

Simplexins J–O, Eunicellin-Based Diterpenoids from a Dongsha Atoll Soft Coral *Klyxum simplex*

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Six new eunicellin-based diterpenoids, simplexins J–O (**1**–**6**), were isolated from a Dongsha Atoll soft coral, *Klyxum simplex*. The structures of these compounds were established by detailed spectroscopic analysis and by comparison with spectroscopic data of related known compounds. Compounds **4**–**6** have been found to significantly inhibit the accumulation of the pro-inflammatory iNOS protein in LPS-stimulated RAW264.7 macrophage cells.

During the course of our search for bioactive metabolites from marine invertebrates of Taiwanese waters, several eunicellin-type compounds also have been isolated from wild-type octocorals *Pachyclavularia violacea*,[1,2] *Cladiella australis*,[3] *Cladiella hirsuta*,[4] *Vigularia juncea*,[5] and a cultured octocoral *Klyxum simplex* by our group.[6–8] Recently, we have isolated a series of new eunicellin-based diterpenoids, simplexins A–I, from a Dongsha Atoll soft coral *Klyxum simplex* Thomson and Dean (Alcyonacea, Alcyoniidae).[9] Our continuing investigation on the chemical constituents of the EtOAc extract of this soft coral has again afforded six new eunicellin-based diterpenoids, simplexins J–O (**1**–**6**) (Chart 1). The molecular structures of these compounds were established by detailed spectroscopic analysis and by comparison with spectroscopic data of other known compounds. The cytotoxicity of compounds **4**–**6** against human medulloblastoma (Daoy), human breast adenocarcinoma (MCF-7), human cervical epitheloid carcinoma (HeLa), and human laryngeal carcinoma (HEp2) cell lines was studied, and the ability of **4**–**6** to inhibit upregulation of the pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells was also evaluated, in the search for bioactive marine natural products.

Simplexin J (**1**) was obtained as a white powder. The HR-ESI-MS (m/z 635.3404 $[M + Na]^+$) of **1** established a molecular formula $C_{32}H_{52}O_{11}$, appropriate for seven degrees of unsaturation. The IR absorptions bands at ν_{max} 3471 and 1734 cm^{-1} revealed the presence of hydroxy and ester carbonyl functionalities. The ^{13}C NMR spectrum measured in $CDCl_3$ 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 carbonyls, and three oxygenated quaternary carbons. The presence of two acetate groups were indicated by the ^1H NMR spectroscopic data (Table 2) at δ 2.09 (s, 3H) and 1.98 (s, 3H) and the ^{13}C NMR signals at δ 21.4 ($2CH_3$), 170.3 (C), and 172.0 (C). In addition, the ^1H NMR spectroscopic data (Table 2) of **1** showed the presence of two secondary methyls (δ 0.96 and 0.99, 3H each, d, $J = 7.0$ Hz) of an isopropyl group and two *n*-butyryloxy moieties, one of them showed the signals at δ 0.99 (3H, t, $J = 7.5$ Hz), 1.67 (2H, m), 2.26 (1H, m), and 2.39 (1H, m) and the other one resonated at δ 0.96 (3H, t, $J = 7.5$ Hz), 1.65 (2H, m), and 2.29 (2H, m). The remaining three degrees of unsaturation identified **1** as a tricyclic diterpenoid. Analysis of the HMQC spectrum showed that proton signals appearing at δ 2.41 (1H, m), 2.62 (1H, br t, $J = 7.5$ Hz), 3.53 (1H, s), and 4.29 (1H, ddd, $J = 11.0$, 7.5, 3.5 Hz) correlated to two ring-juncture methine carbons at δ 43.0 and 56.5 and two oxymethine carbons at δ 75.5 and 93.0, respectively. An ^1H–^1H COSY experiment further assigned two isolated consecutive proton spin systems (Figure 1). The above evidence and the analysis of the HMBC spectrum (Figure 1) suggested that **1** is an eunicellin-based diterpenoid. The placement of two acetoxy groups at both C-6 and C-13 were confirmed from the HMBC connectivities of H-6 (δ 5.60, d, $J = 5.5$ Hz) and H-13 (δ 5.47, dd, $J = 10.5$, 9.5 Hz), and two acetate methyls (δ 2.09 and 1.98) to the ester carbons resonating at δ 172.0 and 170.3, respectively. Finally, two *n*-butyryloxy groups had to be positioned at C-3 and C-12, and were confirmed from the HMBC connectivities of H-2 (δ 3.53) and H-12 (δ 5.04, d, $J = 9.5$ Hz) to the ester-carbonyl carbons resonating at δ 172.2 and 172.5, respectively. Furthermore, the proton resonances of H_3-16 (δ 1.18) and H_3-17 (δ 1.11), and

