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Acetate labeling patterns of dinoflagellate polyketides, amphidinols 2, 3 and 4

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Abstract—Amphidinols, which are polyketide metabolites chiefly comprising a long linear chain with polyhydroxyl groups and polyolefins, are produced by marine dinoflagellates *Amphidinium carterae* and *A. klebsii*. The acetate incorporation experiments of amphidinols 2, 3 and 4 revealed that they are built up with five regular C_2-elongation sequences, which are separated by continuous acetate-methyl derived carbons. The findings support the C1-deletion mechanism from the regular sequence, which could be accounted for either by Favorski-type reaction or by Tiffeneau–Demjanov rearrengement.

1. Introduction

Dinoflagellates are a rich source of bioactive secondary metabolites.[1] Particularly, polyketide derivatives bearing unique structures and powerful bioactivities have been reported for the last two decades; e.g. brevetoxins, ciguatoxins, maitotoxin, palytoxin and okadaic acid. Amphidinols (AMs) are a group of polyene–polyhydroxy compounds, among which AM1 was first reported by Yasumoto et al. in 1991. The structures are best characterized by a long carbon chain encompassing multiple hydroxyl groups and polyolefins.[2] Progress in the biochemistry of actinomycetous and fungal polyketides has been tremendously accelerated since the genes encoding polyketide synthases (PKSs) were identified and sequenced.[3] These findings led to the allocation of enzymatic functions in PKS gene, which could account for the incorporation patterns of acetate, propionate and other small building blocks; carbon skeletons are basically built up by C_2-elongation reactions. In contrast to these prokaryotic and fungal metabolites, polyketides from microalgae, particularly from dinoflagellates, showed markedly different biosynthetic paths as seen for okadaic acid,[4] dinophysistoxins,[5] brevetoxins,[6] amphidinolides,[7] goniodomin A[8] and yessotoxins.[9] Despite the prominent differences in their biosynthesis, which imply unexploited functions in dinoflagellate PKSs, genetic studies have been hampered by their large genome size and phylogenic distance from fungi or bacteria. A considerable number of dinoflagellate polyketides, however, have been identified with their acetate labeling patterns.[4–9] Polycyclic ethers such as brevetoxins and okadaic acid have been extensively investigated while there are only a few examples for acyclic or macrocyclic compounds; e.g. amphidinolides H and J[7b] and polyhydroxy moieties of dinophysistoxin-4.[4] In this paper we present the acetate incorporation patterns of AM2, AM3 and AM4, which possess the longest carbon skeleton among the polyketides so far identified with their carbon origins.

2. Results and discussion

Amphidinium carterae was collected in New Zealand, and *A. klebsii* in Japan. The former was used for acetate-incorporations for AM4, and the latter was for AM2[10] and AM3.[11] ^{13}C NMR signal assignments for AM2 and AM3, which were necessary to allocate labeled atoms, were previously reported.[10,11c] Those of AM4 was assigned this time by usual 2D NMR methods. *A. carterae* from New Zealand was fed with [1-^{13}C]acetate, [2-^{13}C]acetate and [1,2-$^{13}C_2$]acetate (all sodium salts). Additional [methyl-^{13}C]methionine was fed to *A. klebsii*. The labeled acetates were efficiently incorporated in AM2, AM3 and AM4 for most carbons except C1–C2, which corresponds to a starter unit (its origin is currently investigated). The average incorporation ratio from [1,2-$^{13}C_2$]acetate in AMs was estimated to be ca. 30% on the basis of mass spectra. For both AM2 and AM4, ^{13}C NMR signals labeled from [1-^{13}C]acetate increased 2–5 times whilst feeding with

