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The absolute configuration of prunioside A from *Spiraea prunifolia* and biological activities of related compounds

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Abstract

The stereochemistry of prunioside A isolated from *Spiraea prunifolia* was determined by chemical transformations and NMR spectral data analysis. The configurations at C-5 and C-6 were determined to be 5*S* and 6*R* by application of the modified Mosher's method, CD analysis, and ^{13}C NMR spectroscopic data analysis of an acetonide derivative. Other compounds related to prunioside A have inhibitory effects on the synthesis of nitric oxide in LPS-stimulated macrophage-like RAW 264.7 cells.

Keywords: *Spiraea prunifolia*; Rosaceae; Prunioside A; Mosher's method; Inhibitory effects on nitric oxide (NO) production

1. Introduction

Recently, prunioside A (**1**) has been isolated from the methanol extract of *Spiraea prunifolia* var. *simpliciflora* (Oh et al., 2001). It is a unique, highly oxidized monoterpene glycoside composed of coumaroyl, monoterpene-type, and glucosyl units. However, the stereochemistry of C-5 and C-6 positions in prunioside A (**1**) has not been reported. Although naturally occurring prunioside A (**1**) showed no inhibitory effect, its acetylated derivative (**2**) have a suppressive effect on the synthesis of nitric oxide by murine macrophage-like RAW 264.7 cells stimulated with interferon-γ (IFN-γ) plus lipopolysaccharide (LPS) (Oh et al., 2001).

Nitric oxide (NO) is produced by nitric oxide synthases in certain cells, and has been implicated in a wide range of physiological and pathological processes (Kilbourn et al., 1990). NO synthases can be classified into two major groups. NO produced by neuronal and endothelial NO synthases (cNOSs) is a key regulator of homeostasis. On the other hand, the inducible isoform of NO synthase (iNOS) plays important roles in macrophage-mediated cytotoxicity. Stimuli such as pro-inflammatory cytokines and/or endotoxins induce iNOS expression in various inflammatory cells, and once synthesized, iNOS is responsible for the prolonged, high-output production of NO. Despite its beneficial role in host defense, sustained NO production by iNOS can be deleterious to the host, and, in some cases, has been implicated in the pathogenesis of various inflammatory and autoimmune diseases (McCartney-Francis et al., 1993). Therefore, the modulation of iNOS could be a valuable approach to the treatment of diseases related to the overexpression of iNOS.

Based on the above considerations, further chemical modifications of prunioside A (**1**) were conducted to assign its absolute stereochemistry, and the effects of the prunioside A-related compounds on NO synthesis were evaluated.

2. Results and discussion

In order to assign the stereochemistry of C-5 and C-6 in prunioside A (**1**), cleavage of the coumaroyl and glucosyl units was necessary. Incubation of glycoside-possessing galloyl and glucosyl moieties with tannase at 30 °C for 3 h has been reported to yield the corresponding

