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PAPER

Pore formation in phospholipid bilayers by amphiphilic cavitands

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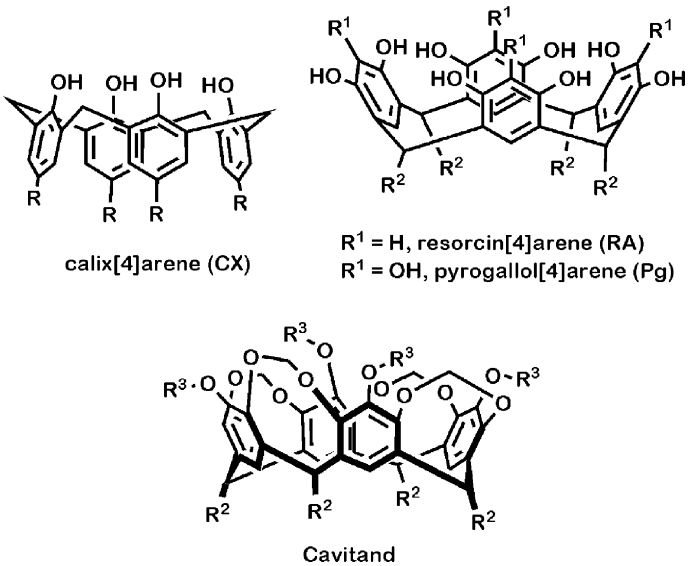
Five new cavitands were prepared that have four pendant *n*-undecyl chains and "headgroups" connected by 2-carbon spacers. The headgroups were ~OCH_2CONH-Ala-OCH_3, **1**; ~OCH_2CONH-Phe-OCH_3, **2**; ~OCH_2CONH-Ala-OH, **3**; ~OCH_2CONH-Phe-OH, **4**; and ~$OCH_2CONHCH_2CH_2$-thyminyl, **5**. Pore formation by each cavitand was studied by use of the planar bilayer conductance experiment. All five compounds were found to form pores in asolectin bialyer membranes. Compounds **1–3** behaved in a generally similar fashion and exhibited open-close dynamics. Compounds **4** and **5** formed pores more rapidly, were more dynamic, and led more quickly to membrane rupture. Differences in the ion transport activity are rationalized in terms of structure and aggregate cavitand assemblies.

Introduction

The chemistry leading to the molecules that Gutsche called calixarenes[1] has been known for more than a century[2] as have their nitrogenated analogs,[3] the calixpyrroles.[4] The extensive study of calixarenes alone is barely summarized in at least half a dozen monographs.[1,5] Analogs of calixarenes have also evolved into two closely related molecular macrocyclic families: the resorcinarenes[6] and the pyrogallolarenes.[7] Each of these basic systems has been elaborated and hybridized into complex multi-element structures. An example is the combination of calixarenes with crown ethers that merge into calixcrowns.[8] In some cases, the better known calixarene name is used to describe related derivatives, *e.g.* calix[4]resorcinarenes.[9] Cavitands[10] are structurally similar to calixarenes and resorcinarenes, but they present additional linkages in their structures.

Macrocycles of the calixarene, resorcinarene, and pyrogallolarene types (Scheme 1) have been studied as carrier molecules and for their ability to form ion-conducting pores within bilayer membranes. McKervey and coworkers demonstrated ion binding and "ion transfer" properties of functionalized calixarenes as early as 1985.[11] Shinkai,[12] Jin *et al.*,[13] and Beer *et al.*[14] reported carrier function and de Mendoza *et al.*[15] reported transport activity in phospholipid bilayers in the 1990s. Siderov, Davis, and coworkers reported ion channel activity for calixarenes in 2002.[16]



Scheme 1 Four related families of supramolecular scaffolds.

Coleman and coworkers reported aqua channel activity from a self-assembled calixarene.[17] Maulucci *et al.* reported calix–steroid conjugates as ionophores.[18] Davis and coworkers reported ion regulation by calixarenes and "acyclic calixarenes."[19] Our own efforts to incorporate calixarenes as elements in our successful hydraphile design met with limited success.[20]

Resorcinarenes are analogs of calixarenes that have been formed from 1,3-dihydroxybenzene and a carbonyl compound.[6] Several examples of transport mediated by resorcinarenes have been reported.[21–23] Most relevant to the present studies is the report by Tanaka, Kobuke, and Sokabe of a resorcinarene that formed K^+-selective pores.[24] The pyrogallolarenes form from carbonyl compounds and 1,2,3-trihydroxybenzene. Studies conducted by

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one of us have shown that these compounds can also form membrane-active ion transporters.[25,26]

Cavitands are similar in many respects both to calixarenes and to resorcinarenes. In cavitands, the two hydroxyl groups of resorcinarenes are linked covalently. This leads to structures that are more conformationally rigid than their calixarene or resorcinarene counterparts. We therefore prepared a group of cavitands as described in detail in the Experimental Section. To our knowledge, no example of a cavitand pore-former has previously been reported. The new cavitands disclosed here are membrane active and they form pores that show unequivocal evidence for ion transport function.

Results and Discussion

In work reported some years ago, Reinhoudt and co-workers[27] described the preparation of several functionalized cavitands. These compounds were elaborated with a variety of headgroups and were shown to bind steroids with association constants in $CDCl_3$ of ~10^2–10^3 M^{-1}. The structural similarities between these cavitands and the calixarene family of structures combined with the membrane activity of calixarene-type macrocycles having hydrophobic chains, suggested that pore formation was likely by suitably functionalized cavitands.

Design rationale

Cavitands are macrocycles that comprise a short tube-like structure and have four attachment points on the nonpolar side to which hydrocarbon chains may be linked, as has been done previously.[27] On the polar side, there are four hydroxyl groups that can connect to a variety of polar elements. The selectivity filter of the voltage gated potassium KcsA channel comprises four aligned tyrosine residues that interact with transient K^+ ions.[28] It has been shown previously that long-chained resorcinarenes can form conducting pores that the authors inferred mediated transport through the macrocycle.[24] We therefore prepared amphiphilic cavitands having amino acids linked through acetic acid chains that could form an extended polar head group and a tetrad of amino acids conceptually similar to that seen in the natural channel protein. Phenylalanine, rather than tyrosine, was chosen for the first studies to avoid synthetic complications caused by the phenolic hydroxyl group. The alanine acid and ester compounds were prepared for comparison.

Compounds prepared for the present study

The cavitands Reinhoudt and coworkers reported had four *n*-undecyl ($C_{11}H_{23}$) sidechains as do the compounds discussed here. In that work, four acetic acid residues appended to the upper rim permitted functionalization to, for example, other calixarene-type macrocycles.[27] The compounds prepared for the present study used the acetic acid bridge to connect alanine methyl ester (**1**), phenylalanine methyl ester (**2**), alanine (**3**), or phenylalanine (**4**). These amino acids and esters were chosen in part so that the effect of hydrophobicity (Ala *vs*. Phe) and charge (carboxylic acid *vs*. ester) could be assessed. A fifth compound having a thymine terminus (**5**), was prepared to examine the effect of a basic residue on membrane activity.

