

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

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Abstract—A series of methyl $7\alpha,12\alpha$ -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.

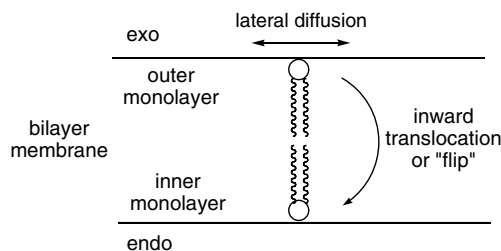
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1. Introduction

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 aminophospholipid 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 apop-

totic 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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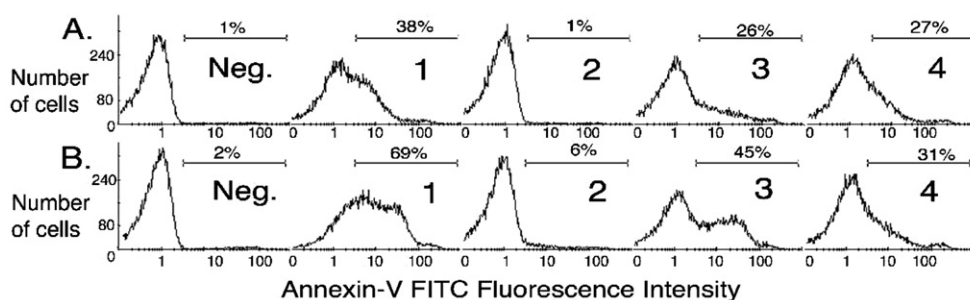


Figure 1. Flow cytometry analysis of annexin V-FITC staining of (A) normal erythrocytes or (B) NEM-pretreated erythrocytes. Before exposure to annexin V-FITC, the erythrocytes were incubated for 3 h at 37 °C with scramblase candidate (10 μ M).

separate samples of erythrocytes were incubated with each of the cholates (from concentrated DMSO stock solutions; final concentration 10 μ M) 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 head-groups, 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 CHCl₃/CH₃OH, using a PS analogue with two short, C₆-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 $(3.4 \pm 0.9) \times 10^5 \text{ M}^{-1}$ and $(0.9 \pm 0.2) \times 10^5 \text{ M}^{-1}$, respectively.¹⁰ Repeating the UV titrations with the new cholates lead to K_{PS} values of $(3.3 \pm 1.8) \times 10^5 \text{ M}^{-1}$ for **3** and $(6.0 \pm 2.0) \times 10^4 \text{ M}^{-1}$ 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 μ M of scramblase candidate to a dispersion of POPC/cholesterol (7:3) vesicles (25 μ M 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.

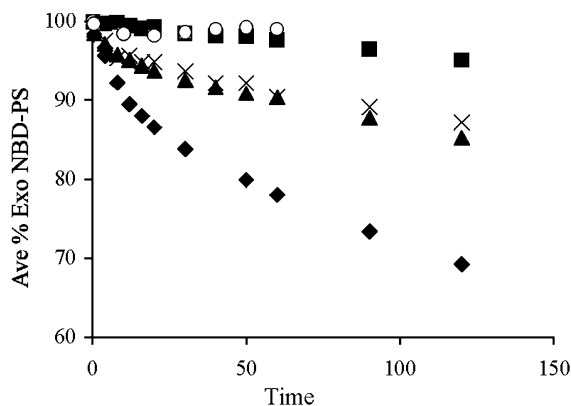


Figure 2. Change in the percent NBD-PS in the outer monolayer (% *exo* NBD-PS) of POPC/cholesterol vesicles (7:3) at room temperature and pH 7.4. Inward translocation induced at $t=0$ min by adding 1.25 μ M of cholate **1** (◆), **2** (■), **3** (▲), **4** (×) and DMSO control (○) to vesicles (25 μ M) 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 μ M scramblase with an error of $\pm 5\%$ (25 μ M 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 3 α -[(2-(chloro)methylcarbonyl)amino]-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -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 3 α -amino-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -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

CH₂Cl₂, washed with aq 1 N HCl, satd aq NaHCO₃, water, dried (Na₂SO₄), and the solvent removed in vacuo to give 0.0421 g of the crude material. Column chromatography (SiO₂), 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, CDCl₃) δ 0.80 (s, 3H, 18-CH₃), 0.94 (br s, 6H, 21-CH₃ and 19-CH₃), 3.35 (br s, 1H, 12 β -H), 3.65 (s, 3H, CO₂CH₃), 3.87 (m, 1H, 7 β -H), 3.96 (br s, 2H, CH₂Cl), 4.13 (br s, 3H, 3 β -H), 5.38 (br s, 2H, NH), 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 3 α -[(2-(bromo)methylcarbonyl)amino]-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -cholan-24-oate (**8**).

