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ARTICLE

Synthesis of dimeric 3α-hydroxy-7α,12α-diamino-5β-cholan-24-oate conjugate and its derivatives, and the effect of lipophilicity on their anion transport efficacy

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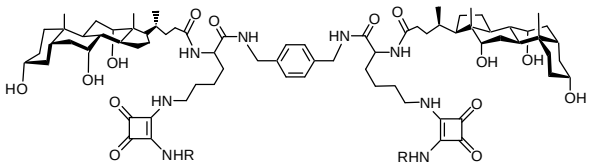
Dimeric 3α-hydroxy-7α,12α-diamino-5β-cholan-24-oate conjugate and its derivatives having alkyl chains of varying length from methyl to *n*-pentyl groups on the amido bonds were synthesized and fully characterized on the basis of NMR (^{1}H and ^{13}C) and ESI MS (LR and HR) data. Their transmembrane anion transport activities were investigated in detail by means of chloride ion selective electrode technique and pyranine assay. The data indicate that this set of compounds is capable of promoting the transmembrane transport of anions, presumably *via* an anion exchange process and a mobile carrier mechanism. Detailed kinetic analysis on the data obtained from both chloride efflux and pH discharge experiments reveals that an optimum log*P* range may exist for the transport effectiveness in term of both k_2/K_{diss} and EC_{50} values. The present finding highlights the importance of high anionophoric activity in clarifying the effect of lipophilicity on ion-transport effectiveness.

1. Introduction

It is known that anion transport across cell membranes plays a crucial role at some levels in almost every conceivable biochemical operation. [1] Dysfunction in this process may lead to some serious disorders, such as cystic fibrosis that is caused by the malfunction in natural chloride ion channels. [2] Therefore, during the past decades, increasing interest has been attracted in identifying small-molecule organic compounds that are capable of efficiently mediating the transport of anions, in particular chloride anions across lipid bilayer membranes. [3] These so-called synthetic anion transporters may serve as alternatives for defective natural anion channels. [4] As a consequence, various non-peptidic structures have been reported to exhibit promising anion transport properties. [5]

Anion translocation through a lipid membrane is a complex event involving multiple equilibria and steps. [6] Identifying the key structural parameter that affects anion transport activity is crucial to the rational design of effective anion transporters. Given the amphiphilic nature of phospholipid bilayer membranes, lipophilicity, widely measured by a log*P* value (the logarithm of *n*-octanol/water partition coefficient *P*), is recognized as one of the major structural factors that affect the activity of synthetic anion transporters. [7] It has been reported that for a given compound series, the anion transport activity may be optimized by varying the lipophilicity. [8] In some cases, an optimal log*P* value has been identified above or below which the transport activity diminishes. [9, 10]

To gain insight into the effect of lipophilicity on the effectiveness of a given anion transporter series, in a previous study we have reported six squaramido-functionalized bis(choloyl) conjugates **A-F** (Fig. 1) that have *clogP* values ranging from 3.90 to 8.32. [11, 12] We have found that the anionophoric activity changes with the lipophilicity in a concentration-dependent fashion. Specifically, at low concentrations the lipophilicity has little effect on the ion transport activity. When the concentration increases, the effect of lipophilicity becomes apparent. This finding makes us reasoning that high anionophoric activity is desired in clarifying the effect of lipophilicity on ion transport activity. In addition, though the flexible alkyl chains on the squaramido subunits are not involved in the recognition of anions, their effect on the interaction of compounds **A-F** with anions may not be ignored.



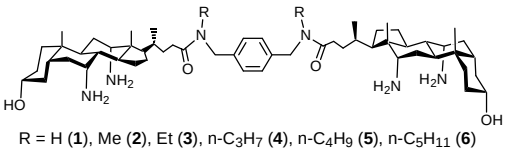


Figure 1. Structures of compounds **A-F** and **1-6**.

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To test this and also to have a closer look at the impact of lipophilicity on transport effectiveness, we designed dimeric 3α-hydroxy-7α,12α-diamino-5β-cholan-24-oate conjugate **1** linked with *p*-bis(aminomethyl)benzene, and its derivatives **2-6** having alkyl chains of varying length (Fig. 1). Here, the 7-, and 12-hydroxyl groups of the choloyl skeleton are transformed into amino substituents. This transformation is expected to strengthen the hydrogen-bonding ability toward anions and thereby lead to an enhancement in anion transport activity. [13] As a matter of facts, such transformation has been demonstrated powerful in the generation of effective anion receptors and transporters. [14] Modification of compound **1** with alkyl chains from methyl to *n*-pentyl groups (to give compounds **2-6**), is made on the amido bonds. This modification should have no effect on the interaction of the 7-, and 12-amino substitutents with anions, but gradually increases the lipophilicity as the predominant factor that regulates the activity of compounds **1-6**. [15] Thus, such modification makes it feasible to assess how lipophilicity is correlated with the transport efficiency of compounds **1-6**. Herein we report the synthesis of compound **1** and its derivatives **2-6** and their transmembrane anionophoric activity investigated by means of chloride ion selective electrode technique and pyranine assays. The effect of lipophilicity on the ion transport activity is discussed in detail.

2. Results and discussion

2.1 Chemistry

Compounds **1-6** were synthesized according to the approach shown in Scheme 1. Thus, methyl 3α-acetoxy-7α,12α-di[N-(t-butyloxycarbonyl)amino]-5β-cholan-24-oate **7** was prepared using literature protocols [13, 14] and hydrolyzed by LiOH to give 3α-hydroxy-7α,12α-di[N-(t-butyloxycarbonyl)amino]-5β-cholan-24-oic acid **8**. Activation of compound **8** with N-hydroxysuccinimide (NHS) or 1-hydroxybenzotriazole (HOBt) and subsequent reaction with *p*-bis(aminomethyl)benzene **9** or *p*-bis(alkylaminomethyl)benzene **16-20** afforded Boc-protected dimeric conjugates **21-26**. Final deprotection of the Boc groups in compounds **21-26** with TFA gave compounds **1-6**. Compounds **1-6** were fully characterized on the basis of NMR (^{1}H and ^{13}C) and ESI MS (LR and HR) data (see experimental section and Fig. S1-51).

2.2 Anionophoric activity of compounds **1**-**6**

To test whether compounds **1-6** possess anion transport activity, firstly, we carried out chloride efflux experiments by using chloride ion selective electrode (ISE). [16, 17] In the experiments, a series of large unilamellar egg-yolk phosphatidylcholine (EYPC)-based vesicles (100 nm diameter, extrusion) loaded with NaCl were prepared and suspended in an external isotonic $NaNO_3$ solution. A sample of compounds **1-6** (of varying concentration in molar percent with respect to the total concentration of lipid) was added as a DMSO solution. The efflux of chloride from the vesicles was detected by ISE. After 300 s, 5 wt% aqueous Triton X-100 solution was added to release the residual NaCl in the vesicles and the final reading of the electrode was used to calibrate the 100% release of chloride. The data are shown in Fig. 2a and S52-53, and indicate that compounds **1-6** are capable of promoting chloride efflux from the EYPC vesicles and the chloride efflux rate is concentration dependent.

