Sesquiterpene Chromones from *Ferula fukanensis* and Their Nitric Oxide Production Inhibitory Effects[1]

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Five new sesquiterpene chromone derivatives, fukanefurochromones A−E (**1**−**5**), were isolated from a 80% aqueous methanol extract of the roots of *Ferula fukanensis*. The structures were elucidated on the basis of spectroscopic evidence, especially heteronuclear multiple-bond connectivity (HMBC) and high-resolution MS. The sesquiterpene chromone derivatives inhibited nitric oxide (NO) production and inducible NO synthase (iNOS) gene expression by a murine macrophage-like cell line (RAW 264.7), which was activated by lipopolysaccharide (LPS) and recombinant mouse interferon-γ (IFN-γ).

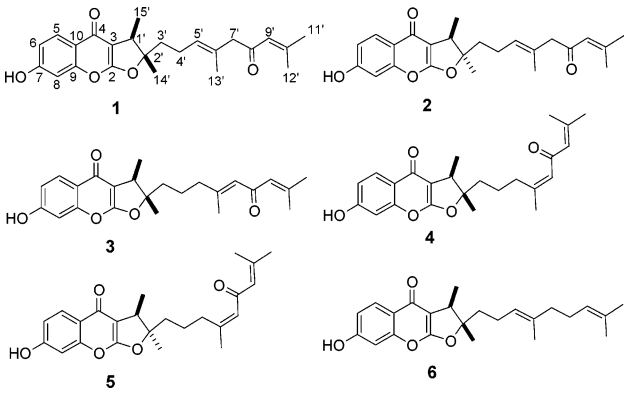
Ferula fukanensis grows on arid land in Central Asia and has been used as a traditional medicine for treatment of rheumatoid arthritis and bronchitis. Previous studies have analyzed the polysulfanes in this plant by GC-MS (CI/EI),[2] and the chemical constituents of plants in the genus *Ferula* (Umbelliferae) have been studied by many groups. In previous papers,[3−5] we reported the isolation of sesquiterpene coumarin derivatives and sesquiterpene phenylpropanoids from *F. fukanensis*. Compounds commonly found in this genus are sesquiterpenes[6−12] (especially daucanes, humulanes, and guaianes), sesquiterpene coumarins, and sesquiterpene chromones.[13−18] In our continuing investigations of in vitro anti-inflammatory effects of medicinal herbal extracts, the 80% aqueous methanol extract of the roots of *F. fukanensis* was observed to inhibit nitric oxide (NO) production in a lipopolysaccharide (LPS)-activated murine macrophage-like cell line.[3]

Macrophages play major roles in the immunity and inflammatory responses involved in host defense. Once activated, they initiate the production of cytokines, oxygen and nitrogen species, and eicosanoids. In macrophages, bacterial lipopolysaccharide (LPS), alone or in combination with recombinant mouse interferon-γ (IFN-γ), is the stimulus best known to induce the transcription of gene-encoding pro-inflammatory proteins. Such stimulation results in cytokine release and the synthesis of enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The nitric oxide (NO) radical is known to play a central role in inflammatory and immune reactions. However, excessive production of NO may cause tissue damage. In inflammatory diseases such as rheumatoid arthritis, excessive NO production by activated macrophages has been observed.

We discovered that an 80% aqueous MeOH extract of the roots of *F. fukanensis* (FFE) inhibited activated macrophage NO production (IC_{50} = 21.9 μg/mL).[3] Therefore, analysis of FFE was undertaken to identify the active compounds in the extract. Through bioactivity-guided fractionation, active compounds that inhibited NO production were isolated from the $CHCl_3$ fraction. These compounds also inhibited iNOS mRNA expression in RAW 264.7 cells treated with LPS and IFN-γ.

Results and Discussion

FFE was partitioned by successive extraction with $CHCl_3$, EtOAc, and H_2O. The $CHCl_3$-soluble fraction inhibited 60% of NO production at 30 μg/mL. Therefore, the $CHCl_3$-soluble fraction was chromatograghed on a silica gel column to yield 11 fractions. Fraction 6 inhibited 96.2% of NO production at 30 μg/mL, and fraction 8 inhibited 84.7% of NO production at 30 μg/mL. Compounds **1**−**6** were isolated from fractions 6 and 8.



