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Steroidal glycosides from the underground parts of *Trillium erectum* and their cytotoxic activity

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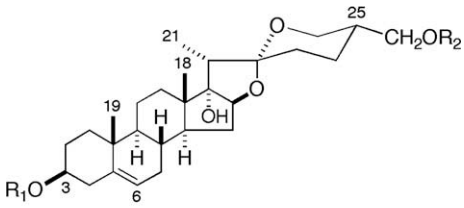
ABSTRACT

Six steroidal glycosides, along with 14 known compounds, were isolated from the underground parts of *Trillium erectum* L. (Liliaceae). The structures of **1**–**6** were determined on the basis of extensive spectroscopic analysis, including two-dimensional (2D) NMR data, and a few chemical transformations. The isolated compounds were evaluated for their cytotoxic activity against HL-60 human promyelocytic leukemia cells.

1. Introduction

Trillium erectum L. belongs to the family Liliaceae and is mainly distributed in Canada and the northeastern region of America (Tsukamoto, 1989). The rhizomes of *T. erectum* are called bethroot and have been used in folk medicine for the treatment of hemorrhages from uterus, urinary tract, and lungs (The Royal Horticultural Society, 1995). Nohara and co-workers made phytochemical examinations on domestic *Trillium* plants in Japan such as *T. tschonoskii* and *T. kamtschaticum* and isolated several steroidal saponins (Ono et al., 1986, 2007a). However, a literature survey concerning the secondary metabolites of *T. erectum* showed that no systematic chemical work has been carried out on the plant and only a few steroidal sapogenins such as diosgenin, kryptgenin, and trillagenin has been isolated and identified (Marker and Crueger, 1940; Marker et al., 1943). Therefore, our attention was directed to the constituents of the underground parts of *T. erectum*, on which a detailed phytochemical investigation was carried out. As a result, six new spirostanol saponins (**1**–**6**), along with 14 known steroidal glycosides (**7**–**20**) were isolated. This paper deals with the structural determination of the six new glycosides on the basis of spectroscopic analysis, including various two-dimensional (2D) NMR spectroscopic data, and a few chemical transformations. The cytotoxic activity of the isolated compounds against HL-60 leukemia cells is also reported.



	R_1	R_2
1	H	Glc
1a	H	H
2	S_1	Glc
3	S_2	Glc
4	S_3	Glc
5	Glc	H
6	S_2	H
7	S_1	H

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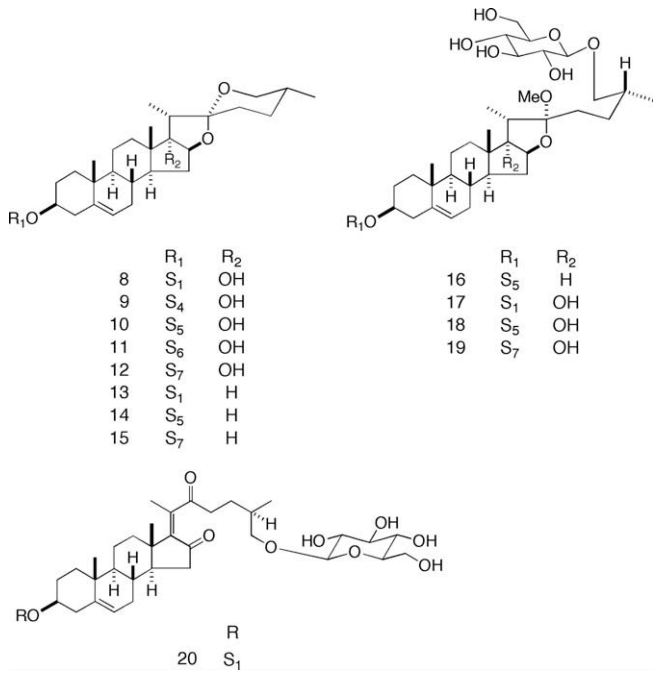
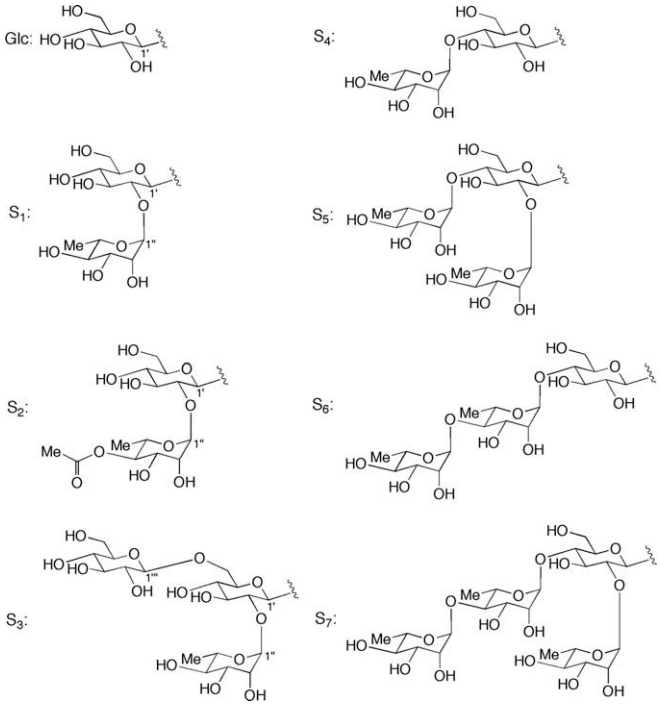