The resorcinarene was prepared from *n*-dodecanal and resorcinol and brominated with NBS. The hydroxyl groups were then coupled through a methylene group by treatment with $ClCH_2Br$ and K_2CO_3. The aryl bromide was converted into a hydroxyl group by lithium–halogen exchange followed by oxidation. The hydroxyl groups were etherified by treatment with methyl bromoacetate. The esters were hydrolyzed and then coupled to alanine methyl ester or to phenylalanine methyl ester to give **1** and **2**. Part of each sample of **1** and **2** was hydrolyzed to give the corresponding acids, **3** and **4**. The synthetic sequence is illustrated in Scheme 2.

Each new, synthetic amphiphile was prepared from the same tetra-acid cavitand previously reported by Reinhoudt *et al.* The appropriate amino acid or thymine[29] derivative was then appended to this structure as described in the Experimental Section. The preparation of thymine cavitand **5** required a strategy somewhat different from that used to prepare cavitands with amino acid headgroups (Scheme 3). In the first step, the ethylene linker was appended to phthalimide and then thymine was connected through the pyrimidine 1-position. After removal of the phthalimide protecting group, the cavitand acid was coupled to 1-(2-aminoethyl)thymine using EDC.

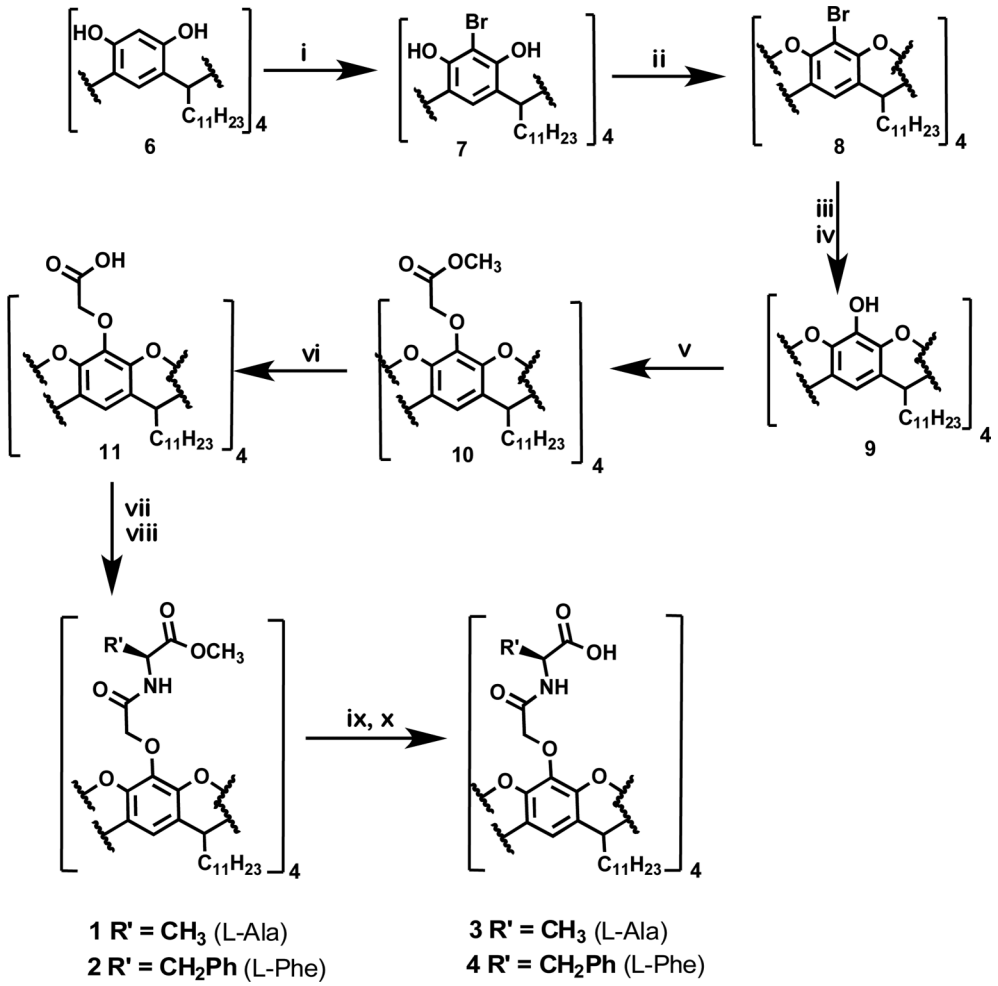
The yields given are for the last step in which the L-amino acid or pyrimidine is attached to the linker. The melting points are also recorded for each compound. The yields and melting points are: **1**, -Ala-OCH_3 (68%, mp 76–79 °C); **2**, -Phe-OCH_3; (70%, mp 72–74 °C); **3**, -Ala-OH; (88%, mp 139–142 °C); **4**, (90%, mp 150–152 °C); -Phe-OH; and **5**, -thymine, (71%, mp 161–163 °C). Each product was fully characterized by NMR and mass spectrometry. Details may be found in the Experimental Section.

Planar bilayer conductance

Compounds **1–5** were assayed for the ability to form pores by examining their behavior in a planar phospholipid bilayer membrane. The voltage clamp experiment[30] used two aqueous KCl solutions buffered at pH 7 that were separated by an asolectin membrane. Cavitand samples were dissolved either in trifluoroethanol or DMSO and then added to one (the *cis*) of the buffer solutions. Each of compounds **1–5** inserted into the bilayer and ion current could be detected. Addition of the pore-former from the bulk phase is important to note because some compounds, such as Triton X-100, can exhibit channel-like behavior only when embedded in the planar bilayer during its formation.[31] The planar bilayer voltage clamp experiment detects a pore formed by compounds that are present in the bilayer. Thus, factors such as aggregation of monomers in the aqueous buffer[32] are expected to alter insertion rates into the membrane.

Pore formation behavior

There are four obvious questions to consider with respect to the amphiphilic cavitands described here. First, do the cavitands insert in the bilayer membrane and form functioning pores? Second, if they form pores, are these what might be expected based on literature precedent? Third, if the cavitands are pore formers, do they show the same or different behavior? Fourth, can the behavior apparent in the voltage clamp experiment be rationalized in terms of structure? The answer to the first question is obvious from the



Scheme 2 Experimental conditions: (i) NBS, 2-butanone, 0 °C. ii. K_2CO_3, $ClCH_2Br$, DMF, 65 °C; (iii) dry THF, *n*-BuLi, $B(OCH_3)_3$, −78 °C; (iv) 1 : 1 15% H_2O_2 : 1.5 M NaOH; (v) $BrCH_2COOMe$, K_2CO_3, dry MeCN, reflux 48 h; (vi) 2 M KOH, THF; (vii) ClCOCOCl, dry CH_2Cl_2, rt; (viii) amino acid ester, Et_3N, dry CH_2Cl_2, 0 °C; (ix) LiOH, THF/H_2O, rt, (x) 4 N HCl.

data presented in Fig. 1. In fact, **1–5** all form functioning pores in asolectin bilayers.

