Simplexins A–I, Eunicellin-Based Diterpenoids from the Soft Coral *Klyxum simplex*

Shwu-Li Wu,†‡ Jui-Hsin Su,* Zhi-Hong Wen,† Chi-Hsin Hsu,* Bo-Wei Chen,* Chang-Feng Dai,* ‡ Yao-Haur Kuo,‡ and Jyh-Hong Sheu*††

Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 804, Taiwan, Republic of China, Center of General Studies, National Kaohsiung Marine University, Kaohsiung 811, Taiwan, Republic of China, Department of Biological Science and Technology, Mei-Ho Institute of Technology, 23 Ping Kuang Road, Neihu Hsiung, Pingtung 912, Taiwan, Republic of China, Institute of Oceanography, National Taiwan University, Taipei 112, Taiwan, Republic of China, National Research Institute of Chinese Medicine, Taipei 112, Taiwan, Republic of China, and Asia-Pacific Ocean Research Center, National Sun Yat-sen University, Kaohsiung 804, Taiwan, Republic of China

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Nine new eunicellin-based diterpenoids, simplexins A–I (1–9), were isolated from a Dongsha Atoll soft coral, *Klyxum simplex*. The structures of these compounds were established by detailed spectroscopic analysis (IR, MS, 1D and 2D NMR) and by comparison with the physical and spectral data of related known compounds. The absolute configuration of 1 was determined by a modified Mosher’s method. Compounds 1, 4, and 5 were found to be cytotoxic toward a limited panel of cancer cell lines. Compound 5 was shown to significantly inhibit the accumulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophage cells.

The first eunicellin-based diterpenoid was isolated from the Mediterranean gorgonian *Eunicella singularis* in 1968. Since then, many eunicellin-based diterpenoids were isolated from various marine organisms, and some of these metabolites were shown to possess interesting biological activities (e.g., anti-inflammatory, cytotoxic, inhibition of cell division of fertilized starfish eggs, hemolytic activity, antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum*, and antibacterial activity against *Mycobacterium tuberculosis*). We have previously isolated several eunicellin-based diterpenoids, named australins A–D,4 vigulariol,8 and pachyclavulariaenones A+G,10,14 from soft corals. Our study on the chemical constituents of a Dongsha Atoll soft coral, *Klyxum simplex* Thomson and Dean (phylum Cnidaria, class Anthozoa, order Alocynacea, family Alcyoniidae), has yielded nine new eunicellin-based diterpenoids, simplexins A–I (1–9).

These compounds possess the more common C-2, C-9-ether linkage, characteristic of the eunicellin-based diterpenoids. The molecular structures of these compounds, including their relative configurations, were established by detailed spectroscopic analysis and by comparison with the physical and spectroscopic data of related known compounds. The absolute structure of 1 was determined by using a modified Mosher’s method. The cytotoxicity of compounds 1–9 against human cervix cancer (MCF-7), human cervical epitheloid (HeLa), and human cervix epithelial (H-35) cell lines was determined.

