Possible conformation of amphotericin B dimer in membrane-bound assembly as deduced from solid-state NMR

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Head-to-Tail Interaction between Amphotericin B and Ergosterol Occurs in Hydrated Phospholipid Membrane

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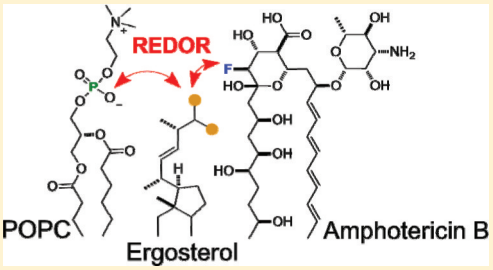
Supporting Information

ABSTRACT: Amphotericin B (AmB) is thought to exert its antifungal activity by forming an ion-channel assembly in the presence of ergosterol. In the present study we aimed to elucidate the mode of molecular interactions between AmB and ergosterol in hydrated phospholipid bilayers using the rotational echo double resonance (REDOR) spectra. We first performed $^{13}C\{^{19}F\}$REDOR experiments with C14-^{19}F-labeled AmB and biosynthetically ^{13}C-labeled ergosterol and implied that both "head-to-head" and "head-to-tail" orientations occur for AmB−ergosterol interaction in the bilayers. To further confirm the "head-to-tail" pairing, ^{13}C-labeled ergosterol at the dimethyl terminus (C26/C27) was synthesized and subjected to the REDOR measurements. The spectra unambiguously demonstrated the presence of a "head-to-tail" orientation for AmB−ergosterol pairing. In order to obtain information on the position of the dimethyl terminus of ergosterol in membrane, $^{13}C\{^{31}P\}$REDOR were carried out using the labeled ergosterol and the phosphorus atom of a POPC headgroup. Significant REDOR dephasing was observed at the C26/C27 signal of ergosterol in the presence of AmB, but not in the absence of AmB, clearly indicating that the side-chain terminus of ergosterol in the AmB complex comes close to the bilayer surface.



Amphotericin B (AmB, Figure 1) is still the drug of choice for treating deep-seated infections despite the fact that its clinical use over 50 years has been hampered by serious side effects.[1,2] It is generally accepted that AmB forms an ion-permeable channel in cell membrane in the presence of sterol,[3] and its higher affinity to ergosterol-containing fungal membranes than cholesterol-containing mammalian membranes is thought to be responsible for the pharmacological activity.[4] As the model of an AmB ion channel, De Kruijff et al.[3] have originally proposed the well-known barrel-stave assembly, which is comprised of about 8 pairs of AmB and sterol molecules. In order to improve the drug's efficacy and reduce its side effects, AmB/AmB and AmB/sterol interactions in membrane have been extensively investigated by biophysical, chemical, electrophysiological, and spectroscopic methods.[4−8] Recently, to explore the structure−activity relationship of the drug, chemical syntheses of AmB analogues have been achieved by two leading laboratories; Carreira's group successfully synthesized epimerized mycosamine derivatives[9] and demonstrated that an epimer at C2′−OH of AmB methyl ester (AME) shows comparable antimicrobial activity to that of AME while O-methylation of the epimeric C2′−OH significantly reduces the activity. They also synthesized 35-deoxy AME,[10] which was less active to fungi, suggesting that the tail part of AmB was also important for the activity. Meanwhile, Burke's group synthesized AmB derivatives lacking some of functional groups[11,12] for evaluating their effects on the activity. While the carboxylic acid-deficient derivative retained the antifungal activity, the mycosamine-deficient derivative was totally devoid of the activity. These results suggest that interaction between the carboxyl and amino groups on a neighboring pair of AmB molecules is not essential for channel formation, whereas the latter is critical for the activity.

Regarding the sterol selectivity of AmB upon ion channel formation, a great number of studies have been carried out. Ergosterol is known to effectively stabilize a membrane-bound form of AmB as evidenced by AFM imaging,[13] surface pressure measurements,[14] and surface plasmon resonance,[15] consequently leading to the higher affinity of ergosterol to AmB than cholesterol.[16,17] Cohen and co-workers[18,19] have reported that two types of ion channels are formed by AmB in the presence of sterol; transmembrane channels with high conductance are comprised of AmB and sterol while the other channels with low permeability occur without sterol. A recent study by Neumann et al.[20] proposed the possible structure of an AmB−ergosterol bimolecular complex formed in PC membrane on the basis of MD calculations, which is in line with experimental results obtained from ^2H NMR.[21,22] These reported results can largely be accounted for by two major interactions of AmB−sterol occurring in membrane; one is $\pi-\pi$ interaction between the heptaene group of AmB and the diene-containing ring system of ergosterol, and the other is hydrogen bond between the mycosamine moiety of AmB and 3-OH of ergosterol. In these notions, it is assumed as a prerequisite that AmB and ergosterol