Generally, the behavior of **1–3** was similar (see Fig. 1). Pore formation was observed within 1 h after addition of the compound to the *cis* buffer for **1–5** and well-behaved open states were recorded. We note that all five compounds eventually formed large pores that ruptured the membrane. Compounds **4** (~CH_2CONH-Phe-OH) and **5** (~CH_2CONH-thymine) formed pores more quickly (within ~10 min), exhibited open–closed states that showed more frequent openings than those observed for **1–3**, and more rapidly led to a membrane-rupturing large pore.

Fig. 1, panels a and b, shows the results of planar bilayer conductance studies for the Ala-OMe terminated cavitand, **1**, at positive (+30 mV) and negative (–20 mV) potentials, respectively and at a concentration of **1** in the *cis* chamber of 0.5 μM. Multiple conductance levels were observed both at positive and negative potentials, likely arising from differences in aggregation states, as discussed in more detail below. The positive potential recording in panel 1a shows well-behaved open states for **1**. At the left of the trace, a single opening is apparent (13 pA), followed by a state that corresponds to 26 pA. It is tempting to conclude that the latter represents two open channels, each of which has a conductance of 13 pA. This seems unlikely, however, as both channels would be required to open and close exactly at the same time for the trace shown in the Figure to be observed.

The 13 pA channel was also observed at negative potentials, but it is not the predominant channel at negative potentials and it is not shown in Fig. 1. The calculated conductance for the 13 pA pore (aggregation state) is ~400 picoSiemens (pS), for which the calculated (Hille equation[33]) diameter is ~15 Å. This size corresponds to measurements made with molecular models on hexamers of amphiphilic cavitands. Panel 1b shows open states having currents of –21 pA and –33 pA, which are the predominant open states observed at –20 mV.

The data shown in panel 1c for the phenylalanyl ester, **2**, were recorded on a current scale more than twice that for **1** (panel a). The time scale (~48 s) is also longer than in panel a (~15 s). The concentration of **2** added to the *cis* chamber in this case is higher than in the alanyl ester case (1.5 μM *vs*. 0.5 μM), and the applied potential is +70 mV (*vs*. +30 mV in panel a) so the open state conductances differ substantially. The predominant conductance state shows a current of ~55 pA and remains open for ~30 s. A second open state is apparent at the end of the trace shown. Its current is ~45 pA. The sharp transition to the lower conductance

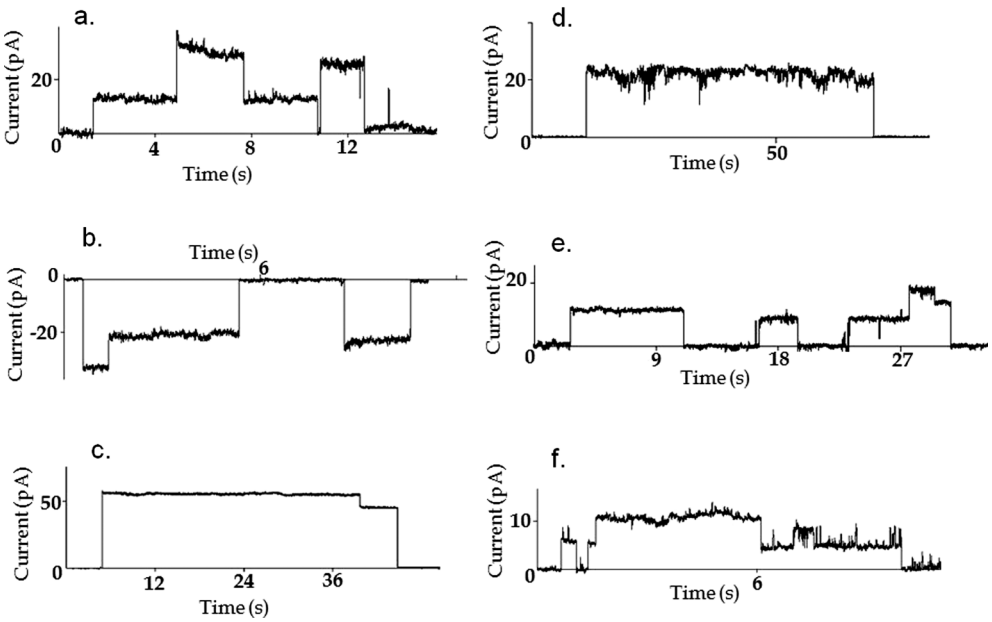
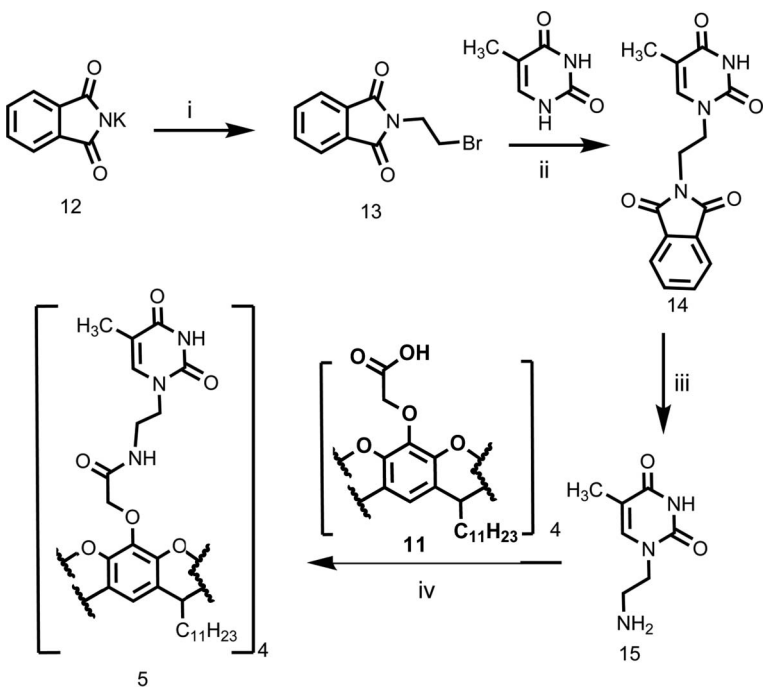


Fig. 1 Planar bilayer traces for compounds **1–5**. Panel a: ~Ala-OMe, [**1**] = 0.5 μM, applied potential +30 mV. Panel b: ~Ala-OMe, [**1**] = 0.5 μM, applied potential −20 mV. Panel c: ~Phe-OMe, [**2**] = 1.5 μM, applied potential +70 mV. Panel d: ~Ala-OH, [**3**] = 0.5 μM, applied potential +50 mV. Panel e: ~Phe-OH [**4**] = 0.5 μM, applied potential +30 mV. Panel f: ~thyminyl, [**5**] = 1.5 μM, applied potential 10 mV.



Scheme 3 Experimental conditions: (i) $BrCH_2CH_2Br$, DMF, rt; (ii) K_2CO_3, DMSO, rt; (iii) 1 : 4 *n*-$C_4H_9NH_2$-MeOH, reflux 2 d; (iv) EDC, DMAP, 0 °C, rt, 18 h.

state is consistent with a change in aggregation state, *i.e.*, loss of one cavitand from a pore-forming aggregate containing multiple cavitands (Fig. 2).