The results of chloride efflux experiments suggest that compounds **1-6** transport chloride by either Cl^-/NO_3^- antiport or Cl^-/cation symport mechanism. To distinguish the probable mechanism of action, we changed the external solution from $NaNO_3$ to Na_2SO_4 and re-measured the chloride efflux. The sulfate anion is strongly hydrated and is assumed to be unable to readily pass across a lipid bilayer. [18] If a compound functions as a Cl^-/anion exchanger, its chloride efflux activity should be inhibited in the presence of sulfate anions. The results of compounds **1-6** are shown in Fig. 2b and S54, and indicate that the chloride efflux rates of compounds **1-6** were significantly inhibited by sulfate anions, suggesting that compounds **1-6** function as anion exchangers.

To gain further insights into the probable mechanism of action and ion selectivity of compounds **1-6**, we carried out the chloride efflux experiments in the presence of the chloride salts of group I alkali metal ions, including Li^+, Na^+, K^+, Rb^+ and Cs^+. The results are shown in Fig. 3a and S55 and indicate that the chloride efflux activity of compounds **1-6** is essentially independent of group I alkali metal ions, excluding any meaningful role of those metal ions in the permeation process. [19] Then, we measured the pH discharge activity of compounds **1-6** in the presence of the sodium salts of different anions (i.e., NO_3^-, Cl^-, Br^-, and I^-). In this test, a series of EYPC vesicles containing sodium salts and pyranine buffered to pH 7.0 were prepared and suspended in the same sodium salt solution buffered to pH 8.0. The fluorescence of pyranine was monitored upon the addition of compounds **1-6**. The results are shown in Fig. 3b and S56 and suggest that compounds **1-6** are capable of inducing pH discharge across the membrane. The activity follows the order of $I^- > NO_3^- \approx Br^- \approx Cl^-$, correlating well with a halide transport based on the lyotropic sequence. [20] These observations imply that pH gradient decay correlates with OH^-/Cl^- antiport.

Finally, to determine the mechanism of action of compounds **1-6**, we measured their pH discharge activity across the lipid membranes derived from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol. It is reported that the addition of cholesterol to the membrane reduces membrane fluidity, therefore cholesterol assays have been frequently used as evidence for a mobile carrier mechanism. [21] As shown in Fig. 4 and S57, the pH discharge activity of compounds **1-6** was found to be significantly reduced across the POPC/cholesterol membranes. This supports a mobile carrier over a channel mechanism.

Taken together, the above-mentioned observations suggest that compounds **1-6** are capable of mediating the transmembrane transport of chloride *via* an anion exchange process and a mobile carrier mechanism.

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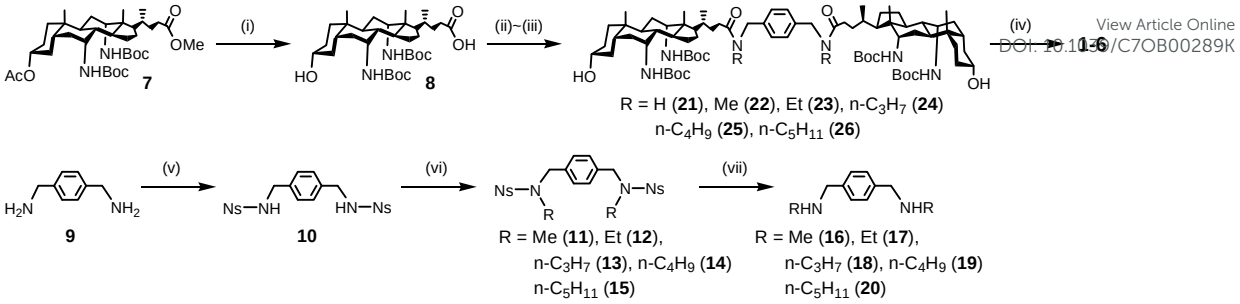

Scheme 1. Synthesis of compounds **1-6**. Reagents and conditions: (i) LiOH, MeOH, reflux; (ii) NHS (or HOBt), DCC, $CHCl_3$ or THF, room temperature; (iii) *p*-bis(aminomethyl)benzene **9** or *p*-bis(alkylaminomethyl)benzene **16-20**, Et_3N, $CHCl_3$ or THF, room temperature; (iv) TFA, CH_2Cl_2, room temperature; (v) *p*-nitrobenzene sulfonyl chloride, DMF; (vi) RI (R = Me, Et, *n*-C_3H_7, *n*-C_4H_9, or *n*-C_5H_{11}), K_2CO_3, DMF; (vii) 4-methoxythiophenol, K_2CO_3, DMF.

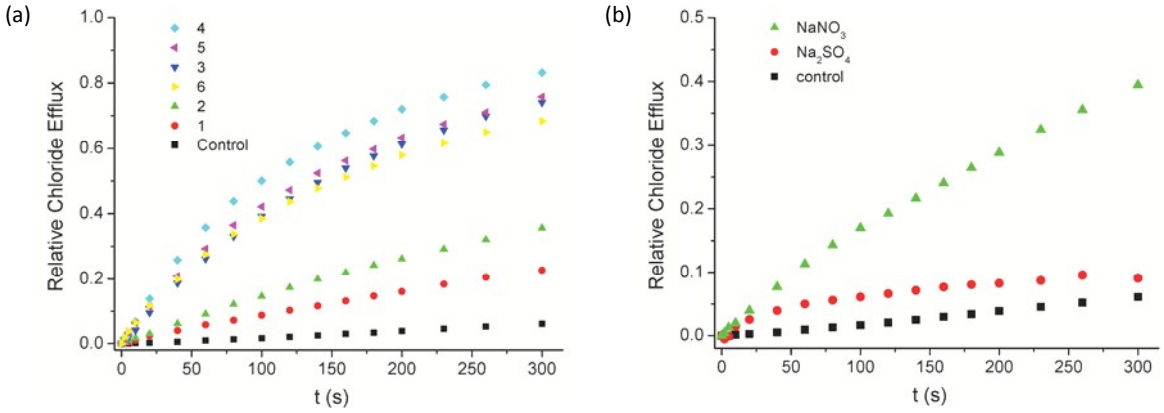

Figure 2. (a) Relative chloride efflux promoted by compounds **1-6** (2 mol%) in EYPC vesicles loaded with 500 mM NaCl buffered to pH 7.0 with 25 mM HEPES. The vesicles were dispersed in 500 mM $NaNO_3$ buffered to pH 7.0 with 25 mM HEPES. (b) Chloride efflux promoted by compound **1** (5 mol%) in the same EYPC vesicles loaded with 500 mM NaCl buffered to pH 7.0 with 25 mM HEPES. The vesicles were dispersed in 25 mM HEPES buffer (pH 7.0) containing 500 mM $NaNO_3$ and 250 mM Na_2SO_4, respectively. The control experiments were conducted in $NaNO_3$ media and in the absence of compound **1**.

