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Membrane-permeabilizing activities of amphidinol 3, polyene-polyhydroxy antifungal from a marine dinoflagellate

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Abstract

Amphidinols, which are polyene-polyhydroxy metabolites produced by the marine dinoflagellate *Amphidinium klebsii*, possess potent antifungal and hemolytic activities. The membrane permeabilizing actions of amphidinol 3, the most potent homologue, were compared with those of polyene antibiotics, amphotericin B (AmB) and filipin, in hemolytic tests, ^{23}Na nuclear magnetic resonance (NMR)-based membrane permeabilizing assays, and UV spectroscopy for liposome-bound forms. In Na^+ flux experiments using large unilamellar vesicles (LUVs), ion efflux by amphidinol 3 was inhibited by cholesterol or ergosterol, which was opposed to previous results [J. Mar. Biotechnol., 5 (1997) 124]. When the effect of the agents on the size of vesicles was examined by light scattering experiments, amphidinol 3 did not significantly alter their size while filipin and synthetic detergent Triton X-100 did. The observations implied that the activity of amphidinol 3 was mainly due to formation of large pores/lesions in liposomes rather than detergent-like disruption of membrane. The pore/lesion size was estimated to be 2.0–2.9 nm in diameter on the basis of osmotic protection experiments using blood cells. The UV spectra in liposomes, which revealed the close interaction of polyene moieties in a lipid bilayer, further implied that the membrane activity of amphidinol 3 is caused by the molecular assemblage formed in biomembrane. These results disclose that amphidinol 3 is one of few non-ionic compounds that possess potent membrane permeabilizing activity with non-detergent mechanism.

Keywords: Amphidinol; *Amphidinium klebsii*; Amphotericin B

1. Introduction

Dinoflagellates are a rich source of bioactive secondary metabolites [1,2]. Particularly, polyketide metabolites bearing unique structures and powerful bioactivities have been frequently found from marine epiphytic species; e.g. ciguatoxins, maitotoxin, palytoxin and okadaic acid. Among these, a family of amphidinols (AMs) has been isolated as a potent antifungal agent from the dinoflagellate *Amphidinium klebsii*. AM1 was first reported by Satake et al. in 1991 [3]. Six congeners have been reported so far [3–6], among which the absolute configuration of AM3 (1, Fig. 1) was recently determined by our group [7]. Their structures are best characterized by a long carbon chain encompassing multiple hydroxyl groups and polyolefins, and the lopsided distribution of these hydrophilic and hydrophobic moieties may be reminiscent of polyene macrolides such as amphotericin B (AmB, 2). Besides the antifungal activity, AMs possess potent toxicity against diatoms [4], implying the allelochemical roles against other epiphytic microbes in marine ecosystems. Their powerful membrane-disrupting activity is supposed to originate from

Abbreviations: AM, amphidinol; AmB, amphotericin B; eggPC, egg yolk phosphatidylcholine; $DyCl_3$, dysprosium chloride; PPP, tripolyphosphate; PEG, polyethylene glycol; MS, mass spectrometry; $Na_7Dy(PPP)_2$, sodium bis(tripolyphosphate) dysprosium; LUV, large unilamellar vesicles; MLV, multi-lamellar vesicles; SUV, small unilamellar vesicles; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; UV, ultraviolet–visible

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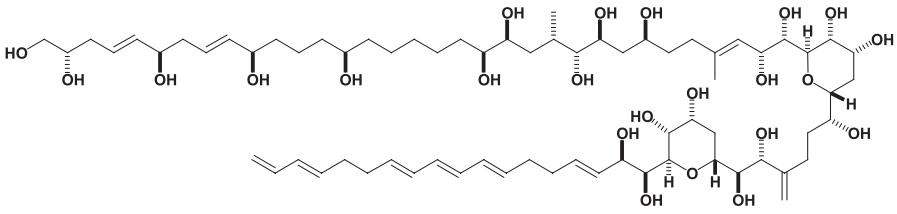
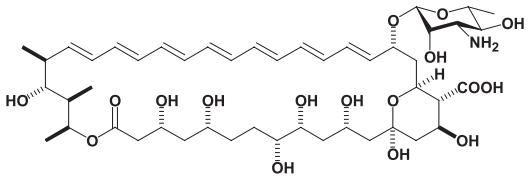
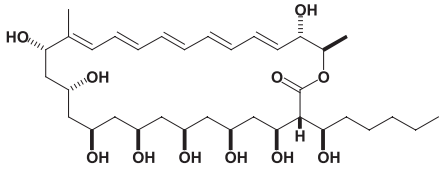


Amphidinol 3 (**1**)



Amphotericin B (**2**)



Filipin (**3**)

Fig. 1. Structures of AM3 (1), AmB (2) and filipin III (3).

a channel-like assemblage [4]. Efflux assays of a fluorescent dye using small unilamellar vesicles (SUV) [4,6] have demonstrated the enhancement of the activity by cholesterol and ergosterol, which further infers the resemblance between AMs and AmB in their membrane activities.

