Amphidinolide N, a Novel 26-Membered Macrolide with Remarkably Potent Cytotoxicity from the Cultured Marine Dinoflagellate *Amphidinium* sp.

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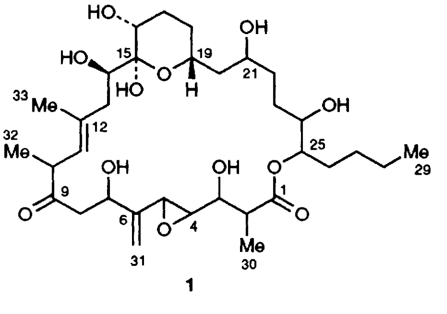
Amphidinolide N (**1**), a novel 26-membered macrolide possessing an unprecedented carbon framework, has been isolated as an extremely potent cytotoxic compound from the cultured marine dinoflagellate *Amphidinium* sp., which was living inside of the Okinawan marine flatwork *Amphiscolops* sp., and its structure elucidated by extensive analyses of its spectroscopic data.

Marine dinoflagellates of the genus *Amphidinium* have proven to be a rich source of unique bioactive secondary metabolites with unprecedented structures.[1,2] We now succeeded in isolating a novel 26-membered macrolide, amphidinolide N (**1**), possessing extremely potent cytotoxicity from extracts of the dinoflagellate (strain number Y-5)† with IC$_{50}$ values against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro* being 0.00005 and 0.00006 μg ml^{-1}, respectively. The cytotoxicity of compound **1** is the most potent among amphidinolides previously isolated,[3] and almost comparable to those of spongistatins (altohyrtins, or cinachyrolide),[4]‡ which were recently isolated from marine sponges and reported to be 'the most potent of all substances tested to date in the NCI screen.'[5] Amphidinolide N (**1**), on the other hand, bears a chemically unique structure with an hitherto unknown carbon framework. Here we describe the isolation and structural elucidation of **1**.

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 portion 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% MeCN) afforded amphidinolide N (**1**, 0.0009% yield, wet weight); colourless amorphous solid: $[\alpha]_D^{20}$ +20° (*c* 0.5, MeOH); IR (film) ν_{max} 3400 and 1710 cm^{-1}; FABMS [matrix, diethanolamine (DEA)] *m/z* 748 (M + DEA + H)$^+$ and 730 (M + DEA + H − H_2O)$^+$.

The molecular formula of amphidinolide N (**1**) was revealed as $C_{33}H_{54}O_{12}$ by high-resolution FABMS data [*m/z* 748.4464 (M + DEA + H)$^+$ for $C_{37}H_{66}O_{14}N$, Δ −1.9 mmu. The chromatographic behaviour of compound **1** suggested that it contains more hydroxyl groups than previously isolated amphidinolides (containing 2–4 OH).§ Interpretation of the ^{1}H and ^{13}C NMR spectral data of **1** (Table 1) indicated the presence of one isolated ketone, one ester carbonyl, one *exo*-methylene, one trisubstituted double-bond, ten oxymethines, two unoxygenated methines, ten sp^3 methylenes, and four methyls. A characteristic ^{13}C signal was observed at δ_C 98.2, which was assignable to a hemiketal group. Since four out of seven unsaturations were thus accounted for, compound **1** was inferred to contain three rings (*viz*., one lactone, one epoxide, and one ether ring).



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Several types of recently developed 2D NMR techniques (^{1}H–^{1}H COSY, NOESY, HOHAHA,[6] HSQC,[7] and HMBC[8]) were applied using a 600 MHz spectrometer and results are given in Fig. 1. The ^{1}H–^{1}H COSY spectrum of **1** clearly showed the proton connectivities for seven partial structural units (**a–g**) designated by bold lines.

