Contributed Reviews

Bioactive Macrolides and Polyketides from Marine Dinoflagellates of the Genus *Amphidinium*[⊥]

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Marine microorganisms such as bacteria, cyanobacteria, dinoflagellates, and others have attracted many natural product chemists as the real producers of marine toxins such as fish and algal poisons as well as bioactive substances isolated from marine invertebrates such as sponges and tunicates. Among marine microorganisms, dinoflagellates have proved to be important sources of marine toxins and have been investigated worldwide by natural product chemists. We have continued investigations on chemically interesting and biologically significant secondary metabolites from *Amphidinium* spp., of a genus of symbiotic marine dinoflagellates separated from inside cells of Okinawan marine flatworms. This review covers the results described in our recent publications on a series of cytotoxic macrolides, designated amphidinolides, and long-chain polyketides isolated from *Amphidinium* spp. In this review, topics include the isolation, structure elucidation, synthesis, biosynthesis, and bioactivity of amphidinolides and long-chain polyketides.

Introduction

In a search for bioactive substances from marine organisms, we started a research project for secondary metabolites from symbiotic marine microorganisms in early 1980. When a number of microorganisms were subjected to biological screening, extracts of symbiotic dinoflagellates of the genus *Amphidinium*, which were isolated from the inner cells of acoel flatworms, *Amphiscolops* species, living on algae or seaweeds in Okinawa coral reefs, were found to exhibit potent cytotoxic activity (70−90% inhibition at 3 μg/mL) against murine lymphoma L1210 cells and human epidermoid carcinoma KB cells.

Up to 2003, 34 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**), a linear short polyketide, amphidinin A (**35**), and eight long-chain polyketides, colopsinols A−E (**36**−**40**) and luteophanols A−C (**41**−**43**), have been isolated from *Amphidinium* spp. by our group (Figures 1 and 2).[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 a challenging target for total synthesis. Total syntheses of amphidinolides A (**1**), E (**5**), 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.

In our studies of the biosynthesis of amphidinolides, incorporation patterns of ^{13}C-labeled acetate for amphidinolides B (**2**), C (**3**), G (**7**), H (**8**), J (**9**), T1 (**19**), W (**22**), X (**23**), and Y (**24**) were investigated (Figure 3).[6] The incorporation patterns for 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 revealed that all C_1 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 patterns, which might be generated from nonsuccessive mixed polyketide biosynthesis, could be found in most dinoflagellate polyketides in which their biosyntheses have been studied so far.[7]

Recently, three new amphidinolides, amphidinolides B4 (**44**), B5 (**45**), and C2 (**46**) (Figures 4 and 5), a new linear polyketide, amphidinin B (**47**) (Figure 6), and two new long-chain polyketides, amphezonol A (**48**) (Figure 7) and luteophanol D (**49**) (Figure 8), have been isolated from four strains (Y-52, Y-56, Y-71, and Y-100) of *Amphidinium* sp., which were obtained from different collections of flatworms. This review covers recent progress on the isolation and structure elucidation of cytotoxic macrolides (**44**, **45**) and linear polyketides (**47**−**49**) from marine symbiotic dinoflagellates of the genus *Amphidinium*, as well as the absolute stereochemistry of amphidinolide A (**1**), the molecular target of amphidinolide H (**8**), and cloning of the putative polyketide synthase (PKS) gene for amphidinolide biosynthesis.

Culture of Dinoflagellates *Amphidinium* spp. and Production of Bioactive Macrolides and Polyketides

Large-scale cultures of the dinoflagellates of the genus *Amphidinium* have been performed using seawater medium enriched with Provasoli's Erd-Schreiber (ES) supplement. Static incubation with illumination in a cycle of 16 h of light and 8 h of darkness was carried out for 2 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 methanol−toluene. The extracts were subjected to a systematic separation using several chromatographies to yield bioactive macrolides and polyketides.

Amphidinolides B4 (44) and B5 (45). Two new cytotoxic 26-membered macrolides, amphidinolides B4 (**44**) and B5 (**45**), have been generated from the marine dinoflagellate *Amphidinium* sp. (strain Y-100), which was isolated from the marine acoel flatworm *Amphiscolops* sp., collected off Ma'eda Cape, Okinawa, Japan.[8] *Amphidinium* sp. (strain Y-100) was cultured in the seawater medium enriched with 1% Provasoli's Erd-Schreiber (ES) supplement and 1% $NaH_{13}CO_3$ to give ^{13}C-enriched samples of amphidinolides B4 (**44**) and B5 (**45**). Amphidinolide B4 (**44**), $C_{32}H_{50}O_7$, showed a pseudomolecular ion peak at *m*/*z* 569.5 $(M + Na)^+$ in the ESIMS, and the ^{13}C-enrichment was estimated as 38% by the pattern of the pseudomolecular ion peak. The ^{1}H NMR spectrum of **44** was similar to that of amphidinolide B (**2**). Detailed analyses of the HMQC and INADEQUATE spectra of **44** established the carbon chain from C-1 to C-26 and six C1 branches including five

[⊥] Dedicated to the late Dr. Kenneth L. Rinehart of the University of Illinois at Urbana−Champaign for his pioneering work on bioactive natural products.

