Amphidinolide C: The First 25-Membered Macrocyclic Lactone with Potent Antineoplastic Activity from the Cultured Dinoflagellate *Amphidinium* sp.

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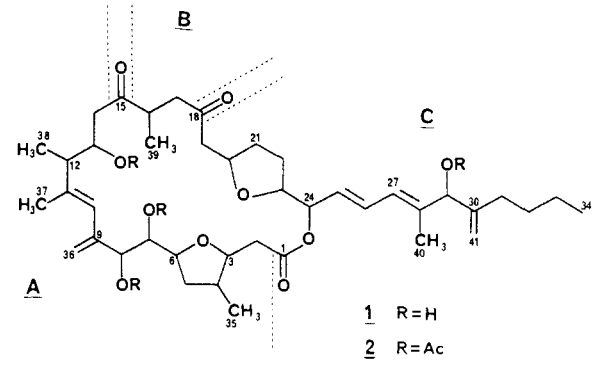
Abstract: Investigation on the extract of the laboratory cultured dinoflagellate *Amphidinium* sp., which was symbiotically associated with an Okinawan flatworm *Amphiscolops* sp., has resulted in the isolation of amphidinolide C, the first 25-membered macrocyclic lactone with potent antineoplastic activity. The structure has been elucidated mainly on the basis of spectroscopic data, especially several types of two-dimensional NMR spectra including ^{1}H-detected heteronuclear multiple-bond correlation method.

Symbiotic microorganisms associated with marine animals are of current interest from the development of natural resources or food hygienic problems. Recently some marine microorganisms such as bacteria,[2] dinoflagellates,[3] or cyanophytes[4] have been demonstrated to be responsible for the production of some marine natural products previously obtained from host or higher animals. Neurotoxins[5-8] from symbiotic dinoflagellates have proved to be essential tools in understanding the molecular basis of cellular excitability.[9] In spite of the growing significance of the research in this field, the chemistry of the metabolites of symbiotic microorganisms has not been well studied[10] because of the difficulty in the isolation and cultivation of those microorganisms. During our investigations on bioactive substances from marine organisms,[11] we have successfully cultivated a dinoflagellate *Amphidinium* sp. isolated from an Okinawan flatworm *Amphiscolops* sp. and previously obtained two antineoplastic macrolides, amphidinolide A[12] and -B.[13] This paper describes the isolation and structure determination of amphidinolide C, another component of antitumor activity in this dinoflagellate. Amphidinolide C, the first 25-membered macrocyclic lactone, has powerful antineoplastic activity (IC_{50} 5.8 ng/mL) against L1210 murine leukemia cells in vitro.

Isolation and Characterization. The dinoflagellate *Amphidinium* sp. was isolated from the inside of the cells of the Okinawan flatworm *Amphiscolops* sp. and mass cultured in the laboratory. The harvested cells were extracted with methanol/toluene, and the extract was subjected to repeated chromatographies on silica gel with methanol/chloroform and hexane/acetone, followed by reversed-phase HPLC on ODS with 88% methanol to give amphidinolide-C (**1**, 0.0015% wet weight) as a colorless amorphous



solid, $[\alpha]^{26}_D$ -106° (*c* 1, $CHCl_3$). The molecular formula, $C_{41}H_{62}O_{10}$, was determined by FABMS ($M^+ + H$, *m/z* 715) and HREIMS ($M^+ - H_2O$, *m/z* 696.4236 for $C_{41}H_{60}O_9$, Δ -0.1 mmu). ^{1}H and ^{13}C NMR studies of **1** revealed the presence of two isolated ketones, an ester carbonyl, five olefins, 12 methines (nine of them bearing oxygen atoms), ten methylenes, and six methyl groups. Multiplicities of the ^{13}C signals were determined by DEPT experiments.[14] Considering that **1** has eleven unsaturations, three rings (two ethers and one lactone) were suggested to be included

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Table I. ^1H and ^{13}C NMR Chemical Shifts (ppm) of the Tetraacetate **2** and Protons to Which a Long-Range Connectivity Was Observed in the HMBC Experiment[c]

