Transport of chloride and carboxyfluorescein through phospholipid vesicle membranes by heptapeptide amphiphiles

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Seven synthetic anion transporters (SAT) of the general form $R_2N\text{-}COCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\text{-}OR'$ were prepared. Three pairs of compounds each contained twin *n*-hexyl, *n*-decyl, and *n*-octadecyl (R) groups at the N-terminus and one contained twin *n*-tetradecyl groups. Three of the compounds were C-terminated by benzyl and three by heptyl (R′) residues. The ability of these compounds to mediate ion release from phospholipid vesicles was assessed. Chloride release was measured by ion selective electrode measurements and by chloride quenching of the fluorescent dye lucigenin. Transport of the anion carboxyfluorescein (CF) was measured by fluorescence dequenching. Differences in both the C- (R′) and N-terminal (R) residues within the ionophores affected anion transport. The chloride release data acquired by ion selective electrode and fluorescence methods were similar but not identical. A possible carrier mechanism for Cl^- transport was discredited. Both Cl^- and CF anions were released from vesicles by these compounds. The results of CF and Cl^- transport showed good consistency when the ionophore's N-terminal chains were either decyl or octadecyl but not when they were hexyl. The transport of CF and Cl^- appears to be fundamentally different when R is C_6 compared to C_{10} or C_{18}. Differences between the behavior of SATs with Cl^- and CF were also reflected in negative ion mass spectrometric studies.

Introduction

Natural bilayer membranes exist in an almost infinite variety. The possibilities for different fatty acid tails and head groups alone in phospholipid monomers are enormous. More than that, natural membranes incorporate integral proteins, receptors, transporters, and a variety of small molecules such as sterols, sphingomyelin, ceramide, and others. During the past two decades, novel synthetic structures have been devised that transport cations, anions, and small molecules through bilayer membranes. These transporters may function as carriers or channels. In the latter case, they may form a unimolecular channel or an oligomeric pore. The challenge to the chemist to design, synthesize, and characterize compounds that have membrane active properties is significant. Beyond that, however, lies the requirement to demonstrate the function or efficacy of the new structure. Such methods as planar bilayer voltage clamp and whole cell patch clamp that are well known to electrophysiologists[1] require complex apparatus and considerable training.

A number of analytical approaches have been developed to evaluate ion transport through phospholipid bilayers. Notable among these are the NMR methods developed by Riddell and coworkers to detect lithium,[2] sodium,[3] and potassium[4] cations, and chloride anion.[5] Ion selective electrodes (ISEs) have been used to detect the egress of both cations[6] and anions from liposomes. Fluorescent methods have proved to be particularly popular, in part because of their sensitivity.[7]

In a recent series of papers, we described several properties of a family of synthetic anion transporters (SATs) that function in phospholipid bilayers. Our initial success was with an amphiphilic heptapeptide having the structure $(H_{37}C_{18})_2NCOCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\text{-}OCH_2Ph$, **1**.[8,9] Planar bilayer voltage clamp (BLM) experiments showed that **1** exhibited at least one conductance state that was selective for Cl^- over K^+ by ≥10-fold. Encouraging as these results were, the complexity of the planar bilayer method led us to assay most of the compounds prepared by monitoring anion release from liposomes. This can be done in a variety of ways. Release of Cl^- from vesicles was measured directly by using a chloride-selective electrode.[10,11] In addition, Cl^- complexation by **1** and several relatives was demonstrated by NMR methods.[12–14]

Recently, an alternative method has been developed that is based on a fluorescent dye, lucigenin.[15] It has already found application in other transporter systems.[16] The method relies upon the chloride-induced fluorescence quenching of lucigenin. Thus, the dye is enclosed within a liposome that is free of Cl^- and baseline fluorescence is observed. Addition of an anion transporter permits the entry of chloride from the external medium through the vesicular bilayer membrane. The resulting quenching is observed as a decrease in lucigenin fluorescence that is proportional to the amount of chloride transported. Whether an ion selective electrode or lucigenin is used analytically, it is Cl^- that is detected passing either into or out of the vesicle.

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A fluorescent method to assess pore formation and anion transport that has been used in a variety of biological applications is carboxyfluorescein (CF) release from liposomes.[17–21] Such studies often quantitate CF release but do not compare the results with the transport of other species. In several studies of the SAT family of transporters developed in our lab, we have used carboxyfluorescein release to assay anion transport through vesicular bilayers.[22–24] Other variations on this theme have also been reported.[25] The carboxyfluorescein (CF) release method relies on the self-quenching of encapsulated CF. When a pore forms in a liposome containing internal CF, its release is detected quantitatively as an increase in fluorescence.

Planar bilayer voltage clamp (BLM) experiments and anion release from liposomes differ in the information that can be obtained from them. The BLM experiment permits an assessment of one or a few pores that form in a planar membrane of limited dimensions. In fact, more than one type of pore may occur and be detected simultaneously and this will be manifested as different conductance states.[26] Release of anions such as chloride or carboxyfluorescein from liposomes gives an overall view of the process, but lacks the detail available from the BLM experiment. Indeed, in liposome experiments, the average of many pores (and perhaps many different pores) is reflected in the results. Because of the macroscopic nature of this measurement, the data show good reproducibility.

Hill plots[27] showed that pores formed by **1** had a molecularity of $\geq$2.[24] Additional support for a dimer pore was obtained by preparing derivatives in which two molecules of **1** were covalently linked at either the C- or N-terminal ends of the heptapeptide to give an analog of the presumed pore. Both "covalent analog dimers" were found to release Cl^- from liposomes more readily than twice the concentration of **1**.[28] Studies with molecular models suggested that a non-covalently linked dimeric pore should be 6–8 Å across, a size appropriate to accommodate Cl^- in its hydrated state. The size of Cl^- as the "free" ion is typically quoted at 3.5 Å. The hexahydrated ion (see below) was calculated to be ~6.5 Å in diameter.[29]

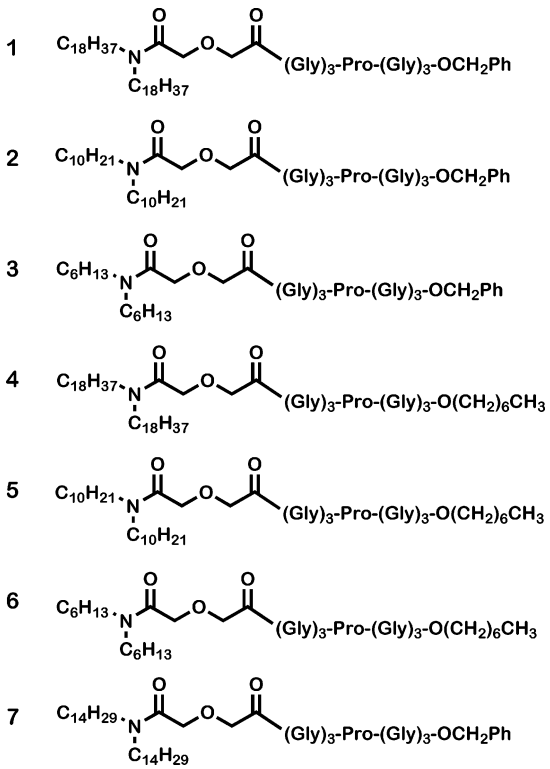
In the studies reported here, amphiphilic heptapeptide ionophores mediated transport of the ions from within phospholipid vesicles. Chloride ion release was assayed by both ion selective electrode methods and by use of the fluorescent dye lucigenin. Fluorescence was also used to quantitate carboxyfluorescein release. We report here an exploration of three issues. First, what is the effect on transport efficacy in changing the C- and N-terminal anchors of amphiphilic heptapeptides? Second, how comparable are the ion selective electrode and lucigenin methods for assaying chloride release? Third, do chloride and carboxyfluorescein anion release from vesicles give similar information about transport efficacy?

