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ORIGINAL PAPER

New cholestane glycosides and sterols from the underground parts of *Chamaelirium luteum* and their cytotoxic activity

Akihito Yokosuka · Kenichi Takagi · Yoshihiro Mimaki

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Abstract Six new cholestane glycosides (**1**, **5**, **6**, **10**, **12**, and **13**) and two new sterols (**9** and **11**), along with five known compounds (**2**–**4**, **7**, and **8**), were isolated from the underground parts of *Chamaelirium luteum* (Liliaceae). The structures of these new compounds were determined by spectroscopic analysis and the results of hydrolytic cleavage. The isolated compounds and aglycones were evaluated for their cytotoxic activity against HL-60 human leukemia cells. Compounds **6a**, **10a**, **12a**, **13**, and **13a** were cytotoxic to HL-60 cells, with IC_{50} values of 12.8, 9.8, 15.3, 6.2, and 10.2 μM, respectively.

Keywords *Chamaelirium luteum* · Liliaceae · Cholestane glycosides · Cytotoxic activity · HL-60 cells

Introduction

Previously, we investigated chemical components in several traditional medicinal plants and isolated steroidal saponins from *Trillium erectum* (Liliaceae) [1], triterpene glycosides from *Curculigo orchioides* (Hypoxidaceae) [2, 3], and lignan glycosides and triterpene glycosides from *Larrea tridentata* (Zygophyllaceae) [4, 5]. *Chamaelirium luteum* (L.) A. Gray (Liliaceae) is a perennial plant distributed throughout North America. Its roots and rhizomes are called 'False Unicorn Root' and have been used as a folk medicine in the United States and Canada to treat female disorders of the reproductive organs, including menstrual and menopausal symptoms [6]. A few phytochemical investigations have been carried out with *C. luteum*, and some steroidal glycosides have been isolated and identified [6–10]. In our continuing phytochemical studies of traditional medicinal plants, we have now investigated the chemical components of the underground parts of *C. luteum*. As a result, six new cholestane glycosides (**1**, **5**, **6**, **10**, **12**, and **13**) and two new sterols (**9** and **11**), along with five known compounds (**2**–**4**, **7**, and **8**), were isolated (Structure 1). This paper deals with the isolation and structural determination of the new glycosides based on spectroscopic analysis, including two-dimensional (2D) NMR spectroscopy and the results of hydrolytic cleavage. The cytotoxic activities of the isolated compounds and derivatives against HL-60 human promyelocytic leukemia cells are also reported.

Results and discussion

The underground parts of *C. luteum* were extracted with MeOH. After solvent removal, the crude extract was passed through a porous-polymer polystyrene resin (Diaion HP-20) column and successively eluted with 30 % MeOH, 50 % MeOH, MeOH, EtOH, and EtOAc. The 50 % MeOH eluted fraction was repeatedly subjected to silica gel and octadecylsilanized (ODS) silica gel column chromatography to yield compounds **1**–**13**. Compounds **2**–**4**, **7**, and **8** were identified as (22*S*,25*R*)-11α,16β,22-trihydroxycholest-5-ene-3β,26-diyl bis-β-D-glucopyranoside (heloside A, **2**) [7], (22*S*,25*R*)-11α,16β,22,26-tetrahydroxycholest-5-en-3β-yl β-D-glucopyranoside (heloside B, **3**) [7], (22*S*,25*R*)-cholest-5-ene-3β,11α,16β,22,26-pentol (helogenin, **4**) [7], (23*R*,24*S*)-6β,16β,23,24-tetrahydroxy-5α-cholestan-3β-yl *O*-β-D-glucopyranosyl-

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(1 → 6)-β-D-glucopyranoside [(23*R*,24*S*)-chamaeliroside A, **7**] [6], and (23*R*,24*S*)-24-[(β-D-fucopyranosyl)oxy]-6β, 16β,23-trihydroxy-5α-cholestan-3β-yl *O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside [(23*R*,24*S*)-chamaeliroside B, **8**] [6], based on physical and spectroscopic data. As for **4**, its structure was confirmed by X-ray crystallographic analysis.

Compound **1** was obtained as an amorphous solid with a molecular formula of $C_{33}H_{56}O_{10}$, as determined from HR-ESITOFMS (*m/z* 613.3956 $[M + H]^+$, calc. for $C_{33}H_{57}O_{10}$, 613.3952) and ^{13}C-NMR spectral data. The ^{1}H-NMR spectrum of **1** showed two three-proton singlet signals at δ 1.42 and 1.25 (each s), two three-proton doublet signals at δ 1.20 (3H, d, $J = 7.0$ Hz) and 1.00 (3H, d, $J = 6.7$ Hz), and an olefinic proton signal at δ 5.51 (1H, br d, $J = 5.3$ Hz), which are all characteristic of a steroidal skeleton. Signals for a β-D-glucopyranosyl group (Glc) were also observed in the ^{1}H- and ^{13}C-NMR spectra [δ_H 4.82 (1H, d, $J = 7.8$ Hz); δ_C 104.9, 75.2, 78.6, 71.7, 78.5, and 62.8]. Enzymatic hydrolysis of **1** with naringinase gave helogenin (**4**) and D-glucose. The identification of D-glucose, including its absolute configuration, was carried out by direct HPLC analysis of the hydrolysate. The above data suggest that **1** is a helogenin monoglucoside. In the HMBC spectrum of **1**, a correlation was observed between H-1 of Glc at δ 4.82 and C-26 of the aglycone at δ 75.3. Thus, the structure of **1** was characterized as (22*S*,25*R*)-3β,11α,16β,22-tetrahydroxycholest-5-en-26-yl β-D-glucopyranoside.

