Structure/Activity Study of Tris(2-aminoethyl)amine-Derived Translocases for Phosphatidylcholine

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Sulfonamide and amide derivatives of tris(aminoethyl)amine (TREN) are known to facilitate phospholipid translocation across vesicle and erythrocyte membranes; that is, they act as synthetic translocases. In this report, a number of new TREN-based translocases are evaluated for their abilities to bind phosphatidylcholine and translocate a fluorescent phosphatidylcholine probe. Association constants were determined from ^{1}H NMR titration experiments, and translocation half-lives were determined via 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)/dithionite quenching assays. A rough correlation exists between translocase/phosphatidylcholine association constants and translocation half-lives. The tris-sulfonamide translocases are superior to the tris-amide versions because they associate more strongly with the phospholipid headgroup. The stronger association is due to the increased acidity of the sulfonamide NHs as well as a molecular geometry (as shown by X-ray crystallography) that is able to form tridentate complexes with one of the phosphate oxygens. Two fluorescent translocase analogues were synthesized and used to characterize membrane partitioning properties. The results indicate that the facilitated translocation of phospholipids by TREN-derived translocases is due to the formation of hydrogen-bonded complexes with the phospholipid headgroups. In the case of zwitterionic phosphatidylcholine, it is the neutral form of the translocases that rapidly associates with the phosphate portion of the phosphocholine headgroup. Complexation masks the headgroup polarity and promotes diffusion of the phospholipid−translocase complex across the lipophilic interior of the membrane.

Introduction

Although the metal coordination chemistry of tris(2-aminoethyl)amine (TREN) derivatives has been studied extensively,[1] less is known about their anion recognition properties.[2] Reinhoudt and co-workers were the first to characterize anion receptors based on the TREN scaffold.[3] In 1993, they reported that TREN tris-sulfonamides and tris-amides have a binding preference in organic solvents of $H_2PO_4^-$ > HSO_4^- > Cl^-. More recently, tris-amide receptors have been employed in combination with crown ethers as dual host systems for the enhanced extraction of $CsNO_3$ into organic solvents,[4] and in aiding the detection of nitrates by MicroITIES analysis.[5] Similarly, a tripodal tris(amido benzo-15-crown-5) conjugate has been used to extract and transport $NaTcO_4$ through a liquid organic membrane.[6] TREN tris-urea and tris-thiourea derivatives have also been synthesized and shown to bind a range of anions in highly polar organic solvents.[7,8]

Our research group has examined a number of TREN derivatives as synthetic receptors for phospholipid headgroups. In particular, we have previously reported that compounds **1** and **2** facilitate the translocation or "flip-flop" of fluorescent phosphatidylcholine probes across the membranes of surface differentiated vesicles and red blood cells (Figure 1).[9,10] We have proposed that these synthetic translocases form hydrogen-bonded complexes with the phosphate portion of the phosphocholine headgroup, which decreases headgroup polarity and promotes diffusion across the lipophilic interior of the bilayer membrane (Figure 2). With this contribution we provide

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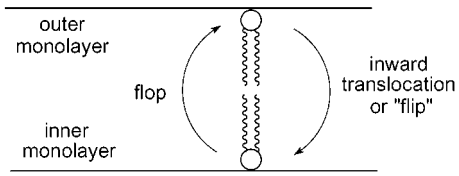


Figure 1. Phospholipid translocation or flip-flop.

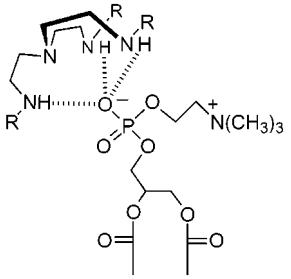
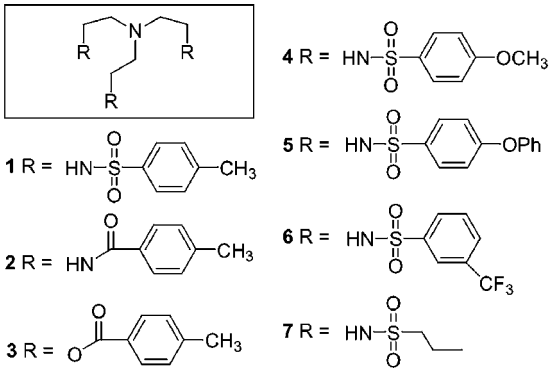
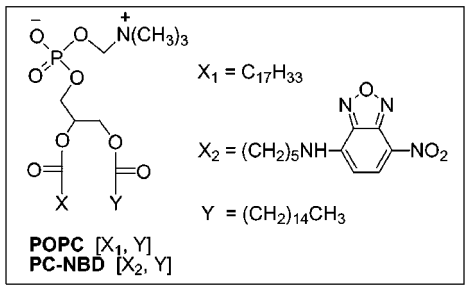


Figure 2. Proposed supramolecular complex between TREN-sulfonamide translocases and the phosphocholine headgroup.

structure/function data that further support our hypothesis. Specifically, we describe the synthesis and structure of a series of related TREN derivatives and correlate the structural information with phosphatidylcholine binding constants, membrane partitioning values, and phospholipid translocation rates. Although the focus of this report is on phosphatidylcholine recognition, our results help explain the more general anion-binding properties of TREN-derived receptors. In particular, a comparison of the X-ray crystal structures of sulfonamide **1** and amide **2** shows why the sulfonamide **1** is a much better anion binder.

