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Article

# Simplexins P–S, Eunicellin-Based Diterpenes from the Soft Coral *Klyxum simplex*

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**Abstract:** Four new eunicellin-based diterpenes, simplexins P–S (1–4), and the known compound simplexin A (5), have been isolated from the soft coral *Klyxum simplex*. The structures of the new metabolites were determined on the basis of extensive spectroscopic analysis, particularly 1D and 2D NMR experiments. Compounds 1 and 3–5 were shown to exhibit cytotoxicity against a limited panel of cancer cell lines, 3 being the most cytotoxic.

Keywords: soft coral; eunicellin-based diterpenes; cytotoxicity; Klyxum simplex

## 1. Introduction

In the investigation of the bioactive metabolites from soft corals of Taiwanese waters, many bioactive eunicellin-based diterpenoids have been isolated from wild-type octocoral *Pachyclavularia violacea* [1,2], *Cladiella australis* [3], *Vigularia juncea* [4], *Cladiella hirsute* [5], *Cladiella krempfi* [6],

*Klyxum molle* [7], and a cultured soft coral *Klyxum simplex* [8–11]. Our previous study on the secondary metabolites of a Dongsha Atoll soft coral *K. simplex* Thomson & Dean (Alcyonacea, Alcyoniidae) has resulted in the isolation of a series of new eunicellin-based diterpenoids, simplexins A–O [12,13]. In continuation of our search for metabolites from the Dongsha Atoll soft coral *K. simplex*, we have isolated another four new eunicellin-type metabolites, simplexins P–S (1–4) (Chart 1) and a known compound simplexin A (5). The structures of 1–4 were established by extensive spectroscopic analysis, including careful examination of 2D NMR ( $^{1}H-^{1}H$  COSY, HMQC, HMBC and NOESY) correlations. The cytotoxicity of 1–5 against human erythroleukemia (K562), human leukemia (CCRF-CEM), human breast earcinoma (T47D), and human lymphoid T (MOLT 4) cell lines was investigated. The results showed that compound 3, being the most cytotoxic, is worthy of further biomedical investigation.





#### 2. Results and Discussion

Simplexin P (1) was obtained as a white powder. Its molecular formula  $C_{26}H_{42}O_7$  was determined by the HRESIMS (*m/z* 489.2827 [M + Na]<sup>+</sup>) was which deduced six degrees of unsaturation. The IR absorptions bands at  $v_{max}$  3255 and 1717 cm<sup>-1</sup> revealed the presence of hydroxy and ester carbonyl functionalities. The <sup>13</sup>C NMR spectrum measured in CDCl<sub>3</sub> showed signals of 26 carbons (Table 1) which were assigned as six methyls, six sp<sup>3</sup> methylenes, one sp<sup>2</sup> methylene, eight sp<sup>3</sup> methines (including four oxymethines), two sp<sup>3</sup> and three sp<sup>2</sup> quaternary carbons (including two ester carbonyls) by DEPT. In the <sup>13</sup>C NMR spectrum of 1, two carbonyl resonances at  $\delta$  172.6 and 170.2 ppm confirmed the presence of two ester groups. In the <sup>1</sup>H NMR spectrum of 1 (Table 2), one acetate methyl ( $\delta$  2.12) and one *n*-butyryloxy [ $\delta$  0.92 (3H, t, *J* = 7.5 Hz), 1.60 (2H, m), and 2.13 (2H, m)] groups were observed. Moreover, two <sup>1</sup>H NMR singlet signals at  $\delta$  5.13 and 5.46 revealed the presence of one olefinic methylene. In addition, the diagnostic signals at  $\delta$  4.17 and 3.58 implied the presence of an ether linkage between C-9 and C-2. On the basis of the above results and by the assistance of <sup>1</sup>H–<sup>1</sup>H COSY and HMBC experiments (Figure 1), the molecular framework of **1** could be established as an eunicellin-type skeleton. Furthermore, the acetoxy group positioned at C-12 was confirmed by HMBC correlations from oxymethine [ $\delta$  4.89 (H-12)] and acetate methyl ( $\delta$  2.12) to the ester carbonyl carbon at  $\delta$  170.2 (C). Thus, the remaining one *n*-butyryloxy group was located at C-3, an oxygen-bearing quaternary carbon resonating at  $\delta$  84.4 ppm. On the basis of the above analysis, the planar structure of **1** was established unambiguously.

