Isoetin 5'-Methyl Ether, A Cytotoxic Flavone from *Trichosanthes kirilowii*

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Received April 9, 2007

Bioassay-directed fractionations of the seed extracts of *Trichosanthes kirilowii*, have resulted in the isolation of two new compounds, 2-(4-hydroxy-3-methoxyphenyl)-3-(2-hydroxy-5-methoxyphenyl)-3-oxo-1-propanol (**2**) and isoetin 5'-methyl ether (5,7,2',4'-tetrahydroxy-5'-methoxyflavone) (**3**), together with two known compounds, 7-hydroxychromone (**1**) and 5,7,4'-trihydroxy-3',5'-dimethoxyflavone (tricin, **4**). Their structures were characterized by spectroscopic analysis such as 2D-NMR, HRTOFMS, and UV. Compound **3** showed cytotoxicity against human lung cancer cell line A549, human skin melanoma SK-Mel-2, and mouse melanoma B16F1, with IC_{50} of 0.92, 8.0, and 7.23 μg/mL, respectively.

Key Words : *Trichosanthes kirilowii*, Isoetin 5'-methyl ether, Tricin, Cytotoxicity

Introduction

Trichosanthes kirilowii (hanultari, Korean common name) is a perennial climber of the Cucurbitaceae family, growing throughout East Asia, Korea, China, and Japan. The medicinal plant has been known to show a wide variety of pharmacological activities such as antibacterial, antituberculotic, expectorant, antidiabetic, abortifacient, antineoplastic, and anti-inflammatory activities.[1,2] Phytochemical studies on this plant have identified several different types of biologically interesting constituents. Trichosanic acid (punicic acid) has been long known from this plant as conjugated linolenic acid with recent evidence showing cytotoxic effect on tumor cells and beneficial effect on lipid metabolism.[3-6] A ribosome-inactivating protein trichosanthin with 27 kDa consisting of 289 amino acids has been found to have abortifacient, antitumor, immunosuppressive, and anti-HIV activities.[7-12] Glycan trichosans isolated from the aqueous extract of this plant have been shown to reduce the plasma glucose level in mice.[13] Diterpene geranylgeranoic acid was shown to have cancer-preventive activity inducing apoptosis in a human hepatoma-derived cell line.[14] Triterpene 7-oxo-10α-cucurbitadienol and multiflorane-type triterpenoid karounidiol derivatives showed anti-inflammatory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear inflammation in mice and were proven to be potential anti-tumor promoters (cancer chemopreventive agents).[15,16] Sterols such as campesterol, sitosterol, stigmasterol, and ethylcholestadienol derivatives and flavonoids such as luteolin glycosides and apigenin glycosides were also reported.[17,18] Recently, antibacterial and antioxidant 1-(4-hydroxyphenyl)-glycerol was reported.[19]

With the aim of searching for antitumor agents from traditional medicinal plants, we led to identify cytotoxic flavonoids from the seed extracts of the plant *T. kirilowii* against human small lung cancer cell line A549. Herein, we report new propanol derivative, 2-(4-hydroxy-3-methoxyphenyl)-3-(2-hydroxy-5-methoxyphenyl)-3-oxo-1-propanol (**2**) and cytotoxic flavone isoetin 5'-methyl ether (5,7,2',4'-tetrahydroxy-5'-methoxyflavone) (**3**), along with two known flavonoids, 7-hydroxychromone (**1**) and tricin (**4**).

