CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

STEREOSELECTIVE SYNTHESIS OF FLUOROGENIC PHOSPHOLIPASE A_2 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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LIST OF ABBREVIATIONS

DCC	N,N'-dicyclohexylcarbodiimide
DHP	3,4-Dihydro-2H-pyran
DMAP	4-(Dimethylamino)-pyridine
DNFB	2,4-Dinitrofluorobenzene
LPC	Lysophosphatidylcholine
PL	Phospholipid
PC	Phosphatidylcholine
PLA_2	Phospholipase A_2
PPTS	Pyridium-*p*-toluenesulfonate
THF	Tetrahydrofuran
THP	Tetrahydropyran

ABSTRACT

STEREOSELECTIVE SYNTHESIS OF FLUOROGENIC PHOSPHOLIPASE A_2 SUBSTRATES

By

Duan Trinh

Master of Science in Biochemistry

Two fluorogenic phosphatidylcholines (PC-1 and PC-2) were synthesized and characterized for PLA_2'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 PLA_2 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*-butylamine. 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

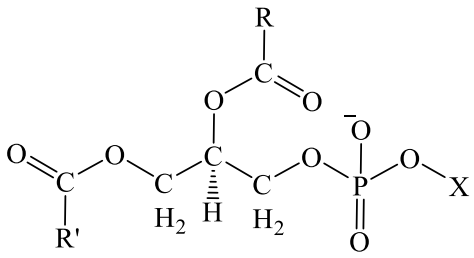
carbonate phospholipids, and opened the way to study the enzymology of new synthetic phospholipids in future.

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.



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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.[20] The phospholipases can be found widely from bacteria to mammals, and they act on the phospholipids that form vesicles and micelles in aqueous solution.[21] 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.[20, 22-25] Phospholipase A_1 cleaves the *sn*-1 acyl chain of phospholipid. Phospholipase A_2 cleaves the *sn*-2 acyl chain of phospholipid. The hydrolysis catalyzed by phospholipase A_1 and phospholipase A_2 produces free fatty acid and lysophospholipid.[25] Phospholipase B can cleave both acyl chains at the *sn*-1 and *sn*-2 positions on the phospholipid.[23-25] Phospholipase C cleaves the phosphodiester bond at the *sn*-3 position to release a phosphate-containing head group and a diacylglycerol.[25-27] Phospholipase D cleaves the head group of phospholipid to release a choline and a phosphatidic acid.[25,28,29]

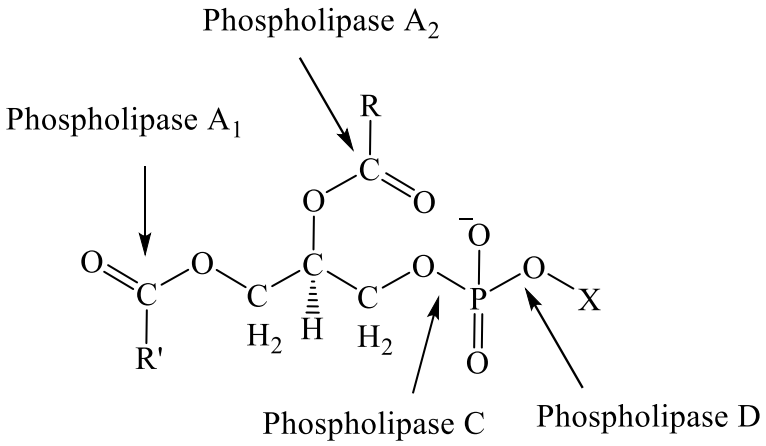
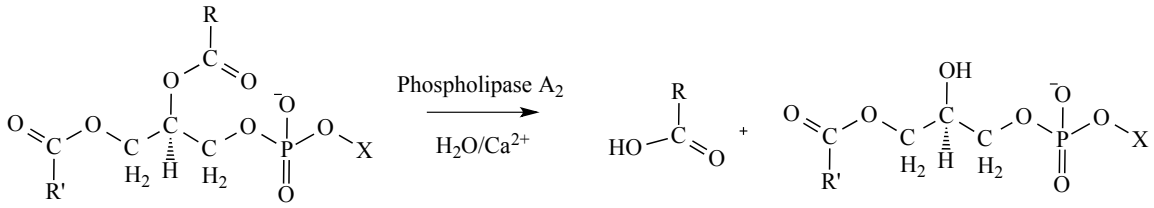


Figure 1. Cleavage sites of phospholipases on phosphatidylcholine

1.3 Phospholipase A_2

Phospholipase A_2 **1** (PLA_2s) have the systematic name of phosphatide 2-acylhydrolase.[30] 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]



1 **2** **3**

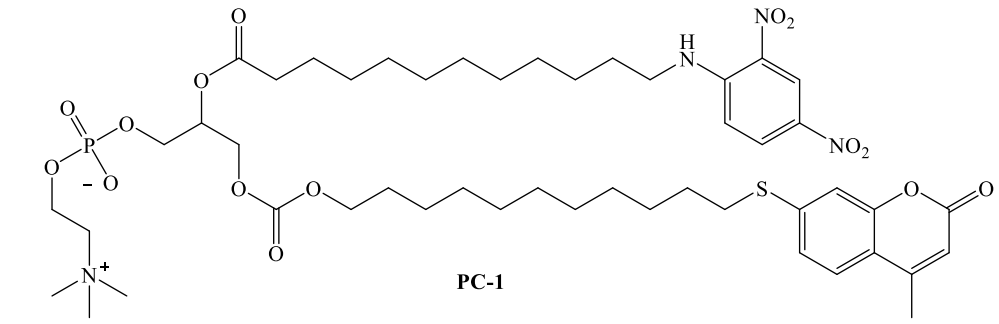
Furthermore, the lysophospholipid products of PLA_2 hydrolysis reaction play important roles in cell migration and survival.[33] The PLA_2s have 15 separate groups based on the ability to cleave *sn*-2 ester bond of phospholipid substrate.[34-36] They are classified into appropriate groups based on their molecular weight, amino acid sequence,

and requirement for Ca^{2+}. The PLA_2s 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 $sPLA_2$s 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.



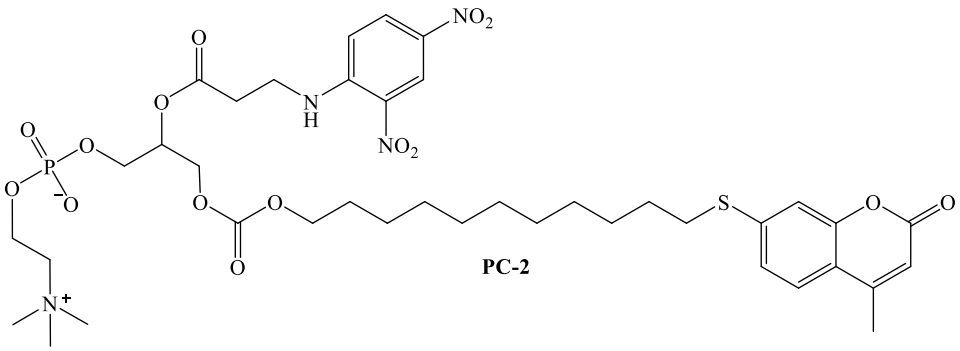


Figure 2. Structures of two final phosphatidylcholines **PC-1** and **PC-2**

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.

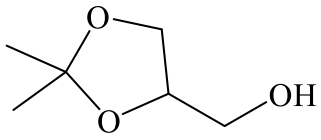


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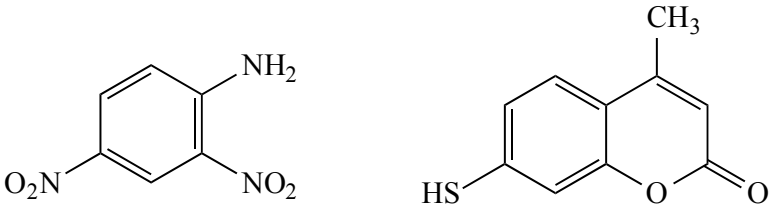
Figure 3. Structure of precursor of chiral glycerol

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 R_f 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-dintitroaniline **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-dintitroaniline **5** and 7-mercapto-4-methyl coumarin **6** were employed as chain terminal reporter groups for the final molecules.



