Hirsutalins A−H, Eunicellin-Based Diterpenoids from the Soft Coral *Cladiella hirsuta*

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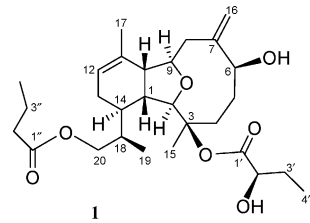
Eight new eunicellin-base diterpenoids, hirsutalins A−H (**1**−**8**), were isolated from the soft coral *Cladiella hirsuta*. Their structures were elucidated by spectroscopic methods, particularly in 1D and 2D NMR experiments. The absolute configuration of **1** was determined by Mosher's method. Compounds **1**, **5**, and **6** have been shown to exhibit cytotoxicity toward several cancer cell lines. Compounds **2**−**4** and **8** were found to display significant *in vitro* anti-inflammatory activity in LPS-stimulated RAW264.7 macrophage cells by inhibiting the expression of the iNOS protein, with compound **2** also effectively reducing the level of COX-2 protein.

Previous reports on the chemical constituents of soft corals belonging to the genus *Cladiella*[1−11] have illustrated the predominance of eunicellin-based diterpenoids as secondary metabolites in these marine organisms. In continuation of our investigation of bioactive eunicellins from marine invertebrates,[12−17] a study of the chemical constituents of the soft coral *Cladiella hirsuta* was carried out. This study led to the isolation of eight new eunicellin-based metabolites, hirsutalins A−H (**1**−**8**). The structures of **1**−**8** were established by extensive spectroscopic analysis, including 2D NMR (^{1}H−^{1}H COSY, HSQC, HMBC, and NOESY). The absolute cofiguration of **1** was further determined by application of Mosher's method. The cytotoxicity of metabolites **1**−**8** against a variety 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) was investigated. The ability of **1**−**8** 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. Herein, we report the isolation, structure elucidation, and bioactivities of these natural products.



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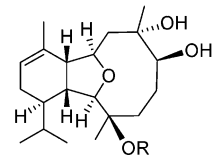

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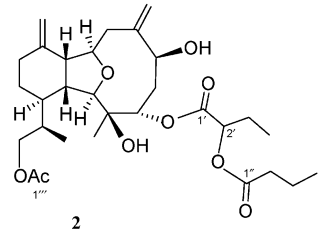
9:R=H



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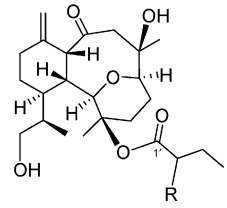
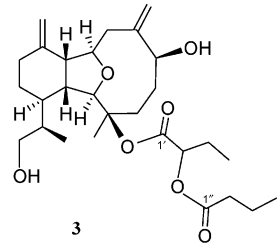


6:R=$OCOCH_2CH_2CH_3$
10:R=H



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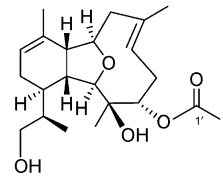

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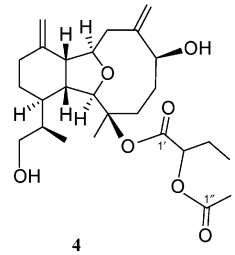

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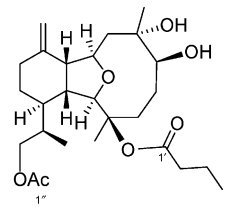

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Results and Discussion

The frozen bodies of the octocoral *C. hirsuta* were minced and extracted exhaustively with acetone. The combined organic extract was concentrated to an aqueous suspension, which was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was concentrated under reduced pressure, and the residue was repeatedly purified by chromatography to yield metabolites **1**−**8**. The absolute configuration of compound **1** was completely assigned on the basis of NOE correlations and Mosher ester analysis. Compounds **2**−**8** are likely in the same enantiomeric series as **1** on the basis of a shared biosynthetic pathway.

Hirsutalin A (**1**) was isolated as a colorless oil. The HRESIMS spectrum of **1** exhibited an $[M + Na]^+$ peak at *m/z* 515.2981, which established a molecular formula of $C_{28}H_{44}O_7$, implying seven degrees of unsaturation. The IR spectrum of **1** revealed the presence of hydroxy and carbonyl functionalities from absorptions at 3417 and 1733 cm^{-1}, respectively. The ^{13}C NMR spectroscopic data of

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Table 1. ^{13}C NMR Spectroscopic Data for Compounds **1**−**8**

