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Ceramide and Cerebrosides from the Octocoral *Sarcophyton ehrenbergi*[#]

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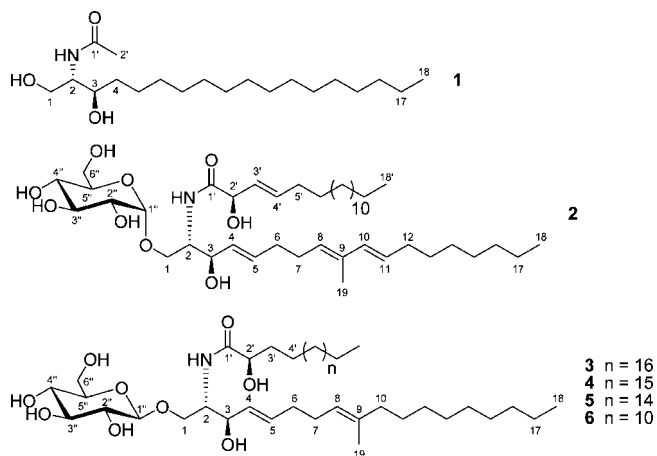
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Chemical investigation of the octocoral *Sarcophyton ehrenbergi*, collected at the Dongsha Islands, Taiwan, has led to the isolation of a known ceramide (**1**) and two new cerebrosides, sarcoehrenosides A (**2**) and B (**4**), along with three known cerebrosides (**3**, **5**, and **6**). The structures of the new compounds were established by spectroscopic and chemical methods. Sarcoehrenoside A (**2**) differs from previously known marine cerebrosides in that it possesses a rare α -glucose moiety. Compounds **1–6** were evaluated for antimicrobial activity against a small panel of bacteria and for anti-inflammatory activity using RAW 264.7 macrophages.

Cerebrosides are a large group of molecules containing a ceramide unit and one or more sugars. The hydrophobic ceramide moiety includes a sphingoid base and an amide-linked fatty acid chain. Cerebroside derivatives have been isolated from various marine invertebrates, such as sea stars,¹ soft corals,² sponges,^{2a,3} sea anemones,⁴ and ascidians.⁵ In recent years several different types of biological activities have been found for these compounds, including antifungal, antitumor, antiviral, cytotoxic, and immunomodulatory properties.⁶

In the course of a search for bioactive substances from marine sources,⁷ chromatographic separation of an alcyonacean soft coral, *Sarcophyton ehrenbergi* Marenzeller (Octocorallia: Alcyonacea), collected at the Dongsha Islands, Taiwan, has afforded two new cerebrosides, sarcoehrenosides A (**2**) and B (**4**). Also obtained were a known ceramide (**1**)⁸ and three known cerebrosides (**3**, **5**, and **6**),^{3a,9,10} which were isolated from *S. ehrenbergi* for the first time. To our knowledge, sarcoehrenoside A (**2**), in possessing a rare α -D-glucose moiety at C-1 of the glycerol ether unit, differs from previous cerebrosides isolated from marine organisms. In this paper, we describe the isolation, structural elucidation, and evaluation of the antimicrobial and anti-inflammatory activity of these metabolites.

Sarcoehrenoside A (**2**) was isolated as a white, amorphous powder and exhibited a $[M + Na]^+$ peak at m/z 774.5490, corresponding to a molecular formula of $C_{43}H_{77}NO_9$ in the positive HRESIMS. The IR absorptions at 1638 and 1547 cm^{-1} and ^{13}C NMR signals at δ 54.6 (C-2) and 175.4 (C-1') suggested a secondary amide in the molecule. In the 1H and ^{13}C NMR spectra of **2**, the characteristic signals of an amide linkage (a nitrogenated methane proton at δ 3.93 and an amide carbonyl carbon at δ 175.4), a long



