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Self-Assembled Amphotericin B Is Probably Surrounded by Ergosterol: Bimolecular Interactions as Evidenced by Solid-State NMR and CD Spectra

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Abstract: Amphotericin B (AmB) is thought to exert its pharmacological effects by forming a barrel-stave assembly with ergosterol in fungal membranes. To examine the interaction between AmB and ergosterol (Erg) or cholesterol (Cho), ^{13}C- and ^{19}F-labelled covalent conjugates were prepared as reported previously (N. Matsumori et al. *Chem. Biol.* **2004**, *11*, 673–679). The CD spectra of the conjugates in a membrane-bound form suggested that the distance between the heptaene moieties of the ergosterol conjugates AmB–C_2–(6-F)Erg **2** and AmB–C_2–Erg **3** is similar to that of AmB in ergosterol-containing membranes, but significantly larger than that of AmB in nonsterol or cholesterol-containing membranes. These observations suggest that, as is the case with ergosterol-containing membranes, the conjugated sterol moiety prevents the close contact between the heptaene moieties within the membrane that would reduce channel conductivity of the AmB assemblies. To further investigate this bimolecular interaction, we recorded the solid-state NMR spectra of conjugates **2** and AmB–C_2–(6-F)Cho **4**, which are composed of uniformly ^{13}C-labelled AmB and 6-fluorinated ergosterol or cholesterol; the conjugates were expected to facilitate the estimation of distances between the fluorine and carbon atoms. By using rotor-synchronous double resonance (rotational echo double resonance of X cluster; RDX) experiments, we deduced the distance between the fluorine atom and its nearest carbon atom in the heptaene moiety of **2** to be less than 8.6 Å. This indicates that the B ring of ergosterol comes close to the AmB polyene moiety. A conformational search of the AmB–ergosterol conjugate by using distance constraints derived from the RDX results suggested that ergosterol molecules possibly surround the AmB assembly, which is in contrast with the conventional image in which ergosterol is inserted into AmB molecules.

Keywords: amphotericin B · antibiotics · ion channels · self-assembly · solid-state NMR spectroscopy

Introduction

Interactions between drugs and membrane-bound biomolecules often provide fundamental information that is essential for understanding the drug's mechanism of action. The structural bases underlying these interactions, however, remain largely unknown owing to a lack of appropriate characterisation methodologies. Powerful structural-biology techniques such as X-ray crystallography and solution-state NMR spectroscopy are difficult to apply to membrane systems owing to their poor crystalline nature and highly anisotropic nature. Membrane-active agents such as antifungal antibiotics and antimicrobial peptides often exert their effects by interacting with lipids and increasing the permeability of microbial plasma membranes. Amphotericin B (AmB, **1**) may be the best-known membrane-active agent as it has been the drug of choice for treating deep-seated systemic fungal infections for nearly 50 years.[1] Its pharmacological action is attributed largely to the formation of an ion channel that is believed to be a "barrel-stave" complex that spans the phosphatidylcholine (PC) bilayer.[2,3] The selective toxicity of AmB to fungi compared with mammalian cells is thought to be due to its higher affinity for ergosterol, an abundant sterol in fungal membranes, than for cholesterol, the major sterol in mammalian membranes.[4,5] However, despite extensive investigations into the structure of this membrane assembly by spectroscopic[6,7] and computational[8–11] methods, details of its molecular architecture remain unclear.

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Knowledge of the bimolecular interactions occurring in membranes between AmB–AmB,[12,13] AmB–PC[14–17] and AmB–sterol[18–20] is essential for better understanding of the structural basis by which AmB induces membrane permeability. The rapid dissociation–association equilibria between AmB–PC and AmB–sterol, however, make it extremely difficult to directly observe these interactions. As membrane sterols are known to stabilise the channel assembly, covalent conjugation of sterol with AmB should further stabilise the assembly and thus facilitate elucidation of their interactions by shifting the equilibrium towards the association state.[12] Based on this hypothesis, we prepared AmB–sterol covalent conjugates (**3** and **5**) in which the amino group of AmB was connected to the hydroxy group of the sterol and demonstrated that the AmB–sterol interaction could be reproduced with these conjugates.[18]

Recently, solid-state NMR spectroscopy has been widely used for the structural elucidation of membrane-bound entities.[21–23] Rotor-synchronous methods such as rotational echo double resonance (REDOR)[24] and rotational resonance[25] are potentially powerful tools for accurately measuring interatomic distances in membrane environments. Compared with other NMR-active nuclei, ^{19}F has distinct advantages owing to its 1/2 nuclear spin, high gyromagnetic ratio, 100% natural abundance and low background signal in biological systems.[26] Thus, ^{13}C and ^{19}F double-isotope enrichment of membrane-bound molecules allows interatomic distances as large as 10 Å to be measured in biological systems.[27] However, these methodologies have primarily been applied to integral membrane polypeptides, leaving their application to nonpeptidic compounds virtually unexplored. Thus, we were interested in examining the utility of these techniques for studying AmB–membrane complexes.

CD spectra of AmB have been extensively examined to deduce the interactions between AmB–AmB and AmB–lipid in membranes. CD measurements have provided important findings: Vertut-Croquin et al.[7] reported that ergosterol-containing membranes give rise to CD features that are different from those observed from cholesterol-containing membranes, and Fujii et al.[6] proposed that the typical Cotton effect at shorter wavelengths is ascribable to AmB assemblies. In addition, Ernst and Grange[28] reported that this Cotton effect is probably caused by the proximity of the heptaene groups of AmB and that the distance is even smaller in cholesterol-containing or sterol-free membranes than in ergosterol membranes in which AmB molecules are arranged in the barrel-stave assembly. In this study, we measured the CD spectra of AmB–sterol conjugates to examine the effects of the sterol on bimolecular interaction between AmB molecules and recorded the solid-state NMR spectra of ^{13}C- and ^{19}F-double-labelled conjugates to deduce their conformation in membranes. The results support a new model that could account for the sterol-dependent formation of AmB assemblies in biomembranes.

