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PHYTOCHEMISTRY

Saundersiosides C–H, rearranged cholestane glycosides from the bulbs of *Ornithogalum saundersiae* and their cytostatic activity on HL-60 cells

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Abstract

Six novel rearranged cholestane glycosides with a six-membered hemiacetal ring system, designated as saundersiosides C–H, were isolated from the bulbs of *Ornithogalum saundersiae*. Their structures were determined on the basis of spectroscopic analysis and the result of hydrolysis. The conformation of the six-membered hemiacetal ring of the rearranged cholestanes was shown to be almost a boat-form by molecular mechanics and molecular dynamics calculation studies. Among the isolated compounds, saundersioside E, F, G and H with an aromatic acid ester group at the glycoside moiety were found to be highly cytostatic to human leukemia HL-60 cells, showing IC_{50} values of 0.021, 0.019, 0.063 and 0.052 μM, respectively, which are as potent as those of the clinically applied anticancer agents, etoposide and methotrexate. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Ornithogalum saundersiae; Liliaceae; Bulbs; Rearranged cholestane glycosides; Saundersiosides C–H; Cytostatic activity; HL-60 cells

1. Introduction

Ornithogalun saundersiae (Liliaceae) is a perennial plant, which is native to Natal, Swaziland and the eastern Transvaal and widely cultivated as an excellent garden plant. Previously, we have reported the structural assignment of two novel rearranged cholestane glycosides, saundersiosides A and B (Kuroda et al., 1997; Kuroda, Mimaki, Sashida, Nikaido & Ohmoto, 1993), a new 16,23-epoxy-cholestane glycoside (Kuroda et al., 1997, 1995) and a new cholestane bisdesmoside (Mimaki et al., 1996), isolated from the bulbs of *O. saundersiae*. Further analysis of the *n*-BuOH-soluble phase of the MeOH extract of the plant material resulted in the isolation of six novel rearranged cholestane glycosides with a six-membered hemiacetal ring system, designated as saundersiosides C–H (**1**–**6**). In this paper, we report the structural determination of **1**–**6** on the basis of spectroscopic analysis and the result of acid hydrolysis, and the conformation of the six-membered hemiacetal ring of the rearranged aglycone moiety revealed by molecular mechanics and molecular dynamics calculation methods. The cytostatic activity exhibited by **1**–**6** on human promyelocytic leukemia HL-60 cells is also described.

2. Results and discussion

The concentrated *n*-BuOH-soluble phase of the MeOH extract of *O. saundersiae* bulbs was fractionated by the combined use of repeated chromatography on porous polymer resin (Diaion HP-20), silica gel and on octadecylsilanized (ODS) silica gel, as well as preparative HPLC to yield saundersioside C (**1**) (131 mg), D (**2**) (51.2 mg), E (**3**) (60.4 mg), F (**4**) (65.2 mg), G (**5**) (20.8 mg) and H (**6**) (76.3 mg).

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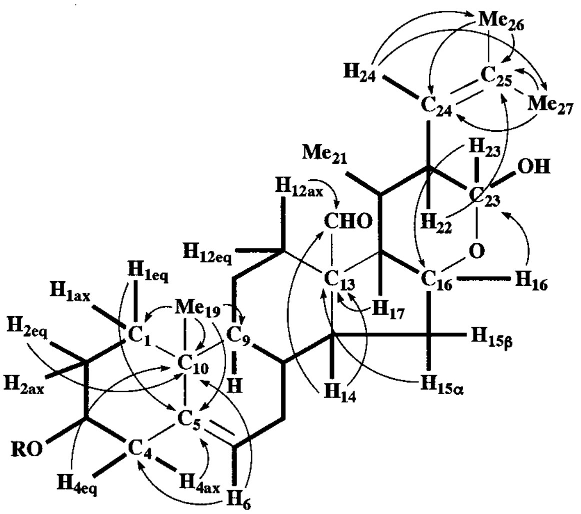


Fig. 1. HMBC correlations of aglycone moiety of **1**.

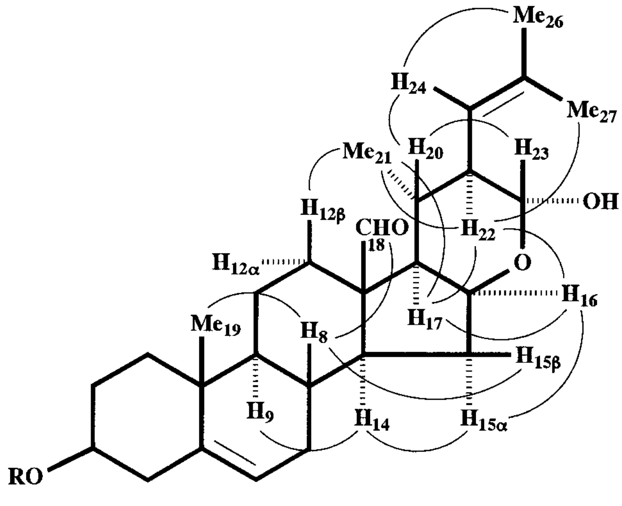


Fig. 2. NOE correlations of aglycone moiety of **1**.

