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Synthesis of phosphatidylcholine analogues derived from glyceric acid: a new class of biologically active phospholipid compounds

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Abstract

Synthesis of a new class of phosphatidylcholine analogues derived from glyceric acid is reported for spectroscopic studies of phospholipases and the conformation of phospholipid side-chains in biological membranes, using fluorescence resonance energy transfer (FRET) techniques.

Keywords: Phosphatidylcholine analogue synthesis; Glyceric acid ester; Orthogonal O-protection; Fluorescence resonance energy transfer (FRET)

Biologically active synthetic phospholipid compounds are required for structural and dynamic studies of biomembranes for the establishment of structure–activity relationships with respect to phospholipid–phospholipid and phospholipid–protein interactions, as well as for mechanistic studies of phospholipid metabolizing enzymes.[1–3] Specifically, with the discovery that phospholipases generate a number of physiologically important lipid metabolites, including second messengers that are involved in cell signaling,[1] the development of new synthetic methods for the preparation of phospholipid derivatives became a key step in advancing membrane biochemistry. Phospholipid analogues incorporating spectroscopically active reporter groups have been shown to be valuable structural probes to study the conformation and the dynamics of phospholipids in aggregates (e.g., micelles, bilayers, and vesicles) as well as for the development of spectroscopic assay systems of lipolytic enzymes,[4–6] and for in vivo monitoring of the fate of products generated by phospholipids metabolizing enzymes.[7]

As a part of our work in this area we focused on the development of highly specific fluorescent phospholipase A_2 substrates for kinetic studies and for the detection of in vivo activity of the enzyme. Phospholipases A_2 (PLA_2, EC 3.1.1.4) comprise a large group of intracellular and secreted enzymes that catalyze the hydrolysis of the *sn*-2 ester function of glycerophospholipids to produce free fatty acids, such as arachidonic acid and lysophospholipids.[8] Both products are the precursors of signaling molecules with a multitude of biological functions.[8–13] Specifically, arachidonic acid is converted to eicosanoids that have been shown to be involved in immune response, inflammation, pain perception, and sleep regulation,[8,9] while lysophospholipids are the precursors of lipid mediators such as lysophosphatidic acid (LPA) and platelet activating factor (PAF). LPA is involved in cell proliferation, survival, and migration, and PAF is particularly involved in inflammatory processes.[8] Thus, mammalian secreted phospholipase A_2s ($sPLA_2$s) have been implicated in a series of physiological and pathophysiological functions including lipid digestion, cell proliferation, neurosecretion, antibacterial defense, cancer, and inflammatory diseases.[10] Kinetic characterization and mechanistic elucidation of $sPLA_2$ enzymes should contribute to better understanding of their exact