Results and discussion

We may consider the family of SATs described here in the semi-schematic form $(anchor)_2NCOCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\text{-}(ester)$. The N- and C-anchor (ester) designations represent a variety of chain lengths and both aromatic and aliphatic residues. We have previously reported the effect on Cl^- and CF release from liposomes of changes in the N- and C-terminal anchor chains. We concluded, based on those studies, that when the N-terminal anchor was bis(octadecyl), medium-sized C-terminal alkyl groups, such as *n*-heptyl, gave the highest anion release rates. In this report, we compare and contrast the effects of N- and C-terminal anchors on Cl^- and CF transport.

Compounds studied

Seven amphiphilic heptapeptides (**1–7**) were considered in the present study. Typically, a dialkylamine that will become the N-terminal anchor is heated with diglycolic anhydride to give the amphiphilic oxyacetic acid derivative apparent in structures **1–7**. This is coupled to a triglycine residue, then to proline, and finally to another triglycine. The latter is coupled on the N-terminal side to $(Gly)_3Pro$ and this product is protected on the C-terminal side by a residue that will become the C-terminal anchor.[10]



Compounds **1–4** and **7** have been described previously.[10] The syntheses of **5** and **6** are described in the Experimental section. All of the compounds incorporate the same $\sim COCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\sim$ sequence, which includes a heptapeptide and the midpolar regime mimic. These compounds comprise three pairs of structures that are identical except for the C-terminal anchor: **1**,**4**; **2**,**5**; and **3**,**6**. Compound **7** is intermediate in alkyl chain length between **1** and **2** and was used for mass spectrometric studies (see below).

Analytical methods

Anions such as Cl^- and CF are typically well-contained by liposomes. Transport through the phospholipid bilayer occurs with the assistance of a carrier molecule or through a pore formed by appropriate ionophores. Influx or egress of anions may be detected in a variety of ways. If anion release is studied, it is necessary to charge the liposomes with the anion of interest and free the surrounding medium of it. When the ionophore is added

to the liposome suspension, detection of the anion where it was previously absent is an indication of transport. In the lucigenin experiment, the liposomes contain no internal chloride, but are created in the presence of the dye to encapsulate it. Thus, the three analytical methods used are release of CF detected by fluorescence dequenching, Cl^- influx detected by lucigenin quenching, and chloride efflux detected by an ion selective electrode. In all cases, anion release was mediated by a member of the family **1–6**.

Chloride release from phospholipid vesicles: ISE method

Phospholipid liposomes (7 : 3 w/w DOPC–DOPA, see Experimental section) of approximate diameter 200 nm were prepared in the presence of KCl and HEPES buffer. External Cl^- was removed by gel filtration. The external solution contained K_2SO_4 buffer. The ionophore (**1–6**) was added to the liposome suspension as a 2-propanol solution and release of chloride was monitored by using an Accumet chloride combination electrode. The data obtained are graphed in Fig. 1.

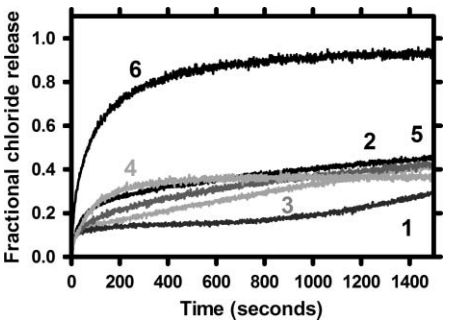


Fig. 1 Chloride ion release from DOPC–DOPA liposomes (310 μM lipids) mediated by **1–6**, [ionophore] = 65 μM.

The key observation in this set of experiments is that **6**, which has N-terminal hexyl groups and a C-terminal heptyl ester, is the most active ionophore. In fact, it is significantly more active than **1–5**, which all exhibit a generally similar transport efficacy. Because different ionophores exhibit different curve shapes, we have chosen an arbitrary time point to compare ion release. The values obtained for fractional chloride release from phospholipid liposomes, mediated by compounds **1–6**, are shown in Table 1.

Although the curves in the graph of Fig. 1 show that compounds **1–5** mediate similar levels of chloride release, the tabular data allow some distinction. The order of transport efficacy can be summarized as **6** > **2**, **3**, **5** > **1**, **4**. Two key features are apparent in these data. First, dihexylamide/heptyl ester **6** is significantly better at transporting Cl^- than is any of its relatives studied here.

Table 1 Percentage of chloride released from liposomes mediated by **1–6**[a]

Compound number	N-terminal alkyl group	Ester	% Cl^- release at 1500 s
1	Octadecyl	Benzyl	29
2	Decyl	Benzyl	45
3	Hexyl	Benzyl	42
4	Octadecyl	*n*-Heptyl	36
5	Decyl	*n*-Heptyl	43
6	Hexyl	*n*-Heptyl	92

[a] 3:7 DOPC:DOPA liposomes, 310 μM lipids and [ionophore] = 65 μM.

Second, the two poorest ionophores for Cl^- are **1** and **4**, which have twin N-terminal octadecyl groups. The two octadecyl derivatives, **1** and **4**, are clearly the poorest ion transporters, although their long alkyl chains suggest that, of the compounds in this group, they should form the most stable pores.

Chloride release from phospholipid vesicles: lucigenin assay

In recent work, Smith, Davis, and their coworkers[15] have used the fluorescent dye lucigenin to monitor chloride transport through a bilayer membrane. Lucigenin fluorescence is quenched by the presence of halide ions. In a typical application, the dye is encapsulated within a phospholipid vesicle in a chloride-free medium. Chloride ions are added to the external medium and chloride leakage is assessed.[16] When the transporter is encapsulated within the liposomes, addition of external chloride results in fluorescence quenching if Cl^- transport occurs. Detergent-induced vesicular lysis gives a final Cl^- concentration, to which the data are normalized.

