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Note

New Neuritogenic Steroidal Saponin from *Ophiopogon japonicus* (Thunb.) Ker-Gawl

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A new steroidal saponin was isolated from *Ophiopogon japonicus*. This saponin possesses a modification by 2-hydroxy-3-methylvalerylation of the hydroxyl group at C-4′ of the sugar, linked to C-1 of the aglycone. It exhibited significant neuritogenic activity for PC12 cells. The structure-activity relationship revealed the aglycone, rather than the sugar moieties and acylation, to be important for the neuritogenic activity.

Key words: steroidal saponin; *Ophiopogon japonicus*; neuritogenic activity; PC12 cells; structure-activity relationship

Nerve growth factor (NGF)[1)] is one of the most important neurotrophic factors essential for the growth, survival, and functional maintenance of neurons. It can be considered as a candidate for treating Alzheimer's disease, the most common neurodegenerative disorder.[2)] NGF also plays an important role in preventing the loss of cholinergic neurons, stimulating the cholinergic function, preventing cholinergic degeneration, and improving memory functions in animal models of Alzheimer's disease.[3,4)] However, NGF is unable to cross the blood-brain barrier because of its large molecular size and hydrophilic properties.[5)] The use of NGF for treating neurodegenerative disorders is thus difficult. Small molecules that mimic the physiological action of NGF are therefore sought.[6)] The PC12 cell line derived from rat pheochromocytoma cells exhibits characteristic neuronal responses to NGF and has been used as a bioassay model to screen for small-molecule candidates.[7)] Previous studies on screening for these compounds with the PC12 cell line have isolated six novel cerebrosides,[8)] a series of steroidal glycosides,[9)] and eleven alkyl benzoates.[10,11)] Bioassay-guided purification of an *Ophiopogon japonicus* (Liliaceae) (Thunb.) Ker-Gawl methanol extract resulted in the isolation of a new neuritogenic steroidal saponin in the present study (**1**, Fig. 1A). Interestingly, spicatoside A, a steroidal saponin, was reported to show neuritogenic activity by J. Hur *et al.* in 2009.[12)] We describe here the isolation, structure, biological activity, and brief structure-activity relationship of this new compound (**1**).

Commercially available dried roots of *O. japonicus* (9.5 kg, dry wt.) were purchased in Sichuan Province.

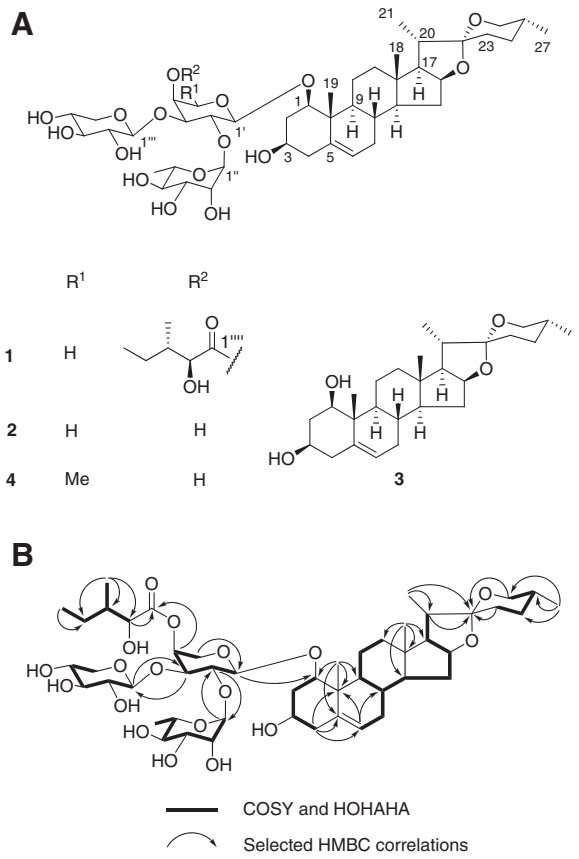

Fig. 1. Structural Elucidation of Compound **1**.
A, Structures of compounds **1**–**4**. B, Gross structure of compound **1**, with selected HMBC correlations.