A mixture of the methyl 3-[(2-(chloro)methylcarbonyl)amino]-7,12-bis[(phenylaminocarbonyl)amino]-5 β -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 CHCl₃, washed with water, dried (Na₂SO₄), 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, CDCl₃) δ 0.79 (s, 3H, 18-CH₃), 0.93 (br s, 6H, 21-CH₃ and 19-CH₃), 3.30 (br s, 1H, N-H), 3.65 (s, 3H, CO₂CH₃), 3.80 (br s, 1H, 7 β -H), 3.93 (br s, 3H, CH₂Br and 12 β -H), 4.12 (br s, 3H, 3 β -H), 5.44 (br s, 1H, NH), 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 3 α -[(2-(N,N,N-trimethylammonium)methylcarbonyl)amino]-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -cholan-24-oate bromide (3**).** Methyl 3 α -[(2-(bromo)methylcarbonyl)amino]-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -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-Br⁺); ¹H NMR (300 MHz, CDCl₃) δ 0.83 (s, 3H, 18-CH₃), 0.96 (s, 3H, 19-CH₃), 0.99 (t, $J=6.0$ Hz, 3H, 21-CH₃), 3.09 (d, 9H, NCH₃), 3.62 (s, 3H, OCH₃), 3.77 (br s, 1H, 7 β -H), 3.81 (d, $J=3.0$ Hz, 2H, C(O)CH₂N), 3.91 (br s, 1H, 12 β -H), 4.32 (br d, $J=9.9$ Hz, 1H, 3 β -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); ¹³C NMR (75 MHz, CDCl₃) *Selected Diagnostic Data* δ 118.0 (Ar-CH), 118.1 (Ar-CH), 118.2 (Ar-CH), 118.4 (Ar-CH), 121.4 (Ar-CH), 128.0 (Ar-CH), 128.8 (Ar-CH), 140.7 (Ar-C), 140.8 (Ar-C), 155.2 (NHCONH), 155.4 (NHCONH), 160.2 (3 α -O₂CCH₂), 174.9 (CO₂CH₃).

4.1.4. Methyl 3 α -[2-(*N*-(*t*-butyloxycarbonyl)amino)-acetoxyl-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -cholan-24-oate (10). A solution of EDC/HCl (0.146 g, 0.761 mmol) in CH₂Cl₂ (8 mL) was added dropwise over ~30 min to a solution of the methyl 3 α -hydroxy-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -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 CH₂Cl₂ (5 mL) at rt. The resulting solution was allowed to stir at rt for 21 h, after which time it was diluted with CH₂Cl₂, washed with 4% aq NH₄HCl, satd aq NaHCO₃, water, dried (Na₂SO₄), and the solvent removed in vacuo. Radial chromatography (SiO₂), 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, CDCl₃) δ 0.79 (s, 3H, 18-CH₃), 0.87 (s, 3H, 19-CH₃), 0.90 (d, *J* = 3.3 Hz, 3H, 21-CH₃), 1.49 (s, 9H, *t*-Bu), 3.63 (s, 3H, CO₂CH₃), 3.84–4.20 (m, 3H, NCH₂ and 7 β -H), 4.22 (s, 2H, 12 β -H), 4.31 (br s, 1H, 3 β -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 3 α -[2-(amino)-acetoxyl-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -cholan-24-oate (4). To a solution of methyl 3 α -[2-(*N*-(*t*-butyloxycarbonyl)amino)-acetoxyl-7 α ,12 α -bis[(phenylaminocarbonyl)amino]-5 β -cholan-24-oate **10** (0.288 g, 0.353 mmol) in dichloromethane (3 mL) at 0 °C under N₂ 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 CH₂Cl₂, washed with satd aq NaHCO₃, dried (Na₂SO₄), 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, CDCl₃) δ 0.72 (s, 3H, 18-CH₃), 0.80 (s, 3H, 19-CH₃), 0.88 (s, 3H, 21-CH₃), 3.10 (br s, 2H, NH₂), 3.26 (br s, 3H, NCH₂), 3.63 (s, 3H, CO₂CH₃), 3.86 (br s, 1H, 7 β -H), 4.12 (br s, 1H, 12 β -H), 4.47 (br s, 1H, 3 β -H), 4.81 (br s, 1H, 7 α -NH), 5.55 (br s, 1H, NH), 5.74 (br s, 1H, NH), 6.84–7.00 (m, 2H, Ar-H), 7.20–7.40 (m, 8H, Ar-H); ¹³C NMR (125 MHz, CDCl₃) Selected Diagnostic Data δ 119.1 (Ar-CH), 120.0 (Ar-CH), 122.7 (Ar-CH), 123.4 (Ar-CH), 129.0 (Ar-CH), 129.2 (Ar-CH), 138.5 (Ar-C), 139.0 (Ar-C), 139.4 (Ar-C), 154.2 (NHCONH), 155.4 (NHCONH), 172.9 (3 α -O₂CCH₂), 175.1 (CO₂CH₃).