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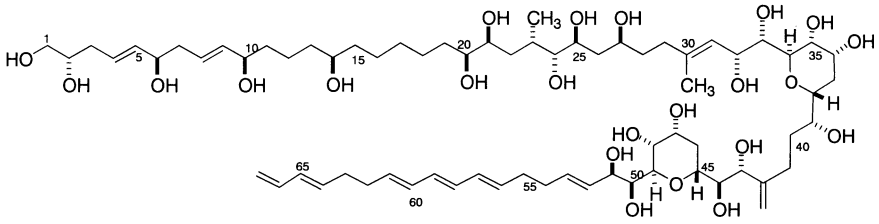
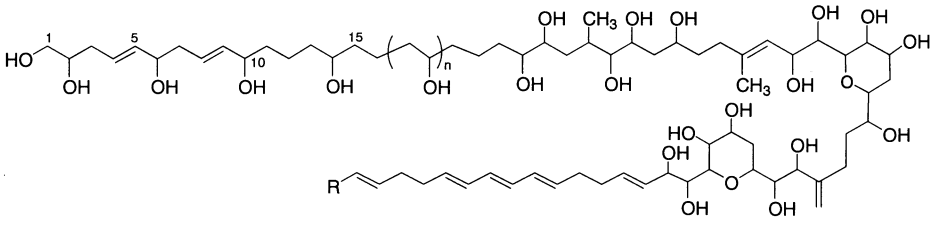
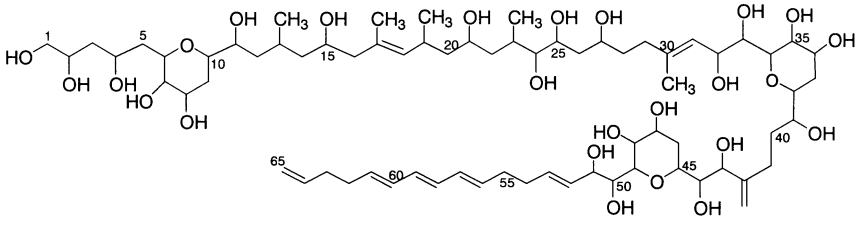


Absolute Configuration of AM3



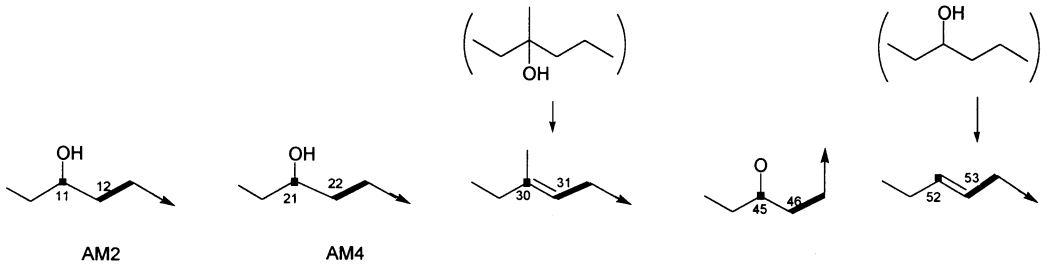
AM3 n=0, R: CH_2=CH-
AM4 n=0, R: H
AM5 n=1, R: CH_2=CH-
AM6 n=1, R: H



AM2

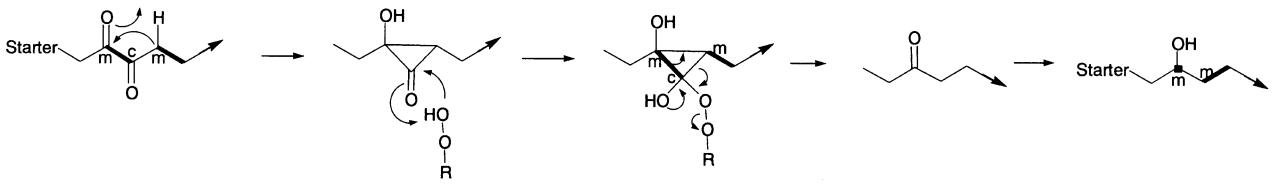
[2-^{13}C]acetate led to 4–20 times enhancement (Fig. 1). [1-^{13}C]acetate generally showed the higher non-specific incorporation compared with [2-^{13}C]acetate.

The biosynthesis of AMs turned out to start from the C1–C2 terminus on the basis of the intact incorporation pattern of [1,2-$^{13}C_2$]acetate in AM4 (Fig. 2), which was readily assigned from $^1J_{C,C}$ values (Table 1). There are four irregular labeling sites both in AM2 and AM4, where acetate methyl-derived carbons resided in the vicinal position (m–m and m–m–m, Fig. 2) as frequently seen for other dinoflagellate polyketides.[4–9] C_1-branching groups such as pendent methyls and exomethylene were all labeled from methyl carbon of acetate as was the case with okadaic acid and amphidinolides while methionine methyl carbon, which was incorporated in brevetoxins[6] and yessotoxin,[9]



AM2 AM4

Scheme 1.



Scheme 2.

