Monanchosterols A and B, Bioactive Bicyclo[4.3.1]steroids from a Korean Sponge Monanchora sp.

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Monanchosterols A and B, Bioactive Bicyclo[4.3.1]steroids from a Korean Sponge *Monanchora* sp.

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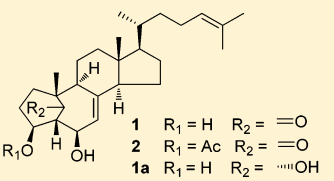
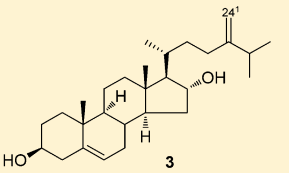
Supporting Information

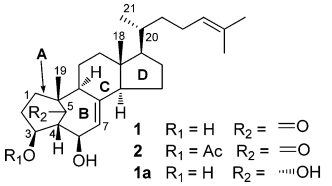
ABSTRACT: Chemical investigation of a Korean marine sponge, *Monanchora* sp., led to the isolation of three new steroids (**1**−**3**). Compounds **1** and **2**, designated as monanchosterols A and B, respectively, represent the first examples of steroids possessing the bicyclo[4.3.1] A/B ring system from a natural source. Compounds **1**−**3** were investigated for their anti-inflammatory activity by evaluating their inhibitory effects on the mRNA expression of IL-6, TNF-α, and COX-2 in the LPS-stimulated murine RAW264.7 macrophage cells. Compounds **2** and **3** exhibited significant inhibitory effects on the mRNA expression of IL-6 without notable cytotoxicity to the cells in a dose-dependent manner.

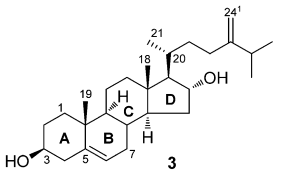




A significant number of steroids have been isolated from marine organisms, especially the Porifera. One major group of steroids is characterized by the polyhydroxylation of the tetracyclic structure with different degrees of substitution and unsaturation in the side chain. A second major group involves steroid derivatives such as steroid esters, sulfated steroids, steroidal alkaloids, and steroid-taurine conjugates. Moreover, marine organisms have also given rise to a small number of steroids with unique carbon skeletons, such as 9,11-secosterols,[1] ring-A-contracted steroids,[2] A-nor steroids,[3] steroids with a bicyclo[4.4.1] A/B ring system,[4] steroids with a spiro-ring A/B system,[5] and dimeric steroids.[6] However, perhaps more significant than their diverse structures, the sponge-derived steroids exhibit a range of biological activities in a variety of therapeutic areas.[7]

In the course of an ongoing program toward the isolation of biologically active metabolites from Korean marine organisms, a sponge of the genus *Monanchora* (class Demospongiae, order Poecilosclerida, family Crambeidae) was examined. ^{1}H NMR-guided fractionation yielded three new steroids, monanchosterols A (**1**) and B (**2**) and compound **3**. Herein, the details of the isolation, structural elucidation, and biological activity of these compounds are described.





RESULTS AND DISCUSSION

Monanchosterol A (**1**) was isolated as a white, amorphous solid. The molecular formula $C_{27}H_{42}O_3$ was confirmed from its HRFABMS and NMR data. A pseudomolecular ion peak at *m*/*z* 437.3013 $[M + Na]^+$ (calcd 437.3032) was observed in the HRFABMS. The presence of one or more hydroxy groups was supported by a strong OH stretching absorption band at 3416 cm^{-1} in the IR spectrum. A ^{13}C NMR resonance at δ 215.0 and the strong IR absorption at 1693 cm^{-1} indicated the presence of a saturated ketone. The ^{1}H NMR spectrum revealed two olefinic signals at δ 5.08 (t, J = 7.1 Hz) and 5.38 (d, J = 5.0 Hz), which were attributed to two sets of isolated trisubstituted double-bond systems. The two double bonds and the ketone group accounted for three of the seven degrees of unsaturation required by the molecular formula, and the remaining degrees of unsaturation required a tetracyclic structure.

In addition to the signals assigned for two double bonds, the ^{1}H NMR spectrum also revealed typical steroid angular methyl singlets at δ 0.57 (s, H_3-18) and 1.11 (s, H_3-19) and one methyl doublet at δ 0.93. The two olefinic methyl signals (δ 1.60 and 1.67) were reminiscent of an unsaturated (Δ^{24E}) side chain. The ^{1}H NMR data readily implied a steroid skeleton, but detailed analysis of the COSY, HSQC, and HMBC data indicated significant changes in the A and B rings. The COSY correlations defined a linear ^{1}H spin system starting with a

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diastereotopic methylene at δ 1.93 and 1.81 (H-1) and continuing in sequence to another diastereotopic methylene at δ 2.23 and 1.50 (H-2), an oxymethine at δ 4.10 (H-3), a methine at δ 2.87 (H-4), an oxymethine at δ 4.31 (H-6), and ending with an olefinic methine at δ 5.38 (H-7). The H_3-19 angular methyl singlet showed HMBC correlations to methine, methylene, quaternary, and carbonyl carbon resonances at δ 57.6 (C-9), 38.2 (C-1), 49.9 (C-10), and 215.0 (C-5), respectively, confirming the location of the carbonyl group. The HMBC correlations from H-1, H-3, H-4, H-6, H-9, and H-19 to C-5 were also observed. These data suggested the occurrence of an unusual bicyclo[4.3.1]decan-10-one moiety, found for the first time among naturally occurring steroids.[8] The bicyclo[4.3.1] moiety was further corroborated by reduction **1** to **1a** (see below). The analysis of the COSY spectrum of **1a** confirmed the presence of a C-1−C-2−C-3−C-4(C-5)−C-6−C-7 spin system.

