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Article

Krempfielins N–P, New Anti-Inflammatory Eunicellins from a Taiwanese Soft Coral *Cladiella krempfi*

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Abstract: Three new eunicellin-type diterpenoids, krempfielins N–P (**1**–**3**), were isolated from a Taiwanese soft coral *Cladiella krempfi*. The structures of the new metabolites were elucidated by extensive spectroscopic analysis and by comparison with spectroscopic data of related known compounds. Compound **3** exhibited activity to inhibit superoxide anion generation. Both **1** and **3**, in particular **1**, were shown to display significant anti-inflammatory activity by inhibiting the elastase release in FMLP/CB-induced human neutrophils.

Keywords: *Cladiella krempfi*; eunicellin-type diterpenoid; anti-inflammatory agent; elastase

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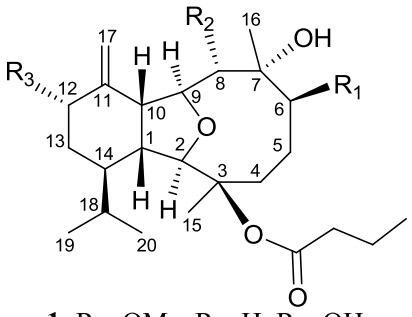
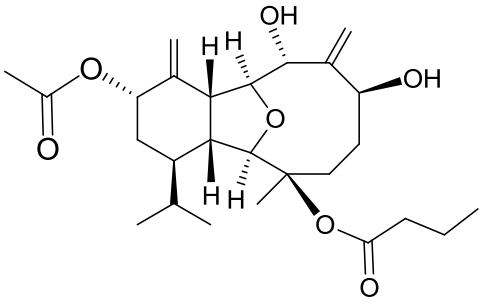
1. Introduction

Soft corals have been known to be rich sources of terpenoid metabolites [1]. For the purpose of discovering bioactive agents from marine organisms, we have previously investigated the chemical constituents and reported a series of bioactive natural products from Taiwanese soft corals [2–5]. In recent studies a series of bioactive eunicellin-based diterpenoids, have been isolated from the soft corals of the genera *Cladiella*, *Klysum* and *Litophyton* sp. [6–14]. The soft coral *Cladiella krempfi* has been found to produce several types of metabolites including eunicellin-type diterpenoids [15–17] and pregnane-type steroids [18,19]. Our previous chemical investigation of the Formosan soft coral *Cladiella krempfi* also resulted in the isolation of a series of new eunicellin-type diterpenoids, krempfielins A–M [20–22]. In this paper, we further report the discovery of three new eunicellin-based diterpenoids, krempfielins N–P (**1**–**3**) (Chart 1 and Supplementary Figures S1–S9). The ability of these compounds to inhibit the superoxide anion generation and elastase release in FMLP/CB-induced human neutrophils was also evaluated. The results showed that compound **3** could inhibit superoxide anion generation while **1** and **3**, especially **1**, effectively inhibited the generation of the elastase release in FMLP/CB-induced human neutrophils.

Chart 1. Structures of metabolites **1**–**3**.



