Journal of Natural Products
Vol. 58, No. 7, pp. 1126-1130, July 1995

SINUGIBBEROL, A NEW CYTOTOXIC CEMBRANOID DITERPENE FROM THE SOFT CORAL *SINULARIA GIBBEROSA*

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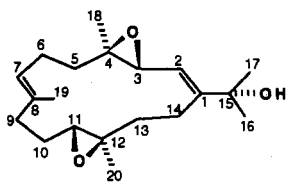
ABSTRACT.—Bioactivity-guided fractionation of a chloroform extract of the soft coral *Sinularia gibberosa* afforded a new cytotoxic cembranoid diterpene, sinugibberol [**1**]. The structure of **1** was determined by spectral and X-ray crystallographic analysis.

As part of our search for bioactive substances from marine organisms, the soft coral *Sinularia gibberosa* Tixier-Durivault (Alcyoniidae) was selected for study when a $CHCl_3$ extract of the species was found to exhibit significant cytotoxicity in the A549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma), KB-16 (human epidermoid carcinoma), and P-388 (mouse lymphocytic leukemia) cell culture systems, as assessed using standard protocols (1). Cytotoxicity-guided chromatographic fractionation led to isolation of a new cytotoxic cembranoid diterpene, sinugibberol [**1**].

The $CHCl_3$-soluble material from an MeOH extract of *Sinularia gibberosa* was chromatographed over Si gel with $CHCl_3$-MeOH (90:10) to obtain cembranoid **1**, as colorless prisms, mp 142–144°, $[\alpha]^{25}D$ +30.6° (c=0.011, $CHCl_3$). Hrms established a molecular formula of $C_{20}H_{32}O_3$. The ^{13}C-nmr spectrum showed the presence of four olefinic carbons (δ 150.9, s, C-1; 134.9, s, C-8; 125.8, d, C-2; 121.2, d, C-7) due to two trisubstituted double bonds, five oxygen-bearing carbons (δ 73.4, s, C-15; 62.1, d, C-11; 61.7, s, C-4; 61.2, s, C-12; 59.3, d, C-3), six methylene carbons (δ 40.2, t; 38.0, t; 36.8, t; 26.0, t; 24.5, t; 22.3, t), and five methyl carbons (δ 29.7, 2C, q, C-16, C-17; 18.0, q, C-18; 16.1, q, C-20; 14.7, q, C-19). The ir (KBr) spectra indicated the presence of an OH group (ν max 3490 cm^{-1}). These data suggested that **1** possessed a 14-membered monocyclic ring and bore a cembrane skeleton. The ^1H-nmr spectrum of **1** also revealed the presence of a methyl-bearing trisubstituted double bond (δ 1.66, 3H, s, H-19; 5.37, 1H, m, H-7), four methyl groups attached to quaternary carbon atoms, probably vicinal to oxygen atoms (δ 1.37, 6H, s, H-16, H-17; 1.30, 3H, s, H-18; 1.26, 3H, s, H-20) (2), one trisubstituted double bond (δ 5.31, 1H, d, J=6.9 Hz, H-2), two epoxy methine protons (δ 3.30, 1H, d, J=6.9 Hz, H-3; 2.69, 1H, dd, J=2.9 and 10.4 Hz, H-11) (3), and six methylene protons (δ 2.03–2.34, m, H-5, H-6, H-9, H-10, H-13, H-14). The assignments of the ^1H-



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and ^{13}C-nmr signals of **1** were conducted by the application of COSY and HETCOR experiments (4). The relative but not the absolute configuration of **1** was determined by X-ray diffraction analysis (Figure 1).

Di-epoxides of this type have been shown to form by oxidation of cembranes which contain a conjugated diene between C-1 and C-4 and an epoxide at C-11–C-12 (3). Compound **1** may be formed by oxidation of (1*E*,3*E*,7*E*)-11,12-epoxycembra-1,3,7-triene-15-ol.

Cembranoid **1** showed significant cytotoxicity against the growth of HT-29 and P-388 cells, with ED_{50} values of 0.50 and 11.7 μg ml^{-1}, respectively.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The mp was determined using a Yanagimoto micro-melting point apparatus and is reported uncorrected. The optical rotation was determined on a Jasco DIP-181 polarimeter. The uv spectrum was obtained on a Shimadzu UV-160A spectrophotometer, and the ir spectrum was recorded on a Hitachi 26-30 spectrophotometer. ^{1}H- and ^{13}C-nmr spectra were recorded with a 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 JEOL JMS-SX/SX 102A mass spectrometer at 70 eV. Si gel 60 (Merck, 230–400 mesh) was used for cc and precoated Si gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for prep. tlc.

