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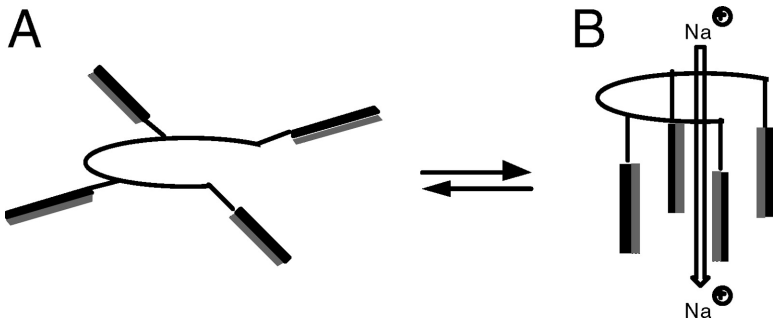

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Kinetic Evidence for the Existence and Mechanism of Formation of a Barrel Stave Structure from Pore-Forming Dendrimers

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Abstract: A dendritic approach to the construction of a homologous series of pore-forming amphiphiles has been developed, based on the use of spermidine, spermine, lysine, and cholic acid. A kinetic analysis of Na^+ transport across bilayers of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine by three dendritic amphiphiles has provided the strongest evidence to date for a barrel stave structure.

The mechanism by which amphotericin B and related facially amphiphilic antibiotics create pores in lipid bilayers and the structure of such pores remain a matter of debate.[1] Although the barrel stave model has proven popular, where it has been postulated that individual amphiphiles (staves) assemble into two "half-barrels" that connect across the bilayer, as noted by Gennis, such a structure is "purely conjecture".[2] In this paper, we report the synthesis of a series of pore-forming amphiphiles in which multiple staves (choloyl groups) have been covalently linked to low-generation dendrimers (i.e., conjugates **1**, **2**, and **3**; Chart 1). We also report kinetic results that not only provide the strongest evidence to date for a barrel stave structure but also indicate that *cooperative forces play a major role in its formation*. The relevance of these findings for the design of novel classes of antibiotics is briefly discussed.

Interest in the design and synthesis of pore-forming agents continues to grow.[3] Our own interest in such molecules stems from the belief that they may lead to new classes of antibiotics, which circumvent common mechanisms of drug resistance.[4] Recently, we showed that a conjugate made from spermine and four choloyl groups (i.e., **4**) promotes the transport of Na^+ across bilayers of 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (**C**14).[5,6] The design principle upon which **4** was based is illustrated in Chart 2. In brief, a molecule containing an array of facially amphiphilic units attached to a flexible chain is expected to favor conformation **B** when inserted into a lipid bilayer. Here, the hydrophobic faces (darkened rectangles) lie in contact with the acyl chains of neighboring phospholipids, and the hydrophilic faces (lightly shaded rectangles) point toward each other. Subsequent dimerization across the bilayer then affords a barrel stave.

Here, we report the results of a structure−activity study that was aimed at gaining greater insight into the nature of the pores that are generated by polyamine-cholic acid conjugates in phospholipid bilayers. This investigation was motivated by our discovery that **4** is ca. 400 times more active for Na^+ transport than a spermidine analogue bearing only three choloyl groups, a finding which suggested that cooperative forces may be involved in pore formation.[5a] For this purpose, we used a dendritic approach to construct a homologous family of conjugates containing four, six, and eight choloyl groups. *Low generation* dendrimers were of special interest due to their greater flexibility, which allows for easier access to conformations approaching **B** (Chart 2).[7] In this work, we have used bilayers of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) as model systems because this lipid closely resembles those that are found in natural membranes.

