Maleimide and Maleic Anhydride Derivatives from the Mycelia of *Antrodia cinnamomea* and Their Nitric Oxide Inhibitory Activities in Macrophages

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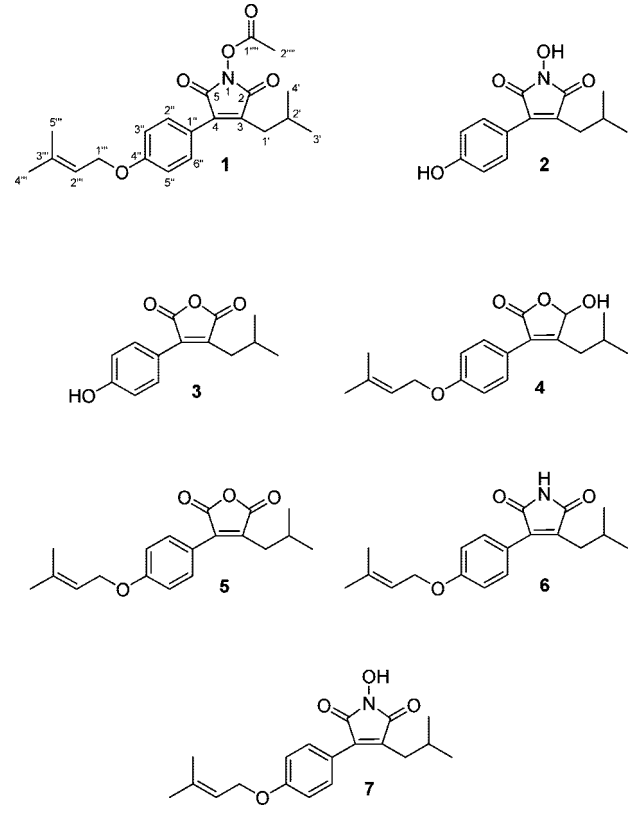
On cultivation of the fungus *Antrodia cinnamomea* (BCRC 36799) on a medium, the mycelium was extracted and evaluated for nitric oxide (NO) inhibitory activity. Bioactivity-directed fractionation led to the isolation of two new maleimide derivatives, antrocinnamomins A (**1**) and B (**2**), and two new maleic anhydride derivatives, antrocinnamomins C (**3**) and D (**4**), along with three known compounds, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione (**5**), 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrole-2,5-dione (**6**), and 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrol-1-ol-2,5-dione (**7**). Structural elucidation of compounds **1**−**4** was carried out by spectroscopic data. Compound **1** displayed significant inhibitory effect on nitric oxide (NO) production.

The fruiting body of the fungus *Antrodia cinnamomea* Chang & Chou (syn. *A. camphorata*, Polyporaceae, Aphyllophorales) is a very rare and expensive mushroom. The fungus is known only in Taiwan and grows on the endemic species *Cinnamomum kanehirai*. It grows slowly on the inner cavity of heart wood wall of this species.[1] The basidioma that grow on the infested wood have long been used as a herbal medicine in Taiwan. The fruiting body of *A. cinnamomea* contains polysaccharide,[2] sesquiterpene lactone,[3] steroid,[4,5] diterpenoid,[6] triterpenoid,[7,8] and benzenoid[9] constituents. There are several biological studies on *A. camphorata* mycelium that have shown anti-inflammatory,[10] protective antioxidative,[11] and cytotoxic properties[12,13] and antihepatitis B virus activities.[2] A number of maleic and succinic acid derivatives were isolated recently from the mycelia of *A. camphorata*.[14] The mycelia of *A. cinnamomea* were extracted with MeOH. The extract was then partitioned with *n*-hexane, ethyl acetate, and water, successively. The *n*-hexane layer showed strong NO production inhibitory activity. Further bioassay-directed fractionation of this fraction led to the isolation of two new maleimide derivatives, antrocinnamomins A (**1**) and B (**2**), and two new maleic anhydride derivatives, antrocinnamomins C (**3**) and D (**4**), along with one known maleic anhydride derivative, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione (**5**),[14] and two known maleimide derivatives, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrole-2,5-dione (**6**)[14] and 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrol-1-ole-2,5-dione (**7**).[14] The similarities of **1**−**4** were established using spectroscopic data analysis, including 1D and 2D NMR techniques (COSY, NOESY, HSQC, and HMBC). Moreover, all isolates (**1**−**7**) were evaluated for their inhibitory effects on nitric oxide production by macrophages.

