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Anti-Inflammatory Polyoxygenated Steroids from the Soft Coral *Sinularia* sp.

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Four new polyhydroxylated steroids (**1**–**4**) were isolated from a Formosan soft coral *Sinularia* sp. The structures of these metabolites were determined on the basis of spectroscopic (IR, MS, and 1D and 2D NMR) analyses. Among these metabolites, **3** and **4** are rarely found marine steroids with a C-9/C-10 double bond. Compounds **1** and **2** have shown significant inhibition of the accumulation of the pro-inflammatory COX-2 protein of LPS-stimulated RAW264.7 macrophage cells at 10 μM.

Previous chemical investigations on the Formosan soft corals of the genus *Sinularia* have afforded several polyoxygenated steroids.[1–5] Recently, we have investigated the chemical constituents of a Taiwanese soft coral *Sinularia* sp. and have isolated six new sesquiterpenoids.[6,7] Our continuing study on the chemical content of this soft coral also has resulted in the isolation of four new polyhydroxylated steroids (**1**–**4**) (Chart 1). The structures of the new metabolites were determined on the basis of extensive spectroscopic analysis, including 2D NMR (^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) spectroscopy. Furthermore, at a concentration of 10 μM both compounds **1** and **2** demonstrated an ability to inhibit the accumulation of two pro-inflammatory proteins, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells.

The soft coral *Sinularia* sp. was kept at −20 °C immediately after collection. The frozen animals were extracted exhaustively with EtOH, and then the concentrated EtOH extract was partitioned between EtOAc and H_2O. The combined EtOAc-soluble fraction was concentrated under reduced pressure and the residue was repeatedly chromatographed to yield metabolites **1**–**4**.

The HR-ESI-MS of **1** revealed a pseudomolecular ion peak at m/z 439.3554 $[M + Na]^+$, corresponding to the molecular formula $C_{28}H_{48}O_2$ (calcd for $C_{28}H_{48}O_2Na$ m/z 439.3552). The IR spectrum displayed absorption bands at 3437 and 1655 cm^{-1} for hydroxy group and carbon–carbon double-bond, respectively. The ^{1}H NMR spectrum (Table 1) revealed six methyl signals [δ 1.04 (s), 0.92 (d, $J = 7.0$ Hz), 0.86 (d, $J = 7.0$ Hz), 0.79 (d, $J = 7.0$ Hz), 0.77 (d, $J = 7.0$ Hz), and 0.68 (s)], two oxymethine signals [δ 3.99 (m) and 3.85 (s)], and an olefinic proton signal at δ 5.60 (d, $J = 5.5$ Hz). In the ^{13}C NMR spectrum (Table 2), compound **1** showed 28 carbon resonances, with multiplicities determined by DEPT 90 and 135 experiments. The olefinic carbon signals appearing at δ_C

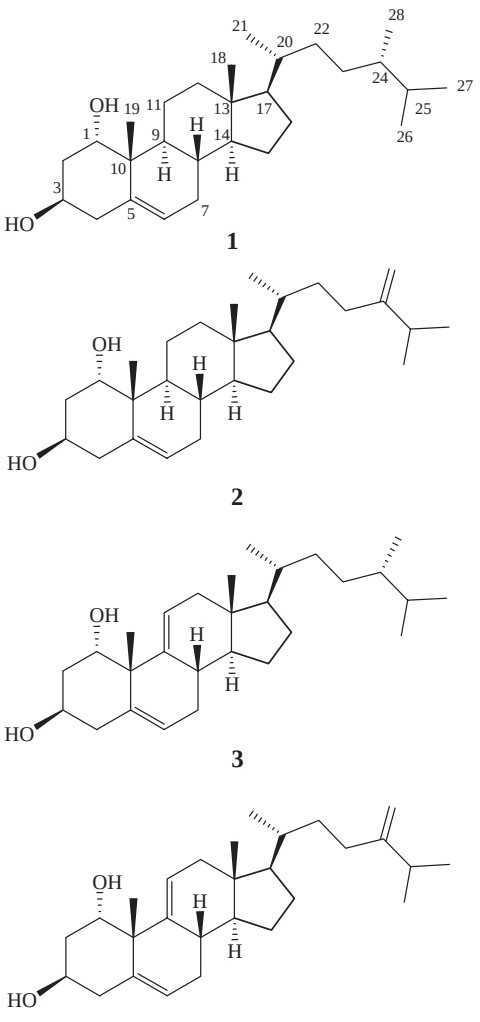

Chart 1.

