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N,N-Didansyl-4,13-diaza-18-Crown-6: A Fluorescence-sensitive, Weakly Complexing Macrocycle Used to Probe the Phospholipid Vesicle Environment

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N,N-Didansyl-4,13-diaza-18-Crown-6: A Fluorescence-sensitive, Weakly Complexing Macrocycle Used to Probe the Phospholipid Vesicle Environment

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_N,N_-Didansyl-4,13-diaza-18-crown-6 has been prepared and characterized by standard chemical techniques. The fluorescence emission of the dansyl sidearms are sensitive to the environment and the macrocycle has been used to probe the interior of a phospholipid bilayer. An experiment to probe the environment experienced by metal cation complexes of this macrocycle failed, apparently due to its extremely low cation binding strength. This macrocycle was also ineffective in transporting Na^+ through a bulk $CHCl_3$ membrane. A solid state structure of the free macrocycle suggests why binding is so poor for this compound.

Keywords: Cation binding, cation transport, crown ether, fluorescence, lariat ether, x-ray structure

INTRODUCTION

During recent years, the study of crown ethers [1] as probes of physical processes has proliferated at a remarkable rate. In particular, the ability of crowns to transport ions through various membranes has been extensively explored [2]. By far, this work has been directed predominantly to synthetic membranes such as bulk chloroform or dichloromethane. Examples of transport in phospholipid bilayers that involve ionophores have also been reported but are far less common [3].

Fluorescent probes have been used extensively in biological systems for some time but their application as sensors in host-guest chemistry has been relatively limited. De Silva [4], Czarnik [5], and others [6] have championed this approach. In this connection, several recent papers have appeared that were designed for use either as a fluorescent probe [7] or as part of a photo-switchable crown system [8]. Our particular interest was to develop a fluorescence-sensitive ionophore that could be used to probe the chemical environment experienced by a host and/or by a host-guest complex during transport through a

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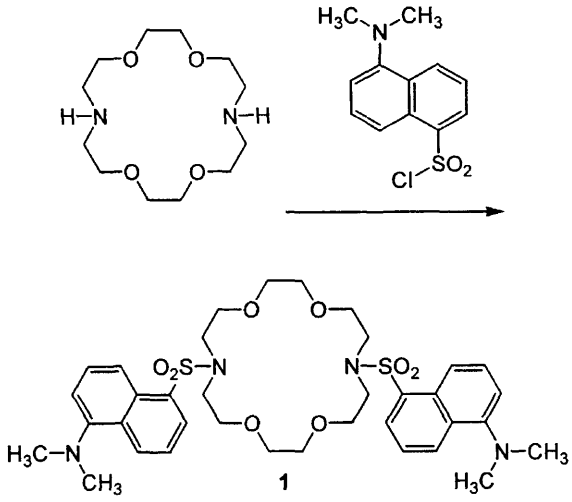
phospholipid bilayer. In previous studies, we demonstrated that lipophilic lariat ether compounds could effectively transport cations through either a bulk organic membrane [9] or in phospholipid liposomes [10].

Such fluorescent polyaromatic hydrocarbons as pyrene [7] and especially anthracene have been used recently in macrocyclic probe systems [8, 11]. We favored the use of the dansyl residue as the flourescent probe for a number of reasons. It has been used extensively as a label for proteins. Dansyl chloride is commercially available and can be readily attached to the nitrogen of macrocycles. The dansyl residue has a fluorescent lifetime of about 10 ns and the emission spectrum is known to be sensitive to its environment [12]. Finally, the nitrogen electron pairs should be unavailable for photoelectron transfer [11]. We thus settled on the use of *N,N*-didansyldiaza-18-crown-6 (**1**) as our probe molecule. We report here our efforts to assess the environment experienced by **1** and the unusual properties of this macrocycle.



RESULTS AND DISCUSSION

Synthesis of 1

Dansyl-lariat **1** was prepared by alkylation of 4,13-diaza-18-crown-6 [13]. Compound **1** was obtained by heating the diaza macrocycle with dansyl chloride at reflux for 24 h in acetonitrile: triethylamine 10:1 (v/v). Compound **1**, the didansyl crown, was obtained after crystallization from CH_2Cl_2: hexanes in 41% yield as a shiny, light green solid, mp 164–166°C.

