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An Amphotericin B-Ergosterol Covalent Conjugate with Powerful Membrane Permeabilizing Activity

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Summary

Amphotericin B-sterol conjugates were synthesized and examined for their membrane permeabilizing activity. Ergosterol and cholesterol, each connected with amphotericin B via an ethylenecarbamate or hexamethylenecarbamate linker, were examined by K^+ flux assays using liposomes and by single-channel recording across phospholipid membrane. Among four conjugates tested, AmB-ergosterol bearing an ethylenecarbamate linker exhibited the most powerful activity, which substantially exceeded that of the cholesterol homolog. Single-channel recording clearly exhibited that the ergosterol conjugate elicited channel current with the conductance of 28 pS, which was comparable with those by AmB, and revealed a higher channel open probability than the cholesterol conjugate. These results imply that direct interaction between amphotericin B and ergosterol is reproduced by their conjugate, which may serve as a model compound for understanding the drug's selective toxicity.

Introduction

Amphotericin B (AmB, **1**) has been the drug of choice for the treatment of systemic fungal infections for over 40 years. Its antibiotic action is generally attributed to the higher affinity for ergosterol, principal fungal sterol, than cholesterol occurring in mammalian membranes. Their structural differences are, however, rather minute: in ergosterol, the presence of two additional carbon-carbon double bonds in ring B and a side chain that possesses one more methyl branching. This preference of fungus sterol over human sterol is only 10-fold, which is presumably responsible for serious side effects of the drug including nephrotoxicity and hypokalemia. Knowing the molecular interactions between AmB and sterol [1, 2] is essential for establishing the structural basis of the drug's toxicity in terms of both its medicinal virtues and side effects. However, no experimental evidence has been obtained for the direct interaction between AmB and ergosterol/cholesterol, and, in particular, regarding the interaction between AmB and cholesterol in biomembrane, negative experimental results have often been reported [3–5].

Not only out of scientific interest but because of pharmacological importance, we became interested in the mechanism underlying AmB-sterol and AmB-AmB interactions in biomembrane; particularly, we have been focusing on the bimolecular interactions after AmB's binding to the membrane. For this purpose, we have prepared several AmB conjugates and have demonstrated in previous studies that covalently liked dimers of AmB retain sterol selectivity similar to that of AmB [6–8]. In this study, we report the preparation of membrane-active AmB-sterol conjugates and their membrane permeabilizing activities determined by K^+ flux assays and channel current recording experiments.

Results and Discussion

Preparation of AmB-Sterol Conjugates

According to molecular dynamics simulations carried out for a barrel-stave model [1, 2] (Figure 1), 3-OH of ergosterol presumably resides close to the mycosamine moiety of AmB [9]. Therefore, we attempted to connect the hydroxyl group of ergosterol/cholesterol with 3′-NH_2 of AmB. This molecular design was expected to force AmB and sterol parts to take a parallel position in close vicinity upon binding to membrane. Taking into consideration the chemical properties of functional groups [10, 11], AmB and sterol were connected with an alkyl-carbamate linker to provide conjugates **2–5** (Figure 2). Besides feasibility in syntheses, these compounds were designed to retain the basic amino group in AmB, which was reportedly essential for membrane activity [12], and to furnish a carbamate functionality attached to C3 of the sterol part. This hydrogen-bonding group may facilitate the sterol moiety to take the upright position in membrane, as reported by Regen et al. [13]. The synthetic route of these compounds was modified depending on the length of linkers and ergosterol/cholesterol. For an ethylene-carbamate linker, the coupling reaction between 3-amino-1,2-propanediol and sterol carbonate followed by periodate oxidation provided the sterol-conjugated aldehyde, which was then subjected to reductive aminoalkylation with the amino group of AmB to furnish conjugates **2** and **4** (Figure 3). For a hexamethylene-carbamate linker, different routes were adopted for cholesterol and ergosterol, since unsuccessful oxidation of an alcohol in the presence of an ergosterol moiety obliged us to do the aminoalkylation of AmB prior to formation of the carbamate linkage (see Experimental Procedures for details).

UV Spectra of AmB-Sterol Conjugates

The interaction of the AmB conjugates in membrane was first examined for their UV spectra using phospholipid

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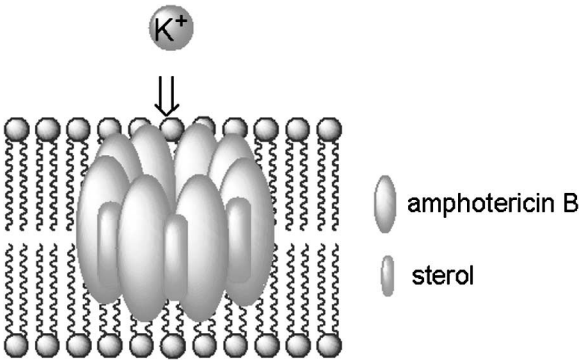