AmB and filipin (3) are polyene antibiotics, both of which are known to interact with sterols in biomembranes. However, they permeabilize the membrane by different mechanisms [8–10]; AmB exerts its action by forming ion channels (channel-type) while filipin induces the damage-type of membrane disruption [9]. In the 1970s, the well known "barrel-stave model" was proposed for an ion channel of AmB, in which about eight pairs of AmB/sterol comprise a single ion channel [11,12]. Its selective toxicity against microorganisms could be attributed to a higher affinity for ergosterol, a principal fungal sterol, over cholesterol comprising mammalian plasma membranes [13,14]. Recent investigations using covalent conjugate analogues have demonstrated that the direct bimolecular interactions of AmB/AmB and AmB/lipid play an important role in channel formation in phospholipid membranes [15–18]. In the present study, we examined the membrane permeabilizing actions of AM3, the most potent amphidinol homologue [6,7], by using hemolytic tests, Na^+ efflux assays, and ultraviolet–visible (UV) spectra; and compared its mode of membrane action with those of AmB, filipin and synthetic detergent Triton X-100.

2. Materials and methods

2.1. Materials

AmB, dysprosium chloride hexahydrate ($DyCl_3 \cdot 6H_2O$), sodium tripolyphosphate (Na_5PPP), raffinose and polyethylene glycols (PEG 600, 1000, 1540, 2000, and 4000) were purchased from Wako Pure Chemical Industries (Osaka, Japan), egg yolk phosphatidylcholine, cholesterol, ergosterol, glucose and sucrose from Nacalai Tesque (Osaka, Japan). Filipin complex comprising 70% filipin III and 5% other filipin homologues was obtained from Sigma Chemicals (St. Louis. MO), and the concentrations of filipins were regarded as 75% as reported [19,20] and expressed as equivalents of filipin III. Polycarbonate filters were purchased from Nuclepore (Pleasanton, CA). Filipin III was purified by high-performance liquid chromatography (HPLC, YMC-Pack ODS-AM, methanol/H_2O, 13:7). The purity of ergosterol was checked by HPLC and MS. Other chemicals were used without further purification.

2.2. Culture and isolation

The marine dinoflagellate *A. klebsii* was separated from Aburatsubo Bay, Kanagawa, Japan, and deposited in the National Institute of Environmental Studies (NIES 613). The culture medium was artificial seawater (Marin Art Hi, Tomita Pharmaceutical, 3% w/v) enriched with ES-1 supplement. Extract and purification of amphidinols were

carried out as previously reported [21]. Briefly, the cultured cells were extracted with methanol and acetone, and the combine extract, after the solvents were removed, was subjected to ethyl acetate/H_2O partition and the resultant aqueous layer was extracted with 1-butanol. The butanol layer was further purified by chromatography over HW-40F (Toyopearl, methanol/H_2O, 1:1) and then HPLC (YMC-Pack ODS-AM, MeCN/H_2O, 1:2) to furnish 1.0 mg of AM3 from 10 l of the culture media.

2.3. Antifungal assays, hemolytic tests, and osmotic protection experiments

The fungi *Aspergillus niger* was cultured in a GP liquid medium (2% glucose, 0.2% yeast extract, 0.5% polypeptone, 0.05% $MgSO_4$, and 0.1% KH_2PO_4) at 25 °C for 2 days. An aliquot of the broth was then spread onto a GP agar plate (1.5% agar). Samples dissolved in methanol or DMSO were spotted on paper disks (8 mm in diameter). They were then placed on an agar plate spread with *A. niger* mycelia. After incubation at 25 °C for 2 days, the diameter of the inhibitory zone on each paper disk was measured. For each hemolytic test, human blood cells were collected in 3.13% sodium citrate and 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 methanol (10 μl) was added to 190 μl of the blood cell suspension in 1% hematocrit PBS buffer and incubated for 12 h at 30 °C. After incubation, the resultant supernatant was subjected to colorimetric measurements at 450 nm on microplate reader (Molecular Devices) to determine the absorbance (As_{450}). Total hemoglobin in the suspension was obtained from the value on complete hemolysis (Ah_{450}). The percentage amount of hemoglobin released from erythrocytes was calculated as $As_{450}/Ah_{450}\times100$. From dose–response curves, the concentration that caused 50% hemolysis (EC_{50}) was determined. The time dependence of hemolytic activity was measured by the optical density of erythrocyte suspension (0.35 cm of light path length) at the wavelength of 650 nm, at which the absorption of hemoglobin was very low, and that was mostly due to light scattering by intact blood cells [9]. In osmotic protection experiments, a blood cell suspension with 1% hematocrit was mixed with 90% PBS buffer, and added with 30 mM of the following solutes as an osmotic protectant; glucose, sucrose, raffinose, and PEGs (600, 1000, 1540, 2000, and 4000), whose molecular diameters are estimated to be 0.7, 0.9, 1.1, 1.6, 2.0, 2.4, 2.9, and 3.8 nm, respectively [22,23].

2.4. Liposome preparations

EggPC with a certain amount of sterol (no sterol, 20% cholesterol, and 10% ergosterol) was dissolved in chloroform to prepare stock solutions. The solvent was then evaporated to form a thin film on the bottom of a flask. To ensure complete removal of solvent, the flask was placed under vacuum overnight. The filmy residue was hydrated with a 100 mM NaCl solution containing 10 mM Tris–HCl at pH 7.2 or 9% sucrose, and incubated for 2 h to form multi-lamellar vesicle (MLV). The MLV solution was frozen and thawed three times. The large unilamellar vesicles (LUV) thus obtained was extruded through polycarbonate membrane with a pore size of 800 or 50 nm (AVESTIN Liposofast®).

