AMPHIDINOLIDES: UNIQUE MACROLIDES FROM MARINE DINOFLAGELLATES†

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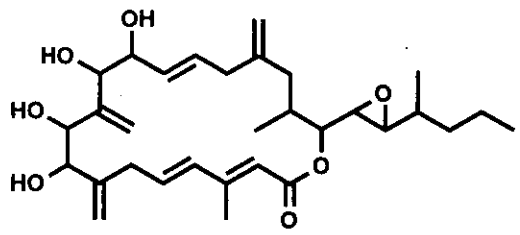
Abstract—A series of macrolides, named amphidinolides, have been isolated from the laboratory-cultured marine dinoflagellates *Amphidinium* sp., which were symbionts of the Okinawan marine flatworm *Amphiscolops* sp. These macrolides possess unique chemical structures as well as cytotoxic activities. Here we describe our recent results on the isolation, structure elucidation, and biosynthesis of these unique macrolides.

1. INTRODUCTION

Marine microalgae are of considerable current interest as new promising source of bioactive substances. We previously obtained amphidinolides A ~ H (**1** ~ **8**), a series of cytotoxic macrolides possessing unique structural features, from the laboratory-cultured dinoflagellates *Amphidinium* sp., which were isolated from the inside of the cells of the Okinawan flatworm *Amphiscolops* sp., and those works were reviewed previously published in 1993.[1] The present review provides an update of the 1993's review describing further investigation on the isolation, structure elucidation, and

†Dedicated to Professor Shigeru Oae on the occasion of his 77th birthday

biosynthesis of these unique macrolides.



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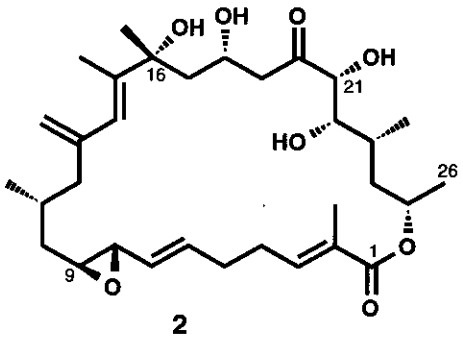

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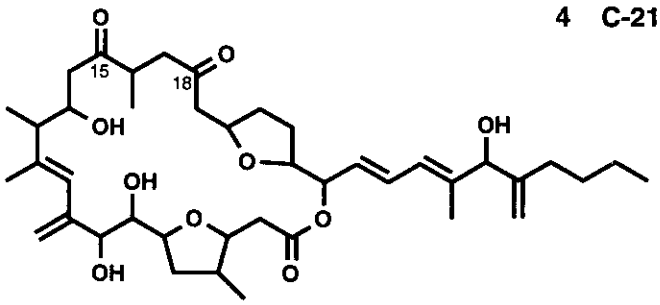

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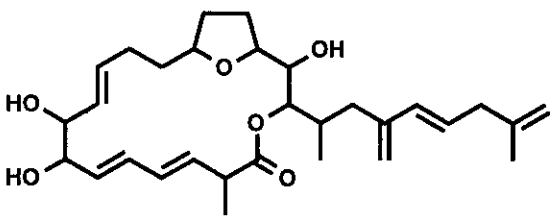

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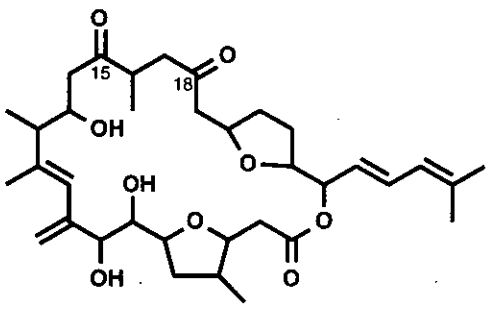

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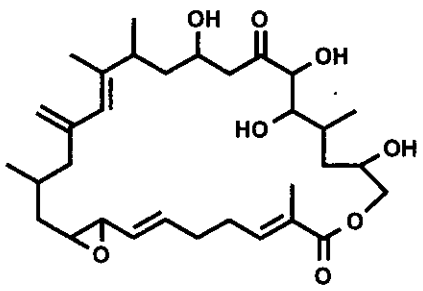

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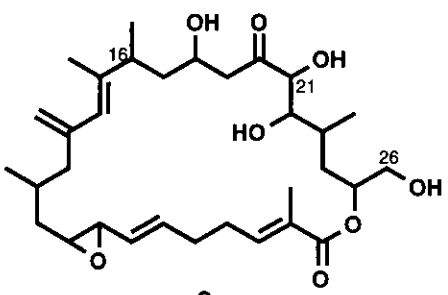

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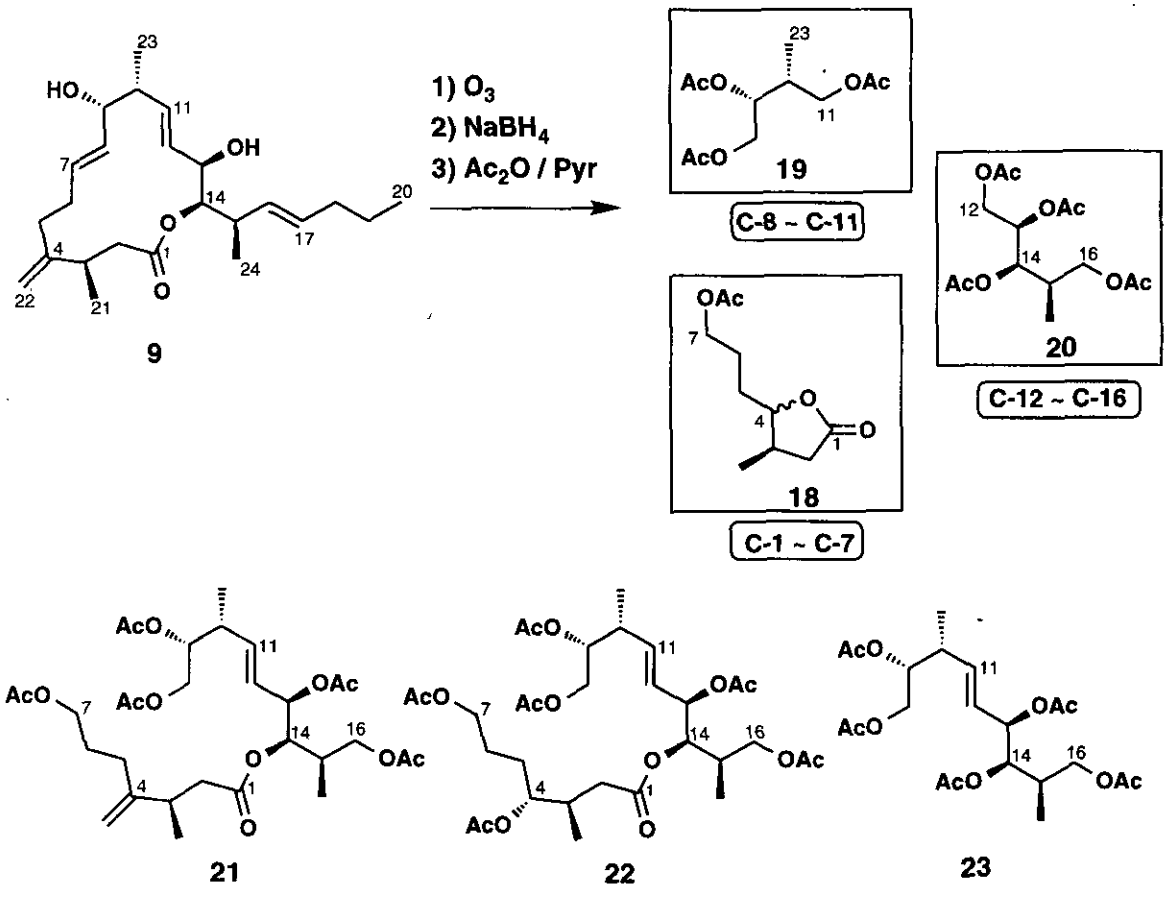
2. AMPHIDINOLIDE J

After obtaining the series of macrolides, amphidinolides A ~ H (**1** ~ **8**), we still continued the mass-culturing of the dinoflagellates *Amphidinium* sp. (strain number, Y-

5 and Y-25).[1] Our previous studies have revealed that fractionation by silica gel chromatography of the toluene-soluble portion of the extracts of these microalgae afforded several fractions exhibiting extremely potent cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells in vitro with the inhibition values at 10 μg/ml being more than 90%. These inhibition values cannot be fully accounted for by estimating from the IC_{50} values of previously isolated amphidinolides. Thus, further investigations continued to search for other cytotoxic components of these dinoflagellates. As a result, we succeeded in isolating several novel cytotoxic macrolides with various ring numbers, amphidinolides J (**9**), K (**10**), M (**12**), N (**13**), O (**14**), P (**15**), and Q (**16**), together with a new linear metabolite amphidinin A (**17**) from the strain of Y-5, while a new 27-membered macrolide, amphidinolide L (**11**), was isolated from the strain of Y-25. This chapter deals with isolation and structure elucidation of amphidinolide J (**9**),[2] a novel 15-membered macrolide, the absolute stereochemistry of which was determined by combination of degradation experiments and synthesis of optically active compounds.

The dinoflagellate *Amphidinium* sp. was cultured at 25 °C for 2 weeks in a sea water medium enriched with ES nutrients. The details of the cultivation procedures were described previously.[1] The harvested algal cells (920 g, wet weight, from 3300 l of culture) were extracted with MeOH/toluene (3:1) and the extracts were partitioned between toluene and water. The toluene-soluble fraction was subjected to a silica gel column ($CHCl_3$/MeOH, 95:5) followed by gel filtration on Sephadex LH-20 ($CHCl_3$/MeOH, 1:1). Subsequent separation by reversed-phase hplc (ODS, 88% MeOH) afforded amphidinolide J (**9**, 0.0002% yield, wet weight) as a colorless oil.

