Anion Transporters

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Regulating Supramolecular Function in Membranes: Calixarenes that Enable or Inhibit Transmembrane Cl^- Transport**

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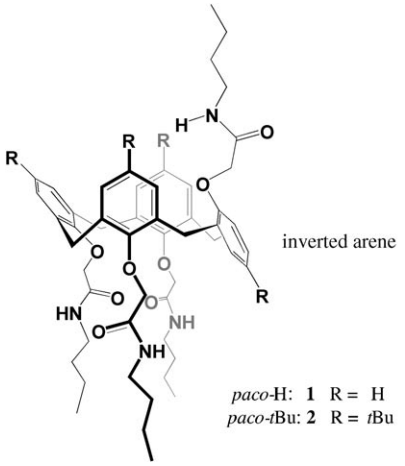
Self-assembly is an attractive strategy for building synthetic ion channels.[1] Solid-state structures, when combined with functional data, can provide clues about how self-assembly regulates transmembrane ion transport.[2,3] We show that modest changes in the primary structure of a calix[4]arene amide lead to dramatic differences in the transport of chloride ions across phospholipid membranes. Comparison of solid-state structures for partial-cone calix[4]arene (*paco*-H) **1** and its *para*-substituted analogue (*paco*-*t*Bu) **2**, suggested that



self-association and Cl^- ion transport activity might be controlled by the conformation of the side chain on the inverted arene of the partial cone. Using this structural information as a basis for probing function, we describe a novel regulatory system, wherein the inactive **2** inhibits transmembrane transport of Cl^- ions by *partial-cone* **1**. This last finding is timely, given the interest in regulating the activity of synthetic transporters using external stimuli such as light, temperature, voltage, or ligand-gating to turn ion transport on or off.[4]

Various "small molecules" transport Cl^- ions across phospholipid membranes. These compounds range from ion carriers such as prodigiosins and cholapods,[5,6] to sterols and peptides that self-assemble in the membrane.[7,8] Our contributions to the field have involved calix[4]arenes[9]—scaffolds previously used for synthetic cation channels.[10] We found that a 1,3-*alt*-calix[4]arene tetraamide and an acylic analogue could transport Cl^- ions across liposomal membranes.[11,12] Solid-state structures revealed that amide NH···Cl^- hydrogen bonds mediated self-assembly of the 1,3-*alt*-calix[4]arene tetraamide and voltage clamp experiments confirmed formation of stable ion channels in phospholipid and cell membranes.[11]

We now compare the structure and function of **1** and **2**, both prepared from conformationally fixed esters.[13] Proton NMR titrations showed that both calixarene tetraamides bind tetrabutylammonium chloride weakly in $CDCl_3$, with similar values ($K_a = 10–20\,M^{-1}$).[14,15] Although both compounds bind Cl^- ions in organic solution, only **1** transports Cl^- ions across phospholipid membranes. Figure 1 shows Cl^- transport activity in the presence of **1** or **2** in dipalmitoyl phosphatidylcholine (DPPC) liposomes at 43 °C. Liposomes (100 nm) containing 1 mM of the Cl^--sensitive dye, lucigenin,[16] and 100 mM $NaNO_3$ were suspended in a solution of 100 mM $NaNO_3$ and 10 mM sodium phosphate (pH 6.4). Calixarenes **1** or **2** were added to the liposome suspension to give a 2:100 calixarene:lipid ratio. Evidence for transmembrane transport of Cl^- ions was obtained from the quenching of lucigenin's fluorescence after NaCl was added to give an extravesicular

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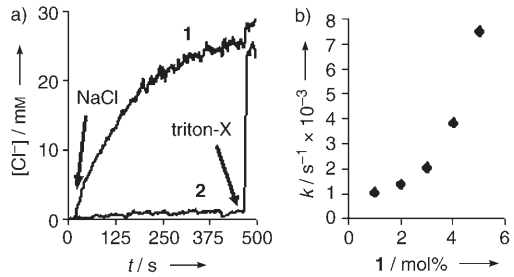