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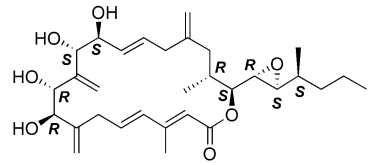
Chart 1



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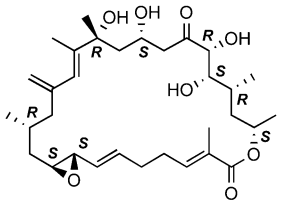

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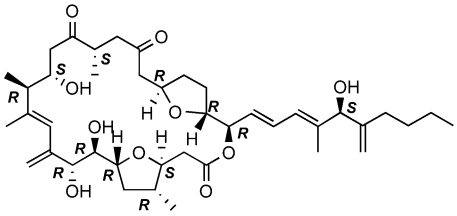

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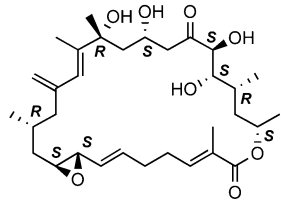

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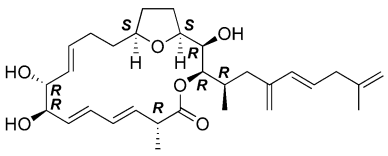

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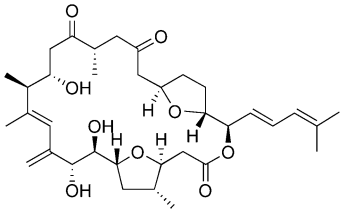

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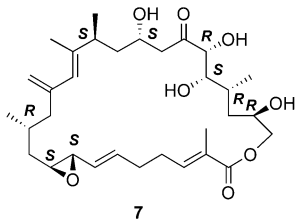

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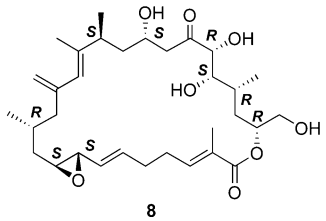

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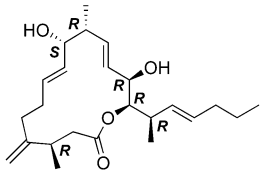

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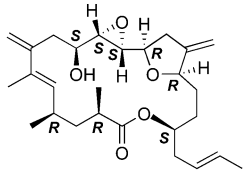

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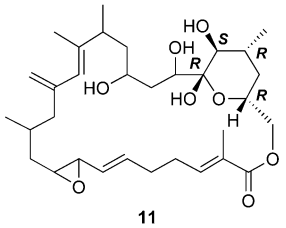

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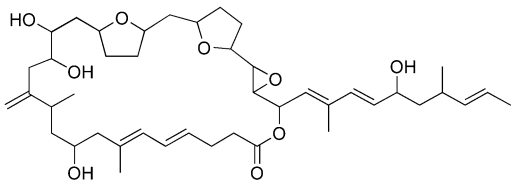

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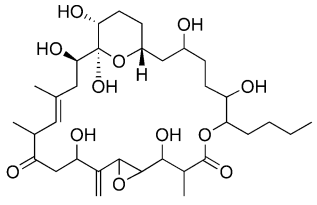

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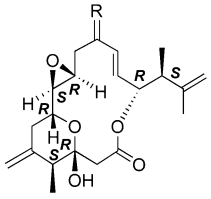
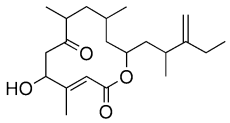


14 R=O

15 R=CH_2

methyls (C-27, C-28, C-30, C-3, and C-32) and an exomethylene (C-29). HMBC correlations for H-3 to C-1, H_3-27 to C-1, and H-25 to C-1 revealed that C-25 is involved in an ester linkage with C-1. Thus, the gross structure of amphidinolide B4 was assigned as **44**.

Chart 2



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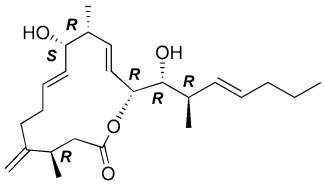

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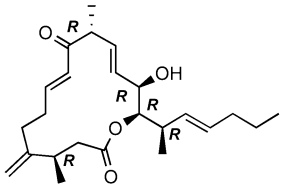

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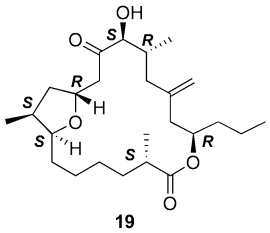

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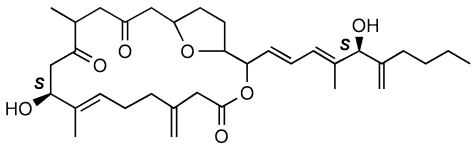

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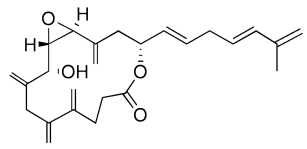

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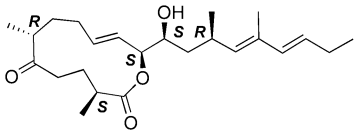

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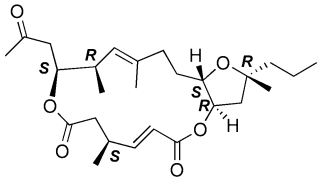

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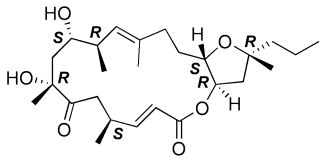

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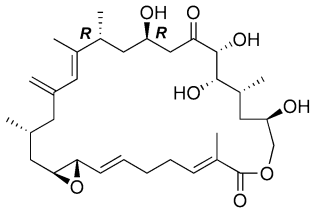

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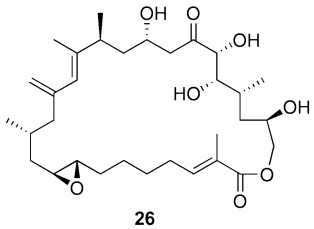

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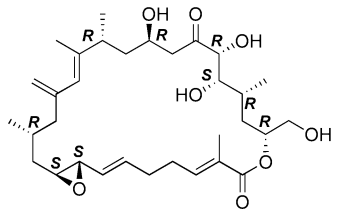