Results

Design and preparation of ^{13}C- and ^{19}F-double-labelled covalent conjugates of AmB and sterol: Conjugation of AmB with sterol was carried out by a reductive N-alkylation reaction to furnish **2–5** (Figure 1).[18] As this linkage method retains ion-channel activities and sterol selectivity of the conjugates,[18] conjugates **3** and **5** were used for CD measurements and double-labelled **2** and **4** were subjected to solid-state NMR spectroscopic experiments. To prepare **2** and **4**, 6-fluoroergosterol and 6-fluorocholesterol[29] were derivatised from the parent sterols, and uniformly ^{13}C-labelled AmB (50% isotope enrichment) was obtained by growing *Streptomyces nodosus* on ^{13}C-enriched medium as reported previously.[17,30] The C6 position in the sterol B ring was chosen as the fluorination site as this rigid aliphatic ring was expected to interact with the polyene moiety of AmB. Furthermore, 6-fluorocholesterol has been reported to exhibit intramembrane spatial occupancy that is nearly identical to that of cholesterol in PC membranes.[31,32] In our experiments with 6-fluoroergosterol, a relatively small effect arising from the fluorine substitution was demonstrated; although 6-fluoroergosterol significantly enhanced the membrane-permeability activity of AmB, its efficacy was somewhat reduced as compared with ergosterol (see the Supporting Information).

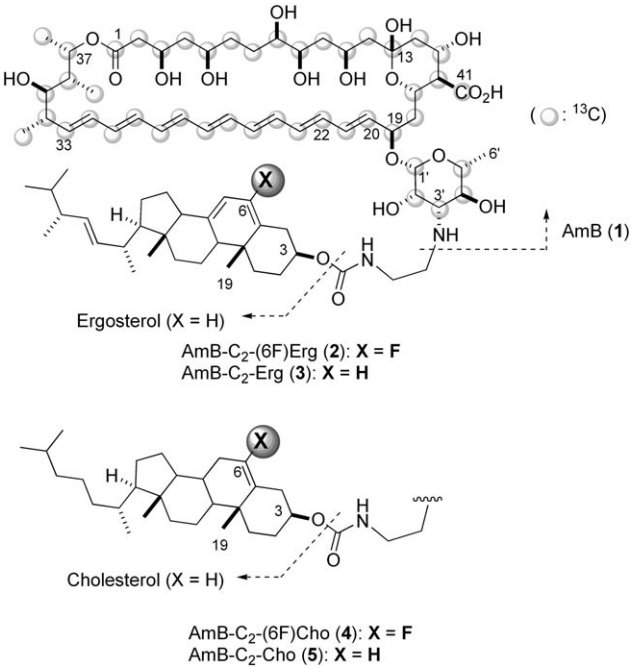

Figure 1. Structures of amphotericin B–sterol covalent conjugates **2–5**. Cho = cholesterol, Erg = ergosterol.

Membrane-permeability activity of AmB–sterol covalent conjugates 2–5: To evaluate the effect of the fluorine substitution on membrane-permeability activity, the K^+ ion flux

activities of conjugates **2** and **4** were measured by using egg phosphatidylcholine (EPC) liposomes.[33] We previously reported that nonfluorinated AmB–ergosterol conjugate **3** exhibits a higher activity than its cholesterol counterpart **5** in liposome assays.[18] In the present experiments, fluorine-labelled conjugate **2** showed a somewhat lower efficacy than nonfluorinated conjugate **3**; at 3.6 μM, amphotericin B, conjugate **2** and conjugate **4** induced 32%, 50% and 49% permeabilisation of liposomes, respectively, whereas **3** induced 80% permeabilisation at the same concentration (see the Supporting Information for details).

CD spectra of AmB–sterol conjugates and AmB in the presence of ergosterol: The CD spectra of the conjugates were obtained to examine the interaction of membrane-bound AmB molecules. The spectra of **2** and **3** in EPC membranes were essentially identical (Figure 2), indicating that fluorine-

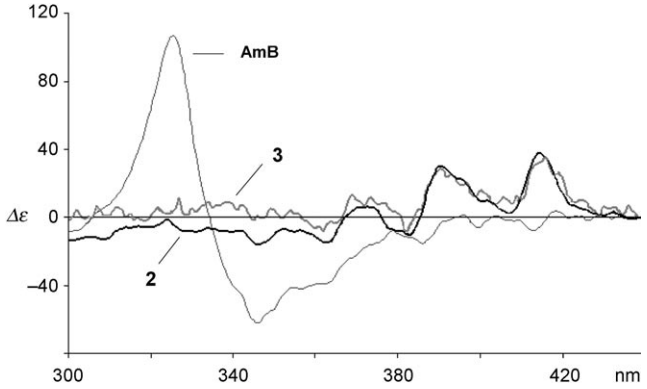

Figure 2. CD spectra of AmB–Erg covalent conjugates in EPC membranes prepared by the mixed-with-lipid method.[19] The AmB (or conjugate) to PC ratio was 10^{-3}:1 and the concentration of PC was 5 mM.

substitution at C6 of ergosterol had little influence on the AmB–AmB interactions in membranes, particularly on the orientation of the heptaene chromophores. A strong split Cotton effect centred at 335 nm was observed for AmB bound to EPC membranes, whereas no corresponding peak was found at 335 nm in the spectra of **2** and **3** (Figure 2). The CD spectrum of AmB in cholesterol-containing liposomes showed split peaks similar to that observed with EPC, whereas the spectrum of AmB in ergosterol-containing liposomes (Figure 3) lacked these split peaks.