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Table 1. ^{1}H NMR Data for Sterols **1**–**4**[a)]

No.	1	2	3	4
1	3.85 s	3.85 s	4.20 s	4.20 br s
2	α: 2.10 m; β: 1.74 m	α: 2.09 m; β: 1.74 m	α: 2.22 m; β: 1.77 m	α: 2.23 m; β: 1.78 m
3	3.99 m	3.99 m	4.03 m	4.04 m
4	α: 2.38 m; β: 2.33 m	α: 2.39 m; β: 2.30 m	α: 2.40 m; β: 2.30 m	α: 2.40 m; β: 2.30 m
6	5.60 br d (5.5)[b)]	5.60 d (5.5)	5.72 d (5.5)	5.72 d (6.0)
7	2.00 m; 1.60 m	2.01 m; 1.62 m	2.20 m; 1.62 m	2.22 m; 1.62 m
8	1.47 m	1.48 m	2.05 m	2.06 m
9	1.58 m	1.60 m		
11	1.48 m	1.49 m	5.60 d (6.0)	5.61 d (6.0)
12	α: 1.19 m; β: 2.02 m	α: 1.20 m; β: 2.01 m	α: 2.12 m; β: 2.25 m	α: 2.14 m; β: 2.25 m
14	1.06 m	1.06 m	1.31 m	1.32 m
15	1.60 m; 1.06 m	1.62 m; 1.08 m	1.78 m	1.76 m
16	1.87 m; 1.29 m	1.88 m; 1.30 m	1.89 m; 1.32 m	1.92 m; 1.32 m
17	1.11 m	1.14 m	1.21 m	1.23 m
18	0.68 s	0.69 s	0.64 s	0.65 s
19	1.04 s	1.04 s	1.21 s	1.21 s
20	1.39 m	1.41 m	1.42 m	1.46 m
21	0.92 d (7.0)	0.95 d (7.0)	0.92 d (7.0)	0.92 d (6.5)
22	1.41 m; 0.96 m	1.56 m; 1.17 m	1.41 m; 0.95 m	1.57 m; 1.17 m
23	1.39 m; 0.96 m	2.12 m; 1.88 m	1.39 m; 0.96 m	2.14 m; 1.90 m
24	1.21 m		1.22 m	
25	1.58 m	2.24 m	1.57 m	2.24 m
26	0.86 d (7.0)	1.02 d (7.0)	0.86 d (6.5)	1.03 d (7.0)
27	0.79 d (7.0)	1.03 d (7.0)	0.79 d (6.5)	1.04 d (7.0)
28	0.77 d (7.0)	4.71 s; 4.66 s	0.78 d (7.0)	4.73 s; 4.67 s

a) Spectra recorded at 500 MHz in $CDCl_3$. b) J values (in Hz) parenthese.

Table 2. ^{13}C NMR Data for Sterols **1**–**4**[a)]

No.	1		2		3		4	
1	73.0	(CH)[b)]	73.0	(CH)	72.8	(CH)	72.8	(CH)
2	38.2	(CH_2)	38.2	(CH_2)	36.5	(CH_2)	36.5	(CH_2)
3	66.5	(CH)	66.5	(CH)	67.4	(CH)	67.4	(CH)
4	41.4	(CH_2)	41.6	(CH_2)	41.5	(CH_2)	41.5	(CH_2)
5	137.2	(C)	137.2	(C)	135.9	(C)	135.9	(C)
6	125.7	(CH)	125.7	(CH)	124.5	(CH)	124.5	(CH)
7	31.8	(CH_2)	31.8	(CH_2)	31.2	(CH_2)	32.2	(CH_2)
8	31.8	(CH)	31.9	(CH)	34.6	(CH)	34.6	(CH)
9	41.7	(CH)	41.7	(CH)	142.2	(C)	142.2	(C)
10	41.6	(C)	41.7	(C)	45.2	(C)	45.2	(C)
11	20.3	(CH_2)	20.3	(CH_2)	119.3	(CH)	119.3	(CH)
12	39.4	(CH_2)	39.5	(CH_2)	41.7	(CH_2)	41.7	(CH_2)
13	42.3	(C)	42.3	(C)	41.1	(C)	41.1	(C)
14	56.6	(CH)	56.6	(CH)	52.8	(CH)	52.8	(CH)
15	24.3	(CH_2)	24.3	(CH_2)	25.2	(CH_2)	25.2	(CH_2)
16	28.1	(CH_2)	28.2	(CH_2)	28.3	(CH_2)	28.3	(CH_2)
17	56.0	(CH)	56.0	(CH)	56.2	(CH)	56.0	(CH)
18	11.8	(CH_3)	11.8	(CH_3)	11.4	(CH_3)	11.4	(CH_3)
19	19.4	(CH_3)	19.4	(CH_3)	26.7	(CH_3)	26.7	(CH_3)
20	36.2	(CH)	35.7	(CH)	35.6	(CH)	35.6	(CH)
21	18.9	(CH_3)	18.7	(CH_3)	18.4	(CH_3)	18.4	(CH_3)
22	33.7	(CH_2)	34.6	(CH_2)	33.6	(CH_2)	34.5	(CH_2)
23	30.6	(CH_2)	31.0	(CH_2)	30.5	(CH_2)	30.9	(CH_2)
24	39.0	(CH)	156.9	(C)	39.0	(CH)	156.8	(C)
25	31.4	(CH)	33.8	(CH)	31.5	(CH)	33.8	(CH)
26	20.5	(CH_3)	21.9	(CH_3)	20.5	(CH_3)	21.9	(CH_3)
27	17.6	(CH_3)	22.0	(CH_3)	17.6	(CH_3)	22.0	(CH_3)
28	15.4	(CH_3)	106.0	(CH_2)	15.4	(CH_3)	106.0	(CH_2)

