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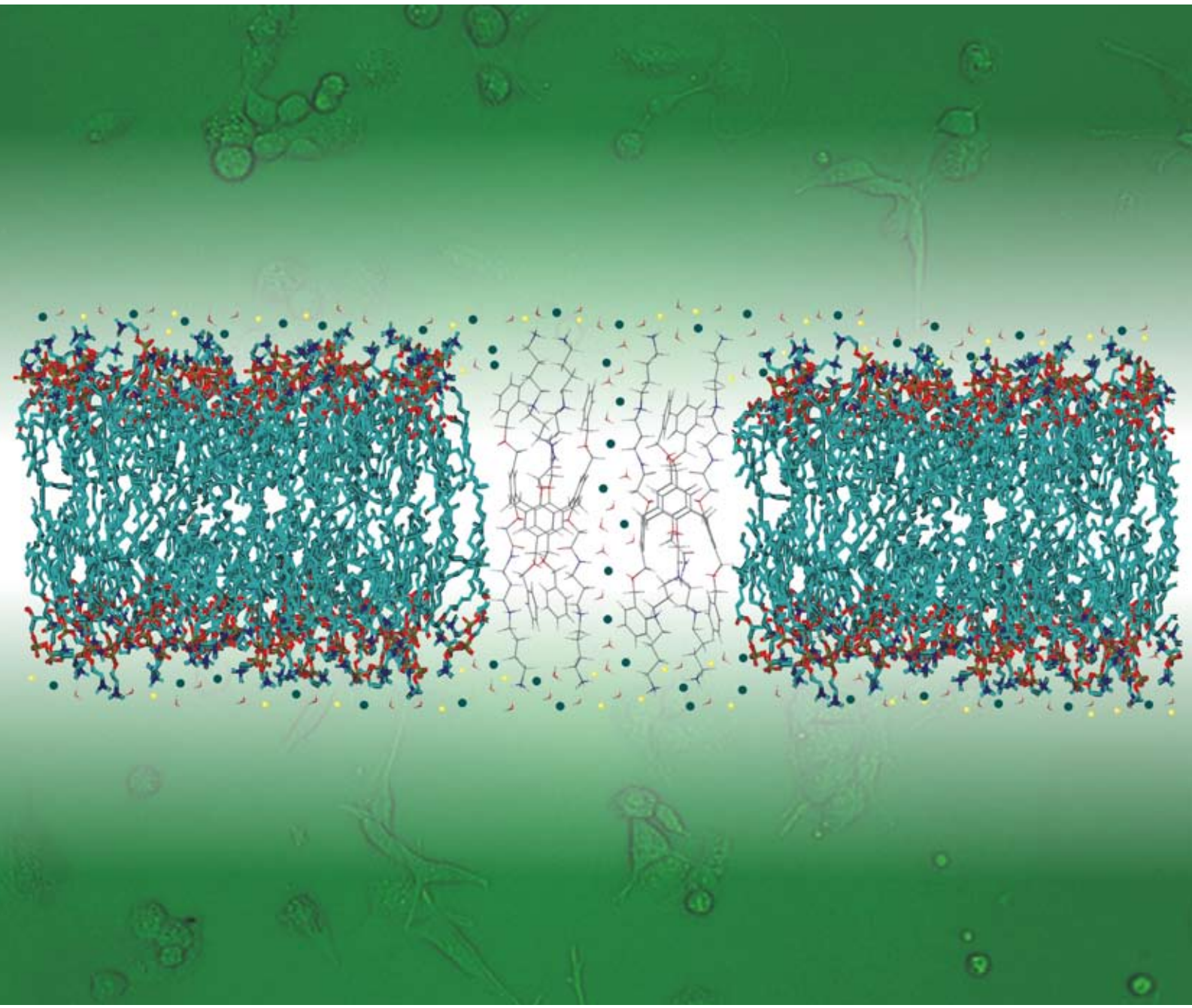

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FEATURE ARTICLE
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Organic semiconductors based on small molecules with thermally or photochemically removable groups

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Cationic calix[4]arenes as anion-selective ionophores†

Irene Izzo,*[a] Sabina Licen,[b] Nakia Maulucci,[a] Giuseppina Autore,[c] Stefania Marzocco,[c] Paolo Tecilla*[b] and Francesco De Riccardis*[a]

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1,3-*Alternate* cationic calix[4]arene 1 proved highly selective for proton/halogens symport transport and showed antiproliferative activity against murine monocyte/macrophage J774.A1 cancer cells.

