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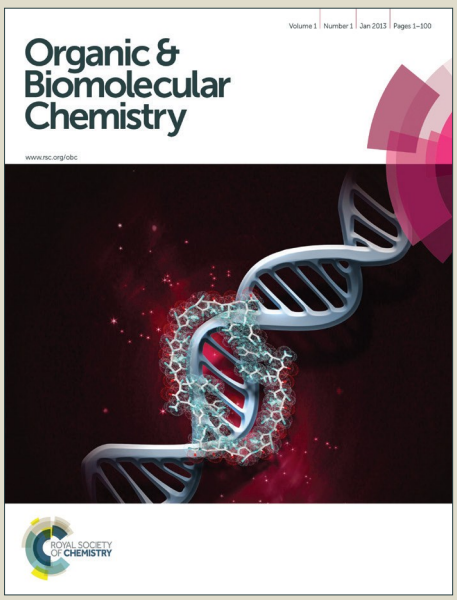

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ARTICLE TYPE

Does lipophilicity affect the effectiveness of a transmembrane anion transporter? Insight from squaramido-functionalized bis(choloyl) conjugates

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Six squaramido-functionalized bis(choloyl) conjugates were synthesized and fully characterized on the basis of NMR (^{1}H and ^{13}C) and ESI MS (LR and HR) data. Their transmembrane anionophoric activity was 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* proton/anion symport and anion exchange processes, and that lipophilicity in terms of *c*logP from 3.90 to 8.32 affects the apparent ion transport rate in a concentration-dependent fashion. Detailed kinetic analysis on the data obtained from both the chloride efflux and pH discharge experiments reveals that there may exist an optimum *c*logP range for the intrinsic ion transport rate. However, lipophilicity exhibits little effect on the effectiveness of this set of compounds in terms of either k_2/K_{diss} or EC_{50} values.

1. Introduction

Identification of small-molecule organic compounds that are capable of mediating the transport of anions, in particular chloride across lipid membranes has received increasing interest at the frontiers of chemistry and biology. [1] Such compounds, namely, anion transporters are thought to have high potentials as therapeutic agents for the treatment of channelopathies (e.g. cystic fibrosis) and cancers by replacing the activity of defective anion channels. [2] As a consequence, a number of effective anion transporters have been designed based on a diverse range of non-peptidic skeletons, such as (thio)ureas, [3] steroids, [4] squaramides, [5] prodigiosins and analogs, [6] calix[4]pyrroles, [7] imidazoliums, [8] cyclodextrins [9] and others. [1, 2] Interestingly, some of anion transporters exhibit potent anti-cancer and antimicrobial activity. [10]

With the aid of those successful endeavours, some key structural factors that control anion transport have been elucidated. Of them, lipophilicity, which is widely measured by a log*P* (the logarithm of octanol/water partition coefficient *P*) value, is recognized as one of the important parameters for maximising the transport rate for a given compound series. [11] It has been reported that carrier activity is influenced by lipophilicity with an optimal value of this parameter giving a maximum in transmembrane transport activity. [12] For example, Quesada *et al* have shown that for tamjamine class of transporters, there exists an optimum log*P* (~4.2±0.5) where the peak activity is observed. [12a] More recently, Gale *et al* have demonstrated that for alkyl-substituted phenylthioureas, though changes in the composition of the lipid bilayers tested affect the rate of transport, the ideal *c*log*P* (= 5~6) for peak activity does not change. [12b]

In this regard, we are concerned about how lipophilicity is correlated with the effectiveness of an anion transporter. In addition, the reported effect of lipophilicity on ion transport activity was assessed at a single concentration. [12a,b] The insights from such single point screening may be limited, because ion translocation through a lipid membrane is a complex event that involves and is controlled by multiple equilibria and steps. [8a, 13] In response to those issues and with the inspirations from our previous findings on squaramide-linked bis(choloyl) conjugate **A** [14] and amino-functionalized bis(choloyl) conjugate **B**, [15] we synthesized squaramido-functionalized bis(choloyl) conjugates **1**-**6** of varying lipophilicity (Fig. 1) and conducted a detailed study on the effect of lipophilicity on the ion transport activity.

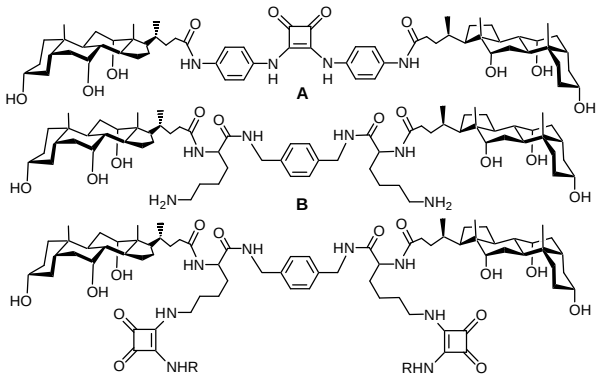


Figure 1. Structures of compounds **A**, **B** and **1**-**6**.

