Spirostanol Pentaglycosides from the Underground Parts of *Polianthes tuberosa*

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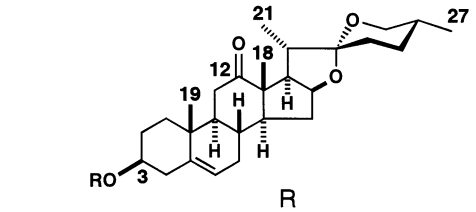
Phytochemical analysis of the underground parts of *Polianthes tuberosa* has resulted in the isolation of four new spirostanol saponins with five monosaccharides (**1**−**4**). Their structures were determined by spectroscopic analysis, including extensive 1D and 2D NMR data, and the results of hydrolytic cleavage. The cytotoxic activities of **1**−**4** against HL-60 human promyelocytic leukemia cells and HSC-2 human oral squamous cell carcinoma cells are reported.

The genus *Polianthes* belongs to the Agavaceae family with some 12 species and contains one of the longest cultivated species of cut flowers, the tuberosa, *Polianthes tuberosa* L., indigenous to Mexico.[1] The flowers are fragrant and used for a raw material of perfume. The flower volatile compounds were identified as benzyl benzoate, methyl (*E*)-isoeugenol, ethyl myristate, and methyl anthranilate.[2] The underground parts of *P. tuberosa* have pharmacological significance in Chinese folk medicine and have been used for the treatment of burns and swellings, being applied externally.[3] A few glycosides, such as (22*S*)-2β,3β,22-trihydroxycholest-5-en-16β-yl β-D-apiofuranoside,[4] 29-hydroxystigmast-5-en-3β-yl β-D-glucopyranoside,[5] and diribofuranosyl ethyleneglycol,[6] were isolated from the underground parts of *P. tuberosa*. Recently, we isolated a new cholestan glycoside and three new steroidal saponins from the aerial parts of *P. tuberosa*.[7] In a continuation of a search for the secondary metabolites produced by *P. tuberosa*, we have now investigated its underground parts, resulting in the isolation of four new spirostanol saponins with five monosaccharides (**1**−**4**). This paper deals with the structural determination of **1**−**4** on the basis of spectroscopic analysis, including extensive 1D and 2D NMR data, and the results of hydrolytic cleavage. The cytotoxic activities of **1**−**4** against HL-60 human promyelocytic leukemia cells and HSC-2 human oral squamous cell carcinoma cells are also described.



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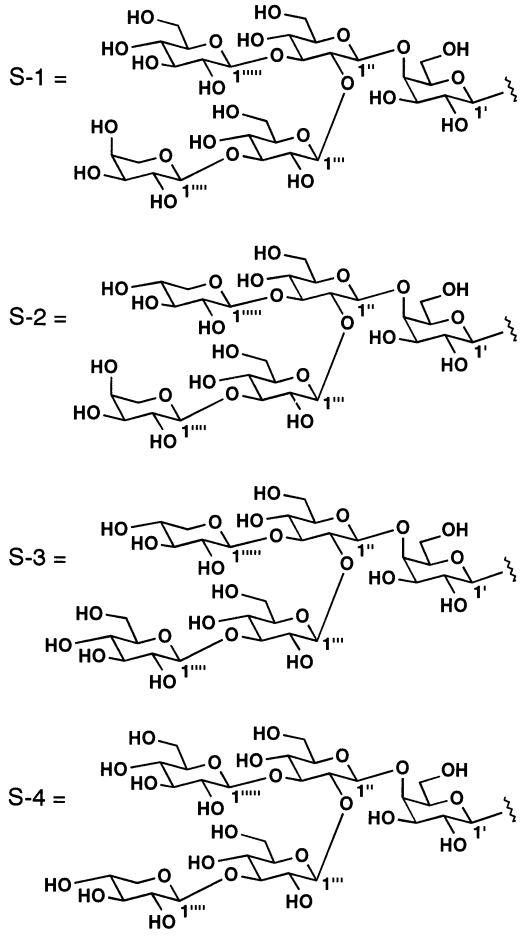

Results and Discussion

The dry, underground parts of *P. tuberosa* (1.3 kg) were extracted with MeOH under reflux. The MeOH extract was passed through a porous-polymer polystyrene resin (Diaion HP-20) column eluted with MeOH−H_2O mixtures followed by MeOH. The 80% MeOH eluate portion was subjected to multiple chromatographic steps over Si gel and octadecylsilanized (ODS) Si gel, giving compounds **1**−**4**.