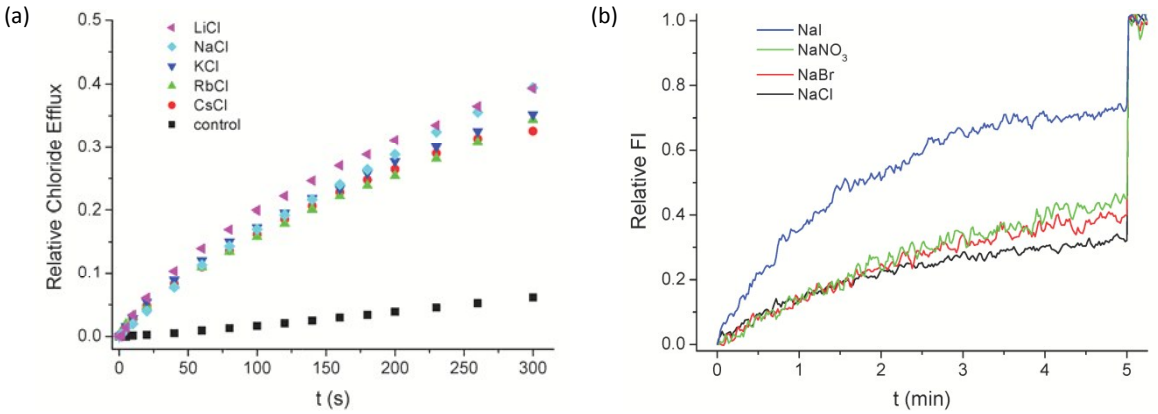

Figure 3. (a) Relative chloride efflux promoted by compound **1** (5 mol%) in EYPC vesicles loaded with 500 mM MCl (M = Li, Na, K, Rb and Cs) buffered to pH 7.0 with 25 mM HEPES. The vesicles were dispersed in 500 mM $NaNO_3$ buffered to pH 7.0 with 25 mM HEPES. The control experiments were conducted in NaCl media with DMSO. (b) Discharge of a pH gradient by compound **1** (0.5 mol%) across EYPC-based liposomal membranes, under the measuring conditions of internal vesicles: 0.1 mM pyranine in 25 mM HEPES (50 mM NaX, pH 7.0) and external vesicles: 25 mM HEPES (50 mM NaX, pH 8.0) (X = NO_3, Cl, Br and I). λ_{Ex} 460 nm; λ_{em} 510 nm. Each profile represents the real increment in the presence of compound **1**.

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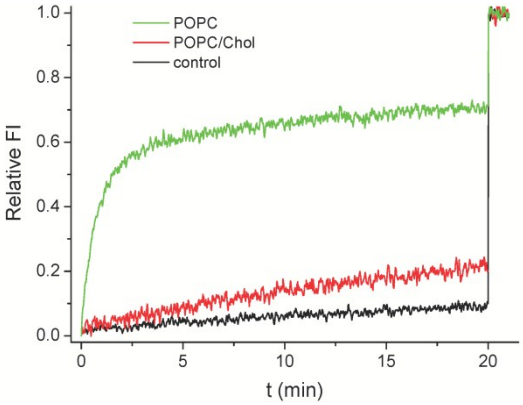

Figure 4. Discharge of a pH gradient promoted by compound **1** (1 mol%) across POPC and POPC-cholesterol (7/3)-based liposomal membranes. Measuring conditions for internal vesicles: 0.1 mM pyranine in 25 mM HEPES (50 mM NaCl, pH 7.0); and for external vesicles: 25 mM HEPES (50 mM NaCl, pH 8.0). λ_{Ex} 460 nm; λ_{em} 510 nm.

2.3 Effect of lipophilicity on the anionophoric activity of compounds **1**-**6**

As shown above, compounds **1-6** exhibit different chloride efflux activity, suggesting that lipophilicity has significant effect on the anion-transport activity. To evaluate this effect, we firstly measured the log*P* values of compounds **1-6**. [22] Specifically, compounds **1-6** were partitioned in a mixture of *n*-octanol and water. After partition equilibrium was achieved, the concentrations of each of compounds **1-6** in organic and aqueous phases were measured by means of gray value analysis on TLC by ImageJ2X software. The measured log*P* values of compounds **1-6** are listed in Table 1. The log*P* values from 0.40 to 0.93 suggest that the lipophilicity of compounds **1-6** increases gradually with alkyl chains of varying length from methyl to *n*-pentyl groups.

Then, we performed detailed analysis on the kinetic data of compounds **1-6** obtained from chloride selective electrode techniques. Analysis of the relationship between the chloride efflux at 260 s and the concentrations of each of compounds **1-6** according to equation (1) afforded two kinetic parameters, [23] i.e., Hill coefficient n and specificity constant k_2/K_{diss}. The former represents the stoichiometry of the transport process, whereas the latter describes the transporter's ability to facilitate the diffusion of a given ion and can be used to measure the specificity of a transporter for a given anion. Here k_2 and K_{diss} represent the intrinsic rate constant and the dissociation constant of the self-association process, respectively. [24]

$$k_{obsd} = k_0 + k_2[\text{monomer}]^n/K_{diss} \quad (1)$$

Because of the high sensitivity of pyranine assays, we conducted the concentration-dependent pH discharge experiments of compounds **1-6** (Fig. S58-59) and calculated the initial rate constants (k_{in}'s) at each concentration. [25] Nonlinear curve fitting of the initial rate constants against the concentrations of each of compounds **1-6** according to equation (2), gave the Hill coefficient n and the EC_{50} value of each compound. [26] Here EC_{50} is defined as effective transporter loading that needs to reach 50% of the maximum rate (k_{max}) after a specified time period and thereby a measure of the effectiveness of a given transporter. [27]

$$K_{in} = k_0 + k_{max}[\text{monomer}]^n/([\text{monomer}]^n + EC_{50}{}^n) \quad (2)$$

The kinetic parameters obtained from both analyses are listed in Table 1. Several observations can be abstracted from these data. Firstly, compounds **1-6** have EC_{50} values of 0.11~0.89 mol%, up to 25-fold lower than those of compounds **A-F**, suggesting that transformation of the 7- and 12-hydroxyl groups into amino groups greatly enhances the anionophoric activity. The enhancement in the transport effectiveness might be ascribed to the following aspects. Firstly, as already stated in the Introduction, the modification of compounds **A-F** with alkyl chains was made on the squaramido functional groups, which may affect the interaction with anions. In contrast, the amino groups in compounds **1**-**6** are not substituted, which would be favorable to the multiple interactions with anions. [14] Secondly, because both compounds **1-6** and **A-F** function, most possibly as carriers, [11] the former are smaller in size than the latter and would be more ready to shuttle back and forth within the interior of the membranes. In addition, it should be noted that compounds **1-6** are comparable to (thio)urea-based anion transporters (EC_{50} = 0.42 mol%), [28] and much more active than cholic acid-based molecular umbrellas (EC_{50} = 5.7 mol%). [29] Thus, compounds **1-6** are considered to be a very effective class of anion transporters.

