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Klysimplexins U–X, Eunicellin-Based Diterpenoids from the Cultured Soft Coral *Klyxum simplex*

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New eunicellin-based diterpenoids, klysimplexins U–X (**1**–**4**), were isolated from a cultured soft coral *Klyxum simplex*. Their structures were elucidated by spectroscopic methods, particularly in 1D and 2D NMR experiments. The absolute configuration of **2** was determined by Mosher's method. Compounds **1** and **2** are the first example of 4-oxygenated eunicellin-type diterpenes isolated from soft corals of this genus.

Previously reported eunicellin-based diterpenoids were isolated mostly from octocorals (Alcyonaceae) belonging to the genera *Acalycigorgia*,[1] *Alcyonium*,[2] *Astrogorgia*,[3] *Briareum*,[4] *Cladiella*,[5–9] *Eleutherobia*,[10] *Eunicella*,[11] *Klyxum*,[12] *Litophyton*,[13] *Muricella*,[14] *Pachyclavularia*,[15,16] *Sclerophytum*,[17] *Sinularia*,[18] and *Solenopodium*.[19] During the course of our investigation on new natural substances from the cultured and wild-type soft corals *K. simplex*, new eunicellin-type metabolites klysimplexins A–H,[20] I–T[21] and klysimplexin sulfoxides A–C[22] were isolated from cultured soft coral, and simplexins A–I,[23] J–O[24] were obtained from the wild-type soft coral. In continuing our effort toward discovering new and bioactive substances from marine invertebrates, the chemical constituents of the cultured soft coral *Klyxum simplex* were further studied. This investigation again led to the isolation of four new eunicellin-based metabolites, klysimplexins U–X (**1**–**4**) (Chart 1). The relative structures of compounds **1**–**4** were established by extensive spectroscopic analysis, including 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY) spectroscopy, and the absolute structure of **2** was determined by a modified Mosher's method.

Results and Discussion

The soft coral (1.5 kg fresh wt) was collected and freeze-dried. The freeze-dried material was minced and extracted exhaustively with EtOH (3 × 10 L). The organic extract was concentrated to an aqueous suspension and was further partitioned between CH_2Cl_2 and water. The combined CH_2Cl_2-soluble fraction was concentrated under reduced pressure and the residue was repeatedly purified by chromatography to yield metabolites **1**–**4**.

Klysimplexin U (**1**) was obtained as a colorless oil that gave a pseudomolecular ion peak at m/z 591.3149 $[M + Na]^+$ in the HRESIMS, consistent with the molecular formula $C_{30}H_{48}O_{10}$, implying seven degrees of unsaturation. The IR absorptions at 3426 and 1734 cm^{-1} revealed the presence of hydroxy and ester functionalities. The ^{13}C NMR spectroscopic data of **1** included 30 carbon signals (Table 1), which were assigned by the assistance of a DEPT spectrum to seven methyls, seven methylenes (including one exomethylene), ten methines (including six oxymethines), three sp^2 ester carbonyls, and two sp^3 and one sp^2 quaternary carbons. The NMR spectra data of **1** (Tables 1 and 2) showed the appearance of a 1,1-disubstituted carbon–carbon double bond (δ_C 151.4 (qC) and 115.2 (CH_2); δ_H 5.36 (s) and 5.02 (s)). Three ester carbonyls (δ_C 175.2, 172.7, and 170.1) were also assigned from the ^{13}C NMR spectrum and were HMBC correlated with the methylenes (δ_H 2.35 and 2.28 (m, 2H) and 1.69 (m, 2H); 2.22 (m, 2H) and 1.61 (m, 2H)) of two butyrate units and an acetate methyl (δ_H 2.08 (s, 3H)), respectively. The remaining three degrees of unsaturation identified **1** as a tricyclic compound. Two 3H singlets appearing in the ^{1}H NMR spectrum (Table 2) at δ_H 1.17 and 1.29 were assigned to two methyls bonded to quaternary oxygenated carbon, respectively. Signals resonating at δ_H 2.50 (1H, dd, J = 12.0, 6.8 Hz), 2.72 (1H, dd, J = 11.2, 6.8 Hz), 3.74 (1H, s), and 4.21 (1H, m); and at δ_C 43.1, 50.2, 89.2, and 77.2, indicated the presence of a tetrahydrofuran structural unit.[1–4] The planar structure of metabolite **1** was elucidated by analysis of ^{1}H–^{1}H COSY and HMBC correlations (Figure 1). Key HMBC correlations from H-2 to C-1, C-9, and C-10; H_3-15 to C-2, C-3, and C-4; H_2-16 to C-6, C-7, and C-8; H_3-17 to C-10, C-11, and C-12; and both H_3-19 and H_3-20 to C-14 and C-18 permitted the assembly of the carbon skeleton. The placement of two *n*-butyrate at C-13 and C-3 were proven from the HMBC correlations from H-13 (δ 5.49) and H-2

