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Brief Article

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Design and Synthesis of a Stable Oxidized Phospholipid Mimic with Specific Binding Recognition for Macrophage Scavenger Receptors

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Supporting Information

ABSTRACT: Macrophage scavenger receptors appear to play a major role in the clearance of oxidized phospholipid (OxPL) products. Discrete peptide−phospholipid conjugates with the phosphatidylcholine headgroup have been shown to exhibit binding affinity for these receptors. We report the preparation of a water-soluble, stable peptide−phospholipid conjugate (**9**) that possesses the necessary physical properties to enable more detailed study of the role(s) of OxPL in metabolic disease.

INTRODUCTION

Oxidized low-density lipoprotein (OxLDL) is believed to play an important role in the pathogenesis of atherosclerosis.[1] Unregulated uptake of OxLDL by macrophages within the arterial wall leads to foam cell formation followed by development of the fatty streak that is typical of early atherosclerotic lesions.[1,2] Macrophages express a number of scavenger receptors that bind OxLDL.[3] Among these is CD36,[4] which recent evidence suggests is important in the uptake of OxLDL by macrophages and may have a significant role in inflammation and in foam cell formation in vivo.[5] The epitope(s) responsible for recognition of OxLDL by CD36, and all of the other scavenger receptors, have been only partially defined. Identification of the detailed structural features on OxLDL that are responsible for recognition by CD36 could provide a template for the design of compounds with highly specific interactions with these macrophages and other immune system components with the eventual goal of developing new strategies for the treatment of inflammation and atherosclerosis.

A wide variety of biologically active phospholipid oxidation products can be formed upon oxidation of phospholipids.[6] For example, oxidation of 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (PAPC, **1**) yields an oxidized phospholipid (OxPL) byproduct, 1-palmitoyl-2-(5′-oxo)valeroyl-*sn*-glycero-3-phosphoryl-choline (POVPC, **2**), with a reactive aldehyde at the ε-carbon (Figure 1).[7] This reactive "phospholipid core aldehyde" in turn forms adducts with lysine residues of apoB, or other proteins, as well as with other amine containing phospholipids.[8]

Previous work has shown that binding of OxLDL to CD36 is mediated by oxidized phospholipids (OxPLs).[8a,9] Several different epitopes on OxPLs have been described as ligands for CD36. One set of ligands results from oxidized moieties on *sn*-2 side chain that have the common motif of oxidized and truncated *sn*-2 fatty acids that terminate in γ-hydroxy (or oxo)-

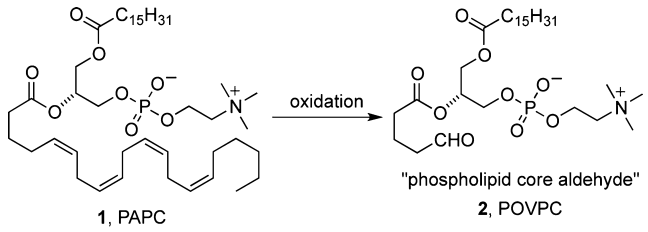

Figure 1. Biological oxidation of PAPC to POVPC.

α,β-unsaturated carbonyl groups.[9c,d] Our laboratory has shown that the phosphocholine (PC) headgroup of oxidized phosphatidylcholines (but not native PL) forms a different and sufficient ligand. Detailed studies of POVPC and variously modified conjugates revealed that the PC headgroup is sufficient for binding to CD36, and furthermore, this activity was retained even after the conjugation of the OxPL to a peptide or protein, mediated by the *sn*-2 aldehyde to yield a Schiff's base.[10] Initial experiments demonstrated that Schiff's base conjugates of POVPC with bovine serum albumin (BSA) inhibited binding of OxLDL to CD36. In a similar manner, POVPC−BSA inhibited the binding of the monoclonal antibody (mAb) E06, which specifically binds the PC headgroup of OxPL but not native PL. Thus, both CD36 and E06 specifically bind the PC of OxPL. A POVPC conjugate with a short peptide chain (**3**, Figure 2) containing a single lysine residue for imine formation also inhibited binding of OxLDL to both CD36 and E06.[9]

The aqueous solution of **3** slowly lost its activity in the assay as the imine hydrolyzed. In an effort to improve the aqueous stability of the Schiff's base conjugate, the imine intermediate was reduced to the corresponding amine (**4**) with sodium cyanoborohydride. This compound retained its competitive