Fig. 1d shows the behavior of alanyl acid cavitand **3**, [**3**] = 0.5 μM), at an applied potential of +50 mV. The time scale in panel a (**1**, Ala-OMe) is ~15 s and in panel 1d it is ~75 s. The open state shows a current similar to those in panel a but the applied potential is +50 mV rather than +30 mV and the open state duration is substantially longer than either open state shown for the corresponding ester.

The behavior of **4**, the phenylalanyl acid, at a concentration of 0.5 μM and an applied potential of +70 mV, is shown in panel 1e. Compounds **2** (~Phe-OMe) and **4** (~Phe-OH) differ only by the absence of the methyl ester in the latter. Their behavior in the

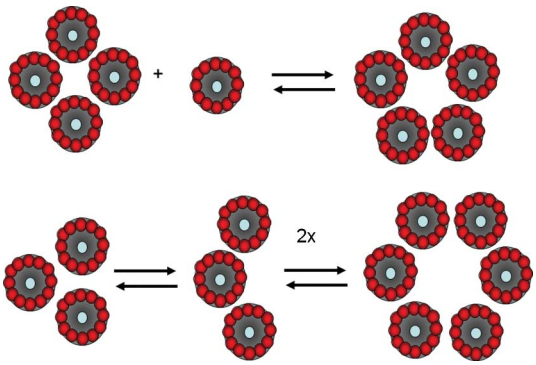


Fig. 2 Schematic representation of aggregation presumed to lead to pore formation by **1–5**.

bilayer is substantially different, however. The currents observed for the open states of **4** are ~15 pA and much shorter open times are observed for the acid compared to the ester.

Thymine-terminated cavitand **5** differs in head group structure and type from **1–4**. Compound **5** was added to the *cis* buffer in a concentration of 1.5 μM and the recordings were obtained at an applied potential of 10 mV. The somewhat noisy trace shows an open state of ~4.5 s duration and a current of ~14 pA. This appears to be the result of a 9 pA pore superimposed on the 5 pA open state that persists nearly to the end of the trace that is illustrated. A hint that this is so can be found at the beginning of the 14 pA state, where the step from 5 pA is obvious.

The second question posed above concerns whether or not the formation of pores correlates to literature precedent. There is precedent from studies of amphiphilic resorcinarenes[24] and pyrogallolarenes.[26] As discussed below, a previous report of pore formation by an amphiphilic resorcinarene[24] concluded that K^+ passed through the macrocycle. The larger currents and longer open times observed in the present case are more in concert with an aggregation mechanism.[26]

As noted in the introduction, studies of transport activity by a variety of calix[4]arenes and their relatives have appeared. The earliest of these was the report by Sokabe *et al.*[24] of a resorcinarene having either undecyl or heptadecyl sidechains. In the **RA** structure (Scheme 1), R^1 is hydrogen and R^2 is either $(CH_2)_{10}CH_3$ or $(CH_2)_{16}CH_3$. The former is most closely related to the structures reported here but the latter is discussed in greater detail in that report. An analogous ion-conducting resorcinarene having properties similar to the heptadecyl-sidechained structure was later reported in which the sidechains were steroids.[34] The key findings presented for the *n*-heptadecyl derivative were as follows. First, rapid open–closed behavior was observed; transitions were on the order of 200 ms. Second, the currents observed for the open states corresponded to a conductance of 6.1 ± 0.8 pS. Third, the channel was cation selective and ~3-fold selective for K^+ over Na^+. Fourth, and most relevant to the present work, the tetraundecylresocinarene, "which has shorter alkyl chains, did not produce stable channel currents, showing that only when the amphiphile has alkyl tails capable of spanning half the membrane thickness it can form a stable channel structure."

The authors proposed that the two *n*-heptadecyl-sidechained amphiphiles located in opposite leaflets of the bilayer formed a channel. The channel conducted ions when the two amphiphiles were aligned, creating a pore that spanned the (soybean lecithin) bilayer membrane. They also proposed that Na^+ or K^+ passed through the interior of the macrocycle. This notion was supported by the fact that too large Rb^+ blocked ion transport.

The behavior observed for **1–5** stand in striking contrast to the observations reported for the resorcinarenes. First, the undecyl side chained cavitands form pores that the undecyl-resorcinarene does not. Second, the pore size calculated from the experimentally observed conductances suggests that the pores form by aggregation of several cavitand monomers and that ions pass through the oligomeric pore and not through the central opening of any monomer. We attribute the difference in behavior to the greater rigidity of the cavitands compared to the resorcinarenes and possibly to the longer polar segment present in the former.

Aggregation behavior analogous to that surmised for the cavitands was observed with the structurally related pyrogallolarenes. One of us recently reported that pyrogallol[4]arenes (Scheme 1, **Pg**, $R^1 = OH$, $R^2 = (CH_2)_{10}CH_3$)[26] form ion-conducting pores in planar soybean asolectin membranes. These pores showed continuous open states that conducted ions at either positive or negative potentials. The side chains in these pyrogallolarene derivatives are the same length as those in the resorcinarenes described above that failed to form channels. Based on models and conductance values, we concluded that the pores were 13 ± 2 Å in diameter and that the pore was formed by supramolecular assembly of six monomers. Addition of cholesterol contracted the pore size to ~5 Å (corresponding to four Pg monomers) but made the channels effective over a wide range of potentials.

Evidence accumulated in the study referenced above of pyrogallol[4]arenes in phospholipid bilayers suggested that the pores were oligomeric aggregates.[26] Addition of cholesterol to the membrane altered the conductance states. In the absence of cholesterol, pore size corresponded to a hexamer arrangement and in the steroid's presence, the pore appeared to be tetrameric. We infer from the structural similarity of these cavitands to pyrogallolarenes that a similar dynamic equilibrium can take place. We therefore expect to observe multiple pore sizes and thus multiple conductance states resulting from self-assembly within the bilayer. Fig. 2 shows a schematic view of how the cavitands may assemble. A top view of a tetrameric pore is shown in the upper left corner and a hexameric pore in the lower right corner of the Figure. The other assemblies are included to suggest the types of equilibria expected to occur both in the bulk phase and in the bilayer.

It is not surprising that thyminyl derivative **5** behaves differently from **1–4**. Compounds **1–4** all have amino acid headgroups but **5** is terminated by a pyrimidine residue. It was unexpected, however, that phenylalanine acid **4** also differs from **1–3**. We surmise that the combination of four additional arenes in **4** (compared to **1** or **3**) and the organizing ability of the carboxylic acid group (compared to **2**) enforces a significantly different conformation on **4** than on **1–3**. This could lead either to differences in aggregation in the aqueous buffer or to more rapid insertion in the bilayer.

