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Amphirionin-4 with Potent Proliferation-Promoting Activity on Bone Marrow Stromal Cells from a Marine Dinoflagellate *Amphidinium* Species

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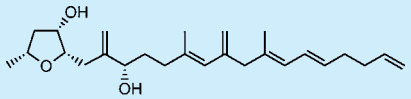
Supporting Information

ABSTRACT: A linear polyketide, amphirionin-4 (**1**), has been isolated from cultivated algal cells of the marine dinoflagellate *Amphidinium* species. The structure was elucidated on the basis of detailed analyses of 1D and 2D NMR data, and the absolute configurations of C-4 and C-8 were determined using the modified Mosher's method. Amphirionin-4 (**1**) exhibited extremely potent proliferation-promoting activity on murine bone marrow stromal ST-2 cells (950% promotion) at a concentration of 0.1 ng/mL.



amphirionin-4
950% proliferation promotion
of ST-2 cells at 0.1 ng/mL

A number of polyketide metabolites with various biological activities have been isolated from marine dinoflagellates.[1] The dinoflagellates of the genus *Amphidinium* are rich sources of bioactive polyketides of macrolides and long-chain compounds. The *Amphidinium* macrolides, designated as amphidinolides,[2] caribenolide I,[3] amphidinolactones,[4] and iriomoteolides,[5] possess unique chemical structures consisting of a variety of backbone skeletons and different sizes of macrolactone ring, and most of them exhibit cell-growth inhibitory activity against tumor cells. Nearly 30 polyketide compounds related to amphidinol-1[6] have been isolated from free-swimming and symbiotic *Amphidinium* dinoflagellates so far.[7−13] These amphidinol-related compounds are characterized by long-chain structures over 1200 atomic mass units having a common C_{16} middle section sandwiched between polyhydroxyl and polyene termini and have been reported to display various biological activities, e.g., antifungal, ichthyotoxic, hemolytic, cytotoxic, antiprotozoan, or antidiatom activities. In contrast, colopsinols A−E[14] and amphezonol A[15] are another structural class of long-chain polyol polyketides isolated from endosymbiotic dinoflagellate *Amphidinium* species.

The third structural class found in the *Amphidinium* dinoflagellates consists of nonpolar polyketides represented by amphidinin A[16] and amphidinoketides.[17] Compounds of this class have characteristic linear polyketide structures not exceeding 600 molecular units with some oxygen functionalities (e.g., hydroxyl group, ketone, epoxide, and/or tetrahydrofuran ring) and a few methyl and/or exomethylene branches. Recently, we have also discovered new polyketides, named amphirionins-1,[18] -2,[19] and -5,[20] from the marine benthic dinoflagellate *Amphidinium* species (HYA024, KCA09051, and KCA09053 strains, respectively) that were separated from sediments collected off Iriomote Island, Okinawa Prefecture, Japan. Amphirionins-1 and -2, as well as amphidinin A and amphidinoketides, exhibited cytotoxic activities against human tumor cells. In addition, amphirionin-2 showed in vivo activity against murine tumor. On the other hand, amphirionin-5 showed no inhibition of cell growth but an increase in the proliferation of murine bone marrow stromal ST-2 cells, murine osteoblastic MC3T3-E1 cells, and murine embryo fibroblastic NIH3T3 cells at a dose range of 0.001−10 ng/mL. Our continuous investigation of cell growth promoters from marine dinoflagellate *Amphidinium* species resulted in the isolation of a novel linear polyketide, amphirionin-4 (**1**) (Figure 1), from the *Amphidinium* KCA09051 strain. Interestingly, **1** showed an intensive proliferation promotion (950 ± 210%) of ST-2 cells at a concentration of 0.1 ng/mL but not of MC3T3-E1 and NIH3T3 cells. This study describes the isolation, structure

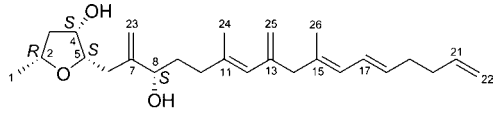


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Figure 1. Structure of **1**.

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elucidation, and biological activity combined with acetate labeling patterns of **1**.

