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Synthesis of Trifunctional Phosphatidylserine Probes for Identification of Lipid-Binding Proteins

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Phosphatidylserine (PS) lipids play a number of roles in cell biology, one of which is to mark apoptotic cells for clearance by macrophages. PS recognition triggers macrophage recognition and clearance, which occurs concomitantly with active suppression of an inflammatory response. A small number of proteins in different tissues have been identified as PS receptors, and we hypothesize that many more PS receptors have yet to be found. We have designed and synthesized a set of phosphatidylserine lipid mimics with a PS lipid headgroup for recognition, a benzophenone moiety for photoaffinity cross-linking, and an alkyne for post-labeling readout. These probes may be useful tools to identify new PS receptors (PSRs). The PS lipid probes thus have potential impact in the areas of discovery biology, anti-inflammatory therapeutics, and cellular delivery. We present herein a versatile and robust synthetic approach to trifunctional phosphatidyl serine lipid mimics for identification of novel PSR proteins.

Introduction

Engulfment of apoptotic cells during tissue remodeling and concomitant suppression of inflammation are complex processes that are still not completely understood.[1,2] A lynchpin event in the clearance of apoptotic and necrotic cells is phosphatidylserine (PS) lipid recognition. PS lipids are primarily found on intracellular membrane surfaces, modulating electrostatic targeting of proteins to the membrane.[3,4] This membrane asymmetry is lost when the cell is activated or apoptotic; surface presentation of PS lipid on apoptotic cells provides a recognition trigger for engulfment by neighboring cells and professional phagocytes such as macrophages during the course of tissue remodeling. As receptors that directly bind to PS have only recently been found and validated,[5–8] opsonin receptors[2] have been the subject of the majority of studies on PS-triggered phagocytosis. Three macrophage opsonin receptors have been well studied and have been shown to be important in some, but not all, cell clearance processes, leaving open the question of how the remaining processes are mediated. The distinct PS lipid recognition proteins implicated in these findings suggest redundant and compensatory roles in apoptotic cell clearance to ensure stable homeostatic cell populations. We hypothesize that there are many more unknown PS opsonizing proteins as well as tissue-specific receptors. Discovery of the key players in these early stages of phagocytosis remains a limiting step in understanding this critical process, as only a handful of mutually exclusive receptors have been found in different cell lines. Phosphatidylserine affinity probes would provide an unbiased screen for cell-surface and PS-opsonin receptors across all cell lines and serum types; this would greatly facilitate advances in understanding phagocytosis and related biological processes. Given the generality of PS-lipid-mediated engulfment, we believe that PS recognition and the associated biological response has been underexploited in biotechnology. PS recognition could provide a cell-entry pathway of therapeutic importance. Synthetic entry to PS-derivatized materials would enable exploration along the lines of discovery biology through the identification of novel PS receptors (PSRs). We present herein the synthesis of lipid affinity probes that mimic phosphatidylserine lipid.

Our synthetic approach to mixed 1,2-*O*-diacyl-*sn*-glycerol PS lipid photoaffinity probes expands on procedures for the preparation of phosphoinositol lipid derivatives previously reported by Prestwich[9] and utilizes the concept of trifunctional photocross-linking probes developed for activity-based protein profiling by Cravatt and coworkers.[10,11] A protected phosphatidylserine glycerol core is first synthesized with a protected terminal amino group on one of the two lipid acyl chains. The PS glycerol core is then acylated to install a benzophenone photoaffinity module and an alkyne group. This general strategy was used to prepare the set of lipid probes shown in Figure 1. The phosphatidylserine headgroup is envisioned to bind directly to the PSRs, which are then captured by photoaffinity labeling with the benzophenone group; this labeled PSR is then identified on a cell lysate gel through in-gel reaction of a fluorophore azide with the probe alkyne by using azide–alkyne [2+3] cycloaddition. The alkyne permits post-label-

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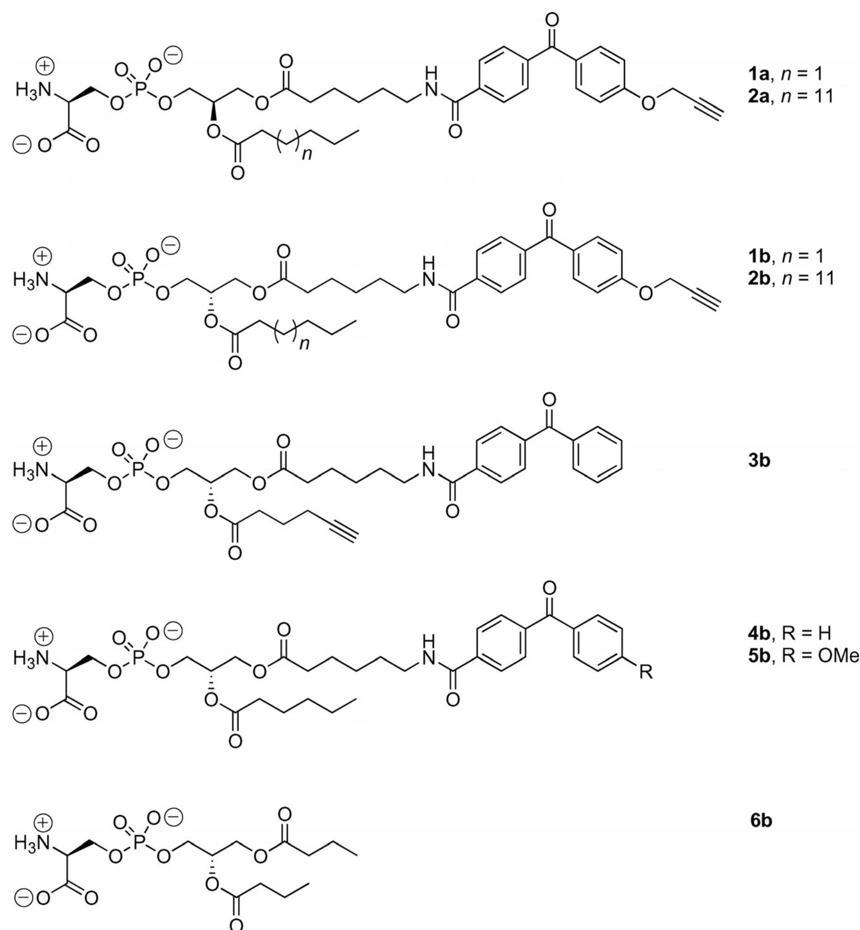

