Amphidinolides and Its Related Macrolides from Marine Dinoflagellates

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Abstract This review covers the recent results described in our publications on several new cytotoxic macrolides isolated from dinoflagellates of the genus *Amphidinium* in addition to an overview of the isolation, structure elucidation, synthesis, biosynthesis, and bioactivity of a series of cytotoxic macrolides, named amphidinolides, reported so far.

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Keywords marine dinoflagellates, *Amphidinium* sp., macrolides, amphidinolides, stereochemistry, total synthesis

1. Introduction

Marine dinoflagellates, a diverse group of unicellular eukaryotes, have been recognized as real producers of marine toxins responsible to fish and algal poisoning, as well as biologically unique organisms due to their taxonomic position and unusual chromosome structure and composition. Marine dinoflagellates have also proved to be one of the most important source of bioactive natural products, which have been investigated worldwide. We have continuously studied structurally intriguing and biologically interesting bioactive macrolides and polyketides from dinoflagellates *Amphidinium* sp., which are symbionts of Okinawan marine flatworms *Amphiscolops* sp.

Thirty-four cytotoxic macrolides, designated amphidinolides A~H (1~8), J~S (9~18), T1 (19), U~Y (20~24), G2 (25), G3 (26), H2~H5 (27~30), and T2~T5 (31~34), have been isolated from *Amphidinium* sp. by our group until 2003, (Fig. 1) [1~6]. Isolation yields and cytotoxicity of the amphidinolides are shown in Table 1. Due to their unique structures and potent cytotoxicity, amphidinolides have been fascinating targets for total synthesis. Total syntheses of amphidinolides A (1), J (9), K (10), P (15), R (17), T1 (19), T3~T5 (32~34), W (22), X (23), and Y (24) have been achieved so far.

Recently, eleven new macrolides, amphidinolides B4 (35), B5 (36), and C2 (37), amphidinolactones A (38) and B (39), iriomoteolides 1a~1c (40~42) and 3a (43), and amphidinolides B6 (45) and B7 (44), (Table 1) have been isolated from five strains (Y-100, Y-71, Y-25, HYA024, and HYA002) of the genus *Amphidinium*, which were collected at different location in Okinawa. This review covers recent progress on the isolation and structure elucidation of these cytotoxic macrolides as well as the molecular target of amphidinolide H (8), cloning of the putative polyketide synthase (PKS) gene of amphidinolide biosynthesis, and synthesis of some amphidinolides recently achieved by other groups. This review follows our early reviews published in 1993 [1], 1997 [2], 1999 [3, 4], 2003 [5], 2004 [6], and 2007 [7]. Synthetic works on amphidinolides up to 2000 were reviewed by Chakraborty and Das [8]. Other reviews concerning amphidinolides have been also reported [9~14].

2. Culture of Dinoflagellates *Amphidinium* sp.

Large-scale cultures of the dinoflagellates of the genus *Amphidinium* have been performed using seawater medium

enriched with Provasoli's Erd-Schriber (ES) supplement. Static incubation with illumination in a cycle of 16 hours of light and 8 hours of darkness was carried out for two weeks at 25°C. The cultures were harvested by removal of the supernatant through suction and then centrifugation to obtain algal cells. Harvested cells were extracted with MeOH-toluene, and the extracts were subjected to a systematic separation using several chromatographies to yield cytotoxic macrolides [15~26].

3. Isolation of New Macrolides

3-1. Amphidinolactones A and B

A new 13-membered macrolide, amphidinolactone A (38), $C_{20}H_{30}O_4$, was isolated from a strain (Y-25) of a dinoflagellate *Amphidinium* sp. [15]. Detailed analyses of the 1H - 1H COSY spectrum of 38 revealed connectivities of a long carbon chain from C-2 to C-20. 1H and ^{13}C chemical shifts of CH_2 -2 and CH -12 suggested that C-12 was involved in an ester linkage with C-1. 1H - 1H couplings of the two disubstituted double bonds at C-5 and C-9 indicated the *Z* and *E* geometries, respectively. Geometries of two disubstituted double bonds at C-14 and C-17 were assigned as both *Z* by NOESY correlations and the carbon chemical shift of C-16, which was a typical value for a methylene carbon between two *Z* double bonds. Thus, the gross structure of amphidinolactone A was elucidated to be 38. The relative configurations at C-8, C-11, and C-12 in 38 were deduced from NOESY correlations.

