INDIANA UNIVERSITY BLOOMINGTON

CHEMISTRY DEPARTMENT

Synthesis of a novel lipid analogue

Angela K. Carrillo Alocen

Advisor: Ph D Martha Oakley

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ABSTRACT

Phosphatidylinositols are membrane phospholipids that play an important role in the regulation of vital functions in the cell. They are implicated in cell motitlity, cell proliferation, programmed cell death, response to insulin and other hormones. However, their interaction with the proteins they regulate is difficult to study due to their inclusion in membranes. The present work propose the synthesis of a phosphatidylinositol 3,4-bisphosphate analogue that will be covalently bond to the surface of PANAM dendrimers. Due to the spherical micelle shape that these dendrimers possess it will be possible to simulate the interaction between the lipids and a variety of proteins implicated in different diseases like cancer and diabetes.

The preliminary steps in the synthesis of the a phosphatidylinositol 3,4-bisphosphate analogue starting from a racemic mixture of the starting material 5,6-di-*O*-cyclohexylidene-*myo*-inositol, are shown. The products have been purified by flash chromatography and characterized by ^{1}H NMR.

1. INTRODUCTION

Membranes consisting of phospholipids bilayers are a ubiquitous component of all cells. There are different kinds of phospholipids, depending on the characteristics of the head-group, as well as the length, saturation, and branching of the hydrocarbon chains (**Figure 1**). Because of the different structures of lipids, the composition of biological membranes is complex and variable, and depends both on the type of cell and the organelle of interest.

Some of the more abundant phospholipids include the neutral zwitterions phophatidylcholine (PC) and phosphatidylethanolamine (PE), which constitute 60% of total phospholipids in the inner leaflet. Additionally the negatively charged lipids phosphatidylserine (PS) and phosphatidylinositol (PI) constitute 25% and 4% respectively. PC is found both in the inner and the outer leaflet of plasma membrane bilayers, whereas PS, PE and PI are located in the inner, cytosolic leaflet[xi].

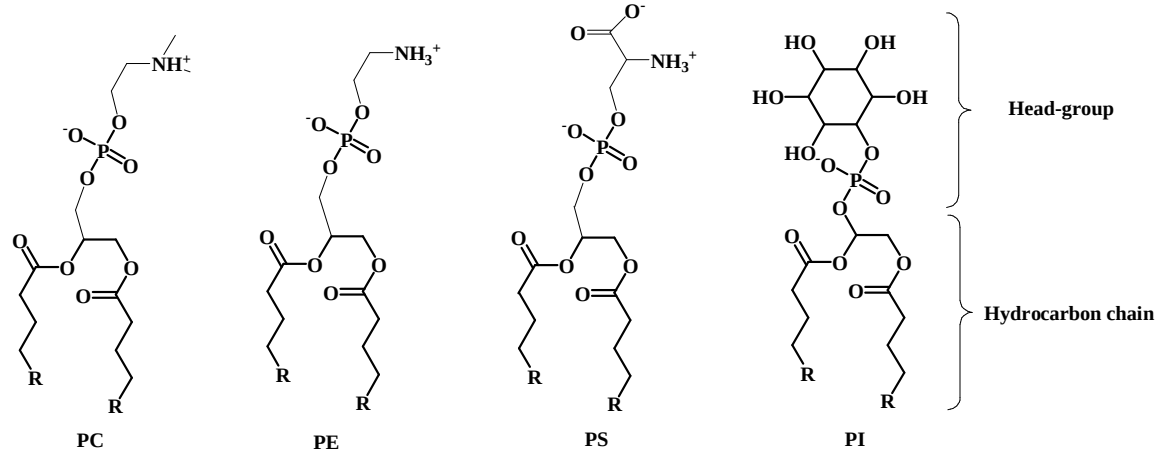


Figure 1. Phospholipids found in cells membranes.

Mono- and polyphosphorylated derivatives of phosphatidylinositol, referred to as phosphoinositides, for example phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$] and phosphatidylinositol 3,4-

bisphosphate [PI(3,4)P_2], represent <1% of total membrane phospholipids. They are unique among phospholipids in their ability to be modified rapidly by phosphorylation/dephosphorylation, to create (or eliminate) membrane-targeting signals at particular locations. Two phosphoinositide-mediated signaling pathways have been demonstrated, both of which originate from PI(4,5)P_2, (**Figure 2**).