The freeze-dried samples were ground to a powder and then stirred in MeOH (50 L) for 3 d at room temperature. A supernatant was separated by filtration and concentrated to obtain 1.2 kg of a crude extract. This extract was chromatographed on ODS (Cosmosil 75 C18-OPN, Nacalai Tesque) and eluted stepwise with MeOH/H_2O (50:50, 60:40, 70:30, 80:20, 90:10, 100:0) to yield six fractions. The active fourth fraction (3.8 g) eluted with MeOH/H_2O (8:2) was chromatographed on silica gel (200–300 mesh, Yantai Chemical Industry Research Institute) and eluted stepwise with MeOH/$CHCl_3$ (10:90, 15:85, 20:80, 25:75, 30:70, 100:0) to yield six

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Table 1. ^{1}H-NMR (compound **1**) and ^{13}C-NMR Data (compounds **1** and **2**) in Pyridine-d_5

Carbon no.	**1** ^{1}H[a]	**1** ^{13}C[b]	**2** ^{13}C[b]	Carbon no.	**1** ^{1}H[a]	**1** ^{13}C[b]	**2** ^{13}C[b]
1	3.79 dd (12.1, 3.8)	83.1	83.7	1′	4.68 d (7.8)	99.9	100.4
2a	2.66 m	37.3	37.4	2′	4.45 dd (9.5, 7.8)	74.4	74.2
2b	2.35 m			3′	4.14 dd (9.5, 3.5)	82.1	84.4
3	3.85 m	68.1	68.2	4′	5.70 m	73.2	69.6
4a	2.68 m	43.6	43.8	5′a	4.23 dd (12.7, 1.5)	64.2	67.0
4b	2.56 dd (12.8, 4.2)			5′b	3.68 d (12.7)		
5	—	139.3	139.5				
6	5.57 d (5.6)	124.6	124.6	1″	6.32 br s	101.9	101.8
7a	1.88 m	31.8	32.3	2″	4.77 m	72.4	72.5
7b	1.51 m			3″	4.55 dd (9.5, 3.6)	72.5	72.5
8	1.57 m	33.0	33.1	4″	4.28 dd (9.5, 9.6)	74.1	74.0
9	1.49 m	50.1	50.3	5″	4.79 m	69.5	69.4
10	—	42.6	42.8	6″	1.73 d (6.2)	19.2	19.1
11a	2.90 m	24.2	24.0				
11b	1.67 m			1‴	4.89 d (7.5)	106.9	106.4
12a	1.82 m	40.2	40.1	2‴	3.93 dd (8.4, 7.9)	74.7	74.6
12b	1.37 m			3‴	4.03 t (8.4)	78.6	78.2
13	—	40.5	40.2	4‴	4.11 m	70.8	70.9
14	1.10 m	56.9	56.8	5‴a	4.25 dd (10.8, 5.6)	67.1	66.9
15a	1.99 m	32.3	31.9	5‴b	3.62 t (10.8)		
15b	1.43 m						
16	4.46 m	81.0	81.1	1⁗	—	174.9	
17	1.76 m	62.9	62.9	2⁗	4.53 m	75.5	
18	0.92 s	16.7	16.6	3⁗	2.14 m	39.5	
19	1.40 s	14.9	14.9	4⁗a	1.93 m	24.5	
20	1.96 m	41.9	41.9	4⁗b	1.47 m		
21	1.19 d (7.0)	14.9	15.0	5⁗	0.93 t (7.4)	11.9	
22	—	109.2	109.2	6⁗	1.13 d (6.8)	15.8	
23a	1.84 m	31.8	31.8				
23b	1.64 m						
24a	1.66 m	29.2	29.2				
24b	1.53 m						
25	1.53 m	30.5	30.5				
26a	3.54 dd (10.5, 2.5)	66.7	66.7				
26b	3.47 t (10.5)						
27	0.67 d (5.4)	17.2	17.2				

[a] 500 MHz, coupling constants (J in Hz) are in parentheses.
[b] 125 MHz.

fractions. The most active sample (190.1 mg), which was eluted with MeOH/$CHCl_3$ (25:75), was subjected to HPLC [Develosil ODS-HG-5 (ϕ 20 × 250 mm), Nomura Chemical, 8 mL/min flow rate, MeOH/H_2O (8:2) mobile phase] to give an active fraction and one pure active compound. The pure compound was identified as ophiopogonin D (**4**) (Fig. 1A) by precise agreement of the specific rotation and NMR data with those reported.[13,14] The active fraction (15.0 mg) was again purified by HPLC [Develosil ODS-HG-5 (ϕ 10 × 250 mm), Nomura Chemical, 3 mL/min flow rate, MeCN/H_2O (5:5) mobile phase] to yield pure compound **1** (5.7 mg, 0.00006% of dry wt., t_R = 45 min).

