Anti-inflammatory eunicellin-based diterpenoids from the cultured soft coral *Klyxum simplex*†

Bo-Wei Chen,[a] Chih-Hua Chao,[a] Jui-Hsin Su,[b] Zhi-Hong Wen,[a] Ping-Jyun Sung[c] and Jyh-Horng Sheu*[a,d]

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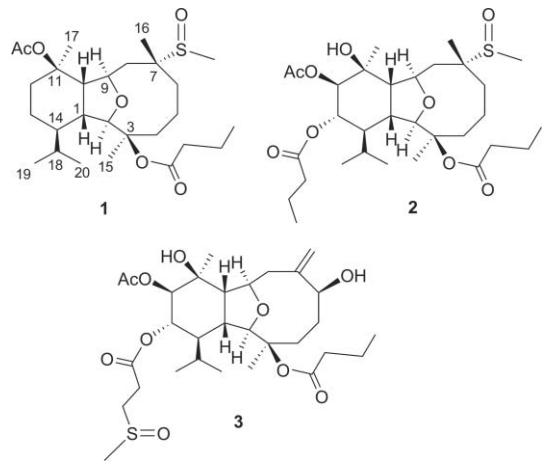
Three novel eunicellin-based diterpenoids, namely klysimplexin sulfoxides A–C (**1–3**), were isolated from the cultured soft coral *Klyxum simplex*. Their structures were elucidated by spectroscopic methods, particularly 1D and 2D-NMR experiments. Compounds **1–3** significantly inhibited the accumulation of the pro-inflammatory iNOS protein in LPS-stimulated RAW264.7 macrophage cells. Compound **3** also showed marked activity in inhibiting the expression of COX-2 protein in the same cells.

Introduction

Bioactive eunicellin-based compounds have been isolated previously from the soft corals of the genera *Astrogorgia*,[1] *Cladiella*,[2,3] *Briareum*,[4] *Eunicella*,[5] *Litophyton*,[6] *Pachy-clavularia*,[7] and *Sclerophytum*.[8] During the course of our investigation on new natural substances from wild[9] and cultured[10] soft coral, *Klyxum simplex*, a number of eunicellin-type metabolites were discovered and some were found to be bioactive. In order to investigate the molecular diversity and biological activities of natural eunicellins, we continued our investigation on the chemical constituents of cultured *K. simplex*. This study has again led to the isolation of three sulfoxyl eunicellins, klysimplexin sulfoxides A–C (**1–3**) (Scheme 1). All the three compounds were found to be able to significantly reduce the level of pro-inflammatory iNOS protin in the LPS-stimulated RAW264.7 macrophage cells. Compound **3** also showed an impressive activity in inhibiting the expression of COX-2 protein.



Scheme 1 Structures of formulae klysimplexin sulfoxides A(**1**), B(**2**), and C(**3**).

Results and discussion

The organic extract was concentrated to an aqueous suspension and was further partitioned between CH_2Cl_2 and water. The combined CH_2Cl_2-soluble fraction was concentrated under reduced pressure and the residue was repeatedly purified by chromatography to yield metabolites **1–3**.