As such, the objective of the work reported herein was two-fold. Firstly, we have shown in previous studies that compound **A** exhibits potent anionophoric activity presumably *via* an anion-cation co-transport/symport process, [14] and that the ion transport activity of a functionalized bis(choloyl) conjugate (e.g. compound **B**) may be regulated by the functional groups. [15] These

observations, together with the findings by Gale *et al* that squaramide derivatives function as potent anion transporters, [5] encouraged us to explore the potentials of squaramido groups as the functional groups of a bis(choloyl) conjugate in facilitating the transmembrane transport of anions.

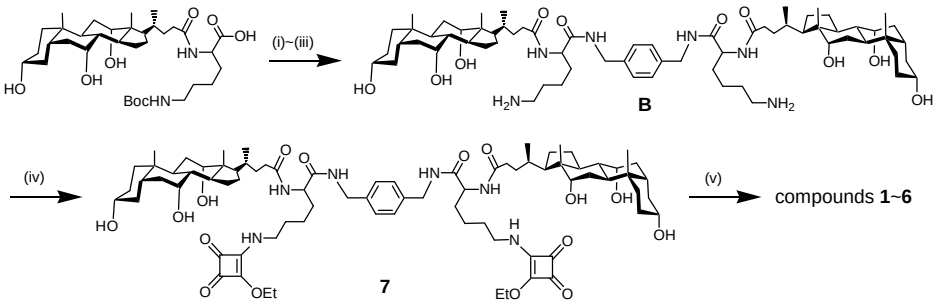
The second objective was to perform a systematic study on the effect of lipophilicity on the ion transport activity of compounds **1**-**6**. The variations in the alkyl substituents from methyl to n-hexyl groups gradually increase the lipophilicity in terms of *c*log*P* from 3.90 to 8.32. [16] Because these flexible alkyl chains are unbranched, they should have little effect on the interaction of anions with the squaramido subunits. These structural features make it feasible to assess how lipophilicity is correlated with the transport efficiency of compounds **1**-**6**.

Herein we report the synthesis of compounds **1**-**6** and their transmembrane anionophoric activity investigated by means of chloride ion selective electrode 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 depicted in Scheme 1. Thus, compound **B** was prepared from *N*-α-choloyl-*N*-ε-(*tert*-butyloxycarbonyl)-*L*-lysine [17] according to the protocols previously described by us [15] and reacted with excess 3,4-diethoxy-cyclobut-3-ene-1,2-dione in EtOH, in the presence of Et_3N, [5d] to afford compound **7** in 78% yield. Reaction of compound **7** with the corresponding alkylamines in the presence of zinc trifluoromethanesulfonate or Et_3N, gave compounds **1**-**6** in 45~84% yields. Compounds **1**-**6** were fully characterized on the basis of ESI MS (LR and HR) and NMR (^1H and ^{13}C) data (See experimental sections and Fig. S1~18 in the Supporting Information).



Scheme 1. Synthesis of compounds **1**-**6**. Reagents and conditions: (i) HOBt, DCC, THF; (ii) *p*-bis(aminomethyl)benzene, THF; (iii) HCl, MeOH; (iv) 3,4-diethoxy-cyclobut-3-ene-1,2-dione, Et_3N, EtOH; (v) RNH_2 (R = Me, Et, n-C_3H_7, n-C_4H_9, n-C_5H_{11} or n-C_6H_{13}), Et_3N or $Zn(CF_3SO_3)_2$, EtOH.

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

The anionophoric activity of compounds **1**-**6** across egg-yolk phosphatidylcholine (EYPC)-based liposomal membranes was investigated by means of chloride ion selective electrode technique and pH discharge assay. [18, 19]

To test whether compounds **1**-**6** are capable of mediating anion transport, we firstly measured chloride efflux from large unilamellar EYPC vesicles (100 nm diameter, extrusion) by using a chloride ion selective electrode. The 100% efflux was obtained by lysing the vesicles. The results are shown in Fig. 2a and S19 and indicate that compounds **1**-**6** are capable of facilitating the chloride efflux and that the rate shows strong concentration dependence. When the external sodium nitrate was replaced with sodium sulfate, the chloride efflux rate of compound **1** was enhanced, whereas those of compounds **2**-**6** were significantly inhibited (Fig. 2b and S21). These are indicative of a chloride/cation co-transport process for compound **1** and an anion exchange process for compounds **2**-**6**, because sulfate is a strongly hydrated anion and it is assumed that it cannot be readily transported across a lipid bilayer. [20]

To gain insight into the probable mechanism of action and ion selectivity of compounds **1**-**6**, we repeated the chloride efflux experiments in the presence of the chloride salts of group I alkali metal ions (Li^+, Na^+, K^+, Rb^+ and Cs^+). The results are shown in Fig. 3a and S22, and indicate that the chloride efflux activity of compounds **1**-**6** is essentially independent of group I alkali metal ions, excluding the role of those metal ions in the permeation process. [21] This result suggests that the chloride efflux was due to a proton/chloride co-transport process for compound **1**, whereas an anion exchange process for compounds **2**-**6**.