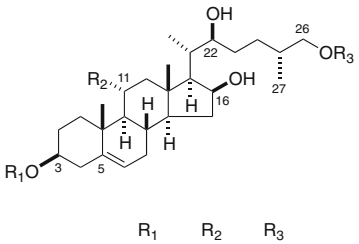
Compound **5** was isolated as an amorphous solid with a molecular formula of $C_{45}H_{76}O_{18}$, based on HR-ESITOFMS (*m/z* 927.4988 $[M + Na]^+$). The ^{1}H-NMR spectrum of **5** contained signals for three anomeric protons at δ 6.40 (1H, br s), 5.08 (1H, d, $J = 7.2$ Hz), and 4.84 (1H, d, $J = 7.8$ Hz), and the methyl group of a 6-deoxyhexopyranosyl moiety at δ 1.79 (1H, d, $J = 6.2$ Hz), as well as signals due to a steroidal skeleton. Further analysis of the ^{1}H-^{1}H COSY, HMQC, and HMBC spectra of **5** revealed that the aglycone moiety of **5** was (22*S*,25*R*)-cholest-5-ene-3β,16β,22,26-tetrol [8] and that the sugar moiety was composed of a terminal α-L-rhamnopyranosyl unit (Rha) [δ_H 6.40 (1H, br s); δ_C 102.1, 72.6, 72.8, 74.2, 69.5, 18.7], a C-2 substituted β-D-glucopyranosyl unit (Glc) [δ_H 5.08 (1H, d, $J = 7.2$ Hz); δ_C 100.4, 77.8, 79.7, 71.8, 78.3, 62.7], and a terminal β-D-glucopyranosyl unit (Glc′) [δ_H 4.84 (1H, d, $J = 7.8$ Hz); δ_C 104.9, 75.3, 78.6, 71.7, 78.5, 62.8]. Acid hydrolysis of **5** with 1 M HCl gave L-rhamnose and D-glucose. In the HMBC spectrum of **5**, long-range correlations were observed between H-1 of Rha at δ 6.40 and C-2 of Glc δ 77.8 H-1 of Glc at δ 5.08 and C-3 of the aglycone at δ 78.0, and between H-1 of Glc′ at δ 4.84 and C-26 of the aglycone at δ 75.3. Accordingly, **5** was formulated as (22*S*,25*R*)-26-[(β-D-glucopyranosyl)oxy]-16β,22-dihydroxycholest-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside.

Compound **6** was obtained as an amorphous solid and its molecular formula $C_{39}H_{68}O_{15}$ was deduced from HR-ESITOFMS (*m/z* 799.4421 $[M + Na]^+$). The ^{1}H-NMR spectrum of **6** showed two anomeric proton signals at δ 5.12 (1H, d, $J = 7.7$ Hz) and 4.73 (1H, d, $J = 7.6$ Hz), as well as two three-proton singlet signals at δ 1.24 and 0.86 (3H, each s), and three three-proton doublet signals at δ 1.28 (3H, d, $J = 7.0$ Hz) and 1.14 (3H × 2, d, $J = 6.7$ Hz), which indicated that **6** is a steroidal diglycoside. Acid hydrolysis of **6** with 1 M HCl gave 5α-cholestane-3β,6β,16β,23,24-pentol (**6a**) (chiograsterol B) and D-glucose. When the ^{1}H- and ^{13}C-NMR spectra of **6a** were compared with those of (23*R*,24*S*)- and (23*S*,24*R*)-chiograsterol B [6], **6a** was completely in accordance with (23*R*,24*S*)-chiograsterol B. Thus, **6** was shown to be a diglucoside of (23*R*,24*S*)-chiograsterol B. In the HMBC spectrum of **6**, one anomeric proton signal at δ 5.12 showed a long-range correlation with C-3 of the aglycone at δ 77.7, whereas another anomeric proton signal at δ 4.73 correlated with C-16 of the aglycone at δ 83.1, indicating that the C-3 and C-16 hydroxy groups of the aglycone were glucosylated. The structure of **6** was determined to be (23*R*,24*S*)-6β,23,24-trihydroxy-5α-cholestane-3β,16β-diyl bis-β-D-glucopyranoside.

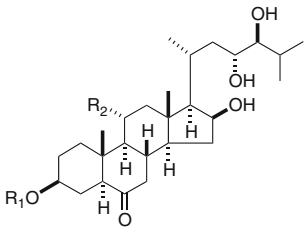
Compound **9** was shown to have a molecular formula of $C_{27}H_{48}O_6$ by HR-ESITOFMS (*m/z* 469.3511 $[M + H]^+$) analysis. The ^{1}H- and ^{13}C-NMR spectral data of **9** were essentially analogues of **6a**. However, the molecular formula of **9** was found to be higher than that of **6a** by one oxygen atom, and the ^{1}H-NMR spectrum of **9** contained signals for six exchangeable protons at δ 6.45 (1H, br s), 5.99 (1H, br s), 5.77 (1H, br s), 5.63 (1H, br s), 5.59 (1H, br s), and 5.45 (1H, br d, $J = 3.2$ Hz), which were removed by the addition of HCl vapor. Comparison of the ^{1}H- and ^{13}C-NMR assignments of **9** with those of **6a** showed that the C-12 methylene carbon signal, which was observed at δ 40.4 in **6a**, was displaced by an oxymethine carbon signal at δ 72.5 in **9**, and the H-12 oxymethine proton signal at δ 4.32 (1H, br s) exhibited spin-couplings with the H_2-11 methylene proton signals at δ 1.90 (1H, m) and 1.89 (1H, m). The HMBC spectrum of **9** exhibited long-range correlations between the oxymethine carbon at δ 72.5 (C-12) and H-17 (δ 2.38)/Me-18 (δ 1.26). These data suggested that **9** is a derivative of **6a**, with one more hydroxy group at C-12 in addition to the C-3 (δ_H 3.95; δ_C 71.4), C-6 (δ_H 4.05; δ_C 71.2), C-16 (δ_H 4.75; δ_C 71.7), C-23 (δ_H 4.12; δ_C 71.7), and C-24 (δ_H 3.67; δ_C 79.5) hydroxy moieties. Thus, the structure of **9** was determined to be (23*R*,24*S*)-5α-cholestane-3β,6β,12α,16β,23,24-hexol.