1: R_1=OMe, R_2=H, R_3=OH
2: R_1=OH, R_2=OAc, R_3=OAc



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2. Results and Discussion

The new metabolite krempfielin N (**1**) showed the molecular ion peak $[M + Na]^+$ at *m*/*z* 461.2882 in the HRESIMS and established a molecular formula of $C_{25}H_{42}O_6$, implying five degrees of unsaturation. The IR absorptions bands at ν_{max} 3445 and 1733 cm^{-1} revealed the presence of hydroxy and ester carbonyl functionalities. The ^{13}C NMR spectrum measured in $CDCl_3$ showed signals of 25 carbons (Table 1) which were assigned by the assistance of the DEPT spectrum to six methyls (including one oxgenate methyl δ_C 57.0), six sp^3 methylenes, one sp^2 methylene, eight sp^3 methines (including four oxymethines), four quaternary carbons (including one ester carbonyl). The NMR spectroscopic data of **1** (Tables 1 and 2) showed the presence of one 1,1-disubstituted double bond (δ_C 112.5 CH_2 and 148.0 C; δ_H 5.03 s, and 4.86 s), one methoxy group (δ_H 3.34, 3H, s) and one *n*-butyryloxy group (δ_C 172.3 C; 37.4 CH_2; 18.4 CH_2; and 13.7 CH_3; δ_H 2.30 m, 2H; 1.67 m, 2H; and 0.98 t, 3H, J = 7.6 Hz). Therefore, taking account of the two degrees of unsaturation from double bonds, it was suggested that **1** should be a tricyclic compound from the remaining three degrees of unsaturation. 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 two hydroxy groups attaching at C-7 and C-12 were confirmed by the HMBC correlations from one methyl (δ_H 1.12 s, H-16) and one oxymethine (δ_H 4.12 m, H-6) to the oxygenated quaternary carbon appearing at δ 75.8 (C-7), and one methine (δ_H 2.91 t, H-10) and one proton of H_2-17 (δ_H 5.03 s) to the oxymethine carbon appearing at δ 71.0 (C-12). Thus, the remaining one *n*-butyryloxy group had to be positioned at C-3, an oxygen-bearing quaternary carbon resonating at δ 86.5 ppm. On the basis of above analysis, the planar structure of **1** was established. The stereochemistry of **1** was finally confirmed by the very similar NOE correlations of both **1** and krempfielin L [22].

Table 1. ^{13}C NMR data for compounds **1**–**3**.

	1 [a]	**2** [b]	**3** [a]
	δ_C	**δ_C**	**δ_C**
1	44.4, CH [c]	45.1, CH	43.2, CH [c]
2	91.0, CH	91.5, CH	90.7, CH
3	86.5, C	85.7, C	84.5, C
4	36.0, CH_2	35.7, CH_2	28.7, CH_2
5	26.7, CH_2	28.9, CH_2	35.4, CH_2
6	89.7, CH	77.5, CH	67.0, CH
7	75.8, C	79.4, C	152.5, C
8	45.2, CH_2	79.0, CH	77.2, CH
9	80.0, CH	79.4, CH	85.0, CH
10	51.4, CH	50.1, CH	46.6, CH
11	148.0, C	143.6, C	141.4, C
12	71.0, CH	73.5, CH	73.2, CH
13	30.7, CH_2	29.2, CH_2	29.0, CH_2
14	36.6, CH	37.2, CH	36.9, CH
15	23.2, CH_3	23.1, CH_3	22.1, CH_3
16	23.6, CH_3	18.0, CH_3	118.1, CH_2
17	112.5, CH_2	115.1, CH_2	119.4, CH_2
18	28.8, CH	28.6, CH	26.9, CH
19	16.0, CH_3	15.6, CH_3	15.3, CH_3
20	21.8, CH_3	21.7, CH_3	21.6, CH_3
3-*n*-butyrate	172.3, C	173.0, C	172.5, C
	37.4, CH_2	36.7, CH_2	37.4, CH_2
	18.4, CH_2	18.5, CH_2	18.5, CH_2
	13.7, CH_3	13.5, CH_3	13.6, CH_3
6-OMe	57.0, CH_3		
8-OAc		170.7, C	
		21.4, CH_3	
12-OAc		170.2, C	170.1, C
		21.6, CH_3	21.7, CH_3

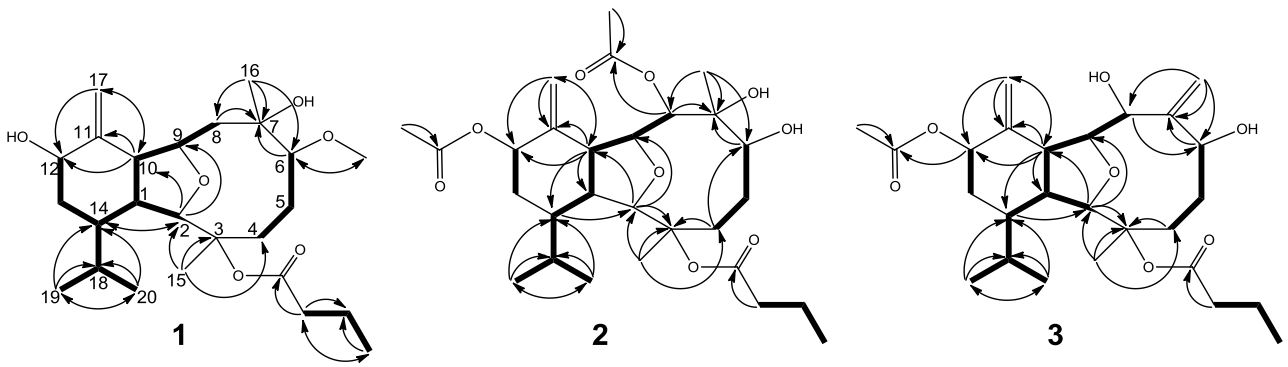
[a] ^{13}C spectra recorded at 100 MHz in $CDCl_3$; [b] ^{13}C spectra recorded at 125 MHz in $CDCl_3$; [c] Deduced from DEPT.