Solid State Structure of 1

The solid state structure of *N,N'*-didansyl-4,13-diaza-18-crown-6 was obtained from a single crystal grown in Et_2O:CH_2Cl_2 solution (see experimental section). The structure obtained was refined to $R = 0.0491$ (R_{int} 0.0345) and is shown in Figure 1 as an ORTEP plot. The conformation of the macrocycle is more or less that expected for an uncomplexed crown ether in that methylenes on opposite sides of the ring are turned inward. In this case, the methylene groups are those adjacent to nitrogen in both cases. This is apparent in panel a of Figure 2. The sidearms are nearly parallel to each other and approximately perpendicular to the mean plane of the crown macroring.

Unfortunately, all efforts to grow crystals of the Li^+, Na^+, K^+, or Cs^+ complex of **1**, using a variety of counteranions, failed. This may be due to the surprising lack of cation binding discussed below.

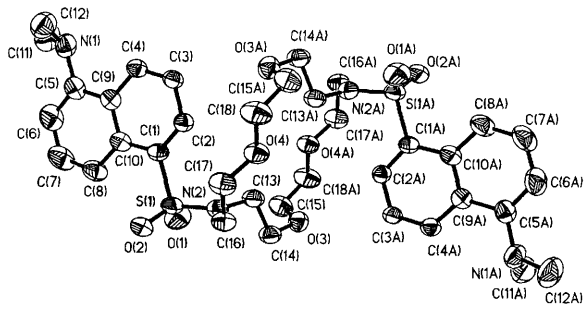


FIGURE 1 ORTEP plot of *N,N'*-didansyl-4,13-diaza-18-crown-6, **1**.

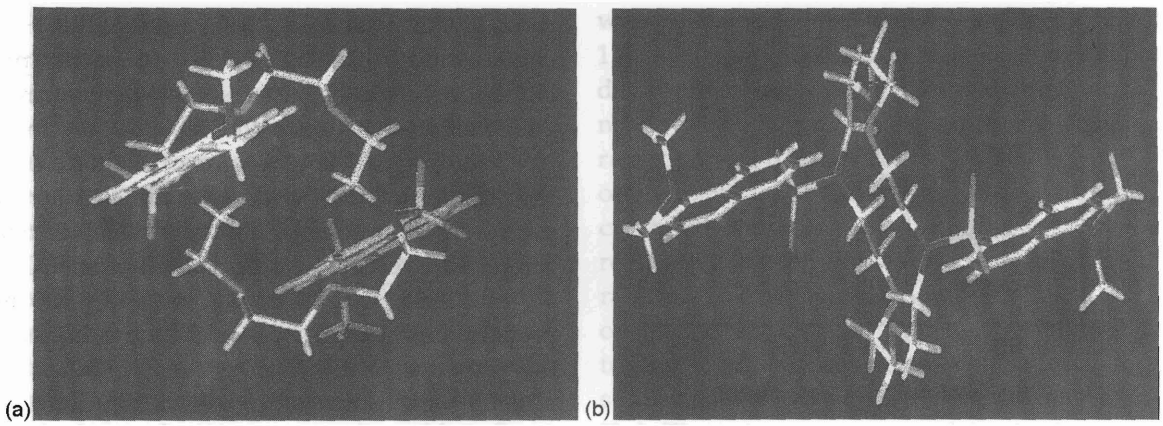


FIGURE 2 (a) Solid state structure of **1** viewed perpendicular to the mean plane of the macrocycle. (b) Solid state structures of **1** viewed parallel to the mean plane of the macrocycle. (See Color Plate I at the end of this issue).

A problem with this compound as a potential cation binder is that both macroring nitrogen atoms are formally positive because of the adjacent sulfonyl residues. Rotation of the two methylene groups out of the macroring will make available a cation binding pocket but unless this is augmented by additional donors, the crown will have only four oxygen donors available for cation complexation. This is apparent from a comparison of Na^+ binding constants determined in anhydrous methanol solution. for the three simplest crowns 12-crown-4, 15-crown-5, and 18-crown-6, the binding constant (K_s) increases as follows: 50, 1740 and 22,400 [14]. We thus anticipate poor cation binding for Na^+ if only the four macroring donors are available. The solid state structure suggests that even if the two methylenes rotated outward to vacate the macroring, the sulfonyl donors would remain conformationally unavailable for complexation. A CPK molecular model, prepared prior to synthesis of **1**, suggested that Na^+ could be accommodated by six oxygens provided by the macroring and one sidearm. This is apparently not the case.

