Role of polyol moiety of amphotericin B in ion channel formation and sterol selectivity in bilayer membrane

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ABSTRACT

Amphotericin B (AmB) is a polyene macrolide antibiotic widely used to treat mycotic infections. In this paper, we focus on the role of the polyol moiety of AmB in sterol selectivity using 7-oxo-AmB, 7 α -OH-AmB, and 7 β -OH-AmB. The 7-OH analogues were prepared from 7-oxo-AmB. Their K⁺ flux activity in liposomes showed that introduction of an additional ketone or hydroxy group on the polyol moiety reduces the original activity. Conformational analyses of these derivatives indicated that intramolecular hydrogen-bonding network possibly influenced the conformational rigidity of the macrolactone ring, and stabilized the active conformation in the membrane. Additionally, the flexible polyol leads to destabilization of the whole macrolactone ring conformation, resulting in a loss of sterol selectivity.

Amphotericin B (AmB, **1** in Figure 1) is a polyene macrolide antibiotic isolated from *Streptomyces nodosus*. Since its discovery in the early 1950s, AmB has been used widely for the treatment of fungal infection because of its efficacy and reduced development of resistance.^{1,2} The mechanism of its anti-fungal activity, involving a barrel-stave-type ion channel model is widely accepted, where AmB forms a stable ion channel in the presence of ergosterol found in the fungal cell membrane.³ The higher selectivity for ergosterol over the mammal counterpart, cholesterol, is thought to account for its pharmacological activity. Recently, another mechanism called the “AmB sponge model” has also been proposed, in which AmB is thought to extract ergosterol from the fungal membrane to form extra-membranous aggregates.⁴

Regarding functional groups responsible for AmB’s physicochemical features, the chemical structure can be divided into five fragments: heptaene, mycosamine, the polar head and tail parts, and the polyol. To elucidate the role of the functional moieties in pharmacological activity and side effects, numerous structure activity relationship (SAR) studies have been carried out for both AmB and sterols. For example, amphotericin A and nystatin, which have a saturated bond between C28-C29, show lower anti-fungal activity than AmB.⁵ Furthermore, substitution of the heptaene moiety with a biphenyl linker markedly decreases the activity of AmB,⁶ indicating the importance of the conjugated polyene in the drug’s efficacy. In addition, detailed SAR studies on sterols were conducted to evaluate their hydrophobic interactions.^{7,8} For the mycosamine moiety, chemical modification of the C3’-amine group revealed that the positive charge of the amine was necessary for its activity.⁹ More recent SAR studies disclosed that the C2’-OH group affects the sterol selectivity.^{10,11} For the polar head moiety, AmB derivatives whose carboxylic acid was esterified or reduced to a methyl group are reported to retain the membrane-permeabilizing activity.^{9,12-15} However, removal of the

hydroxy group at C35 of the tail moiety markedly reduced anti-fungal and membrane-permeabilizing activities.

The role of a polyol moiety has been discussed previously on the basis of fluorescence spectroscopy¹⁶⁻¹⁸ and molecular dynamics (MD) simulations.^{19,20} These studies suggest that, in addition to the function as the channel lining to facilitate ion passage,²¹ the polyol moiety forms intermolecular hydrogen bonds that play a role in formation of a transmembrane channel while other intermolecular hydrogen bonds result in the formation of inactive aggregates.²⁰ However, SAR studies for the polyol moiety are rather limited primarily owing to difficulty in selective modifications of the multiple secondary hydroxy groups. Genetic engineering is a potential approach to induce structural diversity in polyketide metabolites such as polyene macrolide antibiotics.^{22,23} The biosynthetic pathway of AmB has been disclosed,²⁴ which enables the production of polyol-modified analogues such as 7-oxo-AmB **2** and 8-deoxy-AmB.^{25,26} More recently, the SAR studies of AmB derivatives with modification of the polyol moiety have revealed that repositioning of the C8 hydroxy of AmB to the C7 position significantly decreased both anti-fungal and hemolytic activities.^{27,28} However, the structural diversity of AmB analogues derived from genetic engineering is rather limited. In addition, no experimental results on the role of the polyol portion in sterol selectivity have been reported so far. Therefore, in this study, we aim to combine the genetic technique and chemical modification to synthesize new AmB derivatives, 7 α -OH-AmB **3** and 7 β -OH-AmB **4**, and evaluate their membrane-permeabilizing activity and sterol selectivity.

