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Correlation of bilayer membrane cation transport and biological activity in alkyl-substituted lariat ethers

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Dialkyldiaza-18-crown-6 lariat ethers having twin *n*-octyl, *n*-decyl, *n*-dodecyl, *n*-tetradecyl, *n*-hexadecyl, 1-oxodecyl and 1-oxododecyl side arms were prepared and studied. Cation transport in liposomes mediated by these compounds showed discontinuous activity that correlated with toxicity to the bacteria *E. coli* and *B. Subtilis*, and the yeast *S. Cerevisiae*. Transport, toxicity and membrane depolarization studies all suggest that side chain length affords very different interactions in a bilayer membrane compared with bulk phases. An explanation for activity in terms of carrier transport and restricted transverse relaxation is proposed.

Introduction

Crown ethers have been studied extensively since their discovery nearly four decades ago.[1] Literally thousands of crown ether derivatives[2] have been prepared and their ability to complex cations[3] under equilibrium conditions[4] has been evaluated. In addition, there are numerous reports of cation transport through bulk liquid membranes mediated by crowns of widely varying structures.[5] Sodium and potassium are the two most common cations in solution *in vivo* and agents that complex and alter their natural balance are expected to exhibit biological effects. Indeed, the toxicity of certain crown ethers was recognized shortly after their discovery. Pedersen showed that dicyclohexano-18-crown-6 was toxic to dogs[6] and Hendrixson *et al.* reported a general survey of crown toxicity,[7] but these studies did not address the mechanism of action. Several reports on crown ether toxicity have appeared that studied a group of crown derivatives in a particular context.[8,9,10,11,12] Studies dealing with azacrowns or lariat ethers have also been reported.[13,14] Although Tso and coworkers correlated antibacterial activity and alkali metal ion transport efficiency,[15] general evidence to support the reasonable assumption that crown toxicity relates to ion binding and balance has been lacking.

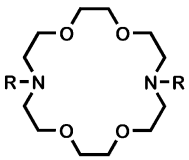
The toxic effect of a crown ether on an organism presumably involves its penetration of a bilayer membrane. Menger *et al.* found that 18-crown-6 did not transport K^+ ions through egg lecithin membranes.[16] Ranganathan *et al*. demonstrated similar results with a series of cyclodepsipeptide carriers containing ethyleneoxy units.[17] Recently though, Figaszewski and coworkers used impedance measurements to show that dibenzo-18-crown-6 can alter the resistance of unilamellar lecithin membranes, allowing for K^+ transport across them.[18] In a previous study conducted by one of us,[23] Na-NMR was used to assess the Na^+ transport ability of a series of ester and amide side chain lariat ethers[19] in liposomes.[20] The studies conducted in phospholipid bilayers showed that these simple lariat ethers rivalled the natural ionophore monensin in efficacy, which suggests that bilayer transport activity may also influence toxicity.

We now report a study involving a series of *N*,*N*′-dialkyldiaza-18-crown-6 ethers that exhibit similar but discontinuous toxicity behavior to *E. coli*, *B. subtilis* and *S. cerevisiae*. Although their binding constants are similar, their ability to release cations from phospholipid vesicles differs dramatically but correlates with the observed toxicity.

Results and discussion

Compounds studied

Eight compounds were prepared for this study. Six of them (**1–6**) are normal alkyl derivatives of 4,13-diaza-18-crown-6 in which the hydrocarbon chains are attached to macroring nitrogen. Compounds **1–3** were obtained by acylation followed by reduction and **4–6** were prepared by dialkylation of diaza-18-crown-6. Diamides **7** and **8** were obtained by acylation of diaza-18-crown-6. The structure of each compound was confirmed by spectral analysis and comparison with literature data. The structures of the compounds studied are illustrated in the adjacent panel.



