Sterol−Polyamine Conjugates as Synthetic Ionophores

Manette Merritt, Marion Lanier, Gang Deng, and Steven L. Regen*

Contribution from the Department of Chemistry and Zettlemoyer Center For Surface Studies, Lehigh University, Bethlehem, Pennsylvania 18015

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Abstract: A design principle has been devised for the construction of sterol−polyamine conjugates that function as synthetic ionophores. For feasibility studies, a prototype (**1**) was synthesized from 3β-hydroxybisnor-5-cholenic acid via sequential activation of its carboxylic acid moiety, condensation with spermine, and sulfation of the 3β-hydroxyl group. Closely related analogues were also prepared in which the terminal amine group was acetylated (**2**), the 3β-hydroxyl group was left unsulfated (**3**), and each of the two remaining secondary amines was replaced with oxygen atoms (**4**). Incorporation of each conjugate into egg phosphatidylglycerol-based vesicles showed that **1** functions as an ionophore by discharging a pH difference across the vesicle membrane, but that **2**, **3**, and **4** do not. A kinetic analysis of the ionophoric activity of **1** has provided evidence that the majority of the conjugate exists as membrane-bound monomer and that *dimers* are the active species that are responsible for ion transport. Comparative experiments have also shown that **1** exhibits greater activity in negatively charged phospholipid membranes relative to ones that are electrically neutral. The implications of these findings, with regard to the design of new classes of antibacterial agents, are briefly discussed.

Introduction

We have begun a program that is aimed at creating new classes of synthetic ionophores.[1] Our motivation for such work is based on the hypothesis that molecules, which can kill bacterial and fungal cells by destroying the barrier properties of their plasma membrane, should have considerable potential as therapeutic agents. In particular, by not having to enter a cell in order to kill it, two of the more common mechansims of drug resistance would be circumvented, i.e., enzymatic degradation within the cell and export processes.[2] Thus, by operating at the membrane level, resistance by a microorganism would be expected to be greatly reduced. With this idea in mind, we have begun to "decode" naturally occurring ionophores, and to use such knowledge in the design of mimics that are suitable for structure/activity studies.[1] Specifically, our immediate goal has been to identify the minimal structural elements that are necessary for ionophoric activity as well as membrane selectivity, i.e., the ability to discriminate between bacterial-like, fungal-like, and mammalian-like plasma membranes.

In previous studies, we have shown that certain sterol−oligo(ethylene glycol) conjugates exhibit significant ionophoric activity.[1b,e] Recently, we reasoned that if sterol−polyamine conjugates could also function as ionophores, then they might distinguish between membranes on the basis of surface charge. In particular, we hypothesized that proton-ionized forms of such ionophores would have stronger interactions with negatively charged lipid membranes, relative to ones that are electrically neutral (as a result of electrostatic forces), and that such interactions could be exploited. The fact that the outer surface of bacterial cell membranes is negatively charged, while that of mammalian cells is electrically neutral, further suggested to us that such an ionophore might have potential as an antibacterial agent, i.e., that it would exhibit significant cellular selectivity and thus low toxicity in vivo.[3] The primary aim of the work that is described in this paper was to test the feasibility of constructing a sterol−polyamine ionophore.[4] A secondary objective was to examine the possibility that such a conjugate would exhibit membrane selectivity based on surface charge.[5]

Experimental Section

General Methods. All reagents and chemicals were obtained from commerical sources and used without further purification. Egg

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(4) For a review on non-peptide ion channel models, see: (a) Gokel, G. W.; Murillo, O. *Acc. Chem. Res.* **1996**, *29*, 425. For selected examples, see: (b) Weiss, L. A.; Sakai, N.; Ghebremariam, B.; Ni, C.; Matile, S. *J. Am. Chem. Soc*. **1997**, *119*, 12142. (c) Murillo, O.; Suzuki, I.; Abel, E.; Murray, C. L.; Meadows, E. S.; Jin, T.; Gokel, G. W. *J. Am. Chem. Soc*. **1997**, *119*, 5540. (d) Meillon, J.-C.; Voyer, N. *Angew. Chem., Int. Ed. Engl*. **1997**, *36*, 967. (e) Sakai, N.; Matile, S. *Tetrahedron Lett*. **1997**, *38*, 2613. (f) Wagner, H.; Harms, K.; Koert, U.; Meder, S.; Boheim, G. *Angew. Chem., Int. Ed. Engl*. **1996**, *35*, 2643. (g) Seebach, D.; Brunner, A.; Burger, H. M.; Reusch, R. N.; Bramble, L. L. *Helv. Chim. Acta* **1996**, *79*, 507. (h) Fyles, T. M.; Loock, D.; van Straaten-Nijenhuis, W. F.; Zhou, X. *J. Org. Chem*. **1996**, *61*, 8866. (i) Dubowchik, G. M.; Firestone, R. A. *Teterahedron Lett*. **1996**, *37*, 6465. (j) Tanaka, Y.; Kobuke, Y.; Sokabe, M. *Angew. Chem., Int. Ed. Engl*. **1995**, *34*, 693. (k) Pregel, M. J.; Jullien, L.; Canceill, J.; Lacombe, L.; Lehn, J.-M. *J. Chem. Soc., Perkin Trans.* 2 **1995**, 417. (l) Nolte, R.; J. M. *Chem. Soc. Rev*. **1994**, 11. (m) Jullien, L.; Lazrak, T.; Canceill, J.; Lacombe, L.; Lehn, J.-M., *J. Chem. Soc., Perkin Trans*. 2 **1993**, 1011. (n) Menger, F. M.; Davis, D. S.; Persichetti, R. A.; Lee, J.-J. *J. Am. Chem. Soc*. **1990**, *112*, 2451. (o) Fuhrhop, J.-H.; Liman, U.; Koesling, V. *J. Am. Chem. Soc*. **1988**, *110*, 6840. (p) Tabushi, I.; Kuroda, Y.; Yokota, K. *Tetrahedron Lett*. **1982**, *23*, 4601.

(5) A preliminary account of this work has previously been reported (see ref 1a).

