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Structures of new amphidinols with truncated polyhydroxyl chain and their membrane-permeabilizing activities

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Abstract—Two new homologues of amphidinols (AM14 and AM15) were isolated from the cultured dinoflagellate *Amphidinium klebsii*. The structures were elucidated on the basis of 2D NMR and collision-induced dissociation MS/MS and turned out to be closely related homologues of AM7. Their weak membrane-disrupting activity indicates that the hydrophobic polyene chain is essential for the potent biological activities. Structure–activity relationship for the polyhydroxyl part was then examined with use of AM homologues possessing various chain lengths, indicating that the pore size of the channel/lesion formed by AMs was not greatly affected by the length of the polyhydroxyl chain.

1. Introduction

Dinoflagellates are known to be one of the most promising sources of diverse natural products with intriguing biological/pharmacological activities,[1,2] such as amphidinolides,[3] ciguatoxins, maitotoxin, okadaic acid, and saxitoxins. Amphidinols (AMs),[4–9] a class of polyhydroxy-polyene compounds isolated from the dinoflagellate *Amphidinium* species, mainly consist of a linear polyene-polyhydroxyl structure and showed potent antifungal activity, which significantly exceeds that of commercial drugs such as amphotericin B. Among known antifungal agents, AMs are unique in possessing neither nitrogenous polycycles as seen for synthetic drugs, nor macrocyclic structures commonly found in polyene-macrolide antibiotics. Therefore, a hitherto unknown mechanism of action should be involved in their biological action. Thus, AMs may provide an intriguing model to gain a better understanding of the mechanism of antifungal activities, which eventually helps develop better drugs for treatment of AIDS-related diseases and those upon transplantation.

In previous studies,[10,11] we have elucidated that AMs increase membrane permeability by binding not to membrane proteins but to lipids, and proposed the molecular mode of action of AMs as follows: (a) AMs bind to bilayer membrane chiefly with the polyene part (C52–C67 of AM3); (b) the size of pore/lesion is dependent on the structures of the polyhydroxyl region (C1–C20 of AM3); (c) the central region (C20–C51 of AM3) takes hairpin-shaped conformation that is stabilized by hydrogen bonds in amphipathic environments. We have recently isolated a new amphidinol, AM7,[9] possessing the smallest molecular weight among known AMs. In this paper, we report the isolation and structure elucidation of two new AMs closely related to AM7 and compared their biological activities with those of the known AMs to gain a better understanding of the structure–activity relationship in the polyhydroxyl chain (see Fig. 1).

2. Results and discussion

2.1. Structure elucidation of AM14

AM14 (**1**) was isolated as a pale yellow amorphous solid (10.5 mg) from 400 L of the cultured dinoflagellate *Amphidinium klebsii* (NIES 613); $[\alpha]_D^{20}$ −12.4 (*c* 0.174, MeOH). Electrospray ionization (ESI) MS *m*/*z* 1287 $[M+Na]^+$ and 1241 $[M-Na]^-$; UV λ_{max} (MeOH), 279 (ε 28,400), 268 (ε 35,500), and 258 (ε 28,100). The MS

