 

# Oppositane-Type Sesquiterpenoids from the Formosan Soft Coral *Sinularia leptoclados*

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Two new natural products, the oppositane-type sesquiterpenoids leptocladolins A and B (**1** and **2**), along with two known compounds 1$\beta$-hydroxy-6$\alpha$-acetoxyeudesm-4(15)-ene (**3**) and 1$\beta$,6$\alpha$-dihydroxyeudesm-4(15)-ene (**4**) were obtained from a Formosan soft coral *Sinularia leptoclados*. The relative structures of **1** and **3** were confirmed by X-ray diffraction analysis. The absolute structures of **1** and **3** were determined by the Mosher's method. It was found that **4** significantly inhibited the upregulation of pro-inflammatory iNOS protein in LPS-stimulated RAW264.7 macrophage cells.

Formosan soft corals of the genus *Sinularia* have been shown to be rich sources of structurally unique and bioactive natural products.[1–6] Our previous studies on the chemical constituents of the soft coral *Sinularia leptoclados* have afforded metabolites such as norcembranoids[7] and steroids.[8] Recently, our chemical examination of a Dongsha Atoll soft coral *S. leptoclados* has resulted in the isolation of six metabolites including two new natural products of the rarely found oppositane-type sesquiterpenoids, leptocladolins A and B (**1** and **2**), and two known compounds, 1$\beta$-hydroxy-6$\alpha$-acetoxyeudesm-4(15)-ene (**3**)[9] and 1$\beta$,6$\alpha$-dihydroxyeudesm-4(15)-ene (**4**) (Chart 1).[9] The relative structures of **1**–**4** were established by detailed spectroscopic analysis and the relative structures of **1** and **3** were unambiguously proven by X-ray diffraction analysis. The absolute structures of **1** and **3** were further determined by the Mosher's method.[10] Oppositol, a rare natural product of this class, was isolated previously from a alga,[11] and compounds **1** and **2** are the first two oppositane-type metabolites discovered from soft corals. The cytotoxicity of compounds **1**–**4** against human medulloblastoma (Daoy), human laryngeal carcinoma (HEp2), human breast adenocarcinoma (MCF-7), and human cervical epitheloid carcinoma (HeLa) cancer cell lines, and the ability of **1**–**4** to inhibit the upregulation of pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells were also evaluated.

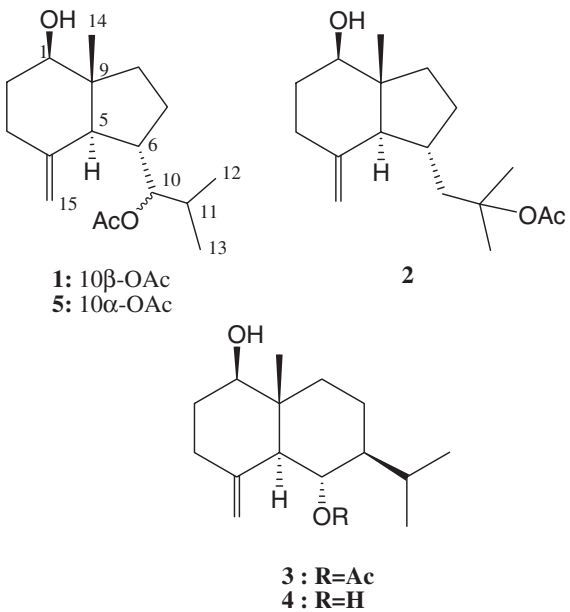



**Chart 1.**

Leptocladolin A (**1**) was obtained as colorless crystals. The HR-ESI-MS of **1** exhibited a $[M + Na]^+$ peak at $m/z$ 303.1935 and established a molecular formula $C_{17}H_{28}O_3$, implying four degrees of unsaturation. The IR absorptions at $\nu_{max}$ 3404 (broad) and 1713 $cm^{-1}$ revealed the presence of hydroxy and carbonyl functionalities. The $^{13}C$ NMR spectroscopic data of **1** exhibited seventeen carbons (Table 1), which were assigned with the assistance of a DEPT experiment to four methyls, four $sp^3$ methylenes, one $sp^2$ methylene, five $sp^3$ methines (including two oxymethines), one $sp^3$ quaternary carbon and two $sp^2$ quaternary carbons. From $^1H$ and $^{13}C$ NMR spectroscopic data, **1** was found to possess an acetoxy group [$\delta_H$ 1.92 (3H, s), $\delta_C$