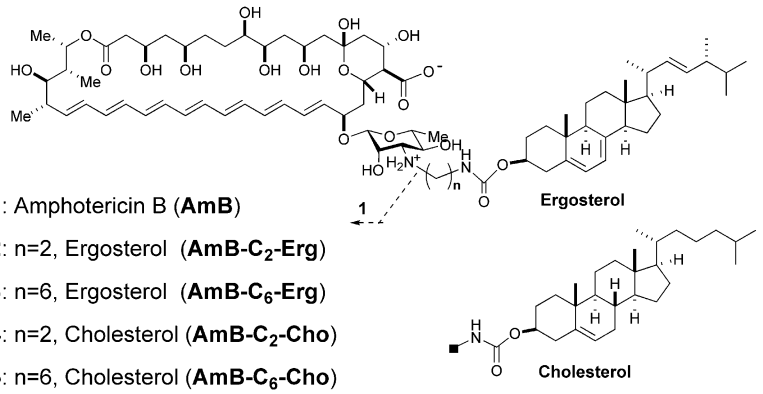
Figure 1. Schematic Representation of Barrel-Stave Model for AmB Ion Channel

liposomes. AmB-C_2-Erg **2** showed smaller absorption maxima compared with those of AmB-C_2-Cho **4** (Figure 4). These spectral differences can be accounted for by the stable complex formation with **2**, where heptaene choromophores reside in close vicinity [14, 15]. In comparison with the UV spectrum of AmB in cholesterol-containing liposomes (data not shown; the absorbance at 412 nm was 0.042), the absorption by AmB-C_2-Cho **4** was markedly enhanced. These findings mean that the cholesterol part of **4** hampers formation of AmB complex in membrane. A similar enhancement in UV spectra has been observed for AmB-phospholipid conjugates [8, 18], which implies that a phospatidyl substituent on the amino group of AmB again decreases the stability of AmB assemblages. Conversely, AmB-C_2-Erg **2**, which also has a hydrophobic bulky substituent similar to AmB-C_2-Cho, showed contrasting results: the UV absorption at 412 nm was comparable with that of AmB in ergosterol-containing liposomes (data not shown; the absorbance at 412 nm was 0.038). These results suggest that, unlike cholesterol or phospholipid conjugates, the ergosterol part in **2** does not prevent the complex formation but facilitates the self-assembly of AmB in association/dissociation equilibrium. The similar changes in UV absorptions of AmB in ergosterol- or cholesterol-containing liposomes have been reported for phospholipid micelles [19].

Membrane Permeabilizing Activity of AmB-Sterol Conjugates

We next examined the membrane permeabilizing activity using a method reported by Gary-Bobo et al. [20, 21]. K^+ influx into liposomes induces proton/K^+ exchange in the presence of proton-transporter FCCP, leading to a rise in inner pH, which can be monitored by chemical shift changes in ^{31}P NMR. In the spectra of Figure 5A, a downfield signal at δ 3.1 was derived from phosphate entrapped in liposomes on which AmB formed ion-permeable channels and H^+ leaked out at the expense of K^+ influx, while an upfield signal at δ 1.2 was due to intact liposomes. In these experiments, AmB or AmB-sterol conjugate was mixed with lipids prior to preparation of liposomes, since the conjugates could not be suspended well and thus would hardly bind to liposome membrane when added to a liposome-containing aqueous suspension. By using this procedure, the step of AmB incorporation into membrane was skipped, and the formation of channel assemblages in membrane was selectively observed. As shown in Figure 5A, AmB permeabilized ergosterol-containing membrane most efficiently while showing a low activity for cholesterol liposomes, even less efficacious than that for sterol-free liposomes.

As shown in Figure 5A, AmB-C_2-Erg **2** revealed the most powerful membrane permeabilizing activity for sterol-free liposomes among the conjugates tested. In the ^{31}P NMR spectrum of **2**, a single peak was observed at δ 3.1, which demonstrated that liposomes were mostly permeabilized. The efficacy of these conjugates shows the tendency AmB-Erg $>$ AmB $>$ AmB-Cho, which is parallel to the potency of AmB in sterol-containing membrane. The concentration-activity relationship in Figure 5B again reveals that AmB-C_2-Erg **2** is much more efficacious than AmB-C_2-Cho **4** but somewhat less potent than AmB in ergosterol-containing liposomes. These results imply that the specific recognition of ergosterol by AmB is reproduced to a certain extent by AmB-C_2-Erg **2**, and also the tether between AmB and ergosterol somewhat hampers their interaction. Nevertheless, the marked difference in the activity between **2** and **4** clearly indicates that minute differences in sterol structure greatly influence the molecular recognition by AmB. These observations may support the well-known hypothesis that the antibiotic activity of AmB is attributed to its specific interaction with ergosterol upon forming an ion channel complex. A great number of experiments previously carried out for AmB-sterol interactions mainly focused on their intermolecular recognition in membrane, where clear distinction between direct interactions and indirect effects was sometimes difficult [16].



