Cytotoxic Homoisoflavonoids from Ophiopogon japonicus Tubers

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A phytochemical fractionation of a methanol extract of Ophiopogon japonicus tubers led to the isolation of a new homoisoflavonane, homoisopogon A (1), and three new homoisoflavanes, homoisopogon B–D (2–4). Their chemical structures were elucidated by mass, NMR, and circular dichroism (CD) spectroscopic methods. Homoisopogon A (1) exhibited potent cytotoxicity against human lung adenocarcinoma LU-1, human epidermoid carcinoma KB, and human melanoma SK-Mel-2 cancer cells with IC50 values ranging from 0.51 to 0.66 μM.

Key words Ophiopogon japonicus; homoisoflavonane; homoisoflavane; homoisopogon; cytotoxicity

Ophiopogon japonicus (L.f) Ker-Gawl. (Convallariaceae) is distributed widely in Southeast Asia. In traditional Vietnamese medicine, it has been used widely as a tonic agent for the treatment of cough, fever, epistaxis, inflammation, respiratory disease, constipation, and gastrointestinal disorders.1) Previous phytochemical investigations have revealed that O. japonicus contains steroidal saponins, homoisoflavonoids, polysaccharides, phenolic acids, and sesquiterpenes.2,3) Anti-inflammatory, antitumor, antidiabetic, anti-oxidant activities of homoisoflavonoids have been reported.2) Homoisoflavonoids belong to a special flavonoid class which their B-ring connected to C-ring by an additional CH2 group. Homoisoflavonoids isolated from Ophiopogon have been shown to exert various biological activities including protective effects on cerebral ischemia/reperfusion (I/R) injury, antioxidant, anti-inflammation and cytotoxic activities.4–8)

In the present study, a methanol extract of O. japonicus tubers showed strong cytotoxicity against human lung cancer cell lines. Phytochemical fractionation of the methanol extract of O. japonicus tubers led to the isolation of a homoisoflavonane (I), and three homoisoflavanes (2–4) (Fig. 1). The cytotoxic activity against human lung adenocarcinoma LU-1, human epidermoid carcinoma KB, and human melanoma SK-Mel-2 cancer cells of the isolated compounds was investigated.

Results and Discussion

The high resolution-electrospray ionization (HR-ESI)-MS spectrum of compound 1 had a molecular ion peak at m/z 345.1329 [M+H]+, corresponding to the molecular formula C19H20O6. Its 1H-NMR spectrum showed the characteristic resonances of a homoisoflavonane at δH 11.84 (1H, s, OH-5), 4.16 (1H, dd, J=10.0, 11.0 Hz, Ha-2), 4.43 (1H, dd, J=5.0, 11.0 Hz, Hb-2), 3.06 (1H, m, H-3), 2.82 (1H, dd, J=6.0, 14.0 Hz, Ha-9), and 3.00 (1H, dd, J=5.0, 14.0 Hz, Hb-9) (Table 1). Moreover, the 2’-OH and 4’-OCH3 substitution patterns in the B-ring were deduced from the ABX type aromatic proton resonances at δH 6.46 (1H, d, J=2.5 Hz, H-3’), 6.41 (1H, dd, J=2.5, 8.0 Hz, H-5’), 6.94 (1H, d, J=8.0 Hz, H-6’). In the 13C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra, one methyl, two methoxys, two methenes, one aliphatic methine, four aromatic methines, eight aromatic quaternary carbons and a carbonyl group and were observed. The heteronuclear single quantum coherence (HSQC) spectrum of 1 established the connectivity between the carbons and respective protons, which confirmed the presence of twelve direct H–C correlations corresponding to twelve proton signals of one methyl, two methoxys, two methenes, one aliphatic methine, four aromatic methines, eight aromatic quaternary carbons and a carbonyl group and were observed. The heteronuclear multiple bond connectivity (HMBC) correlations with C-4a, C-6, C-7, and C-8a. The HMBC correlations from methyl protons (δH 1.97) to C-5, C-6, and C-7 indicated the presence of methyl group at C-6. The ABX system of B-ring was confirmed by the HMBC correlations from H-6’ to C-2’, C-4’, from H-3’ to C-1’, C-5’, and from H-5’ to C-1’, C-3’. Furthermore, the coupling from H-9 to C-2, C-4, C-1’, C-2’, and from H-6’ to C-9 indicated that the
B-ring connected to C-ring via a methylene bridge. Additionally, the location of two methoxyl groups (δ_H 3.83 and 3.75) at C-7 and C-4 was deduced from the HMBC correlations (Fig. 2). The absolute configuration of C-3 was determined to be R in accordance with the positive Cotton effect at 290–294 nm in the circular dichroism (CD) spectra. Finally, the structure of compound 1 was elucidated as (3R)-2,5-dihydroxy-4,7-dimethoxy-6-methylhomoisoflavanone; named homoisopogon A.