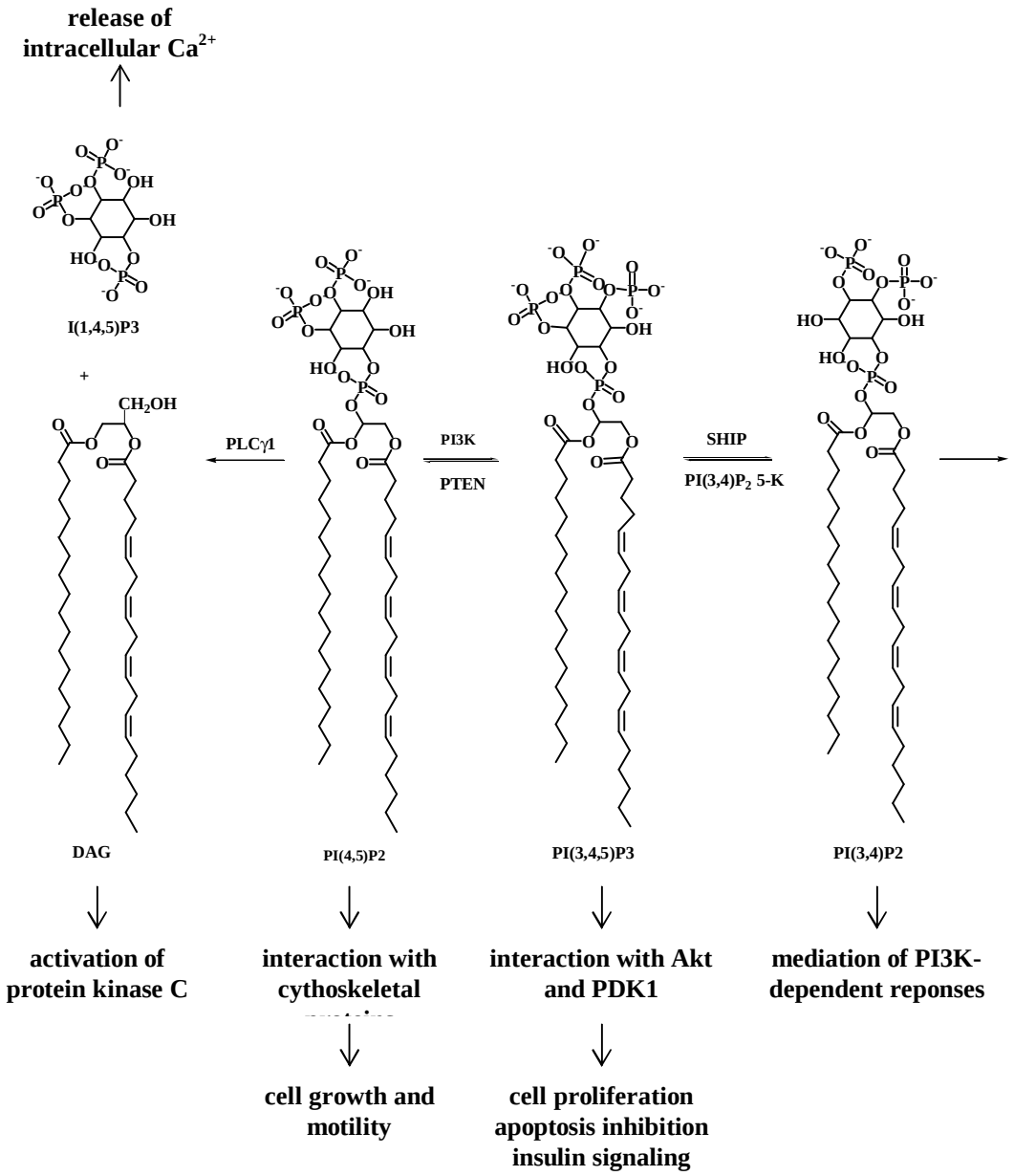


Figure 2. Overview of the major roles of mono- and polyphosphorylated derivatives of phosphatidylinositol in cellular signaling.

The first pathway involves the hydrolysis of $PI(4,5)P_2$ by phosphatidylinositol-specific phospholipase C (PLCγ1) in response to agonist stimulations. This process produces two signaling molecules: diacylglycerol (DAG) and D-*myo*-inositol-1,4,5-trisphosphate [$I(1,4,5)P_3$][xii]. DAG acts by stimulating protein kinase C, whereas $I(1,4,5)P_3$ releases calcium from internal sources[xiii], mediating the effects of several neurotransmitters, hormones and growth factors.

However, the activity of PLC-γ on $PI(4,5)P_2$ can be inhibited by the binding of the cytoskeletal protein profilin to $PI(4,5)P_2$[xiv]. In addition, this phosphoinositide-profilin association can prevent the formation of profilactin (profilin-G-actin complex) which is necessary in actin polymerization and cell motility[xv].

The second pathway that involves $PI(4,5)P_2$ entails phosphoinositide 3-kinase (PI3K), which is activated by growth factors, cytokines and insulin. PI3K phosphorylates $PI(4,5)P_2$ at the D-3 position of the inositol ring, converting it into $PI(3,4,5)P_3$. Proteins that bind directly to $PI(3,4,5)P_3$ are serine-threonine kinases Akt (also known as protein kinase B, PKB) and phosphoinositide-dependent kinase 1 (PDK1)[xvi]. The binding of $PI(3,4,5)P_3$ to the membrane facilitates phosphorylation of Akt by PDK1, a process that stimulates the catalytic activity of the former protein. Akt plays a key role in cancer progression by stimulating cell proliferation and inhibiting apoptosis, and is also probably a key mediator of insulin signaling[xvii]. The termination of PI3K signaling is obtained by 5-

dephosphorylation of PI(3,4,5)P_3 with Src-homology 2-containiing phosphatases (SHIP)[6]. This produces PI(3,4)P_2, an important phosphoinositide because it mediates PI3K-dependent responses. Finally, the PI(3,4,5)P_3 3-phosphatase (PTEN) dephosphorylates the 3 position of PI(3,4,5)P_3 to produce PI(4,5)P_2. Loss of PTEN has been found in a large fraction of advanced human cancers, indicating that uncontrolled signaling through PI3K contributes to metastatic cancers[xviii].

Because of the role that phosphoinositides play in regulating the vital functions mentioned (cell motitlity, cell proliferation, programmed cell death, response to insulin and other hormones), the comprehension of the molecular basis of the interaction of these lipids with the proteins they regulate is crucial to understand how the cell machinery works, and also to obtain alternative ways to treat diseases as cancer and diabetes.

Domains that are known to recognize phosphoinositides include PH (pleckstrin homology), PX (phox homology), FYVE, FERM, ANTH, ENTH domains and Tubby proteins[xix,xx], (**Figure 3**). Recently, the generalities of the interactions found between these domains and the phosphoinositides have been determined:

PI(3,4,5)P_3 and PI(3,4)P_2 targeted PH domains from such proteins as Akt (in the case of PI(3,4,5)P_3), are electrostatically polarized, with the positive charge in the phosphoinositide binding site. These domains interact specifically and strongly with the negative head-group of their phospholipid ligand. Similarly, PH domain of PLC-γ binds to PI(4,5)P_2 electrostatically with short basic and polar residues. In addition, hydrophobic

interactions can improve the membrane association. In the case of PX and FYVE domains, they can recognize the lipid head-group of phosphatidylinositol 3-phosphate [PI(3)P] specifically, but they don't bind it with very high affinity. In order to increase their affinity, these domains insert hydrophobic side-chains into the membrane bilayer or increase their membrane-binding avidity through oligomerization. FERM domains of cytoskeletal proteins, interact with $PI(4,5)P_2$ lipids through regions that contain clusters of basic residues and also hydrophobic interactions. ANTH and ENTH domains also interact with $PI(4,5)P_2$, but the latter induces curvature of its target membranes. Finally, Tubby proteins have been found to bind to $PI(4,5)P_2$, as well as $PI(3,4,5)P_3$ and $PI(3,4)P_2$, by electrostatic interactions with their head-group.

