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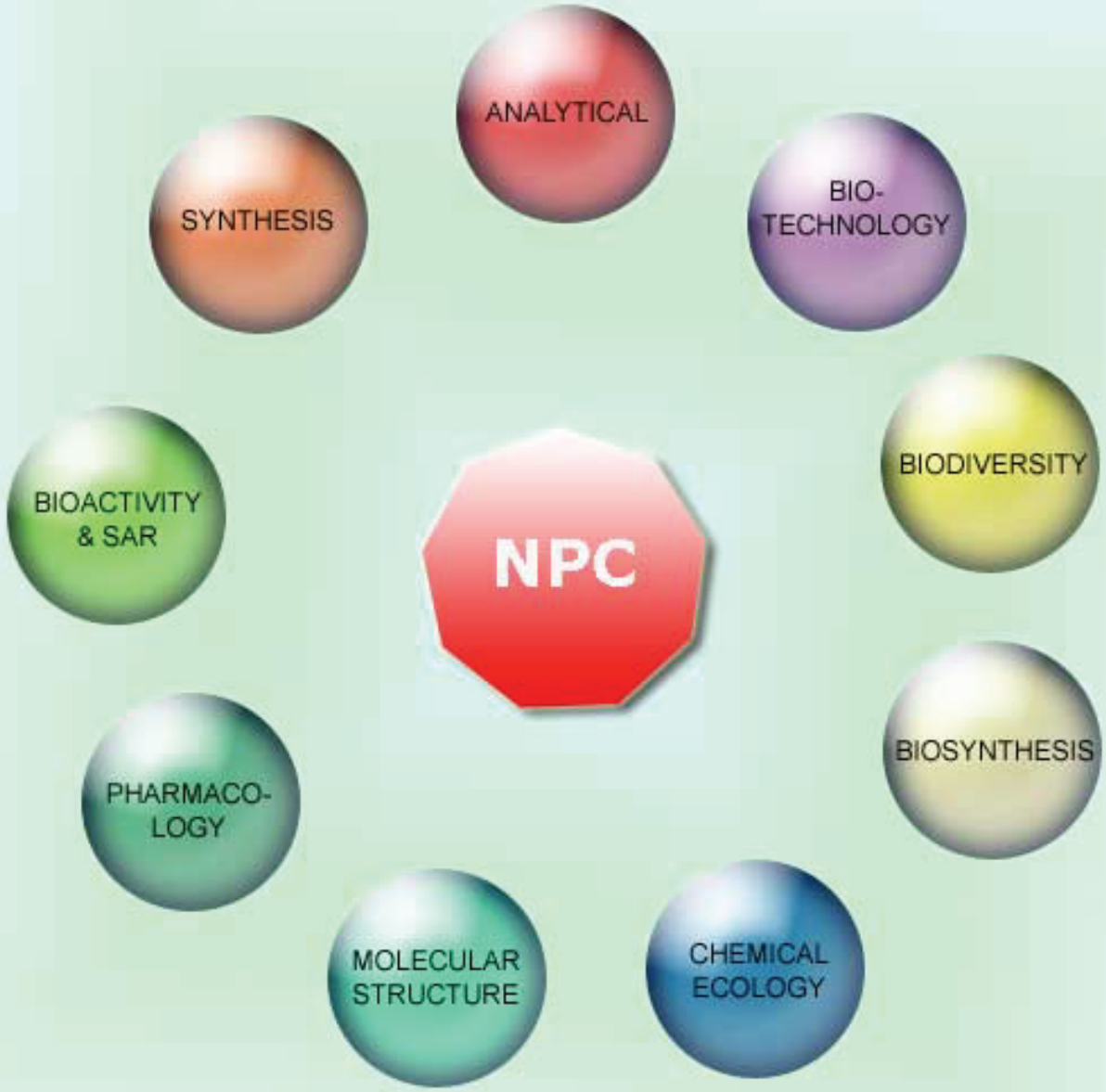

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New Steroidal Glycoside and Flavonoid Constituents from *Ophiopogon japonicus*