position	H	C	HMBC (^1H)
1		170.34 s	H-2, H′-1, H-3, H-24
2	2.58 dd	49.12 t	
2′	2.45 dd		
3	3.81 m	80.79 d	H-2, H′-2, H-5, H-6, H_3-35
4	1.95 m	40.59 d	H-2, H′-2, H_3-35
5	2.16 m	38.00 t	H_3-35
5′	1.32 m		
6	4.08 m	75.74 d	H-7, H-8
7	5.01 dd	75.48 d	H-6, H-8
Ac-7		170.20 s	H-7
	2.08 s[a]	21.12 q[b]	
8	5.35 d	74.98 d	H-7, H-36, H′-36
Ac-8		169.55 s	H-8
	2.05 s[a]	20.73 q[f]	
9		140.93 s	H-7, H-8, H-36
10	5.78 br s	124.59 d	H-8, H-12, H-36, H′-36, H_3-37
11		141.06 s	H-10, H-12, H-13, H_3-37, H_3-38
12	2.63 m	45.31 d	H-10, H′-14, H_3-37, H_3-38
13	5.41 dt	72.20 d	H-12, H-14, H′-14, H_3-38
Ac-13		170.52 s	H-13
	2.09 s[a]	21.14 q[b]	
14	2.83 dd	41.36 t	
14′	2.74 dd		
15		210.25 s	H-13, H-14, H′-14, H-16, H-17, H′-17, H_3-39
16	3.03 m	42.00 d	H-17, H′-17, H_3-39
17	2.90 dd	44.63 t	H-16, H_3-39
17′	2.54 dd		
18		207.11 s	H-16, H-17, H′-17, H-19, H′-19
19	2.64 dd	39.87 t	
19′	2.51 dd		
20	4.40 m	75.37 d	H-19, H′-19
21	2.12 m	32.03 t	
21′	1.54 m		
22	2.00 m	28.26 t	
22′	1.66 m		
23	4.08 m	79.73 d	H-24
24	5.20 t	77.02 d	H-25, H-26
25	5.57 dd	128.56 d	H-24, H-27
26	6.51 dd	130.03 d	H-24
27	6.05 d	126.83 d	H-25, H-26, H-29, H_3-40
28		135.99 s	H-26, H-29, H_3-40
29	5.55 s	80.30 d	H-27, H_3-40, H-41, H′-41
Ac-29		169.69 s	H-29
	2.05 s[a]	20.99 q[b]	
30		145.53 s	H-29, H-41
31	1.91 m (2 H)	31.92 t	H-29, H_2-32, H_2-33, H-41, H′-41
32	1.43 m (2 H)	29.76 t	H_2-33, H_3-34
33	1.32 m (2 H)	22.38 t	H_2-33, H_3-34
34	0.90 t (3 H)	13.93 q	H_2-32, H_2-33
35	1.04 d (3 H)	16.11 q	
36	5.29 s	116.56 t	H-8, H-10
36′	5.08 s		
37	1.84 s (3 H)	16.75 q	H-10, H-12
38	1.10 d (3 H)	14.06 q	H-12, H-13
39	1.16 d (3 H)	16.41 q	H-16, H-17, H′-17
40	1.68 s (3 H)	12.98 q	H-27, H-29
41	5.05 s	111.28 t	H-29
41′	4.96 s		

[a,b] Signals may be reversed. [c] J (H, H) in Hz: 2, 2′ = 15.4; 2′, 3 = 5.0; 4, 35 = 6.5; 6, 7 = 6.8; 7, 8 = 5.1; 12, 38 = 7.0; 12, 13 = 4.6; 13, 14 = 7.8; 13, 14′ = 4.6; 14, 14′ = 16.7; 16, 39 = 7.2; 16, 17 = 6.4; 17, 17′ = 17.9; 19, 19′ = 14.7; 19, 20 = 8.2; 19′, 20 = 4.7; 23, 24 = 7.6; 24, 25 = 7.6; 25, 26 = 15.9; 26, 27 = 10.3; 33, 34 = 7.3.

in the molecule. The ultraviolet spectrum of **1** in methanol, λ_{max} 240 nm (ϵ 26 000), was similar to those of fasciculatin derivatives,[15] indicating that the absorption was due to a diene chromophore.

Outline for the Structure Determination. Extensive 400 and/or 500 MHz NMR analyses were carried out by using the tetra-

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Table II. ^1H NMR Chemical Shifts (ppm) of Amphidinolide C (**1**)[a]

position	H	position	H
1		21	2.10 m
2	2.52 m	21′	1.50 m
2′	2.52 m	22	1.95 m
3	3.83 m	22′	1.65 m
4	1.82 m	23	4.10 dd
5	2.10 m	24	5.25 t
5′	1.45 m	25	5.53 dd
6	3.88 m	26	6.56 dd
7	3.57 m	27	6.11 dd
8	4.14 d	28	
9		29	4.48 s
10	6.01 s	30	
11		31	1.90 m (2 H)
12	2.28 m	32	1.42 m (2 H)
13	3.97 t	33	1.32 m (2 H)
14	2.76 dd	34	0.90 t (3 H)
14′	2.55 dd	35	1.10 d (3 H)
15		36	5.19 s
16	3.16 m	36′	4.97 s
17	3.06 dd	37	1.73 s (3 H)
17′	2.32 dd	38	1.04 d (3 H)
18		39	1.11 d (3 H)
19	2.74 dd	40	1.67 s (3 H)
19′	2.51 dd	41	5.13 s
20	4.39 m	41′	4.96 s

