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

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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.[1] 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,[2] cytotoxic,[3−10] inhibition of cell division of fertilized starfish eggs,[11] hemolytic activity,[12] antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum* W2, and antituberculosis activity against *Mycobacterium tuberculosis* H_{37}-Rv[13]). 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 Alcyonacea, 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 medulloblastoma (Daoy), human breast carcinoma (MCF-7), human cervical epitheloid (HeLa), and human laryngeal (Hep 2) carcinoma cells was studied, and the ability of **1**−**6** and **9** to inhibit up-regulation 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.

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 $C_{26}H_{42}O_6Na$, appropriate for six degrees of unsaturation. The IR absorptions at ν_{max} 3432 (broad) and 1723 cm^{-1} revealed the presence of hydroxy and carbonyl functionalities. The ^{13}C NMR spectrum measured in $CDCl_3$ 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 oxymethines), two carbonyls, two sp^2 oxygenated quaternary carbons, and one sp^2 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 (δ_C 116.8, CH_2 and 150.3, qC; δ_H 5.21, s and 5.47, s). The presence of one acetoxy group was indicated by the ^1H NMR signal at δ 2.01 (s, 3H) and ^{13}C NMR signals at δ 22.5 (CH_3) and 170.1 (qC) in compound **1**. In addition, the ^1H NMR spectroscopic data of **1** showed the presence of one *n*-butyryloxy moiety, which shows signals at δ 0.94 (3H, t, J = 7.5 Hz), 1.60 (2H, tq, J = 7.5, 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 HMQC 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-juncture methine carbons at δ 41.5 and 46.1 and two oxymethine 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 H_2-8 to both H-12 and the isopropyl moiety through H-14. The other was shown to extend from H_2-4 to H-6. 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 H_3-20 (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 mostly confirmed to be the same as that of **10** by comparison

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Chart 1

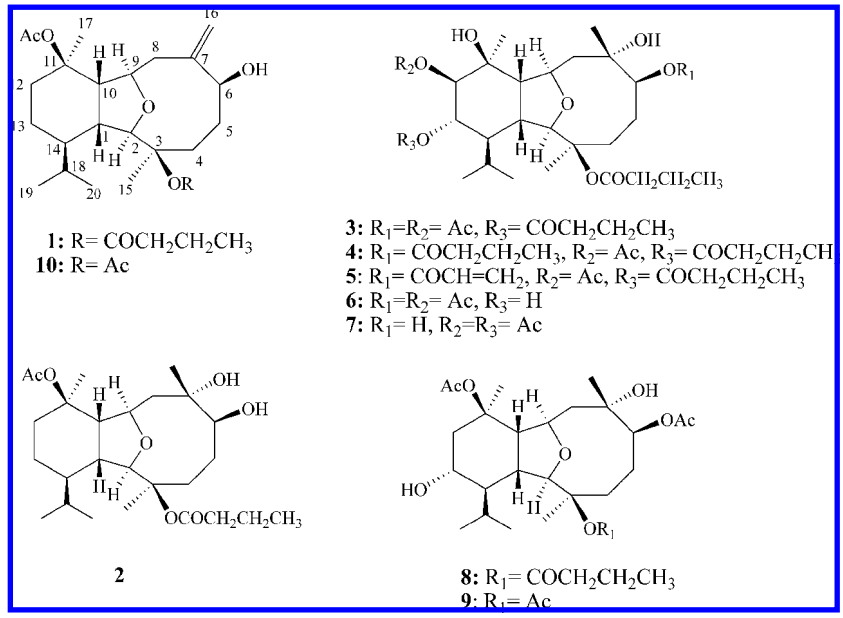

Table 1. ^{13}C NMR Data for Compounds **1**−**6**[a]