A new 26-membered macrolide, amphidinolactone B (39), $C_{32}H_{54}O_8$, has been isolated from the same dinoflagellate *Amphidinium* sp. (strain Y-25) as described above [16]. Detailed analyses of the 1H - 1H COSY and TOCSY spectra as well as HMBC correlations indicated connectivities of a long carbon chain from C-2 to C-26. 1H and ^{13}C chemical shifts of C-25 indicated that C-25 was involved in an ester linkage with C-1. The NOESY correlation for H-2/H-25 also supported the connectivity of C-25 to C-2. The connectivity of C-19 to C-21 through a remaining keto carbonyl at C-20 was deduced from the molecular formula of 39 and the NOESY correlation. The presence of a tetrahydrofuran ring was deduced from deuterium-induced shift of oxymethine carbons in the HSQC spectra of 39. The 1H - 1H coupling of the disubstituted double bond at C-7 indicated the *E* geometry. The *E* geometry of the double bond at C-13 was suggested from the NOESY correlation and the ^{13}C chemical shift of C-29. Thus, the gross structure of amphidinolactone B was elucidated to be 39. The relative stereochemistry of the tetrahydrofuran ring was deduced from NOESY

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14 R=O
15 R=CH₂

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Fig. 1 Structures of amphidinolides A~H, J~S, T1, U~Y, G2~G3, H2~H5, and T2~T5 (1~8, 9~18, 19, 20~24, 25~26, 27~30, and 31~34, respectively).

correlations, while that of C-2, C-22, C-23, and C-25 was assigned from ^1H - ^1H couplings and NOESY correlations. Furthermore, considering conformation of the macrocyclic ring, the relative stereochemistries of the C-21~C-25 and C-1~C-2 moieties were elucidated.

3-2. Iriomoteolides 1a~1c and 3a

A potent cytotoxic 20-membered macrolide, iriomoteolide

1a (**40**), $\text{C}_{29}\text{H}_{46}\text{O}_7$, has been isolated from a benthic dinoflagellate *Amphidinium* sp. (strain HYA024) [17]. The gross structure of **40** was mainly elucidated on the basis of 2D NMR data. The relative stereochemistry of **40** was deduced from detailed analyses of bond rotations based on ^1H - ^1H and ^{13}C - ^1H coupling constants and ROESY correlations. The absolute stereochemistry of **40** was assigned by a modified Mosher's method.

Table 1 Lactone ring size, isolation yields, and cytotoxicity data for amphidinolides A~H, J~S, T1, U~Y (**1**~**8**, **9**~**18**, **19**, and **20**~**24**, respectively), B4 (**35**), B5 (**36**), B6 (**45**), B7 (**44**), and C2 (**37**), amphidinolactones A (**38**) and B (**39**), and iriomoteolides 1a (**40**), 1b (**41**), 1c (**42**), and 3a (**43**)

Compd.	Lactone ring size	Isolation yields (10 ^{−4} %)										Cytotoxicity (IC ₅₀ ^a , ^b μg/ml)	
		Strain no. ^a										L1210 ^c	KB ^d
		Y-5	Y-25	Y-26	Y-42	Y-56	Y-71	Y-72	Y-100	HYA002	HYA024		
1	20	20										2.0	5.7
2	26	10	0.8				17					0.00014	0.0042
35	26								8			0.00012	0.001
36	26								2			0.0014	0.004
45	26									30		^g	^g
44	26									30		^g	^g
3	25	15		0.3			9	12				0.0058	0.0046
37	25						1.5					0.8	3
4	26	4										0.019	0.08
5	19	4										2.0	10
6	25			0.1			6					1.5	3.2
7	27		20		8			46				0.0054	0.0059
8	26		17		7			82		300		0.00048	0.0005
9	15	60										2.7	3.9
10	19	0.3										1.65	2.9
11	27		2									0.092	0.1
12	29	4										1.1	0.44
13	26	9										0.00005	0.00006
14	15	1										1.7	3.6
15	15	2										1.6	5.8
16	12	0.5										6.4	>10
17	15	5										1.4	0.67
18	16	1										4.0	6.5
19	19					50	9.2					18	>20
20	20					2						12	>20
21	14	0.5										3.2	7
22	12				90							3.9	>10
23	16 ^e				4							0.6	7.5
24	17				7							0.8	8.0
38	13	0.1										8	>10
39	26	0.1										3.3	5.3
40	20										280 ^f	^g	^g
41	20										20 ^f	^g	^g
42	20										70 ^f	^g	^g
43	15										150 ^f	^g	^g