In the studies concerning lucigenin, liposomes were prepared from DOPC phospholipids. The DOPC–DOPA mixture described for vesicles used in the ISE method afforded less reproducible results. The final lipid concentration was maintained at 0.31 mM. The liposomes (pH ~6) contained lucigenin (1 mM) and $NaNO_3$ (225 mM). Aqueous NaCl was incorporated to give a chloride gradient (190 mM external, 0 mM internal). The compound of interest was added as a 2-propanol solution and the change in fluorescence was monitored. A final Cl^- value was determined after detergent lysis.

As noted by Seganish, *et al.* for their system,[16] some Cl^- leakage was apparent in the absence of any ionophore. Significant fluorescence changes were noted subsequent to addition of the transporter. Chloride release data as a function of time observed for compounds **1–6** are shown in the graph of Fig. 2. Lucigenin fluorescence decreases as Cl^- is transported through the bilayer into the vesicular compartment containing the dye.

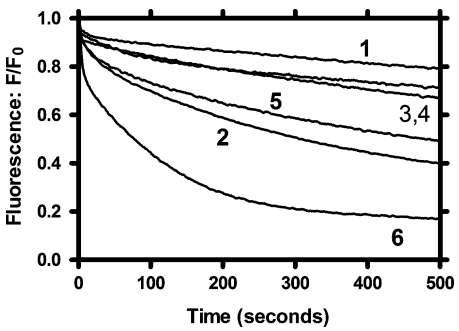


Fig. 2 Chloride ion release from DOPC vesicles (310 μM lipids) mediated by **1–6** (0.135 μmoles) assayed by lucigenin dye fluorescence.

It is interesting to compare the results of the Cl^- transport experiments shown in Fig. 1 and Fig. 2. In principle, of course, they should be identical because both the ISE and fluorescent dye methods purport to measure the same thing. In fact, they are only similar. Some differences are inevitable owing to experimental variation. A difference that should be noted is the lipid composition used to form the vesicles (DOPA–DOPC mixture *vs.* DOPC) in the two different cases. Chloride release mediated by **6** in

DOPA–DOPC vesicles, when assayed by the ISE method, is clearly most effective and poorest when the ionophore is **1**. We obtained the same result whether the ISE or the dye method (in DOPC vesicles) was used. In the data shown in Fig. 2, the release rates are clustered, with Cl^- release being similar for compounds **2** and **5**, and slightly greater than for **3** and **4**. When assayed by the ISE method (Fig. 1), Cl^- release by **2–5** was essentially similar. The two methods therefore give comparable results notwithstanding the differences in vesicle composition or concentration ratios.

Carboxyfluorescein release from vesicles

As noted in the introduction, CF release from phospholipid vesicles has been widely used to monitor pore formation and membrane stability. The rapidity of detection and sensitivity of the fluorescence technique make it useful for monitoring ion release at micromolar concentrations. The structure of carboxyfluorescein is shown in Fig. 3 both in its lactone form and with the free carboxyl protonated.

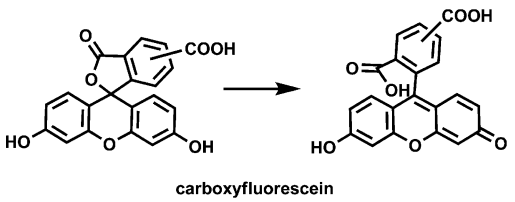


Fig. 3 Structures of carboxyfluorescein in the lactone (left) and open forms.

Release of carboxyfluorescein from phospholipid vesicles

Fig. 4 plots the results of CF release mediated by **1–6**. We note that the vesicles have the same lipid composition in this experiment as they do in the ISE method experiments. The time scale is different because, in part, the higher concentration of ionophore relative to lipids used in this experiment leads to faster ion release. It is obvious from the line trends that the order of relative release rates would not change if the times were identical. Differences in experimental conditions are required to achieve stability and to obtain reproducible results. Despite such differences, the trends apparent for both Cl^- and CF^- release have proved to be generally similar.[10]

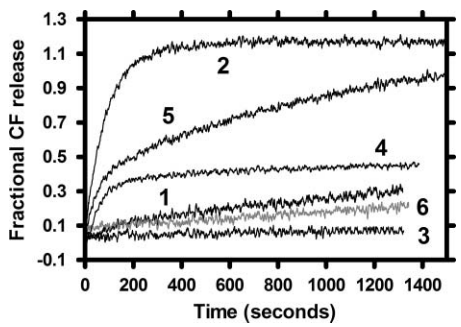


Fig. 4 Carboxyfluorescein release from 7 : 3 DOPC–DOPA vesicles mediated by **1–6**; [lipids] = 0.9 μM; [ionophore] = 3.5 μM.

The data obtained in these experiments may be compared in two different ways. Compounds **1–3** and **4–6** have the same C-terminal ester groups and vary in N-terminal chain length. The pairs of compounds **1**,**4**; **2**,**5**; and **3**,**6** have identical N-terminal chains, but differ in their C-terminal ester residues. For the benzyl ester compounds, decreasing CF release occurs in the following order: **2** > **1** > **3**. Expressed in terms of N-terminal side chains, this is didecyl > dioctadecyl > dihexyl. For the *n*-heptyl esters, the order is equivalent: **5** > **4** > **6** or didecyl > dioctadecyl> dihexyl. The magnitude of CF release is clearly different for the benzyl and heptyl esters, but the activity in both cases is $C_{10} > C_{18} > C_6$ in terms of N-terminal anchor chains.

The alternate series of comparisons gives the following results: **4** > **1**, **2** > **5**, and **6** > **3**. Thus, when the N-terminal side chains are dioctadecyl or dihexyl, the *n*-heptyl esters are more active than the benzyl esters. The situation is reversed for didecyl derivatives **2** and **5**, which are the most active compounds assessed in this study. A further comparison reveals that compounds **2**, **4**, and **5** are more active than are **1**, **3**, and **6**. It is significant that two of the least active CF-release compounds have hexyl anchors.

Carboxyfluorescein carrier transport through a $CHCl_3$ membrane

Evidence obtained in previous studies was consistent with CF release as a result of pore formation.[23] It is also possible that CF transport could occur in whole or in part by a carrier mechanism. Carboxyfluorescein release from liposomes is normally very rapid, suggesting that carrier transport is playing little or no role in ion release. We have not previously explored this possibility, however, nor has any assessment of host-CF transport by a carrier system been reported to our knowledge.

The transport experiments were conducted by using a concentric tube device of a type previously reported.[30] It was charged with $CHCl_3$, which serves as a hydrophobic membrane. An ion gradient was created by charging the aqueous source phase with CF, which was absent from the receiving phase. Two different experiments were undertaken with [ionophore] = 12 mM. In the first, the source phase was a neutral aqueous solution containing CF (20 mM), KCl (100 mM) and HEPES buffer (10 mM, pH 7). The receiving phase was identical except that CF was absent. This mimics the conditions of Cl^- release from vesicles. Chloride transport can occur simultaneously but there is no chloride gradient and hence no driving force for transport.

