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An artificial ionophore based on a polyhydroxylated steroid dimer

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The Na^+ transporting properties of the first member of a new class of artificial ionophores, based on a C_2-symmetric polyhydroxylated steroid dimer, are described.

Interest in the design of membrane-spanning artificial ionophores (*i.e.* ion conductors) has intensified in recent years[1] because they may lead to new classes of antibiotics that are less susceptible toward resistance.[2,3] The design of several of these ionophores has been inspired by the naturally occurring antifungal macrolide Amphotericin B (AmB).[4] This molecule has an amphipatic nature with a hydrophobic and a hydrophilic face defined, respectively, by a rigid heptaene subunit and a polyhydroxylated alkyl moiety. It is thought, that, in the membrane, several AmB monomers aggregate to form a cluster where the hydroxylated face of each macrolide points inward, toward a water filled pore, and the hydrophobic one points outward, toward the hydrocarbon portion of the bilayer (″barrel stave″ structure). Since AmB is too short to span the bilayer the alignment of two of these clusters in the membrane is needed to form a pore. Artificial ionophores mimic these features and, for instance, amphipatic systems based on short peptides,[5] cholic acid derivatives,[6] sterol–polyether conjugates,[7]and polyhydroxylated *p*-biphenyl oligomers[8] have been described.

In this context, polyhydroxylated steroids appear to be a very promising class of compounds. These derivatives, mainly isolated from marine sources,[9] are characterized by a flat lipophilic steroid nucleus and by the presence of several hydroxyl groups. As a consequence, the molecule is amphipatic and presents the key structural requirements to modify the permeability of a lipid membrane, acting as a ionophore.

On these premises, and owing to the experience of some of us in the stereoselective synthesis of such compounds,[10] we designed and synthesised the (*Z*)-5α-pregn-17(20)-en-3β,6α,7β-triol (**1**)† and the C_2-symmetric bis-(20*S*)-5α-23,24-bisnorchol-16-en-3β,6α,7β-triol-22-terephthaloate (**2**) in which all the free hydroxyl groups are aligned on the same side of the steroid backbone, defining a hydrophilic surface opposed to the hydrophobic one (Fig. 1). Compound **1** was obtained in 12 steps and 22% overall yield, starting from the commercially available androst-5-en-3β-ol-17-one. Further elaboration of the side chain and its regioselective esterification with terephthaloyl chloride, led to compound **2** (16 steps and 10% overall yield from androst-5-en-3β-ol-17-one). While the monomer **1** is too short to span the membrane, dimer **2**, in the extended conformation, measures about 42 Å and, therefore, is long enough to completely span the lipid bilayer (~ 40 Å). Moreover, the terephthaloyl diester spacer was intentionally chosen because the four oxygen atoms should ensure the continuity of the hydrophilic surface.

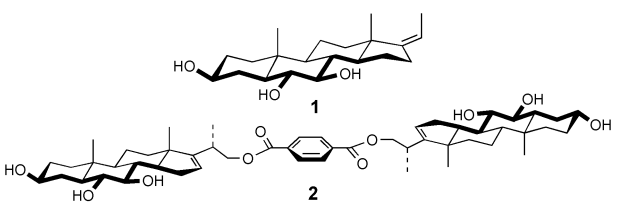

Fig. 1 Structures of polyhydroxylated steroid **1** and its dimer **2**.

To investigate the ionophoric properties of steroids **1** and **2** we studied their ability to promote the transport of Na^+ across a lipid bilayer using a $^{23}Na^+$ NMR based methodology.[11] In brief, a solution of NaCl (75 mM) plus a membrane-impermeable paramagnetic shift reagent ($DyCl_3$–tripolyphosphate complex, 4.0 mM) was added to a 95:5 egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG) dispersion (100 nm diameter, large unilamellar vesicles) that has been prepared in aqueous LiCl (100 mM). Varying mole percentages of steroids **1** or **2** were incorporated in the lipid mixture before the formation of vesicles (double-side addition)[11] which were then prepared by extrusion through polycarbonate filters with a 100 nm pore diameter. Ultrafiltration experiments and UV-Vis analysis of the water solution, free of vesicles, showed that more than 85% of the steroid derivatives were bound to the aggregate. Because the shift reagent is confined in the external bulk aqueous phase, the Na^+ entering the vesicular compartment appears as a separate (unshifted) resonance and integration of internal Na^+ signal as a function of time yields the kinetic profiles shown in Fig. 2.

