PERGAMON

Phytochemistry 55 (2000) 715–720

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Steroidal saponins from the leaves of *Cestrum sendtenerianum*

Mitsue Haraguchi [a], Yoshihiro Mimaki [b,*], Mario Motidome [c], Hiroshi Morita [b], Koichi Takeya [b], Hideji Itokawa [b], Akihito Yokosuka [b], Yutaka Sashida [b]

[a] *Centro de Sanidade Animal, Instituto Biológico, Av. Conselheiro Rodrigues Aves, 1252, CEP 04014-002, São Paulo, SP, Brazil*
[b] *School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1, Horinouchi, Hachioji, Tokyo 192-0392, Japan*
[c] *Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes, 748, CEP 05508-900, São Paulo, SP, Brazil*

Received 29 December 1999; received in revised form 14 February 2000

Abstract

Five steroidal saponins were isolated from the EtOH extract of *Cestrum sendtenerianum* (Solanaceae), as confirmed by detailed analysis of their ^{1}H, ^{13}C, and two-dimensional NMR spectral data, and by the results of hydrolytic cleavage. The saponins were revealed to contain three hydroxyl groups at the C-1β, C-2α, and C-3β positions in the spirostanol skeleton, and to bear a di- or triglycoside at C-3 as the common structural features. One of the compounds, a spirostanol triglycoside, showed weak cytotoxic activity on HL-60 human promyelocytic leukemia cells, with an IC_{50} value of 7.7 μg/ml.

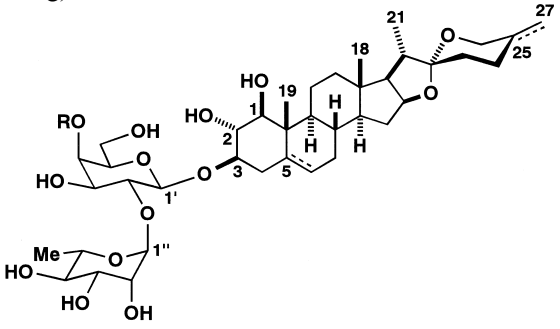
Keywords: *Cestrum sendtenerianum*; Solanaceae; Spirostanol saponins; Cytotoxic activity; HL-60 cells

1. Introduction

The genus *Cestrum* (Solanaceae), with more than 300 species, is mainly distributed in South America. The occurrence of steroidal saponins in several *Cestrum* species has been documented (Ahmad et al., 1991, 1993, 1995). Recently, steroidal saponins have received scientific attention because of their structural diversity and significant biological activities (Waller and Yamasaki, 1996). As part of our systematic study of steroidal constituents with biological activity (Mimaki et al., 1999a,b), we have now undertaken an investigation of the leaves of *Cestrum sendtenerianum* and isolated five new spirostanol saponins. This paper deals with the structural determination of the new saponins on the basis of spectroscopic analysis, including various two-dimensional (2D) NMR spectroscopic techniques, and of the results of hydrolytic cleavage.

2. Results and discussion

The EtOH extract of the leaves of *C. sendtenerianum* (dry weight 500 g) was partitioned between *n*-BuOH and H_2O. The *n*-BuOH-soluble portion was fractionated by column chromatography on silica gel followed by preparative medium-pressure liquid chromatography (MPLC) and preparative HPLC to furnish compounds **1** (9.9 mg), **2** (7.0 mg), **3** (21.6 mg), **4** (4.4 mg), and **5** (12.3 mg).



	R	A/B	
1	H	-	$\Delta^{5,25(27)}$
2	H	-	Δ^{5}
3	H	*trans* (H-5α)	$\Delta^{25(27)}$
4	H	*trans* (H-5α)	
5	β-D-Glcp	-	$\Delta^{5,25(27)}$

Compound **1**, isolated as an amorphous solid, $[\alpha]_D^{25}$ −70.7° (MeOH), showed an $[M+H]^+$ peak at *m/z* 753.4081 in the HR-FABMS, corresponding to an empirical molecular formula $C_{39}H_{60}O_{14}$, which was also deduced by analysis of the ^{13}C NMR spectrum combined

* Corresponding author. Tel.: +81-426-76-4577; fax: +81-426-76-4579.

E-mail address: mimakiy@ps.toyaku.ac.jp (Y. Mimaki).