Figure 1. Phosphatidylserine lipid mimics synthesized in this study. Compounds with the natural glycerol configuration are denoted with the suffix “a” (**1a**, **2a**), whereas compounds with the nonnative configuration at glycerol are denoted with the suffix “b” (**1**–**6b**).

ing readout of captured lipid-binding protein targets, thereby excluding contributions of fluorophore or affinity tag binding to the labeling process. PS lipid mimics **4b** and **5b** were also prepared for use as competitive photocross-linking probes without alkyne readout; **6b** was prepared for use as a soluble PS lipid competitive binding inhibitor to assay for PS probe binding selectivity (Figure 1). As PSRs important in cell clearance are expected to be surface-expressed, cellular uptake is not an issue. Therefore, photoaffinity tagging by these PS lipid probes may be performed on intact, living cells as well as with fractionated cell lysates, followed by in-gel readout of the labeled fraction by using azide–alkyne cycloaddition with a fluorophore azide. To date, there have been relatively few phosphatidylserine lipid syntheses reported,[12–16] and while the general synthesis and application of lipid photoaffinity probes to identify biological partners has been well established,[17–30] none of these prior efforts are focused on the use of PS lipid mimics as affinity capture reagents for PS binding partners.[26] Photoaffinity probes based on PS lipids have been previously reported by using azido-photoactivated cross-linking prepared through enzyme-catalyzed choline–serine headgroup exchange.[26,31] Phosphatidylserine lipid derivatives for delivery and imaging applications have also been synthesized through enzymatic and synthetic manipulation of lysophosphatidylcholine lipid, an expensive starting material that restricts modification to the *sn*-2 position.[32] Glycidyl derivatives have also been used as a starting point to ether phospholipids through Lewis acid catalyzed epoxide ring opening;[12–14] addition to the epoxide with a long-chain carboxylic acid may yield phospholipid diesters.[33] To our surprise, regioselective stepwise acylation[34,35] of monoprotected glycerol, a robust approach that has been widely used to prepare mixed 1,2-*O*-diacylglycerol phospholipids, has not been previously used to prepare phosphatidylserine lipid derivatives. We describe herein our application of this known, versatile, and scalable strategy for lipid synthesis to produce new PS lipid affinity probes for discovery biology (Figure 1).

Results and Discussion

Synthesis of a Selectively Protected Phosphatidylserine Lipid Core

The regioselective glycerol acylation approach allows installation of the benzophenone photocross-linking module and the alkyne readout tag at either the *sn*-1 or *sn*-2 ester

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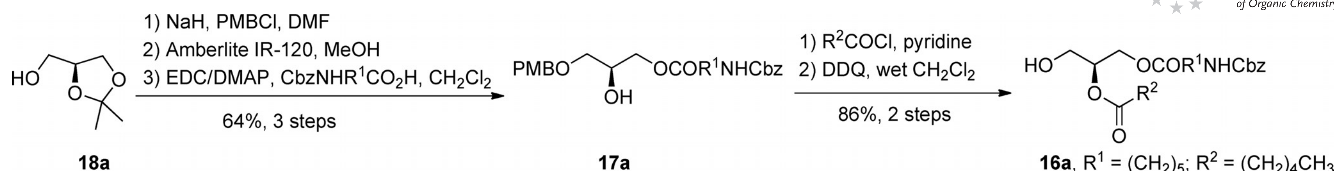
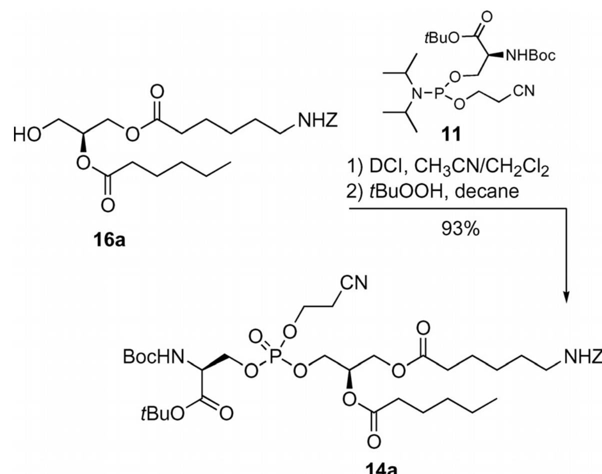
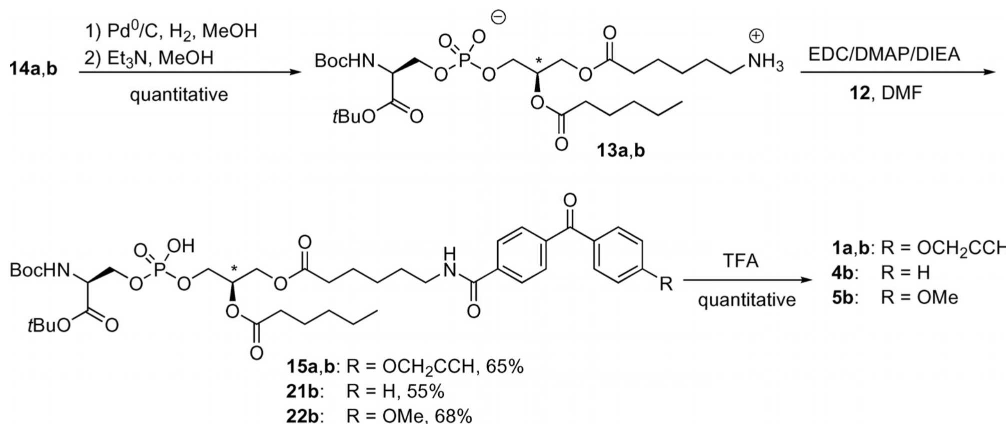


