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A spirostanol saponin from the underground parts of *Ruscus aculeatus*

Yoshihiro Mimaki*, Minpei Kuroda, Akihito Yokosuka, Yutaka Sashida

School of Pharmacy, Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-0392, Japan

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Abstract

A new spirostanol saponin was isolated from the underground parts of *Ruscus aculeatus* and the structure was assigned as (23*S*,25*R*)-spirost-5-ene-3β,23-diol 23-*O*-{*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside} on the basis of spectroscopic analysis, including two-dimensional NMR spectroscopic techniques and the result of acid hydrolysis. The saponin is unique in structure having a diglycoside unit at C-23 of the spirostanol skeleton. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Ruscus aculeatus*; Liliaceae; Underground parts; Steroidal saponin; Spirostanol saponin

1. Introduction

The genus *Ruscus* with three species is distributed throughout Europe. An alcoholic infusion of the rhizomes of *Ruscus aculeatus* has been used for the treatment of some veinous ailments for decades (Hostettmann & Marston, 1995). Previously, we have carried out phytochemical screening of the underground parts of *R. aculeatus* and isolated 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) as the sapogenin structures, some of which showed considerable cytostatic activity on leukaemia HL-60 cells (Horikawa et al., 1994; Mimaki, Kuroda, Yokosuka, & Sashida, 1998; Mimaki, Kuroda, Kameyama, Yokosuka, & Sashida, 1998a; Mimaki, Kuroda, Kameyama, Yokosuka, & Sashida, 1998b). Further analysis of the saponin constituents in *R. aculeatus* led to the isolation of a new spirostanol saponin having a diglycoside moiety at C-23 of the aglycone. In this paper, we report the structural assignment of **1** on the basis of spectroscopic analysis, including two-dimensional NMR techniques and the result of acid hydrolysis.

2. Results and discussion

Fractionation of the methanolic extract, obtained from the underground parts of *R. aculeatus*, by a combination of column chromatographic methods over a porous polymer resin (Diaion HP-20), silica gel and octadecylsilanized (ODS) silica gel resulted in the isolation of compound **1**.

Compound **1** ($C_{39}H_{62}O_{14}$, negative-ion FABMS *m/z* 753 $[M-H]^-$) was obtained as an amorphous solid, $[\alpha]_D$ −44.0° (methanol). The glycosidic nature of **1** was shown by strong IR absorptions at 3420 and 1055 cm^{-1}. The ^1H NMR spectrum of **1** (pyridine-d_5) showed two three-proton singlet signals at δ 1.19 and 0.99, and two three-proton doublets at δ 1.22 ($J=7.0$ Hz) and 0.78 ($J=6.5$ Hz), indicative of the steroidal character of **1**. Furthermore, two anomeric proton signals at δ 5.17 (d, $J=7.8$ Hz) and 4.94 (d, $J=7.7$ Hz), and an olefinic proton signal at δ 5.35 (br d, $J=4.9$ Hz) were also noted in a preliminary inspection of the ^1H NMR spectrum of **1**. Acid hydrolysis of **1** with 1 M hydrochloric acid in dioxane–H_2O (1:1) at 100°C for 1.5 h resulted in the production of D-glucose, as

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

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

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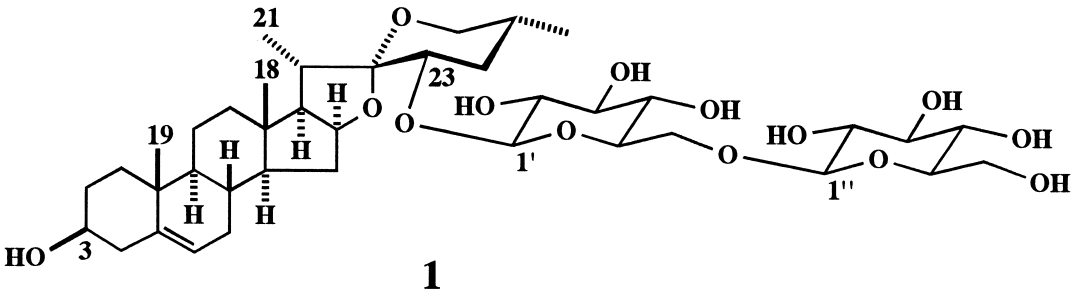