Published on the web May 28, 2010; doi:10.1246/bcsj.20090263

**Table 1.** $^{1}H$ and $^{13}C$ NMR Data for Compounds **1** and **2**

| C/H | **1** | | **2** | |
|---|---|---|---|---|
| | $^{1}H$[a)] | $^{13}C$[b)] | $^{1}H$[a)] | $^{13}C$[b)] |
| 1 | 3.54 dd (11.5, 5.0)[c)] | 78.9 (CH)[d)] | 3.52 dd (12.0, 4.5) | 79.3 (CH) |
| 2 | 1.78 m; 1.44 m | 31.8 ($CH_2$) | 1.81 m; 1.50 m | 31.8 ($CH_2$) |
| 3 | 2.22 ddd (13.5, 5.5, 1.5) | 34.6 ($CH_2$) | 2.30 ddd (14.0, 5.5, 2.0) | 34.6 ($CH_2$) |
| | 1.98 ddd (13.5, 13.5, 5.5) | | 2.03 m | |
| 4 | | 146.3 (C) | | 145.9 (C) |
| 5 | 1.85 m | 55.7 (CH) | 1.54 m | 57.6 (CH) |
| 6 | 2.43 ddd (10.5, 10.5, 5.0) | 38.1 (CH) | 2.17 m | 32.4 (CH) |
| 7 | 1.92 m; 1.40 m | 25.8 ($CH_2$) | 2.05 m; 1.45 m | 29.9 ($CH_2$) |
| 8 | 1.73 dd (10.0, 8.5); 1.41m | 36.9 ($CH_2$) | 1.72 m; 1.41 m | 37.3 ($CH_2$) |
| 9 | | 49.3 (C) | | 47.5 (C) |
| 10 | 4.71 dd (10.5, 3.0) | 82.0 (CH) | 1.95 m; 1.63 dd (14.0, 10.5) | 46.8 ($CH_2$) |
| 11 | 1.89 m | 30.2 (CH) | | 82.8 (C) |
| 12 | 0.94 d (7.0) | 15.3 ($CH_3$) | 1.48 s | 26.3 ($CH_3$) |
| 13 | 0.87 d (7.0) | 20.2 ($CH_3$) | 1.50 s | 26.5 ($CH_3$) |
| 14 | 0.63 s | 12.1 ($CH_3$) | 0.65 s | 12.0 ($CH_3$) |
| 15 | 4.86 d (1.5); 4.68 d (1.5) | 107.4 ($CH_2$) | 4.86 d (1.5); 4.59 d (1.5) | 106.5 ($CH_2$) |
| OAc | 1.92 s | 21.1 ($CH_3$) | 1.98 s | 22.7 ($CH_3$) |
| | | 171.4 (C) | | 170.6 (C) |

a) Spectra recorded at 500 MHz in $CDCl_3$. b) 125 MHz in $CDCl_3$. c) $J$ values (in Hz) parentheses. d) Attached protons determined by DEPT experiments.

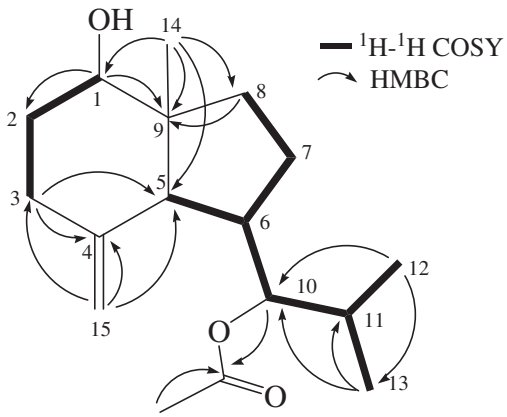
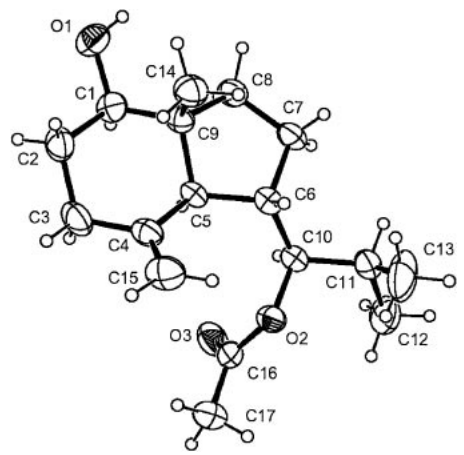


