Calix[4]arene-cholic acid conjugates: a new class of efficient synthetic ionophores†

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The synthesis of a new class of amphiphilic calix[4]arene-based ionophores, relying on direct reductive amination as a key step, and the evaluation of their H^+ and Na^+ transporting properties is described.

The control of membrane permeability is of paramount importance for all living organisms[1] and, since the publication of the first successful model, reported by Tabushi and co-workers in 1982,[2] synthetic chemists have designed and constructed a variety of chemically diverse and effective low molecular weight ion conductors.

A recent survey from Matile[3] shows that among non-peptide systems, the rigid steroid scaffold is gaining more attention: monomeric and dimeric steroids self-assemble into barrel-like supermolecules and exert powerful ionophoric activities. However, in this context, only one example is known where a sterol-based ionophore forms a membrane spanning, tunnel-like, unimolecular channel.[4]

Unimolecular ion channels are considered a particularly attractive model to study, in that most of the biochemical processes that characterize life (signal transduction, muscle contraction, proton-coupled ATP-synthesis, and so on), depend on it. In cells they can be found in high molecular weight transmembrane proteins ("pore helix").[1]

With the idea to synthesize new steroid-based transmembrane unimolecular ionophores, we designed a new class of compounds incorporating the versatile calix[4]arene platform, an ideal molecular scaffold offering easy protocols of derivatization, different preorganized structures and showing ion affinity.[5]

In this paper, we describe the synthesis and the H^+ and Na^+ transporting activity of five conformationally restricted calix[4]-arene-3α,12α-dihydroxy-24-amino-cholane conjugates synthesized in both the *1,3-alternate* and the *cone* conformations in order to understand the influence of the overall length of compounds on the ion transporting properties. Preliminary molecular mechanics studies indicated a 35.0 ± 2.0 Å length for derivatives **1–3**, and a 25.0 ± 2.0 Å length for derivatives **4–5**, in their fully extended conformation (Fig. 1).[6]

The synthetic strategy chosen to assemble the four amine units with the *1,3 alternate* calix[4]arene scaffold, relied on the reductive amination reaction. To this end the steroid amine **8** was prepared according to Scheme 1 in five steps and 37% overall yield.‡ The tetraaldehyde **9** (Fig. 2) was synthesized through Cs_2CO_3 tetraalkylation (known to induce *1,3-alternate* calix[4]arene conformation) from commercially available calix[4]arene and 2-methoxyethyl *p*-toluensulfonate.[7] High yield tetraformylation[8] furnished the expected, stable, tetraaldehyde (61% overall yield).

Direct reductive amination of **9**, in the presence of a slight excess (0.5 eq. per functional group) of amine **8** and the mild reducing agent sodium triacetoxyborohydride ($NaBH(OAc)_3$)[9] afforded in good yield (78%) the tetraaminated **1**. To evaluate the effect of the steroid substituents in the H^+ and Na^+ transporting essays, two related compounds (**2** and **3**) were also prepared. K_2CO_3-mediated C-3 selective hydrolysis gave the C-12 tetracetylated **2** (92%). Full deprotection was obtained with methanolic KOH and gave **3** (89%).

The synthesis of the calix[4]arene *cone* analogues **4** (63% yield) and **5** (98%) was realized in a similar manner starting from the tetraalkylated aldehyde **10** (Fig. 2). This was obtained through

