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Inhibitors of Nitric Oxide Production from the Bark of *Myrica rubra*: Structures of New Biphenyl Type Diarylheptanoid Glycosides and Taraxerane Type Triterpene†

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Abstract—Three new biphenyl type diarylheptanoid glycosides, myricanol 11-*O*-β-D-glucopyranoside, myricanone 5-*O*-β-D-glucopyranoside, and neomyricanone 5-*O*-β-D-glucopyranoside, and a new taraxerane type triterpene, myricetrione, were isolated from the bark of Chinese *Myrica rubra*. Their structures were elucidated on the basis of chemical and physicochemical evidence. Biphenyl type diarylheptanoids, triterpene, and their polyphenols showed potent inhibitory effects on nitric oxide production in lipopolysaccharide-activated macrophages. Furthermore, diarylheptanoids, myricanol and myricanone, were found to inhibit induction of inducible nitric oxide synthase.

Introduction

The Myricaceae plant, *Myrica rubra* Sieb. et Zucc. is widely distributed in China, Taiwan, Korea, and Japan. The bark of this plant is used as an astringent, antidote, and antidiarrhetic in Japanese folk medicine, and is also applied externally for burns and skin diseases in Chinese traditional medicine. Several chemical constituents such as flavonoids, tannins, triterpenes, and diarylheptanoids were isolated from the bark of *M. rubra*.[2–8] In the pharmacological studies of this natural medicine, it has been reported that the methanolic extract showed protective effects on CCl_4- and α-naphthylisothiocyanate-induced liver injury, and the 50% aqueous ethanolic extract and some constituents showed melanin biosynthesis inhibitory and anti-androgenic activities.[9–11] Recently, we reported the structure elucidation of three diarylheptanoid glycosides, (+)-*S*-myricanol 5-*O*-β-D-glucopyranoside (**5**), myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**9**), and myricanene B 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**10**), from the methanolic extract of the bark of *M. rubra* in addition to the inhibitory effect on an immediate allergic reaction by monitoring the release of β-hexosaminidase from rat basophilic leukemia (RBL-2H3) cells.[12]

In the continuing study of this natural medicine, we found that the methanolic extract and its *n*-BuOH soluble fraction showed an inhibitory effect on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages. From the *n*-BuOH soluble fraction, we isolated three new biphenyl type diarylheptanoid glycosides called myricanol 11-*O*-β-D-glucopyranoside (**1**), myricanone 5-*O*-β-D-glucopyranoside (**2**), and neomyricanone 5-*O*-β-D-glucopyranoside (**3**) and a new taraxerane type triterpene, myricetrione (**4**), together with 21 known compounds. The present study deals with the isolation and structure elucidation of new constituents (**1**–**4**) from the bark of *M. rubra*. Furthermore, we describe the inhibitory effect of the chemical constituents from this natural medicine on NO production in LPS-activated mouse peritoneal macrophages and induction of inducible NO synthase (iNOS).

Results and Discussion

Isolation from the bark of *M. rubra*

The methanolic extract of the bark of *M. rubra* (cultivated in Guang Dong province, China) was suspended

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in MeOH and filtered using a Kiriyama funnel to give a soluble fraction and insoluble residue. The insoluble residue was recrystallized with aqueous MeOH to furnish myricitrin (**19**). The soluble fraction was partitioned in an *n*-BuOH–H_2O (1:1) mixture to give an *n*-BuOH soluble fraction and H_2O soluble fraction as described, previously.[12] The *n*-BuOH soluble fraction was found to show an inhibitory effect on NO production as shown in Table 1, while the H_2O soluble fraction lacked the activity. The *n*-BuOH soluble fraction was subjected to ordinary- and reversed-phase silica gel column chromatography, and finally HPLC to furnish myricanol 11-*O*-β-D-glucopyranoside (**1**, 0.0018% from natural medicine), myricanone 5-*O*-β-D-glucopyranoside (**2**, 0.019%), neomyricanone 5-*O*-β-D-glucopyranoside (**3**, 0.0013%), and myricetrione (**4**, 0.00076%) together with 21 known compounds (Chart 1).

Structures of myricanol 11-*O*-β-D-glucopyranoside (1), myricanone 5-*O*-β-D-glucopyranoside (2), and neomyricanone 5-*O*-β-D-glucopyranoside (3)