Bioassay-directed nitric oxide (NO) inhibitory activities from the mycelia of *A. cinnamomea* (BCRC 36799) resulted in the isolation of several maleimide and maleic anhydride derivatives (**1**−**7**). Compound **1** was purified as an oil, and a molecular formula $C_{21}H_{25}NO_5Na$, as measured by HRESIMS. The IR spectrum showed adsorption bands for ester carbonyl (1783 cm^{-1}), amidocarbonyl (1733 cm^{-1}), and aromatic (1604 and 1510 cm^{-1}) groups. The general nature of the structure



of **1** was deduced from ^1H and ^{13}C NMR spectra that showed a *para*-substituted aromatic ring [δ 7.52 (2H, d, J = 8.8 Hz for H-2″ and H-6″), 6.98 (2H, d, 8.8 Hz for H-3″ and H-5″), and 120.6, 131.1, 114.9, 160.4 for C-1″; -2″, 6″; -3″, 5″, and -4″, respectively]. The ^1H NMR spectrum of **1**, together with the $^1H-^1H$ COSY and HSQC spectra, displayed characteristic signals including an isobutyl moiety [δ 0.88 (6H, d, J = 6.9 Hz), 2.04 (1H, sept., J = 6.9 Hz), 2.53 (2H, d, J = 6.9 Hz)] and a 3-methyl-2-butenyloxy moiety [δ 1.74 (3H, s), 1.79 (3H, s), 4.55 (2H, d, J = 6.6 Hz), 5.47 (1H, br t, J = 6.6 Hz)]. In addition, a singlet methyl group [δ 2.32 (3H, s)] and a carbonyl carbon group (δ 167.1) were found in the ^1H and ^{13}C NMR spectra. The remaining signals included two quaternary olefinic carbons (δ 136.6 and 136.7) and two carbonyl groups (δ 165.9 and 166.7). On inspection of the HMBC spectrum (Figure

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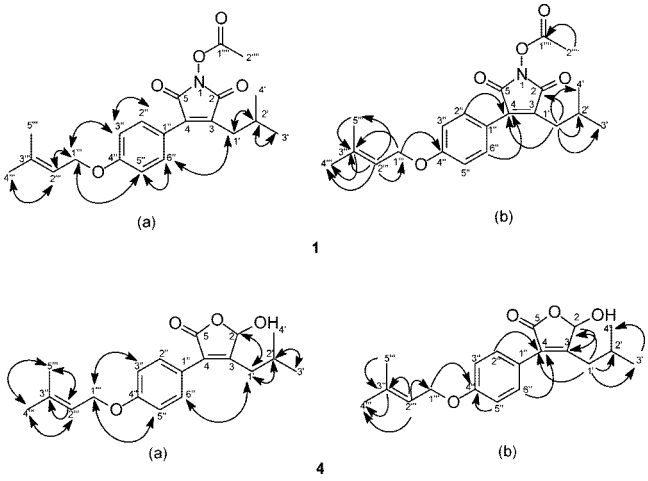

Figure 1. Major NOESY (a) and HMBC connectivities (b) of compounds **1** and **4**.

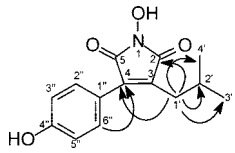

Figure 2. Major NOESY connectivities of compound **2**.

