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Steroid-derived phospholipid scramblases induce exposure of phosphatidylserine on the surface of red blood cells

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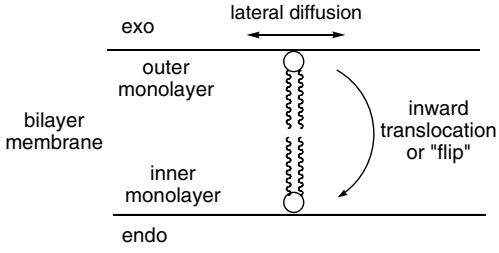
Abstract—A series of methyl 7α,12α-bis(phenylurea) cholate derivatives with different cationic substituents at the 3α-position were prepared and evaluated for an ability to increase the level of endogenous phosphatidylserine (PS) on the surface of red blood cells (erythrocytes). Some of the compounds induced large fractions of erythrocytes to expose sufficient PS to become stained by the protein annexin V-FITC. In addition, the compounds were found to bind PS in homogeneous solution, and to promote the translocation of fluorescent NBD-labeled phospholipids across vesicle membranes, which supports the hypothesis that cholate-induced exposure of endogenous PS on the erythrocyte surface is due to the ability of the cationic cholates to promote anionic phospholipid flip-flop.

1. Introduction

Most mammalian plasma membranes possess an asymmetric transbilayer distribution of phospholipids.[1] In particular, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are found primarily in the inner leaflet of the bilayer while phosphatidylcholine (PC) is mainly found in the outer leaflet. The asymmetric distribution, a fundamental feature of normal cell operation, is generated and maintained by translocase enzymes which vary in lipid specificity, energy requirements, and direction of translocation.[1–6] The best known translocase enzyme is the aminophosphilipid translocase or flippase, which consumes ATP and selectively pumps PS from the membrane outer monolayer to the inner monolayer.[6] During the process of programmed cell death (apoptosis), a phospholipid scrambling function is activated, and the level of PS on the surface of a cell membrane is increased, which in turn induces cell clearance by macrophages.[7–9] Indeed, a recent, high precision study of PS surface levels in apoptotic and nonapoptotic Jurkat cells concluded that nonapoptotic cells externalize 0.9 pmol of endogenous PS/10^6 cells, and that apoptotic cells externalize 240 pmol of endogenous PS/10^6 cells.[5] Furthermore, the concentration threshold for phagocytic response was found to be 5 pmol/10^6 cells. In other words, only 2% of the internalized PS have to be moved to the outer cell surface to trigger phagocytosis. With this data in mind, we are attempting to develop organic molecules that have an ability to scramble the asymmetric transmembrane phospholipid distribution and make PS appear on a cell surface with the expectation that this will induce cell clearance by the body's innate immune system.[10–16]

Our general strategy is to design synthetic phospholipid scramblases that promote translocation or flip-flop across the membrane (Scheme 1). While lateral diffusion of a phospholipid in a membrane bilayer is quite fast,



Scheme 1.

Keywords: Phosphatidylserine; Translocation; Molecular recognition; Flip-flop; Membrane scramblase.
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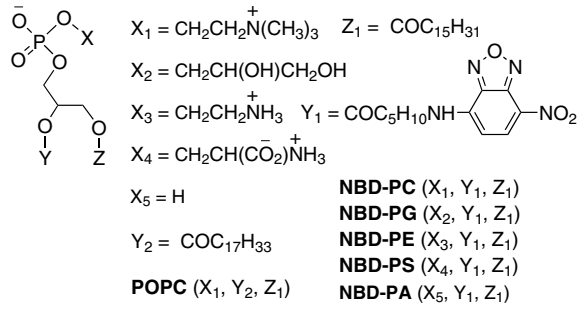
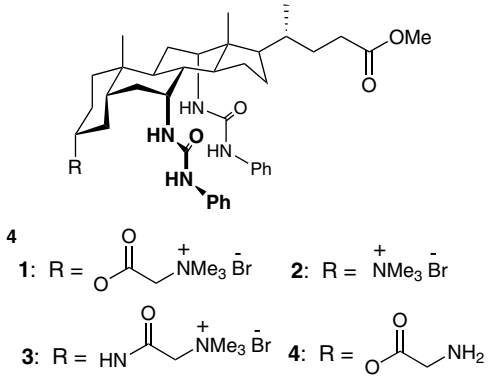


Scheme 2.



