Effects of Constituents from the Bark of *Magnolia obovata* on Nitric Oxide Production in Lipopolysaccharide-Activated Macrophages

Hisashi MATSUDA, Tadashi KAGEURA, Mamiko ODA, Toshio MORIKAWA, Yasuko SAKAMOTO, and Masayuki YOSHIKAWA*

Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607–8412, Japan.
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The methanolic extract from a Japanese herbal medicine, the bark of *Magnolia obovata*, was found to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages. By bioassay-guided separation, three neolignans (magnolol, honokiol, obovatol) and three sesquiterpenes (α-eudesmol, β-eudesmol, γ-eudesmol) were obtained as active constituents. A trineolignan (magnolianin), a phenylpropanoid glycoside (syringin), lignan glycosides (liriodendrin, (+)-syringaresinol 4′-*O*-β-D-glucopyranoside) and a sesquiterpene (caryophyllene oxide) did not show any activity. On the other hand, sesquiterpene-neolignans (eudesmagnolol, clovanemagnolol, caryolanemagnolol, eudeshonokiol A, eudesobovatol A) showed the strong cytotoxic effects. Active constituents (magnolol, honokiol, obovatol) showed weak inhibition for inducible NO synthase (iNOS) enzyme activity, but potent inhibition of iNOS induction and activation of nuclear factor-κB.

Key words *Magnolia obovata*; neolignan; nitric oxide; inducible nitric oxide synthase; nuclear factor-κB; inhibitor

Inducible nitric oxide synthase (iNOS) is involved in pathological processes related to overproduction of nitric oxide (NO), and is expressed in response to pro-inflammatory agents such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS) in various cell types including macrophages, endothelial cells, and smooth muscle cells.[1] Nuclear factor (NF)-κB is a major transcription factor involved in iNOS, TNF-α, IL-1β, and IL-8 genes expression. NF-κB activation involves dissociation of an inhibitory subunit, IκB, which keeps NF-κB in the cytoplasm, thereby preventing activation of the target gene in the nucleus. Cellular signals lead to phosphorylation of IκB following elimination of IκB from NF-κB by proteolytic degradation. Then, the activated-NF-κB is released and translocated into the nucleus to activate transcription of its target genes.[2] Inhibition of iNOS enzyme activity or iNOS induction and inhibition of NF-κB activation may be of therapeutic benefit in various types of inflammation.[2,3]

Magnoliae Cortex, the dried bark of *Magnolia* (*M.*) *obovata* THUNBERG and *M. officinalis* REHDER et WILSON, has been used for treatment of gastrointestinal disorders, anxiety and allergic diseases including bronchial asthma in Japanese and Chinese traditional medicines. There have been many pharmacological reports of the activities of extracts or constituents from the bark of *M. obovata* or *M. officinalis* such as muscle relaxation,[4] central depressant effect,[5] anti-gastric ulcer,[6] vasorelaxant,[7] antiallergic,[8] antibacterial,[9] and neurite spouting activities.[10] In the course of our studies on constituents with NO production inhibitory activity from natural medicines,[11] the methanolic extract from the dried bark of *M. obovata* was found to inhibit nitrite (NO_2^-, a product of NO) accumulation in LPS-activated mouse macrophages (IC_{50}=25 μg/ml).

Previous reports demonstrated that two neolignans [magnolol (**1**), honokiol (**2**)] showed inhibitory effects on NO production from LPS-activated RAW 264.7 cells.[12] However, effects of other constituents on NO production from LPS-activated mouse macrophages and their cytotoxicities for macrophages have not been examined. This report describes the effects of the constituents from the bark of *M. obovata* on NO production in LPS-stimulated macrophages. In addition, we describe the effects of principal active neolignan constituents [magnolol (**1**), honokiol (**2**), obovatol (**3**)] on iNOS enzyme activity, induction of iNOS, and activation of NF-κB to clarify their action mechanisms.