**Compound 2** was obtained as a yellow powder with the molecular formula C_{19}H_{22}O_{4}, which was established from the HR-ESI-MS data (m/z 315.1602 [M+H]+). The 1H-NMR spectrum showed characteristic resonances at δ_H 4.06 (1H, dd, J=2.0, 11.0 Hz) and 3.83 (1H, dd, J=6.5, 11.0 Hz) corresponding to H-2 protons, δ_H 2.25 (1H, m) corresponding to H-3, δ_H 2.44 (1H, dd, J=5.5, 16.0 Hz) and 2.50 (1H, dd, J=6.5, 16.0 Hz) corresponding to H-4 protons, and δ_H 6.76 (1H, d, J=8.5 Hz) and 6.92 (1H, d, J=2.8, 11.0 Hz) corresponding to H-9 protons. The 1H-NMR spectrum also showed signals at δ_H 6.38 (1H, d, J=2.5 Hz, H-3), 6.40 (1H, d, J=2.5, 8.0 Hz, H-5), and 6.98 (1H, d, J=8.0 Hz, H-6) suggesting a 1,2,4-trisubstituted pattern for the B ring. Additionally, two aromatic singlet protons at δ_H 6.76 (1H, s, H-5) and 6.34 (1H, s, H-8) were detected, indicating the presence of a tetrasubstituted A ring. In the 13C and DEPT spectra, one methyl, two methoxyls, three methylenes, one aliphatic methine, five aromatic methines, and seven aromatic quaternary carbons were observed. These data suggested that 2 possesses a homoisoflavane skeleton.

The HMBC correlations from the aromatic methyl group at δ_H 2.10 (3H, s) to C-5 (δ_C 131.4), C-6 (δ_C 119.1), and C-7 (δ_C 156.7), and from the methoxy signals δ_H 3.74 to C-7, and δ_H 3.73 to C-4 (δ_C 159.3), indicated that the methyl and methoxyl groups attached to C-6, C-7, and C-4, respectively. The absolute configuration of C-3 was determined to be R based on the Cotton effect at 230 nm (negative) and 285 nm (positive) in the CD analysis. Accordingly, the structure of 2 was elucidated as (3R)-4,7-dimethoxy-2-hydroxy-6-methylhomoisoflavanone, named homoisopogon B.

**Compound 3** was obtained as a yellow powder with the molecular formula C_{19}H_{22}O_{4}, which was established from the HR-ESI-MS data (m/z 315.1602 [M+H]^+). The 1H-NMR spectrum showed characteristic resonances at δ_H 4.06 (1H, dd, J=2.0, 11.0 Hz) and 3.83 (1H, dd, J=6.5, 11.0 Hz) corresponding to H-2 protons, δ_H 2.25 (1H, m) corresponding to H-3, δ_H 2.44 (1H, dd, J=5.5, 16.0 Hz) and 2.50 (1H, dd, J=6.5, 16.0 Hz) corresponding to H-4 protons, and δ_H 6.76 (1H, d, J=8.5 Hz) and 6.92 (1H, d, J=2.8, 11.0 Hz) corresponding to H-9 protons. The 1H-NMR spectrum also showed signals at δ_H 6.38 (1H, d, J=2.5 Hz, H-3), 6.40 (1H, d, J=2.5, 8.0 Hz, H-5), and 6.98 (1H, d, J=8.0 Hz, H-6) suggesting a 1,2,4-trisubstituted pattern for the B ring. Additionally, two aromatic singlet protons at δ_H 6.76 (1H, s, H-5) and 6.34 (1H, s, H-8) were detected, indicating the presence of a tetrasubstituted A ring. In the 13C and DEPT spectra, one methyl, two methoxyls, three methylenes, one aliphatic methine, five aromatic methines, and seven aromatic quaternary carbons were observed. These data suggested that 2 possesses a homoisoflavane skeleton.