In addition, with the aim to investigate the ion selectivity of compounds **1**-**6** among anions, we carried out pH discharge experiments in the presence of sodium salts of different anions (i.e., NO_3^-, Cl^-, Br^- and I^-). In this test, a pH gradient is established across the liposomal membranes and the increase in the fluorescence of the entrapped pyranine indicates the transmembrane transport of proton or hydroxide. As shown in Fig. 3b and S23, addition of compounds **1**-**6** to EYPC liposomal dispersions containing an internal pH of 7.0 and an external aqueous phase of pH of 8.0, led to an increase in the pyranine fluorescence, indicating that these compounds were capable of inducing pH discharge across the membrane. The activity in the order of $I^- > Br^- \approx NO_3^- \approx Cl^-$ indicates that compounds **1**-**6** exhibit moderate selectivity for iodide over the other three monovalent anions. These data correlates well with a halide transport based on the lyotropic sequence (with the less hydrated anions transported more efficiently), [21] and imply that the pH

gradient decay correlates with OH^-/Cl^- antiport and H^+/Cl^- symport.

Finally, to gain insight into the probable mechanism of action of compounds **1-6**, we conducted pH discharge experiments using vesicles derived from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol. Because the condensing and ordering effects of cholesterol on the membrane slows the diffusion of an ion across a more rigid membrane, cholesterol assays have been frequently used as evidence for a mobile carrier mechanism in anion transport. [22] As shown in Fig. 4, compound **3** displayed significantly decreased pH discharge activity across lipid membranes derived from POPC with 30% cholesterol. 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* a mobile carrier mechanism. Of them, compound **1** acts mostly as a H^+/Cl^- symporter with a slight amount of Cl^-/NO_3^- antiport mechanism, whereas compounds **2-6** function mainly as anion exchangers.

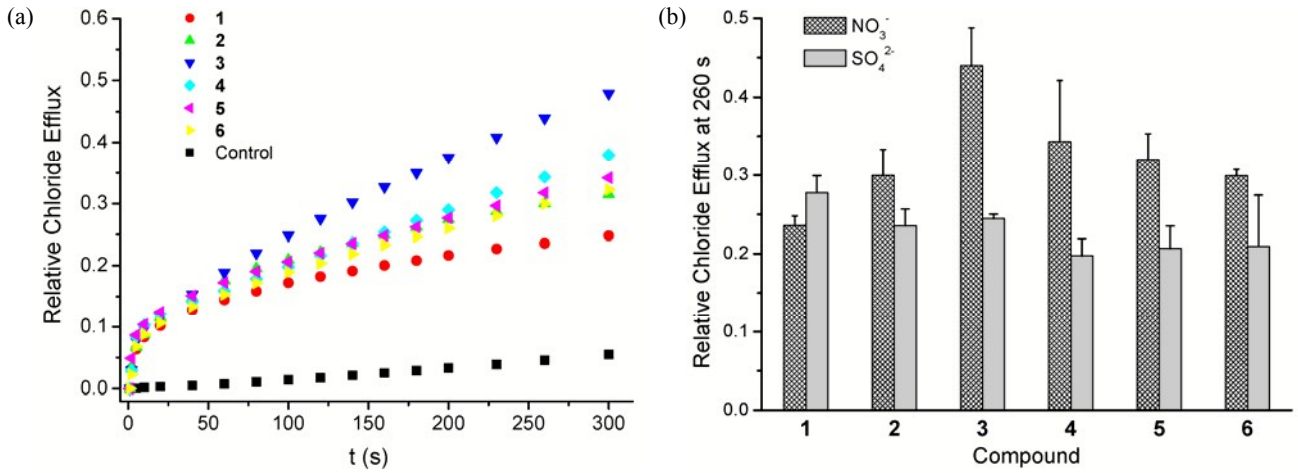


Figure 2. (a) Relative chloride efflux promoted by 5 mol% of compounds **1-6** in EYPC vesicles loaded with 500 mM NaCl buffered to pH 7.0 with 25 mM HEPES. The vesicles were dispersed in 500 mM $NaNO_3$ buffered to pH 7.0 with 25 mM HEPES. (b) Relative chloride efflux at 260 s, promoted by 5 mol% of compounds **1-6** in 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 experiment that was conducted in $NaNO_3$ media with DMSO was used as a control. These and the after-mentioned experiments were conducted at least three time and the average values were taken.