Results and Discussion

Isolation of Chemical Constituents from the Dried Bark of *M. obovata* The bark of Japanese *M. obovata* was extracted with methanol under reflux. The methanolic extract was subjected to ordinary- and reversed-phase silica gel column chromatography and finally HPLC to furnish five neolignans, magnolol (**1**,[13] 2.1% from the natural medicine), honokiol (**2**,[14] 0.43%), obovatol (**3**,[15] 0.26%), 4-*O*-methylhonokiol (**4**,[16] 0.0031%), and 6′-*O*-methylhonokiol (**5**,[10,16] 0.0031%), seven sesquiterpene-neolignans, eudesmagnolol (**6**,[17] 0.096%), clovanemagnolol (**7**,[18] 0.0061%), caryolanemagnolol (**8**,[10] 0.0044%), eudeshonokiols A (**9**,[10] 0.0056%) and B (**10**,[10] 0.0054%), eudesobovatols A (**11**,[19] 0.043%) and B (**12**,[19] 0.024%), a trineolignan, magnolianin (**13**,[20] 0.27%), a phenylpropanoid glycoside, syringin (**14**,[21] 0.39%), two lignan glycosides, liriodendrin (**15**,[22] 0.15%) and (+)-syringaresinol 4′-*O*-β-D-glucopyranoside (**16**,[23] 0.029%), and four sesquiterpenes, caryophyllene oxide (**17**,[24] 0.0049%), α-eudesmol (**18**,[11g,24] 0.096%), β-eudesmol (**19**,[11g,25] 0.061%), and γ-eudesmol (**20**,[11g,26] 0.030%).

Effects on NO Production First, the effects of neolignans (**1**—**5**), sesquiterpene-neolignans (**6**—**12**) and sesquiterpenes (**17**) isolated from the methanolic extract on nitrite accumulation from LPS-activated macrophages were examined. Nitrite, an oxidative product of NO, was accumulated in the medium after 20-h of incubation with LPS. Nitrite concentration in the medium without inhibitors (control group) was 36.9±9.5 μM, and that in the medium without LPS (unstimulated group) was 0.4±0.8 μM (mean±S.D. of 18 experiments). IC_{50} of reference compounds [caffeic acid phenethyl ester (CAPE, an inhibitor of NF-κB activation),[3b] N^G-monomethyl-L-arginine (L-NMMA, a non-selective in-

