Ion Conductors Derived from Cholic Acid and Spermine: Importance of Facial Hydrophilicity on Na^+ Transport and Membrane Selectivity

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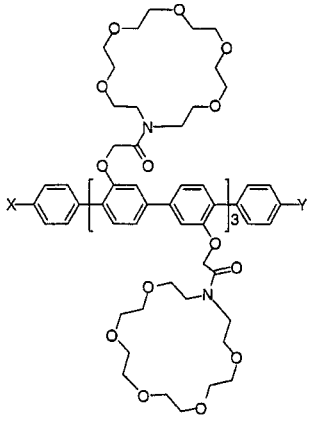
Abstract: A series of ion conductors have been synthesized in which the degree of facial hydrophilicity has been systematically varied. Specifically, conjugates have been prepared from cholic acid and spermine in which the hydrophilic face of each sterol bears methoxy (**1**), hydroxy (**2**), carbamate (**3**), or sulfate groups (**4**). The ability of these conjugates to promote the transport of Na^+ across phosphatidylcholine membranes of varying thickness has been investigated by ^{23}Na NMR spectroscopy. Examination of observed activities in three different phosphatidylcholine membranes has provided evidence for membrane-spanning dimers as the transport-active species. In the thinnest membranes investigated, made from 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (**C***14*), Na^+-transport activity was found to increase, substantially, with increasing facial hydrophilicity. In thicker membranes, made from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (**C***18*), observed activities were found to decrease with increasing facial hydrophilicity; with a membrane of intermediate thickness, prepared from 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (**C***16*), ion-conducting activity increased and then decreased, with continuous increases in facial hydrophilicity. The possible origins for these variations in activity are briefly discussed.

Introduction

Interest in the design and synthesis of ion conductors (i.e., molecules that are capable of transporting ions across phospholipid bilayers) has intensified in recent years.[1,2] Part of this increase derives from practical considerations. In particular, it has been noted that such molecules could lead to new classes of antibiotics that destroy the integrity of the plasma membrane of bacteria and fungi while circumventing the problem of drug resistance.[3] It has also been suggested that synthetic ion conductors could find use in the development of novel ionic devices.[4]

Three recent examples from the literature illustrate the types of approaches that are currently being taken to prepare synthetic ion conductors. Matile and co-workers have introduced molecules termed, "rigid push-pull rods" (e.g., **5**) that can recognize polarized bilayer membranes.[2a] We, ourselves, have taken a more biomimetic approach to the design of ion conductors. Specifically, we have been preparing a variety of sterol conjugates that mimic the structure of certain naturally occurring antibiotics such as amphotericin B and squalamine; for example, **6**.[3,5] Recently, Kobuke and co-workers have begun to report certain cholic acid-based, ion conductors, for example, **7**,[4] in which the hydroxyl groups of the sterol have been replaced by methoxy groups.

The primary objective of the work that is described in this paper was 2-fold: (i) to test a new design principle for the synthesis of ion conductors that uses facial amphiphiles (molecules that maintain a hydrophobic and a hydrophilic face) as "building blocks" and (ii) to define the importance of facial hydrophilicity on the ion-transport properties of such molecules.[6,7]



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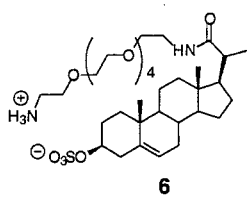

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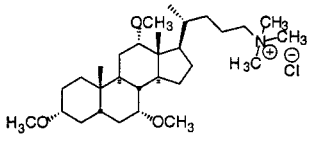

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(1) For general reviews on non-peptide ion channels and channel models, see: (a) Kobuke, Y. In *Advances in Supramolecular Chemistry*; Gokel, G. W., Ed.; JAI: Greenwich, London, 1997; Vol. 4, p 163. (b) Koert, U. *Chem. Unserer Zeit* **1997**, *31*, 20. (c) Gokel, G. W.; Murillo, O. *Acc. Chem. Res.* **1996**, *29*, 425.

(2) For selected examples from the recent literature, see: (a) Sakai, N.; Gerard, D.; Matile, S. *J. Am. Chem. Soc.* **2001**, *123*, 2517. (b) Renkes, T.; Schafer, H. J.; Siemens, P. M.; Neumann, E. *Angew. Chem., Int. Ed.* **2000**, *39*, 25112. (c) Forman, S. L.; Fettinger, J. C.; Pieraccini, S.; Gottarelli, G.; Davis, J. T. *J. Am. Chem. Soc.* **2000**, *122*, 4060. (d) Otto, S.; Janout, V.; DiGiorgio, A. F.; Young, M. C.; Regen, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 1200. (e) Perez, C.; Espinola, C. G.; Foces-Foces, C.; Nunez-Coello, P.; Carrasco, H.; Martin, J. D. *Org. Lett.* **2000**, *2*, 1185. (f) Das, S.; Kurok, P.; Jedlinski, Z.; Reusch, R. N. *Macromolecules* **1999**, *32*, 8781. (g) Abel, E.; Maguire, G. E. M.; Murillo, O.; Suzuki, I.; De Wall, S. L.; Gokel, G. W. *J. Am. Chem. Soc.* **1999**, *121*, 9043. (h) Qi, Z.; Sokabe, M.; Donowaki, K.; Ishida, H. *Biophys. J.* **1999**, *76*, 631. (i) Hall, C. D.; Kirkovits, G. J.; Hall, A. C. *Chem. Commun.* **1999**, 1897. (j) Fritz, M. G.; Walde, P.; Seebach, D. *Macromolecules* **1999**, *32*, 574.

(3) (a) Stadler, E.; Dedek, P.; Yamashita, K.; Regen, S. L. *J. Am. Chem. Soc.* **1994**, *116*, 6677. (b) Sadownik, A.; Deng, G.; Janout, V.; Bernard, E. M.; Kikuchi, K.; Armstrong, D.; Regen, S. L. *J. Am. Chem. Soc.* **1995**, *117*, 6138.

