Polyoxygenated Sterols from the Formosan Soft Coral *Sinularia gibberosa*

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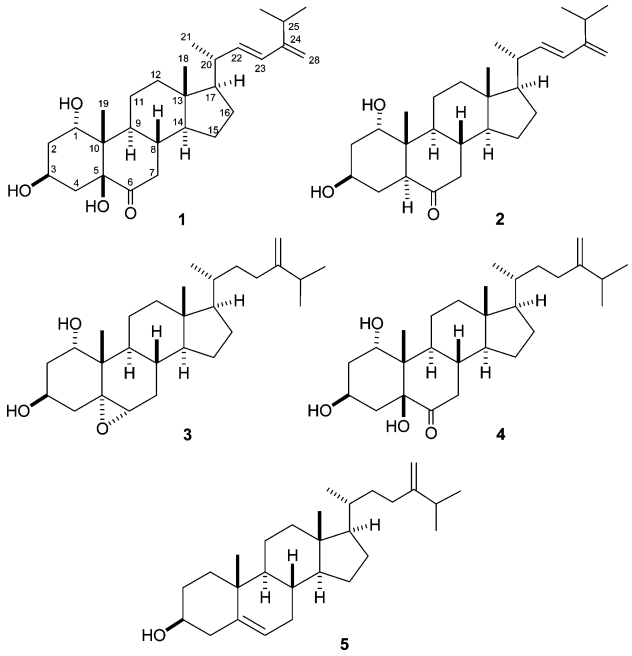
Chemical investigation on the dichloromethane-soluble fraction from the EtOH extract of *Sinularia gibberosa* Tixier-Durivault has led to the isolation of three new polyoxygenated sterols, gibberoketosterols B (**1**) and C (**2**) and gibberoepoxysterol (**3**), along with two known steroids, gibberoketosterol (**4**) and 24-methylenecholest-5-en-3β-ol (**5**). These cholestane-type sterols possessing a 22,24(28)-conjugated diene (**1** and **2**) in the side chain or a 5α,6α-epoxide in the B-ring (**3**) were isolated for the first time from marine sources. Compound **4** showed significant inhibition against the up-regulation of the pro-inflammatory iNOS and COX-2 proteins of LPS-stimulated RAW264.7 macrophage cells, while **1** was found to be inactive. The cytotoxicity of **1**−**4** toward a limited panel of cancer cell lines is also reported.

Marine organisms, including octocorals (Coelenterata), have been well-recognized as a rich source of 3β-hydroxy sterols and their polyoxygenated analogues.[1,2] Worldwide chemical investigations on the steroidal contents of soft corals of the genus *Sinularia* have afforded various polyoxygenated steroids as derivatives of 24-methyl- and 24-methylenecholestan-3β-ol[3−12] and their glycosides.[11,12] Some of these compounds have been shown to exhibit *in vitro* cytotoxic activities toward several cancer cell lines.[7,8] Our current chemical investigation on *S. gibberosa* has again led to the isolation of three new polyoxygenated sterols, gibberoketosterols B (**1**)[13] and C (**2**) and gibberoepoxysterol (**3**), along with the known steroids gibberoketosterol (**4**)[8,13] and 24-methylenecholest-5-en-3-ol (**5**).[8] The structures of **1**−**3** were determined utilizing extensive spectroscopic analyses, including 2D NMR (^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY) spectroscopy, and by comparison of their NMR data with those of related compounds. The ability of **1** and **4** to inhibit up-regulation of the pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells has been evaluated. The cytotoxic activity of metabolites **1**−**4** against HepG2 (human liver carcinoma), MCF7, MDA-MB-23 (human breast carcinoma), and A-549 (human lung carcinoma) cells also is reported herein.



