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Synthesis and Supramolecular Properties of Conformationally Restricted and Flexible Phospholipids

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Short synthetic routes are described to produce structurally related phospholipids that are either conformationally restricted or flexible. The conformationally restricted structures have a cyclopropyl ring in the interfacial region of the phospholipid. The key synthetic step is a cyclopropanation reaction between 2(5*H*)-furanone and a stabilized chloroallylic phosphonate anion. The synthetic routes enable the incorporation of different polar headgroups as well as nonpolar tails at late stages in the sequence. The phosphoethanolamine derivatives **1b** and **2b** readily form encapsulating vesicles, however, dye leakage from vesicles composed of the restricted phospholipid **1b** is significantly slower than from vesicles composed of flexible analogue **2b**. Physicochemical analyses using ^{31}P NMR, differential scanning calorimetry, fluorescence anisotropy, and Langmuir−Blodgett films suggest that the decreased permeability of membranes composed of conformationally restricted **1b** is due to its ability to pack more closely in a bilayer assembly.

Introduction

The structure of a typical glycerophospholipid can be divided into three parts, a highly polar phosphate diester headgroup, a moderately polar interfacial region containing the glycerol carboxylic esters, and the two nonpolar tails. Numerous studies have shown how bilayer membrane assembly is dependent on phospholipid headgroup and tail structure, but there are very few published reports on the importance of the interfacial structure.[1] We have initiated a project to evaluate how flexibility of the phospholipid interfacial region affects various dynamic membrane processes such as membrane packing, pore formation, membrane fusion, and peripheral protein binding. Our strategy is to investigate the supramolecular properties of structurally related phospholipids. We have designed two types of symmetrical, nonnatural phospholipids: conformationally restricted, cyclopropyl-containing compounds **1** and more-flexible, control analogues **2** (Figure 1). The only difference between the two structures is the substitution of two C−H bonds for a C−C bond in the interfacial region of the molecule. Nonetheless, this subtle change in structural flexibility is enough to induce a significant difference in supramolecular properties.

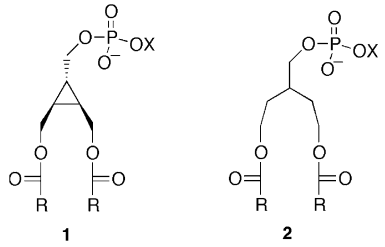

FIGURE 1. Generic structures of conformationally restricted phospholipids **1** and flexible analogues **2**.

A major challenge for this multidisciplinary project is the phospholipid synthesis. Here, we describe, in full detail, our methods for preparing phospholipids **1** and **2**. The synthetic routes are versatile and enable the incorporation of different polar headgroups as well as nonpolar tails at late stages in the sequences. Also discussed are comparative physicochemical studies of the phosphoethanolamine versions of **1** and **2** which we find can readily self-assemble in water to form vesicles, but with substantially different abilities to retain encapsulated dyes. Vesicles composed of the cyclopropyl-containing phosphoethanolamine are less permeable than vesicles composed of the more flexible analogue, apparently because the former can pack more closely in a bilayer membrane.[2]

Results and Discussion

Reterosynthetic Analysis. Despite their biochemical importance, there are relatively few general methods for preparing unnatural phospholipids.[3] In the case of *meso*-

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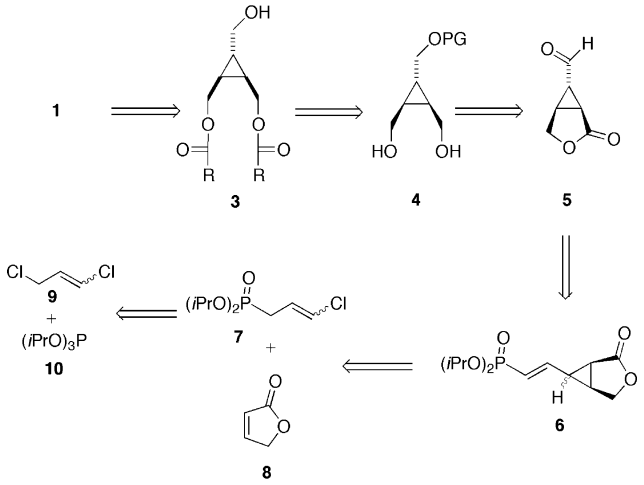
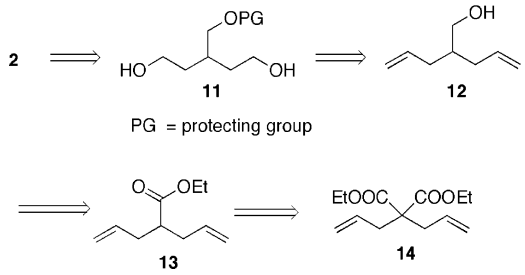
SCHEME 1



