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Amphotericin B covalent dimers with carbonyl-amino linkage: a new probe for investigating ion channel assemblies

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Abstract—Based on an amphotericin B (AmB) ion-channel model where the close proximity of neighboring molecules is effected by interaction between carboxyl and amino groups, we prepared covalent dimers of AmB connected between these functionalities. While directly connected and short-tethered derivatives (**2** and **3**) lacked the activities, dimer **4** with a longer linker revealed K^+ ion flux activity, suggesting that some distance and/or flexibility between the carboxyl and amino groups in adjacent molecules is required for the formation of ion-permeable complex in biomembranes.

Amphotericin B (AmB, **1**), first isolated in 1955 from *Streptomyces nodosus*,[1] has been used for the treatment of fungal infections for over 50 years. It is now widely accepted that AmB molecules associate with sterols in phospholipid membranes to form barrel-stave type channels, in which their polyhydroxy portions point inward and lipophilic heptaene groups direct outward.[2] According to the computer simulations of the ion channel by Baginski et al.,[3] the amino group of AmB is thought to interact with the carboxylic acid of a neighboring AmB molecule through a hydrogen-bond.

Recently some functionalized derivatives of AmB were designed and synthesized to explore the mechanism of action of AmB in membranes.[4] We have also prepared some AmB derivatives based on the following idea: AmB channel is probably in an association–dissociation equilibrium in membranes, and therefore it is necessary to shift the equilibrium to the associated state for precisely determining the channel structure. We have already succeeded in stabilizing the channel aggregates by connecting two AmB molecules; AmB dimers cross-linked between the amino groups (*N*,*N*-dimers)[5,6] and between carboxylic acids (*C*,*C*-dimers)[7] showed similar or greater ion channel activities as compared with AmB. In this Letter, we report preparation and membrane-permeabilizing activities of dimers with a linker between the amino group and carboxylic acid (**2**–**4**, *C*,*N*-dimers, Fig. 1), which are supposed to mimic the original intermolecular interaction.[3]

The preparation methods of dimers **2**–**4** are summarized in Scheme 1 (see Supplementary data for details). After extensive optimization of reaction conditions, we established an effective coupling method although yields for **3** and **4** were still low mainly due to multiple reaction sites and low recovery during HPLC purification. Dimer **2** was prepared by coupling *N*-Fmoc-AmB **5** with AmB methyl ester (AME, **6**), followed by removal of the Fmoc group. Dimers **3** and **4** were obtained through the following steps: reductive aminoalkylation of AME **6** with aldehydes **8** or **11**, removal of Fmoc group, intermolecular amidation with *N*-Fmoc-AmB **5** in the presence of PyBOP, and deprotection of the amino group. All the dimers were purified by HPLC.

We first evaluated the biological activities of dimers **2**–**4** (Table 1). While dimers **2** and **3** were virtually devoid of hemolytic and antifungal activities, dimer **4** induced hemolysis at a concentration comparable to AmB. In addition, dimer **4** revealed antifungal action against *Aspergillus niger*, though being less efficacious than AmB (Table 1). We then assessed the K^+ permeabilizing activity using artificial liposomes composed of egg phosphatidyl-choline.[8] The liposomes possess a higher external K^+ concentration and trans-membrane pH gradient with pH 5.5 inside and pH 7.5 outside. K^+ influx into the liposomes through ion channels results in H^+

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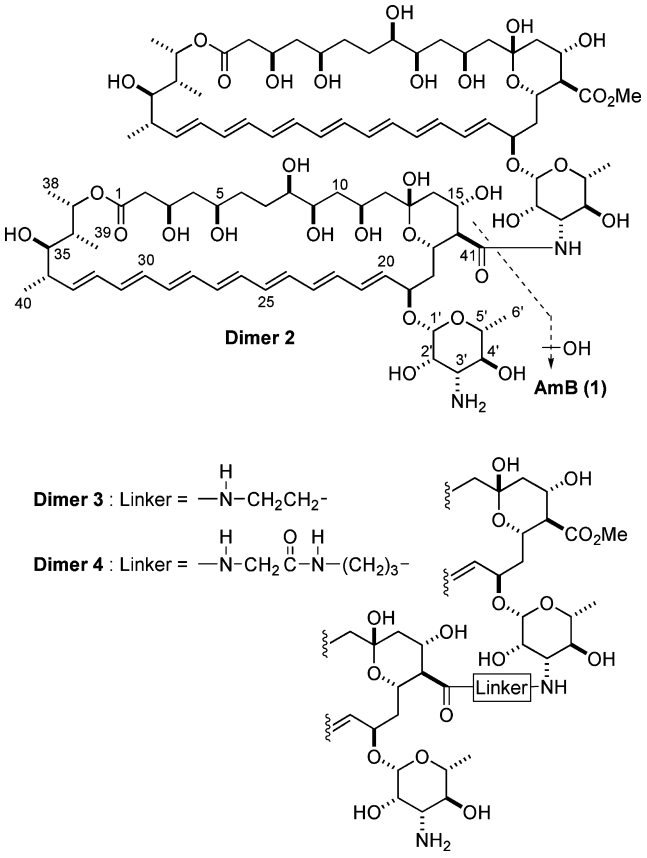