Scheme 3.

spontaneous flip-flop is quite slow with half-lives typically on the order of hours or days. The synthetic scramblases are designed to form charge-neutral, hydrogen bonded complexes with the phospholipid head-group, which reduces head-group polarity and accelerates diffusion through the lipophilic interior of the bilayer membrane to the opposite interface. Since the focus is on PS flip-flop, a primary goal is the development of PS-scramblases. This is a difficult supramolecular challenge because the PS head group is a triple ion at neutral pH with a net anionic charge (Scheme 2).[17] Furthermore, the PS head-group may be able to form intermolecular hydrogen bonds which raises the activation barrier for membrane flip-flop.[18] Recently, we reported that the cationic cholate derivative **1** is able to accelerate the translocation of PS across vesicle membranes, and also increase the concentration of PS on the surface of erythrocytes.[10] Mechanistic studies indicate that the 7α,12α-bis(phenylurea) groups in **1** form a hydrogen bonding pocket for the anionic residues in the PS head-group. The next step in the research is to test for PS-scramblase activity in other cells, however, a concern with compound **1** is that its 3α-ester linkage may be cleaved during these experiments,† which would produce a PS-inactive cholate (Boon, J. M.; Lambert, T. N.; Smith, B. D. *unpublished results*). Therefore, we decided to develop a more stable analogue of compound **1**. Our first attempt was the quaternary ammonium **2**, but this compound was found to have no PS-scramblase activity in vesicles or erythrocytes (red blood cells).[10] This surprising result highlights how a relatively minor change in cholate structure can induce a large change in PS-scramblase activity. In this report, we describe two new analogues of **1**, namely cholates **3** and **4** (Scheme 3). The amide-linked **3** was designed to be a more stable version of **1** but with minimal structural change; whereas, ester amine **4** was a test of the need to have a permanent cationic charge at the cholate 3α-position. We report the results of flow cytometry experiments, indicating that both compounds can induce PS exposure on the surface of erythrocytes. Phospholipid translocation studies in vesicles suggest that this occurs because the compounds promote the transmembrane flip-flop of anionic phospholipids.

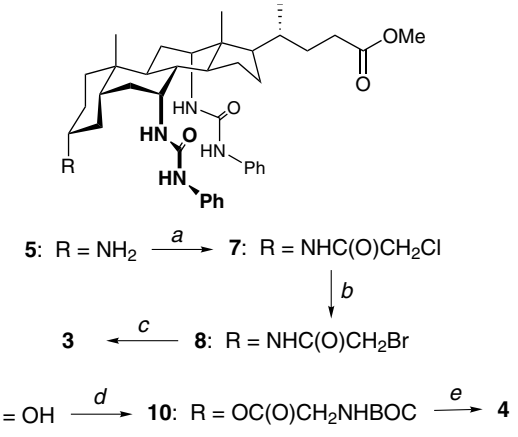
2. Results and discussion

2.1. Synthetic chemistry

Compounds **1** and **2** were prepared as described previously.[10] Compound **3** was prepared from methyl 3α-(amino)-7α,12α-bis(phenylurea) cholate **5**,[10] while compound **4** was prepared from methyl 3α-(hydroxy)-7α,12α-bis(phenylurea) cholate **6**,[10] as described in Scheme 4.