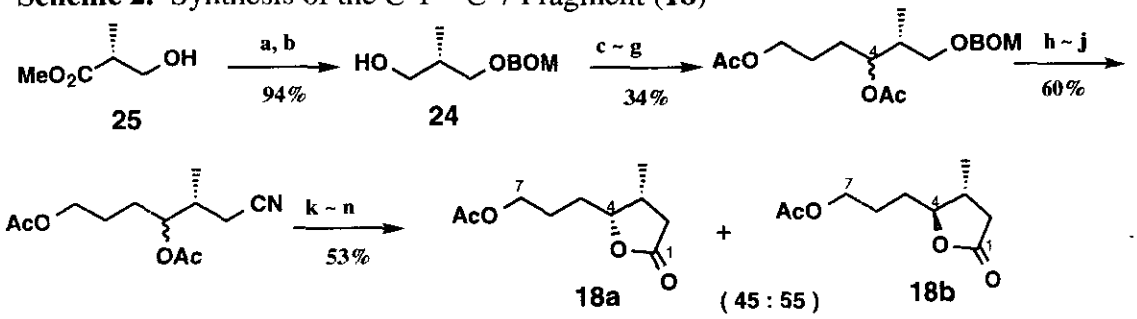
The planar structure of amphidinolide J (**9**), $C_{24}H_{38}O_4$, was studied by detailed analyses of its ^1H and ^{13}C nmr data aided with 2D nmr experiments (^1H-^1H COSY, HSQC, HMBC, and NOESY), thereby leading to a gross structure of **9** consisting of a 15-membered lactone ring with three disubstituted *E*-olefins ($J_{7,8}$=15.0, $J_{11,12}$=15.8, and $J_{16,17}$=15.0 Hz). This gross structure was further confirmed by the structures of the degradation products (**18** ~ **23**) obtained by the following ozonolysis experiments.



Scheme 1. Ozonolysis of Amphidinolide J (**9**)

Treatment of **9** with ozone (-78 °C, 1 min) followed by $NaBH_4$ reduction and acetylation (Scheme 1) afforded a complex mixture, from which the normal and reverse-phase hplc separations were carefully carried out to obtain degradation products (**18** ~ **20**), corresponding to C-1 ~ C-7, C-8 ~ C-11, and C-12 ~ C-16 moieties of **9**, respectively. In addition, partial-degradation products (**21** ~ **23**) were also obtained and their structures provided further evidences for the proposed planar structure of **9**. For unambiguous determination of the absolute configurations of six chiral centers of **9**, the fragments (**18** ~ **20**) together with their all possible diastereomers were prepared in optically active forms.

Scheme 2. Synthesis of the C-1 ~ C-7 Fragment (**18**)



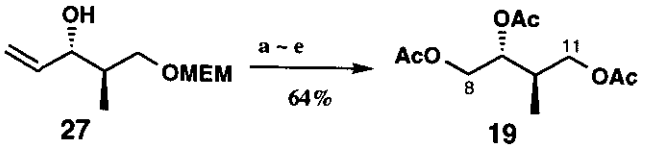
(**a**): BOMCl, i-Pr_2NEt, CH_2Cl_2, room temperature, 44 h; (**b**): $LiAlH_4$, ether, room temperature, 30 min; (**c**): DMSO, $(COCl)_2$, CH_2Cl_2, -78°C, 30 min, then Et_3N, 0°C, 30 min; (**d**): CH_2=$CHCH_2CH_2MgBr$, ether, 50°C, 40 min; (**e**): O_3, MeOH, -78°C, 2.5 h; (**f**): $NaBH_4$, MeOH, 0°C, 1 h; (**g**): Ac_2O, pyridine, room temp, 12 h; (**h**): H_2, Raney Ni (W-2), EtOH, room temp, 48 h; (**i**): TsCl, pyridine, room temperature, 44 h; (**j**): NaCN, DMSO, 85-90°C, 2 h; (**k**): NaOH, H_2O_2, EtOH, 65°C, 1.5 h, then 90°C, 7 h; (**l**): 2M HCl, room temperature; (**m**): Ac_2O, pyridine, room temp, 11 h; (**n**): Hplc separation

The C-1 ~ C-7 fragment (**18**) was synthesized as shown in Scheme 2, starting with monoprotected 2(*S*)-methylpropane-1,3-diol (**24**), which was readily supplied from (-)-methyl 3-hydroxy-2(*R*)-methylpropionate (**25**). The Grignard addition to the corresponding aldehyde from **24** afforded the diastereomeric mixture at C-4 in the ratio of 45:55, which was separated in the final step by silica gel hplc. The 3,4-syn (**18a**) and 3,4-anti (**18b**) isomers thus obtained were completely identical with those from natural specimen including the sign of optical rotations [synthetic, **18a**: $[\alpha]_D$ +17° (*c* 1.0, $CHCl_3$); **18b**: $[\alpha]_D$ -22° (*c* 1.0, $CHCl_3$); natural, **18a**: $[\alpha]_D$ +17° (*c* 0.06, $CHCl_3$); **18b**: $[\alpha]_D$ -34° (*c* 0.2, $CHCl_3$)] to establish 3*R*-configuration for **9**.

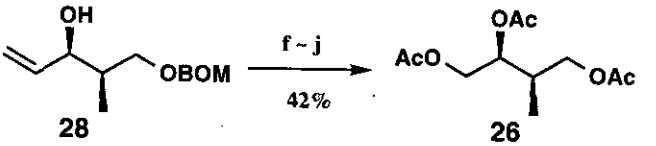
The C-8 ~ C-11 fragment (**19**) and its syn-isomer (**26**) were readily prepared [(1) reductive ozonolysis, (2) deprotection, and (3) acetylation; Scheme 3] from allyl alcohols (**27** and **28**, respectively), which were obtained from **25** *via* modifications of literature procedures.[3] The spectral data of the C-8 ~ C-11 fragment obtained by degradation of **9** were identical with those of the anti-isomer (**19**) and their optical data [synthetic, $[\alpha]_D$ +5.0° (*c* 1.0, $CHCl_3$); natural, $[\alpha]_D$ +2.8° (*c* 0.22, $CHCl_3$)] revealed the 9*R*,10*R*-configurations for **9**.

Preparations of the C-12 ~ C-16 fragment (**20**) and its diastereomers (**29** ~ **31**) were achieved by applying Kishi's methods for pentose synthesis[4] (Scheme 4). The epoxy alcohol (**32**), obtained from D-glyceraldehyde acetonide (**33**), was treated with

Scheme 3. Synthesis of the C-8 ~ C-11 Fragment (**19**) and Its syn Isomer (**26**)

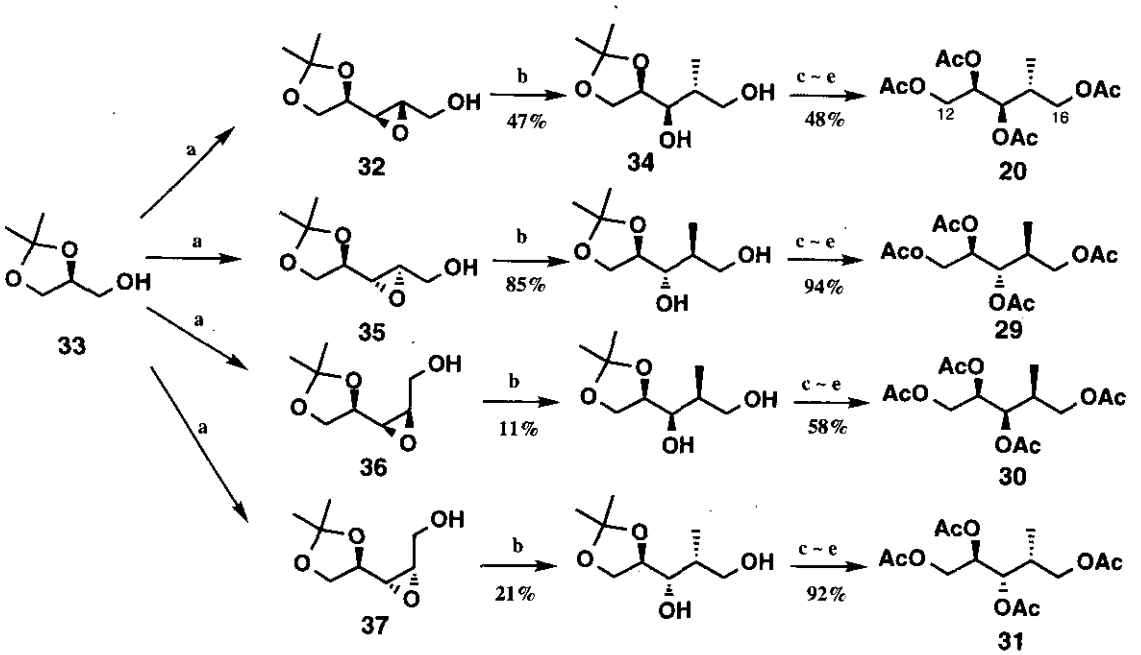


(**a**): O_3, MeOH, -78°C, 5 min; (**b**): $NaBH_4$, MeOH, 0°C, 1 h; (**c**): Ac_2O, pyridine, room temp, 38 h; (**d**): 4M HCl, THF, 50°C, 2 h; (**e**): Ac_2O, pyridine, room temperature, 20 h



(**f**): O_3, MeOH, -78°C, 1 min; (**g**): $NaBH_4$, MeOH, 0°C, 45 min; (**h**): Ac_2O, pyridine, room temperature, 17 h; (**i**): H_2, 10% Pd-C, MeOH, room temperature, 11h; (**j**): Ac_2O, pyridine, room temperature, 20 h

Scheme 4. Synthesis of the C-12 ~ C-16 Fragment (**20**) and Its Diastereomers (**29 ~ 31**)



(**a**): ref. 4 (4 steps); (**b**): CuI (12 eq), MeLi (24 eq), Et_2O, -40°C, 4-9 h, then -23°C, 30 min; (**c**): 1M HCl, THF, room temperatrue, 7-25 h or AcOH/H_2O (4:1), 40 °C, 4 h; (**d**): Ac_2O, pyridine, room temperature, 11-20 h; (**e**): Hplc separation

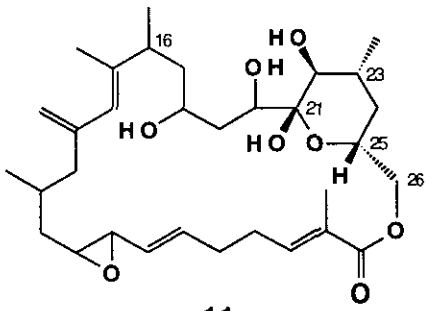
dimethyl cuprate to give 1,3-diol (**34**) together with undesired 1,2-diol in the ratio of 1:1, which was separated in the final step by silica gel hplc (hexane/EtOAc, 2:1). The diastereomers (**29 ~ 31**) were also obtained by the similar procedures from the

corresponding epoxy alcohols (**35** ~ **37**, respectively). The C-12 ~ C-16 fragment derived from **9** was identical with the syn-anti isomer (**20**) including the sign of optical rotation [synthetic, $[\alpha]_D$ +41° (*c* 1.0, $CHCl_3$); natural, $[\alpha]_D$ +44° (*c* 0.23, $CHCl_3$)], thus determining the 13*R*,14*R*,15*R*-configurations for **9**. From these results the structure of amphidinolide J was firmly established as **9** including the absolute stereochemistry of the six chiral centers.

