

Amphidinolides B4 and B5, Potent Cytotoxic 26-Membered Macrolides from Dinoflagellate *Amphidinium* Species

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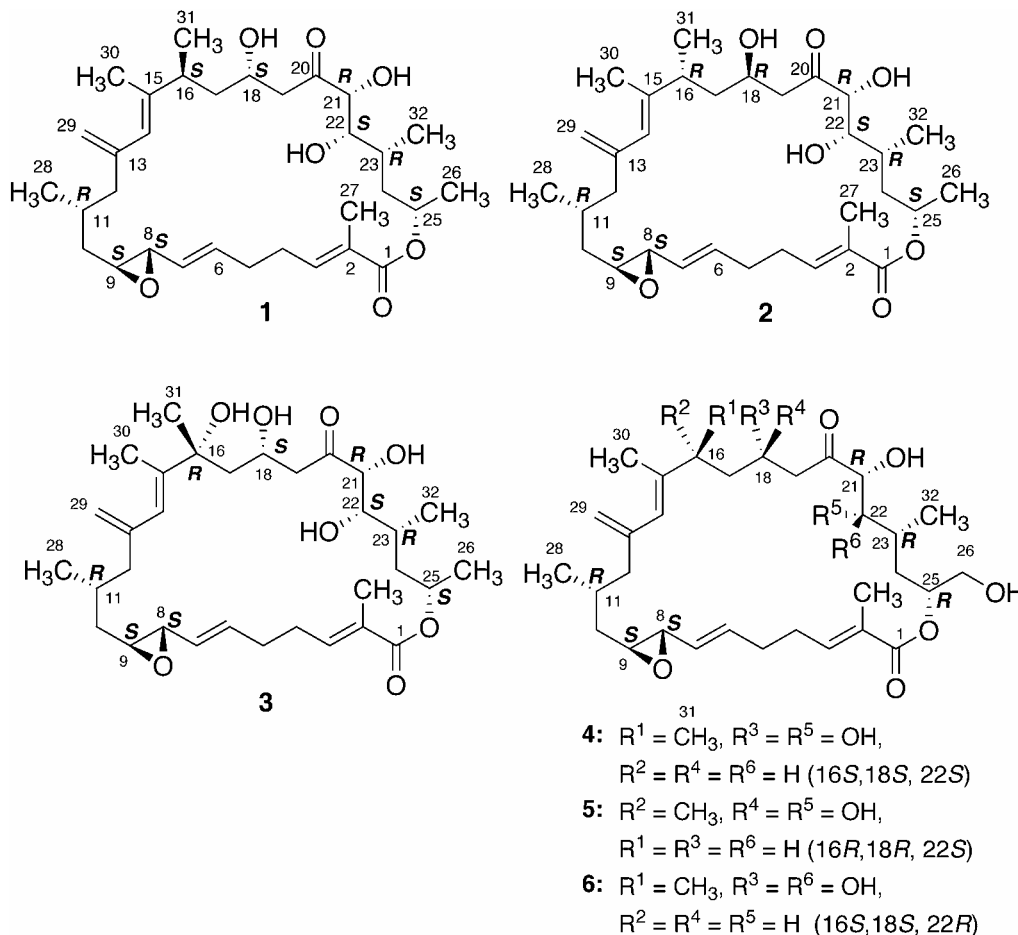
Abstract: Two new cytotoxic 26-membered macrolides, amphidinolides B4 (**1**) and B5 (**2**), have been isolated from a marine dinoflagellate *Amphidinium* sp. (strain Y-100), and the structures were elucidated on the basis of detailed analyses of 2D NMR data including ¹³C–¹³C correlations.

Keywords: dinoflagellate; *Amphidinium* sp.; amphidinolides B4 and B5; cytotoxic

Introduction

Amphidinolides are a series of unique cytotoxic macrolides isolated from dinoflagellates *Amphidinium* species, which were separated from marine acoel flatworms *Amphiscolops* species [1]. The 26- or 27-membered macrolides, which were represented by amphidinolides B (**3**) and H (**4**) [2-7], possess unique structural features such as an allyl epoxide and vicinally located one-carbon branches, and exhibit potent cytotoxicity against tumor cell lines. From our previous studies, the presence of an allyl epoxide, an *S-cis*-diene moiety, and the ketone at C-20 in **3** and **4** was indicated to be important for the cytotoxicity of amphidinolide H-type macrolides [7]. More recently, it was indicated that one of the mechanism of action for the potent cytotoxicity of **4** was due to bind to actin covalently [8].

