Studies on Formosan Soft Corals, II. Cytotoxic Cembranolides from the Soft Coral Lobophytum michaelae

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STUDIES ON FORMOSAN SOFT CORALS, II. CYTOTOXIC CEMBRANOLIDES FROM THE SOFT CORAL *LOBOPHYTUM MICHAELAE*[1]

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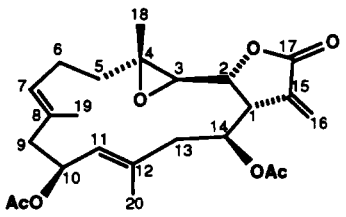
ABSTRACT.—Bioactivity-guided fractionation of a $CHCl_3$ extract of the soft coral *Lobophytum michaelae* afforded a new cytotoxic cembranolide, lobomichaolide [**1**], and a known cytotoxic cembranolide, crassolide [**2**]. The structure of **1** was determined by spectral and X-ray crystallographic analysis.

As part of our search for bioactive substances from marine organisms, the soft coral *Lobophytum michaelae* Tixier-Durivault (Alcyoniidae) was selected for study when a $CHCl_3$ extract of the species was found to exhibit significant cytotoxicity in A-549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma), KB (human nasopharyngeal carcinoma), and P-388 (mouse lymphocytic leukemia) cell culture systems when assessed using standard protocols (2). Cytotoxicity-guided chromatographic fractionation led to the isolation of a new cytotoxic cembranolide, lobomichaolide [**1**], and a known cytotoxic cembranolide, crassolide [**2**].

The $CHCl_3$-soluble material from an MeOH extract of *L. michaelae* was chromatographed over Si gel with $CHCl_3$-MeOH (98:2) to obtain cembranolide **1**, colorless prism, mp 180–181°, $[\alpha]^{25}D$ +54.9° (c = 0.16, $CHCl_3$). Hrms established the molecular formula of $C_{24}H_{32}O_7$. The ^{13}C-nmr spectrum showed the presence of three ester carbonyl carbons (δ 169.5, s, C-17; 170.6, s; 170.9, s), six olefinic carbons (δ 123.4, t, C-16; 127.7, s, C-8; 128.4, d, C-11; 129.7, d, C-7; 135.4, s, C-15; 137.5, s, C-12) due to one exo-methylene and two trisubstituted double bonds, five oxygen-



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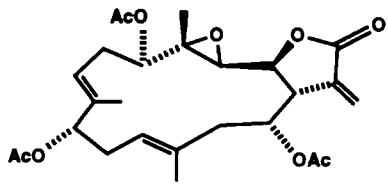

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[1]For the previous paper in this series, see Wu *et al.* (1).

bearing carbons (δ 76.1, d, C-2; 69.3, d, C-10; 67.8, d, C-14; 64.4, s, C-4; 59.6, d, C-3), one methine carbon (δ 46.7, d, C-1), four methylene carbons (δ 44.6, t, C-9; 41.3, t, C-13; 33.4, t, C-6; 23.9, t, C-5), and five methyl carbons (δ 21.5, q; 21.1, q; 20.5, q, C-18; 15.8, 15.7, q, C-19, 20). The ir (KBr) and ^{1}H-nmr spectra indicated the presence of two acetoxy groups (ν 1720, 1716 cm^{-1}; 2.02, 3H, s; 2.05, 3H, s) and an α-methylene-γ-lactone (ν 1760, 1660 cm^{-1}; δ 5.68 and 6.36, both 1H, d, J = 3.4 Hz, H_a-16, H_b-16). These data suggested that **1** possessed a 14-member monocarbocyclic ring with the cembrane skeleton and a γ-lactone ring. The ^{1}H-nmr spectrum also revealed the presence of a tertiary methyl group (δ 1.45, 3H, s, H-18), two methyl-bearing trisubstituted double bonds (δ 1.56, 3H, br s, H-19; 1.79, 3H, br s, H-20; 5.20, 1H, br d, J = 8.2 Hz, H-7; 5.57, 1H, m, H-11), one allylic methine proton (δ 3.01, 1H, m, H-1), a lactonic methine proton (δ 4.36, 1H, t, J = 7.2 Hz, H-2), an oxygen-carrying methine proton (δ 2.71, 1H, d, J = 6.8 Hz, H-3), two acetoxy methine protons (δ 5.67, 2H, m, H-10, -14) and eight methylene protons (δ 2.50–2.44, 2.21–2.00, m, H-5, -6, -9, -13). The assignments of proton and carbon signals were achieved by application of COSY, HETCOR, and long-range HETCOR experiments (3). The relative, not the absolute, configuration of **1** was determined by X-ray diffraction analysis (Figure 1).