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phosphatidylglycerol (egg PG) and egg phosphatidylcholine (egg PC) were obtained from Avanti Polar Lipids (Birmingham, AL) as chloroform solutions; 3β-hydroxybisnor-5-cholenic acid was purchased from Steraloids Inc. (Wilton, NH). Gramicidin A (*Bacillus brevis*) was purchased from Fluka (>90%) and used directly. Vesicle dispersions were prepared by standard extrusion methods by use of a Lipex Biomembrane apparatus (Vancouver, BC) and procedures similar to those previously described.[1e] Unless stated otherwise, the buffer that was used for all pH discharge experiments was composed of 25 mM HEPES plus 100 mM NaCl, which was adjusted to pH 7.0 with NaOH solutions. All gel filtrations were carried out via minicolumn centrifugation using prepacked Sephadex G-25, PD-10 (Pharmacia Biotech) that was equilibrated to the appropriate pH with buffer. Fluorescence measurements were made using a Perkin-Elmer LS 50 luminescence spectrometer. Excitation of pyranine was at 460 nm; the observed emission was at 510 nm. Unless noted otherwise, a spectral bandwidth of 10 nm was used in all measurements. All ^{1}H NMR spectra were recorded on a Bruker 360 MHz instrument; chemical shifts are reported in parts per million and were referenced to residual solvents. High-resolution mass spectra (fast atom bombardment) were obtained at the Mass Spectrometry Facility of the University of California, Riverside.

***N*-Succinimidyl Ester of 3β-Hydroxybisnor-5-cholenic Acid (I).** A solution that was prepared from dicyclohexylcarbodiimide (0.419 g, 2.04 mmol) and 5 mL of anhydrous THF was added dropwise to a stirred suspension of 3β-hydroxybisnor-5-cholenic acid (0.702 g, 2.03 mmol) and *N*-hydroxysuccinimide (0.235 g, 2.04 mmol) in 30 mL of THF at 50 °C. The reaction mixture was stirred for an additional 3 h at 50 °C and left overnight at ambient temperature. The supernatant was then separated from the product mixture by filtration and concentrated under reduced pressure. The solid residue was then dissolved in 40 mL of chloroform and washed, sequentially, with saturated sodium bicarbonate (20 mL), water (20 mL), and brine (10 mL). The chloroform solution was dried over anhydrous sodium sulfate and concentrated under reduced pressure. Recrystallization from acetone/petroleum ether afforded 0.577 g (64%) of **I** as a colorless powder having mp 214−216 °C: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.35 (m, 1 H), 3.53 (m, 1 H), 2.82 (s, 4 H), 2.73 (dq, J = 10.9, 6.9 Hz, 1 H), 2.27 (m, 2 H), 1.38 (d, J = 6.9 Hz, 3 H), 1.02 (s, 3 H), 0.74 (s, 3 H), and 2.0−0.95 (m, 18 H); HRMS-FAB (M + Na^+) calcd for $C_{26}H_{36}O_5N + Na^+$ 466.2569, found 466.2564.

Pyridinium Sulfate of the *N*-Succinimidyl Ester of 3β-Hydroxybisnor-5-cholenic Acid (II). To a solution of **I** (0.445 g, 1.00 mmol) in 20 mL of anhydrous chloroform was added, in a single portion, 0.481 g (3.03 mmol) of sulfur trioxide/pyridine complex. After the heterogeneous mixture was stirred for 14 h at ambient temperature, an additional 20 mL of chloroform was then added and the mixture cooled to ca. −10 °C and filtered. Concentration of the filtrate under reduced pressure, followed by crystallization from acetone/petroleum ether, afforded 0.355 g (75%) of **II** as colorless crystals having mp 220−224 °C: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.92 (m, 2 H), 8.57 (m, 1 H), 8.05 (m, 2 H), 5.28 (m, 1 H), 3.84 (m, 1 H), 2.80 (s, 4 H), 2.68 (dq, J = 10.5, 6.8 Hz, 1 H), 2.38 (m, 2 H), 1.28 (d, J = 6.8 Hz, 3 H), 1.10 (s, 3 H), 0.72 (s, 3 H), 2.14−0.95 (m, 19 H) ppm; HRMS-FAB (MH^+) calcd for $C_{31}H_{42}O_8N_2S$ 603.2740, found 603.2741.

Sulfate of the Spermine Conjugate of 3β-Hydroxybisnor-5-cholenic Acid (1). To a stirred solution of spermine (0.215 g, 1.06 mmol) in 15 mL of anhydrous DMF, which was maintained at 0 °C, was added a solution of **II** (0.380 g, 0.63 mmol) in 2.5 mL of anhydrous DMF over a 20 min period. The mixture was then stirred at ambient temperature for 1 h, quenched with 20 mL of 0.1 M NaOH, and extracted with 1-butanol (2 × 20 mL). The combined extracts were washed with water (3 × 20 mL) and brine (2 × 20 mL) and dried over anhydrous Na_2SO_4. Removal of solvent under reduced pressure (45 °C) afforded 218 mg of solid residue, which was then purified by column chromatography [silica, CH_3OH/30% NH_4OH (4/1, v/v)] and filtration (0.45 mm Millipore) to give 140 mg (35%) of **1** as a cream-colored solid having mp 212−221 °C dec: ^{1}H NMR (500 MHz, CD_3OD) δ 5.39 (m, 1 H), 4.14 (m, 1 H), 3.19−3.24 (m, 2 H), 2.85 (t, J = 7.0 Hz, 2 H), 2.78 (t, J = 7.2 Hz, 2 H), 2.68−2.72 (m, 6 H), 2.54 (m, 1 H), 2.31 (m, 2 H), 1.75 (m, 4 H), 1.60 (m, 4 H), 1.15 (d, J = 6.7 Hz, 3 H), 1.04 (s, 3 H), 0.75 (s, 3 H), 2.10−0.96 (m, 19 H) ppm; HRMS-FAB (MH^+) calcd for $C_{32}H_{58}O_5N_4S$ 611.4206, found 611.4211.

Acetylated Derivative of 1 (2). A solution of acetic acid *N*-hydroxysuccinimide ester (0.053 g, 0.35 mmol) in 3 mL of dry CH_2Cl_2 was added dropwise to a stirred suspension of **1** (0.192 g, 0.3 mmol) in 3 mL of CH_2Cl_2 plus 2 mL of DMF. The mixture was stirred for 12 h at room temperature. After removal of solvent under reduced pressure, the solid residue was dissolved in a minimum amount of CH_3OH, and then precipitated by addition to 50 mL of a saturated aqueous solution of $NaHCO_3$. The filtrate was then purified by column chromatography [silica, CH_3OH/30% NH_4OH (4/1, v/v)] to give 50 mg (25%) of **2** having R_f 0.8: ^{1}H NMR (360 MHz, $CDCl_3/CD_3OD$, 2/3) δ 5.32 (m, 1 H), 4.17 (m, 1 H), 3.16 (m, 4 H), 2.50 (m, 8 H), 1.89 (s, 3 H), 0.95−2.41 (m, 33 H), 0.98 (s, 3 H), 0.68 (s, 3 H); HRMS-FAB (MH^+) calcd for $C_{34}H_{61}N_4O_6S$ 653.4318, found 653.4312.