C#	1	2	3	4	5	6
1	41.5, CH[b]	42.2, CH	42.9, CH	43.0, CH	43.0, CH	43.1, CH
2	90.5, CH	92.1, CH	92.9, CH	93.0, CH	93.0, CH	92.8, CH
3	84.6, qC	86.0, qC	85.8, qC	85.9, qC	85.9, qC	85.9, qC
4	29.7, CH_2	36.3, CH_2	35.8, CH_2	35.9, CH_2	35.8, CH_2	35.9, CH_2
5	35.4, CH_2	30.5, CH_2	29.1, CH_2	29.1, CH_2	29.1, CH_2	29.1, CH_2
6	73.7, CH	80.6, CH	84.6, CH	84.5, CH	85.0, CH	84.7, CH
7	150.3, qC	77.1, qC	75.6, qC	75.7, qC	75.8, qC	75.7, qC
8	41.3, CH_2	47.6, CH_2	47.5, CH_2	47.5, CH_2	47.5, CH_2	47.7, CH_2
9	78.8, CH	75.6, CH	75.5, CH	75.5, CH	75.5, CH	75.6, CH
10	46.1, CH	53.1, CH	56.5, CH	56.5, CH	56.5, CH	56.8, CH
11	82.3, qC	82.2, qC	72.6, qC	72.7, qC	72.7, qC	72.7, qC
12	32.3, CH_2	31.9, CH_2	76.6, CH	76.7, CH	76.7, CH	79.0, CH
13	18.1, CH_2	17.6, CH_2	70.2, CH	70.2, CH	70.2, CH	69.4, CH
14	43.1, CH	42.6, CH	47.3, CH	47.3, CH	47.3, CH	50.0, CH
15	22.6, CH_3	23.1, CH_3	23.0, CH_3	23.1, CH_3	23.1, CH_3	23.1, CH_3
16	116.8, CH_2	22.8, CH_2	23.7, CH_3	23.8, CH_3	23.9, CH_3	23.7, CH_3
17	25.4, CH_3	24.7, CH_3	25.6, CH_3	25.7, CH_3	25.8, CH_3	25.9, CH_3
18	27.5, CH	29.0, CH	30.1, CH	30.2, CH	30.2, CH	30.8, CH
19	21.7, CH_3	21.8, CH_3	23.3, CH_3	23.3, CH_3	23.4, CH_3	24.5, CH_3
20	15.2, CH_3	15.3, CH_3	16.0, CH_3	16.1, CH_3	16.1, CH_3	15.9, CH_3
6-acetate			21.4, CH_3			21.4, CH_3
			171.9, qC			172.0, qC
11-acetate	22.5, CH_3	22.5, CH_3				
	170.1, qC	170.1, qC				
12-acetate			20.7, CH_3	20.7, CH_3	20.7, CH_3	20.9, CH_3
			169.9, qC	169.9, qC	169.9, qC	171.3, qC
3-*n*-butyrate	13.6, CH_3	13.6, CH_3	13.8, CH_3	13.7, CH_3	13.8, CH_3	13.7, CH_3
	18.5, CH_2	18.6, CH_2	18.2, CH_2	18.5, CH_2	18.3, CH_2	18.3, CH_2
	37.3, CH_2	37.3, CH_2	37.2, CH_2	37.3, CH_2	37.3, CH_2	37.3, CH_2
	172.7, qC	172.6, qC	172.2, qC	172.2, qC	172.2, qC	172.2, qC
6-*n*-butyrate				13.8, CH_3		
				18.3, CH_2		
				36.6, CH_2		
				174.5, 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	36.6, CH_2	36.6, CH_2	
			172.9, qC	172.8, qC	172.8, qC	
6-acrylate					128.8, CH	
					130.7, CH_2	
					166.9, qC	

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

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 (1*R**, 2*R**, 3*R**, 6*S**, 9*R**, 10*S**, 11*R**, 14*R**) configuration. The absolute structure of **1** was finally determined by using a modified Mosher's esterification methed.[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 2. ^{13}C NMR Data for Compounds **7**−**9**[a]

C#	7	8	9
1	44.9, CH[b]	44.2, CH	44.2, CH
2	93.3, CH	93.2, CH	93.1, CH
3	85.8, qC	85.9, qC	86.0, qC
4	36.4, CH_2	36.0, CH_2	35.8, CH_2
5	30.4, CH_2	29.1, CH_2	29.1, CH_2
6	80.5, CH	85.0, CH	84.9, CH
7	77.0, qC	75.8, qC	75.8, qC
8	47.5, CH_2	47.6, CH_2	47.5, CH_2
9	75.7, CH	75.9, CH	76.0, CH
10	56.8, CH	52.0, CH	51.3, CH
11	72.6, qC	83.6, qC	83.6, qC
12	76.7, CH	42.0, CH_2	42.3, CH_2
13	70.5, CH	66.4, CH	66.4, CH
14	47.4, CH	50.2, CH	50.1, CH
15	23.3, CH_3	23.2, CH_3	23.1, CH_3
16	22.7, CH_3	23.8, CH_3	23.8, CH_3
17	25.7, CH_3	24.7, CH_3	24.6, CH_3
18	30.2, CH	30.4, CH	30.4, CH
19	23.4, CH_3	23.8, CH_3	24.5, CH_3
20	16.0, CH_3	16.1, CH_3	16.2, CH_3
3-acetate			22.2, CH_3
			169.8, qC
6-acetate		21.4, CH_3	21.4, CH_3
		172.0, qC	172.0, qC
11-acetate		22.4, CH_3	22.5, CH_3
		169.9, qC	170.1, qC
12-acetate	20.6, CH_3		
	170.0, qC		
13-acetate	21.4, CH_3		
	170.2, qC		
3-*n*-butyrate	13.7, CH_3	13.6, CH_3	
	18.3, CH_2	18.6, CH_2	
	37.2, CH	37.2, CH_2	
	172.1, qC	172.5, qC	

