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Transmembrane ion transport meditated by bis-macrocyclic bolaamphiphiles

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A suite of fourteen bolaamphiphiles was prepared by a modular construction approach from a set of subunits. Macrocyclic tetraesters from maleic anhydride were linked by bis sulfide 'linkage' units to one of the two equivalent alkenes. The remaining alkene reacted with sulfur nucleophiles to add polar 'head' groups. The structural variables included head group type and charge, wall unit functionality, and variable hydrogen bonding capability at the linkage unit. The transport activities of the suite of compounds were assessed in a vesicle bilayer system. The most active materials had hydrogen bonding capability at the linkage unit, and ammonium or carboxylate charged head groups. The apparent kinetic orders greater than one and the selectivities observed are consistent with pore formation by aggregates in the bilayer membrane. The influence of structural variables on the transport activity suggest that the aggregates have defined properties that are amenable to structural control via synthesis.

Natural ion transporters are large protein aggregates containing multiple transmembrane segments which act in concert to control transmembrane ion and potential gradients.[1] Structural information is now emerging from molecular biology,[2] but much of the molecular scale detail has been inferred from low molecular weight ionophores such as gramicidin[3] or amphotericin.[4] These same sources suggest that artificial channels for the transport of ions across bilayer membranes could be designed according to the following principles: (i) A channel would have a polar core surrounded by a non-polar exterior layer for simultaneous stabilization of an ion in transit and favorable interaction with membrane lipids; (ii) A channel would have the overall length and shape to fit into a bilayer membrane approximately 40 Å thick. Functional artificial ion channels have been reported which illustrate the general criteria. The most obvious course is to prepare oligopeptides with high helical content.[5] Other reported systems are based on cyclodextrin,[6] polymeric crown ether,[7] and 'bouquet' shaped crown ether and cyclodextrin motifs.[8] One of the most active systems is a simple tris-crown ether derivative reported by Gokel for the transport of sodium cation.[9] Some time ago we reported the synthesis and activity of a functional ion channel[10] and a preliminary mechanistic study[11] which established the channel-like behavior of one compound. A suite of twenty-one related materials has now been prepared,[12] and a structure-activity based elucidation of the modes of action of this class of ion channels has shown the structural constraints on ion channel formation.[13] All of these systems envisage unimolecular transmembrane structures, similar to the gramicidin structural paradigm.

An appealing alternative would exploit multi-component aggregates, akin to an amphotericin pore,[4] to achieve structures of the required size from modest molecular weight components. Simple functionalized amphiphiles apparently act using this mechanism to increase the permeability of synthetic[14] and natural phospholipid membranes.[15] Specifically designed membrane disrupting agents also employ the same strategy.[16] Fuhrhop has reported an intriguing system which can be reversibly switched, and which acts via aggregated structures in synthetic monolayer membranes.[17] The best characterized system to date is an oligoethylene glycolic acid which forms single channels in planar bilayer membranes.[18] Kobuke reports that this system is not voltage gated, but does show the marked voltage and concentration dependence expected of an aggregate channel. Our own preliminary results in this area were reported some time ago.[19] This report describes both synthetic and transport experiments which lead to a structure-activity exploration of the 'aggregate-pore' strategy for ion channel formation.

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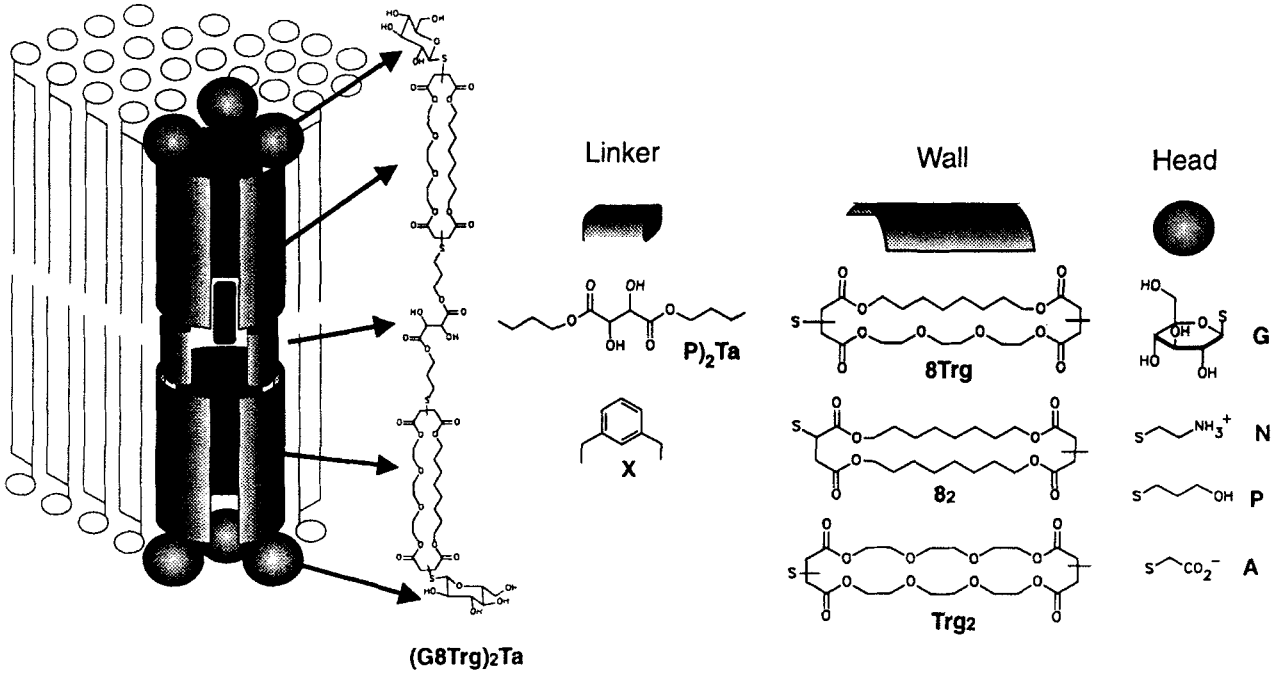


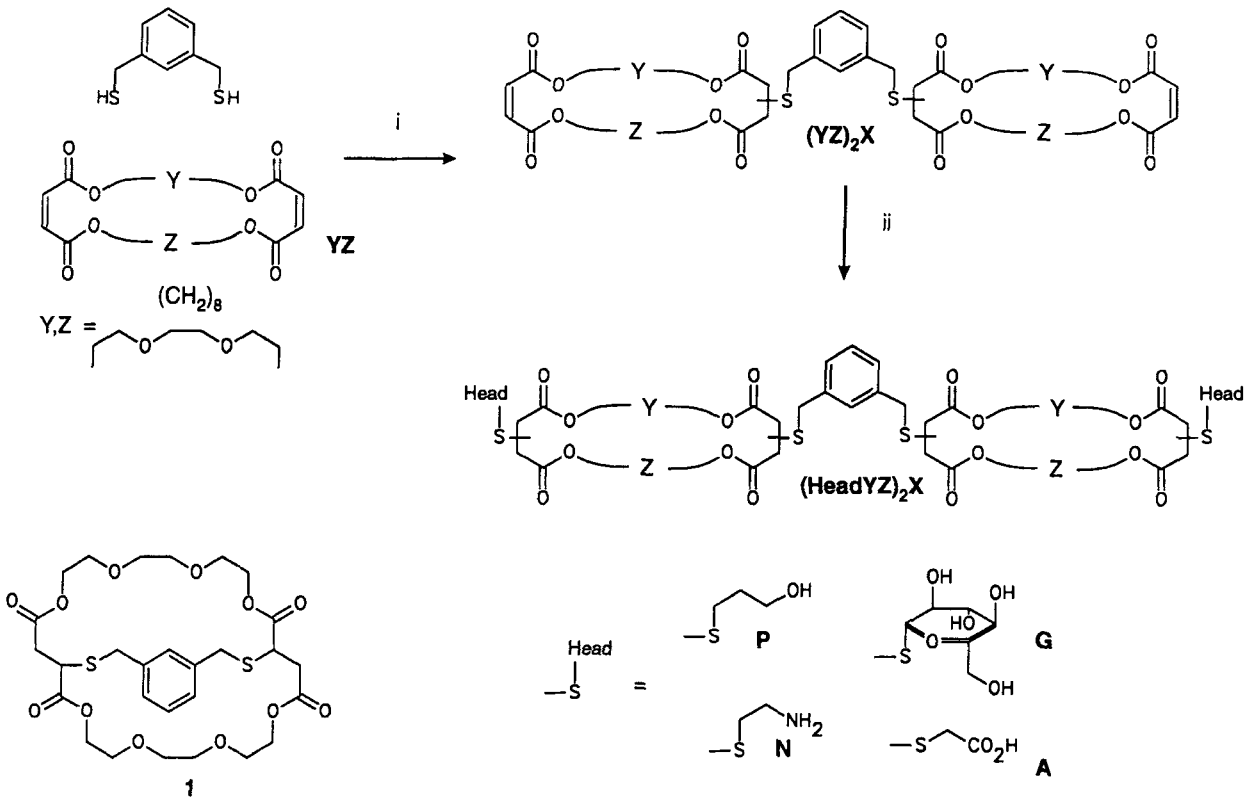
Figure 1 Design proposal for pore formation by aggregation of bolaamphiphiles, and components of the modular construction set. See text for naming conventions.