Table 2. ^{1}H NMR data for compounds **1**–**3**.

	1 [a]	**2** [b]	**3** [a]
	δ_H	**δ_H**	**δ_H**
1	2.25 m	2.28 dd (10.0, 7.0)	2.26 m
2	3.70 br s	3.67 br s	3.84 br s
3			
4	1.86 m, 2.64 m	1.85 m, 2.66 m	1.68 m, 2.66 m
5	1.33 m, 1.65 m	1.49 m, 1.65 m	1.76 m 2.21 m
6	4.12 m	4.66 d (6.5)	4.75 dd (10.8, 4.4) [d]
7			
8	1.82 m	5.19 d (10.0)	4.20 s
9	4.53 m	4.31 dd (10.0, 6.5)	4.43 d (10.8)
10	2.91 t (6.4) [c]	3.38 dd (7.0, 7.0)	2.90 dd (10.8, 8.4)
11			
12	4.39 s	5.43 dd (4.0, 3.0)	5.49 t (2.8)
13	1.36 m, 1.86 m	1.37 m, 1.93 m	1.30 m, 1.98 m
14	1.86 m	1.70 m	1.71 m
15	1.45 s	1.45 s	1.65 s
16	1.12 s	1.08 s	5.23 s, 5.55 s
17	4.86 s, 5.03 s	4.84 s, 5.10 s	4.96 d (1.6) 5.27 d (1.6)
18	1.81 m	1.80 m	1.96 m
19	0.82 d (7.6)	0.80 d (7.0)	0.75 d (6.8)
20	0.99 d (7.2)	0.95 d (7.0)	0.95 d (6.8)
3-*n*-butyrate	2.30 m	2.60 m, 2.50 m	2.12 m
	1.67 m	1.67 m	1.58 m
	0.98 t (7.6)	1.00 t (7.5)	0.92 t (7.6)
6-OMe	3.34 s		
8-OAc		2.07 s	
12-OAc		2.08 s	2.05 s

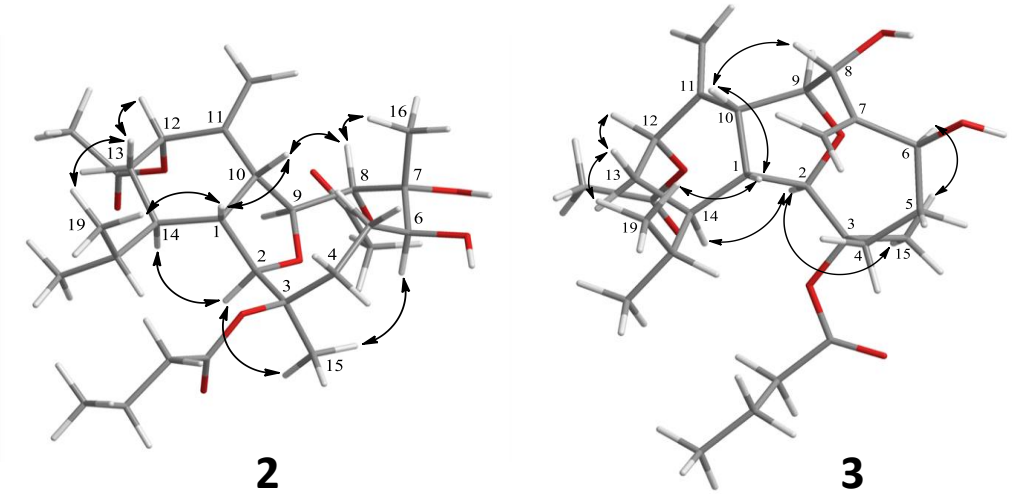
[a] ^{1}H spectra recorded at 400 MHz in $CDCl_3$; [b] ^{1}H spectra recorded at 500 MHz in $CDCl_3$; [c] *J* values (Hz) in parentheses.