Spermine Conjugate of 3β-Hydroxybisnor-5-cholenic Acid (3). To a stirred solution of spermine (0.068 g, 0.34 mmol) in 1 mL of anhydrous chloroform was added, dropwise, a solution that was prepared from **I** (0.086 g, 0.19 mmol) plus 4 mL of chloroform, over a 5 min period at ambient temperature. After 30 min of reaction, some precipitate appeared in the flask. The heterogeneous mixture was stirred overnight, and then transferred to a test tube and washed, sequentially, with 0.1 M NaOH (1 × 2 mL), water (1 × 2 mL), and saturated sodium chloride (1 mL). After drying over anhydrous K_2CO_3, and subsequent solvent removal under reduced pressure, 84 mg of crude product was obtained; chromatographic purification [silica, CH_3OH/30% NH_4OH (4/1, v/v)] afforded 42 mg of **3**: ^{1}H NMR (500 MHz, CD_3OD) δ 5.33 (s, 1 H), 3.40 (m, 1 H), 3.15−3.30 (m, 2 H), 2.82 (t, 2 H), 2.77 (t, 2 H), 2.68 (m, 4 H), 2.29−2.19 (m, 3 H), 1.99 (m, 2 H), 1.90−0.95 (m, 27 H), 1.15 (d, 3 H), 1.03 (s, 3 H), 0.75 (s, 3 H); HRMS-FAB calcd for $C_{32}H_{59}N_4O_2$ 531.4647, found 531.4638.

Sulfate of the Conjugate of 3β-Hydroxybisnor-5-cholenic Acid plus 4,9-Dioxa-1,12-dodecanediamine (4). A solution of 4,9-dioxa-1,12-dodecanediamine (256 mL, 1.2 mmol) in 5 mL of dry DMF was added dropwise to a stirred suspension of **II** (0.500 g, 0.8 mmol) in 4 mL of DMF. The mixture was then stirred for 12 h at room temperature. After addition of 50 mL of $CHCl_3$, the solution was washed with 20 mL of aqueous $NaHCO_3$ (saturated). The aqueous washings were then extracted with 1-butanol (2 × 50 mL), and the butanol extract was combined with the chloroform phase. Removal of solvent under reduced pressure (after drying over anhydrous Na_2SO_4) afforded 700 mg of crude product, which was purified by column chromatography [silica, $CHCl_3/CH_3OH$/30% NH_4OH 60/40/10, (v/v/v)] to give 100 mg (20%) of **4**, having R_f 0.7: ^{1}H NMR (360 MHz, $CDCl_3/CD_3OD$; 10/1) δ 5.26 (m, 1 H), 3.98 (m, 1 H), 3.26−3.59 (m, 8 H), 3.15 (m, 2 H), 2.99 (t, 2 H), 0.95−2.40 (m, 34 H), 0.98 (s, 3 H), 0.68 (s, 3 H); HRMS-FAB calcd for $C_{32}H_{57}N_2O_7S$ 613.3901, found 613.3887.

pH Discharge Experiments. In a typical experiment, 1.6 mL of a chloroform solution of egg phosphatidylglycerol (egg PG, 25 mg/mL) was mixed with 520 μL of a 2 mM solution of **1** (2.0 mol %) in $CHCl_3/CH_3OH$ (1/1, v/v). The solvent was then evaporated under a stream of nitrogen, and the resulting thin film dried (12 h, 23 °C, 0.1 mmHg). Dispersal of the mixture (vortex mixing) in 2.0 mL of buffer containing 0.1 mM pyranine, followed by freeze−thawing (77 K/298 K, five cycles), sequential extrusion through 0.4, 0.2, and 0.1 μm Nuclepore membranes (10 times in each case), removal of nonentrapped pyranine by gel filtration (minicolumn centrifugation), and dilution to a final volume of 10 mL with buffer afforded a stock dispersion.[6] A 100 mL aliquot of this dispersion was then directly injected into a stirred cuvette within the fluorimeter, which contained 3 mL of buffer. The fluorescence emission intensity was monitored, continuously, at 25 °C. After a period of 180 s, a pH gradient of 0.6 unit was then established by addition of 50 μL of 0.5 M NaOH through an injector port. Finally, the fluorescence intensity of the dispersion was measured after complete pH discharge via addition of 20 μL of a 0.25 mM solution of gramicidin A in trifluoroethanol. In a separate experiment, the fluorescence intensity of a similar dispersion was measured after addition of 10 μL

(6) *Liposomes: A Practical Approach*; New, R. R. C., Ed.; Oxford University Press: New York, 1990; p 126.

of a 5% (v/v) aqeuous solution of Triton X-100. No changes in fluorescence intensity were observed when a second equivalent of either gramicidin A or Triton X-100 was added to the dispersion.

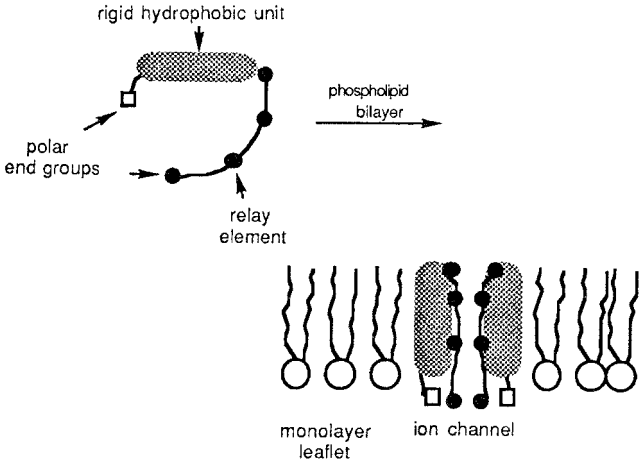
A control experiment was carried out to ensure that pyranine did not escape from the vesicles during the course of the pH discharge. Specifically, a second gel filtration was performed after complete discharge with gramicidin A. Within experimental error (±5%), the fluorescence intensity before and after this second gel filtration was the same. A similar control confirmed the absence of the release of pyranine from egg PG vesicles containing 1 mol % **3**.