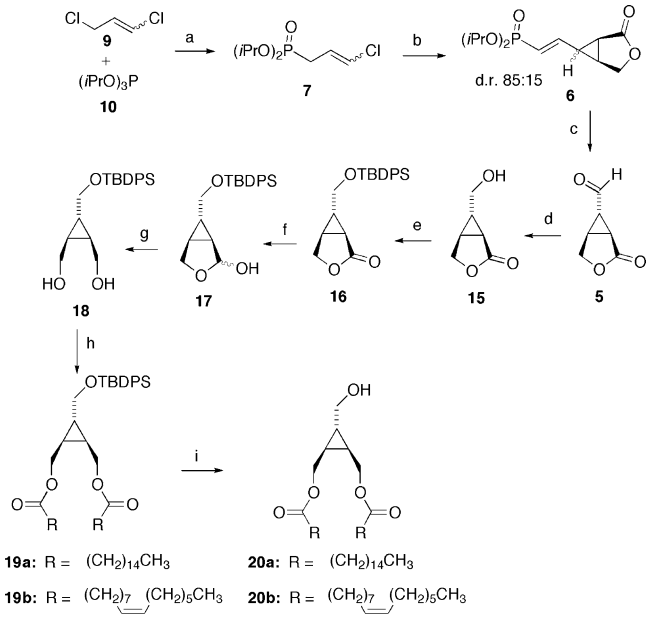
SCHEME 2



phospholipids **1** and **2**, a new synthetic approach had to be developed. The reterosynthetic analyses are shown in Schemes 1 and 2. Both schemes allow the preparation of phospholipids bearing different headgroups and/or nonpolar tails from a late-stage intermediate. In the case of conformationally restricted phospholipid **1**, the key step in the sequence is cyclopropanation of 2(5*H*)-furanone **8** using the methodology developed by Hanessian and coworkers.[4] Since our target compounds, **1**, possess *meso* stereochemistry, we can use the achiral phosphonate **7**. At the time of our studies, the precursor 1,3-dichloropropene, **9**, was only available as a mixture of *cis* and *trans* isomers. Thus, we expected the formation of cyclopropane derivative **6** as a mixture of diastereomers. However, there was literature precedent to suggest that after transformation to **5**, the undesired diastereomer could be converted to the desired one via equilibration.[5]

Synthesis of Conformationally Restricted Phospholipids 1. Chloroallylic phosphonate **7** was prepared via Arbuzov reaction in 88% yield on a multigram scale (Scheme 3). The compound was roughly a 3:2 mixture of two inseparable geometric isomers. The (*Z*)-**7** isomer was

SCHEME 3 [a]



[a] Reaction conditions: (a) reflux, 20 h, 88%; (b) *n*-BuLi, 2-(5*H*)-furanone, THF, −78 °C, 2−3 h, 55−60%; (c) ozone, −78 °C, then Me_2S, 61%; (d) $NaBH_4$, THF/MeOH, 0 °C, 15 min; (e) TBDPSCl, imidazole, DMF, rt, 68% (two steps); (f) DIBALH, CH_2Cl_2, −78 °C, 97%; (g) $NaBH_4$, EtOH, rt, 1.5 h, 87%; (h) **19a**: palmitoyl chloride, Et_3N, CH_2Cl_2, rt, 40 min, 99%; **19b**: palmitoleoyl chloride, Et_3N, CH_2Cl_2, rt, 1 h, 99%; (i) TBAF, THF, rt, 14 h, 93% (**20a**), >99% (**20b**).

expected to give the cyclopropane derivative with desired *trans* stereochemistry, whereas in the case of (*E*)-**7** all three substituents on the cyclopropane ring would have undesired *cis* stereochemistry.[4] Treatment of **7** with *n*-BuLi at −78 °C followed by rapid addition of furan **8** afforded the cyclopropane derivative **6** in 55−60% yield as a mixture of two diastereomers (diastereoselectivity ca. 85:15). A reason that the diastereoselectivity is higher than the starting *E*/*Z* ratio of **7** might be that (Z)-**7** reacts faster than (*E*)-**7**.