(4) Kobuke, Y.; Nagatani, T. *Chem. Lett.* **2000**, 298.

(5) (a) Otto, S.; Osifchin, M.; Regen, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 7276. (b) Otto, S.; Osifchin, M.; Regen, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 10440.

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Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. All of the phospholiplids that were used were obtained from Avanti Polar Lipids (Birmingham, AL). House-deionized water was purified using a Millipore Milli-Q-filtering system containing one carbon and two ion-exchange stages. Vesicle extrusions were carried out using a Lipex Biomembrane apparatus (Vancouver, BC). All ^{1}H NMR and ^{23}Na NMR spectra were recorded on 360 and 500 MHz instruments, respectively, where chemical shifts are reported in ppm relative to residual solvent. The pH of the NaCl solution used in these transport experiments was typically ca. 6.

3α,7α,12α-Trimethoxy-5β-cholanic Acid. To a solution of 0.550 g (1.28 mmol) of cholic acid methyl ester in 5.0 mL of 1,4-dioxane was added 3.50 mL of CH_3I (56.2 mmol) and 98 mg of NaH (60% oil suspension, 2.5 mmol). The mixture was then stirred at 40 °C for 48 h under an argon atmosphere. An additional 0.100 g of NaH (60% oil suspension) (2.5 mmol) was then added and the mixture allowed to stir for 24 h at 40 °C. Additional NaH was added in the same manner three additional times for a total of 0.4 g (10 mmol), over the course of 120 h. [Note: this method of four consecutive addition of "small" portions of NaH afforded yields of the desired trimethoxy methyl ester, which were ca. 10 times greater, as compared to analogous reactions in which the entire amount of NaH was added in one step.] The product mixture was then diluted by adding 100 mL of CH_2Cl_2 and washed with 50 mL of 1 M HCl and 2 × 50 mL of H_2O. After concentration under reduced pressure, the crude product mixture (0.820 g) was purified by column chromatography (SiO_2, $CHCl_3$/CH_3OH; 30/1, v/v) and then preparative thin-layer chromatography (SiO_2, $CHCl_3$/acetone, 20/1, v/v) to give 0.321 g (53%) of the title compound having R_f 0.55; ^{1}H NMR ($CDCl_3$): δ 3.65 (s, 3 H), 3.34 (bs, 1 H), 3.31 (s, 3 H), 3.23 (s, 3 H), 3.19 (s, 3 H), 3.12 (d, 1 H), 2.96 (m, 1 H), 0.86−2.35 (m, 30 H), 0.64 (s, 3 H).

To a solution of 321 mg (0.692 mmol) of 3α,7α,12α-trimethoxy-5β-cholanic acid, methylester in 14 mL of 1,4-dioxane was added 5.0 mL of a methanolic solution of 0.300 g (7.5 mmol) of NaOH, and the mixture was stirred at 45 °C for 2 h. The product mixtures was concentrated under reduced pressure, and the crude product was dissolved in 50 mL of CH_2Cl_2. This solution was then washed, sequentially, with 20 mL of 1 M HCl and 2 × 20 mL of H_2O, and the product was then purified by column chromatography (SiO_2, $CHCl_3$/MeOH; 10/1,v/v) to give 199 mg (63%) of the desired acid having R_f = 0.52; ^{1}H NMR ($CDCl_3$): δ 3.33 (bs, 1 H), 3.29 (s, 3 H), 3.22 (s, 3 H), 3.18 (s, 3 H), 3.11 (d, 1 H), 2.96 (m, 1 H), 0.86−2.35 (m, 30 H), 0.65 (s, 3 H).

***N*$_1$,*N*$_2$,*N*$_3$,*N*$_4$-Spermine-tetrakis-3,7,12-trimethoxy-5-choleamide (1).** To a stirred solution of 60 mg (0.133 mmol) of 3α,7α,12α -trimethoxy-5β-cholanic acid, 19.4 mg (0.15 mmol) of *N*,*N*-diisopropyl-*N*-ethyl amine in 0.5 mL of anhydrous DMF was added 51.1 mg (0.145 mmol) of *O*(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, and the reaction mixture was stirred at room temperature for 2 h. A solution of 5.6 mg (0.028 mmol) of spermine and 0.074 g (0.57 mmol) of *N*,*N*-diisopropyl-*N*-ethyl amine in 0.2 mL of anhydrous DMF was then added dropwise and the resulting solution stirred at 55 °C for 6 h. The mixture was then concentrated under reduced pressure, and the resulting solid was dissolved in 2 mL of CH_3OH. Subsequent precipitation, by addition to an aqueous solution that was saturated with $NaHCO_3$, and preparative thin-layer chromatography (SiO_2, $CHCl_3$/MeOH; 15/1, v/v) afforded 42.9 mg (79%) of **1**, having R_f = 0.45; ^{1}H NMR ($CDCl_3$): δ 6.75 (t, 2 H), 3.34 (s, 4 H), 3.31 (s, 12 H), 3.23 (s, 12 H), 3.19 (s, 12 H), 3.12 (s, 4 H), 3.25−3.39 (m, 12 H), 2.99 (m, 4 H), 0.91−2.32 (m, 128 H), 0.66 (s, 12 H). HRMS for $C_{118}H_{202}N_4O_{16}N_4Na$ Calcd: 1954.5008. Found: 1954.4995.

