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2-Phenoxychromones and Prenylflavonoids from *Epimedium koreanum* and Their Inhibitory Effects on LPS-Induced Nitric Oxide and Interleukin-1β Production

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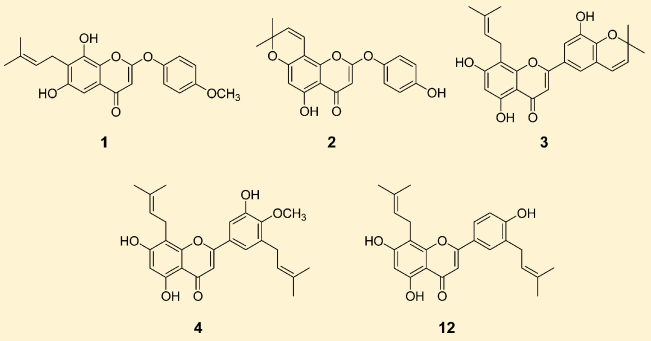
Supporting Information

ABSTRACT: Two new 2-phenoxychromones **1** and **2** and two prenylflavonoids **3** and **4** along with 12 known compounds (**5**−**16**) were isolated from the CH_2Cl_2-soluble fraction of a methanol extract of *Epimedium koreanum*. Compounds **1**, **4**, **6**, **7**, **9**, **10**, **12**, and **15** exhibit inhibitory effects on nitric oxide production with IC_{50} values ranging from 16.8 to 49.3 μM. Compounds **1**, **4**, **7**, and **12** also showed inhibitory effects on interleukin-1β production with IC_{50} values ranging from 8.6 to 38.9 μM in RAW 264.7 macrophages.



Epimedium koreanum Nakai (Berberidaceae) is a deciduous, perennial plant, which is distributed mainly in Korea, mainland China, and Japan. The aerial parts of *E. koreanum* have been widely used as a general tonic and for the treatment of cardiovascular diseases, infertility, impotence, amnesia, lumbago, arthritis, numbness, and weakness of the limbs.[1,2] Chemical investigations of members of this genus have resulted in the isolation of several classes of compounds, including flavonoids, lignans, ionones, sesquiterpenoids, phenols, and phenylethanoid glycosides. Prenylflavonoids are the major constituents, of which some have biological activities, such as anti-osteoporosis, immunomodulatory, antioxidative, anticancer, neuroprotective, and antihepatotoxic activities.[3−11] However, there are only a few reports of the anti-inflammatory activity of plants from the genus *Epimedium*.[8,12−14] A preliminary bioassay showed that the MeOH extract of *E. koreanum* had significant inhibitory effects against LPS-induced nitric oxide (NO) production (IC_{50} value of 15.8 μg/mL), which prompted further study of the active constituents of this plant. The bioassay-guided investigation of the CH_2Cl_2-soluble fraction resulted in the isolation of two 2-phenoxychromones **1** and **2** and two prenylflavonoids **3** and **4** together with 12 known compounds (**5**−**16**). This paper reports the isolation and structural determination of the four new compounds as well as the inhibitory activities of the isolates against LPS-induced NO and interleukin-1β (IL-1β) production in macrophage RAW 264.7 cells.