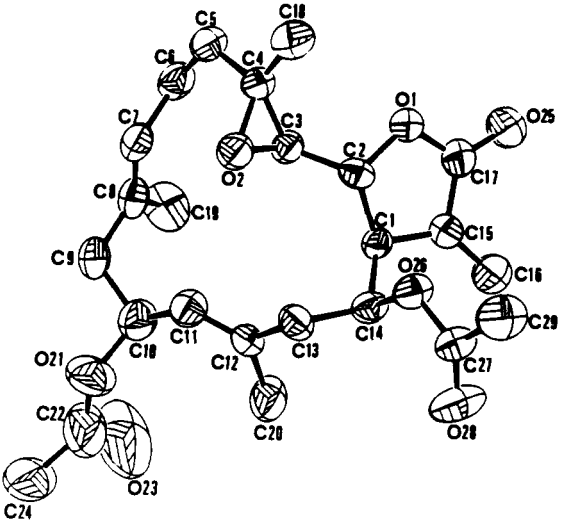

FIGURE 1. Molecular structure (relative configuration) of lobomichaolide [**1**].

The identity of **2** was determined by comparison of physical and spectral data reported previously (4). The assignments of proton and carbon signals were established by application of COSY, HETCOR, and long-range HETCOR experiments (3).

Cembranolides **1** and **2** showed significant cytotoxicity against the growth of A-549, HT-29, KB, and P-388 cells (Table 1). The cytotoxicities of marine cembranolides against P-388, KB, and Hela cells have been reported previously (5,6). Here we report the cytotoxicity of marine cembranolides against A-549 and HT-29 cells for the first time.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined using a Yanagimoto micro-melting point apparatus and were uncorrected. The uv spectra were obtained on a Hitachi 200-20

TABLE 1. Cytotoxicity[a] of Cembranolides **1** and **2**.

Compound	ED_{50} (μg/ml) ($n = 8$)			
	Cell line			
	A-549	HT-29	KB	P-388
1	0.38	0.37	0.59	0.34
2	0.39	0.26	0.85	0.08

[a]For significant activity of pure compounds, an ED_{50} value of ≤4.0 μg/ml is required (2).

spectrophotometer, and ir spectra were measured on a Hitachi 260-30 spectrophotometer. ^1H- and ^{13}C-nmr spectra were recorded with Varian Gemini NMR spectrometer at 200 MHz and 50.3 MHz, respectively, in $CDCl_3$ using TMS as internal standard. Eims spectra were obtained with a Joel JMS-HX110 mass spectrometer at 70 eV. Si gel 60 (Merck, 230–400 mesh) was used for cc, precoated Si gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical tlc, and precoated Si gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative tlc.

SOFT CORAL MATERIAL.—The soft coral *L. michaelae* was collected near Kenting of Taiwan at a depth of 12 m and was stored in a freezer until extraction. A voucher specimen was deposited in the Institute of Marine Biology, National Sun Yat-sen University.

EXTRACTION AND SEPARATION.—The bodies of the soft coral (4 kg, wet wt) were sliced into pieces and then homogenized with MeOH (3 liters × 5) and then CH_2Cl_2 (3 liters × 5). After removal of solvent in vacuo, the residue (35.6 g) was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ extract (19.2 g) was found to exhibit significant cytotoxicity against KB cell lines with ED_{50} of 3.42 μg/ml. Cc of the $CHCl_3$ extract was undertaken using $CHCl_3$ and $CHCl_3$/MeOH mixtures of increasing polarity. Elution by $CHCl_3$-MeOH (98:2) afforded fractions containing **1** and **2** from which these two cembranolides were separated by cc over Si gel with *n*-hexane–EtOAc (1:1) as eluting solvent.

Lobomichaolide [**1**].—Colorless prisms (70 mg): mp 180–181°; $[\alpha]^{25}D$ +54.9° ($c = 0.16$, $CHCl_3$); uv

TABLE 2. ^1H-nmr Chemical Shifts (δ) and Coupling Constants (Hz, in parentheses) of Cembranolides **1** and **2**.[a]