Results and Discussion

The sliced bodies of the soft coral *S. gibberosa* were homogenized exhaustively with EtOH. The concentrated EtOH extract was partitioned between CH_2Cl_2 and water. The combined CH_2Cl_2-soluble fraction was concentrated under reduced pressure, and the residue was repeatedly chromatographed to yield sterols **1**−**5** (see Experimental Section). The physical and spectral data of **4** were found to be in full agreement with those previously reported for gibberoketosterol isolated from the same organism.[8]

Gibberoketosterol B (**1**) was isolated as a white powder. Its molecular formula was established as $C_{28}H_{44}O_4$ by HRESIMS (467.3138 *m/z*, $[M + Na]^+$) and NMR data (Tables 1 and 2), implying seven degrees of unsaturation. The UV spectrum of **1** showed an absorption maximum at 232 nm (log ϵ 4.06) due to a conjugated diene moiety in the molecule. The strong absorptions at ν_{max} 3422 and 1714 cm^{-1} in the IR spectrum suggested the existence of hydroxy and ketone functionalities. Three hydroxyls in the molecule were estimated from the ion peaks appearing at *m/z* 426 $(M - H_2O)^+$, 408 $(M - 2\ H_2O)^+$, and 390 $(M - 3\ H_2O)^+$ in the EIMS spectrum. Two of these hydroxyls exhibited the D_2O-exchangeable proton signals in the ^{1}H NMR spectrum of **1** (in $CDCl_3$, see Table 2, and in C_5D_6N, see Experimental Section). The ^{13}C NMR spectral data of **1** (Table 1), measured in $CDCl_3$, indicated the presence of 28 carbon atoms of an oxosteroid attributable to five methyl, eight methylene (including one olefinic), 10 methine (including two olefinic and two oxygenated), and five quaternary carbons (including one olefinic, one oxygenated, and one ketonic). Moreover, the ^{1}H NMR spectral data of **1** (Table 2), particularly those of the two hydroxyl-bearing methines, one exomethylene, and five methyls, revealed that **1** was a derivative of **4**.[8] The only difference was the appearance of the proton signals at δ 5.94 (1H, d, J = 15.7 Hz) and 5.56 ppm (1H, dd, J = 15.7, 8.8 Hz) due to a *trans* 1,2-disubstituted double bond in the side chain of **1**. The ^{1}H−^{1}H COSY correlations observed between the olefinic proton H-22 (δ 5.56) and H-20 (δ 2.15, m) and between H-20 and H_3-21 (δ 1.05, d, J = 6.6 Hz) indicated the C-22 and C-23 position of the double bond. This was further supported by the ^{1}H−^{1}H COSY correlation between H-22 and H-23 (δ 5.94) and the HMBC correlations found between the exomethylene protons H_2-28 (δ 4.83

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Table 1. ^{13}C NMR Data for Sterols **1**−**4**

C#	**1**[a]	**2**[a]	**3**[a]	**4**[a,8]
1	70.8 (CH)[b]	72.4 (CH)	73.2 (CH)	70.7 (CH)
2	37.6 (CH_2)	38.1 (CH_2)	39.1 (CH_2)	37.5 (CH_2)
3	67.7 (CH)	66.0 (CH)	64.4 (CH)	67.7 (CH)
4	37.3 (CH_2)	30.0 (CH_2)	39.2 (CH_2)	37.3 (CH_2)
5	83.7 (qC)	50.5 (CH)	64.6 (qC)	83.7 (qC)
6	211.3 (qC)	211.7 (qC)	56.8 (CH)	211.5 (qC)
7	41.0 (CH_2)	46.5 (CH_2)	28.7 (CH_2)	41.0 (CH_2)
8	37.4 (CH)	37.3 (CH)	29.9 (CH)	37.4 (CH)
9	43.4 (CH)	46.5 (CH)	36.8 (CH)	43.3 (CH)
10	49.2 (qC)	44.6 (qC)	38.9 (qC)	49.1 (qC)
11	23.6 (CH_2)	20.9 (CH_2)	19.9 (CH_2)	23.6 (CH_2)
12	39.6 (CH_2)	39.2 (CH_2)	39.1 (CH_2)	39.7 (CH_2)
13	42.4 (qC)	43.0 (qC)	42.4 (qC)	42.4 (qc)
14	56.9 (CH)	56.7 (CH)	56.8 (CH)	56.8 (CH)
15	24.2 (CH_2)	24.0 (CH_2)	24.1 (CH_2)	24.2 (CH_2)
16	28.0 (CH_2)	28.2 (CH_2)	28.0 (CH_2)	27.7 (CH_2)
17	56.1 (CH)	56.0 (CH)	55.7 (CH)	56.0 (CH)
18	12.2 (CH_3)	12.4 (CH_3)	11.8 (CH_3)	11.9 (CH_3)
19	13.4 (CH_3)	13.6 (CH_3)	16.5 (CH_3)	13.4 (CH_3)
20	40.2 (CH)	40.3 (CH)	35.7 (CH)	35.6 (CH)
21	20.4 (CH_3)	20.5 (CH_3)	18.6 (CH_3)	18.5 (CH_3)
22	135.5 (CH)	135.6 (CH)	34.6 (CH_2)	34.5 (CH_2)
23	129.5 (CH)	129.4 (CH)	30.9 (CH_2)	30.9 (CH_2)
24	152.9 (qc)	152.9 (qC)	156.8 (qc)	156.7 (qC)
25	29.4 (CH)	29.4 (CH)	33.8 (CH)	33.8 (CH)
26	22.0 (CH_3)	22.0 (CH_3)	21.8 (CH_3)	21.8 (CH_3)
27	22.4 (CH_3)	22.4 (CH_3)	22.0 (CH_3)	22.0 (CH_3)
28	109.7 (CH_2)	109.8 (CH_2)	105.9 (CH_2)	106.0 (CH_2)

