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Article

Eunicellin-Based Diterpenoids, Hirsutalins N–R, from the Formosan Soft Coral *Cladiella hirsuta*

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Abstract: New eunicellin-type hirsutalins N–R (**1**–**5**), along with two known eunicellins, (**6** and **7**) were isolated from the soft coral *Cladiella hirsuta*. The structures of the metabolites were determined by extensive spectroscopic analysis. Cytotoxic activity of compounds **1**–**7** against the proliferation of a limited panel of cancer cell lines was measured. The *in vitro* anti-inflammatory activity of compounds **1**–**7** was evaluated by measuring their ability in suppressing superoxide anion generation and elastase release in fMLP/CB-induced human neutrophils.

Keywords: soft coral; *Cladiella hirsuta*; eunicellins: cytotoxic activity; anti-inflammatory activity

1. Introduction

The chemical investigations on soft corals of the genus *Cladiella* and *Klyxum* [1–30] have afforded several eunicellin-based diterpenoids, of which many have been shown to exhibit interesting bioactivities [8,10–30]. Our recent chemical study of a Taiwanese soft coral *Cladiella hirsuta* has led to the discovery of 13 eunicellin-based diterpenoids hirsutalins A–M [29,30] and seven steroids hirsutosterols A–G [31] some of which have been found to possess cytotoxic [29] and anti-inflammatory activities [29,30]. In this paper we further report the isolation of five new eunicellin-based compounds, hirsutalins N–R (Chart 1), along with two known compounds, (1*R**,2*R**,3*R**,6*S**,7*S**,9*R**,10*R**,14*R**)-3-butanoyloxycladiell-11(17)-en-6,7-diol (**6**) [6], and hirsutalin E (**7**) [29] from *C. hirsuta* (Chart 2). The structures of new compounds were determined by extensive spectroscopic analysis. Cytotoxicity of **1**–**7** against a limited panel of cancer cell lines and their anti-inflammatory activity, determined by their ability to inhibit the generation of super oxide anion and elastase release in *N*-formyl-methionyl-leucylphenylalanine/cytochalasin B(fMLP/CB)-induced human neutrophiles, were studied in order to discover bioactive compounds for future new drug development.

Chart 1. Structures of metabolites **1**–**5**.

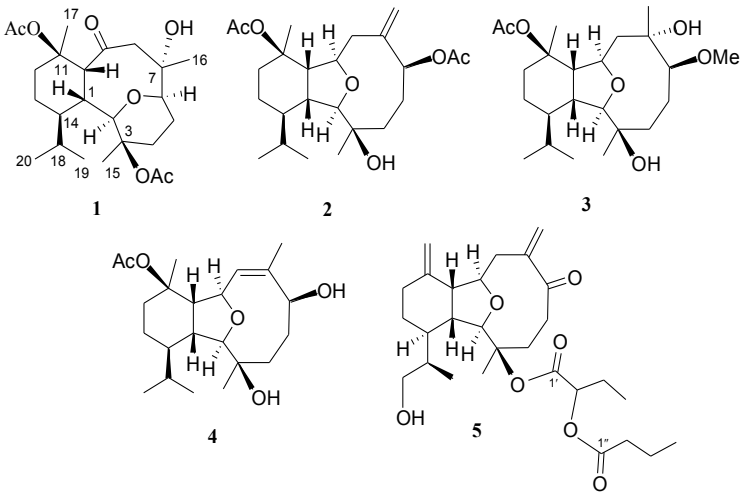
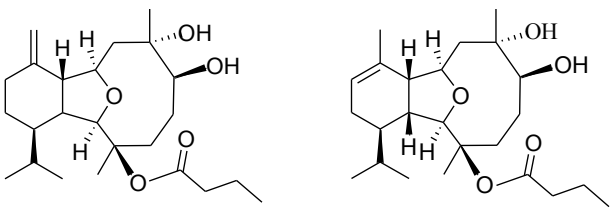


Chart 2. Structures of metabolites **6** and **7**.