The *Amphidinium* KCA09051 strain was monoclonally separated from benthic sea sand collected off Iriomote Island.[19] Large-scale algal cultivation was carried out using polycarbonate bioreactors (1000 and 200 L) with mechanical stirring and illumination. The dinoflagellate was cultured in sterilized deep-seawater, pumped up offshore of Muroto, Kochi Prefecture, Japan, containing 1% Provasoli's enriched seawater (PES) supplement for 2 weeks. The collected algal cells were extracted with MeOH/toluene (3:1). The toluene-soluble materials were subjected to a SiO_2 gel, ODS, and amino-SiO_2 gel columns, followed by C_{18} HPLC to obtain **1** (0.0031% from dry weight). Compound **1** promoted cell proliferation of human cervix adenocarcinoma HeLa cells by 127% compared with controls (= 100%) at a dose of 1 ng/mL, although most nonpolar secondary metabolites isolated from the dinoflagellate *Amphidinium* species showed growth inhibition of HeLa cells at doses of 1−10 μg/mL.

Compound **1** was obtained as colorless oil and an optically active molecule [$[\alpha]^{20}_{D}$ +6 (*c* 0.29, $CHCl_3$)]. The molecular formula of **1** was established to be $C_{26}H_{40}O_3$ by HRESIMS data [*m*/*z* 423.28615 $(M + Na)^+$, Δ −1.37 mmu]. ^1H and ^{13}C NMR data (Table S1, Supporting Information) in C_6D_6 assigned using the HSQC spectrum disclosed the presence of a total of 26 carbons containing 4 sp^2 quaternary carbons, 5 sp^2 methines, 3 sp^2 methylenes, 4 sp^3 oxymethines, 7 sp^3 methylenes, and 3 methyls. Six out of 7 degrees of unsaturation were noted, implying that **1** possessed one ring in the molecule.

The planar structure of **1** was elucidated on the basis of 1D and 2D NMR data measured in C_6D_6. Analyses of $^1H-^1H$ COSY and TOCSY spectra disclosed three proton−proton networks from H_3-1 to H_2-6, from H-8 to H_2-10, and from H-16 to H_2-22 together with one singlet olefin (H-12), one singlet methylene (H_2-14), two singlet methyls (H_3-24 and H_3-26), and two exomethylenes (H_2-23 and H_2-25) (Figure 2).

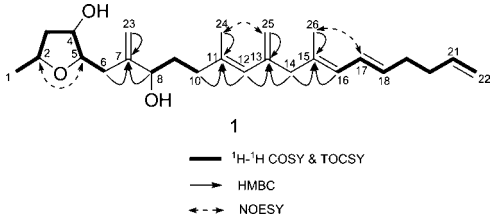

Figure 2. Selected 2D NMR correlations for amphirionin-4 (**1**).

Connectivities between these structural units via four quaternary carbons revealed by HMBC correlations to C-7 from H_2-6, H-8, and H_2-23, to C-11 from H_2-10, H-12, and H_3-24, to C-13 from H-12, H_2-14, and H_2-25, and to C-15 from H_2-14, H-16, and H_3-26. A disubstituted double bond at C-17−C-18 was assigned to be *E* from the $J(^1H-^1H)$ value (15.2 Hz), while geometries of trisubstituted double bonds at C-11−C-12 and C-15−C-16 were deduced as both *E* from NOESY correlations for H_3-24/H-25b[21] and H-17/H_3-26, respectively. The presence of a tetrahydrofuran ring at C-2−C-5 was suggested by the NOESY correlation for H-2/H-5 and was supported by lack of the downfield shifts for H-2 and H-5 by MTPA esterification as described below. Thus, the planar structure of amphirionin-4 was elucidated to be **1**.

The relative configurations among H-2, H-4, and H-5 in **1** were indicated as all β as follows: intensive NOESY correlations were observed for H-2/H-3a, H-2/H-5, H-3a/H-4, and H-4/H-5, while NOESY correlations for H_3-1/H-3a, H-2/H-3b, and H-3b/H-4 were relatively weak (Figure 3). To elucidate the

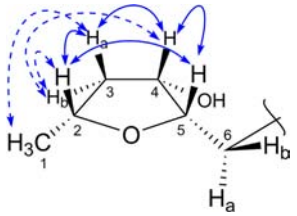

Figure 3. Stereostructure of the tetrahydrofuran ring at C-2−C-5 in amphirionin-4 (**1**). Arrows point to NOESY correlations, while dotted ones show relatively weak NOESY correlations.

absolute stereochemistries of C-4 and C-8, the modified Mosher's method[22] was applied for two hydroxyl groups at C-4 and C-8. The ^1H NMR data for 4,8-bis(*S*)- and (*R*)-MTPA esters (**2a** and **2b**, respectively) of **1** were assigned by analysis of the $^1H-^1H$ COSY and/or NOESY spectra; the experimental difference data ($\Delta\delta_{SR}$) were provided by the comparison of ^1H chemical shifts for **2a** and **2b** (Figure 4). Negative $\Delta\delta_{SR}$ values