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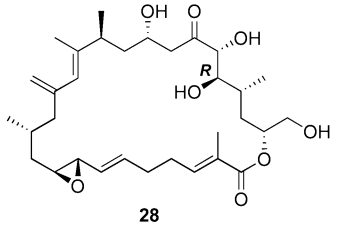

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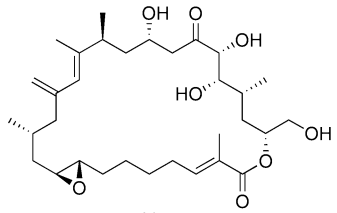

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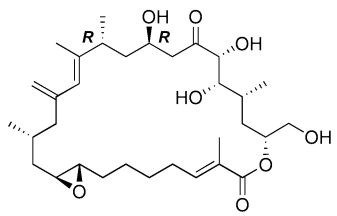

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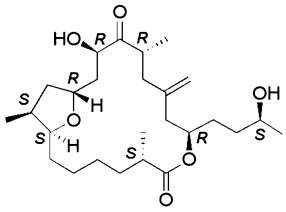

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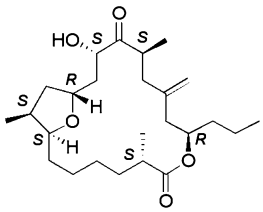

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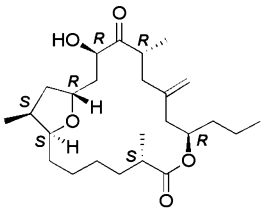

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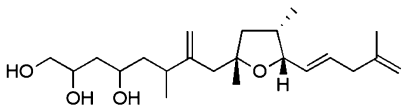

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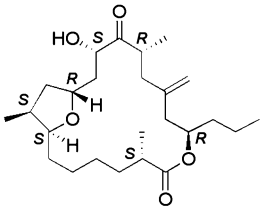

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Figure 1. Structures of amphidinolides A−H, J−S, T1, U−Y, G2−G3, H2−H5, and T2−T5 (**1**−**8**, **9**−**18**, **19**, **20**−**24**, **25**−**26**, **27**−**30**, and **31**−**34**, respectively) and amphidinin A (**35**).

Table 1. Lactone Ring Size, Isolation Yields, and Cytotoxicity Data for Amphidinolides A−H, J−S, T1, U−Y, B4, B5, and C2 (**1**−**8**, **9**−**18**, **19**, **20**−**24**, **44**, **45**, and **46**, respectively) and Amphidinins A (**35**) and B (**47**)

		isolation yields (10^{-4} %)								cytotoxicity (IC_{50},[b] μg mL^{-1})	
		strain no.[a]									
compd	lactone size	Y-5	Y-25	Y-26	Y-42	Y-56	Y-71	Y-72	Y-100	L1210[c]	KB[d]
1	20	20								2.0	5.7
2	26	10		0.8			17			0.00014	0.0042
3	25	15		0.3		9	12			0.0058	0.0046
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		0.00048	0.00052
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
35	*f*	0.6								3.6	3.0
44	26								8	0.00012	0.001
45	26								2	0.0014	0.004
46	25						1.5			0.8	3
47	*f*					2					

[a] *Amphidinium* sp. [b]50% inhibition concentration. [c]Murine lymphoma cell. [d] Human epidermoid carcinoma cells. [e]Macrodiolide. [f]Linear polyketide.

Amphidinolide B5 (**45**) was demonstrated to have the same molecular formula, $C_{32}H_{50}O_7$, as **44** by HRESIMS. Profiles of the ^{1}H and ^{13}C NMR spectra of **45** were reminiscent of those of **44**. The gross structure of **45** was elucidated as being the same as that