Saundersioside C (**1**) was obtained as an amorphous powder, $[\alpha]_D$ −49.6° (MeOH). Its molecular formula was determined to be $C_{45}H_{70}O_{18}$ by the negative-ion FABMS showing an $[M-H]^-$ ion at *m/z* 897 and elemental analysis. The IR spectrum of **1** was consistent with the presence of hydroxyl groups (3400 cm^{-1}) and an aldehyde group (1700 cm^{-1}), the latter was further supported by the ^{13}C NMR resonance at δ 207.5 (CH). The ^1H NMR spectrum (C_5D_5N–CD_3OD, 1:1) displayed signals arising from a tertiary methyl group at δ 0.76 (s), two secondary methyl groups at δ 1.73 (d, J = 6.2 Hz) and 0.93 (d, J = 5.7 Hz), two methyl groups on a double bond at δ 1.74 and 1.66 coupled to an olefinic proton at δ 4.85 (br d, J = 9.7 Hz) with small J values of less than 0.5 Hz, an aldehyde proton at δ 10.10 (s), an olefinic proton at δ 5.32 (br d, J = 4.4 Hz) and three anomeric protons at δ 6.23 (br s), 5.76 (d, J = 7.5 Hz) and 5.05 (d, J = 7.9 Hz). The signal at δ 1.73 was assignable to the methyl group of 6-deoxyhexose. The presence of two trisubstituted double bonds in **1** was shown by the carbon signals at δ141.3 (C), 134.5 (C), 126.2 (CH) and 121.2 (CH). Acid hydrolysis of **1** with 1 M HCl in dioxane–H_2O (1:1) resulted in the production of D-glucose and L-rhamnose in a ratio of 2:1 as the carbohydrate components, while the genuine aglycone was decomposed under acidic conditions. The monosaccharides were identified by HPLC analysis following their conversion to the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives (Oshima, Yamauchi & Kumanotani, 1982). The ^{13}C NMR spectrum of **1** showed a total of 45 resonance lines, 27 of which were assigned to the aglycone moiety and 18 to three monosaccharides. This implied a $C_{27}H_{40}O_4$ composition for the aglycone moiety, possessing 8 degrees of unsaturation. The presence of an aldehyde group, two double bonds accounted for 3 degrees. Consequently, the aglycone of **1** was predicted to be a C_{27} steroid with a five-ring system. The C-1–C-17 portion (A–D rings) of **1** was readily revealed to be identical to that of usual cholest-5-en-3-ol by analysis of the pulsed field gradient (FG)-DQF COSY, selective 1D TOCSY, HMQC and HMBC spectra. The three-proton singlet signal at δ 0.76 showed long-range correlations with the carbon signals at δ 37.6 (C-1), 141.3 (C-5), 50.3 (C-9) and 37.0 (C-10) in the HMBC spectrum and was assigned to Me-19. The quaternary carbon signal at δ 59.5 was assigned to C-13, at which the aldehyde group was shown to be located by the observation of $^3J_{C,H}$ correlations from the H-12ax proton signal at δ 1.00 (ddd, J = 12.4, 12.4, 3.7 Hz) and H-14 at δ 1.28 to the aldehyde carbon signal at δ 207.5. The downfield-shifted proton signal at δ 4.82 was assigned to H-16, which was correlated to the one-bond coupled carbon at δ 69.8 in the HMQC spectrum. This indicated that the C-16 carbon had an oxygen atom. Tracing out the proton spin coupling system from the distinctive H-16 proton signal through the FG-DQF COSY and selective 1D TOCSY spectra allowed us to construct the structure of the rearranged cholestane portion. The proton signal due to H-23 appeared at δ 5.03 (d, J = 7.7 Hz), which was correlated to the δ 98.3 resonance in the HMQC spectrum and was shifted downfield by 1.02 ppm [δ 6.05 (d, J = 7.8 Hz)] on acetylation. The above data and a HMBC correlation from the H-16 proton to the C-23 carbon gave confirmative evidence for the formation of a six-membered hemiacetal ring between C-16 and C-23. The H-22 proton at δ 2.37 was coupled to not only the H-20 and H-23 protons, but also an olefinic proton assignable to H-24 at δ 4.85 with a J value of 9.7 Hz. The H-24 proton, in turn, showed long-range correlations with the two methyl carbons at δ 26.1 and 19.0 (C-26 and C-27). A long-range correlation from H-22 to the olefinic carbon at 134.5 (C-25) was also observed. This information indicated that a 2-methyl-1-propenyl group was attached at C-22. Thus, the plane structure of the aglycone of **1** was shown to be 16,23-epoxy-23-hydroxy-22-(2-methyl-1-propenyl)-24-norchol-5-en-18-al (Fig. 1).

Analysis of the phase-sensitive NOESY spectrum made the stereochemistry assignable. NOE correlations from H-8 to H-15β, H-18 and Me-19, and H-14 to H-9 and H-15α indicated that **1** had the usual B/C *trans* and C/D *trans* steroidal ring junctions. Other NOE networks from H-16 to H-15α, H-17 and H-22, H-17 to Me-21 and H-22, Me-21 to H-22, H-20 to H-23 and H-24 provided evidence for the 16*S**, 17*R**, 20*S**, 22*S**, and 23*R** configurations (Fig. 2). The orientation of the C-3 oxygen atom was ascertained to be β from the multiplicity of the H-3 proton ($W_{1/2} = 20.0$ Hz).

A FG-DQF COSY experiment allowed the sequential assignment of the resonances for each monosaccharide to be made, starting from the easily distinguished anomeric protons. Multiplet patterns and measurements of coupling constants confirmed the presence of two β-D-glucopyranosyl units ($^4C_1$) and an α-L-rhamnopyranosyl unit ($^1C_4$). The HMQC spectrum correlated all the proton resonances with those of the corresponding one-bond coupled carbons. The long-range correlation from each anomeric proton across the glycosidic bond to the carbons of another substituted monosaccharide or the aglycone confirmed the sugar sequence. The anomeric proton signals at δ 6.23 (rhamnose), 5.76 (glucose) and 5.05 (glucose) were correlated to the δ 79.1 (C-2 of glucose), 80.3 (C-2 of glucose) and 78.8 (C-3 of aglycone) resonances, respectively, in the HMBC spectrum. From the data presented above, the structure of **1** was determined to be 3-[(*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]-16,23-epoxy-23-hydroxy-22-(2-methyl-1-propenyl)-(3β,16β,22*S*,23*R*)-24-norchol-5-en-18-al.

The ^{1}H NMR spectrum of saundersioside D (**2**) ($C_{39}H_{60}O_{13}$) showed signals for a tertiary methyl group at δ 0.96 (s), a secondary methyl group at δ 1.00 (d, J = 5.6 Hz), two methyl groups on a double bond at δ 1.80 and δ 1.71 (each br s), a hemiacetal proton at δ 5.12 (d, J = 7.0 Hz), an aldehyde proton at δ 10.14 (s), and two olefinic protons at δ 5.32 (br d, J = 4.7 Hz) and 4.91 (br d, J = 9.5 Hz). The above functional group data and the ^{13}C NMR spectrum confirmed that the aglycone of **2** was identical to that of **1**. The ^{1}H NMR of **2** displayed two anomeric proton signals at δ 6.32 (br s) and 5.02 (d, J = 7.2 Hz), and acid hydrolysis gave D-glucose and L-rhamnose in a ratio of 1:1. In the HMBC spectrum, the anomeric proton signal of the rhamnose at δ 6.32 showed a $^3J_{C,H}$ correlation with C-2 of the glucose (δ 77.9) and that of the glucose at δ 5.02 with C-3 of the aglycone (δ 78.0), indicating the structure of the glycosyl moiety of **2** as α-L-rhamnosyl-(1 → 2)-β-D-glucosyl and its linkage to C-3 of the aglycone. Thus, the structure of **2** was established as 3-[(*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]-16,23-epoxy-23-hydroxy-22-(2-methyl-1-propenyl)-(3β,16β,22*S*,23*R*)-24-norchol-5-en-18-al.

