Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 10, pp 1893–1901, 1997
© 1997 Elsevier Science Ltd
All rights reserved. Printed in Great Britain
PII: S0968-0896(97)00085-0
0968-0896/97 $17.00 + 0.00

The Design, Synthesis and Transmembrane Transport Studies of a Biomimetic Sterol-Based Ion Channel

Anthony D. Pechulis,[a,†] Richele J. Thompson,[a] John P. Fojtik,[a] Herbert M. Schwartz,[a] Carol A. Lisek[b] and Leah L. Frye*[,a,‡]

[a]*Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180, U.S.A.*
[b]*Searle, Skokie, IL 60077, U.S.A.*

Abstract—A model sterol-based ion channel was rationally designed and synthesized. The potential ion channel is comprised of a tartrate-derived crown ether to which six steroids are appended. Macromolecule **1a** was incorporated into phospholipid vesicles and shown to facilitate the transmembrane transport of sodium and lithium ions using alkali metal NMR spectroscopy. © 1997 Elsevier Science Ltd.

Introduction

The control of membrane permeability is of fundamental importance to biological systems. Ions do not readily diffuse through the phospholipid bilayers that surround cells and yet they play essential roles in such physiological processes as the transmission of nerve impulses, the control of muscular function, protein biosynthesis, and certain enzymatic transformations. Biological systems have evolved two general strategies for the selective transport of metal ions across cellular membranes: ion carriers and ion channels.[1] Of the two, ion channels are the more prevalent. However, less is known about the important aspects of ion translocation via the channel mechanism than is known about carriers. This may be a result of the fact that model ion channels are generally larger than carriers since the active channel must span the width of the lipid bilayer for transport to occur.

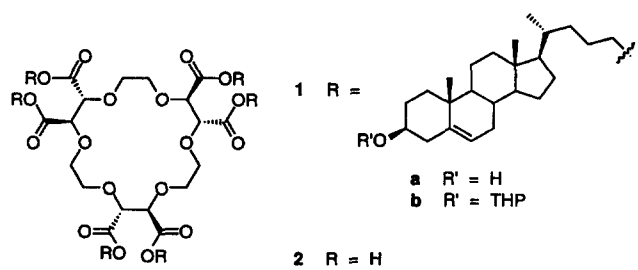
A number of synthetic models of ion channels have appeared in the literature. These include compounds which presumably require aggregation prior to transport[2] as well as a variety of macromolecules designed to act via a unimolecular mechanism.[3–5] This latter group may be better suited for transport rate comparisons, since aggregation should not be a necessary requirement for ion transport to occur and the determination of relative transport constants should be more straightforward.

We wish to report the preparation and evaluation of a unimolecular potential biomimetic ion channel, macromolecule **1a** (Scheme 1), the design of which was based on the well-characterized interaction of sterols with phospholipid bilayers.

Background

Our potential channel consists of an 18-crown-6 to which six steroids are appended via a six-atom linker. The choice of an 18-crown-6 ether as the core of our channel was an obvious one; it provides a metal binding site and, with a cavity diameter of 2.6–3.2 Å, it should allow for the transport of a wide variety of metal cations.[6,7] In addition, appendages of 18-crown-6 systems have been found to adopt 'quasi-axial' orientations,[6] placing them perpendicular to the plane of the crown ether, a situation which is ideal for channel formation.

Analogues of cholesterol were utilized for the walls of the channel. Cholesterol is a natural component of all mammalian cellular membranes and its interaction with the phospholipid bilayer is relatively well understood.[8,9] In the membrane, cholesterol orients itself perpendicular to the bilayer surface with its hydrocarbon region immersed in the interior of the bilayer and its hydroxyl group near the water interface.[10–12] The work of Huang,[13] Lala et al.,[9] Dahl et al.,[9] and Yeagle[9,14]



Scheme 1.

†Current address: Albany Molecular Research, Inc., Albany, NY 12203, U.S.A.
‡Current address: Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT 06877-0368, U.S.A.

suggests that the planar α-face of each steroid is critical for interaction with the side chains of the bilayer's phospholipids. Therefore, it was anticipated that the incorporation of our channel into a lipid bilayer would result in the adoption of a conformation which has the α-face of each steroid directed towards the phospholipid bilayer and the β-face towards the center of the proposed channel. The steroidal nuclei are relatively wide and flat and therefore, if the appropriate number of appendages is chosen, a rigid wall between the bilayer and the interior of the channel would result (Fig. 1). Such a rigid wall should eliminate the possibility of collapse of the macromolecule–cation complex during transport, a mechanism which is more characteristically carrier-like. The choice of a rigid wall unit in our model is in contrast to models reported previously in which either a more flexible branched alkyl chain[4] or a macrocycle is used.[5]

The optimal number of steroidal appendages and the length of the linker between the steroid nuclei and the crown ether were determined using a model study. The model was generated by entering the X-ray crystallographic data for the fully extended form of cholesterol[15] and K^+-tetraamido-18-crown-6[16] into MacroModel. Differing numbers of cholesterol molecules were then docked with the crown ether and placed in a conformation in which the α-faces of the steroids were facing the exterior of the channel. This model study indicated that the arrangement which provides the optimal barrier between the inside and the outside of the channel was one with three steroids equally spaced about the top of the crown ether and three about the bottom. Therefore, the use of a hexasubstituted 18-crown-6 as the core of our macromolecule was indicated, as seen in Figure 2. Ester linkages between the sterol and the crown ether were chosen primarily because of their ease of preparation from known hexaacid **2** (Scheme 1).[17]

In its natural state, cholesterol is immersed in the hydrocarbon region of phosphatidylcholine bilayers with its hydroxyl group 19.5±1.5 Å from the center of the bilayer.[14] Our model study indicated that a four-carbon link between C-17 of the steroids and the oxygen of the ester would yield a distance of 19–20 Å from the central plane of the crown ether to the C-3 hydroxyl of the steroid, as seen in Figures 1 and 2. Thus, with a six-atom linker (four carbons from the steroid side chain and the carbonyl and the oxygen from the ester of the crown ether moiety), the C-3 hydroxyl groups of the steroids should be located at the proper position within the lipid bilayer for hydrogen bonding to the carbonyls of the C-1 chain of the fatty acid side chains to occur.