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identified by HPLC analysis following its conversion to a 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivative (Oshima & Kumanotani, 1981; Oshima, Yamauchi, & Kumanotani, 1982), while the genuine aglycone was decomposed under acidic conditions. The ^{13}C NMR spectrum of **1** contained a total of 39 resonance lines. After subtraction of 12 carbons for the two hexoses, 27 signals, which were assigned to Me × 4, CH_2 × 9, CH × 10 and C × 4 by DEPT experiments, remained for the aglycone moiety. The above data, as well as the two anomeric carbon signals at δ 106.4 and 105.6 and one distinctive quaternary carbon resonance at δ 110.8 (Agrawal, Jain, Gupta, & Thakur, 1985), led to the hypothesis that **1** was a spirostanol saponin with two glucose units. Complete ^1H and ^{13}C NMR assignments of the aglycone moiety of **1** (Table 1), which were established by analysis of the ^1H–^1H COSY spectrum combined with the HOHAHA and HMQC data, were compared with those of a typical plant sapogenin, (25*R*)-spirost-5-en-3β-ol (diosgenin) (Agrawal et al., 1985). This revealed that the structure of the A–E ring parts (C-1–C-21) of **1** was identical that of diosgenin, including orientation of the C-3 oxygen atom (3β-equatorial) and ring junctions (B/C *trans*, C/D *trans*, D/E *cis*). The presence of the C-3 hydroxyl group was confirmed by comparison of the ^1H NMR spectral data of **1** with those of the corresponding octaacetate (**1a**) prepared by the treatment of **1** with acetic anhydride in pyridine. The H-3 proton, which was observed at δ 3.83 (m) in the ^1H NMR spectrum of **1**, was shifted downfield by 0.95 ppm upon *O*-acetylation to appear at δ 4.78 in that of **1a**. Detailed inspection of the ^1H–^1H COSY spectrum combined with the HOHAHA data of **1** led us to propose the F-ring as –CH(O)–CH_2–CH(Me)–CH_2–O– and evidence for the connectivities of the terminal free bonds of the fragment to C-22 was obtained by observation of a $^1H/^{13}C$ long-range correlation from H-26eq (δ 3.45) to C-22 (δ 110.8). Thus, the presence of a C-23 oxygen atom was evident. NOE correlations between the protons of H-26ax (δ 3.51) and H-16 (δ 4.61), H-26ax and Me-27 (δ 0.78), and H-26eq and H-25 (δ 2.14) in the phase-sensitive NOESY spectrum and spin-coupling constants between H-23 and H_2-24 ($^3J_{H\text{-}23,H\text{-}24ax} = 11.6$ Hz, $^3J_{H\text{-}23,H\text{-}24eq} = 5.1$ Hz), and between H-25 and H_2-26 ($^3J_{H\text{-}25,H\text{-}26ax} = 11.0$ Hz, $^3J_{H\text{-}25,H\text{-}26eq} = 4.2$ Hz) were consistent with the 22α, 23*S* and 25*R* configurations. The ^1H–^1H COSY and HOHAHA experiments also allowed the sequential assignment of the resonances for the glycosidic residue, starting from the easily distinguished anomeric protons at δ 5.17 and 4.94. Multiplet patterns and measurements of coupling constants confirmed the presence of two β-D-glucopyranosyl units ($^4C_1$). The HMQC spectrum correlated all the proton resonances with those of the corresponding carbons. The one glucose residue was shown to be a terminal unit, as suggested by the absence of any glycosylation shift for its carbon reson-

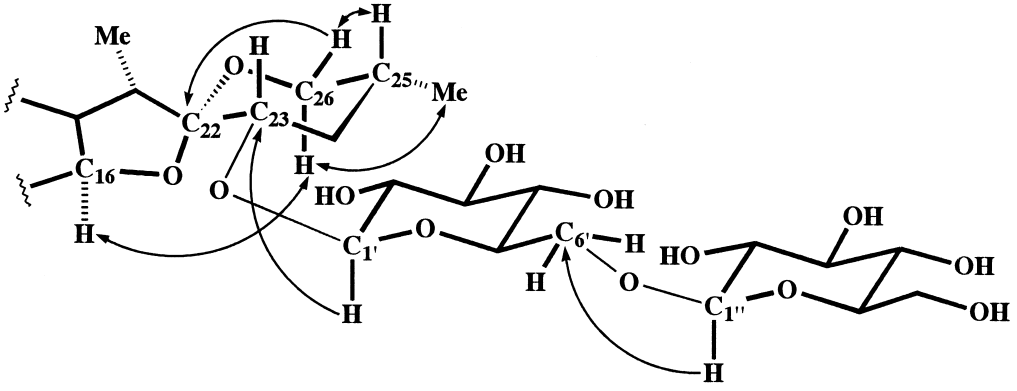

Fig. 1. Arrows indicate HMBC correlations (from H to C) and double-headed arrows indicate NOE correlations.

ances. In the HMBC spectrum, the anomeric proton signals of the glucosyls at δ 5.17 and 4.94 showed $^3J_{C,H}$ correlations with the δ 70.1 (C-6 of inner glucosyl) and 76.2 (C-23 of aglycone) resonances, respectively (Fig. 1). Thus, the diglycoside, glucosyl-(1 → 6)-glucosyl unit was shown to be attached to C-23 of the aglycone. Accordingly, the structure of **1** was characterized as (23*S*,25*R*)-spirost-5-ene-3β,23-diol 23-*O*-{*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside}.

