

Facilitated Phosphatidylserine (PS) Flip-Flop and Thrombin Activation Using A Synthetic PS Scramblase

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Abstract: A cationic steroid with a hydrogen-bonding pocket that has an affinity for anionic phospholipid headgroups was synthesized and shown to strongly promote the translocation or flip-flop of a fluorescent, C₆NBD-labeled phosphatidylserine probe (C₆NBD-PS) across vesicle membranes. In addition, the synthetic PS scramblase increases the levels of endogenous PS on the surface of erythrocytes as monitored by flow cytometry analysis of annexin V-FITC binding. The PS scrambling effect is enhanced when the cells are pretreated with *N*-ethylmaleimide (NEM), an inhibitor of the endogenous aminophospholipid flippase. The combination of NEM and synthetic PS scramblase enhances the ability of erythrocytes to promote the conversion of prothrombin to thrombin by a factor of 4. An analogous cationic steroid with a smaller binding pocket has no measurable PS translocation activity, a result that is attributed to its inability to sufficiently diminish the hydrophilicity of the multiply charged PS headgroup.

Introduction

The asymmetric transmembrane distribution of phospholipids is a fundamental feature of normal cell operation.¹ For example, the phosphatidylserine (PS) that is normally localized in the inner monolayer of the plasma membrane is vital not only for exocytosis and intracellular fusion processes but also for lipid-protein interactions and signal transduction pathways.² The appearance of PS in the membrane outer monolayer correlates with cell death and clearance by phagocytosis, a process where apoptotic cells are removed from the bloodstream by macrophages that specifically recognize the PS on the cell surface.³ Similarly, aging erythrocytes and platelets slowly externalize PS, culminating in engulfment by macrophages.⁴ Another consequence of phospholipid randomization is the activation of hemostasis and thrombosis, where the binding of activated platelets to proteins involved in the coagulation cascade is controlled by the amount of exposed PS.⁵ The tenase and prothrombinase complexes bind to patches of anionic lipid on

the cell surface, and through a series of activation steps, the fibrin matrix of the clot is formed. There is also evidence suggesting that the Alzheimer's amyloid- β -peptide targets cells with exposed PS.⁶

The transmembrane asymmetry is maintained by the concerted action of phospholipid translocases that vary in lipid specificity, energy requirements, and direction of translocation. The translocases can be divided into three classes: bidirectional "scramblases" and energy-dependent transporters that move phospholipids toward ("flippases") or away from ("floppases") the inner surface of the membrane. The best known member of this family is the aminophospholipid flippase which transports PS and to a lesser extent phosphatidylethanolamine (PE) from the outer to the inner monolayer.⁷

The aim of our research is to develop synthetic, low-molecular-weight scramblases that facilitate the translocation of phospholipids across cell membranes and as a consequence alter the endogenous distribution.^{1,8} Previously, we have reported that the neutral bis(phenylurea) cholate derivative, **1** (Scheme 1), can greatly facilitate the translocation of a fluorescent phosphatidylcholine (PC) probe across the membrane of surface-differentiated erythrocytes.⁹ The two urea residues in **1** make an effective hydrogen-bonding pocket for the phosphate diester

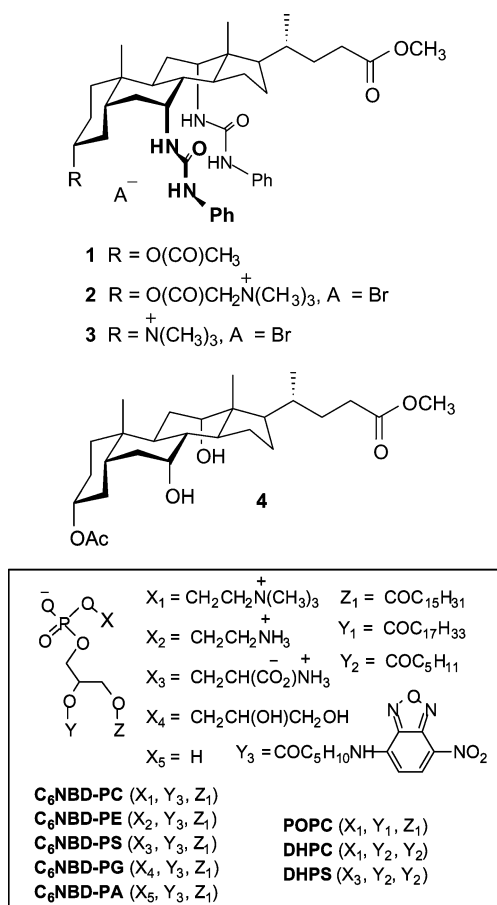
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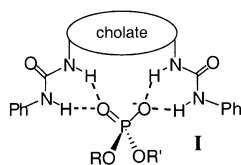
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Scheme 1