Experimental Section

Melting points were measured on a Fisher melting point apparatus and are uncorrected. UV spectra were measured on a Shimadzu UV-2401 PCR Spectrometer. NMR spectra were recorded on a Varian Mercury 400 spectrometer with standard pulse sequences operating at 400 and 100 MHz in ^{1}H and ^{13}C NMR, respectively. ^{1}H and ^{13}C NMR spectra were referenced relative to either methanol-d_4 (δ 3.30 and 49.15 ppm for ^{1}H and ^{13}C NMR, respectively) or DMSO-d_6 (δ 2.50 and 39.51 ppm for ^{1}H and ^{13}C NMR, respectively). 2D NMR spectra (COSY, NOESY, HSQC, and HMBC) were recorded at 400 MHz using the manufacturer's software VNMR 6.1C. Flash column chromatography was carried out with silica gel 60 (70-230 mesh, Merck, 50 id × 220 mm). C_{18} medium-pressure liquid chromatography (MPLC) was carried out on a Yamazen MPLC instrument (model GR-200, pump 540) using a prepacked column (Ultra pack, ODS-S-50B, 26 id × 300 mm, Yamazen corp., Japan). Thin-layer chromatography (TLC) was performed on pre-coated silica gel plates (Kieselgel 60, F_{254}, 20 × 20 cm, 0.25 mm thick, Merck). Spots were detected under UV light at 254 and 365 nm or by staining in solution of p-anisaldehyde-sulfuric acid in methanol followed by heating. Reverse phase HPLC was performed on a Waters 600 model system with a photodiode array detector 996 (variable-wavelength UV) using a C_{18} column (Senshu pak, Pegasil ODS, 20 id × 250 mm) with gradient elution of MeOH/H_2O at a flow rate of 7 mL/min. High resolution TOF mass spectra were measured on a Waters LCT Premier mass spectrometer coupled with a waters AQUITY HPLC system and data acquisition was achieved using MassLynx version 4.0 software. Optical density for a 96-well microplate was measured on a Tecan Sunrise microplate reader (model: A-5080, Austria) at 520 nm. Human lung cancer cell line A-549, human melanoma SK-Mel-2, and mouse melanoma

B16-F1 were purchased from the Korean Cell Line Bank (Seoul, Korea).

Plant Material. The seeds of *T. kirilowii* were purchased from the local market at Geumsan, Chungnam, Korea in August, 2005 and the plant was identified by Dr. Eunkyu Lim at the Busong Clinic of Medicinal Herbs (Iksan, Korea). A voucher specimen (SM1376) has been deposited at the Natural Product Chemistry Laboratory, Department of Chemistry, Kongju National University, Korea.

Extraction and Isolation. The seeds (10 kg) were pulverized and soaked with a series of extraction solvents: 80% aqueous MeOH (20 L) at room temperature for one week, EtOAc (18 L) for one week, and a mixture of EtOAc-Hexane (6:1, 14 L) for 5 days. The extracts were pooled and evaporated under reduced pressure to yield brownish oily syrup (2.6 Kg). The residue was suspended in 30% aqueous MeOH (1.4 L) and was extracted with hexane (1.2 L × 10). Aqueous methanolic layer was concentrated to give brownish residue (98 g) that was partitioned between H_2O (1.2 L) and CH_2Cl_2 (1.2 L × 5). The CH_2Cl_2 layer (30 g, IC_{50} ~100 μg/mL against A549) was chromatographed on a flash column with elution of a mixture of CH_2Cl_2, MeOH, and H_2O of increasing polarity to yield fourteen fractions (1-14). Among these, fraction 4 (6.9 g) showed cytotoxicity with an IC_{50} range of 20-40 μg/mL, was further subjected to silica column chromatography with gradient elution of a mixture of hexane, EtOAc, and MeOH to give ten subfractions. Cytotoxic subfractions 7, 8, and 9 (IC_{50}: 6-10 μg/mL) were combined (3.0 g) and were further fractionated using C_{18} MPLC (30-50% aqueous MeOH for 60 min, 50-70% for 60 min, 70-100% for 100 min, 8 mL/min) to give eight portions (1-8). Purification of portion 1 using C_{18} HPLC (18-24% aqueous MeCN, 40 min, 7 mL/min) afforded compounds **1** (4 mg, t_R = 22.4 min) and **2** (7 mg, t_R = 32 min). Purification of portion 4 (30-60% aqueous MeCN, 60 min, 7 mL/min) afforded compounds **3** (10 mg, t_R = 22.5 min) and **4** (15 mg, t_R = 30.5 min).

