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Synthesis and Evaluation of Fluorogenic Substrates for Phospholipase D and Phospholipase C

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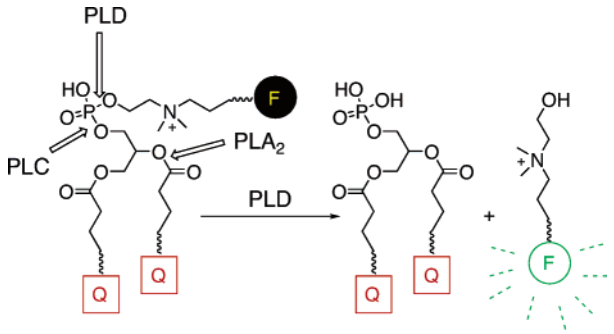
ABSTRACT



Fluorogenic analogues of phosphatidylcholine and lysophosphatidylcholine, DDPB and lysoDDPB, were synthesized by an enzyme-assisted strategy. The analogues were evaluated as substrates for phospholipases C and D and lysophospholipase D. DDPB was cleaved by bacterial and plant phospholipase D (PLD) enzymes and represents the first direct fluorogenic substrate for real-time measurement of PLD activity. Both fluorogenic substrates have potential in screening for PLD and PC–PLC inhibitors and for monitoring spatiotemporal changes in PLD activity in cells.

PLC, PLD, and lysoPLD are three phospholipases that catalyze hydrolysis of phospholipid (PL) headgroups. PLC catalyzes the hydrolysis of the PL phosphodiester glyceryl P−O bond to give diacylglycerol and a phosphomonoester. PLD cleaves the headgroup P−O bond of the phosphodiester linkage, to produce phosphatidic acid (PA) and an alcohol. LysoPLD catalyzes the same reaction as PLD but is selective for lysophospholipids. Most PLDs also catalyze transphosphatidylation,[1] in which a primary alcohol replaces water as the cleaving nucleophile.

PLC isozymes selectively hydrolyze PLs with either inositol or choline headgroups. There are at least 11 different phosphoinositide-selective PLCs (PI−PLC)[2] and two putative phosphatidylcholine-selective PLCs (PC−PLC)[3] in mammals and one PC−PLC in plants.[4]

PLD isozymes found in mammals are involved in a diversity of normal and disease-related biological processes.[5−7] The PLD enzymes found in mammals, plants, and some bacteria are called "HKD PLD" because they share a consensus amino acid sequence ($HxKx_4Dx_6GG/S$) and employ a common catalytic mechanism involving a covalent histidine intermediate.[8] The "non-HKD PLD" enzymes lack an HKD consensus sequence and have a catalytic mechanism

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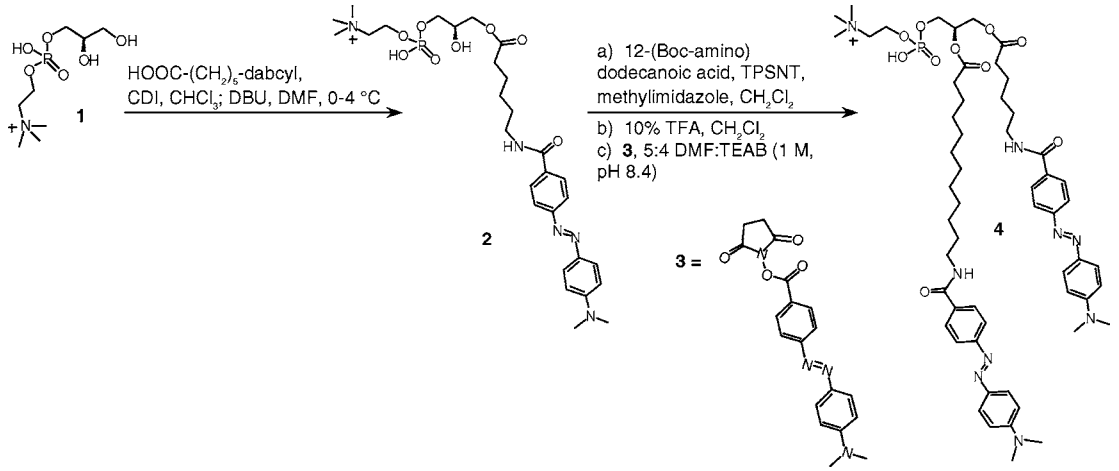
Scheme 1. Synthesis of Didabcyl Intermediate **4**