The CD spectra of **2** and **3** exhibited two positive peaks and one split peak at 414, 390 and 373 nm, respectively, whereas two negative peaks and one split peak were observed in the spectrum of AmB in a 10% ergosterol-containing membrane (Figure 3). These observed differences may be due to the sterol contents of the membrane as the CD sign at these wavelengths is known to depend on the concentrations of ergosterol or AmB in PC.[7]

Solid-state NMR spectroscopic measurements of AmB–sterol conjugates 2 and 4: To gain structural information on

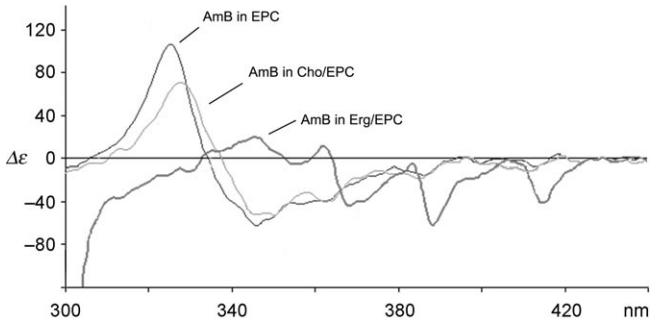

Figure 3. CD spectra of AmB in EPC membrane containing 10% ergosterol or 10% cholesterol prepared by the mixed-with-lipid method.[19] The AmB to lipids (PC plus sterol) ratio was 10^{-3}:1 and the concentration of lipids was 5 mM. The negative peak at around 300 nm for AmB in Erg–EPC is due to ergosterol.

AmB–sterol interaction in membranes, we next attempted to record the solid-state NMR spectra of labelled conjugates **2** and **4**. Application of the original REDOR methodology[24] to ^{13}C-multiple-labelled conjugates is generally difficult as NMR signals are distorted by ^{13}C homonuclear spin–spin coupling. Recently, a modified methodology called RDX (REDOR of X cluster) has been reported by Mehta and Schaefer[36] in which homonuclear interactions are suppressed by a combination of Hahn echo and solid echo. We adopted this technique for estimating the ^{13}C–^{19}F distances of the ergosterol conjugate **2** in dimyristoyl–PC (DMPC) membranes (Figure 4). The difference spectrum revealed

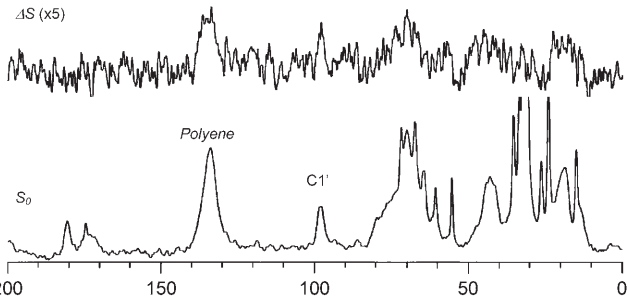

Figure 4. $^{13}C\{^{19}F\}$ RDX spectra of [U-^{13}C]AmB–C_2–(6-F)Erg **2** in a DMPC membrane. The membrane preparation contained conjugate **2** and DMPC at a molar ratio of 1:10 and 10 mM HEPES/D_2O buffer solution (50% wt; pH 7.0). The spectra were obtained after 40 rotor cycles of ^{19}F dephasing (8 ms) with magic-angle spinning at 5 kHz, 30°C. The number of scans was 32896. The top trace is the RDX difference spectrum, ΔS.

significant dephasing effects for the polyene moiety of AmB, indicating its proximity to the B ring of the ergosterol portion. Integration of the signal in the full echo spectrum (S_0) was reduced by 12% ($\Delta S/S_0 = 0.12$) upon fluorine irradiation. Prominent reduction of the signal ($\Delta S/S_0 = 0.15$) was also observed for C1′ of the mycosamine moiety (Figure 4). A diluted membrane preparation comprised of a 1:1 mixture of labelled conjugate **2** and nonlabelled conjugate **3** in

DMPC showed similar dephasing effects, whereas a nonconjugated pair of uniformly ^{13}C-labelled AmB and 6-fluoroergosterol produced virtually no dephasing (see the Supporting Information). These observations indicate that the dephasing effects of the polyene moiety are largely derived from intramolecular ^{13}C–^{19}F dipolar interactions. In the case of the (6-F)Cho conjugate **4**, the $\Delta S/S_0$ value was similar to that of the (6-F)Erg congener (see the Supporting Information), suggesting that, as with the membrane-permeability activity, the sterol selectivity of AmB may be attributable to a small preference for ergosterol over cholesterol, which is easily compensated for by the covalent linkage and fluorine substitution.[20]

Measuring accurate ^{13}C–^{19}F distances for **2** from the RDX spectra was extremely difficult because the polyene peak was comprised of multiple-spin systems with overlapping ^{13}C signals and the biosynthetic ^{13}C-labelled AmB was a mixture of many isotopomers labelled at different sites. Nevertheless, we assumed that the maximum possible ^{13}C–^{19}F distance can be determined from the $\Delta S/S_0$ values. Total dephasing and refocusing effects in RDX experiments are known to be significantly attenuated when compared with those in normal REDOR experiments,[37] indicating that a ^{13}C–^{19}F distance calculated from RDX data with an REDOR dephasing curve[24] will be larger than the actual distance. According to conventional REDOR calculations,[37] a $\Delta S/S_0$ value of 12% corresponds to a ^{13}C–^{19}F distance of 8.6 Å, indicating that the interatomic distance between the ^{19}F atom and its nearest ^{13}C atom in the polyene moiety should be less than 8.6 Å; if smaller dephasing effects in RDX[36] are taken into account, the maximum distance would be 7.0–7.5 Å (see the Supporting Information for details). Thus, the actual distance between the fluorine atom and one of the polyene carbon atoms is likely to be much smaller than 8.6 Å. In a similar manner, the interatomic distance between the fluorine and the C1′ of mycosamine was estimated to be less than 8.3 Å. These distance constraints were utilised in conformational-search calculations.