2.2. Cell studies

Erythrocytes are excellent cell systems for screening and mechanism studies because their structural simplicity and lack of endocytic activity minimizes the chances of confusing artifacts. The abilities of cholates **1**–**4** to induce PS exposure on the outer membrane of erythrocytes were evaluated using flow cytometry.[6] Briefly,



Scheme 4. Reagents and conditions: (a) $ClCH_2CO_2H$, DMAP, HOBt, EDC/HCl, DIPEA, CH_2Cl_2, rt, 18 h, 66%. (b) NaBr, acetone, reflux, 19 h, 64%. (c) NMe_3, acetone, reflux (sealed tube), 24 h, 65%. (d) $HOOCCH_2NHBOC$, DMAP, EDC/HCl, CH_2Cl_2, rt, 21 h, 78%. (e) TFA/CH_2Cl_2, 0 °C to rt, 2 h, then satd aq $NaHCO_3$ workup 67%.

†The goal at this early point is to study stable scramblases that will give unambiguous results. However, clearance of cells with exposed PS is a fairly rapid event, so it is possible that scramblases with short chemical lifetimes may have more selective toxicity. Thus, potentially labile compounds such as **1** or **4** with 3α-ester linkages also need to be investigated.

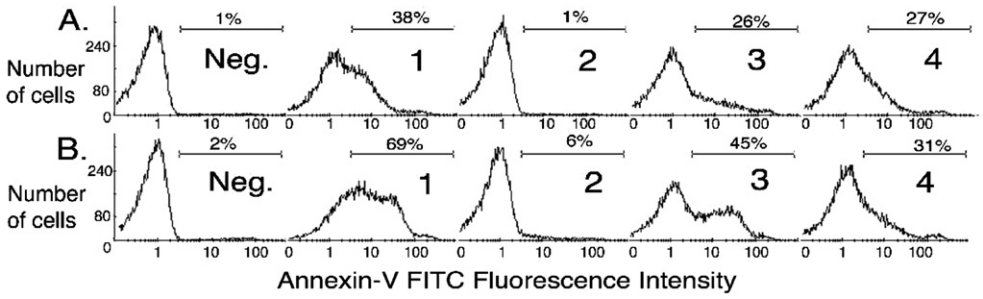


Figure 1. Flow cytometry analysis of annexin V-FITC staining of (A) normal erythrocytes or (B) NEM-pretreated erythrocytes. Before exposure to annexin V-FITC, the erythrocytes were incubated for 3 h at 37 °C with scramblase candidate (10 μM).

separate samples of erythrocytes were incubated with each of the cholates (from concentrated DMSO stock solutions; final concentration 10 μM) for 3 h at 37 °C. The cells were then treated with the fluorescein labeled protein annexin V-FITC which selectively stains cells that have an increased amount of externalized PS. As shown by the representative flow cytometry profiles in Figure 1A, the fraction of cells that were stained by the annexin V-FITC was **1** (38%), **2** (1%), **3** (26%), and **4** (27%). No staining was observed when the cells were treated with the DMSO solvent. As shown in Figure 1B are typical flow cytometry profiles obtained when erythrocytes were first pre-treated with *N*-ethylmaleimide (NEM) an inhibitor of the endogenous aminophospholipid translocase.[6] As expected, the fraction of cells that were stained by the annexin V-FITC increased to **1** (69%), **2** (6%), **3** (45%), and **4** (31%). The data confirms the strong ability of cholate **1**, and the inability of cholate **2**, to induce the appearance of PS on the surface of erythrocytes.[10] The new cholates **3** and **4** have intermediate abilities to externalize PS, but nonetheless, their scramblase activities appear to be high enough to induce phagocytosis.[5]

