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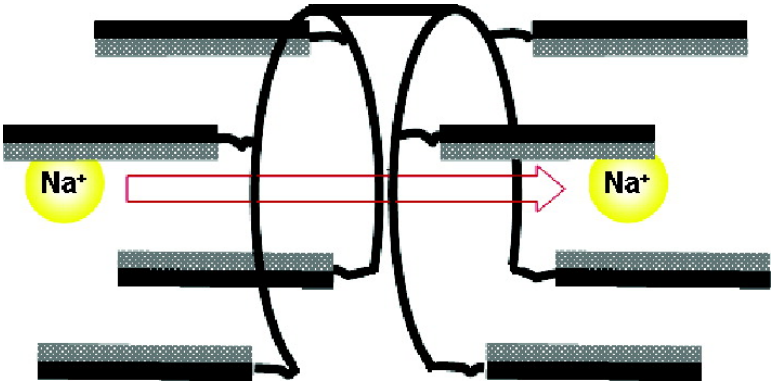

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Poly(choloyl)-Based Amphiphiles as Pore-Forming Agents: Transport-Active Monomers by Design

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Abstract: This paper describes the design and synthesis of a family of pore-forming amphiphiles. Two of these amphiphiles, which are derived from cholic acid, lysine, and *p*-phenylenediamine, can produce pores in lipid bilayers as individual molecules. In sharp contrast, analogous amphiphiles that do not contain a rigid 1,4-phenylenediamide moiety favor the formation of dimer-based pores. Kinetic evidence in support of monomer- and dimer-based pores has been obtained from Na^+ transport measurements across bilayers made from 1-palmitoyl-2-oleoyl-2-*sn*-glycero-3-phosphocholine (POPC). Structure−activity studies that have been carried out with pore-forming, dimer-based amphiphiles have also revealed a significant activity dependence on their overall compactness. The practical potential of pore-forming amphiphiles with controllable supramolecular properties is briefly discussed.

Introduction

The ability to fine tune the pore-forming properties of synthetic amphiphiles offers the promise of a multitude of applications. Pore-forming amphiphiles that can selectively insert into the plasma membrane of bacterial and fungal cells relative to mammalian cells, for example, may lead to new classes of antibiotics having minimal susceptibility toward drug resistance.[1,2] The inclusion of pore-forming amphiphiles in liposomes loaded with enzymes or nonbiogenic catalysts has the potential for use as nanoreactors and chemical sensors.[3] The recent demonstration that a pore-forming protein can recognize a specific base along an individual strand of DNA suggests that large families of pores of different sizes and shapes could result in devices for DNA sequencing.[4,5] Pore-forming amphiphiles that can enhance the release of agents from thermally sensitive liposomes (i.e., thermally gated liposomes) could also be considered for applications ranging from the targeted delivery of drugs to the controlled release of flavors in foods.[6] Despite such potential, the ability to create pore-forming amphiphiles with perfect control over the size, shape, and supramolecular structure of the resulting pores remains as a major synthetic challenge.[7]

In recent studies we have shown that certain conjugates derived from cholic acid, putrescine, spermidine, spermine, and lysine form dimer-based pores in lipid bilayers having Na^+ transport activities that increase, *exponentially*, with increasing numbers of choloyl units up to 12 sterols per conjugate (e.g., **1**, Chart 1).[6,8] Because of this unusually wide range in activity, we have become keenly interested in developing this class of compounds in the broadest possible sense. In the present investigation our primary goal was to design and synthesize pore-forming poly(choloyl)-based amphiphiles which do not rely on supramolecular behavior (i.e., aggregation) for pore formation; that is, they can form pores as *individual* molecules. A secondary objective of this work was to measure the sensitivity of ion transport activity of poly(choloyl)-based amphiphiles with respect to their overall compactness.

Since a poly(choloyl)-based amphiphile that can form a pore as an individual molecule does not involve monomer−aggregate equilibria, its activity is expected to be more controllable. For an analogous amphiphile that requires aggregation for pore formation, however, the number, size, and composition of the resulting pores is expected to be a function of the microenvironment that exists within the membrane. Thus, depending upon the composition of a given target or host membrane, aggregation and pore formation may or may not be favored. In certain instances, such a dependency may be desirable. For example, an amphiphile that favors pores in bacterial membranes that are devoid of sterols, relative to mammalian membranes, which are rich in cholesterol, could lead to useful antibiotics. For other applications, where control over pore size and the number of pores is important (e.g., nanoreactors, chemical sensors, and DNA sequencing), unimolecular pore-forming amphiphiles that do not depend on aggregation would clearly be preferred.

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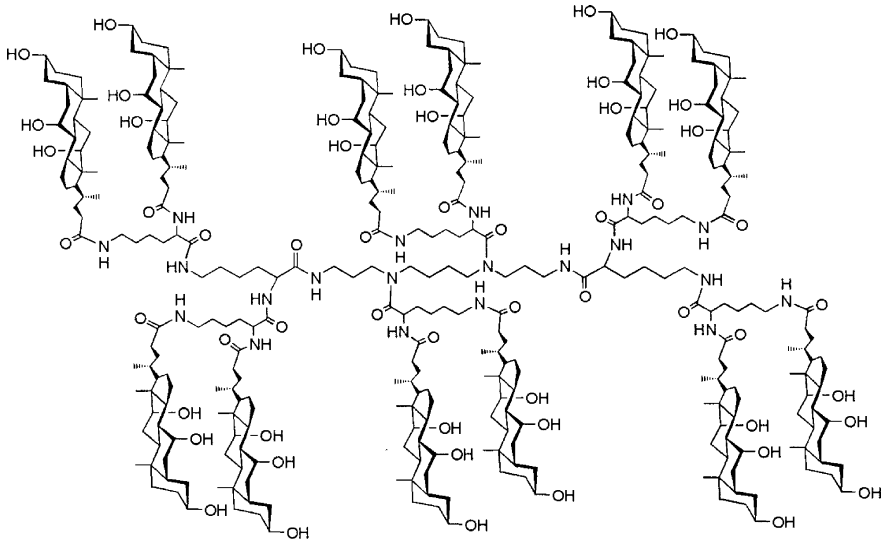
Chart 1



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In addition to controlling the supramolecular properties of a poly(choloyl)-based amphiphile, we have also become interested in the possibility of modulating ion transport activity by adjusting the overall compactness of such molecules. To our knowledge, such a parameter has not previously been examined for any synthetic ionophore reported to date.[7]

Experimental Section

General Methods. ^{1}H NMR spectra were recorded in either CD_3OD or $CDCl_3$ using a Bruker 360 or 500 spectrometer, using the solvent as a reference. Silica gel 60 (Fluka, particle size 0.035−0.070 mm, 220−440 mesh) was used for all column chromatographic purifications. Thin-layer chromatography (TLC) was performed on a glass plate precoated with silica gel and a fluorescence indicator (Whatman). Detection on TLC was made by use of sulfuric acid 10% in water, iodine, and UV (254 or 365 nm). All reagents and chemicals were obtained from commercial sources and used as received unless otherwise stated. Conjugates **1** and **3** were prepared according to the procedures recently described by us.[6,8]

