STEREOSELECTIVE SYNTHESIS OF FLUOROGENIC
PHOSPHOLIPASE A₂ SUBSTRATES

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biochemistry

by

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DEDICATION

TO MY FAMILY
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<tr>
<td>DCC</td>
<td>N,N’-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DHP</td>
<td>3,4-Dihydro-2H-pyran</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(Dimethylamino)-pyridine</td>
</tr>
<tr>
<td>DNFB</td>
<td>2,4-Dinitrofluorobenzene</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
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<td>PC</td>
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<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>PPTS</td>
<td>Pyridium-&lt;i&gt;p&lt;/i&gt;-toluenesulfonate</td>
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<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
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<td>THP</td>
<td>Tetrahydropyran</td>
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ABSTRACT

STEREOSELECTIVE SYNTHESIS OF FLUOROGENIC PHOSPHOLIPASE A₂ SUBSTRATES

By

Duan Trinh

Master of Science in Biochemistry

Two fluorogenic phosphatidylcholines (PC-1 and PC-2) were synthesized and characterized for PLA₂’s kinetic studies. The compound (R) - (-) 2,2- dimethyl-1,3-dioxolane-4-methanol was chosen as the chiral precursor to synthesize the two optically active phosphatidylcholine molecules. 7-Mercapto-4-methylcoumarin was introduced as a fluorophore, and 2,4-dinitroaniline was employed as a quencher of the two PLA₂ substrates. PC-1 was constructed with a long chain acyl group carrying a quencher at the sn-2 position while PC-2 was synthesized with a short chain attached to a quencher at the sn-2 position. The synthesis relied on using tosylate to protect the incipient sn-3 alcohol function that was elaborated in a two-step sequence by displacement with tetramethyl ammonium acetate or tetraethylammonium methoxyacetate, and subsequent hydrolysis catalyzed by t-butyramine. The phosphocholine group was introduced to the glycerol skeleton using 2-chloro-2-oxo-1, 3, 2- dioxaphospholane at sn-3 position. An sn-2 THP protecting group was removed using 0.3M HCl in aqueous dioxane, and the final acylation of the sn-2 position was done using sonication with ultrasound to produce the target phospholipids. The new synthesis provided a flexible route to preparation of
carbonate phospholipids, and opened the way to study the enzymology of new synthetic phospholipids in future.
1. INTRODUCTION

1.1 Phospholipids

Phospholipids are recognized as critical constituents of all biological membranes. They play an important role as essential molecules for the regulations of cell functions by neurotransmitters, inflammatory cytokines, and growth factors.\(^1,2\) Furthermore, membrane phospholipids contribute as lipid metabolites’ precursors and serve as substrates for lipid kinases, phosphatases and phospholipases to generate lipid molecules.\(^2-5\) Glycerophospholipids are composed of a hydrophilic head and two hydrophilic tails in their structure. This amphipathic characteristic helps them to assemble into cell membrane bilayers. Phospholipids also play significant role in cell membranes’ signaling.\(^6-9\) For instance, the lysophospholipids are single-chain phospholipids that form lysophosphatidic acid and sphingosine-1-phosphate that bind to activate G protein coupled receptors from Ras gene family.\(^10-13\) The tail-oxidized phospholipids play a critical role in innate immune system and plasma membrane’s regulation.\(^13,14\) Furthermore, endogenous and exogenous PLs are considered ligand antigens that activate the natural killer T cells in the presence of CD1 proteins in human antigen presenting cells.\(^13,15,16\) PL binding domains of the proteins can recognize PLs, and nuclear receptors can engulf the tail of PLs to create elongated hydrophobic contact with more than fifteen residue structures and three hydrogen bonds that are closed to receptor’s surface.\(^13,17-19\) On the other hand, the binding energies are obtained from the communication with aliphatic tails to binding pocket from the protein’s core and terminates at the surface of protein and increases the stability of protein.\(^13\) The synthetic phosphatidylcholines help
to create a very useful picture and excellent methods to have a deep understanding of
biological functions of phospholipids.

![Phospholipid Structure](image)

R, R' = hydrocarbon groups
X = serine, ethanolamine, inositol, choline

### 1.2 Phospholipases

Phospholipases are an important group of enzymes that selectively catalyze the
hydrolysis of phospholipids. The phospholipases can be found widely from bacteria to
mammals, and they act on the phospholipids that form vesicles and micelles in aqueous
solution. They are classified into 4 major groups A, B, C, and D. These enzymes can
selectively identify one of four ester bonds on phospholipid, except phospholipase B.

- **Phospholipase A<sub>1</sub>** cleaves the \( sn-1 \) acyl chain of phospholipid. **Phospholipase A<sub>2</sub>**
cleaves the \( sn-2 \) acyl chain of phospholipid. The hydrolysis catalyzed by phospholipase
A<sub>1</sub> and phospholipase A<sub>2</sub> produces free fatty acid and lysophospholipid.

- **Phospholipase B** can cleave both acyl chains at the \( sn-1 \) and \( sn-2 \) positions on the phospholipid.

- **Phospholipase C** cleaves the phosphodiester bond at the \( sn-3 \) position to release a
phosphate-containing head group and a diacylglycerol.

- **Phospholipase D** cleaves the head group of phospholipid to release a choline and a phosphatidic acid.
Figure 1. Cleavage sites of phospholipases on phosphatidylcholine

1.3 Phospholipase A₂

Phospholipase A₂ 1 (PLA₂s) have the systematic name of phosphatide 2-acylhydrolase. They hydrolyze the sn-2 postion of phospholipid to release fatty acids 2 and lysophospholipids 3 such as leukotrienes and prostaglandins that are important precursors of cell’s biological activities.⁷⁻¹², 3⁰⁻³⁵

Furthermore, the lysophospholipid products of PLA₂ hydrolysis reaction play important roles in cell migration and survival.³³ The PLA₂s have 15 separate groups based on the ability to cleave sn-2 ester bond of phospholipid substrate.³⁴⁻³⁶ They are classified into appropriate groups based on their molecular weight, amino acid sequence,
and requirement for Ca\(^{2+}\). The PLA\(_2\)s are grouped into 5 different categories: the Ca\(^{2+}\)-independent (iPLA\(_2\)), the secreted low molecular weight (sPLA\(_2\)), the large cytosolic Ca\(^{2+}\)-dependent (cPLA\(_2\)), the platelet activating factor acetylhydrolases (PAF), and the lysosomal PLA\(_2\).\(^{34-38}\) They can be found in bee-venom, cobra venom, and snake venom.\(^{40-42}\) In comparison to phospholipase C and phospholipase D, the PLA\(_2\) family is larger.\(^{39,43}\) The sPLA\(_2\)s are 14-18 kDa proteins that contain 6-8 disulfide bonds on their structures.\(^{21,44}\) The sPLA\(_2\)s function during secretion of the cells, and their function can be controlled by binding of specific proteins.\(^{43,45}\) The small molecular weight sPLA\(_2\)s have been used extensively to study the biological roles of sPLA\(_2\)s. An His/Asp (histidine/aspartic acid) dyad is found at the active site on sPLA\(_2\)s, and mM concentration of Ca\(^{2+}\) is required for hydrolytic activity of the enzyme.\(^{21,46,47}\) Ten members of the sPLA\(_2\)s family form a group based on their disulfide linkage and belong to mammals. The human genomes have 9 sPLA\(_2\)s.\(^{21,48,49}\) Most of sPLA\(_2\)s have a catalytic site and Ca\(^{2+}\) binds to the loop for their hydrolytic activity. Interestingly, the sPLA\(_2\)s use the His with assistance of Asp on catalytic site to polarize the water molecule to attack the carbonyl group of the substrate. The presence of Ca\(^{2+}\) helps to balance the transition state of negative charge of phosphate oxygen and carbonyl group. Groups II, V, and X of sPLA\(_2\)s are associated with inflammatory diseases.\(^{21}\) For example, the high concentration of group II sPLA\(_2\)s are found in the synovial fluid of patient associated with diagnostic rhyematoid arthritis.\(^{27,50}\) In addition, groups I, II, III, V, and X of sPLA\(_2\)s show strong antibacterial functions, specially in antibacterial defense against Gram-positive bacteria.\(^{51}\) Furthermore, groups II, V, and X of sPLA\(_2\)s have crucial role in atherosclerosis in
humans. A high level of these groups of sPLA₂s in bloodstream increase the chance of the lipid accumulation.²¹,⁵²

1.4 Specific Targets

Our target was to synthesize the two new phosphatidylcholine molecules (PC-1 and PC-2) with the sn-1 carbonate group in their glycerol structure.