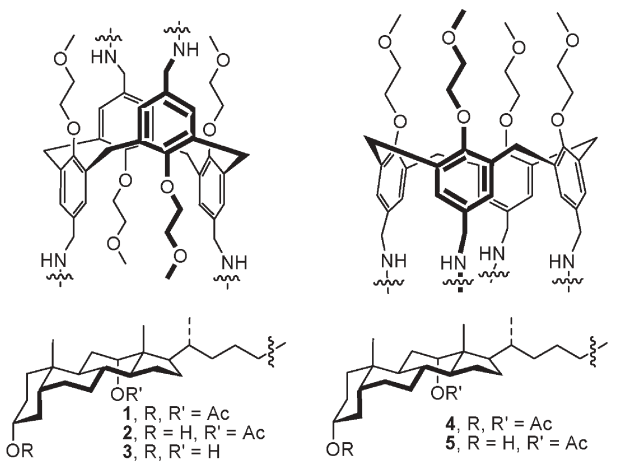
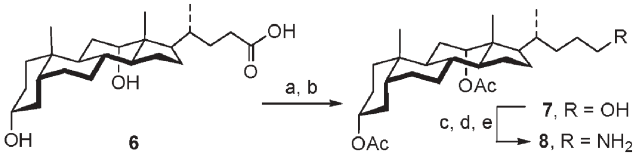


Fig. 1 Calix[4]arene-cholic acid conjugates **1–5**.



Scheme 1 *Reagents and conditions*: a. Ac_2O, Py, DMAP, CH_2Cl_2; b. BH_3·THF, THF, 0 °C; c. TsCl, Py, CH_2Cl_2; d. NaN_3, DMF, 60 °C; e. H_2, PtO_2, EtOH (37%, 5 steps).

† Electronic supplementary information (ESI) available: full synthetic and analytical details, and results of permeability studies on liposomes. See http://www.rsc.org/suppdata/cc/b4/b415908j/

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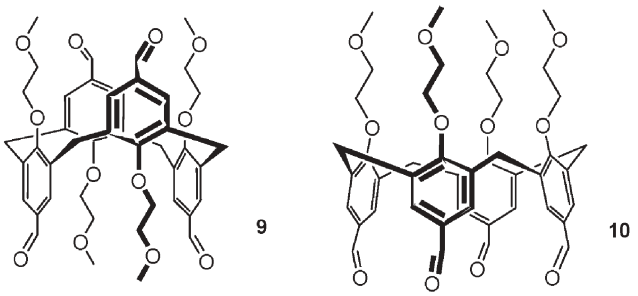

Fig. 2 Calix[4]arene scaffolds.

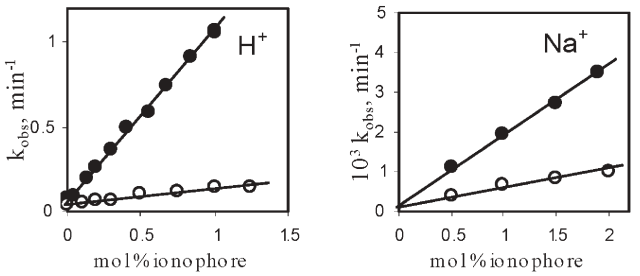

Fig. 3 Plot of k_{obs} for the transport of H^+ (left panel) and Na^+ (right panel) as a function of mol% of **2** (●) and **5** (○).

NaH mediated alkylation[10] of calix[4]arene and subsequent formylation.[8]

The ionophoric properties of compounds **1–5** were investigated in large unilamellar vesicles (100 nm diameter) with a 95 : 5 egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG) lipid composition. Ultrafiltration experiments and UV-Vis analysis of the water solution, free of vesicles, showed that more than 95% of the calixarene derivatives were bound to the aggregate. The ability of the ionophores to mediate a pH or Na^+ gradient collapse was measured using two different essays based, respectively, on the response of the intravesicular pH-sensitive pyranine fluorophore,[11] and on a $^{23}Na^+$ NMR experiment.[12] Kinetic experiments were run at 25 °C and in both processes we found first order kinetic profiles. The fitting of the data[13] allows one to obtain the apparent first order rate constants (k_{obs}, min^{-1}) for the transport of H^+ or Na^+ across the membrane. The data obtained at a fixed 1% molar ratio of calixarene derivatives with respect to the total concentration of lipid are reported in Table 1.