L-Lysine-Dicholamide. The procedure used for the synthesis of L-lysine-dicholamide was similar to that previously described.[6,8,9] Thus, to a solution of cholic acid (6.08 g, 14.9 mmol) and *N*-hydroxysuccinimide (1.90 g, 16.5 mmol) in anhydrous THF (50 mL) was added DCC (3.60 g, 17.4 mmol). The resulting mixture was stirred at room temperature. After 4 h the product mixture was filtered to remove the insoluble urea. The filtrate was then added, dropwise, to a solution of L-lysine (950 mg, 6.50 mmol) and triethylamine (6.2 mL) in H_2O (20 mL) and stirred overnight. The solution was concentrated under reduced pressure and poured into 1 M hydrochloride acid (200 mL). The resulting solid was collected by filtration. The residue was then purified by chromatography on a silica gel column, eluting with $CHCl_3$/MeOH/H_2O (40/10/1, v/v/v), to afford L-lysine-dicholamide (4.51 g, 75%) having the expected ^{1}H NMR spectrum.[6,8,9]

Compound 7. A solution of Boc-L-Lys-OH (200 mg, 0.812 mmol) and $N_\alpha N_\epsilon$-di-Boc-L-lysine hydroxylsuccinimide ester (Boc-L-Lys(Boc)-OSu) (317 mg, 0.834 mmol) in THF−H_2O (16.5 mL, 10/1, v/v) was stirred at room temperature for 2 h, and the product mixture was then concentrated under reduced pressure. The resulting residue was partitioned between water and chloroform, and the organic layer was separated and combined with chloroform extracts (3×) of the aqueous layer. After washing with water, the chloroform layer was dried over anhydrous sodium sulfate. Removal of solvent under reduced pressure and purification by column chromatography on a silica gel column, eluting with $CHCl_3$/MeOH (40/1, v/v), afforded **7** (369 mg, 79%). ^{1}H NMR (500 MHz, CD_3OD, 298 K) δ: 4.01 (br, 1 H), 3.93 (br, 1 H), 3.20 (m, 2H), 3.02 (t, ^3J = 6.78 and 6.91 Hz, 2 H), 1.79−1.30 (m, 39 H).

Compound 8. To a solution of **7** (364 mg, 0.642 mmol) and 3-hydroxy-1,2,3-benzotriazine-4(3*H*)-one (DHBT) (115 mg, 0.705 mmol) in anhydrous THF (10 mL) was added DCC (165 mg, 0.80 mmol). After the mixture was stirred for 5 h, a solution of *p*-phenylenediamine (31 mg, 0.287 mmol) and triethylamine (0.5 mL) in anhydrous THF (1.0 mL) was added. After stirring at room temperature for 12 h, another portion of DCC (165 mg) was added and the stirring continued at room temperature for 12 h. The urea that formed was removed by filtration. The filtrate was then concentrated and purified by chromatography on a silica gel column, eluting with $CHCl_3$/MeOH (40/1, v/v), to afford **8** (334 mg, 95%). ^{1}H NMR (500 MHz, CD_3OD, 318 K) δ: 7.50 (s, 4 H), 4.11 (br, 2 H), 3.94 (br, 2 H), 3.22−3.18 (m, 4 H), 3.01 (t, ^3J = 6.74 and 6.85 Hz, 4 H), 1.69−1.33 (m, 78 H). MALDI-TOF MS *m*/*z* 1244 ([M + Na]$^+$).

Conjugate 2. A solution of **8** (60 mg, 0.049 mmol) in trifluoroacetic acid (3 mL) was stirred at room temperature for 3 h and then concentrated under reduced pressure. The residue was dried under reduced pressure for 4 h and used directly in the following reaction without further purification. The complete removal of Boc, yielding **9**, was confirmed by ^{1}H NMR (500 MHz, CD_3OD, 318 K) δ: 7.57 (s, 4 H), 4.02 (t, 2 H, ^3J = 6.68 and 6.44 Hz), 3.83 (t, 2 H, ^3J = 7.14 and 6.58 Hz), 3.27−3.20 (m, 2H), 2.92 (t, 4 H, ^3J = 7.63 and 7.77 Hz), 2.04−1.82 (m, 8 H), 1.70−1.45 (m, 16 H).

To a solution of L-lysine-dicholamide (340 mg, 0.367 mmol) and *N*-hydroxysuccinimide (60 mg, 0.522 mmol) in anhydrous DMF (2 mL) was added DCC (95 mg, 0.46 mmol). After the mixture was stirred for 3 h, a solution of **9**, which was prepared from **8** (60 mg, 0.049 mmol), and 4-(dimethylamino)pyridine (180 mg, 1.473 mmol) in anhydrous DMF (2.0 mL) was added. The reaction mixture was stirred at room temperature for 24 h, concentrated under reduced pressure, and poured into 1 M hydrochloride acid (200 mL). The resulting precipitate was collected by filtration and purified twice by chromatography on a silica gel column, eluting with $CHCl_3$/CH_3OH/H_2O (30/10/1, v/v), to give **2** (69 mg, 23%). ^{1}H NMR (500 MHz, CD_3OD, 328 K) δ: 7.66 and 7.55 (s, 4 H), 4.54−4.25 (m, 10 H), 3.93 (s, 12 H), 3.78 (s, 12 H), 3.37−3.34 (m, 12 H), 3.15 (m, 20 H), 2.31−0.91 (m,

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396 H), 0.69 (s, 36 H). MALDI-TOF MS *m*/*z* 6099 ([M + Na]$^+$). The sample was judged to be homogeneous based on the appearance of a single TLC spot (developed by two or more eluents) and a comparison of the integrated NMR signals for the lysine protons (4.54−4.25 ppm) relative to the 3-position protons of cholyl units (3.93 ppm).

Compound 10. To a solution of 1,4-diaminobutane (57 mg, 0.65 mmol) and triethylamine (0.5 mL) in anhydrous DMF−THF (4 mL, 1/1, v/v) was added Boc-L-Lys(Boc)-OSu (620 mg, 1.40 mmol). The resulting suspension was stirred at room temperature for 24 h. Removal of solvent under reduced pressure and purification by chromatography on a silica gel column, eluting with $CHCl_3$/MeOH (10/1, v/v), gave **10** (432 mg, 90%). ^{1}H NMR (500 MHz, $CDCl_3$, 298K) δ: 6.89 (br, 2H, CON*H*), 5.29 (br, 2 H, CON*H*), 4.67 (br, 2 H, CON*H*), 4.06 (unresolved, 2 H), 3.36 (unresolved, 2 H), 3.08−3.07 (unresolved, 6 H), 1.75−1.28 (m, 52 H). MALDI-TOF MS *m*/*z* 767 ([M + Na]$^+$).

Conjugate 4. A solution of **10** (50 mg, 0.067 mmol) and trifluoroacetic acid (1 mL) was stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure, and the residue (**11**) was dried under vacuum for 4 h and used directly in the following reaction without further purification. The complete removal of Boc groups, giving **11**, was confirmed by ^{1}H NMR (500 MHz, CD_3OD, 298 K) δ: 3.84 (t, 2 H, ^3J = 6.63 and 6.63 Hz), 3.26−3.20 (m, 4 H), 2.93 (t, 4 H, ^3J = 7.66 and 7.79 Hz), 1.93−1.80 (m, 4 H), 1.73−1.68 (m, 4 H), 1.59−1.53 (m, 4 H), 1.49−1.43 (m, 4 H).

