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CYTOTOXIC MACROLIDES FROM A CULTURED MARINE DINOFLAGELLATE OF THE GENUS *AMPHIDINIUM*

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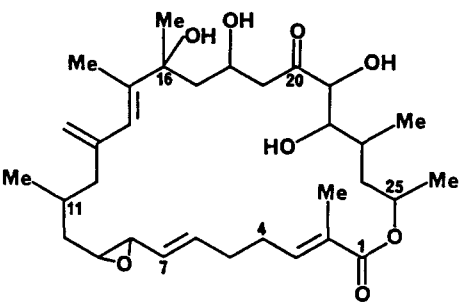
ABSTRACT.—A fourth cytotoxic macrolide, amphidinolide D [**1**], together with known amphidinolide B [**2**], has been isolated from a different batch of the cultured dinoflagellate *Amphidinium* sp., which was symbiotically associated with an Okinawan flatworm *Amphiscolops* sp. Two-dimensional nmr experiments including ^{1}H-detected heteronuclear multiple-bond correlation (HMBC) resulted in the structure assignment of **1** and structure revision of the diene moiety (C-13–C-15) of amphidinolide B.

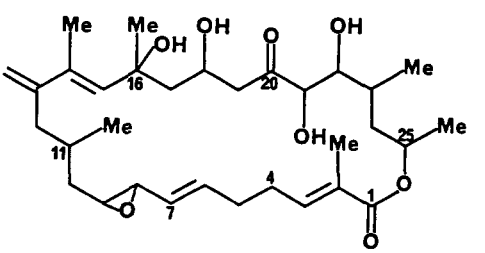
Marine microorganisms such as blue-green algae (1), dinoflagellates (2), and bacteria (3,4) have proven to be a valuable new source of bioactive compounds, including neurotoxins. During our studies on bioactive substances from Okinawan marine organisms (5–8), we investigated a cultured symbiotic dinoflagellate *Amphidinium* sp. and obtained three antineoplastic macrolides, amphidinolides A (9), B (10), and C (11). This paper deals with the isolation and structure elucidation of amphidinolide D [**1**], another cytotoxic component of this dinoflagellate, and the structure revision of amphidinolide B from structure **3** to structure **2**.

RESULTS AND DISCUSSION

A different batch of the cultured dinoflagellate *Amphidinium* sp. was investigated to obtain more pharmacologically useful substances from the symbiotic alga that had been isolated from an Okinawan flatworm *Amphiscolops* sp. (11). Extraction of the harvested cells with MeOH-toluene (3:1) and subjecting the extract to solvent partitioning, Si gel



1
2 } epimers at C-21



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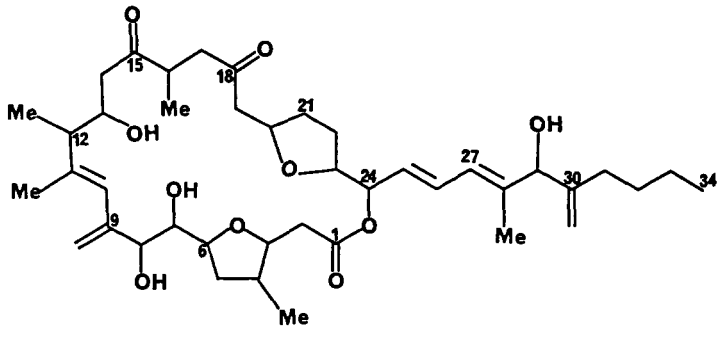

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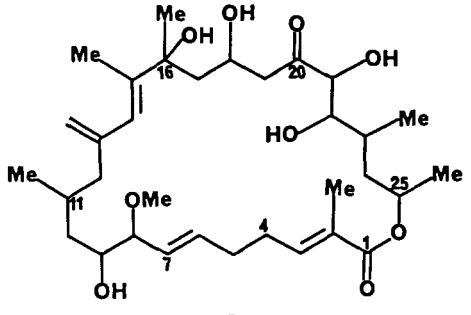

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chromatography, and reversed- and normal phase hplc afforded amphidinolide D [**1**] together with amphidinolide B [**2**] previously isolated (10) and compound **5**, an artifact of isolation, in 0.0004, 0.0007, and 0.003% yield (wet wt), respectively.

