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Synthesis of mixed-chain phosphatidylcholines including coumarin fluorophores for FRET-based kinetic studies of phospholipase A₂ enzymes

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Abstract

Phospholipase A₂ (PLA₂) enzymes catalyze the hydrolysis of the *sn*-2 ester linkage of glycerophospholipids to produce fatty acids and lysophospholipids. A significant number of mammalian phospholipases comprise a family of secreted PLA₂ enzymes, found in specific tissues and cellular locations, exhibiting unique enzymatic properties and distinct biological functions. Development of new real-time spectrofluorimetric PLA₂ assays should facilitate the kinetic characterization and mechanistic elucidation of the isozymes in vitro, with the potential applicability to detect and measure catalytic PLA₂ activity in tissues and cellular locations. Here we report a new synthesis of double-labeled phosphatidylcholine analogues with chain-terminal reporter groups including coumarin fluorophores for fluorescence resonance energy transfer (FRET)-based kinetic studies of PLA₂ enzymes. The use of coumarin derivatives as fluorescent labels provides reporter groups with substantially decreased size compared to the first generation of donor-acceptor pairs of fluorescent phospholipids. The key advantage of the design is to interfere less with the physicochemical properties of the acyl chains, thereby improving the substrate quality of the synthetic probes. In order to assess the impact of the fluorophore substituents on the catalytic hydrolysis and on the phospholipid packing in the lipid-water interface of the assay, we used the experimentally determined specific activity of bee-venom phospholipase A₂ as a model for the secretory PLA₂ enzymes. Specifically, the rate of PLA₂ hydrolysis of the coumarin labeled phosphatidylcholine analogues was less than three times slower than natural substrate dipalmitoyl phosphatidylcholine (DPPC) under the same experimental conditions. Furthermore, variation of the mole fraction of the synthetic phosphatidylcholine vs. that of the natural DPPC substrate showed nearly ideal mixing behavior in the phospholipid-surfactant aggregates of the assay. The synthesis provides a rapid and efficient method for preparation of new synthetic phosphatidylcholines with the desired target structures for enzymatic and physicochemical studies.

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1. Introduction

Phospholipases A₂ (PLA₂s) comprise a superfamily of intracellular and secreted enzymes that catalyze the hydrolysis of the *sn*-2-ester bond of glycerophospholipids to yield fatty acids such as arachidonic acid, and lysophospholipids (Dennis et al., 2011, Eq. (1)):

(1)

The products are precursors of signaling molecules with a broad range of biological functions (Murakami and Lambeau, 2013). For example, arachidonic acid is converted to eicosanoids that have been shown to be involved in immune response, inflammation, pain perception and sleep regulation (Funk, 2001; Murakami et al., 2011a), while lysophospholipids are precursors of lipid mediators such as lysophosphatidic acid (LPA) and platelet activating factor (PAF). Lysophosphatidic acid has been shown to be involved in cell proliferation, survival and migration, while PAF is particularly involved in inflammatory processes (Prescott et al., 2000).

Secreted phospholipases A₂ (sPLA₂s) are widespread in nature (Murakami et al., 2011b). While early studies focused on members of the sPLA₂ family isolated from insect and snake venoms, sPLA₂s were subsequently found in plants, bacteria, fungi, viruses, and mammals as well. To date more than 30 isozymes have been identified in mammals, and they have been classified based on their structures, catalytic mechanisms, localizations, and evolutionary relationships (Schaloske and Dennis, 2006). Specifically, the mammalian sPLA₂ family includes 10 catalytically active isoforms (Lambeau and Gelb, 2008). Isolated from a variety of sources, the sPLA₂s share a series of common structural features. They are low-molecular weight (14–18 kDa) secreted proteins, with a compact structure stabilized by six conserved disulfide bonds and two additional disulfides that are unique to each member (Dennis et al., 2011). Studies focusing on their mechanism of action have shown that an active site histidine and a highly conserved neighboring aspartate form the catalytic dyad involved in the reaction, with absolute dependence on Ca²⁺ for activation (Murakami et al. 2011b).

There is renewed current interest in elucidation of the *in vivo* biological functions of mammalian sPLA₂s as they have been implicated in a series of physiological and pathophysiological processes including lipid digestion, cell proliferation, neurosecretion, antibacterial defense, cancer, tissue injury, and atherosclerosis (Murakami and Lambeau, 2013). Furthermore, it has become apparent that individual secretory phospholipase A₂ enzymes play important and diverse roles in biological events by acting through multiple mechanisms: 1) involving generation of lipid mediators, and 2) executing their own unique action on their specific extracellular lipid targets (Murakami, 2011b). Kinetic characterization and mechanistic elucidation of sPLA₂s should contribute to better understanding of their exact physiological role(s) in these events that are yet to be delineated. In addition, phospholipase A₂ assays should also be applicable for high-throughput screening of potential PLA₂ inhibitors (Rose and Prestwich, 2006, Manna and Cho, 2007), as recent studies have indicated therapeutic potential of PLA₂ inhibitors in cardiovascular disease (Fujioka and Kugiyama, 2009), in the treatment of cancer (Dong et al., 2006).

In this communication we report the synthesis of new phospholipase A₂ targeted substrate analogues for fluorescence resonance energy transfer (FRET)-based kinetic studies of the enzyme. Specifically, we have introduced chain-terminal donor-acceptor pairs including coumarin-based fluorophores, for real-time spectrophotofluorimetric PLA₂ assay for kinetic studies of the enzyme. We hypothesized that relying on the platform of coumarins for substrate development provides a number of advantages over the methods previously employed for the design of fluorescent probes (Cho, 2006; Rose and Prestwich, 2006; Wichmann et al., 2007): 1) the fluorophores with the desired excitation/emission wavelengths are readily available, or can easily prepared by variation of the ring substituents using well-established synthetic methods, (Bennett et al., 1999; Berthelot et al., 2004; Wang and Li, 2004), 2) they have high extinction coefficients and quantum yields, approaching the detection levels of radioactive labels (Bennett et al.), 3) they exhibit good photochemical and chemical stability (Concalves, 2009) and 4) because of their small size compared to other fluorophores they are expected to interfere less with phospholipid packing when the probes are incorporated into lipid assemblies of natural phospholipid bilayers and micelles (Skaug et al., 2011). Thus, availability of the new probes should open the way to address key questions regarding: 1) the substrate quality of the synthetic analogues compared to the natural phospholipid substrates under the same set of experimental conditions, and 2) the impact of addition of the modified phospholipid analogues on the packing of the bilayer and/or the micellar assembly of natural phospholipids.