Steroid **1** behaves as a very poor ionophore: in the presence of 2% of **1** (percent of steroid with respect to the total concentration of lipid, curve ●) and after more than 40 h the amount of Na^+ entering the internal vesicular compartment is only slightly higher than in the absence of any additive (curve ◆). On the contrary, dimer **2** efficiently induces the transport of the Na^+ ion across the lipid bilayer and in the presence of only 0.7% of **2** the Na^+ transmembrane gradient is fully discharged after about 24 h.‡ This process follows first order kinetics and the fitting of the data allows one to obtain the apparent first order rate constant (k_{obs}, h^{-1}) for the entry of Na^+. Fig. 3 shows the dependence of k_{obs} *versus* the mol % of **2**.

The strong dependency of k_{obs} on the concentration of **2** suggests the involvement of steroid aggregates in the Na^+

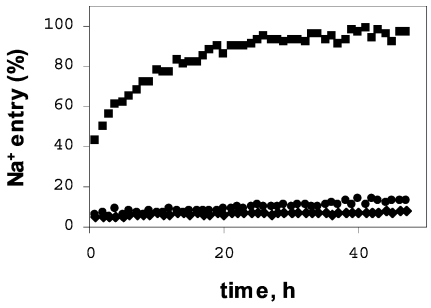

Fig. 2 Kinetic profiles for the entry of Na^+ into 95:5 egg PC/PG vesicles containing **2** (0.7%, ■), **1** (2% ●), and without additives (◆) at 25 °C. The total concentration of lipids was 10 mM.

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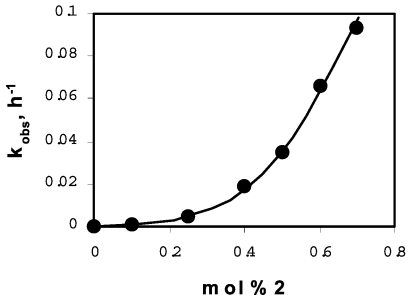

Fig. 3 Plot of k_{obs} as a function of mol % of **2**. The solid curve represents a non-linear least squares fit of the data based on eqn. 1.

transport process. A similar behavior has been observed by Regen and co-workers in the case of sterol-polyether conjugates[7,11] and the authors have shown that this type of profile can be fitted using eqn. 1 where K is the dissociation constant of the aggregate active as an ionophore, k_2 is the intrinsic rate constant for the Na^+ entry process, and n is the number of monomers which form the aggregate. The fit of our data with eqn. 1 (see Fig. 3) gives a n value of 2.8, suggesting that the active ionophore is an aggregate containing an average of three steroid monomers.

$$k_{obs} = k_2[\text{monomer}]^n/K \quad (1)$$

The above results taken together with the inactivity of compound **1** suggest a mechanism in which three steroid molecules self-assemble in the membrane bilayer. This process is probably driven by the amphipatic nature of the steroid derivatives that form a cluster in which the hydrophilic face of the molecules points inward while the hydrophobic one points outward, toward the hydrocarbon portion of the bilayer. In the case of **2**, the cluster is long enough to span entirely across the membrane, forming a pore trough which the Na^+ ion may permeate. On the contrary, steroid **1** is too short and the cluster, if formed, cannot span the membrane and modify its permeability.

The efficacy of **2** as ionophore is strongly dependent from the temperature and, in particular, from the membrane fluidity. Using vesicles made from 1,2-dipalmitoyl-*s*,*n*-glycero-3-phosphocholine (DPPC) which are characterized by a relatively high phase transition temperature (T_c = 41.4 °C) the ionophoric activity of **2** is almost suppressed at a temperature below the T_c (T = 25° C), where the membrane is in the gel-state, while it is maintained at a temperature above the T_c (T = 50° C), where the membrane is in the fluid-state. This strong dependence of the rate of Na^+ transport from the fluidity of the bilayer may support a carrier-type mechanism.[12] However, in the present case, a carrier mechanism seems unlikely because the ionophoric activity of **2** depends strongly on the formation of a cluster of molecules and the length of the steroid derivative is crucial for its activity. More probably, the inactivity of **2** in a gel-phase membrane is related to other phenomena like, for example, a change from an extended to a closed conformation which reduces the length of the steroid derivative or to the squeezing of the ionophore out of the lipid bilayer below the T_c, as recently observed by Regen and co-workers for sterol-polyether conjugates.[11]