Fukanefurochromone A (**1**) was obtained as a colorless oil, with a molecular weight of 396 based on fast atom bombardment mass spectrometry (FABMS) data that showed a protonated molecular ion peak at *m/z* 397 $(M + H)^+$ and a deprotonated molecular ion at *m/z* 395 $(M - H)^-$ in the negative mode. These data, together with the ^1H and ^{13}C NMR data (Tables 1, 2), suggest a molecular formula of $C_{24}H_{28}O_5$, which was supported by HRFABMS in the positive mode ($C_{24}H_{29}O_5$, *m/z* 397.2015). The MS data for compounds **1**−**5** showed the same molecular ions (see Experimental Section), which indicated that they were isomeric compounds.

The ^1H NMR data of **1** showed the presence of a 1,2,4-trisubstituted benzene ring at δ_H 8.01 (1H, d, J = 8.8 Hz, H-5), δ_H 6.99 (1H, dd, J = 2.2, 8.8 Hz, H-6), and δ_H 6.91 (1H, d, J = 2.2 Hz, H-8), and other signals similar to those of the sesquiterpene unit of 2,3-dihydro-7-hydroxy-2*S**,3*R**-dimethyl-2-[4,8-dimethyl-3(*E*),7-nonadien-6-onyl]furo[3,2-*c*]coumarin.[3,15] The remaining ^1H and ^{13}C NMR data of **1**, except for those of the sesquiterpene unit, indicated a 7-hydroxy-substituted chromone compound.[17,18]

In the HMBC spectrum of **1**, the correlations of H-5 with C-4, C-7 and C-9; H-15′ with C-1′, C-2′ and C-3; and H-1′ with C-2, C-4, C-3′, and C-14′ confirmed that C-1′ of the sesquiterpene unit was attached to C-3 of the chromone. On the basis of the above analysis, and combined with the ^{13}C NMR (δ_{C-2} 167.8, $\delta_{C-2'}$ 96.4) and the molecular formula

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of **1**, it was deduced that C-2 and C-2′ were linked by an oxygen atom.

The relative configuration of the dimethyldihydrofuran moiety at C-1′ and C-2′ and the configuration of the double bond of the sesquiterpene unit were determined on the basis of difference NOE experiments. Compound **1** showed significant NOE correlations between H-14′ and H-15′, between H-1′ and H-3′, and between H-5′ and H-7′. Thus, **1** is 2,3-dihydro-7-hydroxy-2*S**,3*R**-dimethyl-2-[4,8-dimethyl-3(*E*),7-nonadien-6-onyl]furo[3,2-*b*]chromone.

Fukanefurochromone B (**2**) was obtained as a colorless oil. HMBC experiments suggested that the structure of **2** was similar to that of **1**. However, the NMR spectra of **2** differed slightly from those of **1**, especially at C-3′ (δ_C 35.2 for **2**, δ_C 41.7 for **1**) and C-14′ (δ_C 25.8 for **2**, δ_C 20.7 for **1**), suggesting that **2** was a diastereomer of **1** at chiral centers C-1′ and C-2′. The relative configuration of the dimethyldihydrofuran moiety at C-1′ and C-2′ was determined on the basis of difference NOE experiments. Compound **2** showed significant NOE correlations between H-3′ and H-15′ and between H-1′ and H-14′, indicating a *trans* relationship between H-14′ and H-15′. Thus, **2** is 2,3-dihydro-7-hydroxy-2*R**,3*R**-dimethyl-2-[4,8-dimethyl-3(*E*),7-nonadien-6-onyl]furo[3,2-*b*]chromone.

Fukanefurochromone C (**3**) was obtained as a colorless oil. The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of **1**. However, the NMR spectra of **3** differed slightly from those of **1**, especially the H-13′ signal (δ_H 2.13 s for **3**, δ_H 1.60 s for **1**), C-6′ (δ_C 156.2 for **3**, δ_C 130.8 for **1**), and the C-8′ signal (δ_C 191.2 for **3**, δ_C 199.5 for **1**).