2. Results and Discussion

Hirsutalin N (**1**) was isolated as a colorless oil. The HRESIMS (*m/z* 461.2518) of **1** established a molecular formula of $C_{24}H_{38}O_7$. The IR spectrum of **1** showed the presence of hydroxy and carbonyl groups from absorptions at 3451 and 1733 cm^{-1}, respectively. The ^{13}C NMR of **1** exhibited 24 carbon signals as expected which were found to be similar to these of a known metabolite hirsutalin I (**8**, Chart 3) [30], the difference being that the hydroxymethyl group attached at C-18 in hirsutalin I was replaced by a methyl group in **1**. This was confirmed by ^1H NMR spectrum of **1** which shows the presence of two isopropyl methyls at δ 0.73 (d, *J* = 7.2 Hz) and 0.97 (d, *J* = 7.2 Hz) (Table 1). Also, NMR data revealed that the *n*-butanoyloxy group at C-3 in **8** was replaced by an acetoxy group in **1**. Key HMBC correlations from H-2 to C-6; H-1, H_2-8, and H-10 to C-9; 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_3-20 to C-14 and C-18, permitted the assembly of the carbon skeleton of **1**. Based on above results and HMBC correlations (Figure 1), the planar structure of **1** was established. Further, comparison of the NOE correlations of **1** (Figure 2) with those of hirsutalin I, the relative configuration of **1** was thus determined to be the same.

Table 1. NMR spectroscopic data for hirsutalins N–P (**1**–**3**).

	1		**2**		**3**	
Position	**δ_C, mult.** [a,b]	**δ_H (*J* in Hz)** [c]	**δ_C, mult.** [a,b]	**δ_H (*J* in Hz)** [c]	**δ_C, mult.** [a,b]	**δ_H (*J* in Hz)** [c]
1	49.6, CH	2.55, dd (12.0, 4.4)	41.4, CH	2.25, m	41.9, CH	2.18, m
2	78.0, CH	3.80, s	91.3, CH	3.56, s	90.8, CH	3.56, s
3	81.3, C	-	74.0, C	-	74.7, C	-
4	27.7, CH_2	1.36, m	34.9, CH_2	1.75, m	41.0, CH_2	1.83, m
	-	2.92, dd (11.8, 4.4)	-	-	-	-
5	20.6, CH_2	1.34, m	32.0, CH_2	1.99, m	25.7, CH_2	1.98, m
	-	1.66, m	-	-	-	-
6	80.4, CH	3.82, dd (11.4, 6.0)	76.4, CH	5.19, dd (12.0, 6.0)	90.8, CH	4.07, m
7	85.4, C	-	149.0, C	-	76.6, C	-
8	49.5, CH_2	2.00, d (12.0)	41.4, CH_2	3.12, dd (13.6, 6.0)	47.0, CH_2	1.73, m
	-	2.78, d (12.0)	-	2.47, d (13.6)	-	2.30, dd (12.8, 11.6)
9	211.4, C	-	78.3, CH	4.09, dd (11.2, 6.0)	75.6, CH	4.07, m
10	55.2, CH	4.14, dd (4.4, 2.0)	46.4, CH	2.95, dd (11.2, 7.2)	54.4, CH	2.82, t (7.6)
11	83.3, C	-	82.3, C	-	82.9, C	-
12	31.4, CH_2	2.10, m	32.5, CH_2	1.43, m	30.5, CH_2	1.38, m
	-	2.24, m	-	2.24, m	-	2.40, m
13	19.3, CH_2	1.61, m	18.2, CH_2	1.34, m	17.7, CH_2	1.20, m
	-	1.25, m	-	1.45, m	-	1.40, m
14	36.5, CH	1.98, m	42.8, CH	1.20, m	42.6, CH	1.22, m
15	23.6, CH_3	1.53, s	27.4, CH_3	1.19, s	30.3, CH_3	1.16, s