Compound **10** was deduced to be $C_{33}H_{56}O_{10}$, based on HR-ESITOFMS (*m/z*: 635.3766 $[M + Na]^+$) results. The presence of a carbonyl group in **10** was revealed by the IR (1708 cm^{-1}) and ^{13}C-NMR spectra (δ 209.7). Enzymatic

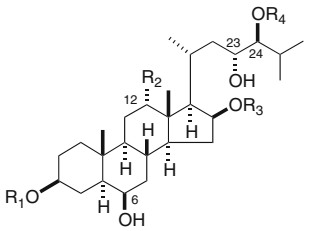
Structure 1



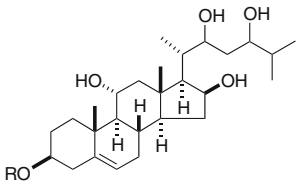
	R_1	R_2	R_3
1	H	OH	Glc
2	Glc	OH	Glc
3	Glc	OH	H
4	H	OH	H
5	S_1	H	Glc



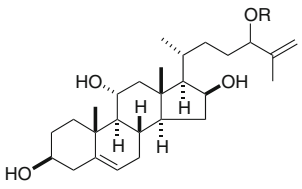
	R_1	R_2
10	Glc	H
10a	H	H
11	H	OH



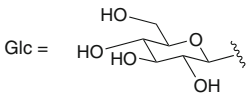
	R_1	R_2	R_3	R_4
6	Glc	H	Glc	H
6a	H	H	H	H
7	S_2	H	H	H
8	S_2	H	H	Fuc
9	H	OH	H	H

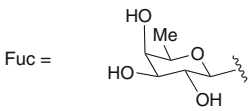


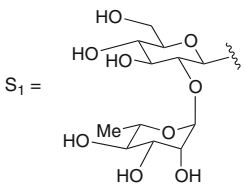
	R
12	Glc
12a	H

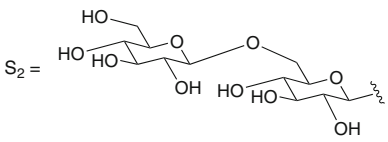


	R
13	Glc
13a	H









hydrolysis of **10** using naringinase gave an aglycone (**10a**) and D-glucose. The ^{1}H- and ^{13}C-NMR data for **10a** were identical to those from **6a**, except for the signals from C-6 and its neighboring carbons, and **10a** was, therefore, identified as 3β,16β,23,24-tetrahydroxycholestan-6-one (chiograsterol A) [9, 10]. An HMBC correlation was observed between the anomeric proton signal of the β-D-glucopyranosyl unit at δ 5.06 (d, $J = 7.7$ Hz) and C-3 of the aglycone at δ 76.7. Accordingly, **10** was determined to be (23*R*,24*S*)-3-[(β-D-glucopyranosyl)oxy]-16β,23,24-trihydroxy-5α-cholestan-6-one.

Compound **11** had a molecular formula of $C_{27}H_{46}O_6$ based on HR-ESITOFMS (*m/z*: 467.3372 $[M + H]^+$) analysis, and the spectral properties of **11** were shown to be very similar to those of **10a**. However, the molecular formula of **11** was higher than that of **10a** by one oxygen atom. Comparison of the ^{1}H- and ^{13}C-NMR assignments of **11** with those of **10a** revealed that the C-11 methylene carbon signal at δ 21.5 in **10a** was displaced by an oxymethine carbon signal at δ 67.9 in **11**, and the H-11 oxymethine proton signal at δ 4.22 (1H, ddd, $J = 10.4$, 10.4, 4.9 Hz) exhibited spin-couplings with H_2-12 methylene proton signals at δ 2.62 (1H, m) and 1.60 (1H, m) and an H-9 methine proton signal at δ 1.65 (1H, dd, $J = 10.4$, 10.4 Hz). The configuration of the C-11 hydroxy group was determined to be α based on its proton spin-coupling constants ($J = 10.4$, 10.4, 4.9 Hz) and NOE correlations between H-11 and Me-19 (δ 1.07)/Me-18 (δ 1.07). Thus, **11** was determined to be (23*R*,24*S*)-3β,11α,16β,23,24-pentahydroxy-5α-cholestan-6-one.

Compound **12** was determined to be $C_{33}H_{56}O_{10}$ by HR-ESITOFMS (613.3969 $[M + H]^+$). In the ^{1}H-NMR

spectrum of **12**, signals from five steroidal methyl groups at δ 1.27 (3H, s), 1.21 (3H, d, $J = 7.3$ Hz), 1.20 (3H, s), 1.06 (3H, d, $J = 6.8$ Hz), and 1.02 (3H, d, $J = 6.8$ Hz) and an anomeric proton at δ 5.06 (1H, d, $J = 7.7$ Hz) were observed. Enzymatic hydrolysis of **12** with naringinase gave an aglycone (**12a**) with the molecular formula ($C_{27}H_{46}O_5$) as that of **4**, along with D-glucose. When the ^{13}C-NMR assignments of **12a** were compared with those of **4**, the signals arising from the ring A–D portions (C-1–C-19) were identical to those of **4**; however, significant differences were recognized in the signals from the two compounds, which could be attributed to side-chain moiety differences (C-20–C-27). The methylene proton signals at δ 2.02 and 1.81, assignable to H_2-23, showed spin-coupling correlations with oxymethine proton signals at δ 4.54 and 3.88 in the ^{1}H-^{1}H COSY spectrum of **12a**. The C-20 methine proton signal at δ 2.62 correlated with the oxymethine proton signal at δ 4.54 (C-22), whereas the C-25 proton signal exhibited a correlation with the other oxymethine proton signal at δ 3.88 (C-24). Thus, two hydroxy groups were shown to be located at C-22 and C-24, respectively. The structure of **12a** was characterized as cholest-5-ene-3β,11α,16β,22,24-pentol. The stereochemistry of the steroidal ring moieties was ascertained by NOE correlations between H-3 (δ 3.93) and H-1ax (δ 1.53), Me-19 (δ 1.43) and H-4ax (δ 2.75)/H-8 (δ 1.37)/H-11 (δ 4.39), Me-18 (δ 1.26) and H-8 (δ 1.63)/H-11 (δ 4.39), and between H-14 (δ 1.12) and H-9 (δ 1.37)/H-17 (δ 1.78) observed in the phase-sensitive NOESY spectrum (Fig. 1). The large J value between H-17 and H-20 ($J = 11.2$ Hz) indicates that the H_{17}–C_{17}–C_{20}–H_{20} part is preferably *trans*-oriented, and an NOE correlation between H-20 and Me-18 made it possible to determine the 17β and 20*S* configurations. An HMBC correlation was observed between the anomeric proton signal of the β-D-glucopyranosyl group at δ 5.06 (d, $J = 7.7$ Hz) and C-3 of the aglycone at δ 78.3. Accordingly, **12** was determined to be 11α,16β,22,24-tetrahydroxycholest-5-en-3β-yl β-D-glucopyranoside.