Figure 1. a) Chloride transport assay in DPPC liposomes in 100 mM $NaNO_3$ solution, 10 mM sodium phosphate (pH 6.4) at 43 °C in the presence of 2 mol% of **1** or **2**. A transmembrane Cl^- gradient was initiated by adding NaCl to the extravesicular buffer. The chloride concentration inside the vesicles was calculated from the fluorescence of the lucigenin dye. b) Concentration dependence of the transport of Cl^- ions by **1** in DPPC liposomes at 37 °C.

chloride concentration of 25 mM. Substituents on the calixarene's upper-rim clearly have an effect on the function (Figure 1a); **1** is a potent transporter of Cl^- ions, whereas **2** is inactive. Furthermore, in experiments done at 37 °C (where DPPC is in its gel phase) the rate of Cl^- transport by **1** was nonlinear with concentration, a result that is consistent with the formation of membrane-active aggregates $(\mathbf{1})_n$ (Figure 1b).[17] The difference in Cl^- transport activity for the calixarenes, and the evidence that **1** self-assembles in the DPPC membrane, prompted us to search for a structural basis that might help rationalize this mechanistic data.

Solid-state structures for **1** and **2** were determined from X-ray analysis of single crystals.[18,19] The conformation of the calixarene core was similar in both structures.[20] The most significant conformational difference for **1** and **2** was the orientation of the *n*-butylamide side chain connected to the inverted arene (Figure 2). For paco-H **1** the amide NH proton on this inverted arm forms an intramolecular H bond with its ether oxygen atom and is buried in a pocket formed by two flanking arenes. In this conformation, the amide NH proton on the inverted arene of **1** is unavailable for intermolecular interactions. In contrast, the inverted amide side chain in **2** is rotated 90° relative to the conformation in **1**. Presumably, the *tert*-butyl substituents on the flanking arenes block folding of the amide group into the macrocycle's pocket. Such a conformation ensures that this NH proton in **2** is available for intermolecular interactions, a feature that we think helps regulate the function of **1** (see below).

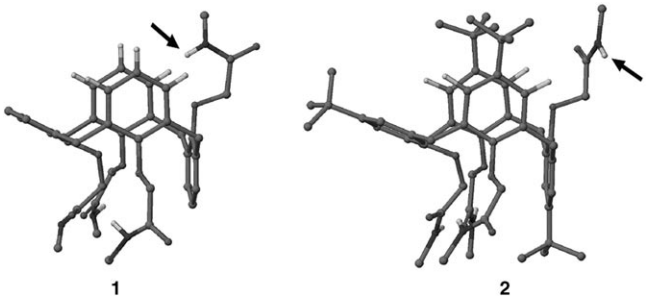

Figure 2. The conformation of the butylamide side chain on the inverted arene is different in solid-state structures of **1** (left) and **2** (right). In **1** the NH proton is buried in a pocket formed by neighboring arenes. In **2** this side chain is rotated 90°, thus making the amide NH proton accessible for intermolecular interactions. The *n*-butyl side chains in **1** and **2** are removed for clarity.

The solid-state conformations for **1** and **2** are retained in solution, as judged by ^{1}H NMR spectroscopic studies (Figure 3). For **1** the shielding environment of the arene pocket caused a significant upfield shift ($\delta = 5.97$ ppm in $CDCl_3$) for the inverted amide NH proton. We also observed a diagnostic NOE interaction between this NH proton and the *ortho* protons on the flanking arenes. The chemical shift for this inverted amide NH proton in **1** was also relatively insensitive to temperature, concentration, and the addition of Cl^- ions, all consistent with the presence of a buried NH proton in solution. In contrast, the chemical shift for the inverted amide NH proton in **2** was shifted relatively far downfield ($\delta = 7.65$ ppm), and had no NOE interactions between the NH group and the arene protons, both consistent with the amide NH group on the inverted arene being exposed to the solvent.

