Two New Bisdesmosidic Steroidal Saponins from the Underground Parts of *Ruscus aculeatus*

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Two new bisdesmosidic spirostanol saponins were isolated from the underground parts of *Ruscus aculeatus*. Their structures were determined to be (23*S*)-spirosta-5,25(27)-diene-1β,3β,23-triol 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl} 23-*O*-β-D-glucopyranoside and (23*S*)-spirosta-5,25(27)-diene-1β,3β,23-triol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl} 23-*O*-β-D-glucopyranoside on the basis of spectroscopic analysis, including two-dimensional NMR techniques, and the result of hydrolysis.

Key words *Ruscus aculeatus*; Liliaceae; underground part; spirostanol saponin; bisdesmoside

Ruscus aculeatus (Liliaceae) is a widely distributed European plant. An alcoholic infusion of its rhizome has been used for the treatment of some vascular conditions for decades.[1] Previously, we have carried out a phytochemical examination of the underground parts of *R. aculeatus* and identified a series of steroidal saponins based upon (25*R*)-spirost-5-ene-1β,3β-diol (ruscogenin) or spirosta-5,25(27)-diene-1β,3β-diol (neoruscogenin).[2] Our additional investigations into the saponin constituents in *R. aculeatus* have resulted in the discovery of two new bisdesmosidic spirostanol saponins, and this paper provides evidence to support the structural assignment of the new saponins.

The concentrated 1-butanol-soluble fraction of the methanolic extract of *R. aculeatus* (fresh weight 3.1 kg) was fractionated by the combined use of repeated column chromatography on Diaion HP-20, silica-gel, octadecylsilanized (ODS) silica-gel and Sephadex LH-20, as well as preparative HPLC to yield compounds **1** (33.3 mg) and **2** (14.0 mg).

Compound **1**, isolated as an amorphous solid, showed an $[M-H]^-$ ion at *m/z* 1045 in the negative-ion FAB-MS, corresponding to the empirical formula $C_{50}H_{78}O_{23}$, also deduced on the basis of the ^{13}C-NMR spectrum combined with distortionless enhancement by polarization transfer (DEPT) data and elemental analysis. The glycosidic nature of **1** was shown by strong IR absorptions at 3400 and 1045 cm^{-1}. The ^{1}H-NMR spectrum in pyridine-d_5–methanol-d_4 (11 : 1) displayed the following representative signals: three steroid methyl protons at δ 1.35 (s), 1.12 (d, J= 7.3 Hz) and 1.11 (s); exomethylene protons at δ 4.81 and 4.77 (each br s); an olefinic proton at δ 5.52 (br d, J= 5.5 Hz); four anomeric protons at δ 6.19 (br s), 5.43 (d, J=7.8 Hz), 4.86 (d, J=7.8 Hz) and 4.58 (d, J=7.5 Hz); methyl protons of a 6-deoxyhexopyranose at δ 1.60 (d, J=6.1 Hz). Acid hydrolysis of **1** with 1 M hydrochloric acid in dioxane–H_2O (1 : 1) at 100 °C for 2 h resulted in the production of D-glucose, L-arabinose and L-rhamnose as the carbohydrate components, while the genuine sapogenin was decomposed under acidic conditions. The above data, and the four anomeric carbon signals at δ 106.3, 106.2, 101.4 and 101.0 and one distinctive quaternary carbon signal at δ 110.8[3] led to the hypothesis that **1** was a spirostanol saponin with four monosaccharides. Comparison of the ^{1}H- and ^{13}C-NMR assignment of the aglycone moiety of **1**, which was established by analysis of ^{1}H–^{1}H shift correlation spectroscopy (^{1}H–^{1}H COSY) data and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) spectrum, with those of spirosta-5,25(27)-diene-1β,3β-diol (neoruscogenin) 1-*O*-glycosides abundantly present in *R. aculeatus*[2b] revealed that the structure of the A—E ring parts (C-1—C-21) of **1** was identical to that of the reference compounds, including the orientation of the C-1 and C-3 oxygen atoms (1β-equatorial, 3β-equatorial) and ring junctions (B/C *trans*, C/D *trans*, D/E *cis*), but with significant differences in the signals from the F-ring. Interpretation of the ^{1}H–^{1}H COSY spectrum showed that the structural fragments of the F-ring were an oxymethine adjacent to a methylene group appearing as an ABX-like spin system (AB part: δ 3.14, 2.97; X part: δ 4.02), as well as exomethylene (δ 4.81, 4.77) and oxymethylene (δ 4.38, 3.94) groups assignable to 27-H_2 [C-25(27)] and 26-H_2, respectively. The methylene protons were correlated to the carbon signal at δ 37.8 by the HMQC spectrum, and three-bond coupled correlations from the exomethylene protons to the methylene carbon in the ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectrum allowed the methylene to be assigned as 24-H_2. Consequently, the oxymethine group was located at 23-H. Nuclear Overhauser effect (NOE) correlations between 23-H and 21-Me, and 26(ax)-H and 16-H in the phase-sensitive NOE correlation spectroscopy (PHNOESY) spectrum, and spin-coupling constants between 23-H and 24-H_2 ($^3J_{23\text{-H,24(ax)-H}}$=12.2 Hz, $^3J_{23\text{-H,24(eq)-H}}$=4.8 Hz) were consistent with the 22α and 23*S* configurations. Thus, the structure of the aglycone of **1** was shown to be (23*S*)-spirosta-5,25(27)-diene-1β,3β,23-triol. The ^{1}H–^{1}H COSY experiment allowed the sequential assignment of the resonances for each monosaccharide, starting from the easily distinguished anomeric proton. Multiplet patterns and measurements of coupling constants confirmed the presence of two β-D-glucopyranosyl units ($^4C_1$), an α-L-arabinopyranosyl unit ($^4C_1$) and an α-L-rhamnopyranosyl unit ($^1C_4$). The HMQC spectrum correlated all the proton resonances with those of the corresponding carbons. The two glucosyl residues were shown to be terminal units, as suggested by the absence of any glycosylation shifts for