Membrane Binding. The extent of binding of **1**, **2**, **3**, and **4** to egg PG bilayers was evaluated by comparing the amine content of appropriate vesicle dispersions before and after gel filtration. Typically, stock dispersions of egg PG vesicles (5 mM) containing 2 mol % sterol−polyamine conjugate were prepared using procedures similar to those used for the pH discharge experiments. In this case, however, pyranine was omitted from the buffer. Prior to gel filtration, the minicolumns were conditioned by filtering 500 μL of a 5 mM egg PG vesicles that were devoid of conjugate, followed by rinsing with 50 mL of buffer (pH 7.0). Samples were assayed for amine content using fluorescamine for **1**, **3**, and **4**, and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) for **2**.[7] The percentage of the fluorescence intensity that remained after gel filtration was taken as a measure of bound conjugate. For vesicles containing **1**, **3**, or **4**, 500 μL aliquots of the dispersion (before and after filtration) were mixed with a solution that was prepared from 100 μL of a 5% aqueous solution of Triton X-100 and 1 mL of 1 mM fluorescamine in acetone. The resulting solution was then stirred for 45 min at room temperature, and its fluorescence intensity measured at l_{max} 480 nm, using a spectral bandwidth of 7 nm and an excitation wavelength of l_{ex} 395 nm. These analyses indicated that 96%, 92%, and 91% of **1**, **3**, and **4** were membrane-bound. For vesicles containing **2**, 500 μL aliquots of the dispersion (before and after filtration) were first stirred with 500 μL of ethanol for 10 min at 70 °C. After addition of 500 μL of a 2 mM ethanolic solution of NBD-F, the mixture was stirred for 5 min at 70 °C, quickly cooled to 0 °C, and allowed to reach room temperature. After addition of 0.1 mL of 3 M HCl, the fluorescence emission intensity of a 100 μL aliquot, which had been diluted with 3 mL of ethanol, was measured at l_{max} 520 nm, using a spectral bandwidth of 9 nm and an excitation wavelength of 470 nm. Such an analysis indicates that 93% of **2** is membrane-bound.

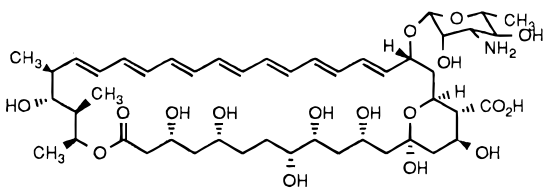
Results

Design Principle. The design principle that we have used for the construction of a prototype (i.e., **1**) incorporates three structural elements that are found in the naturally occurring, pore-forming antibiotic amphotericin B.[8] Specifically, it includes a long and rigid hydrophobic (sterol) unit, an amphiphilic (polyamine) chain that can extend across this unit, and a zwitterionic (ammonium sulfate) headgroup, i.e., analogues of amphotericin B's heptaene, polyol, and carboxyl/mycosamine moieties, respectively.[9] Our working hypothesis was that **1** would favor a macrocyclic conformation by forming a salt bridge between the terminal primary amine group and the sulfate moiety, which is located at the opposite end of the molecule. Examination by CPK models indicates that the effective length of such a macrocycle is ca. 20 Å, which is sufficient to span the hydrocarbon region of a phospholipid monolayer. We further hypothesized that strong hydrophobic interactions between one face of the sterol and the alkyl chains of neighboring phospholipids would drag the pendant amphiphilic polyamine chain into the membrane, thereby leaving a zwitterionic headgroup exposed at the membrane surface (Scheme 1). Subsequent aggregation within each monolayer leaflet, and alignment across the bilayer (possibly assisted by hydrogen bonding via the amide moiety), would then produce a contiguous pore. In principle, the polyamine chain could serve as a conduit for ion transport, whereby each proton-ionizable nitrogen functions as a "relay" element that moves protons and/or anions across the lipid bilayer.

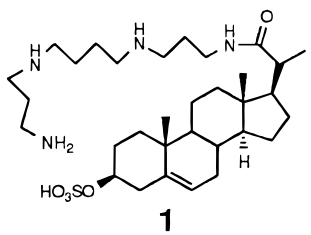
Scheme 1



Sterol−Polyamine Conjugates. To test our design principle, closely related "acetyl-capped" (**2**), nonsulfated (**3**), and ether-substituted (**4**) analogues were also chosen as synthetic targets. With **2**, the possibility of salt bridge formation with the terminal primary amine group is eliminated. Similarly, replacement of the sulfate moiety with a hydroxyl group leaves significant polarity at the C-3 position, but also eliminates the possibility of salt bridge formation. Finally, substitution of both secondary amine groups with ether units (i.e., **4**) tests whether a "string" of proton-ionizable nitrogens is necessary for ionophoric activity.



Amphotericin B



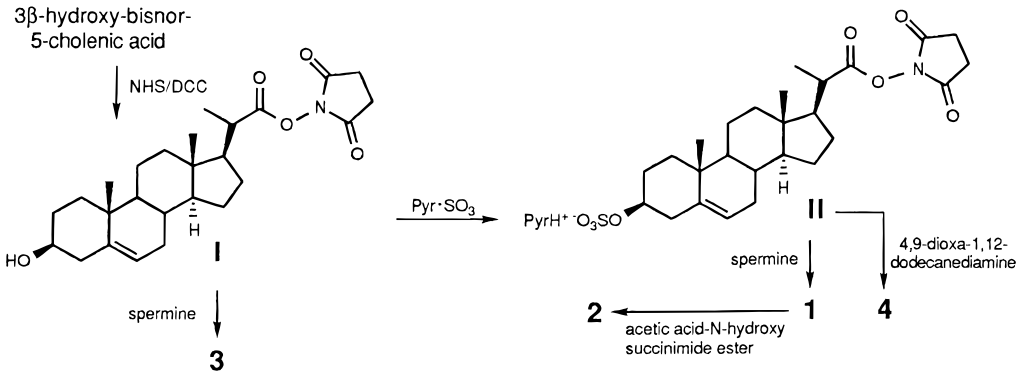
1

(7) (a) Udenfriend, S.; Stein, S.; Bohlen, P.; Dairman, W. *Science* **1972**, *178*, 871. (b) Imai., K.; Watanabe, Y. *Anal. Chim. Acta*. **1981**, 130, 377.

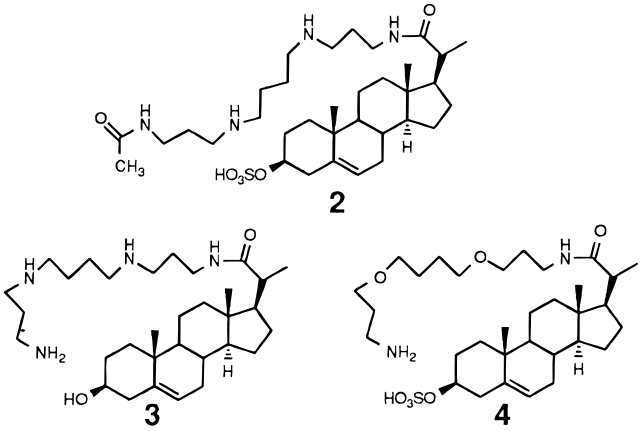
(8) (a) Medoff, G.; Brajtburg, J.; Kobayashi, G. S.; Bolarad, *J. Annu. Rev. Pharmacol. Toxicol*. **1983**, *23*, 303. (b) Finkelstein, A.; Holz, R. In *Membranes 2: Lipid Bilayers and Antibiotics*; Eisenman, G., Ed.; Marcel Dekker: New York, 1973; Chapter 5. (c) Gale, E. F. In *Macrolide Antibiotics: Chemistry, Biology, and Practice*; Omura, S., Ed.; Academic Press: New York, 1984; Chapter 11. (d) Hahn, F. E. *Antibiotics Vol II, Mechanism of Action of Antieukaryotic and Antiviral Compounds*; Springer-Verlag: New York, 1979.