1: R = C_8H_{17} 5: R = $C_{16}H_{33}$

2: R = $C_{10}H_{21}$ 6: R = $C_{18}H_{37}$

3: R = $C_{12}H_{25}$ 7: R = COC_9H_{19}

4: R = $C_{14}H_{29}$ 8: R = $COC_{11}H_{23}$

Toxicity of dialkyldiazacrowns

The potassium ion transporter valinomycin is probably the best known example of a natural ion carrier.[21] Of course, numerous natural peptides have been identified that conduct ions through membranes[22] and/or are toxic to microbial organisms.[23] The toxicity of such ionophores has been attributed to their ability to mediate unregulated ion transport through the host cell's membrane, disrupting ion homeostasis and causing osmotic and physiologic stress.[24] Previous studies from our laboratory showed that hydraphile ion channels function as antimicrobial agents.[25,26] The initial intent of this study was to assay the activity of *N*,*N*′-dialkyl-4,13-diaza-18-crown-6 compounds **1–6** as controls in the hydraphile channel toxicity project.[27]

Previous studies of *N*,*N*′-didodecyldiaza-18-crown-6, **3**, did not show toxicity to *E. coli* DH5α cells when a disk diffusion method was used.[24] The present study confirms that result. However, additional experiments showed that **3** was lethal to *B. subtilis* and to *S. cerevisiae* at minimum inhibitory concentrations

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Table 1 Toxicity data (MIC values) for dialkyldiaza-18-crown-6 lariat ethers

		Toxicity to organism/μM		
Compound number	Side chain	*B. subtilis*	*E. coli*	*S. cerevisiae*
1	*n*-Octyl	26	206	103
2	*n*-Decyl	2.8	11	2.8
3	*n*-Dodecyl	2.5	360[a]	2.5
4	*n*-Tetradecyl	360	360	360
5	*n*-Hexadecyl	360	360	360
6	*n*-Octadecyl	360	360	360
7	CO-*n*-nonyl	360	360	360
8	CO-*n*-undecyl	360	360	360
Valinomycin	—	50	50	n/d[b]

[a] If no toxic effect is observed below 360 μM, the compound is considered to be inactive. [b] N/d means that a value was not determined for this compound.

(MICs) of 2.5 μM in both cases. An organism is considered *susceptible* to a compound when the MIC is ≤10 μM.[28] Penicillin, for example, is effective at about 8 μM. The significant level of toxicity observed for **3** prompted us to explore the activity exhibited by the family of compounds **1–6**. The data obtained with a Gram-negative bacterium, a Gram-positive bacterium and common yeast are recorded in Table 1.

Hydrophobicity of 1–8

We expected the hydrophobicity of **1–6** to vary systematically and to be greater than for **7** or **8** when an equivalent number of carbon atoms was present. This was confirmed by calculating $\log_{10}P$ by using the AlogP interactive website.[29] The calculated values, shown in Table 2, show that incremental differences between adjacent members of the series is modest. The overall change from **1** to **6** is over a thousand-fold but reflects a difference in side chains from 16 to 36 carbon atoms. We note that the presence of the polar amide function reduced hydrophobicity by about a power of ten (compare **7** and **8** with **2** and **3**).

The hydrophobicity P is defined as the partition coefficient between water and octanol, where the latter approximates the polarity of a membrane. Compounds **1–8** all favor the membrane over water by at least five orders of magnitude. Moreover, the calculated hydrophobicities of **1–6** increase in a nearly linear fashion from dioctyl lariat **1** to dioctadecyl lariat **6**. We had previously studied complexation of dialkyldiazacrowns in methanol solution. When the sidearms were propyl, butyl, hexyl, nonyl or dodecyl, the binding constants ($\log_{10}$ K_S) were all 2.9 ± 0.1.[30] Based on the binding and hydrophobicity data, we anticipated that any change in biological activity would be small and gradual, if not linear.

The surprising discontinuities in toxicity observed in the series **1–6** can be appreciated in the graph presented in Fig. 1. This remarkable behavior is apparent in the activity difference between **3** (C_{12}) and **4** (C_{14}) with both *B. subtilis* and *S. cerevisiae*.

Table 2 Calculated[a] hydrophobicities of compounds **1–8**

Compound number	Side chain	$\log_{10}P$
1	*n*-Octyl	5.54
2	*n*-Decyl	6.74
3	*n*-Dodecyl	7.72
4	*n*-Tetradecyl	8.35
5	*n*-Hexadecyl	8.80
6	*n*-Octadecyl	9.19
7	CO-*n*-nonyl	5.64
8	CO-*n*-undecyl	6.65

[a] (http://146.107.217.178/lab/-alogps/index.html).