The modified Mosher's method was applied to determine the absolute configuration of C-3 and C-6.[9] The mono-Mosher esters, 3-*O* and 6-*O*-MTPA esters, of compound **1** were obtained by treatment of **1** with two equivalents of α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride [(*R*)-MTPA-Cl gives the (*S*)-MTPA ester and vice versa]. The *S* configuration at C-3 was defined by the interpretation of the ^{1}H NMR data of its 3-*O*-MTPA ester derivatives, showing negative $\Delta\delta_{S-R}$ values for H-1, H-2, and H_3-19 and positive values for H-4, H-6, H-7, and H-14. Similarly, the $\Delta\delta_{S-R}$ value distribution pattern of 6-*O*-MTPA ester derivatives indicated the *R* configuration at C-6 (Figure 1).

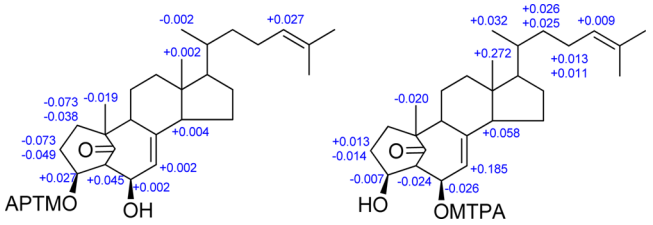

Figure 1. Delta values ($\Delta\delta_{S-R}$) in ppm for 3-*O*- and 6-*O*-MTPA esters of compound **1** in $CDCl_3$.

Although one synthetic steroid with the unusual bicyclo[4.3.1]decan-10-one A/B ring system was obtained by the radical-induced ring opening of epoxides,[8] an unambiguous determination of the absolute configuration of C-4 by the comparison of the NMR data with those of the synthetic steroid could not be made, because of the different substitution pattern and incomplete spectroscopic data. Therefore, compound **1** was modified chemically to determine the absolute configuration at C-4. The reduction of **1** with $NaBH_4$ in MeOH in an ice−water bath overnight afforded monanchosterol C (**1a**). The molecular formula of compound **1a** was determined as $C_{27}H_{44}O_3$ on the basis of a combination of HRFABMS (*m*/*z* 439.3183 measured for $[M + Na]^+$) and the ^{13}C spectroscopic data. Compared with **1**, the obvious difference seen was the appearance of a new hydroxy group along with the absence of the carbonyl group in compound **1a**. The structure was confirmed by HMBC correlations of the new oxymethine signal at δ 4.10 with C-4 (δ 51.1), C-6 (δ 73.3), C-9 (δ 60.4), C-10 (δ 41.0), and C-19 (δ 29.9). Given the defined *S* configuration at C-3, the absolute configuration of C-4 was established from the analysis of the coupling constant values and NOE correlations. The coupling constants of H-1β (td, *J* = 14.1, 4.6 Hz) indicated the axial orientation of H-1β. The key H-1β/H_3-19/H-5 NOE correlations (Figure 2) strongly suggested that all of these

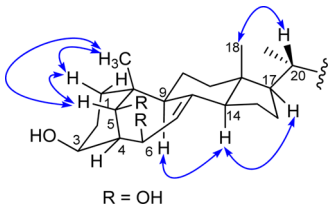

Figure 2. Selected key NOE correlations of **1a**.

protons were present on the β side of the molecule and the axial orientation of H-5. The H-5 signal was coupled to H-4 with a relatively small *J* value (5.6 Hz), confirming the equatorial orientation of H-4, and thus the *S* configuration at C-4 was deduced. The configuration assignment, where both H-4 and H_3-19 were configured in the equatorial position, would explain the absence of any NOE correlation of H-4/H_3-19 in **1**.

Comparison of the ^{1}H NMR data of **1** with those of **1a** showed the upfield shifts of H-11β (δ 1.92 → 1.32), H-11α (δ 1.95 → 1.82), and H_3-18 (δ 0.65 → 0.57) for compound **1**, implying that H_2-11 and H_3-18 are located in the shielding zone of the C-5 ketone group in **1** and establishing the configuration in which the C-5 ketone group occurs at the β face of the skeleton, whereas H-1, H-2, H-3, H-4, and H-6 in **1** showed significant downfield shifts relative to those of **1a**. These observations indicated that all of them fell into the deshielding zone of the C-5 ketone group. Therefore, it can be concluded that ring A does not lie on the same plane as ring B, but is twisted to the α side of the skeleton. This geometry was consistent with the *S* configuration at C-4.

The large coupling constants of H-14 in compounds **1** (*J* = 12.5, 6.3 Hz) and **1a** (*J* = 12.7, 6.4 Hz) defined unequivocally the common *trans* C/D ring fusion. The NOE correlations observed between H_3-18/H-20 and H-14/H-17 showed a β orientation of the substituent at C-17 and further supported a *trans* C/D ring junction. The α orientation of H-9 was determined by the key H-9/H-14 NOE correlation (Figure 2).

