

To the Graduate Council:

I am submitting herewith a thesis written by Ashdeep Kaur Rajpal entitled "Design and Synthesis of Metabolically Stabilized Lipid Probes for the Investigation of Protein–Lipid Binding Interactions." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with a major in Chemistry.

Michael Best

Major Professor

We have read this thesis and
recommend its acceptance:

David Baker

Ziling (Ben) Xue

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records)

Design and Synthesis of Metabolically Stabilized Lipid Probes
for the Investigation of Protein–Lipid Binding Interactions

A Thesis Presented for

The Master of Science Degree

University of Tennessee, Knoxville

Ashdeep Kaur Rajpal

May 2011

Acknowledgements

It is a pleasure to thank those who made this thesis possible by providing constant support, guidance and valuable assistance. First and foremost, I would like to thank my husband for his unconditional love and support through these years. I would also like to thank my parents for all the sacrifices they have made and for standing by my side in all my life's decision. Without their support I would not have reached this far.

I am also grateful to Dr. Best for giving me the opportunity to be a part of his research group. Thank you for being so understanding and patient. Without your able guidance this thesis would not have been complete.

I am also grateful to my group members who in one way or another have contributed in the completion of my thesis. I would like to make a special reference to Heidi, who has helped me immensely from the day I joined the lab till now. Heidi you are an amazing person and a great friend. I would also like to thank Meng Meng for patiently answering my numerous questions. Chi-Linh, Leah and Cheema for never saying no whenever I needed their help. Thank you Chi-Linh for all your help before and during writing my thesis. Thanks Andrew and Ritu for providing me with assistance despite being new to the lab.

I would also like to thank my family and all my friends for always being there whenever I needed them.

Abstract

Protein–lipid binding interactions play crucial roles in various physiological and pathological processes, making it very important to study these interactions at the molecular level. However, investigation of these interactions is complicated by several issues, including the inherent complexity of membranes as well as the diverse mechanisms by which proteins interact with the membrane surfaces. As a result, many of these interactions remain poorly characterized.

Synthetic probes are useful tools employed for studying protein–lipid binding interactions. This thesis will detail the design and synthesis of metabolically stabilized analogues of various signaling lipids, which mimic the natural species and are not easily modified by enzymes present in biological systems. A modular approach is employed for synthesizing these lipid probes, giving access to a wide range of derivatized lipid probes that can then be used for several studies.

Although a wide variety of metabolically stabilized lipid analogues have been synthesized, their activity has not yet been characterized and quantified in detail. So, there is a great need to synthesize biologically active phosphorothioate and phosphonate analogues of various signaling lipids in order to properly characterize and compare the binding affinities and activity of these analogues. Synthesis of metabolically stabilized lipid analogue would take us one step closer towards understanding protein–lipid interactions in biological systems and in trying to find answers to the myriad of questions pertaining to these systems.

Table of Contents

<u>Chapter</u>	<u>Page</u>
Chapter 1: Introduction.....	1
Metabolically Stabilized Lipid Analogues	9
Chapter 2: Design and Synthesis of Metabolically Stabilized Phosphatidic Acid Analogues.....	14
Background and Significance.....	14
Metabolically Stabilized PA Analogues	17
Synthesis of Stabilized PA Lipid Analogues.....	19
Experimental	25
Chapter 3: Design and Synthesis of Azide-Labeled Diacylglycerol (DAG) Analogues with Ether Chains.....	37
Background and Significance.....	37
Synthesis of DAG Analogues with ether tails	40
Experimental	49
Chapter 4: Design and Synthesis of Metabolically Stabilized Phosphatidylinositol Phosphate (PIP_n) Analogues	60
Background and Significance.....	61
Metabolically Stabilized Analogs of PIP _n s	63
Synthesis of Metabolically Stabilized PI(3,4,5)P ₃ Analogue	66