Conclusions

The data presented in this report confirm that a range of structurally related cavitands can form ion-conducting pores. These differ in behavior from previously reported resorcinarenes but show similarities to pyrogallolarenes. We infer from the data

obtained that these compounds insert into the bilayer and form oligomeric aggregates. To the extent these aggregates vary in size, their transport efficacies differ. These inferences are supported by three observations. First, multiple conductance states may be observed for a single molecule in the same or different experiments. Typically, there is a most common conductance state that is observed in replicate experiments. Second, pore formation does not always occur immediately upon addition of the ionophore solution. In some instances/experiments, there was considerable delay in the formation of a conductance state although the pore ultimately proved to be very stable and well-behaved. Third, clear evidence was obtained for open-closed behavior, but over time, all of the pores enlarged until the membrane ruptured. We believe that the family of structures reported here, along with other derivatives that are in hand, will permit us to better understand the factors that lead to stable pore formation by these amphiphilic macrocycles. Studies on these novel synthetic ion channels are ongoing.

Experimental

General information

Reactions requiring anhydrous conditions were conducted in flame-dried glass apparatus under an atmosphere of nitrogen. Commercially available chemicals and reagents were used as supplied. Solvents were dried using standard procedures. Thin layer chromatography (TLC) was performed on aluminium-backed, precoated silica gel plates (Merck, silica gel 60 F_{254}, 20 cm × 20 cm). Mobile phases are reported as volume ratios or volume percents. Compounds were visualized using UV light, *p*-anisaldehyde, or iodine stains. Column chromatography was performed on silica gel 60 (Merck, particle size 0.040–0.063 mm). Eluting solvents are reported as volume ratios or volume percents.

Infrared spectra were recorded on a Perkin Elmer spectrum 100 instrument with a universal ATR attachment. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz in $CDCl_3$ at room temperature unless otherwise stated. ^{1}H NMR spectra are recorded in parts per million (δ, ppm) relative to the peak of $CDCl_3$ (7.26 ppm) and DMSO-d_6 (2.49 ppm). ^{13}C NMR spectra were recorded in $CDCl_3$ at 100 MHz (unless otherwise indicated) relative to the central peak of the chloroform-d_1 triplet (77.0 ppm) and the DMSO-d_6 septet (39.7 ppm). Standard abbreviations indicating multiplicity in the NMR spectra were used as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad; coupling constants (J) are reported in hertz (Hz). High resolution mass spectrometric data were obtained using a Bruker microTOF-Q II instrument operating at room temperature. Optical rotations were recorded on a Perkin Elmer Model 341 Polarimeter Model 341 (λ = 589 nm) at 20 °C.

Synthesis and characterization of compounds 1, 2, 3, and 4. Resorcin[4]arene (6)[35]. To a stirring solution of 95% ethanol (150 mL) and 37% aqueous HCl (50 mL), resorcinol, (39.60 g, 0.36 mol) was added. The reaction solution was cooled to 0 °C in an ice-bath. Dodecanal, (66.40 g, 0.36 mol) in (100 mL) of 95% ethanol was added slowly over a period of 2 h. The resulting solution was allowed slowly to warm to 25 °C and then refluxed at 70 °C. After 18 h of reflux, the reaction mixture was cooled to room temperature, and the precipitate that separated was filtered and washed with cold methanol until the washings were light yellow. The solid was crystallized from methanol, to yield **6** as yellow crystals. The material was suitable for use in subsequent reactions without further purification. (75 g, 75%); mp 288–290 °C; ^{1}H NMR [CD_3COCD_3]: δ = 8.48 (s, 8 H, ArOH), 7.55 (s, 4 H, ArH), 6.25 (s, 4 H, ArH), 4.30 (t, 4H, CH (methine)), 2.29 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.20–1.28 (m 72 H, $CH_2(CH_2)_9CH_3$), 0.89 (t, 12 H, CH_3) ppm; ^{13}C NMR [CD_3COCD_3]: δ = 152.72, 152.62, 125.40, 125.21, 125.15, 103.64, 34.40, 32.72, 30.64, 29.90, 29.85, 29.65, 29.46, 29.27, 29.09, 23.39, 14.41 ppm; FT-IR/ATR: 3355, 2923, 2853 cm^{-1}.

Tetrabromoresorcin[4]arene (7)[35]. To a stirring solution of octol **6** (55 g, 50 mmol) in 2-butanone (300 mL) was added *N*-bromosuccinimide (42.70 g, 240 mmol) during 30 min, the temperature was maintained below 25 °C by cooling (ice bath). The resulting solution was stirred for 18 h at 25 °C in the dark under N_2, then poured directly into boiling methanol (1.00 L) to remove excess succinimide, reheated to boiling, and filtered while hot. The solid was washed with hot methanol and dried, to yield compound, **7**, as an off-white powder. The compound was pure enough for use in subsequent reactions. (62 g, 87%); mp 286–289 °C; ^{1}H NMR [CD_3COCD_3]: δ = 8.28 (s, 8 H, ArOH), 7.60 (s, 4 H, ArH), 4.45 (t, 4 H, CH (methine)), 2.31 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.20–1.28 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.88 (t, 12 H, CH_3) ppm; ^{13}C NMR [CD_3COCD_3]: δ = 150.05, 126.01, 124.49, 101.21, 36.43, 34.56, 32.69, 30.55, 29.82, 29.63, 29.44, 29.25, 28.84, 23.36, 14.38 ppm; FT-IR/ATR: 3383, 2921, 2851 cm^{-1}.

Tetrabromocavitand (8)[38]. To a solution of octol, **7** (42.50 g, 30 mmol) and oven-dried (110 °C) K_2CO_3 (62.10 g, 450 mmol) in dry, degassed DMF (800 mL), bromochloromethane (29.3 mL, 450 mmol) was added over 30 min. The mixture was refluxed at 65 °C under a nitrogen atmosphere. After 24 h, more bromochloromethane (4 mL, 60 mmol) was added. After 48 h, the DMF was removed *in vacuo* to give a dark brown gum. The residue was dissolved in diethyl ether (250 mL), and then 2*N* HCl (400 mL) was slowly added to neutralize K_2CO_3, with stirring. After the mixture was stirred for 20 min, the ether phase was collected and the aqueous phase was extracted with more ether. The combined ether phases were washed with saturated NaCl, then dried over anhydrous $MgSO_4$, and filtered. The solvent was evaporated to give a clear brown gum. The crude gum was chromatographed on silica gel using hexane–dichloromethane (1 : 1). The eluant was concentrated on a rotary evaporator to give a cream-coloured residue. Trituration of this residue with methanol yielded a cream-coloured solid, which was recrystallized from THF–methanol (1 : 1) to yield the title compound as white crystals (31.70 g, 72%); mp 74–76 °C, ^{1}H NMR]: δ = 7.02 (s, 4 H, ArH), 5.96 (d, J = 7.2 Hz, 4 H, outer of OCH_2O), 4.84 (t, J = 8.1 Hz, 4 H, CH (methine)), 4.39 (d, J = 7.2 Hz, 4 H, inner of OCH_2O), 2.30 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.20–1.28 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.88 (t, 12 H, CH_3) ppm, ^{13}C NMR: δ = 152.05, 139.28, 119.07, 113.51, 98.48, 37.63, 31.94, 29.87, 29.70, 29.68, 29.40, 27.73, 25.61, 22.70, 14.12 ppm; FT-IR/ATR: 2921, 2851 cm^{-1}.

