Cytotoxic Steroidal Glycosides from the Whole Plant of *Calamus acanthophyllus**

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Key words
- *Calamus acanthophyllus*
- Arecaceae
- steroidal glycoside
- cytotoxic activity

Abstract

A new steroidal glycoside, callaphylloside (**1**), together with seven known glycosides (**2**–**8**), was isolated from the whole plant of *Calamus acanthophyllus*. The structure of the new compound was elucidated by spectral data analyses and chemical transformations. Compounds **5** and **8** exhibited strong cytotoxic activity against four cancer cell lines ($0.7 \leq IC_{50} \leq 3.4\,\mu M$). Evaluation of the structure-activity relationship among steroidal glycosides revealed that the structure of spirostanol with an α-L-rhamnopyranosyl linked to C-2 of the inner glucopyranosyl residue both play a critical role in the effects of these compounds on the cancer cell lines.

Supporting information available online at http://www.thieme-connect.de/products

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Introduction

Calamus acanthophyllus Becc. (Arecaceae), a small palm, is known in Thailand as "Wai Nang". Fresh young twigs of this plant have traditionally been chewed to relieve pain from insect bites. A previous phytochemical study on the stem of *Calamus insignis* Griff. led to the isolation of five steroidal glycosides, including four spirostanol-type and one furostanol-type, some of which showed cell growth and cell cycle inhibitory activities [1]. In addition, steroidal glycosides were also reported to possess bioactivities, including antiproliferative activity [1–3], a cyclic AMP phosphodiesterase inhibitor [4], and a Na+/K+ ATPase inhibitor [5]. An initial investigation revealed that the water-soluble portion from the MeOH extract of the whole plant of *C. acanthophyllus* showed cytotoxic activity, therefore, we have thus undertaken the chemical investigation of bioactive compounds of the methanolic extract of this plant. Herein we report the isolation and characterization of a new compound, callaphylloside (**1**), together with seven known glycosides (**2**–**8**; **Fig. 1**). The structures of the isolated compounds **1**–**8** were established on the basis of spectral studies and chemical evidence.

* This paper is dedicated to Professor Dr. Dr. h.c. mult. Kurt Hostettmann for his outstanding lifetime achievements.

Results and Discussion

Solvent partition and repeated chromatographic analysis of the glycosides mixture obtained from a methanol extract of the whole plant of *C. acanthophyllus* yielded one new steroidal glycoside (**1**), together with seven known glycosides. Compounds **2**–**8** were identified as roseoside [(+)-(6*S*,9*R*)-9-*O*-*β*-D-glucopyranosyloxy-6-hydroxy-3-oxo-α-ionol] (**2**) [6,7], diosgenin-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-*β*-D-glucopyranoside (**3**) [8–10], diosgenin-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-[α-L-rhamnopyranosyl-(1 → 4)]-*β*-D-glucopyranoside (**4**) [8,11–13], diosgenin-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-[*β*-D-glucopyranosyl-(1 → 4)]-*β*-D-glucopyranoside (**5**) [9,10,13], diosgenin-3-*O*-[α-L-rhamnopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 4)]-*β*-D-glucopyranoside (**6**) [14], diosgenin-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-[α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)]-*β*-D-glucopyranoside (**7**) [11,15], and diosgenin-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-[α-L-rhamnopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 4)]-*β*-D-glucopyranoside (**8**) (causiaroside I) [1,16] by comparison of their NMR data with literature values.

