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Amphotericin B Mimics: A Sterol-Based Ionophore[1]

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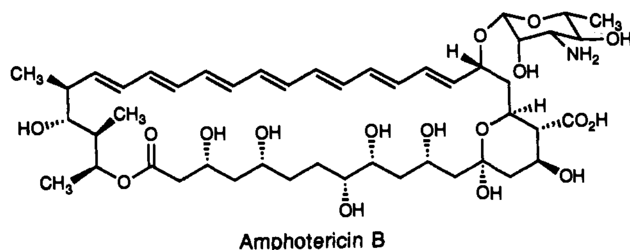
Abstract: A sterol–polyether conjugate, 5-androstene–3β,17β-bis[(oxycarbonyl)hexaethylene glycol] (**1**), has been synthesized via condensation of the bis-chloroformate derivative of 5-androstene-3β,17β-diol with hexaethylene glycol mono(triphenylmethyl ether), followed by deprotection. This conjugate, which is composed of a long and rigid hydrophobic unit, two flexible hydrophilic chains that are linked to the hydrophobic unit, and a pendant polar head group, was designed as a prototype for new classes of compounds that are intended to serve as functional equivalents of the polyene macrolide antibiotic amphotericin B. Analysis of the surface pressure–area isotherm of **1**, generated at the air–water interface, indicates a limiting area of ca. 60 Å^2 per molecule. This value is fully consistent with a model in which the sterol nucleus (ca. 40 Å^2) and *one* pendant polyether chain (ca. 20 Å^2) define the collisional area of the surfactant; that is, it supports the existence of a "folded" conformation at the air–water interface. Incorporation of **1** into egg PC vesicular membranes leads to ion channel formation, as demonstrated by ^{23}Na NMR spectroscopy. Operationally, **1** has been found to have an ionophoric activity that is very similar to that found for a synthetic "bouquet" molecule, but significantly less than amphotericin B, itself.

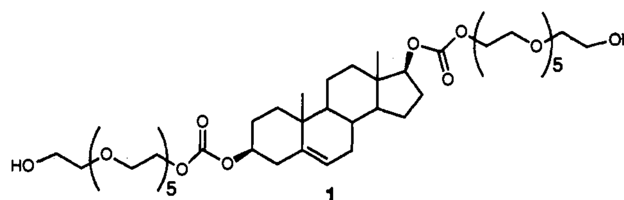
Introduction

The recent emergence of life-threatening microorganisms, together with the growing problem of drug resistance, provides considerable incentive for devising fundamentally new classes of antimicrobial agents.[2] Our own efforts in this area are based on the hypothesis that membrane-disrupting drugs should be ideally suited as therapeutic agents, because they circumvent the problems associated with enzymatic degradation within the cell and also export mechanisms of drug resistance.[3] Previous clinical results that have been obtained with the polyene macrolide antibiotic amphotericin B (AmpB) strongly support this view; that is, the development of drug resistance toward AmpB during its therapeutic use is extremely rare.[4] Although AmpB is now widely used for the treatment of systemic fungal infections in immunocompromised AIDS and cancer patients, its high toxicity and its apparent inability to be metabolized are of particular concern.[5,6] Biodegradable alternatives that exhibit a higher degree of membrane selectivity would clearly be desirable.



Amphotericin B

We have recently begun a synthetic program which is aimed at preparing functional equivalents of AmpB. The general strategy that we have adopted follows, in a sense, Ockham's razor; that is, we sought the simplest molecules that are capable of forming ion channels and wished to avoid unnecessary multiplicities in structure.[7] Consideration of the AmpB molecule suggested to us that the *minimum structural elements* that would be necessary in order to create functional equivalents would consist of (i) a long and rigid hydrophobic unit, (ii) a flexible hydrophilic chain that is linked to the hydrophobic unit, which can extend across its "face", and (iii) a pendant polar head group, i.e., analogs of the heptaene, polyol, and carboxyl/mycosamine components of AmpB, respectively. The sterol conjugate (**1**) was expected to



fulfill these requirements and to serve as a prototype for testing our design principle.[8] Here, we envisioned that strong hydrophobic interactions between *one face of the rigid sterol* and neighboring lipid molecules would "pull" the sterol into parallel alignment with the bilayer, thereby "dragging" one polyether moiety into the membrane; that is, a "folded" conformation would result (Scheme 1).[9] Subsequent self-assembly, in a manner that is

