Amphidinolides B6 and B7, Cytotoxic Macrolides from a Symbiotic Dinoflagellate *Amphidinium* Species

Keiko Oguchi,[†] Masashi Tsuda,*[,‡] Rie Iwamoto,[†] Yumiko Okamoto,[†] Taeko Endo,[†] Jun'ichi Kobayashi,[†] Tomoko Ozawa,[§] and Atsunori Masuda[§]

Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan, Center for Advanced Marine Core Research, Kochi University, Kochi 783-8502, Japan, and Marine Farm, Yanmar Co. Ltd., Oita 873-0421, Japan

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Two 26-membered macrolides, amphidinolides B6 (**2**) and B7 (**1**), have been isolated from a marine symbiotic dinoflagellate *Amphidinium* sp., and the structures were elucidated on the basis of detailed analyses of 2D NMR data. The relative and absolute configurations for **1** and **2** were assigned by comparison of NMR data and CD data with those of known amphidinolides.

The amphidinolides are a series of unique cytotoxic macrolides isolated from the dinoflagellates *Amphidinium* sp., which were separated from marine acoel flatworms of the genus *Amphiscolops*.[1] Amphidinolides H[2,3] (**3**) and B,[4–6] which were initially isolated from marine dinoflagellates *Amphidinium* spp. (strains Y-25 and Y-5, respectively), are 26-membered macrolides possessing unique structures such as an allyl epoxide and an *S-cis*-diene. A 27-membered macrolide, amphidinolide G,[2] is the regioisomeric form of **3** at C-26, while amphidinolide L[7] corresponds to a 20-dihydro-21-dehydro derivative of amphidinolide G. Eight amphidinolide H congeners, amphidinolides H2–H5,[8] and B2–B5[6,9] and three amphidinolide G congeneres, amphidinolides G2–G4,[8] have been isolated so far. Among these macrolides, amphidinolides H (**3**) and B exhibit potent cytotoxicity (IC_{50} 0.0045–0.00014 μg/mL) against cultured tumor cells in vitro. In SAR studies of **3**, it was revealed that the presence of an allyl epoxide, an *S-cis*-diene moiety, and a ketone at C-20 was important for the cytotoxicity.[8] Recently, it was revealed that amphidinolide H binds to actin covalently.[10]

During our search for *Amphidinium* strains that produce amphidinolide B or H in significant quantity,[8,9] we have found a new symbiotic *Amphidinium* strain (named HYA002), which produces large amounts of amphidinolide H-related macrolides (>0.1% of dry cell mass). Detailed investigation of the HYA002 extract resulted in the isolation of two new macrolides, amphidinolides B6 (**2**) and B7 (**1**). Herein we describe the isolation and structure elucidation of **1** and **2**.

The dinoflagellate *Amphidinium* sp. (strain HYA002) was isolated from an acoel flatworm (*Amphiscolops* sp.) collected off Sunabe, Okinawa, and was brought into large-scale unialgal culture at 23 °C for 2 weeks in a 3% Provasoli's enriched seawater (PES) medium (enriched with 3 mM $NaHCO_3$). The algal cells (52.3 g, dry weight) obtained from 540 L of culture were extracted with MeOH–toluene (3:1), and the extracts were partitioned between toluene and 1 M aqueous NaCl. The toluene-soluble materials were subjected to SiO_2 gel and C_{18} columns followed by C_{18} HPLC to afford amphidinolides B6 (**2**, 0.003%) and B7 (**1**, 0.003%), together with known related macrolides, amphidinolides H[2,3] (**3**, 0.03%) and H4[8] (**4**, 0.11%).

Amphidinolide B7 [**1**, $[\alpha]_D^{17}$ −22 (*c* 0.2, $CHCl_3$)] was assigned the molecular formula $C_{32}H_{52}O_7$, corresponding to the deoxy form of **4**, from HRESIMS data [*m/z* 571.3618 $(M + Na)^+$, Δ +0.7 mmu]. Although the ^{13}C NMR (Table 1) spectrum of **1** was similar to those of amphidinolides H4[8] (**4**) and H5[8] (**5**), an additional methyl signal (δ_C 21.2) was observed for **1** in place of the oxymethylene signal at δ_C 66.2 and 66.6 (C-26) for **4** and **5**, respectively. The planar structure of **1** was assigned as the 26-deoxy form of **4** or **5** on the basis of ^1H–^1H COSY, TOCSY, HMQC, and HMBC data (Figure 1).