^a *Amphidinium* sp. ^b 50% inhibition concentration. ^c Murine lymphoma cells. ^d Human epidermoid carcinoma cells. ^e Macrodiolide. ^f Dry weight. ^g not tested.

Two cytotoxic 20-membered macrolides, iriomoteolides 1b (**41**) and 1c (**42**), C₂₉H₄₆O₇ and C₃₀H₄₈O₇, respectively, have been also isolated from the same dinoflagellate *Amphidinium* sp. as described above (strain HYA024) [18].

The gross structure of **42** was assigned as a homologue

of **40** with a 4-hydroxy-3-methylpentyl side chain. Comparing ROESY data and ¹H-¹H coupling constants of **42** with those of **40**, the relative stereochemistry of the macrocyclic ring portion for **42** was elucidated to be the same as that for **40**. Although iriomoteolide 1b (**41**) might

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be an artifact generated from **40**, interchange between **40** and **41** in CHCl_3 or MeOH was not observed.

A cytotoxic 15-membered macrolide, iriomoteolide 3a (**43**), $\text{C}_{25}\text{H}_{38}\text{O}_6$, has been isolated from the same dinoflagellate *Amphidinium* sp. as described above (strain HYA024) [27]. The gross structure of **43** was mainly elucidated on the basis of 2D NMR data. The relative stereochemistry of **43** was deduced from detailed analyses of bond rotations based on ^1H - ^1H and ^{13}C - ^1H coupling constants and NOESY correlations. The absolute stereochemistry of **43** was assigned by a modified Mosher's method.

3-3. Amphidinolides B6 and B7

Two cytotoxic 26-membered macrolides, amphidinolides B6 (**45**) and B7 (**44**), have been isolated from a symbiotic dinoflagellate *Amphidinium* sp. (strain HYA 002) [21]. The molecular formula of amphidinolide B7 (**44**) was assigned as $\text{C}_{32}\text{H}_{52}\text{O}_7$, corresponding to the deoxy form of amphidinolide H4 (**29**). The gross structure of **44** was elucidated to be the 26-deoxy form of amphidinolide H4 (**29**) or amphidinolide H5 (**30**) on the basis of the ^1H - ^1H COSY, TOCSY, HMQC, and HMBC spectra. The relative stereochemistry of amphidinolide B7 (**44**) was elucidated by comparison of the ^1H and ^{13}C chemical shifts and ^1H - ^1H coupling constants of **44** with those of amphidinolides H4 (**29**) [22] and H5 (**30**) [22]. The CD spectrum for **44**

corresponded to those for amphidinolides H (**8**) and B4 (**35**) [23]. Therefore, the absolute configurations of amphidinolide B7 (**44**) were proposed to be as shown.

The molecular formula of amphidinolide B6 (**45**), $C_{32}H_{54}O_8$, was revealed by HRESI-MS data [m/z 589.3718 ($M+Na$)⁺, Δ +0.2 mmu]. The gross structure of amphidinolide B6 was assigned as **45** on the basis of the 1H - 1H COSY, TOCSY, HMQC, and HMBC spectra. The relative stereochemistry of amphidinolide B6 (**45**) was elucidated by comparison of the 1H and ^{13}C chemical shifts and 1H - 1H coupling constants of **45** with those of amphidinolides H4 (**29**) and H5 (**30**). The CD spectrum of **45** was similar to those of amphidinolides H (**8**) and B4 (**35**) [23]. Therefore, the absolute configurations of **45** were proposed to be as shown.