Conformational search: By using the distance constraint (<8.6 Å) between the fluorine atom at C6′ of the ergosterol moiety and the nearest carbon atom in the heptaene chain, fourteen C–C bonds, which encompass the mycosamine moiety, alkylcarbamate linker and the side chain of the ergosterol, were allowed to rotate during the conformational search. By using the Macromodel program, we obtained the 116 minimum-energy conformations within 5 kcal mol^{-1} from the ground minimum; without the constraint, diverse conformers were obtained, most of which lacked interaction between the ergosterol alicycles and the AmB heptaene. The conformers thus obtained were first screened for gross structural features that were relevant to membrane integral assemblies such as the parallel molecular axes for the AmB and sterol moieties. Five conformers **a**, **b**, **x**, **y** and **z** were selected for further analysis (Figure 5B). Conformers **y** and **z** were subsequently eliminated owing to ^{13}C–^{19}F dephasing effects observed for the C1′ of the mycosamine moiety ($\Delta S/S_0$ is approximately 15%), indicating that the C1′–F distance is likely to be less than 8.3 Å. In addition, segregation of the polyhydroxy portion from the hydrophobic ergosterol moieties, which is necessary for stabilising a membrane-integral assembly, is not sufficiently attained in conformer **x**. Ultimately, therefore, two conformers, **a** and **b** in Figure 5A, remain as plausible stereostructures (for details, see the Supporting Information).

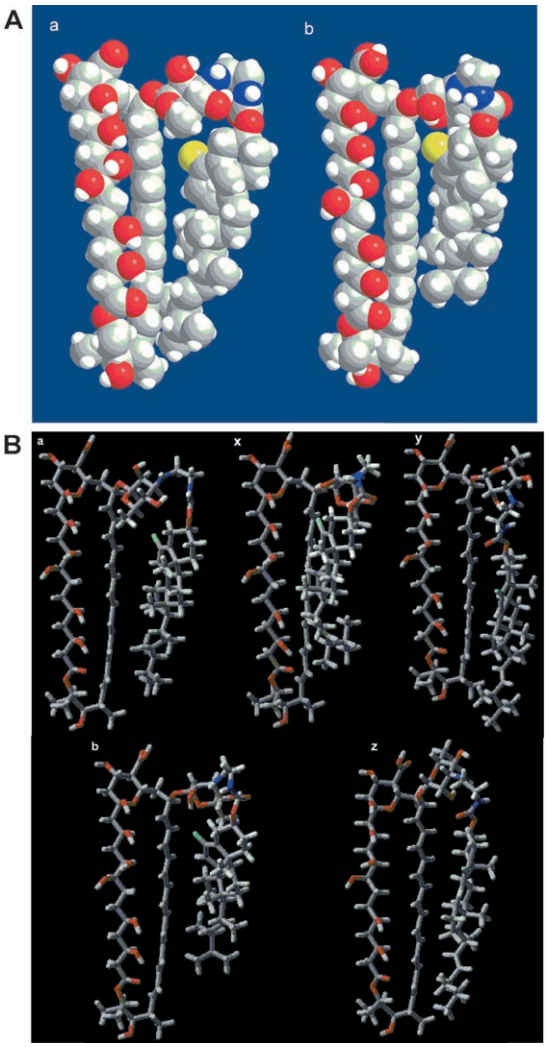

Figure 5. Minimum-energy conformations of AmB–C_2–(6-F)Erg **2**. A total of 116 conformers were generated by a conformational search (Macromodel 8.6)[40] by using the distance constraints between the heptaene group and the fluorine site derived from the RDX spectra. The conformers were first screened for gross structural features relevant to membrane assembly, such as parallel molecular axes of the AmB and sterol moieties. REDOR results provided the five candidates (**a**, **x**, **y**, **b** and **z**; in the order of their calculated stability). These structures were further examined, leading to the elimination of some candidates and leaving **a** and **b** as the more plausible conformers (space-filling models in (A)). Atom colours are white: hydrogen, grey: carbon, red: oxygen, blue: nitrogen, yellow: fluorine.

Discussion

The membrane-permeability potency of fluoroergosterol conjugate **2** is lower than that of nonfluorinated **3**. This may be due to the effect of fluorine substitution on the electron density of the ergosterol diene group as evidenced by the grossly deshielded ^{13}C chemical shifts of the corresponding carbon atoms in **2** (C5 113.1; C6 151.9; C7 112.6; C8 145.1). As 7,8-dehydrocholesterol has a higher affinity for AmB than does cholesterol,[34] the electronic state of the sterol B ring may play a more important role in AmB–sterol interactions than does the sterol side chain. Electrophysiological studies have revealed that the mean open time of AmB channels is increased by ergosterol, whereas their conductance is not greatly affected by the sterol species,[35] suggesting that a similar-sized channel is formed by AmB regardless of sterol variation. Single-channel current recordings for AmB–sterol conjugates **3** and **5** provided essentially the same results.[18] These findings indicate that the structures of the ion-channel assemblies formed by **3** and **5** are similar but that their stabilities are different. The CD spectra of fluorinated conjugate **2** was essentially the same as that of **3** (Figure 2), suggesting that both **2** and **3** form similar assemblies in PC membranes.

The present results, together with previous studies, provide insights into the bimolecular interactions that occur between AmB and ergosterol in membrane-bound assemblies. The split Cotton effect centred at 335 nm, which was not observed in the spectra of AmB–ergosterol conjugates **2** and **3**, is caused by very close interactions between the heptaene moieties of AmB.[6,28] Ernst et al.[28] reported that the distance between the heptaene groups should be less than 6 Å to give rise to this Cotton effect; the distance between neighbouring polyene groups within a barrel-stave assembly may be too large to produce this strong Cotton effect.[28] The CD spectrum of AmB in ergosterol-containing liposomes reveals no such split peaks (Figure 3).[7] In contrast, the spectrum of AmB in cholesterol-containing or pure PC liposomes, in which the membrane-permeability activity of AmB is much less efficacious than that in ergosterol-containing membranes, showed this prominent Cotton effect (Figure 3). These observations suggest that the close proximity of the heptaene moieties is due to the nearly direct contact of π planes, which is impossible within a "barrel-stave" assembly but is feasible at the interface between membrane-integrated assemblies or aggregates.[28] The present and previous results indicate that AmB activity is reduced when this CD effect becomes large. Close heptaene interactions in the absence of ergosterol, therefore, hamper formation of ion-channel assemblies; that is, ergosterol probably prevents close contact between AmB molecules at the heptaene moiety.