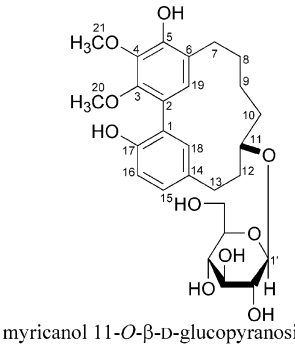
Myricanol 11-*O*-β-D-glucopyranoside (**1**) was isolated as a white powder with negative optical rotation ($[\alpha]_D^{22}$ −3.0°, EtOH). The positive- and negative-ion fast atom bombardment (FAB)-MS of **1** showed quasimolecular ion peaks at *m*/*z* 543 $(M+Na)^+$ and 519 $(M-H)^-$ and a fragment ion peak at *m*/*z* 357 $(M-C_6H_{11}O_5)^-$. The molecular formula $C_{27}H_{36}O_{10}$ of **1** was determined from quasimolecular ion peaks and by high-resolution MS measurement. In the UV spectrum of **1**, absorption maxima were observed at 215 (log ε 4.4), 260 (3.9), and 295 (3.8) nm, which were suggestive of a biphenyl type diarylheptanoid structure.[8] The IR spectrum of **1** showed absorption bands at 3400, 1610, 1559, 1506, 1456, and 1071 cm^{-1}, ascribable to hydroxyl, benzene ring, and ether functions. The ^1H NMR (C_5D_5N) and ^{13}C NMR (Table 2) spectra of **1** showed signals assignable to two methoxyl groups [δ 3.80, 3.99 (both s, 21 and 20-H_3)], a methine bearing a hydroxyl group [δ 4.42 (m, 11-H)], an anomeric proton [δ 4.84 (d, J = 7.5 Hz, 1′-H)], and four aromatic protons [δ 7.12 (s, 19-H), 7.25 (d, J = 8.0 Hz, 16-H), 7.30 (dd, J = 2.0, 8.0 Hz, 15-H), 7.46 (d, J = 2.0 Hz, 18-H)], together with six methylenes (7, 8, 9, 10, 12, 13-H_2). The proton and carbon signals of the ^1H and ^{13}C NMR spectra of **1** were very similar to those of (+)-*S*-myricanol 5-*O*-β-D-glucopyranoside (**5**) and myricanol glucoside (**6**), except for the signals due to the 5- and 11-positions. On acid hydrolysis with 5% aqueous sulfuric acid (H_2SO_4)–1,4-dioxane (1:1, v/v), **1** liberated D-glucose, which was identified by GLC analysis of the trimethylsilyl thiazolidine derivative.[13] Enzymatic hydrolysis of **1** with β-glucosidase gave myricanol (**7**)[4,5] as its aglycon. Comparison of the ^{13}C NMR data for **1** with those for **5**–**7** revealed a glycosidation shift around the 11-position of the myricanol moiety of **1**. The position of a glycosidic linkage and the structure of the myricanol moiety were confirmed on the basis of ^1H–^1H correlation spectroscopy (H–H COSY) and heteronuclear multiple bond correlation (HMBC) experiments. As shown in Figure 1, the H–H COSY experiment on **1** indicated the presence of two partial structures written in the bold line. In the HMBC experiment, long-range correlations were observed between the following protons and carbons of **1** (7-H_2 and 6-C, 19-C; 13-H_2 and 14-C; 18-H and 1-C, 13-C; 19-H and 1-C, 2-C, 7-C; 20-H_3 and 3-C; 21-H_3 and 4-C; 1′-H and 11-C). On the basis of the above-mentioned evidence, the structure of **1** was determined as shown.

Myricanone 5-*O*-β-D-glucopyranoside (**2**) was isolated as a white powder with positive optical rotation ($[\alpha]_D^{22}$ +25.4° in EtOH), and neomyricanone 5-*O*-β-D-glucopyranoside (**3**) was also isolated as a white powder with negative optical rotation ($[\alpha]_D^{22}$ −1.1° in EtOH). In the positive- and negative-ion FAB-MS of **2** and **3**, quasimolecular ion peaks were observed at *m*/*z* 541 $(M+Na)^+$ and *m*/*z* 517 $(M-H)^-$, together with a fragment ion peak at *m*/*z* 355 $(M-C_6H_{11}O_5)^-$, and the common molecular formula $C_{27}H_{34}O_{10}$ was determined by high-resolution MS measurement. The UV spectra (EtOH) of **2** and **3** showed absorption maxima [nm (log ε): **2**, 217 (4.6), 254 (4.1), 295 (3.8); **3**, 213 (4.3), 254 (3.8), 297 (3.5)] suggestive of a biphenyl type diarylheptanoid moiety.[8] The IR spectra of **2** and **3** showed absorption bands due to hydroxyl, carbonyl, benzene ring, and ether functions (**2**: 3568, 1701, 1560, 1508 and 1078 cm^{-1}; **3**: 3400, 1701, 1588, 1506, and 1076 cm^{-1}). On acid hydrolysis with 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v), **2** and **3** liberated D-glucose, which was identified by GLC analysis of their trimethylsilyl thiazolidine derivatives.[13] The ^1H NMR (C_5D_5N) and ^{13}C NMR (Table 2) spectra of **2** showed signals assignable to two methoxyl groups [δ 3.85, 4.07 (both s, 20 and 21-H_3)], a D-glucopyranosyl moiety [δ 5.73 (d, J = 7.5 Hz, 1′-H)], and four aromatic protons [δ 6.85 (s, 19-H), 7.01 (d, J = 2.0 Hz, 18-H), 7.14 (dd, J = 2.0, 8.0 Hz, 15-H), 7.18 (d, J = 8.0 Hz, 16-H)], together with six methylenes (7, 8, 9, 10, 12, 13-H_2). The proton and carbon signals in the ^1H NMR (C_5D_5N) and ^{13}C NMR (Table 2) spectra

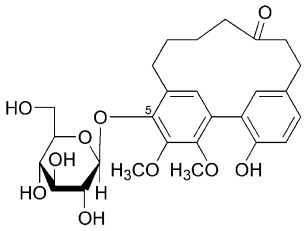
Table 1. Inhibitory effects of MeOH extract and *n*-BuOH- and H_2O-soluble fractions from *M. rubra* on NO production in LPS–activated mouse peritoneal macrophages

	Inhibition (%)				
	0 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	300 μg/mL
MeOH extract	0.0±4.9	6.1±3.8	14.7±4.9	56.0±2.6**	82.5±0.7**
n-BuOH-soluble fraction	0.0±5.0	−1.8±8.7	13.6±2.6	59.8±1.4**	89.3±1.9**[a]
H_2O-soluble fraction	0.0±4.4	−6.2±1.7	2.9±5.4	37.8±3.5**	60.2±0.9**

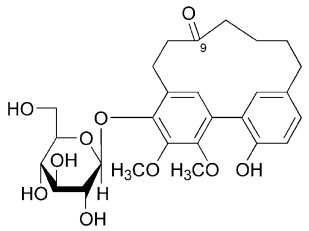
Each value represents the mean±SEM (N = 4). Significantly different from the control, **p < 0.01.
[a]Cytotoxic effect was observed.



