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Direct and Stereospecific Interaction of Amphidinol 3 with Sterol in Lipid Bilayers

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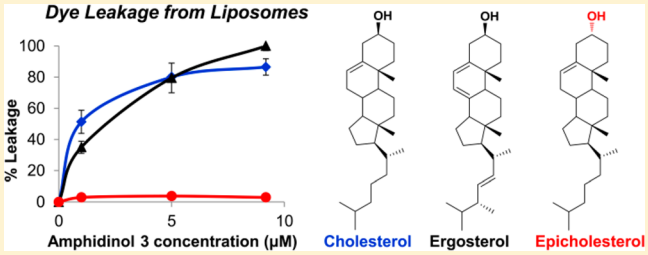
Supporting Information

ABSTRACT: Amphidinol 3 (AM3), a polyhydroxy-polyene metabolite from the dinoflagellate *Amphidinium klebsii*, possesses potent antifungal activity. Although AM3 permeabilizes phospholipid membranes only in the presence of sterol, the detailed molecular basis by which AM3 recognizes sterols in membranes remains unknown. Here, we investigated the molecular interaction between sterols and AM3 in membranes from the viewpoint of stereospecific molecular recognition using ergosterol, cholesterol, and epicholesterol, which is the 3-OH epimer of cholesterol. Dye leakage assays, surface plasmon resonance experiments, ^{2}H and ^{31}P NMR measurements, and microscopic observations revealed that AM3 directly interacts with membrane sterols through the strict molecular recognition of the stereochemistry of the sterol 3-OH group. The direct interaction enhances the membrane binding efficiency of AM3, which subsequently permeabilizes membranes without altering membrane integrity.



INTRODUCTION

Dinoflagellates belonging to the genus *Amphidinium* are widely considered to be a prolific source of polyketide metabolites with unique and intriguing structures and fascinating bioactivities, which include amphidinolides,[1,2] amphidinolactones,[1,3] iriomoteolides,[4,5] and caribenolide.[1] In 1991, screening of the dinoflagellate *Amphidinium klebsii* collected at Ishikagi Island, Japan, for bioactive compounds afforded the very first member of the polyhydroxy-polyene metabolite family, which was subsequently named amphidinol 1.[6] Since then, nearly 20 closely related homologues collectively termed amphidinols have been isolated.[7−14] In addition, numerous amphidinol congeners, including luteophanols,[15] lingshuiol,[16] symbiopolyol,[17] karatungiols,[18] karlotoxins,[19] and prorocentrol,[20] have been reported both from *Amphidinium* sp. and other species of dinoflagellates. The defining structural features of these natural products include polyhydroxy and polyene chains, conferring to them amphiphilic properties, which are separated by an intermediate section that contains two tetrahydropyran rings. This middle region of the molecule containing the tetrahydropyran rings is conserved among the different amphidinols, and the variation in their structures mainly occurs in the length and substitution pattern on both the polyhydroxy and polyene chains.[14]

Amphidinols exhibit antifungal and hemolytic actions, which are believed to arise from their interaction with phospholipid bilayers, ultimately leading to membrane permeabilization.[8,9] Amphidinol 3 (AM3, Figure 1),[9,10,21] which has the most potent antifungal and hemolytic activities among amphidinols, displayed potent membrane permeabilizing activity on artificial liposomes,[9,22] which is absolutely dependent on membrane sterol; i.e., the absence of sterol renders AM3 inactive.[22]

Our previous study using surface plasmon resonance (SPR) measurements showed that AM3 has 5300- and 1000-fold higher affinity for membranes containing ergosterol and cholesterol (Figure 2), respectively, compared with those without sterol.[23] Although these SPR results suggested a functional role of the sterols in increasing the affinity of AM3 for phospholipid bilayers,[23] it is unclear whether direct molecular interactions between AM3 and sterol or sterol-elicited membrane ordering enhances the membrane affinity of AM3.

Hence in this study, from the viewpoint of stereospecific molecular recognition, we investigated molecular interactions between AM3 and membrane sterol with the aid of epicholesterol (3α-cholesterol, Figure 2), using dye leakage assay, SPR, and solid-state ^{2}H NMR. Finally, the effects of AM3 on bilayer morphology was examined by solid-state ^{31}P NMR and microscopy.