Monanchosterol B (**2**) gave a molecular formula of $C_{29}H_{44}O_4$, as inferred from its HRFABMS and NMR data. The HRFABMS exhibited a pseudomolecular ion peak at *m*/*z* 479.3130 $[M + Na]^+$ corresponding to the monosodium salt of the molecule. The low-resolution ESIMS showed the fragment ion peaks at *m*/*z* 439 $[M - OH]^+$ and 397 $[M - OAc]^+$, corresponding to the loss of one hydroxy group and one acetoxy group, respectively. The presence of the acetoxy group was also supported by the NMR signals (δ_H: 2.02, δ_C: 21.2; δ_C: 171.9). The downfield shift of H-3 to δ 5.14 (δ 4.10 in **1**) and the interpretation of the 2D NMR spectroscopic data indicated that compound **2** is a 3-*O*-acetylated congener of **1**. The absolute configuration at C-6 was determined by the advanced Mosher's method.[9] The calculation of $\Delta\delta_{S-R}$ values, derived from ^{1}H NMR data of the MTPA esters, established the absolute configuration of C-6 as *R* (Figure 3).

The molecular formula of **3** was established as $C_{28}H_{46}O_2$ by its HRFABMS and NMR data. The HRFABMS showed a pseudomolecular ion peak at *m*/*z* 437.3390 [M + Na]. The ^{1}H NMR spectrum of **3** showed characteristic steroid signals: two methyl singlets at δ 1.02 (s, H_3-19) and 0.74 (s, H_3-18) and three methyl doublets δ 1.04 (d, *J* = 6.8 Hz, H_3-27), 1.03 (d, *J* = 6.8 Hz, H_3-26), and 0.99 (d, *J* = 6.5 Hz, H_3-21). The analysis of

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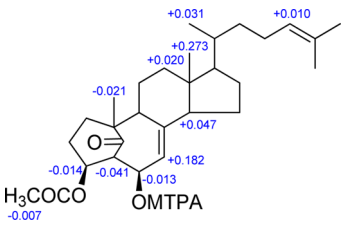

Figure 3. Delta values ($\Delta\delta_{S-R}$) in ppm for MTPA esters of compound **2** in $CDCl_3$.

the ^{13}C and HSQC spectroscopic data revealed the presence of two olefinic quaternary carbons (δ 158.0 and 142.4), one olefinic methine (δ 122.5), one terminal methylene (δ 107.0), and two secondary oxygenated carbons (δ 72.6 and 77.7). The assignments of ^1H and ^{13}C NMR signals of the tetracyclic ring system were established by the interpretation of the COSY, edited HSQC, and HMBC data. The HMBC correlations from the angular methyl H_3-19 to the carbon resonances at δ 38.6 (C-1), 142.4 (C-5), 51.8 (C-9), and 37.9 (C-10) and from H-4 to C-5 and C-6 confirmed a trisubstituted double bond at C-5, whereas the HMBC correlations of the protons at δ 4.72 (H-24^{1}a) and 4.70 (H-24^{1}b) with the carbons at δ 32.9 (C-23), 158.0 (C-24), and 35.0 (C-25) indicated a 24-methylenecholestane side chain. The relative configurations of **3** were assigned by the analysis of the NOESY data and comparison of the NMR data with the reported values.[10,11] The strong H-16/H_3-18/H-20 NOE correlations indicated that the hydroxy group at C-16 occurred on the α side of the molecule. A careful comparison of the ^1H and ^{13}C NMR data with those of natural steroids with OH-16α (e.g., aragusterols F−H) and OH-16β functionalities further corroborated the assignments.[10,11] Thus, the structure of **3** was defined as 24-methyl-5α-cholest-5,24(24^1)-diene-3β,16α-diol.

To the best of our knowledge, monanchosterols A and B (**1** and **2**) represent the first examples of steroids possessing the bicyclo[4.3.1] A/B ring system from a natural source. The first and only report on a steroid with this unusual carbon skeleton was a radical-reduced ring-opening product of a 4β,5β-epoxysteroid.[8] The biosynthesis of monanchosterols A and B presumably commenced from a co-occurring 4β,5β-epoxysteroid.

Interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), and cyclooxygenase 2 (COX-2) have been demonstrated as inflammatory mediators that play important roles in inflammation and various related diseases. Compounds **1**−**3** were investigated for their anti-inflammatory activity by evaluating their inhibitory effects on the mRNA expression of IL-6, TNF-α, and COX-2 in the LPS-stimulated murine RAW264.7 macrophage cells. Compound **1** was cytotoxic against RAW 264.7 cells with an IC_{50} of 65 μM, and therefore we did not investigate its inflammatory inhibition activity at the tested concentrations (0.1, 1, 10, and 100 μM). Interestingly, compound **2** differs from **1** simply by acetylation at C-3, but is devoid of cytotoxicity and exhibited potent inhibitory effect on the mRNA expression of IL-6. Compound **3** also inhibited LPS-induced IL-6 expression. The initial bioassay showed that inhibitory rates of **2** and **3** reached 69% and 67% at concentrations of 10 μM, respectively, more potent than that of SB203580[12] (53% at the concentration of 10 μM). In a more detailed study, **2** and **3** showed inhibitory effects on the mRNA expression of IL-6 in a dose-dependent manner with IC_{50} values of 5.0 ± 0.17 and 5.2 ± 0.30 μM, respectively (Figure 4). However, **2** and **3** did not exhibit significant inhibitory effects against the LPS-induced up-regulation of TNF-α and COX-2 transcripts.