During our continuing search for bioactive metabolites from marine dinoflagellates, two new amphidinolide B-type macrolides, amphidinolides B4 (**1**) and B5 (**2**), have been isolated from a marine dinoflagellate *Amphidinium* species (strain Y-100). In this paper, we describe the isolation, structure elucidation, and cytotoxicity of **1** and **2**.



Results and Discussion

The dinoflagellate *Amphidinium* sp. (strain Y-100) was isolated from a marine acoel flatworm *Amphiscolops* sp. collected off Ma'eda Cape, Okinawa, and mass cultured uniaxially at 25 °C for 2 weeks in a seawater medium enriched with 1% Provasoli's Erd-Schreiber (ES) supplement and 1% NaH¹³CO₃. The mass cultured algal cells (60.5 g, wet weight) obtained from 30 L of culture were extracted with MeOH/toluene (3:1), and the extracts were partitioned between toluene and 1M NaCl aq. The toluene soluble materials were subjected to a silica gel column followed by SiO₂ and/or C₁₈ HPLC to afford amphidinolides B4 (**1**, 0.0008 %, wet weight) and B5 (**2**, 0.0002 %) together with known related macrolides, amphidinolides B [2-4], (**3**, 0.0016 %), C [9,10] (0.0025 %), and T1 [11-13] (0.0028 %).

Amphidinolide B4 (**1**, [α]_D²³ -13° (c 0.2, CHCl₃)) showed the pseudomolecular ion peak at *m/z* 569.5 (M+Na)⁺ in the ESIMS spectrum, and the ¹³C-enrichment was estimated as 32% by the pattern of the pseudomolecular ion peak. The molecular formula of C₃₂H₅₀O₈ was revealed on the basis of