5 **6**

absorption: 415 nm
emission: does not emit

absorption: 330 nm
emission: 415 nm

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-dintitroaniline **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 lysophospholipds 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.[55, 56] The final products **PC-1** and **PC-2** display perfect mixing behavior with components of unlabeled phospholipids in the cell membranes.[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 *sn*-1 position of the glycerol structure. The *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 *sn*-1 position. The fluorescent products are synthesized in two-step method through base catalyzed nucleophilic displacement of bromine of 11-bromoundecanol by mercapto methylcourmarin, then activation with *p*-nitrophenyl chloroformate to create the corresponding carbonate. UV light is used to detect the presence of flourescent products. The coupling reaction is carried out to form a product that has carbonate functional group at *sn*-1 position, THP protecting group at *sn*-2 position, and tosylate group at *sn*-3 position. The *p*-toluenesulfonate functional group is replaced by either tetramethylammonium acetate or tetraethylammonium methoxy acetate in anhydrous acetonitrile. The phosphocholine is introduced into the *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 *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.**

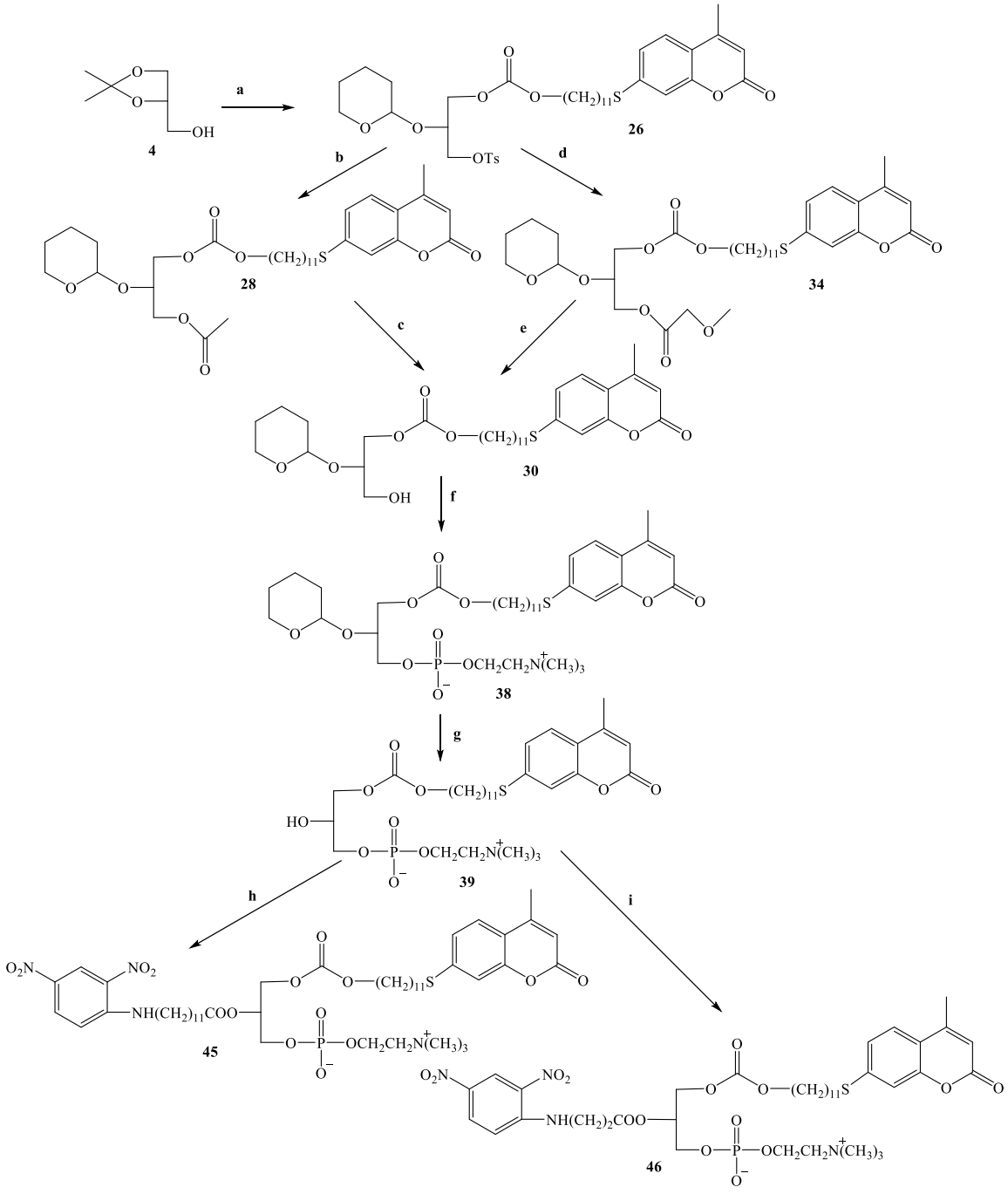


Figure 5. The synthetic strategies for target **PC-1** (**45**) and **PC-2** (**46**)

Reagents and conditions:(a) (i) *p*-toluenesulfonyl chloride/DMAP, CH_2Cl_2, (ii) 0.4 M HCl/CH_3OH, (iii) pivalic acid/DCC/DMAP, CH_2Cl_2, (iv) DHP/PPTS, CH_2Cl_2, (v) $LiBH_4$/THF, Et_2O, (vi) 11-((4-methyl-2-oxo-2H-chromen-7-yl)thio)undecyl (4-nitrophenyl) carbonate/DMAP, CH_2Cl_2; (b) $CH_3COO^-Me_4N^+$,CH_3CN; (c) Bu^tNH_2, $CHCl_3$/MeOH (1:4); (d) $CH_3OCH_2COO^-Et_4N^+$,CH_3CN; (e) Bu^tNH_2, $CHCl_3$/MeOH (1:4); (f) (i) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Me_3N, MeCN, rt., 12 h, (ii) Me_3N, MeCN, 67^{o}C, 24 h; (g) 0.3M HCl, aq.dioxane; (h) N-(2,4-dinitrophenyl)aminododecanoic aicd/DCC/DMAP, $CHCl_3$; (i) N-(2,4-dinitrophenyl)aminopropionic acid/DCC/DMAP, $CHCl_3$.

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-butylamine, 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*-toluenesulfonate, anhydrous Na_2SO_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 R_f 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. ^{1}H-NMR and ^{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-s*n*-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 $CHCl_3$. The product **8** (1.09g, 50%) was obtained as a white solid after freeze-drying from benzene.

IR ($CHCl_3$): 1053 cm^{-1}. ^1H NMR ($CDCl_3$, 400Hz), 1.05(s, 6H), δ 2.34 (s, 3H), 3.65-4.19 (m, 5H), 7.25 (d, 2H), 7.70 (d, 2H). ^{13}C-NMR ($CDCl_3$, 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). R_f ($CHCl_3$) = 0.29. MS MH^+ $C_{13}H_{19}O_5S$ 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 $CHCl_3$/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 ($CHCl_3$): 1354, 1177, 1053, 755 cm^{-1}. ^1H-NMR ($CDCl_3$, 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). ^{13}C-NMR ($CDCl_3$, 400 MHz) δ(142.7, 129.8, 127.4, 125.4, 68.1, 67.5, 60.1, 19.1). R_f ($CHCl_3$/MeOH 9:1) = 0.49. $[\alpha]_D^{25}$: -2.255 (c 1.02, $CHCl_3$-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** (1.58g, 15.5 mmol) in 15ml 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 $CHCl_3$/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 ($CHCl_3$): 1217, 1176, 750, 669 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400Hz), 1.05 (s, 9H), δ 2.34 (s,3H), 3.89-4.11 (m,5H), 7.25 (d,2H), 7.70 (d,2H). ^{13}C –NMR ($CDCl_3$, 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). R_f ($CHCl_3$/MeOH 9.4:0.6) = 0.52. R_f (Cyclohexane/ EtOAc 3:2) = 0.41. $[\alpha]_D^{25}$: -3.673 (c 0.98, $CHCl_3$-MeOH 4:1).

2.3.4 1-pivalyol-2-(tetrahydropyranyl)-3-(tolyene-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** (0.76g, 9.0 mmol), and pyridium *p*-toluenesulfonate **16** (0.23g, 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 ($CHCl_3$): 2937, 1729, 1361, 1175, 984, 813, 667 cm^{-1} . ^{1}H-NMR ($CDCl_3$, 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). ^{13}C-NMR ($CDCl_3$, 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^+ $C_{20}H_{30}O_7NaS$ Calcs: 437.1604, Found 437.1605.

2.3.5 2-(tetrahydopyranyl)-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 $LiBH_4$ 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. $NaHCO_3$ 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 Na_2SO_4 and evaporated. If necessary, the lower layer could be dried with anhydrous Na_2SO_4 to check if there was any organic

solvent in 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 ($CHCl_3$): 2938, 1729, 1358, 1175, 971, 935, 829, 758, 666 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 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). ^{13}C-NMR ($CDCl_3$, 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) R_f (Cyclohexane/EtOAc 1:3) = 0.58. MS MNa^+ $C_{15}H_{22}O_6NaS$ 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 dearated 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 Na_2SO_4. The solution was evaporated and loaded to column chromatography using $CHCl_3$/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 ($CHCl_3$): 2917, 2851, 1712, 1605, 1470, 1392, 1175, 1054, 850, 666 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 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).^{13}C-NMR ($CDCl_3$, 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) R_f ($CHCl_3$/EtOAc 8.5:1.5) = 0.28. MS MH^+ $C_{21}H_{31}O_3S$ 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 $CHCl_3$/EtOAc (8.5:1.5). After freeze-drying from benzene, the product was obtained as a white solid **25** (1.72 g, 78%).