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

respectively. The determination of $\Delta\delta$ 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 $[M + Na]^+$ peak at *m/z* 491.2987 and established a molecular formula of $C_{26}H_{44}O_7$, 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, tq, $J = 7.5, 7.5$ 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 correlations observed from H_3-16 (δ 1.16, 3H, s) to C-6 (δ 80.6, CH), C-7 (δ 77.1, qC), and C-8 (δ 47.6, CH_2). A more detailed 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 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_3-16 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 $[M + Na]^+$ at *m/z* 635.3409 in the HRESIMS, corresponding to the molecular formula $C_{32}H_{52}O_{11}$ and seven degrees of unsaturation. 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 sp^3 oxygenated quaternary carbons. The presence of two acetoxy groups was

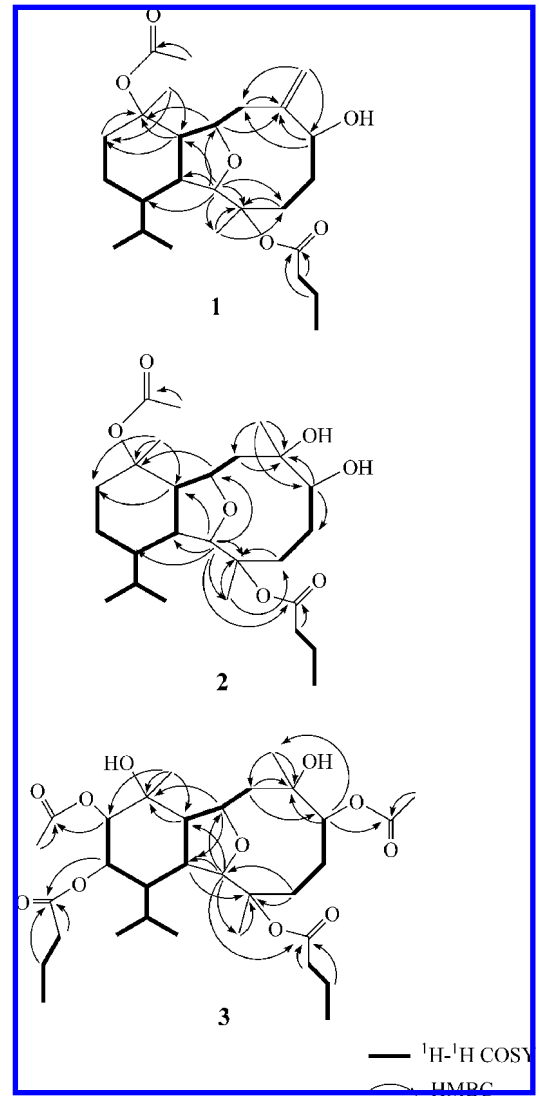

Figure 1. $^1H-^1H$ COSY and HMBC correlations for **1**−**3**.

indicated by the ^1H NMR signals at δ 2.05 (s, 3H) and 2.07 (s, 3H) and the ^{13}C NMR signals at δ 20.7 (CH_3), 21.4 (CH_3), 169.9 (qC), and 171.9 (qC). In addition, the ^1H and ^{13}C NMR spectra of **3** showed the presence of two *n*-butyryloxy groups. Among them one showed signals at δ_H 0.97 (3H, t, $J = 7.5$ Hz), 1.67 (2H, m), 2.25 (1H, m), 2.37 (1H, m) and δ_C 13.8 (CH_3), 18.2 (CH_2), 37.2 (CH_2), 172.2 (qC), and the other resonated at δ_H 0.92 (3H, tq, $J = 7.5, 7.5$ Hz), 1.60 (2H, sex, $J = 7.5$ Hz), 2.19 (2H, dt, $J = 2.0, 7.5$ Hz) and δ_C 13.6 (CH_3), 18.1 (CH_2), 36.6 (CH_2), 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*-butyryloxy 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_3-16 (δ 1.17) and H_3-17 (δ 1.10) determined the positions of two tertiary hydroxy groups at C-7 and C-11. 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

Table 3. ^{1}H NMR Data for Compounds **1**−**5**[a]