residue within the PC headgroup (see supramolecular complex **I**) which diminishes PC headgroup hydrophilicity and promotes its diffusion across the lipophilic interior of the bilayer membrane. In this current contribution, we evaluate the phospholipid translocation ability of two cationic derivatives of **1**, namely, compounds **2** and **3**. We find that compound **2** is the first example of an effective, synthetic scramblase that promotes the translocation of anionic PS across vesicle and cell membranes. This synthetic PS–scramblase increases the level of PS on the surface of erythrocytes and promotes the production of the critical blood coagulation enzyme, thrombin. In contrast, compound **3** has no measurable PS translocation activity, a result that is attributed to the inability of **3** to sufficiently diminish the hydrophilicity of the multiply charged PS headgroup.



Experimental Section

Syntheses. The syntheses of compounds **1–3** and their eicosanyl ester analogues are described in the Supporting Information.

Phospholipid Binding Constants. UV titration experiments were conducted by adding DHPS (1,2-dihexanoyl-*sn*-glycero-3-[phospho-L-serine]) or DHPC (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine) to solutions of compounds **2** or **3** in 99:1 CHCl₃:CH₃OH at 295 K. Specifically, small aliquots of phospholipid stock solution (0.315 mM

phospholipid in 15 μM scramblase solution) were added to a cuvette containing a solution of scramblase (15 μM, 1 mL), followed by the acquisition of a UV spectrum. The total volume of added phospholipid was 400 μL or 6 mol equiv. Titration isotherms were generated from the changes in absorbance, and the data were fitted to a 1:1 binding model.¹⁰ An iterative curve-fitting method yielded association constant and maximum change in absorbance. The association constants listed in the text are the average of three independent measurements.

NBD-Lipid Translocation into Vesicles. A film of the vesicle lipids was dried under vacuum for at least 1 h. Hydration was performed at room temperature with an appropriate amount of TES buffer (5 mM TES, 100 mM NaCl, pH 7.4). 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. All fluorescence measurements were conducted on a Perkin-Elmer LS 50B fluorimeter equipped with a jacketed water cooler. NBD excitation was set at 470 nm, and fluorescence emission was measured at 530 nm using a 515 nm filter.

The inward translocation assay using phospholipids with 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) labels was adapted from the original paper by McIntyre and Sleight.^{8a,11} Exo-labeled vesicles were generated upon addition of a concentrated ethanolic solution of C₆NBD-lipid (final C₆NBD-lipid concentration was 0.125 μM) to a 45 mL solution of (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) POPC/cholesterol (7:3) vesicles (final total lipid concentration was 25 μM) at room temperature. To each vesicle solution, a synthetic scramblase candidate (1.25 μM) was added from a DMSO stock solution (DMSO alone does not induce translocation). Over the time course of 3 h, a series of 3 mL aliquots were removed and assayed for extent of translocation. The 200 s assay consisted of a dithionite injection (60 mM, 180 μL of a 1 M solution) at *t* = 50 s and a Triton X-100 injection (0.5%, 75 μL of a 20% solution) at *t* = 150 s. Data points were collected every second. The percentage of exo C₆NBD-lipid located in the vesicle outer monolayer was calculated according to the following equation, % exo C₆NBD-lipid = [(F_i - F_f)/F_i]100, where F_i and F_f are the intensities just prior to the additions of dithionite and Triton X-100, respectively. All % exo C₆NBD-lipid values contain ±5% error. In some cases, the translocation curves appeared to have biexponential character, but because of the uncertainty in the data a double exponential analysis was not attempted. Instead, the reported translocation half-lives simply indicate the time taken to reach 80% exo C₆NBD-lipid, which is halfway toward an equilibrium value of 60% exo C₆NBD-lipid.