![Structure of PC-1](image1)

![Structure of PC-2](image2)

**Figure 2.** Structures of two final phosphatidylcholines PC-1 and PC-2

The compound (R) – (-)-2,2–dimethyl-1,3- dioxolane- 4- methanol 4 was used as a commercial available precursor of chiral glycerol for the synthesis of phospholipids.⁵³
Many protection and deprotection steps were carried out to prevent acyl migration and construct the two final phosphatidylcholines as expected.

![Structure of precursor of chiral glycerol](image)

**Figure 3.** Structure of precursor of chiral glycerol

The two final phosphatidylcholine molecules were planned to have the same quencher group but different chain lengths, and same fluorescent group on their glycerol structure. The fluorescent groups are UV-absorbing and appear as blue spots to help to detect the movement of products on thin layer chromatography (TLC) plates. The completion and the duration of reactions throughout the synthesis are easily determined by following the Rf of products and starting materials. The presence of quencher groups will be recognized by the appearance of a yellow spot on TLC. The two final structures of phosphatidylcholines should have the same chirality as the starting material 4. The long and short chain linked quencher groups from 2,4-dinitroaniline 5 will be introduced to sn-2 position of the glycerol structure. The fluorophore from mercapto-4-methyl coumarin 6 will be introduced to sn-1 position of the glycerol structure. The 2,4-dinitroaniline 5 and 7-mercapto-4-methyl coumarin 6 were employed as chain terminal reporter groups for the final molecules.
Figure 4. Structures of quencher and fluorophore used in the synthesis of PLA$_2$ substrates.$^{54}$

The synthesis of final phosphatidylcholines (PC-1 and PC-2) is shown in figure 5, and two phospholipids are designed with the carbonate functional group at the same $sn$-1 position carrying the chain terminal fluorophore. The long chain and short chain linked quenchers derived from 2,4-dinitroaniline 5, and form fluorogenic molecules at the end of synthesis. The selection of $sn$-1-carbonate group on target phosphatidylcholines is to make it specific for PLA$_2$ because PLA$_2$ selectively recognizes and hydrolyzes $sn$-2 position. The ester group of the naturally occurring phospholipid is replaced by the carbonate function to interfere with PLA$_1$ catalyzed hydrolysis. The new synthetic compounds are designed to measure the PLA$_2$ activities in the presence of Ca$^{2+}$. They are also used to trace the presence of lysophospholipids and free fatty acids as products of PLA$_2$ hydrolysis. In addition, the results from PLA$_2$ hydrolysis reactions lead to the new study of kinetics of this enzyme in the future. The coumarins of new PLA$_2$ substrates are incorporated as fluorescent probes. The new designed phosphatidylcholines have small-size terminal reporter groups to minimize the impact on biochemical
functions of fatty acyl side-chains.\textsuperscript{55, 56} The final products PC-1 and PC-2 display perfect mixing behavior with components of unlabeled phospholipids in the cell membranes.\textsuperscript{57}

1.5 Synthetic Strategies

The isopropylidene glycerol with tosylate group is deprotected using HCl in methanol to open the ring structure for further elongation and attachment of desired functional groups. The pivaloylation is done introducing pivalic acid at \textit{sn}-1 position of the glycerol structure. The \textit{sn}-2 position of glycerol is protected using DHP and PPTS to achieve tetrahydropyranylation. The reduction of the ester group of pivalic acid is designed to form hydroxyl group at \textit{sn}-1 position. The fluorescent products are synthesized in two-step method through base catalyzed nucleophilic displacement of bromine of 11-bromoundecanol by mercapto methylcoumarin, then activation with \textit{p}-nitrophenyl chloroformate to create the corresponding carbonate. UV light is used to detect the presence of fluorescent products. The coupling reaction is carried out to form a product that has carbonate functional group at \textit{sn}-1 position, THP protecting group at \textit{sn}-2 position, and tosylate group at \textit{sn}-3 position. The \textit{p}-toluenesulfonate functional group is replaced by either tetramethylammonium acetate or tetrylammonium methoxy acetate in anhydrous acetonitrile. The phosphocholine is introduced into the \textit{sn}-3 position using 2-chloro-2-oxo-1,3,2-dioxaphospholane in two-step method. Trimethylamine plays catalytic role in first step, and contributes as nucleophile in ring-opening final step of phosphorylation. The displacement of THP protecting group at \textit{sn}-2 position is done using 0.3M HCl in aqueous dioxane. The acylation of lysophosphatidylcholines are done
with acyl group carrying chain-terminal quencher at sn-2 position of glycerol structure to give the two final PC-1 and PC-2.

**Figure 5.** The synthetic strategies for target PC-1 (45) and PC-2 (46)
Reagents and conditions: (a) (i) p-toluenesulfonyl chloride/DMAP, CH₂Cl₂, (ii) 0.4 M HCl/CH₃OH, (iii) pivalic acid/DCC/DMAP, CH₂Cl₂, (iv) DHP/PPTS, CH₂Cl₂, (v) LiBH₄/THF, Et₂O, (vi) 11-((4-methyl-2-oxo-2H-chromen-7-yl)thio)undecyl (4-nitrophenyl) carbonate/DMAP, CH₂Cl₂; (b) CH₃COO’Me₄N⁺,CH₃CN; (c) Bu’NH₂, CHCl₃/MeOH (1:4); (d) CH₃OCH₂COO’Et₄N⁺,CH₂CN; (e) Bu’NH₂, CHCl₃/MeOH (1:4); (f) (i) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Me₃N, MeCN, rt., 12 h, (ii) Me₃N, MeCN, 67°C, 24 h; (g) 0.3M HCl, aq.dioxane; (h) N-(2,4-dinitrophenyl)aminododecanoic acid/DCC/DMAP, CHCl₃; (i) N-(2,4-dinitrophenyl)aminopropionic acid/DCC/DMAP, CHCl₃.
2. EXPERIMENTAL SECTION

2.1 Materials

(R) - (-) -2,2- dimethyl-1,3-dioxolane-4-methanol, p-toluenesulfonyl chloride, 11-bromo-1-undecanol, 7-mercapto-4-methylcoumarin, trimethylamine, KOH, Tris buffer, Triton X-100, CaCl$_2$, bee venom phospholipase A$_2$ in powder form, and phosphorus pentoxide were purchased from Sigma Aldrich and used as received. Spectrograde chloroform, diethyl ether, dioxane, and acetone were obtained from Fisher Chemical and used as received. Methanol-d$_4$ was obtained from Cambridge Isotope Laboratories and used as received. Benzene, LiBH$_4$ in THF, p-nitrophenyl chloroformate, methoxyacetic acid, t-butyramine, 2-chloro-2-oxo-1,3,2-dioxaphospholane, 1-fluoro-2,4-dinitrobenzene, 12-aminododecanoic acid, 3-aminopropionic acid were purchased from TCI and used as received. Methanol, acetyl chloride, chloroform-d, molecular sieves, tetramethylammonium acetate, tetraethylammonium hydroxide, and acetonitrile were obtained from from Acros Organics and used as received. N,N’-dicyclohexylcarbodiimide, 4-(dimethylamino)pyridine, pivalic acid, pyridium p-toluenesulphonate, anhydrous Na$_2$SO$_4$ were purchased from Fluka Chemical Corporation and used as received. Thin layer chromatography plates were purchased from Sorbtech and used as received. Acid acetic, HCl, dichloromethane, NaHCO$_3$, NaCl, sodium carbonate, and potassium carbonate were obtained from Fisher Scientific and used as received. Glass beads were purchased from Chemglass and used as received.
2.2 General Methods

Thin layer chromatography (TLC) and UV light were used to analyze the Rf of starting materials to determine the time of completion for reactions. Chromatographic purifications were done using silica gel 60. The presence of phospholipids was identified by molybdic acid spray. \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR were recorded using Varian 400Hz. IR spectra were recorded by using PerkinElmer Spectrum Version 10.00.00. The optical rotation of chiral compounds was recorded by using PerkinElmer Model 341 Polarimeter. The samples were freeze-dried using Labconco FreeZone 4.5. Benzene was dried over sodium wire. Acetonitrile was dried over activated molecular sieves. The acylation was done using Lauda Brinkmann Ecoline RE106. High-resolution mass spectra data were recorded by University of California, Riverside, Mass Spectrometry Facility.