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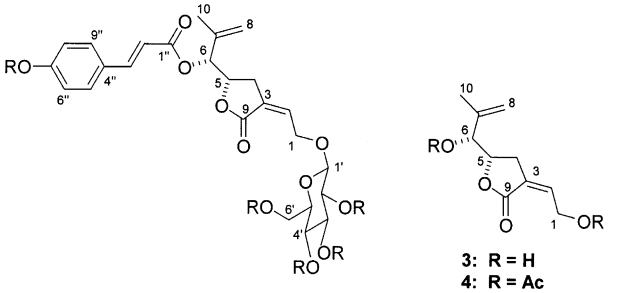
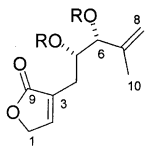
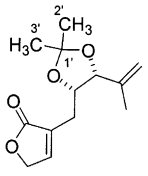
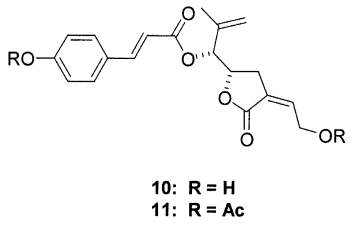
hydrolysis product that retains the glucosyl moiety (Kikuzaki et al., 2000). Therefore, prunioside A (**1**) was incubated with tannase at 30 °C for 12 h. In this case, however, this enzymatic hydrolysis procedure yielded compound **3**, in which both the coumaryl and glucosyl moieties have been removed. The structure of **3** was confirmed by analysis of MS and NMR (1D and 2D) data. Since product **3** possesses a secondary alcohol group, assignment of the absolute configuration at C-6 of **3** was attempted by conversion to Mosher's esters, and by application of Mosher's rules (Ohtani et al., 1991). Thus, compound **3** was converted into the two diastereomeric Mosher esters (**5** and **6**) by reaction with the *S* (+) and *R* (−) enantiomers of α-methoxy-α-(trifluoromethyl)phenyl acetyl chloride (MTPCl), respectively. Formation of the MTPA esters was confirmed by a significant downfield shift of H-6, appearance of NMR signals corresponding to MTPA groups, and by analysis of ESIMS data. Chemical shifts for the proton signals of the (*S*)- and (*R*)-MTPA esters of **3** were assigned by comparison with the NMR spectral data for **3**. The differences in ^{1}H NMR chemical shifts between the diastereoisomers **5** and **6** are illustrated in Fig. 1. Based on Mosher's theory, these data suggested the configuration at C-6 to be *R*. Having assigned the absolute configuration at C-6, it was envisioned that the relative stereochemistry of C-5 and C-6 positions could be determined by hydrolysis of the lactone unit to afford an alcohol group at C-5, thereby producing an 1,2-diol functionality at the C-5 and C-6 positions. It has been reported that acetonides of *syn* and *anti*-1,2-diols can be unambiguously distinguished by the ^{13}C NMR chemical shifts of the acetonide methyl groups (Dana and Danechpajouh, 1980; Solladié et al., 1997). Thus, compound **3** was hydrolyzed with 1N NaOH in MeOH, followed by acidic work-up (1N HCl). The resulting product was separated by HPLC to yield compound **7**, which clearly arose from **3** by hydrolysis of the γ-lactone ring and subsequent esterification of the resulting carboxylic acid group with primary alcohol group at the C-1 position. The structure of compound **7** was assigned by analysis of its MS and NMR (1D and 2D) spectral data. In order to determine the relative configuration at C-5 and C-6, compound **7** was transformed to the corresponding 1,2-diol acetonide (**9**) by treatment with *p*-toluenesulfonic acid and 2,2-dimethoxypropane. Formation of the acetonide was confirmed by analysis of MS and 1D NMR spectral data. Chemical shifts for the carbons in **9** were assigned by analysis of HMQC and HMBC data. The chemical shifts for the two acetonide methyl groups (δ 26.4 and δ 26.8) in the ^{13}C NMR spectrum of **9** were typical of gem-dimethyl groups of *erythro*-diol acetonides (Dana and Danechpajouh, 1980; Solladié et al., 1997). Furthermore, the CD spectrum of di-*p*-bromobenzoate (**8**) showed a clear positive exciton split [first Cotton effect at 256 nm ($\Delta\epsilon = +4.48$), and a second Cotton effect at 239 nm (($\Delta\epsilon = -5.95$)], affording clear evidence for the 5*S* and 6*R* configurations. Thus, the absolute stereochemistry of prunioside A was proposed as shown in **1**.

Since the acetylated derivative (**2**) of prunioside A has previously been shown to have an inhibitory effect on NO production, in contrast to naturally occurring prunioside A (Oh et al., 2001), the activity of compound **3**, which is closely related to prunioside A, was assessed. When the LPS-stimulated murine macrophage-like









Scheme 1. Compounds **1**–**11**.