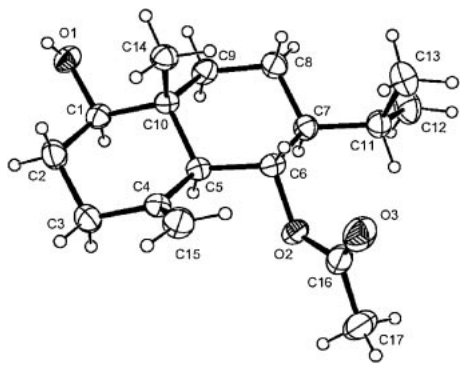

**Figure 1.** The COSY and HMBC correlations for **1**.

171.4 (C), 21.1 ($CH_3$)], in addition to one 1,1-disubstituted carbon–carbon double bond [$\delta_H$ 4.68 (1H, d, $J = 1.5$ Hz), 4.86 (1H, d, $J = 1.5$ Hz), $\delta_C$ 107.4, ($CH_2$), 146.3, (C)]. With the assistance of extensive 2D NMR study (COSY, HMQC, and HMBC), the oppositane-type skeleton of **1** was proposed (Figure 1). Moreover, the relative structure of **1** was fully established by a single-crystal X-ray diffraction analysis (Figure 2). The absolute structure of **1** was determined by the use of the Mosher's method.[10] Thus, the (*S*)- and (*R*)-MTPA esters of **1** (**1a** and **1b**, respectively) were prepared using the corresponding (*R*)-(−)- and (*S*)-(+)-$\alpha$-methoxy-$\alpha$-(trifluoromethyl)phenylacetyl chlorides, respectively. The determination of the chemical shift differences ($\delta_S - \delta_R$) for the protons neighboring C-1 led to the assignment of the *R* configuration at C-1 in **1** (Figure 3).

A structurally similar metabolite **2** was further isolated as a white solid and showed a $[M + Na]^+$ ion peak in the HR-ESI-MS corresponding to a molecular formula $C_{17}H_{28}O_3$, the same

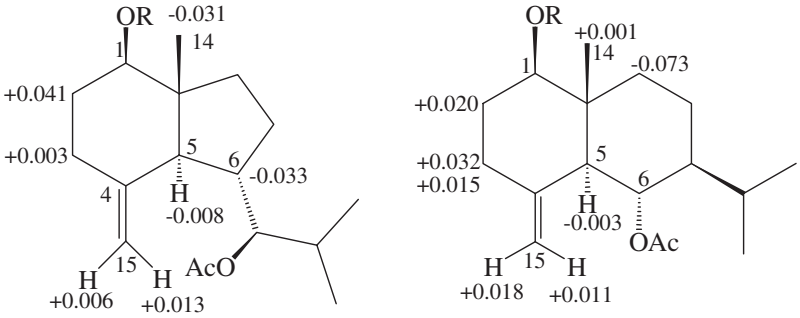



**1**




**3**

**Figure 2.** Molecular structures of **1** and **3** based on X-ray analysis.




**1a:** R= (*S*)-MTPA
**1b:** R= (*R*)-MTPA

**3a:** R= (*S*)-MTPA
**3b:** R= (*R*)-MTPA

**Figure 3.** $^{1}$H NMR chemical shift differences $\Delta\delta$ ($\delta_S - \delta_R$) in ppm for the MTPA esters of **1** and **3**.

as that of **1**. As in the case of **1**, the IR absorption bands of **2** at 3438 and 1733 cm$^{-1}$ suggested the presence of hydroxy and carbonyl functionalities, respectively. The ester functionality was also represented by one acetoxy group from the NMR signals at $\delta_C$ 170.6 (C) and 22.7 ($CH_3$); $\delta_H$ 1.98 (s). Comparison of the $^{13}$C NMR spectroscopic data of compound **2** with those of compound **1** (Table 1) indicated that **2** has the same carbon skeleton as that of **1**, while the acetoxy group was found to be attached at C-11 in **2** compared to C-10 in **1**. After the structure determination of **1**, we found that a structurally similar compound **5** had been obtained previously by an acid-catalyzed reaction of epoxygermacrene-D.[9] However, the relative configuration of C-10 in **5** was not known at that time. By further inspection of the $^{1}$H NMR data of both **1** and **5**, it was found that **5** showed an upfield shift at one of the two olefinic methylene protons ($\delta$ 4.73 and 4.65) relative to the corresponding proton of **1** with a 10*R*-configuration ($\delta_H$ 4.86 and 4.68). Thus, **5** should be the 10-epimer of **1**. Also, we found that compound **2** had been obtained previously by the same chemical reaction of epoxygermacrene-D.[9] Our present study however has led to the isolation of **2** for the first time from natural sources. In addition, our present work successfully led to the elucidation of the absolute structures of **1** and **3**, along with the full assignment of the $^{1}$H and $^{13}$C NMR spectroscopic data for both **1** and **2**.