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form a barrel-stave assembly in a "head-to-head" (or parallel) manner. These interactions are often discussed in molecular modeling studies.[20,23] However, no direct experimental evidence has yet been obtained for the mode of AmB−sterol interaction. Regarding the stability of the AmB−ergosterol complex, Fournier et al.[24] confirmed it by calorimetric measurements and also the high affinity of saturated phospholipids to AmB. Paquet et al.[25] measured the ^{2}H NMR of deuterated phosphatidylcholine (PC) in hydrated membranes containing AmB and sterol and revealed the complexation of ergosterol with AmB in molecular assemblies. Using solid-state NMR techniques, we have been investigating the molecular basis of AmB−ergosterol complexes formed in hydrated PC bilayers,[22,26−30] which are a more physiologically relevant system as compared with freeze-dried or frozen membrane preparations. Previously, we reported "a surrounding model" for AmB−sterol complex on the basis of the ^{13}C{^{19}F} rotational echo double resonance (REDOR) method for covalently linked AmB−sterol conjugates.[26] Recently, we demonstrated the close proximity between ^{13}C-labeled AmB and ^{19}F-labeled AmB in hydrated POPC−sterol membranes using ^{13}C{^{19}F}REDOR experiments and found that the distance between AmB molecules increases by ca. 2 Å in the presence of ergosterol.[27] More recently, the ^{2}H NMR spectra of deuterated sterols in hydrated lipid bilayers showed that the fast lateral movement of ergosterol is markedly reduced by AmB while cholesterol mobility is virtually unchanged by AmB.[22] Regarding AmB−phosphlipid interactions, REDOR experiments have disclosed the strong affinity between AmB and PC; the single-length channels were predominantly formed in DMPC membrane in either the presence or absence of ergosterol,[28−30] and POPC was found to be incorporated in an AmB−ergosterol complex.[27] In this study we prepared site-specifically ^{13}C-labeled ergosterols and examined their interactions with AmB and POPC by the REDOR technique, aiming to elucidate the mode of interaction between AmB, ergosterol, and PC in hydrated lipid bilayers.

MATERIALS AND METHODS

Materials. AmB and cholesterol were purchased from Nacalai Tesque (Kyoto, Japan). Ergosterol was from Tokyo Kasei (Tokyo, Japan) and palmitoyloleoylphosphatidylcholine (POPC) was from Avanti Polar Lipid Inc. (Alabaster, AL). All other chemicals were obtained from standard venders. 14-F AmB (**2**) was derivatized from AmB as reported previously.[31] ^{13}C ergosterol **3** was prepared by the biosynthetic method;[32] *Saccharomyces cerevisiae* were grown in media containing singly ^{13}C(CH_3)-labeled sodium acetate to produce ergosterol with a skipped ^{13}C-labeling pattern (Figure 1a). The ^{13}C-enrichment ratio was estimated to be 58% by ^{13}C NMR.

Preparation of 26,27-$^{13}C_2$ Ergosterol. Synthetic details of 26,27-$^{13}C_2$ ergosterol are provided as Supporting Information. White powder; R_f 0.45 (2/1-hexane/AcOEt). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.55 (1H, dd, J = 5.58, 2.43 Hz, H-6), 5.38 (1H, dt, J = 5.58, 2.72 Hz, H-7), 5.22 (1H, dd, J = 15.25, 8.16 Hz, H-22), 5.17 (1H, dd, J = 15.25, 7.66 Hz, H-23), 3.64 (1H, m, H-3), 2.43 (1H, dd, J = 2.2, 14.3 Hz, H-4e), 2.25 (1H, d, J = 14.3 Hz, H-4a), 1.04 (3H, d, J = 6.7 Hz, H-21), 0.94 (3H, s, H-19), 0.92 (3H, d, J = 6.87 Hz, H-28), 0.82 (3H, dt, J = 124.4, 6.73 Hz, H-26), 0.83 (3H, dt, J = 124.4, 5.73 Hz, H-27), 0.63 (3H, s, H-18). MS m/z 421.3347 [$(M + Na)^+$; calcd for $C_{26}{}^{13}C_2H_{44}NaO$: 421.3357].

Preparation of Multilamellar Vesicles for Solid-State NMR Measurements. AmB (**1** or **2**), ^{13}C ergosterol (**3** or **4**), and POPC were dissolved in $CHCl_3$/MeOH, and the solvent was evaporated to afford a thin film. After left in vacuo for 8 h, the membrane was hydrated with 31.4 μL of 10 mM HEPES buffer (pH 7.0) and 1 mL of H_2O and dispersed by vortexing and sonication. Then the lipid suspension was freeze−thawed five times to produce large vesicles. The suspension was lyophilized, rehydrated with D_2O (31.4 μL), and packed into a glass tube. The glass tube was sealed with epoxy glue and inserted into a φ 5 mm MAS rotor for CP-MAS/^{13}C{^{19}F} REDOR and a φ 4 mm MAS rotor for ^{13}C{^{31}P} REDOR experiments.