Table 1. ^{13}C NMR Spectroscopic Data (δ_C ppm, mult.) for Amphidinolides B7 (**1**), B6 (**2**), H4 (**4**), and H5 (**5**) ($CDCl_3$, 150 MHz)

position	**1**		**2**		**4**		**5**	
1	167.8	C	177.4	C	168.7	C	168.6	C
2	127.8	C	48.2	CH	127.4	C	127.2	C
3	141.8	CH	71.2	CH	143.1	CH	143.6	CH
4	27.9	CH_2	33.5	CH_2	28.1	CH_2	27.1	CH_2
5	25.3	CH_2	24.9	CH_2	25.2	CH_2	25.0	CH_2
6	28.0	CH_2	25.1	CH_2	27.9	CH_2	27.9	CH_2
7	32.1	CH_2	32.2	CH_2	32.1	CH_2	31.6	CH_2
8	60.0	CH	60.0	CH	59.9	CH	59.1	CH
9	58.3	CH	58.6	CH	58.2	CH	58.9	CH
10	40.5	CH_2	40.6	CH_2	40.5	CH_2	40.1	CH_2
11	29.0	CH	29.3	CH	29.0	CH	28.8	CH
12	47.1	CH_2	47.0	CH_2	47.2	CH_2	47.0	CH_2
13	144.0	C	144.1	C	144.1	C	144.0	C
14	126.2	CH	126.3	CH	126.3	CH	126.0	CH
15	141.5	C	141.5	C	141.4	C	141.0	C
16	41.1	CH	41.2	CH	41.1	CH	41.1	CH
17	40.3	CH_2	40.7	CH_2	40.5	CH_2	41.0	CH_2
18	67.0	CH	67.2	CH	67.5	CH	65.6	CH
19	44.7	CH_2	44.6	CH_2	44.9	CH_2	44.0	CH_2
20	210.8	C	209.9	C	210.8	C	210.2	C
21	77.4	CH	77.9	CH	77.5	CH	78.8	CH
22	75.1	CH	74.7	CH	75.0	CH	76.3	CH
23	32.5	CH	32.4	CH	32.4	CH	32.7	CH
24	40.0	CH_2	40.2	CH_2	34.2	CH_2	34.1	CH_2
25	68.0	CH	67.7	CH	73.4	CH	73.2	CH
26	21.2	CH_3	21.2	CH_3	66.2	CH_3	66.6	CH_3
27	12.3	CH_3	14.2	CH_3	12.3	CH_3	12.3	CH_3
28	17.6	CH_3	17.9	CH_3	17.6	CH_3	17.6	CH_3
29	114.9	CH_2	114.9	CH_2	114.9	CH_2	114.9	CH_2
30	12.6	CH_3	12.7	CH_3	12.7	CH_3	12.5	CH_3
31	20.3	CH_3	20.5	CH_3	20.3	CH_3	20.5	CH_3
32	15.7	CH_3	16.2	CH_3	15.6	CH_3	15.3	CH_3

The relative stereochemistry of **1** was deduced by comparison of ^1H and ^{13}C chemical shifts and ^1H–^1H coupling constants with those of amphidinolides H4[8] (**4**) and H5[8] (**5**) as follows. The ^{13}C NMR data (Table 1) for C-1–C-23 and C-27–C-32 portions of **1** were more closely similar to those of **4** rather than those of **5**. Specifically, the chemical shifts for C-17, C-18, C-19, C-20, C-21, and C-22 of **1** were more similar to those of **4** (chemical shift differences for **1** vs **4**; C-17: −0.2, C-18: −0.5, C-19: −0.2,

* To whom correspondence should be addressed. Tel: +81-88-864-6720. Fax: +81-88-864-6713. E-mail: mtsuda@kochi-u.ac.jp.
[†] Hokkaido University.
[‡] Kochi University.
[§] Yanmar Co Ltd.