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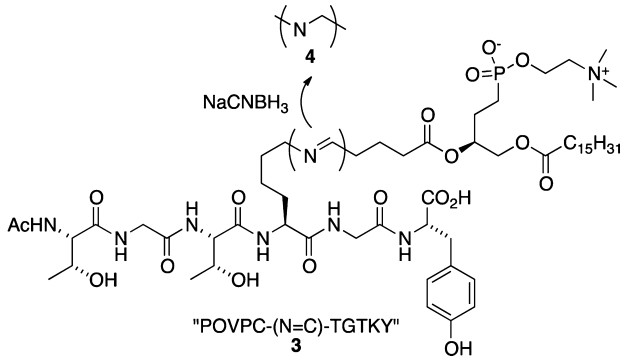


Figure 2. Imine and amine adducts of POVPC.

binding activity but was still prone to a slow decomposition in aqueous solution, presumably the result of delipidation arising from intramolecular *O′N*-acyl transfer (Figure 3).

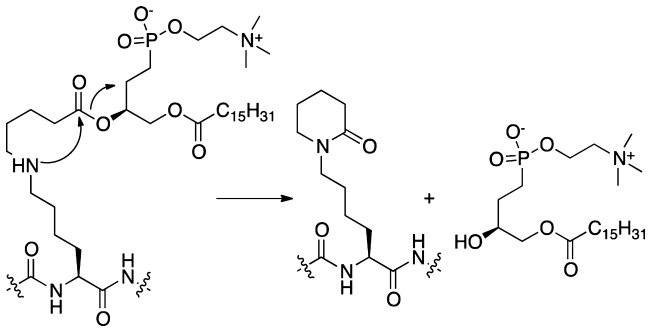


Figure 3. Proposed mechanism for delipidation.

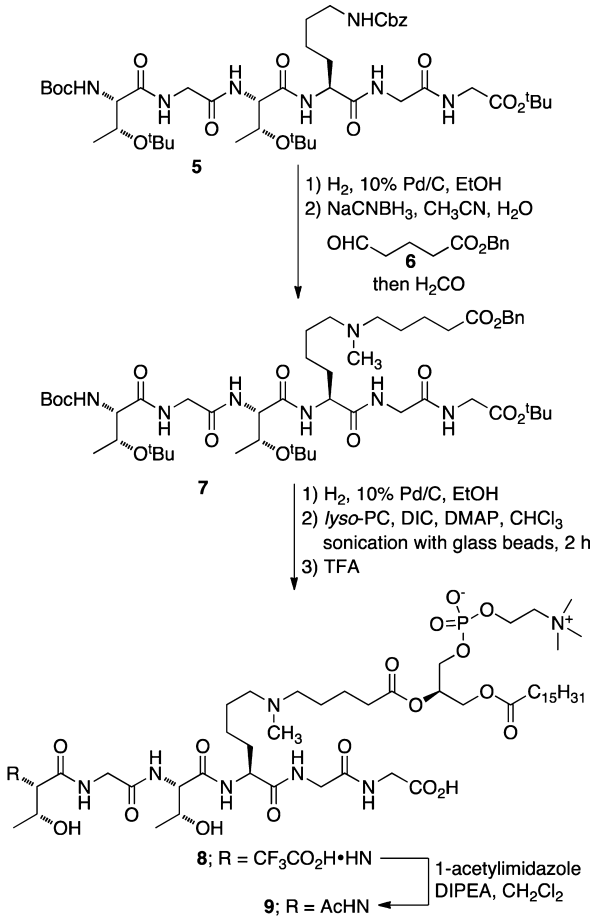
We desired a stable compound with high selectivity and suitable aqueous solubility which would bind to CD36 and to mAb E06 and could be used as a probe in future biological studies and models of atherosclerosis. Instability of both the imine and amine adducts meant that the compounds generated to this point did not meet this goal. Therefore, we set out to find a water-soluble, more stable analogue that maintained the binding specificity of the original POVPC conjugates.

RESULTS

To achieve the desired stability, we felt that modification of the ε-amine of lysine would provide the simplest solution. Conversion of the secondary amine to a tertiary amine should eliminate the acyl transfer reaction that led to the delipidated compound. Our previous work suggested that the amino acid sequence or length of the peptide portion of the mimic was not critical for activity, so we chose to slightly alter the peptide sequence of **4** by replacing the tyrosine with smaller, less-lipophilic glycine to optimize water solubility. These changes produced a new target compound **9**, which was synthesized by the route shown Scheme 1.

