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Detecting Cross-Talk Between Two Halves of a Phospholipid Bilayer

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Abstract

One of the most challenging questions that relates to the structure and function of biological membranes is whether the two halves of the bilayer "talk" to each other. In this paper, we show how the pertubation of the lateral organization of one leaflet of a fluid phospholipid bilayer by an external agent also alters the lateral organization of the adjoining leaflet. In addition, we show that the energy involved in such cross-talk corresponds to ca. 100 cal/mol of phospholipid. These findings provide a basis for expecting similar cross-talk to exist in cell membranes.

Introduction

Lipids serve as the major building blocks for all biological membranes.[1] Although the bilayer character and fluid-like properties of cell membranes are well-established, only recently has attention begun to focus on their two-dimensional organization.[2] In particular, a growing body of experimental observations has emerged, which suggests that lipids are not randomly arranged and that their lateral distribution may be linked to specific processes; e.g., signal transduction and membrane trafficking.[3–8] A closely related structural issue, and one that has proven to be extremely difficult to address by experiment, is whether lipids can "talk" to each other across a bilayer. Specifically, *if external agents interact with phospholipids in one leaflet, resulting in a perturbation of their lateral distribution, will this also affect the lateral organization of the phospholipids in the adjoining leaflet?* Here, we show that such "cross-talk" exists in a model membrane by use of the nearest-neighbor recognition method. These findings provide a basis for expecting that analogous cross-talk should also exist in cell membranes.

Nearest-neighbor recognition (NNR) experiments take molecular-level snapshots of bilayer organization by detecting and quantifying the thermodynamic tendency of exchangeable monomers to become nearest-neighbors of one another.[9–15] Typically, two lipids of interest (**A** and **B**) are converted into exchangeable dimers (**AA, AB** and **BB**), which are then allowed to undergo monomer interchange via thiolate-disulfide interchange. The resulting equilibirum that is established, whereby one molecule of **AA** reacts with one molecule of **BB** to give two molecules of **AB**, is then governed by an equilibrium constant, K, in which $K=[\mathbf{AB}]^2/([\mathbf{AA}][\mathbf{BB}])$. When monomers **A** and **B** mix ideally, this is reflected by an equilibrium constant that equals 4.0. When homo-associations are favored, the equilibrium constant is less than 4.0; favored hetero-associations are indicated by a value that is greater than 4.0.

In previous NNR studies, short phospholipids were found to favor long phospholipids as nearest-neighbors in adjoining monolayers.[11–13] Based on such transbilayer complementarity, it was postulated that structural or compositional changes in one leaflet of a

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cell membrane, *brought about by external stimuli*, should induce a compensating change in the adjoining leaflet. In other words, the two halves of the bilayer should "talk" to each other through changes in their lateral organization. This paper records, what is believed to be, the first experimental evidence for such cross-talk in a model membrane, and provides a quantitative measure of this effect. In essence, our approach uses electrostatic forces between a water-soluble, organic dication and exchangeable phospholipids that are negatively charged, to promote their segregation from analogous zwitterionic phospholipids in fluid bilayers. Comparison of the effectiveness of this segregation, in the absence and in the presence of a chain length mismatch, has then allowed us to detect phospholipid cross-talk.