Buffer, but no chloride ion, was present in the second type of experiment. A previous study demonstrated that heptapeptides of the type $R_2N\text{-}COCH_2OCH_2CO\text{-}(Gly)_3\text{-}Pro\text{-}(Gly)_3\text{-}OR'$ bind Cl^- in $CDCl_3$ with an equilibrium constant of ~1700.[13] It was therefore possible that very little CF transport would be observed under carrier conditions because the host molecule was bound by Cl^- rather than CF^-. Of course, if the binding constant for $\mathbf{1}\cdot Cl^-$ is less than for $\mathbf{1}\cdot CF^-$, CF transport will dominate, but only in proportion to the respective complexation constants.

During the transport experiment, the receiving phase solution was continuously pumped (8 mL min^{-1}) into a cuvette within a Perkin Elmer LS 50B fluorimeter and the solution's fluorescence was monitored. The results, obtained over a period of 6 hours, are shown in Fig. 5. Transport of CF (20 mM in the source phase) was mediated by compounds **1** and **2**. The upper and middle traces show the result when 100 mM KCl is present in both the source

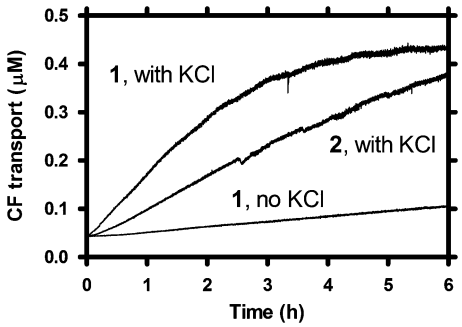


Fig. 5 Comparison of carboxyfluorescein (20 mM) transport mediated by **1** in the presence and absence of KCl (100 mM).

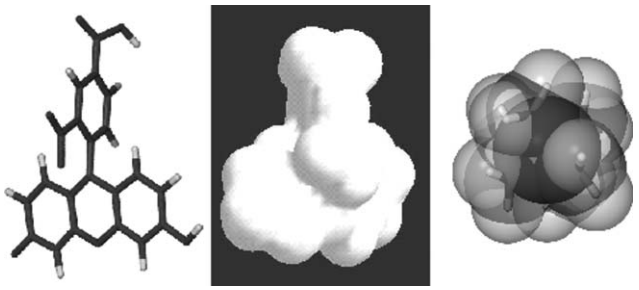


Fig. 6 Left panel: calculated structure of carboxyfluorescein. Center: calculated solvent exposed surface of CF. Right panel: calculated hydrated chloride ion.

and receiving phases. The lower trace shows the result when no KCl is present.

Two observations can be made about these experiments. First, the presence of KCl causes the transport rate of CF to be higher than when it is absent. This is true for either **1** or **2**. This is surprising and counter-intuitive. Second, the amount of CF transported during 6 h is extremely small. Carboxyfluorescein transport is ultimately limited by the amount present in the source phase. Only half of the initial 20 mM concentration will transport because there is no ion gradient when the source and receiving phase each reach 10 mM. In fact, over 5 hours, the receiving phase is only about 1 μM in CF in the absence of Cl^-. In the presence of Cl^-, the concentration reaches about 4 μM. The concentration ratios are such that well under 0.1% of the available CF is transported in 5 hours. We conclude that the carrier mechanism may occur but does not contribute significantly to CF release.

The structure of carboxyfluorescein

A goal of this study was to compare and contrast the release of chloride and carboxyfluorescein ions from liposomes mediated by **1**–**6**. Chloride ion is certainly transported through bilayer membranes by protein channels. Carboxyfluorescein release has been used to detect pore formation or the loss of membrane integrity. Chloride ion and carboxyfluorescein seem impossibly dissimilar in size and shape. In order to gain insight into similarities, we undertook a search of the Cambridge Structural Database. It failed to reveal any structure of CF although several structures of fluorescein derivatives were identified.

We therefore initiated a computational study of CF in its lactone and open forms, with the carboxyl groups *meta* or *para* to the xanthene-phenyl bond, and with the system in its neutral, monoanionic, or dianionic form. The computational package, Gaussian 03, was used in these studies. All structures were optimized using the DFT method (B3LYP/6-31g) without imaginary frequencies. The results are shown in the left panel of Fig. 6.

When the benzoic acid residue is lactonized to the xanthene, the two ring systems are calculated to be perpendicular. This is consistent with the published structure of fluorescein·acetone (CSD: FLSCAC).[31] In the ring-open form shown in Fig. 6, the torsion angle from the *ortho*-carboxyl group to xanthene is −84.9° (lower left panel). The corresponding torsion angle observed in the solid state structure of fluorescein perchlorate (CSD: BALZIK)[32] is 92°. Notwithstanding this deviation from perpendicularity, the approximate size of CF is similar whether lactonized or not. These calculations are for the gas phase and do not show the presence, or effect, of solvent. Even so, the results are consistent with the solid state structures reported for close relatives.

The center panel of Fig. 6 shows the calculated solvent exposed surface of CF at the left and compares it with an hydrated chloride anion. The right panel of the figure was generated by considering Cl^- to be a symmetrical, octahedrally hydrated anion. It was rendered in X-Seed[33] to achieve a format that accurately conveys its overall size. The circle apparent in the center is the van der Waals volume of the chloride anion. The $Cl(H_2O)_6]^-$ structure is nearly spherical and has a diameter of about 6.5 Å. Although CF looks far larger, it is no more than ~10 Å in any dimension, at least in the absence of solvent. We surmise that the carboxylate anion is strongly solvated by water but that hydration of the molecule's hydrophobic surface is less organized.

Comparison of anion transport

We considered two issues in comparing the results of the experiments presented here. The first is the difference in the anions. Chloride and CF are obviously different in size and shape and probably in solvation. Second, their charge densities and charge states differ. Indeed, the number of charges in CF is an issue because one or both carboxyls may be ionized. We use the reported pK_A value of 6.4 for the lactone. The experiments described here were conducted in HEPES buffer at pH = 7.0. Under these conditions, an acid of pK_A 6.4 would be approximately 80% ionized. Chloride will be completely ionized at neutral pH, but the solvation state of neither ion is known.

There is a further difference between the experimental determinations of CF and Cl^-, despite the effort to maintain comparability. The conditions used for the CF experiments are 0.9 μM lipids and 3.5 μM solutions of ionophores compared to 310 μM lipids and 65 μM solutions of ionophores for the Cl^- determinations. The excess of ionophore is required for experimental reasons in the CF release experiments. The fact that different concentration ratios are required suggests that the transport process is different. Notwithstanding, we have generally observed a good correlation between data obtained by the two methods. For example, we directly compared Cl^- and CF release from liposomes mediated by C_8 to C_{18} variants of $(C_n)_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph and found the parallel almost exact.[10] Of course, the present case extends the question to different esters, but more importantly, to the hexyl N-terminal group.