2.3. Phospholipid binding

The synthetic scramblases are designed to form charge-neutral, hydrogen bonded complexes with the PS headgroups, however, it is difficult to directly observe this molecular recognition in a heterogenous cell membrane system. Experimental verification is more tractable in homogenous solution, and previously we have measured association constants in 99:1 $CHCl_3/CH_3OH$, using a PS analogue with two short, C_6-acyl chains (DHPS) so as to minimize any potential problems due to phospholipid aggregation.[10] Hydrogen bonding of the DHPS with the cholate bis(phenylurea) units in **1**–**4** can be monitored by UV absorption, and PS association constants, K_{PS} can be extracted from standard UV titrations experiments. In the case of **1** and **2**, the values of K_{PS} at 295 K are $(3.4 \pm 0.9) \times 10^5\ M^{-1}$ and $(0.9 \pm 0.2) \times 10^5\ M^{-1}$, respectively.[10] Repeating the UV titrations with the new cholates lead to K_{PS} values of $(3.3 \pm 1.8) \times 10^5\ M^{-1}$ for **3** and $(6.0 \pm 2.0) \times 10^4\ M^{-1}$ for **4**. Thus, changing from the ester linkage in **1** to amide linkage in **3** results in no apparent change in PS affinity. In the case of **4**, there is a 5-fold drop in PS affinity but this is expected because the measurement is done under non-protonating conditions, and so **4** is not in its cationic form. Overall, the new cholates **3** and **4** appear to have PS affinities that are comparable to the original compounds **1** and **2**.

2.4. Vesicle studies

The hypothesis that the cholates induce PS exposure on the cell surface by promoting phospholipid translocation was tested by measuring the abilities of cholates **1**–**4** to translocate fluorescent phospholipid probes across surface differentiated vesicle membranes. The well-established NBD/dithionite quenching assay was employed.[19,20] In short, the assay is initiated by adding 1.25 μM of scramblase candidate to a dispersion of POPC/cholesterol (7:3) vesicles (25 μM total lipid concentration) that already have 0.5% of NBD-lipid inserted in the outer monolayer (100% *exo* labeled vesicles). Upon treatment with sodium dithionite, the NBD fluorescence is chemically quenched. The vesicles are effectively impermeable to the dithionite, so only the NBD-lipid in the outer monolayer is quenched. At any given time, the amount of *exo* NBD-lipid can be determined from the drop in fluorescence emission when a portion of the vesicles is subjected to dithionite quenching. Thus, the experiment monitors the change in the percentage *exo* NBD-lipid as the system moves towards an equilibrium value of around 60% *exo* NBD-lipid. Shown in Figure 2 are typical inward translocation curves for NBD-PS induced by the addition of cholates **1**–**4** at room temperature and pH 7.4. These curves allow the estimation of translocation half-lives, and listed in Table 1 are average half-lives for experiments conducted with the probes NBD-PS, NBD-PC, NBD-PE, NBD-PG, and NBD-PA. As above, the data are independent confirmation of the strong ability of cholate **1**, and the inability of cholate **2**, to promote phospholipid translocation. The new cholates **3** and **4** have intermediate abilities to translocate NBD-PS, but they are quite effective at translocating the other anionic probes, NBD-PG and NBD-PA. The inability to translocate zwitterionic NBD-PC demonstrates the selectivity of **3** and **4** for anionic phospholipids. The vesicle data supports the hypothesis that cholate-induced exposure of endogenous PS on the erythrocyte surface is due to the ability of the cationic cholates to promote anionic phospholipid translocation, especially the outward translocation of PS.