Experimental Section

Nearest-Neighbor Recognition Analysis

In a typical liposome preparation, a thin film was prepared from 0.18 μmol {**A',A'**}, 0.30 μmol {**A,A**} and 0.24 μmol of **A'**SH in chloroform. [A similar film was also prepared from 0.36 μmol {**A,A'**}, 0.12 μmol {**A,A**} and 0.24 μmol **A'**SH]. After drying overnight under reduced pressure, 2.0 mL of 10 mM MOPS buffer (10 mM MOPS, 150 mM NaCl and pH = 5.0) were added to the dried film. The mixture was then vortex mixed for 1 min and incubated for 5 min at 60°C, followed by an additional 1 min of vortex mixing and additional 20 min of incubation. The dispersion was then subjected to five freeze/thaw cycles (liquid nitrogen/60°C water bath). Then, the dispersion was incubated for 30 min at 60°C prior to initiating the thiolate disulfide exchange reaction. Thiolate-disulfide interchange reactions were initiated after the dispersions were equilibrated at 60°C by 24 μL of a MOPS-buffer solution that was 8.4 μM in monensin (0.202 nmol), with brief vortex mixing, and finally increasing the pH to 7.0 via addition of ca. 10 μL of 1.0 M NaOH. All dispersions were maintained under an argon atmosphere throughout the course of the interchange reactions. Aliquots (0.30 mL) were withdrawn as a function of time and quenched by addition to a 5.0 mL test tube containing 50 μL of 30 mM HCl (final pH 5.0), followed by brief (10 s) vortex mixing and immediate cooling to −20°C. The frozen samples were then lyophilized and the lipid portion dissolved in 2 mL of chloroform with vortex mixing for 30 s, followed by centrifugation (20 min) using a clinical centrifuge. The clear chloroform solution was poured into another test tube and the chloroform was evaporated under reduced pressure [40 min, 0.4 Torr, 23°C]. The resulting clear film was dissolved in a solution made from 10 μL of chloroform plus 90 μL of the mobile phase that was used for HPLC analysis. This solution was then immediately analyzed by HPLC using a C18 reverse phase column and a mobile phase that was composed of 19 mM tetrabutylammonium acetate in denatured ethanol/water/hexane (82/12/6, v/v/v) with a flow rate of 0.9 min. The column was maintained at 31°C and the components were monitored at 205 nm using a Waters 996-photodiode-array detector. *For those NNR experiments that were carried out in the presence of **C**, a 10 mM MOPS buffer was used in forming the vesicles, which contained 10 mM N,N,N,N',N',N'-hexamethyl-1,6-hexanediammonium dichloride (i.e., **C**)*. Values of *K* that were found using 2 mM **C** were the same as those found using 10 mM **C**. For all NNR experiments, the total lipid concentration was 0.3 mM. Analogous NNR experiments that were carried out for the mixing of **B** with **B'** and **A** with **B'** used the same molar quantities of lipids as that used in measuring the mixing of **A** with **A'**.

Results and Discussion

Experimental Design

Consider a bilayer that is made from a short phospholipid (**A**) and a longer analog (**B'**) that bears a binding site in its head group. Figure 1 shows a hypothetical membrane, **I**, in which **A** and **B'** are randomly arranged within each monolayer, but favor each other as nearest-neighbors across the bilayer. Now consider the effect that a divalent ligand, **C** is expected to

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have on the lateral distribution of these lipids. Direct association of one molecule of **C** with two molecules of **B'** induces homo-phospholipid association within that monolayer. For simplicity, we refer to this process as “direct-talk”. In turn, these pairs of **B'** then act as hydrophobic templates for the pairing of neighboring **A** molecules in the adjoining leaflet (Figure 1, membrane **II**). This secondary recognition process is what we define as “cross-talk”. Thus, the overall effectiveness of **C** in promoting homo-phospholipid association in bilayers made from **A** with **B'** results from a combination of direct-talk and cross-talk.

Now consider the effect that **C** can have on lipid mixing in bilayers made, exclusively from **A** and **A'**, and also ones made from **B** and **B'**. In both of these cases, since there is no chain length mismatch, only direct-talk is possible. Thus, if lipid mixing in all three of these systems (**A/B', A/A'** and **B/B'**) were dominated by head group interactions, then it should be possible to separate, quantitatively, direct-talk from cross-talk. Specifically, the effectiveness of **C** in promoting homo-phospholipid association in these systems should be **A/B'**> **A/A'** = **B/B'**, and the difference between **A/B'** and **A/A'** (or **B/B'**) would be a quantitative measure of the cross-talk.

Testing for Cross-talk

To test for cross-talk, four exchangeable phospholipids were synthesized: one short and one long phosphocholine mimic (**A** and **B**, respectively), and two negatively-charged analogs, **A'** and **B'** (Figure 2).[16] A diquaternary ammonium salt, **C**, was chosen as a divalent ligand. In this case, ion pairing is possible only with **A'** and **B'**.