***N*$_1$,*N*$_2$,*N*$_3$,*N*$_4$-Spermine−tetracholeamide (2).** A solution, which was prepared from 0.560 g (1.37 mmol) of cholic acid, 0.255 g (1.56 mmol) of *N*-3-hydroxy-1,2,3-benzotriazine-4(3H)-one, 1.93 g (1.49 mmol) of *N*,*N*-diisopropylethylamine, 0.309 g (1.49 mmol) of dicyclohexylcarbodiimide, and 5.0 mL of anhydrous DMF was stirred for 15 min at room temperature. To this solution was then added 38.0 mg (0.188 mmol) of spermine. After stirring for 5 h at 45 °C, the mixture was then concentrated at 40 °C (10 Torr). The residue was then dissolved in 3 mL of CH_3OH, and the solution was poured into an aqueous solution that was prepared from 15 mL of saturated $NaHCO_3$ and 5 mL of saturated Na_2CO_3. The precipitate was purified by preparative thin-layer chromatography (silica, $CHCl_3$/CH_3OH/H_2O, 120/73/2, v/v/v; R_f 0.55) to give a colorless solid, which was dried (23 °C, 24 h, 0.1 Torr) and redissolved in 3 mL of CH_3OH. Subsequent precipitation, via dropwise addition into deionized water, centrifugation, and freeze-drying for 24 h afforded 0.251 g (76%) of N_1,N_2,N_3,N_4-spermine-tetracholeamide as a colorless solid having ^{1}H NMR (CD_3OD): δ 3.97 (d, 4 H), 3.79 (bs, 4 H), 3.33 (m, 4 H), 3.22−3.36 (m, 8 H), 3.17 (m, 4 H), 0.90−2.35 (m, 128 H), 0.71 (s, 12 H). HRMS for $C_{106}H_{178}N_4O_{16}Na$ Calcd: 1786.3130. Found: 1786.3131.

3,7,12-Triscarbamyl Cholic Acid. To a solution of 1.37 g (3.24 mmol) of methyl choleate in 7 mL of $CHCl_3$ was added 1.96 g (13.8 mmol) of chlorosulfonyl isocyanate, and the reaction mixture was stirred in a closed flask. After the mixture stirred for 16 h, 30 mL of THF/H_2O (1/1, v/v) was added, followed by 10 mL of an aqueous solution that was saturated with $NaHCO_3$. After stirring for an additional 2 h at room temperature, the product mixture was diluted by adding 40 mL of $CHCl_3$, and the organic phase was separated and washed with 2 × 20 mL of H_2O. Removal of solvent under reduced pressure afforded 1.610 g (91%) of 3,7,12 -triscarbamyl cholic acid methyl ester as a white solid having R_f = 0.40 (SiO_2, $CHCl_3$/MeOH; 10/1,v/v) and ^{1}H NMR (CD_3OD): 4.88 (d, 1 H), 4.72 (d, 1 H), 4.36 (m, 1 H), 3.64 (s, 3 H), 0.88−2.34 (m, 30 H), 0.77 (s, 3 H). To 35 mL of a methanolic solution of 1.610 g of 3,7,12 -triscarbamyl cholic acid, methylester was added 20 mL of 10% Na_2CO_3/H_2O, and the mixture then stirred at 55 °C. After the mixture stirred for 3 h, the acidity was adjusted to pH ∼5 by the addition of a few drops of concentrated HCl. The mixture was then concentrated under reduced pressure, and the resulting solid was purified by column chromatography (SiO_2,$CHCl_3$/MeOH/H_2O; 103/27/3,v/v/v) to give 1.045 g (67%) of the title compound having R_f 0.5 and ^{1}H NMR (CD_3OD): δ 4.87 (d, 1 H), 4.72 (d, 1 H), 4.35 (m, 1 H), 0.88−2.30 (m, 30 H), 0.76 (s, 3 H).

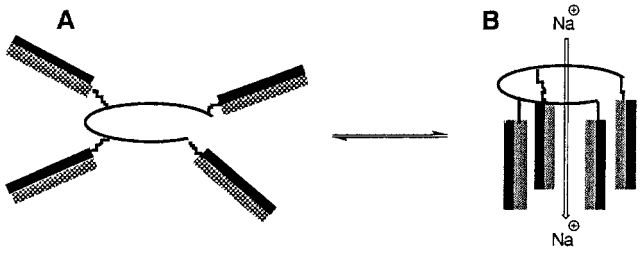
***N*$_1$,*N*$_2$,*N*$_3$,*N*$_4$-Spermine−tetrakis-3,7,12-tricarbamyl-tetracholeamide (3).** To a 0.30 mL DMF solution of 74 mg (0.137 mmol) of 3,7,12 -triscarbamyl cholic acid and 26 μL (19.3 mg, 0.167 mmol) of *N*,*N*-diisopropyl-*N*-ethyl amine was added 52 mg (0.149 mmol) of *O*(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-*N*,*N*,*N*′*N*′-tetramethyluroniumtetrafluoroborate, and the mixture then stirred at room temperature under an argon atmosphere. After the mixture stirred for 2 h, a solution of 7.7 mg (0.033 mmol) of spermine in a mixture of 0.20 mL of anhydrous DMF, and 0.10 mL of *N*,*N*-diisopropyl-*N*-ethyl amine was added dropwise. After stirring at room temperature for 24 h under an argon atmosphere, the mixture was concentrated under reduced pressure (6 Torr, 50 °C), and the resulting solid was dissolved in 0.50 mL of CH_3OH. Subsequent precipitation via addition into 20 mL of 10% $NaHCO_3$/H_2O, washing with 20 mL of H_2O, and purification by preparative thin-layer chromatography (SiO_2, $CHCl_3$/MeOH/H_2O; 100/27/3, v/v/v) afforded 37 mg (49%) of the title compound having R_f = 0.49 and ^{1}H NMR (CD_3OD): 4.89 (bs, 4 H), 4.72 (s, 4 H), 4.37 (m, 4 H), 3.34 (m, 8 H), 3.16 (m, 4 H), 0.92−2.31 (m, 128 H), 0.77 (s, 12 H). HRMS for $C_{118}H_{190}N_{16}O_{28}Na$ Calcd: 2302.3828. Found: 2302.3654.