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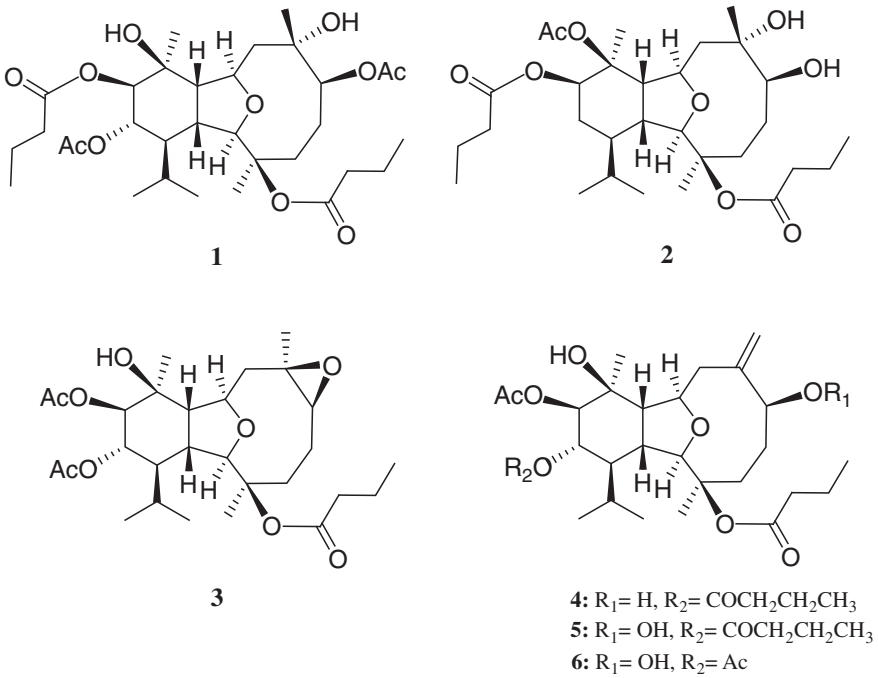

1 **2**

3

4: R_1= H, R_2= $COCH_2CH_2CH_3$
5: R_1= OH, R_2= $COCH_2CH_2CH_3$
6: R_1= OH, R_2= Ac

Chart 1.

the carbon shifts of C-7 (δ 75.7, C) and C-11 (δ 72.7, C), determined the positions of two tertiary hydroxy groups at C-7 and C-11, respectively. Therefore, the gross structure of **1** was established.

The relative stereostructure of **1** was determined by analysis of correlations observed in the NOESY spectrum (Figure 2), which showed NOE correlations between H-1 with both H-10 and H-13, revealing that H-1, H-10, and H-13 are β-oriented. Also, correlations between H-2 with both H_3-15 and H-14; H-12 with H-9, H-14, and H_3-17; H-6 with H-5α (δ 1.52); H-5α with H-2 and H_3-15 suggested that H-2, H-6, H-9, H-12, H-14, H_3-15, and H_3-17 are all α-oriented. Furthermore, H-5β (δ 1.43) showed NOE correlation with H_3-16, revealing the β-orientation of H_3-16. Thus, the structure of diterpenoid **1** was established.

HR-ESI-MS of simplexin K (**2**) exhibited a $[M + Na]^+$ peak at m/z 577.3350 (calcd for $C_{30}H_{50}O_9Na$, 577.3352) and established a molecular formula $C_{30}H_{50}O_9$, implying six degrees of unsaturation. The IR spectrum of **2** disclosed the presence of hydroxy (ν_{max} 3426 cm^{-1}) and carbonyl (ν_{max} 1734 cm^{-1}) functionalities. The NMR spectroscopic data showed that **2** possessed an acetoxy and two *n*-butyryloxy groups (Tables 1 and 2) and the locations of two *n*-butyryloxy groups at C-3 and C-12 were proven from the HMBC connectivities from H-2 (δ 3.46) and two methylene protons of one *n*-butyryloxy group (δ 2.34, 2H) to the carbonyl carbon resonating at δ 172.7 (C), and H-12 (δ 4.88) and two methylene protons of the other *n*-butyryloxy group (δ 2.34, 2H) to a carbon resonating at δ 173.1 (C). Moreover, the upfield chemical shift for H_3-16 (δ 1.16) and the more downfield shift for H_3-17 (δ 1.52) determined the positions of one hydroxy and one acetoxy groups at C-7 and C-11, respectively. Other analysis of the ^1H and ^{13}C 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 configuration of **2** was deduced by comparison of the very similar NOE correlations between the corresponding protons of both **1** and **2**.