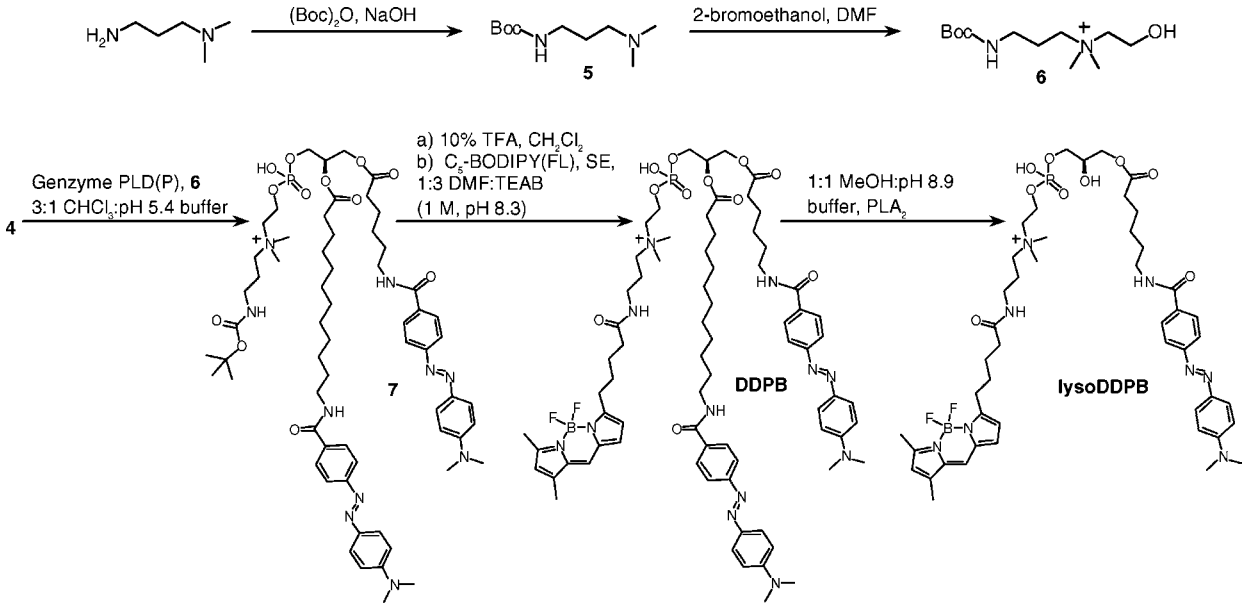
in which metal ions are required to position the PL substrate and activate the attacking nucleophile.[9] *Streptomyces chromofuscus* PLD (scPLD) is an archetypical non-HKD PLD. Similar to the non-HKD PLD, lysoPLD requires metal ions for catalysis and does not contain an HKD sequence.[10] The lysoPLD autotaxin, a nucleotide pyrophosphatase/phosphodiesterase, catalyzes hydrolysis via an active site threonine.[11]

HKD PLDs can be detected in cell biological studies by inhibition with exogenous ethanol, 1-propanol, or 1-butanol. Biochemical assays employ radioactive[12] or fluorescent[13] PLs, requiring extraction, separation, and detection. Alternatively, PLD products can be detected in vitro using direct[14] or indirect[15−18] methods. There are also indirect assays for PC−PLC[18,19] and lysoPLD;[20] in particular, *p*-nitrophenyl phosphate analogues have also been used for monitoring non-HKD PLD and lysoPLD[21,22] and PC−PLC[23] in vitro. Fluorogenic substrates for lysoPLD have recently been described.[24,25]

We report here the synthesis of fluorogenic PC and lysoPC analogues that contain a fluorescence quencher (dabcyl, a.k.a, *p*-methyl red) at each acyl chain terminus and a fluorophore appended to the PL headgroup through a choline-mimetic linker. These PL analogues, denoted **DDPB** and **lysoDDPB**, were evaluated in microtiter plate assays as substrates for lysoPLD, scPLD, PLC, and phospholipase A_2 (PLA_2), as well as several commercially available HKD PLD enzymes.