Experimental	69
References	77
Appendix	
NMR Spectra.....	88
Vita.....	110

List of Figures

Figure 1.1. Lipid bilayers of cell membranes showing the general phospholipid	2
Figure 1.2. A peripheral protein targeting two signaling lipids via different binding	3
Figure 1.3. Bioorthogonal labeling of azide tagged lipid probe with reporter group	5
Figure 1.4. An illustration of ABPP using an ABP consisting of a reactive group,	7
Figure 1.5. Various Activity-Based Probes (ABPs).....	8
Figure 1.6. Various phosphomimetic groups.	10
Figure 1.7. Various metabolically stabilized lipid analogues.....	11
Figure 2.1. General structure of phosphatidic acid.	15
Figure 2.2. Synthesis and degradation of cellular PA by various enzymes. ⁶	18
Figure 2.3. Synthesis of phosphorothioate-stabilized PA probe attached to a	21
Figure 2.4. Migration of the <i>sn</i> -2 acyl chain upon removal of the <i>sn</i> -3 protecting	24
Figure 2.5. Modular synthetic scheme for the synthesis of metabolically stabilized	25
Figure 3.1. Structure of diacylglycerol molecule (DAG).....	38
Figure 3.2. Structure of azide-tagged DAG scaffold with ether chains, 42.	40
Figure 3.3. Use of click chemistry to attach reporter tags to the DAG scaffold.....	42

Figure 3.4. An example of Activity Based Protein Profiling (ABPP) using a bifunctional	42
Figure 3.5. A bifunctional lipid probe.	43
Figure 3.6. Alternate synthetic routes followed for the synthesis of azide-tagged DAG	44
Figure 3.7. Final synthetic scheme for azide-tagged DAG scaffold.	46
Figure 3.8. Synthetic scheme for y-shaped handle having benzophenone as	48
Figure 3.9. Coupling of DAG scaffold with amine at the <i>sn</i> -1 position, 43 to the acid ...	48
Figure 4.1. Various metabolically stabilized PIP _n s analogues.	65
Figure 4.2. Synthetic scheme for phosphorothioate PI(3,4,5)P ₃ headgroup analogue..	68

List of Abbreviations

ABPP	Activity Based Protein Profiling
ABPs	Activity Based probes
DAG	Diacylglycerol
LC-MS	Liquid Chromatography Mass Spectrometry
LPA	Lysophosphatidic Acid
PA	Phosphatidic Acid
PH	Pleckstrin Homology
PIP	Phosphoinositide Phosphate
PLD	Phospholipase D

Chapter 1: Introduction

Lipid bilayers that comprise the core of cellular membranes have highly polarized structures consisting of central non-polar hydrocarbon core regions and two polar flanking interfacial regions.¹ The interfacial regions consist of a complex mixture of water, phosphate groups, headgroups, and the polar portion of the acyl chains.² (Figure 1.1.)

The lipids comprising cellular membranes not only serve roles in controlling the structure and fluidity of the membrane, but also play critical role as signaling molecules and modifiers of membrane protein function.^{3,3b} Based on their function, lipids can be categorized into two types: (a) bulk lipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) that comprise for majority of the lipid composition and are responsible for providing structure to the cell membranes, and (b) signaling lipids, such as diacylglycerol (DAG),^{2,4,5} phosphatidic acid (PA),^{6,7,8} and the phosphoinositides (PIP_ns)^{6, 9,10} which are present in low percentages in cellular membranes, but are known to acts as key regulators in numerous biological processes.

One important role that signaling lipids play is their involvement as site-specific ligands for attachment of peripheral protein receptors onto the membrane surface, a process that generally regulates protein function either by controlling enzyme activity directly, or by localizing proteins to particular intracellular compartments where they