Simplexin L (**3**) showed the pseudomolecular ion peak $[M + Na]^+$ at m/z 547.2880 in the HR-ESI-MS, corresponding to a molecular formula $C_{28}H_{44}O_9$ and seven degrees of unsaturation. The IR absorptions at 3480 and 1734 cm^{-1} indicated the presence of hydroxy and carbonyl functionalities in **3**. The NMR spectroscopic data (Tables 1 and 2) of **3** showed the presence of a trisubstituted epoxide (δ 60.8, C; 64.3, CH) and three ester carbonyls at δ_C 170.1, 170.3, and 172.4. The placement of acetates at C-12 and C-13 were confirmed from the HMBC connectivities of acetate methyls (δ 2.11 and 2.01), H-12 (δ 5.04) and H-13 (δ 5.47) with the carbonyl carbons resonating at δ 170.3 (C) and 170.1 (C), respectively. Also, the location of an *n*-butyryloxy group at C-3 was proven by the HMBC correlations from both H-2 (δ 3.53) and methylene protons (δ 2.27 and 2.32) to the carbonyl carbon resonating at 172.4 (C). Furthermore, the HMBC correlations found from H_3-16 to C-6 (δ 64.3, CH) and C-7 (δ 60.8, C) unambiguously established the C-6/C-7 position of the epoxy group. The stereochemistry of **3** was determined by the NOESY spectrum, which exhibited NOE correlations (Figure 2) between H-10 and H-8β (δ 1.51); H-8β (δ 1.51) and one of the methylene proton at C-5 (δ 2.11), which was thus assigned as H-5β, while the other (δ 2.15, m) was denoted as H-5α. Thus, the NOE correlations observed between H_3-16 and H-6, and H-6 and H-5α reflected the α-orientation of both H-6 and H_3-16. Thus, the structure of **3** was established.

Simplexin M (**4**) was found to have the molecular formula $C_{30}H_{48}O_9$, as indicated from the HR-ESI-MS (m/z 575.3193 $[M + Na]^+$) and NMR spectroscopic data (Tables 1 and 2). The ^{13}C NMR spectrum measured in $CDCl_3$ showed signals of 30

Table 1. ^{13}C NMR Data for Compounds **1**–**6**[a)]