H#	1	2	3	4	5
1	2.25, dd (12.0, 7.1)[b]	2.17, dd (11.2, 7.5)	2.38, m	2.41, dd (11.5, 7.5)	2.42, dd (11.0, 7.5)
2	3.57, s	3.49, s	3.53, s	3.55, s	3.56, s
4α	2.14, d (8.5)	1.79, d (14.5)	1.99, dd (13.5, 11.0)	2.02, dd (14.5, 10.0)	2.05, m
4*β*	1.87, m	2.65, dd (14.5, 8.5)	2.62, dd (13.5, 8.5)	2.65, dd (14.5, 8.5)	2.67, m
5α	2.10, m	1.57, m	1.50, dd (16.5, 8.5)	1.52, dd (16.7, 8.5)	1.57, m
5*β*	1.74, m	1.46, m	1.40, m	1.41, m	1.47, dd (10.5, 5.5)
6	4.30, dd (12.5, 3.0)	4.57, d (6.0)	5.58, d (6.0)	5.60, d (5.5)	5.69, d (5.5)
8α	2.82, dd (13.5, 4.5)	1.86, dd (14.5, 3.5)	1.80, dd (14.5, 3.5)	1.82, dd (14.5, 3.5)	1.84, dd (14.5, 4.0)
8*β*	2.48, d (13.5)	1.99, t (13.0)	1.93, dd (14.5, 11.0)	1.95, dd (14.5, 11.0)	1.97, dd (14.5, 11.0)
9	4.10, dd (10.5, 4.5)	4.06, ddd (11.0, 7.5, 3.5)	4.27, ddd (11.0,7.5, 3.5)	4.29, ddd (11.5, 7.5, 3.5)	4.31, ddd (11.0, 7.5, 3.5)
10	3.06, br t (8.8)	3.08, t (7.5)	2.60, t (7.5)	2.63, t (7.5)	2.64, t (7.5)
12	α: 1.44, m *β*: 2.27, d (12.0)	α: 1.44, m *β*: 2.21, d (14.0)	5.00, d (10.0)	5.03, d (9.5)	5.03, d (9.5)
13	α: 1.46, m *β*: 1.35, t (12.0)	α: 1.42, m *β*: 1.33, m	5.49, t (10.0)	5.50, dd (11.5, 9.5)	5.50, dd (11.0, 9.5)
14	1.20, br t (12.0)	1.14, m	1.73, t (11.5)	1.75, m	1.76, t (11.0)
15	1.54, s	1.39, s	1.37, s	1.39, 3H, s	1.40, 3H, s
16	5.21, s; 5.47, s	1.16, s	1.17, s	1.19, 3H, s	1.22, 3H, s
17	1.55, s	1.49, s	1.10, 3H, s	1.17, 3H, s	1.12, 3H, s
18	1.86, m	1.73, m	1.69, m	1.71, m	1.74, m
19	0.95, 3H, d (7.0)	0.95, d (7.0)	0.97, 3H, d (7.0)	0.99, 3H, d (7.0)	0.99, 3H, d (7.0)
20	0.78, 3H, d (7.0)	0.82, d (7.0)	0.94, 3H, d (7.0)	0.96, 3H, d (7.0)	0.96, 3H, d (7.0)
6-acetate			2.07, 3H, s		
11-acetate	2.01, 3H, s	1.99, 3H, s			
12-acetate			2.05, 3H, s	2.07, 3H, s	2.07, 3H, s
3-*n*-butyrate	0.94, 3H, t (7.5) 1.60, 2H, tq (7.5, 7.5) 2.16, 2H, t (7.5)	1.00, 3H, t (7.5) 1.68, 2H, tq (7.5, 7.5) 2.30, m 2.35, m	0.97, 3H, t (7.5) 1.67, 2H, m 2.25, m 2.37, m	0.97, 3H, t (7.5) 1.68, 2H, m 2.27, m 2.39, m	0.99, 3H, t (7.5) 1.68, 2H, m 2.28, m 2.40, m
6-*n*-butyrate				0.94, 3H, t (7.5) 1.66, 2H, m 2.32, 2H, t (7.5)	
13-*n*-butyrate			0.92, 3H, t (7.5) 1.60, 2H, tq (7.5, 7.5) 2.19, 2H, td (7.5, 2.0)	0.96, 3H, t (7.5) 1.61, 2H, tq (7.5, 7.5) 2.21, td (7.5, 2.0)	0.95, 3H, t (7.5) 1.60, 2H, tq (7.5, 7.5) 2.21, td (7.5, 2.0)
6-acrylate					5.84, dd (10.5, 1.5) 6.15, dd (17.5, 10.5) 6.40, dd (17.5, 1.5)

[a] Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C. [b] The *J* values are in Hz in parentheses.