The appropriately protected hexapeptide (**5**) was prepared using a manual Fmoc solid phase peptide synthesis followed by esterification of the acid terminus (see Supporting Information (SI)). The ε-amine of the lysine unit of **5** was revealed by cleavage of the carbonyl benzyloxy (Cbz) protecting group, and a double reductive alkylation was performed to generate the tertiary amino compound (**7**). This single-pot double alkylation was conducted by first treating the amine with 1.3 equiv of aldehyde **6** ($NaCNBH_3$, AcOH, CH_3CN, H_2O, 1 h), giving

Scheme 1. Synthetic Route to 9



predominately monoalkylation followed by the addition of excess 37% aqueous formaldehyde solution to provide the second alkylation and the tertiary amine. Using this technique, mixtures with about 80% of the appropriate mixed dialkylated amine could be generated with only minor amounts of the homo-bis-dialkylation product(s). After purification, the benzyl ester of **7** was removed by hydrogenation to unmask the acid group needed for coupling to *lyso*-PC. A survey of the literature uncovered a carbodiimide ester coupling technique using sonication that greatly improved reaction yields and shortened reaction times.[11] The carboxylic acid was converted to the corresponding ester using *lyso*-PC (3 equiv), diisopropylcarbodiimide (DIC) (2 equiv), and 4-dimethylaminopyridine (DMAP) (2 equiv) in chloroform in only 2 h with a yield of 68% using sonication in the presence of glass beads to increase the glass surface area. Couplings done using more traditional solution methodology took several days with yields of 20−30%. The product was globally deprotected (TFA, 0 °C, 2 h) to give **8**, and the amino terminus of the peptide was acylated (1-acetylimidazole, DIPEA, CH_2Cl_2) to give target compound **9**.

The competition for binding of **9** was measured as shown in Figure 4. Compound **9** efficiently competes for binding of biotinylated CuOxLDL to J774 murine macrophages. Compound **9** as well as the positive control, unlabeled CuOxLDL, compete >99% and >90%, respectively, with biotinylated CuOxLDL binding to macrophages, whereas the control peptide TGTKGG and native LDL do not compete. The fixed concentration of biotinylated CuOxLDL (1.8 pmol apoB/mL, corresponding to 1 μg apoB/mL or 0.14 nmol PC-

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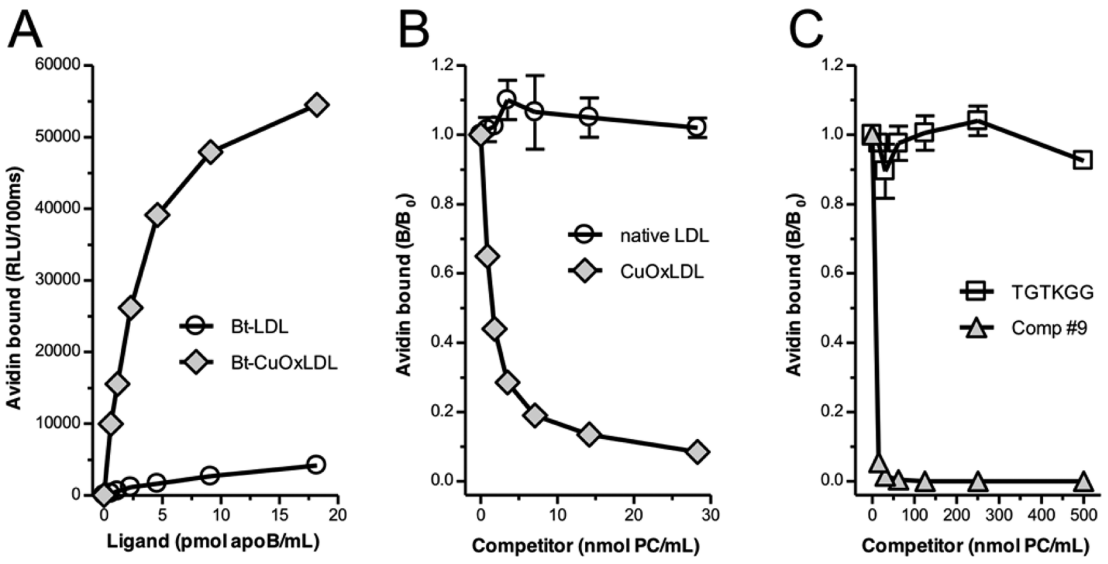