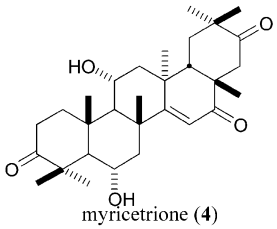
myricanol 11-*O*-β-D-glucopyranoside (**1**)



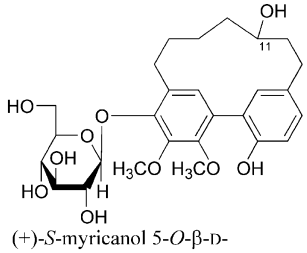
myricanone 5-*O*-β-D-glucopyranoside (**2**)



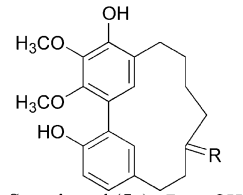
neomyricanone 5-*O*-β-D-glucopyranoside (**3**)



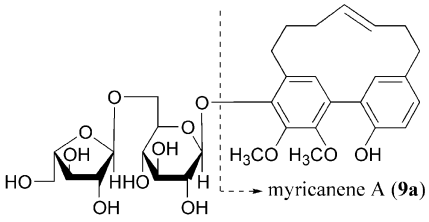
myricetrione (**4**)



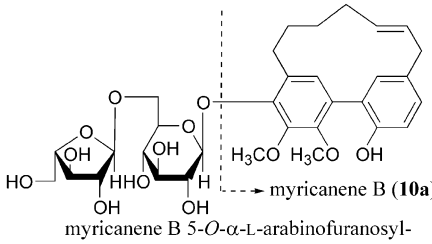
(+)-*S*-myricanol 5-*O*-β-D-glucopyranoside (**5**) : 11α-OH
myricanol glucoside (**6**) : 11β-OH



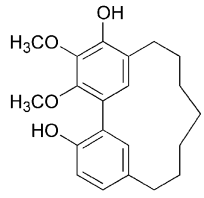
(+)-*S*-myricanol (**5a**) : R=α-OH
myricanol (**7**) : R=β-OH
myricanone (**8**) : R= =O



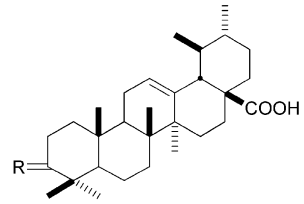
myricanene A 5-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside (**9**)



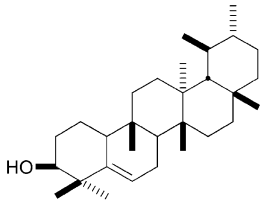
myricanene B 5-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside (**10**)



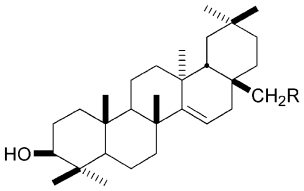
myricanane (**10b**)



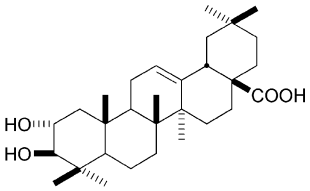
ursolic acid (**11**):R=α-H, β-OH
acetylursolic acid (**12**):R=α-H, β-OAc
ursonic acid (**13**):R= O



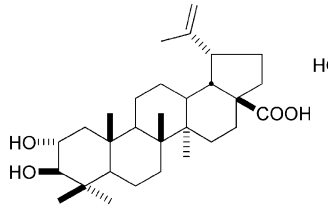
rhoiptelenol (**14**)



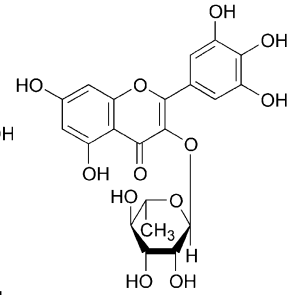
taraxerol (**15**):R=H
myricadiol (**16**):R=OH



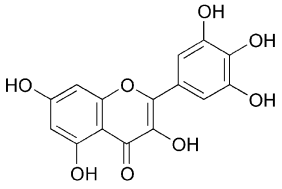
maslinic acid (**17**, =crategolic acid)



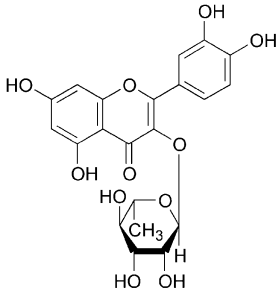
alphitolic acid (**18**)



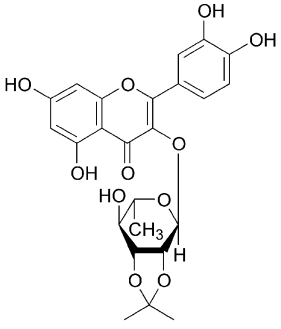
myricitrin (**19**)



myricetin (**20**)



quercitrin (**21**)



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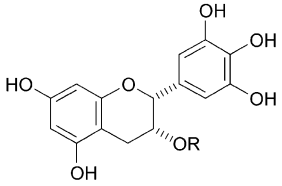
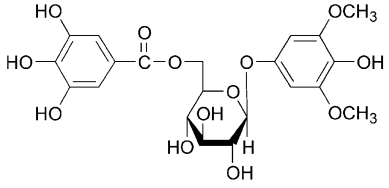


(–)-epigallocatechin (**23**):R=H
(–)-epigallocatechin 3-*O*-gallate (**24**):R=galloyl



3,5-dimethoxy-4-hydroxyphenol 1-*O*-β-D-(6'-*O*-galloyl)glucopyranoside (**25**)

Chart 1.