Scheme 1.

position. We designed a convergent synthetic scheme that begins with protected glycerol **18** (Scheme 1). Both enantiomers are commercially available, allowing ready access to the natural lipid stereochemistry (i.e., **18a**) as well as its stereoisomers. We prepared probes with native stereochemistry (i.e., **1a**, **2a**) as well as probes with the inverted glycerol center (i.e., **1b**, **2b**) to facilitate examination of stereoselectivity of probe recognition; the routes to the diastereomers were found to be identical in terms of isolated yield. Thus, a series of known synthetic manipulations of D-(+)-1,2-isopropylideneglycerol, which has the natural configuration at C2, led to **17a**, an enantiopure glycerol nucleus[9] with *p*-methoxybenzyl (PMB)-protection on the primary hydroxy group, free secondary hydroxy group, and the 6(*N*-Cbz-amino)hexanoate primary ester (Scheme 1). DMAP-catalyzed acylation of the secondary alcohol with hexanoyl chloride or palmitoyl chloride yielded diacyl glycerol frameworks for water-soluble and lipid-soluble probes, **1a** and **2a**, respectively. Oxidative cleavage of the PMB protecting group from water-soluble probe precursor **16a** freed the primary hydroxy group of the mixed 1,2-*O*-diacyl-*sn*-glycerols for O–P coupling with preassembled serine phosphoramidite **11** (Scheme 2).[9,36–38] Intermediate **11** was derived by condensation of Boc-Ser-O*t*Bu and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, yielding a mixture of epimers at the phosphorus center. Coupling of **11** with glycerol **16a** generated the serine glyceryl phosphite, which was oxidized in situ to obtain protected phosphate **14a** as a mixture of diastereomers at the chiral phosphorus center, which were distinguishable by ^{31}P NMR spectroscopy as a pair of singlets at –1.6 and –1.7 ppm. The fully constructed and differentially protected phosphatidylserine nucleus formed in Scheme 2 was then subjected to CBz hydrogenolysis to free the primary amine at the acyl chain terminus. This resulted in partial cyanoethyl deprotection at the phosphorus center, which could be suppressed with formic acid during hydrogenolysis or driven to completion with an excess amount of triethylamine (Scheme 3).[39] Acylation of the resulting zwitterionic intermediate **13** with benzophenone carboxylic acid derivatives gave isolated yields of 55–68% of the serine-protected PS lipid probes. We suspect that these modest yields are due to inefficient recovery from silica gel of the free phosphate intermediates, as purification on ion-exchange resin instead of silica afforded pure products with higher recovery (Supporting Information). To broaden options for variation at one glycerol ester position,



Scheme 2.



Scheme 3. Identical separate routes employed for either glycerol stereoisomer at the chiral center marked with *. Structure is shown with native configuration as found in **1a**, **13a**, **14a**, and **15a**, whereas **1b**, **4b**, **5b**, **15b**, **21b**, and **22b** have inverted stereochemistry. **12** = $HO_2CC_6H_4COC_6H_4$-R.

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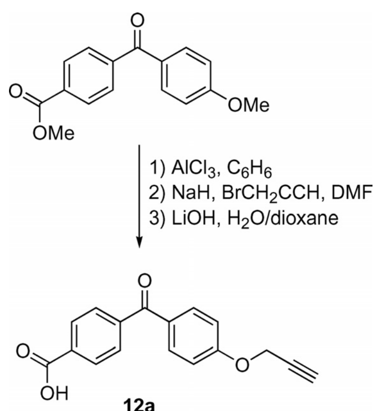
we combined benzophenone and the alkyne in one module by installation of a propargyl ether substituent the on benzophenone carboxylic acid (compound **12a**, Scheme 4); this approach potentially allows wide variation at the remaining ester position. Serine deprotection with trifluoroacetic acid then yielded the free lipid probes. Using this straightforward acylation approach, PS lipid derivatives were constructed with benzophenone and alkyne groups for cross-linking and post-labeling readout. Water-soluble and lipid-soluble PS lipid probe derivatives were prepared simply by varying the chain length of the fatty acid ester at the 2-glycerol position; hexanoates **1a** and **1b** were water-soluble and nonaggregated, whereas palmitate derivatives **2a** and **2b** were not water soluble but partitioned readily into lipid vesicles. Probes were incubated with lipid vesicles then sedimented at 10^5 g; **1a** concentration in the supernatant was unchanged as judged by benzophenone absorption, whereas **2a** was sedimented completely with the lipid pellet. Lipid-soluble probes may be desirable to mimic the context in which PSRs for cell-clearance encounter PS lipid on the surfaces of apoptotic cells. Though we have only prepared derivatives with straight chain saturated acylation at the central glycerol position, these procedures could be used to install other lipid anchors with different biophysical properties, such as sterol and sphingosine anchors.[40–42] Probes were prepared with the native glycerol configuration (i.e., **1a**, **2a**) as well as probes with inverted glycerol stereochemistry (i.e., **1b**, **2b**, **4b**, and **5b**). Compounds with nonnative glycerol stereochemistry were prepared to test whether or not native stereochemistry was required at both serine and glycerol chiral centers for recognition and function.