Tetrahydroxy cavitand (9)[36]. A solution of vacuum-dried **8** (15 g, 10.20 mmol) in dry THF (900 mL) was stirred in an oven-dried round-bottomed flask under N_2, sealed with a septum. The solution was cooled to −78 °C, and treated with n-butyllithium (~1.6 M solution in hexane, 96 mL, 153 mmol), which was slowly

added *via* the septum using a syringe during 30 min. After a further 20 min, trimethylborate (34.2 mL, 306 mmol) was added slowly during 15 min, so turning the solution into a yellow emulsion. The flask and its contents were removed from the cooling bath, and allowed to equilibrate to room temperature slowly, during which time the emulsion dissolved to yield a clear yellow solution. After stirring at room temperature for 2 h, the solution was again cooled to −78 °C, and treated with a 1 : 1 mixture of 15% H_2O_2 and 1.5 M NaOH (300 mL) to give a viscous, white emulsion. The solution was allowed to warm to room temperature and stirred for 18 h. $Na_2S_2O_5$ (60 g) was carefully added to the stirring solution, resulting in the formation of two layers. The THF layer was subsequently removed *in vacuo* to yield a yellow solid in the residual water, which was filtered and air dried. The mixture of alcohols was pre-adsorbed onto silica before chromatography on silica. The chromatography column was gradient eluted, starting with 1 : 2 EtOAc–hexane, accompanied by slow addition of EtOAc towards a final EtOAc–hexane of 8 : 2. Compound **9** was isolated as the most polar fraction (R_f 0.30, by TLC in 8 : 2 EtOAc–hexane) and found to be pure (6.60 g, 53%); mp 188–190 °C; ^{1}H NMR: δ = 6.60 (s, 4 H, ArH), 5.94 (d, J = 6.9 Hz, 4 H, outer of OCH_2O), 5.35 (s, 4 H, ArOH), 4.68 (t, J = 8.1 Hz, 4 H, CH (methine)), 4.42 (d, J = 6.9 Hz, 4 H, inner of OCH_2O), 2.17 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.30–1.52 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.88 (t, 12 H, CH_3) ppm; ^{13}C NMR: δ = 141.95, 140.77, 138.52, 110.17, 99.79, 36.79, 31.94, 29.84, 29.76, 29.72, 29.60, 29.40, 27.86, 22.70, 14.12 ppm; FT-IR/ATR: 3425, 2921, 2851 cm^{-1}.

Tetraester cavitand 10[27]. To a stirring solution of tetrol, **9** (3.10 g, 2.55 mmol) and oven-dried (110 °C) K_2CO_3 (3.50 g, 25.50 mmol) in dry CH_3CN (350 mL), methylbromoacetate (1.50 mL, 12.75 mmol) was added and the reaction mixture refluxed at 82 °C for 24 h under a nitrogen atmosphere, then the reaction mixture was cooled to room temperature and the solvent was removed *in vacuo.* The residue was dissolved in CH_2Cl_2 (200 mL). The organic layer was washed with 1*N* HCl (50 mL) followed by water (100 mL, 3×), and then washed with saturated brine (100 mL), and dried over anhydrous $MgSO_4$. After filtration, the solution was evaporated under reduced pressure to yield the title compound, which was triturated in methanol to give a white, crystalline solid. (3.80 g, 98%), mp 184–187 °C, ^{1}H NMR: δ = 6.77 (s, 4 H, ArH), 5.70 (d, J = 7.4 Hz, 4 H, outer of OCH_2O), 4.67 (t, J = 8.0 Hz, 4 H, CH (methine), 4.52 (s, 8 H, $ArOCH_2$), 4.39 (d, J = 7.4 Hz, 4 H, inner of OCH_2O), 3.76 (s, 12 H, OCH_3), 2.18 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.27–1.40 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.88 (t, 12 H, CH_3) ppm; ^{13}C NMR: δ = 169.75, 147.35, 143.93, 138.86, 114.37, 99.91, 69.99, 51.91, 36.85, 31.94, 29.90, 29.86, 29.75, 29.72, 29.41, 27.41, 22.69, 14.11 ppm; FT-IR/ATR: 2919, 2851, 1758, 1737 cm^{-1}.

Tetrahydroxycarbonyl cavitand 11. To a solution of compound **10** (1.5 g, 1 mmol) in THF (70 mL) was added 2 M KOH (30 mL), and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was acidified with 2 M HCl (60 mL), and the THF was removed *in vacuo.* The precipitate formed was filtered and thoroughly washed with water. The solid obtained was dried at 80 °C under vacuum for 3 h, and then suspended in THF and filtered. The solution was evaporated to dryness to yield **11** as white solid. (1.38 g, 96%), mp 214–216 °C, ^{1}H NMR [DMSO-d_6]: δ = 7.23 (s, 4 H, ArH), 5.70 (d, J = 7.5 Hz, 4 H, outer of OCH_2O), 4.54 (t, J = 7.9 Hz, 4 H, CH (methine)), 4.44 (s, 8 H, $ArOCH_2$), 4.27 (d, J = 7.5 Hz, 4 H, inner of OCH_2O), 2.29 (m, 8H, $CH_2(CH_2)_9CH_3$), 1.21–1.42 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.87 (t, 12 H, CH_3) ppm; ^{13}C NMR [DMSO-d_6]: δ = 171.95, 146.24, 143.44, 138.63, 115.38, 99.62, 69.40, 36.86, 31.30, 29.20, 29.05, 28.73, 27.64, 22.06, 21.00, 13.86 ppm; FT-IR/ATR: 3353, 2922, 2852, 1665 cm^{-1}.

General procedure for the synthesis of amino acid cavitand derivatives (1 and 2)[37]. To a suspension of **11** (0.50 g, 0.35 mmol) in dry CH_2Cl_2 (20 mL) was added freshly distilled oxalyl chloride (0.60 mL, 7.00 mmol), and the mixture was refluxed for 18 h at 40 °C under N_2. Unreacted oxalyl chloride and solvent were removed *in vacuo*, and the product was dried under vacuum for 1 h, dissolved in dry CH_2Cl_2 (10 mL), and slowly added to a cooled solution (0 °C) of amino acid methyl ester hydrochloride (1.75 mmol) and Et_3N (0.25 mL, 1.75 mmol) in dry CH_2Cl_2 (20 mL). The reaction mixture was allowed to warm up to room temperature and stirred under N_2 for 18 h. The solution mixture was treated with 1 N HCl (20 mL), and the organic layer was separated, washed with water (30 mL), and brine (30 mL). The organic layer was dried (Na_2SO_4), filtered, and the solvent was removed under reduced pressure, yielding a sticky residue. The residue obtained was purified silica gel using a 3% MeOH in $CHCl_3$. Evaporation of the solvent followed by addition of MeOH afforded the product after filtration and drying under vacuum.