Table 1. *Cont.*

16	22.9, CH_3	1.13, s	118.3, CH_2	5.29, s	23.8, CH_3	1.16, s
	-	-	-	5.53, s	-	-
17	24.3, CH_3	1.45, s	25.5, CH_3	1.52, s	24.5, CH_3	1.46, s
18	27.2, CH	1.87, m	27.9, CH	1.80, m	29.1, CH	1.71, m
19	14.5, CH_3	0.73, d (7.2)	15.0, CH_3	0.78, d (6.8)	15.0, CH_3	0.78, d (6.8)
20	21.7, CH_3	0.97, d (7.2)	21.8, CH_3	0.94, d (6.8)	21.8, CH_3	0.94, d (6.8)
3-OAc	22.4, CH_3	2.00, s	-	-	-	-
	169.7, C	-	-	-	-	-
11-OAc	22.3, CH_3	2.19, s	22.6, CH_3	2.00, s	22.6, CH_3	2.00, s
	170.1, C	-	170.3, C	-	170.2, C	-
6-OAc	-	-	21.4, CH_3	1.99, s	-	-
	-	-	170.5, C	-	-	-
6-OMe	-	-	-	-	57.1, CH_3	3.37, s

[a] Spectra recorded at 100 MHz in $CDCl_3$; [b] multiplicity deduced from DEPT; [c] spectra recorded at 400 MHz in $CDCl_3$.

Figure 1. COSY and HMBC correlations for **1**, **2**, **4** and **5**.

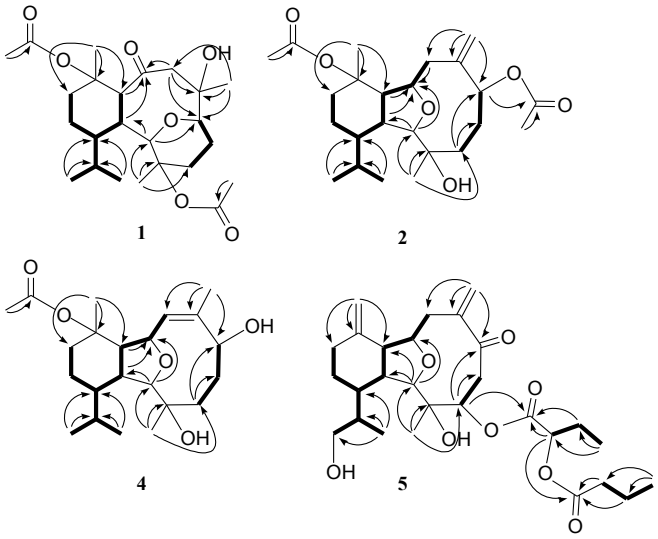
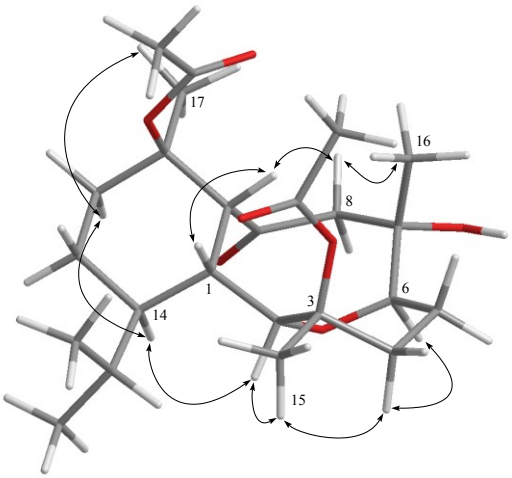
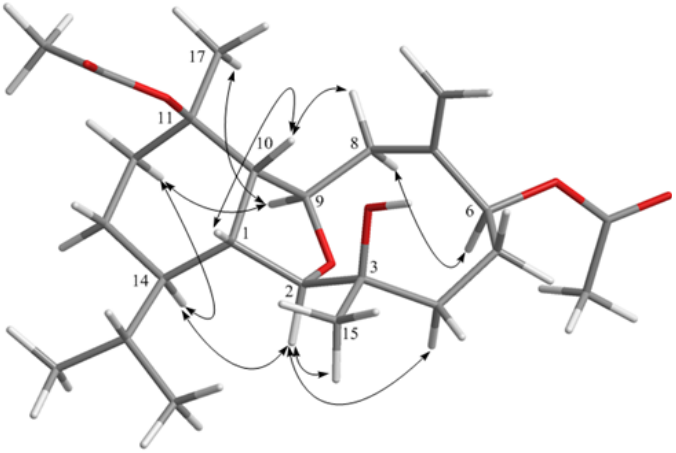


Figure 2. Key NOESY correlations for 1.