Table 2. ^{13}C NMR data for myricanol 11-*O*-β-D-glucopyranoside (**1**), myricanone 5-*O*-β-D-glucopyranoside (**2**), neomyricanone 5-*O*-β-D-glucopyranoside (**3**), and myricetrione (**4**)

	1	2	3		4
C-1	127.0	127.0	126.5	C-1	33.6
C-2	123.1	128.8	129.2	C-2	38.9
C-3	148.5	149.3	149.1	C-3	218.6
C-4	140.7	146.2	146.4	C-4	47.9
C-5	150.5	149.6	149.2	C-5	59.2
C-6	123.9	130.6	131.1	C-6	66.4
C-7	26.6	28.3	22.8	C-7	50.2
C-8	26.1	22.2	41.1	C-8	40.3
C-9	23.3	25.0	212.4	C-9	55.5
C-10	37.1	46.0	44.3	C-10	39.8
C-11	77.0	213.0	22.3	C-11	64.7
C-12	27.8	42.5	26.1	C-12	43.1
C-13	27.7	29.1	31.8	C-13	37.9
C-14	131.6	132.1	131.3	C-14	175.8
C-15	130.0	129.3	130.2	C-15	118.4
C-16	117.1	117.0	117.2	C-16	203.5
C-17	153.0	153.6	153.3	C-17	46.5
C-18	134.5	134.0	134.4	C-18	46.4
C-19	130.6	129.6	129.5	C-19	36.8
C-20	61.0	61.0	61.2	C-20	42.5
C-21	60.8	61.5	61.6	C-21	217.4
				C-22	45.4
C-1′	103.3	105.6	105.8	C-23	20.7
C-2′	75.2	75.8	75.8	C-24	32.0
C-3′	77.4	78.4	78.5	C-25	17.9
C-4′	72.0	71.4	71.7	C-26	25.4
C-5′	78.6	78.5	78.5	C-27	28.9
C-6′	63.0	62.5	62.8	C-28	33.3
				C-29	28.4
				C-30	24.4

Measured in C_5D_5N at 125 MHz.

of **3** were found to be similar to those of **2** {two methoxyl groups [δ 3.79, 4.09 (both s, 20 and 21-H_3)], a D-glucopyranosyl moiety [δ 5.73 (d, J = 7.0 Hz, 1′-H)], and four aromatic protons [δ 6.72 (s, 19-H), 7.12 (dd, J = 2.0, 8.0 Hz, 15-H), 7.13 (br s, 18-H), 7.18 (d, J = 8.0 Hz, 16-H)], together with six methylenes (7, 8, 10, 11, 12, 13-H_2)}. The position of the glycosidic linkage in **2** and **3** was elucidated by HMBC experiment. Namely, a long-range correlation was observed between the 1′-proton of the glucopyranosyl moiety and the 5-carbon of the aglycon moiety. The position of the carbonyl function of **2** and **3** were elucidated by H–H COSY experiment and following HMBC correlations (**2**: 10-H_2, 12-H_2 and 11-C; **3**: 8-H_2, 10-H_2 and 9-C) as shown in Figure 1. Finally, enzymatic hydrolysis of **2** and **3** with β-glucosidase furnished myricanone (**8**)[4,5] and a new diarylheptanoid, neomyricanone (**3a**), respectively. The NMR data of **3a** was also supported in the position of the carbonyl function. Consequently, the structures of **2** and **3** were characterized as shown.

Structure of myricetrione (4)

Myricetrione (**4**) was isolated as a white powder with positive optical rotation ($[\alpha]_D^{22}$ +121.0°, EtOH). The electron impact (EI)-MS of **4** showed a molecular ion peak at m/z 484 (M^+) together with fragment ion peaks at m/z 466 ($M^+ - H_2O$) and m/z 346 (base peak). The molecular formula $C_{30}H_{44}O_5$ of **4** was determined from the molecular ion peak and by high-resolution MS measurement. In the UV spectrum of **4**, absorption maximum was observed at 245 (log ε 4.1) nm, which was suggestive of an enone moiety. The IR spectrum of **4** showed absorption bands at 3500, 1700, and 1610 cm^{-1} ascribable to hydroxyl, carbonyl, and conjugated carbonyl functions. The ^1H NMR (C_5D_5N) and ^{13}C NMR (Table 2) spectra of **4** showed signals assignable to eight methyl groups [δ 1.10, 1.14, 1.14, 1.24, 1.27, 1.47, 1.74, 1.80 (all s, 25, 28, 29, 30, 26, 27, 23, and 24-H_3)], two methines bearing a hydroxyl group [δ 4.32 (m, 6-H), 4.52 (m, 11-H)], and an olefin [δ 6.30 (s, 15-H)], together with six methylenes (1, 2, 7, 12, 19, 22-H_2), three methines (5, 9, 18-H), and 10 quaternary carbons including three carbonyl carbons (4, 8, 10, 13, 14, 17, 20-C and 3, 16, 21-C=O).

The planar structure of **4** was constructed on the basis of H–H COSY and HMBC experiments. Thus, the H–H COSY experiment on **4** indicated the presence of four partial structures written in the bold lines, whereas, in the HMBC experiment, long-range correlations were observed between the protons and carbons, as shown in Figure 2. Next, the stereostructure of **4** was elucidated by nuclear Overhauser enhancement spectroscopy (NOESY) experiment, which showed the NOE correlations between the following proton pairs: 5-H and 9-H, 23-H_3; 6-H and 26-H_3; 11-H and 25, 26-H_3; 18-H and 28, 30-H_3; 25-H_3 and 24, 26-H_3 (Fig. 2). Consequently, the stereostructure of myricetrione was determined to be 6α,11α-dihydroxy-14-taraxerene-3,16,21-trione (**4**).

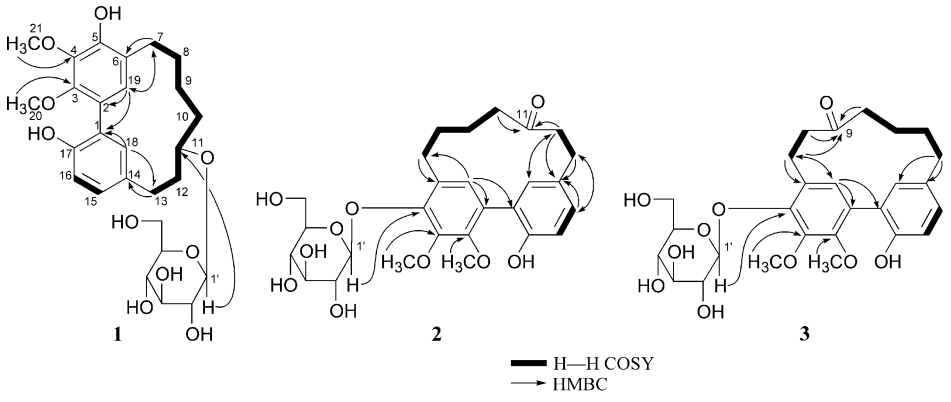


Figure 1.