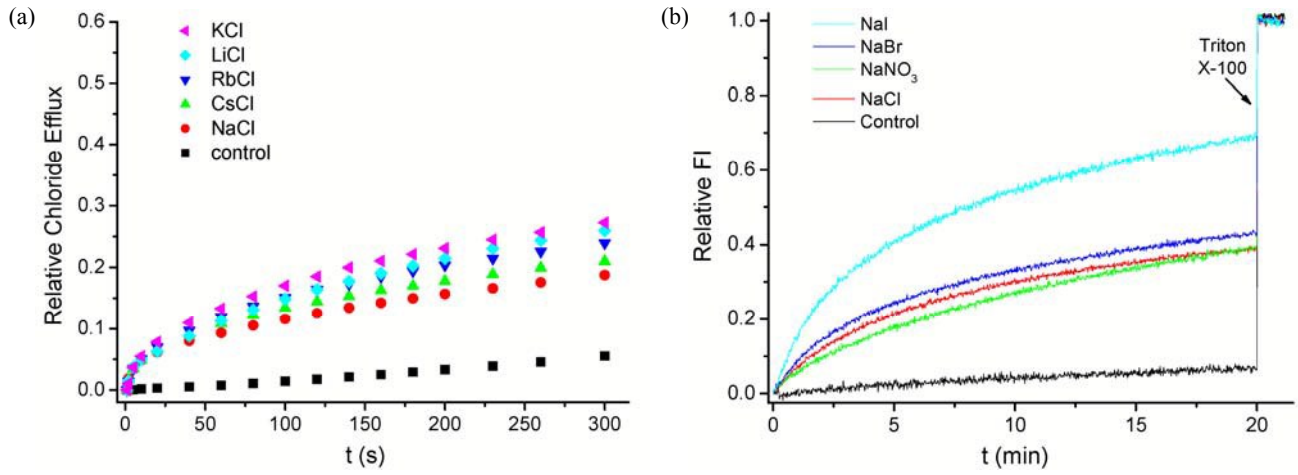


Figure 3. (a) Relative chloride efflux promoted by 3 mol% of compound **1** in EYPC vesicles loaded with 500 mM MCl (M = Li, Na, K, Rb and Cs) buffered to pH 7.0 with 25 mM HEPES. The vesicles were dispersed in 500 mM $NaNO_3$ buffered to pH 7.0 with 25 mM HEPES. (b) Discharge of a pH gradient by 3 mol% of compound **1** 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). Ex 460 nm; em 510 nm. The experiment that was conducted in NaCl media with DMSO was used as a control.

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

As shown above, compounds **1-6** are capable of mediating the transmembrane transport of chloride and inducing pH discharge, however their activity is apparently different. This is a likely consequence of the variation in the lipophilicity of compounds **1-6** caused by the structural variations in the alkyl substituents of the squaramido groups. [16] Therefore, to evaluate the effect of lipophilicity on the ion transport activity, we firstly analysed the relationship between the relative chloride efflux at 260 s of compounds **1-6** at each concentration and the *c*logP values. It is clear from Fig. 5a that lipophilicity affects the chloride efflux activity in a concentration-dependent fashion. Specifically, at low concentrations (for example, 1 mol% and 2 mol%), it appears that lipophilicity has little effect on the ion transport activity. When the concentration increases, the effect of lipophilicity becomes apparent. At 5 mol%, compound **3** in the series exhibits the peak activity. Thus, the ideal *c*log*P* appears to be around 5~6 for this set of compounds.

Because chloride selective electrode assay requires high concentrations of chloride within the vesicles to ensure an adequate signal and may not be as sensitive as fluorescence assay, [23] we measured the pH discharge activity of compounds **1-6** by using pyranine-based fluorescence assay (Fig. S24). In this case, the initial rate constants (k_{in}'s) at each concentration were plotted against the *c*log*P* values (Fig. 5b). As a consequence, similar observations were obtained, that is, the pH discharge activity was affected by the lipophilicity in a concentration-dependent fashion, and compound **3** is the most active one in this set of compounds.

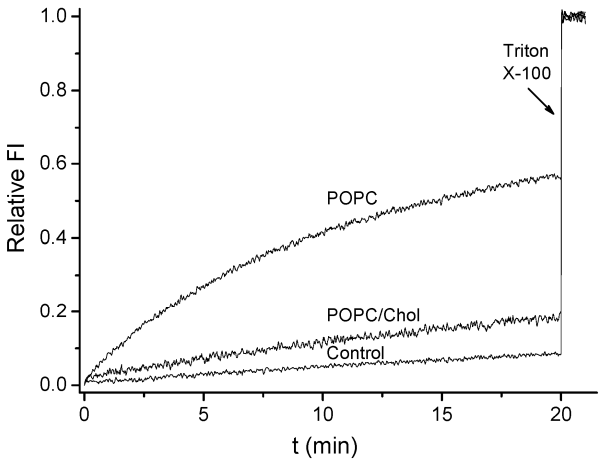


Figure 4. Discharge of a pH gradient promoted by 1 mol% of compound **3** across POPC and POPC-cholesterol (7/3)-based liposomal membranes. Measuring conditions for internal vesicles: 0.1 mM pyranine in 25 mM HEPES (50 mM NaCl, pH 7.0); and for external vesicles: 25 mM HEPES (50 mM NaCl, pH 8.0). Ex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