1: Amphotericin B (**AmB**)

2: n=2, Ergosterol (**AmB-C_2-Erg**)

3: n=6, Ergosterol (**AmB-C_6-Erg**)

4: n=2, Cholesterol (**AmB-C_2-Cho**)

5: n=6, Cholesterol (**AmB-C_6-Cho**)

Figure 2. Structures of AmB-Sterol Conjugates (**2–5**)

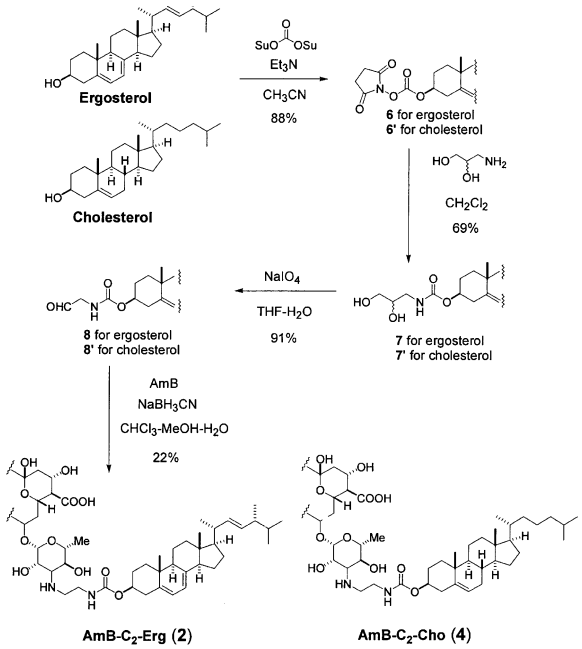


Figure 3. Synthesis of AmB-C_2-Erg (**2**) and AmB-C_2-Cho (**4**)

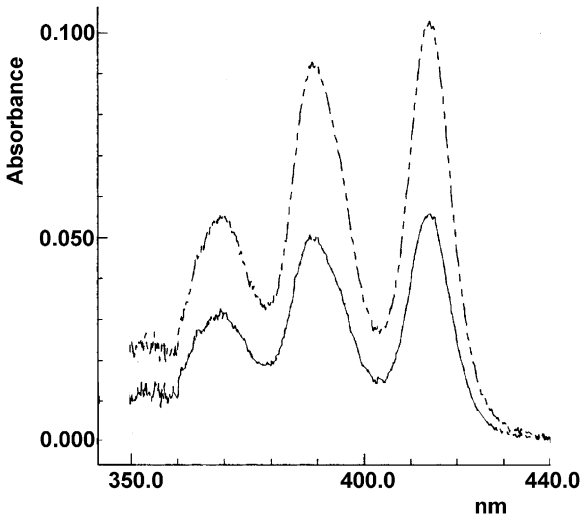


Figure 4. UV Spectra of Ergosterol-Conjugated AmB (**2**) and Choresterol-Conjugated AmB (**4**)

The spectra of AmB-C_2-Erg (**2**) (solid line) and AmB-C_2-Cho (**4**) (dashed line) were measured in liposomes consisting of egg phosphatidylcholine. Concentration of conjugate **2** or **4** was 1.4 μM with the ratio of conjugate/lipid (R) of 3×10^{-4}.

In this study, the interaction is intramolecular, where ergosterol always stays very close to AmB. Thus, the enhanced membrane activity by AmB in ergosterol-containing membrane should be ascribable to the direct AmB-sterol interaction (not by indirect effects such as membrane fluidity or ordering, as suggested for cholesterol [22]).

The conjugated cholesterol part worked in a rather inhibitory manner. This is the opposite of the generally accepted idea. We have recently demonstrated that cholesterol prevents ion channel formation by membrane-bound AmB using similar preparations of liposomes [3]. The present results further support that cholesterol does not participate in formation of a channel complex [3, 4, 5, 23, 24].

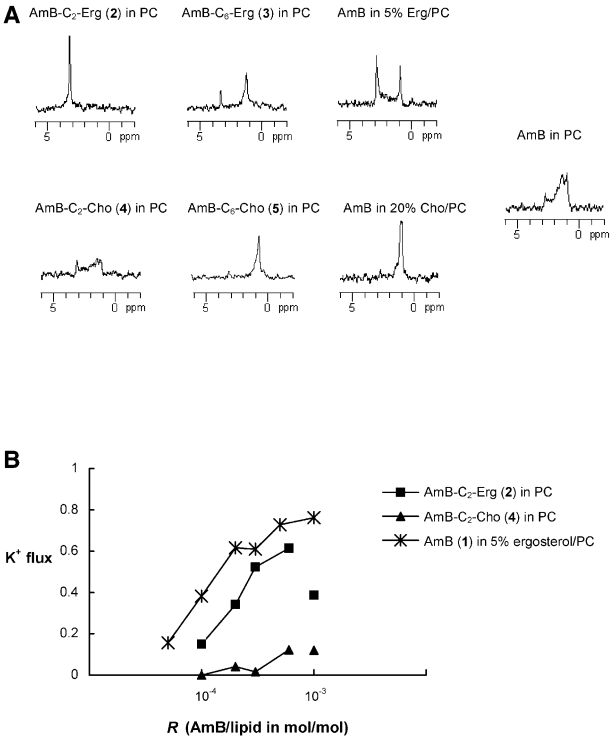


Figure 5. Membrane Permeabilizing Activities of AmB (**1**) and AmB-Sterol Conjugates (**2** and **4**)

(A) ^{31}P NMR spectra of liposome-entrapped phosphate for AmB (**1**) or AmB-sterol conjugates **2–5**. AmB or conjugate was added to lipids prior to liposome preparation and incubated for 3 hr. Lipid concentration in a liposome suspension was 12 mM. Liposomes used for conjugates were composed only of egg-phosphatidylcholine (PC), whereas 5% ergosterol- or 20% cholesterol-containing PC was also used for AmB. The peak around δ 1.2 corresponds to $H_2PO_4^-$ at pH 5.5 (intact liposomes) and that around δ 3.1 corresponds to HPO_4^{2-} at pH 7.5 (permeabilized liposomes). Signals between δ 1.2 and 3.1 are derived from liposomes with inside pH between 5.5 and 7.5. R, a molar ratio between AmB or AmB conjugate and lipids, was 3×10^{-4} for all the spectra.