[a]Spectra recorded at 125 MHz in $CDCl_3$ at 25 °C. [b]Attached protons were determined by DEPT experiments. The values are in ppm downfield from TMS.

and 4.86, each 1H, s) and C-23 (δ 129.5, CH). The detailed analyses of the $^1H-^1H$ COSY and HMBC correlations (Figure 1) further established the structure of **1**, including the C-1, C-3, and C-5 positions of the three hydroxyls and the location of a carbonyl at C-6.

The inspection of the NOESY spectrum of **1** revealed that both **1** and **4**[8] possess the same configurations at C-1, C-3, C-5, C-8, C-9, C-10, C-13, C-14, C-17, and C-20 (Figure 2). By measuring the ^1H NMR spectrum of **1** in C_5D_5N (see Experimental Section), large pyridine-induced downfield shifts ($\Delta\delta = \delta$ $CDCl_3 - \delta$ C_5D_5N)[14] were observed for the β-oriented H-1 ($\Delta\delta = -0.61$ ppm) and H_3-19 ($\Delta\delta = -0.41$ ppm), as in case of compound **4**.[8] Thus, the β-axial orientation for the hydroxy groups at C-3 and C-5 was determined.[8] The NOE correlation displayed by H-4β (δ 1.67 br d, $J = 14.5$ Hz) with 5-OH (δ 4.50, s) further confirmed the β-orientation of the hydroxy group at C-5. Moreover, the coupling constant ($J = 15.7$ Hz) between H-22 and H-23, and the NOE interactions found between H-23 and both H-20 and H-28, and between H-22 and H-25, confirmed the *E* geometry of the 1,2-disubstituted double bond in the side chain. These findings established the structure of gibberoketosterol B (**1**) as 24-methyl-enecholest-22*E*-en-1α,3β,5β-triol-6-one.

The most polar compound, gibberoketosterol C (**2**), was obtained as a white amorphous solid and exhibited a quasimolecular ion peak at *m/z* 451.3190 $(M + Na)^+$ in the HRESIMS, appropriate for a molecular formula of $C_{28}H_{44}O_3$. It was found that the IR, UV, and ^{13}C NMR spectral data of **2** were almost the same as those of **1**, except the replacement of the signal of the quaternary oxycarbon C-5 (δ 83.7, qC) in **1** by a methine carbon in **2** (δ 50.5, CH). This was supported by the significant upfield shifts occurring at C-4 (δ 30.0, CH_2) and C-10 (δ 44.6, qC) relative to those of **1** (δ 37.3 and 49.2, respectively). Therefore, **2** was suggested to be the 5-deoxy derivative of **1**. This was further evidenced by the HMBC correlations (Figure 1) found from H-5 (δ 2.73, dd, $J = 12.5$, 2.5