Exchangeable phospholipid homodimers {**A,A**} and {**B,B**} were synthesized using an approach that is outlined in Figure. 3. In brief, oxidation of 2-(dimethylamino)ethanethiol hydrochloride with I_2 in methanol followed by neutralization with NaOH, quaternization with 2-bromoethanol, ion exchange with $NaBPh_4$, and direct coupling with 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid (DMPA) afforded {**A,A**}. A similar reaction sequence was used to synthesize {**B,B**}. These homodimers were subsequently cleaved with tris-(2-carboxyethyl) phosphine (TCEP) and reacted with the 2′-(pyridyldithio)propionamide derivative of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) or 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) to give the requisite heterodimers {**A,A'**}, {**A,B'**} and {**B,B'**}. Homodimers {**A',A'**) and {**B',B'**} were synthesized by established methods.[15] The gel to liquid-crystalline phase transition temperatures (T_m) for the longest dimers, {**B,B**} and {**B',B'**}, were 53.5°C and 54.4°C, respectively, as determined by high-sensitivity differential scanning calorimetry. Based on analogy, the T_m value for {**A',A'**) is expected to be similar to that of {**A,A**}, which is 22.7°C.[15]

In one set of NNR experiments, multilamellar vesicles were prepared from an equimolar mixture of **A** and **A'** in the form of heterodimers, homodimers and thiol monomer; that is, using 0.36 μmol of {**A,A'**}, 0.12 μmol of {**A,A**}, and 24 μmol of **A'**SH and a 10 mM MOPS buffer (10 mM MOPS, 150 mM NaCl, pH 5.0). A similar dispersion was also prepared using only homodimers and thiol monomer; that is, 0.18 μmol of {**A',A'**}, 0.30 μmol of {**A,A**}, and 0.24 μmol of **A'**SH. Thiolate-disulfide exchange of the monomer units in both membranes at 60°C (pH 7.0) resulted in the identical equilibrium mixture of dimers within 1 h. In the absence of **C**, a preference for heterodimer formation was observed; that is, *K* was equal to 6.55± 0.36 (Table 1). Similar experiments that were carried out in the presence of 10 mM of **C** resulted in a significant reduction in *K*, reflecting an increased preference for homodimer formation.

In the absence of **C**, the mixing behavior of **A** with **B'**, and also **B** with **B'**, was the same as that found for the mixing of **A** with **A'**; that is, all three systems showed the same preference for heterodimer formation. Whereas the presence of **C** in bilayers made from **B** and **B'** reduced *K* to the same extent as in membranes derived from **A** and **A'**, its effect on the mixing of **A**

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with **B'** was *significantly greater*; that is ΔΔG = 390 cal/mol versus ca. 200 cal/mol (Table 1). Thus, the net difference in free energy, corresponding to ca. 200 cal/mol of dimer or ca. 100 cal/mol of phospholipid, which is a direct measure of the phospholipid cross-talk in bilayers made from **A** with **B'**.

The thermodynamic preference for hetero-phospholipid association in the absence of **C** is a likely consequence of electrostatic repulsion between two negatively charged lipids; that is, the homodimers of **A'** and **B'** are disfavored. Association with **C** between two molecules of **A'** or two molecules of **B'** can eliminate this repulsion, leading to favored homo-phospholipid association. An alternative explanation for the action of **C** in decreasing *K* is that it simply reduces charge repulsion via a local ionic strength effect and not ionic bridging. To test for this possibility, the value of *K* was determined for the mixing of **B** with **B'**, where 10 mM **C** was replaced by 20 mM of a monomeric analog; that is, *n*-propyl-trimethylammonium chloride (**D**). The fact that **D** was found to have a negligible effect on the mixing of these lipids (i.e., K=6.45 ±0.15) implies that ionic bridging between the two charged protomers is dominant.[16]

Cell membranes are composed of a rich assortment of lipids of varing length, as well as proteins that span the bilayer (integral proteins), and ones that lie on the inner or outer surface of the membrane (peripheral proteins). Although, the systems reported herein are highly simplified mimics of these natural enclosures, our detection of cross-talk between the two halves of these bilayers shows that similar cross-talk should also exist within cell membranes. Thus, it is now reasonable to expect that any interaction between the inner or outer leaflet of a cell membrane with a water-soluble agent--which results in a perturbation of its lateral organization--would alter the lateral organization of the adjoining leaflet. In principle, such biological cross-talk could play a significant role in some of the most important cellular processes that are required to maintain the living state, such as signal transduction, membrane fusion or membrane trafficking, to cite just a few.