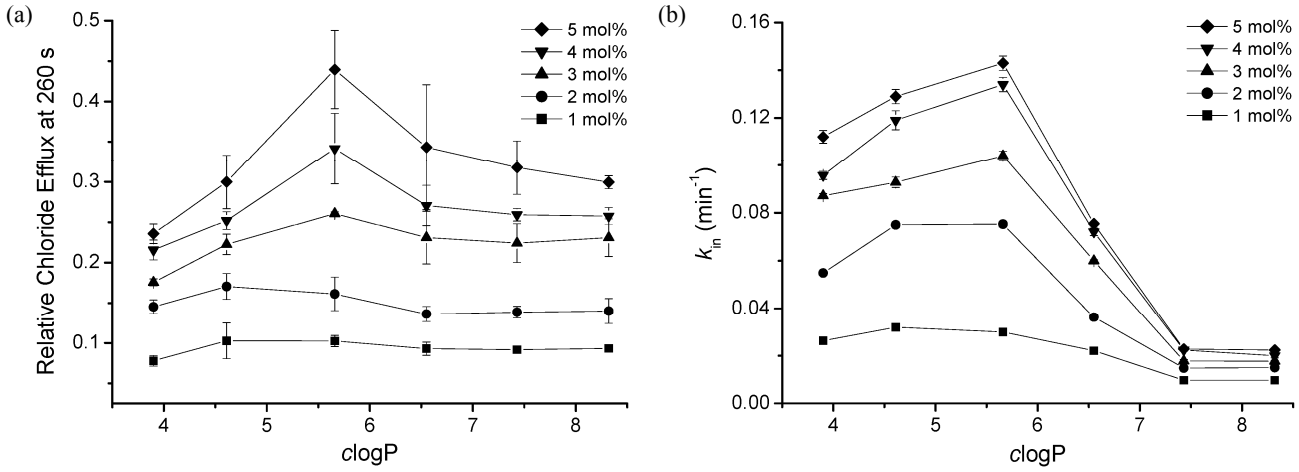


Figure 5. Plots of the relative chloride efflux at 260 s (a) and the initial rate constants (k_{in}'s, b) against the *c*logP of compounds **1-6** at different concentrations.

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

Compound	*c*logP [a]	Chloride selective electrode assay [b]		pH discharge [c]		
		n	k_2/K_{diss} ($s^{-1}\cdot mol\%^{-1}$)	n	k_{max} (min^{-1})	EC_{50} (mol%)
1	3.90	0.84±0.15	0.052±0.013	1.90±0.49	(1.34±0.28)×10^{-1}	2.62±0.63
2	4.61	0.81±0.08	0.069±0.010	1.67±0.44	(1.64±0.37)×10^{-1}	2.60±0.77
3	5.66	1.21±0.07	0.057±0.006	1.91±0.34	(1.76±0.24)×10^{-1}	2.58±0.41
4	6.55	1.08±0.13	0.054±0.013	1.96±0.72	(9.00±2.65)×10^{-2}	2.67±0.87
5	7.43	0.99±0.12	0.058±0.012	1.88±0.70	(2.17±0.68)×10^{-2}	2.74±1.00
6	8.32	0.89±0.15	0.065±0.017	1.62±0.37	(2.16±0.46)×10^{-2}	2.81±0.80

[a] For more details, see reference 16.
[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. S19-20).

[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. S24-25). The rate constant for the background (k_0) was $(6.67\pm0.97)\times10^{-3}$ min^{-1}.

In order to gain further insight into the effect of lipophilicity on the transporting activity, we performed detailed analysis on the kinetic data of compounds **1-6** obtained from both chloride selective electrode and pyranine assays. For the former assay, analysis of the relationship between the relative chloride efflux at 260 s and the concentrations of each of compounds **1-6** according to equation (1), [24] afforded the *n* value that reveals the stoichiometry of the transport process and the parameter k_2/K_{diss}, wherein k_2 is the intrinsic rate constant and K_{diss} is the dissociation constant of the self-association process (Fig. S25).

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

For the pyranine assay, nonlinear fitting of the initial rate constants (k_{in}'s) against the concentrations of each of compounds **1-6** according to equation (2), [25] gave the EC_{50} value of each compound that is defined as effective transporter loading that needs to reach 50% of the maximum rate (k_{max}) after a specified time period (Fig. S20).