Figure 4. Compound **9** efficiently competes with binding of OxLDL to macrophages. (A) In vitro binding experiment showing that biotinylated CuOxLDL (diamonds) bound in a dose-dependent and saturable manner to J774 macrophages, whereas native LDL (circles) did not. Data are given as relative light units (RLU) per 100 ms. Plot is a representative experiment of >4. (B) CuOxLDL (diamonds), but not native LDL (circles), competes for Bt-CuOxLDL (1.5 μg/mL) binding to J774 macrophages. Data are shown as mean ± SD of B/B_0 from two independent experiments with triplicate determinations. (C) Compound **9** (triangles), but not control peptide TGTKGG (squares), competes for Bt-CuOxLDL (1.5 μg/mL) binding to J774 macrophages. Data are shown as mean ± SD of B/B_0 from two independent experiments with triplicate determinations. Data in (B) and (C) were produced in parallel experiments.

epitopes/mL) was preincubated with compound **9**, the control peptide TGTKGG, CuOxLDL, or native LDL, added at the concentrations indicated on the graphs, and then tested for binding as described in the Experimental Section. The highest CuOxLDL concentration tested was 200 μg/mL, which corresponds approximately 28.3 nmoles of PC-epitopes/mL, as estimated from our previous observation that 1 mol of apoB-100 from CuOxLDL contains 78 ± 15 mol of covalently attached phosphorus,[10] which we assume are OxPL-PC-epitopes only. The concentration of native LDL is plotted at equal protein concentration to CuOxLDL (and thus not as molar concentration of PC-epitopes). Each data point represents the mean and SD of two independent experiments of triplicate wells. In data provided in the SI (Figure S1), we specifically demonstrate that compound **9** competes with high affinity for the binding of CuOxLDL to the CD36 scavenger receptor. These data imply that compound **9** should provide a practical and useful agent for further biological studies in this area. In addition, we show that mAb E06 specifically binds to compound **9** as expected (Figure S2, SI).

CONCLUSION

These data demonstrate that compound **9** represents a water-soluble, stable POVPC−peptide adduct[12] that binds to macrophage scavenger receptors and is able to compete with the binding of OxLDL with high affinity. This compound appears to meet the requirements specified. Because increasing data support a major proinflammatory role for OxPL in disease, this stable model POVPC−peptide adduct should be highly useful in future studies to understand many of the biological roles of oxidized phospholipids and their adducts with proteins. In addition, it may be used to target imaging agents or drugs to macrophages, thus enabling macrophage-specific diagnostic and therapeutic possibilities.

EXPERIMENTAL SECTION

General. See SI for details. All final compounds were confirmed to be of >95% purity based on HPLC analysis.