0031-9422/00/$ - see front matter
PII: S0031-9422(00)00109-6

with various DEPT data. The glycosidic nature of **1** was shown by strong IR absorptions at 3371 and 1043 cm^{-1}. Preliminary inspection of the ^1H NMR spectrum of **1** in pyridine-d_5 led to the identification of the following representative signals: three steroid methyl protons at δ 1.36 (*s*), 1.07 (*d*, $J=7.0$ Hz), and 0.90 (*s*); exomethylene protons at δ 4.83 and 4.80 (each *br s*); an olefinic proton at δ 5.53 (*br d*, $J=5.1$ Hz); two anomeric protons at δ 6.31 (*br s*) and 5.02 (*d*, $J=7.8$ Hz); methyl protons of a 6-deoxyhexopyranose at δ 1.55 (*d*, $J=6.2$ Hz). The ^{13}C NMR spectrum displayed signals for two pairs of olefinic groups at δ 138.0 (C) and 125.1 (CH), and 144.5 (C) and 108.6 (CH_2), as well as signals for three steroid methyl carbons at δ 16.5, 15.0, and 14.8, and two anomeric carbons at δ 101.9 and 101.7. Acid hydrolysis of **1** with 1 M HCl in dioxane–H_2O (1:1) at 100° for 1 h resulted in the production of the aglycone (**1a**: $C_{27}H_{40}O_5$) in low yield, and D-galactose and L-rhamnose as the carbohydrate components. The identification of the monosaccharides, including their absolute configurations, was carried out by direct HPLC analysis of the hydrolysate using a combination of RI and optical rotary (OR) detectors. The above mentioned data, and one diagnostic acetalic carbon signal at δ 109.4 (Agrawal et al., 1985) led to the hypothesis that **1** was a spirostanol saponin with two monosaccharides.

The complete structure of **1** was established by detailed interpretation of various 2D NMR spectra such as ^1H–^1H COSY, TOCSY, HSQC, HMBC, and ROESY spectra (Figs. 1 and 2). The olefinic carbon signals at δ 138.0 (C) and 125.1 (CH) were assigned to C-5 and C-6 of the spirostanol skeleton, and the resonance at δ 125.1 was correlated to the corresponding proton signal at δ 5.53 (*br d*, $J=5.1$ Hz). Tracing out the proton spin-coupling connectivities by analysis of the ^1H–^1H COSY and TOCSY spectra, with the olefinic proton being used as the starting point of analysis, combined with the observation of the one-bond coupled carbon correlations in the HSQC spectrum, allowed the assignment of the proton and carbon signals from H-6/C-6 to Me-21/C-21, except for the two quaternary carbons at δ 43.1 and 40.3, both bearing a methyl group. The quaternary carbon signal observed at δ 43.1 showed $^2J_{C,H}$ and $^3J_{C,H}$ correlation peaks with the proton signals at δ 5.53 (H-6) and 1.36 (Me), and was assigned to C-10. Another quaternary carbon signal at δ 40.3 was assignable to C-13, which was correlated to the δ 2.05 (H-15α), 4.55 (H-16), 1.82 (H-17), 1.97 (H-20), and 0.90 (Me) resonances. The complete assignment from H-6/C-6 to Me-21/C-21 of **1** were established (Table 1). The proton signals of an oxygen-bearing methylene group were observed at δ 4.48 and 4.04 as an ABq spin system with a *J* value of 12.1 Hz. The carbon signal at δ 109.4 attributable to C-22 showed long-range correlations with one of the methylene protons at δ 4.04, along with the H-20 and Me-21 protons. The δ 4.04 resonance, in turn, displayed HMBC correlations with the olefinic carbons at δ 144.5 (C) and 108.6 (CH_2). Two adjacent methylene groups were assigned to C-23 and C-24. Thus, the assignment from C-22 to C-27 was carried out, and the presence of a double bond at C-25(27) was evident. Finally, attention focused on the A-ring (C-1–C-5). The oxymethine proton signal at δ 4.12 was revealed to be coupled with the two oxymethine proton resonances at δ 3.58 and 3.96. The signal at δ 3.96 had spin-coupling links with a geminal pair of the protons appearing at δ 2.95 and 2.89, while the resonance at δ 3.58 showed no additional correlation. In the HMBC spectrum, the methine proton signal at δ 3.58 was correlated with the carbon signals at δ 51.2 (C-9), 43.1 (C-10) and 14.8 (C-19). The signal of one of the geminal pair protons at δ 2.89 exhibited HMBC correlations with the olefinic carbon signals at δ 138.0 (C-5) and 125.1 (C-6), as well as with the C-10 carbon. The geminal protons were shown to be attached to a carbon with a resonance at δ 37.7, from which a long-range correlation was detected to H-6. The above findings allowed placement of the oxygen atoms at the C-1, C-2, and C-3 positions. The plane structure of the aglycone moiety of **1** was thus proposed to be spirosta-5,25(27)-diene-1,2,3-triol. This was consistent with the molecular formula of the aglycone obtained by acid hydrolysis of **1**. ROE correlations from Me-19 to H-8 and H-11ax, H-14 to H-9 and H-16, H-16 to H-17 and Me-26ax, H-17 to H-12ax and Me-21, and Me-18 to H-8, H-11ax and H-20 in the ROESY spectrum indicated that **1** had the usual spirostanol ring junctions and configurations of B/C *trans*, C/D *trans*, D/E *cis*, C-20α, and C-22α. The C-1, C-2, and C-3 oxygen atoms were revealed to be present in the equatorial orientations by