We thought that it would be useful to undertake either a Monte Carlo conformational search or a molecular dynamics simulation of didansyl crown **1** so that the calculated structure could be compared to the solid state result. This effort was thwarted by the fact that neither Sybyl v. 6.2 or Macromodel v. 4.5 includes parameters for a sulfonamide bond. According to a recent report, efforts are underway to develop appropriate parameters by using *ab initio* methods [15] but as yet, these are unavailable.

Fluorescence Sensitivity of 1

Because of the presence of the dansyl residues, it was anticipated that the fluorescence emission spectrum of **1** would be solvent dependent. This was assessed for 1.0 μM **1** in a variety of solvents by irradiating at 340 nm and observing the emission spectrum between 360 – 650 nm. In each case, background fluorescence was determined and the value for each blank subtracted from spectrum containing **1**.

It was found that both the fluorescence intensity (data not shown) and the wavelength varied with solvent polarity. The solvent dependence of the fluorescence emission wavelength is presented graphically in Figure 3. Each data point represents three independent determinations. In most cases, the values were so similar that the error bars are not apparent as they are

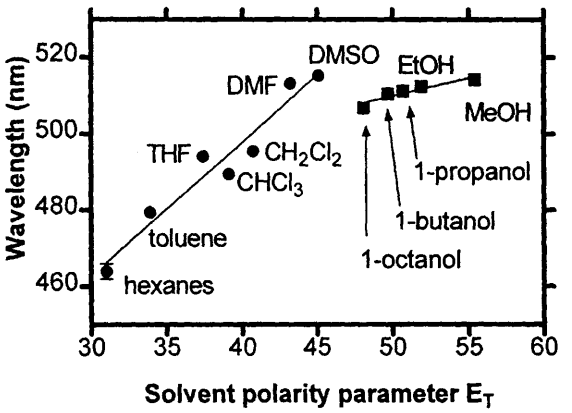


FIGURE 3 Fluorescence emission wavelengths determined for **1** in alcohols and various aprotic solvents.

for the case of hexanes. The correlation coefficient for the aprotic solvents (closed cricles) was $R^2 = 0.94$ (7 points) and the slope of the line was 3.5.

The correlation observed for the alcohols was poorer ($R^2 = 0.86$ for 5 points) than in the aprotic case and the slope of the line more shallow (0.9). The poorer quality of the fit may not be apparent in the alcohol case because the polarity range is relatively small.

A goal of the present study was to determine the polarity of the environment experienced by the host molecule within the bilayer. This was accomplished with somewhat greater difficulty than anticipated. First, the commercial lecithin preparations that we have used in channel studies proved to have significant fluorescence emission. This was surprising since, nominally at least, there is no fluorescent residue present in the membrane components. Although not identified, the fluorescent component was readily observed under UV irradiation during thin layer chromatography of the mixture. Ultimately, the difficulty was resolved by using pure dioleoyl-phosphatidylcholine (DOPC) [16].

Although the use of a pure amphiphile eliminated the fluorescence problem, formation of vesicles from this system proved to be difficult. In particular, the use of PIPES in combination with Bu_4NOH led to partial precipitation of the aggregates that were formed. An attempt to stabilize the vesicles was made by including 10% by weight of cholesterol in the mixture. This did not ameliorate the precipitation problem. Ultimately, stable, nonfluorescent vesicles were prepared from DOPC by the lipid hydration method [17]. In this preparation, HEPES was used as the buffer in the presence of dilute EDTA and pH 7.2. The vesicles were prepared by sonication and then filtered (1 μm polycarbonate). Laser light scattering showed that the average vesicular diameters were in the range 1600–1880 Å (see experimental section).

Compound **1** was added to the vesicle suspension from a 1.0 mM stock solution in trifluoroethanol. The final concentration of **1** in the vesicle suspension is 1.0 μM. The sample was vortexed to ensure even mixing and then incubated at room temperature for 30 min to allow **1** to be completely incorporated and to reach equilibrium within the bilayer. The emission spectrum of **1** (1 μM) was then determined in this vesicle system. The spectrum of the vesicles in the absence of **1** was also determined and background emission was subtracted. The average maximum of the emission wavelength was 510.8 ± 0.7 nm.