2-(4-Hydroxy-3-methoxyphenyl)-3-(2-hydroxy-5-methoxyphenyl)-3-oxo-1-propanol (2): light brown powder; mp. 47-53 °C; UV (CH_3OH): λ_{max} (log ε) 279 (4.35), 229 (4.57), 204 (4.83); ^{1}H-NMR (CD_3OD, 400 MHz): δ 7.60 (1H, dd, J = 8.4, 2.0 Hz, H-4"), 7.55 (1H, d, J = 2.0 Hz, H-6"), 6.88 (1H, d, J = 2.0 Hz, H-2'), 6.79 (1H, d, J = 8.4 Hz, H-3"), 6.75 (1H, dd, J = 8.0, 2.0 Hz, H-6'), 6.71 (1H, d, J = 8.0 Hz, H-5'), 4.74 (1H, dd, J = 8.8, 5.2 Hz, H-2), 4.24 (1H, dd, J = 10.8, 8.8 Hz, H_b-1), 3.85 (3H, s, OCH_3 at C-5"), 3.81 (3H, s, OCH_3 at C-3'), 3.70 (1H, dd, J = 10.8, 5.2 Hz, H_a-1); ^{13}C NMR (CD_3OD, 100 MHz): δ 199.4 (C-3), 152.9 (C-2"), 149.2 (C-3'), 148.8 (C-5"), 146.8 (C-4'), 130.3 (C-1"), 129.8 (C-1'), 125.1 (C-4"), 122.1 (C-6'), 116.5 (C-5'), 115.6 (C-3"), 112.7 (C-2'), 112.5 (C-6"), 65.6 (C-1), 56.5 (OCH_3 at C-3'), 56.46 (C-2), 56.40 (OCH_3 at C-5"); HRTOFMS: $[M+H]^+$ m/z 319.1155 (calcd. for $C_{17}H_{18}O_6$+H, 319.1182).

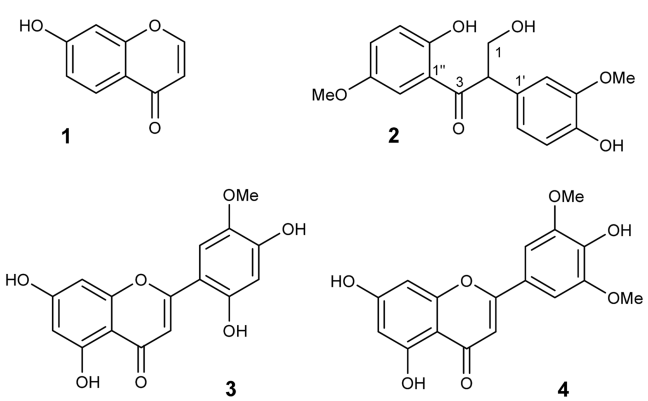
Isoetin 5'-Methyl Ether (3): yellowish powder; mp. > 300 °C; UV (CH_3OH): λ_{max} (log ε) 367 (3.80), 263 (3.78), 209 (4.08); ^{1}H-NMR (CD_3OD, 400 MHz): δ 7.47 (1H, s, H-6'), 7.16 (1H, s, H-3), 6.59 (1H, s, H-3'), 6.55 (1H, d, J = 2.0 Hz, H-8), 6.27 (1H, d, J = 2.0 Hz, H-6), 3.93 (3H, s, OCH_3 at C-5'); ^{13}C NMR (CD_3OD, 100 MHz): δ 183.5 (C-4), 165.3 (C-7), 163.2 (C-2), 162.7 (C-5), 159 (C-9), 154.2 (C-2'), 152.8 (C-4'), 142.9 (C-5'), 112.6 (C-6'), 108.9 (C-1'), 108.7 (C-3), 105.4 (C-3'), 105.1 (C-10), 99.8 (C-6), 95.1 (C-8), 57.6 (OCH_3 at C-5'); HRTOFMS $[M+H]^+$ m/z 317.0629 (calcd. for $C_{16}H_{12}O_7$+H, 317.0661).

Evaluation of Cytotoxic Potential. Cytotoxicity was measured by colorimetric sulforhodamine B (SRB) assay method.[20] Briefly, exponentially growing cells were harvested and suspended in the culture medium (100 μL, RPMI-1640) in a 96-well plate. Following seeding density was used: 1×10^5, 1×10^5, and 2×10^4 cells/mL for A-549, SK-Mel-2, and B16-F1, respectively. After 24 h incubation at 37 °C under humidified 5% CO_2, serially-diluted test solutions (100 μL in RPMI medium) were added to the wells and incubated further for 48 h. The cells were fixed with 50% trichloroacetic acid and stained for 30 min with SRB solution. Unbound dye was removed by 1% acetic acid (four times) and protein-bound dye was then extracted with 10 mM tris base (pH 10.5) for 5 min. Optical density of the released dye was measured at 520 nm in a microplate reader. The results were expressed in terms of IC_{50} value. Cisplatin was used as a reference sample.