No.	**1**	**2**	**3**	**4**	**5**	**6**
1	43.0 (CH)[b)]	41.8 (CH)	43.6 (CH)	41.7 (CH)	41.7 (CH)	41.7 (CH)
2	93.0 (CH)	92.0 (CH)	91.3 (CH)	91.2 (CH)	91.0 (CH)	91.0 (CH)
3	85.9 (C)	85.7 (C)	85.4 (C)	84.3 (C)	84.2 (C)	84.1 (C)
4	35.9 (CH_2)	36.6 (CH_2)	29.6 (CH_2)	28.5 (CH_2)	28.4 (CH_2)	28.0 (CH_2)
5	29.1 (CH_2)	30.3 (CH_2)	25.9 (CH_2)	35.2 (CH_2)	29.6 (CH_2)	29.6 (CH_2)
6	84.7 (CH)	80.7 (CH)	64.3 (CH)	72.8 (CH)	86.3 (CH)	86.4 (CH)
7	75.7 (C)	77.1 (C)	60.8 (C)	150.0 (C)	145.6 (C)	145.7 (C)
8	47.5 (CH_2)	47.6 (CH_2)	41.6 (CH_2)	40.9 (CH_2)	41.5 (CH_2)	41.5 (CH_2)
9	75.5 (CH)	75.6 (CH)	75.8 (CH)	75.6 (CH)	78.5 (CH)	78.5 (CH)
10	56.5 (CH)	51.5 (CH)	56.7 (CH)	49.2 (CH)	49.2 (CH)	49.3 (CH)
11	72.7 (C)	81.9 (C)	72.1 (C)	72.6 (C)	72.5 (C)	72.5 (C)
12	76.3 (CH)	74.3 (CH)	77.3 (CH)	76.8 (CH)	76.8 (CH)	76.9 (CH)
13	70.5 (CH)	23.4 (CH_2)	70.8 (CH)	70.6 (CH)	70.6 (CH_2)	71.0 (CH_2)
14	47.3 (CH)	42.4 (CH)	46.3 (CH)	47.7 (CH)	47.5 (CH)	47.4 (CH)
15	23.1 (CH_3)	23.2 (CH_3)	23.8 (CH_3)	22.2 (CH_3)	22.3 (CH_3)	22.4 (CH_3)
16	23.8 (CH_3)	22.7 (CH_3)	22.5 (CH_3)	117.0 (CH_2)	118.1 (CH_2)	118.1 (CH_2)
17	25.8 (CH_3)	20.6 (CH_3)	24.8 (CH_3)	26.6 (CH_3)	26.1 (CH_3)	26.2 (CH_3)
18	30.2 (CH)	29.0 (CH)	29.8 (CH)	28.0 (CH)	28.0 (CH)	28.0 (CH)
19	23.3 (CH_3)	21.7 (CH_3)	23.4 (CH_3)	23.6 (CH_3)	23.5 (CH_3)	23.6 (CH_3)
20	16.0 (CH_3)	15.2 (CH_3)	15.9 (CH_3)	15.8 (CH_3)	15.7 (CH_3)	15.7 (CH_3)
6-acetate	21.4 (CH_3)					
	172.0 (C)					
11-acetate		22.5 (CH_3)				
		170.1 (C)				
12-acetate			20.7 (CH_3)	20.7 (CH_3)	20.7 (CH_3)	20.6 (CH_3)
			170.3 (C)	172.7 (C)	172.8 (C)	170.2 (C)
13-acetate	21.4 (CH_3)		21.3 (CH_3)			21.4 (CH_3)
	170.3 (C)		170.1 (C)			170.2 (C)
3-*n*-butyrate	13.7 (CH_3)	13.7 (CH_3)	13.6 (CH_3)	13.8 (CH_3)	13.8 (CH_3)	13.6 (CH_3)
	18.3 (CH_2)	18.5 (CH_2)	18.5 (CH_2)	18.1 (CH_2)	18.1 (CH_2)	18.4 (CH_2)
	37.3 (CH_2)	36.3 (CH_2)	37.2 (CH_2)	36.7 (CH_2)	36.6 (CH_2)	37.3 (CH_2)
	172.2 (C)	172.7 (C)	172.4 (C)	172.4 (C)	172.5 (C)	172.5 (C)
12-*n*-butyrate	13.7 (CH_3)	13.6 (CH_3)				
	18.4 (CH_2)	18.7 (CH_2)				
	35.9 (CH_2)	36.9 (CH_2)				
	172.5 (C)	173.1 (C)				
13-*n*-butyrate				13.6 (CH_3)	13.5 (CH_3)	
				18.4 (CH_2)	18.4 (CH_2)	
				37.3 (CH_2)	37.3 (CH_2)	
				172.7 (C)	172.8 (C)	

a) Spectra recorded at 125 MHz in $CDCl_3$ at 25 °C. b) Attached protons determined by DEPT experiments.

carbons (Table 1) which were assigned by the assistance of the DEPT spectrum to seven methyls, eight methylenes, nine methines (including five oxymethines), three carbonyls, two sp^3 oxygenated quaternary carbons, and one sp^2 quaternary carbon of an olefinic group. The NMR spectroscopic data of **4** (Tables 1 and 3) showed the presence of an exocyclic double bond (δ_C 117.0, CH_2 and 150.0, C; δ_H 5.12, s and 5.46, s). The ^1H NMR spectroscopic data of **4** reveal the presence of one acetoxy group (δ_H 2.08, 3H, s) and two *n*-butyryloxy moieties, which shows signals at δ 0.92 (3H, t, $J = 7.5$ Hz) and 0.95 (3H, t, $J = 7.5$ Hz), 1.58 (2H, m) and 1.63 (2H, m), and 2.22 (2H, m) and 2.12 (2H, m), respectively. The molecular framework was also established by ^1H–^1H COSY and HMBC experiments (Figure 1). Furthermore, NOE correlation observed between H_3-15 and H-6 assigned the β-orientation of the hydroxy group at C-6. A detailed analysis of other key NOE correlations (Figure 2) further established the relative structure of **4**.