The cytotoxicity of compounds **1**–**4** against the proliferation of a limited panel of cancer cell lines, including Daoy, HEp2, MCF-7, and HeLa was studied. The results showed that **1**–**4** are not cytotoxic ($ED_{50}$'s $>20\,\mu g\,mL^{-1}$) toward the above cancer cells. The in vitro anti-inflammatory effects of compounds **1**–**4** were tested. In this assay, the inhibition of LPS-induced upregulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophage cells was evaluated using immunoblot analysis. At a concentration of 10 μM, compound **4** was found to significantly reduce the level of iNOS protein to 22.4 ± 1.5% relative to the control cells stimulated with LPS only (Figure 4). However, none of these compounds could effectively inhibit the accumulation of COX-2 protein.

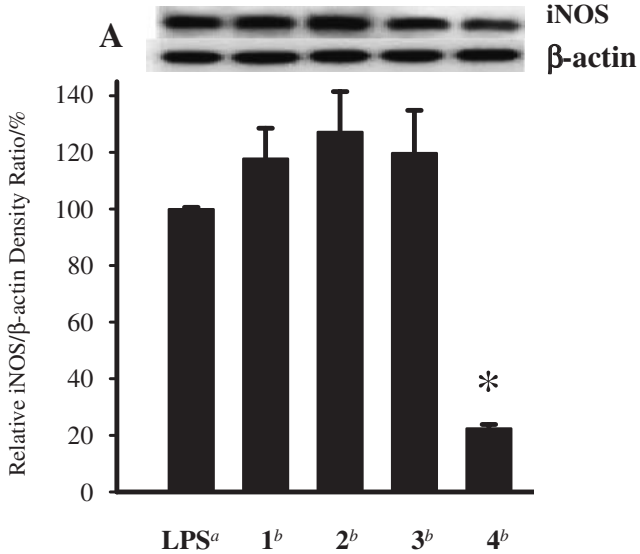



**Figure 4.** Effect of compounds **1**–**4** on iNOS protein expression of RAW264.7 macrophage cells by immunoblot analysis. (**A**) Immunoblots of iNOS and $\beta$-actin; The values are mean ± SEM ($n = 6$). Relative intensity of the LPS alone stimulated group was taken as 100%. Under the same experimental condition CAPE (caffeic acid phenylethyl ester, 10 μM) reduced the levels of the iNOS (22.4 ± 1.5%) *Significantly different from LPS alone stimulated group (*$P < 0.05$). [a]Stimulated with LPS. [b]Stimulated with LPS in the presence of **1**–**4** (10 μM).

## Experimental

**General Experimental Procedures.** Melting points were determined using a Fisher–Johns melting point apparatus. Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. ESI-MS and HR-ESI-MS were obtained with a Bruker APEX II mass spectrometer. NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR at 500 MHz for $^{1}$H and 125 MHz for $^{13}$C, respectively. Silica gel (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC. High-performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with a C-18 column (250 × 21.2 mm$^2$, 5 μm) and a Merck Hibar Si-60 column (250 × 21 mm$^2$, 7 μm).

**Animal Material.** *Sinularia leptoclados* was collected by hand using SCUBA off the coast of Dongsha Atoll, in April,

2007, at a depth of 10–15 m, and stored in a freezer until extraction. A voucher sample (specimen No. 20070429-1) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

**Extraction and Isolation.** The frozen bodies of *S. leptoclados* (720 g, wet wt) were minced and exhaustively extracted with EtOH (1 L × 4). The organic extract was concentrated to an aqueous suspension and was further partitioned between EtOAc and $H_2O$. The EtOAc layer was dried with anhydrous $Na_2SO_4$. After removal of solvent in vacuo, the residue (6.6 g) was subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0–100% of EtOAc, gradient) to yield 24 fractions. Fraction 9 (940 mg), eluted with EtOAc–*n*-hexane (1:8), was further purified over silica gel using acetone–*n*-hexane (1:9 to 1:6) to afford 2 subfractions. Subfraction 9-1 was further purified over silica gel using acetone–*n*-hexane (1:9) to afford **3** (15.2 mg). Subfraction 9-2 was separated by normal phase HPLC using acetone–*n*-hexane (1:7) to afford **4** (2.6 mg). Fraction 11 (390 mg), eluted with EtOAc–*n*-hexane (1:4), was further separated by normal phase HPLC using EtOAc–*n*-hexane (1:3) to yield **1** (9.7 mg) and **2** (3.0 mg).