2.3 Synthetic Procedures

2.3.1 1,2-isopropylidene-3-tosyl-\textit{sn}-glycerol [8]

To a stirred solution of compound 4 (1.00 g, 7.6mmol), p-toluenesulfonyl chloride 7 (1.73 g, 9.1 mmol) in 20 mL of dichloromethane, DMAP (1.11 g, 9.1 mmol) was added. The reaction solution was kept overnight at room temperature. Once the reaction completed, the solution was evaporated at 35°C. The product 8 was purified by column chromatography on silica gel using CHCl\textsubscript{3}. The product 8 (1.09g, 50%) was obtained as a white solid after freeze-drying from benzene.
IR (CHCl₃): 1053 cm⁻¹. ¹H NMR (CDCl₃, 400Hz), δ 2.34 (s, 3H), 3.65-4.19 (m, 5H), 7.25 (d, 2H), 7.70 (d, 2H). ¹³C-NMR (CDCl₃, 400 MHz) δ (145.1, 132.6, 129.9, 128.0, 110.0, 72.9, 69.5, 66.2, 26.7, 25.1, 21.6). R₇(CHCl₃) = 0.29. MS MH⁺ C₁₃H₁₉O₅S Calcs: 287.0948, Found 287.0961.

2.3.2 3-(toluene-4’-sulfonyl)-sn-glycerol [12]

Acetyl chloride 9 (2.84 mL, 39.9 mmol) was added dropwise to 100 mL methanol 10 with ice water bath under reaction flask. The solution was mixed using a magnetic stirring bar for 30 min. 100 mL of 0.4M HCl in methanol was ready, then immediately transferred 40 mL of 0.4M HCl in methanol in 100mL round bottom flask. To a stirred solution of 40 mL of 0.4 M HCl in MeOH 11, compound 8 (4.00 g, 14.7 mmol) was added. The reaction completed in 1 h at room temperature. When reaction completed, the reaction solution was evaporated with additional 80mL of MeOH to neutralize this solution at 35°C. The product 12 was loaded into silica gel chromatography using CHCl₃/MeOH (9:1) to remove the impurities. The fractions of product were collected. The solution was evaporated then freeze dried from benzene overnight to give a white solid 12 (3.21g, 94%).

IR (CHCl₃): 1354, 1177, 1053, 755 cm⁻¹.¹H-NMR (CDCl₃, 400Hz), δ 2.34 (s, 3H), 3.46-3.58 (m, 2H), 3.84 (m, 1H), 3.95 (m, 2H), 7.25 (d, 2H), 7.69 (d, 2H). ¹³C-NMR (CDCl₃, 400 MHz) δ(142.7, 129.8, 127.4, 125.4, 68.1, 67.5, 60.1, 19.1). R₇(CHCl₃/MeOH 9:1) = 0.49.[α]D²⁵: -2.255 (c 1.02, CHCl₃-MeOH 4:1).
2.3.3 1-pivaloyl-3-(toluene-4'-sulfonyl)-sn-glycerol [14]

To a stirred solution of 12 (3.78 g, 15.5 mmol) in 15 mL dichloromethane, DCC (3.2 g, 15.5 mmol) in 15 mL of dichloromethane and pivalic acid 13 (1.58 g, 15.5 mmol) in 15 mL dichloromethane were added dropwise. The reaction solution was stirred using magnetic stirring bar for 10 min. followed by addition of DMAP (0.10 g, 0.8 mmol). The solution was run for 24 h at room temperature. The mixture was filtrated by addition of 90 mL dichloromethane. The two columns for chromatography was used to purify product 14. The first column was run with CHCl₃/MeOH (9.4:0.6), then followed by second column using Cyclohexane/EtOAc (3:2). The product was freeze dried from benzene and became a white solid 14 (3.07 g, 60%).

IR (CHCl₃): 1217, 1176, 750, 669 cm⁻¹. ¹H-NMR (CDCl₃, 400 Hz), 1.05 (s, 9H), δ 2.34 (s, 3H), 3.89-4.11 (m, 5H), 7.25 (d, 2H), 7.70 (d, 2H). ¹³C-NMR (CDCl₃, 400 MHz) δ 178.2, 145.3, 132.4, 130.0, 128.0, 70.1, 67.9, 64.2, 38.8, 27.0, 21.6). Rᵣ (CHCl₃/MeOH 9.4:0.6) = 0.52. Rᵣ (Cyclohexane/EtOAc 3:2) = 0.41. [α]₀²⁵ = -3.673 (c 0.98, CHCl₃-MeOH 4:1).

2.3.4 1-pivaloyl-2-(tetrahydropyranyl)-3-(toluene-4'-sulfonyl)-sn-glycerol [17]

To a solution of 14 (1.00 g, 3.0 mmol) in 50 mL dichloromethane, 3,4-dihydro-2H-pyran 15 (0.76 g, 9.0 mmol), and pyridium p-toluenesulfonate 16 (0.23 g, 0.9 mmol) were added. The reaction solution was stirred using magnetic stirring bar at room temperature for 30 min. The reaction was evaporated and white solid that was catalyst converted. The solid was filtered with suction and reaction solution was evaporated then
loaded to column chromatography using Cyclohexane/EtOAc (2:1) for purification. The fractions of product were collected and evaporated. The product was freeze dried from benzene to give a clear oil 17 (2.53 g, 97%).

IR (CHCl₃): 2937, 1729, 1361, 1175, 984, 813, 667 cm⁻¹. ¹H-NMR (CDCl₃, 400Hz), 1.16 (s, 9H), 1.38-1.68 (m, 9H), δ 2.34 (s, 3H), 3.27-3.53 (m, 2H), 3.64-3.74 (m,2H), 3.89-4.16 (m,5H), 4.60 (m,1H), 7.25 (d,2H), 7.72 (t,2H). ¹³C-NMR (CDCl₃, 400 MHz) δ (177.8, 144.9, 132.7, 129.9, 128.2, 98.3, 71.6, 68.7, 62.1, 61.6, 38.7, 30.3, 27.1, 25.1, 21.7, 18.9) R_f (Cyclohexane/EtOAc 2:1) = 0.56. MS MNa⁺ C₂₀H₃₀O₇NaS Calcs: 437.1604, Found 437.1605.

2.3.5 2-(tetrahydropyranyl)-3-(toluene-4′-sulfonyl)-sn-glycerol [20]

To a stirred solution of compound 17 (3.51 g, 8.5 mmol) in 40 mL of diethyl ether, kept in an ice water bath, 18 and 19 (4.3 mL of 3.0M LiBH₄ in THF) were added. The reaction solution was mixed using magnetic stirring bar for 10 min. and methanol (0.40 g, 12.5 mmol) was added dropwise. The reaction mixture was stirred at 0°C for 30 min. then let it warm to room temperature. The reaction completed in 1 h at room temperature. NaHCO₃ was added to reaction solution with ice water bath under reaction flask and mixed for 15 min. After mixing, the solution was transferred to separatory funnel with additional 40 mL diethyl ether and 40 mL brine (saturated NaCl in water). The solution was settled until the two layers completely separated for better yield. The upper (organic) layer was dried by anhydrous Na₂SO₄ and evaporated. If necessary, the lower layer could be dried with anhydrous Na₂SO₄ to check if there was any organic
solvent in this layer to collect more products. The solution was evaporated and loaded to silica gel chromatography using Cyclohexane/EtOAc (1:3) for purification. After freeze drying from benzene, the sample was obtained as clear oil 20 (2.08 g, 74%).

IR (CHCl₃): 2938, 1729, 1358, 1175, 971, 935, 829, 758, 666 cm⁻¹. ¹H-NMR (CDCl₃, 400Hz), 1.31-1.47 (m, 5H), 1.54-1.69 (m, 2H), δ 2.34 (s, 3H), 3.30-4.12 (m, 8H), 4.60 (d, 1H), 7.25 (d, 2H), 7.69 (d, 2H). ¹³C-NMR (CDCl₃, 400 MHz), δ (145.0, 132.8, 129.9, 128.0, 100.8, 74.8, 69.6, 64.6, 31.0, 25.2, 20.7, 19.4) Rf(Cyclohexane/EtOAc 1:3) = 0.58. MS MNa⁺ C₁₅H₂₂O₆NaS Calcs: 353.1029, Found: 353.1038.