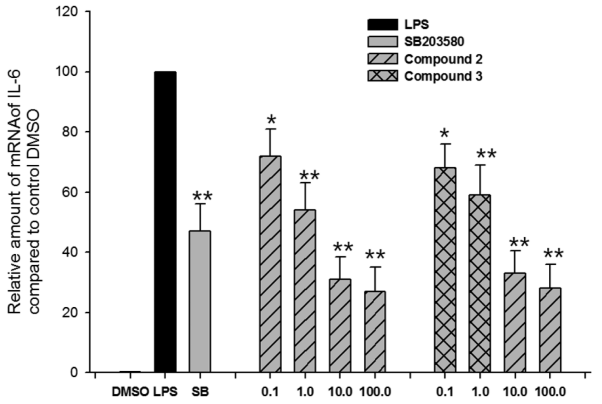

Figure 4. Compounds **2** and **3** inhibited LPS-stimulated mRNA expression of IL-6 in a dose-dependent manner in the murine RAW 264.7 macrophage cells. The cells were treated for 24 h with pure LPS (0.1 μg/mL) or with LPS (0.1 μg/mL) containing the test samples at their indicated concentrations (0.1, 1, and 100 μM). The mRNA levels of IL-6 were quantified by RT-qPCR. The mRNA values for each gene were normalized to internal control 18s mRNA and expressed as a ratio to DMSO. SB203580 (10 μM) was used as a reference standard. Each bar represents the mean ± SD of three independent experiments. Statistical significance relative to LPS group is indicated: *, $p < 0.05$; **, $p < 0.01$.

Steroids may be classified into cholestanes, cholanes, pregnanes, androstanes, and estranes based upon their chemical composition. Steroidal drugs, which are commonly used to treat a variety of illnesses that involve inflammatory symptoms, are usually based on a pregnane carbon skeleton. Hydrocortisone and dexamethasone, two examples of such steroidal drugs, demonstrated significant inhibitory effects on the production of IL-6 in LPS-stimulated human cells in a dose-dependent manner.[13,14] Recently, some naturally occurring steroids of the cholestane subclass were reported to show potent inhibitory effects on the production of IL-6 in LPS-stimulated cells.[15,16] Monanchosterol B (**2**), bearing a bicyclo[4.3.1] A/B ring moiety, also displayed an inhibitory effect on the mRNA expression of IL-6 in a dose-dependent manner with an IC_{50} value of 5.0 ± 0.17 μM and, thus, provides a scaffold for discovering new anti-inflammatory agents.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations were measured in MeOH using a 1.0 cm cell on a Rudolph Research Autopol III polarimeter. UV spectra were obtained with a Hitachi JP/U-3010 UV spectrophotometer. IR spectra were acquired on a JASCO FT/IR 4200 spectrophotometer. All NMR spectra were recorded on a Bruker Avance DPX-600 or Ascend 700 spectrometer using methanol-d_4 or $CDCl_3$ as the solvent. Chemical shifts were reported with reference to the respective solvent peaks [δ_H 3.31 and δ_C 49.0 for CD_3OD; δ_H 7.26 and δ_C 77.2 for $CDCl_3$]. Electrospray ionization source (ESI) low-resolution mass spectra were recorded on an Agilent Technologies 6120 quadrupole mass spectrometer coupled with an Agilent Technologies 1260 series HPLC. High-resolution mass spectrometric data were collected on a JEOL JMS-700 double-focusing (B/E configuration) instrument. The pyridinyl imidazole SB203580 (>98%) was purchased from Sigma and dissolved in DMSO.

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Table 1. NMR Spectroscopic Data for Compounds 1 and 1a in MeOH-d_4

	1[a]			1a[b]		
position	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC
1a	38.2, CH_2	1.93, m	2, 3, 5, 9, 10, 19	39.4, CH_2	1.78, td (14.1, 4.6)	2, 3, 5, 9, 10, 19
1b		1.81, m			1.33, m	
2a	29.0, CH_2	2.23, m	1, 3, 4, 10	28.3, CH_2	2.02, m	1, 3, 4, 10
2b		1.50, m			1.32, m	
3	73.9, CH	4.10, br dd (6.5, 4.2)	1, 2, 4, 5, 6	71.8, CH	3.82, br d (1.9)	1, 2, 4, 5, 6
4	66.9, CH	2.87, br s	2, 3, 5, 6, 7, 10	51.1, CH	2.60 br s	2, 3, 5, 6, 7, 10
5	215.0, C			78.7, CH	4.10, d (5.7)	4, 6, 9, 10, 19
6	69.3, CH	4.31, dd (5.0, 4.3)	3, 4, 5, 7, 8	73.3, CH	4.18, dd (7.0, 2.7)	3, 4, 5, 7, 8
7	121.1, CH	5.38, d (5.0)	4, 6, 8, 9, 14	120.5, CH	5.35, d (7.0)	4, 6, 8, 9, 14
8	145.0, C			147.5, C		
9	57.6, CH	1.94, m	5,7, 8, 10, 11	60.4, CH	1.92, m	7, 8, 10, 11
10	49.9, C			41.0, C		
11a	28.8, CH_2	1.82, m	9, 12	32.3, CH_2	1.95, m	9, 12
11b		1.32, m			1.92, m	
12a	42.5, CH_2	1.97, m	11, 13, 18	44.1, CH_2	1.98, m	11, 13, 18
12b		1.32, m			1.25, m	
13	48.7, C			48.9, C		
14	60.7, CH	2.18, dd (12.5, 6.3)	7, 8, 9, 12, 13, 15, 18	61.1, CH	2.15, dd (12.7, 6.4)	7, 8, 9, 12, 13, 15, 18
15a	23.4, CH_2	1.62, m	14, 16	23.8, CH_2	1.61 m	14, 16
15b		1.39, m			1.37 m	
16a	29.1, CH_2	1.94, m	15, 17	29.2, CH_2	1.92, m	15, 17
16b		1.32, m			1.32, m	
17	57.9, CH	1.32, m	13, 18, 20	58.0, CH	1.31, m	13, 18, 20
18	12.5, CH_3	0.57, s	12, 13, 14, 17	12.7, CH_3	0.65, s	12, 13, 14, 17
19	24.3, CH_3	1.11, s	1, 5, 9, 10	29.9, CH_3	1.09, s	1, 5, 9, 10
20	37.1, CH	1.37, m	17, 21	37.2, CH	1.39, m	17, 21
21	19.1, CH_3	0.93, d (6.4)	17, 20, 22	19.1, CH_3	0.93, d (6.5)	17, 20, 22
22a	37.1, CH_2	1.42, m	21, 23, 24	37.1, CH_2	1.43, m	21, 23, 24
22b		1.03, m			1.04, m	
23a	25.7, CH_2	2.02, m	20, 22, 24, 25	25.8, CH_2	2.02, m	20, 22, 24, 25
23b		1.87, m			1.88, m	
24	126.1, CH	5.08, t (7.1)	22, 23, 26, 27	126.1, CH	5.08, t (7.1)	22, 23, 26, 27
25	131.9, C			131.8, C		
26	17.7, CH_3	1.60, s	24, 25, 27	17.7, CH_3	1.59, s	24, 25, 27
27	25.9, CH_3	1.67, s	24, 25, 26	25.9, CH_3	1.66, s	24, 25, 26