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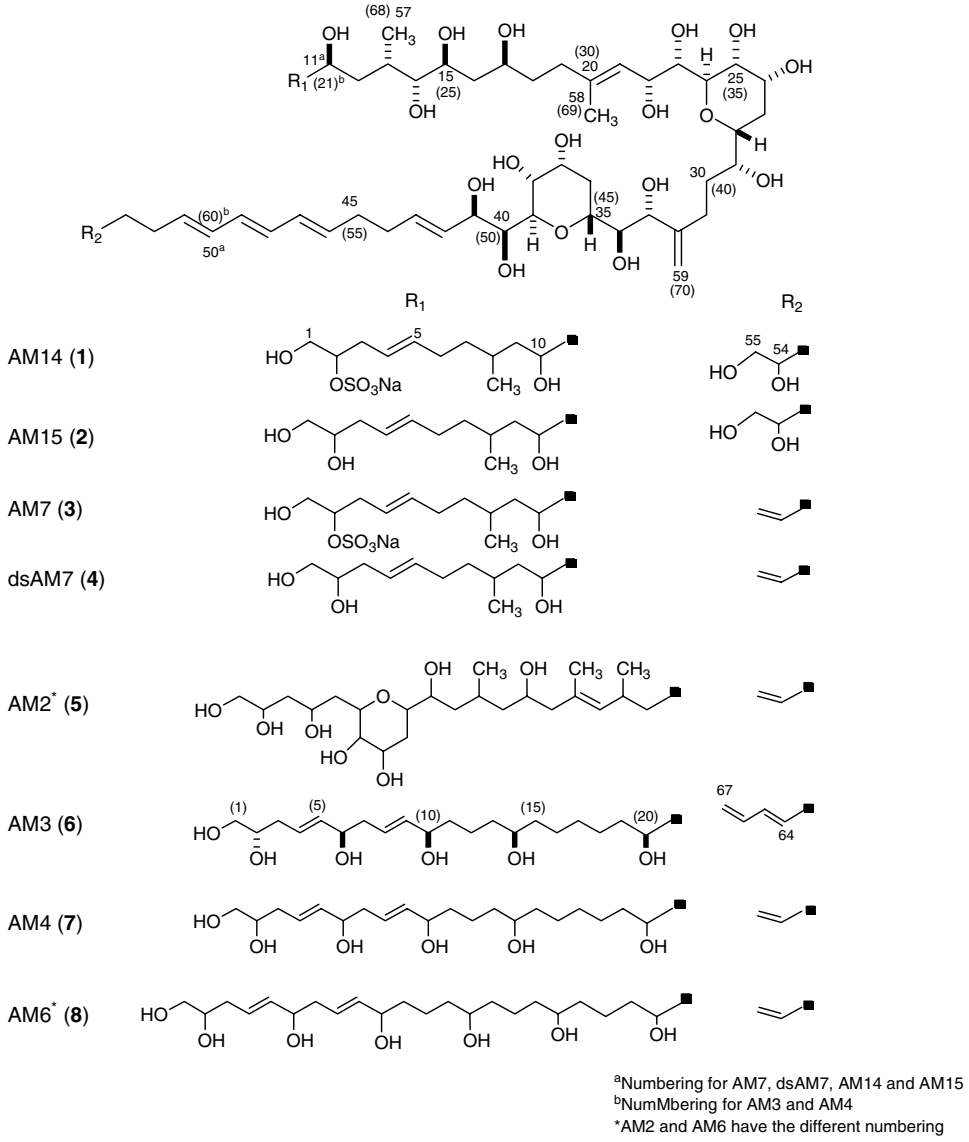

Figure 1. Structures of some new and known amphidinols.

and $^{1}H/^{13}C$ NMR data suggested the molecular formula $C_{59}H_{101}O_{25}SNa$. The UV spectrum was indicative of the presence of a conjugated triene chromophore, which was characteristic of AM family. After mild acid hydrolysis,[12] AM14 was converted to a less polar compound with *m/z* 1185 $[M+Na]^{+}$. A loss of 102 mass unit suggested the presence of a sulfate ester in AM14. Detailed analysis of HMQC, HSQC, DQF-COSY, and TOCSY spectra revealed the structure of AM14 to be identical with that of AM7 except for the terminal polyene moiety (Table 1). The close similarity in the ^{1}H and ^{13}C NMR chemical shifts indicated that the stereochemistry of the common part of AM14 should be identical with that of AM7[9] (and partly with that of AM3); the universal database approach[13] by Kishi's group has demonstrated that alternation in configuration of methyl or hydroxyl substituted alkyl chains causes more than 1 ppm difference in ^{13}C NMR. ^{13}C chemical shift differences between AM7 and AM14 for stereogenic centers (C11–C41) of the common part were much smaller than 1 ppm. The structural difference between AM7 and AM14 turned out to reside in the terminal C54/C55 part, where NMR data suggested the presence of vicinal dihydroxyl group. This structure was further confirmed by collision-induced dissociation (CID) MS/MS experiments.[14] CID is known to produce fragment ions chiefly due to cleavages at α and β positions to hydroxyl groups.[4] Negative ion FAB CID MS/MS spectra of AM14 (Fig. 2) measured for the precursor ion at *m/z* 1241 $[M-Na]^{-}$ showed the characteristic patterns of charge-remote fragmentation due to the presence of a sulfate group.[15,16] Product ion peaks generated by fissions at α positions to a hydroxyl group or at allylic/homoallylic sites were prominently observed in the spectrum (Fig. 2). The product ions smaller than *m/z* 1166 were virtually identical with those of AM7,[9] thereby indicating their structural identity except for the olefinic terminus. AM14 is the first AM homologue that possesses a terminal dihydroxyl group in the olefinic chain (Fig. 3).