KB-16 and P-388 cells were kindly supplied by Prof. J.M. Pezzuto, University of Illinois at Chicago; A-549 and HT-29 cells were purchased from the American Type Culture Collection.

ANIMAL MATERIAL.—The soft coral *S. gibberosa* was collected near Kenting, Taiwan at a depth of 12 meters and was stored in a freezer until extraction. A voucher specimen (NSUMR-1031) was deposited in the Department of Marine Resources, National Sun Yat-sen University.

EXTRACTION AND ISOLATION.—The bodies of the soft coral (4 kg, wet wt) were sliced and then homogenized with MeOH (3 liters×3), Me_2CO (3 liters), and then CH_2Cl_2 (3 liters×2). After removal of solvent *in vacuo*, the residue (300 g) was partitioned between $CHCl_3$ and H_2O. Cc of the $CHCl_3$ extract (60 g) was undertaken using $CHCl_3$ and $CHCl_3$/MeOH mixtures of increasing polarity. Elution by $CHCl_3$-MeOH (9:1) afforded fractions containing cembranoid **1** which was purified by cc over Si gel with *n*-hexane-EtOAc (2:1) as eluting solvent.

Sinugibberol [**1**].—Colorless prisms (10 mg): mp 142–144°; $[\alpha]^{25}D$ +30.6° (*c*=0.011, $CHCl_3$); uv (MeOH) λ max (log ε) 210 (4.3) nm; ir (KBr) ν max 3490, 2920, 1080, 960, 880, 820 cm^{-1}; ^{1}H nmr ($CDCl_3$, 200 MHz) δ 5.37 (1H, m, H-7), 5.31 (1H, d, *J*=6.9 Hz, H-2), 3.30 (1H, d, *J*=6.9 Hz, H-3), 2.69 (1H, dd, *J*=2.9 and 10.4 Hz, H-11), 2.03–2.34 (12H, m, H-5, H-6, H-9, H-10, H-13, H-14), 1.66 (3H, d, s, H-19), 1.37 (6H, s, H-16, H-17), 1.30 (3H, s, H-20), 1.26 (3H, s, H-18); ^{13}C nmr ($CDCl_3$, 50.4 MHz) δ 150.9 (s, C-1), 134.9 (s, C-8), 125.8 (d, C-2), 121.2 (d, C-7), 73.4 (s, C-15), 62.1 d, C-11), 61.7 (s, C-4), 61.2 (s, C-12), 59.3 (d, C-3), 40.2 (t), 38.0 (t), 36.8 (t), 29.7 (q, C-16, C-17), 26.0 (t), 24.5 (t), 22.3 (t), 18.0 (q, C-18), 16.1 (q, C-20), 14.7 (q, C-19); eims *m/z* [M]$^+$ 320 (1), 302 (11), 287 (10), 261 (9), 209 (53), 193 (42), 151 (47), 107 (100), 81 (96), 59 (76); hreims *m/z* found 320.2385, calcd 320.2347 for $C_{20}H_{32}O_3$.

Single crystal X-ray analysis of sinugibberol

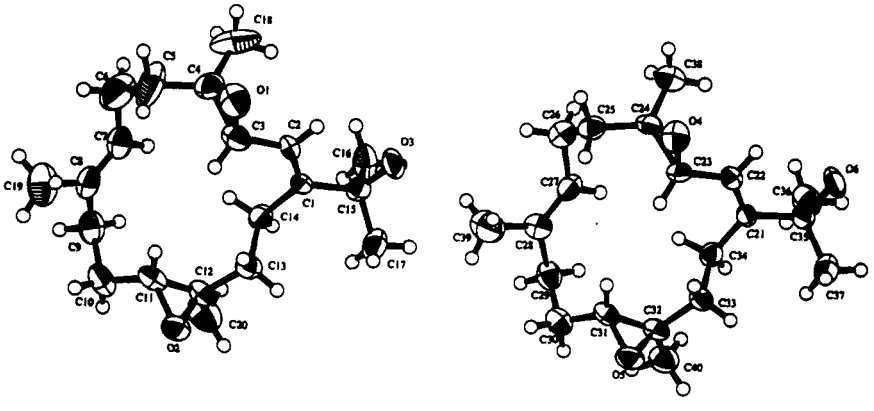

FIGURE 1. Molecular structure (relative configuration) of sinugibberol [**1**].