To a solution of L-lysine-dicholamide (311 mg, 0.335 mmol) and DHBT (68 mg, 0.417 mmol) in anhydrous DMF (1 mL) was added DCC (108 mg, 0.523 mmol). After stirring for 3 h, a solution of **11,** which was prepared from **10** (50 mg, 0.067 mmol), and triethylamine (0.5 mL) in anhydrous DMF (1 mL) was added. The mixture was stirred for 24 h and then poured into 1 M hydrochloride acid (200 mL). The resulting precipitate was collected by filtration and purified twice by chromatography on a silica gel column, eluting with $CHCl_3$/CH_3OH/H_2O (40/10/1, v/v), to give **4** (120 mg, 45%). ^{1}H NMR (500 MHz, CD_3OD, 323 K) δ: 4.27 (m, 6 H), 3.94 (s, 8 H), 3.79 (s, 8 H), 3.36−3.39 (m, 8 H), 3.20−3.15 (m, 16 H), 2.29−0.91 (m, 280 H), 0.70 (s, 24 H). MALDI-TOF MS *m*/*z* 4004 ([M + Na]$^+$). The sample was judged to be homogeneous based on the appearance of a single TLC spot (developed by two or more eluents) and a comparison of the integrated NMR signals for the lysine protons (4.27 ppm) relative to the 3-position protons of cholyl units (3.94 ppm).

Compound 12. To a solution of L-lysine-dicholamide (3.7 g, 4 mmol) in anhydrous DMF (10 mL) was added DHBT (1 g, 6.4 mmol) and DCC (825 mg, 4 mmol). After stirring for 3 h at room temperature, Boc-L-Lys-OH (790 mg, 3.2 mmol) and triethylamine (0.2 mL) were added. The reaction mixture was stirred for 12 h at room temperature and then poured into diluted aqueous HCl (50 mL). The resulting precipitate was collected by filtration and purified by chromatography on a silica gel column, eluting with $CHCl_3$/CH_3OH/H_2O (65/25/4, v/v/v), to give **12** (2.51 g, 68%). ^{1}H NMR (CD_3OD, 500 MHz) δ: 4.25 (m, 1 H), 4.00 (br, 1 H), 3.94 (s, 2 H), 3.78 (s, 2 H), 3.31 (m, 2 H), 3.16−3.14 (m, 4 H), 2.29−1.38 (m, 75 H), 1.02−0.91 (m, 16 H), 0.70 (s, 6 H). MALDI-TOF MS *m*/*z* 1178 ([M + Na]$^+$).

Compound 13. To a solution of **12** (302 mg, 0.26 mmol) in anhydrous DMF (5 mL) was added DHBT (100 mg, 0.64 mmol) and DCC (100 mg, 0.5 mmol). After stirring for 3 h at room temperature, spermine (26 mg, 0.13 mmol) and triethylamine (0.1 mL) were added. The mixture was stirred overnight at room temperature and then poured into diluted aqueous HCl (50 mL). The resulting precipitate was collected by filtration and purified by chromatography on a silica gel column, eluting with $CHCl_3$/CH_3OH/H_2O (65/25/4, v/v/v), to give **13** (212 mg, 66%). ^{1}H NMR (CD_3OD, 500 MHz) δ: 4.23 (br, 2 H), 3.95 (br, 6 H), 3.80 (s, 4 H), 3.35−3.17 (m, 4 H), 3.16 (br, 8 H), 2.60 (br, 8 H), 1.97−1.41 (m, 166 H), 1.10−0.92 (m, 32 H), 0.71 (s, 12 H). MALDI-TOF MS *m*/*z* 2478 ([M + H]$^+$).

Conjugate 5. A solution of **13** (200 mg, 0.08 mmol) in TFA and chloroform (5 mL, 1:1, v/v) was stirred for 4 h at room temperature. After removal of solvent under reduced pressure, the deprotected product (appearing as an oil) was used directly in the next step. To a solution of cholic acid (410 mg, 1 mmol) in anhydrous DMF (5 mL) was added DHBT (200 mg, 1.2 mmol) and DCC (240 mg, 1.2 mmol). After stirring the mixture for 3 h at room temperature, the deprotected form of **13** and triethylamine (0.1 mL) were added. The reaction mixture was stirred overnight at room temperature and then poured into diluted aqueous HCl (50 mL). The resulting precipitate was collected by filtration and purified by chromatography on a silica gel column, eluting with $CHCl_3$/CH_3OH/H_2O (65/25/4, v/v/v), to give **5** (112 mg, 36%). ^{1}H NMR (CD_3OD, 500 MHz) δ: 4.26 (br, 4 H), 3.95 (br, 8 H), 3.81 (s, 8 H), 3.35−3.30 (br, 16 H), 3.18 (br, 12 H), 2.30−1.40 (m, 242 H), 1.05−0.92 (m, 64 H), 0.72 (s, 24 H). MALDI-TOF MS *m*/*z* 3862 ([M + Na]$^+$). The sample was judged to be homogeneous based on the appearance of a single TLC spot (developed by two or more eluents) and a comparison of the integrated NMR signal for the lysine protons (4.26 ppm) relative to the 3-position protons of cholyl units (3.95 ppm).

Compound 14. To a solution of 1,4-phenylenediamine (20 mg, 0.19 mmol) and triethylamine (0.30 mL) in anhydrous THF (2 mL) was added Boc-L-Lys(Boc)-OSu (205 mg, 0.46 mmol). After the reaction mixture was stirred at room temperature for 24 h, another portion of Boc-L-Lys(Boc)-OSu (100 mg, 0.23 mmol) was added. The reaction was stirred for an additional 48 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by chromatography on a silica gel column, eluting with $CHCl_3$/MeOH (40/1, v/v), to give **14** (125 mg, 88%). ^{1}H NMR (500 MHz, CD_3OD, 323 K) δ: 7.49 (s, 4 H), 4.12 (br, 2 H), 3.04 (t, ^3J = 6.66 and 6.76 Hz, 4 H), 1.79−1.37 (m, 48 H). MALDI-TOF MS *m*/*z* 787 ([M + Na]$^+$).

Conjugate 6. A solution of **14** (50 mg, 0.065 mmol) in trifluoroacetic acid (2 mL) was stirred at room temperature for 2 h and then concentrated under reduced pressure. Removal of solvent under reduced pressure and drying under vacuum for 4 h gave **15**, which was used directly without further purification. The complete removal of the Boc groups was confirmed by ^{1}H NMR (500 MHz, CD_3OD, 298 K) δ: 7.59 (s, 4H), 4.03 (t, 2 H, ^3J = 6.60 and 6.50 Hz), 2.93 (t, 4 H, ^3J = 7.55 and 7.85 Hz), 2.01−1.95 (m, 4 H), 1.74−1.69 (m, 4 H), 1.561.51 (m, 4 H).