DDPB and **lysoDDPB** were synthesized efficiently as illustrated in Schemes 1 and 2. The lysophosphatidylcholine intermediate **2** containing the shorter dabcyl acyl chain was obtained by a selective monoacylation of the commercially available glycerol phosphocholine, **1**, followed by installation of the longer-chain dabcyl quencher at the *sn*-2 position (**4**,

Scheme 2. Synthesis of **DDPB** and **LysoDDPB**



Scheme 1). Although the longer, dodecanoyl acyl chain at *sn*-2 improved the lipophilicity of the analogue over compounds with two hexanoyl chains, adding a second C_{12} linker at *sn*-1 afforded a less-soluble analogue that did not exhibit either improved micelle insertion or in vitro enzyme activity (data not shown).

The key step was the modification of the headgroup of intermediate **4** by transphosphatidylation, using Genzyme PLD(P) and a designed "choline-like" primary alcohol **6**, prepared as shown in Scheme 2. Transphosphatidylation has been exploited previously[26−28] to generate PL analogues with both natural and unnatural headgroups. In this case, the headgroup remodeling allowed the installation of a phosphodiester linkage bearing an internal quaternary amine similar to choline as well as a protected primary amine that could be used for further conjugation reactions (**7**, Scheme 2). The primary amine was deprotected and allowed to react with an activated ester of BODIPY−FL (Molecular Probes) to give the fluorogenic PC analogue **DDPB** (Scheme 2). Removal of the *sn*-2 ester of **DDPB** by cobra venom PLA_2 gave **lysoDDPB**, a fluorogenic lysoPC analogue.

Mixed micelles containing **DDPB** or **lysoDDPB** in Triton X-100 (reduced) were incubated with commercial PLDs from various sources for 3 min in buffers near each enzyme's pH optimum. Over this period, **DDPB** (Figure 1a) gave robust fluorescence increases with the enzyme used for its synthesis, Genzyme PLD(P), and with scPLD. **LysoDDPB** (Supporting Information, Figure S2), over 3 min, gave a signal comparable to that of scPLD but only a very small response to Genzyme PLD(P). Over 60 min (Figure 1b), **DDPB** mixed micelles with HKD PLD from peanut, cabbage, and *Streptomyces PMF* (BIOMOL) evolved fluorescence to a degree approaching that observed in 3 min with scPLD, but bee and cobra venom $sPLA_2$ did not generate a fluorescent signal over the same time period, consistent with the retention of an intramolecular quencher even after cleavage of the *sn*-2 quenched acyl chain. A longer incubation time with **lysoDDPB** mixed micelles (Supporting Information, Figure S3) only amplified fluorescence from Genzyme PLD(P). These results support the idea that lysophospholipids are substrates for scPLD[14] but are not substrates for HKD PLD.[29,30]

