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Fluorogenic Phospholipid Substrate to Detect Lysophospholipase D/Autotaxin Activity

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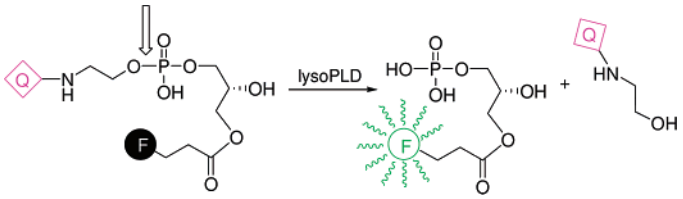
ABSTRACT



Lysophospholipase D (lysoPLD), also known as autotaxin (ATX), is an important source of the potent mitogen lysophosphatidic acid (LPA). Two fluorogenic substrate analogues for lysoPLD were synthesized in nine steps from (*S*)-PMB-glycerol. The substrates (FS-2 and FS-3) show significant increases in fluorescence when treated with recombinant ATX and have potential applications in screening for this emerging drug target.

Bioactive lysophospholipids, such as lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), exhibit pleiomorphic effects on multiple cell lineages, including ovarian cancer cells.[1] LPA and S1P signal through specific cell surface receptors of the endothelial cell differentiation gene (formerly known as edg) family of cell surface seven-transmembrane-domain G-protein-coupled receptors.[2] The purification, characterization, and identification of the ovarian cancer activating factor (OCAF) from ascites of ovarian cancer patients demonstrated that OCAF is comprised of numerous forms of LPA and accounts for the ability of ascites to activate ovarian cancer cells.[3] LPA, at concentrations present in ascites from ovarian cancer patients (1−80 μM), increases proliferation under anchorage-dependent and -independent conditions, prevents apoptosis and anoikis, increases invasiveness, induces cytoskeletal reorganization and change of shape, and decreases sensitivity to cisplatin.[4]

LPA can arise through at least two routes: the loss of the *sn*-2 acyl chain by phosphatidic acid-specific PLA_2 or cleavage of the choline group of lysophosphatidylcholine (LPC) by lysophospholipase D (lysoPLD) (Figure 1A). LysoPLD activity was first characterized over 16 years ago and has important roles in normal physiology as a source of plasma LPA.[5] Two laboratories have independently deter-

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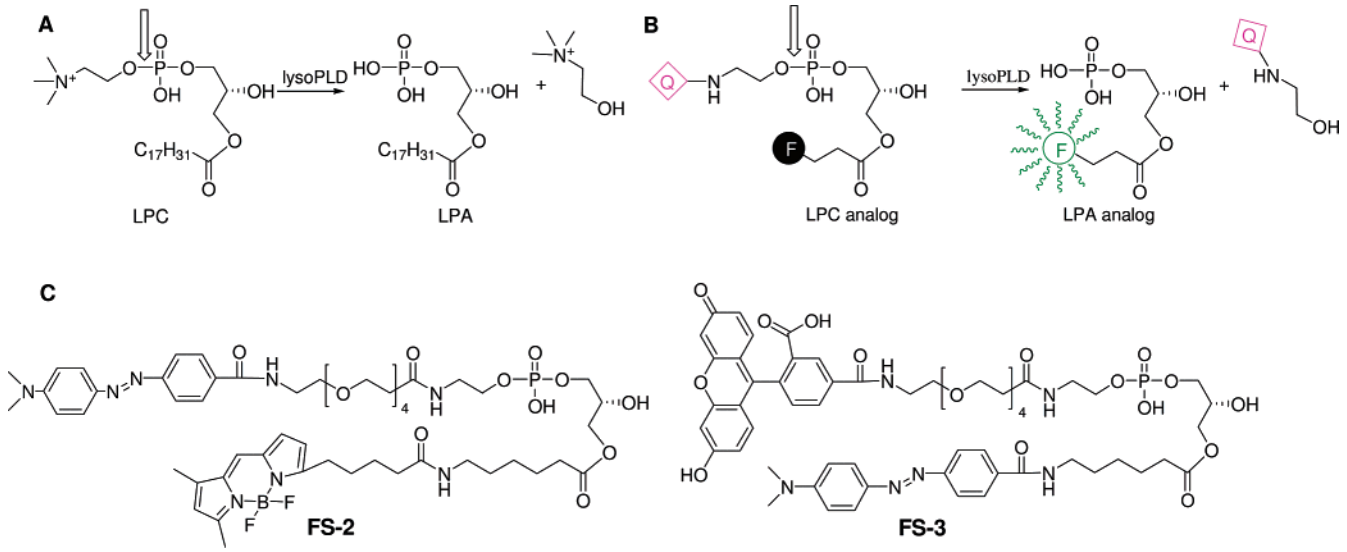