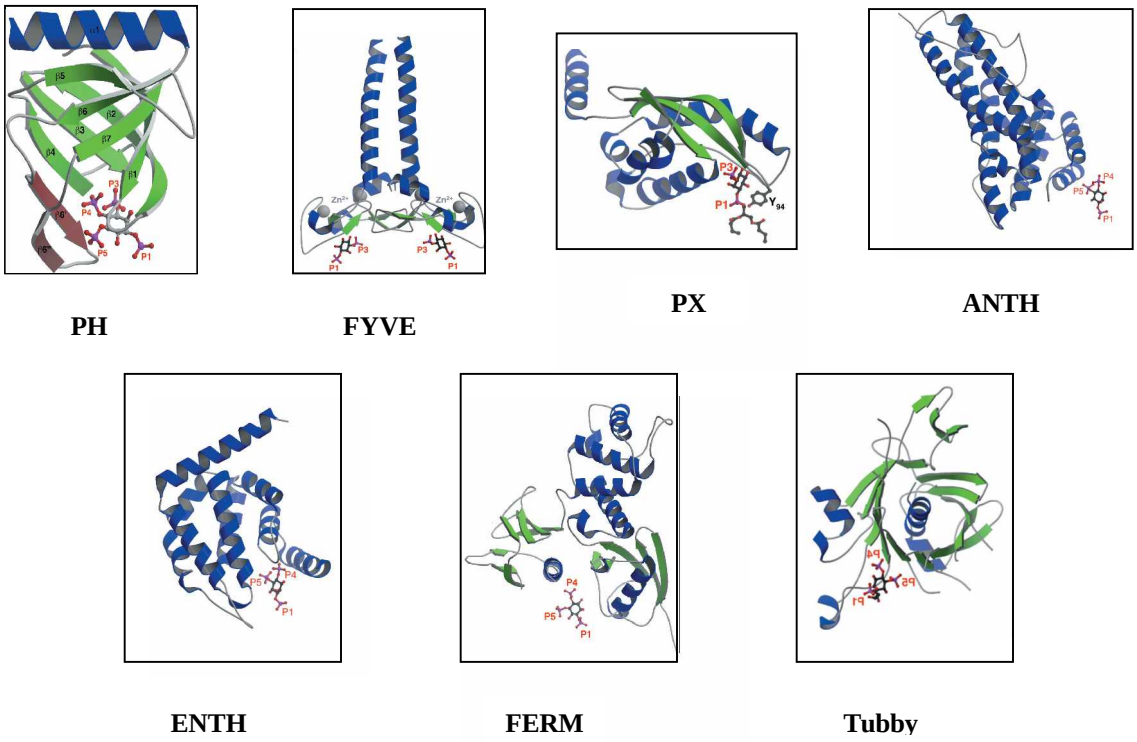


Figure 3. Phosphoinositide recognition domains.

Therefore, it has been found that electrostatic interactions between a positive charge located in phosphoinositide recognition domains, and the negative head-groups of these

lipids, are necessary upon binding. In addition, it has been proven that some of these interactions occur by more than one binding site in the protein with different arrays of lipids, suggesting that multivalency could be an important mechanism for specific protein-lipid molecular recognition.

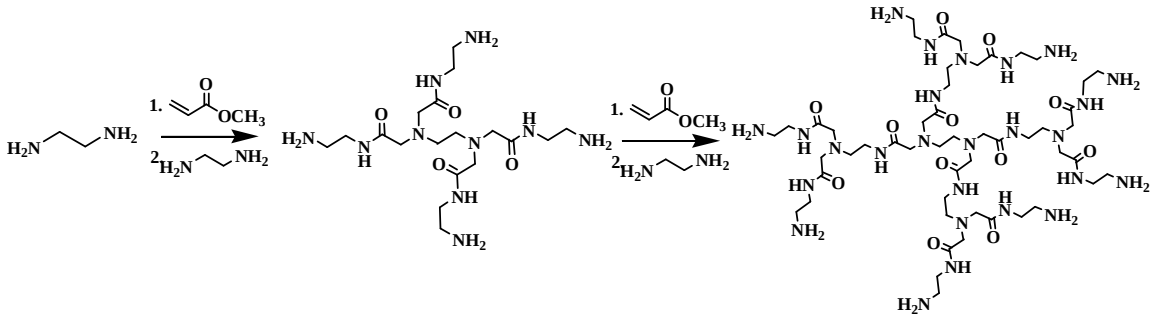
Although the improvements obtained in this field, it is still necessary to recognize the details involved in these interactions in order to understand how the proteins mentioned discriminate among the different kind of lipids during the processes they regulate.

Two of the reasons for which the insights of protein-lipid interactions are not well understood involve the difficulties concerning the synthesis of lipids and their analogues and the difficulty in studying protein-lipid interaction due to their inclusion in membranes. Many proteins interact with phosphoinositides that are arranged in a specific array. Lipids are able to diffuse in model membranes, therefore the spatial requirements for the interaction are difficult to study.

In order to minimize these problems, the present project proposes the usage of a novel class of lipid analogues that allow the display of multiple copies of the polar head-groups on a spherical surface, to mimic the interaction of phosphoinositides with proteins. These analogues are a derivatized form of polymers known as poliamidoamine dendrimers (PAMAM™, Aldrich)[xxi].

Dendrimers are characterized for being formed starting from initiator cores that posses N_c reactive sites. These react with molecules possessing N_b reactive sites, which introduce

multiplicity and produce what is known as dendrimers of generation 0. Repetition of this sequence leads to generation 1, 2, 3, 4, etc. Each increase in integer value of the generation number indicates a doubling in the number of termini on the surface of the dendrimer. In the case of PAMAM dendrimers, $NH_2CH_2CH_2NH_2$ (N_c=3) can be used as the initiator core and $-CH_2CH_2CONHCH_2CH_2NH_2$ (N_b=2) as the repeating units (**Scheme 1**). PAMAM dendrimers increase their diameter by approximately 10 Å per generation, evolving from a dislike shape (generations 0-2) to an oblate spheroid (generations 3,4) to a symmetrical spheroid at generations 5 and higher[xxii].



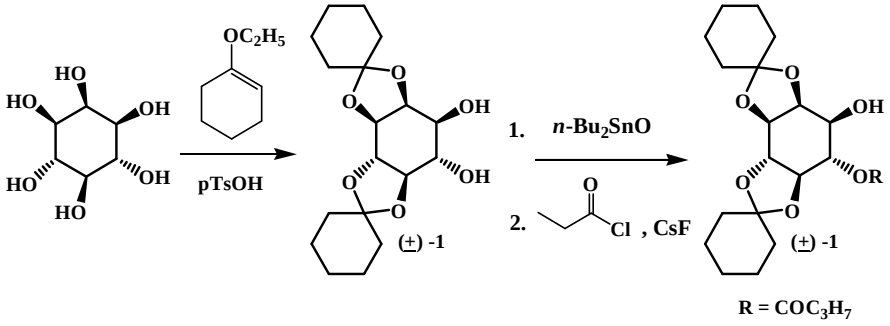
Scheme 1. Synthesis of PAMAM dendrimers starting from ethylene diamine, $NH_2CH_2CH_2NH_2$.

PAMAM dendrimers (starting from generation 4) have been compared to micelle systems[xxiii]. They are similar in size and shape, and also simulate surface-group proximity effects like the ones found in spherical micelles and also in liposomes or bilayers. However, these polymers have the advantage that the head-group multiplicity is determined by synthesis, whereas in self-adjusting micelle systems the head-groups are determined by free-energy minimization.

The surface head-groups of PAMAM dendrimers can be derivatized and this property will be used to obtain dendrimers mimicking phosphoinositide lipid head-groups.