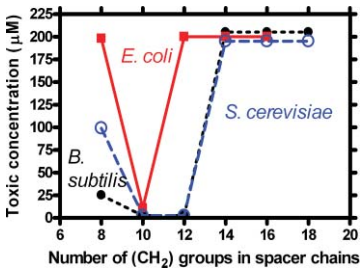


Fig. 1 Toxicity of compounds **1–6** to *B. subtilis*, *E. coli* and *S. cerevisiae*. Maximum toxic concentrations are shown at 200 μM for graphical clarity (see Table 1 for concentration data).

Compound **3** (C_{12} side arms) is active at 2.6 μM against *B. subtilis* and *S. cerevisiae*, whereas **4** (C_{14} side chains) is inactive against either microorganism. In short, similar results are noted for compounds **1–6** with all three organisms. The shortest chain dialkylcrown (**1**, C_8) is modestly active against *B. subtilis*, slightly active against *S. cerevisiae* and inactive to *E. coli*. The 10-carbon side chain compound (**2**) is active against all three organisms. The C_{12} side-chained lariat, **3**, is active against *Bacillus* and yeast but not against *E. coli*. Compounds **4–6** are inactive to all three organisms. The approximately parallel activity of these compounds to different organisms suggests that the effect is real and not an artifact either of the testing method or compound purity. Compound purity was established by standard chemical methods and the sensitivity of toxicity to chain length was confirmed by conducting the biological analysis at least in triplicate in all cases. Further, there was no visual evidence for unexpected differences in solubility or aggregation behavior.

Molecules **7** and **8** were included in this study to assay the effect of a carbonyl group adjacent to the macroring nitrogen. The presence of such a linkage should rigidify the macrocycle. In addition, the donicity of the macroring nitrogen should be reduced owing to its formal positive charge in the enol resonance form. Of course, the amide oxygen becomes a potential extra-annular donor, but when macroring nitrogen is part of an amide residue, binding of cations within the macrocycle is reduced. This expectation is based on previous studies in which aza-15-crown-5 was linked to a cholesteryl ester either in the form ⟨15 N⟩CH_2COO–cholesteryl or ⟨15 N⟩COO–cholesteryl. The equilibrium binding constant for sodium cation complexation in methanol solution in the former case was ~12 000 compared to the urethane-linked compounds: $K < 30$.[30] Despite their overall similarity in structure to the alkyl derivatives, the two macrocycles having amide side chains (**7** and **8**) proved to be inactive to all three organisms tested in these studies.

We assume that compounds **1–6** integrate into the membranes of the microbes. These compounds are amphiphilic and the monomers are known to self-assemble into stable bilayers.[31] We speculate that the active dialkylcrowns can insert into the bilayer and conduct cations as expected for carriers. When the side chain length increases, the crowns become more like amphiphilic membrane monomers than carriers. When a cation is bound in the crown's macroring, transport is impeded in the same fashion as is transverse relaxation of a phospholipid monomer. Storch and coworkers have similarly noted that anthroyloxy fatty acids (AOFA) undergo a 50-fold reduction in "flip-flop" activity when the single chain fatty acid length is increased from C_{12} to C_{18}.[32] Indeed, the reduction in flip-flop activity may be greater in the present case due to the presence of two alkyl chains per lariat ether, rather than one for AOFA. We further surmise that the difference in activity seen for different organisms results from differences in the microbial membranes themselves. Gram-negative *E. coli* possess a membrane, a cell wall and an additional exterior glycomembrane. Gram-positive *Bacillus subtilis* lacks the second membrane, as does the eukaryote *Saccharomyces*. These are different organisms with very different membrane

structures. Even so, there is remarkable consistency in the killing ability of this group of compounds.

Transport studies

The proposed mechanism for toxicity depends on the ability of these compounds to transport ions. We therefore assayed sodium cation release using the ISE method we recently reported.[33] In this assay, sodium chloride containing liposomes were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (DEPC). The liposomes were filtered to ensure uniform size of about 200 nm (see Experimental). An isopropyl alcohol solution of the lariat ether was added and Na^+ release was detected using a sodium-selective electrode. The results obtained in these studies are shown in the graphs presented in Fig. 2. The upper panel shows the concentration dependent release of Na^+ over a concentration range for **3** of 60–360 μM.