IR ($CHCl_3$): 2928, 1716, 1601, 1367, 1215, 1176, 974, 750, 666 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400Hz), 1.32-1.80 (m, 21H), 2.42 (s, 3H), 3.00 (t, 2H), 4.31 (t, 2H), δ 6.23 (s, 1H), 7.18 (d, 2H), 7.39-7.48 (d, 3H), 8.29 (d, 2H). ^{13}C-NMR ($CDCl_3$, 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, 29.1, 28.7, 28.5, 28.5, 25.6, 18.5) R_f (CHCl3/EtOAc 8.5:1.5) = 0.82. MS MH^+ $C_{28}H_{34}NO_7S$ 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 (**1.64g, 3.1mmol) 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 ($CHCl_3$/EtOAc 9:1). After freeze drying from benzene, the product became a white solid **26** (1.00 g, 54%).

IR ($CHCl_3$): 2925, 2853, 1719, 1601, 1524, 1387, 1253, 1177, 956, 856, 750, 666 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400Hz), 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). ^{13}C-NMR ($CDCl_3$, 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) R_f ($CHCl_3$/EtOAc 9:1) = 0.65. MS MNa^+ $C_{37}H_{50}O_{10}NaS_2$ 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, 1601, 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^+ $C_{32}H_{46}O_9NaS$ Calcs: 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, 26.0, 23.1, 22.4, 18.1, 16.0) R_f ($CHCl_3$/EtOAc 8:2) = 0.43. MS MNa^+ $C_{30}H_{44}O_8NaS$ 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, 21.7mmol) 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. ^{1}H-NMR ($CDCl_3$, 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 $CHCl_3$/EtOAc (9:1). The sample was freeze-dried from benzene to give a white solid **34** (0.58 g, 82 %).

IR ($CHCl_3$): 2927, 2855, 1718, 1601, 1456, 1256, 1215, 956, 854, 750, 666 cm^{-1} . ^{1}H-NMR ($CDCl_3$, 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).^{13}C-NMR ($CDCl_3$, 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) R_f ($CHCl_3$/EtOAc 9:1) = 0.61. MS MNa^+ $C_{33}H_{48}O_{10}NaS$ Calcs: 659.2860, Found: 659.2843.

2.3.12 1-[11'-(7''-mercapto-4''-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-*sn*-glycerol [30]

To a solution of **34** (0.41 g, 0.6 mmol) in 10mL of chloroform/methanol (1:4), *t*-butylamine **29 (**0.53 g**,** 7.2 mmol) was added in a ice water bath at 0°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°C using rotatory evaporator. The sample was eluted and loaded to silica gel column using $CHCl_3$/EtOAc (1:1) for purification. The product **30** was freeze-dried from benzene to give a white solid (0.32 g, 95%).

IR ($CHCl_3$): 2924, 2854, 1736, 1601, 1455, 1386, 1253, 1176, 1034, 989, 955, 851,813, 790, 751, 666 cm^{-1} . ^{1}H-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 (t, 1H), δ 6.23 (s, 1H), 7.18 (d, 2H), 7.48 (d, 1H).^{13}C-NMR ($CDCl_3$, 400 MHz) δ (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, 26.8, 26.5, 26.0, 23.1, 23.0, 22.4, 16.9, 16.0) R_f ($CHCl_3$/EtOAc 1:1) = 0.69. MS MNa^+ $C_{30}H_{44}O_8NaS$ Calcs: 587.2649, Found: 587.2629.

2.3.13 1-[11'-(7''-mercapto-4''-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-phosphocholine-*sn*-glycerol [38]

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 $CHCl_3$/MeOH/H_2O (65:25:4) and loaded in a silica gel column chromatography using $CHCl_3$/MeOH/H_2O (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 ($CHCl_3$): 2924, 2855, 1737, 1717, 1601, 1387, 1246, 1085, 1053, 956, 851, 748, 666 cm^{-1} . ^{1}H-NMR ($CDCl_3$, 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).^{13}C-NMR ($CDCl_3$, 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) R_f ($CHCl_3$/MeOH/H_2O 65:25:4) =0.37. MS MH^+ $C_{35}H_{57}NO_{11}PS$ 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 $CHCl_3$/MeOH/H_2O (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 ($CHCl_3$): 3020, 2963, 1740, 1602, 1214, 1190, 1054, 744, 666 cm^{-1} . ^{1}H-NMR ($CDCl_3$, 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 ($CDCl_3$, 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 ($CHCl_3$/MeOH/H_2O 65:25:4) = 0.19. MS MH^+ $C_{30}H_{49}NO_{10}PS$ Calcs: 646.2809, Found: 646.2826. $[\alpha]_D^{25}$: - 0.833 (c 0.96, $CHCl_3$-MeOH 4:1).

2.3.15 N-(2,4-dinitrophenyl)aminododecanoic aicd [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 ($CHCl_3$): 2921, 2851, 1702, 1618, 1523, 1328, 1150, 923, 745, 715, 610 cm^{-1} . ^{1}H-NMR ($CDCl_3$, 400Hz), 1.29-1.82 (m, 20H), 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). ^{13}C-NMR ($CDCl_3$, 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). R_f ($CHCl_3$/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 dearated 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 ($CHCl_3$): 3020, 2929, 1710, 1621, 1524, 1338, 1214, 751 cm^{-1} . ^{1}H-NMR ($CDCl_3$, 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,

1H). ^{13}C-NMR ($CDCl_3$/Methanol-d_4 3:1, 400 MHz), δ(177.1, 152.0, 139.9, 134.3, 128.2, 117.9, 42.7, 37.0). R_f ($CHCl_3$/MeOH 9:1) = 0.32.

2.3.17 1-[11'-(7''-mercapto-4''-methyl-coumarin)-undecacarbonate]-2-[(2,4-dinitrophenyl)aminododecanoicacid]-3-phosphocholine-*sn*-glycerol [45]

To a solution of **39** (0.20g, 0.30 mmol) in 5mL of spectrograde chlololform, **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 30ºC, then diluted by $CHCl_3$/MeOH/H_2O (65:25:4) and loaded to silica gel column chromatography using $CHCl_3$/MeOH (9:1). The solvent system was switched to $CHCl_3$/MeOH/H_2O (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 ($CHCl_3$): 3020, 2931, 1734, 1621, 1525, 1338, 1428, 1406, 1215, 1174, 750, 667cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400Hz), 1.30 -1.82 (m, 40H), 2.31-2.44 (m, 7H), 3.01 (t, 2H), 3.42 (s, 9H), 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). ^{13}C-NMR ($CDCl_3$, 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, 28.8, 28.7,

28.6, 26.9, 26.8, 25.7, 25.7, 25.6, 25.7, 24.9, 24.8, 24.6, 18.6). R_f ($CHCl_3$/MeOH/H_2O 65:25:4)= 0.54. MS MH^+ $C_{48}H_{74}N_4O_{15}PS$ Calcs: 1009.4604, Found: 1009.4622. $[\alpha]_D^{25}$: +10.098 (c 1.02, $CHCl_3$-MeOH 4:1).

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 chlololform, **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 $CHCl_3$/MeOH/H_2O (65:25:4) and loaded to chromatography column of $CHCl_3$/MeOH (9:1). After all of impurities were washed away, the solvent system was switched to $CHCl_3$/MeOH/H_2O (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 ($CHCl_3$): 2925, 2854, 1718, 1619, 1524, 1424, 1335, 1054, 955, 814, 745, 666 cm^{-1}. ^{1}H-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). ^{13}C-NMR ($CDCl_3$, 400 MHz), δ(160.1, 15.0, 153.8, 152.4, 148.1, 143.9, 136.0, 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). R_f ($CHCl_3$/MeOH/H_2O 65:25:4) = 0.42.

MS MH^+ $C_{39}H_{56}N_4O_{15}PS$ Calcs: 883.3195, Found: 883.3207. $[\alpha]_D^{25}$: -0.707 (c 0.99, $CHCl_3$-MeOH 4:1).

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 $CaCl_2$. Each test tube was well-mixed by vortex for 15 sec. 20 μL of bee-venom PLA_2 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 PLA_2's hydrolysis on new synthetic phosphatidylcholines. $CHCl_3$/MeOH/H_2O (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 PLA_2 hydrolysis on samples **45** and **46**.