Figure 1 (a) Chemical structures of AmB, ergosterol, and cholesterol. (b) AmB derivative used in this study.

Materials and Methods

Materials

As previously reported, 7-oxo-AmB was synthesized by genetic engineering methods.²⁵ AmB, cholesterol, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), 2',7'-bis(2-carboxyethyl)-4(5)-carboxyfluorescein (BCECF), NaBH₄, and valinomycin were purchased from Nacalai Tesque (Kyoto, Japan). Ergosterol was obtained from Tokyo Kasei (Tokyo, Japan), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was obtained from NOF Corp (Tokyo, Japan). All other chemicals were obtained from standard vendors and were of analytical grade. Reverse-phase column chromatography was performed with Cosmosil, 75 C₁₈-OPN (Nacalai Tesque). HPLC purification was performed by COSMOSIL Packed Column, 5C₁₈-MS-2 (Nacalai Tesque, 10ID × 250 mm). Solution NMR spectra were recorded on ECA-500 spectrometer (JEOL, Tokyo, Japan) and AVANCE 700 (Bruker, Massachusetts, USA). Mass spectra was measured on an LTQ-Orbitrap XL (Thermo Fisher Scientific, Massachusetts, USA)

Preparation of 7-OH-AmB

To a stirred solution of 7-oxo-AmB (12.7 mg, 13.5 μ mol) in 1.6 mL DMSO/THF (1:1) was added NaBH₄ (2.05 mg, 4.0 equivalents) at 0 °C. The reaction mixture was stirred for 10 min and then quenched with 100 μ L saturated aqueous NH₄Cl. To remove the salt, the product was purified with short reverse-phase column chromatography, and the solvent was evaporated. Further purification was performed with HPLC (Figure S1, COSMOSIL Packed Column, 5C₁₈-MS-2, 10ID $\times$ 250 mm, solvent gradient: 30% 0.5 mM ammonium acetate buffer in MeOH to 0% for 30 min). 7 α -OH-AmB and 7 β -OH-AmB was obtained with a yield of 5% and 18%, respectively. These yields were calculated from UV spectral absorption at 415 nm (ϵ_{415} =1.28 $\times$ 10⁵). 7 α -OH-AmB: yellow solid; HRMS (ESI) calcd for C₄₇H₇₃NO₁₈Na 962.4725, found 962.4729. 7 β -OH-AmB: yellow solid; HRMS (ESI) calcd for C₄₇H₇₃NO₁₈Na 962.4725, found 962.4728. ¹H and ¹³C chemical shifts are shown in Table S1.

Antifungal and hemolytic activity assays

Antifungal tests were carried out by the standard method with the liquid culture of fungi according to the protocol 27-A of Clinical and Laboratory Standards Institute. *Candida albicans* (ATCC 36082) was precultured in Sabouraud dextrose agar at 30 °C for 24 h. A series of DMSO solutions of a sample tested were prepared by 2-fold dilution steps and its 2- μ L aliquot was added to the *Candida* culture (198 μ L) in a 96-microwell plate (Costar) containing a culture medium consisting of PRMI1640 (1 w/v%, Sigma R6504), MOPS (0.165 M, Sigma M3183), and glucose (2 w/v%, Nacalai) at PH 7. After incubation at 35 °C for 2 days, MIC (minimum inhibition concentration) was obtained by a standard method.

To obtain a suspension of erythrocytes, human blood was centrifuged at 2000 rpm and supernatant liquid was decanted off. Precipitated erythrocytes were washed with PBS buffer

three times. Obtained erythrocytes were suspended to PBS buffer to a 100-fold volume of original human blood. To a 190 μ l volume of this suspension was added 10 μ l of AmB derivative solution in DMSO. These suspensions were incubated with gradually shaking at 38 °C for 18 h. Suspensions were centrifuged at 2000 rpm for 5 min, and absorbance of supernatant at 450 nm was measured with microplate reader (Molecular Devices). For the positive control, 1 % erythrocyte solution was prepared with distilled water. And for the negative control, 10 μ l of DMSO was added to a 1% suspension of erythrocyte with PBS buffer.