Figure 1. Structures of amphotericin B dimers with carbonyl-amino linkage.

Table 1. Biological activities of AmB **1**, and dimers **2**–**4**

	AmB (**1**)	Dimer **2**	Dimer **3**	Dimer **4**
Hemolytic activity, EC_{50} (μM)[a]	1.4	>18	>18	1.0
Antifungal activity (μg)[b]	10	>50	>50	50

[a] Against 1% human erythrocytes.
[b] The minimal amount of a sample on a paper disk that shows inhibitory zone on the culture of *Aspergillus niger*.

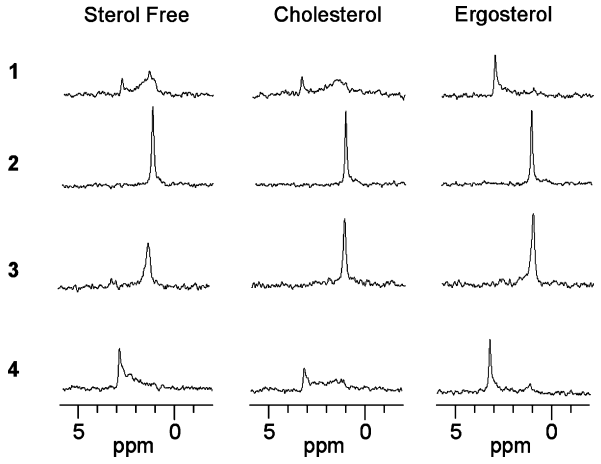
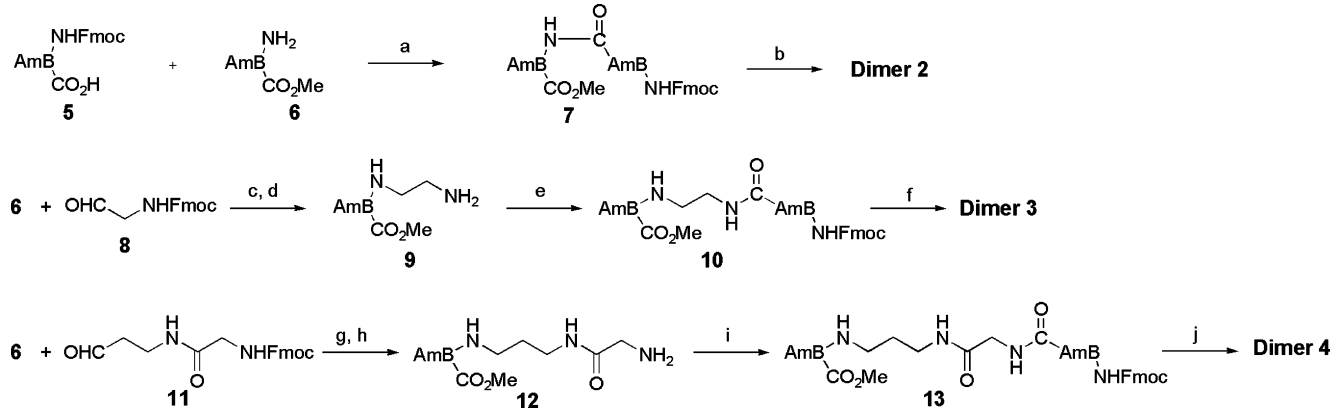