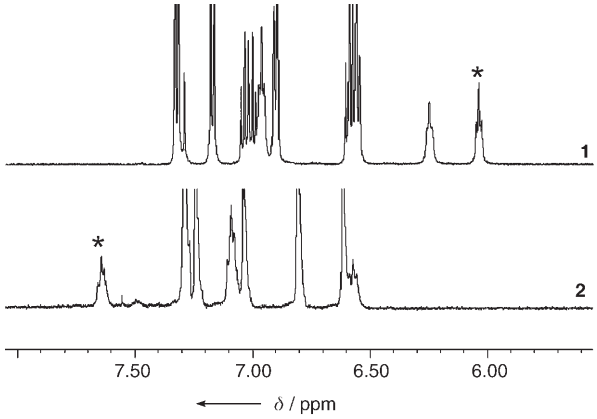

Figure 3. ^{1}H NMR data for **1** and **2**. The signal for the amide NH proton on the inverted arene is marked by an asterisk. Note the upfield shift for this NH proton in **1**, which is consistent with the solid-state conformation shown in Figure 2a, where this NH proton is located within the electron-rich arene pocket.

The conformation of the inverted arene's side chain contributes to different crystal packing for **1** and **2**. As depicted in Figure 4, paco-H **1** forms an extended, parallel-oriented chain of hydrogen bonds by having the downward-pointing amide groups of one molecule interact with the downward-pointing amide groups on neighboring molecules. Since it is buried in the calixarene's cavity, the amide group of the inverted arene is not involved in any intermolecular hydrogen bonding in this self-association of **1**. In marked contrast, the exposed amide group on the inverted arene of **2** forms intermolecular hydrogen bonds with the downward pointing amide groups of adjacent molecules to give an antiparallel, centrosymmetric, "up-down" arrangement of calixarenes in the solid state. This packing of **2** gives chains that are more staggered than the chains observed for **1** (Figure 4). The major point here is that **2** self-associates much differently than does **1**, presumably because the amide NH proton on its inverted arene forms intermolecular hydrogen

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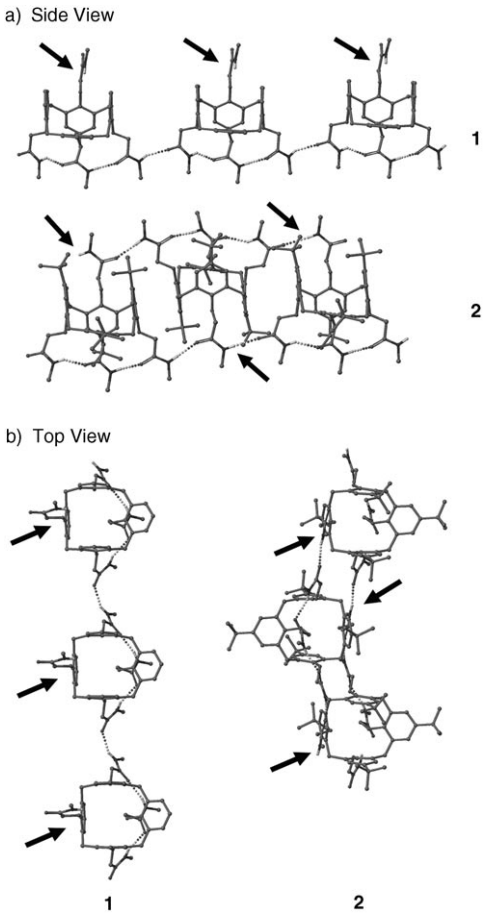