A convergent synthesis of **2** and **3** was readily accomplished by acylating both amino groups of lysine with cholic acid, followed by acylation of spermidine and spermine with the resulting lysine-dicholamide; (Scheme 1). The synthesis of **1** has previously been reported.[6] Ion transport properties were then determined by measuring their ability to promote Na^+ transport across POPC liposomes (ca. 200 nm diameter, unilamellar) using $^{23}Na^+$ NMR methods similar to those previously described.[5]

Plots made of the pseudo first-order rate constants, k_{obsd}, for Na^+ transport versus (mol % of conjugate)[2] for **1**, **2**, and **3** were

(1) (a) Bolard, J. *Biochim. Biophys. Acta* **1986**, *864*, 257. (b) Kleinberg, M. E.; Finkelstein, A. *J. Membr. Biol*. **1984**, *80*, 257.
(2) Gennis, R. B. *Biomembranes: Molecular Structure and Function*; Springer-Verlag: New York, 1989; Chapter 8.
(3) For reviews of synthetic ionophores, see: (a) Kobuke, Y. *Advances in Supramolecular Chemistry*; Gokel, G. W., Ed.; JAI Press: Greenwich, CT, 1997; Vol 4, pp 163−210. (b) Fyles, T. M.; van Straaten-Nijenhuis, W. F. *Comprehensive Supramolecular Chemistry*; Reinhoudt, D. N., Ed.; Elsevier Science: Amsterdam, New York, 1996; Vol. 10, pp 53−77. (c) Gokel, G. W.; Mukhopadhyay, A. *Chem. Soc. Rev*. **2001**, *30*, 274. (d) Matile, S. *Chem. Soc. Rev*. **2001**, *30*, 158.
(4) Stadler, E.; Dedek, P.; Yamashita, K.; Regen, S. L. *J. Am. Chem. Soc*. **1994**, *116*, 6677.
(5) (a) Bandyopadhyay, P.; Janout, V.; Zhang, L. H.; Sawko, J. A.; Regen, S. L. *J. Am. Chem. Soc*. **2000**, *122*, 12888. (b) Bandyopadhyay, P.; Janout, V.; Zhang, L. H. *J. Am. Chem. Soc*. **2001**, *123*, 7691.
(6) Bandyopadhyay, P.; Bandyopadhyay, P.; Regen, S. L. *Bioconjugate Chem*. **2002**, *13*, 1314.
(7) For general texts on dendrimers, see: (a) Frechet, J. M. J.; Tomalia, D. A. *Dendrimers and Other Dendritic Polymers*; John Wiley & Sons: New York, 2001. (b) Newkome, G. R.; Moorefliedl, C. N.; Vogtle, F. *Dendrimers and Dendrons*; Wiley-VCH: Weinheim, 2002.