Table 2. ^1H NMR Data for Sterols **1**−**3**

H#	**1**[a]	**2**[a]	**3**[a]
1	4.26 dd (13.0, 3.5)[b]	3.90 br s	3.89 br s (*w*1/2 8.5)
2α	1.97 ddd (13.0, 13.0, 3.5)	1.97 br d (12.0)	2.23 m
2β	2.09 m	1.66 ddd (12.0, 12.0, 2.5)	1.82 ddd (12.0, 12.0, 2.5)
3	4.20 br s	4.01 m	4.29 m
4α	2.34 dd (14.5, 4.0)	1.51 m	1.37 ddd (13.5, 4.0, 2.0)
4β	1.67 br d (14.5)	1.94 m	2.34 dd (13.5, 12.0)
5		2.73 dd (12.5, 2.5)	
6			2.84 d (4.5)
7α	2.24 dd (14.2, 14.)	1.96 dd (13.0, 12.0)	1.47 dd (15.0, 10.5)
7β	2.42 dd (14.2, 4.7)	2.31 dd (13.0, 3.5)	1.92 m
8	1.79 ddd (12.0, 12.0, 4.7)	1.76 m	1.41 m
9	1.92 dd (11.5, 4.0)	1.75 m	1.69 ddd (12.0, 12.0, 5.0)
11α	2.24 m	1.62 m	1.50 m
11β	1.47 m	1.39 ddd (15.0, 13.0, 2.0)	1.29 ddd (12.0, 12.0, 4.0)
12α	1.23 m	1.30 ddd (12.5, 12.5, 3.5)	1.18 ddd (13.0, 12.0, 4.0)
12β	2.01 (12.5, 3.0)	2.03 ddd (12.5, 3.5, 3.5)	1.97 ddd (13.0, 4.0,3.0)
14	1.29 m	1.28 m	0.99 m
15α	1.52 m	1.51 m	1.57 m
15β	1.52 m	1.09 m	0.99 m
16α	1.67 m	1.68 m	1.84 m
16β	1.29 m	1.27 m	1.25 m
17	1.22 m	1.26 m	1.11 m
18	0.69 3H, s	0.70 3H, s	0.63 3H, s
19	0.98 3H, s	0.77 3H, s	1.09 3H, s
20	2.15 m	2.13 m	1.38 m
21	1.05 3H, d (6.6)	1.06 3H, d (6.6)	0.93 3H, d (6.5)
22	5.56 dd (15.7, 8.8)	5.57 dd (15.5, 8.5)	1.14 m; 1.53 m
23	5.94 d (15.7)	5.94 d (15.5)	1.86 m; 2.09 ddd (15.5, 11.5, 5.0)
25	2.54 septet (7.0)	2.55 septet (7.0)	2.21 m
26	1.06 3H, d (7.0)	1.06 3H, d (7.0)	1.02 3H, d (7.0)
27	1.08 3H, d (7.0)	1.08 3H, d (7.0)	1.02 3H, d (7.0)
28	4.83 s; 4.86 s	4.83 s; 4.85 s	4.65 s; 4.71 s
1-OH	4.07 br s		2.35 d (8.5)
3-OH	*c*		
5-OH	4.50 s		

[a]Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C. [b]The *J* values are in Hz in parentheses. [c]Hydroxy proton signal is not observable.

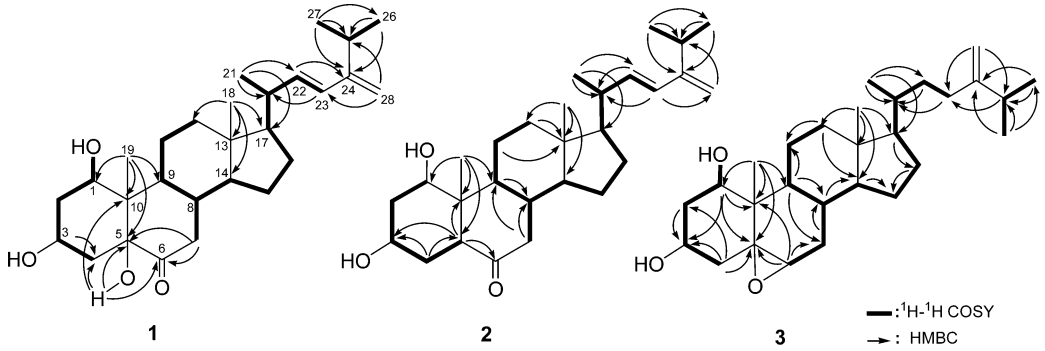

Figure 1. ^{1}H−^{1}H COSY and HMBC correlations for **1**−**3**.