chain (terminal methyl protons at δ 0.90 and methylene protons at δ 1.29–1.38), and a sugar (an anomeric proton at δ 4.87) were observed, strongly suggesting the glycosphingolipid nature of **2**. The α -glucopyranose moiety was indicated by the anomeric proton at δ 4.87 (1H, d, $J = 4.5$ Hz, H-1'') and the chemical shifts in the 1H and ^{13}C NMR spectra (Table 1). The anomeric proton of the α -glucose unit exhibited a long-range $^1H-^{13}C$ correlation with C-1 (Figure 1), demonstrating attachment of the glucose moiety at C-1. The relative configuration of the α -glucopyranose moiety was determined by a NOESY experiment (Figure 2) and from the 1H NMR J values. In the 1H NMR spectrum, a small coupling constant ($J = 4.5$ Hz)¹¹ was observed for H-1'', which required an equatorial-axial relationship between the anomeric proton (H-1'') and H-2''. The large coupling constants ($J = 7.5$ Hz) between H-2'' and H-3'', H-3'' and H-4'', and H-4'' and H-5'' as well as the NOESY correlations of H-2''/H-1'', H-4'' and H-3''/H-5'' suggested that H-2'', H-3'', H-4'', and H-5'' are all axial. Thus, the relative configuration of the α -glucose moiety at the C-1 glycerol ether could be established. Methanolysis of **2** gave a mixture of α - and β -methyl glucopyranoside. The specific rotation of the methyl glucoside mixture, $[\alpha]_D^{24} +74.3$ (c 0.2, MeOH), was close to that of an

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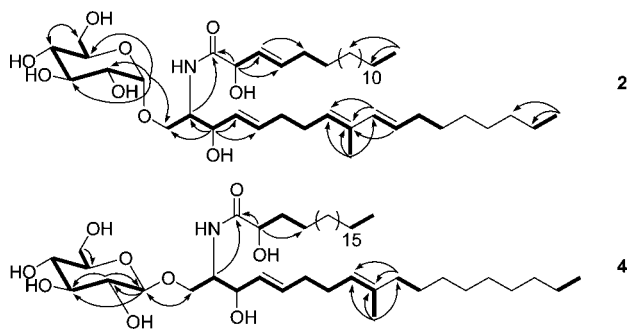
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Table 1. ^1H and ^{13}C NMR Spectroscopic Data of Compounds **2** and **4**^a