(9) The sterol conjugate **1** bears an intriguing structural similarity to squalamine, a naturally occurring sterol−spermidine conjugate that is known to possess potential antimicrobial acitivity. The mechanism of biological action of squalamine, however, remains to be established; see: (a) Moore, K. S.; Wehrli, S.; Roder, H.; Rogers, M.; Forrest, J. N., Jr.; McCrimmon, D.; Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1354. (b) Moriarity, R. M.; Tuladhar, S. M.; Guo, L.; Wehrli, S. *Tetrahedron Lett.* **1994**, *35*, 8103.

Scheme 2

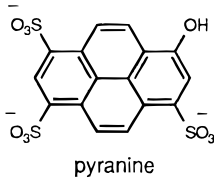


Thus, according to our design principle, **2**, **3**, and **4** would not be expected to function as ionophores.



The synthetic approach that was used to prepare each of these target molecules is outlined in Scheme 2. In brief, activation of the carboxylic acid moiety of 3*β*-hydroxybisnor-5-cholenic acid with *N*-hydroxysuccinimide afforded a common precursor (**I**). Thus, sulfation of **I** (to give **II**), followed by condensation with spermine and 4,9-dioxa-1,12-dodecanediamine, produced **1** and **4**, respectively; acetylation of **1** with acetic acid *N*-hydroxysuccinimide ester yielded **2**. Alternatively, direct condensation of **I** with spermine afforded **3**.

Ionophoric Activity Assayed by pH Discharge. To test for ionophoric activity, we examined the ability of each sterol conjugate to promote the discharge of a pH difference across vesicle membranes derived from egg phosphatidylglycerol (egg PG). For this purpose, a pH-sensitive dye (pyranine, pK_a = 7.2) was entrapped within large unilamellar vesicles (1000 Å



pyranine

diameter) and used as a fluorescence-responsive reporter of pH changes within the vesicle interior.[10,11] The buffer that was used throughout this study was composed of 25 mM HEPES (pK_a = 7.5) plus 100 mM NaCl, which had been adjusted to pH 7.0 by use of a 0.5 M NaOH solution. Typically, a pH difference of 0.6 unit across the membrane was established by rapid injection of an aqueous solution of NaOH (pH "shock"). Under such conditions, ionophoric activity is reflected by an increase in the fluorescence intensity with time.

Two general methods that have been used to incorporate ionophores into vesicles are (i) the addition of preformed vesicles to an aqeuous solution of the ionophore, or vice versa ("single-sided" experiment) and (ii) the inclusion of the ionophore with the phospholipids during vesicle formation ("double-sided" experiment).[12,13] Although single-sided experiments are simpler to carry out, the observed kinetics can be complex due to contributions of diffusion by the ionophore from the outer to the inner monolayer leaflet, from time-dependent pore formation within the membrane, and from multiple mechanisms of ionophore−membrane interaction that can result from the presence of micelles and monomer.[14−16] To simplify our interpretations, we have chosen to carry out double-sided experiments in all of the work reported herein; the one exception, however, involves membrane-selectivity studies (vide infra).

Ionophoric Properties of 1. Typical pH discharge profiles that were observed in the presence of varying concentrations of **1** are shown in Figure 1. In each instance, a small "burst" in fluorescence intensity was followed by a dominant, time-dependent pH discharge. Final fluorescence intensity values, which were obtained by destroying the vesicles with excess Triton X-100, were essentially the same (±5%) as those that were determined by adding a small amount (1.2 mol %) of the naturally occurring ion channel-forming peptide gramicidin A (gA, *Bacillus brevis*).[17] Increasing concentrations of **1** resulted in increasing rates of pH discharge.

Control experiments that were carried out established that the extent of dye adsorption on the outer membrane surface was low. Specifically, when similar egg PG vesicles (2 mL, 25 mM) were prepared in the absence of dye, and then allowed to incubate with 1.0 mL of a 0.1 mM pyranine solution in buffer for 1 h, fluorescence measurements that were made after gel filtration revealed that the vesicles retained ca. 5% of the fluorescence emission intensity of what was found for analogous vesicles that were formed in the presence of pyranine (Table 1). Similar control experiments, which were carried out in the presence of 2 mol % **1**, revealed a relative dye content of ca. 6%. Taken together, these results show that adsorption of dye

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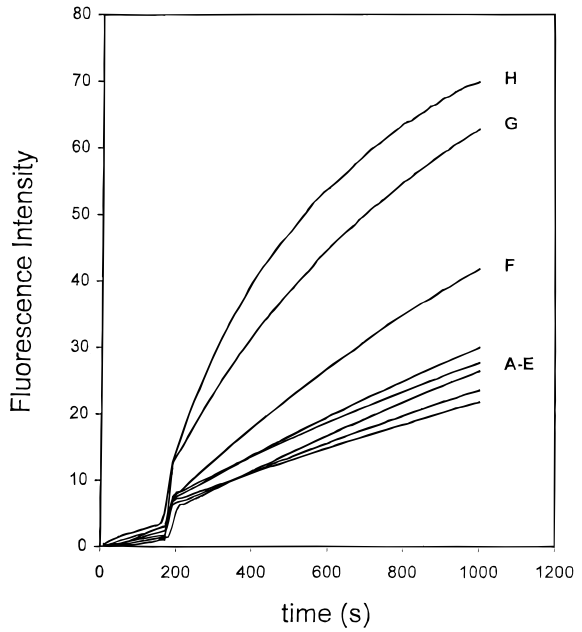

Figure 1. Changes in fluorescence intensity as a function of time for egg PG vesicles containing varying mole percentages of **1**: (A) 0%, (B) 0.13%, (C) 0.25%, (D) 0.38%, (E) 0.50%, (F) 1.0%, (G) 1.5%, (H) 2.0%. A through E are indicated in ascending order at time 1000 s. Values for 100% fluorescence intensity were obtained after complete pH discharge by use of gramicidin A. In all cases initial and final pH values of the external aqueous phase were 7.0 and 7.6, respectively.

