Amphidinolide K, a New 19-Membered Macrolide from the Cultured Symbiotic Dinoflagellate *Amphidinium* sp.

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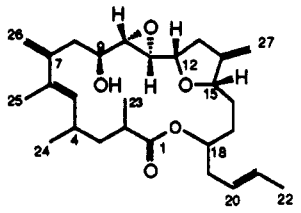
We have previously isolated amphidinolides A–J, cytotoxic macrolides with unique structural features, from the laboratory-cultured dinoflagellates *Amphidinium* sp. which are symbionts of Okinawan marine flatworms *Amphiscolops* sp.[1] Our previous studies have revealed that fractionation by silica gel chromatography of the toluene-soluble portion of the extract of this microalga afforded several fractions exhibiting extremely potent cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells in vitro with the inhibition values at 10 μg/mL being more than 90%. These inhibition values cannot be fully accounted for by estimation from the IC_{50} values of previously isolated amphidinolides. Thus, our investigations in search of other cytotoxic components of this dinoflagellate have continued. As a result, we recently isolated a novel 19-membered macrolide, amphidinolide K (1), as a minor constituent possessing cytotoxic activity with IC_{50} values of 1.65 and 2.9 μg/mL against L1210 and KB cells in vitro, respectively. This paper describes the isolation and structure elucidation of 1.



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The dinoflagellate *Amphidinium* sp. was cultured at 25 °C for 2 weeks in a sea water medium enriched with ES nutrients.[1a] The harvested algal cells (920 g, wet weight, from 3300 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)) followed by gel filtration on Sephadex LH-20 ($CHCl_3$/MeOH (1:1)). Subsequent separation by reversed-phase HPLC (ODS, 88% MeOH) afforded a cytotoxic fraction, which was finally purified with a short silica gel column (hexane/EtOAc (2:1)) to give amphidinolide K (1, 0.0002% yield, wet weight) as a colorless oil.

The molecular formula of 1 was suggested as $C_{27}H_{40}O_5$ by the HRFABMS data [matrix: diethanolamine (DEA), *m/z* 550.3711 $(M + DEA + H)^+$ for $C_{31}H_{52}O_7N$, Δ −3.3 mmu]. The IR spectrum of 1 was indicative of the presence of hydroxyl (3450 cm^{-1}) and carbonyl (1725 cm^{-1}) groups, and the UV absorption maximum at 229 nm suggested the presence of a diene chromophore. Analysis of the ^1H and ^{13}C NMR spectral data of 1 (Table I) provided evidence for the presence of one ester carbonyl, two *exo*-methylenes, one disubstituted and one trisubstituted olefin, six oxygenated methines, two unoxygenated methines, six aliphatic methylenes, and four methyl groups. Five out of the eight unsaturations were thus characterized, and compound 1 was therefore inferred to contain three rings. Although the available sample size of amphidinolide K (1) was no more than 0.3 mg, the gross structure of 1 was elucidated as follows by applying several types of modern 2D NMR techniques (^1H–^1H COSY, NOESY, HOHAHA,[3] HSQC,[4] and HMBC[5] experiments) using a 600-MHz spectrometer. The ^1H–^1H COSY spectrum of 1 clearly showed proton connectivities for three partial structures for C-2–C-5, C-8–C-13, and C-15–C-22. The diene unit (C-5–C-7) was shown to be located between C-4 and C-8

Table I. ^1H and ^{13}C NMR Data of Amphidinolide K (1) in C_6D_6.[a]

position	δ_H	*J* (Hz)	δ_C	HMBC (^1H)[b]
1			175.4 s[c]	H_3-23
2	2.48 m		38.0 d	H_3-23
3 (a)	1.96 ddd	13.8, 11.6, 4.6	41.4 t	H_3-23, H_3-24
(b)	1.08 ddd	13.8, 10.9, 4.2		
4	2.98 m		30.9 d	H_3-24
5	5.36 d	10.6	134.4 d	H_3-24, H_3-25
6			134.2[d] s	H-8b, H_3-25, H-26a, H-26b
7			145.7[d] s	H-5, H-8a, H-8b, H_3-25
8 (a)	3.27 dd	14.0, 6.9	39.9 t	H-26a, H-26b
(b)	2.34 dd	14.0, 8.7		
9	4.37 br t	7.5	65.6 d	H-8a, H-8b
10	3.22 t	2.3	56.8 d	H-8a
11	2.86 br s		56.0 d	H_2-13
12	4.05 br t	6.2	74.2 d	H-11
13 (2H)	2.55 d	6.2	36.0 t	H-27a, H-27b
14			151.5 s	H_2-13
15	4.19 br d	10.3	80.8 d	H-27a, H-27b
16 (a)	1.86 m		29.1 t	
(b)	1.60 m			
17 (a)	1.89 m		30.0 t	
(b)	1.58 m			
18	5.27 m		71.6 d	
19 (a)	2.39 m		35.9 t	
(b)	2.16 m			
20	5.39 m		126.9 d	H-19a, H-19b, H_3-22
21	5.41 m		128.8 d	H-19a, H-19b, H_3-22
22 (3H)	1.56 d	5.8	18.0 q	
23 (3H)	1.14 d	7.1	19.2 q	
24 (3H)	0.92 d	6.6	21.3 q	
25 (3H)	1.84 s		14.5 q	H-5
26 (a)	5.12 s		114.0 t	H-8a, H-8b
(b)	4.96 s			
27 (a)	4.92 d	2.1	104.1 t	
(b)	4.75 d	2.1		

