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A New Stilbene Glucoside from the Roots of *Polygonum multiflorum* Thunb.

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One new stilbene glucoside (**6**), along with five known compounds (**1-5**), were isolated from the roots of *Polygonum multiflorum* Thumb., and their chemical structures established based on physicochemical and spectroscopic data. Of the compounds, compound **3** showed DNA topoisomerase I and II inhibitory activities.

Key words: *Polygonum multiflorum*, NMR, Stilbene glucoside

INTRODUCTION

Polygonum multiflorum Thumb, a perennial vine-like herb of the family Polygonaceae, is one of the most important and widely used Chinese medicinal herbs. The vine and root tubers are called *Ya-Gyo* and *Ha-soo-Oh*, respectively, and are ethnopharmacologically a tonic for anemia, neurasthenia and hypercholesterolemia, and used clinically for the treatment of coronary heart disease, hyperlipidemedia and neurosis, as well as other diseases commonly associated with aging (Xiao *et al.*, 1993). The genus *Polygonum* is the source of a wide range of phenolic compounds, flavanoids anthraquinones, stilbenes and tannins (Lin *et al.*, 2003).

As a part of our continuing search for novel topoisomerases inhibitors from natural plants, the activity-guided fractionation and purification of active compounds were sought. This paper reports the isolation and structural elucidation of a new stilbene glucoside (**6**) and five known compounds (**1-5**) from the roots of *P. multiflorum*.

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MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured using a JASCO DIP-1000 (Tokyo, Japan) automatic digital polarimeter. FT-IR spectra were recorded on a JASCO FT-IR 300E spectrophotometer, and UV spectra were recorded on a JASCO V-550 spectrophotometer. The NMR spectra were recorded on a Bruker 250MHz (DMX 250) spectrometer, using the Bruker standard pulse program. Samples were dissolved in methanol-d_4 or pyridine-d_5, and the chemical shifts reported in ppm downfield from TMS. The MS spectra were measured using a VG TRIO 2A mass spectrometer. The stationary phases for column chromatography (Silica gel 60, 70-230 and 270-400 mesh and Lichroprep RP-18 gel, 40-63 μm, Merck) and TLC plates (Silical gel 60 F_{254} and RP-18 F_{254}, Merck) were purchased from Merck KGaA (Darmstadt, Germany). Spots were detected under UV radiation and by spraying with 10% H_2SO_4, followed by heating. All other chemicals and solvents were of analytical grade and used without further purification.

Plant material

Dried roots of *P. multiflorum* were purchased from a folk medicine market "Yakryong-si" in Daegu, Republic of Korea in September 2001. These materials were taxonomically confirmed by Professor Ki-Hwan Bae, Chungnam National

University, Daejeon, Republic of Korea. A voucher specimen (YNPM 2001) has been deposited at the College of Pharmacy, Yeungnam University.

Extraction and isolation

The roots of *P. multiflorum* (10 kg) were extracted twice with MeOH, by refluxing for 12 h, with the MeOH solution obtained evaporated to dryness (2.4 kg). The MeOH extract was suspended in H_2O, partitioned successively with CH_2Cl_2 and EtOAc, with the resulting CH_2Cl_2 and EtOAc solutions evaporated to dryness *in vacuo*. The CH_2Cl_2 extract (60 g) was loaded onto a silica-gel column (60 × 9 cm, 70-230 mesh), and eluted with two stepwise-gradient solvent mixtures; *n*-hexane-EtOAc (from *n*-hexane 100% to EtOAc 100%) followed by EtOAc-MeOH (from EtOAc 100% to MeOH 100%). The eluent was combined based on the results of TLC, giving 15 fractions (PMM1~15). The fraction PMM-3 was subjected to chromatography on a silica-gel column (4 × 60 cm), and eluted with *n*-hexane-EtOAc (gradient from 100% *n*-hexane to 100% EtOAc), to give compound **1** (21 mg). Fraction PMM-8 was subjected to chromatography on silica-gel column (4 × 60 cm), and eluted with CH_2Cl_2-MeOH (gradient from 100% CH_2Cl_2 to 100% MeOH), to give compounds **3** (15 mg) and **4** (16 mg).