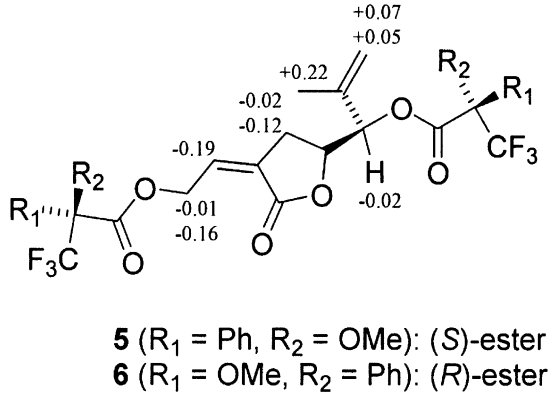


Fig. 1. Observed ^{1}H NMR resonance differences ($\Delta\delta = \delta_S - \delta_R$; ppm) for selected protons of the (*S*)-MTPA ester **5** and (*R*)-MTPA ester **6**.

RAW 264.7 cells were treated with compound **3**, dose-dependent inhibition of NO production was observed with an IC_{50} value of 3.0 μg/ml (Fig. 2A). Furthermore, this inhibitory activity was correlated with the suppression of iNOS mRNA expression as confirmed by the dose dependently reduced amount of iNOS mRNA on reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Fig. 2B). As this monoterpene-type compound **3**, derived from enzymatic hydrolysis of the natural product, has dramatically enhanced inhibitory activity compared to that of prunioside A, additional compounds related to prunioside A were prepared and evaluated in this assay. Thus, compound **10**, which lacks the glucosyl moiety compared to the natural product, was prepared by incubation with β-glucosidase. The formation of the desired product was confirmed by analysis of MS and NMR spectroscopic data. Furthermore, compounds **3** and **10** were converted to **4** and **11**, respectively, by acetylation. Compounds **4** and **11** showed dose-dependent inhibition of NO production, with IC_{50} values of 2.2 and 5.1 μg/ml, respectively, while compound **10** showed a very weak inhibitory effect (12% inhibition at 10 μg/ml). The suppressive effects of **4** and **11** on the expression of iNOS mRNA were also confirmed by RT-PCR analysis (data not shown).

In summary, the stereochemistry of prunioside A (**1**) was assigned by chemical modifications and NMR analysis of the modified compounds. Upon analysis of the biological activities of prunioside A (**1**) and its related compounds, acetylation of the hydroxyl groups tends to enhance the activity, although the magnitude of enhancement depends on the nature of the compounds, as seen in the activities of compounds **1**, **3**, and **10** versus compounds **2**, **4**, and **11**, respectively. Furthermore, strong inhibitory activity of compound **3** as compared to no or weak inhibitory activities of compounds **1** and **10** suggested that the monoterpene-type moiety of prunioside A (**1**) might be the key structural unit exhibiting inhibitory effects on the production of NO by the stimulated cells.

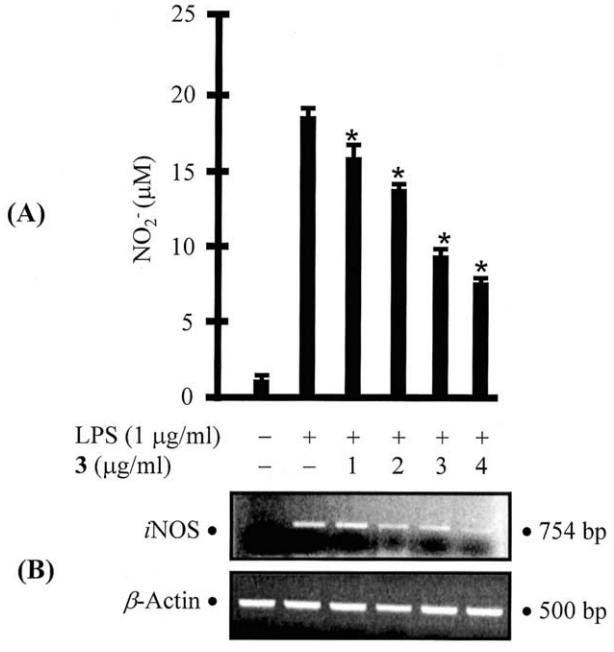


Fig. 2. Dose-dependent effect of compound **3** on NO production as well as iNOS mRNA expression in stimulated RAW 264.7 cells. (A) The cells were cultured with various concentrations of compound **3** for 18 h. NO released was measured by using the Griess reagent and expressed as the means±S.D. of three independent experiments. *$P < 0.05$. (B) The inhibition of iNOS mRNA expression was analyzed by RT-PCR. The figure shows the agarose-gel electrophoresis of the RT-PCR products for iNOS and β-actin (control).

