New Steroidal Constituents of the Underground Parts of *Ruscus aculeatus* and Their Cytostatic Activity on HL-60 Cells

Yoshihiro MIMAKI,* Minpei KURODA, Aiko KAMEYAMA, Akihito YOKOSUKA, and Yutaka SASHIDA*

School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432–1, Horinouchi, Hachioji, Tokyo 192–03, Japan. Received September 8, 1997; accepted October 28, 1997

Phytochemical examination of the underground parts of *Ruscus aculeatus* has led to the isolation of a total of twelve steroidal saponins, including seven new ones. The structures of the new saponins were determined by spectroscopic analysis and chemical evidence. The furostanol saponin, having a diglycoside moiety modified with a (2*S*,3*S*)-2-hydroxy-3-methylpentanoic acid group and an acetic acid group, and its corresponding spirostanol saponin exhibited cytostatic activity on leukemia HL-60 cells.

Key words *Ruscus aculeatus*; Liliaceae; spirostanol saponin; furostanol saponin; cytostatic activity; HL-60 cell

Ruscus (*R.*) *aculeatus* (Liliaceae) is distributed throughout Europe. Preparations from an alcoholic extract of its rhizome have been used for the treatment of veinous insufficiency, haemorrhoids, and capillary fragility. Recently, sulfated steroidal derivatives,[1] triterpenes,[2] and sterols[2] were isolated from the rhizome of *R. aculeatus*. However, studies of the plant to date have been so fragmentary, and there has been no systematic exploration of its secondary metabolites. Our detailed investigation of the underground parts of *R. aculeatus* has led to the isolation of a total of twelve steroidal saponins, including seven new ones. In this paper, we wish to report the identification and structural assignment of these isolated compounds and their cytostatic activity on leukemia HL-60 cells.

The concentrated 1-butanol-soluble fraction of the methanolic extract of *R. aculeatus* (fresh weight of 3.1 kg) was repeatedly subjected to column chromatography on silica-gel, octadecylsilanized (ODS) silica-gel, Diaion HP-20, and Sephadex LH-20, as well as preparative HPLC to yield compounds **1** (757 mg), **2** (10.3 g), **3** (153 mg), **4** (1.89 g), **5** (65.1 mg), **6** (349 mg), **7** (1.25 g), **8** (161 mg), **9** (340 mg), **10** (14.7 mg), **11** (37.6 mg), and **12** (33.2 mg).

Compounds **1**—**5** were known constituents and identified as spirosta-5,25(27)-diene-1β,3β-diol (neoruscogenin) 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside},[3] 26-*O*-β-D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1β,3β,22ξ,26-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside},[3c,4] neoruscogenin 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside},[3a,c] 26-*O*-β-D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1β,3β,22ξ,26-tetrol 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside},[4] and 26-*O*-β-D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1β,3β,22ξ,26-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside},[5] respectively, by comparison of their spectral data and physical properties with literature values.

Compound **6** was obtained as an amorphous solid, $[\alpha]_D$ −84.0° (methanol). The molecular formula of **6** was found to be $C_{38}H_{57}O_{15}NaS$ by elemental analysis, negative-ion FAB-MS, ^{13}C-NMR spectrum, and quantitative atomic flame photometry analysis. The glycosidic nature of **6** was suggested by strong absorption bands at 3420 and 1040 cm^{-1}. The ^{1}H-NMR spectrum of **6**,[6] which showed signals for three steroid methyl protons at δ 1.38 (3H, s), 1.02 (3H, d, J=7.0 Hz) and 0.82 (3H, s), exomethylene protons at δ 4.80 and 4.76 (each 1H, br s), and two anomeric protons at δ 6.13 (1H, d, J=0.9 Hz) and 4.65 (1H, d, J=7.4 Hz), was essentially analogous that of **1**. Acetylation of **6** with acetic anhydride in pyridine introduced five acetyl groups into **6** (**6a**), and solvolysis with a mixture of pyridine and dioxane (4:1) at 100 °C for 4 h gave **1** and sulfuric acid,[7] indicating **6** was a sulfate of **1**. On comparison of the ^{1}H- and ^{13}C-NMR spectra of **6** with those of **1**, the 4-H proton and C-4 carbon signals arising from the arabinose moiety were displaced downfield by 1.03 and 5.7 ppm, respectively, indicating the sulfate group linkage to the C-4 hydroxyl group of the arabinose. Thus, the structure of **6** was determined to be neoruscogenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-4-*O*-sulfo-α-L-arabinopyranoside}.[8]

Compound **7** was shown to be a 22-methoxyfurostanol saponin from Ehrlich's test,[9] and the ^{1}H- and ^{13}C-NMR spectra [δ_H 3.23 (3H, s); δ_C 112.2 (C) and 47.1 (Me)].[10] Enzymatic hydrolysis of **7** with β-glucosidase gave D-glucose and **6**. The structure of **7** was shown to be 26-*O*-β-D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1β,3β,22ξ,26-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-4-*O*-sulfo-α-L-arabinopyranoside}.

The spectral features of compound **8** were similar to those of **7**. The presence of an acetyl group in **8** was shown by the IR (1725 cm^{-1}), ^{1}H-NMR [δ 2.06 (3H, s)], and ^{13}C-NMR [δ 170.9 (C=O) and 21.0 (Me)] spectra. Alkaline treatment of **8** with 10% ammonia solution gave **7**. Therefore, **8** must be a monoacetate of **7**. In the ^{1}H-NMR spectrum of **8**, the signal due to 3-H of the arabinose was shifted downfield by 1.27 ppm to be observed at δ 5.46 (dd, J=8.3, 3.7 Hz), compared with that of **7**, indicating that the acetyl group was linked to the arabinose C-3 hydroxy position. The structure of **8** was formulated as 26-*O*-β-D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1β,3β,22ξ,26-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-3-*O*-acetyl-4-*O*-sulfo-α-L-ara-

* To whom correspondence should be addressed.