	R_1	R_2
8	S_1	OH
9	S_4	OH
10	S_5	OH
11	S_6	OH
12	S_7	OH
13	S_1	H
14	S_5	H
15	S_7	H

	R_1	R_2
16	S_5	H
17	S_1	OH
18	S_5	OH
19	S_7	OH

	R
20	S_1



2. Results and discussion

The MeOH extract of the underground parts of *T. erectum* was subjected to Diaion HP-20, silica gel, and ODS silica gel column chromatography to give compounds **1**–**20**. Compounds **7**–**20** were identified as (25*S*)-17α,27-dihydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**7**) (Ono et al., 2007b), (25*R*)-17α-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**8**) (Nohara et al., 1975), (25*R*)-17α-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (**9**) (Mahato et al., 1981), (25*R*)-17α-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (**10**) (Nohara et al., 1975; Nakano et al., 1989), (25*R*)-17α-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (**11**) (Mimaki et al., 2000), (25*R*)-17α-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-α-D-glucopyranoside (**12**) (Nohara et al., 1975), (25*R*)-spirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**13**) (Nohara et al., 1975; Espejo et al., 1982), (25*R*)-spirost-5-en-3β-yl *O* -α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (dioscin) (**14**) (Nohara et al., 1975; Espejo et al., 1982; Nakano et al., 1989), (25*R*)-spirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (**15**) (Nohara et al., 1975), (25*R*)-26-[β-D-glucopyranosyl]oxy]-22α-methoxyfurost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (methylprotodioscin) (**16**) (Hirai et al., 1984a,b; Teshima et al., 1986), (25*R*)-26-[β-D-glucopyranosyl]oxy]-17α-hydroxy-22β-methoxyfurost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**17**) (Nakano et al., 1984), (25*R*)-26-[β-D-glucopyranosyl]oxy]-17α-hydroxy-22α-methoxyfurost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (**18**) (Nakano et al., 1982), (25*R*)-26-[β-D-glucopyranosyl]oxy]-17α-hydroxy-22α-methoxyfurost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (**19**) (Nakano et al., 1982), and (25*R*)-26-[β-D-glucopyranosyl]oxy]-3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl)oxy]-cholesta-5,17-diene-16,22-dione (**20**) (Nohara et al., 1975), respectively.

Compound **1** was isolated as an amorphous solid. The HRESI-TOFMS of **1** showed an accurate $[M+H]^+$ ion peak at *m*/*z* 631.3455, corresponding to the empirical molecular formula of $C_{33}H_{52}O_{10}$, which was supported by analysis of the ^{13}C NMR and DEPT spectroscopic data. The ^1H NMR spectrum of **1** showed two three-proton singlet resonance at δ 1.08 and 0.98, a three-proton doublet resonance at δ 1.08 (*J* = 7.2 Hz), a trisubstituted olefinic proton signal at δ 5.39 (*br d*, *J* = 4.2 Hz), and an anomeric proton resonance at δ 4.77 (*d*, *J* = 7.6 Hz). Enzymatic hydrolysis of **1** with β-D-glucosidase gave an aglycone (**1a**) and D-glucose. Analysis of the ^1H and ^{13}C NMR spectroscopic data of **1a** allowed the structure to be identified as (25*S*)-spirost-5-ene-3β,17α,27-triol (Chen and Zhou, 1992; Zhang et al., 2005). Identification of D-glucose, including its absolute configuration, was carried out by direct HPLC analysis of the hydrolysate, which was performed on an aminopropyl-bonded silica gel column using MeCN–H_2O (17:3) as solvent system. Detection was carried out using a combination of refractive index (RI) and optical rotation (OR). The ^{13}C NMR spectrum of **1** showed the presence of a β-D-glucopyranosyl unit [δ 105.1, 75.2, 78.6, 71.7, 78.6, and 62.8] in **1**. A long-range correlation was observed between the anomeric proton of the glucosyl moiety at δ 4.77 and the C-27 oxymethylene carbon at δ 71.9 in the HMBC spectrum of **1**. Thus, the structure of **1** was elucidated as (25*S*)-3β,17α-dihydroxyspirost-5-en-27-yl β-D-glucopyranoside.

Compound **2** was shown to have the molecular formula of $C_{45}H_{72}O_{19}$ from its HRESI-TOFMS and ^{13}C NMR data. The ^1H NMR spectrum of **2** contained signals for three anomeric protons at δ 6.31 (*br s*), 4.97 (*d*, *J* = 7.4 Hz), and 4.73 (*d*, *J* = 7.7 Hz), as well as resonances for three methyl groups at δ 1.18 (*d*, *J* = 7.1 Hz), 1.05 (*s*), 0.92 (*s*), and an olefinic proton at δ 5.29 (*br d*, *J* = 4.2 Hz) derived from the steroidal skeleton. Comparison of the ^1H and ^{13}C NMR spectra of **2** with those of **1** indicated that the aglycone of **2** is identical to that of **1**. Acid hydrolysis of **2** with 1 M HCl gave L-rhamnose and D-glucose. Thus, **2** is suggested to be a triglycoside of

1a. The ^1H–^1H COSY experiment with **2** allowed the sequential assignments from H-1 to H_2-6 or Me-6 of the monosaccharides, including identification of their multiplet patterns and coupling constants. The HMQC correlated the proton resonances with those of the one-bond coupled carbons, leading to unambiguous assignments of the carbon shifts. The ^1H and ^{13}C NMR signals thus assigned were indicative of the presence of a terminal α-L-rhamnopyranosyl unit (Rha) [δ_H 6.31 (*br s*); δ_C 101.9, 72.4, 72.7, 74.0, 69.4, and 18.5], a C-2 substituted β-D-glucopyranosyl unit (Glc) [δ_H 4.97 (*d*, *J* = 7.4 Hz); δ_C 100.2, 78.0, 79.4, 71.7, 77.9, and 62.5], and a terminal β-D-glucopyranosyl unit (Glc′) [δ_H 4.73 (*d*, *J* = 7.7 Hz); δ_C 104.8, 75.0, 78.4, 71.5, 78.3, and 62.7] in **2** (Agrawal et al., 1985; Agrawal, 1992). The β-orientations of the anomeric centers of the glucosyl moieties were supported by the relatively large *J* values of their anomeric protons (*J* = 7.4 Hz and 7.7 Hz). For the rhamnopyranosyl residues, the large $^1J_{C\text{-}1,H\text{-}1}$ value (171.3 Hz) indicated that the anomeric proton was equatorial thus possessing an α-pyranoid anomeric form (Jia et al., 1998). In the HMBC spectrum, long-range correlations were observed brtween H-1 of Rha at δ 6.31 and C-2 of Glc at δ 78.0, H-1 of Glc at δ 4.97 and C-3 of the aglycone at δ 77.9, and between H-1 of Glc′ at δ 4.73 and C-27 of the aglycone at δ 71.8. Accordingly, the structure of **2** was characterized as (25*S*)-27-[(β-D-glucopyranosyl)oxy]-17α-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.