Figure 1. Selected ^{1}H–^{1}H COSY (▬) and HMBC (→) correlations of **1**, **2** and **3**.



Krempfielin O (**2**) was shown by HRESIMS to possess the molecular formula $C_{28}H_{44}O_9$ (*m*/*z* 547.2880 $[M + Na]^+$). The NMR spectroscopic data of **2** (Tables 1 and 2) showed the presence of two acetoxy groups (δ_H 2.07, s and 2.08, s, each 3H; and δ_C 170.7, C and 170.2, C; 21.4, CH_3 and 21.6, CH_3), and an *n*-butyryloxy group (δ_H 2.60 m and 2.50 m, each 1H; 1.67 m, 2H and 1.00 t, 3H, J = 7.5 Hz; and δ_C 173.0, C; 36.7, CH_2; 18.5, CH_2 and 13.5, CH_3). As demonstrated by the HMBC correlation from oxymethine proton H-8 (δ 5.19) to the ester carbonyl carbon appearing at δ_C 170.7 (Figure 1), one acetoxy group was positioned at C-8. The position of an *n*-butyryloxy group at C-3 was established by NOE interaction between the methylene protons (δ 1.67) of *n*-butyryloxy group with H-5 (δ 1.49). The remaining one acetoxy group was thus positioned at C-12. The relative configuration of **2** was further confirmed by NOE correlations (Figure 2).

Figure 2. Key NOESY correlations for **2** and **3**.



The related metabolite, krempfielin P (**3**), had a molecular formula of $C_{26}H_{40}O_7$ as indicated by the HRESIMS (*m*/*z* 487.2675, $[M + Na]^+$) and NMR data (Tables 1 and 2). The ^{13}C NMR spectrum of **3** revealed the appearance of two ester carbonyls (δ_C 172.5 and 170.1), which were correlated with one methylene (δ_H 2.12 m, 2H; and δ_C 37.4) of an *n*-butyrate and the methyl (δ_H 2.05 s, 3H; δ_C 21.7CH_3) of an acetate group, respectively. The planar structure of **3** was determined by ^1H–^1H COSY and HMBC correlations (Figure 1). Comparison of the NMR data of **3** with those of the compound krempfielin A [20] revealed that the only difference is the replacement of one methyl and one hydroxy group at C-7 in krempfielin A by the substitution of one olefinic methylene (δ_C 118.1, CH_2; δ_H 5.55, s and 5.23, s) in **3**. The placement of one *n*-butyryloxy group and one acetoxy group at C-3 and C-12, respectively was established by comparison of the spectroscopic data with those of krempfielin A. The relative configuration of **3** was mostly determined to be the same as that of krempfielin A by comparison of the chemical shifts of both compounds and was further confirmed by NOE correlations (Figure 2).

Recently, we discovered several eunicellins showed anti-inflammatory activity by significantly inhibiting superoxide anion generation and elastase release in human neutrophiles induced by *N*-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (FMLP/CB) [22,23]. The same *in vitro* anti-inflammatory effects of the diterpenoids **1**–**3** also were tested in this study (Table 3). At a concentration of 10 μM, **1** and **2** could not significantly reduce the generation of superoxide anion,

however, **3** inhibited 23.32% ± 5.88% generation of superoxide anion, relative to the control cells stimulated with FMLP/CB only. At the same concentration, all of **1**–**3** were found to show anti-inflammatory activity by inhibiting the elastase release. Compound **1** displayed significant inhibition (73.86% ± 14.18%) at this concentration with IC_{50} of 4.94 ± 1.68 μM in this assay.