* To whom correspondence should be addressed. e-mail: shoyaku@mb.kyoto-phu.ac.jp

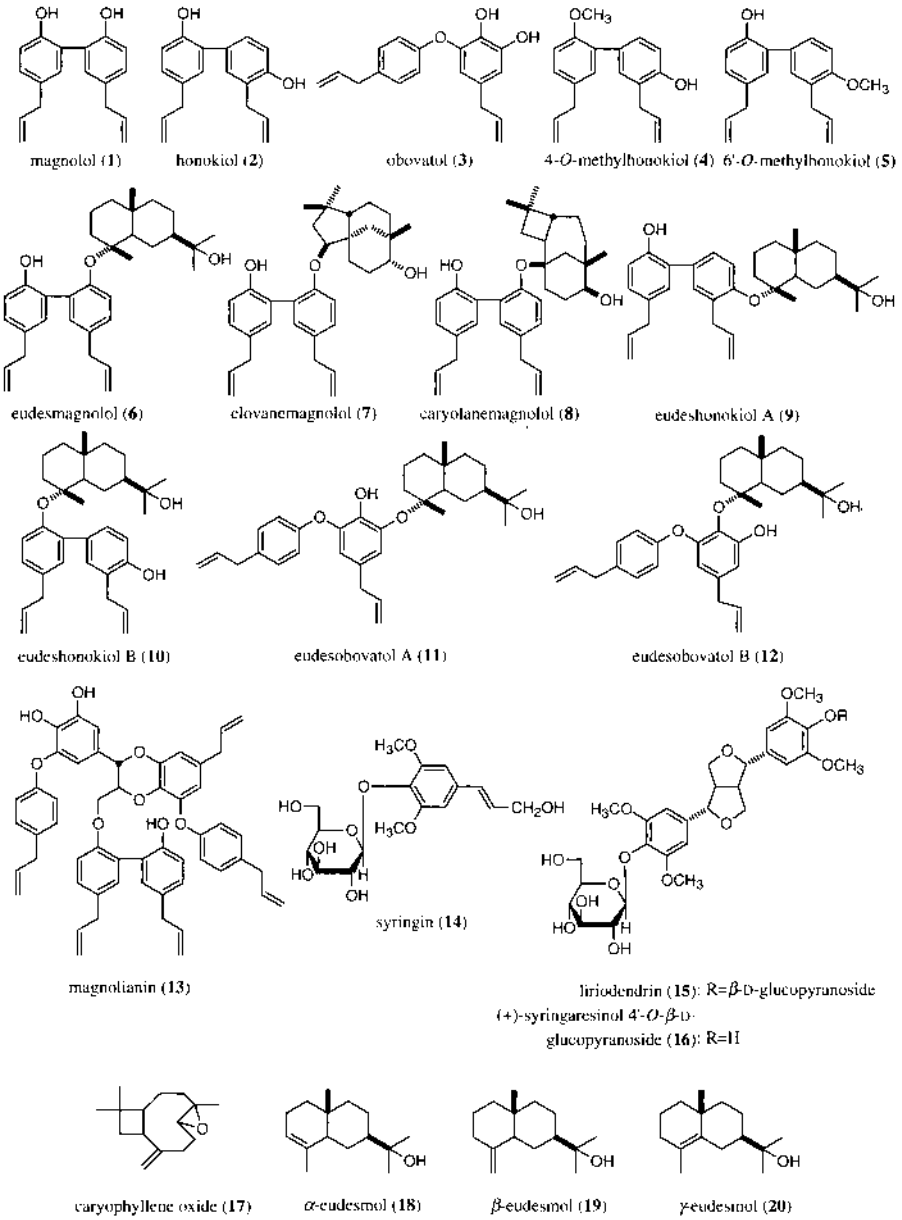

Chart 1

hibitor of NOS),[27] and guanidinoethyldisulfide (GED, an inhibitor of iNOS)[28]] were 4.0, 28, and 1.4 μM, respectively.[11g] Three neolignans [magnolol (**1**), honokiol (**2**), obovatol (**3**)] inhibited the LPS-induced NO production (IC_{50}=21—34 μM). 4-*O*-Methylhonokiol (**4**) and 6′-*O*-methylhonokiol (**5**) showed less activity than **2**. Eudesmagnolol (**6**), clovanemagnolol (**7**), caryolanemagnolol (**8**), eudeshonokiols A (**9**) and B (**10**), and eudesobovatols A (**11**) and B (**12**) showed the inhibition due to their cytotoxic effects. Magnolianin (**13**), syringin (**14**), liriodendrin (**15**), (+)-syringaresinol 4′-*O*-β-D-glucopyranoside (**16**), and caryophyllene oxide (**17**) had no effect. In our previous study of NO production inhibitors from the leaves of *Laurus nobilis*, three sesquiterpene constituents [α-eudesmol (**18**), β-eudesmol (**19**), and γ-eudesmol (**20**)] were found to show the activity. Under the same conditions, their IC_{50} values were 37, 44, and 53 μM, respectively.[11g] These results indicated that the conjugation of methyl or sesquiterpene to the hydroxyl group of neolignan (**1**—**3**) reduced the activity, although the detailed structure–activity relationships still have to be clarified.

Eudesmols (**18**—**20**) did not show any cytotoxic effect, and neolignans (**1**—**3**) showed cytotoxic effects only at 100 μM. However, cytotoxic effects of sesquiterpene-neolignan constituents (**6**—**9**, **11**) were observed at lower concentrations in the MTT assay.

Effects of 1—3 on iNOS Enzyme Activity, iNOS Protein Induction, and NF-κB Activation Next, the effects of three active constituents (**1**—**3**) on iNOS enzyme activity and iNOS induction were examined. A reference compound, L-NMMA, inhibited iNOS enzyme activity with an IC_{50} of 13 μM, but **1**—**3** showed weak inhibition for iNOS activity; inhibitory effects of **1**, **2**, and **3** at 100 μM were 16, 18, and 6%, respectively. iNOS was detected at 130 kDa after a 12-h incubation with LPS by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE)–Western blotting analysis (Fig. 1). In agreement with the results of a previous

Table 1. Inhibitory Effects of Constituents from the Bark of *M. obovata* on NO_2^- Accumulation in LPS-Activated Mouse Macrophages