The possible conformations of AmB–Erg conjugate **2**, which were calculated by using the distance constraints obtained from solid-state NMR spectroscopy, show that the π plane of the AmB heptaene resides close to the ergosterol ring system (Figure 5A). These configurations of the sterol moiety may be compatible with prevention of the close proximity of two heptaene groups between the AmB assemblies. Ergosterol is believed to stabilise a molecular assembly by inserting its rings between two AmB molecules.[2] Baginski et al.[9] carried out simulation studies on an AmB–ergosterol–PC complex and evaluated sterol functions with respect to interatomic distances. When the distance between C9 in the polyhydroxy chain of AmB and C19 of ergosterol agrees within 2 Å with the distance between C22 in the heptaene chain of AmB and the C19 of ergosterol, the lactone ring of AmB is nearly parallel to the ring system of ergosterol (and is inserted between AmB molecules). When the distance difference exceeds 2 Å, these two ring systems are not parallel (not inserted).[9] The differences in these interatomic distances in conformers **a** and **b** in Figure 5 are 2.35 and 2.36 Å, respectively, which rules out the insertion of the ergosterol rings into the AmB macrolactones. In these conformers, the angular methyl side (the β face) of the ergosterol moiety is directed to the heptaene π plane, whereas conformers **y** and **z** orient the other side (the α face) to the π plane. The latter two conformers are less plausible because the fluorine site is too far away from the mycosamine moiety to give rise to the dipole interaction observed for C1′ in the RDX experiment. In addition, mycosamine orientation is known to play a major role in enhancing the binding of AmB molecules for ion-channel formation.[8–11,20] Conformer **b** (but not **a**) satisfies the requirements for glycosidic φ-ψ angles and, among the conformers in Figure 5B, **b** is considered to be the most appropriate for producing the required bimolecular interactions in a conductive channel (see the Supporting Information). The AmB–sterol complex may represent one of the stable conformers that occurs in dynamic conformational changes, however, this must be confirmed by further experiments. Nevertheless, the proximity of ergosterol to AmB that is observed in conformer **b** could reproduce the intermolecular recognition of the sterol ring system by the polyene group of AmB in membranes.

Based on these considerations, we propose a new model for the AmB–ergosterol complex, as is depicted in Figure 6. In contrast with the conventional model in which ergosterols are inserted into AmB molecules,[2,9] in the new model, the sterol molecules surround the AmB assembly. Unlike cholesterol, erogsterol is thought to directly interact with AmB in membranes.[9,28,38] Recently, Silva et al. reported[39] that a close relative of AmB, nystatin, efficiently forms a compact ion-conducting oligomer only in the presence of ergosterol. The present results suggest that AmB–ergosterol interactions are important in stabilising conductive ion channels but that the sterol may not be firmly integrated into the channel assembly. The primary role of the sterol in the antibiotic action of AmB may be to prevent the formation of nonconducting aggregates by AmB, some of which give rise to the observed strong split CD peaks. The relatively weak affinity of ergosterol for AmB is evidenced by the solid-state NMR spectroscopy results; dipolar interactions between (6-F)ergosterol and ^{13}C-labelled AmB, which were not detected in an intermolecular manner by the RDX ex-

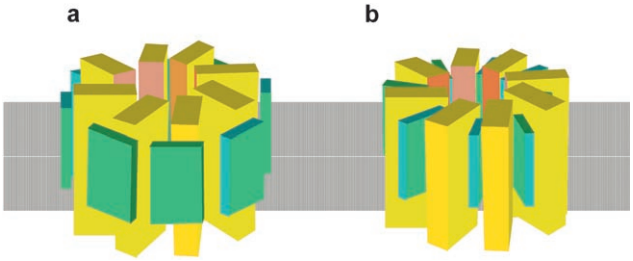

Figure 6. The surrounding model (A) versus the inserted model (B). The solid-state NMR and CD spectra of AmB–ergosterol conjugates, coupled with conformational search results, support model (A) in which ergosterol molecules (blue green) surround a barrel-stave channel of AmBs (yellow) rather than the previous model (B) in which ergosterol is inserted between two AmB molecules.[2] The orange face and its opposite (yellow) face of each yellow box correspond to the polyhydroxy group and heptaene portion of AmB, respectively. Single-length channels, which were reported to occur in DMPC membranes,[16] are presented.

periments, were observed for their covalent conjugates **1** and **3**. Under the same conditions, we previously observed the intermolecular ^{13}C–^{31}P interactions between DMPC and AmB.[16] The question is raised as to what molecules, other than sterols, occupy the space between AmB macrolactones that are arranged in a radial orientation. We hypothesize that this space could be occupied by PC molecules and as such, we are presently conducting NMR spectroscopic experiments to examine the interaction between AmB and PC.

Conclusion

The CD spectra of the AmB–ergosterol conjugates in a membrane-bound form suggested that the distances between the heptaene moieties of conjugates **2** and **3** are similar to that of AmB in ergosterol-containing membranes but are significantly larger than that of AmB in nonsterol or cholesterol-containing membranes. These observations imply that, as in the case with ergosterol-containing membranes, the conjugated sterol moiety prevents contact of the heptaene moieties in the membrane that destabilise ion-channel assemblies. The solid-state NMR spectroscopic measurements of ^{13}C, ^{19}F labelled AmB–sterol conjugates **2** and **4** in DMPC membrane showed a significant dephasing effect for the heptaene portion, indicating that the sterol closely approaches the hydrophobic side of AmB in membrane assemblies. Another notable feature of the present study deals with nonpeptidic agents, which are generally difficult to isotope label or otherwise chemically modify. By using the present method, however, we could not determine precise interatomic distances, mainly owing to signal overlap of polyene ^{13}C resonances. To address this problem, selectively ^{13}C- or ^{19}F-labelled AmB and sterol are essential and are currently being prepared in our laboratory.

Experimental Section

Materials: Amphotericin B (AmB, **1**), cholesterol and egg phosphatidylcholine were purchased from Nacalai Tesque. Ergosterol was from Tokyo Kasei (Tokyo, Japan), carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) from Tocris Cookson (Bristol, UK) and DMPC from Avanti Polar Lipid (Alabaster, AL). All other chemicals were obtained from standard vendors and used without further purification.