Compound **13** had a molecular formula of $C_{33}H_{54}O_9$ based on HR-ESITOFMS (617.3690 $[M + Na]^+$) results. The ^{1}H-NMR spectrum contained signals for four steroidal methyl groups at δ 1.87 (3H, s), 1.41 (3H, s), 1.19 (3H, s), and 1.06 (3H, d, $J = 6.6$ Hz), and a pair of exomethylene protons at δ 5.17 (1H, br s) and 4.93 (1H, br s), as well as an anomeric proton at δ 4.93 (1H, d, $J = 7.9$ Hz). Enzymatic hydrolysis of **13** with naringinase gave an aglycone (**13a**, $C_{27}H_{44}O_4$) and D-glucose. Analysis of the ^{1}H- and ^{13}C-NMR spectra of **13a** revealed that the ring A–D portions (C-1–C-19) were identical to those of **4**. However, differences were recognized in the signals from the side-chain moiety (C-20–C-27) that suggested the presence of a hydroxy group, a tertiary methyl group, and an exomethylene group. The hydroxymethine proton signal at δ 4.44 correlated with the methylene proton signals at δ 1.86 (2H, m, H_2-23) in the ^{1}H-^{1}H COSY spectrum and showed long-range correlations with the olefinic carbon signals at δ 149.7 and 109.7, and a methyl carbon signal at δ 18.4 in the HMBC spectrum. On the other hand, the tertiary methyl proton signals at δ 1.87 showed long-range correlations with the olefinic carbon signals at δ 149.7 and 109.7, and an oxymethine carbon signal (C-24) at δ 74.1, while exomethylene proton signals at δ 5.24 and 4.93 exhibited HMBC correlations with the oxymethine carbon signal (C-24) and the tertiary methyl carbon signal at δ 18.4 (C-27) (Fig. 2). These data are consistent with the presence of a hydroxy group at C-24 and a double-bond between C-25 and C-26, and they enabled the structure of **13a** to be characterized as cholest-5,25(26)-diene-3β,11α,16β,24-tetrol. The large J value between H-17 and H-20 ($J = 11.0$ Hz), and an NOE correlation between H-20 and Me-18 accounted for the 17β and 20*S* configurations. An HMBC correlation was observed between the anomeric proton of a β-D-glucopyranosyl group at δ 4.93 (d, $J = 7.9$ Hz) and C-3 of the aglycone at δ 83.4. Therefore, **13** was found to be 3β,11α,16β-trihydroxycholest-5,25(26)-dien-24-yl β-D-glucopyranoside.

Compounds **1**, **5**, **6**, **10**, **12**, and **13** are new cholestane glycosides, and **9** and **11** are new sterols. The aglycones (**12a** and **13a**) of **12** and **13** are also new polyhydroxylated cholestane derivatives. Compound **9** is the first representative of a C_{27}-sterol with six hydroxy groups from the plant kingdom, whereas hexahydroxylated sterols have been reported from a marine organism [11]. The absolute configurations at C-22 and C-24 of **12** and at C-24 of **13** remain to be determined due to low yields.

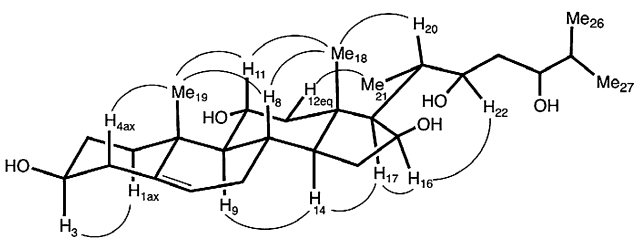


Fig. 1 Important NOE correlations of **12a**

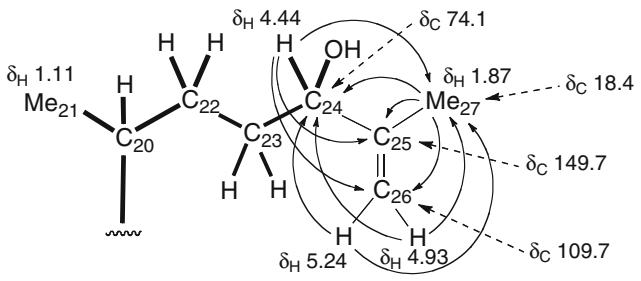


Fig. 2 ^{1}H-^{1}H correlations (*bold lines*) and ^{1}H/^{13}C long-range correlations (*arrows*) of the side-chain moiety of **13a**

Compounds **1**–**13** and the aglycones (**6a**, **10a**, **12a**, and **13a**) were evaluated for their cytotoxic activities against HL-60 cells. Compounds **6a**, **10a**, **12a**, **13**, and **13a** were cytotoxic to HL-60 cells, with IC_{50} values of 12.8, 9.8, 15.3, 6.2, and 10.2 μM, respectively, whereas etoposide, which was used as a positive control, gave an IC_{50} value of 0.34 μM. Compounds **1**–**12** did not show cytotoxicity at sample concentrations of 20 μM.

Experimental

General

Optical rotation was measured using a JASCO P-1030 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 (500 MHz for ^{1}H-NMR, Karlsruhe, Germany) spectrometer using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard. HR-ESITOFMS data were obtained by using a Waters-Micromass LCT mass spectrometer (Manchester, UK). Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan), silica gel (Fuji Silysia Chemical, Aichi, Japan), and ODS silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography (CC). TLC was carried out on silica gel 60 F_{254} (thickness: 0.25 mm, Merck, Darmstadt, Germany) and RP_{18} F_{254S} plates (thickness: 0.25 or 0.5 mm, Merck), and spots were visualized by spraying the plates with 10 % H_2SO_4 aqueous solution, followed by heating. HPLC was performed with a system composed of a DP-8020 pump (Tosoh, Tokyo, Japan), an RI-8021 (Tosoh) or a Shodex OR-2 (Showa Denko, Tokyo, Japan) detector, and a Rheodyne injection port. A TSKgel ODS-100Z column (10 mm i.d. × 250 mm, 5 μm, Tosoh) was employed for preparative HPLC. The following materials and reagents were used for cell culture assay: 96-well flat-bottom plate (Iwaki Glass, Chiba, Japan); RPMI 1640 medium, etoposide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO); fetal bovine serum (FBS) (BioWhittaker, Walkersville, MO); and penicillin G sodium salt and streptomycin sulfate (Gibco, Grand Island, NY). All other chemicals used were of biochemical reagent grade.

Plant material

The underground parts of *Chamaelirium luteum* were obtained from a wholesale firm in Richters, Ontario, Canada, in 2000. A voucher specimen has been deposited in our laboratory (voucher no. CL-00-001, Department of Medicinal Pharmacognosy).