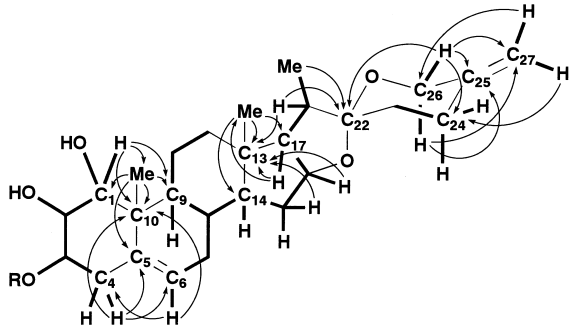


Fig. 1. HMBC correlations of the aglycone moiety of **1**.

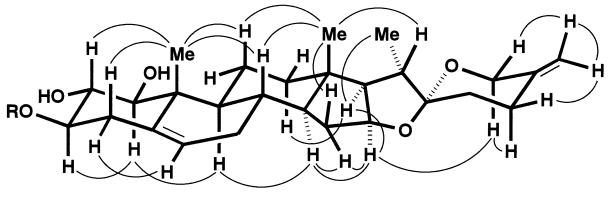


Fig. 2. ROE correlations of the aglycone moiety of **1**.

Table 1
^{1}H and ^{13}C NMR spectral data for compound **1** in pyridine-d_5

Position	^{1}H	J (Hz)	^{13}C
1	3.58 *d*	8.9	82.3
2	4.12 *dd*	8.9, 8.9	75.7
3	3.96 *ddd*	12.3, 8.9, 6.0	81.9
4 eq	2.89 *dd*	12.3, 6.0	37.7
ax	2.95 *dd*	12.3, 12.3	
5	–		138.0
6	5.53 *br d*	5.1	125.1
7 α	1.52		32.2
β	1.87 *br dd*	13.8, 5.1	
8	1.55		32.4
9	1.33 *ddd*	10.7, 10.7, 3.5	51.2
10	–		43.1
11 eq	2.87		23.9
ax	1.70		
12 eq	1.74 *ddd*	12.8, 3.1, 3.1	40.4
ax	1.22 *ddd*	12.8, 12.8, 3.2	
13	–		40.3
14	1.12		56.7
15 α	2.05		32.4
β	1.49		
16	4.55 *q*-like	7.3	81.4
17	1.82 *dd*	7.3, 6.7	63.2
18	0.90 *s*		16.5
19	1.36 *s*		14.8
20	1.97		41.9
21	1.07 *d*	7.0	15.0
22	–		109.4
23	1.80 (2H)		33.2
24 eq	2.27 *br d*	12.7	29.0
ax	2.74 *ddd*	12.7, 12.7, 5.7	
25	–		144.5
26 eq	4.04 *d*	12.1	65.0
ax	4.48 *d*	12.1	
27 a	4.83 *br s*		108.6
b	4.80 *br s*		
1′	5.02 *d*	7.8	101.7
2′	4.66 *dd*	9.5, 7.8	75.7
3′	4.27 *dd*	9.5, 3.3	76.6
4′	4.46 *br d*	3.3	70.8
5′	4.09 *br dd*	6.7, 5.2	76.9
6′ a	4.42 *dd*	11.1, 6.7	62.2
b	4.35 *dd*	11.1, 5.2	
1″	6.31 *br s*		101.9
2″	4.80 *br d*	3.7	72.5
3″	4.64 *dd*	9.6, 3.7	72.8
4″	4.28 *dd*	9.6, 9.6	74.2
5″	4.89 *dq*	9.6, 6.2	69.3
6″	1.55 *d*	6.2	18.4