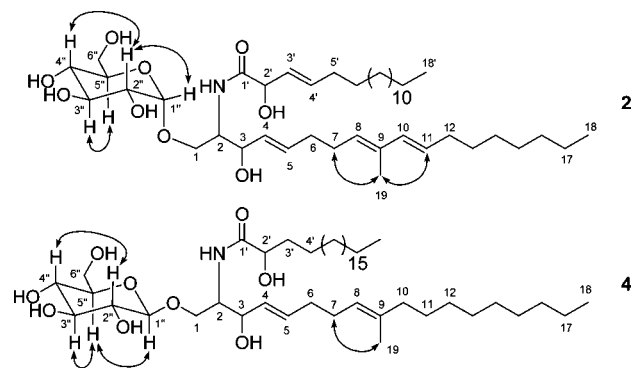
| C/H | 2 | | 4 | |
|--------|---------------------|---|---------------------|---|
| | ^{13}C | ^1H | ^{13}C | ^1H |
| | | lipid base unit | | lipid base unit |
| 1 | 68.8 t ^b | a b | 69.9 t ^b | a b |
| | | 3.88 dd (10.5, 4.5) ^c 3.83 dd (10.5, 4.5) | | 4.12 dd (10.5, 3.5) ^c 3.71 dd (10.5, 3.5) |
| 2 | 54.6 d | 3.93 dd (8.0, 4.5) | 54.7 d | 3.99 dd (7.5, 3.5) |
| 3 | 72.9 d | 4.14 t (8.0) | 73.0 d | 4.13 t (7.5) |
| 4 | 131.6 d | 5.47 dd (15.5, 8.0) | 131.3 d | 5.48 dd (15.5, 7.5) |
| 5 | 134.3 d | 5.72 dd (15.5, 6.5) | 134.8 d | 5.74 dd (15.5, 6.0) |
| 6 | 33.7 t | 2.08 td (7.5, 6.5) | 34.0 t | 2.06 m |
| 7 | 29.0 t | 2.20 td (7.5, 7.0) | 28.8 t | 2.07 m |
| 8 | 130.6 d | 5.35 t (7.0) | 125.0 d | 5.14 t (6.5) |
| 9 | 135.3 s | | 136.9 s | |
| 10 | 136.3 d | 6.02 d (15.5) | 40.9 t | 1.97 t (7.5) |
| 11 | 128.7 d | 5.55 dt (15.5, 7.0) | 29.3 t | 1.39 m |
| 12 | 34.1 t | 2.08 td (7.5, 7.0) | | |
| 13 | 30.4 t | 1.38 m | | |
| 12–15 | | | 30.5–30.9 t | 1.29 m |
| 14, 15 | 30.4–31.1 t | 1.29 m | | |
| 16 | 33.2 t | 1.29 m | 33.2 t | 1.29 m |
| 17 | 23.9 t | 1.29 m | 23.9 t | 1.29 m |
| 18 | 14.6 q | 0.90 t (6.0) | 14.6 q | 0.90 t (6.5) |
| 19 | 12.9 q | 1.71 s | 16.3 q | 1.59 s |
| | | N-acyl unit | | N-acyl unit |
| 1' | 175.4 s | | 177.4 s | |
| 2' | 74.3 d | 4.45 d (6.0) | 73.2 d | 3.99 dd (8.0, 4.0) |
| 3' | 129.1 d | 5.50 dd (15.5, 6.0) | 36.0 t | 1.70 m; 1.55 m |
| 4' | 135.0 d | 5.83 dd (15.5, 6.5) | 26.3 t | 1.41 m |
| 5' | 33.6 t | 2.03 td (7.5, 6.5) | | |
| 5'–19' | | | 30.5–30.9 t | 1.29 m |
| 6' | 30.5 t | 1.38 m | | |
| 7'–15' | 30.4–31.1 t | 1.29 m | | |
| 16' | 33.2 t | 1.29 m | | |
| 17' | 23.9 t | 1.29 m | | |
| 18' | 14.6 q | 0.90 t (6.0) | | |
| 20' | | | 23.9 t | 1.29 m |
| 21' | | | 14.6 q | 0.90 t (6.5) |
| 22' | | | | |
| | | α -D-glucose unit | | β -D-glucose unit |
| 1'' | 103.6 d | 4.87 d (4.5) | 104.9 d | 4.27 d (8.0) |
| 2'' | 79.1 d | 3.96 dd (7.5, 4.5) | 75.1 d | 3.19 dd (9.5, 8.0) |
| 3'' | 76.3 d | 4.10 t (7.5) | 78.1 d | 3.34 m |
| 4'' | 74.4 d | 3.63 m | 71.7 d | 3.30 m |
| 5'' | 83.6 d | 3.69 m | 78.1 d | 3.28 m |
| 6'' | 64.3 t | a b | 62.8 t | a b |
| | | 3.63 m 3.56 m | | 3.87 dd (12.0, 1.5) 3.67 dd (12.0, 5.0) |

^a Spectra were measured in CD_3OD (^1H , 500 MHz; ^{13}C , 125 MHz). ^b Multiplicities were deduced by HSQC and DEPT experiments. ^c J values (in Hz) are in parentheses.

**Figure 1.** Key ^1H – ^1H COSY (—) and HMBC (---) correlations of **2** and **4**.

authentic sample ($[\alpha]_{\text{D}}^{25} +77.3$).^{1f} Therefore, the absolute configuration of the α -glucose moiety was deduced to be the D-isomer.

The large vicinal coupling constants ($J = 15.5$ Hz) between H-4 and H-5, H-10 and H-11, and H-3' and H-4' clearly indicated *E* geometry for $\Delta^{4,10,3'}$ in **2**. The key HMBC correlations from H-3 to C-4, from H-2' to C-3', and from H₃-19 to C-8, C-9, C-10, and C-11 helped locate the positions of the double bonds (Figure 1). The UV absorption maximum at 224 nm indicated the presence of