3. RESULTS AND DISCUSSIONS

3.1 Synthesis of 2-(tetrahydopyranyl)-3-(toluene-4'-sulfonyl)- *sn*- glycerol

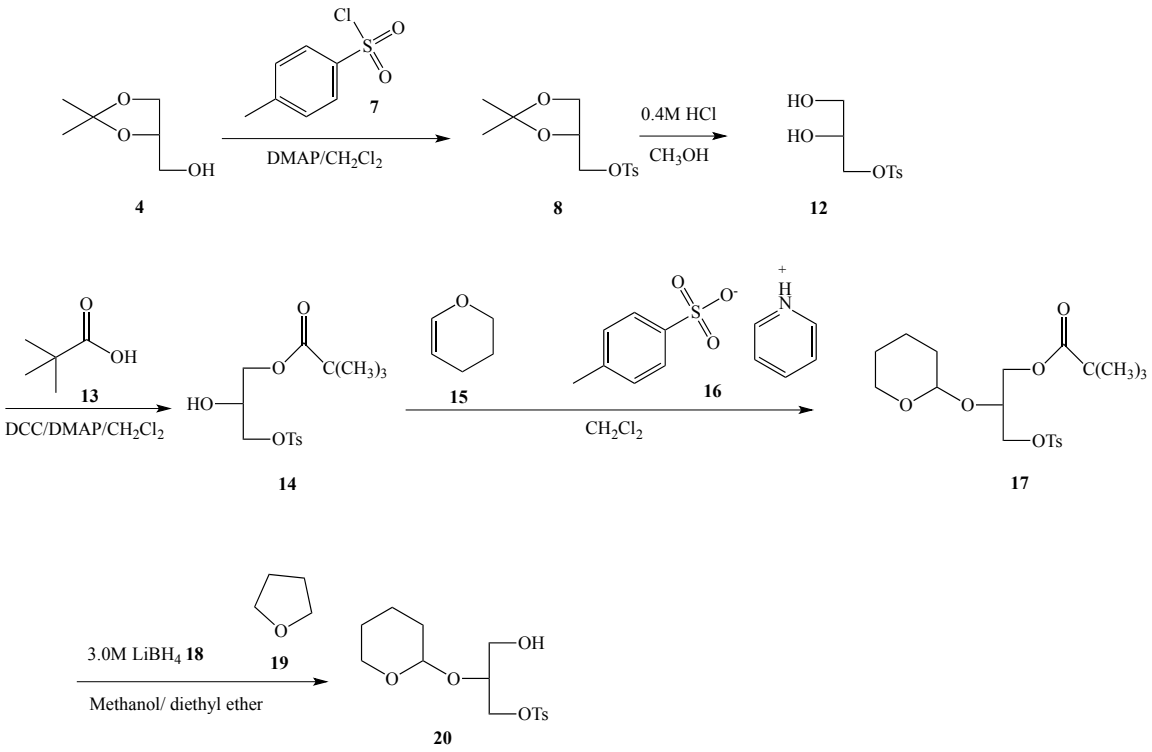
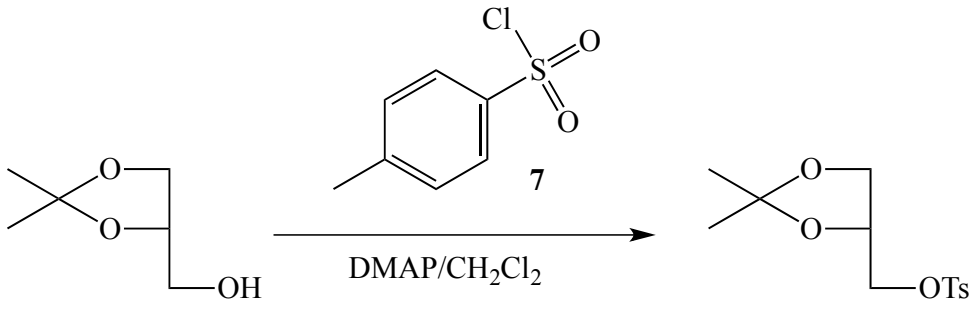


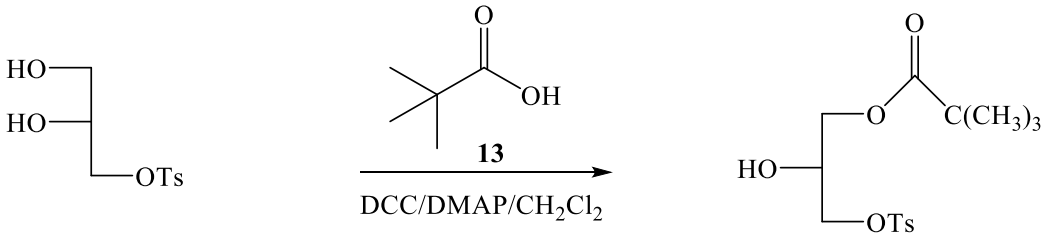
Figure 6: Scheme 1 for the synthesis of 2-(tetrahydropyranyl)-3-(toluene-4'-sulfonyl)-*sn*-glycerol

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.



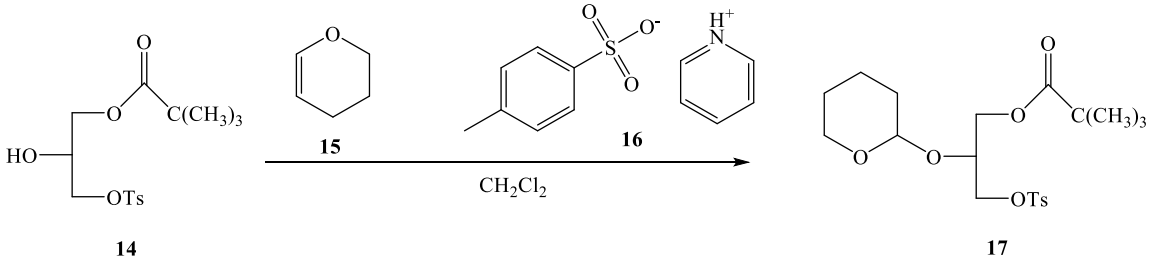
4 **8**

In the first step preparation of the 0.4 M HCl, solution was accomplished by mathanolysis of acetyl chloride. The isopropylidene group was removed using 0.4M 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%.



12 **14**

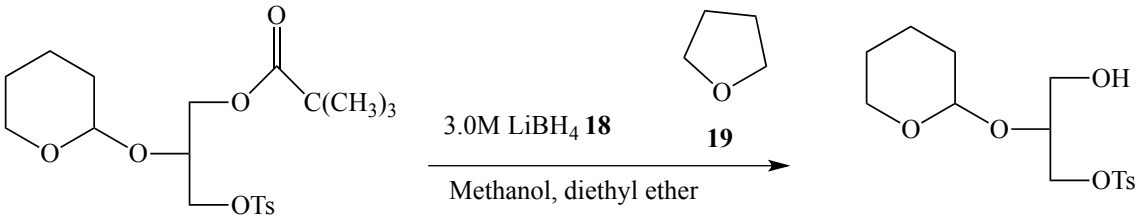
The secondary hydroxyl group of the product **14** was protected at the *sn*-2 glycerol position using 3-fold excess of DHP. PPTS contributed as a catalyst in the tetrahydropyranylation to give a yield of 97% at room temperature. Reaction was completed within 30 min. The product was loaded to silica 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.



14 **17**

The product **17** was then reacted with 3.0 M $LiBH_4$ in THF to create the hydroxyl group at *sn*-1 position. This hydroxyl group was prepared for the introduction of flourophore 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 $LiBH_4$ as a reducing agent instead of $LiAlH_4$ was to increase the recovery of product **20** to have a better yield. [58] 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.



17 **20**

3.2 Synthesis of 1-[11'-(7''-mercapto-4''-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-tosylate-*sn*-glycerol

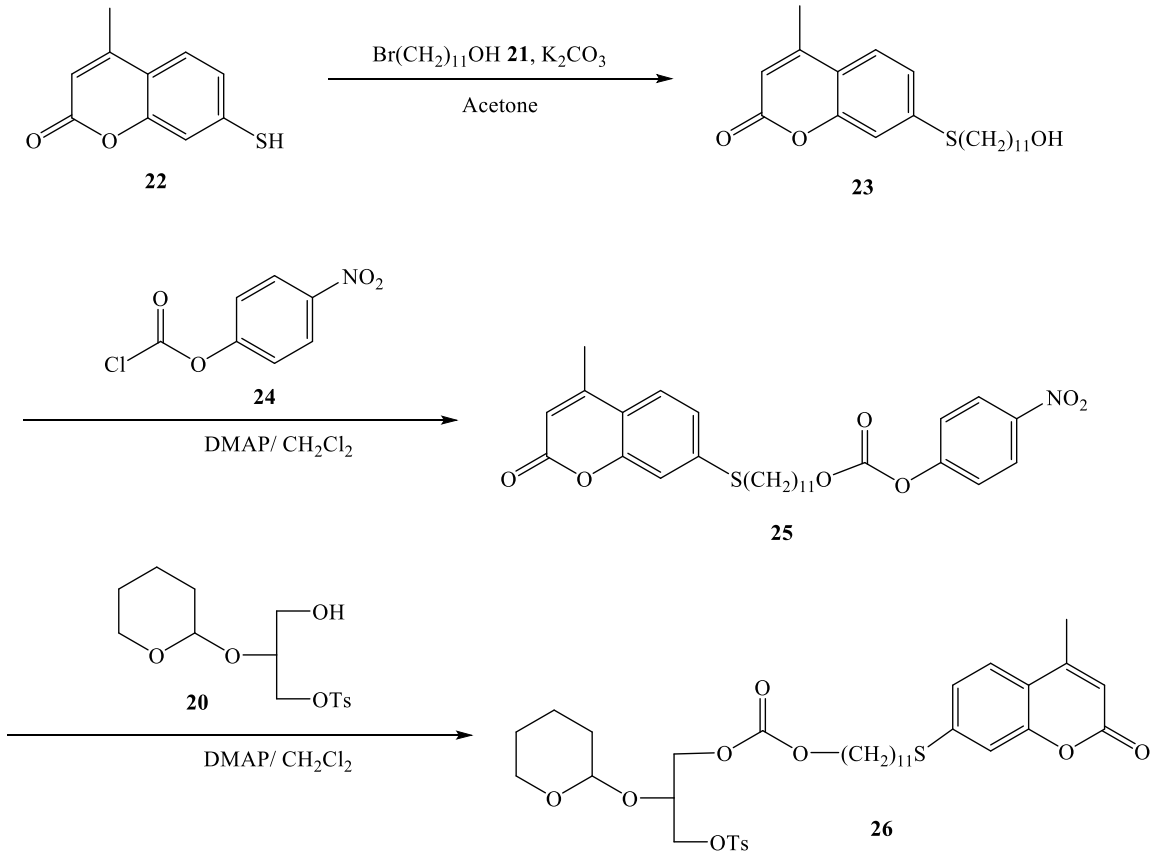
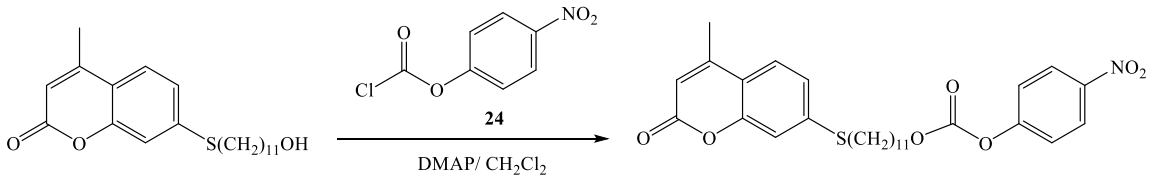