The EtOAc extract (85.7 g) was loaded onto a silica-gel column (60 × 9 cm, 70-230 mesh), and eluted with CH_2Cl_2-MeOH saturated with H_2O (gradient from CH_2Cl_2 100% to MeOH 100 %), yielding 30 fractions (PME1~30). Fraction PME-17 was obtained as a pure solid (compound **2**, 13 mg). Fraction PME-12 (1.3 g) was subjected to chromatography on a RP-18 column (4 × 60 cm), and eluted with MeOH-H_2O (gradient from 10% to 100% MeOH in H_2O), to give 19 subfractions (PME12-1~12-19). Subfractions PME12-2 and PME12-3 were further purified, separately, on a reverse column (4 × 60 cm) with MeOH-H_2O (gradient from 10% to 100% MeOH in H_2O) elution, which afforded compounds **5** (67 mg) and **6** (18 mg), respectively.

Physcion (1)

Yellow needles, mp 203-205°C; the ^{1}H- and ^{13}C-NMR data were consistent with the literature values (Qin *et al.*, 1999); positive FAB-MS *m/z* 285 $[M+H]^+$.

Physcion-8-*O*-β-D-glucopyranoside (2)

Yellow needles, mp 230-232°C; the ^{1}H- and ^{13}C-NMR data were consistent with the literature values (Takeshi and Yutaka, 1987); EI-MS *m/z*: 284 $(M\text{-glc})^+$.

β-Sitosterol (3)

White crystals, mp. 277-278°C, $[\alpha]_D^{25}$ -34.1° (*c* 0.2, $CHCl_3$), the ^{1}H- and ^{13}C-NMR data were consistent with the literature values (Chang *et al.*, 1981), Negative FAB-MS *m/z* 413 $[M-H]^-$ (Kim *et al.*, 2005).

β-Sitosterol-3-*O*-β-D-glucoside (4)

White amorphous powder, $[\alpha]_D^{25}$ -40.7° (*c* 0.1, $CHCl_3$); the ^{1}H- and ^{13}C-NMR data were consistent with the literature values (Chang *et al.*, 1981), positive FAB-MS *m/z* 577 $[M+H]^+$.

(*E*)-2,3,5,4'-Tetrahydroxy-stilbene-2-*O*-β-D-glucopyranoside (5)

Brown powder; $[\alpha]_D^{24}$ +22.35° (*c*, 0.32, MeOH); ^{1}H-NMR (CD_3OD, 250 MHz) δ 7.73 (1H, d, *J* = 16.5 Hz H-b), 7.46 (2H, d, *J* = 8.6 Hz, H-2', 6'), 6.93 (1H, d, *J* = 16.5 Hz H-a), 6.79 (2H, d, *J* = 8.6 Hz H-3', 5'), 6.65 (1H, d, *J* = 2.8 Hz, H-4), 6.29 (1H, d, *J* = 2.8 Hz, H-6), 4.69 (1H, d, *J* = 7.8 Hz, H-1"), 3.81 (2H, m, H-6"), 3.5 (1H, m, H-4"), 3.4 (1H, m, H-2"); 3.3 (1H, m, H-5"), 3.2 (2H, m, H-3"); ^{13}C-NMR (CD_3OD, 62.9 MHz) δ 158.1 (C-4'), 155.8 (C-5), 151.9 (C-3), 137.8 (C-2), 133.6 (C-1), 130.7 (C-1'), 130.0 (C-b), 129.1 (C-2', 6'), 121.6 (C-a), 116.4 (C-3', 5'), 108.0 (C-4), 103.5 (C-1"), 102.7 (C-6), 78.0 (C-5"), 77.8 (C-3"), 75.4 (C-2"), 70.7 (C-4"), 61.9 (C-6) (Chen *et al.*, 1999; Nonaka *et al.*, 1982; Ryu *et al.*, 2002); positive FAB-MS *m/z* 407 $[M+H]^+$.