Analysis of the HMBC spectrum (between H-5′ and C-3′, C-4′, C-7′, between H-13′ and C-5′, C-6′, C-7′, between H-7′ and C-8′, C-9′, C-13′, between H-9′ and C-8′, C-7′) established the sesquiterpene unit. The relative configuration of the dimethyldihydrofuran moiety at C-1′ and C-2′ was determined on the basis of the difference NOE experiments. Compound **3** showed significant NOE correlations between H-14′ and H-15′, between H-1′ and H-3′, and between H-5′ and H-7′, indicating a *cis* relationship between H-14′ and H-15′. Furthermore, the double bond of the sesquiterpene unit of **3** has the *E* configuration. Thus, **3** is 2,3-dihydro-7-hydroxy-2*S**,3*R**-dimethyl-2-[4,8-dimethyl-4(*E*),7-nonadien-6-onyl]furo[3,2-*b*]chromone.

Fukanefurochromone D (**4**) was obtained as a colorless oil. The HMBC experiments suggested that the structure of **4** was similar to that of **3**. However, the NMR spectra of **4** differed slightly from those of **3**, especially C-13′ (δ_C 26.3 for **4**, δ_C 19.0 for **3**). The double bond of the sesquiterpene unit was determined on the basis of difference NOE experiments. Compound **4** showed significant NOE correlations between H-7′ and H-13′, indicating that the double bond of the sesquiterpene unit of **4** has the *Z* configuration. Thus, **4** is 2,3-dihydro-7-hydroxy-2*S**,3*R**-dimethyl-2-[4,8-dimethyl-4(*Z*),7-nonadien-6-onyl]furo[3,2-*b*]chromone.

Fukanefurochromone E (**5**) was obtained as a colorless oil. The ^{1}H and ^{13}C NMR spectra of **5** were similar to those of **4**. However, the NMR spectra of **5** differed slightly from those of **4**, especially at C-3′ (δ_C 34.7 for **5**, δ_C 41.9 for **4**) and C-14′ (δ_C 25.3 for **5**, δ_C 21.2 for **4**), suggesting that **5** was a diastereomer of **4** at chiral centers C-1′ and C-2′. The relative configuration of the dimethyldihydrofuran moiety at C-1′ and C-2′ was determined on the basis of difference NOE experiments. Thus, the structure of **5** is 2,3-dihydro-7-hydroxy-2*R**,3*R**-dimethyl-2-[4,8-dimethyl-4(*Z*),7-nonadien-6-onyl]furo[3,2-*b*]chromone.

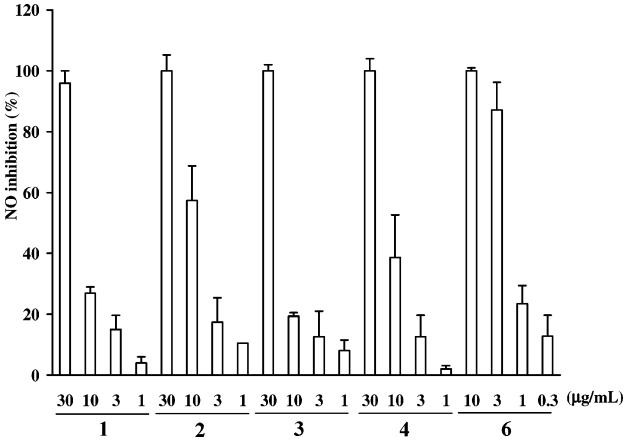

Figure 1. Inhibitory effects of compounds **1**−**4** and **6** on NO production stimulated by LPS and IFN-γ. RAW 264.7 cells were treated with LPS/IFN-γ alone or together with each compound at the concentrations indicated. After 16 h incubation, the supernatants were tested by Griess assay and the inhibitory rates were calculated. The experiment was performed three times, and the data are expressed as mean ± SD values.

Figure 2. Inhibitory effect of **4** and **6** on gene expression on stimulation with LPS and IFN-γ. RAW 264.7 cells were treated with LPS/IFN-γ alone or together with each compounds at the concentrations indicated. The untreated and treated cells were collected and examined by RT-PCR. The experiment was repeated twice, and a representative result is shown.

