Novel Cytotoxic Cembranoids from the Soft Coral *Sinularia flexibilis*

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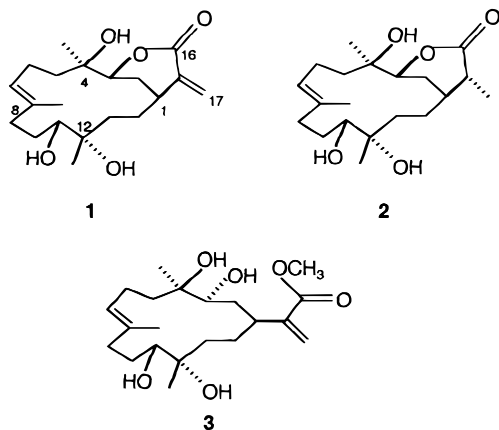
Three new cytotoxic cembranoid diterpenes, sinuflexolide (**1**), dihydrosinuflexolide (**2**), and sinuflexibilin (**3**), have been isolated from the soft coral *Sinularia flexibilis.* The structures of compounds **1**−**3** were determined by spectral and X-ray crystallographic analysis.

In a search for bioactive substances from marine organisms, the soft coral *Sinularia flexibilis* (Quoy and Gaimard) (Alcyoniidae) was selected for study since its CH_2Cl_2 extracts showed significant cytotoxicity in A549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma), KB (human epidermoid carcinoma), and P-388 (mouse lymphocytic leukemia) cell cultures as determined by standard procedures.[1,2] Bioassay-guided fractionation resulted in the isolation of three new cytotoxic cembranoid diterpenes, sinuflexolide (**1**), dihydrosinuflexolide (**2**), and sinuflexibilin (**3**).



Compound **1** was obtained as colorless prisms, mp 172−173 °C. HRMS established a molecular formula of $C_{20}H_{32}O_5$, and its IR spectrum (ν_{max} 3406, 1716 cm^{-1}) suggested the presence of hydroxy groups and an α,β-unsaturated ester or six-menbered lactone group, respectively. The ^{1}H NMR spectrum of **1** showed two doublets at δ 6.42 and 5.69 (1H each) with allylic couplings of 2.4 and 2.2 Hz, indicating the presence of a conjugated *exo*-methylene lactone. Other features of the spectrum included a doublet of doublet at δ 5.22 (J = 4.8, 11.7 Hz) and a broad methyl singlet at δ 1.64 indicative of a −C(CH_3)=CH− group, two sharp three-protons singlets at δ 1.37 and 1.19 indicating two tertiary methyl groups, a one-proton doublet of doublet at δ 4.06 (J = 2.0, 11.6 Hz), suggesting a hydrogen atom on the carbon bearing the lactone oxygen, and a one-proton doublet of doublet at δ 3.42 (J = 5.8, 11.2 Hz) indicative of a hydrogen on a carbon bearing a second hydroxyl group. The ^{13}C NMR spectrum exhibited 20 signals (3CH_3, 8CH_2, 4CH, and 5C) whose chemical shift values and multiplicity confirmed the presence of a conjugated exocyclic methylene lactone [δ 168.3 (s), 85.2 (d), 140.9 (s), 126.1 (t)], a trisubstituted olefin [δ 127.1 (d), 136.0 (s)] and three oxygen-bearing carbons [δ 73.8 (d), 78.1 (s), 77.6 (s)]. Spectral evidence thus demanded that compound **1** was bicyclic with two olefins and a carbonyl group. Thus, in common with the majority of alcyonarian metabolites, it apppeared that compound **1** contained a cembrane skeleton.[3−5] The structure of **1** was determined and the relative stereochemistry asssigned from a single-crystal X-ray diffraction analysis (Figure 1).[6]