**Figure 2.** Selected NOESY correlations of **2** and **4**.

a conjugated diene moiety.^{1f} Furthermore, the stereochemistry of the conjugated diene moiety was assigned as *s-trans* from NOE correlations (Figure 2) from Me-19 to H-7 and H-11. The ^1H – ^1H COSY correlations observed between H-2 and both H-1 and H-3 were used to define the 2-amino-1,3-dioxygenated fragment. The relative configurations of C-2 (δ 54.6) and C-3 (δ 72.9) were predicted to be the D-*erythro* stereochemistry at C-2 and C-3, which

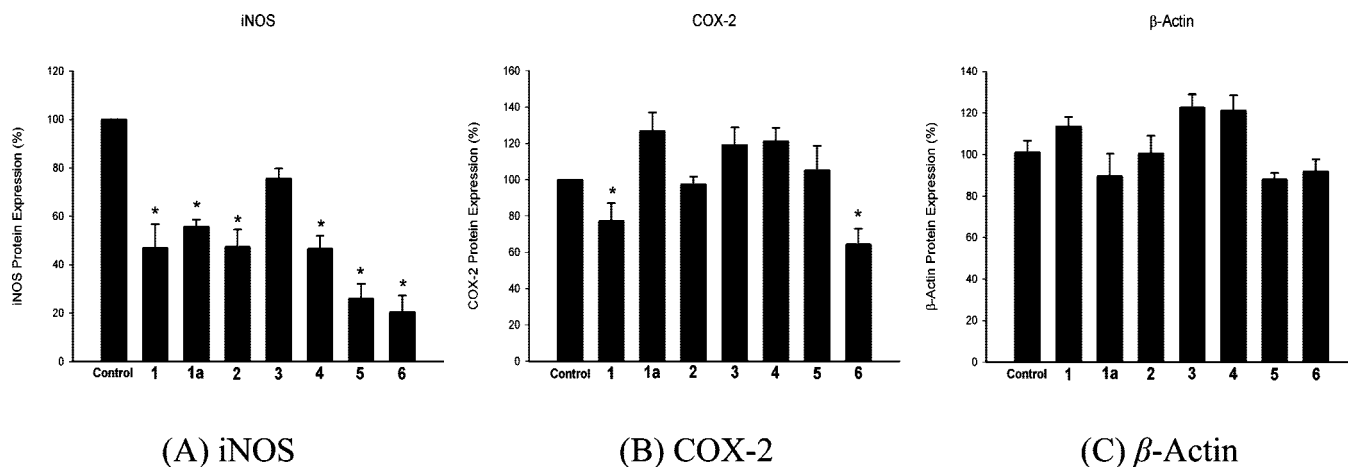


Figure 3. Effect of compounds **1–6** at 10 μ M on the LPS-induced pro-inflammatory iNOS and on COX-2 protein expression of RAW 264.7 macrophage cells by immunoblot analysis. (A) Immunoblot of iNOS. (B) Immunoblot of COX-2. (C) Immunoblot of β -actin. A and B values are mean \pm SEM ($n = 5$). The relative intensity of the LPS alone stimulated group was taken as 100%. *Significantly different from LPS-stimulated (control) group (* $P < 0.05$).

was consistent with those reported for other (2*S*,3*R*,2'*R*) sphingosine moieties.^{12,13} The stereochemistry of **2** (2*S*,3*R*), as shown in its structure, was confirmed after determination of the stereochemistry of **1** on the basis of biosynthetic reasoning. Methanolysis of **2** yielded methyl (2'*R*,3'*E*)-2'-hydroxyoctadec-3'-enoate (**2a**), for which the molecular formula C₁₉H₃₆O₃ was determined by ESIMS-MS (m/z 335.0 [M + Na]⁺) as C₁₉H₃₆O₃. On the basis of the aforementioned observations, sarcoehrenoside A (**2**) was characterized unambiguously as 1-*O*-(α -D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*E*,10*E*)-2-[(2'*R*,3'*E*)-2'-hydroxyoctadec-3'-enoylamino]-9-methyloctadeca-4,8,10-triene-1,3-diol.