position	**1**[a]	**2**[b]	**3**[a]	**4**[c]	**5**[b]	**6**[a]	**7**[a]	**8**[a]
1	40.5, CH[d]	43.1, CH	43.6, CH	43.7, CH	40.3, CH	54.4, CH	39.5, CH	45.0, CH
2	88.3, CH	91.3, CH	90.6, CH	90.6, CH	86.7, CH	77.4, CH	89.8, CH	92.3, CH
3	86.9, C	73.6, C	86.3, C	86.0, C	86.1, C	83.0, C	77.0, C	86.3, C
4	27.0, CH_2	74.5, CH	28.1, CH_2	28.1, CH_2	31.4, CH_2	27.8, CH_2	74.1, CH	36.2, CH_2
5	33.2, CH_2	41.0, CH_2	35.4, CH_2	29.7, CH_2	30.4, CH_2	19.6, CH_2	29.0, CH_2	30.5, CH_2
6	73.0, CH	69.9, CH	72.3, CH	86.3, CH	75.9, CH	80.8, CH	123.4, CH	80.3, CH
7	149.8, C	149.0, C	150.7, C	146.3, C	75.8, C	85.0, C	131.5, C	76.9, C
8	41.4, CH_2	38.1, CH_2	38.9, CH_2	39.6, CH_2	46.7, CH_2	48.1, CH_2	44.4, CH_2	45.7, CH_2
9	82.8, CH	80.3, CH	80.1, CH	79.9, CH	76.9, CH	211.1, C	81.2, CH	78.4, CH
10	44.5, CH	46.8, CH	47.2, CH	47.5, CH	48.4, CH	56.7, CH	46.8, CH	53.7, CH
11	131.1, C	145.4, C	145.5, C	145.4, C	132.2, C	147.2, C	132.7, C	147.0, C
12	122.3, CH	31.7, CH_2	31.5, CH_2	31.4, CH_2	121.9, CH	31.1, CH_2	121.2, CH	31.2, CH_2
13	23.4, CH_2	25.8, CH_2	25.9, CH_2	25.9, CH_2	22.8, CH_2	26.1, CH_2	22.1, CH_2	25.2, CH_2
14	34.8, CH	39.8, CH	38.8, CH	38.6, CH	39.3, CH	32.8, CH	32.6, CH	38.8, CH
15	21.9, CH_3	23.4, CH_3	22.1, CH_3	22.2, CH_3	23.4, CH_3	23.3, CH_3	22.3, CH_3	23.3, CH_3
16	116.3, CH_2	117.8, CH_2	116.8, CH_2	118.0, CH_2	22.8, CH_3	23.0, CH_3	19.1, CH_3	22.7, CH_3
17	23.1, CH_3	111.9, CH_2	111.7, CH_2	111.8, CH_2	21.9, CH_3	109.5, CH_2	21.9, CH_3	109.8, CH_2
18	32.6, CH	32.7, CH	36.2, CH	36.2, CH	29.0, CH	35.9, CH	36.4, CH	34.1, CH
19	12.2, CH_3	10.3, CH_3	10.7, CH_3	10.6, CH_3	21.5, CH_3	9.9, CH_3	15.7, CH_3	10.8, CH_3
20	67.8, CH_2	68.1, CH_2	66.5, CH_2	66.5, CH_2	20.0, CH_3	67.3, CH_2	66.3, CH_2	67.8, CH_2
1′	174.4, C	170.2, C	169.1, C	169.0, C	172.5, C	169.4, C	171.4, C	172.2, C
2′	71.3, CH	73.8, CH	73.7, CH	73.9, CH_2	37.4, CH_2	74.2, CH	21.4, CH_3	37.3, CH_2
3′	27.9, CH_2	24.4, CH_2	24.4, CH_2	24.4, CH_2	18.4, CH_2	24.5, CH_2		18.4, CH_2
4′	8.8, CH_3	9.4, CH_3	9.6, CH_3	9.6, CH_3	13.6, CH_3	9.7, CH_3		13.7, CH_3
1″	173.7, C	173.6, C	173.4, C	170.8, C		173.6, C		171.2, C
2″	36.2, CH_2	35.7, CH_2	35.8, CH_2	20.6, CH_3		35.7, CH_2		21.1, CH_3
3″	18.5, CH_2	18.3, CH_2	18.3, CH_2			18.4, CH_2		
4″	13.7, CH_3	13.6, CH_3	13.6, CH_3			13.6, CH_3		
1‴		171.3, C						
2‴		21.0, CH_3						

[a] Spectra recorded at 100 MHz in $CDCl_3$ at 25 °C. [b] Spectra recorded at 75 MHz in $CDCl_3$ at 25 °C. [c] Spectra recorded at 125 MHz in $CDCl_3$ at 25 °C. [d] Multiplicities deduced by DEPT.