2. Results and Discussion

2.1. Design of the synthetic target compounds

Design and development of new fluorogenic phospholipase A₂ substrates suitable for real-time spectrophotometric assays of the enzyme are required for *in vitro* kinetic studies, and for detection and characterization of PLA₂ activity *in vivo*, to better understand the role of the respective sPLA₂ isozymes in health and disease (Cho, 2006). In this context a number of fluorescence-based assays have been developed, however, the first generation of PLA₂ substrates yielded mostly qualitative information, falling short of providing detailed kinetic parameters, as the probes were “limited by a window of robust PLA₂ activities” (Rose and Prestwich, 2006; Cho, 2006). Specifically, beyond this window of substrate concentrations the observed PLA₂ activity failed to follow the kinetics shown by the enzyme acting on natural phospholipid substrates (Rose and Prestwich, 2006). One likely cause of this behavior could be attributed to the disproportionately large size of the fluorophores incorporated into the chain-terminals, that modified the physicochemical properties of the acyl chains of the synthetic phospholipid analogues, thereby interfering with the protein-lipid interactions involved in the catalysis (Wichmann et al., 2007).

Our design of fluorescent PLA₂ substrates relies on fluorophores of substantially reduced size, while exhibiting efficient donor-acceptor behavior of fluorescence resonance energy transfer (FRET). The probes were designed to be capable of addressing two key parameters that have yet to be elucidated: 1) what impact introduction of the fluorophores will have on the rate of the reaction catalyzed by the enzyme compared to the rates of hydrolysis of the natural substrates measured under the same experimental conditions, and 2) how will the presence of the chain-terminal reporter groups impact on the packing / mixing of the phospholipid substrate in the aggregates used for measuring the catalytic activity of the enzyme.

We have designed two phosphatidylcholine analogues PC-1 and PC-2 for FRET-based kinetic studies of PLA₂ enzymes.

The first compound PC-1 incorporates a pair of chain-terminal fluorophores that consists of the naphthylacetyl donor and 7-mercapto-4-methylcoumarin as the acceptor. Specifically, the naphthylacetyl group has a well-established track record as donor in FRET studies in carbohydrate chemistry (Lee and Lee 1995), while the mercaptocoumarin group provides a highly fluorescent, photostable acceptor (Brogan et al., 2003), having the requisite spectroscopic properties of overlapping excitation spectrum ($\lambda_{\text{exc}} = 330$ nm) with the emission of the naphthylacetyl group ($\lambda_{\text{em}} = 335$ nm). The same fluorophore that serves as the acceptor in PC-1 acts as a donor in PC-2 ($\lambda_{\text{em}} = 390$ nm), used in conjunction with 7-diethylaminocoumarin-3-carboxylate ($\lambda_{\text{exc}} = 405$ nm) incorporated at the *sn*-2-chain end of the phospholipid molecule. In addition to the substantial overlap between the emission of the donor and the excitation spectrum of the acceptor, both coumarin derivatives exhibit large extinction coefficients, high quantum yields, and large Stokes shifts (Concalves, 2009). As pointed out, the reporter groups are relatively small in size in order to minimize the impact on the properties of the hydrocarbon chains. Finally, the synthesis of the fluorescent phospholipids is designed in such a sequence that includes formation of lysophospholipid intermediates, to allow incorporation of a wide range of substituents, including those that would not survive the reaction conditions involved in phosphorylation.

2.2 Syntheses

The synthetic strategy, outlined in Scheme 1, is based upon the following key elements: 1) 2,3-*O*-isopropylidene-*sn*-glycerol **3** is used to provide the chiral three-carbon skeleton to construct the target phospholipid compound, 2) introduction of the *sn*-1-acyl substituent is followed by the acid-catalyzed hydrolysis of the acetonide function to produce the corresponding diol, 3) orthogonal protection of the hydroxyl groups of the diol is achieved by using the base-labile phenoxyacetyl group at the primary glycerol position and the acid-labile tetrahydropyranyl function at the secondary hydroxyl group, and finally 4) elaboration of the phosphocholine headgroup is accomplished using 2-chloro-2-oxo-1,3,2-dioxaphospholane for phosphorylation, followed by cleavage of the 5-membered phosphodiester ring with anhydrous trimethylamine.

The synthesis starts with preparation of the fluorescently labeled fatty acid **4**, in reaction between 7-mercapto-4-methylcoumarin and 11-bromoundecanoic acid in deaerated acetone, in the presence of potassium carbonate as catalyst. Treatment of the product with aqueous HCl, followed by extraction with chloroform and chromatography on silica gel produced the purified target compound **4** in essentially quantitative yield. Acylation of 2,3-*O*-isopropylidene-*sn*-glycerol **3** with stoichiometric amount of the fluorescent fatty acid **4** was carried out in reaction with DCC and catalytic amount of DMAP in dichloromethane at room temperature for 2 h. The resulting *sn*-1-glyceryl ester **5** purified on silica gel chromatography was isolated in high yield (98%).

Next, acid catalyzed cleavage of the isopropylidene group of the acetonide **5** was carried out with 0.4 M HCl in aqueous dioxane at room temperature for 2 h. The product **6**, was isolated as a white solid in 78% yield following purification on silica gel chromatography. We have observed that in order to achieve efficient isopropylidene cleavage it is critically important to control the acidity of the solution; higher acidity leads to substantial decomposition of the diol **6**, while at lower acid concentrations less cleavage occurs resulting in poor yields of the deprotected product **6**.