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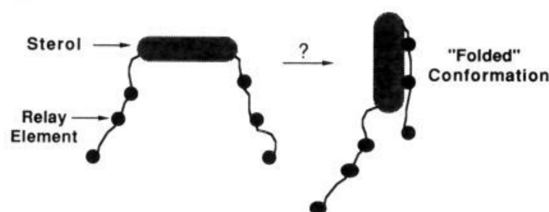
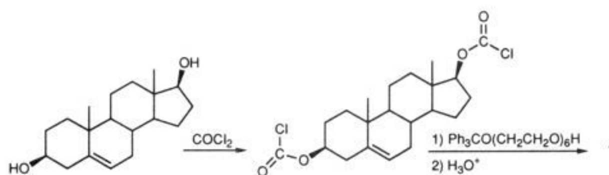
Scheme 1



Scheme 2



similar to what has been proposed for AmpB, was then expected to lead to a functional ion channel.[10] Our choice of a rigid sterol nucleus as the hydrophobic component was also based on the belief that it would be well-suited for future studies that focus both on membrane selectivity and activity. In particular, we envisioned that the interactions of such an ionophore with membrane sterols of biological importance (i.e., cholesterol in mammalian cells versus ergosterol in fungi) could be fine-tuned and that this control could be exploited from a drug-design standpoint. Finally, the potential for biodegradability and/or excretability was also taken into account in the choice of **1**, by virtue of its sterol, carbonate, and poly(ethylene glycol) composition.

The primary objective of the work that is described herein was to establish, unequivocally, whether or not a simple sterol conjugate such as **1** could, in fact, produce ion channels. In essence, we sought to establish the viability of **1** as a prototype for functional equivalents of AmpB.

Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. The sterol 5-androstene-3β,17β-diol was purchased from Steraloids Inc. (Wilton, NH). Egg phosphatidylcholine (egg PC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were obtained from Avanti Polar Lipids (Birmingham, AL) as a chloroform solution and used directly. 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 $^{23}Na^+$ NMR measurements were performed using a Bruker 500-MHz NMR spectrometer. Amphotericin B was purchased from Sigma Chemical Co. (St. Louis, MO) and used directly. Hexaethylene glycol mono(triphenylmethyl ether) was prepared using procedures previously described.[11] The shift reagent that was used in this work, dysprosium(III) bis(tripolyphosphate) ($Dy(P_6O_{20})^{-7}$), was prepared by mixing an aqueous solution of $DyCl_3$ with 1 equiv of pentakis-(tetramethylammonium) tripolyphosphate that was dissolved in water. The latter was prepared by ion exchange of $Na_5P_3O_{10}$ (Aldrich), using a Dowex-50X 2–200 cation exchange resin that was loaded with tetramethylammonium ion and was recrystallized five times (ethanol/acetone, 1/1, v/v) prior to use.[12] 5-Androstene-3β,17β-bis(chloroformate) was prepared using methods similar to those previously described.[13]

5-Androstene-3β,17β-bis[(oxycarbonyl)hexaethylene glycol], 1. To a solution of 2.167 g (4.13 mmol) of hexaethylene glycol mono(triphenylmethyl ether) in 5.5 mL of $CHCl_3$ were added 0.33 mL (4.10 mmol) of pyridine and a catalytic amount of 4-(dimethylamino)pyridine (DMAP). To the resulting solution was then added (dropwise at 23 °C) a solution that was made from 0.851 g (2.05 mmol) of 5-androstene-3β,17β-bis-(chloroformate) plus 4 mL of $CHCl_3$. After the addition was complete, the mixture was allowed to stir for 12 h at 23 °C. Subsequent dilution with 10 mL of $CHCl_3$, washing with water (2 × 20 mL), drying (Na_2SO_4), concentration under reduced pressure, and purification by column chromatography and preparative thin layer chromatography (silica, CH_2Cl_2/CH_3OH (20/1,v/v) afforded 0.54 g (19%) of 5-androstene–3β,-17β-bis[(oxycarbonyl)hexaethylene glycol]mono(triphenylmethyl ether), having R_f = 0.27 and ^{1}H NMR ($CDCl_3$) δ 0.80–2.50 (m, 25 H), 3.25 (t, 4 H), 3.30–3.50 (m, 40 H), 4.25 (m, 4 H), 4.50 (m, 2 H), 5.40 (m, 1 H), 7.10–7.60 (m, 30 H); IR (film) ν_{CO_3}1741 cm^{-1}. Subsequent deprotection was accomplished by dissolving 0.54 g of 5-androstene–3β,17β-bis(oxycarbonylhexaethylene glycol)mono(triphenylmethyl ether) in 5 mL of CH_2Cl_2/CH_3OH (3/2,v/v), which contained a crystal of *p*-toluenesulfonic acid. After stirring at 23 °C for 1 h, a few crystals of Na_2CO_3 were then added followed by additional stirring for 10 min, dilution with 20 mL of CH_2Cl_2, filtration, and concentration under reduced pressure. Subsequent purification by column chromatography (silica, CH_2Cl_2/CH_3OH (20/1,v/v) afforded 0.070 g (20%) of 5-androstene–3β,17β-bis(oxycarbonylhexaethylene glycol), **1**, having ^{1}H NMR ($CDCl_3$) δ 0.80–2.50 (m, 25 H), 2.80 (s, 2 H), 3.50–3.80 (m, 44 H), 4.25 (t, 4 H), 4.50 (m, 2 H), 5.40 (m, 1 H); IR (film) ν_{CO_3}1741 cm^{-1}. Anal. Calcd for $C_{45}H_{78}O_{18}$: C, 59.58; H, 8.66. Found: C, 59.15; H, 8.64.