Flow Cytometry. Blood was obtained daily from a single healthy donor by venipuncture and treated with EDTA solution (dipotassium salt). Erythrocytes were isolated by centrifugation at 7900 rpm for 5 min, washed three times with 4 volumes of ice cold 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4 (PBS), and suspended in PBS at a density of 1.5 × 10⁸ cells/mL. A portion of the cells were pretreated with *N*-ethylmaleimide (NEM, 10 mM in PBS) for 30 min at room temperature. After 2 PBS washings, the NEM-pretreated cells were suspended in PBS at a density of 1.5 × 10⁸ cells/mL. A 10 μM translocase solution was prepared by dilution of a DMSO or ethanol stock solution into PBS. A 50 μL aliquot of either normal or NEM-pretreated cells was added to 450 μL of this translocase solution (1.5 × 10⁷ cells/mL). The resulting solutions were incubated at 37 °C for 3 h.

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To prepare the samples for flow cytometry analysis,¹² the above solutions were resuspended in 500 μL of 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4 (binding buffer). In a 5 mL culture tube, 15 μL annexin V-FITC (BD Biosciences/PharMingen) was added to 100 μL of the erythrocyte solution (1.5×10^6 cells), and the mixture was incubated at 37 $^\circ\text{C}$ for 15 min. An additional 600 μL of binding buffer was added before measuring fluorescence intensity and size with a Beckman Coulter Epics XL flow cytometer. Control experiments indicate that ethanol or DMSO alone has no effect on the amount of bound annexin V-FITC.

Prothrombinase Assay.^{5,13} Erythrocyte stock solutions were prepared as described above. A 10 μM translocase solution was prepared by dilution of a DMSO or ethanol stock solution into PBS. A 50 μL aliquot of either normal or NEM-pretreated cells was added to 450 μL of this translocase solution (1.5×10^7 cells/mL). The resulting solutions were incubated at 37 $^\circ\text{C}$ for 3 h. Then, the solutions were resuspended in 50 mM Tris, 120 mM NaCl, 6 mM CaCl_2 , pH 7.8 (prothrombinase buffer). The prothrombinase complex enzymes (Enzyme Research Laboratories, South Bend, IN) were added (0.33 units/mL bovine factor V/Va, 0.33 U/mL human factor Xa, 1.3 U/mL human prothrombin), and the mixture was incubated at 37 $^\circ\text{C}$ for 3 min. The reaction was stopped upon the addition of 15 mM EDTA. After centrifugation, a 250 μL aliquot of supernatant was added to 100 μL of the chromogenic substrate, sarcosine-Pro-Arg-*p*-nitroanilide (500 μM stock solution, 50 μM final concentration) in 650 μL prothrombinase buffer. The initial rate of appearance of the substrate cleavage product *p*-nitroaniline was monitored at 405 nm for 2 min. Control experiments showed that no thrombin hydrolysis activity is observed if either factor V/Va or factor Xa is absent. Also the addition of ethanol or DMSO alone has no effect on the thrombin hydrolysis rate.

Results

$\text{C}_6\text{NBD-Lipid Translocation into Vesicles.$

The well-established (NBD)/dithionite quenching assay^{8,11} was used to measure the abilities of compounds **1–4** to facilitate the inward translocation of five types of $\text{C}_6\text{NBD-lipids}$ across surface differentiated POPC/cholesterol (7:3) vesicles (25 μM total lipid concentration). In short, the assay involves addition of 1.25 μM of scramblase candidate to vesicles that already have 0.5 mol % (0.125 μM) of $\text{C}_6\text{NBD-labeled phospholipid}$ in the membrane outer monolayer. Upon treatment with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), the NBD fluorescence is quenched due to chemical reduction of the nitro group. Vesicle membranes are effectively impermeable to dithionite, therefore, only NBD-lipid located in the outer leaflet is chemically quenched. At any given time, the percentage of NBD-lipid located in the outer monolayer can be determined from the drop in fluorescence intensity when a portion of the vesicles is subjected to dithionite quenching. The system progresses to an equilibrated state with the outer monolayer containing about 60% NBD-lipid. The data are shown in Figure 1, and the translocation half-lives (average of three independent runs) are listed in Table 1. Interestingly, compound **2** decreases the half-life for $\text{C}_6\text{NBD-PS}$ translocation from much greater than 180 min to 30 min, whereas structurally related **3** hardly affects PS translocation at all and has essentially the same effect as the negative control, **4**. In fact, compound **3** is only able to weakly promote the translocation of $\text{C}_6\text{NBD-labeled phosphatidylglycerol (PG)}$ and phosphatidic acid (PA), anionic phospholipids with singly charged headgroups at neutral pH. Control experiments verified that compounds **1–4** do not