Callaphylloside (**1**) was obtained as colorless granules (from MeOH-acetone), m.p. 247–249 °C, $[\alpha]_D^{33}$ −70.5° (pyridine). The IR spectrum of **1** showed absorption bands at 3364 (OH), 1710 (C=O), and

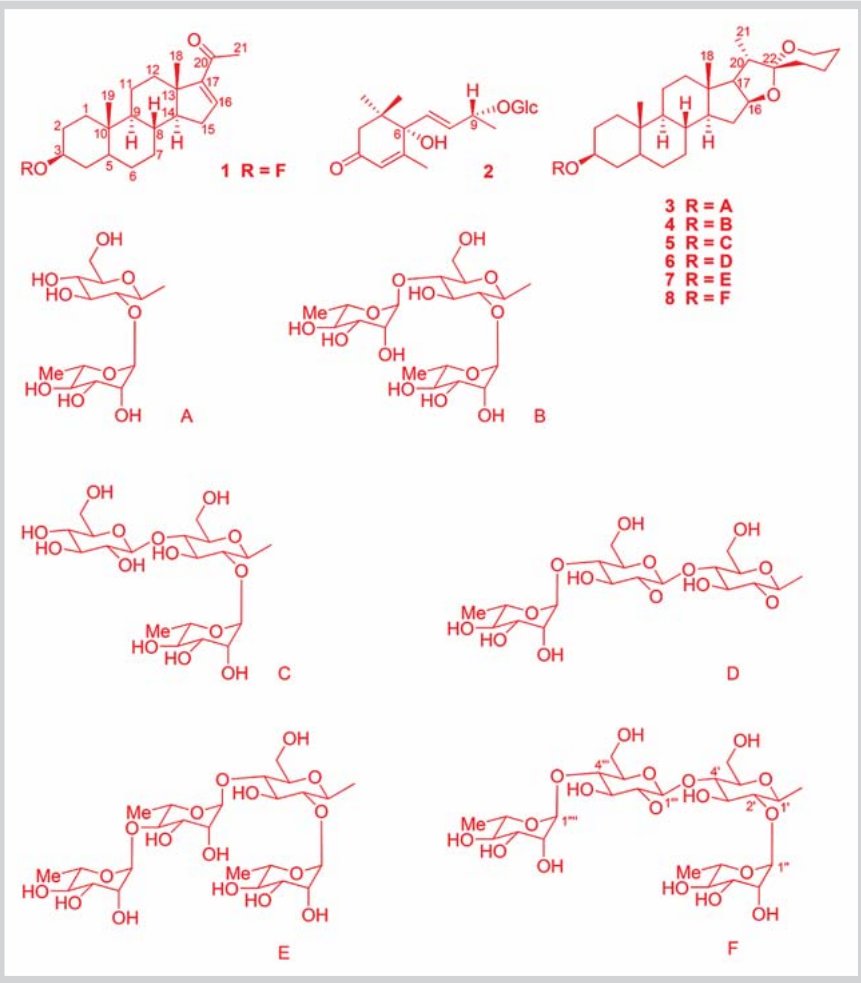

Fig. 1 Structures of the compounds isolated from *C. acanthophyllus*. (Color figure available online only.)