Compound **1** was obtained as an amorphous solid with a molecular formula of $C_{56}H_{90}O_{28}$, as determined by data of the negative-ion FABMS, showing an $[M - H]^-$ ion at *m*/*z* 1209, the ^{13}C NMR spectrum with a total of 56 carbon signals, and the results of elemental analysis. The ^1H NMR spectrum of **1** showed two three-proton singlet signals at

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δ 1.07 and 0.76 and two three-proton doublet signals at δ 1.25 (J = 6.9 Hz) and 0.73 (J = 6.1 Hz), which were characteristic of the spirostanol skeleton, as well as signals for five anomeric protons at δ 5.42 (d, J = 7.9 Hz), 5.08 (d, J = 7.8 Hz), 4.95 (d, J = 7.8 Hz), 4.94 (d, J = 7.0 Hz), and 4.73 (1H, d, J = 7.7 Hz). When **1** was submitted to acid hydrolysis with 1 M HCl in dioxane−H_2O (1:1), it was hydrolyzed to yield a sapogenin identified as (25*R*)-3β-hydroxy-5α-spirostan-12-one (hecogenin),[8] and L-arabinose, D-galactose, and D-glucose as the carbohydrate moieties. The monosaccharides, including their absolute configurations, were identified by HPLC analysis following their conversion to the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives.[9] The negative-ion FABMS of **1** showed fragment ion peaks at *m*/*z* 1077 and 1047; the former was assignable to the loss of an arabinosyl group from the parent ion at *m*/*z* 1209, and the latter corresponded to the loss of a hexosyl, that is, a glucosyl or a galactosyl group. Furthermore, two prominent fragment ion peaks at *m*/*z* 915 and 591 were due to the elimination of an arabinosyl and a hexosyl, and an arabinosyl and three hexosyl groups, respectively. This was suggestive of a branched pentaglycoside. The severe overlapping of the proton signals for the pentaglycoside moiety excluded the possibility of a complete assignment in a straightforward way using a conventional heteronuclear COSY spectrum in **1**. An HSQC-TOCSY technique was applied to solve the sugar sequence of **1**, which correlates the anomeric protons with their respective skeleton carbons atoms.[10] This allowed the identification of the ^{13}C NMR signals for all the monosaccharides and the substituted positions of the inner glycosyl moieties, with only little knowledge of the sequential ^{1}H NMR assignment. On the H-1 track through the anomeric ^{1}H/^{13}C correlations at $\delta_{H/C}$ 5.08 (d, J = 7.8 Hz)/104.4, five relayed cross-peaks extending to C-6 were observed, with the carbon chemical shifts of δ 75.3 (C-2‴″), 78.5 (C-3‴″), 71.5 (C-4‴″), 78.3 (C-5‴″), and 62.4 (C-6‴″), confirming the presence of a terminal β-D-glucopyranosyl unit in **1**.[8,11] The anomeric proton signal at δ 4.94 (d, J = 7.0 Hz) showed relayed correlation peaks with the five carbon signals in sequence at δ 105.6 (C-1‴), 72.7 (C-2‴), 74.1 (C-3‴), 69.2 (C-4‴), and 67.0 (C-5‴″), indicative of a terminal α-L-arabinopyranosyl group.[8,11] The HSQC-TOCSY spectrum showed that the anomeric proton signal at δ 5.42 (d, J = 7.9 Hz) was correlated to the δ 104.0 signal (C-1‴) and then established that the next five carbon resonances in sequence for this sugar were δ 75.1 (C-2‴), 86.2 (C-3‴), 69.2 (C-4‴), 78.1 (C-5‴), and 62.2 (C-6‴). These shift values gave an excellent match with literature data for β-D-glucopyranose glycosylated at C-3.[12,13] The anomeric proton at δ 5.95 (d, J = 7.8 Hz) was correlated to the six carbon signals at δ 104.7 (CH), 88.3 (CH), 81.1 (CH), 77.4 (CH), 70.7 (CH), and 63.0 (CH_2). In the ^{1}H−^{1}H COSY spectrum, the anomeric proton had a proton spin-coupling with the δ 4.14 (dd, J = 8.7, 7.8 Hz) signal, which was connected to the one-bond coupled carbon signal at δ 81.1 using the HSQC spectrum. The δ 4.14 resonance showed, in turn, a $^3J_{H,H}$ correlation with δ 4.04 (dd, J = 8.7, 8.7 Hz), and it was associated with δ 88.3. These correlations led to the assignment of δ 81.1 and 88.3 as C-2 and C-3, respectively, of a β-D-glucopyranosyl unit, to which the other sugars were linked.[8,11] On the H-1 track of a monosaccharide with an anomeric proton resonance at δ 4.73 (d, J = 7.7 Hz), four relayed cross-peaks were observed in sequence at δ 102.6 (C-1′), 73.1 (C-2′), 75.4 (C-3′), and 80.1 (C-4′), and these signals together with the two remaining sugar signals at δ 75.3 (C-5′) and 60.8 (C-6′) corresponded to a β-D-galactopyranosyl group glycosylated at C-4.[8,11] The above spectral information provided evidence for a terminal β-D-glucopyranosyl unit, a terminal α-L-arabinopyranosyl unit, a C-3 substituted β-D-glucopyranosyl unit, a C-2 and C-3 disubstituted β-D-glucopyranosyl unit, and a C-4 substituted β-D-galactopyranosyl unit present in **1**. In the HMBC spectrum, correlation peaks were observed from δ 5.42 (H-1 of 3-substituted glucosyl) to 81.1 (C-2 of 2,3-disubstituted glucosyl), δ 5.08 (H-1 of terminal glucosyl) to 88.3 (C-3 of 2,3-disubstituted glucosyl), δ 4.95 (H-1 of 2,3-disubstituted glucosyl) to 80.1 (C-4 of galactosyl), δ 4.94 (H-1 of arabinosyl) to 86.2 (C-3 of 3-substituted glucosyl), and δ 4.73 (H-1 of galactosyl) to 77.9 (C-3 of aglycon). All of these data for **1** were consistent with the structure (25*R*)-3β-[(*O*-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)-oxy]-5α-spirostan-12-one.