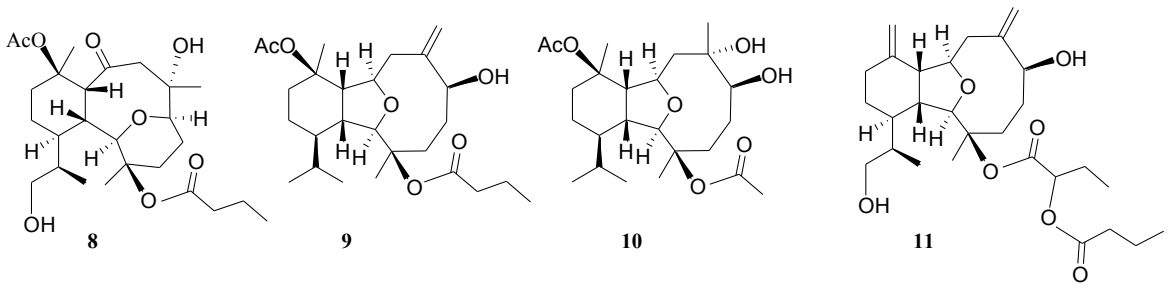
Hirsutalin O (**2**) was also afforded as a colorless oil. Compound **2** has a molecular formula $C_{24}H_{38}O_6$, as determined by HRESIMS. In comparing NMR data of **2** with those of the known compound simplexin A (**9**, Chart 3) [11], it was found that the *n*-butanoyloxy group at C-3 and the hydroxy group at C-6 in simplexin A (**9**) were replaced by a hydroxy group and acetoxy group in **2**, respectively, as confirmed by the downfield shift of C-3 (δ_C 81.3) of **1**, relative to that of **2** (δ_C 74.0), and the HMBC connectivity from H-6 (δ 5.19) to the carbonyl carbon resonating at δ 170.5 (C) (Table 1). The relative configuration of **2** was confirmed to be the same as that of **9** by analysis of NOE correlations (Figure 3).

Figure 3. Key NOESY correlations for 2.



The new eunicellin, hirsutalin P (**3**), has a molecular formula $C_{23}H_{40}O_6$ as determined by HRESIMS. The spectroscopic data (IR, ^{1}H NMR, and ^{13}C NMR) of **3** were similar to those of a known one, klysimplex G (**10**, Chart 3) [12], except that the acetoxy group at C-3 and the hydroxy group at C-6 in **10** were replaced by a hydroxy group and methoxy group, respectively, in **3**. The similar ^{1}H NMR data and the analysis of NOE correlations of **3** further revealed the same relative configuration of both compounds. Thus, the structure of **3** was established.

Chart 3. Structures of known compounds **8**–**11**.



Hirsutalin Q (**4**) was obtained as a colorless oil and exhibited a molecular formula $C_{22}H_{36}O_5$. IR absorptions of **4** showed the presence of hydroxy and carbonyl groups at 3421 and 1724 cm^{-1}, respectively. The NMR spectroscopic data revealed the presence of a trisubstituted double bond (δ_H 5.28, s, 1H; δ_C 128.4, CH and 139.4, C) (Table 2). One ester carbonyl (δ_C 170.2) was assigned from the ^{13}C NMR spectrum and was HMBC correlated with an acetate methyl (δ_H 1.99 s). The

chemical shift of H_3-15 at δ 1.18 indicated the presence of a hydroxy group substitution at C-3, the same as that in compounds **2** and **3**. The presence of an acetoxy group at C-11 could be seen from the more downfield shift of H_3-17 (δ 1.53), in comparison with that of H_3-15 (δ 1.18). The planar structure of metabolite **1** was elucidated by analysis of COSY and HMBC correlations (Figure 1). The *Z* geometry of the double bond at C-7 and C-8 was evidenced by the presence of NOE correlation between H-8 and H_3-16. In the NOESY spectrum of **4**, observation of the NOE correlation between H-1 with H-10 suggested that H-1 and H-10 are β-oriented. Also, correlations between H-2 with both H-14 and H_3-15; H-9 with both H-14 and H_3-17; and H-6 with H_3-15 suggested that all of H-2, H-6, H-9, H-14, H_3-15 and H_3-17 are α-oriented. Thus, the structure of diterpenoid **4** was established.