Compounds	Inhibition (%)				
	3	10	30	100	IC_{50} (μM)
Neolignans					
Magnolol (**1**)	1.4±2.9	9.2±1.9**	40.3±2.8**	100.8±1.0**,*a)*	34
Honokiol (**2**)	−1.2±3.8	11.2±2.1*	73.2±3.3**	102.0±1.4**,*a)*	21
Obovatol (**3**)	−1.8±7.6	1.1±13.5	50.2±6.0**	80.9±4.4**,*a)*	28
4-*O*-Methylhonokiol (**4**)	8.1±2.2	2.5±6.5	9.8±2.7	56.5±8.6**	88
6′-*O*-Methylhonokiol (**5**)	−3.4±3.7	−3.4±5.7	31.0±3.3	32.8±2.0	>100
Sesquiterpene-neolignans					
Eudesmagnolol (**6**)	−16.3±1.8	−12.1±4.0	28.4±4.8**,*a)*	98.3±1.1**,*a)*	—
Clovanemagnolol (**7**)	6.9±6.9	10.1±3.0	76.3±5.5**,*a)*	99.0±2.7**,*a)*	—
Caryolanemagnolol (**8**)	2.9±0.8	11.4±1.5*a)*	20.6±1.8**,*a)*	97.5±0.5**,*a)*	—
Eudeshonokiol A (**9**)	−2.7±3.1	−3.5±2.8	25.9±3.0**,*a)*	99.7±0.2**,*a)*	—
Eudeshonokiol B (**10**)	−9.6±2.9	−7.0±2.1	10.9±2.6**	97.3±0.4**,*a)*	—
Eudesobovatol A (**11**)	−13.4±1.3	9.8±1.4	12.2±3.3*a)*	93.7±3.0**,*a)*	—
Eudesobovatol B (**12**)	−1.2±5.0	12.6±6.3**	37.2±2.5**	89.2±1.9**,*a)*	—
Trineolignan					
Magnolianin (**13**)	−12.9±2.4	−9.2±2.3	−8.6±4.1	−12.9±2.6	>100
Phenylpropanoid glycoside					
Syringin (**14**)	−2.4±2.6	−1.6±3.3	−3.4±2.0	9.4±3.1	>100
Lignan glycosides					
Liriodendrin (**15**)	−0.1±4.6	−5.2±3.7	−3.8±4.6	−3.5±3.6	>100
(+)-Syringaresinol					
4′-*O*-β-D-glucopyranoside (**16**)	7.6±2.9	4.4±4.1	9.8±2.7	13.5±6.6	>100
Sesquiterpene					
Caryophyllene oxide (**17**)	11.1±7.3	−4.6±7.7	0.5±9.6	26.0±6.4**	>100

Values represent means±S.E.M. (n=4), and IC_{50} values were determined graphically. Asterisks denote significant differences from each control at $*p<0.05$, $**p<0.01$. *a*) Cytotoxic effects were observed.

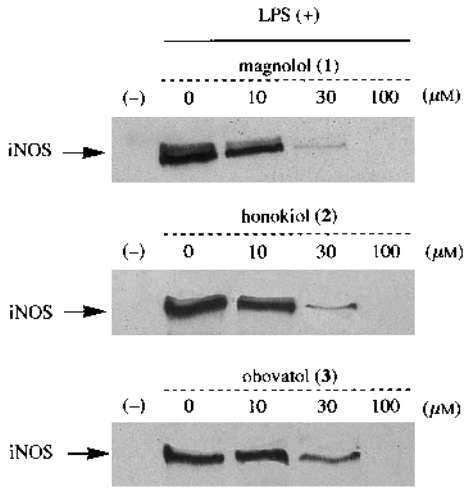

Fig. 1. Effects of Neolignans (**1**—**3**) on iNOS Induction in LPS-Activated Mouse Macrophages

study using RAW 264.7 cells,[12] iNOS induction of LPS-activated macrophages was suppressed by **1** and **2** in a concentration-dependent manner. In addition, another principal neolignan, obovatol (**3**), showed similar effects.