Surface Pressure–Area Isotherms. Surface pressure–area isotherms were recorded by use of an MGW Lauda film balance that was maintained at 25 °C and was equipped with a computerized data acquisition station. Water (ca. 1 L), which was used as a subphase, was purified via a Milli-Q filtration system and purged with nitrogen for 15 min. Before addition to the film balance, the surface of this degassed water was removed via aspiration in order to remove surface-active contaminants. Surfactant solutions were spread onto the aqueous subphase having a surface area of 600 cm^2, using a 50-μL Hamilton syringe. Chloroform ($CHCl_3$) solutions of **1** were prepared as stock solutions, and their concentrations (typically 0.51 mM) were determined by direct weighing (Cahn electrobalance). The spreading solvents were allowed to evaporate for at least 30 min prior to compression. Compression speeds used in the present study were 11.5 Å^2/min. Isotherms were recorded after two successive compression/expansion cycles.

Na^+ Entry Experiments. Specific procedures that were used were similar to those previously described. Large unilamellar vesicles (1000-Å diameter) were first prepared from egg PC plus 1 or 2 mol % of **1** via extrusion methods using a 100 mM LiCl solution.[14,15] Typically, a 1.5-mL aliquot of this dispersion, containing 28 μmol of lipid, was diluted with 1.65 mL of an isoosmolar shift reagent solution that was 8.2 mM in $(Me_4N)_5P_3O_{10}$, 2.7 mM in $DyCl_3$, and 72.7 mM in NaCl, and the $^{23}Na^+$ NMR absorbance for internal and external ions was recorded as a function of time at 25 °C. For AmpB transport experiments, a DMSO stock solution of the ionophore (5 mM) was injected directly into an egg PC vesicle dispersion, prior to dilution with shift reagent. The final DMSO concentration was 1% (v/v). Control experiments carried out in the absence of AmpB showed no evidence of Na^+ entry over a 10-h period.

Results and Discussion

5-Androstene–3β,17β-bis[(oxycarbonyl)hexaethylene Glycol] (1). The synthetic route that we have used to prepare **1** was straightforward and unexceptional in character (Scheme 2). Conversion of 5-androstene–3β,17β-diol to its bis-chloroformate, followed by condensation with hexaethylene glycol mono(triphenylmethyl ether) and deprotection, afforded the requisite sterol conjugate.

Monolayer Properties. In order to test our hypothesis that **1** would favor a folded conformation in an assembled state, we examined its monolayer behavior at the air–water interface. Thus,

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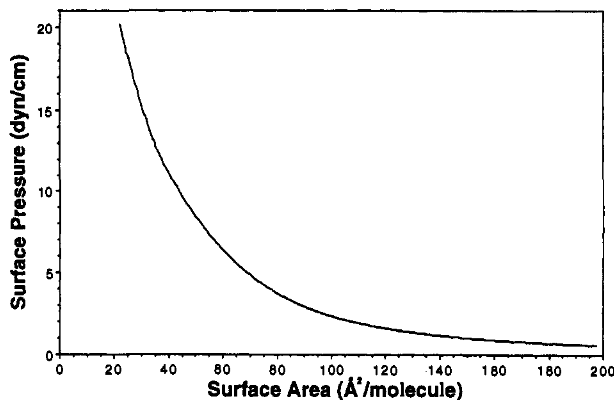

Figure 1. Surface pressure–area isotherm for **1** over a pure water subphase at 25 °C.