Figure 4. Depiction of the solid-state packing in the crystal structures of **1** and **2** Individual calixarene units in $(\mathbf{1})_n$ are connected by intermolecular hydrogen bonds between the three downward-pointing *n*-butylamide chains of the calixarene. The buried amide NH proton on the inverted arene does not perturb the packing of $(\mathbf{1})_n$. In the self-association of **2**, the amide group on the inverted arene participates in intermolecular interactions with the downward-pointing *n*-butylamide chains of neighboring molecules, thus resulting in a staggered, antiparallel, up-down arrangement of individual calixarenes within the hydrogen-bonded chains of $(\mathbf{2})_n$. Arrows point to the NH amide proton on the inverted arene of **1** or **2**.

bonds. In addition, the *tert*-butyl groups also likely influence the packing geometry for $(\mathbf{2})_n$. The assembly depicted in Figure 4 for $(\mathbf{1})_n$, with its hydrogen-bonded chain, suggests a plausible mechanism for transmembrane transport of Cl^- ions; a solvent-exposed amide NH proton could hydrogen bond to a Cl^- ion at the water–lipid interface and, upon rotation of the side chain, the Cl^- ion could be passed to the next NH group in the chain.

Whether the solid-state structures for **1** and **2** are related to 1) membrane-active structures or to 2) Cl^- transport activity remain open questions. The solid-state structures provided, however, a logical basis for experiments aimed at regulating anion transport using these calixarenes. Since the amide NH proton on the inverted arene of **2** is available for intermolecular interactions, we hypothesized that combining inactive **2** with active **1** might form heteroaggregates $\mathbf{1}_n{\cdot}\mathbf{2}_m$ in the membrane. We also reasoned that such $\mathbf{1}_n{\cdot}\mathbf{2}_m$ heteroaggregates might have attenuated Cl^- transport activity (either enhanced or diminished) relative to any self-assembled structures $\mathbf{1}_n$.[21] In contrast, if **1** functions by a carrier mechanism then one would not expect addition of an inactive analogue such as **2** to influence the transport rate of Cl^- ions. In the experiments, the concentration of active **1** was fixed at a 2:100 ligand-to-DPPC lipid ratio and the concentration of **2** was varied from a 0:100 to a 6:100 ligand:lipid ratio. The anion gradient assay, in which lucigenin was used as an intravesicular sensor,[16] was again used to monitor transmembrane transport of Cl^- ions. As shown in Figure 5, a plot

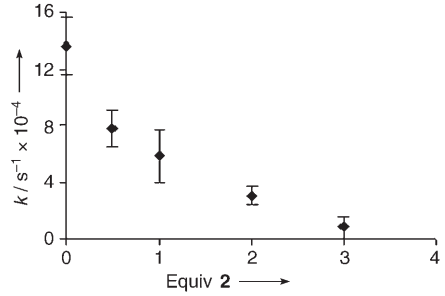

Figure 5. Inhibition of Cl^- transport by the inactive analogue **2**. Pseudo-first-order rate constants for Cl^- transport by **1** (2 mol% to lipid) are plotted against the concentration of **2** (0–6 mol% to lipid). Lucigenin was used in the assays to measure intravesicular [Cl^-] in DPPC liposomes (37°C) suspended in 100 mM $NaNO_3$ and 10 mM sodium phosphate (pH 6.4). A Cl^- gradient was initiated by adding NaCl to give an external concentration of 25 mM.

of pseudo-first-order rate constants versus concentration of **2** shows dose-dependent inhibition of the transport of Cl^- ions. One explanation for this data is that inactive **2** partitions into the membrane in preference to **1**. Another explanation, which we favor, is that **2** and **1** form inactive heteroaggregates $\mathbf{1}_n{\cdot}\mathbf{2}_m$ in the membrane, thus shifting the equilibrium away from active $\mathbf{1}_n$ structures.[22] This type of inhibition within a membrane favors a channel or hopping transport mechanism (versus a carrier mechanism) and it has recent precedent in that self-assembled ceramide ion channels in both liposomes and mitochondria are inhibited by addition of inactive dihydroceramide.[23,24]

