Article

Cytotoxic and Anti-Inflammatory Eunicellin-Based Diterpenoids from the Soft Coral *Cladiella krempfi*

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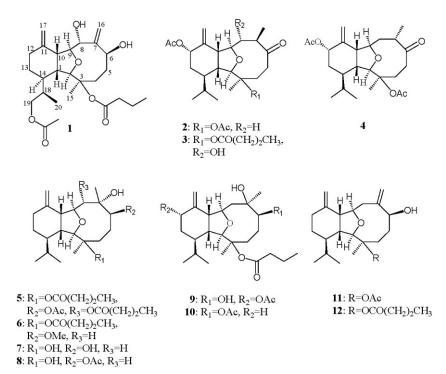
Abstract: Five new eunicellin-based diterpenoids, krempfielins E–I (**1**–**5**) and seven known compounds (**6**–**12**) were isolated from the organic extract of a Taiwanese soft coral *Cladiella krempfi*. The structures of the new metabolites were elucidated on the basis of extensive spectroscopic analysis. Metabolites **5**, **6**, **10** and **12** were shown to exhibit cytotoxicity against a limited panel of cancer cell lines. Furthermore, compounds **6** and **10** could potently inhibit the accumulation of the pro-inflammatory iNOS protein, and **6** and **12** could significantly reduce the expression of COX-2 protein in LPS-stimulated RAW264.7 macrophage cells.

1. Introduction

Previous studies showed that many eunicellin-based diterpenes discovered from soft corals exhibited cytotoxic and anti-inflammatory activities [1–14]. The soft coral *Cladiella krempfi* has been found to generate several types of metabolites including eunicellin-type diterpenoids [15] and pregnane-type steroids [16–18]. Our previous chemical investigation of *C. krempfi* had led to the isolation of four new eunicellin-based diterpenoids, krempfielins A–D [19]. In this paper, we further report the isolation of five new eunicellin-based diterpenoids, krempfielins E–I (**1–5**) (Chart 1) and known compounds 6-methyl ether of litophynol B (**6**) [13], sclerophytin A (**7**) [20], sclerophytin B (**8**) [20], litophynin I monoacetate (**9**) [21], 6-acetoxy litophynin E (**10**) [22], (1*R**, 2*R**, 3*R**, 6*S**, 9*R**, 10*R**, 14*R**)-3-acetoxycladiell-7(16),11(17)-dien-6-ol (**11**) [23] and litophynin F (**12**) [24]. The structures of metabolites **1–12** were characterized by extensive spectroscopic analysis. Cytotoxicity of all compounds against five human tumor cell lines, lung adenocarcinoma (A549 and H1299), breast carcinoma (BT483), liver carcinoma (HepG2), oral cancer (SAS) and one human lung bronchial cell (BEAS2B) lines was studied. The ability of them to inhibit the up-regulation of pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells was also evaluated. The results showed that compounds **5**, **6**, **10** and **12**, in particular **10**, are cytotoxic towards the above cancer cell's compounds. Except **2**, **4** and **11**, these compounds were found to significantly reduce the levels of iNOS protein; among them, **6** and **10** are most active. Furthermore, **6** and **12** also could significantly reduce the expression of COX-2 protein.

Chart 1. Structures of metabolites **1–12**.

2: R$_1$=OAc, R$_2$=H
3: R$_1$=OCO(CH$_2$)$_2$CH$_3$, R$_2$=OH

5: R$_1$=OCO(CH$_2$)$_2$CH$_3$, R$_2$=OAc, R$_3$=OCO(CH$_2$)$_2$CH$_3$
6: R$_1$=OCO(CH$_2$)$_2$CH$_3$, R$_2$=OMe, R$_3$=H
7: R$_1$=OH, R$_2$=OH, R$_3$=H
8: R$_1$=OH, R$_2$=OAc, R$_3$=H