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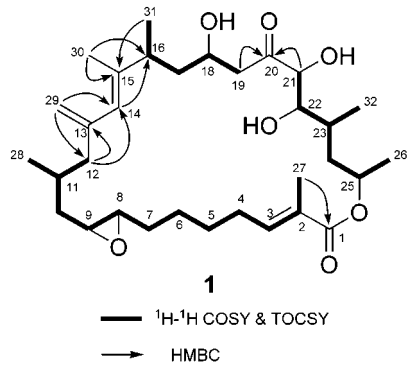
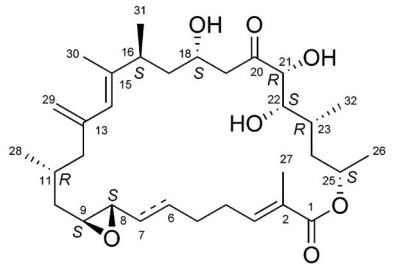
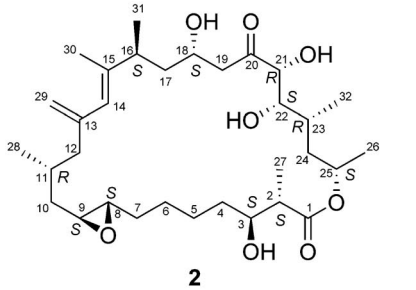
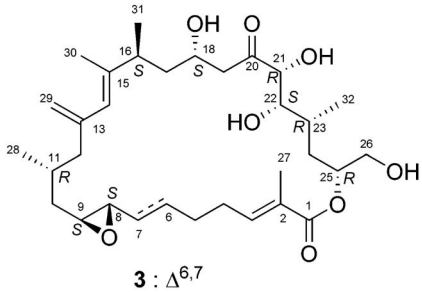
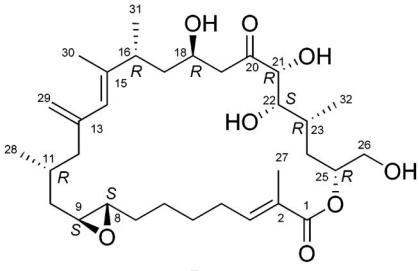


Figure 1. Selected 2D NMR correlations for amphidinolide B7 (**1**).









C-20: 0.0, C-21: −0.1, C-22: +0.1; rmsd 0.1) and different from those of **5** (chemical shift differences for **1** vs **5**; C-17: −0.7, C-18: +1.4, C-19: +0.7, C-20: +0.6, C-21: −1.4, C-22: −1.2; rmsd 0.9). Furthermore, ^{1}H chemical shifts and coupling constants (Table 2) for C-7–C-12 and C-16–C-25 portions of **1** also showed the same trend as those of **4**, while *J*(H-18/H-19a) and *J*(H-18/H-19b) values for **1** (9.0 and 2.0 Hz, respectively) were quite different from those for **5** (2.5 and 10.4 Hz, respectively). Thus, the relative configuration of nine chiral centers, C-8, C-9, C-11, C-16, C-18, C-21, C-22, C-23, and C-25, for **1** was considered to be the same as that for **4**. This was supported by NOESY correlations observed for the C-7–C-26 part of **1** as shown in Figure 2. The CD spectrum [λ_{max} 261 ($\Delta\varepsilon$ +0.1) and 234 nm (−0.18)] for **1** matched those for amphidinolides H (**3**) [λ_{ext} 261 ($\Delta\varepsilon$ +0.1) and 234 nm (−0.15)] and B4[9] (**6**) [λ_{ext} 262 ($\Delta\varepsilon$ +0.1) and 234 nm (−0.22)]. Therefore, the absolute configuration of amphidinolide B7 (**1**) is proposed to be 8*S*, 9*S*, 11*R*, 16*S*, 18*S*, 21*R*, 22*S*, 23*R*, and 25*S*.

Table 2. ^{1}H NMR Spectroscopic Data (δ_H ppm) for Amphidinolides B7 (**1**), B6 (**2**), H4 (**4**), and H5 (**5**) ($CDCl_3$, 600 MHz)