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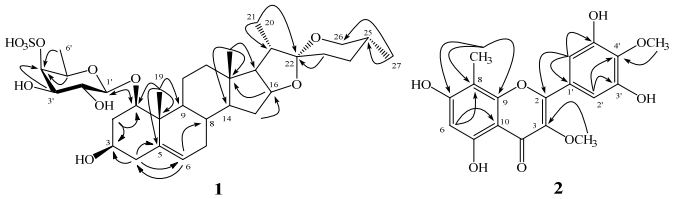
Chromatographic separations of a methanolic extract *of Ophiopogon japonicus* tubers resulted in the isolation of a new steroidal glycoside, (25*R*)-ruscogenin 1-*O*-(4-*O*-sulfo)-β-D-fucopyranoside (**1**), a new *C*-methylflavonol, 3,4′-dimethoxy-3′,5,5′,7-tetrahydroxy-8-methylflavone (**2**), and a known flavonol, 3,4′-dimethoxy-3′,5,5′,7-tetrahydroxyflavone (**3**). The *C*-methylflavonol was reported for the first time from the genus *Ophiopogon*. Compound **1** showed moderate cytotoxicity against SK-Mel-2 and KB cells with IC_{50} values of 24.3 and 28.8 μM, respectively. Compound **2** was active on SK-Mel-2 cells (IC_{50} 20.3 μM).

Keywords: *Ophiopogon japonicus*, Steroidal glycoside, *C*-Methylflavone, Cytotoxicity.

Ophiopogon japonicus (Thunb.) Ker-Gawl, an herbaceous plant in the family Convallariaceae, has a high medicinal value [1]. In our search for anticancer agents from natural sources, a methanol extract of the tubers of *O. japonicus* was found to possess cytotoxicity against human lung cancer cell lines (72.4 and 67.6% inhibition against KB and SK-Mel-2 cells at 30 μg/mL). In this paper, we describe the isolation and structural elucidation as well as the cytotoxicity of a new steroidal glycoside (**1**), a new *C*-methyl-flavone (**2**), and a known flavonoid (**3**) from *O. japonicus* tubers.

Compound **1** was obtained as white amorphous powder. Its HR-ESI-MS showed a molecular ion peak at *m/z* 657.3305 $[M + H]^+$ corresponding to the molecular formula $C_{33}H_{52}O_{11}S$. The IR spectrum of **1** displayed the S-O stretching band at 1228 cm^{-1} characteristic for the sulfate group [2]. The ^1H NMR spectrum of **1** exhibited two tertiary methyls at δ_H 0.81 (H_3-18) and 1.11 (H_3-19), and three secondary methyls at δ_H 1.01 (d, J = 7.0 Hz, H_3-21), 1.28 (d, J = 6.0 Hz, H_3-27), and 1.52 (d, J = 6.0 Hz, H_3-6′). In addition, an anomeric proton signal [δ_H 4.43 (d, J = 7.5 Hz)] and an olefinic proton signal [δ_H 5.55 (d, J = 5.0 Hz)] showed correlations in the HSQC spectrum with carbon signals at δ_C 99.7 (C-1′) and 125.7 (C-6), respectively. These NMR data, together with olefinic carbon signals at δ_C 139.9 (C-5), 125.7 (C-6) and a carbon signal at δ_C 111.0 (C-22) in the ^{13}C NMR spectrum suggested that **1** was a $\Delta^{5,6}$-spirostanol glycoside. The sugar part of **1** was determined to be a D-fucose on the basis of the NMR data and acid hydrolysis. The β-configuration of the fucopyranosyl unit was assigned based on the large coupling constant (J = 7.5 Hz) of the anomeric proton. The HMBC correlations (Figure 1) showed the coupling from H-1 (δ_H 3.40) to C-1′ (δ_C 99.7) and from H-1′ (δ_H 4.43) to C-1 (δ_C 84.8) indicating the location of sugar moiety at C-1. These NMR data of **1** were in good agreement with those of (25*R*)-ruscogenin 1-*O*-β-D-fucopyranoside [3], except for the difference at C-4′ of the fucopyranosyl unit. The downfield chemical shift at C-4′ of **1** (δ_C 79.6) suggested that a sulfate group attached to C-4′ [4]. With the above evidence, compound **1** was elucidated as (25*R*)-ruscogenin 1-*O*-(4-*O*-sulfo)-β-D-fucopyranoside.



1 **2**

Figure 1: Structures and key HMBC correlations of **1** and **2**.