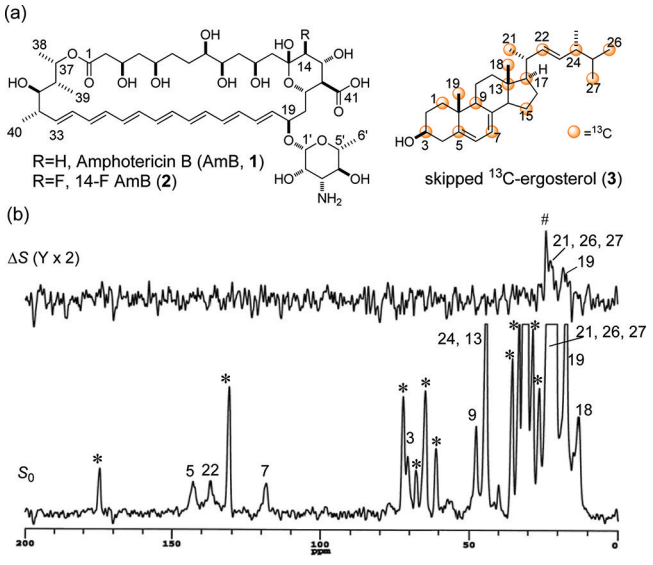

Figure 1. (a) Structures of AmB **1**, 14-F AmB **2** and skipped ^{13}C-labeled ergosterol **3**. (b) ^{13}C{^{19}F}REDOR spectra of **2** and **3** in hydrated POPC bilayers. The spectra were obtained at 38 °C after the ^{19}F-dephasing period of 64 rotor cycles (12.8 ms) with 35 456 scans for each; ΔS is a difference spectrum obtained by subtracting a ^{19}F-irradiated spectrum S from a nonirradiated one, S_0 (full echo). The sample was prepared at the ratio of **2**/**3**/POPC, 1:1:9, in 10 mM HEPES/D_2O (50 wt %) at pH 7.0. Numbers in the spectra denote the carbon atoms of ergosterol. (∗) Signals are due to POPC. (#) Signal is caused by the subtraction of spectra.

Solid-State NMR Measurements. All solid-state NMR spectra were recorded on a CMX300 (Chemagnetics) spectrometer at 75 MHz ^{13}C resonance frequency with the MAS frequency of 5000 ± 2 Hz. The temperature controlling air was maintained at 30 ± 1 °C. The temperature in a sample rotor was measured separately in the essentially same conditions on the basis of ^{31}P NMR chemical shift that is temperature dependent[33] and turned out to be around 38 °C (see Figure S6 in Supporting Information for details). The spectral width was 30 kHz. Typically, the $\pi/2$ pulse width for ^{1}H was 5 μs, and the π pulse widths for ^{13}C, ^{19}F, and ^{31}P were 8.2, 11, and 7.4 μs, respectively. The contact times for cross-polarization transfer were set to be 1.0 ms for ^{13}C{^{19}F}REDOR and 3.0 ms for ^{13}C{^{31}P}REDOR. The REDOR spectra were acquired with a recycle delay of 3 s under TPPM ^{1}H-decoupling[34] with field strength of 63 kHz, and xy-8 phase cycling[35] was used for ^{19}F and ^{31}P irradiation.

RESULTS AND DISCUSSION

^{13}C{^{19}F}REDOR of 14-F AmB and Skipped ^{13}C Ergosterol in Hydrated POPC Membrane. Labeled compounds **2**, **3**, and **4** used in this study are shown in Figure 1a and Scheme 1. 14-F AmB **2** was derivatized from AmB as

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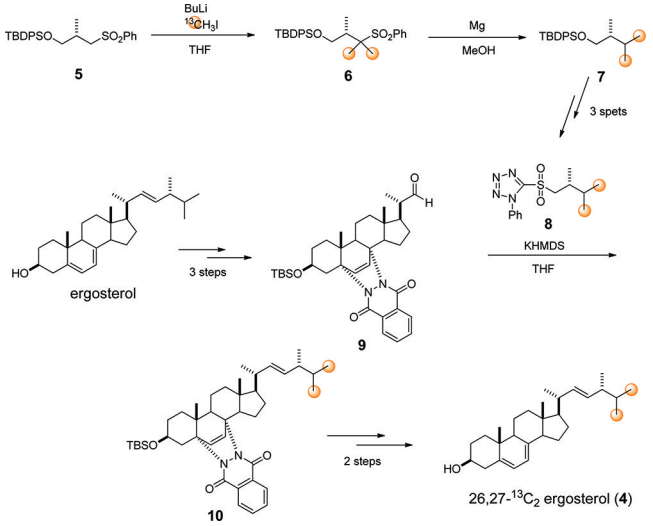
Scheme 1. Preparation of 26,27-$^{13}C_2$ Ergosterol