2.5. Na^+ efflux assays by using ^{23}Na NMR

Na^+ efflux assays were carried out basically following a method by Kimura et al. [24]. LUV (800 nm in diameter) in 100 mM NaCl/10 mM Tris–HCl (pH 7.2) buffer was dialyzed against 2 l of 120 mM KCl/10 mM Tris–HCl buffer for 12 h to replace external Na^+ with K^+. A nuclear magnetic resonance (NMR) sample was made by mixing 100 μl of D_2O, 2.0 μl of 89.3 mM sodium bis(tripolyphosphate) dysprosium, $Na_7Dy(PPP)_2$, and 500 μl of LUVs suspension. The shift reagent was prepared by mixing 50 μl of 250 mM $DyCl_3\cdot6H_2O$ and 90 μl of 500 mM $Na_5(PPP)$ at pH 7.0 [24]. Na^+ efflux was measured by ^{23}Na NMR at 30 °C with the lipid concentration of 25 mM. The ^{23}Na NMR spectra were determined with an LUV suspension containing 100 mM NaCl inside and 100 mM KCl outside the vesicles. The residual Na^+ outside LUV was shifted up-field around −1.0 ppm by shift reagent $Na_7Dy(PPP)_2$ [25], whereas leaving a signal due to the inside ions at 0 ppm unaffected. An aliquot of a sample solution in methanol (10 μl) was added to LUV suspensions (600 μl) and incubated for 2 h at room temperature. When an agent permeabilizes the membrane, the peak around −1.0 ppm increases while a peak at 0 ppm decreases, which clearly shows that inside Na^+ and outside K^+ was exchange through liposomal membrane. Percentage of a reduction of peak area at 0 ppm was expressed as $(Na_0^+–Na_t^+)/Na_0^+\times100$, where Na_t^+ is the molar quantity of Na^+ inside the vesicles in the presence of a sample, and Na_0^+ is that in the absence of a sample.

2.6. Evaluation of detergent-like activity

Detergent-like activity of AM3, AmB and filipin complex were measured by the method described in the literature [9] with some modifications. In the present study, detergent-like activity denotes that an agent permeabilizes membrane by disrupting lipid bilayers into small micelles as is the case with typical detergent such as Triton X. A methanol stock solution of AM3, AmB or filipin complex, or an aqueous stock solution of Triton X-100 (2 μl) was added to sterol-free LUV (800 nm of diameter) in Tris–HCl buffer (120 μl). After incubation for 2 h at room

temperature, the optical density of the liposome suspension (0.35 cm of light path length) was measured with a spectrophotometer at the wavelength of 650 nm, on a 96-well microplate. Detergent activity was expressed as $(Aa_{650}-As_{650})/Aa_{650}\times100$, where As_{650} is the optical density at 650 nm in the presence of a sample, Aa_{650} is in the absence of a sample.

2.7. Measurement for UV spectra in lipid bilayers

The powder of AM3 (8.4 nmol) and AmB (5.5 nmol) were obtained by lyophilizing the methanol stock solutions, and added with 30 mM eggPC LUV (50 nm of diameter) in 9% sucrose buffer (10, 100 μl), followed by incubation for 3 h at room temperature. After dilution to 750 μl with the same buffer, UV spectra were measured. Because liposome suspension caused light scattering, the UV traces in Fig. 7 were obtained by subtracting the spectra of liposomes from those of AM3-containing liposomes. The molar extinction coefficients of AM3 and AmB in methanol were 4.19×10^4 l/mol cm (270 nm) [3] and 1.20×10^5 l/mol cm (406 nm) at 25 °C, respectively.

3. Results

3.1. Antifungal and hemolytic activities

We re-examined the antifungal and hemolytic activities of AM3, AmB and filipin III (Table 1) [4,6]. AM3 revealed very potent hemolytic activity against human erythrocytes with the EC_{50} value of 0.25 μM, which was somewhat less potent than the reported value [4], but still significantly exceeded those of AmB and filipin III. Next, we attempted to measure the time course of hemolysis, following a method by Knopik-Skrocka and Bielawski [9], by which hemolytic process could be monitored as light scattering by intact erythrocytes (not as leakage of hemoglobin). As shown in Fig. 2a, AM3 caused rapid hemolysis at 1.3 μM, mimicking the process of filipin III (Fig. 2c). On the contrary, at 0.42 μM, AM3 showed slow hemolysis similar to that of AmB (Fig. 2b). In the erythrocyte membrane, AmB forms selective channels permeable to Na^+ and K^+, which induces colloid osmotic stress, ultimately leading to slow hemolysis. These results seemingly suggest that hemolysis of AM3 could be either damage-type or channel-type depending on concentrations.