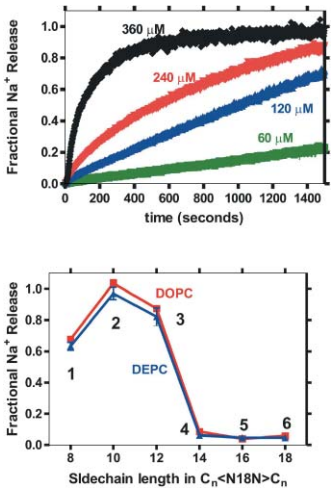


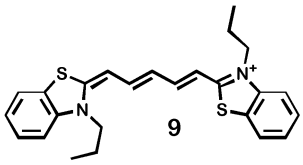
Fig. 2 (Top panel) concentration dependent release of Na^+ from DOPC vesicles mediated by **3**. (Bottom panel) sodium release from DOPC and DEPC vesicles at 1500 s mediated by 240 μM **1–6**.

The lower panel of Fig. 2 shows Na^+ release for compounds **1–6** from either DOPC or DEPC vesicles. The data plotted are the observed release values at 1500 s in the presence of each compound at a concentration of 240 μM. This value was chosen because the electrode is not sensitive enough to respond reproducibly at the very low concentrations that kill the living organisms. We note that the identical transport results observed in DOPC and DEPC liposomes suggest that membrane thickness is not a critical variable in the ionophoretic activity.

The parallel between the results shown in Fig. 2 and the *in vivo* data shown in Fig. 1 is remarkable. In both cases, compounds **4–6** were inactive in their separate contexts. Peak transport activity was observed for **2** (C_{10} side chains), which was the most toxic compound in the study to all three microbes.

Fluorescence studies of membrane depolarization

If the family of compounds presented here is toxic owing to disruption of ion homeostasis, it should be possible to detect the associated membrane depolarization. The membrane dye, 3,3′-dipropylthiadicarbocyanine [$DiSC_3(5)$], **9**, is known to exhibit different fluorescence properties in polarized *versus* depolarized bilayers.[34] If the membrane of an organism depolarizes owing to unregulated ion transport (*i.e.* disruption of ion gradients), the dye will be released and increased fluorescence intensity will be detected. The bacteria *B. subtilis* and *E. coli* exhibit an internal, negative membrane potential[35] and readily absorb **9**. This membrane potential reflects both proton transport and a concentration gradient of K^+ from inside the cell to outside.[36] In short, the dye accumulates in negatively polarized membranes and self quenches.



The experiments reported here were conducted as follows. The fluorescent dye, $DiSC_3(5)$ (**9**, final concentration = 0.5 μM) was added to a cuvette containing buffer solution (0.5 mM HEPES, 200 mM dextrose). This is shown in Fig. 3 as point "A." A rapid increase in fluorescence ("INT" for intensity on the ordinate) was detected. After equilibration (~5 min, "B"), bacterial cells were added to the cuvette, whereupon fluorescence rapidly decreased owing to dye absorption (>90% for *B. subtilis* and >80% for *E. coli*) and stabilized. At point "C," 60 mM KCl is added to the external buffer (see below). Addition of ionophore **2** at point "D" leads to rapid membrane depolarization and increased fluorescence. Essentially identical behavior was observed at this point when **1**, **3**, **4** or valinomycin was used instead of **2**. The cells were destabilized (or perhaps lysed, "E") by treatment with 10% Triton-X solution to obtain a final fluorescence value to which other readings were normalized.

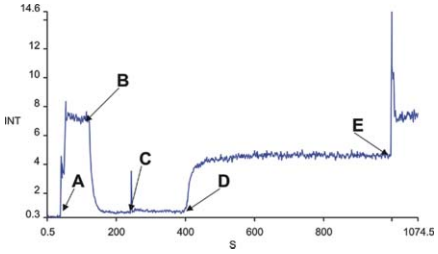


Fig. 3 Membrane depolarization indicated by increased fluorescence of **9** for *B. subtilis*. Fluorescence intensity (ordinate labeled "INT") is shown as a function of time (s). See text for additional details.