Compound **6** is a known compound, whose structure was confirmed by comparison with literature reports.[17]

Inhibitory effects of these compounds on the production of NO induced by LPS/IFN-γ were examined. Compounds **1**−**4** and **6** showed inhibitory activity [**1**: IC_{50} = 9.8 μg/mL (24.7 μM); **2**: IC_{50} = 8.9 μg/mL (22.5 μM); **3**: IC_{50} = 11.5 μg/mL (29.0 μM); **4**; IC_{50} = 9.5 μg/mL (24.0 μM); **6**: IC_{50} = 4.1 μg/mL (10.7 μM)] (Figure 1). The amount of **5** was so little that it could not be tested. Compounds **1**, **2**, **4**, and **6** showed stronger inhibition than quercetin on NO production. Quercetin exhibited a similar effect (IC_{50} = 26.8 μM).[19,20] Quercetin is reported to have an inhibitory effect on the production of NO by LPS-stimulated macrophage cell RAW 264.7. Cytotoxic effects of these compounds were measured using the MTT assay.[22] Compounds **1**−**4** (1−30 μg/mL) and **6** (0.3−10 μg/mL) did not show any significant cytotoxicity with LPS/IFN-γ treatment for 24 h. Furthermore, the RT-PCR analysis in the present study indicated that LPS/IFN-γ treatment increased the level of iNOS mRNA expression and that **4** inhibited this increase in a concentration-dependent manner (Figure 2). Compound **6** did not inhibit iNOS mRNA expression.

Inhibitors of NO production by macrophages act mainly through two mechanisms: one is the inhibition of iNOS expression, and the other is the inhibition of enzyme activity. In the case of **4**, it appears to be the former and **6** the latter.

Experimental Section

General Experimental Procedures. UV spectra were obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and IR spectra were recorded on a JASCO IR A-2 spectrophotometer. The NMR spectra were taken on a Mercury-300BB

Table 1. ^{1}H NMR Data for Compounds **1**−**4** (300 MHz) and **5** (500 MHz) ($CDCl_3$, TMS, δ (ppm), J = Hz)[a]

H	1	2	3	4	5
5	8.01 d (8.8)	8.03 d (8.8)	8.02 d (8.8)	8.00 d (8.4)	8.04 d (8.5)
6	6.99 dd (2.2,8.8)	7.00 dd (2.1, 8.8)	7.01 dd (2.3, 8.5)	6.97 dd (2.2,8.8)	7.00 dd (2.1, 8.5)
8	6.91 d (2.2)	6.91 d (2.1)	6.91 d (2.3)	6.88 d (2.2)	6.88 d (2.1)
1′	3.38 q (7.0)	3.30 q (7.0)	3.35 q (6.7)	3.37 q (7.0)	3.27 q (7.0)
3′	1.85 m	1.93 m	1.78 m	1.82 m	1.74, 1.94 m
4′	2.19 m	2.26 m	1.64 m	1.63 m	1.72 m
5′	5.20 t (7.5)	5.27 t (6.9)	2.16 m	2.63 t (7.4)	2.65, 2.73 m
7′	3.03 s	3.07 s	6.00 brs	6.02 m[b]	6.08 brs
9′	6.08 brs	6.11 brs	6.04 m	6.02 m[b]	6.05 brs
11′	1.87 s	1.89 s	1.88 s	1.87 s	1.88 s
12′	2.13 s	2.15 s	2.15 s	2.13 s	2.15 s
13′	1.60 s	1.66 s	2.13 s	1.86 s	1.91 s
14′	1.46 s	1.46 s	1.45 s	1.43 s	1.47 s
15′	1.35 d (6.6)	1.31 d (6.7)	1.35 d (6.7)	1.34 d (7.0)	1.30 d (7.0)

[a] Assignments were confirmed by decoupling, ^{1}H−^{1}H COSY, HMQC, HMBC, and difference NOE spectra. [b] Overlapped signal.

Varian spectrometer, with TMS as an internal standard. MS were obtained on a JEOL GCmate spectrometer. Column chromatography was carried out on silica gel (Wako gel C-300, WAKO Pure Chemical Industry Ltd.). TLC was performed on Merck TLC plates (0.25 mm thickness), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in EtOH and then heating on a hot plate. HPLC was performed on a JASCO PU-2089 apparatus equipped with a JASCO UV-2075 detector. YMC-Pak SIL-06 (10 × 150 mm i.d.) columns and YMC-PAK Pro-C_{18} (10 × 150 mm i.d.) were used for preparative purposes.