Amphidinolide D [**1**], a colorless amorphous solid, $[\alpha]^{30}D$ −30° (c = 0.5, $CHCl_3$), was shown to possess the same molecular formula, $C_{32}H_{50}O_8$, as amphidinolide B [**2**] by fabms (*m/z* 563 $[M+H]^+$). High resolution analysis of the $[M-H_2O+H]^+$ ion revealed the composition $C_{32}H_{49}O_7$ (*m/z* 545.3461, Δ −1.8 mmu). The uv and ir absorptions are analogous to those of **2**. The ^1H- and ^{13}C-nmr data of amphidinolide D [**1**] and amphidinolide B [**2**] are presented in Tables 1 and 2. All protons were assigned on the basis of a COSY spectrum, and the ^{13}C signals were assigned by comparison with those of **2** along with the ^1H-detected heteronuclear multiple-bond correlation (HMBC) (12) spectrum of **1**. The proton and long-range C-H connectivities obtained by the COSY and HMBC spectra indicated the same location of functional groups between **1** and **2**. The ^1H-^1H coupling constants and the ^{13}C chemical shifts of the olefinic methyl groups showed that the geometries of the double bonds and the epoxide also were identical to each other. The ^1H chemical shifts for OH-18, H-19, OH-22, and H-24 revealed relatively large differences between **1** and **2** (Table 1). The NOESY spectrum of **1** gave cross peaks for H-19 (δ 3.32)/H-21 (δ 4.21) and H-21/23-Me (δ 0.94), while the NOESY spectrum of **2** showed no such cross peaks. To account for these observations, amphidinolide D [**1**] is considered to be a stereoisomer at C-21 position of amphidinolide B [**2**], a 26-membered macrocyclic lactone.

In a previous paper (10), the structure of amphidinolide B was assigned as **3** on the basis of COSY and nOe results. But the diene moiety, C-13–C-15, became questionable, since amphidinolide C [**4**] obtained from the same dinoflagellate possessed a similar diene unit (C-9–C-11) whose structure was established by its HMBC spectrum and the formation of a cycloaddition product with oxygen (11). In contrast to **4**, am-

TABLE 1. ^{1}H-nmr Spectra of Amphidinolides D [**1**] and B [**2**].

Proton	Compound		Proton	Compound	
	1[a]	**2**		**1**[a]	**2**
1			15		
2			15-Me	1.84 brs	1.83 brs
2-Me	1.82 brs	1.82 brs	16		
3	6.73 ddd	6.77 td	16-Me	1.41 s	1.42 s
4	2.45 m	2.42 m	16-OH	1.97 s	2.21 s
4′	2.15 m	2.20 m	17	1.98 dd	1.95 m
5	2.40 m	2.40 m	17′	1.80 dd	1.78 dd
5′	2.08 m	2.15 m	18	4.19 m	4.19 m
6	5.88 ddd	5.92 ddd	18-OH	3.04 d	3.91 d
7	5.22 dd	5.16 dd	19	3.32 dd	2.87 dd
8	3.10 dd	3.14 dd	19′	2.64 dd	2.79 dd
9	2.95 dt	2.93 dt	20		
10	1.59 m	1.49 ddd	21	4.21 dd	4.33 dd
10′	1.12 t	1.27 m	21-OH	3.74 d	3.87 d
11	1.63 m	1.65 m	22	3.66 td	3.71 td
11-Me	0.92 d	0.89 d	22-OH	3.51 d	3.16 d
12	2.29 dd	2.19 m	23	1.90 m	1.85 m
12′	1.85 m	1.95 m	23-Me	0.94 d	1.01 d
13			24	2.26 ddd	1.95 m
13-H_2C=	5.02 s	5.03 s	24′	1.21 ddd	1.28 m
	4.81 s	4.83 s	25	5.09 qd	5.06 dd
14	5.94 s	5.97 s	25-Me	1.28 d	1.28 d

[a]$J_{H,H}$ in Hz for **1**: $J_{2\text{-Me},3}=1.4$, $J_{3,4}=9.3$, $J_{3,4'}=4.9$, $J_{5,6}=9.8$, $J_{5',6}=4.9$, $J_{6,7}=15.4$, $J_{7,8}=8.8$, $J_{8,9}=2.3$, $J_{9,10}=7.0$, $J_{9,10'}=2.3$, $J_{11,11\text{-Me}}=6.2$, $J_{11,12}=4.0$, $J_{12,12'}=13.4$, $J_{17,17'}=14.5$, $J_{17,18}=5.1$, $J_{17',18}=7.6$, $J_{18,18\text{-OH}}=3.9$, $J_{18,19}=2.6$, $J_{18,19'}=8.7$, $J_{19,19'}=17.7$, $J_{21,21\text{-OH}}=3.9$, $J_{21,22}=2.0$, $J_{22,22\text{-OH}}=10.8$, $J_{22,23}=10.8$, $J_{23,23\text{-Me}}=6.6$, $J_{23,24}=11.3$, $J_{23,24'}=2.7$, $J_{24,24'}=14.1$, $J_{24,25}=2.8$, $J_{24',25}=11.2$, $J_{25,26}=6.1$.