[a] J (H, H) in Hz: 4, 35 = 6.5; 7, 8 = 4.2; 12, 38 = 7.0; 13, 14 = 8.9; 13, 14′ = 2.4; 14, 14′ = 15.2; 16, 39 = 7.1; 16, 17 = 9.2; 16, 17′ = 4.1; 17, 17′ = 17.3; 19, 19′ = 16.2; 19, 20 = 8.5; 19′, 20 = 3.6; 23, 24 = 7.9; 24, 25 = 7.9; 25, 26 = 15.1; 26, 27 = 11.2; 33, 34 = 7.2.

acetate **2**. Two-dimensional NMR techniques, in particular, conventional COSY[16] coupled with double relayed coherence transfer (RCT2) experiment[17] were very efficient for presenting the proposed partial structures: C-2 ~ C-14 (**A**), C-16 ~ C-17 (**B**), and C-19 ~ C-34 (**C**). The assignments of the carbons bearing hydrogens were established by ^1H–^{13}C COSY via one-bond coupling.[18] The phase-sensitive 2D NOESY[19] facilitated by one-dimensional difference NOE experiments[20] afforded useful information to determine the geometries of double bonds. Three segments (**A**–**C**) were separated by three carbonyls (210.25, 207.11, and 170.34 ppm). Since the studies on C–H long-range couplings were quite essential to connect those segments through the carbonyls, the technique of ^1H-detected heteronuclear multiple-bond correlation (HMBC) method[21] was then applied by using 5 mg of **2**.[22] A large number of long-range (two- and three-bond) couplings were detected in the HMBC spectrum, which enabled assignment of the carbons without hydrogens and afforded additional proof for the structures of **A**–**C**. Moreover, the HMBC spectrum clearly established the connectivities of three segments (**A**–**C**) through three carbonyls to construct a whole molecule.

The following detailed discussions on the structure elucidation of amphidinolide C are mainly based on the combination of those NMR spectroscopic analyses. The complete assignments of the ^1H and ^{13}C NMR signals of **2** and long-range connectivities obtained by the HMBC spectrum are presented in Table I. ^1H NMR data on **1** are given in Table II.

Partial Structure A. First, the structure of segment **A** was disclosed as follows. In the COSY spectrum protons at 2.58 and 2.45 ppm (labeled H-2 and H′-2, respectively) showed couplings

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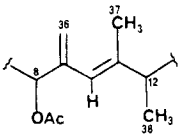
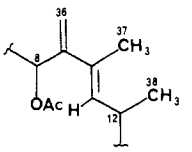
to the proton at 3.81 ppm (H-3). The chemical shifts of H-2 and H′-2 along with the fact that they showed no other couplings in the COSY spectrum suggested that these protons were attached to the carbon adjacent to a carbonyl group. In Figure 1 (Supplementary Material), H-3 was coupled to H-4 at 1.95 ppm, which in turn showed couplings to methyl protons at 1.04 ppm (H_3-35) and one of the methylene protons on C-5 (H′-5 at 1.32 ppm). Although the cross-peak between H-4 (1.95 ppm) and H-5 (2.16 ppm) was overlapped with other signals, further evidences for the connection from C-4 to C-5 were obtained by the RCT2 and HMBC spectra. In the RCT2 spectrum a very weak cross-peak was observed between H-5 and H_3-35 (1.04 ppm), and the HMBC spectrum showed an obvious cross-peak between C-5 (38.00 ppm) and H_3-35.[23] In the RCT2 spectrum the signals for H-5 or H′-5 did not afford clear cross-peak indicating the relayed coupling to H-3, and in the HMBC spectrum H-5 or H′-5 did not show the long-range connectivity to C-4 (40.59 ppm) or C-35 (16.11 ppm) either. Probably the lack of these cross-peaks was due to very broad unresolvable patterns of multiplet proton signals resulting in very low intensity in the relayed or long-range connectivity spectrum. There were some other signals that lacked cross-peaks that one could expect to observe in the RCT2 or HMBC spectrum. In the COSY spectrum H-5 and H′-5 showed couplings to H-6 (4.08 ppm),[24] H-6 was coupled to H-7 (5.01 ppm),[24] and H-7 was coupled to H-8 (5.35 ppm). The RCT2 spectrum showed a cross-peak for H-6/H-8. From these observations the proton connectivities from H-2 and H′-2 to H-8 were established. The comparison of the ^{1}H NMR of **1**[25] and **2** indicated that H-3 and H-6 were observed at nearly the same chemical shifts (H-3: 3.83 ppm for **1** and 3.81 ppm for **2**; H-6: 3.88 ppm for **1** and 4.08 ppm for **2**), whereas H-7 and H-8 were shifted to lower field by acetylation (H-7: 3.57 ppm for **1** and 5.01 ppm for **2**; H-8: 4.14 ppm for **1** and 5.35 ppm for **2**). This fact suggested that C-3 and C-6 bore an ether oxygen atom to form a tetrahydrofuran ring and C-7 and C-8 bore acetoxyl groups, which was confirmed by the HMBC spectrum. The one-dimensional proton spectrum corresponding to the carbon at 80.79 ppm (C-3) showed a small peak at 4.08 ppm (H-6) which was slightly but consistently above noise level, indicating the connectivity between C-3 and H-6 through an oxygen atom.[26] In the HMBC experiment carried out with folded signals to increase the resolution, H-7 (5.01 ppm) and H-8 (5.35 ppm) were clearly shown to be coupled to acetyl carbonyl carbons at 170.20 and 169.55 ppm, respectively.