In order for the bacterial cell membrane to depolarize, the potassium cation must be at equal concentrations across the cell boundary. Thus, KCl is subsequently added to the cuvette at a final concentration of 60 mM (point "C" in Fig. 3). This concentration effectively equilibrates the K^+ across the cellular bilayer but the bacteria maintain their membrane potential by transport of other ions, *e.g.* protons.[37] The addition of a K^+-ionophore causes the K^+ exchange rate to increase. This, in turn, makes the membrane potential approximately equal across the cell membrane. Thus, the membrane is depolarized to the diffusion potential of K^+, which is zero, and the rapid increase in fluorescence is detected as the dye exits from the cells.

Fig. 4 shows the results of membrane potential depolarization experiments using **1–4** and valinomycin with *B. subtilis* cells.

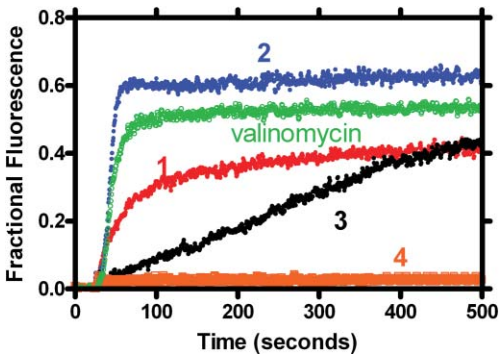


Fig. 4 Membrane depolarization indicated by increased fluorescence of **9** for *B. subtilis* with **1–4** and valinomycin (see text for concentration data).

The increase in fluorescence shown for each compound begins as point "D" in Fig. 3. The presence of **4** causes no change in the membrane potential when used at 10 μM, while **3** shows a linear, steady depolarization over about 10 min at 5 μM. The C_{10} side chained crown, **2**, is the most effective at depolarizing the membrane (2.5 μM). Its influence on the cell, as manifested in these fluorescence experiments, is similar in magnitude and time course to valinomycin (2 μM). Octyl side chained **1** also shows K^+ transport at 2.5 μM.

It is important to note that when K^+ is absent from the external buffer, **1–4** show dye release only through non-specific effects such as membrane disruption. In addition, depolarization does not directly correlate to the level of toxicity. This is evident from the high fluorescence release engendered by valinomycin, which is relatively non-toxic (see Table 1). It is interesting to note that studies using Na^+ in the external buffer elicited results similar to those shown in Fig. 4 (data not shown).

Fig. 5 shows the change in fluorescence for compounds **1–3** and valinomycin on *E. coli.* As noted above, ionophore **2** was the only compound toxic to *E. coli* and surpassed valinomycin in depolarization activity as noted with the *B. subtilis* (Fig. 4). Compound **1** reproducibly shows a small initial increase in fluorescence intensity followed by a decrease in fluorescence. Thus, depolarization of the *E. coli* membrane occurs but the cell apparently counters the weak effects of **1** and repolarizes its membrane to absorb the dye. When the alkyl side chains are two carbons shorter (**1**) or two carbons longer (**3**), the compounds are not toxic and fail to significantly alter the potential of the *E. coli* membrane. Compound **2**, which has decyl side arms, is the best ion transporter in both *E. coli* and *B. subtilis*.

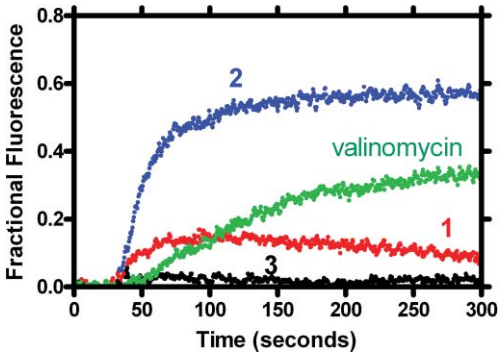


Fig. 5 Membrane depolarization indicated by increased fluorescence of **9** for *E. coli* with **1–3** and valinomycin.