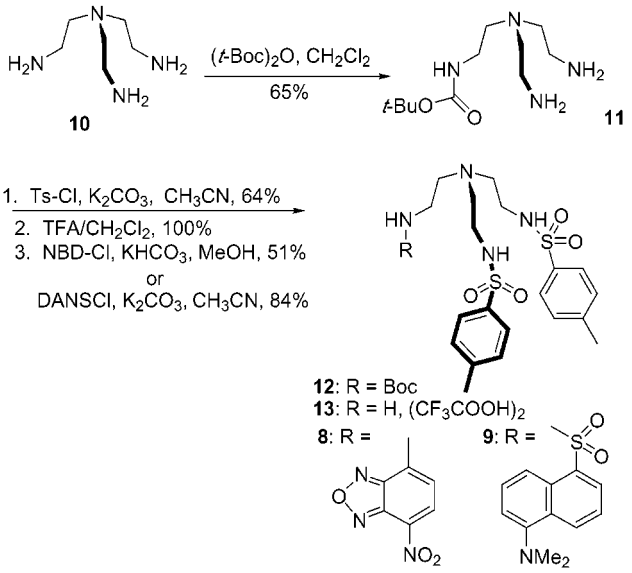




Results and Discussion

Synthesis of Receptors 1−9. Compounds **1**, **2**, and **4**−**6** were prepared in 45−70% yield by reacting TREN (**10**), with a slight excess of the corresponding aryl sulfonyl or acyl chloride. The yield for tris(propylsulfonamide) **7** was substantially lower (7%), although this reaction was only run once. Compound **3** was synthesized (66% yield) by reacting triethanolamine with a slight excess of the corresponding acyl chloride. The fluorescent derivatives **8** and **9** were prepared in a convergent manner (Scheme 1). Treatment of TREN (**10**, 10 molar equiv) with di-*tert*-butyl dicarbonate (1 molar equiv) at −78 °C in CH_2Cl_2 under high-dilution conditions,[11] followed by warming to room temperature and stirring overnight, gave the desired mono-Boc-protected TREN derivative **11** in 65% yield after careful chromatography.[1d,12] Subsequent treatment of **11** with toluenesulfonyl chloride gave **12**, which was deprotected with 50% TFA to give **13** as its bis-trifluoroacetate salt. Compound **13** was coupled with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to give **8** or with 5-(dimethylamino)-1-naphthalenesulfonyl chloride[1b] (DANS-Cl) to give **9**.

Scheme 1



Phosphatidylcholine Binding in $CDCl_3$. The ability of compounds **1**−**7** to complex with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in $CDCl_3$ at 25 °C was evaluated using ^{1}H NMR spectroscopy. Titration isotherms were generated by adding aliquots of POPC to solutions of the corresponding receptor, and association constants were extracted by fitting the curves to a 1:1 binding model using iterative computer methods.[13] The validity of the 1:1 binding model was confirmed in the case of **1** with a Job plot (data not shown). As discussed previously,[10] the complexed-induced shifts (along with the X-ray structural data described below) are consistent with the hydrogen-bonded complex shown in Figure 2.

The order of observed association constants with POPC was found to be **6** [$(9.1 \pm 1.4) \times 10^3$ M^{-1}] > **5** [$(2.5 \pm 0.4) \times 10^3$ M^{-1}] ≈ **4** [$(2.2 \pm 0.3) \times 10^3$ M^{-1}] ≈ **1** [$(2.1 \pm 0.3) \times 10^3$ M^{-1}] > **7** [$(6.4 \pm 0.9) \times 10^2$ M^{-1}] > **2** [$(2.0 \pm 0.3) \times 10^1$ M^{-1}] ≫ **3** (<1 M^{-1}). Broadly speaking, the association constants correlate with the acidity of the receptor NH groups. For example, all of the aryl sulfonamides have a greater affinity than that of the propyl sulfonamide **4**,

(11) Jacobsen, A. R.; Makris, A. N.; Sayre, L. M. *J. Org. Chem.* **1987**, *52*, 2592−2594.
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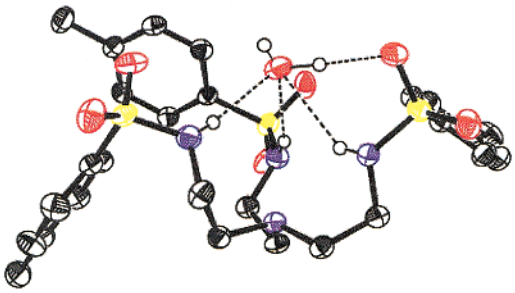
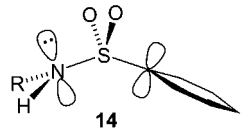


Figure 3. Crystal structure of sulfonamide **1**·H_2O with 50% ellipsoid probability. Only sulfonamide and water hydrogen atoms are shown for clarity. Dashed lines indicate hydrogen bonds.

which in turn binds better than amide **2**; ester **3** has no discernible binding ability. The higher affinity displayed by sulfonamide **6** relative to the other aryl sulfonamides can be attributed to its lower pK_a.[14] The binding constants for **1**, **4**, and **5** are within the experimental error. On the basis of acidity, a lower binding constant is predicted for **5** because the order of electron-donating ability is phenoxy > methoxy > methyl.[14] It is possible that there are additional weak interactions between the receptor and the phospholipid headgroup. For example, π-stacking of the phenoxy rings in **5** with the cationic choline portion may compensate for the weakened hydrogen bonding to the phosphate group.[15]

X-ray Crystallography.[16] The crystal structure of tris-sulfonamide **1**·H_2O is shown in Figure 3. The three secondary arylsulfonamides adopt their characteristic conformation **14**, where the nitrogen lone pair and the



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aromatic p-orbital lie in a plane that bisects the O−S−O internuclear angle.[17,18] The overall molecular geometry of **1** is roughly a C_3-symmetric tripodal arrangement with all three NHs hydrogen-bonded to a water molecule (NH···O distances are 2.43, 2.48 and 2.18 Å), which is also hydrogen-bonded to a sulfonyl oxygen in the same molecule (SO···H distance of 1.89 Å). Overall, the solid-state structure of **1** provides a clear visual picture of its "anion binding pocket" and supports the supramolecular complex proposed in Figure 2.

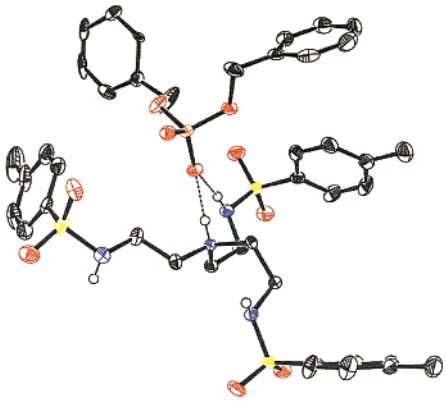

Figure 4. Crystal structure of (**1** + H)$^+$:(dibenzyl phosphate)$^-$ ionic complex with 50% ellipsoid probability. Only relevant hydrogen atoms are shown. Included $CHCl_3$ has been omitted for clarity. Dashed lines indicate hydrogen bonds.