a) Spectra recorded at 125 MHz in $CDCl_3$. b) Attached protons were deduced by DEPT experiments.

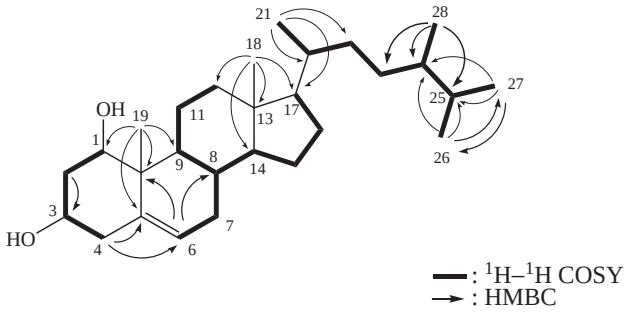

Figure 1. Key ^{1}H–^{1}H COSY and HMBC correlations for **1**.

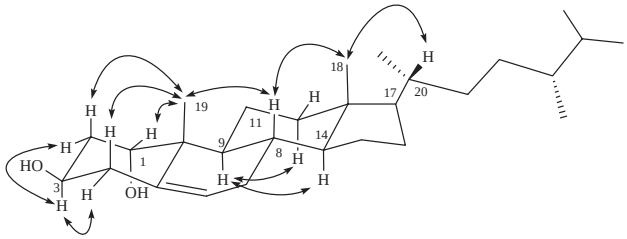

Figure 2. Selective NOESY correlations of **1**.

Table 3. Protons to Which Long-Range Correlations Were Observed in the HMBC Experiments on Sterols **1**–**4**

Carbon	**1**	**2**	**3**	**4**
1	H_3-19	H_3-19	H_3-19	H_3-19
2		H-4α		
3	H-2α	H-2α, H-4α		
4		H-6		
5	H-4α, H_3-19	H-4α, H_3-19	H_3-19	H-1, H_3-19
6	H-4α	H-4α		
8	H-6	H-6		
9	H_3-19	H_3-19	H-12β, H_3-19	H-12β, H_3-19
10	H-6, H_3-19	H-6, H_3-19	H_3-19	H_3-19
11			H-12β	H-12β
12	H_3-18	H_3-18	H_3-18	H_3-18
13	H_3-18	H_3-18	H_3-18	H_3-18
14	H_3-18	H-12α, H_3-18	H_3-18	H_3-18
17	H_3-18, H_3-21	H_3-18, H_3-21	H_3-18, H_3-21	H_3-18, H_3-21
20	H_3-21	H_3-21	H_3-21	H_3-21
22	H_3-21	H_3-21, H_2-23	H_3-21	H_3-21
23	H_3-28	H_2-28	H_3-28	H_2-28
24	H_3-26, H_3-27, H_3-28	H_2-23, H_3-26, H_3-27	H_3-26, H_3-27, H_3-28	H_2-23, H_3-26, H_3-27
25	H_3-26, H_3-27, H_3-28	H_3-26, H_3-27, H_2-28	H_3-26, H_3-27, H_3-28	H_3-26, H_3-27, H_2-28
26	H_3-27	H_3-27	H_3-27	H_3-27
27	H_3-26	H_3-26	H_3-26	H_3-26