(B) Concentration and activity relationship of K^+ flux activity for AmB (**1**), conjugates **2** and **4**. Sterol-free liposomes were used for conjugates, whereas 5% ergosterol was contained in the liposome for AmB. At higher concentrations above $R = 10^{-3}$, the K^+ flux activity could not be determined because conjugates **2** and **4** were significantly lost due to absorption to a filter membrane during liposome preparation. The y axis is a ratio of a peak area at δ 3.1 out of the integration for δ 1.2–3.1.

When comparing ergosterol conjugates with different tether lengths, **2** with an ethylene linker showed significantly stronger activity than **3** with a longer C_6 linker (Figure 5A); the same tendency can been seen for the cholesterol conjugates **4** and **5**. This difference in activity may reflect the close proximity of C3-OH of sterol and C3′-NH_2 of AmB in the channel complex, which is in parallel with the molecular dynamics calculation by Baran and Mazerski [9]. The observations further imply the direct interaction between AmB and ergosterol, probably through hydrogen bonding, near the membrane surface of the channel complex.

Single-Channel Current Recording and Antibiotic Assays

To investigate the behaviors of these sterol conjugates at the level of single ion channels, we measured channel currents by the tip-dip method. Figure 6A shows examples of current recordings for AmB, **2**, and **4** at 150 mV potential. In this experiment, diphytanoylphosphatidylcholine (DPhPC) was used as a membrane lipid, since this phospholipid has good stability suitable for channel recordings. DPhPC was also used for channel recordings of AmB in the previous report [25], which facilitates the comparison of our results. Because of low binding ability of conjugates to the membrane, AmB or conjugate was premixed with membrane lipids, and channel recordings were started after further addition of the drug to the bath solution at a concentration of 3 μM. Current fluctuations between open and closed channel states were clearly observed for conjugates **2** and **4** in DPhPC as well as for AmB in ergosterol-DPhPC (Figure 6A); particularly, formation of stable channels by AmB can be seen in the presesnce of ergosterol, as reported previously [25]. The ergosterol preference over cholesterol in conjugates is seen in Figure 6B, where AmB-C_2-Erg **2** has higher channel open probability than AmB-C_2-Cho **4**. The average open probability of **2** in DPhPC membrane is comparable with that of AmB in ergosterol-DPhPC membrane (Figure 6B). The conductance of **2** was determined to be 28 pS (Figure 6C), which is similar to or even higher than that of AmB, which is reported to be around 20 pS by Brutyan et al. [25]. These findings from K^+ ion-flux assays and ion channel recordings indicate that properties and behaviors of AmB-ergosterol conjugates in sterol-free membrane are similar to those of AmB in ergosterol-containing phospholipid bilayers.

We then attempted to evaluate the membrane permeabilizing activity in biological systems. The sterol conjugates **2–5** did not induce hemolysis up to 50 μM against 1% human erythrocytes, whereas AmB was active at 1.5 μM. The conjugates did not reveal significant antifungal action even at 20 μM against *Candida albicans* and *Paecilomyces varioti*, where the EC_{50}s of AmB were around 0.1 μM. These weak biological activities may be partly attributable to their poor solubility in water, as seen for the K^+ flux assays using liposomes.

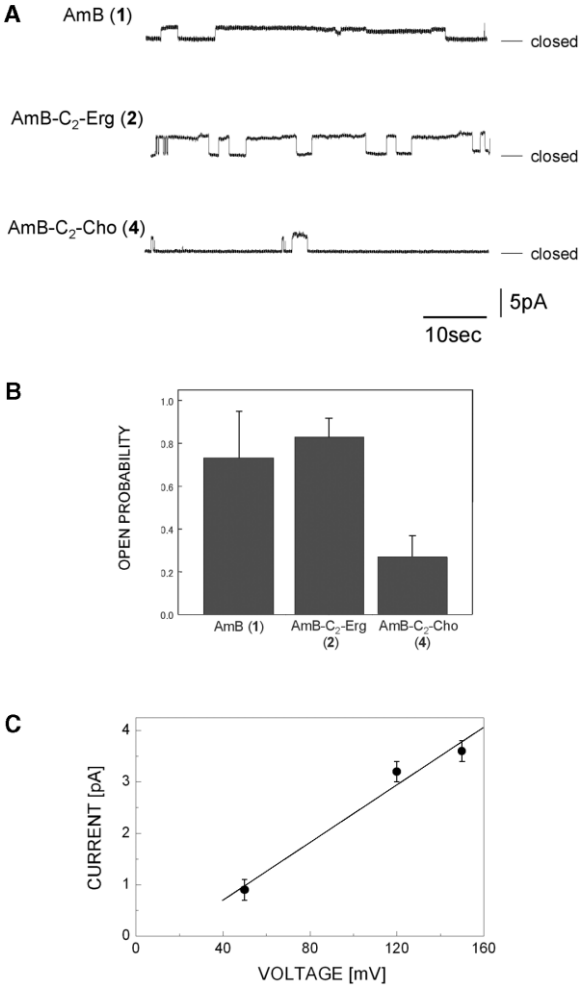


Figure 6. Single-Channel Recording of AmB (**1**) and AmB-Sterol Conjugates (**2** and **4**)