The crystal structure of the protonated form of sulfonamide **1** complexed with dibenzyl phosphate is shown in Figure 4. Again, the secondary sulfonamides adopt the characteristic conformation **14**.[19] In this case, only one sulfonamide NH and the protonated tertiary amine are hydrogen-bonded to one of the phosphate oxygens (NH···O distances of 1.94 and 1.77 Å, respectively). While this ionic structure is not so relevant to the translocation of zwitterionic phosphatidylcholine (which we believe involves a neutral translocase/phospholipid complex), it is relevant to the facilitated translocation of anionic phospholipids such as phosphatidylglycerol,[9] which most likely requires a protonated translocase/phospholipid complex.

Acyclic secondary amides are well-known to prefer a *Z* conformation about the amide C−N bond,[20] a preference that is seen in the crystal structure of tris-amide **2** (Figure 5).[21] Because of this conformational constraint, the putative tripodal "anion binding pocket" in receptor **2** is effectively closed as a result of the steric bulk of the three aromatic rings, which are oriented edge-to-face with each other. While the amide *E*/*Z* conformational equilibrium can be altered by noncovalent interactions,[22] it is an energetically costly process.[20] Thus, it appears that aromatic TREN tris-amides such as **2** cannot bind anions in the same tridentate fashion as the analogous tris-sulfonamide **1**, a point that has not previously been noted in the literature.[3−6] We conclude that tris-amide **2** has a relatively weak affinity for phosphatidylcholine for two reasons: (1) the amide hydrogen atoms are less acidic,

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(16) X-ray data for **1**·H_2O, **2** and the [(**1** + H)$^+$:(dibenzyl phosphate)$^-$·$CHCl_3$] complex can be retrieved from the Cambridge Crystallographic Data Center using deposition numbers CCDC 175856 (**1**), CCDC 175858 (**2**), and CCDC 175857 [(**1** + H)$^+$:(dibenzyl phosphate)$^-$·$CHCl_3$].

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(18) The torsion angles Φ [C(2)−C(1)−S−N] and ψ [C(1)−S−N−H] are used to describe the conformation of a secondary arylsulfonamide skeleton,[17b] and torsion angles of $\Phi \approx 90°$ and $\psi \approx 90°$ are generally observed. For tris-sulfonamide **1**, the torsion angles are $\Phi = 97.5°$, 84.8°, 79.4° and $\psi = 94.0°$, 69.5°, 76.3°, consistent with the literature.

(19) Torsion angles:[18] $\Phi = 77.8°$, 115.8°, 79.4° and $\psi = 75.0°$, 62.9°, 53.2°.

(20) The *Z* conformation of *N*-methylacetamide is more stable than the *E* conformation by 2.1−2.5 kcal/mol. Furthermore, the free energy barrier to rotation about the OC−N bond in *N*-methylacetamide is 21.3 kcal/mol. (a) Eliel, E. E.; Wilen, S. H.; Doyle, M. P. *Basic Organic Stereochemistry*; Wiley-Interscience: New York, 2001; p 392. (b) Stewart, W. E.; Siddall, T. H. *Chem. Rev.* **1970**, *70*, 517−551.

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(22) For examples of hydrogen bonded stabilization of *E* secondary amides, see: (a) Deetz, M. J.; Fahey, J. E.; Smith, B. D. *J. Phys. Org. Chem.* **2001**, *14*, 463−467. (b) Beijer, F. H.; Sijbesma, R. P.; Vekemans, J. A. J. M.; Meijer, E. W.; Kooijman, H.; Speck, A. L. *J. Org. Chem.* **1996**, *61*, 6371−6380. (c) Perni, G. J.; Kilburn, J. D.; Essex, J. W.; Mortshire-Smith, R. J.; Rowley, M. *J. Am. Chem. Soc.* **1996**, *118*, 10220−10227. (d) Bairaktari, E.; Mierke, D. F.; Mammi, S.; Peggion, E. *J. Am. Chem. Soc.* **1990**, *112*, 5383−5384.

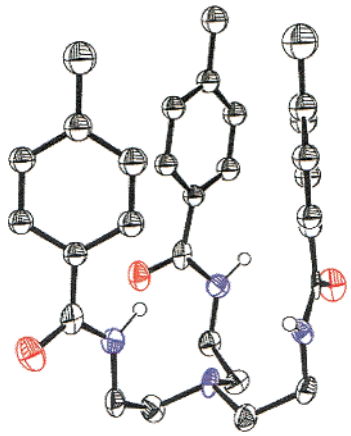

Figure 5. Crystal structure of amide **2** with 50% ellipsoid probability. Only amide hydrogen atoms are shown for clarity.

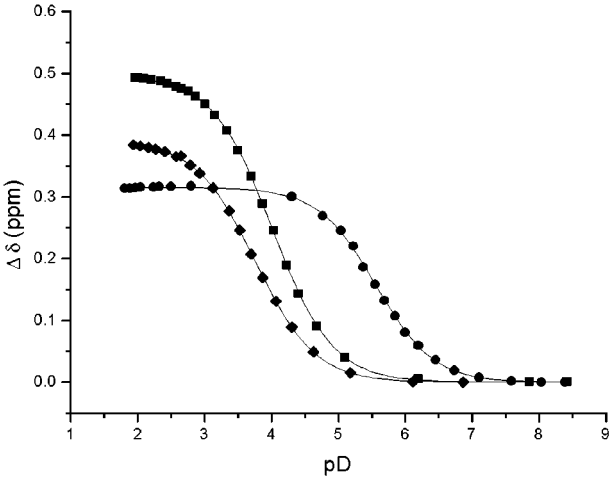

Figure 6. ^{1}H NMR titration curves for sulfonamide **1** (■, CH_2NH), amide **2** (●, CH_2NH), and ester **3** (◆, CH_2O) in 9:1 CD_3OD/D_2O.

and (2) it cannot achieve tridentate complexation with the phosphate portion of the headgroup.