Treatment of alkene **6** with ozone, followed by the reduction of the ozonide with Me_2S, afforded the aldehyde **5** as a single diastereomer in 61% yield.[6] The product **5** was isolated together with an unidentified phosphorus-containing byproduct (the ratio of **5**:byproduct ca. 2:1).[7] Subsequently, the aldehyde was reduced to the corresponding alcohol by sodium borohydride and the hydroxyl group protected as a silyl ether (**16**) in 68% overall yield. NOE experiments confirmed the relative stereochemistry of **16**. Strong cross relaxation was observed between the *tert*-butyl hydrogens and the bridgehead methines, indicating their *cis* relationship, whereas there was no change in the intensity of the remaining *trans*-cyclopropyl methine. Attempts were made to open the lactone in **16** with alkoxide, but the cyclopropane ring was not stable under the reaction conditions. However, when **16** was treated with DIBALH at low temperature, lactol **17** was formed cleanly. The lactol, which is in equilibrium with the corresponding hydroxyaldehyde,[8] was reduced to diol

(3) (a) Bittman, R. In *Phospholipid Handbook*, Cevc, G., Ed.; Marcel Dekker: New York, 1993; p 141. (b) Herbert, N.; Beck, A.; Lennox, R. B.; Just, G. *J. Org. Chem.* **1992**, *57*, 1777. (c) Martin, S. F.; Josey, J. A.; Wong, V.-L.; Dean, D. W. *J. Org. Chem.* **1994**, *59*, 4805. (d) Martin, S. F.; Josey, J. A. *Tetrahedron Lett.* **1988**, *29*, 3631. (e) Srisiri, W.; Lamparski, H. G.; O'Brien, D. F. *J. Org. Chem.* **1996**, *61*, 5911. (f) Burgos, C. E.; Ayer, D. E.; Johnson, R. A. *J. Org. Chem.* **1987**, *52*, 4973. (g) Ali, S.; Bittman, R. *J. Org. Chem.* **1988**, *53*, 5547. (h) Roodsari, F. S.; Wu, D.; Pum, G. S.; Hajdu, J. *J. Org. Chem.* **1999,** *64*,7727. (i) Lindberg, J.; Ekeroth, J.; Kondrasson, P. *J. Org. Chem.* **2002**, *67*, 194.

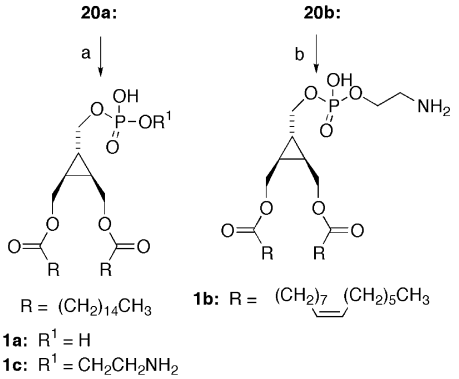
(4) Hanessian, S.; Andreotti, D.; Gomtsyan, A. *J. Am. Chem. Soc.* **1995**, *117*, 10393.

(5) Hanessian, S.; Griffin, A.; Devasthale, P. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 3119.

(6) We cannot state with certainty whether the minor diastereomer equilibrated to the major one or it was separated during chromatography.

(7) The yield given (61%) reflects the actual amount of **5**.

SCHEME 4[a]

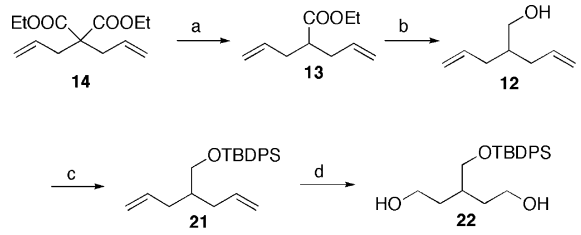


[a] Reaction conditions: (a) **1a**: diphenylchlorophosphate, imidazole, CH_2Cl_2, rt, 14 h, then cat. PtO_2, H_2, 8 h, rt, 91%; **1c**: 2-chloro-1,3,2-dioxaphospholane 2-oxide, Et_3N, benzene, rt, 20 h, then NH_3, benzene/acetonitrile 4 h, rt, 62%; (b) 2-chloro-1,3,2-dioxaphospholane-2-oxide, Et_3N, benzene, rt, 20−21 h, then NH_3, benzene 5.5 h, rt, 62%.