[a]The ^{1}H NMR spectra were recorded at 700 MHz, while the ^{13}C NMR spectra were recorded at 175 MHz. [b]The ^{1}H NMR spectra were recorded at 600 MHz, while the ^{13}C NMR spectra were recorded at 150 MHz.

Animal Material. Specimens of the sponge *Monanchora* sp. (QM G331990) were collected by scuba diving at 15−20 m depth off the shore of Gageo Island, southwestern Korea, in July 2009. The specimens were massive (120 mm × 100 mm × 80 mm) with a undulating surface covered in small papillae, with numerous scattered oscula (0.5−4 mm in diameter). The sponge was bright orange externally and pale orange internally and turned dark brown in alcohol. The texture of the sponge was soft and compressible and could easily be torn or cut. The ectosomal skeleton was composed of a thin spongin layer encrusted with anchorate isochelae (30−40 μm). The choanosomal skeleton was composed of spongin containing spicules of tylostyles to subtylostyles (300−400 μm) as well as anchorate and unguiferous isochelae (30−40 μm), interspersed with aquiferous channels lined with unidirectional styles (300−400 μm). The specimen was identified taxonomically as *Monanchora* sp. 4831 (class Demospongiae, order Poecilosclerida, family Crambeidae) by Dr. Merrick Ekins from the Queensland Museum, Australia. The voucher specimen (QM G331990) is deposited at the Queensland Museum, Australia, and at the Center for Marine Natural Products and Drug Discovery, Seoul National University, Korea (registered as CMDD09A0401).

Extraction and Isolation. The lyophilized specimens (dry wt 400 g) were cut into small pieces and extracted three times with 50% MeOH in CH_2Cl_2 at room temperature. These extracts (32 g) were combined and partitioned three times between H_2O and CH_2Cl_2. The CH_2Cl_2 layer was further partitioned between aqueous MeOH (90%) and *n*-hexane to afford an aqueous MeOH-soluble fraction (17 g) and an *n*-hexane-soluble fraction (15.8 g). Then, the aqueous MeOH fraction was subjected to reversed-phase silica gel flash column chromatography (YMC Gel ODS-A, 120 Å, 40−60 μm), eluting with a step gradient solvent system of 50% to 0% H_2O/MeOH and washing with acetone to afford 14 fractions (1−14). Fraction 7 was further separated by normal-phase MPLC (silica gel 60, 400/230 mesh), eluting with a solvent system of 2.5% to 100% ethyl acetate in *n*-hexane, to afford 10 fractions. Compound **1** (10.5 mg, 0.03%) was obtained by separation of subfraction 7-8 using reversed-phase HPLC (Xterra column 5 μm, 100 Å, 2.0 mL/min, UV detection at 210 nm) eluting with 65% CH_3CN (retention time: 26 min). Compound **2** (3.0 mg, 0.009%) was obtained by purification of subfraction 7-7 by reversed-phase HPLC (Watchers 120 ODS-BP (5 μm), 250 × 4.6 mm, 2.0 mL/min, 210 nm) eluting with 90% CH_3CN (retention time: 23 min). Compound **3** (3.5 mg, 0.01%) was obtained from subfraction 7-8 using reversed-phase HPLC (Xterra column 5 μm, 100 Å, 2.0 mL/min, 210 nm) eluting with 70% CH_3CN (retention time: 39 min).

*Monanchosterol A (**1**):* white, amorphous solid; $[\alpha]^{20}_D$ +69.3 (*c* 0.4 MeOH); UV (MeOH) λ_{max} (log ε) 203 (5.49) nm; IR (film) ν_{max}

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3416, 2957, 2931, 2871, 1693, 1682, 1657, 1452, 1377, 1079 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRESIMS *m/z* 397, 437 $[M + Na]^+$; HRFABMS *m/z* 437.3013 (calcd for $C_{27}H_{42}O_3Na$, 437.3032).