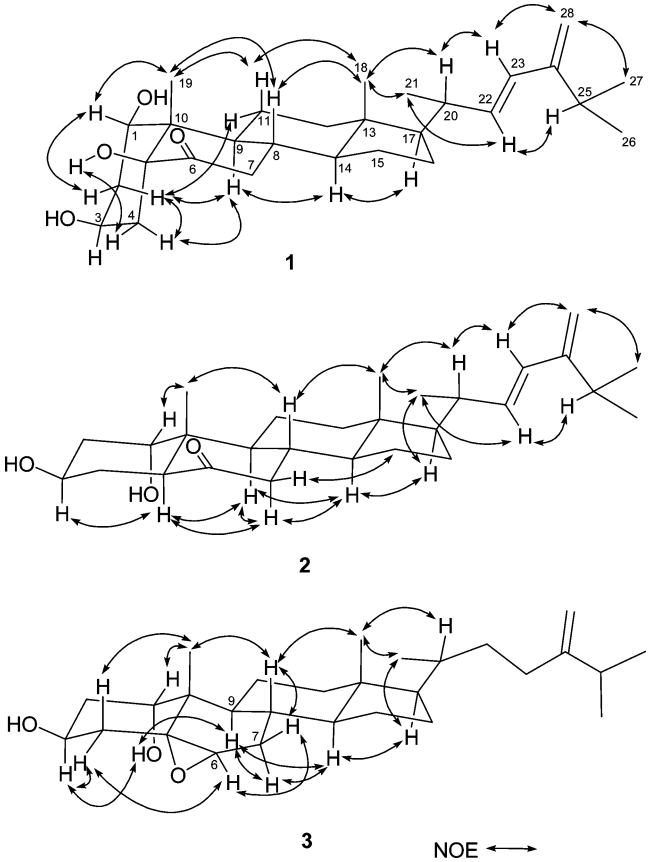

Figure 2. Key NOESY correlations for **1**−**3**.

Hz) to C-3, C-6, and C-10 and that observed from H_2-4 to C-5. On the basis of NOESY correlations (Figure 2), it was found that H-5 exhibited NOE interactions with the α-oriented H-3, H-7, and H-9, but not with the *β*-oriented H_3-19. Thus, H-5 was α-oriented. Furthermore, the NOE correlation displayed by H_3-19 with H-1 reflected the α-orientation of the hydroxy group at C-1. On the basis of the above results, together with the detailed analysis of 2D NMR spectra of **2** (Figures 1 and 2), the structure of compound **2** was unambiguously established as 5α-H-24-methylenecholest-22*E*-ene-1α,3*β*-diol-6-one.

