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The presence of cholestanyl substituents in hydraphile channels inhibits cation transport in the phospholipid bilayer

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Abstract

The presence of steroids within a synthetic, cation channel model compound restricts or prevents the passage of Na^+ through the phospholipid bilayer owing to collapse of the pore induced by hydraphobic contacts

During the past decade, we have designed and developed a family of cation-conducting channel compounds that function effectively in phospholipid bilayer membranes.[1] The compounds we call hydraphile channels have four structural elements. These are: (1) headgroups; (2) spacer chains; (3) a central relay; and (4) sidechains. For most of the compounds we have prepared thus far, the headgroups and central relay have been based on the 4,13-diaza-18-crown-6 macrocycle. In a number of studies, we have attempted to characterize the family of channel structures. We have demonstrated that the hydraphile channels span the phospholipid bilayer and that the headgroups are separated by about 30 Å, the approximate width of the so-called hydrocarbon slab. Fluorescence studies have confirmed the headgroup separation and thus the span of the channel as well as indicating that the hydraphiles are not significantly aggregated in the bilayer. As a result of these studies and additional efforts summarized recently, we have developed a workable structure–activity relationship for these compounds. Notwithstanding this progress, one observation that perplexed us was the effect of cholestanol that was incorporated into the hydraphiles as sidechains. We now report a combination of solution and solid state data in concert with calculations that suggest an appealing explanation for what we previously regarded as anomalous behavior.

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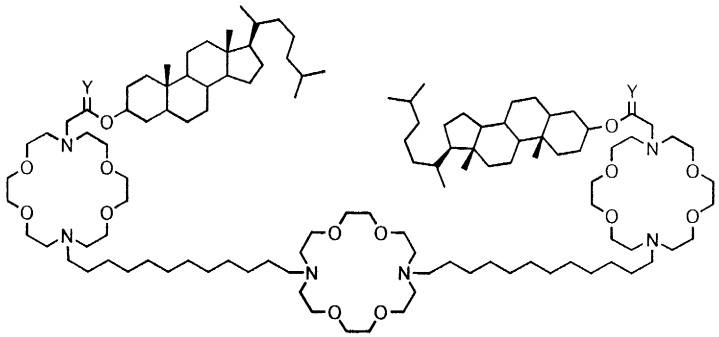
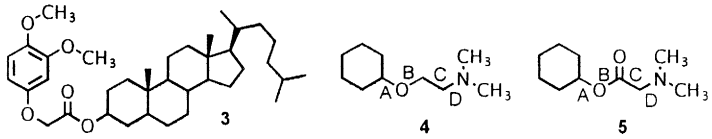


1, Y = H_2; **2**, Y = O



3 **4** **5**

Three compounds were prepared for the present study. All are steroid derivatives. Two are hydraphile[2] channels. They are shown as **1** and **2** and differ only in the means by which the steroids are attached to the macrocyclic headgroups. The third compound was prepared in the hope of obtaining a solid state structure that would guide our understanding of the interactions leading to the observed cation transport behavior.

Compound **1** was prepared by a sequence as follows.[3] 3-Cholestanone was protected as its ethylene ketal and then reduced with $BH_3 \cdot THF$ to give $CholOCH_2CH_2OH$. The alcohol was tosylated and $CholOCH_2CH_2OTs$ was allowed to react with H<N18N>C_{12}<N18N>C_{12}<N18N>H to afford **1** as a white solid (mp 71–72°C). A more elaborate route was required for the preparation of **2**. Thus, chloroacetyl chloride was treated with dihydrocholesterol to afford $ClCH_2COOChol$. Reaction of the chloroacetic acid derivative with 4,13-diaza-18-crown-6 gave the two-armed crown and $CholOCOCH_2$<N18N>H. This, in turn, was alkylated with 1,12-dibromododecane to give $CholOCOCH_2$<N18N>$(CH_2)_{12}Br$. Alkylation of diaza-18-crown-6 with this bromide gave **2** (mp 71–73°C). Compound **3** was prepared by treating cholestanyl chloroacetate[4] with 3,4-dimethoxyphenol in the presence of K_2CO_3 in acetone.[5] Compounds **4** and **5** were models used only for calculation but they are known substances.[6]

Solid state structure: Many phospholipid bilayer membranes, particularly plasma membranes, are rich in cholesterol.[7] In part, because of the importance of cholesterol as a membrane component, several structural studies have been conducted.[8] The compounds discussed here utilize cholestanol, the product of cholesterol hydrogenation. This relative was preferred in our studies because synthetic methods involving strong bases were sometimes found to isomerize the $\Delta^{4,5}$-bond in the natural product when it was used.