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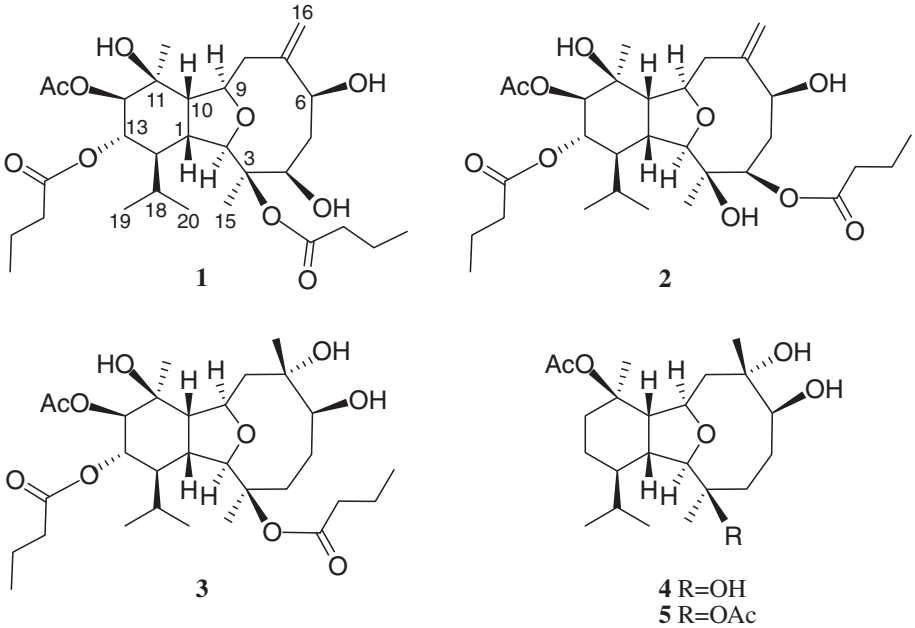

Chart 1.

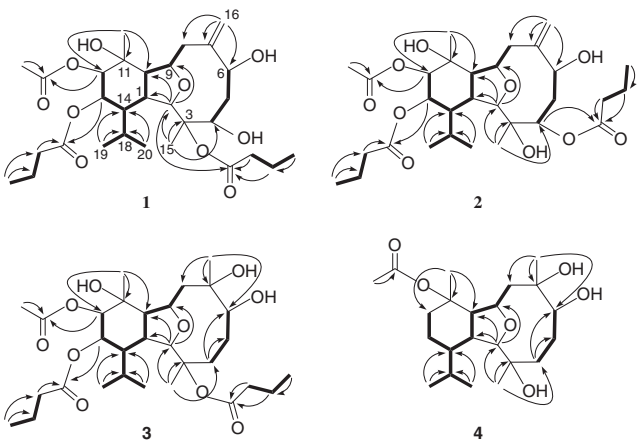

Figure 1. Key ^{1}H–^{1}H COSY and HMBC correlations for **1**–**4**.