Table 1. Binding of Pyranine to Phospholipid Vesicles[a]

phospholipid	conjugate	pyranine present[b]	relative fluorescence intensity[c]
egg PG		inside and outside	100
egg PG		outside only	5
egg PG	**1**	outside only	6
egg PG	**3**	outside only	7
egg PC		inside and outside	100
egg PC		outside only	14
egg PC	**1**	outside only	73

[a] Large unilamellar vesicles were prepared using procedures described in the Experimental Section; conjugates, when included, were present in 2 mol %. [b] "inside and outside" means that pyranine was present during vesicle formation and that external dye was removed by gel filtration; "outside only" means that vesicles were prepared in the absence of pyranine, and then incubated with 0.1 mM dye for 1 h at room temperature prior to gel filtration. [c] Fluorescence intensities (λ_{em} 510 nm, λ_{ex} 460 nm, slit width 10 nm) were measured at pH 7.6 after addtion of Triton X-100, and were normalized to account for variations in phospholipid concentrations.

to the membrane exterior occurs to a small extent, and that such adsorption can account for the majority of the "burst" phase that is observed.

Additional control experiments established that the observed increase in fluoresence intensity reflects ionophoric activity and not the leakage of dye out of the vesicles. Specifically, gel filtration of vesicles containing 2 mol % **1**, after being fully discharged by addition of gA, confirmed that >95% of the dye remained entrapped within the vesicles. To establish that **1** was fully bound to the vesicles, a similar dispersion containing 2 mol % conjugate (in the absence of pyranine) was analyzed for primary amine content before and after gel filtration by use of a fluorescamine-based assay (see the Experimental Section).[18] From such analyses, we conclude that >95% of **1** is bound to the vesicles.

(18) See ref 6, p 105.

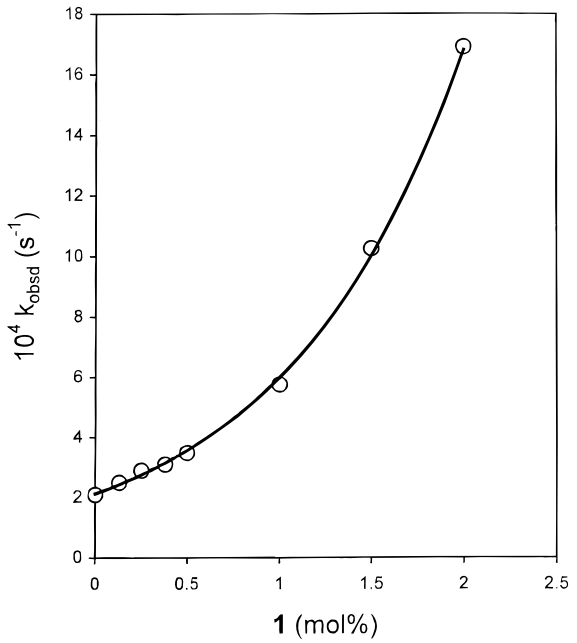

Figure 2. Observed pseudo-first-order rate constants as a function of mole percentage of **1**. The solid line represents a nonlinear least-squares fit of the data based on eq 5.

Pseudo first-order rate constants (k_{obsd}) that were calculated as a function of mol % **1** are shown in Figure 2. To ensure that changes in fluorescence intensity accurately reflect changes in pH within the vesicles, titration experiments were carried out with egg PG vesicles containing entrapped pyranine plus membrane-bound gA. Here, the gA was present in order to ensure rapid and complete equilibration. As expected, plots of fluorescence intensity versus molar hydroxide concentration were found to be linear over the same pH range that was used to test **1** for ionophoric activity (not shown). Thus, the pseudo-first-order rate constants for the fluorescence change are an accurate measure of transmembrane proton and/or hydroxide fluxes.

In a separate set of experiments, a methanolic solution of a protonophore [5 mol %, carbonyl cyanide [*p*-(trifluoromethoxy)-phenyl]hydrazone (FCCP)] was directly injected into a vesicle dispersion containing 2 mol % **1**, and allowed to incubate for a period of 3 min prior to pH shock.[12,13,19] This weak organic acid has been shown to effectively increase the permeability of lipid bilayers toward protons.[11,19] In the presence of FCCP, a 4-fold increase in rate of the pH discharge was observed (Figure 3).[20] Addition of an identical volume of methanol (minus FCCP) to a similar vesicle dispersion, and also addition of a methanolic solution of FCCP (5 mol %) to vesicles that were devoid of **1**, had a negligible effect on the rate of pH discharge (not shown). These results strongly suggest that proton transport across egg PG membranes is at least partially rate-limiting when **1** is present. In view of the negligible activity that has been found for **1** in promoting Na^+ transport across phospholipid membranes, we currently favor a H^+/Cl^- symport mechanism over a H^+/Na^+antiport process for the pH discharge process.[1a]

Ionophoric Properties of 2 and 4. Analogous pH discharge experiments that were carried out over a similar concentration range with **2** and **4** showed *negligible ionophoric activity* (Figure

(19) Gennis, R. B. *Biomembranes: Molecular Structure and Function;* Springer-Verlag: New York, 1989; Chapter 8.

(20) In our preliminary studies, we noted that FCCP did not increase the rate of pH discharge in the presence of **1**. These results have since been found to be incorrect.

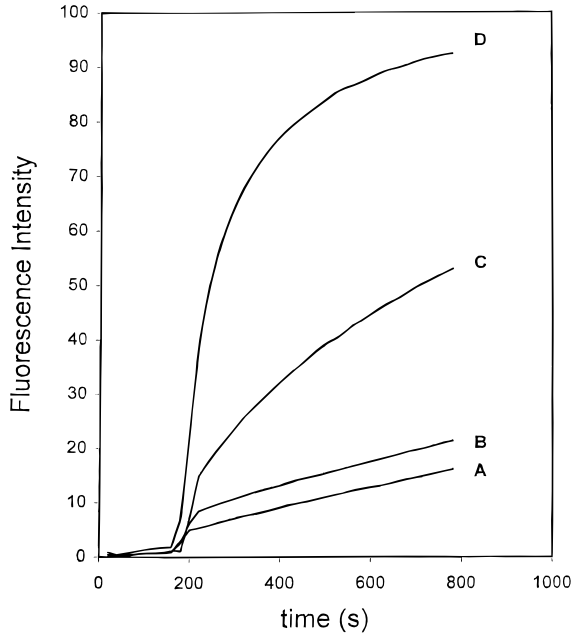

Figure 3. Changes in fluorescence intensity as a function of time for vesicles composed of (A) egg PG, (B) egg PG + 5 mol % FCCP, (C) egg PG + 2 mol % **1**, and (D) egg PG + 2 mol % **1** + 5 mol % FCCP. Initial and final pH values were 7.0 and 7.6, respectively; FCCP, when used, was added as a methanolic solution to the vesicle dispersion, and allowed to incubate for 3 min prior to pH shock.