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**Compound 4** was obtained as a yellow powder with the molecular formula C_{19}H_{22}O_{4}, which was established from the HR-ESI-MS data (m/z 315.1602 [M+H]^+). The 1H-NMR spectrum showed characteristic resonances at δ_H 4.06 (1H, dd, J=2.0, 11.0 Hz) and 3.83 (1H, dd, J=6.5, 11.0 Hz) corresponding to H-2 protons, δ_H 2.25 (1H, m) corresponding to H-3, δ_H 2.44 (1H, dd, J=5.5, 16.0 Hz) and 2.50 (1H, dd, J=6.5, 16.0 Hz) corresponding to H-4 protons, and δ_H 6.76 (1H, d, J=8.5 Hz) and 6.92 (1H, d, J=2.8, 11.0 Hz) corresponding to H-9 protons. The 1H-NMR spectrum also showed signals at δ_H 6.38 (1H, d, J=2.5 Hz, H-3), 6.40 (1H, d, J=2.5, 8.0 Hz, H-5), and 6.98 (1H, d, J=8.0 Hz, H-6) suggesting a 1,2,4-trisubstituted pattern for the B ring. Additionally, two aromatic singlet protons at δ_H 6.76 (1H, s, H-5) and 6.34 (1H, s, H-8) were detected, indicating the presence of a tetrasubstituted A ring. In the 13C and DEPT spectra, one methyl, two methoxyls, three methylenes, one aliphatic methine, five aromatic methines, and seven aromatic quaternary carbons were observed. These data suggested that 2 possesses a homoisoflavane skeleton.
corresponding to the molecular formula C_{18}H_{18}O_{4}. The absolute configuration of C-3 was determined to be R based on the CD analysis.\(^{11}\) Thus, the structure of 3 was established as (3R)-4’7-dimethoxy-2’-hydroxyhomoisoflavane, named homoisopogon C.

Compound 4 was formulated as C_{18}H_{18}O_{4} based on HR-ESI-MS (m/z 299.1271 [M+H]+). The \(^1H\) and \(^13C\)-NMR data for 4 were similar to those for 2 except for the presence of a methylenedioxy group [\(\delta_H 5.93 \ (2H, s)\)] instead of two methoxy groups. Also, the HMBC correlations of H-2’/C-9, C-4’, and C-6’, and H-5’/C-1’, C-3’, and C-4’, as well as H-6’/C-9, C-2’, and C-4’, indicated the presence of a 1,3,4-trisubstituted benzene ring in 6, instead of the 1,2,4-trisubstituted benzene ring in 2. The position of the methylenedioxy group at C-3’-C-4’ was confirmed by HMBC correlations (Fig. 2). The absolute configuration of C-3 was determined to be R based on the CD analysis.\(^{11}\) Accordingly, compound 4 was elucidated as (3R)-7-hydroxy-3’,4’-methylenedioxy-6-methoxyisoflavane, named homoisopogon D.

Compounds 1–4 were evaluated for their cytotoxic effect against LU-1, KB, and SK-Mel-2 cells. As indicated in Table 3, homoisopogon A (1) was the most potent, followed by homoisopogon B (2) and homoisopogon C (3). Interestingly, compound 1 exhibited a strong cytotoxic effect on all tested cell lines with the IC\(_{50}\) values of 0.51–0.66 \(\mu\)M. The activity is comparable to that of the positive control, ellipticine (Table 3). Compound 1 was probably responsible for the cytotoxic effect observed in the MeOH extract since other isolated components showed weak or null effect. The cytotoxic effect of homoisoflavonoids has been indicated elsewhere,\(^2\) and the structure–activity relationship has been investigated\(^{13}\). Accordingly, the 2’-hydroxy and 4’-methoxy groups seem to have a contribution to the activity. In our study, compounds 1, 2, and 3 possessing 2’-hydroxy and 4’-methoxy substituent showed positive effect on at least one cancer cell line. Compound 4 with a methylenedioxy group at C-3’-C-4’ and lack of hydroxyl group at C-2’, was inactive against all tested cells.

In conclusion, four new homoisoflavonoids including one homoisoflavonane (1) and three homoisoflavanes (2, 3, and 4) were isolated from tubers of Ophiopogon japonicus Ker-Gawler. In a cytotoxic activity investigation, compound 1 exhibited potent effect against three cancer cells LU-1, KB, and SK-Mel-2. The results indicated that homoisoflavonoids from O. japonicus may be a potential material for the development of anticancer agents.

### Experimental

**General Experimental Procedures** TLC was performed using precoated Kiesel gel 60 (Merck, 70–230 mesh). Optical rotation was recorded on a JASCO P-2000 digital polarimeter. The IR spectra were obtained from a Tensor 37 FT-IR spectrometer (Bruker, Ettlingen, Germany). CD spectra were obtained with a JASCO J-1100 spectropolarimeter. NMR experiments were carried out on a Bruker AM500 FT-NMR spectrometer (Bruker, Rheinstetten, Germany) using tetramethylsilane (TMS) as internal standard. The HR-ESI-MS were recorded on a Waters Q-TOF micromass spectrometer Waters Q-TOF micromass spectrometer. Absorbance of bioassay solutions was read on an xMark microplate spectrophotometer.

**Plant Materials** The tubers of O. japonicus were collected in Feb. 2014 at Me Linh, Hanoi and identified by Prof. Tran Huy Thai, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The voucher specimens were deposited at the Department of Bioactive Products, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology.