The spectral data of saundersioside E (**3**) ($C_{46}H_{64}O_{15}$) were almost identical with those of **2**. The existence of a *p*-hydroxybenzoyl ester group in the molecule was indicated by the IR (1700 cm^{-1}), UV [λ_{max} 260 nm (log ε 4.07)], ^{1}H NMR [δ 8.29 and 7.16 (each 2H, d, J = 8.6 Hz)] and ^{13}C NMR [δ 122.0 (C), 132.6 (CH) × 2, 116.1 (CH) × 2, 163.6 (C) and 166.7 (C=O)] spectra and by alkaline hydrolysis of **3** with 4% KOH in EtOH, giving **2** and *p*-hydroxybenzoic acid. On comparison of the ^{1}H NMR spectrum of **3** with that of **2**, the H-4 proton of the rhamnose of **3** was shifted downfield by 1.83 ppm and observed at δ 6.14 (dd, J = 9.4, 9.4 Hz). Accordingly, **3** was revealed to be a *p*-hydroxybenzoyl ester of **2** at the rhamnose C-4 hydroxy position.

The NMR data of saundersioside F (**4**) ($C_{47}H_{66}O_{15}$) were completely superimposable on those of **3**, except for the aromatic region signals due to the substituted benzoyl moiety. The aromatic acid linked to the glycoside moiety of **4** was suggested to be *p*-methoxybenzoic acid by the ^{1}H NMR [δ 8.27 and 7.01 (each 2H, d, J = 8.8 Hz) and 3.78 (3H, s)] and ^{13}C NMR [δ 123.5 (C), 132.2 (CH) × 2, 114.1 (CH) × 2, 163.8 (C), 166.4 (C=O) and 55.5 (OMe)] spectra. This was confirmed by alkaline hydrolysis of **4**, which gave *p*-methoxybenzoic acid and **2**. The linkage position of *p*-methoxybenzoyl group to C-4 of the rhamnosyl moiety was ascertained by the downfield-shifted proton signal at δ 6.13 (dd, J = 9.8, 9.8 Hz) assignable to H-4 of the rhamnose. Thus, **4** was shown to be a *p*-methoxybenzoyl ester of **2** at the rhamnose C-4 hydroxy position.

The ^{1}H NMR spectrum of saundersioside G (**5**) ($C_{46}H_{66}O_{15}$) exhibited signals for a tertiary methyl group, a secondary methyl group, two methyl groups on a double bond, a hemiacetal proton, two olefinic protons, two anomeric protons and *p*-disubstituted aromatic protons, which were essentially analogous to those of **3**. However, the signal due to an aldehyde proton, which was observed at δ 10.13 (s) in the ^{1}H NMR of **3**, could not be detectable in that of **5**. Furthermore, in the ^{13}C NMR spectrum of **5**, the signal due to C-18, which appeared at δ 207.3 and was assigned to an aldehyde carbon in **3**, was displaced by a hydroxymethyl signal at δ 60.4 (CH_2). Treatment of **3** with $NaBH_4$ gave **5**. Accordingly, **5** was assigned as a C-18 hydroxymethyl derivative of **3**.

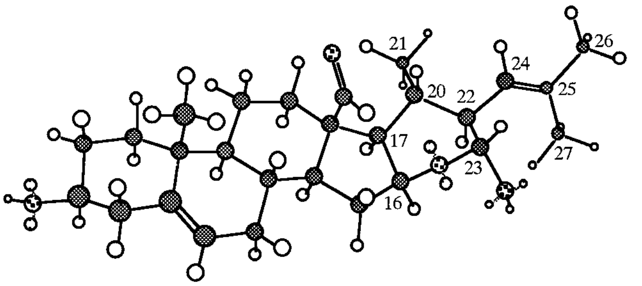
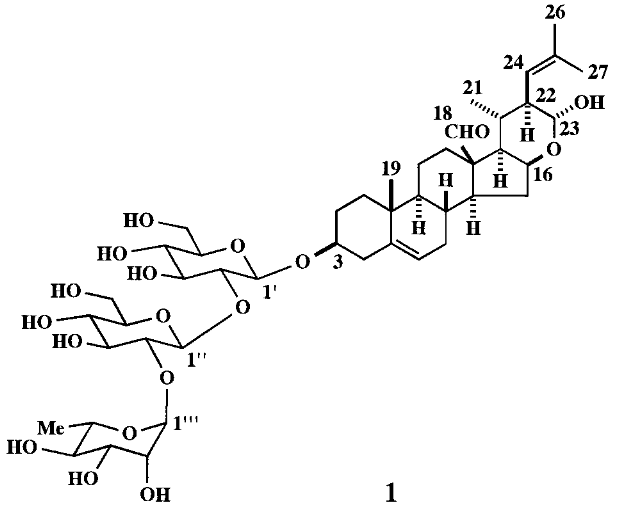


Fig. 3. The lowest energy conformer of the aglycone moiety of **1**.

The spectral data of saundersioside H (**6**) ($C_{47}H_{68}O_{15}$) indicated that the aglycone was related to that of **5** and that the diglycoside moiety attached to C-3 of the aglycone had a *p*-methoxybenzoyl group at the rhamnose C-4 hydroxyl group. Reduction of **4** with $NaBH_4$ furnished **6**. Compound **6** was defined as a hydroxymethyl derivative of **4** at C-18.