Results and Discussion

Simplexin A (1) was obtained as a colorless oil. The HRESIMS (m/z 473.2879 [M + Na]+) of 1 established a molecular formula of C26H42O6Na, appropriate for six degrees of unsaturation. The IR absorptions at νmax 3432 (broad) and 1723 cm−1 revealed the presence of hydroxy and carbonyl functionalities. The 13C NMR spectrum measured in CDCl3 showed signals of 26 carbons (Table 1), which were assigned by the assistance of the DEPT spectrum to six methyls, eight methylenes, seven methines (including three oxyxmethines), two carbonyls, two sp2 oxygenated quaternary carbons, and one sp2 quaternary carbon of an olefinic group. The NMR spectroscopic data of 1 (Tables 1 and 3) showed the appearance of a 1,1-disubstituted double bond (δC 116.8, CH2, and 150.3, qC; δH 5.21, s, and 5.47, s). The presence of one acetoxy group was indicated by the 1H NMR signal at δ 2.01 (3H, t, J = 7.5 Hz), 1.60 (2H, t, J = 7.5 Hz), and 2.16 (2H, t, J = 7.5 Hz). Therefore, the remaining three degrees of unsaturation identified compound 1 as a tricyclic molecule. Furthermore, the 1H NMR data of 1 showed two secondary methyls (δ 0.78 and 0.95, 3H each, d, J = 7.0 Hz) of an isopropyl moiety. Inspection of the HMBC spectrum showed that proton signals appearing at δ 2.25 (1H, dd, J = 12.0, 7.1 Hz), 3.06 (1H, br t, J = 8.8 Hz), 3.57 (1H, s), and 4.10 (1H, dd, J = 10.5, 4.5 Hz) were correlated to two ring-junctue methine carbons at δ 41.5 and 46.1 and two oxymethylene carbons at δ 90.5 and 78.8, respectively. The gross structure of 1 was further established by 2D NMR experiments, especially by analysis of 1H–1H COSY and HMBC correlations (Figure 1). The 1H−1H COSY experiment assigned two isolated consecutive proton spin systems. One was found to extend from H2-8 to both H-12 and the isopropyl moiety through H-14. The other was shown to extend from H3-20 to H-2, 36). The HMBC correlations observed from H-2 to C-1, C-9, C-10, and C-14 and H-9 to C-7 and C-11 established the 2,9-ether linkage of the tetrahydrofuran moiety. On the basis of the above observations, compound 1 possessed the common C-2, C-9-ether linkage characteristic of eunicellin-based compounds. Furthermore, the position of the n-butyryloxy group attached at C-3 was confirmed from the NOE correlations (Figure 2) between H1-20 (δ 0.78) and H-18 (δ 1.86) with the α-methylene protons of the n-butyryloxy group (δ 2.16). From the above results, the structure of compound 1 was shown to be very similar to that of a known compound, palmonine F (10).7 The relative configuration of 1 was confirmed by the same as that of 10 by comparison.
of the chemical shifts and coupling constants for protons of both compounds and was further confirmed by NOE correlations (Figure 2). The structure of 1 was thus found to possess the \((1R^*, 2R^*, 3R^*, 6S^*, 9R^*, 10S^*, 11R^*, 14R^*)\) configuration. The absolute structure of 1 was finally determined by using a modified Mosher's esterification method.\(^{15}\) The \((S)-\) and \((R)-\)MTPA esters of 1 (1a and 1b, respectively) were prepared by using the corresponding \((R)-(-)\)- and \((S)-(+)-\)MTPA chloride,

### Table 1. \(^{13}\)C NMR Data for Compounds 1–6<sup>a</sup>

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<sup>a</sup> Spectra recorded at 125 MHz in CDCl<sub>3</sub> at 25 °C. <sup>b</sup> Attached protons were determined by DEPT experiments.
Table 2. $^{13}$C NMR Data for Compounds 7–9

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<sup>a</sup>Spectra recorded at 125 MHz in CDCl<sub>3</sub> at 25 °C. <sup>b</sup>Attached protons were determined by DEPT experiments.

respectively. The determination of Δδ values ($\delta_S - \delta_R$) for protons neighboring C-6 led to the assignment of the S configuration at C-6 in 1 (Figure 3).

The HRESIMS of simplexin B (2) exhibited a [M + Na]<sup>+</sup> peak at m/z 491.2987 and established a molecular formula of C<sub>39</sub>H<sub>54</sub>O<sub>11</sub>, implying five degrees of unsaturation. The NMR spectroscopic data (Tables 1 and 3) of 2 showed the presence of two ester carbonyls (δ 170.1, qC and 172.6, qC), which were HMBC correlated with the acetate methyl (δ 1.99, 3H, s) and methylenes (δ 1.68, 2H, t, J = 7.5, 7.5 Hz, 2.30, m and 2.35, m) of an n-butyrate, respectively. By comparison of the NMR data of 2 with those of 1 (Tables 1 and 2), it was found that a C-7/C-16 double bond in 1 was replaced by an oxymethine bearing a methyl and a hydroxy group in 2, as confirmed by HMBC connectivities observed from H-16 (δ 1.16, 1H, t, J = 8.0 Hz, 3H, s) to C-6 (δ 80.6, CH<sub>3</sub>), C-7 (δ 77.1, qC), and C-8 (δ 47.6, CH<sub>2</sub>). A more detailed analysis of the 1H and 13C NMR spectroscopic data and the detected 2D correlations in the 1H−1H COSY and HMBC spectra led to the establishment of the gross structure of 2 (Figure 1). The relative configurations of all chiral centers except C-7 of 2 were confirmed to be the same as those of 1 by comparison of the proton shifts, coupling constants, and NOE correlations (Figure 2). H-16 was found to exhibit an NOE correlation with H-5β but not with H-6, revealing the α-orientation of hydroxy group at C-7. Thus, the structure of diterpenoid 2 was established.