a chloroform solution of **1** was spread onto the surface of water, resulting in the formation of a stable monolayer (Figure 1). The limiting area for **1**, which was estimated by taking a small segment of the high-pressure region of the isotherm (20–17 dyn/cm) and extrapolating to zero pressure via simple linear regression, was 59 Å^2. This isotherm was further analyzed in the low-pressure region (1–3 dyn/cm) by use of the semiempirical two-dimensional gas equation (1).[16] Here, π is the experimentally measured surface pressure, π_0 is the position of the horizontal asymptote of the hyperbola that characterizes the liquid-expanded region, A is the measured surface area for a given surface pressure, A_0 is the limting area of the surfactant, T is the absolute

$$\pi = \pi_0 + kT/(A - A_0) \tag{1}$$

temperature, and k is the product of the gas constant and an empirical parameter that is thought to reflect segmental motion. A nonlinear least-squares fit of the data was excellent, affording a limiting area of 62 Å^2/molecule; the calculated values of π_0 and kT were –0.9 dyn/cm and 280, respectively. Thus, both the high- and low-pressure data indicate a limiting area of ca. 60 Å^2/molecule. This value is fully consistent with a model in which the sterol nucleus (ca. 40 Å^2) and *one* pendant polyether chain (ca. 20 Å^2) define the collisional area of the surfactant; that is, it supports the existence of a "folded" conformation at the air–water interface. Examination of CPK molecular models indicates that a folded polyether unit (fully extended) covers the complete length of the sterol, but that a negligible amount would remain for penetration into an adjoining monolayer. Although it is important to realize that extrapolation of results from monolayer experiments to lipid membranes must always be viewed with caution, these findings indicate that, in an assembled state, this sterol favors a conformation in which one face is covered by polyether and the other is not.

Ionophoric Activity. Several experimental protocols have previously been used for investigating the transport of ions across lipid bilayers. Some of the more common methods are based on atomic absorption, conductance, fluorescence, and pH measurements.[7e,g,i,k] In the present study we have employed NMR spectroscopy as a means of monitoring the transport of $^{23}Na^+$ across a liposomal membrane.[7a,c] As has been pointed out by other researchers, a major advantage of this method is that transmembrane transport cannot be confused with vesicle rupture, i.e., a "catastrophic" process in which there is rapid and complete loss in membrane integrity in a fraction of the vesicles that are present.[7a,17] Thus, it provides definitive evidence for ion transport.

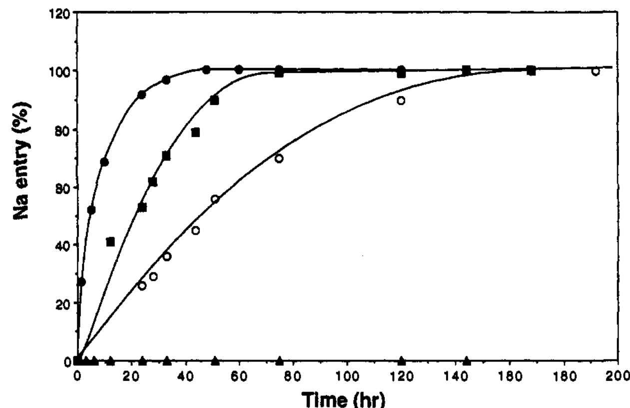

Figure 2. Plot of Na^+ ion entry into egg PC vesicles containing 1 mol % of **1** (○), egg PC vesicles containing 2 mol % of **1** (●), and DPPC vesicles containing 2 mol % of **1** (■) as a function of time at 25 °C. Control experiments carried out with egg PC vesicles, in the absence of **1**, showed no detectable Na^+ entry (▲).

Experimentally, a NaCl solution (containing a paramagnetic shift reagent) is mixed with an aqueous vesicle dispersion that is prepared using an isoosmolar solution of LiCl. If Na^+ ions are able to enter the vesicles, and the shift reagent is not, then one can distinguish between internal (unshifted signal) and external (shifted signal) Na^+ ions. The rate of ion transport can then be followed, directly and unambiguously, by measuring the ratio of internal/external Na^+ as a function of time. In practice, the time scale for such transport experiments ranges from ca. 10 to 10^4 min.

Using procedures that are described in the Experimental Section, large unilamellar vesicles (1000-Å diameter) composed of egg PC plus 2 mol % of **1** were examined with respect to their permeability toward Na^+ ions. In Figure 2, we show a plot of the extent of Na^+ entry as a function to time, where the extent of entry represents a normalized parameter in which maximum entry is defined as the 100% value.[7a] At 25 °C, approximately 5 h was required to reach 50% entry. When the concentration of **1** was reduced to 1 mol %, the time that was required for 50% entry increased to ca. 50 h (Figure 2). Control experiments that were carried out in the absence of **1**, established that *no detectable Na^+ entry was observed over this same time period*!