2.3.6 11-(7'-mercapto-4'-methyl-coumarin)-undecanol [23]

To a solution of 11-bromo-1-undecanol 21 (1.31 g, 5.2 mmol) in 25 mL of deareted nitrogen, 7-mercapto-4-methylcoumarin 22 (1.00 g, 5.2 mmol) and potassium carbonate (1.43 g, 10.4 mmol) were added. At the beginning, the reaction mixture turned to yellow solution then turned to white solution with a lot of solid. The reaction completed in 1 h at room temperature. The reaction solution was extracted by 3 times with 25 mL of water followed by 3 times with 25 mL of chloroform. The lower layer was collected and dried by anhydrous Na₂SO₄. The solution was evaporated and loaded to column chromatography using CHCl₃/EtOAc (8.5:1.5) for purification. After freeze drying from benzene, the product was obtained as white solid 23 (1.66 g, 88 %).

IR (CHCl₃): 2917, 2851, 1712, 1605, 1470, 1392, 1175, 1054, 850, 666 cm⁻¹. ¹H-NMR (CDCl₃, 400Hz), 1.27-1.77 (m, 22H), 2.43 (s, 3H), 3.01 (t, 2H), 3.67 (m, 2H), δ 6.23 (s, 1H), 7.18 (d, 2H), 7.49 (d, 2H). ¹³C-NMR (CDCl₃, 400 MHz), δ (18.5, 25.7, 28.7, 28.8,
29.0, 29.1, 29.4, 29.5, 32.1, 32.8, 32.8, 63.0, 113.7, 113.8, 116.9, 122.9, 124.5, 143.8, 152.3, 153.9, 160.7) Rf(CHCl₃/EtOAc 8.5:1.5) = 0.28. MS MH⁺ C₂₁H₃₁O₃S Calcs: 363.1988 Found: 363.1996.

2.3.7 11-((4-methyl-2-oxo-2H-chromen-7-yl)thio)undecyl (4-nitrophenyl) carbonate [25]

To a stirred solution of 23 (1.51 g, 4.2 mmol) in 20 mL of dichloromethane was kept under an ice-water bath, p-nitrophenyl chloroformate 24 (1.02 g, 5.1 mmol) and DMAP (0.62 g, 5.1 mmol) in 15 mL dichloromethane were added. The reaction solution was mixed using stirring bar and kept in ice-water bath for 10 min. then let it warm to room temperature. The reaction mixture turned to yellow solution. The reaction completed within 3 h at room temperature. The sample was evaporated and turned to a white yellow solid. The silica gel column chromatography was used to purify the product 25 using CHCl₃/EtOAc (8.5:1.5). After freeze-drying from benzene, the product was obtained as a white solid 25 (1.72 g, 78%).

IR (CHCl₃): 2928, 1716, 1601, 1367, 1215, 1176, 974, 750, 666 cm⁻¹. ¹H-NMR (CDCl₃, 400Hz), 1.32-1.80 (m, 21H), 2.42 (s, 3H), 3.00 (t, 2H), 4.31 (t, 2H), 6.23 (s, 1H), 7.18 (d, 2H), 7.39-7.48 (d, 3H), 8.29 (d, 2H). ¹³C-NMR (CDCl₃, 400 MHz) δ (160.7, 155.6, 153.9, 152.5, 152.2, 143.8, 125.3, 124.5, 122.9, 121.8, 116.9, 113.8, 113.7, 69.6, 32.1, 29.4, 29.1, 28.7, 28.5, 28.5, 25.6, 18.5) Rf(CHCl₃/EtOAc 8.5:1.5) = 0.82. MS MH⁺ C₂₈H₃₄NO₇S Calcs: 528.2051, Found: 528.2063.
2.3.8 1-[(11’-(7’’-mercapto-4’’-methyl-coumarin)undecacarbonate]-2-
(tetrahydropyranyl)-3-tosylate-sn-glycerol [26]

To a solution of compound 25 (0.86 g, 2.6 mmol) in 20 mL dichloromethane, compound 20 (1.64 g, 3.1 mmol) and DMAP (0.38 g, 0.31 mmol) were added. The reaction solution was stirred using magnetic stirring bar for 24 h at room temperature. After the reaction completed, it was evaporated using a rotary evaporator. The sample was purified using two silica gel chromatography columns (CHCl₃/EtOAc 9:1). After freeze drying from benzene, the product became a white solid 26 (1.00 g, 54%).

IR (CHCl₃): 2925, 2853, 1719, 1601, 1524, 1387, 1253, 1177, 956, 856, 750, 666 cm⁻¹. ¹H-NMR (CDCl₃, 400 Hz), 1.28-1.81 (m, 28H), 2.43 (d, 6H), 23.01 (t, 2H), 3.49 (m, 1H), 3.78 (m, 1H), 4.06-4.30 (m, 7H), 4.67-4.76 (d, 1H), δ 6.23 (s, 1H), 7.18 (d, 2H), 7.37 (d, 2H), 7.47 (d, 1H) 7.82 (t, 2H). ¹³C-NMR (CDCl₃, 400 MHz) δ (160.7, 153.9, 151.7, 143.28, 143.1, 129.1, 127.8, 127.5, 124.5, 122.9, 116.9, 113.8, 113.8, 113.7, 98.2, 71.5, 71.2, 69.9, 69.8, 68.1, 32.2, 30.3, 30.3, 29.4, 29.2, 29.1, 28.9, 28.6, 25.6, 25.2, 22.9, 21.5, 18.9, 18.6, 18.5) Rf (CHCl₃/EtOAc 9:1) = 0.65. MS MNa⁺ C₃₇H₅₀O₁₀Na₂S Calcs: 741.2738, Found: 741.2755.

2.3.9 1-[(11’-(7’’-mercapto-4’’-methyl-coumarin)undecacarbonate]-2-
(tetrahydropyranyl)-3-acetyl-sn-glycerol [28]

To a solution of 10 mL of 0.3 M tetramethylammonium acetate 27, compound 26 (0.70 g, 0.9 mmol) was added. The reaction solution was mixed using magnetic stirring bar at room temperature for 48 h. The solution was bright yellow. After the reaction
completed, it was purified using silica gel column using CHCl$_3$/EtOAc (9:1). After
freeze-drying from benzene, the product was obtained as a white solid 28 (0.22 g, 41%).

IR (CHCl$_3$): 2925, 2853, 1739, 1386, 1232, 1122, 1077, 1034, 954, 752 cm$^{-1}$. $^1$H-
NMR (CDCl$_3$, 400Hz), 1.28- 1.84 (m, 26H), 2.08 (s, 3H), 2.43 (s, 3H), 3.00 (t, 2H), 3.54
(m, 1H), 3.91 (t, 1H), 4.12-4.33 (m, 8H), 4.82 (t, 1H), δ 6.23 (s, 1H), 7.18 (d, 2H), 7.49
(d, 1H). $^{13}$C-NMR δ (170.1, 152.2, 144.4, 124.5, 122.9, 113.8, 113.7, 71.9, 68.4, 66.3,
63.9, 32.2, 30.5, 29.4, 29.2, 29.1, 28.9, 28.7, 28.6, 25.6, 25.3, 19.1, 18.6) R$_f$
(CHCl$_3$/EtOAc 9:1) = 0.61. MS MNa$^+$ C$_{32}$H$_{46}$O$_9$NaS Cales: 629.2755, Found: 629.2741.

2.3.10 1-[11'--(7''-mercapto-4''-methyl-coumarin)undecacarbonate]-2
(tetrahydropyranyl)-sn-glycerol [30]

To a stirred solution of compound 28 (0.30 g, 0.49 mmol) in 10 mL of
CHCl$_3$/MeOH (1:4), t-butylamine 29 (0.04 g, 0.49 mmol) was added. The reaction
completed in about 2 h at room temperature. The solution was evaporated using
evaporator and loaded to silica gel chromatography using chloroform/ethyl acetate (8:2)
to purify the reaction product. After freeze drying from benzene, the product became a
white solid 30 (0.21 g, 77 %).

IR (CHCl$_3$): 2924, 2853, 1736, 1600, 1387, 1252, 1161, 1053, 1021, 954, 850, 812, 750,
667 cm$^{-1}$. $^1$H-NMR (CDCl$_3$, 400Hz), 1.26-2.00 (m, 30H), 2.42 (s, 3H), 3.03 (t, 2H), 3.54
(m, 3H), 3.95 (m, 2H), 4.21-4.46 (m, 4H), 4.95 (t, 1H), δ 6.23 (s, 1H), 7.22 (d, 2H), 7.49
(d, 1H). $^{13}$C-NMR (CDCl$_3$, 400 MHz) δ (158.1, 151.4, 149.7, 142.1, 121.9, 120.4, 111.3,
111.1, 98.2, 95.9, 76.3, 72.5, 65.9, 65.8, 64.2, 64.1, 62.0, 60.4, 60.2, 29.7, 28.6, 26.8,
26.6, 26.5, 26.3, 26.1, 22.4, 18.1, 16.0) Rf (CHCl₃/EtOAc 8:2) = 0.43. MS MNa⁺ C₃₀H₄₄O₈NaS Calcs: 587.2649, Found: 587.2629.