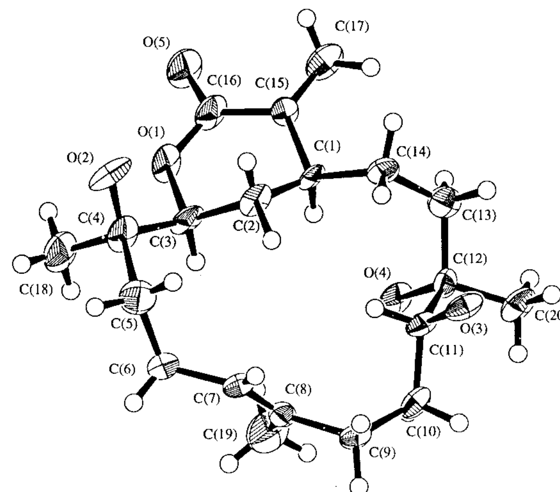


Figure 1. Molecular Structure (Relative Configuration) of Compound **1**.

The ^{1}H NMR spectrum (Table 1) of compound **2** was almost identical with that of compound **1** with the exception that the *exo*-methylene proton resonances of the latter were replaced by a methyl doublet at δ 1.31 (3H, J = 6.3 Hz) coupled to a signal at δ 2.06 (^{1}H−^{1}H 2D COSY). The replacement of the exocyclic methylene group of **1** by a methyl group was further confirmed by the ^{13}C NMR spectrum, which showed a signal at δ 15.7 (s) corresponding to a methyl doublet δ 1.31 in the ^{1}H NMR spectrum (^{1}H−^{13}C 2D HETCOR). The relative

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Table 1. ^{1}H NMR Data of **1**-**3**[a]

proton	compd 1	2	3
H-1	2.82 m	2.10 m	2.97 d (12.3)[b]
H-2α	1.36 m	1.46 m	1.25 m
H-2β	2.12 m	2.02 m	2.00 m
H-3	4.06 dd (2.0, 11.6)	4.04 dd (2.7, 11.7)	3.66 d (11.8)
H-5α	1.83 m	1.72 m	1.98 m
H-5β	1.62 m	1.70 m	1.50 m
H-6α	2.38 m	2.42 m	2.35 m
H-6β	2.28 m	2.31 m	2.24 m
H-7	5.22 dd (4.8, 11.7)	5.25 dd (4.5, 9.0)	5.44 t (5.7)
H-9	2.16 m	2.19 m	2.18 m
H-10α	1.65 m	1.50 m	1.79 m
H-10β	1.46 m	1.48 m	1.42 m
H-11	3.42 dd (5.8, 11.2)	3.44 d (8.7)	3.48 d (11.4)
H-13α	1.56 m	1.85 m	1.46 m
H-13β	1.32 m	1.54 m	1.21 m
H-14	1.66 m	1.90 m	1.48 m
H-15		2.06 m	
H-17	5.69 d (2.0)	1.31 d (6.3)	5.52 s
	6.42 d (2.4)		6.15 s
H-18	1.37 s	1.36 s	1.09 s
H-19	1.64 s	1.63 s	1.67 s
H-20	1.19 s	1.13 s	1.08 s
OMe			3.74 s

[a] Chemical shifts were determined at 400 MHz in CD_3OD. The values are in ppm downfield from TMS. [b] J values (in Hz) in parentheses.

Table 2. ^{13}C NMR Data of **1**−**3**[a]

carbon	compd 1	2	3
C-1	36.5 d	37.1 d	36.6 t
C-2	29.8 t	32.1 t	33.7 t
C-3	85.2 d	86.2 d	71.2 d
C-4	78.1 s	75.4 s	75.8 s
C-5	38.5 t	39.7 t	39.5 t
C-6	22.5 t	23.3 t	24.3 t
C-7	127.1 d	128.7 d	128.9 d
C-8	136.0 s	135.7 s	133.9 s
C-9	36.1 t	36.5 t	35.9 t
C-10	30.0 t	32.0 t	27.8 t
C-11	73.8 d	73.3 d	70.7 d
C-12	77.6 s	74.8 s	75.3 s
C-13	35.6 t	38.9 t	34.9 t
C-14	29.2 t	28.8 t	24.4 t
C-15	140.9 s	45.7 d	146.6 s
C-16	168.3 s	177.8 s	169.6 s
C-17	126.1 t	15.7 q	124.3 t
C-18	24.8 q	24.9 q	23.7 q
C-19	16.3 q	16.2 q	16.8 q
C-20	24.7 q	24.5 q	24.1 q
OMe			52.4 q