***N*$_1$,*N*$_2$,*N*$_3$,*N*$_4$-Spermine−tetrakis-3,7,12 -tetracholeamide-*O*$_1$,*O*$_2$,*O*$_3$-persulfate, Sodium Salt (4).** A solution of 124.8 mg (0.07 mmol) of N_1,N_2,N_3,N_4-spermine-tetrakis-3,7,12 -tetra choleamide and 0.255 g (1.6 mmol) of Py·SO_3 in 2.5 mL of anhydrous DMF was stirred at room temperature for 24 h. The solvent was then removed under reduced pressure (6 Torr, 50 °C), and the crude product was dissolved in 4 mL of an aqueous solution that was saturated with $NaHCO_3$. Water was then removed under reduced pressure (6 Torr, 30 °C), and the resulting solid was extracted with 3 × 5 mL of MeOH. Concentration of the

(6) (a) Stein, T. M.; Gellman, S. H. *J. Am. Chem. Soc*. **1992**, *114*, 3943. (b) McQuade, D. T.; Barrett, D. G.; Desper, J. M.; Hayashi, R. K.; Gellman, S. H. *J. Am. Chem. Soc*. **1995**, *117*, 4862. (c) Cheng, Y.; Ho, D. M.; Gottlieb, C. R.; Kahne, D.; Bruck, M. A. *J. Am. Chem. Soc*. **1992**, *114*, 7319. (d) Venkatesan, P.; Cheng, Y.; Kahne, D. *J. Am. Chem. Soc*. **1994**, *116*, 6955.

(7) A preliminary account of this work has appeared: Bandyopadhyay, P.; Janout, V.; Zhang, L. H.; Sawko, J. A.; Regen, S. L. *J. Am. Chem. Soc*. **2000**, *122*, 12888.

Chart 1



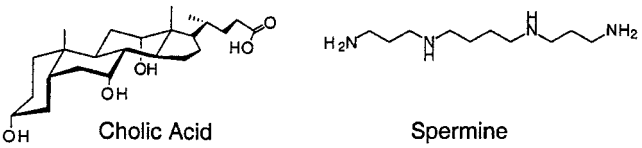
methanol extract under reduced pressure afforded 240 mg of crude product, which was purified by column chromatography (SiO_2,$CHCl_3$/MeOH/H_2O; 60/55/20, v/v/v) to give 95 mg (46%) of the title compound having $R_f = 0.57$) and ^{1}H NMR (D_2O): δ 4.64 (bs, 4 H), 4.45 (bs, 4 H), 4.18 (m, 4 H), 3.33 (m, 8 H), 3.16 (m, 4 H), 0.92−2.35 (m, 128 H), 0.73 (s, 12 H). Calcd for $C_{106}H_{166}N_4O_{52}S_{12}Na_{12}\cdot 12H_2O$: C, 39.72; H, 5.97; N, 1.75. Found: C, 40.27; N, 5.62; N, 1.77.

Vesicle Formation and Na^+/Li^+ Transport Measurements. Typically, 1.77 mL of a 25 mg/mL solution of 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (C14:1) PC in chloroform was transferred to a Pyrex test tube. The desired amount of ion conductor was then added from a stock solution in chloroform/methanol (1/1, v/v). While rotating the tube, the organic solvents were evaporated using a stream of nitrogen, resulting in a thin film of (C14:1) PC/ionophore. The last traces of solvent were removed under reduced pressure (25 °C, 12 h, <0.2 Torr). To the dried film was added 1.0 mL of a 150 mM LiCl solution that was 10% in D_2O and 90% in H_2O, and the mixture was then vortexed for 30 s (in cases where not all of the material had come off of the wall, vortexing was continued). The dispersion was then incubated for 5 min, followed by another 30 s 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 sample was incubated for 1.25 h. In a test tube, 1.5 mL of a 150 mM NaCl solution in 10% D_2O plus 90% H_2O was mixed with 0.200 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 15 s. The mixture was then transferred to a quartz NMR tube, and NMR spectra were recorded continuously at 35 °C overnight on a Bruker AMX 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.

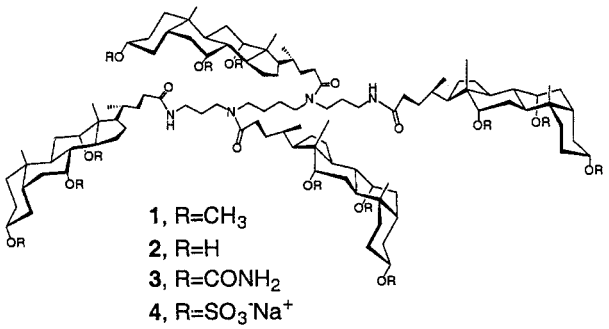
Results

Molecular Design Principle. The design principle that we have sought to test is illustrated in Chart 1. In brief, two or more facial amphiphiles (appearing as doubly shaded rectangles) are covalently attached to a linear backbone. When incorporated into a lipid bilayer, such a conjugate is expected to favor conformation **B**, where each hydrophobic face (darkened region) is in intimate contact with the alkyl chains of neighboring phospholipids and each hydrophilic face (lightly shaded region) points toward a hydrophilic face of a nearest-neighbor.[8] Conformation **A**, on the other hand, is expected to be favored when the conjugate is adsorbed to the membrane surface.[9] In principle, dimerization of **B** across a lipid bilayer should produce a contiguous pathway that permits a flow of ions. Very recently, Kobuke et al. have reported a related strategy in which a macrocyclic resorcin[4]arene was used as rigid template for binding four methoxylated, cholic acid units.[10]

Target Molecules. Specific molecules that were chosen as synthetic targets were **1**, **2**, **3,** and **4**. Complete acylation of spermine with cholic acid was expected to produce a conjugate (**2**) that fulfills the basic requirements of our design principle.