Plant Material. Dried roots of *F. fukanensis* were collected in the Urumqi, Xinjiang, People's Republic of China, in October 2002. Voucher specimens (NK03044) have been deposited at College of Pharmacy, Nihon University.

Extraction and Isolation. Dried roots of *F. fukanensis* (5.9 kg) were chopped and extracted twice with 80% MeOH (18 L). Solvent was evaporated under reduced pressure, and the extract (448 g) was suspended in H_2O (3.0 L) and partitioned with $CHCl_3$ (3 × 3 L) and EtOAc (3 × 3 L), successively. Evaporation of the solvent yielded a $CHCl_3$ fraction (272 g), an EtOAc fraction (142 g), and the aqueous fraction (96 g). The $CHCl_3$ fraction was subjected to silica gel column chromatography (12 × 17 cm, eluted with hexane and EtOAc in increasing polarity). The fractions (200 mL each) were combined according to TLC monitoring into 11 portions. Fraction 8, eluted with hexane−EtOAc (1:1), was isolated and further purified by column chromatography and reversed-phase HPLC (CH_3CN−H_2O, 56:44) to give **1** (23.8 mg), **2** (5.5 mg), **3** (19.6 mg), **4** (7.9 mg), and **5** (1.5 mg). Fraction 6, eluted with hexane−EtOAc (4:1), was further purified by column chromatography and reversed-phase HPLC (CH_3CN−H_2O, 65:35) to give **6** (65.6 mg).

Fukanefurochromone A (1): colorless oil; $[\alpha]^{23}_D$ ±0 (*c* 0.99, MeOH); UV (MeOH) λ_{max} (log ϵ) 293 (4.15), 241 (4.30), 211 (4.21) nm; IR (LF) ν_{max} 3114, 2978, 1682, 1617, 1554, 1502, 1451, 1385, 1255, 1166, 1091, 1056, 966, 895, 850, 775, 721 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.2015 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.2015).

Fukanefurochromone B (2): colorless oil; $[\alpha]^{23}_D$ ±0 (*c* 1.81, MeOH); UV (MeOH) λ_{max} (log ϵ) 293 (4.10) 243 (4.24), 208 (4.23) nm; IR (KBr) ν_{max} 3423, 2973, 1684, 1623, 1559, 1444, 1379, 1254, 1090, 964, 848, 775, 720 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.2016 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.2015).

Fukanefurochromone C (3): colorless oil; $[\alpha]^{23}_D$ −2.7 (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ϵ) 277 (4.32) 253 (4.29), 209 (4.14) nm; IR (LF) ν_{max} 3100, 2938, 1668, 1621, 1556, 1503, 1454, 1385, 1331, 1255, 1166, 1108, 1054, 968, 851, 755, 722 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.2017 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.2015).

Fukanefurochromone D (4): colorless oil; $[\alpha]^{23}_D$ +10.3 (*c* 0.64, MeOH); UV (MeOH) λ_{max} (log ϵ) 275 (4.38) 252 (4.35), 210 (4.21) nm; IR (KBr) ν_{max} 3099, 2977, 2938, 1668, 1628, 1554, 1502, 1451, 1383, 1332, 1254, 1165, 1110, 1090, 1057, 968, 851, 773, 721 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.2007 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.2015).

Table 2. ^{13}C NMR Data for Compounds **1**−**4** (75 MHz) and **5** (125 MHz) ($CDCl_3$, TMS, δ (ppm))[a]

C	1	2	3	4	5
2	167.8	167.7	167.2	168.2	167.7
3	99.0	99.4	98.3	99.3	99.2
4	176.1	176.1	175.6	176.5	176.0
5	126.9	127.0	126.3	127.28	126.7
6	115.0	115.0	114.5	115.3	114.6
7	161.7	161.5	161.2	161.9	161.2
8	103.5	103.5	102.9	103.9	103.3
9	155.0	155.1	154.5[c]	155.4[c]	155.03
10	116.7	116.8	116.0	117.1	116.8
1′	41.6	43.7	41.1	42.1	43.2
2′	96.4	95.9	95.7	97.1	96.0
3′	41.7	35.2	40.8	41.9	34.7
4′	22.7	23.4	21.2	22.8	22.7
5′	128.0	128.3	40.9	34.1	33.5
6′	130.8	130.8	156.2	157.9	157.6
7′	55.5	55.5	125.7[b]	127.3	126.9
8′	199.5	199.5	191.2	191.6	191.4
9′	123.0	123.1	125.7[b]	126.8	126.2
10′	156.7	156.6	154.7[c]	155.5[c]	154.96
11′	28.3	28.3	27.7	28.7	27.8
12′	21.4	21.4	20.6	21.6	20.7
13′	17.0	17.1	19.0	26.3	25.5
14′	20.7	25.8	20.1	21.2	25.3
15′	15.1	14.7	14.5	15.5	14.1