1), the H-1′ signal in the isobutyl moiety correlated with one of the olefinic carbons (C-4, δ 136.7) and one of amidocarbonyl carbons (δ 166.7), and H-2″ and H-6″ in the aromatic ring correlated with the other olefinic carbon, suggesting the connectivities among the pyrrole, isobutyl, and *para*-substituted aromatic moieties for the skeleton of **1** as shown. In addition, a ^3J correlation between H-1‴ in the 3-methyl-2-butenyloxy moiety and the oxygenated carbon (C-4″) in the aromatic ring was also observed. This revealed that **1** is similar to 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrol-1-ole-2,5-dione (**7**) except for an acetoxy moiety [δ 2.32 (3H, s)] found in **1**. Moreover, the IR spectrum of **7** showed a characteristic N-OH [IR (neat) $\nu_{max} = 3244$ cm^{-1}] peak, while this signal was lacking in **1**, consistent with the OH in **7** being replaced by an acetoxy group in **1**. According to the above data, together with further confirmation by NOESY (Figure 1) experiments, the structure of **1** was elucidated as 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrol-1-acetoxyl 2,5-dione and was named antrocinnamomin A.

Compound **2** revealed a molecular formula of $C_{14}H_{15}NO_4$ from a molecular ion at *m*/*z* 261 [M]$^+$ by HREIMS. The IR spectrum of **2** showed strong absorption bonds at 3424 (OH), 3244 (N−OH), 1711 (N−C═O), and 1606 and 1513 (aromatic) cm^{-1}. The ^{1}H NMR signals of **2** were similar to those of compound **1**, the major difference being the absence of a 3-methyl-2-butenyloxy unit and an acetate group in **2**. Like **1**, the ^{1}H and ^{13}C NMR chemical shifts of **2** were assigned completely, on the basis of the ^{1}H−^{1}H COSY, HSQC, and HMBC spectra (Figure 2). Moreover, on comparison of their mass spectra, the lower molecular weight by 110 amu was also consistent with the absence of an acetate and a 3-methyl-2-butenyloxy in **2**. Thus, the structure of **2** (antrocinnamomin B) was determined to be 3-isobutyl-4-(4-hydroxyphenyl)-1*H*-pyrrol-1-ole-2,5-dione.

Compound **3** was isolated as a yellowish oil, and the molecular formula, $C_{14}H_{14}O_4$, was determined by EIMS ([M]$^+$, *m*/*z* 246) and HREIMS. The IR spectrum showed that the compound had a carbonyl absorption (1760 cm^{-1}) of acid anhydride and a hydroxyl absorption at 3424 cm^{-1}. Most of the ^{1}H and ^{13}C NMR spectroscopic signals of **3** were very similar to those of **2** except that two carbonyl carbons and olefinic carbons of **3** were shifted to high field and low field, respectively. On the basis of the lack of a characteristic IR signal at 3244 cm^{-1} (N−OH), together with the molecular ion at *m*/*z* 246 in **3** rather than *m*/*z* 261 in **2**, the N−OH of the pyrrole-2,5-dione moiety in **2** was replaced by an oxygen of a furan-2,5-dione unit in **3**. Moreover, the structure of **3** was also similar to the known 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione (**5**), except for the absence of a 3-methyl-2-butenyloxy moiety in **3**. Accordingly, the structure of **3** was elucidated as 3-isobutyl-4-(4-hydroxyphenyl)furan-2,5-dione and was designated as antrocinnamomin C.

Compound **4** was isolated as a white, amorphous solid, and the molecular weight of 339, corresponding to the molecular formula $C_{19}H_{24}NO_4Na$, was measured by HRESIMS. The IR spectrum showed adsorption bands for hydroxyl (3389 cm^{-1}), a carbonyl of an acid anhydride (1738 cm^{-1}), and aromatic (1607 and 1511 cm^{-1}) groups. The ^{1}H and ^{13}C NMR data as well as mass spectra of **4** were similar to those of 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)-phenyl]furan-2,5-dione (**5**), except that a ketone moiety of **5** was replaced by an oxymethine moiety [δ 6.07 (1H, s)] and the molecular weight of **4** had more two more mass units than that of **5**. In addition, the structure of **4** showed an optical inactivity with $[\alpha]^{24}_D \pm 0$ (*c* 0.18, $CHCl_3$). The zero [α] value of **4** suggests that this compound may also exist as a racemate. The structure of **4** was confirmed using NOESY, HSQC, and HMBC (Figure 1) spectroscopic experiments. On the basis of the above evidence, **4** (antrocinnamomin D) was deduced as 2-hydroxy-3-isobutyl-4-[4-(3-methylbut-2-enyloxy)phenyl]-2*H*-furan-5-one, unambiguously.