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

In the above analyses, the k_2/K_{diss} and EC_{50} values are widely used to characterize the ion transport properties of an ionophore. The former parameter 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, [26] whereas the latter is a measure of activity of a given transporter in terms of effective concentration. [27]

The obtained parameters are listed in Table 1 and indicate that compounds **1-4** display similar rate constants that are 4~8-fold greater than those of compounds **5-6**, suggesting that there may exist an optimum *c*log*P* range for the intrinsic transport ability. However, all the compounds show comparable k_2/K_{diss} and EC_{50} values within experimental errors (Fig. 6), suggesting that for this set of compounds, the effect of lipophilicity on the effectiveness in terms of k_2/K_{diss} and EC_{50}, appears weak. There is another possibility that there exists no simple relationship between lipophilicity and the k_2/K_{diss} or EC_{50} values. [27]

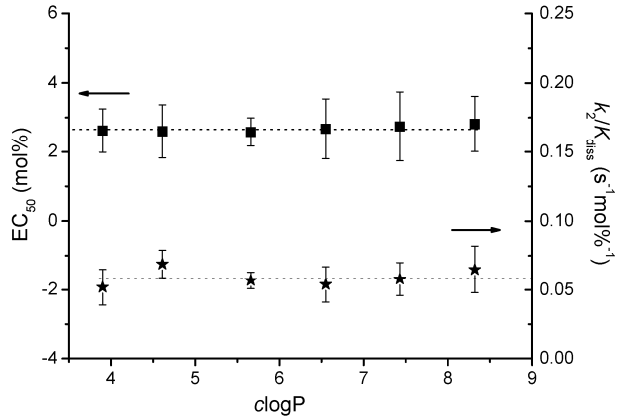


Figure 6. Plots of the EC_{50} (■) and k_2/K_{diss} (★) values against the *c*logP of compounds **1-6**.

Though the k_2 and K_{diss} terms cannot be separated, [26] the comparable k_2/K_{diss} values of compounds **1-6** reveal that both terms show the same variation trend. That is, increase in the lipophilicity lowers the mobility of these compounds within the lipid membrane (resulting in small k_2), but at the same time is favourable for the molecules to partition from the aqueous phase into the lipid bilayers (expressed as small K_{diss}). [12a] In a sense, this lipophilic/hydrophilic balance plays a crucial role in the efficient transport of anions by this set of compounds. [13]

In addition, the EC_{50} values (2.6~2.8 mol%) of compounds **1-6** are comparable to those obtained for bis-indolylureas (1.3~3.4 mol%), [22a] and smaller than that obtained for imidazolium salts (5.7 mol%). [26] Thus, compounds **1-6** are considered to be an effective class of anion transporters. However, it is noteworthy that compounds **1-6** are much less active than squaramide derivatives (EC_{50} = 0.01~1.38 mol%), [5d] steroidal squaramides (activity for chloride/nitrate exchange is observable at 0.004 mol%) [5b] and functionalized cholapods (activity for chloride/nitrate exchange is observable at 0.0004 mol%). [23, 28] This may be because compounds **1-6** are too large to readily shuttle within the lipid bilayers. The *n* values indicate that in the chloride selective electrode assay, compounds **1-6** do not aggregate to function. In the pyranine assay, on average, two molecules of each of compounds **1-6** are assembled into the transport-active species. This difference in the aggregation is simply due to the difference in the assay conditions.

3. Conclusions

In this study, we have successfully synthesized six bis(choloyl) conjugates functionalized with squaramido groups as the potential anion binding sites, 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 anionophoric 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* proton/anion symport and anion exchange processes and with moderate selectivity with respect to monoanionic ions. Detailed analysis of the effect of lipophilicity on the ion-transport activity indicates that there may exist an optimum *c*logP range for the intrinsic ion transport rate. However, lipophilicity has little effect on the effectiveness (in terms of k_2/K_{diss} and EC_{50}) of this set of compounds, or there may be no simple relationship between lipophilicity and the k_2/K_{diss} or EC_{50} values. This finding highlights a new vision for the impact of lipophilicity on the ion-transport activity, which is presently under active investigations in our laboratories by using anion transporters of other types. The results will be reported in due course.

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. Silica gel 60 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). The 100 nm polycarbonate membranes were Nuclepore track-etched polycarbonate membranes from Whatman (Florham Park, New Jersey, USA). Chloride efflux was measured on a Mettler-Toledo PerfectIon™ 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). 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 **7***

To a mixture of 3,4-diethoxy-cyclobut-3-ene-1,2-dione (64 mg, 0.38 mmol) and Et_3N (1.0 mL) in anhydrous EtOH (4.5 mL) was added compound **B** (176 mg, 0.15 mmol). The resulting mixture was stirred at room temperature and monitored with TLC (CH_2Cl_2/MeOH = 8/1, v/v). 2 h later, the reaction mixture was concentrated under reduced pressure. The obtained residue was purified by chromatography on a silica gel column, eluted with CH_2Cl_2/MeOH (8/1, v/v) to give compound **7** (166 mg, 78%) having ^{1}H NMR (400 MHz, CD_3OD) δ 7.25 (s, 4H), 4.77-4.70 (m, 4H), 4.42-4.33 (m, 6H), 3.98 (s, 2H), 3.82 (s, 2H), 3.61-3.58 (m, 2H), 3.45-3.37 (m, 4H), 2.38-1.00 (m, 72H), 0.94 (s, 6H), 0.72 (s, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 188.6, 188.5, 183.2, 183.0, 176.7, 176.2, 175.4, 173.3, 173.2, 172.8, 137.4, 127.2, 72.6, 71.4, 69.3, 67.6, 53.2, 48.4, 46.6, 46.1, 43.8, 43.7, 42.3, 41.8, 41.6, 39.6, 39.0, 35.6, 35.5, 35.1, 34.5, 32.4, 31.8, 31.7, 31.2, 29.8, 28.1, 27.3, 26.4, 22.8, 22.4, 21.8, 16.4, 14.8, 14.7, 11.6; ESI-MS: *m/z* 1444.8 ([M+Na]$^+$) and negative HR-ESI-MS for $C_{80}H_{119}O_{16}N_6$ ([M-H]$^-$) Calcd: 1419.86880; Found: 1419.86816.