Table 1. Translocation Half-Lives for Compounds **1–4**

cmpd	half-lives for translocation into vesicles (min)				
	$\text{C}_6\text{NBD-PS}$	$\text{C}_6\text{NBD-PC}$	$\text{C}_6\text{NBD-PE}$	$\text{C}_6\text{NBD-PG}$	$\text{C}_6\text{NBD-PA}$
1	130	30	30	<1	<1
2	30	120	20	8	<1
3	>180	$\gg 180$	$\gg 180$	120	120
4	$\gg 180$	$\gg 180$	$\gg 180$	>180	>180
none	$\gg 180$	$\gg 180$	$\gg 180$	$\gg 180$	$\gg 180$

Table 2. Effect of Compounds **1–4** on PS Translocation and Thrombin Activation

cmpd	erythrocytes bound by annexin V-FITC (%)		thrombin hydrolysis activity ($\times 10^{-3}$ au/s)	
	normal cells	NEM-pretreated	normal cells	NEM-pretreated
1	14 \pm 4	33 \pm 3	2.2 \pm 0.3	2.6 \pm 0.3
2	38 \pm 5	79 \pm 2	3.3 \pm 0.3	6.8 \pm 0.7
3	1.7 \pm 0.2	4 \pm 0.8	2.7 \pm 0.3	2.9 \pm 0.1
4	0.9 \pm 0.4	2.3 \pm 0.3	1.8 \pm 0.2	2.1 \pm 0.4
none	1.3 \pm 0.5	2.1 \pm 0.6	1.4 \pm 0.1	1.9 \pm 0.3

induce leakage of entrapped carboxyfluorescein from the vesicles.

The different capabilities of **2** and **3** to translocate the various $\text{C}_6\text{NBD-lipids}$ or endogenous PS (see below) is notable. To test if the difference was due to an inability of **3** to partition into the vesicle membrane, we prepared and measured the translocation abilities of the corresponding eicosanyl ($\text{C}_{20}\text{H}_{41}$) esters of **1–3**. In this case, the vesicles were prepared with the highly lipophilic scramblase candidates (5 mol % or 1.25 μM) premixed with the other membrane lipids (POPC/cholesterol, 7:3, 25 μM total lipid). The subsequent $\text{C}_6\text{NBD-PS}$ translocation experiments with these vesicles produced almost identical results to those listed in Table 1, indicating that the poor translocation activity with **3** is not because it cannot partition into the membrane. Furthermore, we examined vesicle systems containing a mixture of 3 mol % each of the eicosanyl esters of **2** and **3** and found that the $\text{C}_6\text{NBD-PS}$ translocation half-life was equal to that expected with 3 mol % of **2** alone. In other words, compound **3** is not an inhibitor of the action of **2**.

Phospholipid Binding. We considered if compounds **2** and **3** have different affinities for the PS phospholipid headgroups. This is a difficult question to answer experimentally because it is not obvious what solvents and counterions most closely mimic the chemical environment at the membrane surface. Since the physical barrier to PS translocation is the lipophilic interior of the bilayer membrane, we decided to measure association constants in 99:1 $\text{CHCl}_3:\text{CH}_3\text{OH}$. In addition, we used PC and PS analogues with two short, C_6 -acyl chains so as to minimize any potential problems due to phospholipid aggregation. Titration of DHPS or DHPC into solutions of **2** or **3** produced enhanced UV absorptions by the urea *N*-phenyl groups (presumably because the phospholipid headgroups form hydrogen bonds with the urea NH groups). This led to titration isotherms such as those shown in Figure 2, and curve-fitting methods were used to extract association constants. In the case of **2**, K_{PS} is $(3.4 \pm 0.9) \times 10^5 \text{ M}^{-1}$ and K_{PC} is $(2.2 \pm 0.5) \times 10^5 \text{ M}^{-1}$ in 99:1 $\text{CHCl}_3:\text{CH}_3\text{OH}$ at 295 K, whereas in the case of **3**, K_{PS} is $(0.9 \pm 0.2) \times 10^5 \text{ M}^{-1}$ and K_{PC} is $(1.1 \pm 0.5) \times 10^5 \text{ M}^{-1}$. Thus, compound **2** binds the PS headgroup 3 to 4 times better and the PC headgroup two times better than compound **3**.