Extraction and isolation

The plant material (dry weight, 3.0 kg) was extracted with MeOH (each 6 L). The MeOH extract was concentrated under reduced pressure, and viscous concentrate (530 g) was passed through a Diaion HP-20 column (80 mm i.d. × 350 mm) and successively eluted with 30 % MeOH, 50 % MeOH, MeOH, EtOH, and EtOAc. CC of the 50 % MeOH eluted fraction (67 g) on silica gel (80 mm i.d. × 350 mm), followed by elution with a stepwise gradient mixture of $CHCl_3$–MeOH–H_2O (90:10:1; 40:10:1; 30:10:1; 20:10:1; 1:1:0), and finally with MeOH alone, gave 13 fractions (A–M). Fraction D (1.26 g) was chromatographed on ODS silica gel eluted with MeCN–H_2O (2:7; 2:5; 1:2) to give 9 subfractions (D-1–D-9). Fraction D-4 was further separated by preparative HPLC using MeOH–H_2O (1:1) to yield **11** (8.8 mg). Fraction D-6 was suspended in MeCN–H_2O (1:2), and the insoluble solid was filtered to give **4** (63.3 mg). Fraction F (3.65 g) was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (80:10:1; 60:10:1; 40:10:1; 20:10:1; 1:1:0) to give 13 subfractions (F-1–F-13). Fraction F-6 was separated by ODS silica gel CC eluted with MeOH–H_2O (2:3; 1:1, 3:2; 2:1) to yield **13** (5.7 mg). Fraction F-8 was separated by ODS silica gel CC eluted with MeOH–H_2O (2:3; 1:1, 3:2; 2:1) to yield **10** (3.1 mg). Fraction G (2.19 g) was separated by ODS silica gel CC eluted with MeCN–H_2O (1:3; 1:2) and silica gel CC eluted with $CHCl_3$–MeOH–H_2O (60:10:1; 50:10:1) to yield **1** (61.7 mg), **3** (37.9 mg), **9** (10.0 mg), and **12** (3.1 mg). Fraction K (26.6 g) was chromatographed on ODS silica gel eluted with MeCN–H_2O (2:7; 1:2) to give 16 subfractions (K-1–K-16). Fraction K-2 was separated by ODS silica gel CC eluted with MeCN–H_2O (1:4; 4:11; 1:2) and MeOH–H_2O (3:2) to yield **2** (262 mg) and **5** (37.8 mg). Fraction K-15 was separated by silica gel CC eluted with $CHCl_3$–MeOH–H_2O (20:10:1; 7:4:1) and ODS silica gel CC eluted with MeOH–H_2O (2:1) to yield **6** (10.0 mg), **7** (12.5 mg), and **8** (28.9 mg).

Compound **1**

Amorphous powder; $[\alpha]_D^{26}$ −21.1 ($c = 0.10$, MeOH); IR (film) ν_{max} cm^{-1}: 3367 (OH), 2933 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 5.51 (1H, br d, $J = 5.3$ Hz, H-6), 4.82 (1H, d, $J = 7.8$ Hz, H-1′), 4.77 (1H, ddd, $J = 7.6$, 7.0, 4.3 Hz, H-16), 4.38 (1H, ddd, $J = 11.5$, 10.2, 4.8 Hz, H-11), 4.16 (1H, br d, $J = 4.9$ Hz, H-22), 3.93 (1H, dd, $J = 9.5$, 6.6 Hz, H-26a), 3.92 (1H, overlapping, H-3), 3.63 (1H, dd, $J = 9.5$, 5.9 Hz, H-26b), 1.42 (3H, s, Me-19), 1.25 (3H, s, Me-18), 1.20 (3H, d, $J = 7.0$ Hz, Me-21), 1.00 (3H, d, $J = 6.7$ Hz, Me-27); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (*m*/*z*): 613.3956 $[M + H]^+$ (calc. for $C_{33}H_{57}O_{10}$, 613.3952).

Table 1 ^{13}C-NMR chemical shift assignments for compounds **1**, **4–6**, **9–12**, **12a**, **13**, and **13a** in C_5D_5N

Position	**1**	**4**	**5**	**6**	**9**	**10**	**11**	**12**	**12a**	**13**	**13a**
1	40.0	40.0	37.5	38.8	39.0	36.7	39.4	39.6	40.1	40.0	40.1
2	32.9	32.9	30.2	30.1	32.7	29.5	32.4	30.4	33.0	32.9	33.0
3	71.7	71.7	78.0	77.7	71.4	76.7	70.0	78.3	71.7	71.7	71.7
4	44.1	44.2	39.0	32.8	37.3	27.0	31.7	39.8	44.2	44.2	44.2
5	142.9	142.9	140.9	47.8	48.7	56.4	57.2	141.7	142.9	142.9	142.9
6	120.9	121.0	121.9	70.9	71.2	209.7	210.4	121.5	120.9	120.9	120.9
7	32.4	32.4	32.3	40.7	41.0	46.7	46.6	32.2	32.4	32.4	32.4
8	31.9	32.0	31.9	30.6	31.2	37.5	36.4	31.7	31.9	31.9	31.9
9	57.3	57.3	50.5	54.7	48.3	53.8	60.1	57.0	57.3	57.3	57.3
10	38.9	38.9	37.0	36.0	35.8	40.9	43.4	38.8	38.9	38.9	38.8
11	68.2	68.2	21.1	21.2	29.5	21.4	67.9	68.1	68.2	68.1	68.1
12	52.4	52.4	40.4	40.3	72.5	39.8	52.2	52.1	52.3	52.2	52.2
13	43.4	43.4	40.6	42.9	47.0	43.0	43.0	43.2	43.4	43.3	43.2
14	54.5	54.6	55.0	54.5	45.9	54.5	54.1	54.3	54.5	54.3	54.3
15	37.2	37.2	37.2	36.6	36.5	36.0	36.2	36.8	36.9	37.8	37.2
16	71.7	71.7	71.4	83.1	71.7	71.6	71.8	71.9	72.0	71.3	71.9
17	58.0	58.1	58.1	62.8	53.8	62.4	62.4	57.7	57.9	62.2	62.0
18	14.7	14.7	13.4	13.5	14.4	13.4	14.1	14.5	14.6	14.6	14.6
19	19.3	19.3	19.4	16.0	16.2	13.0	14.6	19.0	19.3	19.3	19.3
20	36.0	36.0	36.1	26.4	27.8	27.6	27.5	34.9	35.0	30.0	28.8
21	14.9	15.1	15.1	18.1	19.3	20.5	20.4	14.3	14.5	18.4	18.6
22	75.1	75.3	75.2	39.1	40.4	40.3	40.1	74.6	74.8	30.5	31.8
23	31.8	31.9	31.8	69.4	71.7	71.6	71.5	37.1	37.1	30.2	32.2
24	31.5	31.5	31.6	80.4	79.5	79.5	79.5	76.4	76.5	83.4	74.1
25	34.2	37.0	34.2	30.1	30.0	30.0	30.0	34.7	34.8	147.1	149.7
26	75.3	67.6	75.3	17.3	17.2	17.2	17.2	19.0	19.1	111.7	109.7
27	17.6	17.6	17.6	20.4	20.4	20.5	20.4	17.8	17.9	18.4	18.4
1′	104.9		100.4	102.0		102.2		102.3		103.4	
2′	75.2		77.8	75.4		75.4		75.3		75.5	
3′	78.6		79.7	78.6		78.6		78.5		78.6	
4′	71.7		71.8	71.8		71.8		71.6		71.7	
5′	78.5		78.3	78.6		78.6		78.3		78.3	
6′	62.8		62.7	63.0		63.0		62.7		62.9	
1″			102.1	107.4							
2″			72.6	75.8							
3″			72.8	78.5							
4″			74.2	71.7							
5″			69.5	78.0							
6″			18.7	63.0							
1‴			104.9								
2‴			75.3								
3‴			78.6								
4‴			71.7								
5‴			78.5								
6‴			62.8								