137.2 (C) and 125.7 (CH) corresponded to one trisubstituted double bond. The resonances appearing at δ 73.0 (CH) and 66.5 (CH) confirmed the presence of two oxymethine carbons. The ^{1}H–^{1}H COSY correlations revealed that **1** has three separated proton sequences. Interpretation of the COSY and HMQC spectra led to the placement of two hydroxy groups at C-1 (δ 73.0) and C-3 (δ 66.5). This assignment was also supported by HMBC cross-peaks of H_2-2/C-3 and H_3-19/C-1. Detailed analyses of the ^{1}H–^{1}H COSY and HMBC correlations (Figure 1 and Table 3) further established the planar structure of **1**.

In the NOESY spectrum of **1** (Figure 2), the NOE correlations between H-8 and H_3-18 and H_3-19 as well as between H_3-18 and H-20 indicated that these protons adapt a β-orientation. The hydroxy groups at C-1 and C-3 were determined to have the α and β orientations, respectively, on the basis of strong NOE interactions of H_3-19 (δ 1.04, s) with H-1 (δ 3.85, s), H_3-19 with H-4β (δ 2.33, m) and H-4α (δ 2.38, m) with H-3 (δ 3.99, m) (Figure 2). Furthermore, the 24S configuration of **1** was determined by comparison of NMR data with those of yonarasterol B which was isolated from the soft coral *Clavularia viridis*[8] as the proton shift of H_3-28, δ_H 0.77, was found to be identical with that of yonarasterol B. Also, the carbon shifts of C_{24}–C_{28} are in excellent agreement with those of yonarasterol B and (24*S*)-methylcholestanol (vs. those of (24*R*)-24-methylcholestanol).[9] On the basis of the above findings and other detailed NOE correlations (Figure 2), the structure of **1** was fully established as (24*S*)-24-methylcholest-5-ene-1α,3β-diol.

Compound **2** also obtained as a white powder. The HR-ESI-MS (m/z 437.3394, $[M + Na]^+$) and NMR data of **2** indicated the molecular formula, $C_{28}H_{46}O_2$. Both the ^{1}H and ^{13}C NMR signals of **2** were found to be very closely related to those of compound **1**, suggesting the very similar steroidal skeleton. By comparison of NMR data of **2** with those of **1** (Tables 1 and 2), it was found that a methyl proton signal (δ_H 0.77 d, $J = 7.0$ Hz) in **1** was replaced by two exomethylene proton signals (δ_H 4.71 and 4.66, each s) in **2**. This was further confirmed by the HMBC correlations (Table 3) from H_2-28 to C-23, C-24, and C-25. Thus, the structure of steroid **2** was established as 24-methylenecholest-5-ene-1α,3β-diol.

Metabolite **3** was obtained as a white powder and exhibited a pseudomolecular ion peak at m/z 437.3399 $[M + Na]^+$ in the HR-ESI-MS, appropriate for a molecular formula of

$C_{28}H_{46}O_2$. The IR spectrum of **3** was found to exhibit absorptions of a hydroxy (3384 cm^{-1}) group. The spectroscopic data of **3** (IR, ^1H and ^{13}C NMR) were similar to those of **1**, except that the presence of a C-9/C-10 double bond in **3** could be observed. This was confirmed by HMBC correlations (Table 3) from H-12β (δ 2.25), H_3-19 (δ 1.21), and C-9 (δ 142.2). On the basis of above analysis, the structure of **3** was established as (24*S*)-24-methylcholest-5,9-diene-1α,3β-diol.

The new metabolite **4** was isolated as a white powder. It's molecular formula $C_{28}H_{44}O_2$ was established by HR-ESI-MS (m/z 435.3242, $[M + Na]^+$). The ^{13}C NMR spectrum of **4** (Table 2) showed the presence of 28 carbons and the chemical shifts (δ_H and δ_C) of the tetracyclic system of **4** were close to those of **3** (Tables 1 and 2). The chemical shifts of the side chain from C-20 to C-28 in **4** are nearly identical with those of **2**. Thus, the structure of steroid **4** was established as 24-methylenecholest-5,9-diene-1α,3β-diol.