Regioselective manipulation of the diol **6**, required orthogonal protection of the *sn*-2- and *sn*-3-hydroxyl groups. To that effect we used the base-labile phenoxyacetyl group at the primary alcohol position and the acid-labile tetrahydropyranyl protection at the *sn*-2-position of the glycerol skeleton. First, the base-labile phenoxyacetyl protecting group was

introduced in reaction of compound **6** with phenoxyacetyl chloride in the presence of 2,4,6-trimethylpyridine as catalyst in chloroform at 0°C for 5 h, to produce compound **7** in 68% yield. Traces of the *sn*-2-acylated regioisomeric byproduct could readily be separated from the main product by triphenylmethylation of the unreacted primary hydroxyl group. Specifically, the purified *sn*-3-phenoxyacetyl ester **7** showed baseline ¹H-NMR absorption in the δ 5.00–5.10 range, indicating that the *sn*-2-hydroxyl group remained intact in the course of the reaction. It should be noted that selection of the base-labile phenoxyacetyl protection, in this case, turned out to be preferable to the alternative Fmoc-carbonate strategy (Bibak and Hajdu, 2003), because isolation, purification, and manipulation of the phenoxyacetyl esters was much easier than the handling of the respective fluorenyloxycarbonyl derivatives.

Tetrahydropyranylation of the hydroxyl group at the *sn*-2-glycerol position was carried out using 3-fold excess of 3,4-dihydro-2*H*-pyran with PPTS as catalyst in chloroform at room temperature for 1 h. The product **8** was purified on silica gel chromatography, and isolated in 84% yield. Although introduction of the tetrahydropyranyl group creates a second chiral center in the molecule **8**, it does not complicate the synthesis, because carrying this protecting group is only temporary. In fact, as a hydrophobic neighboring group it provides a nonpolar local environment that facilitates the phosphorylation step in the synthesis.

For elaboration of the *sn*-3-phosphocholine headgroup we have modified the traditional procedure (Kim and Hajdu, 1993; Roodsari et al., 1999) by using anhydrous trimethylamine both as a catalyst in phosphorylation and as a nucleophile in the subsequent ring-opening step. Significantly, the two-step one-pot procedure eliminates the need to isolate the reactive dioxaphospholane intermediate. Thus, the alcohol **9** was phosphorylated using 2-chloro-2-oxo-1,3,2-dioxaphospholane in the presence of trimethylamine, in anhydrous acetonitrile at room temperature in a pressure bottle, to yield the cyclic phosphate intermediate formed as a single phosphate-positive product. The solution was cooled and treated directly with a second portion of trimethylamine, and kept at 67°C in the pressure bottle for 12 h. The phospholipid product precipitated from the reaction mixture on cooling, and it was purified by silica gel chromatography to afford compound **10** in 50% overall yield from the alcohol **9**.

Finally, the *sn*-2-tetrahydropyranyl group was removed by hydrolytic cleavage in 0.15 M hydrochloric acid in aqueous dioxane to yield the fluorescent lysophosphatidylcholine **11** (79%) for subsequent acylation at the *sn*-2-position. We have used two alternative routes to install the second fluorescently labeled fatty acyl substituent: either by direct acylation, or by relying on the strategy of using a chain-terminal protecting group to be subsequently exchanged with the designated fluorophore (Scheme 1). The second strategy should be particularly useful for incorporating chain-terminal labels that are only available for small-scale syntheses.

2.3. Enzymological characterization of the synthetic fluorescent phospholipids

2.3.1 Substrate properties of PC-1—To determine the substrate quality of the synthetic phospholipids we have measured the rates of catalytic hydrolysis of PC-1 and dipalmitoyl phosphatidylcholine (DPPC) by bee-venom phospholipase A₂ for comparison. Using a well-characterized assay system of mixed micelles with the surfactant 3-(N,N-dimethyldodecylammonio) propanesulfonate (DPS), the rates of PLA₂ catalyzed hydrolyses were measured at pH 8.00, in the presence of 10 mM CaCl₂ at 38°C, based on the volume of 0.01 M NaOH needed to neutralize the fatty acid produced in the reaction (Singh et al., 2009). The specific activities were found to be 679 μmol·min⁻¹·mg⁻¹ for DPPC/DPS, and 257 μmol·min⁻¹·mg⁻¹ for PC-1/DPS mixed micelles as substrates, under the same

experimental conditions (Figure 2, Table 1). These results indicate that the substrate quality of PC-1 measured in this assay is close to that of the natural phospholipid: modifying the structure by introduction of the chain-terminal fluorophores caused less than 3-fold decrease in the catalytic rate by the enzyme.

2.3.2. Incorporation of PC-1 in DPPC/DPS mixed micelles—To assess how addition of the synthetic phosphatidylcholine affects the substrate properties of phospholipid-surfactant mixed micelles, a series of micelles were prepared, keeping the phospholipid-to-surfactant mole ratio constant (2 mM / 8 mM), while varying the mole fractions within the lipid portion of the micelle from zero to 2 mM (Table 1). Using the specific activity of the enzyme to measure the substrate quality of the aggregates, the experimental results and the predicted values based upon the mole fractions of the components are summarized in Table 1.

The calculated values to predict the specific activities in the series of assay mixtures were determined based on the sum of the individual contributions of each phospholipid component as a function of its mole fraction in the micelle. Plotting the experimentally determined specific activities vs. the mole fraction of PC-1 yielded a straight line very close to the calculated linear dependence on the mole fraction of PC-1 in the assay (Figure 3), *indicating a close to ideal mixing behavior of the two phospholipid components in the micelle*. Thus, using the enzyme activity as the parameter to measure the impact on the packing behavior, addition of the fluorescent substrate analogue PC-1 seemed to cause only minor changes in the packing of the components in the micelle.

Finally, we have also measured the enzymatic hydrolysis of PC-1 following the loss of FRET to the acceptor by the decrease in fluorescence emission at 390 nm (Figure 4). Formation of the lysophosphatidylcholine product was determined from the change in the donor's fluorescence in mixed micelles of PC-1 with DPS and plotted vs. time.