Klysimplexin sulfoxide A (**1**), $[\alpha]_D^{25}$ −33 (*c* 0.20, $CHCl_3$) was obtained as a colorless oil. The HRESIMS of **1** exhibited a peak at *m/z* 521.2917 and established a molecular formula of $C_{27}H_{46}O_6S$ based in the pseudomolecular ion of $[M + Na]^+$. The IR spectrum of **1** revealed the presence of ester and sulfoxide functionalities from absorptions at 1738 and 1053 cm^{-1}, respectively. The ^{13}C NMR data of **1** showed the presence of twenty-seven carbon signals (Table 1), which were characterized by DEPT as eight methyls, eight sp^3 methylenes, six sp^3 methines (including two oxymethines), two sp^2 carbonyls, and three sp^3 quaternary carbons (including two oxygenated carbons). Two ester carbonyls (δ_C 172.6 and 170.2) were assigned from the ^{13}C NMR spectrum and were long-range correlated with the methylenes (δ_H 2.35 m, 2H and 1.76 m, 2H) of an *n*-butyrate and an acetate methyl (δ_H 2.00, 3H, s), respectively. Inspection of the HSQC spectrum showed that the proton signal appearing at δ_H 2.42 ppm (3H, s) was correlated to the methyl carbon resonating at δ_C 32.0, suggesting the presence of a methylsulfoxyl moiety.[11] The ^1H NMR of **1** (Table 1) showed the presence of a methyl (δ 1.08, 3H, s) attached to a sulfoxide-bearing quaternary carbon, two tertiary methyls bonded to oxygenated carbons (δ 1.46 and 1.38, each 3H, s), and two secondary methyls (δ 0.95 and 0.81, each 3H, d, $J = 6.8$ Hz) of an isopropyl moiety. Signals resonating at δ_H 2.16 ppm (1H, dd, 11.6 and 7.2), 3.13 (1H, br t, 8.0), 3.53 (s), and 3.96 ppm (1H, ddd, 14.8, 7.6 and 3.2), and at δ_C 42.3, 53.8, 92.0, and 76.1 ppm, suggested the presence of a tetrahydrofuran structural unit.[3] The COSY and HMBC correlations (Fig. 1) were further used to establish the molecular skeleton of **1**. The placement of an *n*-butyrate at C-3 was supported by the HMBC connectivity from

[a]*Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 804, Taiwan*
[b]*Department of Biological Science and Technology, Mei-Ho Institute of Technology, 23 Ping Kuang Road, Neipu Hsiang, Pingtung 912, Taiwan*
[c]*Taiwan Coral Research Center, National Museum of Marine Biology & Aquarium, Checheng, Pingtung 944, Taiwan*
[d]*Asia-Pacific Ocean Research Center, National Sun Yat-sen University, Kaohsiung 804, Taiwan*

† Electronic supplementary information (ESI) available: ^1H NMR, ^{13}C NMR and HRESIMS spectra of compounds **1–3**. See DOI: 10.1039/b926353e

Table 1 ^{1}H and ^{13}C NMR chemical shifts for compound **1**

C	^{1}H [a](ppm)	^{13}C [b](ppm)
1	2.16 (dd, 11.6, 7.2)[c]	42.3 (CH)[b]
2	3.53 (s)	92.0 (CH)
3	—	86.7 (qC)
4	2.64 (dd, 14.4, 8.0); 1.65, m	38.4 (CH_2)
5	1.84 (m); 1.04 (m)	17.4 (CH_2)
6	2.50 (dd, 11.2, 6.0) 1.85 (m)	34.5 (CH_2)
7	—	59.3 (qC)
8	2.15 (dd, 15.2, 3.6); 1.71 (d, 14.8)	42.2 (CH_2)
9	3.96 (ddd, 14.8, 7.6, 3.2)	76.1 (CH)
10	3.13 (br t, 8.0)	53.8 (CH)
11	—	81.8 (qC)
12	2.14 (m); 1.34 (m)	32.4 (CH_2)
13	1.41 (m); 1.35 (m)	17.4 (CH_2)
14	1.19 (m)	42.3 (CH)
15	1.38 (s)	23.2 (CH_3)
16	1.08 (s)	20.1 (CH_3)
17	1.46 (s)	25.0 (CH_3)
18	1.76 (m)	28.9 (CH)
19	0.95 (d, 6.8)	21.6 (CH_3)
20	0.81 (d, 6.8)	15.1 (CH_3)
3-*n*-Butyrate	1.01 (t, 7.2)	13.7 (CH_3)
	1.76 (m)	18.7 (CH_2)
	2.35 (m)	37.3 (CH_2)
		172.6 (qC)
11-OAc	2.00 (s)	22.5 (CH_3)
		170.2 (qC)
7-SOMe	2.42 (s)	32.0 (CH_3)

[a] Spectrum recorded at 400 MHz in $CDCl_3$. [b] 100 MHz in $CDCl_3$. [c] *J* values (in Hz) in parentheses.

