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Amphidinolide M, a Novel 29-Membered Macrolide from the Cultured Marine Dinoflagellate *Amphidinium* sp.

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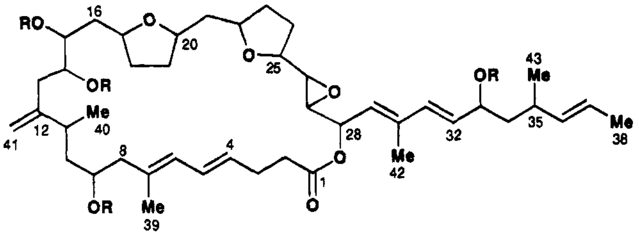
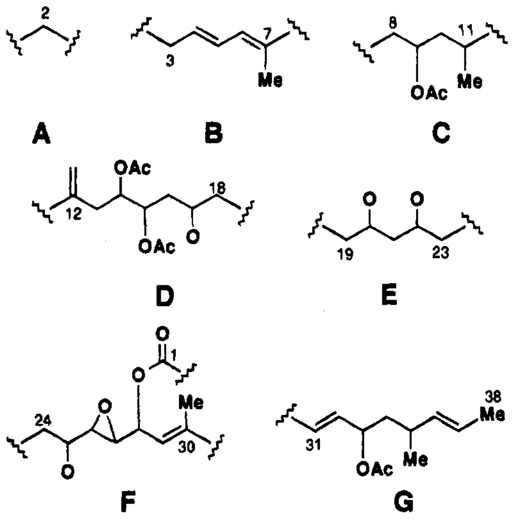
Amphidinolides are a series of cytotoxic macrolides isolated from the laboratory-cultured dinoflagellates *Amphidinium* sp. which live inside of Okinawan marine flatworms *Amphiscolops* sp.[1] These macrolides are full of chemically unique structural features: (i) amphidinolides possess a variety of new carbon skeletons different from each other, which are produced by one genus of microalga, and (ii) many of them contain odd-numbered macrocyclic lactone rings, which are seldom found from natural products of terrestrial origins. The toluene-soluble fraction of extracts of the dinoflagellate *Amphidinium* sp. (Y-5) proved to exhibit extremely potent cytotoxicity, and it was strongly expected that other cytotoxic components of interest are still contained in the extract. We therefore have continued mass cultivation of the dinoflagellate and further investigation on the extract. Here we describe isolation of a novel 29-membered macrolide, amphidinolide M (1),[2] and elucidation of its structure on the basis of spectroscopic data. Amphidinolide M (1) exhibited cytotoxic activity against murine lymphoma L1210 and human epidermoid carcinoma KB cells in vitro with IC_{50} values of 1.1 and 0.44 µg/mL, respectively.



1 R = H
2 R = Ac

The harvested algal cells (878 g, wet weight, from 3240 L of culture) were extracted with MeOH/toluene (3:1), and the extracts were partitioned between toluene and water. The toluene-soluble fraction was subjected to a silica gel column ($CHCl_3$/MeOH, 95:5), and the cytotoxic fractions were subsequently separated by gel filtration on Sephadex LH-20 ($CHCl_3$/MeOH, 1:1). Further purification by reversed-phase HPLC (ODS, 60% CH_3CN) afforded amphidinolide M (1, 0.0004% yield, wet weight).

Amphidinolide M (1) had a molecular formula of $C_{43}H_{66}O_9$, established by HRFABMS [matrix: diethanolamine (DEA), m/z 832.5548 $(M + DEA + H)^+$ for $C_{47}H_{78}O_{11}N$, Δ −2.7 mmu]. The UV spectrum showed an absorption maximum at 238 nm with a relatively large molar absorptivity (ε 29 000) to be suggestive of the presence of two conjugated diene chromophores, which was supported by NMR data. Interpretation of the ^1H and ^{13}C NMR spectral data of 1 (Table 1) indicated the presence of one ester carbonyl, one *exo*-methylene, two trisubstituted and three disubstituted olefins, 10 oxygenated methines, two unoxygenated methines, 12 aliphatic methylenes, and five methyl groups, which accounts for seven out of 11 unsaturations. Compound 1 was therefore inferred to contain four rings, viz., one lactone, one epoxide, and two ether rings. Although the ^1H NMR signals due to oxymethine protons of 1 heavily overlapped in C_6D_6, the ^1H NMR of the tetraacetate (2) in C_6D_6 fortunately afforded well-separated signals. Analyzing the $^1H-^1H$ COSY spectrum of 2 straightforwardly led to seven partial structural units **A**−**G**. The chemical shifts of H_2-2 (**A**), H_2-8 (**C**), and H-11 (**C**) (δ_H 2.2−2.6 for **2**) implied that C-2, C-8, and C-11 were adjacent to sp^2 carbons, while C-18 (**D**), C-19 (**E**), C-23 (**E**), and C-24 (**F**) were suggested to be connected to sp^3 carbons by their ^1H chemical shifts (δ_H 1.3−2.0 for **2**).[3] The low-field resonance of H-28 for 1 (δ_H 5.77) indicated that C-28 was participating in an ester linkage with the lactone carbonyl (C-1). The HOHAHA[4] spectrum of 2 (mixing time, 61.3 msec) afforded cross-peaks (H-2a/H-3a, H_2-2/H-4, H_2-2/H-5, and H_2-2/H-6; H-17/H_2-19, H_2-18/H-20, and H-17/H-20; H-22/H_2-24, H_2-23/H-25, and H-22/H-25), which provided evidences for connection of units **A/B**, **D/E**, and **E/F**. It was therefore deduced that two ether linkages were present between C-17 and C-20 as well as C-22 and C-25, thereby constructing two tetrahydrofuran (THF) rings. The $^1H-^1H$ coupling constants between the THF-ring angular position (H-25) and two adjacent THF-ring methylene protons (H_2-24) were the same (**2**: $J_{24a,25} = J_{24b,25} = 7.2$ Hz); this phenomenon was also observed in