the proton coupling constants of the H-1, H-2, and H-3 hydrogens ($^3J_{H\text{-}1,H\text{-}2}=8.9$ Hz, $^3J_{H\text{-}2,H\text{-}3}=8.9$ Hz, $^3J_{H\text{-}3,H\text{-}4ax}=12.3$ Hz, and $^3J_{H\text{-}3,H\text{-}4eq}=6.0$ Hz). This was supported by ROEs from H-1 to H-3 and H-9. All of these data were consistent with the structure spirosta-5,25(27)-diene-1β,2α,3β-triol for the aglycone. The structure of the diglycoside moiety of **1** was also revealed by analysis of 2D NMR spectral data. The ^{1}H–^{1}H COSY and TOCSY experiments allowed the sequential assignment of the resonances for the glycoside residue, starting from the easily distinguished anomeric protons at δ 6.31 and 5.02. Multiplet patterns and measurements of coupling constants confirmed the presence of an α-L-rhamnopyranosyl unit ($^1C_4$) and a β-D-galactopyranosyl unit ($^4C_1$). The HSQC spectrum correlated all of the proton resonances with those of the corresponding carbons. The rhamnosyl residue was shown to be a terminal unit, as suggested by the absence of any glycosylation shift for its carbon resonances (Agrawal et al., 1985; Agrawal, 1992). In the HMBC spectrum, the anomeric proton signals at δ 6.31 and 5.02 showed $^3J_{C,H}$ correlations with the δ 75.7 (C-2 of galactosyl) and 81.9 (C-3 of aglycone) resonances, respectively. The diglycoside, rhamnosyl-(1→2)-galactosyl unit, was shown to be attached to C-3 of the aglycone. Accordingly, the structure of **1** was characterized as 1β,2α-dihydroxyspirosta-5,25(27)-dien-3β-yl *O*-α-D-rhamnopyranosyl-(1→2)-β-L-galactopyranoside. Spirostanol saponins are biosynthetically derived from furostanol saponins (Mahato et al., 1982). Several steroidal saponins based upon spirost-5,25(27)-diene have been isolated by us from *Ruscus aculeatus*, together with the corresponding furostanol saponins (Mimaki et al., 1998). Although the furostanol saponin relative to **1** was not isolated in this investigation, the 25(27)-exomethylene group is considered to be biosynthetically formed in the furostanol stage.

Compound **2** was obtained as an amorphous solid, which was shown to have a molecular formula $C_{39}H_{62}O_{14}$ from the HR-FABMS and ^{13}C NMR data. The ^{13}C NMR spectrum of **2**, which included signals for an α-L-rhamnosyl and a β-D-galactosyl group, along with 27 carbon signals due to the aglycone moiety, was very similar to that of **1** with the exception of the signals due to the aglycone F-ring part (C-22–C-27). On comparison of the ^{1}H and ^{13}C NMR spectra of **2** with those of **1**, the signals due to the C-25(27) exomethylene group in **1** were replaced by the signals assignable to a Me-CH group at δ_H 0.70 (3H, *d*, $J=5.3$ Hz) and 1.59 (1H, *m*), and δ_C 17.3 (Me) and 30.6 (CH) in **2**. Furthermore, the molecular formula of **2** was greater by two hydrogen atoms than that of **1**. These data strongly indicated that **2** was a reduced derivative of **1** with regard to the C-25(27) position. The proton coupling constant between H-25 and H-26ax ($J=10.0$ Hz), the ^{13}C NMR shifts of the F-ring part (Agrawal et al., 1985), and ROE correlations from Me-27 to both of the H_2-26 protons gave evidence for the C-25*R* configuration. The structure of **2** was formulated as (25*R*)-1β,2α-dihydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranoside.

Compound **3** was obtained as an amorphous solid, and its molecular formula $C_{39}H_{62}O_{14}$ deduced from the HR-FABMS and ^{13}C NMR spectral data was identical to that of **2**. The spectral features of **3** also showed a close similarity to those of **1**, as was seen for **2**. On comparison of the ^{1}H NMR spectrum **3** with that of **1**, the olefinic proton signal due to H-6 was missing in that of **3**, accompanied by an upfield shift of Me-19 by 0.18 ppm. Furthermore, the olefinic carbon signals due to

C-5 and C-6 observed in the ^{13}C NMR spectrum of **1** were displaced by aliphatic carbon signals at δ 41.9 (CH) and 28.4 (CH_2) in that of **3**. These data indicated that **3** was a 5,6-dihydro derivative of **1**. The ^{13}C NMR shift of Me-19 at δ 8.7 (Agrawal et al., 1985) and a ROE correlation between H-2 and Me-19 accounted for the A/B *trans* ring junction (H-5α). The structure of **3** was determined to be 1β,2α-dihydroxy-5α-spirost-25(27)-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranoside.

Compound **4** was obtained as an amorphous solid. Its molecular formula of $C_{39}H_{64}O_{14}$ was four hydrogen atoms larger than that of **1**. The ^1H NMR spectrum contained signals for four steroid methyl groups at δ 1.19 (*s*), 1.11 (*d*, *J* = 7.0 Hz), 0.88 (*s*), and 0.70 (*d*, *J* = 5.4 Hz), and two anomeric protons at δ 6.28 (*d*, *J* = 1.4 Hz) and 5.04 (*d*, *J* = 7.8 Hz). The spectral features of **4** were essentially analogous to those of **1**. However, no olefinic carbon signals could be detected in the ^{13}C NMR spectrum of **4**. The ^{13}C NMR shifts of the A and B ring parts, including the C-19 carbon, and the ^1H NMR signals due to H-1, H-2, H-3, and Me-19 corresponded well to those of **3**. On the other hand, the NMR shifts of the F-ring part of **4** were superimposable on those of **2**. These data implied that **4** was a tetrahydro derivative of **1** with the H-5α and C-25*R* configurations. The structure of **4** was characterized as (25*R*)-1β,2α-dihydroxy-5α-spirostan-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranoside.