Simplexin C (3) showed the pseudomolecular ion peak [M + Na]<sup>+</sup> at m/z 635.3409 in the HRESIMS, corresponding to the molecular formula C<sub>36</sub>H<sub>54</sub>O<sub>11</sub> and seven degrees of unsaturation. The 13C NMR spectrum measured in CDCl<sub>3</sub> showed signals of 32 carbons (Table 1), which were assigned by the assistance of the DEPT spectrum to nine methyls, seven methylenes, nine methines (including five oxymethines), four carboxyls, and three sp<sup>3</sup> oxygenated quaternary carbons. The presence of two acetoxy groups was indicated by the 1H NMR signals at δ 2.05 (s, 3H) and 2.07 (s, 3H) and the 13C NMR signals at δ 20.7 (CH<sub>3</sub>), 21.4 (CH<sub>3</sub>), 169.9 (qC), and 171.9 (qC). In addition, the 1H and 13C NMR spectra of 3 showed the presence of two n-butyroxy groups. Among them one showed signals at δ<sub>R</sub> 0.97 (3H, t, J = 7.5 Hz), 1.67 (2H, m), 2.25 (1H, m), 2.37 (1H, m) and δ<sub>S</sub> 13.8 (CH<sub>3</sub>), 18.2 (CH<sub>3</sub>), 37.2 (CH<sub>2</sub>), 172.2 (qC), and the other resonated at δ<sub>R</sub> 0.92 (3H, t, J = 7.5, 7.5 Hz), 1.60 (2H, sex, J = 7.5 Hz), 2.19 (2H, dt, J = 2.0, 7.5 Hz) and δ<sub>S</sub> 13.6 (CH<sub>3</sub>), 18.1 (CH<sub>2</sub>), 36.6 (CH<sub>3</sub>), 172.9 (qC). Therefore, the remaining three degrees of unsaturation identified compound 3 as a tricyclic diterpenoid. The molecular framework was established by 1H−1H COSY and HMBC experiments (Figure 1). The placement of acetates at C-6 and C-12 was confirmed from the HMBC connectivities of acetate methyls (δ 2.07 and 2.05), H-6 (δ 5.58) and H-12 (δ 5.00) with the carbonyl carbons resonating at δ 171.9 (qC) and 169.9 (qC), respectively. Also, the locations of two n-butyroxy groups at C-3 and C-13 were proven from the HMBC connectivities from H-2 (δ 3.53) and H-13 (δ 5.49) to the carbonyl carbons resonating at δ 172.2 (qC) and 172.9 (qC), respectively. The upfield chemical shifts for H-3 (δ 5.58) and H-12 (δ 5.00) were determined by NOE correlations between H-5β and H-6, revealing the α-orientation of hydroxy group at C-7. Therefore, the gross structure of 3 was established. The relative configuration of compound 3 was determined from analysis of correlations observed in the NOESY spectrum (Figure 2), which exhibited NOE correlations between H-12 and H-9, and H-13 and H-1, and established the α-orientation
of H-12 and the \( \beta \)-orientation of H-13. By comparison of the NMR spectroscopic data of 2 and 3 and by detailed analysis of other key NOE correlations (Figure 2), the structure of compound 3 was determined unambiguously.

Simplexin D (4) was found to have the molecular formula C\(_{33}\)H\(_{52}\)O\(_{11}\), as indicated from the HRESIMS (m/z 663.3718 [M + Na\(^{+}\)]) and NMR data (Tables 1 and 3). Comparison of the NMR data of 4 with those of 3 revealed the replacement of one acetoxy group (\( \delta_{H} 2.07, 3 \mathrm{H}, \delta_{C} 171.9, q \mathrm{C} \) and 21.4, CH\(_3\)) in 3 by an n-butyryloxy group in 4. The \( \delta_{H} 2.32, 2 \mathrm{H}, t (7.5) \) and 2.16, 2H, t (7.5) resonances in 4 showed the presence of two acetoxy and one n-butyryloxy group. The correlations observed in the NOESY spectrum of 4 also showed that the configuration of this metabolite is identical with that of 3. Thus, simplexin D (4) was found to be the 6-deacetyl-6-O-n-butyryl derivative of 3.

