Aculeoside B, a New Bisdesmosidic Spirostanol Saponin from the Underground Parts of *Ruscus aculeatus*

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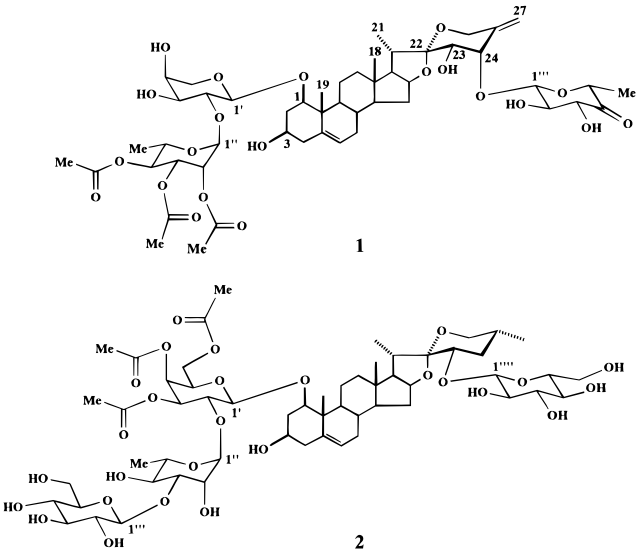
From the underground parts of *Ruscus aculeatus*, a new bisdesmosidic spirostanol saponin named aculeosides B (**2**) was isolated, and its structure was determined on the basis of spectroscopic analysis, including 2D NMR techniques. Aculeoside A (**1**), which was previously isolated from the same plant source, exhibited inhibitory activity on cell growth of leukemia HL-60 cells with an IC_{50} value of 0.48 μg mL^{-1}, while aculeoside B (**2**) was inactive.

Ruscus aculeatus L. is a widely distributed European plant belonging to the family Liliaceae. An alcoholic extract of its rhizomes has been reputed to have important pharmacological properties, the most notable being antiinflammatory activity and the treatment of venous insufficiency. It was reported by Capra that the steroidal saponins and sapogenins from the rhizomes demonstrated antiinflammatory effect on carageenin-induced rat-paw edema after an intraperitoneal administration.[1] A survey of the literature showed that saponins abundantly contained in *R. aculeatus* were spirosta-5,25(27)-diene-1β,3β-diol (neoruscogenin) glycosides and furosta-5,25(27)-diene-1β,3β,22,26-tetrol glycosides.[2−4] A communication with the characterization of two minor sulfated steroidal derivatives from this plant has also recently been published.[5] Previously, we briefly reported the structure of a new bisdesmosidic spirostanol saponin, aculeoside A (**1**, Chart 1), isolated from the underground parts of *R. aculeatus*.[6] It is unique in structure having 6-deoxy-D-glycero-L-threo-4-hexosulose linked to the C-24 hydroxyl group of the aglycon. Further analysis of *R. aculeatus* resulted in the isolation of an additional new bisdesmosidic spirostanol saponin, designated as aculeoside B (**2**). This paper describes the identification procedures of 6-deoxy-D-glycero-L-threo-4-hexosulose group of **1** by chemical methods and structural assignment of **2** on the basis of spectroscopic analysis, including 2D NMR techniques. Inhibitory activity of **1** and **2** on cell growth of leukemia HL-60 cells is also discussed.

The concentrated *n*-BuOH-soluble phase of the MeOH extract of *R. aculeatus* was subjected to Si gel, octadecylsilanized (ODS) Si gel, Diaion HP-20, and Sephadex LH-20 column chromatography to yield aculeosides A (**1**) (156 mg) and B (**2**) (14.5 mg). Aculeoside A (**1**), isolated as an amorphous solid and identified as previously reported,[6] was distinguished by the presence of 6-deoxy-D-glycero-L-threo-4-hexosulose linked to the C-24 hydroxyl group of the aglycon. The 2D NMR data allowed the identification of the unusual deoxyhexosulose, which was well supported by the following chemical and spectral evidence. Treatment of **1** with $NaBH_4$ in MeOH reduced the C-4 carbonyl group of the deoxyhexosulose, accompanied by removing the three acetyl groups linked to the rhamnosyl residue (**1a**). The ^{13}C NMR spectrum of **1a** displayed the six signals assignable to a β-fucopyranosyl unit,[7,8] along with the signals due to a terminal α-L-rhamnopyranosyl unit and a 2-substituted α-L-arabinopyranosyl unit. Acid hydrolysis of **1a** gave L-rhamnose, L-arabinose, and D-fucose, which were identified by HPLC analysis following their conversion to the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives.[9] Thus, the structure of the deoxyhexosulose was unequivocally assigned, including its absolute configuration.