The ion-coupled processes occurring in the plasma membrane regulate most of the physiologically relevant cell processes,[1] however, in contrast with the inspiring specificity of natural ion channels[2] and despite the advances in the ion-transport field, only a restricted number of synthetic ionophores demonstrated efficient anion transport and selectivity.[3] The quest for the design and synthesis of artificial anion conductors, is justified by the surprising scarcity of anion-transporting secondary metabolites[4] and by the concurrent unfortunate high number of diseases due to insufficient chloride transport.[5] In this context, newly conceived transporters may represent interesting leads for the treatment of these channelopathies.[3]

Calix[4]arene represents a versatile scaffold for the design of synthetic ionophores.[6,7] Recent work from the group of J. T. Davis[8] showed that a 1,3-*alternate* derivative of a tetrabutylamide calix[4]arene, transports HCl across a liposome membrane.[8a] We reasoned that cationic spermidine appendages, serving as anion extracting devices and intrapore water stabilizers, could regulate transmembrane anion traffic and elicit biological activity. We thus designed the D_{2d} symmetric 1,3-*alternate* calix[4]arenes **1** and **2** (Fig. 1), confiding in a favourable balance between the polar set of spermidine chains and the lipophilic calixarene scaffold, once decorated with four non-polar benzyloxymethyl (**1**) or methyl (**2**) substituents.

Membrane spanning[9] derivatives **1** and **2** were assembled, both in 19% overall yield, from the known tetrachloromethyl calix[4]arene **3**,[10] as shown in Scheme 1.

The synthesis started with a sodium benzylate-induced chlorines substitution, to give the ether **4**. The lower rim phenolic groups were subsequently alkylated, in the presence of cesium carbonate, to afford the conformationally-immobilized 1,3-*alternate* 5,11,17,23-tetrabenzyloxymethyl-25,26,-27,28-tetrakis(ethoxycarbonylmethoxy)calix[4]arene (**5**).

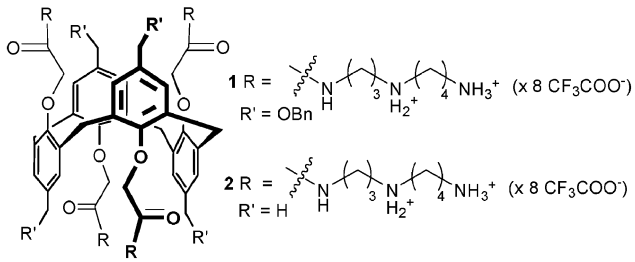
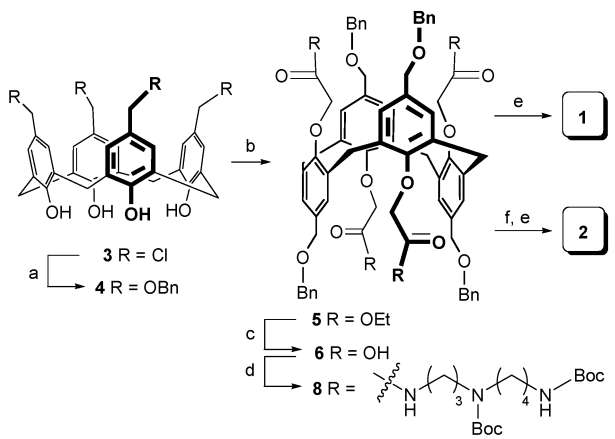


Fig. 1 Chemical structures of ionophores **1** and **2**.

Carboxyethyl hydrolysis and subsequent reaction of the tetra-acid **6** with the bis-Boc protected spermidine **7**,[11] yielded the fully protected adduct **8**. Finally, amino groups deprotection furnished target **1**. The more polar conjugate **2** was similarly obtained from **8**, once effected a Pd-mediated benzyl deoxygenation.