To a solution of L-lysine-dicholamide (290 mg, 0.31 mmol) and DHBT (64 mg, 0.39 mmol) in anhydrous DMF (2 mL) was added DCC (100 mg, 0.51 mmol). After the mixture was stirred for 3 h, a solution of **15,** which was prepared from **14** (50 mg, 0.065 mmol), and triethylamine (0.5 mL) in anhydrous DMF (0.5 mL) was added. The reaction mixture was stirred at room temperature for 24 h and then poured into 1 M hydrochloride acid (150 mL). The resulting precipitate was collected by filtration and purified twice by chromatography on a silica gel column, eluting with $CHCl_3$/CH_3OH/H_2O (40/10/1, v/v/v), to give **6** (147 mg, 56%). ^{1}H NMR (500 MHz, CD_3OD, 328 K) δ: 7.69 (unsolved, 2H), 7.56 (unsolved, 2H), 4.44 (unresolved, 2 H), 4.31 and 4.23 (unresolved, 4 H), 3.94 (s, 8 H), 3.79 (s, 8 H), 3.36−3.34 (m, 8 H), 3.15 (m, 12 H), 2.29−0.91 (m, 276 H), 0.70 (s, 24 H). MALDI-TOF MS *m*/*z* 4024 ([M + Na]$^+$). The sample was judged to be homogeneous based on the appearance of a single TLC spot (developed by two different eluents) and a comparison of the integrated NMR signals for the lysine protons (4.44−4.23 ppm) relative to the 3-position protons of cholyl units (3.94 ppm).

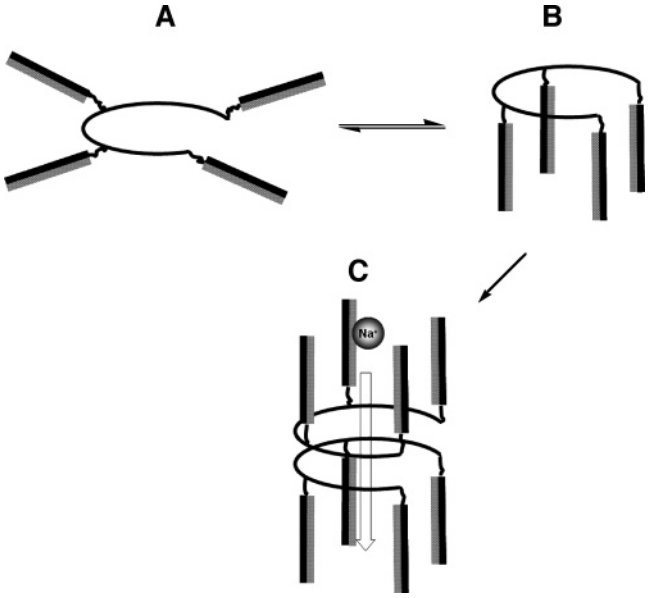
Vesicle Formation and Na^+/K^+ Transport Measurements. Typically, 2.5 mL of a 20 mg/mL solution of 1-palmitoyl-2-oleoyl-2-*sn*-glycero-3-phosphocholine (POPC) in chloroform was transferred to a Pyrex test tube. The desired amount of pore-forming conjugate was then added from a stock solution in methanol. While rotating the tube, the organic solvents were removed under a stream of nitrogen, resulting in a thin lipid film. The last traces of solvent were then removed under reduced pressure (25 °C, 12 h, <0.2 Torr). To the dried film was added 1.0 mL of a 150 mM KCl solution that was 10% D_2O and 90% H_2O, and the mixture was vortexed for 1 min. The dispersion was then

incubated for 5 min, followed by another 1 min of vortexing and 20 min of incubation at ambient temperature. The sample was subjected to five freeze/thaw cycles (77/325 K), followed by sequential extrusion through a 400 nm Nuclepore membrane (10 times) and a 200 nm membrane (10 times). After extrusion the dispersion was incubated at room temperature for 1.25 h. In a quartz NMR tube 1.5 mL of a 150 mM NaCl solution in 10% D_2O plus 90% H_2O was mixed with 0.3 mL of a shift reagent solution (10 mM $DyCl_3$; 30 mM $Na_5P_3O_{10}$ in 10% D_2O plus 90% H_2O). To this solution was added 0.75 mL of the vesicle dispersion, and the resulting mixture was vortexed for 30 s. ^{23}Na NMR spectra were recorded continuously at 35 °C overnight on a Bruker AMX 360 MHz NMR instrument. Pseudo-first-order rate constants were calculated from the change in the percentage of encapsulated Na^+ as a function of time using a curve-fitting procedure.

Results and Discussion

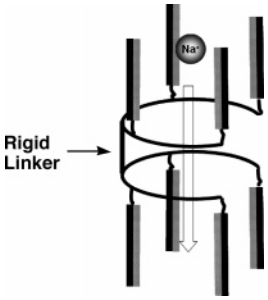
Design Principles. The design principle that we have previously used in devising **1** and related homologues is shown in Chart 2.[6,8,9] In essence, a collection of facial amphiphiles is covalently attached to a flexible backbone. When inserted into a lipid bilayer, such a conjugate is expected to favor conformation **B** over **A**. In the case of **B**, the hydrophobic faces (darkened rectangles) lie in contact with the acyl chains of neighboring phospholipids (not shown) and the hydrophilic faces (lightly shaded rectangles) point toward each other. Subsequent dimerization across the bilayer is then expected to produce a "barrel stave" structure (**C**). Kinetic studies that have been carried out for the transport of Na^+ across liposomal membranes made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) for conjugates of this type have provided support for such a model.[6,8]

Chart 2



An alternate design principle that we have recently become attracted toward is illustrated in Chart 3. Here, a small, rigid, and planar linker is used to connect two "half barrels" to yield a "whole barrel". It should be noted that this design principle bears a strong resemblance to that previously used in creating an artificial ionophore via the attachment of two hydroxylated sterols to a terephthaloyl diester spacer.[10] In that study it was postulated that such a molecule would favor a membrane-spanning orientation in the bilayer. On the basis of a kinetic analysis for Na^+ transport, approximately *three* such amphiphiles appear to combine to form a transport-active species.[10] A key difference with the approach reported herein is that our conjugates have the potential for acting as *individual* molecules; that is, aggregation is not required. We also note that the use of small, rigid, and planar moieties has been used in the design of certain other artificial ionophores, where the transport-active species appears to exist in an aggregated form.[11−15]

Chart 3



Synthetic Targets. To test our design principle we first chose conjugate **2** as a synthetic target (Chart 4). Here, a 1,4-phenylenediamide moiety was selected as a small, rigid, and planar linker. To explore the sensitivity of ion transport activity toward the compactness of a poly(choloyl)-based amphiphile, we compared the activity of **3** with two less compact analogues; that is, **4** and **5** (Chart 5). Thus, whereas **3** consists of four lysine−dicholamide moieties that are covalently attached to a spermine backbone, conjugate **4** has the same four lysine−dicholamides attached to a putrescine backbone, which has been extended by two lysine groups. In the case of conjugate **5**, the eight choloyl units are now spaced further apart by use of a spermine core that has been extended on both ends by two lysine units. Finally, as a further test of our new design principle, conjugate **6** was chosen as a synthetic target since it can be directly compared with **4** (Chart 6). In this case, the flexible putrescine core of **4** has been replaced by a 1,4-phenylenediamide moiety.