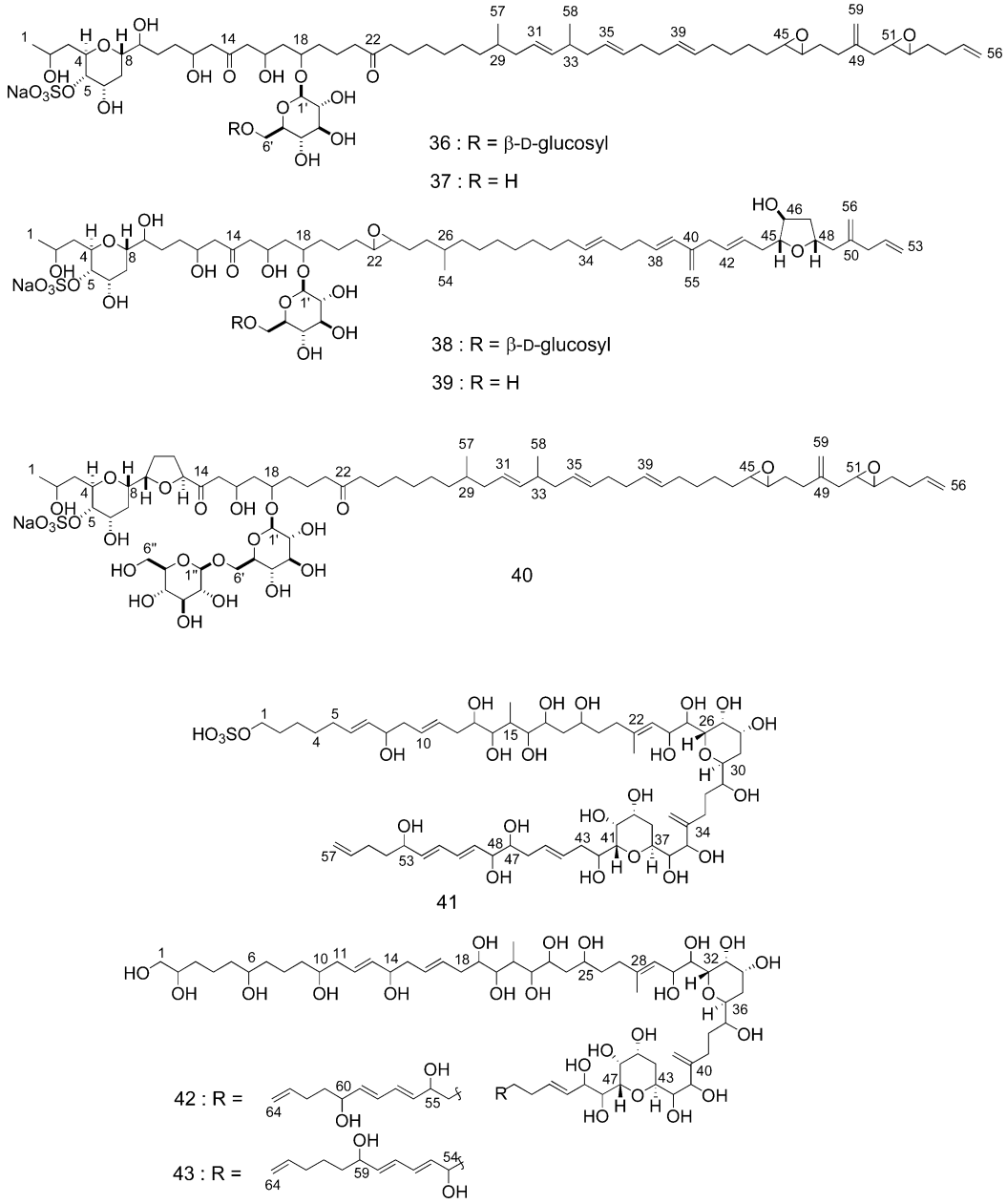


Figure 2. Structures of colopsinols A−E (**36**−**40**) and luteophanols A−C (**41**−**43**).

of **44** from analyses of the HMQC, HMBC, and INADEQUATE spectra. Comparison of the ^{13}C NMR data and CD spectra of **44** and **45** with those of amphidinolides B (**2**), H (**8**), H2 (**27**), and H3 (**28**) allowed the determination to be made that amphidinolides B4 (**44**) and B5 (**45**) are the 16-deoxy and 16-deoxy-16,18-epi forms of amphidinolide B (**2**), respectively (Figure 4).[9−12] Amphidinolides B4 (**44**) and B5 (**45**) exhibited potent cytotoxicity against L1210 cells (IC_{50}, 0.00012 and 0.0014 μg/mL, respectively) and KB cells (IC_{50}, 0.001 and 0.004 μg/mL, respectively).

Amphidinolide C2 (46). A new cytotoxic 25-membered macrolide, amphidinolide C2 (**46**), has been purified from the marine dinoflagellate *Amphidinium* sp. (strain Y-71), which was isolated from the marine acoel flatworm, *Amphiscolops* sp., collected off Sunabe, Okinawa.[13] Amphidinolide C2 (**46**), $C_{43}H_{64}O_{11}$, exhibited a UV absorption maximum at 230 nm, implying the presence of a diene chromophore. The ^1H and ^{13}C NMR data of amphidinolide C2 (**46**) were similar to those of amphidinolide C (**3**) except for the presence of additional methyl and ester carbonyl carbons. The $^1H-^1H$ COSY and HOHAHA spectra revealed proton connectivities of five fragments, C-2−C-11 (C-35, C-36, and C-37), C-12−C-14 (C-38), C-16−C-17 (C-39), C-19−C-29 (C-40), and C-30−C-34 (C-41). The connectivities of these five partial structures through two quaternary carbons (C-11 and C-30) and two carbonyl groups (C-15 and C-18) were established from HMBC correlations. The geometry of three internal olefins was assigned as all *E* on the basis of NOESY data. HMBC cross-peaks from H-6 to C-3 and H-20 to H-23 suggested that two tetrahydrofuran rings were formed between C-3 and C-6 and between C-20 and C-23, and their relative stereochemistries were implied by analysis of NOESY correlations. The existence of the 25-membered macrocyclic ring was implied by HMBC correlations from H_2-2 and H-24 to C-1. HMBC correlations from an oxymethine proton at C-29 and a single methyl proton (C-43) to an ester carbonyl carbon (C-42) suggested that an acetoxy group was attached to C-29. Thus, the gross structure of amphidinolide C2 (**46**) was elucidated to be the 29-*O*-acetyl form of amphidinolide C (**3**).[14,15] Amphidinolide C2 (**46**) was converted into its 7,8,13-*O*-triacetate, the spectroscopic data of which were identical with those of the tetraacetate of amphidinolide C (**3**). Therefore, the absolute configurations at all 12 chiral centers in amphidinolide C2 (**46**) have been elucidated as 3*S*, 4*R*, 6*R*, 7*R*,