Figure 2. Membrane permeabilizing activity of AmB (**1**) and dimers (**2**–**4**) evaluated by the ^{31}P NMR spectra of liposome-entrapped phosphate. Each compound **1**–**4** was premixed with egg-phosphatidylcholine (PC) to prepare liposomes and incubated for 3 h. The lipid concentration of all the liposome suspensions was 12 mM. Liposomes comprised PC only (sterol free), 10% cholesterol-containing PC, or 10% ergosterol-containing PC. The peak around δ 1.2 is derived from $H_2PO_4^-$ at pH 5.5 (intact liposomes) and that around δ 3.1 is due to HPO_4^{2-} at pH 7.5 (permeabilized liposomes). Broad signals between δ 1.2 and 3.1 reveal the presence of liposomes with inside pH between 5.5 and 7.5. The molar ratio of drug/lipid was 1×10^{-4} for all the experiments.

leakages in the presence of a proton-transporter FCCP, which raises inner pH and induces chemical shift changes of inorganic phosphate (Fig. 2). NMR signals of phosphate ions outside the liposomes were quenched by paramagnetic Mn^{2+}. Dimers **2** and **3** show no significant channel activity in all the membranes at the drug/lipid ratio of 10^{-4} (Fig. 2), which were consistent with the lack of biological activities in **2** and **3**. On the other hand, dimer **4** revealed a distinct signal at δ 3.1 in ergosterol-containing membrane with a similar potency to that of AmB. The dimer also shows reduced ion flux in cholesterol-containing and sterol-free



Scheme 1. Reagents and conditions: (a) PyBOP, HOBt, DIPEA, DMF, rt, 18 h, 62%; (b) piperidine, DMSO–MeOH, rt, 2 h, 63%; (c) $NaBH_3CN$, DMF–MeOH, rt, 18 h, 31%; (d) piperidine, DMSO–MeOH, rt, 1 h, quant.; (e) **5**, PyBOP, HOBt, DIPEA, DMF, rt, 18 h, 38%; (f) piperidine, DMSO–MeOH, rt, 1 h, 12%; (g) $NaBH(OAc)_3$, DMF-*i*-PrOH, rt, 5.5 h, 66%; (h) piperidine, DMSO–MeOH, rt, 0.5 h, 85%; (i) **5**, PyBOP, DIPEA, DMF, rt, 4 h, 62%; (j) piperidine, DMSO–MeOH, rt, 2 h, 22%.

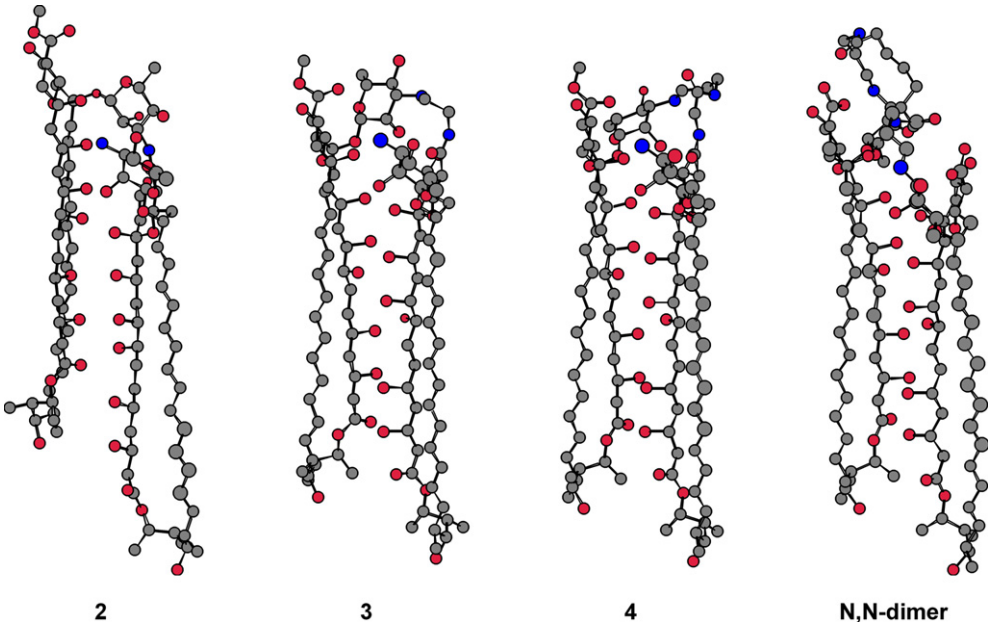


Figure 3. The lowest energy conformations of dimers **2**–**4** and *N*–*N* dimer generated by the Monte-Carlo conformation search method. Hydrogen atoms were omitted for clarity. The *N*,*N*-dimer has an amide-type linker $(CH_2)_3$–$NHCO(CH_2)_6CONH$–$(CH_2)_3$ between amino groups of AmB.[5]

preparations as compared to that in the ergosterol membrane (Fig. 2). However, as is the case with *C*–*C* and *N*–*N* dimers,[5,7] the selectivity of ergosterol over cholesterol is significantly reduced compared with that of AmB.