2.3.11 1-[11’-(7’’-mercapto-4’’-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-methoxyacetyl-sn-glycerol [34]

To a solution of tetraethylammonium hydroxide 32 (3.2 g, 21.7 mmol) in water, methoxy acetic acid 98% 31 (1.96 g, 21.7 mmol) was added dropwise. The reaction was mixed for 2 h using magnetic stirring bar and completed at room temperature. The reaction solution was freeze dried itself first to remove the water. In the following day, the product was added 40mL dioxane for freeze drying. The product 33 was obtained as a clear yellow oil. ¹H-NMR (CDCl₃, 400Hz), 1.24 (s, 16H), δ 2.21 (s, 2H), 3.25 (s, 4H). To a solution of 45 mL of 0.3M tetraethylammonium methoxy acetate 33 in acetonitrile, compound 26 (0.80 g, 1.1 mmol) was added. The reaction was mixed using magnetic stirring bar for 48 h at room temperature. After reaction completed, the solution was evaporated using rotatory evaporator at 25°C. The silica gel column chromatography was used to purify product 34 using CHCl₃/EtOAc (9:1). The sample was freeze-dried from benzene to give a white solid 34 (0.58 g, 82%).

IR (CHCl₃): 2927, 2855, 1718, 1601, 1456, 1256, 1215, 956, 854, 750, 666 cm⁻¹. ¹H-NMR (CDCl₃, 400Hz), 1.24-1.85 (m, 32H), 2.43 (s, 3H), 3.00 (t, 2H), 3.98-4.24 (m, 12H), 4.77 (s, 1H), δ 6.23 (s, 1H), 7.18 (d, 2H), 7.47 (d, 1H). ¹³C-NMR (CDCl₃, 400 MHz) δ (18.6, 19.0, 19.1, 25.3, 25.6, 28.6, 28.7, 28.9, 29.1, 29.2, 29.4, 30.5, 32.2, 62.8, 64.1, 66.8, 69.6, 71.8, 97.8, 98.6, 113.8, 115.6, 116.9, 122.9, 124.5, 143.8, 152.3, 153.9,
155.1, 160.8, 170.0) \text{R}_f (\text{CHCl}_3/\text{EtOAc} 9:1) = 0.61. \text{ MS } \text{MNa}^+ \text{C}_{33}\text{H}_{48}\text{O}_{10}\text{NaS} \text{ Cales: 659.2860, Found: 659.2843.}

\section*{2.3.12 1-[(7''-mercapto-4''-methyl-coumarin)undecacarbonate]-2-(tetrahydropranyl)-sn-glycerol [30]}

To a solution of 34 (0.41 g, 0.6 mmol) in 10mL of chloroform/methanol (1:4), \text{t}-butyamine 29 (0.53 g, 7.2 mmol) was added in a ice water bath at 0\degree C. The reaction was stirring using magnetic stirring bar, and the ice water bath was removed after 20 min. The reaction completed within 2 h at room temperature. The reaction solution was evaporated at 30\degree C using rotatory evaporator. The sample was eluted and loaded to silica gel column using \text{CHCl}_3/\text{EtOAc} (1:1) for purification. The product 30 was freeze-dried from benzene to give a white solid (0.32 g, 95%).

\text{IR (CHCl}_3): 2924, 2854, 1736, 1601, 1455, 1386, 1253, 1176, 1034, 989, 955, 851, 813, 790, 751, 666 \text{ cm}^{-1}. \text{^1H-NMR (CDCl}_3, 400Hz), 1.30-1.88 (m, 30H), 2.42 (s, 3H), 3.00 (t, 2H), 3.66 (m, 3H), 3.99 (m, 2H), 4.12-4.31 (m, 4H), 4.61-4.81 (s, 1H), \delta 6.23 (t, 1H), 7.18 (d, 2H), 7.48 (d, 1H). \text{^13C-NMR (CDCl}_3, 400 MHz) \delta (158.4, 153.3, 141.2, 121.9, 120.4, 114.4, 111.3, 111.1, 98.4, 95.8, 95.3, 76.3, 72.5, 65.9, 64.0, 62.0, 60.5, 60.4, 60.2, 60.0, 29.6, 26.9, 26.8, 26.5, 26.0, 23.1, 23.0, 22.4, 16.9, 16.0) \text{R}_f (\text{CHCl}_3/\text{EtOAc} 1:1) = 0.69. \text{ MS } \text{MNa}^+ \text{C}_{30}\text{H}_{44}\text{O}_8\text{NaS} \text{ Cales: 587.2649, Found: 587.2629.}
To a stirred solution of compound 30 (0.62 g, 1.1 mmol) in 20mL of anhydrous acetonitrile at 0°C in a pressure bottle, 2-chloro-2-oxo-1,3,2-dioxaphospholane 35 (0.24 g, 1.65 mmol) and 2 mL of pre-cooled trimethyl amine 36 was added. The reaction solution was mixed using magnetic stirring bar. The reaction completed in 12 h at room temperature. The white precipitate was formed. The pressure bottle was frozen at -10°C using dry ice, and another 2 mL of trimethylamine 36 was added. The pressure bottle was sealed and heated at 67°C for 24 h. The solid was formed. The precipitate was filtered with suction. The solution was evaporated using rotatory evaporator. The product was dissolved in CHCl₃/MeOH/H₂O (65:25:4) and loaded in a silica gel column chromatography using CHCl₃/MeOH/H₂O (65:25:4), then ran another column to separate product 38. The benzene was used for freeze-drying, and product 38 became a white solid (0.34g, 42%).

IR (CHCl₃): 2924, 2855, 1737, 1717, 1601, 1387, 1246, 1085, 1053, 956, 851, 748, 666 cm⁻¹. ¹H-NMR (CDCl₃, 400Hz), 1.21-1.83 (m, 26H), 2.44 (s, 3H), 3.01 (t, 2H), 3.21 (s, 9H), 3.29 (m, 3H), 3.85 – 4.41 (m, 18H), 4.77-4.84 (t, 1H), δ 6.23 (s, 1H), 7.21 (d, 2H), 7.54 (d, 1H). ¹³C-NMR (CDCl₃, 400 MHz), δ(161.2, 153.7, 152.9, 144.0, 141.2, 124.5, 123.0, 116.9, 113.7, 113.3, 113.3, 65.1, 65.1, 59.3, 59.2, 54.2, 49.6, 49.4, 49.2, 49.0, 48.8, 44.7, 32.1, 30.5, 30.5, 29.4, 29.4, 29.3, 29.2, 29.0, 28.8, 28.6, 25.5, 18.4) Rf (CHCl₃/MeOH/H₂O 65:25:4) =0.37. MS MH⁺ C₃₅H₅₇NO₁₁PS Calcs: 730.3392. Found: 730.3407.
2.3.14 1-[11’-(7’’-mercapto-4’’-methyl-coumarin)undecacarbonate]-3-phosphocholine-sn-glycerol [39]

To a cloudy solution of compound 38 (0.33 g, 0.46 mmol) in 25 mL of 1,4-dioxane, 0.15 mL 12M aqueous HCl was added. The reaction mixture was stirred at room temperature and completed in 30 min. To this solution, 30 mL of 1,4-dioxane was added and then the reaction mixture was freeze dried. The white residue was dissolved in CHCl3/MeOH/H2O (65:25:4) and loaded into short column of silica gel. The fractions of product were collected and evaporated. After freeze-drying from benzene, the product was obtained as a white solid 39 (0.24g, 79%).

IR (CHCl3): 3020, 2963, 1740, 1602, 1214, 1190, 1054, 744, 666 cm\(^{-1}\). \(^1\)H-NMR (CDCl₃, 400Hz), 1.27-1.72 (m, 18H), 2.44 (s, 3H), 3.02 (t, 2H), 3.22 (s, 9H), 3.63 (t, 2H), 3.81 – 4.33 (m, 9H), δ 6.23 (s, 1H), 7.18 (d, 2H), 7.55 (d, 1H). \(^{13}\)C-NMR (CDCl₃, 400 MHz), δ(161.7, 155.2, 153.7,153.2, 152.2, 144.0, 128.2, 124.5, 123.0, 116.8, 113.7, 113.3, 68.3, 68.1, 66.8, 66.3, 58.3, 54.1, 49.3, 49.1, 48.9, 48.7, 48.4, 44.7, 32.0, 29.4, 29.3, 29.3, 29.3, 29.1, 29.0, 28.7, 28.5, 28.5, 25.5, 18.4) R\(_f\) (CHCl₃/MeOH/H₂O 65:25:4) = 0.19. MS MH\(^+\) C\(_{30}\)H\(_{49}\)NO\(_{10}\)PS Calcs: 646.2809, Found: 646.2826. [α]\(_D\)\(^{25}\): - 0.833 (c 0.96, CHCl₃-MeOH 4:1).