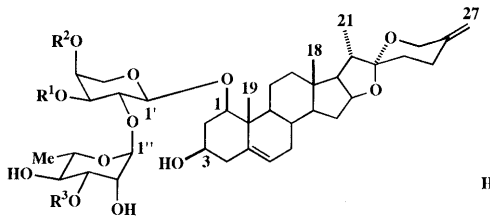
Table 1. ^{13}C-NMR Spectral Data of Compounds **1**—**12**[a]

C	1	2	3	4	5	6	7	8	9	10	11	12
1	83.5	83.4	84.8	84.7	83.8	83.5	83.5	83.8	83.2	83.2	83.2	84.3
2	37.4	37.4	38.1	38.1	37.4	36.9	36.9	37.1	37.2	37.2	37.5	38.1
3	68.2	68.2	68.2	68.2	68.2	67.9	67.9	67.9	68.1	68.1	68.2	68.3
4	43.9	43.9	43.8	43.9	43.8	43.5	43.4	43.6	43.7	43.7	43.7	43.7
5	139.7	139.6	139.6	139.6	139.5	139.2	139.2	139.2	139.2	139.3	139.4	139.4
6	124.7	124.7	124.8	124.8	124.7	124.8	124.7	124.9	124.8	124.8	124.6	124.7
7	32.0	32.0	32.0	32.0	31.9	31.9	31.8	31.9	31.9	31.9	31.9	31.9
8	33.2	33.1	33.1	33.0	33.1	33.0	32.9	33.0	33.0	33.2	33.0	33.1
9	50.4	50.4	50.5	50.5	50.3	50.2	50.2	50.2	50.2	50.3	50.3	50.4
10	42.9	42.9	43.0	42.9	42.9	42.8	42.7	42.8	42.7	42.8	42.6	42.7
11	24.1	24.0	23.9	23.9	24.0	23.8	23.7	23.9	24.3	24.3	24.3	24.3
12	40.3	40.2	40.3	40.4	40.2	39.7	39.6	39.7	40.5	40.4	40.5	40.5
13	40.3	40.5	40.2	40.5	40.5	40.0	40.3	40.4	40.7	40.6	40.6	40.4
14	56.9	56.7	56.9	56.7	56.7	56.5	56.4	56.5	56.8	57.0	57.0	57.1
15	32.4	32.4	32.4	32.3	32.3	32.2	32.2	32.3	32.3	32.4	32.3	32.3
16	81.5	81.5	81.5	81.4	81.4	81.3	81.3	81.4	81.5	81.5	81.5	81.5
17	63.1	64.2	63.0	64.2	64.1	62.7	63.9	63.9	64.2	63.2	64.3	63.2
18	16.7	16.6	16.7	16.6	16.6	16.4	16.3	16.4	16.8	16.9	16.8	16.9
19	15.1	15.1	15.1	15.1	15.0	14.6	14.8	14.8	15.0	15.0	15.0	15.1
20	41.9	40.4	41.8	40.5	40.4	41.7	40.3	40.4	40.5	42.0	40.4	41.9
21	15.0	16.1	15.0	16.1	16.1	14.8	15.9	16.0	16.1	15.0	16.1	15.0
22	109.5	112.4	109.5	112.4	112.4	109.2	112.2	112.3	112.5	109.5	112.4	109.5
23	33.3	31.6	33.2	31.6	31.6	33.1	31.5	31.6	31.6	33.3	31.6	33.3
24	29.0	28.1	29.0	28.1	28.0	28.8	27.9	28.0	28.1	29.0	28.1	29.0
25	144.6	146.9	144.5	146.8	146.8	144.4	146.6	146.8	146.8	144.5	146.9	144.5
26	65.0	72.0	65.0	72.0	72.0	64.8	71.8	72.0	72.0	65.0	72.1	65.0
27	108.6	111.0	108.6	111.0	111.0	108.3	111.0	111.0	111.0	108.6	111.1	108.7
OMe		47.3		47.3	47.3		47.1	47.2	47.3		47.3	
1′	100.3	100.3	101.4	101.4	100.5	100.0	100.0	99.5	99.5	99.5	100.0	100.7
2′	75.2	75.2	74.3	74.3	74.2	75.7	75.7	71.5	73.1	73.2	75.3	74.6
3′	75.8	75.9	76.1	76.1	84.5	74.5	74.6	74.5	74.9	74.9	73.2	73.2
4′	70.1	70.1	70.3	70.2	69.5	75.8	75.8	73.4	70.1	70.1	73.7	73.8
5′	67.3	67.3	67.7	67.7	67.0	65.5	65.5	64.5	64.1	64.1	64.3	64.6
1″	101.7	101.7	101.2	101.1	101.7	101.3	101.3	101.8	102.1	102.1	101.9	101.7
2″	72.5	72.5	72.1	72.0	72.5	71.9	71.9	72.0	72.3	72.3	72.5	72.0
3″	72.7	72.7	82.7	82.7	72.5	72.0	72.0	72.0	72.4	72.5	72.7	82.8
4″	74.3	74.2	73.3	73.3	74.0	73.8	73.8	73.7	73.8	73.9	74.2	73.2
5″	69.4	69.4	69.3	69.3	69.6	69.2	69.2	70.0	69.5	69.5	69.6	69.5
6″	19.0	19.0	18.7	18.7	19.1	18.6	18.6	18.7	19.0	19.0	19.1	18.8
1‴			106.5	106.5	106.4				174.7	174.7		106.6
2‴			76.1	76.1	74.6				75.6	75.6		76.1
3‴			78.3	78.3	78.2				39.7	39.7		78.4
4‴			71.7	71.7	70.9				24.7	24.7		71.1
5‴			78.4	78.4	67.0				12.2	12.2		78.4
6‴			62.6	62.6					16.1	16.1		62.6
1⁗		103.9		103.8	103.8		103.4	103.6	103.9		103.9	
2⁗		75.2		75.1	75.1		74.7	74.9	75.2		75.2	
3⁗		78.5		78.5	78.5		78.0	78.3	78.5		78.6	
4⁗		71.7		71.7	71.7		71.3	71.5	71.7		71.7	
5⁗		78.6		78.6	78.6		78.0	78.3	78.6		78.6	
6⁗		62.9		62.9	62.8		62.4	62.6	62.9		62.9	
Ac								170.9	170.1	170.1	170.8	170.9
								21.0	20.7	20.7	21.0	20.9