3. AMPHIDINOLIDE L

In connection of our studies on cytotoxic macrolides from the dinoflagellate *Amphidinium* sp. (strain Y-5), as described in the beginning of the preceding chapter, we also continued examining the extract of the strain of Y-25, *Amphidinium* sp., which was isolated from the Okinawan flatworm *Amphiscolops breviviridis* to result in the isolation of a new 27-membered macrolide, amphidinolide L (**11**).[5]

Amphidinolide L (**11**), $C_{32}H_{50}O_8$, was isolated as a colorless oil in 0.0004% yield from *ca.* 1800 g (wet weight) of the harvested cells obtained from 1750 l of culture of this alga. Detailed analysis of the 2D nmr data (^1H-^1H COSY, HOHAHA, HMQC, and HMBC) led to the planar structure of amphidinolide L (**11**) as constructed from a 27-membered lactone ring with an epoxide and a tetrahydropyran moieties, which corresponded to 20-dihydro-21-dehydro derivative of amphidinolide G (**7**). The relative stereochemistry of the tetrahydropyran moiety (C-21, C-22, C-23, and C-25 positions) was elucidated on the basis of NOE and coupling constant data, and the absolute configurations of C-23 and C-25 positions were established by synthesis of the tetraacetate (**38**), corresponding to the C-21 ~ C-26 fragment of **11**, starting from the optically active epoxy alcohol (**39**)[6] (Scheme



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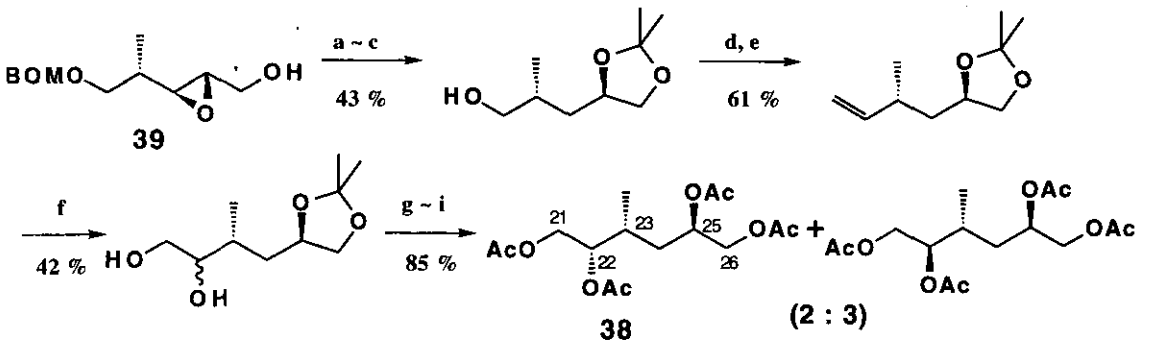
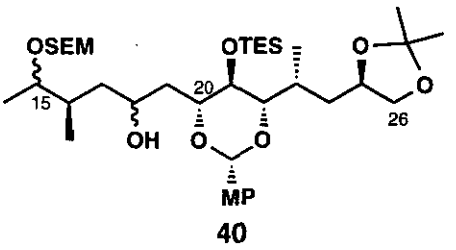
Scheme 5. Synthesis of the C-21 ~ C-26 Fragment (**38**) of Amphidinolide L (**11**)



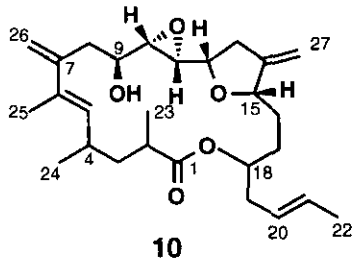
(**a**) DIBALH, benzene, room temperature, 1 h; (**b**) $(MeO)_2CMe_2$, PPTS, CH_2Cl_2, room temperature, 5 h; (**c**) H_2, $Pd(OH)_2$, EtOH, room temperature, 18 h; (**d**) DMSO, $(COCl)_2$, CH_2Cl_2, -78 °C, 30 min, then Et_3N, -20 °C, 30 min; (**e**) Ph_3PCH_3Br, *n*-BuLi, THF, room temperature, 2 h; (**f**) OsO_4, pyridine, THF, room temperature, 4 h; (**g**) 1N HCl, THF, room temperature, 5 h; (**h**) Ac_2O, pyridine, room temperature, 18 h; (**i**) Hplc separation

5). The synthetic tetraacetate (**38**) showed completely identical spectral data including the sign of the optical rotations [synthetic, $[\alpha]_D$ +64° (*c* 0.2, $CHCl_3$); natural, $[\alpha]_D$ +72±8° (*c* 0.01, $CHCl_3$)], with those of compound (**38**), which was obtained from **11** by treatment with $NaIO_4$ followed by $NaBH_4$ reduction and acetylation, indicating the 21*R*,22*S*,23*R*,25*R*-configurations. We are currently further investigating the synthesis of the diastereomers of the C-15 ~ C-26 fragment of **11** to determine the stereochemistry of that moiety; preparation of a diastereomer (**40**) possessing 16*R*,20*R*-configuration from (-)-methyl 3-hydroxy-2(*R*)-methylpropionate (**25**, Scheme 2) has been achieved by efficient manners with good yields.[7,8]



4. AMPHIDINOLIDES K, M, N, AND AMPHIDININ A

This and the following sections again deal with the studies on the cytotoxic metabolites isolated from the Y-5 strain of *Amphidinium* sp. The cytotoxic fraction mainly containing amphidinolide J (**9**) was further examined carefully by separation using reversed-phase hplc to give four new compounds, amphidinolides K (**10**), M (**12**), N (**13**), and amphidinin A (**17**).



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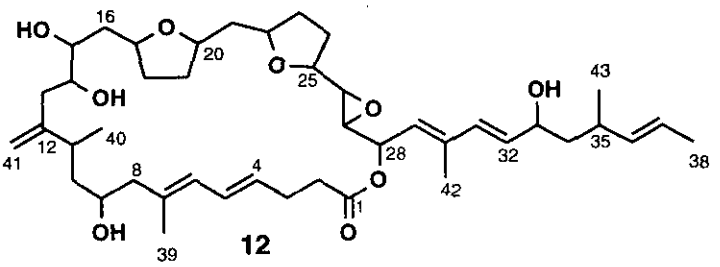

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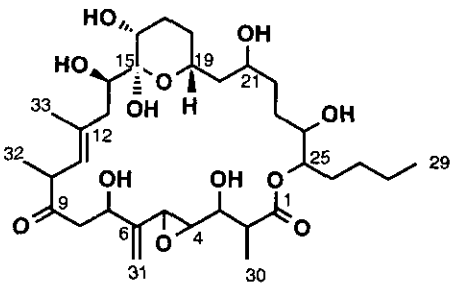
Amphidinolide K (**10**),[9] $C_{27}H_{40}O_5$, was isolated in 0.0002% yield (wet weight), and its gross structure was elucidated by applying several types of 2D nmr techniques using a 600 MHz spectrometer to deduce the planar structure as **10** containing a 19-membered macrocyclic lactone along with a diene, an epoxide, and a tetrahydrofuran moieties. We proposed the relative stereochemistry of the epoxide-tetrahydrofuran portion (C-9, C-10, C-11, C-12, and C-15) on the basis of the NOESY and coupling constant data, and the diene moiety was inferred to have *S-trans* conformation from the NOESY correlations (H-5 and one of H_2-8; H_3-25 and one of H_2-26).

Amphidinolide M (**12**),[10] $C_{43}H_{66}O_9$, was first isolated in 1986 from the dinoflagellate of this species (strain number Y-5) in *ca.* 0.0005% yield (wet weight). Unfortunately, the sample of **12** decomposed extensively during storage as a $CDCl_3$ solution, and the structural studies were interrupted.[1] The quantity of these macrolides contained in the extracts of the cultured cells varied a little during the course of time and amphidinolide M (**12**) was not isolated for several years. We recently reisolated amphidinolide M (**12**) fortunately from the same strain of this cultured alga by careful hplc examination. Spectral studies of **12** and its tetraacetate were extensively carried out to suggest that compound (**12**) was a 29-membered macrolide with two dienes, two tetrahydrofuran (THF) rings, an exomethylene, and an epoxide. The stereochemistry of **12** remained undetermined; the NOESY data of **12**, however, may have implied that the angular hydrogens of two THF portions were both *trans* since NOESY cross-peaks were significantly observed for H-15/H-20 and H-22/H-27 while no correlations between angular protons (H-17/H-20 and H-22/H-25) were visible.



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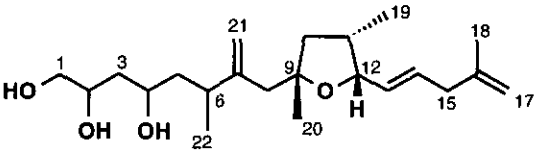

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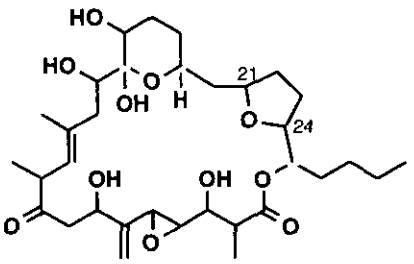
Amphidinolide N (**13**)[11] was isolated from relatively polar fraction by reversed-phase hplc (Develosil ODS-5, 60% CH_3CN) separation. This compound (**13**) was extremely cytotoxic against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro* (*vide infra*); the cytotoxicity of **13** was the most potent of all amphidinolides that have ever been isolated. The structure of this macrolide (**13**, $C_{33}H_{54}O_{12}$) was interpreted by the extensive analysis of its spectroscopic data and was proposed to be **13**, which was composed of a 26-membered macrolide containing a tetrahydropyran (THP) moiety with an hemiketal group, an epoxide, and an exomethylene group. The NOESY cross-peak observed between H-14 and H-19 might suggest that C-14 and H-19 were both axially oriented on the THP ring. The hydroxyl group on C-16 was deduced to be axial from the coupling constants ($J_{16,17a} = J_{16,17b} = 2.5$ Hz). After the isolation and gross structure of amphidinolide N (**13**) was published, isolation and structure of caribenolide I (**41**),[12] a related compound to **13**, was described by Shimizu and coworkers; they isolated compound (**41**) from a cultured free-swimming Caribbean dinoflagellate *Amphidinium* sp. Caribenolide I (**41**) was reported to show strong cytotoxicity (IC_{50} 0.001 μg/ml or 1.6 nM) against both human colon tumor cell line (HCT 116 and its drug-resistant cell line, HCT 116/VM 46). This cytotoxicity was about 100 times higher than that of amphidinolide B (**2**, IC_{50}, HCT 116, 0.122 μg/ml). Compound (**41**) was also described to exhibit *in vivo* activity against murine tumor P388 (T/C: 150 at a dose of 0.03 mg/kg).



