

Stabilizing Guanosine-Sterol Ion Channels with a Carbamate to Urea Modification in the Linker

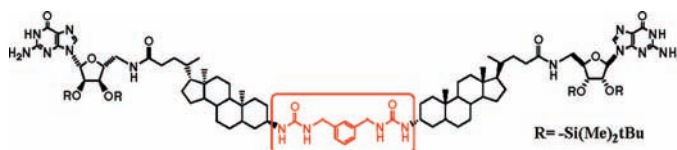
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ABSTRACT



The use of a bis-urea lithocholamide linker within a guanosine-sterol dimer resulted in formation of large and stable ion channels. The channels were longer-lived than those formed by the corresponding bis-carbamate.

Numerous synthetic compounds form transmembrane ion channels in lipid bilayers.^{1,2} Usually the conductance of these channels is a few to tens of picoSiemens (pS), corresponding to pore diameters of less than 3–4 Å.³ Whereas some peptides and lipids self-associate into “large” (>1 nm) and stable channels,^{4,5} there are fewer examples of such channels made from synthetic compounds. Matile and colleagues showed that rigid rods formed “giant” nS channels that stayed open for seconds to minutes.⁶ More recently, Fyles and Tong described long-lived and highly conducting nS ion channels formed by a lipophilic ethylenediamine–Pd(II) coordination complex.⁷

We reported that guanosine-lithocholamide **1** forms large and long-lived ion channels in planar lipid bilayers, with nS conductances and “open” times of seconds.⁸ Our design was based on the hypothesis that self-assembled G-quartets might be formed upon attachment of guanosines to a membrane-spanning lithocholate dimer.^{9,10} This spacer was inspired by Kobuke’s studies that had shown that bis-cholic acid derivatives formed transmembrane ion channels, presumably by self-assembling in the membrane.¹¹ More recently, another membrane-active lithocholate dimer has been generated by using coordination chemistry to bring together the sterol fragments.¹² In addition to these examples of channels, a variety of other cholic acid derivatives are membrane-active ionophores.¹³

We originally envisioned that membrane insertion of guanosine-sterol **1**, followed by G-quartet formation, might

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provide functional pores. Indeed, we found that the guanosine end-groups were essential for channel formation by **1**, as a bis-lithocholamide lacking the guanosines was inactive.⁸ We concluded, however, that ion transport was not occurring through the G-quartet cavity, as most conductance events for **1** were in the 1–5 nS range. Pores that conduct in the nS range must be much larger than a G-quartet, with a diameter of 3.6–3.7 Å. We estimated that the diameter for a single channel of 2.5 nS, one of the most frequently observed conductances for **1**, must be greater than 10 Å.^{3,8} To rationalize these large conductances, we proposed that the hydrophobic bis-lithocholate linker in **1** might provide the walls for the pore and that a cation-filled G-quadruplex could serve as a structural pillar to support the assembly within the membrane. In this way, we proposed that self-assembled structures like the one shown in Figure 1 might be responsible for the function of **1**.¹⁴

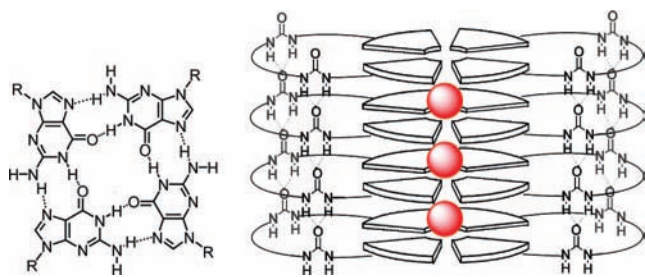
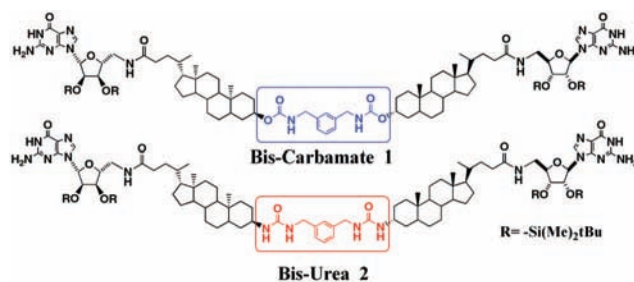


Figure 1. A G-quartet (left) and one possible self-assembled structure (right) formed by bis-urea **2**, showing intermolecular H-bonds between urea units in an assembly stabilized by a cation-filled G-quadruplex (for other possible assemblies, see ref 8).