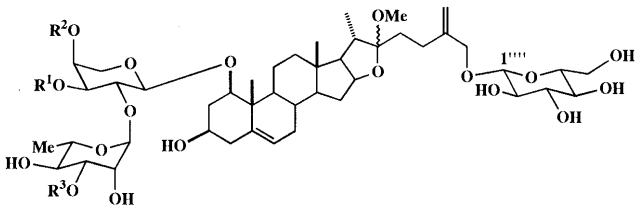
a) Spectra of **1**—**5** and **9**—**12** were measured in pyridine-d_5, and those of **6**—**8** in pyridine-d_5 with the addition of a small amount of methanol-d_4 to increase solubility in pyridine-d_5.

binopyranoside}.

Compound **9** was also a furostanol saponin, based on furosta-5,25(27)-diene-1β,3β,22ξ,26-tetrol. The presence of an acetyl group in **9** was readily apparent from the spectral data. In addition, the ^{13}C-NMR spectrum showed the presence of a six carbon substituent, the signals of these carbons being two methyls (δ 16.6 and 12.2), a methylene (δ 24.7), a methine (δ 39.7), a hydroxy methine (δ 75.6), and an ester carbonyl (δ 174.7). Signals in the ^{1}H-NMR spectrum that could be ascribed to the substituent included two methyl groups at δ 1.31 (d, J=6.9 Hz) and 1.14 (t, J=7.4 Hz). These data suggested that 2-hydroxy-3-methylpentanoic acid was a candidate for the substituent. Alkaline hydrolysis of **9** gave **2** and 2-hydroxy-3-methylpentanoic acid (**9a**). Thus, **9** was shown to be a furostanol saponin with two ester linkages consisting of an acetyl group and a 2-hydroxy-3-methylpentanoyl group. On comparison of the ^{1}H-NMR spectrum of **9** with that of **2**, the signals assignable to the arabinose 3-H and 4-H were shifted downfield by 1.29 and

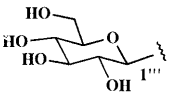


	R^1	R^2	R^3
1	H	H	H
3	H	H	β-D-Glc*p*
6	H	SO_3^-	H
10	Ac	HMP	H
12	H	Ac	β-D-Glc*p*

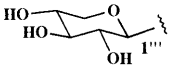


	R^1	R^2	R^3
2	H	H	H
4	H	H	β-D-Glc*p*
5	β-D-Xyl*p*	H	H
7	H	SO_3^-	H
8	Ac	SO_3^-	H
9	Ac	HMP	H
9b	H	HMP	H
11	H	Ac	H

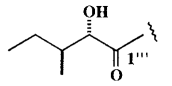
β-D-Glc*p*:



β-D-Xyl*p*:



HMP:



1.52 ppm, respectively, due to *O*-acylation, leading to two possible structures, 3-*O*-acetyl-4-*O*-(2-hydroxy-3-methylpentanoyl)arabinoside or 3-*O*-(2-hydroxy-3-methylpentanoyl)-4-*O*-acetylarabinoside. Mild alkaline hydrolysis of **9** liberated acetic acid to yield a furostanol saponin (**9b**) with only a 2-hydroxy-3-methylpentanoyl group. A downfield shift due to the acyl group linkage was identified at the 4-H proton of the arabinose (+1.39 ppm) by comparison of the ^{1}H-NMR spectrum of **9b** with that of **2**. These data provided evidence for the location of the acetyl group at C-3 and the 2-hydroxy-3-methylpentanoyl group at C-4 of the arabinose. The precise agreement between the specific rotation, and the ^{1}H- and ^{13}C-NMR spectra of 2-hydroxy-3-methylpentanoic acid from **9** and that prepared by deamination of (2*S*,3*S*)-2-amino-3-methylpentanoic acid (L-isoleucine) following treatment of nitrous acid[11] confirmed that the absolute configurations of the asymmetric center of the 2-hydroxy-3-methylpentanoyl group in **9** were 2*S* and 3*S*. All the data presented above were consistent with the structure 26-*O*-β-D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1β,3β,22ξ,26-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-3-*O*-acetyl-4-*O*-[(2*S*,3*S*)-2-hydroxy-3-methylpentanoyl]-α-L-arabinopyranoside} for compound **9**.

Enzymatic hydrolysis of **9** gave the corresponding spirostanol saponin, and its physical properties and spectral data were identical with those of compound **10**. The structure of **10** was shown to be neoruscogenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-3-*O*-acetyl-4-*O*-[(2*S*,3*S*)-2-hydroxy-3-methylpentanoyl]-α-L-arabinopyranoside}.

The presence of an acetyl group in compounds **11** and **12** was confirmed from spectral data. Alkaline hydrolysis of **11** and **12** yielded **2** and **3**, respectively. On comparison of the ^{1}H-NMR spectra of **11** and **2**, and **12** and **3**, the downfield shift produced by *O*-acetylation was identified at the 4-H proton of the arabinose in both compounds. The respective structures of **11** and **12** were assigned as 26-*O*-β-D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1β,3β,22ξ,26-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-4-*O*-acetyl-α-L-arabinopyranoside} and neoruscogenin 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-4-*O*-acetyl-α-L-arabinopyranoside}.