MATERIALS AND METHODS

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Figure 2) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, ergosterol, and epicholesterol were obtained

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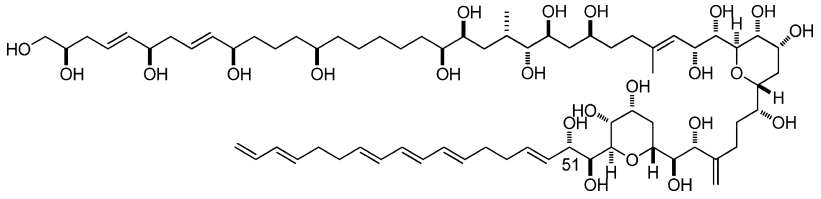
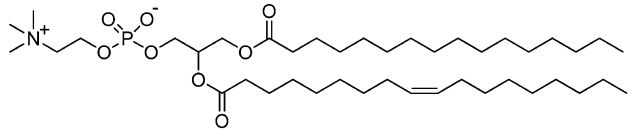
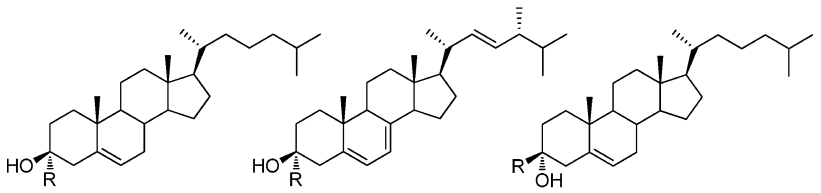


Figure 1. Chemical structure of AM3. The stereochemistry at C51 was recently revised.[21]



1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)



Cholesterol : R = H Ergosterol : R = H Epicholesterol : R = H
Cholesterol-d_1 : R = D Ergosterol-d_1 : R = D Epicholesterol-d_1 : R = D

Figure 2. Chemical structures of the lipids used in this study.

from Nacalai Tesque (Kyoto, Japan), Tokyo Kasei (Tokyo, Japan), and Steraloids (Newport, RI), respectively. Cholesterol-d_1, ergosterol-d_1, and epicholesterol-d_1 (Figure 2) were synthesized as previously reported.[24,25]

Isolation of AM3. The culture of the dinoflagellate *A. klebsii* and the isolation of AM3 were performed as reported previously.[22]

In this study, large-scale culture of the dinoflagellate in a 1000-L fermenter was also conducted using deep-seawater enriched with Pravasoli's ES supplement. *A. klebsii* was first grown in a 10-L medium for 2 weeks and then transferred to a 200-L medium for another 2 weeks and finally to a 1000-L medium for 3 weeks, after which the cells were harvested. In brief, the culture medium was filtered and concentrated with a MOLSEP Fiber FS03-FC-FUS1582 (Daicen Membrane-Systems Ltd., Tokyo, Japan). The concentrated cells (approximately 80 L) were centrifuged at 5000 rpm, 20 °C for 2−3 h using a Hitachi CR 22GIII high-speed refrigerated centrifuge (Hitachi Koki Co., Ltd., Tokyo, Japan) to facilitate sedimentation of the cells. The subsequent isolation of AM3 from the cells followed a previously reported protocol.[22]

Calcein Leakage Experiments. The extent of calcein leakage from liposomes was assessed as reported previously.[22] Large unilamellar vesicles (LUVs) were prepared as follows: POPC (10 mg), with or without sterol (10 mol %), was dissolved in $CHCl_3$ in a round-bottom flask. The solvent was removed under vacuum for approximately 2 h at 30 °C and further dried *in vacuo* overnight. The lipid film obtained was rehydrated with 1 mL of 60 mM calcein in Tris−HCl containing 1 mM EDTA and 150 mM NaCl (pH 7.4) and subjected to two cycles of vortexing (1 min) and warming (65 °C) followed by five cycles of freezing (−20 °C) and thawing (65 °C) to obtain multilamellar vesicles (MLVs). Then, the suspension was passed through a polycarbonate membrane filter (pore size, 200 nm) 19 times using a LiposoFast-Basic (AVESTIN Inc., Ottawa, Canada) to prepare LUVs of homogeneous size. Excess calcein was removed by passing the suspension through a Sepharose 4B column (GE Healthcare BioSciences AB, Uppsala, Sweden) with Tris−HCl containing 1 mM EDTA and 150 mM NaCl (pH 7.4). The lipid concentration in the LUV fraction was quantified using phospholipid C-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Resulting LUV suspension was stored at 4 °C under N_2 gas.