Further development of anion transporters, based on results from these inhibition studies, could lead to a "switch" for regulating function if self-assembly could be triggered by reversibly controlling the conformation of compounds such as **1** and **2**. Additionally, our data suggests that the inverted amide side chain is not necessary for transport function in **1**. Thus, this side chain could be used as a connection to make dimeric or oligomeric calixarenes, compounds that may show enhanced transmembrane Cl^- transport activity, especially if a channel mechanism is operative. Such synthetic iterations, based on the structure–function relationships revealed herein, are currently being pursued.

Experimental Section

Preparation of DPPC liposomes: DPPC lipid (50 mg, Avanti) was dissolved in a chloroform/methanol mixture (5% MeOH, 5 mL). The

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resulting solution was evaporated under reduced pressure at 45 °C to produce a thin film that was dried in vacuo for 2 h. The lipid film was hydrated with a solution (1 mL) of 10 mM sodium phosphate (pH 6.4) containing 100 mM $NaNO_3$ and 1 mM lucigenin. After 5 freeze/thaw cycles (thawing and then warming to 45 °C) the liposomes were extruded through a 100-nm polycarbonate membrane 21 times between 45–55 °C. The liposome solution was passed through a sephadex (G-25) column to remove excess dye. The isolated liposomes were diluted in 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a concentration of 11 mM in DPPC, assuming 100% retention of lipid during the gel filtration process.

Chloride transport assay: In a typical experiment, the stock DPPC liposomes (0.1 mL) were diluted into 10 mM sodium phosphate (2 mL, pH 6.4, 100 mM $NaNO_3$) to give a solution that was 0.5 mM in lipid. A solution of calixarene **1** or **2** in DMSO was added to this solution to give a 2:100 ligand:lipid ratio. The sample was incubated for 10 minutes at either 43 °C or 37 °C. 4.0 M NaCl (20 μL) was added to the cuvette containing the DPPC/calixarene mixture through an injection port to give a final extravesicular Cl^- concentration of 25 mM. The fluorescence of the intravesicular lucigenin was monitored at an excitation wavelength of 372 nm and emission wavelength of 504 nm for 500 s. After 470 s, 10% triton-X detergent (0.04 mL) was added to destroy the liposomes and to determine the maximal fluorescence quenching of lucigenin by Cl^- ions. Experiments at two different temperatures (37 °C and 43 °C) were done in triplicate. Lucigenin fluorescence was converted into chloride concentration by using the Stern–Volmer constant determined under the assay conditions. To measure the Stern–Volmer constant, liposomes prepared as above were lysed immediately with triton-X. 4.0 M NaCl (5 μL) was titrated in every 30 s through the injection port. The slope of a plot of f_0/f versus chloride concentration gave the Stern–Volmer constant for lucigenin.

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[1] For reviews of synthetic ion channels, see: S. Matile, A. Som, N. Sorde, *Tetrahedron* **2004**, *60*, 6405; U. Koert, L. Al-Momani, J. R. Pfeeifer, *Synthesis* **2004**, 1129.

[2] Crystal structures provided insight into the function of the K^+ ion channel: R. MacKinnon, *Angew. Chem.* **2004**, *116*, 4363; *Angew. Chem. Int. Ed.* **2004**, *43*, 4264.

[3] Solid-state structures also helped rationalize the function of a synthetic ion channel: J. D. Hartgerink, J. R. Granja, R. A. Milligan, M. R. Ghadiri, *J. Am. Chem. Soc.* **1996**, *118*, 43.

