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# Direct Interaction between Amphotericin B and Ergosterol in Lipid Bilayers As Revealed by $^2$H NMR Spectroscopy

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***Abstract:*** Although amphotericin B (AmB) is thought to exert its antifungal activity by forming transmembrane ion-permeable self-assemblies together with ergosterol, no previous study has directly proven AmB−ergosterol interaction. To establish the interaction, we measured $^2$H NMR using deuterium-labeled sterols and AmB. The $^2$H NMR spectra of deuterated ergosterol in palmitoyloleoylphosphatidylcholine (POPC) bilayers showed that fast axial diffusion of erogosterol was almost completely inhibited by the coexistence of AmB. Conversely, cholesterol mobility in POPC membrane was essentially unchanged with or without AmB. These results unequivocally demonstrate that ergosterol has significant interaction with AmB in POPC bilayers. In addition, we examined the mobility of AmB using deuterium-labeled AmB, and found that, although AmB is almost immobilized in sterol-free and cholesterol-containing POPC membranes, a certain ratio of AmB molecules acquires mobility in the presence of ergosterol. The similar mobility of AmB and ergosterol in POPC bilayers confirmed the idea of the direct intermolecular interaction between ergosterol and AmB.

Amphotericin B (AmB, Chart 1) has been a standard drug for treatment of deep-seated systemic fungal infections for nearly 50 years.[1−3] For lack of better alternatives, as well as the rare occurrence of resistant strains,[4] the clinical importance of AmB has remained unchanged. It is presently widely accepted that AmB molecules in lipid bilayers form a barrel-stave channel, with their polyhydroxy side pointing inward and their lipophilic heptaene part directing outward.[5,6] The occurrence of such molecular assemblies in fungal cell membranes increases ion permeability and alters membrane potentials, ultimately leading to cell death.[6−9] The pharmacological action of AmB is based on its selective toxicity against fungi over mammalians, which is thought to result from its stronger interaction to ergosterol (Chart 1), an abundant sterol in fungal membranes, than to cholesterol (Chart 1), the major sterol in mammalian membranes.[10,11] The interaction between AmB and sterol, particularly ergosterol, has also been supported by a number of experimental results: (a) higher affinity of AmB to ergosterol-containing membranes than to sterol-free and cholesterol membranes;[10,12,13] (b) more potent ion-permeability of AmB in ergosterol-membranes;[8,14−16] and (c) spectroscopic changes of AmB in sterol-containing membranes, as seen in UV,[17−19] CD,[8,20−22] and IR spectra.[23,24] None of these studies, however, have directly proven the AmB−sterol interaction in membrane environments. Although we have recently demonstrated close contacts between polyene carbon atoms of AmB and a fluorine atom of 6-F-ergosterol using an AmB-6-F-ergosterol covalent conjugate by a solid state NMR technique,[25] it might not be so straightforward to

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*Chart 1.* Structures of AmB, Ergosterol, Cholesterol and Their Deuterium-Labeled Counterparts

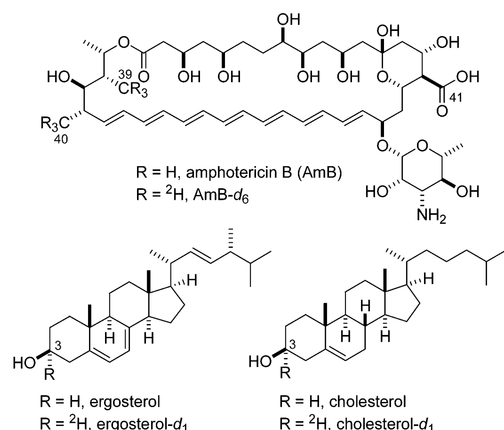
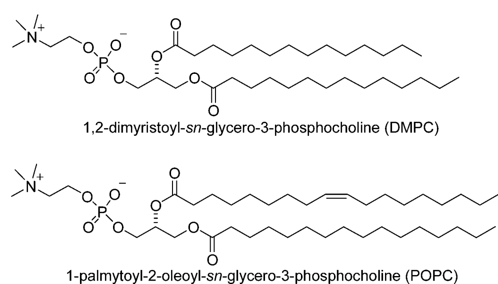