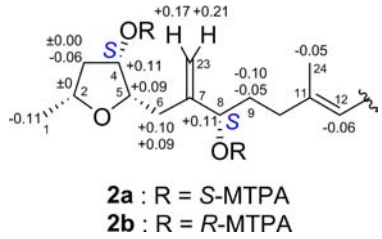

Figure 4. $\Delta\delta_{SR}$ values [$\Delta\delta_{SR}$ (in ppm)] $\delta_S - \delta_R$] obtained for the 4,8-bis(*S*)- and (*R*)-MTPA esters (**2a** and **2b**, respectively) of amphirionin-4 (**1**).

were observed for H_3-1, H-3b, H_2-9, H-12, and H_3-24, while H-5, H_2-6, and H_2-23 showed positive $\Delta\delta_{SR}$ values. The distribution pattern of the signs around C-4 and C-8 suggested both are *S*-configurations. Both positive $\Delta\delta_{SR}$ values for H-4 and H-8 also agreed with the diagnostic pattern for diesters of *S*,*S*-1,3-diol with chiral anisotopic reagents reported by Riguera and co-workers.[23] Considering the relative stereochemistries of the tetrahydrofuran ring, the absolute configurations at C-2 and C-5 were interpreted as *R* and *S*, respectively. Therefore, the structure of amphirionin-4 was determined to be **1** as shown in Figure 1.

The feeding experiments with [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$] sodium acetates were carried out by using a 50 L culture of the dinoflagellate *Amphidinium* species in addition to 5 g of each labeled precursor. Carbon-13 enrichments derived from labeled precursors were estimated to be ca. 6% on the basis of the relative intensity of satellite peaks in the ^1H NMR spectra. Isotopic incorporation results of **1** are described in Table S2 (Supporting Information). In the ^{13}C NMR spectrum of **1** enriched by [1-^{13}C] sodium acetate, significant enrichments of 10 out of 26 carbons were observed as follows: C-2, C-4, C-7, C-9, C-11, C-13, C-15, C-17, C-19, and C-21. Enrichments by [2-^{13}C] sodium acetate were observed for the remaining 16 carbons (C-1, C-3, C-5, C-6, C-8, C-10, C-12, C-14, C-16, C-18, C-20, C-22, C-23, C-24, C-25, and C-26). Analyses of the INADEQUATE spectrum revealed that 10 acetate units were directly incorporated for C-1−C-2, C-3−C-4,

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C-6−C-7, C-8−C-9, C-10−C-11, C-12−C-13, C-14−C-15, C-16−C-17 C-18−C-19, and C-20−C-21 (Figure 5). The acetate

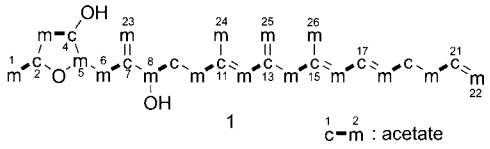

Figure 5. Labeling patterns of amphirionin-4 (**1**) resulting from feeding experiments with ^{13}C-labeled acetates.

incorporation pattern indicated that **1** was a nonsuccessive polyketide with two irregular C_1 sites (C-5 and C-22) and four C_1 branches (C-23, C-24, C-25, and C-26), both of which were labeled by the C-2 of acetate. These features are characteristic for acetate-labeling patterns of dinoflagellate polyketides.[24−26] The labeling pattern of the tetrahydrofuran portion (C-1−C-6) in **1** corresponded well to that of the C-11−C-6 portion in amphidinolide-T1,[27] while the labeling pattern for the polyene portion (C-11−C-22) in **1** was reminiscent of that for the polyene terminus in amphidinols-2, -4,[28] and -17.[8]

The cell proliferation promoting activity of **1** on normal cell lines was evaluated using ST-2, MC3T3-E1, and NIH3T3 cells seeded at a density of 10^3 cells per well during a 3 day incubation period. Compound **1** exhibited potent proliferation-promoting activity ranging from 378 ± 130 to 950 ± 210% for ST-2 cells at the dose range of 0.001−1000 ng/mL, with maximum proliferation rate observed at a dose of 0.1 ng/mL (Figure 6). In contrast, **1** showed no proliferation promotion