Preparation of AmB–C_2–(6-F)sterol covalent conjugates: AmB–sterol covalent conjugates **2**–**5** were prepared according to the previous report.[18] ^{13}C-labelled AmB was obtained by the laboratory cultures of the drug-producing organism, *Streptomyces nodosus* with media containing the uniformly ^{13}C-labelled glucose ([U-^{13}C]).[17,30]

***N*-(6-Fluoroergosteryloxycarbonyloxy)-3-amino-1,2-propanediol (7a)**: Triethylamine (0.33 mL, 2.352 mmol) was added to a stirred solution of 6-fluoroergosterol (48.8 mg, 0.118 mmol) and *N*,*N*′-disuccinimidyl carbonate (301.5 mg, 1.176 mmol) in acetonitrile (2.4 mL). The reaction mixture was sonicated for 4 h and then diluted with aqueous saturated $NaHCO_3$ followed by extraction with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. Purification by silica gel column chromatography by using hexane/ethyl acetate (3:1) as a mobile phase afforded *N*-succinimidyl carbonate (53.2 mg, 81%). 3-Amino-1,2-propanediol (43.6 mg, 0.479 mmol) was added to a stirred solution of *N*-succinimidyl carbonate at room temperature. After 2.5 h, the solution was diluted with chloroform, washed with aqueous saturated NH_4Cl and dried over Na_2SO_4. Removal of the solvent under reduced pressure gave *N*-(6-fluoroergosteryloxycarbonyloxy)-3-amino-1,2-propanediol **7a** (47.6 mg, 94%) as a diastereomeric mixture; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.37$ (br d, $J = 8.5$ Hz, 1H), 5.31 (br t, $J = 5$ Hz, 1H), 5.19 (dd, $J = 15.0$, 7.5 Hz, 1H), 5.14 (dd, $J = 15.0$, 7.5 Hz, 1H), 4.53 (dddd, $J = 11.3$, 11.3, 4.3, 4.3 Hz, 1H), 3.76 (br s, 1H), 3.62–3.53 (m, 2H), 3.34–3.20 (m, 2H), 2.98 (dd, $J = 14.5$, 4.0 Hz, 1H), 2.03–1.22 (m, 21H), 1.00 (d, $J = 6.5$ Hz, 3H), 0.94 (s, 3H), 0.89 (d, $J = 7.0$ Hz, 3H), 0.81 (d, $J = 7.0$ Hz, 3H), 0.80 (d, $J = 7.0$ Hz, 3H), 0.59 ppm (s, 3H); ESI-MS: m/z: 554.7 $[M+Na]^+$.

***N*-(6-Fluoroergosteryloxycarbonyloxy)aminoacetoaldehyde (8a)**: An aqueous solution of $NaIO_4$ (0.113 mmol in 0.23 mL) was added to the THF solution of diol **7a** (24.1 mg, 0.0453 mmol; 0.9 mL). The reaction mixture was stirred vigorously at room temperature for 2.5 h, then 1.0 M ethylene glycol solution (0.23 mL) was added. After extraction with AcOEt, the resulting organic layer was washed with water, dried over $MgSO_4$ and evaporated in vacuo. Purification by silica gel column chromatography with hexane/ethyl acetate (3:1) afforded aldehyde **8a** (16.6 mg, 75%); ^1H NMR (500 MHz, $CDCl_3$): $\delta = 9.65$ (s, 1H), 5.37 (ddd, $J = 8.5$, 2.4, 2.4 Hz, 1H), 5.31 (t, $J = 4.9$ Hz, 1H), 5.19 (dd, $J = 15.3$, 7.3 Hz, 1H), 5.14 (dd, $J = 15.3$, 7.3 Hz, 1H), 4.57 (dddd, $J = 11.3$, 11.3, 4.5, 4.5 Hz, 1H), 4.13 (d, $J = 4.9$ Hz, 2H), 3.00 (ddd, $J = 14.3$, 4.9, 1.5 Hz, 1H), 2.05–1.23 (m, 19H), 1.01 (d, $J = 6.7$ Hz, 3H), 0.95 (s, 3H), 0.89 (d, $J = 7.0$ Hz, 3H), 0.81 (d, $J = 7.0$ Hz, 3H), 0.80 (d, $J = 7.0$ Hz, 3H), 0.59 ppm (s, 3H).

AmB–C_2–(6-F)Erg (2): Aldehyde **8a** (16.6 mg, 0.0339 mmol) and AmB (34.5 mg, 0.0373 mmol) were dissolved in DMF/MeOH/$CHCl_3$ (9:3:1; 3.9 mL) and stirred vigorously for 11 h. $NaBH_3CN$ (10.6 mg, 0.170 mmol) was added to the solution and stirred at room temperature. After 36 h, the reaction mixture was poured into Et_2O. The resulting suspension was filtered through celite and the precipitate was washed with Et_2O. The resulting yellow precipitate was dissolved in $CHCl_3$/MeOH (3:1) and this organic layer was washed with water, dried over $MgSO_4$ and evaporated in vacuo. Purification by silica gel column chromatography with $CHCl_3$/MeOH/H_2O (10:6:1) afforded AmB–C_2–(6-F)Erg **2**. Further purification of conjugate **2** was performed by a gel-permeation chromatography column, JAIGEL-GS310 (φ21.5×300 mm, Japan Analytical Industry Co. Ltd.) and a $CHCl_3$/MeOH (3:1) solvent system with a LC-918 recycling preparative HPLC system. Finally, AmB–C_2–(6-F)Erg (2.5 mg, 0.00178 mmol, 5%) was obtained: HPLC retention time = 27.5 min; ^1H NMR (500 MHz, [D_6]DMSO): $\delta = 5.34$–5.30 (m, 1H; sterol 7-H), 5.26–5.15 (m, 2H; sterol 22-H and 23-H), 0.88 (d, $J = 7.0$ Hz, 3H; sterol 28-H), 0.81 (d, $J = 7$ Hz, 3H; sterol 26-H or 27-H), 0.79 ppm (d, $J = 7$ Hz, 3H; sterol 26-H or 27-H). All other signals are practically identical with

those of AmB–C_2–(6-F)Cho **4**; ^{19}F NMR (470.4 MHz, [D_6]DMSO): $\delta = -125.0$ (br s); ESI-MS: m/z: 1407.5 $[M+H]^+$ ([U-^{13}C]AmB–C_2–(6-F)Erg: m/z: 1431.7 $[M+H]^+$).