Measurement of calcein leakage was performed on a JASCO FP 6500 spectrofluorometer (JASCO Corp., Tokyo, Japan) with an excitation wavelength of 490 nm (slit 1.5 nm) and an emission wavelength of 517 nm (slit 5 nm). To monitor calcein leakage, 20 μL of the LUV suspension was diluted to 980 μL with the same Tris buffer, to which a 20-μL aliquot of AM3 in MeOH was added. Subsequently, 20 μL of 10% Triton X-100 (v/v) (Nacalai Tesque) was added to obtain the condition of 100% leakage. All measurements were performed at room temperature with a final lipid concentration of 27 μM.

SPR Experiments. LUVs were prepared and immobilized on the CM5 sensor chip surface, as previously described.[26] AM3 solutions at concentrations of 30, 40, and 50 μM, which were prepared by diluting aqueous AM3 solution (1 mg/mL) with PBS buffer, were then injected at a flow rate of 10 μL min^{-1}, and the association of AM3 was monitored for 300 s. Then, PBS running buffer was injected at the same flow rate, and the dissociation process of AM3 from the surface was monitored for 300 s. The sensor chip surface was regenerated after each analysis using our previously described protocol.[26]

Solid-State NMR Experiments. The membrane samples for the ^2H NMR measurements, consisting of POPC (46 μmol), AM3 (0 or 2.6 μmol), and 3-*d*-sterol (2.6 μmol), were prepared as described previously.[26] The ^2H NMR spectra for these samples were recorded on a 400 MHz ECA400 (JEOL,

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Tokyo, Japan) spectrometer at 30 °C using our previously described protocol.[26]

For the measurement of ^{31}P NMR spectra, the membrane samples were prepared by mixing POPC (7.2 μmol), cholesterol or ergosterol (0 or 0.4 μmol), and AM3 (0 or 0.4 μmol). An additional sample with a higher content of AM3 was also made by mixing POPC (6.4 μmol), cholesterol (0.4 μmol), and AM3 (1.2 μmol). The same procedures as in ^{2}H NMR sample preparation were performed except that deuterium oxide (50% w/w) was used to rehydrate the sample, instead of deuterium-depleted water. The hydrated membrane samples were transferred to 7 mm Teflon tubes. The ^{31}P NMR spectra were recorded on a 400 MHz ECA400 (JEOL) using a 7 mm CP-MAS probe (Doty Scientific Inc., Columbia, SC, USA) without rotation at 30 °C. A single pulse sequence with proton decoupling was employed with the following spectra parameters: acquisition time was 18 ms, 90° pulse width was 5.6 μs, relaxation delay was 2 s, and total number of scans was approximately 25 000.

Giant Unilamellar Vesicle (GUV) Preparation. GUVs were obtained by electroformation as described by Angelova and Dimitrov.[27] In brief, POPC-cholesterol-AM3 solutions (18:1:1) were prepared in $CHCl_3$–MeOH (4:1 v/v) to a final phospholipid concentration of 1 mg mL^{-1}. Aliquots (15 μL) were subsequently deposited on parallel aligned electrodes (Pt wires, φ = 100 μm) attached to glass slides, after which the solvent was evaporated under vacuum overnight. Milli-Q water (300 μL, Simplicity UV) was then added to completely immerse the electrodes, which were then sealed with another glass slide using a rubber spacer with a small fill port for drug injection. This slide was maintained at 50 °C on a temperature-controlled objective plate (Tokai Hit ThermoPlate, Tokai Hit Co., Ltd.), and an alternating current (10 V, 10 Hz) was applied (Arbitrary Waveform Generator 33220A, Agilent Technologies) for 40–50 min to form GUVs.

Differential Interference Microscopy. After formation, the morphological changes of GUVs were observed using a FluoView FV1000-D scanning unit with an IX81 inverted microscope (Olympus Corp, Tokyo, Japan). A LUCPLFLN 60× universal semiapochromat objective with an NA of 0.70 (Olympus Corp.) was used for differential interference observations. The acquisition speed was 8 μs pixel^{-1}, and images were visualized using an FV10-ASW-3.0 software. Contrast was edited using Adobe Photoshop CS6 to provide clear images. The sample temperature was maintained at 30 °C using the temperature-controlled objective plate.