Table 2 Summary of ion release data obtained for **1–6** by various methods

	Terminal groups		% Ion release[a] (rank)[b]		
Compound number	N-chain	Ester	Cl^- [by ISE]	Cl^- [by dye]	CF^-
1	C_{18}	OCH_2Ph	32 (6)	22 (6)	19 (4)
2	C_{10}	OCH_2Ph	49 (2)	90 (2)	100 (1)
3	C_6	OCH_2Ph	45 (4)	50 (5)	7 (6)
4	C_{18}	C_7H_{15}	39 (5)	62 (4)	37 (3)
5	C_{10}	C_7H_{15}	47 (3)	85 (3)	72 (2)
6	C_6	C_7H_{15}	100 (1)	100 (1)	16 (5)

[a] Normalized for data points at 1000 s for CF and 1500 s for Cl^-. [b] Anion transport efficacy rank within the series for the method.

A direct comparison of the data can be made by considering release at fixed time points. Table 2 was prepared from data obtained for CF or Cl^- release. An arbitrary time point was chosen in each case. The time points were: ISE-chloride, 1500 s; lucigenin-chloride, 500 s; and carboxyfluorescein dequenching, 1000 s. The data shown in Table 2 were normalized in each case so that release by the most effective ionophore was set to 100%. This expanded the scale to facilitate comparisons. This approach fosters the problem of increasing the apparent difference between compounds that may be essentially similar and the data should be considered with this in mind. Adjacent to each percent value, the rank within the series is given in parentheses.

The data recorded in Table 2 show that the first compound prepared in this series, **1**,[8] is at or near the bottom of ion transporter efficacy as judged by any of the three methods. Compound **2**, which was previously found to be much more effective as a chloride transporter than **1**,[24] is the best of the benzyl ester compounds. Compound **2**, as a chloride transporter, is superior to other benzyl ester compounds in this study although only slightly better than its counterpart *n*-heptyl ester, **5**. Compounds **2** and **5**, which have twin, *N*-terminal decyl chains, are also the most effective CF transporters.

The variations in analytical methodology notwithstanding, significant differences in ion transport are observed as the N- and C-terminal chains are varied. Both Cl^- and CF release from liposomes appear to be very favorable when $(C_{10}H_{21})_2N$-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph, **2**, is added to the suspension. The results obtained for compound **6**, $(C_6H_{13})_2N$-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$O(CH_2)_6CH_3$, are surprising. Chloride release is highest when it is the ionophore but CF release is second lowest. It was already known that the C-terminal heptyl ester gave Cl^- release results superior to those observed for the corresponding benzyl esters. Two results are surprising concerning **6**. First, it was not expected that **6** would be superior in Cl^- transport to the other five compounds. That dihexyl-heptyl **6** is so much more effective at Cl^- release than is dihexyl-benzyl **3** was also unanticipated. It is unclear why the combination of two hexyl and a heptyl group should be so effective for Cl^- release and so unfavorable for CF release.

The data of Table 2 have been plotted in Fig. 7 with the omission of compounds **3** and **6**. These two structures have N-terminal hexyl chains and their interaction with CF is clearly different from the other ionophores studied here. There is general consistency between the ISE and dye methods used to measure Cl^- transport, although the ISE method gives a somewhat more compressed data set. Carboxyfluorescein is clearly subject to a different set of variables, although the data obtained for the octadecyl and decyl compounds is consistent with data obtained for Cl^- release. It is only the six-carbon sidechained ionophores **3** and **6** that behave very differently with CF than with Cl^-. A possible, but speculative, explanation for this is that the hexyl sidechains do not anchor effectively in the bilayer and therefore interact more strongly with the hydrophobic surface of CF. In principle, of course, any of the methods can be used within any given series. The challenge is comparing the results when the liposomal compositions, concentrations, and detection methods all differ.

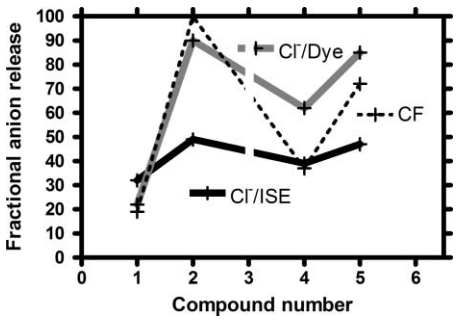


Fig. 7 Comparison of ion release from vesicles mediated by **1**, **2**, **4**, and **5**. The blank area in each graph indicates the omission of data for **3**.

Electrospray mass spectrometry

In order to gain additional insight about the SAT-anion complexation process, we undertook a negative ion (low resolution) mass spectrometric analysis (see Experimental section). The upper panel of Fig. 8 shows the spectrum observed for a 1 : 1 : 1 mixture of $(H_{21}C_{10})_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2Ph$ (**2**), $(H_{13}C_6)_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OC_7H_{15}$ (**6**), and Bu_4NCl. The spectrum was scanned over the mass range (*m*/*z*) 600–1529 (600–1200 shown). The base peak in the spectrum was observed at *m*/*z* 873.2, which corresponds to $[\mathbf{6}\cdot Cl]^-$. A peak (93%) was observed at *m*/*z* 977.0, which corresponds to $[\mathbf{2}\cdot Cl]^-$. The masses of non-ionized **6** and **2** are 838.5 and 950.6, respectively. Both peaks show the expected isotope distributions for chloride in the respective complexes. The spectrum shows that both SATs effectively bind Cl^- in the gas phase and in CH_3CN, the solvent in which the complexation initially occurs. The fact that the **2**–**6**–Cl^- ratio was 1 : 1 : 1 means that **2** and **6** must compete for Cl^-. The nearly identical peak heights for $[\mathbf{2}\cdot Cl]^-$ and $[\mathbf{6}\cdot Cl]^-$ suggest similar complex stabilities under these conditions. When detected by lucigenin quenching, the transport of Cl^- by **2** and **6** were similar.