Table 1. ^{13}C NMR Data of Amphidinolides B4 (**1**), B5 (**2**), B (**3**), H (**4**), H2 (**5**), and H3 (**6**) in CDCl_3 .

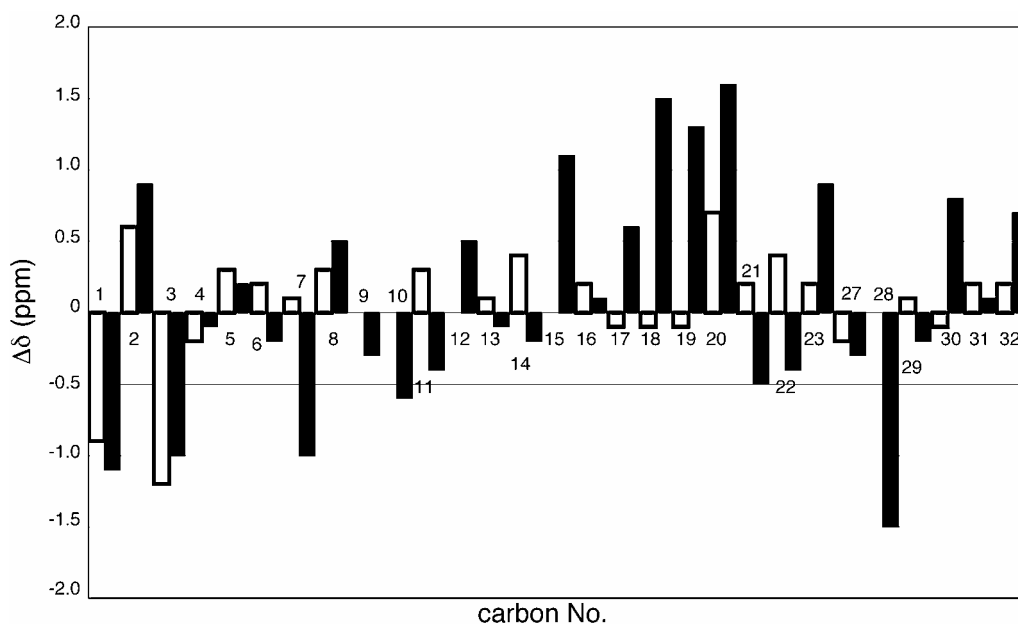
positn	1	2	3	4	5	6
1	167.8	167.8	167.7	168.7	168.9	168.8
2	128.5	128.1	128.3	127.9	127.6	128.1
3	139.8	139.4	139.9	141.0	140.8	140.7
4	26.8	26.9	26.8	27.0	26.9	26.8
5	31.2	31.0	30.8	30.9	31.0	30.7
6	135.9	136.1	135.4	135.7	136.1	136.1
7	128.7	129.7	128.5	128.6	129.7	127.9
8	60.6	60.1	60.0	60.3	60.1	60.5
9	59.5	59.9	59.3	59.5	59.8	59.7
10	39.8	40.4	39.4	39.8	40.4	39.6
11	29.4	29.9	29.1	29.1	29.8	29.3
12	47.1	46.7	46.7	47.1	46.6	47.1
13	144.2	144.3	144.4	144.1	144.3	143.9
14	126.5	126.6	124.3	126.1	126.7	125.8
15	141.7	140.6	143.1	141.7	140.6	141.5
16	40.9	40.9	75.9	40.7	40.8	40.9
17	40.8	40.2	45.2	40.9	40.2	40.8
18	67.4	65.9	66.5	67.5	65.9	67.1
19	45.1	43.9	45.9	45.2	43.8	46.6
20	212.9	212.0	212.4	212.2	211.3	215.1
21	77.9	78.8	77.7	77.7	78.4	77.0
22	75.8	76.3	75.5	75.4	76.2	77.2
23	33.2	32.9	33.2	33.0	32.3	30.1
24	39.2	39.4	39.3	33.5	33.9	30.2
25	68.3	73.4	68.3	73.4	73.3	73.7
26	21.2	21.2	21.0	66.1	66.6	66.4
27	12.4	12.5	12.4	12.6	12.7	12.6
28	18.0	19.5	18.2	18.0	19.5	18.2
29	114.8	114.9	114.8	114.7	115.0	114.8
30	13.1	12.3	15.6	13.2	12.3	12.8
31	20.5	20.2	28.3	20.3	20.4	20.8
32	15.8	15.2	15.0	15.6	15.1	16.2