Compound **3** was analyzed for $C_{33}H_{52}O_{10}$ by HRESI-TOFMS. The ^1H and ^{13}C NMR spectra of **3** were quite similar to those of **2**. In addition, the presence of an acetyl group in **3** was shown by the IR (1731 cm^{-1}), ^1H NMR [δ 2.12 (3H, *s*)], and ^{13}C NMR [δ 170.8 (C=O) and 21.2 (Me)] spectra. Alkaline treatment of **3** with 7% NaOMe in MeOH furnished **2**, indicating that **3** is a monoacetate of **2**. In the HMBC spectrum of **3**, a correlation peak was observed between H-4 of Rha at δ 5.87 (*t*, *J* = 9.8 Hz) and the acetyl carbonyl carbon at δ 170.8. The structure of **3** was assigned as (25*S*)-27-[(β-D-glucopyranosyl)oxy]-17α,27-dihydroxyspirost-5-en-3β-yl *O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-β-D-glucopyranoside.

Compound **4** was shown to have the molecular formula $C_{51}H_{82}O_{24}$ on the basis of HRESI-TOFMS. The deduced molecular formula was higher than that of **2** by $C_6H_{10}O_5$, corresponding to one hexose unit. The ^1H and ^{13}C NMR spectra of **4** contained resonance for four anomeric protons at δ 6.31 (*br s*), 5.06 (*d*, *J* = 7.3 Hz), 4.94 (*d*, *J* = 7.7 Hz), and 4.76 (*d*, *J* = 7.4 Hz), as well as resonance for three steroidal methyl groups at δ 1.19 (*d*, *J* = 7.0 Hz), 1.07 (*s*), and 0.93 (*s*), and an olefinic proton at δ 5.28 (*d*, *J* = 3.9 Hz). Acid hydrolysis of **4** with 1 M HCl gave L-rhamnose and D-glucose. On comparison of the ^{13}C NMR spectrum of **4** with that of **2**, a set of six additional signals corresponding to a terminal β-D-glucopyranosyl unit (Glc′) could be observed at δ 105.3, 75.1, 78.4, 71.6, 78.4, and 62.7, and the resonance due to C-6 of the Glc moiety attached at C-3 of the aglycone and its neighboring carbons varied, while all other signals remained almost unaffected. In the HMBC spectrum, H-1 of Glc′ at δ 5.06 showed a correlation peak with C-6 of Glc at δ 69.8, of which H-1 at δ 4.94 exhibited a correlation with C-3 of the aglycone at δ 78.3, giving evidence for a linkage of the Glc′ group to C-6 of the Glc unit. HMBC correlations between H-1 of Rha at δ 6.31 and C-2 of Glc at δ 77.5, and between H-1 of Glc″ at δ 4.76 and C-27 of aglycone at δ 71.9 were also noted. Thus, the structure of **4** was characterized as (25*S*)-27-[(β-D-glucopyranosyl)oxy]-17α,27-dihydroxyspirost-5-en-3β-yl *O*-α-D-glucopyranosyl-(1→6)-*O*-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside.

Compound **5** showed an accurate $[M+H]^+$ ion peak at *m/z* 609.3588 in HRESI-TOFMS, corresponding to the molecular formula $C_{33}H_{52}O_{10}$, which was the same as that of **1**. However, differences between the two compounds were recognized in the ^1H and ^{13}C NMR signals arising from the ring A and F parts of the aglycone moiety. Enzymatic hydrolysis of **5** with β-D-glucosidase gave **1a** and D-glucose. In the HMBC spectrum, a long-range correlation was observed between H-1 of Glc at δ 5.04 (*d*, *J* = 7.7 Hz) and C-3 of the aglycone at δ 78.1. Thus, the structure of **5** was formulated as (25*S*)-17α,27-dihydroxyspirost-5-en-3β-yl β-D-glucopyranoside.

Compound **6** exhibited a molecular formula $C_{41}H_{64}O_{15}$ based on HRESI-TOFMS. Its ^1H and ^{13}C NMR spectral properties were in good agreement with those of **7**, except for the presence of the signals for one acetyl group [δ_H 2.12 (3H, *s*); δ_C 170.8 (C=O) and 21.2 (Me)]. Alkaline treatment of **6** with 7% NaOMe in MeOH furnished **7**, and the HMBC spectrum showed correlation peaks between H-4 of Rha at δ 5.89 (*t*, *J* = 9.8 Hz) and the acetyl carbonyl carbon at δ 170.8. The structure of **6** was shown to be (25*S*)-17α,27-dihydroxyspirost-5-en-3β-yl *O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-β-D-glucopyranoside.