pK_a Measurements. Previously, pK_a* values for the protonated forms of compounds **1**−**3** were obtained by a potentiometric method in 9:1 CH_3OH/H_2O and found to be **1** (4.4), **2** (5.7), and **3** (4.2).[10] We have now verified these measurements with ^{1}H NMR titrations in 9:1 CD_3OD/D_2O (Figure 6), and the numbers are in good agreement: **1** (4.00 ± 0.01), **2** (5.52 ± 0.02), **3** (3.75 ± 0.01). These values suggest that compounds **1**−**3** exist primarily in their free base form at neutral pH and indicate that during the phospholipid translocation assay (pH = 7.4) a smaller fraction of sulfonamide **1** is protonated, as compared to amide **2**. This is another reason why sulfonamide **1** is a better translocase of zwitterionic phosphatidylcholine than amide **2** (translocation involves a neutral translocase/phospholipid complex). It also explains why amide **2** is a better translocase of anionic phosphatidylglycerol than sulfonamide **1** (translocation involves a protonated translocase/phospholipid complex).[9]

Inward Translocation of Phospholipids. Phospholipid translocation was monitored via the well-established 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)/dithionite quenching assay, which uses a phosphatidylcholine probe containing an NBD group in one of its acyl chains (PC-NBD).[9,10,23,24] Exo-labeled vesicles, used to measure inward translocation (flip), were prepared by addition of a small aliquot of PC-NBD (0.5 mol % of total phospholipid) in ethanol to a solution of unlabeled POPC vesicles (25 mM). The PC-NBD readily inserts into the vesicle outer monolayer, and upon treatment with sodium dithionite ($Na_2S_2O_4$), the NBD fluorescence is quenched as a result of reduction of the NBD nitro group. Vesicle membranes are effectively impermeable to dithionite, therefore only PC-NBD located in the outer leaflet is chemically quenched. Lysis of the vesicles with detergent allows the dithionite to gain access to the remaining PC-NBD, and thus the percent exo PC-NBD can be computed. The inward translocation experiment starts with 100% exo PC-NBD and progresses to an equilibrium value of around 60%. The translocation half-life is the time taken to reach 80% exo PC-NBD. All translocation measurements were conducted at pH 7.4 and 25 °C with 120 nm unilamellar vesicles that were prepared by membrane extrusion.

We have already shown that sulfonamide **1** is more effective than amide **2** (and the control ester **3**) at translocating PC-NBD across surface differentiated vesicles.[9] In the absence of translocase the half-life for inward PC-NBD translocation is many hours, but in the presence of compound **1** (38 μM) the half-life is about 5 min.[9] A goal of this present study was to measure the relative abilities of translocases **1**−**7** to transport PC-NBD across the membranes of surface differentiated vesicles and to determine how the translocation correlates with the POPC binding constants. On the basis of the binding constants, 3-(trifluoromethyl)phenyl sulfonamide **6** was expected to be the most effective translocase. However, it facilitated PC-NBD translocation by approximately the same amount as tolyl sulfonamide **1** (uncertainty for the measured translocation half-lives is around ±33%). Unfortunately, sulfonamides **4** and **5** suffered from poor aqueous solubility, and reproducible translocation data could not be obtained. Propyl sulfonamide **7** is water-soluble up to 100 μM but is nonetheless ineffective at PC-NBD translocation; this behavior is likely due to its poor phosphocholine binding ability. As previously reported, amide **2** is also ineffective at PC-NBD translocation.[9] In all cases, carboxyfluorescein leakage experiments showed that the translocases do not induce vesicle leakage.

Although there is a rough correlation between translocase/phosphatidylcholine association constants and PC-NBD translocation half-lives, the trend is not exact. It appears that other factors such as membrane partitioning values, membrane diffusion constants, etc. may also be influential.

Fluorescent Translocase Analogues. In an effort to gain some insight into how the TREN sulfonamides interact with vesicle membranes, two fluorescent analogues, **8** and **9**, were prepared (Scheme 1) and used in binding and partitioning experiments. A fluorescence resonance energy transfer (FRET) assay was performed to determine how quickly mono-NBD derivative **8** (38 μM) comes in contact with the headgroup region of POPC vesicles (25 μM) containing 0.6% of the energy acceptor Rhodamine-PE (Rh-PE). The fluorescence emission pro-

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(24) (a) McIntyre, J. C.; Sleight, R. G. *Biochemistry* **1991**, *30*, 11819−11827. (b) Balch, C.; Morris, R.; Brooks, E.; Sleight, R. G. *Chem. Phys. Lipids* **1994**, *70*, 205−212.

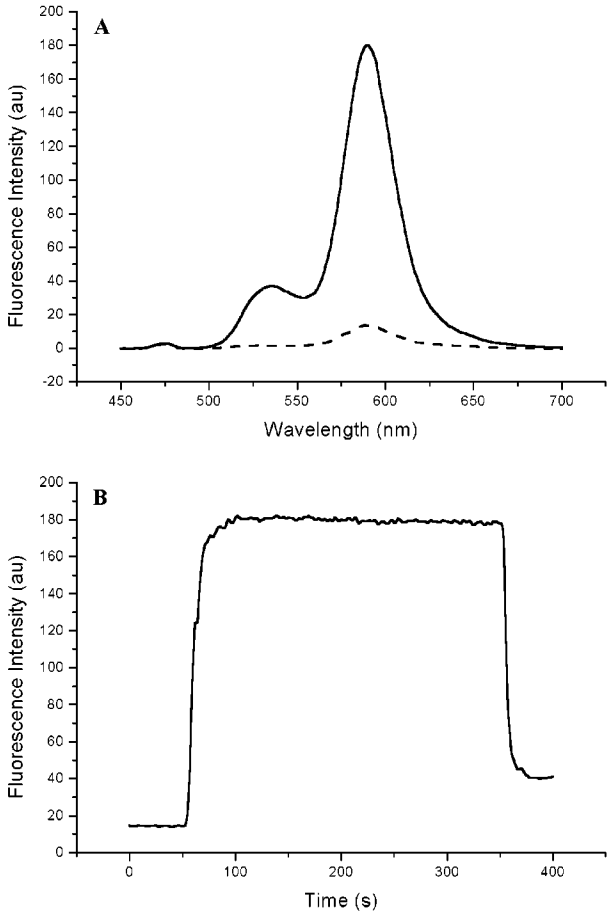

Figure 7. Fluorescence resonance energy transfer assays. (A) Fluorescence emission of POPC/Rh-PE (0.6%) vesicles (25 μM) with (solid line) or without (dashed line) addition of mono-NBD **8** (38 μM). (B) Time drive spectrum of POPC/Rh-PE (0.6%) vesicles (25 μM) with mono-NBD **8** (38 μM) added at t = 50 s and detergent added at t = 350 s.

file in Figure 7a shows that the excitation energy of the NBD chromophore in **8** (excitation 470 nm, emission 530 nm) is transferred to the Rh-PE molecules, resulting in a Rh-PE emission peak at 590 nm. Furthermore, the time-drive spectrum in Figure 7b demonstrates that **8** moves rapidly (within seconds) into close contact with the headgroup portion of the phospholipid bilayer. Disruption of the membrane by detergent addition at t = 350 s leads to immediate loss of the energy transfer.