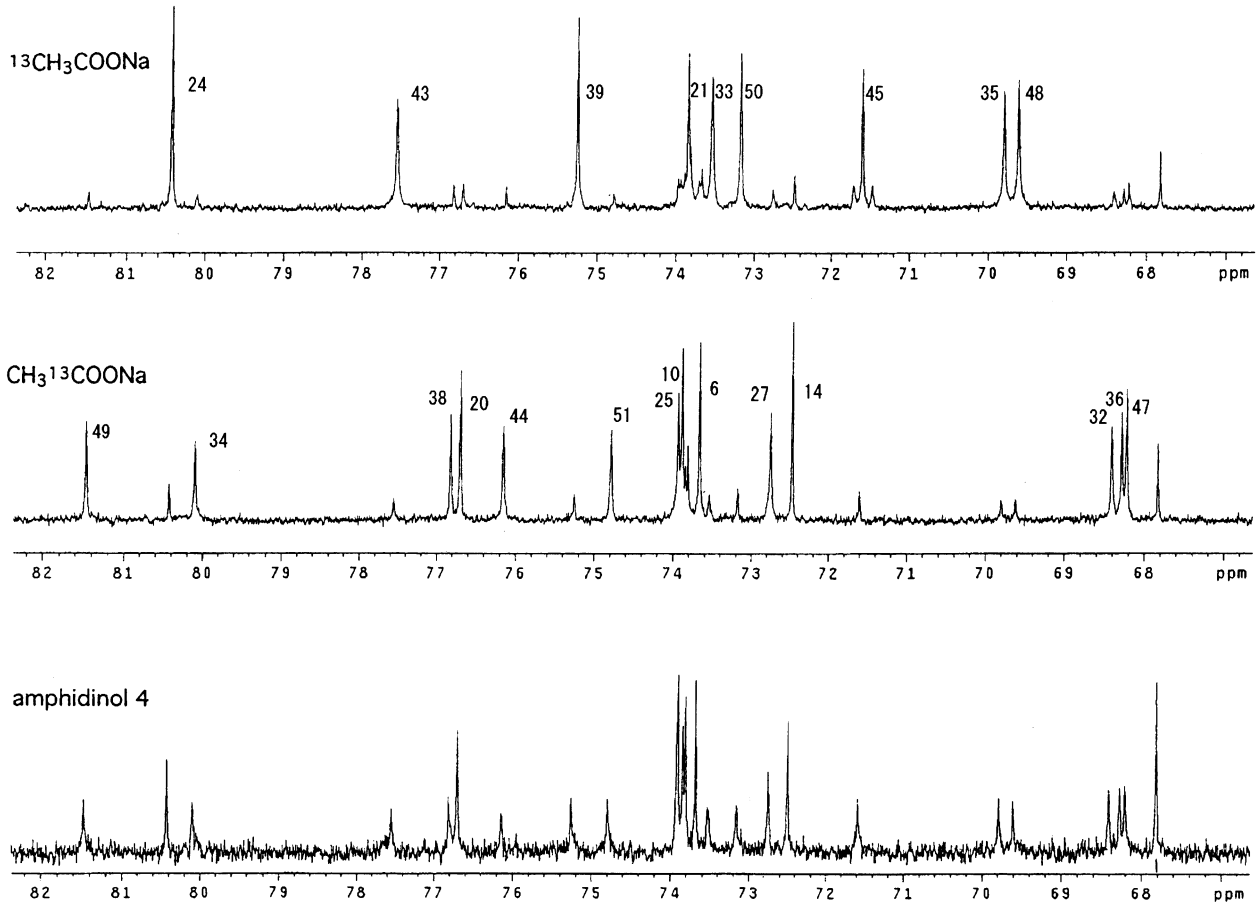
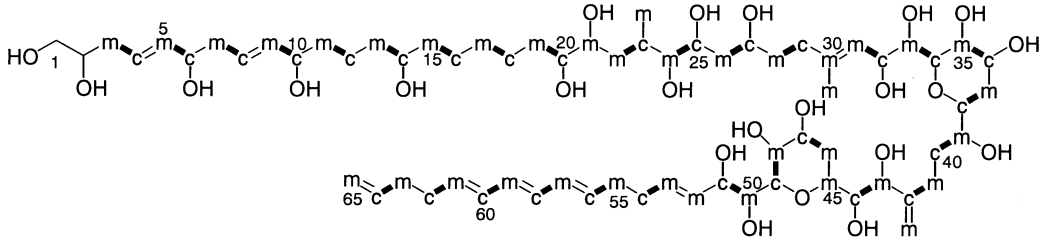


Figure 1. Partial ^{13}C NMR spectra of AM4 incorporated from [2-^{13}C]acetate (top), [1-^{13}C]acetate (middle), and control (bottom). The spectra were measured at 150 MHz in CD_3OD–C_5D_5N, 1:2 solutions.