(*Z*)-2,3,5,4'-Tetrahydroxy-stilbene-2-*O*-β-D-glucopyranoside (6)

Brown powder; $[\alpha]_D^{24}$ +17.83° (*c*, 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.29), 290 (4.03); IR (KBr) ν_{max}: 3363 (OH), 1607, 1513, 1012, 837, 610 cm^{-1}; ^{1}H-NMR (CD_3OD, 250 MHz) δ 7.05 (2H, d, *J* = 8.5 Hz, H-2', 6'), 6.70 (1H, d, *J* = 12.0 Hz, H-b), 6.61 (2H, d, *J* = 8.59 Hz, H-3', 5'), 6.47 (1H, d, *J* = 12.0 Hz, H-a), 6.21 (1H, d, *J* = 2.8Hz, H-4), 6.12 (1H, d, *J* = 2.8 Hz, H-6), 4.56 (1H, d, *J* = 7.6 Hz, H-1"), 3.7 (1H, m, H-3"), 3.5 (1H, m, H-4"), 3.4 (1H, m, H-2"), 3.3 (1H, m, H-5"), 3.2 (2H, m, H-6"); ^{13}C-NMR (CD_3OD, 62.9 MHz) δ 157.7 (C-4'), 155.3 (C-5), 151.8 (C-3), 137.8 (C-2), 133.9 (C-1), 131.4 (C-2', 6'), 131.3 (C-b), 129.8 (C-1'), 125.5 (C-a), 115.9 (C-3', 5'), 108.2 (C-4), 107.6 (C-1"), 103.6 (C-6), 78.3 (C-5"), 77.9 (C-3"), 75.4 (C-2"), 70.9 (C-4"), 61.2 (C-6"); positive HR-FAB-MS *m/z* 407.1339 $[M+H]^+$ (calcd. 407.1345 for $C_{20}H_{23}O_9$).

Determination of sugar in 6

A solution of compound **6** (6 mg) in 0.4 N HCl (in dioxane-H_2O, 1:1, 10 mL) was heated to 90°C for 2 h. After cooling, the reaction mixture was extracted with $CHCl_3$ (20 mL × 3). The $CHCl_3$ extract was evaporated, and the residue purified on a silica gel column, using a discontinuous gradient of $CHCl_3$-MeOH (99:1 to 1:1) as eluent, to give an aglycone. The H_2O layers was neutralized with Ag_2CO_3 and analyzed by TLC (Kieselgel, eluting

solvent *n*-BuOH-AcOH-H_2O, 4:1:1, sprayed with 10% H_2SO_4 and heated) to reveal the presence of glucose (*Rf* = 0.30). The *Rf* value was coincident with that of an authentic sample. The optical rotation value of the above sugar fraction {$[\alpha]_D^{21}$ + 52.5° (*c* = 0.08, H_2O)} was in good agreement with that of D-glucose (Crublet *et al.*, 2003, Kiem *et al.*, 2003).

Assay for DNA topoisomerase I inhibition *in vitro*

A DNA topoisomerase I inhibition assay was carried out according to the method reported by Fukuda *et al.* (1996), with minor modifications. The topoisomerase I activity was assessed by measuring the relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixture consisted of 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 0.01% bovine serum albumin (BSA), 250 ng pBR 322 plasmid DNA and 0.3 U calf thymus DNA topoisomerase I. The reaction mixture, with the addition of a test compound solution (less than 0.25% DMSO), was used to measure the inhibition of the DNA relaxation due to the topoisomerase I, in a final volume of 10 μL. The reaction mixtures were incubated for 30 min at 37°C, and the reaction terminated by the addition of a dye solution comprised of 2.5% SDS, 15% ficoll-400, 0.05% bromophenol blue, 0.05% xylene cyanole and 25 mM EDTA (pH 8.0). The reaction products were determined by electrophoresis on 1% agarose gels in TBE (Tris-borate-EDTA) running buffer, at 1.5 V/cm for 10 h. The gels were stained with ethidium bromide (0.5 μg/mL) and destained in water for 30 min each. For the visualization and quantitative analyses of the topoisomerase I activity, the gels were directly scanned with an image analyzer, and the area representing the supercoiled DNA calculated.