A qualitative measure of the extent that the translocases partition across the membrane was gained by conducting dithionite quenching experiments. If mono-NBD derivative **8** binds only to the external leaflet of the bilayer or remains in the surrounding aqueous media, then treatment with dithionite will quench the fluorescence. On the other hand, if **8** diffuses into the inner leaflet of the bilayer or enters the aqueous vesicle interior, then no quenching will be observed. We find that when **8** (38 μM) is added to a solution of unlabeled POPC vesicles (25 μM), roughly 20% of **8** is partitioned inside the vesicles and protected from dithionite reduction. The fluorescence intensity for a vesicle/**8** mixture treated with dithionite gradually decreases over time (drops to 5% after 1 h), indicating that the protected probe **8** slowly diffuses out of the vesicles and into the outer aqueous environment. Control experiments with vesicles containing PC-NBD indicate that these results are not due to penetration of dithionite into the vesicles. We conclude that the initial binding of the translocases to the membrane surface is relatively rapid and that subsequent movement across the membrane, as either the free species or the translocase-phospholipid complex, is significantly slower.

The mono-dansyl sulfonamide **9** was prepared and used to gain information concerning the equilibrium position of the translocases within the membrane. The fluorescence emission wavelength of the dansyl chromophore is known to be sensitive to solvent polarity, and dansyl derivatives are widely used to obtain membrane location information.[25,26] In this case, the fluorescence emission spectra of **9** in a number of solvents (chosen to mimic the different polarities of the bilayer regions) were acquired and the observed λ_{max} values plotted against known solvent polarity values (E_T).[27] A value of λ_{max} = 510 nm was obtained for mono-dansyl **9** (5 mol %) in POPC vesicles (25 mM), which corresponds to a polarity value of E_T = 52 (ethanol-like polarity). This suggests that **9** resides, on average, in the mid-polar/headgroup region of bilayer membranes.

Summary

The results in this paper support our hypothesis that the facilitated translocation of phospholipids by TREN-derived translocases is due to the formation of hydrogen-bonded complexes with the phospholipid headgroups. In the case of zwitterionic phosphatidylcholine, it is the neutral form of the translocase that rapidly associates with the phosphate portion of the phosphocholine headgroup (Figure 2). This hydrogen-bonded complex masks the headgroup polarity and promotes diffusion of the phospholipid−translocase complex across the lipophilic interior of the membrane. The tris-sulfonamide translocases (such as **1**) are superior to the tris-amide versions (such as **2**) because they associate more strongly with the phospholipid headgroup. The stronger association is due to the increased acidity of the tris-sulfonamide NHs as well as a molecular geometry that is able to form a tridentate complex with one of the phosphate oxygens. In the case of facilitated translocation of anionic phospholipids, it is the protonated form of the translocase that binds to the phosphate portion of the headgroup, producing a complex that is analogous to that shown in Figure 4. These results provide structural insight into the supramolecular chemistry behind the facilitated translocation process; however, a number of kinetic questions remain that will require additional mechanistic study.

Experimental Section

General Synthetic Procedure for Translocases 4−7. Compounds **1**−**3** were prepared as previously described.[9,28] Compounds **4**−**7** were prepared in a manner similar to the method of Reinhoudt and co-workers.[3] In general, a solution of tris(2-aminoethyl)amine **10** (3.68 mmol) in CH_2Cl_2 or THF (50 mL) was added dropwise to a solution of the corresponding sulfonyl chloride (12.08 mmol) and triethylamine (12.08 mmol) in CH_2Cl_2 or THF (5−50 mL) at room temperature under an atmosphere of N_2. The reaction mixture was then stirred at

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room temperature for 12 h, after which time it was gravity filtered to remove the white precipitate (triethylammonium chloride). The solvent was removed *in vacuo* to give the crude product, which was purified by column chromatography using silica gel.

Tris[2-(4-methoxy(phenylsulfonamido))ethyl]amine (4). Column chromatography with 0−5% $CH_3OH/CHCl_3$ gradient elution, followed by recrystallization from $CHCl_3$, gave **4** as a white solid (45%): mp 156−157 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.50 (t, J = 5.0 Hz, 6H), 2.92 (q, J = 6.5 Hz, 6H), 3.86 (s, 9H), 5.71 (t, J = 6.2 Hz, 3H), 6.99 (d, J = 9.0 Hz, 6H), 7.87 (d, J = 9.0 Hz, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 40.2, 53.0, 55.6, 114.4, 128.6, 132.2, 162.1; MS(FAB$^+$) mass calcd for $C_{24}H_{36}N_4O_9S_3$ [M + H]$^+$ 658, found 658.

Tris[2-(4-phenoxy(phenylsulfonamido))ethyl]amine (5). The requisite 4-phenoxybenzenesulfonyl chloride was prepared in 31% overall yield in two steps following known procedures.[29,30] Column chromatography with 0−5% $CH_3OH/CHCl_3$ gradient elution, followed by recrystallization from $CHCl_3$, gave **5** as a white solid (70%): mp 52−56 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.58 (t, J = 5.0 Hz, 6H), 3.02 (q, J = 5.8 Hz, 6H), 5.78 (t, J = 6.5 Hz, 3H), 7.03 (d, J = 8.5 Hz, 6H), 7.06 (d, J = 8.0 Hz, 6H), 7.20 (t, J = 7.5 Hz, 3H), 7.20 (t, J = 7.5 Hz, 3H), 7.38 (t, J = 8.0 Hz, 6H), 7.87 (d, J = 9.0 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 40.8, 54.1, 117.7, 120.0, 124.6, 129.3, 130.0, 133.3, 155.1, 161.2; MS(FAB$^+$) mass calcd for $C_{42}H_{42}N_4O_9S_3$ [M + H]$^+$ 843, found 843.