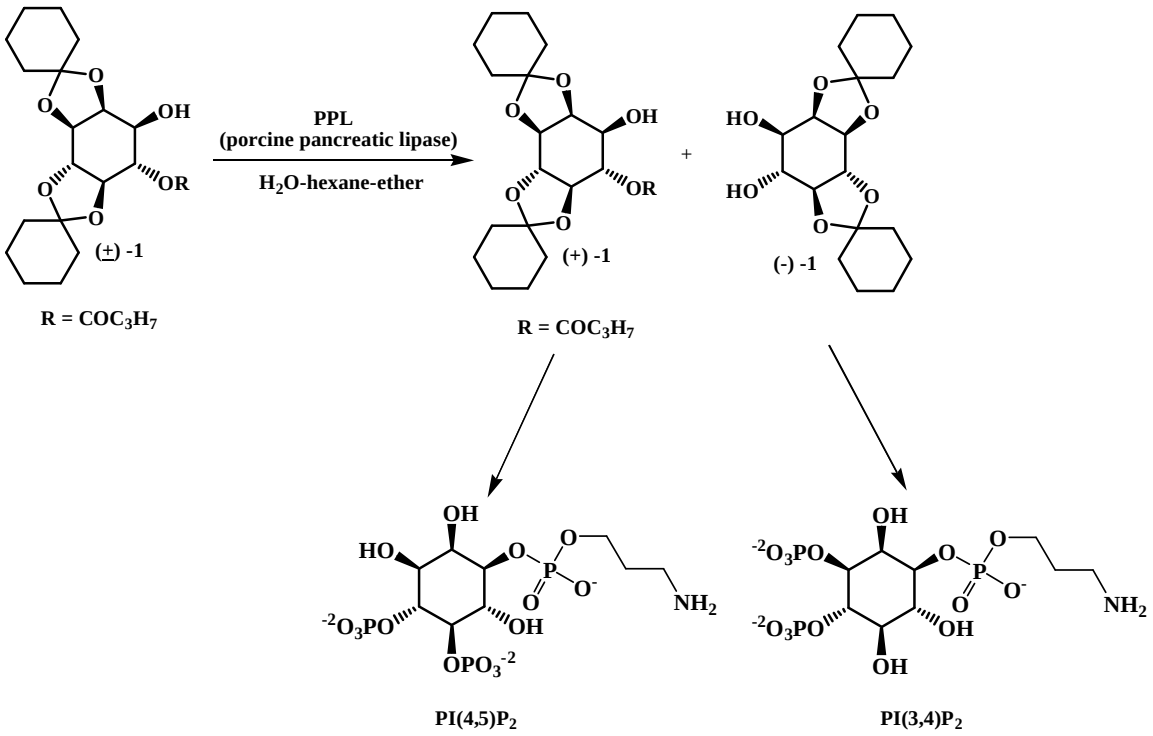
Among the phosphoinositides analogues of interest, it is the $PI(3,4)P_2$ analogue. The goal of the current research work will be the production of the $PI(3,4)P_2$ analogue by an appropriate derivatization of a PANAM dendrimer.

The chiral starting material necessary for the synthesis was prepared by Nichole Stewart at the Oakley laboratory. Firstly, the (±)-6-O-*Butyril*-1,2,4,5-di-O-*cyclohexylidene*-myo-*inositol* was obtained protecting inositol in the 1, 2, 4 and 5 positions[xxiv], following by its esterification at position 6 (**Scheme 2**).



Scheme 2. Obtainment of (±)-6-O-*Butyril*-1,2,4,5-di-O-*cyclohexylidene*-myo-*inositol*.

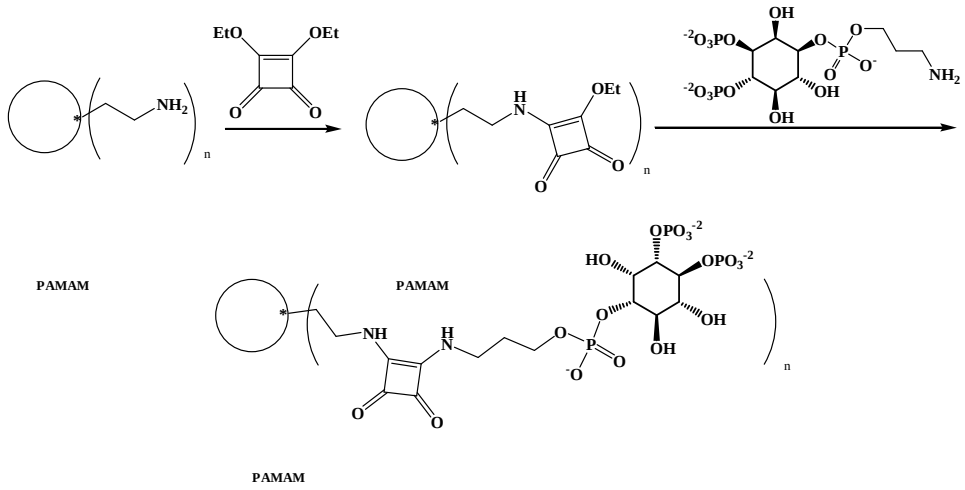
The butyril ester of the racemic mixture (±)-1, was subjected to enantioselective hydrolysis by porcine pancreatic lipase applying the procedure developed by Chen and co-workers[13], **Scheme 3**.



Scheme 3. Kinetic resolution of butyryl ester using porcine pancreatic lipase.

In **Scheme 3** is also shown that the (+)-1 enantiomer is the precursor of the $PI(4,5)P_2$ analogue that Sarah Webb synthetized at the Oakley laboatory. From the kinetic resolution, the (-)-1 enantiomer, which is the precursor for the $PI(3,4)P_2$ analogue, was also obtained. Because the enantiomeric material is very valuable, the current research will convert the racemic mixture into the $PI(3,4)P_2$ analogue as a preliminary step.