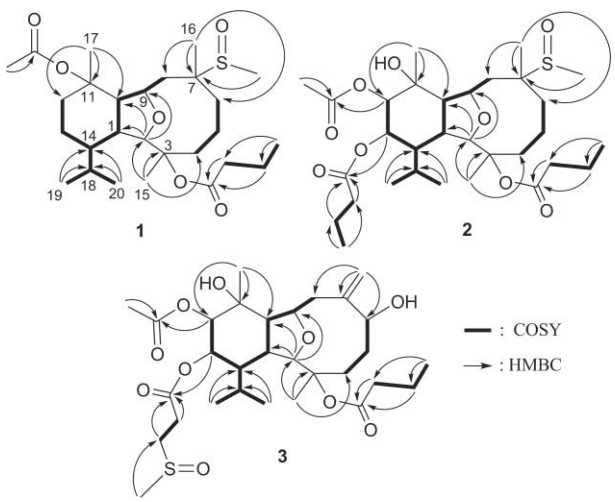


Fig. 1 Key ^{1}H-^{1}H COSY and HMBC correlations of **1–3**.

H-2 (δ 3.53) to the carbon resonating at δ 172.6 (qC).[9,10] Also, the location of a sulfoxyl group at C-7 was supported by the HMBC connectivity from the methylsulfoxyl protons (δ_H 2.42) to the carbon resonating at δ_C 59.3 (qC). The downfield chemical shift for H_3-17 (δ 1.46) determined the position of an acetate group at C-11. Therefore, the planar structure of **1** was established unambiguously. In the NOESY spectrum of **1** (Fig. 2), observation of the NOE correlations between H-10 with one proton of H_2-8 (δ_H 2.15) and H-1; and H_3-16 with H_2-8 (δ_H 2.15 and 1.71), suggested the β-orientation of H-1, H-10, and H_3-16. Also, correlations between H-2 with both H_3-15 and H-14; H-9 with H-14, H_3-17, and protons of methylsulfoxyl group (δ_H 2.42) suggested that

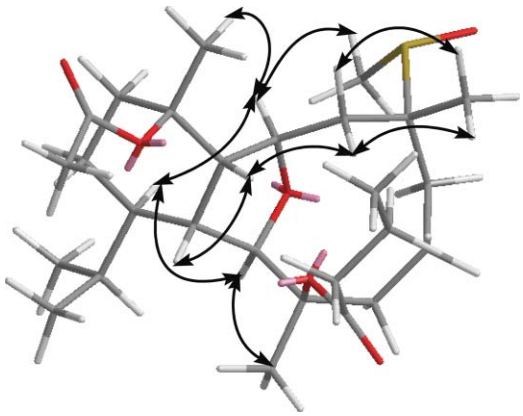


Fig. 2 Key NOESY correlations of **1**.

H-2, H-9, H-14, H_3-15, H_3-17, and the methylsulfoxyl group are α-oriented. Thus, the structure of diterpenoid **1** was established.