9: R$_1$=OH, R$_2$=OAc
10: R$_1$=OAc, R$_2$=H

11: R=OAc
12: R=OCO(CH$_2$)$_2$CH$_3$

2. Results and Discussion

The new metabolite krempfielin E (**1**) showed the molecular ion peak [M + Na]$^+$ at *m/z* 487.2669 in the HRESIMS and established a molecular formula of $C_{26}H_{40}O_7$, implying seven degrees of unsaturation. The IR absorptions bands at v_{max} 3421 and 1732 cm^{-1} revealed the presence of hydroxy and ester carbonyl functionalities. The ^{13}C NMR spectrum measured in CDCl$_3$ showed signals of twenty-six carbons (Table 1) which were assigned by the assistance of the DEPT spectrum to four methyls (including one acetate methyl δ_C 21.0), seven sp^3 methylenes, two sp^2 methylenes, eight sp^3 methines (including four oxymethines), one sp^3 and four sp^2 quaternary carbons (including two ester carbonyls).The NMR spectroscopic data of **1** (Tables 1 and 2) showed the presence of two 1,1-disubstituted double bonds (δ_C 118.1 CH$_2$, 112.0 CH$_2$, 152.2 C, and 145.2 C; δ_H 5.51 s, 5.22 s, 4.81 s, and 4.65 s). Two ester carbonyls (δ_C 172.5 and 171.2) were assigned from the ^{13}C NMR spectrum and their signals were correlated with the methylene protons (δ_H 2.10, 2H, m) of an *n*-butyrate and protons of an acetate methyl (δ_H 2.07 s, 3H), respectively. Therefore, the remaining three degrees of unsaturation identified **1** as a tricyclic molecule. The ^1H–^1H COSY and HMBC correlations (Figure 1) were further used for establishing the molecular skeleton of **1**. The COSY experiment assigned three isolated consecutive proton spin systems. Above evidences and the analysis of HMBC spectrum (Figure 1) suggested that **1** is an eunicellin-based diterpenoid. Furthermore, the acetoxy group attaching at C-19 was confirmed by the HMBC correlations from oxymethylene [δ_H 3.94 (H$_2$-19)] and acetate methyl protons (δ_H 2.07) to the ester carbonyl carbon appearing at δ 171.2 (C). Thus, the remaining one *n*-butyryloxy group had to be positioned at C-3, an oxygen-bearing quaternary carbon resonating at δ 84.4 ppm. On the basis of above analysis, the planar structure of **1** was established.

Table 1. ^{13}C NMR Data for Compounds **1**–**5**.

C	1 [a]	2 [a]	3 [b]	4 [b]	5 [a]
1	43.3, CH [c]	45.8, CH	44.7, CH	44.9, CH	46.6, CH
2	91.4, CH	90.6, CH	90.9, CH	90.8, CH	92.6, CH
3	84.4, C	84.7, C	84.2, C	84.6, C	85.6, C
4	28.4, CH$_2$	33.2, CH$_2$	33.2, CH$_2$	32.6, CH$_2$	35.8, CH$_2$
5	35.3, CH$_2$	30.2, CH$_2$	29.7, CH$_2$	38.3, CH$_2$	28.5, CH$_2$
6	66.9, CH	214.8, C	217.0, C	213.4, C	82.3, CH
7	152.2, C	48.3, CH	40.7, CH	40.6, CH	78.0, C
8	77.3, CH	42.4, CH$_2$	78.5, CH	37.7, CH$_2$	78.5, CH
9	83.7, CH	81.9, CH	85.5, CH	78.9, CH	78.3, CH
10	47.9, CH	52.1, CH	47.5, CH	49.4, CH	52.0, CH
11	145.2, C	142.1, C	141.0, C	141.6, C	148.5, C
12	31.3, CH$_2$	73.1, CH	72.8, CH	73.0, CH	31.6, CH$_2$
13	25.7, CH$_2$	28.5, CH$_2$	28.9, CH$_2$	28.7, CH$_2$	25.5, CH$_2$
14	39.1, CH	35.3, CH	35.6, CH	35.7, CH	44.0, CH
15	22.2, CH$_3$	22.3, CH$_3$	22.6, CH$_3$	22.6, CH$_3$	22.8, CH$_3$
16	118.1, CH$_2$	17.8, CH$_3$	14.1, CH$_3$	15.4, CH$_3$	18.5, CH$_3$
17	112.0, CH$_2$	118.1, CH$_2$	119.5, CH$_2$	118.7, CH$_2$	109.2, CH$_2$
18	32.6, CH	28.0, CH	27.2, CH	27.5, CH	29.0, CH
19	67.5, CH$_2$	21.5, CH$_3$	21.6, CH$_3$	21.5, CH$_3$	21.9, CH$_3$

