Isolation and Structure of Caribenolide I, a Highly Potent Antitumor Macrolide from a Cultured Free-Swimming Caribbean Dinoflagellate, *Amphidinium* sp. S1-36-5

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A large number of highly active antitumor compounds have been found in marine organisms, especially in invertebrates. In most cases, however, the yields of these potentially important compounds as therapeutic agents are prohibitively low. On the other hand, there is the speculation that some of the animal constituents have their origins in microalgae, which may serve as culturable sources of the compounds.[1] In our screening efforts to find such progenitors, potent antineoplastic activity was discovered in the cell extract of a free-swimming dinoflagellate isolated from the water at Brewers Bay, St. Thomas, US Virgin Islands.

A single-cell isolate of the organism, *Amphidinium* sp. S1-36-5, was cultivated in enriched seawater, K-medium,[3] under fluorescent illumination, and the cells were harvested at the stationary phase. The freeze-dried cells were extracted with a mixture of toluene and methanol (3:1), and the extract was partitioned between 90% methanol and n-hexane. The 90% aqueous methanol fraction, which had shown strong cytotoxic activity (IC_{50} 0.35 μg/mL) against human colon tumor cell line HCT 116, was chromatographed successively on silica gel with methylene chloride–methanol (95:5), C18 silica gel with 80% aqueous acetonitrile, Hamilton PRP-1 with 80% aqueous acetonitrile, and Econosil CN (Alltech) with isooctane-2-propanol 8:1 to afford a pure active compound (named caribenolide I) in a 0.026% yield from the dried cells.

Caribenolide I (**1**) was obtained as a colorless amorphous or microcrystalline solid, $[\alpha]^{25}_D = +91.4 \pm 0.8°$ (c = 0.13, CH_2Cl_2). High resolution mass spectroscopy (HRFABMS) suggested a molecular formula of $C_{33}H_{52}O_{11}$, which was consistent with the carbon and hydrogen numbers counted in the NMR spectra. The compound has no UV maximum above 200 nm, indicating the absence of a conjugated system.

Table 1. Assignment of Proton and Carbon NMR Signals and Proton COSY Correlations of Caribenolide I (1) (500 MHz, in CD_2Cl_2)

pos	^{13}C	^1H	J [Hz], COSY corr
1	173.3		
2	45.1	2.66 dq	8.0 (H3), 7.1 (H30)
3	72.7	3.86 dt	8.0 (H2), 4.6 (3OH), 4.6 (H4)
		OH: 4.69 d	4.6 (H3)
4	61.5	3.06 dd	4.6 (H3), 2.0 (H5)
5	54.4	3.43 d (2.0)	2.0 (H4), H31a, H31b
6	145.5		
7	69.4	4.53 br m	H31a, 7OH, H8a, H8b
		OH: 3.69 br d	6.2 (H7)
8	45.0	a: 2.93 dd	10.1 (H7), 16.7 (H8b)
		b: 2.64 dd	2.4 (H7), 16.7 (H8a)
9	211.3		
10	47.7	3.36 dq	9.9 (H11), 6.6 (H32)
11	126.4	5.02 br d	10.1 (H10), (13a, 33)
12	136.4		
13	39.8	a: 2.38 br d	13.3 (H13b), H14, H33, 14OH
		b: 2.17 dd	13.3 (H13a), 11.4 (H14), H33
14	70.4	4.06 br dd	11.4, 2.9 (H13a, 13b)
		OH: 3.72 br s	H14
15	97.7	OH: 3.99 s	
16	65.6	3.57 br s	H17a, H17b, 16OH
		OH: 4.49[a]	H16
17	26.4	a: 2.08 ddt	13.9, 4.6, 2.8, (H16, 17b, 18a,18b)
		b: 1.69 m	H16, 17a, 18a, 18b
18	25.2	a: 1.50 m	H18b, 17a, 17b, 19
		b: 1.31 m	H18a, 17a, 17b, 19
19	65.8	4.09 m	H18a, H18b, H20a, H20b
20	40.8	a: 1.65 m	H19, H20b, H21
		b: 1.57 m	H19, H20a, H21
21	74.5	4.13 m	H20a, H20b, H22a, H22b
22	32.2	a: 1.99 m	H21, H22b, H23a, H23b
		b: 1.50 m	H21, H22a, H23a, H23b
23	27.9	a: 2.02 m	H22a, H22b, H23b, H24
		b: 1.60 m	H22a, H22b, H23a, H24
24	80.5	3.94 ddd	8.4 (H25), 7.1, 5.7 (H23a, H23b)
25	75.1	4.79 dt	8.4 (H24), 6.3 (H26)
26	30.3	1.47 m (2H)	H25, H27
27	27.3	1.27 m (2H)	H26, (H28)[b]
28	22.4	1.29 m (2H)	H29, (H27)[b]
29	13.9	0.86 br t (3H)	6.9 (H28)
30	13.9	1.24 d (3H)	7.1 (H2)
31	112.7	a: 5.23 br s	H31b, H5, H7
		b: 5.14 br s	H31a, H5
32	15.4	1.12 d (3H; 6.7)	6.7 (H10)
33	15.7	1.79 s (3H)	H11, H13a, H13b

