Int. J. Mol. Sci. **2013**, *14*, 4317-4325; doi:10.3390/ijms14024317

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Article

Oxygenated Cembranoids from the Soft Coral *Sinularia flexibilis*

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Received: 9 January 2013; in revised form: 1 February 2013 / Accepted: 4 February 2013 / Published: 21 February 2013

Abstract: Chemical examination of the Taiwanese soft coral *Sinularia flexibilis* led to the isolation of five cembrane-based diterpenoids **1**–**5**, including two new metabolites, 11-acetylsinuflexolide (**1**) and 11-acetyldihydrosinuflexolide (**2**). The structures of the new metabolites were determined based on extensive spectroscopic analysis, particularly mass spectrometry and 2D NMR (^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) spectroscopy. Metabolites **1**, **3** and **4** exhibited moderate to weak cytotoxicity to human tumor cell lines, HeLa, HEp-2, MCF-7 and MDA-MB-231.

Keywords: diterpenoid; soft coral; *Sinularia flexibilis*

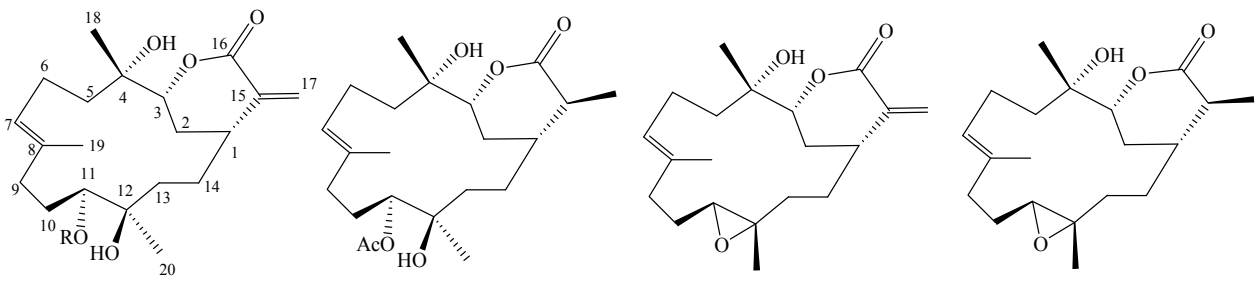
1. Introduction

Soft corals have attracted a great deal of attention in light of the structural diversity and wide range of biological activities of their metabolites [1]. Recently, in the investigation of the bioactive metabolites from the Formosan soft corals, many bioactive cembranoids have been isolated from soft

corals (Alcyonaceae) belonging to the genera *Sinularia* [2–11], *Sarcophyton* [12–16] and *Lobophytum* [17–19]. Some of these metabolites have been found to possess several kinds of biological activities, such as cytotoxic [4,9–18] and anti-inflammatory activity [2–8,12–14,18,19]. During the course of our investigation on new natural substances from wild and cultured soft coral *Sinularia flexibilis*, a number of cembrane-based diterpenoids were discovered, and some were found to be bioactive [20]. In continuation of our search for biologically active secondary metabolites from a soft coral *Sinularia flexibilis* (Figure 1), we have isolated two new cembrane-based diterpenoids, 11-acetylsinuflexolide (**1**) and 11-acetyldihydrosinuflexolide (**2**), along with three known cembranoids, sinuflexolide (**3**) [21], sinularin (**4**) [22] and dihydrosinularin (**5**) [22] (Figure 2). The structures of **1** and **2** were established by extensive spectroscopic analysis, including careful examination of 2D-NMR (^{1}H–^{1}H COSY, HMQC, HMBC and NOESY) (Figures S1–S10) correlations and by comparison of their NMR data with those of related compounds. The cytotoxicity of compounds **1**–**5** against human cervical epitheloid carcinoma (HeLa), laryngeal carcinoma (HEp-2) and breast carcinoma (MCF-7 and MDA-MB-231) cell lines was also investigated.

Figure 1. The soft coral *Sinularia flexibilis*.

Figure 2. Structures of metabolites **1**–**5**.



1: R = Ac
3: R = H

2 **4** **5**

2. Results and Discussion

Frozen samples of *Sinularia flexibilis* were extracted with EtOAc. The dry EtOAc extracts were fractionated by silica gel gravity column chromatography, and the eluted fractions were further purified by HPLC to yield cembranoids **1**–**5**.