We originally prepared **3** in connection with our studies of steroidal crown ethers that are capable of forming vesicles.[9] As the compound was already available and nicely crystalline, we felt that a solid state structure of it would provide us with an important general reference and in particular as a model for the channel sidechains. We had previously obtained the structure of *N*-(cholesteryloxycarbonyl)aza-15-crown-5[4] and report the structure of the cholestanoyl ester **3** here. Crystals were obtained by slow evaporation of solvent (MeOH:CH_2Cl_2, 1:1 (v/v)) at ambient temperature. The structure was then determined by standard methods using a CAD4 diffractometer. The unit cell contains two crystallographically independent molecules that differ by 180° rotation around the C–O bond at the 3-position.

Transport: Sodium cation flux was measured[3] for **1** and **2** in bilayer membranes formed from

phosphatidyl choline and phosphatidyl glycerol (4:1) by using the ^{23}Na NMR-based method of Riddell.[10] The sodium cation transport rate observed for **2** was about 5% that observed for the known peptide channel former and control molecule, gramicidin (k_{rel}=100). No transport could be detected for **1**. In contrast, when the sidearm was dodecyl or benzyl, the relative transport rates were 28 or 39 compared to that observed for gramicidin. The results obtained for **1** and **2** were perplexing because we anticipated that the presence of the steroid as the 'wall' of the channel would afford a higher level of organization within the bilayer. If both hydraphiles **1** and **2** were inactive, it would have been difficult to assess any chemical differences between them. Fortunately, **2** showed a small, but reproducible level of transport efficacy.

We hypothesized that contact between the flat α-face of the steroid and the opposing dodecyl spacer might block the channel. If so, it seemed reasonable that the extent of surface contact and pore blockage would be affected by linker flexibility. The glycinyl residue (of **2**) is expected to be more rigid than ethylene (in **1**). Esters (RCOOR′) are known to favor a planar conformation, with the carbonyl oxygen and R′ *cis*. (Experimental results have demonstrated that the *cis* conformation of methyl acetate is favored by approximately 4 kcal/mol over the *trans*.[11]) Compounds **1** and **2** each have more than 125 non-hydrogen atoms and molecular weights very near 2 kDa. We therefore chose to model the critical fragments by use of the conformational search routine in Macromodel. Structures **4** and **5** correspond to the linker regions between the crown head group and the cholestanyl sidearm. The nitrogen atom corresponds to the crown nitrogen and the cyclohexyl ring models the cholestanol A ring.

Structures retrieved in the search were superimposed to reveal the differences in conformational space available to each molecule. In both cases, the overlaid structures were within 5 kcal/mol of the calculated global minimum. Fig. 1 shows the conformational search for **4**. The high level of conformational flexibility reflects the adaptability of the ethylene linker. The three-fold symmetric minima for the ester, **5**, may be rationalized by inspection of a Newman projection. The global minimum structure is depicted. The three families of conformers found for **5** result from 120° rotations about bond D (see structures for letter designations). Other structures observed are dihedral rotamers of the bonds C and A.