Table 3. Effect of pure compounds on elastase release in *N*-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (FMLP/CB)-induced human neutrophils.

Compound	Elastase		
	Inhibition (%)		IC_{50} (μM)
1	73.86 ± 14.18	**	4.94 ± 1.68
2	13.33 ± 3.56	*	>10
3	35.54 ± 3.17	***	>10

Percentage of inhibition (%) was measured at 10 μM; results are presented as mean ± S.E.M. (n = 3 or 4); * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control value.

3. Experimental Section

3.1. General Experimental Procedures

Melting point was determined using a Fisher-Johns melting point apparatus. 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. The NMR spectra were recorded either on a Varian UNITY INOVA-500 FT-NMR and a Varian 400MR FT-NMR. Silica gel (Merck, Darmstadt, Germany, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Darmstadt, Germany, Kieselgel 60 F-254, 0.2 mm) were used for analytical thin layer chromatography (TLC). High performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with an octadecylsilane (ODS) column (250 × 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 31, eluted with *n*-hexane–EtOAc (1:10), was rechromatoraphed over a silica gel open column using *n*-hexane–acetone (3:1) as the mobile phase to afford eight subfractions (A1–A8). Subfraction

A4 was repeatedly separated by reverse phase HPLC (CH_3CN–H_2O, 0.8:1 to 1:1) to afford compound **1** (3.2 mg). Subfraction A5 separated by reverse phase HPLC (CH_3CN–H_2O, 1:1 to 1:1.6) to afford compound **2** (1.2 mg). Subfraction A6 by reverse phase HPLC (CH_3CN–H_2O, 1:1.5) to afford compound **3** (3.9 mg).

3.3.1. Krempfielin N (1)

Colorless oil; $[\alpha]_D^{25}$ = +27.3 (*c* 0.91, $CHCl_3$); IR (neat) ν_{max} 3445, 2961, 1733, 1457, 1370, 1180, and 1084 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESIMS *m*/*z* 461 $[M + Na]^+$; HRESIMS *m*/*z* 461.2882 $[M + Na]^+$ (calcd. for $C_{25}H_{42}O_6Na$, 461.2879).

3.3.2. Krempfielin O (2)

Colorless oil; $[\alpha]_D^{25}$ = −56.7 (*c* 0.3, $CHCl_3$); IR (neat) ν_{max} 3461, 2960, 1735, 1464, 1372, 1238, 1177, 1076, and 1026 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESIMS *m*/*z* 547 $[M + Na]^+$; HRESIMS *m*/*z* 547.2880 $[M + Na]^+$ (calcd. for $C_{28}H_{44}O_9Na$, 547.2883).

3.3.3. Krempfielin P (3)

Colorless oil; $[\alpha]_D^{25}$ = +13.1 (*c* 3.8, $CHCl_3$); IR (neat) ν_{max} 3419, 2959, 1733, 1437, 1371, 1237, 1182, and 1072 cm^{-1}; ^{13}C and ^{1}H 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).

3.4. In Vitro *Anti-Inflammatory Assay—Superoxide Anion Generation and Elastase Release by Human Neutrophils*

Human neutrophils were obtained by means of dextran sedimentation and Ficoll centrifugation. Measurements of superoxide anion generation and elastase release were carried out according to previously described procedures [24,25]. LY294002, a phosphatidylinositol-3-kinase inhibitor, was used as a positive control for inhibition of superoxide anion generation and elastase release with IC_{50} values of 1.88 ± 0.45 and 4.12 ± 0.92 μM, respectively. Briefly, superoxide anion production was assayed by monitoring the superoxide dismutase-inhibitable reduction of ferricytochrome c. Elastase release experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate.

4. Conclusions

New eunicellin-based diterpenoids were isolated from the soft coral *Cladiella krempfi*. Compounds **1** and **3**, especially **1**, could significantly inhibit the release of elastase in FMLP/CB-induced human neutrophils, and **3** inhibited 23% generation of superoxide anion. Thus, compounds **1** and **3** are promising anti-inflammatory agents and may warrant further biomedical investigation.

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Conflicts of Interest

The authors declare no conflict of interest.

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