RESULTS

Effect of the Stereochemistry of the 3-OH Group of Sterol on AM3 Activity. To examine whether the stereochemistry of the 3-OH group of sterol is critical for the membrane-permeabilizing activity of AM3, we first performed calcein leakage assays using epicholesterol (3α-cholesterol)-containing POPC liposomes (for lipid structures, see Figure 2). As shown in Figure 3, AM3 did not display any significant pore forming activity in epicholesterol-containing POPC liposomes, which was almost identical to that in pure POPC membranes. This was in contrast to its high activity in cholesterol- or ergosterol-containing liposomes (Figure 3).[22] These data suggest that membrane permeabilization by AM3, assumed to proceed via formation of pores, requires the β configuration of the 3-OH group of sterols.

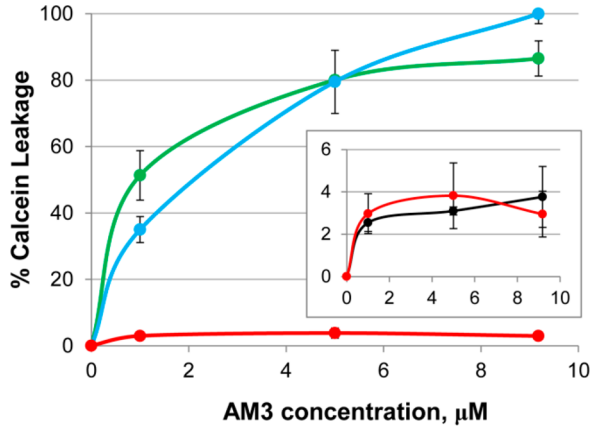

Figure 3. AM3-induced calcein leakage from POPC-cholesterol (green), POPC-ergosterol (blue), and POPC-epicholesterol (red) liposomes. The mole ratio of POPC and sterol was 9:1. In all cases, the final lipid concentration was 27 μM. The inset shows the leakage from pure POPC (black) and POPC-epicholesterol (red) liposomes.

Next, we investigated the affinity of AM3 for epicholesterol-containing lipid membranes using SPR experiments. A dodecylamine-modified CM5 sensor chip was prepared to immobilize POPC liposomes on the chip as we previously reported.[23,26,28] As shown in Figure 4, very weak binding was observed between AM3 and epicholesterol-containing POPC liposomes, which was comparable to that in sterol-free liposomes. In contrast, as we reported previously,[23] liposomes containing cholesterol or ergosterol exhibited dramatically increased binding of AM3 and its extremely slow dissociation (Figure 4A,B). This result clearly indicated that AM3 preferentially binds to liposomes containing 3β-sterols with high affinity. Because the membrane permeabilization of AM3 is assumed to proceed via its membrane binding and subsequent pore formation, the good correlation between dye-leakage assays and SPR experiments suggests that the membrane binding of AM3 is a determinant of its membrane permeabilizing potency.

Stereospecific and Direct Binding of AM3 to Sterols Examined by Solid-State ^{2}H NMR. Although the aforementioned results strongly suggest that AM3 directly and selectively targets 3-β-hydroxysterols in membranes, alternative mechanism cannot be discounted: 3-β-hydroxysterols change the physical properties of membranes such as fluidity and thickness, which may indirectly promote AM3 binding. Hence, to obtain evidence of a direct interaction between AM3 and sterols, solid-state ^{2}H NMR experiments were conducted using deuterated sterols incorporated into POPC liposomes.[24,26] In the absence of AM3 (Figure 5A), cholesterol-d_1 in membranes undergoes fast rotational motion as revealed by the characteristic Pake doublet. This splitting pattern was significantly reduced in the presence of AM3 (Figure 5B), implying that molecular motion was considerably slowed to the range of correlation times of between 100 and 10 μs.[24] A similar change in the splitting pattern was recorded with ergosterol-d_1 (Figure 5C,D). These observations were in stark contrast to the spectra of epicholesterol-d_1, in which the intensity of the Pake doublet was comparable both in the absence and presence of AM3 (Figure 5E,F). These changes in the spectral pattern clearly indicate that the fast rotational motion of both cholesterol-d_1 and ergosterol-d_1 was considerably attenuated because of a direct intermolecular interaction with AM3 in membranes. In

