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# Amphotericin B–phospholipid covalent conjugates: dependence of membrane-permeabilizing activity on acyl-chain length

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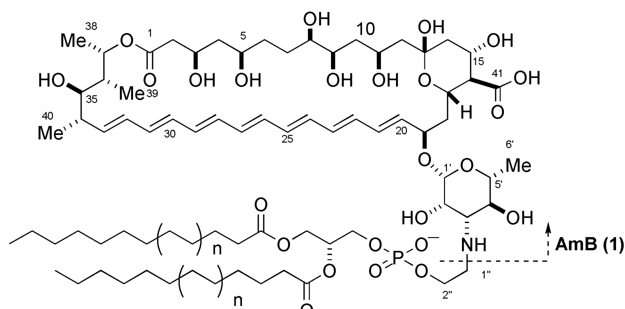
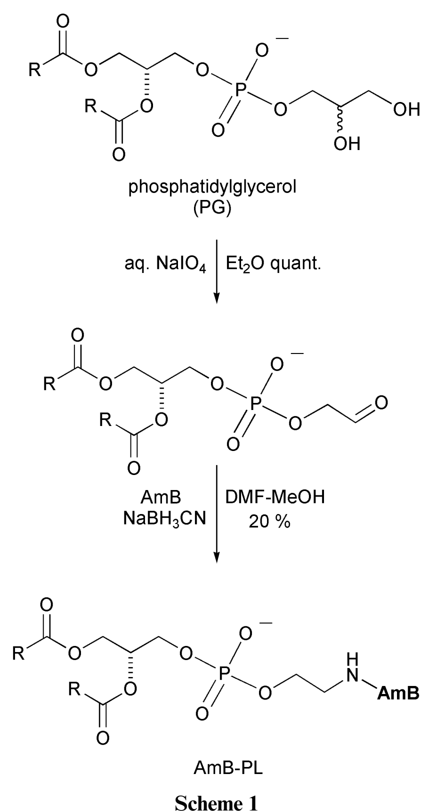
**The interaction between amphotericin B and phospholipid upon forming ion channels across a biomembrane was investigated using their covalent conjugates. The membrane permeabilizing activity was greatly affected by the chain length of the fatty acyl groups, suggesting that their interaction is involved in ion channel assemblages.**

Amphotericin B (AmB, **1**) has been the drug of choice for treating systemic infections for over forty years.[1] Its selective toxicity against fungi and other eukaryotic microbes is generally accounted for by the formation of sterol-specific ion-permeable channels across a plasma membrane, where ergosterol, a fungus sterol, has higher affinity for AmB than mammalian cholesterol.[2] A widely accepted model for this channel is a barrel-stave complex comprising about eight pairs of AmB and sterol.[3] This idea has attracted scientists' interest, leading to numerous biochemical/biophysical investigations on the interaction between AmB and sterols. Yet, the precise structure of the channel or its formation mechanism remains unknown. More recently, it has been demonstrated that AmB at higher concentrations forms ion permeable channels in phospholipid (PL) membranes in the absence of sterols.[4] The interaction between AmB and PL became a target for research of the drug's mode of action.[5] In plasma membranes containing ergosterol, the composition of PL greatly affects the drug's activity,[6] which implies that PL directly participates in the channel assemblage. In previous studies, we produced AmB–AmB conjugates to observe the bimolecular interaction between AmB–AmB in the channel.[7] To follow these precedents, we attempted to prepare AmB–PL conjugates. In this communication we report a versatile method for the preparation of conjugates with various fatty acyl chains and a comparison of their membrane-permeabilizing activities.