[a] Signal with coupling to H16 appeared in 500 MHz COSY spectrum in $CDCl_3$, but not in 1D spectrum. [b] Assignments tentative due to overlapping signal.

Analysis of the NMR data (Table 1)[4] indicated the presence of one ketone carbon, one ester or lactone carbonyl carbon, one terminal methylene, and one trisubstituted double bond. The molecular formula suggested the presence of a total of eight unsaturation equivalents in the molecule. Thus the remaining four unsaturation equivalents should be due to cyclic forms.

The tracking of cross peaks in the ^1H, ^1H-COSY, and HETCOR NMR spectra led to three partial structures (**a**, **b**, and **c**) which are bounded by a quaternary carbon, a ketone carbonyl, and a lactone group (Figure 1). The connectivity of the fragments **a** and **b** was established by the HMBC spectrum, which showed cross peaks between the keto carbon at δ 211.3 and the H8 methylene protons at δ 2.93 and 2.64 in fragment **a**. Additional correlation with the allylic methine proton at δ 3.36 and

(1) Shimizu, Y. *Chem. Rev.* **1993**, *93*, 1685–1698. Shimizu, Y. In *Marine Biotechnology*; Attaway, D. H., Zaborsky, O. R., Ed.; Plenum Press: New York, 1993; Vol. 1, 391–410, and references therein.

(2) Although the ultrastructural characters of the organism best fit the description of *Amphidinium carterae* Hulburt, the shape of the cell (length, 31–43; width, 19–23 μm) does not. Further morphological and ultrastructural studies are underway and will be published elsewhere.

(3) Keller, M. D.; Guillard, R. L. In *Toxic Dinoflagellates*; Anderson, D. M., White, A. W., Baden, D., Ed.; Elsevier: New York, 1985; pp 113–116.

(4) Experiments at 500 MHz were carried out at Bristol Myers Squibb NMR laboratory. Other data were collected on a Bruker 300 MHz instrument at Department of Chemistry, University of Rhode Island.

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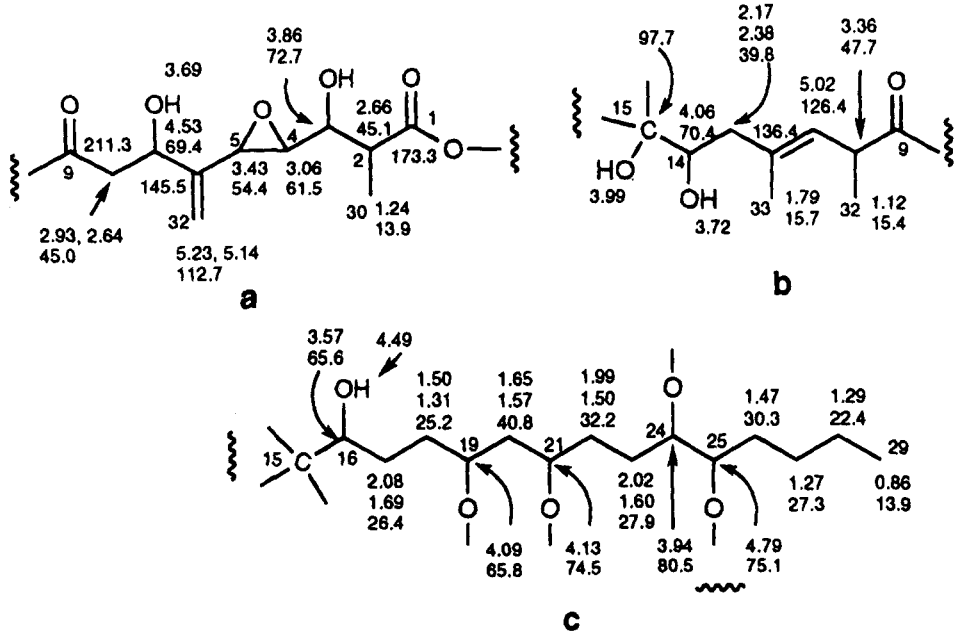

Figure 1. The partial structures of caribenolide I (1) deduced from HETCOR, $^1H-^1H$ COSY.