phidinolide B affords no oxygen adduct. The structure of amphidinolide B was thus reinvestigated by the HMBC nmr technique. As a result, the long-range connectivity from the methyl protons on C-16 (16-Me, δ 1.42) to the carbon at δ 143.1 (s) was observed but that from 16-Me to the carbon at δ 124.3 (d) was not detectable. Both of the exomethylene protons on C-13 (δ 5.03 and 4.83) were coupled to the latter carbon but not to the former carbon. Thus the former [δ 143.1 (s)] is assigned to C-15 and the latter [δ 124.3 (d)] to C-14. In addition, the olefinic proton at δ 5.97 shows a cross peak with the exomethylene carbon [δ 114.8 (t)] on C-13, implying that the olefinic proton is present within three bonds from the exomethylene carbon; namely, it was labeled H-14, and the methyl group is, therefore, on C-15. The diene has to adopt the *S*-cis conformation to account for the NOESY data [cross peaks: H-12 (δ 2.19)/H-14 (δ 5.97); one of H_2C= on C-13 (δ 5.03)/15-Me (δ 1.83)] (10). Accordingly the structure of amphidinolide B is revised to **2**. The stereostructures of amphidinolides D [**1**] and B [**2**] remain to be resolved.

The molecular formula of compound **5**, a colorless amorphous solid, $[\alpha]^{25}D$ −28° (*c*= 1, $CHCl_3$), was determined as $C_{33}H_{54}O_9$ by fabms (*m/z* 617 $[M+Na]^+$) and hreims (*m/z* 576.3660 $[M-H_2O]^+$, Δ −0.3 mmu: $C_{33}H_{52}O_8$). The spectral data suggested that compound **5** is an MeOH adduct of amphidinolide B [**2**]. The signals for protons on the epoxide-bearing carbon were lost, while a methoxy proton signal appeared at δ 3.20 (3H, s). Decoupling experiments revealed that the olefinic proton at δ 5.38 (H-7) is coupled to the double doublet at δ 3.24 (H-8) by 8.8 Hz, which in turn is

TABLE 2. ^{13}C-nmr Spectra of Amphidinolides D [**1**] and B [**2**].

Carbon	Compound		Carbon	Compound	
	1[a]	**2**		**1**[a]	**2**
1	167.7 s	167.7 s	14	124.5 d	124.3 d
2	128.1 s	128.3 s	15	142.7 s	143.1 s
2-Me	12.6 q	12.4 q	15-Me	15.3 q	15.6 q
3	139.7 d	139.9 d	16	75.9 s	75.9 s
4	26.8 t	26.8 t	16-Me	29.3 q	28.3 q
5	31.1 t	30.8 t	17	44.8 t	45.2 t
6	135.9 d	135.4 d	18	65.6 d	66.5 d
7	129.5 d	128.5 d	19	45.5 t	45.9 t
8	59.9 d	60.0 d	20	212.5 s	212.4 s
9	59.8 d	59.3 d	21	78.2 d	77.7 d
10	40.0 t	39.4 t	22	76.4 d	75.5 d
11	29.7 d	29.1 d	23	32.8 d	33.2 d
11-Me	19.5 q	18.2 q	23-Me	15.3 q	15.0 q
12	46.6 t	46.7 t	24	39.5 t	39.3 t
13	144.6 s	144.4 s	25	68.1 d	68.3 d
13-CH_2=	115.1 t	114.8 t	25-Me	21.1 q	21.0 q

[a]^{1}H-^{13}C long-range connectivities obtained by the HMBC spectrum of **1** (proton/carbon): 2-Me/C-1, 2-Me/C-2, 2-Me/C-3, H-7/C-5, H-9/C-7, H′-10/C-9, 11-Me/C-11, 13-H_2C=/C-12, 13-H_2C=/C-14, H-14/13-CH_2=, H-14/15-Me, H-14/C-16, 15-Me/C-14, 15-Me/C-15, 15-Me/C-16, 16-Me/C-16, 16-Me/C-17, H-17/C-16, H-17/C-18, H-17/C-19, H′-19/C-18, H-19/C-20, OH-21/C-20, 23-Me/C-22, 23-Me/C-23, 23-Me/C-24, 25-Me/C-24, and 25-Me/C-25 (very clear cross peaks only).