Secondly, it is clear that compounds **1-6** have different k_2/K_{diss} and EC_{50} values. A plot of the k_2/K_{diss} and $1/EC_{50}$ values of compounds **1-6** against their log*P* values shows that the transport effectiveness of this set of compounds increases with lipophilicity on going from compound **1** to compound **4**, and tend to decrease on going from compound **4** to compound **6** (Fig. 5). This reveals that an optimum log*P* range exists for the transport effectiveness. This may be rationalized if the effect of lipophilic/hydrophilic balance on the transport process is taken into account. [30] Increasing the lipophilicity is favourable for compounds **1-6** to partition from aqueous phases into membranes and leads to an increase in the transport activity. However, because compounds **1-6** function *via* a mobile carrier mechanism, increasing the lipophilicity may lower their mobility within the lipid membranes and results in a decrease in the activity. In a sense, the lipophilic/hydrophilic balance plays a crucial role in the efficient transport of anions by this set of compounds.

The Hill coefficients n of compounds **1-6** obtained from both experiments are close to 1, which suggests that they do not aggregate to function.

3. Conclusions

In conclusion, we have successfully synthesized a dimeric 3α-

hydroxy-7α,12α-diamino-5β-cholan-24-oate conjugate and its five derivatives having varying lipophilicity, and fully characterized them on the basis of NMR (^{1}H and ^{13}C) and ESI MS (LR and HR) data. We have measured their transmembrane ionophoric activity and ion selectivity by means of pyranine assay and chloride ion selective electrode technique. The data indicate that these conjugates exhibit potent anionophoric activity across EYPC-based liposomal membranes, presumably *via* an anion exchange process and a mobile carrier mechanism. Of these compounds, the ones with ethyl, *n*-propyl and *n*-butyl groups display top activity within experimental errors. Detailed kinetic analysis of the effect of lipophilicity on the ion-transport activity indicates that this set of compounds has an optimal log*P* range for the effectiveness in term of both k_2/K_{diss} and EC_{50}. This finding highlights the importance of high anionophoric activity in addressing the effect of lipophilicity on transport effectiveness. Further efforts aimed at creating more effective anion transporters, guided by the present observations, are currently under active investigation in our laboratory. The outcome will be reported in due course.

Table 1. Kinetic parameters for the chloride efflux and pH discharge by compounds **1**-**6**

Compound	log*P* [a]	Chloride selective electrode assay [b]		pH discharge [c]	
		n	k_2/K_{diss} (s^{-1}·mol%$^{-1}$)	*n*	EC_{50} (mol%)
1	0.40±0.021	0.74±0.06	0.059±0.004	1.82±0.18	0.887±0.016
2	0.55±0.045	1.29±0.08	0.173±0.008	1.44±0.22	0.457±0.091
3	0.64±0.066	0.68±0.04	0.320±0.025	1.13±0.11	0.115±0.022
4	0.77±0.048	0.74±0.06	0.374±0.026	1.59±0.14	0.108±0.011
5	0.82±0.043	0.75±0.04	0.329±0.019	1.26±0.34	0.135±0.007
6	0.93±0.077	0.77±0.03	0.297±0.015	1.38±0.17	0.138±0.028

[a] See experimental section for the detailed procedures.

[b] Measured in EYPC vesicles under the measuring conditions of internal vesicles: 500 mM NaCl in 25 mM HEPES (pH 7.0) and external vesicles: 500 mM $NaNO_3$ in 25 mM HEPES (pH 7.0) (Fig. S53).

[c] Measured in EYPC vesicles under the measuring conditions of internal vesicles: 0.1 mM pyranine in 25 mM HEPES (50 mM NaCl, pH 7.0) and external vesicles: 25 mM HEPES (50 mM NaCl, pH 8.0) (Fig. S59). The average rate constant for the background (k_0) was (6.46±3.10)×10^{-3} min^{-1}.

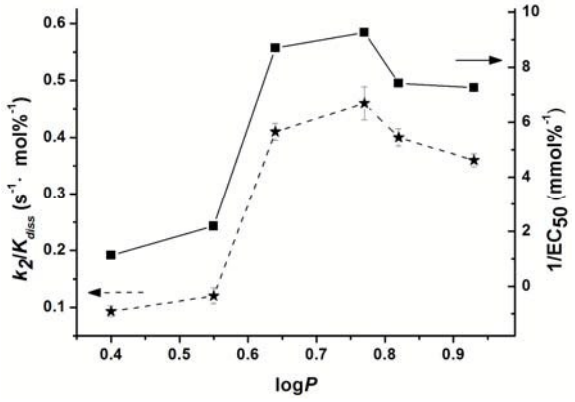

Figure 5. Plots of the 1/EC_{50} (■) and k_2/K_{diss} (★) values against the log*P* of compounds **1**-**6**.

4. Experimental

Generals. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance AV 400 spectrometer and the deuterium solvents as standards. LR and HR ESI mass spectra were measured on Waters UPLC/Quattro Premier XE and Bruker maXis 4G ESI-Q-TOF mass spectrometers, respectively. LR and HR EI mass spectra were measured on a Thermo DSQ and Thermo MAT 95XP mass spectrometers, respectively. Silica gel 80-100 Å (reagent pure, Qingdao Haiyang Chemical Co. Ltd) was used for column chromatography. Analytical thin-layer chromatography (TLC) was performed on silica gel plates 60 GF254. Detection on TLC was made by use of iodine, UV (254 or 365 nm) and 20% aqueous H_2SO_4. Liposomes were prepared by extrusion using an Avanti's Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA). Nuclepore track-etched polycarbonate membranes (100 nm) were obtained from Whatman (Florham Park, New Jersey, USA). Chloride efflux was measured on a Mettler-Toledo PerfectIon^{TM} chloride ion selective electrode. Fluorescence spectra were measured on a PE LS55 spectrofluorimeter.

EYPC, POPC and pyranine were purchased from Sigma Chemical Co. (St Louis, USA). Compound **7** was synthesized starting from cholic acid according to reported protocols. [13, 14] All the other chemicals and reagents were obtained from commercial sources and used without further purification. Buffer solutions were prepared in triply distilled deionized water.