reported previously;[31] a fluorine atom was stereoselectively introduced to a C13/C14-glycal derivative of AmB to furnish 14-fluorinated AmB **2**. ^{13}C ergosterol **3** was prepared by a biosynthetic method as reported in the literature;[32] the yeast *Saccharomyces cerevisiae* was grown in media containing singly $^{13}CH_3$-labeled sodium acetate, consequently producing ergosterol with a skipped ^{13}C-labeling pattern as shown in Figure 1a. The average ^{13}C-enrichment was estimated to 58% by ^{13}C NMR.

For intermolecular REDOR experiments, the labeled compounds **2** and **3** were mixed in palmitoyloleoylphosphatidylcholine (POPC) at the one-to-one ratio to form hydrated bilayers; in the previous study, we performed the REDOR experiments for measuring AmB−AmB distance at the same ratio and successfully observed the effects of ergosterol on AmB complex formation. Figure 1b shows the full-echo spectrum (S_0) without irradiation at ^{19}F frequency and the difference spectra (ΔS) of $^{13}C\{^{19}F\}$REDOR.[36,37] It is well-known that multiple-labeled compounds such as uniformly ^{13}C-labeled products cause a serious problem in the REDOR experiments since homonuclear ^1J coupling induces heavy phase distortion. However, the ^{13}C-labeled ergosterol **3** with a skipped labeling pattern largely escaped from this problem because the homonuclear scalar coupling over two bonds gives rise to negligibly small values (largely less than 4 Hz).[38] In fact, there was no apparent phase distortion in the REDOR spectra as seen in Figure 1b, clearly demonstrating the merit of the skipped labeling of ergosterol.

In the ΔS spectrum, a significant dephasing effect was observed for an angular methyl carbon of C19 upon irradiation at ^{19}F frequency, indicating that the head groups of AmB and ergosterol come close together to form a "head-to-head" complex. The dephasing effect supports the barrel-stave model, where "head-to-head"-type interaction is considered to be one of the prerequisites.[3] Unexpectedly, the other methyl signals around 24 ppm were also dephased. According to the previous report,[39] these signals are assignable to the terminal methyl groups C26/C27 or C21 in an ergosterol side chain. In the "head-to-head" model, the estimated distances from the ^{19}F-14 of AmB to the C26/C27 dimethyl group and to the C21 methyl group of ergosterol would be 19 and 15 Å, respectively, both of which are beyond the reach of REDOR dephasing effects. As seen in a great number of previous reports,[12,23,40] AmB and ergosterol have been thought to take the "head-to-head" orientation in a membrane-bound assembly. However, the REDOR results strongly suggests that a different mode of bimolecular interaction, most likely "head-to-tail", is partly involved in the AmB−ergosterol complex under the experimental conditions.

Solid-State NMR Measurements Using 26,27-$^{13}C_2$ Ergosterol. The REDOR experiments of the skipped-labeled ergosterol **3** unexpectedly implied a "head-to-tail" interaction. However, heavy overlapping of NMR signals prevented detailed examination of AmB/ergosterol orientation. Thus, to obtain clearer evidence for the possible "head-to-tail" pairing, we synthesized regioselectively 100% ^{13}C-labeled ergosterol at C26/C27 **4** as shown in Scheme 1 (see Supporting Information for synthetic details).

Briefly, chiral sulfone **5** was prepared as reported previously.[41] Introduction of the ^{13}C-dimethyl group was achieved by repeated treatment of sulfone **5** with BuLi and ^{13}C-methyl iodide. Then ^{13}C-labeled sulfone **7** was converted to phenyltetrazole-sulfone (PT-sulfone) **8** by three steps. Construction of the side chain was performed by Julia−Kocienski coupling between the PT-sulfone **8** and aldehyde **9**, which was prepared from ergosterol as reported previously.[42] Removal of protecting groups of the diene portion and the hydroxyl group furnished 26, 27-$^{13}C_2$ ergosterol **4**.