The conformation of the six-membered hemiacetal ring part of the rearranged cholestanes was examined through molecular mechanics (MM) calculations using the MM2* force field as implemented in MacroModel 6.0. The starting geometries were generated by a systematic Monte Carlo conformation search. The most stable conformer thus found was taken as starting structures for molecular dynamics (MD) calculation in vacuo at 296 K with a path length of 1000 ps and following by minimizing random structures sampled after multiple 1 ps intervals. In this run, two conformers were obtained; the most stable conformer, whose bolzmann population was 94.5% at 296 K, showed 176° for the H_{20}–C_{20}–C_{22}–H_{20}, 153.9° for the H_{22}–C_{22}–C_{23}–H_{23} and 152.8° for the H_{22}–C_{22}–C_{24}–H_{24} torsion angles (Fig. 3). The observed proton coupling constants, $^3J_{H\text{-}20,H\text{-}22}=11.0$ Hz, $^3J_{H\text{-}22,H\text{-}23}=7.7$ Hz and $^3J_{H\text{-}22,H\text{-}24}=9.7$ Hz, almost corresponded to those (12.5, 7.4 and 9.7 Hz, respectively) calculated through the application of the given dihedral angles to the advanced Karplus-type equation proposed by Haasnoot, De Leeuw & Altona (1980). The above data confirmed that the six-membered hemiacetal ring was almost a boat-form.

The cytostatic activity of **1**–**6** on human promyelocytic leukemia HL-60 cells was evaluated. The cells were continuously treated with each compound for 72 h and the cell growth was measured with an MTT assay procedure. Compounds **3**–**6** possessed an aromatic acid ester group at the glycoside moiety and strongly suppressed the growth of HL-60 cells in a dose-dependent manner. Their IC_{50} values were calculated from a dose-dependent curve of each compound as 0.021, 0.019, 0.063 and 0.052 μM, respectively, which were as potent as those of the clinically applied anticancer agents, etoposide (IC_{50} 0.025 μM) and methotrexate (IC_{50} 0.012 μM). Compound **1** showed no activity ($IC_{50}>10$ μM) and **2** was moderately cytostatic with an IC_{50} value of 4.4 μM. Thus, the aromatic acid group attached to the diglycoside moiety was considered to be a structural requirement for the potent activity.



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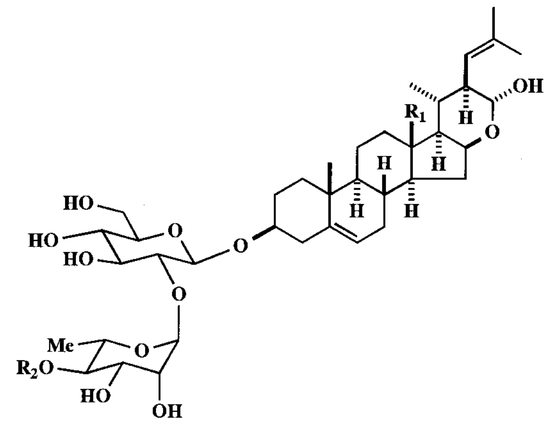

	R_1	R_2
2	**CHO**	**H**
3	**CHO**	***p*-hydroxybenzoyl**
4	**CHO**	***p*-methoxybenzoyl**
5	$\mathbf{CH_2OH}$	***p*-hydroxybenzoyl**
6	$\mathbf{CH_2OH}$	***p*-methoxybenzoyl**

3. Experimental

3.1. General

FABMS: VG AutoSpec E (matrix: Magic Bullet, a mixture of dithiothreitol and dithioerythritol, 3:1; Tokyo-Kasei, Japan). NMR (ppm, *J* Hz): Bruker AM-400 (400 MHz for ^1H NMR), Bruker AM-500 (500

MHz for ^1H NMR) or JEOL JNM-A600 (600 MHz for ^1H NMR). CC: silica gel (Fuji-Silysia Chemical, Japan), ODS silica gel (Nacalai Tesque, Japan) and Diaion HP-20 (Mitsubishi-Kasei, Japan). TLC: precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck, Germany) and RP-18 $F_{254}S$ (0.25 mm thick, Merck). HPLC: a Tosoh HPLC system (pump, CCPM; controller, CCP controller PX-8010; detector, RI-8010 or UV-8000) equipped with a Capcell Pak C_{18} column (10 mm i.d. × 250 mm, ODS, 5 μm, Shiseido, Japan) for preparative HPLC and a TSK-gel ODS-Prep column (4.6 mm i.d. × 250 mm, ODS, 5 μm, Tosoh, Japan) for analytical HPLC. Computer calculation: molecular-modeling software MacroModel 6.0 on a Silicon Graphics work station, COMTEC 4D/O2 10000SC (USA). Cell culture and assay for cytostatic activity: microplate reader, Corona MT P-32 (Japan); 96-well flat-bottom plate, Corning (USA); HL-60 cells, ICN Biomedicals (USA); RPMI 1640 medium, GIBCO BRL (USA); MTT, Sigma (USA). All other chemicals used were of biochemical reagent grade.

3.2. Plant material

The bulbs of *O. saundersiae* were purchased from Heiwaen, Japan. The bulbs were cultivated and the plant specimen is on file in our laboratory.

3.3. Extraction and isolation

The fresh plant material (16.2 kg) was extracted with hot MeOH. The MeOH extract was concentrated under reduced pressure and the viscous concentrate was partitioned between H_2O and *n*-BuOH. The *n*-BuOH-soluble phase was passed through a Diaion HP-20 column eluting with increased amounts of MeOH in H_2O. Fractions eluted with 80% MeOH and MeOH were combined and further fractionated by silica gel CC, eluting with stepwise gradients of $CHCl_3$–MeOH (9:1; 4:1; 2:1) and finally with MeOH, to collect six fractions (I–VI). Fraction III was subjected to ODS silica gel CC eluting with MeOH–H_2O (4:1) and divided into four additional fractions (IIIa–IIId). Fraction IIIb was subjected to silica gel CC eluting with $CHCl_3$–MeOH (9:1) and preparative HPLC with MeCN–H_2O (1:1; 9:11) and MeOH–H_2O (1:1) to yield **3** (60.4 mg), **5** (20.8 mg), and **6** (76.3 mg). Fraction IIIc was submitted to preparative HPLC using MeOH–H_2O (3:2) to furnish **4** (65.2 mg). Fraction IV was subjected to ODS silica gel CC eluting with MeOH–H_2O (7:3) and preparative HPLC with MeCN–H_2O (9:11) to afford **2** (51.2 mg). Fraction V was chromatographed on silica gel eluting with $CHCl_3$–MeOH–H_2O (40:10:1) and ODS silica gel with MeOH–H_2O (4:1; 7:3) to give **1** with a few impurities. Final purification of **1** was carried out by pre-