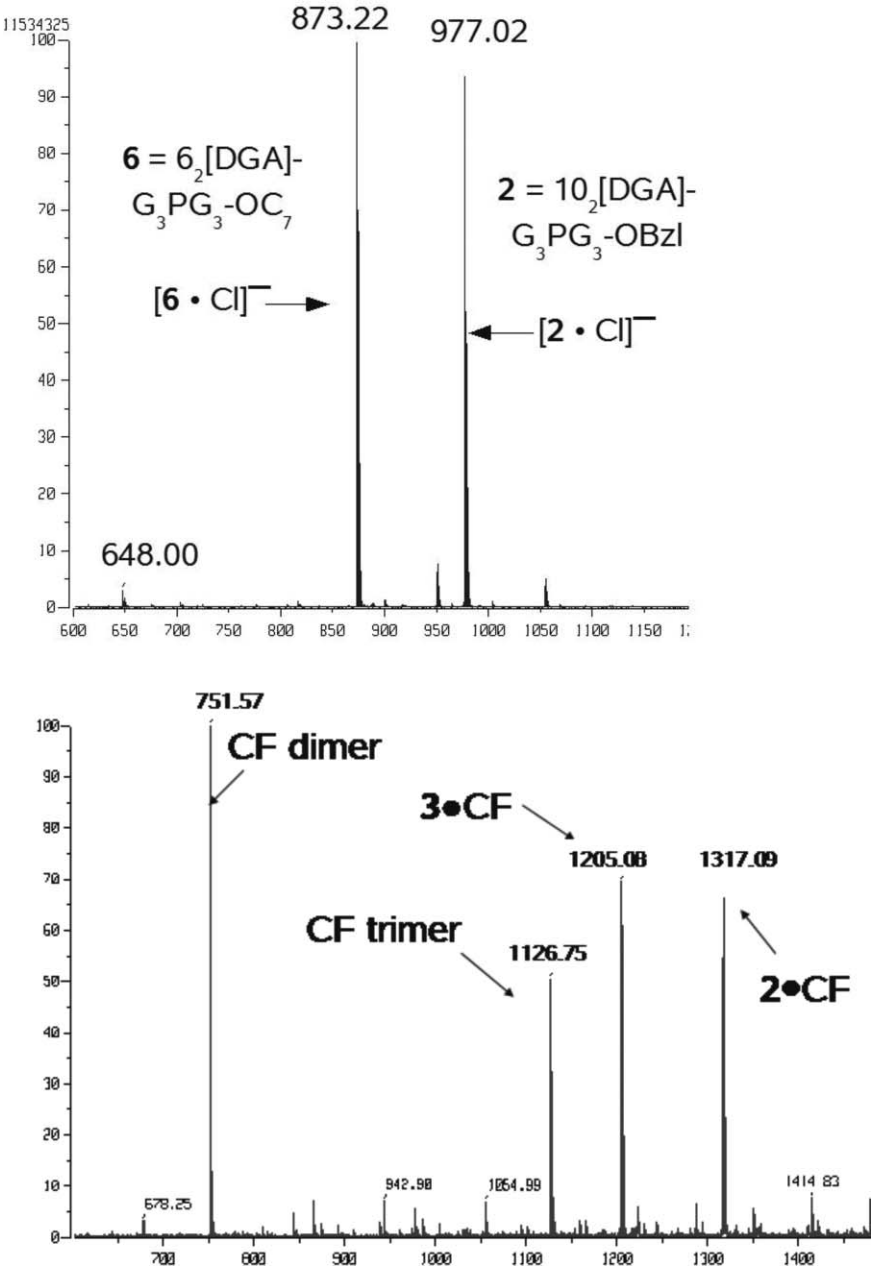


Fig. 8 Top panel: negative ion mass spectrum of **2**–**6**–Bu_4NCl in CH_3CN in a ratio of 1 : 1 : 1. Lower panel: negative ion mass spectrum of **2**–**3**–carboxyfluorescein (CF) in CH_3CN in a ratio of 1 : 1 : 4. [DGA] represents diglycoyl; 10_2[DGA]G_3PG_3OBzl represents $(C_{10}H_{21})_2$N-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph.

The lower panel of Fig. 8 shows results obtained when benzyl ester SATs **2** and **3** were sprayed in CH_3CN with carboxyfluorescein. The use of compound **2** in both studies provides a point of comparison. In this case, a 1 : 1 : 4 mixture of $(C_{10}H_{21})_2$N-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph (**2**), $(C_6H_{13})_2$N-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OCH_2Ph (**3**), and carboxyfluorescein in CH_3CN were sprayed as described above. The base peak in this spectrum is observed at *m/z* 751.6, which corresponds to $[(CF)_2]^-$. A substantial peak (49% of base) is also observed at *m/z* 1126.8 that is assigned to $[(CF)_3]^-$. Two additional prominent peaks are observed at *m/z* 1205.1 (69%) and 1317.1 (66%). These correspond to $[\mathbf{3}\cdot CF]^-$ and $[\mathbf{2}\cdot CF]^-$, respectively. Thus, a considerable difference is observed in the ability of SATs to complex Cl^- and CF^-. Table 2 shows that CF^- transport by **2** and **3** is significantly different. Since complexation, expected to principally involve the heptapeptide, is similar, we surmise that aggregation involving the hydrocarbon chains and CF or its aggregates plays a significant role in transport.

Conclusion

The results obtained in this study show that assay of Cl^- release from liposomes will give generally similar, but not identical, results if determined by ISE or lucigenin detection methods.

The results obtained for carboxyfluorescein release are consistent with previous studies and consistent with Cl^- release for longer-chained ionophores. These studies also show that $(C_6H_{13})_2N$-$COCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$O(CH_2)_6CH_3$, **6**, is superior to longer-chained relatives in Cl^- transport but not effective at all in releasing CF from liposomes. A limited study has shown that a carrier mechanism is not likely for transport in these cases but not all compounds have been examined. Thus, the length and disposition of alkyl chains must impact either the interaction between host and guest or pore formation and stability. Studies are in progress to gain insight into the latter question.

Experimental

General

^{1}H-NMR spectra were recorded at 300 MHz in $CDCl_3$ and are reported in ppm (δ) downfield from internal $(CH_3)_3Si$. ^{13}C-NMR spectra were recorded at 75 MHz in $CDCl_3$ unless otherwise stated. Infrared spectra were recorded on a Perkin-Elmer 1710 Fourier Transform Infrared Spectrophotometer and were calibrated against the 1601 cm^{-1} band of polystyrene. Melting points were determined on a Thomas Hoover apparatus in open capillaries and are uncorrected. Thin layer chromatographic (TLC) analyses were performed on aluminium oxide 60 F-254 neutral (Type E) with a 0.2 mm layer thickness or on silica gel 60 F-254 with a 0.2 mm layer thickness. Preparative chromatography columns were packed with activated aluminium oxide (MCB 80–325 mesh, chromatographic grade, AX 611) or with Kieselgel 60 (70–230 mesh).

All reactions were conducted under dry N_2 unless otherwise stated. All reagents were the best (non-LC) grade commercially available and were distilled, recrystallized, or used without further purification, as appropriate. Molecular distillation temperatures refer to the oven temperature of a Kugelrohr apparatus. Combustion analyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and are reported as percents. Where water is factored into the analytical data, spectral evidence is presented for its presence.

In the experimental descriptions below, $R_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-OR' may be abbreviated as R_2[DGA]-GGGPGGG-OR', in which [DGA] represents the diglycolic acid unit and R is the chain length of the dialkylamine.

$[CH_3(CH_2)_{17}]_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2C_6H_5$, 1

was prepared as previously described.[10]

$[CH_3(CH_2)_9]_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2C_6H_5$, 2

was prepared as previously described.[10]

$[CH_3(CH_2)_5]_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2C_6H_5$, 3

was prepared as previously described.[10]

$[CH_3(CH_2)_{17}]_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$O(CH_2)_6CH_3$, 4

was prepared as previously described.[10]

$[CH_3(CH_2)_9]_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$O(CH_2)_6CH_3$, 5.