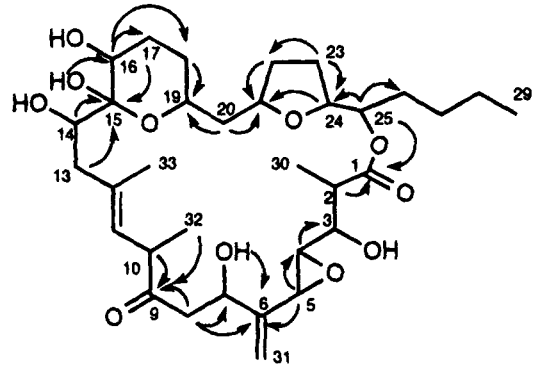

Figure 2. The structure of caribenolide I (1) and major HMBC correlations in NMR (arrowhead – carbon, tail of arrow – proton).

the homoallylic methyl protons at δ 1.12 in fragment **b** further confirmed the connection of these fragments.

Fragment **b** and fragment **c** were connected in the following manner. The connectivity of fragment **b** in the proton COSY spectrum ends at the oxygen-substituted methine proton (H14, δ 4.06). An HMBC connectivity was observed between H14 in fragment **b** and a quaternary carbon at a chemical shift of δ 97.7, which is a characteristic value for a ketal or hemiketal carbon. Long-range C–H correlation in this part of the molecule was also observed between one of the H13 methylene protons at δ 2.17 and the δ 97.7 carbon (C15). The hydroxyl proton at δ 3.99, which did not have any $^1H-^1H$ coupling, showed a correlation with C15 in the HMBC spectrum. Thus, **1** has a hemiketal group. A long-range HMBC connectivity from the hydroxyl proton to the oxygen-bearing carbon at δ 65.6 of fragment **c** confirmed the connection of **b** and **c** via the hemiketal carbon.

Four methine protons showed coupling with the corresponding hydroxyl protons, as shown by the cross peaks (*OH/CH* δ: 4.69/3.86, 3.69/4.53, 3.72/4.06, and 4.49/3.57) in the $^1H-^1H$ COSY spectrum. The carbon NMR spectrum in CD_2Cl_2 taken after partial exchange of the hydroxyl protons with deuterium by addition of a 1:1 mixture of D_2O and H_2O, showed well-resolved split signals for C3, C7, C14, C15, and C16 due to the deuterium isotope effects; Δ ppm: 0.13, 0.14, 0.11, 0.08, and 0.11, respectively. No other carbon signals were affected by the deuterium exchange. These results, coupled with the HETCOR results, conclusively established the positions of the open hydroxyl groups at these carbon positions. Consequently, the remaining six oxygen-substituted methine carbons at C4, C5, C19, C21, C24, and C25 must be involved in ring formations. The proton signals C4 and C5 were found to be shifted upfield with respect to the normal oxygen-bearing methine protons, which suggested an epoxide structure. The coupling constant of the two protons, 2.0 Hz, is typical of a *trans* substituted epoxide.[5] The carbon chemical shifts of δ 61.5 and 54.4 are also in agreement with the values reported for similar structures.[6]

The site of lactone attachment was speculated to be C25, the methine proton of which was apparently shifted downfield to δ 4.79 by esterification. The presence of a distinct HMBC cross peak observed between the lactone carbonyl carbon (δ 173.3) and H25 confirms this assignment. The HMBC spectrum also showed the connection between the carbonyl carbon and C2 methine proton.

A long-range HMBC correlation was observed between H24 at δ 3.94 and C21 at δ 74.5 implicating the presence of an ether bridge between C21 and C24. The COSY and HMBC spectra showed connectivity between the oxygen bearing carbons, C21 and C24, via two methylene groups, which completes the connectivity around the tetrahydrofuran ring. The above assignments left C15–C19 as the only site for the hemiketal ring, which would result in a six-membered hemiketal structure. In fact, connectivity could be followed from C15 through C19 in the COSY and HMBC spectra. However, no long-range coupling was observed between C15 and H19 in HMBC spectra taken under various parameters. We believe that this is due to the unfavorable dihedral angle, ϕ, of H19–C19–

(5) Jackman, L. M.; Sternhell, S. *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, 2nd ed.; Pergamon Press: Oxford, 1969; p 272.