Finally, the effects of **1**—**3** on activation of NF-κB were examined by electrophoretic mobility shift assay. Cells were incubated with or without LPS and the test sample for 4 h, and proteins of the cell lysate were added to reaction mixtures containing NF-κB consensus oligonucleotide labeled with ^{32}P-ATP. The oligonucleotide–protein complex was separated electrophoretically. Detection of oligonucleotide–NF-κB was prevented by **1**—**3** in a concentration-dependent manner (Fig. 2). Moreover, the TNF-α gene is known to be a target gene of NF-κB,[2] and TNF-α expression was reported to be suppressed by **1** and **2**.[12] These findings indicate that the active constituents (**1**—**3**), at least in part, inhibit the upstream signaling pathway of NF-κB activation following iNOS expression, thereby preventing NO production.

NO produced by constitutive NOS (cNOS) is important for vasodilation as an endothelium-derived relaxing factor (EDRF).[29] The present study demonstrated that constituents (**1**—**3**, **18**—**20**) inhibited NO by iNOS in LPS-activated macrophages. However, the effects of the constituents on cNOS have yet to be determined. The principal constituents, magnolol (**1**), honokiol (**2**), and β-eudesmol (**19**), were reported to exhibit vasodilation due to Ca^{2+}-blocking or EDRF-like activities in the isolated rat thoracic aorta.[8,30] In conclusion, neolignans (**1**—**3**) and sesquiterpenes (**18**—**20**) from *M. obovata* may be effective for treatment of pathological processes including inflammation and endotoxic shock without vasocontraction.

Experimental

The following instruments were used to obtain physical data : specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); UV spectra, Shimadzu UV-1200 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H-NMR spectra, JNM-LA500 (500 MHz), JEOL EX-270 (270 MHz) spectrometer; ^{13}C-NMR spectra, JNM-LA500 (125 MHz), JEOL EX-270 (68 MHz) spectrometers with tetramethylsilane as an internal standard; MS and high-resolution MS, JEOL JMS-GCMATE mass spectrometer, JEOL JMS-SX 102A mass spectrometer.

The following experimental conditions were used for chromatography: ordinary-phase column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh), reversed-phase column chromatography; Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh): TLC, pre-coated TLC plates with Silica gel $60F_{254}$ (Merck, 0.25 mm) (normal-phase) and Silica gel RP-18 $60F_{254}$ (Merck, 0.25 mm) (reversed-

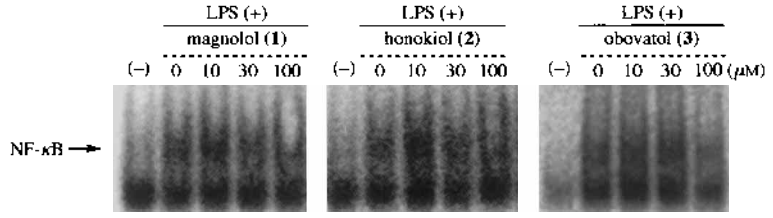

Fig. 2. Effects of Neolignans (**1**—**3**) on LPS-Induced Activation of NF-κB in Macrophages

phase); HPTLC, pre-coated TLC plates with Silica gel RP-18 $60WF_{254s}$ (Merck, 0.25 mm) (reversed-phase). Detection was done by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