According to previous simulation studies of an AmB assembly,[3] the ion channel is thought to have intermolecular hydrogen bonds between carboxyl and amino groups of the neighboring molecules. Therefore, we assumed that directly-linked dimer **2** or short-tethered dimer **3** was able to reproduce the intermolecular interactions and thereby retained potent ion-channel activity. Both of the dimers, however, turned out to be virtually devoid of the activities, suggesting that the direct or short linkage inhibited the channel formation. In contrast, dimer **4** having a longer spacer elicited potent biological and ion-channel activities. These results may be accounted for by the following notions; the amino and carboxyl groups of neighboring AmBs are not in proximity in the ion-channel complex, or although the functional groups are closely interacting in the channel complex, a certain distance and/or flexibility of the linker are required during the transient process to form the channel complex as suggested for the binding of AmB to membrane from aqueous media.[9]

To gain further insight into bimolecular interaction, we carried out conformational analysis for **2**–**4** using the Monte–Carlo multiple minimization method.[10] One of the *N*,*N*-dimers that exhibited potent ion-channel activity[5] was subjected to the same conformational search. Due to the immobility of the macrolide ring,[11] it was treated as a motion-restricted portion, where ±30° allowance from the crystal structure[12] was given to each C–C single bond upon calculations. Randomly generated 5000 conformations were minimized by the conjugate gradient method with an MMFF force field in vacuo.[13] Figure 3 shows the lowest energy conformations for dimers **2**–**4** and the *N*,*N*-dimer. These conformations are stabilized by multiple hydrogen bonds among polyhydroxy groups although the structure of **2** with fewer hydrogen bonds is distorted due to the strain around the direct linkage. Moreover, close similarity between **4** and the *N*,*N*-dimer in conformation of the macrolide pair may possibly account for their potent biological activities. A similar *head-to-head* structure to those of **3**, **4** and the *N*,*N*-dimer was reported as a most stable non-covalent dimer on the basis of molecular mechanics calculations.[14] These structures are thought to be suitable for penetration into the membrane interior because polar polyhydroxy regions are mostly shielded from the hydrophobic lipid environment. With this respect, the calculated conformation for dimer **2** might not be suitable due to the imperfect segregation of hydroxyl groups from hydrophobic surroundings. The conformations in Figure 3 may not precisely reproduce the bimolecular interaction of AmB since they were obtained in vacuo without experimental data. Thus spectroscopic data including those of solid state NMR should be necessary to verify the proposed structures.

Meanwhile, in the barrel-stave model,[2,3] AmB molecules are supposed to be arranged in radial orientation with their polyhydroxy side pointing inward to constitute the pore lining and their lipophilic heptaene part directing outward to interact with the membrane interior. Assuming that dimers **2**–**4** also form the barrel-stave pores, transformation from dimer structures as shown in Figure 3 to the barrel-stave complex within the membrane may require a considerable conformational change. Bond rotations, while being inserted in membrane interior, probably require a flexible linker with a

certain length. This notion may be another possible account for the weak activities of dimers **2** and **3**. We prepared three dimers **2**–**4** with the linker of 0, 3 and 7 C/N atom lengths, respectively. To further elucidate the structure–activity relationship of *C*,*N*-dimers, the length and/or functionality of a linker should be extensively examined. The present results may, however, provide a clue to design a molecular probe for investigating the mechanism of action of this unique drug.

In conclusion, we prepared new AmB dimers connected between amino and carboxyl groups, and revealed that dimer **4** with a longer linker reproduced biological and ion-channel activities of AmB to a certain degree. This *C*,*N*-dimer together with *N*,*N*-dimers[5,6] will provide useful information on the bimolecular interactions of AmB in biomembranes. Synthesis of the dimers with ^{13}C label and ^{19}F-label in each AmB unit is currently underway in our laboratory for measuring the interatomic distances among AmB units in a membrane assembly using solid-state NMR techniques.[15]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.03.058.

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