Hydraphile Channels: Models for Transmembrane, Cation-Conducting Transporters

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Abstract: A completely synthetic, non-peptide channel has been prepared and shown to conduct cations across a phospholipid bilayer membrane. Studies have been undertaken to assess the compound's location within the bilayer and to better understand its function. These studies are described along with background on the design and concept of the channel.

Keywords: cations · cation transport · membranes · peptides · synthetic channels

Introduction

The membranes of cells constitute an important barrier between the cytosol (the cell's internal fluid) and the external medium. One important reason that this barrier must be maintained is because there is typically an ionic imbalance between the inside and the outside of the cell. In red blood cells, for example, the concentration of K^+ is 150 mM inside the cell and 5 mM outside. The concentration of the sodium cation, on the other hand, is only 10 mM within and 150 mM without. This is but one example of the non-equilibrium conditions that must be maintained for cells to survive.

Channels of many types play a critical role in transporting ions and molecules through the phospholipid bilayer.[1] Channels may be formed from peptides or from proteins. It is currently the consensus that channels that form from peptides do so as a result of monomers, such as alamethicin (Table 1), that organize into a pore.[2] The number of monomers required and the structures of such pores remain elusive.[3] Peptides such as gramicidin (Table 1) that consist of alternating D- and L-amino acids form a channel within the helix and dimerize to achieve the appropriate span rather than the appropriate pore diameter.

Table 1. Amino acid sequences for gramicidin and alamethicin.

gramicidin D[a]	OHCNH-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-xxx-D-Leu-L-Trp-D-Leu-L-Trp-$CONHCH_2CH_2OH$
alamethicin	Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol

[a] Gramicidin D (Dubos) is a mixture of gramicidin A, B, and C (~80:5:15),[4] in which xxx (above) is Trp in gramicidin A (gA), Phe in gB, and Tyr in gC.

Protein channels have considerably higher molecular weights than do peptides channels. They are thought to have multiple, α-helical strands that can cross the membrane repeatedly. These α-helices are connected by peptide loops that may be constricting or quite loose. In most cases, it is thought that the protein crosses the membrane several times arranging multiple helices into a pore. Cations then pass through this assembly. The formation of an organized assembly by this type of arrangement is known from the structure of bacteriorhodopsin.[5] Bacteriorhodopsin is in the family of molecules known as G-coupled protein receptors and, although it is not a cation-conducting channel, its seven-transmembrane helix structure was the best model available until very recently.

Solid-State Structures of Channel-Forming Proteins

A breakthrough has occurred during the past few years in our understanding of cation-channel structures. This can be attributed to the publication of the structure of the KcsA channel from *Streptomyces lividans* reported by Doyle et al. working in the MacKinnon laboratory.[6] A second structure, that of the so-called "mechanosensitive" channel isolated from *Mycobacterium tuberculosis* has further enhanced our knowledge in this area.[7] Both of these channels have pores formed from multiple α-helices, but the K^+ channel has fourfold symmetry and the mechanosensitive channel has fivefold symmetry, as has the acetylcholine receptor.[8] The structures of channels have raised innumerable questions and

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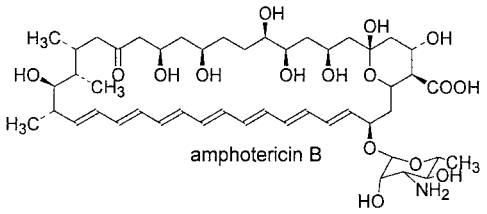
will continue to inspire study for many years. The goal of the organic chemist is to use this new structural information along with existing biophysical results to design and prepare simple models of channel function.

Natural Products that Are Channel Models

The organic chemist's view of channel function has been influenced by a variety of natural products. These include polyene antibiotics such as amphotericin B and nystatin. For example, amphotericin B is thought to form an aggregate that



is rather like a barrel and the arrangement has been termed the "barrel-stave model."[9] Gramicidin is a pentadecapeptide that dimerizes in a tail-to-tail fashion to form the most studied of all cation-selective channel compounds. Although it has been the subject of more than 4000 literature reports,[10] gramicidin's functional structure and mode of action continue to inspire study and controversy.[11] It seems remarkable that such simple, relatively low-molecular-weight (MW $\sim$ 12 kD) structures exhibit behavior typical of many protein channels: ion selectivity, voltage dependence, sub-conductance states, and blocking.