3. Experimental

3.1. General

ESIMS data were obtained on a MicroMass Quatro LC with electro-spray ionization. FABMS data were obtained on a JEOL JMS HX-110 spectrometer using 3-nitrobenzyl alcohol as a matrix. CD spectra were measured on a JASCO J-715 spectropolarimeter. NMR spectra were recorded in either acetone-d_6 or $CDCl_3$ using a JEOL Eclipse-500 MHz spectrometer (500 MHz for ^1H and 125 MHz for ^{13}C), and chemical shifts were referenced relative to the corresponding residual solvent signals (acetone-d_6: δ 2.04/29.9, $CDCl_3$: δ 7.24/77.0). HMQC and HMBC data were optimized for $^1J_{CH} = 140$ Hz and $^nJ_{CH} = 8$ Hz, respectively. HPLC separations were performed on a Symmetry Prep C_{18} column (1.9 × 30 cm; 7-μm particle size; flow rate of 4 ml/min). Compounds were detected by UV absorption at 210 and 254 nm.

3.2. Extraction and isolation of prunioside A (1)

The air-dried roots (1 kg) of *S. prunifolia* were collected from Iksan City, Chonbuk Province, Korea in May 2000, and extracted with MeOH for 24 h. The MeOH extract was concentrated, suspended in H_2O, and sequentially partitioned with *n*-hexane, CH_2Cl_2, and EtOAc. The EtOAc-soluble fraction (4.6 g) was subjected to C_{18} flash CC with a stepwise gradient of 40 to 100% (v/v) MeOH in H_2O. The fraction eluted at 60% MeOH in H_2O (1.2 g) was subjected to reversed-phase HPLC, using a gradient from 20 to 30% CH_3CN in H_2O over 50 min, then 100% CH_3CN for 10 min, to yield **1** (648 mg).

3.3. Enzymatic hydrolysis of prunioside A (1) with tannase

A solution of **1** (22.4 mg) in H_2O (5 ml) was incubated with 15 mg of tannase (49 units/mg, *Aspergillus oryzae*,

Wako) at 30 °C for 12 h. The reaction mixture was extracted with EtOAc (3 × 5 ml). The dried organic residue was subjected to reversed-phase HPLC to afford compound **3** (5.9 mg) and cinnamic acid (4.0 mg).

3.4. Compound 3

^{1}H NMR (500 MHz, acetone-d_6): δ 1.77 (3H, *s*, H-10), 2.81 (1H, *m*, H-4), 2.91 (1H, *m*, H-4), 4.08 (1H, *m*, H-6), 4.59 (2H, *m*, H-1), 4.65 (1H, *ddd*, J = 8.3, 5.1, 5.1 Hz, H-5), 4.91 (1H, *br s*, H-8), 5.05 (1H, *br s*, H-8), 6.27 (1H, *m*, H-2); ^{13}C NMR: δ 17.8 (*q*, C-10), 30.8 (*t*, C-4), 58.1 (*t*, C-1), 76.6 (*d*, C-6), 78.8 (*d*, C-5), 112.5 (*t*, C-8), 125.2 (*s*, C-3), 142.4 (*d*, C-2), 144.6 (*s*, C-7), 169.3 (*s*, C-9); ESI-MS: *m*/*z* 221 (100%, M + Na$^+$); LRFABMS: *m*/*z* 221 [M + Na]$^+$; HRFABMS: *m*/*z* 221.0787 (calc. for $C_{10}H_{14}O_4Na$, 221.0790).

3.5. Acetylation of compound 3

To a solution of **3** (2 mg) in CH_3CN (2 ml) was added acetic anhydride (0.5 ml) and triethylamine (2 ml), and the resulting solution was stirred at room temperature for 12 h. The solvent was then evaporated under N_2. The residue was redissolved in 2 ml of CH_2Cl_2 and extracted with H_2O (2 ml). The organic phase was dried and subjected to reversed-phase HPLC to afford diacetate **4** (2 mg).