1043 (C–O) cm^{-1}. The negative ion HRFAB-MS gave an $[M - H]^-$ ion at *m/z* 929.43799, corresponding to a molecular formula of $C_{45}H_{70}O_{20}$. The TOF-MS (negative ion) showed a pseudo-molecular ion at *m/z* 965.4131 $[M + Cl]^-$ and fragment ions at *m/z* 819.36 $[M + Cl - 146.06]^-$, 673.30 $[M + Cl - 146.06–146.06]^-$, 657.30 $[M + Cl - 146.06–162.05]^-$, and 511.25 $[M + Cl - 146.06–146.06–162.05]^-$. The fragment ions corresponded to the successive losses of a deoxyhexose, two deoxyhexoses, a deoxyhexose and a hexose, and two deoxyhexoses and a hexose, respectively. The ^{13}C NMR spectrum of the saponin (**Table 1**) exhibited signals for four anomeric carbons (δ_C 99.8, 101.5, 102.4, and 104.8). This was consistent with the ^1H NMR of **1**, which contained resonances from four anomeric protons at δ_H 4.91 (d, J = 7.5 Hz), 5.06 (d, J = 7.7 Hz), 5.82 (br s), and 6.16 (br s). The peak at δ_C 78.0, showing a glycosidation shift, was suggestive of the linkage of the sugar moiety to the aglycone at C-3. Acid hydrolysis of compound **1** gave a sugar fraction containing L-rhamnose and D-glucose, which were analyzed by TLC in EtOAc-*n*-BuOH-H_2O (20 : 70 : 10, v/v), together with the authentic sugar samples. The aglycone of **1** was identified as pregna-5,16-diene-3β-ol-20-one by comparison of its NMR data with literature values [17,18]. The TOF (positive ion) mass spectrum of **1** gave a $[M + H-616]^+$ ion at *m/z* 315.2318 and the HMBC correlations of **1** (**Fig. 2**) confirmed the structure of the aglycone. The complete structure of **1** was determined by extensive 2D NMR experiments at 600 MHz using a pyridine-d_5 solution. The ^1H NMR spectrum was fully assigned from a combination of the ^1H-^1H COSY, HSQC, ROESY, and HMBC experiments. The assignments are shown in **Table 1**. The ^{13}C NMR spectrum was then assigned by a combination of DEPT, HSQC, and HMBC experiments. Finally, the positions of the glycosidic linkages were established from the connectivities indicated in the HMBC and ROESY experiments (**Fig. 2**). The HMBC experiment showed a correlation between aglycone C-3 (δ 78.0) and glucose I, H-1′ (δ 4.91). The ^1H NMR signals of glucose I, H-2′ and H-4′ at δ 4.16 and 4.17, showed long-range correlations with the anomeric ^{13}C NMR signals of rhamnose I (δ 101.5; C-1″) and of glucose II (δ 104.8; C-1‴), implying glycosidic substitutions at C-2′ and C-4′ of glucose I. Similar long-range correlations were also observed between glucose II, H-4‴ at δ 4.43 and rhamnose II, C-1⁗ (δ 102.4). The ^1H NMR spectrum of the peracetate derivative of **1** (see Materials and Methods) showed no significant changes in the signals of H-2′ (δ 4.02) and H-4′ (δ 4.17) of glucose I and H-4‴ (δ 4.14) of glucose II, confirming the glycosidic substitutions at C-2′ and C-4′ of glucose I and C-4‴ of glucose II. The ROESY experiment showed cross-peaks between H-1, H-3, and H-5 of the glucoses, thus pointing to β-D-configurations of the two glucoses, I and II. The large coupling constants of the anomeric protons [J = 7.5 Hz (H-1′) and 7.7 Hz (H-1‴)] also reflected the β-configurations of the two glucoses. The α-configuration of the two rhamnoses, I and II of **1**, were inferred from the carbon signals for C-3 [δ 72.6 (C-3″) and 72.5 (C-3⁗)] and for C-5 [δ 69.2 (C-5″) and 70.2 (C-5⁗)] [19]. The structure of callaphylloside (**1**) was thus deduced as pregna-5,16-diene-3-β-ol-20-one-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-[α-L-

Table 1 NMR data (pyridine-d_5) of compound **1** (^{1}H NMR 600 MHz, ^{13}C NMR 100 MHz).