Figure 7. Scheme 2 for the synthesis of 1-[11'-(7''-mercapto-4''-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-tosylate-*sn*-glycerol

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 methylcourmarin 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

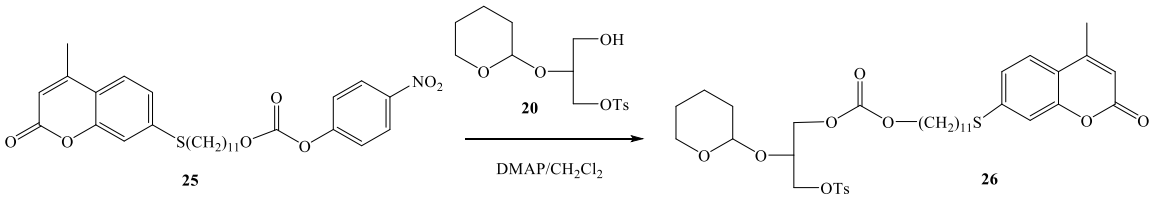
was UV-absorbing and turned to blue under UV light. The product **23** was obtained as white solid after silica gel chromatography purification using $CHCl_3$/EtOAc (8.5:1.5) and freeze-drying from benzene. In the next step of synthesis of product **25**, molecule **23** was reacted to *p*-nitrophenyl chloroformate to create the carbonate functional group. DMAP was employed as a catalyst to help reaction complete in a faster duration, and created a flourescent 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**.



23 **25**

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.



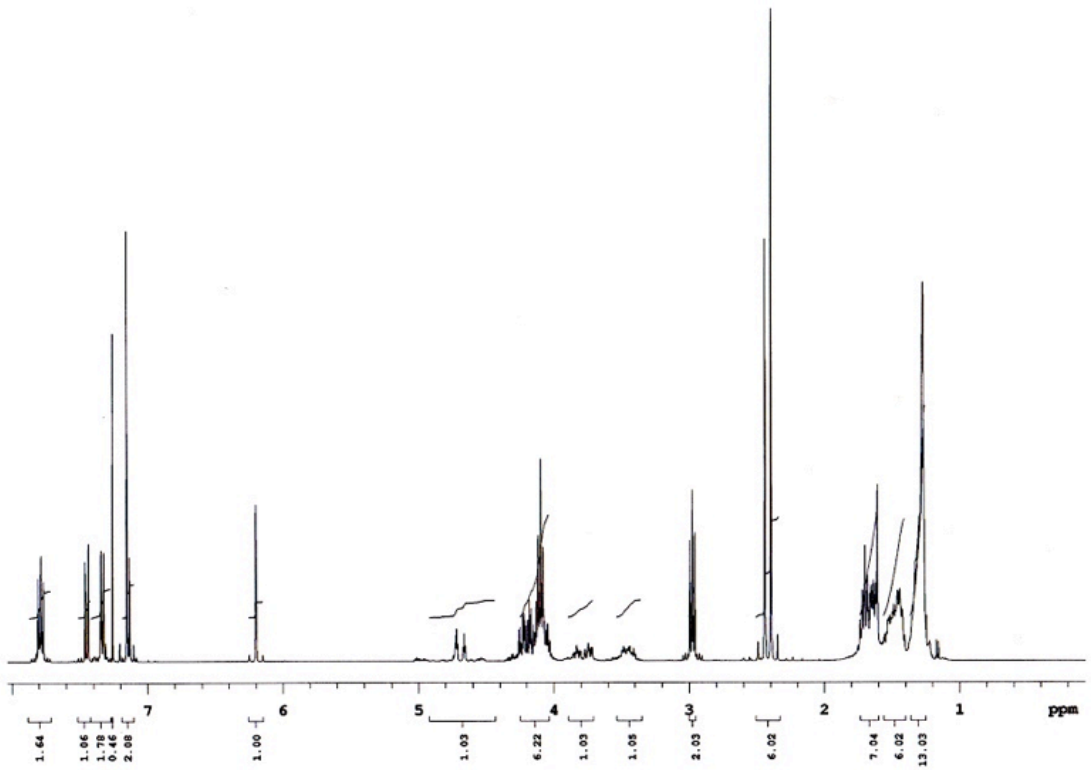


Figure 8. The coupling product **26** showed THP, coumarin and tosylate peaks on ^{1}H-NMR.

3.3 Synthesis of 1-[11'-(7''-mercapto-4''-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-phosphocholine-*sn*-glycerol

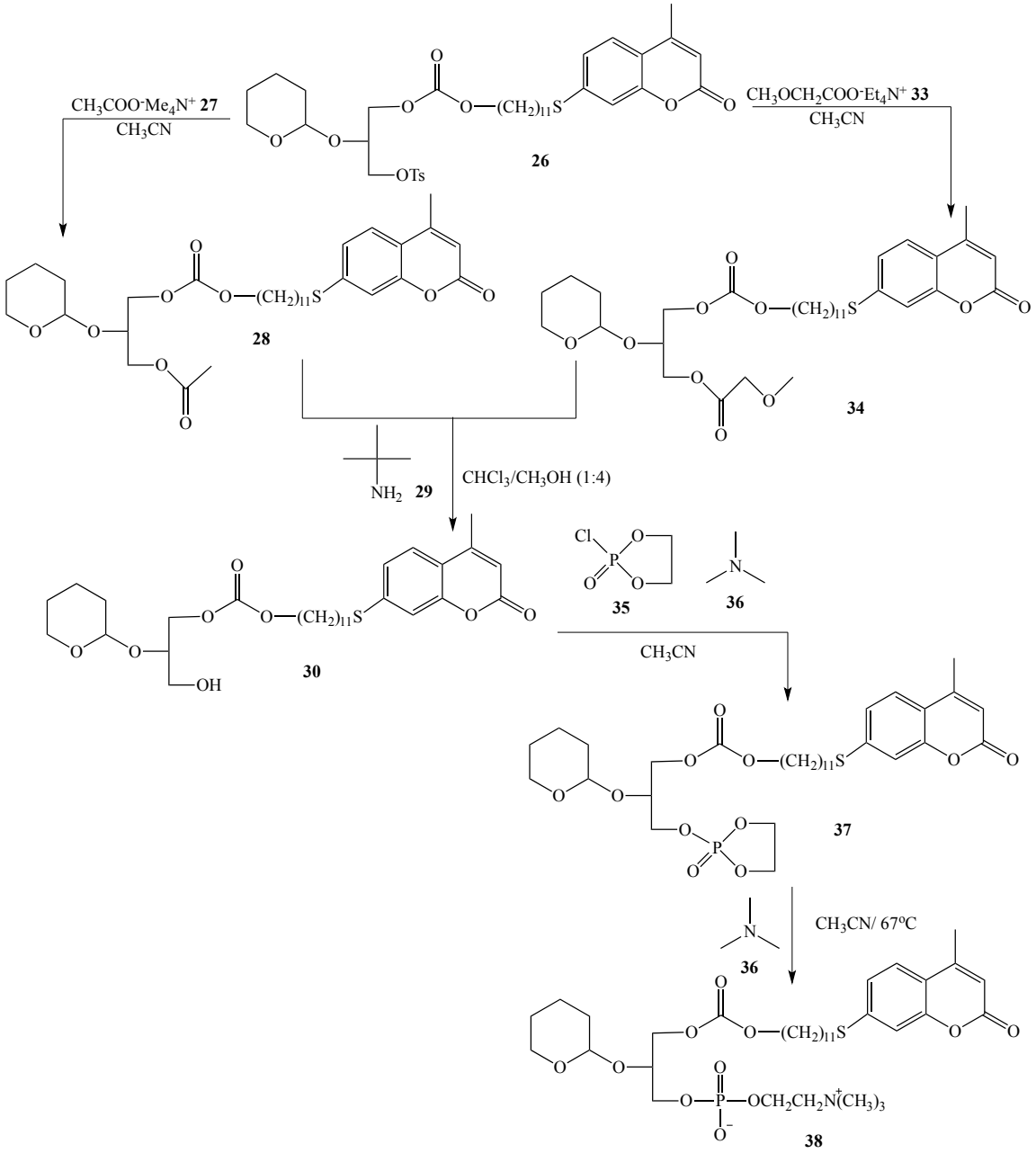
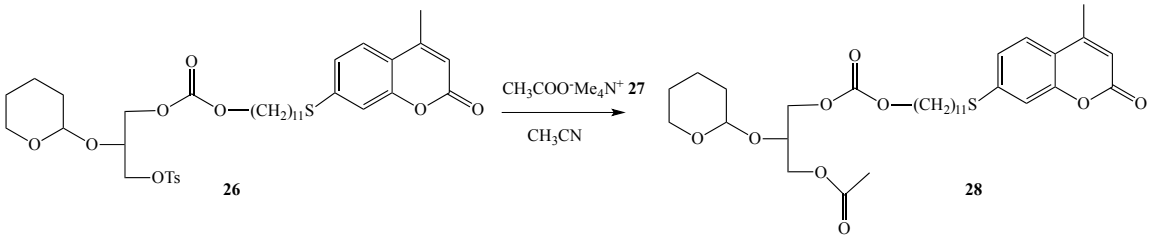