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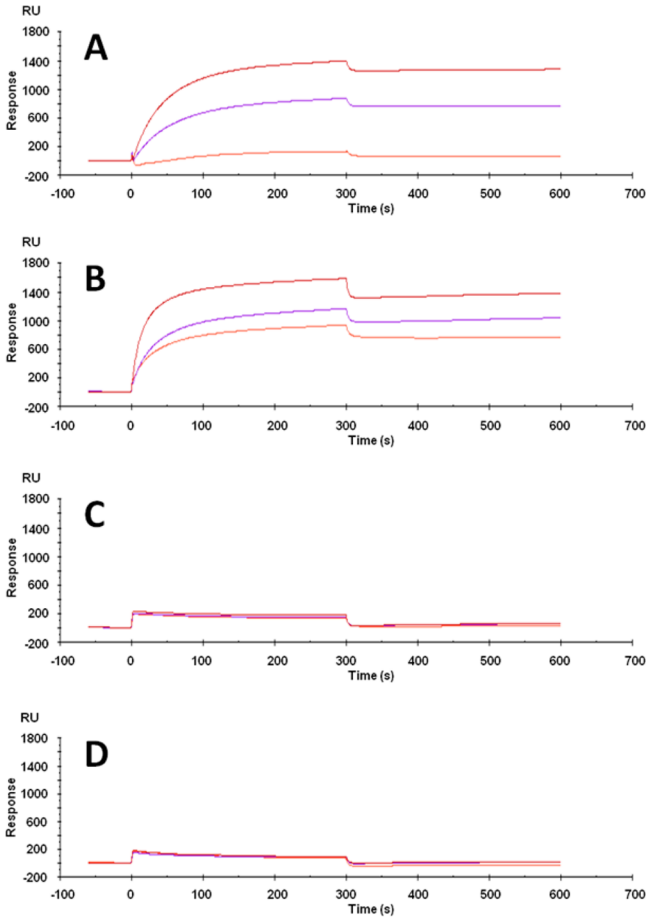

Figure 4. SPR sensorgrams for the binding of AM3 to liposomes immobilized on a dodecylamine-modified CM5 sensor chip: POPC liposomes containing 10 mol % cholesterol (A), 10 mol % ergosterol (B), 10 mol % epicholesterol (C), and pure POPC liposomes (D). The concentrations of AM3 are 50 (red), 40 (purple), and 30 (orange) μM. The association of AM3 was monitored from 0 to 300 s, and its dissociation from the surface was recorded from 300 to 600 s.

contrast, the lack of noticeable inhibition of epicholesterol's rotation indicates a weaker interaction with AM3. This is consistent with its lack of membrane permeabilization in, and its lower binding affinity to, epicholesterol-containing liposomes. These ^{2}H NMR results explicitly demonstrate the direct intermolecular interaction between AM3 and 3β-hydroxysterols in membranes.

Effect of AM3 on Membrane Morphology Examined by Solid-State ^{31}P NMR and Microscopy. To investigate morphological changes of membrane upon the incorporation of AM3, the solid-state ^{31}P NMR spectra of POPC liposomes were recorded in the absence and presence of AM3 (Figure 6). In the absence of AM3 (Figure 6A), cholesterol-containing POPC liposomes exhibited a typical bilayer lamellar structure as evidenced by the characteristic powder pattern. Interestingly, in the presence of 5 mol % AM3 (Figure 6B), the spectra did not change, and no isotropic signals were observed. This was the case with a higher concentration of AM3 at 15 mol % (Figure 6C). A similar observation was recorded in ergosterol-containing POPC liposomes (Figure S1 in the Supporting Information) as well as in sterol-free POPC membranes (Figure S2 in the Supporting Information). AM3, at this sterol concentration (5 mol %), is expected to efficiently permeabilize the membrane;[22] therefore, the lack of distinctive isotropic signals suggests that AM3 binds to the membrane without substantially changing the orientation of the surrounding lipids.

Next, to further observe macroscopic changes in membrane morphology upon AM3 incorporation, we used differential interference microscopy for giant unilamellar vesicles (GUVs). The GUVs are suitable models for microscopic observations of the structural details of membrane organization at a resolution exceeding approximately 300 nm.[29,30] However, mixing of AM3 with GUVs composed of POPC and cholesterol did not induce any distinct morphological changes (Figure 7), which is consistent with the aforementioned ^{31}P NMR data. The observation that AM3 produced no changes in ^{31}P NMR spectra and the microscopic image of membranes suggests that AM3 binds to the membrane without significantly disturbing its integrity.