If such a molecule were active in transporting ions, it would also hold the distinction of being the first synthetic ion conductor to be prepared entirely from biogenic starting material. Analogues of **2**, having varying degrees of facial hydrophilicity, were also of interest to us. In particular, we reasoned that facial hydrophilicity could have a strong influence on their ion-conducting properties by altering the extent to which the conjugates dimerize across the bilayer, and their intrinsic activity of the dimers. Specifically, we hypothesized that if **B** (Chart 1) were made from amphiphilic units having a high degree of facial hydrophilicity, the possibility of producing water-filled pores would exist.[11] Subsequent dimerization might then be driven by favorable hydrophilic/hydrophilic association between the "ends" of two such pores at the center of the bilayer, thereby avoiding exposure of a hydrophilic end to the hydrocarbon core of the membrane. In addition, contiguous, water-filled pores would be well-suited for the transport of ions, since water of hydration would not have to be shed in order for the ions to enter the membrane. With these ideas in mind, analogues **1**, **3**, and **4**, bearing methoxy, carbamate, and sulfate groups, respectively, were also chosen as synthetic targets.



Direct condensation of spermine and cholic acid, using dicyclohexylcarbodiimide in the presence of *N*-3-hydroxy-1,2,3-benzotriazine-4(3H)-one, afforded **2** in 76% isolated yield (Scheme 1). Alkylation of methyl choleate with methyl iodide, subsequent saponification, and condensation with spermine, using *O*(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-*N*,*N*,*N*′,*N*′,-tetramethyluronium tetrafluoroborate, afforded **1** in a 26% overall yield (Scheme 2). The carbamate derivative was synthesized by first converting the hydroxyl groups of methyl choleate to carbamate groups by reaction with chlorosulfonyl-isocyanate. Subsequent hydrolysis of the ester moiety, followed by condensation with spermine produced **3** (27% overall yield)

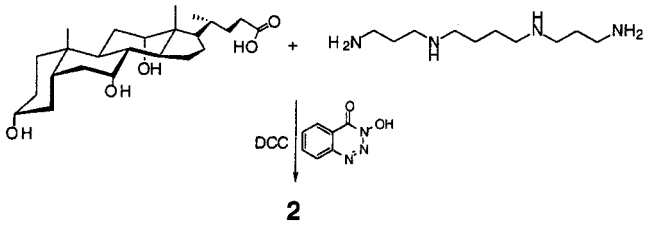
(8) The elements of this design principle resemble those that have been used to construct molecular umbrellas: (a) Janout, V.; DiGiorgio, C.; Regen, S. L. *J. Am. Chem. Soc*. **2000**, *122*, 2671. (b) Shawaphun, S.; Janout, V.; Regen, S. L. *J. Am. Chem. Soc*. **1999**, *121*, 5860.

(9) (a) Fahey, D. A.; Carey, M. C.; Donovan, J. M. *Biochemistry* **1995**, *34*, 10886. (b) Leonard, M. R.; Bogle, M. A.; Carey, M. C.; Donovan, J. M. *Biochemistry* **2000**, *39*, 16064.

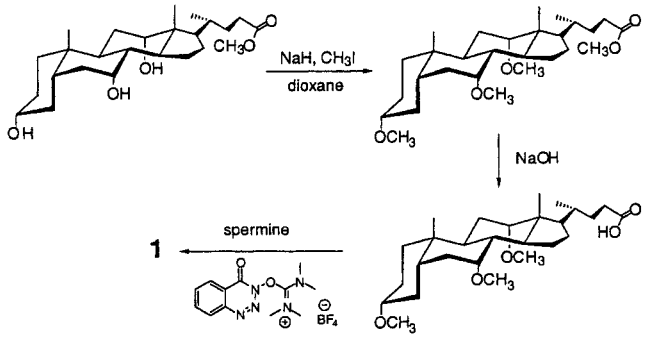
(10) Yoshino, N.; Satake, A.; Kobuke, Y. *Angew. Chem., Int. Ed*. **2001**, *40*, 457.

(11) It has been proposed that amphotericin B self-assembles in lipid bilayers to form water-filled pores. Such pores bear a resemblance to the ones that proposed herein: Gennis, R. B. *Biomembranes: Molecular Structure and Function*; Springer-Verlag: New York, 1989; p 288.

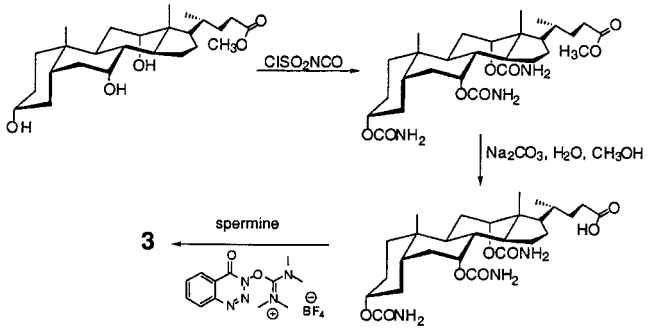
Scheme 1



Scheme 2



Scheme 3

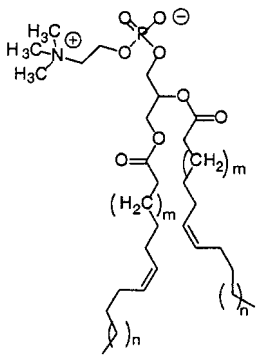


(Scheme 3). Finally, direct sulfation of **2** with pyr·SO_3 afforded the desired persulfated analogue, **4**, in 46% isolated yield (Scheme 4). Qualitative evidence for the relative differences in facial hydrophilicity was obtained by measuring the mobility of these conjugates on silica gel by thin-layer chromatography. Thus, the R_f values that were found for **1**, **2**, **3**, and **4**, using $CHCl_3/CH_3OH/H_2O$ (100/27/3, v/v/v) as an eluting solvent, were 0.99, 0.65, 0.49, 0.01, respectively, reflecting relative polarities of **4** > **3** > **2** > **1**.