Inspection of Table 1 shows that all the calixarene derivatives are effective in promoting the transport of both ions although to a different extent. In particular, compounds **1–3**, characterized by the *1,3-alternate* conformation, are clearly more active than compounds **4**, **5** which display the *cone* conformation. As a matter of fact, the activity of compounds **1–3** are comparable in the case of Na^+ or higher in the case of H^+ than that of amphotericin B, a natural occurring ionophore which is often taken as a reference compound.[14] The tetracetylated derivative **2** is the most active both in the transport of H^+ and Na^+ while an inverse selectivity is observed between **1** and **3** with the first more active than the second in the transport of H^+ and slightly less active in that of the Na^+ ion. However, these activity differences are very small indicating that in these systems there is not a clear preference toward free or acetylated hydroxyl moieties.

Fig. 3 reports the dependence of k_{obs} *versus* the concentration of **2** and, for comparison, of **5**. Increasing the concentration of ionophore the rate of transport of H^+ and Na^+ increases linearly and the same linear dependence is observed with the other calixarene derivatives. This linear dependence of transport rate on ionophore concentration, taken together with the higher activity of the *1,3-alternate* derivatives characterized by a length that roughly matches the thickness of the hydrophobic core of the membrane, is in full accord with a unimolecolar transmembrane active structure.[3] On the contrary, the *cone* derivatives are too short to span the membrane and the formation of an active structure probably requires the assembly of more than one molecule.[15] This process is clearly less favoured with respect to a unimolecular pore formation and, therefore, the *cone* derivatives are less active than the *1,3 alternate* isomers.

In conclusion the first example of artificial ionophores presenting calix[4]arene-cholic acid substructures is reported.[16] These compounds seem to act through a unimolecolar mechanism; this result, together with the different activity of compounds **2** and **5**, suggest that the overall length plays a critical role in the ion transporting properties. Studies to better evaluate the mechanism of action, the ion selectivity, and towards the synthesis of more efficient ionophores, using different steroidal scaffolds, are in progress.

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Table 1 Observed rate constants (min^{-1}) for the entry of H^+ (k_H) and Na^+ (k_{Na}) into 95 : 5 egg PC/PG vesicles in the presence of the different ionophores (1% respect to the total concentration of lipids)[a]

Ionophore	k_H	k_{Na}
None	0.043	3.3×10^{-5}[b]
1	0.90	1.21×10^{-3}
2	1.06	1.96×10^{-3}
3	0.47	1.92×10^{-3}
4	0.14	6.01×10^{-4}
5	0.13	6.53×10^{-4}
Amphotericin B	0.14	2.67×10^{-3}[c]

[a] The total concentration of lipid was 0.17 mM and 10 mM in the H^+ and Na^+ experiments, respectively. T = 25 °C. [b] Estimated from initial rate. [c] Ref. 13.

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Notes and references

‡ Compounds **1–10** have been fully characterized (^1H and ^{13}C NMR, HRESI-MS) for details see ESI.† *Selected data and procedure* for **1**: Direct reductive amination. General procedure: To a solution of amine **8** (0.332 g,

0.720 mmol) and tetraaldehyde **9** (0.091 g, 0.120 mmol) in 1,2-dichloroethane (2.5 ml) $NaBH(OAc)_3$ (0.152 g, 0.720 mmol) and AcOH (30 μl, 0.480 mmol) were added. The reaction mixture was stirred at r.t. overnight, quenched with 1 N NaOH solution and extracted with methylene chloride. The combined organic phases were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The crude residue was flash-chromatographed (5–25% of methanol in dichloromethane to give **1** (0.240 g, 78%) as an amorphous solid. **1**: R_f: 0.38 (20% methanol in dichloromethane); $[\alpha]_D$: 70 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, TCDE, 110 °C) δ 0.60 (3 H, s, CH_3-18), 0.72 (3 H, bd, CH_3-21), 0.78 (3 H, s, CH_3-19), 1.84 (3 H, s, COCH_3), 2.72 (2 H, CH_2CH_2NH), 3.30 (3 H, s, OCH_3), 3.55–3.97 (8 H, m, CH_2OCH$_3$, ArOCH_2, ArCH_2Ar, HNCH_2Ar, 4.53 (1 H, m, H-3), 4.91 (1 H, bs, H-12), 7.13 (2 H, s, Ar*H*); ^{13}C NMR (400 MHz, TCDE, 110 °C) δ 10.7, 16.2, 19.2, 19.3, 20.9, 21.1, 21.7, 23.9, 24.2, 24.9, 25.2, 25.6, 30.7, 31.5, 32.3, 32.9, 33.1, 33.2, 33.7, 34.2, 40.3, 43.6, 45.4, 46.5, 47.8, 48.0, 56.6, 69.6, 70.5,72.4, 74.1, 121.4, 130.4 (×2), 132.1 (×2), 155.8, 167.9, 168.2. HRESI-MS (*m*/*z*): calcd. for $C_{156}H_{236}N_4O_{24}$ $[M + 2H]^{2+}$ 1275.8763; found 1275.8676.