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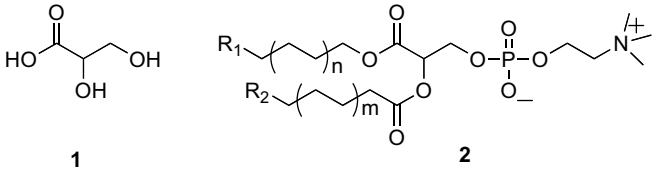
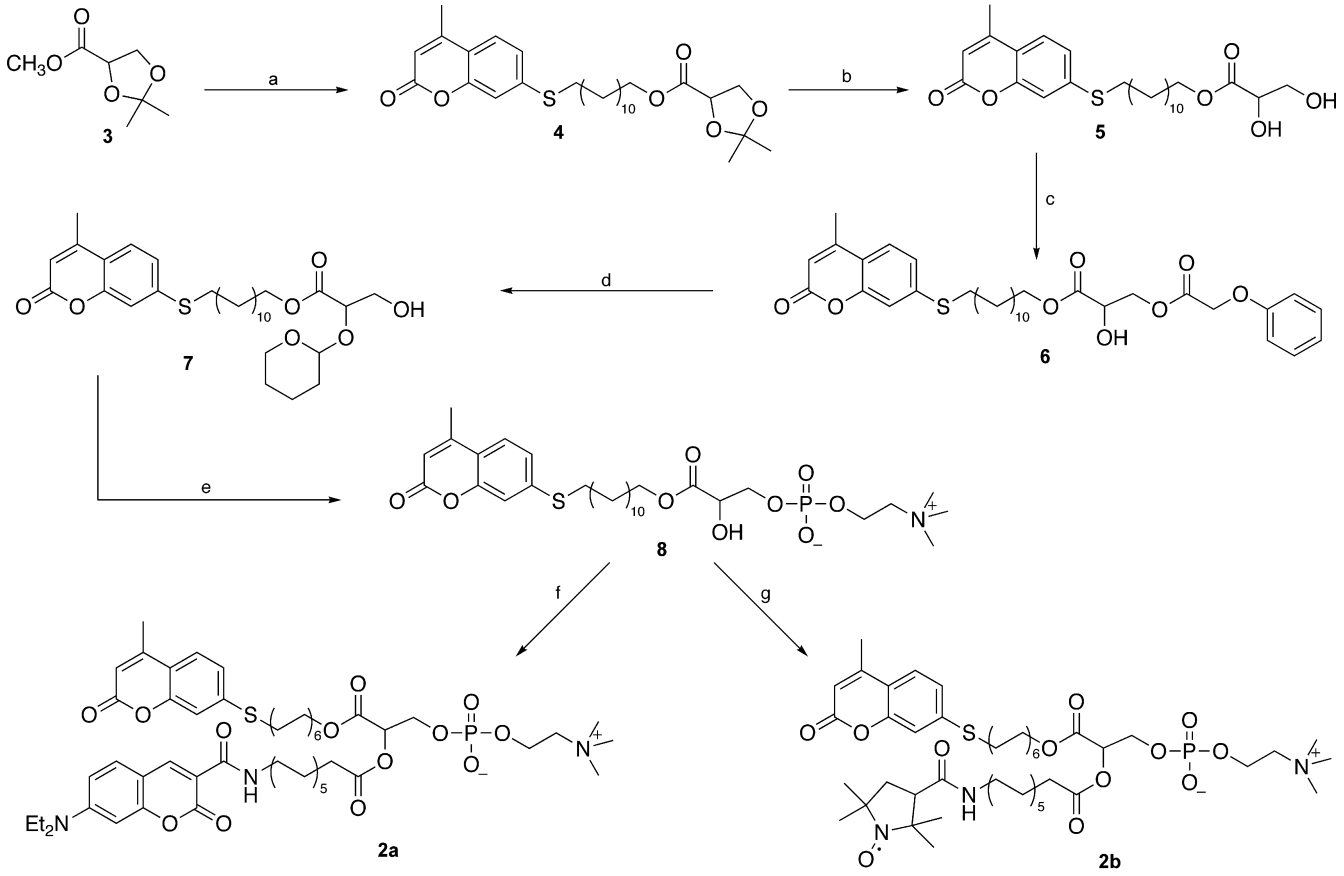
physiological role in these events, which remain to be elucidated.[11] In addition, fluorescence-based phospholipase A_2 assay methods should also be applicable for high-throughput screening of potential PLA_2 inhibitors.[4] Significantly, recent studies indicated therapeutic potential of phospholipase A_2 inhibitors in cardiovascular disease,[12] and it has also been suggested that select isoforms of the enzyme may be targets for drugs in the treatment of cancer.[13]

In the present Letter, we report the synthesis of a new series of phospholipid compounds derived from glyceric acid (**1**), incorporating an 'inverse ester' function at the *sn*-1-position preventing hydrolysis by PLA_1, while keeping the *sn*-2-ester group intact for cleavage by phospholipase A_2 enzymes (**2**).



1 **2**

Phospholipid analogues such as compound **2** should also be applicable to detect the activity of PLA_2 enzymes, because the change in fluorescence depends on specific cleavage of the ester linkage at the *sn*-2-position of the substrate. In designing the target structure we selected two photostable coumarin fluorophores with the requisite spectroscopic properties to provide a suitable donor–acceptor pair.[14] In addition, the chain-terminal reporter groups are relatively small in size in order to minimize the impact on the properties of the hydrocarbon chains. Using the 'inverse ester' strategy instead of relying on the *sn*-1-alkyl ether substitution to achieve PLA_2 selectivity brings the compound closer to the naturally occurring glycerophospholipid structure.[1] Furthermore, the synthetic sequence is designed in such a way that includes lysophospholipid intermediates, to allow the incorporation of substituents with chain-terminal functional groups, including those that would not survive the reaction conditions involved in phosphorylation. Significantly, fluorescent lysophospholipid compounds have been shown to be important biologically active lipid analogues in their own right.[1,15]