[**1**][1].—Crystal data: $C_{20}H_{32}O_3$, space group $P2_12_12_1$, a=18.606(3) Å, b=19.121(2) Å, c=10.726(2) Å, V=3815.9(9) Å^3, Z=8, D_{calcd}=1.116 g/cm^3, λ (CuKα)=1.54178 Å. Intensity data were measured on a AFC6S diffractometer up to 2 θ of 120.3°. A total of 3225 reflections was collected, from which 2072 reflections were observed [I>3.00σ(I)]. The structure was solved by the direct method, and the final structure parameters were obtained by a full-matrix least squares process. The agreement indices were R(F)=0.056, Rw(F)=0.038 and were anistropic on all non-hydrogen atoms. There were two independent molecules in the asymmetric unit. Basically they have the same conformation and differ slightly only on the ring skeleton due to a small conformation variation. Close examination of the two molecules reveals two possible types of intermolecular hydrogen bonding with the OH groups as donors and epoxy O atoms as acceptors. The distances are O3...O5 2.95 Å and O6...O2 2.99 Å. Hydroxyl hydrogen atoms found in the difference-Fourier map (H-63, H-64) (Figure 2) were included in the final structural calculation but not refined. All other hydrogen atoms were fixed at their ideal positions with a C-H distance of 0.95 Å in the final calculation. Final atomic coordinates are listed in Table 1.

CYTOTOXICITY TESTING.—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's Minimum Essential Medium (EMEM) containing 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 Roswell 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 assays, all four cell lines were adapted to one single medium, RPMI 1640 supplemented with 10% FCS and 1 μM glutamate.

The cytotoxic activities of the test compounds or fractions against P-388, KB-16, A549, and HT-29 cells were assayed using a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method (5). For P-388 cells, 200 μl of culture were established at 1500 cells/well in 96-well tissue culture plates (Falcon). Test compounds were dispensed subsequently to the established culture plate at eight concentrations each with three replicates. After 3 days of incubation, P-388 cells were evaluated with MTT.

To measure the cytotoxic activities of pure compounds or crude fractions against KB-16, A-549, and HT-29, each cell line was initiated at 2000, 750, 750 cells/well, respectively, in 96-well

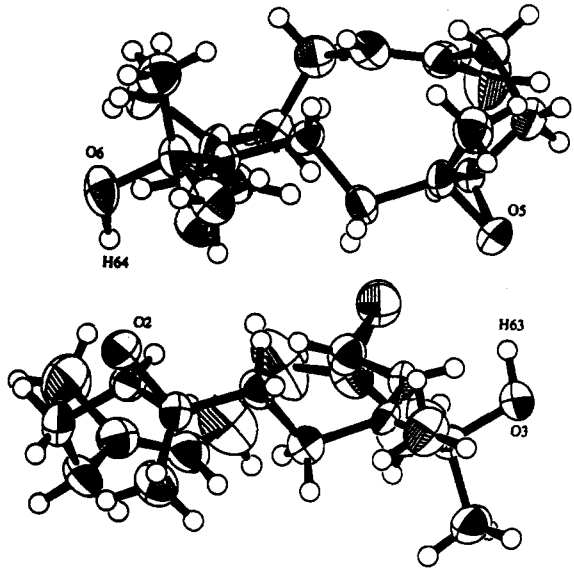

FIGURE 2. Difference-Fourier map (H-63, H-64) of sinugibberol [**1**].

[1]Hydrogen coordinates, thermal parameters, bond distances and angles, and observed and calculated structure factors have been deposited with the Cambridge Crystallographic Data Centre and can be obtained upon request from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge CB2 1EZ, UK.

TABLE 1. Atomic Parameters for Sinugibberol [**1**].