Channel-facilitated transport, as illustrated in nature by the gramicidins and other transport proteins, is thought to be promoted by the presence of metal ion binding sites. Ion transport presumably proceeds via binding of the ion to the channel followed by 'hopping' from one binding site to the next with the ultimate release of the ion into the medium on the opposite side. The use of steroids as appendages of a model channel allows for the easy access of analogues of compound **1a** possessing additional binding sites within the channel. The

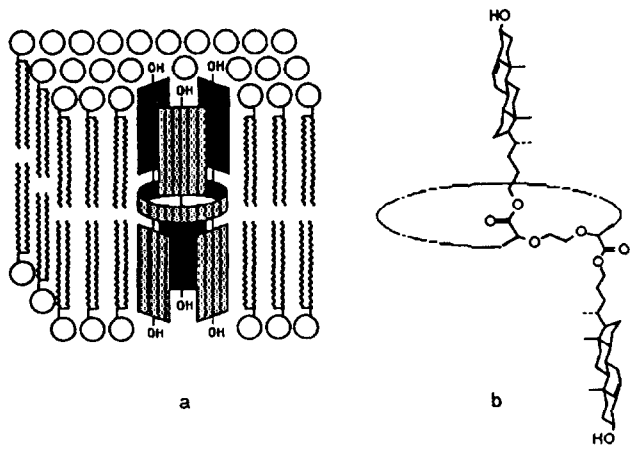


Figure 1. Biomimetic ion channel design: (a) Model embedded in phospholipid bilayer; (b) portion of the channel fully drawn to indicate clearly each component.

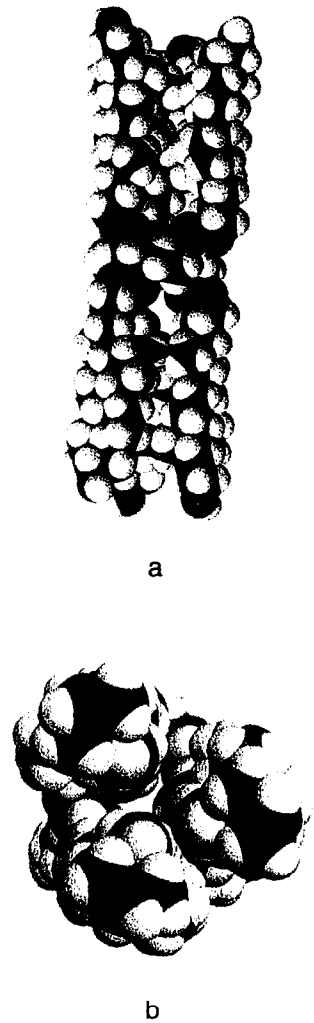


Figure 2. Space-filled model of the ion channel: (a) Long view; (b) top view, as generated from MacroModel.

introduction of functionalities onto the β-face of the steroid would be possible at many locations along its nonpolar region prior to connection of the crown ether. Such modifications on the structure of compound **1a** may eventually provide information as to what functionality is important within the channel and where it is best located.

The most extensively studied natural ion channel, gramicidin A, exists in two different ion conducting forms: the 'channel' form and the 'pore' form.[18–20] The 'channel' form is a helical dimer in which the metal ion binding sites are thought to be 20 Å apart and the 'pore' form is a double helix in which the binding sites are 11.6 Å apart. This information suggested that the simplest version of our proposed ion channel (i.e., compound **1a**) may itself be capable of facilitating metal ion transport across phospholipid bilayers since it was designed so that the C-3 hydroxyl and the crown ether are approximately 19 Å apart.

Results and Discussion

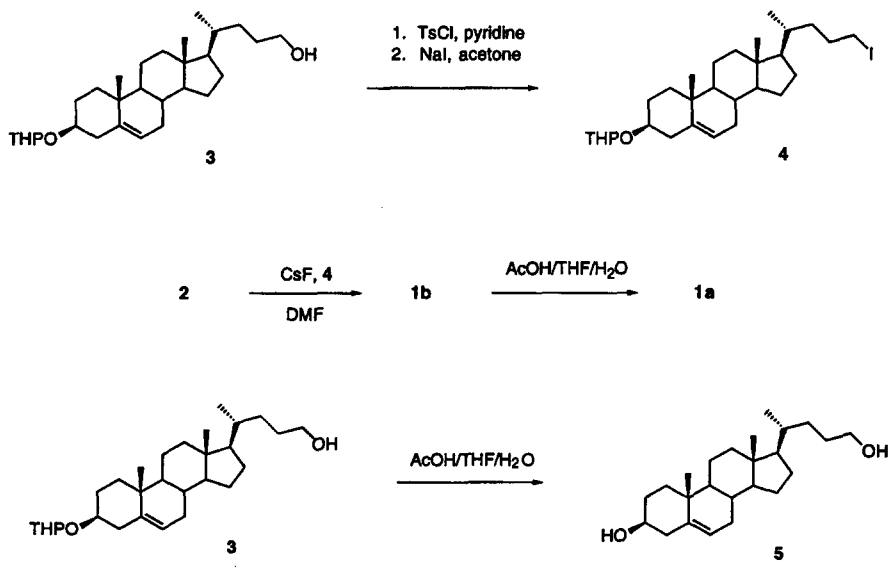
We envisioned the preparation of potential channel **1a** by the alkylation of tartrate-derived hexaacid **2**[17] with an appropriately protected iodosterol in the presence of a suitable base. The requisite 24-iodosterol **4** was prepared from the known C-24 alcohol **3** via the tosylate (84% yield from **3**, Scheme 2). Compound **3** was synthesized from cholenic acid, as previously described.[21,22] Known hexaacid **2**[17] was treated with iodosterol **4** and cesium fluoride in DMF[23] at 40 °C for three days to give protected channel **1b** in 58% yield. Removal of the THP protecting groups with acetic acid:THF:H_2O[24] (4:2:1) at 50 °C provided potential channel **1a** in 82% yield.

Diol **5** was used for a control as described in 'Transport Studies'. Compound **5** was synthesized from C-3-protected sterol **3** using acetic acid/THF/H_2O.

Transport Studies

The ability of compound **1a** to facilitate the transmembrane transport of sodium cations was studied using ^{23}Na NMR spectroscopy.[25] Phosphatidylcholine large unilamellar vesicles (LUVs) were prepared by the reverse-phase evaporation technique of Szoka and Papahadjopoulos.[26] Potential ion channel **1a** was incorporated into the vesicles during the LUV preparation to ensure its incorporation into the phospholipid bilayer. This 'pre-incorporation' was a necessary step since no effect was seen on the addition of compound **1a** to a solution of preformed vesicles. The LUVs were prepared in a buffer containing only lithium and potassium cations.