DISCUSSION

AM3 activity was previously demonstrated to strictly require cholesterol or ergosterol in membranes.[9,22] This was supported by previous SPR experiments detailing more than 3 orders of magnitude increase in the affinity of AM3 toward sterol-containing POPC membranes compared with sterol-free liposomes.[23] Despite these findings, an in-depth understanding of AM3−sterol interactions is still lacking; in particular, it remained unclear whether direct molecular recognition between AM3 and sterol or ordering effect of sterol on lipid bilayers enhances the membrane binding of AM3. In this study, we demonstrated the direct interaction between sterols and AM3 by investigating the effect of the stereochemistry of the sterol 3-OH group. Furthermore, it is worth mentioning that the AM3:lipid molar ratios used in our experiments were similar (1:27 for calcein leakage, 1:17 for the lowest concentration in SPR, and 1:19 for ^{2}H and ^{31}P NMR and microscope observations); thus, these results are reasonably comparable.

The calcein leakage experiments revealed that pore formation by AM3 requires both sterol in the membrane and the correct orientation of the 3-OH group. In addition, pore formation was also closely correlated with the extent of membrane binding, as demonstrated by SPR sensorgrams in Figure 4. The increase in the binding of AM3 to 3β-hydroxysterol-containing membranes was induced by direct intermolecular interactions between AM3 and the sterol, as evidenced by characteristic changes in the patterns of solid-state ^{2}H NMR spectra (Figure 5). Given that the OH group of epicholesterol, which resides around the water−membrane interface,[31−33] is accessible by AM3 in membranes, the weaker interaction of AM3 with epicholesterol implies that AM3 strictly recognizes the stereochemistry of the sterol 3-OH group, probably via hydrogen bonding. As well, given that epicholesterol was reported to produce ordered lipid bilayers, although only slightly less effective than cholesterol,[34] it is less likely that the ordering effect of sterol plays a crucial role in the enhanced membrane binding of AM3. In addition, we previously reported the membrane-permeabilizing activity of AM3 at different cholesterol and ergosterol contents (0 to 60 mol %) in POPC liposomes, which showed that, although no more than 1 mol % of sterol in POPC significantly promoted the AM3 activity, the phase state of the membranes was less associated with its activity.[22] Collectively, it is speculated that the direct molecular interaction of AM3 with sterols through the stereospecific recognition of the 3β-OH group results in increased AM3 binding and subsequent membrane permeabi-

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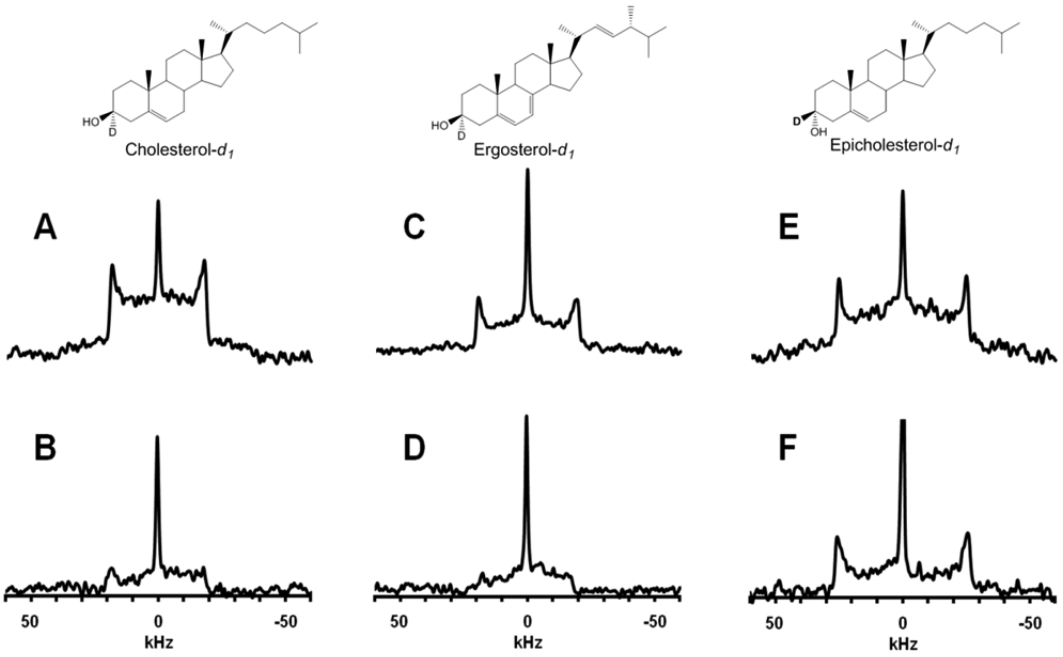