**Extraction and Isolation** The air-dried and powdered tubers of O. japonicus (2.4 kg) were extracted with methanol (4L×3 times) in a sonic bath for 30min at 40°C. The combined extracts were concentrated under a vacuum to obtain a crude residue (360 g), which was then resuspended in water (2L), and extracted by chloroform (1L×3 times) to obtain chloroform (8 g) and water residues. The chloroform residue was chromatographed on a silica gel column eluted with a gradient of 1–100% ethyl acetate in hexane to afford nine fractions F1–F9. Fraction F3 was fractionated on a silica gel column eluted with hexane–ethyl acetate (9:1, v/v) to give four fractions F3.1–F3.3. Compound 2 (125.6 mg) and 4 (46.3 mg) were isolated from F3.1 by using a reverse phase C\(_{18}\) column eluted with acetone–water (1:1, v/v). The fraction F3.2 was passed through a C\(_{18}\) column (acetone–water 3:2, v/v) to obtain 3 (31.5 mg). Compound 1 (40.4 mg) was purified from F3.3 by using a reverse phase C\(_{18}\) column (acetone–water 3:2, v/v) and a silica gel column (hexane–ethyl acetate 7:1,
v/v).

Homoisopogon A (1)
Yellow amorphous powder; $[\alpha]_D^{25} = -25.0$ (c = 0.1, MeOH); CD (c = 0.29 mm, MeOH) $\lambda_{max} (\Delta\varepsilon)$ nm 290 (+16.8); IR $\nu_{max}$ (KBr): 3368, 2925, 1644, 1588, 1280 cm$^{-1}$; $^1$H-NMR (500 MHz, CDCl$_3$) and $^{13}$C-NMR (125 MHz, CDCl$_3$); see Tables 1 and 2; HR-ESI-MS: m/z 345.1329 [M+H]$^+$ (Calcd for C$_{19}$H$_{21}$O$_6$ 345.1338).

Homoisopogon B (2)
Yellow amorphous powder; $[\alpha]_D^{25} = +18.0$ (c = 0.1, MeOH); CD (c = 0.31 mm, MeOH) $\lambda_{max} (\Delta\varepsilon)$ nm 230 (–8.8), 285 (+10.4); IR $\nu_{max}$ (KBr): 3393, 1620, 1509, 1190 cm$^{-1}$; $^1$H-NMR (500 MHz, CDCl$_3$) and $^{13}$C-NMR (125 MHz, CDCl$_3$); see Tables 1 and 2; HR-ESI-MS: m/z 315.1602 [M+H]$^+$ (Calcd for C$_{18}$H$_{21}$O$_3$ 315.1596).

Homoisopogon C (3)
Yellow amorphous powder; $[\alpha]_D^{25} = +23.0$ (c = 0.1, MeOH); CD (c = 0.33 mm, MeOH) $\lambda_{max} (\Delta\varepsilon)$ nm 230 (–12.1), 285 (+8.4); IR $\nu_{max}$ (KBr): 3430, 1620, 1508, 1159 cm$^{-1}$; $^1$H-NMR (500 MHz, CDCl$_3$) and $^{13}$C-NMR (125 MHz, CDCl$_3$); see Tables 1 and 2; HR-ESI-MS: m/z 301.1425 [M+H]$^+$ (Calcd for C$_{19}$H$_{21}$O$_4$ 301.1440).

Homoisopogon D (4)
Yellow amorphous powder; $[\alpha]_D^{25} = +12.5$ (c = 0.1, MeOH); CD (c = 0.33 mm, MeOH) $\lambda_{max} (\Delta\varepsilon)$ nm 290 (+9.1); IR $\nu_{max}$ (KBr): 1625, 1501, 1242 cm$^{-1}$; $^1$H-NMR (500 MHz, CDCl$_3$) and $^{13}$C-NMR (125 MHz, CDCl$_3$); see Tables 1 and 2; HR-ESI-MS: m/z 299.1271 [M+H]$^+$ (Calcd for C$_{19}$H$_{21}$O$_4$ 299.1283).

Cell Culture and Cytotoxic Assay
KB (human epidermoid carcinoma), LU-1 (human lung adenocarcinoma), and SK-Mel-2 (human melanoma) cell lines, obtained from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.), were maintained as monolayers in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Grant Island, NY, U.S.A.), sodium bicarbonate, penicillin G, and streptomycin at 37°C under a humidified atmosphere of 5% CO$_2$. The cytotoxicity of the isolated compounds was determined using the sulforhodamine B (SRB) method as previously reported. 14

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Conflict of Interest
The authors declare no conflict of interest.

Supplementary Materials
The online version of this article contains supplementary materials (spectroscopic data for compounds including 1D-, 2D-NMR and CD spectra).

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