Synthetic Peptides as Models for Channel Function

A number of groups have constructed channels from various peptides. Mutter and Montal used what they refer to as template-assembled synthetic proteins (TASPs).[12] DeGrado and co-workers[13] studied the aggregation of α-helices by using model peptides that contain only leucine and serine residues: $H_2N(LSSLLSL)_3CONH_2$.

Synthetic Organic Compounds as Model Ion Channels

From the biological perspective, it is transport of H^+, Na^+, K^+, Ca^{2+}, and a few other ions that are of interest. Pioneers in this field have, however, studied transport of ions such as Co^{II} in order to assess their designs within a bilayer. Early channel designs were reported by Tabushi et al.,[14] Lehn et al.,[15] Fyles et al.,[16] Menger et al.,[17] Kobuke et al.,[18] and also from our own laboratory.[19] These designs varied widely but the issues addressed were similar. In all cases, it was essential to span the membrane and to provide a pathway so that the ion could traverse it.

Designs diverged at this point into essentially two groups.[20] We have described these as "half-channel" units and full-membrane spans. Half-channel elements have a span of approximately half the thickness of the membrane. The molecules reside in one or the other of the two leaflets that comprise the bilayer.[21] They move about (lateral relaxation) within the bilayer and when opposite half-channels align, a transmembrane pore forms. This is illustrated in Figure 1 with an aggregate in the "barrel-stave" arrangement.

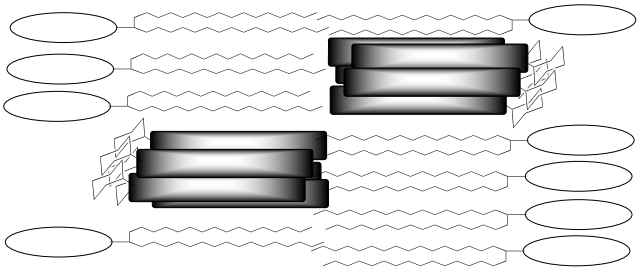


Figure 1. Schematic diagram showing two half-channel elements, each residing in a separate membrane leaflet; they can form a transmembrane pore when they are aligned.

The approach that more closely mimics the transmembrane pores formed by proteins involves a single structure as wide as the membrane. At a minimum, such a design must span the insulator regime ("hydrocarbon slab") of the bilayer (Figure 2). This distance is typically 30 – 35 Å.[22] The bilayer contains two other structural elements that together comprise the "headgroup." In fact, between the insulator and the exposed headgroup, there is a region of intermediate polarity involving the glyceryl esters. We referr to this space as the "midpolar regime" (Figure 2). We believe that important interactions occur between protein channels and this portion of the membrane.[23]

The Design and Synthesis of Synthetic Channel Compounds

The design and synthesis of transmembrane ion conductors presents two challenges. The first is that the design involves numerous assumptions and guesses because the mechanism of channel transport is not well understood. Evidently, the biophysics of what happens has been extensively characterized, but the chemical mechanisms underlying these phenomena are poorly understood. The second challenge is to prepare the designed structure as economically as possible. The elegant synthesis of a nonfunctional structure is folly. The design must be realized quickly and, if possible, in a modular approach so that structural variations can be implemented as the analytical data accumulate.