Conclusions

The NNR experiments reported herein provide strong support for the existence of phospholipid cross-talk between two halves of a fluid bilayer. As such, they provide a basis for expecting that analogous cross-talk should also exist in cell membranes. To the extent that lateral organization of lipids controls cellular processes, phospholipid cross-talk may now be expected to play an important contributing role, and one that has not previously been recognized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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REFERENCES and NOTES

1. Gennis, RB. Biomembranes: Molecular Structure and Function. New York: Springer-Verlag; 1989.
2. Singer SJ, Nicolson GL. Science 1972;175:720–731. [PubMed: 4333397]
3. Simons K, van Meer G. Biochemistry 1988;27:6197–6202. [PubMed: 3064805]
4. Simons K, Ikonen E. Nature 1997;387:569–572. [PubMed: 9177342]
5. Simons K, Vaz WLC. Annu. Rev. Biomol. Struct 2004;33:269–295.
6. Edidin M. Annu. Rev. Biomol. Struct 2003;32:257–283.
7. Munro S. Cell 2003;115:377–388. [PubMed: 14622593]
8. Veatch SL, Keller SL. Biochim. Biophys. Acta 2005;1746:172–185. [PubMed: 16043244]

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9. Davidson SKM, Regen SL. Chem. Rev 1997;97:1269–1280. [PubMed: 11851451]
10. Regen SL. Curr. Opin. In Chem. Biol 2002;6:729–735. [PubMed: 12470724]
11. Zhang J, Jing B, Tokutake N, Regen SL. J. Am. Chem. Soc 2004;126:10856–10857. [PubMed: 15339166]
12. Zhang J, Jing B, Tokutake N, Regen SL. Biochemistry 2005;44:3598–3603. [PubMed: 15736969]
13. Zhang J, Jing B, Regen SL. Langmuir 2005;21:8983–8986. [PubMed: 16171319]
14. Uragami M, Miyake Y, Tokutake N, Zhang LH, Regen SL. Langmuir 2000;16:8010–8015.
15. Krisovitch SM, Regen SL. J. Am. Chem. Soc 1992;114:9828–9835.
16. Although we cannot rule out the possibility that other factors may also be contributing to **C'**s ability to promote homodimer formation (e.g., membrane curvature effects) the effectiveness of **C** in inducing lipid segregation is clearly **A/B'**>**A/A'**=**B/B'**, and it is this difference that is a direct measure of cross-talk in bilayers made from **A** and **B'**.
17. The fact that exchangeable phospholipids that differ by four methylene groups produce statistical mixtures of dimers in the fluid bilayer state (e.g., the mixing of **A** with **B**) argues against intramonolayer clustering as being the source of the effects of C on the mixing of **A** with **B'**.[15]