Once prepared, the LUVs were added to a sodium cation buffer containing dysprosium tripolyphosphate. The dysprosium-based reagent acts as an alkali metal NMR shift reagent. The addition of this reagent to only one side of the vesicle bilayer allows one to distinguish between the sodium cations on the inside of the vesicles from those on the outside by creating a shift differential in the two absorptions of about 8 ppm in the ^{23}Na NMR spectrum. Relative integration of both peaks in the ^{23}Na NMR spectrum served to indicate the percentage of Na^+ present inside ($\%Na^+_{in}$) and outside the vesicles at various time intervals. Approximately 11% of the aq volume was encapsulated by the vesicles, as observed by the addition of a known ion channel (gramicidin) and equilibration followed by integration of the two resulting peaks. Thus, 11% proved to be the maximum



Scheme 2.

(equilibrium) percentage of Na^+ that can be incorporated into the vesicles.[27]

As can be seen in Figure 3, macromolecule **1a** was found to facilitate the transmembrane transport of sodium cations across the lipid bilayer of the LUVs. The effect is clearly dependent on the concentration of the potential ion channel **1a** incorporated into the vesicles. The graph of samples containing 2.00 mol% and 0.30 mol% of compound **1a** (based on the moles of lipid used) tended to plateau between 10 and 11% Na^+_{in}. The sample containing 0.01 mol% of compound **1a** had not yet reached a plateau after 100 h. At a concentration of 2.00 mol%, approximately 10 h were required to reach half of the maximum value of $\%Na^+_{in}$, whereas at 0.01 mol%, approximately 70 h were required.

It should be noted that no Na^+ transport was observed for vesicles prepared in the absence of macromolecule **1a**, as seen in Figure 3. Furthermore, the fact that the $\%Na^+_{in}$ for samples containing larger concentrations of channel plateaus without falling off indicates that there is little or no movement of the shift reagent through the membrane or the potential channel.

Control Studies

We were also interested in determining the importance of various components of potential ion channel **1a**. Incorporation of diol **5** into vesicles did not result in any transport of Na^+ well beyond the time frame of the study. Diol **5** is a sterol with a structure similar to the appendages of the potential biomimetic ion channel **1a**, as well as polarity which is very similar to that of the channel. The control study using diol **5** served two purposes. Most importantly, it illustrated that preincorporation of a compound that is similar in polarity and structure to the potential biomimetic ion channel does not cause ion transport across the lipid bilayer. Diol **5** lacks the critical crown ether, the ionophoric portion of the potential ion channel, and since diol **5** did not transport, this lends support to the validity of the channel design. Also, since diol **5** had been added to the lipids as a solution in THF, we were confident that a THF/sterol complex would not cause ion translocation. It should be noted that, as in all transport experiments, a solution of gramicidin was added to the vesicle suspension after an adequate period of time to confirm the presence of vesicles. Indeed, on addition of gramicidin to the vesicle suspension, the $\%Na^+_{in}$ increased rapidly to the previously determined maximal value of 11%.

Further control studies, as plotted in Figure 4, indicate that the steroid portion as well as the hydroxyl functionality are both critical for transport to occur in our experiment. Both 18-crown-6 and the hexaacid intermediate **2** were incorporated into the vesicle suspension at a concentration of 1.0 mol% in the same fashion as was the potential ion channel **1a**. Both showed a much slower rate of transport over the time period of the study. Specifically, after 100 h, both crown ethers facilitated the transport of sodium at levels well below 4%, while at the same time interval, the incorporation of the potential ion channel **1a** at 0.01 mol% (100-fold lower concentration) caused sodium transport at over 6% (Fig. 3). Furthermore, the incorporation of the fully protected form of the ion channel (**1b**) caused transport to occur at levels significantly lower than those observed for the potential ion channel **1a**, but consistently greater (at least 1.5-fold) than observed for the hexaacid **2**. This study implies that the steroid backbone enhances transport, however, the presence of the THP groups somehow impedes transport. This may be an effect of the bulky nature of this protecting group or its nonpolar nature. This series of controls is evidence that each component of our potential ion channel is important for transport to occur across the bilayer of phospholipid vesicles.

Data Analysis

In order to further analyze and effectively compare the data, all samples were normalized to take into account any variances in vesicle sizes from one batch to the next. Thus, at the end of each run, gramicidin was added at a concentration of 0.001 mol% and the samples were allowed to equilibrate. Since gramicidin rapidly equilibrates the system,[28] it was assumed that analysis of the ^{23}Na NMR spectrum after equilibration would provide a

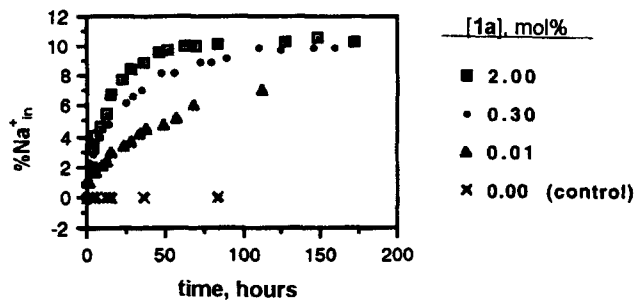


Figure 3. Plots of growth of $\%Na^+_{in}$ over time resulting from the presence of potential ion channel **1a** in vesicles. Concentrations of compound **1a** (based on the moles of lipid used) are indicated.

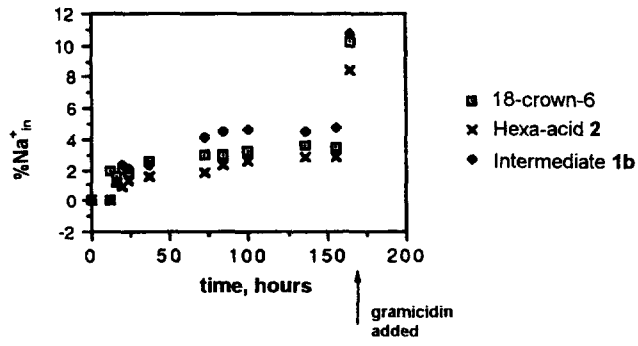


Figure 4. Plots of growth of $\%Na^+_{in}$ over time resulting from the presence of 18-crown-6, hexaacid **2**, and fully protected channel **1b** in vesicles, at concentrations of 1.0 mol%.

value for the maximal $\%Na^+_{in}$. The effect is seen in Figure 4, where on the addition of gramicidin, the value for $\%Na^+_{in}$ increases dramatically. All values obtained for $\%Na^+_{in}$ inside the vesicles at any given time were corrected by dividing by the maximal $\%Na^+_{in}$ and multiplying by 100. Variations in vesicle size have been noted by Lehn, but have not been addressed in any literature data analysis of which we are aware. This normalized $\%Na^+_{in}$ was subtracted from 100% for each datum point to provide $\%Na^+_{corr}$, a value for the percentage of Na^+ that will enter the vesicles, but that has not yet entered. Such a data analysis is necessary since the initial rate of Na^+ transport depends on the concentration of Na^+ outside of the vesicles. The data were then plotted on a log scale vs. time, as shown in Figure 5. It is clear from Figure 5 that the transport of Na^+ mediated by compound **1a** exhibits first-order kinetics.[29]

The value of the rate constant k may be obtained from the slope of each line (the slope is $-k$).[29] The values for k at various concentrations of compound **1a** are listed in Table 1. The lipid concentration for each sample was 8.96 μmol mL^{-1} (6.81 mg mL^{-1}).