The interpretation of this λ_{max} value presents an interesting dilemma. A horizontal line projected from the ordinate of Figure 3 intersects both the alcohol and the aprotics lines. The points of intersection are E_T values of 43 on the aprotics line and 50 on the alcohol line. Which of these observations is more directly relevant to the environment experienced by the dansyl crown in DOPC vesicles is therefore an interesting question.

Let us consider the alcohol solvents. We think that the combination of hydrophobic organic chains and hydroxyl groups better represents the environmental possibilities present in a natural phospholipid bilayer than do the apro-

tics shown in Figure 3. The environment experienced by **1** in a bilayer is most similar to 1-butanol (510.5 ± 0.4 nm) and 1-propanol (511.3 ± 0.2 nm). These values correspond to the environmental assessment reported by Zachariasse *et al.* [18] using pyridinium *N*-phenolbetaine ("Reichardt's dye" [19]) in a dimyristoylphosphatidylcholine bilayer. Their focus was primarily on micellar systems and they offered no judgement about the "location" of the dye in the bilayer. We presume that didansyl crown **1** diffuses throughout the bilayer and the medium-polarity butanol environment reasonably reflects that average. Unfortunately, our original goal of assessing the environment experienced by a $Na^+ \bullet 1$ host-guest complex was thwarted by the poor complexing and transport ability of **1** as discussed in detail below.

Attempted Cation Transport by 1

The effort to assess the polarity of the environment experienced by a $1 \bullet M^+$ complex was predicated on the expectation that **1** would bind and transport cations. First, we attempted to transport sodium picrate in the fashion we had previously reported [20]. The technique involves the use of $CHCl_3$ as the bulk organic membrane and water as the source and receiving phases. The source phase contained 1 mM picric acid and 100 mM NaOH in distilled, deionized H_2O. The concentric tube membrane system was created by suspending a glass tube (10 mm i.d.) in a 20 mL beaker within 5 mm of the beaker floor. The $CHCl_3$ solution was stirred at 100 rpm using a 7 mm Teflon-coated magnetic stirrer driven by a four-site synchronous motor at ambient temperature. Transport of each cation was assumed to be accompanied by a picrate anion. The amount of the latter present was assessed by UV analysis using the 354 nm peak.

The efficacy of the transport system was tested by using *N,N'*-dibenzyldiaza-18-crown-6 as a control compound. Transport of sodium picrate was observed to occur at a rate of 1.33×10^{-6} mol/h. This modest rate was reproducible in more than a dozen experiments. In nearly 30 experimental attempts, the transport rate for **1** was found to be only 5.4×10^{-8} mol/h, or about 5% that observed for dibenzyldiaza-18-crown-6. The study of **1** in this system was replicated so many times precisely because the rate was so unexpectedly low. This value can be compared to 3.3×10^{-9} mol $\bullet$ h^{-1}, the rate for transport of sodium picrate determined under similar conditions in the absence of any carrier [9a]. The rates are compared in the bar graph shown in Figure 4.

One concern was that the dansyl residues of **1** are UV-active. The UV maxima observed for picrate ($\lambda_{max} = 354$ nm) and dansyl ($\lambda_{max} = 340$ nm) are similar enough that misinterpretation of the results might have occurred. We thus undertook a simple extraction experiment and used NMR to assess the extraction ability of the two crowns. Thus, picric acid in base was dissolved in H_2O (see experimental section) and contacted by an equal volume of a solution containing either **1** or **2** in $CDCl_3$. The progress of the extraction was monitored (^{1}H-NMR) by integration of the picrate proton ($\delta = 8.83$ ppm). The results are shown in Figure 5.

During the course of spinning the sample tube, picrate (presumably as the sodium salt) was extracted into the $CHCl_3$ phase by dibenzyldiaza-18-crown-6 but not by its didansyl

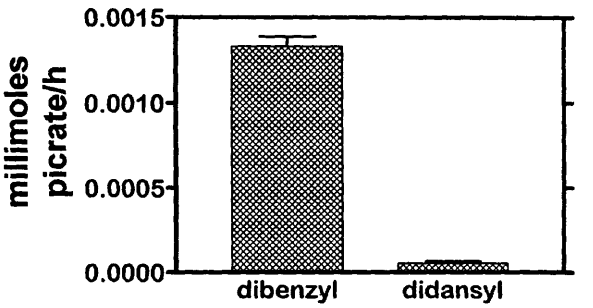


FIGURE 4 Sodium picrate transport rates ($H_2O/CHCl_3/H_2O$) for **1** and **2** (top error bar apparent in both cases).