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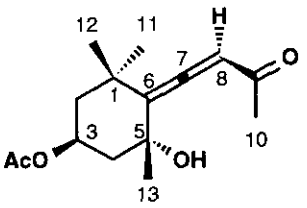

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Amphidinin A (**17**, $C_{22}H_{38}O_4$)[13] was isolated from the macrolide-containing fraction by the reversed-phase hplc (Develosil ODS-5; 10 x 250 mm; 59% MeCN; flow rate: 2.5 ml/min; detection: RI and UV at 220 nm); under this separation condition, compound (**17**) had a very close retention time (t_R 27.6 min) to those of amphidinolides A (**1**, t_R 29.1 min) and E (**5**, t_R 26.0 min). Being different from all other cytotoxic metabolites isolated from this microalga, amphidinin A (**17**) did not have the macrolide-structure; the ir spectrum of **17** showed no characteristic band due to carbonyl group. Extensive nmr studies revealed that compound (**17**) possessed a linear backbone skeleton with one tetrahydrofuran (THF) moiety. Three hydroxyl groups are located on one end of the molecule, while a 2-methyl-1,4-pentadiene unit on the other end, constructing a hydrophilic and hydrophorbic moieties, respectively, in a linear molecule. The relative stereochemistry of the THF portion was suggested by the NOESY data. As a result, the gross structure of amphidinin A was deduced as **17**, but the relative and absolute configurations of the chiral centers of **17** remained undefined. Although amphidinin A (**17**) is a non-macrolide, this compound has several structural relationships to previously isolated amphidinolides, implying that biogenesis of amphidinin A (**17**) may be closely related to those amphidinolides.

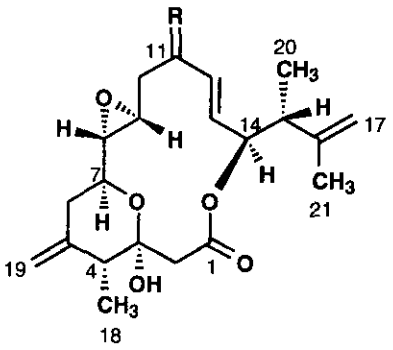
During our continuing examinations of the cytotoxic fraction of the extract of the Y-5 strain of this microalga, we also isolated an allenic compound (**42**);[14] this compound was identified including the CD spectral data as apo-9'-fucoxanthinone,[15] which was previously reported as a permanganate oxidation product of fucoxanthin. The deacetyl derivative of **42** was known as a grasshopper ketone isolated from ant repellent

secretions of the large flightless grasshopper *Romalea microptera*[16] and also isolated from *Edgeworthia chrysantha*.[17]

5. AMPHIDINOLIDES O, P, AND Q

During our continuing studies on the Y-5 strain of *Amphidinium* sp., we recently examined the cytotoxic fractions being less polar than amphidinolide J (**9**). The ^{1}H nmr spectra of these crude fractions exhibited significantly the exomethylene signals. Previously isolated amphidinolides all contain exomethylene groups, which were detected as sharp singlets around 5 ppm in the ^{1}H nmr spectra of crude fractions in the latter stage of the isolation process. Thus, we further purified these fractions by reversed-phase hplc to result in isolating three novel macrolides, amphidinolides O (**14**), P (**15**), and Q (**16**).

Amphidinolides O (**14**, $C_{21}H_{28}O_6$) and P (**15**, $C_{22}H_{30}O_5$)[18] were both novel 15-membered macrolides, and these two compounds are structurally related to each other. They both contain a tetrahydropyran (THP) moiety with a hemiketal group, an epoxide, and at least two exomethylene groups. The structural difference was found at the C-11 position; the C-11 ketone group of **14** was replaced by an exomethylene group for **15**, which was indicated from the following observations: (i) two IR absorption bands due to carbonyl groups were observed for **14**, but one (ν_{max} 1730 cm^{-1}) for **15**; (ii) compound(**14**)showed a UV absorption maximum at 231 nm due to an enone moiety while the UV absorption of **15** underwent a blue-shift (λ_{max} 225 nm), which was assignable to a diene chromophore; (iii) the ^{13}C nmr of **15** showed no signal due to a conjugated ketone, instead of which nmr signals for another exomethylene groups were observed [δ_C 118.1 (C-22) and 142.3 (C-11); δ_H 4.98 (1H, br s) and 4.85 (1H, br s) for H_2-22].



14 R = O
15 R = CH_2
22

Amphidinolides O (**14**) and P (**15**) possess seven chiral centers; the relative configurations of five chiral centers contained in the THP and epoxide ring portion were both elucidated on the basis of the nmr data as 3S*, 4R*, 7S*, 8S*,[19] and 9S* for **14** and 3S*, 4R*, 7S*, 8R*,[19] and 9S* for **15**. The relative stereochemistries of remaining two chiral centers (C-14 and C-15) were investigated by the combination of the ^{1}H nmr data and molecular mechanics calculations. We considered four diastereomers (**14a ~ 14d** and **15a ~ 15d**) for each compound (**14** and **15**), respectively (**14a/15a**: 14R*15R*; **14b/15b**: 14R*15S*; **14c/15c**: 14S*15R*; **14d/15d**: 14S*15S*), and the Monte Carlo lowest-energy conformations calculated with the MM2 force field were represented in Figure 1, and summary results of the calculation were shown in Table 1.

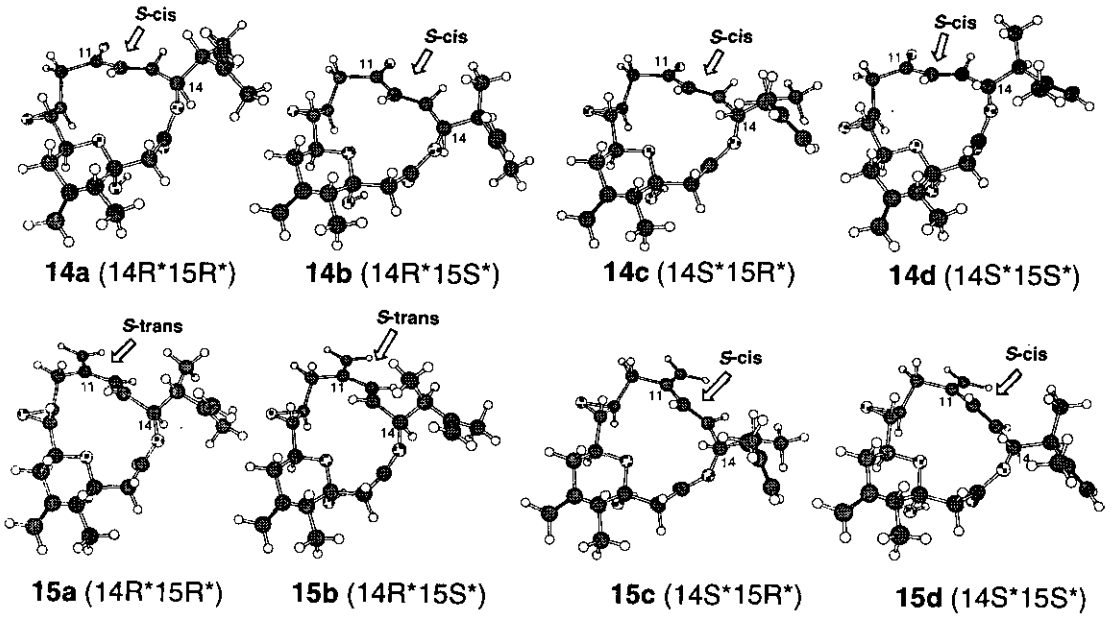
The NOESY spectrum of amphidinolide O (**14**) in C_6D_6 solution revealed clearly cross-peaks due to H-8/H-12 and H-10b/H-12 with no correlations for H-8/H-13 or H-10b/H-13 observed. On the other hand, the NOESY spectra of amphidinolide P (**15**) in C_6D_6 showed substantial correlations for H-8/H-13, H-10b/H-13, and H-12/H-22a, but no cross-peaks due to H-8/H-12, H-10b/H-12, or H-13/H-22a were visible. These observations implied that, in the C_6D_6 solution states, the C-11 ~ C-13 enone moiety of **14** is abundantly *S*-cis while the *S*-trans conformation is predominant for the C-11 ~ C-13 diene moiety of **15**. This result was consistent with the calculation data of 14R*-diastereomers (**14a/14b** and **15a/15b**, Table 1); for **14a** and **14b**, the lowest-energy

Table 1. Summary Data of the Calculations for Diastereomers (**14a-14d**) and (**15a-15d**)

Diastereomer	the major C-11~C-13 confomation	calculated average of $J_{14,15}$ (Hz)	Distance[a] H-8/H-12 (Å)	Distance[a] H-8/H-13 (Å)	Distance[a] H-12/H-14 (Å)	Distance[a] H-13/H-14 (Å)
14a (14R*15R*)	*S*-cis	9.2	3.02	5.81	3.77	2.39
14b (14R*15S*)	*S*-cis	2.5	3.04	5.88	3.71	2.42
14c (14S*15R*)	*S*-cis	4.8	2.99	5.78	2.75	2.87
14d (14S*15S*)	*S*-cis	10.3	3.03	5.80	2.99	2.82
15a (14R*15R*)	*S*-trans	9.5	5.34	2.74	2.38	3.13
15b (14R*15S*)	*S*-trans	3.7	5.38	2.72	2.40	3.11
15c (14S*15R*)	*S*-cis	6.3	3.09	5.73	2.59	2.94
15d (14S*15S*)	*S*-cis	10.4	3.18	5.55	2.63	2.97

[a]Distances for the lowest energy confomers

Figure 1. Three-Dimensional Structures of the Lowest-Energy Conformers of the Diastereomers (**14a - 14d**) and (**15a - 15d**)



conformers comprise the *S*-cis enone and those for **15a** and **15b** have the *S*-trans diene. In Table 1, the distance H-8/H-12 of the conformers having the *S*-cis enone or diene (*ca.* 3.0 Å) is much shorter than the distance H-8/H-13 (*ca.* 5.7 Å), and conversely, for those having *S*-trans conformation the distance H-8/H-13 (*ca.* 2.7 Å) is significantly shorter than that of H-8/H-12 (*ca.* 5.3 Å). In the NOESY spectrum of **14** a cross-peak due to H-13/H-14 was intensely observed but that for H-12/H-14 appeared indistinct. On the contrary, the NOESY spectrum of **15** showed a significant cross-peak for H-12/H-14 while the H-13/H-14 correlation was obscure. These findings also coincided with the calculation data of the **14a/14b** and **15a/15b** diastereomers (Table 1); the calculated distance H-13/H-14 of the lowest conformer of **14a/14b** (*ca.* 2.4 Å) is quite shorter than that of H-12/H-14 (*ca.* 3.7 Å), whereas the latter (*ca.* 2.4 Å) is considerably shorter than the former (*ca.* 3.1 Å) for **15a/15b** diastereomers. In addition, for the 14S*-diastereomers (**14c/14d** and **15c/15d**), the calculated distances of H-12/H-14 and H-13/H-14 are not significantly different from each other (*ca.* 2.6-2.9 Å, Table 1), which appears to be inconsistent with the NOESY data of **14** and **15**

described above. From all of these results, the 14R*-configuration was strongly suggested for both **14** and **15**.