Tris[2-(3-trifluoromethyl(phenylsulfonamido))ethyl]amine (6). Column chromatography with 0−5% $CH_3OH/CHCl_3$ gradient elution, followed by recrystallization from $CHCl_3$, gave **6** (69%): mp 121−124 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.63 (t, J = 5.0 Hz, 6H), 3.09 (q, J = 5.5 Hz, 6H), 5.98 (t, J = 6.0, 3H), 7.70 (t, J = 8.2, 3H), 7.84 (d, J = 8.0 Hz, 3H), 8.16 (d, J = 6.8 Hz, 3H), 8.17 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) 40.8, 54.1, 122.9 (q, $^1J_{CF}$ = 270.0 Hz), 123.8 (q, $^3J_{CF}$ = 3.8 Hz),129.0 (q, $^3J_{CF}$ = 3.2 Hz), 130.1, 130.3, 131.4 (q, $^2J_{CF}$ = 32.5 Hz), 141.6; MS(FAB$^+$) mass calcd for $C_{27}H_{27}F_9N_4O_6S_3$ [M + H]$^+$ 771, found 771.

Tris[2-(propylsulfonamido)ethyl]amine (7). Column chromatography with ethyl acetate gave the product **7** (7%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.05 (t, J = 7.5 Hz, 9H), 1.79−1.92 (m, 6H), 2.61 (t, J = 5.2 Hz, 6H), 3.05 (t, J = 8.0 Hz, 6H), 3.22 (q, J = 6.2, 6H), 5.71 (t, J = 6.3 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.1,17.4, 40.9, 54.4, 55.4; MS(FAB$^+$) mass calcd for $C_{15}H_{36}N_4O_6S_3$ [M + H]$^+$ 465, found 465.

***N,N*-Bis(2-aminoethyl)-*N*-[2-(*tert*-butylcarbamoyl)ethyl]amine (11).** To a solution of tris-(2-aminoethyl)amine (**10**) (2.93 g, 20.0 mmol) in CH_2Cl_2 (300 mL) at −78 °C under N_2 was added a solution of di-*tert*-butyl dicarbonate (0.444 g, 2.03 mmol) in CH_2Cl_2 (100 mL) dropwise over 1 h. The reaction mixture was allowed to warm to room temperature and stirred overnight after which time the solvent was removed *in vacuo*. Column chromatography, eluting with a ternary eluent (10:4:1) of $CHCl_3$/MeOH/concentrated aqueous NH_4OH gave the product **11** (0.324 g, 65% based on Boc_2O) as a viscous oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.43 (s, 9H), 1.58 (s, 4H), 2.52 (t, J = 6.0 Hz, 4H), 2.55 (t, J = 5.8 Hz, 2H), 2.75 (t, J = 6.0 Hz, 4H), 3.18 (unresolved t, 2H), 5.34 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 28.4, 38.8, 39.6, 54.1, 57.1, 78.8, 156.3; MS(FAB$^+$) exact mass calcd for $C_{11}H_{26}N_4O_2$ [M + H]$^+$ 247.2134, found 247.2130.

***N,N*-Bis[2-(4-methyl(phenylsulfonamido))ethyl]-*N*-[2-(*tert*-butylcarbamoyl)ethyl]amine (12).** To a mixture of **11** (0.168 g, 0.684 mmol) and K_2CO_3 (0.292 g, 2.11 mmol) in CH_3CN (10 mL) at room temperature under N_2 was added a solution of toluenesulfonyl chloride (0.322 g, 1.69 mmol) in CH_3CN (5 mL) dropwise over 5 min. The reaction mixture was allowed to stir at room temperature for 23 h after which time the solvent was removed *in vacuo*. The resulting residue was extracted with CH_2Cl_2 and filtered to remove the suspended solids, and the solvent was removed *in vacuo* to give 0.331 g of crude product. Column chromatography (SiO_2) with ethyl acetate/hexane gradient elution gave the product **12** (0.241 g, 64%) as a viscous oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.43 (s, 9H), 2.38 (t, J = 5.8 Hz, 2H), 2.41 (s, 6H), 2.46 (t, J = 5.0 Hz, 4H), 2.86−2.92 (m, 4H), 3.02 (dt, J = 5.8 and 5.5 Hz, 2H), 5.12 (t, J = 5.5 Hz, 1H), 5.89 (t, J = 5.2 Hz, 2H), 7.29 (d, J = 8.0 Hz, 4H), 7.78 (d, J = 8.0 Hz, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.5, 28.4, 38.4, 40.8, 53.8, 54.3, 79.5, 127.1, 129.7, 137.0, 143.2, 156.6; MS(FAB$^+$) exact mass calcd for $C_{25}H_{38}N_4O_6S_2$ [M + H]$^+$ 555.2311, found 555.2297.

***N,N*-Bis[2-(4-methyl(phenylsulfonamido))ethyl]-*N*-(2-aminoethyl)amine bis(trifluoroacetate) Salt (13).** To a solution of **12** (0.118 g, 0.213 mmol) in CH_2Cl_2 (6 mL) at 0 °C under N_2 was added TFA (6 mL) dropwise over 2−3 min. The reaction was stirred at 0 °C for 45 min after which time the solvents were removed *in vacuo* to give **13** (0.144 g, 100%) as a hygroscopic pale white solid: ^{1}H NMR (500 MHz, CD_3OD) δ 2.42 (s, 6H), 2.72 (t, J = 6.2 Hz, 4H), 2.87 (t, J = 6.0 Hz, 2H), 2.93 (t, J = 6.2 Hz, 4H), 3.07 (t, J = 6.0 Hz, 2H), 7.38 (d, J = 7.5 Hz, 4H), 7.73 (d, J = 8.5 Hz, 4H); ^{13}C NMR (125 MHz, CD_3OD) δ 21.6, 36.7, 40.3, 52.3, 54.8, 117.6 (q, $^1J_{CF}$ = 288.9 Hz), 128.3, 131.1, 138.0, 145.4, 162.1 (q, $^2J_{CF}$ = 36.2 Hz); MS(FAB$^+$) exact mass calcd for $C_{20}H_{30}N_4O_4S_2$ [M + H]$^+$ 455.1787, found 455.1786.