Position	1	2	3	4
1	41.7 (CH) <sup>b</sup>	43.0 (CH)	43.1 (CH)	41.5 (CH)
2	89.8 (CH)	91.6 (CH)	91.4 (CH)	91.4 (CH)
3	84.4 (C)	84.5 (C)	84.6 (C)	74.1 (C)
4	28.7 (CH <sub>2</sub> )	29.4 (CH <sub>2</sub> )	30.0 (CH <sub>2</sub> )	35.2 (CH <sub>2</sub> )
5	35.3 (CH <sub>2</sub> )	35.4 (CH <sub>2</sub> )	30.0 (CH <sub>2</sub> )	35.0 (CH <sub>2</sub> )
6	73.0 (CH)	73.5 (CH)	87.3 (CH)	74.1 (CH)
7	150.3 (C)	150.3 (C)	145.6 (C)	152.0 (C)
8	41.0 (CH <sub>2</sub> )	41.2 (CH <sub>2</sub> )	41.9 (CH <sub>2</sub> )	41.5 (CH <sub>2</sub> )
9	78.4 (CH)	79.2 (CH)	78.9 (CH)	78.2 (CH)
10	50.2 (CH)	45.5 (CH)	45.0 (CH)	46.4 (CH)
11	71.1 (C)	83.5 (C)	83.3 (C)	82.1 (C)
12	75.5 (CH)	42.2 (CH <sub>2</sub> )	42.7 (CH <sub>2</sub> )	32.4 (CH <sub>2</sub> )
13	24.2 (CH <sub>2</sub> )	66.8 (CH)	66.8 (CH)	18.1 (CH <sub>2</sub> )
14	43.4 (CH)	50.3 (CH)	49.8 (CH)	42.7 (CH)
15	22.2 (CH <sub>3</sub> )	22.7 (CH <sub>3</sub> )	22.9 (CH <sub>3</sub> )	27.6 (CH <sub>3</sub> )
16	116.9 (CH <sub>2</sub> )	117.0 (CH)	118.2 (CH)	117.2 (CH <sub>2</sub> )
17	26.2 (CH <sub>3</sub> )	25.2 (CH <sub>3</sub> )	25.3 (CH <sub>3</sub> )	25.4 (CH <sub>3</sub> )
18	27.4 (CH)	28.4 (CH)	28.5 (CH)	28.0 (CH)
19	21.7 (CH <sub>3</sub> )	24.8 (CH <sub>3</sub> )	24.8 (CH <sub>3</sub> )	21.8 (CH <sub>3</sub> )
20	15.5 (CH <sub>3</sub> )	15.8 (CH <sub>3</sub> )	15.7 (CH <sub>3</sub> )	15.0 (CH <sub>3</sub> )
3-Ac			22.3 (CH <sub>3</sub> )	
			169.9 (C)	
11-Ac		22.4 (CH <sub>3</sub> )	22.4 (CH <sub>3</sub> )	22.6 (CH <sub>3</sub> )
		170.0 (C)	170.0 (C)	170.3 (C)
12-Ac	21.2 (CH <sub>3</sub> )			
	170.2 (C)			
3- <i>n</i> -Butyrate	13.6 (CH <sub>3</sub> )	13.6 (CH <sub>3</sub> )		
	18.4 (CH <sub>2</sub> )	18.6 (CH <sub>2</sub> )		
	37.3 (CH <sub>2</sub> )	37.4 (CH <sub>2</sub> )		
	172.6 (C)	172.6 (C)		

**Table 1.** <sup>13</sup>C NMR data for compounds  $1-4^{a}$ .

<sup>a</sup> Spectra recorded at 125 MHz in CDCl<sub>3</sub> at 25 °C; <sup>b</sup> Attached protons were determined by DEPT experiments.