Gibberoepoxysterol (**3**) was found to be more polar than **1** and less polar than **2** (see Experimental Section). Its HRESIMS (453.3342 *m/z*, $[M + Na]^+$) and NMR data are consistent with a molecular formula of $C_{28}H_{46}O_3$. The IR spectrum (ν_{max} 3392 cm^{-1}) indicated the presence of a hydroxy functionality in **3**. Moreover, the ion peak appearing in the EIMS at *m/z* 394 $[M - 2\ H_2O]^+$) revealed the possible presence of two hydroxy groups in the molecule. The NMR data of **3** (Tables 1 and 2) revealed that **3** is a derivative of 24-methylenecholestane-1,3-diol. The presence of a hydroxy group at C-1 was also concluded from the D_2O-exchangeable proton signal at *δ* 2.35 (1H, d, $J = 8.5$ Hz) and its ^{1}H−^{1}H COSY correlation with H-1 (*δ* 3.89, 1H, br s, $w_{1/2} = 8.5$ Hz). The α-orientation of this hydroxy group was elucidated from the large pyridine-induced downfield shift induced at H-3 ($\Delta\delta = -0.72$ ppm). Comparison of the ^{13}C NMR spectral data of **3** with those of **2** revealed that carbons of the ring-juncture methine (50.5, CH, C-5), the keto-carbonyl (*δ* 211.7, qC, C-6), and the 1,2-disubstituted olefinic bond (135.6, CH, C-22 and 129.4, CH C-23) in **2** were replaced by those of an epoxide (*δ* 64.6, qC and 56.8, CH) and an ethylene (*δ* 34.6, CH_2 and 30.9, CH_2) in **3**, respectively. The consecutive proton spin system, established by ^{1}H−^{1}H COSY correlations (Figure 1), which extends from the epoxymethine proton H-6 (*δ* 2.84, d, $J = 4.5$ Hz) to H-8 through H_2-7, supported the C-5/C-6 location of the epoxide. This was further confirmed by the HMBC correlations (Figure 1) found from H-1, H_3-19, H-6, and H_2-4 to C-5 (*δ* 64.6, qC). Comparison of the NMR data of the epoxide group in **3** with those of 5*β*,6*β*-epoxy-24-methylenecholestan-3*β*-ol, a sterol isolated from the sponge *Haliclona oculata*[15] (δ_H 3.06, br s, H-6; δ_C 62.9, qC, C-5 and 63.7, CH, C-6), suggested the α-orientation of the epoxide in **3**. This was further supported by the NOE correlations (Figure 2) observed for H-6 with H-4α (1.37, ddd, $J = 13.5$, 4.0, 2.0 Hz) and H-7*β* (*δ* 1.92, m). On the basis of above results together with the detailed analyses of 2D NMR spectra of **3** (Figures 1 and 2), the structure of gibberoepoxysterol was unambiguously established as 5α,6-epoxy-24-methylenecholest-1α,3*β*-diol.

The *in vitro* anti-inflammatory effect of the sterols **1** and **4** was tested. 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. It was found that compound **4** reduced the levels of the iNOS and COX-2 proteins at a concentration of 10 *μ*M to 44.5 ± 14.5% and 68.3 ± 12.2%, respectively, relative to the control cells stimulated with LPS. However, the same concentration of the related sterol **1** did not produce any inhibition of LPS-induced iNOS and COX-2 expression (Figure 3). The toxicity of **1** and **4** to RAW264.7 cells was also assessed by trypan blue staining. Cell survival was not changed by the presence of **1** and **4** at the concentration of 10 *μ*M. Both cell loss (38 .4 ± 10.2%) and *β*-actin decrease occurred at high concentration (100 *μ*M) of **4** (Figure 3).

The cytotoxicity of compounds **1**−**4** against the Hep G2, MCF-7, MDA-MB-231, and A-549 cell lines was studied. Steroid **4** exhibited moderate cytotoxicity against the growth of all cancer cell lines (IC_{50} 13.0, 14.1, 14.4, and 14.5 *μ*g/mL, respectively), which was in agreement with its reported cytotoxicity against other cell lines.[8] The 5,6-epoxide-possessing sterol **3** also showed moderate cytotoxicity but only against MDA-MB-231 and A-549 cells (IC_{50} 15.9 and 15.5 *μ*g/mL, respectively). The other two sterols (**1**, **2**), possessing the conjugated diene in the side chain, did not exhibit cytotoxicity against the tested cancer cells ($IC_{50} > 30$ *μ*g/mL).