(A) Examples of current recordings of single channels induced by AmB or AmB-sterol conjugate (**2** or **4**) with the tip-dip method at 150 mV applied potential. The aqueous solution contains 3 M KCl, 2.5 mM HEPES, and 1 mM $CaCl_2$ (pH 7.4). Membrane conditions are as follows: top, diphytanoylphosphatidylcholine (DPhPC), ergosterol, and AmB (molar ratio, 15000:3000:1); middle and bottom, DPhPC and AmB-C_2-Erg (**2**) or AmB-C_2-Cho (**4**) (molar ratio, 15000:1). Current recordings were started after further addition of AmB, **2**, or **4** to the bath solution at a concentration of 3 μM.

(B) Average channel open probability at 150 mV for AmB (0.73 ± 0.22, $n = 6$), **2** (0.83 ± 0.09, $n = 8$), and **4** (0.27 ± 0.10, $n = 5$).

(C) Current/voltage relationship for AmB-C_2-Erg (**2**). From a linear fit to the I/V relationship, the single-channel conductance of **2** was determined to be 28 pS.

Significance

In the present study, we have demonstrated that, in membrane, the bimolecular interaction of AmB with ergosterol is stronger than that with cholesterol. To our knowledge, this may be the first experimental evidence that the direct interaction between amphotericin B and ergosterol in a lipid bilayer results in the marked enhancement of membrane permeabilizing activity. Some of the present conjugates, such as AmB-C_2-Erg 2, may possibly serve as models for investigating the precise mechanism of antibiotic action of AmB. Currently, we are attempting to prepare isotope-labeled conjugates for solid-state NMR measurements. Interatomic distance estimated from the

NMR data would help us elucidate the structure of a complicated molecular assemblage formed in membrane by AmB and sterol, which may provide invaluable information to reduce the drug's side effects and improve its pharmacological efficacy.

Experimental Procedures

Preparation of Amphotericin B-Sterol Conjugates

N-*(3-Ergosteryloxycarbonyloxy)succinimide (6)*

Triethylamine (5 ml) was added to a stirred solution of ergosterol (1190 mg, 3.0 mmol) and *N,N′*-disuccinimidyl carbonate (3300 mg, 13 mmol) in acetonitrile (50 ml). After being stirred at 23°C for 15 hr under sonication, the mixture was diluted with aqueous saturated $NaHCO_3$ (100 ml) and extracted with ethyl acetate. The organic layer was washed with brine and water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. Purification by column chromatography (SiO_2) using hexane-ethyl acetate (3:1) as a mobile phase afforded *N*-(3-ergosteryloxycarbonyloxy)succinimide (1420 mg, 88%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.54 (dd, 1H), 5.32 (m, 1H), 5.13 (m, 1H), 5.12 (m, 1H), 4.64 (m, 1H), 2.77 (s, 4H), 2.61 (dd, 1H), 2.46 (t, 12 Hz, 1H), 0.97 (d, 3H), 0.90 (s, 3H), 0.85 (d, 3H), 0.76 (d, 6H), 0.56 (s, 3H).

N-*(3-Ergosteryloxycarbonyl)-3-Amino-1,2-Propanediol (7)*

A mixture of *N*-(3-ergosteryloxycarbonyloxy)succinimide **6** (100 mg, 186 μmol) and 3-amino-1,2-propanediol (56 mg, 615 μmol) in CH_2Cl_2 (5 ml) was stirred for 40 min at room temperature. Subsequently, the reaction mixture was mixed with saturated aqueous NH_4Cl (10 ml) and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated in vacuo to furnish *N*-(3-ergosteryloxycarbonyl)-3-amino-1,2-propanediol (66 mg, 69%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.50 (dd, 1H), 5.31 (m, 1H), 5.15 (q, 1H), 5.11 (q, 1H), 4.53 (m, 1H), 3.71 (m, 1H), 3.53 (m, 2H), 3.27 (m, 2H), 2.46 (dd, 1H), 2.27 (t, 1H), 0.97 (d, 3H), 0.88 (s, 3H), 0.85 (d, 3H), 0.76 (d, 6H), 0.56 (s, 3H).

N-*(3-Ergosteryloxycarbonyl)aminoacetoaldehyde (8)*

To a solution of *N*-(3-ergosteryloxycarbonyl)-3-amino-1,2-propanediol **7** (54 mg, 105 μmol) dissolved in THF (1 ml), 0.5 M aqueous $NaIO_4$ (0.5 ml) was added. After being stirred vigorously for 1 hr, the solution was treated with 1 M ethylene glycol (450 μl) to reduce excess $NaIO_4$. The resulting solution was diluted with ethyl acetate, washed with water, and dried over $MgSO_4$. Removal of solvent under reduced pressure gave *N*-(3-ergosteryloxycarbonyl)aminoacetoaldehyde (46 mg, 91%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.60 (s, 1H), 5.50 (dd, 1H), 5.32 (dd, 1H), 5.13 (q, 1H), 5.11 (q, 1H), 4.08 (d, 2H), 2.47 (dd, 1H), 2.28 (t, 1H), 0.97 (d, 3H), 0.88 (s, 3H), 0.85 (d, 3H), 0.76 (d, 6H), 0.56 (s, 3H).