Table 2. ^1H NMR Data for Compounds **1**−**5**

position	**1**[a]	**2**[b]	**3**[a]	**4**[c]	**5**[b]
1	2.35, dd (7.6, 2.8)[c,d]	2.49, m	2.33, m	2.31, m	2.59, dd (12.4, 6.2)
2	3.84, d (2.8)	3.78, s	3.71, s	3.71, s	3.89, d (6.2)
4	2.35, m	4.97, m	2.29, m	2.30, m	2.22, m
	1.82		1.83, m	1.91, m	2.15, m
5	2.25, m	2.45, m	2.14, m	2.16, m	1.85, m
	1.75, m	1.89, m	1.75, m	1.58, m	1.56, m
6	4.34, dd (9.6, 3.6)	4.43, m	4.37, dd (10.8, 4.4)	4.72, dd (8.8, 3.5)	4.52, br d (8.5)
8	α 2.79, dd (14.0, 4.8)	α 2.65, m	α 2.77, dd (13.2, 4.8)	2.75, dd (13.5, 5.0)	1.87, m
	β 2.47, d (14.0)	*β* 2.32, m	*β* 2.27, d (13.6)	2.34, d (14.0)	1.70, m
9	4.08, m	4.15, m	4.16, dd (10.8, 4.4)	4.16, dd (10.5, 5.0)	4.31, br d (9.5)
10	2.73, t (7.6)	2.99, t (9.5)	3.11, dd (10.0, 8.4)	3.08, dd (10.5, 8.0)	2.32, m
12	5.50, m	2.26, m	*β*: 2.27, m	*β*: 2.29, m	5.44, m
		2.05, m	α: 2.08, m	α: 2.12, m	
13	1.93,m	1.65, m	1.66, m	1.68, m	2.11, m
	1.89, m	1.12, m	1.12, m	1.12, m	1.97, m
14	1.63, m	1.50, m	1.65, m	1.65, m	1.38, m
15	1.67, s	1.33, s	1.61, s	1.61, s	1.63, s
16	5.45, s	5.61, s	5.48, s	5.48, s	1.16, s
	5.17, s	5.17, s	5.12, s	5.23, s	
17	1.73, s	4.82, s	4.83, s	4.84, s	1.67, s
		4.72, s	4.69, s	4.71, s	
18	2.08, m	2.04, m	1.89, m	1.86, m	1.60, m
19	0.84, d (6.8)	0.75, d (6.8)	0.81, d (6.8)	0.81, d (7.0)	0.96, d (7.5)
20	4.01, d (6.8)	3.93, d (7.5)	3.54, d (7.2)	3.54, d (6.5)	0.86, d (6.6)
	3.97, d (6.8)				
2′	3.95, t (4.8)	4.84, t (6.9)	4.64, t (7.2)	4.64, dd (7.0, 5.5)	2.22, m
3′	1.73, m; 1.54, m	1.90, m	1.79, m	1.80, m	1.65, m
4′	0.96, t (7.6)	1.02, t (7.5)	0.98, t (7.5)	0.99, t (7.5)	0.94, t (7.6)
2″	2.29, m	2.37, m	2.38, m	2.13, s	
3″	1.66, m	1.66, m	1.68, m		
4″	0.95, t (7.6)	0.95, t (7.4)	0.97, t (7.2)		
2‴		2.05, s			

[a] Spectra recorded at 400 MHz in $CDCl_3$ at 25 °C. [b] Spectra recorded at 300 MHz in $CDCl_3$ at 25 °C. [c] Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C. [d] *J* values (in Hz) in parentheses.

1 included 28 carbon signals (Table 1), which were assigned by the assistance of a DEPT spectrum to five methyls, nine methylenes (including one exomethylene), nine methines (including four oxymethines and one vinylic methine), two sp^2 ester carbonyls, and one sp^3 and two sp^2 quaternary carbons. The ^{13}C NMR data for **1** (Table 1) revealed one trisubstituted and one 1,1-disubstituted

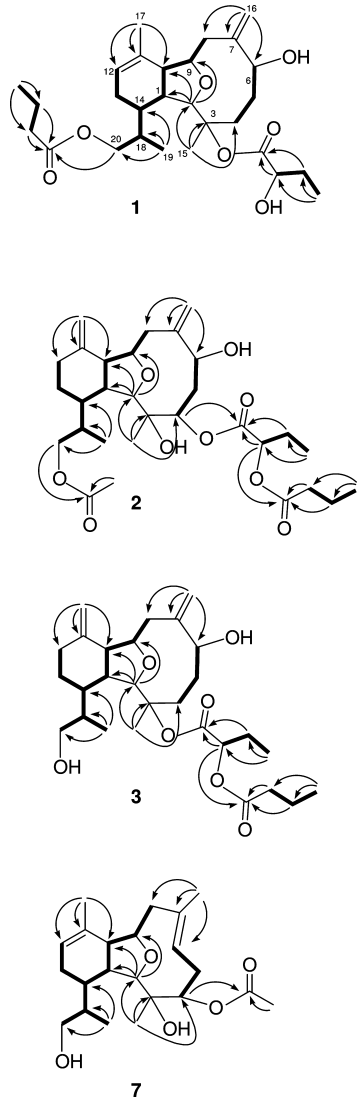


Figure 1. ^{1}H−^{1}H COSY (solid lines) and HMBC correlations (arrows) for **1**−**3** and **7**.

carbon−carbon double bond [δ_C 149.8 (C) and 116.3 (CH_2); 131.1 (C) and 122.3 (CH)]. Two ester carbonyls (δ_C 174.4 and 173.7) were also assigned from the ^{13}C NMR spectrum and were correlated by an HMBC spectrum with the methine and methylene protons (δ_H 3.95 t, J = 4.8 Hz, 1H; δ_H 1.73 and 1.54 m, 2H) of a 2-hydroxybutyrate group and with two methylenes (δ_H 2.29 m, 2H and 1.66 m, 2H) of an *n*-butyrate, 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 1.73 and 1.67 were assigned to one olefinic methyl and one methyl bonded to a quaternary oxygenated carbon, respectively. Signals resonating at δ_H 2.35 (1H, dd, J = 7.6, 2.8 Hz), 2.73 (1H, t, J = 7.6), 3.84 (1H, d, J = 2.8), and 4.08 (1H, m) and at δ_C 40.5, 44.5, 88.3, and 82.8 indicated the presence of a tetrahydrofuran structural unit.[15,16] 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_2-20 to C-14 and C-18 permitted the assembly of the carbon skeleton. The placement of an *n*-butyrate at C-20 was proven by the HMBC correlation from H_2-20 (δ 4.01 and 3.97) to the carbonyl carbon resonating at δ 173.7 (C). The downfield chemical shifts for H_3-15 (δ 1.67) and C-3 (δ 86.9) determined the C-3 location of the 2-hydroxybutyrate. 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 one proton (δ 2.47) at C-8 and H-1, and of H-1 with H_3-19, suggested that H-1, H-10, and this H-8 are β-oriented. Also, correlations of H-2 with both H_3-15 and H-14; H-9 with H-14; and H-6 with both H-8α (δ 2.79) and H_3-15 suggested that H-2, H-6, H-9, H-14, and H_3-15 are all α-oriented. Furthermore, the asymmetric center at C-18 was suggested to be *R**-configured on the basis of NOE correlations between the β-oriented H-1 and H_3-19 and between the α-oriented H-2 and H-18. Thus, the relative configuration of diterpenoid **1** was established. The absolute configurations of C-6 and the 2-hydroxybutyrate functionality were determined using Mosher's method.[18,19] The (*S*)- and (*R*)-MTPA esters of **1** (**1a** and **1b**, respectively) were prepared using the corresponding (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chlorides, respectively. The determination of the chemical shift differences ($\delta_S - \delta_R$) for the protons neighboring C-6 and 2-hydroxybutyrate led to the assignment of *S* and *R* configurations for C-6 and the α-carbon of 2-hydroxybutyrate of **1**, respectively (Figure 3). On the basis of these results, **1** was found to possess the 1*R*, 2*R*, 3*R*, 6*S*, 9*R*, 10*R*, 14*R*, 18*R*, 2′*R* configuration.