In an effort to gain more quantitative insight into this ion transport process, we have compared an egg PC vesicle dispersion (made in the presence of NaCl) with a similar dispersion that was allowed to reach 100% Na^+ entry. Thus, analysis of a mixture that was made from 1.5 mL of an egg PC dispersion (prepared in 100 mM NaCl) and 1.65 mL of a shift reagent solution that was 73 mM in NaCl revealed an internalized Na^+ content that was 2% of the total amount. If this percentage is corrected for the small difference between the internal and external Na^+ concentrations that results from a dilution by the shift reagent (that is, actual internal and external concentrations are 100 and 86 mM, respectively), then the volume of the aqueous phase that has been captured by these 1000-Å vesicles is 1.7% of the total. On the basis of the final lipid concentration that is present (10.2 mM), the captured volume is estimated to be 1.7 L/mol, which is in excellent agreement with previous estimates for other 1000-Å-diameter unilamellar phosphatidylcholine vesicles.[18,19] Two similar egg PC dispersions that contained 2 mol % of **1**, which were allowed to reach 100% Na^+ entry (see Experimental Section), encapsulated very similar percentages of the total Na^+ ion content. If one assumes that all of these vesicles are active toward ion transport and that the Na^+ concentration gradient has been

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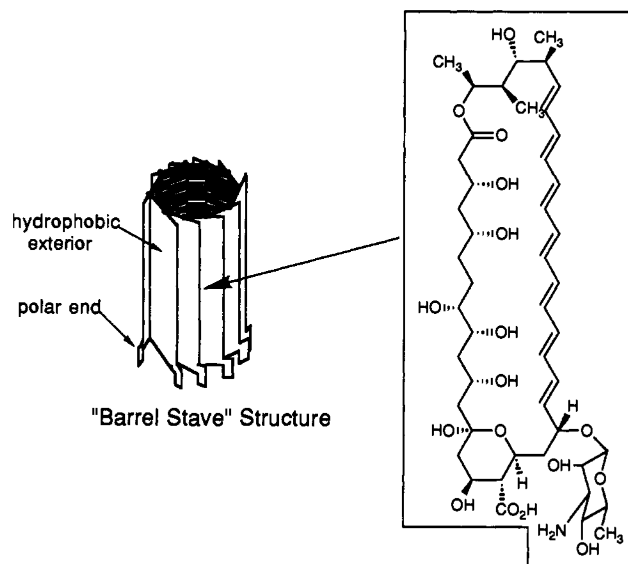
eliminated, then the apparent captured volumes for the two dispersions were 1.7 and 1.9 L/mol. From these results, we conclude that Na^+ entry throughout these channelized dispersions is extensive.

Sterol Conjugate Remains Fully Bound to the Lipid Membrane Throughout Ion Transport. A related question that was of interest to us was whether or not the sterol conjugate remains fully bound to the lipid membrane throughout the transport experiment. In order to answer this question, we carried out the following additional experiment: A "channelized" egg PC dispersion (2 mol % of **1**) that had reached 100% Na^+ entry (as determined by ^{23}Na NMR spectroscopy) was mixed with an equal volume and equal concentration of a similar egg PC dispersion, but one that was devoid of ionophore. These latter vesicles were, in fact, the "control" vesicles that were used to demonstrate that Na^+, in the absence of ionophore, does not enter egg PC vesicles; that is, Na^+ was present only in the exterior bulk phase. If the ionophore were fully bound to the original "channelized" vesicles, and if its on/off rate was very slow, then the internal to external Na^+ ratio should be reduced by a factor of 2 since the "internal" (vesicular) Na^+ would, in effect, be diluted by a factor of 2. If, however, the ionophore were free to migrate between these two vesicle populations, then one would expect that the "control" vesicles would become active toward ion transport and that an "apparent" entry of 50% (after the vesicles are initially mixed) would return to a 100% value. Analysis of the resulting mixture, after 92 h at 25 °C, revealed that the *internal to external Na^+ ratio was, in fact, reduced by a factor of 2.* This result clearly shows that this second population of vesicles, which are devoid of ionophore at the onset of this experiment, remains inactive throughout the entire incubation period. Thus, we conclude that the ionophore is fully bound to egg PC and that the ionophore's on/off rate is slow.