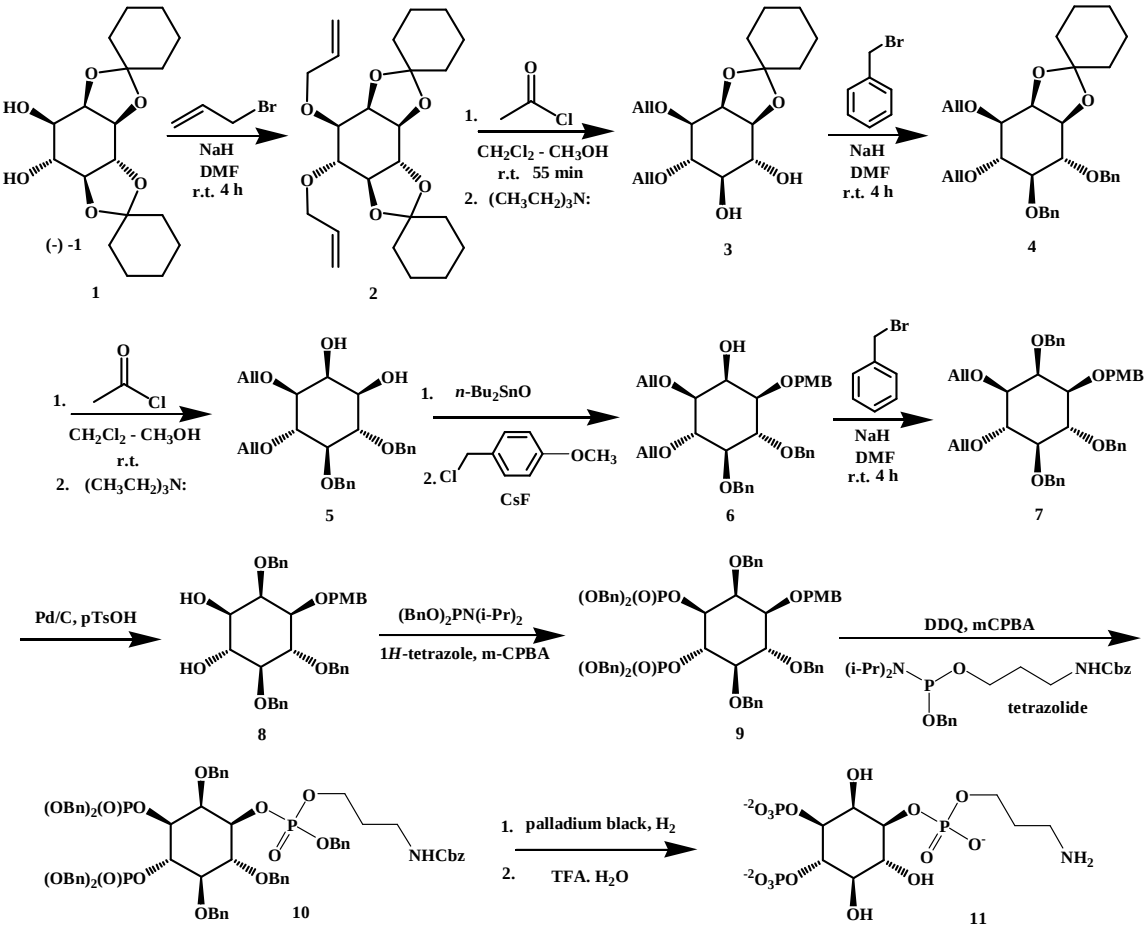
After obtaining the $PI(3,4)P_2$ head-group analogue, it will be reacted with the vinilogous amide ester dendrimer of choice using the reaction sequence that follows (**Scheme 4**).



Scheme 4. Synthesis of PI(3,4)P_2 micelle mimic.

The reaction sequence shown (**Scheme 4**) was used and optimized by Sarah Webb to obtain the PI(4,5)P_2 analogue as part of her research work to pursue the degree of Doctor in Philosophy[xxv].

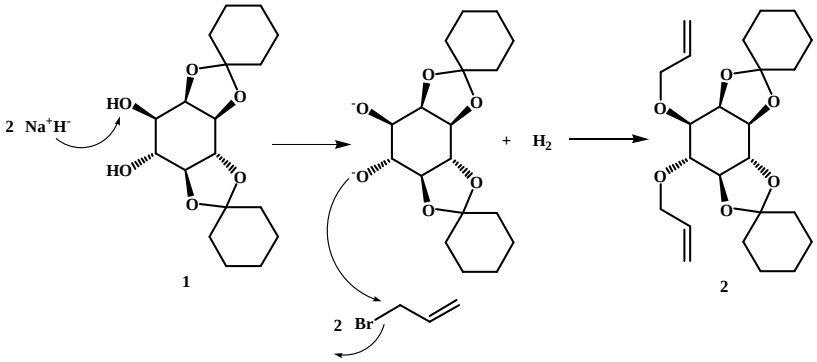
The tetherable head-group was proposed to be prepared using the synthetic route[xxvi] shown in **Scheme 5**.



Scheme 5. Synthetic route of PI(3,4)P_2 analogue.

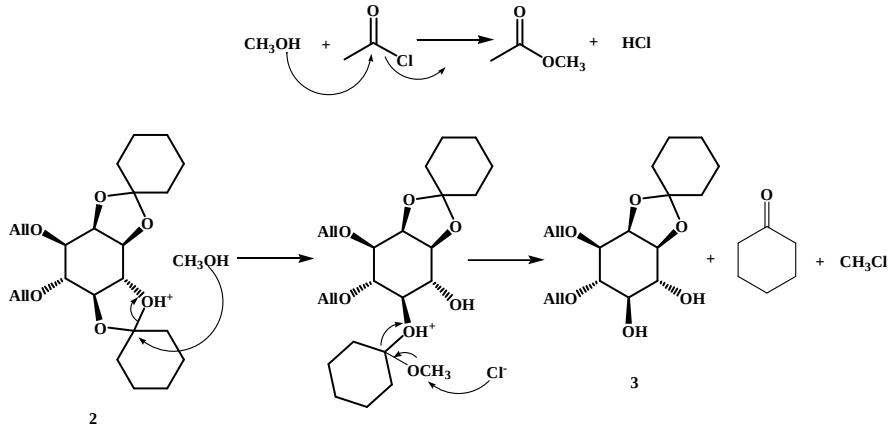
At the time this document was written compounds 2, 3, and 4 were synthesized. The mechanistic insights and experimental discussion will be focus on the synthesis of these molecules.

The obtainment of the racemic mixture of 3,4-di-O-*allyl*-1,2,5,6-di-O-*cyclohexylidene*-myo-*inositol*, compound 2, involves a S_N2 reaction type. The nucleophile is obtained by dehydrogenation of the OH groups with NaH as the base. Compound 2 is produced when the strong electrophile allyl bromide is attacked by this nucleophile.



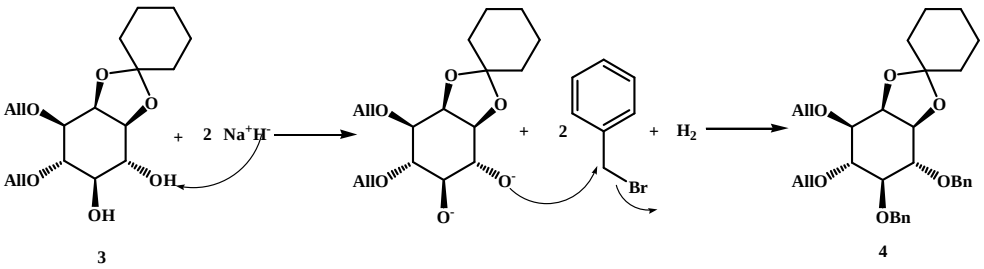
Scheme 6. Mechanism of the synthesis of 3,4-di-O-*allyl*-1,2,5,6-di-O-*cyclohexylidene*-myo-*inositol* (2).