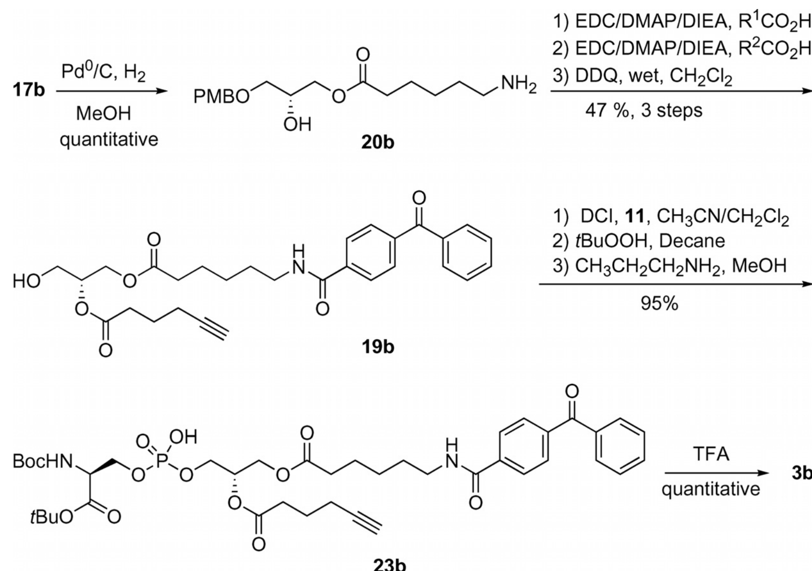
While the combination of photocross-linking and readout at one position is a versatile design, this may also result in greater steric barriers to labeling and readout. To address this possibility, we also prepared PS probe molecules with photolabeling and readout moieties separately displayed at two different ester positions (Scheme 5). This approach is essentially the same, with the exception that Cbz protection is cleaved prior to acylation at *sn*-2, allowing regioselective amidation with benzophenone carboxylic acid, followed by esterification with hexynoic acid. Reaction with phosphoramidite **11** and global deprotection with an base/acid sequence then yields probe **3b** with photoaffinity and alkyne tags at separate glycerol positions.



Scheme 4.

Biotin- and Fluorescein-Derivatized Phosphatidylserine Lipid Mimics

Lipid-based probes have potential utility as intracellular probes.[43,44] Derivatization of PS lipid mimics with affinity and fluorescence tags may also be useful in target enrichment and diagnostic applications. Intermediate **13b** was subjected to Cbz hydrogenolysis and directly treated with fluorescein isothiocyanate (FITC) to yield fluorescein derivative **7b** (Scheme 6); this compound may be useful to follow PSR cellular localization[32] or as a reagent to screen chemical libraries for PS lipid binding elements.



Scheme 5. $R^1 = C_6H_4COC_6H_5$, $R^2 = (CH_2)_3CCH$. Intermediate **17b** is the enantiomer of **17a** in Scheme 2.

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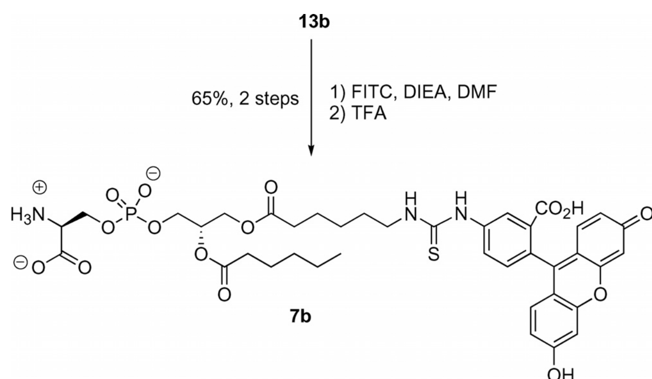


Scheme 6.

coupling FITC with 2-azidoethylamine. Biotin azide derivatives were similarly prepared through acylation of amino azide linkers with biotin active esters to facilitate isolation and enrichment of labeled species on an avidin support; a biotin thiolester azide derivative was also prepared to increase the efficiency of elution of support-bound proteins through thiolester cleavage. Under known copper-catalyzed azide–alkyne [2+3] cycloaddition conditions in aqueous solvent, each of these azides coupled cleanly to soluble probe **1b** within 2 h, as indicated by HPLC and mass spectrometric analysis (Scheme 7). These validated molecular tools are currently being used in our search for new cell-surface PSRs.

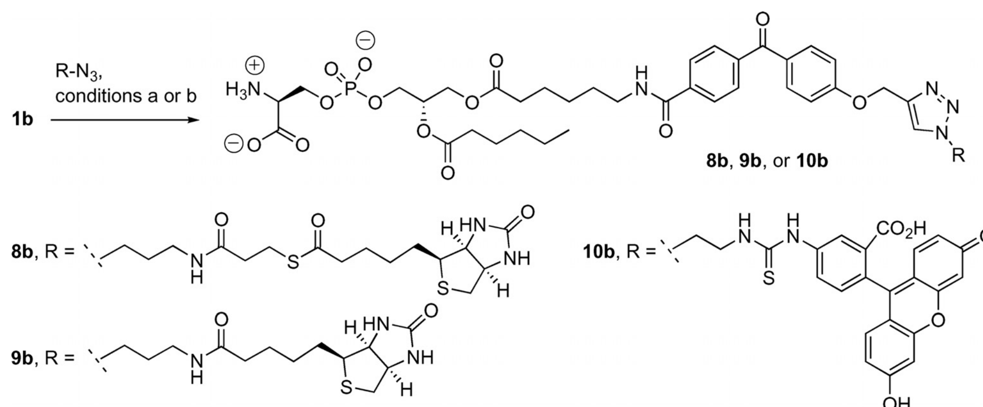
Probe Readout by [2+3] Azide–Alkyne Cycloaddition

Photoaffinity probe derivatization with fluorescent and enrichment tags is of particular importance to their utility in discovery biology, as the chemistry for readout of affinity labeling of cell lysate fractions and subsequent target identification must be robust. While [2+3] azide–alkyne click cycloaddition has been used extensively for this purpose,[32,45–49] the propargyloxybenzophenone probe module has not been previously validated as an efficient substrate. To address this issue, we carried out the desired readout and isolation reactions on soluble probe **1b** (Scheme 7).