of H-12 and the *β*-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 $C_{34}H_{56}O_{11}$, 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 (δ_H 2.07, 3H, s; δ_C 171.9, qC and 21.4, CH_3) in **3** by an *n*-butyryloxy group in **4** (δ_H 0.94, 3H, t, $J = 7.5$ Hz; 1.66, 2H, m; 2.32, 2H, t, $J = 7.5$ Hz; δ_C 174.5, qC; 36.6, CH_2; δ_C 18.3, CH_2 and 13.8, CH_3). It was further confirmed by the HMBC experiment, which showed a correlation between H-6 and the carbonyl carbon (δ_C 174.5, qC) of an *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-*O*-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 $C_{33}H_{52}O_{11}$, consistent with eight degrees of unsaturation. The IR spectrum of **5** showed the presence of hydroxy (ν_{max} 3478 cm^{-1}), carbonyl (ν_{max} 1733 cm^{-1}), and double-bond (ν_{max} 1636 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 (δ_H 5.84, dd, $J = 10.5$, 1.5 Hz; 6.15, dd, $J = 17.5$, 10.5 Hz; 6.40, dd, $J = 17.5$, 1.5 Hz; δ_C 166.9, qC; 128.8, CH and 130.7 CH_2) in **5**. The attachment of this acrylate at C-6 was confirmed by the HMBC correlation between H-6 and the carbonyl carbon (δ_C 166.9, qC) of the acrylate. H-6 exhibited an NOE interaction with H-4α, revealing the *β*-orientation of the acrylate. A detailed analysis of other key NOE correlations further showed that **5** has the same relative configuration as those of **3** and **4**. A structurally similar metabolite, simplexin F (**6**), was also isolated 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 G (**7**) was obtained as a white powder. Its molecular formula, $C_{28}H_{46}O_{10}$, was determined by HRESIMS (*m*/*z* 565.2991 $[M + Na]^+$). The NMR spectra of **7** (Tables 2 and 4) showed the presence of two acetoxy and one *n*-butyryloxy group. The HMBC correlations of H-12 (δ 5.01) and H-13 (δ 5.47) with the carbonyl carbons resonating at δ 170.0 (qC) and 170.2 (qC), respectively, revealed the location of two acetoxy groups at C-12 and C-13. Thus, **7** is the 6-*O*-deacetyl-13-*O*-acetyl derivative of **6**.

Simplexin H (**8**) was isolated as a white powder and has a molecular formula of $C_{28}H_{46}O_9$, 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 oxymethine carbon bearing a hydroxy group (δ_H 3.88, 1H m; δ_C 66.4, CH) in **8**. Also, H-6 and C-6 of **8** were downfield shifted to δ 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 (δ_H 2.08, 3H, s) to a carbonyl carbon (δ 172.0, qC). Thus, the planar structure of **8** was fully established. The relative configuration of **8** was also deduced using a NOESY spectrum and was found to be similar to that of **2**. In addition, H-13 shows NOE

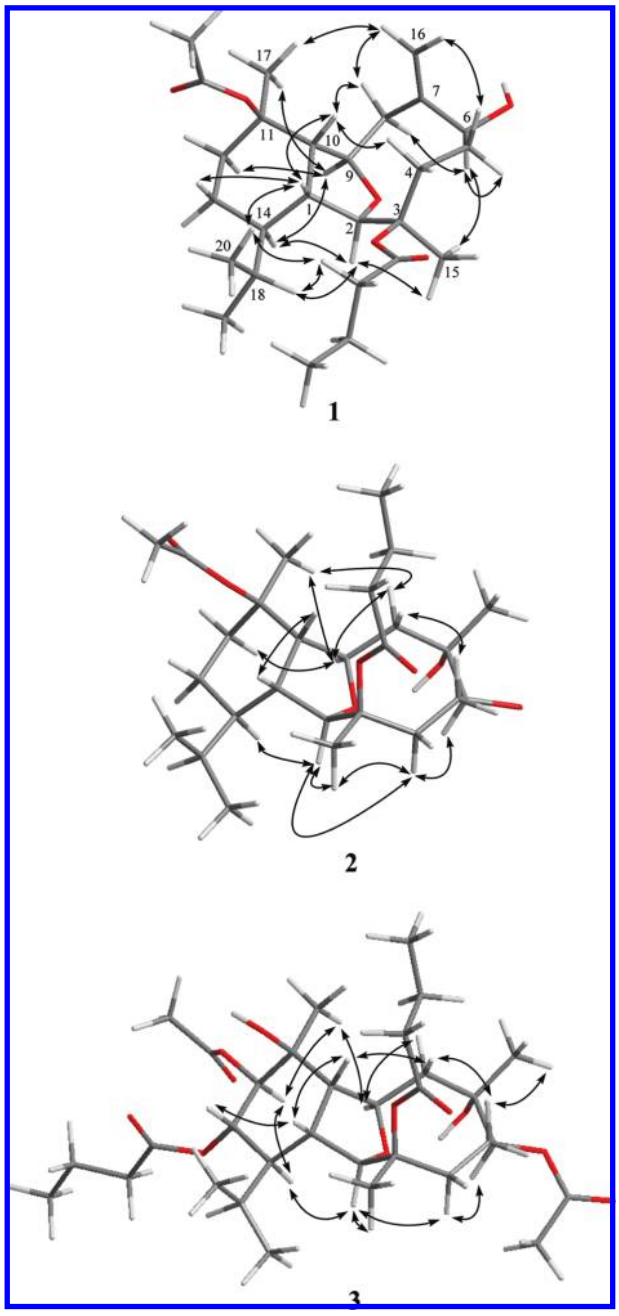

Figure 2. Selective NOE correlations for **1**−**3**.

response with H-1 and H-12β, but not with H-14 (Figure 2), revealing the β-orientation of H-13. Therefore, the structure of **8** was found to possess the (1*R**, 2*R**, 3*R**, 6*S**, 7*S**, 9*R**, 10*S**, 11*R**, 13*R**, 14*R**) configuration.