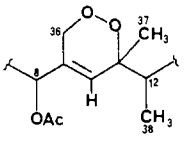
Next to C-8, a diene part C-9 ~ C-11, depicted as **D**, was shown to be connected. The COSY spectrum afforded the following proton connectivities: H-8/H-36 (5.29 ppm); H-8/H′-36 (5.08 ppm); H-8/H-10 (5.78 ppm); H-36/H′-36; H-36/H_3-37 (1.84 ppm); H′-36/H-10; H′-36/H_3-37; H-10/H-12 (2.63 ppm). The *E* configuration of $\Delta^{10(11)}$-double bond and *S*-cis conformation of this diene were deduced by NOE experiments. The NOESY spectrum showed cross-peaks for H′-36/H_3-37, H-10/H-8, and H-10/H-12. Difference NOE experiments were also carried out by using **1**.[25] Irradiation of H_3-37 (1.73 ppm) caused an enhancement in the area of H′-36 (4.97 ppm), while irradiation of



D



E



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H-10 (6.01 ppm) yielded appreciable NOE at H-8 (4.14 ppm) and H-12 (2.28 ppm). By these COSY and NOE data alone, however, one cannot rule out the possibility of structure **E** for the diene part, viz., a methyl group is present on C-10 and the diene adopts *S*-trans conformation. In this case the ^{1}H NMR signal of **2** at 5.78 ppm is assigned to H-11, and H_3-37 at 1.84 ppm is corresponding to the methyl on C-10. The HMBC spectrum gave information to support the structure **D**. The long-range connectivity from H_3-38 (1.10 ppm) to the carbon at 141.06 ppm (s)[27] was observed but that from H_3-38 to the carbon at 124.59 ppm (d) was not detectable.[23] The former carbon bore a methyl group (H_3-37), and the latter carbon bore an olefinic proton resonating at 5.78 ppm. This finding suggested that the former carbon was present within three bonds from H_3-38, namely, assignable to C-11, while the latter carbon was apart from H_3-38 via more than three bonds; therefore, it was labeled C-10. This assignment was unambiguously confirmed by identifying a decomposition product of **2**; in a $CDCl_3$ solution of **2**, the diene moiety C-9 ~ C-11 was slowly oxidized by air to afford a [4 + 2] cycloaddition product **3** [FABMS *m/z* 1020 (M + diethanolamine + H)$^+$]. In the ^{1}H NMR spectrum of **3**, an AB quartet signal was observed at 4.82 and 4.44 ppm (J = 15.8 Hz), which was corresponding to H-36 and H′-36. The singlet methyl signal for H_3-37 resonated at 1.30 ppm, showing upfield shift by 0.54 ppm from that for **2** (1.84 ppm), while H-10 remained in the region of the olefinic proton (5.91 ppm). These observations were completely consistent with the proposed structure **D** for C-9 ~ C-11 position of **2**.

As described above, C-12 bearing a methyl group (C-38) had been demonstrated to be connected to C-11. The COSY spectrum afforded cross-peaks for H-12/H-13 (5.41 ppm), H-13/H-14 (2.83 ppm), and H-13/H′-14 (2.74 ppm). The chemical shift of H-13 (5.41 ppm) implied that an acetoxyl group was attached to C-13. The HMBC spectrum actually showed connectivity from H-13 to an acetyl carbonyl at 170.52 ppm. H-14 and H′-14, the methylene protons on C-14, revealed no other coupling in the COSY spectrum. Their chemical shifts (2.83 and 2.74 ppm) suggested that C-14 was α to a carbonyl group. Therefore, C-14 was considered to be a terminus of partial structure **A**.