Tris-macrocycles and Hydraphiles

A number of decisions were required at the beginning of the project. For example, we decided that spanning the insulator regime was more important than spanning the entire bilayer. We therefore devised a structure with an overall length of

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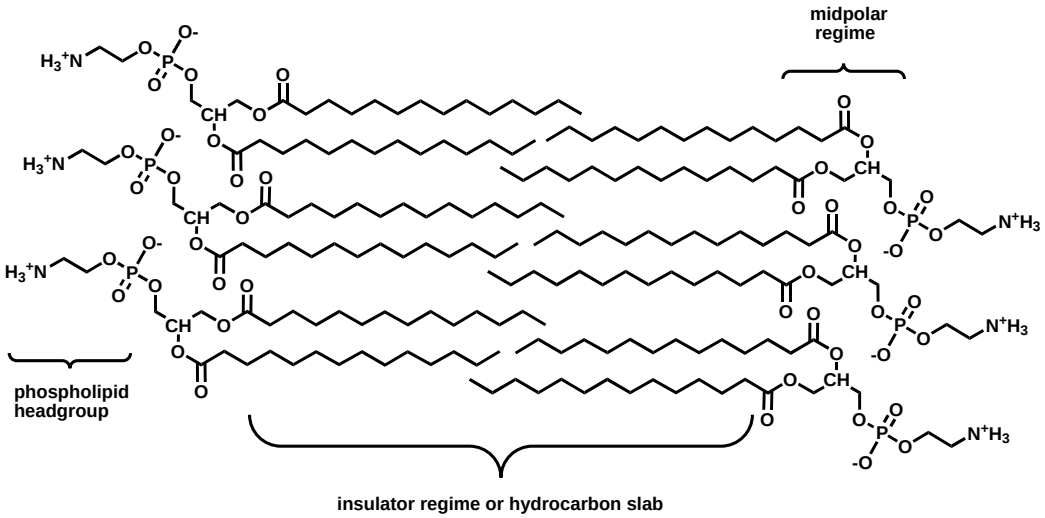

Figure 2. Representation of a phospholipid bilayer membrane.

~40 Å rather than 50–60 Å. We have long taken the view that it is prudent to combine structural features thought to be important with structural flexibility. By doing so, an incorrect guess about distance or orientation may be corrected by conformational adjustment. A rigid compound may be more intellectually appealing, but a minor misalignment in an inflexible structure may portend disaster.

Based on our experience with macrocycles,[24] we decided to incorporate crown ethers[25] into the design as headgroups. A separate research effort by others[26] and by us[27] established that appropriately substituted crowns could function as headgroups in the amphiphilic sense. The crowns would anchor the channel at the polar/insulator boundary and also serve as entry portals. We envisioned that they would also play a second role: a central ion relay. Chemical intuition suggested that ions were unlikely to "jump" 30 Å or more in the absence of any stabilization. The midplane of the bilayer is clearly nonpolar and presents a significant barrier to cation transport. We felt that a crown ether could serve as a relay station for the transient ion. Indeed, the existence of just such a relay station[28] was revealed in the KcsA channel structure.[6] Our own studies have confirmed this function as discussed below.

Our original design is illustrated in Figure 3. Note that we envisioned water being associated with all alkali metal cations essentially in a chain within the pore. The compound shown here may be represented in shorthand as $C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}$ (**1**). As our experience evolved, we learned that the central macrocycle was parallel to the membrane's lipid axis rather than parallel to the other macrocycles (discussed below). When we ultimately eliminated the central macrocycle, we found that our designation of these compounds as "tris-macrocycles" was no longer appropriate. We dubbed them "hydraphiles", in part because of the dictionary definition of hydra: "Any of several small freshwater polyps of the genus *Hydra* and related genera, having a naked cylindrical body and an oral opening surrounded by tentacles."[29] This definition seemed to describe the chemical structures as well as the creatures.