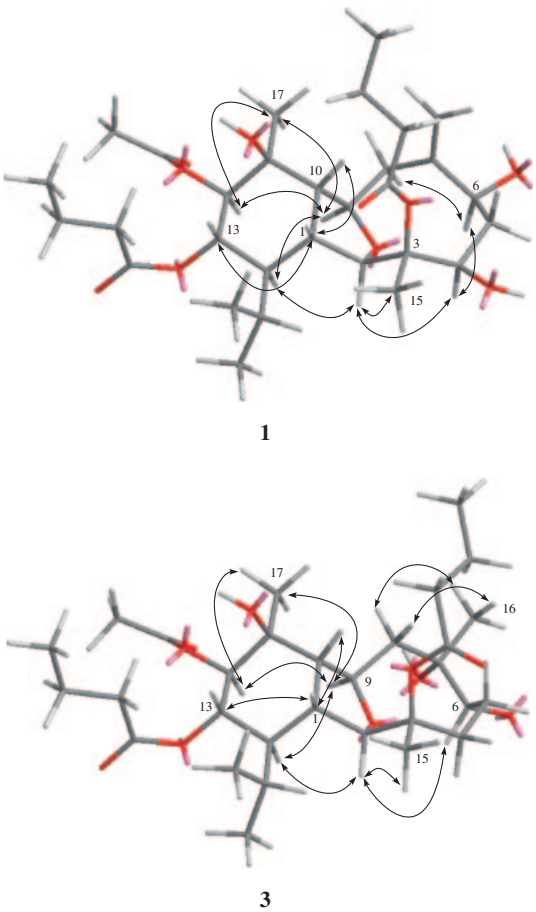

Figure 2. Key NOESY correlations of **1** and **3**.

(δ 3.74) to the carbonyl carbons resonating at δ 172.7 (qC) and 175.2 (qC).[1] Also, the position of the acetate at C-12 was confirmed by the HMBC correlations of the acetate methyl (δ_H 2.08 (s, 3H)) and H-12 (δ 5.03) with the carbonyl carbon resonating at δ_C 170.1 (C). The proton resonance for H_3-17 (δ 1.17) also determined the position of the hydroxy group at C-11. Therefore, the planar structure of **1** was established. In the NOESY spectrum of **1** (Figure 2), observation of the NOE correlations between H-10 with H-1, and H-1 with H-13, suggested that H-1, H-10, and H-13 are β-oriented. Also, correlations of H-2 with H_3-15, H-14, and H-4; H-9 with H_3-17, H-14, and H-12; and H-6 with both H-8α (δ 2.46) and H-4 suggested that H-2, H-4, H-6, H-9, H-12, H-14, H_3-15, and H_3-17 are all α-oriented. Thus, the relative configuration of diterpenoid **1** was established.

The HRESIMS of klysimplexin V (**2**) exhibited a pseudomolecular ion peak at m/z 591.3147 $[M + Na]^+$, consistent with a molecular formula $C_{30}H_{48}O_{10}$. Thus, **1** and **2** have the same molecular formula. IR absorption bands, ESIMS ion peak, and the NMR spectral data suggested that both **1** and **2** possess the same substituents and are geometric isomers. By comparison of NMR data of **2** with those of **1** (Tables 1 and 2), it was found that an butyrate at C-3 and the hydroxy group at

Table 1. ^{13}C NMR Data for Compounds **1**–**4**

Position	**1**[a)]	**2**[a)]	**3**[a)]	**4**[a)]
1	43.1 (CH)[b)]	42.4 (CH)	42.9 (CH)	41.9 (CH)
2	89.2 (CH)	89.8 (CH)	93.3 (CH)	90.7 (CH)
3	90.0 (qC)	77.0 (qC)	85.8 (qC)	74.6 (qC)
4	72.2 (CH)	76.2 (CH)	36.5 (CH_2)	40.0 (CH_2)
5	46.7 (CH_2)	41.8 (CH_2)	30.4 (CH_2)	29.2 (CH_2)
6	73.3 (CH)	72.4 (CH)	80.5 (CH)	80.3 (CH)
7	151.4 (qC)	151.7 (qC)	77.2 (qC)	77.2 (qC)
8	40.3 (CH_2)	40.6 (CH_2)	47.5 (CH_2)	46.9 (CH_2)
9	77.2 (CH)	77.5 (CH)	75.7 (CH)	75.5 (CH)
10	50.2 (CH)	50.6 (CH)	56.7 (CH)	54.2 (CH)
11	72.1 (qC)	72.4 (qC)	72.7 (qC)	82.8 (qC)
12	77.0 (CH)	76.9 (CH)	76.7 (CH)	30.6 (CH_2)
13	70.7 (CH)	70.6 (CH)	70.1 (CH)	17.8 (CH_2)
14	45.5 (CH)	46.0 (CH)	47.5 (CH)	42.5 (CH)
15	19.9 (CH_3)	23.8 (CH_3)	23.3 (CH_3)	30.2 (CH_3)
16	115.2 (CH_2)	116.8 (CH_2)	22.7 (CH_3)	23.2 (CH_3)
17	26.5 (CH_3)	26.1 (CH_3)	25.7 (CH_3)	24.4 (CH_3)
18	28.8 (CH)	29.0 (CH)	30.2 (CH)	29.1 (CH)
19	23.6 (CH_3)	23.5 (CH_3)	23.4 (CH_3)	21.8 (CH_3)
20	15.5 (CH_3)	15.6 (CH_3)	16.1 (CH_3)	15.6 (CH_3)
3-*n*-butyrate	13.8 (CH_3)		13.8 (CH_3)	
	18.2 (CH_2)		18.3 (CH_2)	
	37.2 (CH_2)		37.3 (CH_2)	
	175.2 (qC)		172.2 (qC)	
4-*n*-butyrate		13.8 (CH_3)		
		18.4 (CH_2)		
		36.4 (CH_2)		
		172.9 (qC)		
11-OAc				22.6 (CH_3)
				170.3 (qC)
12-OAc	20.8 (CH_3)	20.7 (CH_3)	20.7 (CH_3)	
	170.1 (qC)	170.2 (qC)	170.0 (qC)	
13-*n*-butyrate	13.6 (CH_3)	13.7 (CH_3)	13.7 (CH_3)	
	18.1 (CH_2)	18.1 (CH_2)	18.1 (CH_2)	
	36.6 (CH_2)	36.4 (CH_2)	36.6 (CH_2)	
	172.7 (qC)	172.8 (qC)	172.8 (qC)	