Compound **1** was obtained as a yellow, amorphous powder. Its molecular formula ($C_{21}H_{20}O_6$) was established on the basis of high-resolution electrospray ionization mass spectrometry (HRESIMS) (*m*/*z* 391.1155 [M + Na]$^+$; calcd for $C_{21}H_{20}O_6Na$, 391.1152) in combination with ^{13}C nuclear magnetic resonance (NMR) data (Table 1). The IR spectrum exhibited characteristic absorptions for hydroxy (3117 cm^{-1}), carbonyl (1646 cm^{-1}), and aromatic (1615 and 1414 cm^{-1}) functionalities. In the ^1H and ^{13}C NMR data (Table 1), the presence of a 2-phenoxychromone skeleton was evident from the resonances at δ_H 5.11 (1H, s, H-3), δ_C 88.2 (C-3), and δ_C 169.7 (C-2).[15,16] The ^1H and ^{13}C NMR data showed resonances for a 1,4-disubstituted aromatic ring [δ_H 7.19 (2H, d, J = 9.0 Hz, H-2′,6′), 7.03 (2H, d, J = 9.0 Hz, H-3′,5′); δ_C 159.9, 146.3, 122.9, 116.5], a methoxy group [δ_H 3.83 (3H, s); δ_C 56.3], and a γ,γ-dimethylallyl group [δ_H 5.20 (1H, t, J = 7.5 Hz, H-12), 3.28 (2H, m, H-11), 1.76 (3H, s, H-15), 1.65 (3H, s, H-14); δ_C 123.3, 22.2, 25.9, 17.9]. The remaining

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Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds 1−4[a]

	1[b]		2[c]		3[c]		4[b]	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2		169.7, C		168.1, C		163.1, C		165.7, C
3	5.11, s	88.2, CH	5.05, s	87.1, CH	6.66, s	103.1, CH	6.53, s	104.8, CH
4		185.6, C		183.2, C		181.6, C		184.3, C
5	6.34, s	94.2, CH		153.8, C		158.9, C		160.9, C
6		163.3, C	6.48, s	94.8, CH	6.24, s	99.5, CH	6.25, s	99.7, CH
7		113.6, C		158.3, C		163.1, C		163.6, C
8		160.1, C		105.2, C		106.1, C		108.3, C
9		154.8, C		155.5, C		154.3, C		156.5, C
10		103.4, C		102.8, C		103.2, C		105.4, C
11	3.28, m	22.2, CH_2	6.57, d (10.0)	114.4, CH	3.44, m	21.3, CH_2	3.50, br d (7.0)	22.6, CH_2
12	5.20, t (7.5)	123.3, CH	5.78, d (10.0)	129.1, CH	5.21, m	122.3, CH	5.28, m	123.7, CH
13		132.2, C		78.1, C		131.7, C		132.8, C
14	1.65, s	25.9, CH_3	1.42, s	27.7, CH_3	1.63, s	25.3, CH_3	1.67, s	26.0, CH_3
15	1.76, s	17.9, CH_3	1.42, s	27.7, CH_3	1.77, s	17.7, CH_3	1.75, s	17.9, CH_3
1′		146.3, C		143.1, C		121.3, C		128.2, C
2′	7.19, d (9.0)	122.9, CH	7.18, d (9.0)	121.7, CH	7.35, br d (2.0)	113.7, CH	7.28, br d (2.0)	113.5, CH
3′	7.03, d, (9.0)	116.5, CH	6.86, d (9.0)	116.5, CH		145.8, C		151.9, C
4′		159.9, C		156.1, C		143.6, C		150.7, C
5′	7.03, d (9.0)	116.5, CH	6.86, d (9.0)	116.5, CH		121.4, C		137.5, C
6′	7.19, d (9.0)	122.9, CH	7.18, d (9.0)	121.7, CH	7.30, br d (2.0)	115.6, CH	7.31, br d (2.0)	119.9, CH
7′					6.42, d (8.5)	121.7, CH	3.39, br d (7.0)	29.5, CH_2
8′					5.85, d (8.5)	131.7, CH	5.28, m	123.6, CH
9′						77.1, C		134.1, C
10′					1.42, s	27.6, CH_3	1.79, s	18.2, CH_3
11′					1.42, s	27.6, CH_3	1.75, s	25.9, CH_3
OCH_3	3.83, s	56.3, CH_3					3.85, s	60.9, CH_3

[a]Assignments were made by an analysis of the HMQC and HMBC data. The chemical shifts (δ) are expressed in parts per million; J values (in hertz) are given in parentheses. [b]Data recorded in methanol-d_4. [c]Data recorded in dimethyl sulfoxide-d_6.