Sarcoehrenoside B (**4**) was found to have a molecular formula of C₄₆H₈₇NO₉, as indicated by HRESIMS. In addition to the pseudomolecular ion peak at m/z 820.6 [M + Na]⁺, the ESIMS-MS of **4** also exhibited an intense fragment peak at m/z 496.7, produced by elimination of the *N*-acyl unit from the molecular ion. The spectroscopic data for **4** (Table 1) exhibited the presence of an amide linkage, a long chain, and a sugar, consistent with the C-9 methyl cerebroside nature of **4**. In the ¹H and ¹³C NMR spectra of **4**, an anomeric proton appeared at δ 4.27 (1H, d, $J = 8.0$ Hz, H-1'') and ¹³C NMR signals resonated at δ 104.9 (H-1''), 75.1 (H-2''), 78.1 (H-3''), 71.7 (H-4''), 78.1 (H-5''), and 62.8 (H-6''), supporting the presence of a β -glucopyranose moiety.^{3b} Methanolysis of **4** also yielded an α - and β -glucopyranosyl mixture, the specific rotation ($[\alpha]^{24}_D +71.4$, MeOH) of which was used to identify β -glucose as a D-isomer. The glycerol ether linkage between the anomeric proton (δ 4.27, H-1'') and C-1 was confirmed by HMBC correlations (Figure 1) of H-1'' with C-1 and H-1 with C-1''. The coupling constant (15.5 Hz) indicated an *E* geometry for Δ^4 . A NOE correlation from Me-19 to H-7 supported the presence of an (*E*)-8,9 double bond. Therefore, **3** was defined as a C-9 methyl 4*E*,8*E*-sphingadiene-type cerebroside. The connectivity between the acyl moiety and the NH of the sphingosine base was confirmed by HMBC correlations of H-2 with C-1' and H-2' with C-1'. Methanolysis of **4** yielded methyl (2'*R*)-2'-hydroxyhenicosanoate (**4a**), for which the molecular formula, C₂₂H₄₄O₃, was identified by ESIMS-MS analysis (m/z 379.3 [M + Na]⁺). The specific rotation value ($[\alpha]^{24}_D -3.8$) of **4a** indicated a 2'*R* absolute configuration in **4**.^{1f} The relative configuration at C-2 and C-3 was determined as 2*S*,3*R* (erythro) based on their ¹³C NMR chemical shifts and the specific rotation of this compound.^{1f} Consequently, sarcoehrenoside B (**4**) was fully assigned as 1-*O*-(β -D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*E*)-2-[(2'*R*)-2'-hydroxyhenicosanoylamino]-9-methyl-4,8-octadecadiene-1,3-diol.

Compounds **1–6** were tested against five bacterial strains, comprising *Enterobacter aerogenes* (ATCC13048), *Serratia marce-*

scens (ATCC25419), *Salmonella enteritidis* (ATCC13076), *Yersinia enterocolitica* (ATCC23715), and *Shigella sonnei* (ATCC11060). None of these compounds exhibited any antibacterial activity at a concentration of 100 μ g/disk.