A structurally-related metabolite, hirsutalin R (**5**), was also isolated as a colorless oil with a molecular formula of $C_{28}H_{42}O_7$. Two ester carbonyl carbons (δ_C 169.0 and 173.5) were correlated in the HMBC spectrum with the methine proton (H-2′, δ_H 4.76 t, J = 6.8 Hz) of a 2-butyryloxybutanoate unit. Moreover, the ^{13}C NMR spectroscopic data (Table 2) of **5** showed the presence of two 1, 1-disubstituted carbon–carbon double bonds (δ_C 147.7 (C) and 118.4 (CH_2); 145.2 (C) and 111.6 (CH_2)). Comparison of the NMR data of **5** with those of hirsutalin C (**11**, Chart 3) [29] revealed that the only difference between both compounds is the replacement of the hydroxy group in hirsutalin C by a ketone (δ_C 206.5) at C-6 in **5**. The absolute configuration of hirsutalin A [29] and hirsutalin J [30] have been completely assigned based on NOE correlations and Mosher's method. Compounds **1**–**5** are likely in the same enantiomeric series as hirsutalin A and hirsutalin J, based on a shared biosynthetic pathway. Thus, these compounds are suggested to possess the absolute configurations as shown in formula **1**–**5**.

Table 2. NMR spectroscopic data for hirsutalins Q and R (**4** and **5**).

	4		**5**	
Position	**δ_C, mult.** [a,b]	**δ_H (J in Hz)** [c]	**δ_C, mult.** [a,b]	**δ_H (J in Hz)** [c]
1	40.9, CH	2.35, m	45.0, CH	2.25, m
2	90.8, CH	3.57, s	90.8, CH	3.69, s
3	74.7, C	-	86.0, C	-
4	37.2, CH_2	1.83, m;	32.2, CH_2	2.12, m
5	25.7, CH_2	1.81, m	36.4, CH_2	2.68, m
	-	1.90, m	-	2.28, m
6	70.6, CH	5.48, d (8.8) [d]	206.5, CH	-
7	139.4, C	-	147.7, C	-
8	128.4, CH	5.28, s	37.3, CH_2	3.22, dd (13.2, 5.6)
	-	-	-	2.34, m
9	78.6, CH	4.47, d (6.0)	78.4, CH	4.08, m
10	54.9, CH	2.70, t (7.2)	48.8, CH	3.08, dd (9.6, 7.6)
11	83.0, C	-	145.2 , C	-
12	30.4, CH_2	1.32, m	31.2, CH_2	2.08, m
	-	1.52, m	-	2.27, m
13	18.4, CH_2	1.35, m	25.9, CH_2	1.10, m
	-	1.45, m	-	1.65, m
14	42.1, CH	1.26, m	37.5, CH	1.66, m

Table 2. *Cont.*

15	27.7, CH_3	1.18, s	22.7, CH_3	1.48, s
16	17.9, CH_3	1.79, s	118.4, CH_2	5.27, s
	-	-	-	5.62, s
17	23.7, CH_3	1.53, s	111.6, CH_2	4.72, s
	-	-	-	4.85, s
18	29.2, CH	1.72, m	36.4, CH	1.78, m
19	16.5, CH_3	0.83, d (7.2)	16.3, CH_3	0.79, d (7.2)
20	21.9, CH_3	0.96, d (7.2)	66.4, CH_2	3.52, d (7.2)
11-OAc	22.6, CH_3	1.99, s	-	-
	170.2, C	-	-	-
2-butanoyloxybutanoate	-	-	-	-
1′	-	-	169.0, C	-
2′	-	-	73.6, CH	4.76, t (6.8)
3′	-	-	24.5, CH_2	1.83, m
4′	-	-	9.7, CH_3	1.03, t (7.2)
1″	-	-	173.5, C	-
2″	-	-	35.8, CH_2	2.40, m
3″	-	-	18.3, CH_2	1.66, m
4″	-	-	13.6, CH_3	0.98, t (7.2)

[a] Spectra recorded at 100 MHz in $CDCl_3$; [b] Multiplicity deduced from DEPT; [c] Spectra recorded at 400 MHz in $CDCl_3$.