*Synthesis of compound **8***

To a solution of compound **7** (200 mg, 0.32 mmol) in methanol (3.5 mL) was added lithium hydroxide aqueous solution (0.2 mol/L, 3 mL). The reaction was conducted under reflux and monitored by TLC (CH_2Cl_2/CH_3OH = 30/1, v/v). After 4 h, the reaction mixture was concentrated under reduced pressure, dissolved with EtOAc (150 mL) and washed with pH 3 HCl aqueous solution (100 mL × 3). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The obtained residues were purified by chromatography on a silica gel column, eluted with a mixture of CH_2Cl_2 and CH_3OH (85/1, v/v) to afford compound **8** (116 mg, 63%) as a white foam having ^{1}H-NMR (400 MHz, CD_3OD): δ 3.97-3.95 (m, 1H), 3.57 (br, 1H), 3.50-3.42 (m, 1H), 2.41-2.17 (m, 2H), 1.95-1.12 (m, 40H), 0.97-0.96 (m, 6H), 0.84 (s, 3H) ppm; ^{13}C-NMR (100 MHz,

CD_3OD): δ 176.8, 156.5, 78.4, 71.0, 53.3, 48.3, 44.5, 43.8, 41.4, 38.0, 36.5, 34.8, 34.7, 34.0, 31.3, 30.7, 30.6, 29.3, 28.4, 27.5, 27.4, 27.0, 26.7, 22.6, 21.6, 16.8, 12.3 ppm; negative ESI-MS: *m/z* 605.8 ($[M-H]^-$) and HR-ESI-MS for $C_{34}H_{59}O_7N_2$ ($[M+H]^+$) Calcd: 607.43168; Found: 607.43188.

*Synthesis of compound **10***

To a solution of *p*-bis(aminomethyl)benzene **9** (500 mg, 3.67 mmol) in DMF (4.5 mL) was added slowly a solution of *p*-nitrobenzene sulfonyl chloride (2.44 g, 11.01 mmol) in DMF (9.5 mL). The reaction mixture was stirred at room temperature. The reaction was monitored with TLC (CH_2Cl_2/CH_3OH = 35/1, v/v). After 12 h, the reaction mixture was added to saturated sodium bicarbonate aqueous solution (250 mL) with vigorous stirring. The resulting yellow precipitates were collected by filtration and washed thoroughly with water to afford compound **10** (965 mg, 52%) as a pale yellow powder. Mp 194-195 °C; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.32 (d, *J* = 8 Hz, 4H), 7.95 (d, *J* = 8 Hz, 4H), 7.09 (s, 4H), 3.95 (s, 4H) ppm; negative ESI-MS: *m/z* 505.3 ($[M-H]^-$) and negative HR-ESI-MS for $C_{20}H_{17}O_8N_4S_2$ ($[M-H]^-$) Calcd: 505.04933; Found: 505.04941.

*Syntheses of compounds **11-15***

*Compound **11***. A mixture of compound **10** (250 mg, 0.49 mmol) and anhydrous potassium carbonate (684 mg 4.94 mmol) in anhydrous DMF (9 mL) was stirred at room temperature for 30 min. Then methyl iodide (1.5 mL, 9.88 mmol) was added. The resulting mixture was stirred at room temperature. The reaction was monitored with TLC (petroleum ether/ CH_2Cl_2 = 1/15, v/v). After 40 h, the reaction mixture was added to water (25 mL). The formed yellow precipitates were collected by filtration, washed thoroughly with water and dried in vacuum to afford compound **11** (156 mg, 59%) as a yellow powder. Mp 205-206 °C; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.45 (d, *J* = 7.6 Hz, 4H), 8.12 (d, *J* = 7.6 Hz, 4H), 7.31 (s, 4H), 4.22 (s, 4H), 2.62 (s, 6H) ppm; EI MS: *m/z* 534.09 (M^+) and HR-EI-MS for $C_{22}H_{22}O_8N_4S_2$ ($[M]^+$) Calcd: 534.0874; Found: 534.0866.

*Compound **12***. Procedure as described for **11**; from **10** (500 mg, 0.99 mmol) and ethyl iodide (1.45 mL, 19.76 mmol). Yield: 365 mg (66%), a yellow powder. Mp 209-210 °C; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.36 (d, *J* = 8.4 Hz, 4H), 8.01 (d, *J* = 8.4 Hz, 4H), 7.27 (s, 4H), 4.39 (s, 4H), 3.28-3.23 (m, 4H), 0.97 (t, 6H, *J* = 7.2 Hz) ppm; EI MS: *m/z* 562.12 (M^+) and HR-EI-MS for $C_{24}H_{26}O_8N_4S_2$ (M^+) Calcd: 562.1187; Found: 562.1180.

*Compound **13***. Procedure as described for **11**; from **10** (500 mg, 0.99 mmol) and 1-iodopropane (1.90 mL, 19.76 mmol). Yield: 355 mg (61%), a yellow powder. Mp 214-215 °C; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.36 (d, *J* = 8.4 Hz, 4H), 8.00 (d, *J* = 8.4 Hz, 4H), 7.25 (s, 4H), 4.37 (s, 4H), 3.12 (t, *J* = 7.6 Hz, 4H), 1.39-1.34 (m, 4H), 0.73 (t, *J* =7.6 Hz, 6H) ppm; EI MS: *m/z* 590.15 (M^+) and HR-EI-MS for $C_{26}H_{30}O_8N_4S_2$ (M^+) Calcd: 590.1500; Found: 590.1507.

*Compound **14***. Procedure as described for **11**; from **10** (300 mg, 0.59 mmol) and 1-iodobutane (1.35 mL, 11.86 mmol). Yield: 230 mg (63%), a yellow powder. Mp 220-221 °C; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.36 (d, *J* = 8.8 Hz, 4H), 8.00 (d, *J* = 8.8 Hz, 4H), 7.25 (s, 4H), 4.37 (s, 4H), 3.15 (t, *J* = 7.6 Hz, 4H), 1.36-1.28 (m, 4H), 1.19-1.09 (m, 4H), 0.77 (t, *J* =7.2 Hz, 6H) ppm; EI MS: *m/z* 618.19 (M^+) and HR-EI-MS for $C_{28}H_{34}O_8N_4S_2$ (M^+) Calcd: 618.1813; Found: 618.1805.

*Compound **15***. Procedure as described for **11**; from **10** (300 mg, 0.59 mmol) and 1-iodopentane (1.55 mL, 11.86 mmol). Yield: 236 mg (62%), a yellow powder. Mp 226-227 °C; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.36 (d, *J* = 8.8 Hz, 4H), 8.00 (d, *J* = 8.8 Hz, 4H), 7.25 (s, 4H), 4.37 (s, 4H), 3.14 (t, *J* = 7.6 Hz, 4H), 1.37-1.30 (m, 4H), 1.21-1.04 (m, 8H), 0.78 (t, *J* = 7.2 Hz, 6H) ppm; EI MS: *m/z* 646.21 (M^+) and HR-EI-MS for $C_{30}H_{38}O_8N_4S_2$ (M^+) Calcd: 646.2126; Found: 646.2136.

