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IDENTIFICATION OF NEW POLYPRENYL HYDROQUINONE DERIVATIVES FROM TROPICAL MARINE SPONGE *IRGINIA* SP.

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Abstract – Chemical investigations of the tropical marine sponge of the genus *Ircinia* have resulted in the isolation of three new polyprenyl chromenes (**1–3**), together with known hydroquinone derivatives (**4–9**). Their structures were elucidated on the basis of mass spectrometry and detailed 2D NMR spectroscopic data. The anti-inflammatory activity of these compounds was evaluated. Compounds **7**, **8**, and **9** exhibited moderate inhibition of LPS-induced NO production in RAW 264.7 macrophages.

Polyprenyl quinones and chromenes are widely distributed among various marine organisms, including sponges¹⁻⁷ and brown algae.⁸⁻¹³ These metabolites, which are abundant in marine sponge *Ircinia* sp. and *Sarcotragus spinulosus*, exhibit brine shrimp lethality,⁵ anti-inflammatory activity,⁷ and inhibition activity against various enzymes such as Na⁺/K⁺-ATPase,² tyrosine protein kinase,⁴ and HIV-integrase.⁴ Quinones and chromenes from other marine organisms also possess diverse bioactive properties such as antimicrobial activity,⁹ cytotoxicity,¹¹ antioxidant activity,¹²⁻¹⁵ and phospholipase A₂ activity.¹⁶ During the course of our search for biologically active constituents from tropical marine sponges,^{17,18} we encountered a sponge of the genus *Ircinia* from the Federated States of Micronesia, and a crude extract from the sponge exhibited moderate anti-inflammatory activity. Guided by the results of ¹H NMR analyses, the organic extracts from this animal were separated by employing solvent partitioning followed by silica vacuum flash chromatography and HPLC to afford several polyprenyl quinones and chromenes

(1-9) (Figure 1). Herein, we describe the structure elucidation and biological evaluation of these secondary metabolites.

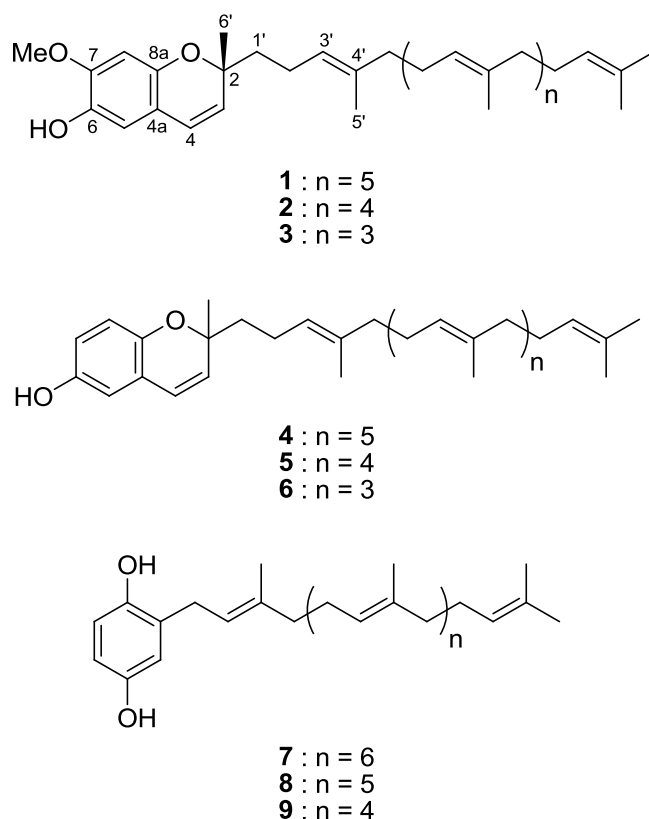


Figure 1. Structures of compounds **1–9** isolated from marine sponge *Ircinia* sp.