Assay for DNA topoisomerase II Inhibition *in vitro*

The DNA topoisomerase II inhibition activity was measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixtures contained 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 300 ng pBR 322 plasmid DNA and 0.3 U human DNA topoisomerase II, with the indicated compound concentrations (less than 0.25% DMSO), in a final volume of 20 μL. The reaction mixtures were incubated for 30 min at 37°C and terminated by the addition of 5 μL of a mixture containing 0.77% SDS, 77 mM EDTA (pH 8.0), 30% sucrose, 0.5% bromophenol blue and 0.5% xylene cyanole. The reaction products were subjected to electrophoresis on 1% agarose gels in TBE (Tris-borate-EDTA) running buffer, at 1.5 V/cm for 10 h. Gels were stained with 0.5 μg/mL ethidium bromide and destained in water for 30 min each. For visualization and quantitative analyses of the topoisomerase II activity, the gels were directly scanned with an image analyzer, and the area representing supercoiled DNA calculated.

Assay for cytotoxicity

The tetrazolium-based colorimetric assay (MTT assay) was used to assess the cytotoxicity towards human colon

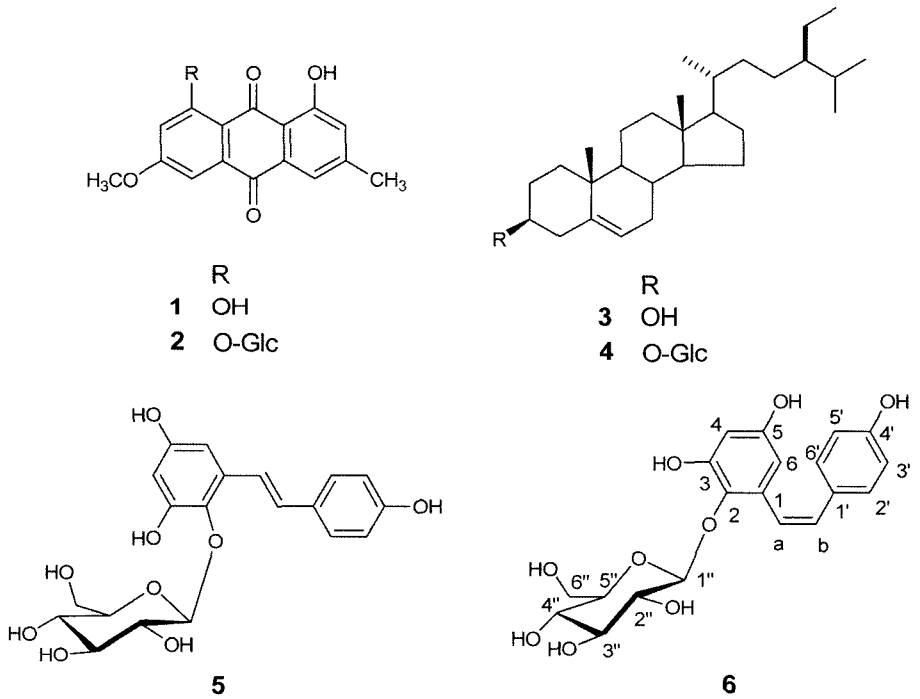

Fig. 1. Structures of compounds **1-6** from the roots of *Polygonum multiflorum*

carcinoma (HT-29), human breast carcinoma (MCF-7) and human hepatoblastoma (HepG2) cell lines (Rubinstein *et al.*, 1990).