3. Concluding remarks and biological activity

The new compounds **1**–**6** have the common aglycone of (25*S*)-spirost-5-ene-3β,17α,27-triol, among which **2**–**4** are bisdesmosides with the sugar units at the C-3 and C-27 hydroxy groups. This type of spirostanol glycosides has not been isolated from natural sources, except for polygonatosides A and B, and pratioside C from *Polygonatum* spieces (Jin et al., 2004; Li et al., 1993). The isolated compounds were evaluated for their cytotoxic activity against HL-60 human promyelocytic leukemia cells (Table 3). The known spirostanol saponins (**8**, **10**, **12**–**15**) and the furostanol saponin (**16**) showed moderate cytotoxic activity with IC_{50} values of 1.68–8.85 μg/ml, when etoposide used as a positive control had an IC_{50} value of 0.21 μg/ml. α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranoside, α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside, and α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside of (25*R*)-spirost-5-en-3β-diol (diosgenin) (**13**, **14**, and **15**) and (25*R*)-spirost-5-en-3β,17α-diol (pennogenin) (**8**, **10**, and **12**) exhibited moderate cytotoxic activity. Compounds **9** and **11**, which are structurally related to **10** and **12** without the terminal rhamnosyl group linked to C-2 of the inner glucosyl residue, did not show cytotoxic activity. Although pennogenin α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**8**) showed cytotoxic activity, the corresponding C-27 hydroxy derivative (**7**) and its 27-*O*-glycoside (**2**) did not show cytotoxic activity, suggesting that introduction of a polar substituent to the C-27 methyl group diminishes cytotoxic activity. These data imply that the structures of both aglycones and sugar moieties contribute to the appearance of cytotoxic activity in steroidal glycosides.

4. Experimental

4.1. General

Optical rotations were 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 ^1H NMR, Karlsruhe, Germany) and a Bruker DRX-600 (600 MHz for ^1H NMR) spectrophotometer using standard Bruker pulse programs. Chemical shifts are given as *d* values with reference to tetramethylsilane (TMS) as an internal standard. HRESI-TOFMS were recorded on a 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. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck, Darmstadt, Germany) and RP_{18} F_{254} S plates (0.25 mm thick, Merck), and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. HPLC was performed by using a system composed of a

CCPM pump (Tosoh, Tokyo, Japan), a PX-8010 controller (Tosoh), an RI-8010 (Tosoh) or a Shodex OR-2 (Showa-Denko, Tokyo, Japan) detector, and a Rheodyne injection port. A Capcell Pak C_{18} UG120Å column (10 mm i.d. × 250 mm, 5 mm, Shiseido, Tokyo, Japan) was employed for preparative HPLC. HL-60 cells were obtained from the Human Science Research Resources Bank (JCRB 0085, Osaka, Japan). The following reagents were obtained from the indicated companies: RPMI 1640 medium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA); FBS (Bio-Whittaker, Walkersville, MO, USA); penicillin G sodium salt and streptomycin sulfate (Gibco, Grand Island, NY, USA). All other chemicals used were of biochemical reagent grade.

4.2. Plant material

The plant material, defined as the underground parts of *T. erectum* L., was obtained from a wholesale firm in Richters, Ontario, Canada in 2000. A voucher specimen has been deposited in our laboratory (Voucher No. TE-00-001, Laboratory of Medicinal Pharmacognosy).

4.3. Extraction and isolation

The underground parts of *T. erectum* (2.5 kg) were extracted with MeOH (6 l) under reflux, and the MeOH extract (360 g) was passed through a Diaion HP-20 column successively eluted with MeOH–H_2O (3:7, v/v) MeOH–H_2O (1:1, v/v), MeOH–H_2O (1:1, v/v), EtOH, and EtOAc (each 10 l). The MeOH eluate portion (110 g) was subjected to silica gel CC (70 mm i.d. × 380 mm) eluted with a stepwise gradient mixture of $CHCl_3$–MeOH–H_2O (90:10:1; 40:10:1; 30:10:1; 20:10:1), and finally with MeOH alone, giving nine fractions (A–I). Fraction D was subjected to ODS silica gel CC (45 mm i.d. × 250 mm) eluted with MeOH–H_2O (2:1; 7:3; 4:1), and then silica gel (30 mm i.d. × 200 mm) eluted with $CHCl_3$–MeOH–H_2O (80:10:1; 60:10:1) to give **1** (5.4 mg), **3** (24 mg), **5** (6.7 mg), **6** (16 mg), **8** (4.5 mg), **9** (13 mg), **11** (4.9 mg), **13** (13 mg), and **14** (12 mg). Fraction G was applied to a ODS silica gel column (45 mm i.d. × 280 mm) this being eluted with MeOH–H_2O (2:1; 7:3; 4:1) and then further silica gel CC (30 mm i.d. × 230 mm) using $CHCl_3$–MeOH–H_2O (50:10:1) as eluent to give **7** (130 mg), **10** (55 mg), **12** (117 mg), and **15** (110 mg). Fraction I was subjected to ODS silica gel CC (45 mm i.d. × 280 mm) eluted with MeOH–H_2O (2:1; 7:3; 9:1) and MeCN–H_2O (1:3), and than silica gel (22 mm i.d. × 230 mm) CC using $CHCl_3$–MeOH–H_2O (30:10:1; 20:10:1) as eluent to give **2** (49 mg), **16** (500 mg), **17** (22 mg), **19** (6.0 mg) and **20** (26 mg), and **18** with a few impurities. The latter was further purified by preparative HPLC using MeCN–H_2O (2:7) to give **18** (8.0 mg). The MeOH–H_2O (1:1, v/v) eluate portion (50 g) was applied to a silica gel column (70 mm i.d. × 380 mm) eluted with a stepwise gradient mixture of $CHCl_3$–MeOH–H_2O (40:10:1; 30:10:1; 20:10:1; 7:4:1), and finally with MeOH, giving nine fractions (a–g). Fraction g was subjected to ODS silica gel CC (47 mm i.d. × 250 mm) eluted with MeOH–H_2O (1:1; 3:2) and preparative HPLC using MeCN–H_2O (1:3) to give **4** (13 mg).