***N*-(6-Fluorocholesteryloxycarbonyloxy)-3-amino-1,2-propanediol (7b)**: Triethylamine (0.27 mL, 1.980 mmol) was added to a stirred solution of 6-fluorocholesterol (24.0 mg, 0.0593 mmol) and *N,N'*-disuccinimidyl carbonate (253.6 mg, 0.990 mmol) in acetonitrile (1.2 mL). The reaction proceeded under sonication. After 3 h, the mixture was diluted with aqueous saturated $NaHCO_3$ then extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. Purification by silica gel column chromatography with hexane/ethyl acetate (3:1) afforded *N*-succinimidyl carbonate (25.1 mg, 78%). 3-Amino-1,2-propanediol (37.6 mg, 0.413 mmol) was added to a stirred solution of *N*-succinimidyl carbonate at room temperature. After 2.5 h, the solution was diluted with chloroform, washed with aqueous saturated NH_4Cl and dried over Na_2SO_4. Removal of the solvent under reduced pressure gave *N*-(6-fluoroergosteryloxycarbonyloxy)-3-amino-1,2-propanediol **7b** (25.5 mg, quantitative) as a diastereomeric mixture; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.19$ (br s, 1H), 4.44 (dddd, $J = 11.3$, 11.3, 4.5, 4.5 Hz, 1H), 3.75 (br s, 1H), 3.62–3.53 (m, 2H), 3.33–3.20 (m, 2H), 3.03 (dd, $J = 13.5$, 4.0 Hz, 1H), 2.12–1.05 (m, 29H), 0.98 (s, 3H), 0.89 (d, $J = 6.5$ Hz, 3H), 0.84 (d, $J = 6.5$ Hz, 3H), 0.84 (d, $J = 6.5$ Hz, 3H), 0.65 ppm (s, 3H); ESI-MS: m/z: 544.4 $[M+Na]^+$.

***N*-(6-Fluorochoresteryloxycarbonyloxy)aminoacetoaldehyde (8b)**: The THF solution of diol **7b** (25.5 mg, 0.0489 mmol; 0.5 mL) was added to 0.5 M aqueous solution of $NaIO_4$ (0.24 mL, 0.122 mmol). The reaction mixture was stirred vigorously at room temperature for 2 h, then 1.0 M ethylene glycol solution (0.25 mL) was added. After extraction with AcOEt, the resulting organic layer was washed with water, dried over $MgSO_4$ and evaporated in vacuo. Purification by silica gel column chromatography with hexane/ethyl acetate (3:1) as the mobile phase afforded aldehyde **8b** (20.1 mg, 84%); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 9.64$ (s, 1H), 5.28 (br s, 1H), 5.31 (t, $J = 4.9$ Hz, 1H), 4.48 (dddd, $J = 11.6$, 11.6, 3.7, 3.7 Hz, 1H), 4.11 (d, $J = 4.0$ Hz, 2H), 3.05 (ddd, $J = 13.0$, 5.0, 2.0 Hz, 1H), 2.12–2.05 (m, 1H), 1.99 (ddd, $J = 12.5$, 3.5, 3.5 Hz, 1H), 1.89–1.76 (m, 5H), 1.60–1.23 (m, 13H), 1.17–1.03 (m, 7H), 0.99 (s, 3H), 0.89 (d, $J = 6.5$ Hz, 3H), 0.84 (d, $J = 6.5$ Hz, 3H), 0.84 (d, $J = 6.5$ Hz, 3H), 0.65 ppm (s, 3H).

AmB–C_2–(6-F)Cho (4): Aldehyde **8b** (17.3 mg, 0.0353 mmol) and AmB (32.6 mg, 0.0353 mmol) were dissolved in DMF/MeOH/$CHCl_3$ (9:3:1; 3.9 mL) and stirred vigorously for 2.5 h. $NaBH_3CN$ (11.1 mg, 0.177 mmol) was added to the solution and stirred at room temperature. After 37.5 h, the reaction mixture was poured into Et_2O. The resulting suspension was filtered through celite and the precipitate was washed with Et_2O. The resulting yellow precipitate was dissolved in $CHCl_3$/MeOH (3:1) and the organic layer was then washed with water, dried over $MgSO_4$ and evaporated in vacuo. Purification by silica gel column chromatography with $CHCl_3$/MeOH/H_2O (10:6:1) afforded AmB–C_2–(6-F)Erg **2**. Further purification of conjugate **2** was performed by a column chromatography with JAIGEL-GS310 (φ21.5 × 300 mm, Japan Analytical Industry Co., Ltd.) and a $CHCl_3$/MeOH (3:1) solvent system with a LC-918 recycling preparative HPLC system. Finally, AmB–C_2–(6-F)Erg (3.8 mg, 0.00272 mmol, 8%) was obtained: HPLC retention time = 27.5 min; all ^{1}H NMR signals (500 MHz, [D_6]DMSO) are practically identical with those of AmB–C_2–Cho;[18] ^{19}F NMR (470.4 MHz, [D_6]DMSO): $\delta = -109.8$ (br s); ESI-MS: m/z: 1397.7 $[M+H]^+$ ([U-^{13}C]AmB–C_2–(6-F)Cho: m/z: 1421.5 $[M+H]^+$).