HRESIMS data [m/z 569.3467 ($\text{M}+\text{Na}$) $^+$, Δ +1.3 mmu]. The ^1H NMR spectrum of **1** was similar to that of amphidinolide B (**3**). The ^{13}C NMR data (Table 1) of **1** revealed total 32 carbon signals due to a ketone, an ester carbonyl, three sp^2 quaternary carbons, four sp^2 methines, an sp^2 methylene, nine sp^3 methines consisting of six oxygenated ones, seven sp^3 methylenes, and six methyls. Detailed analyses of the HMQC and INADEQUATE spectra of **1** established the carbon chain from C-1 to C-26 and six

C₁ branches including five methyls (C-27, C-28, C-30, C-3, and C-32) and an exomethylene (C-29). The relatively lower-field resonance of H-25 (δ_{H} 5.08) indicated that C-25 was involved in an ester linkage with C-1. This was supported by HMBC correlations for H-3/C-1, H₃-27/C-1, and H-25/C-1. Thus, the gross structure of amphidinolide B4 was assigned as **1**.

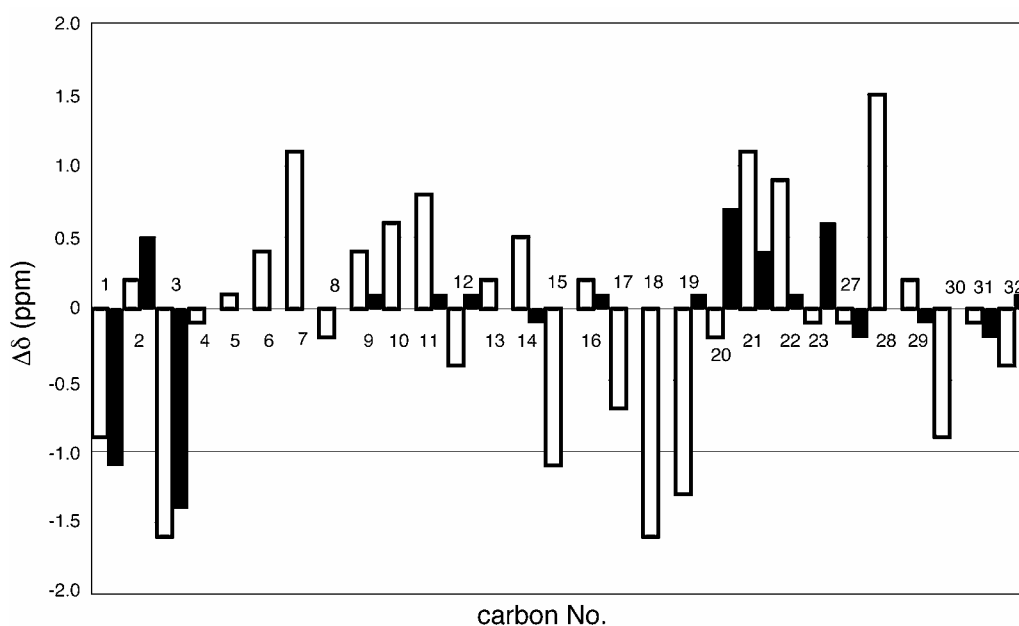
The relative stereochemistry of **1** was deduced from detailed comparison of the ¹³C NMR data (Table 1) of **1** with those of amphidinolides B (**3**), H (**4**), H2 (**5**), and H3 (**6**). The ¹³C chemical shifts for C-1 ~ C-13 part with two methyls (C-27 and C-28), and an exomethylene (C-29) of **1** corresponded well to those of **3** ~ **5**, and the ¹³C chemical shifts for C-20 ~ C-26 and C-32 of **1** were also close to those of **4** and **5**, suggesting that the relative stereochemistry for these parts of **1** might be common to that of amphidinolides B (**3**), H (**4**), and H2 (**5**). On the other hand, the ¹³C NMR data of the corresponding portion of amphidinolide H3 (**6**) were different from those of **1**, while the ¹³C NMR data for C-14 ~ C-23 and C-28, C-29, C-31 of **1** were similar to those for the corresponding parts of amphidinolide H (**4**) rather than those of amphidinolide H2 (**5**) as shown in Figure 1. The chemical shifts for C-18 (δ_{C} 67.4), C-19 (δ_{C} 45.1), and C-30 (δ_{C} 13.1) of **1**; those of **4** (C-18: δ_{C} 67.5, C-19: δ_{C} 45.2, and C-30: δ_{C} 13.2); those of **5** (C-18: δ_{C} 65.9, C-19: δ_{C} 43.8, and C-30: δ_{C} 12.3). The ¹H-¹H coupling constants for H-18/H-19a (8.2 Hz) and H-18/H-19b (small) of **1** resembled those of **4** (H-18/H-19a: 8.5 Hz and H-18/H-19b: 1.8 Hz), while those for H-18/H-19a (2.6 Hz) and H-18/H-19b (9.8 Hz) of **5** were different from those of **1**. These observations indicated the possibility that the relative stereochemistry of nine chiral centers in **1** might be the same as that in **4**. The CD spectrum [λ_{ext} 262 ($\Delta\epsilon$ +0.1) and 235 nm (-0.22)] of **1** was similar to those of **4** [λ_{ext} 261 ($\Delta\epsilon$ +0.1) and 235 nm (-0.15)]. Considering the biosynthetic relationship among **1** and amphidinolides B (**3**) and H (**4**), the absolute configurations of **1** were elucidated to be the same as those of **3** and **4**. Thus, amphidinolide B4 (**1**) was assigned as 16-deoxy form of amphidinolide B (**3**).

Figure 1. Chemical shift differences between amphidinolides B4 (**1**) and H (**4**: frame) or H2 (**5**: solid).