Our design proposal is sketched in Fig 1. We envisage an aggregate of membrane spanning bolaamphiphilic units, functionalized and oriented to create an aggregate which would be polar inside and non-polar outside. The aggregate would provide a defect or aqueous fjord deep into the bilayer membrane. The sketch implies a pore formed from three molecules, but a suite of related structures in a dynamic equilibrium controlled by the interactions between the components and with the lipid membrane is more likely. We envisaged building the structures using a modular construction approach from a suite of sub-units.[12] Suitable functionalized wall units would provide some rigidity, and would be linked near the bilayer mid-plane by simple, bifunctional linking groups. The structures would be completed by addition of polar head groups to impart overall bolaamphiphilic character. Some structural variables are presented in Fig 1.

Given the number of combinations possible, we use a structure-based semi-systematic naming system. Names and sub-structures are equated in Fig 1. Each synthon was assigned a simple letter or number name: **G** = 1-mercapto-β-D-glucose,[20] **P** = 3-mercaptopropyl, **8** = from 1,8-octanediol, **Trg** = from triethylene glycol, **X** = *meta*-xylyl, **Ta** = tartaric acid, etc. Each intermediate was named as a combination of its synthon abbreviations with the exception that the maleate esters of the wall units are implied: $\mathbf{8_2}$ = the macrocyclic tetraester from 2 moles of 1,8-octanediol (and 2 moles of maleic anhydride), **8Trg** = the macrocyclic tetraester derived from 1 mole of 1,8:octanediol and 1 mole of triethylene glycol (and 2 moles of maleic anhydride), etc. The final structures were ordered from head to linkage unit: (head + wall (+ spacer if needed))$_2$ + linkage unit. Thus **(G8TrgP)$_2$Ta**, as illustrated in Fig 1, is (a mercapto glucose head group + a tetraester wall unit derived from 1,8-octanediol and triethylene glycol + a propyl spacer) as the bis ester of tartaric acid.

SYNTHESIS

The synthesis of bolaamphiphiles derived from the *meta*-xylyl linkage unit is outlined in Scheme 1. The required macrocyclic tetraester dienes (**YZ**) were prepared by acid-catalysed dehydration of maleic anhydride plus diol mixtures. The procedures are described in detail elsewhere.[12] The Michael addition of sulfur nucleophiles to these maleate esters occur readily, and the cases in Scheme 1 proved to be no exception. Oligomerization was inhibited by use of the macrocycle in excess, but the dimerization yields of the required products were poor (ca. 20%). Unreacted **YZ** was recovered in good yield in all cases. The diene products were characterized by NMR, FAB MS, and elemental analysis. Analytical gel permeation chromatography established that contamination from oligomers was absent in all cases. As drawn, the



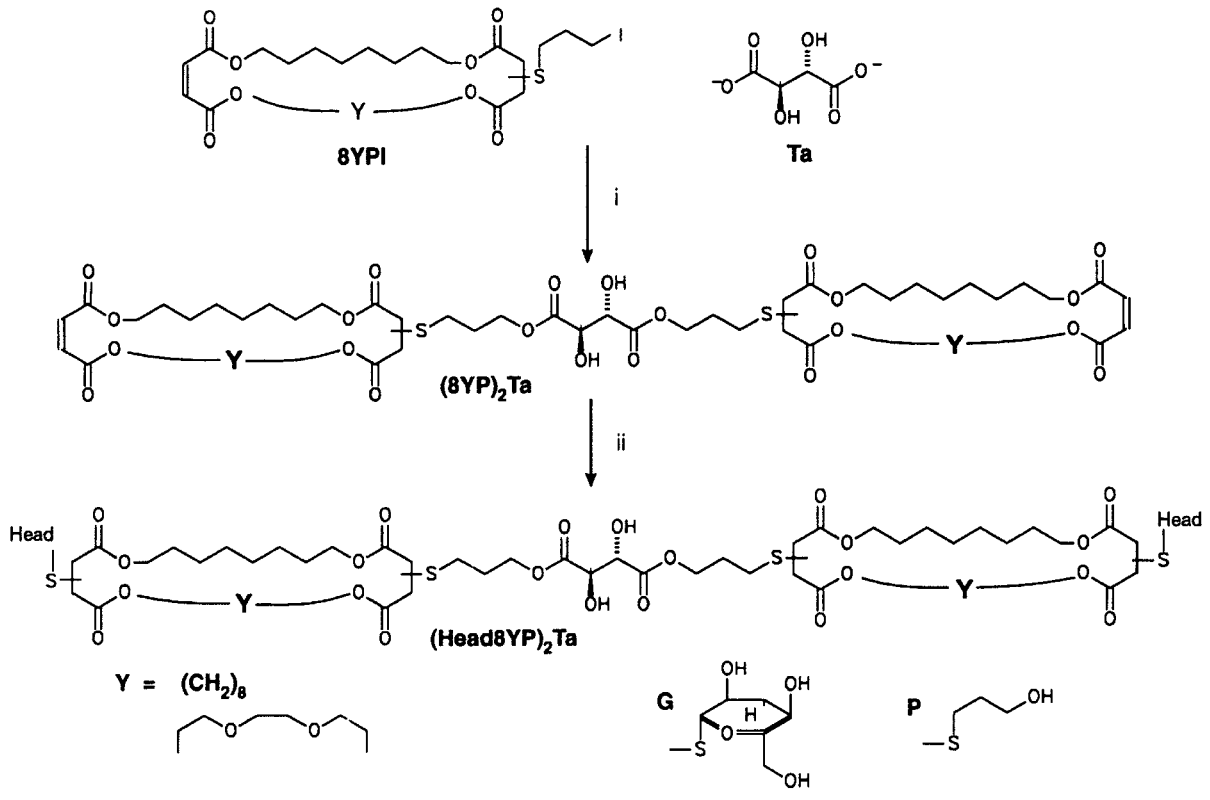
Scheme 1 (i) Addition of dithiol in THF/iPrOH containing tetramethylpiperidine to 10-fold excess of diene, 80°C, 14 hr. 13–24%; (ii) HeadSH (5 fold excess) added to diene in iPrOH containing piperidine, reflux, 1 hr, 75–95%.

maleate extremities appear to be remote from one another. However, a capping reaction to form **1** was a significant competing reaction in the case of the macrocycle $\mathbf{Trg_2}$ under conditions of insufficient excess of the diene. This suggests that a 'collapsed' conformation is accessible. This is consistent with the competing preferences of polymethylene and oligoethylene glycol chains. The former prefer an all *anti* conformation leading to linear chains and macrocycles as drawn. The latter prefer *gauche* conformations about the OCH_2CH_2O units resulting in 'crown' type turns as pictured in **1**.

Addition of the head groups, again via a Michael reaction, completes the synthesis of candidate pore-former molecules. All of the combinations of $\mathbf{(8_2)_2X}$, $\mathbf{(8Trg)_2X}$, and $\mathbf{(Trg_2)_2X}$, with 1-mercapto-β-D-glucose (**G**), 3-mercapto-1-propanol (**P**), and 2-mercaptoethyl amine (**N**) were prepared. Only one example of the **A** head group was prepared in this series, but several other examples[12] suggest the additional two cases would be well behaved. The average yield for the full suite of compounds is 90% for the addition of two head groups. This is consistent with an average yield of >90% per group added observed in another extensive series.[12]

The synthesis of bolaamphiphiles derived from the tartaric acid linkage unit is outlined in Scheme 2. The synthesis closely parallels our modular synthesis of ion channel mimics based on 18-crown-6-polycarboxylic acids.[12] Suitably functionalized wall units bearing iodide or mesylate leaving groups on a thiopropyl spacer (**8YPI** or **8YPOMs**) react with the bis tetramethyl ammonium salt of tartaric acid to give the diesters $\mathbf{(8_2P)_2}$ and $\mathbf{(8TrgP)_2Ta}$ in 45% and 52% yield respectively. Ironically, the yields are better for higher molecular weight cases, as the gel permeation purification is more efficient for larger molecular weight differences between the desired product and the main by-product **8YPOH**.[12] Only the **G** and **P** head groups were added in this series. Ammonolysis of the tartarate esters is a competing reaction with the **N** head group,[21] but addition of the **A** groups should occur readily.

In total, fourteen of the possible twenty four compounds represented in Fig 1 were made. The main structural variables are hydrogen-bonding capability at the linkage unit (**X** vs **Ta**), neutral head group size (**G** vs **P**), and head group charge (**N** – positively charged at pH 7.6, vs **A** – anionic at pH 7.6). The wall unit series $\mathbf{8_2}$, **8Trg**, and $\mathbf{Trg_2}$ offers a range of lipophilicities, and as noted above, many involve some conformational differences.



Scheme 2 (i) Excess iodide in DMSO plus $Me_4N^+OH^-$, 60 °C, 3 hr, 45–40%. (ii) HeadSH (5 fold excess) added to diene in iPrOH containing piperidine, reflux, 1 hr, 75–95%.