Simplexin E (5) was obtained as a colorless oil. On the basis of its HRESIMS (m/z 647.3410 [M + Na\(^{+}\)]) along with the \( ^1\)H and \( ^{13}\)C NMR spectroscopic data (Tables 1 and 3), the molecular formula of 5 was established as C\(_{34}\)H\(_{56}\)O\(_{11}\), consistent with eight degrees of unsaturation. The IR spectrum of 5 showed the presence of hydroxy (\( \nu_{\text{max}} 3478 \text{ cm}^{-1} \)), carbonyl (\( \nu_{\text{max}} 1733 \text{ cm}^{-1} \)) and double-bond (\( \nu_{\text{max}} 1636 \text{ cm}^{-1} \)) functionalities. Comparison of its \( ^1\)H NMR (Table 1) and \( ^{13}\)C NMR (Table 3) data with those of 3 revealed that an acetate in 3 was replaced by an acrylate (\( \delta_{H} 5.84, \delta_{C} 166.9, q \mathrm{C} \)) in 5. The attachment of this acrylate at C-6 was confirmed by the HMBC correlation between H-6 and the carbonyl carbon (\( \delta_{C} 174.5, q \mathrm{C} \)).

Simplexin F (6) was also isolated as a white powder. Its molecular formula, C\(_{28}\)H\(_{46}\)O\(_{9}\), determined unambiguously.

Simplexin G (7) was obtained as a white powder. Its molecular formula, C\(_{28}\)H\(_{46}\)O\(_{10}\), was established by HRESIMS (m/z 565.2987 [M + Na\(^{+}\)]). The \( ^1\)H and \( ^{13}\)C NMR data (Tables 1 and 4) revealed that 6 is simply the 13-O-debutyryl derivative of 3.

Simplexin H (8) was isolated as a white powder and has a molecular formula of C\(_{28}\)H\(_{46}\)O\(_{10}\), appropriate for six degree of unsaturation. Comparison of the NMR data of 8 with those of 2 showed that the methylene group of C-13 in 2 was converted to an oxymethylene group bearing a hydroxy group (\( \delta_{H} 3.88, 1 \mathrm{H} \) m; \( \delta_{C} 66.4, \mathrm{CH} \)) in 8. Also, H-6 and C-6 of 8 were downfield shifted to \( \delta \) 5.60 and 85.0, respectively, in comparison with those of 2. Thus, an acetoxy group was attached at C-6. This was further confirmed by the HMBC correlations from H-6 and the methyl protons of an acetate (\( \delta_{H} 2.08, 3 \mathrm{H}, s \)) to a carbonyl carbon (\( \delta \) 172.0, qC). Thus, the planar structure of 8 was fully established.

Table 3. \( ^1\)H NMR Data for Compounds 1–5

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<th>4</th>
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<td>3.55, s</td>
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<td>1.57, m</td>
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<tr>
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<td>1.46, m</td>
<td>1.40, m</td>
<td>1.41, m</td>
<td>1.47, dd (10.5, 5.5)</td>
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<td>1.12, 3H, s</td>
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<td>1.69, m</td>
<td>1.71, m</td>
<td>1.74, m</td>
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<td>0.96, 3H, d (7.0)</td>
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</table>

*a Spectra recorded at 500 MHz in CDCl\(_3\) at 25 °C. b The J values are in Hz in parentheses.*
Hep2) was studied. The results (Table 5) showed that human cervical epithelioid carcinoma (HeLa), and human laryngeal medulloblastoma (Daoy), human breast adenocarcinoma (MCF-7), and human esophageal adenocarcinoma (ACH-2) showed weak cytotoxicity toward the above four cancer cell lines, and compounds I and 4 displayed weak cytotoxicity toward Daoy and MCF-7 cancer cell lines.