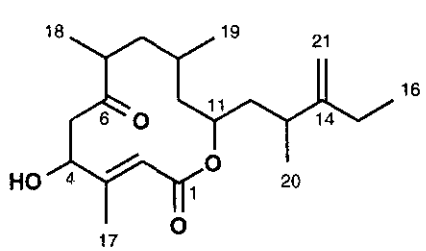
For the remaining one chiral center at C-15, two diastereomers [**14a** and **15a** (15R*); **14b** and **15b** (15S*)] now had to be considered for **14** and **15**, respectively, and the relative stereochemistry of C-15 was analyzed on the basis of comparison of the proton-proton coupling constant ($J_{14,15}$) between the observed and the calculated values. The observed $J_{14,15}$-values were 7.4 and 9.3 Hz for **14** and **15**, respectively. The calculated average values of $J_{14,15}$ were shown in Table 1, and those for the 15R*-diastereomers (**14a**: 9.2 Hz; **15a**: 9.5 Hz) corresponded better than those of 15S*-diastereomers (**14b**: 2.5 Hz; **15b**: 3.7 Hz). Thus, in conclusion, the structures of amphidinolides O and P were deduced as **14** (=**14a**) and **15** (=**15a**), respectively, including the relative configurations (3S*, 4R*, 7S*, 8S*,[19] 9S*, 14R*, and 15R* for **14** and 3S*, 4R*, 7S*, 8R*,[19] 9S*, 14R*, and 15R* for **15**). The stereoviews of the Monte Carlo lowest-energy conformations of **14** and **15** were shown in Figures 2 and 3, respectively. The structural difference between **14** and **15** is found only at the C-11 position, viz., the

Figure 2. Stereoview of the Monte Carlo lowest-energy conformation of **14**

Figure 3. Stereoview of the Monte Carlo lowest-energy conformation of **15**

ketone group for **14** and the exomethylene group for **15**. To the best of our knowledge, natural product analogs with this type of structural difference (ketone/exomethylene) are quite rare. Compounds **(14)** and **(15)** are likely to be biogenetically related to each other; one may be a precursor of the other, but it is unknown which one preceded the other.

Amphidinolide Q (**16**, $C_{21}H_{34}O_4$)[20] was revealed to possess one ketone, one exomethylene, and four methyl groups by spectral data. The selected 2D nmr data were represented in Figure 4. The ^{13}C chemical shift of the C-17 methyl (δ_C 16.6) argued that the Δ^2-olefin was *E*, and this double bond was suggested to be conjugated with the C-1 ester carbonyl from the ^{13}C chemical shifts (C-2: δ_C 117.4; C-3: δ_C 155.4), which was also consistent with the UV absorption data of **16** (MeOH, λ_{max} 222 nm, ε 10300). Since the molecule of **16** was inferred to contain one ring from the unsaturation degrees, the C-1 carbonyl had to be linked to the C-11 oxymethine to form a 12-membered lactone ring, which was coincident with the low-field resonance of H-11 (δ_H 5.28). The gross structure of amphidinolide Q was thus elucidated as **16** having a novel backbone skeleton with a 12-membered macrocylcic lactone ring. Among the NOESY correlations considerably observed for **16**, cross-peaks for H-2/H-8a, H-7/H-9, H-8a/H-10a, and H-9/H-11 were noteworthy, which may suggest that the H-7, H-9, and H-11 are oriented to the same side of the macrocycle plane whereas the H-2, H-8a, and H-10a are directed otherwise. Further convincing evidences, however, have not been provided thus far for stereochemical assignment of the molecule of **16**.



16

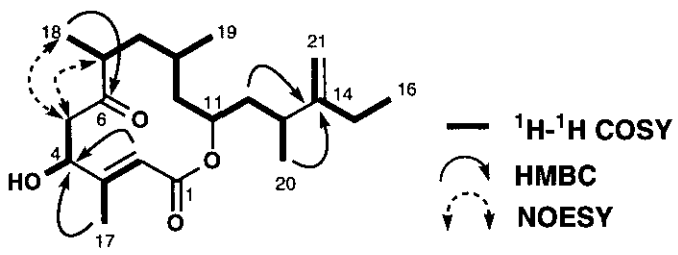
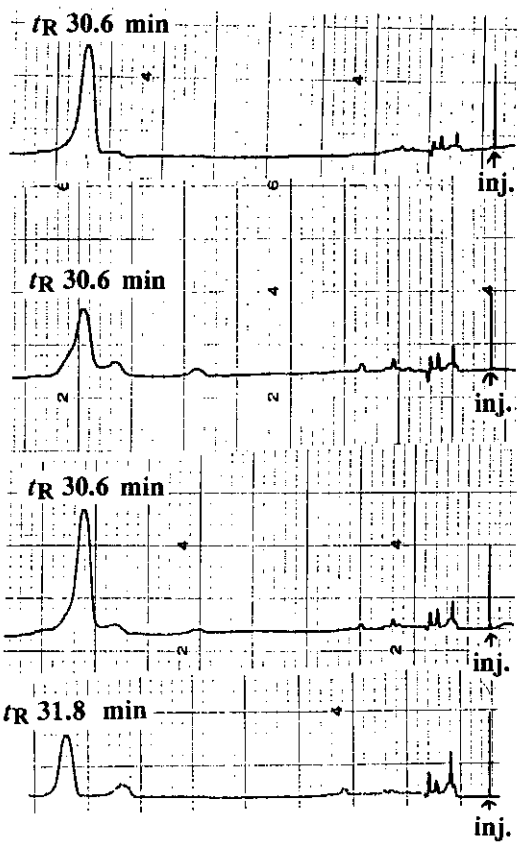


Figure 4. *Selected 2D NMR Data of* ***16****.*

6. STEREOCHEMISTRY OF AMPHIDINOLIDE B

In 1987 we first reported the isolation and planar structure of amphidinolide B (**2**) from the Y-5 strain of *Amphidinium* sp.,[21] and the planar structure was later revised partially.[22] In 1994 Shimizu and coworkers (The University of Rhode Island) isolated three macrolides belonging to the amphidinolide B group [amphidinolides B_1 (**2**), B_2 (**43**), and B_3 (**44**)] from a free-swimming dinoflagellate *Amphidinium* sp. and reported their relative stereochemistry on the basis of X-ray crystal structure of amphidinolide B_1.[23] Identity of amphidinolides B and B_1 was unambiguously established by direct comparison of hplc (Figure 5) and ^{1}H nmr (Figure 6) data using each authentic sample.[24] The signs of the optical rotations of these two samples were the same. We thereupon studied the absolute stereochemistry of amphidinolide B (**2**) based on synthesis of a degradation product (**45**) and chiral hplc analysis as follows.

Figure 5. Hplc Comparison of Amphidinolide B and Amphidinolide B_1



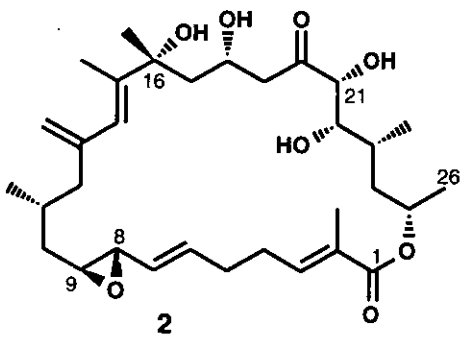
[Develosil ODS-5; Nomura Chemical; 10 x 250 mm; eluent: 60% MeCN; flow rate: 2.5 ml/min; uv detection at 220 nm]

Amphidinolide B_1 (Rhode Island)

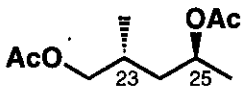
Amphidinolide B (Hokkaido)

Coinjection of Amphidinolide B (Hokkaido) and Amphidinolide B_1 (Rhode Island)

Amphidinolide D (Hokkaido)