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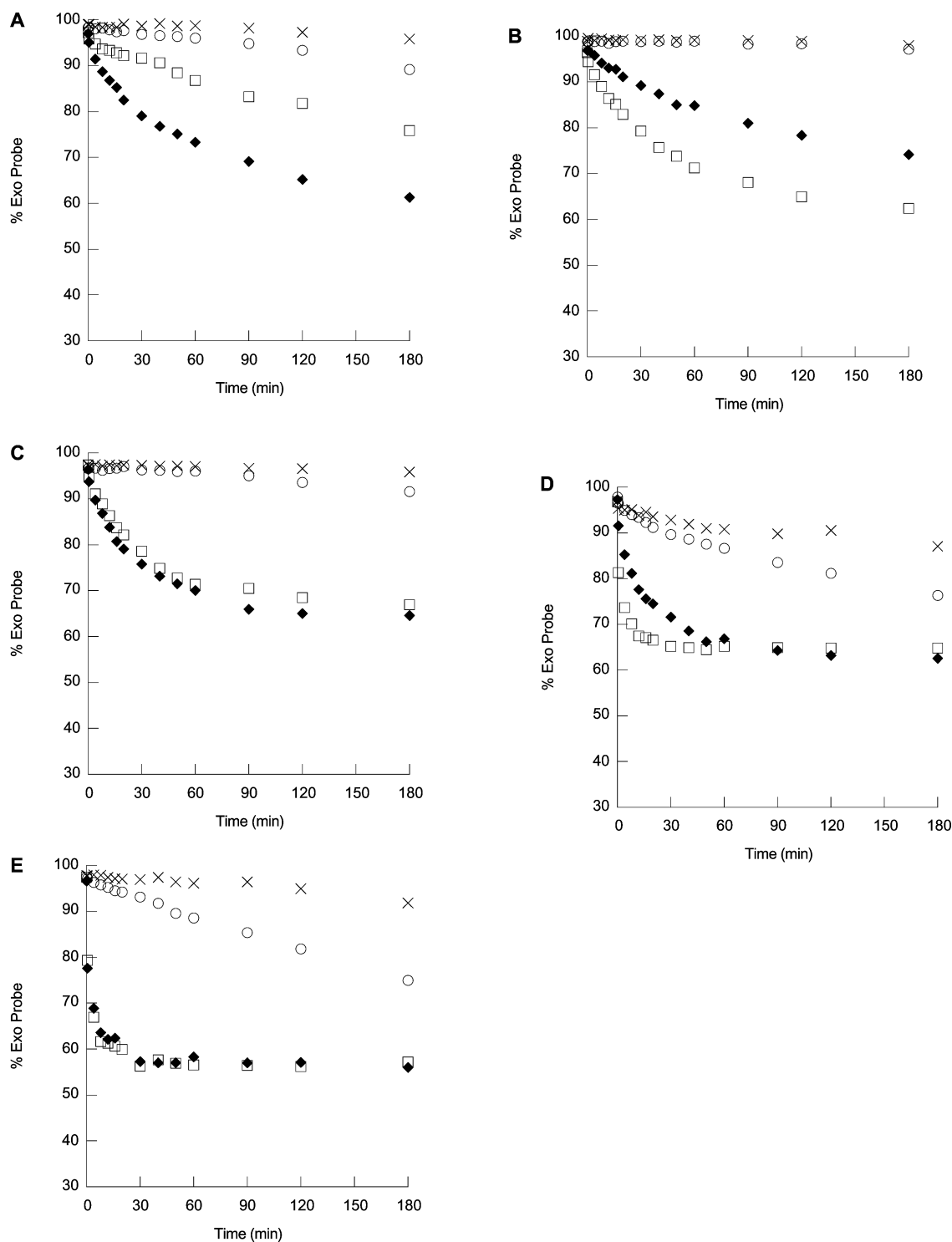


Figure 1. Change in the percent C₆NBD-lipid in the outer monolayer (% exo probe) of POPC/cholesterol (7:3) vesicle membranes at room temperature, pH 7.4. Inward translocation induced at $t = 0$ min by adding 1.25 μM of scramblase candidate **1** (□), **2** (◆), **3** (○), or **4** (×) to vesicles (25 μM) with 0.5 mol % of C₆NBD-PS (A), C₆NBD-PC (B), C₆NBD-PE (C), C₆NBD-PG (D), or C₆NBD-PA (E) already inserted in the outer monolayer.