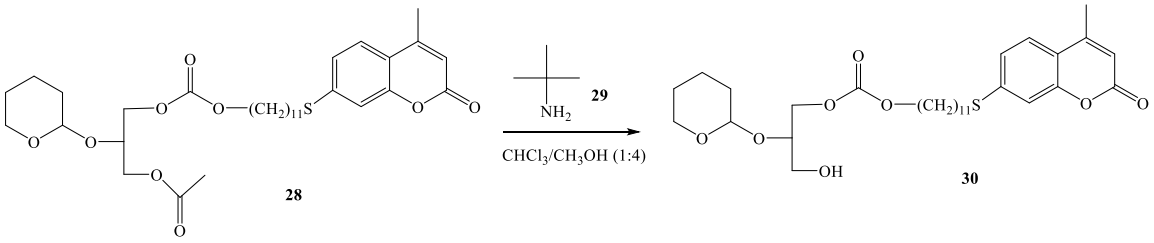
Figure 9. Scheme 3 for synthesis of 1-[11'-(7''-mercapto-4''-methyl-coumarin)undecacarbonate]-2-(tetrahydropyranyl)-3-phosphocholine-*sn*-glycerol.

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.

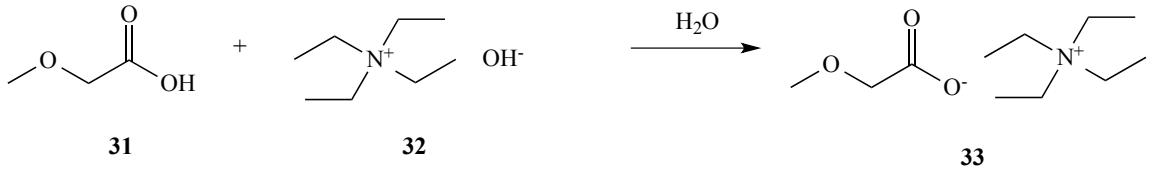


The hydrolysis of molecule **28** took placed in chloroform/methanol (1:4) with *tert*-butylamine as a base-catalyst to replace acetate group with a hydroxyl group at *sn*-3 position of glycerol structure. This hydroxyl group was prepared for the introduction of phosphocholine group in the phosphorylation 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%.

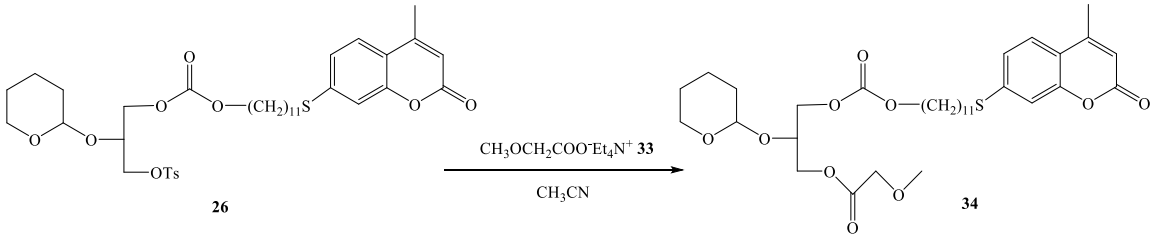


The second strategy in replacing tosylate group of product **26** was carried out to improve the efficiency and the yield of the synthesis. Tetraethylammonium methoxy acetate **33** was synthesized from reaction of methoxy acetic acid **31** and tetraethylammonium hydroxide **32** in water. The product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

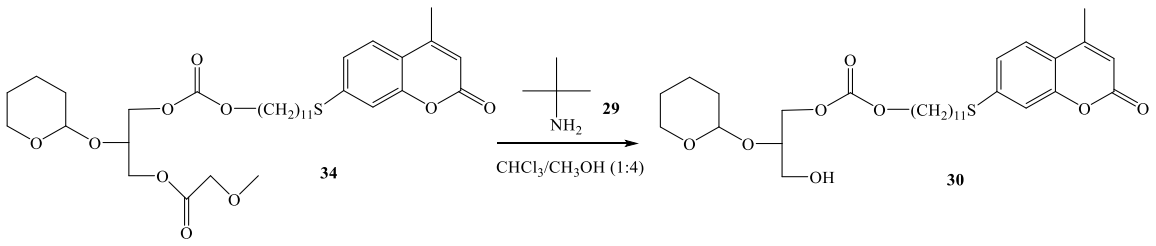


The replacement of the tosylate of product **26** using methoxyacetate **33** in anhydrous acetonitrile completed in 2 days at room temperature. A solution of freshly made 0.3M tetraethylammonium methoxyacetate **33** anhydrous acetonitrile was prepared and used for displacement of toluene-4-sulfonyl 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.

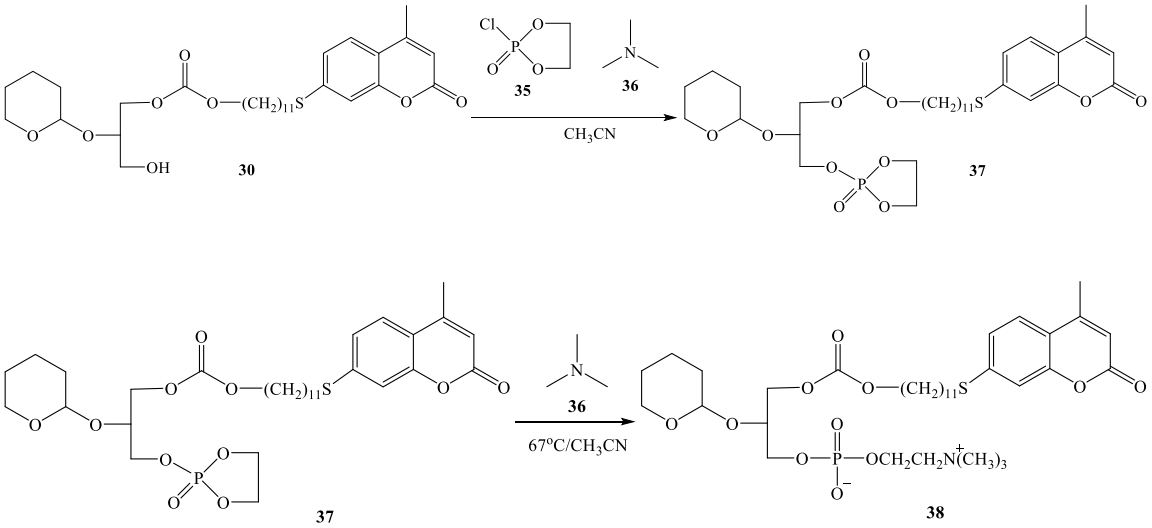


The product **34** was reacted with a mixture of chloroform/methanol (1:4) with *t*-butylamine **29** as a catalyst in hydrolysis reaction to produce **30** with a yield of 95%. The product **30** had a hydroxyl group that was introduced into *sn*-3 position of the glycerol structure. The reaction completed 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.



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 ^{1}H-NMR as singlet indicated the successful introduction of phosphocholine into the glycerol structure. The addition of some methanol-d_4 was required to dissolve the product **38** in combining chloroform-d for ^{1}H-NMR and ^{13}C-NMR.