*Monanchosterol B (**2**):* white, amorphous solid; $[\alpha]^{20}_D$ +65.0 (*c* 0.2 MeOH); UV (MeOH) λ_{max} (log ε) 204 (5.54) nm; IR (film) ν_{max} 3396, 2958, 2931, 2871, 1710, 1694, 1689, 1672, 1456, 1376 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 5.41 (1H, d, *J* = 5.4 Hz, H-7), 5.14 (1H, br s, H-3), 5.09 (1H, t, *J* = 6.9 Hz, H-24), 4.42 (1H, dd, 5.4, 4.3 Hz, H-6), 2.90 (1H, br s, H-4), 2.32 (1H, m, H-2a), 2.22 (1H, dd, *J* = 12.4, 6.4 Hz, H-14), 2.03 (1H, m, H-23a), 2.02 (3H, s, 3-*O*-$COCH_3$), 1.98 (1H, m, H-9), 1.97 (1H, m, H-12a), 1.95 (1H, m, H-16a), 1.90 (2H, br t, *J* = 6.3 Hz, H-1), 1.88 (1H, m, H-23b), 1.84 (1H, m, H-11a), 1.67 (3H, s, H-27), 1.64 (1H, m, H-15a), 1.60 (3H, s, H-26), 1.44 (1H, m, H-22a), 1.42 (1H, m, H-15b), 1.59 (1H, m, H-2b), 1.38 (1H, m, H-20), 1.34 (2H, m, H-12b,16b), 1.33 (2H, m, H-17,11b), 1.13 (3H, s, H-19), 1.04 (1H, m, H-22b), 0.94 (3H, d, *J* = 6.3 Hz, H-21), 0.57 (3H, s, H-18); ^{13}C NMR (CD_3OD, 150 MHz) δ 213.4 (C, C-5), 171.9 (C, 3-*O*-$COCH_3$), 145.7 (C, C-8), 132.0 (C, C-25), 126.2 (CH, C-24), 121.0 (CH, C-7), 76.9 (CH, C-3), 69.0 (CH, C-6), 63.7 (CH, C-4), 60.9 (CH, C-14), 58.1 (CH, C-17), 57.7 (CH, C-9), 50.1 (C, C-10), 42.7 (CH_2, C-12), 38.5 (CH_2, C-1), 37.3 (CH, C-20), 37.2 (CH_2, C-22), 29.3 (CH_2, C-16), 29.0 (CH_2, C-11), 26.5 (CH_2, C-2), 26.1 (CH_3, C-27), 25.9 (CH_2, C-23), 24.4 (CH_3, C-19), 23.5 (CH_2, C-15), 21.2 (CH_3, 3-*O*-$COCH_3$), 19.3 (CH_3, C-21), 17.9 (CH_3, C-26), 12.7 (CH_3, C-18); LRESIMS *m/z* 397 $[M - OAc]^+$, 439 $[M - OH]^+$, 479 $[M + Na]^+$; HRFABMS *m/z* 479.3130 (calcd for $C_{29}H_{44}O_4Na$, 479.3137).

*Compound **3**:* white needles; $[\alpha]^{20}_D$ −8.0 (*c* 0.4 MeOH); UV (MeOH) λ_{max} (log ε) 203 (5.42) nm; IR (film) ν_{max} 3418, 2933, 2900, 2870, 1660, 1651, 1455, 1152 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; LRESIMS *m/z* 415 $[M + H]^+$, 437 $[M + Na]^+$; HRFABMS *m/z* 437.3390 (calcd for $C_{27}H_{42}O_3Na$, 437.3396).

Preparation of MTPA Esters. The mono-Mosher esters, 3-*O* and 6-*O*-MTPA esters, of compound **1** were prepared by treatment of **1** (2.0 mg, 4.8 μmol) with 2 equiv of MTPA-Cl (1.85 μL, 9.6 μmol) in dry pyridine (25 μL) for 24 h at room temperature [(*R*)-MTPA-Cl gives the (*S*)-MTPA ester and vice versa]. The reaction was monitored by TLC (ODS, MeOH). After removal of solvent, the resulting products were purified by reversed-phase HPLC on an Xterra column (250 × 4.6 mm, 5 μm, 100 Å) and analyzed by ^1H NMR spectroscopy. The procedure for the preparation of MTPA esters of compound **2** was similar to that used for compound **1**. Compound **2** (1.0 mg, 2.2 μmol) was treated with (*R*)-(−)- and (*S*)-(+)-MTPA-Cl (4 μL, 21 μmol) in dry pyridine (25 μL) for 24 h at room temperature to afford the (*S*)-(−)- and (*R*)-(+)-MTPA ester, respectively.

*3-O-(S)-MTPA ester of **1**:* ^1H NMR (600 MHz, $CDCl_3$) δ 7.505 (2H, br d, 7.6 Hz, Ar-H), 7.462 (3H, m, Ar-H), 5.507 (1H, br s, H-3), 5.454 (1H, br d, *J* = 5.1 Hz, H-7), 5.108 (1H, t, *J* = 6.9 Hz, H-24), 4.465 (1H, br s, H-6), 3.546 (3H, s, OMe), 3.098 (1H, br s, H-4), 2.430 (1H, br t, *J* = 13.2 Hz, H-2a), 2.262 (1H, dd, *J* = 11.6, 5.7 Hz, H-14), 2.060 (1H, m, H-23a), 1.916 (1H, m, H-23b), 1.868 (1H, m, H-1a), 1.697 (1H, m, H-1b), 1.693 (3H, s, H-27),1.681 (1H, m, H-15a), 1.675 (1H, m, H-2b), 1.622 (3H, s, H-26), 1.473 (1H, m, H-15b), 1.016 (3H, s, H-19), 0.951 (3H, d, *J* = 6.3 Hz, H-21), 0.593 (3H, s, H-18); LRESIMS *m/z* 631 $[M + H]^+$, 653 $[M + Na]^+$.