Position	δ_C	δ_H, mult.	Sugar	Position	δ_C	δ_H, mult.
1	37.2	0.92, overlapped; 1.67, m	Glc I	1′	99.8	4.91, d (7.5)
2	29.9	1.82, m; 2.06, m		2′	76.9	4.16, overlapped
3	78.0	3.83, m		3′	76.2	4.13, overlapped
4	38.8	2.68, m; 2.73, m		4′	81.6	4.17, m
5	141.1			5′	76.0	3.80, m
6	121.3	5.30, br s		6′	61.5	4.42, overlapped; 4.50, m
7	31.6	1.55, m; 1.88 m	Rha I	1′′	101.5	6.16, br s
8	30.2	1.53, m		2′′	72.2	4.71, br s
9	50.6	0.93, m		3′′	72.6	4.56, overlapped
10	37.0	–		4′′	73.8	4.34, overlapped
11	20.7	1.49, 2 H, m		5′′	69.2	4.90, m
12	34.9	1.35, m; 2.58, br d (11.7)		6′′	18.4	1.73, d (6.2)
13	46.1	–	Glc II	1′′′	104.8	5.06, d (7.7)
14	56.3	1.30, m		2′′′	74.9	3.97, t (8.3)
15	32.1	1.90, m; 2.17, m		3′′′	77.4	4.15, overlapped
16	144.4	6.64, br s		4′′′	77.6	4.43, overlapped
17	155.1	–		5′′′	77.0	3.72, br d (8.2)
18	15.7	0.92, s		6′′′	60.8	4.05, br d (10.1); 4.16, overlapped
19	19.0	1.03, s	Rha II	1′′′′	102.4	5.82, overlapped
20	196.1	–		2′′′′	72.3	4.64, br s
21	26.9	2.25, s		3′′′′	72.5	4.53, overlapped
				4′′′′	73.7	4.33, overlapped
				5′′′′	70.2	4.96, m
				6′′′′	18.3	1.68, d (6.2)

Assignments are based on HSQC, HMBC, DEPT, and ROESY experiments; δ in ppm; proton coupling constants (J) in Hz are given in parentheses.

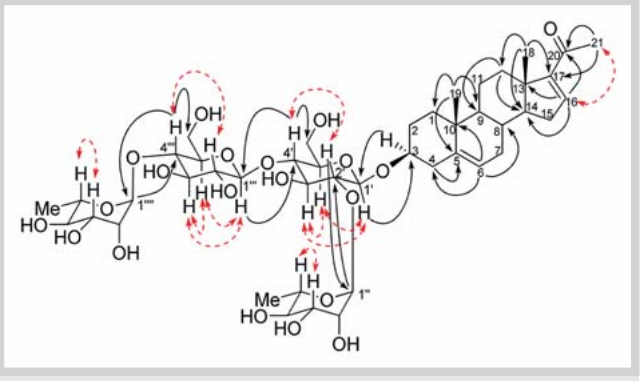

Fig. 2 Selected HMBC (→) and ROESY (red ←----→) NMR correlations for compound **1**. (Color figure available online only.)

rhamnopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 4)]-*β*-D-glucopyranoside.

It is interesting to note that the aglycone in **1** is different from that of other glycosides (**3**–**8**), which contain diosgenin as the aglycone. The skeleton of the pregnane in **1** may arise from the oxidative cleavage at C20–C22 of diosgenin [20]. Since the saccharide of **1** is the same as that of **8**, it is assumed that **1** may be derived from the C20–C22 oxidative cleavage of the aglycone of **8**.

The isolated compounds (**1**–**8**) and diosgenin were evaluated for their cytotoxicity against four cancer cell lines, HuCCA-1, KB, HeLa, and MDA-MB 231. Diosgenin and glycoside **2** showed no cytotoxicity and the pregnastanol saponin **1** (callaphylloside) and the diosgenin (spirostanol) saponin **6** were weakly toxic, whereas the spirostanol saponins (**3, 4, 5, 7**, and **8**) were highly toxic (**Table 2**). The cytotoxicity of **6** was much weaker than that of **8**. These findings show that the structure of spirostanol and an *α*-L-rhamnopyranosyl linked to C-2′ of the inner glucopyranosyl residue both play a critical role in the effects of these compounds on these cancer cell lines. The attachment of a *β*-D-glucopyranosyl to C-4′ of the inner glucopyranosyl residue of **3** increased the cytotoxicity (**5**). When an *α*-L-rhamnopyranosyl or two *α*-L-rhamnopyranosyls were linked to C-4 of the inner glucopyranosyl residue of **3**, no profound effect on the activity (**4** and **7**) was observed.