Simplexin N (**5**), was isolated as a colorless oil and exhibited a pseudomolecular ion peak at m/z 591.3144 $[M + Na]^+$ by HR-ESI-MS, appropriate for a molecular formula of $C_{30}H_{48}O_{10}$, with one more oxygen atom than that of **4**. The NMR spectra data were found to be very similar to those of **4** (Tables 1 and 3) except for CH-6, which become more downfield shifted (δ_C 86.3 and δ_H 4.63) relative to that of **4** (δ_C 72.8 and δ_H 4.33), revealing the presence of a hydroperoxy group at C-6. This was confirmed by the signal of a hydroperoxy proton at δ 8.31 (br s). The relative configuration of **5** was found to be the same as that of **4** by comparison of chemical shifts, coupling constants and NOE correlations. HR-ESI-MS of simplexin O (**6**) exhibited a $[M + Na]^+$ peak at m/z

Table 2. ^{1}H NMR Data for Compounds **1**–**3**[a)]

No.	**1**	**2**	**3**
1	2.41 m	2.04 m	2.50 dd (12.0, 7.0)
2	3.53 s	3.46 s	3.53 s
4α	2.00 m	1.78 m	1.78 m
4β	2.65 m	2.66 dd (14.5, 8.5)	2.47 dd (14.5, 11.5)
5α	1.52 m	1.57 m	2.15 m
5β	1.43 m	1.41 m	2.11 dd (12.2, 4.5)
6	5.60 d (5.5)[b)]	4.54 d (5.5)	2.79 dd (10.5, 4.0)
8α	1.82 dd (14.5, 4.0)	1.90 dd (14.5, 4.0)	2.06 m
8β	1.93 m	1.99 m	1.51 t (12.0)
9	4.29 ddd (11.0, 7.5, 3.5)	4.17 ddd (11.0, 7.5, 4.0)	4.10 td (10.0, 4.5)
10	2.62 t (7.5)	3.79 t (7.5)	2.72 dd (9.0, 7.0)
12	5.04 d (9.5)	4.88 dd (13.5, 4.0)	5.04 d (9.5)
13	5.47 dd (10.5, 9.5)	1.56 2H, m	5.47 t (10.5)
14	1.75 m	1.76 m	1.73 m
15	1.39 3H, s	1.36 3H, s	1.39 3H, s
16	1.18 3H, s	1.16 3H, s	1.38 3H, s
17	1.11 3H, s	1.52 3H, s	1.11 3H, s
18	1.72 m	1.75 m	1.75 m
19	0.99 3H, d (7.0)	0.96 3H, d (7.0)	1.01 3H, d (7.0)
20	0.96 3H, d (7.0)	0.84 3H, d (7.0)	0.96 3H, d (7.0)
6-acetate	2.09 3H, s		
11-acetate		2.05 3H, s	
12-acetate			2.11 3H, s
13-acetate	1.98 3H, s		2.01 3H, s
3-*n*-butyrate	0.99 3H, t (7.5)	0.99 3H, t (7.5)	0.95 3H, t (7.5)
	1.67 2H, m	1.67 2H, m	1.66 2H, m
	2.26 m	2.34 2H, m	2.27 m
	2.39 m		2.32 m
12-*n*-butyrate	0.96 3H, t (7.5)	0.97 3H, t (7.5)	
	1.65 2H, m	1.65 2H, m	
	2.29 2H, m	2.34 2H, m	

a) Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C. b) *J* values in Hz in parentheses.

563.2831 (calcd for $C_{28}H_{44}O_{10}Na$, 563.2832) and established the molecular formula, $C_{28}H_{44}O_{10}$, implying six degrees of unsaturation. The presence of a hydroperoxy proton was also indicated by the ^{1}H NMR signal at δ 8.00 (1H, s) in **6**. Comparison of the NMR data of **6** (Tables 1 and 3) with those of **5** revealed the replacement of an *n*-butyryloxy group at C-13 in **5** by an acetoxy group in **6**. It was further confirmed by the HMBC spectrum of **6** which showed correlations between both H-12 and H-13 to two carbonyl carbons (δ_C 170.2) of the acetoxy groups. The very similar NMR data of **5** and **6** further revealed that both compounds have the same relative configuration.