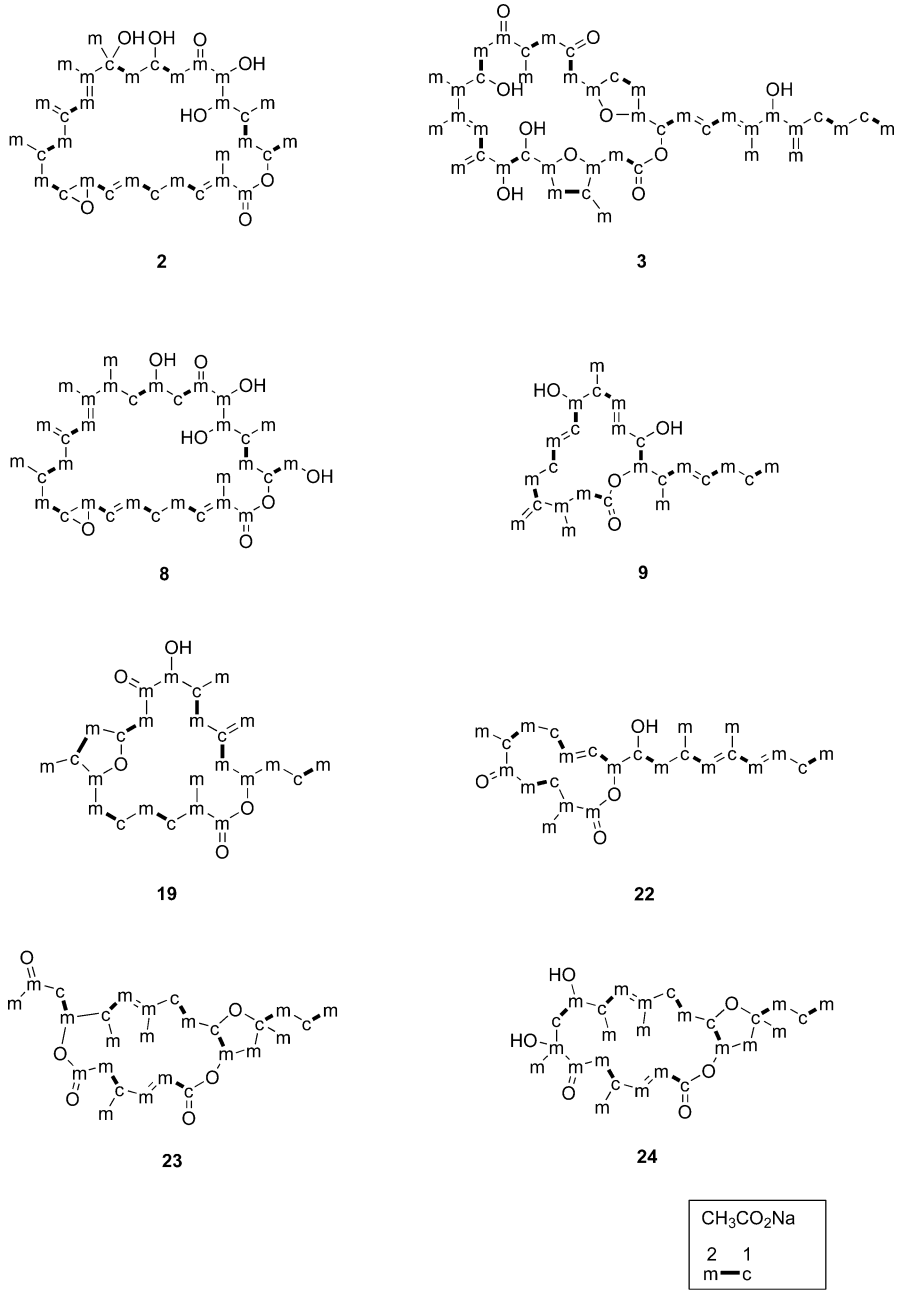


Figure 3. Acetate-incorporation patterns of amphidinolides B (**2**), C (**3**), H (**8**), J (**9**), T1 (**19**), W (**22**), X (**23**), and Y (**24**).

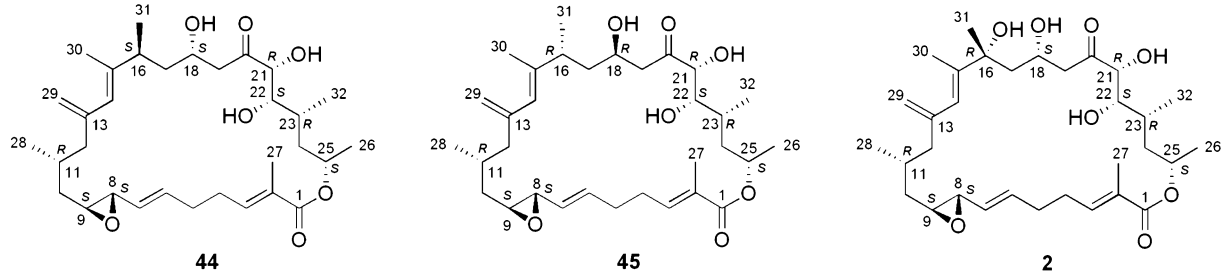
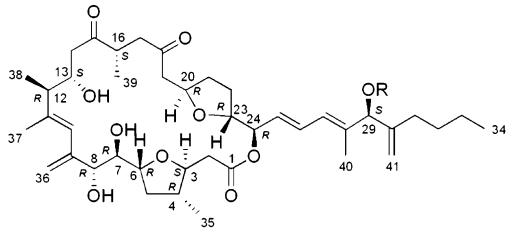


Figure 4. Structures of amphidinolides B4 (**44**), B5 (**45**), and B (**2**).

8*R*, 12*R*, 13*S*, 16*S*, 20*R*, 23*R*, 24*R*, and 29*S* (Figure 5). Amphidinolide C2 (**46**) exhibited cytotoxicity against L1210 and KB cells (IC_{50}, 0.8 and 3 μg/mL, respectively).

Amphidinin B (47). A new linear polyketide, amphidinin B (**47**), has been afforded from the marine dinoflagellate *Amphidinium* sp. (strain Y-56), which was isolated from a marine acoel flatworm, *Amphiscolops* sp., collected off Zanpa, Okinawa.[16] Amphidinin B (**47**), $C_{25}H_{42}O_7$, exhibited IR absorptions at 3400−3000, 2700−2500, and 1714 cm^{-1} and indicated the presence of a carboxylic acid functionality. Three partial structures, C-2 to C-3 (C-22), C-5 to C-9, and C-11 to C-20 (C-24 and C-25), were elucidated by analysis of the $^1H-^1H$ COSY and HOHAHA spectra. Connections among these units and the remaining four carbons (C-1, C-4, C-10, and C-21) were assigned on the basis of correlations observed in



3: R = H
46 : R = Ac

Figure 5. Structures of amphidinolides C (**3**) and C2 (**46**).

