Note

Geranylated Flavanones from *Paulownia coreana* and Their Inhibitory Effects on Nitric Oxide Production

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The activity-guided fractionation of the MeOH extract of the flower of *Paulownia coreana* led to the isolation of a new geranylated flavanone, 3′-*O*-methyl-5′-hydroxydiplacol (1), along with 10 known compounds (2–11). Their structures were determined using spectroscopic techniques, which included one and two dimensional (1- and 2D)-NMR. Among the isolates, compounds 1–6 showed potent inhibitory activities against lipopolysaccharide (LPS)-induced nitric oxide production with IC_{50} values ranging 1.48 to 16.66 μM.

Key words *Paulownia coreana*; Paulowniaceae; geranylated flavanone; nitric oxide inhibitor

Paulownia coreana Uyeki, which belongs to the Paulowniaceae, is a fast growing ornamental tree widely distributed in Korea, China, and Japan. It has been used traditionally to treat cough, phlegm, bronchitis, asthma, hemorrhoid, gonorrhea, and erysipelas.[1)] Previous phytochemical studies of the genus *Paulownia* have reported the isolation of iridoids,[2,3)] lignans,[4)] furanoquinones,[5)] phenolic glycosides,[6)] and flavonoids.[7,8)] Recently, geranylated flavonoids isolated from the genus *Paulownia* have been reported to possess a series of bioactivities including antiviral,[9)] antioxidant,[10)] antimicrobial,[11)] cytotoxic,[12)] and anti-cholinesterase activities.[13)] However, little information is available on the anti-inflammatory constituents of the *P. coreana*. In our continuing search for the plant-derived inhibitors of nitric oxide (NO) production, the methanolic extract of *P. coreana* was found to inhibit the lipopolysaccharide (LPS)-induced NO production in RAW264.7 cells. Subsequent bioassay-guided fractionation of the CH_2Cl_2-soluble fraction led to the isolation of a new geranylated flavanone, 3′-*O*-methyl-5′-hydroxydiplacol (**1**), together with 10 known compounds (**2**–**11**). The structures of known isolates were elucidated on the basis of their spectroscopic analysis (^{1}H-, ^{13}C-NMR, and electrospray ionization (ESI)-MS) by comparison with those of published data. Ten known compounds were determined to be 3′-*O*-methyl-5′-hydroxydiplacone (**2**),[14)] diplacol (**3**),[15)] diplacone (**4**),[15)] 3′-*O*-methyldiplacol (**5**),[15)] 6-geranyl-4′,5,7-trihydroxy-3′,5′-dimethoxyflavanone (**6**),[7)] mimulone B (**7**),[16)] dihydrotricin (**8**),[8)] 5,7-dihydroxy-3′,4′,5′-trimethoxyflavanone (**9**),[17)] (+)-pinoresinol (**10**),[18)] and (+)-dehydrovomifoliol (**11**),[19)] respectively (Fig. 1). Herein, we described the isolation and structure elucidation of the new compound and the inhibitory activities of all isolates against the LPS-induced NO production in murine macrophage RAW264.7 cells.

Results and Discussion

Compound **1** was obtained as a yellow amorphous powder. The high-resolution (HR)-ESI-MS spectrum showed a pseudomolecular ion at *m/z* 493.1833 (Calcd for $C_{26}H_{30}O_8Na$: 493.1832), consistent with a molecular formula of $C_{26}H_{30}O_8$. The ^{1}H-NMR spectrum (Table 1) revealed two proton signals of an AB system at δ_H 4.55 (1H, d, J=12.0 Hz, H-3), and 4.96 (1H, d, J=12.0 Hz, H-2), assignable to *trans*-diaxial H-2 and H-3 protons of the C ring of a flavanonol skeleton. In addition, two *meta*-coupled doublet signals at δ_H 6.68 (1H, br d, J=1.7 Hz, H-2′) and 6.84 (1H, br d, J=1.7 Hz, H-6′) assignable to the aromatic protons of a 1,3,4,5-tetrasubstituted aromatic B ring, a downfield proton at δ_H 11.59 (1H, s) assignable to chelated OH group at C-5, and an additional aromatic proton at δ_H 6.04 (1H, s) were observed. The remaining signals of ^{1}H-NMR spectrum implied the presence of a geranyl group δ_H 3.41 (2H, d, J=7.0 Hz, H-1″), 5.28 (1H, t, J=7.0 Hz, H-2″), 1.84 (3H, s, H-4″), 2.10 (2H, t, J=7.3 Hz, H-5″), 2.13 (2H, t, J=7.3 Hz, H-6″), 5.07 (1H, t, J=7.0 Hz, H-7″), 1.70 (3H, s, H-9″) and 1.62 (3H, s, H-10″)[7)] and a methoxy group δ_H 3.95 (3H, s). The ^{13}C- and distortionless enhancement by polarization transfer (DEPT) NMR spectra displayed resonances for 26 carbons, comprising four methyl (including one methoxy), three methylene, seven methine, and twelve quaternary carbons. Of which, 16 signals were assigned to a 3-hydroxyflavanol with a methoxy group and 10 signals to a geranyl group. The location of the geranyl and methoxy groups was assigned by the heteronuclear multiple bond correlation (HMBC) experiment. The HMBC correlations between 5-OH and C-5, C-6, and C-10; H-1″ and C-5, C-6, and C-7 suggested that the geranyl group was placed at C-6, whereas the HMBC correlation of OCH_3/C-3′ also established that a methoxy group was located at C-3′ (Fig. 2). The absolute configuration of **1** was determined by analyzing circular dichroism (CD) data. A positive Cotton effect at 342 ($\Delta\varepsilon$ +0.35) nm and a negative Cotton effect at 302 ($\Delta\varepsilon$ −2.15) nm suggested the 2*R* and 3*R* configuration.[7,8)] Therefore, the structure of compound **1** was determined to be 3′-*O*-methyl-5′-hydroxydiplacol.