position	1	2	4	5
2		2.45 (quint, 7.0)		
3	6.88 (brt, 7.0)	3.76 (br t, 7.0)	6.97	6.81
4	2.28	1.51	2.31	2.32
	2.16	1.34	2.18	2.22
5	1.56	1.61	1.59	1.60
	1.43	1.50	1.44	1.54
6	1.60	1.54	1.66	1.60
	1.42	1.32	1.46	1.52
7	1.83	1.88	1.90	1.84
	1.15	1.13	1.15	1.21
8	2.74 (dt, 9.0, 2.4)	2.71 (dt, 9.1, 2.4)	2.75	2.83
9	2.91 (dt, 9.5, 2.4)	2.87 (dt, 9.3, 2.4)	2.90	2.97
10	1.50	1.48	1.55	1.52
	1.13	1.11	1.17	1.00
11	1.61	1.63	1.63	1.52
12	2.13 (dd, 4.0, 13.5)	2.13 (dd, 4.2, 13.3)	2.16	2.16
	1.78	1.79	1.80	1.84
14	5.50 (s)	5.48 (s)	5.52	5.56
16	2.20	2.18 (ddq, 4.5, 10.8, 6.6)	2.21	2.22
17	1.80	1.83	1.81	1.84
	1.42	1.42 (ddd, 4.3, 9.7, 14.0)	1.42	1.42
18	3.97	4.01 (ddt, 1.8, 4.0, 9.7)	3.98	4.03
19	2.73 (dd, 9.0, 15.6)	2.73 (dd, 9.7, 15.7)	2.72	2.89
	2.54 (br d, 15.6)	2.54 (dd, 1.8, 15.7)	2.54	2.56
21	4.43 (br s)	4.46 (br s)	4.44	4.27
22	3.69 (br d, 9.0)	3.69 (br d, 9.4)	3.74	3.57
23	1.93	1.90	1.95	1.92
24	1.82	1.79 (br dd, 11.6, 13.8)	2.01	2.42
	1.24	1.20	1.28	1.17
25	5.06 (dq, 10.8, 6.7)	5.18 (ddq, 1.3, 11.6, 6.7)	5.07	5.15
26	1.26[b] (d, 6.7)	1.22[b] (d, 6.7)	3.76	3.76
			3.68	3.72
27	1.80[b] (br s)	1.12[b] (d, 7.0)	1.83[b]	1.83[b]
28	0.86[b] (d, 6.5)	0.86[b] (d, 6.5)	0.88[b]	0.84[b]
29	5.00 (s)	5.00 (s)	5.00	4.97
	4.81 (s)	4.82 (s)	4.83	4.81
30	1.73[b] (br s)	1.74[b] (br s)	1.75[b]	1.75[b]
31	1.05[b] (d, 6.7)	1.06[b] (d, 6.6)	1.07[b]	1.07[b]
32	1.01[b] (d, 6.7)	1.06[b] (d, 6.8)	1.06[b]	0.97[b]

[a] Figures in parentheses denote *J* values (Hz). [b] 3H.

Amphidinolide B6 [**2**, $[\alpha]_D^{17}$ +29 (*c* 0.01, $CHCl_3$)] showed a pseudomolecular ion peak at *m/z* 567 $(M + H)^+$ in the ESIMS spectrum, and the molecular formula of $C_{32}H_{54}O_8$ was assigned on the basis of HRESIMS data [*m/z* 589.3718 $(M + Na)^+$, Δ +0.2 mmu]. The ^{13}C NMR data (Table 1) assigned by HMQC and multiplicity-edited HSQC spectra disclosed 32 carbon signals in total: a ketone, an ester carbonyl, two quaternary sp^2 carbons, an sp^2 methine, an sp^2 methylene, 11 sp^3 methines including seven oxygenated ones, nine sp^3 methylenes, and six methyls. Although the ^{1}H NMR (Table 2) spectrum of **2** was comparable to that of amphidinolide B7 (**1**), the C-3 sp^2 methine and the C-27 allylic methyl resonances typical for amphidinolide B- and H-type macrolides were not observed in the ^{1}H NMR data for **2**, indicating that the trisubstituted double bond at C-2–C-3 was absent from **2**.

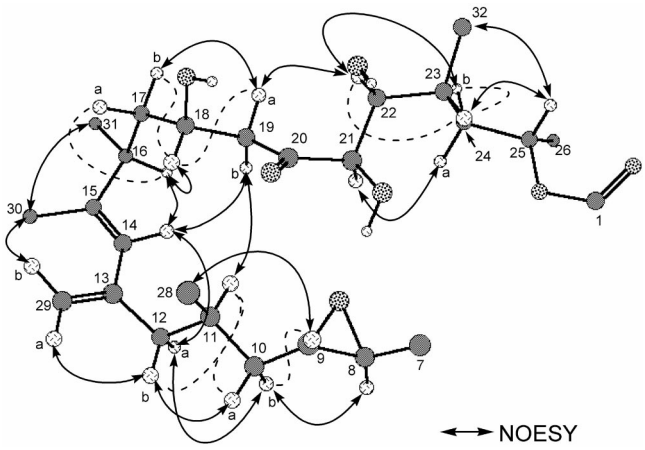


Figure 2. NOESY correlations and stereostructure for C-7–C-26 portion of amphidinolide B7 (**1**). Dotted lines showed anti relations for geminal proton pairs.