Extraction and Isolation The dried bark of *Magnolia obovata* Thunberg (5.0 kg, cultivated in Japan and purchased from MAE CHU Co., Ltd., Nara, Japan) were finely minced and extracted with methanol (20 l) under reflux three times. Evaporation of the solvent under reduced pressure gave the methanolic extract (885 g). The methanolic extract (200 g) was subjected to ordinary-phase silica gel column chromatography [3.0 kg, *n*-hexane–AcOEt (7 : 1→3 : 1)→$CHCl_3$–MeOH (10 : 1)→$CHCl_3$–MeOH–H_2O (6 : 4 : 1)→MeOH] to afford six fractions [fr. 1 (19.2 g), fr. 2 (52.0 g), fr. 3 (12.4 g), fr. 4 (23.7 g), fr. 5 (30.5 g), fr. 6 (62.2 g)]. Fraction 1 (12.5 g) was further subjected to reversed-phase silica gel column chromatography [MeOH–H_2O (75 : 25)→MeOH] and finally HPLC [YMC-pack SIL, *n*-hexane–AcOEt (20 : 1)] to furnish 4-*O*-methylhonokiol (**4**, 23 mg), 6′-*O*-methylhonokiol (**5**, 23 mg), caryophyllene oxide (**17**, 36 mg), β-eudesmol (**19**, 449 mg), and γ-eudesmol (**20**, 221 mg). Fraction 2 (11.6 g) was separated by reversed-phase silica gel column chromatography [MeOH–H_2O (80 : 20→90 : 10)→MeOH] and finally HPLC [YMC-pack ODS-A, (1) MeOH–H_2O (80 : 20), (2) MeOH–H_2O (90 : 10), or (3) CH_3CN–H_2O (70 : 30)] to furnish magnolol (**1**, 5.3 g), honokiol (**2**, 1.1 g), obovatol (**3**, 656 mg), eudesmagnolol (**6**, 242 mg), clovanemagnolol (**7**, 15 mg), caryolanemagnolol (**8**, 11 mg), eudesobovatols A (**11**, 108 mg) and B (**12**, 61 mg), and α-eudesmol (**18**, 242 mg). Fraction 3 (9.0 g) was purified by reversed-phase silica gel column chromatography [MeOH–H_2O (70 : 30→80 : 20→90 : 10)→MeOH] to furnish magnolianin (**13**, 2.2 g) and the other fraction. The other fraction was finally purified by HPLC [YMC-pack ODS-A, MeOH–H_2O (65 : 35) and CH_3CN–H_2O (80 : 20), and YMC-pack SIL, *n*-hexane–AcOEt (5 : 1)] to furnish eudeshonokiols A (**9**, 46 mg) and B (**10**, 44 mg). Fraction 5 (5.0 g) was subjected to ordinary-phase silica gel column chromatography [$CHCl_3$–MeOH–H_2O (10 : 3 : 1, lower layer→65 : 35 : 10, lower layer)→MeOH], reversed-phase silica gel column chromatography [MeOH–H_2O (30 : 70→40 : 60→50 : 50→90 : 10)→MeOH], and finally HPLC [YMC-pack ODS-A, (1) MeOH–H_2O (25 : 75), (2) MeOH–H_2O (35 : 65), or (3) MeOH–H_2O (40 : 60)] to furnish syringin (**14**, 722 mg), liriodendrin (**15**, 278 mg), and (+)-syringaresinol 4′-*O*-β-D-glucopyranoside (**16**, 54 mg). These constituents were identified by comparison of their physical data with reported value.[10,13—26]

Reagents LPS (from *Salmonella enteritidis*) and L-NMMA were purchased from Sigma; 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) was from Dojin; RPMI 1640 was from Gibco; protease inhibitor cocktail (Complete Mini) was from Boehringer Mannheim; fetal calf serum (FCS) was from Bio Whittaker; anti-mouse iNOS antibody (monoclonal) was from Transduction Laboratories; anti-mouse IgG antibody conjugated to horseradish peroxidase and the enhanced chemiluminescense (ECL) kit, L-[U-^{14}C]-arginine, γ-[^{32}P]-ATP were from Amersham; thioglycolate (TGC) medium was from Nissui Seiyaku; iNOS was from OXIS International; NF-κB consensus oligonucleotide and T4 polynucleotide kinase were from Promega; Aquasol-2 was from Packard, and all other chemicals were from Wako. Nitrocellulose membranes (0.25 μm) were purchased from Bio Rad; 96-well microplates and culture dishes (6 cm) were from Nunc; and spin column (UFC30SV00) were from Millipore.