Compound 3, the racemic mixture of 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol*, is obtained by catalytic acidic deprotection of the alcohol groups as shown in **Scheme 7**. The acidic media necessary for the reaction is produced catalytically by reaction of methanol with acetyl chloride. Triethylamine is used to quench the acidic mixture at the end of the reaction.



Scheme 7. Mechanism of the synthesis of 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol* (3).

Similarly to the obtainment of compound 2, the racemic mixture of 3,4-di-O-*allyl*-5,6-di-O-*benzyl*-1,2-di-O-*cyclohexylidene*-myo-*inositol*, compound 4, is obtained using NaH to generate the nucleophile which can attack the reactive benzyl bromide.



Scheme 8. Mechanism of the synthesis of 3,4-di-O-*allyl*-5,6-di-O-*benzyl*-1,2-di-O-*cyclohexylidene*-myo-*inositol* (4).

2. MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich.

The reactions were followed by thin layer chromatography (TLC) using silica gel 60 $F_{254.}$

Silica gel (Whatman-Fisher) for flash chromatography was 230-400 mesh (60 Å).

NMR spectra were collected using either a 300 (^{1}H) or 400 (^{1}H) MHz Varian Inova NMR spectrometer.

2.1. Synthesis of 3,4-di-O-*allyl*-1,2,5,6-di-O-*cyclohexylidene*-myo-*inositol* (2)

A solution of the racemic mixture 1 (0.5 g, 1.47 mmol) in N,N-dimethylformamide, (DMF) (3 mL) was treated with NaH 60% (0.352 g, 8.8mmol, 6 equiv.) in an ice bath (-72°C). The mixture was stirred for 15 min. Allyl bromide (0.45 mL, 5.13 mmol, 3.5 equiv.) was added at room temperature and the reaction was stirred under Ar atmosphere for 4 h. The excess of NaH was destroyed with methanol 0 °C. CH_2Cl_2 was added and the solution was washed with water and $NaCl_{(sat)}$. The organic layer was dried with $MgSO_4$,

and concentrated under reduced pressure. Column chromatography (hexanes:ethyl acetate, 10:0.85) of the residue gave 0.5 g of 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol* (80 % yield).

^{1}H-NMR in $CHCl_3$-d_1, δ (ppm): 1.37-1.72 (m, 20 H, 10 C**H**$_2$); 3.42 (dd, 1 H, C**H**); 3.63 (t, 1 H, C**H**); 3.75 (dd, 1 H, C**H**); 4.04-4.2 (m, 5 H, C**H** and 2 OC**H**$_2$); 4.29 (m, 1 H, C**H**); 4.35 (m, 1 H, C**H**); 5.14-5.18 (m, 2 H, C**H**$_2$=C); 5.25-5.32 (m, 2 H, C**H**$_2$=C); 5.84-5.95 (m, 2 H, 2 C=C**H**).

2.2. Synthesis of 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol* (3)

A solution of 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol* (0.05 g, 0.118 mmol) in CH_2Cl_2-CH_3OH (1:1, 1.4 mL) was stirred with acetyl chloride (0.0015 mL, 0.0213 mmol, 0.18 equiv.) at room temperature for 55 min. Triethylamine (0.049 mL, 0.335 mmol, 3 equiv.) was added and the solution was concentrated under reduced pressure. Column chromatography (hexanes:ethyl acetate, 0.8:1) of the residue gave 0.032 g of 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol* (80 % yield).

^{1}H-NMR in $CHCl_3$-d_1, δ (ppm): 1.22-1.75 (m, 10 H, 5 C**H**$_2$); 2.86 (s, 2 H, O**H**); 3.24-3.32 (m, 1 H, C**H**); 3.55-3.6 (m, 2 H, C**H**); 3.71 (dd, 1 H, C**H**); 3.966 (dd, 1 H, C**H**); 4.18-4.22 (m, 3 H, C**H** and OC**H**$_2$); 4.37-4.43 (m, 2 H, OC**H**$_2$); 5.14-5.19 (m, 2 H, C**H**$_2$=C); 5.25-5.31 (m, 2 H, C**H**$_2$=C); 5.86-6.0 (m, 2 H, 2 C=C**H**).

2.3. Synthesis of 3,4-di-O-*allyl*-5,6-di-O-*benzyl*-1,2-di-O-*cyclohexylidene*-myo-*inositol* (4)

A solution of 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol* (0.088 g, 0.258 mmol) in N,N-dimethylformamide (0.89 mL) was treated with NaH 60 % (0.062 g, 1.55 mmol, 6 equiv.) in an ice bath (-72°C). The mixture was stirred for 15 min. Benzyl bromide (0.109 mL, 0.904 mmol, 3.5 equiv.) was added at room temperature and the reaction was stirred under Ar atmosphere for 4 h. The excess of NaH was destroyed with methanol at 0 °C. CH_2Cl_2 was added and the solution was washed with water and $NaCl_{(sat).}$ The organic layer was dried with $MgSO_4$, and concentrated under reduced pressure. Column chromatography (hexanes:ethyl acetate, 5:0.8) of the residue gave 0.113 g of 3,4-di-O-*allyl*-5,6-di-O-*benzyl*-1,2-di-O-*cyclohexylidene*-myo-*inositol* (89% yield).

^{1}H-NMR in $CHCl_3$-d_1, δ (ppm): 1.1.58-1.71 (m, 10 H, 5 C**H**$_2$); 3.36 (dd, 1 H, C**H**); 3.58 (dd, 1 H, C**H**); 3.75 (dd, 2 H, C**H**); 4.14 (dd, 1 H, C**H**); 4.19-4.3 (m, 4 H, 2 OC**H**$_2$); 4.33-4.37 (m, 1 H, C**H**); 4.71-4.89 (m, 4 H, 2 OC**H**$_2$C$_6$H$_5$); 5.13-5.19 (m, 2 H, C**H**$_2$=C); 5.25-5.32 (m, 2 H, C**H**$_2$=C); 5.92-5.99 (m, 2 H, 2 C=C**H**); 7.23-7.35 (m, 10 H, 2 **C$_6$H$_5$**).

3. RESULTS AND DISCUSSION

There were not major complications in the obtainment of compounds 2, 3 and 4. These were obtained pure as their proton magnetic resonance spectra (^{1}H-NMR) revealed.