NO plays an important role in the inflammatory process, and inhibitors of NO production may be potential anti-inflammatory agents. All isolates were tested for their inhibi-

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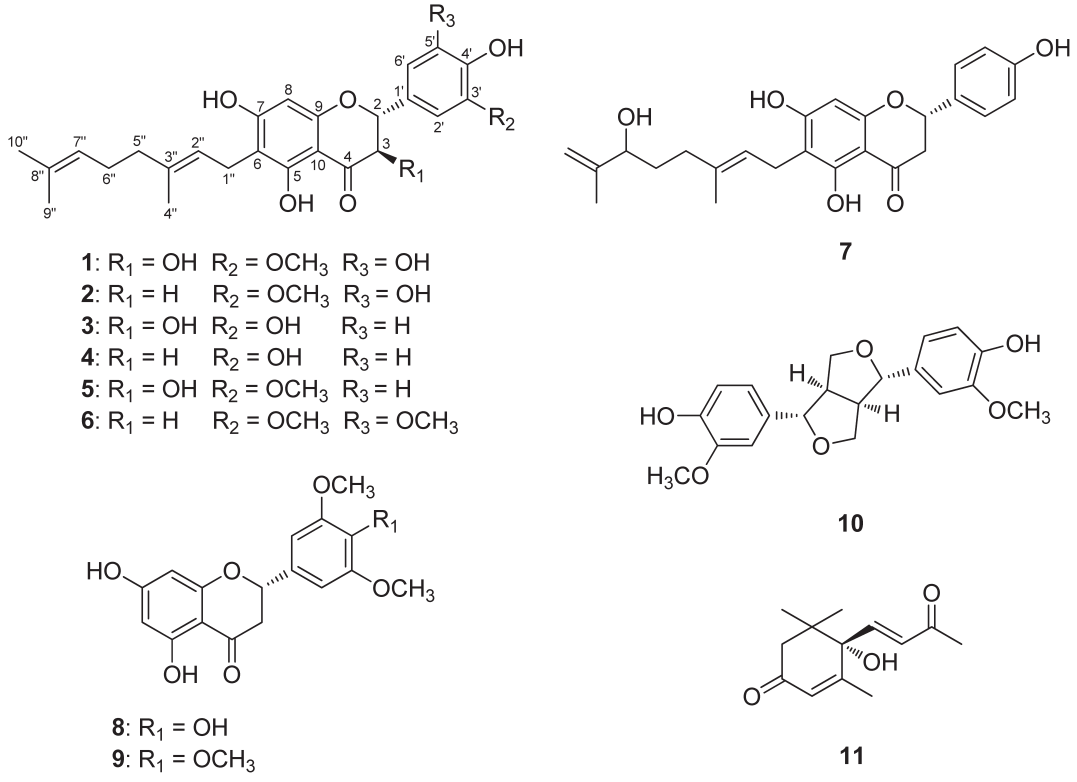