1 R. MacKinnon, *Angew. Chem. Int. Ed.*, 2004, **43**, 4265–4277.
2 I. Tabushi, Y. Kuroda and K. Yokota, *Tetrahedron Lett.*, 1982, **23**, 4601–4604.
3 For recent and general reviews, see: G. W. Gokel and A. Mukhopadhyay, *Chem. Soc. Rev.*, 2001, **30**, 274–286; S. Matile, A. Som and N. Sordé, *Tetrahedron*, 2004, **60**, 6405–6435.
4 A. D. Pechulis, R. J. Thompson, J. P. Fojtik, H. M. Schwartz, C. A. Lisek and L. L. Frye, *Bioorg. Med. Chem.*, 1997, **5**, 1893–1901.
5 V. Böhmer, *Angew. Chem. Int. Ed.*, 1995, **34**, 713–745.
6 The thickness of a phosphatidylcholine/phosphatidylglicerol membrane bilayer model is around 30–35 Å.
7 W. Verboom, S. Datta, Z. Asfari, S. Harkema and D. N. Reinhaudt, *J. Org. Chem.*, 1992, **57**, 5394–5398.
8 A. Arduini, S. Fanni, G. Manfredi, A. Pochini, R. Ungaro, A. R. Sicuri and F. Ugozzoli, *J. Org. Chem.*, 1995, **60**, 1448–1453.
9 A. F. Abdel-Magid, K. G. Carson, B. D. Harris, C. A. Marynoff and R. D. Shah, *J. Org. Chem.*, 1996, **61**, 3849–3862.
10 L. C. Groenen, B. H. M. Ruel, A. Casnati, P. Timmerman, W. Verboom, S. Harkema, A. Pochini, R. Ungaro and D. N. Reinhaudt, *Tethraedron Lett.*, 1991, **32**, 2675–2687.
11 N. R. Clement and J. M. Gould, *Biochemistry*, 1981, **20**, 1534–1538.
12 S. Otto, M. Osifchin and S. L. Regen, *J. Am. Chem. Soc.*, 1999, **121**, 10440–10441 and references cited therein.
13 The pseudo-first order rate constants were obtained by non-linear regression analysis of the fluorescence or the Na^+ NMR peak areas *vs.* time data and the fit error on the rate constant was always less than 1%.
14 F. De Riccardis, M. Di Filippo, D. Garrisi, I. Izzo, F. Mancin, L. Pasquato, P. Scrimin and P. Tecilla, *Chem. Commun.*, 2002, 3066–3067.
15 The formation of pores by the assembly of hydrophobic calix[4]arene amide has been reported: V. Sidorov, F. W. Kotch, G. Abdrakhmanova, R. Mizani, J. C. Fettinger and J. T. Davis, *J. Am. Chem. Soc.*, 2002, **124**, 2267–2278.
16 A ionophore based on a resorcin[4]arene-cholic acid ether assembly has been reported. In this case, however, only the cone-like *rccc* isomer was prepared. N. Yoshino, A. Satake and Y. Kobuke, *Angew. Chem. Int. Ed.*, 2001, **40**, 457–459.