Figure 1. A: Hydrolysis of oleoyl LPC by lysoPLD. B: Schematic of fluorescence-dequenching lysoPLD/ATX substrate. The fluor in the substrate is quenched through intramolecular energy transfer until it is hydrolyzed by the enzyme at which point the fluorescent product can be observed. C: Structures of fluorogenic substrates **FS-2** and **FS-3**.

mined purified and cloned plasma lysoPLD and showed that there was no sequence homology to other PLD enzymes. Instead, it was identical to secreted autotaxin (ATX), a member of the nucleotide pyrophosphatase/phosphodiesterase (NPP) family of ecto/exo enzymes, that stimulates tumor cell motility and has an in vivo role in tumor progression and invasion.[6] Recent reviews suggest that inhibition of increased lysoPLD/ATX activity by small molecules could be an attractive new avenue for anticancer chemotherapy; however, for that to become reality, a simple assay for high throughput screening is needed.[7]

A number of different methods have been employed to assay lysoPLD activity.[8] Early work was performed with ^{14}C-palmitoyl-LPC and radio-thin-layer chromatography (TLC). This was supplanted by a TLC purification of unlabeled LPA with GC analysis of methyl esters or by measurement of LPA-trimethylsilyl ether by gas chromatography−mass spectrometry (GC-MS). An endpoint-type dual-enzymatic photometric assay has recently been employed to detect choline liberated from exogenously added LPC.[6c] However, these methods are complex and are not suited to high throughput screening. During the course of this project, the application of a doubly labeled ATX substrate, CPF4, was published.[9] CPF4 is a fluorescence resonance energy transfer (FRET) substrate derived from bis-*p*-nitrophenyl phosphate, a common colorimetric phosphodiesterase substrate, and although it was a significant improvement over the previously described assays, it does not share many structural features of LPC. In this report, the synthesis and validation of two simple fluorogenic phospholipid substrates for lysoPLD/ATX activity are demonstrated.

The envisioned substrates employ a "fluorescence dequenching" motif, in which a fluorophore that is "silent" because of intramolecular FRET to a nonfluorescing quencher becomes fluorescent once enzymatic hydrolysis cleaves the substrate (Figure 1B). This FRET paradigm has been applied to fluorogenic assays for ceramidase,[10] DNA ligase,[11] PLA_2,[12] and nucleic acid hybridization.[13] Because ATX is reported to hydrolyze a variety of acyl chain modifications and is also tolerant to changes in the backbone and headgroup,[14] a labeled ethanolamido headgroup was chosen for **FS-2** and **FS-3** (Figure 1C), rather than a synthetically more complex choline analogue. In addition, a PEG tether was appended to the headgroup to move the bulkier fluor or