*Syntheses of compounds **21-26***

*Compound **21***. To a solution of **8** (116 mg, 0.19 mmol) and NHS (43 mg, 0.37 mmol) in $CHCl_3$ (6 mL) was added DCC (134 mg, 0.65 mmol). The resulting mixture was stirred at room temperature. The reaction was monitored by TLC (CH_2Cl_2/CH_3OH = 25/1, v/v). After 5.5 h, a solution of *p*-bis(aminomethyl)benzene **9** (11 mg, 0.081 mmol) in $CHCl_3$ (2.5 mL) was added. The resulting mixture was stirred at room temperature. The reaction was monitored with TLC (CH_2Cl_2/CH_3OH = 18/1, v/v). After 24 h, the reaction mixture was diluted with $CHCl_3$ (150 mL) and washed subsequently with saturated $NaHCO_3$ (150 mL × 2), water (150 mL) and saline (150 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The obtained residues were purified by chromatography on a silica gel column, eluted with a mixture of CH_2Cl_2 and CH_3OH (40/1, v/v) to afford compound **21** (59 mg, 56% based on compound **9**) as a white solid. Mp 157-158 °C; ^{1}H-NMR (400 MHz, $CDCl_3/CD_3OD$, 10/1, v/v): δ 7.22 (s, 4H), 4.36 (s, 4H), 3.92 (br, 2H), 3.57 (br, 2H), 3.36 (br, 2H), 2.32-0.93 (m, 92H), 0.90 (s, 6H), 0.77 (s, 6H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3/CD_3OD$, 10/1, v/v): δ 174.5, 156.3, 137.4, 127.7, 78.6, 78.5, 77.4, 71.2, 52.8, 47.2, 44.8, 43.8, 42.9, 41.3, 38.0, 36.5, 35.0, 34.9, 34.2, 33.6, 31.7, 31.6, 29.5, 28.5, 28.3, 28.2, 27.0, 23.0, 22.4, 17.6, 13.2 ppm; ESI-MS: *m/z* 1335.7 ($[M+Na]^+$) and negative HR-ESI-MS for $C_{76}H_{123}O_{12}N_6$ ($[M–H]^–$) Calcd: 1311.92045; Found: 1311.91748.

*Compound **22***. A solution of compound **11** (250 mg, 0.47 mmol) and potassium carbonate (324 mg, 2.34 mmol) in DMF (2 mL) was stirred at room temperature for 30 min. Then 4-methoxythiophenol (460 µL, 3.75 mmol) was added. The reaction mixture was stirred at room temperature. The reaction was monitored with TLC ($CH_3OH/NH_3{\cdot}H_2O$ = 30/1, v/v). After 9.5 h, the reaction mixture was diluted with $CHCl_3$ (150 mL) and washed with saturated Na_2CO_3 (100 mL × 3). The organic layer was dried over anhydrous Na_2SO_4. Purification was achieved by flash chromatography on a silica gel column, eluted with a mixture of CH_3OH and $NH_3{\cdot}H_2O$ (100/1, v/v) to give compound **16** (42 mg, 55%). The removal of 4-nitrobenzenesulfonyl groups was confirmed with ESI-MS: *m/z* 165.5 ($[M+H]^+$). Because of the potential sensitivity to oxidation, compound **16** was used in the next step without further characterization. Then, to a solution of compound **8** (150 mg, 0.25 mmol) and HOBt (100 mg, 0.74 mmol) in anhydrous THF (3.5 mL) was added DCC (179 mg, 0.87 mmol). The resulting mixture was stirred at room temperature. The reaction was monitored by TLC

(CH_2Cl_2/CH_3OH = 25/1, v/v). After 5.5 h, a solution of compound **16** (18 mg, 0.11 mmol) and Et_3N (0.5 mL) in anhydrous THF (440 μL) was added to the above reaction mixture. The resulting mixture was stirred at room temperature. The reaction was monitored with TLC (CH_2Cl_2/CH_3OH = 20/1, v/v). After 14 h, the reaction mixture was diluted with $CHCl_3$ (150 mL) and washed with saturated $NaHCO_3$ (150 mL × 2). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The obtained residues were purified by preparative TLC (petroleum ether/EtOAc = 1/2, v/v) to afford compound **22** (99 mg, 67%) as a white solid. Mp 160-161 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.30-7.18 (m, 4H), 4.64-4.59 (m, 4H), 3.96-3.91 (m, 2H), 3.57 (br, 2H), 3.48-3.42 (m, 2H), 3.00-2.95 (m, 6H), 2.53-2.30 (m, 4H), 1.95-0.80 (m, 98H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 174.7, 156.5, 136.4, 136.1, 128.1, 127.7, 126.7, 126.3, 78.4, 70.9, 53.1, 53.0, 50.1, 44.6, 43.7, 41.4, 38.1, 36.5, 35.0, 34.8, 34.7, 34.3, 34.0, 31.3, 31.0, 30.1, 29.4, 28.4, 27.5, 27.4, 26.9, 26.7, 22.7, 21.6, 17.1, 12.4 ppm; ESI-MS: *m/z* 1363.6 ($[M+Na]^+$) and HR-ESI-MS for $C_{78}H_{129}O_{12}N_6$ ([M+H]+) Calcd: 1341.9663; Found: 1341.96655.

*Compound **23***. Compound **17** was prepared using the procedures as described for compound **16**. The removal of 4-nitrobenzenesulfonyl group was confirmed with ESI-MS: *m/z* 193.4 ($[M+H]^+$). The rest procedure as described for **22**; from **8** (150 mg, 0.25 mmol) and **17** (21 mg, 0.11 mmol). Yield: 86 mg (57%), a white solid. Mp 162-163 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.29-7.18 (m, 4H), 4.64-4.58 (m, 4H), 3.96-3.88 (m, 2H), 3.57 (br, 2H), 3.48-3.36 (m, 6H), 2.48-2.26 (m, 4H), 1.95-0.80 (m, 104H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3/CD_3OD$, 10/1, v/v): δ 174.1, 156.2, 156.0, 137.1, 136.6, 128.2, 127.9, 126.8, 126.4, 78.6, 71.3, 52.8, 50.8, 48.9, 47.9, 47.1, 45.8, 44.8, 43.9, 41.3, 38.0, 36.6, 35.1, 34.9, 34.2, 31.6, 30.6, 29.5, 28.3, 28.2, 27.2, 26.9, 23.0, 22.4, 19.9, 17.8, 13.6, 13.2 ppm; ESI-MS: *m/z* 1391.7 ($[M+Na]^+$) and HR-ESI-MS for $C_{80}H_{133}O_{12}N_6$ ($[M+H]^+$) Calcd: 1369.9976; Found: 1369.99915.