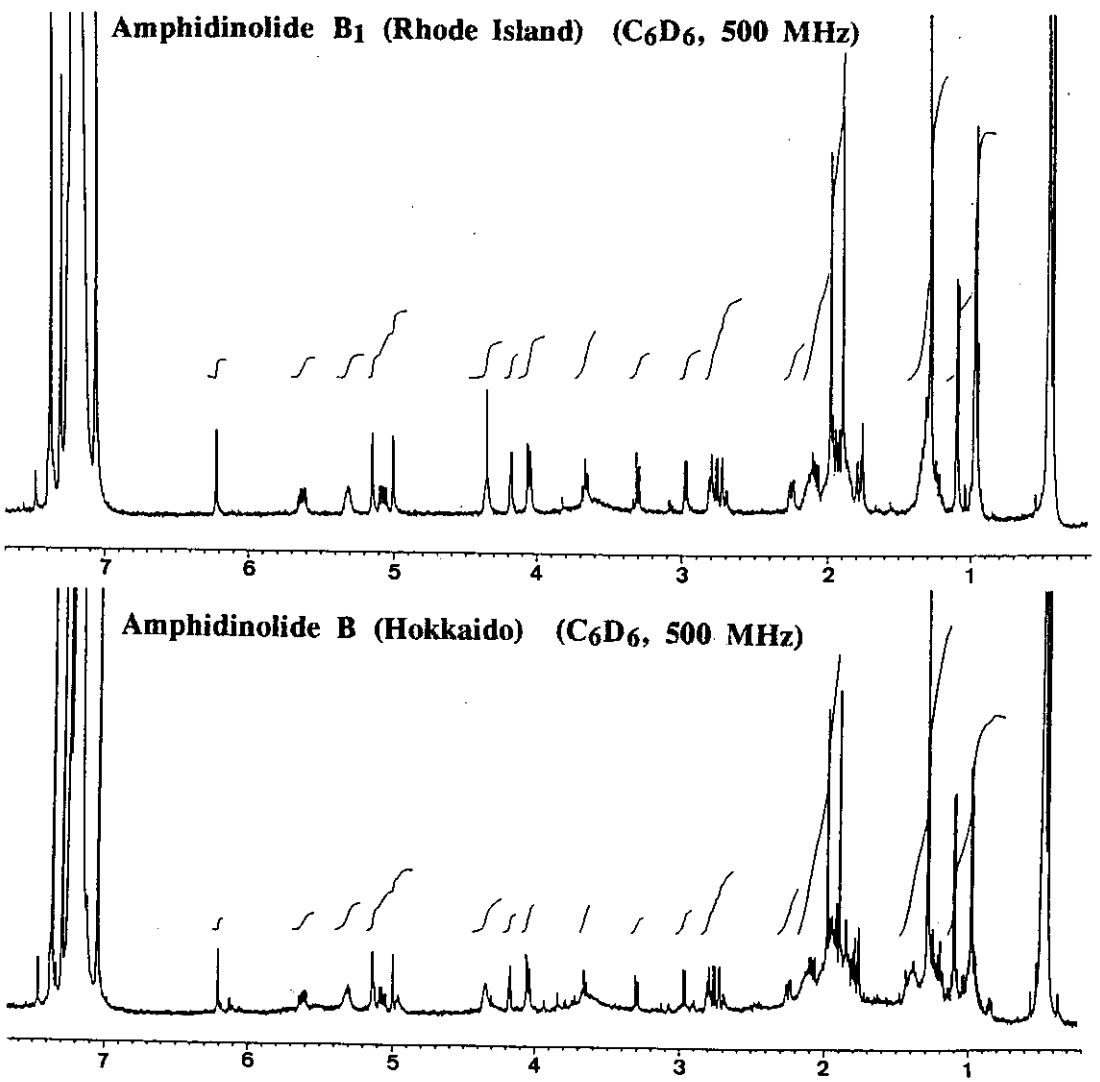
2
43 C-18 epimer
44 C-22 epimer



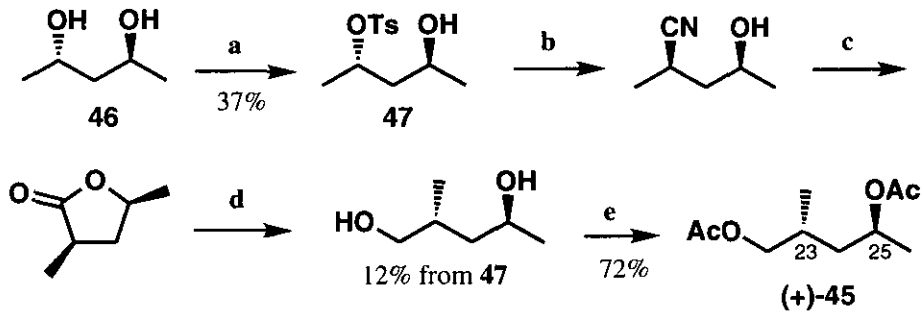
(+)-45

23*R*, 25*S*

Figure 6. ^{1}H Nmr Comparison of Amphidinolide B and Amphidinolide B_1



Scheme 6. Preparation of (+)-**45**.



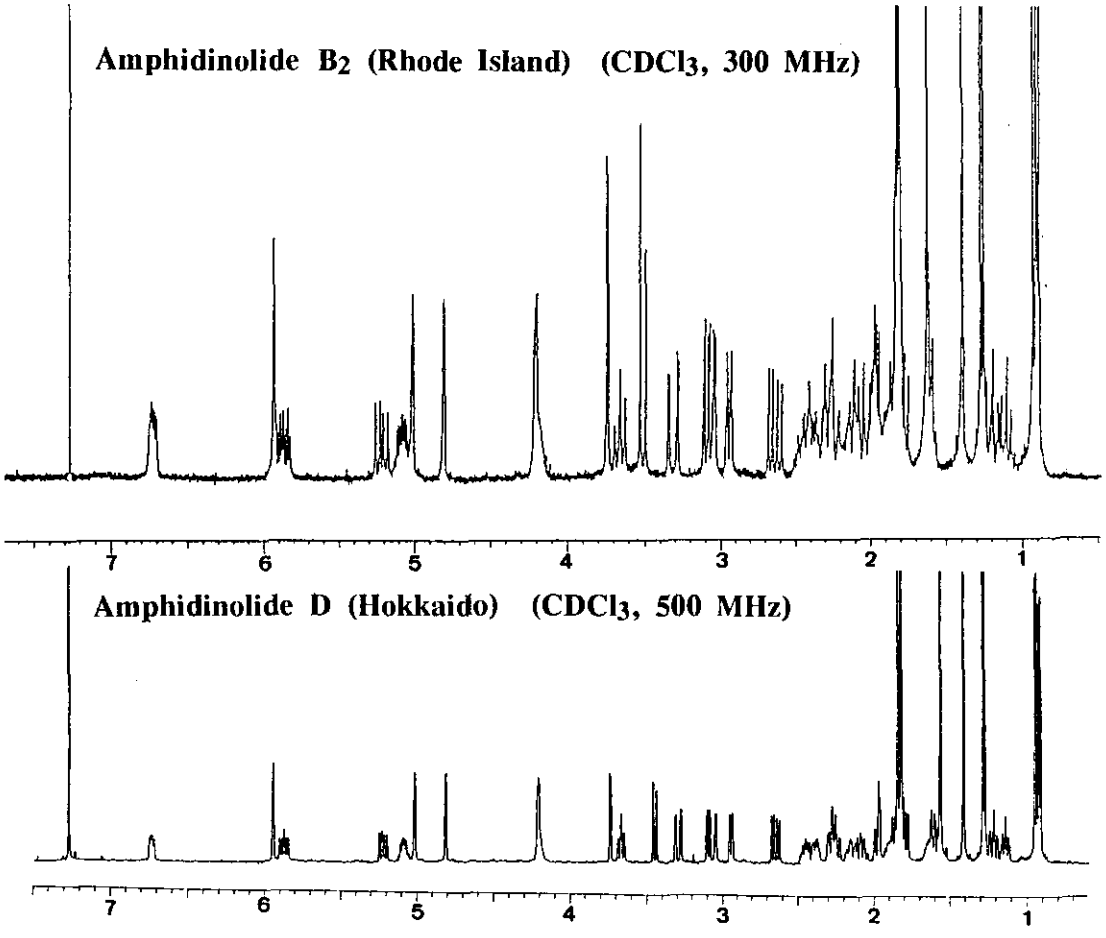
(a) TsCl, pyridine; (b) NaCN, DMSO; (c) (1) NaOH, H_2O_2, EtOH; (2) 2N HCl; (d) $LiAlH_4$, Et_2O; (e) Ac_2O, pyridine.

In advance of the degradation experiment we prepared both enantiomers of the C-22 ~ C-26 fragment, (+)-**45** and (-)-**45**, as shown in Scheme 6 from (2*S*,4*S*)-(+)-pentanediol (**46**) and (2*R*,4*R*)-(-)-pentanediol, respectively, both of which were available commercially. The chiral hplc analysis [CHIRALCEL OD, Daicel Chemical Ind., Ltd.; 4.6 x 250 mm; flow rate: 1.0 ml/min; eluent: hexane/2-propanol (500:1); uv detection at 215 nm] of the enantiomers (+)- and (-)-**45** showed that they were separable [(+)-**45**, t_R 23.2 min; (-)-**45**, t_R 22.3 min]. A MeOH adduct of amphidinolide B (**2**), which was obtained as an artifact of isolation and has a structure with a methoxyl and a hydroxyl groups at C-8 and C-9 positions,[22] was treated with $NaIO_4$ followed by $NaBH_4$ reduction and acetylation (Ac_2O/pyridine) to give the C-22 ~ C-26 fragment (**45**) after separation by normal-phase hplc. This fragment (**45**) thus obtained was subjected to chiral hplc analysis as above and proved to be identical with (+)-**45** (t_R 23.2 min), thus revealing that the C-22 ~ C-26 fragment (**45**) has (23*R*, 25*S*)-configurations. Since the relative stereochemistry of amphidinolide B_1 identical with **1** is known,[23] the absolute configurations of amphidinolide B (**2**) were concluded as 8*S*, 9*S*, 11*R*, 16*R*, 18*S*, 21*R*, 22*S*, 23*R*, and 25*S*, which was in agreement with our results on the absolute configurations of amphidinolide L (**11**).[5]

Shimizu and coworkers reported that amphidinolides B_2 (**43**), and B_3 (**44**), which they isolated concurrently with amphidinolide B_1 (**2**), were C-18 and C-22 epimer of **2**,

respectively.[23] The ^1H nmr spectra of amphidinolide B_2 (**43**) and amphidinolide D (**4**) resembled each other very well (Figure 7), indicating that these two compounds were identical. We had assigned the struture of amphidinolide D (**4**) was C-21 epimer of **2**,[22] and the conclusion of this different structural assignment (**4** or **43**) was not obtained presently. In addition to the presently available nmr spectral data (NOE or ^1H-^1H coupling constants), further evidences seemed to be required for unambiguous stereochemical assignment of this molecule.