Table 1
^1H and ^{13}C NMR spectral data for compound **1**[a]

Position	^1H	*J* (Hz)	^{13}C
1 eq	1.62 br d	13.4	37.6
1 ax	0.94		
2 eq	2.05 br d	11.9	30.1
2 ax	1.69		
3	3.88 m	20.0[b]	78.8
4 eq	2.78 dd	13.3, 2.6	39.4
4 ax	2.52 br dd	13.3, 13.3	
5	–		141.3
6	5.32 br d	4.4	121.2
7α	1.48		32.2
7β	1.92 br dd	13.4, 4.7	
8	1.53 dddd	10.5, 10.5, 10.5, 4.7	33.6
9	0.92		50.3
10	–		37.0
11 eq	1.50		22.7
11 ax	1.41 dddd	12.4, 12.4, 12.4, 3.7	
12 eq	2.64 br d	12.4	33.6
12 ax	1.00 ddd	12.4, 12.4, 3.7	
13	–		59.5
14	1.28		52.8
15α	2.34 ddd	13.2, 7.0, 7.0	33.7
15β	1.91 ddd	13.2, 13.2, 4.9	
16	4.82		69.8
17	1.46		59.7
18	10.10 s		207.5
19	0.76 s		19.5
20	1.43		31.4
21	0.93 d	5.7	18.6
22	2.37 ddd	11.0, 9.7, 7.7	47.6
23	5.03 d	7.7	98.3
24	4.85 br d	9.7	126.2
25	–		134.5
26	1.66 br s		26.1
27	1.74 br s		19.0
1′	5.05 d	7.9	101.2
2′	4.26 dd	8.8, 7.9	80.3
3′	4.39 dd	8.8, 8.8	79.2
4′	4.03 dd	8.8, 8.8	71.7
5′	3.84 ddd	8.8, 6.1, 2.5	78.1
6′a	4.40 dd	11.9, 2.5	62.7
6′b	4.22 dd	11.9, 6.1	
1″	5.76 d	7.5	102.2
2″	4.18 dd	8.9, 7.5	79.1
3″	4.13 dd	8.9, 8.9	79.2
4″	4.01 dd	8.9, 8.9	72.2
5″	3.79 ddd	8.9, 5.5, 2.3	77.8
6″a	4.34 dd	11.6, 2.3	62.9
6″b	4.19 dd	11.6, 5.5	
1‴	6.23 br s		102.1
2‴	4.64 br d	3.3	72.2
3‴	4.57 dd	9.2, 3.3	72.4
4‴	4.21 dd	9.2, 9.2	74.2
5‴	4.89 dq	9.2, 6.2	70.1
6‴	1.73 d	6.2	18.8

[b] $W_{1/2}$.

[a] Spectra were measured in C_5D_5N–CD_3OD (11:1). Assignment was established by interpretation of the FG-DQF COSY, 1D selective TOCSY, HMQC and HMBC spectra.

parative HPLC using MeCN–H_2O (3:1) to yield **1** (131 mg) as a pure compound.

3.4. Saundersioside C (*1*)

Amorphous solid. $[\alpha]_D^{30}$ −49.6° (MeOH: *c* 0.10). (Found: C, 57.56; H, 7.94. Calc. for $C_{45}H_{70}O_{18}\cdot 2H_2O$: C, 57.80; H, 7.98%.) Negative-ion FABMS *m*/*z* 897 $[M-H]^-$. IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2920 (CH), 1700 (C=O), 1650, 1620, 1380, 1250, 1130, 1070, 1030, 990, 900, 800, 700. ^1H NMR (C_5D_5N): δ 10.12 (1H, br s, H-18), 6.37 (1H, br s, H-1‴), 5.85 (1H, d, J = 7.4 Hz, H-1″), 5.37 (1H, br d, J = 4.7 Hz, H-6), 5.13 (1H × 2, d, J = 7.4 Hz, H-23 and H-1′), 4.92 (1H, d, J = 9.6 Hz, H-24), 3.95 (1H, m, $W_{1/2}$ = 18.0 Hz, H-3), 1.80 (3H, d, J = 6.2 Hz, Me-6‴), 1.79 (3H, br s, Me-27), 1.71 (3H, br s, Me-26), 0.99 (3H, d, J = 5.9 Hz, Me-21), 0.82 (3H, s, Me-19). ^1H and ^{13}C NMR (C_5D_5N–CD_3OD, 1:1): Table 1).

3.5. Acid hydrolysis of *1*

A solution of **1** (20 mg) in 1 M HCl (dioxane–H_2O, 1:1, 6 ml) was heated at 100° for 2 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo, Japan) column and fractionated using a Sep-Pak C_{18} cartridge (Waters, USA), eluting with H_2O (10 ml) followed by MeOH (10 ml), to give a sugar fraction (10.1 mg) and an aglycone fraction (8.5 mg). TLC analysis of the aglycone fraction showed that it contained several unidentified artifactual sapogenols. The sugar fraction was suggested to be composed of glucose and rhamnose by direct TLC comparison with authentic samples. R_f (*n*-BuOH–Me_2CO–H_2O, 4:5:1): 0.65 (rhamnose); 0.39 (glucose). The monosaccharide mixture (2 mg) was dissolved in H_2O (1 ml), to which (−)-α-methylbenzylamine (5 mg) and Na[BH_3CN] (8 mg) in EtOH (1 ml) were added. After being set aside at 40° for 4 h followed by addition of AcOH (0.2 ml) and evaporation to dryness, the reaction mixture was acetylated with Ac_2O (0.3 ml) in pyridine (0.3 ml) at room temperature for 12 h. The crude mixture was passed through a Sep-Pak C_{18} cartridge with H_2O–MeCN (4:1; 1:1; 1:9, each 5 ml) mixture as solvents. The H_2O–MeCN (1:9) eluate was further passed through a Toyopak IC-SP M cartridge (Tosoh) with EtOH (10 ml) to give a mixture of the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides (Oshima et al., 1982), which was then analyzed by HPLC under the following conditions: solvent, MeCN–H_2O (2:3); flow rate, 0.8 ml/min; detection, UV 230 nm. The derivatives of D-glucose and L-rhamnose were detected. R_t (min): 23.0 (derivative of D-glucose); 26.1 (derivative of L-rhamnose).