Amphidinolide B5 (**2**, $[\alpha]_D^{23} -25^\circ$ (c 0.2, CHCl_3)) was elucidated to have the same molecular formula, $\text{C}_{32}\text{H}_{50}\text{O}_8$, as **1**. Profiles of the ^1H and ^{13}C NMR (Table 1) spectra of **2** were reminiscent of those of **1**. The gross structure of **2** was elucidated to be the same as that of **1** from analyses of the HMQC, HMBC, and INADEQUATE spectra. The relative stereochemistry of **2** was deduced from comparison of the carbon chemical shifts with those of **3**, **4**, and **5** as follows. The ^{13}C chemical shifts for C-1 ~ C-13 and C-27 ~ C-29 of **2** were similar to those of **3** ~ **5**. The ^{13}C NMR data for C-1 ~ C-23 and C-27 ~ C-32 of **2** were closer to those of **5** than those of **4** (Figure 2), indicating that the relative stereochemistry of this part in **2** was the same as that of the corresponding part in **5**. The chemical shifts for C-18 (δ_{C} 65.9), C-19 (δ_{C} 43.9), and C-30 (δ_{C} 12.3) of **2** corresponded well to those of **5** (C-18: δ_{C} 65.9, C-19: δ_{C} 43.8, and C-30: δ_{C} 12.3), while those of **2** were slightly different from those of **4**. The absolute configurations of **2** were elucidated to be the same as those of **5**, since the CD spectrum of **2** [λ_{ext} 262 ($\Delta\epsilon$ +0.16) and 220 nm (0)] was quite similar to that of **5** [λ_{ext} 262 ($\Delta\epsilon$ +0.17) and 221 nm (0)]. Thus, amphidinolide B5 (**2**) was assigned as 26-deoxy form of amphidinolide H2 (**5**) or 16-deoxy-16,18-epi form of amphidinolide B (**3**).

Figure 2. Chemical shift differences between amphidinolides B5 (**2**) and H (**4**: frame) or H2 (**5**: solid).



Amphidinolides B4 (**1**) and B5 (**2**) exhibited potent cytotoxicity against murine lymphoma L1210 (IC_{50} : 0.00012 and 0.0014 $\mu\text{g}/\text{mL}$, respectively) and human epidermoid carcinoma KB cells (IC_{50} : 0.001 and 0.004 $\mu\text{g}/\text{mL}$, respectively).

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Experimental

General

The IR and UV spectra were taken on a FT/IR-5300 and a UV-1600PC spectrophotometers, respectively. NMR spectra were recorded on a Bruker AMX-600 spectrometer. Positive-mode ESI mass spectra were obtained on a JEOL JMS-700TZ spectrometer.

Cultivation and Isolation

The dinoflagellate *Amphidinium* species (strain number Y-100) was separated from inside cells of a marine acoel flatworm *Amphiscolops* species, which was collected off Ma'eda Cape, Okinawa. The dinoflagellate was unialgally cultured at 25 °C for two weeks in seawater medium enriched with 1% ES supplement, 16 h light and 8 h dark. The harvested cells (60.5 g, wet weight, from 30 L of culture) were extracted with MeOH/toluene (3:1, 200 mL x 3). After addition of 1 M NaCl aq. (100 L), the mixture was extracted with toluene (100 mL x 3). The toluene-soluble fractions (730 mg) were subjected to a silica gel column (CHCl₃/MeOH, 98:2) to afford a macrolide-containing fraction, which was separated with a Sep-Pak cartridge C₁₈ (MeOH/H₂O, 8:2) followed by C₁₈ HPLC [YMC-Pack Pro C₁₈, 5 μm, YMC Co., Ltd., 10 x 250 mm; eluent, CH₃CN/H₂O (75:25); flow rate, 3 mL/min; UV detection at 210 nm] to give amphidinolides B4 (**1**, 0.42 mg, 0.0008 %, wet weight, *t*_R 23.9 min) and B5 (**2**, 0.10 mg, 0.0002 %, *t*_R 27 min) together with amphidinolide B (**3**, 0.23 mg, 0.0005 mg). Amphidinolides C (0.0025 %) and T1 (0.0028 %) were isolated from another fraction.