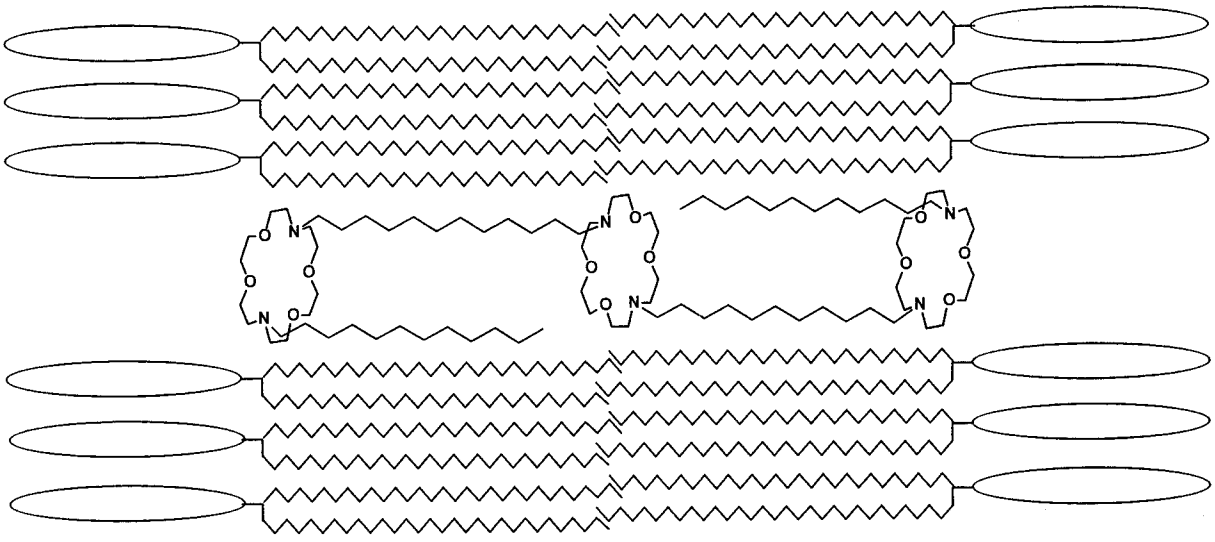

Figure 3. Semi-schematic representation of the original design concept for hydraphile channels.

Analytical Chemistry: Confirming Structure and Function

The initial challenge is to confirm function. The hydraphiles were designed to transport cations and the ability to do so can be confirmed in several ways. Proton transport in the bilayer was assessed by using fluorescence methods. Alkali-metal cation transport was also assessed by use of the electrophysiological method of "patch clamping" or more correctly in our case, planar bilayer conductance measurements. The method that we used most extensively is a dynamic NMR method developed originally by Riddell.[30] In this technique, the exchange rate of Na^+ between the inside and the outside of phospholipid vesicles is assessed by using ^{23}Na NMR spectroscopy. The equilibrium exchange ($K = 1/\tau$) is detected as a concentration dependent change. Channel concentrations in the latter experiments were typically in the range 0–20 μM. Gramicidin (see above) was used as the control in all experiments ($K = 100\,\%$). Under these conditions, channel **1** transports Na^+ at a rate about 27 % of that for gramicidin ($K \sim 175\ s^{-1}$) under identical conditions.

Control experiments: A number of control experiments were run to establish whether the observed function was in agreement with the concepts. Changing sidearm groups altered transport efficacy in ways that could be readily correlated with structure. Removal of the sidearms by replacement of the distal *N*-alkyldiaza[18]crown-6 compounds by aza[18]crown-6 eliminated the cation-transport function. If transport occurred by a carrier mechanism, removal of the sidearms was not expected to matter much. Indeed, the family of hydraphiles was found to transport sodium picrate in a $CHCl_3/H_2O$ artificial membrane system, but there was no correlation with transport rates determined in the bilayer. This is in concert with, but does not prove, the assumption that the hydraphiles function as channels in bilayers.[31]

If cations passed through the central macrocycle (see Figure 2), diminishing the size of macroring (or eliminating it) would nullify the transport efficacy. When $C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}$ was converted into $C_{12}\langle N18N\rangle C_{12}\langle N15N\rangle C_{12}\langle N18N\rangle C_{12}$, little change in rate was observed. Likewise, cleavage of the central macrocycle did not obviate function. We thus concluded that the arrangement of **1** in the bilayer was as shown in Figure 4.

The use of fluorescence measurements: The dodecyl sidechains of **1** can be replaced by fluorescent dansyl groups $Dn\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle Dn$ to give **2**. When **2** was dissolved in solvents with polarities (E_T) of 35–60 D, its fluorescence maximum (λ_{max}) was observed between about 500 and 525 nm (see Figure 5). When **2** was studied in a phospholipid bilayer, λ_{max} for the dansyl groups was observed at 516 nm. This corresponds to a relative polarity between that of methanol and ethanol, but closer to the latter. This is the environment expected if the dansyl groups are near the bilayer's ester carbonyl groups. The interior of the membrane would be much less polar and the membrane's headgroup region would be more polar.