coupled to the double-double doublet at δ 3.65 (H-9) by 5.5 Hz. During hplc separation (ODS, 87% MeOH), the ratio of compound **5** to **2** increased, implying that **5** was an artificial product generated from **2**. Because the cationic center would rather be formed at C-8 (the allylic position) than at C-9, the methoxyl group was assigned to C-8 and the hydroxyl group to C-9.

Amphidinolide D [**1**] and compound **5** exhibited potent cytotoxicity against L1210 murine leukemia cells in vitro with IC_{50} values of 19 and 81 ng/ml, respectively. It should be noted that the cytotoxicity of amphidinolide B [**2**], a stereoisomer of **1** at C-21, was more than 100 times as great as that of **1** or **5**, suggesting that the stereochemistry at C-21 and the presence of the epoxide at C-8 and C-9 are important for the activities of these compounds.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Optical rotations were measured on a JASCO DIP-360 polarimeter. Uv and ir spectra were taken on a JASCO UVIDEC-660 and a Hitachi 260-50 spectrometer, respectively. Mass spectra were obtained on a JEOL HX-100 spectrometer. ^{1}H- and ^{13}C-nmr spectra were recorded on Bruker AM-500 and JEOL GX-500 spectrometers in $CDCl_3$. The 7.27 ppm resonance of residual $CHCl_3$ and 76.9 ppm of $CDCl_3$ were used as internal references, respectively. HMBC spectra were obtained from 256 × 2048 data matrix sizes, with 256 and 320 scans per t_1 value, and scan delays were 1.4 and 1.0 sec. Aquisition times were 18 and 241 msec in the t_1 and t_2 dimensions, respectively, and two durations were 50 and 90 msec.

MICROALGAL COLLECTION AND TAXONOMY.—The dinoflagellate identified as *Amphidinium* sp. I was symbiotically associated inside of the apocyte or vacuole of a marine flatworm *Amphiscolops* sp. (0.3 mm wide and 1.2 mm long, brown color), which was collected at Okinawa, Japan. The dinoflagellate was isolated out of the flatworm, washed well with sterilized sea water. *Amphidinium* sp. I (Mitsubishi Kasei Institute of Life Sciences Collection #850822-Y-5) is an undescribed Okinawan dinoflagellate of the family Gymnodinaceae, order Gymnodinales. The species is similar in overall morphology to the type species *Amphidinium klebsii* Kof. et Swezy (13,14). The body provides a small triangle epicone, a large ovoidal

hypocone, a clear girdle, and two flagellae. Widths range from 16.4 to 22.2 μm [average, 20.51 ± 1.30 μm (SD), $n = 157$]. Lengths range from 20.9 to 29.1 μm [average 25.26 ± 0.56 μm (SD), $n = 157$]. The nucleus, located near the posterior end, contains a peripheral nucleolus and chromosomes. The single pyrenoid located just anterior to the nucleus connects radially to several branches of the chromophore, which is separated into lobes and occupies the peripheral part of the algal cytoplasm. Thyllakoids of the chromophore penetrate into the pyrenoid. The cytoplasm contains numerous small vacuoles and occasionally an assimilation body with an electron-dense multilayered structure (5 μm in diameter) besides the usual organelles and inclusions.