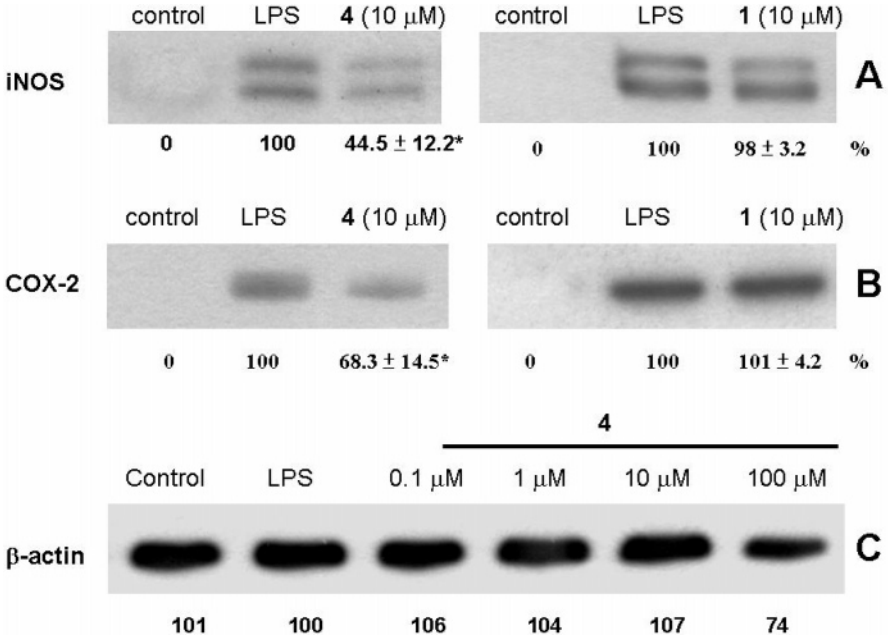

Figure 3. Effect of compound **1** and **4** on the pro-inflammatory iNOS and COX-2 expression of RAW264.7 macrophage cells by immunoblot analysis: (A) Immunoblot of iNOS; (B) immunoblot of COX-2; (C) immunoblot of β-actin. A and B values are mean ± SD ($n = 3$). Relative intensity of the LPS alone stimulated group was taken as 100%. *Significantly different from LPS-stimulated group (*$P < 0.05$).

Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting point apparatus. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter. IR spectra were recorded on a Hitachi I-2001 infrared spectrophotometer. NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR or on a Bruker AVANCE 500 FT-NMR at 500 MHz for ^{1}H and 125 MHz for ^{13}C, in $CDCl_3$, unless otherwise stated. Low-resolution mass spectral data were obtained by EI or ESI with a VG QUATTRO GC/MS spectrometer. HRMS were recorded by ESI FT-MS on a Bruker APEX II mass spectrometer. Silica gel (Merck, 230−400 mesh) and Sephadex LH 20 (Pharmacia) were used for open CC. Precoated Si gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC. Isolation by HPLC was performed by a Shimadzu SPD-10A instrument equipped with a normal-phase column (Lichrosorb Si-60, 7 μm, 250 × 25 mm).

Animal Material. The soft coral *S. gibberosa* was collected by hand using scuba off the coast of Kenting, Taiwan, in June 2004, at depths of 15 to 20 m, and stored in a freezer until extraction. A voucher sample (SC-61) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Separation. The sliced bodies of the soft coral *S. gibberosa* (1.3 kg, wet wt) were exhaustively extracted with EtOH (4 L). The organic layer was filtered and concentrated under vacuum and then partitioned between CH_2Cl_2 and H_2O. The solvent-free CH_2Cl_2 extract (10 g) was subjected to CC on Si gel and eluted with EtOAc in *n*-hexane (0−100%, gradient) to yield 23 fractions. Fraction 6, eluted with EtOAc−*n*-hexane (1:4), yielded **5** (20 mg). Fraction 10, eluted with EtOAc−*n*-hexane (4:1), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford 12 subfractions. The sixth subfraction was separated by normal-phase HPLC, using EtOAc−*n*-hexane (1:3), to afford **4** (13.6 mg) and **1** (8.5 mg), respectively. Fraction 12, eluted with EtOAc−*n*-hexane (10:0), was similarly chromatographed over Sephadex LH-20, using acetone as the mobile phase, to afford nine subfractions. The sixth subfraction was isolated by normal-phase HPLC, using acetone−*n*-hexane (1:5), to afford **3** (4.7 mg) and **2** (1.3 mg), respectively.