The HR-ESI-MS (*m*/*z* 417.2250 $[M + Na]^+$) of 11-acetylsinuflexolide (**1**) established the molecular formula $C_{22}H_{34}O_6$, appropriate for six degrees of unsaturation. Inspection of the ^{13}C-NMR and DEPT spectroscopic data (Table 1) (Figures S1 and S2) showed signals of four methyls (including one acetate methyl), seven sp^3 methylenes, one sp^2 methylene, three sp^3 methines (including two oxymethines), one sp^2 methine, two sp^3 and four sp^2 quaternary carbons (including two ester carbonyls). The ^{13}C NMR signals appearing at δ_C 166.6 (C), 140.4 (C), 125.5 (CH_2), 84.5 (CH), 36.7 (CH), and 29.3 (CH_2) were assigned to an α-exomethylenic–δ-lactone ring functionality by comparing the very similar NMR data of the cembranoids with the same six-membered lactone ring [23,24]. Resonances in the ^{13}C NMR spectrum of **1** at δ_C 170.6 (C) supported the presence of one additional ester group (Table 1). The ester was identified as acetate by the presence of one methyl resonance in the ^{1}H NMR spectrum at δ_H 2.11 (3H, s) (Table 1). Furthermore, carbon signals of three methyls (δ_C 16.1, 25.4 and 25.5), one trisubstituted double bond (δ_C 135.1, C; 127.2, CH), two oxygen-bearing methines (δ_C 84.5 and 77.5), and two oxygenated quaternary carbons (δ_C 74.8 and 73.7) were also determined. The ^{1}H-NMR spectral data revealed the presence of two olefinic methylene protons (δ 6.43, d, J = 2.0 Hz and 5.63, d, J = 2.0 Hz) and one olefinic methine proton (δ 5.26, dd, J = 7.5, 7.5 Hz). Furthermore, two oxygenated methines (δ 4.79, dd, J = 6.5, 2.5 Hz and 4.05, d, J = 11.5 Hz) were also designated from the ^{1}H NMR signals. By interpretation of ^{1}H-^{1}H COSY correlations (Figure S5), it was possible to establish three partial structures from H-1 to H-3, from H_2-5 to H-7, from H_2-9 to H-11, and from H_2-13 to H-1 through H_2-14 (Figure 3). These data, together with the HMBC correlations (Figure 3) (Figure S4) from H_2-5 to C-3 and C-4, H_2-9 to C-7 and C-8, and H_2-13 to C-11 and C-12 established the connectivity within the 14-membered ring. Three methyl groups attached at C-4, C-8 and C-12 were confirmed by the HMBC correlations from H_3-18 to C-3, C-4 and C-5, H_3-19 to C-7, C-8 and C-9, H_3-20 to C-11, C-12 and C-13. A 1,1-disubstituted double bond attached at C-15 was confirmed by the HMBC correlations from H_2-17 to C-1, C-15 and C-16. Moreover, one acetoxy group positioned at C-11 was confirmed from the HMBC correlations of H-11 (δ 4.79) and protons of an acetate methyl (δ 2.11) to the ester carbonyl carbon at δ 170.6 (C). The *E*–configuration of one double bond at C-7/C-8 was assigned based on the ^{13}C NMR chemical shifts at C-19 (δ_C 16.1). Thus, **1** was revealed as a cembranoid possessing an α-exomethylenic–δ-lactone ring, based on the above analysis. Furthermore, the relative stereochemistry of **1** was mostly confirmed to be the same as that of the known metabolite sinuflexolide (**3**) by comparison of the proton chemical shifts and coupling constants [24]. Further comparison of the ^{1}H and ^{13}C NMR data of **1** with those of **3**, showed that **1** contains an extra acetyl group relative to **3**. The chemical shift of H-11 in **3** (δ_H 3.47, dd, J = 6.4, 2.4 Hz) was shifted downfield (δ_H 4.79, dd, J = 6.5, 2.5 Hz) in **1**, suggesting that **1** is the 11-acetyl derivative of **3**. This was further supported by acetylation of **3** with acetic anhydride in pyridine to yield **1**. Thus, compound **1** was established as the 11-acetyl derivative of **3**.