Enzymatic hydrolysis of **1**

Compound **1** (15.0 mg) was treated with naringinase (EC 232-962-4, Sigma-Aldrich, 35.2 mg) in HOAc/KOAc buffer (pH 4.3, 20 mL) at room temperature for 24 h. The reaction mixture was diluted with H_2O (10 mL) and extracted with EtOAc (10 mL × 3). After concentration of the EtOAc-soluble phase, the mixture was chromatographed on silica gel eluted with $CHCl_3$–MeOH (9:1) to yield **4** (10.2 mg). The H_2O-soluble phase was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (7:4:1) to yield a sugar fraction (3.6 mg). The sugar fraction was analyzed by HPLC under the following conditions: column, Capcell Pak NH_2 UG80 (4.6 mm i.d. × 250 mm, 5 μm, Shiseido, Tokyo, Japan); solvent, MeCN–H_2O (17:3); flow rate, 1.0 mL/min; detection, OR. Identification of D-glucose present in the sugar fraction was carried out by comparing the retention time and optical rotation with those of an authentic sample. t_R (min): 17.31 (D-glucose, positive optical rotation).

Compound **4**

Orthorhombic crystal; mp 235–240 °C; $[\alpha]_D^{25}$ −32.9 ($c = 0.10$, MeOH); IR (film) ν_{max} cm^{-1}: 3334 (OH), 2926 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 5.52 (1H, br d, $J = 5.0$ Hz, H-6), 4.78 (1H, ddd, $J = 7.7$, 7.0, 4.4 Hz, H-16), 4.39 (1H, ddd, $J = 10.8$, 10.3, 4.3 Hz, H-11), 4.22 (1H, m, H-22), 3.93 (1H, m, $W_{1/2} = 23.2$ Hz, H-3), 3.78 (1H, dd, $J = 10.2$, 5.6 Hz, H-26a), 3.71 (1H, dd, $J = 10.2$, 5.6 Hz, H-26b), 1.43 (3H, s, Me-19), 1.27 (3H, s, Me-18), 1.22 (3H, d, $J = 7.0$ Hz, Me-21), 1.11 (3H, d, $J = 6.6$ Hz, Me-27); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESI-TOFMS (*m/z*): 451.3452 $[M + H]^+$ (calc. for $C_{27}H_{47}O_5$, 451.3424).

Compound **5**

Amorphous powder; $[\alpha]_D^{27}$ −56.2 ($c = 0.10$, MeOH); IR (film) ν_{max} cm^{-1}: 3367 (OH), 2933 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 6.40 (1H, br s, H-1″), 5.32 (1H, br d, $J = 4.6$ Hz, H-6), 5.08 (1H, d, $J = 7.2$ Hz, H-1′), 4.84 (1H, d, $J = 7.8$ Hz, H-1‴), 4.76 (1H, ddd, $J = 7.4$, 6.9, 4.4 Hz, H-16), 4.18 (1H, overlapping, H-22), 3.96 (1H, overlapping, H-3), 3.95 (1H, dd, $J = 9.3$, 6.8 Hz, H-26a), 3.64 (1H, dd, $J = 9.3$, 6.0 Hz, H-26b), 1.79 (3H, d, $J = 6.2$ Hz, Me-6″), 1.20 (3H, d, $J = 7.0$ Hz, Me-21), 1.16 (3H, s, Me-18), 1.10 (3H, s, Me-19), 1.02 (3H, d, $J = 6.7$ Hz, Me-27); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (*m/z*): 927.4988 $[M + Na]^+$ (calc. for $C_{45}H_{76}O_{18}Na$, 927.4929).

Acid hydrolysis of **5**

A solution of **5** (30.3 mg) in 1 M HCl (dioxane-H_2O, 1:1, 4 mL) was heated at 95 °C for 1.5 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-96SB column (Organo, Tokyo, Japan) and chromatographed on a Diaion HP-20 column eluted with MeOH–H_2O (2:3), followed by EtOH–Me_2CO (1:1). The MeOH–H_2O eluted fraction was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (7:4:1) to yield the sugar fraction (16.6 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of **1** showed the presence of D-glucose and L-rhamnose. t_R (min): 16.41 (D-glucose, positive optical rotation), 9.07 (L-rhamnose, negative optical rotation).