Table 2
^{13}C NMR spectral data for compounds **2**–**6**[a]

C	**2**	**3**	**4**	**5**	**6**
1	37.5	37.4	37.2	37.5	37.4
2	30.2	30.0	30.3	30.5	30.5
3	78.0	78.4	78.3	78.5	78.6
4	39.0	39.4	39.3	39.4	39.6
5	141.0	141.0	141.0	141.0	141.1
6	121.2	121.5	121.4	122.0	122.0
7	32.2	32.3	32.3	32.8	32.8
8	33.5	33.6	33.6	31.8	31.9
9	50.2	50.3	50.1	50.7	50.6
10	37.0	36.9	37.0	37.1	37.2
11	22.6	22.6	22.5	21.4	21.4
12	33.7	33.6	33.8	35.2	35.2
13	59.3	59.3	59.2	46.8	46.8
14	52.7	52.7	52.5	53.3	53.2
15	33.5	33.5	33.4	33.8	33.9
16	69.9	69.9	69.9	70.7	70.6
17	59.6	59.6	59.5	60.6	60.6
18	207.3	207.3	207.3	60.4	60.4
19	19.4	19.5	19.6	19.4	19.4
20	31.3	31.3	31.2	31.5	31.5
21	18.6	18.1	18.0	18.1	18.1
22	47.6	47.6	47.6	48.2	48.2
23	98.3	98.3	98.3	98.7	98.7
24	126.3	126.3	126.2	127.3	127.3
25	134.3	134.3	134.3	133.5	133.5
26	26.0	26.1	26.0	26.1	26.1
27	18.8	18.9	18.8	19.5	19.5
1′	100.5	100.8	100.7	100.7	100.9
2′	77.9	77.3	77.3	77.3	77.4
3′	79.7	79.7	79.6	79.6	79.6
4′	71.9	71.8	71.7	71.8	71.8
5′	78.3	78.4	78.1	78.3	78.3
6′	62.7	62.7	62.6	62.6	62.6
1″	102.1	101.6	101.6	101.6	101.6
2″	72.6	72.7	72.6	72.6	72.5
3″	72.9	70.6	70.5	70.5	70.5
4″	74.2	76.4	76.5	76.3	76.7
5″	69.5	67.1	67.0	67.1	66.9
6″	18.7	18.7	18.9	18.9	18.9
1‴		122.0	123.5	121.9	123.5
2‴		132.6	132.2	132.6	132.3
3‴		116.1	114.1	116.1	114.1
4‴		163.6	163.8	163.5	163.7
5‴		116.1	114.1	116.1	114.1
6‴		132.6	132.2	132.6	132.3
7‴		166.7	166.4	166.7	166.4
OMe			55.5		55.4

[a] Spectra were measured in pyridine-d_5.

3.6. Acetylation of *1*

Compound **1** (25 mg) was acetylated with Ac_2O (0.5 ml) in pyridine (0.5 ml) and the crude acetate was chromatographed on silica gel eluting with hexane–Me_2CO (2:1) to yield the corresponding decaacetate (**1a**) (28 mg).

*3.7. Compound **1a***

Amorphous solid. IR ν_{max} (KBr) cm^{-1}: 2920 (CH), 1750 (C=O), 1360, 1230, 1030, 930, 790. ^{1}H NMR (C_5D_5N): δ 10.04 (1H, s, H-18), 6.05 (1H, d, J = 7.8 Hz, H-23), 5.76 (1H, dd, J = 9.0, 9.0 Hz, H-3″), 5.75 (1H, dd, J = 9.9, 3.2 Hz, H-3‴), 5.70 (1H, dd, J = 8.5, 8.5 Hz, H-3′), 5.65 (1H, dd, J = 3.2, 1.9 Hz, H-2‴), 5.62 (1H, dd, J = 9.9, 9.9 Hz, H-4‴), 5.48 (1H, dd, J = 9.0, 9.0 Hz, H-4″), 5.43 (1H, dd, J = 8.5, 8.5 Hz, H-4′), 5.42 (1H, br d, J = 5.3 Hz, H-6), 5.36 (1H, d, J = 1.9 Hz, H-1‴), 5.27 (1H, d, J = 7.5 Hz, H-1″), 5.11 (1H, d, J = 7.0 Hz, H-1′), 4.80 (1H, m, H-16), 4.79 (1H, br d, J = 9.5 Hz, H-24), 4.67 (1H, dd, J = 12.5, 4.5 Hz, H-6a″), 4.65 (1H, dq, J = 9.9, 6.2 Hz, H-5‴), 4.59 (1H, dd, J = 12.8, 4.7 Hz, H-6a′), 4.43 (1H, dd, J = 12.8, 2.4 Hz, H-6b′), 4.40 (1H, dd, J = 12.5, 2.3 Hz, H-6b″), 4.35 (1H, dd, J = 8.5, 7.0 Hz, H-2′), 4.19 (1H, ddd, J = 9.0, 4.5, 2.3 Hz, H-5″), 4.11 (1H, dd, J = 9.0, 7.5 Hz, H-2″), 4.10 (1H, ddd, J = 8.5, 4.7, 2.4 Hz, H-5′), 3.86 (1H, m, $W_{1/2}$ = 21.9 Hz, H-3), 2.18, 2.17, 2.15, 2.12, 2.05, 2.04, 2.03, 2.02, 2.01 and 1.98 (each 3H, s, Ac), 1.69 (3H, br s, Me-27), 1.65 (3H, br s, Me-26), 1.59 (3H, d, J = 6.2 Hz, Me-6‴), 0.97 (3H, d, J = 6.0 Hz, Me-21), 0.90 (3H, s, Me-19).