Compounds **6**—**12** are new steroidal saponins.

The effect of the isolated saponins on the *in vitro* growth of human promyelocytic leukemia HL-60 cells was evaluated. Cells were continuously treated with each sample for 72 h, and cell growth was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The percentage inhibition at a sam-

Table 2. Inhibitory Activity of the Isolated Saponins on the Growth of Leukemia HL-60 Cells

Compound	Inhibition (%)[a]	IC_{50} (μg/ml)
1	30.5	—[b]
2	8.3	—
3	16.8	—
4	6.2	—
5	5.8	—
6	13.6	—
7	7.8	—
8	2.1	—
9	92.4	3.5
10	98.2	3.0
11	27.9	—
12	39.8	—

a) Data expressed as a percentage of cell growth inhibition at a sample concentration of 10 μg/ml. *b*) Not measured.

ple concentration of 10 μg/ml is given in Table 2. The furostanol saponin, having a diglycoside moiety modified with a (2*S*,3*S*)-2-hydroxy-3-methylpentanoic acid group and an acetic acid group (**9**), and its corresponding spirostanol saponin (**10**) exhibited 92.4% and 98.2% inhibition at 10 μg/ml, respectively, while the other saponins were far less potent. The IC_{50} values of **9** and **10** were calculated from the dose–response curve as about 3.5 and 3.0 μg/ml, respectively. The above facts suggest that the acetyl and 2-hydroxy-3-methylpentanoyl groups attached to the diglycoside moiety contribute to the cytostatic activity.

Experimental

Optical rotations were measured using a JASCO DIP-360 automatic digital polarimeter. IR spectra were recorded on a Hitachi 260-30 spectrophotometer and MS on a VG AutoSpec E instrument. Elemental analysis was carried out with an Elementar Vario EL elemental analyzer for C and H analysis, and a Yokokawa IC-7000S ion-chromatography system for S analysis. Atomic flame photometry was performed on a Hitachi Z-8100 polarized Zeeman atomic absorption spectrophotometer. NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz for ^{1}H-NMR) using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. Silica-gel (Fuji-Silysia Chemical), Diaion HP-20 (Mitsubishi-Kasei), Sephadex LH-20 (Pharmacia), and ODS silica-gel (Nacalai Tesque) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck) 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 Tosoh HPLC system comprising of a CCPM pump, a CCP controller PX-8010, an RI-8010 detector, and Rheodyne injection port with a 2 ml sample loop for preparative HPLC. A CAPCELL PAK C_{18} column (Shiseido, 10 mm i.d. × 250 mm, ODS, 5 μm) was linked to the HPLC system. The following materials and reagents were used for cell culture and assay of cytostatic activity: microplate reader, Immuno-Mini NJ-2300 (Inter Med, Japan); 96-well flat-bottom plate, Iwaki Glass (Japan); HL-60 cells, ICN Biomedicals (U.S.A.); RPMI 1640 medium, GIBCO BRL (U.S.A.); MTT, Sigma (U.S.A.). All other chemicals used were of biochemical reagent grade.

Plant Material The underground parts of *R. aculeatus* used for this experiment were collected in Chiba Prefecture, Japan, in June 1992, and a plant specimen is on file in our laboratory.

Extraction and Isolation The plant material (fresh weight, 3.1 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. Column chromatography of the *n*-BuOH-soluble phase was performed on silica-gel and elution with a gradient mixture of $CHCl_3$–MeOH (9 : 1; 6 : 1; 4 : 1; 2 : 1), and finally with MeOH, to give six fractions (fr. I—VI). 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; 3 : 1) to give **10** (14.7 mg). Fraction VI, after removal of considerable amounts of monosaccharides by passing it through a Diaion HP-20 column eluting with increased amounts of MeOH in H_2O, was further separated into three fractions (fr. VIa—VIc) on a silica-gel column eluting with $CHCl_3$–MeOH (4 : 1) . Fraction VIa was chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (40 : 10 : 1), ODS silica-gel with MeOH–H_2O (4 : 1) and MeCN–H_2O (2 : 3), and on Sephadex LH-20 with MeOH to yield **1** (757 mg), **3** (153 mg) and **12** (33.2 mg). Fraction VIb was subjected to column chromatography on silica-gel eluting with $CHCl_3$–MeOH–H_2O (30 : 10 : 1) and $CHCl_3$–Et_2O–MeOH–H_2O (18 : 10 : 7 : 1), and ODS silica-gel with MeOH–H_2O (7 : 3; 3 : 2) and MeCN–H_2O (3 : 7) to give **9** (340 mg), and **11** with a few impurities. Final purification of **11** was carried out by preparative HPLC using MeCN–H_2O (3 : 7) to give **11** (37.6 mg) as a pure compound. Fraction VIc was chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (30 : 10 : 1; 25 : 10 : 1; 20 : 10 : 1), and ODS silica-gel with MeOH–H_2O (13 : 7; 3 : 2) and MeCN–H_2O (3 : 7; 2 : 3) to give **2** including a few impurities, and a crude mixture of **4**—**8**. The impurities in **2** were removed by preparative HPLC using MeCN–H_2O (2 : 3) to give **2** (10.3 g) as a pure compound. The mixture of **4**—**8** was also separated by preparative HPLC using MeCN–H_2O (3 : 7) to give **4** (1.89 g), **5** (65.1 mg), **6** (349 mg), **7** (1.25 g) and **8** (161 mg).