Klysimplexin sulfoxide B (**2**), $[\alpha]_D^{25}$ −67 (*c* 0.22, $CHCl_3$) was obtained as a colorless oil that gave a pseudomolecular ion peak at *m/z* 623.3226 $[M + Na]^+$ in the HRESIMS, consistent with the molecular formula of $C_{31}H_{52}O_9S$. The IR absorptions at 3452, 1734, and 1052 cm^{-1} revealed the presence of hydroxy, ester, and sulfoxide functionalities. Two 3H singlets appearing at δ_H 2.42 and 1.40 ppm were assigned to a methylsulfoxyl group and one methyl bonded to an acyloxy group-containing quaternary carbon, respectively. The above observations and the similar NMR data from C-1 to C-10 of both **1** and **2** (Table 2) suggested that both compounds have the same planar structure of the ten-membered rings. Furthermore, COSY and HMBC correlations revealed that an acetate, an *n*-butyrate and an isopropyl group were found to be attached at C-12, C-13, and C-14, respectively. C-11 was further found to be an oxygenated quaternary carbon bearing a methyl and a hydroxy group. Therefore, the planar structure of **2** was established. NOE correlations observed for H-10 with one proton of H_2-8 (δ 2.09) and H-1; H-1 with H-13; and H_3-16 with H_2-8 suggested that H-1, H-10, H-13 and H_3-16 are β-oriented. Also, correlations observed for H-2 with both H_3-15 and H-14; H-9 with H-12, H-14, H_3-17, and methylsulfoxyl protons suggested that all of H-2, H-9, H-12, H-14, H_3-15, H_3-17, and the methylsulfoxyl group are α-oriented. Thus, the structure of diterpenoid **2** was established.

The HRESIMS of Klysimplexin sulfoxide C (**3**) exhibited a pseudomolecular ion peak at *m/z* 623.2863 $[M + Na]^+$, consistent with a molecular formula of $C_{30}H_{48}O_{10}S$. Similar to **1** and **2**, the IR spectrum of **3** indicated the presence of hydroxy (3478 cm^{-1}), ester (1735 cm^{-1}), and sulfoxide (1054 cm^{-1}) functionalities. A comparison of the NMR data of **3** (Table 3) with those of klysimplexin C[10] and **2** showed that **3** has the same ten-membered ring as that of klysimplexin C and the very similar six-membered ring as that of **2**. Also, **3** possessed a 3-methylsulfoxylpropionate substituent at C-13, instead of an *n*-butyrate as in the case of **2**, which was evidenced by COSY and HMBC correlations (Fig. 1). The relative configurations for all asymmetric carbons in **3** were elucidated by the analysis of NOE correlations, as shown in Fig. 3.

The anti-inflammatory activity of **1–3** against the accumulation of pro-inflammatory iNOS and COX-2 proteins in RAW264.7 macrophage cells stimulated with LPS was evaluated using immunoblot analysis. At a concentration of 10 μM (Fig. 4),

Table 2 ^{1}H and ^{13}C NMR chemical shifts for compound **2**

C	^{1}H [a](ppm)	^{13}C [b](ppm)
1	2.41 (dd, 11.6, 7.2)[c]	43.5 (CH)
2	3.53 (s)	92.6 (CH)
3	—	86.3 (qC)
4	2.64 (dd, 14.8, 6.8); 1.66 (m)	38.9 (CH_2)
5	1.89 (m); 1.04 (m)	18.6 (CH_2)
6	2.50 (dd, 12.0, 7.2); 1.87 (m)	35.5 (CH_2)
7	—	59.5 (qC)
8	2.09 (dd, 15.2, 3.2); 1.62 (d, 14.4)	42.3 (CH_2)
9	4.10 (ddd, 14.4, 7.6, 3.2)	76.0 (CH)
10	2.59 (br t, 7.6)	58.1 (CH)
11	—	72.6 (qC)
12	4.97 (d, 9.6)	77.2 (CH)
13	5.49 (dd, 11.2, 10.0)	70.2 (CH)
14	1.76 (m)	47.6 (CH)
15	1.40 (s)	24.3 (CH_3)
16	1.09 (s)	20.7 (CH_3)
17	1.07 (s)	26.8 (CH_3)
18	1.72 (m)	30.9 (CH)
19	0.96 (d, 7.2)	24.3 (CH_3)
20	1.01 (d, 7.2)	17.1 (CH_3)
3-*n*-Butyrate	0.99 (t, 7.6)	15.0 (CH_3)
	1.69 (m)	19.4 (CH_2)
	2.39 (m); 2.31 (m)	38.1 (CH_2)
		171.1 (qC)
12-OAc	2.08 (s)	21.8 (CH_3)
		168.4 (qC)
13-*n*-Butyrate	0.95 (t, 7.6)	14.0 (CH_3)
	1.60 (m)	19.2 (CH_2)
	2.21 (m)	37.3 (CH_2)
		170.4 (qC)
7-SOMe	2.42 (s)	32.8 (CH_3)

[a] Spectrum recorded at 400 MHz in $CDCl_3$. [b] 100 MHz in $CDCl_3$. [c] *J* values (in Hz) in parentheses.