Units **a** and **b** were connected through an sp^2 carbon atom (C-6) to which is connected the *exo*-methylene group; this fact was revealed by the HMBC (H-5/C-6, H-5/C-31, H-7/C-6, H-7/C-31, H-31a/C-5, and H-31b/C-7) as well as NOESY correlations (H-4/H-7, H-5/H-7, H-5/H-8a, H-5/H-31b, H-7/H-31a).¶ The isolated ketone (C-9) showed HMBC connectivities with H-8a, H-8b, H-10, and H_3-32, thus placing it between C-8 (**b**) and C-10 (**c**). The hemiketal carbon (C-15) was revealed to be adjacent to C-14 by the HMBC correlation for H-13b/C-15, and was connected to C-16 (**d**) by the NOESY cross-peaks for H-13a/H-16 and H-13b/H-16. These observations were coincident with the fact that the ^{1}H-^{1}H COSY spectrum of **1** had suggested that C-14 and C-16 were vicinal to a quaternary carbon.

In the HOHAHA spectrum of **1** H-16 was correlated to H_2-18 and H-19, while H-19 showed cross-peaks with H_2-17. Units **d** and **e** were thus shown to be adjoining. A NOESY cross-peak was observed between H-14 and H-19, thus suggesting that the C-19 oxymethine and the C-15 hemiketal

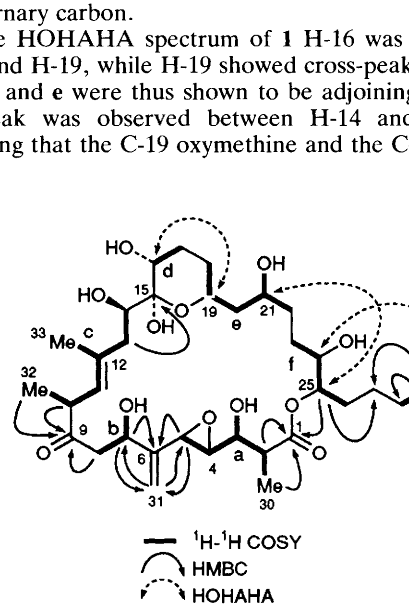

Fig. 1 Partial structural units (**a–g**) based on the ^{1}H–^{1}H COSY data and selected HMBC and HOHAHA correlations

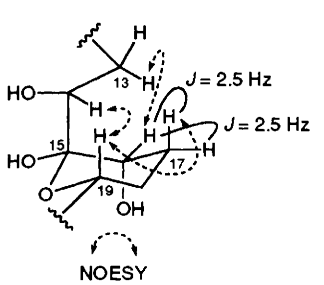

Fig. 2 Conceivable relative stereochemistry of the tetrahydropyran portion (C-13–C-19) of **1**

were linked through an oxygen to form a tetrahydropyran (THP) ring and H-19 and C-14 were both axially oriented on the THP ring (Fig. 2). The hydroxyl group on C-16 was deduced to be axial on the basis of the coupling constants ($J_{16,17a} = J_{16,17b} = 2.5$ Hz).

Connection between units **e** and **f** was substantiated by the HOHAHA correlations observed for H-21/H$_2$-23, H-21/H-24, H-21/H-25, H$_2$-22/H-24 and H$_2$-22/H-25. The C-25 oxymethine proton, resonating at a relatively low field (δ_H 4.84), showed an HMBC correlation with the ester carbonyl carbon (δ_C 173.5, C-1), which in turn showed HMBC connectivities with H-2 and H$_3$-30. Thus, C-25 (**f**) and C-2 (**a**) were connected through the C-1 carbonyl to construct a 26-membered macrocyclic lactone ring.

The H-25 showed an HMBC cross-peak to an sp^3 methylene at δ_C 27.8 (C-27), which was also correlated with the terminal methyl protons (H$_3$-29) by the HMBC spectrum. A *n*-butyl group was therefore suggested to be attached on the C-25 oxymethine carbon. The ^{13}C chemical shifts for C-27 (δ_C 27.7), C-28 (δ_C 22.7), and C-29 (δ_C 14.3) were very similar to those of the corresponding carbons of hexan-2-ol.[9][‖]

The configuration of the 4,5-epoxide was deduced to be *trans* by the coupling constant ($J_{4,5} = 1.9$ Hz),[10] whereas the ^{13}C chemical shift of the C-33 methyl group (δ_C 15.7) implied that the geometry of the $\Delta^{11,12}$-double bond was E, which was supported by the NOESY correlations for H-10/H$_3$-33 and H-11/H-13b. From all of these results the structure of amphidinolide N was concluded as **1**.