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Chart 1

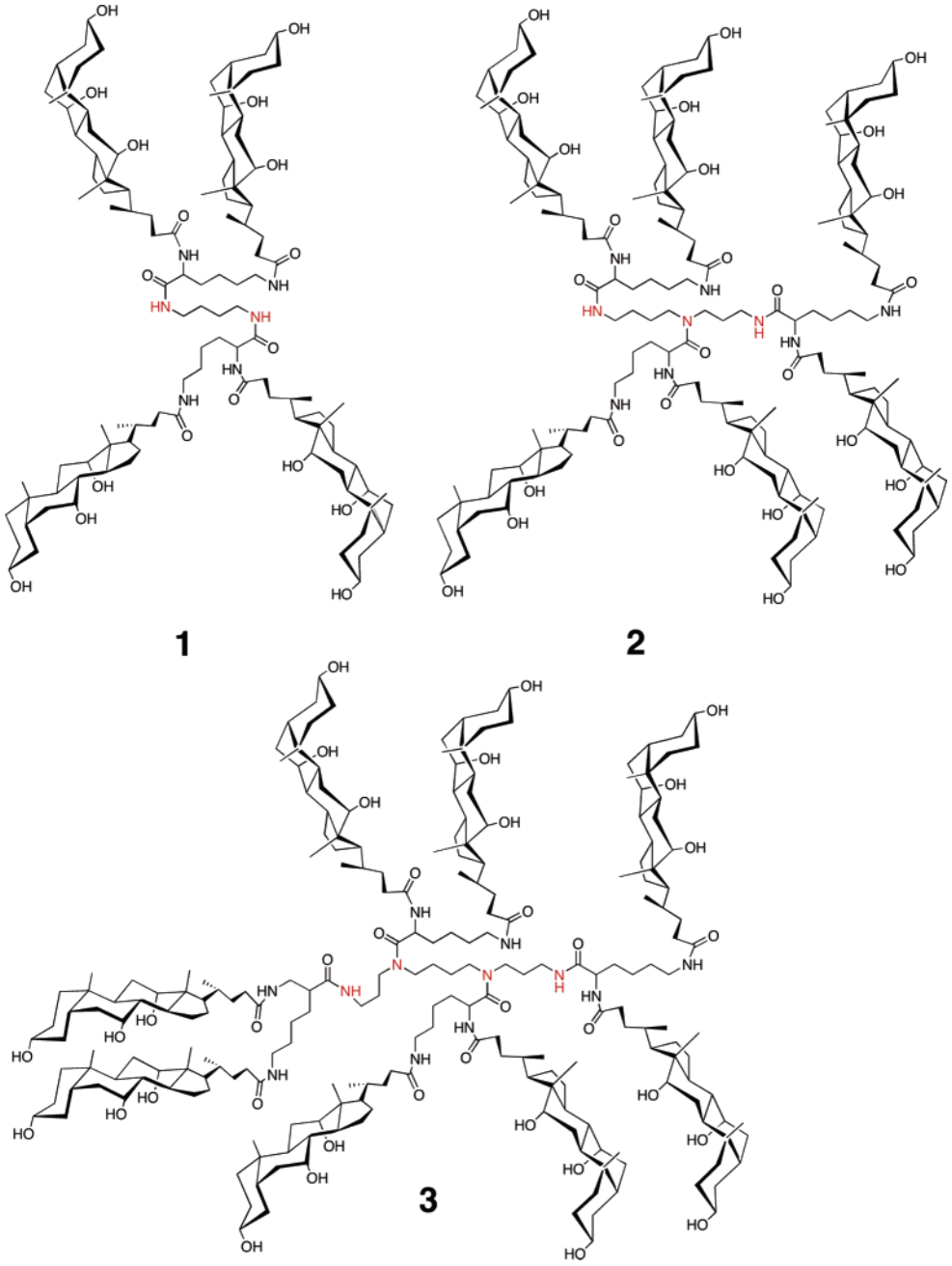
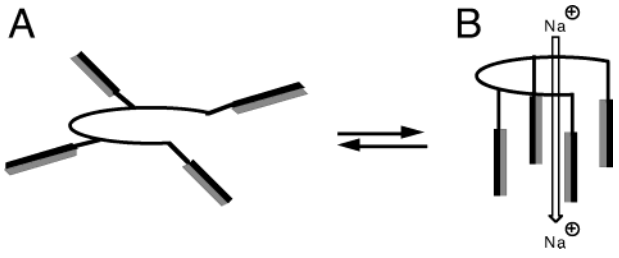
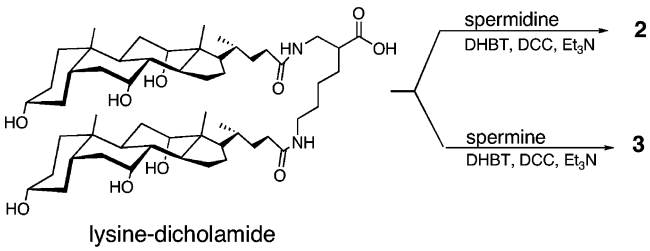


Chart 2



Scheme 1



found to be linear, indicating that *transport-active dimers* are involved. (Figure 1A−C).[4,5,8] Here, it is assumed that only a small fraction of the conjugate is in the dimer form, where it can be shown that

$$k_{obsd} = k_2[\text{monomer}]^2/K \qquad (1)$$

where K is the equilibrium constant for dissociation of the dimer,

(8) Liposomes prepared in aqueous LiCl with 0.005 mol % of **3**, followed by dilution with aqueous NaCl, resulted in $k_{obsd} = 0.0069$ min^{-1} for $^{7}Li^{+}$ efflux and $k_{obsd} = 0.0071$ min^{-1} for $^{23}Na^{+}$ influx in the same dispersion. These results are consistent with an antiport mechanism of transport.