Table 1. ^{1}H and ^{13}C chemical shift data of AM7, AM14, and AM15 in CD_3OD–C_5D_5N (2:1)

Position	AM7		AM14		AM15	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	3.78, 3.80	64.48	3.77, 3.80	64.58	3.52, 3.68	67.00
2	4.54	80.57	4.56	80.55	3.70	74.58
3	2.47	35.73	2.48	35.79	2.16, 2.26	38.19
4	5.49	134.72	5.50	134.98	5.50	134.99
5	5.47	126.33	5.48	126.38	5.48	127.43
6	1.92, 1.95	30.79	1.94, 1.98	30.88	1.94, 1.98	30.92
7	1.16, 1.28	38.91	1.16, 1.28	39.00	1.18, 1.30	39.04
8	1.76	29.73	1.75	29.78	1.75	29.80
9	1.20, 1.56	41.46	1.20, 1.56	41.47	1.20, 1.56	41.39
10	3.56	73.63	3.56	73.82	3.56	73.69
11	3.60	73.74	3.59	73.71	3.59	73.37
12	1.58, 1.63	38.91	1.58, 1.63	39.00	1.58, 1.62	39.04
13	2.34	31.68	2.35	31.75	2.35	31.72
14	3.44	79.79	3.45	79.77	3.45	79.83
15	3.86	72.88	3.86	72.93	3.86	72.95
16	1.63, 2.08	41.46	1.63, 2.08	41.47	1.63, 2.08	41.53
17	3.95	71.84	3.96	71.90	3.96	71.89
18	1.62	36.91	1.62	37.00	1.62	37.01
19	2.08, 2.22	36.75	2.08, 2.22	36.76	2.08, 2.22	36.78
20	—	138.72	—	138.74	—	138.70
21	5.63	126.81	5.63	126.86	5.63	126.89
22	4.72	67.85	4.73	67.95	4.73	67.91
23	3.84	72.59	3.84	72.77	3.84	72.57
24	4.22	79.26	4.22	79.24	4.22	79.30
25	4.30	68.97	4.31	69.02	4.31	69.03
26	4.12	67.45	4.12	67.51	4.12	67.53
27	1.92, 2.00	31.18	1.92, 2.00	31.18	1.92, 2.00	30.92
28	3.60	75.84	3.60	75.86	3.60	75.91
29	3.70	74.54	3.70	74.58	3.70	74.20
30	1.67, 2.07	32.63	1.67, 2.07	32.69	1.66, 2.07	32.70
31	2.22, 2.60	28.04	2.22, 2.60	28.12	2.22, 2.60	28.09
32	—	151.95	—	152.03	—	152.06
33	4.38	76.75	4.38	76.79	4.38	76.80
34	3.50	75.25	3.50	75.37	3.50	75.38
35	4.19	70.62	4.20	70.73	4.20	70.70
36	1.64, 2.32	31.90	1.64, 2.32	31.96	1.64, 2.32	31.97
37	4.17	67.34	4.17	67.39	4.17	67.43
38	4.30	68.77	4.31	68.82	4.31	68.83
39	3.98	80.68	4.00	80.59	4.00	80.70
40	4.19	72.19	4.19	72.29	4.19	72.27
41	4.60	74.17	4.60	74.23	4.60	73.84
42	5.76	129.76	5.80	129.89	5.80	129.86
43	5.82	134.28	5.76	134.20	5.76	134.12
44	2.04	33.58	2.04	33.57	2.04	33.60
45	2.04	33.58	2.04	33.57	2.04	33.60
46	5.62	134.51	5.62	134.41	5.62	134.25
47	6.00	132.11	6.00	132.17	6.00	132.13
48	6.04	132.26	6.04	132.17	6.04	132.17
49	5.99	132.36	6.05	132.37	6.05	132.21
50	6.00	132.26	6.05	132.17	6.05	132.17
51	5.60	134.30	5.62	134.69	5.62	134.43
52	2.07	33.23	2.04	33.57	2.04	33.60
53	2.04	34.61	1.50, 1.60	34.34	1.50, 1.60	34.36
54	5.72	139.25	3.68	63.47	3.68	63.47
55	4.87, 4.95	115.42	3.52, 3.58	66.99	3.52, 3.58	67.00
56	0.83	19.54	0.83	19.60	0.85	19.61
57	1.02	13.24	1.02	13.34	1.03	13.26
58	1.70	17.44	1.70	17.48	1.71	17.49
59	5.00, 5.12	112.86	5.00, 5.12	112.83	5.00, 5.12	112.84