(6) Bauer, I.; Maranda, L.; Shimizu, Y.; Peterson, R. W.; Cornell, L.; Steiner, J. R.; Clardy, J. *J. Am. Chem. Soc.* **1994**, *116*, 2657–2658.

O−C15.[7] The stereochemistry of the compound is still under investigation. However, the trisubstituted double bond was judged to have an *E*-configuration from the chemical shift value for C33 (δ 16.1).[8]

There are several reports on the isolation of cytotoxic macrolides derived from dinoflagellates.[9−12] The best known examples are amphidinolide B and its congeners, which also have a 26-membered lactone ring.[6,13,14] However, caribenolide I represents a new type of macrocyclic lactone,[15] which, with an α-methylene epoxide and a furan ring, is quite different from other known macrolides.[1,2] Caribenolide I showed strong cytotoxic activity (IC_{50} 0.001 μg/mL or 1.6 nM) against both human colon tumor cell line: HCT 116 and its drug-resistant cell line, HCT 116/VM 46. This cytotoxicity is about 100 times higher than that of amphidinolide B, the most potent dinoflagellate macrolide previously reported (IC_{50}, HCT 116, 0.122 μg/mL).[6,13,14] Most importantly, the compound showed *in vivo* activity against murine tumor P388 (T/C: 150 at a dose of 0.03 mg/kg). Testing against other tumors is in progress.

Experimental Section

All solvents used in this work were of HPLC grades. Except for the initial extraction and crude fractionation, glass-distilled solvents were used. Throughout the work, precautions were taken to prevent photo and oxidative degradation by using argon gas and avoiding exposure to light. The high resolution FAB mass spectrum was taken in 3-nitrobenzyl alcohol at the University of Ilinois/Urbana−Champaign Mass Spectrometry Laboratory.

Culturing of Organism. *Amphidinium* sp. strain S1-36-5 was isolated as a single cell from the water collected at Brewers Bay, St. Thomas, US Virgin Islands on April 1, 1990. The organism was cultured at 25−27 °C in sterilized seawater enriched with K supplement under fluorescent lighting with a 16 h/8 h light and dark cycle. The culture was gradually scaled up from a test tube to 150 L tanks. For a large tank culture, a rectangular HDPE tank was sterilized with alcohol, filled with autoclaved medium, which had been filtered first through a 0.45 μm membrane. The necessary nutrients were added after sterile filtration. The inoculation was usually done with an inoculum of about 10% of the total volume. Healthy culture took approximately 4−5 weeks to reach the maximum cell density of 35 000−45 000 mL^{-1}. The average yield from a 150 L culture tank was about 50 g of wet cells, which gave about 10−12 g dry cells upon lyophylization.

Extraction. Several different extraction procedures were tried. Typically, 20−25 g of the dried cells was mixed with 200 mL of the solvent in a 400 mL beaker. The slurry mixture was sonicated for 40 min under ice-cooling, added with 50 mL of Celite, and filtered through a Büchner funnel. The Celite cake was reextracted until the filtrate turned pale-green. The combined extracts were concentrated to dryness under reduced pressure. The crude extract (6−8 g) was then partitioned between n-hexane (200 mL) and 90% aqueous methanol (200 mL). The methanol layer was backwashed three times with 150 mL of n-hexane. The n-hexane layers were combined and reextracted with 90% methanol. The methanol fraction was backwashed with n-hexane and combined with the first extract. The combined methanol fraction gave 2−4 g of a residue after evaporation *in vacuo*.

Crude Fractionation on Silica Gel. The above crude methanol extract was dissolved in a mixture of CH_2Cl_2/MeOH 97:3 and mixed with 20−30 mL of silica gel. The solvent was removed *in vacuo*, and the powdery mixture was placed on a silica gel column (3.5 × 35 cm, packed in CH_2Cl_2/MeOH 97:3). The column was successively eluted with CH_2Cl_2/MeOH 97:3, 90:10 and 50:50. Caribenolide I was eluted with CH_2Cl_2/MeOH (97:3) together with the amphidinolide B group compounds just after the major pigment, peridinin.

Reverse-Phase Chromatography on C18 Silica Gel. The above amphidinolide B fraction (940 mg) was loaded on a C18 silica gel column (2.2 × 33 cm, Bakerbond C18) and eluted with 80% aqueous CH_3CN. Caribenolide I (first code-named Q10) (**1**) was eluted with an allene ketone degradation product of peridinin in the first fraction followed by amphidinolide B1, B2, and B3. The R_f of **1** on TLC (HPKF, $CHCl_3$/MeOH 95:5) was 0.5, while that of amphidinolide B1 was 0.43. Evaporation of the combined fractions gave a residue (74.7 mg), which contained **1** as the major component.