It is noteworthy to mention that metabolites **3** and **4** are rarely found marine steroids with a double bond at C-9/C-10.[10,11] The in vitro anti-inflammatory effect of the steroids **1** and **2** was tested as they were isolated in larger quantities relative to **3** and **4**. In this assay, the up-regulation of the pro-inflammatory iNOS and COX-2 proteins of the LPS-stimulated RAW 264.7 macrophage cells was evaluated using the immunoblot analysis. At a concentration of 10 μM, compounds **1** and **2** could reduce the levels of the iNOS to $53.9 \pm 4.6\%$ and $45.7 \pm 5.6\%$, respectively, and COX-2 to $35.8 \pm 6.0\%$ and $26.4 \pm 5.0\%$, respectively, relative to the control cells stimulated with LPS only (Figure 3). The housekeeping protein, β-actin was not changed notably by the presence of **1** and **2** at the concentration of 10 μM. Both compounds **1** and **2** at 10 μM significantly inhibited the expression of LPS-induced pro-inflammatory proteins, iNOS and COX-2, in macrophage cells. Thus, **1** and **2** were found to be active anti-inflammatory steroids.

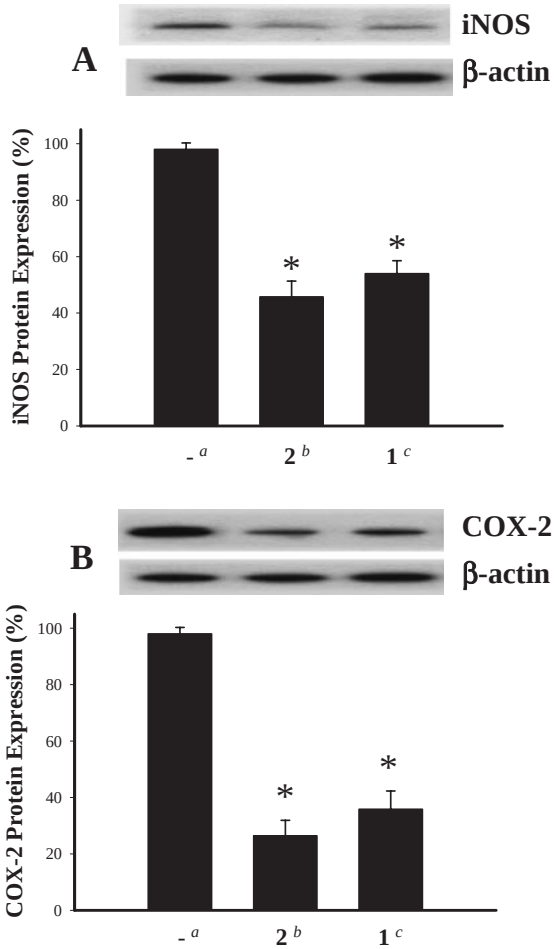

Figure 3. Effect of compounds **1** and **2** on iNOS and COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis. (**A**) Immunoblots of iNOS and β-actin; (**B**) Immunoblots of COX-2 and β-actin. The values are mean ± SEM. ($n = 6$). Relative intensity of the LPS alone stimulated group was taken as 100%. Under the same experimental condition CAPE (caffeic acid phenylethyl ester, 10 μM) reduced the levels of the iNOS and COX-2 to $2.5 \pm 3.7\%$ and $67.2 \pm 13.4\%$, respectively. *Significantly different from LPS alone stimulated group ($^*P < 0.05$). [a]Stimulated with LPS alone, [b]stimulated with LPS in the presence of **2** (10 μM), [c]stimulated with LPS in the presence of **1** (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. NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^1H and 125 MHz for ^{13}C, respectively, in $CDCl_3$. LRMS and HRMS were obtained by ESI on a Bruker APEX II mass spectrometer. 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 (HPLC) was performed on a Hitachi L-7100 apparatus equipped with a Bischoff refractive index detector, or a Hitachi L-7400 UV detector and with a Merck Hibar Si-60 column (250 × 21 mm^2, 7 μm).