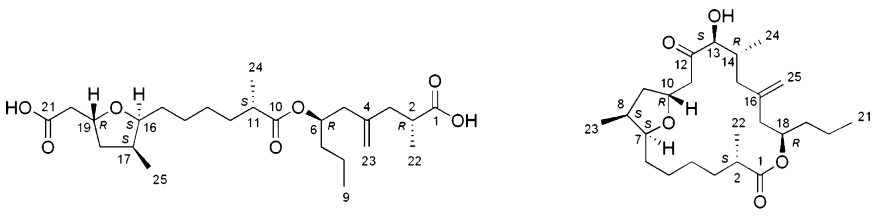
the HMBC spectrum. The HMBC correlation from H-19 to C-16 revealed the presence of an ether linkage between C-16 and C-19. The presence of an ester linkage between C-6 and C-10 was implied by the HMBC correlation from H-6 to C-10. Thus, the gross structure of amphidinin B was assigned as **47**. The ^{1}H NMR data of the bis-(*S*)-MTPA esters of the C-1 to C-9 and C-11 to C-21 segments, which were obtained by the successive treatment of **47** with TMS−CHN_2, $LiAlH_4$, and (*R*)-MTPACl, were identical with those of the bis-(*S*)-MTPA esters of C-13 to C-21 and C-1 to C-12 segments obtained from natural amphidinolide T1 (**19**),[20,21] respectively. Thus, the absolute configurations at all six chiral centers in **47** were elucidated as 2*R*, 6*R*, 11*S*, 16*S*, 17*S*, and 19*R*. Amphidinin B (**47**) is a new polyketide metabolite consisting of two linear carbon-chain units of C-1 to C-9 (C-22 and C-23) and C-10 to C-21 (C-24 and C-25) through an ester linkage (C-6 and C-10), possessing a tetrahydrofuran ring, one exomethylene, three branched methyls, and two carboxylic acid groups (Figure 6). This is the third isolation of a linear polyketide metabolite with a low molecular weight and no macrocyclic lactone ring from the marine dinoflagellate *Amphidinium* sp., although amphidinin A (**35**)[17] and amphidinoketides[18,19] have been isolated from dinoflagellates of the same genus. The backbone framework of **47** was the same as those of amphidinolides T1 (**19**) and T3−T5 (**32**−**34**).[20−22] Biogenetically, amphidinin B (**47**) may be related to amphidinolides T1 (**19**) and T3−T5 (**32**−**34**).

Amphezonol A (48). Amphezonol A (**48**), a novel polyhydroxyl linear carbon-chain metabolite, has been obtained from the cultured marine dinoflagellate *Amphidinium* sp. (strain Y-72), which was isolated from a marine acoel flatworm, *Amphiscolops* sp., collected off Zanpa, Okinawa.[23] The structure of amphezonol A (**48**), $C_{62}H_{114}O_{24}$, was elucidated by detailed analyses of the 2D NMR spectra including HSQC-TOCSY and INADEQUATE. The ^{1}H−^{1}H COSY and HOHAHA spectra of **48** revealed connectivities of five partial structures, C-1−C-8, C-10−C-23, C-33−C-44, C-49−C-53, and C-56−C-60 (C-62). HMBC correlations of H_2-61 to C-8, C-9, and C-10 and H-8 to C-10 implied that an exomethylene (C-61) was connected to C-8 and C-10 via C-9. Connections between C-23−C-33, C-44−C-49, and C-56−C-60 were deduced from correlations obtained from the HSQC-TOCSY and INADEQUATE spectra. The disubstituted double bond at C-51 was indicated to have an *E* geometry by the ^{1}H−^{1}H coupling constant. The presence of a tetrahydrofuran and two tetrahydropyran rings was deduced from deuterium-induced shift analysis of the oxymethine carbon signals in the ^{13}C NMR spectra of **48**, observed in CD_3OD and CD_3OH, respectively. The relative configurations of a tetrahydrofuran ring (C-3−C-6) and two tetrahydropyran rings (C-13−C-17 and C-39−C-43) in **48** were elucidated on the basis of ROESY correlations of **48**. Amphezonol A (**48**) possesses one tetrahydrofuran ring, two tetrahydropyran rings, and 21 hydroxyl groups on a C_{60}-linear aliphatic chain with one exomethylene and one methyl branch (Figure 7). The successive hydroxylated moiety of the carbon chain (C-23−C-33) is a characteristic of **48**. Amphezonol A (**48**) exhibited inhibitory activity against DNA polymerase α (IC_{50}, 15 μM).