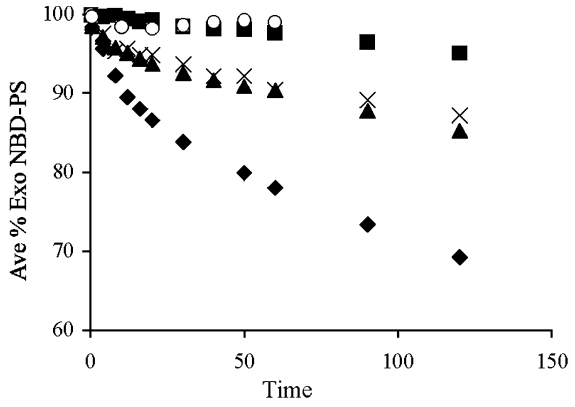


Figure 2. Change in the percent NBD-PS in the outer monolayer (% *exo* NBD-PS) of POPC/cholesterol vesicles (7:3) at room temperature and pH 7.4. Inward translocation induced at $t = 0$ min by adding 1.25 μM of cholate **1** (♦), **2** (■), **3** (▲), **4** (×) and DMSO control (○) to vesicles (25 μM) with 0.5 mol% of NBD-PS already inserted in the outer monolayer.

Table 1. Half-lives (min) for inward translocation[a]

Scramblase	NBD-PS	NBD-PC	NBD-PE	NBD-PG	NBD-PA
1	30	130	25	<1	<1
2	≫180	≫180	≫180	120	120
3	150	≫180	>120	8	12
4	150	≫180	>120	20	20

[a] Half-lives are the average of three independent experiments using 1.25 μM scramblase with an error of ±5% (25 μM 7:3 POPC/cholesterol vesicles; 0.5 mol% NBD-lipid).

3. Conclusion

Although not as active as cholate **1**, the new analogues **3** and **4** are still able to effectively promote the transmembrane flip-flop of anionic phospholipids, and induce large fractions of erythrocytes to expose sufficient endogenous PS on their membrane surfaces to become stained by annexin V-FITC. The translocation success with primary amine **4** shows that a quaternary ammonium cation is not a necessary substituent for PS-scramblase activity, but nonetheless, it is a beneficial feature. The amide-linked **3** is a promising PS-scramblase and warrants further testing in nucleated human cells. These studies will be reported in due course.

4. Experimental details

4.1. Synthesis

4.1.1. Methyl 3α-[(2-(chloro)methylcarbonyl)amino]-7α,12α-bis[(phenylaminocarbonyl)amino]-5β-cholan-24-oate (7). A solution of EDC/HCl (0.031 g, 0.162 mmol) in THF (5 mL) was added dropwise over 30 min to a solution of methyl 3α-amino-7α,12α-bis[(phenylaminocarbonyl)-amino]-5β-cholan-24-oate **5**[10] (0.0737 g, 0.112 mmol), DMAP (0.0114 g, 0.093 mmol), chloroacetic acid (0.013 g, 0.138 mmol), and HOBt (0.025 g, 0.185 mmol) in THF (8 mL) at rt. The resulting solution was allowed to stir at rt for 18 h, after which time it was diluted with CH_2Cl_2, washed with aq 1 N HCl, satd aq $NaHCO_3$, water, dried (Na_2SO_4), and the solvent removed in vacuo to give 0.0421 g of the crude material. Column chromatography (SiO_2), eluting with 1–5% methanol/chloroform gradient elution, gave the pure compound **7** (0.055 g). White solid, yield 66%; MS (FAB^+) exact mass 734.4054, theoretical 734.4048 (M^+); ^1H NMR (300 MHz, $CDCl_3$) δ 0.80 (s, 3H, 18-C*H*$_3$), 0.94 (br s, 6H, 21-C*H*$_3$ and 19-C*H*$_3$), 3.35 (br s, 1H, 12β-*H*), 3.65 (s, 3H, CO_2CH_3), 3.87 (m, 1H, 7β-*H*), 3.96 (br s, 2H, C*H*$_2$Cl), 4.13 (br s, 3H, 3β-H), 5.38 (br s, 2H, N*H*), 6.50 (br s, 1H, N-*H*), 7.00–7.15 (m, 4H, N-*H* and Ar-*H*), 7.25–7.42 (m, 8H, Ar-*H*).