resonances at δ_H 6.34 and δ_C 94.2 indicated the presence of a pentasubstituted aromatic ring A. These results suggested that compound **1** was a 2-phenoxychromone possessing a γ,γ-dimethylallyl and a methoxy group. The HMBC correlations between H-11 (δ_H 3.28) and C-6 (δ_C 163.3), C-7 (δ_C 113.6), and C-8 (δ_C 160.1) and between OCH_3 (δ_H 3.83) and C-4′ (δ_C 159.9) indicated the γ,γ-dimethylallyl and the methoxy groups are attached at C-7 and C-4′, respectively (Figure 1). Therefore, the structure of compound **1** was defined as 2-(4-methoxyphenoxy)-6,8-dihydroxy-7-(γ,γ-dimethylallyl)chromen-4-one and given the trivial name epimedonin A.

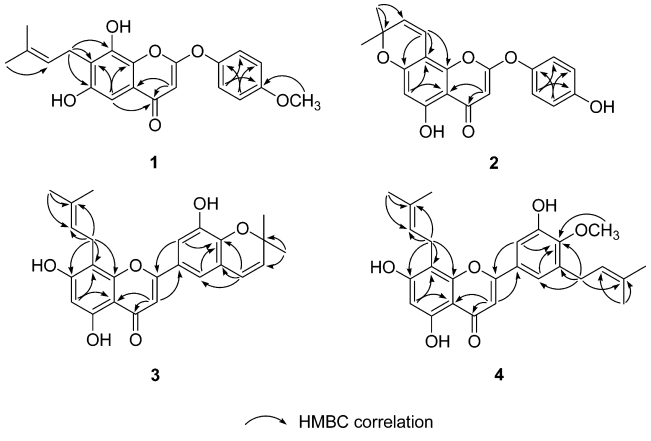

Figure 1. Key HMBC correlations of compounds **1**−**4**.

Compound **2** was obtained as a yellow, amorphous powder. Its molecular formula ($C_{20}H_{16}O_6$) was established on the basis of ^{13}C NMR and high-resolution fast atom bombardment mass spectrometry (HRFABMS) data (m/z 351.0885 [M − H]$^-$; calcd for $C_{20}H_{15}O_6$, 351.0869). The ^{1}H and ^{13}C NMR data (Table 1) of **2** were similar to those of **1**, suggesting that compound **2** was also a 2-phenoxychromone derivative.[15,16] Notable differences included the absence of a methoxy group and the presence of a 2,2-dimethylpyran ring instead of a γ,γ-dimethylallyl group. In the HMBC experiment (Figure 1), the proton at δ_H 6.57 (1H, d, J = 10.0 Hz, H-11) was correlated with three quaternary carbons at C-7 (δ_C 158.3), C-8 (δ_C 105.2), and C-9 (δ_C 155.5), which indicated the 2,2-dimethylpyran ring was fused to C-7 and C-8 in ring A. Therefore, the structure of compound **2** was defined as 2-(4-hydroxyphenoxy)-5-hydroxy-7,8-(2,2-dimethylpyrano)-chromen-4-one and given the trivial name epimedonin B.

Compound **3** was obtained as a yellow, amorphous powder. Its molecular formula ($C_{25}H_{24}O_6$) was established on the basis of ^{13}C NMR and HRESIMS data. The ^{1}H NMR data (Table 1) showed a γ,γ-dimethylallyl group [δ_H 5.21 (1H, m, H-12), 3.44 (2H, m, H-11), 1.77 (3H, s, H-15), 1.63 (3H, s, H-14)], a 2,2-dimethylpyran ring [δ_H 6.42 (1H, d, J = 8.5 Hz, H-7′), 5.85 (1H, d, J = 8.5 Hz, H-8′), 1.42 (6H, s, H-10′, 11′)], two *meta*-coupled aromatic protons [δ_H 7.35 (1H, br d, J = 2.0 Hz, H-2′), 7.30 (1H, br d, J = 2.0 Hz, H-6′)], and two isolated aromatic protons [δ_H 6.66 (1H, s, H-3), 6.24 (1H, s, H-6)]. The ^{13}C NMR data, along with HMQC data, exhibited 25 carbon resonances (Table 1), corresponding to a flavone derivative with a γ,γ-dimethylallyl and a 2,2-dimethylpyran group. In the