Chart 1



Aculeoside B (**2**) ($C_{57}H_{88}O_{27}$, positive ion FABMS *m*/*z* 1227 $[M + Na]^+$, negative ion FABMS *m*/*z* 1203 $[M - H]^-$), was obtained as an amorphous solid, $[\alpha]_D$ −24.0° (MeOH). Signals for protons of four steroid methyls at δ 1.34 (s), 1.23 (s), 1.22 (d, J = 6.9 Hz), and 0.63 (d, J = 6.1 Hz); four anomeric protons at δ 5.58 (br s), 5.44 (d, J = 7.8 Hz), 4.93 (d, J = 7.7 Hz), and 4.76 (d, J = 7.7 Hz), methyl protons of 6-deoxyhexopyranose at δ 1.67 (*d*, J = 6.1 Hz); and three acetyl methyl protons at δ 2.06 and 1.99 × 2 (each s), completed the distinctive features of the ^1H NMR spectrum of **2**. The above ^1H NMR data and a quaternary carbon signal at δ 110.8 in the ^{13}C NMR spectrum suggested that **2** was also a steroid saponin with spirostanol skeleton.[7] Acid hydrolysis of **2** gave D-glucose, D-galactose, and L-rhamnose. On comparison of the proton and carbon chemical shifts of **2** with those of **1**, the structure of the A−E ring part (C-1−C-21) of **2** was shown to be identical to that **1**, including the orientations of C-1 and C-3 oxygen atoms and the ring junctions, but significant differences were recognized in the signals from the F-ring part and

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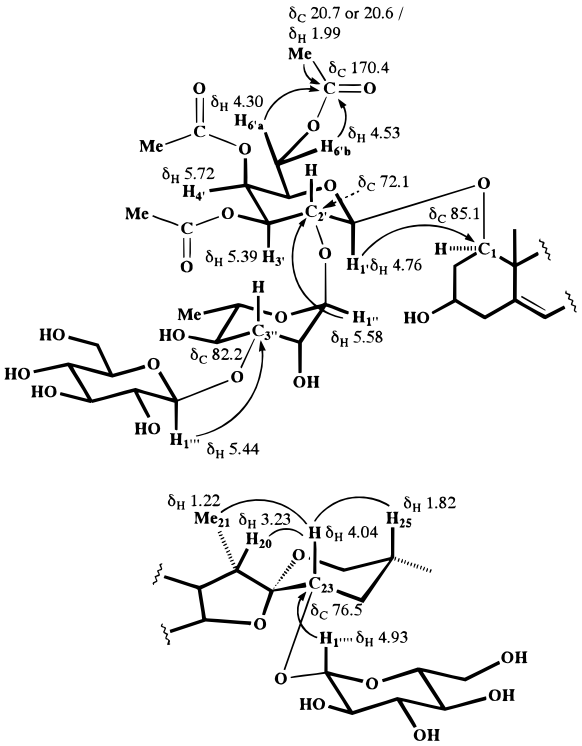

Figure 1. NOE (⌒) and HMBC (↷) correlations of aculeoside B (**2**).

the saccharide moieties between them. Inspection of the $^1H-^1H$ COSY spectrum combined with the HOHAHA data of **2** led us to assemble the structure of the F-ring as −CH(O−)−CH_2−CH(Me)−CH_2−O−, which differed from **1** by the absence of the C-24 glycosyloxy group and the saturation of the 25(27)-ene group. The 23*S* and 25*R* configurations were revealed by the clear NOEs from H-23 to H-20, Me-21 and H-25 observed in the phase-sensitive NOESY spectrum (Figure 1). The coupling constants between H-23 and H_2-24 ($^3J_{H-23,H-24ax}$ = 11.1 Hz and $^3J_{H-23,H-24eq}$ = 4.5 Hz) were consistent with the 23*S* configuration.