AmB-C_2-Erg (2)

A solution of *N*-(3-ergosteryloxycarbonyl)aminoacetoaldehyde **8** (8.5 mg, 17 μmol) and AmB (17 mg, 18 μmol) in $CHCl_3$-MeOH-H_2O (10:6:1, 1.5 ml) was stirred for 2 hr, and $NaBH_3CN$ (9 mg, 143 μmol) was added to the solution. After being stirred overnight, the solution was diluted with $CHCl_3$-MeOH (10:1, 10 ml) and washed with 1M HCl, 0.7% NH_4OH, and then distilled water. The organic layer was concentrated in vacuo and purified by column chromatography (SiO_2, $CHCl_3$-MeOH-H_2O 10:6:1) to afford AmB-C_2-Erg (**2**) (5.3 mg, 22%). HPLC retention time (YMC-ODS AM-323 ϕ 10 × 250 mm): 44.7 min (flow rate, 1.5 ml/min; eluent, MeOH-5 mM ammonium acetate [pH 5.3] changing linearly from 8:2 to 10:0 in 20 min). ESI-MS *m/z*: 1389.5 $(M+H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6:$CDCl_3$ = 19:1): δ 5.52 (dd, *J* = 6, 2 Hz, 1H, 6‴), 5.33 (m, 1H, 7‴), 5.20 (m, 2H, 22‴ and 23‴). All other ^{1}H NMR signals are practically identical with those of AmB-C_2-Cho (**4**) described below.

AmB-C_6-Erg (3)

To a DMSO solution (1 ml) of ergosterol (48 mg, 90 μmol) and *N*-6-aminohexyl-amphotericin B (56 mg, 55 μmol), which was prepared by following the same procedure as described previously [6], triethylamine (50 μl) was added. After being stirred overnight at room temperature, a yellow precipitate was formed by addition of diethyl ether to the solution. The precipitate was isolated on Celite, washed with diethyl ether, and purified by column chromatography (SiO_2, $CHCl_3$-MeOH-water 10:6:1) to give AmB-C_6-Erg (**3**) (15 mg, 18%). HPLC retention time (YMC-ODS AM-323 ϕ 10x250 mm): 44.7 min (flow rate, 1.5 ml/min; eluent, 1.5 ml of MeOH-5 mM ammonium acetate [pH 5.3] changing linearly from 8:2 to 10:0 in 20 min). ESI-MS *m/z*: 1445.5 $(M+H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6:$CDCl_3$ = 19:1): δ 5.52 (dd, *J* = 6, 2 Hz, 1H, 6‴), 5.33 (m, 1H, 7‴), 5.20 (m, 2H, 22‴ and 23‴), 1.45-1.55 (m, 8H, 2″-5″). All other ^{1}H NMR signals are practically identical with those of AmB-C_2-Cho (**4**).

N-*(3-Cholesteryloxycarbonyloxy)succinimide (6′)*

This compound was obtained by a procedure similar to the one applied for the preparation of *N*-(3-ergosteryl- oxycarbonyloxy)succinimide **6**, starting from cholesterol (135 mg, 0.35 μmol) instead of ergosterol. Yield: 167 mg (90%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.36 (d, 1H), 4. 54 (m, 1H), 2.78 (s, 4H), 0.98 (s, 3H), 0.87 (d, 3H), 0.82 (d, 6H), 0.63 (s, 3H).

N-*(3-Cholesteryloxycarbony)-3-Amino-1,2-Propanediol (7′)*

A CH_2Cl_2 solution (2 ml) of *N*-(3-cholesteryloxycarbonyloxy)succinimide **6′** (103 mg, 195 μmol) and 3-amino-1,2-propanediol (38 mg, 417 μmol) was stirred overnight at 23°C. Subsequently, the mixture was concentrated under reduced pressure and purified by column chromatography on silica gel using hexane-ethyl acetate (1:1), providing *N*-(3-cholesteryloxycarbony)-3-amino-1,2-propanediol (95 mg, 97%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.36 (d, 1H), 4. 48 (m, 1H), 3.74 (m, 1H), 3.57 (m, 2H), 3.31 (m 2H), 2.33 (dd, 1H), 2.26 (t, 1H), 0.99 (s, 3H), 0.89 (d, 3H), 0.84 (d, 6H), 0.66 (s, 3H).

N-*(3-Cholesteryloxycarbony)aminoacetoaldehyde (8′)*

This compound was obtained by a procedure similar to the one used for the preparation of *N*-(3-ergosteryl- oxycarbonyl)aminoacetoaldehyde **8**, starting from *N*-(3-cholesteryloxycarbony)-3-amino-1,2-propanediol **7′** (95 mg, 189 μmol). Yield: 64 mg (72%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.64 (s, 1H), 5.35 (d, 1H), 4.49 (m, 1H), 4.10 (m, 2H), 2.34 (dd, 1H), 2.28 (t, 1H), 0.99 (s, 3H), 0.89 (d, 3H), 0.84 (d, 6H), 0.65 (s, 3H).