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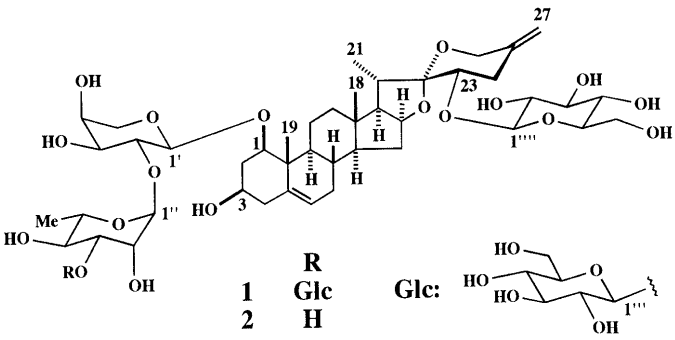
Table 1. ^{1}H- and ^{13}C-NMR Chemical Shift Assignment of **1**[a)]

Position	^{1}H	J (Hz)	^{13}C
1	3.69 dd	11.8, 3.8	84.4
2eq	2.66 br dd	11.8, 3.8	37.9
ax	2.30 q-like	11.8	
3	3.76 m		68.2
4eq	2.48 dd	12.3, 3.9	43.7
ax	2.60 dd	12.3, 12.3	
5	—		139.4
6	5.52 br d	5.5	125.0
7α	1.50		32.3
β	1.82		
8	1.48		33.1
9	1.45		50.6
10	—		43.0
11eq	2.87 br d	11.1	23.9
ax	1.55		
12eq	1.58 br d	12.3	40.8
ax	1.28		
13	—		40.8
14	1.10		56.9
15α	1.97		32.2
β	1.50		
16	4.54 q-like	7.7	81.9
17	1.81 dd	8.1, 7.7	62.4
18	1.11 s		17.4
19	1.35 s		15.1
20	3.15		35.8
21	1.12 d	7.3	14.7
22	—		110.8
23	4.02 dd	12.2, 4.8	77.2
24eq	3.14 dd	12.2, 4.8	37.8
ax	2.97 br dd	12.2, 12.2	
25	—		144.0
26eq	3.94 d	12.6	64.2
ax	4.38 br d	12.6	
27a	4.81 br s		109.9
b	4.77 br s		
1′	4.58 d	7.5	101.0
2′	4.44 dd	8.8, 7.5	74.5
3′	4.01 dd	8.8, 3.5	75.9
4′	4.09 br d	3.5	70.2
5′a	4.20 br d	12.6	67.6
b	3.94 br d	12.6	
1″	6.19 br s		101.4
2″	4.85 br d	3.0	71.9
3″	4.69 dd	9.5, 3.0	82.6
4″	4.35 dd	9.5, 9.5	73.1
5″	4.77 dq	9.5, 6.1	69.3
6″	1.60 d	6.1	18.7
1‴	5.43 d	7.8	106.3
2‴	3.97 dd	8.8, 7.8	76.0
3‴	4.11 dd	8.8, 8.8	78.1
4‴	4.07 dd	8.8, 8.8	71.4
5‴	3.97 m		78.2
6‴a	4.38 br d	11.8	62.4
b	4.20 dd	11.8, 5.2	
1‴	4.86 d	7.8	106.2
2‴	3.88 dd	8.0, 7.8	75.2
3‴	4.08		78.7
4‴	4.08		71.5
5‴	3.86 m		78.3
6‴a	4.40 br d	11.7	62.6
b	4.25 dd	11.7, 5.2	

a) Spectra were measured in pyridine-d_5–methanol-d_4 (11 : 1).