The specimens of *Ircinia* sp. were collected along the offshore of Weno Island, Chuuk State, Federated States of Micronesia, in 2009. The collected specimens were extracted with methanol and dichloromethane. The combined extracts were concentrated and fractionated into water, *n*-butanol, 15% aqueous methanol and *n*-hexane. The residue of the *n*-hexane layer was subjected to silica vacuum flash chromatography using gradient mixtures of *n*-hexane and ethyl acetate followed by silica HPLC to afford nine polyprenyl quinones and chromenes, including three new compounds. The six known metabolites were readily identified as 2-heptaprenylmethyl-2-methylchromen-6-ol (**4**),⁵ 2-hexaprenylmethyl-2-methylchromen-6-ol (**5**),⁵ 2-pentaprenylmethyl-2-methylchromen-6-ol (**6**),⁷ 2-octaprenyl-1,4-hydroquinone (**7**),¹ 2-heptaprenyl-1,4-hydroquinone (**8**),¹ and 2-hexaprenyl-1,4-hydroquinone (**9**)¹ by a combination of spectroscopic analysis and comparison with data reported for these compounds.

Compounds **1**, **2**, and **3** were each obtained as a yellow gum, and analyzed for $C_{47}H_{70}O_3$, $C_{42}H_{62}O_3$, and $C_{37}H_{54}O_3$, respectively, by combined HREIMS and ^{13}C NMR spectrometry. The analysis of the ^{13}C NMR data of these compounds revealed that the compounds had quite similar carbon signals in the

aromatic/olefinic region. The chromenol and polyprenyl natures of these compounds were evident from the eight individual carbon signals (δ_C 100.1 – 146.7) and the two clustered carbon signals (δ_C 124.0 – 124.4 and δ_C 134.8 – 135.2) in the ^{13}C NMR data. There were subtle differences in the relative intensities of the clustered carbon signals in this region of the ^{13}C NMR spectra of each compound; these differences resulted from the difference in the identities of repeated prenyl units. In the ^1H NMR spectra, two singlet aromatic protons (δ_H 6.57, 6.39) and two sets of doublet protons at δ_H 6.26 (d, $J = 9.8$ Hz) and 5.46 (d, $J = 9.8$ Hz) were found, which revealed the presence of a tetra-substituted styrene moiety in the molecule. The presence of a chromene skeleton was supported by an observed absorption maximum at 326 nm in the UV spectrum.

The structures of the metabolites (**1–3**) were determined by detailed interpretation of 2D NMR data. Long-range correlations of the aromatic protons at δ_H 6.57 and 6.39, the olefinic proton signals at δ_H 6.26 and 5.46, and the upfield singlet protons at δ_H 3.85 (3H) and 1.37 (3H) with chromene carbons in the HMBC data readily established the presence of a 7-methoxy-2-methylchromen-6-ol moiety (Table 1). Similarly, long-range correlations of the vinyl methyl protons at δ_H 1.59 – 1.68 with neighboring carbons, combined with the ^1H COSY correlations of the olefinic protons with aliphatic protons, defined the structure of the prenyl portions. The important HMBC interactions of compound **1** are shown in Figure 2.

Table 1. Selected NMR data for compound **1** in CDCl_3 (δ in ppm)

| Position | δ_H (mult., J) | δ_C | HMBC (δ_H to δ_C) |
|--------------------|--------------------------|------------|-----------------------------------|
| 2 | | 78.2 | |
| 3 | 5.46 (d, 9.8) | 127.6 | C2, C4a |
| 4 | 6.26 (d, 9.8) | 122.5 | C2, C5, C8a |
| 4a | | 113.9 | |
| 5 | 6.57 (s) | 111.7 | C4, C6, C7, C8a |
| 6 | | 139.2 | |
| 7 | | 146.7 | |
| 8 | 6.39 (s) | 100.1 | C4a, C6, C7, C8a |
| 8a | | 146.7 | |
| 1' | 1.59-1.62 (m) | 40.9 | |
| 2' | 1.98-2.08 (m) | 22.6 | |
| 3' | 5.09-5.14 (m) | 124.1 | |
| 4' | | 135.2 | |
| 5' | 1.59 (s) | 16.0 | |
| 6' | 1.37 (s) | 25.7 | |
| 7-OCH ₃ | 3.85 (s) | 56.0 | C7 |

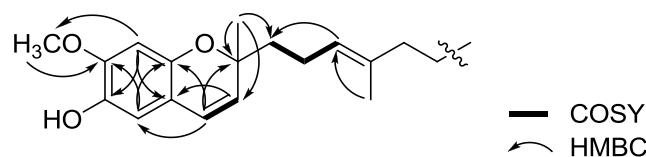


Figure 2. COSY and selected HMBC (H→C) correlations of compound **1**.