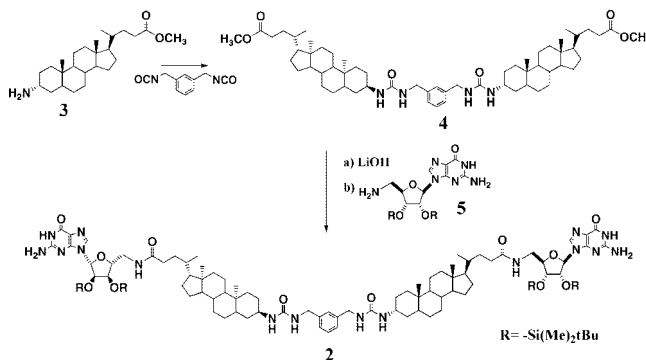
To test this hypothesis we have now modified the bis-lithocholate linker so that it should be better able to self-associate and form structures that are even more stable than those formed by **1**. We reasoned that such stabilization could be accomplished by replacing the carbamates in **1** with urea groups to give **2** (Scheme 1). The urea group, a better hydrogen bonding group than a carbamate, has been used to direct self-association of many supramolecular structures,^{15,16} including nanotubes.¹⁷ Of particular relevance to this study, was the finding that a self-associating ureido-crown ether gave single channels in planar bilayer membranes.¹⁸ Below,

Scheme 1. Guanosine-Sterol Conjugates **1** and **2**



we describe the synthesis and function of the bis-urea G-lithocholamide **2**, a compound that like bis-carbamate **1** forms large nS pores.

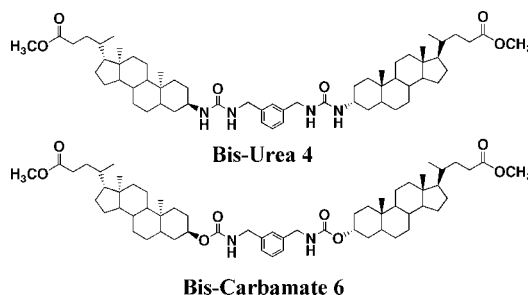
Scheme 2. Synthesis of Bis-Urea **2**



The synthesis of bis-urea **2** is shown in Scheme 2. The 3α-hydroxy group of lithocholic acid was converted, with retention of stereochemistry, to the known 3α-amino derivative **3**.¹⁹ Bis-urea lithocholate **4** was prepared by reacting **3** with *m*-xylene diisocyanate.²⁰ Hydrolysis of diester **4** to the diacid, followed by coupling to the known 5'-amino-G **5**,²¹ gave the target compound, bis-urea **2**.

The impact of the carbamate to urea modification on self-association was first evaluated using ¹H NMR spectroscopy. We compared bis-lithocholate esters **4** and **6** (Scheme 3),

Scheme 3. Compounds Used for NMR and IR Study



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rather than **1** and **2**, so as to eliminate complications in the spectra that arise from guanosine's NH protons. We monitored chemical shifts for NH protons in **4** and **6** as a function of concentration and temperature. As shown in Figure 2, an

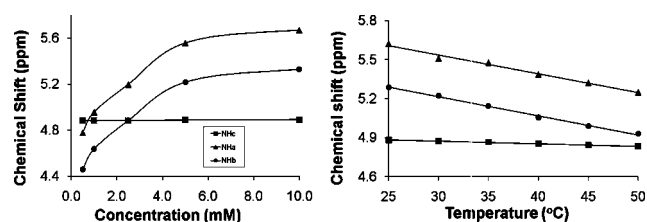


Figure 2. (Left) ^1H NMR chemical shifts of NH protons in CDCl_3 at 25 °C plotted against concentration of bis-urea **4** (triangles and circles for the two NH protons) and bis-carbamate **6** (squares). (Right) ^1H NMR chemical shifts of NH protons in CDCl_3 at 10 mM concentration plotted against temperature for bis-urea **4** (triangles and circles for the two NH protons) and bis-carbamate **6** (squares).