3.6. Compound 4

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.78 (3H, *s*, H-10), 2.08 (3H, *s*, *Ac*), 2.10 (3H, *s*, *Ac*), 2.70 (1H, *m*, H-4), 2.97 (1H, *m*, H-4), 4.72 (1H, *ddd*, J = 8.3, 5.5, 5.5 Hz, H-5), 5.07 (1H, *br s*, H-8), 5.08 (1H, *br s*, H-8), 5.22 (3H, *m*, H-1 and H-6), 6.17 (1H, *m*, H-2); ^{13}C NMR: δ 19.1 (*q*, C-10), 20.9 (*q*, *Ac*-Me), 20.95 (*q*, *Ac*-Me), 30.9 (*t*, C-4), 60.7 (*t*, C-1), 76.6 (*d*, C-6), 77.7 (*d*, C-5), 116.4 (*t*, C-8), 126.2 (*s*, C-3), 136.9 (*d*, C-2), 139.1 (*s*, C-7), 168.5 (*s*, C-9), 170.0 (*s*, *Ac*-C), 170.8 (*s*, *Ac*-C); ESI-MS: *m*/*z* 305 (100%, M + Na$^+$).

3.7. Preparation of (S)-MTPA ester 5

A sample of compound **3** (2.5 mg) was dissolved in CH_2Cl_2 (0.5 ml). Added to the stirred solution were 4-*N*,*N*-dimethylaminopyridine (0.5 mg), triethylamine (35 μl), and (*S*)-MTPA-Cl (20 μl). The reaction mixture was stirred for 12 h, concentrated, and separated by reversed-phase HPLC to yield 2.7 mg of compound **5**.

3.8. Compound 5

^{1}H NMR (500 MHz, acetone-d_6): δ 1.85 (3H, *s*, H-10), 2.71 (1H, *m*, H-4), 3.12 (1H, *m*, H-4), 3.52 (3H, *s*, O*Me*), 3.57 (3H, *s*, O*Me*), 4.91 (1H, *ddd*, J = 8.3, 6.0, 6.0 Hz, H-5), 5.19 (1H, *br s*, H-8), 5.23 (1H, *m*, H-1), 5.24 (1H, *br s*, H-8), 5.46 (1H, *m*, H-1), 5.60 (1H, *d*, J = 6.0 Hz, H-6), 6.12 (1H, *m*, H-2), 7.36–7.64 (10H, *series of m*, Ar-Hs); ESI-MS (positive ion detection): *m*/*z* 631 (100%, M + H$^+$).

3.9. Preparation of (R)-MTPA ester 6

Compound **6** was prepared from compound **3** and (*R*)-MTPA-Cl using a procedure analogous to the one described above for the preparation of compound **5**.

3.10. Compound 6

^{1}H NMR (500 MHz, acetone-d_6): δ 1.63 (3H, *s*, H-10), 2.83 (1H, *m*, H-4), 3.14 (1H, *m*, H-4), 3.55 (3H, *s*, O*Me*), 3.57 (3H, *s*, O*Me*), 4.93 (1H, *ddd*, J = 7.4, 6.4, 6.4 Hz, H-5), 5.14 (1H, *br s*, H-8), 5.17 (1H, *br s*, H-8), 5.39 (1H, *m*, H-1), 5.47 (1H, *m*, H-1), 5.57 (1H, *d*, J = 7.4 Hz, H-6), 6.31 (1H, *m*, H-2), 7.25–7.56 (10H, *series of m*, Ar-Hs); ESI-MS: *m*/*z* 631 (100%, M + H$^+$).

3.11. Hydrolysis of compound 3 under mild alkaline condition

A solution of compound **3** (5 mg) in THF (2 ml) was added to 2 ml of NaOH (1 N), and stirred at 25 °C for 80 min. The reaction mixture was concentrated, partitioned between CH_2Cl_2 and H_2O (1N HCl). The organic phase was dried and subjected to reversed-phase HPLC to afford compound **7** (3.4 mg).