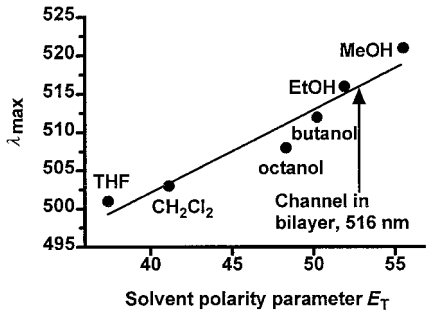

Figure 5. Plot of fluorescence emission for dansyl channel **2**.

Another means by which the headgroup placement can be assessed is the use of fluorescence resonance energy transfer (FRET). By placing the fluorescence quenching doxyl group at various positions on phospholipid fatty acid chains, it is possible to triangulate the location of the fluorescent residue.

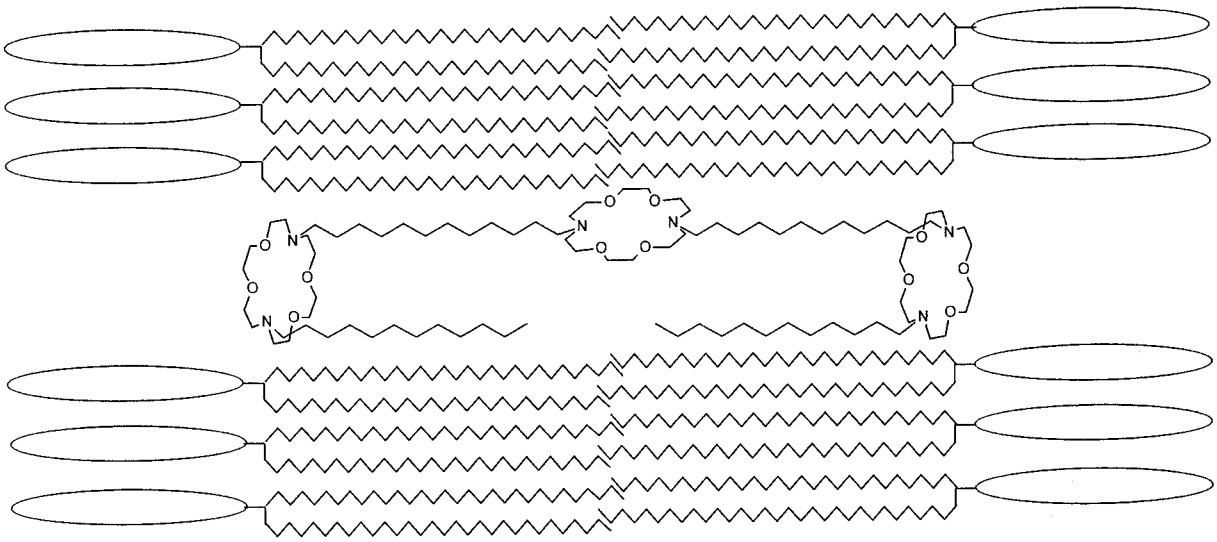

Figure 4. Semi-schematic representation of the current understanding of the hydraphile channel **1** arrangement within the bilayer.