The other isolated compounds **5**−**7** were identified as the known maleic anhydride derivative 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione (**5**)[14] and the known maleimide derivatives 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrole-2,5-dione (**6**)[14] and 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrol-1-ole-2,5-dione (**7**),[14] respectively, by comparison with literature data.

Compounds **1**−**7** were evaluated for their anti-inflammatory activity, using RAW264.7 cells, supplemented with LPS, which induce cell inflammation and cause nitrite accumulation in the medium. Among the tested compounds, compound **1** showed strong inhibition of NO production of macrophages consistent with anti-inflammatory activity (Table 3).[15] The high cell viability (>90%, Table 3) indicated that the inhibitory activity of LPS-induced nitrite production by compound **1** did not result from its cytotoxicity. Compound **6** also showed strong inhibition of NO production of macrophages, but the low cell viability (69.1%) suggested the possibility of cytotoxicity.

Experimental Section

General Experimental Procedures. All melting points were determined on a Yanaco micromelting point apparatus and are uncorrected. IR spectra (KBr or neat) were taken on a Perkin-Elmer System 2000 FT-IR spectrometer. 1D and 2D NMR spectra were established on Bruker 400, Varian Unity 400, and Varian Gemini 200 spectrometers. Chemical shifts are given in ppm (δ), with TMS as the internal standard. EIMS were recorded on a Micromass TRIO-2000 spectrometer. HREIMS spectra recorded on a Finnigan/Thermo Quest NAT mass spectrometer; HRESIMS spectra, on a Bruker Daltonics APEX II 30e spectrometer. Silica gel (70−230, 230−400 mesh) (Merck) was used for CC, and silica gel 60 F-254 (Merck) was used for TLC and preparative TLC.

Fungal Material. *Antrodia cinnamomea* BCRC 36799 was used throughout this study, identified by one of the coauthors (B.-C.W.), and specimens were deposited at the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI). *A. cinnamomea* BCRC 36799 was maintained on PDA (potato dextrose agar, DIFCO) plates and incubated at 28 °C. Mycelia harvested from a 14-day-old PDA plate were homogenized with 50 mL of sterile water and used as the inoculum for submerged cultivation in flasks. The seed culture for the fermentor was prepared in 500 mL flasks containing 300 mL of medium on a rotary shaker at 100 rpm and 30 °C. The 5-day-old culture broth was inoculated into a

Table 1. ^{1}H NMR (400 MHz) Data of Compounds **1**−**4**[a]

H	**1**[b]	**2**[c]	**3**[b]	**4**[b]
2				6.07 (1H, s)
3				
4				
5				
1′	2.53 (2H, d, J = 6.9)	2.52 (2H, d, J = 7.0)	2.59 (2H, d, J = 6.8)	2.49 (2H, d, J = 6.8)
2′	2.04 (1H, sept, J = 6.9)	1.99 (1H, sept, J = 7.0)	2.12 (1H, sept, J = 6.8)	1.99 (1H, sept, J = 6.8)
3′	0.88 (6H, d, J = 6.9)	0.88 (6H, d, J = 7.0)	0.95 (6H, d, J = 6.8)	0.97 (3H, d, J = 6.8)
4′				0.86 (3H, d, J = 6.8)
1″				
2″, 6″	7.52 (2H, d, J = 8.8)	7.46 (2H, d, J = 8.4)	7.59 (2H, d, J = 8.8)	7.36 (2H, d, J = 8.8)
3″, 5″	6.98 (2H, d, J = 8.8)	6.88 (2H, d, J = 8.4)	6.97 (2H, d, J = 8.8)	6.96 (2H, d, J = 8.8)
4″				
1‴	4.55 (2H, d, J = 6.6)			4.54 (2H, d, J = 6.8)
2‴	5.47 (1H, br t, J = 6.6)			5.50 (1H, br t, J = 6.8)
3‴				
4‴	1.79 (3H, s)			1.80 (3H, s)
5‴	1.74 (3H, s)			1.75 (3H, s)
1⁗				
2⁗	2.32 (3H, s)			

[a] Values in ppm (δ), with J (in Hz) in parentheses. [b] In $CDCl_3$. [c] In CD_3OD.