*Chart 2.* Structures of Phospholipids




extrapolate the data of the conjugate to the native AmB−sterol interaction, because the conjugate can be structurally constrained by the linkage. Moreover, AmB turned out to have ion permeability without sterols under certain situations, such as higher concentrations of AmB, osmotic gradient, and gel phase membranes.[9,26−32] Therefore, there is another notion that sterol enhances AmB channel activity by changing structures and properties of membranes rather than by directly participating in AmB ion-channel complex.[33,34]

Meanwhile, detecting sterol dynamics affected by the presence of AmB would provide a direct clue to the AmB−sterol interaction. Until now, however, very few studies have been conducted on the influence of AmB on dynamic properties of sterols; the only example has been reported by Dufourc et al.,[35] but they did not detect the change of cholesterol dynamics in the presence of AmB upon measuring the $^{2}$H NMR of deuterium-labeled cholesterol in dimyristoylphosphatidylcholine (DMPC, Chart 2) bilayers. Hence, in the present study, we also used $^{2}$H NMR and examined whether the dynamic properties of ergosterol in lipid bilayers are affected by the presence of AmB. We also investigated the motional property of AmB in the presence or absence of sterols. The merit of applying the $^{2}$H NMR method is that the deuterium labeling in molecules of interest is essentially nonperturbing. The findings provided the first experimental evidence of the intermolecular interactions between AmB and ergosterol. Additionally, we have found that the strength of AmB−ergosterol interactions varies depending on the type of phospholipid used.

## Results and Discussion

The preparation of erogosterol-$d_1$ (Chart 1) commenced with protection of the 5,7-diene of ergosterol with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Scheme 1). Oxidation of alcohol **1** to ketone **2** followed by reduction with $NaBD_4$ afforded a mixture of the desired alcohol **3** and its $C_3$-epimer, which were separable by silica gel column chromatography. Removal of the PTAD group of **3** using diisobutylaluminum hydride (DIBAL), however, unexpectedly led to replacement of the deuterium on C3 by a hydrogen atom, probably via a Meerwein−Ponndorf−Verley reaction. Thus, prior to removal of the PTAD group, the alcohol group of **3** was protected by a *t*-butyldimethylsilyl (TBDMS) group to produce **4**. Removal of the PTAD group of **4** with DIBAL gave rise to **5**, which was further treated with tetrabutylammonium fluoride (TBAF) to provide the desired ergosterol-$d_1$. Cholesterol-$d_1$[36] (Chart 1) was also prepared as previously reported.[37]

Deuterium-labeled AmB (AmB-$d_6$, Chart 1), in which hydrogen atoms on C39 and C40 methyl groups were substituted with deuterium atoms, was biosynthetically prepared with a slightly modified procedure for producing [39,40,41-$^{13}C_3$]-AmB.[38] Briefly, the AmB-producing actinomycete *Streptomyces nodosus* (ATCC14899) was cultured in a modified FCA medium in which the nutrient contents were reduced to half the amount of the original medium to decrease hydrocarbon sources. To the medium was added sodium [3-$^{2}H_3$]propionate to produce site-specifically 39- and 40-$^{2}$H-labeled AmB-$d_6$. From 50 mL of the culture, about 15 mg of AmB-$d_6$ was obtained with 30% deuterium enrichment at C39 and C40 positions.