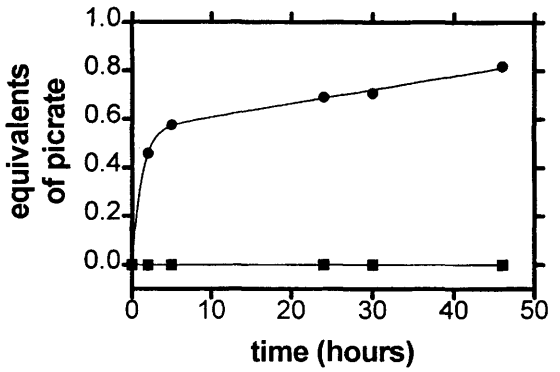


FIGURE 5 Sodium picrate extraction by **1** and **2** determined by ^{1}H-NMR in $D_2O/CDCl_3$.

counterpart (**1**). In the latter case, no evidence at all was observed for the extraction of picrate even after two days. The attempted extraction of potassium picrate by **1** also failed. Extraction of sodium picrate by dibenzyldiaza-18-crown-6 was more or less as expected. Although a maximum value of about 80% of theory is shown in Figure 5, the extraction was still slowly increasing after more than 40 hours. Presumably, this value would increase until all of the crown was ion-paired with salt but the experiment was discontinued as shown.

Cation Binding Studies of 1

In previous studies, we showed that there was a reasonable correlation between cation • picrate transport rate in a $CHCl_3$:H_2O system and the cation binding constant determined either by picrate extraction or by ion selective electrode (ISE) methods in homogeneous solution. The poor transport rate observed for 1 is confirmed by the lack of picrate extraction in the NMR experiment described above. We attempted, however, to further confirm this surprising lack of cation binding affinity by the ISE technique in anhydrous methanol solution. The effort was complicated by limited solubility of the macrocycle in methanol but the binding constant appeared to be at or near the lower limit of detection by using this method.

CONCLUSION

We report the preparation, characterization, and solid state structure of *N,N'*-didansyldiaza-18-crown-6, **1**. This compound has two, fluorescence-active sidearms that have been used to probe the environment it experiences when within a phospholipid bilayer. Remarkably, Na^+ and K^+ picrate extraction and transport experiments with **1** were unavailing despite numerous attempts under different conditions. Homogeneous binding constant measurements for Na^+ confirmed that **1** has a negligible binding constant in anhydrous methanol solution. This may be due to a conformation that is unfavourable for cation binding possibly as a result of the rigidifying effect of the sulfonyl groups. Why cation complexation involving the four macroring oxygens and some or all of the four sulfonyl oxygen atoms is not more favorable remains unclear. The relatively low solubility of this compound may also contribute to its lack of efficacy in binding and transport. Alkali metal binding by some of the other bibracchial lariat ethers that have been designed to serve as fluorescent probes has not been described and the present work suggests that this should be examined.

EXPERIMENTAL SECTION

^{1}H-NMR were recorded at 300 MHz in $CDCl_3$ solvents and are reported in ppm (δ) downfield from internal $(CH_3)_4Si$. ^{13}C-NMR spectra were recorded at proportional frequencies as noted above. Infrared spectra were recorded on a Perkin-Elmer 1710 Fourier Transform Infrared Spectrophotometer and were calibrated against the 1601 cm^{-1} band of polystyrene. Melting points were determined on a Thomas Hoover

apparatus in open capillaries and are uncorrected. Thin layer chromatographic (TLC) analyses were performed on aluminium oxide 60 F-254 neutral (Type E) with a 0.2 mm layer thickness or on silica gel 60 F-254 with a 0.2 mm layer thickness. Preparative chromatography columns were packed with activated aluminum oxide (MCB 80–325 mesh, chromatographic grade, AX 611) or with Kieselgel 60 (70–230 mesh).

All reactions were conducted under dry N_2 unless otherwise stated. All reagents were the best (non-LC) grade commercially available and were distilled, recrystallized, or used without further purification, as appropriate. Combustion analyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and are reported as percents.