a) Spectra recorded at 100 MHz in $CDCl_3$ at 25 °C. b) Multiplicities deduced by DEPT.

C-4 in **1** were replaced by a hydroxy group and *n*-butyrate ester in **2**, respectively, as confirmed by the downfield shifted δc value of C-3 (δc 90.0) of **1**, relative to that of **2** (δc 77.0), and the HMBC connectivity from H-4 (δ 5.24) to the carbonyl carbon resonating at δ 172.9 (qC). In order to resolve the absolute structure of **2**, we determined the configuration at C-6 using Mosher's method.[25,26] The (*S*)- and (*R*)-α-methoxy-α-trifluoromethylphenylacetic (MTPA) esters of **2** (**2a** and **2b**, respectively) were prepared by using the corresponding (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chlorides, respectively. The values of $\Delta\delta$ [δ((*S*)-MTPA ester) − δ((*R*)-MTPA ester)] for H-8, H-9, and H_2-16 were positive, while the values of $\Delta\delta$ for H-2, H-4, H_2-5, and H_3-15 were negative, revealing the *S*-configuration at C-6 in Figure 3.

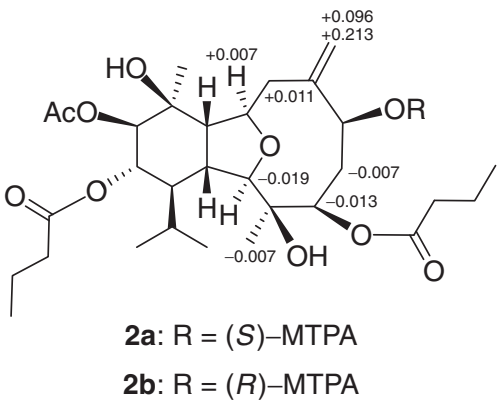

Figure 3. ^{1}H NMR chemical shift differences $\Delta\delta$ ($\delta_S - \delta_R$) in ppm for the MTPA esters of **2**.

On the basis of its HRESIMS spectrum (*m*/*z* 593.3308 $[M + Na]^+$), the molecular formula of klysimplexin W (**3**) was established as $C_{30}H_{50}O_{10}$, implying six degrees of unsaturation. A comparison of the NMR data of **3** (Tables 1 and 2) with those of **1** and klysimplexin F[1] showed that **3** has the same six-membered ring as that of **1** (including the identical substituent at C-14) and the same ten-membered ring as that of