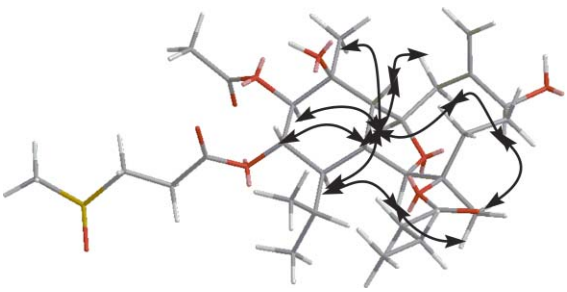


Fig. 3 Key NOESY correlations of **3**.

compounds **1–3** were found to significantly reduce the levels of iNOS protein to 8.8 ± 1.0%, 17.8 ± 4.7%, and 11.3 ± 1.5%, respectively, relative to control cells stimulated with LPS only. At the same concentration, metabolite **3** also significantly reduced COX-2 expression (7.2 ± 2.5%) by LPS treatment

Conclusion

Our investigation demonstrated that the cultured soft coral, *K. simplex*, could be a good source of bioactive substances. The isolated compounds **1–3**, in particular **3**, are potentially anti-inflammatory and may become lead compounds in the future drug development. Also, it is worthwhile to mention that eunicellin-type metabolites containing a sulfoxide, such as **1–3**, were discovered for the first time in this study.

Table 3 ^{1}H and ^{13}C NMR chemical shifts for compound **3**

C	^{1}H [a](ppm)	^{13}C [b](ppm)
1	2.56 (dd, 11.6, 7.6)[c]	42.3 (CH)
2	3.60 (s)	90.9 (CH)
3	—	84.1 (qC)
4	2.25 (m); 1.71 (m)	30.6 (CH_2)
5	2.15 (m); 1.74 (m)	35.9 (CH_2)
6	4.32 (dd, 10.4, 4.0)	72.7 (CH)
7	—	148.7 (qC)
8	2.87 (dd, 14.8, 4.4); 2.44 (d, 14.0)	41.5 (CH_2)
9	4.30 (ddd, 14.0, 10.4, 4.4)	78.7 (CH)
10	2.67 (dd, 10.8, 8.0)	49.8 (CH)
11	—	72.9 (qC)
12	5.04 (d, 9.6)	76.6 (CH)
13	5.55 (dd, 10.8, 9.6)	72.2 (CH)
14	1.77 (m)	48.2 (CH)
15	1.60 (s)	23.3 (CH_3)
16	5.46 (s); 5.12 (s)	116.3 (CH_2)
17	1.19 (s)	27.1 (CH_3)
18	2.01 (m)	28.9 (CH)
19	0.99 (d, 7.2)	24.7 (CH_3)
20	0.93 (d, 7.2)	16.9 (CH_3)
3-*n*-Butyrate	0.92 (t, 7.2)	14.7 (CH_3)
	1.56 (m)	19.5 (CH_2)
	2.12 (m)	38.1 (CH_2)
		170.7 (qC)
12-OAc	2.13 (s)	21.7 (CH_3)
		168.6 (qC)
13-Methylsulfoxyl-propioate	2.62 (s)	39.7 (CH_3)
	2.83 (m); 2.80 (m)	28.3 (CH_2)
	3.05 (m)	49.4 (CH_2)
		168.9 (qC)

[a] Spectrum recorded at 400 MHz in $CDCl_3$. [b] 100 MHz in $CDCl_3$. [c] *J* values (in Hz) in parentheses.