The absolute configuration of simplexin A[9] has been completely assigned based on NOE correlations and Mosher ester analysis. Compounds **1**–**6** are likely in the same enantiomeric series as simplexin A based on a shared biosynthetic pathway. Thus, these compounds are suggested to possess the absolute configurations as shown in formula **1**–**6**.

The cytotoxicity of more abundant compounds **4**–**6** against the proliferation of a limited panel of cancer cell lines, including human medulloblastoma (Daoy), human breast adenocarcinoma (MCF-7), human cervical epitheloid carcinoma (HeLa), and human laryngeal carcinoma (HEp2) was studied. The results showed that compounds **4** and **6** are not cytotoxic toward the above cancer cells ($ED_{50} > 20\,\mu g\,mL^{-1}$). Compound **5** has been shown to exhibit a weak cytotoxicity toward the Daoy cancer cell line ($ED_{50} = 15.3\,\mu g\,mL^{-1}$). In vitro anti-inflammatory effect of compounds **4**–**6** was also tested. In this assay, the upregulation 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 **6** was found to effectively reduce the levels of iNOS and COX-2 proteins to $31.1 \pm 5.2\%$ and $67.5 \pm 6.4\%$, respectively, relative to the control cells stimulated with LPS only. At the same concentration, metabolites **4** and **5** did not inhibit the COX-2 protein expression, but could significantly reduce iNOS expression ($28.3 \pm 6.7\%$ and $32.5 \pm 7.7\%$, respectively) by LPS stimulation. Also, the levels of β-actin, a house-keeping protein, were found to be $100.5 \pm 6.5\%$, $88.0 \pm 8.7\%$, and $113.5 \pm 4.1\%$ in the presence of 10 μM of **4**–**6**, respectively, relative to the cells treated with LPS only. Thus, **5** showed weak cytotoxicity toward RAW264.7 cells and **4** and **6** were not cytotoxic toward these cells.

Experimental

General Experimental Procedures. Melting points were determined using a Fisher–Johns melting point apparatus and

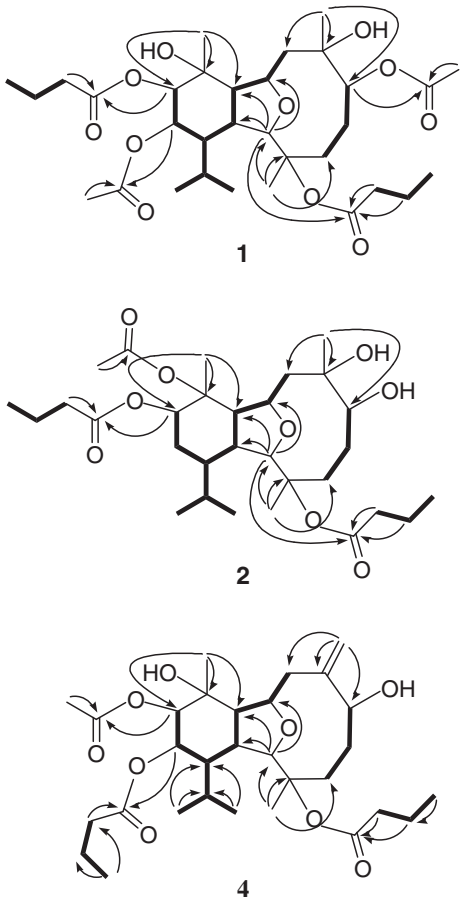

Figure 1. Selective ^{1}H–^{1}H COSY and HMBC correlations for **1**, **2**, and **4**.