Compound 1 Amorphous solid, $[\alpha]_D^{26}$ −75.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 705 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 6.34 (1H, d, J=0.9 Hz, 1″-H), 5.59 (1H, br d, J=5.5 Hz, 6-H), 4.85 (1H, dq, J=9.4, 6.2 Hz, 5″-H), 4.81 and 4.77 (each 1H, br s, 27-H_2), 4.74 (1H, dd, J=3.0, 0.9 Hz, 2″-H), 4.73 (1H, d, J=7.2 Hz, 1′-H), 4.63 (1H, dd, J=9.4, 3.0 Hz, 3″-H), 4.59 (1H, dd, J=8.4, 7.2 Hz, 2′-H), 4.52 (1H, q-like, J=7.3 Hz, 16-H), 4.46 and 4.02 (each 1H, br d, J=12.0 Hz, 26-H_2), 4.31 (1H, dd, J=9.4, 9.4 Hz, 4″-H), 4.27 and 3.67 (each 1H, br d, J=12.0 Hz, 5′-H_2), 4.16 (2H, overlapping, 3′-H and 4′-H), 3.87 (1H, m, 3-H), 3.84 (1H, dd, J=11.8, 4.1 Hz, 1-H), 1.75 (3H, d, J=6.2 Hz, 6″-Me), 1.45 (3H, s, 19-Me), 1.05 (3H, d, J=7.0 Hz, 21-Me), 0.87 (3H, s, 18-Me).

Compound 2 Amorphous solid, $[\alpha]_D^{26}$ −54.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 899 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 6.34 (1H, br s, 1″-H), 5.59 (1H, br d, J=5.4 Hz, 6-H), 5.35 and 5.06 (each 1H, br s, 27-H_2), 4.92 (1H, d, J=7.9 Hz, 1‴-H), 4.73 (1H, d, J=7.1 Hz, 1′-H), 4.16 (2H, overlapping, 3′-H and 4′-H), 3.25 (3H, s, OMe), 1.74 (3H, d, J=6.1 Hz, 6″-Me), 1.44 (3H, s, 19-Me), 1.13 (3H, d, J=6.9 Hz, 21-Me), 0.84 (3H, s, 18-Me).

Compound 3 Amorphous solid, $[\alpha]_D^{26}$ −64.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 867 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 6.38 (1H, d, J=1.1 Hz, 1″-H), 5.67 (1H, d, J=7.8 Hz, 1‴-H), 5.58 (1H, br d, J=5.6 Hz, 6-H), 4.81 and 4.78 (each 1H, br s, 27-H_2), 4.63 (1H, d, J=7.6 Hz, 1′-H), 4.14 (2H, overlapping, 3′-H and 4′-H), 1.69 (3H, d, J=6.1 Hz, 6″-Me), 1.47 (3H, s, 19-Me), 1.05 (3H, d, J=6.9 Hz, 21-Me), 0.86 (3H, s, 18-Me).

Compound 4 Amorphous solid, $[\alpha]_D^{26}$ −52.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 1061 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 6.36 (1H, br s, 1″-H), 5.65 (1H, d, J=7.8 Hz, 1‴-H), 5.58 (1H, br d, J=5.5 Hz, 6-H), 5.34 and 5.05 (each 1H, br s, 27-H_2), 4.92 (1H, d, J=7.8 Hz, 1⁗-H), 4.63 (1H, d, J=7.4 Hz, 1′-H), 4.13 (2H, overlapping, 3′-H and 4′-H), 3.25 (3H, s, OMe), 1.67 (3H, d, J=6.1 Hz, 6″-Me), 1.46 (3H, s, 19-Me), 1.12 (3H, d, J=6.9 Hz, 21-Me), 0.84 (3H, s, 18-Me).

Compound 5 Amorphous solid, $[\alpha]_D^{26}$ −46.6° (c=0.31, MeOH). Negative-ion FAB-MS m/z: 1031 $[M-H]^-$. ^{1}H-NMR (pyridine-d_5) δ: 6.33 (1H, br s, 1″-H), 5.59 (1H, br d, J=5.6 Hz, 6-H), 5.35 (1H, br s, 27-Ha), 5.06 (overlapping with H_2O signal, 27-Hb), 4.98 (1H, d, J=7.5 Hz, 1‴-H), 4.92 (1H, d, J=7.7 Hz, 1⁗-H), 4.72 (1H, d, J=7.4 Hz, 1′-H), 3.25 (3H, s, OMe), 1.73 (3H, d, J=6.1 Hz, 6″-Me), 1.43 (3H, s, 19-Me), 1.13 (3H, d, J=6.9 Hz, 21-Me), 0.83 (3H, s, 18-Me).

Compound 6 Amorphous solid, $[\alpha]_D^{26}$ −84.0° (c=0.10, MeOH). *Anal.* Calcd for $C_{38}H_{57}O_{15}NaS \cdot H_2O$: C, 55.19; H, 7.19; S, 3.88. Found: C, 55.27; H, 7.29; S, 3.73. Negative-ion FAB-MS m/z: 807 $[M-H]^-$, 785 $[M-Na]^-$. IR (KBr) : 3420 (OH), 2930 (CH), 1255, 1225, 1040 cm^{-1}. ^{1}H-NMR (pyridine-d_5 + methanol-d_4) δ: 6.13 (1H, d, J=0.9 Hz, 1″-H), 5.57 (overlapping with H_2O signal, 6-H), 5.19 (1H, br d, J=2.8 Hz, 4′-H), 4.80 and 4.76 (each 1H, br s, 27-H_2), 4.70 (1H, dq, J=9.4, 6.2 Hz, 5″-H), 4.65 (1H, d, J=7.4 Hz, 1′-H), 4.62 and 3.73 (each 1H, br d, J=11.8 Hz, 5′-H_2), 4.61 (1H, dd, J=3.3, 0.9 Hz, 2″-H), 4.49 (1H, dd, J=9.4, 3.3 Hz, 3″-H), 4.48 (1H, overlapping, 16-H), 4.42 and 3.98 (each 1H, br d, J=12.0 Hz, 26-H_2), 4.41 (1H, dd, J=9.1, 7.4 Hz, 2′-H), 4.19 (1H, dd, J=9.1, 2.8 Hz, 3′-H), 4.18 (1H, dd, J=9.4, 9.4 Hz, 4″-H), 3.77 (1H, m, 3-H), 3.74 (1H, overlapping, 1-H), 1.68 (3H, d, J=6.2 Hz, 6″-Me), 1.38 (3H, s, 19-Me), 1.02 (3H, d, J=7.0 Hz, 21-Me), 0.82 (3H, s, 18-Me).