Cytotoxicity of compounds **1**–**7** against the proliferation of a limited panel of cancer cell lines, including P388 (murine leukemia), K562 (human erythro myeloblastoid leukemia), A549 (human lung adenocarcinoma), and HT-29 (human colon adenocarcinoma), was evaluated. Compound **5** was found to exhibit cytotoxicity toward P388 and K562 cell lines with IC_{50} values of 13.8 and 36.3 μM (Table 3). Compound **7** displayed cytotoxicity toward A549 cell line with IC_{50} value of 37.2 μM. Other metabolites were found to be inactive against the four cancer cells. The neutrophil pro-inflammatory responses to compounds **1**–**7** were evaluated by suppressing *N*-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (fMLP/CB)-induced superoxide anion ($O_2^{\bullet-}$) generation and elastase release in human neutrophils, as shown in Table 4. At a concentration of 10 μg/mL, none of compounds were able to significantly reduce the expression of superoxide anion generation, relative to the control cells stimulated with fMLP/CB only. At the same concentration, compound **1** was found to significantly inhibit the elastase release (31.7% ± 3.2% inhibition) in the same fMLP/CB-stimulated neutrophils.

Table 3. Cytotoxicity (IC_{50} μM) of compounds **5** and **7**.

Compound	P388	K562	HT-29	A-549
5	13.8	36.3	(–) [a]	(–)
7	(–)	(–)	(–)	37.2
5-Fluorouracil	8.5	24.6	20.8	38.5

[a] IC_{50} > 40 μM.

Table 4. Effect of compounds **1**–**7** on superoxide anion generation and elastase release in fMLP/CB-induced human neutrophils at 10 μg/mL.

Compounds	Superoxide Anion		Elastase Release		
	IC_{50} (μg/mL) [a]	Inhibition %	IC_{50} (μg/mL) [a]	Inhibition %	
1	>10	1.0 ± 5.5	>10	31.7 ± 3.2	***
2	>10	9.6 ± 5.5	>10	11.5 ± 5.0	-
3	>10	1.7 ± 0.7	>10	17.9 ± 6.9	*
4	>10	6.1 ± 2.6	>10	6.4 ± 2.4	-
5	>10	6.5 ± 2.9	>10	13.6 ± 4.9	*
6	>10	1.0 ± 1.9	>10	6.1 ± 5.6	-
7	>10	4.2 ± 3.8	>10	3.1 ± 6.9	-

Percentage of inhibition (Inh %) at 10 μM concentration. Results are presented as mean ± S.E.M. (n = 3 or 4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control value. [a] Concentration necessary for 50% inhibition (IC_{50}).

3. Experimental Section

3.1. General Experimental Procedures

Silica gel (230–400 mesh, Merck, Darmstadt, Germany) 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 Hitachi L-2455 HPLC apparatus (Hitachi Ltd., Tokyo, Japan) with a Supelco C18 column (250 × 21.2 mm, 5 μm). NMR spectra were recorded on a Varian 400MR FT-NMR instrument (Varian Inc, Palo Alto, CA, USA) at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$. LRMS and HRMS were obtained by ESI on a Bruker APEX II mass spectrometer (Bruker, Bremen, Germany). Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer (Japan Spectroscopic Corporation, Tokyo, Japan).

3.2. Animal Material

The animal *Cladiella hirsuta* was 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 was stored in a freezer until extraction. A voucher sample (PI-20080610-17) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3. Extraction and Separation

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 ethyl acetate (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 18, eluting with *n*-hexane–EtOAc (1:1), 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 combined and separated by reversed-phase HPLC (MeOH–H_2O, 3:1 and 2:1) to afford compounds **4** (1.8 mg), **5** (1.4 mg), **6** (27.7 mg) and **7** (5.6 mg). Fraction 19, eluting 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 combined and separated by reversed-phase HPLC (acetonitrile–H_2O, 3:1 and 2:1) to afford compounds **1** (9.2 mg), **2** (4.0 mg) and **3** (1.8 mg).