Scheme 1. Reagents and conditions: (a) (i) 1.2 M NaOH, 80% aq dioxane, (ii) Bio-Rad AG 50-X (H^+), (iii) 12-(7′-mercapto-4′methylcoumarin)dodecanol–DCC–DMAP, $CHCl_3$; (b) 0.4 M HCl, aq dioxane, 2 h; (c) 1.4 equiv $C_6H_5OCH_2COCl$, 2,4,6-trimethylpyridine, $CHCl_3$, 0 °C, 3 h; (d) (i) DHP, PPTS, CH_2Cl_2, rt, 3 h, (ii) 5 equiv *tert*-butylamine, $CHCl_3$–MeOH (1:1), 0 °C, 14 h; (e) ethylene chlorophosphate, Et_3N, benzene, (ii) $(CH_3)_3N$, MeCN, 65 °C; (iii) 0.3 M HCl, rt, 6 h; (f) (i) FMOC-12-aminododecanoic acid–DCC–DMAP, $CHCl_3$, (ii) DBU, rt, 1 h, (iii) *p*-nitrophenyl-7-diethylaminocoumarin-3-carboxylate, DMAP, $CHCl_3$; (g) (i) and (ii) as in (f), then *p*-nitrophenyl-PROXYL-3-carboxylate, DMAP, $CHCl_3$.

The synthetic strategy, outlined in Scheme 1, is based upon two key elements. First, commercially available 2,3-*O*-isopropylidene-L-methyl glycerate **3** is used to provide the three-carbon skeleton to construct the target phospholipid compound. Specifically, we have discovered that while glyceric acid itself is rather intractable due to its poor solubility in organic solvents, base-catalyzed hydrolysis of acetonide **3** readily affords the corresponding sodium glycerate in essentially quantitative yield. Treatment of the sodium salt with ion exchange resin provides the corresponding free acid, which in turn can be converted to the long-chain fluorescent ester **4** in condensation with the desired alcohol.[16] The second principal element involves the orthogonal protection of the diol portion of the glyceric acid skeleton. Here, we selected the phenoxyacetyl group to provide base-labile protection at the *sn*-3-position, since we have found that the acylation of compound **5** using a combination of phenoxyacetyl chloride/collidine in $CHCl_3$ proceeds with high regioselectivity at the primary hydroxyl group, the only byproduct being the diacyl derivative that is readily separated from product **6** on column chromatography. On the other hand, for acid labile protection at the *sn*-2-alcohol function tetrahydropyranylation turned out to be the most suitable choice. Specifically, both the introduction of the tetrahydropyranyl function as well as its cleavage later on in the sequence, can be carried out under mild acidic conditions, and most importantly *without acyl group migration* from the adjacent ester function. Although the protection step introduces a second chiral center, it does not complicate the synthesis because the intermediate can easily be carried through the sequence as a diastereoisomeric mixture up to the deprotection step.

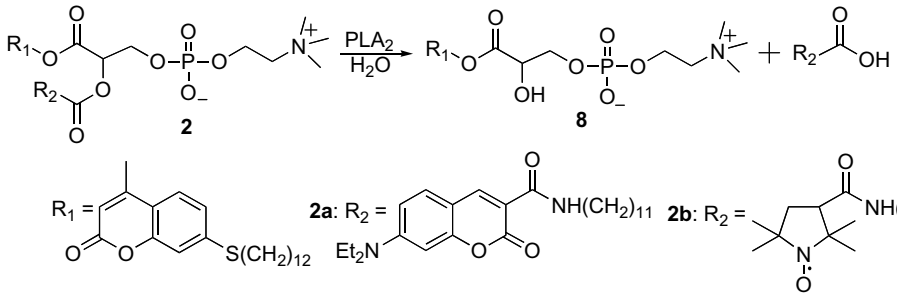
Thus, the hydrolysis of methyl 2,3-*O*-isopropylidene-L-glycerate **3** with stoichiometric amount of 1.2 M NaOH in 80% aqueous 1,4-dioxane at room temperature for 2 h yielded the sodium salt of the protected glyceric acid as a white powder (97%). The compound was isolated from the reaction mixture by freeze-drying. The product was treated with 30 mL Bio-Rad AG50-X (H^+) ion exchange resin in dioxane for 15 min at room temperature to obtain the carboxylic acid as a colorless oil, which was converted to the fluorescent ester **4** in reaction with 12-(7′-mercapto-4′-methylcoumarin)dodecanol–DCC–DMAP in chloroform at room temperature for 6 h. The resulting ester **4** was purified by silica gel chromatography ($CHCl_3$–EtOAc 9.5:0.5), and freeze-dried from benzene as a white solid (88%). Acid-catalyzed hydrolysis of the isopropylidene function with 0.4 M HCl in 97% aq dioxane at room temperature for 2 h followed by freeze-drying and subsequent chromatography ($CHCl_3$–EtOAc 5:1) led to the pure diol **5** in 78% yield.