Simplexin I (**9**) was assigned the molecular formula $C_{26}H_{42}O_9$ 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 (δ_C 169.8, qC; 170.1, qC; 172.0, qC; 21.4, CH_3; 22.2, CH_3 and 22.5, CH_3; δ_H 2.00, 3H, s; 2.09, 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 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 epitheloid carcinoma (HeLa), and human laryngeal carcinoma (Hep2) was studied. The results (Table 5) showed that

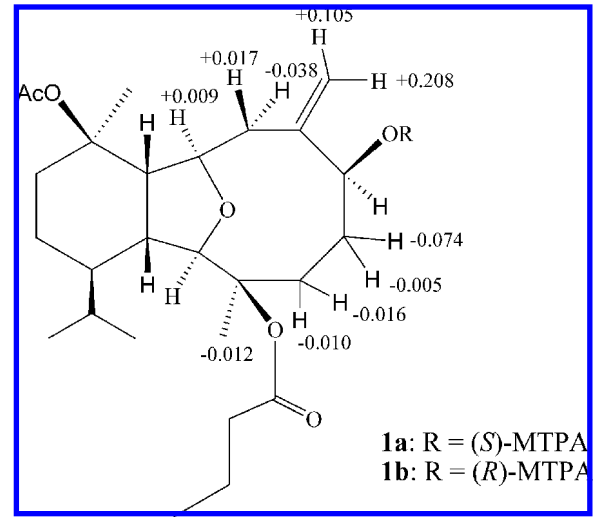

Figure 3. H NMR chemical shift differences $\Delta\delta$ ($\delta_S - \delta_R$) in ppm for the MTPA esters of **1**.

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.

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 spectrometer. 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 or on a Varian 400 MR FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively, in $CDCl_3$ 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 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).

Animal Materials. *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 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 (1:6) 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

Table 4. ^{1}H NMR Data for Compounds **6**−**9**[a]

H#	6	7	8	9
1	2.33, dd (11.5, 7.0)[b]	2.38, m	2.13, dd (10.8, 7.0)	2.11, m
2	3.54, s	3.55, s	3.55, s	3.54, s
4α	2.00, dd (15.0, 10.0)	1.82, m	1.99, m	1.99, m
4*β*	2.64, dd (15.0, 7.5)	2.65, dd (13.8, 9.0)	2.64, dd (14.5, 8.0)	2.62, dd (14.5, 8.5)
5α	1.52, dd (16.5, 8.5)	1.59, m	1.54, m	1.51, m
5*β*	1.43, m	1.42, m	1.45, m	1.44, m
6	5.60, d (6.0)	4.56, d (6.0)	5.60, d (5.5)	5.60, d (5.5)
8α	1.83, dd (14.5, 4.0)	1.86, m	1.86, dd (15.0, 3.5)	1.87, dd (15.0, 4.0)
8*β*	1.94, dd (14.5, 11.0)	1.95, m	2.00, m	2.00, m
9	4.26, ddd (11.0, 7.5, 4.0)	4.24, m	4.12, ddd (11.0, 7.5, 3.5)	4.11, ddd (11.0, 8.0, 3.5)
10	2.59, t (7.5)	2.60, t (7.0)	3.17, t (7.5)	3.26, t (7.5)
12	4.87, d (9.5)	5.01, d (10.0)	α: 1.48, m *β*: 2.32, m	α: 1.46, m *β*: 2.26, dd (13.0, 2.5)
13	2.99, dd (11.5, 9.5)	5.47, t (10.0)	3.88, m	3.90, td (11.0, 3.5)
14	1.44, t (11.5)	1.71, m	1.22, m	1.19, m
15	1.40, 3H, s	1.40, 3H, s	1.39, 3H, s	1.41, 3H, s
16	1.18, 3H, s	1.16, 3H, s	1.19, 3H, s	1.20, 3H, s
17	1.15, 3H, s	1.12, 3H, s	1.51, 3H, s	1.50, 3H, s
18	1.74, m	1.69, m	1.71, m	1.72, m
19	1.16, 3H, d (7.0)	0.99, 3H, d (7.0)	1.18, 3H, d (7.0)	1.18, 3H, d (6.5)
20	1.03, 3H, d (7.0)	0.96, 3H, d (7.0)	1.00, 3H, d (7.0)	1.00, 3H, d (6.5)
3-acetate				2.10, 3H, s
6-acetate	2.08, 3H, s		2.08, 3H, s	2.09, 3H, s
11-acetate			1.98, 3H, s	2.00, 3H, s
12-acetate	2.18, 3H, s	2.18, 3H, s		
13-acetate		2.08, 3H, s		
3-*n*-butyrate	0.99, 3H, t (7.5)	0.98, 3H, t (7.0)	0.99, 3H, t (7.5)	
	1.69, 2H, m	1.67, 2H, m	1.67, 2H, m	
	2.28, m	2.27, m	2.30, m	
	2.37, m	2.39, m	2.34, m	

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

Table 5. Cytotoxicity (ED_{50} μg/mL) of Compounds **1**−**6** and **9**

compound	Daoy	MCF-7	HeLa	Hep2
1	10.37	12.06	(−)[a]	12.10
2	(−)	(−)	(−)	(−)
3	(−)	(−)	(−)	(−)
4	15.34	14.54	(−)	(−)
5	12.76	7.19	17.36	10.72
6	(−)	(−)	(−)	(−)
9	(−)	(−)	(−)	(−)
mitomycin	0.09	0.14	0.08	0.02

[a] (−): $ED_{50} > 20$ μg/mL.