Monolayer Properties. To gain insight into the interaction of such conjugates with phospholipid membranes, we examined the monolayer properties of **2** in the absence, and in the presence, of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (**C***18*) at the air−water interface. Unfortunately, comparative monolayer experiments with the more hydrophilic analogue, **4**, were not possible due to the fact that this surfactant did not form stable monolayers. As shown in Figure 1, **2** exhibits significant compressibility from 0 to 24 mN·m^{-1}. Extrapolation of the condensed portion of this part of the isotherm to zero surface pressure yields a limiting area of 4.5 nm^2·$molecule^{-1}$, which is consistent with a structure having the hydrophilic face of each sterol in intimate contact with water; that is, the entire molecule lies flat on the subphase (conformation **A** in Chart 1). At 24 mN·m^{-1}, a very broad, first-order phase transition occurs. Such a transition implies that a radical change in conformation has taken place. Given the low surface area that **2** occupies at the completion of this transition, it is likely that two of the sterol units have "flipped" up into air or down into water. In sharp contrast, mixed monolayers of **2** and **C***18* were much less compressible and *did not exhibit a phase transition*. Extrapolation of the isotherms made from pure **C***18*, **C***18*/**2** (95.6/4.4, mol/mol), and **C***18*/**2** (91.7/8.3, mol/mol) to zero surface pressure yielded limiting areas of 0.70, 0.87, and 1.02 nm^2·$molecule^{-1}$, respectively. If one assumes ideal mixing, then the calculated limiting area of **2** in these mixed monolayers is 4.57 and 4.55 nm^2·$molecule^{-1}$, respectively.[12] At biologically relevant surface pressures (ca. 30 mN·m^{-1}), the calculated areas for **2** are 2.89 and 2.51 nm^2·$molecule^{-1}$, respectively.[13] The significant reduction in the occupied area of **2**, at this higher surface pressure, is consistent with a model in which each sterol has lifted upward from the water surface such that only the C-3 hydroxyls remain in contact with aqueous subphase. Thus, these results indicate that **2** *is readily taken up into a compressed phospholipid monolayer in a conformation that approaches* **B**.

Ion-Conducting Properties. Phosphatidylcholine bilayers, which were used as model membranes to characterize the ion-conducting properties of each conjugate, were in the form of 200-nm diameter unilamellar vesicles. In addition to **C***18*, 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (**C***14*), and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (**C***16*) were used as vesicle-forming lipids. Due to the presence *cis*-double bonds, each of these membranes exhibit gel to liquid-crystalline phase transition temperatures that are well below room temperature.[14] Thus, at the temperature that was used for all of our Na^+-transport experiments (35 °C), each bilayer was maintained in the physiologically relevant fluid phase.

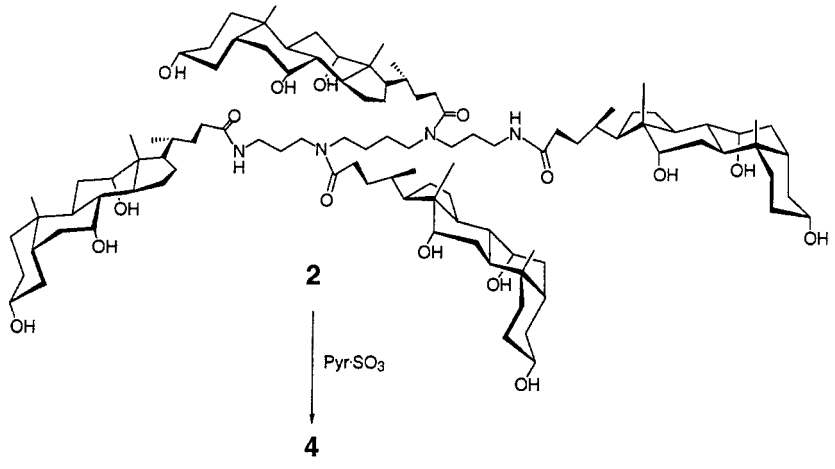
Using procedures that are described in the Experimental Section, the rate of Na^+ transport across each membrane type was monitored via ^{23}Na NMR spectroscopy. In these experiments, a membrane-impermeable paramagnetic shift reagent was first added to the dispersion in order to distinguish external from internalized Na^+. In all cases, pseudo-first-order rate constants (k_{obsd}) showed a second-order dependency on the mol % of the conjugate that was present. In one such experiment, where vesicles were made from **C***16* and **2** was used as an ion conductor, both the rate of $^{23}Na^+$ influx and the rate of $^7Li^+$ efflux were monitored simultaneously. Within experimental error, the rate constants for both transport processes were identical. This finding supports an antiport mechanism of transport. Figure 2 shows typical plots for k_{obsd} versus (mol % of **2**)^2 for the three different membranes investigated (**C***14*, **C***16*, and **C***18*).



***C*14, m=4; n=1**
***C*16, m=4; n=3**
***C*18, m=4; n=5**

As discussed previously, a second-order dependency of k_{obsd} on the mol % of an ion conductor indicates the existence of transport-active dimers, if it is assumed that only a small fraction

Scheme 4



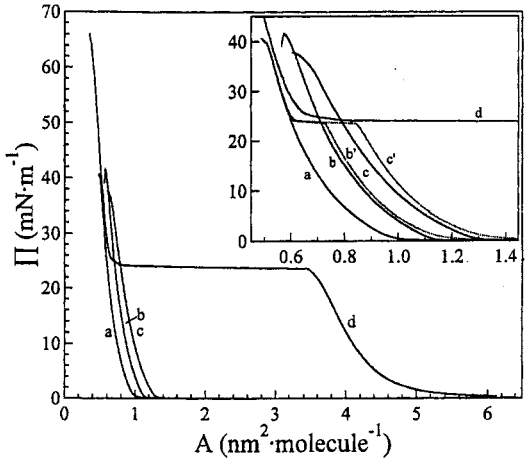


Figure 1. Surface pressure−area isotherms for mixtures of **C***18*/**2** having the following mol % of **C***18*: (a) 100, (b) 95.6, (c) 91.7, and (d) 0 at 25 °C. Inset shows expanded scale plus theoretical curves (b′ and c′ correspond to b and c, respectively), assuming ideal mixing.