4.4. Compound *1*

(25*S*)-3β,17α-Dihydroxyspirost-5-en-27-yl β-D-glucopyranoside (**1**); amorphous powder; $[\alpha]_D^{24}$ −47.2 (*c* 0.19; MeOH); IR ν_{max} (film) cm^{-1}: 3389 (OH), 2922 (CH), 1026; ^1H NMR (500 MHz, C_5D_5N): δ 5.39 (1H, *br d*, *J* = 4.2 Hz, H-6), 4.41 (1H, *m*, H-16), 3.98 (1H, *dd*, *J* = 11.1, 4.0 Hz, H-26eq), 3.93 (1H, *dd*, *J* = 9.7, 5.6 Hz, H-27a), 3.74 (1H, *t*, *J* = 11.1 Hz, H-26ax), 3.45 (1H, *dd*, *J* = 9.7, 7.8 Hz, H-27b), 3.82 (1H, *m*, H-3), 1.21 (3H, *d*, *J* = 7.2 Hz, Me-21), 1.08 (3H, *s*, Me-19), 0.98 (3H, *s*, Me-18); For ^1H NMR spectroscopic data of sugar moiety, see Table 1; for ^{13}C NMR (125 MHz, C_5D_5N) spectroscopic data, see Tables 1 and 2; HRESI-TOFMS *m/z*: 631.3455 $[M+Na]^+$ (calculated for $C_{33}H_{52}O_{10}Na$, 631.3458).

4.5. Enzymatic hydrolysis of *1*

Compound **1** (4.7 mg) was treated with β-D-glucosidase (Sigma, St. Louis, MO, USA, 12 mg) in HOAc/NaOAc buffer (pH 5.0, 10 ml) at room temperature for 13 h. The reaction mixture was applied to a silica gel column eluted with $CHCl_3$–MeOH (19:1) followed by MeOH to yield **1a** (1.5 mg) and a sugar fraction (0.5 mg). The sugar fraction was passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) and a Toyopak IC-SP M cartridge (Tosoh), which was then analyzed by HPLC under the following conditions: column, Capcell Pak NH_2 UG80 (4.6 mm i.d. × 250 mm, 5 μm, Shiseido); solvent, MeCN–H_2O (17:3); flow rate, 0.9 ml/min; detection, RI and OR. Identification of D-glucose present in the sugar fraction was carried out by comparison of its retention time and optical rotation with those of an authentic sample. R_t (min): 18.26 (D-glucose, +).

4.6. Compound *1a*

(25*S*)-Spirost-5-ene-3β,17α,27-triol; amorphous solid, $[\alpha]_D^{24}$ -26.9 (*c* 0.08; MeOH); IR ν_{max} (film) cm^{-1}: 3358 (OH), 2922 (CH), 1028; ^1H NMR (500 MHz, C_5D_5N): δ 5.39 (1H, *br d*, *J* = 4.5 Hz, H-6), 4.50 (1H, *dd*, *J* = 7.5, 6.4 Hz, H-16), 4.08 (1H, *dd*, *J* = 11.1, 4.3 Hz, H-26eq), 3.91 (1H, *t*, *J* = 11.1 Hz, H-26ax), 3.82 (1H, *m*, H-3), 3.73 (1H, *m*, H-27a), 3.65 (1H, *m*, H-27b), 1.27 (3H, *d*, *J* = 7.2 Hz, Me-21), 1.08 (3H, *s*, Me-19), 1.01 (3H, *s*, Me-18); For ^{13}C NMR (125 MHz, C_5D_5N) spectroscopic data, see Table 2; HRESI-TOFMS *m/z*: 469.2958 $[M+Na]^+$ (calculated for $C_{27}H_{42}O_5Na$, 469.2930).

4.7. Compound *2*

(25*S*)-27-[(β-D-Glucopyranosyl)oxy]-17α-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**2**); amorphous powder; $[\alpha]_D^{24}$ −77.9 (*c* 0.10; MeOH); IR ν_{max} (film) cm^{-1}: 3389 (OH), 2922 (CH), 1049; ^1H NMR (500 MHz, C_5D_5N): δ 5.29 (1H, *br d*, *J* = 4.2 Hz, H-6), 4.41 (1H, *dd*, *J* = 7.1, 6.5 Hz, H-16), 3.98 (1H, *overlapping*, H-26eq), 3.91 (1H, *overlapping*, H-27a), 3.89 (1H, *m*, H-3), 3.72 (1H, *t*, *J* = 11.1 Hz, H-26ax), 3.43 (1H, *dd*, *J* = 9.4, 8.1 Hz, H-27b), 1.74 (3H, *d*, *J* = 6.1 Hz, Me-6″), 1.18 (3H, *d*, *J* = 7.1 Hz, Me-21), 1.05 (3H, *s*, Me-19), 0.92 (3H, *s*, Me-18); For ^1H NMR spectroscopic data of sugar moieties, see Table 1; for ^{13}C NMR (125 MHz, C_5D_5N) spectroscopic data, see Tables 1 and 2; HRESI-TOFMS *m/z*: 939.4563 $[M+Na]^+$ (calculated for $C_{45}H_{72}O_{19}Na$, 939.4563).

4.8. Acid hydrolysis of *2*

A solution of **2** (10.2 mg) in 1 M HCl (dioxane-H_2O, 1:1, 2 ml) was heated at 95 °C for 1 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo, Tokyo, Japan) column (10 mm i.d. × 100 mm) and chromatographed on Diaion HP-20 (10 mm i.d. × 100 mm), eluted with H_2O–MeOH (3:2) followed by EtOH–Me_2CO (1:1), to yield a sugar fraction (2.5 mg). HPLC analysis of the sugar fraction under the same condition as in the case of **1** showed the presence of D-glucose and L-rhamnose. R_t (min): 8.90 (L-rhamnose, −); 18.73 (D-glucose, +).

