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Synthesis of tagged phospholipids analogs for studying protein-lipid binding

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Senior Thesis

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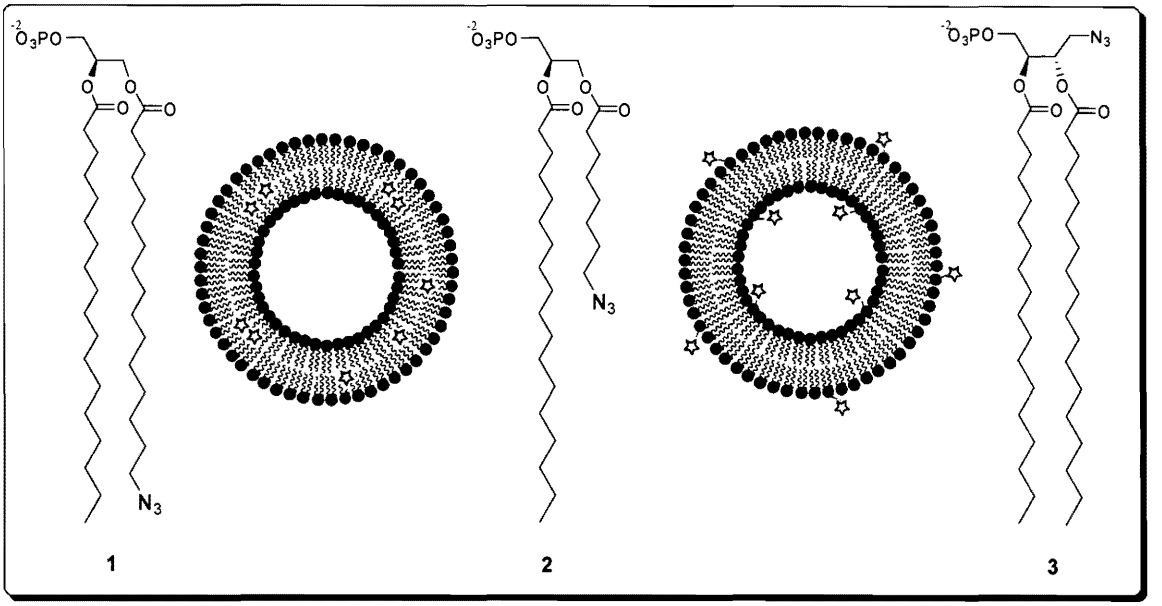
Abstract

The purpose of this project was to synthesize analogs of the phospholipid phosphatidic acid (PA) for use in investigating protein-lipid binding interactions. Using multiple organic synthesis steps, we produced a phospholipid containing a shortened lipid tail with a terminal azide group. The azide tag was attached in order to allow for introduction of reporter groups such as fluorophores. These tags will allow us to track the interaction between a specific protein and phospholipid using techniques such as fluorescence resonance energy transfer (FRET).

Introduction

The cell membrane has multiple components including phospholipids and membrane proteins. Interactions between proteins and phospholipids are part of numerous physiological and pathophysiological events. Binding acts as a regulatory mechanism that causes proteins to dock onto the cell surface. This allows them to perform specific functions needed by the cell. Phosphatidic acid (PA) has been directly implicated in activities such as diabetic cardiomyopathy[9] and protooncogene regulation[4]. PA is also involved in the immune response[3], phospholipid biosythesis[5], intracellular trafficking[1], and cell proliferation[2]. Tracking the specific mechanism can help in studying diseases caused by malfunctions in some of these crucial cellular processes. To study these processes, we are developing phospholipid analogs with azide reactive tags attached at different locations of the phospholipid, including the head or tail group (Figure 1). These will serve as versatile probes for examining cellular events.

Figure 1. Target lipid probes containing azide tags at different locations.