With deuterium-labeled molecules available, we first measured the solid-state $^{2}$H NMR of ergosterol-$d_1$ and cholesterol-$d_1$ in a membrane to observe the influence of AmB on dynamic properties of sterols. In fast axially rotating substances such as sterol molecules in lipid bilayers, quadrupolar splittings observed in $^{2}$H NMR spectra depend on the tilt angle of the C−$^{2}$H bond and the wobbling of a molecule with respect to the rotation axis. As described previously, Dufourc et al. showed that the $^{2}$H NMR spectra of deuterated cholesterol in DMPC bilayers were substantially unchanged in the presence of AmB.[35] Hence, we carried out similar experiments using deuterium-labeled ergosterol in place of cholesterol. Figure 1 shows the $^{2}$H NMR spectra of ergosterol-$d_1$ in DMPC bilayers in the presence or absence of AmB. The spectra show typical Pake doublet patterns with quadrupolar splittings of ca. 40 kHz, which is significantly reduced from the rigid-limit quadrupolar splitting value (ca. 114 kHz). This value is almost equivalent to that of cholesterol in

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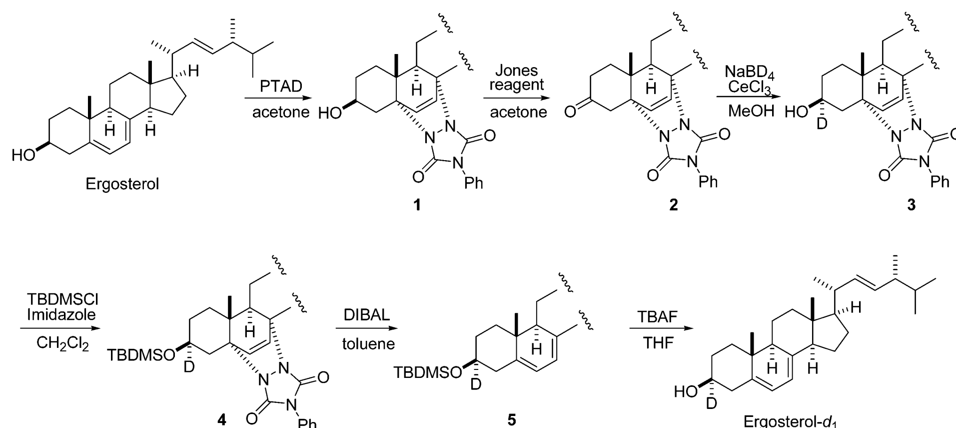
***Scheme 1.*** Preparation of Ergosterol-$d_1$




the DMPC bilayers,[37] suggesting that ergosterol also undergoes fast rotational averaging in the DMPC membrane as is the case with cholesterol. Unexpectedly, the doublet pattern is still clearly observable in the presence of AmB (Figure 1B), while the peak intensity seems to be slightly reduced. This indicates that a considerable portion of ergosterol does not interact with AmB in the DMPC bilayers, and gives rise to the splitting peaks in Figure 1B. Figure 1 also shows large isotropic components in the $^2$H NMR spectra, which are most likely due to coexisting small vesicles tumbling rapidly in water, although the samples also contain a small amount of residual deuterium from HDO in the water.

Since it is known that saturated phospholipids such as DMPC and dipalmytolylphosphatidylcholine (DPPC) have strong interactions with AmB,[24,39] probably via hydrophobic contacts between the saturated acyl chains of PC and the polyene portion of AmB, we postulate that the strong AmB−DMPC interaction rather impedes the interaction between AmB and ergosterol. On the other hand, we have recently shown by surface plasmon resonance (SPR) experiments that AmB exhibits much higher affinity for sterol-containing palmitoyloleoylphosphatidylcholine (POPC, Chart 2) membranes than those without sterol.[13] Hence, to selectively observe the spectral changes of deuterated sterols in the presence or absence of AmB, we altered the membrane constituent from DMPC to POPC, and measured the $^2$H NMR spectra of sterols in the membrane. Figure 2 shows the spectra of ergosterol-$d_1$ in POPC bilayers with increasing ratios of AmB. In the absence of AmB (Figure 2A), ergosterol-$d_1$ shows the splitting pattern (ca. 39 kHz) as seen in the DMPC membranes, thus, indicating the fast axial rotation of ergosterol in POPC bilayers. Unlike the results in DMPC bilayers, the splitting signal of ergosterol-$d_1$ broadened and almost disappeared with the increase in AmB ratios (Figure 2B,C). The vanishing of the doublet signal and the peak broadening are generally characterized by intermediate molecular motion with correlation times