Partial Structure B. The COSY spectrum clearly showed that the proton at 3.03 ppm labeled H-16 was coupled to the methyl protons at 1.16 ppm (H_3-39) and also to two protons at 2.90 and 2.54 ppm, labeled H-17 and H′-17, which are methylene protons on C-17. The RCT2 spectrum afforded cross-peaks for H-17/H_3-39 and H′-17/H_3-39. These protons, viz., H-16, H-17, H′-17, and H_3-39, constituted an isolated proton network with no other proton couplings being observed. The chemical shifts of H-16, H-17, and H′-17 suggested that both C-16 and C-17 were adjacent to carbonyl groups.

Partial Structure C. Starting from methylene protons at 2.64 ppm (H-19) and 2.51 ppm (H′-19),[28] whose chemical shifts

(23) The presence of methyl groups was very helpful in interpretating the HMBC spectrum, because three methyl protons enhanced the sensitivity of the long-range correlation by a factor of 3 (see ref 21b).

(24) The signal for H-6 was very severely overlapped with that of H-23, but one can distinguish the pair of cross-peaks for H-6/H-5 and H-6/H′-5 from the pair of cross-peaks for H-23/H-22 and H-23/H′-22, the former resonating at a slightly higher field than the latter. Similarly, the cross-peak for H-6/H-7 was at a slightly higher field than that for H-23/H-24.

(25) The ^{1}H NMR signals of **1** (Table II) were also firmly assigned by the COSY spectrum at 500 MHz (spectrum not shown).

(26) Since the H-6 signal was heavily overlapped with H-23, the observation of the connectivity between C-3 and the proton at 4.08 ppm might mean that C-3 was coupled to H-23, viz., C-3 was connected to C-23 through an oxygen and, in that case, C-6 had to be connected to C-20 through an oxygen. However, formation of such seven-membered and 16-membered ether rings is very unlikely from the biogenetical point of view. Although we were not able to see cross-peaks for C-6/H-3 or C-23/H-20, presumably because of the small coupling through oxygen, two tetrahydrofuran rings were expected to be entropically most preferred.

(27) Although the signal at 141.06 ppm (s) was severely overlapped with C-9 (140.93 ppm), the connectivity from H_3-38 to C-9 via five bonds may be ruled out. The possibility of inverse connection of the diene C-9 ~ C-11 between C-8 and C-12 can be excluded since the connection from C-8 to C-9 was firmly established by the COSY and RCT2 spectra. The relayed coupling from H-7 to H-36, especially, was observed in Figure 2 (Supplementary Material).

suggested that this methylene (C-19) was also α to a carbonyl, connectivity was observed in the COSY spectrum to the proton at 4.40 ppm (H-20), which was then coupled to two protons at 2.12 ppm (H-21) and 1.54 ppm (H′-21). It was relatively difficult to identify the connectivity from C-21 to C-22 in the COSY, RCT2, or HMBC spectrum of **2**, since cross-peaks were very weak or absent due to the broadness of the multiplet signals for these methylene protons. However, in the COSY spectrum very weak cross-peaks for H-21/H′-22 (1.66 ppm) and H′-21/H′-22 were detected, and in the RCT2 spectrum a weak cross-peak for H′-21/H-23 was observed. In addition, significant cross-peaks for H-21/H-22, H-21/H′-22, H′-21/H-22, and H′-21/H′-22 were obtained by the COSY spectrum of **1**,[25] confirming the connection between C-21 and C-22. Then, the COSY spectrum afforded cross-peaks for H-22 (2.00 ppm)/H-23 (4.08 ppm) and H′-22 (1.66 ppm)/H-23.[24] The chemical shifts of H-20 (4.40 ppm) and H-23 (4.08 ppm) implied that these protons were attached to oxygen-bearing carbons, which strongly suggested that C-20 and C-23 bore an ether oxygen atom to form another tetrahydrofuran ring[26] since the presence of two ether rings had to be considered from the unsaturation number as described before. In the COSY spectrum H-23 showed coupling to H-24 (5.20 ppm),[24] which in turn was coupled to H-25 (5.57 ppm) and H-26 (6.51 ppm). Starting from H-25, the connectivity of the second diene part C-25 ~ C-28 with a methyl group (C-40) on C-28 was easily deduced by analyzing the COSY and RCT2 spectra. The COSY spectrum showed cross-peaks for H-25/H-26, H-26/H-27 (6.05 ppm), and H-27/H_3-40 (1.68 ppm), and the RCT2 spectrum afforded additional cross-peaks for H-24/H-27, H-25/H-27, and H-26/H_3-40. The stereochemistry of this diene was determined to be 25*E* and 27*E* by the ^1H–^1H coupling constant ($J_{25,26}$ = 15.9 Hz) and difference NOE experiment with use of **1**.[29] On irradiation of H_3-40 (1.67 ppm), an obvious NOE was observed at H-26 (6.56 ppm).