Comparison of the data in Table 1 with literature values suggests that potential ion channel **1a** causes ion translocation at a rate between 10 and 50 times more slowly than that reported by Lehn et al. for the 'bouquet' macromolecules.[4] For example, at a channel to lipid molar ratio of 0.66 (μmol L^{-3} mg^{-1} mL^3) (corresponding to 0.05 mol% channel in Table 1) the k value was 2.57×10^{-4} min^{-1} (0.0154 h^{-1} in Table 1), whereas Lehn reports a k value of 51×10^{-4} min^{-1} observed at a channel to lipid ratio of 0.42 μmol L^{-1} mg^{-1} mL^3. This comparison is relatively straightforward, since Lehn also uses a modified ^{23}Na NMR technique. However, it should be noted that for Lehn's polyether-containing 'bouquet' macromolecule, transport was not dependent on macromolecule concentration. In a further comparison, the potential ion channel **1a** appears to cause transport approximately 1000 times more slowly than that reported for similar compounds synthesized by Fyles et al.[5] It should be noted that Fyles uses the pH Stat method of data collection, very different from our own method. This makes any comparison of the two results cumbersome and problematic.

Table 1. The rate constant k for a variety of potential ion channel **1a** concentrations

[channel], mol%	k, h^{-1}
2.00	0.0237
1.00	0.016
0.30	0.0130
0.10	0.0087
0.05	0.0154
0.04	0.0079
0.01	0.0047

Li^+ Transport Studies

The transport mechanism was probed further by observing any movement of the counterion, Li^+, to the outside of the vesicles. The Li^+ absorption is shifted upfield in the presence of the dysprosium-based shift reagent in the same manner as described for Na^+. The gradient for Li^+ is in the opposite direction as compared with Na^+, that is a larger initial concentration of Li^+ exists inside the vesicles. As in the ^{23}Na NMR studies, integration of the two ^7Li NMR peaks provides $\%Li^+_{in}$ at any time interval. This value of $\%Li^+_{in}$ was then subtracted from the initial value of $\%Li^+_{in}$ to provide an adjusted value for the $\%Li^+_{in}$ ($\%Li^+_{adj}$).

Next, $\%Na^+_{in}$ and $\%Li^+_{adj}$ were multiplied by either the total moles of Na^+ used or the total moles of Li^+ used in the sample (37.84 μmol or 70.60 μmol, respectively) to provide values for the total moles of Na^+ transported into and the total moles of Li^+ transported out of the vesicles, over time. Such an analysis provides a direct comparison between the Na^+ transported into the vesicle and the Li^+ transported out of the vesicle. These values, plotted together in Figure 6, clearly show that

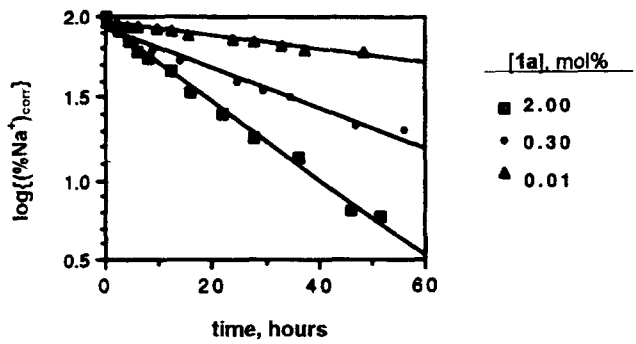


Figure 5. First-order plot of log (% Na^+) vs time, resulting from the presence of potential ion channel **1a** in vesicles. Concentrations of compound **1a** are indicated.

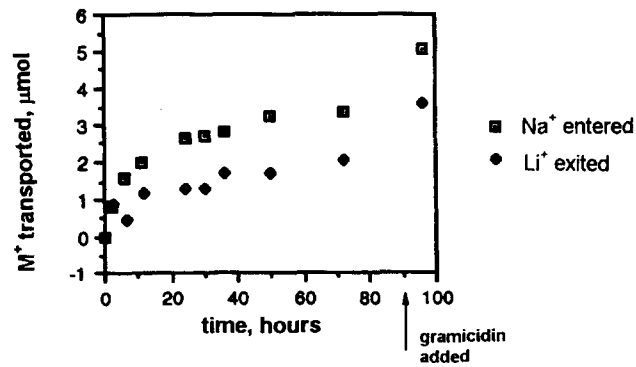


Figure 6. A direct comparison of the amounts of Na^+ and Li^+ transported through vesicles containing compound **1a** and initially containing LiCl in the internal solution and LiCl and NaCl in the external solution. Initially, $[Li^+]_{in} > [Li^+]_{out}$.

the transport of Na^+ resulting from the presence of the potential ion channel is coupled to a counter-transport of Li^+ to the external solution. Such a coupling of transport of charged species maintains electroneutrality across the membranes of the vesicles, and has been documented with one other synthetic ion channel.[6] The difference in amounts of Na^+ entered and of Li^+ exited in our case may be attributed to a number of mechanisms. For example, Li^+ may be co-transported with H^+, or Na^+ may be co-transported with Cl^- in order to maintain electroneutrality across the vesicle membrane.

Conclusions

A rationally designed potential biomimetic ion channel was synthesized and its ability to transport sodium and lithium ions across the membranes of phospholipid vesicles was shown. The design of the compound lends itself to the synthesis of analogues bearing additional ion binding sites, the study of which may ultimately shed some light on the relationship between the structure and the function of ion channels.

We found the alkali metal NMR technique using the localized dysprosium-based shift reagent to be the most convenient and straightforward method for the determination of ion transport. The isolation and observation of both internal and external ion signals has a major benefit over other methods which measure a change occurring on only one side of the membrane: the integrity of the vesicles is never in question, therefore, vesicle rupture cannot be confused with ion transport. Using this technique, it is possible to observe the movement of different ions in the same sample.