Conjugate Synthesis. The synthetic approach that was used for the preparation of **2** is outlined in Scheme 1. Thus, acylation of Boc-L-Lys-OH with $N_\alpha N_\epsilon$-di-Boc-L-lysine hydroxylsuccinimide ester (Boc-L-Lys(Boc)-OSu) afforded **7**. Subsequent activation of the carboxylic group and condensation with 1,4-phenylenediamine produced **8**, which was then deprotected and acylated with L-lysine-dicholamide to give **2**. The synthesis of **4** was carried out by a similar sequence of reactions. Specifically, acylation of putrescine with Boc-L-Lys(Boc)-OSu, followed by deprotection, and acylation with L-lysine-dicholamide produced the desired conjugate (Scheme 2). Conjugate **5** was synthesized by acylating L-lysine-dicholamide with Boc-L-Lys-OH, followed by activation, condensation with spermine, deprotection, and, finally, condensation with an activated form of cholic acid (Scheme 3).

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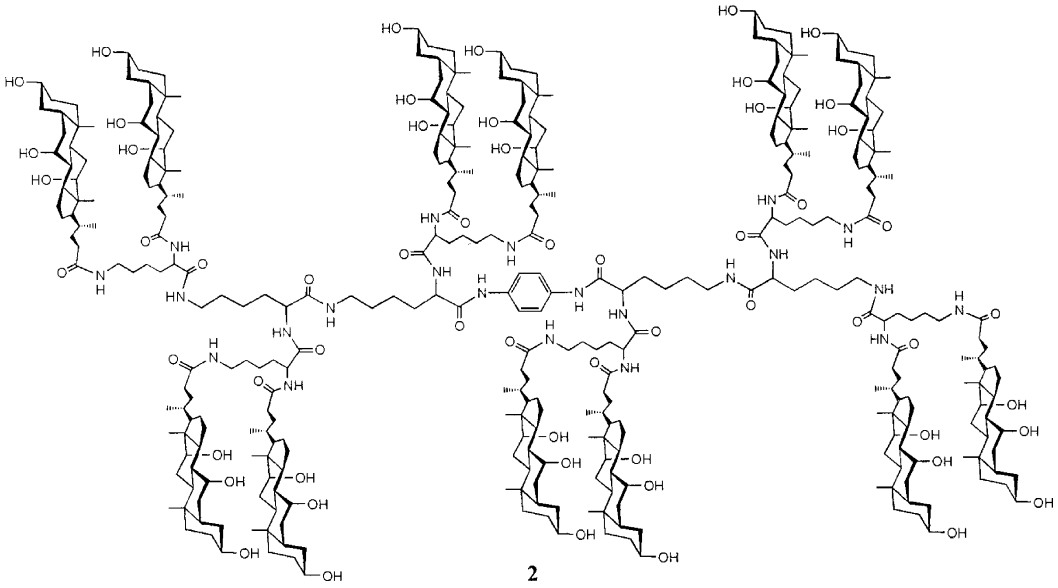
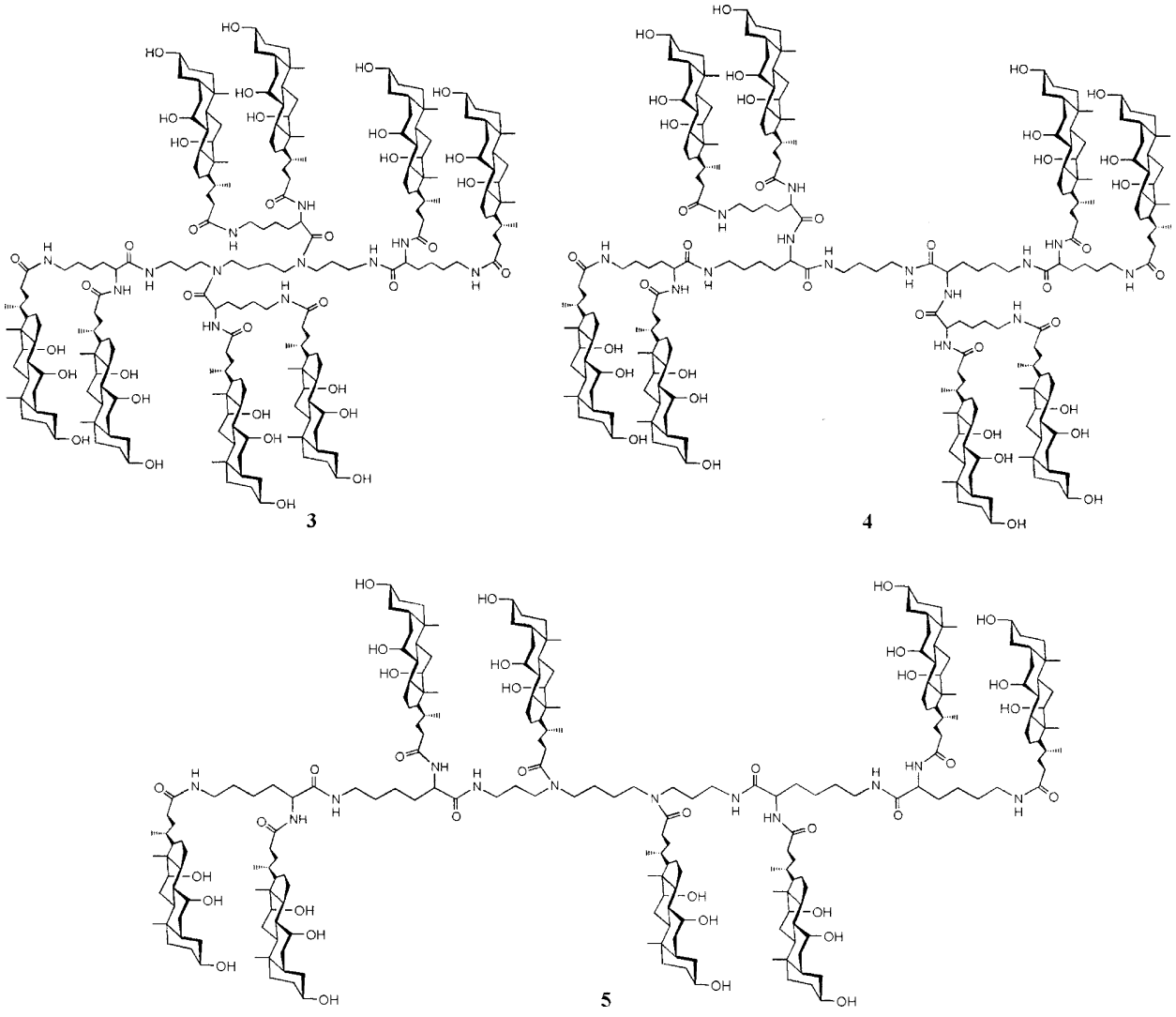
Chart 4