150 L fermentor (B. Braun Melsungen, Germany) operated at 100 rpm and 30 °C with an aeration rate of 0.3 vvm. After 12 days of cultivation, the pellet mycelium harvested from the culture broth was freeze-dried, stored at −80 °C, and used as a sample for further extraction.

Extraction and Isolation. The dried mycelium of *A. cinnamomea* BCRC 36799 (1205 g) was extracted three times with MeOH (1.5 L) at room temperature. Then the crude methanol syrup was evaporated under reduced pressure and partitioned with *n*-hexane−H_2O (1:1) three times to give *n*-hexane and H_2O layers. The *n*-hexane layer extract (15.2 g) was chromatographed on a silica gel column with a stepwise gradient of *n*-hexane−EtOAc [15:1, 12:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1, EtOAc, acetone, methanol, each 1 L] to give 15 fractions. Fraction 3 was purified by preparative TLC (*n*-hexane−EtOAc, 8:1) to afford compounds **1** (1.3 mg, R_f 0.62) and **4** (4.2 mg, R_f 0.43). Fraction 5 was further purified by column chromatography on silica gel eluting with *n*-hexane−CH_2Cl_2 [8:1, 6:1, 4:1, 2:1, 1:1 and CH_2Cl_2 (each 500 mL)] to yield six fractions (5.1−5.6). Compounds **6** (12.5 mg, R_f 0.62) and **7** (20.3 mg, R_f 0.32) were furnished from fraction 5.3 by preparative TLC (*n*-hexane−EtOAc, 3:1). Compound **5** (4.2 mg, R_f 0.59) was obtained from fraction 5.1. Fraction 7 was chromatographed on a silica gel column with *n*-hexane−EtOAc [6:1, 4:1, 2:1, 1:1, EtOAc] to give six fractions (7.1−7.6). Fraction 7.4 was purified with preparative TLC (*n*-hexane−EtOAc, 1.5:1) to yield compounds **2** (4.6 mg, R_f 0.38) and **3** (1.6 mg, R_f 0.65).

Antrocinnamomin A (1): yellowish oil; UV (MeOH) λ_{max} (log ϵ) 231 (4.18), 284 (3.57), 370 (3.66) nm; IR (neat) ν_{max} 1783 (ester), 1733 (N−C═O), 1604 and 1510 (aromatic) cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; EIMS *m*/*z* 371 ([M]$^+$, 8), 303 (82), 261 (80), 244 (100), 228 (15), 202 (22), 173(15); HRESIMS *m*/*z* 394.1629 [M + Na]$^+$ (calcd for $C_{21}H_{25}NO_5Na$, 394.1630).

Antrocinnamomin B (2): yellowish solid; UV (MeOH) λ_{max} (log ϵ) 232 (4.05), 298 (3.48), 378 (3.42) nm; IR (KBr) ν_{max} 3424 (OH), 3244 (N−OH), 1711 (N−C═O), 1606 and 1513 (aromatic) cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; EIMS *m*/*z* 261 ([M]$^+$, 50), 244 (100), 202 (55), 159 (38), 131 (30); HREIMS *m*/*z* 261.1001 [M]$^+$ (calcd for $C_{14}H_{15}NO_4$, 261.1000).

Antrocinnamomin C (3): yellowish oil; UV (MeOH) λ_{max} (log ϵ) 230 (3.80), 358 (3.71) nm; IR (neat) ν_{max} 3424 (OH), 1760 (carbonyl absorption of acid anhydride), 1606 and 1513 (aromatic) cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; EIMS *m*/*z* 246 ([M]$^+$, 100), 218 (21), 204 (65), 176 (30); HREIMS *m*/*z* 246.0888 [M]$^+$ (calcd for $C_{14}H_{14}O_4$, 246.0892).

Antrocinnamomin D (4): white, amorphous solid; $[\alpha]^{24}_D$ ±0 (*c* 0.18, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 210 (4.02), 278 (3.73) nm; IR (KBr) ν_{max} 3389 (OH), 1738 (carbonyl absorption of acid anhydride), 1607 and 1511 (aromatic) cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; EIMS *m*/*z* 316 ([M]$^+$, 4), 248 (100), 220 (52), 188 (25), 164 (67), 131 (27); HRESIMS *m*/*z* 339.1571 [M + Na]$^+$ (calcd for $C_{19}H_{24}O_4Na$, 339.1572).