Table 1. *Cont.*

20	10.6, CH$_3$	16.6, CH$_3$	14.9, CH$_3$	14.9, CH$_3$	15.4, CH$_3$
	172.5, C		172.5, C		173.1, C
3-*n*-butyrate	37.4, CH$_2$		37.3, CH$_2$		36.5, CH$_2$
	18.5, CH$_2$		18.4, CH$_2$		18.4, CH$_2$
	13.6, CH$_3$		13.7, CH$_3$		13.8, CH$_3$
3-OAc		169.6, C		169.7, C	
		22.4, CH$_3$		22.3, CH$_3$	
6-OAc					171.6, C
					21.4, CH$_3$
					173.3, C
8-*n*-butyrate					36.7, CH$_2$
					18.2, CH$_2$
					13.5, CH$_3$
12-OAc		170.2, C	170.1, C	170.2, C	
		21.6, CH$_3$	21.5, CH$_3$	21.4, CH$_3$	
19-OAc	171.2, C				
	21.0, CH$_3$				

[a] Spectra recorded at 100 MHz in CDCl$_3$. [b] Spectra recorded at 125 MHz in CDCl$_3$. [c] Deduced from DEPT.

Table 2. ^{1}H NMR Data for Compounds **1**–**5**.

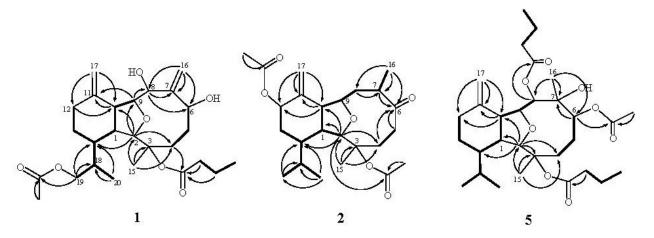| H	1 [a]	2 [a]	3 [b]	4 [b]	5 [a]
1	2.25 m	2.25 dd (12.4, 7.2)	2.28 m	2.29 m	2.17 m
2	3.71 br s	3.67 br s	3.81 br s	3.73 br s	3.63 br s
4	1.64 m 2.25 m	2.03 m 2.75 dd (14.4, 8.8)	2.23 m 2.46 m	2.19 m 2.52 dd (5.5, 2.0)	1.96 m 2.65 m
5	1.74 m 2.19 m	1.91 m 2.48 dd (13.2, 11.2)	1.25 m	2.25 m 2.43 t (10.5)	1.56 m
6	4.73 dd (11.2, 4.0) [c]				5.71 d (5.6)
7		2.58 m	2.32 m	2.68 m	
8	4.18 d (3.2)	1.88 m 2.00 m	3.77 d (8.5)	1.87 t (5.0) 2.24 m	5.27 d (9.2)
9	4.19 d (6.8)	4.12 ddd (11.6, 8.8, 4.4)	4.43 d (11.0)	4.23 dt (10.0, 5.0)	4.11 t (8.8)
10	2.87 dd (10.8, 8.0)	3.12 t (7.6)	2.91 t (7.5)	3.07 td (10.0, 1.5)	3.39 t (7.2)
12	2.10 m 2.27 m	5.49 t (2.8)	5.52 br s	5.49 t (2.5)	2.03 m 2.23 m
13	1.11 m 1.70 m	1.26 m 1.92 m	1.32 m 1.97 dt (14.5, 3.0)	1.29 m 1.94 dt (14.5, 3.0)	1.08 m 1.76 m