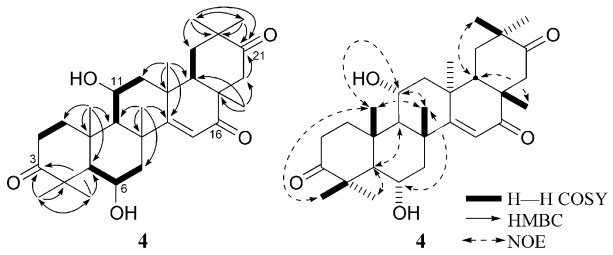


Figure 2.

Inhibitory effects on NO production and iNOS induction in LPS-activated mouse peritoneal macrophages

The inorganic free radical NO has been implicated in physiological and pathological processes, such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine by NO synthase (NOS). In the family of NOS, iNOS in particular is involved in pathological aspects with overproduction of NO, and can be expressed in response to pro-inflammatory agents such as interleukin-1β, tumor necrosis factor-α, and LPS in various cell types including macrophages, endothelial cells, and smooth muscle cells.

As a part of our characterization studies on the bioactive components of natural medicines, we previously reported various NO production inhibitors: higher unsaturated fatty acids,[14] polyacetylenes,[15,16] coumarins,[15] flavonoids,[16] stilbenes,[17] lignans,[18] sesquiterpenes,[19,20] and triterpenes.[20] As a continuation of these studies, the effects of the 17 constituents from the bark of *M. rubra* (**2**, **5**–**11**, **14**–**16**, **19**, **20**, **22**–**25**) and four related compounds (**5a**, **9a**, **10a**, **10b**) on NO production from LPS-activated mouse peritoneal macrophages were examined, and the results were summarized in Table 3. Among them, six biphenyl type diarylheptanoids (**5a**, **7**, **8**, **9a**, **10a**, **10b**) showed an inhibitory effect on NO production (IC_{50} = 19–ca. 30 μM), and their activities were equivalent to that of N^G-monomethyl-L-arginine (L-NMMA), a non-selective NOS inhibitor (IC_{50} = 28 μM). However, their glycosides (**2**, **5**, **6**, **9**) showed weak or no activity. In addition, a triterpene [rhoiptelenol (**14**)], and polyphenols [**22**, (−)-epigallocatechin (**23**), (−)-epigallocatechin 3-*O*-gallate (**24**), and 3,5-dimethoxy-4-hydroxyphenol 1-*O*-β-D-(6′-*O*-galloyl)-glucopyranoside (**25**)] also inhibited it (IC_{50} = 3.0–65 μM). In the previous study, glycosides such as stilbene glycosides (e.g., rhaponticin) showed less activity than their corresponding aglycons.[15–18] We assumed that glycosides (**2**, **5**, **6**, **9**) were hardly permeable to cell membranes and could not reach to the active site and, therefore, showed less activity.

Next, the effects of two principal active constituents (**7**, **8**) and the most potent constituent, 3,5-dimethoxy-4-hydroxyphenol 1-β-D-(6′-*O*-galloyl)glucopyranoside (**25**), on iNOS induction were examined. iNOS was detected at 130 kDa after a 12-h incubation with LPS by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)–Western blotting analysis as shown in Figure 3. iNOS inductions of LPS-activated macrophages were shown to be suppressed by two biphenyl type diarylheptanoid constituents (**7**, **8**) in closely related to their inhibitions of NO. On the other hand, **25** also suppressed the iNOS induction, but the effect was weaker than that on the NO production. These results suggested that **7** and **8** inhibited NO production mainly due to their inhibitory activities against iNOS induction

Table 3. Inhibitory effects of constituents from *M. rubra* on NO production in LPS–activated mouse peritoneal macrophages