[a] Chemical shifts were determined at 100.6 Hz in CD_3OD. The values are in ppm downfield from TMS.

configuration of the secondary methyl group at C-15 was assigned to be on the same side as the H-1 proton of the δ-lactone ring by comparison of ^{1}H NMR spectral data with those of dihydrosinularin [δ 1.35 (3H, d, J = 7 Hz, H_3-17), 2.2 (1H, m, H-15)] and its 15-epimer [δ 1.21 (3H, H_3-17; 2.80 (1H, m, H-15)].[7,8] The structure of **2** was further supported by 2D NMR spectra including COSY, NOESY, HMQC, and HMBC.

A molecular formula of $C_{21}H_{36}O_6$ was established for compound **3** from HREIMS plus ^{1}H and ^{13}C NMR data. The IR spectrum contained a carbonyl band at 1718 cm^{-1} consistent with the presence of an ester group in addition to a strong hydroxyl stretching band at 3440 cm^{-1}. As with **2**, the ^{1}H NMR (Table 1) and ^{13}C NMR (Table 2) chemical shifts of **3** were very similar to those of **1** except for the disappearance of the lactone functionality. The ^3J HMBC couplings of C-16 with methoxyl protons and H_2-17 supported the replacement of the lactone functionality by a methyl ester. The ^{1}H−^{1}H COSY spectrum of **3** showed coupling between 1α-H at δ 2.97 and 2β-H at δ 2.00 and between 3-H at δ 3.66 and 2α-H at δ 1.25. In the NOESY spectrum of **3**, coupling between 1α-H and 2α-H and 3β-H and 2β-H justifies the assigned relative stereochemistry at C-1 and C-3. The relative positions of other functional groups were clearly supported by ^{1}H−^{1}H COSY, NOESY (Figure 2), HMQC, and HMBC (Figure 3) results.

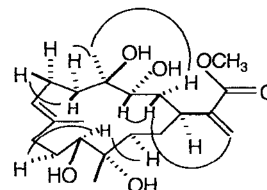

Figure 2. NOESY correlations of compound **3**.

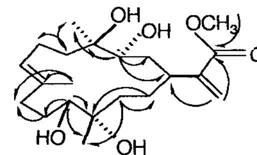

Figure 3. HMBC correlations of compound **3**.

Table 3. Cytotoxicitiy[a] of **1**−**3** (N = 8)

compd	ED50 (μg /mL) in indicated cell line A549	HT-29	KB	P-388
1	0.68	0.39	0.46	0.16
2	16.8	32.4	>50	3.86
3	0.72	0.22	1.73	0.27

[a] For significant activity of pure compounds, an ED_{50} of ≤4.0 μg/mL is required.[1]

Compounds **1** and **3** exhibited significant cytotoxicity toward the growth of A549, HT-29, KB, and P-388 cells. Compounds **2** exhibited significant cytotoxicity toward the growth of P-388 cells. The cytotoxicity values for compounds **1**−**3** are presented in Table 3.

Experimental Section

General Experimental Procedures. Melting points were determined using a Yanagimoto micro-melting point apparatus and are reported uncorrected. Optical rotations were determined on a JASCO DIP-181 polarimeter. UV spectra were obtained on a Shimadzu UV-160A spectrophotometer, and IR spectra were recorded on a Hitachi 26−30 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded with a Varian Unity Plus 400 NMR spectrometer at 400 and 100.6 MHz, respectively, in $CDCl_3$ using TMS as internal standard. EIMS spectra were obtained with a JEOL JMS-SX/SX 102A mass spectrometer at 70 eV. Silica gel 60 (Merck, 230−400 mesh) was used for column chromatography; precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative TLC.