Determination of NO Production and Cell Viability Assay. The murine macrophage cell line RAW264.7 (BCRC 60001 = ATCC TIB-71) was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and incubated at 37 °C in a humidified 5% CO_2 atmosphere with a 96-well flat-bottomed culture

Table 2. ^{13}C NMR (100 MHz) Data of Compounds **1**−**4**[a]

carbon	**1**[b]	**2**[c]	**3**[b]	**4**[b]
2	166.7	170.2	166.3	97.1
3	136.6	136.2	140.1	129.4
4	136.7	137.0	140.2	159.0
5	165.9	169.4	165.4	171.7
1′	33.1	33.7	33.6	35.4
2′	28.1	29.2	27.9	27.0
3′	22.7	22.9	22.7	23.3
4′	22.7	22.9	22.7	22.2
1″	120.6	121.3	120.2	121.5
2″	131.1	132.3	131.4	130.3
3″	114.9	116.5	116.0	114.7
4″	160.4	160.4	157.9	159.0
5″	114.9	116.5	116.0	114.7
6″	131.1	132.3	131.4	130.3
1‴	64.9			64.8
2‴	119.1			119.3
3‴	138.9			138.5
4‴	25.8			25.8
5‴	18.2			18.2
1⁗	167.1			
2⁗	17.6			

[a] Values in ppm (δ), with J (in Hz) in parentheses. [b] In $CDCl_3$. [c] In CD_3OD.

Table 3. Effects of Compounds **1**−**7** on LPS-Induced NO Production and Cell Viability in RAW264.7 Macrophages[a]

compound	nitrite (μM)	cell viability (% control)
control	3.0 ± 0.1	100 ± 5.5
LPS	25.7 ± 0.2	95.5 ± 3.3
1	12.5 ± 0.2[b]	91.1 ± 4.2
2	18.0 ± 0.3[b]	82.2 ± 3.2[c]
3	21.8 ± 0.5[b]	76.3 ± 5.4[c]
4	20.0 ± 1.5[b]	88.2 ± 3.3[c]
5	20.8 ± 1.5[b]	67.9 ± 5.3[c]
6	10.6 ± 0.5[b]	69.1 ± 5.0[c]
7	18.3 ± 2.6[b]	86.8 ± 2.9[c]

[a] RAW264.7 cells were incubated with compounds in the presence of LPS (1 μg/mL). The medium was harvested 24 h later and assayed for nitrite production. NO released was measured by using Griess reagent. Cell viability was evaluated with the MTT assay. The results are presented as a percentage of the control value obtained from nontreated cells. Values are expressed as mean ± SD of three individual experiments, performed in triplicate. [b] Statistically significant difference compared to LPS-activated cells (p < 0.05). [c] Statistically significant difference compared to untreated cells (p < 0.05).

plate. After 24 h, the condition medium was replaced with fresh DMEM and FBS. Then compounds **1**−**7** (0, 1, 5, 10, or 20 μg/mL) were added respectively in the presence of lipopolysaccharide (LPS, 1 μg/mL; Sigma, cat. no. L-2654) and incubated at the same conditions for 24 h. The cultured cells were then centrifuged and the supernatants used for NO production measurement, with a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay used to determine cell viability.

The supernatant was mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid solution) and incubated for 10 min at room temperature. Nitrite concentration was determined by measuring the absorbance at 540 nm using an ELISA plate reader (μ Quant).[15]

The MTT colorimetric assay was modified from that of Mosmann.[16] The test is based on the selective ability of living cells to reduce the yellow, soluble salt, MTT, to a purple-blue, insoluble formazan MTT (Merck; dissolved in phosphate-buffered saline at 5 mg/mL) solution added onto the attached cells mentioned above (10 μL per 100 μL culture) and incubated at 37 °C for 4 h. Then, DMSO was added, and amount of colored formazan metabolite formed was determined by absorbance at 550 nm. The optical density of formazan formed in control (untreated) cells was taken as 100% viability.

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