Detailed kinetic studies using a series of coumarin fluorophores including the synthetic analogues PC-1 and PC-2 here reported will be conducted by real-time spectroscopic monitoring of the changes in FRET in the course of the enzymatic reaction. The results emerging from these studies will establish the scope and limitation of the method including its applicability for cellular assay of the enzyme. The results from these studies will be reported separately, in due course.

In conclusion, the synthesis here described provides an efficient method for the preparation of a wide range of functionalized phospholipid analogues with chain-terminal reporter groups. The method should be applicable to develop new, real-time spectrofluorimetric assays of phospholipases as well as membrane probes for the study of phospholipid-phospholipid and phospholipid-protein interactions in aggregates including bilayer vesicles. Furthermore, availability of the new fluorescent probes may be applicable for detection and measurement of phospholipase activity in cellular assays and related in vivo studies as well. Work toward these goals is underway in our laboratory.

3. Experimental procedures

3.1. Syntheses

11-(7'-mercapto-4'-methylcoumarin)-undecanoic acid (4)—To a suspension of potassium carbonate (0.680 g, 5 mmol) in 20 mL deaerated acetone was added 7-mercapto-4-methylcoumarin (0.481 g, 2.5 mmol), followed by stirring at room temperature for 1 h, whereupon a cloudy orange solution formed. To this solution was added 11-bromoundecanoic acid (0.796 g, 3.0 mmol) in 10 mL acetone, and on continued stirring at

room temperature for 2 h the color of the reaction mixture changed to light yellow. The solvent was then evaporated and the residue was washed by acetone (3×50 mL), treated with 100 mL H₂O, and acidified by addition of 1 M HCl to pH 4. The resulting solution was extracted with 100 mL CHCl₃, the organic layer was washed with 100 mL H₂O, then the solvent was evaporated and the solid residue was chromatographed on silica gel using CHCl₃-EtOAc (9:1) as eluent. The fractions of the product were combined and freeze-dried from benzene to give **4** as a light yellow powder (0.998 g, 99.5 %). IR (CHCl₃): 1738, 1732, 1721, 1603 cm⁻¹; ¹H-NMR (CDCl₃, 200 MHz) δ 1.16-1.82 (br m, 16H), 2.36 (t, *J* = 7.6 Hz, 2H), 2.41 (s, 3H), 2.99 (t, *J* = 7.4 Hz, 2H), 6.22 (s, 1H), 7.16 (d, *J* = 8.1 Hz, 2H), 7.47 (d, *J* = 8.9 Hz, 1H); ¹³C-NMR (CDCl₃, 200 MHz) δ 18.72, 24.80, 28.82, 29.01, 29.24, 29.34, 29.46, 29.52, 29.61, 32.32, 34.23, 113.85, 113.99, 117.09, 123.10, 124.63, 143.97, 152.43, 154.06, 160.94, 179.35; Anal. Calcd for C₂₁H₂₈O₄S: C 66.99, H 7.50; Found: C 67.29, H 7.77; MS M⁺ (C₂₁H₂₈O₄S) Calcd: 376.1700; Found: 376.1708; R_f(CHCl₃-EtOAc 9:1) = 0.18.

1-[11'-(7"-mercapto-4"-methylcoumarin)-undecanoyl]-*sn*-2,3-isopropylidene-glycerol (5)—To a solution of compound **4** (0.493 g, 1.31 mmol) in 15 mL CH₂Cl₂ was added *sn*-2,3-isopropylidene-glycerol **3** (0.173 g, 1.31 mmol), followed by 50 mg DMAP and DCC (0.270 g, 1.31 mmol) in 10 mL CH₂Cl₂, dropwise. The reaction mixture was stirred for 2 h at room temperature, and then the DCC-urea was removed by filtration, the solvent was evaporated and the residue was purified by silica gel chromatography using CHCl₃-EtOAc (9:1) as eluent. The fractions of the product were collected and the light yellow residue was freeze-dried from benzene to give a white powder **5** (0.634 g, 98.7 %). IR (CHCl₃): 1738, 1731, 1721, 1621, 1603 cm⁻¹; ¹H-NMR (CDCl₃, 200 MHz) δ 1.05-2.05 (br m, 22H), 2.38 (t, *J* = 6.2 Hz, 2H), 2.42 (s, 3H), 2.99 (t, *J* = 7.2 Hz, 2H), 3.65-4.35 (m, 4H), 4.35-4.65 (m, 1H), 6.21 (s, 1H), 7.17 (d, 2H), 7.44 (d, 1H); ¹³C-NMR (CDCl₃, 200 MHz) δ 18.73, 25.01, 25.55, 26.84, 28.82, 29.01, 29.24, 29.34, 29.46, 29.52, 29.61, 32.34, 34.24, 64.68, 66.50, 70.80, 109.97, 113.07, 113.87, 117.10, 123.09, 124.63, 143.94, 152.36, 154.08, 160.94, 173.76; Anal. Calcd: for C₂₇H₃₈O₄S: C 66.09, H 7.81; Found: C 65.95, H 8.01; MS M⁺ (C₂₇H₃₈O₄S) Calcd: 490.2400; Found: 490.2383; R_f(CHCl₃-EtOAc 9:1) = 0.75. [α]_D²⁵: +3.4° (c 1.00, CHCl₃-MeOH 4:1).