Compound **5** was obtained as an amorphous solid. It gave an $[M+Na]^+$ ion peak at *m*/*z* 974.4405 in the HR-FABMS, suggesting a molecular formula of $C_{45}H_{70}O_{19}$. The ^1H NMR spectrum contained signals for three anomeric protons at δ 6.25 (*br s*), 5.17 (*d*, *J* = 7.8 Hz), and 4.95 (1H, *d*, *J* = 7.8 Hz), as well as signals for two tertiary methyl groups at δ 1.38 and 0.90 (each *s*), a secondary methyl group at δ 1.07 (*d*, *J* = 6.9 Hz), and an exomethylene group at δ 4.82 and 4.79 (each *br s*) due to the aglycone moiety. Acid hydrolysis of **5** with 1 M HCl in dioxane–H_2O (1:1) gave **1a**, D-glucose, D-galactose, and L-rhamnose. On comparison of the ^{13}C NMR spectrum of **5** with that of **1**, a set of six additional signals corresponding a terminal β-D-glucopyranosyl group appeared at δ 107.2 (CH), 75.6 (CH), 78.9 (CH), 72.1 (CH), 78.6 (CH), and 63.0 (CH_2). The signal due to C-4 of the galactosyl moiety was displaced downfield by 10.5 ppm and observed at δ 81.3, suggesting that the C-4 hydroxyl group of the galactosyl moiety was the glycosylated position to which the additional D-glucose was linked. This was confirmed by a long-range correlation from the anomeric proton of the glucosyl at δ 5.17 to the galactose C-4 oxymethine carbon at δ 81.3. The structure of **5** was shown to be 1β,2α-dihydroxyspirosta-5,25(27)-dien-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside.

Compounds **1–5** are new steroidal saponins. They contain three hydroxyl groups at the C-1β, C-2α, and C-3β positions in the spirostanol skeleton, and bear a di- or triglycoside at C-3 as the common structural features.

Compound **5** exhibited weak cytotoxic activity against HL-60 human promyelocytic leukemia cells with an IC_{50} value of 7.7 μg/ml; that of etoposide used as a positive control was 0.75 μg/ml.

3. Experimental

3.1. General

FABMS: VG AutoSpec E (matrix: Magic Bullet, a mixture of dithiothreitol and dithioerythritol, 3:1; Tokyo-Kasei, Japan). NMR: Bruker DPX-400 (400 MHz for ^1H NMR) or Bruker AM-500 (500 MHz for ^1H NMR). CC: Silica-gel 60 (Merck, Germany). TLC: precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck, Germany) and RP-18 $F_{254}S$ (0.25 mm thick, Merck). MPLC: a CIG column system (Kusano Scientific, Japan) equipped with a glass column [22 mm i.d.×300 mm, octadecylsilanized (ODS) silica gel, 20 μm]. Preparative HPLC: Inertsil PREP-ODS column (20 mm i.d.×250 mm, ODS, 10 μm, GL Science, Japan). Cell culture and assay for cytotoxic activity: microplate reader, Inter Med Immuno-Mini NJ-2300 (Japan); 96-well flat-bottom plate, Iwaki Glass (Japan); HL-60 cells (JCRB 0085), Human Science Research Resources Bank (Japan); RPMI 1640 medium, GIBCO BRL (USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), Sigma (USA). All other chemicals used were of biochemical reagent grade.

3.2. Plant material

Cestrum sendtenerianum was collected in the fields of Itapetininga City, São Paulo State. It was identified by Dr. Sylvio Panizza, Universidade de São Paulo, and a voucher specimen of the plant is on file in our laboratory.

3.3. Extraction and isolation

The dried leaves of the plant (500 g) were extracted with hot EtOH three times (each 960 ml). The EtOH extract was concentrated under reduced pressure to give a crude residue (71 g). This was partitioned between H_2O-saturated *n*-BuOH and H_2O. CC of the *n*-BuOH-soluble phase (39 g) and elution with stepwise gradients of $CHCl_3$–MeOH (9:1; 4:1; 7:3), followed by $CHCl_3$–MeOH–H_2O (20:10:1; 7:4:1), and finally with MeOH alone, gave six fractions, I (0.112 g), II (0.123 g), III (0.177 g), IV (0.737 g), V (4.79 g), and VI (18.4 g). Fraction V was further fractionated by submitting it to MPLC using MeOH–H_2O (4:1) to collect 22 fractions (V-1–V-22). Fractions V-15–V-17 were combined (0.224 g) and repeatedly subjected to preparative HPLC using

MeCN–H_2O (2:3) to afford **1** (9.9 mg), **2** (7.0 mg), **3** (21.6 mg), **4** (4.4 mg), and **5** (12.3 mg).