*3-O-(R)-MTPA ester of **1**:* ^1H NMR (600 MHz, $CDCl_3$) δ 7.494 (2H, br d, 7.0 Hz, Ar-H), 7.464 (3H, m, Ar-H), 5.480 (1H, br s, H-3), 5.453 (1H, br d, *J* = 5.5 Hz, H-7), 5.081 (1H, t, *J* = 6.5 Hz, H-24), 4.463 (1H, br s, H-6), 3.544 (3H, s, OMe), 3.053 (1H, br s, H-4), 2.479 (1H, br t, *J* = 11.5 Hz, H-2a), 2.258 (1H, dd, *J* = 10.8, 6.2 Hz, H-14), 2.070 (1H, m, H-23a), 1.948 (1H, m, H-23b), 1.941 (1H, m, H-1a), 1.748 (1H, m, H-2b), 1.735 (1H, m, H-1b), 1.693 (3H, s, H-27),1.665 (1H, m, H-15a), 1.623 (3H, s, H-26), 1.504 (1H, m, H-15b), 1.035 (3H, s, H-19), 0.953 (3H, d, *J* = 6.5 Hz, H-21), 0.591 (3H, s, H-18); LRESIMS *m/z* 631 $[M + H]^+$, 653 $[M + Na]^+$.

*6-O-(S)-MTPA ester of **1**:* ^1H NMR (600 MHz, $CDCl_3$) δ 7.558 (2H, br d, 7.6 Hz, Ar-H), 7.457 (3H, m, Ar-H), 5.728 (1H, dd, *J* = 6.1, 2.9 Hz, H-6), 5.456 (1H, br d, *J* = 6.1 Hz, H-7), 5.113 (1H, t, *J* = 6.9 Hz, H-24), 4.249 (1H, br s, H-3), 3.500 (3H, s, OMe), 3.076 (1H, br s, H-4), 2.338 (1H, dd, *J* = 12.2, 6.8 Hz, H-14), 2.297 (1H, m, H-2a), 2.044 (1H, m, H-23a), 1.898 (1H, m, H-23b), 1.698 (3H, s, H-27), 1.624 (3H, s, H-26), 1.576 (1H, m, H-2b), 1.430 (1H, m, H-22a), 1.128 (3H, s, H-19), 1.062 (1H, m, H-22b), 0.928 (3H, d, *J* = 6.5 Hz, H-21), 0.400 (3H, s, H-18); LRESIMS *m/z* 631 $[M + H]^+$, 653 $[M + Na]^+$.

Table 2. NMR Spectroscopic Data for Compound 3 in MeOH-d_4

position	δ_C, type	δ_H (*J* in Hz)	HMBC
1a	38.6, CH_2	1.87, m	2, 3, 10, 19
1b		1.08, m	
2a	32.4, CH_2	1.78, m	1, 3, 4, 10
2b		1.48, m	
3	72.6, CH	3.40, m	1, 2, 4, 5
4	43.2, CH_2	2.23, m	3, 5, 6
5	142.4, C		
6	122.5, CH	5.35, br d (5.1)	4, 5, 7, 8, 10
7a	33.2, CH_2	2.20, m	5, 6, 8, 9, 14
7b		1.58, m	
8	32.7, CH	1.96, m	7, 9, 14
9	51.8, CH	1.00, m	8, 10, 11
10	37.9, C		
11a	22.0, CH_2	1.55, m	9, 12
11b		1.50, m	
12a	41.4, CH_2	2.02, m	11, 13, 18
12b		1.29, m	
13	45.1, C		
14	55.1, CH	1.39 s	8, 9, 12, 13, 15, 18
15a	38.4, CH_2	1.63 m	14, 16
15b		1.52 m	
16a	77.7, CH	3.96, br t (6.7)	14, 17, 20
16b			
17	66.9, CH	1.09, m	13, 18, 20
18	14.0, CH_3	0.74, s	12, 13, 14, 17
19	20.0, CH_3	1.02, s	1, 5, 9, 10
20	35.6, CH	1.57, m	17, 21
21	19.8, CH_3	0.99, d (6.5)	17, 20, 22
22a	35.6, CH_2	1.89, m	21, 23, 24
22b		1.28, m	
23a	32.9, CH_2	2.23, m	22, 24, 25, 24^1
23b		1.93, m	
24	158.0, C		
25	35.0, CH	2.27, m	
26	22.7, CH_3	1.03, d (6.8)	24, 25, 27, 24^1
27	22.4, CH_3	1.04, d (6.8)	24, 25, 26, 24^1
24^1a	107.0, CH_2	4.72, s	23, 25, 24^1
24^1b		4.70, s	23, 25, 24^1

*6-O-(R)-MTPA ester of **1**:* ^1H NMR (600 MHz, $CDCl_3$) δ 7.597 (2H, br s, Ar-H), 7.441 (3H, m, Ar-H), 5.754 (1H, br d, *J* = 6.2 Hz, H-6), 5.271 (1H, br d, *J* = 6.2 Hz, H-7), 5.104 (1H, t, *J* = 6.8 Hz, H-24), 4.256 (1H, br s, H-3), 3.634 (3H, s, OMe), 3.100 (1H, br s, H-4), 2.280 (1H, dd, *J* = 11.2, 6.5 Hz, H-14), 2.284 (1H, m, H-2a), 2.031 (1H, m, H-23a), 1.887 (1H, m, H-23b), 1.707 (3H, s, H-27), 1.627 (3H, s, H-26), 1.590 (1H, m, H-2b), 1.405 (1H, m, H-22a), 1.148 (3H, s, H-19), 1.036 (1H, m, H-22b), 0.896 (3H, d, *J* = 6.2 Hz, H-21), 0.128 (3H, s, H-18); LRESIMS *m/z* 631 $[M + H]^+$, 653 $[M + Na]^+$.