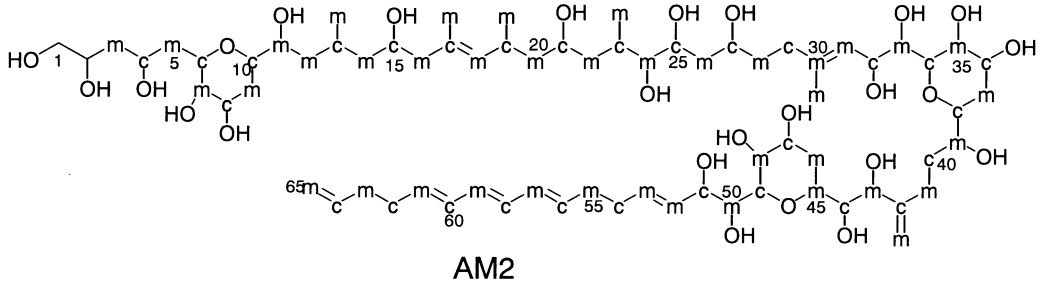


Figure 2. Acetate-labeling patterns of AM4 and AM2. c: carbons efficiently labeled from C1(CO) of acetate (middle). m: those from C2(Me) of acetate. c–m: intact acetate incorporation from [1,2-$^{13}C_2$]acetate. C1 and C2 in both AM4 and AM2 were not labeled from the acetates above the nonspecific incorporation levels. AM3 revealed the same incorporation pattern as that of AM4 (data not shown).

Table 1. ^{13}C NMR data of amphidinol 4 (AM4) (CD_3OD–C_5D_5N, 1:2 as $^{13}CD_3OD$ at 49.8 ppm)

Carbon No.	δ_C	$^1J_{C,C}$ (Hz)	Carbon No.	δ_C	$^1J_{C,C}$ (Hz)
1	67.8	s[a]	35	69.8	37
2	73.8	s	36	68.3	36
3	38.7	43	37	32.2	35
4	129.0	43	38	76.8	35
5	137.5	-[b]	39	75.3	39
6	73.6	45	40	33.5	39
7	42.7	43	41	28.8	42
8	128.6	43	42	153.4	42
9	138.3	47	43	77.6	44
10	73.9	47	44	76.2	44
11	39.5	35	45	71.6	s
12	23.8	35	46	33.0	36
13	39.5	38	47	68.2	36
14	72.5	38	48	69.6	38
15	39.6	35	49	81.5	38
16	27.7	35	50	73.2	41
17	31.8	35	51	74.8	41
18	28.0	35	52	131.2	s
19	35.1	38	53	134.1	43
20	76.7	38	54	34.2	42
21	73.8	s	55	34.2	44
22	40.0	35	56	135.3	44
23	32.4	35	57	132.6	–
24	80.4	44	58	133.0	–
25	73.9	44	59	132.8	–
26	42.4	38	60	132.8	–
27	72.8	38	61	134.9	44
28	38.1	34	62	33.8	44
29	37.5	34	63	35.2	42
30	138.7	s	64	139.8	42
31	128.1	50	65	116.2	s
32	68.4	50	66	14.2	s
33	73.5	43	67	18.3	s
34	80.1	43	68	113.1	s

[a] Height of doublet peaks is less than 20% of a center peak.
[b] NOT determined.

was not transferred into AM2. The linear polyhydroxy part (C3–C20) of AM4 is apparently built by Claisen condensation to yield simply repeated acetate (m–c) units with hydroxy groups at carbonyl-derived carbons, C6, C10, C14 and C20. Conversely, in AM2, the m–c repetition was interrupted at C10–C11, which, together with three additional pendent methyl groups, suggested the different genetic origins for these starting units. Dissimilarity in the labeling patterns between AM2 and AM4 was again recognized in the middle part, where repetition of the m–c sequence continued to C29 in AM2, whereas being interrupted at C20–C21 in AM4. Among the four m–m sites in AM4, the hydroxy-bearing carbons of C21 and C45 biosynthetically precede their following methylene carbons of C22 and C46, respectively; similar situation can be seen for C11–C12 of AM2 (Scheme 1). The olefinic m–m sites, C30–C31 and C52–C53, are probably formed by dehydration from a hydroxyl-bearing methine and its adjacent methylene (Scheme 1). These observations would support the hypotheses proposed by Wright et al.[5b] that Favorski-type reaction results in these m–m connections by extruding a carbonyl-derived carbon from the normal c–m repetition sequence (Scheme 2). This reaction leaves the preceding carbon as ketone and the following one as methylene, corresponding to the m–m pairs in AM2 and AM4. Another possible explanation of the m–m pairs by Rawlings[12] is that Tiffeneau–Demjanov reaction could give rise to the m–m sequence bearing a hydroxy group at the biosynthetically preceding carbon.