Animal Material. *Sinularia* sp. was collected by hand via scuba off the northern east coast of Taiwan, in May 2004, at depths of 15 to 20 m, and stored in a freezer until extraction. A voucher sample (20040516-6) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Isolation. The sliced bodies of the soft coral *Sinularia* sp. (1.0 kg, wet wt) were exhaustively extracted with EtOH (1 L × 5). The organic layer was filtered and concentrated under vacuum, and the residue of aqueous suspension was partitioned between EtOAc and H_2O. The solvent-free EtOAc extract (9.8 g) was subjected to CC on silica gel and eluted with EtOAc in hexane (0–100%, gradient) to yield 14 fractions. Fraction 9, eluting with hexane–EtOAc (4:1), was purified on a Sephadex LH-20 column using acetone as the mobile phase to afford two subfractions. Subfraction 2 was separated by normal phase HPLC using hexane–acetone (8:1) to yield **1** (3.0 mg), **2** (3.6 mg), **3** (0.7 mg), and **4** (0.6 mg), respectively.

24*S*-24-Methylcholest-5-ene-1α,3β-diol (1): White powder; mp 140–142 °C; $[\alpha]_D^{25} = -58$ (*c* 0.3, $CHCl_3$); IR (neat) ν_{max} 3437, 1655 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Tables 1 and 2; ESIMS m/z 439 (80, $[M + Na]^+$); HRESIMS m/z 439.3554 (calcd for $C_{28}H_{48}O_2Na$, 439.3552).

24-Methylenecholest-5-ene-1α,3β-diol (2): White powder; mp 141–142 °C; $[\alpha]_D^{25} = -66$ (*c* 0.4, $CHCl_3$); IR (neat) ν_{max} 3437 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Tables 1 and 2; ESIMS *m*/*z* 437 (80, $[M + Na]^+$); HRESIMS *m*/*z* 437.3394 (calcd for $C_{28}H_{46}O_2Na$, 437.3395).

(24*S*)-24-Methylcholest-5,9-diene-1α,3β-diol (3): White powder; mp 126–127 °C; $[\alpha]_D^{25} = -40$ (*c* 0.5, $CHCl_3$); IR (neat) ν_{max} 3384, 1653 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Tables 1 and 2; ESIMS *m*/*z* 437 (50, $[M + Na]^+$); HRESIMS *m*/*z* 437.3393 (calcd for $C_{28}H_{46}O_2Na$, 437.3393).

24-Methylenecholest-5,9-diene-1α,3β-diol (4): White powder; mp 126–128 °C; $[\alpha]_D^{25} = -30$ (*c* 0.3, $CHCl_3$); IR (neat) ν_{max} 3437, 1653 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Tables 1 and 2; ESIMS *m*/*z* 435 (90, $[M + Na]^+$); HRESIMS *m*/*z* 435.3242 (calcd for $C_{28}H_{44}O_2Na$, 435.3239).

In Vitro Anti-Inflammatory Assay. The anti-inflammatory assay was modified from Ho et al.[12] and Park et al.[13] Murine RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC, No. TIB-71) and cultured in Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum, at 37 °C in a humidified 5% CO_2–95% air incubator under standard conditions. Inflammation in macrophages was induced by incubating them for 16 h in a medium containing only LPS (0.01 μg mL^{-1}; Sigma, USA) without the presence of test compounds. For the anti-inflammatory activity assay, compounds **1** and **2** were added to the cells 5 min before LPS challenge, respectively. Then, cells were washed with ice-cold PBS, lysed in ice-cold lysis buffer, and then centrifuged at 20000*g* for 30 min at 4 °C. The supernatant was decanted from the pellet and retained for Western blot analysis. Protein concentrations were determined by a DC protein assay kit (Bio-Rad) modified by the method of Lowry et al.[14] Samples containing equal quantities of protein were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore, 0.45 μm pore size). The resultant PVDF membranes were incubated with blocking solution, and then incubated for 180 min at room temperature with antibodies against inducible nitric oxide synthase (iNOS; 1:1000 dilution; Transduction Laboratories) and cyclooxygenase-2 (COX-2; 1:1000 dilution; Cayman Chemical) proteins. The blots were detected using ECL detection reagents (Perkin-Elmer, Western Blot Chemiluminescence Reagent Plus) according to the manufacturer instructions and finally exposed to X-ray film (Kodak X-OMAT LS, Kodah, U.S.A.). The membranes were reprobed with a monoclonal mouse anti-β-actin antibody (1:2500, Sigma) as the loading control. For the immunoreactivity data, the intensity of each drug-treated band is expressed as the integrated optical density (IOD) calculated with respect to the average optical density of the corresponding control (treated with LPS only) band. For statistical analysis, all the data were analyzed by a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post hoc test for multiple comparisons. A significant difference was defined as a *P* value of < 0.05.

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