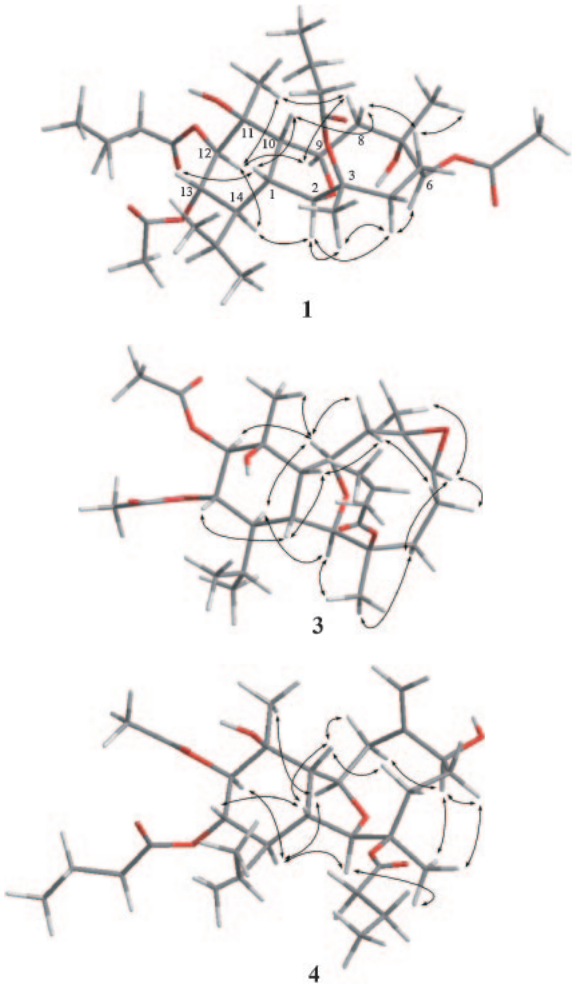

Figure 2. Selective NOE correlations for **1**, **3**, and **4**.

are uncorrected. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. 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 ^{1}H and 125 MHz for ^{13}C in $CDCl_3$. Silica gel 60 (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.2 mm) were used for analytical TLC. High-performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with a Merck Hibar Si-60 column (250 × 21 mm, 7 µm) and on a Hitachi L-2455 diode array detector apparatus with a ODS-3 column (250 × 20 mm, 5 µm).

Animal Material. *Klyxum 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 silica 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), was further purified over silica gel using EtOAc–*n*-hexane (1:3 to 1:1) to afford 46 subfractions. Subfraction 34 was separated by normal phase HPLC using acetone–*n*-hexane (1:3) to afford **3** (1.8 mg) and **5** (22.0 mg), and subfractions 36 and 37 were also purified by normal phase HPLC using acetone–*n*-hexane (1:2) to afford **6** (9.9 mg). Fractions 13–15 (0.47 g), eluted with EtOAc–*n*-hexane (1:1), were further resolved over silica gel using EtOAc–*n*-hexane (1:1) to afford 19 subfractions. Subfractions 14, 15, and 17 were separated by normal phase HPLC using acetone–*n*-hexane (1:2) to yield **1** (1.4 mg) and **2** (2.7 mg), respectively. Fractions 16–19 (0.51 g) eluted with EtOAc–*n*-hexane (2:1), were further resolved over silica gel using EtOAc–*n*-hexane (2:1) to afford 4 subfractions. Subfractions 3 and 4 were separated by normal phase HPLC using MeOH–CH_2Cl_2 (1:30) to afford **4** (7.4 mg).

Simplexin J (1): White powder; mp 70–71 °C; $[\alpha]_D^{26} = +39.6$ (*c* 0.2, $CHCl_3$); IR (neat): ν_{max} 3471 and 1734 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESI-MS *m/z* 635 [100, $(M + Na)^+$]; HR-ESI-MS *m/z* 635.3404 (calcd for $C_{32}H_{52}O_{11}Na$, 635.3407).

Simplexin K (2): Colorless oil; $[\alpha]_D^{26} = +22.2$ (*c* 1.1, $CHCl_3$); IR (neat): ν_{max} 3426 and 1734 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESI-MS *m/z* 577 [100, $(M + Na)^+$]; HR-ESI-MS *m/z* 577.3350 (calcd for $C_{30}H_{50}O_9Na$, 577.3352).

Table 3. ^{1}H NMR Data for Compounds **4**–**6**[a)]