Diosgenin glycosides were previously reported [1,2] to possess cytotoxicity against HeLa and HL-60 cell lines with IC_{50} values ranging from 1.5 to 13.9 and 2.0 to 9.7 µM, respectively. Our study supported that the diosgenin glycosides, compounds **3, 4, 5, 7**, and **8**, from *C. acanthophyllus,* exhibited cytotoxic activity against four cancer cell lines, HuCCA-1, KB, HeLa, and MDA-MB 231, with IC_{50} values ranging from 0.7 to 8.4 µM.

Materials and Methods

Melting points were determined on a Büchi 535 and were uncorrected. Optical rotations were recorded on a JASCO DIP 1020 polarimeter. FT-IR was obtained using a universal attenuate total reflectance (ATR) attached to a Perkin-Elmer Spectrum One spectrometer. FABMS (recorded in glycerol matrix) was measured on a Finigan MAT 90 mass spectrometer. TOF-MS spectra (APCI) were measured on a Bruker microTOF mass spectrometer. ^{1}H and ^{13}C NMR measurements were performed on a Bruker AM400, AVANCE500, or AVANCE600 spectrometer. Chemical shifts were referred to the residual solvent signal (pyridine-d_5: δ_H 7.21; δ_C 123.5). Silica gel 60 (Merck, 63–200 µm) was used for column chromatography, and precoated silica gel plates (Merck, Kieselgel 60 F_{254}) were used for TLC analysis. Gel permeation

Table 2 Cytotoxic activity of glycosides **1**–**8** and diosgenin.

Compounds	Cytotoxicity (IC_{50} μM)			
	HuCCA-1	KB	HeLa	MDA-MB 231
1	32.3	> 50	34.4	29.0
2	> 50	> 50	> 50	> 50
3	5.8	4.2	3.7	3.5
4	8.4	6.2	3.6	1.7
5	3.4	1.2	0.9	1.1
6	41.9	33.9	43.0	33.9
7	3.0	6.2	3.0	3.1
8	2.6	0.9	0.7	0.9
Diosgenin	> 50	> 50	> 50	> 50
Etoposide	4.8	0.9	0.5	–
Doxorubicin	–	–	–	0.4

was visualized by spraying the chromatograms with *p*-anisaldehyde-ethanol-H_2SO_4-H_2O (2 : 98 : 3 : 4, v/v) and heating at 110 °C.

Plant material

Calamus acanthophyllus was collected from Ubon Rachathani Province, Thailand. The plant was identified by Dr. Wongsasit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. A voucher specimen (CRI 24) is deposited at the Laboratory of Natural Products, Chulabhorn Research Institute, Bangkok, Thailand.