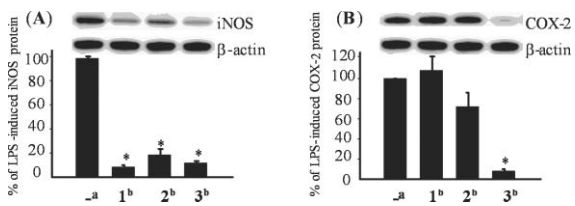


Fig. 4 Effect of compounds **1–3** on iNOS and COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis. (**A**) Immunoblots of iNOS and β-actin; (**B**) Immunoblots of COX-2 and β-actin. The values are mean ± SEM. ($n = 6$). Relative intensity of the LPS alone stimulated group was taken as 100%. Under the same experimental condition CAPE (caffeic acid phenylethyl ester, 10 μM) reduced the levels of the iNOS and COX-2 to 2.5 ± 3.7% and 67.2 ± 13.4%, respectively. *Significantly different from LPS alone stimulated group (*$P < 0.05$). [a]stimulated with LPS, [b]stimulated with LPS in the presence of **1–3** (10 μM).

Experimental

General experimental procedures

Melting points were 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 on a Varian 400 MR FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C. 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 (250 × 21.2 mm, 5 μm).

Extraction and isolation

Specimens of the cultured soft coral *K. simplex* were collected by hand in a 30 ton cultivating tank located in the National Museum of Marine Biology and Aquarium, Taiwan, in July 2005. A voucher sample (CSC-2) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University. The octocoral (1.5 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 (15.2 g) was subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0–100% of EtOAc, gradient) and then further with MeOH in EtOAc with increasing polarity to yield 40 fractions. Fraction 37, eluted with EtOAc–MeOH (3 : 1), was rechromatographed over a Sephadex LH-20 column using MeOH as the mobile phase to afford five subfractions (A1–A4). Subfraction A3 was separated by reverse-phase HPLC (CH_3CN–H_2O, 1 : 3 to 1 : 1) to afford compounds **1** (2.0 mg) and **2** (2.2 mg). Fraction 38, eluted with EtOAc–MeOH (2 : 1), was rechromatographed over a Sephadex LH-20 column using MeOH as the mobile phase to afford five subfractions (B1–B5). Subfraction B2 was separated by reverse-phase HPLC (CH_3CN–H_2O, 1 : 3 to 1 : 1) to afford compound **3** (1.0 mg).

Klysimplexin sulfoxide A (1). Colorless oil; $[\alpha]_D^{25}$ −33 (*c* 0.20, $CHCl_3$); IR (neat) ν_{max} 1738 and 1053 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CHCl_3$), see Table 1; ESIMS *m/z* 521 $[M + Na]^+$; HRESIMS *m/z* 521.2917 $[M + Na]^+$ (calcd. 521.1913 for $C_{27}H_{46}O_6SNa$).

Klysimplexin sulfoxide B (2). Colorless oil; $[\alpha]_D^{25}$ −67 (*c* 0.22, $CHCl_3$); IR (neat) ν_{max} 3452, 1734 and 1052 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CHCl_3$), see Table 2; ESIMS *m/z* 623 $[M + Na]^+$; HRESIMS *m/z* 623.3226 $[M + Na]^+$ (calcd. 623.3230 for $C_{31}H_{52}O_9Na$).

Klysimplexin sulfoxide C (3). Colorless oil; $[\alpha]_D^{25}$ −84 (*c* 0.10, $CHCl_3$); IR (neat) ν_{max} 3478, 1735 and 1054 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Table 3; ESIMS *m/z* 623 $[M + Na]^+$; HRESIMS *m/z* 623.2863 $[M + Na]^+$ (calcd 623.2867 for $C_{30}H_{48}O_{10}SNa$).

In vitro anti-inflammatory assay

Assay procedure was as previously reported.[12,13]

Acknowledgements

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