3.4. Compound *1*

Amorphous solid, $[\alpha]_D^{25}$ −70.7° (MeOH: *c* 0.20). HR-FABMS (positive mode) *m/z*: 753.4081 $[M+H]^+$ ($C_{39}H_{61}O_{14}$ requires 753.4061); IR ν_{max} (film) cm^{-1}: 3371 (OH), 2903 and 2848 (CH), 1449, 1373, 1230, 1130, 1043, 983, 920, 876; ^1H and ^{13}C NMR spectral data: Table 1.

3.5. Acid hydrolysis of *1*

A solution of **1** (8 mg) in 1 M HCl (dioxane–H_2O, 1:1, 4 ml) was heated at 100° for 1 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU column (Organo, Japan) and chromatographed on silica gel using a gradient mixture of $CHCl_3$–MeOH (12:1 to 1:1) to give an aglycone **1a** (1.2 mg) and a sugar fraction (3.1 mg). HPLC analysis of the sugar fraction under the following conditions showed the presence of D-galactose and L-rhamnose. Column: Capcell Pak NH_2 UG80 (4.6 mm i.d. × 250 mm, Shiseido, Japan); detector: Shodex OR-2 (Showa-Denko, Japan); solvent: MeCN–H_2O (3:1); flow rate: 0.35 ml/min. R_t (min): 13.33 (L-rhamnose, negative optical rotation); 18.20 (D-galactose, positive optical rotation).

3.6. Compound *1a*

Amorphous solid, $[\alpha]_D^{24}$ −32.0° (EtOH: *c* 0.10). FABMS (positive mode) *m/z*: 487.38 $[M+Na]^+$, 445.32 $[M+H]^+$; IR ν_{max} (film) cm^{-1}: 3387 (OH), 2925, 2902, and 2850 (CH), 1452, 1375, 1230, 1071, 1044, 1012, 993, 959, 939, 921, 896, 876; ^1H NMR spectral data (pyridine-d_5): δ 5.63 (1H, *br d*, *J* = 5.6 Hz, H-6), 4.83 and 4.80 (each 1H, *br s*, H_2-27), 4.55 (1H, *ddd*, *J* = 7.7, 7.7, 6.4 Hz, H-16), 4.48 and 4.05 (each 1H, *d*, *J* = 12.3 Hz, H_2-26), 4.06 (1H, *dd*, *J* = 9.2, 9.2 Hz, H-2), 3.93 (1H, *ddd*, *J* = 11.8, 9.2, 5.6 Hz, H-3), 3.66 (1H, *d*, *J* = 9.2 Hz, H-1), 1.36 (3H, *s*, Me-19), 1.09 (3H, *d*, *J* = 7.0 Hz, Me-21), 0.93 (3H, *s*, Me-18); ^{13}C NMR spectral data: Table 2.

3.7. Compound *2*

Amorphous solid, $[\alpha]_D^{25}$ −57.1° (MeOH: *c* 0.14). HR-FABMS (positive mode) *m/z*: 777.4000 $[M+Na]^+$ ($C_{39}H_{62}O_{14}Na$ requires 777.4037); IR ν_{max} (film) cm^{-1}: 3377 (OH), 2951 and 2926 (CH), 1454, 1375, 1260, 1242, 1130, 1054, 982, 920, 899, 867; ^1H NMR spectral data (pyridine-d_5): δ 6.32 (1H, *d*, *J* = 1.3 Hz, H-1″), 5.52 (1H, *br d*, *J* = 4.8 Hz, H-6), 5.02 (1H, *d*, *J* = 7.7 Hz, H-1′), 4.56 (1H, *q*-like, *J* = 7.1 Hz, H-16), 4.13 (1H, *dd*, *J* = 8.8, 8.8 Hz, H-2), 3.97 (1H, *ddd*, *J* = 12.1, 8.8, 6.0 Hz, H-3), 3.58 (1H, *d*, *J* = 8.8 Hz, H-1), 3.58 (1H, *br d*, *J* = 10.0 Hz, H-26eq), 3.51 (1H, *dd*, *J* = 10.0, 10.0 Hz, H-26ax), 1.55 (3H, *d*, *J* = 6.0 Hz, Me-6″), 1.36 (3H, s, Me-19), 1.12 (3H, *d*, *J* = 6.9 Hz, Me-21), 0.90 (3H, *s*, Me-18), 0.70 (3H, *d*, *J* = 5.3 Hz, Me-27); ^{13}C NMR spectral data: Table 2.