To confirm the chemical shift assignment of C26/C27 in POPC membrane, we first carried out conventional CP-MAS experiments for the labeled ergosterol **4** with the different concentrations of nonlabeled AmB. As shown in Figure 2, in

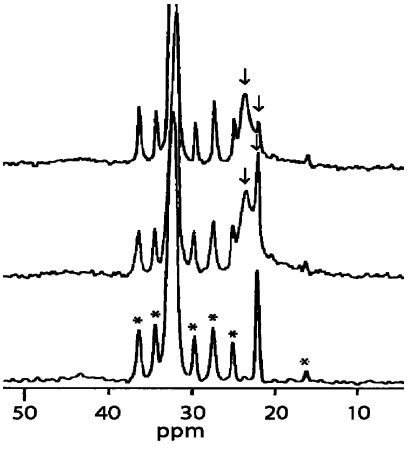


Figure 2. CP-MAS ^{13}C NMR spectra of 26,27-$^{13}C_2$ ergosterol **4** in the presence and absence of AmB in hydrated POPC membrane at 30 °C. The membrane preparation contained **1**/**4**/POPC at the ratios of 2:1:18 (top), 1:1:9 (middle), and 0:1:9 (bottom) in 10 mM HEPES/D_2O (50 wt %) at pH 7.0. Arrows depict signals due to C26/C27 of ergosterol. (*) Signals are due to POPC.

the absence of AmB, the C26/C27 dimethyl group of **4** gave rise to a sharp peak at 21.5 ppm, indicating the high mobility of ergosterol in the membrane. Upon addition of AmB, a broad peak appeared at 23.8 ppm while the signal intensity at 21.5 ppm decreased. Thus, the broad peak was assignable to the dimethyl group of the ergosterol complexed with AmB. We previously reported that AmB forms relatively large aggregates in POPC bilayers, where AmBs were mostly immobilized.[22] Therefore, the broadening of the ergosterol signal can be accounted for by the notion that formation of the AmB−ergosterol complex prevents the fast lateral and rotational diffusion of the sterol in membrane.

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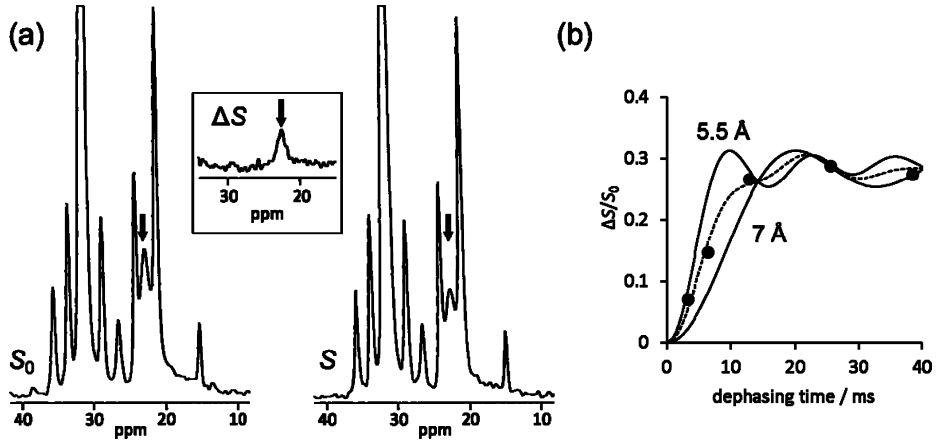

Figure 3. (a) $^{13}C\{^{19}F\}$REDOR spectra of nonirradiated (full-echo) (S_0), ^{19}F-irradiated experiments (S), and difference spectrum (ΔS) for 14-F AmB **2** and 26,27-$^{13}C_2$ ergosterol **4** at the ratio of **2**/**4**/POPC (1:1:9); hydrated POPC bilayers were prepared in 10 mM HEPES/D_2O (50 wt %) at pH 7.0. The data were obtained after ^{19}F dephasing period of 64 rotor cycles (12.8 ms) at 38 °C with 29 488 scans for each experiment. Arrows depict the C26/C27 signals. (b) Experimental $^{13}C\{^{19}F\}$REDOR dephasing values ($\Delta S/S_0$) for the C26/C27 signal at 23.8 ppm (●), and simulation curves (solid lines) for the $^{13}C-^{19}F$ distances of 5.5 and 7.0 Å, assuming that 30% of 26,27-$^{13}C_2$ ergosterol **4** is involved in a "head-to-tail" pairing. The simulation curve (dashed line) derived from two different distances 5.5 Å and 7.0 Å (1:1) nicely fits the experimental data.

Additionally, lower magnetic field shift of the dimethyl signal was probably caused by the close contact of ergosterol with AmB,[43] and this "shifted" chemical shift of C26/C27 agrees well with that of the dephased peak in the REDOR shown in Figure 1b. The residual narrow peak at 21.5 ppm in the presence of excess AmB indicates that a small amount of ergosterol molecules diffuse rapidly in POPC membrane without forming the AmB–ergosterol complex.