11-acetyldihydrosinuflexolide (**2**) obtained as a white powder. The HRESIMS (*m*/*z* 419.2411, $[M + Na]^+$) and NMR data of **2** indicated the molecular formula, $C_{22}H_{36}O_6$. Both the ^{1}H and ^{13}C NMR

signals of **2** were found to be very closely related to those of compound **1**, suggesting the same skeleton. Further comparison of NMR data of **2** with those of **1** (Table 1) (Figures S1–S10), revealed that the two exomethylene proton signals (δ_H 6.43 and 5.63) in **1** was replaced by a methyl proton signal (δ_H 1.35 d, J = 7.0 Hz) in **2**. This was confirmed by the HMBC correlations (Figure 3) from H_3-17 to C-1, C-15 and C-16. The relative stereochemistry of all stereocenters except C-15 of **2** was determined to be the same as that of **1** by comparison of the proton shifts and coupling constants. The methyl group at C-15 was assigned the β-configuration primarily due to the NOE correlation between H_3-17 and H-1. Furthermore, comparison of the NMR data between **2** and **5** confirmed both compounds have the same relative stereochemistry at C-15 [22]. Thus, the structure of **2** was established.

Finally, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to examine the cytotoxic activities of compounds 1–5 against four cancer cell lines, including human cervical epitheloid carcinoma (HeLa), laryngeal carcinoma (HEp-2) and breast carcinoma (MCF-7 and MDA-MB-231) cancer cells. Cells were treated with different concentrations of 1–5 for 72 h. The results show that compound 3, the most potent of compounds 1–5, exhibited cytotoxicity against the HeLa, HEp-2, MCF-7 and MDA-MB-231 cancer cell lines with IC50 values of 8.6, 8.2, 16.0 and 11.3 μg/mL, respectively. Furthermore, compounds 1 and 4 were found to exhibit weak cytotoxicity towards some of the cell lines (Table 2).

Table 1. ^{1}H and ^{13}C NMR data for **1** and **2**.

C/H	1		2	
	δ_H (J in Hz) [a]	δ_C (mult.) [b]	δ_H (J in Hz) [a]	δ_C (mult.) [b]
1	2.75 m	36.7 (CH)	1.71 m	38.4 (CH)
2	2.25 m; 1.57 m	29.3 (CH_2)	2.24 m; 1.44 m	29.8 (CH_2)
3	4.05 d (11.5)	84.5 (CH)	4.05 d (11.5, 2.5)	85.0 (CH)
4		73.7 (C)		73.7 (C)
5	1.83 m; 1.77 m	37.8 (CH_2)	1.77 m	37.5 (CH_2)
6	2.28 m; 2.02 m	22.1 (CH_2)	2.28 m; 1.98 m	22.1 (CH_2)
7	5.26 dd (7.5, 7.5)	127.2 (CH)	5.23 dd (7.0, 7.0)	127.2 (CH)
8		135.1 (C)		135.0 (C)
9	2.31 m; 1.93 m	35.3 (CH_2)	2.27 m; 1.92 m	35.1 (CH_2)
10	1.92 m; 1.72 m	27.9 (CH_2)	1.90 m; 1.72 m	28.1 (CH_2)
11	4.79 dd (6.5, 2.5)	77.5 (CH)	4.80 dd (7.0, 2.0)	77.2 (CH)
12		74.8 (C)		74.8 (C)
13	1.74 m; 1.53 m	35.2 (CH_2)	1.68 m; 1.48 m	36.1 (CH_2)
14	1.92 m; 1.36 m	28.6 (CH_2)	1.68 m; 1.12 m	28.7 (CH_2)
15		140.4 (C)	2.09 m	43.5 (CH)
16		166.6 (C)		174.8 (C)
17	6.43 d (2.0); 5.63 d (2.0)	125.5 (CH_2)	1.35 d (7.0)	15.3 (CH_3)
18	1.38 s	25.5 (CH_3)	1.39 s	25.6 (CH_3)
19	1.62 s	16.1 (CH_3)	1.62 s	16.4 (CH_3)
20	1.19 s	25.4 (CH_3)	1.17 s	25.4 (CH_3)
OAC		170.6 (C)		170.6 (C)
	2.11 s	21.1 (CH_3)	2.11 s	21.1 (CH_3)

[a] 500 MHz in $CDCl_3$; [b] 125 MHz in $CDCl_3$.

Figure 3. The structures of metabolites **1** and **2** and selected ^{1}H-^{1}H COSY (−) and HMBC (→) correlations.