4. Synthetic Studies of Amphidinolides

4-1. Amphidinolide E

Amphidinolide E (**5**) is a cytotoxic 19-membered macrolide possessing a tetrahydrofuran ring, four C_1 branches, and three hydroxyl groups [24]. The stereochemistry of eight chiral centers in **5** remained unresolved due to lack of sample. Recently, we assigned the absolute stereochemistry of **5** by using a sample (2.0 mg) obtained through repeated cultivation [28]. The total synthesis of amphidinolide E (**5**) was achieved by Roush group, *via* a [3+2] annulation reaction of aldehyde **47** and allylsilane **48** followed by olefin metathesis of **46** as a key step (Scheme 1) [29]. Recently, Lee and co-workers synthesized amphidinolide E (**5**) using macrolactonization of **49** through Julia coupling of **50** and **51** derived from **52** *via* a radical cyclization of a β -alkoxy acrylate (Scheme 2) [30]. The total synthesis by

Scheme 1 Retrosynthetic analysis of amphidinolide E (**5**) by Roush's group.

Scheme 2 Retrosynthetic analysis of amphidinolide E (**5**) by Lee's group.

Scheme 3 Retrosynthetic analysis of amphidinolide H (**8**) by Fürstner's group.

Scheme 4 Retrosynthetic analysis of amphidinolide V (**21**) by Fürstner's group.

these two groups confirmed the proposed structure [28].

4-2. Amphidinolide H

Amphidinolide H (**8**) is a 26-membered macrolide possessing an epoxide, six C_1 branches, and four hydroxyl groups with potent cytotoxicity against L1210 and KB cells (IC_{50} : 0.00048 and 0.00052 $\mu\text{g/ml}$, respectively) [25, 26]. The relative configurations at nine chiral centers in **8** were deduced from a single crystal X-ray diffraction analysis. The absolute stereochemistry of amphidinolide H (**8**) was assigned by comparison of the ^1H -NMR data of the tris-(*S*)-MTPA ester of the C-22~C-26 segment derived from natural **8** with those of tris-(*S*)- and -(*R*)-MTPA esters of the C-22~C-26 segment synthesized from methyl (2*S*)-3-hydroxy-2-methyl-propionate [26]. Recently, Fürstner and co-workers synthesized amphidinolide H (**8**) using ring closing metathesis of **53** through sp^2 - sp^2 Stille coupling reaction between **54** and **55** derived from **56** and **57**, respectively, *via* aldol reaction (Scheme 3) [31].

4-3. Amphidinolide V

A cytotoxic 14-membered macrolide, amphidinolide V (**21**), possesses five *exo*-methylenes and one epoxide. The relative configurations at four chiral centers in **21** were deduced from ^1H - ^1H coupling constants and NOESY data [32].

Fürstner and co-workers synthesized amphidinolide V (**21**) using ring-closing alkyne metathesis of **58** through chelation-controlled additions of **59** and **60** derived from **61** and **62**, respectively, *via* an esterification (Scheme 4) [33]. As a result, the relative stereochemistry of amphidinolide V (**21**) was proposed as shown.

4-4. Amphidinolide N

The structure of amphidinolide N (**13**) [34] was interpreted to be a 26-membered macrolide containing a 6-membered hemiacetal ring, an epoxide, a ketone carbonyl, four C_1 branches, and seven hydroxyl groups. This compound was extremely cytotoxic against L1210 and KB cells (IC_{50} : 0.00005 and 0.00006 $\mu\text{g/ml}$, respectively). Although the relative configurations at C-14, C-15, C-16, and C-19 were

Scheme 5 Retrosynthetic analysis of iso-epoxy-amphidinolide N (**63**) by Nicolaou's group.

Scheme 6 Retrosynthetic analysis of des-epoxy-caribenolide I (**68**) by Nicolaou's group.

indicated as shown, the other relative stereochemistry as well as the absolute stereochemistry of **13** have not been determined. Shimizu and co-workers isolated an amphidinolide N-type macrolide, named caribenolide I [35], from a free-swimming dinoflagellate *Amphidinium operculatum* ver nov *Gibbosum*, which showed antitumor activity against murine leukemia P388 *in vivo*.