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(1) (a) Kobayashi, J.; Ishibashi, M. *Chem. Rev.* **1993**, *93*, 1753−1769. (b) Ishibashi, M.; Sato, M.; Kobayashi, J. *J. Org. Chem.* **1993**, *58*, 6928−6929 and references cited therein.

(2) Amphidinolide M (1) was first isolated in 1986 from the dinoflagellate of this species (strain number Y-5) in *ca.* 0.0005% yield (wet weight). Unfortunately, the sample of 1 decomposed extensively during storage as a $CDCl_3$ solution, and the structural studies were interrupted.[1a] The quantity of these macrolides contained in the extracts of the cultured cells varied a little during the course of time and amphidinolide M (1) was not isolated for several years. We reisolated amphidinolide M (1) recently from the same strain of this cultured alga by careful HPLC examination.

(3) For the assignment of nonequivalent methylene protons, lower field methylene protons were suffixed by "a" (*e.g.*, H-2a), while higher field ones were suffixed by "b" (*e.g.*, H-2b).

(4) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355−360.

0022-3263/94/1959-4698$04.50/0

Table 1. ^{1}H and ^{13}C NMR Data of Amphidinolide M (1) and ^{1}H NMR Data of Its Tetraacetate 2 in C_6D_6

	1			2	
position	δ_H	J (Hz)	δ_C	δ_H	J (Hz)
1			171.8		
2 (a)	2.24, m		34.4	2.32, m	
(b)	2.24, m			2.19, m	
3 (a)	2.35, m		28.6	2.43, m	
(b)	2.25, m			2.25, m	
4	5.58, dt	15.2, 7.0	130.5	5.71, m	
5	6.45, dd	15.2, 10.7	128.5	6.47, dd	15.1, 10.7
6	6.03, d	10.7	128.2	6.00, d	10.7
7			134.6		
8 (a)	2.20, m		48.7	2.56, m	
(b)	1.90, m			2.22, m	
9	3.93, m		67.8	5.26, m	
10 (a)	1.65, m		43.6	1.78, m	
(b)	1.53, m			1.64, m	
11	2.69, m		36.1	2.52, m	
12			152.1		
13 (a)	2.57, m		39.2	2.41, m	
(b)	2.31, m			2.41, m	
14	3.91, m		72.8	5.80, ddd	8.2, 5.8, 2.3
15	3.91, m		72.5	5.61, ddd	8.9, 3.8, 2.3
16 (a)	1.85, m		37.8	1.82, m	
(b)	1.70, m			1.82, m	
17	4.22, m		76.5	4.06, m	
18 (a)	1.65, m		30.2	1.73, m	
(b)	1.32, m			1.38, m	
19 (a)	1.70, m		32.3	1.80, m	
(b)	1.18, m			1.29, m	
20	4.11, m		76.5	4.13, m	
21 (a)	1.45, m		42.3	1.61, m	
(b)	1.45, m			1.56, m	
22	4.12, m		77.7	4.27, m	
23 (a)	1.82, m		32.6	1.99, m	
(b)	1.25, m			1.30, m	
24 (a)	1.70, m		28.3	1.72, m	
(b)	1.63, m			1.65, m	
25	3.93, m		76.5	3.79, td	7.2, 4.5
26	2.90, dd	3.9, 2.0	58.4	2.83, dd	4.5, 2.0
27	3.32, dd	5.9, 2.0	56.1	3.22, dd	6.5, 2.0
28	5.77, dd	9.4, 5.9	71.4	5.65, dd	9.4, 6.5
29	5.64, d	9.4	125.3	5.54, d	9.4
30			138.7		
31	6.36, d	15.8	132.9	6.40, d	15.2
32	5.73, dd	15.8, 5.8	134.5	5.70, dd	15.2, 7.1
33	4.21, m		70.5	5.66, m	
34 (a)	1.65, m		45.1	1.80, m	
(b)	1.38, m			1.50, m	
35	2.39, m		33.9	2.24, m	
36	5.39, dd	15.3, 7.7	137.9	5.34, dd	15.3, 8.9
37	5.40, m		123.3	5.40, dq	15.3, 6.2
38 (3H)	1.62, d	5.0	18.0	1.61, d	6.2
39 (3H)	1.81, s		17.5	1.92, s	
40 (3H)	1.14, d	6.9	22.2	1.17, d	7.0
41 (a)	5.03, s		110.9	5.16, s	
(b)	4.97, s			5.06, s	
42 (3H)	1.86, s		13.5	1.84, d	1.0
43 (3H)	1.04, d	6.7	20.8	1.01, d	6.7
CH_3CO (3H) × 4				1.88 s, 1.86 s, 1.85 s, 1.76 s	