increase in concentration of **4** in CDCl_3 resulted in downfield changes for chemical shifts of both urea NH protons ($\Delta\delta \approx 0.8$ ppm from 0.5 to 10 mM). In contrast, the chemical shift of the carbamate NH in **6** was insensitive to changes in concentration. This data indicates that bis-urea **4** has greater propensity to self-associate than the analogous carbamate **6**.²²

The NH chemical shifts in **4** were also sensitive to temperature, another signature of intermolecular hydrogen bonding. As shown in Figure 2, increasing temperature caused a significant upfield shift for both urea NH protons in **4**, consistent with increased dissociation of hydrogen bonds with increasing temperature. On the contrary, the carbamate NH in **6** displayed little dependence on temperature, consistent with it being monomeric in CDCl_3 .

We confirmed intermolecular H-bonding for bis-urea **4** by FT-IR spectroscopy. Unlike NMR, which gives time-averaged signals, FT-IR gives separate signals for the monomeric and self-associated species. The urea N–H stretch moves toward lower wavenumber on formation of intermolecular hydrogen bonds.²³ Figure 3 shows FT-IR results for **4** and **6** in CHCl_3 . As the concentration of bis-urea **4** was increased, a broad N–H absorption band (~ 3360

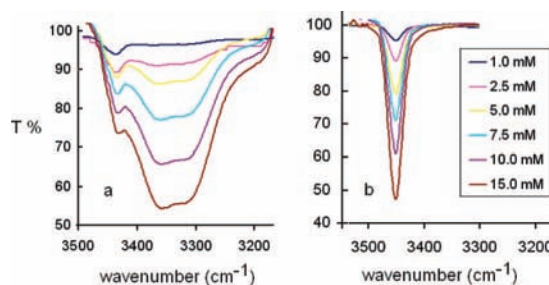


Figure 3. N–H stretching region of the IR spectra as a function of concentration for bis-urea **4** (left) and bis-carbamate **6** (right) measured in CDCl_3 at 25 °C.

cm^{-1}) grew in on the lower frequency side of the band for the free N–H stretch (~ 3430 cm^{-1}). Clearly, increased concentration leads to self-association of **4**. In contrast, bis-carbamate **6** revealed no intermolecular hydrogen bonding in CHCl_3 , remaining monomeric even at 15 mM.

Ion channel formation was characterized using the voltage clamp technique.^{1a,8} Compound **2** (50 nM final concentration) was added to the cis side of a planar membrane (1.0 M KCl and a -10 mV applied potential). Figure 4 shows representa-

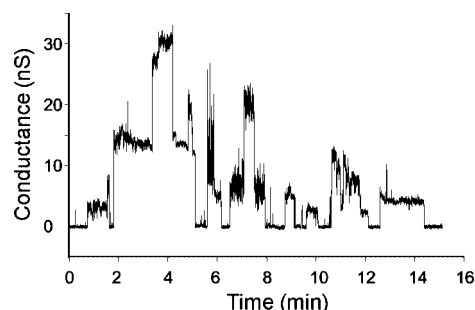


Figure 4. Representative traces from voltage-clamp experiments indicating distinct conductance values recorded in the presence of bis-urea **2** at -10 mV in 1 M KCl. The experiment was carried out by adding compound **2** (50 nM final concentration) to the cis side of the chamber after the planar bilayer membrane was formed.

tive conductance changes generated after addition of **2**. The data indicate formation of discrete channels, the majority showing conductances in the 1–20 nS range with lifetimes of seconds to minutes. Transitions from “open” to “closed” states indicates the dynamic nature of these self-assembled ion channels.