4.1.2. Methyl 3α-[(2-(bromo)methylcarbonyl)amino]-7α,12α-bis[(phenylaminocarbonyl)amino]-5β-cholan-24-oate (8). A mixture of the methyl 3-[(2-(chloro)methylcarbonyl)amino]-7,12-bis[(phenylaminocarbonyl)amino]-5ß-cholan-24-oate **7** (0.055 g, 0.075 mmol) and NaBr (0.120 g, 1.17 mmol) in acetone (5 mL) was heated at reflux under an atmosphere of nitrogen for 19 h. The solvent was then removed in vacuo, and the resulting residue suspended in $CHCl_3$, washed with water, dried (Na_2SO_4), and the solvent removed in vacuo to give the product **8** (0.037 g), which was used immediately without further purification. White solid, yield 64%; ^1H NMR (300 MHz, $CDCl_3$) δ 0.79 (s, 3H, 18-C*H*$_3$), 0.93 (br s, 6H, 21-C*H*$_3$ and 19-C*H*$_3$), 3.30 (br s, 1H, N-*H*), 3.65 (s, 3H, CO_2CH_3), 3.80 (br s, 1H, 7β-*H*), 3.93 (br s, 3H, C*H*$_2$Br and 12β-*H*), 4.12 (br s, 3H, 3β-H), 5.44 (br s, 1H, N*H*), 5.51 (br s, 1H, N-*H*), 6.43 (br s, 1H, N-*H*), 6.92–7.16 (m, 4H, N-*H* and Ar-*H*), 7.20–7.44 (m, 8H, Ar-*H*).

4.1.3. Methyl 3α-[(2-(*N*,*N*,*N*-trimethylammonium)methylcarbonyl)amino]-7α,12α-bis[(phenylaminocarbonyl)amino]-5β-cholan-24-oate bromide (3). Methyl 3α-[(2-(bromo)-methylcarbonyl)amino]-7α,12α-bis[(phenylaminocarbonyl)-amino]-5β-cholan-24-oate **8** (0.037 g, 0.048 mmol) was dissolved in an anhydrous solution of saturated trimethylamine in acetone (5 mL) and then refluxed in a sealed reaction tube for 24 h. The solvent was removed in vacuo, and the compound was washed once with a small portion of with hexanes. The compound was re-crystallized from ethyl acetate/diethyl ether to provide the pure compound **3** (0.0259 g). No attempts to obtain a second crop were made. White solid, yield 65%; MS (FAB^+) exact mass 757.5033, theoretical 757.5016 ($M\text{-}Br^+$); ^1H NMR (300 MHz, $CDCl_3$ δ 0.83 (s, 3H, 18-C*H*$_3$), 0.96 (s, 3H, 19-C*H*$_3$), 0.99 (t, $J = 6.0$ Hz, 3H, 21-C*H*$_3$), 3.09 (d, 9H, NC*H*$_3$), 3.62 (s, 3H, OC*H*$_3$), 3.77 (br s, 1H, 7β-*H*), 3.81 (d, $J = 3.0$ Hz, 2H, C(O)C*H*$_2$N), 3.91 (br s, 1H, 12β-*H*), 4.32 (br d, $J = 9.9$ Hz, 1H, 3β-*H*), 6.06 (d, $J = 11.4$ Hz, 1H, N-*H*), 6.56 (d, $J = 8.4$ Hz, 1H, N-*H*), 6.91 (t, $J = 7.2$ Hz, 2H, Ar-*H*), 7.19–7.26 (m, 4H, Ar-*H*), 7.59 (unresolv q, 4H, Ar-*H*), 8.28 (s, 1H, N-*H*), 8.45 (d, $J = 8.1$ Hz, 1H, N-*H*), 8.55 (s, 1H, N-*H*); ^{13}C NMR (75 MHz, $CDCl_3$) *Selected Diagnostic Data* δ 118.0 (Ar-*C*H), 118.1 (Ar-*C*H), 118.2 (Ar-*C*H), 118.4 (Ar-*C*H), 121.4 (Ar-*C*H), 128.0 (Ar-*C*H), 128.8 (Ar-*C*H), 140.7 (Ar-*C*), 140.8 (Ar-*C*), 155.2 (NH*C*ONH), 155.4 (NH*C*ONH), 160.2 (3α-O_2CCH_2), 174.9 (CO_2CH_3).