*Synthesis of compounds **1-6***

*Compound **1***. To a solution of compound **7** (45 mg, 0.032 mmol) and zinc trifluoromethanesulfonate (5 mg, 0.013 mmol) in anhydrous EtOH (1.5 mL) was added methylamine (234 μL, 30-35% wt, 1.5-1.8 mmol). The resulting mixture was stirred at room temperature and monitored with TLC (CH_2Cl_2/MeOH = 8/1, v/v). 1 h later, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The obtained residue was purified by preparative TLC (CH_2Cl_2/MeOH, 5/1, v/v) to give compound **1** (20 mg, 45%) having ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD, 2/1, v/v) δ 7.18-7.17 (m, 4H), 4.37-4.25 (m, 6H), 3.94 (s, 2H), 3.81 (s, 2H), 3.55-3.54 (m, 4H), 3.41-3.39 (m, 2H), 3.25-3.23 (m, 6H), 2.34-0.93 (m, 66H), 0.89 (s, 6H), 0.67 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD, 2/1, v/v) δ 182.3, 182.0, 175.3, 172.7, 168.4, 137.1, 127.3, 77.5, 72.8, 71.4, 68.1, 49.3, 46.2, 43.7, 42.6, 41.5, 41.4, 39.4, 39.1, 35.6, 35.5, 35.1, 34.6, 34.4, 32.9, 31.8, 31.7, 30.6, 30.2, 29.8, 28.0, 27.5, 26.3, 23.0, 22.2, 16.9, 12.2; ESI-MS: *m/z* 1414.7 ([M+Na]$^+$) and negative HR-ESI-MS for $C_{78}H_{117}O_{14}N_8$ ([M-H]$^-$) Calcd: 1389.86947; Found: 1389.86536.

*Compound **2***. Procedure as described for compound **1**; from compound **7** (50 mg, 0.035 mmol) and ethylamine (500 μL, 30-35% wt, 2.3-2.7 mmol). Yield: 25 mg (50 %). ^{1}H NMR (400 MHz, CD_3OD) δ 7.24 (s, 4H), 4.42-4.32 (m, 6H), 3.97 (s, 2H), 3.82 (s, 2H), 3.65-3.61 (m, 8H), 3.43-3.39 (m, 2H), 2.37-1.03 (m, 72H), 0.94 (s, 6H), 0.72 (s, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 182.1, 182.0, 175.5, 175.4, 172.9, 168.0, 167.8, 137.4, 129.4, 127.2, 72.6, 71.4, 67.6, 53.4, 53.3, 46.7, 46.6, 46.1, 43.6, 42.3, 41.8, 41.6, 39.6, 39.0, 38.9, 35.6, 35.5, 35.1, 34.5, 32.5, 31.8, 31.7, 31.2, 30.3, 29.8, 29.4, 29.3, 29.2, 29.0, 28.9, 28.2, 27.3, 26.7, 26.4, 22.8, 22.4, 21.8, 16.4, 15.6, 11.7, 11.6; ESI-MS: *m/z* 1442.6 ([M+Na]$^+$) and negative HR-ESI-MS for $C_{80}H_{121}O_{14}N_8$ ([M-H]$^-$) Calcd: 1417.90077; Found: 1417.89722.

*Compound **3***. Procedure as described for compound **1**, except that Et_3N was used instead of zinc trifluoromethanesulfonate; from compound **7** (50 mg, 0.035 mmol) and n-propylamine (21 mg, 0.350 mmol). Yield: 42 mg (84 %). ^{1}H NMR (400 MHz, CD_3OD) *δ* 7.25 (s, 4H), 4.42-4.33 (m, 6H), 3.97 (br, 2H), 3.82 (br, 2H), 3.61 (br, 8H), 3.39 (br, 2H), 2.32-0.97 (m, 76H), 0.94 (s, 6H), 0.72 (s, 6H); ^{13}C NMR (100 MHz, *d*$_6$-DMSO) *δ* 182.7, 173.2, 172.3, 168.1, 138.3, 127.4, 71.4, 70.9, 66.7, 52.8, 46.6, 46.2, 45.4, 43.6, 42.2, 41.9, 41.7, 35.7, 35.6, 35.3, 34.8, 32.8, 32.1, 30.9, 30.8, 29.4, 29.0, 27.7, 26.6, 24.5, 23.2, 23.0, 22.9, 17.6, 12.7, 11.2; ESI-MS: *m/z* 1470.6 ([M+Na]$^+$) and negative HR-ESI-MS for $C_{82}H_{125}O_{14}N_8$ ([M-H]$^-$) Calcd: 1445.93207; Found: 1445.93152.