TRANSPORT EXPERIMENTS

Vesicles were prepared by a reverse evaporation process[22,23a] from an 8:1:1 mole ratio of egg phosphatidyl choline (PC): egg phosphatidic acid (PA) and cholesterol. Lipid in ether was dispersed in a pH 6.6 buffer by sonication, the ether was removed under reduced pressure, and the vesicles were suspended in unbuffed isoosmolal choline sulfate solution. The vesicles were filtered to remove large aggregates, and the residual buffer solution was replaced by unbuffered choline sulfate solution by gel filtration. Electron microscopy[23b] showed predominantly unilamellar vesicles of approximately 150 nm diameter, with some smaller unilamellar vesicles (50 nm diameter), and a small proportion of multilamellar structures (MLVs). Particle sizing by dynamic light scattering[23c] subsequently confirmed a bimodal size distribution. Vesicle solutions were stable as prepared for periods in excess of 96 hours, but were typically used within 24–36 hours of preparation.

Aliquots of vesicle solution (1.9 mg phospholipid, approx. 5×10^{12} vesicles) were suspended in unbuffered choline sulfate solution, and the pH was adjusted to 7.6 by addition of choline hydroxide. This is the origin of the pH-stat experimental curves illustrated in Fig 2. Addition of the proton carrier FCCP ensures

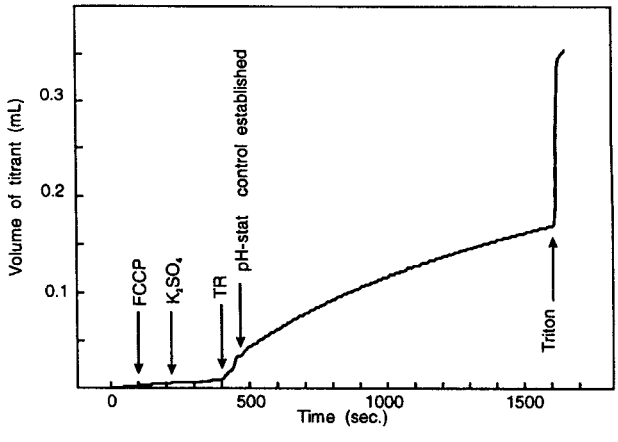


Figure 2 Experimental pH-state curve of volume of choline hydroxide titrant added as a function of time for the transporter **(G8TrgP)$_2$Ta** (entry of Table 1).

that proton transport can occur rapidly.[22] A counter gradient in alkali metal sulfate is also supported by the bilayer as the system lacks pathways for cation-proton antiport or anion-proton symport. Upon addition of a transporter, proton efflux occurs, and choline hydroxide titrant is added by the pH-stat to maintain the set pH of 7.6. Addition of the detergent Triton X-100 at any point results in vesicle lysis with the release any remaining entrapped buffer.

Control experiments establish the following: (1) The initial pH gradient is stable in the absence of cations for times in excess of 8 hours. (2) Coupled cation/proton gradients in the presence of FCCP collapse slowly at a rate of 0.1–1 μL of titrant added per minute corresponding to a base 'leakage' rate of $< 1 \times 10^{-10}$ mol H^+ sec^{-1}. (3) The 'leakage' is not accelerated by addition of solvents (methanol, THF, or DMSO) at ten times the volumes added with the transporter solutions. (4) Lysis using Triton X-100 is very fast, but the response time of the pH-state instrumentation limits the rate at which pH control can be reestablished. The apparent upper limit on proton efflux rate is $< 1.0 \times 10^{-8}$ mol H^+ sec^{-1}. (5) Melittin assays[24] indicated that 95% of the entrapped proton titer is contained in unilamellar vesicles (LUVs). (6) Electron microscopy and dynamic light scattering analyses indicate that there is no change in vesicle morphology or size provoked by any of the transporters at the concentrations used in the transport experiments. At a concentration ten times higher than any discussed below, **(N8Trg)$_2$X** and **(A8Trg)$_2$X** both acted as detergents to form micellar solutions of phospholipid containing few intact vesicles. None of the **Ta** derivatives acted as detergents at these concentrations.

Fig 2 illustrates 'typical' transport results for **(G8TrgP)$_2$Ta**. The slow 'leakage' reaction is evident in the first 400 seconds. Immediately following addition of **(G8TrgP)$_2$Ta**, a brief burst of proton efflux occurs and the pH-state instrumentation loses control of the pH. Eventually, the instrument reestablishes control, and thereafter the proton efflux approximates a first-order process. Linear regression of log (volume added) as a function of time gives good correlation ($r^2 = 0.9998$). As in other cases,[13,22] the extent of transport does not reach the 'limit' of 95% based on the MLV content of the solutions. In principle, the apparent initial proton efflux rate can be derived from the first-order rate constant and the value for the experimental extent of transport ($rate_0 = k \times$ experimental extent (vol.) $\times$ choline hydroxide concentration).[13] Unfortunately, the brief burst event hides the experimental initial volume required for this calculation. Since the relative activities of the transporters are the first issue, the rate was derived from the experimental curves by direct linear regression of volume added as a function of time for the points immediately following the burst event, where the pH-stat instrumentation was in control of the cell pH. It is clear from Fig 2, that the experimental data are not linear, so the calculated slope will vary with the number of points selected. A fixed time window of 400 seconds (80 points) was used in all cases. For the case of Fig 2, the slope is 1.66×10^{-4} mL sec^{-1} ($r^2 = 0.993$) corresponding to an initial proton efflux rate of 8.9×10^{-10} mol H^+ sec^{-1}. Volumetric differences between vesicle preparations, coupled with the approximate method used to determine initial rate both increase experimental uncertainty. The reproducibility between different vesicle preparations is estimated to be $\pm 15\%$. Replicates within a single vesicle preparation are better than $\pm 8\%$. Experiments involving a range of concentrations were done using a single vesicle preparation to improve precision.

The activity of the suite of compounds is summarized in Table 1. Some transporters showed very marked concentration dependent behaviors (see below), so the rate data in Table 1 was determined for a fixed amount of transporter. Under these conditions **(N8Trg)$_2$X** and **(G8$_2$P)$_2$Ta** are very active and the rates are near the limit of the experiment. Conversely, the least active materials **(G8$_2$)$_2$X** and **(G8Trg)$_2$X**, are not within a concentration range where they are active; previous work used a different lipid preparation which apparently enhanced activities of all transporters.[19] Three structural trends are evident in the data. The most marked is the effect of the linkage unit. Compounds with the **Ta** linkage unit are clustered at the top of the rank order. In all cases of a common (head group + wall) combination, the **Ta** derivatives are more active than the **X** derivatives by a factor of > 3. The head group activity falls in the series **N > (A) > P > G**, in contrast to the sequence observed with unimolecular ion channels from the wall and head group components (**G > A > P**).[12] The most active wall unit at a fixed head group and linkage combination (**X**) is **8Trg** followed by **8$_2$** and **Trg$_2$**. The exception is

Table 1 Rank order of activity of the pore formers studied[a]

Transporter	Moles of transporter ($\times 10^7$)	Initial proton efflux rate ($\times 10^{10}$ mol H^+ sec^{-1})	Vesicle batch
(N8Trg)$_2$X	2.85	fastest	3
(G8$_2$P)$_2$Ta	2.80	fast	4
(P8TrgP)$_2$Ta	2.84	12	5
(P8$_2$P)$_2$Ta	2.84	9.1	5
(G8TrgP)$_2$Ta	2.97	8.9	4
(A8Trg)$_2$X	2.83	8.0	5
(N8$_2$)$_2$X	2.81	6.3	5
(P8Trg)$_2$X	2.81	6.0	5
(NTRg$_2$)$_2$X	2.77	4.9	5
(PTrg$_2$)$_2$X	2.90	4.6	5
(P8$_2$)$_2$X	2.81	3.2	4
(GTrg$_2$)$_2$X	2.76	1.6	4
(G8$_2$)$_2$X	2.84	< 1.0	3
(G8Trg)$_2$X	2.79	< 1.0	3

[a] Transport across vesicle bilayer membranes at 298 °K in the presence of $1.5–1.8 \times 10^{-7}$ M FCCP and $4.7–4.9 \times 10^{-2}$ M K_2SO_4. Rates determined for the 400 seconds following an initial burst of proton efflux as described in the text.