No.	**4**	**5**	**6**
1	2.55 dd (11.5, 8.0)[b)]	2.53 dd (11.8, 7.5)	2.54 dd (11.5, 7.5)
2	3.59 s	3.58 s	3.59 s
4α	2.23 m	2.21 m	2.22 m
4β	1.76 m	1.85 m	1.86 t (12.5)
5α	2.13 m	2.16 m	2.17 m
5β	1.71 m	1.51 m	1.52 m
6	4.33 dd (11.0, 4.0)	4.63 dd (11.5, 3.0)	4.65 dd (11.5, 3.5)
8α	2.86 dd (14.0, 4.5)	2.84 dd (14.0, 5.0)	2.85 dd (14.0, 5.0)
8β	2.44 d (14.5)	2.49 d (14.0)	2.50 d (14.0)
9	4.29 dd (11.0, 4.0)	4.27 dd (11.0, 4.5)	4.27 dd (11.0, 5.0)
10	2.66 dd (11.0, 7.5)	2.65 dd (11.0, 7.5)	2.66 dd (11.0, 7.5)
12	5.02 d (10.0)	5.00 d (10.0)	5.01 d (9.5)
13	5.51 t (10.0)	5.50 t (10.0)	5.48 t (10.5)
14	1.74 m	1.73 t (11.5)	1.74 t (11.5)
15	1.60 3H, s	1.58 3H, s	1.59 3H, s
16	5.12 s	5.20 s	5.21 s
	5.46 s	5.42 s	1.74 t (11.5)
17	1.17 3H, s	1.16 3H, s	1.18 3H, s
18	1.97 m	1.94 m	1.96 m
19	0.99 3H, d (7.0)	0.98 3H, d (7.0)	0.99 3H, d (7.0)
20	0.92 3H, d (7.0)	0.91 3H, d (7.0)	0.91 3H, d (7.0)
12-acetate	2.08 3H, s		2.09 3H, s
13-acetate		2.07 3H, s	2.00 3H, s
3-*n*-butyrate	0.95 3H, t (7.5)	0.94 3H, t (7.5)	0.92 3H, t (7.5)
	1.63 2H, m	1.60 2H, m	1.58 2H, m
	2.22 2H, m	2.21 2H, m	2.13 2H, m
13-*n*-butyrate	0.92 3H, t (7.5)	0.91 3H, t (7.5)	
	1.58 2H, m	1.57 2H, m	
	2.12 2H, m	2.10 2H, m	
6-OOH		8.31 br s	8.00 s

a) Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C. b) *J* values in Hz in parentheses.

Simplexin L (3): White powder; mp 86.5–87.0 °C; $[\alpha]_D^{26} = +21.5$ (*c* 0.7, $CHCl_3$); IR (neat): ν_{max} 3480 and 1734 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESI-MS *m*/*z* 547 [100, $(M + Na)^+$]; HR-ESI-MS *m*/*z* 547.2880 (calcd for $C_{28}H_{44}O_9Na$, 547.2883).

Simplexin M (4): White powder; mp 193–194 °C; $[\alpha]_D^{26} = -1.6$ (*c* 0.7, $CHCl_3$); IR (neat): ν_{max} 3273 (broad), 1723 and 1647 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 3; ESI-MS *m*/*z* 547 [100, $(M + Na)^+$]; HR-ESI-MS *m*/*z* 575.3193 (calcd for $C_{30}H_{48}O_9Na$, 575.3196).

Simplexin N (5): Colorless oil; $[\alpha]_D^{26} = -4.2$ (*c* 4.4, $CHCl_3$); IR (neat): ν_{max} 3258 and 1733 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 3; ESI-MS *m*/*z* 591 [100, $(M + Na)^+$]; HR-ESI-MS *m*/*z* 591.3144 (calcd for $C_{30}H_{48}O_{10}Na$, 591.3145).

Simplexin O (6): White powder; mp 196.0–197.5 °C; $[\alpha]_D^{26} = -9.8$ (*c* 1.0, $CHCl_3$); IR (neat): ν_{max} 3410 and 1731 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 3; ESI-MS *m*/*z* 563 [100, $(M + Na)^+$]; HR-ESI-MS *m*/*z* 563.2831 (calcd for $C_{28}H_{44}O_{10}Na$, 563.2832).

Cytotoxicity Testing. Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of compounds **4**–**6** were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[10,11]

In Vitro Anti-Inflammatory Assay. Macrophage (RAW264.7) cell line was purchased from ATCC. In vitro anti-inflammatory activities of compounds **4**–**6** were measured by examining the inhibition of lipopolysaccharide (LPS) induced upregulation of iNOS (inducible nitric oxide synthetase) and COX-2 (cyclooxygenase-2) proteins in macrophages cells using western blotting analysis.[12,13]

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Supporting Information

The ^{1}H and ^{13}C NMR spectra of **1**–**6** are available free of charge via the Internet at http://www.csj.jp/journals/bcsj/.

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