The difference in activity engendered by the addition or subtraction of two methylene units is remarkable. The altered activity is dramatic in terms of toxicity but also significant in terms of liposomal transport and membrane depolarization. In order to assess if the bilayer itself was affecting transport by the lariat ethers, we measured ion transport in a bulk organic membrane. If the bilayer is critical for the selectivity that is observed here, then such selectivity should be absent in bulk phase transport experiments. We compared the transport of NaCl by **3** and **5** in a bulk water–chloroform membrane (concentric tube) system. The more hydrophobic **5** transported Na^+ (detected by ISE) about 16-fold more rapidly than did **3** (data not shown).

Conclusions

The remarkable selectivity of compounds **1–6** is exhibited in phospholipid bilayers but not in bulk phases. Transport of cations through an organic bulk membrane occurs readily with **1–6** and shows no discontinuity of the type observed in both liposomes and *in vivo*. We interpret the selectivity pattern, which is similar both in the organisms studied here and in liposomes, to reflect compound–membrane interactions. When the lariat ethers have relatively short side chains, but are sufficiently hydrophobic, they transport cations through a bulk, liposomal or vital membrane. In the latter case, this leads to a disruption of ion homeostasis and toxicity to the organism. Compound **1**, which has the shortest side chains in this family, shows little toxicity to *E. coli* and moderate toxicity to *Bacillus* and to yeast. Compound **2**, which has twin decyl side chains, is toxic to all three organisms. When the side chains are long enough, the dialkyl crowns apparently insert as if they were amphiphilic membrane monomers and remain relatively static rather than functioning as carriers. The ability of dialkyl crown ethers to form stable bilayer membranes is well documented.[31] When these lariat ethers bind cations, they become membrane monomers that have charged head groups. As such, transverse relaxation ("flip flop") is prohibited. When the charge can be compensated by a complexing agent such as Smith's synthetic translocase,[38] flip flop can occur. If the side arms possess a donor group such as those we previously studied in bilayers,[20] transport is also possible in liposomes. When there is no donor or complexing agent to compensate for or attenuate the charge, the dialkyl crowns insert in the bilayer and remain as membrane components where they neither serve as transport agents or induce toxicity.

Experimental

^{1}H- and ^{13}C-NMR spectra [in ppm downfield from internal Me_4Si] were recorded at 300 and 75 MHz respectively, in $CDCl_3$, unless otherwise stated. Melting points were determined in open capillaries and are uncorrected. All reagents were the best grade commercially available and were distilled, crystallized or used without further purification, as appropriate.

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-dierucoyl-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids as chloroform solutions. Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and the inorganic salts NaCl and cholineCl were all purchased from Sigma-Aldrich. The water that was used for all buffer preparation was of Milli-Q Plus quality, which is essential to avoid salt contamination in the buffer systems. *N*-Octylglucoside was purchased from CalBioChem.

N,*N*′-Dioctyldiaza-4,13-crown-6, 1

Prepared as reported previously, but purified by crystallization from acetone (yield: 65%). Crystals slowly melt at room temperature to form a colorless oil (lit. 63% as a colorless oil).[39]

N,*N*′-Didecyldiaza-4,13-crown-6, 2

Prepared as reported previously, but purified by crystallization from acetone (yield: 63%), mp 36–37 °C (lit. mp 34.5–36.5 °C).[39]

N,*N*′-Didodecyldiaza-4,13-crown-6, 3

Compound **3** was prepared as reported in detail in reference 26.

N,*N*′-Ditetradecyldiaza-4,13-crown-6, 4

Compound **4** was obtained in 36% yield after alkylation of *N*,*N*-diaza-18-crown-6 with 1-bromotetradecane as reported for **5**, mp 54 °C (lit. mp 54–55 °C).[39]

N,*N*'-Dihexadecyldiaza-4,13-crown-6, 5

Prepared in 44% yield as reported previously, mp 61–63 °C (lit. 23% with mp 63–64 °C).[39]

N,*N*'-Dioctadecyldiaza-4,13-crown-6, 6

Prepared as reported in detail in reference 1. Purity was verified by ^{1}H-NMR and mp 66–67 °C (lit. mp 66–67.5 °C).[39]

N,*N*'-Bis(1-oxodecyl)diaza-4,13-crown-6, 7

Compound **7** was prepared in 50% yield as previously reported, mp 67–68 °C (lit. mp 65.5–67 °C).[39]

N,*N*'-Bis(1-oxododecyl)diaza-4,13-crown-6, 8

Compound **8** was prepared as reported in detail in reference 26.