Table 2. *Cont.*

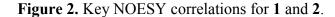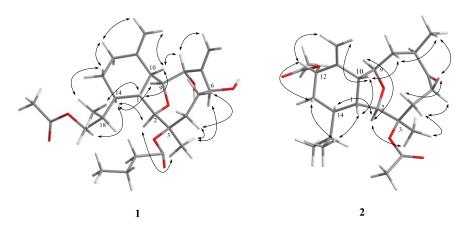|					
14	1.55 m	1.71 m	1.76 m	1.71 m	1.23 m
15	1.63 s	1.40 s	1.49 s	1.48 s	1.38 s
16	5.22 s / 5.51 s	1.06 d (7.2)	1.26 d (6.5)	1.07 d (7.0)	1.08 s
17	4.65 s / 4.81 s	4.99 d (1.6) / 5.91 d (1.6)	4.96 s / 5.27 s	4.97 s / 5.28 s	4.51 s / 4.64 s
18	2.08 m	1.76 m	1.84 m	1.81 m	1.69 m
19	3.94 t (6.4)	0.91 d (6.8)	0.96 d (7.0)	0.93 d (6.5)	0.96 d (6.8)
20	0.80 s	0.75 d (6.8)	0.78 d (7.0)	0.77 d (6.5)	0.77 d (6.8)
3-*n*-butyrate	2.10 m / 1.57 m / 0.92 t (7.2)		2.28 m / 1.68 m / 0.99 t (7.5)		2.25 m / 1.68 m / 0.98 t (7.6)
3-OAc		2.18 s		2.14 s	
6-OAc					2.09 s
8-*n*-butyrate					2.55 m / 1.61 m / 0.96 t (7.2)
12-OAc		2.00 s	2.03 s	1.98 s	
19-OAc	2.07 s				

[a] Spectra recorded at 400 MHz in CDCl$_3$. [b] Spectra recorded at 500 MHz in CDCl$_3$. [c] *J* values (Hz) in parentheses.

Figure 1. Selected ^{1}H–^{1}H COSY (━) and HMBC (→) correlations of **1**, **2** and **5**.
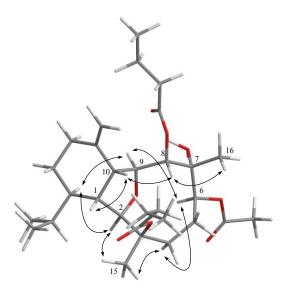

The relative structure of **1** was elucidated by the analysis of NOE correlations, as shown in Figure 2. The observation of the NOE correlations of H-1 with H-10 and H$_3$-20 suggested that these protons had the same orientation and were assumed to be β-oriented. The NOE interactions found between the oxymethine proton H-8 and H-10 assigned the α-orientation of the hydroxy group. Furthermore, the NOE correlations of H-2 with both H-14 and H$_3$-15; H-14 with both H-9 and H$_2$-19; and H$_3$-15 with H-6, suggested that H-2, H-6, H-9, H-14, and H$_3$-15 are α-oriented. Furthermore, the configuration of C-18 was to be *R** on the basis of NOE correlations of H-1/H$_3$-20 and H-14/H$_2$-19. The relative configuration of **1** was thus established. Comparison of the ^{1}H and ^{13}C NMR spectroscopic data of **1** with those of its 19-deacetoxyl derivative, litophynol A [21], further confirmed the structure of **1**.

Figure 2. Key NOESY correlations for **1** and **2**.

1   **2**

Krempfielin F (**2**) was found to have the molecular formula $C_{24}H_{36}O_6$ and seven degrees of unsaturation, as indicated from the HRESIMS (*m/z* 443.2408 [M + Na]$^+$). The ^{13}C NMR spectrum of **2** showed signals of twenty-four carbons (Table 1), which were characterized by the DEPT spectrum as six methyls, five methylenes (including one sp^2 methylene), eight methines (including three oxygenated carbons), and five quaternary carbons (including one ketone carbonyl, two ester carbonyls, and one sp^2 quaternary carbon of an olefinic group). The presence of two acetoxy groups was indicated by the ^{1}H NMR signals (Table 2) the ^{1}H NMR signals at δ_H 2.18 (s, 3H) and 2.00 (s, 3H), and the ^{13}C NMR signals at δ_C 22.4 (CH$_3$), 21.6 (CH$_3$), 169.6 (C), and 170.2 (C). The remaining three degrees of unsaturation again identified **2** as a tricyclic diterpenoid. The molecular framework was established by ^{1}H–^{1}H COSY and HMBC experiments (Figure 1). The stereochemistry of compound **2** was also determined by the NOESY spectrum (Figure 3), which exhibited NOE correlations of H-1 and with H-10 and H$_3$-20, H-13β (δ_H 1.26) and with H-12 and H$_3$-20, H-8β (δ_H 1.88) with H-5β (δ_H 2.48), H-10 and H$_3$-16, H-5α with H-4α and H$_3$-15, and H$_3$-16 with both H-8β (δ_H 1.88) and H-5β (δ_H 2.48), establishing the β-orientation of H-12 and H$_3$-16. On the basis of these results and other observed NOE correlations (Figure 2), the structure of metabolite **2** was determined.