2.3.15 N-(2,4-dinitrophenyl)aminododecanoic acid [42]

To a solution of 12-aminododecanoic acid 40 (1.5 g, 6.96 mmol) of in 15 mL 1,4-dioxane, 15 mL of 1M KOH was added dropwise. The magnetic stir bar was used to mix reaction solution. 2,4-dinitrofluorobenzene 41 (1.30 g, 6.96 mmol) was added to solution
mixture. The solution turned to yellow upon addition of 2,4-dinitrobenzene, and let it run for 24 h at room temperature. After reaction completed, 1.0M HCl was added to solution mixture dropwise. The pH level was checked by pH paper to 2. The yellow precipitate appeared upon addition of HCl. The yellow precipitate was filtered and air-dried by itself for 24 h. The reaction product was obtained as a yellow solid 42 (2.26 g, 85%).

IR (CHCl₃): 2921, 2851, 1702, 1618, 1523, 1328, 1150, 923, 745, 715, 610 cm⁻¹. ¹H-NMR (CDCl₃, 400Hz), δ 2.34 (t, 3H), 3.41 (t, 2H), 3.71 (d, 2H), 6.92 (d, 1H), 8.26 (d, 1H), 8.56 (s, 1H), 9.19 (s, 1H). ¹³C-NMR (CDCl₃, 400 MHz), δ(179.7, 148.4, 135.9, 130.3, 124.3, 113.9, 67.0, 43.6, 34.0, 29.4, 29.3, 29.3, 29.2, 29.1, 29.0, 28.7, 26.9, 24.6). Rᶠ(CHCl₃/MeOH 9.8:0.2) = 0.38.

2.3.16 N-(2,4-dinitrophenyl)aminopropionic acid [44]

To a solution of 2,4-dinitrofluorobenzene 41 (2.1 g, 11.28 mmol) in 15mL deaerated nitrogen, 3-aminopropionic acid 43 (2.01 g, 22.56 mmol) in 15 mL of 2.0 M aqueous sodium carbonate was added dropwise. The solution was mixed using stirring bar for 90 min and to it was added 1.0 M HCl to decrease to pH 2 at room temperature. The yellow precipitate appeared after adding HCl. The solid was filtered with suction, and washed by cooled acetone and 0.1M HCl. The reaction product was a yellow solid 44 (2.13 g, 74%).

IR (CHCl₃): 3020, 2929, 1710, 1621, 1524, 1338, 1214, 751 cm⁻¹. ¹H-NMR (CDCl₃, 400Hz), δ 2.61 (t, 2H), 3.79 (q, 2H), 6.98 (d, 1H), 8.78 (d, 8.33), 8.78 (s, 1H), 9.16 (s,
To a solution of 39 (0.20 g, 0.30 mmol) in 5mL of spectrograde chloroform, 42 (0.58 g, 1.50 mmol), DCC (0.30, 1.50 mmol), DMAP (0.18 g, 1.50 mmol), and 1.3 g of glass beads were added. The reaction solution was mixed using sonicator. The reaction completed within 5 h at room temperature. The solution was evaporated using rotatory evaporator at 30°C, then diluted by CHCl₃/MeOH/H₂O (65:25:4) and loaded to silica gel column chromatography using CHCl₃/MeOH (9:1). The solvent system was switched to CHCl₃/MeOH/H₂O (65:25:4) after impurities were washed away. The main reaction product was collected and freeze-dried from benzene overnight. The product became a yellow sticky solid 45 (0.15 g, 50%).

IR (CHCl₃): 3020, 2931, 1734, 1621, 1525, 1338, 1406, 1215, 1174, 750, 667cm⁻¹.

¹H-NMR (CDCl₃, 400Hz), 1.30-1.82 (m, 40H), 2.31-2.44 (m, 7H), 3.41-3.44 (m, 7H), 3.57-3.73 (m, 4H), 4.12-4.80 (m, 11H), 5.15 (m, 1H), δ6.23 (s, 1H), 6.95 (d, 2H), 7.15 (d, 2H), 7.55 (d, 1H), 8.34 (d, 1H), 8.59 (s, 1H), 9.00 (s, 1H) 9.20 (s, 1H). ¹³C-NMR (CDCl₃, 400 MHz), δ(173.8, 160.8, 154.7, 153.9, 152.4, 148.4, 143.8, 135.9, 130.3, 130.2, 128.8, 124.5, 124.5, 124.4, 124.3, 122.9, 122.9, 122.4, 116.9, 114.0, 113.9, 113.8, 113.7, 70.9, 68.7, 68.6, 67.1, 65.0, 63.9, 57.8, 54.5, 43.6, 34.1, 33.9, 32.2, 32.1, 29.7, 29.6, 29.5, 29.5, 29.4, 29.4, 29.3, 29.2, 29.2, 29.1, 29.1, 28.9, 28.9, 28.9, 28.9, 28.9, 28.9, 28.8, 28.7,

2.3.18 1-[11’-(7’’-mercapto-4’’-methyl-coumarin)-undecacarbonate]-2-[(2,4-dinitrophenyl)aminopropionicacid]-3-phosphocholine-sn-glycerol [46]

To a solution of 39 (0.48 g, 0.74 mmol) in 10mL of chloroform, 44 (0.94 g, 3.70 mmol), DCC (0.76g, 3.70 mmol), DMAP (0.45g, 3.70 mmol), and 2.3 g of glass beads were added. The reaction solution was mixed using sonicator at room temperature. The reaction completed within 5 h. The solution was evaporated using rotatory evaporator at 30ºC, then diluted by CHCl3/MeOH/H2O (65:25:4) and loaded to chromatography column of CHCl3/MeOH (9:1). After all of impurities were washed away, the solvent system was switched to CHCl3/MeOH/H2O (65:25:4) to purify the product 46. The fractions of product were collected and evaporated. The final product was freeze-dried from benzene to give a yellow sticky solid 46 (0.26 g, 40 %).

IR (CHCl3): 2925, 2854, 1718, 1619, 1524, 1424, 1335, 1054, 955, 814, 745, 666 cm⁻¹.

1H-NMR (CDCl3, 400Hz), 1.29-1.74 (m, 18H), 2.44 (m, 5H), 2.84 (t, 2H), 3.02 (t, 2H), 3.39 (s, 9H), 3.81 (m, 4H), 4.09 (m, 4H), 4.20-4.44 (m, 4H), 5.30 (m, 1H), 6.23 (s, 1H), 7.09 (d, 1H), 7.19 (d, 1H), 7.50 (d, 1H), 8.30 (d, 1H), 8.98 (d, 1H), 9.04 (d, 1H). 13C-NMR (CDCl3, 400 MHz), δ(160.1, 15.0, 153.8, 152.4, 148.1, 143.9, 136.0, 130.5, 130.5, 128.3, 124.5, 122.9, 116.9, 114.1, 113.7, 68.5, 68.4, 66.2, 66.1, 54.5, 38.8, 32.1, 29.4, 29.4, 29.2, 29.1, 28.8, 28.6, 28.6, 25.6, 18.6). Rf (CHCl3/MeOH/H2O 65:25:4) = 0.42.