Animal Materials. The soft coral *S. flexibilis* was collected near Kenting, Taiwan, at a depth 12 m and was stored in a freezer until extraction. The voucher specimen (NSUMR-1040) was deposited in the Department of Marine Resources, National Sun Yat-sen University.

Extraction and Isolation. The bodies of the soft coral *S. flexibilis* (2 kg, wet wt) were frozen and extracted with CH_2Cl_2 (3 L × 3). After removal of solvent in vacuo, the residue (300 g) was chromatographed over silica gel 60 using $CHCl_3$ and $CHCl_3$–MeOH mixtures of increasing polarity. Elution by $CHCl_3$–MeOH (19:1) afforded fractions containing cembranoids **1** and **2**, which were purified by centrifugal partition chromatography (ascending mode) using *n*-hexanes–EtOAc–acetone–water (1:1:1:1) (upper layer) as eluting solvent system. Elution by $CHCl_3$–MeOH (9:1) afforded fractions containing cembranoid **3**, which was purified by column chromatography over silica gel with *n*-hexanes–EtOAc as eluting solvents.

Sinuflexolide (1): 20 mg; colorless prisms; mp 172–173 °C; $[\alpha]^{25}_D$ −8.6° (*c* 0.17, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 242 (4.1) nm; IR (CCl_4) ν_{max} 3460, 2972, 1716 cm^{-1}; ^1H NMR see Table 1; ^{13}C NMR see Table 2; EIMS (70 eV) *m/z* $[M]^+$ 352 (2), 334 (5), 317 (4), 301 (5), 275 (6), 209 (12), 123 (24), 55 (68), 43 (100); HREIMS *m/z* 352.2238 (calcd for $C_{20}H_{32}O_5$ 352.2241).

Dihydrosinuflexoilde (2): 8 mg; colorless prisms; mp 165–167 °C; $[\alpha]^{25}_D$ −3.8° (*c* 0.066, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (4.4) nm; IR (KBr) ν_{max} 3404, 2924, 1732, 1450, 710 cm^{-1}; ^1H NMR see Table 1; ^{13}C NMR see Table 2; EIMS *m/z* $[M]^+$ 354 (5), 336 (5), 319 (4), 301 (5), 275 (8), 209 (10), 123 (26), 55 (80), 43 (100); HREIMS *m/z* 354.2398 (calcd for $C_{20}H_{34}O_5$ 354.2397).

Sinuflexibilin (3): 8 mg; colorless prisms; mp 169–170 °C; $[\alpha]^{25}_D$ −3.9° (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ϵ) 206 (4.01) nm; IR (KBr) ν_{max} 3440, 2936, 1718, 1635 cm^{-1}; ^1H NMR see Table 1; ^{13}C NMR see Table 2; EIMS *m/z* $[M]^+$ 384 (1), 366 (2), 348 (2), 330 (1), 316 (3), 299 (3), 298 (2), 273 (4), 43 (100); HREIMS *m/z* 384.2506 (calcd for $C_{21}H_{36}O_6$ 384.2502).