	Inhibition (%)						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
Myricanone 5-*O*-β-D-glucopyranoside (**2**)	0.0±0.9	−4.6±2.2	5.2±0.9	−0.2±1.7	6.4±1.6	8.3±4.1	–
(+)-*S*-Myricanol 5-*O*-β-D-glucopyranoside (**5**)	0.0±1.6	5.6±1.7	3.1±0.7	10.8±2.6**	7.3±0.7	33.5±2.8**	>100
(+)-*S*-Myricanol (**5a**)	0.0±2.5	2.9±2.3	−1.8±3.8	23.2±1.8**	68.3±2.0**	92.9±0.4**	19
Myricanol glucoside (**6**)	0.0±2.3	−9.6±2.4	−4.3±3.5	−10.7±3.7	4.1±1.7	4.2±1.9	–
Myricanol (**7**)	0.0±2.2	8.5±2.4	7.5±1.8	20.6±1.7**	61.4±1.2**	86.9±0.6**	23
Myricanone (**8**)	0.0±0.9	7.2±0.6	12.2±2.7**	17.8±2.4**	65.2±4.6**	95.3±0.3**	23
9	0.0±2.6	2.8±1.6	2.1±2.2	−2.9±2.5	−8.2±2.4	31.0±1.3**	>100
Myricanene A (**9a**)	0.0±1.8	6.4±2.4	6.3±2.7	17.5±1.5**	61.5±0.4**	101.3±0.2**[a]	23
10	0.0±3.4	9.4±1.4	11.6±5.8	7.0±3.0	6.1±1.5	72.2±0.5**[a]	>30
Myricanene B (**10a**)	0.0±2.5	2.2±1.5	4.9±4.0	7.8±2.3	49.3±2.1**	99.9±0.4**[a]	ca. 30
Myricanane (**10b**)	0.0±2.2	6.3±1.9	3.3±1.7	18.9±1.0**	55.4±0.8**	100.9±0.2**[a]	26
Ursolic acid (**11**)	0.0±2.0	4.5±1.3	10.5±1.3**	58.5±0.7**[a]	92.8±0.7**[a]	100.6±0.3**[a]	>3
Rhoiptelenol (**14**)	0.0±3.4	1.9±5.0	−1.3±2.3	15.3±3.3	59.3±2.2**	101.3±0.4**[a]	24
Taraxerol (**15**)	0.0±2.9	−3.2±3.2	−7.8±2.3	−4.6±1.7	−11.6±1.8	−4.9±3.8	–
Myricadiol (**16**)	0.0±6.2	−9.4±6.5	−8.6±4.4	−1.5±9.9	−0.2±1.8	−5.4±8.4	–
Myricitrin (**19**)	0.0±0.9	2.6±1.5	0.6±0.3	0.6±0.9	6.2±0.8**	14.9±0.6**	>100
Myricetin (**20**)	0.0±0.7	−0.2±0.7	0.3±1.4	1.9±0.6	10.6±0.4**	50.4±1.4**	99
22	0.0±0.7	1.0±3.7	5.9±1.8	10.1±3.6*	34.9±0.8**	67.7±0.3**	49
(−)-Epigallocatechin (**23**)	0.0±2.3	2.3±3.6	1.5±2.2	15.1±1.2**	37.9±2.2**	53.4±2.4**	65
(−)-Epigallocatechin 3-*O*-gallate (**24**)	0.0±2.3	8.5±1.7	13.4±2.0**	32.0±2.8**	50.5±2.3**	75.5±1.1**	27
25	0.0±1.2	23.1±2.7**	50.7±1.8**	80.8±2.1**	100.1±0.3**	99.5±0.4**[a]	3.0
L-NMMA	0.0±1.1	4.4±2.0	2.0±1.6	17.7±2.8**	52.3±1.5**	79.2±0.9**	28

Each value represents the mean±SEM (N = 4). Significantly different from the control, $^{**}p < 0.01$.
[a]Cytotoxic effect was observed.

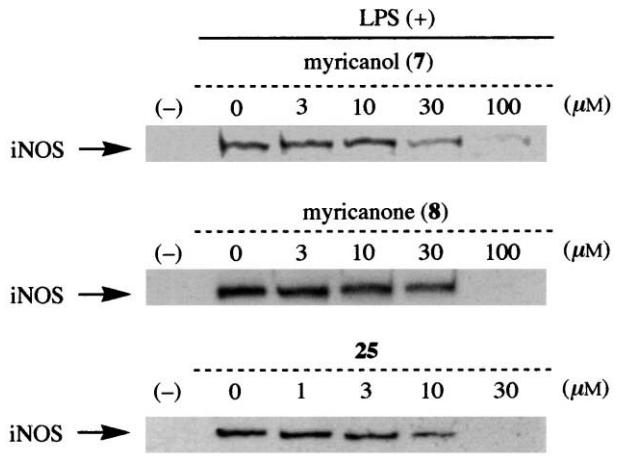


Figure 3. Effects of biphenyl type diarylheptanoids (**7**,**8**) and **25** on iNOS induction in LPS-activated mouse macrophages.

in LPS-activated macrophages, and the inhibitory activity of **25** against iNOS induction was partly involved in its mechanism of action.

The inhibitory activities of these components against NO production in LPS-activated macrophages appeared to substantiate the traditional effects of this natural medicine for the treatment of inflammation.

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; EI–MS and high-resolution MS, JEOL JMS-GCMATE mass spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H NMR spectra, JNM-LA500 (500 MHz) spectrometer; ^{13}C NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index detector.

The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh); reversed-phase silica gel 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) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, pre-coated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Isolation of myricanol 11-*O*-β-D-glucopyranoside (1), myricanone 5-*O*-β-D-glucopyranoside (2), and neomyricanone 5-*O*-β-D-glucopyranoside (3), and myricetrione (4)

The dried bark of *M. rubra* (5.0 kg, cultivated in Guang Dong Province, China and purchased from Mae Chu Co., Ltd., Nara, Japan) was finely cut and extracted three times with MeOH at room temperature for 1 day. Evaporation of the solvent under reduced pressure provided a MeOH extract (1600 g). The MeOH extract (1400 g) was suspended in a small amount of MeOH, and the suspension was filtered to give a soluble fraction (1200 g) and an insoluble fraction, which was recrystallized with aqueous MeOH to furnish myricitrin (**19**, 200 g). The soluble fraction (722 g) was partitioned in an *n*-BuOH–H_2O (1:1) mixture to give *n*-BuOH- and H_2O-soluble fractions (380 and 342 g), respectively. The *n*-BuOH–soluble fraction (50 g) was subjected to ordinary-phase silica gel column chromatography [1.5 kg, $CHCl_3$–MeOH (10:1 → 5:1) → $CHCl_3$–MeOH–H_2O (6:4:1) → MeOH] to give seven fractions {Fr. 1 (5.5 g), Fr. 2 (1.0 g), Fr. 3 (4.8 g), Fr. 4 (1.2 g), Fr. 5 [= myricitrin (**19**, 19.4 g)], Fr. 6 (1.4 g), Fr. 7 (16.7 g)}. Fraction 2 (1.0 g) was subjected to reversed-phase silica gel column chromatography [30 g, MeOH–H_2O (25:75 → 40:60 → 60:40 → 90:10 → 95:5) → MeOH] to give six fractions [Fr. 2-1 (141 mg), Fr. 2-2 (114 mg), Fr. 2-3 (113 mg), Fr. 2-4 (56 mg), Fr. 2-5 (150 mg), Fr. 2-6 (426 mg)] as reported, respectively.[12]

Fraction 2–2 (114 mg) was further purified by HPLC [detection RI, YMC-Pack ODS-A (250 × 20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (35:65)] to give myricanone 5-*O*-β-D-glucopyranoside (**2**, 71 mg, 0.019%). Fraction 2–3 (113 mg) was further purified by HPLC [detection RI, YMC-Pack ODS-A (250 × 20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (35:65)] to give myricanol 11-*O*-β-D-glucopyranoside (**1**, 8 mg, 0.0022%), neomyricanone 5-*O*-β-D-glucopyranoside (**3**, 7 mg, 0.0020%), and 6α,11α-dihydroxy-14-taraxerene-3,16,21-trione (**4**, 3 mg, 0.00076%).