The in vitro anti-inflammatory effects of compounds 1–6 and 9 were tested. In this assay, the up-regulation of the pro-inflammatory iNOS and COX-2 proteins of LPS-stimulated RAW264.7 macrophage cells was evaluated using immunoblot analysis. At a concentration of 10 µM, compound 5 was found to significantly reduce the levels of iNOS and COX-2 proteins to 4.8 ± 1.8% and 37.7 ± 4.7%, respectively, relative to the control cells stimulated with LPS only. At the same concentration, metabolites 1 and 4 did not inhibit the COX-2 expression, but could significantly reduce iNOS expression (15.9 ± 14.5% and 37.7 ± 7.2%, respectively) by LPS treatment (Figure 4).

**Experimental Section**

**General Experimental Procedures.** Melting points were determined using a Fisher-Johns melting point apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. The UV spectrum of 5 was taken in MeOH on a Hitachi U-3210 UV spectrophotometer. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. ESIMS were obtained with a Bruker APEX II mass spectrometer. The NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR at 500 MHz for 1H and 125 MHz for 13C or on a Varian 400 MR FT-NMR at 400 MHz for 1H and 100 MHz for 13C, respectively, in CDCl3 using TMS as an internal standard. Si gel 60 (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F254, 0.2 mm) were used for analytical TLC. High-performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with a Merck Hlibar Si-60 column (250 × 21 mm, 7 µm).

**Animal Materials.** *Klyssum simplex* (230 g, wet wt) was collected by hand using scuba off the coast of Dongsha Atoll, in September 2006, at a depth of 10.9 m, and stored in a freezer until extraction. A voucher sample (specimen no. 20060901-1) was deposited at the Department of Marine Biotechnology and Resources, Sun Yat-sen University.

**Extraction and Isolation.** The frozen bodies of *K. simplex* (230 g, wet wt) were minced and exhaustively extracted with EtOAc (1 L × 4). The organic extract was evaporated under reduced pressure to give a residue (2.5 g), which was subjected to Si gel column chromatography and eluted with EtOAc in n-hexane (0–100%, gradient) to yield 22 fractions. Fractions 10–12 (1.05 g), eluted with EtOAc–n-hexane (1:3), were further purified over silica gel using EtOAc–n-hexane (1:3 to 1:1) to afford 46 subfractions. Subfraction 13 was separated by normal-phase HPLC using acetone–n-hexane (1:1) to afford 7 (1.6 mg), subfraction 27 was purified by normal-phase HPLC using acetone–n-hexane (1:5) to afford 1 (4.6 mg), subfraction 34 was also purified by normal-phase HPLC using acetone–n-hexane (1:2) to afford 2 (0.5 mg), and subfraction 46 was purified by normal-phase HPLC using acetone–n-hexane (1:10) to afford 3 (0.2 mg).

The cytotoxicity of compounds 1–6 and 9 against the proliferation of a limited panel of cancer cell lines, including human medulloblastoma (Daoy), human breast adenocarcinoma (MCF-7), human cervical epithelioid carcinoma (HeLa), and human laryngeal carcinoma (Hep2) was studied. The results (Table 5) showed that compounds 2, 3, 6, and 9 are not cytotoxic toward the above cancer cells. Compound 5 exhibited moderate to weak cytotoxicity toward the above four cancer cell lines, and compounds 1 and 4 displayed weak cytotoxicity toward Daoy and MCF-7 cancer cell lines.

Simplexin I (9) was assigned the molecular formula C25H42O9 from its HRESIMS data. Thus, six degrees of unsaturation were determined for 9. NMR spectroscopic data of 9 (Tables 2 and 4) showed the presence of three acetoxy groups (δC 169.8, qC; 170.1, qC; 172.0, qC; 21.4, CH3; 22.2, CH3 and 22.5, CH3; δH 2.00, 3H, s and 2.10, 3H, s). Comparison of the NMR data of 9 with those of 8 revealed the only difference between both compounds arises from the replacement of the n-butyryloxy moiety at C-3 in 8 by an acetoxy group in 9.