[4] Examples of stimuli-responsive transmembrane transporters: T. M. Fyles, D. Lock, X. Zhou, *J. Am. Chem. Soc.* **1998**, *120*, 2997; C. Goto, M. Yamamura, A. Satake, Y. Kobuke, *J. Am. Chem. Soc.* **2001**, *123*, 12152; V. Gorteau, F. Perret, G. Bollot, J. Mareda, A. N. Lazar, A. W. Coleman, D.-H. Tran, N. Sakai, S. Matile, *J. Am. Chem. Soc.* **2004**, *126*, 13592; W. H. Chen, S. L. Regen, *J. Am. Chem. Soc.* **2005**, *127*, 6538.

[5] P. A. Gale, M. E. Light, B. McNally, K. Navakhum, K. I. Sliwinski, B. D. Smith, *Chem. Commun.* **2005**, 3773; J. L. Sessler, L. R. Eller, W.-S. Cho, S. Nicolaou, A. Aguilar, J. T. Lee, V. M. Lynch, D.J Magda, *Angew. Chem.* **2005**, *117*, 6143; *Angew. Chem. Int. Ed.* **2005**, *44*, 5989; J. L. Seganish; J. T. Davis, *Chem. Commun.* **2005**, 5781.

[6] A. V. Koulov, T. N. Lambert, R. Shukla, M. Jain, J. M. Bood, B. D. Smith, H. Y. Li, D. N. Sheppard, J. B. Joos, J. P. Clare, A. P. Davis, *Angew. Chem.* **2003**, *115*, 5081; *Angew. Chem. Int. Ed.* **2003**, *42*, 4931.

[7] S. Otto, M. Osifchin, S. L. Regen, *J. Am. Chem. Soc.* **1999**, *121*, 7276.

[8] P. H. Schlesinger, R. Ferdani, J. Liu, J. Pajewska, R. Pajewki, M. Saito, H. Shabany, G. W. Gokel, *J. Am. Chem. Soc.* **2002**, *124*, 1848.

[9] For a lead to the extensive calixarene literature, see: V. Böhmer, *Angew. Chem.* **1995**, *107*, 785; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 713.

[10] Transmembrane cation transporters from calix[4]arenes or resorcin[4]arenes: Y. Tanaka, Y. Kobuke, M. Sokabe, *Angew. Chem.* **1995**, *107*, 717; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 693; J. de Mendoza, F. Cuevas, P. Prados, E. S. Meadows, G. W. Gokel, *Angew. Chem.* **1998**, *110*, 1650; *Angew. Chem. Int. Ed.* **1998**, *37*, 1534; N. Yoshino, A. Satake, Y. Kobuke, *Angew. Chem.* **2001**, *113*, 471; *Angew. Chem. Int. Ed.* **2001**, *40*, 457; A. J. Wright, S. E. Matthews, W. Fischer, P. D. Beer, *Chem. Eur. J.* **2001**, *7*, 3474; M. Mauleucci, F. De Riccardis, B. Botta, A. Casapullo, E. Cressina, E. Fregonese, P. Tecilla, I. Izzo, *Chem. Commun.* **2005**, 1354.

[11] V. Sidorov, F. W. Kotch, G. Abdrakhmanova, R. Mizani, J. C. Fettinger, J. T. Davis, *J. Am. Chem. Soc.* **2002**, *124*, 2267.

[12] V. Sidorov, F. W. Kotch, Y. Lam, J. L. Kuebler, J. T. Davis, *J. Am. Chem. Soc.* **2003**, *125*, 2840.

[13] K. Iwamoto, S. Shinkai, *J. Org. Chem.* **1992**, *57*, 7066.

[14] For reviews on calixarene–anion receptors, see: P. Lhotak, *Top. Curr. Chem.* **2005**, *255*, 65; S. E. Matthews, P. D. Beer, *Supramol. Chem.* **2005**, *17*, 411; P. D. Beer, P. A. Gale, *Angew. Chem.* **2001**, *113*, 502; *Angew. Chem. Int. Ed.* **2001**, *40*, 486.