Compound **2** was shown to have the molecular formula $C_{55}H_{88}O_{27}$ on the basis of the negative-ion FABMS (*m*/*z* 1179 [M − H]$^-$), ^{13}C NMR data (55 carbon signals), and elemental analysis. Analysis of the ^{1}H and ^{13}C NMR spectra of **2** and comparison of those of **1** implied that **2** differed from **1** in terms of the terminal monosaccharide constituent. Instead of the signals for the terminal β-D-glucopyranosyl group, five carbon signals assignable to a terminal β-D-xylopyranosyl group were newly observed at δ 104.9 (C-1‴″), 75.1 (C-2‴″), 78.2 (C-3‴″), 70.8 (C-4‴″), and 67.3 (C-5‴″) in **2**, and the anomeric carbon signal was associated with the δ 5.01 (d, J = 7.8 Hz) signal by the HMQC spectrum. The anomeric proton signal at δ 4.92 (d, J = 7.1 Hz) and five carbon signals arising from a terminal α-L-arabinopyranosyl group appeared at almost the same positions as those observed in **1**. Acid hydrolysis of **2** with 1 M HCl in dioxane−H_2O (1:1) gave (25*R*)-3β-hydroxy-5α-spirostan-12-one, L-arabinose, D-galactose, D-glucose, and D-xylose. In the HMBC spectrum of **2**, the anomeric proton signal of the xylosyl group at δ 5.01 showed a long-range correlation with C-3 of a 2,3-branched β-D-glucopyranosyl moiety at δ 87.3, whereas that of the arabinosyl at δ 4.92 with C-3 of a β-D-glucopyranosyl at δ 86.2, which was attached at C-2 of a 2,3-branched β-D-glucopyranosyl. Thus, the structure of **2** was assigned as (25*R*)-3β-[(*O*-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)oxy]-5α-spirostan-12-one.