2.3.19 Enzymological Studies

10 mg of each sample of 45 and 46 was separately transferred to 18x150 mm test tube. To this phospholipid was added 4 mL of 0.05 M Tris buffer (pH 8.5) followed by 10mM Trion X-100 and 10 mM CaCl₂. Each test tube was well-mixed by vortex for 15 sec. 20 µL of bee-venom PLA₂ was added to each solution. The solution was vortexed again twice then placed in water bath at 40°C for incubation. The reaction was followed every 5 min. by TLC and UV light, and molybdic acid spray was applied to trace the process of PLA₂’s hydrolysis on new synthetic phosphatidylcholines. CHCl₃/MeOH/H₂O (65:25:4) was chosen as solvent system to check the TLC. 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) was used as standard to compare the duration of PLA₂ hydrolysis on samples 45 and 46.
3. RESULTS AND DISCUSSIONS

3.1 Synthesis of 2-(tetrahydropyryl)-3-(toluene-4′-sulfonyl)-sn-glycerol

Figure 6: Scheme 1 for the synthesis of 2-(tetrahydropyryl)-3-(toluene-4′-sulfonyl)-sn-glycerol

Molecule 4 was employed as chiral precursor of glycerol and reacted with $p$-toluenesulfonyl chloride to produce isopropylidene tosylate sn-glycerol 8 at room temperature overnight. The tosylate was introduced into sn-3 position of glycerol structure for protection of the hydroxyl group.
In the first step, preparation of the 0.4 M HCl solution was accomplished by methanolysis of acetyl chloride. The isopropylidene group was removed using 0.4 M HCl in MeOH. The reaction completed for 1 h to give a yield of 94% at room temperature. The tosyl glycerol product 12 was obtained as a white precipitate with all of corresponding peaks on $^1$H-NMR, and it was reacted with pivalic acid, DCC and DMAP as catalyst. The hydroxyl group at the sn-2 position was prepared for the introduction of THP protecting group in tetrahydropyranylation step. The pivaloylation was carried out using a 1:1 ratio of the reactants to maximize the yield of the reaction. The bulky group from pivalic acid was introduced into skeleton of 12 at the sn-1 position. Two different solvent systems chloroform/methanol (9.4:0.6) and cyclohexane/ethyl acetate (3:2) were used in silica gel chromatography columns for better purification to give a yield of 60%.
The secondary hydroxyl group of the product 14 was protected at the sn-2 glycerol position using 3-fold excess of DHP. PPTS contributed as a catalyst in the tetrahydropyranylation to give a yield of 97% at room temperature. Reaction was completed within 30 min. The product was loaded to silica gel column chromatography using cyclohexane/ethyl acetate (2:1) for purification. The structure of tetrahydropyranyl product 17 was confirmed by the presence of multiplet peak at 4.60 ppm on $^1$H-NMR indicating the secondary THP protecting group.

![Chemical structures](image)

The product 17 was then reacted with 3.0 M LiBH₄ in THF to create the hydroxyl group at sn-1 position. This hydroxyl group was prepared for the introduction of fluorophore group to the glycerol structure at sn-1 position in the coupling reaction. Lithium borohydride acted as a reducing agent in anhydrous ether. Methanol contributed as a catalyst to shorten reaction’s duration. The selection of LiBH₄ as a reducing agent instead of LiAlH₄ was to increase the recovery of product 20 to have a better yield. The product 20 was purified using silica gel column chromatography with cyclohexane/ethyl acetate (1:3) and freeze-dried from benzene to give a yield of 74%. Appropriate data from $^1$H-NMR, $^{13}$C-NMR and mass spectra indicated that a pure reduced alcohol 20 was
obtained. With the secondary hydroxyl group protected at $sn$-2 position, and primary hydroxyl group deprotected at $sn$-1 position, product 20 was ready for the next coupling reaction. The bulkiness of THP protecting group at $sn$-2 position might cause steric hinderance on hydroxyl group at $sn$-1 position of the glycerol structure and slower the rate of coupling reaction.

![Chemical diagram showing the reaction of 17 with 3.0M LiBH$_4$ and 18 to form 20 in the presence of methanol and diethyl ether.]
3.2 Synthesis of 1-[11’-(7’’-mercapto-4’’-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-tosylate-\(sn\)-glycerol

![Chemical structures and reactions](image)

**Figure 7.** Scheme 2 for the synthesis of 1-[11’-(7’’-mercapto-4’’-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-tosylate-\(sn\)-glycerol

The fluorescent reagent in the synthesis was synthesized in a two-step model. First, the base catalyzed nucleophilic replacement of bromine in 21 by mercapto methylcoumarin produced a stable fluorescent product 23. Potassium carbonate was used as a catalyst to speed up the forward reaction, and gave a yield of 88%. The product
was UV-absorbing and turned to blue under UV light. The product 23 was obtained as white solid after silica gel chromatography purification using CHCl₃/EtOAc (8.5:1.5) and freeze-drying from benzene. In the next step of synthesis of product 25, molecule 23 was reacted to p-nitrophenyl chloroformate to create the carbonate functional group. DMAP was employed as a catalyst to help reaction complete in a faster duration, and created a fluorescent product as expected. The reaction completed in 3 h at room temperature. The coumarin group was chain-terminal reporter group of both target structures PC-1 and PC-2.

![Chemical structure](image)

The product 20 previously prepared was coupled with product 25 to form a fluorescent product that had carbonate functional group at sn-1 position, THP protecting group at sn-2 position, and tosylate group at sn-3 position. The reaction was followed by TLC using chloroform/ethyl acetate (9:1) and UV light. The newly formed product 26 was less polar than reducing alcohol 20, but more polar than fluorescent product 25. The reaction completed in 24 h at room temperature to give a yield of 54%. The loss of product 26 was due to the second purification using silica gel chromatography. The coupling product 26 was characterized by the presence of multiplet peak at 4.67-4.76
ppm from THP protecting group, the singlet peak at 6.23 ppm of the coumarin group and doublet peak at 2.43 ppm of tosylate group on $^1$H-NMR.

**Figure 8.** The coupling product 26 showed THP, coumarin and tosylate peaks on $^1$H-NMR.
3.3 Synthesis of 1-[11’-(7’’-mercapto-4’’-methyl-
 coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-phosphocholine-sn-glycerol

**Figure 9.** Scheme 3 for synthesis of 1-[11’-(7’’-mercapto-4’’-methyl-
coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-phosphocholine-sn-glycerol.
The $p$-toluenesulfonate functional group of product 26 was replaced by acetate using tetramethylammonium acetate in anhydrous acetonitrile. The anhydrous condition was used to prevent the further hydrolysis and avoid the unnecessary low yield result. The yield and duration of this replacing-tosylate reaction mostly depended on the substrate, the dryness of reagent and dipolar medium of reaction. The presence of water or other impurities showed a significant decrease in yield of reaction and increase reaction’s duration to complete. The reaction was complete in 2 days at room temperature. The product 28 was purified using silica gel chromatography with chloroform/ethyl acetate (9:1) to give a white solid. A yield of 41% indicated that tetramethylammonium acetate was not a good substrate for this reaction. The absence of doublet peak of tosylate group at 7.47 ppm, and the presence newly formed singlet peak at 2.08 ppm on $^1$H-NMR showed the characteristic of acetate and indicated that the replacement of tosylate group was successful, but new strategy needed to be carried out to improve the yield of synthesis.

The hydrolysis of molecule 28 took placed in chloroform/methanol (1:4) with tert-butylamine as a base-catalyst to replace acetate group with a hydroxyl group at $sn$-3 position of glycerol structure. This hydroxyl group was prepared for the introduction of phosphocholine group in the phosphorylation step. The hydrolysis completed in 2 h at
room temperature, and the product 30 was purified using silica gel column chromatography with chloroform/ethyl acetate (8:2) to give a yield of 77%.

The second strategy in replacing tosylate group of product 26 was carried out to improve the efficiency and the yield of the synthesis. Tetraethylammonium methoxy acetate 33 was synthesized from reaction of methoxy acetic acid 31 and tetraethylammonium hydroxide 32 in water. The product was dissolved in acetonitrile dried with 3Å molecular sieves.

The replacement of the tosylate of product 26 using methoxyacetate 33 in anhydrous acetonitrile completed in 2 days at room temperature. A solution of freshly made 0.3M tetraethylammonium methoxyacetate 33 anhydrous acetonitrile was prepared and used for displacement of toluene-4-sulfonyl group. This reaction greatly depended on dryness of the solvent and degree of impurities. The product 34 was purified using same chloroform/ethyl acetate (9:1) on silica gel chromatography to give a yield of 82%. The product 34 was also obtained as white solid, but in comparison to product 28, the yield was doubled. The product 34 was a heat-sensitive molecule. Therefore, after reaction
completed, the solution was evaporated at 25°C to avoid decomposing and losing any product.

![Chemical structure](image)

The product 34 was reacted with a mixture of chloroform/methanol (1:4) with t-butylamine 29 as a catalyst in hydrolysis reaction to produce 30 with a yield of 95%. The product 30 had a hydroxyl group that was introduced into sn-3 position of the glycerol structure. The reaction completed in 2 h at room temperature, and the product 30 was purified using silica gel chromatography with chloroform/ethyl acetate (1:1) instead of chloroform/ethyl acetate (8:2) from the first strategy. In comparison to the first strategy of synthesizing product 30, this hydrolysis reaction improved 18% yield. The product 30 must be completely dried for subsequent step of phosphorylation, so it was freeze-dried from benzene to give a white solid.