*3.8. Saundersioside D (**2**)*

Amorphous solid. $[\alpha]_D^{28}$ −60.0° (MeOH; c 0.10). (Found: C, 61.21; H, 8.51. Calc. for $C_{39}H_{60}O_{13}\cdot 3/2\,H_2O$: C, 61.32; H, 8.31%.) Negative-ion FABMS m/z 735 $[M-H]^-$. IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2950, 2920 (CH), 2870, 1700 (C=O), 1380, 1260, 1080, 1040, 1030, 900, 800 cm^{-1}. ^{1}H NMR (C_5D_5N): δ 10.14 (1H, s, H-18), 6.32 (1H, br s, H-1″), 5.32 (1H, br d, J = 4.7 Hz, H-6), 5.12 (1H, d, J = 7.0 Hz, H-23), 5.02 (1H, d, J = 7.2 Hz, H-1′), 4.91 (1H, br d, J = 9.5 Hz, H-24), 4.31 (1H, dd, J = 9.3, 9.3 Hz, H-4″), 3.95 (1H, m, $W_{1/2}$ = 20.0 Hz, H-3), 1.80 (3H, br s, Me-27), 1.74 (3H, d, J = 6.2 Hz, Me-6″), 1.71 (3H, br s, Me-26), 1.00 (3H, d, J = 5.6 Hz, Me-21), 0.96 (3H, s, Me-19). ^{13}C NMR: Table 2).

*3.9. Acid hydrolysis of **2***

Compound **2** (5 mg) was subjected to acid hydrolysis by the same procedures as described for **1** to give a sugar fraction (1.9 mg). The monosaccharide constituents in the sugar fraction were converted to the corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxylalditol acetate derivatives, which were then analyzed by HPLC. The derivatives of D-glucose and L-rhamnose were detected.

*3.10. Saundersioside E (**3**)*

Amorphous solid. $[\alpha]_D^{27}$ −32.8° (MeOH; c 0.25). (Found: C, 61.96; H, 7.96. Calc. for $C_{46}H_{64}O_{15}\cdot 2H_2O$: C, 61.87; H, 7.68%.) Negative-ion FAB-MS m/z 855 $[M-H]^-$. IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2920 (CH), 1700 (C=O), 1600 and 1505 (aromatic ring), 1420, 1360, 1300, 1260, 1160, 1110, 1090, 1050, 1020, 840, 760. UV λ_{max} (MeOH) nm (log ε): 260 (4.07). UV λ_{max} (MeOH + NaOH) nm: 299. ^{1}H NMR (C_5D_5N): δ 10.13 (1H, s, H-18), 8.29 (2H, d, J = 8.6 Hz, H-2‴, -6‴), 7.16 (2H, d, J = 8.6 Hz, H-3‴, -5‴), 6.46 (1H, br s, H-1″), 6.14 (1H, dd, J = 9.4, 9.4 Hz, H-4″), 5.46 (1H, br d, J = 4.8 Hz, H-6), 5.13 (1H, d, J = 7.1 Hz, H-23), 5.07 (1H, d, J = 7.4 Hz, H-1′), 4.89 (1H, br d, J = 9.8 Hz, H-24), 3.97 (1H, m, $W_{1/2}$ = 20.5 Hz, H-3), 1.81 (3H, br s, Me-27), 1.72 (3H, br s, Me-26), 1.57 (3H, d, J = 6.2 Hz, Me-6″), 1.00 (3H, d, J = 5.3 Hz, Me-21), 0.95 (3H, s, Me-19). ^{13}C NMR: Table 2).

*3.11. Alkaline hydrolysis of **3***

Compound **3** (10 mg) was treated with 4% KOH (4 ml) at room temperature for 2 h. The reaction mixture was neutralized by passage through an Amberlite IR-120B (Organo) column and then chromatographed over Sephadex LH-20 eluting with MeOH to yield *p*-hydroxybenzoic acid (1.2 mg) and **2** (8 mg).

*3.12. Saundersioside F (**4**)*

Amorphous solid. $[\alpha]_D^{26}$ −4.0° (MeOH; c 0.10). (Found: C, 61.82; H, 7.88. Calc. for $C_{47}H_{66}O_{15}\cdot 5/2\,H_2O$: C, 61.62; H, 7.81%.) Negative-ion FABMS m/z 869 $[M-H]^-$. IR ν_{max} (KBr) cm^{-1}: 3425 (OH), 2950, 2925 and 2870 (CH), 1710 and 1695 (C=O), 1600 and 1510 (aromatic ring), 1450, 1415, 1370, 1315, 1255, 1165, 1120, 1070, 1050, 1025, 1005, 990, 890, 840, 800, 765. UV λ_{max} (MeOH) nm (log ε): 256 (4.20). ^{1}H NMR (C_5D_5N): δ 10.15 (1H, s, H-18), 8.27 (2H, d, J = 8.8 Hz, H-2‴, -6‴), 7.01 (2H, d, J = 8.8 Hz, H-3‴, -5‴), 6.45 (1H, br s, H-1″), 6.13 (1H, dd, J = 9.8, 9.8 Hz, H-4″), 5.48 (1H, br d, J = 5.2 Hz, H-6), 5.15 (1H, d, J = 6.9 Hz, H-23), 5.08 (1H, d, J = 7.5 Hz, H-1′), 5.02 (1H, overlapping with H_2O signal, H-24), 3.98 (1H, m, $W_{1/2}$ = 20.5 Hz, H-3), 3.78 (3H, s, OMe), 1.81 (3H, br s, Me-27), 1.72 (3H, br s, Me-26), 1.56 (3H, d, J = 6.1 Hz, Me-6″), 1.01 (3H, d, J = 4.2 Hz, Me-21), 0.97 (3H, s, Me-19). ^{13}C NMR: Table 2).

*3.13. Alkaline hydrolysis of **4***

Compound **4** (5 mg) was treated with 4% KOH in EtOH (2 ml) at room temperature for 1 h to yield *p*-methoxybenzoic acid (0.4 mg) and **2** (3.8 mg).