Quantitative Analysis of the Na Content of 6 Compound **6** (0.9 mg, 1.1×10^{-6} mol) was dissolved in 0.2 ml concentrated HCl, to which was added H_2O to make the volume up to 10 ml. The Na content of the sample solution was determined by atomic flame photometry. The lamp was filled with Ar gas and the Na was ionized in an air-acetylene flame (wavelength, 589 nm; slit width, 0.4 nm). A calibration curve was prepared from standard Na solutions (Wako Pure Chemical Industries). The sample solution was found to contain 3.53 ppm (1.4×10^{-6} mol in 10 ml) Na.

Solvolysis of 6 Compound **6** (10 mg) was refluxed in a mixture of pyridine and dioxane (4 : 1, 5 ml) for 4 h. The reaction mixture was passed through a Sep-Pak C_{18} cartridge (Waters), and eluted successively with H_2O (10 ml) and MeOH (10 ml). The MeOH eluate fraction was chromatographed on silica-gel using $CHCl_3$–MeOH–H_2O (30 : 10 : 1) to yield **1** (6.7 mg). The H_2O phase was examined by paper chromatography (Toyo Roshi, No 50) employing MeOH–H_2O (1 : 1). H_2SO_4 was detected as a light yellow spot after spraying the paper with a solution of $BaCl_2$ (100 mg/50 ml in 70% MeOH) followed by spraying with a solution of potassium rhodizonate (10 mg/50 ml in 50% MeOH). *Rf* 0.74.

Acetylation of 6 Compound **6** (25 mg) was acetylated with Ac_2O (0.5 ml) in pyridine (0.5 ml), and the crude acetate was purified by silica-gel column chromatography using $CHCl_3$–MeOH (9 : 1) to yield the corresponding pentaacetate (**6a**) (29.7 mg) as an amorphous solid. Compound **6a**: IR (KBr): 2955 (CH), 1750 (C=O), 1255, 1235, 1080, 1040, 1015 cm^{-1}. ^{1}H-NMR (pyridine-d_5 + methanol-d_4) δ: 5.73 (1H, dd, J=10.0, 3.4 Hz, 3″-H), 5.64 (1H, br d, J=5.4 Hz, 6-H), 5.58 (1H, d, J=1.7 Hz, 1″-H), 5.53 (1H, dd, J=10.0, 10.0 Hz, 4″-H), 5.44 (1H, dd, J=3.4, 1.7 Hz, 2″-H), 5.34 (1H, br d, J=3.7 Hz, 4′-H), 5.28 (1H, dd, J=9.8, 3.7 Hz, 3′-H), 4.83 and 4.79 (each 1H, br s, 27-H_2), 4.82 (1H, m, 3-H), 4.74 (1H, dq, J=10.0, 6.2 Hz, 5″-H), 4.64 (1H, d, J=7.7 Hz, 1′-H), 4.62 and 3.77 (each 1H, br d, J=12.0 Hz, 5′-H_2), 4.50 (1H, q-like, J=7.3 Hz, 16-H), 4.43 and 4.00 (each 1H, br d, J=12.0 Hz, 26-H_2), 4.36 (1H, dd, J=9.8, 7.7 Hz, 2′-H), 3.67 (1H, dd, J=11.8, 4.0 Hz, 1-H), 2.24, 2.23, 2.18, 2.07 and 2.05 (each 3H, s, Ac), 1.45 (3H, d, J=6.2 Hz, 6″-Me), 1.29 (3H, s, 19-Me), 0.98 (3H, d, J=7.0 Hz, 21-Me), 0.84 (3H, s, 18-Me).

Compound 7 Amorphous solid, $[\alpha]_D^{26}$ −52.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 947 $[M-Na-OMe-H]^-$. IR (KBr): 3425 (OH), 2920 (CH), 1255, 1225, 1070, 1045 cm^{-1}. ^{1}H-NMR (pyridine-d_5 + methanol-d_4) δ: 6.13 (1H, br s, 1″-H), 5.57 (overlapping with H_2O signal, 6-H), 5.30 and 5.04 (each 1H, br s, 27-H_2), 5.20 (1H, br d, J=2.9 Hz, 4′-H), 4.83 (1H, d, J=7.8 Hz, 1‴-H), 4.65 (1H, d, J=7.2 Hz, 1′-H), 4.19 (1H, dd, J=9.2, 2.9 Hz, 3′-H), 3.23 (3H, s, OMe), 1.67 (3H, d, J=6.1 Hz, 6″-Me), 1.38 (3H, s, 19-Me), 1.10 (3H, d, J=6.8 Hz, 21-Me), 0.81 (3H, s, 18-Me).

Enzymatic Hydrolysis of 7 Compound **7** (10 mg) was dissolved in an AcOH–AcONa buffer (pH 5, 5 ml) with β-D-glucosidase (Sigma, EC 3.2.1.21) (20 mg), and incubated at room temperature for 48 h. The crude mixture was chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (30 : 10 : 1) to yield **6** (6.5 mg) and D-glucose. D-Glucose was identified by direct TLC comparison with an authentic sample. *Rf* 0.40 (*n*-BuOH–Me_2CO–H_2O, 4 : 5 : 1).