(6*S*,12*S*,15*S*)-*tert*-Butyl-15-(4-((5-(benzyloxy)-5-oxopentyl)-(methyl)amino)butyl)-6,12-bis((*R*)-1-(*tert*-butoxy)ethyl)-2,2-dimethyl-4,7,10,13,16,19-hexaoxo-3-oxa-5,8,11,14,17,20 hexaazadocosan-22-oate 2,2,2-trifluoroacetate Salt (7). A solution of **5** (2.5 g, 2.7 mmol) in ethanol (60 mL) to a slurry of 10% Pd/C (660 mg) in 20 mL of ethanol. Acetic acid (185 μL) was added, and the solution was put under a balloon of hydrogen gas for 3 h. Completion of the Cbz removal was confirmed by HPLC. The catalyst was removed by filtration through a filter disk, and the solvent was removed in vacuo to give the acetic acid salt of the amine intermediate as a foam (2.07 g, 2.44 mmol, 90% yield). The foam was dissolved in acetonitrile (15 mL) and water (2 mL). Benzyl 5-oxopentanoate (6) (0.654 g, 3.17 mmol, 1.3 equiv) was dissolved in acetonitrile and added to the reaction mixture (total volume 20 mL). Acetic acid (0.559 mL, 9.76 mmol, 4 equiv) was added next, followed by a solution of sodium cyanoborohydride (0.92 g, 14.65 mmol, 6 equiv) in 1 mL of water. The reaction was stirred at room temperature, and the pH was checked (pH 5−6). After 1 h, a 37% formaldehyde solution in water (0.5 mL, ~6 mmol) was added and stirring was continued for an additional hour. The reaction solution was made basic with 2N NaOH solution, brine was added, and the mixture was extracted with ether (3×). The aqueous layer was next extracted with dichloromethane (2×). The combined organic extracts were dried over magnesium sulfate and reduced in vacuo to give 1.13 g of crude product as a white foam. HPLC analysis (Phenomenex C18(2) reverse-phase column, 50−90% acetonitrile/water over 10 min, buffer 0.1% trifluoroacetic acid, product retention time 7.25 min) indicated that 79% of the crude material was the desired product. The crude product was dissolved in 50% ACN/water with 0.1% TFA (20 mL), and trifluoroacetic acid (181 μL) was added to protonate the amine. Purification over a preparative C18 reverse-phase column, using the same conditions as used for the analytical runs, and lyophilization gave **7** (1.24 g, 46% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.99 (d, 3H), 1.02 (d, 3H), 1.10 (s, 9H), 1.13 (s, 9H), 1.20−1.35 (m, 4H), 1.39 (s, 18H), 1.52−1.67 (m, 5H), 1.67−1.79 (m, 1H), 2.43 (t, 2H), 2.70 (d, 3H), 2.87−3.15 (m, 4H), 3.75 (m, 4H), 3.83 (m, 2H), 3.88 (m, 2H), 3.96 (m, 1H), 4.30−4.36 (m, 2H), 5.10 (s, 2H), 7.30−7.40 (m,

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5H), 7.75 (d, 1H), 7.97 (d, 1H), 8.07 (t, 1H), 8.14−8.21 (m, 2H), 9.33 (br s, 1H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 19.2, 20.1, 21.8, 23.2, 28.1, 28.3, 28.4, 31.9, 33.2, 41.6, 41.9, 42.6, 52.6, 54.9, 55.2, 57.6, 59.5, 65.9, 67.6, 68.0, 74.0, 74.4, 78.8, 81.1, 128.4, 128.5, 128.9, 136.6, 155.5, 158.4, 158.8, 168.9, 169.2, 169.3, 169.5, 170.65, 171.7, 172.8. HRMS (ESI) $C_{50}H_{86}N_7O_{13}$ (M + H$^+$) calcd 992.6284, found 992.6251.