18 in 87% yield. Diol **18** is a versatile intermediate, since different nonpolar tails can be incorporated. For example, palmitoyl and palmitoleoyl tails were attached each in 99% yield. The desilylation of **19a** and **19b** afforded the corresponding alcohols **20a** and **20b** in 93% and 99% yield, respectively. Our first choice for phospholipid headgroup was a phosphatidic acid which was introduced by treating first the alcohol **20a** with diphenyl chlorophosphate followed by the catalytic hydrogenation to afford **1a** in 92% yield (Scheme 4). The phosphoethanolamine derivatives **1b** and **1c** were obtained by converting the corresponding alcohols **20a** and **20b** to rather unstable dioxaphospholane intermediates which were ring-opened in 62% yield using ammonia.[9] The phospholipids with saturated tails, **1a** and **1c**, were practically insoluble in common organic solvents which prevented complete characterization by NMR. However, unsaturated **1b** was quite soluble in chloroform or methanol.

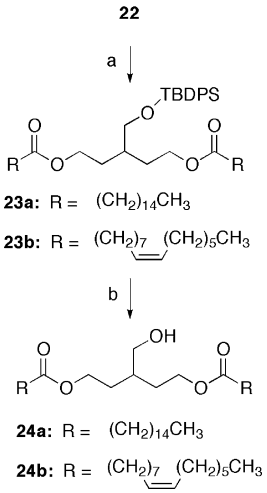
Synthesis of Flexible Phospholipids 2. Alcohol **12** was prepared by modification of the method described by Nugent and co-workers.[10] Diethyl diallylmalonate **14** was first decarboxylated in 86% yield (Scheme 5).[11] Reduction of ethyl ester **13** to the corresponding alcohol **12** was achieved with DIBALH followed by $NaBH_4$ in 69% yield.[12] After the hydroxyl group was protected as a silyl ether in 78% yield, the terminal alkenes in **21** were cleaved by first treating the diene with ozone followed by reduction of the ozonide with $NaBH_4$ to give diol **22** in quantitative yield. The diol **22** is a versatile intermediate that can be conjugated with different nonpolar tails

SCHEME 5[a]



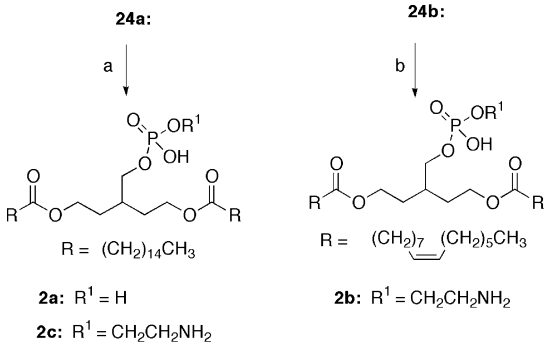
[a] Reaction conditions: (a) LiCl, DMSO, H_2O, reflux, 6 h, 86%; (b) DIBAL, CH_2Cl_2, −78 °C, then $NaBH_4$, MeOH, rt, 30 min, 69%; (c) TBDPSCl, imidazole, DMF, rt, 20 h, 78%; (d) ozone, −78 °C, then $NaBH_4$, >99%.

SCHEME 6[a]



[a] Reaction conditions: (a) **23a**: palmitoyl chloride, Et_3N, CH_2Cl_2, 1 h, rt, 70%; **23b**: palmitoleic acid, DCC, DMAP, CH_2Cl_2, 20 h, 77%; (b) TBAF, THF, rt, 4−16 h, rt, 79% (**24a**), 60% (**24b**).