Extraction and isolation

The whole plant of *C. acanthophyllus* was air-dried, powdered (1.74 kg), and extracted at room temperature with MeOH (3 × 5 L, 24 h each). The MeOH extract was concentrated under reduced pressure and partitioned between CH_2Cl_2 and H_2O. The aqueous layer was evaporated *in vacuo* and freeze-dried to give a brown solid (213.2 g), which exhibited anticancer activity (IC_{50} of 15.0 μg/mL). A portion of the aqueous fraction (90 g) was subjected to column chromatography on silica gel (15 × 17 cm) eluting with CH_2Cl_2-MeOH-H_2O, lower layer [12 : 3 : 1 (3 L), 10 : 3 : 1 (10 L), 7 : 3 : 1 (3 L), 7 : 3.2 : 1 (33 L), 6.5 : 3.5 : 1 (12 L), 6 : 4 : 1 (10 L)]. The collected fractions (500 mL, each) were combined on the basis of their TLC characteristics to afford seven fractions (A–G). Fraction B (20–28 L, 800 mg) was further subjected to column chromatography on silica gel (3.5 × 27 cm) eluting with CH_2Cl_2-MeOH-H_2O, lower layer [10 : 3.5 : 1 (3 L)], to give four fractions (B1–B4). Fraction B2 (1.2–1.8 L, 390 mg) was chromatographed on a column of silica gel (3 × 50 cm) eluting with hexane-EtOAc-MeOH (2 L) to yield **2** (40 mg) and **3** (41 mg), while B3 (1.9–2.4 L, 300 mg) was separated on a column of silica gel (1 × 60 cm) eluting with EtOAc-acetone-MeOH-H_2O (10 : 10 : 1 : 1, 2.5 L) to yield **4** (63 mg) and **5** (69 mg). Fraction C (28–39 L, 1.4 g) was subjected to column chromatography on silica gel (3 × 25 cm) eluting with MeOH-acetone-CH_2Cl_2 (1 : 4 : 6, 6 L) and to yield compound **6** (60 mg). Fraction D (41.5–45.5 L, 1.2 g) was subjected to column chromatography on silica gel (3.5 × 30 cm) eluting with CH_2Cl_2-MeOH-H_2O, lower layer [15 : 3 : 1 (10 L)], to give four fractions (D1–D4). Fraction D3 (7.0–8.8 L, 590 mg) was chromatographed on a column of silica gel (3.5 × 30 cm) eluting with CH_2Cl_2-MeOH-H_2O, lower layer [12 : 3 : 1 (10 L)], to yield **7** (24.1 mg). Fraction E (46.0–49.5 L, 3.5 g) was crystallized from MeOH-H_2O to give compound **8** (2.1 g). Fraction F (64.5–66 L, 13.3 g) was subjected to column chromatography on silica gel (9 × 25 cm) eluting with CH_2Cl_2-MeOH-H_2O, lower layer [12 : 3 : 1 (3 L), 10 : 3 : 1 (5 L), 7 : 3 : 1 (3 L), 6.5 : 3.5 : 1 (3 L), 6 : 4 : 1 (3 L)]. The collected fractions (300 mL, each) were combined on the basis of their TLC characteristics to afford three fractions (F1–F3). F2 (11–12.6 L, 1.5 g) was chromatographed on a column of silica gel (3.5 × 25 cm) eluting with CH_2Cl_2-MeOH-H_2O [12 : 3 : 1 (lower layer, 6.7 L)] to afford compound **1** (11 mg) as colorless granules from MeOH-acetone.

Callaphylloside (**1**): colorless granules (MeOH-acetone), m. p. 247–249 °C, $[\alpha]_D^{33}$ −70.5° (*c* 0.13, pyridine); IR (ATR) ν_{max} 3364, 2923, 2850, 1710, 1652, 1370, 1117, 1043, 835, 811 cm^{-1}; ^1H and ^{13}C NMR: (**Table 1**); HRFABMS (negative ion): *m/z* 929.4379 $[M - H]^-$ (calcd. for $C_{45}H_{69}O_{20}$ 929.4382), TOF-MS-MS (negative ion): *m/z* 965.4131 $[M + Cl]^-$, 819.3586 $[M + Cl - 146]^-$ (calcd. for $C_{39}H_{60}O_{16}Cl$, 819.3575), 673.2971 $[M + Cl - 292]^-$ (calcd. for $C_{33}H_{50}O_{12}Cl$, 673.2996), 657.3036 $[M + Cl - 308]^-$ (calcd. for $C_{33}H_{50}O_{11}Cl$, 657.3047), 511.2468 $[M + Cl - 454]^-$ (calcd. for $C_{27}H_{40}O_7Cl$, 511.2474); TOF-MS-MS (positive ion): *m/z* 315.2318 $[M + H - 616]^+$ (calcd. for $C_{21}H_{31}O_2$ (aglycone part), 315.2319).

Acetylation of 1

Pyridine (0.5 mL) and acetic anhydride (2 mL) were added to compound **1** (5 mg) and the reaction mixture was allowed to stand overnight. Workup as usual gave peracetate **1** (4.5 mg).