Gibberoketosterol B (1): white powder; mp 163−164 °C; R_f = 0.27 (Si, EtOAc−hexane, 1:1); $[\alpha]^{25}_D$ −25 (*c* 1.0, $CHCl_3$); UV λ_{max} MeOH nm (log ϵ) 232 (4.06); IR (neat) ν_{max} 3422, 2961, 2935, 2870, 1714, 1647, 1458, 1419, 1377 1269, 1086, 1055 cm^{-1}; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; ^{1}H NMR (C_5D_5N, 300 MHz, selected data) δ 6.68 (1H, s, 5-OH), 6.38 (1H, br s, 1-OH), 6.07 (1H, d, J = 15.6 Hz, H-23), 5.67 (1H, dd, J = 15.6, 8.5 Hz, H-22), 5.01 and 4.92 (each 1H, s, H_2-28), 4.87 (1H, dd, J = 12.3, 3.5 Hz, H-1), 4.46 (1H, br s, H-3), 1.39 (3H, s, H_3-19), 1.06 (6H, d, J = 6.9 Hz, H_3-26 and H_3-27), 1.02 (3H, d, J = 6.9 Hz, H_3-21), 0.66 (3H, s, H_3-18); EIMS *m/z* 444 (1.6, $[M]^+$), 426 (0.5, $[M - H_2O]^+$, 408 (0.6, $[M - 2\ H_2O]^+$, 390 (0.2, $[M - 3\ H_2O]^+$; ESIMS *m/z* 467 (50, $[M + Na]^+$), 413 (100); HRESIMS *m/z* 467.3138 $[M + Na]^+$ (calcd for $C_{28}H_{44}O_4Na$, 467.3137).

Gibberoketosterol C (2): white powder; mp 142−143 °C; R_f = 0.09 (Si, EtOAc−hexane, 1:1); $[\alpha]^{25}_D$ −38 (*c* 0.5, $CHCl_3$); UV λ_{max} MeOH nm (log ϵ) 231 (3.67); IR (neat) ν_{max} 3362, 2960, 2943, 2868, 1699, 1635, 1458, 1420, 1375, 1053 cm^{-1}; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; HRESIMS *m/z* 451.3190 $[M + Na]^+$ (calcd for $C_{28}H_{44}O_3Na$, 451.3188).

Gibberoepoxysterol (3): white powder; mp 156−157 °C; R_f = 0.15 (Si, EtOAc−hexane, 1:1); $[\alpha]^{25}_D$ −25 (*c* 1.9, $CHCl_3$); IR (neat) ν_{max} 3392, 2958, 2941, 2870, 1375 1254, 1161, 1057 cm^{-1}; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; ^{1}H NMR (C_5D_5N, 300 MHz, selected data) δ 6.39 (1H, br s, 1-OH), 5.01 (1H, m, H-3), 4.84 and 4.81 (each 1H, s, H_2-28), 4.12 (1H, br s, H-1), 2.79 (1H, d, J = 4.0 Hz, H-6),1.10 (3H, s, H_3-19), 1.03 (6H, d, J = 6.3 Hz, H_3-26 and H_3-27), 0.90 (3H, d, J = 6.3 Hz, H_3-21), 0.61 (3H, s, H_3-18); EIMS *m/z* 430 (2.9, $[M]^+$), 412 (5.3, $[M - H_2O]^+$), 394 (2.2, $[M - 2\ H_2O]^+$), 378 (0.4, $[M - 2\ H_2O - O]^+$) 341 (8.9), 328 (17.5), 285 (13.4), 267 (16.4); ESIMS *m/z* 453 (100, $[M + Na]^+$), 413 (40, $[M - H_2O + H]^+$), 395 (13, $[M - 2\ H_2O + H]^+$); HRESIMS *m/z* 453.3342 $[M + Na]^+$ (calcd for $C_{28}H_{46}O_3$-Na, 453.3344).

***In Vitro* Anti-inflammatory Assay.** The anti-inflammatory assay was modified from Ho et al.[16] and Park et al.[17] 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. Macrophage cells were activated by incubation in medium containing *Escherichia coli* LPS (0.01μg/mL; Sigma) for 16 h in the presence or absence of various compounds. 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 the DC protein assay kit (Bio-Rad) modified by the method of Lowry et al.[18] 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 incubated for 180 min with antibody against inducible nitric oxide synthase (iNOS; 1:1000 dilution; Transduction Laboratories) and cyclooxygenase-2 (COX-2; 1:1000 dilution; Cayman

Chemical) protein. The blots were detected using ECL detection reagents (Perkin-Elmer, Western Blot Chemiluminescence Reagent Plus) according to the manufacturer instructions. The membranes were reprobed with a monoclonal mouse anti-β-actin antibody (1:2500, Sigma) as the loading control.

Cytotoxicity Testing. Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of the test compounds **1**−**4** were performed using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[19,20]

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References and Notes

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