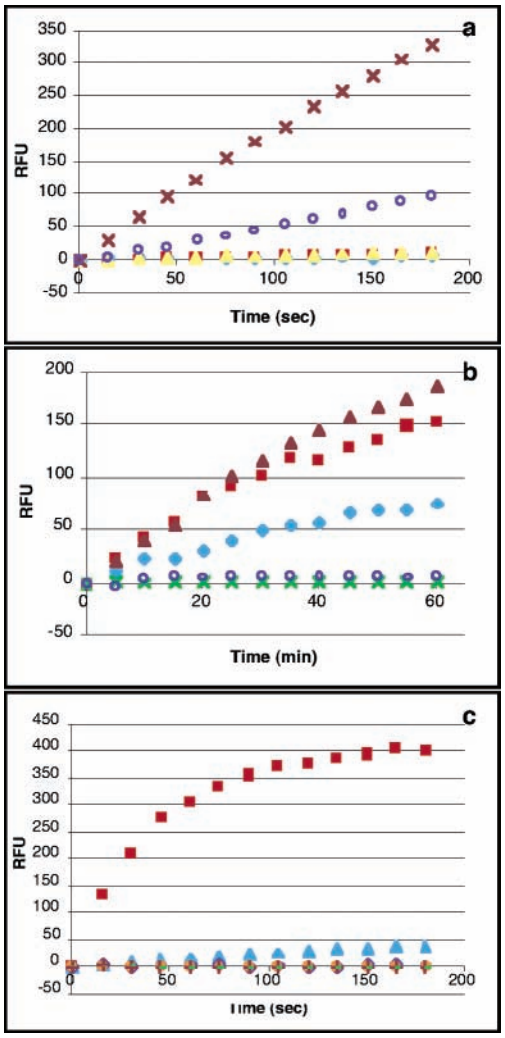


Figure 1. Fluorescence evolution ($\lambda_{Ex}/\lambda_{Em}$ = 500/530 nm) during (a) 3 min incubation of **DDPB** mixed micelles with PLD from various sources (blue ♦ = peanut PLD; red ■ = cabbage PLD; yellow ▲ = *Strep. PMF* PLD; brown × = Genzyme PLD(P); ○ = scPLD), (b) 60 min incubation of **DDPB** mixed micelles with PLD and PLA_2 from various sources (blue ♦ = peanut PLD; red ■ = cabbage PLD; brown ▲ = *Strep. PMF* PLD; green × = cobra venom PLA_2; ○ = bee venom PLA_2), and (c) 3 min incubation of **DDPB** or **lysoDDPB** mixed micelles with PC−PLC or PI−PLC from *B. cereus* or *C. perfringens* (red ■ = *B. cereus* PC−PLC + **DDPB**; blue ▲ = *B. cereus* PC−PLC + **lysoDDPB**; ◊ = *C.* perfingens PC−PLC + **DDPB**; orange ● = *C. perfringens* PC−PLC + **lysoDDPB**; brown + = *B. cereus* PI−PLC + **DDPB**; green rectangle = *B. cereus* PI−PLC + **lysoDDPB**).

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Next, mixed micelles of the PC analogues were tried as substrates for PC−PLC, from *Bacillus cereus* and *Clostridium perfringens*, and PI−PLC, from *B. cereus* (Figure 1c). Over 3 min, **DDPB** gave an overwhelmingly large fluorescence response with *B. cereus* PC−PLC and virtually none with *B. cereus* PI−PLC or *C. perfringens* PC−PLC. **LysoDDPB** gave a small but detectable signal with *B. cereus* PC−PLC but none with the other PLC tested.

We anticipated that **lysoDDPB** would be accepted as a fluorogenic substrate by lysoPLD, but neither **lysoDDPB**- nor **DDPB**-containing mixed micelles produced fluorescence when assayed in the presence of FBS, an abundant source of lysoPLD activity,[31] even after 60 min incubation. On the other hand, the commercial lysoPLD substrates *p*NP-TMP[32] and FS-3[25] both generated significant UV and fluorescence signals, respectively (Supporting Information, Figure S4). **LysoDDPB** was assayed with 50% FBS at 37 °C, but no fluorescence increase was observed even when the concentration of **lysoDDPB** was quadrupled and the FBS concentration was increased to 80% (data not shown). Incubation of **lysoDDPB**-containing micelles with up to 2 μg of venom from *Loxosceles reclusa*, another known source of lysoPLD,[33] failed to show a fluorescence increase (data not shown).

The inability of lysoPLD to hydrolyze **lysoDDPB** was unexpected, considering the close structural similarity among lysoPC, **lysoDDPB**, and FS-3 (Supporting Information, Figure S4). The phosphate linker of FS-3, despite its lack of a quaternary amine, is nearly twice the length of that in **lysoDDPB**. This extra length may give the bulky fluorescent group of FS-3 flexibility to avoid unfavorable interactions in the binding site. Increasing the chain length between the phosphate and the fluorophore in future fluorogenic analogues of lysoPC could test this supposition.

DDPB and **lysoDDPB** were both designed to be PC-like, amphiphilic probes that could be used in living cells by insertion into lipid bilayers; neither is freely soluble in water. **DDPB** was further engineered to have fluorescence quenchers at the end of each acyl chain to prevent fluorescence increases resulting from cellular PLA_2 or PLA_1 activity. Indeed, no fluorescent signal was observed even after incubation of **DDPB** with venom PLA_2 at 37 °C for 1 h (Figure 1c).

Given the well-established importance of the HKD PLDs and the growing importance of PC−PLC in cell signaling, **DDPB** and **lysoDDPB** have the potential to be valuable tools in cellular and molecular biology. The utility of these fluorogenic substrates with mammalian HKD PLD has been observed and will be reported elsewhere.[34] Both **DDPB** and **lysoDDPB** are cell permeant (data not shown) and may be applicable for cell-based assays, as previously described for PLA_2.[35,36] Further studies examining the use of **DDPB** and **lysoDDPB** in living systems are merited and will be reported in due course.

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Supporting Information Available: Figures S2, S3, and S4, full experimental details, and NMR and MS spectroscopic data for the characterization of **DDPB** and **lysoDDPB** and their intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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