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HMBC spectrum (Figure 1), correlations between H-11 (δ_H 3.44) and C-7 (δ_C 163.1), C-8 (δ_C 106.1), and C-9 (δ_C 154.3) indicated that the γ,γ-dimethylallyl group was located at C-8. In addition, the HMBC correlations between H-7′ and C-6′, C-5′, and C-4′ and between H-8′ and C-5′ confirmed that the 2,2-dimethylpyran moiety was fused at C-4′ and C-5′ (Figure 1). Therefore, the structure of compound **3** was defined as 5,7,3′-trihydroxy-8-(γ,γ-dimethylallyl)-4′,5′-(2,2-dimethylpyrano)-flavone and given the trivial name epimedonin C.

Compound **4** was obtained as a yellow, amorphous powder. Its molecular formula ($C_{26}H_{28}O_6$) was established on the basis of ^{13}C NMR and HRFABMS data (*m*/*z* 435.1816 $[M - H]^-$; calcd for $C_{26}H_{27}O_6$, 435.1808). The ^1H and ^{13}C NMR data (Table 1) of **4** were similar to those of **3**, except for the presence of a methoxy group and an additional γ,γ-dimethylallyl group instead of a 2,2-dimethylpyran ring. Two γ,γ-dimethylallyl groups were located at C-8 and C-5′, which were supported by the HMBC correlations between H-11 and C-7, C-8, and C-9 and between H-7′ and C-4′, C-5′, and C-6′. The HMBC correlation between OCH_3 and C-4′ suggested the methoxy group was located at C-4′ (Figure 1). Therefore, the structure of compound **4** was defined as 5,7,3′-trihydroxy-8,5′-bis(γ,γ-dimethylallyl)-4′-methoxyflavone and given the trivial name epimedonin D.

The 12 known compounds were identified by comparison of their reported spectroscopic data: 2,3,4,6,7-pentamethoxy-9,10-dihydrophenanthrene (**5**),[17] 2,3,4,6,7-pentamethoxyphenanthrene (**6**),[17] magnolol (**7**),[18] xylogranatinin (**8**),[19] (−)-syringaresinol (**9**),[18] dehydrovomifoliol (**10**),[20] loliolide (**11**),[20] 5,7,4′-trihydroxy-8,3′-diprenylflavone (**12**),[21] (+)-epiloliolide (**13**),[20] (3*S*,5*R*,6*S*,7*E*)-3,5,6-trihydroxy-7-megastigmen-9-one (**14**),[22] (+)-epipinoresinol (**15**),[23] and olivil monoacetate (**16**).[24]

The overproduction of NO caused by activated macrophages has been implicated in the pathology of a variety of inflammatory disorders, including sepsis and tissue damage after inflammation. Therefore, the isolated compounds were examined for their inhibitory effects on NO production in LPS-stimulated macrophage RAW 264.7 cells (Table 2). Compounds **1**, **4**, **6**, **7**, **9**, **10**, **12**, and **15** inhibited NO production with IC_{50} values ranging from 16.8 to 49.3 μM. The prenylflavones, **4** and **12** possessing a prenyl moiety in both rings A and B, most strongly inhibited NO production (IC_{50} values of 16.8 and 19.7 μM, respectively). Neolignan **7** and lignans **9** and **15** exhibited moderate inhibitory activity. In contrast, compound **3**, in which the prenyl moiety in ring B was cyclized into a six-membered pyran ring, exhibited no inhibitory activity, suggesting that the two prenyl moieties play key roles in the inhibitory activity of these compounds. In addition, the 2-phenoxychromone **1** (IC_{50} value of 27.5 μM) possessing a prenyl moiety in ring A exhibited better activity than compound **2** (IC_{50} value of >50 μM), suggesting that the presence of a 2,2-dimethylpyran moiety in ring A might be responsible for the loss of activity. These results suggest that the prenyl moiety is essential for inhibitory activity.