Nicolaou and co-workers have succeeded in synthesis of iso-epoxy-amphidinolide N (**63**) using macrolactonization of **64** via Enders hydrazone alkylation of three subunits **65**–**67** (Scheme 5) [36, 37]. Nicolaou's group has also achieved the synthesis of des-epoxy-caribenolide I (**68**) using macrolactonization of **69** via Enders hydrazone alkylation of three subunits **65**, **66**, and **70** (Scheme 6) [36, 37]. Figadere and co-workers synthesized the C-1~C-11 [38] and the C-13~C-29 segments of caribenolide I [39].

4-5. Amphidinolides X and Y

Amphidinolide X (**23**) [40] is a cytotoxic 16-membered macrolide consisting of polyketide-driven diacid and diol units. The absolute configurations at C-10 and C-17 were

elucidated to be *S* and *R*, respectively, by application of a modified Mosher's method. A 4*S*-configuration was deduced from comparison of ¹H-NMR data of MTPA esters of the C-1~C-6 segments of the synthetic 1,6-bis-(*R*)-MTPA ester. The structure of amphidinolide Y (**24**) [41] was interpreted to be a cytotoxic 17-membered macrolide existing as a 9:1 equilibrium mixture of 6-keto and 6(9)-hemiacetal forms (**24a** and **24b**, respectively). The absolute stereochemistry of the 6-keto form (**24a**) was assigned on the basis of the spectroscopic data and chemical conversion of **24** into amphidinolide X (**23**) by Pb(OAc)₄ oxidation.

Fürstner and co-workers synthesized amphidinolide X (**23**) using macrolactonization of **71** through alkyl-Suzuki reaction of **73** and **72** derived from **74** and **75**, respectively, via esterification (Scheme 7) [42]. Amphidinolide Y (**24**) was also synthesized by Fürstner's group via macrolactonization of **76** through alkyl-Suzuki reaction of **73** and **77** (Scheme 8) [42]. The absolute stereochemistry of amphidinolides X (**23**) and Y (**24**) was confirmed through these total synthesis. Recently, Dai and co-workers also synthesized amphidinolide Y (**24**) via ring-closing

Scheme 7 Retrosynthetic analysis of amphidinolide X (**23**) by Fürstner's group.

Scheme 8 Retrosynthetic analysis of amphidinolide Y (**24**) by Fürstner's group.

Scheme 9 Retrosynthetic analysis of amphidinolide Y (**24**) by Dai's group.

metathesis of **78** through esterification of **79** and **80** (Scheme 9) [43].

5. Mechanism of Action of Amphidinolides B and H

Amphidinolide B (**2**) increased the ATPase activity of myofibrils and natural actomyosin. The ATPase activity of actomyosin reconstituted from actin and myosin was enhanced in a concentration-dependent manner in the presence or absence of troponin-tropomyosin complex. Ca^{2+} -, K^{+} -EDTA- or Mg^{2+} -ATPase of myosin was not affected by **2**. These results suggest that amphidinolide B (**2**) enhances an interaction of actin and myosin directly and increases Ca^{2+} sensitivity of the contractile apparatus

mediated through troponin-tropomyosin system, resulting in an increase in the ATPase activity of actomyosin and thus enhances the contractile response of myofilament [44].

The molecular target of amphidinolide H (**8**) [25, 26] has been investigated as follows [45, 46]. The analysis of phenotypes of amphidinolide H-treated cells suggested that amphidinolide H (**8**) disrupt the actin organization in the cells, and the polymerization/depolymerization assay using purified actin indicated that amphidinolide H (**8**) stimulated actin polymerization and stabilized F-actin. MALDI-TOF mass analysis and the halo assay using the yeast harboring site-directed mutagenized actin revealed that the covalent binding of amphidinolide H (**8**) to actin and the binding site was Tyr200 of actin subdomain 4. Time-lapse analyses showed that amphidinolide H (**8**) stimulated the formation

of small actin patches, followed by F-actin rearrangement into aggregates *via* the retraction actin fibers. These results indicated that amphidinolide H (**8**) is a novel F-actin stabilizer that covalently binds on actin [45].