Compound **3** was analyzed for $C_{56}H_{90}O_{28}$ by combined negative-ion FABMS (*m*/*z* 1209 [M − H]$^-$), ^{13}C NMR data (56 carbon signals), and elemental analysis. The ^{1}H NMR spectrum of **3** contained signals for five anomeric protons at δ 5.40 (d, J = 7.9 Hz), 4.99 (d, J = 7.8 Hz), 4.96 (d, J = 7.8 Hz), 4.95 (d, J = 7.8 Hz), and 4.73 (1H, d, J = 7.6 Hz), along with signals for four steroid methyls. Acid hydrolysis of **3** with 1 M HCl in dioxane−H_2O (1:1) gave (25*R*)-3β-hydroxy-5α-spirostan-12-one, D-galactose, D-glucose, and D-xylose. Since the ^{1}H NMR signals for the pentaglycoside moiety were highly overlapped, the unequivocal ^{1}H and ^{13}C NMR shift assignments were carried out by analysis of the HSQC-TOCSY spectrum combined with the ^{1}H−^{1}H COSY and HSQC spectra, as established for those of **1**. Comparison of the carbon chemical shift thus assigned with those of the reference methyl glycosides,[11] taking into account the known effects of *O*-glycosylation, indicated that **3** contained a terminal β-D-glucopyranosyl unit, a terminal β-D-xylopyranosyl unit, a C-3 substituted β-D-glucopyranosyl unit, a C-2 and C-3 disubstituted β-D-glucopyranosyl unit, and a C-4 substituted β-D-galactopyranosyl unit. The

sugar sequences were confirmed by HMBC correlations from δ 5.40 (H-1 of 3-substituted glucosyl) to 80.8 (C-2 of 2,3-disubstituted glucosyl), δ 4.99 (H-1 of terminal xylosyl) to 87.3 (C-3 of 2,3-disubstituted glucosyl), δ 4.96 (H-1 of 2,3-disubstituted glucosyl) to 79.9 (C-4 of galactosyl), δ 4.95 (H-1 of terminal glucosyl) to 87.5 (C-3 of 3-substituted glucosyl), and δ 4.73 (H-1 of galactosyl) to 77.9 (C-3 of aglycon). The structure of **3** was shown to be (25*R*)-3β-[(*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)oxy]-5α-spirostan-12-one.

Compound **4** was deduced as $C_{56}H_{90}O_{28}$ from the negative-ion FABMS (*m/z* 1209 $[M - H]^-$), ^{13}C NMR (56 signals), and elemental analysis data. Acid hydrolysis of **3** with 1 M HCl in dioxane−H_2O (1:1) gave (25*R*)-3β-hydroxy-5α-spirostan-12-one, D-galactose, D-glucose, and D-xylose. Comparison of the ^1H and ^{13}C NMR spectra of **4** with those of **3** showed their considerable structural similarity and confirmed that the pentaglycoside moiety included a terminal β-D-glucopyranosyl unit, a terminal β-D-xylopyranosyl unit, a C-3 substituted β-D-glucopyranosyl unit, a C-2 and C-3 disubstituted β-D-glucopyranosyl unit, and a C-4 substituted β-D-galactopyranosyl unit, as in **3**. The FABMS fragment ion at *m/z* 915 corresponded to the loss of a glucosyl and a xylosyl from the $[M - H]^-$ ion. From the above data, **4** was presumed to be a positional isomer of **3** with regard to the terminal monosaccharides linked to the inner sugar moieties. In the HMBC spectrum, the anomeric proton signal due to the terminal glucosyl group at δ 5.07 (d, $J = 7.8$ Hz) showed a long-range correlation with the δ 88.3 resonance assignable to C-3 of a 2,3-branched β-D-glucopyranosyl moiety, whereas that of the xylosyl at δ 4.92 (d, $J = 7.5$ Hz) with C-3 of a β-D-glucopyranosyl at δ 87.1, which was attached at C-2 of a 2,3-branched β-D-glucopyranosyl. Thus, the structure of **4** was determined to be (25*R*)-3β-[(*O*-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)oxy]-5α-spirostan-12-one.

Compounds **1**−**4** are newly described spirostanol saponins with five monosaccharides, and as far as we know, the branched pentaglycosides of **1**, **2**, and **4** have not been reported as sugars of either steroidal saponins or triterpene saponins. Compounds **1**−**4** were evaluated for their cytotoxic activities against HL-60 cells and HSC-2 cells (Table 2). Although the cytotoxicity of **1**−**4** against HL-60 cells was moderate (IC_{50} 3.9−9.0 μg/mL) compared with that of etoposide used as a positive control (IC_{50} 0.30 μg/mL), HSC-2 cells, which were resistant to etoposide (IC_{50} 24.4 μg/mL), were relatively sensitive to **1**−**4**, showing IC_{50} values ranging between 1.5 and 13.0 μg/mL.