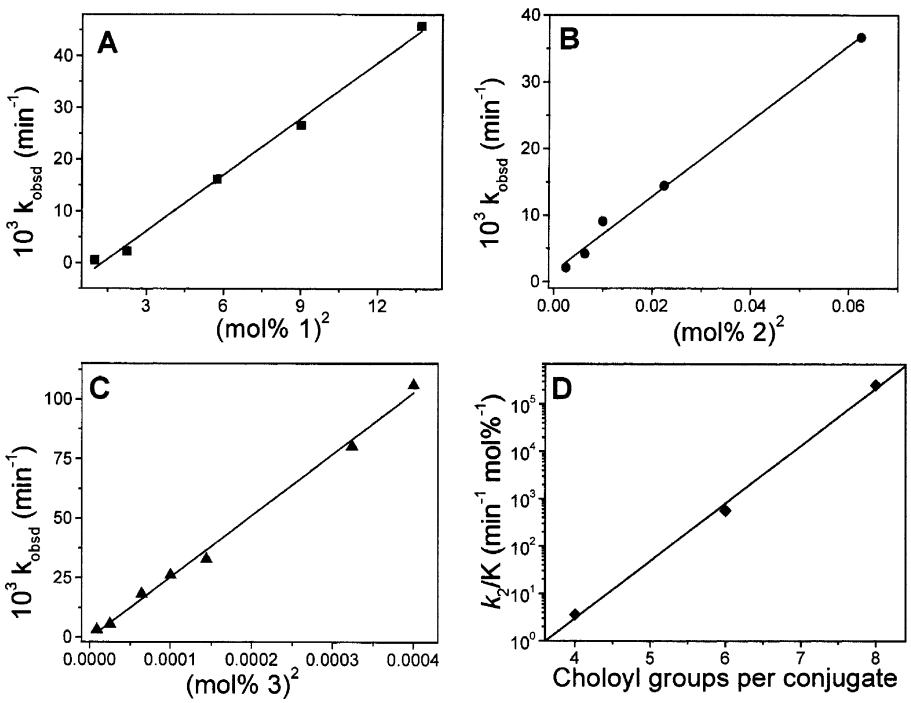


Figure 1. Plot of k_{obsd} versus (mol % dendrimer)^2 in POPC liposomes at 35 °C for (A) **1**, (B) **2**, and (C) **3**. Semilogarithmic plot of k_2/K versus the number of choloyl groups per dendrimer (D).

k_2 is the rate constant for ion transport, and [monomer] is the analytical concentration of the conjugate that is present in the dispersion.[4,5] A plot of dendrimer activity (expressed as k_2/K) versus the number of choloyl groups in **1**, **2**, and **3** further reveals an exponential relationship (Figure 1D).

The involvement of transport-active dimers with **1**, **2**, and **3**, together with a correlation between their activity and their choloyl content, make a strong case that *the pores that are generated within this family of conjugates are similar in structure*. That **1** functions as a membrane-spanning agent has already been indicated by its high sensitivity toward bilayer thickness. Specifically, its activity in **C**14 bilayers was found to be 2300 times greater than in bilayers made from 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (**C**16).[9] The exponential increase in activity, on going from **1** to **2** to **3**, further implies that cooperative forces play a major role in pore formation. That this effect is not the result of a change in pore size is supported by simple geometric considerations. Thus, to a first approximation, a doubling of the number of choloyl groups per dendrimer should lead to a doubling of the circumference and the radius of the pore and to a quadrupling of the pore's surface area. Since the rate of permeation across a pore is directly proportional to its surface area, the Na^+ transport rate is also expected to quadruple. This predicted increase pales in comparison with the ca. 10^5 increase in activity on going from **1** to **3**! In addition, simple combinatorial statistics would also not account for the large rate increases that accompany increased numbers of sterol units per dendrimer.[10]