Compound 8 Amorphous solid, $[\alpha]_D^{26}$ −34.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 1021 $[M-Na-H]^-$. IR (KBr): 3420 (OH), 2930 (CH), 1725 (C=O), 1255, 1045 cm^{-1}. ^{1}H-NMR (pyridine-d_5 + methanol-d_4) δ: 5.72 (1H, br s, 1″-H), 5.58 (1H, br d, J=5.6 Hz, 6-H), 5.46 (1H, dd, J=8.3, 3.7 Hz, 3′-H), 5.43 (1H, br d, J=3.7 Hz, 4′-H), 5.31 and 5.05 (each 1H, br s, 27-H_2), 4.87 (1H, d, J=7.8 Hz, 1‴-H), 4.83 (1H, d, J=6.7 Hz, 1′-H), 3.24 (3H, s, OMe), 2.06 (3H, s, Ac), 1.72 (3H, d, J=6.1 Hz, 6″-Me), 1.36 (3H, s, 19-Me), 1.10 (3H, d, J=6.9 Hz, 21-Me), 0.82 (3H, s, 18-Me).

Alkaline Hydrolysis of 8 Compound **8** (3 mg) in 10% NH_3 aq. (5 ml) was kept at room temperature for 4 h. The reaction mixture was evaporated to dryness under reduced pressure and chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (20 : 10 : 1) to yield **7** (2.1 mg).

Compound 9 Amorphous solid, $[\alpha]_D^{26}$ −44.0° (c=0.10, MeOH). *Anal.* Calcd for $C_{53}H_{84}O_{21}\cdot 2H_2O$: C, 58.23; H, 8.11. Found: C, 58.13; H, 8.15. Negative-ion FAB-MS m/z: 1055 $[M-H]^-$. IR (KBr): 3430 (OH), 2930 (CH), 1745 (C=O), 1045 cm^{-1}. ^{1}H-NMR (pyridine-d_5) δ: 5.68 (1H, br dd, J=3.3, 1.9 Hz, 4′-H), 5.66 (1H, br s, 1″-H), 5.61 (1H, br d, J=5.7 Hz, 6-H), 5.45 (1H, dd, J=9.7, 3.3 Hz, 3′-H), 5.37 and 5.08 (each 1H, br s, 27-H_2), 4.93 (1H, d, J=7.7 Hz, 1‴-H), 4.77 (1H, d, J=7.3 Hz, 1′-H), 3.26 (3H, s, OMe), 2.00 (3H, s, Ac), 1.75 (3H, d, J=6.2 Hz, 6″-Me), 1.40 (3H, s, 19-Me), 1.31 (3H, d, J=6.9 Hz, 6‴-Me), 1.24 (3H, d, J=6.9 Hz, 21-Me), 1.14 (3H, t, J=7.4 Hz, 5‴-Me), 0.94 (3H, s, 18-Me).

Alkaline Hydrolysis of 9 and Preparation of Authentic (2*S*,3*S*)-2-Hydroxy-3-methylpentanoic Acid Compound **9** (100 mg) was treated with 20% NH_3 aq. (10 ml) at room temperature for 9 h. The reaction mixture was evaporated to dryness and chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (30 : 10 : 1) and Sephadex LH-20 with MeOH to yield **2** (40 mg) and 2-hydroxy-3-methylpentanoic acid (**9a**) (6.7 mg). 2-Hydroxy-3-methylpentanoic acid: colorless oil, $[\alpha]_D^{26}$ +22.0° (c=0.10, $CHCl_3$). ^{1}H-NMR (chloroform-*d*) δ: 4.18 (1H, d, J=3.6 Hz, 2-H), 1.89 (1H, m, 3-H), 1.43 and 1.30 (each 1H, m, 4-H_2), 1.03 (3H, d, J=6.9 Hz, 6-Me), 0.93 (3H, t, J=7.4 Hz, 5-Me). ^{13}C-NMR (chloroform-*d*) δ: 178.8 (C-1), 74.6 (C-2), 38.9 (C-3), 23.7 (C-4), 15.3 (C-6), 11.7 (C-5). The above data agreed exactly with those of authentic (2*S*,3*S*)-2-hydroxy-3-methylpentanoic acid prepared from L-isoleucine by the following method. L-Isoleucine (1.31 g) was dissolved in $NaNO_2$/1 N H_2SO_4 (1.6 eq) solution, and the reaction mixture was allowed to stand at 0 °C for 2 h then set aside at room temperature for 3 h. The reaction solution, after dilution with H_2O, was extracted with Et_2O, and the Et_2O phase was chromatographed on silica-gel eluting with $CHCl_3$–MeOH (9 : 1) to give the authentic acid (133 mg).

Mild Alkaline Hydrolysis of 9 Compound **9** (19 mg) was treated with 10% NH_3 aq. (10 ml) at room temperature for 10 min. The reaction mixture was evaporated to dryness and chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (40 : 10 : 1) to yield **9b** (3.5 mg). Compound **9b**: amorphous solid, $[\alpha]_D^{26}$ −44.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 1013 $[M-H]^-$. IR (KBr): 3400 (OH), 2920 (CH), 1730 (C=O), 1045 cm^{-1}. ^{1}H-NMR (pyridine-d_5) δ: 6.25 (1H, br s, 1″-H), 5.60 (1H, br d, J=4.8 Hz, 6-H), 5.55 (1H, br dd, J=3.1, 1.3 Hz, 4′-H), 5.37 (1H, br s, 27-Ha), 5.05 (overlapping with H_2O signal, 27-Hb), 4.94 (1H, d, J=7.9 Hz, 1⁗-H), 4.72 (1H, d, J=6.9 Hz, 1′-H), 4.31 (1H, dd, J=9.7, 3.1 Hz, 3′-H), 3.26 (3H, s, OMe), 1.78 (3H, d, J=6.2 Hz, 6″-Me), 1.45 (3H, s, 19-Me), 1.29 (3H, d, J=6.9 Hz, 6‴-Me), 1.24 (3H, d, J=6.8 Hz, 21-Me), 1.09 (3H, t, J=7.4 Hz, 5‴-Me), 0.93 (3H, s, 18-Me).