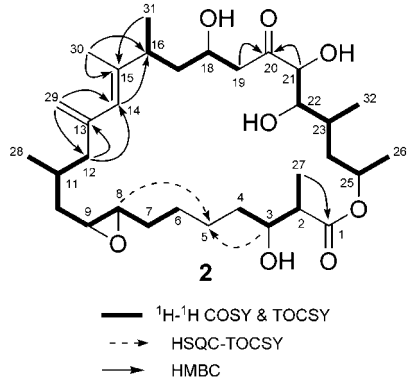


Figure 3. Selected 2D NMR correlations for amphidinolide B6 (**2**).

Detailed analyses of ^{1}H–^{1}H COSY and TOCSY spectra disclosed four spin systems, from H-2 to H_2-4 and H_3-27, from H_2-6 to H_2-12 and H_3-28, from H-16 to H_2-19 and H_3-31, and from H-21 to H_3-26 and H_3-32 (Figure 3). Connectivity between C-4 and C-7 through two methylene carbons (C-5 and C-6) was deduced from HSQC-TOCSY correlations for H-3/C-5 and H-8/C-5. The C-7–C-26 portion of **2** including five methyls (C-28, C-29, C-30, C-31, and C-32) was common to that of **1** and was delineated by HMBC correlations as shown in Figure 3. An HMBC correlation from H_3-27 to the ester carbonyl carbon (C-1: δ_C 177.4) indicated that the ester group was attached to the methine carbon at C-2. The relatively deshielded resonance of H-25 (δ_H 5.18) suggested that C-25 was involved in an ester linkage with C-1. Thus, the planar structure of amphidinolide B6 was assigned as **2**.

Comparison of ^{13}C and ^{1}H chemical shifts and ^{1}H–^{1}H coupling constants (Tables 1 and 2, respectively) for **2** with those of **1**, **4**, and **5** suggested that the relative configuration of the nine chiral centers (C-8, C-9, C-11, C-16, C-18, C-21, C-22, C-23, and C-25) in the C-7–C-26 portion of **2** was the same as that of **1** and **4**, which was supported by NOESY correlations. *Erythro* rotation for the C-2–C-3 bond in **2** was elucidated by a relatively large $J_{(H\text{-}2,H\text{-}3)}$ value (7.2 Hz) and NOESY correlations for H-2/H_2-4, H-3/H_3-27, and H-4a/H_3-27 (Figure 4a). NOESY correlation for H-3/H-25 indicated that H-3 and H-25 were oriented to the same side of the plane across C-3 and C-25, as shown in Figure 4b. Thus, the relative configuration for amphidinolide B6 was assigned as **2**. The CD spectrum [λ_{ext} 262 ($\Delta\varepsilon$ +0.1) and 235 nm (−0.18)] of **2** was similar to those of amphidinolides H (**3**) and B4 (**6**). Therefore, the absolute configuration of **2** was proposed to be 2*S*, 3*S*, 8*S*, 9*S*, 11*R*, 16*S*, 18*S*, 21*R*, 22*S*, 23*R*, and 25*S*.

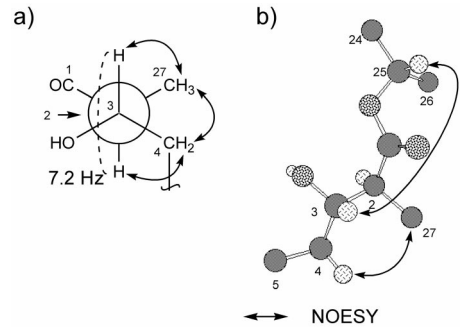


Figure 4. (a) Rotation for C-2–C-3 bond and (b) streostructure for C-24–C-4 portion of amphidinolide B6 (**2**).

Amphidinolide B6 (**2**) is the first oxygenated congener at C-3 in the amphidinolides B- and H-type macrolides, while amphidinolide B7 (**1**) is a 6,7-dihydro form of amphidinolide B4 (**6**). Amphidinolides B6 (**2**) and B7 (**1**) exhibited cytotoxicity against human B lymphocyte DG-75 cells (IC_{50}: 0.02 and 0.4 μg/mL, respectively), both of which were considerably weaker than that (IC_{50}: 0.001 μg/mL) of amphidinolide H (**3**). This is considered to be due to the absence of the double bond at C-6 and agrees with previous structure–activity relationship studies.[8]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-370 polarimeter. IR spectra were recorded on a JASCO FT/IR-5300 spectrophotometer. ^{1}H, ^{13}C, and 2D NMR spectra were measured on a Bruker AMX-600 or AMX-500 spectrometer using 2.5 mm micro cells for $CDCl_3$ (Shigemi Co., Ltd.). Positive mode ESIMS spectra were obtained on a JEOL JMS 700-TZ spectrometer (−80 V as a focus voltage) using a sample dissolved in MeOH with a flow rate of 200 μL/min.