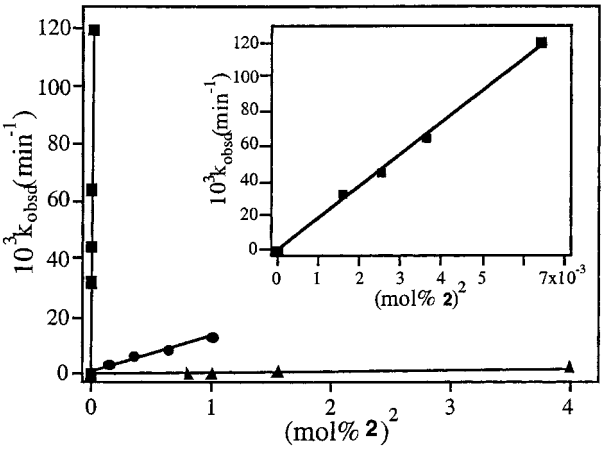


Figure 2. Plot of k_{obsd} versus (mol % **2**)^2 for vesicles made from (■)-**C***14*, (●) **C***16*, and (▲) **C***18* at 35 °C. The concentration ranges used in **C***14*, **C***16*, and **C***18* were 0.04−0.08, 0.4−1, and 0.9−2 mol %, respectively. Inset shows expanded *X*- and *Y*- axes.

of the conjugate is aggregated. Specifically, it can be shown that:

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

where K is the equilibrium constant for dissociation of the dimer, k_2 is the rate constant for Na^+ transport, and [monomer] is the analytical concentration of the ion conductor that is present in the dispersion.[5a] Although k_2 and K cannot be separated by such analyses, one can make operational comparisons among the different conjugates. Specific values of k_2/K that have been determined for **1**, **2**, **3**, and **4** are listed in Table 1. Also listed are membrane-selectivity factors, S, which represent the ratio of $(k_2/K)_m/(k_2/K)_n$, where m and n refer to two different bilayers that are being compared.

From these experiments, the least hydrophilic member of this series (**1**) was found to have only moderate Na^+-transport activity in **C***14* membranes; in bilayers that were prepared from either **C***16* or **C***18*, no ion-transport activity could be detected (Table 1). In contrast, the hydroxylated analogue, **2**, exhibited ion-transport activity that was more than 3 orders of magnitude higher in the **C***14*-based membranes; on going to the thicker **C***16*- and **C***18*-based membranes, however, the activity of the conjugate substantially decreased. The carbamate analogue **3** showed a ca. 20-fold increase in activity in **C***14* membranes, relative to **2**. In **C***16* bilayers, **3** was ca. 1 order of magnitude greater in activity than **2**; in **C***18* membranes, the difference was less. Finally, the conjugate having the greatest degree of facial hydrophilicity, **4**, showed the highest ion-conducting activity in the thin, **C***14* membranes, which was more than a factor of 10^5 greater than that of **1**; in the thicker bilayers,

Table 1. Ion-Conducting Activities and Membrane Selectivities[a]

ion conductor	phospholipid	$10^4k_2/K$ (min^{-1} mol %$^{-1}$)	*S*
1	**C***14*	113	
2	**C***14*	190 000	1500 (**C***14*/**C***16*)
	C*16*	130	22 (**C***16*/**C***18*)
	C*18*	5.8	33 000 (**C***14*/**C***18*)
3	**C***14*	3 360 000	2900 (**C***14*/**C***16*)
	C*16*	1180	787 (**C***16*/**C***18*)
	C*18*	1.5	2 240 000 (**C***14*/**C***18*)
4	**C***14*	21 620 000	124 000 (**C***14*/**C***16*)
	C*16*	174.5	698 (**C***16*/**C***18*)
	C*18*	0.25	86 480 000 (**C***14*/**C***18*)

[a] All kinetic experiments were carried out at 35 °C; the error in k_{obsd} is estimated to be ±10%.

(12) For ideal mixing, $A_p = X_1A_1 + (1 - X_1)A_2$, where A_p is the average area per molecule at a given surface pressure (p), X_1 is the mole fraction of the phospholipid, $(1 - X_1)$ is the mole fraction of the conjugate, and A_1 and A_2 are the areas occupied by each pure surfactant at the specified pressure, p.

(13) Georgallas, A.; Hunter, D. L.; Lookman, T.; Zuckerman, M. J.; Pink, D. A. *Eur. Biophys. J.* **1984**, *11*, 79.

(14) Lewis, B. A.; Engelman, D. M. *J. Mol. Biol.* **1983**, *166*, 211.

however, its ion-conducting activity was less than that of **3**. Two general trends that emerge from these data are: (i) in the thin **C***14* membranes, ion-conducting activity increases, substantially, with increasing facial hydrophilicity; (ii) in the thick **C***18* bilayers, however, the opposite is found to be the case. For the intermediate bilayers made from **C***16*, the ion-conducting activity increases on going from **2** to **3** but then decreases on going from **3** to **4**. As a consequence of these trends, **C***14*/**C***16* and **C***14*/**C***18* membrane selectivity increases with increasing facial hydrophilicity; for **C***16*/**C***18* selectivity, however, a maximum is reached with **3**.