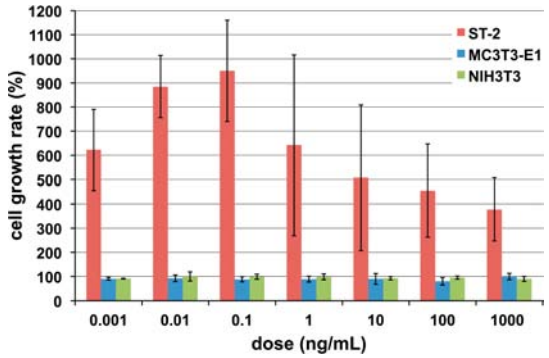

Figure 6. Proliferation-promoting activity of amphirionin-4 (**1**) on ST-2, MC3T3-E1, and NIH3T3 cells. The number of viable cells was counted by absorption at 450 nm using the Cell Counting Kit-8 assay, and cell growth rates were calculated as a percentage of that (100%) in the corresponding control group.

for the proliferation of MC3T3-E1 cells (proliferation rates: 80 ± 15 to 100 ± 14%) and NIH3T3 cells (proliferation rates: 90 ± 10 to 100 ± 19%).[29] On the other hand, amphirionin-5 showed a potent proliferation-promoting activity on all of these three cell lines at a dose range of 0.001−10 ng/mL.[20] The mechanism and specificity on cell proliferation promotion of **1** are of interest and currently being researched.

Preliminarily, we examined the effects of **1** on the growth of ST-2 cells by assessing cell morphology using fluorescence microscopy. Nuclei and actin filaments and tubulin filaments were stained by DAPI (4′,6-diamidino-2-phenylindole), rhodamine−phalloidins, and Alexa Fluor488-conjugated anti-β-tubulin antibodies, respectively. Figure 7 shows triple-stained ST-2 cells treated with **1** (a), untreated cells (b), tubulin

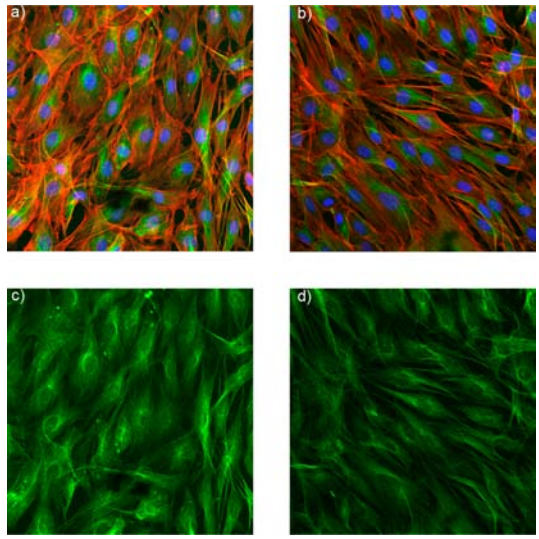

Figure 7. Effects of amphirionin-4 (**1**) on the nuclei and tubulin/actin cytoskeletons in ST-2 cells. ST-2 cells were treated with **1** (a and c) or not (b and d). (a and b) Merged images with DNA (blue), tubulin (green), and actin (red) stained with DAPI, rabbit anti-β-tubulin antibodies/Alexa Fluor 488-conjugated antirabbit IgG antibodies, and rhodamine−phalloidins, respectively. (c and d) Tubulin images.

cytoskeletons in **1**-treated ST-2 cells (c), and untreated cells (d), and images (a) and (b) were captured by choosing the views with the same cell density. Although there are almost no morphological changes in the nuclei between treated and untreated cells, actin and tubulin filaments were developed extensively in ST-2 cells treated with **1** compared with that in untreated ST-2 cells, suggesting that compounds **1** may promote the syntheses of cytoskeleton proteins in ST-2 cells. An increase of actin and tubulin filaments was not observed for the **1**-treated NIH3T3 cells as shown in Figure S1 (Supporting Information). Since ST-2 cells support the development of lymphocytes from bone marrow cells,[30] **1** may enhance ability of the immune system for infection or disease. The proliferation-promoting ability of **1** may be applicable for the regeneration of bone, cartilage, and other organs using mesenchymal stem cells derived from multipotent marrow stromal cells.[31−33]

Amphirionin-4 (**1**) is a novel polyketide consisting of a linear C_{22} carbon chain with four C_1 branches. Cell proliferation activators such as **1** and amphirionin-5[20] are a new class of bioactive polyketides isolated from marine dinoflagellate *Amphidinium* species. Coexistence of cell growth inhibitors of amphirionin-2[19] and promoters of **1** in the same dinoflagellate may be important for an ecological means, including chemical defense.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental details and characterization of data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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