According to NOESY experiments in the solution state by Balakrishnan and Easwaran,[8] the head group of phosphatidylcholine resides close to the ionic groups of AmB in a plausible molecular complex probably because of the electrostatic interaction between amphoteric ion pairs present in both AmB and PC. Therefore, we attempted to obtain AmB–PL conjugates by linking the two molecules at 3′-$NH_2$ of AmB and phosphate of PL (Scheme 1). AmB–PL conjugates **2**–**5** were prepared simply in a one-pot reaction: oxidation of phosphatidylglycerol (PG) by sodium metaperiodate and coupling between the resulting PL aldehyde and the amino group of AmB by a reductive aminoalkylation reaction. According to the scheme, four AmB–PL conjugates were prepared with different lengths of fatty acyl chains from $C_{12}$ to $C_{18}$. Separation of the products from the reaction mixture, which was not straightforward, was only effected by HPLC using THF-containing mobile phases.† ‡ §




**Scheme 1**

The myristoyl conjugate **3** was subjected to hemolysis and fungicidal bioassays. The conjugate showed weaker hemolytic activity than AmB (~1/6) and was practically devoid of antifungal activity (<1/100). These weak activities are probably due to poor solubility in water. Membrane permeabilizing action was thus assessed by a $K^+$ flux assay using artificial liposomes, in which AmB–PL was mixed with lipids prior to membrane preparation. In this method, the activity can be evaluated with-

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out the step of drug binding to phospholipid bilayers from an aqueous phase.[9] The ion flux was then measured according to a method reported by Gary-Bobo's group.[10] $K^+$ entry in liposomes induces proton–$K^+$ exchange across the liposome membrane, and raises the inner pH, which can be monitored by chemical shift changes in a $^{31}P$ NMR signal of inorganic phosphate.

All the conjugates **2**–**5** elicited ion permeabilizing actions (Figs. 1 and 2). In the dose–response relationship in Fig. 2, the curves for **2** and **3** were steeper than that for AmB while those for **4** and **5** were similar to that of AmB. Moreover, $^{31}P$ NMR spectra of short-chain conjugates **2** and **3** show the graded type of ion flux, in which broad peaks appear at the sample/lipid ratio of $2 \times 10^{-4}$ (Fig. 1) because gradual ion flux in liposomes slowly changes pH of the encapsulated buffer. In contrast, **4** and **5** reveal the all-or-none type of ion flux in which clear two peaks are observed because the ion flux 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. These distinctions between **3** ($C_{14}$) and **4** ($C_{16}$) are due to a difference in acyl chain length of only two carbons. We have recently demonstrated that a small difference in phosphatidylcholine composition significantly influences the membrane activity of AmB.[11] In the present study the similar effects by acyl chain length were observed as intramolecular events.

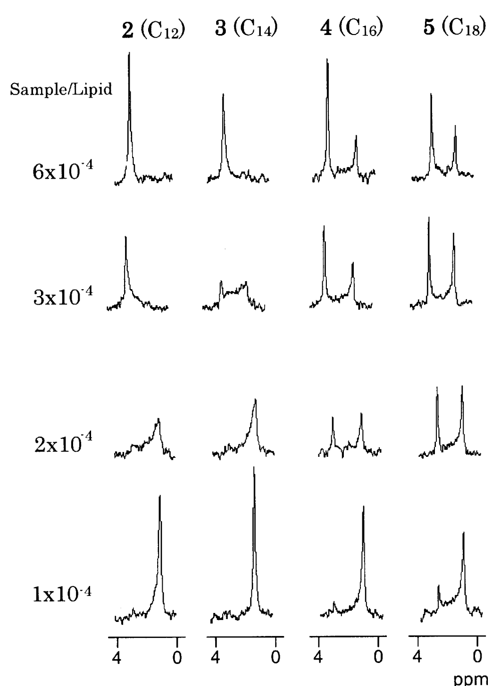


**Fig. 1** $^{31}P$ NMR signals of liposome-entrapped phosphate in eggPC LUV containing AmB–di($C_{12}$)PL (**2**), AmB–di($C_{14}$)PL (**3**), AmB–di($C_{16}$)PL (**4**) and AmB–di($C_{18}$)PL (**5**). Signals of external phosphate and phospholipids disappeared due to addition of $Mn^{2+}$. The liposomes were incubated for 6 h at 25 °C under a pH gradient across the membrane: internal pH = 5.5, external pH = 7.5. The peak around $\delta$ 1.2 corresponds to $H_2PO_4^-$ at pH 5.5 (initial pH) and that around $\delta$ 3.1 corresponds to $HPO_4^{2-}$ at pH 7.5 (due to permeabilized liposomes). AmB–PL conjugate was added to lipid prior to liposome preparation.