Chart

their carbon resonances, and the anomeric proton signals of the glucosyls at δ 5.43 and 4.86 showed $^3J_{C,H}$ correlations with the δ 82.6 (C-3 of rhamnosyl) and 77.2 (C-23 of aglycone) resonances, respectively, in the HMBC spectrum. The other anomeric proton signals at δ 6.19 (rhamnosyl) and 4.58 (arabinosyl) were correlated to the δ 74.5 (C-2 of arabinose) and 84.4 (C-1 of aglycone) resonances, respectively. Thus, one glucosyl unit was directly attached to the C-23 hydroxyl group of the aglycone, and the triglycoside, glucosyl-(1→3)-rhamnosyl-(1→2)-arabinosyl unit was attached to C-1 of the aglycone. Accordingly, the structure of **1** was characterized as (23*S*)-spirosta-5,25(27)-diene-1β,3β,23-triol 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl} 23-*O*-β-D-glucopyranoside.

The spectral features of **2** ($C_{44}H_{68}O_{18}$) were essentially analogous to those of **1** and suggestive of a bisdesmosidic spirostanol saponin of the same type. The ^{1}H-NMR spectrum of **2** showed signals for three anomeric protons at δ 6.33 (br s), 4.97 (d, J=7.9 Hz) and 4.73 (d, J=7.5 Hz), three steroid methyl protons at δ 1.41 (s), 1.18 (d, J=6.9 Hz) and 1.17 (s), exomethylene protons at δ 4.80 and 4.74 (each br s), and an olefinic proton at δ 5.54 (br d, J=5.5 Hz). Acid hydrolysis of **2** yielded D-glucose, L-arabinose and L-rhamnose. On comparison of the whole ^{13}C-NMR spectrum of **2** with that of **1**, the six signals assignable to the terminal glucopyranosyl moiety and the downfield shift produced by *O*-glycosylation at C-3 of the rhamnosyl unit attached to C-2 of the inner arabinopyranosyl residue could not be identified in the ^{13}C-NMR spectrum of **2**. These findings indicated that **2** was a bisdesmosidic spirostanol saponin related to **1** without the terminal glucosyl moiety. Confirmative evidence for this was obtained by partial acid hydrolysis of **1** with 0.2 M hydrochloric acid at 100 °C for 1 h, which produced **2**. The structure of **2** was assigned as (23*S*)-spirosta-5,25(27)-diene-1β,3β,23-triol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl} 23-*O*-β-D-glucopyranoside.

Compounds **1** and **2** are new bisdesmosidic spirostanol saponins.

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. One dimensional (1D) NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz for ^{1}H-NMR) and two dimensional (2D) NMR on a Bruker AM-500 (500 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 a CCPM pump, a CCP controller PX-8010, a UV-8000 or an RI-8010 detector, and Rheodyne injection port with a 2 ml sample loop for preparative HPLC and a 20 μl loop for analytical HPLC. A CAPCELL PAK C_{18} column (Shiseido, 10 mm i.d. × 250 mm, ODS, 5 μm) was used for preparative HPLC and a TSK-gel ODS-Prep column (Tosoh, 4.6 mm i.d. × 250 mm, ODS, 5 μm) employed for analytical HPLC.

Plant Material The underground parts of *R. aculeatus* used in this study 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 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 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, gave six fractions (I—VI). Fraction VI, after removal of considerable amounts of monosaccharides by passing it through a Diaion HP-20 column eluting with increasing amounts of MeOH in H_2O, was further separated on a silica-gel column eluting with $CHCl_3$–MeOH (4 : 1) to give three fractions (fr. VIa—VIc). Fraction VIb was chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (30 : 10 : 1), ODS silica-gel with MeOH–H_2O (7 : 3; 3 : 2) and MeCN–H_2O (2 : 3), and on Sephadex LH-20 with MeOH to give **2** (14.0 mg). Fraction VIc was subjected to column chromatography on silica-gel eluting with $CHCl_3$–MeOH–H_2O (20 : 10 : 1), ODS silica-gel with MeOH–H_2O (3 : 2) and MeCN–H_2O (3 : 7) to give **1** with a few impurities. Final purification of **1** was achieved by preparative HPLC using MeCN–H_2O (3 : 7) to furnish **1** (33.3 mg) as a pure compound.