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−CH_2Cl_2 (1:30) to afford **3** (37.2 mg).

Simplexin A (1): colorless oil (4.6 mg); $[\alpha]_D^{26}$ −8.9 (*c* 0.8, $CHCl_3$); IR (neat) ν_{max} 3432 (broad) and 1723 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 3; ESIMS *m*/*z* 473 [100, (M + Na)$^+$]; HRESIMS *m*/*z* 473.2879 (calcd for $C_{26}H_{42}O_6Na$, 473.2877).

Simplexin B (2): colorless oil (8.0 mg); $[\alpha]_D^{26}$ +26 (*c* 0.7, $CHCl_3$); IR (neat) ν_{max} 3442 (broad) and 1731 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 3; ESIMS *m*/*z* 491 [100, (M + Na)$^+$], 451 (16); HRESIMS *m*/*z* 491.2987 (calcd for $C_{26}H_{44}O_7Na$, 491.2985).

Simplexin C (3): white powder (37.2 mg); mp 93.0−94.0 °C; $[\alpha]_D^{26}$ +29.7 (*c* 0.64, $CHCl_3$); IR (KBr) ν_{max} 3478 (broad) and 1732 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 3; ESIMS *m*/*z* 635 [100, (M + Na)$^+$], 381 (21); HRESIMS *m*/*z* 635.3409 (calcd for $C_{32}H_{52}O_{11}Na$, 635.3407).

Simplexin D (4): colorless oil (10.1 mg); $[\alpha]_D^{26}$ +9.0 (*c* 1.0, $CHCl_3$); IR (neat) ν_{max} 3489 (broad) and 1732 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 3; ESIMS *m*/*z* 663 [100, (M + Na)$^+$]; HRESIMS *m*/*z* 663.3718 (calcd for $C_{34}H_{56}O_{11}Na$, 663.3720).

Simplexin E (5): colorless oil (10.4 mg); $[\alpha]_D^{26}$ +14.0 (*c* 2.3, $CHCl_3$); IR (neat) ν_{max} 3478 (broad), 1733 and 1636 cm^{-1}; UV (MeOH) λ_{max} 216 (log ε = 3.50); ^{1}H and ^{13}C NMR data, see Tables 1 and 3; ESIMS *m*/*z* 647 [100, (M + Na)$^+$]; HRESIMS *m*/*z* 647.3410 [M + Na]$^+$ (calcd for $C_{33}H_{52}O_{11}Na$, 647.3407).

Simplexin F (6): white powder (9.6 mg); mp 113.0−113.5 °C; $[\alpha]_D^{26}$ +18.1 (*c* 2.68, $CHCl_3$); IR (neat) ν_{max} 3450 (broad) and 1728 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 4; ESIMS *m*/*z* 565 [100, (M + Na)$^+$]; HRESIMS *m*/*z* 565.2987 (calcd for $C_{28}H_{46}O_{10}Na$, 565.2989).

Simplexin G (7): white powder (1.6 mg); mp 101.5−102.5 °C; $[\alpha]_D^{26}$ +34 (*c* 0.4, $CHCl_3$); IR (neat) ν_{max} 3451 (broad) and 1734 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 4; ESIMS *m*/*z* 565 [100, (M + Na)$^+$]; HRESIMS *m*/*z* 565.2991 (calcd for $C_{28}H_{46}O_{10}Na$, 565.2989).

Simplexin H (8): white powder (2.8 mg); mp 88.0−89.0 °C; $[\alpha]_D^{26}$ +30 (*c* 0.6, $CHCl_3$); IR (neat) ν_{max} 3449 (broad) and 1730 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 4; ESIMS *m*/*z* 549 [100, (M + Na)$^+$], 467 (16); HRESIMS *m*/*z* 549.3042 (calcd for $C_{28}H_{46}O_9Na$, 549.3039).