Position	1	2	3	4	
1	2.42 dd (12.0, 7.5)	2.24 dd (11.5, 7.0)	2.20 dd (12.5, 7.0)	2.27 dd (11.5, 7.5)	
2	3.58 s	3.59 s	3.58 s	3.56 s	
	2.20 m	2.17 m	2.10 m	1.72	
4	1.80 m	1.84 m	1.97 m	1./3 m	
F	2.12 m	2.10 m	2.13 m	2.06 m	
3	1.71 m	1.73 m	1.54 m	1.95 m	
6	4.33 dd (11.0, 4.0)	4.30 <i>br</i> d (10.5)	4.62 dd (10.5, 2.0)	4.32 br d (10.5)	
8	2.84 dd (14.0, 4.5)	2.83 dd (14.0, 5.0)	2.83 dd (13.5, 5.0)	2.86 dd (13.5, 5.5)	
	2.47 d (14.0)	2.47 d (14.0)	2.55 d (13.5)	2.51 d (13.5)	
9	4.17 dd (11.0, 4.0)	4.13 dd (10.5, 4.5)	4.11dd (11.0, 5.0)	4.09 dd (10.0, 5.5)	
10	2.66 dd (11.0, 7.5)	3.08 dd (10.5,7.5)	3.17 dd (10.5, 7.0)	2.96 dd (10.0, 7.5)	
12	4.89 dd (11.7, 4.2)	1.52 m	1.54 m	1.44 m	
		2.41 m	2.34 dd (13.5, 3.5)	2.25 m	
13	1.61 m 1.70 m	3.90 ddd (15.0, 13.2, 4.5)	3.90 ddd (16.0, 11.0, 5.0)	1.34 m	
				1.46 m	
14	1.41 m	1.26 m	1.26 t (11.0)	1.19 m	
15	1.59 s	1.56 s	1.53 s	1.16 s	
16	5.13 s	5.22 s	5.35 s	5.30 s	
10	5.46 s	5.47 s	5.46 s	5.61 s	
17	1.21 s	1.57 s	1.58 s	1.54 s	
18	1.92 m	1.92 m	1.89 m	1.79 m	
19	0.95 d (7.0)	1.18 d (7.0)	1.19 d (7.0)	0.94 d (7.0)	
20	0.83 d (7.0)	0.96 d (7.0)	0.97 d (7.0)	0.78 d (7.0)	
3-acetate			1.95 s		
11-acetate		2.00 s	2.01 s	2.01 s	
12-acetate	2.12 s				
3-n-butyrate	0.92 t (7.5)	0.94 t (7.5)			
	1.60 m	1.59 m			
	2.13 m	2.15 m			
6-OOH			7.78 s		

**Table 2.** <sup>1</sup>H NMR Data for compounds 1–4<sup>*a*</sup>.

<sup>*a*</sup> Spectra recorded at 500 MHz in CDCl<sub>3</sub> at 25 °C; <sup>*b*</sup> J values are (in Hz) in parentheses.

The relative configuration of **1** was determined by analysis of NOE correlations observed in the NOESY spectrum (Figure 2), which showed NOE interactions between H-1 and H-10, revealing they were both  $\beta$ -oriented. Also, correlations between H-2 and both H<sub>3</sub>-15 and H-14; H-14 and both H-9 and H-12; H-9 and both H-12 and H<sub>3</sub>-17; and H<sub>3</sub>-15 and H-6 suggested that of all of H-2, H-6, H-9, H-12, H-14, H<sub>3</sub>-15 and H<sub>3</sub>-17 were all  $\alpha$ -oriented. Thus, the NOESY spectrum indicated that **1** was found to possess the (1*R*\*, 2*R*\*, 3*R*\*, 6*S*\*, 9*R*\*, 10*S*\*, 11*S*\*, 12*R*\*, 14*R*\*)-configuration.



Figure 1. Key  ${}^{1}H-{}^{1}H$  COSY and HMBC correlations of 1 and 2.

Figure 2. Selective NOESY correlations of 1 and 3.



Compound **2**, simplexin Q, was assigned as the molecular formula  $C_{26}H_{42}O_7$  from its HRESIMS data, appropriate for six degrees of unsaturation. <sup>1</sup>H and <sup>13</sup>C NMR spectral data of **2** (Tables 1 and 2) also showed the presence of one acetoxy group ( $\delta_C$  170.0, C; 22.4, CH<sub>3</sub>;  $\delta_H$  2.00, 3H, s) and one *n*-butyryloxy group ( $\delta_C$  172.6, C; 37.4, CH<sub>2</sub>; 18.6, CH<sub>2</sub>; 13.6, CH<sub>3</sub>;  $\delta_H$  2.15, 2H, m; 1.59, 2H, m; 0.94, 3H, t, *J* = 7.5 Hz). Comparison of the NMR data of **2** with those of the known compound simplexin A (**5**) [12], revealed that the only difference was the presence of an oxymethine ( $\delta_H$  3.90;  $\delta_C$  66.8) at C-13 in **2**, instead of the methylene ( $\delta_H$  2.27 and 1.44;  $\delta_C$  18.1) in **5** arising from the substitution of a hydroxy moiety at C-13 in **2**, instead of a methylene moiety at the same carbon in **1**. Furthermore, the molecular framework was also established by <sup>1</sup>H–<sup>1</sup>H COSY and HMBC experiments (Figure 1). The relative configuration of **2**, deduced using a NOESY spectrum, is similar to that of **5**. In addition, H-13 was found to exhibit a NOE correlation with H-1 but not with H-14, revealing the  $\alpha$ -orientation of the hydroxyl group at C-13. Therefore, the structure of **2** was found to possess the (1*R*\*, 2*R*\*, 3*R*\*, 6*S*\*, 9*R*\*, 10*S*\*, 11*R*\*, 13*R*\*, 14*R*\*)-configuration.