The relatively slow rate observed for sodium ion translocation by potential ion channel **1a** may be a result of the known interactions between crown ethers and alkali metal cations. The 18-crown-6 ether was chosen as the core of potential ion channel **1a** because of its ionophoric properties. However, the notable stability of 18-crown-6–metal cation complexes[30] may, in fact, impede transport. Specifically, if the complex between the sodium ions and the crown ether portion of potential ion channel **1a** is overwhelming, the channel may become 'blocked' and bulk movement of ions will be slowed. Syntheses and transmembrane transport studies of analogues of compound **1a** bearing various types of ionophoric constituents with a diverse array of ion association constants may eventually allow scientists to determine what type of correlation exists between ion association properties and transport phenomena.

Experimental

Synthesis

^1H NMR spectra were obtained on a Varian Unity-500 (500 MHz) NMR spectrometer with tetramethylsilane as an internal reference for samples dissolved in $CDCl_3$. ^{13}C NMR spectra were obtained on a Varian Unity-500 (125 MHz) NMR spectrometer, and referenced on the middle peak of the solvent, deuterochloroform, at 77 ppm. Infrared spectra were recorded on a Perkin–Elmer 298 spectrometer. Direct insertion probe (DIP) chemical ionization or electron ionization mass spectral data were obtained on a Hewlett–Packard HP 5087 GC-MS. Mass spectral data for compound **2** were obtained by Dr Carol Lisek of Searle. The sample was added to a lithium 3-NBA matrix; the matrix was prepared by adding a small amount of solid LiCl to 3 mL of 3-nitrobenzylalchohol. One drop of water was added and the mixture vortexed. If solid LiCl remained, the supernatant was decanted and used as the matrix. If no solid remained, additional LiCl was added until the solution was saturated after vortexing; the supernatant was then used as the matrix. Desorption of molecular ions was induced by fast ion bombardment, using 35 KV Cs^+; mass spectral data for compounds **1a** and **1b** were obtained by Joseph Lech, Rutgers University, NJ, using a similar procedure. Melting points were obtained on a Thomas Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses were performed by Quantitative Technologies, NJ or Atlantic Microlab, GA. Optical rotations were recorded in a 0.1 dm cell on a Perkin–Elmer 241 polarimeter.

5-Cholenic acid was obtained from Steraloids and used as received. All other reagents were purchased from Aldrich Chemical Company and were used as received unless otherwise indicated. THF and Et_2O were distilled from sodium/benzophenone ketyl. DMF was distilled from BaO at reduced pressure. Pyridine was distilled from KOH. Acetone was dried by standing over anhyd Na_2SO_4. Silica gel (EM Science Silica Gel 60, 230–400 Mesh) or neutral alumina (Universal Scientific, 32–63) were used for flash chromatography.

24-Iodo-3β-tetrahydropyranyloxychol-5-ene (4). Alcohol **3** (417 mg, 0.94 mmol) was dissolved in dry, distilled pyridine (30 mL) in a round-bottom flask. The solution was magnetically stirred at 0 °C for 15 min, after which time *p*-toluenesulfonyl chloride (536 mg, 2.81 mmol) was added. The reaction solution was stirred for an additional 15 min and allowed to stand at 0 °C for 20 h. The resultant solution was poured over crushed ice (50 g) and the mixture was stirred for 1.25 h. The resultant aq suspension was extracted with EtOAc (3 × 150 mL). The combined organic extracts were washed with satd aq $NaHCO_3$ solution (50 mL) and satd aq NaCl solution (50 mL), dried over anhyd Na_2SO_4, and filtered. The solvent was removed in vacuo and the flask was evacuated overnight to remove residual pyridine. The primary tosylate was provided as a yellow solid, which was used without further purification. ^1H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, J = 8 Hz, 2H, tosylate Ar H), 7.34 (d, J = 8 Hz, 2H, tosylate Ar H), 5.34 (m, 1H, C-6 H), 4.71 (brs, 1H, THP methine H), 4.70–3.89 (m, 3H, THP CH_2O and C-24 H), 3.53–3.45 (m, 2H, THP CH_2O and C-3 H), 2.45 (s, 3H, tosylate CH_3), 1 (s, 3H, C-19 H), 0.85 (d, J = 5 Hz, 3H, C-21 H), 0.65 (s, 3H, C-18

H); MS (CI/isobutane) *m/z* 600 (M+1, 0.15%), 497 (M+1−THPOH, 3.2%), 157 (tosylate).

The crude tosylate was dissolved in dry acetone (35 mL). Sodium iodide (1.41 g, 9.38 mmol) was added as a solid followed by a catalytic amount of *N,N*-diisopropylethylamine (0.029 mL, 0.17 mmol). The resultant yellow solution was stirred at room temperature for 20 h. The solvent was removed in vacuo. The yellow residue was dissolved in EtOAc (250 mL) and the solution was washed with 5% aq $Na_2S_2O_3$ solution (100 mL), satd aq NaCl solution (100 mL), and dried over anhyd $MgSO_4$. Filtration and removal of the solvent in vacuo produced a yellow solid. The crude material was dissolved in CH_2Cl_2, preadsorbed onto SiO_2, and added to a column of SiO_2 packed with hexanes:EtOAc 50:1. Flash chromatography (hexanes:EtOAc 50:1) followed by combination and concentration in vacuo of the fractions containing the least polar component and evacuation of the flask overnight yielded the iodide **4** as a white crystalline solid (439 mg, 0.79 mmol, 84% from alcohol **3**). R_f 0.55 in hexanes/EtOAc on SiO_2; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.36–5.33 (m, 1H, C-6 H), 4.72–4.71 (m, 1H, THP methine H), 3.94–3.90 (m, 1H, THP C$\underline{H}_2$O), 3.54–3.47 (m, 2H, THP C$\underline{H}_2$O and C-3 H), 3.22–3.10 (m, 2H, C-24 H), 1.01 (s, 3H, C-19 H), 0.93 (d, J = 6.6 Hz, 3H, C-21 H), 0.68 (s, 3H, C-18 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 140.91, 140.73, 121.38, 121.30, 96.79, 96.65, 75.84, 75.82, 62.68, 62.59, 56.58, 55.73, 50.01, 49.98, 42.21, 40.11, 39.60, 38.65, 37.31, 37.07, 36.75, 36.64, 36.60, 34.93, 31.78, 31.74, 31.16, 31.13, 30.22, 29.55, 28.09, 27.87, 25.38, 24.14, 20.92, 20.90, 19.93, 19.86, 19.26, 18.65, 11.75, 7.64; IR ($CHCl_3$) 2900 cm^{-1}; MS (CI/isobutane) *m/z* 556 (M+1, 2%), 453 (M+1−THPOH, 50%), 327 (M+1−THPOH-I, 100%); anal. calcd for $C_{29}H_{47}O_2I$: C, 62.79; H, 8.55; I, 22.9. Found: C, 63.10; H, 8.53; I, 22.99; mp 118–125 °C.