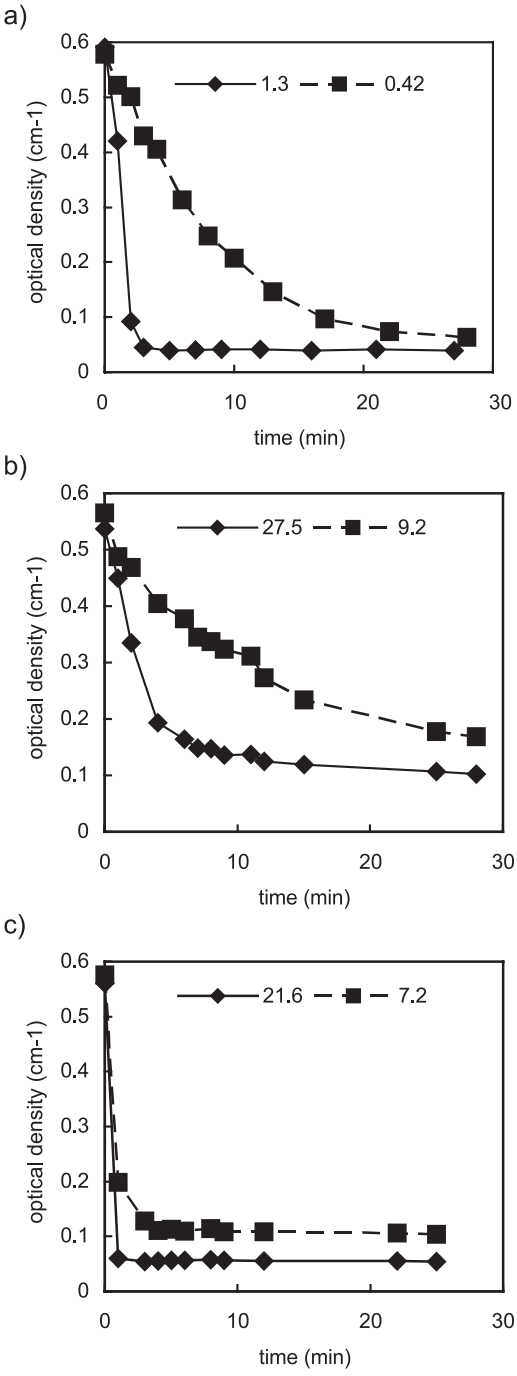


Fig. 2. Time courses of hemolysis elicited by AM3 (a), AmB (b) and filipin III (c) Hemolytic activities of AM3, AmB and filipin III in two concentrations were monitored by the absorbance of erythrocyte suspension at 650 nm as described in Section 2, and plotted against incubation time. The numbers in figures show the concentrations of the agent in micromoles.

Table 1
Biological activities of AM3, AmB and filipin III

Activity	AM3	AmB	Filipin III
Antifungal (MEC[a], in μg/disk; *A. niger*)	9.0	6.3	1.4
Hemolysis (EC50, in μM; human erythrocytes)	0.25	2.0	2.0

[a] Minimal effective concentration.

3.2. Na^+ efflux assays by using ^{23}Na NMR

The selective toxicity of AmB against fungi and yeasts is generally attributed to higher affinity for ergosterol over

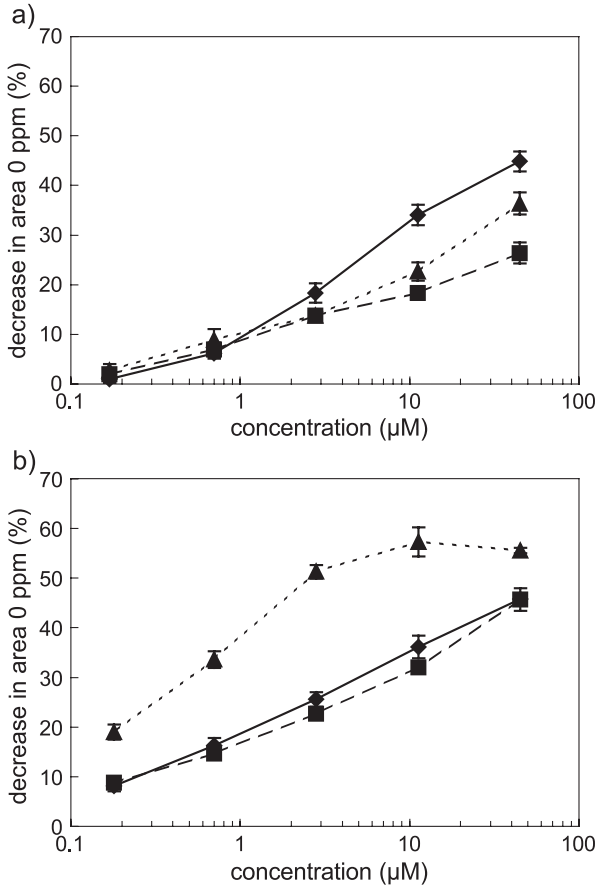


Fig. 3. Does–response curves of Na^+ efflux from LUV for AM3 (a) and AmB (b) The curves show the effect of cholesterol and ergosterol on the channel activities for AM3 and AmB at the lipid concentration of 25 mM. The *X*-axis indicates their concentration. The *Y*-axis represents the rate of decrease in area at 0 ppm. The diamonds show the activity in sterol-free liposomes, the squares show eggPC/cholesterol (8:2) and the triangles show eggPC/ergosterol (9:1) in liposome membrane. Percentage of decrease in area 0 ppm was determined as described in Section 2.