Synthesis of *N,N'*-(didansyl)diaza-18-crown-6

(This preparation was designed to obtain a mixture of the mono- and the disubstituted crown, **1**.) Diaza-18-crown-6 (2.0 g, 7.6 mmol) and dansyl chloride (2.0 g, 7.4 mmol) were dissolved in 100 mL CH_3CN and 10 mL Et_3N. The reaction was heated to reflux temperature for 24 h. Column chromatography (alumina, 3%MeOH/CH_2Cl_2) afforded two products. The monosubstituted compound was obtained as a yellow oil (0.78 g, 21% yield). The desired, disubstituted compound was obtained as a pale yellow solid. This solid was dissolved in refluxing CH_2Cl_2. Hexane was added until the solution became slightly cloudy. The suspension was cooled in a freezer. Filtration of the resulting crystals afforded a shiny light green solid (2.27 g, 41% yield), mp 164–166°C. ^{1}H-NMR ($CDCl_3$): 2.85 (s, NCH_3, 12H), 3.46(m, OCH_2CH_2O, 8H), 3.57 (m, S-NCH_2CH_2O and NCH_2CH_2O, 56H), 7.14 (d, dansyl, 2H), 7.49 (m, dansyl, 4H), 8.11 (d, dansyl, 2H), 8.27 (d, dansyl, 2H), 8.49 (d, dansyl, 2H). *Anal.* calcd for $C_{36}H_{48}N_4O_8S_2$: C, 59.32%; H, 6.64%; Found: C, 59.26%; H, 6.6%.

***N,N'*-(Dibenzyl)diaza-18-crown-6,2,** was prepared as previously described [21].

Solid State Structure Determination for 1

X-ray quality crystals were obtained by suspending *N,N'*-dansyldiaza-18-crown-6 in hot diethyl ether and adding CH_2Cl_2 dropwise until all was dissolved, followed by slow crystallization at room temperature. A crystal was mounted on a glass fiber and diffraction was measured at room temperature by using a Siemens P4 X-ray spectrometer by ω-2θ scans, $3.5° < \theta < 113.5°$. The crystal data obtained for $C_{36}H_{48}N_4O_8S_2$, MW. 728.92 g/mol, space group P2(1)/n, $a = 9.1727(16)$ Å, $b = 22.2501(39)$ Å, $c = 9.9149(20)$ Å, $Z = 2$, $\alpha = 90.000(0)°$, $\beta = 115.763(16)°$, $\gamma = 90.000(0)°$, CuKα ($\mu = 1.79\ mm^{-1}$), $R = 0.0491$ ($R_{int} = 0.0345$) for 2253 unique reflections with $F(o) > 4\sigma F(o)$ of 2424 unique data.

Fluorescence Behavior of 1

All solvents used in the fluorescence study were HPLC grade (Aldrich) and used without further purification. The purity of dioleoylphosphatidylcholine (DOPC, Sigma) was assessed by TLC (silica, $CHCl_3$/MeOH/H_2O 9:0.9:0.1 (v/v/v)). Examination of the TLC plate under a UV lamp at 365 nm and 254 nm revealed the absence of any UV-active or fluorescent impurities in the lipid. The plate was developed by dipping in 10% phosphomolybdic acid in EtOH (w/v) followed by heating to reveal a single spot ($R_f = 0.31$).

Preparation of Lipid Vesicles

Vesicles were prepared from DOPC by the lipid hydration method. DOPC (2.0 mg) was weighed into a 15 mL Corex test tube. The lipid was dissolved in approximately 2 mL of $CHCl_3$, and then the solvent was removed by rotary evaporation to leave an even film of lipid on the inner wall of the test tube. The lipid was dried under high vacuum for 45 min. to remove additional solvent. After drying, the sample was diluted to 10 mL with 10 mM HEPES,

0.1 mM EDTA (pH 7.2) buffer and sonicated for 20 min with a tip sonicator (Branson sonifier 450). The vesicle preparation was allowed to cool, it was filtered (1.0 μm polycarbonate filter), and then characterized by dynamic light scattering (Coulter N4MD). Vesicles prepared by this method have diameters of 1560 ± 590 Å (unimodal) and, by SDP intensity, 1770 ± 720 Å.