*Compound **24***. Compound **18** was prepared using the procedures as described for compound **16**. The removal of 4-nitrobenzenesulfonyl group was confirmed with ESI-MS: *m/z* 221.8 ($[M+H]^+$). The rest procedure as described for **22**; from **8** (150 mg, 0.25 mmol) and **18** (25 mg, 0.11 mmol). Yield: 114 mg (72%), a white solid. Mp 165-166 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.29-7.17 (m, 4H), 4.66-4.56 (m, 4H), 3.96-3.90 (m, 2H), 3.57 (br, 2H), 3.48-3.40 (m, 2H), 3.29-3.24 (m, 4H), 2.48-2.27 (m, 4H), 1.95-0.80 (m, 108H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3/CD_3OD$, 10/1, v/v): δ 174.1, 156.1, 137.1, 136.6, 128.2, 128.0, 126.7, 126.4, 78.6, 71.3, 52.8, 50.8, 47.9, 47.1, 46.1, 44.8, 43.9, 41.3, 38.0, 36.6, 35.2, 34.9, 31.6, 30.3, 29.5, 29.0, 28.8, 28.5, 28.3, 28.2, 27.2, 26.9, 23.0, 22.4, 22.2, 17.8, 13.7, 13.3 ppm; ESI-MS: *m/z* 1420.0 ($[M+Na]^+$) and HR-ESI-MS for $C_{82}H_{137}O_{12}N_6$ ($[M+H]^+$) Calcd: 1398.0289; Found:1398.03052.

*Compound **25***. Compound **19** was prepared using the procedures as described for compound **16**. The removal of 4-nitrobenzenesulfonyl group was confirmed with ESI-MS: *m/z* 249.8 ($[M+H]^+$). The rest procedure as described for **22**; from **8** (162 mg, 0.27 mmol) and **19** (30 mg, 0.12 mmol). Yield: 69 mg (40%), a white solid. Mp 168-169 °C; ^{1}H-NMR (400 MHz, $CDCl_3/CD_3OD$, 10/1, v/v): δ 7.13-7.00 (m, 4H), 4.48-4.42 (m, 4H), 3.87-3.83 (m, 2H), 3.50 (br, 2H), 3.27-3.06 (m, 6H), 2.38-2.11 (m, 4H), 1.81-0.80 (m, 106H), 0.71-0.66 (m, 6H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3/CD_3OD$, 10/1, v/v): δ 174.3, 156.4, 137.4, 136.9, 128.5, 128.2, 127.0, 126.6, 78.8, 78.7, 71.5, 53.0, 51.1, 48.1, 47.4, 46.1, 45.0, 44.1, 41.5, 38.3, 36.8, 35.4, 35.1, 34.5, 31.8, 30.8, 30.5, 29.8, 29.7, 28.6, 28.5, 27.4, 27.2, 23.2, 22.7, 20.3, 20.2, 18.0, 13.8, 13.5 ppm; ESI-MS: *m/z* 1447.9 ($[M+Na]^+$) and HR-ESI-MS for $C_{84}H_{141}O_{12}N_6$ ($[M+H]^+$) Calcd: 1426.0602; Found: 1426.06018.

*Compound **26***. Compound **20** was prepared using the procedures as described for compound **16**. The removal of 4-nitrobenzenesulfonyl group was confirmed with ESI-MS: ESI-MS: *m/z* 277.7 ($[M+H]^+$). The rest procedure as described for **16**; from **8** (125 mg, 0.21 mmol) and **20** (26 mg, 0.093 mmol). Yield: 58 mg (44%), a white solid. Mp 169-170 °C; ^{1}H-NMR (400 MHz, CD_3Cl/CD_3OD, 10/1,v/v): δ 7.16-7.04 (m, 4H), 4.52-4.45 (m, 4H), 3.91-3.87 (m, 2H), 3.54 (br, 2H), 3.47-3.37 (m, 2H), 3.23-3.09 (m, 4H), 2.38-2.16 (m, 4H), 1.85-0.70 (m, 116H) ppm; ^{13}C-NMR (100 MHz, $CDCl_3/CD_3OD$, 10/1, v/v): δ 174.3, 156.4, 137.4, 136.9, 128.5, 128.2, 127.0, 126.6, 78.8, 71.5, 53.0, 51.1, 48.1, 47.4, 46.3, 45.0, 44.1, 41.5, 38.3, 36.8, 35.4, 35.1, 34.5, 31.8, 30.5, 29.8, 29.2, 29.1, 28.7, 28.6, 28.5, 27.4, 27.1, 23.2, 22.7, 22.5, 22.4, 18.0, 14.0, 13.5 ppm; ESI-MS: *m/z* 1475.9 ($[M+Na]^+$) and HR-ESI-MS for $C_{86}H_{145}O_{12}N_6$ ($[M+H]^+$) Calcd: 1454.0915; Found: 1454.09412.

*Syntheses of compounds **1-6***

*Compound **1***. To a solution of **21** (49 mg, 0.037 mmol) in CH_2Cl_2 (2 mL) was added TFA (0.57 mL, 7.42 mmol). The resulting mixture was stirred at room temperature. The reaction was monitored with TLC (CH_2Cl_2/CH_3OH = 18/1, v/v). After 4 h, the reaction mixture was evaporated under reduced pressure. The obtained yellow residues were dissolved in MeOH (0.4 mL) and transferred to a centrifuging tube. Ammonia solution (25%, 12 mL) was then added and the resulting mixture was centrifuged for 10 min. The clear upper solution was disposed and water (12 mL) was added. The resulting mixture was re-centrifuged for 5 min and the clear upper solution was disposed. The obtained precipitates were dissolved in CH_3OH (1 mL) and concentrated under reduced pressure. The obtained residue was purified by chromatography on a silica gel column, eluted with a mixture of CH_3OH and $NH_3{\cdot}H_2O$ (150/1, v/v) to give compound **1** (23 mg, 67%) as a yellow solid. Mp 117-118 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.25 (s, 4H), 4.35 (s, 4H), 3.43 (br, 2H), 3.31 (br, 2H), 3.17 (br, 2H), 2.35-1.14 (m, 56H), 1.05 (s, 6H), 0.98 (s, 6H) , 0.81 (s, 6H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 174.9, 137.7, 127.3, 71.0, 54.3, 45.5, 42.3, 41.3, 38.9, 38.3, 35.3, 34.9, 34.5, 32.7, 32.6, 31.6, 29.7, 27.4, 25.9, 25.8, 22.9, 21.3, 16.1, 12.3 ppm; ESI-MS: *m/z* 914.4 ($[M+H]^+$) and negative HR-ESI-MS for $C_{56}H_{91}O_4N_6$ ($[M–H]^–$) Calcd: 911.71073; Found: 911.7078.