1-[11'-(7"-mercapto-4"-methylcoumarin)-undecanoyl]-*sn*-glycerol (6)—To a solution of compound **5** (0.597g, 1.22 mmol) in 25 mL 1,4-dioxane was added a solution of 25 mL 0.8 M HCl (1.6 mL of 12 M aqueous hydrochloric acid diluted in 23.5 mL dioxane) at room temperature. The reaction mixture was stirred at room temperature for 3 h, and it was freeze-dried to give a pale yellow powder. The solid was washed by EtOAc (3×20 mL) and benzene (3×20 mL), and chromatographed on silica gel using CHCl₃-EtOAc (1:1) as eluent. The fractions of the product were collected, the solvents were evaporated, and the residue was freeze-dried from benzene to give a yellowish white powder **6** (0.428g, 78.1%). IR (CHCl₃): 1738, 1731, 1621, 1603 cm⁻¹; ¹H-NMR (CDCl₃, 200 MHz) δ 1.15-2.05 (br m, 16H), 2.36 (t, *J* = 7.5 Hz, 2H), 2.49 (s, 3H), 2.99 (t, 2H), 3.55-3.80 (m, 2H), 3.85-4.05 (m, 1H), 4.15-4.45 (m, 2H), 6.21 (s, 1H), 7.18 (d, 2H), 7.45 (d, 1H); ¹³C-NMR (CDCl₃, 200 MHz) δ 18.72, 25.01, 28.82, 29.01, 29.24, 29.34, 29.46, 29.52, 29.61, 32.30, 34.28, 63.49, 65.33, 70.40, 113.07, 113.87, 117.10, 123.17, 124.63, 143.94, 152.36, 154.08, 160.94, 173.76; Anal. Calcd for C₂₄H₃₄O₆S: C 63.97, H 7.61; Found: C 64.02, H 7.72; MS M⁺ (C₂₄H₃₄O₆S) Calcd: 450.2100; Found: 450.2076; R_f(CHCl₃-EtOAc 1:1) = 0.23. [α]_D²⁵: +4.3° (c 1.00, CHCl₃-MeOH 4:1).

1-[11'-(7"-mercapto-4"-methylcoumarin)-undecanoyl]-3-phenoxyacetyl-*sn*-glycerol (7)—To a solution of **6** (1.030 g, 2.29 mmol) and phenoxyacetyl chloride (0.508 g, 3 mmol) in 40 mL CHCl₃ was added 2,4,6-trimethylpyridine (0.555 g, 4.6 mmol) in 10

mL CHCl_3 at 0°C . After stirring for 5 h at 0°C , the reaction mixture was evaporated to dryness and the residue was chromatographed on silica gel using CHCl_3 -EtOAc (1:1) as eluent. The fractions of the product were combined, the solvent was evaporated, and the solid was freeze-dried from benzene to give a white solid (0.904 g, 67.5 %, which contained 91% of **7** and 9% of the *sn*-2-phenoxyacetyl regioisomer. To the solution of this crude product **7** (0.500 g, 0.86 mmol) in 10 mL CH_2Cl_2 were added triethylamine (0.12 mL, 0.86 mmol) and triphenylchloromethane (0.240 g, 0.86 mmol). The reaction mixture was stirred for 24 h at 30°C . Then it was chromatographed directly on a silica gel column using CHCl_3 -EtOAc (1:1) as eluent. The fractions of the *sn*-2-alcohol were combined and the product was freeze-dried from benzene to give **7** (0.452 g, 90.4 %) as a white solid. IR (CHCl_3): 1738, 1731, 1722, 1716, 1694, 1619, 1604 cm^{-1} ; ^1H -NMR (CDCl_3 , 200 MHz) δ 1.20-2.12 (br m, 16H), 2.33 (t, J = 6.9 Hz, 2H), 2.41 (s, 3H), 2.98 (t, J = 6.8 Hz, 2H), 4.03-4.49 (m, 5H), 4.71 (s, 2H), 6.22 (s, 1H), 6.85-7.12 (m, 3H), 7.15 (d, 2H), 7.28-7.42 (d, 2H), 7.47 (d, J = 6.3 Hz, 1H); ^{13}C -NMR (CDCl_3 , 200 MHz) δ 18.72, 25.01, 28.82, 29.01, 29.24, 29.34, 29.46, 29.52, 29.61, 32.30, 34.28, 65.13, 65.75, 67.98, 75.52, 113.07, 113.87, 114.56, 114.57, 117.10, 120.65, 123.17, 124.63, 130.43, 130.43, 143.94, 152.36, 154.08, 157.24, 160.74, 169.84, 173.76; Anal. Calcd for $\text{C}_{32}\text{H}_{40}\text{O}_8\text{S}$: C 65.73, H 6.90; Found: C 65.43, H 7.12; MS MNa^+ ($\text{C}_{32}\text{H}_{40}\text{O}_8\text{SNa}^+$) Calcd: 607.2298; Found: 607.2367; R_f (CHCl_3 -EtOAc 1:1) = 0.61; $[\alpha]_D^{25}$: +10.3° (c 1.0, CHCl_3 -MeOH 4:1).

1-[11'-(7"-mercapto-4"-methylcoumarin)-undecanoyl]-2-(tetrahydropyranyl)-3-phenoxyacetyl-*sn*-glycerol (8**)**—To a solution of compound **7** (1.00 g, 1.7 mmol) and PPTS (0.215 g, 0.86 mmol) in 10 mL CHCl_3 , was added 3,4-dihydro-2*H*-pyran (0.720 g, 8.6 mmol). After stirring for 1 h at room temperature, the reaction mixture was loaded directly on a silica gel column, and chromatographed with CHCl_3 -EtOAc (5:1) as eluent. The fractions of the product were combined, the solvent was evaporated, and the residue was freeze-dried from benzene to give **8** (0.952 g, 84 %) as an oil. IR (CHCl_3): 3466, 2913, 2847, 1738, 1731, 1722, 1716, 1694, 1731, 1619, 1606 cm^{-1} ; ^1H -NMR (CDCl_3 , 200 MHz) δ 1.18-2.05 (br m, 22H), 2.31 (t, J = 7.0 Hz, 2H), 2.38 (s, 3H), 2.96 (t, J = 7.0 Hz, 2H), 3.52-4.00 (d, 2H), 4.05-4.39 (m, 7H), 4.41-5.50 (d, 1H), 6.21 (s, 1H), 6.85-7.09 (m, 3H), 7.15 (d, 2H), 7.28-7.42 (d, 2H), 7.47 (d, 1H); ^{13}C -NMR (CDCl_3 , 200 MHz) δ 18.72, 19.05, 25.01, 28.02, 28.82, 29.01, 29.24, 29.34, 29.46, 29.52, 29.61, 32.30, 33.99, 34.28, 62.22, 63.49, 65.26, 69.65, 71.59, 98.62, 113.07, 113.87, 114.56, 114.57, 117.10, 120.65, 123.17, 124.63, 129.43, 129.43, 143.64, 152.10, 153.74, 157.62, 160.49, 168.44, 173.25; Anal. Calcd for $\text{C}_{37}\text{H}_{48}\text{O}_9\text{S}$: C 66.44, H 7.23; Found: C 66.31, H 7.37; MS MNa^+ ($\text{C}_{37}\text{H}_{48}\text{O}_9\text{SNa}^+$) Calcd: 691.2898; Found: 691.2879; R_f (CHCl_3 -EtOAc 5:1) = 0.69.