Table 2 Survey of transport behaviors of the most active transporters

Transporter	Apparent kinetic order[a]	Cation selectivity[b]	Migration ratio[c]	FCCP acceleration ratio[d]
$(N8Trg)_2X$	3.8 ± 0.2	$Cs \sim Na \sim K > Rb \sim Li$	0.9 ± 0.1	1.3 ± 0.1
$(G8_2P)_2Ta$	2.9 ± 0.2	$Rb > Na \sim Cs \sim K > Li$	0.4 ± 0.1	1.8 ± 0.2
$(G8TrgP)_2Ta$	2.6 ± 0.2	$K > Cs \sim Na > Rb \sim Li$	0.6 ± 0.1	2.5 ± 0.3
$(P8_2P)_2Ta$	1.6 ± 0.2	[f]	1.0 ± 0.2	[e]
$(P8TrgP)_2Ta$	1.5 ± 0.3	[f]	0.9 ± 0.2	[e]
$(A8Trg)_2X$	1.6 ± 0.5	[f]	0.15 ± 0.15	1.1 ± 0.2

[a] Transport across vesicle bilayer membranes at 298 °K in the presence of $1.5–1.8 \times 10^{-7}$ M FCCP and $4.7–4.9 \times 10^{-2}$ M K_2SO_4. Variable [Tr] in the range $0.5–15 \times 10^{-6}$ M; slope of log(rate) as a function of log([Tr]) ± statistical error, $r^2 > 0.98$ on > 3 points in all cases.
[b] Transport across vesicle bilayer membranes at 298 °K in the presence of $1.5–1.8 \times 10^{-7}$ M FCCP and $4.7–4.9 \times 10^{-2}$ M M_2SO_4. Transporter amounts as in Table 1 except for **$(N8Trg)_2X$** (1.71×10^{-7} mol) and **$(G8_2P)_2Ta$** (1.56×10^{-7} mol).
[c] Transport across vesicle bilayer membranes at 298 °K in the presence of $1.5–1.8 \times 10^{-7}$ M FCCP and $4.7–4.9 \times 10^{-2}$ M K_2SO_4. Transporter amounts 35–50% of the amounts in Table 1. Rate ratio defined in the text.
[d] Conditions as in footnote [c]. Order of addition as illustrated in Fig 4. Rate ratio defined in the text.
[e] No transport observed in the absence of added K_2SO_4 *and* FCCP.
[f] All cations transported at the same rate within experimental error.

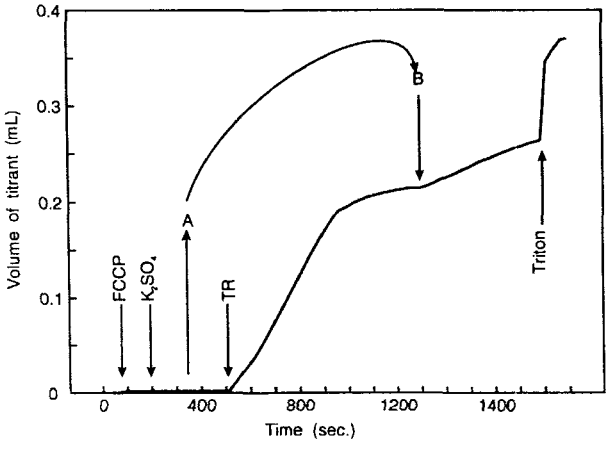


Figure 3 Experimental curve of volume of choline hydroxide titrant added as a function of time illustrating a migration experiment for the transporter **$(G8_2P)_2Ta$** (entry of Table 2). Vesicle solution (2.0 mL) removed at point A was returned to the cell at point B.

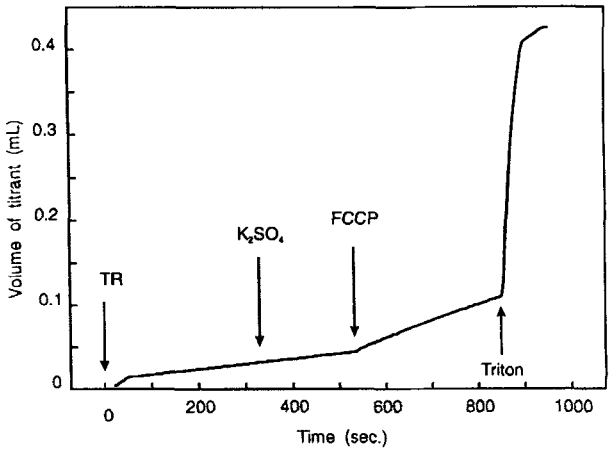


Figure 4 Experimental curve of volume of choline hydroxide titrant added as a function of time illustrating transport acceleration by addition of FCCP for the transporter **$(G8TrgP)_2Ta$** (entry of Table 2).

the **G + Ta** combination since **$(G8_2P)_2Ta$** is very active at this concentration.

The six most active materials were investigated in more detail and the results are presented in Table 2, and Figs 3 and 4. The apparent kinetic order of the transport processes was determined from the variation in initial proton efflux rate as a function of added transporter concentration.[13,25] Linear regression of log (initial rate) as a function of log (transporter concentration) gave good straight lines in all cases ($r^2 > 0.988$) with slopes as reported in Table 2. All active cases have apparent orders well above one, consistent with the rate limiting processes involving a small aggregate of transporter molecules. The variation in initial efflux rate as a function of added cation concentration was investigated for K_2SO_4 concentrations in the range 1×10^{-3} to 1×10^{-1} M. The rate variation in all cases was less than 20% and there was no evidence that the transport behaved as a saturable process in cation concentration.[13] The cation selectivity of the transport was explored for the alkali metal sulfates. The results are summarized in Table 2. The main feature is the low selectivity in all cases; no transporter showed differences greater than a factor of two between the slowest and the fastest. In three cases the differences were smaller than the estimated error of ±8%, and are shown as 'unselective'. The modest selectivity differences in the other cases is consistent with the formation of some structured aggregate that mediates the transport.

The 'migration ratio', the ability of transporters to shift between vesicles[13] was assessed from experiments such as the one pictured in Fig 3. The experiment establishes the pH and cation gradients as previously, and approximately half of the vesicles are removed from the cell (point A). Transporter is added and the

transport processes proceeds to a plateau. The removed vesicles are reintroduced to the cell (point B) and further transport is recorded. The dilution at point B results in a rate decrease which depends on the kinetic order for each transporter (n). The rate in the second portion of the curve ($rate_2$) can be calculated from the rate in the first section of the curve ($rate_1$) according to $rate_2 = rate_1 \times ([Tr]_2/[Tr]_1)^n$. The migration ratio is then given by the ratio of the experimental and calculated values of $rate_2$. For transporters which can move freely between vesicles in the solution, the ratio will approach one. For transporters which are held in the vesicle membranes they first enter, the ratio is lower. These results have no mechanistic implications as there is no relationship of activity to migration between vesicles.

The experiment illustrated in Fig 4 reveals that some transporters mediate proton efflux in the absence of added cations. This could be due to an anion transport process with the available sulfate from the buffer solution, or could be a proton-cation exchange process based on the choline present. The four cases discovered are listed on Table 2. In each case, addition of K_2SO_4 did not accelerate the transport process, but subsequent addition of the proton carrier FCCP resulted in a marked increase in the proton efflux rate. The acceleration factor observed is reported in Table 2. One interpretation of this factor views it as a measure of the selectivity of the pore formed. A higher value would indicate that the pore supports proton transport to a smaller extent than a case having a lower value.

SUMMARY

The transport survey results indicate that this suite of compounds apparently form pores in vesicle bilayer membranes that support ion transport. Activity related to structural variables, the varying apparent kinetic orders, and the interesting selectivity differences all support the view that structured aggregates of the transporters are formed. The differences between related compounds in this suite, and with respect to related unimolecular ion channels from the same wall and head group components,[13] are also consistent with the formation of relatively well controlled structures. It is unlikely that discrete pores of well defined, and invariant structure are formed. Rather, the population of pores formed by a given transporter, and their mean properties, are apparently well behaved and reproducible. This vesicle survey serves to define the active materials. More detailed probes of the mechanisms will require more direct probes of ion conductivity and dynamics.[18]

ACKNOWLEDGMENTS

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EXPERIMENTAL SECTION

Melting points were taken on a Reichart hotstage microscope (uncorrected). Proton NMR spectra were recorded with a Perkin Elmer R32 (90 MHz, CW), a Bruker WM250 (250 MHz, FT) or a Bruker AMX 360 (360.14 MHz, FT) spectrometers in $CDCl_3$, CD_2Cl_2, or CD_3OD as solvent, 90 MHz ^1H NMR spectra (R32) were referenced to Me_4Si as internal standard, and all 360 MHz ^1H NMR spectra (AMX 360) were referenced with the central solvent line as standard (7.24 ppm for $CDCl_3$, 5.32 ppm for CD_2Cl_2 and 3.30 ppm for CD_3OD all relative to Me_4Si). Carbon spectra were recorded with either a Bruker WM 250 (62.89 MHz) or Bruker AMX 360 (90.57 MHz) spectrometer with the central solvent line as standard (77.0 ppm for $CDCl_3$, 53.8 ppm for CD_2Cl_2 and 49.0 ppm for CD_3OD all relative to Me_3Si). Methane chemical ionization mass spectra were recorded on a Finnegan 3300 GC-MS instrument. FAB mass spectra were recorded with a Kratos Concept IH mass spectrometer using thioglycerol as matrix. Elemental analysis were performed by Canadian Microanalytical Services, New Westminster, B.C., and are quoted as percentages.