Compound **1**[15] has the molecular formula $C_{49}H_{78}O_{18}$ as determined by HR FT-ICR MS measurements. The IR spectrum of **1** shows an absorption band at 3425 cm^{-1} due to hydroxyl groups. The ^{1}H-NMR, ^{13}C-NMR, and DEPT spectra of **1** indicate the presence of 17 oxymethines, 3 oxymethylenes, 1 oxygenated quaternary carbon, 1 trisubstituted double bond (δ_H 5.57, δ_C 124.6, and 139.3), 2 quaternary sp^3 carbons (δ_C 42.6 and 40.5), and 1 carboxyl group (δ_C 174.9) (Table 1). The remaining proton and carbon signals are attributable to 7 methyls, 7 methines, and 9 methylenes. The signal at δ_C 174.9 supports the presence of one carboxyl group. The anomeric resonance of protons and carbons (δ_H/δ_C: 4.68/99.9, 6.32/101.9, and 4.89/106.9) reveals the presence of three sugar moieties. An analysis of the COSY and HOHAHA spectra led to the determination of the partial structures depicted by the bonds (in boldface type) in Fig. 1B. These partial structures were connected by the following long-range H-C correlations obtained by an HMBC experiment to give the overall structure of **1** (Fig. 1B): methyl protons (H-18) to C-12, C-13, C-14, and C-17; methyl protons (H-19) to C-1, C-5, C-9, and C-10; methyl protons (H-21) to C-20 and C-22; methyl protons (H-27) to C-24, C-25, and C-26; methyl protons (H-5⁗) to C-4⁗; methyl protons (H-6⁗) to C-2⁗, C-3⁗, and C-4⁗; H-4 to C-5, C-6, and C-10; H-6 to C-8 and C-10; H-20 to C-22; H-23 to C-22; H-26 to C-22; and H-5′ to C-1′. The correlations from anomeric H-1′ to C-1, H-1″ to C-2′, and H-1‴ to C-3′ indicate the location of three sugar moieties. The important HMBC correlations H-4′ to C-1⁗ and H-2⁗ to C-1⁗ reveal the sugar linked to C-1 of the aglycone to be acylated at the hydroxyl group of C-4′ which has a 2-hydroxy-3-methylvaleryl group.

Further validation was done by the degradation of **1** to confirm the structure and determine the stereochemistry. The hydrolysis of **1** under weakly basic conditions

produced compound **2** and potassium 2-hydroxy-3-methylpentanoate.[16] The ^{1}H-NMR and ^{13}C-NMR spectra, MS data, and specific rotation of compound **2** agree with those reported.[17] The ^{1}H-NMR data for 2-hydroxy-3-methylpentanoic acid obtained precisely agree with those for (2*S*,3*S*) and (2*R*,3*R*)-2-hydroxy-3-methylpentanoic acid.[18] Although the concentration was low, the specific rotation of 2-hydroxy-3-methylpentanoic acid obtained almost agrees with that of the reported value.[16,18,19] These results respectively indicate the 2*S*,3*S* configuration for the hydroxyl group at C-2$''''$ and the methyl group at C-3$''''$. The same modification has been reported for the steroidal saponin from *Ruscus auleatus* which belongs to the same Liliaceae group as *O. japonicus*.[19] The absolute stereochemistry of the 2-hydroxy-3-methylvaleryl group of compound **1** was therefore determined to be 2*S*,3*S*. The degradation of **2** enabled the aglycone to be obtained to identify the structure-activity relationship and further confirm the structure. Acid hydrolysis of **2** in MeOH resulted in the production of an aglycone which was identified as ruscogenin (**3**).[20,21]

The neuritogenic activities of these compounds were evaluated according to the methods described in previous papers.[8,9] A suspension of 20,000 PC12 cells in 1 mL of a DMEM medium was inoculated in a 24-well microplate and then pre-cultured under a humidified atmosphere of 5% CO_2 at 37 °C. The medium was replaced after 24 h by 1 mL of the serum-free DMEM medium containing a test sample and DMSO (0.5%), and the morphological changes in the cells were monitored 48 h later under a phase-contrast microscope. About 100 cells were counted in a randomly chosen field, this being conducted in triplicate.