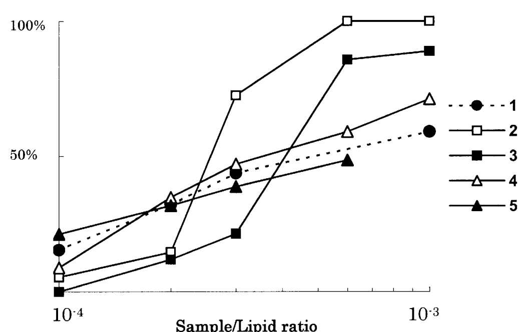


**Fig. 2** Dependence of $K^+$ flux activity of AmB (**1**), AmB–di($C_{12}$)PL (**2**), AmB–di($C_{14}$)PL (**3**), AmB–di($C_{16}$)PL (**4**) and AmB–di($C_{18}$)PL (**5**) on sample/lipid molar ratios. Liposomes consisting of eggPC were used. The *y*-axis is a ratio (in percentage) of a peak area at $\delta$ 3.1 relative to the total peak area between $\delta$ 1.2 and $\delta$ 3.1.

In the two-state models proposed for equilibrium between monomeric and oligomeric states, the formation of molecular assemblages is known to depend heavily on the concentrations. Fig. 2 reveals the tendency for dose dependency to become smaller as the chain length increases. The steeper dose-dependence of **2** and **3** may suggest that a certain number of the molecules assemble to form a structured ion channel as suggested for the barrel-stave model. These channel assemblages may be formed more easily with **2** and **3**. On the other hand, the gentle dose-dependence of **4** and **5** with longer chains implies that the large aggregates of the conjugates as reported previously for AmB[12] are mainly responsible for ion flux. It can be suggested that the ion channel assemblage with definitive stoichiometry can be stabilized by the conjugated phospholipid moiety, and this effect is more prominent with $C_{12}$ or $C_{14}$ acyl chains than $C_{16}$ or $C_{18}$.

The all-or-none type of $K^+$ flux is more prominent for longer acyl conjugates **4** and **5** than **2** and **3** (Fig. 1). This difference can be accounted for by formation of aggregates. As **4** and **5** easily gather together during liposome preparations, their distribution among liposomes should be uneven; vesicles with more AmB or AmB-PL molecules undergo rapid $K^+$ flux while those with fewer molecules are unchanged, resulting in the coexistence of fully permeabilized and intact liposomes. This may be the case with AmB, particularly in the absence of sterol. Further investigations on ion channels by AmB–PLs including the sterol-dependence and single channel recording are currently under way.

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## Notes and references

† **2**: This compound was obtained by a procedure analogous to that described for AmB–di($C_{14}$)PL (**3**) (see below). Reagent amounts: di($C_{12}$)PG (9 mg, 14 µmol), 0.25 M aqueous $NaIO_4$ (20.6 µL), AmB (19 mg, 21 µmol) and $NaBH_3CN$ (6 mg, 90 µmol). Yield: 1.0 mg (5%). In the improved method, 5.4 mg of **2** (8.5 µmol) was treated with $NaIO_4$ (11.4 µmol) on silica gel (12.2 mg)[13] in 300 µL of $CHCl_3$ containing 2.7 µL of $H_2O$. After being stirred for 1 h, the precipitate was filtered to give aldehyde bearing PL, and then subjected to the reductive aminoalkylation under the same conditions. Yield: 25%. HPLC retention time (YMC-ODS AM-323 $\phi$ 10 × 250 mm): 41 min (flow rate: 1.0 mL $min^{-1}$, eluent: THF : $H_2O$ 80 : 20 – 10 mM formic acid THF : EtOH : $H_2O$ 26 : 24 : 50 changing linearly from 1 : 9 to 10 : 0 in 50 min). ESI-MS *m*/*z*: 1484 $(M + H)^+$, Calc. for $C_{76}H_{127}NO_{25}P$ $[M - H]^-$ 1484. $^1H$ NMR signals were practically identical with those of **3** except for the signals of equivalent polymethylene groups in fatty acyl moieties.