Proton	Compound	
	1	**2**
H-1	3.01 m	3.16 m
H-2	4.36 t (7.2)	5.00 dd (3.6, 3.6)
H-3	2.71 d (6.8)	2.83 d (3.6)
H-5	2.50 m	4.45 dd (11.0, 4.0)
H-6	2.46 m	2.50 m
H-7	5.20 br d (8.2)	5.10 m
H-9	2.00 m	4.94 dd (11.0, 4.0)
H-10	5.67 m	2.30 m
H-11	5.57 m	5.30 m
H-13	2.00 m	2.20 m
H-14	5.67 m	5.25 m
H_a-16	5.68 d (3.4)	5.78 d (2.4)
H_b-16	6.36 d (3.4)	6.43 d (2.4)
H-18	1.45 s	1.45 s
H-19	1.56 s	1.84 s
H-20	1.79 s	1.73 s
OAc	2.02 s, 2.05 s	2.01 s, 2.07 s, 2.12 s

[a]200 MHz, $CDCl_3$, δ-scale, relative to TMS.

(MeOH) λ max (log ε) 215 nm (3.8); ir (KBr) ν max 2900, 1760, 1720, 1716, 1660, 1420, 1380, 1330 cm^{-1}; ^1H nmr see Table 2; ^{13}C nmr see Table 3; eims *m/z* $[M]^+$ 432 (1%), 390 (3), 373 (7), 330 (6), 284 (2), 245 (30), 217 (43), 191 (100); hreims found 432.2166, calcd 432.2148 for $C_{24}H_{32}O_7$.

Crassolide [**2**].—Amorphous solid (50 mg): $[\alpha]^{25}D - 18°$ ($c = 0.32$, $CHCl_3$); uv (MeOH) λ max (log ε) 210 nm (3.5); ir (KBr) ν max 2905, 1780, 1745, 1670, 1410, 1360, 1320 cm^{-1}; ^1H nmr see Table 2; ^{13}C nmr see Table 3; eims *m/z* $[M]^+$ 490 (1%), 448 (1), 431 (5), 405 (1), 388 (4), 328 (30), 223 (29), 207 (5), 191 (100).

TABLE 3. ^{13}C-nmr Chemical Shifts of Cembranolides **1** and **2**.[a]

Carbon	Compound	
	1	**2**
C-1	46.7 d	42.6 d
C-2	76.1 d	73.9 d
C-3	59.6 d	62.5 d
C-4	64.4 s	61.8 s
C-5	23.9 t	79.3 d
C-6	33.4 t	42.6 t
C-7	129.7 d	125.0 d
C-8	127.7 s	135.0 s
C-9	44.6 t	77.8 d
C-10	69.3 d	30.5 t
C-11	128.4 d	123.1 d
C-12	137.5 s	138.3 s
C-13	41.3 t	29.7 t
C-14	67.8 d	74.0 d
C-15	135.4 s	136.3 s
C-16	123.4 t	125.8 t
C-17	170.0 s	170.4 s
C-18	20.5 q	13.2 q
C-19	15.7 q	16.0 q
C-20	15.9 q	11.4 q
OAc	21.5 q, 21.1 q, 170.6 s, 170.9 s	21.1 q, 21.4 q, 21.6 q, 171.7 s, 171.6 s

[a]Chemical shifts were determined at 50.3 MHz in $CDCl_3$. The values are in ppm downfield from TMS.

SINGLE CRYSTAL X-RAY ANALYSIS OF LOBOMICHAOLIDE [**1**][2].—Crystal data: $C_{24}H_{32}O_7$, space group $P2_1$; $a = 10.975$ (7), $b = 8.039$ (3), $c = 13.884$ (6) Å, V = 1203 (1) Å^3, Z = 2, $D_{calcd} = 1.20$ g/cm, $\lambda(M_DK_\alpha) = 0.71069$ Å. Intensity data were measured on a CAD4 diffractometer up to 2θ of 50°. A total of 2279 reflections were collected, from which 1724 reflections were observed [$I \geq 2\sigma(I)$]. The structure was solved by direct method, and the final structure parameters were obtained by a full-matrix least squares process. The agreement indices were R(F) = 0.049, Rw(F) = 0.036 with anistropic on all non-hydrogen atoms. Final atomic coordinates are listed in Table 4.

CYTOTOXICITY TESTING.—KB and P-388 cells were kindly provided by Prof. J.M. Pezzuto, Univeristy of Illinois at Chicago; A-549 and HT-29 were purchased from the American Type Culture Collection.

The P-388 cells were cultured in Fisher's medium supplemented with 10% heat-inactivated fetal calf serum (FCS). The KB cells were maintained in Basal Medium Eagle (BME) containing 10% heat-inactivated FCS. The A-549 cell line was cultured in Eagle Minimum Essential Medium (EMEM) containing

[2]Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained from Dr. Olga Kennard, 12 Union Road, Cambridge, CB2 1EZ, UK.