The identities of the repeated prenyl units were determined to be linear heptaprenyl (**1**), hexaprenyl (**2**), and pentaprenyl (**3**) on the basis of MS and NMR analysis. *E* geometry of C-3' olefin was determined by the upfield shifts of the vinyl methyl carbons at δ_c 16.0 (C-5'). The absolute stereochemistry at the C-2 asymmetric center was determined to be *R* on the basis of CD measurement and application of Crabbe's rule.^{12,19} Thus, the structures of **1**, **2** and **3** were determined to be (2*R*)-2-heptaprenylmethyl-7-methoxy-2-methylchromen-6-ol, (2*R*)-2-hexaprenylmethyl-7-methoxy-2-methylchromen-6-ol, and (2*R*)-2-pentaprenylmethyl-7-methoxy-2-methylchromen-6-ol, respectively.

Compounds **1–9** were evaluated for their cytotoxicity on human leukemia HL-60 cells *via* a colorimetric MTT assay. The absorbance was measured in triplicate and the percentage of cell viability was calculated.²⁰ *In vitro* growth inhibition activity was examined after 72 h treatment of cells, and the results are summarized in Figure 3. As can be seen from the figure, compounds **2**, **7**, **8**, and **9** exhibited significant growth inhibition at 50 μ M concentration against HL-60 with 76.9%, 66.0%, 71.5%, and 72.8% inhibition, respectively. The growth inhibition rate was determined by the MTT assay. Each value represents that the mean \pm SE from three independent experiments.

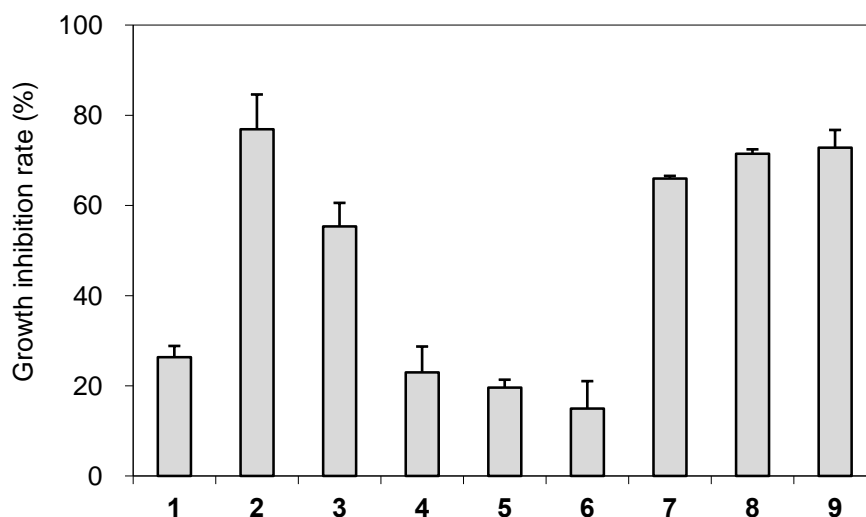


Figure 3. Inhibitory effect of compounds against growth of HL-60 cells.

The anti-inflammatory effects of these nine compounds were also evaluated by determining their

inhibitory effects on pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells. NO is a short-lived free radicals and plays an important role in the pathogenesis of various inflammatory diseases.²¹ LPS induced NO production, and the increase was inhibited markedly by three compounds. As shown in Figure 4, polyprenyl-1,4-hydroquinones **7**, **8**, and **9** inhibited LPS-induced NO production by 89.0%, 86.6%, and 71.8% at 50 μ M, respectively. Therefore, the inhibitory effect on NO production suggests anti-inflammatory action of polyprenyl-1,4-hydroquinones **7**, **8**, and **9**. The production of NO was assayed in the culture medium of macrophages stimulated with LPS (1 μ g/mL) for 24 h in the presence of compounds (50 μ M). Each value indicates the mean \pm SE from three independent experiments.