The presence of an α-L-rhamnopyranosyl unit ($^1C_4$), two β-D-glucopyranosyl units ($^4C_1$), and a β-D-galactopyranosyl unit ($^4C_1$) was readily revealed by tracing out the proton networks through the $^1H-^1H$ COSY spectrum combined with the HOHAHA data, starting from the anomeric proton signals at δ 5.58 (br s), 5.44 (d, *J* = 7.8 Hz), 4.93 (d, *J* = 7.7 Hz), and 4.76 (d, *J* = 7.7 Hz) (Table 1). The HMQC spectrum correlated all the proton resonances with those of the corresponding carbons. The two glucopyranosyl residues were shown to be terminal units, as suggested by the absence of any glycosylation shifts for their carbon resonances, and the anomeric proton signals of the glucose moieties at δ 5.44 and 4.93 showed $^3J_{C,H}$ correlations with the δ 82.2 (C-3 of rhamnose) and 76.5 (C-23 of aglycon) resonances, respectively. The other anomeric proton signals at δ 5.58 (rhamnosyl) and 4.76 (galactosyl) were correlated to the δ 72.1 (C-2 of galactose) and 85.1 (C-1 of aglycon) resonances, respectively. Thus, one glucosyl unit was revealed to be directly attached to the C-23 hydroxyl group of the aglycon, and the triglycoside, glucosyl-(1→3)-rhamnosyl-(1→2)-galactosyl unit attached to C-1 of the aglycon. Long-range correlations between the acetyl carbonyl carbon signal at δ 170.4 and each of the proton signals at δ 4.53 (dd, *J* = 11.3, 7.0) and 4.30 (dd, *J* = 11.3, 6.1 Hz) due to H_2-6 of the galactose, and downfield-shifted proton signals at δ 5.39 (dd, *J* = 9.9, 3.2 Hz) and 5.72 (br d, *J* = 3.2 Hz) assignable to H-3 and H-4 of the galactose confirmed the linkage of the three acetyl ester groups to the galactose C-3, C-4, and C-6 hydroxy positions (Figure 1). All of these data were consistent with the structure, (23*S*,25*R*)-spirost-5-ene-1β,3β,23-triol 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-β-D-galactopyranosyl}-24-*O*-β-D-glucopyranoside for compound **2**. The structural peculiarities of aculeoside B (**2**) were the presence of three acetyl esters at the inner galactose unit and the glucosyloxy group attached to C-23 of the aglycon.

Table 1. ^1H and ^{13}C NMR Chemical Shift Assignments of the Saccharide Moieties of Aculeoside B (**2**)[a]

position	^1H	*J* (Hz)	^{13}C
1′	4.76 d	7.7	100.0
2′	4.37 dd	9.9, 7.7	72.1
3′	5.39 dd	9.9, 3.2	75.4
4′	5.72 br d	3.2	68.5
5′	4.19 br dd	7.0, 6.1	70.9
6′	4.53 dd	11.3, 7.0	62.3
	4.30 dd	11.3, 6.1	
1″	5.58 br s		101.7
2″	4.65 br d	2.8	71.1
3″	4.55 dd	9.6, 2.8	82.2
4″	4.38 dd	9.6, 9.6	72.8
5″	4.94 dq	9.6, 6.1	69.8
6″	1.67 d	6.1	18.7
1‴	5.44 d	7.8	106.2
2‴	3.99 dd	8.2, 7.8	75.9
3‴	4.13		78.1
4‴	4.12		71.6
5‴	3.97		78.2
6‴	4.41 dd	11.8, 1.4	62.4
	4.24 dd	11.8, 5.1	
1⁗	4.93 d	7.7	106.2
2⁗	3.93 dd	8.1, 7.7	75.3
3⁗	4.15		78.7
4⁗	4.10		71.5
5⁗	3.91		78.4
6⁗	4.45 dd	11.7, 1.8	62.7
	4.29 dd	11.7, 6.1	
Ac	2.06 s		170.8
			20.3
	1.99 s		170.4
			20.7
	1.99 s		170.4
			20.6

[a] Spectra were measured in C_5D_5N-CD_3OD (11:1).

The cytostatic activity of **1** and **2** on human promyelocytic leukemia HL-60 cells was evaluated. The cells were continuously treated with each sample for 72 h, and the cell growth was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay procedure. Compound **1** showed considerable cytostatic activity with an IC_{50} value of 0.48 μg mL^{-1}, while **2** was inactive.