Table 2. Inhibition of LPS-Induced NO and IL-1β Production in Macrophage RAW 264.7 Cells of Compounds 1−16[a]

compound	IC_{50} value (μM)[b]	
	NO	IL-1β
1	27.5	28.9
4	16.8	8.8
6	49.3	>50
7	35.4	38.9
9	45.6	>50
10	38.5	>50
12	19.7	8.6
15	39.5	>50
aminoguanidine[c]	21.4	not determined
indomethacin[c]	15.8	18.7

[a]Compounds **2**, **3**, **5**, **8**, **11**, **13**, **14**, and **16** were inactive (IC_{50} values of >50 μM). [b]Results are expressed as the mean IC_{50} values from triplicate experiments. [c]Positive control.

Levels of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), are elevated during the development of the inflammatory disease and play important roles in immune and inflammatory responses. To further analyze the anti-inflammatory effects of compounds **1**−**16**, the level of IL-1β production by LPS-stimulated RAW 264.7 cells was determined by an enzyme-linked immunosorbent assay (ELISA) (Table 2). Compounds **1**, **4**, **7**, and **12** reduced the level of IL-1β production with IC_{50} values ranging from 8.6 to 38.9 μM. The results are consistent with the profile of the inhibitory effects of NO production.

To determine whether the inhibitory effects on NO and IL-1β production are caused by the inhibition of the transcriptional level of the corresponding genes, the mRNA levels of iNOS and IL-1β were analyzed by using reverse transcriptase polymerase chain reaction (RT-PCR). As shown in Figure 2,

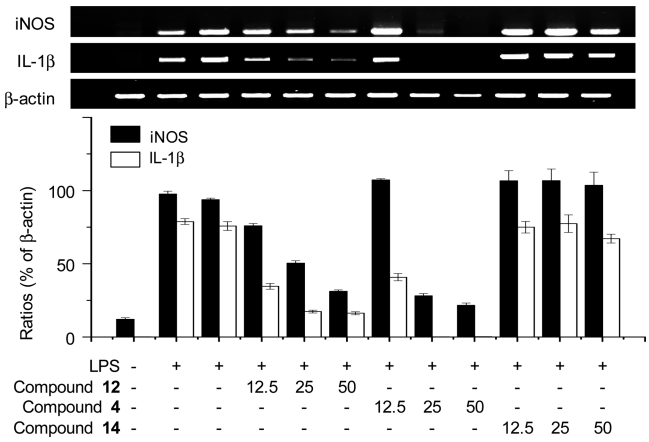

Figure 2. Effects of compounds **4** and **12** on iNOS and IL-1β mRNA expression in LPS-induced RAW 264.7 cells.

compounds **4** and **12**, the most active compounds, suppressed the expression of iNOS and IL-1β mRNA levels in a dose-dependent manner. However, compound **14**, which displayed no inhibitory activity on NO and IL-1β production (IC_{50} value of >50 μM), did not affect the iNOS and IL-1β mRNA levels. These data suggest that the decreases in the levels of LPS-induced NO and IL-1β production by compounds **4** and **12** are mediated by the suppression of transcriptional expression of the iNOS and IL-1β genes.