Tetra-alanyl methyl ester cavitand 1. L-Alanine methyl ester hydrochloride (0.25 g, 1.75 mmol) was used as described in the general procedure. Compound **1** was obtained as a white, crystalline solid (0.42 g, 68%), R_f 0.42 (3% MeOH/CH_3Cl), mp 76–79 °C; ^{1}H NMR: δ = 8.08 (d, J = 7.6 Hz, 4 H, NH), 6.81 (s, 4 H, ArH), 6.03 (d, J = 7.1 Hz, 4 H, outer of OCH_2O), 4.62 (t, 4 H, CH (methine)), 4.62 (m, 4 H, Ala-α H), 4.50 (d, J = 5.0 Hz, 8 H, $ArOCH_2$), 4.39 (d, J = 7.1 Hz, 4 H, inner of OCH_2O), 3.71 (s, 12 H, OCH_3), 2.07 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.38 (d, 12 H, Ala-CH_3), 1.20–1.28 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.84 (t, 12 H, CH_3) ppm; ^{13}C NMR: δ = 173.24, 168.66, 147.03, 143.98, 139.30, 114.83, 99.64, 73.05, 52.52, 47.64, 36.91, 31.94, 29.86, 29.74, 29.72, 29.40, 27.87, 22.69, 18.56, 14.11 ppm; FT-IR/ATR: 3355, 2923, 2852, 1742, 1680 cm^{-1}. MS (ESI-TOF) Calcd for $C_{100}H_{148}O_{24}N_4$: 1789.2916. Found: 1790.0560. $[\alpha]_D^{20}$ = 41.7° (c = 0.12, $CHCl_3$).

Tetra-phenylalanyl methyl ester cavitand 2. L-Phenylalanine methyl ester hydrochloride (0.38 g, 1.75 mmol) was used as described in the general procedure. Compound **2** was obtained as a white, crystalline solid (0.51 g, 70%), R_f 0.52 (3% MeOH–$CHCl_3$), mp 72–74 °C. ^{1}H NMR: δ = 8.06 (d, J = 8.2 Hz, 4 H, NH), 7.19–7.10 (m, 20 H, Phe-ArH). 6.82 (s, 4 H, ArH), 5.69 (d, J = 7.1 Hz, 4 H, outer of OCH_2O), 4.97 (m, 4 H, Phe-α H), 4.67 (t, 4 H, CH (methine)), 4.51 (d, J = 7.0 Hz, 8 H, $ArOCH_2$), 4.28 (d, J = 7.1 Hz, 4 H, inner of OCH_2O), 3.68 (s, 12 H, OCH_3), 3.21 (dd, 4 H, Phe-$ArCH_2$), 3.11 (dd, 4 H, Phe-$ArCH_2$), 2.17 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.20–1.28 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.87 (t, 12 H, CH_3) ppm; ^{13}C NMR: δ = 171.80, 168.59, 146.96, 146.68, 143.89, 139.15, 138.88, 135.92, 129.32, 128.40, 126.97, 114.70, 99.30, 72.85, 52.93, 52.34, 38.42, 36.93, 31.93, 29.97, 29.80, 2976, 29.71, 29.41, 28.01, 22.69, 14.11 ppm; FT-IR/ATR: 3355, 2923, 2852, 1742, 1680 cm^{-1}. MS (ESI-TOF) Calcd for $C_{124}H_{164}O_{24}N_4$: 2093.4444. Found: 2094.1762. $[\alpha]_D^{20}$ = 36.42° (c = 0.11, $CHCl_3$).

General procedure for the hydrolysis of amino acid methyl ester cavitand derivatives (1 and 2)[38] to 3 and 4. A solution of $LiOH{\cdot}H_2O$ (63 mg, 1.50 mmol) in H_2O (2 mL) was added to a solution of amino acid methyl ester cavitand (**1** and **2**) (0.15 mmol) in THF (10 mL), cooled at 0 °C. The reaction mixture was allowed to warm to rt while stirring under N_2 for 18 h. The mixture was quenched by addition of 4 N HCl (10 mL). Evaporation of the organic solvent followed by filtration afforded the desired compound was obtained in quantitative yield by filtration and without further purification.

Tetra-alanyl acid cavitand 3. Tetra-alanyl methyl ester cavitand, **1** (0.27 g, 0.15 mmol) was used as described in the general procedure to afford **3** as a white solid, (0.23 g, 88%), mp 139–142 °C. ^{1}H NMR [DMSO-d_6]: δ = 7.98 (d, 4 H, NH), 7.28 (s, 4 H, ArH), 6.06 (d, 4 H, outer of OCH_2O), 4.55 (t, 4 H, CH (methine)), 4.36 (q, 8 H, $ArOCH_2$), 4.29 (d, 4 H, inner of OCH_2O), 4.14 (m, 4 H, Ala-α H), 2.30 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.26 (d, 12 H, Ala-CH_3), 1.19–1.24 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.82 (t, 12 H, CH_3) ppm; ^{13}C NMR [DMSO-d_6]: 173.72, 167.27, 146.36, 143.65, 138.74, 138.59, 116.03, 99.59, 72.15, 48.19, 31.29, 30.38, 29.15, 29.03, 28.99, 28.77, 27.55, 22.05, 18.00, 13.83 ppm; FT-IR/ATR: 3355, 2923, 2852, 1742, 1680 cm^{-1}. MS (ESI-TOF) Calcd for $C_{96}H_{140}O_{24}N_4$: 1733.2240. Found: 1733.9856. $[\alpha]_D^{20}$ = 13.9° (*c* = 0.12, DMSO).

Tetra-phenylalanyl acid cavitand 4. Tetra-phenylalanyl methyl ester cavitand, **2**, (0.31 g, 0.15 mmol) was used hydrolyzed as described in the general procedure to afford **4** a white solid, (0.28 g, 90%), mp 150–152 °C. ^{1}H NMR [DMSO-d_6]: δ = 7.92 (d, 4 H, NH), 7.27 (s, 4 H, ArH), 7.19–7.10 (m, 20 H, Phe-ArH), 5.68 (d, 4 H, outer of OCH_2O), 4.53 (m, 4 H, Phe-α H), 4.53 (t, 4 H, CH (methine)), 4.33 (q, 8 H, $ArOCH_2$), 4.20 (d, 4 H, inner of OCH_2O), 3.09 (dd, 4 H, Phe-$ArCH_2$), 2.94 (dd, 4 H, Phe-$ArCH_2$), 2.32 (m, 8 H, $CH_2(CH_2)_9CH_3$), 1.20–1.26 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.86 (t, 12 H, CH_3) ppm; ^{13}C NMR [DMSO-d_6]: δ = 172.44, 167.26, 146.37, 146.25, 143.33, 138.78, 138.60, 137.24, 129.17, 127.89, 126.18, 116.09, 99.33, 71.83, 53.46, 37.17, 36.85, 31.28, 30.39, 29.20, 29.02, 28.71, 27.63, 22.05, 13.84 ppm; FT-IR/ATR: 3385, 2922, 2852, 1731, 1659 cm^{-1}. MS (ESI-TOF) Calcd for $C_{120}H_{156}O_{24}N_4$: 2037.3768. Found: 2033.1461. $[\alpha]_D^{20}$ = 8.3° (*c* = 0.12, DMSO).