**Leptocladolin A (1).** Colorless crystals; mp 122.0–124.0 °C; $[\alpha]_D^{25}$ +7.4 (*c* 0.50, $CHCl_3$); IR (neat) $\nu_{max}$ 3404, 2958, 2870, 1713, 1465, 1376, and 1265 $cm^{-1}$; $^{13}C$ and $^1H$ NMR data, see Table 1; ESI-MS *m/z* 303 $[M + Na]^+$; HR-ESI-MS *m/z* 303.1935 $[M + Na]^+$ (calcd for $C_{17}H_{28}O_3Na$, 303.1936).

**Leptocladolin B (2).** White solid; mp 117.0–119.0 °C; $[\alpha]_D^{25}$ +6.3 (*c* 0.30, $CHCl_3$); IR (neat) $\nu_{max}$ 3438, 2938, 2869, 1732, 1715, 1367, and 1261 $cm^{-1}$; $^{13}C$ and $^1H$ NMR data, see Table 1; ESI-MS *m/z* 303 $[M + Na]^+$; HR-ESI-MS *m/z* 303.1934 $[M + Na]^+$ (calcd for $C_{17}H_{28}O_3Na$, 303.1936).

**Preparation of (*S*)- and (*R*)-MTPA Esters of 1.** To a solution of **1** (2.0 mg) in pyridine (100 μL) was added (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μL), and the solution was allowed to stand overnight at room temperature. The reaction mixture was added to 1.0 mL of $H_2O$, followed by extraction with EtOAc (1.0 mL × 3). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$ and evaporated. The residue was purified by a short silica gel column using EtOAc–*n*-hexane (1:3) to yield the (*S*)-MTPA ester **1a** (1.2 mg, 32%). The same procedure was applied to obtain the (*R*)-MTPA ester **1b** (0.7 mg, 19%) from the reaction of (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride with **1** in pyridine. Selected $^1H$ NMR ($CDCl_3$, 500 MHz) data of **1a**: δ 4.875 (1H, brs, H-15a), 4.697 (1H, brs, H-15b), 2.397 (1H, dd, *J* = 10.8, 4.8 Hz, H-6), 1.999 (1H, m, H-5), 0.631 (1H, s, H-14); Selected $^1H$ NMR ($CDCl_3$, 500 MHz) data of **1b**: δ 4.862 (1H, brs, H-15a), 4.691 (1H, brs, H-15b), 2.430 (1H, dd, *J* = 10.4, 5.2 Hz, H-6), 2.007 (1H, m, H-5), 0.662 (1H, s, H-14).

**Preparation of (*S*)- and (*R*)-MTPA Esters of 3.** To a solution of **3** (2.0 mg) in pyridine (100 μL) was added (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μL), and the solution was allowed to stand overnight at room temperature. The reaction mixture was added to 1.0 mL of $H_2O$, followed by extraction with EtOAc (1.0 mL × 3). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$ and evaporated. The residue was purified by a short silica gel column using acetone–*n*-hexane (1:5) to yield the (*S*)-MTPA ester **3a** (0.6 mg, 16%). The same procedure was applied to obtain the (*R*)-MTPA ester **3b** (0.4 mg, 11%) from the reaction of (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride with **3** in pyridine. Selected $^1H$ NMR ($CDCl_3$, 500 MHz) data of **3a**: δ 4.844 (1H, brs, H-15a), 4.627 (1H, brs, H-15b), 2.338 (1H, m, H-3a), 2.146 (1H, m, H-3b), 2.077 (1H, m, H-5), 1.161 (1H, m, H-9a), 1.731 (1H, m, H-2), 0.775 (1H, s, H-14); Selected $^1H$ NMR ($CDCl_3$, 500 MHz) data of **3b**: δ 4.826 (1H, brs, H-15a), 4.616 (1H, brs, H-15b), 2.306 (1H, m, H-3a), 2.131 (1H, m, H-3b), 2.080 (1H, m, H-5), 1.234 (1H, m, H-9a), 1.711 (1H, m, H-2), 0.774 (1H, s, H-14).