Fluorescence Measurements

Fluorescence measurements were made using a Perkin-Elmer LS-50B luminescence spectrophotometer. All *N,N'*-didansyldiaza-18-crown-6 solutions were excited at 340 nm and the emission spectra were recorded from 360 to 650 nm.

Fluorescence of N,N'-didansyldiaza-18-crown-6 in a series of solvents. The emission spectrum of *N,N'*-didansyldiaza-18-crown-6 was determined in seven aprotic solvents (hexanes, toluene, THF, $CHCl_3$, CH_2Cl_2, DMF, and DMSO) and five protic solvents (1-octanol, 1-butanol, 1-propanol, ethanol, and methanol). Samples were prepared by adding 10 μL of a 1.0 mM *N,N'*-didansyldiaza-18-crown-6 in $CHCl_3$ to a 10 mL volumetric flask. The flask was purged with dry N_2 and then diluted to volume with solvent to give a final concentration of *N,N'*-didansyldiaza-18-crown-6 of 1.0 μM. For each solvent, the solutions were made in triplicate and then the emission spectrum was recorded for each. Background fluorescence of each solvent was determined by recording the emission spectrum of a blank. For each solvent the fluorescence in the region of interest was small but it was subtracted from the solution spectra.

Fluorescence of N,N'-didansyldiaza-18-crown-6 in DOPC Vesicles. Three 2.4975 mL aliquots of the 10 mL vesicle preparation (see above) were added to three vials. Next, 2.5 μL of 1.0 mM *N,N'*-didansyldiaza-18-crown-6 in CF_3CH_2OH was added to each vial to give a final volume of 2.5 mL and a final concentration of 1.0 μM. The samples were vortexed, incubated at room temperature for 30 min, and the emission spectra recorded. The emission spectrum of the DOPC vesicles was also recorded and subtracted from the sample spectrum in each case.

The size of the vesicles was measured after the fluorescence measurements were obtained. Vesicle size was found to be identical before and after addition of the fluorophore.

Bulk Membrane Transport Study

The concentric tube method was as previously described [20]. In brief, a $CHCl_3$ layer separated two aqueous phases. The source phase consisted of a 1 mM solution of sodium picrate which was prepared by dissolving picric acid in 0.100 M NaOH. The receiving phase was initially distilled, deionized water. A layer of 6.0 mL of $CHCl_3$ (1.0 mM in ionophore) was stirred (Magne-4 synchronous stirrer, 100 rpm) using a 7 mm stir bar. The 10 mm (i.d.) glass tube separated the source (1 mL) and receiving (6 mL) phases in a 20 mL beaker. The transport rate of sodium picrate was calculated from the picrate concentration in the receiving phase. The picrate concentration was determined by measuring the absorbance ($\lambda = 354$ nm) of a diluted 250 μL aliquot from the aqueous receiving phase. UV-visible spectra were recorded on a Beckman DU-8 spectrophotometer.

^{1}H-NMR Sodium Picrate Extraction Experiment

The NMR-monitored extraction of sodium picrate was performed by suspending 0.5 mL of an aqueous phase containing sodium picrate above an equal volume of a chloroform solution of the ionophore. The sample tube was positioned in the spinner such that only the chloroform layer would be in the range of the receiver coil. The extent of extraction was determined by comparing the peak height of the 8.83 ppm singlet from picrate to the 4.89 ppm singlet of iodoform, which was added as an internal standard at a concentration of 100 mM.

The chloroform phase was a 50 mg/mL (85 mM) solution of *N,N'*-didansyldiaza-18-crown-6 prepared in $CDCl_3$. An identical experiment was done with 85 mM *N,N'*-dibenzyldiaza-18-crown-6 for comparison. The aqueous phase (120 mM in picrate) was prepared from picric acid and 200 mM aqueous NaOH.

Binding Constant Determination

Cation binding constants were measured in absolute CH_3OH at 25.0 ± 1.0°C using a Ross Model 8411 electrode and an Orion model 701A "ionalyzer" meter according to the method developed originally by Frensdorff and described in detail by us [22]. The reference electrode was a Model 476370 Corning double junction electrode. Values for the equilibrium constants are reported as log K_S. The electrodes were checked for Nerstian response prior to use and the method was verified by determining the binding constant for **2**, which was within experimental error to the value determined previously.

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