Table 1. ^{13}C NMR Data for the Sugar Moiety of **1**−**4**[a]

carbon	1	2	3	4
1′	102.6	102.6	102.6	102.6
2′	73.1	73.1	73.1	73.1
3′	75.4	75.5	75.5	75.4
4′	80.1	80.0	79.9	80.1
5′	75.3	75.4	75.3	75.3
6′	60.8	60.8	60.8	60.8
1″	104.7	104.8	104.8	104.8
2″	81.1	80.9	80.8	81.0
3″	88.3	87.3	87.3	88.3
4″	70.7	70.4	70.3	70.7
5″	77.4	77.5	77.5	77.4
6″	63.0	62.9	62.9	63.0
1‴	104.0	103.9	103.9	104.0
2‴	75.1	75.1	74.9	75.0
3‴	86.2	86.2	87.5	87.1
4‴	69.2	69.4	69.6	69.2
5‴	78.1	78.3	78.2	78.1
6‴	62.2	62.3	62.3	62.2
1⁗	105.6	105.6	105.3	106.0
2⁗	72.7	72.7	75.5	75.2
3⁗	74.1	74.1	77.9	77.6
4⁗	69.2	69.3	71.5	70.8
5⁗	67.0	67.1	78.3	67.1
6⁗			62.5	
1⁗′	104.4	104.9	104.9	104.4
2⁗′	75.3	75.1	75.1	75.3
3⁗′	78.5	78.2	78.1	78.5
4⁗′	71.5	70.8	70.7	71.5
5⁗′	78.3	67.3	67.3	78.3
6⁗′	62.4			62.4

[a] Spectra were measured in C_5D_5N−CD_3OD (2:1).

Table 2. Cytotoxic Activities of **1**−**4** and Etoposide against HL-60 Cells and HSC-2 Cells

	IC_{50} (μg/mL)	
compound	HL-60	HSC-2
1	9.0	13.0
2	4.4	2.2
3	5.9	1.5
4	3.9	7.8
etoposide	0.30	24.4

Experimental Section

General Experimental Procedures. Optical rotations were measured by using a JASCO DIP-360 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO A-100 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^1H NMR, Karlsruhe, Germany) using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. MS were recorded on a Finnigan MAT TSQ-700 (San Jose, CA) mass spectrometer, using a dithiothreitol and dithioerythritol (3:1) matrix. Elemental analysis was carried out using an Elemental Vario EL (Hanau, Germany) elemental analyzer. Si gel (Fuji-Silysia Chemical, Aichi, Japan), ODS Si gel (Nacalai Tesque, Kyoto, Japan), and Diaion HP-20 (Mitsubishi-Kasei, Tokyo, 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 $F_{254}S$ (0.25 mm thick, Merck) plates, and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. HPLC was performed using a system comprised of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), a UV-8000 detector (Tosoh), and Rheodyne injection port with a 20 μL sample loop. A Capcell Pak C_{18} column (4.6 mm i.d. × 250 mm, 5 μm, Shiseido, Tokyo, Japan) was employed for HPLC analysis. The following reagents were obtained from the indicated companies: RPMI 1640 medium (Gibco, Gland Island, NY); FBS (Bio-Whittaker, Walkersville, MD, or JRH Biosciences, Lenexa, KS); Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY); MTT (Sigma, St. Louis, MO); penicillin and streptomycin (Sigma or Meiji-Seika, Tokyo, Japan). All other chemicals used were of biochemical reagent grade.

Plant Material. *P. tuberosa* was purchased from a nursery in Daiichi Seed (Tokyo, Japan) and was identified by one of the authors (Y.S.). A voucher specimen has been deposited in our laboratory (voucher No. PT-00-005, Laboratory of Medicinal Plant Science).

Extraction and Isolation. The plant material (dry weight, 1.3 kg) was extracted with hot MeOH (2.2 L, twice). The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (205 g) was passed through a Diaion HP-20 column, successively eluting with 30% MeOH, 50% MeOH, 80% MeOH, and MeOH only. Column chromatography of the 80% MeOH eluate portion (35 g) on Si gel and elution with a mixture of $CHCl_3$−MeOH−H_2O (40:10:1) gave five fractions (I−V). Fraction IV was chromatographed on Si gel eluting with

$CHCl_3$−MeOH−H_2O (40:10:1; 30:10:1; 20:10:1) and ODS Si gel with MeOH−H_2O (2:1) and MeCN−H_2O (5:6; 5:8) to yield **1** (52.8 mg), **2** (111 mg), **3** (84.8 mg), and **4** (77.5 mg).