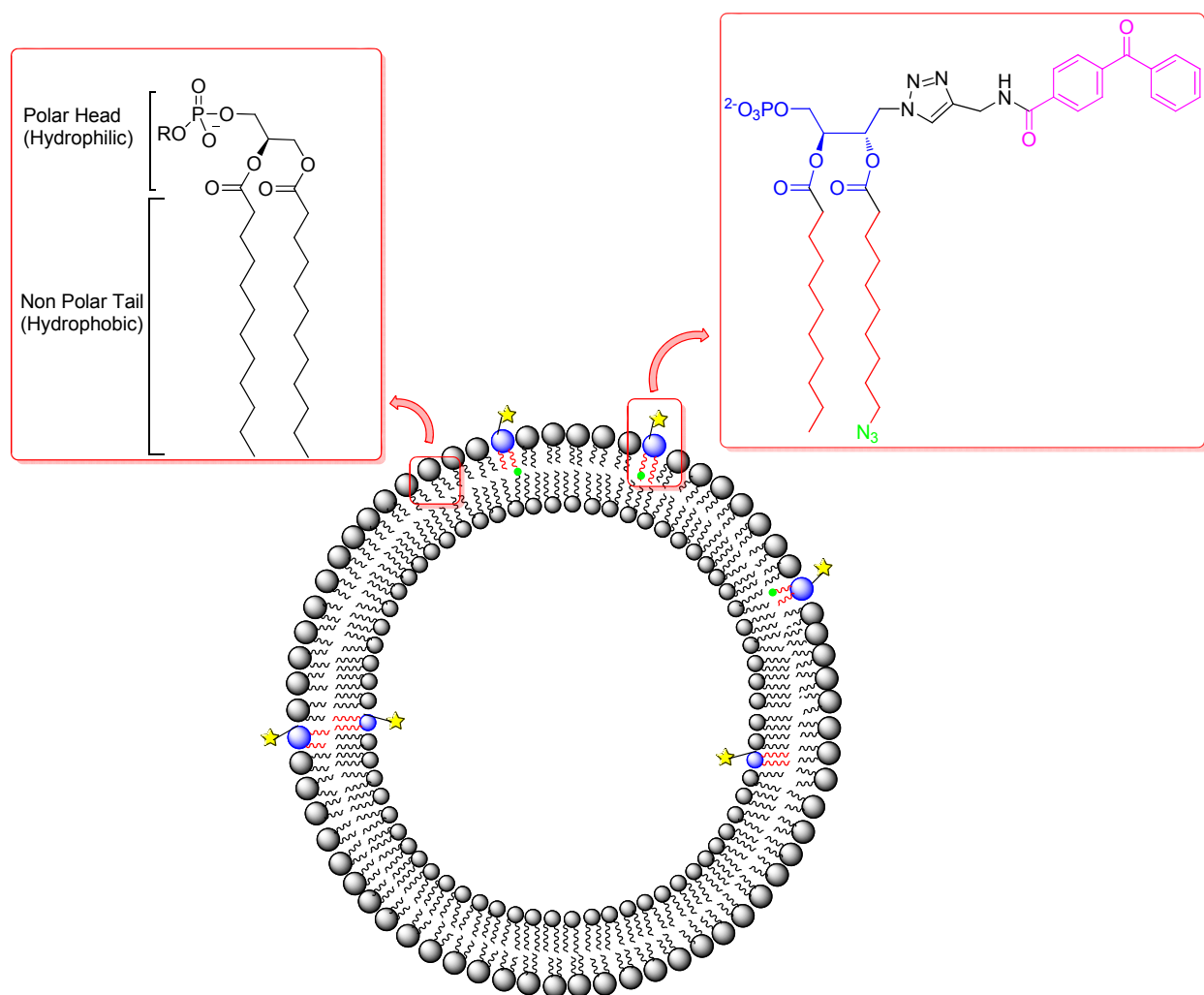


Figure 1.1. Lipid bilayers of cell membranes showing the general phospholipid structure and a PA lipid probe containing a photoaffinity tag.

perform a specialized role.^{2,5,11} A large number of peripheral proteins contain one or more modular domains specialized in lipid binding. These lipid-binding domains, including the C1, C2, PH, and PX domains, recognize specific lipid molecules in the membranes and achieve signal transduction and subcellular localization. (Figure 1.2.)

Such protein–lipid binding interactions play diverse roles in the regulation of protein trafficking, signaling and in disease onset. In order to fully understand the role these interactions play in essential biological processes, in cell signaling pathways, and in diseases such as cancer and diabetes, it becomes crucial to understand the nature of these interactions at the molecular level.

However, investigation of these interactions is complicated by several issues, including the inherent complexity of membranes due to their heterogeneous nature as well as the diverse mechanisms by which proteins interact with membrane surfaces. Lipids comprising the membrane are targeted by a diverse array of protein-binding