1,3-Bis-(2-thia-2-(3- and/or 4-(1,6,15,20-tetraoxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)) ethyl) benzene, $(\mathbf{8_2})_2\mathbf{X}$

Meta-xylylene dithiol (113 mg, 663 μmol, in 3 mL of THF:isopropanol, 1:1) was added dropwise over a 1.5 hr period into a solution of $\mathbf{8_2}$ (3.0 g, 663 mmol, 10 eq) and 2,2,6,6-tetramethylpiperidine (0.5 mL) in 15 mL of THF at 80°C under a N_2 atmosphere. The reaction was heated for a total of 14 hr. The solvent was evaporated under reduced pressure and the residue was purified on a silica-gel column (50 g) to remove unreacted $\mathbf{8_2}$. The product was eluted with dichloromethane:methanol (95:5), concentrated under reduced pressure and the oily residue was further purified on a gel permeation column (Sephadex LH-20, 3 × 20 cm, chloroform:methanol (4:3) eluent). Evaporation of the solvent gave $\mathbf{(8_2)_2X}$ as a colorless oil (172 mg, 24%); ^1H NMR (δ, CD_2Cl_2): 7.3–7.2 (br m, 4H, aromatic CH), 6.8, 6.2 (s, 4H, trans and cis CH=CH), 4.2 (m, 16H, CO_2CH_2), 3.9–3.5 (m, 6H, $ArCH_2SCH$), 2.9–2.5 (m, 4H, $CH_2C{=}O$), 1.6 (m, 16H, $CO_2CH_2CH_2$), 1.3 (br s, 32H, $CH_2CH_2CH_2$); ^{13}C

NMR 62.89 MHz (δ, CD_2Cl_2): 171.3, 170.3, 165.5 (**C**=O), 138.1, 133.7, 130.0, 129.8, 128.9, 128.3 (**CH**=**CH**), 65.6, 65.5, 65.2 (CO_2CH_2), 41.7, 41.6 (**CHS**), 36.8, 36.0 (CH_2C=O, ArCH_2), 29.5, 28.9, 28.8, 26.1, 25.6 ($CH_2CH_2CH_2$); MS: 1075.3 $(M+H)^+$; Analysis calculated for $C_{56}H_{82}O_{16}S_2$: C 62.55%, H 7.69%, S 5.96%; Found: C 62.80%, H 7.52%, S 5.80%.

1,3-Bis-(2-thia-2-(3- and/or 4-(1,6,9,12,15,20-hexaoxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)) ethyl) benzene, $(8Trg)_2X$

Prepared as described for $(8_2)_2X$ from *meta*-xylylene dithiol (112 mg, 657 μmol) and **8Trg** (3.0 g, 657 mmol, 10 eq), as a colorless oil (92 mg, 13%); ^1H NMR 250 MHz (δ, CD_2Cl_2): 7.3–7.2 (br m, 4H, aromatic **CH**), 6.8, 6.2 (s, 4H, trans and cis **CH**=**CH**), 4.2 (m, 16H, CO_2CH_2), 4.0–3.5 (m, 22H, $CO_2CH_2CH_2OCH_2$-CH_2O, ArCH_2SCH), 2.9–2.5 (m, 4H, CH_2C=O), 1.6 (m, 8H, $CO_2CH_2CH_2$), 1.3 (br, s, 16H, $CH_2CH_2CH_2$); ^{13}C NMR 90.57 NHz: (δ CD_2Cl_2) 171.5, 171.3, 170.7, 170.6, 170.3, 165.5, 165.4, 165.2, 165.1 (**C**=O), 138.0, 134.3, 133.8, 133.2, 130.5, 130.4, 130.3, 130.1, 129.8, 129.5, 128.9, 128.4 (**CH**=**CH**), 72.8, 71.0, 70.8, 70.6 (OCH_2CH_2O), 69.2, 69.1 ($CO_2CH_2CH_2O$), 65.6, 65.5, 65.1, 65.0, 64.8, 64.6, 64.3 (CO_2CH_2), 41.8, 41.7, 41.6, 41.5 (**CHS**), 36.7, 36.5, 36.1, 35.9 (CH_2C=O, ArCH_2), 29.4, 29.2, 29.1, 29.0, 28.9, 28.8, 28.7, 28.6, 26.2, 26.1, 25.9, 25.8 (two peaks), 25.7, 25.6 (CH_2CH_2-CH_2); MS: 1083.3 $(M+H)^+$; Analysis calculated for $C_{52}H_{74}O_{20}S_2$: C 57.65%, H 6.88%, S 5.91%; Found: C 57.57%, H 6.92%, S 5.84%.

1,3-Bis(2-thia-2-(3-and/or4-(1,6,9,12,15,20,23, 26-octa-oxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)) ethyl)-benzene, $(Trg_2)_2X$

Prepared as described for $(8_2)_2X$ from *meta*-xylylene dithiol (100 mg, 587 μmol) and Trg_2 (2.7 g, 587 mmol, 10 eq), as a colorless oil (151 mg, 24%); ^1H NMR 250 MHz (δ, $CDCl_2$): 7.3–7.2 (br m, 4H, aromatic **CH**), 6.8, 6.2 (s, 4H, trans and cis (**CH**=**CH**), 4.2 (m, 16H, CO_2CH_2), 3.9–3.5 (m, 54H, $CO_2CH_2CH_2OCH_2CH_2O$, ArCH_2SCH), 3.0–2.6 (m, 4H, CH_2C=O); ^{13}C NMR 62.89 MHz (δ, CD_2Cl_2): 171.0, 170.9, 170.0, 169.9, 164.9, 164.5 (**C**=O), 137.3, 133.4, 129.6, 129.5, 128.5, 128.0 (**CH**=**CH**), 70.6, 70.4 (OCH_2CH_2O), 68.8, 68.7, 68.6 ($CO_2CH_2CH_2O$), 64.3, 64.2, 63.8 (CO_2CH_2), 41.2, 41.0 (**CHS**), 36.0, 35.5 (CH_2C=O, ArCH_2); MS: 1091.2 $(M+H)^+$; Analysis calculated for $C_{48}H_{66}O_{24}S_2$: C 52.84%, H 6.09%, S 5.87%; Found: C 52.61%, H 6.10%, S 5.50%. As described in the text, slower addition of more closely stoichiometric amounts of diene results in the macrobicyclic product **1**, prepared from *meta*-xylylene dithiol (70 mg, 412 μmol) added dropwise at 80°C over 24 hr to a solution of Trg_2 (380 mg, 826 μmol, 2 eq) and 2,2,6,6-tetramethyl-piperidine (0.5 mL) in a 1:1 mixture of THF:isopropanol (10 mL). Purification as above gave **1** as a colorless oil (160 mg, 62%); ^1H NMR 360 MHz (δ, CD_2Cl_2): 7.3–7.2 (br m, 4H, aromatic **CH**), 4.2 (m, 8H, CO_2CH_2), 3.9–3.5 (m, 22H, $CO_2CH_2CH_2OCH_2CH_2O$, ArCH_2SCH), 2.9 (dd, J = 17 Hz and J = 10 Hz, 2H, CH_AH_BC=O), 2.6 (dd, J = 17 Hz and J = 10 Hz, 2H, CH_AH_BC=O); ^{13}C NMR 90.57 MHz (δ, CD_2Cl_2): 171.1, 170.2 (**C**=O), 137.5, 129.8, 128.7, 128.2 (**C**=**C**), 70.6, 70.5 (OCH_2CH_2O), 69.0 ($CO_2CH_2CH_2O$), 64.5, 64.1 (CO_2CH_2), 41.3 (**CHS**), 36.1, 35.7 (CH_2C=O, ArCH_2); MS (CI, m/e) 631 (M + 1).

2R,3R-Bis(3- and/or 4-(1,6,15,20-tetraoxa-2,5,16,19-tetraoxo(cyclooctacosa-17-3nyl)-4-thiabutyl-tartarate, $(8_2P)_2Ta$

Compound 8_2PI (500 mg, 764 μmol), 4 eq) in DMSO (10 mL) was added to a solution of 2R,3R-(+)-tartaric acid (29 mg, 191 μmol) and tetramethyl ammonium hydroxide pentahydrate (69 mg, 382 μmol, 2 eq) in DMSO (10 mL) at 60°C under a N_2 atmosphere. The reaction mixture was heated for 3 hr and the solvent was removed under reduced pressure. The product was purified by gel permeation (Sephadex LH-20, 3 × 20 cm, eluted with chloroform:methanol (4:3)). Evaporation of the solvent gave $(8_2P)_2Ta$ as a clear oil (103 mg, 45%); ^1H NMR 250 MHz (δ, $CDCl_3$): 6.8 (s, 4H, trans **CH**=**CH**), 4.5 (s, 2H, **CHOH**), 4.4–4.0 (m, 20H, CO_2CH_2), 3.6 (m, 2H, **CHS**), 2.9–2.6 (m, 8H, CH_2C=O, CH_2S), 1.9 (m, 2H, $SCH_2CH_2CH_2O$), 1.6 (m, 16H, $CO_2CH_2CH_2(CH_2)_4$), 1.3 (br s, 32H, $CO_2CH_2CH_2(CH_2)_4$); ^{13}C NMR 62.89 MHz (δ, $CDCl_3$): 171.5, 171.2, 170.5, 164.9 (**C**=O), 133.5 (**C**=**C**), 72.2 (**CHOH**), 65.3, 64.9, 64.4 (CO_2CH_2), 41.6 (**CHS**), 36.5 (CH_2C=O), 28.8, 28.3, 27.6, 25.9, 25.3 (CH_2CH_2S, $CH_2CH_2CH_2$); MS: 1203.5 $(M+H)^+$; Analysis calculated for $C_{58}H_{90}O_{22}S_2$: C 57.88%, H 7.53%; Found: C 57.86%, H 7.50%.

