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

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Received: 6 January 2005 / Accepted: 19 February 2005 / Published: 1 March 2005

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 $^{13}$C–$^{13}$C 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 vicinaly 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 unialgally at 25 °C for 2 weeks in a seawater medium enriched with 1% Provasoli’s Erd-Schreiber (ES) supplement and 1% NaH$^{13}$CO$_3$. 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 SiO$_2$ and/or C$_{18}$ 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, $[\alpha]_D^{23}$ -13° (c 0.2, CHCl$_3$)) showed the pseudomolecular ion peak at m/z 569.5 (M+Na)$^+$ in the ESIMS spectrum, and the $^{13}$C-enrichment was estimated as 32% by the pattern of the pseudomolecular ion peak. The molecular formula of C$_{32}$H$_{50}$O$_8$ 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$_3$.

| posn | 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 (M+Na)$^+$, $\Delta$ +1.3 mmu]. The $^1$H 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 (δH 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 (δC 67.4), C-19 (δC 45.1), and C-30 (δC 13.1) of 1; those of 4 (C-18: δC 67.5, C-19: δC 45.2, and C-30: δC 13.2); those of 5 (C-18: δC 65.9, C-19: δC 43.8, and C-30: δC 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-19b: 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 [λext 262 (Δε +0.1) and 235 nm (-0.22)] of 1 was similar to those of 4 [λext 261 (Δε +0.1) and 235 nm (-0.15)].

Considering the biosynthetical 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° (c 0.2, CHCl$_3$)\} was elucidated to have the same molecular formula, C$_{32}$H$_{50}$O$_8$, as 1. Profiles of the $^1$H 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 (\(\delta_C\) 65.9), C-19 (\(\delta_C\) 43.9), and C-30 (\(\delta_C\) 12.3) of 2 corresponded well to those of 5 (C-18: \(\delta_C\) 65.9, C-19: \(\delta_C\) 43.8, and C-30: \(\delta_C\) 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 \([\lambda_{ext}\;262 (\Delta\varepsilon +0.16)\;and\;220\;nm\;(0)]\) was quite similar to that of 5 \([\lambda_{ext}\;262 (\Delta\varepsilon +0.17)\;and\;221\;nm\;(0)]\). 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 µg/mL, respectively) and human epidermoid carcinoma KB cells (IC$_{50}$: 0.001 and 0.004 µg/mL, respectively).

**Acknowledgments**

We thank Mr. A. Masuda, Yanmar Co., Ltd., for help with dinoflagellate collection, Ms. Y. Fukuda for assistance of dinoflagellate cultivation, and Ms. S. Oka, Center for Instrumental Analysis,
Hokkaido University, for ESIMS measurements. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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 acelo flatworm *Amphiscolops* species, which was collected off Ma’eda Cape, Okinawa. The dinoflagellate was unialgically 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, tₚ 23.9 min) and B₅ (2, 0.10 mg, 0.0002 %, tₚ 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; [α]²³° D -13° (c 0.2, CHCl₃); UV (EtOH) λₘᵢ₇ 209 nm (ε 6800); IR (neat) νₘᵢ₇ 3436, 2956 and 1704 cm⁻¹; CD (EtOH) λₑₓₗ 262 (Δε +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_{7}Na require (M+Na)^{+}, 569.3454).

Amphidinolide B5 (2): colorless oil; [α]D -25° (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 (Δε +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); 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 569.3448 (C_{32}H_{50}O_{7}Na require (M+Na)^{+}, 569.3454).

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Sample availability: Not available.

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