The H NMR chemical shift differences Δδ (δR − δS) in ppm for the MTPA esters of 1.
Table 5. Cytotoxicity (ED50 µg/mL) of Compounds 1–6 and 9

<table>
<thead>
<tr>
<th>Compound</th>
<th>Daoy</th>
<th>MCF-7</th>
<th>HeLa</th>
<th>Hep2</th>
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<td>(--)</td>
<td>(--)</td>
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<tr>
<td></td>
<td>0.09</td>
<td>0.14</td>
<td>0.08</td>
<td>0.02</td>
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</table>

*a* Spectra recorded at 500 MHz in CDCl3 at 25 °C. *b* J values are (in Hz) in parentheses.

purified by normal-phase HPLC using acetone–n-hexane (1:3) to afford 4 (10.1 mg), and subfraction 37 was also separated by normal-phase HPLC using acetone–n-hexane (1:2) to give 5 (10.4 mg), 8 (2.8 mg), and 9 (8.0 mg). Fractions 13–15 (0.47 g), eluted with EtOAc–n-hexane (1:1), were further purified over silica gel using EtOAc–n-hexane (1:1) to afford 19 subfractions. Subfractions 17 and 19 were separated by normal-phase HPLC using acetone–n-hexane (1:2) to yield 2 (8.0 mg) and 6 (9.6 mg), respectively. Fractions 16–19 (0.51 g), eluted with EtOAc–n-hexane (2:1), were further purified over silica gel using EtOAc–n-hexane (2:1) to afford four subfractions. Subfraction 2 was separated by normal-phase HPLC using MeOH–CHCl3 (1:3) to afford 3 (37.2 mg).

**Simplexin A (1):** colorless oil (4.6 mg); [α]D26 +8.9 (c 0.8, CHCl3); IR (neat) νmax 3438 (broad) and 1723 cm⁻¹; H and 13C NMR data, see Tables 1 and 3; ESIMS m/z 473 [100, (M + Na)⁺]; HRESIMS m/z 473.2879 (calcd for C26H42O9Na, 473.2877).

**Simplexin B (2):** colorless oil (8.0 mg); [α]D25 +26 (c 0.7, CHCl3); IR (neat) νmax 3444 (broad) and 1731 cm⁻¹; H and 13C NMR data, see Tables 1 and 3; ESIMS m/z 491 [100, (M + Na)⁺]; HRESIMS m/z 491.2987 (calcd for C26H42O9Na, 491.2985).

**Simplexin C (3):** white powder (37.2 mg); mp 93.0–94.0 °C; [α]D26 +29.7 (c 0.64, CHCl3); IR (KBr) νmax 3478 (broad) and 1732 cm⁻¹; H and 13C NMR data, see Tables 1 and 3; ESIMS m/z 635 [100, (M + Na)⁺], 381 (21); HRESIMS m/z 635.3409 (calcd for C35H54O13Na, 635.3407).

**Simplexin D (4):** colorless oil (10.1 mg); [α]D25 +9.0 (c 1.0, CHCl3); IR (neat) νmax 3489 (broad) and 1732 cm⁻¹; H and 13C NMR data; see Tables 1 and 3; ESIMS m/z 663 [100, (M + Na)⁺]; HRESIMS m/z 663.3718 (calcd for C36H56O14Na, 663.3720).

**Simplexin E (5):** colorless oil (10.4 mg); [α]D25 +14.0 (c 2.3, CHCl3); IR (neat) νmax 3478 (broad), 1733 and 1636 cm⁻¹; UV (MeOH) λmax 216 (log ε = 3.50); H and 13C NMR data, see Tables 1 and 3; ESIMS m/z 647 [100, (M + Na)⁺]; HRESIMS m/z 647.3410 [M + Na]⁺ (calcd for C28H46O9Na, 647.3407).

**Simplexin F (6):** white powder (9.6 mg); mp 113.0–113.5 °C; [α]D26 +18.1 (c 2.68, CHCl3); IR (neat) νmax 3450 (broad) and 1728 cm⁻¹; H and 13C NMR data, see Tables 1 and 3; ESIMS m/z 565 [100, (M + Na)⁺]; HRESIMS m/z 565.2987 (calcd for C28H46O9Na, 565.2989).