Figure 3. Key NOESY correlations for **5**.

5

The HRESIMS (m/z 487.2675 [M + Na]$^+$) of **3** established the molecular formula of $C_{26}H_{40}O_7$. Comparison of the NMR data of **3** with those of **2** revealed the replacement of one acetoxy group (δ_H 2.18, 3H, s; δ_C 169.6, C and 22.4, CH$_3$) in **2** by an *n*-butyryloxy group in **3** (δ_H 0.99, 3H, t, J = 7.5 Hz; 1.68, 2H, m; 2.28, 2H, m; and δ_C 172.5, C; 37.3, CH$_2$; 18.4, CH$_2$ and 13.7, CH$_3$), and an additional hydroxy group substituted at C-8 of **3** that downfielded H-8 to δ_H 3.77 and C-8 to δ_C 78.5 ppm. The placement of the *n*-butyryloxy group at C-3 was confirmed by the HMBC experiment which showed a correlation between H-2 and the carbonyl carbon (δ_C 172.5 C) of this *n*-butyryloxy group. The NOE correlations of **3** also showed that the stereochemistry of this metabolite is identical with that of **2** excepted for the presence of the α-oriented hydroxy group at C-8.

The HRESIMS of krempfielin H (**4**) exhibited a [M + Na]$^+$ ion peak at m/z 443.2408, appropriate for a molecular formula of $C_{24}H_{36}O_6$. By analysis of 2D NMR spectra, including ^{1}H–^{1}H COSY, HMQC, and HMBC, compound **4** was shown to possess the same molecular framework as that of **2**. Furthermore, it was found that the NMR data of **4** were very similar to those of **2** (Tables 1 and 2), suggesting that **4** might be an isomer of **2**. From NOESY spectrum, it was found that the β-oriented H-10 showed NOE interactions with both H-7 and H-8β (δ_H 1.87), while H-8β showed NOE interactions with H-7, indicating the β-orientation of H-7. This inferred the α-orientation of methyl substituent at C-7. Further analysis of other NOE interactions revealed that **4** possessed the same relative configuration sat C-1, C-2, C-3, C-9, C-10, C-12 and C-14 as those of **2**. Therefore, **4** was found to be the C-7 epimer of **2**.

The related metabolite, krempfielin I (**5**), had a molecular formula of $C_{30}H_{48}O_8$ as indicated by the HRESIMS (m/z 559.3243, [M + Na]$^+$) and NMR data (Tables 1 and 2). The ^{13}C NMR spectrum of **5** revealed the appearance of three ester carbonyls (δ_C 173.3, 173.1 and 171.6), which were correlated with protons of two methylenes (δ_H 2.55, 2.25, m, each 2H; and δ_C 36.7 and 36.5) of two *n*-butyrates and the methyl protons (δ_H 2.09 s, 3H and δ_C 21.4) of one acetate group, respectively. The planar structure of **5** was established by ^{1}H–^{1}H COSY and HMBC correlations (Figure 2). The HMBC connectivities from H-2 (δ_H 3.63 br s, 1H) and H-8 (δ_H 5.27 d, 1H, J = 9.2 Hz) to two carbonyl carbons δ_C 173.3 (C) and 173.1 (C) determined the positions of the two *n*-butyrates at C-8 and C-3. Also, the location of an acetate group at C-6 was supported by the HMBC connectivities from both of the acetate methyl protons (δ_H 2.09 s, 3H) and oxygenated methine proton (δ_H 5.71 d, 1H, J = 5.6 Hz) to the carbon resonating at δ_C 171.6 (C). The relative configuration of **5** was confirmed by analyzing the key NOE correlations (Figure 3).
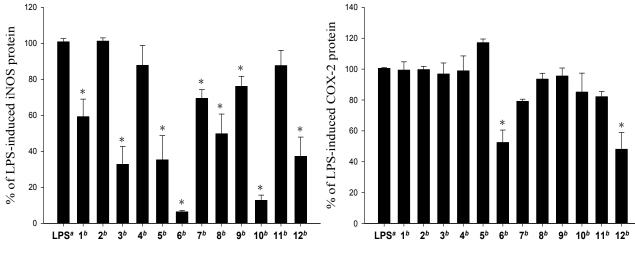
The cytotoxicity of the diterpenoids **1**–**12** against the growth of five human carcinoma cells A549, BT483, H1299, HepG2, SAS and one human normal cell line BEAS2B was studied (Table 3). The results showed that **1**–**4**, **7**–**9** and **11** are not cytotoxic toward the above cancer and normal cells. Compounds **5**, **6**, **10** and **12** exhibited cytotoxicity toward the above five cancer cell lines and the human normal cell line; **10**, being the most cytotoxic. The *in vitro* anti-inflammatory effects of compounds **1**–**12** were also tested by examining the inhibitory activity of these compounds toward the LPS-induced up-regulation of pro-inflammatory proteins iNOS and COX-2, in RAW264.7 macrophage cells (Figure 4). At a concentration of 10 μM, compounds except **2**, **4**, and **11** were found to significantly reduce the expression of iNOS protein, relative to the control cells stimulated with LPS only. Among them, **6** and **10** could potently reduce the levels of iNOS protein to 6.4 ± 0.8% and 12.8 ± 2.9%, respectively. Compounds **6** and **12** also effectively reduced COX-2 expression (52.5 ± 8.0% and 48.1 ± 10.8%, respectively) in the same LPS-stimulated cells. These results revealed that *n*-butyryloxy