cholesterol [13]. To examine the similarity between AM3 and AmB, particularly in the effects of cholesterol and ergosterol as suggested by the previous studies [4,6], we carried out the Na^+ efflux assays by using LUV consisting of PC and sterol. As shown in Fig. 4, AM3 and AmB increased the intensity of an up-field peak at the expense of a decreasing peak at 0 ppm, which clearly indicated that Na^+ in the vesicles effluxed from LUV. The dose–response curves in Fig. 3 revealed the effect of cholesterol and ergosterol on the Na^+ efflux. As expected, Na^+ efflux elicited by AmB was greatly enhanced by ergosterol as compared with cholesterol-containing or sterol-free liposomes. On the other hand, Na^+ efflux by AM3 was rather inhibited by cholesterol or ergosterol.

AM3 induced the drifting of Na^+ NMR signal due to ions outside liposomes with increasing concentrations of AM3 (Fig. 4a), which was also seen for the spectra of filipin complex (21.4 μM–1.73 mM) (Fig. 4c) and Triton X-100 (1.4–5.5 mM) (Fig. 4d). The drifting of the peaks is presumably caused by the dilution of the shift reagent, $Na_7Dy(PPP)_2$ [25,26], by diffusing into the liposome lumen through the pore formed by AM3 or filipin complex.

3.3. Detergent activity

The interaction of detergents with lipid bilayer disrupts lamellar structures and results in formation of lipid–detergent mixed micelles with much smaller size [27,28]. Addition of a detergent to LUVs, therefore, causes reduction in the light scattering of the suspension, which can be monitored by attenuation in the optical density of visible light [29]. Under the experimental conditions, the absorbance should be linearly correlated with the concentrations of intact liposomes. The sample concentrations used in these assays corresponded to those required to permeabilize 50% of liposomes (AM3, 70 μM; AmB, 80 μM; filipin complex, 1.1 mM; Triton X-100, 3.5 mM) in the Na^+ efflux assays in Section 3.2. Filipin complex, as shown in Fig. 5, showed the detergent activity to a similar extent to that of Triton X-100, which is known to disrupt liposomes into smaller micelles. On the other hand, AM3 and AmB do not decrease the optical density of liposome suspensions, clearly indicating that AM3-inducted Na^+ efflux is mediated by a different mechanism from those of filipin complex or Triton X-100.

3.4. Osmotic protection experiments and estimation of pore size

Osmotic protection experiments using erythrocytes is widely applied for measuring the size of channels formed in lipid bilayer membranes [22,23]. In these experiments, when a protecting agent added to the medium is too large in size to pass through a channel formed on erythrocytes, no hemolysis takes place; the osmotic pressure of the intracellular hemoglobin is balanced with that of the agent outside. Thus, the size of the channels in the membrane can be estimated by the size of the efficacious protecting agent. According to the results in Fig. 6, the size of a pore or lesion formed by AM3 was deduced at 2.0–2.9 nm since the hemolysis was observed in the presence of PEG 1000 but suppressed by PEG 2000. The diameter of an AmB channel was estimated from 0.7 to 0.9 nm as previously reported [30,31]. For filipin III, the hemolysis was observed up to PEG 4000, implying leakage of hemoglobin by the direct action of the agent.

3.5. UV spectra

In order to access the state of AM3 in lipid bilayer, we measured the UV spectra and compared with those of AmB. As shown in Fig. 7a and b, the UV spectra of AM3 at 11.2 μM and AmB at 7.3 μM were recorded in methanol, aqueous buffer and liposome preparations with 0.4 and 4 mM eggPC. The spectra of AmB were