Flow Cytometry. Flow cytometry experiments showed that compound **2** also transports PS across erythrocyte membranes. The appearance of PS on the cell surface following treatment with compounds **1–4** (10 μM , 3 h, 1.5×10^7 cells/mL) was detected using the fluorescein-labeled, PS-binding protein, annexin V (annexin V–FITC).⁸ The distribution of fluorescence intensity from a representative flow cytometry experiment is shown in Figure 3, and the average percentages of cells bound by annexin V–FITC from three separate experiments are

provided in Table 2. Results obtained with normal erythrocytes are represented in the top half of Figure 3 (a–e), while the cells in the bottom half (Figure 3, f–j) were pretreated with the aminophospholipid flippase inhibitor *N*-ethylmaleimide (NEM) which prevents endogenous translocation of PS back to the membrane inner monolayer.⁷ As expected, hardly any normal cells were bound by annexin V–FITC (Figure 3a), but ~40% of the cells displayed increased surface PS after treatment with compound **2** (Figure 3b). Identical treatment with compound **3**

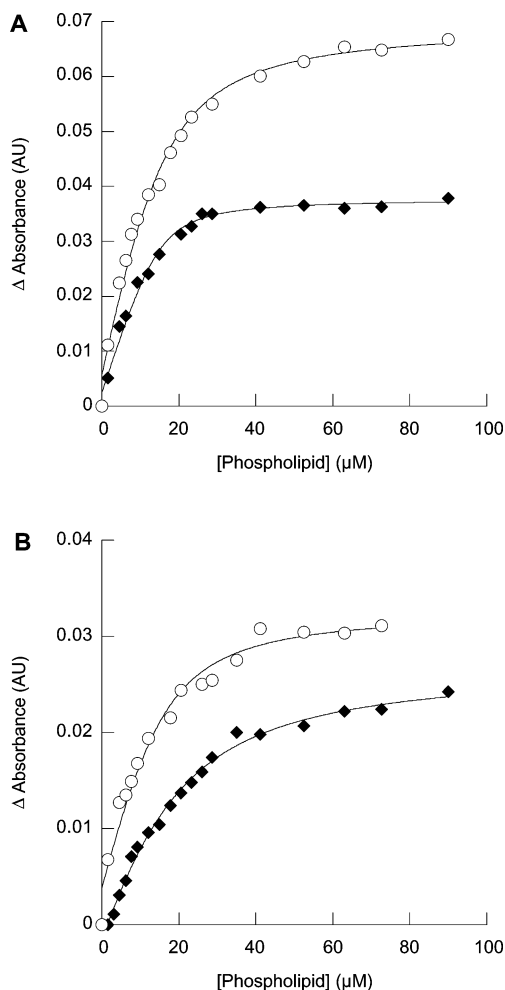


Figure 2. Typical binding isotherms (absorbance at 244 nm) generated from titration of, (A) **2** or (B) **3**, with DHPS (◆), or DHPC (○). Initially the scramblase concentration was 15 μM in 99:1 CHCl_3 : CH_3OH at 295 K. Also shown is the curve-fitting to a 1:1 binding model.

or **4** had virtually no effect on PS distribution (Figure 3, c and e), while compound **1** increased PS exposure in 14% of the cells (Figure 3d). If the endogenous aminophospholipid flippase is inhibited with NEM, then the PS scramblase activities of compounds **1** and **2** are approximately doubled (Figure 3, g and i). The lack of hemoglobin leakage (<7% leakage over 3 h) indicated that the compounds do not induce nonselective membrane transport.

Thrombin Activation. Since PS scramblase **2** can alter the endogenous transmembrane distribution of PS, it should also affect a number of cellular processes. For example, it is well-known that the assembly of the prothrombinase complex (factor Va, factor Xa, prothrombin, Ca^{2+}) requires the presence of a PS-rich membrane surface and that successful complex formation results in the generation of thrombin, an essential step in blood clot formation.⁵ Thus, compounds **1–4** (10 μM , 3 h, 1.5×10^7 cells/mL) were evaluated for their abilities to increase the conversion of prothrombin to thrombin on the surface of erythrocytes.^{5,13} Thrombin activity was determined by measuring the initial rate of hydrolysis of the thrombin-specific chromogenic substrate, sarcosine-Pro-Arg-*p*-nitroanilide (Figure 4). The thrombin hydrolysis activities listed in Table 2 correlate with increased amounts of externalized PS; i.e., treatment with synthetic PS scramblase **2** produces the highest thrombin activity in both normal and NEM-pretreated erythrocytes.