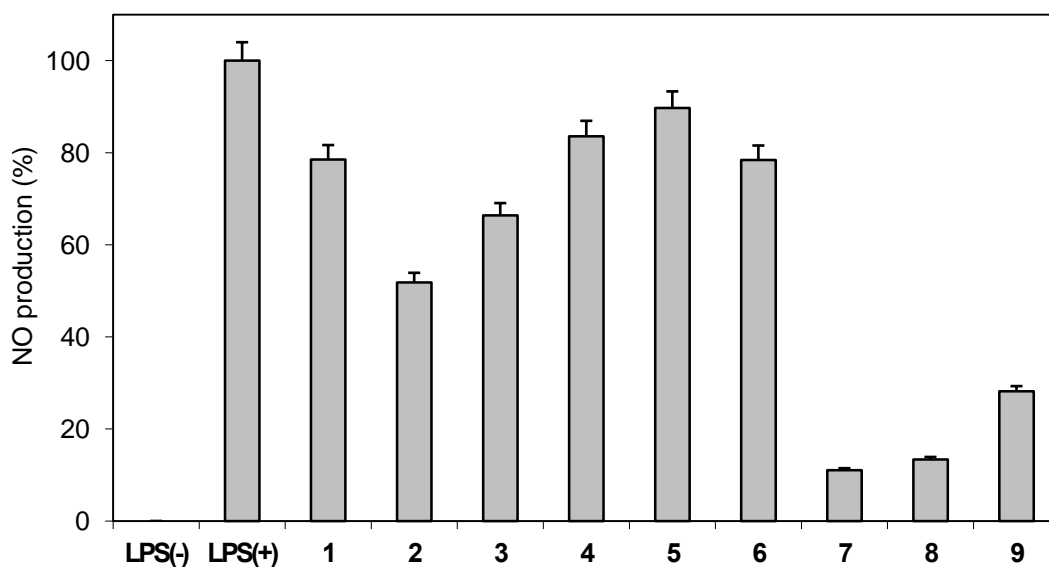


Figure 4. Inhibitory effect of compounds on LPS-induced NO production in RAW 264.7 macrophages.

In conclusion, biological and chemical investigations of the crude extract of the Micronesian marine sponge of the genus *Ircinia* led to the isolation of three new polyprenyl hydroquinone derivatives **1–3**, together with six known related hydroquinone derivatives. Their structures were elucidated on the basis of mass spectrometry and detailed 2D NMR spectroscopic data. Among the isolated polyprenyl hydroquinone derivatives, compounds **7**, **8**, and **9** exhibited moderate inhibition of LPS-induced NO production in RAW 264.7 macrophages.

EXPERIMENTAL

General Experimental Procedures

Optical rotations were measured on a JASCO DIP-1000 digital polarimeter using a 5 cm cell. CD data were obtained on a JASCO J-715 spectropolarimeter. NMR spectra were recorded on a Varian Unity 500

instrument at 500 MHz for ^1H and 125 MHz for ^{13}C . All chemical shifts were recorded with respect to the residual solvent signals as an internal standard (CDCl_3 , δ_{H} 7.26 ppm, δ_{C} 77.0 ppm). IR spectra were recorded on a JASCO FT/IR-4100 spectrophotometer as thin films. UV spectra were obtained in MeOH using a Shimadzu UV-1650PC spectrophotometer. Mass spectral data were obtained at the Korean Basic Science Institute, Seoul, Korea. All solvents used were of spectroscopic grade or were freshly distilled from glass prior to use.

Animal Material

Specimens of *Ircinia* sp. were collected by hand with scuba equipment at a depth of 15–20 m off the shore of Weno Island, Chuuk State, Federated States of Micronesia, in July, 2009. The sample was identified by its morphological character and deposited at the Marine Biotechnology Research Center, Korea Ocean Research & Development Institute, Korea, under the curatorship of Dr. H. S. Park.