ISOLATION OF AMPHIDINOLIDE D [**1**].—The procedure for the algal cultivation has been previously described (11). The harvested cells (452 g) from 1058 liters of culture were extracted with MeOH-toluene (3:1) (500 ml × 3). After addition of 1 M NaCl (0.75 liter), the mixture was extracted with toluene (250 ml × 4). The toluene-soluble fraction was evaporated under reduced pressure to give a crude residue (15 g), which was subjected to Si gel cc (Wako gel C-300, Wako Chemical, 2.8 × 53 cm) eluted with MeOH-$CHCl_3$ (5:95). The fraction eluting from 440 ml to 500 ml was further separated by the second Si gel cc (Wako gel C-300, 1.6 × 41 cm) eluted with MeOH-$CHCl_3$ (3:97). The fraction eluting from 120 ml to 180 ml was then purified by hplc (Develosil ODS-5, Nomura Chemical, 10 × 250 mm; flow rate 2.5 ml/min; uv detection at 254 nm; eluent 87% MeOH) to afford compound **5** (15 mg, Rt 11.5 min) and a mixture of amphidinolides B and D (ca. 12 mg, Rt 12.0 min). The mixture of amphidinolides B and D was separated by hplc [YMC-Pack A-014 SIL, Yamamura Chemical, 7 × 300 mm; flow rate 1.2 ml/min; uv detection at 240 nm; eluent $CHCl_3$-MeOH (2:98)] to give amphidinolide B [**2**] (3.1 mg, Rt 14.9 min) and amphidinolide D [**1**] (1.9 mg, Rt 13.8 min), colorless amorphous solid: $[\alpha]^{30}D$ −32° ($c = 0.5$, $CHCl_3$); uv (MeOH) 221 nm (ε 17000); ir (film) 3450, 2930, 1700, 1380, 1280, 1120 cm^{-1}; ^1H nmr see Table 1; ^{13}C nmr see Table 2; fabms (glycerol) *m/z* $[M + H]^+$ 563, $[M - H_2O + H]^+$ 545, 527, 511, 493. Found *m/z* 545.3461, calcd for $C_{32}H_{49}O_7$ $[M - H_2O + H]^+$ 545.3479.

COMPOUND **5**.—Colorless amorphous solid; $[\alpha]^{25}D$ −28° ($c = 1$, $CHCl_3$); uv (MeOH) 221 nm (ε 13000); ir (film) 3400, 2940, 1700, 1270, 1100 cm^{-1}; ^1H nmr ($CDCl_3$) δ 0.79 (3H, d, $J = 6.5$ Hz, 11-Me), 1.02 (3H, d, $J = 6.7$ Hz, 23-Me), 1.27 (3H, d, $J = 6.1$ Hz, 25-Me), 1.40 (3H, s, 16-Me), 1.82 (6H, s, 2-Me and 15-Me), 2.74 (1H, dd, $J = 15.5$ and 8.1 Hz, H-19), 2.93 (1H, dd, $J = 15.5$ and 3.3 Hz, H′-19), 3.20 (3H, s, MeO), 3.24 (1H, dd, $J = 8.8$ and 5.5 Hz, H-8), 3.65 (1H, ddd, $J = 10.1$, 5.5, and 2.5 Hz, H-9), 3.73 (1H, d, $J = 8.2$ Hz, H-22), 4.20 (1H, br s, H-18), 4.31 (1H, s, H-21), 4.80 and 5.02 (each 1H, s, 13-H_2C=), 5.08 (1H, ddd, $J = 10.3$, 6.3, and 2.7 Hz, H-25), 5.38 (1H, dd, $J = 15.5$ and 8.8 Hz, H-7), 5.67 (1H, dt, $J = 15.5$ and 6.2 Hz, H-6), 5.98 (1H, s, H-14), 6.74 (1H, td, $J = 7.4$ and 1.2 Hz, H-3); ^{13}C nmr ($CDCl_3$) δ 12.5 q (2-Me), 14.9 q (23-Me), 15.5 q (15-Me), 18.4 q (11-Me), 21.0 q (25-Me), 27.3 t (C-4), 27.8 d (C-11), 28.4 q (16-Me), 30.9 t (C-5), 33.1 d (C-23), 40.0 t (C-24), 40.1 t (C-10), 45.5 t (C-17), 46.2 t (C-19), 46.9 t (C-12), 55.8 q (MeO), 66.3 d (C-18), 68.5 d (C-25), 70.7 d (C-9), 75.1 d (C-22), 75.8 s (C-16), 77.7 d (C-21), 86.0 d (C-8), 114.7 t (13-H_2C=), 124.6 d (C-14), 127.4 d (C-7), 128.5 s (C-2), 136.1 d (C-6), 141.0 d (C-3), 142.8 s (C-15), 144.7 s (C-13), 167.5 s (C-1), 212.3 s (C-20); fabms (glycerol) *m/z* $[M + Na]^+$ 617, $[M + K]^+$ 633, $[M - H_2O + H]^+$ 577; eims *m/z* $[M - H_2O]^+$ 576 (1.5), 558 (5), 514 (100), 476 (45), 444 (40), 426 (21). Found *m/z* 576.3660, calcd for $C_{33}H_{52}O_8$ $[M - H_2O]^+$ 576.3663.

BIOASSAY METHOD.—The assay method of determining cytotoxicity in vitro has been previously reported (15).

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