Compound 1: amorphous solid; $[\alpha]_D^{26}$ −24.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3400 (OH), 2930 (CH), 1705 (C=O), 1060 cm^{-1}; ^1H NMR (C_5D_5N−CD_3OD, 2:1) δ 5.42 (1H, d, J = 7.9 Hz, H-1‴), 5.08 (1H, d, J = 7.8 Hz, H-1⁗), 4.95 (1H, d, J = 7.8 Hz, H-1″), 4.94 (1H, d, J = 7.0 Hz, H-1‴), 4.73 (1H, d, J = 7.7 Hz, H-1′), 4.43 (1H, m, H-16), 4.14 (1H, dd, J = 8.7, 7.8 Hz, H-2″), 4.04 (1H, dd, J = 8.7, 8.7 Hz, H-3″), 3.80 (1H, m, H-3), 3.54 (1H, br d, J = 10.8 Hz, H-26a), 3.43 (1H, dd, J = 10.8, 10.8 Hz, H-26b), 2.65 (1H, dd, J = 8.6, 6.8 Hz, H-17), 2.41 (1H, dd, J = 13.8, 13.8 Hz, H-11a), 2.20 (1H, dd, J = 13.8, 4.9 Hz, H-11b), 1.87 (1H, m, H-20), 1.25 (3H, d, J = 6.9 Hz, Me-21), 1.07 (3H, s, Me-18), 0.76 (3H, s, Me-19), 0.73 (3H, d, J = 6.1 Hz, Me-27); ^{13}C NMR (C_5D_5N−CD_3OD, 2:1) δ 37.0 (C-1), 29.9 (C-2), 77.9 (C-3), 34.8 (C-4), 44.9 (C-5), 28.9 (C-6), 32.0 (C-7), 34.7 (C-8), 56.0 (C-9), 36.6 (C-10), 38.2 (C-11), 213.7 (C-12), 55.7 (C-13), 56.3 (C-14), 31.6 (C-15), 80.0 (C-16), 54.5 (C-17), 16.2 (C-18), 11.9 (C-19), 42.9 (C-20), 13.9 (C-21), 109.7 (C-22), 32.0 (C-23), 29.5 (C-24), 30.8 (C-25), 67.3 (C-26), 17.4 (C-27), signals for the sugar moiety, see Table 1; FABMS (negative mode) *m*/*z* 1209 [M − H]$^-$, 1077 [M − arabinosyl]$^-$, 1047 [M − glucosyl]$^-$, 915 [M − arabinosyl − glucosyl]$^-$, 753 [M − arabinosyl − glucosyl × 2]$^-$, 591 [M − arabinosyl − glucosyl × 3]$^-$; *anal*. C 52.63%, H 7.79% (calcd for $C_{56}H_{90}O_{28}\cdot 4H_2O$, C 52.41%, H 7.70%).

Acid Hydrolysis of 1. A solution of **1** (10.4 mg) in 1 M HCl (dioxane−H_2O, 1:1, 5 mL) was heated at 98 °C for 2 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by an Amberlite IRA-93ZU (Organo, Tokyo, Japan) column and passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA) eluting with MeOH−H_2O (1:1, 10 mL) followed by MeOH (10 mL) to give a sugar fraction and an aglycon fraction. The sugar fraction (1 mg) was dissolved in H_2O (1 mL), to which (−)-α-methylbenzylamine (5 mg) and Na[BH_3-CN] (8 mg) in EtOH (1 mL) were added. After being set aside at 40 °C 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) and pyridine (0.3 mL) in the presence of 4-(dimethylamino)pyridine (5 mg) catalyst at 40 °C for 12 h. The crude mixture was passed through a Sep-Pak C_{18} cartridge with H_2O−MeCN (4:1; 1:1, each 10 mL) mixtures as solvents. The H_2O−MeCN (1:1) eluate was further passed through a Toyopak IC-SP M cartridge (Tosoh, Tokyo, Japan) with EtOH (10 mL) to give a mixture of the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides, 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 L-arabinose, D-galactose, and D-glucose were detected as follows: t_R (min) 13.09 (derivative of L-arabinose); 14.90 (derivative of D-galactose); 17.42 (derivative of D-glucose). The aglycon fraction was purified by Si gel column chromatography using $CHCl_3$−MeOH (19:1) to give (25*R*)-3β-hydroxy-5α-spirostan-12-one (hecogenin) (2.5 mg).