Fig. 1. The Structures of Compounds **1**–**11**

tory effects against LPS-induced NO production in RAW264.7 cells with aminoguanidine as the positive control (IC_{50} value: 16.60 μM) (Table 2). Among the isolates, flavanone compounds **1**–**6**, which have geranyl group at C-6 position, exhibited significantly greater inhibitory activity (IC_{50} values: 1.48 to 16.66 μM) than the simple flavanone compounds **8** and **9** (IC_{50} values: 44.92 and >50 μM, respectively). These results indicate that the presence of a geranyl group at C-6 position of the flavanone derivatives is an important factor for the NO inhibitory activity. However, compound **7**, which had hydroxy group at C-7″ position of geranyl moiety, showed moderate inhibitory activity (IC_{50} value: 23.49 μM), suggesting that the hydroxylation of the geranyl group might be responsible for the loss of activity. Compounds **10** and **11** exhibited moderate inhibitory activity (IC_{50} values: 35.31 and 31.82 μM, respectively). The cell viability of all isolates was evaluated using a CCK-8 assay, and the results indicated that there were no significant cytotoxic effects to the RAW 264.7 cells at their effective concentration for the inhibition of NO production (data not shown).

Recently, it was reported that several gernanylated flavanones showed the inhibitory effects of the inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) by suppressing the nuclear factor-kappa B (NF-κB)-related signaling pathways.[20–22]

In conclusion, these results indicate that *P. coreana* is a rich source of geranylated flavanones, which has potential to be used as candidates for the treatment of various inflammatory diseases. However, further investigation is needed to clarify the detailed mechanism of action of these geranylated flavanones.

Experimental

General Experimental Procedures Optical rotations were determined with a JASCO DIP-1000 polarimeter. UV and CD spectra were obtained on JASCO UV-550 and JASCO J-715 spectrometers, respectively. NMR spectra were recorded on a Bruker DRX 800 MHz and AMX 500 MHz NMR spectrometer using CD_3OD, $CDCl_3$ as solvents with the tetramethylsilane as an internal standard. Chemical shift are presented in ppm. HR-ESI-MS and ESI-MS spectra were measured on maXis 4G (Bruker) and LCQ Fleet (Thermoscientific), respectively. Preparative HPLC was performed using a Waters HPLC system equipped with two Waters 515 pumps and a 996 photodiode array detector using a YMC J'sphere octadecyl silica (ODS)-H80 column (4 μm, 150×20 mm, i.d., flow rate 6 mL/min). Open column chromatography was performed using silica gel (70–230 mesh, Merck), and MCI gel (75–150 μm, Mitsubishi). Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F254 (0.24 mm, Merck) plates, and spots were detected under UV and heating after spraying with 10% vanillin H_2SO_4.

Plant Materials The flowers of *P. coreana* were collected from Mooju, in Jeollabuk-do, Korea, in September 2012 and identified by J.-H. Kim of Gachon University, Republic of Korea. A voucher specimen (DiAB2012-007) was deposited at the Herbarium of Korea Institute of Oriental Medicine, Republic of Korea.

Extraction and Isolation The air-dried flowers of *P. coreana* (2 kg) were pulverized and extracted with MeOH (15 L, three times). After removing solvent, the MeOH extract (270 g) was partitioned sequentially with *n*-hexane, CH_2Cl_2, EtOAc and H_2O. The CH_2Cl_2-soluble extract (18 g) was subjected to silica gel (70–230 mesh) column chromatography (CC) and eluted with a mixed solvent of *n*-hexane/

Table 1. ^{1}H- (800 MHz) and ^{13}C-NMR (200 MHz) Data of Compound **1** ($CDCl_3$)[a]

Position	δ_H	δ_C
2	4.96 d (12.0)	83.5
3	4.55 d (12.0)	72.4
4	—	195.9
5	—	160.6
6	—	107.2
7	—	164.9
8	6.04 s	96.2
9	—	161.0
10	—	100.5
1′	—	127.9
2′	6.68 brd (1.7)	102.5
3′	—	146.9
4′	—	133.2
5′	—	143.9
6′	6.84 brd (1.7)	108.4
1″	3.41 d (7.0)	21.0
2″	5.28 t (7.0)	120.9
3″	—	140.0
4″	1.84 s	16.2
5″	2.10 brt (7.3)	39.7
6″	2.13 brt (7.3)	26.3
7″	5.07 brt (7.0)	123.6
8″	—	132.2
9″	1.70 s	25.7
10″	1.62 s	17.7
3′-OCH_3	3.95 s	56.2
5-OH	11.59 s	—
7-OH	6.32 s	—
4′-OH	5.40 s	—
5′-OH	5.51 s	—

a) The assignments were confirmed by DEPT, HMQC, and HMBC spectra.