Ion Channels versus a Mobile Carrier Mechanism. Although **1** was expected to create ion channels, an alternate explanation for its ionophoric activity is that it functions as a "mobile carrier".[20] Specifically, it is possible that this sterol conjugate binds Na^+ at the outer surface of the vesicle, "carries" it to the inner monolayer leaflet, and then releases it to the vesicle interior. In order to distinguish between these two mechanisms, we have compared the activity of **1** in fluid-phase egg PC vesicles with that found for *gel*-phase vesicles derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, DPPC (Figure 2). The fact that the activity of **1** is very similar in both membrane types, together with the fact that transverse movement across a lipid bilayer is greatly retarded in the gel phase, constitutes compelling evidence that the sterol conjugate promotes ion transport via channel formation and not by a carrier mechanism.[7a,20-22]

What kind of ion channel structures are being formed by **1**? At present, we can only speculate. For AmpB, the popularly held view is that "barrel stave" type of structures are formed, where the hydroxylated face of each macrolide points inward toward a water-filled pore and the hydrophobic polyene segments point outward toward the hydrocarbon portion of the bilayer.[10] It is also presumed that two such pores align themselves across the bilayer (one buttressed against another in an adjoining monolayer), such that there is contiguous pore extending across the bilayer. The strong dependency of ion transport 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 serves as the primary "port of



entry". Specifically, if one assumes that the observed first-order rate constant (k_{obsd}) reflects pseudo-first-order kinetics, where the influx of Na^+ requires passage through a pore that is composed of *n* sterol molecules, then k_{obsd} is equal to the product of the pore concentration and the rate constant that characterizes the influx of Na^+ through such pores (eqs 1 and 2). If the pore concentration

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

$$k_{obsd} = k_1[\text{pore}] \quad (2)$$

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

$$k_{obsd} = k_1[\text{sterol monomer}]^n/K \quad (4)$$

is expressed in terms of the sterol monomer concentration and the dissociation constant (K) that defines the aggregate (pore)–monomer equilibrium (eq 3), then k_{obsd} should vary with the sterol monomer concentration to the *n*th power (eq 4). If the sterol conjugate exists primarily in the monomeric form, then the sterol monomer concentration can be approximated by its *analytical* concentration (i.e., the total concentration of sterol conjugate that is present in the dispersion), and k_{obsd} will vary with respect to the analytical concentration to the *n*th power. If the sterol conjugate were extensively aggregated, however, then the concentration of pores is expected to be directly proportional to the analytical concentration of the sterol conjugate. The fact that doubling the concentration of **1** results in a ca. 10-fold increase in the rate of Na^+ entry implies, therefore, that most of the sterol conjugate is in the monomeric form and that each pore is composed of ca. three to four ionophores. The involvement of clusters of **1** in the transport of Na^+, together with its structural similarity to AmpB, lead us to suggest a similar "barrel stave" type of model. Whether these channels, and also those that are produced from AmpB, are flatter and less cylindrical in character than what is illustrated herein, however, remains to be established. We note, in this regard, a semantic problem that sometimes arises with the use of the term *channel*. Other researchers who have discussed this issue have pointed out that "the structure of gramicidin channels makes one associate a tunnel shape with the term 'channel'. The gramicidin channel is an excellent channel, but it is not the only one and should be regarded as an example rather than a definition. The present channel may be flatter and more like, as the dictionary says, a 'groove' or 'trench'".[7c]

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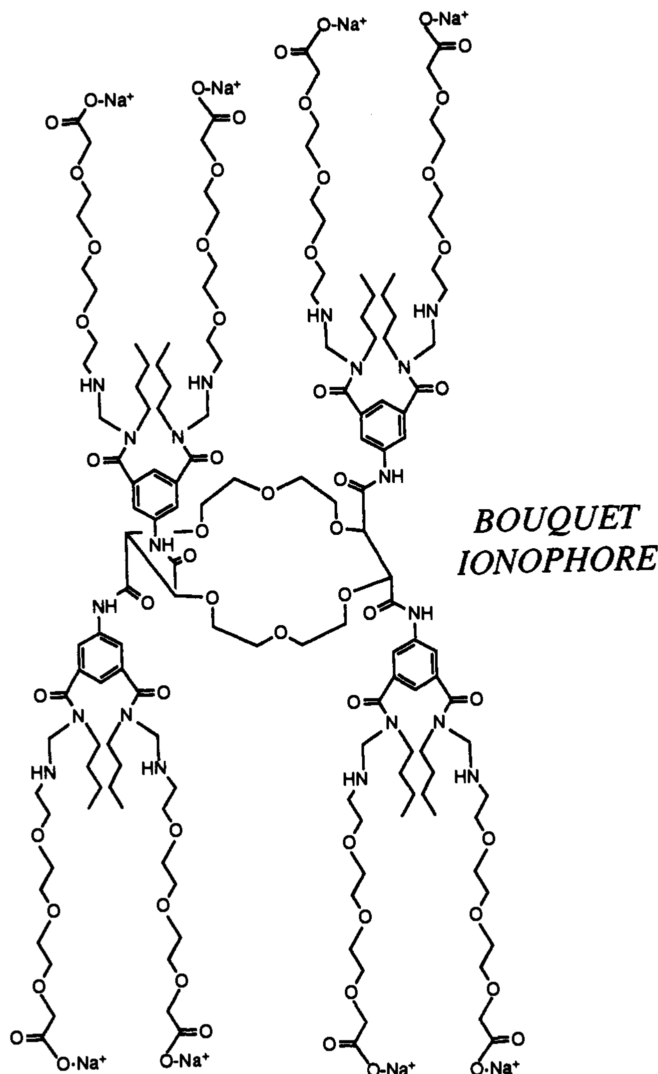
(21) Voet, D.; Voet, J. G. *Biochemistry*; John Wiley & Sons: New York, 1990; p 489.
(22) Inclusion of 2 mol % of **1** in DPPC membranes had no significant effect on the phospholipid's melting behavior, as judged by high-sensitivity differential scanning calorimetry.