K⁺ influx assays using BCECF

K⁺ influx assays were performed following the procedure in a previous report.²⁹ In this experiment, pH change followed by K⁺ influx inside the liposomes was monitored using the pH-sensitive fluorescent dye, BCECF. POPC and sterol (5 mol%), ergosterol or cholesterol, were dissolved in CHCl₃, and solvent was evaporated slowly to form a membrane film inside a round-bottomed flask. The film was dried under vacuum for more than 6 h. BCECF solution (1 mL; 76 μ M) in phosphate buffer (0.15 M, pH 5.6) was added to the film for hydration. The lipid film was suspended in this solution by vortex and sonication and subjected to a freeze/thaw cycle five times. The resulting suspension was sized by extrusion (19 times) using a Liposofast[®] syringe extruder (200 nm pore size, Avestin Inc., Ottawa, Canada) to form large unilamellar vesicles entrapping BCECF (LUVs). The remaining BCECF outside the liposome was removed by gel filtration on Sepharose 4B (Sigma-Aldrich, St. Louis, MO, USA) with 0.15 M potassium phosphate buffer (pH 5.8). After determining the lipid concentration of the suspension by Phospholipid C-test (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), it was diluted with 0.15 M potassium phosphate buffer (pH 5.8) to get an LUV solution containing 0.5 mM POPC. To a 5-mL aliquot of this solution, 50 μ L FCCP in

EtOH (1 mM) was added and the resulting suspension was cooled to 6 °C. The liposome solution was poured into a cuvette containing 1.8 mL aqueous K₂HPO₄ (0.15 M, pH 9.2) and positioned in a fluorescent spectrometer (FP-6600, JASCO, Tokyo, Japan) equipped with a magnetic stirrer. AmB (10 µL; 32 µM) or its derivatives in DMSO (10 µL) were added to the stirred solution. The change in pH caused by the K⁺ influx was monitored by the increase in fluorescence intensity (excitation 500 nm; emission 535 nm) at 6 °C. After recording for 70 s, potassium ionophore valinomycin in MeOH (5 mM, 10 µL) was added to obtain the fluorescence intensity corresponding to 100% exchange ($F_{\max}$). The resulting time-dependent fluorescence intensity change was normalized to the $F_{\max}$ value. For quantitative analysis, the initial K⁺/H⁺ exchange, r , was calculated from the fluorescence intensity change immediately after the addition of AmB by fitting the curve to the exponential curve ($F = A(1-\exp(-t/T))$). The r value (represented as A/T) was expressed as the rate of K⁺ entering the liposomes per second with respect to the initial [K⁺] difference between the outside and inside of the liposomes. The experiments were performed in triplicate and the averages of the r values and standard errors were obtained.

Conformational Search

All calculations were carried out with Macromodel software version 9.9 (Schrödinger, New York, NY, USA) installed on a personal computer running the Windows 7 operating system. Initial atomic coordinates and structure files were generated from the crystal data of *N*-iodoacetyl AmB.³⁰ Initial structures of newly synthesized derivatives were generated by substitution of atoms into the crystal data, and the C41 carbonyl and C3' amino groups were calculated as COO⁻ and NH₃⁺, respectively. The sampling of the conformation was performed with Monte Carlo Multiple Minimum method.³¹ OPLS_2005 force field³² implemented in Macromodel was used and 5000 random conformers were generated and

minimized by the conjugate gradient method. For the partial charges of polar functional groups, the following values were used; NH_3^+ : 0.300(N) and 0.330(H), COO^- : -0.800(O), OH: -0.683(O) and 0.418(H), acetal: -0.583(O). A continuum solvation model using a generalized Born-surface area (GB/SA) method³³ was applied for water through calculation.