2

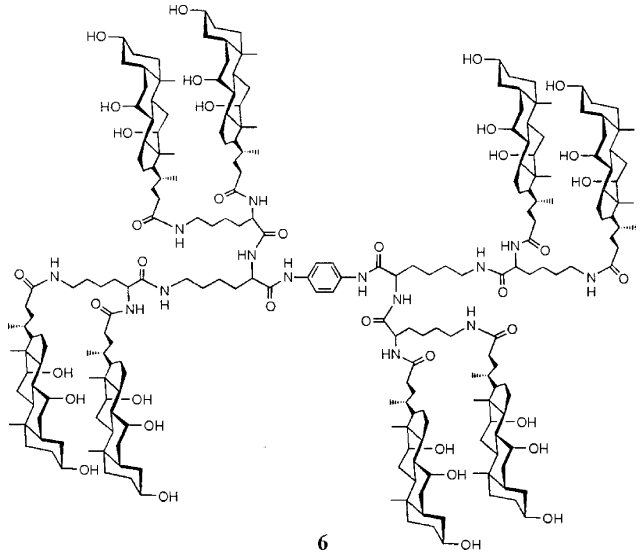
Chart 5



3 **4** **5**

Finally, the synthesis of **6** was accomplished by acylating 1,4-phenylenediamine with Boc-L-Lys(Boc)-OSu, followed by deprotection and acylation with L-lysine-dicholamide (Scheme 4).

Chart 6



6

Kinetics of Na^+ Transport. The pore-forming properties of each of these conjugates were examined by measuring their ability to promote Na^+ transport across POPC-based liposomes (ca. 200 nm diameter, unilamellar), as determined by $^{23}Na^+$ NMR spectroscopy, using methods similar to those previously described.[8] In all cases, the rate of entry of Na^+ into the aqueous compartment of the liposomes obeyed pseudo-first-order kinetics. For **1** the observed pseudo-first-order rate constant (k_{obsd}) was found to have a strong dependence on its concentration in the membrane (Figure 1A). Specifically, k_{obsd} showed a second-order dependence on the mol % of **1**. As discussed elsewhere, such a dependence lends strong support for a model in which (i) monomers of **1** are in equilibrium with dimers, (ii) monomers are thermodynamically favored and their concentration can be approximated by the total concentration of **1** that is present, and (iii) dimers are responsible for ion transport.[16] Thus, for the general case of transport-active aggregates, the rate of ion flow is expected to obey eq 1, where k_{obsd} is a pseudo-first-order rate constant that is the product of the aggregate concentration and a rate constant, k_2 (eq 2). If the aggregate concentration is expressed in terms of the monomer concentration and the dissociation constant (K) that defines the aggregate−monomer equilibrium (eq 3), then k_{obsd} will vary with the monomer concentration to the nth power (eq 4). Thus, the second-order dependence of k_{obsd} on the mol % of **1** in bilayers of POPC supports the existence of transport-active dimers.

$$\text{rate} = k_{obsd}[Na^+] \quad (1)$$

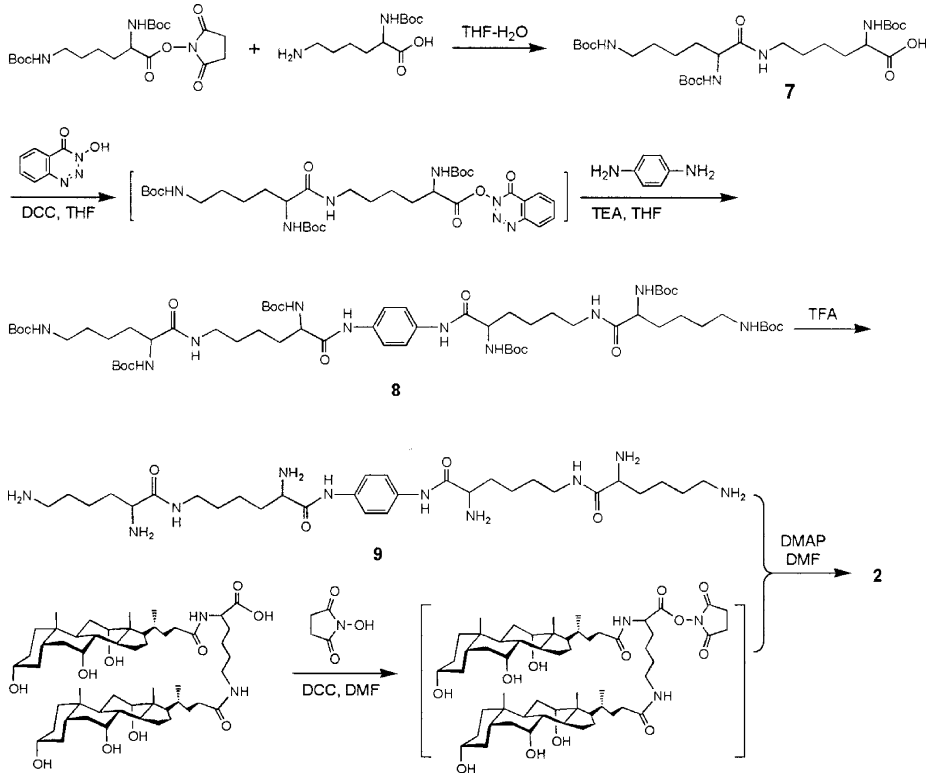
$$k_{obsd} = k_2[\text{aggregate}] \quad (2)$$

$$K = [\text{monomer}]^n/[\text{aggregate}] \quad (3)$$

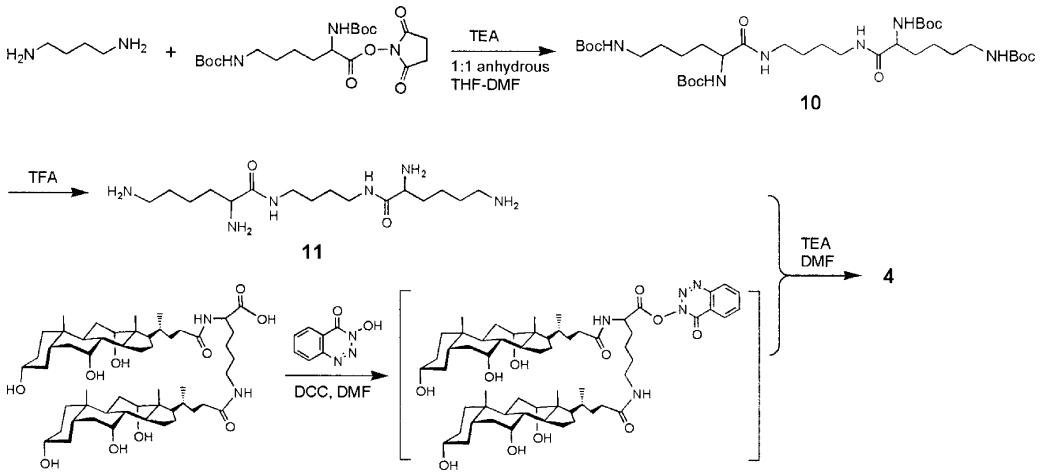
$$k_{obsd} = k_2[\text{monomer}]^n/K \quad (4)$$