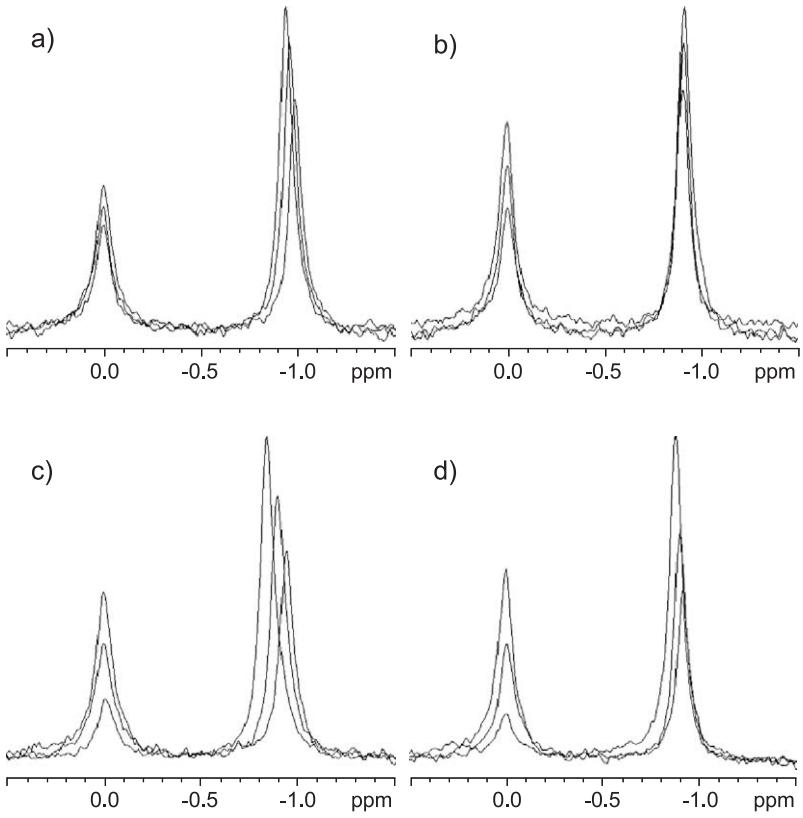


Fig. 4. ^{23}Na NMR spectra of sterol-free liposomes with various concentrations of AM3 (a), AmB (b), filipin complex (c) and Triton X-100 (d). The peaks at 0 ppm are due to Na^+ inside LUV and those at −0.8 to −1.0 ppm are Na^+ outside. The concentrations of AM3 were 2.79, 11.2 and 44.6 μM, those of AmB were 0.18, 2.81 and 45.0 μM, those of filipin complex were 21.4, 192, 1730 μM, and those of Triton X-100 were 0, 2790, 5500 μM, respectively. In all spectral sets (a), (b), (c) and (d), a trace with a high peak at 0 ppm (low at −1 ppm) corresponds to a low concentration of each agent as depicted in (a). The experimental procedure is described in Section 2.

essentially the same as reported in Refs. [32,33]. AM3 exhibited three absorption maxima with slightly different wavelengths depending on the media; in MeOH at 259/270/280 nm, in buffer at 261/272/282 nm, and for LUV at 262/273/285 nm. These shifts in UV maxima are also observed in AmB spectra, although their intensities are markedly different; the bands of AM3 in buffer were greater than those in liposomes, which was in clear contrast to those of AmB. These differences in UV spectra imply that AM3 at low concentration is soluble in aqueous media while AmB tends to form micelles. The lower intensities for the LUV preparations than those in buffer may suggest that AM3 aggregates in membrane as reported for AmB [34,35].

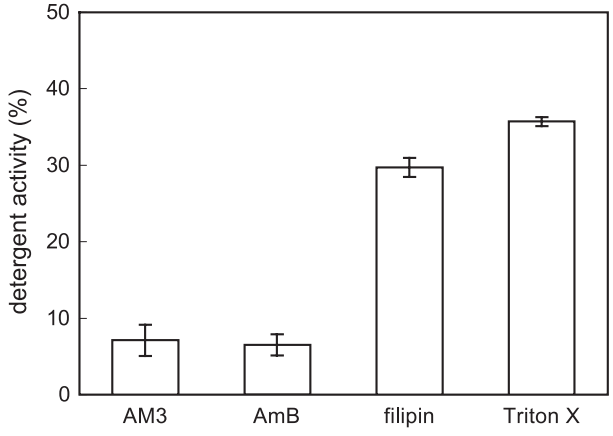


Fig. 5. Evaluation of detergent activity for AM3, AmB, filipin complex and Triton X-100. The optical density of sterol-free LUV suspensions was measured at 650 nm for AM3, AmB, filipin complex and Triton X-100 at the same concentrations of their EC_{50} (AM3: 70 μM, AmB: 80 μM, filipin complex: 1.1 mM, Triton X-100: 3.5 mM) for sterol-free liposomes in ^{23}Na NMR efflux assays (Fig. 3). Most of the optical density at 650 nm is due to light scattering, which is linearly related to remaining LUV regardless of it being permeabilized or not. Experimental details are described in Section 2.

4. Discussion

In the present Na^+ flux experiments, the membrane-permeabilizing activity of AmB was greatly enhanced by ergosterol as expected. On the other hand, the activity of AM3 was not augmented or even inhibited by cholesterol or ergosterol. These results are in contrast to the previous reports [4,6], which describe that AM3 and AM5 induce the leakage of a fluorescent dye, calcein, from SUV and their activity is enhanced by cholesterol or by ergosterol from 0% to 33% (w/w) in a concentration-dependent manner. The conditions of these experiments were, however, signifi-