TABLE 4. Atomic Parameters *x*, *y*, *z* and Biso[a] of Lobomichaolide [**1**] (ESDs refer to the last digit printed).

Position	*x*	*y*	*z*	Biso
C-1	0.1894(3)	1.0367(6)	0.9096(3)	3.16(20)
C-2	0.0547(3)	0.9884(5)	0.8648(3)	3.48(22)
C-3	−0.0075(3)	1.0940(6)	0.7802(3)	3.55(21)
C-4	−0.1307(3)	1.0563(6)	0.7248(3)	3.88(23)
C-5	−0.2126(4)	1.1923(6)	0.6724(3)	4.7(3)
C-6	−0.1504(4)	1.3578(7)	0.6542(3)	4.9(3)
C-7	−0.0510(4)	1.3460(6)	0.5942(3)	4.23(23)
C-8	0.0550(4)	1.4281(6)	0.6063(3)	4.48(24)
C-9	0.1491(4)	1.3933(7)	0.5434(3)	5.2(3)
C-10	0.2710(4)	1.3253(6)	0.6030(3)	5.4(3)
C-11	0.2522(4)	1.1599(6)	0.6507(3)	4.30(23)
C-12	0.3230(3)	1.0995(6)	0.7295(3)	3.72(23)
C-13	0.2914(4)	0.9304(6)	0.7689(3)	3.90(22)
C-14	0.2894(3)	0.9286(6)	0.8775(3)	3.65(21)
C-15	0.1909(3)	1.0338(6)	1.0175(3)	3.93(22)
C-16	0.2834(4)	1.0367(8)	1.0924(3)	5.9(3)
C-17	0.0617(4)	1.0219(8)	1.0315(3)	5.7(3)
C-18	−0.2009(4)	0.9054(8)	0.7455(3)	5.8(3)
C-19	0.0903(5)	1.5642(7)	0.6813(3)	7.1(3)
C-20	0.4422(4)	1.1760(7)	0.7798(3)	5.9(3)
O-21	0.3511(3)	1.3008(5)	0.53099(23)	7.23(22)
C-22	0.4549(5)	1.3864(10)	0.5391(4)	10.1(4)
O-23	0.4950(5)	1.4608(10)	0.6105(3)	18.5(5)
C-24	0.5194(5)	1.3684(11)	0.4575(4)	12.8(5)
O-25	0.0215(3)	1.0278(7)	1.10692(20)	8.9(3)
O-26	0.25978(23)	0.7592(4)	0.90343(19)	4.14(15)
C-27	0.3522(4)	0.6620(6)	0.9455(3)	4.18(23)
O-28	0.4578(3)	0.7009(4)	0.96047(24)	6.60(21)
C-29	0.3042(4)	0.4949(7)	0.9708(4)	6.6(3)
O-1	−0.01508(23)	1.0059(5)	0.94358(18)	5.35(18)
O-2	−0.02009(24)	1.01892	0.68593(18)	4.31(15)

[a]Biso is the mean of the principal axes of the thermal ellipsoid.

Earle's salts and supplemented with 0.1 mM of nonessential amino acids and 10% heat-inactivated FCS. The HT-29 cell lines were maintained in Rosewell Park Memorial Institute (RPMI) 1640 medium containing 10% heat-inactivated FCS. All the cell lines were maintained in an incubator at 37° in humidified air containing 5% CO_2. For routine cytotoxicity assay, all four cell lines were adapted to one single medium, RPMI 1640 supplemented with 10% FCS and 1 mM glutamate.

The cytotoxic activities of tested compounds or fractions against P-388, KB, A-549, and HT-29 were assayed with modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method described by Alley *et al.* (7). 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 KB, A-549, and HT-29, each cell line was initiated at 2000, 750, and 750 cells/well, respectively, in 96-well microtiter plates. Three to eight concentrations encompassing 8- to 128-fold range were performed on each cell line. KB, A-549, and HT-29 cells were enumerated using MTT after the exposure to tested samples for 3, 6, and 6 days, respectively. Fifty μl of 1 mg/ml MTT was added to each well, and plates were incubated at 37° for a further 5 h. Supernatant was aspirated with a Dynatec Automatic Washer. Formazan crystals were redissolved in DMSO (Merck) for 10 min with shaking, and the plate was read immediately on an enzyme-linked immunosorbant assay reader (Microplate Reader, BioRad) at a wavelength of 540 nm. The ED_{50} was defined as 50% reduction of absorbance in the no drug control assay. Results are given in Table 1.

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