Table 2
^{13}C NMR spectral data for compounds **1a** and **2–5** in pyridine-d_5

C	1a	2	3	4	5
1	82.6	82.3	81.7	81.8	82.3
2	78.2	75.7	76.4	76.4	75.4
3	73.2	81.9	81.7	81.8	81.4
4	41.3	37.7	33.5	33.5	37.5
5	139.3	138.0	41.9	41.9	138.0
6	124.4	125.2	28.4	28.5	125.1
7	32.2	32.2	32.3	32.4	32.2
8	32.4	32.4	35.5	35.6	32.4
9	51.4	51.2	55.6	55.6	51.2
10	43.6	43.1	41.7	41.7	43.1
11	24.0	23.9	24.5	24.6	23.9
12	40.5	40.4	40.7	40.8	40.2
13	40.3	40.2	40.5	40.5	40.2
14	56.8	56.7	56.6	56.7	56.6
15	32.4	32.4	32.3	32.5	32.4
16	81.4	81.1	81.4	81.1	81.4
17	63.2	63.2	63.2	63.3	63.1
18	16.6	16.5	16.6	16.7	16.5
19	15.0	14.8	8.7	8.8	14.8
20	41.9	42.0	41.9	42.0	41.9
21	15.1	15.0	14.9	15.0	15.0
22	109.4	109.2	109.4	109.2	109.4
23	33.2	31.9	33.2	31.9	33.2
24	29.0	29.3	28.9	29.3	29.0
25	144.5	30.6	144.5	30.6	144.5
26	65.0	66.9	65.0	66.9	65.0
27	108.7	17.3	108.6	17.3	108.6
1′		101.7	101.4	101.4	101.0
2′		75.7	76.1	76.1	76.4
3′		76.6	76.5	76.6	76.5
4′		70.8	70.8	70.9	81.3
5′		76.9	76.9	77.0	75.4
6′		62.2	62.3	62.4	60.9
1″		101.9	102.1	102.1	102.1
2″		72.5	72.4	72.5	72.4
3″		72.8	72.7	72.8	72.7
4″		74.2	74.1	74.2	74.1
5″		69.3	69.3	69.4	69.4
6″		18.4	18.4	18.5	18.4
1‴					107.2
2‴					75.6
3‴					78.9
4‴					72.1
5‴					78.6
6‴					63.0

3.8. Compound *3*

Amorphous solid, $[\alpha]_D^{25}$ −54.2° (MeOH: *c* 0.43). HR-FABMS (positive mode) *m/z*: 777.4076 $[M+Na]^+$ ($C_{39}H_{62}O_{14}Na$ requires 777.4037); IR ν_{max} (film) cm^{-1}: 3379 (OH), 2925 and 2849 (CH), 1451, 1374, 1258, 1232, 1127, 1047, 921, 878; ^1H NMR spectral data (pyridine-d_5): δ 6.25 (1H, *br s*, H-1″), 5.02 (1H, *d*, *J* = 7.7 Hz, H-1′), 4.82 and 4.79 (each 1H, *br s*, H_2-27), 4.55 (1H, *q*-like, *J* = 7.6 Hz, H-16), 4.48 and 4.04 (each 1H, *d*, *J* = 12.2 Hz, H_2-26), 4.02 (1H, overlapping, H-3), 3.99

(1H, *dd*, $J=9.0$, 9.0 Hz, H-2), 3.51 (1H, *d*, $J=9.0$ Hz, H-1), 1.54 (3H, *d*, $J=6.1$ Hz, Me-6″), 1.18 (3H, *s*, Me-19), 1.07 (3H, *d*, $J=6.9$ Hz, Me-21), 0.88 (3H, *s*, Me-18); ^{13}C NMR spectral data:Table 2.

3.9. Compound 4

Amorphous solid, $[\alpha]_D^{25}$ −56.4° (MeOH: *c* 0.11). HR-FABMS (positive mode) *m/z*: 779.4198 $[M+Na]^+$ ($C_{39}H_{64}O_{14}Na$ requires 779.4194); IR ν_{max} (film) cm^{-1}: 3377 (OH), 2950, 2926, and 2857 (CH), 1453, 1375, 1241, 1129, 1072, 1053, 982, 921, 899, 866; ^{1}H NMR spectral data (pyridine-d_5): δ 6.28 (1H, *d*, $J=1.4$ Hz, H-1″), 5.04 (1H, *d*, $J=7.8$ Hz, H-1′), 4.55 (1H, *q*-like, $J=7.4$ Hz, H-16), 4.03 (1H, overlapping, H-3), 4.01 (1H, *dd*, $J=8.9$, 8.9 Hz, H-2), 3.58 (1H, *br d*, $J=10.3$ Hz, H-26eq), 3.52 (1H, *d*, $J=8.9$ Hz, H-1), 3.51 (1H, *dd*, $J=10.3$, 10.3 Hz, H-26ax), 1.54 (3H, *d*, $J=6.2$ Hz, Me-6″), 1.19 (3H, *s*, Me-19), 1.11 (3H, *d*, $J=7.0$ Hz, Me-21), 0.88 (3H, *s*, Me-18), 0.70 (3H, *d*, $J=5.4$ Hz, Me-27); ^{13}C NMR spectral data: Table 2.