In contrast to **1**, k_{obsd} for **2** was found to be *directly proportional* to the concentration of the conjugate (Figure 1B). This finding supports the existence of a *monomer*-active species and is fully consistent with our new design principle (Chart 3). Similar plots that were made for **3**−**5** showed the expected second-order dependence on the mol % of each conjugate, which also supports the existence of dimer-active species and our earlier design principle (Figure 1C−E). Finally, similar to **2**, a first-order dependence of k_{obsd} on the mol % of the conjugate was found for **6** (Figure 1F). Thus, of the six conjugates that were investigated, those four that had a flexible core (**1**, **3**, **4,**

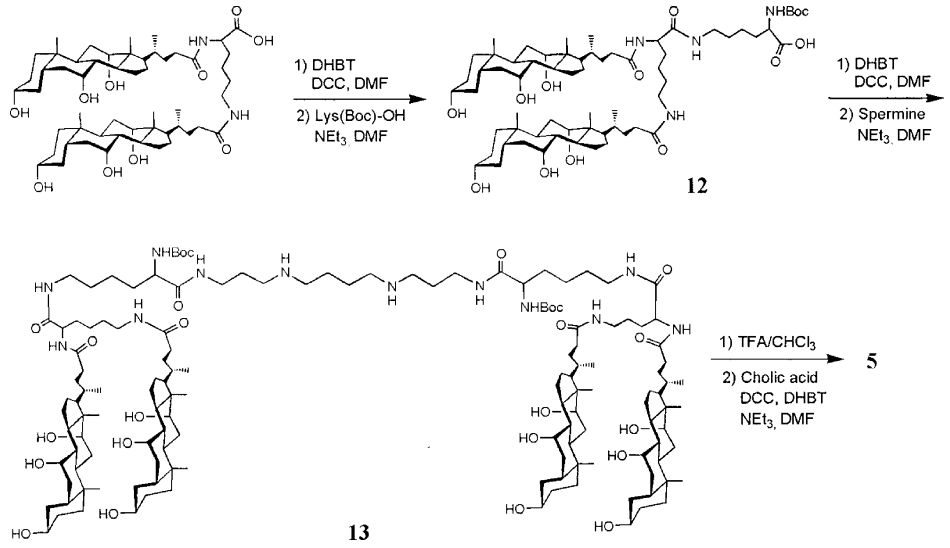
Scheme 1



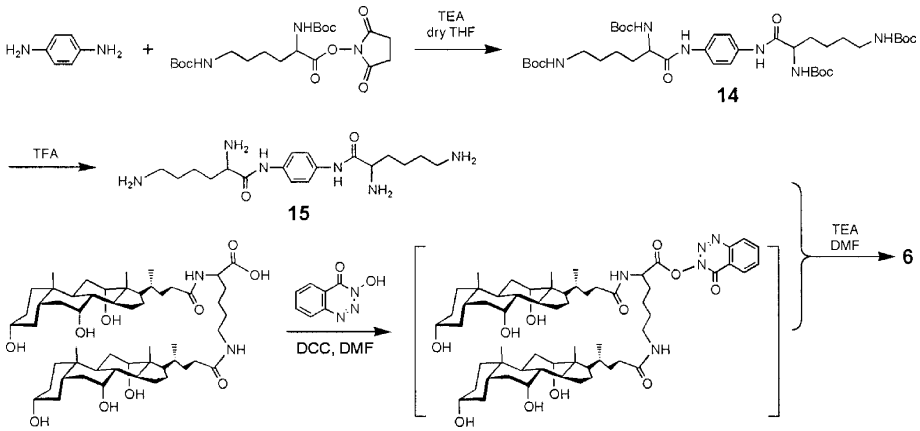
Scheme 2



Scheme 3



Scheme 4



and **5**) showed kinetic features indicative of dimer-active species; the two that possessed a rigid, planar core (**2** and **6**) exhibited kinetic features indicative of a monomer-active species.

On the basis of a nonlinear least-squares fit of the data in Figure 1, values of k_2/K for **3**, **4,** and **5** were found to be 260, 21, and 85 ($min^{-1}mol\ \%^{-2}$), respectively. Although we are presently unable to separate these kinetic and thermodynamic terms, we believe that the much higher value of k_2/K for **3** relative to **4** is a likely consequence of a pore structure that is less deformable due to a higher degree of compactness. In other words, we believe that this difference is largely a reflection of the difference in the intrinsic activities of the pores (i.e., k_2 is dominant). The reason k_2/K for **5** is greater than that of **4** is not presently clear. One possibility is that the greater conformational

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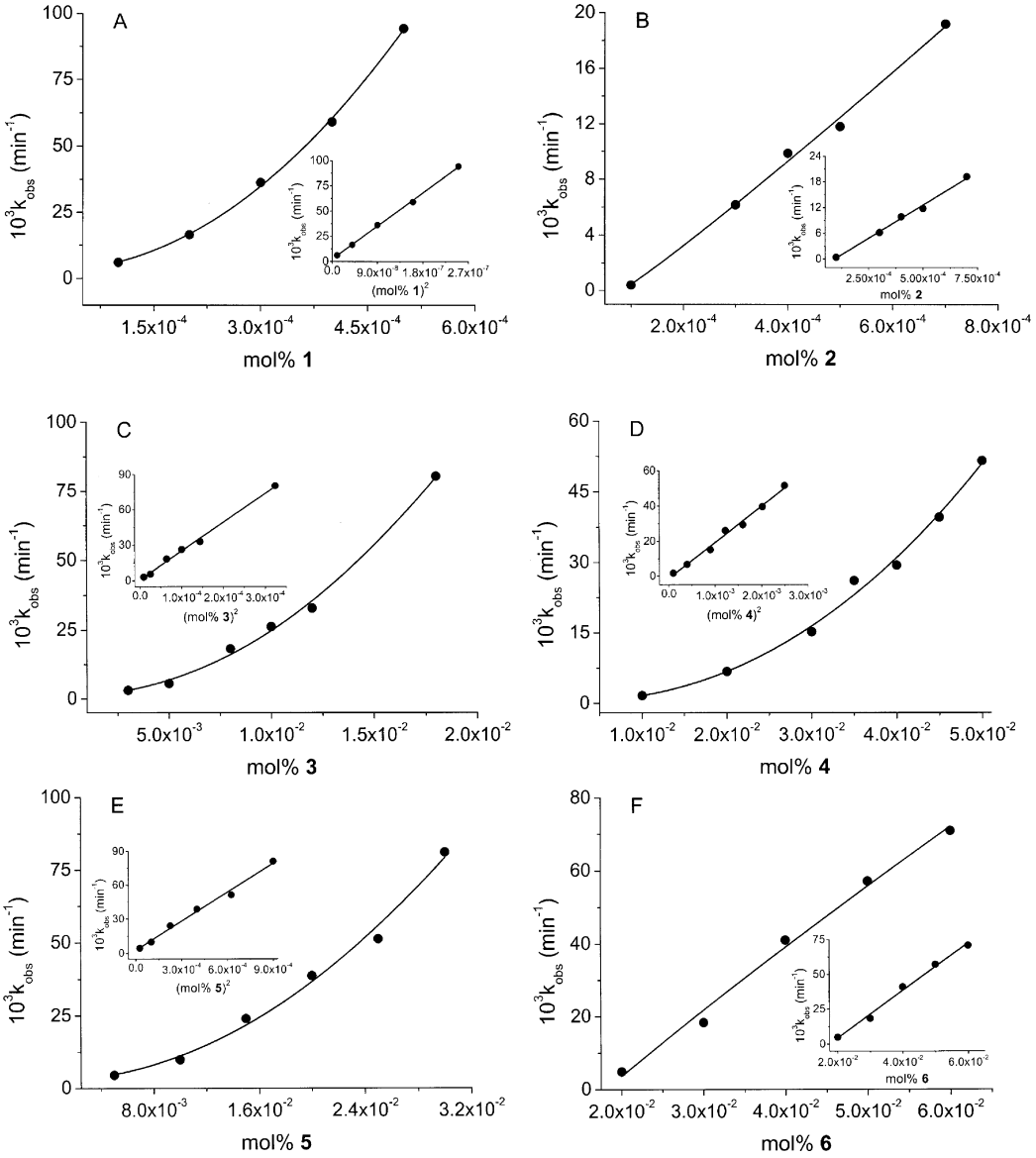
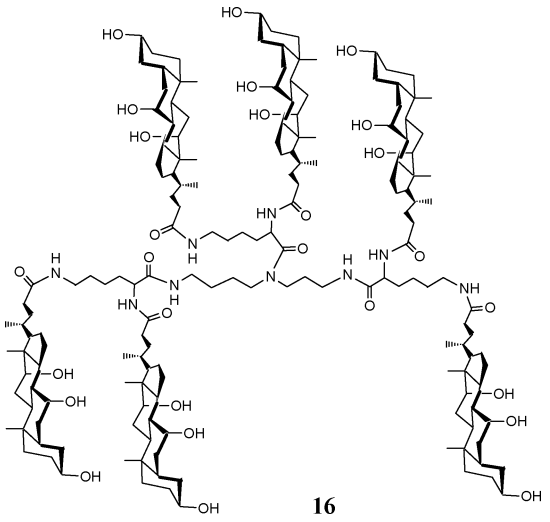