Results

Preparation of 7-OH-AmBs and assignment of stereochemistry

Two diastereomers, 7 α -OH-AmB **3** and 7 β -OH-AmB **4**, were prepared by reduction of 7-oxo-AmB **2**, which has been reported previously²⁵, with sodium borohydride, and purified with HPLC. By various NMR measurements including ^1H - ^1H COSY, NOESY, and HSQC spectra (Figures S2-S10), chemical shifts of ^1H and ^{13}C atoms were assigned and are listed in Table S1. Stereochemistry of the C7 position was determined by ^{13}C NMR chemical shifts (δ_{C}) based on the universal NMR database method; the 1,3,5-triol system³⁴ was applied for the C3-C7 portion of 7-OH-AmBs (Figure 2a, b). In this case, the δ_{C} values of the central OH group is smaller by ~2 ppm for the *syn/anti* triol than that for the *syn/syn* triol (Figure 2c). Assuming that the chemical shifts are not influenced by functional groups that are two bonds or more away, the difference of $\delta_{\text{C}5}$ between **3** and **4** largely reflects the stereochemistry of C7. Thus, their chemical shifts can be subjected to the database for assigning the stereochemistry at C7 (Figure 2a, b). Additionally, an NOE correlation between H-7 and H-9 was observed only for 7 β -OH-AmB. (Figure 2d).

Figure 2. ^{13}C chemical shift of (a) 7 α -OH-AmB **3** and (b) 7 β -OH-AmB **4**. (c) ^{13}C chemical shift of 1,3,5,7-tetraol.³⁴ (d) NOE correlation, which is critical for the determination of stereochemistry

Antifungal and hemolytic activities

Since the sample size of analogues **3** and **4** was not enough to extensively evaluate their antimicrobial activity by standard methods, we run antifungal assays only with *Candida albicans*. The analogues **2**, **3** and **4** showed somewhat lower than AmB, as shown in Table 1;²⁵

7 β -OH-AmB **4** revealed slightly higher activity than 7 α -OH-AmB **3**. Next, the hemolytic activities of **3** and **4** were examined. As compared with AmB, the AmB analogues showed slightly lower, however potent hemolytic activity. On the other hand, 7-oxo-AmB did not induce significant hemolysis in the concentration tested (Table S1).

Table 1. Antifungal and Hemolytic Activities of AmB and its derivatives

	Antifungal (MIC, μM)	Hemolysis (μM) ^{a)}
AmB	0.25	3.2
7-oxo-AmB	2	>15 ^{b)}

7 α -OH-AmB	4	5.4
7 β -OH-AmB	1	6.9

a) 50% hemolysis

b) 10% hemolysis was induced at 15 μ M of 7-oxo-AmB.

K⁺ flux assays

Since we wanted to focus on the role of the polyol moieties in forming ion channels, we adopted K⁺ flux assays with artificial bilayers. In these assays, the ion flux activity of AmB analogues can be examined by changing the membrane sterol without altering any other conditions. To examine the membrane-permeabilizing activity and sterol selectivity of **2**, **3** and **4**, we assessed AmB-induced K⁺ influx using a pH-dependent fluorescent dye, BCECF.²⁹ In this assay, pH change in liposomes caused by K⁺ influx through the AmB channel is monitored by the change in fluorescence intensity of BCECF. We adopted POPC as a membrane phospholipid and evaluated the K⁺ influx activity of AmB analogues using sterol-free and cholesterol- and ergosterol-containing POPC liposomes. Changes in pH in the vesicles induced by AmB derivatives are shown in Figure 3 **a-d**, and the K⁺/H⁺ exchange rate of each experiment is summarized in Figure 3**e**. All the polyol derivatives showed significantly lower activity than AmB. However, both 7 α -OH-AmB and 7 β -OH-AmB retained ergosterol selectivity to a certain extent, and 7 α -OH-AmB was slightly more potent than 7 β -OH-AmB. Meanwhile, 7-oxo-AmB largely lost the K⁺ flux activity and sterol selectivity.