Myricanol 11-*O*-β-D-glucopyranoside (1). A white powder, $[\alpha]_D^{22}$ −3.0° (*c* 0.20, EtOH). High-resolution positive-ion FAB-MS: calcd for $C_{27}H_{36}O_{10}Na$ $(M+Na)^+$: 543.2206. Found: 543.2574. UV (EtOH, nm, log ε): 215 (4.4), 260 (3.9), 295 (3.8). IR (KBr): 3400, 2926, 2857, 1610, 1559, 1506, 1456, 1071 cm^{-1}. ^{1}H NMR (C_5D_5N) δ: 1.43, 1.79 (1H each, both m, 9-H_2), 1.79, 2.09 (1H each, both m, 8-H_2), 1.92, 2.11 (1H each, both m, 10-H_2), 2.11, 2.37 (1H each, both m, 12-H_2), 2.86, 3.45 (1H each, both m, 13-H_2), 2.90, 2.96 (1H each, both m, 7-H_2), 3.80, 3.99 (each 3H, both s, 21 and 20-H_3), 4.42 (1H, m, 11-H), 4.84 (1H, d, $J = 7.5$ Hz, 1′-H), 7.12 (1H, s, 19-H), 7.25 (1H, d, $J = 8.0$ Hz, 16-H), 7.30 (1H, dd, $J = 2.0$, 8.0 Hz, 15-H), 7.46 (1H, d, $J = 2.0$ Hz, 18-H). ^{13}C NMR (C_5D_5N) δc: given in Table 2. Positive-ion FAB-MS *m*/*z*: 543 $(M+Na)^+$. Negative-ion FAB-MS *m*/*z*: 519 $(M-H)^-$, 357 $(M-C_6H_{11}O_5)^-$.

Myricanone 5-*O*-β-D-glucopyranoside (2). A white powder, $[\alpha]_D^{22}$ +25.4° (*c* 0.10, EtOH). High-resolution positive-ion FAB-MS: calcd for $C_{27}H_{34}O_{10}Na$ $(M+Na)^+$: 541.2050. Found: 541.2059. UV (EtOH, nm, log ε): 217 (4.6), 254 (4.1), 295 (3.8). IR (KBr): 3568, 2940, 1701, 1560, 1508, 1078 cm^{-1}. ^{1}H NMR (C_5D_5N) δ: 1.84 (4H m, 8, 9-H_2), 2.62 (2H, m, 10-H_2), 2.75 (2H, m, 12-H_2), 3.04 (2H, m, 13-H_2), 3.05, 3.29 (1H each, both m, 7-H_2), 3.85, 4.07 (each 3H, both s, 20 and 21-H_3), 5.73 (1H, d,

$J=7.5$ Hz, 1′-H), 6.85 (1H, s, 19-H), 7.01 (1H, d, $J=2.0$ Hz, 18-H), 7.14 (1H, dd, $J=2.0$, 8.0 Hz, 15-H), 7.18 (1H, d, $J=8.0$ Hz, 16-H). ^{13}C NMR (C_5D_5N) δ*c* : given in Table 2. Positive-ion FAB-MS *m*/*z*: 541 $(M+Na)^+$. Negative-ion FAB-MS *m*/*z*: 517 $(M-H)^-$, 355 $(M-C_6H_{11}O_5)^-$.

Neomyricanone 5-*O*-β-D-glucopyranoside (3). A white powder, $[\alpha]_D^{22}$ −1.1° (*c* 0.20, EtOH). High-resolution positive-ion FAB-MS: calcd for $C_{27}H_{34}O_{10}Na$ $(M+Na)^+$: 541.2050. Found: 541.2063. UV (EtOH, nm, log ε): 213 (4.3), 254 (3.8), 297 (3.5). IR (KBr) : 3400, 2926, 2853, 1701, 1588, 1506, 1453, 1076 cm^{-1}. ^{1}H NMR (C_5D_5N) δ: 1.72 (2H m, 11-H_2), 1.92 (2H m, 12-H_2), 2.86 (2H m, 8-H_2), 2.59 (2H, m, 10-H_2), 2.70 (2H, m, 13-H_2), 3.35, 3.70 (1H each, both m, 7-H_2), 3.79, 4.09 (each 3H, both s, 20 and 21-H_3), 5.73 (1H, d, $J=7.0$ Hz, 1′-H), 6.72 (1H, s, 19-H), 7.12 (1H, dd, $J=2.0$, 8.0 Hz, 15-H), 7.13 (1H, br s, 18-H), 7.18 (1H, d, $J=8.0$ Hz, 16-H). ^{13}C NMR (C_5D_5N) δc: given in Table 2. Positive-ion FAB-MS *m*/*z*: 541 $(M+Na)^+$. Negative-ion FAB-MS *m*/*z*: 517 $(M-H)^-$, 355 $(M-C_6H_{11}O_5)^-$.