Compound **6**

Amorphous powder; $[\alpha]_D^{25}$ −12.2 ($c = 0.26$, MeOH); IR (film) ν_{max} cm^{-1}: 3219 (OH), 2935 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 5.12 (1H, d, $J = 7.7$ Hz, H-1′), 4.73 (1H, d, $J = 7.6$ Hz, H-1″), 4.30 (1H, overlapping, H-16), 4.21 (1H, overlapping, H-23), 4.14 (1H, m, $W_{1/2} = 20.8$ Hz, H-3), 3.92 (1H, br d, $J = 1.7$ Hz, H-6), 3.80 (1H, m, H-24), 1.28 (3H, d, $J = 6.5$ Hz, Me-21), 1.24 (3H, s, Me-19), 1.14 (3H, d, $J = 6.7$ Hz, Me-27), 0.86 (3H, s, Me-18); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESI-TOFMS (*m/z*): 799.4421 $[M + Na]^+$ (calc. for $C_{39}H_{68}O_{15}Na$, 799.4456).

Acid hydrolysis of **6**

A solution of **6** (5.0 mg) in 1 M HCl (dioxane-H_2O, 1:1, 3 mL) was heated at 95 °C for 1.5 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-96SB column and chromatographed on a Diaion HP-20 column, eluted with MeOH–H_2O (2:3), followed by EtOH–Me_2CO (1:1). The EtOH–Me_2CO eluted fraction was chromatographed on silica gel eluted with $CHCl_3$–MeOH (7:1) to yield **6a** (0.6 mg). The MeOH–H_2O eluted fraction was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (7:4:1) to yield the sugar fraction (2.3 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of **1** showed the presence of D-glucose. t_R (min): 16.41 (D-glucose, positive optical rotation).

Compound **6a**

Amorphous powder; $[\alpha]_D^{25}$ −3.2 ($c = 0.10$, MeOH); IR (film) ν_{max} cm^{-1}: 3359 (OH), 2929 (CH); HR-ESITOFMS (*m/z*): 475.3382 $[M + Na]^+$ (calc. for $C_{27}H_{48}O_5Na$, 475.3399).

Compound **9**

Amorphous powder; $[\alpha]_D^{26}$ −11.8 (c = 0.10, MeOH); IR (film) ν_{max} cm^{-1}: 3288 (OH), 2770 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 4.75 (1H, ddd, J = 7.2, 7.2, 4.4 Hz, H-16), 4.32 (1H, br s, $W_{1/2}$ = 5.7 Hz, H-12), 4.12 (1H, m, H-23), 4.05 (1H, br d, J = 2.0 Hz, H-6), 3.95 (1H, m, $W_{1/2}$ = 22.1 Hz, H-3), 3.67 (1H, dd, J = 6.4, 4.7 Hz, H-24), 2.38 (1H, dd, J = 11.3, 7.2 Hz, H-17), 1.90 (1H, m, H-11eq), 1.89 (1H, m, H-11ax), 1.47 (3H, d, J = 6.6 Hz, Me-21), 1.44 (3H, s, Me-19), 1.27 (3H, d, J = 6.7 Hz, Me-26), 1.26 (3H, s, Me-18), 1.11 (3H, d, J = 6.9 Hz, Me-27); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (m/z): 469.3511 $[M + H]^+$ (calc. for $C_{27}H_{49}O_6$, 469.3529).

Compound **10**

Amorphous powder; $[\alpha]_D^{23}$ −16.4 (c = 0.10, MeOH); IR (film) ν_{max} cm^{-1}: 3375 (OH), 2932 (CH), 1708 (C=O); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 5.06 (1H, d, J = 7.7 Hz, H-1′), 4.57 (1H, ddd, J = 7.4, 7.0, 4.8 Hz, H-16), 4.09 (1H, overlapping, H-23), 3.99 (1H, overlapping, H-3), 3.69 (1H, dd, J = 11.0, 5.3 Hz, H-24), 1.19 (3H, d, J = 6.6 Hz, Me-21), 0.66 (3H, s, Me-19), 1.28 (3H, d, J = 6.7 Hz, Me-26), 1.17 (3H, d, J = 6.9 Hz, Me-27), 1.00 (3H, s, Me-18), 0.66 (3H, s, Me-19); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (m/z): 635.3766 $[M + Na]^+$ (calc. for $C_{33}H_{56}O_{10}Na$, 635.3771).

Compound **10a**

Amorphous powder; $[\alpha]_D^{25}$ +8.4 (c = 0.04, MeOH); IR (film) ν_{max} cm^{-1}: 3375 (OH), 2924 (CH), 1714 (C=O); CD λ_{max} (MeOH) nm ($\Delta\varepsilon$): 294 (−13.6); HR-ESITOFMS (m/z): 473.3244 $[M + Na]^+$ (calc. for $C_{27}H_{46}O_5Na$, 473.3243).

Compound **11**

Amorphous powder; $[\alpha]_D^{25}$ +21.9 (c = 0.10, MeOH); IR (film) ν_{max} cm^{-1}: 3377 (OH), 2929 (CH), 1702 (C=O); CD λ_{max} (MeOH) nm ($\Delta\varepsilon$): 294 (−6.84); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 4.62 (1H, ddd, J = 7.1, 7.1, 4.6 Hz, H-16), 4.22 (1H, ddd, J = 10.4, 10.4, 4.9 Hz, H-11), 4.08 (1H, m, H-23), 3.90 (1H, m, $W_{1/2}$ = 22.1 Hz, H-3), 3.68 (1H, dd, J = 6.4, 4.7 Hz, H-24), 2.62 (1H, dd, J = 12.2, 4.9 Hz, H-12eq), 1.65 (1H, dd, J = 10.4, 10.4 Hz, H-9), 1.60 (1H, dd, J = 12.2, 10.4 Hz, H-12ax), 1.27 (3H, d, J = 6.7 Hz, Me-26), 1.19 (3H, d, J = 6.6 Hz, Me-21), 1.15 (3H, d, J = 6.9 Hz, Me-27), 1.07 (3H, s, Me-18), 1.07 (3H, s, Me-19); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (m/z): 467.3372 $[M + H]^+$ (calc. for $C_{27}H_{47}O_6$, 467.3373).

Compound **12**

Amorphous powder; $[\alpha]_D^{22}$ −26.0 (c = 0.10, MeOH); IR (film) ν_{max} cm^{-1}: 3371 (OH), 2929 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 5.42 (1H, br d, J = 5.5 Hz, H-6), 5.06 (1H, d, J = 7.7 Hz, H-1′), 4.76 (1H, ddd, J = 7.4, 7.0, 4.4 Hz, H-16), 4.52 (1H, overlapping, H-22), 4.30 (1H, overlapping, H-11), 4.01 (1H, overlapping, H-3), 3.87 (1H, m, H-24), 1.27 (3H, s, Me-19), 1.21 (3H, d, J = 7.3 Hz, Me-21), 1.20 (3H, s, Me-18), 1.06 (3H, d, J = 6.8 Hz, Me-27), 1.02 (3H, d, J = 6.8 Hz, Me-26); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (m/z): 613.3969 $[M + H]^+$ (calc. for $C_{33}H_{57}O_{10}$, 613.3952).