Figure 5. ^{2}H NMR spectra of 3-*d*-sterols incorporated into POPC bilayers in the absence (A, C, and E) and presence (B, D, and F) of AM3. The membrane preparations contained cholesterol-d_1 (A and B), ergosterol-d_1 (C and D), and epicholesterol-d_1 (E and F) at AM3:sterol-d_1:POPC molar ratios of 0:1:18 (A, C, and E) and 1:1:18 (B, D, and F). Isotropic signals at 0 ppm are mostly due to residual deuterium water.

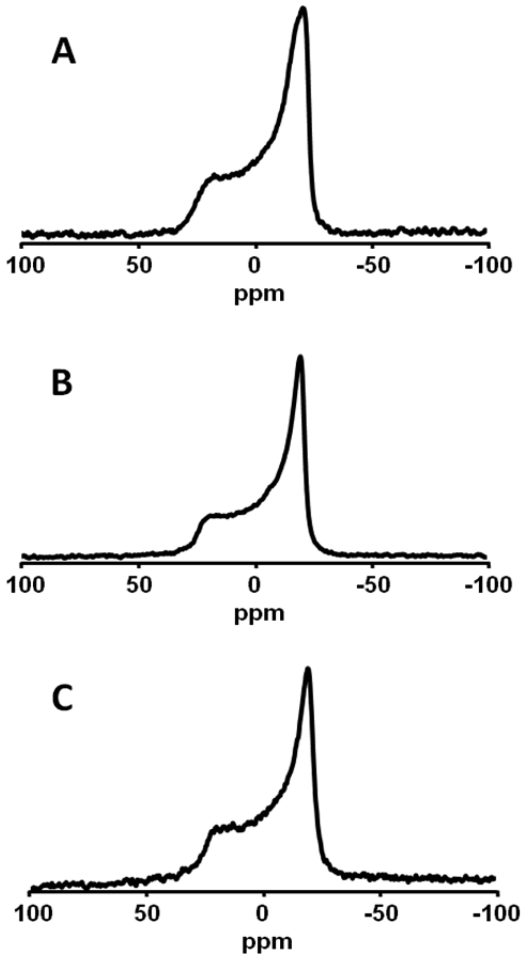

Figure 6. ^{31}P NMR spectra of POPC-cholesterol bilayers in the absence (A) and presence (B and C) of AM3. AM3:cholesterol:POPC molar ratios of 0:1:18 (A), 1:1:18 (B), and 3:1:16 (C) were used.

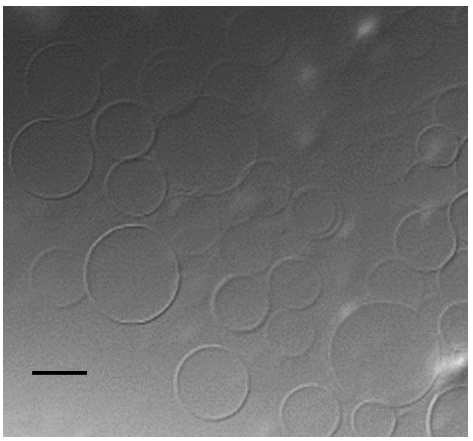

Figure 7. Differential interference micrographs of GUVs containing AM3, cholesterol, and POPC at a 1:1:18 molar ratio. Scale bar = 25 μm.

lization. Since the middle region of AM3 containing the two tetrahydropyran rings is conserved among amphidinols, some of which are reported to have sterol-dependent membrane-permeability,[22] the middle region might be responsible for recognizing the 3β-OH group of sterols.