4.1.4. Methyl 3α-[2-(*N*-(*t*-butyloxycarbonyl)amino)-acetoxy]-7α,12α-bis[(phenylaminocarbonyl)amino]-5β-cholan-24-oate (10). A solution of EDC/HCl (0.146 g, 0.761 mmol) in CH_2Cl_2 (8 mL) was added dropwise over ~30 min to a solution of the methyl 3α-hydroxy-7α,12α-bis[(phenylaminocarbonyl)amino]-5β-cholan-24-oate **6**[10] (0.417 g, 0.634 mmol), DMAP (0.159 g, 1.30 mmol), and *N*-*Boc*-Glycine (0.133 g, 0.760 mmol) in CH_2Cl_2 (5 mL) at rt. The resulting solution was allowed to stir at rt for 21 h, after which time it was diluted with CH_2Cl_2, washed with 4% aq NH_4HCl, satd aq $NaHCO_3$, water, dried (Na_2SO_4), and the solvent removed in vacuo. Radial chromatography (SiO_2), eluting with 1–3% methanol/ethyl acetate gradient elution, gave the pure product **10** (0.404 g). White solid, yield 78%; MS (FAB^+) exact mass 816.4911, theoretical 816.4911 (M+1); ^1H NMR (300 MHz, $CDCl_3$) δ 0.79 (s, 3H, 18-C*H*$_3$), 0.87 (s, 3H, 19-C*H*$_3$), 0.90 (d, $J = 3.3$ Hz, 3H, 21-C*H*$_3$), 1.49 (s, 9H, *t-Bu*), 3.63 (s, 3H, CO_2CH_3), 3.84–4.20 (m, 3H, NC*H*$_2$ and 7β-*H*), 4.22 (s, 2H, 12β-*H*), 4.31 (br s, 1H, 3β-*H*), 5.09 (br s, 1H, N-*H*), 5.40 (br s, 1H, N-*H*), 5.57 (br s, 1H, N-*H*), 6.68 (br s, 1H, N-*H*), 6.83 (br s, 1H, N-*H*), 6.99 (t, $J = 4.4$ Hz, 2H, Ar-*H*), 7.18–7.30 (m, 4H, Ar-*H*), 7.36 (d, $J = 4.5$ Hz, 2H, Ar-*H*), 7.40 (d, $J = 4.2$ Hz, 2H, Ar-*H*).