Hirsutalin B (**2**) was obtained as a colorless oil. Its molecular formula, $C_{30}H_{46}O_9$, was established by HRESIMS (*m*/*z* 573.3036, $[M + Na]^+$). Thus, **2** has eight degrees of unsaturation. Its IR spectrum exhibited strong absorptions at 3503 and 1738 cm^{-1}, indicative of hydroxy and ester carbonyl groups. The 3H singlet appearing at δ 2.05 in the ^{1}H NMR spectrum and the carbonyl signal at δ 171.3 in the ^{13}C NMR spectrum were ascribable to an acetate. Moreover, two ester carbonyl carbons (δ_C 173.6 and 170.2) were correlated in the HMBC spectrum with the methine proton (δ_H 4.84 t, J = 6.9 Hz) of a 2-butyryloxybutanoate unit. Comparison of the NMR data (Tables 1 and 3) of **2** with those of **1** showed the appearance of one additional 1,1-disubstituted double bond in **2**. The position of the acetate at C-20 was confirmed by the HMBC correlations of the acetate methyl (δ_H 2.05 s, 3H) and H_2-20 (δ 3.93) with the carbonyl carbon resonating at δ_C 171.3 (C). Also, the location of one 2-butyryloxybutanoate unit at C-4 was proven from the HMBC correlation of H-4 (δ 4.97) to the carbonyl carbon resonating at δ_C 173.6 (C). A more detailed analysis of the ^{1}H and ^{13}C NMR spectroscopic data and the observed 2D correlations in the ^{1}H−^{1}H COSY and HMBC spectra (Figure 1) led to the establishment of the planar structure of **2**. Furthermore, the analysis of NOE correlations of **2** revealed the same relative configuration at C-1, C-2, C-3, C-6, C-9, C-10, C-14, and C-18 as that of **1**. The H-4 proton was found to exhibit an NOE correlation with both H-1 and H-10, revealing the α-orientation of the 2-butyryloxybutanoate at C-4.

The HRESIMS data for **3** (*m*/*z* 515.2981 $[M + Na]^+$) established the molecular formula $C_{28}H_{44}O_7$. Thus, **1** and **3** have the same molecular formula. The NMR data of **3** were found to be very similar to those of **2**, except that a hydroxy group at C-3 and the 2-butyryloxybutanoate at C-4 in **2** were replaced by a 2-butyryloxybutanoate and a methylene proton in **3**, respectively, as confirmed by the upfield shifted δ_C value of C-3 (δ_C 73.6) of **2** relative to that of **3** (δ_C 86.3) and the HMBC correlations from H_3-15 (δ 1.61) to C-2 (δ 90.6, CH), C-3 (δ 86.3, C), and C-4 (δ 28.1, CH_2). Also, the NMR chemical shifts for H_2-20 and C-20 of **3** (δ_H 3.54, δ_C 66.5) were found to be shifted upfield in comparison with the analogous data of **2** (δ_H 3.93, δ_C 68.1), suggesting that the acetoxy group of **2** was replaced by an hydroxy group in **3**. The assignments of ^{1}H and ^{13}C NMR spectroscopic data of **3** were assisted by a series of 2D NMR (^{1}H−^{1}H COSY, HMQC, and HMBC) experiments (Figure 1). The relative configuration of **3** was confirmed to be identical to that of **1** by the NOE correlations, with the exception that the configuration of C-2′ was not established.

Metabolite **4** was also isolated as a colorless oil with a molecular formula of $C_{26}H_{40}O_7$. The NMR spectroscopic data of **4** (Tables 1 and 2) showed the presence of a 2-acetoxybutanoate moiety [δ_C