*Compound **4***. Procedure as described for compound **1**, except that Et_3N was used instead of zinc trifluoromethanesulfonate; from compound **7** (50 mg, 0.035 mmol) and n-butylamine (26 mg, 0.350 mmol). Yield: 29 mg (56 %). ^{1}H NMR (400 MHz, CD_3OD) *δ* 7.25 (s, 4H), 4.42-4.33 (m, 6H), 3.97 (br, 2H), 3.82 (br, 2H), 3.62 (br, 8H), 3.40 (br, 2H), 2.32-0.96 (m, 80H), 0.94 (s, 6H), 0.72 (s, 6H); ^{13}C-NMR (100 MHz, *d*$_6$-DMSO) *δ* 182.7, 173.2, 172.3, 168.2, 138.3, 127.4, 71.5, 70.9, 66.7, 52.8, 46.6, 46.2, 43.6, 43.4, 42.2, 41.9, 41.7, 35.7, 35.6, 35.3, 34.8, 33.2, 32.8, 32.1, 32.0, 30.9, 30.8, 29.0, 27.7, 26.6, 23.2, 23.0, 22.9, 19.4, 17.6, 13.9, 12.7; ESI-MS: *m/z* 1498.7 ([M+Na]$^+$) and negative HR-ESI-MS for $C_{84}H_{129}O_{14}N_8$ ([M-H]$^-$) Calcd: 1473.96337; Found: 1473.96228.

*Compound **5***. Procedure as described for compound **1**, except that Et_3N was used instead of zinc trifluoromethanesulfonate; from compound **7** (50 mg, 0.035 mmol) and n-pentylamine (31 mg, 0.350 mmol). Yield: 24 mg (45 %). ^{1}H NMR (400 MHz, CD_3OD) *δ* 7.25 (s, 4H), 4.42-4.33 (m, 6H), 3.97 (br, 2H), 3.82 (br, 2H), 3.62 (br, 8H), 3.40 (br, 2H), 2.32-0.97 (m, 84H), 0.94 (s, 6H), 0.72 (s, 6H); ^{13}C-NMR (100 MHz, *d*$_6$-DMSO) *δ* 182.7, 173.2, 172.3, 168.2, 138.3, 127.4, 71.5, 70.9, 66.7, 52.8, 46.6, 46.2, 43.6, 43.5, 42.2, 42.0, 41.8, 35.7, 35.6, 35.3, 34.8, 32.8, 32.1, 32.0, 30.9, 29.4, 29.0, 28.5, 27.7, 26.6, 23.2, 23.0, 22.9, 22.2, 17.6, 14.3, 12.7; ESI-MS: *m/z* 1527.7 ([M+Na]$^+$) and negative HR-ESI-MS for $C_{86}H_{133}O_{14}N_8$ ([M-H]$^-$) Calcd: 1501.99467; Found: 1501.99386.

*Compound **6***. Procedure as described for compound **1**, except that Et_3N was used instead of zinc trifluoromethanesulfonate; from compound **7** (66 mg, 0.046 mmol) and n-hexylamine (47 mg, 0.46 mmol). Yield: 55 mg (77 %). ^{1}H NMR (400 MHz, CD_3OD) *δ* 7.25 (s, 4H), 4.42-4.33 (m, 6H), 3.97 (br, 2H), 3.82 (br, 2H), 3.62 (br, 8H), 3.40 (br, 2H), 2.32-1.04 (m, 88H), 0.94 (s, 6H), 0.72 (s, 6H); ^{13}C NMR (100 MHz, *d*$_6$-DMSO) *δ* 182.7, 173.2, 172.3, 168.2, 168.1, 138.3, 127.4, 71.5, 70.9, 66.7, 52.8, 46.6,

46.2, 43.7, 43.6, 42.2, 42.0, 41.8, 40.8, 35.7, 35.6, 35.3, 34.8, 32.8, 32.1, 32.0, 31.4, 31.3, 31.1, 30.8, 30.7, 29.0, 27.7, 26.6, 26.2, 25.9, 23.2, 23.0, 22.8, 22.5, 17.6, 14.3, 12.7; ESI-MS: *m/z* 1555.7 ($[M+Na]^+$) and negative HR-ESI-MS for $C_{88}H_{137}O_{14}N_8$ ($[M-H]^-$) Calcd: 1530.02597; Found: 1530.02483.

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. [18, 19]

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Notes and references

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