Atom	*x*	*y*	*z*	Beq
O-1	0.3945 (3)	0.2193 (3)	0.0885 (6)	5.4 (2)
O-2	0.6631 (3)	0.4144 (3)	−0.0371 (5)	4.2 (1)
O-3	0.2867 (3)	0.4464 (3)	0.0131 (5)	4.3 (1)
O-4	0.5835 (3)	0.2294 (3)	0.1588 (5)	4.5 (1)
O-5	0.3385 (2)	0.4319 (3)	0.2719 (5)	4.0 (1)
O-6	0.7203 (2)	0.4432 (2)	0.2181 (5)	4.5 (1)
C-1	0.3967 (4)	0.3921 (3)	−0.0676 (7)	2.7 (2)
C-2	0.3772 (4)	0.3343 (3)	−0.0097 (7)	3.1 (2)
C-3	0.4234 (4)	0.2714 (4)	0.0032 (8)	4.0 (2)
C-4	0.4018 (5)	0.2012 (4)	−0.0392 (9)	4.7 (3)
C-5	0.4614 (6)	0.1465 (5)	−0.056 (1)	8.6 (4)
C-6	0.4969 (6)	0.1399 (5)	−0.1711 (10)	7.6 (4)
C-7	0.5397 (5)	0.2006 (4)	−0.2182 (8)	4.4 (2)
C-8	0.6096 (5)	0.2091 (4)	−0.2158 (8)	4.2 (2)
C-9	0.6462 (4)	0.2694 (4)	−0.2782 (8)	4.7 (2)
C-10	0.6894 (4)	0.3185 (4)	−0.1943 (8)	4.8 (2)
C-11	0.6384 (4)	0.3510 (4)	−0.1014 (7)	3.6 (2)
C-12	0.6008 (4)	0.4171 (4)	−0.1210 (7)	3.5 (2)
C-13	0.5298 (4)	0.4253 (4)	−0.0568 (7)	3.8 (2)
C-14	0.4674 (4)	0.3990 (3)	−0.1360 (7)	3.4 (2)
C-15	0.3438 (4)	0.4534 (4)	−0.0754 (7)	3.3 (2)
C-16	0.3063 (4)	0.4529 (4)	−0.2014 (8)	4.8 (2)
C-17	0.3801 (4)	0.5240 (4)	−0.0538 (8)	5.0 (2)
C-18	0.3321 (6)	0.1933 (5)	−0.107 (1)	8.9 (4)
C-19	0.6602 (5)	0.1586 (4)	−0.1527 (10)	7.4 (3)
C-20	0.6120 (4)	0.4601 (5)	−0.2352 (8)	6.0 (3)
C-21	0.6036 (4)	0.4050 (3)	0.3016 (7)	2.7 (2)
C-22	0.6168 (4)	0.3422 (4)	0.2517 (7)	3.2 (2)
C-23	0.5646 (3)	0.2843 (4)	0.2470 (7)	3.2 (2)
C-24	0.5805 (4)	0.2125 (4)	0.2896 (8)	3.2 (2)
C-25	0.5182 (4)	0.1645 (4)	0.3221 (8)	3.7 (2)
C-26	0.4948 (4)	0.1658 (4)	0.4584 (8)	4.3 (2)
C-27	0.4578 (4)	0.2338 (4)	0.4972 (7)	3.7 (2)
C-28	0.3892 (4)	0.2445 (4)	0.5090 (8)	3.8 (2)
C-29	0.3591 (4)	0.3141 (4)	0.5550 (7)	4.1 (2)
C-30	0.3156 (4)	0.3556 (4)	0.4601 (8)	4.1 (2)
C-31	0.3637 (4)	0.3767 (4)	0.3522 (7)	3.1 (2)
C-32	0.4024 (4)	0.4429 (4)	0.3482 (7)	3.3 (2)
C-33	0.4728 (4)	0.4433 (4)	0.2799 (7)	3.4 (2)
C-34	0.5346 (4)	0.4219 (3)	0.3668 (7)	3.1 (2)
C-35	0.6619 (4)	0.4620 (4)	0.2993 (8)	3.4 (2)
C-36	0.6953 (4)	0.4707 (4)	0.4269 (8)	4.9 (2)
C-37	0.6319 (4)	0.5321 (4)	0.2520 (8)	4.7 (2)
C-38	0.6520 (4)	0.1970 (4)	0.3483 (9)	5.5 (3)
C-39	0.3321 (5)	0.1901 (4)	0.4853 (10)	7.0 (3)
C-40	0.3927 (4)	0.4982 (4)	0.4463 (7)	4.6 (2)

microtiter plates. Three to eight concentrations encompassing an 8- to 128-fold range were evaluated on each cell line. KB-16, A-549, and HT-29 cells were enumerated using MTT after the exposure to test samples for 3, 6, and 6 days, respectively. Fifty μl of 1 mg/ml MTT were added to each well, and plates were incubated at 37° for a further 5 h. Supernatant was aspirated with a Dynatech 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 cells in the MTT assay.

ACKNOWLEDGMENTS

We thank Prof. J.M. Pezzuto, Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, for the provision of P-388 and KB-16 cell lines. This work was supported by grants from the National Science Council of the Republic of China (NSC-84-2321-B-110-005-BH) awarded to C.-Y. Duh.

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Received 20 January 1995