Extraction and Isolation

The specimens of *Ircinia* sp. were collected from the Federated States of Micronesia in 2009. The fresh sponge was immediately frozen and kept at $-25\text{ }^\circ\text{C}$ until its chemical investigation. The specimens were lyophilized (wet wt. 512 g) and repeatedly extracted with MeOH (1 L \times 2) and CH_2Cl_2 (1 L \times 1). The extract was filtered and concentrated under reduced pressure to afford 82.7 g of crude extract. The residue was partitioned between H_2O and *n*-BuOH to yield 11.2 g of organic-soluble material. The *n*-BuOH layer was re-partitioned between 15% aqueous MeOH (3.7 g) and *n*-hexane (6.2 g). The residue of the *n*-hexane layer was subjected to silica vacuum flash chromatography using gradient mixtures of *n*-hexane and EtOAc as eluents (elution order: 10%, 20%, 30%, 40%, 50% EtOAc in *n*-hexane, and 100% EtOAc). The fraction eluted with 20% EtOAc in *n*-hexane (1.41 g) was dried and separated by silica HPLC (YMC silica column, 250 \times 10 mm; 5% EtOAc in *n*-hexane) to afford in order of elution, 11.2, 5.3, 8.1, 24, 5.1, and 61 mg of **1**, **2**, **3**, **4**, **5**, and **6**, respectively. The fraction eluted with 30% EtOAc in *n*-hexane (2.65 g) was dried and separated by silica HPLC (10% EtOAc in *n*-hexane) to yield 37.1, 19.2, and 5.0 mg of **7**, **8**, and **9**, respectively. The purity of these compounds was checked by HPLC.

(2R)-2-Heptaprenylmethyl-7-methoxy-2-methylchromen-6-ol (1): $[\alpha]_{\text{D}}^{20} +18.5$ (*c* 0.05, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 6.57 (1H, s, H-5), 6.39 (1H, s, H-8), 6.26 (1H, d, $J = 9.8$, H-4), 5.46 (1H, d, $J = 9.8$, H-3), 5.14–5.09 (7H, m, =CH), 3.85 (3H, s, OCH_3), 2.04–2.09 (14H, m, CH_2), 1.95–2.01 (14H, m, CH_2), 1.67 (3H, s, CH_3), 1.60–1.67 (21H, m, CH_3), 1.37 (3H, CH_3 , H-6'); ^{13}C NMR (CDCl_3 , 125MHz) δ 146.7 (C-7, C-8a), 139.2 (C-6), 135.2 (C-4'), 135.0–134.8 (5C), 131.2 (1C), 127.6 (C-3), 124.4–124.0

(7C), 122.5 (C-4), 113.9 (C-4a), 111.7 (C-5), 100.1 (C-8), 78.2 (C-2), 56.0 (OCH₃), 40.9 (C-1'), 39.7 (6C), 26.8-26.6 (6C), 26.0 (1C), 25.7 (C-6'), 22.6 (C-2'), 17.6 (1C), 16.0 (C6); IR 3350, 2995, 1770, 1759, 1374, 1245, 1056 cm⁻¹; UV (MeOH) λ_{\max} (log ϵ) 324 nm (2.7); HREIMS m/z 682.5316, (calcd for C₄₇H₇₀O₃, m/z 682.5325)

(2R)-2-Hexaprenylmethyl-7-methoxy-2-methylchromen-6-ol (2): $[\alpha]_{\text{D}}^{20} +20.9$ (*c* 0.05, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 6.57 (1H, s), 6.39 (1H, s), 6.26 (1H, d, *J* = 9.8), 5.45 (1H, d, *J* = 9.8), 5.15-5.09 (6H, m), 3.84 (3H, s), 2.04-2.09 (12H, m), 1.95-2.01 (12H, m), 1.67 (3H, s), 1.60-1.67 (18H, m), 1.37 (3H, CH₃); ¹³C NMR (CDCl₃, 125MHz) δ 146.7, 139.2, 135.2, 135.0-134.8 (4C), 131.2, 127.6, 124.4-124.0 (6C), 122.4, 113.9, 111.7, 100.1, 78.1, 56.0, 40.9, 39.7 (5C), 26.8-26.6 (5C), 26.0, 25.7, 22.6, 17.6, 16.0 (C5); IR 3350, 2995, 1770, 1759, 1374, 1245, 1056 cm⁻¹; UV (MeOH) λ_{\max} (log ϵ) 326 nm (3.0); HREIMS m/z 614.4697, (calcd for C₄₂H₆₂O₃, m/z 614.4699)