***N,N*-Bis[2-(4-methyl(phenylsulfonamido))ethyl]-*N*-[2-(7-nitrobenzo-2-oxa-1,3-diazole-4-amino)-ethyl]amine (8).** To a solution of **13** (0.142 g, 0.207 mmol) in CH_3OH (10 mL) at 0 °C under N_2 was added $KHCO_3$ (0.133 g, 1.32 mmol). The resulting solution was stirred at 0 °C for 5 min then at room temperature for 10 min after which time a solution of NBD-Cl (0.056 g, 0.281 mmol) in CH_3OH (10 mL) was added dropwise over 5 min. The reaction was stirred at room temperature for 22 h after which time the solvent was removed *in vacuo*. The residue was extracted with ethyl acetate and concentrated *in vacuo*. and the product was purified with column chromatography (Al_2O_3) using 0.5−1.0% CH_3OH /ethyl acetate gradient elution to give **8** (0.065 g, 51%) as a yellow-green oil: ^{1}H NMR (500 MHz, CD_3CN) δ 2.37 (s, 6H), 2.52 (t, J = 6.0 Hz, 4H), 2.70 (t, J = 6.2 Hz, 2H), 2.83 (t, J = 6.0 Hz, 4H), 3.43 (br s, 2H), 5.58 (s, 2H), 6.25 (s, 1H), 7.30 (d, J = 8.0 Hz, 5H), 7.66 (d, J = 8.0 Hz, 4H), 8.46 (d, J = 4.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3CN) δ 20.2, 40.6, 41.0, 51.3, 53.0, 98.9, 122.3, 126.4, 129.3, 136.9, 143.2, 144.0, 144.2, 144.4; MS(FAB$^+$) exact mass calcd for $C_{26}H_{31}N_7O_7S_2$ [M + H]$^+$ 618.1805, found 618.1824.

***N,N*-Bis[2-(4-methyl(phenylsulfonamido))ethyl]-*N*-[2-(5-(dimethylamino)-1-naphthalenesulfonamido)ethyl]amine (9).** A mixture of **13** (0.148 g, 0.217 mmol) and K_2CO_3 (0.24 g, 1.74 mmol) in CH_3CN (10 mL) was allowed to stir under N_2 at room temperature for 15 min. A solution of dansyl chloride (0.109 g, 0.404 mmol) in CH_3CN (10 mL) was then added dropwise over 15 min. The reaction was allowed to stir for 19 h after which time it was filtered, and the solvent removed *in vacuo*. The product was purified by column chromatography (SiO_2) with ethyl acetate/hexanes gradient elution to give **9** (0.126 g, 84%) as a light yellow semisolid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.38 (s, 6H), 2.38−2.46 (m, 6H), 2.82−2.92 (m, 6H), 2.87 (s, 6H), 5.90 (t, J = 6.0 Hz, 2H), 6.07 (t, J = 5.8 Hz, 1H), 7.13 (d, J = 7.5 Hz, 1H), 7.25 (d, J = 8.0 Hz, 4H), 7.51 (t, J = 7.5 Hz, 2H), 7.79 (d, J = 8.0 Hz, 4H), 8.24 (dd, J = 7.5 and 1.0 Hz, 1H), 8.33 (d, J = 9.0 Hz, 1H), 8.52 (d, J = 8.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.7, 41.1, 41.3, 45.6, 54.4, 54.7, 115.4, 119.3, 123.4, 127.4, 128.7, 129.5, 129.7, 129.9, 130.0, 130.4, 135.2, 137.1, 143.4, 152.0; MS(FAB$^+$) exact mass calcd for $C_{32}H_{41}N_5O_6S_3$ [M + H]$^+$ 688.2297, found 688.2316.

^{1}H NMR Titrations. POPC Binding. Translocases **4**−**7** were titrated with POPC. Four different NMR tubes each contained a solution of translocase in $CDCl_3$ (5 mM, 750 μL). Small aliquots of POPC stock solution (0.375 M) were added, followed by the acquisition of a ^{1}H NMR spectrum. Concentrations and equivalents were adjusted to give the optimum

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change in Weber *r* values (0.2−0.8).[31] Titration isotherms were generated for the sulfonamide NHs. Fitting the data to a 1:1 binding model using an iterative curve-fitting method yielded association constants and maximum change in chemical shift.[13]

pK_a Measurements. Translocases **1**−**3** were dissolved in 9:1 CD_3OD/D_2O (1 mM, 750 mL) in three different NMR tubes. To each tube, 4 equiv of DCl were added to fully protonate the tertiary amine. Chemical shifts of the NCH_2 and NCH_2CH_2 protons were monitored upon the addition of small aliquots (1−3 mL) of NaOD stock solution (0.1 M). A pD reading was also recorded after each addition. The changes in chemical shift were plotted against the pD values, and the pK_a values were extracted from a curve fitting procedure (Figure 6).

Single-Crystal X-ray Diffraction Analysis.[16] Crystal data were collected and integrated using a Bruker Apex system, with graphite monochromated Mo Kα (λ = 0.71073 Å) radiation at 100 K (170 K for sulfonamide **1**). The structures were solved by direct methods using SHELXS-97 and refined using SHELXL-97 (Sheldrick, G. M., University of Göttingen). Crystals of **1**·H_2O deposited directly from the eluent after column chromatography with ethyl acetate. Non-hydrogen atoms were refined anisotropically; full matrix least squares refinement on F^2 converged with ${}_wR_2 = 0.1170$, $R_1 = 0.0424$ [5562 reflections with $I > 2\sigma(I)$], goodness of fit = 1.014. The hydrogen atoms in the structure were found in difference Fourier maps and refined isotropically. Crystallographic summary for **1**·H_2O: triclinic, $M_r = 626.79$, $Z = 2$ in a cell of unit dimensions $a = 9.4007(6)$ Å, $b = 11.5963(7)$ Å, $c = 14.5787(9)$ Å, $\alpha = 92.1160(10)°$, $\beta = 95.6880(10)°$, $\gamma = 105.3620(10)°$, $V = 1521.6(2)$ Å^3, $\rho_{calc} = 1.368$ Mg m^{-3}, $F(000) = 664$.