In a typical protein-labeling procedure based on published protocols with benzophenone photoaffinity probes,[10,11] the PS probe is incubated with protein or a mixture of proteins and then photocross-linked with UV light at 0 °C, followed by readout by "click" [2+3] azide–alkyne cycloaddition with an azide-derivatized fluorophore. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) is then used to visualize the profile of probe binding propensities within the protein mixture as a function of fluorescence intensity of each protein band on SDS-PAGE. We set out to confirm that click functionalization would proceed smoothly with relevant azide derivatives under these conditions. Both rhodamine azide and fluorescein azide were prepared: acylation of 3-azidopropylamine with *N*-hydroxysuccinimidyl ester of rhodamine furnished rhodamine azide, whereas fluorescein azide was prepared by

Protein-Labeling with Soluble Benzophenone–Alkyne-Functionalized PS Lipid Probes

Though propargyloxybenzophenone carboxylic acid **12a** has not been previously explored as a photoaffinity tag for protein labeling, it is well suited for the trifunctional probe strategy described by Cravatt and co-workers and is readily prepared by using adaptations of known procedures (Scheme 4).[50] This is conceptually related to the heterobifunctional cross-linking maleimidobenzophenone affinity probes that combine targeted benzophenone photocross-linking with thiol capture on a pendant maleimide group.[51] Replacement of the maleimide with alkyne results in improved chemoselectivity and probe stability. In preliminary studies, we examined the ability of soluble PS probes **1a** and **1b** to label prothrombin-1 (PT-1), which has a known affinity for PS lipids.[52] Soluble PS probe **1a** has native stereochemistry, whereas **1b** has inverted stereochemistry at the glycerol center and native L-serine configuration, allowing isolation of the effect of phosphoserine recognition from the glycerol backbone. Our studies revealed that **1a** and **1b** both labeled PT-1 with comparable efficiency in a concentration-dependent manner, as judged by in-gel fluorescence intensity of the protein band on SDS-PAGE (Figure 2), whereas no labeling was detected with heat-denatured PT-1 protein. These preliminary labeling studies indicate that protein-labeling with our synthetic probes is indeed possible under standard photolabeling conditions and



Scheme 7. Conditions a: $CuSO_4$, sodium ascorbate, water; conditions b: $CuSO_4$, TCEP, TBTA, water.

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that the glycerol stereochemistry may not be generally required for lipid recognition, though further examination is necessary. Exploration of differential labeling profile of this set of PS-lipid photoaffinity probes in cell lysates of phagocytic and nonphagocytic cell lines is currently underway.

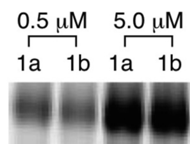

Figure 2. In-gel fluorescent readout of probe-labeled PT-1 using **1a** and **1b** and rhodamine azide at the indicated probe concentrations.

Conclusions

We have demonstrated here a straightforward and flexible synthesis of PS lipid photoaffinity probes to aid in the search for novel cell-surface PSR proteins important in mediating cell clearance of apoptotic cells. Preliminary data has been presented, which demonstrates the ability of these probe molecules to react with lipid binding proteins. Given the limited knowledge currently available regarding PSRs, we anticipate that approaches of this type will be useful in the elucidation of PSR biology, which has an impact on cellular delivery strategies as well as control of inflammation.

Experimental Section

General Procedure for the Purification of Probe Compounds: After complete removal of the Boc and *tert*-butyl ester protecting groups on the headgroup serine, the excess amount of trifluoroacetic acid was removed under reduced pressure to yield the free phosphatidylserine derivatives as oils. The oil was redissolved in a minimum amount of methanol and precipitated with an excess amount of diethyl ether to give white solids that were washed with diethyl ether (3×) to yield analytically pure PS lipid derivatives **1a**–**6b** (Figure 1). Compounds **1a** and **1b** were further purified by RP-HPLC (C_{18}, gradient run from 0% solvent A to 70% solvent B over 20 min, with elution at 15 min; solvent A = 1% CH_3CN in water, 1% TFA; solvent B = 10% CH_3CN in water, 0.1% TFA). All intermediates were purified by silica gel chromatography (unless specifically noted) and all final compounds were judged to be pure by NMR and HRMS.

Procedure for Synthesis of Serine Phosphoramidite 11: To a solution of Boc-Ser-O*t*Bu (0.41 g, 1.57 mmol) in anhydrous CH_2Cl_2 (10 mL) was added diisopropylethylamine (DIEA; 0.24 g, 1.88 mmol) followed by 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.40 g, 1.73 mmol), and the resulting solution was stirred under an atmosphere of nitrogen. The reaction was judged to be complete after 2 h by TLC and ^{31}P NMR spectroscopy, and the mixture was diluted with CH_2Cl_2 (100 mL), washed with aqueous saturated $NaHCO_3$ (2×60 mL), and dried with anhydrous Na_2SO_4. Concentration under reduced pressure and purification by column chromatography (SiO_2; hexanes/EtOAc/Et_3N, 65:34:1) yielded **11** as a colorless oil (0.67 g, 92%). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 5.45 (br., 0.5 H, N-*H*), 5.34 (br., 0.5 H, N-*H*), 4.30 (br., 1 H, CH_2^{α}), 4.02 (m, 1 H, CH_2^{β}), 3.85 (m, 3 H, O-CH_2, C*H*), 3.59 (m, 2 H, *i*Pr-C*H*), 2.64 (m, 2 H, CH_2CN), 1.48 (br., 9 H, CO_2tBu-CH_3), 1.46 (br., 4.5 H, Boc-*t*Bu-CH_3), 1.45 (br., 4.5 H, Boc-*t*Bu-CH_3), 1.18 (br., 12 H, *i*Pr-CH_3) ppm. ^{31}P NMR (161 MHz, $CDCl_3$, 25 °C): δ = 149.4, 149.2 ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 169.2, 155.1, 155.0, 117.5, 81.9, 81.7, 79.4, 64.4 (d, J_{CP} = 16 Hz), 63.9 (d, J_{CP} = 17 Hz), 58.4 (d, J_{CP} = 18 Hz), 58.2 (d, J_{CP} = 18 Hz), 55.0 (d, J_{CP} = 7.6 Hz), 54.9 (d, J_{CP} = 8.0 Hz), 43.0 (d, J_{CP} = 4.1 Hz), 42.8 (d, J_{CP} = 4.1 Hz), 28.2, 27.8, 24.4 (d, J_{CP} = 7.4 Hz), 20.2 (d, J_{CP} = 6.2 Hz), 20.1 (d, J_{CP} = 6.8 Hz) ppm. HRMS (ESI): calcd. for $C_{21}H_{40}N_3O_6PNa$ [M + Na]$^+$ 484.2553; found 484.2555.