Discussion

Phospholipid translocation across a bilayer membrane is known to be promoted by compounds that form water-filled channels,¹⁴ or create local defects that act as flip sites.^{15,16} Our previous studies of $\text{C}_6\text{NBD-PC}$ translocation using compound **1** found kinetic and structural evidence in favor of a carrier diffusion mechanism where the scramblase forms the 1:1, lipophilic hydrogen-bonded complex **I** which promotes diffusion of the polar zwitterionic PC headgroup across the nonpolar interior of the membrane.⁹ Compound **1** has only a modest ability to translocate $\text{C}_6\text{NBD-PS}$.¹⁷ Since the polar PS headgroup has a net anionic charge at neutral pH, we prepared the cationic derivatives **2** and **3**, which we thought may be superior PS-scramblases because they could possibly form lipophilic charge-neutral supramolecular complexes. Indeed, we find that scramblase **2** can quite effectively translocate anionic NBD-labeled phospholipids across vesicle membranes and endogenous PS across erythrocyte membranes. Analogue **3** can weakly translocate NBD-labeled PA and PG, anionic phospholipids with singly charged headgroups, but it cannot translocate NBD-labeled PS, PC, and PE, phospholipids with multiply charged headgroups. Binding studies, in an organic solvent mixture that mimics the interior of a bilayer membrane, show that analogue **3** has a slightly lower affinity for the PS and PC headgroups than compound **2**.

We rationalize our results in the following way. An important factor controlling the rate of phospholipid translocation is headgroup solvation.^{17c} Since the multiply charged phospholipid headgroups such as PC, PS, and PE are more hydrophilic than the singly charged headgroups such as PA and PG, it is intrinsically harder for them to diffuse through the interior of a bilayer membrane.^{16–18} Translocation of the PS headgroup at neutral pH is facilitated if the three charged residues are at least partially desolvated.^{19,20} Furthermore, there is literature evidence that organic and inorganic cations can dehydrate the PS headgroup to different extents.²¹ Compounds **2** and **3** are

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- (17) Although not the focus of this specific study, it is worth noting that compound **1** is remarkably effective at promoting the translocation of $\text{C}_6\text{-NBD-PA}$ and $\text{C}_6\text{-NBD-PG}$ (see Figure 1 and Table 1). This result raises the mechanistic question of whether compound **1** translocates these phospholipids as anions or as neutral acids. The literature is mixed on this topic. Papers that support translocation of the anion include: (a) reference 16, and (b) Haest, C. W. M.; Oslender, A.; Kamp, D. *Biochemistry* **1997**, *36*, 10885–10891. Papers that support translocation of the neutral acid include: (c) Homan, R.; Pownall, H. J. *Biochim. Biophys. Acta* **1988**, *938*, 155–166, and (d) Eastman, S. J.; Hope, M. J.; Cullis, P. R. *Biochemistry* **1991**, *30*, 1740–1745.
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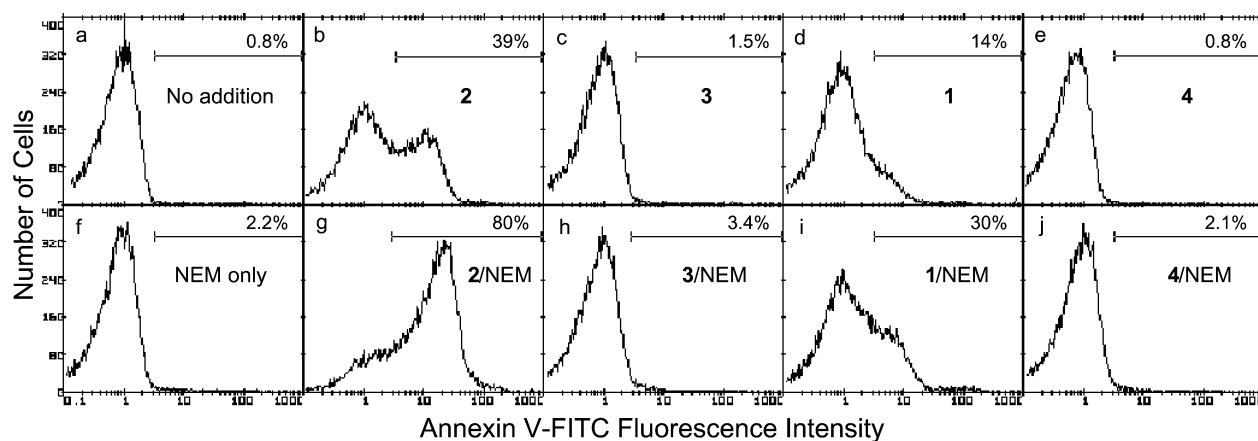