**Simplexin G (7):** white powder (1.6 mg); mp 101.5–102.5 °C; [α]D26 +34 (c 0.4, CHCl3); IR (neat) νmax 3445 (broad) and 1734 cm⁻¹; H and 13C NMR data, see Tables 2 and 3; ESIMS m/z 549 [100, (M + Na)⁺], 467 (16); HRESIMS m/z 549.3042 (calcd for C28H46O9Na, 549.3039).

**Simplexin I (9):** white powder (8.0 mg); mp 85.5–86.0 °C; [α]D25 +28.6 (c 1.5, CHCl3); IR (neat) νmax 3452 (broad) and 1728 cm⁻¹; H and 13C NMR data, see Tables 2 and 3; ESIMS m/z 521 [100, (M + Na)⁺], 439 (23), 381 (37), 353 (11); HRESIMS m/z 521.2727 (calcd for C28H46O9Na, 521.2726).

**Preparation of (S)- and (R)-MTPA Esters of 1.** To a solution of 1 (2.0 mg) in pyridine (100 µL) was added R-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 µL), and the solution was allowed to stand overnight at room temperature. The reaction mixture was added to 1.0 mL of H2O, followed by extraction with EtOAc (1.0 mL × 3). The EtOAc-soluble layers were combined, dried over anhydrous MgSO4, and evaporated. The residue was purified by a short silica gel column using acetone–n-hexane (1:6) to yield the (S)-MTPA ester 1a (1.2 mg). The same procedure was applied to obtain the (R)-MTPA ester 1b (0.8 mg) from the reaction of S-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride with 1. Selected 1H NMR (CDCl3, 400 MHz) data of 1a: δ 5.414 (1H, s, H-16a), 5.388 (1H, d, J = 3.2 Hz, H-6), 5.303 (1H, s, H-16b), 4.109 (1H, dd, J = 10.6, 5.2 Hz, H-8), 3.146 (1H, dd, J = 14.0, 5.2 Hz, H-6), 2.526 (1H, d, J = 14.0 Hz, H-8), 2.179 (1H, m, H-4a), 2.026 (1H, m, H-5a), 1.895 (1H, m, H-5b), 1.854 (1H, m, H-4b), 1.524 (3H, s, Me). Selected 13C NMR (CDCl3, 400 MHz) data of 1a: δ 170.7 (C-16), 134.5 (C-8), 133.0 (C-7), 132.9 (C-15), 130.6 (C-13), 129.1 (C-14, C-26), 128.2 (C-17), 127.7 (C-6), 127.6 (C-27), 127.5 (C-28), 124.5 (C-19), 123.2 (C-1), 114.5 (C-4, C-25), 55.0 (C-16a), 54.9 (C-16b), 54.8 (C-17a), 54.7 (C-17b), 20.5 (C-19a), 20.4 (C-19b).
NMR (CDCl₃, 400 MHz) data of 1b:
δ 5.366 (1H, dd, J = 13.6, 3.6 Hz, H-6), 5.206 (1H, s, H-16a), 5.198 (1H, s, H-16b), 4.100 (1H, dd, J = 10.6, 5.2 Hz, H-9), 3.184 (1H, dd, J = 13.6, 5.2 Hz, H-8), 2.509 (1H, d, J = 13.6 Hz, H-8), 2.189 (1H, m, H-4a), 2.100 (1H, m, H-5a), 1.900 (1H, m, H-5b), 1.870 (1H, m, H-4b), 1.536 (3H, s, Me-15).

Cytotoxicity Testing. Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of compounds 1–6 and 9 were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. In Vitro Anti-inflammatory Assay. Assay procedure was as previously reported. Acknowledgment. Financial support was provided by Ministry of Education (96-C031702) and National Science Council of Taiwan (NSC 95-2113-M-110-011-MY3) awarded to J.-H.S. Supporting Information Available: The ¹H and ¹³C NMR spectra of 1–9 are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

Figure 4. Effect of compounds 1–6 and 9 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 conditions CAPE (caffeic acid phenylethyl ester, 10 µM) reduced the levels of the iNOS and COX-2 to 2.5 ± 3.7% and 67.2 ± 13.4%, respectively. *Significantly different from LPS alone stimulated group (*P < 0.05). a Stimulated with LPS. b Stimulated with LPS in the presence of 1–6 and 9 (10 µM).