What is this origin for this cooperativity? One plausible explanation is that hydrophobic interactions between the individual sterol units of the dendrimer and the acyl chains of neighboring phospholipids cooperate in "pulling" the dendrimer into the leaflet in the form of a half-barrel. Here, it is assumed that the majority of dendrimer lies flat at the membrane−water interface (Chart 2, conformation **A**). An alternative possibility is that cooperative hydrogen bonding between two dendrimers across the bilayer stabilizes the barrel stave, resulting in an increase in the number of pores in the membrane. Although we cannot distinguish between these two scenarios, both involve *two dendrimers coming together from the two adjoining leaflets of the bilayer to form a barrel stave.*

In preliminary studies, we have examined whether the pores that are generated from **3** are large enough for glutathione (γ-Glu-Cys-Gly, GSH) to cross. Using experimental methods similar to those previously described, 2.1 mM GSH was entrapped within POPC liposomes in the presence of 0.01 mol % of **3**.[11] Subsequent dialysis (23 °C) resulted in the release of

(9) When the bilayer thickness begins to exceed the length of a membrane-spanning agent, ion transport activity is expected to be sharply reduced or eliminated due to its inability to provide a contiguous pathway for ion flow. For a carrier mechanism, transport activity is expected to show a modest dependence on membrane thickness: Otto, S.; Osifchin, M.; Regen, S. L. *J. Am. Chem. Soc*. **1999**, *121*, 7276.

(10) If there were some minimum number of choloyl groups (staves) that were required to form a half-barrel, then the larger the number of choloyl groups per dendrimer, the greater the probability of reaching this number. Since we have already shown that a 3-sterol analogue follows pure second-order kinetics, this minimum number would have to be three or less.[5a] If the critical number was 3, then there are 20 different ways to make an intramolecular sterol trimer, 15 ways to make an intramolecular tetramer, 6 ways to make an intramolecular pentamer, and 1 way for an intramolecular hexamer from the 6-sterol dendrimer. In other words, there are $20 + 15 + 6 + 1 = 42$ combinations that can lead to a half-pore. In the case of the 8-sterol dendrimer, there are 56, 70, 56, 28, 8, and 1 ways of making intramolecular trimers, tetramers, pentamers, hexamers, heptamers, and octamers, respectively, corresponding to 219 effective combinations. Thus the rate increase due to increased probabilities is $(219/42)^2 = 27.1$. By a similar analysis, if the minimal number of sterols that were required to form the half-barrel was 2, then the total number of combinations that would lead to half-barrels would be 57 in the case of the 6-sterol dendrimer and 247 in the case of the 8-sterol dendrimer. Thus, the rate enhancement due to increased probabilities is $(247/57)^2 = 18.8$. Since the observed ratio of the second-order rate constants for the 8- and 6-sterol dendrimers is 258 000/565 = 457, simple combinatorial statistics cannot account for the observed rate increase.

(11) Janout, V.; Jing, B.; Staina, I. V.; Regen, S. L. *J. Am. Chem. Soc*. **2003**, *125*, 4436. In the present experiments, the aqueous solution also contained 150 mM KCl and 1 mM EDTA, adjusted to pH 7.0.

8 and 21% of GSH after 18 and 44 h, respectively. Thus, the resulting pores appear to be moderate in size. In the absence of **3**, negligible leakage of the tripeptide (<0.4%) was observed.[12,13]

Studies currently in progress are aimed at defining these pores in greater detail and at developing pore-forming dendrimers with a view toward drug design. The results of these efforts will be reported in due course.