2.2. Structure elucidation of AM15

AM15 (**2**) was isolated as a pale yellow amorphous solid (7 mg) from 400 L of the cultured dinoflagellate *A. kleb-sii* (NIES 613); $[\alpha]_D^{20}$ −12.6° (*c* 0.046, MeOH). Electrospray ionization (ESI) MS *m*/*z* 1185 $[M+Na]^+$ and 1161 $[M-1]^-$; UV λ_{max} (MeOH), 279 (ε 25,700), 268 (ε 32,700), and 258 (ε 25,200). ^{1}H and ^{13}C NMR data of

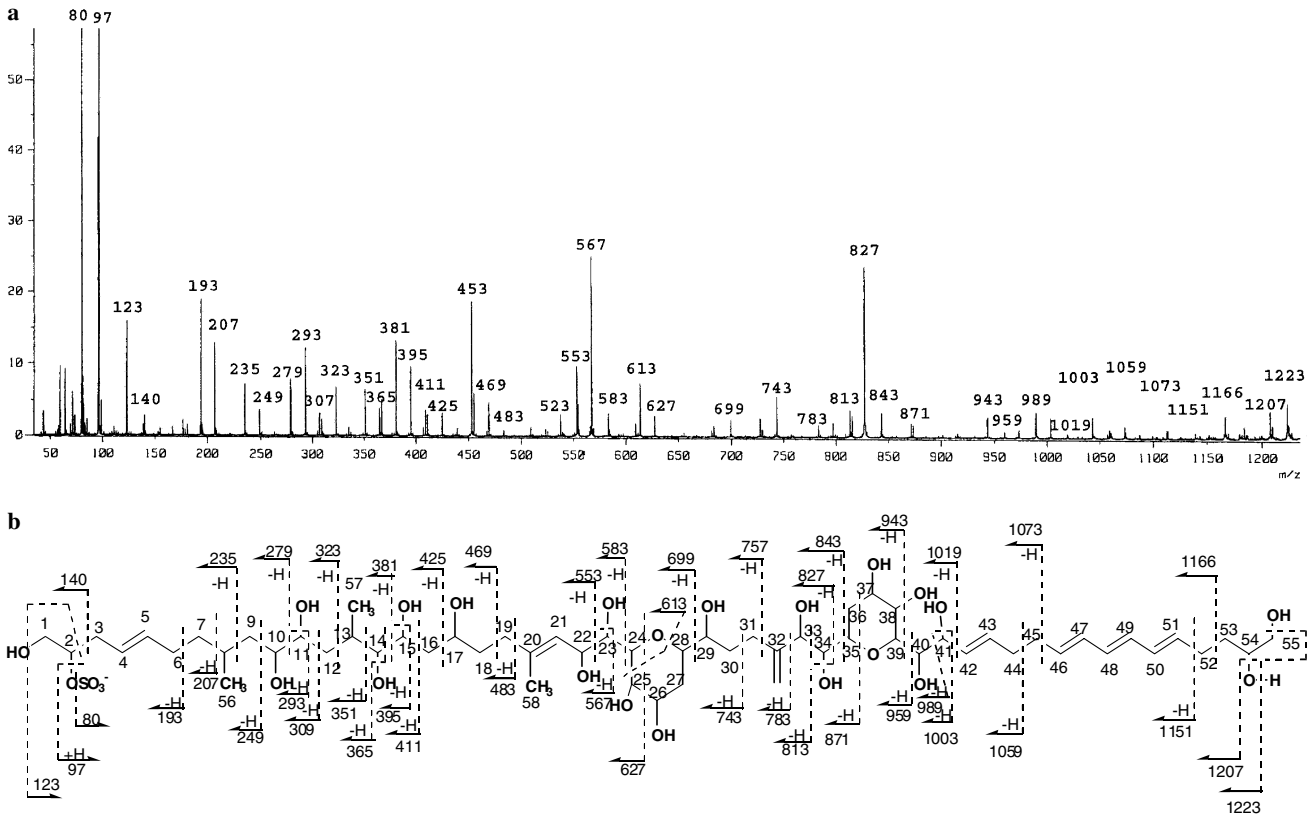

Figure 2. CID MS/MS spectrum (a) and fragmentation pattern (b) observed in negative ion FABMS/MS spectra of AM14, precursor ion *m/z* 1241.

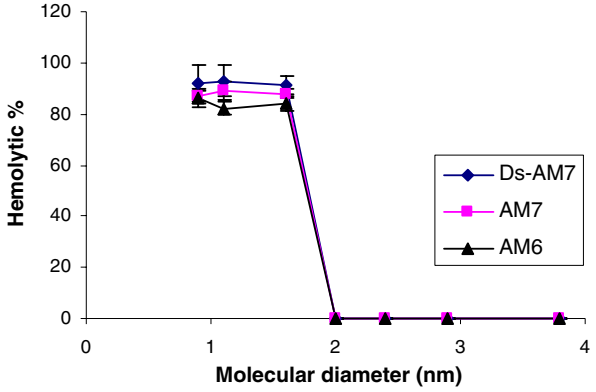

Figure 3. Osmotic protection experiments for AM7, dsAM7, and AM6 using human erythrocytes. The size of a pore formed by these agents was estimated by hemolytic tests in the presence of various osmotic protectants at concentrations where they caused 80–90% hemolysis without a protectant.

AM15 shown in Table 1 were essentially identical with those of AM14 except for the polyhydroxyl terminus. The similarity to AM7 in ^{13}C chemical shifts demonstrates that AM15 has the same configuration as that of AM7 on the basis of the notion described above.