Compound **1** showed maximum neuritogenic activity of 46% at 0.3 μM 48 h after the treatment, compared with the solvent (0.5% DMSO) and the NGF positive control at optimum concentration (40 ng/mL, Fig. 2A). Compound **2** obtained from the hydrolysis of compound **1** showed 52% activity at 0.3 μM; this suggests that acylation at C-4′ of the sugar linked at C-1 to the aglycone had little or no effect on the biological activity of the steroidal saponin. Compound **3** showed similar neuritogenic activity (54% at 0.3 μM), suggesting that the sugar moiety at C-1 of the aglycone did not affect the biological activity of the saponin toward PC12 cells. These results are supported by the neuritogenic activity (47% at 0.3 μM) of compound **4** (Fig. 2A). The structure-activity relationship emphasizes that the aglycone, rather than the sugar moieties and acylation, was important for the neuritogenic activity.

Figure 2B shows the morphological changes to the PC12 cells after treating with **1** and **3** in comparison with the solvent control (0.5% DMSO, a) and positive control (40 ng/mL, b). Cells treated with **1** (c) and **3** (d) at 0.3 μM showed long bipolar and multipolar neurite outgrowth 48 h after the treatment. While the NGF treatment resulted in multipolar neurite outgrowth of the cells, control cells cultured without additional compounds showed few short neurite outgrowths.

The steroidal saponin exhibited significant neuritogenic activity toward PC12 cells. The aglycone showed neuritogenic activity higher than that of the natural compound (the original saponin) at the same concentration. The aglycone, rather than the sugar moieties and other modifications, played a key role in the neuritogenic activity. The aglycone may be useful for studying the mechanism for the neurotrophic effect on PC12 cells.

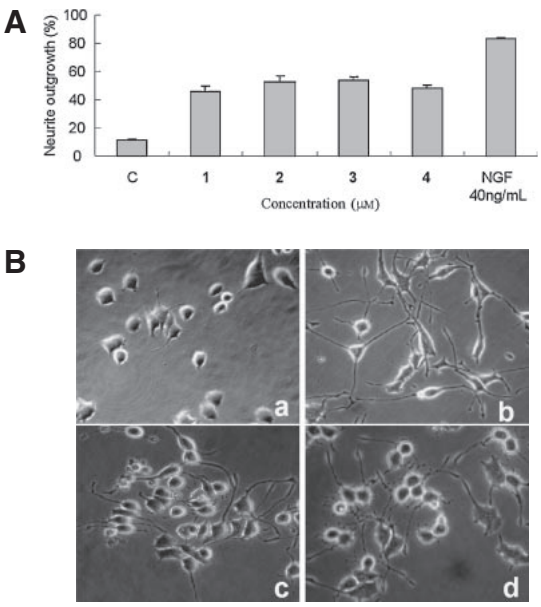

Fig. 2. Biological Activity of Compounds **1**–**4**.

A, Neuritogenic activity of compounds **1**–**4** 48 h after the treatment. The activity is presented as the percentage of PC12 cells with neurite outgrowth longer than the diameter of the cell body. c, control (0.5% DMSO); NGF (40 ng/mL), positive control; **1**–**4** at 0.3 μM. B, Images of the PC12 cells under a phase-contrast microscope 48 h after the treatment. a, solvent control (0.5% DMSO); b, NGF (40 ng/mL); c, **1** at 0.3 μM; d, **3** at 0.3 μM.