Experimental Section

Dendrimer 2. To a solution of lysine-dicholamide (139 mg, 0.15 mmol) in 1 mL of DMF was added dihydro-4-oxo-1,2,3-benzotriazine (DHBT) (26 mg, 0.16 mmol) and DCC (33 mg, 0.16 mmol).[6] After stirring the mixture for 3 h, spermidine (7.3 mg, 0.05 mmol) and triethlyamine (125 μL) were added. The reaction mixture was stirred overnight at room temperature and then poured into 50 mL of 1 M aqueous HCl. The resulting precipitate was collected by filtration and purified by preparative thin-layer chromatography [silica, $CHCl_3/CH_3OH/H_2O$ (65/25/4, v/v/v)] to give 100 mg (67%) of the desired conjugate having R_f 0.59 [silica, $CHCl_3/CH_3OH/H_2O$ (65/25/4, v/v/v)] and ^{1}H NMR (CD_3OD, 360 MHz, 315 K) δ ppm 0.68 (s, 18 H), 0.89−2.20 (m, 204 H), 3.14 (m, 10 H), 3.35 (m, 10 H), 3.80 (s, 6 H), 3.92 (s, 6 H), 4.28 (br, 2 H), 4.74 (br, 1 H). HRMS for $C_{169}H_{283}N_9O_{27}Na^+$: calcd, 2894.0941; found, 2894.1043.

Dendrimer 3. To a solution of lysine-dicholamide (120 mg, 0.129 mmol) in 1 mL of DMF were added dihydro-4-oxo-1,2,3-benzotriazine (DHBT) (25 mg, 0.153 mmol) and DCC (32 mg, 0.155 mmol). After the mixture was stirred for 3 h, spermine (5.5 mg, 0.027 mmol) and triethylamine (54 μL) were added. The reaction mixture was stirred overnight and then poured into 50 mL of 1 M aqueous HCl. The resulting precipitate was collected by filtration and purified by preparative thin layer chromatograph [silica, $CHCl_3/CH_3OH/H_2O$ (40/10/1, v/v/v)] to give 79 mg (76%) of the desired conjugate having R_f 0.43 [silica, $CHCl_3/CH_3OH/H_2O$ (40/10/1, v/v/v)] and ^{1}H NMR (CD_3OD, 500 MHz, 318 K) δ ppm 0.70 (s, 24 H), 0.90−2.31 (m, 272 H), 3.16 (m, 12 H), 3.33−3.50 (m, 16 H), 3.79 (s, 8 H), 3.94 (s, 8 H), 4.25 (br, 2 H), 4.75 (br, 2 H). HRMS for $C_{226}H_{378}N_{12}O_{36}Na^+$: calcd, 3859.8009; found, 3859.8264.

Vesicle Formation and Na^+/K^+ Transport Measurements. Typically, 2.00 mL of a 25 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 ion conductor was then added from a stock solution in methanol. While rotating the tube, the organic solvents were evaporated using 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% in D_2O and 90% in H_2O, and the mixture 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 K/325 K), followed by extrusion through a 400 nm Nuclepore membrane (10 times) and a 200 nm membrane (10 times). After extrusion, the dispersion was incubated 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 0.300 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 0.750 mL of the vesicle dispersion, and the resulting mixture was vortexed for 30 s. NMR spectra were recorded continuously at 35 °C overnight on a Bruker AMX 360 or 500 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.

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Supporting Information Available: Synthesis of **2** and **3** and procedures for measuring Na^+ transport (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) To place the activity of **3** into perspective, 0.01 mol % of this conjugate affords a half-life for Na^+ transport across POPC bilayers of 26 min. In contrast, 1 mol % of amphotericin B in egg PC vesicles has a half-life for Na^+ transport of ca. 60 min: Stadler, E.; Dedek, P.; Yamashita, K.; Regen, S. L. *J. Am. Chem. Soc*. **1994**, *116*, 6677.

(13) Dynamic light scattering measurements that were made at the end of these transport experiments showed that the POPC liposomes containing **3** had the same size and size distribution as those that were prepared in the absence of the conjugate; the average diameter for both dispersions was ca. 170 nm.