group at C-3 could significantly enhance the cytotoxic and anti-inflammatory activities in eunicellin-type compounds. Overall, compounds **6**, **10** and **12** exhibited interesting cytotoxic and anti-inflammatory activity and could become lead compounds in the future drug development.

Table 3. Cytotoxicity (ED$_{50}$ µg/mL) of compounds **5**, **6**, **10** and **12**.

Compounds	Cell Lines					Normal Cell Line
	A549	BT483	H1299	HepG2	SAS	BEAS2B
5	15.0 ± 3.5	11.5 ± 1.8	19.2 ± 4.0	12.9 ± 3.1	10.2 ± 3.5	$-$ [a]
6	16.1 ± 1.2	10.0 ± 1.8	11.8 ± 1.0	$-$ [a]	17.2 ± 0.4	10.4 ± 0.3
10	6.8 ± 1.0	11.6 ± 2.8	6.7 ± 0.7	8.5 ± 1.3	9.5 ± 3.7	4.8 ± 0.7
12	12.2 ± 1.1	6.8 ± 0.6	12.8 ± 1.2	11.1 ± 0.4	10.3 ± 0.5	13.6 ± 0.5
Taxol	1.5 ± 0.9	3.9 ± 0.8	1.2 ± 0.1	1.4 ± 0.7	2.3 ± 1.5	2.3 ± 1.5

[a] $IC_{50} > 20$ µg/mL.

Figure 4. Effect of compounds **1–12** on LPS-induced iNOS and COX-2 proteins expression in RAW264.7 macrophage cells by immunoblot analysis. The values are mean $\pm$ 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 **1–12** (10 µM).

3. Experimental Section

3.1. 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 were obtained with a Bruker APEX II mass spectrometer. NMR spectra were recorded either on a Varian UNITY INOVA-500 FT-NMR, a Varian 400MR FT-NMR. 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 a ODS column (25.0 × 21.2 mm, 5 µm).

3.2. Animal Material

C. krempfi was collected by hand using scuba off the coast of Penghu islands of Taiwan in June 2008, at a depth of 5–10 m, and stored in a freezer until extraction. A voucher sample (specimen no. 200806CK) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3. Extraction and Separation