Table 2. ^{1}H NMR Data for Compounds **1**–**4**

Position	**1**[a)]	**2**[a)]	**3**[a)]	**4**[a)]
1	2.50, dd (12.0, 6.8)	2.49, dd (12.0, 6.8)	2.40, m	2.20, m
2	3.74, s	3.73, s	3.49, s	3.53, s
4	3.96, dd (10.0, 2.8)	5.24, dd (8.4, 2.8)	2.68, dd (14.4, 8.8)	1.85, m
			1.82, m	1.81, m
5	2.19, m	2.47, m	1.41, m	2.02, m
	1.99, m			
6	4.36, d (10.4)	4.45, d (9.6)	4.57, d (6.4)	4.54, d (5.6)
8	α 2.46, d (13.6)	α 2.46, d (14.0)	1.97, m	2.35, d (14.0)
	β 2.79, dd (13.6, 7.2)	β 2.80, dd (14.0, 6.0)	1.83, m	1.74, m
9	4.21, m	4.21, m	4.25, m	4.04, ddd (10.8, 6.8, 3.6)
10	2.72, dd (11.2, 6.8)	2.68, m	2.60, br t (7.6)	2.82, br t (6.8)
12	5.03, d (10.4)	5.03, d (10.0)	5.03, d (9.2)	2.37, m
				1.40, m
13	5.49, dd (11.2, 10.4)	5.49, dd (10.8, 10.0)	5.51 dd (11.2, 9.2)	1.41, m
				1.23, m
14	1.75, t (11.2)	1.73, m	1.75, t (11.2)	1.18, m
15	1.29, s	0.99, s	1.40, s	1.16, s
16	5.36, s	5.62, s	1.16, s	1.20, s
	5.02, s	5.14, s		
17	1.17, s	1.16, s	1.12, s	1.46, s
18	1.79, m	1.71, m	1.71, m	1.69, m
19	1.00, d (7.2)	0.98, d (7.2)	1.00, d (7.2)	0.95, d (6.8)
20	0.93, d (7.2)	0.93, d (7.2)	0.96, d (7.2)	0.82, d (6.8)
3-*n*-butyrate	0.97, t (7.6)		1.01, t (7.2)	
	1.69, m		1.68, m	
	2.35, m; 2.28, m		2.38, m; 2.27, m	
4-*n*-butyrate		0.96, t (7.6)		
		1.68, m		
		2.31, m		
11-OAc				2.00, s
12-OAc	2.08, s	2.08, s	2.07, s	
13-*n*-butyrate	0.95, t (7.2)	0.95, t (7.2)	0.95, t (7.2)	
	1.61, m	1.62, m	1.62, m	
	2.22, m	2.21, m	2.21, m	

a) Spectra recorded at 400 MHz in $CDCl_3$ at 25 °C. b) *J* values in Hz in parentheses.

klysimplexin F, which was evidenced by COSY and HMBC correlations. The relative configuration for all asymmetric carbons in **3** was elucidated by the analysis of NOE correlations, as shown in Figure 2.

A structurally-related metabolite, klysimplexin X (**4**), was also isolated as a colorless oil with a molecular formula $C_{22}H_{38}O_6$, implying four degrees of unsaturation. The ^{13}C NMR spectroscopic data of **4** (Table 1) again showed the presence of an acetate [δ_C 170.3 (C) and 22.6 (CH_3)]. Comparison of the NMR data of **4** with those of a known metabolite klysimplexin G (**5**)[20] revealed that the only difference between the compounds was the replacement of one acetate at C-3 in **5** by the hydroxy group moiety in **4**. This was evidenced from the upfield chemical shifts induced by hydroxy group at C-3 (δ_C 74.6 (C)) and H_3-15 (δ_H 1.16 (CH_3)) in **4** relative to those of **5**. Thus, the structure of diterpenoid **4** was established.

Our study discovered the presence of 4-oxygenated eunicellin-based compounds like **1** and **2** for the first from corals of this genus. Cytotoxicity of metabolites **1**–**4** against a limited panel of human tumor cell lines including human liver carcinoma (Hep G2 and Hep G3B), human breast carcinoma (MDA-MB-231 and MCF-7) human lung carcinoma (A-549), and human oral cancer cells (Ca9-22) were investigated, and the ability of **1**–**4** 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. However, none of these compounds was found to possess satisfactory cytotoxicity and anti-inflammatory activity at 20 μM.

Experimental

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. ESIMS spectra were obtained with a Bruker APEX II mass spectrometer. NMR spectra were recorded on a Varian 400 MR FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Silica gel (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 an ODS column (250 × 21.2 mm, 5 μm).

Organism. Specimens of the cultured soft coral *K. simplex* were collected by hand in a 30 ton cultivating tank located in the National Museum of Marine Biology and Aquarium (NMMBA), Pingtung, Taiwan, in July 2005. A voucher sample (CSC-2) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Separation. The frozen bodies of *K. simplex* (1.5 kg, wet wt) were sliced and exhaustively extracted with EtOH (3 × 10 L). The combined organic layer was filtered and concentrated with a rotorary evaporator, and the residue of the resulting aqueous suspension was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 layer was dried with anhydrous Na_2SO_4. After removal of solvent in vacuo, the residue (15.2 g) was subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0–100% of EtOAc, gradient) and then further with MeOH in EtOAc of increasing polarity to yield 40 fractions. Fraction 30, eluted with *n*-hexane–EtOAc (1:5), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford five subfractions (E1–E4). Subfraction E3 was separated by reverse-phase HPLC (CH_3CN–H_2O, 2:1 to 1:1) to afford compounds **1** (2.8 mg), **2** (7.8 mg), **3** (1.0 mg), and **4** (10.1 mg).

