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Amphotericin B Covalent Dimers Forming Sterol-Dependent Ion-Permeable Membrane Channels

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For over 30 years, the polyene antibiotic amphotericin B (AmB, **1**) has been used for the treatment of systemic fungal infections. Its antifungal action is generally accounted for by the formation of an ion-permeable channel across the lipid bilayer, where the drug has a higher affinity for ergosterol, an abundant sterol in fungal membranes, than for cholesterol, a major constituent of mammalian plasma membranes. Despite extensive investigations by UV/CD spectroscopic methods[1] and by molecular dynamics calculations,[2] details of the molecular architecture of the ion-permeable channel are not fully understood; a generally accepted model is a barrel-stave assemblage comprising around eight pairs of AmB/sterol.[3] The mechanism of bimolecular recognition by AmB/AmB or by AmB/sterol is essential for the better understanding of the drug's action, which may lead to a reduction of serious side effects in mammals. However, the molecular assemblage is thought to reach a dissociation/association equilibrium, resulting in an exchange between conducting and nonconducting states of the channel, hampering spectroscopic observation of the active assemblage. As suggested for channel-forming peptides,[4] if dimers, instead of monomers, participate in this process, the equilibrium should shift to the associated state, thus stabilizing the assemblage long enough for NMR measurements (Figure 1). We therefore attempted to prepare covalently linked conjugates of AmB/AmB and AmB/sterol. In this communication we report preparation of AmB dimers with channel-forming ability that can be utilized in investigating some of the details underlying bimolecular recognition.

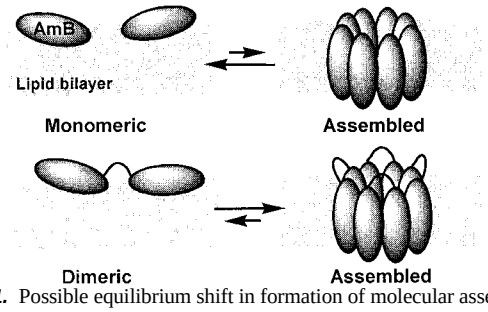
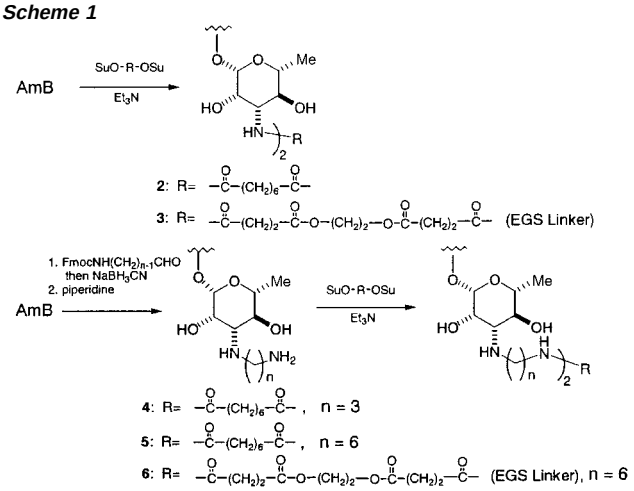


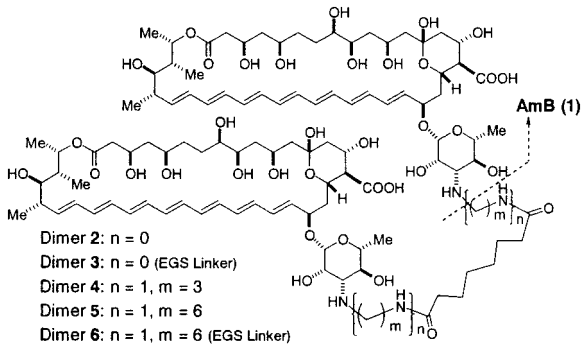
Figure 1. Possible equilibrium shift in formation of molecular assemblages by covalently linked dimers.

AmB dimers **2** and **3** were prepared by linking each amino group of AmB with dicarboxylic acid. These dimers bearing an *N*-acyl substitutent at their amino groups showed no significant bioactivity,[5] as suggested by previous reports.[6] We next prepared dimers that retained amine functionality.[7] After several trials, bioactive conjugates, which had an *N*-aminoalkylamide linkage via dicarboxylic acid, were successfully furnished (Scheme 1). Among other dimers, **4**, **5**, and **6** exhibited the prominent hemolytic activity, exceeding that of AmB.[5] Although the extremely low solubility of these dimers in most solvents hampered product recovery during purification, the coupling reaction itself proceeded in moderate yield of ca. 20% for **4** and **5**.