Regioselective acylation at the primary hydroxyl group of compound **5** was accomplished using 1.4 equiv of phenoxyacetyl chloride in the presence of 2.0 equiv of 2,4,6-trimethyl pyridine in chloroform at 0 °C for 3 h. Product **6** was purified by silica gel chromatography using chloroform–ethyl acetate (5:1) as eluant, then freeze-dried from benzene to give a colorless oil that turned to a white waxy solid on standing (68%). The high-field ^1H NMR spectrum of compound **6** shows baseline resonance in the δ 5.00–5.09 region, confirming that the secondary hydroxyl group remained intact in the isolated product.[17] Next, the tetrahydropyranyl function was elaborated at the *sn*-2-glycerol position using five-fold excess of 3,4-dihydro-2*H*-pyran with pyridinium *p*-toluenesulfonate catalysis in CH_2Cl_2 at room temperature for 3 h, in 96% yield, and base-catalyzed chemoselective cleavage of the phenoxyacetyl ester function was accomplished using 5 equiv of *tert*-butylamine in chloroform–methanol (1:1) at 0 °C for 14 h. Product **7** was purified by column chromatography on silica gel using a stepwise gradient of $CHCl_3$–EtOAc (9:1 followed by 6:1) to obtain the primary alcohol **7** in 97% yield.

Phosphorylation of the substituted glycerol **7** at the *sn*-3-position was carried out using 2 equiv of 2-chloro-2-oxo-1,3,2-dioxaphospholane and triethylamine in sodium-dried benzene at room temperature for 4 h. The phosphorylated intermediate was treated with 5 mL anhydrous trimethylamine in acetonitrile at 65 °C (in a pressure bottle) for 24 h to afford the crude product that separated from the reaction mixture on cooling. This intermediate was purified by silica gel chromatography ($CHCl_3$–CH_3OH–H_2O 65:25:4, 74% yield), and treated with a 0.3 M HCl in a biphasic system of chloroform-water (4:0.1) for 2 h. Freeze-drying from benzene yielded the crude fluorescent lysophospholipid **8**, which was purified by chromatography ($CHCl_3$–CH_3OH–H_2O 65:25:4) and isolated as a white solid (57%). Next, compound **8** was acylated with 2.5 fold excess of FMOC-12-aminododecanoic acid–DCC–DMAP in $CHCl_3$ in the presence of glass beads at 25 °C, with sonication, for 5 h.[18] Purification by silica gel chromatography, first with $CHCl_3$–MeOH (4:1), followed by $CHCl_3$–MeOH–H_2O (65:25:4) as eluant gave the FMOC-protected phosphatidylcholine derivative (60%). Base-catalyzed elimination of the FMOC protecting group using 4 equiv of DBU in $CHCl_3$ at room temperature for 1 h, followed by the treatment with *p*-nitrophenyl-7-diethylaminocoumarin-3-carboxylate in the presence of catalytic amount of DMAP for 4 h in the same reaction mixture[18] afforded the target phospholipid **2a**. Purification by silica gel chromatography using the same solvent systems as mentioned above yielded the fluorescent product **2a** (66% from **8**) as a yellow solid.[19]

With the availability of compound **8** we were able to extend the scope of reporter groups to include a nitroxide spin label introduced at the *sn*-2-ester group's chain-terminal **2b**. Conversion of compound **8** in the same sequence of reactions as outlined for **2a** afforded the double-labeled phosphatidylcholine derivative **2b** in 52% overall yield.

Both compounds **2a** and **2b** were completely hydrolyzed by bee-venom phospholipase A_2 yielding the corresponding lysophospholipid **8** (Eq. 1).



(1)

Specifically, the hydrolysis of **2a** occurs with a decrease in the fluorescence emission of the acceptor at 462 nm, and an increase in the emission of the donor at 397 nm due to loss of fluorescence resonance energy transfer. On the other hand, cleavage of the *sn*-2-ester linkage of **2b** leads to enhanced fluorescence at 397 nm via de-quenching, due to the release of the paramagnetically labeled fatty acid from the molecule.[20]

In conclusion, the synthesis reported here provides a facile and efficient method for the preparation of a new class of phospholipid analogues. The method should be applicable for the preparation of a wide range of functionalized phospholipid derivatives not only for the development of new real-time spectrophotometric assays of phospholipases and high-throughput screening of phospholipase inhibitors, but also for the design and the development of new membrane probes for the study of conformation and interaction of phospholipids in monolayers, bilayers, and micelles. Work toward these goals is underway in our laboratory.

Acknowledgment

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Supplementary data

Characterization of the synthetic compounds by spectroscopic methods including IR, ^{1}H, ^{13}C NMR, HRMS, and elemental analyses. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.03.084.

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