10_2[DGA]-GGGPGGG-OH. 10_2[DGA]-GGGPGGG-OCH_2Ph (0.26 g, 0.27 mmol) was dissolved in absolute EtOH (20 mL) and 10% Pd/C (0.1 g) was added, and this mixture was shaken under 60 psi pressure of H_2 for 3 h. The reaction mixture was heated to reflux and filtered (Celite pad). The solvent was evaporated to afford a crude product, which was used in the next step without further purification.

10_2[DGA]-GGGPGGG-OC_7H_{15}. To 10_2[DGA]-GGGPGGG-OH (0.24 g, 0.28 mmol) suspended in CH_2Cl_2 (30 mL) were added 1,3-diisopropylcarbodiimide (0.05 mL, 0.36 mmol) and DMAP (0.02 g, 0.14 mmol), and the mixture was stirred at room temperature. After 0.5 h, 1-heptanol (0.04 mL, 0.28 mmol) was added and the reaction was stirred at room temperature for 48 h. The reaction mixture was evaporated *in vacuo*, and the residue was chromatographed (SiO_2, 2–10% MeOH–$CHCl_3$) and afforded a white solid (0.15 g, 53%), mp 124–125 °C. ^{1}H NMR: 0.85–1.00 (9H, overlapping signals due to $-CH_2CH_3$), 1.25–1.50 (36H, overlapping signals due to $CH_3(CH_2)_4CH_2CH_2O$ and $CH_3(CH_2)_7CH_2CH_2N$), 1.5–1.7 (6H, overlapping signals due to $CH_3(CH_2)_7CH_2CH_2N$ and $CH_3(CH_2)_4CH_2CH_2O$), 1.9–2.25 (4H, m, Pro $NCH_2CH_2CH_2$), 3.07 (2H, t, J = 7.5 Hz, $CH_3(CH_2)_7CH_2CH_2N$), 3.26 (2H, t, J = 7.5 Hz, $CH_3(CH_2)_7CH_2CH_2N$), 3.50–4.30 (21H, overlapping signals due to Pro $NCH_2CH_2CH_2$, Gly NCH_2, $COCH_2O$, $CH_3(CH_2)_4CH_2CH_2O$ and Pro NCH), 7.6 (1H, bs, Gly CO*NH*), 7.7 (1H, bs, Gly CO*NH*), 7.91 (1H, bs, Gly CO*NH*), 8.0 (1H, bs, Gly CO*NH*), 8.27 (1H, bs, Gly CO*NH*), 8.4 (1H, bs, Gly CO*NH*). ^{13}C NMR: 14.0, 14.1, 22.5, 22.6, 25.1, 25.8, 26.9, 27.0, 27.6, 28.4, 28.8, 28.9, 29.1, 29.3, 29.4, 29.5, 29.6, 31.6, 31.8, 41.2, 41.9, 42.6, 42.8, 43.4, 46.4, 47.0, 61.3, 65.7, 69.3, 71.2, 168.6, 168.9, 170.1, 170.3, 170.4, 170.9, 171.2, 173.5. IR ($CHCl_3$): 3302, 2925, 2854, 1745, 1655, 1549, 1466, 1377, 1337, 1247, 1206, 1129, 1029 cm^{-1}. High resolution mass spectral analysis for $C_{48}H_{86}N_8O_{11}$. Theoretical: $[MNa]^+$ 973.63141. Found: 973.6314.

$[CH_3(CH_2)_5]_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$O(CH_2)_6CH_3$, 6.

6_2-[DGA]-GGGPGGG-OH. 6_2-[DGA]-GGGPGGG-OCH_2Ph (0.37 g, 0.45 mmol) was dissolved in absolute EtOH (20 mL) and 10% Pd/C (0.2 g) was added, and this mixture was shaken under 60 psi pressure of H_2 for 3 h. The reaction mixture was heated to reflux and filtered (Celite pad). The solvent was evaporated to afford a crude product, which was used in the next step without further purification.

6_2[DGA]-GGGPGGG-OC_7H_{15}. To 10_2[DGA]-GGGPGGG-OH (0.31 g, 0.42 mmol) suspended in CH_2Cl_2 (30 mL) were added 1,3-diisopropylcarbodiimide (0.09 mL, 0.55 mmol) and DMAP (0.03 g, 0.21 mmol), and the mixture was stirred at room temperature. After 0.5 h, 1-heptanol (0.06 mL, 0.42 mmol) was added and the reaction was stirred at room temperature for 48 h. The reaction mixture was evaporated *in vacuo*, and the residue was

chromatographed (SiO_2, 10% MeOH–$CHCl_3$) and afforded a white solid (0.31 g, 88%), mp 120–121 °C. ^{1}H NMR: 0.85–0.95 (9H, overlapping signals due to –CH_2CH_3), 1.25–1.40 (16H, overlapping signals due to $CH_3(CH_2)_2CH_2CH_2O$ and $CH_3(CH_2)_3$-CH_2CH_2N), 1.5–1.7 (6H, overlapping signals due to $CH_3(CH_2)_3$-CH_2CH_2N and $CH_3(CH_2)_4CH_2CH_2O$), 1.9–2.25 (4H, m, Pro $NCH_2CH_2CH_2$), 3.08 (2H, t, $J = 7.5$ Hz, $CH_3(CH_2)_3CH_2CH_2N$), 3.27 (2H, t, $J = 7.5$ Hz, $CH_3(CH_2)_3CH_2CH_2N$), 3.50–3.75 (2H, m, Pro $NCH_2CH_2CH_2$), 3.7–4.15 (16H, overlapping signals due to Gly NCH_2, $COCH_2O$, and $CH_3(CH_2)_4CH_2CH_2O$), 4.30 (2H, s, $COCH_2O$), 4.36 (1H, t, $J = 6.5$ Hz, Pro NCH), 7.37 (1H, t, $J = 6.0$ Hz, Gly $CONH$), 7.51 (1H, t, $J = 6.0$ Hz, Gly $CONH$), 7.85 (1H, t, $J = 6.0$ Hz, Gly $CONH$), 7.89 (1H, t, $J = 6.0$ Hz, Gly $CONH$), 7.96 (1H, t, $J = 6.0$ Hz, Gly $CONH$), 8.31 (1H, t, $J = 6.0$ Hz, Gly $CONH$). ^{13}C NMR: 14.0, 14.1, 22.5, 25.2, 26.5, 26.7, 27.5, 28.5, 28.8, 28.9, 29.0, 31.4, 31.5, 31.7, 41.2, 41.9, 42.8, 42.9, 46.2, 46.8, 61.2, 65.6, 69.6, 71.5, 168.4, 168.7, 170.0, 170.2, 170.6, 171.7, 173.3. IR ($CHCl_3$): 3306, 2955, 2928, 2857, 1748, 1657, 1543, 1454, 1410, 1377, 1336, 1245, 1200, 1129, 1029 cm^{-1}. High resolution mass spectral analysis for $C_{40}H_{70}N_8O_{11}$. Theoretical: $[MNa]^+$ 861.50616. Found: 861.5046.