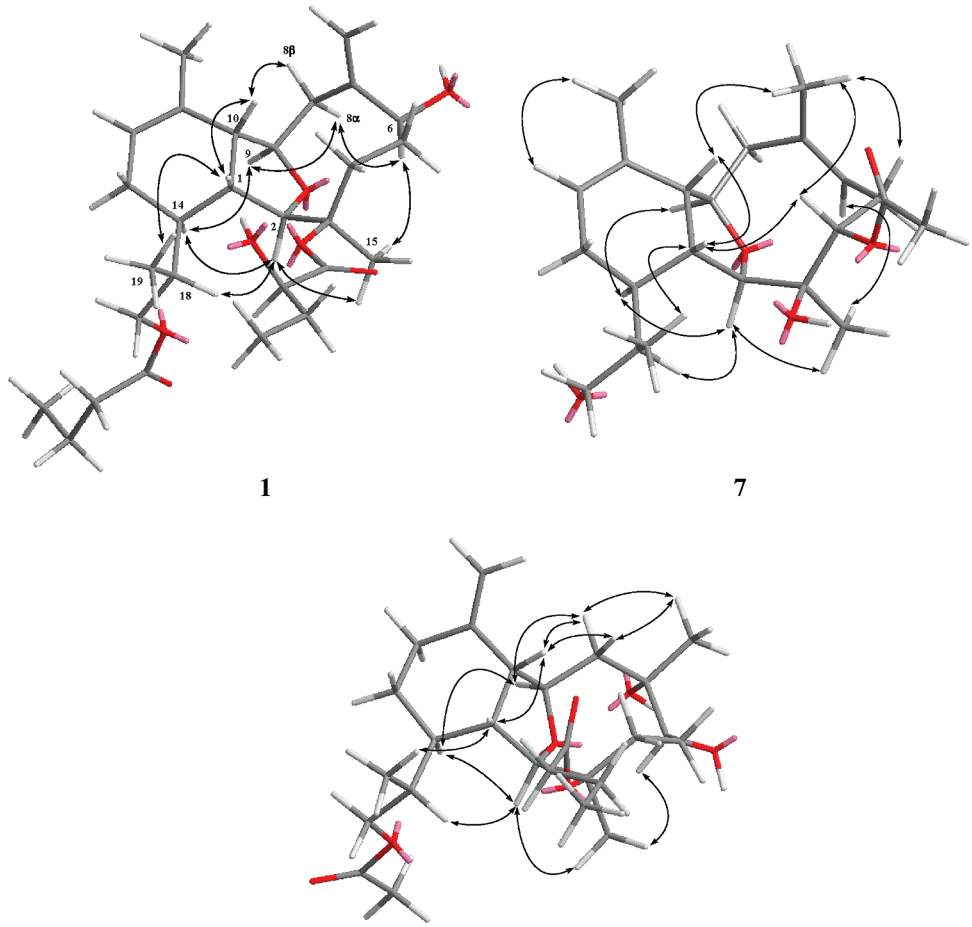
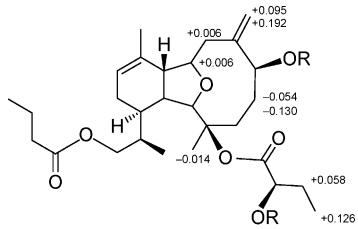


Figure 2. Key NOESY correlations of **1**, **7**, and **8**.



1a: R=(*S*)–MTPA
1b: R=(*R*)–MTPA

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

169.0 (C), 73.9 (CH), 24.4 (CH_2), and 9.6 (CH_3); 170.8 (C) and 20.6 (CH_3)]. Comparison of the NMR data of **4** with those of **3** revealed that the only difference between the compounds was the replacement of the 2-butyryloxybutanoate moiety at C-3 in **3** by the 2-acetoxybutanoate group in **4**.

Hirsutalin E (**5**) was obtained as a colorless oil and exhibited a pseudomolecular ion peak at *m*/*z* 431.2771 $[M + Na]^+$ by HRESIMS, appropriate for the molecular formula $C_{24}H_{40}O_5$. The ^{1}H NMR spectroscopic data (Table 2) of **5** showed the presence of one *n*-butyrate, which showed signals at δ_H 2.22 (2H, m), 1.65 (2H, m), and 0.94 (3H, t, $J = 7.6$ Hz). Comparison of the NMR data of **5** with those of known compound **9**[7] revealed that the only difference between the compounds was the replacement of a hydroxy group at C-3 in **9** by the *n*-butyryloxy moiety in **5**. This was evidenced from the downfield chemical shifts induced by an

Table 3. ^{1}H NMR Data for Compounds **6**−**8**

position	**6**[a]	**7**[a]	**8**[a]
1	2.21 dd (12.0, 4.8)[b]	2.65, m	2.22, dd (11.6, 7.6)
2	3.91, s	4.05, d (8.8)	3.53, s
4	3.00, m	5.27, dd (10.8, 7.2)	2.64, dd (14.4, 8.8)
	1.40, m		1.84, m
5	1.68, m	2.84, dd (10.8, 4.0)	1.60, m
		2.08, m	1.42, m
6	3.85, dd (9.2, 8.8)	5.47, t (8.8)	4.60, d (6.0)
8	2.96, d (12.4)	2.54, dd (13.2, 6.0)	1.91, m
	2.03, d (13.2)	1.99, m	1.84, m
9		4.16, m	4.14, ddd (14.8, 7.2, 4.0)
10	4.23, d (4.4)	2.42, d (8.0)	3.01, t (7.2)
12	2.88, m	5.43, m	2.29, m
	2.23, m		2.05, m
13	1.73, m	2.18, m	1.65, m
	1.14, m	1.88, m	1.10, m
14	2.44, m	1.89, m	1.50, m
15	1.50, s	1.45, s	1.40, s
16	1.17, s	1.91, s	1.17, s
17	4.74, s	1.68, s	4.71, s
	4.61, s		4.66, s
18	1.91, m	1.56, m	1.93, m
19	0.76, d (7.2)	0.92, d (7.2)	0.84, d (6.4)
20	3.66, dd (10.4, 6.0)	3.78, dd (10.4, 4.4)	3.96, d (7.6)
	3.51, dd (10.8, 7.2)	3.58, dd (10.8, 4.4)	
2′	4.76, t (6.8)	2.11, s	2.33, m
3′	1.89, m		1.69, m
4′	1.06, t (7.6)		1.00, t (7.2)
2″	2.41, m		2.08, s
3″	1.70, m		
4″	0.99, t (7.6)		

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

ester group at C-3 (δ_C 86.1, C) and C-15 (δ_H 1.63, CH_3) in **5** relative to those of **9**. Thus, the structure of diterpenoid **5** was established.