Screening for NO Production Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice by washing with 6—7 ml of ice-cold phosphate-buffered saline (PBS), and cells (5×10^5 cells/well) were suspended in 200 μl of RPMI 1640 supplemented with 5% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml), and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing with PBS, and the adherent cells (more than 95% macrophages as determined by Giemsa staining) were cultured in fresh medium containing 10 μg/ml LPS and test compound (3, 10, 30, and 100 μM) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent.[31] Cell viability was determined by MTT colorimetric assay. Briefly, after 20-h incubation with test compounds, MTT (10 μl, 5 mg/ml in PBS) solution was added to the wells. After a further 4 h in culture, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm). Each test compound was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.5%).

$$\text{inhibition (\%)} = \frac{A-B}{A-C}\times 100$$

A—C: NO_2^- concentration (μM)

[A: LPS (+), sample (−);
B: LPS (+), sample (+);
C: LPS (−), sample (−)]

Detection of iNOS In this experiment, peritoneal exudate cells were obtained from the peritoneal cavities of male ddY mice that had been intraperitoneally injected with 4% TGC medium 4 d previously to get a large numbers of cells. Cells (7.5×10^6 cells/3 ml/dish) were pre-cultured in culture dishes (6 cm i.d.) for 1 h, and the adherent cells (more than 95% macrophages) were obtained as described above. After washing, the culture medium was exchanged for fresh medium containing 5% FCS, 20 μg/ml LPS and test compound for 12 h. Cells were collected in lysis buffer [100 mM NaCl, 10 mM Tris, Complete Mini (1 tab/10 ml), 0.1% Triton X-100, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)] and sonicated. After determination of protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer.[32] For SDS-PAGE, aliquots of 50 μg of protein from each sample were subjected to electrophoresis in 10% polyacrylamide gels. Following electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were incubated with 5% nonfat dried milk in Tris–buffered saline (TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with mouse monoclonal IgG (dilution of 1 : 1000) against iNOS. The blots were washed in TBS and probed with secondary antibody, anti-mouse IgG antibody conjugated to horseradish peroxidase (dilution of 1 : 5000). Detection was performed using an ECL kit and X-ray film (Hyper Film, Amersham).

Electrophoretic Mobility Shift Assay TGC-induced peritoneal macrophages (7.5×10^6 cells/3 ml/dish) was prepared as described above. Cells were cultured in RPMI 1640 supplemented with 5% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml), 20 μg/ml LPS and test compound for 4 h. Cells were collected in ice-cold PBS and resuspended in four cell volumes of lysis buffer [420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 25% glycerol, 1% Nonidet P40, 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7.9]. The cell lysate was incubated on ice for 1 h, then centrifuged at 13000 rpm at 4 °C for 5 min. The protein content of each supernatant was determined, and equal amounts of protein (20 μg) were added to reaction mixtures containing 20 μg bovine serum albumine and ^{32}P-labeled NF-κB consensus oligonucleotide. The oligonucleotide–protein complex was separated by non-denaturing polyacrylamide gel electrophoresis (Gel Shift Assay Kit, Promega), and autoradiography was performed using an imaging analyzer (BAS 5000, Fuji Film). ^{32}P-labeled NF-κB consensus oligonucleotide was labeled using γ-[^{32}P]-ATP (3000 Ci/mmol) and T4 polynucleotide kinase.

iNOS Enzyme Activity iNOS enzyme activity was determined as follows. NOS activity was measured by monitoring the conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline. Briefly, test sample solution (5 μl) and 40 μl of substrate and coenzyme solution [100 μM arginine (containing 50 nCi L-[^{14}C]-arginine), 1 mM nicotinamide adenine dinucleotide (reduced form, NADPH), 3 μM tetrahydrobiopterin (BH_4), 1 μM FAD, 1 μM flavin mononucleotide (FMN) in 25 mM Tris–HCl buffer (pH 7.4)] were pre-incu-

bated at 37 °C for 10 min. iNOS (20 mU/5 μl) was then added to the reaction mixture. After incubation at 37 °C for 30 min, the reaction was terminated by addition of 400 μl of ice-cold buffer containing 5 mM EDTA and 50 mM HEPES (pH 5.5). The substrate was adsorbed on AG 50W X-8 ion-exchange resin (Na^+ form, 60—70 mg) packed in spin columns. The L-citrulline, which is ionically neutral at pH 5.5, flowed through the column completely,[33] and was mixed with a scintillation cocktail (Aquasol-2) and radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman). Test compound was dissolved in DMSO and diluted with Tris–HCl buffer (pH 7.4) (final concentration of DMSO: 2%).

Statistical Analysis Values were expressed as means±S.D. or S.E.M. One-way analysis of variance following Dunnett's test for multiple comparison analysis were used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

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