Results and Discussion

The powdered seeds of *T. kirilowii* were successively extracted with 80% aqueous MeOH, MeOH, and CH_2Cl_2. The combined extracts were suspended in a mixture of hexane-aqueous MeOH. The aqueous MeOH layer separated was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 layer (IC_{50}~100 μg/mL against A549) was chromatographed on a flash column of silica gel with gradient elution of a mixture of CH_2Cl_2 and MeOH to yield fourteen fractions. Fraction 4 (IC_{50}~20 μg/mL against A549) was further subjected to a series of chromatograph (silica gel column chromatography and C_{18} HPLC) to afford compounds, **1**, **2**, **3**, and **4**, in the yield of 4, 7, 10, and 15 mg, respectively.

Compound **2** was obtained as light brown powder with a molecular formula of $C_{17}H_{18}O_6$ based on HRTOFMS data ($[M+H]^+$ m/z 319.1155, calcd. 319.1182). UV absorption maxima of **2** in CH_3OH appeared at 279, 229, and 204 nm.



The ^{1}H-NMR spectrum indicated the presence of six aromatic protons at δ 7.60 (1H, dd, J = 8.4, 2.0 Hz), 7.55 (1H, d, J = 2.0 Hz), 6.88 (1H, d, J = 2.0 Hz), 6.79 (1H, d, J = 8.4 Hz), 6.75 (1H, dd, J = 8.0, 2.0 Hz), and 6.71 (1H, d, J = 8.0 Hz). The spectrum also showed two methoxy group signals at δ 3.81 and 3.85 (each 3H, s), one methine proton signal at δ 4.74 (1H, dd, J = 8.8, 5.2 Hz), and two methylene protons at δ 4.24 (1H, dd, J = 10.8, 8.8 Hz) and 3.70 (1H, dd, J = 10.8, 5.2 Hz). The ^{13}C NMR spectrum showed three signals at δ 199.4, 65.6, and 56.4, and twelve aromatic signals at δ 152.9, 149.2, 148.8, 146.8, 130.3, 129.8, 125.1, 122.1, 116.5, 115.6, 112.7, and 112.5. In the ^{1}H-^{1}H COSY spectrum the methine proton at δ 4.74 was coupled with both protons at δ 3.70 and 4.24, which were coupled each other. The aromatic proton at δ 7.60 was coupled with the protons at δ 6.79 and 7.55, and another aromatic proton at δ 6.75 with the protons at δ 6.71 and 6.88. All the protons and carbons were assigned with an aid of interpretation of HSQC data. From these assignments and coupling constants, it was suggested that **2** contained one carbonyl, one HO-CH_2-CH<, and two methylated polyphenolic moieties. Connectivities among these groups were achieved by the ^{1}H-^{13}C heteronuclear correlation spectrum (HMBC).

In HMBC spectrum, the methine proton at δ 4.74 showed correlations with the carbonyl carbon at δ 199.4, the methylene carbon at δ 65.6, the quaternary aromatic carbon at 129.8, and two aryl carbons at 122.1 and 112.7. The carbonyl carbon in turn showed correlations with the aromatic proton at δ 7.55. These correlations clearly indicated the presence of 3-oxo-1-propanol moiety with attachments of one aromatic group at C-2 and another aromatic group at C-3. The positions of methoxy groups on the aromatic moieties were established by the HMBC correlations: methoxy protons at δ 3.81 with the aromatic carbon at 149.2 (C-3'); another methoxy protons at δ 3.85 with another aromatic carbon at δ 148.8 (C-5"). Other correlations shown in Figure 1 confirmed the positions of hydroxyl groups [the aromatic carbon at δ 146.8 (C-4') with the protons at δ 6.71 (C-5') and 6.75 (C-6'), and the aromatic carbon at δ 152.9 (C-2") with the protons at 6.79 (C-3") and 7.60 (C-4")]. Thus, the structure of **2** was determined to be 2-(4-hydroxy-3-methoxyphenyl)-3-(2-hydroxy-5-methoxyphenyl)-3-oxo-1-propanol.