Compound **2** was obtained as light-yellow amorphous powder. The HR-ESI-MS showed a molecular ion at *m/z* 361.0940 $[M + H]^+$ corresponding to the molecular formula $C_{18}H_{16}O_8$. The ^1H NMR spectrum of **2** showed two-proton aromatic singlet at δ_H 7.27 (2H, br s, H-2′, 6′), an aromatic singlet at δ_H 6.22 (1H, s, H-6), an aromatic methyl at δ_H 2.26 (3H, br s, 8-CH_3), and two methoxy groups (δ_H 3.81 and 3.91). The ^{13}C NMR and HSQC-DEPT spectra of **2** revealed a methyl, two methoxyl, two aromatic methine and ten quaternary carbon signals. An α,β-unsaturated carbonyl system at δ_C 156.5 (C-2), 139.9 (C-3) and 179.9 (C-4) suggested the flavonol skeleton. The high intensity of the resonances at δ_C 109.0 (C-2′, 6′) and 152.0 (C-3′, 5′) indicated a 1,3,4,5- tetrasubstituted B-ring. The above data was similar to that of 3,4′-dimethoxy-3′,5,5′,7-tetrahydroxyflavone (**3**) [5], except for the presence of an aromatic proton singlet and a methyl singlet instead of the two aromatic proton doublets. The HMBC correlations (Figure 1) from H-6 (δ_H 6.22) to δ_C 160.2 (C-5), 167.5 (C-7), 104.1 (C-8), and 104.9 (C-10), and from the methyl protons (δ_H 2.26) to δ_C 167.5 (C-7), 104.1 (C-8), and 156.5 (C-9) confirmed that the methyl group was located at C-8. In addition, the HMBC correlations from the methoxy signals at δ_H 3.91 and 3.81 to δ_C 139.3 (C-3) and 139.9 (C-4′), respectively, indicated that two methoxy groups were attached to the C-3 and C-4′. Thus, **2** was determined to be 3,4′-dimethoxy-3′,5,5′,7-tetrahydroxy-8-methylflavone, a new compound from nature. Although a number of *C*-methylhomoisoflavonoids have been isolated from *Ophiopogon* sp. [1], the present study reported for the first time the presence of *C*-methylflavonol in this genus.

The cytotoxicity of the isolated compounds was evaluated against human melanoma (SK-Mel-2), human epidermoid cancer (KB), and human lung cancer (LU-1) cell lines. Among the tested compounds, the *C*-methylflavonol (**2**) showed the strongest effect against SK-Mel-2 cells (IC_{50} 20.3 μM). The saponin (**1**) showed moderate cytotoxicity against SK-Mel-2 and KB cells with IC_{50} values of 24.3 and 28.8 μM, respectively. Compound **1** and **2** were not toxic to LU-1 cells, while **3** was inactive against all three cell lines.

Experimental

General experimental procedures: Optical rotation, JASCO P-2000 polarimeter; IR, Tensor 37 FT-IR spectrometer; NMR, Bruker AM500 FT-NMR; HR-ESI-MS, API Q-STAR PULSAR I of Applied Biosystem.

Plant materials: *Ophiopogon japonicus* were collected at Me Linh, Hanoi in Feb. 2014. The sample was identified by Prof. Tran Huy Thai, Institute of Ecology and Biological Resources, VAST. A voucher specimen was deposited at the Department of Bioactive Products, IMBC, VAST.

Extraction and isolation: The air-dried and powdered tubers of *O. japonicus* (2.4 kg) were extracted with methanol (4L × 3 times) at 40 °C in a sonic bath for 30 min. The combined extracts were concentrated under vacuum to obtain a crude residue (360 g), which was then partitioned in water and chloroform. The water layer was passed through a Diaion HP-20 column and eluted with a gradient system of MeOH–H_2O (0:100, 25:75, 100: 0, v/v) to give three fractions (W-1 to W-3). Fraction W-3 was chromatographed on silica gel column eluted with a gradient of 1-100% MeOH in $CHCl_3$ to give nine fractions (W-3.1 to W-3.9). Compound **1** (365.7 mg) was obtained by purifying fraction W-3.6 on a Sephadex LH-20 column using a solvent system of MeOH–H_2O (1:1). The fraction W-3.4 was subjected to a RP-18 column using a mobile phase of MeOH–H_2O (1:1) to afford six subfractions W-3.4.1–W-3.4.6. Compound **2** (35.5 mg) were isolated from W-3.4.5 by using a Sephadex LH-20 column (MeOH–H_2O 1:1). A silica gel column eluted with $CHCl_3$–MeOH (15:1) was applied to subfraction W-3.4.4 to obtain compound **3** (27.8 mg).