(2*R*,3*R*,8*R*,9*R*,14*R*,15*R*)-1,4,7,10,13,16-Hexaoxacyclooctadecane-2,3,8,9,14,15-hexacarboxylic acid hexa-3β-tetrahydropyranyloxychol-5-en-24-oate (1b). Cesium fluoride (201 mg, 1.32 mmol) was weighed into a flame-dried round-bottom flask. After flushing the flask with argon, dry, distilled DMF (50 mL) was added, followed by the 18-crown-6 hexacarboxylic acid **2** (50 mg, 0.095 mmol), as a solid. The resulting cloudy solution was stirred at room temperature for 1 h and then heated to 40 °C over 30 min. Then the steroidal iodide **4** (735 mg, 1.32 mmol) was added rapidly, as a solid. The reaction mixture was stirred at 40 °C for two days and then at room temperature for one day.

The solvent was removed in vacuo and the flask was evacuated overnight to remove residual DMF. The resulting yellow material was dissolved in CH_2Cl_2, preadsorbed onto SiO_2, and loaded onto a column of SiO_2 packed with 20:1 hexanes:EtOAc. The column was eluted with this solvent followed by an increasingly polar gradient. The desired product was eluted with EtOAc. Appropriate fractions were combined, the solvent was removed in vacuo, and the flask was evacuated overnight to provide product **1b** as an off-white solid (171 mg, 55.4 mmol, 58%). R_f 0.35 in 10% MeOH/EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.36–5.33 (m, 1H, C-6 H), 4.72 (brs, 1H, THP methine H), 4.26 (s, 1H, crown methine H), 4.08 (t, J = 7.0 Hz, 2H, C-24 H), 3.94–3.91 (m, 1H, THP C$\underline{H}_2$O), 3.83 (s, 2H, crown methylene H), 3.55–3.47 (m, 2H, THP C$\underline{H}_2$O and C-3 H), 1.01 (s, 3H, C-19 H), 0.93 (d, J = 6.6 Hz, 3H, C-21 H), 0.67 (s, 3H, C-18 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.28, 140.82, 121.46, 121.38, 96.93, 96.80, 80.19, 75.93, 70.80, 65.75, 62.85, 62.77, 56.66, 55.85, 50.09, 42.28, 40.19, 39.70, 38.72, 37.39, 37.16, 36.73, 35.36, 31.86, 31.25, 29.63, 28.17, 27.94, 25.44, 25.09, 24.22, 20.99, 20.05, 19.98, 19.32, 18.58, 11.83; IR ($CHCl_3$) 2900, 1740 cm^{-1}; MS (LSIMS, Rutgers) *m/z* 3095.2 ((M+Li)$^+$, 100%), 3111.1 ((M+Na)$^+$, 35%), 3126.2 ((M+K)$^+$, 25%), 3006.2 ((M+Li−THP)$^+$, 22%), 2990.2 ((M+Li−THPOH)$^+$, 45%); anal. calcd for $C_{192}H_{300}O_{30}\cdot H_2O$: C, 74.24; H, 9.80. Found: C, 73.98; H, 9.88; mp 115–120 °C.

(2*R*,3*R*,8*R*,9*R*,14*R*,15*R*)-1,4,7,10,13,16-Hexaoxacyclooctadecane-2,3,8,9,14,15-hexacarboxylic acid chol-5-en-3β-ol-24-ate (1a). The tetrahydropyranyl-protected hexasteroidal ester **1b** (104 mg, 0.0337 mmol) was dissolved in a solution of AcOH:THF:H_2O (4:2:1) and stirred at 50 °C overnight. The solvent was removed in vacuo. The crude product was dissolved in a minimum amount of CH_2Cl_2 and loaded onto a column of Al_2O_3 packed with 2% CH_3OH/CH_2Cl_2. Flash chromatography with this solvent followed by an increasing CH_3OH gradient eluted the less polar impurities. The desired product eluted at ca. 4–6% CH_3OH/CH_2Cl_2. Appropriate fractions were combined, the solvent removed in vacuo, and the flask was evacuated overnight to provide product **1a** as a white solid (83 mg, 0.032 mmol, 95%). R_f 0.33 in 7:1 CH_2Cl_2:MeOH on SiO_2. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.35–5.34 (m, 1H, C-6 H), 4.26 (s, 1H, crown methine H), 4.09 (t, J = 7.0 Hz, 2H, C-24 H), 3.84 (s, 2H, crown methylene H), 3.54–3.49 (m, 1H, C-3 H), 1.01 (s, 3H, C-19 H), 0.93 (d, 3H, J = 6.6 Hz, C-21 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.34 (C=0), 140.74 (C-5), 121.56 (C-6), 80.17 (CHO), 71.65 (C-3), 70.78 (CH_2O), 65.79 (C-24), 56.69 (C-14), 55.86 (C-17), 50.04 (C-9), 42.28, 42.18 (C-4, C-13), 39.72 (C-16), 37.22 (C-1), 36.42 (C-10), 35.36 (C-22), 31.86 (C-7), 31.82 (C-8, C-20), 31.53 (C-2), 28.17 (C-12), 25.07 (C-23), 24.21 (C-15), 21.01 (C-11), 19.33 (C-19), 18.58 (C-21), 11.83 (C-18) (C identifications made by comparison to cholesterol's ^{1}H NMR spectrum in Sadtler NMR series); IR ($CHCl_3$) 3600, 2900, 1700 cm^{-1}; MS (LSIMS, Rutgers) *m/z* 2589.7 ((M+Li)$^+$, 25%), 2606.7 ((M+Na)$^+$, 100%), 2622.6 ((M+K)$^+$, 55%); anal. calcd for $C_{162}H_{252}O_{24}\cdot H_2O$: C, 74.78; H, 9.84. Found: C, 74.41; H, 9.70; mp 151–154 °C; $[\alpha]^{26}_D$ −8.1 (*c* 0.3, $CHCl_3$).