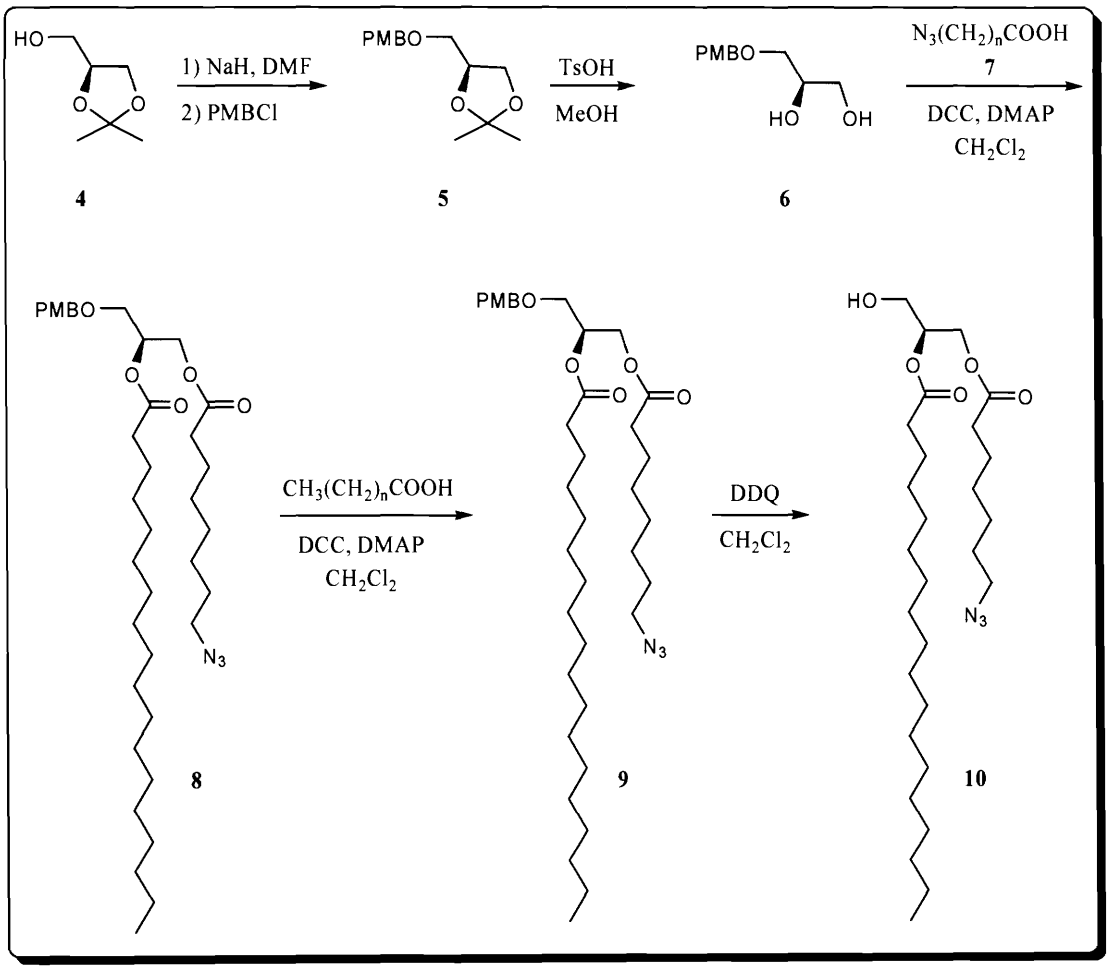


The bioorthogonal reactivity of the azide group allows for specific modification within a complex biological environment[8]. Use of core azide-tagged structures avoids X-ray crystallography, where time-consuming studies are required to study each separate protein. One type of approach to analysis includes Fluorescence Resonance Energy Transfer (FRET). FRET can be used to study lipid organization upon protein binding. Binding of the protein leads to a change in the proximity of lipids, which can be detected by energy transfer between fluorescent dyes incorporated into the lipid structure. These studies can produce a better understanding of receptor-ligand associations at the cellular level. Information from these studies can also provide vital details for improving medical treatments to diseases through the development of multivalent lipid analogs. Multivalent ligands exhibit high affinity and specificity in protein binding[6]. Studying these types of ligands is important because their properties allow for avoidance of side effects during

treatment. Most side effects arise because of interfering proteins that bind the same ligand.

Materials and Methods

Figure 2. Synthesis of the lipid scaffold containing a shortened azide-terminal chain



The synthesis of the phospholipid analog with an incorporated azide tag required multiple steps of organic synthesis. The following is a detailed outline of each step in the process.