^1H NMR of the sugar part of peracetate **1** *(400 MHz in pyridine-d_5)*: δ_H 4.86 (d, *J* = 7.6 Hz, H-1′), 4.02 (m, H-2′), 5.71 (t, *J* = 9.5 Hz, H-3′), 4.17 (t, *J* = 9.5 Hz, H-4′), 3.89 (m, H-5′), 4.86 (m, H-6′) and 4.54 (dd, *J* = 12.0, 5.2 Hz, H-6′), 5.48 (br d, *J* = 1.6 Hz, H-1″), 5.61 (m, H-2″), 5.81 (dd, *J* = 10.2, 3.3 Hz, H-3″), 5.67 (t, *J* = 9.2 Hz, H-4″), 4.86 (m, H-5″), 1.47 (d, *J* = 6.2 Hz, H-6″), 5.03 (m, H-1‴), 5.24 (dd, *J* = 9.5, 9.1 Hz, H-2‴), 5.64 (t, *J* = 9.6 Hz, H-3‴), 4.14 (t, *J* = 9.6 Hz, H-4‴), 4.05 (m, H-5‴), 4.70 (2 H, m, H-6‴), 5.26 (br s, H-1⁗), 5.54 (m, H-2⁗), 5.56 (dd, *J* = 10.0, 3.3 Hz, H-3⁗), 5.51 (m, H-4⁗), 4.02 (m, H-5⁗), 1.30 (d, *J* = 6.2 Hz, H-6⁗), 2.39, 2.26, 2.22, 2.18, 2.15, 2.12, 2.09, 2.06, 2.03, 2.00, 1.99, 1.95 (all s, $COCH_3$ and 11 OAc groups).

Acid hydrolysis of 1

A solution of **1** (2 mg) in 2 N HCl (1 mL) was reflux for 3 h. The reaction mixture was extracted with EtOAc. The aqueous phase was neutralized with $NaHCO_3$ and lyophilized. The lyophilized residue was dissolved in water (0.2 mL) and analyzed by TLC in EtOAc-*n*-BuOH-H_2O (20 : 70 : 10, v/v) together with the authentic sugar samples.

Cell culture

The cell line used in this experiment was human cholangiocarcinoma (HuCCA-1), which was prepared previously from tumor tissues of bile ducts obtained from Thai patients [21]. The HuCCA-1 was cultured in Hams F12 culture medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin. The human epidermoid carcinoma in the mouth (KB) cell line was obtained from University of Illinois. The human cervical carcinoma (HeLa) cell line was purchased from ATCC and cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. The hormone-independent breast cancer (MDA-MB 231) cell line was obtained from M. D. Anderson Cancer Center and cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine and nonessential amino acids (Sigma). Etoposide (Sigma; purity > 98%) and doxorubicin hydrochloride [Sigma; purity 98.0–102.0% (HPLC)] were used as the reference compounds.

Cytotoxicity assay

Bioassays against HuCCA-1, KB, HeLa, and MDA-MB 231 cell lines were based on reported procedures [22]. For the crystal violet staining assay, 100 µL samples of suspension of HeLa, MDA-MB 231, and KB (10 000 cells/well) and HuCCA-1 (15 000 cells/well) cells in the logarithmically growing phase were seeded in each well of the 96-well plates with the appropriately diluted sample. Etoposide and doxorubicin were prepared at concentrations of 0.0005, 0.005, 0.05, 0.5, 5, and 50 µg/mL in 100 µL of culture medium. The cells were cultured at 37 °C under 5% carbon dioxide incubators and cultured for 3 days. For cell fixation, each well received 20 µL glutaraldehyde (25%) and was left for at least 20 min. After being washed with water, the plates were stained with 0.4% crystal violet solution in methanol for 30 min. The plates were then dried. Absorbance at 550 nm was measured by an automatic microtiter plate reader (Multiskan Ascent). Average absorbance of the control wells, which received no chemical, was regarded as 100%, and the percentage cell growth in each well was calculated. The concentration that inhibited the growth of the cells to the level of 50% of the control culture (IC_{50}) was obtained from graphical plots.

Supporting Information

^{1}H, ^{13}C NMR, DEPT, COSY, ROESY, HSQC, and HMBC spectra of compound **1** are available as Supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

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