2R,3R-Bis(3- and/or 4-(1,6,9,12,15,20-hexaoxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)-4-thiabutyl)-tartarate, $(8TrgP)_2Ta$

Prepared as described for $(8_2P)_2Ta$ from **8TrgPI** (500 mg, 760 μmol, 4 eq) and 2R,3R-(+)-tartaric acid (28 mg, 190 μmol), as a clear oil (121 mg, 52%); ^1H NMR 250 MHz (δ, $CDCl_3$): 6.8 (s, 4H, trans **CH**=**CH**), 4.5 (s, 2H, **CHOH**), 4.4–4.0 (m, 20H, CO_2CH_2), 3.7–3.6 (m, 18H, $CH_2OCH_2CH_2O$, **CHS**), 3.0–2.6 (m, 8H, CH_2C=O, CH_2S), 2.0 (m, 2H, $SCH_2CH_2CH_2O$), 1.6 (m, 16H, $CO_2CH_2CH_2(CH_2)_4$), 1.3 (br s, 16H, $CO_2CH_2CH_2(CH_2)_4$); ^{13}C NMR 62.89 MHz (δ, $CDCl_3$): 171.4, 171.3, 170.4, 164.9 (**C**=O), 134.0, 133.1 (**C**=**C**), 72.2 (**CHOH**), 70.8, 70.4 (OCH_2CH_2O), 69.0 (CO_2CH_2 CH_2O), 65.3 ($CO_2CH_2CH_2O$), 64.9, 64.4, 64.0

($CO_2CH_2CH_2CH_2$), 41.7 (CHS), 36.3 ($CH_2C{=}O$), 28.7, 28.6, 28.3, 27.7, 25.7, 25.3 (CH_2CH_2S); MS: 1211.4 $(M+H)^+$; Analysis calculated for $C_{54}H_{82}O_{26}S_2$: C 53.54%, H 6.82%; Found: C 53.56%, H 6.74%.

General procedure for the addition of mercapto-glucose head group (G)

To a stirred solution of the diene (50 μmol) in 50:50 isoproparol/THF (20 mL), at 50°C under nitrogen, methane sulfonic acid (19 mg, 200 μmol) as an isopropanol solution was added. To this solution, 1-thio-β-D-glucose sodium salt dihydrate (2 eq per alkene) was added, followed by 2,2,6,6-tetramethylpiperidine (0.25 mL) to adjust the pH to 8, and the cloudy solution was stirred a further 12 hr at 50°. The solvent was removed under reduced pressure, and the product was dissolved in 4:3 chloroform/methanol (2 mL), filtered, and chromatographed by gel filtration (LH-20, 4 × 20 cm). The product was collected in 1 mL fractions near the void volume. The product containing fractions were identified by TLC (silanized silica, Merck RP-2, 5% CH_3OH in $CHCl_3$ eluent, I_2 stain), combined, and the solvent removed to give the product. Due to aggregation and the large number of exchangeable hydrogens, the ^1H NMR spectra in all cases were broad and relatively uninformative, but confirmed the complete reaction of the olefin, and were consistent with the assigned structures. The following compounds were prepared by this method:

1,3-Bis-(2-thia-2-[17- and/or 18-(β-D-glucopyranosylthio)-3- and/or 4-(1,6,15,20-tetraoxa-2,5,16,19-etraoxocyclooctacosa-17-enyl)]) ethyl)benzene, $(G8_2)_2X$

From $(8_2)_2X$, as a pale oil (90%); ^{13}C NMR 62.89 MHz (δ, CD_3OD): 173.4, 173.1, 172.4, 172.1, 171.9 (C=O), 139.2, 130.9, 129.8, 129.2 (C=C), 86.5, 85.6, 82.0, 81.7, 79.6, 79.5, 74.3, 71.4, 71.3, 63.0 (glucose), 66.9, 66.7, 66.5, 66.1 (CO_2CH_2), 42.3 (two peaks, CHS), 37.6, 37.2, 36.7 ($CH_2C{=}O$, $ArCH_2$), 30.3, 29.7, 27.0, 26.9 ($CH_2CH_2CH_2$); MS: 1467.8 $(M+H)^+$, 1272.7 $(M-G)^+$.

1,3-Bis-(2-thia-2([17- and/or 18-(β-D-glucopyranosylthio)-3- and/or 4-(1,6,9,12,15,20-hexaoxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)]) ethyl)benzene, $(G8Trg)_2X$

From $(8Trg)_2X$, as a pale oil (84.4 mg, 36%); ^{13}C NMR 90.56 MHz (δ, CD_3OD): 173.4, 173.1, 172.3, 172.1, 172.0, 171.9 (C=O), 139.2, 131.0, 129.9, 129.3 (C=C), 86.5, 85.8, 82.0, 81.9, 79.6, 79.5, 74.3, 62.9 (glucose), 71.1, 70.0 (OCH_2CH_2O), 69.2, 69.1 ($CO_2CH_2CH_2O$), 65.2, 65.1, 64.8, 64.4, 64.3, 62.9 (CO_2CH_2), 41.8, 41.7, 41.2 (CHS), 36.7, 36.5, 36.1, 35.9, 35.5 ($CH_2C{=}O$, $ArCH_2$), 30.1, 29.9, 29.6, 28.9, 28.7, 26.7, 26.2, 25.9, 25.6 ($CH_2CH_2CH_2$); MS: 1475.7 $(M+H)^+$, 1280.7 $(M-G)^+$.

1,3-Bis-(2-thia-2-([17- and/or 18-(β-D-glucopyranosylthio)-3- and/or 4-(1,6,9,12,15,20,23,26-octaoxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)]) ethyl)benzene, $(GTrg_2)_2X$

From $(Trg_2)_2X$, as a pale oil (27.3 mg, 29%); ^{13}C NMR 62.89 MHz (δ, CD_3OD): 173.5, 173.4, 173.2, 172.2, 172.0, 171.9 (C=O), 139.2, 131.1, 129.9, 129.3 (C=C), 86.4, 85.7, 82.0, 79.7, 79.5, 74.3, 71.7, 71.3, 62.9 (glucose), 70.0 (OCH_2CH_2O), 65.9, 65.8, 65.3 (CO_2CH_2), 42.6, 41.2 (CHS), 38.6, 37.3, 36.7 ($CH_2C{=}O$, $ArCH_2$); MS: 1483.6 $(M+H)^+$, 1288.6 $(M-G)^+$.

2R,3R-Bis[17- and/or 18-(β-D-glucopyranosylthio)-3- and/or 4-(1,6,15,20-tetraoxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)-4-thiabutyl]tartarate, $(G8_2)_2Ta$

From $(8_2P)_2Ta$, as a pale yellow oil (57 mg, 80%); ^{13}C NMR 62.89 MHz (δ, CD_3OD): 173.5, 173.2, 172.8, 172.1, 172.0 (C=O), 86.5, 85.8, 82.0, 79.6, 79.5, 74.3, 71.6, 71.5, 62.9 (glucose), 73.9 (CHOH), 66.8, 66.6, 66.1, 64.9 (CO_2CH_2), 43.1, 42.8, 41.3 (CHS), 38.8, 38.1, 37.8, 36.0 ($CH_2C{=}O$), 30.3, 29.7, 29.0, 27.7, 27.0 (CH_2CH_2S, $CH_2CH_2CH_2$; MS: 1595.9 $(M+H)^+$, 1400.8 $(M-G)^+$.

2R,3R-Bis[17- and/or 18-(β-D-glucopyranosylthio)-3- and/or 4-(1,6,9,12,15,20-hexaoxa-2,5,16,19-tetraoxocyclooctacosa-17-3nyl)-4-thiabutyl]-tartarate, $(G8TrgP)_2Ta$

From $(8TrgP)_2Ta$, as a pale yellow oil (60.6 mg, 78%). ^{13}C NMR 90.56 MHz (δ, CD_3OD): 173.5, 173.3, 173.2, 172.8, 172.2, 172.1, 172.0 (C=O), 86.4, 86.8, 82.0, 81.9, 79.6, 79.5, 74.3, 71.6, 71.5, 62.9 (glucose), 73.9 (CHOH), 71.3, 71.2, 70.0 (OCC_2CH_2O), 66.7, 66.6, 66.4, 66.0, 65.9, 65.8, 65.2, 64.9 (CO_2CH_2), 43.0, 42.8, 42.4, 41.2, 41.0 (CHS), 39.5, 38.7, 38.6, 37.8, 37.5, 36.0 ($CH_2C{=}O$), 30.2, 29.9, 29.5, 29.4, 29.0, 27.6, 26.9, 26.7 (CH_2CH_2S, $CH_2CH_2CH_2$); MS: 1603.8 $(M+H)^+$, 1408.7 $(M+G)^+$.