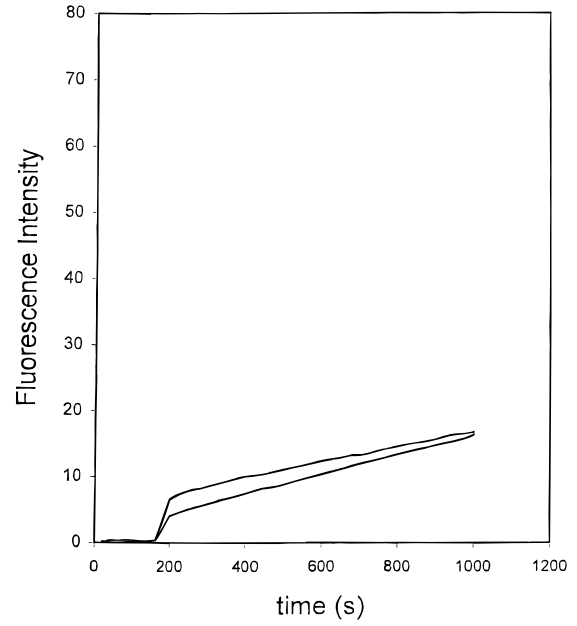

Figure 4. Changes in fluorescence intensity as a function of time for egg PG vesicles containing 2 mol % **2** (lower trace) or **4** (upper trace).

4). Analysis of each dispersion for conjugate content, before and after gel filtration, established that >90% of each sterol was bound to the vesicles (see the Experimental Section).

Ionophoric Properties of 3. In sharp contrast to the behavior of **1**, the burst phase that was associated with **3** was found to sharply increase with increasing concentrations of the conjugate (Figure 5). In addition, the slow phase of the pH discharge was essentially independent of the concentration of the conjugate. Control experiments that were carried out established that >95% of the pyranine remained entrapped within vesicles containing 2 mol % **3**, after the remaining pH gradient was fully discharged with gA. In addition, incubation of preformed vesicles containing 2 mol % **3** with an aqueous pyranine solution

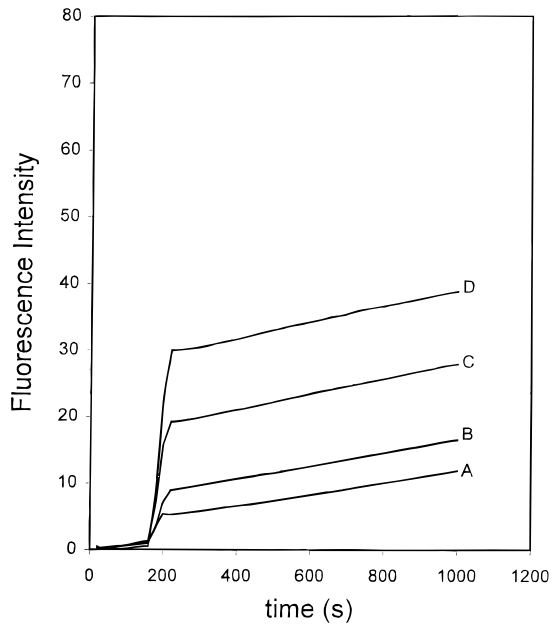

Figure 5. Changes in fluorescence intensity as a function of time for egg PG vesicles containing varying mole percentages of **3**: (A) 0.0%, (B) 0.50%, (C) 1.0%, (D) 2.0%.

showed that the extent of adsorption to the outer membrane surface was low (Table 1). Taken together, these findings, rule out the possibility that the large increases in the burst phase result from increasing amounts of dye that are adsorbed on the vesicle exterior.

If one assumes that the pendant polyamine group of **3** is fully exposed to the aqueous/membrane interface (i.e., that it is not "dragged" into the membrane interior), then the dependency of the burst phase on the concentration of this conjugate can be accounted for in terms of a reduced buffering capacity within the aqueous interior of the vesicles. That such a possibilty exists is readily apparent, if one considers the captured volume of the vesicles, the buffer concentration that has been used, and the ionophore concentration employed. Specifically, on the basis of the vesicle's efficiency in entrapping pyranine, the captured volume for these 1000 Å diameter vesicles is estimated to be 1.7 L per mol of phospholipid. This value is in good agreement with previous estimates of ca. 1.5 L/mol for 1000 Å diameter vesicles made from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.[21,22] Thus, 1 L of vesicle-entrapped aqueous phase (containing 25 mmol of HEPES) is surrounded by ca. 600 mmol of phospholipid. If 2 mol % **1** is present in the bilayer, then a maximum of 36 mmol of proton-ionizable amine groups is available to the vesicle interior [i.e., 600 × 0.02 × 3]. Thus, contributions to the burst phase, which result from electrically uncompensated proton transport, would be expected to result in larger pH changes due to a reduction in the buffer capacity by the exposed polyamine chains.[11] The fact that a 30% burst phase, which is observed with 2 mol % **3** in the presence of 25 mM HEPES, is reduced to 18% when a 100 mM HEPES buffer is used (not shown) is fully consistent with this interpretation. It should be noted that if this burst phase represented an "all or none" pH discharge (i.e., only a fraction of the vesicles are responsive to changes in pH), then the extent of the burst would be expected to be independent of the buffer concentration that is used. The fact that this is not the case, together with the fact that the slow phase of the pH discharge is independent of the

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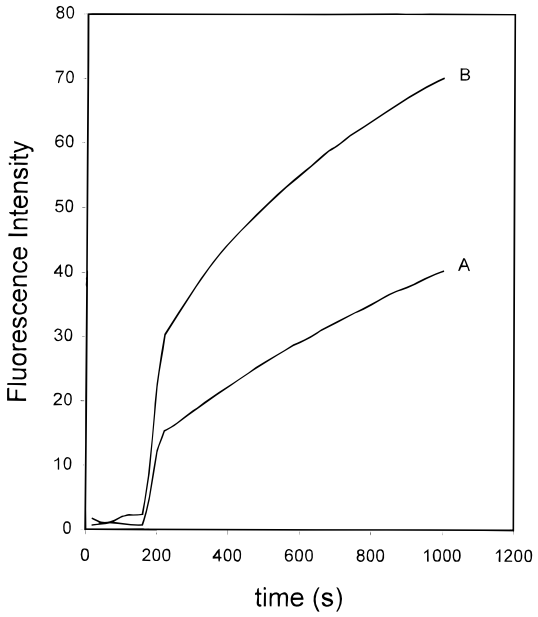

Figure 6. Changes in fluorescence intensity as a function of time for (A) egg PC vesicles and (B) egg PG vesicles, after injection of 5 mol % **1** (single-sided addition of 20 mL of 1 mM methanolic solution of the conjugate) followed by a 4 min incubation period prior to pH shock.

concentration of **3**, provides compelling evidence that this conjugate does not possess ionophoric properties.