A structurally related metabolite, hirsutalin F (**6**), was also isolated as a colorless oil with a molecular formula of $C_{28}H_{44}O_8$. Similar to known metabolite australin D (**10**),[2] the IR spectrum of **6** indicated the presence of hydroxy (3376 cm^{-1}), ester (1735 cm^{-1}), and ketone (1715 cm^{-1}) groups. The ^{13}C NMR spectroscopic data of **6** (Table 1) again showed the presence of one 2-butyryloxybutanoate [δ_C 169.4 (C), 74.2 (CH), 24.5 (CH_2), and 9.7 (CH_3); 173.6 (C), 35.7 (CH_2), 18.4 (CH_2), and 13.6 (CH_3)]. Comparison of the 1D and 2D NMR data of **6** with those of **10** revealed that the only difference between the compounds was the replacement of the *n*-butyryloxy moiety at C-3 in australin D by a 2-butyryloxybutanoate group in **6**, as confirmed by HMBC correlations of both carbonyl carbons, resonating at δ_C 173.6 and 169.4, with the proton of an oxygenated methine (δ 4.76, 1H, t, J = 6.8 Hz).

The HRESIMS spectrum of **7** exhibited a pseudomolecular ion peak at *m/z* 401.2301 $[M + Na]^+$, consistent with a molecular formula of $C_{22}H_{34}O_5$. The NMR spectroscopic data of **7** revealed the presence of two trisubstituted double bonds (δ_H 5.47, 1H, t, J = 8.8 Hz and 5.43, 1H, m; δ_C 132.7 C, 131.5 C, 123.4 CH and 121.2 CH), in addition to one acetoxy group [δ_H 2.05 s; δ_C 171.4 C]. Therefore, **7** is a tricyclic diterpenoid. The planar structure of **7** was 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 proton spin systems. One of them was found to extend from the oxygenated methine proton H-4 to the olefinic proton H-6. 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_3-16 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_2-20 to C-14 and C-18 permitted the assembly of the carbon skeleton. The placement of the acetate at C-4 was proven by the HMBC correlation from H-4 (δ 5.27) to the carbonyl carbon resonating at δ 171.4 (C). The relative configuration at C-1, C-2, C-3, C-4, C-9, C-10, and C-14 in **7** was elucidated by analysis of the NOE correlations (Figure 2). The *E* geometry of the 6,7-endocyclic double bond in **7** was indicated by the NOE correlation of H_3-16 (δ 1.91, s) with one proton of H_2-5 (δ 2.84), but not with the olefinic proton H-6 (δ 5.47), and the upfield shift of C-16 (δ < 20 ppm). Thus, the structure of diterpenoid **7** was established.

On the basis of its HRESIMS spectrum (*m/z* 489.2831 $[M + Na]^+$), the molecular formula of hirsutalin H (**8**) was established as $C_{26}H_{42}O_7$. A comparison of the NMR data of **8** (Tables 1 and 3) with those of **2** and **5** showed that **8** has the same six-membered ring as that of **2** (including the identical substituent at C-14) and the same 10-membered ring as that of **5**, which was evidenced by COSY and HMBC correlations. The relative configuration for all asymmetric carbons in **8** was elucidated by the analysis of NOE correlations, as shown in Figure 2. Although many eunicellin-type natural products have been discovered, eunicellins containing a 2-hydroxybutyrate or a 2-acyloxybutyrate at C-3, such as **1**−**4** and **6**, have been discovered for the first time in this study.

In addition to our studies, previous reports have shown that eunicellin-based diterpenes possess antitumor and anti-inflammatory activities.[20−24] A recent publication showed some eunicellin diterpenes to display significant anti-invasive and antimigratory activities.[25] The cytotoxicity of metabolites **1**−**8** toward a panel of six cancer cell lines was evaluated. The results showed that compound **1** exhibited weak cytotoxicity toward Hep 3B, A549, and Ca9-22 cell lines with IC_{50} values of 29, 28, and 35 μM, respectively. Also, metabolite **5** showed moderate to weak cytotoxicity (IC_{50} values of 14, 41, 35, 34, and 34 μM) toward the growth of Hep 3B, MDA-MB-231, MCF-7, A549, and Ca9-22 cells, respectively, and significant cytotoxicity toward the Hep G2 (IC_{50} value of 4.7 μM) cell line, while compound **6** exhibited weak cytotoxicity toward Hep G2, Hep 3B, and MCF-7 cell lines with IC_{50} values of 29, 29, and 32 μM, respectively. The remaining metabolites were found to be inactive toward the growth of the employed cancer cell lines.