‡ **3**: To a solution of di($C_{14}$)PG (6.8 mg, 9.9 µmol) dissolved in $Et_2O$ (1 mL) was added 0.25 M aqueous $NaIO_4$ (14.6 µL). After being stirred vigorously at rt for 1 h, the solution was treated with 0.1 M ethylene glycol (5.8 µL) to quench excess $NaIO_4$. The resulting solution was diluted with $CHCl_3$, washed with water, and the solvent removed under reduced pressure. A solution of aldehyde-bearing PL (8.5 mg, 17 µmol) and AmB (17 mg, 18 µmol) in dry DMF (250 µL) was stirred for 2 h, and $NaBH_3CN$ (5 mg, 75 µmol) was added to the solution. After being stirred overnight, the solution was diluted two times with

distilled water and purified by HPLC. Yield: 3.1 mg (20%) HPLC retention time (YMC-ODS AM-323 $\phi$ 10 × 250 mm): 40 min (flow rate: 1.0 mL $min^{-1}$, eluent: THF : $H_2O$ 80 : 20–10 mM formic acid THF : EtOH : $H_2O$ 26 : 24 : 50 changing linearly from 1 : 9 to 10 : 0 in 40 min). ESI-MS *m*/*z*: 1541 $(M + H)^+$. $^1H$ NMR signals (500 MHz) are listed in Table 1.

**Table 1** $^1H$ NMR chemical shifts for AmB–di($C_{14}$)PL (**3**) in DMSO-$d_6$ : $D_2O$ = 19 : 1, 50 °C, 500 MHz

| Position | $\delta_H$ | Position | $\delta_H$ | Position | $\delta_H$ |
|---|---|---|---|---|---|
| 2 | 2.19 | 20 | 5.95 | 3′ | 3.20 |
| 3 | 4.06 | 21–31 | 6.1–6.5 | 4′ | 3.32 |
| 4 | 1.39 | 32 | 6.08 | 5′ | 3.22 |
| 5 | 3.55 | 33 | 5.46 | 6′ | 1.20 |
| 9 | 3.48 | 34 | 2.30 | 1″ | 3.23 |
| 10 | 1.56 | 35 | 3.12 | 2″ | 4.00 |
| 11 | 4.22 | 36 | 1.74 | 1‴ | 3.82 |
| 14 | 1.89, 1.13 | 37 | 5.20 | 2‴ | 5.10 |
| 15 | 4.06 | 38 | 1.12 | 3‴ | 4.31, 4.11 |
| 16 | 1.96 | 40 | 1.04 | Acyl α | 2.27 |
| 17 | 4.26 | 41 | 0.92 | Acyl β | 1.50 |
| 18 | 2.05, 1.56 | 1′ | 4.39 | Acyl $CH_2$ | 1.15–1.30 |
| 19 | 4.41 | 2′ | 3.95 | Acyl $CH_3$ | 0.85 |

§ **4** and **5**: These analogues were synthesized and purified essentially by the same methods as for **3**. HPLC chromatograms of **4** and **5** showed virtually only one peak, indicating that the purity should be 95% or higher. **4**: ESI-MS found *m*/*z*: 1595, Calc. for $C_{84}H_{143}NO_{25}P$ $[M - H]^-$ 1595. **5**: ESI-MS found *m*/*z*: 1651, Calc. for $C_{88}H_{151}NO_{25}P$ $[M - H]^-$ 1651.


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