Chol-5-en-3,24-diol (5). Alcohol **3** (44 mg, 0.099 mmol) was magnetically stirred in a solution of acetic acid:THF:water (4:2:1, 7.0 mL) at 65 °C overnight. The solvent was removed in vacuo. The crude product was preadsorbed onto SiO_2 and loaded onto a column of SiO_2 packed with 5:1 hexanes:EtOAc. Flash chromatography (hexanes:EtOAc 5:1 with a polar gradient

to hexanes:EtOAc 1:1) followed by combination and concentration of the appropriate fractions in vacuo provided product **5** as a yellow solid (22 mg, 61%). R_f 0.56 in hexanes:EtOAc 1:1 on SiO_2; ^1H NMR (500 MHz, $CDCl_3$) δ 5.36–5.35 (m, 1H, C-6 H), 3.64–3.60 (m, 2H, C-24 H), 3.56–3.49 (m, 1H, C-3 H), 1.01 (s, 3H, C-19 H), 0.94 (d, J = 6.5 Hz, 3H, C-21 H), 0.68 (s, 3H, C-18 H); IR 3600, 2900 cm^{-1}; mp 190–193 °C (ref 31 mp 191–193 °C); $[\alpha]^{24}_D$ −33 (*c* 0.39, $CHCl_3$ with two drops CH_3OH) (ref 32 $[\alpha]^{25}_D$ −41.8 (*c* 0.17, $CHCl_3$).

Experimental Details of Transport Studies

Materials, instrumentation, and equipment

The following materials were purchased from Sigma: L-α-phosphatidylcholine (#P-2772, Type XI-E, 100 mg mL^{-1} in $CHCl_3$), gramicidin (#G-5002, 1080 μg mg^{-1}), NaCl (#S-7653), tripolyphosphate, pentasodium salt hexahydrate (#T-5633).

Dysprosium(III) chloride hexahydrate (#20,317-3) was purchased from Aldrich. K_2HPO_4 (anhydrous), KH_2PO_4, and LiCl were purchased from Fisher Scientific. D_2O was purchased from NMR Specialties, PA. Diethyl ether and tetrahydrofuran were distilled over sodium/benzophenone immediately prior to use. All aqueous solutions were prepared with distilled, deionized water from a Barnstead nanopure ultrapure water system.

Sonications were performed with a Vibracell probe sonicator (Sonics & Materials, CT). The evaporation of organic solvents was performed with a rotavap attached to a Gast vane pump, with variable pressure and a pressure gauge. ^{23}Na NMR spectra were obtained on a Varian Unity-500 (500 MHz) NMR spectrometer.

Vesicle preparation

Vesicles were formed using a variation of the reverse-phase evaporation procedure.[26] Phosphatidylcholine (0.21 mL, 28 μmol) in chloroform was added via syringe to a 50-mL round-bottom flask. The solvent was removed in vacuo using a rotary evaporator. The flask was then wrapped in aluminum foil and dried under vacuum at 50 °C. After 4 h the flask was removed from the vacuum and the lipid film was dissolved in dry, freshly distilled diethyl ether (5 mL). The lipid/ether mixture was combined with 1.5 mL of the internal aqueous solution (100 mM LiCl, 0.64 mM K_2HPO_4, and 0.14 mM KH_2PO_4, pH 7.3) in a 16 × 100 mm test tube. The test tube was cooled to 0 °C. An appropriate volume of potential synthetic ion channel **1a** in dry THF (7.73 μmol mL^{-1}) was added to the two-phase system. The resulting two-phase suspension was immediately sonicated in an ice bath, using 1 s pulses for 2 min, at 50% power output level.

After vortexing the sample, it was poured into a 100-mL round bottom flask. The flask was attached to a rotavap and all of the organic phase was removed very slowly in vacuo. The pressure was held at 460 mmHg (as read from the vane pump gauge) for 10 min, without the water bath. As the ether evaporated slowly, the solution began to coat the inside walls of the flask. Then, the pressure was slowly reduced to 260 mmHg and evaporation continued for another 10 min. The pressure was lowered further to 60 mmHg. The flask was then immersed in the water bath at 40 °C. Spontaneous and large bubbling ensued, and the pump pressure needed to be controlled so as not to have the solution foam up into the glass rotavap trap (in many cases, the suspension bumped). After the bubbling stopped, the pressure was kept at 60 mmHg for 15 min. This vesicle preparation was stored at 4 °C until the NMR studies were undertaken.

NMR measurements

All ^{23}Na NMR spectra were recorded at a frequency of 132.218 MHz with d1 = 1.000 s. All ^7Li NMR spectra were recorded at 194.264 MHz with a d1 of 2.00 s. The probe temperature was kept at a constant 25 °C throughout the duration of each experiment, and the samples were not spun. Samples were prepared in a 5-mm NMR tube. The vesicle preparation (0.480 mL) was combined with the shift reagent solution (0.520 mL: 0.64 mM K_2HPO_4, 0.14 mM KH_2PO_4, 2.7 mM $DyCl_3$, 10.8 mM $Na_5P_3O_{10}$, 18.76 mM NaCl, 43.44 mM LiCl necessary to keep the overall ion concentration the same on both sides of the membrane, and 10% D_2O by volume, pH 7.3) immediately prior to obtaining the first spectrum. The tube was inverted twice before lowering into the magnet, and before each spectrum was recorded.

Typically, 240 transients were accumulated per spectrum at a probe temperature of 25 °C. (There was a separation of 8 ppm between inside and outside Na^+ NMR peaks.) A spectrum was taken approximately every 2 h for the first 10 h and every 4 h for two days, and intermittently after that.

In the determination of $\%Na^+_{in}$, the peaks corresponding to Na^+_{in} and Na^+_{out} were integrated using a Gaussian Fit, a program available with the Varian Unity 500 NMR software. The $(Na^+_{in})_{INT}$ was then divided by the sum of $(Na^+_{in})_{INT}$ and $(Na^+_{out})_{INT}$, providing a value for the $\%Na^+_{in}$. Note that gramicidin was added to equilibrate the Na^+ by eliminating the Na^+ gradient. Each batch of vesicles may not be the same. This equilibration, then defining the maximum $\%Na^+_{in}$ as 100% is essentially a correction factor.

Acknowledgements

We thank the National Science Foundation and the National Institutes of Health for support of this project. We would also like to thank George W. Gokel, Oscar Murillo, and Steven L. Regen for helpful conversations regarding the preparation of LUVs and the ^{23}Na NMR experiments.