Crystals of the ionic complex [(**1** + H)$^+$:(dibenzyl phosphate)$^-$]·$CHCl_3$] were obtained in the following way. A solution of sulfonamide **1** (0.250 g, 0.411 mmol) and dibenzyl phosphate (0.114 g, 0.410 mmol) in $CHCl_3$ (ca. 1−2 mL) in a small vial was carefully layered with ethyl acetate (ca. 5−8 mL) and allowed to sit loosely capped for 2 days after which time crystals suitable for analysis were obtained. Non-hydrogen atoms were refined anisotropically; full matrix least squares refinement on F^2 converged with ${}_wR_2 = 0.1410$, $R_1 = 0.0545$ [8281 reflections with $I > 2\sigma(I)$] goodness of fit = 1.037. The unit cell contained one tertiary ammonium ion, one phosphate ion, and one 2-fold disordered $CHCl_3$ solvent molecule (relative site occupancies 59%/41%). One of the phosphate benzyl rings was also disordered (51%/49% site occupancies). Hydrogen atom positions were located from difference Fourier maps, except for the hydrogen atoms located on the disordered benzyl rings and disordered solvent molecule which were placed at idealized positions with the appropriate site occupancy factor. Crystallographic summary for [(**1** + H)$^+$:(dibenzyl phosphate)$^-$]·$CHCl_3$]: triclinic, $M_r = 1006.38$, $Z = 2$ in a cell of unit dimensions $a = 10.7898(13)$ Å, $b = 15.1014(18)$ Å, $c = 16.812(2)$ Å, $\alpha = 114.089(2)°$, $\beta = 91.449(2)°$, $\gamma = 105.462(2)°$, $V = 2382.1(5)$ Å^3, $\rho_{calc} = 1.403$ Mg m^{-3}, $F(000) = 1052$.

Amide **2** was recrystallized from ethyl acetate with a few drops of chloroform. Full matrix least squares refinement on F^2 converged with ${}_wR_2 = 0.1410$, $R_1 = 0.0705$ [2823 reflections with $I > 2\sigma(I)$], goodness of fit = 1.100. All non-hydrogen atoms were refined anisotropically, except for the phenyl and methyl carbon atoms which were refined isotropically. Hydrogen atom positions were placed at idealized positions, except for the amide hydrogen atoms, which were located from difference Fourier maps. A riding model with fixed thermal parameters [$u_{ij} = 1.2U_{ij}$(eq) for the atom to which they are bonded], was used for subsequent refinements. Crystallographic summary for amide **2**: monoclinic, $M_r = 500.63$, $Z = 4$ in a cell of unit dimensions $a = 14.391(3)$ Å, $b = 12.212(3)$ Å, $c = 15.413(3)$ Å, $\alpha = 90°$, $\beta = 94.033(5)°$, $\gamma = 90°$, $V = 2701.9(10)$ Å^3, $\rho_{calc} = 1.231$ Mg m^{-3}, $F(000) = 1072$.

Fluorescence Assays. Vesicle Preparation. Lipids were dissolved in chloroform and were then dried *in vacuo* for at least 1 h. Hydration was performed at room temperature with an appropriate amount of 5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, pH 7.4) and 100 mM NaCl. Multilamellar vesicles were generated using a Vortex mixer; use of a Pyrex glass bead ensured complete lipid removal from the flask wall. The multilamellar vesicles were extruded to form large unilamellar vesicles with a hand-held Basic LiposoFast device purchased from Avestin, Inc, Ottawa, Canada. The vesicles were extruded 29 times through a 19-mm polycarbonate Nucleopore filter with 100-nm diameter pores.

NBD-Lipid Translocation Assay. Excitation was set at 470 nm, and fluorescence emission was measured at 530 nm using a 515 nm filter. Exo-labeled vesicles were generated upon addition of an ethanolic solution of NBD-lipid to a 35 mL solution of unlabeled liposomes (25 μM) at room temperature in TES buffer. To each vesicle solution, translocase (38 μM) was added from THF stock solutions. (Control experiments showed that THF does not induce flip-flop on its own.) At varying time intervals, 3-mL samples were removed and assayed for transbilayer movement. The assay consisted of a dithionite injection (60 mM) at $t = 50$ s, and a Triton X-100 injection (0.5%) at $t = 150$ s. Total assay time was 200 s. The amount of probe located in the outer monolayer was calculated according to the following equation: % Exo Probe = $(F_i - F_f)/F_i$, where F_i and F_f are the intensities just prior to the additions of dithionite and Triton X-100, respectively. All % exo probe values contain ±5% error. Experiments were repeated at least three times, and the values reported represent averages.

Carboxyfluorescein Leakage Assay. Excitation was set at 495 nm, and fluorescence emission was measured at 520 nm using an open filter. Vesicles encapsulating carboxyfluorescein were freeze−thawed 10 times prior to extrusion. The fluorescence of a 3-mL sample of vesicles (25 μM) in TES buffer was monitored. After 50 s, an aliquot of translocase (38 μM) was added. Detergent (0.5% Triton X-100) was added at t = 250 s. Total assay time was 300 s. A constant fluorescent intensity up until the point of detergent addition indicated no vesicle leakage.

Mono-NBD 8/Dithionite Protection Assay. The preparation was analogous to that described above for the NBD-lipid translocation assay, with the exception that unlabeled phospholipids were used. In addition, for select samples, Triton X-100 was not added until $t = 3500$ s instead of $t = 150$ s.

Mono-NBD 8/Rh-PE FRET Assay. Excitation was set at 470 nm, and fluorescence emission was measured at 590 nm using a 515 nm filter. An aliquot of **8** (38 μM) was added to a stirring solution of POPC/Rh-PE (0.6%) vesicles (25 μM) in TES buffer at $t = 50$ s. Triton X-100 (0.5%) was added at t = 350 s.

Mono-Dansyl 9 Solvation Study. Excitation was set at 340 nm, and an emission scan was obtained from 400 to 600 nm using an open filter. Spectra were acquired for solutions of **9** (38 μM) in 3 mL of THF, CH_2Cl_2, BuOH, EtOH, and MeOH. A spectrum was acquired of POPC vesicles (25 μM) extruded in the presence of **9** (5 mol %), in TES buffer, pH 7.4.

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Supporting Information Available: NMR spectra of compounds **8** and **9** (4 pages). This material is available free of charge via the Internet at http://pubs.acs.org. This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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