[13] Up-field shifts in ^{1}H signals at C1, C2, and C3, and the smaller molecular ion by 102 mass units compared with those of AM14 clearly revealed a loss of the sulfate group at C2 position. This is further evidenced by hydrolysis of AM14, which provided a less polar compound with *m/z* 1185 (similar to AM15). HPLC comparison (Cosmosil, 5C18-AR-II, waters, 4.6×150 mm, MeCN/H_2O gradient elusion monitored at 270 nm) confirmed the identity of desulfated AM14 with AM15 by giving rise to the same retention time at 17.0 min.

2.3. Biological activities of AM7, AM14, and AM15 in comparison with known AMs

We measured the antifungal and hemolytic activities of AMs with the truncated polyhydroxyl chain, AM7, AM14, and AM15, in comparison with those of known AMs. The antifungal assays on *Aspergillus niger* revealed that AM3, AM2, and AM6 had almost the same potency and dsAM7 and AM7 showed a little less efficacy (Table 2). In contrast, AM14 revealed no antifungal activity and AM15 showed only marginal activity at a higher dose. The hemolytic activity to human erythrocytes disclosed a slightly different tendency from the results of the antifungal assays; AM3 was the most potent followed by AM2, and AM6/AM7, whereas AM14 or AM15 induced no hemolysis up to 50 μM. (In Table 2, the EC_{50} values of AM2, AM3, and AM6 are somewhat different from the previous data[11] since the hemolytic activity was greatly affected by conditions of erythrocytes.)