Acknowledgments

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14) Ophiopogonin D was obtained as amorphous solid, $[\alpha]_D^{25}$ −105.7 (*c* 0.45, pyridine) [lit $[\alpha]_D^{14}$ −107.9 (*c* 0.66, pyridine)]; ^{13}C NMR (125 MHz, in pyridine-d_5) δ: 139.5 (C-5), 124.7 (C-6), 109.2 (C-22), 106.6 (C-1‴), 101.7 (C-1″), 100.4 (C-1′), 85.4 (C-3′), 83.4 (C-1), 81.0 (C-16), 78.3 (C-3‴), 74.6 (C-4″), 74.2 (C-2′), 73.8 (C-2‴), 72.6 (C-3″), 72.5 (C-4′, 2″), 70.9 (C-5′), 70.7 (C-4‴), 69.3 (C-5″), 68.2 (C-3), 67.0 (C-5‴), 66.7 (C-26), 62.9 (C-17), 57.1 (C-14), 50.5 (C-9), 43.8 (C-4), 42.7 (C-10), 41.9 (C-20), 40.4 (C-13), 40.1 (C-12), 38.0 (C-2), 33.0 (C-8), 32.3 (C-7), 32.0 (C-15), 31.7 (C-23), 30.5 (C-25), 29.2 (C-24), 24.0 (C-11), 19.1 (Me-6″), 17.2 (Me-27), 17.0 (Me-6′), 16.8 (Me-18), 14.9 (Me-21), 14.8 (Me-19); ESI-MS *m/z*: 855 $[M+H]^+$. NMR chemical shifts in δ (ppm) were referenced to the solvent peaks of δ_C 123.4 for pyridine-d_5.
15) Compound **1** was obtained as amorphous solid, $[\alpha]_D^{25}$ −49.4 (*c* 0.22, MeOH); IR (KBr): 3425, 1727, 1592, 1055 cm^{-1}; HR FT-ICR MS *m/z* $[M+Na]^+$ Calcd. for $C_{49}H_{78}O_{18}Na$: 977.5080. Found: 977.5095.
16) Hydrolysis of **1** to give **2** and 2-hydroxy-3-methylpentanoate. Compound **1** (3 mg) in methanol (1 mL) was treated with potassium carbonate (4 mg) at room temperature for 24 h. The sample solution was evaporated and then the crude product was dissolved in H_2O (2 mL). The water solution was applied on an ODS (Cosmosil 75 C18-OPN, Nacalai Tesque), which was washed with H_2O (4 mL) and then eluated with a MeOH/H_2O in stepwise (50:50, 70:30, 90:10, 100:0) to afford the compound **2** (1.8 mg), which was eluted with MeOH/H_2O (90:10). Compound **2** was obtained as amorphous solid, $[\alpha]_D^{25}$ −53.8 (*c* 0.10, MeOH); IR (KBr): 3425, 1592, 1060 cm^{-1}; ESI-MS *m/z*: 863 $[M+Na]^+$. 2-Hydroxy-3-methylpentanoic acid (approx. 200 μg) was also eluted with MeOH/H_2O (90:10) (later than that of compound **2**) and obtained as colorless oil, $[\alpha]_D^{25}$ +18.4 (*c* 0.03, $CHCl_3$) [lit $[\alpha]_D^{26}$ +22.0 (*c* 0.1, $CHCl_3$)]; ^{1}H NMR ($CDCl_3$) δ: 4.19 (1H, d, $J = 3.5$ Hz, H-2), 1.91 (1H, m, H-3), 1.43 and 1.30 (2H, m, H-4), 1.03 (3H, d, $J = 7.0$ Hz, Me-6), 0.93 (3H, t, $J = 7.3$ Hz, Me-5); ESI-MS *m/z*: 155 $[M+Na]^+$. NMR chemical shifts in δ (ppm) were referenced to the solvent peaks of δ_H 7.26 for $CDCl_3$.
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20) Acid hydrolysis of **2** to give **3**. Compound **2** (1.4 mg) in anhydrous 2.0 M HCl in MeOH (1 mL) was heated at 80 °C for 10 h in a sealed tube. After being cooled down, the reaction solution was evaporated and partitioned between EtOAc and water. The EtOAc layer was dried up to afford the compound **3** (0.6 mg). Compound **3** was obtained as amorphous solid; ^{1}H NMR (500 MHz, in pyridine-d_5) δ: 5.60 (1H, d, $J = 5.5$ Hz, H-6), 4.53 (1H, q-like, $J = 7.5$ Hz, H-16), 3.96 (1H, m, H-3), 3.81 (1H, br d, $J = 11.5$ Hz, H-1), 3.56 (1H, dd, $J = 10.5$, 3.5 Hz, H-26b), 3.49 (1H, t, $J = 10.5$ Hz, H-26a), 1.33 (3H, s, Me-19), 1.09 (3H, d, $J = 7.0$ Hz, Me-21), 0.90 (3H, s, Me-18), 0.67 (3H, d, $J = 6.0$ Hz, Me-27); ESI-MS *m/z*: 431 $[M+H]^+$. ^{1}H NMR chemical shifts in δ (ppm) were referenced to the solvent peaks of δ_H 7.19 for pyridine-d_5.
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