In the COSY spectrum H-27 and H_3-40 were coupled to the proton at 5.55 ppm (H-29), which was, however, slightly overlapped with H-25. The HMBC spectrum afforded unambiguous connectivity from C-28 to C-29, namely, C-27 (126.83 ppm) and C-28 (135.99 ppm) showed long-range connectivities to H-29 (5.55 ppm) and C-29 (80.30 ppm)[30] and also coupling to H-27 (6.05 ppm). H-29 was shown to be coupled to an acetyl carbonyl at 169.69 ppm, which indicated that C-29 bore an acetoxyl group. The COSY spectrum afforded obvious cross-peaks for H-29/H-41 (5.05 ppm) and H-29/H′-41 (4.96 ppm). C-29 was, therefore, connected to C-30 which bore an exo methylene group (C-41); indeed, in the HMBC spectrum C-30 (145.53 ppm) showed connectivities to H-29 (5.55 ppm) and H-41 (5.05 ppm). In the COSY spectrum H-41 and H′-41 were shown to be coupled to H_2-31 (1.91 ppm), then cross-peaks for H_2-31/H_2-32 (1.43 ppm), H_2-32/H_2-33 (1.32 ppm), and H_2-33/H_3-34 (0.90 ppm) were relatively easily distinguished indicating an *n*-butyl group, which eventually turned out to be a terminus of the molecule.

Whole Structure. Partial structures of **A**–**C** were demonstrated to be connected through three carbonyls by the HMBC spectrum as follows. The lactone carbonyl at 170.34 ppm (C-1) showed connectivities to the methylene protons on C-2 (H-2 and H′-2) and also to H-24. In the ^1H NMR of **1** and **2** H-24 was observed at nearly the same chemical shift (5.25 ppm for **1** and 5.20 ppm for **2**), which had suggested that C-24 was the lactone terminal position. The carbonyl of the lowest resonance (210.25 ppm) was coupled to H-13, H-14, H′-14, H-16, H-17, H′-17, and H_3-39, whereas the carbonyl at 207.11 ppm showed connectivities to H-17, H′-17, H-19, and H′-19. Therefore, the former carbonyl, labeled C-15, was connected to C-14 and C-16; on the other hand, the latter carbonyl, labeled C-18, was connected to C-17 and C-19. Thus the three fragments of **A**–**C** have now achieved to be connected through three carbonyls, and the whole structure of amphidinolide C was, therefore, concluded to be **1**.[31]

Discussion

Amphidinolide C (**1**), the first 25-membered macrocyclic lactone from a natural source,[32] exhibits potent antitumor activity in vitro. Its molecular constitution is new and substitution patterns are different even from those of amphidinolide A[12] or -B[13] previously obtained from the same dinoflagellate. Their biogenetic relationships and biological roles may raise interesting questions, since a large difference in the strength of antitumor activity was observed between **1** and amphidinolide A or -B.[33] Recently, a number of macrolides with structural similarities have been isolated from sponges,[34] nudibranchs,[35] or cyanophytes.[36] There remain questions as to whether those macrolides were produced by host animals or symbiotic microorganisms.

Experimental Section

General Methods. ^1H and ^{13}C NMR spectra were recorded on a Bruker AM-400 or AM-500 spectrometer in $CDCl_3$. The 7.27 ppm resonance of residual $CHCl_3$ and 76.9 ppm of $CDCl_3$ were used as internal references for ^1H and ^{13}C NMR, respectively. Mass spectra were obtained on a JEOL JMS-D300 spectrometer operating at 70 eV (for EI) and a JEOL HX-100 spectrometer by using glycerol or diethanolamine as a matrix (for FAB).