A previous study has reported that glycosphingolipids and sphingolipids possess cyclooxygenase-2 inhibition,¹⁴ which prompted us to evaluate the anti-inflammatory effect of our isolated metabolites. As shown in Figure 3, the in vitro anti-inflammatory activity of compounds **1–6** was tested using LPS-stimulated cells. Stimulation of RAW 264.7 cells with LPS resulted in up-regulation of the pro-inflammatory iNOS and COX-2 proteins. Both compounds **1** and **6** reduced the levels of iNOS to 46.9 \pm 9.7% and 20.3 \pm 6.8%, respectively, and of COX-2 to 77.2 \pm 9.9% and 64.3 \pm 8.6%, respectively, in comparison with those of the control groups. Compounds **1a**, **2**, **4**, and **5** reduced iNOS protein expression (55.6 \pm 2.9%, 47.3 \pm 7.1%, 46.5 \pm 5.3%, and 25.8 \pm 6.2%, respectively), but did not inhibit COX-2 protein expression. All compounds did not affect β -actin protein expression at a 10 μ M concentration. Under the same experimental conditions, 10 μ M CAPE (caffeic acid phenylthyl ester) reduced the levels of iNOS and COX-2 protein to 1.5 \pm 2.1% and 70.2 \pm 11.5%, respectively, relative to the control cells stimulated with LPS.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO P1020 polarimeter. UV spectra were obtained on a Hitachi U-3210 spectrophotometer, and IR spectra were recorded on a JASCO FT/IR-4100 spectrophotometer. The NMR spectra were recorded on a Bruker Avance 300 NMR spectrometer at 300 MHz for ¹H and 75 MHz for ¹³C, or on a Varian MR 400 NMR spectrometer at 400 MHz for ¹H and 100 MHz for ¹³C, or on a Varian Unity INOVA 500 FT-NMR spectrometer at 500 MHz for ¹H and 125 MHz for ¹³C. Chemical shifts are expressed in δ (ppm) referring to the solvent peaks δ_H 3.30 and δ_C 49.0 for CD₃OD and δ_H 7.265 and δ_C 77.0 for CDCl₃, and coupling constants are expressed in Hz. Electrospray ionization MS/MS analysis was recorded using a positive-mode API 4000 tandem mass spectrometer, with a capillary voltage of 5500 V applied, and the spectra were acquired at m/z 400–1000 for parent ion spectra and m/z 50–1000 for fragment ion spectra. ESIMS were recorded by ESI FT-MS on a Bruker APEX II mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) was used for column chromatography; precoated Si gel plates (Merck, Kieselgel 60 F₂₅₄, 0.25 mm) were used for TLC analysis. C₁₈ reversed-phase silica gel (230–400 mesh, Merck) was also used for column chromatography. High-performance liquid chromatography (HPLC) was performed on a Hitachi L-7420 UV detector L-7100 pump apparatus equipped with a Merck Hibar RP-18e column (250 \times 10 mm, 5 μ m).

Animal Material. The octocoral *S. ehrenbergi* was collected by hand using scuba at Dongsha Islands, Taiwan, in April 2007, at a depth of 10 m, and was stored in a freezer for 5 weeks until extraction. This soft coral was identified by one of the authors (C.-F.D.). A voucher specimen (TS-07) was deposited in the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Isolation. Soft coral extracts were prepared by percolating aliquots of 50 g of freeze-dried *S. ehrenbergi* with acetone for 24 h at room temperature. The combined acetone extracts were concentrated to a brown gum, which was partitioned between H₂O and EtOAc. The dried EtOAc-soluble (20.0 g) partition was chromatographed over a silica column using *n*-hexane, *n*-hexane–EtOAc, and EtOAc–MeOH mixtures of increasing polarity to obtain fractions 1–40. Fraction 28 (0.5 g), eluted with EtOAc–MeOH (1:1), was further subjected to RP-18 gravity column chromatography by eluting with 80% MeOH in H₂O, 90% MeOH in H₂O, and 100% MeOH. Altogether, six fractions were obtained, of which fraction 1 (60 mg) was purified further by RP-18 HPLC column chromatography (5% CH₃CN in MeOH, flow rate 5.0 mL/min) to afford **1** (3 mg), **2** (2 mg), **3** (2 mg), **4** (3 mg), **5** (2 mg), and **6** (6 mg). The retention time for each metabolite was as follows: **1** (26.5 min), **2** (35.6 min), **3** (48.6 min), **4** (44.2 min), **5** (41.0 min), and **6** (33.5 min).

Sarcoehrenoside A (2): white, amorphous powder; [α]_D²³ +77.0 (c 0.2, MeOH); IR (KBr) 3387, 2951, 2859, 1638, 1547, 1456, 1387, 1241, 1131, 1035, 737 cm⁻¹; UV λ_{\max} (MeOH) (log ϵ) 224 (3.97) nm; ¹H NMR and ¹³C NMR data, see Table 1; HRESIMS *m/z* 774.5490 [M + Na]⁺ (calcd for C₄₃H₇₇NO₉Na, 744.5496).

Sarcoehrenoside B (4): white, amorphous powder; [α]_D²³ +51.3 (c 0.3, MeOH); IR (KBr) 3396, 2951, 2857, 1638, 1546, 1457, 1385, 1242, 1178, 1131, 1036, 736 cm⁻¹; ¹H NMR and ¹³C NMR data, see Table 1; HRESIMS *m/z* 820.6274 [M + Na]⁺ (calcd for C₄₆H₈₇NO₉Na, 820.6278).

