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), ligan 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.3,4)

*Magnoliae Cortex*, the dried bark of *Magnolia* (M.) obovata THUNBERG and *M. officinalis* REIDER 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,5) central depressant effect,6) anti-gastric ulcer,7) vasorelaxant,8) antiallergic,9) and neu- rite 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 (NO₂⁻), a product of NO) accumulation in LPS-activated mouse macrophages (IC₅₀ = 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 (5,17) 0.0031%, seven sesquiterpene-neolignans, eudesmagnolol (6),18) 0.096%, clovanemagnolol (7,19) 0.0061%, caryolane-magnolol (8,20) 0.0044%, eudeshonokiol A (9),21) 0.0056% and B (10,22) 0.0054%, eudesobovatol A (11,23) 0.043%, and B (12,24) 0.024%, a trineolignan, magnolianin (13,25) 0.27%, a phenylpropanoid glycoside, syringin (14,26) 0.39%, two lignan glycosides, liriodendrin (15,27) 0.15% and (+)-syringaresinol 4'-O-β-D-glucopyranoside (16,28) 0.029%, and four sesquiterpenes, caryophyllene oxide (17,29) 0.0499%, α-eudesmol (18),30) 0.096%, β-eudesmol (19,31) 0.061%), and γ-eudesmol (20,32) 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). IC₅₀ of reference compounds [caffeeic acid phenethyl ester (CAPE), an inhibitor of NF-κB activation),33) N⁵-[monomethyl-L-arginine (L-NMMA, a non-selective in-
hibitor of NOS),27) and guanidinoethylsulfide (GED, an inhibitor of iNOS)28) were 4.0, 28, and 1.4 μM, respectively.11

Three neolignans [magnolol (1), honokiol (2), obovatol (3)] inhibited the LPS-induced NO production (IC<sub>50</sub>=21—34 μM). 4-O-Methylhonokiol (4) and 6'-O-methylhonokiol (5) showed less activity than 2. EudesmaglonoLol (6), clovane-
magnolol (7), caryolanemagnolol (8), eudeshonokiol 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 carophyllene oxide (17) had no effect. In our previous study of NO production inhibitors from the leaves of <i>Laurus nobilis</i>, three sesquiter-
pene constituents [α-eudesmol (18), β-eudesmol (19), and γ-eudesmol (20)] were found to show the activity. Under the same conditions, their IC<sub>50</sub> values were 37, 44, and 53 μM, respectively.11) These results indicated that the conjugation of methyl or sesquiterpene to the hydroxyl group of neo-

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<sub>50</sub> 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–polyacryl-
lam ide 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 NO2\(^{-}\) Accumulation in LPS-Activated Mouse Macrophages

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Inhibition (%)</th>
<th>IC(_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Neolignans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnolol (1)</td>
<td>1.4±2.9</td>
<td>9.2±1.9**</td>
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<tr>
<td>Honokiol (2)</td>
<td>12.2±3.8</td>
<td>11.2±2.1*</td>
</tr>
<tr>
<td>Obovatol (3)</td>
<td>1.8±7.6</td>
<td>1.1±13.5</td>
</tr>
<tr>
<td>4-O-Methylhonokiol (4)</td>
<td>8.1±2.2</td>
<td>2.5±6.5</td>
</tr>
<tr>
<td>6′-O-Methylhonokiol (5)</td>
<td>3.4±3.7</td>
<td>3.4±5.7</td>
</tr>
<tr>
<td>Sesquiterpene-neolignans</td>
<td></td>
<td></td>
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<tr>
<td>Eudesmagnolol (6)</td>
<td>16.3±1.8</td>
<td>12.1±4.0</td>
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<tr>
<td>Clovanemagnolol (7)</td>
<td>6.9±6.9</td>
<td>10.1±3.0</td>
</tr>
<tr>
<td>Caryolannemagnolol (8)</td>
<td>2.9±8.8</td>
<td>11.4±1.5**</td>
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<tr>
<td>Eudesohonokiol A (9)</td>
<td>2.7±3.1</td>
<td>3.5±2.8</td>
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<tr>
<td>Eudesohonokiol B (10)</td>
<td>9.6±2.9</td>
<td>7.0±2.1</td>
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<tr>
<td>Eudesobovatol A (11)</td>
<td>13.4±1.3</td>
<td>9.8±1.4</td>
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<tr>
<td>Eudesobovatol B (12)</td>
<td>12.1±5.0</td>
<td>12.6±6.3**</td>
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<tr>
<td>Trimeolignan</td>
<td></td>
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<tr>
<td>Magnolamin (13)</td>
<td>12.9±2.4</td>
<td>9.2±2.3</td>
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<tr>
<td>Phenylpropanoid glycoside</td>
<td>2.4±2.6</td>
<td>1.6±3.3</td>
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<tr>
<td>Syringin (14)</td>
<td>10.1±4.6</td>
<td>5.2±3.7</td>
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<tr>
<td>Lignan glycosides</td>
<td></td>
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<tr>
<td>Liriodendrin (15)</td>
<td>7.6±2.9</td>
<td>4.4±2.1</td>
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<tr>
<td>(+)-Syringaresinol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4′-O-β-D-glucopyranoside (16)</td>
<td>11.1±7.3</td>
<td>-4.6±7.7</td>
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<tr>
<td>Sesquiterpene</td>
<td></td>
<td></td>
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<tr>
<td>Caryophyllene oxide (17)</td>
<td>11.1±7.3</td>
<td>-4.6±7.7</td>
</tr>
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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.

*S* Cytotoxic effects were observed.

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The following instruments were used to obtain physical data: specific rotation, 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-AX505D mass spectrometer, JEOL JMS-SX102A mass spectrometer.

The following experimental conditions were used for chromatography: ordinary-phase column chromatography; Silica gel GW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh), reversed-phase column chromatography; Chromatore 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-phase) column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh), reversed-phase column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh), reversed-phase column chromatography.

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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 vasoconstriction.

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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,\(^{20}\) 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).\(^{20}\) 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\(^{2+}\)-block or EDRF-like activities in the isolated rat thoracic aorta.\(^{8,20}\) 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 vasoconstriction.
phase); HPTLC, pre-coated TLC plates with Silica gel RP-18 60WF30, 254s/UV, 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–H2O (80:20:90:10)–MeOH] to afford magnololin (1, 5.3 g), honokiol (2, 1.1 g), homovanillic acid (8, 11 mg), eudesmol (9, 242 mg), caryophyllene oxide (17, 36 mg), β-eudesmol (19, 449 mg), and γ-eudesmol (20, 221 mg). Fraction 2 (11.6 g) was further subjected to reversed-phase silica gel column chromatography [MeOH–H2O (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).

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 days previously to get a large number of cells. Cells (7.5×10⁵ 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, 10 mM EDTA, 0.1% Triton X-100, 2 mM ethylene glycol bis-[β-aminethoxy]-ethyl)-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. 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 electrotransferred onto nitrocellulose membranes. The membranes were incubated with 5% nonfat dried milk in Tris–buffered saline (TBS, 50 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 (Hyperl Film, Amersham).

Electrophoretic Mobility Shift Assay TGC-induced peritoneal macrophages (7.5×10⁵ 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 MgCl₂, 0.2 mM ethylene-diaminetetraacetic 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 albumin and 132-P-labeled NF-kB 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). 132-P-labeled NF-kB consensus oligonucleotide was labeled using γ-[32P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase.

Inos E3P/InsP₃-Dependent Activity iNOS enzyme activity was measured as follows. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent. 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%).
bated at 37 °C for 10 min. iNOS (20 μM/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, 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 was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

References