(S)-4-((4-methoxybenzyloxy) methyl)-2,2-dimethyl-1,3-dioxolane (5)

Sodium hydroxide (259 mg, 0.00648 mol) was dissolved to 50 ml of DMF in a round bottom flask at 0 °C and placed under nitrogen. (S)-(2,2-dimethyl-1,3-dioxolan-4-yl)methanol (4) was added to an additional funnel mounted onto the reaction flask. The solution dropped into the reaction slowly over 30 minutes. PMBCl (4-methoxy benzyl chloride) (.823 μL, .006 mol) was then added to the solution at room temperature. The reaction stirred overnight and was quenched with 50 ml of H_20. This was extracted with 2, 40 ml portions of dichloromethane. The organic layer was removed and dried with magnesium sulfate, filtered and rotavaped. Thin layered chromatography (TLC) analysis of the product was performed using 25% ethyl acetate/hexanes solution. The plate was treated with potassium permanganate which stained the product spots. The product was purified by column chromatography over silica gel with gradient elution from 20%-40% ethyl acetate/hexanes. The product came out in fractions 7-11, and was rotovaped then placed on a high vacuum pump to yield a yellow oil (1.05g).

(R)-3-(4-methoxybenzyloxy) propane-1,2-diol (6)

(S)-4-((4-methoxybenzloxy) methyl)-2,2-dimethyl-1,3-dioxolane (5) was quenched with 50 mL of saturated sodium bicarbonate. Then, we did an extraction with 2, 50 ml portion of methylene chloride. The organic layer was then dried with magnesium sulfate, filtered, and rotovaped. TLC analysis of the product was performed using 75% ethyl acetate/hexanes solution. The plate was treated with potassium permanganate which stained the product spots. The product was purified by column chromatography over silica gel with gradient elution from 75%-100% ethyl acetate/hexanes. The product came

out in fractions 14-24, and was rotovaped then placed on a high vacuum pump (.598g, 68%).

8-Azidoctanoic acid (7)

Bromooctonoic acid (450 mg, 2.02 mol) and sodium azide (262 mg, 4.03 mol) were combined with 20 mL of DMF in a round bottom flask. The reaction flask was immersed in an oil bath and heated to 75 °C. The reaction stirred overnight at room temperature. The product was then purified by column chromatography over silica gel with gradient elution from 1%-3% methanol/dichloromethane/1% acetic acid. Fractions collected were rotovaped and then placed on a high vacuum pump to yield a yellow oil (0.157g, 42%).

(S)-2-hydroxy-3-(4-methoxybenzloxy) propyl 8-azidoctanoate (8)

(R)-3-(4-methoxybenzyloxy) propane-1,2-diol (6) (200 mg, 945 μmol), azidoctanoic acid (157mg, 848 μmol), DCC (N,N'-Dicyclohexylcarbodiimide) (233 mg, 1.13 mmol), and DMAP (4-Dimethylaminopyridine) (138 mg, 1.13 mmol) were suspended in 15 mL of dichloromethane in a round bottom flask. The reaction stirred overnight at room temperature. The reaction mixture was quenched with water and extracted twice with dichloromethane, dried with magnesium sulfate, filtered through celite, and then rotovaped. TLC analysis was performed using 35% ethyl acetate/ hexane. The plate was treated with potassium permanganate which stained the product spots. The product was purified by column chromatography over silica gel with gradient elution from 10%-50% ethyl acetate/hexanes. The product came out in fractions 34-38, and was rotovaped and then placed on a high vacuum pump (227mg, 71%).

1-(8-azidoctanoyloxy)-3-(4-methoxybenzyloxy) propan-2-yl hexadecanoate (9)

(S)-2-hydroxy-3-(4-methoxybenzyloxy) propyl 8-azidoctanoate (8) (116mg, 307 μmol), stearic acid (130mg, 460 μmol), DCC (94.9mg, 460 μmol), and DMAP (18.7mg, 153.3 μmol) were suspended in 6 mL of dichloromethane in a round bottom flask. The reaction stirred overnight at room temperature and was extracted with dichloromethane and water. The organic layer was then dried with magnesium sulfate, filtered, and rotovaped. TLC analysis of the product was performed using 20% ethyl acetate/hexanes solution. The plate was treated with potassium permanganate which stained the product spots. The product was purified by column chromatography over silica gel with gradient elution from 20%-30% ethyl acetate/hexanes. The product came out in fractions 8-10, and was rotovaped and then placed on a high vacuum pump (173mg, 87%).