Cultivation. The dinoflagellate *Amphidinium* sp. was symbiotically associated inside of the apocyte or vacuole of a marine flatworm *Amphiscolops* sp. (0.2-mm wide and 0.5-mm long, green color), which was collected at Chatan beach, Okinawa, Japan. The dinoflagellate was isolated out of the flatworm, washed well with sterilized sea water, and designated strain number Y-5. Uni-algal cultures of Y-5 were grown in 3-L glass bottles containing 2 L of sea water medium enriched with Provasoli's ES supplement,[37] which consisted of the following elements in 3 L of distilled water: $NaNO_3$, 10.5 g; $Na_2C_3H_5(OH)_2PO_4 \cdot 5.5H_2O$ (sodium glycerophosphate), 1.5 g; $FeCl_3 \cdot 6H_2O$, 138 mg; $Na_2EDTA \cdot 2H_2O$ (ethylenediaminetetraacetic acid, disodium salt), 900 mg; H_3BO_4, 150 mg; $MnCl_2$, 30 mg; $ZnCl_2$, 3.8 mg; $CoCl_2$, 0.75 mg; vitamin B_{12}, 0.3 mg; thiamine hydrochloride (vitamin B_1), 15 mg; *D*-biotin (vitamin H), 0.15 mg; tris(hydroxymethyl)aminomethane, 15 g. The pH of the supplement was adjusted to 7.8 with 3 M hydrochloric acid, prior to mixing with sea water which was sterilized by autoclaving. Cultures were incubated statically at 25 °C in an apparatus where illumination from a fluorescent light source was supplied in a cycle of 16-h light and 8-h darkness. After 2 weeks the culture was harvested by suction of the supernatant media with an aspirator, followed by centrifugation, to yield harvested cells ranging from 0.3 to 0.5 g/L of culture.

Isolation. The harvested cells (375 g, wet weight) were extracted with methanol/toluene (3:1, 500 mL × 3). After addition of 1 M NaCl (0.75 L), the mixture was extracted with toluene (250 mL × 4). The toluene-soluble fraction was evaporated under reduced pressure to give a crude extract (13 g), which was subjected to silica gel column chromatography (Wako gel C-300, Wako Chemical, 3.4 × 44 cm) eluted with methanol/chloroform (5:95). The fraction eluting from 1000 to 1200 mL was further separated by the second silica gel column chromatography (Wako gel C-300, 1.1 × 30 cm) eluted with hexane/acetone (2:1). The fraction eluting from 80 to 130 mL was finally purified by HPLC (De-

(28) In the ^1H–^{13}C COSY spectrum (Figure 3, Supplementary Material) a weak cross-peak for C-19/H′-19 (2.51 ppm) was observed but that for C-19/H-19 (2.64 ppm) was absent due to low intensity of the C-19 signal. The cross-peak for C-2/H-2 or C-2/H′-2 was also absent, but the assignment of C-2 was able to be made by process of elimination since all other methylene carbons were assigned from Figure 3 (Supplementary Material).

(29) The NOESY spectrum of **2** also afforded strong cross-peaks for H-26/H_3-40.

(30) By the expansion plot of the HMBC spectrum (partly shown in Figure 4b, Supplementary Material), C-29 (80.30 ppm) was easily distinguished from C-3 (80.79 ppm) or C-23 (79.73 ppm).

(31) The stereochemistry of all chiral centers remains undefined.

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velosil ODS-5, Nomura Chemical, 10 × 250 mm; flow rate, 2.5 mL/min; UV detection at 254 nm; eluant, 88% methanol) to afford amphidinolide C (**1**, t_R 10.9 min; 6 mg) in 0.0015% yield (wet weight).

Amphidinolide C (1): colorless amorphous solid; $[\alpha]^{26}_D$ −106° (*c* 1, $CHCl_3$); UV (MeOH) 240 nm (ε 26 000); IR (film) 3400, 2930, 1705, and 1035 cm^{-1}; ^1H NMR (Table II); ^{13}C NMR ($CDCl_3$) δ 12.52 (q), 13.81 (q), 14.45 (q), 15.33 (q), 15.45 (q), 16.09 (q), 22.35 (t), 28.15 (t), 30.02 (t), 31.42 (t), 31.89 (t), 36.81 (t), 38.74 (t), 39.79 (d), 42.54 (d), 45.47 (t), 46.00 (t), 48.34 (t), 49.16 (d), 70.70 (d), 74.91 (d), 76.26 (d), 76.79 (d), 77.20 (d), 78.77 (d), 79.65 (d), 79.77 (d), 81.29 (d), 110.20 (t), 115.71 (t), 124.54 (d), 124.95 (d), 127.29 (d), 130.75 (d), 139.88 (s), 140.11 (s), 144.56 (s), 149.13 (s), 171.02 (s), 207.66 (s), and 213.63 (s); FABMS (positive ion; glycerol as a matrix) *m/z* 715 $(M + H)^+$ and 697 $(M - H_2O + H)^+$; HREIMS *m/z* 696.4236 ($M - H_2O$; calcd for $C_{41}H_{60}O_9$, 696.4237).