RESULTS AND DISCUSSION

The MeOH extract of the roots of *P. multiflorum*, after suspension in H_2O, was successively partitioned with CH_2Cl_2 and EtOAc. The CH_2Cl_2 and EtOAc extracts were repeatedly subjected to column chromatography to afford two known anthraquinones, two known sterols, one known *trans*-stilbene and a new *cis*-stilbene. By comparison of ^{1}H- and ^{13}C-NMR, MS and optical rotation data with the literature values, compounds **1-5** were characterized as physcion (**1**) (Qin *et al.*, 1999), physcion-8-O-β-D-glucopyranoside (**2**) (Takeshi *et al.*, 1987), β-sitosterol (**3**), β-sitosterol-3-*O*-glucoside (**4**) (Chang *et al.*, 1981) and (*E*)-2,3,5,4'-tetrahydroxy-stilbene-2-*O*-β-D-glucopyranoside (**5**) (Chen *et al.*, 1999, Nonaka *et al.*, 1982; Ryu *et al.*, 2002).

Compound **6** was obtained from the EtOAc extract as a brown powder, with the molecular formula established as $C_{20}H_{23}O_9$ based on the HR-FAB-MS, ^{13}C-NMR and DEPT spectral data. The ^{1}H-NMR spectrum of **6** was very similar to that of **5**, with the exception of the coupling constants (J = 12.0 Hz) of the vinylic protons signals (H-a and H-b), indicating the presence of two *cis*-coupled vinylic protons at δ 6.71 (H-a) and 6.47 (H-b). In the aromatic region of the ^{1}H-NMR spectra of **6**, two sets of chemically equivalent protons at δ_H 7.05 (H-2'/H-6', d, J = 8.6 Hz) and δ_H 6.60 (H-3'/H-5', d, J = 8.6 Hz) suggested a 1, 4-disubstituted aromatic ring, with the ^3J coupling between H-4 (δ_H 6.21, d, J = 2.8 Hz) and H-6 (δ_H 6.12, d, J = 2.8 Hz) indicating a 1, 2, 3, 5-tetrasubstituted benzene ring. The ^{13}C-NMR spectrum of **6** exhibited two chemically equivalent aromatic carbons at δ_C 131.4 (C-2'/C-6') and δ_C 115.9 (C-3'/C-5').

In the HMBC spectrum, a linkage between the two aromatic rings and the vinyl group were established by the cross peaks between H-a and C-2 and C-6, and those between H-b and C-2' and C-6'. The position of the sugar

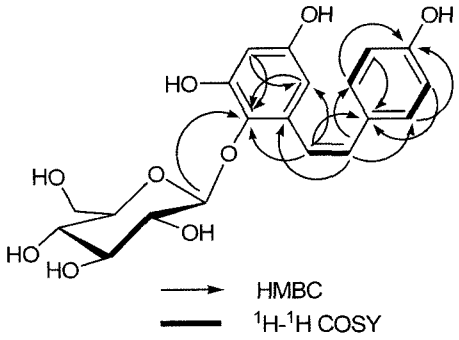

Fig. 2. HMBC and ^{1}H-^{1}H COSY correlations of compound **6**

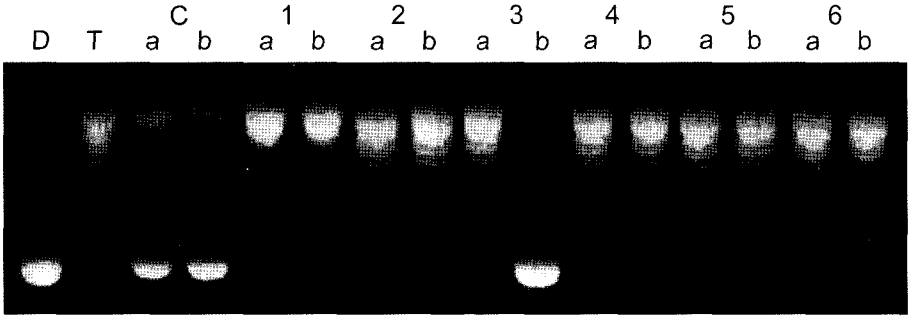

Fig. 3. TOPO inhibitory activities of compounds **1-6** from the roots of *Polygonum multiflorum*. Lane D:supercoiled DNA alone; Lane T: supercoiled DNA + toposiomerase II (calf thymus); Lane C: supercoiled DNA + topoisomerase II (calf thymus) + camptothecin-positive control; Lanes **a** and **b**: **a**: 20 μM, **b**: 100 μM.