Six amphidinol homologues, AM1–AM6, have been isolated and characterized.[2,11] Structural variations among the homologues are restricted in the C1–C22 and terminal olefinic parts. Their structural variability may be accounted for by the recombination of polyketide synthase (PKS) domains; variable regions are responsible for the synthesis of the former part while the relatively constant regions are for the latter part. Moreover, the structural alteration between AM3 and AM5 is only the insertion of $-CH_2-CHOH-$ in AM5 after C16, which corresponds to one module of PKS. The same speculation could be possible for their polyolefinic termini, which again differ in one c–m unit. Thus, variations in skeletal structures among AM1, AM3, AM4, AM5 and AM6 (not AM2) could be accounted for simply by activation/deactivation of certain modules of dinoflagellate PKS. Further labeling experiments are currently underway to gain the better understanding of dinoflagellate polyketide biosynthesis.

3. Experimental

3.1. Chemicals and instruments

[1-^{13}C]acetate sodium salt was purchased from ICON Co. [2-^{13}C]acetate, [1,2-$^{13}C_2$]acetate (both sodium salts); and [methyl-^{13}C]methionine were obtained from Mass Trace Inc and Shoko. Co. Ltd, respectively. All other chemicals were purchased from standard venders and used without further purification. NMR spectra were obtained in CD_3OD–C_5D_5N, 2:1 solutions for AM2 and AM3, and CD_3OD–C_5D_5N, 1:2 solutions for AM4. HSQC and COSY spectra for ^{13}C assignment of AM4 were recorded on a Unity INOVA spectrometer (Varian, ^1H at 600 MHz). 2D INADEQUATE spectrum was recorded on a JEOL L-500 spectrometer (JEOL, ^{13}C at 125 MHz). 1D ^{13}C NMR spectra for determining ^{13}C-incorporation patterns were measured with the Unity instrument (^{13}C at 150 MHz) and a GSX-500 spectrometers (JEOL, ^{13}C at 125 MHz). Atmospheric pressure chemical ionization (APCI) spectra to determine the gross incorporation ratio of ^{13}C were recorded on an API-III Plus (PE-Sciex) mass spectrometer.

3.2. Culture of dinoflagellates and isolation of amphidinols

Amphidinium carterae was separated from Kauaroa, South island, New Zealand, and cultured as described previously.[11] Briefly, the unialgal culture was grown in a three-liter glass flask containing 2 L of 80% seawater enriched with GSe supplements. Isotope-labeled AcONa (0.6 mM) was added simultaneously with inoculation (200 mL) and the culture was harvested in 21 days. *Amphidinium klebsii* was separated from Aburatsubo-Bay, Kanagawa, Japan, and deposited in National Institute of Environmental Studies (NIES 613). This was used for production of AM2 and AM3. The culture medium was

artificial seawater (Marine Art Hi, Tomita Pharmaceutical, 3% w/v) enriched with ES-1 supplements containing antibiotics (penicillin G, 50 unit/mL; streptomycin sulfate, 50 unit/mL) to prevent nonspecific incorporation due to bacterial metabolism. Isotope-labeled AcONa (1.2 mM) or [methyl-^{13}C]methionine (0.17 mM) was added at seven days after inoculation (30 mL) and the culture was continued for more 14 days. Extract and purification of amphidinols were carried out as previously reported.[2,11b] Briefly, the cultured cells were extracted with MeOH and acetone, and the combine extract, after the solvents were removed, was subjected to EtOAc-H_2O partition and the resultant aqueous layer was extracted with 1-butanol. The butanol layer was further purified by chromatography over HW-40 (Toyopearl, MeOH–H_2O, 1:1) and then HPLC (ODS, YMC-AM, MeCN–H_2O, 1:2) to furnish 1.0 mg of AM2 and 2.0 mg of AM3, from 10 L of the culture media. Similar extraction and purification from the cells of *A. carterae* in 10 L of the media provided 1.0 mg of AM4.

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