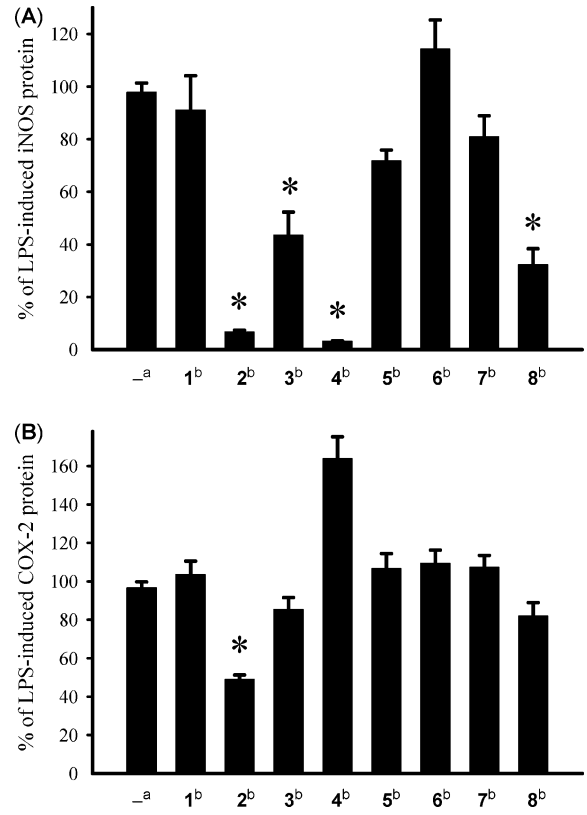


Figure 4. Effect of compounds **1**−**8** on iNOS and COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis. (A) Relative intensities of iNOS immunoblots. (B) Relative intensities of COX-2 immunoblots. The values are mean ± SEM (n = 6). Relative intensity of the LPS alone stimulated group was taken as 100%. *Significantly different from LPS alone stimulated group (*P < 0.05). [a]Stimulated with LPS. [b]Stimulated with LPS in the presence of **1**−**8** (10 μM).

The anti-inflammatory activities of **1**−**8** against the accumulation of pro-inflammatory iNOS and COX-2 proteins in RAW264.7 macrophage cells stimulated with LPS were evaluated using immunoblot analysis. At a concentration of 10 μM (Figure 4), compounds **2**−**4** and **8** were found to significantly reduce the levels of iNOS protein to 6.8 ± 0.6%, 43.6 ± 8.7%, 3.3 ± 0.1%, and 32.3 ± 6.1%, respectively, relative to control cells stimulated with LPS only. At the same concentration, metabolite **2** also significantly reduced COX-2 expression (49.0 ± 2.3% relative to control cells) by LPS treatment. Thus, compounds **2**−**4** and **8**, in particular **2** and **4**, could be promising anti-inflammatory agents and may warrant further study.

Experimental Section

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. The NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^1H and 125 MHz for ^{13}C, or on a Varian 400 MR FT-NMR at 400 MHz for ^1H and 100 MHz for ^{13}C, or on a Bruker AVANCE-DPX 300 FT-NMR at 300 MHz for ^1H and 75 MHz for ^{13}C, respectively. ESIMS spectra were obtained with a Bruker APEX II mass spectrometer. 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 using a Hitachi L-7100 HPLC apparatus with an ODS column (250 × 21.2 mm, 5 μm).

Animal Material. Specimens of the soft coral *Cladiella hirsuta* were collected by hand using scuba off the coast of Sianglu Islet (23°32′ N,

119°38′ E) in the region of Penghu Islands, in June 2008, at a depth of 10 m, and were stored in a freezer until extraction. A voucher sample (PI-20080610-17) is deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Isolation. The frozen bodies of *C. hirsuta* (3.1 kg, wet wt) were sliced and exhaustively extracted with acetone (3 × 10 L). The organic extract was concentrated to an aqueous suspension and was partitioned between EtOAc and H_2O. The EtOAc layer was dried with anhydrous Na_2SO_4. After removal of solvent *in vacuo*, the residue (32.8 g) was subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0−100% of EtOAc, gradient) and further with MeOH in EtOAc of increasing polarity to yield 25 fractions. Fraction 17, eluted with *n*-hexane−EtOAc (2:1), was rechromatographed over a Sephadex LH-20 column using acetone as the mobile phase to afford four subfractions (A1−A4). Subfractions A3 and A4 were separated by reversed-phase HPLC (CH_3CN-H_2O, 5:2 to 7:6) to afford compounds **1** (2.6 mg), **2** (8.1 mg), **3** (3.7 mg), **4** (1.5 mg), and **5** (31.8 mg), respectively. Fraction 20, eluted with *n*-hexane−EtOAc (1:2), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase, to afford four subfractions (B1−B4). Subfractions B2 and B3 were separated by reversed-phase HPLC (CH_3CN-H_2O, 3:1 to 2:3) to afford compounds **6** (1.0 mg), **7** (1.1 mg), and **8** (3.0 mg), respectively.

Hirsutalin A (1): colorless oil; $[\alpha]^{25}_D$ −22 (*c* 0.26, $CHCl_3$); IR (neat) ν_{max} 3417 and 1733 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m/z* 515 $[M + Na]^+$; HRESIMS *m/z* 515.2981 $[M + Na]^+$ (calcd for $C_{28}H_{44}O_7Na$, 515.2985).