4.9. Compound *3*

(25*S*)-27-[(β-D-Glucopyranosyl)oxy]-17α,27-dihydroxyspirost-5-en-3β-yl *O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-β-D-gluco-

Table 1
^{1}H and ^{13}C NMR spectroscopic data for the sugar moieties of compounds **1–6** in $C_5D_5N_5$[a]

1				**2**				**3**				**4**				**5**				**6**			
		^{1}H	^{13}C			^{1}H	^{13}C			^{1}H	^{13}C			^{1}H	^{13}C			^{1}H	^{13}C			^{1}H	^{13}C
Glc	1	4.77 *d* (7.6)	105.1	Glc	1	4.97 *d* (7.4)	100.2	Glc	1	5.03 *d* (7.5)	99.8	Glc	1	4.94 *d* (7.0)	100.6	Glc	1	5.04 *d* (7.7)	102.5	Glc	1	5.05 *d* (7.5)	99.8
	2	4.02 *dd* (8.7, 7.6)	75.2		2	4.21 *dd* (9.0, 7.4)	78.0		2	4.22 *dd* (9.1, 7.5)	77.0		2	4.20 *dd* (8.6, 7.0)	77.5		2	4.06 *dd* (8.7, 7.7)	75.3		2	4.24 *dd* (9.0, 7.5)	77.0
	3	4.25 *t* (8.7)	78.6		3	4.27 *t* (9.0)	79.4		3	4.29 *t* (9.1)	79.5		3	4.21 *dd* (9.1, 8.6)	79.4		3	4.30 *t* (8.7)	78.6		3	4.30 *t* (9.0)	79.6
	4	4.23 *t* (8.7)	71.7		4	4.12 *t* (9.0)	71.7		4	4.15 *t* (9.1)	71.7		4	4.12 *t* (9.1)	71.5		4	4.28 *t* (8.7)	71.7		4	4.17 *t* (9.0)	71.7
	5	3.96 *m*	78.6		5	3.88 *m*	77.9		5	3.91 *m*	78.2		5	3.98 *m*	76.7		5	3.98 *m*	78.4		5	3.90 *m*	78.3
	6 a	4.58 *dd* (11.8, 2.1)	62.8		6 a	4.47 *dd* (11.6, 2.1)	62.5		6 a	4.50 *dd* (11.5, 1.5)	62.5		6 a	4.76 *br d* (11.6)	69.8		6 a	4.56 *dd* (11.7, 1.9)	62.8		6 a	4.51 *dd* (11.8, 2.1)	62.6
	b	4.40 *dd* (11.8, 5.4)			b	4.31 *dd* (11.6, 5.3)			b	4.34 *dd* (11.5, 5.1)			b	4.31 *dd* (11.6, 4.0)			b	4.41 *dd* (11.7, 5.2)			b	4.36 *dd* (11.8, 5.2)	
											101.4	Rha	1	6.31 *br s*	102.0					Rha	1	6.42 *br s*	101.4
				Rha	1	6.31 *br s*	101.9	Rha	1	6.39 *br s*	72.4		2	4.77 *br d* (3.8)	72.5						2	4.76 *br d* (3.2)	72.5
					2	4.78 *br d* (3.1)	72.4		2	4.76 *br d* (3.1)	70.1		3	4.60 *dd* (9.3, 3.8)	72.8						3	4.68 *dd* (9.8, 3.2)	70.2
					3	4.59 *dd* (9.3, 3.1)	72.7		3	4.66 *dd* (9.8, 3.1)	76.1		4	4.34 *t* (9.3)	74.1						4	5.89 *t* (9.8)	76.2
					4	4.33 *t* (9.3)	74.0		4	5.87 *t* (9.8)	66.7		5	4.97 *dq* (9.3, 6.0)	69.4						5	5.06 *dq* (9.8, 6.3)	66.8
					5	4.94 *dq* (9.3, 6.1)	69.4		5	5.05 *m*	17.9		6	1.77 *d* (6.0)	18.6						6	1.51 *d* (6.3)	18.0
					6	1.74 *d* (6.1)	18.5		6	1.50 *d*	(6.3)	Glc'	1	5.06 *d* (7.3)	105.3								
				Glc'	1	4.73 *d* (7.7)	104.8	Glc'	1	4.76 *d* (7.7)	104.9		2	4.02 *dd* (8.3, 7.3)	75.1								
					2	3.99 *dd* (8.8, 7.7)	75.0		2	4.01 *dd* (8.8, 7.7)	75.1		3	4.19 *dd* (9.3, 8.3)	78.4								
					3	4.23 *t* (8.8)	78.4		3	4.25 *t* (8.8)	78.5		4	4.22 *t* (9.3)	71.6								
					4	4.18 *dd* (9.1, 8.8)	71.5		4	4.21 *t* (8.8)	71.6		5	3.90 *m*	78.4								
					5	3.93 *m*	78.3		5	3.95 *m*	78.4		6 a	4.50 *br d* (12.0)	62.7								
					6 a	4.54 *dd* (11.8, 1.7)	62.7		6 a	4.56 *dd* (11.9, 1.7)	62.8		b	4.35 *dd* (12.0, 3.7)									
					b	4.34 *dd* (11.8, 5.5)			b	4.38 *dd* (11.9, 5.4)	62.1	Glc''	1	4.76 *d* (7.4)	105.0								
								Ac		2.12 *s*	170.8		2	4.01 *dd* (9.2, 7.4)	75.1								
											21.2		3	4.24 *t* (9.2)	78.5								
													4	4.23 *t* (9.2)	71.6								
													5	3.95 *m*	78.5								
													6 a	4.57 *br d* (11.9)	62.8								
													b	4.39 *dd* (11.9, 4.0)									
																				Ac			170.8
																						2.12 *s*	21.2

[a] Values in parentheses are coupling constants in Hz.