General procedure for the addition of thiopropanol head groups (P)

The diene (30 μmol) and 3-mercapto-1-propanol (3 eq per alkene) as an isopropanol solution were added to isopropanol (20 mL), piperidine (0.25 mL) was added and the mixture stirred at reflux for 1 hr. The solvent was removed and the product dissolved in 4:3 chloroform/methanol (2 mL) and chromatographed by gel filtration (Sephadex LH 20, 4 × 20 cm). The product was collected in 1 mL fractions near the void volume. Product containing fractions were identified by TLC (silanized silica, Merck RP-2, 5% CH_3OH

in $CHCl_3$ eluent, I_2 stain), combined and the solvent removed to give the product. The ^1H NMR spectra were broad and relatively uninformative but confirmed complete reaction of the olefin and were consistent with the expected structures. The following compounds were prepared by this procedure:

1,3-Bis(2-thia-2-([17- and/or 18-(4-hydroxy-1-thiabutyl)-3- and/or 4-(1,6,15,20-tetraoxa-2,5,16,19-tetraoxo-cyclooctacosa-17-enyl)]) ethyl)benzene, $(P8_2)_2X$
From $(8_2)_2X$, as a colorless oil (81.2 mg, 81%); ^{13}C NMR 90.56 MHz (δ, CD_2Cl_2): 171.8, 171.5, 170.6, 170.5 (C=O), 138.1, 130.1, 129.0, 128.4 (C=C), 65.8, 65.6, 65.3 (CO_2CH_2), 61.3 (CH_2OH), 42.2, 41.7, 41.6 (CHS), 37.1, 36.8, 36.0 (CH_2C=O), $ArCH_2$), 32.4 ($CH_2CH_2CH_2S$), 29.6, 29.5, 29.3, 28.9, 28.4, 26.2, 26.1, 26.0 ($CH_2CH_2CH_2$); MS: 1259.7 $(M+H)^+$, 1168.7 $(M-P)^+$.

1,3-Bis(2-thia-2-([17- and/or 18-(4-hydroxy-1-thiabutyl)-3- and/or 4-(1,6,9,12,15,20-hexaoxa-2,5,16,19-tetra-oxocyclooctacosa-17-enyl)]) ethyl)benzene, $(P8Trg)_2X$
From $(8Trg)_2X$, as a colorless oil (45.3 mg, 89%); ^{13}C NMR 90.56 MHz (δ, CD_2Cl_2): 171.9, 171.7, 171.6, 171.5, 170.7, 170.6, 170.5 (C=O), 138.1, 130.1, 129.0, 128.4 (C=C), 70.9 (OCH_2CH_2O), 69.3 ($CO_2CH_2CH_2O$), 65.7, 65.2, 64.8, 64.4 (CO_2CH_2), 61.3 (CH_2OH) 42.3, 42.0, 41.8, 41.6 (CHS), 37.0, 36.9, 36.7, 36.5, 36.1, 36.0 (CH_2C=O, $ArCH_2$), 32.4, 32.3 ($CH_2CH_2CH_2S$), 29.2, 29.1, 28.8, 28.5, 28.3, 26.1, 25.9, 25.8, 25.7 ($CH_2CH_2CH_2$); MS: 1267.6 $(M+H)^+$, 1176.5 $(M-P)^+$.

1,3-Bis(2-thia-2-([17- and/or 18-(4-hydroxy-1-thiabutyl)-3- and/or 4-(1,6,9,12,15,20,23,26-octaoxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)]) ethyl)benzene, $(PRrg_2)_2X$
From $(Trg_2)_2X$, as a colorless oil (58.4 mg, 94%); ^{13}C NMR 90.56 MHz (δ, CD_2Cl_2): 171.9, 171.5, 170.6, 170.5 (C=O), 138.0, 130.1, 128.9, 128.5 (C=C), 70.9 (OCH_2CH_2O), 69.3 ($CO_2CH_2CH_2O$), 64.9, 64.4 (CO_2CH_2), 61.1 (CH_2OH), 42.1, 41.8 (CHS), 36.8, 36.5, 36.0 (CH_2C=O, $ArCH_2$), 32.3 ($CH_2CH_2CH_2S$), 28.4 ($CH_2CH_2CH_2S$); MS 1275.5 $(M+H)^+$, 1184.5 $(M-P)^+$.

2R,3R-Bis[17- and/or 18-(4-hydroxy-1-thiabutyl)-3-and/or 4-(1,6,15,20-tetraoxa-2,5,16,19-tetraoxocyclo-octacosa-17-enyl)-4-thiabutyl]-tartarate, $(P8_2P)_2Ta$
From $(8_2P)_2Ta$, as a colorless oil (37.5 mg, 94%); ^{13}C NMR 90.56 (δ, $CDCl_3$): 171.4, 171.3, 170.3(C=O), 72.2 (CHOH), 65.5, 65.4, 65.1, 65.0, 64.4 (CO_2CH_2), 61.1 (CH_2OH), 41.8, 41.6 (CHS), 36.7, 36.5 (CH_2C=O), 31.7 ($CH_2CH_2CH_2S$), 29.6, 29.1, 29.0, 28.4, 28.2, 28.0, 27.6, 25.7 ($CH_2CH_2CH_2$); MS: 1387.8 $(M+H)^+$, 1296.7 $(M-P)^+$.

2R,3R-Bis[17- and/or 18-(4-hydroxy-1-thiabutyl)-3-and/or 4-(1,6,9,12,15,20-hexaoxa-2,5,16,19-tetraoxo-cyclooctacosa-17-3nyl)-4-thiabutyl]-tartarate, $(P8TrgP)_2Ta$
From $(8TrgP)_2Ta$, as a colorless oil (57.6 mg, 86%); ^{13}C NMR 90.56 MHz (δ, $CDCl_3$): 171.6, 171.5, 171.3 (two peaks), 171.2, 170.4, 170.3 (C=O), 72.2 (CHOH), 70.5 (OCH_2CH_2O), 68.9, 6.8 ($CO_2CH_2CH_2O$), 65.4, 65.3, 64.9, 64.8, 64.4, 64.3, 64.2, 63.9 (CO_2CH_2), 60.9 (CH_2OH), 41.8, 41.6, 41.5 (CHS), 36.5, 36.4, 36.3 (CH_2C=O), 31.8, 31.7 ($CH_2CH_2CH_2S$), 29.6, 28.7, 28.6, 28.3, 28.2, 28.0, 27.9, 27.7, 25.4, 25.4, 25.3 ($CH_2CH_2CH_2$); MS: 1395.6 $(M+H)^+$, 1304.6 $(M-P)^+$.

General procedure for the addition of thioethylamine head groups (N)
The diene (50 μmol) and 2-mercaptoethyl amine (5 eq per alkene) as an isopropanol solution were added to isopropanol (20 mL), piperidine (.25 mL) was added and the mixture stirred at reflux for 1 hr. The solvent was removed and the product was dissolved in 4:3 chloroform/methanol (2 mL) and chromatographed by gel filtration (Sephadex LH 20, 4 x 20 cm). The product was collected in 1 mL fractions near the void volume. Product containing fractions were identified by TLC (silanized silica, Merck RP-2, 5% CH_3OH in $CHCl_3$ eluent, I_2 stain), combined and the solvent removed to give the product. The ^1H NMR spectra were broad and uninformative, but confirmed complete reaction of the olefin, and were generally consistent with the assigned structures. The following compounds were prepared by this procedure:

1,3-Bis(2-thia-2-([17- and/or 18-(3-amino-1-thiapropyl)-3- and/or 4-(1,6,15,20-tetraoxa-2,5,16,19-tetraoxo-cyclooctacosa-17-enyl)]) ethyl)benzene, $(N8_2)_2X$
From $(8_2)_2X$, as a colorless oil (50.3 mg, 88%); ^{13}C NMR 90.56 MHz (δ, CD_2Cl_2): 171.8, 171.7, 171.4, 170.6, 170.5, 170.4 (C=O), 138.1, 130.0, 129.0, 128.4 (C=C), 66.0, 65.7, 65.4, 65.3 (CO_2CH_2), 43.5 (CH_2N), 42.1, 42.0, 41.7, 41.6 (CHS), 37.2, 37.0, 36.8, 36.0, 35.6 (CH_2C=O, CH_2S), 29.6, 29.5, 28.9, 26.2, 26.1 ($CH_2CH_2CH_2$); MS: 1229.7 $(M+H)^+$.

1,3-Bis(2-thia-2-([17- and/or 18-(3-amino-1-thiapropyl)-3- and/or 4-(1,6,9,12,15,20-hexaoxa-2,5,16,19-tetra-oxocyclooctacosa-17-enyl)]) ethyl)benzene, $(N8Trg)_2X$
From $(8Trg)_2X$, as a colorless oil (64.2 mg, 88%);

^{13}C NMR 90.56 MHz (δ, CD_3OD): 171.9, 171.8, 171.7, 171.4, 171.2, 170.8, 170.7, 170.5 (**C**=O), 138.0, 130.1, 129.0, 128.4 (**C**=**C**), 70.9 (OCH_2CH_2O), 69.3 ($CO_2CH_2CH_2O$), 65.9, 65.7, 65.4, 65.2, 64.9, 64.7, 64.5, 64.4 (CO_2CH_2), 43.5 (CH_2N), 41.8, 41.6, 41.3, 40.5, 40.4 (CHS), 36.9, 36.8, 36.7, 36.5, 36.4, 36.1, 36.0, 35.6 (CH_2C=O, CH_2S), 33.1, 29.6, 29.4, 29.2, 28.9, 28.8 (two peaks), 27.7, 27.2, 26.1, 26.0, 25.9, 25.8; MS: 1237.5 $(M+H)^+$.