HPLC Purification. The above fraction was subjected to further purification on a PRP-1 column (Hamilton, 21.5 × 25 cm, 80% CH_3CN; flow rate 5 mL/min). Caribenolide I was eluted from 14.0 min. to 17.5 min. The final purification of **1** was accomplished by HPLC on an Econosil CN column (Alltech, 1.0 × 25.0 cm). The column was eluted first with isooctane/2-propanol 8:1 at a flow rate, 3.5 mL/min for 16 min and then at a flow rate 6.5 mL/min. Caribenolide I (**1**) (7.0 mg) which was eluted after 26 min was rechromatographed on the same system to a pure specimen of **1** (yield, 6.0 mg).

Caribenolide I (1). Colorless amorphous or microcrystalline powder from isooctane/2-propanol, an oil from dichloromethane, $[\alpha]^{25}_D = +91 \pm 0.8°$ (c = 0.13, CH_2Cl_2), HRFABMS Calcd for $C_{33}H_{53}O_{11}$ $(M+H)^+$ 625.358788; found 625.357483 (Δ = 2.1 ppm), UV: end absorption, no maximum above 200 nm in MeOH. NMR: (For 500 MHz data in $CDCl_3$ and assignment, see Table 1) HMBC (H/C, 500 MHz in $CDCl_3$): 2/1, 3, 4, 30; 3OH/4; 4/2, 3; 5/4, 6, 7, 31; 7OH/6; 8a/6, 7, 9; 8b/6, 7, 9, (32); 10/9, 11, 12, 32;11/10, 13, 33; 13a/11, 12; 13b/11, 12, 14, 15, 33; 14/13; 14OH/13; 15OH/15, 16; 16/17,18; 17b/16, 15; 18a/19, (20); 18b/19; 19/(20); 20a/19, (18, 22); 22b/21, (24); 23b/21,22,24; 24/21; 25/1, 24, (23); 26/27, (28); 27/28; 29/27, 28; 30/1, 2, 3; 31a/5, 6, 7, (10); 31b/5, 6, 7, (10); 32/9, 10, 11.

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Supplementary Material Available: Copies of ^1H, ^{13}C, and ^{13}C-DEPT NMR spectra (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(7) Wehrli, F. W. and Wirthlin, T. *Interpretation of Carbon-13 NMR Spectra*; Heyden & Son: London, 1978; p 56. The broadness of the H19 signal suggests that the proton is axial to the six-membered ketal ring. From molecular modeling the dihedral angle is estimated at ϕ 61.5°, which is within the zero coupling range of similar structures.

(8) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH Publishers: New York, 1990; pp 193−194.

(9) Yasumoto, T.; Murata, M.; Oshima, Y.; Matsumoto, G. K.; Clardy, J. *Tetrahedron* **1985**, *41*, 1019−1025.

(10) Torigoe, K.; Murata, M.; Yasumoto, T.; Iwashita, T. *J. Am. Chem. Soc.* **1988**, *110*, 7876−7877.

(11) Murakami, M.; Makabe, K.; Yamaguchi, K.; Konosu, S.; Wälchli, M. R. *Tetrahedron Lett.* **1988**, *29*, 1149.

(12) Kobayashi, J.; Ishibashi, M. *Chem. Rev.* **1993**, *93*, 1753−1770, and references therein.

(13) Ishibashi, M.; Ohizumi, Y.; Hamashima, M.; Nakamura, H.; Hirata,Y.; Sasaki, T.; Kobayashi, J.; *J. Chem. Soc., Chem. Commun.* **1987**, 1127.

(14) Kobayashi, J.; Ishibashi, M.; Nakamura, H.; Ohizumi, Y.; Hirata, Y.; Sasaki, T.; Ohta, T.; Nozoe, S. *J. Nat. Prod.*, **1989**, *52*, 1036−1041.

(15) In the recently held US/Japan Joint Seminar on Bioorganic Marine Chemistry, we reported the isolation and structure of **1**, but a compound with the same skeleton but no furan ring was also reported by Ishibashi, M.; Kobayashi, M. *Abstracts of Papers*, Japan-U. S. Seminar on Bioorganic Marine Chemistry, Numazu, Japan; Organizing Committee of the 3rd Japan−U. S. Seminar on Bioorganic Marine Chemistry, 1994; p 14. After this manuscript was submitted, the compound was reported in *J. Chem. Soc., Chem. Commun.* **1994**, 1455−1456.