$^{13}C\{^{19}F\}$REDOR of 14-F AmB and 26,27-$^{13}C_2$ Ergosterol. In the next step, REDOR spectra were recorded with 14-F AmB **2** and 26,27-$^{13}C_2$ ergosterol **4**. Figure 3a shows the partial nonirradiated (S_0) and ^{19}F-irradiated spectra (S) of the $^{13}C\{^{19}F\}$-REDOR experiments, where **2** and **4** were mixed at 1:1 ratio in hydrated POPC bilayers. As in the case of the CP-MAS spectra, two peaks were observed at 21.5 ppm (sharp) and 23.8 ppm (broad), which were assigned as the C26/C27 dimethyl group of free and AmB–complexed ergosterol, respectively; lower intensity of the broad peak at 23.8 ppm as compared with that in CP-MAS (the middle spectrum of Figure 2) is probably due to greater relaxation rate (low mobility) of the broader signal during a long dephasing time. As expected, significant REDOR dephasing ($\Delta S/S_0$ = 27%) was observed for the broad peak while the intensity of the sharp peak at 21.5 ppm was unchanged. This dephasing effect further reveals that the ^{13}C atoms in the ergosterol side chain comes close to the ^{19}F atom of the AmB headgroup; e.g., the "head-to-tail" binary interaction between AmB and ergosterol occurs in a hydrated POPC membrane. Figure 3b is a plot of the experimental REDOR dephasing values ($\Delta S/S_0$) for the C26/C27 peak. The dephasing effect is saturated around 30%, which probably indicates that ergosterol molecules partly interact with AmB in a "head-to-tail" manner, and the remaining molecules largely form "head-to-head" complexes as discussed later.

The interatomic distance between ^{19}F-14 and $^{13}C26/^{13}C27$ was estimated by fitting the REDOR plots to theoretical curves[44] on the basis of the following assumptions. First, there is only one type of the AmB–ergosterol complex in head-to-tail pairing that can be treated as a sum of 2 spin systems, ^{19}F-14/$^{13}C26$ and ^{19}F-14/$^{13}C27$; the dephasing effect of the other ^{13}C spin on an observing ^{13}C is negligibly small in most cases, and the influence of ^{19}F-14 of the other AmB molecules in the assembly on the C26/C27 signal should not be significant as suggested by the possible intermolecular distances between AmB and ergosterol (see Figure S7 in Supporting Information). Second, the maximum REDOR dephasing was 30% due to the co-occurrence of "head-to-head" pairing. Lastly, molecular motion including exchanges of AmB–sterol pairing does not significantly influence the dephasing effect; the REDOR at −10 °C gave rise to similar $\Delta S/S_0$ values to those at 38 °C (see Figure S3). As a result, the distances between F-14 and C26/C27 can be estimated to be 5.5 and 7.0 Å, where the values are interchangeable (Figure 3b). In realistic systems, multiple complexes with different $^{13}C-^{19}F$ distances, beside those with head-to-head paring, should be involved, which complicate the interpretation of the REDOR data and frequently cause errors in distance estimation. Nevertheless, the present results clearly reveal that AmB forms a stable complex in membrane with a close proximity between the headgroup of AmB and the side chain of the sterol. We previously reported that AmB–AmB distance was about 12 Å in ergosterol-containing POPC membrane,[27] which was much longer than the F14–C26/C27 distance. This may imply that ergosterol in the "head-to-tail" complex is inserted between AmB molecules.

It has so far been generally believed that the membrane-bound AmB assembly takes a "head-to-head" orientation.[8,40] Previous papers, therefore, have discussed a structural model on the basis of the "head-to-head" configuration for AmB–AmB and AmB–sterol. However, the present results disclosed for the first time the co-occurrence of "head-to-head" and "head-to-tail" interactions for AmB–ergosterol pairing in membrane. Thus, the broad peak at 23.8 ppm is assumed to be the overlapping C26/C27 signals derived from "head-to-head" and "head-to-tail" orientations; as shown in Figure S4 of the Supporting Information, the peak shape of S is somewhat different from that of S_0, which suggested the signal shape and/or chemical shift of the C26/C27 signal are slightly different between the two orientations because the subtraction of the portion due to "head-to-tail" (ΔS) interaction from the overlapping signal increases the ratio of the signal due to "head-to-head" paring (S). Another important feature of the assembly is the orientation of a side chain of ergosterol with respect to the alicyclic molecular stem. It has been reported that the conformation of the ergosterol side chain is highly restricted