[a] The NOESY (mixing time: 800 msec) and the HOHAHA (mixing time: 50 and 90 msec) spectra also afforded correlations fully consistent with the proposed structure (1) of amphidinolide K. [b] The HMBC experiment was carried out with the F1 width (^{13}C NMR axis) of 92.03 ppm, which was reduced to about half of the conventional width to enhance the digital resolution, giving a spectrum with folded signals. [c] Multiplicities were determined by DEPT and HSQC experiments. [d] The signals of these carbons were not observed in the 1D ^{13}C NMR spectrum and assignment of these carbons were based on the cross-peaks observed in the HMBC spectrum.

(1) (a) Kobayashi, J.; Ishibashi, M. *Chem. Rev.* **1993**, *93*, 1753–1769. (b) Kobayashi, J.; Sato, M.; Ishibashi, M. *J. Org. Chem.* **1993**, *58*, 2645–2646 and references cited therein.

(2) Amphidinolides A–E and J were previously isolated[1] from this same isolate (strain number Y-5). For further description of the dinoflagellate of this species, see: Kobayashi, J.; Ishibashi, M.; Nakamura, H.; Ohizumi, Y.; Yamasu, T.; Hirata, Y.; Sasaki, T.; Ohta, T.; Nozoe, S. *J. Nat. Prod.* **1989**, *52*, 1036–1041.

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

(4) Bodenhausen, G.; Ruben, D. J. *Chem. Phys. Lett.* **1980**, *69*, 185–189.

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by the COSY correlations for H-4/H-5, H-5/H_3-25, H-5/H-26b, H_3-25/H-26b, H-8a/H-26b, and H-8b/H-26b as well as the HMBC cross-peaks based on two- or three-bond ^1H–^{13}C long-range couplings for C-5/H_3-24, C-6/H-8b, C-7/H-8a, C-7/H-8b, C-8/H-26a, and C-8/H-26b. The ^{13}C chemical shift of the C-25 methyl group (δ_C 14.5) implied that the $\Delta^{5,6}$-double bond has the *E*-configuration. This diene unit was assumed to adopt the *S*-trans conformation based on NOESY correlations involving H-5/H-8a, H-5/H-9, and H_3-25/H-26a. The presence of an epoxide on the C-10–C-11 position was deduced from the corresponding ^1H and ^{13}C NMR chemical shifts (Table I). The ^1H–^1H coupling constant ($J_{10,11}$ = 2.3 Hz) indicated this epoxide to be *trans*.[6] The second *exo*-methylene group was placed on C-14, since ^1H–^1H COSY cross-peaks due to allylic or long-range ^1H–^1H couplings were observed for H_2-13/H-27a, H_2-13/H-27b, H-15/H-27a, H-15/H-27b, and H_2-13/H-15. The HMBC spectrum revealed connectivities for C-13/H-27a, C-13/H-27b, C-15/H-27a, and C-15/H-27b, further verifying that the *exo*-methylene group is present between C-13 and C-15. The connection of C-12 and C-15 through an ether oxygen to form a tetrahydrofuran ring was inferred from the ^{13}C chemical shifts for the *exo*-methylene group at C-14 [δ_C 151.5 (C-14) and 104.1 (C-27)], which corresponded well to those for the sp^2 carbons of methylenecyclopentane (δ_C 152.3 and 105.2).[7] The ester carbonyl (C-1) was shown to be adjacent to C-2, which bears the C-23 methyl group, by the HMBC correlation between C-1 and H_3-23. The ester oxygen on C-1 was deduced to be connected to C-18 by the low-field resonance of H-18 (δ_H 5.27). The molecule was terminated by a methyl group (C-22) attached on a disubstituted olefin. A homo-spin-decoupling experiment irradiating at H_3-22 indicated $J_{20,21}$ to be 14.9 Hz, implying the 20*E*-configuration. In the FABMS spectrum of **1** (diethanolamine (DEA) matrix) a fragment ion peak was observed at *m/z* 496, which corresponded to the ion (M + DEA + H – butadiene)$^+$, generated by loss of the C-19–C-22 moiety [(CH_2CH=$CHCH_3$ – H); 54 amu]. In order to interpret the stereochemistry of this macrocyclic compound containing eight chiral centers, we examined the NOESY spectral data as well as the ^1H–^1H coupling constants and propose the relative stereochemistry and conformation of the tetrahydrofuran–epoxide portion as presented in Chart I, which appears to sufficiently satisfy the NOESY data and *J* values.[8] From all of these results, the structure of amphidinolide K was concluded to be **1**.