The octocoral (1.1 kg fresh wt) was collected and freeze-dried. The freeze-dried material was minced and extracted exhaustively with EtOH (3 × 10 L). The EtOH extract of the frozen organism was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2-soluble portion (14.4 g) was subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0%–100% of EtOAc, stepwise) and then further with MeOH in EtOAc with increasing polarity to yield 41 fractions. Fraction 28, eluted with *n*-hexane–EtOAc (1:2), was rechromatoraphed over a reversed-phase column (RP-18) using acetone–H_2O (10:1) as the mobile phase to afford six subfractions (A1–A6). Subfraction A1 was repeatedly separated by reverse phase HPLC (CH_3CN–H_2O, 1:1 to 2:1) to afford compounds **1** (3.5 mg), **2** (4.9 mg), **3** (7.6 mg), **4** (4.8 mg), and **9** (3.6 mg). Subfraction A2 separated by reverse phase HPLC (CH_3CN–H_2O, 3.8: 1) to afford compounds **5** (7.1 mg), **6** (16.4 mg), **10** (30.2 mg), **11** (5.2 mg) and **12** (5.4 mg). Subfraction A3 by reverse phase HPLC (CH_3CN–H_2O, 1:1) to afford compound **7** (13.2 mg). Subfraction A4 by reverse phase HPLC (MeOH–H_2O, 2.4:1) to afford compound **8** (5.5 mg).

3.3.1. Krempfielin E (**1**)

Colourless oil; $[\alpha]_D^{25}$ −78.3 (*c* 0.35, $CHCl_3$); IR (neat) ν_{max} 3421, 3072, 2931, 1732, 1648, 1447, 1372, 1238, 1180 and 1039 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m/z* 487 [M + Na]$^+$; HRESIMS *m/z* 487.2669 [M + Na]$^+$ (calcd for $C_{26}H_{40}O_7Na$, 487.2672).

3.3.2. Krempfielin F (**2**)

Colourless oil; $[\alpha]_D^{25}$ −7.3 (*c* 0.49, $CHCl_3$); IR (neat) ν_{max} 3073, 2959, 1736, 1645, 1454, 1369, 1240, 1165 and 1095 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m/z* 443 [M + Na]$^+$; HRESIMS *m/z* 443.2408 [M + Na]$^+$ (calcd for $C_{24}H_{36}O_6Na$, 443.2409).

3.3.3. Krempfielin G (**3**)

Colourless oil; $[\alpha]_D^{25}$ −26.3 (*c* 0.76, $CHCl_3$); IR (neat) ν_{max} 3459, 3078, 2961, 1735, 1645, 1456, 1371, 1238, 1177 and 1075 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m/z* 487 [M + Na]$^+$; HRESIMS *m/z* 487.2675 [M + Na]$^+$ (calcd for $C_{26}H_{40}O_7Na$, 487.2672).

3.3.4. Krempfielin H (**4**)

Colourless oil; $[\alpha]_D^{25}$ −5.8 (*c* 0.48, $CHCl_3$); IR (neat) ν_{max} 3077, 2958, 1734, 1645, 1455, 1369, 1240, 1165 and 1095 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m/z* 443 [M + Na]$^+$; HRESIMS *m/z* 443.2408 [M + Na]$^+$ (calcd for $C_{24}H_{36}O_6Na$, 443.2409).

3.3.5. Krempfielin I (**5**)

Colourless oil; $[\alpha]_D^{25}$ −18.3 (*c* 0.35, CHCl$_3$); IR (neat) v_{max} 3460, 3069, 2960, 1733, 1646, 1448, 1370, 1254, 1181 and 1089 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m/z* 559 [M + Na]$^+$; HRESIMS *m/z* 559.3243 [M + Na]$^+$ (calcd for C$_{30}$H$_{48}$O$_8$Na, 559.3247).

3.4. Cytotoxicity Testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of compounds **1**–**12** were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetra-zolium bromide] colorimetric method [25,26].

3.5. In Vitro *Anti-Inflammatory Assay*

Macrophage (RAW264.7) cell line was purchased from ATCC. In vitro anti-inflammatory activities of compounds **1**–**12** were measured by examining the inhibition of lipopolysaccharide (LPS) induced upregulation of iNOS (inducible nitric oxide synthetase) and COX-2 (cyclooxygenase-2) proteins in macrophages cells using western blotting analysis [27,28].

4. Conclusions

New eunicellin-based diterpenoids were isolated together with known ones from the soft coral *Cladiella krempfi*. Compounds **5**, **6**, **10** and **12** showed cytotoxicity toward the above five cancer cell lines, and one human normal cell line. Also, **6**, **10** and **12** could significantly reduce the accumulation of pro-inflammatory proteins iNOS and COX-2. Thus, these compounds, in particular **6**, **10** and **12** could be promising bioactive agents and may warrant further biomedical investigation.

Acknowledgements

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