(2R)-2-Pentaprenylmethyl-7-methoxy-2-methylchromen-6-ol (3): $[\alpha]_{\text{D}}^{20} +19.5$ (*c* 0.05, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 6.57 (1H, s), 6.39 (1H, s), 6.25 (1H, d, *J* = 9.8), 5.46 (1H, d, *J* = 9.8), 5.15-5.09 (5H, m), 3.85 (3H, s), 2.04-2.09 (10H, m), 1.95-2.01 (10H, m), 1.67 (3H, s), 1.60-1.67 (15H, m), 1.37(3H, CH₃); ¹³C NMR (CDCl₃, 125MHz) δ 146.7, 139.2, 135.2, 135.0-134.8 (3C), 131.2, 127.6, 124.4-124.0 (5C), 122.5, 113.9, 111.7, 100.1, 78.1, 56.0, 40.9, 39.7 (4C), 26.8-26.6 (4C), 26.0, 25.7, 22.6, 17.6, 16.0 (C4); IR 3345, 2995, 1772, 1760, 1378, 1244, 1056 cm⁻¹; UV (MeOH) λ_{\max} (log ϵ) 326 nm (3.1); HREIMS m/z 546.4074, (calcd for C₃₇H₅₄O₃, m/z 546.4073).

2-Heptaprenylmethyl-2-methylchromen-6-ol (4): LRAPCIMS m/z 653.48 (M+H), (calcd for C₄₆H₆₉O₂, m/z 653.53).

2-Hexaprenylmethyl-2-methylchromen-6-ol (5): LRAPCIMS m/z 585.42 (M+H), (calcd for C₄₁H₆₁O₂, m/z 585.46).

2-Pentaprenylmethyl-2-methylchromen-6-ol (6): LRAPCIMS m/z 517.38 (M+H), (calcd for C₃₆H₅₃O₂, m/z 517.40).

2-Octaprenyl-1,4-hydroquinone (7): LRAPCIMS m/z 655.55 (M+H), (calcd for C₄₆H₇₁O₂, m/z 655.55).

2-Heptaprenyl-1,4-hydroquinone (8): LRAPCIMS m/z 587.44 (M+H), (calcd for C₄₁H₆₃O₂, m/z

587.48).

2-Hexaprenyl-1,4-hydroquinone (9): LRAPCIMS m/z 518.41 (M+H), (calcd for C₃₆H₅₃O₂, m/z 519.42).

HL-60 Cell Culture.

The human promyelocytic leukemia cell line (HL-60) was grown on an RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL). Cultures were maintained at 37 °C in a 5% CO₂ incubator.

Cell Growth Inhibitory Assay.

The cytotoxicity of compounds on tumor cells was assessed *via* a colorimetric MTT assay. The suspension of cells was seeded at 5×10^4 cells/mL together with the test compounds and incubated for up to 72 h prior to MTT treatment. MTT stock solution [2 mg/ml in phosphate buffered saline (PBS)] was added to each well to achieve a total reaction volume of 250 µL. After 4 h of incubation, the plates were centrifuged for 10 min at 2000 rpm and the supernatants were aspirated. The formazan crystals in each well were dissolved in DMSO, and the amount of purple formazan was quantified by measuring the absorbance at 540 nm.

RAW 264.7 Cell Culture.

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, KOREA). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO Inc., NY, USA) supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin and 10% fetal bovine serum (FBS; GIBCO). The cells were then incubated in an atmosphere of 5% CO₂ at 37 °C and were subcultured every 3 days.

Determination of NO Production.

LPS-induced NO production was measured in RAW 264.7 cells to determine the anti-inflammatory activities of polyprenyl hydroquinones. The cells (1.5×10^5 cells/mL) were treated with compounds **1–9** (at 50 µM concentration), followed by incubation with LPS (1 µg/mL) for 24 h. The quantity of nitrite that accumulated in the culture medium was measured as an indicator of NO production. In brief, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid); the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. Fresh culture

medium was employed as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

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