Discussion

The fact that pseudo-first-order rate constants for Na^+ transport are second-order with respect to **1**, **2**, **3** and **4** provides strong support for a model in which dimers are the transport-active species. The very strong dependency of ion-transport activity on bilayer thickness further indicates that these ion conductors function as membrane-*spanning* agents. To place this thickness dependency into perspective, the activity of valinomycin (a well-established ion conductor that functions through a carrier mechanism) in lipid membranes made from α-monoglycerides has been found to vary by less than 1 order of magnitude as the acyl chain length was varied from 16 to 20 carbons.[15]

The relatively high activity that has been found for each conjugate in **C***14* membranes is a likely consequence of a proper matching of bilayer thickness with the length of the membrane-spanning dimer. With the thicker **C***16* and **C***18* membranes, the ability of these transport-active dimers to span the bilayer becomes increasingly difficult. The large increase in activity in the **C***14* membranes, which accompanies an increase in facial hydrophilicity, can be readily accounted for by a greater degree of dimerization across the bilayer, or a higher intrinsic activity of the dimer (vide ante). The opposite trend, which has been found in the thicker **C***18* membranes, was unexpected. One possible explanation for this behavior is that in these thicker bilayers, where the ability of the dimer to "reach across" becomes more difficult, a greater percentage of the conjugate becomes adsorbed to the membrane surface (conformation **A**, Chart 1), and a greater degree of facial hydrophilicity results in a greater extent of surface adsorption. Results that have been obtained in **C***16* membranes appear to reflect a combination of two opposing factors, where surface hydrophilicity acts in two different ways.

The fact that the ether-based conjugate **1** was more than 3 orders of magnitude *less* active in **C***16* membranes, as compared with the hydroxylated analogue **2**, is in sharp contrast to Kobuke's ether-based ion conductor **7**, where a hydroxylated analogue was reported to exhibit negligible activity.[4] It would appear, therefore, that the mechanism of action of these different classes of sterol-based ion conductors is fundamentally different.

The ability of an ion conductor to recognize bilayer thickness raises the intriguing possibility that one may be able to use such compounds for exploiting subtle differences in thickness between mammalian membranes and those of microorganisms. One can envision, for example, that fungi and bacteria have regions (i.e., microdomains) within their plasma membrane that are thinner than mammalian membranes, due to the strong condensing (and thickening) effect of cholesterol in the latter.[16−18]

Finally, in preliminary studies, we have found that addition of **4** to preformed vesicles made from **C***16* (single-sided addition) also showed a second-order dependency on the concentration of the conjugate and that transport rates were comparable to those obtained via double-sided additions; that is, where the conjugate is included in the membrane during vesicle formation, as was done in all of the experiments reported in Table 1. This finding implies that insertion of such compounds into a lipid bilayer and self-assembly to form membrane-spanning dimers are relatively fast processes.

Conclusions

Conjugates that have been synthesized from cholic acid and spermine, containing varying degrees of facial hydrophilicity, promote the passive transport of Na^+ across phosphatidylcholine vesicles. In each case, transport activity decreases, substantially, as the thickness of the bilayer is increased. This finding, together with the second-order dependency of activity on the concentration of conjugate that is present, indicates that membrane-spanning dimers are the transport-active species. Systematic increases in facial hydrophilicity have been shown to increase ion-transport activity in thin bilayers (**C***14*), but to decrease activity in ones that are thicker (**C***18*). The ability to modulate membrane-selectivity on the basis of facial hydrophilicity opens a fundamentally new avenue in the area of ion conductors, which warrants detailed exploration.

Efforts aimed at exploiting this new design principle, with a view toward drug design, are currently in progress.[19] The biological properties of these and related conjugates, as well as further modulation of the ion-conducting properties of such molecules (e.g., the introduction of proton-ionizable amine groups for targeting bacterial membranes) will be reported in due course.[20]

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(15) Benz, R.; Frohlich, O.; Lauger, P. *Biochim. Biophys. Acta* **1977**, *464*, 465.

(16) (a) Vist, M. R.; Davis, J. K. *Biochemistry* **1990**, *29*, 451. (b) Ipsen, J. H.; Mouritsen, O. G.; Bloom, M. *Biophys. J.* **1990**, *57*, 405. (c) Nezil, F. A.; Bloom, M., *Biophys. J.* **1992**, *61*, 1176. (d) Urbina, J. A.; Pekerar, S.; Le, H.; Patterson, J.; Montez, B.; Oldfield, E. *Biochim. Biophys. Acta* **1995**, *1238*, 163.

(17) Welti, R.; Glaser, M. *Chem. Phys. Lipids* **1994**, *73*, 121.

(18) (3) Tocanne, J.-F.; Cezanne, L.; Lopez, A.; Piknova, B.; Schram, V.; Tournier, J.-F.; Welby, M. *Chem. Phys. Lipids* **1994**, *73*, 139.

(19) In very recent studies, we have shown that a di-walled molecular umbrella, derived from two cholic acid molecules that contain six sulfate groups, is able to cross phospholipid bilayers (Janout, V.; Zhang, L. Staina, I. V.; DiGiorgio, C.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 5401.). Although our interest in such behavior has centered around the potential of such molecules as drug carriers, this fact also supports the hypothesis of insertion of heavily sulfated **4** into a lipid membrane.

(20) Antimicrobial activity has previously been found in a variety of amine-substituted cholic acid derivatives: (a) Quan, Q.; Li, C.; Schmidt, E. J.; Boswell, J. S.; Walsh, J. P.; Allman, G. W.; Savage, P. B. *Org. Lett.* **2000**, *2*, 2837. (b) Savage, P. B.; Li, C. *Expert Opin. Invest. Drugs* **2000**, *9*, 263. (c) Li, C.; Budge, L. P.; Driscoll, C. D.; Willardson, B. M.; Allman, G. W.; Savage, P. B. *J. Am. Chem. Soc.* **1999**, *121*, 931.