![Chemical structure](image)

The phosphocholine was introduced to glycerol structure at sn-3 position in a two-step method. The degree of dryness of solvent greatly affected the yield of phosphorylation, thus the acetonitrile was dried over 3Å molecular sieves to remove any water. The anhydrous trimethylamine contributed as a base-catalyst in acetonitrile in the
first step of phosphorylation. The primary alcohol 30 was phosphorylated using 2-chloro-2-oxo-1,3,2-dioxaphospholane to give product 37. The cyclic phosphate intermediate 37 was reacted with second portion of anhydrous trimethylamine in the same pressure bottle at 67°C for 24 h to give compound 38. Using same pressure bottle for both first and second steps of phosphorylation reduced the chance of losing product and maintained anhydrous conditions. The product 38 was filtered with suction and purified by silica gel chromatography using chloroform/methanol/water (65:25:4), and freeze-dried from benzene to give a yield of 42%. The narrow long column was used for purification to prevent losing product. The molecule 38 was obtained as a white solid after freeze-drying step. The phosphocholine was introduced to sn-3 position of glycerol structure. The presence of choline peak at 3.21 ppm on ¹H-NMR as singlet indicated the successful introduction of phosphocholine into the glycerol structure. The addition of some methanol-d₄ was required to dissolve the product 38 in combining chloroform-d for ¹H-NMR and ¹³C-NMR.
3.4 Synthesis of N-(2,4-dinitrophenyl)aminododecanoic acid and N-(2,4-dinitrophenyl)aminopropionic acid

Figure 10. Scheme 4 for the synthesis of N-(2,4-dinitrophenyl)aminododecanoic acid and N-(2,4-dinitrophenyl)aminopropionic acid.

2,4-Dinitrofluorobeneze 41 was used in the nucleophilic aromatic substitution with N-terminal amino group of 12-aminolauric acid 40. Fluorine atom on aromatic ring of 41 was replaced by N-terminal amino group. Upon acidification using 1.0M HCl, a yellow precipitate 42 formed. The product 42 was obtained as pure sample without any silica gel chromatography to reduce the loss of product and give a yield of 85%. The
doublet peaks at 6.92 ppm and 8.26 ppm on $^1$H-NMR showed the presence of 2,4-dinitroaniline. The product 44 was synthesized in similar fashion to product 42. The product 44 was also obtained as yellow precipitate without purification using silica gel column chromatography to give a yield of 74%. The presence of doublet peaks at 6.98 ppm and 8.78 ppm on $^1$H-NMR showed the appropriate structure of 2,4-dinitroaniline. Products 42 and 44 both have 2,4-dinitroaniline acting as energy acceptor while 7-mercapto-4-methyl coumarin of product 39 acted as energy donor. The quencher groups were introduced to glycerol structure at the $sn$-2 position as second chain terminal reporter groups of phosphatidylcholines PC-1 and PC-2 through acylation step.
3.5 Synthesis of final PC-1 (45) and PC-2 (46) products

![Chemical structure diagram]

**Figure 11:** Scheme 5 for the synthesis of final PC-1 (45) and PC-2 (46)

The acid-catalyzed cleavage of *sn*-2-tetrahydropyranyl group using 0.3M HCl gave the product 39. This reaction took place in anhydrous dioxane to give a yield of 79%. The product 39 was purified using small silica gel chromatography with chloroform/methanol/water (65:25:4). The product 39 was a lysophosphatidylcholine. Some methanol-d$_4$ was added to product 39 in combining chloroform-d to make it more
soluble for $^1$H-NMR and $^{13}$C-NMR. The absence of triplet peak at 4.77-4.84 ppm on $^1$H-NMR indicated the THP protecting group was displaced.

The product 39 was acylated with carboxylic acid 42 containing quencher group. The product 39 was insoluble in chloroform, and very sticky. The acylation was done in long necked round bottom flask. The glass beads and sonicator were used in this acylation. The reaction took place on the surface of glass beads. DMAP was a catalyst for the reaction, and product 42, DCC and DMAP were used in five fold excess to increase the yield of reaction. The reaction completed in 5 h at 25°C. The product 45 was purified by silica gel column chromatography using chloroform/methanol (9:1) to remove impurities then using chloroform/methanol/water (65:25:4) to obtain a single spot product to give a yield of 50%. About 50 mg of product 45 needed for each run of $^1$H-NMR to have precise data of all required peaks. The singlet at 6.23 ppm indicated the presence of coumarin peak. The choline peak at 3.39 ppm appeared as a sharp singlet. The consistency of $^1$HNMR, $^{13}$CNMR, and mass spectra of 45 indicated that a pure phosphatidylcholine was obtained.
The acylation of product 39 with short chain quencher 44 was done in similar conditions to introduce the quencher to sn-2 position of glycerol structure. After purification with two different solvent systems of chloroform/methanol (9:1) and chloroform/methanol/water (65:25:4), product 46 was obtained as a sticky yellow solid in a yield of 40%. In comparison to product 44, product 46 had a lower $R_f$ because it had a more polar structure. An approximate 40-50 mg of product 46 was used for each run of $^1$H-NMR to have precise data of all functional peaks. The multiplet peak at 5.30 ppm, the choline peak at 3.39 ppm, and the singlet peak at 6.23 ppm on $^1$H-NMR indicated the acylation of lysophosphatidylcholine 39 with short chain quencher was successful. All of structures were confirmed by high-resolution mass spectra data.
3.6 Hydrolysis of PLA$_2$

The hydrolysis of bee-venom PLA$_2$ was tried on natural phospholipid DPPC first to confirm the enzymatic activity.$^{57}$ Ca$^{2+}$ was a cofactor required for PLA$_2$ to catalyze the hydrolysis of phospholipid.$^{34-38,58,59}$ The appearance of lysophospholipid indicated enzymatic activity of PLA$_2$ on DPPC. The reaction completed within 30 min. at 40°C. The PLA$_2$ hydrolysis was done on PC-1, DPPC, and PC-2 on same TLC plate using chloroform/methanol/water (65:25:4) to compare the hydrolytic rate. Upon addition of PLA$_2$, PC-1 was hydrolyzed at $sn$-2 position to released fatty acid 42 and lysophospholipid 39.$^{59}$ The presence of 39 was traced by UV light, and this product turned to blue using molybdic acid spray. PC-2 was hydrolyzed at $sn$-2 position after
adding PLA$_2$. The fatty acid 44 and lysophospholipid 39 were released upon addition of PLA$_2$. The lysophospholipid 39 had a lower $R_f$ because its structure was more polar than the phospholipids PC-1 and PC-2. In comparison to the natural phospholipid DPPC, both synthetic phospholipids PC-1 and PC-2 were hydrolyzed with slower enzymatic rates. Within 30 minutes, DPPC was hydrolyzed completely, but only about 10% of PC-1 and 25% of PC-2 products were hydrolyzed. The PLA$_2$ stereoselectively hydrolyzed $sn$-2 position of phospholipids. The hydrolysis of PLA$_2$ on PC-1 and PC-2 indicated the same chirality of final synthetic products with the R-chiral starting material.

![Enzymatic hydrolysis activity of PC-1 (45).](image)

**Figure 12.** Enzymatic hydrolysis activity of PC-1 (45).
Figure 13. Enzymatic hydrolysis activity of PC-2 (46).
A new stereoselective synthesis of new fluorogenic phospholipase A\(_2\) substrates has been developed. The strategies were carried out to produce two new synthetic phospholipids with chain-terminal reporter groups. In this synthesis, (R) - (-) 2,2-dimethyl-1,3-dioxolane-4-methanol was successfully used as the chiral precursor to synthesize the two optical active phosphatidylcholine molecules. The tosylate group was designed at \(sn\)-3 position of glycerol structure for hydrolysis and introduction of phosphocholine. The \(sn\)-2 THP protecting group was removed to give new lysophosphatidylcholine. Acylation was carried out at \(sn\)-2 position of glycerol structure to introduce the quencher groups as second chain-terminal reporter groups for the target phosphatidylcholines. The chirality of the target phosphatidylcholines was pre-reserved very well through each step. The bee-venom phospholipase A\(_2\) hydrolyzed both new synthetic phospholipids at \(sn\)-2 position. Both \textbf{PC-1} and \textbf{PC-2} were hydrolyzed with slower enzymatic rates than the natural phospholipid DPPC. Further studies needed to be done to determine the PLA\(_2\)' kinetic studies on the new synthetic phospholipids \textbf{PC-1} and \textbf{PC-2}. 
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