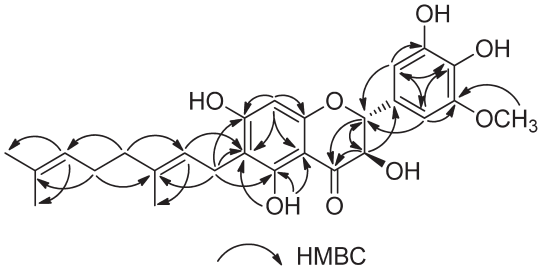

Fig. 2. Selected HMBC Correlations of Compound **1**

CH_2Cl_2 (20 : 1 to 0 : 1) and CH_2Cl_2/MeOH (1 : 0 to 0 : 1) in increasing polarity to give 7 fractions (PC1–PC7). PC1 was purified by preparative HPLC (Waters system, YMC J'sphere ODS-H80, 150×20 mm i.d., MeCN/H_2O=80 : 20, flow rate 6 mL/min) to afford compounds **2** (10.0 mg, t_R=25 min) and **5** (7.0 mg, t_R=21 min). PC2 was further subjected to MCI gel (75–150 μm) CC and eluted with MeOH/H_2O (30 : 70 to 100 : 0) to afford 3 sub-fractions (PC2-1 to PC2-3). PC2-1 was further purified by preparative HPLC (MeCN/H_2O=40 : 60 to 70 : 30) to afford compounds **10** (2.0 mg, t_R=12 min) and **11** (3.5 mg, t_R=14 min). PC2-3 was purified by preparative HPLC (MeCN/H_2O=85 : 15) to afford compounds **3** (8.0 mg, t_R=17 min) and **4** (10.0 mg, t_R=19 min). PC3, PC5, and PC6 were further purifier by Sephadex LH-20 CC and eluted with CH_2Cl_2/MeOH (1 : 1) to afford PC3-1, PC5-1, and PC6-1 sub-fractions, respectively. PC3-1 was purified by preparative HPLC (MeCN/H_2O=80 : 20) to afford compounds **6** (7.0 mg, t_R=15 min) and **7** (5.0 mg, t_R=18 min). PC5-1 was further purified by preparative HPLC (MeCN/H_2O=85 : 15) to afford compounds **8** (6.0 mg, t_R=10 min). PC6-1 was purified by preparative HPLC (MeCN/H_2O=70 : 30 to 85 : 15) to afford compounds **1** (4.5 mg, t_R=19 min) and **9** (3.0 mg, t_R=15 min).

Table 2. Inhibitory Effects of Compounds **1–11** on LPS-Induced NO Production in RAW264.7 Cells

Compound	IC_{50} (μM)
3′-*O*-Methyl-5′-hydroxydiplacol (**1**)	16.66
3′-*O*-Methyl-5′-hydroxydiplacone (**2**)	1.48
Diplacol (**3**)	4.53
Diplacone (**4**)	5.02
3′-*O*-Methyldiplacol (**5**)	5.94
6-Geranyl-4′,5,7-trihydroxy-3′,5′-dimethoxyflavanone (**6**)	6.44
Mimulone B (**7**)	23.49
Dihydrotricin (**8**)	44.92
5,7-Dihydroxy-3′,4′,5′-trimethoxyflavanone (**9**)	>50
(+)-Pinoresinol (**10**)	35.31
(+)-Dehydrovomifoliol (**11**)	31.82
Aminoguanidine (positive control)	16.60

3′-*O*-Methyl-5′-hydroxydiplacol (**1**): Yellow amorphous powder; $[\alpha]_D^{25}$ −2.4 (*c*=1.0, MeOH); CD (MeOH) nm ($\Delta\varepsilon$): 302 (−2.15), 342 (+0.35); ^{1}H-NMR (800 MHz, $CDCl_3$) and ^{13}C-NMR (200 MHz, $CDCl_3$), see Table 1; HR-ESI-MS (positive-ion mode) *m/z*: 493.1833 $[M+Na]^+$ (Calcd for $C_{26}H_{30}O_8Na$, 493.1832).

Measurement of NO Production and Cell Viability The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. Briefly, RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 units/mL)-streptomycin (100 μg/mL) in an incubator at 37°C and 5% CO_2. The cells were seeded into 96-well tissue culture plates (2×10^5 cells/mL) and stimulated with 1 μg/mL of LPS in the presence or absence of compounds. After incubation at 37°C for 24 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess reagent containing equal volumes of 2% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.2% (w/v) *N*-(1-naphthyl)ethylenediamine solution to determine nitrite production. Absorbance was measured in a microplate reader at 550 nm against a calibration curve with sodium nitrite standards. The viability of the remaining cells was determined by CCK (Cell Counting Kit, Dojindo, Kumamoto, Japan)-based colorimetric assay.

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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.

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