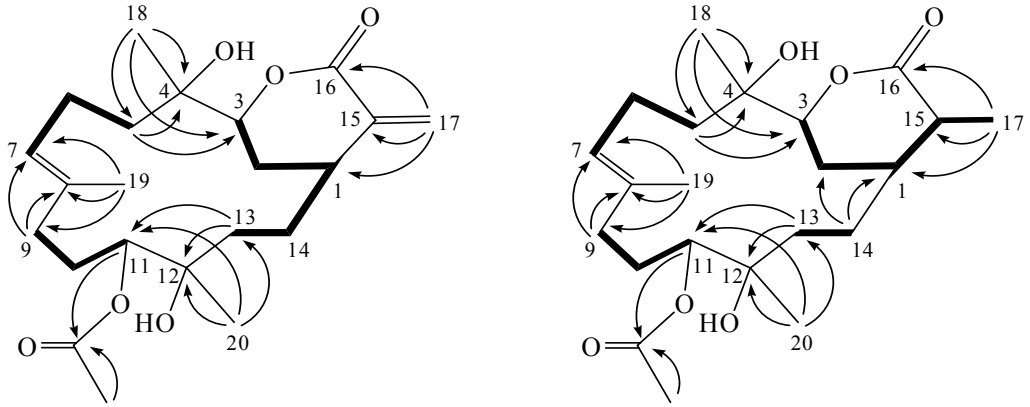


Table 2. Cytotoxicity (IC_{50} μg/mL) of compounds **1**–**5**.

Compound	Cell Lines			
	HeLa	**HEp-2**	**MCF-7**	**MDA-MB-231**
1	9.5	11.3	17.8	15.7
2	NA [b]	NA [b]	NA [b]	NA [b]
3	8.6	8.2	16.0	11.3
4	NA [b]	12.6	17.5	13.5
5	NA [b]	NA [b]	NA [b]	NA [b]
Doxorubicin [a]	0.05	0.1	0.07	0.5

[a] Clinical anticancer drug as positive control; [b] NA, not active at 20 μg/mL.

3. Experimental Section

3.1. General Procedures

Optical rotation values were measured using a Jasco P-1010 digital polarimeter. IR spectra were recorded on a Varian Digilab FTS 1000 Fourier transform infrared spectrophotometer. NMR spectra were recorded on a Varian Mercury Plus 400 FT-NMR (or Varian Unity INOVA 500 FT-NMR) instrument at 400 MHz (or 500 MHz) for ^{1}H-NMR and 100 MHz (or 125 MHz) for ^{13}C-NMR, respectively, in $CDCl_3$. ESIMS and HRESIMS data were recorded with a Bruker APEX II mass spectrometer. Gravity column chomatography was performed on silica gel (230–400 mesh, Merck, Darmstadt, Germany). Thin layer chomatography (TLC) was carried out on precoated Kieselgel 60 F254 (0.2 mm, Merck) and spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. HPLC was performed using a system comprised of a Hitachi L-7100 pump (Tokyo, Japan) and a Rheodyne 7725 injection (Cotati, CA, USA) port. A preparative normal phase column (Hibar 250 × 21.2 mm, Supelco, silica gel 60, 5 μm, Bellefonte, PA, USA) was used for HPLC.

3.2. Animal Material

The marine soft coral *Sinularia flexibilis* (Quoy and Gaimard, 1833) was collected by scuba divers at a depth of around 10 m off the coast of Pingtung County, Taiwan, in July 2012, and the sample was frozen immediately after collection. A voucher sample was deposited at the National Museum of Marine Biology and Aquarium, Taiwan (specimen No. 2012-0709-10).

3.3. Extraction and Separation

The soft coral (2.0 kg, wet wt.) was stored frozen and then freeze dried. The freeze-dried material (450 g) was minced and extracted five times with EtOAc (2 L) for 24 h each time at room temperature. The organic extract was evaporated to yield a residue (60.5 g), which was subjected to open column chomatography on silica gel eluting with gradients of *n*-hexane (H)–EtOAc (E), to give 14 fractions: Fr-1 (eluted by *n*-hexane), Fr-2 (eluted by H–E 100:1), Fr-3 (eluted by H–E 50:1), Fr-4 (eluted by H–E 30:1), Fr-5 (eluted by H–E 20:1), Fr-6 (eluted by H–E 15:1), Fr-7 (eluted by H–E 10:1), Fr-8 (eluted by H–E 8:1), Fr-9 (eluted by H–E 5:1), Fr-10 (eluted by H–E 3:1), Fr-11 (eluted by H–E 2:1), Fr-12 (eluted by H–E 1:1), Fr-13 (eluted by H–E 1:2), and Fr-14 (eluted by EtOAc). Fraction 10 was further separated by silica gel column chomatography with gradient elution (*n*-hexane–EtOAc, 5:1 to 1:1) to yield five subfractions (10A–E). Subfraction 10C was subjected to normal phase HPLC with *n*-hexane–EtOAc (4:1) as the eluent (flow rate 2 mL/min) to obtain compounds **4** (250 mg, 0.41% dry wt. of extract) and **5** (330 mg, 0.55% dry wt. of extract). Fraction 12 was further separated by silica gel column chomatography with gradient elution (*n*-hexane–EtOAc, 1:1 to 1:3) to yield seven subfractions (12A–G). Subfraction 12C was subjected to normal phase HPLC with *n*-hexane–EtOAc (1:1) as the eluent (flow rate 2 mL/min) to obtain compounds **1** (8.0 mg, 0.013% dry wt. of extract) and **2** (6.5 mg, 0.011% dry wt. of extract). Subfraction 12F was subjected to normal phase HPLC with *n*-hexane–acetone (1:1) as the eluent (flow rate 2 mL/min) to obtain compound **3** (6.5 mg, 0.011% dry wt. of extract).