$[CH_3(CH_2)_{13}]_2NCOCH_2OCH_2CO$-(Gly)$_3$-Pro-(Gly)$_3$-$OCH_2Ph$, 7

was prepared as previously reported.[10]

Vesicle preparation. Dry films of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate monosodium salt (DOPA, both from Avanti Polar Lipids, 20 mg, 7 : 3) were dissolved in Et_2O (0.5 mL) and 0.5 mL of aqueous HEPES buffer (600 mM KCl, 10 mM, *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid pH = 7.0) was added. Sonication (10–20 s) gave an opalescent dispersion, which was evaporated *in vacuo* to a suspension that was filtered through a 200 nm filter membrane (5×) using a mini extruder. The suspension was then passed through a Sephadex G25 column, previously equilibrated with external 400 mM K_2SO_4, 10 mM HEPES, pH = 7.0 buffer. Laser light scattering (Coulter N4MD) confirmed their diameter: ~200 nm.

Determination of chloride release by ISE methods. Liposomes were prepared as above, and loaded with 600 mM KCl and 10 mM (HEPES, pH = 7.0), then equilibrated with an external buffer (400 mM K_2SO_4, 10 mM HEPES, pH = 7.0). The pH of the buffers prepared was adjusted using 10% NaOH. An Accumet chloride combination electrode was equilibrated (5 min) in external buffer (2.0 mL) and the vesicle solution was added. After recording the baseline voltage, aliquots of the amphiphile solution (5–9 mM in 2-PrOH) were added. The amount of isopropyl alcohol was limited to ≤20 μL to avoid alcohol-induced leakage. Vesicles were lysed by addition of aqueous Triton X100 (100 μL, 2%). The lipid concentration used in the studies presented here was 0.65 mM.

Determination of chloride release by lucigenin quenching. Vesicles were prepared from a dry film of dioleoylphosphatidylcholine (DOPC). Diethyl ether (375 μL) and 1 mM lucigenin–225 mM $NaNO_3$ (375 μL) solution were added to 15 mg of lipids and then sonicated (2 × 10 s). The ether was subsequently evaporated under mild vacuum at 30 °C. The resulting solution was extruded 5 times through a 200 nm membrane filter and then passed through a Sephadex column equilibrated with 225 mM $NaNO_3$. The size of the collected vesicles was ascertained to be 200 nm by light scattering analysis. The concentration of the lipids in the final vesicle suspension was measured with the colorimetric method previously described.

The vesicles were diluted to a 0.31 mM concentration and then a 2 mL aliquot was placed in a quartz cuvette to be used for the lucigenin quenching experiment. The excitation wavelength was set to 455 nm and the emission wavelength to 506 nm, with both slits set to 5 nm. After a brief initial equilibration phase, 100 μL of a 4 M NaCl solution were added in order to create a chloride gradient between the outside (190 μM) and the inside (0 μM) of the vesicles. When the fluorescence reached a stable reading, 15 μL of a 9 mM solution (0.135 μmoles) of the desired compound in i-PrOH were added. At the end of each experiment the vesicles were lysed with 100 μL of a 2% Triton X-100 solution.

A calibration line for transforming fluorescence intensity into chloride concentration was obtained using the conditions of the described experiments. 2 mL of a 0.31 mM vesicles suspension in 225 mM $NaNO_3$ were lysed with 100 μL of a 2% Triton X-100 solution and then titrated with aliquots of a 4 M NaCl solution. The Stern–Volmer constant was found to be 119.7 M^{-1}.

Determination of carboxyfluorescein release. DOPC–DOPA liposomes (7 : 3 w/w, 20 mg) were prepared as above except 20 mM 5(6)-carboxyfluorescein (CF), 100 mM KCl and HEPES (pH = 7.0) were added. In order to dissolve CF, sodium hydroxide (1 M) was added dropwise until pH = 11. The solution was then carefully acidified to pH = 7.0 (1M aq. HCl). Free CF was removed by gel filtration and exchanged for 100 mM KCl and 10 mM HEPES (pH = 7.0). These were diluted to 3 μM lipid. CF dequenching was followed by emission at 520 nm (excitation at 497 nm). For each experiment, the initial (F_0) and total (F_{triton}, 1% Triton X100) fluorescence were determined and used to determine the final value: $(F - F_0)/(F_{triton} - F_0)$. In the CF release experiments, the concentration of amphiphile is present in large excess compared with the phospholipid concentration. This ratio ensures that pore activation kinetics rather than insertion kinetics are being observed.

Carboxyfluorescein carrier experiments. A concentric tube device was used.[30] The bottom was filled with $CHCl_3$ containing the SAT compound (12 mM) and a stirring bar. A blank experiment was also done in which the SAT was absent. The (external ring) source phase was a neutral aqueous solution containing CF (20mM), KCl (100 mM) and HEPES buffer (10 mM, pH = 7) while the (inner) receiving phase was a neutral aqueous solution containing KCl (100 mM) and HEPES buffer (10 mM, pH = 7). The solution was pumped (8 ml min^{-1}) in a continuous loop from the receiving phase to a cuvette inside a Perkin Elmer LS 50B fluorimeter. The CF released from the receiving phase was detected immediately. All experiments were processed for at least 7 h. The recording time was limited by the data recording capacity of the fluorimeter.

Calculational details. Calculations were performed using the GAUSSIAN 03 suite of programs. Semi-empirical (PM3) and density functional (B3LYP) methods were used to optimize the geometries and perform frequency analysis. The B3LYP method combines Becke's three-parameter function[34] with the non-local

correlation provided by the correlation function of Lee, Yang and Parr,[35] which is suitable for vibrational calculations.

All geometries were completely optimized without any imaginary frequencies (gas phase). The proline amino acid residue based peptide sequence was optimized by using the semi-empirical method (PM3) followed by the DFT method (B3LYP) with 6-31g as the basis set. It has been shown that the semi-empirical method can be a good choice for large systems with much cheaper computational resource cost. The 6-carboxyfluorescein anion was optimized using B3LYP/6-31 g. The complex of the combination of these two molecules was optimized using the PM3 method with appropriate frequency and conformational analysis under gas phase. Considering the entire complex system, other conformations might exist. The conformation presented is the energy minimum of all those computed.

Negative ion mass spectrometry. Mass spectra were obtained using a JEOL MStation [JMS-700] mass spectrometer equipped with an electrospray ionization source, operating in the negative ion mode and scanning from m/z 600 to 2400. Slits were set to achieve a resolution of about 2000. Six different 1 mM stock solutions were prepared in CH_3CN from the amphiphilic peptides **2**, **3** and **6**, tetrabutylammonium chloride, and carboxyfluorescein and mixtures of these, as indicated in the text, were perfused at 50 μL min^{-1} by using a Harvard Syringe Pump. The spray voltage was 2.00 kV, and the capillary temperature (desolvating temperature) was 200 °C. Each trial was processed using the MSMP9020D software supplied by JEOL with a minimum of 10 scans averaged for the final spectral presentation.

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