AmB-C_2-Cho (4)

This conjugate was obtained by a procedure analogous to that described for AmB-C_2-Erg (**2**). Reagent amounts: *N*-(3-cholesteryl-oxycarbony)aminoacetoaldehyde (45 mg, 95 μmol), AmB (120 mg, 130 μmol), and $NaBH_3CN$ (38 mg, 578 μmol). Yield: 46 mg (35%). HPLC retention time (YMC-ODS AM-323 10 × 250 mm): 41.8 min (flow rate, 1.5 ml/min; eluent, MeOH-5 mM ammonium acetate [pH 5.3] changing linearly from 8:2 to 10:0 in 20 min). ESI-MS *m/z*: 1379.6 $(M+H)^+$. ^{1}H NMR signals (500 MHz) are listed in Table 1.

N-*(3-Cholesteryloxycarbony)-6-Amino-1-Hexanol*

A mixture of *N*-(3-cholesteryloxycarbony)succinimide **6′** (87 mg, 0.17 μmol) and 6-amino-1-hexanol (33 mg, 0.28 μmol) in CH_2Cl_2 (5 ml) was stirred overnight at room temperature. Subsequently, the reaction mixture was diluted with ethyl acetate (10 ml) and washed with saturated aqueous NH_4Cl and distilled water. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure to yield *N*-(3-cholesteryl- oxycarbony)-6-amino-1-hexanol (85 mg, 97%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.30 (dd, 1H), 4.54 (m, 1H), 4.42 (m, 1H), 3.57 (t, 2H), 3.10 (q, 2H), 2.29 (dd, 1H), 2.19 (t, 1H), 0.94 (s, 3H), 0.85 (d, 3H), 0.80 (d, 6H), 0.61 (s, 3H).

N-*(3-Cholesteryloxycarbony)-6-Amino-1-Hexanal*

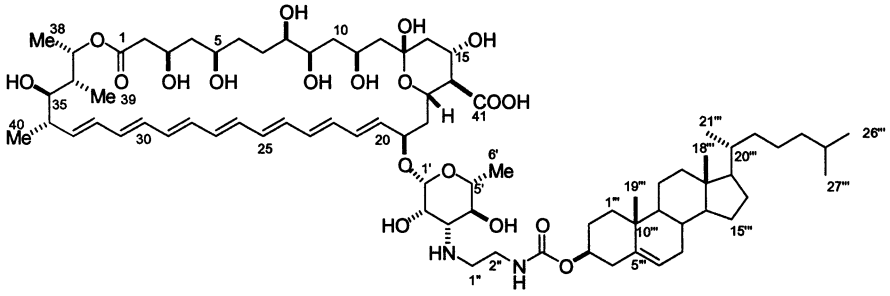
A CH_2Cl_2 (2 ml) solution of DMSO (160 μl) was dropped into a stirred solution of oxalyl chloride (160 μl, 1.83 μmol) in CH_2Cl_2 (2 ml) at −78°C. After 10 min at −78°C, a CH_2Cl_2 solution (5 ml) of *N*-(3-cholesteryl- oxycarbony)-6-amino-1-hexanol (463 mg, 0.87 μmol) was slowly added to the solution, and the resulting mixture was stirred at −78°C for 15 min and then at −45°C for 1 hr. The solution was treated with triethylamine (1 ml) and stirred for 20 min at 0°C. The reaction was quenched with saturated aqueous NH_4Cl (10 ml) and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. Column chromatography on silica gel (hexane: EtOAc 3:1) provided *N*-(3-cholesteryl-oxycarbony)- 6-amino-1-hexanal (281 mg, 61%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.74 (t, 1H), 5.35 (dd, 1H), 4.58 (m, 1H), 4.46 (m, 1H), 3.14 (q, 2H), 2.42 (dt, 2H), 2.34 (dd, 1H), 2.24 (t, 1H), 0.98 (s, 3H), 0.89 (d, 3H), 0.84 (d, 6H), 0.65 (s, 3H).

AmB-C_6-Cho (5)

A solution of *N*-(3-cholesteryloxycarbony)-6-amino-1-hexanal (10 mg, 19 μmol) and AmB (20 mg, 22 μmol) in $CHCl_3$-MeOH-water (10:6:1, 3 ml) was stirred for 2 hr, and $NaBH_3CN$ (30 mg, 0.48 mmol) was added to the solution. After 15 hr, diethyl ether was added to form a yellowish precipitate. The precipitate was collected on Celite and washed with diethyl ether. Purification was achieved by MPLC (gel: YMC ODS-AQ120-S50) eluting with MeOH-5 mM ammonium

Table 1. ^{1}H NMR Chemical Shifts for AmB-C_2-Cho (**4**) in DMSO-d_6:$CDCl_3$ = 19:1