4.1.5. Methyl 3α-[2-(amino)-acetoxy]-7α,12α-bis[(phenylaminocarbonyl)amino]-5β-cholan-24-oate (4). To a solution of methyl 3α-[2-(*N*-(*t*-butyloxycarbonyl)amino)-acetoxy]-7α,12α-bis[(phenylaminocarbonyl)amino]-5β-cholan-24-oate **10** (0.288 g, 0.353 mmol) in dichloromethane (3 mL) at 0 °C under N_2 was added trifluoroacetic acid (3 mL) dropwise over 2–3 min. The reaction was allowed to warm to rt and stirred for 2 h, after which time the solvents were removed in vacuo. The resulting residue was dissolved in CH_2Cl_2, washed with satd aq $NaHCO_3$, dried (Na_2SO_4), and the solvent removed in vacuo to give the pure product **4** (0.17 g) as a white solid, that was stable upon storage; yield (67%). MS (FAB^+) exact mass 716.4352, theoretical 716.4387 (M^+); ^1H NMR (500 MHz, $CDCl_3$) δ 0.72 (s, 3H, 18-C*H*$_3$), 0.80 (s, 3H, 19-C*H*$_3$), 0.88 (s, 3H, 21-C*H*$_3$), 3.10 (br s, 2H, N*H*$_2$), 3.26 (br s, 3H, NC*H*$_2$), 3.63 (s, 3H, CO_2CH_3), 3.86 (br s, 1H, 7β-*H*), 4.12 (br s, 1H, 12β-*H*), 4.47 (br s, 1H, 3β-*H*), 4.81 (br s, 1H, 7α-N*H*), 5.55 (br s, 1H, N*H*), 5.74 (br s, 1H, N*H*), 6.84–7.00 (m, 2H, Ar-*H*), 7.20–7.40 (m, 8H, Ar-*H*); ^{13}C NMR (125 MHz, $CDCl_3$) *Selected Diagnostic Data* δ 119.1 (Ar-*C*H), 120.0 (Ar-*C*H), 122.7 (Ar-*C*H), 123.4 (Ar-*C*H), 129.0 (Ar-*C*H), 129.2 (Ar-*C*H), 138.5 (Ar-*C*), 139.0 (Ar-*C*), 139.4 (Ar-*C*), 154.2 (NH*C*ONH), 155.4 (NH*C*ONH), 172.9 (3α-O_2CCH_2), 175.1 (CO_2CH_3).

4.2. Biochemical assays

4.2.1. Flow cytometry assay. Blood samples were collected from a single healthy donor by venipucture and treated with EDTA solution (di-potassium salt). To collect the erythrocytes, samples were spun at 7500 rpm for 5 min and washed three times with ice cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). A 50% hematocrit solution was made. Samples that were pretreated with NEM used 10 mM NEM in PBS (1.5×10^8 cells/mL). The cells were incubated at room temperature for 30 min and then washed three times with ice cold PBS. The cells were then spun and resuspended at 1.5×10^8 cell/mL in PBS buffer. To separate solutions of 10 μM scramblase in PBS, were added 50 μL of either normal or NEM pretreated erythrocytes (final cell 1.5×10^7 cells/mL) and the samples incubated at 37 °C for 3 h. After incubation the samples were spun down (7500 rpm) and re-suspended in 500 μL of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4).[21] To 100 μL of each sample (1.5×10^6 cells), was added 15 μL of annexin V-FITC (BD Biosciences/PharMingen), followed by incubation at 37 °C for 15 min. An additional 600 μL of binding buffer was added before analysis using a Beckman Coulter Epics XL flow cytometer. The entire experiment was repeated three times for each scramblase.

4.2.2. Vesicle translocation assay.[19,20] A 7:3 POPC/cholesterol mixture in $CHCl_3$ was dried in vacuo for 1 h. A stock solution of 10 mM vesicles was made by rehydration at room temperature with TES buffer (5 mM TES, 100 mM NaCl, pH 7.4). Unilamellar vesicles were formed after extrusion 29 times through a 19-mm polycarbonate Nucleopore filter with 100-nm diameter pores using a Basic LiposoFast device. The fluorescence excitation was set at 470 nm while the emission was measured at 530 nm using a 515 nm cut off filter. *Exo*-labeled vesicles were generated by adding 0.5 mol% NBD-lipid (in 200 proof ethanol) to 25 μM unlabeled vesicles in 45 mL of TES buffer. Subsequently, 1.25 μM translocase (in DMSO) was added and time points were taken. During a 200 s scan, 180 μL of sodium dithionite (60 mM in 1 M Tris, pH ~10) was added at 50 s and 20 μL of 20% Triton X-100 (v/v) was added at 180 s. The percent of *exo* NBD-lipid was calculated by the following equation: (% *exo* NBD-lipid) = $(F_i - F_f)/F_i$, where F_i and F_f are the fluorescence intensities just prior to the additions of sodium dithionite and Triton X-100. The values reported in Table 1 are the average of three independent experiments.

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