Compound **1** is unique in structure having a diglycoside unit at C-23 of the spirostanol skeleton.

3. Experimental

3.1. General

NMR (ppm, *J* Hz): Bruker DRX-500 using XWIN-NMR 2.0 pulse programs, 500 MHz for ^{1}H NMR. CC: silica gel (Fuji-Silysia Chemical, Japan), ODS silica gel (Nacalai Tesque, Japan) and Diaion HP-20 (Mitsubishi-Kasei, Japan). TLC: precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck, Germany) and RP-18 $F_{254}S$ (0.25 mm thick, Merck). HPLC: a Tosoh HPLC system (pump, CCPM; controller, CCP controller PX-8010; detector, UV-8000) equipped with a Kaseisorb LC ODS-120-5 column (Tokyo-Kasei-Kogyo, Japan, 4.6 mmi.d. × 250 mm, ODS, 5 μm).

3.2. Plant material

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

3.3. Extraction and isolation

The plant material (3.1 kg, fresh weight) was extracted with hot MeOH. The MeOH extract was concentrated under red. pres. and the viscous concentrate was partitioned between H_2O and *n*-BuOH. CC of the *n*-BuOH-soluble phase on silica gel and elution with a stepwise gradient mixture of $CHCl_3$–MeOH system (9:1; 6:1; 4:1; 2:1) and finally with MeOH, gave six fractions (I–VI). Fr. VI contained considerable amounts of saccharides, the removal of which was performed by passage through a Diaion HP-20 column using gradients of MeOH in H_2O. The 80% MeOH and MeOH eluate frs were combined and further fractionated by a silica gel column eluting with $CHCl_3$–MeOH (4:1) into three frs (VIa–VIc). Fr. VIa was subjected to ODS silica gel CC eluting with MeOH–H_2O (7:3; 3:1) and MeCN–H_2O (1:1) to give **1** (50.5 mg) as a pure compound.

Table 1
^{1}H and ^{13}C NMR chemical shift assignment for **1** (Spectra were measured in pyridine-d_5)

Position	^{1}H	*J* (Hz)	^{13}C
1eq	1.80		37.8
ax	1.10		
2eq	2.08		32.6
ax	1.78		
3	3.83 m		71.2
4eq	2.61 dd	13.2, 4.6	43.5
ax	2.57 dd	13.2,13.2	
5	–		141.9
6	5.35 br d	4.9	121.1
7α	1.85		32.4
β	1.52		
8	1.57		31.6
9	0.95		50.4
10	–		37.0
11eq	1.47		21.2
ax	1.40		
12eq	1.79		40.4
ax	1.19		
13	–		41.0
14	1.11		56.7
15α	1.99		32.0
β	1.48		
16	4.61 q-like	7.7	81.3
17	1.94 dd	8.5, 7.7	62.1
18	1.19 s		17.3
19	0.99 s		19.5
20	3.19 m		35.7
21	1.22 d	7.0	14.7
22	–		110.8
23	4.16 dd	11.6, 5.1	76.2
24eq	2.64		37.6
ax	2.01 q-like	11.6	
25	2.14 m		31.4
26eq	3.45 dd	11.0, 4.2	65.8
ax	3.51 dd	11.0, 11.0	
27	0.78 d	6.5	16.8
1′	4.94 d	7.7	106.4
2′	3.95 dd	9.2, 7.7	75.1
3′	4.17 dd	9.2, 9.2	78.7
4′	4.02 dd	9.2, 9.2	71.8
5′	4.15 m		77.7
6′a	4.85 dd	11.8, 1.1	70.1
b	4.33 dd	11.8, 6.8	
1″	5.17 d	7.8	105.6
2″	4.04 dd	8.6, 7.8	75.3
3″	4.22 dd	8.6, 8.6	78.4
4″	4.25 dd	8.6, 8.6	71.6
5″	3.93 m		78.5
6″a	4.53 dd	11.7, 2.0	62.8
b	4.39 dd	11.7, 5.1	

3.4. Compound *1*

Amorphous solid. $[\alpha]_D^{25}$ −44.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 753 $[M–H]^-$, 591 [M–glucosyl]$^-$. IR ν_{max} (KBr) cm^{-1}: 3420 (OH), 2930 (CH), 1450, 1370, 1270, 1250, 1155, 1055, 960, 940, 900.