Figure 1. Plots of k_{obsd} versus (mol % conjugate) for (A) **1**, (B) **2**, (C) **3**, (D) **4**, (E) **5,** and (F) **6** in POPC liposomes at 35 °C. The solid lines are nonlinear least-squares fit of the data according to $k_{obsd} = k_0 + k_2(\text{mol \% conjugate})^n/K$, where $n = 2.0$, 1.1, 2.1, 2.2, 2.0, and 0.9, respectively; insets represent similar plots where n has been fixed at 2.0, 1.0, 2.0, 2.0, 2.0, and 1.0 for **1**, **2**, **3**, **4**, **5,** and **6**, respectively.

flexibility of **5** allows for more effective matching of the two halves of the barrel stave; that is, K may be more important than k_2. Despite these uncertainties, the fact that the overall activity of a poly(choloyl)-based amphiphile can be modified to a significant extent by adjusting its compactness adds a new dimension to the design of pore-forming agents in a general sense. It is also noteworthy that since each molecule of **2** (a monomer-active amphiphile) contributes, in effect, six choloyl units per monolayer, one may compare its activity with a dimer-active amphiphile (**16**) containing six choloyl groups linked to a spermidine backbone that we have reported recently.[8] Thus, whereas 0.11 mol % of **16** results in a half-life for the Na^+ transport equaling 70 min, a similar level of activity requires only 0.0004 mol % of **2**. From an operational standpoint, therefore, **2** is ca. 300 times more active than **16**.[8] The greater transport activity found with **2** ($k_1 = 31.0$ min^{-1}mol %$^{-1}$) relative to **6** ($k_1 = 1.7$ min^{-1}mol %$^{-1}$), where $k_{obsd} = k_1$[**2** or **6**], is a likely consequence of a larger pore size resulting from the larger number of sterols per conjugate. Finally, the fact that the activity per mole of **1** (a dimer-active amphiphile) is not very different from that of **2** (a monomer-active amphiphile)



16

implies that the unimolecular pore is less active for cation translocation. The lower activity of the latter is a likely result of a smaller pore size, due to the presence of fewer sterols in each half of the bilayer.

Thermally Gated Release of Carboxyfluorescein. Although a precise determination of the pore sizes that are created by these conjugates lies beyond the scope of the present study, preliminary experiments that we have carried out with the most compact member of this series (i.e., **3**) indicate an effective diameter that is greater than that of carboxyfluorescein (CF), estimated to be ca. 0.96 nm.[17] Thus, using experimental procedures similar to those previously described, CF was encapsulated in liposomes made from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) containing **3**.[6] By raising the temperature of the dispersion from 37 to 43 °C, a rapid efflux of CF was observed (Figure 2).[18]

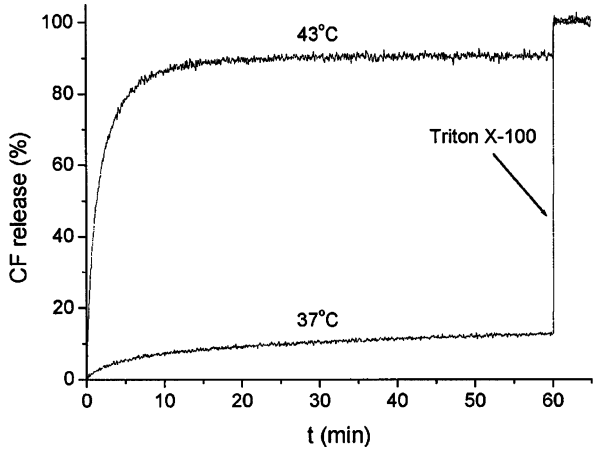


Figure 2. Plot of percentage of CF release from liposomes made from DPPC containing 0.01 mol % of **3** as a function of time at 37 and 43 °C. Complete release of CF was measured after destroying the liposomes with Triton X-100.

Conclusions

A series of poly(cholyl)-based pore-forming amphiphiles have been synthesized in which core flexibility and overall compactness have been modified by synthetic design. Kinetic results obtained from Na^+ transport measurements across POPC bilayers provide strong support for a design principle in which a poly(cholyl)-based conjugate bearing a rigid core can act as a transporter as an individual molecule. Related studies with analogous amphiphiles having a flexible core indicate that overall compactness is an additional variable that can be used to adjust transport activity. Efforts aimed at gaining further control over pore formation by poly(cholyl)-based conjugates are currently in progress with a view toward the design of new antibiotics.

Acknowledgment. We are grateful to the National Science Foundation (CHE-0345248) for support of this research.

Supporting Information Available: Typical $^{23}Na^+$ NMR spectrum used for quantifying internal and external (shifted) Na^+ (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA053527Q

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(18) Dynamic light scanning measurements indicated the DPPC liposomes containing 0.01 mol % of **3** had, essentially, the same size and size distribution before and after CF release at 43 °C. The average diameters for the dispersions were ca. 172 and 179 nm, respectively.