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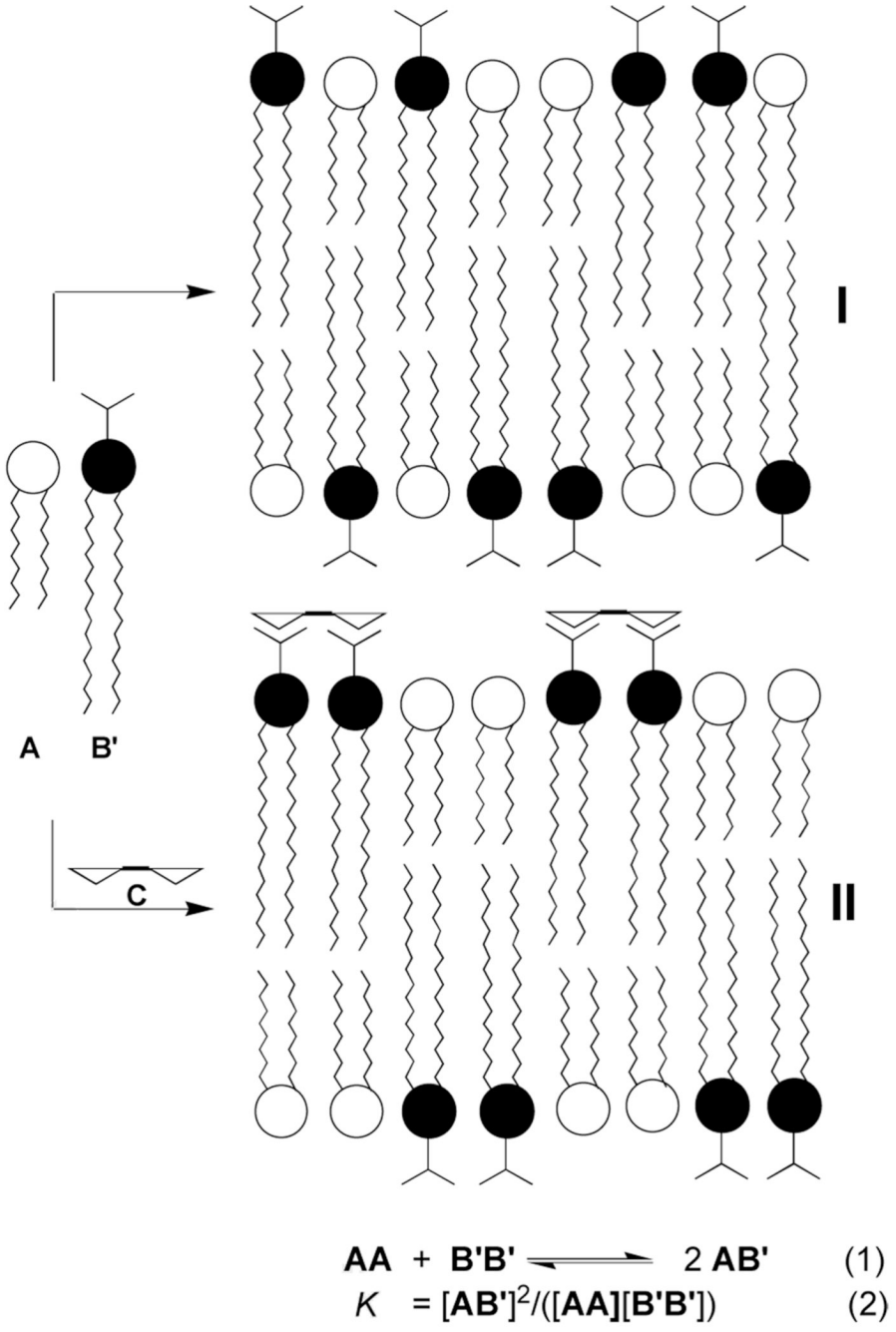

Figure 1.
Stylized illustration showing a random lateral distribution within each leaflet and a favored **A** and **B'** arrrangement across the bilayer (structure **I**), and favored homo-phospholipid association within each leaflet, induced by complexation with a divalent ligand (**C**) plus a favored **A** and **B'** arrrangement across the bilayer (structure **II**)

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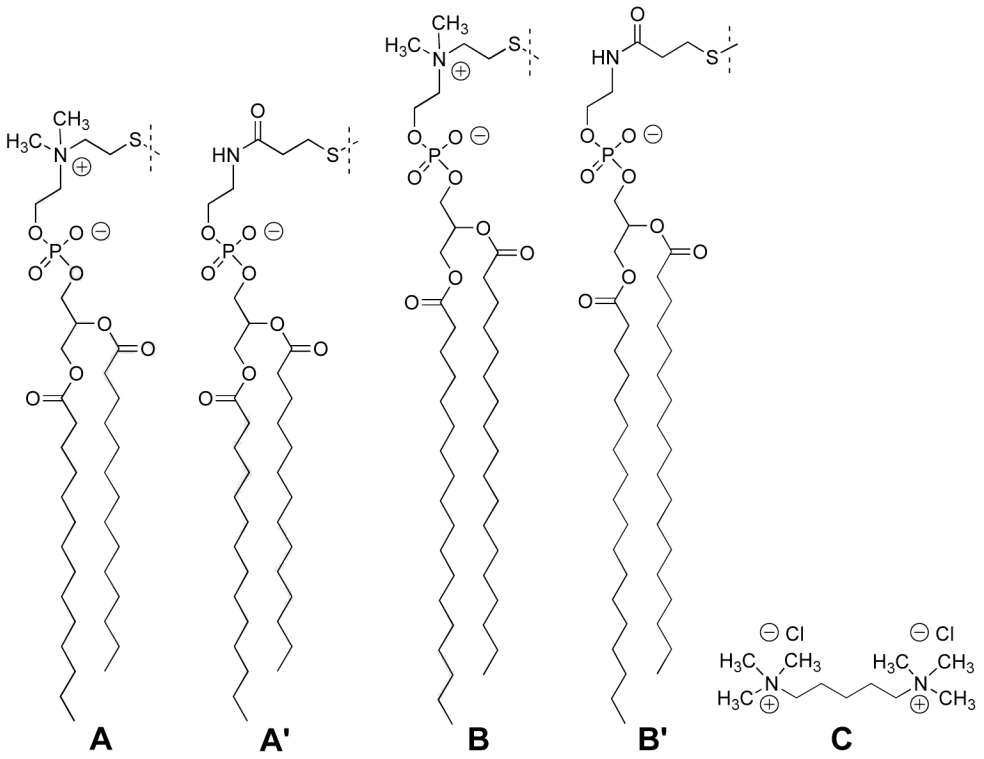