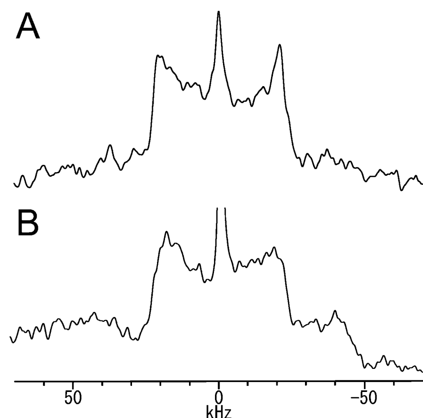



***Figure 1.*** $^2$H NMR spectrum of ergosterol-$d_1$ in DMPC bilayers in the absence (A) and presence (B) of AmB. The molar ratios of ergosterol-$d_1$/AmB/DMPC are 1:0:18 (A) and 1:2:18 (B). Spectra were recorded at 30 °C. The quadrupolar splitting value is 40 kHz. The isotropic center peaks are likely to be attributed to coexisting small vesicles with faster tumbling in water.

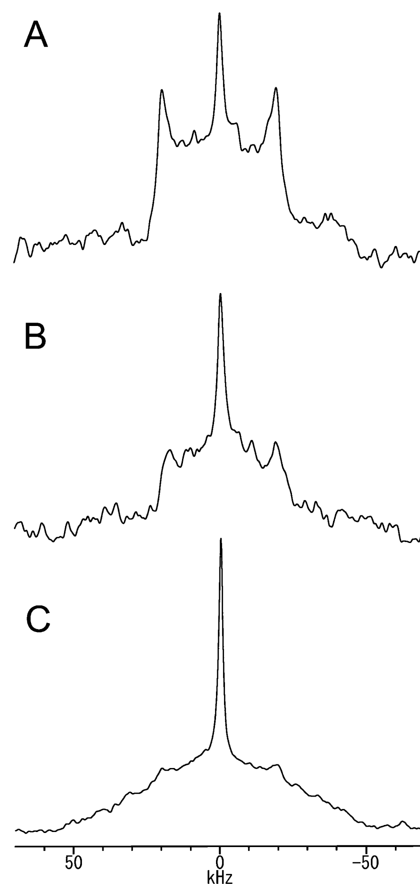



***Figure 2.*** $^2$H NMR spectra of ergosterol-$d_1$ in POPC with increasing ratios of AmB. The molar ratios of ergosterol-$d_1$/AmB/POPC are 1:0:18 (A), 1:1:18 (B), and 1:2:18 (C). Spectra were recorded at 30 °C. The isotropic center peaks are likely to be attributed to coexisting small vesicles with faster tumbling in water.

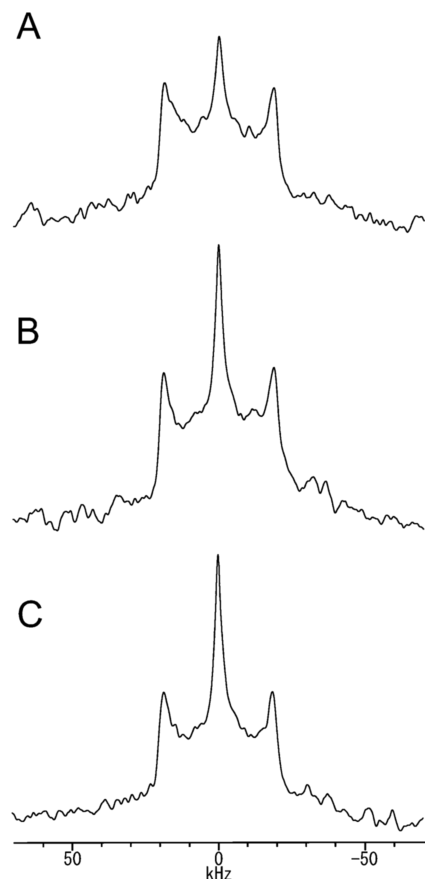



***Figure 3.*** $^2$H NMR spectra of cholesterol-$d_1$ in POPC with increasing ratios of AmB. The molar ratios of cholesterol-$d_1$/AmB/POPC are 1:0:18 (A), 1:1:18 (B), and 1:2:18 (C). Spectra were recorded at 30 °C. The isotropic center peaks are likely to be attributed to coexisting small vesicles with faster tumbling in water.