Figure 3. K⁺ permeability of AmB 1 and its derivatives 2, 3, and 4 in liposomes. pH change induced by K⁺/H⁺ exchange was monitored by pH-dependent fluorescent dye, BCECF. (a)~(d) Time-dependent pH changes inside liposomes were recorded with 5 mol% ergosterol in POPC (blue), 5 mol% cholesterol in POPC (magenta), and POPC only (light green). AmB 1 (a), 7-oxo-AmB 2 (b), 7 α -OH-AmB 3 (c), and 7 β -OH-AmB 4 (d) in DMSO were added at a ratio of $R_{(\text{AmB}/\text{Lipid})}=3.16 \times 10^{-3}$. (e) Exchange rate immediately after adding AmB (r) was calculated as $\Delta F = A/T$. Time-dependent fluorescence change was fitted to the exponential

curve ($F = A(1-\exp(-t/T))$). The initial fluorescence values for 60 s were used for the fitting analysis. * r was calculated by linear fitting. (f)/(g) Ratio of the K^+/H^+ exchange rate between two types of membrane. 5% ergosterol/POPC only (f) and 5% cholesterol/POPC (g). Data in (e)–(g) were obtained from triplicate experiments and expressed as mean $\pm$ S.E.M.

Conformational search

In order to investigate the difference in conformation and hydrogen bond network of the polyol analogues of AmB, we carried out a conformational search in the water environment using the GB/SA solvent model,³³ in which solvation free energy was calculated as a sum of the electrostatic term and non-electrostatic term; the former term was obtained from the Generalized Born equation, and the latter was calculated based on the solvent accessible surface area. Figure 4 illustrates the most stable conformation of AmB and its analogues. The calculations show that 7-oxo-AmB **2** prefers a totally different conformation from those of AmB and the 7-OH-AmBs because of the sp^2 carbon at the C7 position. Consequently, the conformations prevent the polyol moiety from formation of stable intramolecular hydrogen bonding network, therefore markedly increasing molecular flexibility; various conformers were obtained within the 5 kJ/mol energy level (Figure S11).

Regarding AmB **1**, 7 α -OH-AmB **3**, and 7 β -OH-AmB **4**, the conformation of the polyol moiety can be categorized into two types; one is the fully extended conformation, which consists of only the *anti* (extended) conformation, and the other is the C6/C7-*gauche* conformation. The most stable conformation of AmB in water is assumed to be C6/C7 *gauche*, while the extended conformer (Figure 4e) turned out to be less stable by around 10 kJ/mol compared with that of the *gauche* conformation. We previously reported that, based on NMR data, the polyol moiety of AmB predominantly adopts the extended conformation in

SDS micelles,³⁵ which suggested that AmB changes its conformation from the *gauche*-containing conformation to the extended one upon binding to the membrane.

For 7 α -OH-AmB **3**, only the *gauche*-containing conformation was observed within 21 kJ/mol from the most stable conformation. The extended conformation was apparently unfavorable due to a close contact of 7 α -OH and heptaene in this orientation (Figure S12). All the conformers of **3** deduced from the MD calculations had a hydrogen bond between the 5-OH and 8-OH, and were particularly similar to those of AmB. In contrast, the most stable conformation of 7 β -OH-AmB **4** was calculated as the fully extended conformation, which was stabilized by hydrogen bonding between the 5-OH and 7-OH. A C9-C10 *gauche* conformation was also observed in **4** as a less stable conformation.

Figure 4. Most stable conformation based on conformational search by Monte Carlo Multiple Minimum. The force field was OPLS-2005, and the GB/SA water model was used. (a) AmB 1, (b) 7-oxo-AmB 2, (c) 7 α -OH-AmB 3, (d) 7 β -OH-AmB 4. (e) *anti*-Conformer of AmB. Yellow dotted lines indicate hydrogen-bond network.