Table 2
^{13}C NMR spectroscopic data for compounds **1**, **1a**, and **2**–**6** in C_5D_5N

Position	1	la	2	3	4	5	6
1	37.8	37.9	37.4	37.4	37.5	37.5	37.5
2	32.6	32.6	30.1	30.0	30.2	30.2	30.1
3	71.3	71.3	77.9	77.4	78.3	78.1	77.5
4	43.5	43.5	38.8	38.9	39.1	39.3	39.0
5	142.0	142.0	140.7	140.6	140.9	140.8	140.7
6	121.0	121.1	121.7	122.0	121.6	121.7	122.1
7	32.1	32.1	32.3	32.3	32.3	32.3	32.4
8	32.4	32.4	32.2	32.3	32.3	32.3	32.4
9	50.3	50.4	50.1	50.2	50.1	50.2	50.3
10	37.0	37.0	37.0	37.1	37.1	37.0	37.2
11	21.0	21.1	20.8	20.9	20.8	20.9	21.0
12	32.4	32.4	31.9	32.0	32.0	32.1	32.1
13	45.1	45.2	45.0	45.1	45.1	45.1	45.2
14	53.1	53.1	52.9	53.0	52.9	53.0	53.1
15	31.8	31.8	31.6	31.7	31.7	31.8	31.8
16	90.1	90.1	89.9	89.9	90.0	90.0	90.1
17	90.1	90.1	90.0	90.1	90.1	90.1	90.1
18	17.2	17.2	17.0	17.1	17.1	17.1	17.2
19	19.6	19.6	19.3	19.4	19.4	19.4	19.5
20	44.8	44.9	44.7	44.8	44.8	44.8	44.9
21	9.7	9.8	9.6	9.6	9.6	9.7	9.8
22	110.1	110.3	110.0	110.1	110.1	110.2	110.3
23	31.5	31.8	31.4	31.5	31.5	31.8	31.8
24	23.5	23.6	23.3	23.4	23.4	23.6	23.6
25	36.6	39.1	36.4	36.5	36.5	39.0	39.0
26	63.5	64.0	63.4	63.5	63.5	63.9	63.9
27	71.9	64.4	71.8	71.8	71.9	64.3	64.4

Table 3
Cytotoxic activity of the isolated compounds **1**–**20** on HL-60 leukemia cells

Compound	Growth rate (%)[a]	IC_{50} (μg/ml)[b]	Compound	Growth rate (%)	IC_{50} (μg/ml)
1	93.0		**11**	87.2	
2	103.0		**12**	4.1	2.65 ± 0.22
3	91.3		**13**	3.3	2.30 ± 0.08
4	103.0		**14**	45.6	8.85 ± 0.50
5	85.9		**15**	8.5	1.68 ± 0.11
6	91.4		**16**	3.6	2.89 ± 0.24
7	96.7		**17**	76.9	
8	21.8	6.10 ± 0.04	**18**	102.3	
9	74.4		**19**	75.5	
10	4.5	3.58 ± 0.18	**20**	102.3	
Etoposide		0.21 ± 0.02			

[a] Data are expressed as percentage of cell growth at the sample concentration of 10 μg/ml.
[b] Data represent the mean ± SEM of three independent experiments.

pyranoside (**3**); amorphous powder; $[\alpha]_D^{24}$ −42.9 (*c* 0.10; MeOH); IR ν_{max} (film) cm^{-1}: 3357 (OH), 2923 (CH), 1731 (C=O), 1027 (C–O); ^{1}H NMR (500 MHz, C_5D_5N): δ 5.37 (1H, *br d*, *J* = 5.1 Hz, H-6), 4.44 (1H, *dd*, *J* = 7.1, 6.6 Hz, H-16), 3.99 (1H, *dd*, *J* = 11.2, 4.6 Hz, H-26eq), 3.94 (1H, *m*, H-3), 3.93 (1H, *overlapping*, H-27a), 3.74 (1H, *t*, *J* = 11.2 Hz, H-26ax), 3.44 (1H, *dd*, *J* = 9.7, 7.9 Hz, H-27b), 2.12 (3H, *s*, OMe), 1.21 (3H, *d*, *J* = 7.2 Hz, Me-21), 1.14 (3H, *s*, Me-19), 0.99 (3H, *s*, Me-18); For ^{1}H NMR signals of sugar moieties, see Table 1; for ^{13}C NMR (125 MHz, C_5D_5N) spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z*: 631.3455 [M+Na]$^+$ (calculated for $C_{33}H_{52}O_{10}Na$, 631.3458).

4.10. Alkaline methanolysis of *3*

Compound **3** (7.5 mg) was treated with 7% NaOMe in MeOH (5 ml) at room temperature for 4 h. The reaction mixture was neutralized by passage through an Amberlite IR-120B (Organo) column (10 mm i.d. × 100 mm) and purified by silica gel CC (10 mm i.d. × 100 mm) eluted with $CHCl_3$–MeOH–H_2O (20:10:1) to give **2** (5.1 mg).

4.11. Compound *4*

(25*S*)-27-[(β-D-Glucopyranosyl)oxy]-17α,27-dihydroxyspirost-5-en-3β-yl *O*-[β-D-glucopyranosyl-(1→6)]-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside (**4**); amorphous powder; $[\alpha]_D^{25}$ −51.8 (*c* 0.10; MeOH); IR ν_{max} (film) cm^{-1}: 3420 (OH), 2927 (CH), 1042; ^{1}H NMR (500 MHz, C_5D_5N): δ 5.28 (1H, *br d*, *J* = 3.9 Hz, H-6), 4.44 (1H, *dd*, *J* = 7.1, 6.6 Hz, H-16), 3.98 (1H, *overlapping*, H-26eq), 3.93 (1H, *m*, H-3), 3.93 (1H, *overlapping*, H-27a), 3.74 (1H, *t*, *J* = 11.1 Hz, H-26ax), 3.44 (1H, *dd*, *J* = 8.6, 8.2 Hz, H-27b), 1.19 (3H, *d*, *J* = 7.0 Hz, Me-21), 1.07 (3H, *s*, Me-19), 0.93 (3H, *s*, Me-18); For ^{1}H NMR signals of sugar moieties, see Table 1; for ^{13}C NMR (125 MHz, C_5D_5N) spectroscopic data, see Tables 1 and 2; HRESI-TOFMS *m/z*: 1079.5226 [M+H]$^+$ (calculated for $C_{51}H_{83}O_{24}$, 1079.5274).

4.12. Acid hydrolysis of *4*

A solution of **4** (8.1 mg) was subjected to acid hydrolysis as described for **2** to give a sugar fraction (3.0 mg). HPLC analysis of the sugar fraction under the same condition as in the case of **1** showed the presence of D-glucose and L-rhamnose. R_t (min): 8.96 (L-rhamnose, −); 18.34 (D-glucose, +).