3.4 Synthesis of N-(2,4-dinitrophenyl)aminododecanoic aicd and N-(2,4-dinitrophenyl)aminopropionic acid

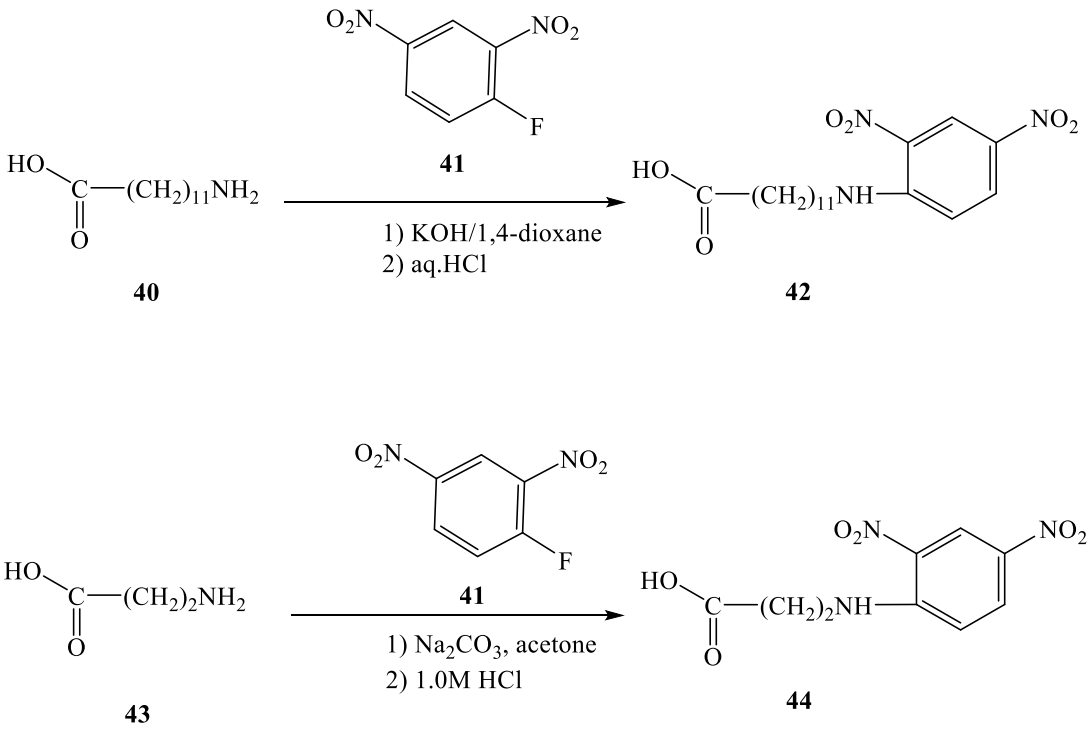


Figure 10. Scheme 4 for the synthesis of N-(2,4-dinitrophenyl)aminododecanoic aicd and N-(2,4-dinitrophenyl)aminopropionic acid.

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.[54] 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

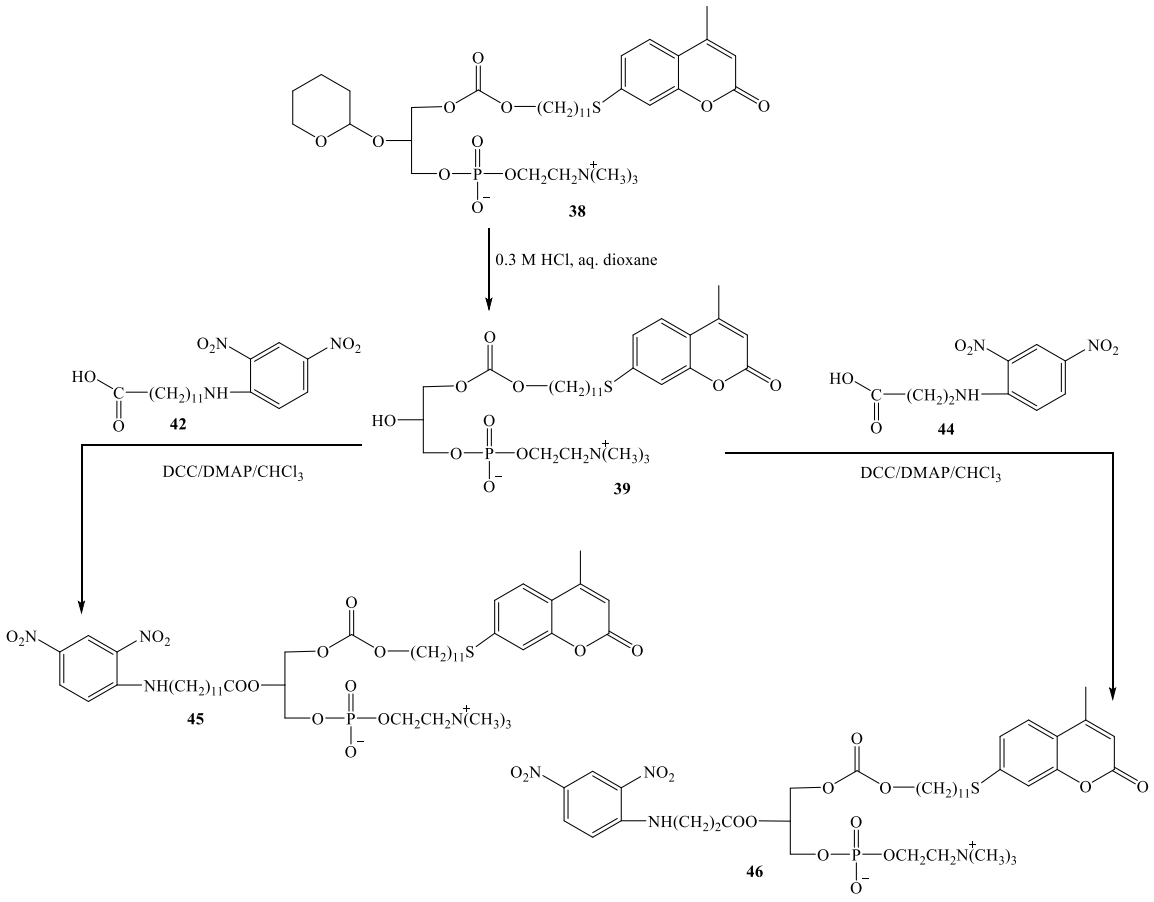
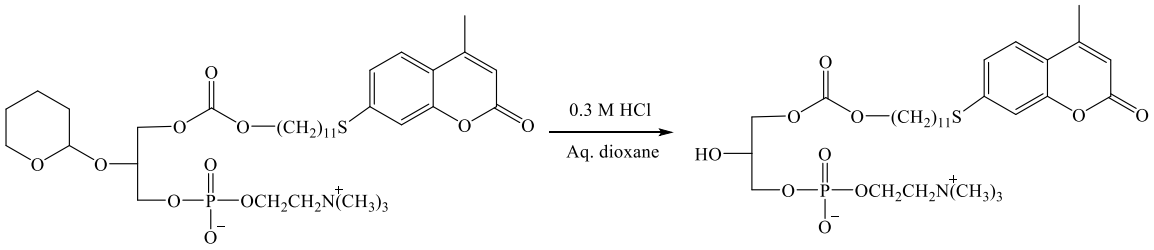


Figure 11: Scheme 5 for the synthesis of final **PC-1** (**45**) and **PC-2** (**46**)

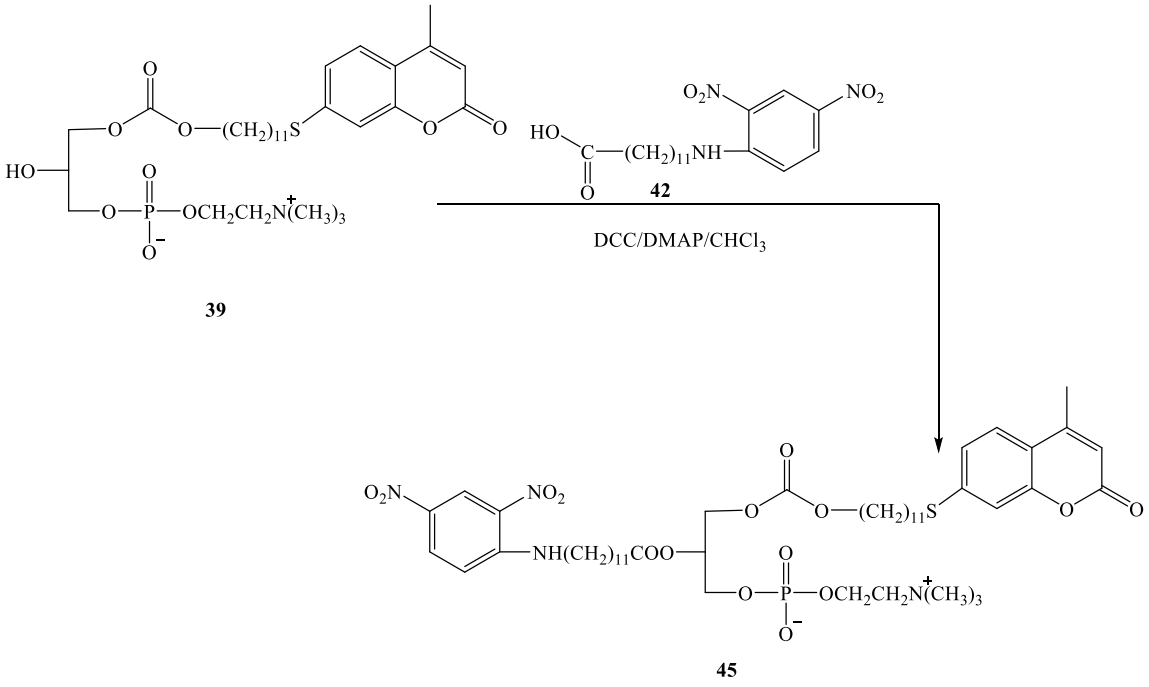
The acid-catalyzed cleavage of *sn*-2-tetrahydropyranyl group using 0.3M HCl gave the product **39**. This reaction took place in anhydrous dioxane to give a yield of 79%. The product **39** was purified using small silica gel chromatography with chloroform/methanol/water (65:25:4). The product **39** 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.