case of amphidinolides C, E, F, and K,[5,6] which may support the presence of the THF ring. To obtain information for connecting the remaining partial structural units, NOESY and HMBC[7] spectra of 1 were recorded and afforded the following cross-peaks showing the connection of units **B/C**, **C/D**, and **F/G** (NOESY: H-6/H-8a, H_3-39/H-9, H-10a/H-41b, H-11/H-41b, H_3-40/H-41b, H-29/H-31, and H_3-42/H-32; HMBC:[8] H_3-39/C-8, H_3-40/C-12, H-41a/C-11, H-41b/C-11, H-29/C-31, H_3-42/C-31,

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[5] For amphidinolide C,[6a] $J_{22a,23} = J_{22b,23} = 7.9$ Hz; for amphidinolide E,[6b] $J_{15a,16} = J_{15b,16} = 7.1$ Hz; for amphidinolide F,[6c] $J_{22a,23} = J_{22b,23} = 7.8$ Hz; for amphidinolide K,[1b] $J_{12,13a} = J_{12,13b} = 6.2$ Hz. There were possibilities for other combinations of ether linkages (C-17/C-22 and C-20/C-25; C-17/C-25 and C-20/C-22). The latter case is very unlikely from the model consideration. In the former case (bicyclo[4.4.1]-dioxaundecane system), different *J* values may be expected, and a long-range coupling due to a *W*-type coupling between H-20 and H-22 corresponding to bridgehead protons may be expected for the former case, but such coupling was not observed. For a *W*-type coupling between bridgehead protons, see: Sakai, T.; Naoki, H.; Takaki, K.; Kameoka, H. *Chem. Lett.* **1981**, 1257–1258.

[6] (a) Kobayashi, J.; Ishibashi, M.; Wälchli, M. R.; Nakamura, H.; Hirata, Y.; Sasaki, T.; Ohizumi, Y. *J. Am. Chem. Soc.* **1988**, *110*, 490–494. (b) Kobayashi, J.; Ishibashi, M.; Murayama, T.; Takamatsu, M.; Iwamura, M.; Ohizumi, Y.; Sasaki, T. *J. Org. Chem.* **1990**, *55*, 3421–3423. (c) Kobayashi, J.; Tsuda, M.; Ishibashi, M.; Shigemori, H.; Yamasu, T.; Hirota, H.; Sasaki, T. *J. Antibiot.* **1991**, *44*, 1259–1261. In refs 6a (Table 2) and 6c (Table 1), the multiplicity of H-23 was described as "dd", which has to be revised to "q" with almost the same three coupling constants ($J_{22a,23} = J_{22b,23} = J_{23,24}$).

[7] Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.

and H-31/C-42). Since the connections of all seven units (**A**–**G**), viz. from C-2 to C-38 throughout the molecule were thus revealed, the C-2 (**A**) had to be connected to the C-1 carbonyl carbon, being consistent with the ^{1}H chemical shifts of H_2-2 (see Table 1). The geometrical configurations of the olefinic bonds of **1** were established as 4*E*, 31*E*, and 36*E* by their vicinal coupling constants ($J_{4,5}$ = 15.2, $J_{31,32}$ = 15.8, and $J_{36,37}$ = 15.3 Hz). The stereochemistries of C-6 and C-29 were determined to be both *E* on the basis of high-field chemical shifts for C-39 (δ_C 17.5) and C-42 (δ_C 13.5), and both dienes were deduced to adopt *S-trans* conformations from the NOESY correlations for H-4/H-6, H-5/H_3-39, H-29/H-31, and H_3-42/H-32. The 26,27-epoxide was shown to be trans by the coupling constant ($J_{26,27}$ = 2.0 Hz).[9] From all of these results structure of amphidinolide M was concluded as **1**. The angular hydrogens of two THF rings were suggested to be both *trans* since characteristic NOESY correlations were observed for H-15/H-20 and H-22/H-27 with no cross-peaks between angular protons (H-17/H-20 and H-22/H-25).