SCHEME 7[a]



[a] Reaction conditions: (a) **2a**: diphenylchlorophosphate, imidazole, CH_2Cl_2, rt, 17 h, then cat. PtO_2, H_2, AcOH, rt, 6 h, 85%; **2c**: 2-chloro-1,3,2-dioxaphospholane 2-oxide, Et_3N, benzene, rt, 18 h, then NH_3, acetonitrile/benzene 1.5 h, 43%; (b) 2-chloro-1,3,2-dioxaphospholane 2-oxide, Et_3N, benzene, rt, 18 h, then NH_3, rt, 4.5 h, 81%.

and polar headgroups. For example, the diol **22** was successfully acylated with palmitoyl chloride in 67% yield (Scheme 6). When palmitoleoyl chloride was used as an acylating agent, the diester **23b** was isolated in less that 50% yield. However, when the corresponding acid was used instead, the desired **23b** was obtained in 77% yield. Removal of silyl protecting groups gave alcohols **24a** and **24b** in 79% and 60% yield, respectively. Different head-

(8) On the basis of ^1H NMR in $CDCl_3$, the lactol/hydroxy-aldehyde ratio in **17** is about 95:5 at room temperature. We have not yet explored the possibility of converting **17** into differentially acylated phospholipids.

(9) Menger, F. M.; Chen, X. Y.; Brocchini, S.; Hopkins H. P.; Hamilton, D. J. *J. Am. Chem. Soc.* **1993**, *115*, 660.

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(12) Even if excess DIBALH was used, some intermediate aldehyde was always obtained. However, subsequent treatment with $NaBH_4$ converted all material to desired alcohol **12**.

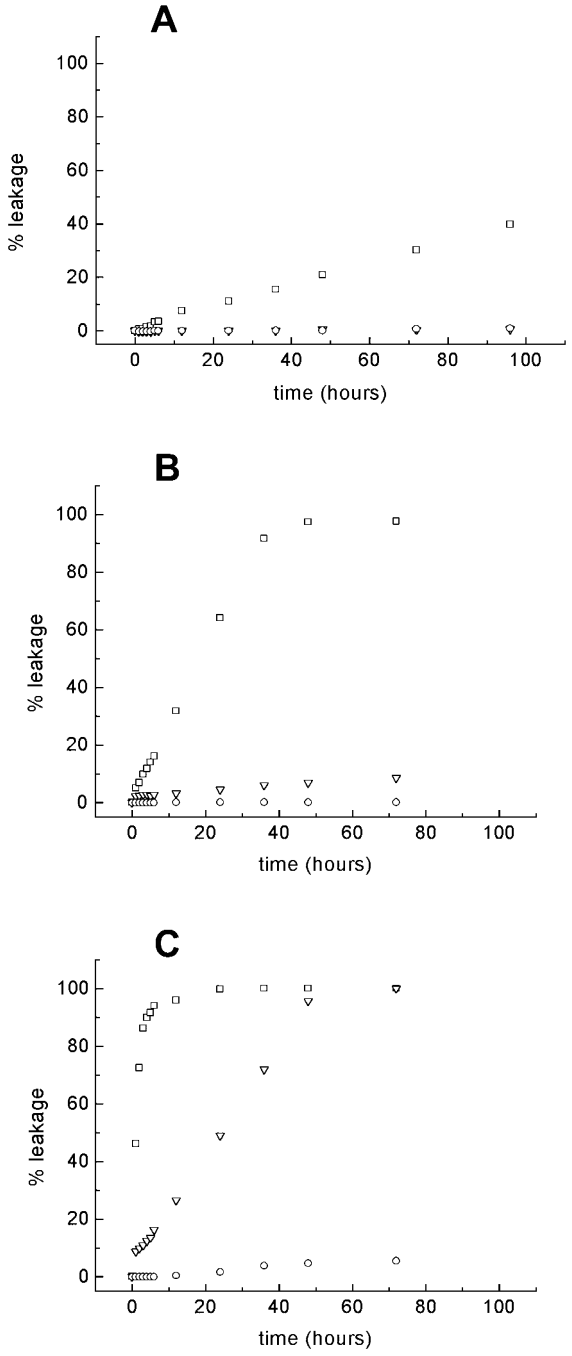

FIGURE 2. Percent leakage of HPTS/DPX from 100 nm diameter unilamellar vesicles (50 μM phospholipid) composed of **1b** (triangles), **2b** (squares), or DOPC (circles). Vesicles contained 5 mM HPTS/7 mM DPX/100 mM NaCl and were dispersed in 5 mM TES/100 mM NaCl, pH 7.4. Experiments were conducted at (A) 25 °C; (B) 37 °C; (C) 50 °C.