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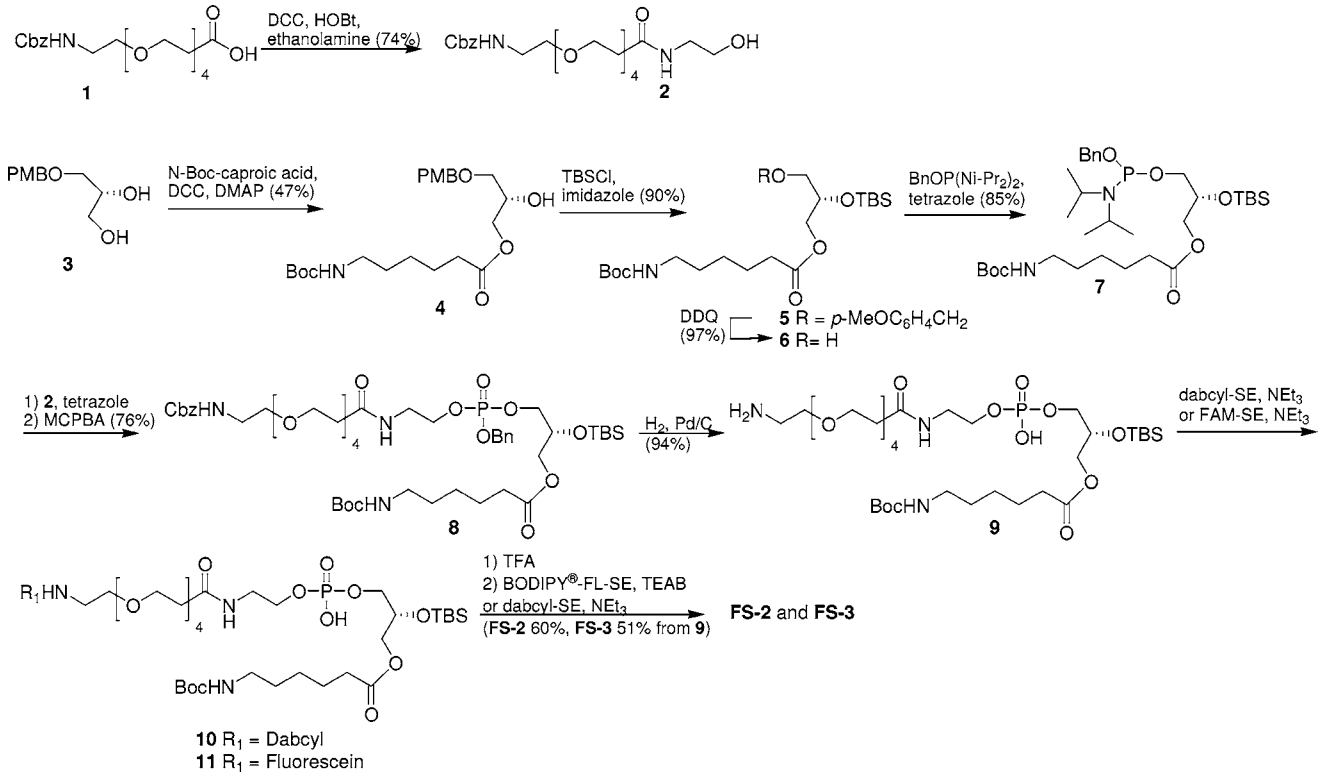
Scheme 1. Synthesis of **FS-2** and **FS-3**



quencher away from the phosphodiester and increase hydrophilicity. Dabcyl was chosen as the quencher for both substrates because it has been shown to effectively quench green fluors in the previously described applications. In **FS-2**, the hydrophobic fluor BODIPY-FL is appended to the *sn*-1 acyl chain with dabcyl on the headgroup. For **FS-3**, the positions of the quencher and fluor are reversed and BODIPY-FL was replaced with more hydrophilic fluorescein.

The synthesis of **FS-2** and **FS-3** is described in Scheme 1. A number of considerations were taken into account when designing the synthetic strategy. Because the majority of commercially available dyes are amine reactive, orthogonally protected amino groups in the headgroup and monoacyl glycerol were required, and in this case, Cbz and Boc were chosen. In addition, a universal intermediate (**9**) was desired so that different combinations of fluors and quenchers could be potentially attached, thus allowing simple alteration of structual and/or fluorogenic properties. The PEG headgroup (**2**) was prepared by coupling commercially available Cbz-protected PEG_4 acid (**1**) with ethanolamine by DCC/HOBt. To prepare the protected monoacyl glycerol segment, *p*-methoxybenzyl glyceryl ether **3** (prepared from (*S*)-isopropylideneglycerol[15]) was first selectively esterified at the primary hydroxyl with *N*-Boc-caproic acid forming **4** in 47% yield.[16] Attempts to protect the *sn*-2 hydroxyl as the *tert*-butyl ether, which could be removed with the Boc group at the end of the synthesis, resulted in poor yields. Instead, it was protected as the *tert*-butyldimethylsilyl ether (**5**) on the basis of examples in the literature showing TBS ethers could be cleaved under acidic conditions. Oxidative cleavage of the PMB group gave the chiral intermediate **6**. Phosphitylation of the hydroxyl with benzyl bisdiisopropylphosphoramidite yielded phosphoramidite **7**. The protected glycerol was converted to the phosphoramidite rather than the PEG headgroup because previous attempts in this lab to prepare phosphoramidites from PEG alcohols had been unsuccessful. Coupling **2** and **7** via tetrazole proceeded smoothly followed by oxidation with MCPBA, forming the orthogonally protected phosphate **8**. Catalytic hydrogenation liberated the PEG amine **9** which was used as a precursor for both **FS-2** and **FS-3**. The PEG amino group was acylated via the *N*-hydroxysuccinimidyl ester (SE) of dabcyl forming **10**. Treatment with TFA in CH_2Cl_2 removed the TBS and Boc groups, and the resulting amine was acylated via BODIPY-FL-SE yielding the fluorogenic substrate **FS-2** (60% from **9**). The route to **FS-3** followed the same sequences of reactions as **FS-2**. Treatment of **9** with FAM-SE gave **11** which was deprotected with TFA and labeled with dabcyl to yield **FS-3** (51% from **9**).