Compound 2: amorphous solid; $[\alpha]_D^{26}$ −30.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3400 (OH), 2930 (CH), 1705 (C=O), 1040 cm^{-1}; ^1H NMR (C_5D_5N−CD_3OD, 2:1) δ 5.41 (1H, d, J = 7.9 Hz, H-1‴), 5.01 (1H, d, J = 7.8 Hz, H-1⁗), 4.96 (1H, d, J = 7.9 Hz, H-1″), 4.92 (1H, d, J = 7.1 Hz, H-1‴), 4.72 (1H, d, J = 7.7 Hz, H-1′), 4.42 (1H, m, H-16), 3.81 (1H, m, H-3), 3.54 (1H, br d, J = 10.8 Hz, H-26a), 3.42 (1H, dd, J = 10.8, 10.8 Hz, H-26b), 2.64 (1H, dd, J = 8.6, 6.7 Hz, H-17), 2.41 (1H, dd, J = 13.8, 13.8 Hz, H-11a), 2.20 (1H, dd, J = 13.8, 4.9 Hz, H-11b), 1.87 (1H, m, H-20), 1.24 (3H, d, J = 6.9 Hz, Me-21), 1.07 (3H, s, Me-18), 0.77 (3H, s, Me-19), 0.73 (3H, d, J = 6.1 Hz, Me-27); ^{13}C NMR, see compound **1**[14] and Table 1; FABMS (negative mode) *m*/*z* 1179 [M − H]$^-$, 1047 [M − arabinosyl (or xylosyl)]$^-$, 915 [M − arabinosyl − xylosyl]$^-$, 753 [M − arabinosyl − xylosyl − glucosyl]$^-$, 591 [M − arabinosyl − xylosyl − glucosyl × 2]$^-$; *anal*. C 51.34%, H 7.74% (calcd for $C_{55}H_{88}O_{27}\cdot 6H_2O$, C 51.31%, H 7.67%).

Acid Hydrolysis of 2. Compound **2** (10.6 mg) was subjected to acid hydrolysis as described for **1** to give hecogenin (3.2 mg) and a sugar fraction. The monosaccharide constituents in the sugar fraction were converted to the corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives, which were then analyzed by HPLC. The derivatives of L-arabinose, D-xylose, D-galactose, and D-glucose were detected as follows: t_R (min) 13.62 (derivative of L-arabinose); 14.00 (derivative of D-xylose); 15.11 (derivative of D-galactose); 17.74 (derivative of D-glucose).

Compound 3: amorphous solid; $[\alpha]_D^{26}$ −30.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3400 (OH), 2930 (CH), 1705 (C=O), 1040 cm^{-1}; ^1H NMR (C_5D_5N−CD_3OD, 2:1) δ 5.40 (1H, d, J = 7.9 Hz, H-1‴), 4.99 (1H, d, J = 7.8 Hz, H-1⁗), 4.96 (1H, d, J = 7.8 Hz, H-1″), 4.95 (1H, d, J = 7.8 Hz, H-1‴), 4.73 (1H, d, J = 7.6 Hz, H-1′), 4.43 (1H, m, H-16), 3.81 (1H, m, H-3), 3.55 (1H, br d, J = 10.8 Hz, H-26a), 3.43 (1H, dd, J = 10.8, 10.8 Hz, H-26b), 2.65 (1H, dd, J = 8.6, 6.7 Hz, H-17), 2.41 (1H, dd, J = 13.8, 13.8 Hz, H-11a), 2.19 (1H, dd, J = 13.8, 4.8 Hz, H-11b), 1.87 (1H, m, H-20), 1.24 (3H, d, J = 7.0 Hz, Me-21), 1.07 (3H, s, Me-18), 0.76 (3H, s, Me-19), 0.73 (3H, d, J = 6.1 Hz, Me-27); ^{13}C NMR, see compound **1**[14] and Table 1; FABMS (negative mode) *m*/*z* 1209 [M − H]$^-$, 591 [M − xylosyl − glucosyl × 3]$^-$; *anal*. C 50.95%, H 7.69% (calcd for $C_{56}H_{90}O_{28}\cdot 6H_2O$, C 50.98%, H 7.79%).