groups were introduced in analogues manner to the above cyclopropane derivatives. Reaction of alcohol **24a** with diphenyl chlorophosphate (Scheme 7) followed by the catalytic hydrogenation afforded the phosphatidic acid **2a**. The treatment of alcohol **24a** with 2-chloro-1,3,2-dioxaphospholane-2-oxide, followed by ring opening with ammonia afforded phosphoethanolamine **2c** in 43% yield.[9] As was the case with corresponding cyclopropane-containing analogues, compounds **2a** and **2c** with saturated tails were practically insoluble in common organic

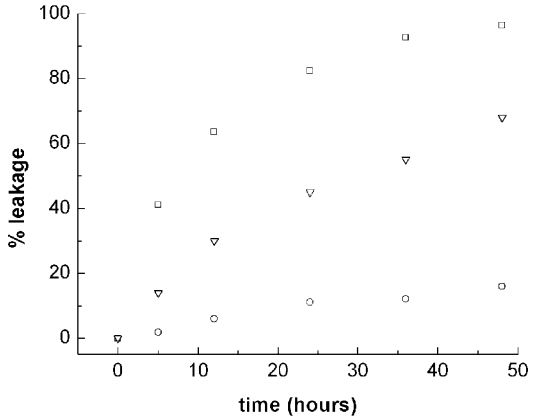

FIGURE 3. Percent leakage of carboxyfluorescein from 100 nm diameter unilamellar vesicles (50 μM phospholipid) composed of **1b** (triangles), **2b** (squares), or DOPC (circles) at 25 °C. Vesicles contained 50 mM carboxyfluorescein/100 mM NaCl and were suspended in 5 mM TES/150 mM NaCl, pH 7.4.

solvents and could not be dispersed in water. However, the unsaturated phosphoethanolamine, **2b**, prepared from **24b** in 81% yield, was readily soluble in chloroform or methanol.

Physicochemical Studies. The leakage of encapsulated fluorescent dyes from vesicles composed of **1b** or **2b** was compared to leakage from control vesicles composed of dioleoylphosphatidylcholine (DOPC).[13] Two sets of leakage experiments were conducted. The data in Figure 2 shows leakage of the fluorophore/quencher pair HPTS/DPX (1-hydroxypyrene-3,6,8-trisulfonic acid/*p*-xylylenebis(pyridinium) bromide) as a function of time. The fluorescence emission of HPTS after excitation at 413 nm is independent of pH, and increases as the HPTS/DPX is diluted upon escape from the vesicles.[14] The leakage experiments were performed at 25, 37, and 50 °C. Vesicles composed of **2b** release about 30% of their encapsulated HPTS/DPX over 3 days at 25 °C, while vesicles composed of **1b** or DOPC were essentially impermeable (Figure 2A). At 37 °C, the leakage rate from vesicles composed of **2b** is twenty-seven times greater that from vesicles composed of **1b** which in turn is higher than leakage from vesicles composed of DOPC (Figure 2B). When the temperature is elevated to 50 °C, the vesicles composed of **2b** release all of their entire aqueous contents within 5 h, compared to 50 h for vesicles composed of **1b**, while DOPC vesicles remain relatively impermeable (Figure 2C). The same relative order of vesicle permeabilities is observed with leakage experiments using encapsulated carboxyfluorescein at neutral pH, although the absolute leakage rates are higher (Figure 3). As previously communicated, the leakage rates do not change with phospholipid concentration, and as leakage progresses there is no increase in vesicle size as judged by dynamic light scattering.[2] Thus, it appears that leakage does not require vesicle collision.

Shown in Figure 4 are ^{31}P NMR spectra of vesicle suspensions composed of **1b**, **2b**, or egg phosphatidyl-

(13) Membranes composed of DOPC are known to adopt a lamellar phase at temperatures below 50 °C. LIPIDAT: http://www.lipidat.chemistry.ohio-state.edu, accessed Oct 15, 2003.

(14) Daleke, D. L.; Hong, K.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1990**, *1024*, 352.