Amphidinolide B4 (**1**): colorless oil; $[\alpha]_D^{23} -13^\circ$ (*c* 0.2, CHCl₃); UV (EtOH) λ_{\max} 209 nm (ϵ 6800); IR (neat) ν_{\max} 3436, 2956 and 1704 cm⁻¹; CD (EtOH) λ_{ext} 262 ($\Delta\epsilon$ +0.1) and 235 nm (-0.22); ¹H NMR (CDCl₃) δ 0.85 (3H, d, *J* = 6.5 Hz, H₃-28), 0.98 (3H, d, *J* = 6.7 Hz, H₃-32), 1.06 (3H, d, *J* = 6.7 Hz, H₃-31), 1.15 (1H, m, H-10), 1.20 (1H, m, H-24), 1.24 (3H, d, *J* = 6.7 Hz, H₃-26), 1.42 (1H, m, H-17), 1.48 (1H, m, H-10), 1.56 (1H, m, H-11), 1.71 (3H, s, H₃-30), 1.82 (3H, s, H₃-27), 1.84 ~ 1.92 (3H, m, H-12, H-17, and H-23), 2.05 ~ 2.16 (3H, m, H-5, H-12, and H-24), 2.18 ~ 2.28 (2H, m, H-4 and H-16), 2.34 (1H, m, H-5), 2.42 (1H, m, H-4), 2.61 (1H, brd, *J* = 16.0 Hz, H-19), 2.74 (1H, dd, *J* = 8.9 and 16.0 Hz, H-19), 2.94 (1H, brd, *J* = 9.7 Hz, H-9), 3.14 (1H, brd, *J* = 8.0 Hz, H-8), 3.27 (1H, brs, OH), 3.55 (1H, brs, OH), 3.71 (1H, m, H-22), 3.86 (1H, brs, OH), 3.92 (1H, m, H-18), 4.31 (1H, brs, H-21), 4.81 (1H, s, H-29), 4.97 (1H, s, H-29), 5.08 (1H, m, H-25), 5.16 (1H, dd, *J* = 15.6 and 8.2 Hz, H-7), 5.56 (1H, s, H-14), 5.89 (1H, m, H-6), and 6.73 (1H, m, H-3); ¹³C NMR (Table 1); (+)-ESIMS *m/z* 569 ([M+Na]⁺, 6%), 570 (7%), 571 (11%), 572 (19%), 573 (32%), 574 (48%), 575 (64%), 576 (77%), 577 (87%), 578 (100%), 579 (96%), 580 (92%), 581 (86%), 582 (76%), 583 (64%), 584 (51%), 585 (41%),

586 (31%), 587 (25%), 588 (18%), 589 (14%), 590 (12%), 591 (13%), and 592 (12%); (+)-HRESIMS m/z 569.3467 ($C_{32}H_{50}O_7Na$ require $(M+Na)^+$, 569.3454).

Amphidinolide B5 (2): colorless oil; $[\alpha]_D -25^\circ$ (c 0.2, $CHCl_3$); UV (EtOH) λ_{max} 209 nm (ϵ 6800); IR (neat) ν_{max} 3740, 2923, and 1706 cm^{-1} ; CD (EtOH) λ_{ext} 262 ($\Delta\epsilon$ +0.16) and 220 nm (0); 1H NMR ($CDCl_3$) δ 0.86 (3H, d, J = 6.5 Hz, H_3 -28), 0.92 (3H, d, J = 6.7 Hz, H_3 -32), 0.98 (1H, m, H-10), 1.05 (3H, d, J = 6.7 Hz, H_3 -31), 1.28 (1H, m, H-24), 1.28 (3H, d, J = 6.7 Hz, H_3 -26), 1.45 (1H, m, H-17), 1.59 (1H, m, H-11), 1.64 (1H, m, H-11), 1.70 (1H, m, H-10), 1.71 (3H, s, H_3 -30), 1.82 (3H, s, H_3 -27), 1.82 (1H, m, H-17), 1.91 (1H, m, H-19), 2.05 (1H, m, H-24), 2.12 (1H, m, H-5), 2.22 ~ 2.40 (4H, m, H-4, H-5, H-12, and H-16), 2.45 (1H, m, H-4), 2.53 (1H, dd, J = 9.8 and 16.0 Hz, H-19), 2.97 (1H, brd, J = 9.7 Hz, H-9), 3.06 (1H, brd, J = 8.0 Hz, H-8), 3.09 (1H, d, J = 16.0 Hz, H-19), 3.57 (1H, m, H-22), 3.60 ~ 3.75 (3H, brs, OH x 3), 4.09 (1H, m, H-18), 4.22 (1H, brs, H-21), 4.80 (1H, s, H-29), 4.96 (1H, s, H-29), 5.09 (1H, m, H-25), 5.22 (1H, dd, J = 15.6 and 8.2 Hz, H-7), 5.56 (1H, s, H-14), 5.87 (1H, m, H-6), and 6.73 (1H, m, H-3); ^{13}C NMR (Table 1); (+)-ESIMS m/z 569 ($[M+Na]^+$, 6%), 570 (7%), 571 (11%), 572 (19%), 573 (32%), 574 (48%), 575 (64%), 576 (77%), 577 (87%), 578 (100%), 579 (96%), 580 (92%), 581 (86%), 582 (76%), 583 (64%), 584 (51%), 585 (41%), 586 (31%), 587 (25%), 588 (18%), 589 (14%), 590 (12%), 591 (13%), and 592 (12%); (+)-HRESIMS m/z 569.3448 ($C_{32}H_{50}O_7Na$ require $(M+Na)^+$, 569.3454).