Scheme 1



It has been reported that cation current across a liposome membrane can be monitored by pH-dependent changes in the ^{31}P NMR chemical shift of phosphate.[8] In spectra **c** and **d** of AmB and dimer **5** (Figure 2), new signals appearing at δ 3.1 were derived from phosphate entrapped in liposomes on which AmB or dimer **5** formed ion-permeable channels and H^+ leaked out at the expense of K^+ influx. This pH rise caused a downfield shift of a phosphate



signal. AmB and dimer **5** elicited an all-or-none ion flux[9] in ergosterol-containing lipid membranes, where the conductance of their channels was so large that, once the channel was formed, K^+/H^+ exchange instantly reached equilibrium, thus leaving only two kinds of liposomes. One kind, which was free of the channels, retained an initial pH (δ 1.2), and the other, which had been permeabilized, showed a higher pH (δ 3.1).[8a] The spectra in the absence of sterol or in the presence of cholesterol (spectra **a** and **b** in Figure 2) showed markedly different features from those in the

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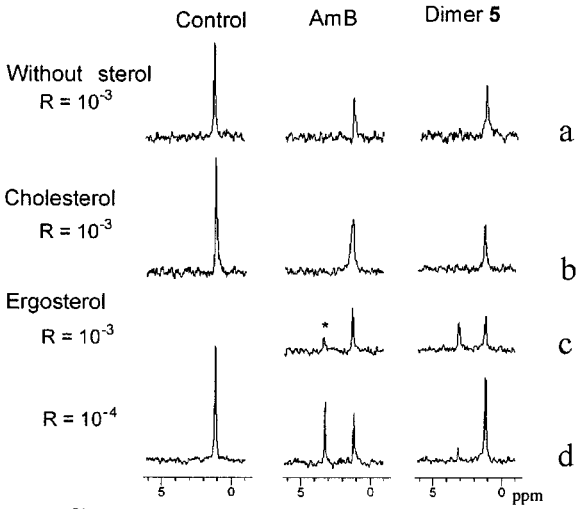

Figure 2. ^{31}P NMR spectra of liposome-entrapped phosphate for AmB and Dimer **5**. The peak around δ 1.2 corresponds to $H_2PO_4^-$ at pH 5.5 of intact liposomes, and that around δ 3.1 corresponds to HPO_4^{2-} at pH 7.5 of the buffer. (a) No sterol was present in liposome membrane, $R = 10^{-3}$ (R: sample/lipid molar ratio). (b) Phosphatidylcholine (PC)-cholesterol, $R = 10^{-3}$. (c) PC-ergosterol, $R = 10^{-3}$. (d) PC-ergosterol, $R = 10^{-4}$. Control, where no AmB or dimer was added, depicted the intensity of the peak at δ 1.2 when liposomes stayed intact. *AmB at $R = 10^{-3}$ showed an unexpected pattern, where the intensity of a phosphate signal due to permeabilized liposomes was smaller than that in trace **d** (AmB, $R = 10^{-4}$). This can be explained by disruption action of higher concentrations of AmB, which should result in not only influx of K^+ but efflux of phosphate.

presence of ergosterol, indicating that ion conductance of these channels was not high enough to induce the all-or-none flux although reductions in the intensity of signals at δ 1.2 suggested that liposome membranes were partly permeabilized by AmB or dimer **5**. These spectral characteristics of **5**, particularly ergosterol-dependent all-or-none type flux, are essentially similar to those of AmB although the efficacy of the dimer was more or less reduced. Dimers **4** and **6** also revealed a typical NMR change of the all-or-none type flux in ergosterol-containing liposomes (see Supporting Information), whereas the directly linked dimers **2** and **3** were found to lack ion-conducting activity. Assemblage formation by dimer **5** was further examined by CD spectra. In ergosterol-containing phospholipid liposomes, **5** showed a larger split Cotton effect than that of AmB (Figure 3).[10] This spectral difference appears to reflect close vicinity of heptaene chromophores of the dimers in a molecular assemblage.[10]

These findings suggest that dimers **4**, **5**, and **6** form a molecular assemblage that is functionally similar to that of AmB. With bioactive dimers in hand, regio-specific labeling with NMR nuclei for each monomer part could facilitate the determination of an interatomic distance even in highly symmetrical structures proposed for AmB channels. For this purpose, we are trying to synthesize dimers that are labeled with ^{13}C and ^{19}F nuclei for use in structure studies based on solid-state NMR.