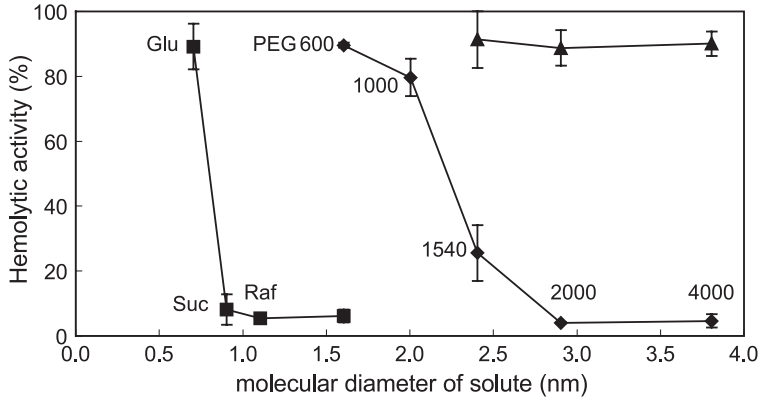


Fig. 6. Osmotic protection experiments for AM3 (diamonds), AmB (squares), and filipin III (triangles) using erythrocytes. The size of a pore or lesion formed by these agents was estimated by hemolytic tests in the presence of various osmotic protectants. A blood cell suspension at 1% hematocrit was suspended in 90% PBS buffer and added with 30 mM of the following solutes as an osmotic protectant; glucose, sucrose, raffinose, PEGs 600, 1000, 1540, 2000, and 4000, whose diameters were estimated as 0.7, 0.9, 1.1, 1.6, 2.0, 2.4, 2.9, and 3.8 nm, respectively. The concentrations of AM3, AmB and filipin III were 0.38, 5.1 and 6.4 μM, respectively, where 100% hemolysis occurs in PBS buffer in the absence of a protectant.

cantly different from those of the present study. In the calcein experiments [4,6], SUV with a diameter of about 20 nm were used, whereas LUVs with a diameter around 800 nm were used in our experiments. The curvature of SUV is much higher than that of LUV. Moreover, the AM/lipid ratio in the calcein assays was very high, while the ratio in the

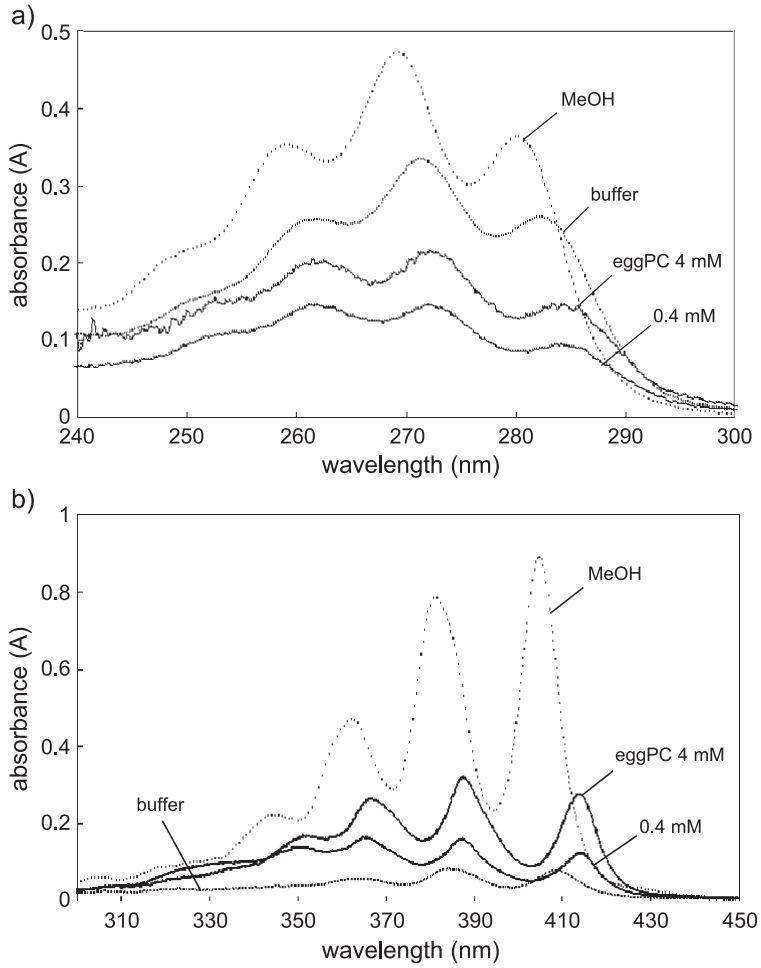


Fig. 7. UV spectra of AM3 (a) and AmB (b) in LUV. The UV spectra of AM3 (11.2 μM) and AmB (7.3 μM) were measured in methanol, 9% sucrose buffer and eggPC liposomes (0.4 and 4 mM) in the same buffer.

Na^+ flux assay system was relatively low (AM/lipid ≈ 0.001). The apparent inconsistency in the dependency on sterol may be attributed to these differences in experimental conditions. One of plausible accounts may be the susceptibility of liposomes to membrane-binding agents. As reported by Paul et al. [6], a higher concentration of AM3 (79 μM) is necessary to induce the leakage of calcein from sterol-free SUV compared with that in the Na^+ efflux assays in LUV (ca. 10 μM, Fig. 3a). This is probably because a certain number of AM3 molecules per vesicle are necessary to form a single pore or lesion regardless of the size of vesicles. Cholesterol is known to enhance the binding of AmB to liposomes [13,33]. If this is the case with AM3, the stimulatory effect by cholesterol in SUV could be accounted for by the notion that in the presence of cholesterol, AM3 can bind to SUV at a concentration enough to induce calcein leakage while in the absence of sterol AM3 in membrane cannot reach this concentration. Moreover, cholesterol is known to enlarge the vesicle size of SUV [36]. AM3/lipid ratios could be reduced to make a single pore on larger vesicles. This may be another reason for the stimulatory effects of cholesterol in SUV. In the present experiments using LUV, of which size is usually unaffected by sterol, cholesterol and ergosterol show rather inhibitory effects. The UV spectra of AM3 in LUV, unlike those of AmB, were not significantly affected by cholesterol or ergosterol (data not shown), which may imply that indirect effects, rather than intermolecular interaction between AM3 and sterols, may be responsible for this inhibition. For example, the thickness and rigidity of PC membrane are known to be significantly affected by sterols [36–39], both of which may well account for a slight inhibitory effect, although further experiments are necessary to figure out the roles of sterols in the AM3 activities.