While both sterol-guanosine conjugates, **2** and **1**, form large channels, there are significant differences between the compounds in terms of their effective concentration, conductance levels and “open” lifetimes. Bis-urea **2** is membrane-active at much lower concentrations than is carbamate **1**. Thus, bis-urea **2** was active at a concentration of 50 nM (Table 1), but compound **1** had to be added to the solution bathing the planar membrane at a much higher concentration (31.4 mM) in order to observe channel formation within a

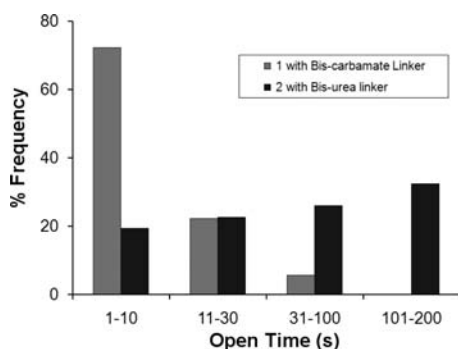
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Table 1. Frequency of Events for Channels Formed from **1** and **2**

conductance (nS)	bis-carbamate 1		bis-urea 2	
	events	% events	events	% events
0.1–1.0	100	20	16	22
1.1–5.0	331	68	26	35
5.1–20	43	9	17	23
>20	13	3	13	20

The number and frequency of ion channel open events for **1** were taken from the three experiments that used Method 1 reported in ref 8. For compound **2**, the data were taken from a total of four experiments. These experiments were conducted by adding either compound **1** (31.4 μ M) or **2** (50 nM) to the cis side of the chamber after the planar bilayer membrane was formed (1.0 M KCl and -10 mV applied potential).

reasonable time. Addition of **2** at these millimolar concentrations typically caused breakage of the planar bilayer membrane during the voltage clamp experiment. We attribute this difference in effective concentrations to the fact that bis-urea **2** is better able to self-associate into some active structure than is bis-carbamate **1**. Second, the bis-urea **2** formed, on average, larger channels than did bis-carbamate **1**. As shown in Table 1, “giant” channels with conductance levels greater than 5 nS were formed more frequently by bis-urea **2** (43% of all events) than by bis-carbamate **1** (12% of all events). Finally, bis-urea **2** formed channels that were kinetically more stable than those formed by **1** (Figure 5).

**Figure 5.** Histogram showing the various open lifetimes for 1–5 nS channels formed from **1** and **2**.

To compare the relative stability of channels formed by the two compounds, we analyzed the “open” lifetimes for channels formed by **1** and **2** that had conductance values between 1 and 5 nS, since these conductances were the most frequent events observed for both guanosine-sterols (see Table 1). As shown in the histogram in Figure 5, about 70% of the 1–5 nS channels formed from bis-carbamate **1** had open lifetimes that were less than 10 s. In contrast, over 80% of the 1–5 nS channels formed by bis-urea **2** had open lifetimes that were greater than 10 s. Remarkably, about 50% of these channels formed by **2** were open for 30 s or longer. Indeed, the average lifetime of 1–5 nS channels formed from bis-urea **2** was 66 s/event, as compared to 16 s/event for the 1–5 nS channels formed from bis-carbamate **1**. Our data indicate that the bis-urea unit in the linker region of **2** helps to stabilize transmembrane ion channels formed by this guanosine-lithocholate derivative.

Guanosine-sterol conjugate **2**, containing a bis-urea group in its linker region, was designed so that it might further stabilize self-assembled ion channels within the phospholipid bilayer. Our hypothesis is that strengthening the intermolecular interactions between the bis-lithocholate linker region might work cooperatively with the guanosine-based self-association to provide highly stable conducting structures. That the bis-urea **2** forms large ion channels whose lifetimes appear to be considerably longer than those channels formed by bis-carbamate **1** is consistent with our structural hypothesis. Our ongoing efforts are now focused on investigating whether this new guanosine-lithocholic acid derivative **2**, which can form channels that must have nm-sized diameter, can be used to transport larger compounds in and out of phospholipid vesicles.

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Supporting Information Available: Experimental details and spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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