11-Acetylsinuflexolide (**1**): white solid; mp 82.0–85.0 °C; $[\alpha]_D^{25}$ −12 (*c* 0.7, $CHCl_3$); IR (neat) ν_{max} 3434, 2974, 2937, 1712, 1622, 1452, 1376 and 1256 cm^{-1}; UV (MeOH) λ_{max} (log ε) 212 (3.9) nm; ^{13}C and ^{1}H NMR data, see Table 1; ESIMS *m*/*z* 417 $[M + Na]^+$; HRESIMS *m*/*z* 417.2250 $[M + Na]^+$ (calcd for $C_{22}H_{34}O_6Na$, 417.2253).

11-Acetyldihydrosinuflexolide (**2**): white solid; mp 75.0–78.0 °C; $[\alpha]_D^{25}$ −15 (*c* 0.6, $CHCl_3$); IR (neat) ν_{max} 3434, 2975, 2938, 1714, 1639, 1458, 1377 and 1245 cm^{-1}; ^{13}C and ^{1}H NMR data, see Table 1; ESIMS *m*/*z* 419 $[M + Na]^+$; HRESIMS *m*/*z* 419.2411 $[M + Na]^+$ (calcd for $C_{22}H_{36}O_6Na$, 419.2409).

Sinuflexolide (**3**): white solid; $[\alpha]_D^{24}$ −7.0 (*c* 0.2, $CHCl_3$); IR (neat) ν_{max} 3400, 2972, 1714, 1458, 1381, and 1235 cm^{-1}; UV (MeOH) λ_{max} 215 (log ε =3.8); [lit. $[\alpha]_D^{25}$ −8.6 (*c* 0.17, $CHCl_3$)] [21].

Sinularin (**4**): white solid; $[\alpha]_D^{25}$ −105 (*c* 1.0, $CHCl_3$); IR (neat) ν_{max} 3404, 2965, 1710, 1455, 1381, and 1237 cm^{-1}; UV (MeOH) λ_{max} 212 (log ε = 3.8); [lit. $[\alpha]_D^{20}$ −127] [22].

Dihydrosinularin (**5**): white solid; $[\alpha]_D^{25}$ −30 (*c* 0.3, $CHCl_3$); IR (neat) ν_{max} 3400, 2960, 1714, 1459, 1385, and 1231 cm^{-1}; [lit. $[\alpha]_D^{20}$ −45] [22].

Acetylation of **3**: A solution of **3** (5.0 mg) in pyridine (0.5 mL) was mixed with Ac_2O (0.1 mL), and stirred at room temperature for 24 h. After evaporation of excess reagent, the residue was subjected to column chromatograph over silica gel using *n*-hexane–EtOAc (1:2) to give the acetyl derivative **1** (4.8 mg, 81%).

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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method [25,26].

4. Conclusions

In the previous reports, cembranoids possessing a δ-lactone have been mostly isolated from soft coral (Alcyonaceae) belonging to the genera *Sinularia* [2,21–23,27,28] and *Lobophytum* [29]. Some of these metabolites have been found to possess several kinds of biological activities, such as cytotoxic [21–23] and anti-inflammatory activity [2,29]. In the present study, compounds **1**, **3** and **4** exhibited moderate or weak cytotoxicity against the growth of HeLa, HEp-2, MCF-7 and MDA-MB-231 cancer cell lines. According to the structures of **1**–**5**, it seems that the α-exomethylenic–ä-lactone ring group in compounds **1**, **3** and **4** is critical for the cytotoxic activity of metabolites **1**–**5**.

Acknowledgements

This study was supported in part by a grant from National Science Council of Taiwan (NSC 98-2313-B-276-001-MY3 and NSC 101-2313-B-276-002) and part by grant from Antai Medical Care Cooperation Antai Tian-Sheng Memorial Hospital Research Fund. (Project No. 101-FI-DBS-IAC-R-003).

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