1-[11'-(7"-mercapto-4"-methylcoumarin)-undecanoyl]-2-(tetrahydropyranyl)-*sn*-glycerol (9**)**—To a solution of compound **8** (0.700 g, 1.05 mmol) in 6 mL CHCl_3 - CH_3OH (1:1), *tert*-butylamine (0.553 mL, 5.25 mmol) was added at 0°C . After being stirred for 24 h at 0°C , the reaction mixture was directly loaded on a silica gel column and chromatographed using CHCl_3 -EtOAc (1:1) as eluent. The fractions of the product were combined, the solvent was evaporated, and the residue was freeze-dried from benzene to give compound **9** as a pale yellow solid (0.453 g, 88 %). IR (CHCl_3): 1738, 1731, 1721, 1716, 1687, 1621, 1604 cm^{-1} ; ^1H -NMR (CDCl_3 , 200 MHz) δ 1.15-2.05 (br m, 22H), 2.36 (t, J = 7.5 Hz, 2H), 2.49 (s, 3H), 2.99 (t, J = 7.3 Hz, 2H), 3.45-4.40 (m, 7H), 4.55-4.85 (d, 1H), 6.21 (s, 1H), 7.18 (d, 2H), 7.45 (d, 1H); ^{13}C -NMR (CDCl_3 , 200 MHz) δ 18.72, 19.81, 25.01, 28.20, 28.82, 29.01, 29.24, 29.34, 29.46, 29.52, 29.61, 31.30, 33.46, 34.28, 63.24, 65.27, 69.12, 70.40, 100.04, 113.07, 113.87, 117.10, 123.17, 124.63, 143.94, 152.36, 153.71, 160.64, 173.74; Anal. Calcd for $\text{C}_{29}\text{H}_{42}\text{O}_7\text{S}$: C 65.14, H 7.92; Found: C 64.94, H 8.21; MS MNa^+ ($\text{C}_{29}\text{H}_{42}\text{O}_7\text{SNa}^+$) Calcd: 557.2598; Found: 557.2543; R_f (CHCl_3 -EtOAc 1:1) = 0.55–0.62 (double spot).

1-[11'-(7''-mercapto-4''-methylcoumarin)-undecanoyl]-2-(tetrahydropyranyl)-3-*sn*-glycerophosphocholine (10)—To a solution of compound **9** (0.40 g, 0.75 mmol) in 25 mL of anhydrous acetonitrile at 0°C was added pre-cooled trimethylamine (1.2 mL, 8.24 mmol), followed by 2-chloro-2-oxo-1,3,2-dioxaphospholane (0.76 mL, 8.24 mmol) in a pressure bottle. The reaction mixture was stirred for 5 h at room temperature, when TLC showed that the alcohol disappeared. The reaction mixture was cooled in a dry ice-acetone bath below −10°C, and 1 mL of pre-cooled trimethylamine was added. The pressure bottle was sealed, and stirred for 12 h in an oil bath at 67°C. The white solid that precipitated on cooling was chromatographed on a silica gel using CHCl₃/CH₃OH/H₂O (65:25:4) as the eluent. The fractions containing the product were combined, the solvent was evaporated and the residue was freeze-dried from benzene to give compound **10** (0.300 g, 50% yield) as a white solid. IR (CHCl₃): 1738, 1731, 1720, 1715, 1698, 1694, 1620, 1603 cm^{−1}; ¹H-NMR (CDCl₃, 200 MHz) δ 1.10-2.05 (br m, 22H), 2.32 (t, 2H), 2.41 (s, 3H), 2.25-3.20 (m, 6H), 3.38 (s, 9H), 3.70-4.45 (m, 8H), 6.22 (s, 1H), 7.15 (d, 2H), 7.45 (d, 1H); ¹³C-NMR (CDCl₃, 200 MHz) δ 18.62, 18.72, 24.70, 25.01, 28.00, 28.82, 29.01, 29.24, 29.34, 29.46, 29.52, 29.61, 32.15, 32.15, 34.28, 54.32, 60.60, 64.40, 66.02, 72.10, 98.10, 113.87, 113.87, 117.10, 123.17, 124.63, 143.94, 152.36, 154.08, 160.94, 173.65; Anal. Calcd for C₃₄H₅₄NO₁₀PS·2H₂O, C 55.50, H 7.87, N 1.91; Found: C 55.54, H 7.40, N 2.14; MS MNa⁺ (C₃₄H₅₄NO₁₀PSNa⁺) Calcd: 722.3098; Found: 722.3098; R_f(CHCl₃-CH₃OH-H₂O 65:25:4) = 0.31.