(2*R*,14*S*,17*S*,23*S*,24*R*)-23-Ammonio-14-((2-((carboxymethyl)-amino)-2-oxoethyl)carbamoyl)-24-hydroxy-17-((*R*)-1-hydroxyethyl)-9-methyl-4,16,19,22-tetraoxo-2-((palmitoyl-oxy)-methyl)-3-oxa-9,15,18,21-tetraazapentacosyl-(2-(trimethylammonio)ethyl)phosphate 2,2,2-Trifluoroacetate (8). A catalyst slurry was formed by placing 370 mg of 10% Pd/C in a dry flask and adding ethanol (3 mL). A solution of 7 (1.24 g, 1.121 mmol) in ethanol (15 mL) was added to the slurry, and the mixture was put under a balloon of hydrogen for 3 h. An aliquot was checked by HPLC (Phenomenex C18(2) reverse-phase column, 50−90% acetonitrile/water over 15 min, buffer 0.1% trifluoroacetic acid, product retention time 4.6 min) and showed complete conversion to product. The catalyst was removed by filtration, and the solution was reduced in vacuo to give 1.07 g (94% yield) of the acid as a foam/oil. (HRMS (ESI) $C_{43}H_{80}N_7O_{13}$ (M + H$^+$) calcd 902.5814, found 902.5836). The acid (1.07 g, 1.053 mmol, 1 equiv) and *lyso*-PC (1.566 g, 3.16 mmol, 3 equiv) were dissolved in toluene with sonication in the reaction flask. The solvent was removed in vacuo to eliminate any residual ethanol (2×). The flask was put under high vacuum using a vacuum pump for 2 days to complete solvent removal. The solid was slurried in ethanol-free chloroform (40 mL), and diisopropyl carbodiimide (DIC) (0.326 mL, 2.1 mmol, 2 equiv) was added followed by DMAP (257 mg, 2.1 mmol, 2 equiv). Glass beads were added to just below the solvent surface, and the reaction flask was sonicated for 2 h. The solution was pipetted away from the glass beads and the beads washed with additional chloroform. The combined solutions were reduced in vacuo to give 4.24 g of crude product. HPLC (Phenomenex C18(2) reverse-phase column, 50−90% acetonitrile/water over 10 min, buffer 0.1% trifluoroacetic acid, product retention time 13.8 min) showed complete conversion to product. The material was dissolved in 50% acetonitrile/water and purified by HPLC (Phenomenex C18(2) reverse-phase column, 10 μm, 250 mm × 30 mm, 40 mL/min, 60−90% acetonitrile/water over 15 min, buffer 0.1% TFA). The product fraction was partially reduced in vacuo to remove most of the acetonitrile and lyophilized. The lyophilate was collected in dichloromethane and reduced again to give the product oil. Drying overnight under high vacuum gave 1.07 g (68% yield) of the coupled product. This material was dissolved in trifluoroacetic acid (5 mL) and stirred in an ice bath for 2.5 h. The solvent was removed in vacuo and the residue dissolved in dichloromethane and reduced in vacuo to give a product oil. HPLC (Phenomenex C18(2) reverse-phase column, 30% acetonitrile/water for 2 min, then to 90% acetonitrile at 10 min, buffer 0.1% trifluoroacetic acid, product retention time 9.59 min (98% pure)) The oil was dissolved in water and lyophilized to give 846.2 mg (88% yield) of **8** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, 3H), 1.04 (d, 3H), 1.19 (d, 3H), 1.19−1.80 (m, 34H), 2.28 (t, 2H), 2.30−2.45 (m, 2H), 2.66 (d, 3H), 2.80−3.08 (m, 5H), 3.13 (s, 9H), 3.57 (s, 2H), 3.62 (br s, 1H), 3.75 (m, 4H), 3.80−4.05 (m, 7H), 4.10−4.35 (m, 7H), 5.18 (s, 1H), 5.5 (br s, 1H), 8.01 (m, 2H), 8.05−8.20 (m, 4H), 8.32 (m, 1H), 8.83 (t, 1H), 10.80 (s, 1H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 14.4, 20.1, 20.2, 22.5, 22.9, 23.2, 24.8, 28.8, 29.1, 29.3, 29.4, 29.5, 31.7, 33.7, 41.0, 42.1, 42.3, 53.5, 58.6, 59.3, 62.4, 64.0, 65.6, 66.3, 67.0, 70.8, 158.3, 158.6, 167.6, 168.9, 169.4, 170.3, 171.5, 172.1, 173.1. HRMS (ESI) $C_{50}H_{96}N_8O_{17}P$ (M + H$^+$) calcd 1111.6631, found 1111.6614.

(4*S*,10*S*,13*S*,25*R*)-13-((2-((Carboxymethyl)amino)-2-oxoethyl)carbamoyl)-4,10-bis((*R*)-1-hydroxyethyl)-18-methyl-2,5,8,11,23-pentaoxo-25-((palmitoyloxy)methyl)-24-oxa-3,6,9,12,18-pentaazahexacosan-26-yl-(2-(trimethylammonio)-ethyl)phosphate (9). Compound **8** (49.8 mg, 0.037 mmol, 1 equiv) was dissolved in 2 mL of anhydrous dichloromethane. 1-Acetylimidazole (6.1 mg, 0.056 mmol, 1.5 equiv) and DIPEA (25.9 μL, 0.149 mmol, 4 equiv) were added, and the reaction was stirred for 18 h. Complete conversion to product was seen when the reaction was checked by HPLC (Phenomenex C18(2) reverse-phase column, 30−90% acetonitrile/water over 10 min, buffer 0.1% TFA, product retention time 9.3 min). The solvent was removed in vacuo, and the residual oil was dissolved in 50% acetonitrile/water and purified over a preparative HPLC column using the same conditions as for the analytical HPLC. The pure fraction was lyophilized overnight. The sample was relyophilized from water to give 25.6 mg (60% yield) of **9** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.71, (t, 3H), 0.89 (d, 3H), 0.92 (d, 3H), 1.05−1.70 (m, 34H), 1.78 (s, 3H), 2.10−2.20 (m, 3H), 2.25−2.40 (m, 6H), 2.80 (s, 4H), 2.99 (s, 9H), 3.42 (s, 3H), 3.54−3.70 (m, 7H), 3.70−3.90 (m, 4H), 3.95−4.05 (m, 4H), 4.05−4.15 (4H), 4.90 (br s, 1H), 5.05 (s, 1H), 7.62 (d, 1H), 7.84 (d, 1H), 7.93 (s, 2H), 8.09 (t, 1H), 8.16 (s, 1H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 14.4, 20.0, 20.2, 22.5, 22.9, 23.2, 24.8, 28.8, 29.1, 29.3, 29.4, 29.5, 31.4, 31.7, 33.7, 33.8, 41.1, 42.2, 42.7, 52.8, 53.6, 55.3, 58.6, 59.7, 59.4, 62.5, 63.9, 65.8, 66.8, 67.2, 71.0, 169.4, 169.6, 170.5, 170.6, 171.4, 171.6, 172.1, 172.4, 173.1. HRMS (ESI) $C_{52}H_{98}N_8O_{18}P$ (M + H$^+$) calcd 1153.6737, found 1153.6749.