Experimental Section

General Experimental Procedures. 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 using an Elementar Vario EL elemental analyzer. 1D NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz for ^1H NMR), and 2D NMR, on a Bruker AM-500 (500 MHz for ^1H NMR) using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. Si gel (Fuji-Silysia Chemical), Diaion HP-20 (Mitsubishi-Kasei), and ODS Si 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 composed of a CCPM pump, a CCP controller PX-8010, a UV-8000 detector, and Rheodyne injection port with a 20-μL sample loop. A TSK-gel ODS−Prep column (Tosoh, 4.6 mm i.d. × 250 mm, ODS, 5 μm) was used for HPLC analysis. The following materials and reagents were used for cell culture and assay for cytostatic activity: microplate reader, Immuno-Mini NJ-2300, Inter Med; 96-well flat-bottom plate, Iwaki Glass; HL-60 cells, ICN Biomedicals; RPMI 1640 medium, GIBCO BRL; and MTT, Sigma. All other chemicals used were of biochemical reagent grade.

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

Extraction and Isolation. The plant material (fresh wt, 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 on Si gel and elution with a gradient mixture of $CHCl_3$−MeOH system (9:1; 6:1; 4:1; 2:1), and finally with MeOH, gave six fractions (I−VI). Fraction II was chromatographed on Si gel eluting with $CHCl_3$−MeOH (9:1) and ODS Si gel with MeOH−H_2O (4:1) to give **1** (156 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 by a Si gel column eluting with $CHCl_3$−MeOH (4:1) into three fractions (Fraction VIa−VIc). Fraction VIb was chromatographed on Si gel eluting with $CHCl_3$−MeOH−H_2O (30:10:1), ODS Si gel with MeOH−H_2O (7:3; 3:2) and MeCN−H_2O (3:7), and on Sephadex LH-20 with MeOH to yield **2** (14.5 mg).

Aculeoside A (1): amorphous solid; $[\alpha]^{25}{}_D$ −50.0° (*c* 0.10, MeOH); IR (KBr) ν_{max} 3450 (OH), 2930 (CH), 1740 (C=O), 1445, 1365, 1255, 1225, 1140, 1075, 1045, 960 cm^{-1}; negative ion FABMS *m/z* 1007 $[M - H]^-$, 863, 821, 779, 737, 591, 459; positive ion HRFABMS *m/z* 1031.4468 $[M + Na]^+$ ($C_{50}H_{72}O_{21}$-Na requires 1031.4464); *anal.* C 57.46%, H 7.06%, calcd for $C_{50}H_{72}O_{21}\cdot 2H_2O$, C 57.51%, H 7.28%.

Reduction of 1 by $NaBH_4$. A mixture of **1** (20 mg) dissolved in MeOH (10 mL) with $NaBH_4$ (60 mg) was allowed to stand at room temperature for 30 min. Purification of the reaction mixture was carried out by Si gel column chromatography, eluting with $CHCl_3$−MeOH−H_2O (40:10:1), to give **1a** (7.2 mg).

Compound 1a: amorphous solid; $[\alpha]^{27}{}_D$ −72.0° (*c* 0.10, MeOH); IR (KBr) ν_{max} 3400 (OH), 2920 (CH), 1445, 1375, 1255, 1045, 960 cm^{-1}; ^{1}H NMR (C_5D_5N) δ 6.33 (1H, br s, H-1″), 5.57 (1H, br d, J = 5.4 Hz, H-6), 5.25 and 5.10 (each 1H, br s, H_2-27), 5.15 (1H, d, J = 8.0 Hz, H-1‴), 4.71 (1H, d, J = 7.6 Hz, H-1′), 1.72 (3H, d, J = 6.1 Hz, Me-6″), 1.48 (3H, d, J = 6.3 Hz, Me-6‴), 1.42 (3H, s, Me-19), 1.07 (3H, d, J = 6.9 Hz, Me-21), 0.94 (3H, s, Me-18); ^{13}C NMR (C_5D_5N) δ 83.5, 37.4, 68.2, 43.9, 139.7, 124.6, 32.0, 33.0, 50.3, 42.9, 24.0, 40.5, 40.7, 56.7, 32.3, 82.2, 61.5, 16.8, 15.1, 37.4, 14.7, 111.7, 70.4, 83.0, 144.0, 61.5 and 113.7 (C-1 − C-27), 100.4, 75.2, 75.9, 70.1 and 67.3 (C-1′ − C-5′), 101.7, 72.5, 72.6, 74.2, 69.4 and 19.0 (C-1″ − C-6″), and 106.3, 73.1, 75.4, 72.8, 71.5 and 17.3 (C-1‴ − C-6‴); negative ion FABMS *m/z* 883 $[M - H]^-$.