3,3′-Dipropylthiadicarbocyanine iodide ($DiSC_3(5)$), 9

Purchased from Sigma Chemicals and used as received.

Antimicrobial activity

The *m*inimum *i*nhibitory *c*oncentration (MIC) for each hydraphile is reported as the lowest serial 2-fold dilution that prevented bacterial growth as outlined by the National Committee for Clinical Laboratory Standards (NCCLS).[40] *E. coli* DH5α cells with a pBluescript plasmid having AMP resistance were tested using the standard inoculum size of 5×10^5 C. F. U.[41] mL^{-1}. Cells were grown at 37 °C in 2 mL of Luria Bertani (LB) Miller media (10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 100 μg mL^{-1} ampicillin) that were 2-fold serially diluted with hydraphile test compound. *Bacillus subtilis* (JH642 WT) cells were tested in similar fashion using regular Luria broth at the same inoculum size, and grown at 30 °C. The MIC was taken as the lowest hydraphile concentration that inhibited growth after 24 h as judged by visual turbidity. Each compound was assayed three times at every reported concentration to each bacterium, using an independent dilution of compound for each experiment. The *m*inimum *b*actericidal *c*oncentration (MBC) was determined as the lowest macrodilution that killed >99.9% of bacteria in the culture. This was determined by plating aliquots of the test suspensions onto Petri dishes and counting the number of C. F. U. after overnight growth.

***Sacharomyces cerevisiae*.** MIC experiments were conducted in similar fashion. Cells were grown in YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} dextrose) at 30 °C. An inoculum of 5×10^3 cells was used for MIC studies, and allowed to grow for 48 hours to achieve turbidity.

Vesicle preparation

Sodium chloride containing vesicles were prepared by reverse evaporation[42] from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (DEPC) as previously described.[32] The vesicles used in the transport studies had diameters of ~200–250 nm depending on lipid tail length.

Na^+ transport measurements

Sodium transport was measured using a Micro-Combination pH/sodium electrode (Thermo-Orion) in aqueous sodium-free buffer (750 mM cholineCl–15 mM HEPES, pH 7.0). Final vesicular lysis was accomplished by treatment with *n*-octylglucoside. Data were collected by Axoscope 7.0 using a Digidata 1322A series interface. The method has previously been described in detail.[32] All experiments in this study were performed at room temperature.

Membrane depolarization studies

The alphabetic designations in this description for valinomycin correspond to Fig. 3. *B. subtilis* cells were grown at 30 °C in Luria Bertani (LB) Miller media to mid-log phase, and collected by centrifugation. Cells were washed once in buffer, centrifuged again and resuspended to O. D. = 0.6 in the same buffer containing HEPES (0.5 mM) and dextrose (200 mM) at a pH of 7.5. A solution of dye was prepared by dissolving 1 mg of 3,3′-dipropylthiadicarbocyanine iodide (Sigma-Aldrich) in 50 mL of DMSO. Fluorescence was monitored in real time using a Perkin-Elmer Model LS 50B fluorescence spectrophotometer, at an excitation wavelength of 640 nm and an emission of 670. To 3 mL of buffer solution in a cuvette was added 50 μL of dye solution (A, 0.5 mM final), followed by 300 μL of the *B. subtilis* stock solution (B) to a final O. D. of 0.05. The fluorescence was allowed to stabilize and 100 μL of 2 M KCl buffer solution was added to give a 60 mM final concentration (C). The next step was to add the desired compound (D, 2 μM valinomycin shown). Finally, 100 μL of a 10% Triton-X-100 solution was added (E) to destabilize the membrane and give the final fluorescence level. *E. coli* experiments were conducted under identical conditions. The solution was vigorously mixed throughout the experiment.

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