The ionophoric activities of compound **1** and **2** were investigated using the HPTS assay (Fig. 2).[8a,12] In this test a pH gradient is established across the liposomial membrane and the increase of the HPTS fluorescence indicates H^+ efflux or OH^- influx. The transmembrane charge translocations depend on four possible overall processes: H^+/Na^+ antiport, OH^-/Cl^- antiport, H^+/Cl^- symport and Na^+/OH^- symport (Fig. 2(a)). The effect of **1** and **2** on ion transport, is reported in Fig. 2(b) and (c), respectively.

Compound **1** shows a powerful ionophoric activity: the pH gradient is completely discharged after about 15 min in the presence of 1% ionophore (it still shows activity at 0.01% concentration, Fig. 2(b)). The more polar **2** requires a 3%



Scheme 1 Synthesis of **1** and **2**. *Reagents and conditions*: (a) Na, BnOH, THF; (b) Cs_2CO_3, $BrCH_2CO_2Et$, acetone, 56%, over two steps; (c) $KOH_{(aq)}$, THF–MeOH (1 : 1), 49%; (d) (i) $SOCl_2$, toluene, 80 °C; (ii) $H_2N(CH_2)_3N(Boc)(CH_2)_4NHBoc$ (**7**), NEt_3, toluene, 68%; (e) CF_3COOH, CH_2Cl_2, quant.; (f) H_2, Pd/C, MeOH, quant.

[a] *Department of Chemistry, University of Salerno, Via Ponte Don Melillo, I-84084 Fisciano Salerno, Italy. E-mail: dericca@unisa.it; iizzo@unisa.it; Fax: +39 089 969603; Tel: +39 089 969552*
[b] *Department of Chemical Sciences, University of Trieste, Via L. Giorgieri, I-34127 Trieste, Italy. E-mail: ptecilla@units.it; Fax: +39 040 5583903; Tel: +39 040 5583925*
[c] *Department of Pharmaceutical Sciences, University of Salerno, Via Ponte Don Melillo, I-84084 Fisciano Salerno, Italy*

† Electronic supplementary information (ESI) available: Complete experimental synthetic procedures, ionophoric activity studies, and biological tests. See DOI: 10.1039/b719482j

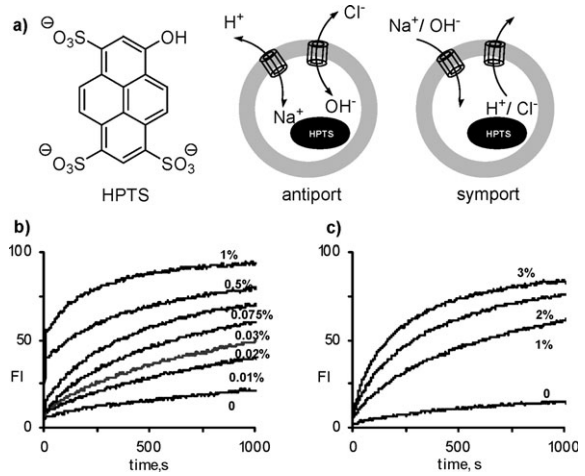


Fig. 2 (a) Schematic representation of the four possible mechanisms for pH gradient collapse in the HPTS assay. (b) and (c) Normalized fluorescence change in HPTS emission (FI, λ_{ex} 460 nm, λ_{em} 510 nm) as a function of time after the addition of base (50 μL of 0.5 M NaOH), in the presence of different concentrations of ionophore **1** (b) and **2** (c), to 95 : 5 EYPC–EYPG LUVs loaded with HPTS (0.1 mM HPTS, 0.17 mM total lipid concentration, 25 mM HEPES, 100 mM NaCl, pH 7.0, total volume 3 mL). The ionophore concentration is given in percent with respect to the total concentration of lipid. (HPTS: 8-hydroxy-1,3,6-pyrenetrisulfonic acid, trisodium salt; HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EYPC: egg yolk phosphatidyl choline; EYPG: egg yolk phosphatidyl glycerol).

concentration to fully collapse the pH gradient in equal times (Fig. 2(c)).[13] Both the kinetic profiles appear complex, being composed by two different processes (both dependent on the