Hirsutalin B (2): colorless oil; $[\alpha]^{25}_D$ −41 (*c* 0.81, $CHCl_3$); IR (neat) ν_{max} 3503 and 1738 cm^{-1}; ^{13}C and ^1H NMR data (300 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m/z* 573 $[M + Na]^+$; HRESIMS *m/z* 573.3036 $[M + Na]^+$ (calcd for $C_{30}H_{46}O_9Na$, 573.3039).

Hirsutalin C (3): colorless oil; $[\alpha]^{25}_D$ −78 (*c* 0.37, $CHCl_3$); IR (neat) ν_{max} 3475 and 1731 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m/z* 515 $[M + Na]^+$; HRESIMS *m/z* 515.2981 $[M + Na]^+$ (calcd for $C_{28}H_{44}O_7Na$, 515.2985).

Hirsutalin D (4): colorless oil; $[\alpha]^{22}_D$ −52 (*c* 0.15, $CHCl_3$); IR (neat) ν_{max} 3409 and 1742 cm^{-1}; ^{13}C and ^1H NMR data (500 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m/z* 487 $[M + Na]^+$; HRESIMS *m/z* 487.2670 $[M + Na]^+$ (calcd for $C_{26}H_{40}O_7Na$, 487.2672).

Hirsutalin E (5): colorless oil; $[\alpha]^{22}_D$ −13 (*c* 3.18, $CHCl_3$); IR (neat) ν_{max} 3412 and 1732 cm^{-1}; ^{13}C and ^1H NMR data (300 MHz; $CDCl_3$), see Tables 1 and 2; ESIMS *m/z* 431 $[M + Na]^+$; HRESIMS *m/z* 431.2771 $[M + Na]^+$ (calcd for $C_{24}H_{40}O_5Na$, 431.2773).

Hirsutalin F (6): colorless oil; $[\alpha]^{22}_D$ −62 (*c* 0.10, $CHCl_3$); IR (neat) ν_{max} 3376, 1735, and 1715 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 3; ESIMS *m/z* 531 $[M + Na]^+$; HRESIMS *m/z* 531.2937 $[M + Na]^+$ (calcd for $C_{28}H_{44}O_8Na$, 531.2934).

Hirsutalin G (7): colorless oil; $[\alpha]^{22}_D$ −29 (*c* 0.11, $CHCl_3$); IR (neat) ν_{max} 3457 and 1731 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 3; ESIMS *m/z* 401 $[M + Na]^+$; HRESIMS *m/z* 401.2301 $[M + Na]^+$ (calcd for $C_{22}H_{34}O_5Na$, 401.2304).

Hirsutalin H (8): colorless oil; $[\alpha]^{22}_D$ −140 (*c* 0.30, $CHCl_3$); IR (neat) ν_{max} 3410 and 1735 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 3; ESIMS *m/z* 489 $[M + Na]^+$; HRESIMS *m/z* 489.2831 $[M + Na]^+$ (calcd for $C_{26}H_{42}O_7Na$, 489.2828).

Preparation of (*S*)- and (*R*)-MTPA Esters of 1. To a solution of **1** (1.0 mg) in pyridine (0.4 mL) was added (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) 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 H_2O, 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 (6:1) to yield the (*S*)-MTPA ester, **1a** (1.1 mg, 59%). The same procedure was used to prepare the (*R*)-MTPA ester, **1b** (1.1 mg, 59%), from the reaction of (*S*)-MTPA chloride with **1** in pyridine. Selected ^1H NMR ($CDCl_3$, 400 MHz) of **1a**: 5.462 (1H, dd, *J* = 11.2 and 4.0, H-6), 5.434 (1H, s, H-16a), 5.241 (1H, s, H-16b), 4.807 (1H, t, *J* = 6.0, 2-hydroxybutyrate), 4.076 (1H, dd, *J* = 10.4 and 3.6, H-9), 2.539 (1H, d, *J* = 13.6 Hz, H-8a), 2.115 (1H, m, H-4a), 1.897 (1H, m, H-4b), 1.697 (1H, s, H-15), 1.870 (2H, m, 2-hydroxybutyrate), 1.010 (3H, s, 2-hydroxybutyrate). Selected ^1H NMR ($CDCl_3$, 400 MHz) of **1b**: 5.432 (1H, dd, *J* = 11.2 and 4.4, H-6), 5.242 (1H, s, H-16a), 5.146 (1H, s, H-16b), 4.763 (1H, t, *J* = 6.4, 2-hydroxybutyrate), 4.070 (1H, dd, *J* = 10.0 and 4.0, H-9), 2.533 (1H, d, *J* = 13.6 Hz, H-8a), 2.245 (1H, m, H-4a), 1.951 (1H, m, H-4b), 1.711 (1H, s, H-15), 1.812 (2H, m, 2-hydroxybutyrate), 0.884 (3H, s, 2- hydroxybutyrate).

Cytotoxicity Testing. Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays were performed in duplicate using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[26,27] Doxorubicin was employed as positive control, which exhibited cytotoxic activity toward Hep G2, Hep 3B, MDA-MB-231, MCF-7, A549, and Ca9-22 cancer cell lines with IC_{50} values of 0.4, 1.3, 2.0, 2.9, 2.6, and 0.2 μM, respectively.

***In Vitro* Anti-inflammatory Assay.** The macrophage (RAW264.7) cell line was purchased from ATCC. *In vitro* anti-inflammatory activities of compounds **1**−**8** were 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.[28,29]

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Supporting Information Available: ^1H and ^{13}C NMR spectra of **1**−**8** are available free of charge via the Internet at http://pubs.acs.org.

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