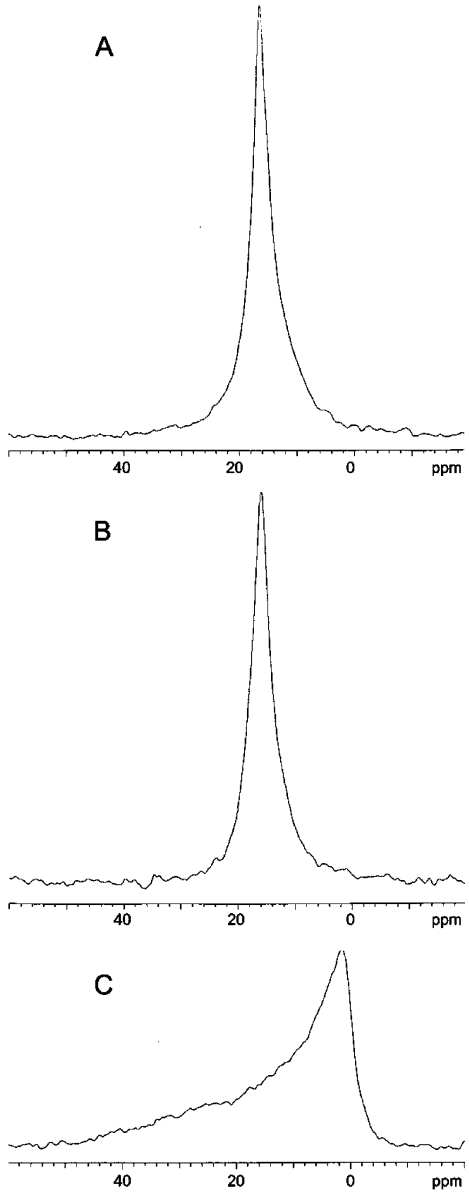

FIGURE 4. ^{31}P NMR spectra of 100 nm diameter unilamellar vesicles (50 mM in 5 mM TES/100 mM NaCl pH 7.4) composed of (A) **1b**, (B) **2b**, (C) egg phosphatidylcholine.

choline at pH 7.4 and 25 °C. The spectrum for egg phosphatidylcholine vesicles exhibits a broad powder pattern that is typical of a glycerophospholipid in a liquid crystalline lamellar phase.[15] In contrast, the spectra for **1b** and **2b** exhibit narrow, isotropic signals. Since all three vesicle systems were prepared by sample extrusion through filters with 100 nm pores, the difference in ^{31}P NMR peak shape is unlikely to be due to differences in vesicle tumbling rates. Instead, we attribute the isotropic signals for vesicles composed of **1b** or **2b** to fast axial diffusion of the structurally symmetrical phospholipids, which leads to motional averaging of the chemical shift anisotropy and dipolar interactions.

Previously communicated fluorescence anisotropy measurements using vesicles containing appropriate fluorescent probes indicate that membranes composed purely of **1b** or **2b** are fluid phase at 25 °C.[2] Furthermore, analysis by differential scanning calorimetry (DSC)

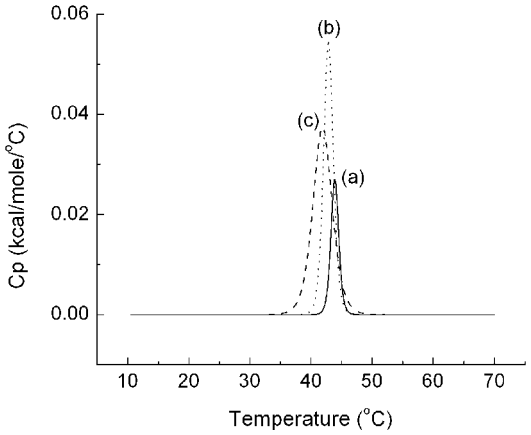

FIGURE 5. Differential scanning calorimetry. Lamellar to inverse hexagonal phase transitions of: (a) pure DiPOPE; (b) 99:1 mixture of DiPOPE/**1b**, and (c) 99:1 mixture of DiPOPE/**2b**. In each case, 14.5 mM phospholipid in 5 mM TES/100 mM NaCl, pH 7.4.

shows that the vesicles do not undergo a measurable phase transition between 10 and 90 °C. Additional DSC measurements were made on samples of dipalmitoleoylphosphatidylethanolamine (DiPOPE), the glycerophospholipid version of **1b** and **2b** with the same palmitoleoyl tails and phosphoethanolamine headgroup. The lamellar-inverse hexagonal phase transition temperature, T_H, for DiPOPE is known to be quite sensitive to amphiphilic additives that influence membrane curvature.[16] For example, we recently reported that the presence of one mol % of various triple-chain amphiphiles can raise or lower the T_H for DiPOPE by up to 10 °C.[17] However, in this present case we find that the presence of one mol % **1b** or **2b** has only a very minor effect on T_H (Figure 5) demonstrating that the nonglycerol interfacial regions in **1b** and **2b** do not significantly alter the effective size or hydration of the phosphoethanolamine headgroups.[18]