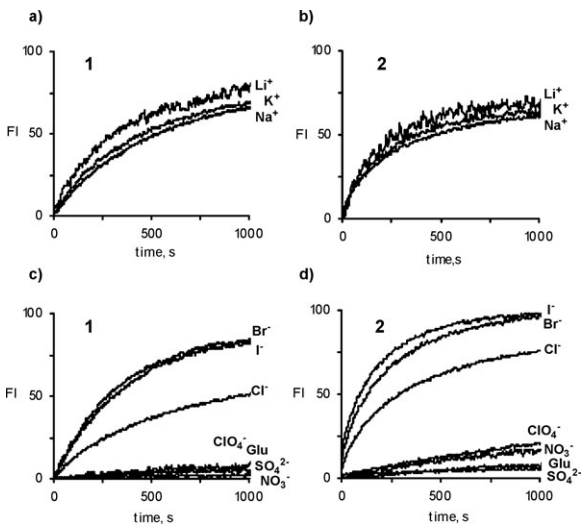


Fig. 3 Determination of cation (a), (b) and anion (c), (d) selectivities for ionophore **1** (0.03% concentration; (a) and (c)) and **2** (2.0% concentration; (b) and (d)), using the HPTS assay (λ_{ex} 460 nm, λ_{em} 510 nm) in 95 : 5 EYPC–EYPG LUVs (0.17 mM total lipid concentration, total volume 3 mL). Conditions: (a) and (b) 25 mM HEPES, 100 mM MCl, pH 7.0, base pulse by addition of 50 μL of 0.5 M MOH; (c) and (d) 25 mM HEPES, 100 mM NaX, pH 7.0, base pulse by addition of 50 μL of 0.5 M NaOH. The rates of transport of anions have been corrected for the membrane permeability of the different anions in the absence of ionophore. (Glu: glutamate).

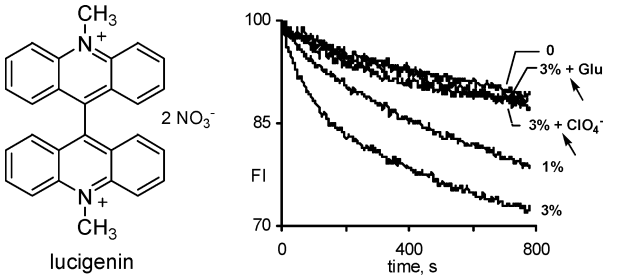


Fig. 4 Normalized fluorescence change in lucigenin emission (FI, λ_{ex} 455 nm, λ_{em} 506 nm) in the presence of different concentrations of ionophore **1** after the addition of NaCl (50 μL of 1.46 M solution, final external concentration 24 mM) to 95 : 5 EYPC–EYPG LUVs loaded with lucigenin (1 mM lucigenin, 0.4 mM total lipid concentration, 25 mM HEPES, 225 mM $NaNO_3$, pH 7, total volume 3 mL). The concentration of ionophore is given in percentage with respect to the total concentration of lipid. In two runs, 5 min before the addition of NaCl, an equimolar amount of sodium glutamate (Glu) and $NaClO_4$ was added to the vesicular suspension (50 μL of 1.46 M solution, final external concentration 24 mM NaX and 24 mM NaCl).

ionophore concentration): an almost instantaneous fluorescence "burst",[14] and a subsequent slower first-order rate law increase.[15]

Selectivity in ion transport was investigated using lower concentrations of ionophores (**1**: 0.03%; **2**: 2%) in order to suppress the intrinsically unselective fluorescence "burst" (Fig. 3). Inspection of profiles (a) and (b) in Fig. 3, attests independence of the ion fluxes from group 1 alkali metals,[16] confirming the unfitness of polypositive ionophores to transport alkaline cations.[17] The observed results imply that the pH gradient decay correlates with OH^-/Cl^- antiport or to H^+/Cl^- symport, excluding the group 1 alkali metals in the ion permeation processes.

The results obtained with anions, evidenced in Fig. 3(c) and (d), demonstrate efficient halide transport (with some selectivity toward iodine and bromide over chloride) and low transmembrane conductance of oxygenated anions (ClO_4^-, glutamate, NO_3^-, SO_4^{2-}).[18] These data correlate well with a halide transport based on the lyotropic sequence (with the less hydrated anion transported more efficiently), but exclude from this trend the little hydrated nitrate ion and some other oxygenated anions, suggesting, for the latter, a certain degree of anion binding with the ionophores. It is interesting to note that the fully protected neutral Boc-derivative **8** shows lower activity with respect to **1** and, more importantly, it demonstrates a low selectivity towards the anion transport.[19] These data attest the relevance of the cationic spermidine chains in the overall transport processes.