To evaluate whether amphidinolide H (**8**) competes at the same binding site with phalloidin, the effect of **8** on phalloidin-actin binding was measured using fluorescein isothiocyanate conjugated phalloidin. Amphidinolide H (**8**) does not compete with, instead enhances, the binding of phalloidin to F-actin, indicating that **8** increased susceptibility of F-actin to phalloidin [46].

6. Biosynthetic Studies of Amphidinolides

The ^{13}C -labeling patterns for amphidinolides B (**2**), C (**3**), G (**7**), H (**8**), J (**9**), T1 (**19**), W (**22**), X (**23**), and Y (**24**) by feeding experiments using ^{13}C -labeled acetates for some amphidinolide producers are shown in Fig. 2 [6]. The incorporation patterns for these amphidinolides revealed that the main chain of these macrolides was generated from unusual units derived only from C-2 of acetates in addition to successive polyketide chains. The experiments also showed that all C1 branched carbons were derived from C-2 of acetates and attached to C-1 of intact acetate or isolated C-2 of acetate. These unusual incorporation

Fig. 2 Acetate-incorporation patterns of amphidinolides C (**3**), H (**8**), and T1 (**19**), and amphidinol 4 (**81**).

patterns could be found in most of dinoflagellate polyketides [9]. For example, a similar labeling pattern of amphidinol 4, a long chain polyene-polyhydroxy compound isolated from *Amphidinium klebsii*, has been reported by Murata and co-workers (Fig. 2) [47].

HCS-like gene cassette has been identified in the curacin A and jamaicamide biosynthetic genes from a cyanobacterium *Lyngbya majuscula* [48, 49], the mupirocin biosynthesis gene from *Pseudomonas fluorescens*, and the bacillaene biosynthesis gene from *Bacillus subtilis*, and is proposed to catalyze addition of C-2 from acetate onto the polyketide chain to generate a pendant functional group [50]. The pendant methyl groups in dinoflagellate polyketides might be constructed by similar genes to HCS-like gene cassette.

Rein and co-workers amplified approximately 700-bp DNA fragments homologous with β -ketoacyl synthase domains in known type I PKSs from seven different species of dinoflagellates by polymerase chain reaction (PCR). They reported the localization of polyketide synthase (PKS) genes by a combination of flow cytometry/PCR and fluorescence *in situ* hybridization (FISH). However, these genes have not yet been linked to dinoflagellate polyketide production [51, 52].

We have attempted to clone the PKS gene responsible for amphidinolide biosynthesis from a dinoflagellate *Amphidinium* sp. (strain Y-42). Fourteen β -ketoacyl synthase genes were obtained by PCR amplification using degenerated primer sets designed from the conserved amino acid sequences of β -ketoacyl synthase domains in known type I PKSs. The PCR analysis revealed that these DNA sequences exist only in the amphidinolide producer. The deduced gene products for insert DNA of a positive clone, which was isolated from the genomic DNA library of *Amphidinium* sp. (strain Y-42), showed similarity to β -ketoacyl synthase, acyl transferase, dehydratase, ketoreductase, acyl carrier protein, and thioesterase in known type I PKSs [53].

7. Conclusions

Recent studies on cytotoxic macrolides, amphidinolides, from dinoflagellates *Amphidinium* sp. indicate that they are expected to be hopeful drug leads or bioprobes useful for basic research in life sciences. The stereochemistry of some amphidinolides remains undefined due to their poor productivity, while the absolute stereochemistry of some amphidinolides has been determined through their total synthesis. Among all the amphidinolides isolated so far, amphidinolides H (8) and N (13) exhibit remarkably potent

cytotoxicity against human tumor cell lines and are expected to be hopeful lead compounds for new anticancer drugs. Though the mechanism of action of amphidinolide H (8) has been studied well, those of other amphidinolides remain to be defined. For further biological testing, the poor productivity of these macrolides needs to be considerably improved. One of the approaches is to identify the polyketide synthase in dinoflagellate *Amphidinium* sp., although it is quite difficult to identify it from the huge genome of the dinoflagellate. Further development of the cell biology and molecular biology of dinoflagellates is required for biomedical and pharmaceutical application of the amphidinolides.

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