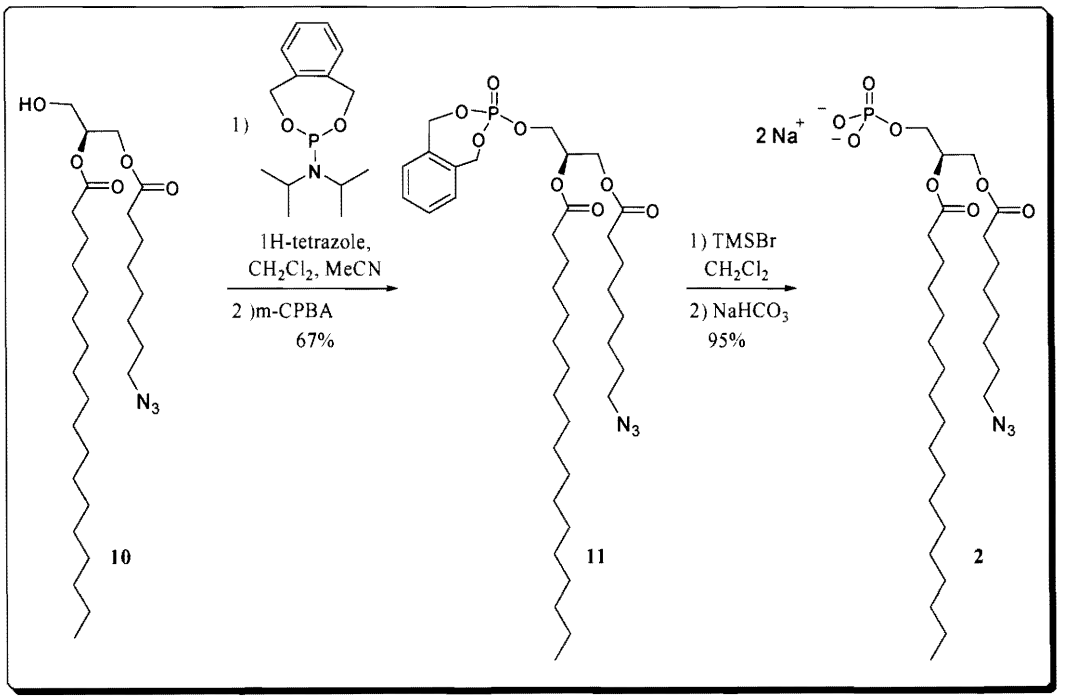
1-(8-azidoctanoyloxy)-3-hydroxypropan-2-yl hexadecanoate (10)

1-(8-azidoctanoyloxy)-3-(4-methoxybenzyloxy) propan-2-yl hexadecanoate (9) (173mg, 267.4 μmol) and DDQ (2,3-Dichloro-5,6-dicyano-p-benzoquinone) (121.4mg, 534.7 μmol) were suspended in a solution of 5 mL of dichloromethane and 500 μL of deionized water. The reaction stirred at room temperature for 2 hours and was then quenched with 20 mL of sodium bicarbonate. The product was extracted using 50 mL of dichloromethane. The organic layer was then dried with magnesium sulfate, filtered, and rotovaped. TLC analysis of the product was performed using 10% ethyl acetate/hexanes solution. The plate was treated with potassium permanganate which stained the product spots. The product was purified by column chromatography over silica gel with gradient

elution from 20%-30% ethyl acetate/hexanes. The product came out in fractions 17-26, and was rotovaped and then placed on a high vacuum pump (80mg, 57%).

The final steps for PA synthesis was installing the phosphate group. This required two steps: phosphotriester formation and deprotection.