The transport of chloride across the lipid bilayer was independently evinced using lucigenin, a dye whose fluorescence emission is quenched by chloride ion.[20] The results, reported in Fig. 4, clearly show that **1** transports the chloride anion across the bilayer.[21] Interestingly, the chloride conductance is almost completely suppressed in the presence of equimolar concentration of perchlorate and glutamate (with respect to the added chloride), as shown in the two top curves of Fig. 4 (indicated by the arrows) suggesting an interference of these two not transported anions.[22] This effect reminds,

respectively, the Cl^- current reduction induced by xenobiotic anions on the cystic fibrosis chloride channel pore,[23] and the intrapore regulatory activity of the Glu148 in the EcClC channels.[24]

Considering the strong correlation between H^+/Cl^- symport transport rates and *in vitro* cytotoxic activity demonstrated for prodigiosin analogues,[4a] we evaluated the cytotoxic potential of calix[4]arenes **1** and **8**, against the J774.A1 (murine monocyte/macrophage) cancer cell line.

In the proliferation assays,[25] compound **1** inhibited the cell growth with an IC_{50} value of 47.2 ± 0.5 μM, while compound **8** showed a much lower potency ($IC_{50} = 99 \pm 0.5$ μM).

In conclusion, in this contribution we demonstrated that cationic calix[4]arenes mediate HX efflux, induce block of the chloride transport in the presence of appropriate interfering anions, and show a moderate antiproliferative activity against murine monocyte/macrophage J774.A1 cancer cells. These last two properties seem to be directly related to the presence of the cationic spermidine chains. Efforts currently in progress are aimed at understanding the complex interplay among ionophore structure, anion permeation, and biological activity.

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9. The length of **1** and **2** in their "extended" conformations (molecular mechanic calculations using the MM3 software) is 3.2 ± 0.2 nm, comparable to the phospholipid membrane thickness ($\sim$3.5 nm).
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13. The lower activity of **2** is ascribable to its higher hydrophilicity, which limits its partition into the membrane (see ESI†).
14. The activity/concentration profiles and control experiments excluding that the "burst" is due to partial lysis or fusion of the liposomes are reported in the ESI†.
15. This trend is compatible with a model recently proposed by Chiu and co-workers: C. L. Kuyper, J. S. Kuo, S. A. Mutch and D. T. Chiu, *J. Am. Chem. Soc.*, 2006, **128**, 3233. In this model the "burst" is associated to ion flux across randomly formed membrane pores while the slower process is governed by the diffusion of the ions into the bilayer. Accordingly, the burst is intrinsically unselective toward anion and cation transport (see ESI†).
16. These data are also confirmed by $^{23}Na^+$-NMR experiments (see ESI†) showing that Na^+ is not transported across the lipid bilayer.
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18. With ionophore **2** we have also tested a slightly different protocol, proposed by Matile (ref. 12) in which liposomes prepared in NaCl solution are diluted in a buffer containing the ion under study. This experiment confirms the independence of the transport process from the cation (Li^+, Na^+, K^+, Rb^+, Cs^+) and shows a similar trend for anions ($I^- > Br^- \approx Cl^- \gg SO_4^{2-} \approx NO_3^- \gg ClO_4^- \approx F^-$ > acetate), see ESI†.
19. The anions selectivity ratios for **1** and **8** are, respectively: Cl^-/Glu = 9.4 and 1.3; Cl^-/ClO_4^- = 5.4 and 1.3; see ESI†.
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21. The chloride transport is associated with an increase in the liposome internal pH as shown by an experiment performed in similar conditions but using HPTS as internal pH probe (see ESI†). This further supports that the anion translocation is coupled with H^+ symport or OH^- antiport.
22. This effect is confirmed by a substantial anomalous mole fraction effect (AMFE) observed with glutamate. Moreover, the glutamate induced blockage is almost absent with compound **8** (see ESI†).
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