Recently, the water extract of *Epimedium brevicornum* was found to have inhibitory effects on pro-inflammatory mediators, including NO, IL-3, and IL-10, in LPS-induced RAW 264.7 cells.[12] Prenylated flavonol glycosides, such as icariin and ikarisoside A, from the genus *Epimedium*, inhibited the LPS-

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induced production of NO, PGE_2, TNF-α, and IL-1β in macrophages through the inhibition of NF-κB signaling.[8,10,13] Icaritin, an intestinal metabolite of a prenylflavonoid from the genus *Epimedium*, also inhibited the production of NO, IL-6, IL-10, and TNF-α both *in vitro* and *in vivo*.[25]

In conclusion, two new 2-phenoxychromones **1** and **2** and two prenylflavonoids **3** and **4** along with 12 known compounds (**5**−**16**) were isolated from *E. koreanum*. The 2-phenoxychromone and flavone compounds with prenyl moieties exhibited promising inhibitory effects on NO and IL-1β production through the suppression of iNOS and IL-1β mRNA in LPS-stimulated RAW 264.7 cells. Further studies are needed to verify the mechanism of action for the inhibition of NO and IL-1β production.

EXPERIMENTAL SECTION

General Experimental Procedures. Infrared (IR) spectra were recorded on a PerkinElmer model LE599 spectrometer. ^{1}H, ^{13}C, and two-dimensional (2D) NMR spectra were recorded on a Bruker DRX 500 spectrometer using dimethyl sulfoxide-d_6 (DMSO-d_6) and methanol-d_4 as the solvent. HRESIMS and HRFABMS spectra were recorded on maXis 4G (Bruker) and JMS 700 (JEOL) instruments, respectively. Preparative high-performance liquid chromatography (HPLC) was conducted on a Waters system (two 515 pumps and a 2996 photodiode array detector) and a YMC J'sphere ODS-H80 column (4 μm, 150 mm × 20 mm), using the MeCN/H_2O mixed solvent system at a flow rate of 6.0 mL/min. Open column chromatography was performed using silica gel (Kieselgel 60, 70−230 mesh, Merck) and Sephadex LH-20 (25−100 μM, Pharmacia), and thin layer chromatography was performed using precoated silica gel 60 F_{254} (0.25 mm, Merck).

Plant Material. The dried aerial parts of *E. koeranum* were collected at the herbal garden of Chungbuk National University (Cheongju, Korea) in October 2012. The plant material was identified by emeritus Professor Kyong Soon Lee, and a voucher specimen of this plant was deposited at the Herbarium of the College of Pharmacy of Chungbuk National University (CBNU1208).

Extraction and Isolation. The dried and powdered leaves of *E. koeranum* (5 kg) were extracted with MeOH (3 × 10 L) at room temperature to yield a MeOH extract (82 g). The extract was filtered, concentrated *in vacuo*, and partitioned sequentially with *n*-hexane, CH_2Cl_2, and H_2O. The CH_2Cl_2-soluble extract (4 g) was chromatographed on a silica gel column eluted with a step gradient of an *n*-hexane/CH_2Cl_2 mixture (from 90:10 to 0:100) to give 13 fractions (EKC1−EKC13). Fraction EKC3 (610 mg) was chromatographed further on a Sephadex LH-20 column (1:1 CH_2Cl_2/MeOH) to afford two subfractions EKC31 and EKC32. Fraction EKC31 (110 mg) was purified by preparative HPLC (MeCN/H_2O gradient from 80:20 to 100:0) to afford compounds **1** (3.5 mg), **5** (4 mg), **6** (6.0 mg), and **7** (5 mg). Fraction EKC32 (64 mg) was purified further by preparative HPLC (MeCN/H_2O gradient from 90:10 to 100:0) to afford compounds **2** (3.2 mg), **8** (3.6 mg), **9** (4 mg), and **10** (4.5 mg). Fraction EKC5 (430 mg) was chromatographed on a Sephadex LH-20 column (1:1 CH_2Cl_2/MeOH) to yield two fractions EKC51 and EKC52. Fraction EKC51 (100 mg) was purified by preparative HPLC (MeCN/H_2O gradient from 75:25 to 90:10) to give compounds **3** (3.0 mg), **4** (5.0 mg), **11** (2.5 mg), **12** (5.0 mg), and **13** (3.0 mg). Fraction EKC52 (60 mg) was further purified by HPLC (MeCN/H_2O gradient from 70:30 to 100:0) to yield compounds **14** (4 mg), **15** (6 mg), and **16** (4 mg).