Discussion

The results of the K⁺-flux assays and conformational search suggest that the activity of the AmB analogues is closely related to the hydrogen-bonding network and the conformation of the polyol moiety. For example, 7 α -OH-AmB stabilizes the *gauche*-containing conformation, which is possibly similar to that of AmB in water, and prevents formation of the extended

conformation. Recently, we have reported the SAR results of various sterol analogues, and found that the AmB-ergosterol interaction is probably stabilized by face-face VDW interaction.^{7,8} Thus, the *gauche*-containing conformation induced by 7 α -OH-AmB may prevent the VDW contact between the AmB macrolide and sterol, consequently altering the sterol selectivity of **3** (Figure 3 f/g). The C6/C7-*gauche* conformation of **3** was suggested by NOE experiments for a derivative of **3** (Figures S13 and S14). On the other hand, 7 β -OH-AmB is stabilized in the fully extended conformation, which is thought to be suitable for the VDW interaction with sterol. Therefore, it seems that 7 β -OH-AmB retains ergosterol selectivity to an extent as suggested by the K⁺ flux assays (Figure 3 f/g) and the antifungal activity (Table 1). Meanwhile, the activities of 7 β -OH-AmB **4** for sterol-containing liposomes were significantly weaker than those of natural AmB. To estimate the affinity between **4** and ergosterol, we measured the UV/VIS spectra of their POPC liposomes containing 5% mol ergosterol; the absorption maximum at 415 nm has been reported to reveal the direct interaction between AmB and sterol in membrane.^{36,37} As shown in Figure S17, this absorption was observed only for the AmB/ergosterol membrane while the **4**/ergosterol preparation showed the peak not at 415 but at 408 nm; the spectra implied that **4** has lower affinity to ergosterol, and thus its channel assembly is probably less stable than that of AmB.^{8,37,38} Another possible explanation is that the extended conformation enhances the stability of large aggregates in water (Figure S16), which prevent the analogue to bind to membrane. A recent spectroscopic study and MD simulation¹⁸ indicated that the AmB dimer in water exhibited the extended conformation.

As suggested by its weak antifungal activity,²⁵ 7-oxo-AmB **2**, which lacks the specific hydrogen-bonding network in the polyol moiety, lost the sterol selectivity. The K⁺-flux assays in Figure 3 f/g indicate that the membrane activity of **2** is comparable with that of natural AmB in the absence of sterol but significantly reduced in the presence of ergosterol. As

described above, it is plausible that the flexibility of the polyol conformation influences the conformation of the polyene moiety (Figures 4d, and S8b), consequently reducing the rigidity of the macrolide ring. A similar observation has been reported for nystatin, which has a saturated site in the polyene moiety and thus shows higher flexibility in the macrolide ring; nystatin shows much less sterol selectivity than AmB on POPC membrane in liposome rupture experiments.³⁹ The reason for the inhibitory effect of ergosterol toward 7-oxo-AmB **2** is, however, unknown.

CONCLUSION

We prepared AmB derivatives **3** and **4** for SAR probe from the AmB analogue **2** produced by genetically engineered microbes. While 7-oxo-AmB **2** completely lost sterol selectivity, 7-OH-AmBs **3** and **4** retained sterol-selective activity to an extent. The conformational search of these AmB analogues implies that the hydrogen-bonding network is crucial for the polyol conformation, which is basically in line with the activity of AmB. These findings suggest that 7 β -OH-AmB prefers the fully extended conformation, which may be similar to the membrane-bound form of natural AmB, and thus analogue **4** may be a useful molecular probe to further investigate the membrane-bound conformation of AmB implicated in its sterol-selective ion channels. The present study implies that the conformational stability in the macrolide ring is particularly important for interaction with ergosterol.

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ABBREVEATIONS

AmB, Amphotericin B; BCECF, 2', 7'-bis(carboxyethyl)-4(5)-carboxyfluorescein; COSY, correlation spectroscopy; FCCP, carbonyl cyanide-*p*-trifluoro-methoxy-phenyl hydrazide; HSQC, hetero-nuclear single quantum coherence; NOESY, nuclear Overhauser effect correlated spectroscopy; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SAR, structure activity relationship

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Role of polyol moiety of amphotericin B in ion channel formation and sterol selectivity in bilayer membrane

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Additional hydroxy-bearing AmB were prepared from bioengineered 7-oxo AmB. Their efficacy and sterol selectivity in K⁺ flux assays revealed the importance of intramolecular hydrogen bonds in the rigidity of the macrolactone ring.