To examine the structure–activity relationship for the polyhydroxyl part, desulfo-AM7 (dsAM7) is prepared from AM7 by hydrolysis of the sulfate ester. DsAM7 possesses the shortest polyhydroxyl chain among the

Table 2. Hemolytic and antifungal activities of some amphidinols

Compound	AM2	AM3	AM6	AM7	AM14	AM15	dsAM7
Antifungal[a] (MEC[a] in μg/disk; *Aspergillus niger*)	6	6	6	10	>60	60	8
Hemolysis[b] (EC_{50} in μM; human erythrocytes)	1.7	0.4	2.9	3	>50	>50	1.2

[a] Maximum amount used 60 μg/disk.
[b] Maximum concentration used was 50 μM.

known AMs, which should help elucidate the function of this moiety in membrane-disrupting activity. The hemolytic activity of dsAM7 was significantly higher than that of AM6, which possessed a much longer polyhydroxyl chain, but should be a little lower than that of AM4 (or AM3) according to the previous results.[11] This tendency in hemolytic activity implies that the length of the polyhydroxyl side chain of AM3 (and AM4) most effectively disrupts erythrocyte membrane among AMs tested since the major structure differences among AM4, AM6 and dsAM7 reside chiefly in the length of the polyhydroxyl chain.

AMs reported so far possess a hydrophobic polyene region, whereas the corresponding portion of AM14 and AM15 is substituted with a hydrophilic dihydroxyl group. Marked reductions in the biological activities of AM14 and AM15 in comparison with their vinyl homologues AM7 and dsAM7 (Table 2) are evidently due to the terminal polar functionality. The importance of the polyene chain for the efficacy can be seen for the case of luteophanol A,[16] which possesses three hydroxyl groups in this region and does not show membrane-disrupting activity.

In the next step, we attempted to estimate the pore size of channels formed by AMs in erythrocyte membrane using colloid osmotic protection experiments.[17,18] When an osmotic-protecting agent added to a medium is too large in size to pass through a membrane channel formed by AMs, no hemolysis occurs; the osmotic pressure by intracellular hemoglobin is balanced by the protecting agent outside. Thus, the size of a pore or lesion can be estimated from the molecular size of the protectant. The hemolytic tests were carried out for AM6, AM7 and dsAM7 at the concentration at which each AM caused 80–90% hemolysis in the absence of a protectant. The pore size of all the AMs ranged between 1.6 and 2.0 nm despite the great variation in length of their polyhydroxyl chains.

On the basis of these results, it is deduced that the pore size of channels formed by AMs is not a determinant factor for their biological activities because all of the homologues tested showed different potency in hemolytic activity but form relatively large pores with a roughly same size. These findings further disclosed that the length of the polyhydroxyl chain does not greatly influence the pore size since AM6 has an 11-carbon longer polyhydroxyl chain than AM7 and dsAM7. The presence of a sulfate group is known to be inhibitory for membrane-disrupting activity as reported by Satake et al.[5b] AM2 that lacks a sulfate group is around 25 times more potent than AM11, which is sulfated-AM2 at C1 position. The inhibitory effect of a sulfate substitution was observed for AM7 and dsAM7, the latter of which is 2.5 times more efficacious than the former in hemolytic activity. The pore size of sulfated AM was first measured in the present experiments, indicating that a sulfate substitution gave rise to no significant effect on the pore size as is the case with the length of the polyhydroxyl chain.

3. Conclusion

We have isolated two new amphidinols and elucidated their structures to be closely related congeners to AM7. The biological activities of these short-chained AMs in comparison with known homologues have added some insight into structure–activity relationship of amphidinols. First, hydrophobicity of the polyene chain of AMs dramatically affects the membrane-disrupting activities. Second, substitution of the sulfate group is generally inhibitory to antifungal and hemolytic activities, whereas giving rise to insignificant effect on the size of pores/lesions formed in membrane by AMs. Third, the polyhydroxyl chain moderately modulates the potency of the biological activities; although AM7 and dsAM7 possessing the shortest chain of all the AMs, they revealed a comparable efficacy as a longer-chained homologue, AM6. The pore size is not greatly affected by the chain length, which suggests that the difference in potency is not accounted for by the size of membrane-permeabilizing channels.

4. Experimental

4.1. Chemical and instruments

Sucrose, raffinose, and polyethylene glycols (PEG 600, 1000, 2000, and 4000) were from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were from standard commercial sources and used without further purification. ^{1}H and ^{13}C NMR spectra including 2D versions were recorded on an INOVA-600 spectrometer. A sample was dissolved in 0.65 ml CD_3OD/pyridine–d_5 (2:1). CID MS/MS spectra were recorded on a JMS HX-110/HX-110A four sector tandem mass spectrometer equipped with 6 kV Xe beam FAB gun and the MS-ADS11 variable mass dispersion array detector. The MS/MS spectra were measured at 10 kV acceleration voltage (MS1), electrically 8 kV floated collision

cell, and 10% dispersion array detector conditions. 2,2′-Dithiodiethanol was used as a matrix. Ar used as collision gas was introduced at the rate such that precursor ion intensity was attenuated to ca. 30%. UV spectra were measured on Shimadzu UV-2500 spectrometer. The hemolytic percentage was measured on precision microplate reader (Molecular devices).