Hirsutalin N (**1**): colorless oil; $[\alpha]^{25}_D$ −98 (*c* 0.54, $CHCl_3$); IR (neat) ν_{max} 3451 and 1733 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Table 1; ESIMS *m/z* 461 $[M + Na]^+$; HRESIMS *m/z* 461.2518 $[M + Na]^+$(calcd for $C_{24}H_{38}O_7Na$, 461.2515) (Supplementary Information, Figures S1–S3).

Hirsutalin O (**2**): colorless oil; $[\alpha]^{25}_D$ −128 (*c* 0.68, $CHCl_3$); IR (neat) ν_{max} 3482 and 1729 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Table 1; ESIMS *m/z* 445 $[M + Na]^+$; HRESIMS *m/z* 445.2564 $[M + Na]^+$(calcd for $C_{24}H_{38}O_6Na$, 445.2566) (Supplementary Information, Figures S4–S6).

Hirsutalin P (**3**): colorless oil; $[\alpha]^{25}_D$ +27 (*c* 0.54, $CHCl_3$); IR (neat) ν_{max} 3426 and 1730 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Table 1; ESIMS *m/z* 435 $[M + Na]^+$; HRESIMS *m/z* 435.2720 $[M + Na]^+$(calcd for $C_{23}H_{40}O_6Na$, 435.2722) (Supplementary Information, Figures S7–S9).

Hirsutalin Q (**4**): colorless oil; $[\alpha]^{25}_D$ +12 (*c* 0.51, $CHCl_3$); IR (neat) ν_{max} 3421 and 1724 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Table 2; ESIMS *m/z* 403 $[M + Na]^+$; HRESIMS *m/z* 403.2457 $[M + Na]^+$(calcd for $C_{22}H_{36}O_5Na$, 403.2460) (Supplementary Information, Figures S10–S12).

Hirsutalin R (**5**): yellow oil; $[\alpha]^{25}_D$ −18 (*c* 0.54, $CHCl_3$); IR (neat) ν_{max} 3437 and 1740 cm^{-1}; ^{13}C and ^{1}H NMR data (400 MHz; $CDCl_3$), see Table 2; ESIMS *m/z* 513 $[M + Na]^+$; HRESIMS *m/z* 513.2831 $[M + Na]^+$(calcd for $C_{28}H_{42}O_7Na$, 513.2828) (Supplementary Information, Figures S13–S15).

3.4. Cytotoxicity Testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of compounds **1**–**7** were performed using the Alamar Blue assay [32,33].

3.5. In Vitro Anti-Inflammatory Assay

Human neutrophils were obtained using dextran sedimentation and Ficoll centrifugation. Measurements of superoxide anion generation and elastase release were carried out according to previously described procedures. [34,35]. LY294002, a phosphatidylinositol-3-kinase inhibitor, was used as a positive control for inhibition of superoxide anion generation and elastase release with IC_{50} 0.6 ± 0.1 and 1.2 ± 0.3 μg/mL [36].

4. Conclusions

Five new eunicellin-type compounds, hirsutalins N–R (**1**–**5**) and two known eunicellin-type compounds (**6** and **7**), were discovered from the soft coral *C. hirsuta*. Compound **5** displayed cytotoxicity against the proliferation of P388 and K562 cancer cells possibly due to the presence of the α,β-unsaturated ketone group. Compound **1** was found to effectively inhibit the elastase release in FMLP/CB-induced human neutrophils.

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Author Contributions

Jyh-Horng Sheu designed the whole experiment and contributed to manuscript preparation. Tzu-Zin Huang and Bo-Wei Chen carried out the experiment and wrote the manuscript. Chiung-Yao Huang and Tsong-Long Hwang performed and analyzed the bioassay. Chang-Feng Dai identified the soft coral.

Conflicts of Interest

The authors declare no conflict of interest.

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