4.2. Biochemical assays

4.2.1. Flow cytometry assay. Blood samples were collected from a single healthy donor by venipuncture 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 Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). A 50% hematocrit solution was made. Samples that were pretreated with NEM used 10 mM NEM in PBS (1.5 \times 10⁸ 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 \times 10⁸ cell/mL in PBS buffer. To separate solutions of 10 μ M scramblase in PBS, were added 50 μ L of either normal or NEM pretreated erythrocytes (final cell 1.5 \times 10⁷ 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 μ L of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4).²¹ To 100 μ L of each sample (1.5 \times 10⁶ cells), was added 15 μ L of annexin V-FITC (BD Biosciences/PharMingen), followed by incubation at 37 °C for 15 min. An additional 600 μ L 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 CHCl₃ 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 μ M unlabeled vesicles in 45 mL of TES buffer. Subsequently, 1.25 μ M translocase (in DMSO) was added and time points were taken. During a 200 s scan, 180 μ L of sodium dithionite (60 mM in 1 M Tris, pH ~10) was added at 50 s and 20 μ L 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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References and notes

- Boon, J. M.; Smith, B. D. *Med. Res. Rev.* **2002**, *22*, 251.
- Williamson, P.; Schlegel, R. A. *Biochim. Biophys. Acta* **2002**, *1585*, 53.
- Serra, M. V.; Kamp, D.; Haest, C. W. M. *Biochim. Biophys. Acta* **1996**, *1282*, 263.
- Op den Kamp, J. A. F. *Ann. Rev. Biochem.* **1979**, *48*, 47.
- Borisenko, G. G.; Matsura, T.; Liu, S.; Tyurin, V. A.; Jianfei, J.; Serinkan, F. B.; Kagan, V. E. *Arch. Biochem. Biophys.* **2003**, *413*, 41.
- Daleke, D. L.; Lyles, J. V. *Biochim. Biophys. Acta* **2000**, *1486*, 108.

7. Schlegel, R. A.; Williamson, P. *Cell Death Differ.* **2001**, *8*, 551.
8. Fadok, V. A.; Bratton, D. L.; Rose, D. M.; Pearson, A.; Ezekewitz, R. A. B.; Henson, P. M. *Nature* **2000**, *405*, 85.
9. Martin, S. J.; Reutelingsperger, C. P. M.; McGahon, A. J.; Rader, J. A.; Vanschie, R. C. A. A.; Laface, D. M.; Green, D. R. *J. Exp. Med.* **1995**, *182*, 1545.
10. Boon, J. M.; Lambert, T. N.; Sisson, A. S.; Davis, A. P.; Smith, B. D. *J. Am. Chem. Soc.* **2003**, *125*, 8195.
11. Smith, B. D.; Lambert, T. N. *Chem. Commun.* **2003**, *18*, 2261.
12. Boon, J. M.; Smith, B. D. *J. Am. Chem. Soc.* **1999**, *121*, 11924.
13. Boon, J. M.; Smith, B. D. *J. Am. Chem. Soc.* **2001**, *123*, 6221.
14. Boon, J. M.; Shukla, R.; Smith, B. D.; Licini, G.; Scrimin, P. *Chem. Commun.* **2002**, 260.
15. Boon, J. M.; Lambert, T. N.; Smith, B. D.; Beatty, A. M.; Ugrinova, V.; Brown, S. N. *J. Org. Chem.* **2002**, *67*, 2168.
16. Lambert, T. N.; Boon, J. M.; Smith, B. D.; Pérez-Payán, M. N.; Davis, A. P. *J. Am. Chem. Soc.* **2002**, *124*, 5276.
17. Lambert, T. N.; Smith, B. D. *Coord. Chem. Rev.* **2003**, *240*, 129.
18. Leberle, K.; Kempf, I.; Zundel, G. *Biophys. J.* **1989**, *55*, 637.
19. McIntyre, J. C.; Sleight, R. G. *Biochemistry* **1991**, *30*, 11819.
20. Moss, R. A.; Bhattacharya, S. *J. Am. Chem. Soc.* **1995**, *117*, 8688.
21. Vermes, I.; Haanen, C.; Steffens-Nakken, H.; Reutelingsperger, C. *J. Immunol. Methods* **1995**, *184*, 39.