3.14. Saundersioside G (**5**)

Amorphous solid. $[\alpha]_D^{26}$ −20.0° (MeOH; *c* 0.10). (Found: C, 61.37; H, 8.14. Calc. for $C_{46}H_{66}O_{15}\cdot 5/2\ H_2O$: C, 61.11; H, 7.92%.) Negative-ion FABMS *m*/*z* 857 $[M-H]^-$. IR ν_{max} (KBr) cm^{-1}: 3425 (OH), 2950, 2920 and 2870 (CH), 1690 (C=O), 1605 and 1510 (aromatic ring), 1420, 1365, 1305, 1265, 1160, 1120, 1020, 890, 845, 830, 805, 795, 765. UV λ_{max} (MeOH) nm (log ε): 257 (4.12). UV λ_{max} (MeOH + NaOH) nm: 297. ^{1}H NMR (C_5D_5N): δ 8.31 (2H, d, *J* = 8.6 Hz, H-2‴, -6‴), 7.17 (2H, d, *J* = 8.6 Hz, H-3‴, -5‴), 6.46 (1H, br s, H-1″), 6.15 (1H, dd, *J* = 9.7, 9.7 Hz, H-4″), 5.49 (1H, br d, *J* = 5.3 Hz, H-6), 5.29 (1H, d, *J* = 6.5 Hz, H-23), 5.07 (1H, d, *J* = 7.7 Hz, H-1′), 5.02 (1H, overlapping with H_2O signal, H-24), 3.99 (1H, m, $W_{1/2}$ = 19.8 Hz, H-3), 1.87 (3H, br s, Me-27), 1.73 (3H, br s, Me-26), 1.57 (3H, d, *J* = 6.2 Hz, Me-6″), 1.28 (3H, d, *J* = 6.1 Hz, Me-21), 1.03 (3H, s, Me-19). ^{13}C NMR: Table 2).

3.15. Saundersioside H (**6**)

Amorphous solid. $[\alpha]_D^{26}$ −16.0° (MeOH; *c* 0.10). (Found: C, 62.66; H, 8.03. Calc. for $C_{47}H_{68}O_{15}\cdot 3/2\ H_2O$: C, 62.72; H, 7.95%.) Negative-ion FABMS *m*/*z* 871 $[M-H]^-$. IR ν_{max} (KBr) cm^{-1}: 3425 (OH), 2950, 2925 and 2850 (CH), 1695 (C=O), 1600 and 1500 (aromatic ring), 1425, 1410, 1365, 1310, 1255, 1160, 1115, 1050, 1015, 890, 840, 800, 765. UV λ_{max} (MeOH) nm (log ε): 256 (4.18). ^{1}H NMR (C_5D_5N): δ 8.27 (2H, d, *J* = 8.8 Hz, H-2‴, -6‴), 6.99 (2H, d, *J* = 8.8 Hz, H-3‴, -5‴), 6.44 (1H, br s, H-1″), 6.13 (1H, dd, *J* = 9.6, 9.6 Hz, H-4″), 5.53 (1H, br d, *J* = 5.5 Hz, H-6), 5.30 (1H, br d, *J* = 6.7 Hz, H-23), 5.06 (1H, d, *J* = 7.5 Hz, H-1′), 4.88 (1H, d, *J* = 10.0 Hz, H-24), 3.98 (1H, m, $W_{1/2}$ = 20.5 Hz, H-3), 3.72 (3H, s, OMe), 1.87 (3H, br s, Me-27), 1.74 (3H, br s, Me-26), 1.56 (3H, d, *J* = 6.2 Hz, Me-6″), 1.28 (3H, d, *J* = 6.3 Hz, Me-21), 1.02 (3H, s, Me-19). ^{13}C NMR: Table 2).

3.16. Reduction of **3** and **4**

Compound **3** (10 mg) and **4** (10 mg) were treated with $NaBH_4$ (4 mg) in MeOH (4 ml) at room temperature for 30 min. Each reaction mixture was purified by silica gel column chromatography eluting with $CHCl_3$–MeOH–H_2O (40:10:1). Compound **3** yielded **5** (8.2 mg) and **4** yielded **6** (8.8 mg).

3.17. Conformational analysis

1000-Step systematic Monte Carlo conformation searches were carried out with the MM2* force field as implemented in MacroModel 6.0 to predict the fully optimized lowest energy structure (Goodman & Still, 1991). Energies were minimized with the PR conjugate gradient minimizer and convergence was obtained when the gradient root mean square was less than 0.001 kJ Å^{-1} M. Through this article, all MM calculations assumed a dielectric constant of 1.0. The MD simulations were carried out with MacroModel beginning with the lowest energy structures obtained by the Monte Carlo conformation search. The following options were used in the MD calculations; time step: 0.001 ps, equilibration time period: 10 ps, and production run time period: 1000 ps. Initial kinetic energy was added to all atoms as random velocities. Translational and rotational momentum was reset to zero every 0.1 ps. To maintain a constant temperature, the system was coupled to an external temperature bath set at 296 K. Coupling between bath and molecule was updated every 0.2 ps. In the production run time at 296 K the conformers were sampled every 1 ps, followed by energy minimizations using the MM2* force field. The final MM calculations provided the fully optimized lowest energy structure as shown in Fig. 3.

3.18. Cell culture and assay for cytostatic activity

HL-60 cells were maintained in the RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin. The leukemia cells were washed and resuspended in the above medium to 3×10^4 cells/ml and 196 µl of this cell suspension was placed in each well of a 96-well flat-bottom plate. The cells were incubated in 5% CO_2/air for 24 h at 37°. After incubation, 4 µl of EtOH–H_2O (1:1) solution containing the sample was added to give the final concentrations of 0.001–10 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 and then cell growth was evaluated by an MTT assay procedure (Sargent & Tayler, 1989). The MTT assay was carried out according to a modified method of Sargent and Taylor as follows. After termination of the cell culture, 10 µl of 5 mg/ml MTT in phosphate buffered saline was added to every well and the plate was further reincubated in 5% CO_2/air for 4 h at 37°. The plate was then centrifuged at 1500*g* for 5 min to precipitate cells and formazan. An aliquot of 150 µl of the supernatant was removed from every well, and 175 µl of DMSO was added to dissolve the formazan crystals. The plate was mixed on a microshaker for 10 min, and then read on a microplate reader at 550 nm. A dose–response curve was plotted for each compound and the concentration giving 50% inhibition (IC_{50})

was calculated. The IC_{50} values of **1**–**6**, thus, obtained were more than 10, 4.4, 0.021, 0.019, 0.063 and 0.052 μM, respectively. These values were the means of three independent experiments and agreed to less than 10% from the mean.

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