Figure 2.
Molecular structures of exchangeable phospholipids **A, A', B, B'**, and divalent ligand, **C**, used in this study.

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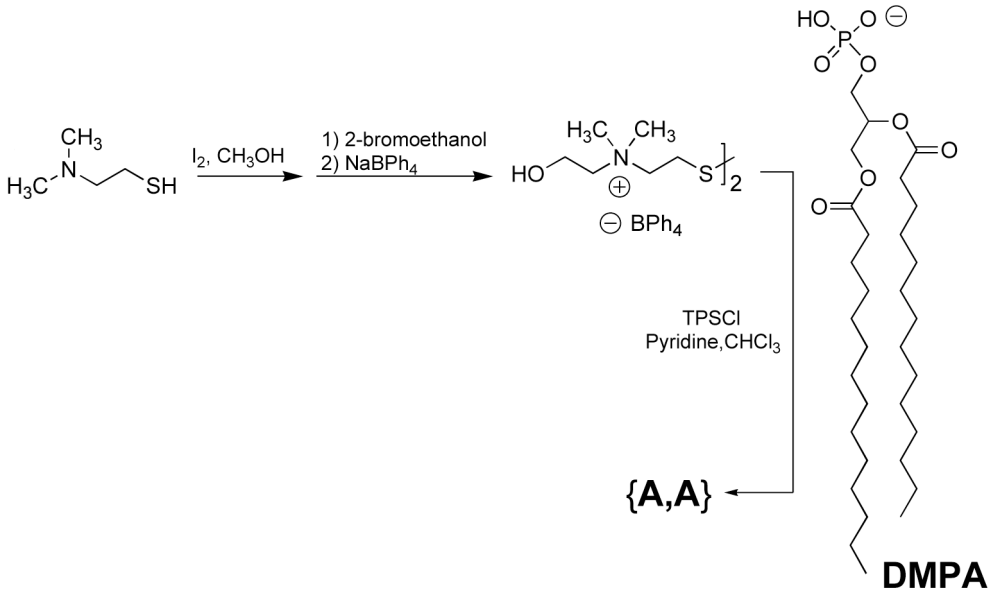

Figure 3.
Synthetic route that was used for the preparation of {**A,A**}.

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Table 1

Effects of C on Dimer Equilibria[a]

Lipids	C[b] (mM)	*K*	ΔG[c] (cal/mol)	ΔΔG (cal/mol)
A + A'	0	6.55 ± 0.36	−1250 ± 36	
A + A'	10.0	4.88 ± 0.27	−1050 ± 36	200 ± 50
A + B'	0	6.92 ± 0.47	−1280 ± 45	
A + B'	10.0	3.84 ± 0.12	−892 ± 20	390 ± 50
B + B'	0	6.76 ± 0.16	−1270 ± 15	
B + B'	10.0	4.86 ± 0.09	−1050 ± 12	220 ± 20

[a] Equilibrium was reached in all cases within 1 h at 60°C.

[b] N,N,N,N',N',N'-hexamethyl-1,6-hexanediammonium dichloride (i.e., hexamethonium dichloride).

[c] The free energy (per mol of dimer) includes a statistical component, R × ln 4 or 2.75 cal/K-mol of lipid dimer, due to the fact that the heterodimer is statistically favored over each homodimer by a factor of two.