Tetraacetate (2). Amphidinolide C (**1**, 4.7 mg) was treated with 0.3 mL of acetic anhydride and 0.3 mL of pyridine at room temperature for 17 h. After evaporation of the solvent, purification by silica gel column chromatography (Wako gel C-300, 7 × 70 mm) eluted with hexane/acetone (3:1) afforded the tetraacetate (**2**, 4.0 mg): ^1H and ^{13}C NMR (Table I); FABMS (positive ion; diethanolamine as a matrix) *m/z* 988 $(M + \text{diethanolamine} + H)^+$.

Compound 3. In the $CDCl_3$ solution of **2** (5 mg in 0.5 mL), **2** was slowly oxidized by air to give the peroxide **3**, which was separated by a silica gel column chromatography (Wako gel C-300, 7 × 70 mm) eluted with hexane/acetone (3:1). After 3-4 months, the ratio of **2** and **3** in the $CDCl_3$ solution was approximately 3:1. **3**: ^1H NMR ($CDCl_3$) δ 6.51 (dd, *J* = 15.3 and 10.9 Hz, H-26), 6.07 (d, *J* = 10.9 Hz, H-27), 5.91 (s, H-10), 5.64 (dd, *J* = 15.3 and 7.7 Hz, H-25), 5.55 (s, H-29), 5.48 (m, H-13), 5.37 (d, *J* = 4.1 Hz, H-7), 5.28 (dd, *J* = 7.7 and 6.8 Hz, H-24), 5.16 (dd, *J* = 6.1 and 4.1 Hz, H-7), 5.05 (s, H-41), 4.96 (s, H′-41), 4.82 (d, *J* = 15.8 Hz, H-36), 4.44 (d, *J* = 15.8 Hz, H′-36), 4.30 (m, H-20), 4.14 (m, H-23), 4.09 (m, H-6), 3.87 (m, H-3), 3.07 (m, H-16), 2.15, 2.10, 2.04, and 2.04 (each 3 H, s, Ac × 4), 1.69 (3 H, s, H_3-40), 1.30 (3 H, s, H_3-37), 1.06 (3 H, d, *J* = 7.1 Hz, H_3-39), 1.05 (3 H, d, *J* = 6.5 Hz, H_3-38), 0.97 (3 H, d, *J* = 7.3 Hz, H_3-35), and 0.90 (3 H, t, *J* = 7.3 Hz, H_3-34); FABMS (positive ion; diethanolamine as a matrix) *m/z* 1020 $(M + \text{diethanoamine} + H)^+$.

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Supplementary Material Available: Figures 1-4 consisting of the COSY, RCT2, ^1H-^{13}C COSY, and HMBC spectra of **2** (6 pages). Ordering information is given on any current masthead page.

Reaction of Singlet Oxygen with Thiirane: Peroxysulfenic Acid Intermediate as a New Oxidizing Species[1]

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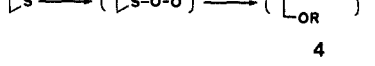
Abstract: The reaction of singlet oxygen with 7-thiabicyclo[4.1.0]heptane (cyclohexene sulfide) in methanol has been investigated. The first example for epoxidation of olefins by the active oxidizing species generated in photooxygenation of the thiirane is provided. It suggests that the active oxidizing species is probably the peroxysulfenic acid intermediate derived from ring opening of a thiirane peroxide intermediate by nucleophilic attack of methanol.

The oxidation of sulfur compounds with singlet oxygen ($^1O_2$) has been extensively studied, and much attention has been devoted to their structures and the reactivities of peroxidic intermediates.[2-7]

Scheme I



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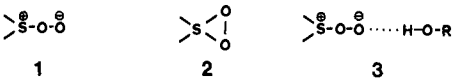
Recently, Foote et al.[4g] elegantly proposed that there are two intermediates in the singlet oxygenation reaction of sulfide in aprotic solvents, in which an initial nucleophilic persulfoxide intermediate **1** reacts with an electrophile such as diphenyl sulfoxide, loses singlet oxygen, or collapses to an electrophilic thiadioxirane intermediate **2** that reacts with a nucleophile such as sulfide. Meanwhile, in protic solvents the persulfoxide intermediate is stabilized by hydrogen bonding as **3**.[4e,7] The function of the alcohol was interpreted as decreasing the negative charge density on the persulfoxide, thus promoting nucleophilic attack by a second sulfide. Since no direct trapping of the intermediates has been achieved,[8] however, the structures of them are still controversial.[2-7] Accordingly, the molecule that may serve as a diagnostic test for a persulfoxide intermediate is clearly desirable for mechanistic studies of the oxidation reaction of sulfides. The candidate is thiirane.[5f,9] The particular advantage of thiirane



1 **2** **3**

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