In conclusion we have shown that polyhydroxylated steroids can be versatile building blocks for the construction of effective ionophores. With respect to other related systems like cholic acid derivatives,[6,13] polyhydroxylated steroids present the advantage of the flat liphophilic steroid nucleus which optimizes the interaction with the lipid bilayer and possibly with other steroids naturally present in the cellular membrane. As a matter of fact, the activity of compound **2** is comparable with that of AmB§ and also with a tetrameric derivative based on cholic acid[13] in spite of the fact that only two steroid moieties are present in its structure. The mechanism of action of this ionophore is still not completely unravelled and work is in progress to better define it and to improve the efficacy of the system.

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Notes and references

† Compounds **1** and **2** have been fully characterized (^{1}H- and ^{13}C-NMR, FAB-MS, elemental analysis) and their synthesis will be reported elsewhere. *Selected data* for **1**: mp 182–184 °C; *Rf*: 0.50 (14% methanol in methylene chloride); $[\alpha]_D^{20}$: 66 (c = 1.0, in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (3 H, s, CH_3-18), 0.90 (3 H, s, CH_3-19), 1.62 (3 H, d, J = 7.2 Hz, CH_3-21), 3.14 (1 H, dd, J = 10.0, 8.9 Hz, H-6 or H-7), 3.27 (1 H, dd, J = 11.1, 8.9 Hz, H-6 or H-7), 3.57 (1 H, m, H-3), 5.16 (1 H, br q, J = 7.2 Hz, H-20); ^{13}C NMR (400 MHz, $CDCl_3$) δ 13.1, 13.6, 17.1, 21.6, 27.4, 30.6, 32.0, 32.5, 35.7, 36.7, 37.2, 40.5, 44.9, 47.9, 52.1, 55.8, 70.9, 74.7, 79.9, 113.7, 149.2; FABMS *m*/*z*: 357 $[M + Na]^+$, 100%; Calc. for $C_{21}H_{34}O_3$: C, 75.41; H, 10.25. Found: C, 75.38; H, 10.23%. Incidentally, compound **1** is a recently synthesised novel potent anti-asthma agent (IPL576,092). See: Y. Shen and D. L. Burgoyne, *J. Org. Chem.*, 2002, **67**, 3908. *Selected data* for **2**: mp 106–107 °C; *Rf*: 0.50 (40% methanol in chloroform); $[\alpha]_D^{20}$: 48 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD) δ 0.84 (6H, s, CH_3-18), 0.92 (6H, s, CH_3-19), 1.18 (6H, d, J = 6.9 Hz, CH_3-21), 2.15 (2H, m, H-4$_{eq}$), 2.22 (2H, m, H-15), 2.31 (2H, m, H′-15), 2.62 (2H, m, H-20), 3.04 (2H, dd, J = 10.0, 8.9 Hz, H-6 or H-7), 3.16 (2H, dd, J = 11.1, 8.9 Hz, H-6 or H-7), 3.51 (2H, m, H-3), 4.24 (2H, dd, J = 10.6, 7.6 Hz, H-22), 4.45 (2H, dd, J = 10.6, 6.5 Hz, H′-22), 5.57 (2H, br s, H-16), 8.10 (4H, s, Ar-*H*); ^{13}C NMR (100 MHz, CD_3OD) δ: 13.9, 16.7, 19.3, 22.4, 31.8, 32.8, 33.2, 35.0, 36.0, 36.9, 38.4, 41.2, 49.0 (×2, overlapping with $^{13}CD_3OD$), 53.9, 57.8, 70.4, 71.8, 75.9, 81.0, 125.0, 130.6 (×2), 135.5, 156.9, 167.0; FABMS *m*/*z*: 881 $[M + Na]^+$, 100%; Calc. for $C_{52}H_{74}O_{10}$: C, 72.70; H, 8.68. Found: C, 72.67; H, 8.66%.

‡ Concentrations of **2** higher than 0.7% gave an unstable vesicular solution, hampering the determination of the kinetic parameters.

§ In our experimental conditions the rate constant for the Na^+ entry in the presence of 1% AmB is 0.16 h^{-1}. In a similar experiment k_{obs} = 0.74 h^{-1} was found. See: E. Stadler, P. Dedeck, K. Yamashita and S. L. Regen, *J. Am. Chem. Soc.*, 1994, **116**, 6677.

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