Simplexin I (9): white powder (8.0 mg); mp 85.5−86.0 °C; $[\alpha]_D^{26}$ +28.6 (*c* 1.5, $CHCl_3$); IR (neat) ν_{max} 3452 (broad) and 1728 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 4; ESIMS *m*/*z* 521 [100, (M + Na)$^+$], 439 (23), 381 (37), 353 (11); HRESIMS *m*/*z* 521.2727 (calcd for $C_{26}H_{42}O_9Na$, 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 H_2O, followed by extraction with EtOAc (1.0 mL × 3). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$, 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 ^{1}H NMR ($CDCl_3$, 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-9), 3.146 (1H, dd, *J* = 14.0, 5.2 Hz, H-8), 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-15). Selected ^{1}H

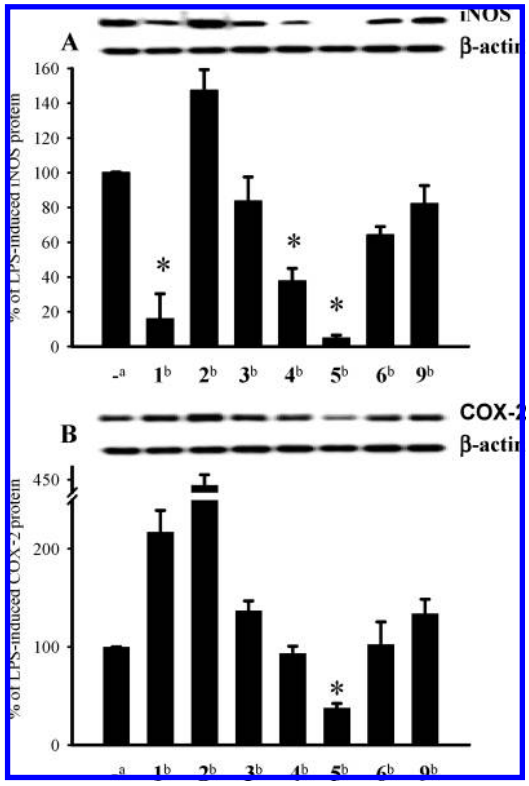

Figure 4. Effect of compounds **1**−**6** and **9** on iNOS and COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis. (A) Immunoblots of iNOS and β-Actin; (B) immunoblots of COX-2 and β-actin. The values are mean ± SEM ($n = 6$). Relative intensity of the LPS alone stimulated 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).

NMR ($CDCl_3$, 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-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[16,17]

***In Vitro* Anti-inflammatory Assay.** Assay procedure was as previously reported.[18]

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Supporting Information Available: The ^1H and ^{13}C NMR spectra of **1**−**9** are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Kennard, O.; Watson, D. G.; Riva di Sanseverino, L.; Tursch, B.; Bosmans, R.; Djerassi, C. *Tetrahedron Lett.* **1968**, *24*, 2879–2884.
(2) Lin, Y.; Bewely, C. A.; Faulkner, D. J. *Tetrahedron* **1993**, *49*, 7977–7984.
(3) Chill, L.; Berrer, N.; Benayahu, Y.; Kashman, Y. *J. Nat. Prod.* **2005**, *68*, 19–25.
(4) Ahmed, A. F.; Wu, M.-H.; Wang, G.-H.; Wu, Y.-C.; Sheu, J.-H. *J. Nat. Prod.* **2005**, *68*, 1051–1055.
(5) Chen, S.-P.; Sung, P.-J.; Duh, C.-Y.; Dai, C.-F.; Sheu, J.-H. *J. Nat. Prod.* **2001**, *64*, 1241–1242.
(6) Kakonikos, C.; Vagias, C.; Roussis, V. *Nat. Prod. Lett.* **1999**, *13*, 89–95.
(7) Ortega, M. J.; Zubia, E.; Salva, J. *J. Nat. Prod.* **1994**, *57*, 1584–1586.
(8) Su, J.-H.; Huang, H.-C.; Chao, C.-H.; Yan, L.-Y.; Wu, Y.-C.; Wu, C.-C.; Sheu, J.-H. *Bull. Chem. Soc. Jpn.* **2005**, *78*, 877–879.
(9) Sharma, P.; Alam, M. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2537–2540.
(10) Wang, G.-H.; Sheu, J.-H.; Duh, C.-Y.; Chiang, M. Y. *J. Nat. Prod.* **2002**, *65*, 1475–1478.
(11) Fusetani, N.; Nagata, H.; Hirota, H.; Tsuyuki, T. *Tetrahedron Lett.* **1989**, *30*, 7079–7082.
(12) Miyamoto, T.; Yamada, K.; Ikeda, N. *J. Nat. Prod.* **1994**, *57*, 1212–1219.
(13) Ospina, C. A.; Rodriguez, A. D. *J. Nat. Prod.* **2006**, *69*, 1721–1727.
(14) Wang, G.-H.; Sheu, J.-H.; Chiang, M. Y.; Lee, T.-J. *Tetrahedron Lett.* **2001**, *42*, 2333–2336.
(15) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.
(16) Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 589–601.
(17) Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827–4833.
(18) Lu, Y.; Huang, C.-Y.; Lin, Y.-F.; Wen, Z.-H.; Su, J.-H.; Kuo, Y.-H.; Chiang, M. Y.; Sheu, J.-H. *J. Nat. Prod.* **2008**, *71*, 1754–1759.

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