Luteophanol D (49). Luteophanol D (**49**), a new polyhydroxyl linear carbon-chain metabolite, has been isolated from the cultured marine dinoflagellate *Amphidinium* sp. (strain Y-52), which was obtained from the Okinawan marine acoel flatworm *Pseudaphanostoma luteocoloris*.[24] The structure of luteophanol D (**49**), $C_{66}H_{114}O_{25}$, was elucidated by extensive 2D NMR experiments. Detailed analyses of the ^{1}H−^{1}H COSY, HOHAHA, and HSQC spectra revealed the connectivities of three partial structures, C-1−C-27, C-28−C-39 (C-65), and C-41−C-63. The HMBC spectrum of **49** showed cross-peaks for H-29 to C-27, H_2-66 to C-39, H_2-66 to C-41, and H_2-39 to C-40, indicating the connectivities of these three partial structures. Two tetrahydropyran rings were assigned by the HMBC cross-peaks for H-32 to C-36 and H-47 to C-43. Five di- and one trisubstituted double bonds were indicated to all have the *E* geometry by ROESY data and ^{1}H−^{1}H coupling constants. The relative configuration of the two tetrahydropyran rings was elucidated on the basis of the ROESY data and ^{1}H−^{1}H coupling constants of **49**. Thus, it was revealed that luteophanol D (**49**) possesses two tetrahydropyran rings and 23 hydroxyl groups on a C_{60}-linear aliphatic chain with one exomethylene and two methyl branches (Figure 8). Luteophanol D (**49**) contained a hydrophilic diene portion at C-54−C-59, whereas the known polyhydroxylated metabolites, amphidinols,[25−29] lingshuiols,[30,31] and karatungiols,[32] isolated from the same genus of dinoflagellates, possess a hydrophobic moiety in this portion. The biosynthesis of the amphidinols has been studied by acetate incorporation experiments (Figure 9).[33] Since the polyketide chain of **49** was shorter than those of luteophanols B (**42**) and C (**43**)[34] by only one carbon, this truncation of the carbon chain might result from an unusual dinoflagellate polyketide biosynthesis. Luteophanol D (**49**) exhibited antibacterial activity against *Micrococcus luteus* (MIC, 33 μg/mL).

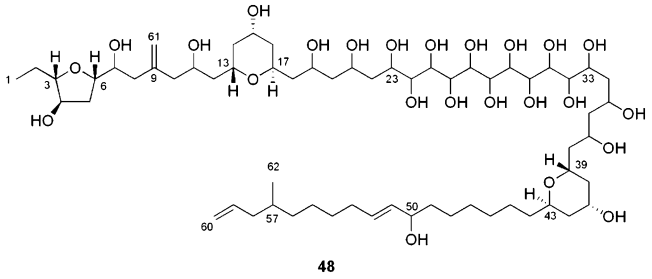
Absolute Stereochemistry of Amphidinolide A (1). Amphidinolide A (**1**) is a cytotoxic 20-membered macrolide, isolated from the cultured dinoflagellate *Amphidinium* sp., which is a symbiont of the Okinawan marine flatworm *Amphiscolops* sp.[35] The relative stereochemistry of the nine stereogenic centers in amphidinolide A was proposed to be **50** on the basis of extensive NMR experiments by our group.[36] The unique structure and bioactivity of amphidinolide A have prompted studies of its total syntheses. First Pattenden and later Maleczka and Trost accomplished total syntheses of the stereostructure (**50**) proposed for amphidinolide A and indicated that the proposed stereostructure (**50**) was incorrect from comparison of the NMR data of their synthetic compounds with those reported for amphidinolide A.

In our efforts to determine the correct stereostructure of amphidinolide A, we have re-examined the relative stereochemistry of this compound. Our re-examination of the ^{1}H and ^{13}C NMR data



47 **19**

Figure 6. Structures of amphidinin B (**47**) and amphidinolide T1 (**19**).



48

Figure 7. Structure of amphezonol (**48**).

have indicated that the correct stereostructure of amphidinolide A could be either the diastereomer **51** or **1** of the structure (**50**) proposed for amphidinolide A. Since the diastereomer **1** has been synthesized by Trost's group,[37−40] we have synthesized the alternative diastereomer **51** to compare the NMR data of the synthetic diastereomers **51** and **1** with those of naturally derived amphidinolide A. The ^{1}H and ^{13}C NMR data for synthetic diastereomer **51** were not coincident with those of amphidinolide A, whereas the NMR data for diastereomer **1** synthesized by Trost's group were close to those of amphidinolide A. Diastereomers **51** and **1** and amphidinolide A were subjected to C_{18} HPLC, and it was found that the retention time of amphidinolide A was identical with that of **1** but not that of **51**. Thus, the relative stereostructure for amphidinolide A was assigned as **1**. Comparisons of the optical rotations of compounds **51** and **1** and amphidinolide A were as follows; $[\alpha]^{21}_{D}$ −11 (*c* 0.6, $CHCl_3$) for **51**, $[\alpha]^{24}_{D}$ +56 (*c* 0.05, $CHCl_3$) for **1**, and $[\alpha]^{24}_{D}$ +68 (*c* 1.0, $CHCl_3$) for amphidinolide A. Therefore, it was concluded that the absolute configurations at the nine chiral centers of amphidinolide A (**1**) were 8*R*, 9*R*, 11*S*, 12*S*, 18*R*, 19*S*, 20*R*, 21*S*, and 22*S* (Figure 10).[41]

Mechanisms of Action of Amphidinolides H (8). The molecular target of amphidinolide H (**8**),[42,43] which shows potent cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells in vitro (IC_{50} 0.00048 and 0.00052 μg/mL, respectively), has been investigated.[44,45] Amphidinolide H (**8**) induced multinucleated cells by disrupting actin organization in the cells and the hyperpolymerization of purified actin into filaments of apparently normal morphology in vitro. Amphidinolide H (**8**) covalently binded

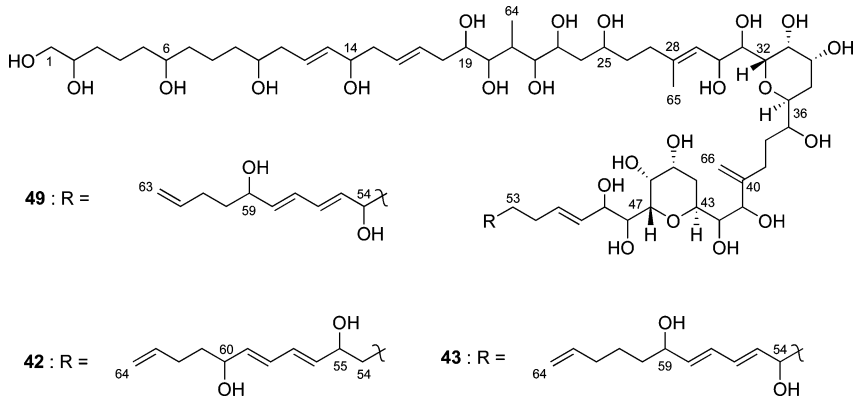


Figure 8. Structures of luteophanols B (**42**), C (**43**), and D (**49**).