Liposome preparations: Large unilamellar vesicles (LUV) were prepared according to the methods reported by Hervé et al.[33] Briefly, egg phosphatidylcholine (12 μmol) and either AmB–C_2–Sterol or AmB (3.6 nmol) were dissolved in chloroform/methanol (3:1) and the mixture was evaporated to a thin film in a 10-mL test tube. After the film was dried under vacuum for over 8 h, 0.4 mM KH_2PO_4 and 1 mM EDTA at pH 5.5 in H_2O/D_2O (6:4; 166.7 μL) was added to the test tube. The lipid mixture was suspended in the buffer solution by using a vortex apparatus and sonication. The resultant suspension was frozen at −20 °C and thawed at 50 °C three times. The LUV thus obtained was passed through a membrane filter (pore size: 0.2 μm) 19 times with a Liposofast apparatus (AVESTIN).

K^+ ion flux assays by using ^{31}P NMR: The LUV suspension was diluted six times with 0.4 mM K_2SO_4 and adjusted to pH 7.5 with KOH. Then FCCP (2.67 nmol) in EtOH (2.67 μL) was added and the LUV was shaken gently for 6 h at 25 °C. The LUV suspension (550 μL) with the lipid concentration of 12 mM was transferred to a 5-mm NMR glass tube and added to 1 mM $MnCl_2$ (4.4 μL). The ^{31}P NMR spectrum at 23 °C was measured at 202.35 MHz (JEOL, GSX-500 Spectrometer) with ^{1}H broadband decoupling. The flux activity was obtained from the NMR spectroscopic peak integrations from 1.1 to 3.2 ppm and was then expressed as the percentage of peak area for 1.3–3.2 ppm in the total area for 1.1–3.2 ppm. In other words, all resonances in this area, other than a peak at 1.2 ppm, were regarded as those of permeabilised liposomes.

CD spectral measurements: The CD spectra of AmB–Erg conjugates were measured for liposomes prepared by the mixed-with-lipid method as reported previously.[19] Briefly, EPC (12 μmol) and either AmB–C_2–sterol or AmB (3.6 nmol, added as a 1.25 mM stock solution in DMSO) were dissolved in $CHCl_3$/MeOH (3:1) and the mixture was evaporated to a thin film in a 10-mL test tube. After the film was dried under vacuum for over 8 h, 9% sucrose solution (166.7 μL) was added to the test tube. The lipid mixture was suspended in the buffer solution by using a vortex apparatus and sonication. The resultant suspension was frozen at −20 °C and thawed at 50 °C three times. The LUV thus obtained was passed through a membrane filter (pore size: 0.2 μm) 19 times with a Liposofast apparatus (AVESTIN). Then, 9% sucrose buffer solution was added so that the final concentration of AmB was adjusted to be 5 μM. CD spectroscopy was performed on a JASCO J-750W spectrometer with a 2-mm cuvette.

Solid-state NMR spectroscopic measurements: For preparing membrane-bound AmB, [U-^{13}C]AmB–C_2–(6-F)Erg **2** (3.2 mg) and DMPC (15.2 mg; 1:10 molar ratio) were dissolved in $CHCl_3$/MeOH (3:1) and the solvent was evaporated in vacuo for 8 h. The membrane preparation was hydrated with 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer solution (18.4 μL; pH 7.0) under Ar and then diluted with H_2O (1 mL). The lipid mixture was suspended in aqueous phase by using a vortex apparatus and sonication. The resultant suspension was frozen and thawed and then stirred vigorously to make multilamellar vesicles. The MLV preparation was lyophilised, rehydrated with D_2O (about 20 μL) and packed into a φ5 mm magic-angle spinning (MAS) rotor within a glass insert. The membrane dispersion of [U-^{13}C]AmB–C_2–(6-F)Cho **4** (1:10) and the diluted dispersion of **2** with nonlabelled AmB–C_2–Erg **3** (0.5:0.5:10) were prepared by the same method. ^{13}C{^{19}F}RDX spectra were recorded at 75.315 Hz for ^{13}C and 281.773 Hz for ^{19}F on a CMX300 (Varian/Chemagnetics) spectrometer with the MAS frequency of (5000 ± 2) Hz. The rotor temperature was maintained at (30 ± 1) °C with a temperature controller. The spectral width was 30 kHz. Typically, the $\pi/2$ pulse width for ^{1}H was 4 μs, and the π pulse width for ^{13}C and ^{19}F were 8 μs and 14 μs, respectively. The contact time for cross-polarisation transfer was set to be 1.5 ms. The REDOR spectra were acquired with a recycle delay of 4 s and a ^{1}H decoupling field strength of 83 kHz and were measured at a dephasing time of 8 ms by using xy-8 phase cycling for ^{19}F irradiation.

Molecular modelling of AmB–C_2–(6F)Erg 2: The conformational search of AmB–C_2–(6-F)Erg was carried out with the Macromodel software[40] version 8.6 installed on a RedHat Linux 8 operating system. Initial atomic coordinates and structure files were generated step by step from the crystal data of *N*-iodoacetyl AmB.[41] The macrolide in the AmB moiety was treated as a semirigid group in which ±30° allowance from the crystal structure was given to each C−C bond upon calculation. The interatomic distance between the fluorine at the ergosterol moiety and C23 of AmB was restricted in the range of (5.6 ± 3.0) Å as C23 should come close to the fluorine atom in ergosterol on the basis of the AmB–ergosterol interaction proposed in our previous study.[18] Sampling of the conformational space was performed with a Monte Carlo multiple minimum method (MCMM).[42] The following 14 C−C bonds that consist of the covalent conjugation parts including the mycosamine moiety of AmB and the side chain of the ergosterol were allowed to rotate during the

MCMM conformational search; C16−C41, C19−O, O−C1′, C3′−N, N−CH_2(linker), CH_2−CH_2(linker), CH_2−N(linker), N−CO(linker), CO−O (linker), O−C3(sterol) and four relevant bonds in a sterol side chain. The AMBER* force field[43] implemented in the Macromodel program was used for the conformational searches in 5000 steps. Continuum solvation models for water by using a generalised Born/surface area (GB/SA)[44] were applied through the calculations. Energy minimisation was carried out by using the Polak–Ribiele conjugate gradient (PRCG) method with 7000 maximum iterations.

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