Acid Hydrolysis of 3. Compound **3** (10.5 mg) was subjected to acid hydrolysis as described for **1** to give hecogenin (3.6 mg) and a sugar fraction. The monosaccharide constituents in the sugar fraction were converted to the corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives, which were then analyzed by HPLC. The derivatives of D-xylose, D-galactose, and D-glucose were detected as follows: t_R (min) 13.70 (derivative of D-xylose); 14.70 (derivative of D-galactose); 17.32 (derivative of D-glucose).

Compound 4: amorphous solid; $[\alpha]_D^{26}$ −34.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3400 (OH), 2930 (CH), 1705 (C=O), 1040 cm^{-1}; ^1H NMR (C_5D_5N−CD_3OD, 2:1) δ 5.43 (1H, d, J = 7.8 Hz, H-1‴), 5.07 (1H, d, J = 7.8 Hz, H-1⁗), 4.96 (1H, d, J = 7.9 Hz, H-1″), 4.92 (1H, d, J = 7.5 Hz, H-1‴), 4.74 (1H, d, J = 7.7 Hz, H-1′), 4.43 (1H, m, H-16), 3.80 (1H, m, H-3), 3.55 (1H, br d, J = 11.1 Hz, H-26a), 3.43 (1H, dd, J = 11.1, 11.1 Hz, H-26b), 2.65 (1H, dd, J = 8.6, 6.7 Hz, H-17), 2.41 (1H, dd, J = 13.8, 13.8 Hz, H-11a), 2.20 (1H, dd, J = 13.8, 4.9 Hz, H-11b), 1.87 (1H, m, H-20), 1.25 (3H, d, J = 7.0 Hz, Me-21), 1.07 (3H, s, Me-18), 0.75 (3H, s, Me-19), 0.73 (3H, d, J = 6.1 Hz, Me-27); ^{13}C NMR, see compound **1**[14] and Table 1; FABMS (negative mode) *m*/*z* 1209 [M − H]$^-$, 915 [M − xylosyl − glucosyl]$^-$, 591 [M − xylosyl − glucosyl × 3]$^-$; *anal*. C 53.38%, H 7.52% (calcd for $C_{56}H_{90}O_{28}\cdot 3H_2O$, C 53.16%, H 7.65%).

Acid Hydrolysis of 4. Compound **4** (10.5 mg) was subjected to acid hydrolysis as described for **1** to give hecogenin (3.3 mg) and a sugar fraction. The monosaccharide constituents in the sugar fraction were converted to the corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives, which were then analyzed by HPLC. The derivatives of D-xylose, D-galactose, and D-glucose were detected as follows: t_R (min) 13.54 (derivative of D-xylose); 14.62 (derivative of D-galactose); 17.04 (derivative of D-glucose).

HL-60 Cell Culture Assay. HL-60 cells, which were obtained from Human Science Research Resources Bank (JCRB 0085, Osaka, Japan), were maintained in the RPMI 1640 medium containing heat-inactivated 10% FBS 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 (Iwaki Glass, Chiba, Japan). The cells were incubated in 5% CO_2/air for 24 h at 37 °C. After incubation, 4 μL of EtOH−H_2O (1:1) solution containing the sample was added to give the final concentrations of 0.1−20 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.[15] 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 5% CO_2/air for 4 h at 37 °C. 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 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. Each assay was done in triplicate, and cytotoxicity was expressed as IC_{50} value, which reduced the viable cell number by 50%.

HSC-2 Cell Culture Assay. HSC-2 cells were maintained as monolayer cultures at 37 °C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Cells were trypsinized and inoculated at 6×10^3 per each 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson, San Jose, CA) and incubated for 24 h. After washing once with PBS, they were treated for 24 h without or with test compounds. They were washed once with PBS and incubated for 4 h with 0.2 mg/mL MTT in DMEM supplemented with 10% FBS. After the medium was removed, the cells were lysed with 0.1 mL of DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using Labsystems Multiskan (Biochromatic, Helsinki, Finland) connected to a Star/DOT Matrix printer JL-10.[16,17]

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