Simplexin R (3), isolated as a white powder, was assigned a molecular formula  $C_{24}H_{38}O_8$  from high resolution ESIMS analysis. The presence of the acetate groups was indicated by IR absorption at 1733 cm<sup>-1</sup>, <sup>1</sup>H NMR signals (Table 2) at  $\delta$  1.95 (s, 3H) and 2.01 (s, 3H) and <sup>13</sup>C NMR (Table 1) signals at  $\delta$  22.3 (CH<sub>3</sub>), 22.4 (CH<sub>3</sub>), 169.9 (C) and 170.0 (C). The NMR spectral data of **3** showed the presence of a 1,1-disubstituted carbon–carbon double bond ( $\delta_C$  118.2, CH<sub>2</sub> and 145.6, C;  $\delta_H$  5.35, s and 5.46, s) and a hydroperoxy proton ( $\delta_H$  7.78, s). Comparison of the NMR data of **3** with those of **2** revealed the replacement of the *n*-butyryloxy moiety at C-3 and the hydroxy group at C-6 in **2** by the acetoxy and the hydroperoxy groups in **3**, respectively. The relative configuration of **3** was determined mainly by the assistance of the NOESY experiment. The NOE correlations of **3** indicated that **3** possessed the same configurations for each chiral center as those of **2**.

Simplexin S (4) showed the pseudomolecular ion peak  $[M + Na]^+$  at m/z 403.2463 in the HRESIMS and the molecular formula was determined as C<sub>22</sub>H<sub>36</sub>O<sub>5</sub>. NMR spectroscopic data of 4 (Tables 1 and 2) showed the presence of one acetoxy group ( $\delta_C$  170.3, C; 22.6, CH<sub>3</sub>;  $\delta_H$  2.01, 3H, s). Comparison of the NMR data of 4 with those of 5 revealed that the only difference between both compounds arises from the replacement of the hydroxy group at C-3 in 4 by one *n*-butyryloxy moiety in 5. The NOESY spectrum indicated that 4 was found to possess the (1*R*\*, 2*R*\*, 3*R*\*, 6*S*\*, 9*R*\*, 10*S*\*, 11*R*\*, 14*R*\*)-configuration.

The cytotoxicity of compounds 1–5 against the proliferation of a limited panel of cancer cell lines, including K562, CCRF-CEM, T47D, and MOLT 4 was evaluated by the Alamar Blue assay, using 5-fluorouracil as a positive control. It was found that **3** showed activity against the proliferation of K-562, CCRF-CEM, T47D, and MOLT 4 cancer cells (ED<sub>50</sub> values of  $7.2 \pm 2.4$ ,  $2.7 \pm 0.1$ ,  $13.5 \pm 2.8$ , and  $3.8 \pm 0.5 \mu g/mL$ , respectively) (Table 3).

	Cell Lines ED <sub>50</sub> (µg/mL)				
Compound	K-562	<b>CCRF-CEM</b>	T47D	MOLT 4	
1	>20	$12.0 \pm 1.6$	>20	$30.3 \pm 3.4$	
2	>20	>20	>20	>20	
3	$7.2 \pm 2.4$	$2.7 \pm 0.1$	$13.5\pm2.8$	$3.8 \pm 0.5$	
4	>20	$13.0\pm0.9$	>20	$16.4 \pm 3.1$	
5	>20	$17.0\pm2.9$	>20	$18.2 \pm 2.6$	
5-Fluorouracil	$2.3 \pm 0.2$	$1.8 \pm 0.3$	$9.8 \pm 1.5$	$2.3 \pm 0.3$	

Table 3. Cytotoxicities of Compounds 1–5.

#### 3. Experimental Section

#### 3.1. General Experimental Procedures

Melting points were determined using a Fisher-Johns melting point apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. 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 on a Varian Unity INOVA 500 FT-NMR at 500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C in CDCl<sub>3</sub>. Si gel 60 (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F<sub>254</sub>, 0.2 mm) were used for

analytical TLC. High-performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with a Merck Hibar Si-60 column ( $250 \times 21 \text{ mm}$ , 7 µm).