Figure 3. Installation of the Phosphate Head Group of PA Analogs **2**



Phosphotriester formation (11)

1-(8-azidoctanoyloxy)-3-hydroxypropan-2-yl hexadecanoate (10) (80mg, 152.1 μmol), phosphoram (N, N-Diethyl-1,5-dihydo 2,4, 3-benzodioxaphosphepin-3-amine (109mg, 456 μmol), tetrazole (31mg, 456 μmol), 0.75 mL of dichloromethane, and 0.75 mL of acetonitrile were combined in a flame-dried round bottom flask. The reaction flask was placed at 0 °C while stirring under nitrogen for 5 minutes. It was then removed and let stir at room temperature for three hours. We then added M-CPBA (chloroperoxy benzoic

acid) (78.7mg, 456 μmol), and let reaction mixture stir at room temperature under air for three hours. The product was extracted with 2x25 mL of dichloromethane and 25 mL of saturated sodium bicarbonate. The organic layer was then dried with magnesium sulfate, filtered, and rotovaped. TLC analysis of the product was performed using 25% ethyl acetate/hexanes solution. The plate was treated with potassium permanganate which stained the product spots. The product was purified by column chromatography over silica gel with gradient elution from 30%-50% ethyl acetate/hexanes. The product came out in fractions 22-25, and was rotovaped and then placed on a high vacuum pump (78mg, 72%).

Deprotection to PA analog 1

The protected 2-tail (33.2mg, 0.048 mmol), TMS-Br (0.0441g, 0.2879 mmol), and 1mL of dichloromethane were combined in a round bottom flask and let stir under nitrogen at 0 °C for three hours. 1 mL of saturated sodium bicarbonate was added to the solution and let stir for 20 minutes. 1 mL of distilled water was used to quench and then solution was placed on rotovap to evaporate off the dichloromethane only. TLC analysis of the product was performed using 50% ethyl acetate/isopropanol solution. The plate was treated with phospray which stained the product spots. The product was purified by reverse column chromatography over silica gel with gradient elution from 0%-100% methanol/water. The product came out in fractions 4-6, and was rotovaped and then placed on a high vacuum pump (9.7mg, 33%).

One of the final parts of the experiment performed was synthesis of the fluorophore, anthracene, for FRET use. We followed the protocol outline by Owton et al. We added dropwise a solution of tetrabutylammonium bromide (4.93g, 15.3 mmol) and 10 mL of deionized water to a solution of potassium permanganate (2.27g, 14.4 mmol) in 30 mL deionized water while continuously stirring. We let the solution stir for 1 hour at room temperature. The solution was filtered using a Büchner funnel. The solid removed was dissolved in 75 mL of pyridine. In another round bottom flask, a mixture of 2-methylanthraquinone (1g, 4.5 mmol) and 30 mL of pyridine was stirred and heated to 75 °C under nitrogen. The tetrabutylammonium permanganate solution was added dropwise (over 1 hour) to the 2-methylanthraquinone solution. The reaction mixture was further stirred for 1 hour at 75 °C. TLC analysis of the product was performed using 10% methanol/dichloromethane solution. The plate was observed under UV light which showed that the starting material was gone. The solution was cooled to 15 °C and sodium metabisulfite (7.36g, 38.7 mmol) was added and let stir overnight at room temperature. The solution was rotovaped down and the residue was dissolved in 100 mL of deionized water. Concentrated HCl was added until we reached a pH of 4. The solution was then cooled down to 2 °C, filtered, then placed on high vacuum[7].

Results and Discussion

The final product was made and confirmed after a proton and phosphorous NMR. The overall yield of the synthesis was low because many steps were repeated due to the incorrect products formed. When making product (7), an additional purification step was done due to the observation of a solid impurity. The product was filtered with celite in

5% methanol/dichloromethane. During the purification of product (8), coupling product came out with the target product. This was confirmed using TLC analysis. The byproduct spot came out slightly below the target product (8) spot. TLC analysis with potassium permanganate sometimes yielded weak spots. TLC analysis was done again using a Ce/Mn stain.

One part of this synthesis that we are continuing to work on is the deprotection step. We have not gotten consistent results or a good yield with the different approaches we have made to this reaction. We have often seen the absence of a peak after the phosphorous NMR. The product has also been unstable in the solvents we use, so it is hard to isolate. We have experimented with a methanol/water mixture and also a chloroform/methanol/water mixture. But the results still show a low product yield and no phosphorous peak.

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