In our previous reports, membrane permeabilization of AM3 was assumed to follow a toroidal pore model based on its hairpin conformation in membranes and independence of its activity from membrane thickness.[22,35,36] However, a recent report argues that the effects of membrane thickness on the permeabilizing activity of antimicrobial peptides are not a reliable criterion to clarify whether their mechanisms of action are based on the barrel-stave or the toroidal model.[37] In addition, although toroidal pore formation should give isotropic signals in solid-state ^{31}P NMR spectra as demonstrated for a number of antimicrobial peptides,[38,39] no spectral changes were observed in cholesterol- or ergosterol-containing POPC

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liposomes even in the presence of a higher AM3 molar ratio (Figure 6 and Figure S1 in the Supporting Information). This clearly indicates that no alteration in the orientation of the phospholipid headgroup was induced by AM3. These data suggest that membrane binding and subsequent permeabilization by AM3 hardly disturbed the arrangement and packing of the surrounding lipids. Interestingly, amphotericn B, a well-known barrel-stave pore-former in ergosterol-containig membranes, did not give isotropic signals in ^{31}P NMR spectrum, either (Figure S3 in the Supporting Information). Accordingly, the mechanism of AM3 action may be accounted for, not by the originally proposed toroidal channel, but rather by a barrel-stave pore lined with highly flexible polyhydroxy chains. We have reported that AM3 takes a hairpin-like conformation with a relatively rigid turn structure at the middle region including the tetrahydropyran rings.[35,36] Given that, as described above, the turned region, which is conserved among other amphidinols, recognizes the 3β-OH group of sterols via hydrogen bonds, a hypothetical barrel-stave model may be proposed to explain the membrane permeabilization activity of AM3 interacting with sterol molecules (Figure 8).

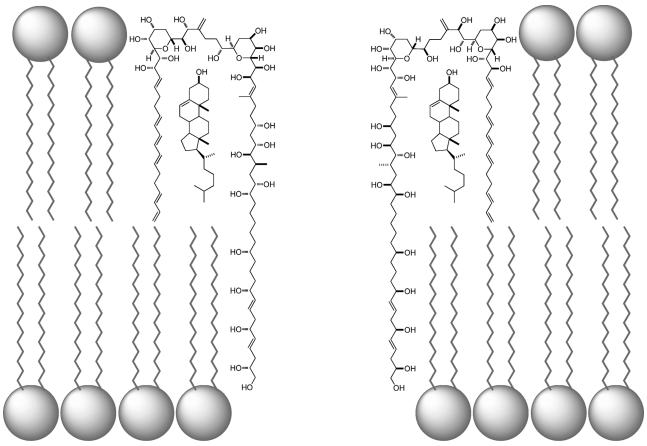

Figure 8. Hypothetical pore model of AM3 interacting with cholesterol. In the model, the relatively rigid turned region of AM3 recognizes the 3β-OH group of cholesterol through hydrogen bonds, and the flexible polyhydroxy chains form the transient pore-lining surface.

In summary, dye leakage and SPR experiments demonstrated that the membrane permeabilizing and membrane-binding activities of AM3 were dependent on both the presence of membrane sterols and, more importantly, the 3-OH group of sterol being in the β-configuration. This enhanced affinity and activity toward 3β-hydroxysterol-containing membranes was explicitly observed to arise from direct intermolecular interactions between AM3 and sterol in membrane, as evidenced by the solid-state ^{2}H NMR spectral patterns. The solid-state ^{31}P NMR data and microscopic images of GUVs unexpectedly revealed that AM3 perforates the membranes without apparent disruption of membrane integrity. Considering together, AM3 directly interacts with membrane sterols through the strict molecular recognition of the 3-OH configuration, resulting in its increased membrane binding and subsequent permeabilization without disturbing the morphology of the membrane. Finally, the ^{31}P NMR observation may imply a different mechanism of action from the originally proposed toroidal pore model, although further experiments are needed to clarify the mechanism of action of AM3. To this end, solid-state NMR experiments are currently underway to probe in more detail the state of AM3 in lipid bilayers.

■ ASSOCIATED CONTENT

Supporting Information

Solid state ^{31}P NMR spectra of ergosterol-containing and sterol-free POPC liposomes in the presence and absence of AM3, and of ergosterol-containing POPC liposomes in the presence of amphotericin B. 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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■ ABBREVIATIONS

AM3, amphidinol 3; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; RU, resonance unit; SPR, surface plasmon resonance

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