Position	δ	Position	δ	Position	δ
2	2.21	32	6.08	6′	1.18
3	4.06	33	5.44	1″	2.52, 2.70
4	1.43	34	2.32	2″	3.08
5	3.58	35	3.13	2‴	1.79
10	1.60	36	1.77	3‴	4.33
11	4.23	37	5.21	4‴	2.28
14	1.93, 1.12	38	1.12	6‴	5.33
15	4.04	40	1.04	7‴	1.93
16	2.02	41	0.92	18‴	0.64
17	4.23	1′	4.35	19‴	0.97
18	2.02	2′	3.80	20‴	1.38
19	4.41	3′	2.80	21‴	0.90
20	6.00	4′	3.19	25‴	1.51
21–31	6.0–6.5	5′	3.19	26‴, 27‴	0.85



acetate buffer (pH 5.3) (linear gradient from 8:2 to 10:0 in 20 min) to furnish AmB-C_6-Cho (**5**) (5.7 mg, 21%). HPLC retention time (column: YMC-ODS AM-323 ϕ 10x250 mm): 53.2 min (flow rate, 1.5 ml/min; eluent, MeOH-5 mM ammonium acetate [pH 5.3] changing linearly from 8:2 to 10:0 in 20 min). ESI-MS *m/z*: 1435.6 $(M+H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6:$CDCl_3$ = 19:1): δ 1.45-1.55 (m, 8H, 2″-5″). All other ^{1}H NMR signals are identical with those of AmB-C_2-Cho (**4**).

Liposome Preparation

Large unilamellar vesicles (LUV) were prepared according to methods reported by Herve et al. [17]. Briefly, 72 μmol of egg phosphatidylcholine and the corresponding amount of AmB or AmB conjugate were dissolved in 1 ml of MeOH-$CHCl_3$, and the mixture was evaporated to a thin film in a round-bottom flask. In the case of 5% ergosterol-containing LUV, 5 mol% of egg phosphatidylcholine was replaced with ergosterol. After drying in vacuo for over 8 hr, 1 ml of pH 5.5 buffer containing 0.4 mM KH_2PO_4 and 1mM EDTA in H_2O-D_2O 6:4 was added to the flask. The lipid mixture was suspended in the buffer by vortexing and sonication. The resultant suspension was frozen at −20°C and thawed at 60°C three times. The LUV suspension was diluted with 5 ml of 0.4 mM K_2SO_4 and mixed with 9.7 μl of 1 mM carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) in ethanol. The LUV thus obtained was passed repeatedly through a membrane filter (pore size 0.2 μm, 19 times) with a Liposofast apparatus (AVESTIN). We checked the retention rate of **2** and **4** after the process for liposome formation, and more than 90% of the drug was retained in the liposome membrane at the sample/lipid ratio below 10^{-3}.

K^+ Flux Assays Using ^{31}P NMR

For K^+ flux assays, an LUV suspension was adjusted to pH 7.5 with KOH. After 3 hr at 23°C, 550 μl of the LUV suspension (lipid concentration was 12 mM) was transferred to a 5-mm NMR glass tube and mixed with 4.4 μl of 100 mM $MnCl_2$. ^{31}P NMR spectrum was recorded at 23°C on a JEOL GSX-500 spectrometer (^{31}P at 202.35 MHz) with ^{1}H-broad band decoupling. The assay was generally repeated more than three times for each sample with good reproducibility. The concentration-activity relationship shown in Figure 5B was depicted by making liposomes with various sample/lipid molar ratios. The incubation time was 6 hr. Sterol-free liposomes were used for the conjugates **2** and **4**, whereas 5% ergosterol was contained in the liposomes for AmB. At higher concentration above $R = 10^{-3}$, the K^+ flux activity could not be determined because the conjugates **2** and **4** were significantly lost due to absorption to a filter membrane during liposome preparation. The y axis is a ratio of a peak area at δ 3.1 out of the integration for δ 1.2–3.1.

UV Spectra

For the UV spectral measurements, LUV was prepared by the same method described above except for using 9% sucrose solution; at low AmB-lipid ratios (*R*) below 0.001, phosphate buffer caused light scattering. Concentration of conjugate **2** or **4** was 1.4 μM with the ratio of conjugate/lipid (*R*) of 3×10^{-4}.

Channel Current Recording

Single-channel recordings from lipid bilayers were obtained using the tip-dip method. In brief, patch-clamp pipettes (Harvard GC-150T-10) were made using a P97 Sutter Instruments puller (Novato, CA) to have a tip diameter of 10 μm. The same solution was used both in the bath and in the pipette (3 M KCl, 2.5 mM HEPES, 1 mM $CaCl_2$, pH 7.4). Monolayer was formed by spreading *n*-hexane solutions of mixed lipids on the surface of the bath. The pipette tip, precoated with hexadecane, was repeatedly passed through the surface of the solution until the pipette resistance rose above 5 gigaohms. In this study, three kinds of premixed lipids were used: (1) diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Inc.), ergosterol, and AmB (15000:3000:1 in molar ratio) for AmB single-channel recording; (2) DPhPC and **2** (15000:1 in molar ratio) for recording of AmB-ergosterol conjugate; and (3) DPhPC and **4** (15000:1 in molar ratio) for AmB-cholesterol conjugate. AmB, **2**, or **4** was further added to the bath at the concentration of 3 μM prior to channel recordings. A patch-clamp amplifier CEZ-2400 (Nihon-Kohden, Japan) was used to record single-channel currents. The applied potential was 150 mV. Current recordings were digitized at 5 kHz and filtered at 1 kHz. Single-channel analyses were carried out using the commercial software pClamp 9 (Axon Instruments, Novato, CA).

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