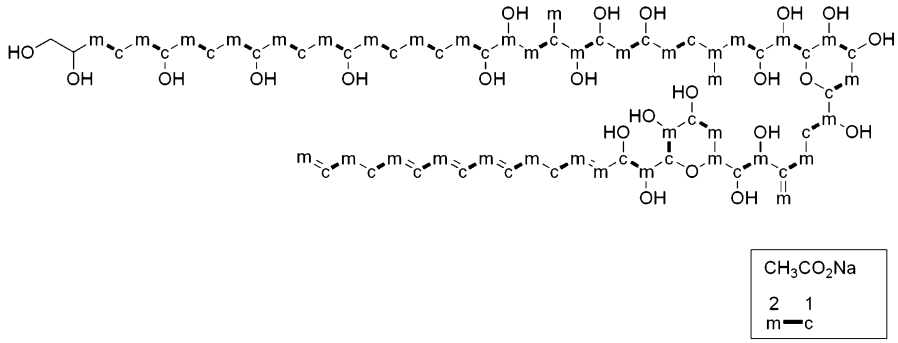
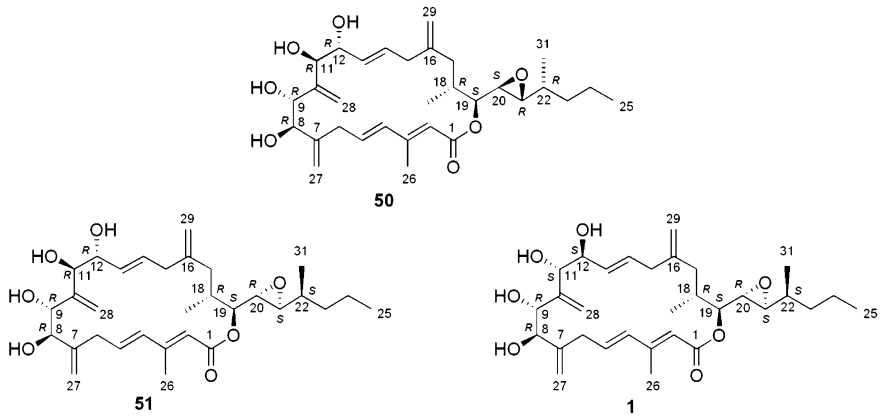


Figure 9. Acetate-incorporation patterns of amphidinol-4.



50 **51** **1**

Figure 10. Structures of amphidinolide A (**1**) and its diastereomers (**50**, **51**).

on actin, and the binding site was determined as Tyr200 of actin subdomain 4 by mass spectrometry and the halo assay using the yeast harboring site-directed mutagenized actins. 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 of actin fibers. These results indicate that amphidinolide H (**8**) is a novel inhibitor that covalently binds on actin.

Biosynthetic Gene of Amphidinolides. Cloning of the polyketide synthase (PKS) gene for amphidinolide biosynthesis was attempted from a dinoflagellate *Amphidinium* sp. (strain Y-42).[46] Fourteen β-ketoacyl synthase gene fragments were obtained by polymerase chain reaction (PCR) amplification from degenerated primer sets designed on the basis of the conserved amino acid sequences of β-ketoacyl synthase domains in known type I PKSs. The PCR analysis using primer sets designed from these 14 β-ketoacyl synthase gene fragments revealed that these DNA sequences exist only in the dinoflagellates producing amphidinolides. The DNA sequence of the positive clone, which was isolated from the genomic DNA library of *Amphidinium* sp. (strain Y-42), was analyzed by shotgun sequencing. The deduced gene products in the positive clone showed similarity to β-ketoacyl synthase (KS), acyl transferase (AT), dehydratase (DH), ketoreductase (KR), acyl carrier protein (ACP) in known type I PKSs, and thioesterase (TE).[46]

In this study, a 36.4 kb genomic DNA fragment has been found from approximately 100 000 clones. However, the total size of six open reading frames was only 5625 bp. Amphidinolide H (**8**), for example, is a 26-membered macrolide and the main chain consists of 16 units derived from acetate. If deduced products of these genes could not work interactively, they might be responsible for only one extension step performed by condensation with a malonate unit. This represents the difficulty of cloning of a whole amphidinolide biosynthesis gene from a genomic DNA library. The construction of a complementary DNA library of amphidinolide-producing dinoflagellates might be the best alternative to clone amphidinolide biosynthesis genes.

Conclusions

Marine dinoflagellates of the genus *Amphidinium* produce a variety of cytotoxic macrolides and/or long-chain polyketides. Among all the amphidinolides, amphidinolide N (**13**) exhibits remarkably potent cytotoxicity against human tumor cell lines and is expected to be a lead compound for new anticancer drugs together with caribenolide I,[47] an amphidinolide N-type macrolide isolated from *Amphidinium operculatum* var. nov. *Gibbosum*. For further biological testing, the poor productivity of these macrolides needs to be considerably improved. Although one of the approaches is to try heterogeneous expression of the polyketide synthase of amphidinolides in some other organisms, it seems to be quite difficult to clone genes responsible for amphidinolide biosynthesis from the huge genome of the dinoflagellate. The construction of a complimentary DNA library might be the best alternative to clone amphidinolide biosynthesis genes. Such an approach is also important for understanding the mechanisms of the unique dinoflagellate polyketide biosynthesis. Further development of the cell biology and molecular biology of dinoflagellates is required for biomedical and pharmaceutical application of metabolites such as the amphidinolides.

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