*(S)-MTPA ester of **2**:* ^1H NMR (600 MHz, $CDCl_3$) δ 7.539 (2H, br d, 6.6 Hz, Ar-H), 7.459 (3H, m, Ar-H), 5.836 (1H, br s, H-6), 5.501 (1H, br d, *J* = 5.5 Hz, H-7), 5.258 (1H, br s, H-3), 5.110 (1H, t, *J* = 6.90 Hz, H-24), 3.495 (3H, s, OMe), 3.050 (1H, br s, H-4), 2.333 (1H, dd, *J* = 12.2, 6.8 Hz, H-14), 2.121 (1H, br d, *J* = 12.7 Hz, H-12a), 2.062 (3H, s, $OCOCH_3$), 1.699 (3H, s, H-27), 1.625 (3H, s, H-26), 1.146 (3H, s, H-19), 1.065 (1H, m, H-22b), 0.931 (3H, d, *J* = 6.1 Hz, H-21), 0.398 (3H, s, H-18); LRESIMS *m/z* 673 $[M + H]^+$, 695 $[M + Na]^+$.

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(R)-MTPA ester of 2: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.569 (2H, br d, 6.6 Hz, Ar-H), 7.442 (3H, m, Ar-H), 5.849 (1H, br s, H-6), 5.319 (1H, br d, J = 5.5 Hz, H-7), 5.272 (1H, br s, H-3), 5.100 (1H, t, J = 6.90 Hz, H-24), 3.615 (3H, s, OMe), 3.091 (1H, br s, H-4), 2.286 (1H, dd, J = 12.2, 6.8 Hz, H-14), 2.101 (1H, br d, J = 12.7 Hz, H-12a), 2.069 (3H, s, $OCOCH_3$), 1.708 (3H, s, H-27), 1.628 (3H, s, H-26), 1.167 (3H, s, H-19), 1.037 (1H, m, H-22b), 0.900 (3H, d, J = 6.1 Hz, H-21), 0.125 (3H, s, H-18); LRESIMS m/z 673 $[M + H]^+$, 695 $[M + Na]^+$.

Reduction of Monanchosterol A (1) to Monanchosterol C (1a). To a solution of compound **1** (5 mg, 12.1 μmol) in 2 mL of MeOH was added $NaBH_4$ (0.5 mg, 13.0 μmol) in an ice−water bath. After 12 h, the solvent was evaporated. Then, 1 M HCl was poured into the mixture, which was then extracted with CH_2Cl_2 and concentrated in vacuo to afford **1a** (4.5 mg, 90%) as a white solid.

Monanchosterol C (1a): white, amorphous solid; $[\alpha]^{20}_D$ 50.0 (c 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 204 (5.42) nm; IR (film) ν_{max} 3382, 2958, 2922, 2874, 1665, 1672, 1451, 1442, 1377, 1252, 1050 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; LRESIMS m/z 417 $[M + H]^+$, 439 $[M + Na]^+$; HRFABMS m/z 439.3183 (calcd for $C_{27}H_{44}O_3Na$, 439.3188).

Cell Culture. The murine RAW 264.7 macrophage cells were purchased from ATCC (Rockville, MD, USA), cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% penicillin−streptomycin (100 U/mL) at 37 °C, and maintained in 5% CO_2 humidified air.

Anti-inflammatory Assay. Murine RAW 264.7 macrophage cells in 10% FBS DMEM were plated in six-well plates (3×10^5 cells/mL) and incubated for 24 h. Next, the cells were treated with 0.1 μg/mL LPS in the absence or presence of each test compound for 24 h. The mRNA levels of inflammatory mediators IL-6, TNF-α, and COX-2 were quantified by real-time quantitative PCR (RT-qPCR). LPS significantly increased the mRNA expression of inflammatory mediators compared to those of the vehicle control as shown in Figure 4. When cells were treated with LPS and test compounds, increases of the inflammatory mRNA levels were prevented to different extents.

Real-Time Quantitative PCR. Cells were homogenized in an easyBLUE kit (iNtRON, Sungnam, Korea) for extraction of RNA according to the manufacturer's protocol. Reverse transcription and quantitative PCR were carried out using Transcriptor First Strand cDNA synthesis kit (Roche) and Quanti Tect SYBR Green PCR kit (Qiagen), respectively. The RotorGene 3000 system (Corbett Research Pty Ltd., Sydney, Australia) was used for q-PCR analysis. The primers of genes including IL-6, TNF-α, COX-2, and β-actin were synthesized by Invitrogen. The primer sequences of mouse genes used are shown as follows: IL-6 sense primer, 5′-CTGCAAGAGACTTC-CATCCAG-3′; IL-6 antisense primer, 5′-AGTGGTATAGA-CAGGTCTGTTGG-3′; TNF-α sense primer, 5′-TCCAGGCGGT-GCCTATGT-3′; TNF-α antisense primer, 5′-CGATCACCCCG-AAGTTCAGT-3′; COX-2 sense primer, 5′-TGCACTATGGTTACAAAAGCTGG-3′; COX-2 antisense primer, 5′-TCAGGAAGCTCCTTATTTCCCTT-3′; 18s sense primer, 5′-AGTCCCTGCCCTTTGTACACA-3′; 18s antisense primer, 5′-CGATCCGAGGGCCTCACTA-3′. The amount of each gene was determined and normalized by the amount of 18s.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra for compounds **1**−**3** and **1a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

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