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Amphotericin B Dimers with Bisamide Linkage Bearing Powerful Membrane-Permeabilizing Activity

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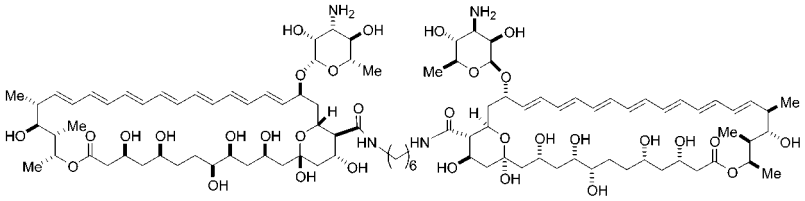
ABSTRACT



Covalently linked dimers of amphotericin B were prepared by cross-linking its carboxylic acid. Among these, a dimer with a linkage of 1,6-hexanediamine revealed potent hemolytic activity (EC_{50}, 0.25 μM) while its *N*-acetyl derivative gave rise to large K^+ ion flux in phosphatidylcholine liposomes, regardless of the presence or absence of sterols, suggesting that the dimers may serve as a tool for elucidating the structure of the ion channel assemblage formed by amphotericin B.

The polyene antibiotic amphotericin B (AmB, **1**) was first reported in 1955 from *Streptomyces* sp.[1] Since then, the drug has been widely used for treatment of systemic fungal infections. Its antibiotic action is generally accounted for by an ion channel complex, where AmB together with sterol forms molecular assemblages. Despite extensive investigations of this channel by spectroscopic and molecular dynamics methods, details of its architecture remain unelucidated. In the1970s, the well-known "barrel-stave model" was proposed, which comprises about eight pairs of AmB/sterol.[2] In this assemblage, a polyhydroxy part of AmB comes close together to form a hydrophilic pore and its hydrophobic polyene region faces outside to interact with sterols and fatty acyl chains of phospholipids. Recent studies, however, disclosed that sterol-independent pores formed by AmB play an important role in its actions.[3] Bimolecular interactions between AmB/AmB and AmB/lipids are essential for understanding the formation mechanism of the ion channel complex. More recently, we reported that AmB dimers possess ion-channel activity, which had a cross-linkage between the amino groups of AmB (*N*-linked dimers).[4] In this Letter, we wish to report the other dimers with a carboxylic acid linker (*C*-linked dimers) and their membrane-permeabilizing activities, which are markedly different from those of *N*-linked dimers.

Dimer **2** with a carboxylic acid linkage was prepared by a simple reaction sequence as shown in Scheme 1 (see Supporting Information for details): protection of an amino group of **1** with Fmoc, coupling of carboxylic acid groups with hexanediamine in the presence of benzotriazole-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), and deprotection of Fmoc. An *N*-acetyl derivative

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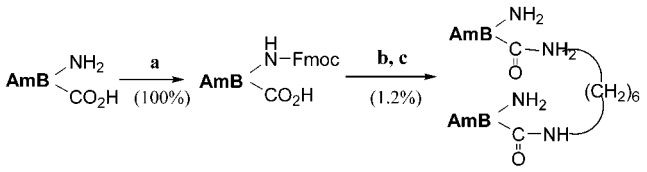
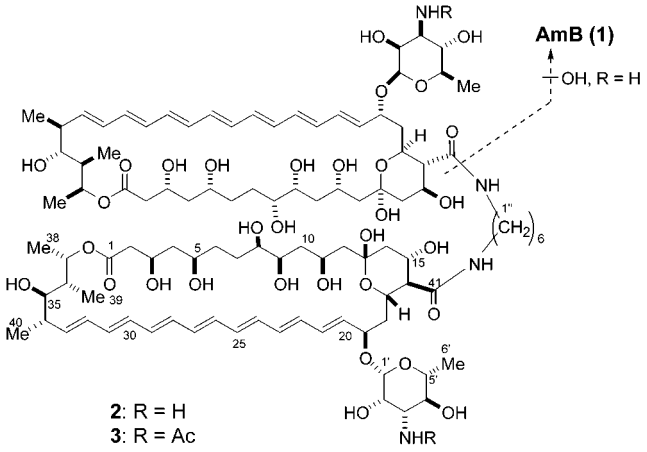
Scheme 1[a]



[a] (a) Fmoc-OSu, Et_3N, DMF/MeOH; (b) $H_2N(CH_2)_6NH_2$, DIPEA, HOBt, PyBOP, DMF; (c) piperidine.

of *C*-linked dimer **2** was prepared by acetylating an amino group with *N*-succinimidyl acetate followed by the same coupling reaction with diamine (5% yield from **1**). Low yields of the dimers were mainly due to poor solubility in solvents and low recovery during HPLC purification.