Klysimplexin U (1): Colorless oil; $[\alpha]_D^{25}$ +82 (*c* 0.28, $CHCl_3$); IR (neat): ν_{max} 3426 and 1734 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz, $CDCl_3$): see Tables 1 and 2; ESIMS: *m/z* 591 $[M + Na]^+$; HRESIMS: *m/z* 591.3149 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_{10}Na$, 591.3145).

Klysimplexin V (2): Colorless oil; $[\alpha]_D^{25}$ +65 (*c* 0.78, $CHCl_3$); IR (neat): ν_{max} 3429 and 1730 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz, $CDCl_3$): see Tables 1 and 2; ESIMS: *m/z* 591 $[M + Na]^+$; HRESIMS: *m/z* 591.3147 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_{10}Na$, 591.3145).

Klysimplexin W (3): Colorless oil; $[\alpha]_D^{25}$ +15 (*c* 0.10, $CHCl_3$); IR (neat): ν_{max} 3436 and 1735 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz, $CDCl_3$): see Tables 1 and 2; ESIMS: *m/z* 593 $[M + Na]^+$; HRESIMS: *m/z* 593.3308 $[M + Na]^+$ (calcd for $C_{30}H_{50}O_{10}Na$, 593.3302).

Klysimplexin X (4): Colorless oil; $[\alpha]_D^{25}$ −15 (*c* 1.01, $CHCl_3$); IR (neat): ν_{max} 3424 and 1732 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz, $CDCl_3$): see Tables 1 and 2; ESIMS: *m/z* 421 $[M + Na]^+$; HRESIMS: *m/z* 421.2567 $[M + Na]^+$ (calcd for $C_{22}H_{38}O_6Na$, 421.2566).

Preparation of (*S*)- and (*R*)-MTPA Esters of 2. To a solution of **2** (0.5 mg) in pyridine (0.4 mL) was added *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MPTA) chloride (25 μL), and the mixture was allowed to stand for 24 h at room temperature. The reaction was quenched by addition of 1.0 mL of water, and the mixture was subsequently extracted with EtOAc (3 × 1.0 mL). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$, and evaporated. The residue was subjected to column chromatography over silica gel using *n*-hexane–EtOAc (3:1) to yield the (*S*)-MTPA ester, **2a**. The same procedure was used to prepare the (*R*)-MTPA ester, **2b** from the reaction of (*S*)-MTPA chloride with **2** in pyridine. Selective ^1H NMR ($CDCl_3$, 400 MHz) of **2a**: δ 5.432 (1H, s, H-16a), 5.418 (1H, d, *J* = 9.2 Hz, H-6), 5.221 (1H, br d, *J* = 9.6 Hz, H-4a), 5.148 (1H, s, H-16b), 4.171 (1H, dd, *J* = 10.4 and 6.8 Hz, H-9), 2.610 (1H, m, H-5a), 2.486 (1H, d, *J* = 14.0 Hz, H-8a), 0.928 (3H, s, H_3-15). Selective ^1H NMR ($CDCl_3$, 400 MHz) of **2b**: δ 5.381 (1H, d, *J* = 9.6 Hz, H-6), 5.234 (1H, br d, *J* = 9.6 Hz, H-4a), 5.219 (1H, s, H-16a), 5.052 (1H, s, H-16b), 4.164 (1H, dd, *J* = 10.4 and 5.6 Hz, H-9), 2.603 (1H, m, H-5a), 2.475 (1H, d, *J* = 13.6 Hz, H-8a), 0.935 (3H, s, H_3-15).

Cytotoxicity Testing. Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[27,28]

In Vitro Anti-Inflammatory Assay. Macrophage (RAW264.7) cell line was purchased from ATCC. In vitro anti-inflammatory activity of compounds **1**–**4** was measured by examining the inhibition of lipopolysaccharide (LPS) induced upregulation of iNOS (inducible nitric oxide synthetase) and COX-2 (cyclooxygenase-2) proteins in macrophage cells using western blotting analysis.[29]

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