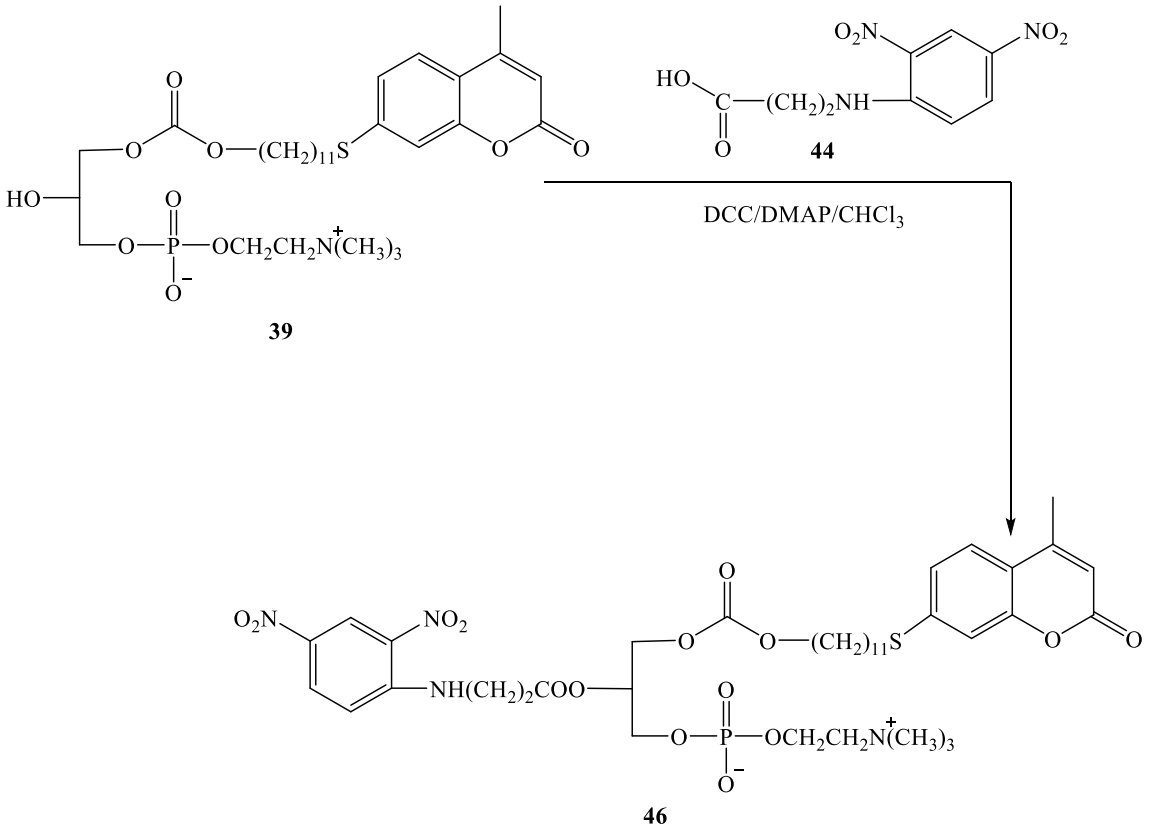


38 **39**

The product **39** was acylated with carboxylic acid **42** containing quencher group. The product **39** was insoluble in chloroform, and very sticky.[56] 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.[60] 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.

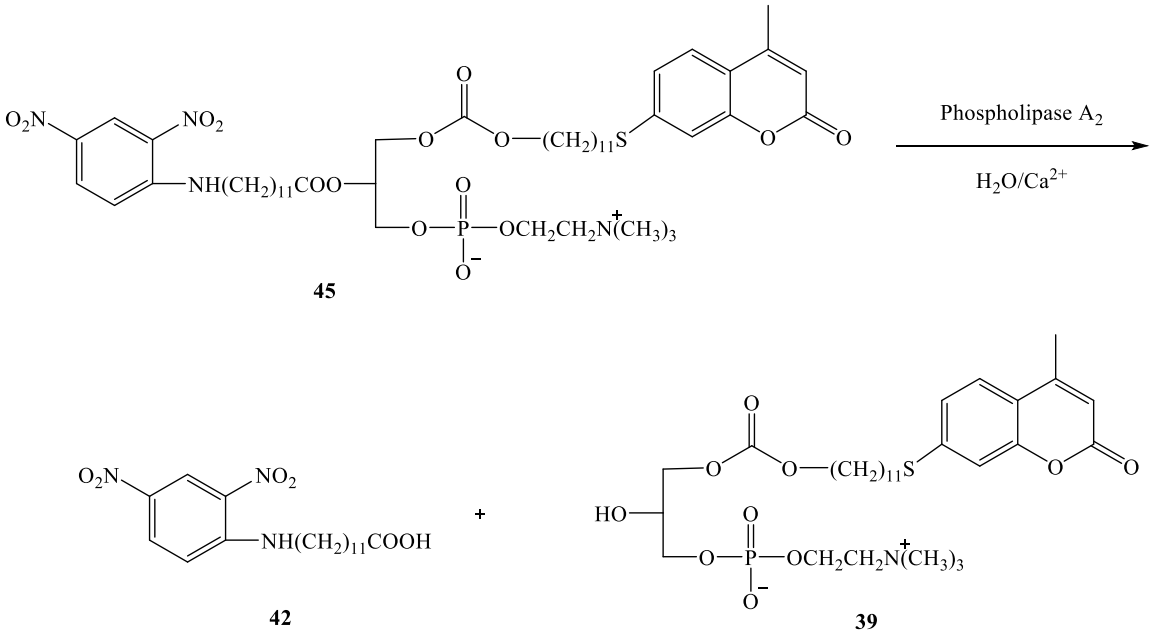
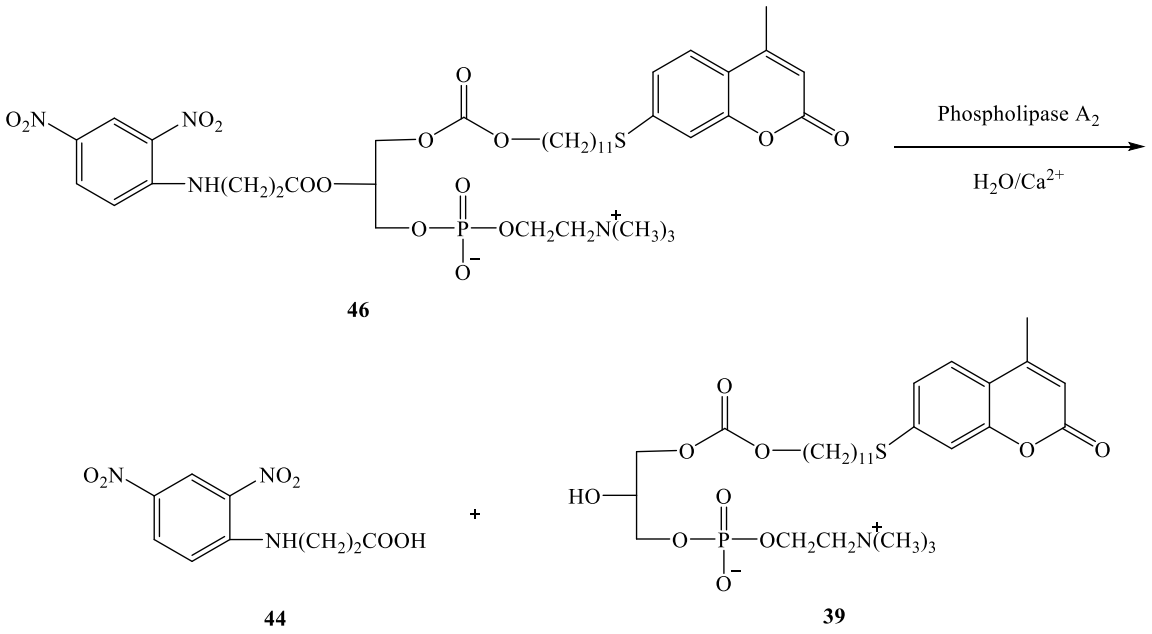


Figure 12. Enzymatic hydrolysis activity of **PC-1** (**45**).



46

44

39

Figure 13. Enzymatic hydrolysis activity of **PC-2** (**46**).

4. CONCLUSIONS

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-venon phospholipase A_2 hydrolyzed both new synthetic phospholipids at *sn*-2 position. Both **PC-1** and **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 **PC-1** and **PC-2**.

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