4.2. Culture of A. klebsii and isolation of AM14 and AM15

The dinoflagellate *A. klebsii* was isolated from the surface wash of several species of seaweed that were abundant at the collection site near the shore of Aburatsubo Bay, Japan, and deposited in National Institute for Environmental Studies as NIES 613. The unialgal culture was grown in a 3-L Fernbach flask containing artificial seawater (Marine Art Hi, Tomita Pharmaceutical, 3% w/v) enriched with 2% ES-1 supplement (Provasoli 1968) at 25 °C for 3–4 weeks under illumination with a 16–8 light–dark photocycle. The cultured cells (400 L) were harvested by filtration with glass filters under reduced pressure. The filtrate was passed through Diaion HP-20 column to collect the organic material, and then it was eluted with methanol. The organic material was applied to an ODS open column and eluted with a stepwise solvent system of water/methanol to collect fractions 1:1 and 1:5 water/methanol which afforded crude fractions of AM14 and AM15, respectively. Both of these fractions were purified separately using HW-40 column using a 1:1 methanol/water eluting system. Then each fraction was applied to HPLC (YMC-pack, ODS-AM, 250×10 mm, MeCN/H_2O gradient elusion UV: 270 nm) to give 10.5 mg of AM14 and 7 mg of AM15.

4.3. Desulfation of AM7 and AM14

Hydrolysis of sulfate ester of AM7 and AM14 was performed by using a mild hydrolysis method[12]; 1 mg of the sample in 1 mL dioxane was added with 5 mg of *p*-toluene sulfonic acid in 0.5 mL dioxane. The solution was stirred for 30 min and then Na_2CO_3 in a 10% aqueous solution was added to neutralize the solution. The product was extracted with ethyl acetate. The extract was evaporated and dried with N_2 gas. The hydrolysate was dissolved in MeOH and purified by HPLC (Cosmosil, 5C18-AR-II, Waters, 4.6×150 mm, MeCN/H_2O gradient elusion UV: 270 nm).

4.4. Biological assays

The fungus *A. niger* was cultured at 25 °C in a glucose–peptone liquid medium (2% glucose, 0.2% yeast extract, 0.5% polypeptone, 0.05% $MgSO_4$, and 0.1% KH_2PO_4) for 2 days. An aliquot of the broth was then spread onto an agar plate containing the same medium with 1.5% agar. Each sample dissolved in MeOH was spotted on a paper disk (8 mm in diameter), which was then placed on an agar plate spread with *A. niger* mycelia. After incubation at 25 °C for 2 days, the diameter of an inhibitory zone on each paper disk was measured. In hemolytic assays, human blood cells in 3.13% sodium citrate were immediately separated from the plasma by centrifugation at 1000*g* for 5 min. Sedimented cells were washed three times with PBS buffer, containing 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 at pH 7.4. A sample dissolved in MeOH (10 μL) was added to 190 mL of the blood cell suspension in 1% hematocrit containing PBS buffer and incubated for 6 h at 37 °C. After incubation, the resultant supernatant was subjected to colorimetric measurements at 450 nm on microplate reader (Molecular devices). The percentage amount of hemoglobin released from erythrocytes was calculated. From dose–response curves, the concentration that caused 50% hemolysis (EC_{50}) was determined. In osmotic protection experiments, a human blood cell suspension with 1% hematocrit was mixed with 90% PBS, in which 30 mM of each of the following solutes was added as an osmotic protectant; sucrose, raffinose, and polyethylene glycols (PEG 600, 1000, 2000, and 4000), whose molecular diameters are estimated to be 0.9, 1.1, 1.6, 2.0, 2.9, and 3.8 nm, respectively.[17,19] The concentrations of AM homologues used in the experiments were taken to be equivalent to cause 80–90% hemolysis without a protactant.

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