Figure 7. ^1H Nmr Comparison of Amphidinolide D and Amphidinolide B_2



7. BIOSYNTHESIS OF AMPHIDINOLIDE J

Macrolide antibiotics from terrestrial microorganisms generally possess even-numbered macrocyclic lactones, which are reasonably derived from the polyketide biosynthesis. However, many of amphidinolides comprise unusual odd-numbered macrocyclic lactone rings [amphidinolides C (**3**; 25-membered), E (**5**; 19-membered), F (**6**; 25-membered), G (**7**; 27-membered), J (**9**; 15-membered), K (**10**; 19-membered), L (**11**; 27-membered), M (**12**; 29-membered), O (**14**; 15-membered), and P (**15**; 15-membered)]. The amphidinolides comprise some other unique structural features: (i) they have a variety of novel backbone-skeletons, isolated from one genus of microalga, (ii) all amphidinolides contain one or more exomethylene units, and (iii) vicinally located one-carbon branches (viz., methyl or exomethylene) are present in amphidinolides B (**2**), C (**3**), D (**4**), F (**6**), G (**7**), H (**8**), J (**9**), K (**10**), L (**11**), M (**12**), O (**14**), P (**15**), and Q (**16**), and amphidinin A (**17**). Particularly, the generation of odd-numbered macrocyclic lactone ring as well as the structural feature (iii), was unable to be accounted for by the classical polyketide biosynthesis. We therefore investigated the biosynthesis of amphidinolides based on stable isotope incorporation experiments, although the sample size of the macrolides produced by the alga was not very high, requiring a large scale of culturing and considerable amount of ^{13}C-labeled precursors. The present experimental results as well as our hypothesis on the biosynthesis of amphidinolide J (**9**), currently the most abundant macrolide in *Amphidinium* sp. (strain Y-5), are described below.[26]

The dinoflagellate *Amphidinium* sp. (strain Y-5) was cultured in 3-l glass bottles containing nutrient-enriched sea water medium as previously described,[1] and feeding experiments were carried out with [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$] sodium acetate and [*methyl*-^{13}C]-L-methionine. Summary of the conditions of feeding experiments was shown in Table 2. The ^{13}C-labeled precursors were fed to the alga (610 μM for labeled sodium acetate and 93 μM for labeled methionine) in one portion 10-12 days after inoculation, and 2 days later the culture was harvested. The extract of the harvested cells was purified by improved procedures to afford ^{13}C-labeled

Table 2. Feeding Experiments of ^{13}C-Labeled Precursors to *Amphidinium* sp. (Y-5)

run	^{13}C-labeled precursors	culture (L)	concentration (μM)	the day after inoculation	
				addition (day)	harvest (day)
1	1-^{13}C-NaOAc	200	610	10	14
2	1-^{13}C-NaOAc	100	610	10	12
3	2-^{13}C-NaOAc	100	610	12	14
4	1,2-$^{13}C_2$-NaOAc	100	610	10	12
5	methyl-^{13}C-(L)-methionine	80	93	10	12

Table 3. Isotope Incorporation Results from the ^{13}C Nmr Data of Amphidinolide J (**9**)[a]

position	δ_C	intensity ratio (labeled/unlabeled)[b]		Assignment of 'c' or 'm'[c]	J_{CC} (Hz)
		[1-^{13}C]-acetate	[2-^{13}C]-acetate		[1,2-$^{13}C_2$]-acetate
1	171.6	1.41	1	c	57.8
2	39.9	1.01	1.72	m	57.8
3	34.6	0.88	1.59	m	-
4	151.9	1.34	0.97	c	42.5
5	36.1	1.05	2.02	m	41.4
6	29.7	1.68	1.36	c	43.6
7	130.8	0.87	1.88	m	43.6
8	136.5	2.10	1.10	c	49.0
9	78.8	0.95	1.66	m	48.0
10	45.7	1.51	0.99	c	43.6
11	133.5	1.03	2.09	m	43.6
12	132.6	0.87	1.51	m	-
13	72.6	1.46	1.05	c	42.5
14	79.9	1.15	2.33	m	41.4
15	39.5	1.52	1.16	c	42.5
16	133.6	0.76	1.61	m	43.6
17	131.5	1.53	0.95	c	42.5
18	35.3	0.94	2.50	m	42.5
19	23.4	1.58	1.01	c	34.9
20	14.2	1	1.98	m	34.9
21	22.2	0.96	1.81	m	-
22	108.7	1.12	1.99	m	-
23	19.0	1.03	1.78	m	-
24	17.5	1.14	2.21	m	-

[a] The ^{13}C nmr spectra were recorded in C_6D_6 solution on a Bruker ARX500 spectrometer at 125 MHz with sweep width of 35700 Hz using Bruker's pulse program 'zgpg30'. Numbers of scans were *ca.* 13000 and 25728, for the samples from feedings of mono- and double-^{13}C labeled precursors, respectively.

[b] Intensity of each peak in the labeled **9** divided by that of the corresponding signal in the unlabeled **9**, normalized to give a ratio of 1 for an unenriched peak (C-20 for [1-^{13}C]-acetate labeling and C-1 for [2-^{13}C]-acetate labeling).

[c] 'c' denotes 'carbon derived from C-1 of acetate', while 'm' indicates 'carbon derived from C-2 of acetate'.

Figure 8. The ^{13}C Nmr spectra of **9** labeled with sodium [1-^{13}C] and [2-^{13}C] acetate and [*methyl*-^{13}C]-L-methionine

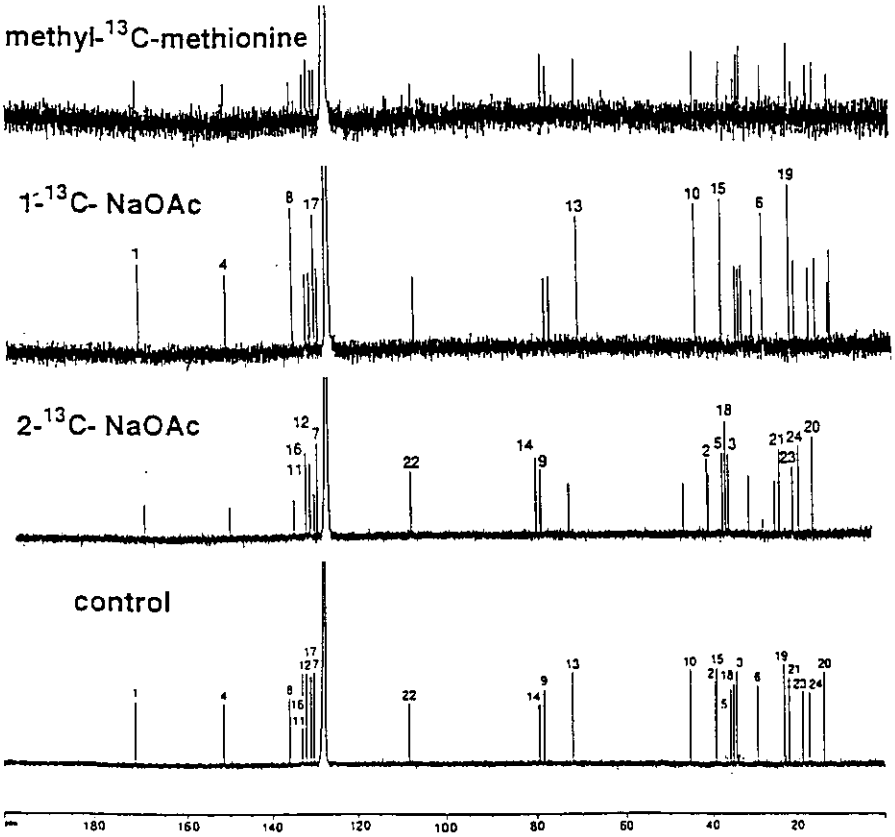
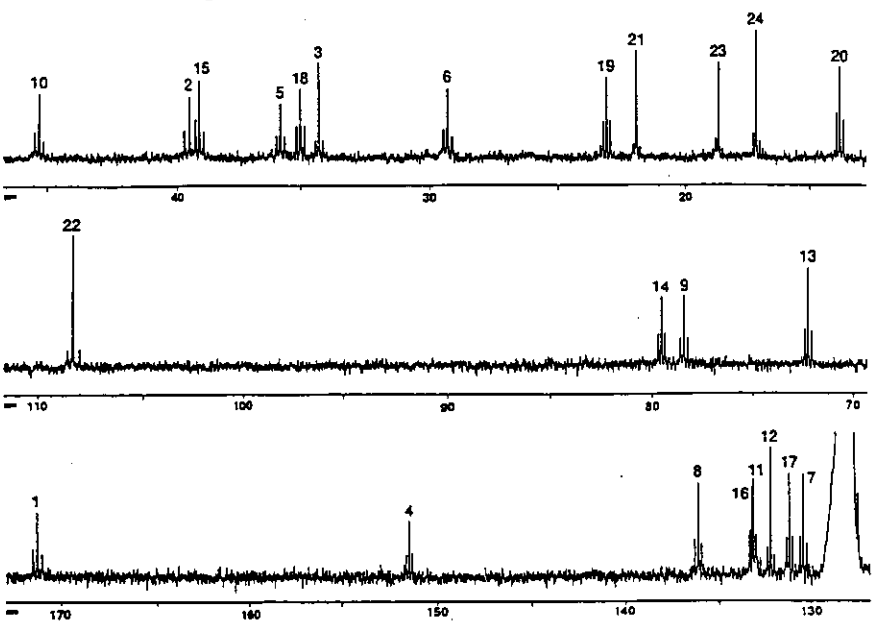
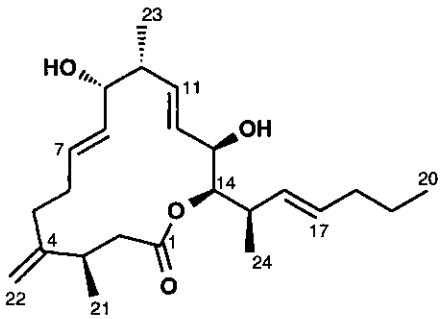


Figure 9. The ^{13}C Nmr spectrum of **9** labeled with sodium [1,2-$^{13}C_2$] acetate





9

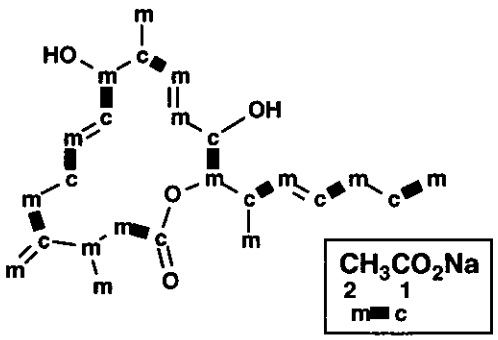
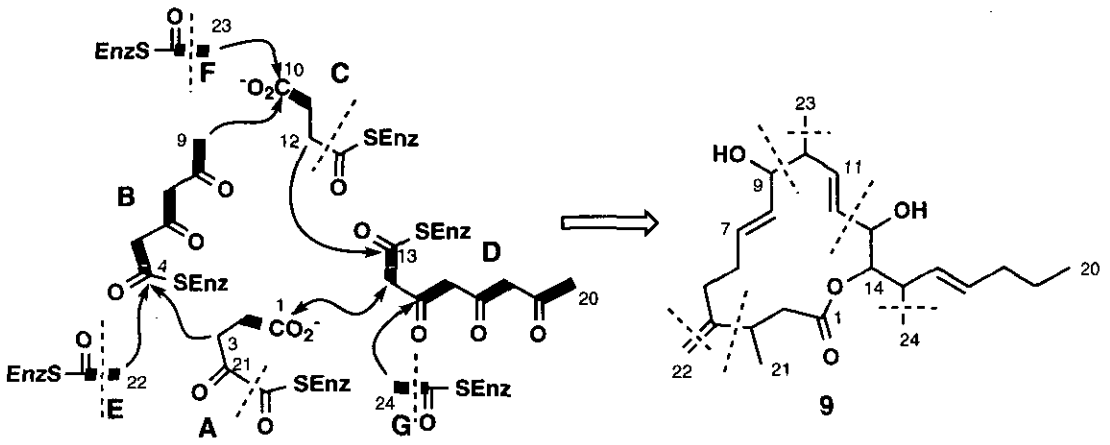