Compound **1**: Amorphous solid, $[\alpha]_D^{26}$ −50.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 1045 $[M-H]^-$, 883 $[M-\text{glucosyl}]^-$. *Anal.* Calcd for $C_{50}H_{78}O_{23}\cdot 2H_2O$: C, 55.44; H, 7.63. Found: C, 55.51; H, 7.70. IR (KBr) ν_{max}: 3400 (OH), 2905 (CH), 1045. ^{1}H-NMR (pyridine-d_5) δ: 6.36 (1H, d, J=1.0 Hz, 1″-H), 5.65 (1H, d, J=7.8 Hz, 1‴-H), 5.53 (1H, br d, J=5.5 Hz, 6-H), 4.96 (1H, d, J=7.9 Hz, 1⁗-H), 4.80, 4.73 (each 1H, br s, 27-H_2), 4.63 (1H, d, J=7.6 Hz, 1′-H), 1.66 (3H, d, J=6.2 Hz, 6″-Me), 1.43 (3H, s, 19-Me), 1.18 (3H, d, J=6.9 Hz, 21-Me), 1.17 (3H, s, 18-Me). ^{13}C-NMR (pyridine-d_5) δ: 84.5, 38.1, 68.2, 43.9, 139.5, 124.8, 32.3, 33.0, 50.5, 43.0, 23.9, 40.8, 40.8, 56.8, 32.1, 81.8, 62.3, 17.3, 15.1, 35.7, 14.7, 110.7, 77.2, 37.7, 144.0, 64.1, 109.8 (C-1—C-27), 101.0, 74.3, 76.2, 70.2, 67.7 (C-1′—C-5′), 101.4, 72.1, 82.8, 73.3, 69.3, 18.7 (C-1″—C-6″), 106.7, 76.2, 78.3, 71.6, 78.4, 62.6 (C-1‴—C-6‴), 106.2, 75.3, 78.9, 71.7, 78.4, 62.8 (C-1⁗—C-6⁗).

Acid Hydrolysis of 1 A solution of **1** (5 mg) in 1 M 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 silica-gel eluting with $CHCl_3$–MeOH (15 : 1; 1 : 1) to give an aglycon fractione (1.9 mg) and a sugar fraction (1.9 mg). TLC analysis of the aglycone fraction showed that it contained several unidentified artifactual sapogenols. 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,[4] which was then analyzed by HPLC under the following conditions: solvent, MeCN–H_2O (2 : 3); flow rate, 0.8 ml/min; detection, UV 230 nm. The derivatives of D-glucose, L-arabinose and L-rhamnose were detected; t_R (min): 18.31 (derivative of L-arabinose); 25.06 (derivative of D-glucose); 28.06 (derivative of L-rhamnose).

Compound **2**: Amorphous solid, $[\alpha]_D^{26}$ −52.0° (c=0.10, MeOH). Positive-ion FAB-MS m/z: 907 $[M+Na]^+$. Negative-ion FAB-MS m/z: 883 $[M-H]^-$. *Anal.* Calcd for $C_{44}H_{68}O_{18}\cdot H_2O$: C, 58.52; H, 7.81. Found: C, 58.43; H, 7.90. IR (KBr) ν_{max}: 3430 (OH), 2930 (CH), 1045. ^{1}H-NMR (pyridine-d_5) δ: 6.33 (1H, br s, 1″-H), 5.54 (1H, br d, J=5.5 Hz, 6-H), 4.97 (1H, d, J=7.9 Hz, 1⁗-H), 4.80, 4.74 (each 1H, br s, 27-H_2), 4.73 (1H, d, J=7.5 Hz, 1′-H), 1.73 (3H, d, J=6.2 Hz, 6″-Me), 1.41 (3H, s, 19-Me), 1.18 (3H, d, J=6.9 Hz, 21-Me), 1.17 (3H, s, 18-Me). ^{13}C-NMR (pyridine-d_5) δ: 83.4, 37.4, 68.2, 43.9, 139.5, 124.7, 32.2, 33.0, 50.4, 42.9, 23.9, 40.7, 40.8, 56.8, 32.1, 81.8, 62.3, 17.3, 15.0, 35.7, 14.6, 110.7, 77.2, 37.7, 143.9, 64.1, 109.8 (C-1—C-27), 100.3, 75.2, 75.9, 70.1, 67.3 (C-1′—C-5′), 101.7, 72.5, 72.6, 74.2, 69.4, 19.0 (C-1″—C-6″), 106.2, 75.3, 78.9, 71.6, 78.3, 62.8 (C-1⁗—C-6⁗).

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 D-glucose, L-arabinose and L-rhamnose were detected; t_R (min): 18.45 (derivative of L-arabinose); 25.64 (derivative of D-glucose); 28.89 (derivative of L-rhamnose).

Preparation of 2 by Partial Acid Hydrolysis of 1 Compound **1** (10 mg) was treated with 0.2 M HCl (dioxane–H_2O, 1 : 1, 5 ml) at 100 °C for 1 h. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU column, and chromatographed on silica-gel eluting with $CHCl_3$–MeOH–H_2O (35 : 10 : 1) to give **2** (1.2 mg).

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