The fluorogenic substrates were evaluated with recombinant ATX. **FS-2** and **FS-3** showed 2.8- and 10.7-fold increases in fluorescence, respectively, at 2.5 μM when incubated with 75 nM ATX (Figure 2). An increase in fluorescence was not observed when either substrate was treated with an inactive ATX mutant (data not shown). K_M and V_{max} were determined for both substrates (Table 1), but

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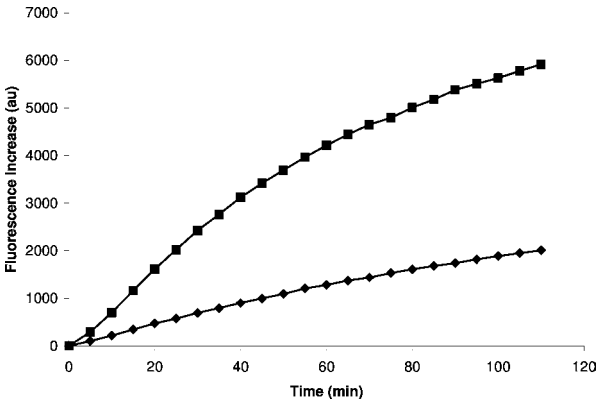

Figure 2. Incubation of **FS-2** (◆) and **FS-3** (■) with recombinant ATX results in a measurable increase in fluorescence (140 mM NaCl, 5 mM KCl, 1mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Tris; pH 8.0, 1 mg/mL fatty acid-free BSA, absorption = 485 nm, emission = 520 nm, 37 °C).

a more detailed kinetic analysis and comparison with CPF4 and bisNPP will be described in a subsequent paper.[17]

Although **FS-2** and **FS-3** are effectively hydrolyzed by ATX (NPP2), neither would be expected to act as substrates for NPP1, NPP3, NPP4, or NPP5, which do not hydrolyze lysophospholipids.[18,19] NPP6 has recently been shown to have lysoPLC activity; however, it is selective for LPC over other lysophospholipids. Therefore, on the basis of its headgroup specificity, it may not process **FS-2** or **FS-3**.[19] NPP7 possesses lysoPLC and sphingomyelinase activities, but there is not enough evidence yet regarding headgroup specificity to preclude **FS-2** or **FS-3** acting as substrates.[20]

Table 1. Michaelis−Menten Kinetics of Hydrolysis of **FS-2** and **FS-3** by Recombinant ATX[a]

substrate	K_M (μM)	V_{max}[b]
FS-2	3.1 ± 0.6	261 ± 12.3
FS-3	6.3 ± 0.2	261 ± 2.3

[a] Experimental details in ref 17. [b] Arbitrary fluorescence in units/min.

In conclusion, two dual-labeled ATX substrate analogues were synthesized featuring a fluor and a quencher on the *sn*-1 acyl chain and the ethanolamino headgroup. Because of the proximity of the two moieties, intramolecular energy transfer effectively quenches the fluorescence. Hydrolysis by recombinant ATX cleaved the phosphodiester bond resulting in a measurable increase in fluorescence. Because lysoPLD/ATX is an emerging potential drug target and biomarker, the substrates provide a simple, sensitive assay that could be applied to high throughput screening for diagnosis and drug discovery.

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Supporting Information Available: Experimental procedures and characterizations for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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