Compound **3** was obtained as yellowish powder with molecular formula of $C_{16}H_{12}O_7$ measured by HRTOFMS ([M+H]$^+$ *m/z* 317.0629, calcd. 317.0661). UV absorption maxima of **3** in methanol appeared at 367 and 263 nm, indicating a characteristic pattern of a typical flavonoidal moeity.[21] The ^{1}H NMR spectrum showed signals due to five sp^2-methine protons at δ 7.47 (1H, s), 7.16 (1H, s), 6.59 (1H, s), 6.55 (1H, d, J = 2.0 Hz), and 6.27 (1H, d, J = 2.0 Hz), and methoxy protons at δ 3.93 (3H, s, OCH_3). The ^{13}C NMR showed sixteen carbon signals at δ 183.5, 165.3, 163.2, 162.7, 159.0, 154.2, 152.8, 142.9, 112.6, 108.9, 108.7, 105.4, 105.1, 99.8, 95.1, and 57.6. These ^{1}H and ^{13}C NMR spectral data except for the methoxy group were consistent with those of isoetin flavonoidal moiety.[21,22] The ^{1}H NMR signals at δ 6.27 and 6.54 (each 1H, d, J = 2.0 Hz) were assignable to the protons at C-6 and C-8 positions of A ring, and the signals at δ 6.59, and 7.47 (each 1H, s) were assignable to the protons at C-3 and C-6 of B ring of the isoetin nucleus, respectively.

The HMBC correlations also supported the presence of the isoetin nucleus, shown in Figure 2. The methoxy protons signal at δ 3.93 was correlated with the quaternary carbon (C-5') at δ 142.9, indicating the attachment of methoxy group at the carbon. This position was also confirmed by 1D NOESY experiment, in which irradiation of the methoxy signal at δ 3.93 enhanced the signal of the aromatic proton H-6' at δ 7.47 (Figure 2). From all the correlations, the structure of **3** was determined to be 5,7,2',4'-tetrahydroxy-5'-methoxyflavone or isoetin 5'-methyl ether. Compound **3** represents an unusually single-methylated 2',4',5'-trihydroxy flavone derivative. Isoetin (5,7,2',4',5'-pentahydroxy flavone) was first reported from *Isoetes durieui*,[22] and it's glycosides from various plants like *Adonis aleppica*,[21] *Taraxacum formosanum*,[23] and *Hispidella hispanica*.[24]

Compounds **1** and **4** were identified as 7-hydroxychromone[25] and tricin[26-27] by comparison of their physical and spectroscopic properties with those reported in the literature. Compound **1** was previously isolated from *Phlojodicarpus villosus*,[28] and **4** from a wide variety of species such as *Medicago sativa*,[26] *Agelaea pentagyna*,[27] and *Wikstroemia indica*.[29] However, these isolates are first reports from *T. kirilowii*.

The *in vitro* cytotoxicity of the isolates was evaluated against human lung cancer cell line A549, human melanoma Sk-Mel-2, and mouse melanoma B16F1 cell lines. Compound **3** showed cytotoxicity with IC_{50} 0.92, 8.0, and 7.23 μg/mL, against A549, SK-Mel-2, and B16F1, respectively, comparable to **4**. Compounds **1** and **2** were inactive below a concentration

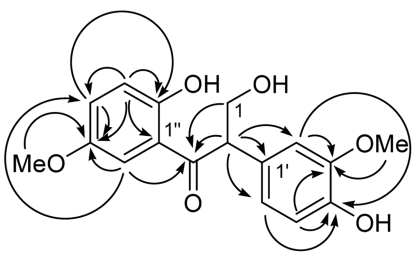


Figure 1. HMBC correlations (H to C) of **2**.

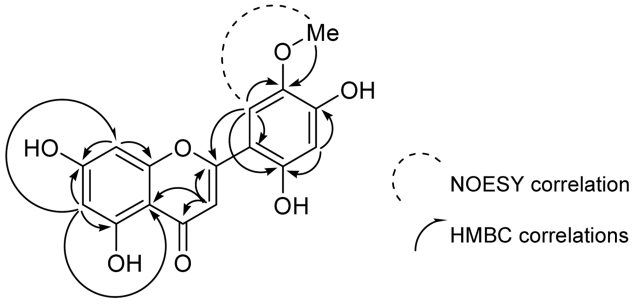


Figure 2. HMBC (H to C) and 1D NOESY correlations of **3**.

of 40 μg/mL. Although several isoetin derivatives have been reported from plants,[21,23,24,30] there was no report on cytotoxicity.

Acknowledgements. This work was supported by grants from the Korea Science and Engineering Foundation (Grant No. KOSEF R01-2003-000-10458-0) and in part by the Korean Research Foundation Grant funded by the Korean Government (MOEHRD, 2004) for M. A. A. Rahman.

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