3.10. Compound 5

Amorphous solid, $[\alpha]_D^{25}$−124.4° (MeOH:*c* 0.25); HR-FABMS (positive mode) *m/z*: 937.4405 $[M+Na]^+$ ($C_{45}H_{70}O_{19}Na$ requires 937.4409); IR ν_{max} (film) cm^{-1}: 3360 (OH), 2923, 2898, and 2844 (CH), 1451, 1372, 1227, 1133, 1067, 1039, 988, 921, 877; ^{1}H NMR spectral data (pyridine-d_5): δ 6.25 (1H, *br s*, H-1″), 5.53 (1H, *br d*, $J=5.3$ Hz, H-6), 5.17 (1H, *d*, $J=7.8$ Hz, H-1‴), 4.95 (1H, *d*, $J=7.8$ Hz, H-1′), 4.82 and 4.79 (each 1H, *br s*, H_2-27), 4.55 (1H, overlapping, H-16), 4.48 and 4.04 (each 1H, *d*, $J=12.2$ Hz, H_2-26), 4.12 (1H, *dd*, $J=9.0$, 9.0 Hz, H-2), 3.93 (1H, *ddd*, $J=12.4$, 9.0, 6.0 Hz, H-3), 3.58 (1H, d, $J=9.0$ Hz, H-1), 1.54 (3H, *d*, $J=6.1$ Hz, Me-6″), 1.38 (3H, *s*, Me-19), 1.07 (3H, *d*, $J=6.9$ Hz, Me-21), 0.90 (3H, *s*, Me-18); ^{13}C NMR spectral data: Table 2.

3.11. Acid hydrolysis of 5

Compound **5** (5 mg) was subjected to acid hydrolysis as described for **1** to give **1a** (0.8 mg) and a sugar fraction (1.8 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of that of **1** showed the presence of D-glucose, D-galactose, and L-rhamnose. R_t (min): 13.35 (L-rhamnose, negative optical rotation); 18.24 (D-galactose, positive optical rotation); 18.63 (D-glucose, positive optical rotation).

3.12. Cell culture assay

HL-60 cells were maintained in the RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. 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. 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 0.1–10 μg/ml and 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 using a modified MTT reduction assay (Sargent and Taylor, 1989). Briefly, 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 $1500\times g$ 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 at 550 nm. A dose response curve was plotted for **5**, which showed more than 50% of cell growth inhibition at the sample concentration of 10 μg/ml, and the concentration giving 50% inhibition (IC_{50}) was calculated.

References

Agrawal, P.K., 1992. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. Phytochemistry 31, 3307–3330.

Agrawal, P.K., Jain, D.C., Gupta, R.K., Thakur, R.S., 1985. Carbon-13 NMR spectroscopy of steroidal sapogenins and steroidal saponins. Phytochemistry 24, 2479–2496.

Ahmad, V.U., Baqai, F.T., Fatima, I., Ahmad, R., 1991. A spirostanol glycoside from *Cestrum nocturnum*. Phytochemistry 30, 3057–3061.

Ahmad, V.U., Baqai, F.T., Ahmad, R., 1993. A tigogenin pentasaccharide from *Cestrum diurnum*. Phytochemistry 34, 511–515.

Ahmad, V.U., Baqai, F.T., Ahmad, R., 1995. A diosgenin tetrasaccharide from *Cestrum nocturnum*. Z. Naturforsch. 50b, 1104–1110.

Mahato, S.B., Ganguly, A.N., Sahu, N.P., 1982. Steroid saponins. Phytochemistry 21, 959–978.

Mimaki, Y., Kuroda, M., Kameyama, A., Yokosuka, A., Sashida, Y., 1998. New steroidal constituents from the underground parts of *Ruscus aculeatus* and their cytostatic activity on HL-60 cells. Chem. Pharm. Bull. 46, 298–303.

Mimaki, Y., Kuroda, M., Fukasawa, T., Sashida, Y., 1999a. Steroidal glycosides from the bulbs of *Allium jesdianum*. J. Nat. Prod. 62, 194–197.

Mimaki, Y., Satou, T., Kuroda, M., Sashida, Y., Hatakeyama, Y., 1999b. Steroidal saponins from the bulbs of *Lilium candidum*. Phytochemistry 51, 567–573.

Sargent, J.M., Taylor, C.G., 1989. Appraisal of the MTT assay as a rapid test of chemosensitivity in acute myeloid leukemia. Br. J. Cancer 60, 206–210.

Waller, G.R., Yamasaki, K. (Eds.), 1996. Saponins Used in Traditional and Modern Medicine. Plenum Press. New York.