^{1}H-NMR spectrum of compound 2, (**Figure 4**) does not show the OH peak located at 2.59 ppm in the ^{1}H-NMR spectrum of the starting diol (**Figure 5**). However, compound 2 does have peaks located around 5.9 ppm which are not in the starting diol, and that correspond to hydrogens of C=CH functional group. These peaks together with the ones between 5.14 and 5.32 ppm revealed that the starting material was successfully allylated.

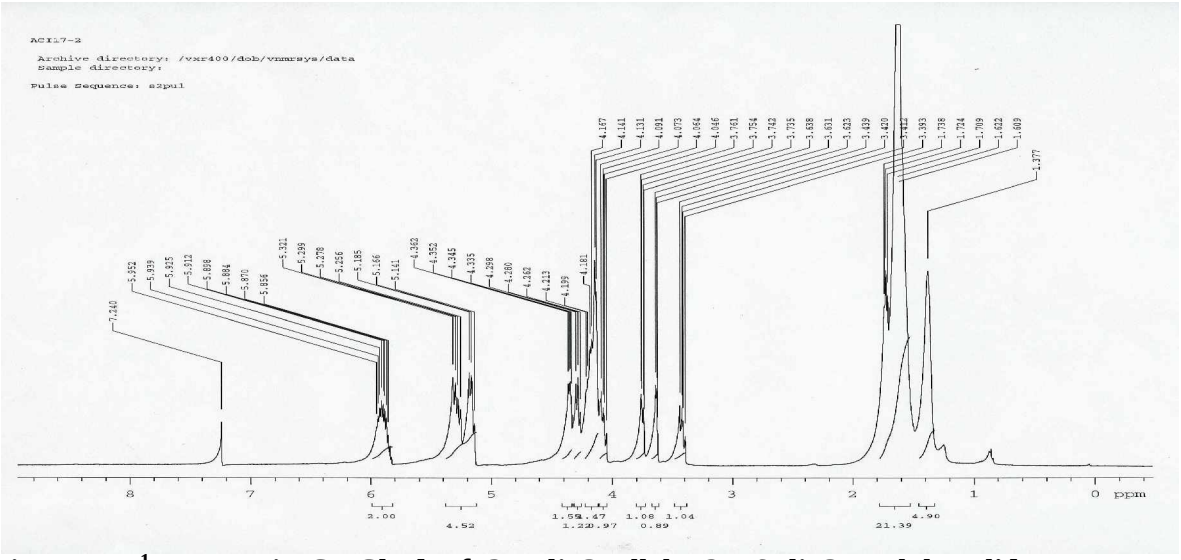


Figure 4. . ^{1}H-NMR in $CHCl_3$-d_1 of 3,4-di-O-*allyl*-1,2,5,6-di-O-*cyclohexylidene*-myo-*inositol* (compound 2).

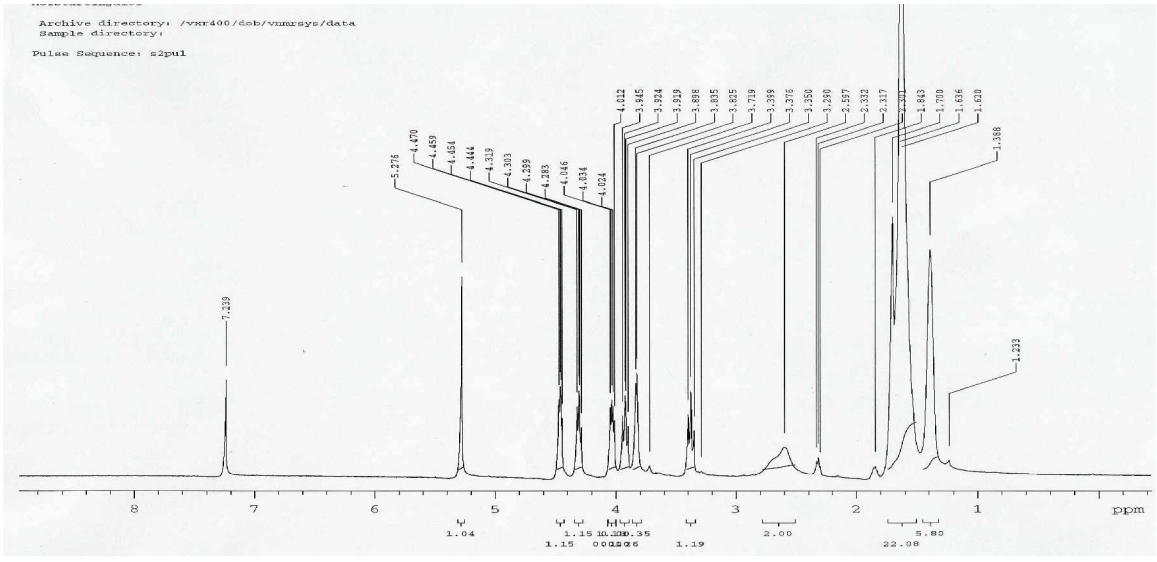


Figure 5. ^{1}H-NMR in $CHCl_3$-d_1 of starting diol (±)-1

^{1}H-NMR spectrum of compound 3 (**Figure 6**) shows a broad peak located at 2.86 ppm that corresponds to the OH groups of the deprotected molecule. In this spectrum it can also be seen that the intensity of the peaks located between 1.22 and 1.75 ppm are half the intensity of the similar peaks shown in the ^{1}H-NMR spectrum of compound 2 (**Figure 4**). These characteristics confirmed the obtainment of compound 3.

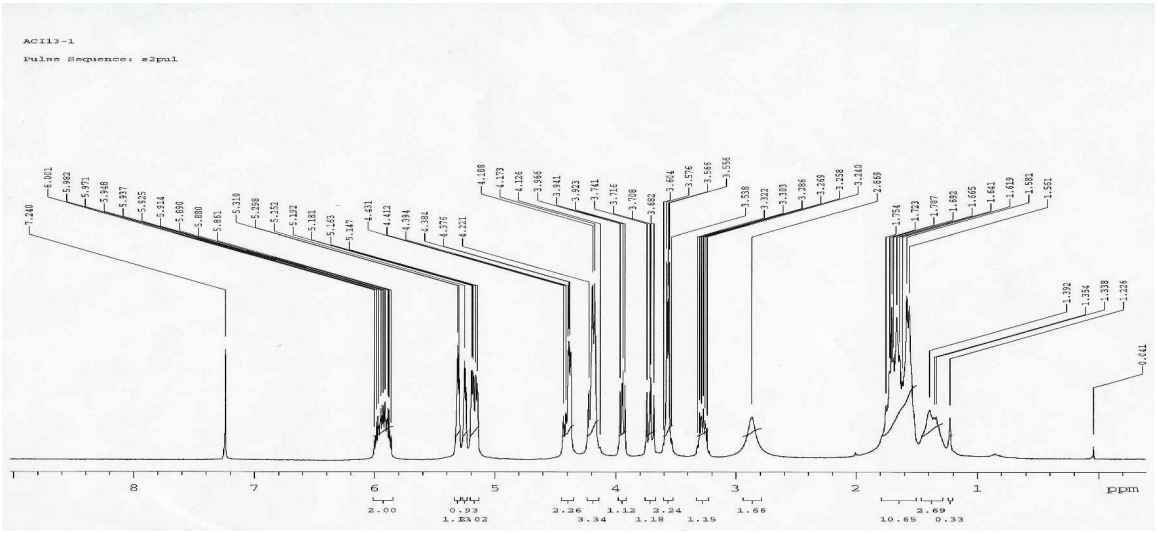


Figure 6. ^{1}H-NMR in $CHCl_3$-d_1 of 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol* (compound 3).