4.13. Compound *5*

(25*S*)-17α,27-Dihydroxyspirost-5-en-3β-yl β-D-glucopyranoside (**5**); amorphous powder; $[\alpha]_D^{24}$ −57.0 (*c* 0.10; MeOH); IR ν_{max} (film) cm^{-1}: 3333 (OH), 2923 (CH), 1019; ^{1}H NMR (500 MHz, C_5D_5N): δ 5.29 (1H, *br d*, *J* = 4.2 Hz, H-6), 4.49 (1H, *dd*, *J* = 6.9, 6.9 Hz, H-16), 4.07 (1H, *dd*, *J* = 11.1, 4.0 Hz, H-26eq), 3.91 (1H, *m*, H-3), 3.90 (1H, *t*, *J* = 11.1 Hz, H-26ax), 3.73 (1H, *t*, *J* = 6.9 Hz, H-27a), 3.64 (1H, *dd*, *J* = 10.7, 7.7 Hz, H-27b), 1.25 (3H, *d*, *J* = 7.1 Hz, Me-21), 0.97 (3H, *s*, Me-18), 0.93 (3H, *s*, Me-19); For ^{1}H NMR signals of sugar moieties, see Table 1; for ^{13}C NMR (125 MHz, C_5D_5N) spectroscopic data, see Tables 1 and 2; HRESI-TOFMS *m/z*: 609.3588 [M+H]$^+$ (calculated for $C_{33}H_{53}O_{10}$, 609.3639).

4.14. Enzymatic hydrolysis of *5*

Compound **5** (4.1 mg) was treated with β-D-glucosidase (20.5 mg) in HOAc/NaOAc buffer (pH 5.0, 10 ml) at room temperature for 37 h to yield **1a** (1.3 mg) and a sugar fraction (0.3 mg). HPLC analysis of the sugar fraction under the same condition as in the case of **1** showed the presence of D-glucose. R_t (min): 18.38 (D-glucose, +).

4.15. Compound *6*

(25*S*)-17α,27-Dihydroxyspirost-5-en-3β-yl *O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-β-D-glucopyranoside (**6**); amorphous powder; $[\alpha]_D^{24}$ −86.1 (*c* 0.10; MeOH); IR ν_{max} (film) cm^{-1}: 3417 (OH), 2933 (CH), 1731 (C=O), 1038 (C–O); ^{1}H NMR (500 MHz, C_5D_5N): δ 5.38 (1H, *br d*, *J* = 5.1 Hz, H-6), 4.52 (1H, *dd*, *J* = 7.5, 6.2 Hz, H-16), 4.08 (1H, *dd*, *J* = 11.1, 3.6 Hz, H-26eq), 3.96 (1H, *m*, H-3), 3.91 (1H, *t*, *J* = 11.1 Hz, H-26ax), 3.73 (1H, *dd*, *J* = 10.7, 5.1 Hz, H-27a), 3.65 (1H, *dd*, *J* = 10.7, 7.9 Hz, H-27b), 2.17 (3H, *s*, OMe), 1.27 (3H, *d*, *J* = 7.2 Hz, Me-21), 1.16 (3H, *s*, Me-19), 1.03 (3H, *s*, Me-18); For ^{1}H NMR signals of sugar moieties, see Table 1; for ^{13}C NMR (125 MHz, C_5D_5N) spectroscopic data, see Tables 1 and 2; HRESI-TOFMS *m/z*: 797.4399 [M+H]$^+$ (calculated for $C_{41}H_{65}O_{15}$, 797.4323).

4.16. Alkaline methanolysis of **6**

Compound **6** (7.6 mg) was treated with 7% NaOMe in MeOH (5 ml) at room temperature for 4 h. The reaction mixture was neutralized by passage through an Amberlite IR-120B column (10 mm i.d. × 100 mm) and purified by silica gel CC (10 mm i.d. × 100 mm) eluted with $CHCl_3$–MeOH–H_2O (30:10:1) to give **7** (3.0 mg).

4.17. HL-60 cell culture assay

HL-60 cells were maintained in RPMI 1640 medium containing heat-inactivated 10% (v/v) fetal bovine serum (FBS) supplemented with 100 units/mL penicillin G sodium salt and 100 μg/mL streptomycin sulfate. For cytotoxicity assay, the leukemia cells were washed and resuspended in the above medium to 4×10^4 cells/ml, and 196 μl of this cell suspension was placed in each well of a 96-well flat-bottom plate (Iwaki Glass, Chiba, Japan). The cells were incubated in a humidified air/CO_2 (19:1) atmosphere for 24 h at 37 °C. After incubation, 4 μl of EtOH–H_2O (1:1) solution containing the sample was added to give the final concentrations of 0.1–10 μg/ml; 4 μl of EtOH–H_2O (1:1) was added into control wells. The cells were further incubated for 72 h in the presence of each agent. Then cell growth was evaluated by the modified MTT assay procedure established by Sargent and Taylor (Sargent and Taylor, 1989) as follows. At the end of incubation, 10 μl of 5 mg/ml MTT in phosphate-buffered saline (PBS) was added to every well, and the plate was further incubated in a humidified air/CO_2 (19:1) atmosphere for 4 h at 37 °C. The plate was then centrifuged at 1500*g* for 5 min to precipitate cells and MTT formazan. An aliquot of 150 μl of the supernatant was removed from every well, and 175 μl of DMSO was added to dissolve the MTT formazan crystals. The plate was mixed on a microshaker for 10 min and then read on a microplate reader (Spectra Classic, Tecan, Salzburg, Austria) at 550 nm. A dose-response curve was plotted for **8**, **10**, and **12**–**16**, which showed less than 50% of cell growth at the sample concentration of 10 μg/ml, and the concentration giving 50% inhibition (IC_{50}) was calculated. Each assay was done in triplicate.

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