A number of macrolides with a variety of new carbon skeletons have been isolated from dinoflagellates of the genus *Amphidinium*.[3] Amphidinolide N (**1**) also possesses an unprecedented carbon framework; some structural features are, however, similar to those of previously isolated amphidinoldes[1,3] (*e.g.* presence of *exo*-methylene and epoxide groups). The most noteworthy-feature is the outstanding cytotoxicity of compound **1**, which may be expected to be a new source of antitumour agent.

Table 1 ^{1}H and ^{13}C NMR data of amphidinolide N (**1**) in C$_6$D$_6$[a]

Position	δ_H	δ_C	Position	δ_H	δ_C
1		171.8	18a	1.39 m	25.6
2	2.73 qnt	45.0	18b	1.03 m	
3	3.86 m	73.0	19	4.23 m	65.9
3-OH	4.85 br s		20 (2H)	1.44 m	41.5
4	3.13 dd	62.3	21	4.16 m	74.5
5	3.59 d	54.6	21-OH	4.36 br s	
6		147.0	22a	1.66 m	32.6
7	4.82 dd	69.7	22b	1.16 m	
7-OH	3.97 br s		23a	1.20 m	27.9
8a	3.00 dd	45.2	23b	1.57 m	
8b	2.65 dd		24	3.77 q	80.5
9		210.5	25	4.84 m	
10	3.25 dq	47.9	26a	1.40 m	
11	5.16 d	127.5	26b	1.35 m	
12		136.3	27 (2H)	1.34 m	27.7
13a	2.49 br d	40.3	28 (2H)	1.33 m	22.7
13b	2.41 dd		29 (3H)	0.91 t	14.3
14	4.32 dd	70.9	30 (3H)	1.22 d	14.1
14-OH	3.98 br s		31a	5.38 s	111.6
15		98.2	31b	5.30 s	
16	3.62 br t	65.8	32 (3H)	1.13 d	15.8
17a	2.20 m	26.9	33 (3H)	1.79 s	15.7
17b	1.53 m				

[a] J(H/H) in Hz: 2/3 = 7.2, 2/30 = 7.2, 3/4 = 4.6, 4/5 = 1.9, 7/8a = 10.4, 7/8b = 2.3, 8a/8b = 16.9, 10/11 = 9.9, 10/32 = 6.7, 13a/13b = 13.1, 13a/14 = 3.4, 13b/14 = 11.2, 16/17a = 2.5, 16/17b = 2.5, 23a/24 = 7.5, 23b/24 = 7.5, 24/25 = 7.5, 28/29 = 7.1.

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Footnotes

[†] This dinoflagellate *Amphidinium* sp. was isolated from the Okinawan marine flatworm *Amphiscolops* sp. Amphidinolides A-E, J, and K were previously isolated from extracts of this species of dinoflagellate.[3]

[‡] The IC$_{50}$ values of altohyrtin A[4b]; L1210, 0.0001 μg mL^{-1}; KB, 0.00001 μg ml^{-1} were determined by T. Sasaki, who also determined the IC$_{50}$ values of **1**. Spongistatin A and altohyrtin A possess the same planar structure and probably are identical compounds.

[§] Retention times on reversed-phase HPLC analysis [Develosil ODS-5, Nomura Chemical, 10 × 250 mm, 60% MeCN; flow rate, 2.5 mL min^{-1}, RI (refractive index) and UV (220 nm) detection]: **1**, 10.0 min; amphidinolide E (containing 3 OH), 24.9 min; amphidinolide A (containing 4 OH), 27.6 min; amphidinolide J (containing 2 OH), 35.7 min; amphidinolide C (containing 4 OH), 41.6 min.

[¶] For the assignment of nonequivalent methylene protons, lower field methylene protons were suffixed by 'a' (*e.g.* H-8a), while higher field ones were suffixed by 'b' (*e.g.* H-8b).

[‖] The ^{13}C chemical shifts for the C-4–C-6 positions of hexan-2-ol occur at δ_C 28.2 (C-4), 23.2 (C-5) and 14.3 (C-6) whereas the ^{13}C chemical shifts for the C-4–C-6 positions of hexan-3-ol occur at δ_C 39.4 (C-4), 19.2 (C-5), and 14.3 (C-6)[9].

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