of $10^{-4}$−$10^{-5}$ s.[40] Therefore, Figure 2C shows that the fast motional averaging of ergosterol is inhibited by the presence of AmB, indicating that ergosterol has a significant interaction with AmB. In contrast, $^2$H NMR spectra of cholesterol-$d_1$ hardly change in the presence or absence of AmB (Figure 3), indicative of weaker intermolecular interaction between AmB and cholesterol. These data strongly support the notion that AmB directly interacts with ergosterol, and further, confirm that ergosterol has much stronger affinity for AmB than does cholesterol.

The above results can be interpreted in terms of the equilibrium of sterol molecules between freely rotating state (unbound to AmB) and AmB-bound state. Although it is difficult to quantitatively evaluate the equilibrium constant between AmB and sterols in POPC membranes from the current $^2$H NMR data, we can safely conclude that in case of cholesterol the equilibrium leans much to the free state, which gives no detectable change in $^2$H NMR spectra (Figure 3), whereas ergosterol is mostly in the interacting state, thus, giving the broadened spectra as shown in Figure 2.

Next, to further characterize the AmB−ergosterol interaction from the standpoint of AmB, we examined the motional properties of AmB in POPC membranes using AmB-$d_6$ that was

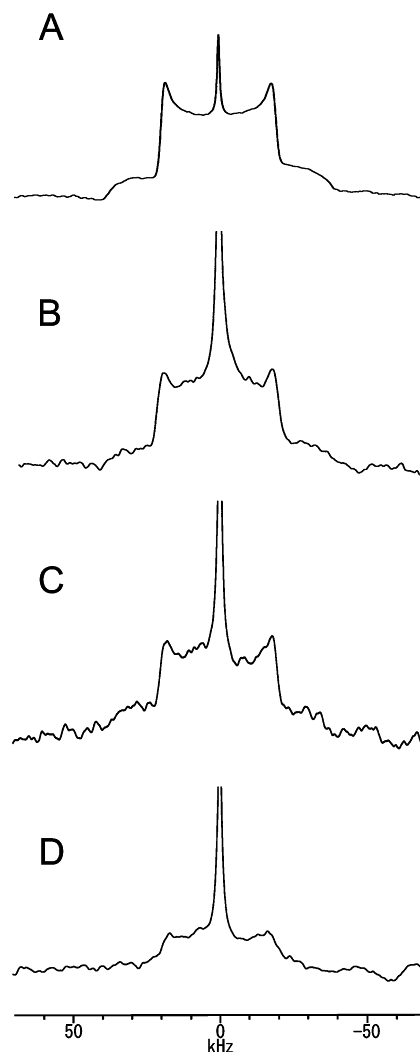



***Figure 4.*** $^2$H NMR spectra of AmB-$d_6$ in powder state (A), in sterol-free POPC bilayers (B), in cholesterol-containing POPC bilayers (C), and in ergosterol-containing POPC bilayers (D). The molar ratios of AmB-$d_6$/sterol/POPC are 1:0:20 (B) and 1:2:18 (C, and D). Spectra were recorded at 30 °C. The isotropic components of panels B−D are likely to be attributed to coexisting small vesicles with faster tumbling in water.

deuterated at the 39- and 40-methyl groups. Although Schaefer's group[41] and our group[42] have reported the dynamics of AmB in membranes using its amide derivatives, this is the first report using virtually nonderivatized AmB. Figure 4A shows the $^2$H NMR spectrum of AmB-$d_6$ in the powder state, where the quadrupolar splitting is intrinsically reduced to 37 kHz due to the methyl rotation. In sterol-free and cholesterol-containing POPC bilayers (Figure 4B,C), the quadrupolar splittings of AmB are clearly observed and the values (37 kHz) are almost identical to that in the powder state, demonstrating that AmB molecules are mostly immobilized in those membrane systems. This corresponds to formation of large aggregates of AmB in these membranes.[42] Meanwhile, the $^2$H NMR of AmB-$d_6$ in ergosterol-containing POPC membrane shows that the split signal, albeit still observed, is markedly reduced (Figure 4D), although we used the same amount of AmB-$d_6$ and almost equalized the number of accumulations for measuring Figure 4B−D spectra.