Compound **12a**

Amorphous powder; $[\alpha]_D^{25}$ −6.6 (c = 0.04, MeOH); IR (film) ν_{max} cm^{-1}: 3346 (OH), 2924 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 5.52 (1H, br d, J = 5.3 Hz, H-6), 4.78 (1H, ddd, J = 7.5, 7.5, 4.6 Hz, H-16), 4.54 (1H, m, H-22), 4.39 (1H, ddd, J = 10.6, 10.6, 4.8 Hz, H-11), 3.93 (1H, m, $W_{1/2}$ = 21.7 Hz, H-3), 3.88 (1H, m, H-24), 2.75 (1H, overlapping, H-4ax), 2.62 (1H, m, H-20), 2.02 (1H, m, H-23a), 1.81 (1H, m, H-23b), 1.78 (1H, dd, J = 11.2, 7.5 Hz, H-17), 1.63 (1H, overlapping, H-8), 1.53 (1H, overlapping, H-1ax), 1.43 (3H, s, Me-19), 1.37 (1H, dd, J = 10.6, 10.1 Hz, H-9), 1.26 (3H, s, Me-18), 1.24 (3H, d, J = 7.1 Hz, Me-21), 1.12 (1H, overlapping, H-14), 1.08 (3H, d, J = 6.8 Hz, Me-26), 1.04 (3H, d, J = 6.8 Hz, Me-27); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (m/z): 473.3202 $[M + Na]^+$ (calc. for $C_{27}H_{46}O_5Na$, 473.3243).

Compound **13**

Amorphous powder; $[\alpha]_D^{25}$ −12.4 (c = 0.10, MeOH); IR (film) ν_{max} cm^{-1}: 3371 (OH), 2929 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 5.51 (1H, br d, J = 6.7 Hz, H-6), 5.17 (1H, br s, H-26a), 4.93 (1H, br s, H-26b), 4.93 (1H, d, J = 7.9 Hz, H-1′), 4.57 (1H, ddd, J = 7.5, 7.5, 4.4 Hz, H-16), 4.47 (1H, m, H-24), 4.36 (1H, ddd, J = 10.3, 10.3, 4.5 Hz, H-11), 3.93 (1H, m, $W_{1/2}$ = 21.7 Hz, H-3), 1.87 (3H, s, Me-27), 1.41 (3H, s, Me-19), 1.21 (3H, d, J = 7.3 Hz, Me-21), 1.19 (3H, s, Me-18); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (m/z): 617.3690 $[M + Na]^+$ (calc. for $C_{33}H_{54}O_9Na$, 617.3666).

Compound **13a**

Amorphous powder; $[\alpha]_D^{23}$ +6.6 (c = 0.10, MeOH); IR (film) ν_{max} cm^{-1}: 3357 (OH), 2926 (CH); ^{1}H-NMR (C_5D_5N, 500 MHz) δ: 5.52 (1H, br d, J = 5.6 Hz, H-6), 5.24 (1H, br s, H-26a), 4.93 (1H, br s, H-26b), 4.62 (1H, ddd, J = 7.6, 7.2, 4.6 Hz, H-16), 4.44 (1H, dd, J = 8.6,

4.7 Hz, H-24), 4.36 (1H, ddd, $J = 10.7$, 10.7, 4.7 Hz, H-11), 3.94 (1H, m, $W_{1/2} = 23.6$ Hz, H-3), 1.87 (3H, s, Me-27), 1.86 (2H, m, H_2-23),1.42 (3H, s, Me-19), 1.20 (3H, s, Me-18), 1.11 (3H, d, $J = 6.7$ Hz, Me-21); ^{13}C-NMR (C_5D_5N, 125 MHz): Table 1; HR-ESITOFMS (*m*/*z*): 455.3118 $[M + Na]^+$ (calc. for $C_{27}H_{44}O_4Na$, 455.3137).

Enzymatic hydrolysis of **10**, **12**, and **13**

Compounds **10** (2.5 mg), **12** (3.0 mg), and **13** (5.5 mg) were independently subjected to enzymatic hydrolysis as described for **1** to give the aglycones (**10a**: 0.7 mg, **12a**: 0.8 mg, **13a**: 2.4 mg) and sugar fractions (**10**: 1.1 mg, **12**: 1.0 mg, **13**: 2.0 mg). HPLC analysis of the sugar fractions under the same conditions as in the case of **1** showed the presence of D-glucose.

Cell culture and assay for cytotoxic activity against HL-60 cells

HL-60 cells were maintained in RPMI 1640 medium containing 10 % heat-inactivated FBS and antibiotics (100 U/mL penicillin sodium salt and 100 μg/mL streptomycin sulfate) in a 5 % CO_2 humidified incubator at 37 °C. The cells were washed and suspended in the medium to a concentration of 4×10^4 cells/mL, and 196 μL of this cell suspension was divided into 96-well flat-bottom plates. The cells were incubated in 5 % CO_2/air for 24 h at 37 °C. After incubation, 4 μL of EtOH–H_2O (1:1) solution containing the sample was added to produce a final concentration of 0.1–20 μM, and 4 μL of EtOH–H_2O (1:1) was added to the control wells. The cells were further incubated for 72 h in the presence of each agent, and cell growth was subsequently evaluated using a modified MTT reduction assay [12]. After incubation, 10 μL of 5 mg/mL MTT in phosphate buffered saline (PBS) was added to each well, and the plate was further incubated in 5 % CO_2/air for 4 h at 37 °C. The plate was then centrifuged at 1500*g* for 5 min to precipitate the MTT formazan. An aliquot of 150 μL of the supernatant was removed from each well, and 175 μL of dimethylsulfoxide (DMSO) was added to dissolve the MTT formazan crystals. The plate was mixed on a microplate mixer for 10 min and read in a microplate reader at 550 nm. Each assay was performed in triplicate. Cytotoxicity is expressed as an IC_{50} value, which indicates the level at which there was a 50 % reduction in the number of viable cells.

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