[15] See the Supporting Information for the NMR titration data. These K_a values (**1**, 28.7 ± 17 and **2**, $31.7 \pm 6\ M^{-1}$) for Cl^- binding by secondary amides in **1** and **2** are consistent with values for *N*-methylacetamide binding tetrabutylammonium chloride (TBACl) in $CDCl_3$: F. Werner, H.-J. Schneider, *Helv. Chim. Acta* **2000**, *83*, 465.

[16] B. A. McNally, A. V. Koulov, B. D. Smith, J. B. Joos, A. P. Davis, *Chem. Commun.* **2005**, 1087.

[17] For examples where concentration dependence indicates membrane-active aggregates with ion-channel characteristics, see: T. M. Fyles, D. Loock, X. Zhou, *J. Am. Chem. Soc.* **1998**, *120*, 2997; A. F. DiGiorgio, S. Otto, P. Bandyopadhyay, S. L. Regen, *J. Am. Chem. Soc.* **2000**, *122*, 11029.

[18] Crystal data for **1** from acetonitrile: $C_{52}H_{68}N_4O_8 \cdot C_2H_3N$, $M_r = 918.16$, dimensions $0.38 \times 0.32 \times 0.13\ mm^3$, triclinic, space group $P\bar{1}$, $a = 12.9335(4)$, $b = 16.6625(5)$, $c = 25.6605(8)$ Å, $V = 5107.6(3)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.1194\ mg\,m^{-3}$, $Mo_{K\alpha} = 0.080\ mm^{-1}$. Data were collected on a Bruker SMART 1000 CCD diffractometer at 173(2) K. The structure was refined to convergence [$\Delta/\sigma \leq 0.001$] with $R(F) = 5.55\%$, $wR(F^2) = 17.73\%$, GOF = 1.095 for all 18,493 reflections. CCDC-293015 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

[19] Data for **2** from acetone/CH_2Cl_2: $C_{68}H_{100}N_4O_8 \cdot 0.77\,CH_2Cl_2 \cdot C_3H_6O$, $M_r = 1180.27$, dimensions $0.05 \times 0.08 \times 0.380\ mm^3$, triclinic, space group $P\bar{1}$, $a = 13.798(3)$, $b = 14.308(3)$, $c = 18.487(4)$ Å, $V = 3438.1(12)$ Å^3, $Z = 2$, $\rho_{calcd} = 1.140\ mg\,m^{-3}$, $Mo_{K\alpha} = 0.131\ mm^{-1}$. Data collected were at 223(2) K. The structure was refined to convergence [$\Delta/\sigma \leq 0.001$] with $R(F) = 5.93\%$, $wR(F^2) = 13.43\%$, GOF = 1.034 for all 9876 unique reflections. CCDC-293016 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

[20] *para* Substitution typically has a minor influence on calix conformation: C. D. Gutsche, L. J. Bauer, *J. Am. Chem. Soc.* **1985**, *107*, 6052; P. D. J. Grootenhuis, P. A. Kollman, L. C.

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Groenen, D. N. Reinhoudt, G. J. van Hummel, F. Ugozzoli, G. D. Andreeti, *J. Am. Chem. Soc.* **1990**, *112*, 4165.
[21] Recently, it was shown that oppositely charged calixarenes combine within a lipid layer to form protein sensors that are more sensitive than either of the individual components: R. Zadmard, T. Schrader, *J. Am. Chem. Soc.* **2005**, *127*, 904.
[22] Solution NMR studies shows that **1** and **2** (both 1 mM) interact in CD_2Cl_2, as judged by changes in the chemical shift of the NH proton.
[23] J. Stiban, D. Fistere, M. Colombini, *Apoptosis* **2006**, in press.
[24] This type of inhibition is also similar to that for HIV-1 protease, wherein an inactive subunit when combined with a native subunit gave inactive heterodimers: J. E. Rozzelle, D. S. Dauber, S. Todd, R. Kelley, C. S. Craik, *J. Biol. Chem.* **2000**, *275*, 7080.

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