Single-Crystal X-ray Analysis of Sinuflexolide (1): Cyrstal data: $C_{20}H_{32}O_5$, space group $P2_1$, a = 10.252(4) Å, b = 10.483(5) Å, c = 10.25(2) Å, β = 116.71(8)°; V = 983(1) Å^3, Z = 2, D_{calcd} = 1.183 g/cm^3, λ (Mo Kα) = 0.710 69 Å. Intensity data were measured on a Rigaku AFL6S diffractometer up to 2θ of 47.2°. A total of 1666 reflections (+*h*, +*k*, ±*l*) were observed [$I > 3\sigma(I)$]. The structure was solved by the direct methods (SIR92),[9] and the final structure parameters were obtained by a full-matrix least-squares process. In view of the absence of heavy atom in the structure, Friedel pairs were not collected and the absolute configuration of sinuflexolide (**1**) was not determined via diffraction method. Calculated hydrogen positions were put in the final cycle of structure factor calculation but not refined. The agreement indices were R(F) = 0.065, Rw(F) = 0.052 with anisotropic refinement done on all non-hydrogen atoms. Experimental details are shown in Table 4. Final atomic coordinates are listed in Table 5.

Cytotoxicity Testing. The cytotoxic activities of tested compounds or fractions against P-388, KB, A549, and HT-29 were assayed with modification of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. For P-388 cells, 200 μL of culture was established at 1500 cells/well in 96-well tissue culture plates (Falcon). Tested compounds were dispensed subsequently to the established culture plate at eight concentrations each with three repetitions. After 3 days of incubation, P-388 cells were enumerated with MTT.

To measure the cytotoxic activities of pure compounds or crude fractions against A549, HT-29, KB, and P-388, each cell line was initiated at 750, 750, 2000, and 1500 cells/well, respectively, in 96-well microtiter plates. Three to eight concentrations encompassing an 8- to 128-fold range were evaluated on each cell line. A549, HT-29, KB, and P-388 cells were evaluated using MTT after exposure to test samples for 6, 6, 3, and 3 days, respectively. Fifty mL of 1 mg/mL MTT was added to each well, and plates were incubated at 37 °C for a further 4 h. Supernatant was aspirated with a Dynat-

Table 4. Crystal Data and Intensity Collection Data for Sinuflexolide (1)

empirical formula	$C_{20}H_{30}O_5$
FW	350.45
color, habit	colorless, prism
diffractormeter used	Rigaku AFL6S
space group	$P2_1$(#4)
a, Å	10.252(4) Å
b, Å	10.483(5) Å
c, Å	10.25(2) Å
β, deg	116.71(8)°
V, Å^3	983(1) Å^3
Z	2
D_{calcd}, g cm^{-3}	1.183 g/cm^3
λ(Mo Kα), Å	0.71069 Å
F(000)	380.00
unit cell detn (no. 2θ range, deg)	20, θ (8.7–14.5°)
scan type	$\omega-2\theta$
2θ range, deg	13–47.2°
h,k,l range	0 ≈ +11, 0 ≈ +11, −11 ∼ +11
μ(Mo Kα), cm^{-1}	0.83 cm^{-1}
cryst size, mm	0.40 × 0.45 × 0.50 mm
transm factor	0.624–1.000
temp, K	23.0 °C
no. of measd reflns	1666
no. of unique reflns	1572 (R_{int} = 0.083)
no. of obsd reflns (N_o)	1134
R,[a] R_w[a]	0.065, 0.052
GOF[a]	3.96
no. of ref params (N_p)	224
maximum peak in final diff. map	0.27 e$^-$/Å^3
minimum peak in final diff. map	−0.25 e$^-$/Å^3

[a] $R = [\sum||F_o| - |Fc||/\sum|F_o|$. $R_w = [\sum w(|F_o| - |F_c|)^2/\sum w|F_o|^2]^{1/2}$. GOF $= [\sum w(|F_o| - |F_c|)^2/(No - N_p)]^{1/2}$.