1,3-Bis(2-thia-2-([17- and/or 18-(3-amino-1-thiapropyl)-3- and/or 4-(1,6,9,12,15,20,23,26-octaoxa-2,5,16,19-tetraoxocyclooctacosa-17-enyl)]) ethyl)benzene, $(NTrg_2)_2X$

From $(Trg_2)_2X$, as a colorless oil (39.0 mg, 82%); ^{13}C NMR 90.56 MHz (δ, CD_2Cl_2): 171.8, 171.1, 170.8, 170.5 (**C**=O), 138.0, 130.2, 129.0, 128.5 (**C**=**C**), 70.8 (OCH_2CH_2O), 69.2 ($CO_2CH_2CH_2O$), 64.9, 64.8, 64.5, 64.3 (CO_2CH_2), 43.4 (CH_2N), 41.2 (two peaks, CHS), 36.8, 36.3, 35.9, 35.8 (CH_2C=O, CH_2S); MS: 1245.4 $(M+H)^+$.

1,3-Bis(2-thia-2([17- and/or 18-(1-thio-2-carboxylethyl)-3- and/or 4-(1,6,15,20-tetraoxa-2,5,16,19-tetraoxo-cyclooctacosa-17-enyl)]) ethyl)benzene, $(A8Trg)_2X$

To a solution of $(8Trg)_2X$ (59.9 mg, 55.3 μmol) and mercaptoacetic acid (12.6 mg, 111 μmol, 2 eq, from a stock solution in THF) in THF (20 mL) at 50°C under a N_2 atmosphere was added 2,2,6,6-tetramethyl-piperidine (10 drops, pH 9). The reaction mixture was stirred for 6 h and the solvent was removed under reduced pressure. The residue was dissolved in a 4:3 mixture of chloroform:methanol (5 mL) and was added to a cation exchange resin (Dowex 50 × 8-100, 1 × 5 cm) which had been activated with 2 M sulfuric acid, washed with water, methanol, and a 4:3 mixture of chloroform:methanol. The acidic fractions were combined, concentrated (~2 mL), and purified on a gel-permeation column (Sephadex LH-20, 3 × 20 cm). The product was eluted with chloroform:methanol (4:3) and was collected in fractions 3–14 (12 mL). Evaporation of the solvent gave $(A8Trg)_2X$ as a pale-yellow oil (67.6 mg, 97%); ^{13}C NMR 90.56 MHz (δ, CD_2Cl_2): 171.6, 171.4, 171.1, 170.8, 170.7, 170.5, 170.3, 170.0, 169.4 (**C**=O), 138.1, 130.1, 129.0, 128.4 (**C**=**C**), 70.9 (OCH_2CH_2O), 69.2 ($CO_2CH_2CH_2O$), 66.0, 65.8, 65.7, 65.5, 65.3, 65.1, 65.0, 64.9, 64.4, 64.3, 64.1 (CO_2CH_2), 42.7, 42.3, 42.1, 41.8, 41.7 (CHS), 36.8, 36.5, 36.4, 36.1, 36.0 (CH_2C=O, CH_2S), 34.0, 33.9 (CH_2CO_2H), 29.5, 29.2, 29.1, 29.0, 28.8, 25.9, 25.8, 25.7 ($CH_2CH_2CH_2$); MS: 1267.5 $(M+H)^+$, 1289.5 $(M+Na)^+$.

TRANSPORT EXPERIMENTS

Materials

Egg phosphatidylcholine and egg phosphatidic acid (egg PC and PA) were purchased from Avanti Polar Lipids, Inc., Pelham, Alabama. Bis-Tris (2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol), carbonyl-cyanide-trifluoromethoxyphenylhydrazone (FCCP), cholesterol, choline hydroxide (20% in water), gramicidin D, melittin, and valinomycin were obtained from Sigma/Aldrich; sulfuric acid (ultrapure) was obtained from Fluka. Only D^3 (deionized, double distilled) water was used.

Choline Sulfate[22] – Choline hydroxide (250 mL of 20% solution in 500 mL of D^3 water) was titrated to pH 6.5 with concentrated sulfuric acid, decolorized with activated charcoal and the solution was concentrated to a thick oil by rotary evaporation. Residual water was removed by azeotropic drying with absolute ethanol, and crystalline choline sulfate was obtained from absolute ethanol/ether at −10° for 16 hrs (2 crops). Combined batches were dried for 24 hours in vacuum.

Solutions

Internal Buffer Solution – 0.20 M. Bis-Tris, and 0.054 M D-mannitol; pH adjusted to 6.60 using 0.45 M H_2SO_4. *External Solution* – 0.110 M choline sulfate, and 0.093 M D-mannitol; cold storage under nitrogen and periodic filtration through Millipore GS 0.22 μm filters was required to stabilize the solution. *Lipid Stock Solution*: 8:1:1 (molar ratio; 16:2:1 weight ratio) of egg PC:egg PA:cholesterol in $CHCl_3$ at a concentration of 50 mg PC per 3 mL. The solution was stored under nitrogen at −15°C shielded from light. *Choline Hydroxide Titrant* – 4.75 mM choline hydroxide, 0.35 M D-mannitol; base titer established versus potassium hydrogen phthalate.

FCCP, Gramicidin D, and valinomycin were dissolved in methanol to concentrations of 9.6×10^{-4} M, 3.3×10^{-5} M, and 2.8×10^{-3} M, respectively. Triton X-100 was diluted by a factor of 20 with D^3 water. The transporters were dissolved in methanol at concentrations between 2.58×10^{-3} M and 5.70×10^{-3} M.

Vesicle preparation

Mixed lipids (3 mL of stock solution containing 50 mg PC) were evaporated onto the walls of a boiling tube (20 mm dia, 50 mm deep, B24 joint) and the lipid film was dried for four hours at 50 °C under vacuum. The dried lipids were dissolved in anhydrous ether (6 mL) and 2 mL of internal buffer was added.

The mixture was sonicated to an opalescent single phase using a Heat Systems W385 Ultrasonic probe sonicator (13 mm tip; forty 2 second pulses at 50% duty cycle and 50% power output). The ether was removed from the mixture by slow rotary evaporation at 150–200 mm Hg pressure with a bath temperature of 25 °C. As the ether begins to evaporate, the solution coats the sides of the flask. Further evaporation at a pressure of 100–150 mm Hg promotes spontaneous bubbling which continues for about 5 minutes. The mixture goes through a gelatinous phase, then becomes a freely flowing liquid. At this point, external buffer solution (3 mL) is added and the remaining ether is removed by rotary evaporation for fifteen minutes at a pressure of 50 mm Hg. The vesicles were 'sized' through Nucleopore filters (1 μm followed by 0.4 μm) using nitrogen pressure. Excess buffer components were removed by gel filtration (Sephadex G-25M, PD-10 column, Pharmacia). The column was equilibrated with external solution, the 'sized' vesicle mixture was added (2.5–4 mL) and the column was eluted with external solution. The first 0.5 mL of cloudy eluent after the exclusion volume (2.5 mL) were discarded. A total of 3–4 mL of eluent containing vesicles was retained. The prepared vesicles were used within 36 hours.

Vesicle characterization

Total phospholipid in the vesicle preparations (typically $1.0–1.3 \times 10^{-2}$ M) was determined by colorimetric analysis of a phosphomolybdate complex of the head groups.[23] Vesicle morphology was assessed by transmission electron microscopy (phosphotungstic acid stain[23b]). Quantitative size distribution analysis of vesicles by dynamic light scattering was performed using a NICOMP C370 Submicron Particle Sizer.[23c] Vesicle entrapment of buffer solution is directly determined by the pH-stat experiment (Triton lysis). The fraction of multilamellar vesicles (MLVs) was assessed from the proportion of the vesicle contents released by a single aliquot of melittin.[24]

pH-stat experiment

The pH-stat system used a Metrohm 655 Dosimat burette and thermostatted titration cell controlled by a Metrohm 614 Impulsomat and Metrohm 632 pH-meter, fitted with a glass electrode and a standard calomel electrode linked to the cell via an electrolyte bridge to give: calomel‖0.11 M choline sulfate, 0.093 M mannitol‖vesicles in 0.11 M choline sulfate, 0.093 M mannitol at a set pH‖glass electrode. The burette was linked to an HP-85 microcomputer for data acquisition of time versus volume curves. *Typical procedure*: External solution (3.8 mL) and vesicles (0.2 mL) were mixed in the cell. A pH gradient was imposed by addition of choline hydroxide titrant solution to a set pH of 7.60 (approx. 0.4 mL). FCCP (7.7×10^{-4} M in methanol, 10 μL) was added, followed by typically 0.5 mL of a cation sulfate salt solution (0.5 M) to create an opposing cation gradient. The transporter ($2.5–5.7 \times 10^{-3}$ M in methanol or THF, 2–50 μL) was added, and the pH and cation gradients began to collapse. The order of addition of cation sulfate and transporter can be reversed. Volume of base added versus time data was accumulated until rate of addition fell to below 1–2 μL/min. The remaining entrapped protons were released by lysis of the vesicles with Triton X-100, and were subsequently titrated. The data were analysed as described in the text.

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