References

1. For general reviews of ion transport in biological systems, see: *Transport Through Membranes: Carriers, Channels, and Pumps*; Pulman, A., Ed.; Marcel Dekker: New York, 1988. Urry, D. W. *Top. Curr. Chem.* **1985**, *128*, 175. *Membrane Transport in Biology*; Giegisch, G.; Tosteson, D. C.; Ussing, H. H., Eds; Springer: New York, 1978, Vol. 1. Chock, P. B.; Titus, E. O. *Current Research Topics in Bioinorganic Chemistry*; Lippard, S. J., Ed.; John Wiley: New York, 1973; Vol. 18, pp 228–382.

2. (a) Menger, F. M.; Davis, D. S.; Persichetti, R. A; Lee, J.-J. *J. Am. Chem. Soc.* **1990**, *112*, 2451. (b) Kobuke, Y.; Ueda, K.; Sokabe, M. *J. Am. Chem. Soc.* **1992**, *114*, 7618. (c) Stadler, E.; Dedek, P.; Yamashita, K.; Regen, S. L. *J. Am. Chem. Soc.* **1994**, *116*, 6677.

3. Nakano, A.; Xie, Q.; Mallen, J. V.; Echegoyen, L.; Gokel, G. W. *J. Am. Chem. Soc.* **1990**, *112*, 1287. Roks, M. F. M.; Nolte, R. J. M. *Macromolecules*, **1992**, *25*, 5398. Voyer, N.; Robitaille, M. *J. Am. Chem. Soc.* **1995**, *117*, 6599.

4. Pregel, M. J.; Jullien, L.; Lehn, J.-M. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1637. Pregel, M. J.; Jullien, L.; Canceill, J.; Lacombe, L.; Lehn, J.-M. *J. Chem. Soc. Perkin Trans. 2*, **1995**, 417.

5. Fyles, T. M.; James, T. D.; Pryhitka, A.; Zojaji, M. *J. Org. Chem.* **1993**, *58*, 7456. Fyles, T. M.; James, T. D.; Kaye, K. C.; *J. Am. Chem. Soc.* **1993**, *115*, 12315.

6. *Host Guest Complex Chemistry/Macrocycles*; Vogtle, F.; Weber, E., Eds; Springer: New York, 1985. Lehn, J.-M. *Pure Appl. Chem.* **1979**, *51*, 979. Lehn, J.-M. *Pure Appl. Chem.* **1978**, *50* 871.

7. Gokel, G. W.; Koezeniowski, S. H. *Macrocyclic Polyether Syntheses*; Springer: New York, 1982.

8. Chapman, D.; Kramers, M. T. C.; Restall, C. J. In *Sterols and Bile Acids*; Danielsson, H.; Sjovall, J, Eds; Elsevier: New York, 1985, pp 152–159.

9. Yeagle, P. L. *Biochim. Biophys. Acta* **1985**, *815*, 33. Lala, A. K.; Lin, H. K.; Bloch, K. *Bioorg. Chem.* **1978**, *7*, 437. Dahl, C. E.; Dahl, J. S.; Bloch, K. *Biochemistry* **1980**, *19*, 1462.

10. Franks, N. P. *J. Mol. Biol.* **1976**, *100*, 345. Worcester, D. L.; Franks, N. P. *J. Mol. Biol.* **1976**, *100*, 359.

11. Dufourc, E. J.; Parish, E. J.; Chitrakorn, S.; Smith, I. C. P. *Biochemistry* **1984**, *23*, 6062.

12. Yeagle, P. L.; Martin, T. B. *Biochem. Biophys. Res. Commun.* **1976**, *69*, 775.

13. Huang, C. *Chem. Phys. Lipids* **1977**, *19*, 150.

14. Yeagle, P. L. *Biochim. Biophys. Acta* **1985**, *822*, 267.

15. Shieh, H.-S.; Hoard, L. G.; Nordman, C. E. *Acta Cryst.* **1981**, *B37*, 1538.

16. Dock, A.-C.; Moras, D.; Behr, J.-P.; Lehn, J.-M. *Acta Cryst.* **1983**, *C39*, 1001.

17. Dutton, P. J.; Fyles, T. M.; McDermid, S. J. *Can. J. Chem.* **1988**, *66*, 1097.

18. Langs, D. A. *Science* **1988**, *241*, 188.

19. Urry, D. W. *Top. Curr. Chem.* **1985**, *128*, 175.

20. Wallace, B. A.; Ravikumar, K. *Science* **1988**, *241*, 182.

21. Pechulis, A. D.; Bellevue, F. H., III; Cioffi, C. L.; Trapp, S. G.; Fojtik, J. P.; McKitty, A. A.; Kinney, W. A.; Frye, L. L. *J. Org. Chem.* **1995**, *60*, 5121.

22. Morisaki, M.; Shibata, M.; Duque, C.; Imamura, N.; Ikekawa, N. *Chem. Pharm. Bull.* **1980**, *28*, 606.

23. Sato, T.; Otera, I.; Nozaki, H. *J. Org. Chem.* **1992**, *57*, 2166.

24. Corey, E. J.; Danheiser, R. L.; Chandrasekaran, S.; Siret, P.; Keck, G. E.; Gras, J.-L. *J. Am. Chem. Soc.* **1978**, *100*, 8031.

25. Pike, M. M.; Simon, S. R.; Balschi, J. A.; Springer, C. S. Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 810. Stadler, E; Dedek, P.; Yamashita, K.; Regen, S. L. *J. Am. Chem. Soc.* **1994**, *116*, 6677. Pregel, M. J.; Jullien, L.; Canceill, J.; Lacombe, L.; Lehn, J.-M. *J. Chem. Soc. Perkin 2* **1995**, 417. Nakano, A.; Xie, Q.; Mallen, J. V.; Echegoyen, L.; Gokel, G. W. *J. Am. Chem. Soc.* **1990**, *112*, 1287.

26. Szoka, F. Jr.; Papahadjopoulos, D. *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 4194.

27. The encapsulated volume was confirmed by forming the vesicles in the presence of Na^+.

28. On addition of gramicidin at this concentration to a typical batch of vesicles, the $\%Na^+_{in}$ reaches a plateau at 150 min and has a half-life of approximately 10 min.

29. Atkins, P. W. *Physical Chemistry*, 3rd edn; W. H. Freeman: New York, 1986, Chapter 28.

30. deJong, F.; Reinhoudt, D. N. *Stability and Reactivity of Crown Ether Complexes*; Academic: London, 1981.

31. Deno, N. C.; Meyer, M. D. *J. Org. Chem.* **1979**, *44*, 3383.

32. McIntosh, A. V. Jr.; Meinzer, E. M.; Levin, R. H. *J. Am. Chem. Soc.* **1948**, *70*, 2955.

(*Received in U.S.A.* 8 *January* 1997; *accepted* 21 *February* 1997)