## 3.2. Animal Material

*Klyxum simplex* (230 g, wet wt), was collected by hand using scuba off the coast of Dongsha Atoll, in September, 2006, at a depth of 11 m, and stored in a freezer until extraction. A voucher sample (specimen No. 20060901-1) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

## 3.3. Extraction and Separation

The frozen bodies of *K. simplex* (230 g, wet wt) were minced and exhaustively extracted with EtOAc (1 L × 4). The organic extract was evaporated under reduced pressure to give a residue (2.5 g) which was subjected to Si gel column chromatography and eluted with EtOAc in *n*-hexane (0–100%, gradient) to yield 22 fractions. Fractions 10–12 (1.05 g) eluted with EtOAc–*n*-hexane (1:3), were further purified over silica gel using EtOAc–*n*-hexane (1:3 to 1:1) to afford 46 subfractions. Subfraction 37 was also purified by normal phase HPLC using acetone–*n*-hexane (1:2) to afford **3** (0.9 mg, 0.036%). Fractions 13–15 (0.47 g), eluted with EtOAc–*n*-hexane (1:1), were further purified over silica gel using acetone–*n*-hexane (1:2) to yield **4** (1.6 mg, 0.064%) whilst subfraction 19 was purified by normal phase HPLC using acetone–*n*-hexane (1:2) to afford **2** (1.3 mg, 0.052%). Fractions 16–19 (0.51 g) eluted with EtOAc–*n*-hexane (2:1), were further purified over silica gel using EtOAc–*n*-hexane (2:1) to afford 4 subfractions. Subfraction 4 was separated by normal phase HPLC using MeOH–CH<sub>2</sub>Cl<sub>2</sub> (1:30) to afford **1** (3.3 mg, 0.132%).

Simplexin P (1): white powder (3.3 mg); mp 179.0–180.0 °C;  $[\alpha]_D^{26} = -27$  (*c* 1.2, CHCl<sub>3</sub>); IR (neat)  $v_{\text{max}}$  3255 (broad) and 1717 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Tables 1 and 2; ESIMS *m*/*z* 489 (100,  $[M + \text{Na}]^+$ ); HRESIMS *m*/*z* 489.2827 (calcd for C<sub>26</sub>H<sub>42</sub>O<sub>7</sub>Na, 489.2828).

Simplexin Q (2): colorless oil (1.3 mg);  $[\alpha]_D^{26} = -11$  (*c* 0.8, CHCl<sub>3</sub>); IR (neat)  $v_{max}$  3410 (broad) and 1732 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Tables 1 and 2; ESIMS *m*/*z* 489 (100,  $[M + Na]^+$ ); HRESIMS *m*/*z* 489.2830 (calcd for C<sub>26</sub>H<sub>42</sub>O<sub>7</sub>Na, 489.2828).

Simplexin R (**3**): white powder (0.9 mg); mp 167–168 °C;  $[\alpha]_D^{26} = -27$  (*c* 0.4, CHCl<sub>3</sub>); IR (neat)  $v_{max}$  3395 (broad) and 1733 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Tables 1 and 2; ESIMS *m/z* 477 (100,  $[M + Na]^+$ ); HRESIMS *m/z* 477.2467 (calcd for C<sub>24</sub>H<sub>38</sub>O<sub>8</sub>Na, 477.2464).

Simplexin S (4): colorless oil (1.6 mg);  $[\alpha]_D^{26} = -41$  (*c* 0.6, CHCl<sub>3</sub>); IR (neat, CHCl<sub>3</sub>)  $v_{max}$  3354 (broad) and 1716 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Tables 1 and 2; ESIMS *m*/*z* 403 (100, [M + Na]<sup>+</sup>); HRESIMS *m*/*z* 403.2463 (calcd for C<sub>22</sub>H<sub>36</sub>O<sub>5</sub>Na, 403.2460).

## 3.4. Cytotoxicity Testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of compounds 1–5 were performed using the Alamar Blue assay [14,15].

## 4. Conclusions

In previous studies, a series of new eunicellin-based diterpenoids were isolated from the cultured and wild-type soft corals *Klyxum simplex*. Our continued investigation on the chemical constituents of wild-type soft coral *K. simplex* has again led to the isolation of four new eunicellin-based diterpenoids, simplexins P–S. Simplexin R (3) exhibited significant cytotoxicity against CCRF-CEM and MOLT 4 cells, and moderate to weak cytotoxicity against K-562 and T47D cells. Also, compounds **1**, **4** and **5** exhibited moderate to weak cytotoxicity toward CCRF-CEM and MOLT 4 cell lines. Besides our research, many recent studies have showed the versatile structures and bioactivities of eunicellin-type compounds [16–21]. These studies along with the new simplexins described here suggest that eunicellin-type compounds, in particular 3, are worthy of further biomedical investigation.

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