Figure 10. Labeling patterns of amphidinolide J (**9**)

amphidinolide J (**9**; 0.5-1 mg from 80-100 l of culture). Assignments of the ^{13}C nmr signals of **9** in C_6D_6 solution were fully established by HMQC and HMBC spectra and are presented in Table 3. Figures 8 and 9 represent the ^{13}C nmr spectra of amphidinolide J (**9**) obtained by the feeding experiments. The ^{13}C nmr spectrum of amphidinolide J (**9**) labeled from sodium [1-^{13}C] acetate showed significant enrichment of 9 carbons (C-1, C-4, C-6, C-8, C-10, C-13, C-15, C-17, and C-19), while those enriched by sodium [2-^{13}C] acetate were 15 carbons (C-2, C-3, C-5, C-7, C-9, C-11, C-12, C-14, C-16, C-18, C-20, C-21, C-22, C-23, and C-24). The ratios of the signal intensities over those of nonlabeled **9** were also described in Table 3. Thus, all 24 carbons contained in amphidinolide J (**9**) were revealed to be derived from acetates. The ^{13}C nmr of **9** obtained from feeding experiment of [*methyl*-^{13}C]-L-methionine did not show appreciable enrichment of any carbon. The ^{13}C-^{13}C coupling constants ($^1J_{CC}$) of **9** labeled with [1,2-$^{13}C_2$]-acetate (Table 3) indicated that the C_2 units for C-1/C-2, C-4/C-5, C-6/C-7, C-8/C-9, C-10/C-11, C-13/C-14, C-15/C-16, C-17/C-18, and C-19/C-20 originate from the same acetates. Interestingly, when the culture was harvested 4 days after feeding of sodium [1-^{13}C] acetate to the alga (run 1 of Table 2), the ^{13}C nmr of isolated **9** showed that all carbon atoms of **9** were enriched and almost all signals were observed with double satellite signals due to vicinal ^{13}C-^{13}C couplings. This phenomenon was considered to be observed probably because C-1 of acetate was cleaved *via* decarboxylation during passage through the TCA cycle and the released

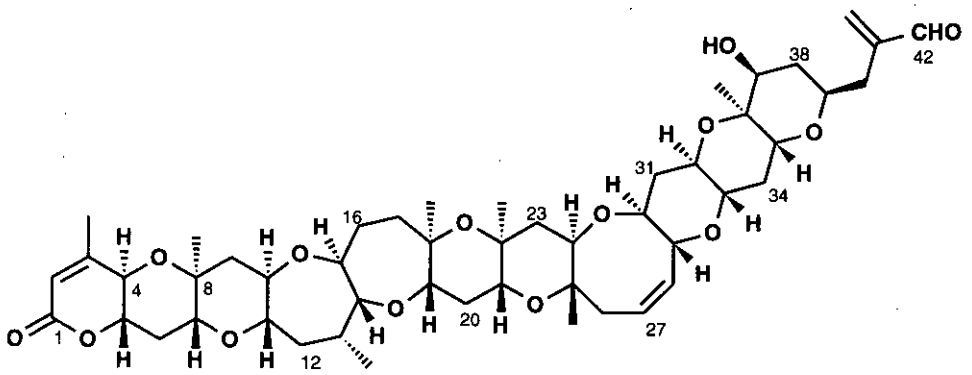
Figure 11. Possible Biosynthetic Building Blocks of Amphidinolide J (**9**)



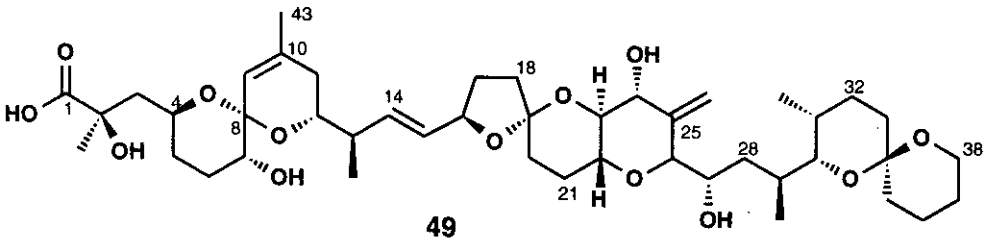
A: α-ketoglutarate; **B**, **D**: classical polyketide;
C: succinate; **E**, **F**, **G**: C-2 of acetate

$^{13}CO_2$ was reincorporated during photosynthesis to give randomly labeled acetates, which led to all-carbon enriched **9**.

The labeling patterns of amphidinolide J (**9**) shown by the feeding experiments were quite unusual and represented in Figure 10. Significantly, the C-3 and C-12 of **9** were derived from the methyl carbons of acetates, and the carboxyl carbons of which were lost. Thus, the carbons constituting the 15-membered lactone ring were not constructed from the consecutive polyketide chain. This finding seems to justify that the lactone ring size of **9** dose not have to be even. The irregular labeling pattern of **9** could be interpreted as one possibility by assuming that the backbone carbons of **9** were biosynthetically derived from the precursors depicted in Figure 11. Units **B** (C-4 to C-9) and **D** (C-13 to C-20) are likely to be classical polyketides derived as a result of the condensation of three and four acetate units, respectively. Unit **A** (C-1/C-2/C-3/C-21) contains the "c-m-m-m" moiety and may come from a dicarboxylic acid like α-ketoglutarate after passage of acetate through the TCA cycle, which has been observed in the biosynthesis of brevetoxin B (**48**, C-6/C-7/C-8/C-9)[27-29] and okadaic acid (**49**, C-8/C-9/C-10/C-43).[30] Unit **C** (C-10/C-11/C-12) labeled as "c-m-m" may be derived from succinate, corresponding to the six units of **48** (*e.g.*, C-10/C-11/C-12). Units **E**,



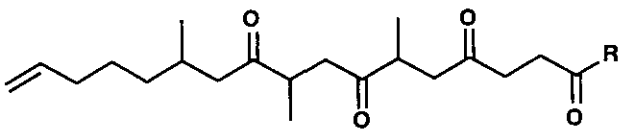
48



49

F, and **G** (C-22, C-23, and C-24) are one-carbon branches (an exomethylene and two secondary methyls), and they were demonstrated to be derived from the C-2 of acetates and attached to carbons in a linear chain derived from the C-1 of acetates (C-4, C-10, and C-15, respectively). One-carbon branching of this type is unusual in polyketide biosynthesis and has been previously reported only in few cases. Another one-carbon branch of C-21 also came from the C-2 of acetate. However, the condensation of this carbon to the linear chain occurred at the carbon (C-3) derived from the C-2 of acetate; thus, the participation of a dicarboxylic acid precursor was proposed for this moiety (*vide supra*). How the vicinal locations of one-carbon branches are brought about in amphidinolides still appears an interesting question, and the present results argued that two vicinal one-carbon branches (C-21 and C-22) of **1** were both derived from the C-2 of acetate but attached to the linear chain through different processes. It should be noted that the oxymethines at C-9 and C-14 of **1** are derived from the C-2 of acetate, and the origins of the oxygen atoms are unknown.

In connection with this our work, unusual 1,4-polyketides, amphidinoketides I (**50**) and II (**51**) were isolated recently from *Amphidinium* sp. by Shimizu's group[31] and a possible biosynthetic pathway involving the condensation of succinates was proposed. The 1,4-polyketide moieties of **50** and **51** may correspond to the C-15 ~ C-18 portion of amphidinolides C (**3**) and F (**6**).



50 R = -CH=CMe$_2$ **51** R = -CH$_2$C(Me)=CH$_2$

8. CYTOTOXICITY AND ISOLATION YIELDS OF AMPHIDINOLIDES

The cytotoxicity data of amphidinolides A (**1**) ~ H (**8**), J (**9**) ~ Q (**16**), and amphidinin A (**17**) against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro* are summarized in Table 4 together with their isolation yields from the four

Table 4. Isolation Yields and Cytotoxicity Data of Amphidinolides

Compounds		lactone ring size	Isolation Yields (x 10^{-4}%)[a] strain number Y-5[b]	Y-5'	Y-25	Y-26	Cytotoxicity (IC_{50}, μg/ml) L1210	KB
Amphidinolide A	**1**	(20)	20	4	-	-	2.0	5.7
Amphidinolide B	**2**	(26)	10	-	-	0.8	0.00014	0.0042
Amphidinolide C	**3**	(25)	15	-	-	0.3	0.0058	0.0046
Amphidinolide D	**4**	(26)	4	-	-	-	0.019	0.08
Amphidinolide E	**5**	(19)	4	3	-	-	2.0	10
Amphidinolide F	**6**	(25)	-	-	-	0.1	1.5	3.2
Amphidinolide G	**7**	(27)	-	-	20	-	0.0054	0.0059
Amphidinolide H	**8**	(26)	-	-	17	-	0.00048	0.00052
Amphidinolide J	**9**	(15)	60	-	-	-	2.7	3.9
Amphidinolide K	**10**	(19)	0.3	-	-	-	1.65	2.9
Amphidinolide L	**11**	(27)	-	-	2	-	0.092	0.1
Amphidinolide M	**12**	(29)	4	-	-	-	1.1	0.44
Amphidinolide N	**13**	(26)	9	-	-	-	0.00005	0.00006
Amphidinolide O	**14**	(15)	1	-	-	-	1.7	3.6
Amphidinolide P	**15**	(15)	2	-	-	-	1.6	5.8
Amphidinolide Q	**16**	(12)	0.5	-	-	-	6.4	>10
Amphidinin A	**17**	(-)	0.6	-	-	-	3.6	3.0

a) Based on the wet weight of the harvested cells. b) Isolation yields vary during course of time. "-" denotes "not isolated"

strain of the dinoflagellates *Amphidinium* sp. The level of the cytotoxic activity of amphidinolides B_1 (**2**), B_2 (**43**), and B_3 (**44**) against a different cell line (human colon tumor cell line HCT 116 (IC_{50} 0.122, 7.5, and 0.206 μg/ml, respectively), reported by Shimizu and coworkers,[23] were significanly lower than those of amphidinolides B (**2**) and D (**4**).

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