Membrane permeabilizing activity of the dimers was evaluated by two methods, hemolysis tests and K^+ flux assays using liposomes. Dimer **2** revealed powerful hemolytic activity, which exceeded that of AmB by six times.[5] Conversely, *N*-acetyl dimer **3** showed virtually no activity. It has been reported that cation current across liposomal membrane can be monitored by pH-dependent changes in the ^{31}P NMR chemical shift of phosphate.[6] In the spectra of **1** and **3** for PC-Erg (Figure 1), a signal appearing at δ 3.1 was derived from phosphate entrapped in liposomes where AmB or dimer **3** formed ion-permeable channels and H^+ leaked out via a proton transporter, FCCP, at the expense of K^+ influx. This pH rise was detected as a downfield shift of a ^{31}P NMR signal; background resonance due to external phosphate was quenched by Mn^{2+} (see Supporting Information for experimental details). The spectra of **3** (Figure 1) demonstrated two peaks for three liposome preparations, while dimer **2** gave rise to no clear peak at δ 3.1. These results indicate that dimer **3** forms K^+ channels with high

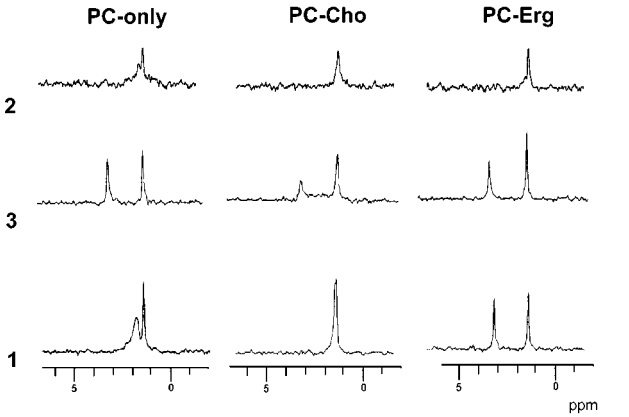


Figure 1. ^{31}P NMR spectra of liposome-entrapped phosphate for AmB **1** and dimers **2** and **3**. The peak around δ 1.2 corresponds to $H_2PO_4^-$ at pH 5.5 (initial pH) and that around δ 3.1 corresponds to HPO_4^{2-} at pH 7.5. PC-only: liposomes were prepared with PC. PC-Cho: with 10% cholesterol in PC. PC-Erg: with 10% ergosterol in PC. AmB or dimer was dissolved in DMF and added to liposome suspensions. For all experiments, molar ratios between AmB or an AmB equivalent within dimers and lipids were 1:1000.

activity; as reported by Gary-Bobo et al.,[5] once an ion channel with high activity is formed, K^+ concentration in liposomes instantly reaches equilibrium, resulting in a rapid rise of lumen pH to give rise to these two peaks. This spectral pattern is characteristic of "all-or-none" type of ion flux.

It was reported that the CD spectra of AmB in liposomes give rise to very small Cotton effects in these AmB-lipid ratios, while those in aqueous media show large CD splits.[4,7] In the CD spectra of dimers **2** and **3** (Figure 2), however, large Cotton effects were observed both in liposomes and in buffer; CD for liposomes were partly due to aqueous micelles of dimers in an aqueous phase.[4] For all conditions tested, the magnitudes of CD extrema in buffer were significantly smaller than those in both ergosterol- and cholesterol-containing liposomes. Moreover, unlike AmB,[6] the dependence of the CD magnitude of **2** and **3** on dimer/lipid ratios was not evident (see Supporting Information). These observations suggest that dimers **2** and **3** bind to membrane effectively to form a stable assemblage even in low concentrations.

The positions of CD extrema of dimer **2** are virtually identical with those of AmB,[6] demonstrating that inter- and intramolecular interactions among the heptaene chromophores mimic that of AmB.[4] Conversely, the spectra of dimer **3** gave rise to large positive Cotton effects both in liposomes and in MeOH, suggesting that **3** forms different assemblage from that of AmB or **2**. Significant CD spectral changes for dimer **3** with ergosterol liposomes, particularly three prominent positive peaks, resembling the UV absorption characteristic of AmB, suggest that **3** has some interaction with ergosterol.