*Compound **2***. To a solution of **22** (83 mg, 0.062 mmol) in CH_2Cl_2 (2 mL) was added TFA (0.95 mL, 12.30 mmol). The resulting solution was stirred at room temperature. The reaction was monitored with TLC (CH_2Cl_2/CH_3OH = 15/1, v/v). After 5.5 h, the reaction mixture was evaporated under reduced pressure. The obtained yellow residues were dissolved in MeOH (0.4 mL) and added into ammonia solution (25%, 18 mL). The resulting yellowish precipitates were collected by filtration and re-dissolved in MeOH. The MeOH solution was evaporated under reduced pressure to afford **2** (34 mg,

59%) as a yellow solid. Mp 121-122 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.30-7.19 (m, 4H), 4.69-4.59 (m, 4H), 3.46-3.41 (m, 2H), 3.21-3.16 (m, 2H), 3.05-2.96 (m, 8H), 2.58-2.38 (m, 4H), 2.19-0.79 (m, 62H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 175.2, 174.7, 136.8, 136.5, 128.0, 127.7, 126.8, 126.4, 71.1, 54.1, 52.8, 50.1, 45.8, 41.6, 41.5, 39.2, 39.0, 35.4, 34.9, 34.6, 34.3, 33.5, 33.3, 30.9, 30.0, 29.8, 27.4, 26.4, 25.8, 23.0, 21.5, 16.2, 12.5 ppm; ESI-MS: *m/z* 941.7 ($[M+H]^+$) and HR-ESI-MS for $C_{58}H_{97}O_4N_6$ ($[M+H]^+$) Calcd: 941.75658; Found: 941.75702.

*Compound **3***. Procedure as described for **2**; from **23** (88 mg, 0.064 mmol). Yield: 37 mg (59 %), a yellow solid. Mp 122-123 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.30-7.19 (m, 4H), 4.67-4.60 (m, 4H), 3.52-3.37 (m, 6H), 3.19-3.14 (m, 2H), 3.03 (br, 2H), 2.56-2.29 (m, 4H), 2.21-0.78 (m, 68H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 174.7, 174.5, 137.3, 136.9, 127.9, 127.6, 126.7, 126.3, 71.1, 54.1, 50.4, 45.9, 41.7, 41.6, 39.3, 39.1, 35.4, 34.9, 34.6, 33.6, 31.5, 31.2, 29.8, 29.5, 29.3, 27.4, 26.5, 25.8, 23.0, 22.3, 21.6, 16.3, 12.6, 11.5 ppm; ESI-MS: *m/z* 969.7 ($[M+H]^+$) and HR-ESI-MS for $C_{60}H_{101}O_4N_6$ ($[M+H]^+$) Calcd: 969.78788; Found: 969.78821.

*Compound **4***. Procedure as described for **2**; from **24** (93 mg, 0.067 mmol). Yield: 48 mg (72%), a yellow solid. Mp 123-124 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.29-7.18 (m, 4H), 4.68-4.60 (m, 4H), 3.45-3.39 (m, 2H), 3.31-3.22 (m, 6H), 3.12 (br, 2H), 2.55-2.30 (m, 4H), 2.21-0.79 (m, 72H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 175.0, 174.6, 137.1, 136.8, 127.9, 127.6, 126.7, 126.2, 71.0, 54.1, 50.9, 49.2, 48.1, 45.7, 41.4, 39.1, 38.7, 35.4, 34.8, 34.6, 33.1, 31.4, 31.2, 29.7, 29.6, 27.4, 26.2, 25.8, 23.0, 21.5, 21.4, 20.3, 16.2, 12.4, 10.2 ppm; ESI-MS: *m/z* 997.8 ($[M+H]^+$) and HR-ESI-MS for $C_{62}H_{105}O_4N_6$ ($[M+H]^+$) Calcd: 997.81918; Found: 997.8183.

*Compound **5***. Procedure as described for **2**; from **25** (64 mg, 0.045 mmol). Yield: 29 mg (63%), a yellow solid. Mp 127-128 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.30-7.18 (m, 4H), 4.69-4.60 (m, 4H), 3.49-3.37 (m, 4H), 3.31-3.17 (m, 4H), 3.06 (br, 2H), 2.52-2.32 (m, 4H), 2.19-0.78 (m, 76H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 174.9, 174.6, 137.2, 136.9, 127.9, 127.6, 126.7, 126.3, 71.1, 54.1, 50.8, 46.2, 45.8, 41.6, 39.2, 38.9, 35.4, 34.9, 34.6, 33.5, 31.5, 31.2, 30.4, 29.8, 29.7, 29.3, 27.4, 26.4, 25.8, 23.0, 21.5, 19.8, 19.6, 16.3, 12.8, 12.5 ppm; ESI-MS: *m/z* 1026.0 ($[M+H]^+$) and HR-ESI-MS for $C_{64}H_{109}O_4N_6$ ($[M+H]^+$) Calcd: 1025.85048; Found: 1025.85156.

*Compound **6***. Procedure as described for **2**; from **26** (62 mg, 0.043 mmol). Yield: 29 mg (65%), a yellow solid. Mp 129-130 °C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.30-7.19 (m, 4H), 4.68-4.60 (m, 4H), 3.46-3.38 (m, 4H), 3.29-3.23 (m, 4H), 3.13 (br, 2H), 2.52-2.30 (m, 4H), 2.21-0.79 (m, 80H) ppm; ^{13}C-NMR (100 MHz, CD_3OD): δ 174.9, 174.6, 137.2, 136.9, 127.9, 127.6, 126.7, 126.3, 71.1, 54.1, 50.8, 46.4, 45.7, 41.4, 39.1, 38.6, 35.5, 35.3, 34.8, 34.5, 33.1, 31.5, 29.7, 29.3, 28.8, 28.6, 28.0, 27.4, 26.8, 26.2, 25.8, 23.0, 22.0, 21.4, 16.2, 12.9, 12.4 ppm; ESI-MS: *m/z* 1053.9 ($[M+H]^+$) and HR-ESI-MS for $C_{66}H_{113}O_4N_6$ ($[M+H]^+$) Calcd: 1053.88178; Found: 1053.87866.

Partitioning between 1-octanol and water

This experiment was conducted according to reported protocols. [22] Specifically, a given compound (1 mg) was partitioned between water-saturated *n*-octanol and *n*-octanol-saturated water in equal volume (0.5 mL each) and allowed to equilibrate at room temperature over 10 h. The aqueous and organic phases were separated, and aliquots (5.0 µL) of each phase were deposited onto a thin layer chromatography plate, dried and stained with ninhydrin alcohol solution (2%, wt). The plate was scanned and transformed into gray. The density of each spot was analyzed by gray value analysis of ImageJ2X software. The ratio of the gray values from organic and aqueous phases represents the oil-water partition coefficient *P*. Experiments were repeated twenty times and the average values were taken.

Measurement of chloride efflux and pH discharge activity

The preparation of vesicles and the measurement of the chloride efflux and pH discharge activity of compounds **1**-**6** were conducted using the protocols previously described by us and others. [16, 17]

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