Methanolysis of 2 and 4. An aliquot (1 mg) of **2** and **4** in a mixture of 1 N aqueous HCl (0.1 mL) and methanol (0.9 mL) was refluxed for 18 h on a magnetic stirrer. The reaction mixture was neutralized with NaHCO₃ and diluted with H₂O (1.5 mL). The aqueous solution was extracted with *n*-hexane three times, and the organic phase was dried with anhydrous MgSO₄. After removal of solvent and purification by silica gel column chromatography, methyl (2'*R*,3'*E*)-2'-hydroxyoctadec-3'-enoate (**2a**, 0.3 mg) and methyl (2'*R*)-2'-hydroxyhenicosanoate (**4a**, 0.4 mg) were obtained and identified by ESIMS-MS peaks at *m/z* 335.0 and 379.3 [M + Na]⁺, respectively. In addition, the aqueous layer was removed and purified on a C₁₈ reversed-phase column, eluted with MeOH–H₂O (4:1), to give **2b** [0.2 mg, [α]_D²⁴ +74.3 (c 0.2, MeOH)] and **4b** [0.2 mg, [α]_D²⁴ +72.5 (c 0.2, MeOH)].

Methyl (2'*R*,3'*E*)-2'-hydroxyoctadec-3'-enoate (2a): white, amorphous powder; [α]_D²⁴ -17.5 (c 0.3, CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ 5.90 (1H, dt, *J* = 15.6, 6.4 Hz, H-4'), 5.50 (1H, dd, *J* = 15.6, 6.4 Hz, H-3'), 4.61 (1H, d, *J* = 6.4 Hz, H-2'), 3.80 (3H, s, COOCH₃), 2.07 (2H, td, *J* = 7.6, 6.4 Hz, H-5'), 1.39 (2H, m, H-6'), 1.26 (22H, brs, H-7'-H-17'), 0.88 (3H, t, *J* = 6.4 Hz, H-18'); ESIMS-MS *m/z* 335.0 [M + Na]⁺.

Methyl (2'*R*)-2'-hydroxyhenicosanoate (4a): white, amorphous powder; [α]_D²⁴ -3.8 (c 0.4, CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ 4.20 (1H, dd, *J* = 7.6, 4.4 Hz, H-2'), 3.79 (3H, s, COOCH₃), 1.76 (1H, m, H-3a'), 1.58 (1H, m, H-3b'), 1.45 (2H, m, H-4'), 1.26 (32H, brs, H-5'-H-20'), 0.88 (3H, t, *J* = 6.4 Hz, H-21'); ESIMS-MS *m/z* 379.3 [M + Na]⁺.

In Vitro Antimicrobial Activity. Bacterial strains were grown in LB (Luria–Bertani) broth medium for 24 h at 37 °C. Then, 17 mL of LB hard agar (1.5% agar) was poured into sterile Petri dishes (9 cm) and allowed to set. Next, 2.7 mL of molten LB soft agar (0.7% agar, 45 °C) was inoculated with 0.3 mL of broth culture of the test organism and poured over the base hard agar plates, forming a homogeneous top layer. Sterile paper disks (Advantec, 8 mm) were placed onto the top layer of the LB agar plates. Ten microliters (2 μ g/ μ L) of the tested compounds was applied onto each filter paper disk. Ampicillin (5 μ g/ μ L) and the same solvents served as positive and negative controls. All plates were incubated at 37 °C, 24 h prior to antibacterial activity evaluation. The antimicrobial activity of compounds **1–6** was tested up to 100 μ g/mL against *E. aerogenes* (ATCC13048), *S. marcescens* (ATCC25419), *S. enteritidis* (ATCC13076), *Y. enterocolitica* (ATCC23715), and *S. sonnei* (ATCC11060). All bacterial strains were obtained from the American Type Culture Collection. The antibiotic activity evaluation method was conducted on the basis of previous reports.¹⁵

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). The cells were activated by incubation in medium containing *Escherichia coli* LPS (0.01 μ g/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 centrifuged at 20000g 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 μ m 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's instructions.

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