CD36 Macrophage Receptor Binding Competition Assay. Binding of biotinylated OxLDL ligands to murine macrophages plated in microtiter wells was assessed by a chemiluminescent binding assay as previously described with modifications.[13] Isolated human LDL was biotinylated according to manufacturer's protocol (catalogue no. 21326; Pierce Biotechnology). Native and biotinylated native LDL were subjected to copper sulfate oxidation[8a] in parallel to prepare unlabeled and biotinylated CuOxLDL ligands, respectively. We have previously shown that CuOxLDL contains approximately 78 mol of PC-epitopes per mol of apoB-100 (the sole protein on LDL).[8a] The biotinylated native LDL and CuOxLDL ligands of equal protein concentration were serially diluted and tested for binding to adherent macrophages. The specificity of the binding of biotinylated CuOxLDL to macrophages was tested in competition experiments, where fixed concentrations of biotinylated CuOxLDL were incubated with the serially diluted competitor (**9**) and controls in PBS at concentrations indicated in Figure 2. The ligand-competitor solutions were incubated overnight at 4 °C. Murine macrophages from the J774 cell line were cultured in 10% fetal bovine serum in DMEM (DMEM-10) and plated in 100 μL of L929-fibroblast conditioned media at 100000 or 25000 cells/well, respectively, in sterile 96-well flat-bottom white plates (Greiner Bio-One). The plating media consisted of 20% L929-fibroblast conditioned DMEM-10 and 80% fresh DMEM-10 and served as a source of growth factors, including macrophage colony-stimulating factor. After 72 h, plates were washed gently 5 times with PBS using a microtiter plate washer (Dynex Technologies, Chantilly, VA), and wells were blocked with ice-cold 200 μL of DMEM for 30 min while plates were kept on ice. After washing, macrophages were incubated with ice-cold ligand and ligand-competitor solutions (100 μL/well) for 2 h on ice, washed again, and fixed with ice-cold 3.7% formaldehyde in PBS for 30 min in the dark. After fixing the macrophages, the remaining of the assay was carried out at room temperature. Macrophage-bound biotinylated OxLDL ligands were detected with NeutrAvidin-conjugated alkaline phosphatase (Pierce Biotechnology), LumiPhos 530 (Lumigen, Southfield, MI), and a Dynex Luminometer (Dynex Technologies). Ligand binding was recorded and expressed as relative light units counted per 100 ms (RLU/100 ms) or in the case of inhibition of binding by competitors as a ratio of binding in the presence of competitor (B) divided by binding in the absence of competitor (B_0). In preliminary studies, we have shown that at the concentrations used, there was no impact of compound **9** on viability of macrophages even at room temperature for up to 72 h as judged by cell number or protein content at the end of experiment or by detailed time course studies of cell function (data not shown). The binding studies shown here, however, were conducted on ice.

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■ ASSOCIATED CONTENT

Supporting Information

Supplemental figures and experimental data for compounds **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

OxLDL, oxidized low-density lipoprotein; CuOxLDL, copper-oxidized low-density lipoprotein; OxPL, oxidized phospholipid; *lyso*-PC, 1-palmitoyl-*sn*-glycero-3-phosphorylcholine; DIC, diiso-propylcarbodiimide; POVPC, 1-palmitoyl-2-(5′-oxo)-valeroyl-*sn*-glycero-3-phosphorylcholine; PAPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine

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