Myricetrione (4). A white powder, $[\alpha]_D^{22}$ +121.0° (*c* 0.10, EtOH). High-resolution EI–MS: calcd for $C_{30}H_{44}O_5$ (M^+): 484.3188. Found: 484.3186. UV (EtOH, nm, log ε): 245 (4.1). IR (KBr): 3500, 2930, 1700, 1610, 1456 cm^{-1}. ^{1}H NMR (C_5D_5N) δ: 1.10, 1.14, 1.14, 1.24, 1.27, 1.47, 1.74, 1.80 (3H each, all s, 25, 28, 29, 30, 26, 27, 23, and 24-H_3), 1.78, 2.30 (1H each, both m, 19-H_2), 1.85, 2.62 (1H each both m, 7-H_2), 1.87 (1H, m, 18-H), 1.93 (1H, m, 9-H), 2.04 (1H, m, 5-H), 2.10 (2H, m, 12-H_2), 2.42 (2H, m, 2-H_2), 2.87 (2H, m, 1-H_2), 2.89 (2H, m, 22-H_2), 4.32 (1H, m, 6-H), 4.52 (1H, m, 11-H), 6.30 (1H, s, 15-H). ^{13}C NMR (C_5D_5N) δc: given in Table 2. EI–MS: *m*/*z* (%): 484 (M^+, 9), 466 (M^+-H_2O, 13), 346 (100).

Enzymatic hydrolysis of 1–3

A solution of **1** or **3** (2.0 mg each, 0.0038 mmol) in 0.2 M acetate buffer (pH 4.4, 2.0 mL) was treated with β-glucosidase (5 mg, from almond, Oriental Yeast Co., Ltd.), and the solution was stirred at 38 °C overnight. After treatment of the reaction mixture with EtOH, the mixture was evaporated to dryness under reduced pressure and the residue was purified by ordinary-phase silica gel column chromatography [500 mg, *n*-hexane–AcOEt (1:1)] to give myricanol (**7**, 1.4 mg, 99%) or neomyricanone (**3a**, 1.1 mg, 80%).

Through a similar procedure, a solution of **3** (10.5 mg, 0.020 mmol) in 0.2 M acetate buffer (pH 4.0, 2.0 mL) was treated with β-glucosidase (10 mg), and the solution was stirred at 38 °C overnight. After treatment of the reaction mixture with EtOH, the mixture was evaporated to dryness under reduced pressure, and the residue was purified by ordinary-phase silica gel column chromatography [1.0 g, *n*-hexane–AcOEt (2:1)] to give myricanone (**8**, 5.7 mg, 66%).

Neomyricanone (3a). A white powder. High-resolution EI–MS: calcd for $C_{21}H_{24}O_5$ (M^+): 356.1636. Found: 356.1624. UV [EtOH, nm, (log ε)]: 217 (4.0), 261 (3.4), 297 (3.2). IR (KBr): 3400, 2924, 2857, 1716, 1620, 1506, 1458 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.79 (2H, m, 11-H_2), 2.05 (2H, m, 12-H_2), 2.63 (2H, m, 10-H_2), 2.74 (2H, m, 13-H_2), 2.92 (2H, m, 8-H_2), 2.99 (2H, m, 7-H_2), 3.85, 3.99 (each 3H, both s, 20 and 21-H_3), 5.86 (1H, br s, 5-OH), 6.46 (1H, s, 19-H), 6.87 (1H, d, $J=8.0$ Hz, 16-H), 6.95 (1H, d, $J=2.0$ Hz, 18-H), 7.03 (1H, dd, $J=2.0$, 8.0 Hz, 15-H), 7.70 (1H, s, 17-OH). EI–MS *m*/*z* (%): 356 (M^+, 100).

Acid hydrolysis of 1–3

A solution of **1**–**3** (2 mg each) in 5% aqueous H_2SO_4–1,4-dioxane (0.5 mL, 1:1, v/v) was heated under reflux for 1 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form), and the residue was removed by filtration. After removal of the solvent from the filtrate under reduced pressure, the residue was transferred to a Sep-Pak C18 cartridge with H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (4 mg) in pyridine (0.5 mL) at 60 °C for 1 h. After the reaction, the solution was treated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (0.2 mL) at 60 °C for 1 h. The supernatant was then subjected to GLC analysis to identify the derivatives of D-glucose from **1**–**3**. GLC conditions: column: Supeluco STB™-1, 30 m × 0.25 mm (i.d.) capillary column; injector temperature: 230 °C; detector temperature: 230 °C; column temperature: 230 °C; He flow rate: 15 mL/min; t_R: 24.2 min.

Bioassay

NO production from macrophages stimulated by LPS

Inhibitory effects of test samples on the NO production in LPS-activated mouse macrophages were evaluated by the method reported previously.[14–20] Briefly, peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice and the cells (5×10^5 cells/well) were suspended in 200 μL of RPMI 1640 supplemented with 5% fetal calf serum (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. Non-adherent cells were removed by washing the cells with phosphate-buffered saline (PBS), and the adherent cells (greater than 95% macrophages as determined by Giemsa staining) were cultured in fresh medium containing 10 μg/mL LPS and test compound (1–100 μM) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent.[21] Cytotoxicity was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay, after 20-h incubation with test compounds. L-NMMA was used as a reference compound. Each test compound was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated using the following formula and IC_{50} was determined graphically ($N=4$):

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

$A-C$: NO_2^- concentration (μM) [A: LPS (+), sample (−); B: LPS (+), sample (+); C: LPS (−), sample (−)].

Detection of iNOS

In this experiment, TGC-induced peritoneal exudate cells (7.5×10^6 cells/3 mL/dish) from male ddY mice were pre-cultured in culture dishes (6 cm i.d.) for 1 h, and the adherent cells (greater than 95% macrophages) were obtained as described previously.[15,17,18,19b,20] 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 the protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer.[22] 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 transferred electrophoretically 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 with horseradish peroxidase (dilution of 1:5000). Detection was performed using an ECL kit and X-ray film (Hyper Film, Amersham).

Statistics

Values are expressed as means ± SEM. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

References and Notes

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