3.12. Compound 7

^{1}H NMR (500 MHz, acetone-d_6): δ 1.74 (3H, *s*, H-10), 2.32 (1H, *m*, H-4), 2.42 (1H, *m*, H-4), 3.86 (1H, *m*, H-5), 3.88 (1H, *d*, J = 6.0 Hz, H-6), 4.86 (1H, *br s*, H-8), 4.80 (2H, *m*, H-1), 4.99 (1H, *br s*, H-8), 7.47 (1H, *m*, H-2); ^{13}C NMR: δ 17.5 (*t*, C-10), 29.0 (*t*, C-4), 70.3 (*t*, C-1 and *d*, C-5), 78.4 (*d*, C-6), 112.3 (*t*, C-8), 130.3 (*s*, C-3), 145.9 (*s*, C-7), 147.7 (*d*, C-2), 174.3 (*s*, C-9); ESI-MS: *m*/*z* 199 (100%, M + H$^+$); LRFABMS: *m*/*z* 199 [M + H]$^+$; HRFABMS *m*/*z*: [M + H]$^+$ 199.0974 (calc. for $C_{10}H_{15}O_4$, 199.0970).

3.13. Formation of p-bromobenzoate 8

To a solution of **7** (2.2 mg) in CH_3CN (2 ml) was added triethylamine (100 μl), 4-*N*,*N*-dimethylaminopyridine (0.5 mg), and *p*-bromobenzoyl chloride (5 mg). The resulting solution was stirred at room temperature for 2 h. The solvent was then evaporated under N_2. The residue was redissolved in 2 ml of EtOAc and extracted with H_2O (2 ml). The organic phase was dried and subjected to reversed-phase HPLC to afford di-*p*-bromobenzoate **4** (1.2 mg).

^{1}H NMR (500 MHz, acetone-d_6): δ 1.89 (3H, *s*, H-10), 2.76 (1H, *m*, H-4), 2.84 (1H, *m*, H-4), 4.75 (2H, *m*, H-1), 5.04 (1H, *br s*, H-8), 5.17 (1H, *br s*, H-8), 5.65 (1H, *d*, $J=6.0$ Hz, H-6), 5.83 (1H, *m*, H-5), 7.52 (1H, *m*, H-2), 7.36 (2H, *dd*, $J=8.5$ Ar-Hs), 7.64 (2H, *dd*, $J=8.5$ Ar-Hs), 7.88 (2H, *dd*, $J=8.5$ Ar-Hs), 7.93 (2H, *dd*, $J=8.5$ Ar-Hs); ESI-MS: *m/z* 563 (100%, $M+H^+$).

3.14. Formation of acetonide 9

To a solution of 2 mg of **7** in anhydrous acetone (1 ml) was added 10 μl of 2,2-dimethoxypropane and 0.2 mg of *p*-toluenesulfonic acid. The resulting solution was stirred at 25 °C for 6 h, and the organic solvents were removed in vacuo. Water was added, followed by extraction with ether (3 × 3 ml). The dried organic residue was subjected to semi-preparative reversed-phase HPLC to afford acetonide **9** (1.4 mg).

3.15. Compound 9

^{1}H NMR (500 MHz, acetone-d_6): δ 1.34 (3H, *s*, H-2′ or H-3′), 1.36 (3H, *s*, H-2′ or H-3′), 1.75 (3H, *s*, H-10), 2.43 (2H, *m*, H-4), 4.06 (1H, *m*, H-5), 4.12 (1H, *d*, $J=8.7$ Hz, H-6), 4.81 (2H, *m*, H-1), 4.94 (1H, *m*, H-8), 5.06 (1H, *m*, H-8), 7.52 (1H, *m*, H-2); ^{13}C NMR: δ 16.6 (*q*, C-10), 26.4 (*q*, C-2′ or C-3′), 26.8 (*q*, C-2′ or C-3′), 28.2 (*t*, C-4), 70.3 (*t*, C-1), 76.3 (*d*, C-5), 84.5 (*d*, C-6), 108.5 (*s*, C-1′), 113.9 (*t*, C-8), 129.3 (*s*, C-3), 142.1 (*s*, C-7), 148.2 (*d*, C-2), 173.8 (*s*, C-9); ESI-MS: *m/z* 261 (100%, $M+Na^+$).