Amphidinolide M (**1**) is the first macrolide natural product having a 29-membered macrocyclic lactone ring and possesses a hitherto unknown carbon framework. Amphidinolide M (**1**) contains an *exo*-methylene unit juxtaposed on a methyl group (C-11–C-12 moiety). This type of partial structural moiety containing two vicinally located methyl or exomethylene groups is also found in other amphidinolides (B, C, D, F, G, H, J, and K).[1] These types of structural moiety are unable to be generated through usual polyketide biosynthesis, viz. acetate–malonate pathway, and may have certain relationships to the biosynthesis of odd-numbered lactone rings.[10]

Experimental Section

General Methods. For each 2D NMR experiment a total of 256 increments of 1K data points was collected. The COSY and HMBC spectra were recorded in the absolute mode, while the NOESY, HOHAHA, and HSQC spectra were in the phase-sensitive mode.

Isolation. The harvested cells of the cultured dinoflagellate *Amphidinium* sp. (878 g, wet weight, from 3240 L of culture) were extracted with MeOH/toluene (3:1; 1 L × 3). After addition of 1 M NaCl (1.5 L), the mixture was extracted with toluene (500 mL × 5). The toluene-soluble fraction was evaporated under reduced pressure to give a residue (40 g), which was partially (26 g) subjected to a flash column chromatography on silica gel (4.5 × 40 cm) eluted with $CHCl_3$/MeOH (95:5). The fraction eluting from 540 to 940 mL (1.6 g) was partially (950 mg) dissolved in 80% MeOH and passed through a Sep-Pak cartridge C_{18} (9 × 12 mm, Waters). The eluate (708 mg) was then separated by gel filtration on Sephadex LH-20 (3.5 × 108 cm; $CHCl_3$/MeOH, 1:1), and the fraction eluting from 270 to 330 mL (86 mg) was subsequently separated by reversed-phase HPLC (Develosil ODS-5, Nomura Chemical, 10 × 250 mm; flow rate, 2.5 mL/min; UV detection at 220 nm; eluant, 60% CH_3CN) to give amphidinolide M (**1**, 1.4 mg, t_R 32.4 min).

Amphidinolide M (1): colorless amorphous solid; $[\alpha]^{26}_{D}$ +4.5° (*c* 1, $CHCl_3$); UV (MeOH) λ_{max} 238 nm (ϵ 29 000); IR (film) ν_{max} 3400, 1730, 1440, and 1050 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); FABMS (matrix: DEA) *m/z* 832 (M + DEA + H)$^+$; HRFABMS *m/z* 832.5548, calcd for $C_{47}H_{78}O_{11}N$ (M + DEA + H) 832.5575.

Tetraacetate 2. The crude fraction (0.7 mg) containing amphidinolide M (**1**) was treated with acetic anhydride (0.2 mL) and pyridine (0.2 mL) at room temperature for 12 h. After evaporation of the solvent, purification by silica gel column chromatography (0.7 × 7 cm) eluted with hexane/acetone (3:1) afforded the tetraacetate (**2**, *ca.* 0.1 mg): ^{1}H NMR (Table 1); FABMS (matrix: DEA) *m/z* 1000 (M + DEA + H)$^+$.

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Supplementary Material Available: NMR (^{1}H–^{1}H COSY, HOHAHA, NOESY, HSQC, HMBC, and ^{13}C NMR for **1**; ^{1}H–^{1}H COSY, HOHAHA, and slice spectra of HOHAHA for **2**) and MS spectra of compounds **1** and **2** (10 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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(8) Other HMBC correlations observed: H_3-39/C-6, H_3-39/C-7, H_3-40/C-10, H_3-40/C-11, H-41a/C-13, H-41b/C-13, H-13a/C-12, H-29/C-42, H_3-42/C-29, H_3-42/C-30, H-31/C-33, H_3-43/C-34, H_3-43/C-35, H_3-43/C-36, H_3-38/C-36, and H_3-38/C-37.

(9) Kobayashi, J.; Ishibashi, M.; Nakamura, H.; Ohizumi, Y.; Yamasu, T.; Sasaki, T.; Hirata, Y. *Tetrahedron Lett.* **1986**, *27*, 5755–5758.

(10) Norhalichondrin A contains this type of structural unit (C-25–C-26 moiety) and also contains a 31-membered macrocyclic lactone ring: Uemura, D.; Takahashi, K.; Yamamoto, T.; Katayama, C.; Tanaka, J.; Okumura, Y.; Hirata, Y. *J. Am. Chem. Soc.* **1985**, *107*, 4796–4798.