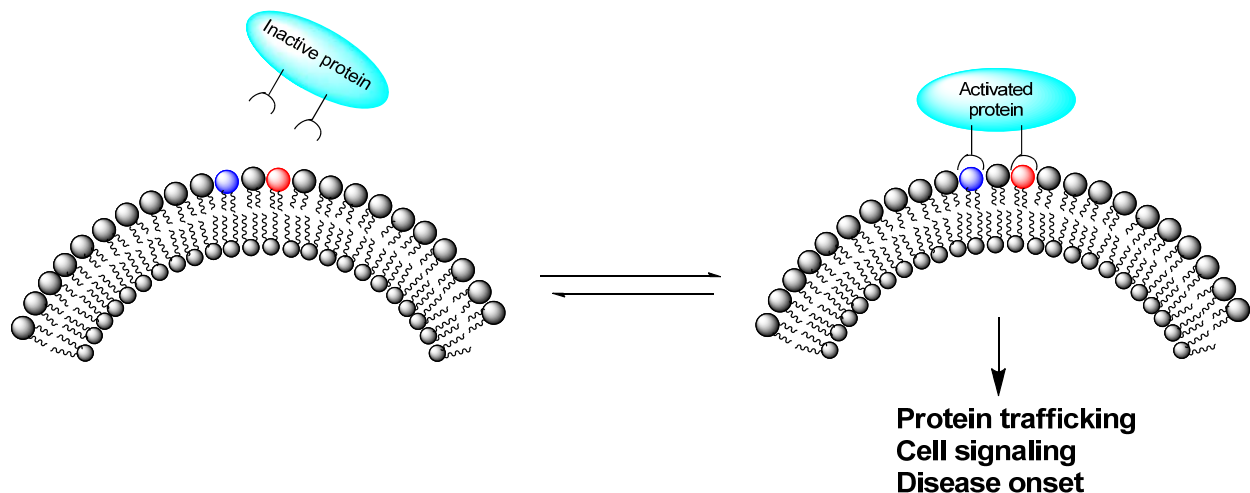


Figure 1.2. A peripheral protein targeting two signaling lipids via different binding domains.

modules, as is the case with PIP_ns which are known to bind to a wide array of proteins containing specialized PIP_n-binding modules, such as the PH,⁹ PX, FYVE, ENTH, ANTH, FERM, Tubby, and PROPPIN¹² domains.^{10, 13,14} Then there are variations even within the domain families as has been exemplified by investigations that indicate there are over 250 PH domains, indicating further complexity.¹⁴ PIP_ns affinities and specificities are also known to vary widely amongst the various PIP_n-binding modules based on the diversity in the binding mode, requiring the careful investigation of each distinct receptor with each PIP_n isomer.

Our approach for studying protein–lipid interactions employs the synthesis of lipid probes mimicking natural species that can be effectively modified at a later stage of synthesis giving access to a wide range of derivatized target probe structures. This approach is advantageous as it provides access to a range of probes that can be used for several studies.

Click chemistry is one such technique utilized for synthesizing derivatized lipid probes. The concept of click chemistry was proposed by Sharpless, Finn, and Kolb¹⁵ in 2001, which employed an innovative approach for the synthesis of target molecules. Instead of focusing on the formation of difficult bonds, their approach involved exploring those reactions that are inherently efficient at linking two components. Such reactions must be fast, efficient, high yielding and should occur under ambient conditions. They termed such reactions as “click reactions”. Since then, click reactions have been applied in diverse areas, including drug discovery,^{16,17} materials science,¹⁸ and chemical biology. In chemical biology, bioorthogonal click reactions have been used in the

selective labeling and detection of biomolecules such as protein,^{19,20} glycans,²¹ DNA, RNA^{22,23,24} and lipids^{25,26} in cell extracts and in living organisms. In order to take advantage of the click reactions for biomolecule labeling, one of the two bioorthogonal functional groups, usually an azide or alkyne, must first be introduced into the biomolecule. Next, reporter groups such as fluorophores or affinity tags are attached to an azide or alkyne and the copper-catalyzed click reaction is used to attach them to the biomolecule for labeling purposes. (Figure 1.3.)

It was in 2000 that Bertozzi and co-workers²⁷ first introduced azide as a functional handle for bioorthogonal click reactions. Since then, azide and alkyne groups have been extensively used in click reactions as their small size results in minor structural perturbation, and they possess high intrinsic reactivity, yet are quite selective in the manner in which they react. They are stable to water, acidic and basic conditions, yet reactive enough to efficiently undergo bioorthogonal 1,3-dipolar cycloaddition (“click” chemistry)²⁸ or Staudinger ligation.^{27, 29} All these factors render these reactions as invaluable tools for the labeling of biomolecules. Schultz and co-workers^{27, 30} recently