Material. The dinoflagellate *Amphidinium* sp. (strain HYA002) was separated from the internal cells of the marine acoel flatworm *Amphiscolops* sp., which was collected off Sunabe beach, Okinawa Island, Japan. The culture was maintained in sterilized seawater medium enriched with 1% PES supplement at 23 °C under an illumination of about 30 μmol photons μm^{-2} μs^{-1} with 16:8 h light:dark cycle. The small subunit rRNA gene (SSU rDNA) was amplified from a single cell using the primer pairs described previously,[11] and both the coding and noncoding strands were sequenced using an Applied Biosystems thermal cycler GeneAmp PCR Systems 9700 DNA sequencer. Three repetitions of the amplification reaction of SSU rDNA from a single cell were carried out. The DNA sequence was compared with those of the SSU rDNA in the databases using BLAST SEARCH and the SSU rDNAs of *Amphidinium gibossum*[12] (as *A. belauense*, strain 324, accession No. L13719). *A. gibossum* was originally described as a symbiont of the flatworm *Haplodiscus* sp.,[13] and *Amphidinium* sp.[14] (strain Y-42, accession No. AB107845) separated from acoel flatworm *Amphiscolops* sp. was found to be the closest relative (>99% identity). The voucher specimen and the SSU rDNA gene are deposited at the Center for Advanced Marine Core Research, Kochi University.

Cultivation and Isolation. The dinoflagellate was cultured unialgally at 25 °C for 2 weeks in sterilized seawater medium enriched with 3% PES supplement, 16 h light and 8 h dark. The harvested cells (dry weight: 196 g from 540 L of culture) were extracted with MeOH–toluene (3:1, 1000 mL × 3). After addition of 1 M aqueous NaCl (1000 mL), the mixture was extracted with toluene (1000 mL × 3). A portion (8 g) of the toluene-soluble partition (24.63 g) was subjected to SiO_2 gel ($CHCl_3$–MeOH, 98:2) and then C_{18} columns (MeOH–H_2O, 8:2) to give a macrolide-containing fraction (230 mg), part (50 mg) of which was separated by C_{18} HPLC [YMC-Pack Pro C_{18}, 5 μm, YMC Co., Ltd., 10 × 250 mm; CH_3CN–H_2O (3:1); flow rate, 3 mL/min; UV detection at 210 nm] to afford amphidinolides B6 (**2**, 0.42 mg, 0.003%, wet weight, t_R 23.9 min) and B7 (**1**, 0.40 mg, 0.003%, t_R 27 min) together with amphidinolides H (**3**, 4.3 mg, 0.03%) and H4 (**4**, 15 mg, 0.11%).

Amphidinolide B6 (2): colorless oil; $[\alpha]^{17}_D$ +29 (*c* 0.01, $CHCl_3$); IR (neat) ν_{max} 3423, 2925, 1719, and 1458 cm^{-1}; ^{1}H NMR (Table 2)

and ^{13}C NMR (Table 1); ESIMS *m/z* 567 $(M + H)^+$; HRESIMS *m/z* 589.3718 [calcd for $C_{32}H_{54}O_8Na$ $(M + Na)^+$, 589.3716].

Amphidinolide B7 (1): colorless oil; $[\alpha]_D$ −22 (*c* 0.01, $CHCl_3$); UV (EtOH) λ_{max} 209 nm (ε 6800); IR (neat) ν_{max} 3740, 2923, and 1706 cm^{-1}; ^1H NMR (Table 2) and ^{13}C NMR (Table 1); EISMS *m/z* 571 $(M + Na)^+$; HRESIMS *m/z* 571.3618 [calcd for $C_{32}H_{52}O_7Na$ $(M + Na)^+$, 571.3611].

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Supporting Information Available: ^{13}C and ^1H NMR data for compounds **1** and **2**. This information is available free of charge via the Internet at http://pubs.acs.org.

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