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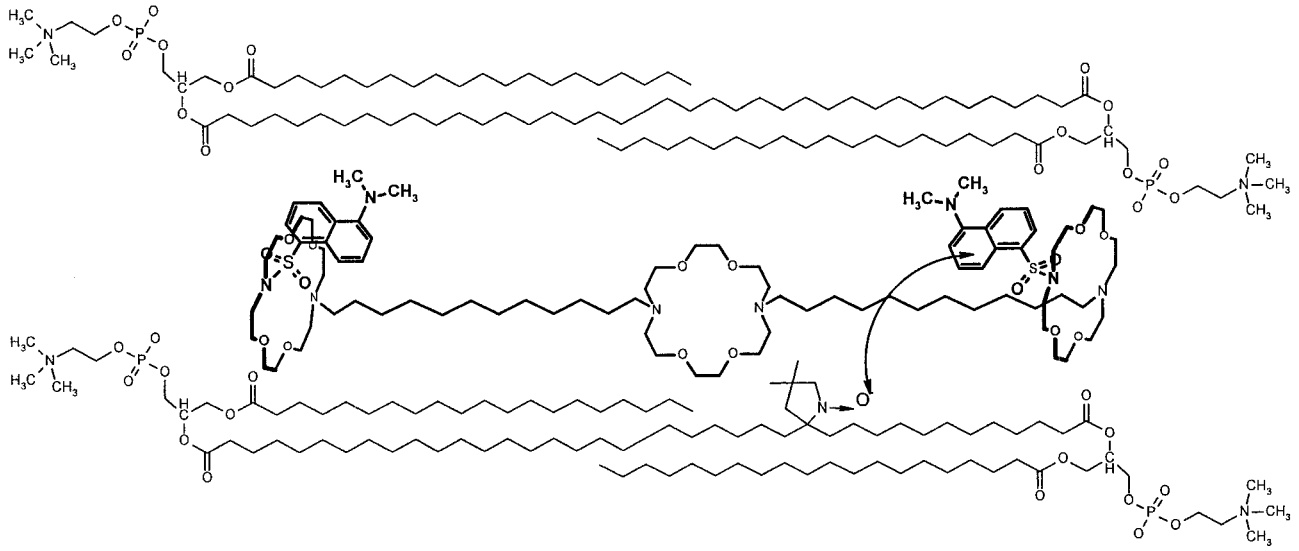

Figure 6. Quenching (indicated by arrow) of dansyl group fluorescence by the doxyl group of a modified phospholipid residue.

Again, the dansyl groups of **2** are the fluorescent residues. In this case, a doxyl group is attached at varying positions on the fatty acid portion of the membrane monomer. The doxyl group's unpaired electron will quench the dansyl group fluorescence according to their separation. The assumption is made that the doxyl-containing fatty acid chains align with the membrane's other monomer chains in a predictable fashion (see Figure 6). The concentration of doxyl lipid is varied within the membrane. A plot is then made of $\ln F/F_0$ for each monomer. The ratio $F/F_0 = \exp[\pi C/70(R_0^2 - X^2 - Z^2]$, in which F and F_0 are the quenched fluorescence and the fluorescence at zero quencher concentration, respectively. The other variables in this equation are as follows: C is the molar ratio of spin-labeled lipid, R_0 is the critical quenching radius, X is the minimum closest allowed lateral approach, and Z is the vertical distance between the fluorophore and the quencher. Values for R_0, X, and Z were taken directly from the literature.[32]

Evaluation of the data shows that the distance from the fluorescent headgroup to the midplane of the bilayer is 14 Å, which suggests a headgroup separation of 28 Å. This is very encouraging because it indicates that the headgroups are near the boundaries of the membrane's insulator regime (~30 Å). Certainly, it comports with the polarity data obtained above and is inconsistent with the headgroups being buried within the bilayer.

Fluorescent channel **2** can be used in conjunction with a closely related channel compound (**3**) in which the dansyl headgroups are replaced by *N*-methylindolyl groups (**3** = $MeIndCH_2CH_2\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle CH_2CH_2IndMe$). Methylndolyl channel **3** absorbs energy at 283 nm and emits at 343 nm. Excitation of **2** occurs at about this wavelength and fluorescence occurs (see graph above) at 516 nm in a phospholipid bilayer (Figure 7). By varying the concentrations of **2** and **3** so that [**2**]+[**3**] = 1 molal, one can evaluate the aggregation state. In this experiment, a plot of $\log F/F_0$ versus log mole fraction of **3** should give a straight line, the slope of which corresponds to the aggregation state. In this case the slope is 1.12, which suggests that the channel functions as a monomer rather than an aggregate, at least for channels **2** and **3**.