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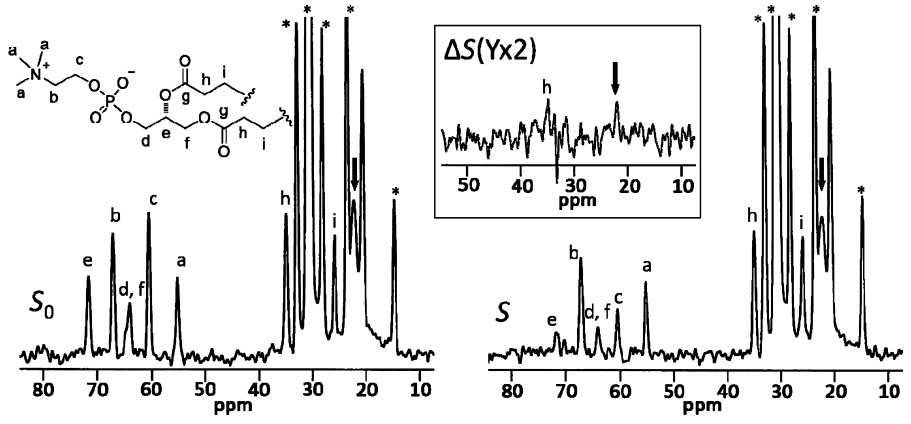

Figure 4. $^{13}C\{^{31}P\}$REDOR spectra of nonirradiated (full-echo) (S_0) and ^{31}P-irradiated experiments (S) with ΔS (inset) in the presence of AmB for 26, 27-$^{13}C_2$ ergosterol **4** and POPC at the ratio of AmB/**4**/POPC (1:0.5:9); hydrated POPC bilayers were prepared in 10 mM HEPES/D_2O (50 wt %) at pH 7.0. The data were obtained after the ^{31}P dephasing period of 160 rotor cycles (32 ms) at 38 °C with 41 424 scans for each experiment. Arrows depict C26/C27 broad signals, where 16% reduction of the peak area was observed upon irradiation at ^{31}P frequency. Small alphabets denote the ^{13}C signals of POPC headgroup. (∗) ^{13}C signals are those of POPC acyl chains.

to take an extended conformation,[45] ruling out the possibility that the sterol takes a U-shaped conformation upon complexation with AmB. It is, therefore, highly plausible that the side chain takes an extended conformation in both "head-to-head" and "head-to-tail" pairings.

$^{13}C\{^{31}P\}$REDOR for Interaction between 26,27-$^{13}C_2$ Ergosterol and Phosphodiester of POPC. To examine the mode of ergosterol/POPC interaction in the AmB complex, we next performed intermolecular $^{13}C\{^{31}P\}$REDOR experiments. Figure 4 shows the nonirradiated (S_0) and ^{31}P-irradiatred spectra (S) of the $^{13}C\{^{31}P\}$REDOR experiments where 26,27-$^{13}C_2$ ergosterol **4** was incorporated in AmB-containing POPC membrane. The two peaks due to the terminal dimethyl group were again observed at 21.5 ppm (sharp) and 23.8 ppm (broad), corresponding to free and bound ergosterol, respectively. The S spectrum reveals the intermolecular dephasing effect ($\Delta S/S_0$ = 16%) on the broad dimethyl signal from the POPC headgroup, whereas the acyl-chain carbons showing virtually no dephasing (detailed REDOR dephasing data are provided as Supporting Information).

This result unambiguously demonstrated that the tail group of AmB-bound ergosterol comes close to the phosphodiester of a POPC headgroup. Generally, short lifetime of phospholipid–sterol pairing markedly attenuate the magnitude of intermolecular dipolar coupling detected by solid-state NMR. However, this result suggests that ergosterol/POPC interaction in an AmB assembly is relatively stable, which agrees with our previous results on an importance of AmB–ergosterol–POPC assemblies for active ion-channel formation.[24] We also carried out a similar $^{13}C\{^{31}P\}$REDOR experiment with AmB-free membrane. However, no REDOR dephasing was observed for the sharp C26/C27 signal (date is not shown).

On the basis of these experimental data together with previous experimental results,[22,27] we attempt to propose possible intermolecular interactions occurring in an AmB/ergosterol/PC complex as depicted in Figure 5. Although it is difficult at the moment to present an accurate image for the complex from only a few distance constraints, the hydrogen bond between 35-OH (or 1-COOR) of AmB and 3-OH of ergosterol may possibly stabilize the "head-to-tail" orientation (Figure 5b). Additionally, heptaene–diene π–π interactions may also contribute the "head-to-tail" orientation as is the case with the "head-to-head". The interaction between ergosterol and POPC is stabilized in the complex (Figure 5c), where the