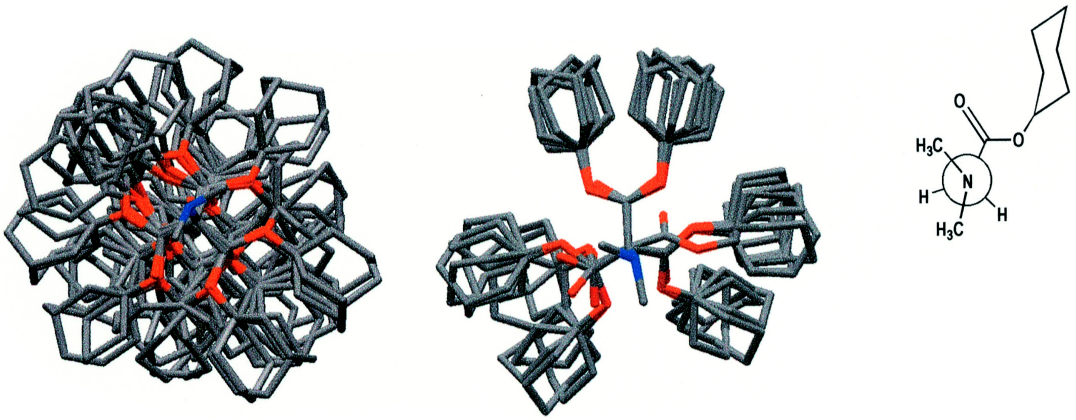


Fig. 1. Conformational spaces for **4** (left), **5** (center), and global minimum for **5** (right)

The calculated results comport with our intuition and the experimentally observed results. We infer that the more flexible ethylene link (**1**, **4**) permits cholestanol to contact the dodecyl sidechain on the opposite wall of the channel and thus occlude the pore and enjoin transport. The glycinyl link (**2**, **5**) permits the same interaction but to a reduced extent owing to its relatively lower flexibility. We envision

the contact as shown in Fig. 2, which shows the crystal structure of **3** docked to the dodecyl chain of *N*,*N*-bis(dodecyl)-4,13-diaza-18-crown-6.[12] The packing is reminiscent of the proposed interaction between the α-face of cholesterol with the alkyl chains of phospholipids in membranes.[13]

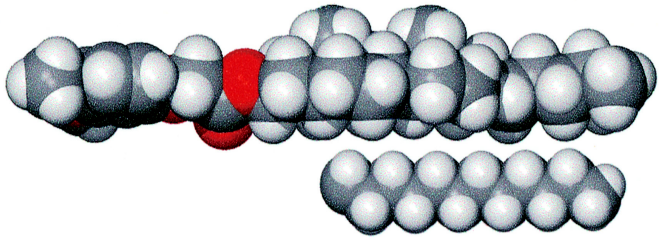


Fig. 2.

An intriguing result obtained by others appears to further confirm this hypothesis. An artificial channel designed and prepared by Frye and coworkers was based on a central macrocycle to which were appended six cholesterol 'walls.' The design appeared very promising but Na^+ transport was extremely low.[14] This system lacks the dodecyl chain at the opposite wall and may be occluded in a different way. If the three cholesteryl residues turn their α-faces 'outward' to the phospholipid bilayer, the six methyl groups on the β-surface of the steroid would be turned inward. A transient cation would be presented with a molecular obstacle course that would certainly reduce the ability of a water-and-cation column to form and organize. Our observations suggest that careful consideration should be made with respect to intrachannel or channel-lipid interactions when sterols are used as design elements in synthetic ion channels.

1. Experimental

Crown-sidearm model system: All molecular mechanics calculations were performed on a Silicon Graphics Indigo^2 workstation equipped with a 100 MHz R4000 processor. Macromodel V5.0[15] and the GB/SA chloroform solvation model[16] were used for all simulations. Energy minimizations utilized the MM2* force field and the TNCG method[17] (1.0 kJ/mol*Å^2 Hessian cutoff). A 1500 step Monte Carlo conformational search was completed for each model compound. The energy cut-off for stored structures was 11.9 kcal/mol (50 kJ/mol) and the lowest 5 kcal/mol (20.92 kJ/mol) were superimposed for visual analysis. The global minima were found 32 and 101 times for the ether (**4**) and ester (**5**), respectively.

*Crystal data for **3***: $C_{37}H_{58}O_5$, M=582.8, triclinic, space group *P*1, *a*=9.627(2), *b*=11.991(3), *c*=15.224(3), α=78.54(2), β=84.19(2), γ=76.24(2) Å, Vol. 1670.2(7) Å^3, *Z*=2, μ=5.4 cm^{-1}, λ=1.5418 Å, 4109 reflections measured of which 3783 were unique, *R1*=6.9% for 3570 reflections with $I>3\sigma$.

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