**X-ray Diffraction Analysis of Leptocladolin A (1).** A suitable colorless crystal (0.8 × 0.6 × 0.6 $mm^3$) of **1** was grown by slow evaporation of the EtOAc solution. Diffraction intensity data was acquired with a Rigaku AFC7S single-crystal X-ray diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). Crystal data for **1**: $C_{17}H_{28}O_3$ (formula weight 280.39), approximate crystal size, 0.8 × 0.6 × 0.6 $mm^3$, orthorhombic, space group, $P2_12_12_1$ (# 19), *T* = 298(2) K, *a* = 8.340(3) Å, *b* = 10.431(4) Å, *c* = 19.572(6) Å, *V* = 1702.7(11) $Å^3$, $D_{calcd}$ = 1.094 Mg $m^{-3}$, *Z* = 4, *F*(000) = 616, $\mu_{(Mo K\alpha)}$ = 0.073 $mm^{-1}$. A total of 1940 reflections were collected in the range 2.08° < θ < 26.00°, with 1940 independent reflections, completeness to $\theta_{max}$ was 100%; ψ-scan absorption correction applied; full-matrix least-squares refinement on $F^2$, the number of data/restraints/parameters were 1940/0/185; goodness-of-fit on $F^2$ = 1.011; final *R* indices [$I > 2\sigma(I)$], $R_1$ = 0.0501, $wR_2$ = 0.1161; *R* indices (all data), $R_1$ = 0.1276, $wR_2$ = 0.1444, largest difference peak and hole, 0.184 and −0.234 e $Å^{-3}$.

**1β-Hydroxy-6α-acetoxyeudesm-4(15)-ene (3).** A suitable colorless crystal (0.8 × 0.6 × 0.6 $mm^3$) of **3** was grown by slow evaporation of the EtOAc solution. Diffraction intensity data was acquired with a Rigaku AFC7S single-crystal X-ray diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). Crystal data for **3**: $C_{17}H_{28}O_3 \cdot 0.25H_2O$ (formula weight 284.90), approximate crystal size, 0.8 × 0.6 × 0.6 $mm^3$, tetragonal, space group, $P4_2$ (# 77), *T* = 298(2) K, *a* = *b* = 23.594(3) Å, *c* = 6.034(1) Å, *V* = 3359.1(9) $Å^3$, $D_{calcd}$ = 1.127 Mg $m^{-3}$, *Z* = 8, *F*(000) = 1252, $\mu_{(Mo K\alpha)}$ = 0.076 $mm^{-1}$. A total of 4090 reflections were collected in the range 2.08° < θ < 26.00°, with 3789 independent reflections [*R*(int) = 0.01], completeness to $\theta_{max}$ was 99.9%; ψ-scan absorption correction applied; full-matrix least-squares refinement on $F^2$, the number of data/restraints/parameters were 3789/1/374; goodness-of-fit on $F^2$ = 1.024; final *R* indices [$I > 2\sigma(I)$], $R_1$ = 0.0390, $wR_2$ = 0.0937; *R* indices (all data), $R_1$ = 0.0995, $wR_2$ = 0.1113, largest difference peak and hole, 0.137 and −0.170 e $Å^{-3}$.

Crystallographic data for compounds **1** and **3** have been deposited with the Cambridge Crystallographic Data Centre (deposition numbers CCDC 690524 and 691495). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12, Union Road, Cambridge, CB2 1EZ, U.K. (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

**Cytotoxicity Testing.** Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of compounds **1**–**4** were performed using the MTT [3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[12,13]

**In Vitro Anti-Inflammatory Assay.** Macrophage (RAW264.7) cell line was purchased from ATCC. Anti-inflammatory activity assay of compounds **1**–**4** (10 μM) were added to the cells 5 min before LPS challenge by western blot analysis. Monoclonal antibody against $\beta$-actin was used as the internal control for protein loading. The immunoreactive bands of iNOS, COX-2 and $\beta$-actin were visualized using the UVP BioChemi Imaging System (UVP, Upland, CA, USA). For the immunoreactivity data, the intensity of each test band is expressed as the integrated optical density. The data were expressed as a ratio of the iNOS or COX-2 protein of interest to $\beta$-actin. Relative variations between the bands of the compound-treatment samples and the LPS sample were calculated using the same image.[14,15]

Financial supports awarded to J.-H. Sheu were provided by Ministry of Education (No. 97C031702) and National Science Council of Taiwan (No. NSC 98-2113-M-110-002-MY3).

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