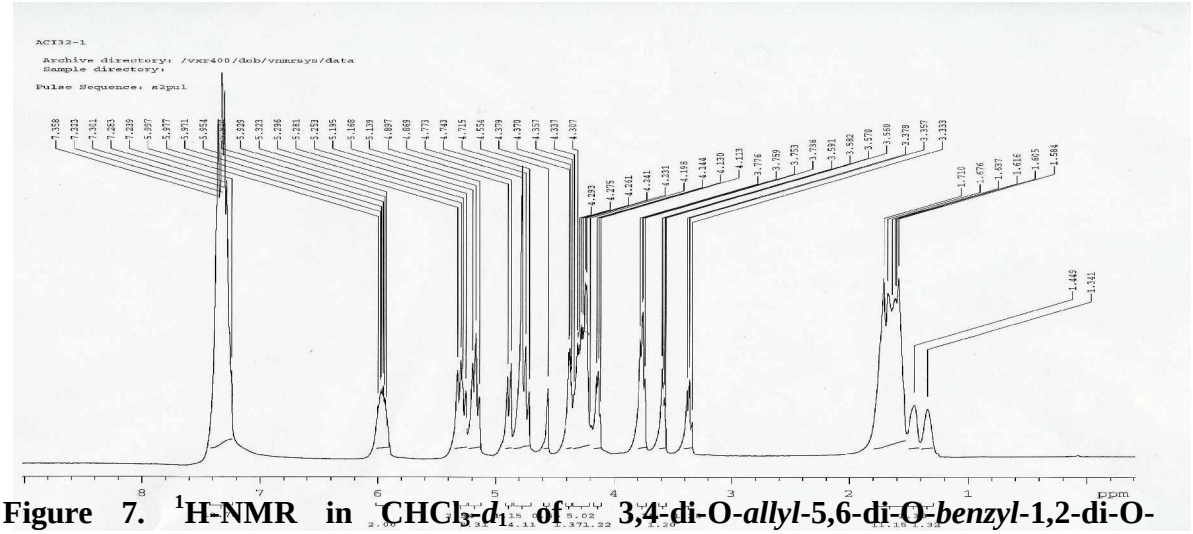


Figure 7. ^{1}H-NMR in $CHCl_3$-d_1 of 3,4-di-O-*allyl*-5,6-di-O-*benzyl*-1,2-di-O-*cyclohexylidene*-myo-*inositol* (compound 4).

Finally, the obtainment of compound 4 could be confirmed because its ^{1}H-NMR spectrum (**Figure 7**) did not show the peak corresponding to the OH groups that can be found in the spectrum of compound 3 (**Figure 6**), and also because it shows a multiplet at 7.23-7.35 ppm that correspond to phenyl hydrogens.

One of the main purposes of this work was to optimize the percentage yields of the reactions mentioned. Table 1 shows a summary of the reactions carried out.

Synthesis of compound 2			
	Starting Material (g)	Product (g)	% yield
Starting diol (+/-)-1	0.1	0.048	40
	0.5	0.158	26
	0.5	0.37	65
	0.5	0.5	80

Synthesis of compound 3			
	Starting Material (g)	Product (g)	% yield
Compound 2	0.05	0.0145	37
	0.05	0.0323	80
	0.2	0.0889	56
	0.34	0.16	60

Synthesis of compound 4			
	Starting Material (g)	Product (g)	% yield
Compound 3	0.088	0.113	89
	0.192	0.39	61
Noah Benjamin pg 70	0.32		

Table 1. Percentage yields achieved in the obtainment of compounds 2, 3 and 4.

Synthesis of compound 2 was done without major complications. The use of 6 equivalents of NaH was found to be necessary to obtain a better yield. The first two low percentages yields shown corresponds to spills during the work up of the reaction mixture, and problems during the purification of the product like the use of an unnecessary amount of silica.

Compound 3 was more difficult to obtain in good yields. The 4,5-cyclohexylidene is more susceptible to be deprotected by this method because of the conformational strain present in the 4,5 trans acetal. However, 1,2-cyclohexylidene group can also be deprotected but at a slower rate. In order to improve the yield, the reaction was carefully monitored by thin layer chromatography (TLC) and stopped when the fully deprotected product started to form. Distillation of the methanol and methylene chloride mixture prior the reaction could improve the yield. The presence of water could produce larger amounts of acidic protons, and consequently, it could allow the deprotection of the 1,2-cyclohexylidene group.

Compound 4 was obtained in good yield. 6 equivalents of NaH were also necessary to carry out the synthesis. 61% yield corresponds to the synthesis using not only compound 3 but also a significant amount of 3,4-di-O-*allyl*-6-O-*benzyl*-1,2-O-*cyclohexylidene*-myo-*inosoitol* produced by Noah Benjamin at the Oakley laboratory. Some fractions collected during the column chromatography, besides the ones that contained the pure product, revealed its presence among the fractions, suggesting that the percentage yield could have been greater.

4. CONCLUSIONS

Racemic mixture of 3,4-di-O-*allyl*-1,2,5,6-di-O-*cyclohexylidene*-myo-*inositol*, 3,4-di-O-*allyl*-1,2-O-*cyclohexylidene*-myo-*inositol*, and 3,4-di-O-*allyl*-5,6-di-O-*benzyl*-1,2-di-O-*cyclohexylidene*-myo-*inositol* were successfully obtained in good yields using the synthetic route proposed. These products were purified by flash chromatography and theirs structures identified by proton magnetic resonance spectrometry (^{1}H-NMR).

5. FUTURE WORK

In the near future, a bigger amount of each of the products already synthesized will be produced in order to continue with the rest of the steps specified in **Scheme 5**. All the products will be purified and characterized. Once each step have been improved to give a good yield the PI(3,4)P_2 analogue will be synthesize using the (-)-1 enantiomer from **Scheme 3**, and the surface of a PANAM dendrimer will be derivitized with it.

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