Comparison of 1 with a Bouquet Molecule and Amphotericin B. In order to put the ion transport activity of 1 into perspective, we can compare it, operationally, with a recently reported *Bouquet* ionophore, as well as with AmpB itself.[7a] The *Bouquet* structure is unique among this set of ionophores in the sense that it has the potential for extending across an entire phopholipid bilayer.



Since the experimental conditions that we have used in the present study were very similar to those that were used to investigate this *Bouquet*, a direct comparison is possible. Thus, incorporation of ca. 0.5 mol % of the *Bouquet* (ca. 2 mol % on the basis of pairs of pendant polyether groups) into egg PC vesicles afforded an apparent first-order rate constant of ca. 0.06 h^{-1} for Na^+ entry. This value is, in fact, slightly less than the apparent first-order rate constant for Na^+ entry that we have found using 2 mol % of 1, i.e., $k_{obsd} = 0.14\ h^{-1}$ (Figure 3). In contrast, inclusion of 1 mol % of AmpB into egg PC vesicles results in an apparent first-order rate constant of ca. 0.72 h^{-1} (Figures 3 and 4). Thus, from an operational standpoint, 1 and the *Bouquet* possess similar activity in promoting Na^+ transport across phospholipid bilayers, and both are significantly less active that AmpB.

The fact that the activity of 1 for inducing Na^+ entry into egg PC vesicles compares favorably with a more elaborate *Bouquet* ionophore is significant. It clearly demonstrates that much simpler surfactant structures, which resemble AmpB, can produce ion channels in phospholipid membranes. Thus, it lends strong support for our Ockham's razor approach to the design and synthesis of amphotericin B mimics.[23] In principle, the use of a

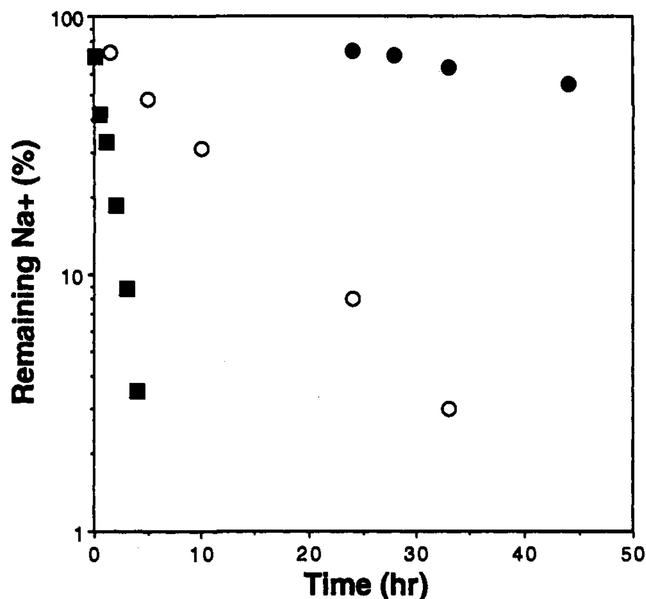
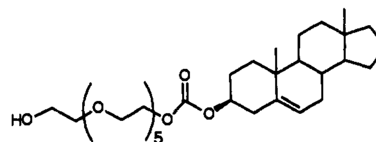


Figure 3. First-order plot of percentage of Na^+ that remains to enter egg PC vesicles containing 2 mol % of 1(○), 1 mol % of 1 (●), and 1 mol % of amphotericin B (■).