Procedure for the Conjugation of 11 with Diacyl Glycerol and Subsequent Oxidation (14a): A solution of primary alcohol **16a** (0.28 g, 0.64 mmol) and **11** (0.36 g, 0.77 mmol) in anhydrous CH_2Cl_2 (10 mL) was treated with a solution of 4,5-dicyanoimidazole (DCI; 0.22 g, 1.93 mmol) in anhydrous CH_3CN (10 mL). The reaction was run under a nitrogen atmosphere and judged to be complete after 3 h by ^{31}P NMR spectroscopy. The reaction mixture was cooled in an ice bath and subjected to dropwise addition of a solution of *tert*-butylhydrogen peroxide (≈5.5 M in decane, 1.42 mL, 3.85 mmol) then warmed to room temperature over 2 h. Complete oxidation was indicated by ^{31}P NMR spectroscopy, which revealed a ^{31}P chemical shift change from δ = 139.2, 139.1 ppm for the interconverting P^{III} diastereomers to δ = –1.61, –1.67 ppm for the oxidized P^{V} diastereomeric products. Dilution with CH_2Cl_2 (100 mL), acidic workup (3×60 mL of 1 N HCl), followed by 10% sodium sulfite wash (2×40 mL), drying over anhydrous Na_2SO_4, and concentration produced a crude product that was purified by column chromatography (SiO_2; EtOAc/hexanes, 7:3) to yield **14a** as a colorless oil (0.48 g, 93%).

Procedure for Protein Photolabeling and Fluorescence Readout by Click Conjugation: Human prothrombin-1 (PT-1) solutions (44 μL of 1.2 mg/mL stock solution in HBSS buffer with 50 μM $CaSO_4$) were incubated with 4.9 μM PS probe (1 μL of 215 μM stock in HBSS) for 15 min then cooled with an ice bath and irradiated with a long-wave UV lamp (centered at 365 nm) for 1 h. After irradiation, each protein sample was treated with rhodamine azide (final concentration 44 μM, from 40× DMSO stock solution) followed by a freshly prepared catalyst cocktail of TCEP (aqueous stock solution), TBTA (DMSO/*t*BuOH, 1:4 stock solution) and $CuSO_4$ (aqueous stock solution) to yield final concentrations of 1 mM, 100 μM, and 1 mM, respectively. All stock solutions and reaction volumes were prepared with degassed solvents. The resulting reaction mixture was briefly vortexed and incubated at room temperature for 1 h, and then a 10 μL aliquot was collected and quenched with the addition of 6 μL standard 2× SDS-PAGE loading buffer (reducing) followed by heat denaturation of the protein sample for 10 min at 70 °C and analysis on SDS-PAGE (12 μg of protein/gel lane). In-gel visualization was performed by using a Typhoon Trio (GE Healthcare) laser-induced fluorescence scanner followed by standard coomassie staining and destaining.

Phosphatidylserine Probe (1a): Intermediate **15a** (0.19 g, 0.22 mmol) was dissolved in TFA (1 mL), and the mixture was stirred for 4 h at room temperature. After completion of Boc and *tert*-butyl deprotection, the excess amount of TFA was removed, and the resulting phosphatidylserine was triturated with diethyl ether to give **1a** (0.16 g) as a white solid in quantitative yield. ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 7.95 (d, $J_{H,H}$ = 8.5 Hz, 2 H, 3-ArC*H*), 7.82 (d, $J_{H,H}$ = 8.5 Hz, 2 H, 2-ArC*H*), 7.79 (d, $J_{H,H}$ = 8.4 Hz, 2 H, 2′-ArC*H*), 7.12 (d, $J_{H,H}$ = 8.5 Hz, 2 H, 3′-ArC*H*), 5.22 (m, 1 H, *sn*-2-C*H*), 4.86 (d, $J_{H,H}$ = 2.4 Hz, 2 H, OCH_2C) 4.42 (dd, $J_{H,H}$ = 3.72, 12 Hz, 1 H, C*H*), 4.28 (m, 3 H, C*H*, O-CH_2),

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4.18 (m, 1 H, C*H*), 4.01 (m, 2 H, CH_2), 3.41 [m, 2 H, C(O)NH-CH_2], 3.02 (t, $J_{H,H}$ = 2.3 Hz, 1 H, CC-*H*), 2.35 (m, 4 H, CO-CH_2), 1.74–1.54 (m, 6 H, CH_2), 1.44 (m, 2 H, CH_2), 1.31 (m, 4 H, CH_2), 0.89 (t, $J_{H,H}$ = 6.6 Hz, 3 H, CH_3) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): δ = 197.1, 175.3, 175.1, 169.7, 163.7, 142.4, 139.5, 134.0, 131.9, 131.2, 128.8, 116.3, 79.6, 78.0, 72.2 (d, $J_{C,P}$ = 7.5 Hz), 65.6 (d, $J_{C,P}$ = 5.1 Hz), 64.8 (d, $J_{C,P}$ = 4.8 Hz), 64.0, 57.4, 55.24 (d, $J_{C,P}$ = 7.3 Hz), 41.4, 35.5, 35.2, 32.8, 30.6, 28.0, 26.1, 23.9, 14.8 ppm. HRMS (ESI): calcd. for $C_{35}H_{45}N_2O_{13}PNa$ [M + Na]$^+$ 755.2551; found 755.2515. Probe **1b** is indistinguishable from **1a** with respect to HRMS and the ^{1}H and ^{13}C NMR spectra.