Reference and Notes

1. Kobayashi, J.; Tsuda, M. Amphidinolides, bioactive macrolides from symbiotic marine dinoflagellates. *Nat. Prod. Rep.* **2004**, *21*, 1-18 and references cited therein.
2. Ishibashi, M.; Ohizumi, Y.; Hamashima, M.; Nakamura, H.; Hirata, Y.; Sasaki, T.; Kobayashi, J. Amphidinolide-B, a novel macrolide with potent antineoplastic activity from the marine dinoflagellate *Amphidinium* sp. *J. Chem. Soc., Chem. Commun.* **1987**, 1127-1129.
3. Kobayashi, J.; Ishibashi, M.; Nakamura, H.; Ohizumi, Y.; Yamasu, T.; Hirata, Y.; Sasaki, T.; Ohta, T.; Nozoe, S. Antineoplastic macrolides from the cultured dinoflagellate of the genus *Amphidinium* sp. *J. Nat. Prod.* **1989**, *52*, 1036-1041.
4. Bauer, I.; Maranda, L.; Shimizu, Y.; Peterson, R. W.; Cornell, L.; Steiner, J. R.; Clardy, J. The structures of amphidinolide B isomers: strongly cytotoxic macrolides produced by a free-swimming dinoflagellate *Amphidinium* sp. *J. Am. Chem. Soc.* **1994**, *116*, 2657-2658.
5. Kobayashi, J.; Shigemori, H.; Ishibashi, M.; Yamasu, T.; Hirota, H.; Sasaki, T. Amphidinolides G and H: New potent cytotoxic macrolides from the cultured symbiotic dinoflagellate *Amphidinium* sp. *J. Org. Chem.* **1991**, *56*, 5221-5224.
6. Kobayashi, J.; Shimbo, K.; Sato, M.; Shiro, M.; Tsuda, M. Absolute stereochemistry of amphidinolides G and H. *Org. Lett.* **2000**, *2*, 2805-2807.

7. Kobayashi, J.; Shimbo, K.; Sato, M.; Tsuda, M. Amphidinolides H2 ~ H5, G2, and G3, New Cytotoxic 26- and 27-Membered Macrolides from Dinoflagellate *Amphidinium* sp. *J. Org. Chem.* **2002**, *67*, 6585-6592.
8. Usui, T.; Kazami, S.; Dohmae, N.; Mashimo, Y.; Kondo, H.; Tsuda, M.; Terasaki, G.; A.; Ohashi, K.; Kobayashi, J.; Osada, H. Amphidinolide H, a potent cytotoxic macrolide, covalently binds on actin subdomain 4 and stabilizes actin filament. *Chemistry & Biology* **2004**, *11*, 1269-1277.
9. Kobayashi, J.; Ishibashi, M.; Wälchli, M. R.; Nakamura, H.; Hirata, Y.; Sasaki, T.; Ohizumi, Y. *J. Am. Chem. Soc.* **1988**, *110*, 490-494.
10. Kubota, T.; Tsuda, M.; Kobayashi, J. Absolute stereochemistry of amphidinolide C. *Org. Lett* **2001**, *3*, 1363-1366.
11. Tsuda, M.; Endo, T.; Kobayashi, J. Amphidinolide T, novel 19-membered macrolide from marine dinoflagellate *Amphidinium* sp. *J. Org. Chem.* **2000**, *65*, 1349-1352.
12. Tsuda, M.; Endo, T.; Kobayashi, J. Amphidinolides T2, T3, and T4, new 19-membered macrolides from dinoflagellate *Amphidinium* sp. and biosynthesis of amphidinolide T1. *J. Org. Chem.* **2000**, *65*, 1349-1352.
13. Kubota, T.; Endo, T.; Tsuda, M.; Shiro, M.; Kobayashi, J. Amphidinolide T5, a new 19-membered macrolide from a dinoflagellate and X-ray structure of amphidinolide T1 *Tetrahedron* **2001**, *57*, 6175-6179.

Sample availability: Not available.

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