Enzymatic Hydrolysis of 9 Compound **9** (30 mg) was subjected to enzymatic hydrolysis with β-D-glucosidase (10 mg) in AcOH–AcONa buffer (pH 5, 10 ml) at room temperature for 144 h. The reaction mixture was chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (50 : 10 : 1) to yield **10** (8.7 mg) and D-glucose.

Compound 10 Amorphous solid, $[\alpha]_D^{26}$ −40.0° (c=0.10, MeOH). *Anal.* Calcd for $C_{46}H_{70}O_{15}\cdot H_2O$: C, 62.71; H, 8.24. Found: C, 62.63; H, 8.29. Negative-ion FAB-MS m/z: 861 $[M-H]^-$. IR (KBr): 3430 (OH), 2930 (CH), 1740 (C=O), 1045 cm^{-1}. ^{1}H-NMR (pyridine-d_5) δ: 5.67 (1H, br dd, J=3.3, 2.1 Hz, 4′-H), 5.66 (1H, d, J=1.0 Hz, 1″-H), 5.60 (1H, br d, J=5.5 Hz, 6-H), 5.44 (1H, dd, J=9.7, 3.3 Hz, 3′-H), 4.81 and 4.78 (each 1H, br s, 27-H_2), 4.77 (1H, d, J=7.4 Hz, 1′-H), 2.00 (3H, s, Ac), 1.75 (3H, d, J=6.2 Hz, 6″-Me), 1.40 (3H, s, 19-Me), 1.30 (3H, d, J=6.9 Hz, 6‴-Me), 1.17 (3H, d, J=7.2 Hz, 21-Me), 1.14 (3H, t, J=7.5 Hz, 5‴-Me), 0.97 (3H, s, 18-Me).

Compound 11 Amorphous solid, $[\alpha]_D^{26}$ −38.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 941 $[M-H]^-$. IR (KBr): 3425 (OH), 2930 (CH), 1735 (C=O), 1045 cm^{-1}. ^{1}H-NMR (pyridine-d_5) δ: 6.32 (1H, br s, 1″-H), 5.60 (1H, br d, J=5.7 Hz, 6-H), 5.39 (1H, br s, 4′-H), 5.35 and 5.07 (each 1H, br s, 27-H_2), 4.93 (1H, d, J=7.6 Hz, 1⁗-H), 4.69 (1H, d, J=7.4 Hz, 1′-H), 3.26 (3H, s, OMe), 2.00 (3H, s, Ac), 1.79 (3H, d, J=6.1 Hz, 6″-Me), 1.43 (3H, s, 19-Me), 1.16 (3H, d, J=6.9 Hz, 21-Me), 0.92 (3H, s, 18-Me).

Alkaline Hydrolysis of 11 Compound **11** (5 mg) was treated with 3% NaOMe in MeOH (5 ml) at room temperature for 1.5 h. After neutralization of the reaction mixture by passage through an Amberlite IR-120B (Organo) column, it was chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (20 : 10 : 1) to yield **2** (4.1 mg).

Compound 12 Amorphous solid, $[\alpha]_D^{26}$ −54.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 909 $[M-H]^-$. IR (KBr): 3430 (OH), 2935 (CH), 1735 (C=O), 1040 cm^{-1}. ^{1}H-NMR (pyridine-d_5) δ: 6.32 (1H, d, J=1.0 Hz, 1″-H), 5.64 (1H, d, J=7.8 Hz, 1‴-H), 5.59 (1H, br d, J=5.4 Hz, 6-H), 5.39 (1H, br d, J=3.3 Hz, 4′-H), 4.81 and 4.78 (each 1H, br s, 27-H_2), 4.59 (1H, d, J=7.6 Hz, 1′-H), 1.96 (3H, s, Ac), 1.72 (3H, d, J=6.1 Hz, 6″-Me), 1.46 (3H, s, 19-Me), 1.07 (3H, d, J=6.9 Hz, 21-Me), 0.92 (3H, s, 18-Me).

Alkaline Hydrolysis of 12 Compound **12** (5 mg) was subjected to alkaline hydrolysis as described for **11** to give **3** (4.2 mg).

Cell Culture and Assay for Cytostatic Activity HL-60 cells were maintained in 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 give 3×10^4 cells/ml, and 196 μl of this cell suspension were placed in each well of a 96-well flat-bottom plate. The cells were incubated for 24 h at 37 °C in 5% CO_2/air. After incubation, 4 μl EtOH–H_2O (1 : 1) solution containing the sample was added to give final concentrations of 0.01—10 μg/ml; 4 μl EtOH–H_2O (1 : 1) was added to control wells. The cells were incubated for a further 72 h in the presence of each agent, and then cell growth was evaluated by MTT assay. The MTT assay was carried out according to a modification of the method of Sargent and Tayler as follows.[12] After termination of cell culture, 10 μl MTT (5 mg/ml in phosphate buffered saline) was added to every well and the plate reincubated at 37 °C in 5% CO_2/air for a further 4 h. The plate was then centrifuged at 1500 × ***g*** for 5 min to precipitate cells and formazan. Then, 150 μl supernatant was removed from every well, and 175 μl dimethyl sulfoxide 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 sample of **9** and **10**, and the concentration giving 50% inhibition of cell growth (IC_{50}) was calculated.

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References and Notes

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8) Compound **6** was obtained as a sodium salt, but there is no evidence to confirm that it is also present as a sodium salt in the plant. It may be formed during the extraction and isolation procedures. From the above reason, the sulfate group of **6**—**8** is shown to be an anion form in their structural drawings.
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