(25*R*)-ruscogenin 1-*O*-(4-*O*-sulfo)-β-D-fucopyranoside (**1**)
R_f: 0.30 (EtOAc-MeOH, 5:1).
$[\alpha]_D^{25}$: – 37.5 (*c* 0.1, MeOH).
IR λ_{max} (KBr): 3467, 1646, 1228, 1066, 1006 cm^{-1}.
^{1}H NMR (500 MHz, CD_3OD): δ 0.81 (3H, br s, H-18), 1.01 (3H, d, *J* = 7.0 Hz, H-21), 1.11 (3H, br s, H-19), 1.16 (1H, m, H-14), 1.28 (3H, d, *J* = 6.0 Hz, H-27), 1.52 (3H, d, *J* = 6.0 Hz, H-6′), 1.58 (1H, m, H-8), 1.71 (1H, m, H-25), 1.72 (1H, m, H-17), 1.88 (1H, m, H-20), 3.29 (1H, d, *J* = 11.0 Hz, H-26a), 3.36 (1H, m, H-3), 3.40 (1H, dd, *J* = 11.0, 4.5 Hz, H-1), 3.61 (1H, m, H-5′), 3.68 (1H, m, H-2′), 3.95 (1H, dd, *J* = 2.5, 6.0 Hz, H-26b), 4.28 (1H, dd, *J* = 8.0, 9.5 Hz, H-4′), 4.40 (1H, m, H-16), 4.43 (1H, d, *J* = 7.5 Hz, H-1′), 5.56 (1H, d, *J* = 5.5 Hz, H-6).
^{13}C NMR (125 MHz, CD_3OD): δ 84.8 (C-1), 37.3 (C-2), 69.1 (C-3), 43.6 (C-4), 139.9 (C-5), 125.7 (C-6), 32.8 (C-7), 34.0 (C-8), 51.8 (C-9), 43.3 (C-10), 24.4 (C-11), 41.3 (C-12), 41.0 (C-13), 58.0 (C-14), 32.9 (C15), 82.3 (C-16), 63.8 (C-17), 17.1 (C-18), 14.9 (19), 43.2 (20), 14.7 (21), 111.0 (22), 26.9 (C23), 26.7 (C-24), 28.4 (C-25), 66.0 (C-26), 16.4 (C-27), 99.7 (C-1′), 72.6 (C-2′), 74.9 (C-3′), 79.6 (C-4′), 71.3 (C-5′), 16.8 (C-6′).
HR-ESI-MS: *m/z* $[M+H]^+$ calcd. for $C_{33}H_{53}O_{11}S$: 657.3309; found: 657.3305.
365.7 mg (1.52×10^{-4}% of dried weight).

3,4′-dimethoxy-3′,5,5′,7-tetrahydroxy-8-methylflavone (**2**)
R_f: 0.40 (CH_2Cl_2-MeOH, 15:1).
IR λ_{max} (KBr): 3450, 1653, 1596, 1380, 1360 cm^{-1}.
^{1}H NMR (500 MHz, CD_3OD): δ 7.27 (2H, s, H-2′, 6′), 6.22 (1H, s, H-6), 3.91 (3H, br s, 3-OMe) 3.81 (3H, br s, 4′-OMe), 2.26 (3H, br s, 8-Me).
^{13}C NMR (125 MHz, CD_3OD): 155.9 (C-2), 139.3 (C-3), 179.9 (C-4), 160.2 (C-5), 100.4 (C-6), 167.5 (C-7), 104.1 (C-8), 156.5 (C-9), 104.9 (C-10), 127.4 (C-1′), 109.1 (C-2′, 6′), 152.0 (C-3′, 5′), 139.9 (C-4′), 7.93(8-Me), 60.8 (3-OMe), 60.6 (4′-OMe).
HR-ESI-MS: *m/z* $[M+H]^+$ calcd. for $C_{18}H_{17}O_8$: 361.0923; found: 361.0940.
35.5 mg (1.48×10^{-5}% of dried weight).

Acid hydrolysis: Compound **1** (5 mg) was heated in 2N HCl (2 mL) at 80 °C for 2 h, then the solution was extracted with ethyl acetate (1 mL × 3). The aglycone was identified as (25*R*)-ruscogenin by its negative optical rotation and similar R_f with an authentic standard in thin layer chromatography (TLC). The sugar product in the aqueous layer was identified as fucose (R_f 0.60) in comparison with the standard by silica gel thin layer chromatography (developed with ethyl acetate-isopropanol-water 3:3:2 and sprayed by 10% sulfuric acid solution containing 2% vanillin). Preparative TLC followed by checking optical rotation led to the assignment of D-fucose (positive value in water).

Cytotoxic assay: The cytotoxicity of the isolated compounds against SK-Mel-2, KB, and LU-1 cell lines was determined as previously reported [6]. Ellipticine was used as a positive control (IC_{50} 0.27, 0.51 and 0.43 μM, respectively).

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