Table 5. Atomic Parameters for Sinuflexolide (1)

atom	x	y	z	B_{eq}
O(1)	0.4116(5)	0.2249	0.5329(6)	3.7(2)
O(2)	0.5146(6)	0.4451(8)	0.7151(7)	4.3(2)
O(3)	1.0391(6)	0.2669(7)	0.4625(6)	2.8(1)
O(4)	0.7890(5)	−0.0025	0.4117(6)	3.1(2)
O(5)	0.2158(6)	0.2078(9)	0.3267(6)	4.7(2)
C(1)	0.5968(7)	0.2412(10)	0.3922(8)	2.6(2)
C(2)	0.6331(8)	0.3142(10)	0.5331(9)	3.1(2)
C(3)	0.5671(8)	0.249(1)	0.6233(9)	3.0(2)
C(4)	0.5752(9)	0.318(1)	0.7550(10)	3.5(3)
C(5)	0.742(1)	0.346(1)	0.8671(10)	4.2(3)
C(6)	0.8431(9)	0.229(1)	0.9151(9)	3.5(2)
C(7)	0.9568(8)	0.224(1)	0.8600(8)	2.8(2)
C(8)	1.0000(9)	0.122(1)	0.8178(8)	2.4(2)
C(9)	1.1247(8)	0.128(1)	0.7747(8)	2.9(2)
C(10)	1.0829(8)	0.081(1)	0.6192(9)	3.0(2)
C(11)	0.9697(9)	0.1625(9)	0.5000(10)	2.4(2)
C(12)	0.8622(9)	0.0916(10)	0.3642(9)	2.4(2)
C(13)	0.7456(9)	0.1817(10)	0.2553(9)	3.1(2)
C(14)	0.6828(8)	0.285(1)	0.3154(8)	2.8(2)
C(15)	0.4327(8)	0.247(1)	0.3045(9)	4.4(3)
C(16)	0.3483(9)	0.227(1)	0.3860(10)	3.5(2)
C(17)	0.3632(8)	0.242(1)	0.162(1)	5.0(3)
C(18)	0.5040(9)	0.242(1)	0.8299(10)	4.8(3)
C(19)	0.940(1)	−0.011(1)	0.810(1)	5.2(3)
C(20)	0.9369(8)	0.021(1)	0.2876(9)	3.3(2)

ech automatic washer. Formazan crystals were redissolved in DMSO (Merck) for 10 min with shaking, and the plate was read immediately on a microtiter plate reader (Dynatech) at a wavelength of 540 nm. The ED_{50} value was defined as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cell in the MTT assay.[10]

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References and Notes

(1) Geran, R. I.; Greenberg, N. H.; MacDonald, M. M.; A. M. Schumacher, A. M.; Abbott, B. J. *Cancer Chemother. Rep.* **1972**, *3*, 1−91.
(2) Hou, R.-S.; Duh, C.-Y.; Chiang, M. Y.; Lin, C.-N. *J. Nat. Prod.* **1995**, *58*, 1126−1130.
(3) Coll, J. C.; Hawes, G. B.; Liyanage, N.; Oberhänsli, W.; Wells, R. J. *Aust. J. Chem.* **1977**, *30*, 1305−1309.
(4) Bowden, B. F.; Coll, J. C.; Hicks, W.; Kazlauskas, R.; Mitchell, S. J. *Aust. J. Chem.* **1978**, *31*, 2707−2712.
(5) Bowden, B. F.; Coll, J. C.; Mitchell, S. J. *Aust. J. Chem.*. **1980**, *33*, 879−884.
(6) Crystallographic data for **1** have been deposited at the Cambridge Crystallographic Data Centre. The data can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge, CB2 1EZ, UK.
(7) Weinheimer, A. J.; Matson, J. A.; Hossain, M. B.; van der Helm, D., *Tetrahedron Lett.*, **1977**, 2923−2926.
(8) Kazlauskas, R.; Murphy, P. T.; Wells, R. J.; Schonholzer, P. *Aust. J. Chem.*, **1978**, *31*, 1817−1824.
(9) Attomare, A.; Cascarano, M.; Giacovazzo, C.; Guagliardi, A. *J. Appli. Cryst.*, **1993**, *26*, 343−348.
(10) Mosmann, T. *J. Immunol. Method* **1983**, *65*, 55−63.

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