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***Table 1.*** Mobility of AmB, Ergosterol, and Cholesterol in POPC Bilayers

| | molecular mobility | | |
|---|---|---|---|
| | ergosterol | AmB | cholesterol |
| single substance in POPC bilayers | high | immobile | high |
| AmB−ergosterol coexisting system | medium | medium | |
| AmB-cholesterol coexisting system | | immobile | high |

In $^2$H NMR spectra, not only the signal broadening, but also the peak intensity is an indicator of molecular mobility. Therefore, the reduction of signal intensity in ergosterol-containing POPC membrane as shown in Figure 4D suggests that, although immobilized aggregates of AmB partly exist, a significant proportion of AmB molecules are in motion on a time scale of quadrupolar coupling ($10^{-4}-10^{-5}$ s). AmB is, therefore, considered to gain some mobility in the ergosterol membrane in contrast to the immobile AmB in cholesterol-containing and sterol-free POPC membranes.

Table 1 summarizes the mobility of AmB, ergosterol, and cholesterol in POPC membranes revealed by the current $^2$H NMR measurements. Interestingly, the coexistence of ergosterol and AmB makes the mobility of ergosterol (Figure 2C) and AmB (Figure 4D) comparable; fast axial diffusion of ergosterol decelerates upon addition of AmB, while immobilized AmB begins to move in the presence of ergosterol. These findings unequivocally demonstrate that AmB and ergosterol interact closely to form a channel complex and move together in POPC bilayers.

We have recently reported that the AmB−AmB intermolecular distance is elongated by 2 Å in an ergosterol-containing POPC membrane as compared with the distance in sterol-free membranes on the basis of rotational-echo double resonance (REDOR) experiments.[43] Those results together with the current findings allow us to propose a simple model on the function of ergosterol. Without ergosterol, AmB molecules are largely in aggregated forms with low mobility due to the tight AmB−AmB complexation. AmB is known to induce slight ion permeation even in the absence of sterol,[9] which might be attributed to membrane defects formed at the phase boundary between lipid bilayers and AmB aggregates.[28] Meanwhile, in the presence of ergosterol, the AmB−AmB binding should be loosened by the interaction with ergosterol, which consequently enhances the mobility of the AmB aggregates and increases the intermolecular distances between AmB molecules.[43] These effects of ergosterol may facilitate the formation of ion channels in the membranes.

Nonetheless, it is still unclear how AmB discriminates the minute difference between ergosterol and cholesterol and interacts preferentially with ergosterol. Previous studies showed that the conjugated double bonds on the sterol skeleton are of critical importance in interacting with AmB,[44,45] and the conjugated diene on ergosterol is thought to contribute to rigidifying the sterol backbone, thus, allowing the molecular proximity necessary to maximize the hydrophobic interactions with AmB.[45] Our recent study, however, revealed that the ion permeability of AmB in the presence of sterols that have a conjugated triene on the sterol skeleton is unexpectedly weaker than that in the presence of sterols having a conjugated diene system such as ergosterol or 7-dehydrocholesterol (to be published in due course). This probably suggests that the rigidification of sterol nucleus by conjugated double bonds is not the only determinant of the interaction with AmB. To gain further insights into the molecular recognition between AmB and sterol, measurements of intermolecular distances between AmB and ergosterol by solid-state NMR are now in progress.