Phosphatidylserine Probe (2a): Lipid-soluble phosphatidylserine probe **2a** (0.15 g) was quantitatively obtained from **42** (0.18 g, Supporting Information) following a procedure analogous to that described for the conversion of **15a** into **1a**. ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 7.95 (d, $J_{H,H}$ = 8.6 Hz, 2 H, 3-ArC*H*), 7.81 (d, $J_{H,H}$ = 8.6 Hz, 2 H, 2-ArC*H*), 7.79 (d, $J_{H,H}$ = 8.4 Hz, 2 H, 2′-ArC*H*), 7.12 (d, $J_{H,H}$ = 8.6 Hz, 2 H, 3′-ArC*H*), 5.23 (m, 1 H, C*H*), 4.85 (d, $J_{H,H}$ = 2.3 Hz, 2 H, O-CH_2C), 4.41 (dd, $J_{H,H}$ = 3.5, 12 Hz, 1 H, C*H*), 4.28 (m, 3 H, C*H*, O-CH_2), 4.18 (dd, $J_{H,H}$ = 6.1, 12 Hz, 1 H, C*H*), 4.02 (m, 2 H, CH_2), 3.42 [m, 2 H, C(O)NH-CH_2], 3.00 (t, $J_{H,H}$ = 2.1 Hz, 1 H, CC-*H*), 2.37 [t, $J_{H,H}$ = 7.0 Hz, 2 H, C(O)-CH_2], 2.34 [t, $J_{H,H}$ = 7.0 Hz, 2 H, C(O)CH_2], 1.73–1.54 (m, 6 H, CH_2), 1.44 (m, 2 H, CH_2), 1.26 (br., 24 H, CH_2), 0.88 (t, $J_{H,H}$ = 6.7 Hz, 3 H, CH_3) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): δ = 197.1, 175.3, 175.2, 169.6, 163.7, 142.3, 139.5, 134.0, 131.9, 131.2, 128.9, 116.3, 79.5, 78.0, 72.2 (d, $J_{C,P}$ = 7.9 Hz), 65.7 (d, $J_{C,P}$ = 5.4 Hz), 64.9 (d, $J_{C,P}$ = 4.8 Hz), 64.0, 61.2, (possible impurity) 57.4, 55.2 (d, $J_{C,P}$ = 6.9 Hz), 41.5, 35.6, 35.2, 33.5, 31.3 (br.), 31.2, 31.1, 31.0, 30.7, 30.6, 28.0, 26.5, 26.1, 24.2, 14.9 ppm. HRMS (ESI): calcd. for $C_{45}H_{64}N_2O_{13}P$ [M – H]$^-$ 871.4146; found 871.4122. Probe **2b** is indistinguishable from **2a** with respect to HRMS and the ^{1}H and ^{13}C NMR spectra.

Phosphatidylserine Probe (3b): Lipid-soluble phosphatidylserine probe **3b** (0.17 g) was quantitatively obtained from **23** (0.22 g, Supporting Information) following a procedure analogous to that described the conversion of **15a** into **1a**. ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 7.95 (m, 2 H, 3-ArC*H*), 7.83 (d, $J_{H,H}$ = 8.3 Hz, 2 H, 2-ArC*H*), 7.78 (d, $J_{H,H}$ = 8.2 Hz, 2 H, 2′-ArC*H*), 7.66 (t, $J_{H,H}$ = 7.4 Hz, 1 H, 4′-ArC*H*), 7.54 (m, 2 H, 3′-Ar-C*H*), 5.24 (m, 1 H, C*H*), 4.42 (dd, $J_{H,H}$ = 3.7, 12 Hz, 1 H, C*H*), 4.28 (m, 3 H), 4.19 (dd, $J_{H,H}$ = 6.1, 12 Hz, 1 H, C*H*), 4.02 (m, 2 H, CH_2), 3.42 [m, 2 H, C(O)NH-CH_2], 2.48 (m, 2 H, C-CH_2), 2.38 [m, 2 H, C(O)-CH_2], 2.25 [m, 2 H, C(O)-CH_2], 2.23 (t, $J_{H,H}$ = 1.7 Hz, 1 H, CC-*H*), 1.80 (m, 2 H, CH_2), 1.67 (m, 4 H, CH_2), 1.44 (m, 2 H, CH_2) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): δ = 196.3, 173.5, 172.6, 167.8, 139.9, 138.0, 137.0, 132.8, 129.7, 129.6, 128.3, 127.0, 82.7, 70.4 (br.), 69.0, 63.8, 63.0 (br.), 62.0, 59.4, 53.4 (br.), 39.6, 33.3, 32.3, 28.7, 26.1, 24.2, 23.5, 17.0 ppm. HRMS (ESI): calcd. for $C_{32}H_{38}N_2O_{12}P$ [M – H]$^-$ 673.2162; found 673.2141.

Phosphatidylserine Probe (4b): Lipid-soluble phosphatidylserine probe **4b** (0.13 g) was quantitatively obtained from **21b** (0.16 g, Supporting Information) following a procedure analogous to that described for the conversion of **15a** into **1a**. ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 7.95 (d, $J_{H,H}$ = 8.1 Hz, 2 H, 3-ArC*H*), 7.83 (d, $J_{H,H}$ = 8.1 Hz, 2 H, 2-ArC*H*), 7.79 (d, $J_{H,H}$ = 7.9 Hz, 2 H, 2′-ArC*H*), 7.66 (t, $J_{H,H}$ = 7.6 Hz, 1 H, 4′-ArC*H*), 7.54 (m, 2 H, 3′-ArC*H*), 5.22 (m, 1 H, C*H*), 4.41 (m, 1 H, C*H*), 4.30 (m, 4 H, C*H*, C*H*, O-CH_2), 4.01 (m, 2 H, CH_2), 3.41 [m, 2 H, C(O)NH-CH_2], 2.37 [t, $J_{H,H}$ = 7.0 Hz, 2 H, C(O)-CH_2], 2.33 [t, $J_{H,H}$ = 6.9 Hz, 2 H, C(O)-CH_2], 1.64 (m, 6 H, CH_2), 1.44 (m, 2 H, CH_2), 1.31 (m, 4 H, CH_2), 0.89 (t, $J_{H,H}$ = 6.5 Hz, 3 H, CH_3) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): δ = 197.9, 174.9, 174.8, 159.3, 158.9, 141.4, 139.5, 138.5, 134.3, 131.2, 131.1, 129.8, 128.5, 117.6, 114.8, 71.8 (br.), 65.6, 63.7, 54.6, 54.3, 41.1, 35.1, 34.9, 32.5, 30.2, 27.6, 25.8, 25.7, 23.5, 14.4 ppm. HRMS (ESI): calcd. for $C_{32}H_{42}N_2O_{12}P$ [M – H]$^-$ 677.2475; found 677.2453.