1-[11'-(7''-mercapto-4''-methylcoumarin)-undecanoyl]-3-*sn*-glycerophosphocholine (11)—To a solution of phospholipid **10** (0.300 g, 0.43 mmol) in 25 mL 1,4-dioxane-H₂O (1:1) was added 0.3 M HCl in 25 mL 1,4-dioxane-H₂O (1:1) dropwise. After stirring for 5 h at room temperature the reaction mixture was freeze-dried to give a pale yellow semisolid. The crude product **11** was chromatographed on silica gel using CHCl₃-CH₃OH-H₂O (65:25:4) as eluent. The fractions of the product were combined, the solvent was evaporated, and the residue was freeze-dried from benzene to give a white solid **11** (0.212 g, 79%). IR (CHCl₃): 1738, 1731, 1721, 1716, 1688, 1620, 1603 cm^{−1}; ¹H-NMR (CDCl₃, 200 MHz) δ 1.12-1.78 (br m, 16H), 2.25 (s, 2H), 2.38 (s, 3H), 2.94 (t, *J* = 6.7 Hz, 2H), 3.33 (s, 9H), 3.60-4.47 (br m, 9H), 6.15 (s, 1H), 7.10 (d, 2H), 7.44 (d, 1H); ¹³C-NMR (CDCl₃, 200 MHz) δ 18.72, 24.70, 25.01, 28.82, 29.01, 29.24, 29.34, 29.46, 29.52, 29.61, 32.30, 34.28, 54.32, 60.60, 66.02, 72.23, 113.87, 113.87, 117.10, 123.17, 124.63, 143.94, 152.36, 154.08, 160.94, 173.65; Anal. Calcd: for C₂₉H₄₆NO₉PS·1.5H₂O, C 54.18, H 7.63, N 2.18; Found: C 54.24, H 7.76, N 2.26; FAB MH⁺ (C₂₉H₄₆NO₉PSH⁺) Calcd: 616.2680; Found: 616.2737; R_f(CHCl₃-CH₃OH-H₂O 65:25:4) = 0.20; [α]_D²⁵: +4.4° (c 1.03, CHCl₃-MeOH 4:1).

1-[11'-(7''-mercapto-4''-methylcoumarin)-undecanoyl]-2-[12'-(2''naphthylacetyl)-N-aminododecanoyl]-3-*sn*-glycerophosphocholine (PC-1)

—(i) *12-N-(2''naphthylacetyl)aminododecanoic acid*. To a solution of 12-*N*-aminododecanoic acid (1.000 g, 4.64 mmol) in 20 mL of 1M methanolic KOH was added 2-naphthylacetyl chloride (1.424 g, 7 mmol) in 20 mL aq. CH₃OH (1:1), followed by stirring at room temperature for 1 h, whereupon a cloudy pale yellow suspension formed. To the reaction mixture was added 100 mL CHCl₃, and it was acidified by addition of 1M HCl to pH 4. The organic layer was extracted and evaporated, and the residue was then treated with CH₃OH (5×20 mL). The combined methanol solution was evaporated to give a light yellow residue that was purified on silica gel column using CHCl₃-MeOH (9:1) as eluent. The fractions of the product were combined, the solvent was evaporated, and the residue was freeze-dried from benzene to give a light yellow powder (1.432 g, 81 %). (ii) *PC-1*. To a solution of compound **11** (0.500 g, 0.812 mmol) in 15 mL CHCl₃ were added 12-*N*-(2''naphthylacetyl)-aminododecanoic acid (1.560 g, 4.06 mmol) and DMAP (0.500 g, 4.06

mmol), followed by 1.5 g glass beads (Rossetto and Hajdu, 2005), and DCC (0.838 g, 4.06 mmol) in 5 mL CHCl_3 dropwise. The reaction mixture was sonicated for 5 h at 25°C. The DCC-urea precipitate, and the glass beads were filtered, and the solvent was evaporated. The residue was dissolved in CHCl_3 - CH_3OH (65:45), and loaded on a silica gel column for chromatography, first with CHCl_3 - CH_3OH (65:25) to elute the impurities, followed by CHCl_3 - CH_3OH - H_2O (65:25:4) to elute the product. The fractions of the product were combined, the solvent was evaporated, and the residue was freeze-dried from benzene to give pure **PC-1** as a white powder (0.450 g, 56.2 %). IR (CHCl_3): 1738, 1731, 1721, 1716, 1660, 1620, 1603 cm^{-1} ; ^1H -NMR (CDCl_3 , 200 MHz) δ 1.12-1.78 (br m, 34H), 2.25-2.45 (m, 4H), 2.38 (s, 3H), 2.94-3.20 (m, 4H), 3.33 (s, 9H), 3.60-4.47 (br m, 10H), 5.05-5.25 (d, 1H), 6.15 (s, 1H), 7.10 (d, 2H), 7.37 (d, 1H), 7.44 (d, 1H), 7.51 (t, 2H), 7.72 (s, 1H), 7.84 (m, 3H); ^{13}C -NMR (CDCl_3 , 200 MHz) δ 18.72, 24.63, 24.70, 25.01, 26.54, 28.32, 28.82, 29.01, 29.01, 29.24, 29.24, 29.34, 29.34, 29.46, 29.46, 29.52, 29.52, 29.61, 29.81, 32.30, 33.85, 34.28, 39.56, 43.10, 54.32 [$4\text{C}, \text{CH}_2\text{-N}(\text{CH}_3)_3$], 60.60, 66.02, 72.23, 113.87, 113.87, 117.10, 123.17, 124.63, 124.95, 125.70, 126.95, 126.95, 127.59, 127.59, 127.59, 131.60, 133.51, 135.20, 143.94, 152.36, 154.08, 160.94, 169.50, 173.65, 177.22; Anal. Calcd for $\text{C}_{53}\text{H}_{77}\text{N}_2\text{O}_{11}\text{PS}\cdot 3.5\text{H}_2\text{O}$, C 60.95, H 8.05, N 2.68; Found: C 60.92, H 7.48, N 2.72; MS MH^+ ($\text{C}_{53}\text{H}_{77}\text{N}_2\text{O}_{11}\text{PSH}^+$) Calcd: 981.5036; Found: 981.5059; R_f (CHCl_3 - CH_3OH - H_2O 65:25:4) = 0.41; $[\alpha]_D^{25}$: +5.9° (c 1.06, CHCl_3 -MeOH 4:1).