## Conclusions

We have achieved the first direct observation of the intermolecular AmB−ergosterol interaction in POPC membranes using deuterated sterols and AmB. The similar mobility of AmB and ergosterol in POPC bilayers as shown in the $^2$H NMR spectra confirms their direct complexation. As mentioned above, there exists another notion that sterol enhances AmB channel activity by changing structures and properties of membranes rather than by directly participating in AmB ion-channel complex.[33,34] Therefore, this study settles the dispute about the effect of ergosterol on AmB channel. In addition, we demonstrated that a certain type of phospholipids interfered with the AmB−ergosterol interaction; DMPC, which has high affinity to AmB through the strong binding between polyene carbons of AmB and saturated acyl chains of DMPC, relatively weakens the AmB−ergosterol interaction, while POPC, which should have weaker interaction with AmB because of the flexible unsaturated acyl chain, allows for the AmB−ergosterol interaction.

To understand the mode of action of AmB in biomembranes, it is necessary to take account of three intermolecular interactions: AmB−AmB, AmB−sterol, and AmB−phospholipid. It is more likely that these interactions are not independent but interdependent; therefore, one interaction could promote and/or prevent the other two. As shown in this study, strong AmB−phospholipid interaction may impede AmB−sterol vicinity. On the other hand, AmB−ergosterol interaction could loosen AmB−AmB contact to prevent the formation of large AmB aggregates in membranes. Previously, a number of research groups had attempted to elucidate the mechanism of the AmB channel, but results were sometimes conflicting and confusing. This is probably because they used different phospholipids, which significantly influenced AmB−AmB and AmB−sterol interactions. The present results clearly show that POPC is more suitable than saturated phospholipids for examining the AmB−sterol interaction. Moreover, since most biological membranes, including fungal ones, contain unsaturated phospholipids as a major constituent, the findings obtained in this study using POPC membranes should largely hold true for the biological activity of AmB.

## Experimental Section

Experimental procedures for the preparation of AmB-$d_6$ and ergosterol-$d_1$ are described in the Supporting Information.

**Sample Preparation for $^2$H NMR.** For measurements of $^2$H NMR spectra of deuterated sterols in DMPC or POPC membranes, 5.0 $\mu$mol of ergosterol-$d_1$ or cholesterol-$d_1$, 90.0 $\mu$mol of phosphocholine (DMPC or POPC), and AmB (0, 5, or 10 $\mu$mol) were dissolved in $CHCl_3$-MeOH, and the solvent was removed in vacuo for 12 h. The dried membrane film was hydrated with 1 mL of water. After a few minutes of sonication, the suspension was freeze−thawed three times and vortexed to make multilamellar vesicles. The vesicle solution was lyophilized, rehydrated with deuterium-depleted water (50% w/w), and freeze−thawed three

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times. The sample was transferred into a 5-mm glass tube (Wilmad), which was sealed with epoxy glue.

For measuring of $^{2}$H NMR spectra of AmB-$d_6$ in POPC membranes, a combination of 3.25 $\mu$mol of AmB-$d_6$ and 58.44 $\mu$mol of POPC (molar ratio 1:18), or that of 3.25 $\mu$mol of AmB-$d_6$, 58.44. $\mu$mol of POPC, and 6.5 $\mu$mol of cholesterol or ergosterol (molar ration 1:18:2), was mixed in $CHCl_3$-MeOH. The following procedure is the same as above.

**$^{2}$H NMR Measurements.** All the $^{2}$H NMR spectra were recorded on a 300 MHz CMX300 spectrometer (Chemagnetics, Varian, Palo Alto, CA). Spectra were recorded at 30 °C with a 5-mm $^{2}$H static probe (Otsuka Electronics, Osaka, Japan) using a quadrupolar echo sequence.[46] The 90° pulse width was 2 $\mu$s, interpulse delay was 30 $\mu$s, and repetition rate was 0.5 s. The sweep width was 200 kHz, and the number of scans was around 100 000.

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**Supporting Information Available:** Experimental details of preparation of AmB-$d_6$ and ergosterol-$d_1$. This material is available free of charge via the Internet at http://pubs.acs.org.

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