, C-2), 23.3 (t, C-11), 23.3 (t, C-16), 23.0 (q, C-30), 21.1 (q, $OCOCH_3$), 17.8 (q, C-24), 17.7 (q, C-26), 16.6 (q, C-25); EIMS (70 eV) *m/z* 466 $[M - H_2O]^+$ (3), 248 (100), 203 (65), 189 (20).

Rotundic acid (7): white amorphous powder (3.4 mg); mp 255−258 °C [lit.[22] 271 °C]; $[\alpha]_D$ +34.2° (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 236 (2.81), 282 (2.80) nm; IR (film) ν_{max} 3724, 2886, 2345, 1734, 1661, 1552, 1498, 1438, 1367, 1053 cm^{-1}; ^1H NMR (500 MHz, C_5D_5N) δ 5.61 (1H, m, H-12), 4.30 (1H, m, H-3), 4.20 (1H, m, H-23a), 3.74 (1H, d, *J* = 10.5 Hz, H-23b), 3.10 (1H, br s, H-18), 1.70 (3H, s, CH_3-25), 1.60 (3H, s, CH_3-27), 1.46 (3H, s, CH_3-29), 1.15 (3H, s, CH_3-26), 1.08 (3H, s, CH_3-24), 1.01 (3H, d, *J* = 4.13 Hz, CH_3-30); ^{13}C NMR (125 MHz, C_5D_5N) δ 180.0 (s, C-28), 144.9 (s, C-13), 128.0 (d, C-12), 73.4 (d, C-3), 72.7 (d, C-19), 67.9 (t, C-23), 54.6 (d, C-18), 48.7 (d, C-5), 47.8 (d, C-9), 42.9 (d, C-17), 42.3 (s, C-20), 42.1 (C-14), 40.3 (s, C-1), 39.9 (s, C-4), 37.4 (t, C-22), 37.2 (s, C-10), 33.3 (t, C-7), 29.3 (t, C-15), 27.7 (t, C-21), 27.1 (q, C-29), 26.9 (t, C-2), 26.4 (t, C-16), 24.9 (q, C-27), 24.1 (t, C-11), 18.7 (t, C-6), 16.8 (q, C-26), 16.0 (q, C-30), 13.1 (q, C-24); EIMS (70 eV) *m/z* 488 $[M]^+$ (6), 470 $[M - H_2O]^+$ (8), 442 (25), 264 (54), 246 (65), 201 (67), 175 (50), 146 (100).

16α-Hydroxyprotobassic acid (8): white amorphous powder (2.5 mg); mp 210−213 °C [lit.[14] 235−237 °C (dec)]; $[\alpha]_D$ +2.5° (*c* 0.5, MeOH) [lit.[14] +5.6°]; UV (MeOH) λ_{max} (log ϵ) 297 (2.81) nm; IR (film) ν_{max} 3671, 3530, 3108, 2912, 2846, 1717, 1652, 1495, 1245, 1156, 1011 cm^{-1}; ^1H, ^{13}C NMR, and EIMS data consistent with literature values.[11]

Acid Hydrolysis of 1−4. Solutions of **1** (5 mg) and **4** (2 mg) in MeOH were refluxed with 2 N HCl for 5 h. After reflux, solvent was removed in vacuo and the residue was partitioned between EtOAc and H_2O. The aqueous layers were dried in vacuo, and a portion of each residue was mixed with 0.2 mL of Sigma-Sil A and heated at 65 °C for 30 min. Authentic sugars (1 mg, Sigma Chemical Co., St. Louis, MO) were treated in a similar manner. Sugar analysis by GC was carried out by injecting a 5-μL aliquot of the trimethylsilyl derivative onto a DB-1 fused silica capillary column.[25] Glucose and rhamnose in the aqueous layer were identified (t_R 18.6 min for glucose, and t_R 16.2 min for rhamnose) for both **1** and **4**.

Sugars were also identified by TLC analysis. MeOH solutions of **1**−**4** and standard D-glucose and l-rhamnose were applied to a Si gel TLC plate and were hydrolyzed with 2 N HCl vapor for 2 h by heating. After removal of the residual HCl, the plate was developed using $CHCl_3$−MeOH (6:4) as solvent. The plate was sprayed with vanillin−10% H_2SO_4 in EtOH−H_2O (4:1) and heated at 100 °C for 10 min to locate sugar spots (R_f 0.3 for glucose, and R_f 0.6 for rhamnose).

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