3.6. Acid hydrolysis of **1**

A soln of **1** (5 mg) in 1 M HCl (dioxane–H_2O, 1:1, 5 ml) was heated at 100°C for 1.5 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU (Organo, Japan) column and this was then subjected to chromatography on silica gel and eluted with $CHCl_3$–MeOH (9:1; 1:1). This gave an aglycone fraction (2.5 mg) and a sugar fraction (2.0 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. The temperature was raised to 40°C, this being held for 4 h, followed by addition of AcOH (0.2 ml) with the whole then evaporated 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 (Waters, USA) with H_2O–MeCN (4:1; 1:1, each 5 ml) mixtures as solvents. The H_2O–MeCN (1:1) eluate was further passed through a Toyopak IC-SP M cartridge (Tosoh) with EtOH (10 ml) to give a 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivative of D-glucose (Oshima & Kumanotani, 1981; Oshima et al., 1982), 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. R_t (min): 25.00.

3.6. Acetylation of **1**

Compound **1** (15 mg) was acetylated with Ac_2O in pyridine and the crude acetate was chromatographed on silica gel eluting with hexane–Me_2CO (3:1) to afford the corresponding octaacetate (**1a**) (13.4 mg). IR ν_{max} (KBr) cm^{-1}: 2955 (CH), 1755 (C=O), 1455, 1435, 1375, 1245, 1220, 1175, 1065, 1035, 965, 945, 905, 800. ^1H NMR (pyridine-d_5): δ 5.71 (1H, dd, $J=9.5$, 9.5 Hz, H-3′), 5.70 (1H, dd, $J=9.5$, 9.5 Hz, H-3″), 5.50 (1H, dd, $J=9.5$, 9.5 Hz, H-4″), 5.49 (1H, dd, $J=9.5$, 8.0 Hz, H-2″), 5.37 (1H, dd, $J=9.5$, 7.9 Hz, H-2′), 5.36 (1H, dd, $J=9.5$, 9.5 Hz, H-4′), 5.33 (1H, br d, $J=5.6$ Hz, H-6), 5.09 (1H, d, $J=7.9$ Hz, H-1′), 5.06 (1H, d, $J=8.0$ Hz, H-1″), 4.78 (1H, m, H-3), 4.58 (1H, dd, $J=12.3$, 4.9 Hz, H-6″a), 4.55 (1H, q-like, $J=7.3$ Hz, H-16), 4.39 (1H, dd, $J=12.3$, 2.2 Hz, H-6″b), 4.22 (1H, ddd, $J=9.5$, 6.7, 2.0 Hz, H-5′), 4.19 (1H, dd, $J=11.8$, 2.0 Hz, H-6′a), 4.08 (1H, ddd, $J=9.5$, 4.9, 2.2 Hz, H-5″), 3.99 (1H, dd, $J=11.8$, 6.7 Hz, H-6′b), 3.90 (1H, dd, $J=11.0$, 4.6 Hz, H-23), 3.56 (1H, dd, $J=10.7$, 3.3 Hz, H-26a), 3.50 (1H, dd, $J=10.7$, 10.7 Hz, H-26b), 2.20, 2.18, 2.09, 2.06, 2.04, 2.02, 2.01 × 2 (each 3H, s, Ac × 8), 1.20 (3H, d, $J=7.0$ Hz, Me-21), 1.04 (3H, s, Me-18), 0.95 (3H, s, Me-19), 0.90 (3H, d, $J=5.6$ Hz, Me-27).

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References

Agrawal, P. K., Jain, D. C., Gupta, R. K., & Thakur, R. S. (1985). *Phytochemistry*, *24*, 2479.

Horikawa, T., Mimaki, Y., Kameyama, A., Sashida, Y., Nikaido, T., & Ohmoto, T. (1994). *Chemistry Letters*, 2303.

Hostettmann, K., & Marston, A. (1995). In *Saponins* (pp. 302–303). London: Cambridge University Press.

Mimaki, Y., Kuroda, M., Yokosuka, A., & Sashida, Y. (1998). *Chemical and Pharmaceutical Bulletin*, *46*, 879.

Mimaki, Y., Kuroda, M., Kameyama, A., Yokosuka, A., & Sashida, Y. (1998a). *Chemical and Pharmaceutical Bulletin*, *46*, 298.

Mimaki, Y., Kuroda, M., Kameyama, A., Yokosuka, A., & Sashida, Y. (1998b). *Phytochemistry*, *48*, 485.

Oshima, R. & Kumanotani, J. (1981). *Chemistry Letters*, 943.

Oshima, R., Yamauchi, Y., & Kumanotani, J. (1982). *Carbohydrate Research*, *107*, 169.