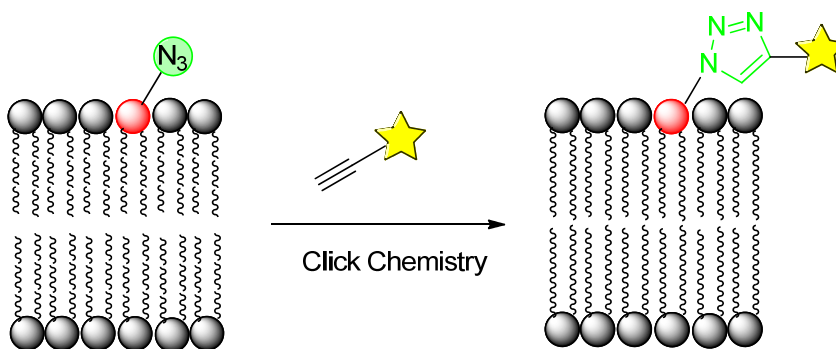


Figure 1.3. Bioorthogonal labeling of an azide tagged lipid probe with reporter group via click reaction.

carried out selective *in vivo* labeling and optical detection of phosphatidic acid probes bearing reactive tags. They attached a cyclooctyne moiety at the end of the *sn*-1 position of the acyl chain. The probe was introduced into the cell, and copper-free click chemistry was used to attach the reporter groups for selective *in vivo* labeling of the lipid probe.

Click chemistry can also be used for derivatization of lipid probes. This involves the synthesis of lipid scaffolds attached to an azide or alkyne tag, which can then be used for late-stage derivatization via bioorthogonal azide–alkyne click reaction. (Figure 1.3.) Our lab has successfully used this approach to derivatize azide-tagged PA probes. For this, click chemistry was utilized to introduce fluorescent reporters or benzophenone photoaffinity tags onto the azide-tagged PA probe to synthesize a wide array of lipid probes.²⁵ Synthesis of a bifunctional PA analogue attached to a benzophenone moiety and an azide moiety was also reported, which will be used for activity based protein profiling (ABPP) to purify, identify and characterize proteins that interact with the target lipid probes.

ABPP is an important approach that makes use of small molecules called activity based probes (ABPs) for specific labeling of the enzyme targets, biochemical profiling of diverse proteomes, and direct imaging and monitoring of enzyme activity in biological system. ABPs are designed to interact with active-site residues of enzymes resulting in the formation of a stable covalent bond, allowing for the enzyme adduct to be visualized or isolated and identified. An ABP is usually comprised of: 1) a reactive

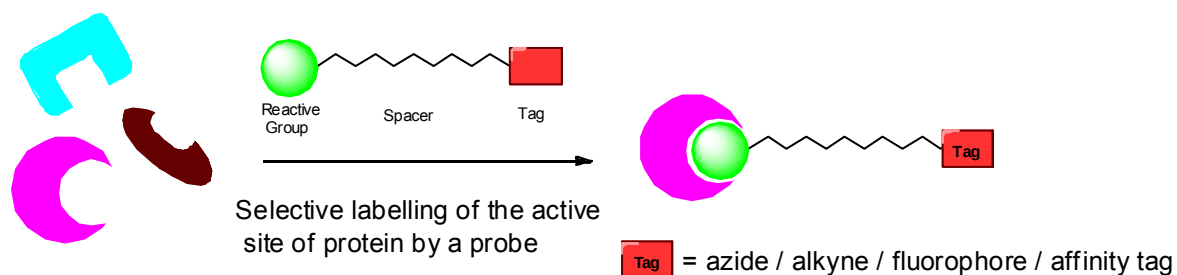
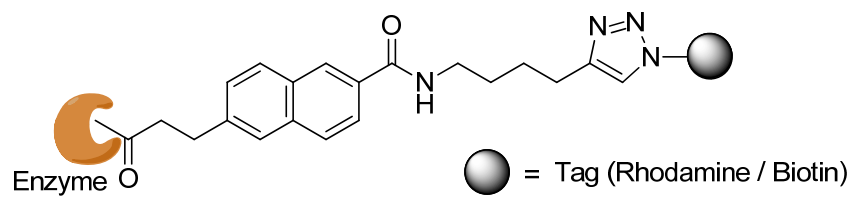