A series of ^{23}Na NMR traces with various concentrations of AM3 (Fig. 4a), filipin complex (Fig. 4c) and Triton X-100 (Fig. 4d) revealed that −1.0 ppm peaks drifted to the down-field direction with increasing concentrations of AM3 or filipin complex. The drifting of the peaks is probably due to dilution of $Na_7Dy(PPP)_2$, which has diffused into the liposome lumen through the pore or lesion formed by the agents. Since ion channels formed by AmB are known to pass only monovalent cations such as Na^+ and K^+, the shift reagent cannot penetrate into the liposomes, thus leaving the peaks unchanged (Fig. 4b). These observations seemingly infer the detergent-like effects of AM3 rather than the formation of ion-permeable pores. To examine this possibility, we next carried out light scattering experiments. If AM3 disrupts LUV into micelles as does a detergent, the reduction in particle size is to be detected as decreasing optical density (Fig. 5). In these experiments, AM3 revealed similar results to those of AmB (Fig. 5), which clearly demonstrated that AM3's activities are due neither to micelle formation nor other possible mechanism leading to reduction of liposome size. Osmotic protection experiments revealed that the size of a pore or lesion formed by AM3 and AmB are approximately 2.3 and 0.8 nm, respectively (Fig. 6). The size of AM3 lesion is much larger than that of AmB lesion, and ranges widely from 2.0 to 2.9 nm. Among the few non-peptidic hemolysins, these features of AM3 are reminiscent of cationic natural compounds such as lipogrammistin-A [40]. These hemolysins manifest their activity through uneven binding to the leaflets of bilayer membranes, which leads to the invagination of erythrocyte, and eventually to hemoglobin release. A shark-derived cationic compound, squalamine, is also reported to induce a pore with a diameter around 2 nm in phospholipid membrane [41]. However, unlike these amine derivatives, a lack of ionic groups in AM3 thus implies that a different, probably unknown, mechanism should underlay its membrane activity.

The hemolytic activities of the antibiotics used in this study can be categorized into the colloid osmotic type and damage type. The former type of agents, as is the case with AmB, induce the Na^+/K^+ flux into erythrocyte to cause hemolysis with osmotic stress, where considerable ion leakage occurs at much lower concentrations than that necessary for hemolysis. In the latter type including filipin, the disruption of erythrocyte membrane leads to the simultaneous leakage of small molecules as well as macromolecules including hemoglobin. The mechanism of action by AM3 appears somewhat similar to that of filipin, particularly in forming a large lesion, but strikingly distinct in non-dependence on sterol and in the size of a pore formed in membrane.

The UV spectra of AM3 in the presence of LUV (Fig. 7) reveal similar features to those of AmB, which may also suggest their resemblance in membrane-bound forms. Binding of the hydrophobic polyene part of AM3 to membrane is inferred by the reduced UV intensity for LUV at 0.4 mM PC, where less AM3 should reside in membrane and more in water in comparison with that of 4 mM PC; if a significant amount of AM3 was present in aqueous media, the UV intensity should have increased for 0.4 mM PC compared with that at 4 mM PC. In addition, the partition coefficient of AM3 to eggPC bilayer, which was obtained in our preliminary experiments using sedimentable membrane preparations, allows us to estimate the molar fraction of AM3 in membrane to be around 90% (experimental details will be published elsewhere). The heptaene group of AmB was deduced to reside mostly in the membrane since red shifts of the triplet peaks in LUV from those in buffer were reportedly due to the interaction of the chromophore with the membrane interior [33,34,42]. Thus, the UV absorptions for the LUV preparations in Fig. 7a and b are assumed to largely come from membrane-bound AM3 and AmB. At the higher AM3/lipid ratio, the UV absorptions significantly decreased, which was in parallel with the spectra of AmB (Fig. 7a and b). These spectral changes could be partly accounted for by the formation of aggregates at a higher concentration in

membrane, which led to the reduction in UV absorption [34,35]. However, further experiments would be necessary to confirm the intermolecular interaction of AM3 in lipid bilayer membrane.

All these findings allow us to deduce the features of AM3-elicited membrane permeabilization for LUV as follows: (a) AM3 efficiently binds to phospholipid membrane without ionic interactions; (b) the formation of a pore or lesion is not greatly affected by membrane sterols; (c) the size of a pore or lesion is 2.0–2.9 nm in diameter; (d) the polyene chain of AM3 binds to lipid core and comes close to each other; and (e) this molecular assemblage presumably causes membrane permeabilization.

We believe that the present study could provide some useful information for future investigations, particularly those for LUV. However, the molecular-based mechanism accounting for potent membrane action of amphidinol is open to question. The bimolecular interactions between AM3/AM3 or AM3/lipid should provide a clue to elucidate how this unique natural product exerts its potent membrane activity. Further investigations including NMR analysis of fluorine-labeled analogues of AM3 are currently underway.

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