sterol nucleus as a hydrophobic framework should provide considerable flexibility in fine-tuning the activity and membrane selectivity of such ionophores. One can envision, for example, that by varying sterol stereochemistry (e.g., *cis* versus *trans* A/B ring fusion), one may be able to control the effective size of a resulting pore. The fact that AmpB can distinguish between cholesterol-rich versus ergosterol-rich bilayers further suggests that some degree of membrane selectivity may also be possible by adjustment of sterol stereochemistry and composition.[4] Moreover, the incorporation of moieties that promote self-association within the bilayer (e.g., hydrogen-bonding functionality) should result in increased ionophoric activity by converting a higher percentage of the sterol conjugate that is present into pore structures. In a simialr vein, the inclusion of proton-ionizable nitrogen atoms and amide functionalities at various positions along the hydrophilic segments offers additional possibilities for

(23) In principle, a single-chain analog of 1 (e.g., 5-androsten-3β-oxycarbonylhexaethylene glycol, shown) might also be expected to exhibit



ionophoric activity. Since poly(ethylene glycol)s have sufficient hydrophobicity to be surface active,[24] it is possible that some fraction of such *membrane-anchored* polyethers may be "drawn" into the bilayer, through hydrophobic interactions with the sterol and/or neighboring lipids. Alternatively, all of the polyethers could reside at the hydrocarbon–water interface. It was, in fact, based on this uncertainty that 1 was specifically chosen as a prototype for detailed study; that is, 1 was viewed as a simpler mimic of AmpB from a mechanistic standpoint. Here, strong hydrophobic interactions of the sterol with neighboring lipids should "drag" at least one of the polyether groups into the bilayer; insertion of a second (covering the remaining face of the sterol) was considered less likely since the overall hydrophobicity of the polyether–sterol conjugate in an assembled state would be significantly reduced. In preliminary studies, we have found that 5-androsten-3β-(oxycarbonyl)-hexaethylene glycol (2 mol %) showed *no ionophoric activity over a 20-h period*. This result lends strong support to the notion that one of the polyether groups of 1 is "dragged" into the bilayer. Control experiments that have been carried out using 1 mol % of hexa(ethylene glycol), and also the parent sterol of 1 (5-androstene-3β,17β-diol), showed no ionophoric activity over a 24-h period. Similarly, 5-androsten-3β-ol (and phenol, which was also tested for calibration purposes) showed no activity.

(24) Shuler, R. L.; Zisman, W. A. *J. Phys. Chem.* **1970**, *74*, 1523. Kawaguchi, M.; Komatsu, S.; Matsuzumi, M.; Takahashi, A. *J. Colloid Interface Sci.* **1984**, *102*, 356.

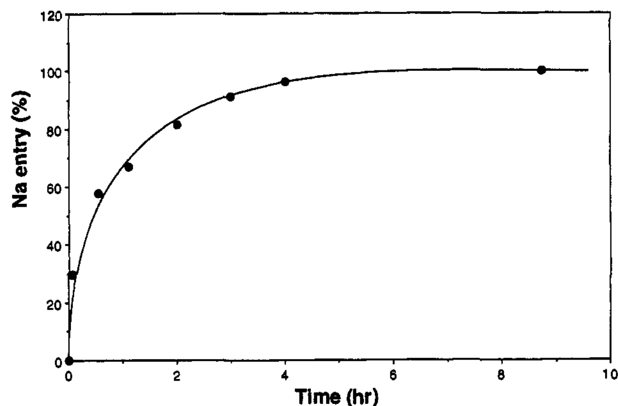


Figure 4. Plot of Na^+ ion entry into egg PC vesicles containing 1 mol % of AmpB as a function of time at 25 °C.

modulating activity and selectivity.[25] In our view, sterol-based ionophores represent a rich new area that warrants detailed exploration and one that holds considerable promise for the creation of novel classes of antimicrobial agents.[26]

Acknowledgment. We are grateful to Dr. Francois Kezdy (The Upjohn Co.) for valuable discussions.

(25) de Wolf, F. A.; Staffhorst, W. H. M.; Smits, H. P.; Onwezen, M. F.; Kruijff, B. D. *Biochemistry* **1993**, *32*, 6688.

(26) These efforts have now intensified as a result of the recent (and for us astonishing) report of the isolation of squalamine from tissues of the dogfish shark, *Squalus acanthias*. This sterol–spermidine conjugate has been found to possess potent antimicrobial activity against a remarkably broad spectrum of microorganisms. While its mechanism of action remains to be clarified, we regard its resemblence to **1** as stunning. On that basis alone, we hypothesize that squalamine functions as a *naturally-occurring sterol-based ionophore*, where its insertion into the cytoplasmic membrane is assisted by partial or complete deprotonation of the spermidine moiety. To our chagrin, we suspect that the dogfish shark may be a bit further along with the synthesis of AmpB equivalents than we are: 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.