1-[11'-(7''-Mercapto-4''-methylcoumarin)-undecanoyl]-2-[12'-N-(7'''-(diethylaminocoumarin)-3'''-carbonyl)-aminododecanoyl]-3-sn-glycerophosphocholine (PC-2)—(i) Acylation of the *sn*-2-glycerol position of

lysophospholipid with Fmoc-protected aminododecanoic acid was carried out using the procedure previously described (Rossetto and Hajdu, 2005). The product 1-[11'-(7''-mercapto-4''-methylcoumarin)-undecanoyl]-2-(12'-Fmoc-aminododecanoyl)-*sn*-3-glycerophosphocholine **12** obtained in 66% yield and it was used directly for the next step. (ii) To a solution of **12** (0.251 g, 0.24 mmol) in 9 mL CH_2Cl_2 was added DBU (0.185 g, 1.2 mmol) in 1 mL CH_2Cl_2 at room temperature. After 1 h TLC CHCl_3 - CH_3OH - H_2O (65:25:4) showed that no more **12** left in the reaction mixture, having been replaced by a new, ninhydrin-positive spot appearing close to the origin. To this solution was added the *p*-nitrophenyl ester of 7-diethylamino-coumarin-3-carboxylate (0.275 g, 0.72 mmol) and DMAP (0.088 g, 0.72 mmol), and the reaction mixture was stirred for 5 h. The solvent was evaporated and the residue was chromatographed on a silica gel column first with CHCl_3 -MeOH (9:1), followed by using CHCl_3 - CH_3OH - H_2O (65:25:4) as eluent. The fractions of the product were combined, the solvent was evaporated, and the residue was freeze-dried from benzene give **PC-2** (0.123 g, 49 %) as an orange solid. IR (CHCl_3): 1738, 1731, 1721, 1717, 1696, 1620 1603 cm^{-1} ; ^1H -NMR (CDCl_3 - CD_3OD , 200 MHz) δ 1.21-1.95 (br m, 40H), 2.18-2.65 (br m, 7H), 2.72-3.03 (t, 2H), 3.05-3.85 (br m, 19H), 3.85-4.41 (br m, 4H), 5.10 (s, 1H), 6.15 (s, 1H), 6.35-6.90 (br m, 2H), 7.10 (d, 2H), 7.45 (d, 1H), 7.60-7.75 (d, 1H), 8.62 (s, 1H); ^{13}C -NMR (CDCl_3 , 200 MHz) δ 12.32, 12.32, 18.72, 24.63, 24.70, 25.01, 26.54, 28.32, 28.82, 29.01, 29.01, 29.24, 29.24, 29.34, 29.34, 29.46, 29.46, 29.52, 29.52, 29.61, 29.81, 32.30, 33.85, 34.28, 47.19, 49.82, 49.82, (53.78, 56.51, 58.81, 60.29) [$4\text{C}, \text{CH}_2\text{-N}(\text{CH}_3)_3$], 62.30, 63.31, 69.92, 107.42, 109.83, 113.87, 113.87, 115.67, 117.10, 117.71, 123.17, 124.63, 131.65, 142.24, 143.94, 149.73, 152.36, 153.65, 154.08, 160.94, 165.11, 165.11, 173.20, 173.59; Anal. Calcd for $\text{C}_{55}\text{H}_{82}\text{N}_3\text{O}_{13}\text{PS}$: C 62.54, H 7.82, N 3.98; for $\text{C}_{55}\text{H}_{82}\text{N}_3\text{O}_{13}\text{PS}\cdot 5\text{H}_2\text{O}$, C 57.62, H 8.03, N 3.67; Found: C 57.59, H 7.75, N 3.12; MS MH^+ ($\text{C}_{55}\text{H}_{82}\text{N}_3\text{O}_{13}\text{PSH}^+$) Calcd: 1056.5380; Found: 1056.5387; R_f (CHCl_3 - CH_3OH - H_2O 65:25:4) = 0.47; $[\alpha]_D^{25}$: +4.7° (c 1.00, CHCl_3 -MeOH 4:1).

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Figure 1.
Structures of fluorescently labeled phosphatidylcholines PC-1 and PC-2

Figure 2.
Reaction progress curves of catalytic hydrolysis by bee-venom phospholipase A₂ of PC1-DPS (2 mM:8 mM) [O] and DPPC-DPS (2mM:8mM) [◆] in mixed micelles.

Figure 3. Specific activity of bee-venom PLA_2 vs. the mole fraction of PC-1 (■) in phosphatidylcholine-DPS mixed micelles. Also shown are the calculated specific activities (○) predicted by assuming ideal mixing between PC-1 and DPPC in the phospholipid component of the micelles. (The data is taken from Table 1).

Figure 4.

Formation of lysophosphatidylcholine by bee-venom PLA₂ acting on PC-1 / DPS (0.005 mM:10 mM) mixed micelles. The product concentrations were calculated from the decrease in the acceptor's fluorescence at 390 nm.

Scheme 1.

Reagents and conditions: (a) DCC-DMAP, CH₂Cl₂; (b) 0.4 M HCl, aq. dioxane, 2 h; (c) C₆H₅OCH₂COCl, 2,4,6-trimethylpyridine, CHCl₃, 0°C; (d) DHP, PPTS, CH₂Cl₂, rt; (e) *tert*-butylamine, CHCl₃-MeOH (1:1), 0°C, 24 h; (f) (i) ethylene chlorophosphate, (C₂H₅)₃N, benzene, rt., 6 h, (ii) (CH₃)₃N, MeCN, 65°C, 24 h; (g) 0.15 M HCl, aq.dioxane; (h) 12-(2'-naphthylacetyl)aminododecanoic acid-DCC-DMAP, CHCl₃; (j) (i) 12-*N*-FMOC-aminododecanoic acid-DCC-DMAP, CHCl₃, (ii) 5 eq. DBU, rt, 1 h, (iii) *p*-nitrophenyl-7-diethylaminocoumarin-3-carboxylate, DMAP, CHCl₃.

Table-1

Specific activities of bee-venom phospholipase A₂ on phosphatidylcholine-DPS (2 mM:8 mM) mixed micelles as a function of PC-1 in the assay mixture.

[PC-1] (mM)	[DPPC] (mM)	[DPS] (mM)		Specific Activity ($\mu\text{mol}/\text{min}/\text{mg}$)
2	0	8	1	257
1	1	8	0.5	455
0.75	1.25	8	0.375	543
0.5	1.5	8	0.25	624
0.1	1.9	8	0.05	680
0	2	8	0	679