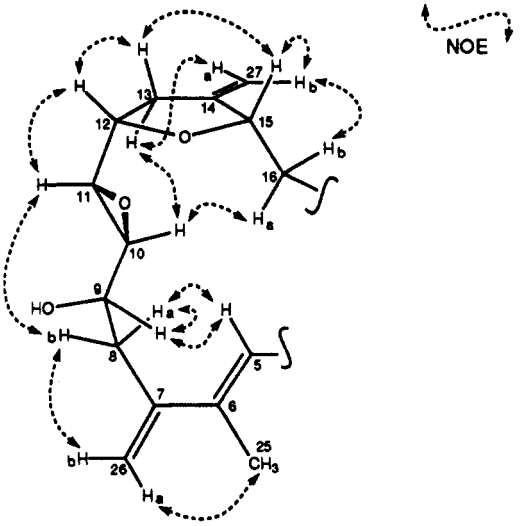
Amphidinolide K (**1**) belongs to a new class of macrolide natural products possessing a novel carbon framework, although some of its structural features are common to previously known amphidinolides (*e.g.*, the odd-numbered macrocyclic lactone ring and the presence of *exo*-methylene, epoxide, and tetrahydrofuran units).[1] The dinoflagellate *Amphidinium* sp. is quite unique because it produces a number of cytotoxic macrolides possessing a variety of carbon skeletons; they, however, have similar structural units as described above, appearing to be biogenetically related to one another.

(6) Ishibashi, M.; Ohizumi, Y.; Hamashima, M.; Nakamura, H.; Hirata, Y.; Sasaki, T.; Kobayashi, J. *J. Chem. Soc., Chem. Commun.* **1987**, 1127–1129.

(7) Kalinowski, H.-O.; Berger, S.; Braun, S. *Carbon-13 NMR Spectroscopy*; John Wiley & Sons: Chichester, 1984; p 136.

(8) The NOESY spectrum of **1** also showed cross-peaks for H-2/H-5 and H-2/H-9. However, the relative configurations of C-2, C-4, and C-18 positions remained unassigned.

Chart I. Relative Stereochemistry for the C-5–C-16 Part of 1 Proposed from the NOESY Data[a]



[a]The coupling constants for this moiety (H/H, in Hz) are as follows: 8a/9 = 6.9, 8b/9 = 8.7, 9/10 = 2.3, 10/11 = 2.3, 11/12 = <1, 12/13a = 12/13b = 6.2, 15/16a = 10.3, and 15/16b = <1.

Experimental Section

General Methods. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-600 spectrometer, and for each 2D NMR experiment, a total of 256 increments of 1 K 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. (920 g, wet weight, from 3300 L of culture) were extracted with MeOH/toluene (3:1; 1 L × 3). After addition of 1 M NaCl (500 mL), the mixture was extracted with toluene (500 mL × 3). The toluene-soluble fraction was evaporated under reduced pressure to give a residue (33 g), which was partially (4.9 g) subjected to a silica gel column (2.7 × 45 cm) eluted with $CHCl_3$/MeOH (95:5). The fraction eluting from 120 to 170 mL (580 mg) was dissolved in MeOH/H_2O (80:20) and passed through a Sep-Pak cartridge C_{18} (9 × 12 mm, Waters). The eluate (267 mg) was then separated by gel filtration on Sephadex LH-20 (2.0 × 120 cm; $CHCl_3$/MeOH (1:1)), and the fraction eluting from 135 to 149 mL (43 mg) was then separated by reversed-phase HPLC (Develosil ODS-5, Nomura Chemical, 10 × 250 mm; flow rate, 2.5 mL/min; UV detection at 254 nm; eluant, MeOH/H_2O (88:12)) to give a cytotoxic fraction (2.5 mg, t_R 14.9 min; inhibition at 10 μg/mL: L1210, 90.6%; KB, 93.5%). This fraction was finally purified by use of a silica gel column (0.5 × 7 cm) eluted with hexane/EtOAc (2:1) to give amphidinolide K (**1**, 0.3 mg) in the 10–15-mL fraction.

Amphidinolide K (**1**): colorless oil; $[\alpha]^{21}_D$ –71° (*c* 0.05, MeOH); UV (MeOH) λ_{max} 229 nm (ϵ 11 000); IR (neat) ν_{max} 3450, 1725, and 1160 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS (matrix: glycerol) *m/z* 445 (M + H)$^+$; FABMS (matrix: diethanolamine (DEA)) *m/z* 550 (M + DEA + H)$^+$; HRFABMS *m/z* 550.3711, calcd for $C_{31}H_{52}O_7N$: (M + DEA + H) 550.3744.

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Supplementary Material Available: 2D NMR spectra of compound **1** (7 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.