Acid Hydrolysis of 1a. A solution of **1a** (3 mg) in 1M HCl (dioxane−H_2O, 1:1, 5 mL) was heated at 100 °C for 2 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo) column, and chromatographed on Si gel, eluting with $CHCl_3$−MeOH (9:1; 1:1), to give a sugar fraction (1 mg). The sugar fraction was dissolved in H_2O (1 mL), to which (−)-α-methylbenzylamine (5 mg) and $Na[BH_3CN]$ (8 mg) in EtOH (1 mL) were added. After being set aside at 40 °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) in pyridine (0.3 mL) at room temperature for 12 h. The crude mixture was passed through a Sep-Pak C_{18} cartridge with H_2O−MeCN (4:1; 1:1; 1:9, each 5 mL) mixtures as solvents. The H_2O−MeCN (1:9) eluate was further passed through a Toyopak IC−SP M cartridge (Tosoh) with EtOH (10 mL) to give a mixture of the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides,[9] which was then analyzed by HPLC under the following conditions: solvent, MeCN−H_2O (2:3); flow rate, 0.8 mL min^{-1}; detection, UV 230 nm. The derivatives of L-arabinose, L-rhamnose, and D-fucose were detected; t_R (min): 17.40 (derivative of L-arabinose); 20.68 (derivative of D-fucose); 26.97 (derivative of L-rhamnose).

Aculeoside B (2): amorphous solid; $[\alpha]^{27}{}_D$ −24.0° (*c* 0.20, MeOH); IR (KBr) ν_{max} 3420 (OH), 2920 (CH), 1745 (C=O), 1450, 1370, 1255, 1060 cm^{-1}; ^{1}H NMR (C_5D_5N−CD_3OD, 11:1) δ 5.56 (overlapping with H_2O signal, H-6), 4.04 (1H, dd, J = 11.1, 4.5 Hz, H-23), 3.75 (1H, m, H-3), 3.68 (1H, dd, J = 12.1, 4.1 Hz, H-1), 3.47 (2H, H_2-26), 3.23 (1H, m, H-20), 2.42 (1H, br dd, J = 10.8, 4.5 Hz, H-24a), 1.87 (1H, H-24b), 1.82 (1H, m, H-25), 1.34 (3H, s, Me-19), 1.23 (3H, s, Me-18), 1.22 (3H, d, J = 6.9 Hz, Me-21), 0.63 (3H, d, J = 6.1 Hz, Me-27). Assignment of the signals due to the saccharide moieties was shown in Table 1; ^{13}C NMR (C_5D_5N−CD_3OD, 11:1) δ 85.1, 38.1, 68.2, 43.4, 139.0, 125.2, 32.1, 33.1, 50.7, 42.8, 24.3, 40.9, 41.0, 57.3, 32.3, 81.6, 62.6, 17.7, 15.0, 35.9, 14.7, 110.8, 76.5, 37.3, 31.6, 65.8, and 16.8 (C-1 − C-27); negative ion FABMS *m/z* 1203 $[M - H]^-$, 1162, 1042, 446; positive ion FABMS *m/z* 1227 $[M + Na]^+$.

Acid Hydrolysis of 2. Compound **2** (3 mg) was subjected to acid hydrolysis as described for **1** to give a sugar fraction (1.2 mg). The monosaccharide constituents in the 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-rhamnose, D-galactose, and D-glucose were detected by comparison of their t_R with those of authentic samples: t_R (min), 19.79 (derivative of D-galactose); 23.50 (derivative of D-glucose); 26.46 (derivative of L-rhamnose).

Cell Culture and Assay for Cytostatic Activity. HL-60 cells were maintained in the RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin. The leukemia cells were washed and resuspended in the above medium to 3 × 10^4 cells mL^{-1}, 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 °C. After incubation, 4 μL of EtOH−H_2O (1:1) solution containing the sample was added to give the final concentrations of 0.01−10 μg mL^{-1}; 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 the cell growth was evaluated with an MTT assay procedure. The MTT assay was carried out according to a modified method of Sargent and Tayler as follows.[10] After termination of cell culture, 10 μL of 5 mg mL^{-1} MTT in phosphate buffered saline was added to every well and the plate was further incubated in 5% CO_2/air at 37 °C for 4 h. The plate was then centrifuged at 1500 *g* for 5 min to precipitate cells and formazan. The supernatant (150 μL) was removed from every well, and 175 μL of DMSO was added to dissolve the formazan crystals. The plate was mixed on a microshaker for 10 min, and then read on a microplate reader at 550 nm. A dose response curve was plotted for the sample of **1**, and the concentration which gave 50% inhibition of cell growth (IC_{50}) was calculated.

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