Acknowledgment. We are grateful to Professor Yuzuru Mikami, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, for antifungal assays. This work was supported by a Grant-In-Aid for Scientific Research on Priority Area (A) from the Ministry of Education, Sciences, Sports, Culture, and Technology, Japan; by a grant from the CREST, Japan Science and Technology Corporation, and by the Yamada Science Foundation.

Supporting Information Available: Experimental sections for synthesis of AmB dimers, the preparation of liposomes, measurements of NMR and CD/UV spectra; ^{31}P NMR spectra of dimers **4**/**6**, and UV spectra of dimer **5** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

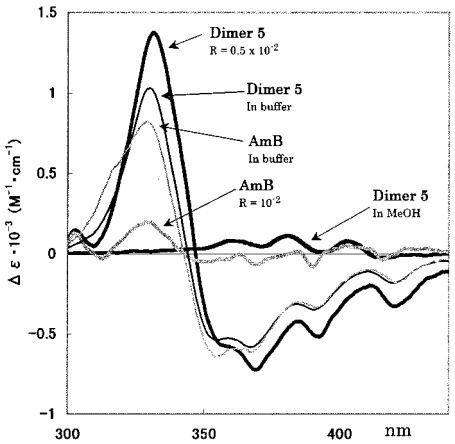

Figure 3. CD spectra of dimer **5** and AmB in phospholipid−ergosterol liposomes and in buffer without liposomes. CD spectra of dimer **5** were measured in MeOH, in buffer and in liposomes $R = 0.5 \times 10^{-2}$ (R: sample/lipid molar ratio), and a trace for AmB was obtained in the same conditions except $R = 10^{-2}$ (the same concentration as monomer AmB). The liposomes were prepared in the same manner as that for ^{31}P NMR measurements except for addition of FCCP.[8] Concentrations of dimer **5** and AmB were kept at 2.5 and 5 μM, respectively. $\Delta\epsilon$ was normalized to the concentration of the monomer part for dimer **5**.

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(5) EC_{50} values of **4**, **5**, and **6** with regard to hemolytic activities were all 0.3 μM (against 1% human erythrocytes), while the potency of AmB was 1.5 μM. Those of directly linked dimers **2** and **3** were over 100 μM. The antifungal activity of **4**, **5**, and **6** was not evident compared with that of **1**. ED_{50} values of **5** and **6** in growth inhibition assays against *Candia albicans* were 8 μM and 4 μM, respectively, which were significantly weaker than that of AmB, 0.3 μM. This low activity may be due to their low solubility or small critical micelle concentrations in aqueous media. Details in the bioactivity of the analogues will be published elsewhere.

(6) For example: Mazerski, J.; Bolard, J.; Borowski, E. *Biochim. Biophys. Acta* **1995**, *1236*, 170.

(7) Preparation of some of the AmB dimers was briefly reported in patents; Griffin, J. H. Int. Appl. PCT/US99/12699, 1999.

(8) Liposomes were prepared from a mixture of egg phosphatidylcholine−ergosterol (or cholesterol) 9:1 in phosphate buffer (pH 5.5). After sonication, freezing, and thawing, the liposomes were passed through a 0.2 μm filter, and the medium was neutralized to pH 7.5. A sample and FCCP (H^+ carrier) were added to the liposomes and left for 6 h at 23 °C. Upon NMR measurements, the concentration of lipids in the buffer was 9 mM. ^{31}P NMR was measured at 202.35 MHz after addition of 1 mM $MnCl_2$. For experimental details, see Supporting Information and the following reference: (a) Hervé, M.; Cybulska, B.; Gary-Bobo, C. M. *Eur. Biophys. J.* **1985**, *12*, 121−128. (b) Hervé, M.; Debouzy, J. C.; Borowski, E.; Cybulska, B.; Gary-Bobo, C. M. *Biochim. Biophys. Acta* **1989**, *980*, 261−272.

(9) An all-or-none ion flux is reported to occur with channel-forming ionophores such as AmB and gramicidin D, which are reported to elicit a biphasic evolution of phosphate NMR signals.[8]

(10) The increase in CD split of dimer **5** by binding to liposome membrane (as seen in Figure 3) was confirmed by different experiments, see Supporting Information. Bolard et al., however, reported that micelles of AmB formed in water give rise to the Cotton effect at 330 nm as seen for the trace for AmB in buffer (Figure 3). The large CD split for dimer **5**, therefore, may be partly due to micelles of dimer **5** in aqueous phase. (a) Bolard, J.; Seigneuret, M.; Boudet, G. *Biochim. Biophys. Acta* **1980**, *599*, 280−293. (b) Bolard, J.; Legrand, P.; Heitz, F.; Cybulska, B. *Biochemistry* **1991**, *30*, 5707−5715.

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