Membrane Selectivity Features of 1. In contrast to egg PG-based vesicles, we have found that egg phosphatidylcholine (egg PC) membranes adsorb significant amounts of pyranine (Table 1). Moreover, addition of 2 mol % **1** significantly increases the extent of dye adsorption.[23] To judge the relative activity of **1** toward egg PG (negatively charged) versus egg PC (electrically neutral) membranes and, at the same time, to minimize ambiguities associated with significant quantities of adsorbed dye, comparative *single-sided* experiments were performed. Thus, egg PC vesicles (100 μL, 5 mM) containing entrapped pyranine were added to 3 mL of a 7 μM solution of **1** in HEPES buffer, and the resulting dispersion was allowed to incubate for 4 min prior to pH shock. As shown in Figure 6, a burst phase, corresponding to ca. 15% of the total change in fluorescence intensity, was followed by a slower second phase. A similar experiment that was performed with egg PG vesicles showed a larger burst component, which was also followed by a slower second phase. Gel filtration of the latter, and analysis for dye content, revealed *a significant release of pyranine from the vesicles* (ca. 30%). This amount of release, therefore, accounts for nearly all of the burst phase. Although we have not investigated this membrane disruption phenomenon in detail, we suspect that it reflects, at least in part, a rupture event.[15] Nonetheless, the second phase of these discharge profiles shows that **1** is, operationally, more active against negatively charged membranes relative to ones that are electrically neutral; i.e., k_{obsd} values for the slow phase in egg PG and egg PC membranes were 10.4×10^{-4} and 4.4×10^{-4} s^{-1}, respectively.

Discussion

The results that have been obtained for the pH discharge within egg PG vesicles clearly show that **1** functions as an ionophore. In sharp contrast, analogous sterol−polyamine conjugates that have their terminal amine group acylated, or their sulfate moiety replaced by a hydroxyl group, or both secondary amines substituted by ether oxygens, do not exhibit such activity. Taken together, these structure/activity results provide strong support for the general design principle upon which **1** was based. In addition, the apparent influence that **3** has on the buffering capacity within egg PG vesicles, relative to that of **1**, provides support for our hypothesis that the polyamine chain of **1** is "dragged" into the lipid membrane to a significant extent as the result of macrocycle formation and strong hydrophobic interactions with neighboring phospholipids.

The strong dependency of k_{obsd} on the concentration of **1** that is present is fully consistent with a model in which monomer is in equilibrium with an aggregated structure (i.e., pore), and where the latter is responsible for ion transport.[1b] Specifically, if one assumes that the observed first-order rate constant (k_{obsd}) reflects pseudo-first-order kinetics, where each ion passes through a pore that is composed of *n* ionophore molecules, then k_{obsd} is equal to the product of the pore concentration and the rate constant that chacterizes ion passage through the pore (eqs 1 and 2). If the pore concentration is expressed in terms of the ionophore concentration and the dissociation constant (K) that defines the aggregate (pore)−monomer equilibrium (eq 3), then k_{obsd} should vary with the ionophore concentration to the *n*th power (eq 4). If the ionophore exists primarily in the monomeric form, then the ionophore concentration can be approximated by its *analytical* concentration (i.e., the total concentration of ionophore that is present in the dispersion), and k_{obsd} will vary with respect to the analytical concentration to the *n*th power. If the ionophore were extensively aggregated, however, then the concentration of pores is expected to be directly proportional to the analytical concentration of the ionophore.

$$\text{rate} = k_{obsd}[\text{ion}] \tag{1}$$

$$k_{obsd} = k_i[\text{pore}] \tag{2}$$

$$K = [\text{ionophore}]^n/[\text{pore}] \tag{3}$$

$$k_{obsd} = k_1[\text{ionophore}]^n/K \tag{4}$$

Since a slow background rate of pH discharge (k_o) is also detected in the absence of ionophore (a likely consequence of transient gaps that develop in the membrane as a result of thermal motion), the pseudo-first-order rate constant that is actually observed would then be given by eq 5. By use of eq

$$k_{obsd} = k[\text{ionophore}]^n/K + k_0 \tag{5}$$

5, a nonlinear least-squares fit of k_{obsd} as a function of ionophore concentration yields $n = 2.1 \pm 0.1$ and $k_o = 2.5 \times 10^{-4} \pm 0.1 \times 10^{-4}$ s^{-1} (Figure 2). Thus, these kinetic results support a model in which the majority of **1** exists as membrane-bound monomer, and where *dimers* are responsible for ion transport.

A much more difficult issue to resolve deals with the question of whether ion transport takes place through ion channels or via a mobile ion carrier mechanism. While conductance experiments can distinguish between these two possibilities when planar bilayer lipid membranes are used, such measurements cannot be performed with 1000 Å diameter unilamellar

(23) In our preliminary studies, we underestimated the activity of **1** in egg PC vesicles by not taking into account adsorbed dye.[1a] Specifically, fluorescence intensities that were observed after disruption of the vesicles with Triton X-100 reflected substantial amounts of adsorbed dye as opposed to ion transport.

vesicles. Moreover, conductance measurements do not have sufficient sensitivity for determining the ion flux associated with **1**.[24] At the present time, we favor a channel model in which dimers of **1** in one monolayer leaflet become aligned with dimers in the adjacement monolayer, thereby producing transient pores (Scheme 1). However, we cannot rule out the possiblity that a mobile carrier mechanism also contributes to the ion transport that is observed.

Conclusions

This study has established a new design principle for creating sterol−polyamine conjugates as synthetic ionophores. A kinetic analysis of the ionophoric properties of **1** has implicated *dimers* as being responsible for the ion transport. At the same time, this analysis has provided strong evidence that the majority of **1** exists as membrane-bound monomer. The greater activity that has been found for **1** within negatively charged membranes, relative to ones that are electrically neutral, further suggests that additional structure/activity experiments may lead the way to new classes of antibacterial agents having therapeutic potential, i.e., compounds possessing antibacterial activity with minimal toxicity toward mammalian cells. Studies that are currently in progress are focusing on the synthesis of flexible and rigid *dimeric analogues* of **1**, with a view toward improving ionophoric activity. The results of these studies will be reported in due course.

(24) For conductance measurements involving black lipid membranes, a minimum of 1 pA/100 μm diameter of membrane-covered hole is necessary for detection; this corresponds to an ion flux of ca. 8×10^{10} ions/(cm^2 s). For a 1000 Å diameter unilamellar vesicle of the type used in this study, where the internal pH is 7.0 and the external pH is raised to 7.6, the initial efflux rate for HCl is estimated to be only 1.2×10^5 ions/(cm^2 s); here, the efflux rate = [k_{obsd}][ion concentration gradient][vesicle volume/vesicle surface area], and $k_{obsd} = 17 \times 10^{-4}$ s^{-1} when 2 mol % **1** is used.

Acknowledgment. We are grateful to the National Science Foundation (Grant CHE-9612702) for support of this research.

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