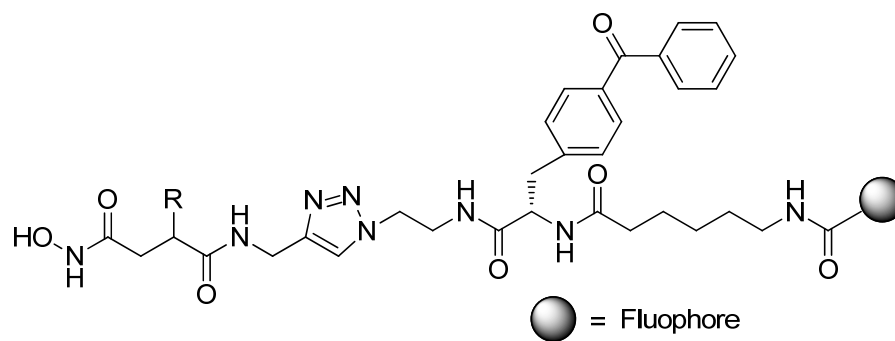
Figure 1.4. An illustration of ABPP using an ABP consisting of a reactive group, spacer and a tag.

group, which could be an electrophile or a photoactive group that attaches the probe to the enzyme active site, 2) a flexible linker/spacer that connects the reactive group with the reporter tag 3) a reporter tag, which could be a fluorophore for visualization or affinity tag like biotin for isolation, purification and characterization. (Figure 1.4.) In order to increase the versatility of ABPs, several groups have utilized synthetic handles such as azide and alkyne to incorporate reporter groups via click chemistry for post-labeling the proteins. An advantage of this approach is that it reduces the size of the ABP, making its incorporation into the cell easy since the ABP is no longer attached to the large fluorophores or affinity tags. Wright and Cravatt³¹ have synthesized ABPs having an alkyne handle. Once the probe was attached to the enzyme cytochrome P450 by reaction of the electrophilic reporter group with the nucleophilic residue of the active site, the alkyne handle was then used to attach rhodamine or biotin reporter tags both for *in vivo* and *in vitro* studies. (Figure 1.5.) Wang and co-workers³² have synthesized bifunctional ABPs for labeling metalloproteases, containing a benzophenone photocrosslinker and a rhodamine fluorophore.

Wright and Cravatt



Wang



Kroon

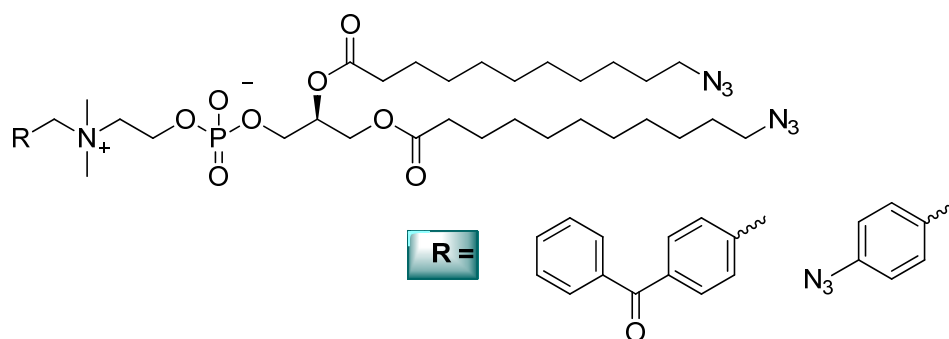


Figure 1.5. Various Activity-Based Probes (ABPs).

Kroon and co-workers³³ have synthesized bifunctional probes of phosphatidylcholine, making use of phenylazide or benzophenone as the photoactivatable group for covalent binding of the protein and an azide tag in the lipid tail to attach biotin or rhodamine for post labeling the protein in order to purify or visualize the proteins respectively. (Figure 1.5.)