(5) Hemolytic activity was measured for 1% human erythrocytes. EC_{50} value of **2** was 0.25 μM, whereas that of *N*-acetyl derivative **3** was over 30 μM. As was the case with the *N*-linked dimers,[3] antifungal activity of **2** and **3** should be weaker than that of AmB as a result of their poor solubility.

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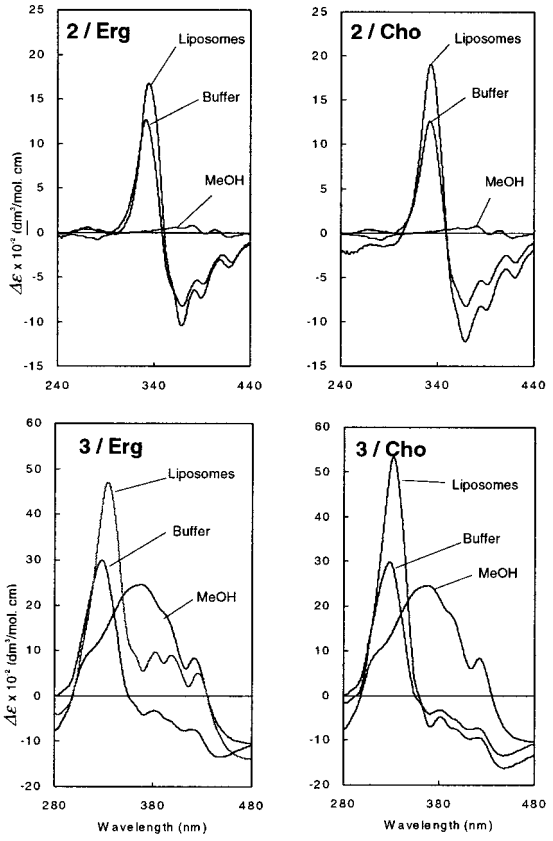

Figure 2. CD spectra of dimers **2** and **3** in ergosterol (Erg)- and cholesterol (Cho)-containing liposomes. CD spectra were recorded for the consistent concentration of dimer (2.5 μM as a dimer, not for a monomer part) with the dimer/lipids molar ratio of 0.005. The molar ratio of EggPC-sterol was 9:1.

According to previous reports by Gary-Bobo et al., *N*-acetyl AmB methyl ester, which resembled the monomeric part of dimer **3**, showed activity far weaker than that of AmB in the same K^+ flux assays.[8] Dimer **3**, however, revealed activity even greater than that of AmB for PC-cholesterol and PC-only liposomes (Figure 1). These results indicate that, by the cross-linking, ion channel activity is augmented in these K^+ flux assays. It is intriguing that ion flux induced by **3** was an all-or-none type for the liposome preparations tested and showed only marginal sterol dependence (Figure 1). Baginski et al. reported[9] that an amino function of AmB probably comes close to a hydroxy group of sterol in the channel assemblage. When the amino group of AmB is acetylated, AmB/sterol interaction hardly occurs, thereby reducing the interaction between AmB and sterol.

Dimer **2** revealed a very potent hemolytic activity to erythrocytes, while showing no indication for large K^+ flux in liposomes (in Figure 1, no clear NMR signal at δ 3.1 was observed). This discrepancy may be accounted for by the surface charge of erythrocyte membrane. Because of the cationic ammonium groups, dimer **2** should bind more efficiently to negatively charged erythrocytes than PC membrane. Yet, once dimers bind to membrane, positive charge of dimer **2** hampers formation of a stable channel aggregate. This may lead to reduction of K^+ current. In fact, the peak at δ 1.2 was markedly reduced in the spectra of **2**, demonstrating that a significant amount of a K^+ flow occurred but the current per channel was not high enough to induce the all-or-none flux.

The present results suggest that *C*-linked dimer **2** with intact amino groups forms a molecular assemblage similar to that of AmB, although its channel activity is smaller. On the other hand, dimer **3** with *N*-acetyl groups revealed potent K^+ flux activity, while its CD spectra implied different structures of ion channel assemblages. These dimers together with *N*-linked dimers[4] may provide useful molecular probes for investigating the molecular mode of action of AmB in biomembrane.

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Supporting Information Available: Experimental details for synthesis of AmB dimers and the preparation of liposomes and ^{31}P NMR, CD, and UV spectra of dimers. This material is available free of charge via the Internet at http://pubs.acs.org.

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