A Langmuir film balance was used to compare the packing of monolayers composed purely of **1b**, **2b**, or DiPOPE.[19] The compression isotherms shown in Figure 6 were recorded at 25.0 °C. The limiting area, A_∞, a parameter approximating the area occupied by one molecule on the monolayer surface at zero pressure, was 72, 82, and 90 Å^2 for compounds **1b**, **2b**, and DiPOPE, respectively. These results support the intuitive idea that the conformationally restricted phospholipid **1b** packs more closely in a condensed monolayer. The lift-off area, A_L, defined as the first point on the isotherm where a monolayer shows detectable resistance to compression, was 115, 120, and 125 Å^2, for **1b**, DiPOPE, and **2b**, respectively. This allows calculation of $A_L - A_\infty$ which reflects the change in molecular area in going from an expanded to a condensed state at the air−water interface.

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(18) The presence of one mol % **1b** or **2b** in DiPOPE increases the transition enthalpy from 47 cal/mol to 114 cal/mol and 155 cal/mol, respectively.
(19) Maget-Dana, R. *Biochim. Biophys. Acta* **1999**, *1462*, 109.

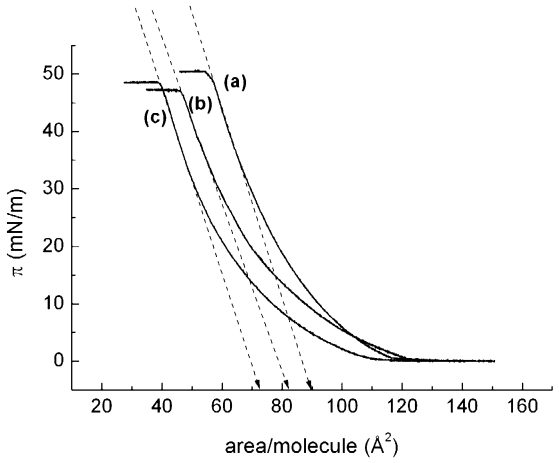

FIGURE 6. Surface pressure (π) versus area/molecule for compounds **1b**, **2b**, or DiPOPE spread on a water subphase at 25.0 °C. The approximate values of A_∞ for each sample are indicated by arrows: (a) DiPOPE, (b) **2b**, and (c) **1b**.

The higher values of $A_L - A_\infty$ for **1b** and **2b** indicate that their expanded states are more "compressible" than DiPOPE which is reasonable since the structures of **1b** and **2b** have three additional carbons in their interfacial regions.

Summary

Practical and versatile routes have been developed to produce structurally related phospholipids that are conformationally restricted or flexible (**1** and **2**, respectively). The conformationally restricted structures have a cyclopropyl ring in the interfacial region of the phospholipid. The phosphoethanolamine derivatives **1b** and **2b** readily form encapsulating vesicles, however, dye leakage from vesicles composed of the conformationally restricted **1b** is significantly slower than from vesicles composed of flexible analogue **2b**. It is notable that such a subtle restriction on conformational flexibility can produce a large decrease in vesicle permeability; particularly, since the membranes are in the fluid phase which is characterized by high acyl chain mobility, as well as rapid rotational and translational motion. The monolayer compression data in Figure 6 demonstrates that **1b** packs more closely in a condensed monolayer than **2b**. The observation that the rate of dye leakage is independent of vesicle concentration suggests that the leakage process is collision independent, and implies that passage of the relatively large, hydrophilic dyes through the membrane involves transient but substantial local reorganization of the bilayer. Thus, we conclude that membranes composed of the restricted phospholipid **1b** are less likely to form local nonbilayer defects. In other words, the degree of defect formation in a bilayer membrane, and hence the membrane permeability, can be lowered by decreasing structural flexibility at the phospholipid interfacial region.

Experimental Section. Full experimental details are provided as Supporting Information.

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Supporting Information Available: Experimental methods, synthetic procedures, and NMR spectra of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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