3.16. Enzymatic hydrolysis of compound 1 with β-glucosidase

A solution of compound **1** (40 mg) in H_2O (10 ml) was incubated at 37 °C for 12 h with β-glucosidase (20 mg) from almonds (Sigma Chemical Co., St. Louis, MO). The reaction mixture was extracted with EtOAc (3 × 10 ml), and the dried organic residue was subjected to reversed-phase HPLC to afford compound **10** (25 mg).

3.17. Compound 10

^{1}H NMR (500 MHz, acetone-d_6): δ 1.82 (3H, *s*, H-10), 2.75 (1H, *m*, H-4), 3.08 (1H, *m*, H-4), 4.58 (1H, *m*, H-1), 4.64 (1H, *m*, H-1), 4.91 (1H, *ddd*, $J=8.3$, 5.5, 5.5, H-5), 5.04 (1H, *br s*, H-8), 5.10 (1H, *br s*, H-8), 5.40 (1H, *d*, $J=5.5$, H-6), 6.32 (1H, *m*, H-2), 6.38 (1H, *d*, $J=16.0$ Hz, H-2″), 6.89 (2H, *d*, $J=8.7$ Hz, H-6″ and H-8″), 7.56 (2H, *d*, $J=8.7$ Hz, H-5″ and H-9″), 7.62 (1H, *d*, $J=16.0$ Hz, H-3″); ^{13}C NMR: δ 18.5 (*q*, C-10), 30.9 (*t*, C-4), 58.1 (*t*, C-1), 76.7 (*d*, C-5), 77.4 (*d*, C-6), 114.1 (*d*, C-2″), 114.4 (*t*, C-8), 115.9 (*d*, C-6″ and C-8″), 124.3 (*s*, C-3), 126.0 (*s*, C-4″), 130.3 (*d*, C-5″ and C-9″), 140.5 (*s*, C-7), 143.0 (*d*, C-2), 145.5 (*d*, C-3″), 160.0 (*s*, C-7″), 165.6 (*s*, C-1″), 168.9 (*s*, C-9); ESI-MS: *m/z* 345 (100%, $M+H^+$); LRFABMS: *m/z* 345 $[M+H]^+$; HRFABMS: *m/z* 345.1335 (calc. for $C_{19}H_{21}O_6$, 345.1338).

3.18. Acetylation of compound 10

A solution of **10** (5 mg), 4-*N*,*N*-dimethylaminopyridine (0.5 mg), and acetic anhydride (0.5 ml) in acetone (2 ml) was stirred for 20 h at room temperature. The solvent was then evaporated under N_2. The residue was redissolved in 1.5 ml of EtOAc, and extracted with H_2O (2 × 2 ml). The organic phase was evaporated, and the dried organic residue was subjected to reversed-phase HPLC to yield diacetate **11** (6 mg).

3.19. Compound 11

^{1}H NMR (500 MHz, acetone-d_6): δ 1.84 (3H, *s*, H-10), 1.93 (3H, *s*, *Ac*), 2.26 (3H, *s*, *Ac*), 2.81 (1H, *m*, H-4), 3.15 (1H, *m*, H-4), 4.98 (1H, *ddd*, $J=8.7$, 4.6, 4.6, H-5), 5.03-5.19 (2H, *m*, H-1), 5.06 (1H, *br s*, H-8), 5.11 (1H, *br s*, H-8), 5.45 (1H, *d*, $J=4.6$ Hz, H-6), 6.23 (1H, *m*, H-2), 6.58 (1H, *d*, $J=16.1$ Hz, H-2″), 7.20 (2H, *d*, $J=8.3$ Hz, H-6″ and H-8″), 7.69 (1H, *d*, $J=16.1$ Hz, H-3″), 7.75 (2H, *d*, $J=8.3$ Hz, H-5″ and H-9″); ^{13}C NMR: δ 18.5 (*q*, C-10), 19.8 (*q*, *Ac*-Me), 20.1 (*q*, *Ac*-Me), 31.0 (*t*, C-4), 60.2 (*t*, C-1), 76.8 (*d*, C-5), 77.5 (*d*, C-6), 114.5 (*t*, C-8), 117.6 (*d*, C-2″), 122.5 (*d*, C-6″ and C-8″), 127.1 (*s*, C-4″), 129.5 (*d*, C-5″ and C-9″), 132.0 (*s*, C-3), 135.6 (*d*, C-2), 140.2 (*s*, C-7), 144.4 (*d*, C-3″), 152.8 (*s*, C-7″), 165.1 (*s*, C-1″), 168.58 (*s*, C-9), 168.59 (*s*, *Ac*-C), 169.9 (*s*, *Ac*-C); ESI-MS: *m/z* 429 (100%, $M+H^+$).