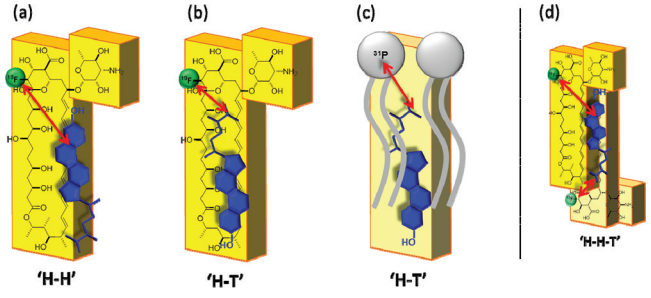

Figure 5. Putative model for bimolecular interactions occurring in an AmB–ergosterol–PC complex. AmB (yellow boxes) bound to the bilayers interacts with ergosterol (blue) in both "head-to-head" (a) and "head-to-tail" (b). The terminus of an ergosterol side chain comes close to the head groups of POPC (silver) in the presence of AmB (c). Red arrows point to the pair of labeled atoms that gave rise to REDOR dephasing. (d) A hypothetical model for one ergosterol interacting with two molecules of AmB in both "head-to-head" and "head-to-tail" pairings.

pairing of the sterol and POPC is in a head-to-tail manner. Recently, a couple of reports suggest a possibility of "head-to-tail" orientation for AmB/AmB[46,47] under more physiologically relevant conditions. Provided that this antiparallel interaction could occur in the model membrane used in this study, one ergosterol molecule might interact with two molecules of AmB in both "head-to-head" and "head-to-tail" pairings (Figure 5d). To examine these intermolecular interactions in more details, preparation of ^{19}F-labeled AmBs at a different position is currently under way; these ^{19}F-labeled AmBs would facilitate a CODEX experiment, which is an excellent methodology to determine the number of monomers for one molecular assembly residing in membrane as reported by Schmidt-Rohr et al.[48] and Hong et al.[49]

The conditions of the present experiments are different from the physiological environment in which AmB exerts its pharmacological activity. In particular, the concentration of the drug is thought to be below 1 μM in human serum upon intravenous administration. AmB content in membrane in our experiments was much greater than the pharmacological relevant doses chiefly due to a low sensitivity of solid-state NMR. Nevertheless, we

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believe that the present results obtained with a higher AmB content can help understand the drug's activity under the physiological environment by the following reasons. The previous REDOR results for AmB/ergosterol-containing POPC membranes[27] indicate that AmB−AmB distance is significantly increased by complexation with ergosterol and POPC; in a separate experiment of ^{2}H NMR, we demonstrated that ergosterol interacts directly with AmB under the essentially same conditions as those of the present study.[22] The results also reveal that AmB molecules are mixed well with the lipids in membrane preparations used for NMR measurements. Meanwhile, we examined the interactions at much lower concentrations of AmB by surface plasmon resonance[15] and disclosed that ergosterol markedly enhances the affinity of AmB to POPC membrane but cholesterol does not, suggesting that the drug is merged in the membrane and then form complexes with ergosterol and POPC even at very low concentrations. These two sets of experimental data obtained with high and low concentrations of AmB imply that, on the association−dissociation equilibrium, AmB molecules in membrane form a similar complex with ergosterol and POPC regardless of the concentrations of the drug. At higher doses, the equilibrium should shift to the complexation from a monomeric form that should be predominant at lower concentrations. In fungus membranes, ergosterol occurs in both the outer and inner leaflets of bilayers, thus inferring that AmB interacts with the sterol in both head-to-head and head-to-tail pairings in micromolar concentrations. The notions imply that a mode of the ternary complexation could be reproduced at higher concentrations of NMR experiments to a certain extent. In particular, in ergosterol-containing PC membranes, the ternary interaction between AmB, ergosterol, and PC, which appears to be robust but somewhat flexible,[27] should be a major force for organizing an ion permeable assembly.

In conclusion, we examined AmB−ergosterol and ergosterol−POPC bimolecular interactions in hydrated POPC bilayers by solid-state NMR using ^{13}C-labled ergosterol and ^{19}F-labeled AmB. The vicinity between the 14-F of AmB and the 26,27-dimethyl group of ergosterol in the membrane detected as an REDOR dephasing effect indicates that "head-to-tail" orientation occurs in an AmB−ergosterol complex. ^{13}C $\{^{31}P\}$ REDOR experiments disclosed that the terminal dimethyl group of ergosterol comes close to membrane surface upon complexation with AmB. These findings should provide new insight into bimolecular interactions necessary for formation of an AmB channel assembly.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details of preparation of 26, 27-$^{13}C_2$ ergosterol, CP-MAS spectra of the AmB−ergosterol−POPC preparation at variable temperatures, additional $^{13}C\{^{19}F\}$ REDOR data at −10 °C, $^{13}C\{^{31}P\}$ REDOR dephasing effects, the temperature-dependent change of ^{31}P chemical shift, and the calculated REDOR curves for F−C−F three spin systems. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AmB, amphotericin B; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MAS, magic angle sample spinning; NMR, nuclear magnetic resonance; REDOR, rotational echo double resonance.

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