Synthesis and characterization of compound 5.[39,40] *N*-(2-Bromoethyl)phthalimide (13). To a well-stirring solution of potassium phthalimide (**12**) (5.0 g, 27.0 mmol) in dry degassed DMF (10 mL) was added 1,2-dibromoethane (6.91 mL, 81.0 mmol). The reaction mixture was stirred at rt for 12 h under N_2. After this period, TLC, using 1 : 1 hexane–EtOAc, showed completion of the reaction (R_f 0.5). The DMF was removed *in vacuo.* The resulting yellow solid was dissolved in EtOAc (200 mL) and extracted with water (2×). The organic layer was washed successively with saturated solution of ammonium chloride and brine, dried (Na_2SO_4), and the EtOAc was removed *in vacuo* to afford **13** as white, crystalline solid (4.8 g, 70%), mp 224–226 °C. ^{1}H NMR: δ = 7.87 (m, 2 H, ArH), 7.74 (m, 2 H, ArH), 4.12 (t, 2 H, NCH_2CH_2), 3.63 (t, 2 H, NCH_2CH_2) ppm; ^{13}C NMR: δ = 167.82, 134.23, 134.00, 131.84, 123.52, 123.35, 36.83, 28.15 ppm, FT-IR/ATR: 3465, 3047, 1765, 1704, 1392 cm^{-1}.

***1*-[*N*-(2-Phthalimidoethyl)]thymine (14).** Thymine (5.0 g, 39.5 mmol) was dissolved in dry DMSO (100 mL) and oven-dried (110 °C) K_2CO_3 (5.50 g, 40 mmol) and **13** (5.20 g, 20.5 mmol) were added. After stirring for 24 h at rt under N_2, the precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to afford a viscous, yellowish liquid. The resulting liquid was diluted with H_2O and the suspension was extracted with $CHCl_3$ (200 mL). The $CHCl_3$ fractions were combined and dried ($MgSO_4$) and solvent was removed *in vacuo.* The resulting oil was dissolved in minimum EtOAc and induced to crystallize with ether to give **14** as an off-white solid which was pure enough for use in subsequent reactions (3.9 g, 63.9%), mp 247–250 °C. ^{1}H NMR [DMSO-d_6: δ = 11.15 (s, 1 H, NH), 7.86–7.77 (m, 4 H, ArH), 7.52 (s, 1 H, thymine-6 H), 3.86 (t, 2 H, NCH_2CH_2N), 3.81 (t, 2 H, NCH_2CH_2N), 1.62 (s, 3 H, thymine-CH_3) ppm; ^{13}C NMR [DMSO-d_6]: δ = 168.79, 141.84, 134.98, 131.68, 123.54, 110.07, 46.52, 37.11, 12.20 ppm; FT-IR/ATR: 3152, 3038, 1767,1682, 1392 cm^{-1}.

Aminolysis of 14. Compound **13** (1.05 g, 3.5 mmol) was dissolved in a solution of *n*-butylamine-MeOH (1 : 4, v/v, 50 mL). The suspension was heated under reflux (N_2) for 2 da. When TLC showed that aminolysis was complete, the mixture was concentrated to dryness *in vacuo*. The residue was dissolved in 0.5 N HCl (50 mL) and extracted with Et_2O (50 mL), the aqueous layer was evaporated under reduced pressure. The residue was dissolved in benzene–MeOH (1 : 1 v/v) and the azeotropic mixture was evaporated. A solid mass that formed was crystallized from a mixture of MeOH–Et_2O–$CHCl_3$ (1 : 1 : 1 v/v/v) to afford **14** as an off-white solid (0.46 g, 78%), mp 292–295 °C. ^{1}H NMR [D_2O]: δ = 7.41 (s, 1 H, thymine-6 H), 4.02 (t, 2 H, NCH2CH2NH2), 3.29 (t, 2 H, NCH2CH2NH2), 1.79 (s, 3 H, thymine-5 CH_3) ppm; ^{13}C NMR [D_2O]: δ = 166.91, 152.77, 142.37, 111.46, 45.94, 38.61, 11.27 ppm; FT-IR/ATR: 3358, 3186, 2971, 1675, 1682, 1477, 1356 cm^{-1}.

Synthesis of thymine-cavitand 5. To a stirring solution of tetra-acid **9** (0.5 g, 0.35 mmol) and aminoethylthymine **13** (0.30 g, 1.75 mmol) in dry degassed DMF (20 mL) at 0 °C was added 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) (0.34 g, 1.75 mmol). The reaction mixture was stirred for 20 min at 0 °C under N_2, 4-dimethylaminopyridine (DMAP, 0.21 g, 1.75 mmol) was added, and the resulting mixture was allowed to warm to rt and stirred for 24 h. The solvent was removed *in vacuo* at 50 °C and the remaining oil was triturated with MeOH. The resulting white precipitate was isolated by centrifugation and washed twice with MeOH. Chromatography on silica gel (95 : 5 v/v $CHCl_3$–MeOH), followed by stirring in MeOH, gave **5** as an Off-white powder (0.51 g, 71%), mp 161–163 °C. ^{1}H NMR [DMSO-d_6]: δ = 11.20 (s, 4 H, thymine-NH), 7.89 (t, 4 H, thymine, CH_2CH_2NH), 7.31 (s, 4 H, thymine-6 H), 7.31 (s, 4 H, ArH), 5.93 (d, 4 H, outer of OCH_2O), 4.60 (t, 4 H, CH (methine)), 4.32 (s, 8 H, $ArOCH_2$), 4.32 (d, 4 H, inner of OCH_2O), 3.78 (t, 8 H, NCH_2CH_2NH), 3.46 (t, 8 H, NCH_2CH_2NH), 2.28 (m, 8H, $CH_2(CH_2)_9CH_3$), 1.63 (s, 12 H, thymine-5 CH_3), 1.21–1.28 (m, 72 H, $CH_2(CH_2)_9CH_3$), 0.81 (t, 12 H, CH_3) ppm; ^{13}C NMR [DMSO-d_6]: δ = 168.30, 164.32, 150.95, 146.49, 143.34, 141.53, 138.78, 116.19, 107.97, 99.71, 71.84, 47.33, 36.99, 36.83, 31.28, 29.17, 29.02, 28.70, 27.53, 22.05, 13.85, 11.66 ppm; FT-IR/ATR: 3355, 3181, 3048, 2923,2852, 1667, 1467, 969 cm^{-1}. MS (ESI-TOF) Calcd for $C_{112}H_{156}O_{24}N_{12}$: 2053.4216. Found: 2054.1344.

Planar bilayer conductance studies. Planar bilayer conductance (also called BLM) experiments were performed by using a Warner BC-525D bilayer clamp apparatus. Planar membranes were formed by painting lipids (asolectin, 25 mg mL^{-1} in *n*-decane] over a 200 μm aperture on the side of a cuvette fitted into a chamber. The cuvettes contained a 450 mM KCl buffer solution (10 mM HEPES, pH = 7). After membrane formation was confirmed (capacitance > 100 pF), an aliquot of a trifluoroethanol or DMSO solution of the compound was stirred into the buffer in the *cis* chamber to achieve the desired concentration. Recordings were acquired with Clampex 9.2 (Axon instruments) and data analyses were performed with Clamp fit 9.2 (Axon Instruments).

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