3.20. Macrophage cell line culture

The murine macrophage cell line RAW264.7 was obtained from the American Tissue Culture Collection (Rockville, MD). The cells were maintained in complete RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids, 1% antibiotic/antimycotic (100 U/ml of penicillin, 25 μg/ml of amphotericin D, and 100 μg/ml of streptomycin), 1.5% sodium bicarbonate, and 1% minimal essential vitamins at 37 °C in a humidified 5% CO_2 atmosphere.

3.21. Cell viability

For the determination of the cell viability, 50 μg/ml of MTT (Sigma) was added to 1 ml of cell suspension treated with LPS in the presence of various concentrations of test compounds (1×10^6 cells/ml in 24-well plates) for 4 h, and the formazan formed was dissolved in acidic 2-propanol; optical density was measured using an assay reader at 590 nm. The optical density of

formazan formed in control (untreated) cells was taken as 100% viability.

3.22. Measurement of nitrite concentration

Experiments were undertaken on cells grown in the presence of various concentrations of test compounds dissolved in DMSO with LPS (1 μg/ml) for 18 h. The final concentration of DMSO in culture media was 0.1%. Supernatants in cultured macrophages were collected and 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. The level of nitrite reflects nitric oxide synthesis. Sodium nitrite was used as a standard. The cell-free medium contained 5–8 μM of nitrite, and this value was determined in each experiment and subtracted from the value obtained with cells.

3.23. Analysis of mRNA levels for iNOS, and β-actin

RAW 264.7 cells grown on 100 mm culture dish were treated with various concentrations of test compounds, and stimulated with LPS for 6 h. Total RNA was extracted from the cells by the acid-guanidinium isothiocyanate phenol chloroform (AGPC) method (Chomczynski and Sacchi, 1987). The reverse transcription reactions (20 μl total volume) were carried out in 5 mM $MgCl_2$, PCR buffer (5 mM KCl, 10 mM Tris–HCl pH 8.3), using 1 mM dNTPs, 1.75 units/μl RNAse inhibitor, 2.5 units/μl M-MLV reverse transcriptase, 25 units/μl oligo (dT) primers and 25 ng total RNA. All the tubes were incubated at room temperature for 10 min before undergoing the following reverse transcription protocol in a thermal cycler: 42 °C for 60 min; 94 °C for 3 min. After generation of the first standard cDNAs, the tubes were placed on ice for 5 min prior to either to being stored at −20 °C or used for the polymerase chain amplification step. The PCR reaction (100 μl total volume) were carried out in 2 mM $MgCl_2$, PCR buffer, using AmpliTag™ DNA polymerase, 5 μg/ml sense and antisense primer pair and a 20 μl reverse transcription volume. For the amplification of the iNOS, the following primers were used: 5′-CAT GGC TTG CCC CTG GAA GTT TCT CTT CAA AG-3′ (sense) and 5′-GCA GCA TCC CCT CTG ATG GTG CCA TCG-3′ (antisense). β-Actin was also amplified as a control using the following primers: 5′-CCT CTA TGC CAA CAC AGT-3′ (sense) and 5′-AGC CAC CAA TCC ACA CAG-3′ (antisense). PCR (35 cycles for iNOS cDNAand 25 cycles for β-actin cDNA) was performed using the GeneAmp PCR system 2400 (Perkin Elmer).

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