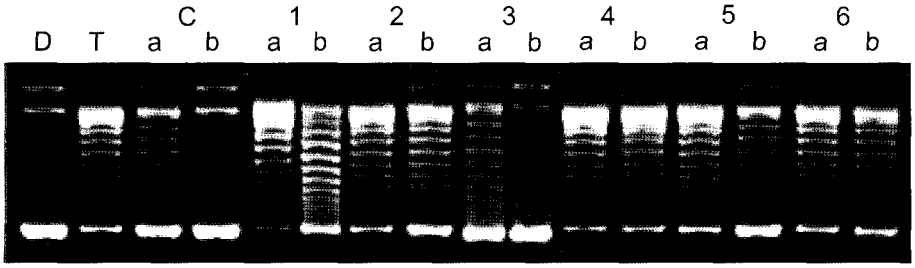

Fig. 4. TOPO inhibitory activities of compounds **1-6** from the roots of *Polygonum multiflorum*. Lane D:supercoiled DNA alone; Lane T: supercoiled DNA + toposiomerase II (human); Lane C: supercoiled DNA + topoisomerase II (human) + etoposide-positive control; Lanes **a** and **b**: **a**: 20 μM, **b**: 100 μM.

Table I. Inhibitory effects of compounds **1-6** from the roots of *Polygonum multiflorum* toward topoisomerase I and II enzymes (%inhibition ratio of relaxation), as well as their IC_{50} values against HT-29, MCF-7 and HepG-2 cell lines.

Compounds	(%) Inhibition ratio for topoisomerase I		(%) Inhibition ratio for topoisomerase II		Cytotoxicity IC_{50} (μg/mL)		
	100 μM	20 μM	100 μM	20 μM	HT-29[a]	MCF-7[b]	HepG-2[c]
1	NA	NA	8	NA	>100	>100	>100
2	NA	NA	NA	NA	>100	>100	>100
3	89	NA	99	39	>100	>100	>100
4	NA	NA	27	NA	>100	>100	>100
5	NA	NA	46	NA	>100	>100	>100
6	NA	NA	10	NA	49	50	>100
CPT[d]	73	55	NA	NA	0.96	1.0	1.06
etoposide[e]	NA	NA	78	58	NA	NA	NA

[a]HT-29: Human colon carcinoma; [b]MCF-7: Human breast carcinoma; [c]HepG-2: Human hepatoblastoma; [d]camptothecin: positive control for topoisomerase I; [e]positive control for topoisomerase II; NA: not applicable.

linkage was confirmed by the cross peak between H-1" (δ_H 4.56) and C-2 (δ_C 137.8).

Acid hydrolysis of **6** liberated aglycone and D-glucose, as identified by TLC analysis. The configuration of the anomeric proton of **6** was deduced to be the β form on the basis of the coupling constant of the proton peak (J = 7.6 Hz).

Of the six isolated compounds, compound **3** showed strong inhibitory activities against DNA topoisomerases I and II, exhibiting an almost equivalent inhibitory activity as that of etoposide against DNA topoisomerase II (99% and 39% inhibition at the concentration of 20 μM and 100 μM, respectively, Fig 3) and 89% inhibition at a concentration of 100 μM against DNA topoisomerase I, Fig. 4. Compounds **1**, **4**, **5**, and **6** showed weak inhibitory activities against DNA topoisomerase II at concentrations of 100 μM (8, 27, 46 and 10% inhibitions, respectively), but no inhibitory activity against DNA topoisomerase I. Only compound **6** showed weak cytotoxicities against the HT29 and MCF-7 cell lines (IC_{50}: 49 and 50 μM, respectively), as shown in Table I.

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