Phosphatidylserine Probe (5b): Water-soluble phosphatidylserine probe **5b** (0.19 g) was quantitatively obtained from **22b** (0.23 g, Supporting Information) following a procedure analogous to that described for the conversion of **15a** into **1a**. ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 7.94 (d, $J_{H,H}$ = 8.2 Hz, 2 H, 3-ArC*H*), 7.80 (d, $J_{H,H}$ = 8.8 Hz, 2 H, 2-ArC*H*), 7.77 (d, $J_{H,H}$ = 8.2 Hz, 2 H, 2′-ArC*H*), 7.05 (d, $J_{H,H}$ = 8.8 Hz, 2 H, 3′-ArC*H*), 5.23 (m, 1 H, C*H*), 4.41 (dd, $J_{H,H}$ = 3.9, 12.2 Hz, 1 H, C*H*), 4.31 (m, 3 H, C*H*, O-CH_2), 4.19 (dd, $J_{H,H}$ = 6.4, 12.0 Hz, 1 H, C*H*), 4.05 (m, 2 H, CH_2), 3.89 (s, 3 H, O-CH_3), 3.41 [m, 2 H, C(O)NH-CH_2], 2.35 [m, 4 H, C(O)CH_2], 1.71–1.56 (m, 6 H, CH_2), 1.44 (m, 2 H, CH_2), 1.31 (m, 4 H, CH_2), 0.89 (t, $J_{H,H}$ = 6.9 Hz, 3 H, CH_3) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): δ = 195.3, 173.3, 167.8, 167.7, 164.0, 140.7, 137.5, 132.3, 129.3, 129.2, 126.9, 113.5, 70.2 (d, $J_{C,P}$ = 7.7 Hz), 64.0, 63.2 (d, $J_{C,P}$ = 5.1 Hz), 62.0, 54.8, 53.3 (d, $J_{C,P}$ = 6.8 Hz), 39.6, 33.6, 33.3, 30.9, 28.7, 26.1, 24.3, 24.2, 22.0, 12.9 ppm. HRMS (ESI): calcd. for $C_{33}H_{44}N_2O_{13}P$ [M – H]$^-$ 707.2586; found 707.2551.

Phosphatidylserine Mimic (6b): Water-soluble phosphatidylserine probe **6b** (0.31 g) was quantitatively obtained from **37** (0.43 g, Supporting Information) following a procedure analogous to that described for the conversion of **15a** into **1a**. ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 5.23 (m, 1 H, C*H*), 4.41 (dd, $J_{H,H}$ = 3.6, 12 Hz, 1 H, C*H*), 4.28 (m, 3 H, C*H*, O-CH_2), 4.20 (dd, $J_{H,H}$ = 6.4, 12 Hz, 1 H, C*H*), 4.00 (m, 2 H, CH_2), 2.32 (m, 4 H, CO-CH_2), 1.64 (m, 4 H, CH_2), 0.95 (m, 6 H, CH_3) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): δ = 175.3, 175.0, 169.7, 72.2 (d, $J_{C,P}$ = 7.1 Hz), 65.6 (d, $J_{C,P}$ = 5.0 Hz), 64.8 (d, $J_{C,P}$ = 5.5 Hz), 64.0, 54.9 (d, $J_{C,P}$ = 7.2 Hz), 37.4, 37.2, 19.5, 14.4 ppm. HRMS (ESI): calcd. for $C_{14}H_{25}NO_{10}P$ [M – H]$^-$ 398.1222; found 398.1217.

Phosphatidylserine Probe (7b): Water-soluble phosphatidylserine probe **7b** (0.14 g) was quantitatively obtained from **29** (0.17 g, Supporting Information) following a procedure analogous to that described for the conversion of **15a** into **1a**. ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 8.25 (s, 1 H, Ar-C*H*), 7.85 (d, $J_{H,H}$ = 8.4 Hz, 1 H, Ar-C*H*), 7.18 (d, $J_{H,H}$ = 8.4 Hz, 1 H, Ar-C*H*), 6.86 (d, $J_{H,H}$ = 8.9 Hz, 2 H, Ar-C*H*), 6.80 ($J_{H,H}$ = 2.5 Hz, 2 H, Ar-C*H*), 6.67 (d, $J_{H,H}$ = 8 Hz, 2 H, Ar-C*H*), 5.15 (m, 1 H, C*H*), 4.36 (dd, $J_{H,H}$ = 3.7, 12 Hz, 1 H, C*H*), 4.21 (m, 4 H, CH, CH, O-CH_2), 3.95 (m, 2 H, CH_2), 3.56 [m, 2 H, C(S)NH-CH_2], 2.29 (m, 4 H, CO-CH_2), 1.67–1.49 (m, 5 H, CH_2), 1.39 (m, 2 H, CH_2), 1.24 (m, 5 H, CH_2), 0.83 (t, $J_{H,H}$ = 6.6 Hz, 3 H, CH_3) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): δ = 171.9, 171.8, 166.3, 152.9, 140.0, 128.4, 126.8, 124.2, 124.1, 112.6, 110.4, 100.6, 68.8 (d, $J_{C,P}$ = 7.0 Hz), 62.2 (br.), 61.4, 60.4, 51.9 (d, $J_{C,P}$ = 5.0 Hz), 42.5, 32.0, 31.9, 29.4, 27.8, 26.7, 24.6, 22.8, 22.7, 20.5, 11.4 ppm. HRMS (ESI-TOF): calcd. for $C_{39}H_{45}N_3O_{15}PS$ [M – H]$^-$ 858.2314; found 858.2318.

Supporting Information (see footnote on the first page of this article): Detailed experimental procedures, mass spectrometric data, and ^{1}H and ^{13}C NMR spectral characterization of all new compounds and synthetic intermediates.

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