[a] Assignments were confirmed by decoupling, ^{1}H−^{1}H COSY, HMQC, HMBC, and difference NOE spectra. [b] Overlapped signal. [c] Assignments may be interchanged.

Fukanefurochromone E (5): colorless oil; IR (KBr) ν_{max} 3398, 2971, 2928, 1625, 1559, 1444, 1377, 1333, 1255, 1161, 1111, 1088, 849, 773, 720 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.2022 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.2015).

Nitrite Assay.[22,23] RAW 264.7 cells, a mouse macrophage-like cell line transformed with the Abelson leukemia virus, were obtained from the American Type Culture Collection (Rockville, MD). The cells were incubated in Ham's F12 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 50 μg/mL kanamycine. Cell concentration was adjusted to 1.2 × 10^6 cells/mL, and 200 μL of cell suspension was seeded in each well of a 96-well flat-bottomed plate. After a 2 h incubation, cells were treated with *Escherichia coli* LPS (100 ng/mL), recombinant mouse IFN-γ (0.33 ng/mL), and test samples dissolved in DMSO (final DMSO concentration 0.2%, v/v) for 16 h at 37 °C. The culture supernatant (100 μL) was placed in each well, along with samples in duplicate, in a 96-well flat-bottomed plate. A standard solution of $NaNO_2$ was also placed in other wells on

the same plate. Griess reagent (50 μL of 1% sulfanilamide in 5% H_3PO_4, and 50 μL of 0.1% *N*-1-naphthylethylenediamide dihydrochloride) was added to each well. After 10 min, the reaction products were quantified at 550 nm, with subtraction of the background absorbance at 630 nm, using a microplate reader. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[20] An MTT solution (200 μg/mL) was added after 16 h treatment, and the incubation was continued for another 8 h at 37 °C. The reduced MTT-formazan was dissolved in 150 μL of DMSO, and the absorbance of the MTT-formazan solution at 550 nm was measured using a microplate reader. The percentage of suppression was calculated by comparing the absorbance of sample-treated cells with that of nontreated cells.

Reverse Transcription-Polymerase Chain Reaction Analysis of iNOS mRNA.[21−23] Cell concentration was adjusted to 1.2×10^6 cells/mL, and 800 μL (200 μL $\times$ 4) of cell suspension was seeded in each well of a 96-well flat-bottomed plate. After a 2 h incubation, cells were treated with LPS (100 ng/mL), IFN-γ (0.33 ng/mL), and test samples dissolved in DMSO (final DMSO concentration 0.2%, v/v) for 8 h at 37 °C. After incubation, the cells were collected in a microfuge tube, chilled on ice, and then centrifuged at 2800*g* for 1 min. Total RNA was isolated from the cell pellet using a RNA isolation kit (QIAGEN, Hilden, Germany). Total RNA (250 ng) was reverse-transcribed into cDNA by oligo $(dT)_{12-18}$ primer. The PCR sample contained 30 μL of the reaction mixture, comprised of 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dNTP, 0.6 units of Ampli Taq GOLD (Applied Biosystems, CA), and 0.4 μmol of sense and antisense primers. The sense primer for iNOS was 5′-ACCTACTTCCTGGACATTACGACCC-3′, and the antisense primer was 5′-AAGGGAGCAATGCCCGTACCAGGCC-3′. The sense primer for glyceraldehydes-3-phosphatedehydrogenase (GAPDH) was 5′-ACCACAGTCCATGCCATCAC-3′, and the antisense primer was 5′-TCCACCACCCTGTTGCTGTA-3′. The PCR reaction was performed under the following conditions: 25 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1.5 min, using a thermal cycler (GeneAmp PCR Systems 9770; PE Applied Biosystems). The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The bands in the gel were then photographed.

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