Epimedonin A (**1**): yellow amorphous powder; FT-IR (KBr) ν_{max} 3117, 1646, 1615, 1492, 1414 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 500 and 125 MHz) data in Table 1; HRESIMS *m*/*z* 391.1155 [M + Na]$^+$ (calcd for $C_{21}H_{20}NaO_6$, 391.1152).

Epimedonin B (**2**): yellow amorphous powder; FT-IR (KBr) ν_{max} 3282, 1652, 1613, 1414 cm^{-1}; ^{1}H and ^{13}C NMR (DMSO-d_6, 500 and 125 MHz) data in Table 1; HRFABMS *m*/*z* 351.0885 [M − H]$^-$ (calcd for $C_{20}H_{15}O_6$, 351.0869).

Epimedonin C (**3**): yellow amorphous powder; FT-IR (KBr) ν_{max} 2924, 1651, 1574, 1507, 1428 cm^{-1}; ^{1}H and ^{13}C NMR (DMSO-d_6, 500 and 125 MHz) data in Table 1; HRESIMS *m*/*z* 419.1486 [M − H]$^-$ (calcd for $C_{25}H_{23}O_6$, 419.1489).

Epimedonin D (**4**): yellow amorphous powder; IR (KBr) ν_{max} 3296, 1651, 1579, 1507, 1425 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 500 and 125 MHz) data in Table 1; HRFABMS *m*/*z* 435.1816 [M − H]$^-$ (calcd for $C_{26}H_{27}O_6$, 435.1808).

Determination of LPS-Induced NO and IL-1β Production in Macrophage RAW 264.7 Cells. The nitrite concentration in the medium was measured as an indicator of NO production as previously described.[26] Briefly, RAW 264.7 cells were seeded into 96-well tissue culture plates at a density of 2 × 10^6 cells/mL and stimulated with 1 μg/mL LPS in the presence or absence of the compounds. After incubation at 37 °C for 24 h, 100 μL of the culture supernatant was incubated with 100 μL of a Griess reagent (reagents A and B, Sigma) at room temperature for 10 min. The absorbance was measured at 550 nm against a calibration curve with sodium nitrite standards. The level of IL-1β in the culture medium was quantified using a commercially available ELISA kit (R&D Systems), according to the manufacturer's instructions. The viability of the remaining cells was determined with a cell counting kit (Dojindo, Tokyo, Japan)-based colorimetric assay.

RT-PCR Analysis. The total RNA was isolated using TRIZOL Reagent (Molecular Research Center, Cincinnati, OH). For RT-PCR, single-stranded cDNA was synthesized from 2 μg of the total RNA. The following primer sequences were used: iNOS, 5′-CCT TCC GAA GTT TCT GGC AGC AGC-3′ (sense) and 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′ (antisense); IL-1β, 5′-ATG GCA ATG TTC CTG AAC TCA ACT-3′ (sense) and 5′-CAG GAC AGG TAT AGA TTC TTT CCT TT-3′ (antisense); β-actin, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ (sense) and 5′-TAA AAC GCA GCT CAG TAACAG TCC G-3′ (antisense). The PCR products were fractionated on 1% agarose gels and stained with 5 μg/mL ethidium bromide.

ASSOCIATED CONTENT

Supporting Information

One-dimensional NMR, 2D NMR, and HRESIMS or HRFABMS spectra of compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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