Figure 3. Flow cytometry analysis of annexin V-FITC binding to normal (a–e) or NEM-pretreated (f–j, 10 mM) erythrocytes. Before exposure to annexin V-FITC, the erythrocytes were incubated at 37 °C for 3 h with 10 μ M of scramblase candidate: (a, f) no addition, (b, g) **2**, (c, h) **3**, (d, i) **1**, or (e, j) **4**.

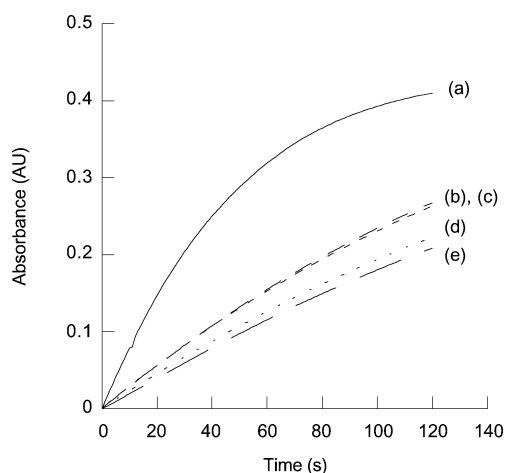
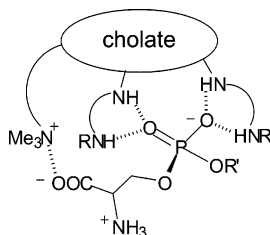


Figure 4. Increase in absorbance observed upon addition of cell supernatants to thrombin-specific chromogenic substrate, sarcosine-Pro-Arg-p-nitroanilide (50 μ M). Supernatants contained thrombin generated from prothrombinase complex (factor Va, factor Xa, prothrombin, Ca^{2+}) formation in the presence of erythrocytes that had been pretreated with NEM (10 mM) prior to a 3 h incubation with 10 μ M of scramblase candidate: (a) **2**, (b) **1**, (c) **3**, (d) **4**, (e) no addition.

Scheme 2. Ditopic Complex II between **2** and the PS Headgroup



reasonably effective PS binders. We propose that when compound **3** binds to the PS headgroup, the resulting supramolecular complex is highly amphiphilic and thus does not readily translocate across the membrane. Compound **2**, however, has a larger, ditopic binding pocket that can simultaneously contact the phosphate and carboxylate residues in the PS headgroup. The schematic supramolecular complex **II** shown in Scheme 2 illustrates how compound **2** may be able to more effectively dehydrate the PS headgroup.^{22,23} The idea that it is easier for a scramblase to dehydrate and translocate a singly charged phos-

pholipid compared to a multiply charged phospholipid is in agreement with recent reports that single, membrane-spanning helical peptides can translocate PA and PG, but not PS and PC.¹⁶ Taken together these studies illustrate the inherent difficulty for multiply charged phospholipid headgroups to diffuse across a bilayer membrane. In essence, the data are consistent with the Hofmeister effect, a commonly observed partitioning selectivity that is widely attributed to differences in solvation.²⁴ In addition, the data suggest that it is easier to maintain an asymmetric transmembrane PS distribution, compared to PA or PG asymmetry, which is likely one of the reasons why PS asymmetries are much more common in biological membranes.¹

We find that the conversion of prothrombin to thrombin on the surface of erythrocytes that have been treated with NEM and scramblase **2** is 4-fold higher than the amount induced by untreated erythrocytes. The flow cytometry data indicate that this is because **2** increases the amount of PS on the erythrocyte surface. It is not possible to analyze this thrombin activation effect in a quantitative manner because the precise concentrations of exposed PS on the erythrocyte surfaces, before and after treatment with **2**, were not determined. Furthermore, the relationship between exposed PS levels and thrombin activation is not linear, but bell-shaped.²⁵ We believe that synthetic PS scramblases, such as **2**, may be a novel way of activating blood clotting by increasing thrombin production.²⁶ It is also possible

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that compound **2** will influence other biological processes that depend on transmembrane PS distribution.

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Supporting Information Available: Synthetic procedures and spectral detail (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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