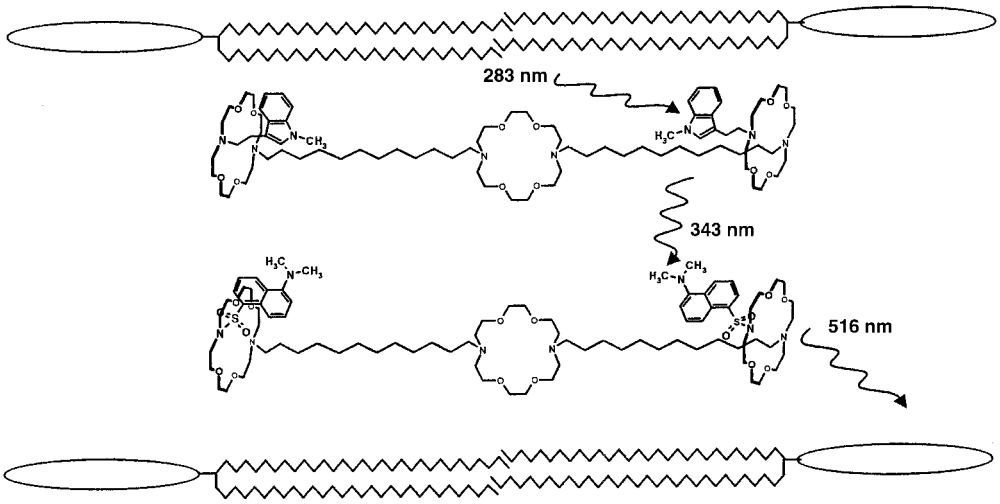

Figure 7. Fluorescence energy transfer from the indolyl-side-chained channel **3** to the dansyl channel **2**.

Other applications of basic physical organic chemistry to the synthetic channel problem: In other studies, we applied traditional physical organic chemistry in an effort to confirm the conformation and function of the channel compounds. We briefly describe two such endeavors here.

First, we[20] and others[33] have speculated that the indole residue of tryptophan (Trp, W) may play a role as a membrane anchor. We thus incorporated indole into the channel compound $InCH_2CH_2\langle N18N\rangle C_{12}\langle N18N\rangle C_{12}\langle N18N\rangle CH_2CH_2In$ (**4**). Remarkably, no Na^+ transport was detected by the ^{23}Na NMR spectroscopic method. Both CPK molecular models and Monte Carlo simulations suggested that hydrogen-bond formation between a macroring oxygen atom and the indole NH could block the "portal" and prohibit channel function. Infrared spectroscopic studies confirmed the presence of an intramolecular hydrogen bond.[34] When **4** was methylated to form **5**, function was fully restored.

Second, we reasoned that if such a modest interaction as a hydrogen bond could obviate function, we might be able to detect passage of the ion through the portal by the classical Hammett analysis. Thus, we prepared three channel compounds of the type $Z\text{-}C_6H_4\text{-}CH_2\langle N18N\rangle C_{12}\langle N18N\rangle\text{-}C_{12}\langle N18N\rangle CH_2\text{-}C_6H_4\text{-}Z$. The *para* substituent Z was varied from NO_2 to H to OCH_3. The transport rates for Na^+ was studied in phospholipid liposomes. A plot of the rates vs. σ^+ gave a straight line with a negative slope.[35] The slope was about half that observed for the related crown ethers[36] as expected for a transient interaction. Although the graph comprises only three points, the results are compelling.

Conclusion

Many compounds can be envisioned that are (or that can adopt) tubular in shape and 30 – 50 Å long. The two key issues, however, are to synthesize the molecules in an economical way and to assess their efficacy. A complex total synthesis of a compound that fails to function is a poor use of time and skill. The demonstration of efficacy should, ideally, be in a phospholipid bilayer (liposomes, planar bilayer, or patch). A compound designed to mimic channel function should transport alkali metal ions at a significant rate. The transport of Co^{2+} is not biologically relevant, and transport equilibration rates of hours rather than milliseconds are also of marginal interest. Once these conditions have been met, a variety of physical organic and spectroscopic tests can be used to assess location, conformation, and function.

Acknowledgement

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