Metabolically Stabilized Lipid Analogues

An important concern regarding the use of synthetic probes in biological systems is that these probes can be easily modified by various enzymes present in the biological systems so that the original probe is no longer being studied. There has been considerable amount of interest in synthesizing “metabolically stabilized analogues” where the labile structural elements of naturally occurring compounds are chemically stabilized. The phosphate group, an integral part of many important biomolecules such as nucleotides and phospholipids, is considered to be a very labile moiety and is susceptible to hydrolysis by several enzymes. Attempts have been made at making the phosphate group resistant to enzymes without greatly affecting the binding properties. In this regard, phosphorothioates were the first metabolically stabilized phosphomimetic group to be used to replace the phosphate group. This involves replacing P=O with a P=S bond in the phosphate group. (Figure 1.6.) Eckstein and co-workers³⁴ first introduced this group into nucleosides and found the corresponding analogues resistant to phosphate hydrolysis without greatly affecting the binding properties.³⁵

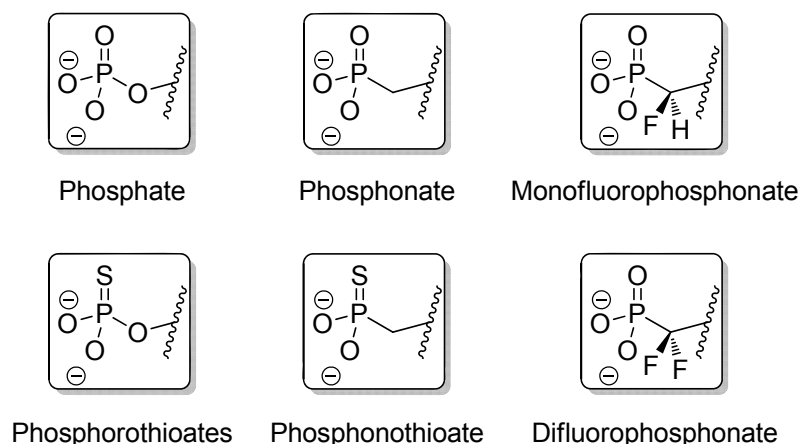
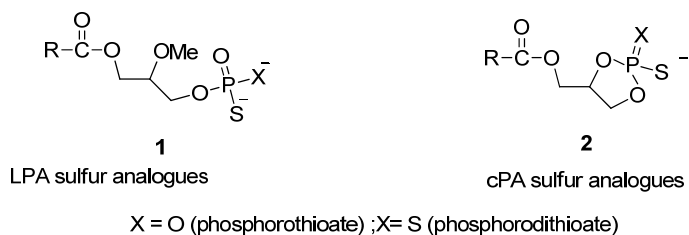


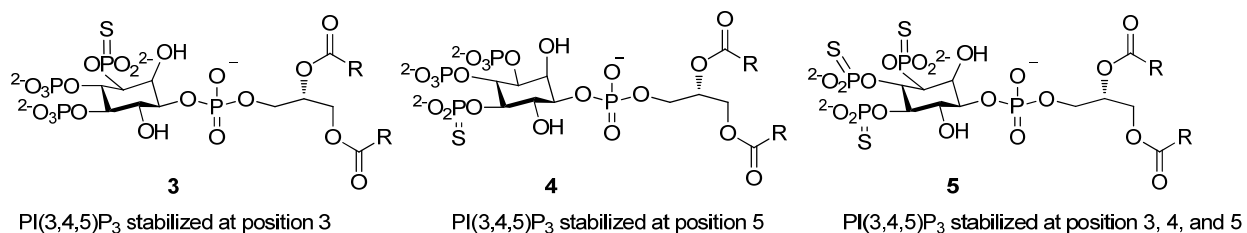
Figure 1.6. Various metabolically stabilized phosphomimetic groups.

Since then, interest in enzymatically resistant analogues has risen substantially, and many phosphorothioate analogues, particularly those of signaling lipids such as PIP_nS ,^{36,36b} LPA ^{37,38} and cPA ³⁷ have been synthesized. Okruszek and co-workers³⁷ have recently synthesized phosphorothioate/phosphorodithioate derivatives of lysophosphatidic acids (LPA) **1** and cyclic phosphatidic acids (cPA) **2**. (Figure 1.7) The preliminary experiments performed on unmodified phosphate and phosphorothioate cPA show both analogues to be stable after 48h incubation in thermally inactivated bovine fetal serum or in human serum. Prestwich and co-workers^{36b} have synthesized and studied the binding affinities of various phosphorothioate stabilized $\text{PI}(3,4,5)\text{P}_3$ towards the pH domain containing protein GRP. These metabolically stabilized $\text{PI}(3,4,5)\text{P}_3$ analogues have phosphorothioate groups in place of the phosphate group at position 3, position 5, and position 3, 4 and 5. (Figure 1.7. structure **3**, **4** and **5** respectively)

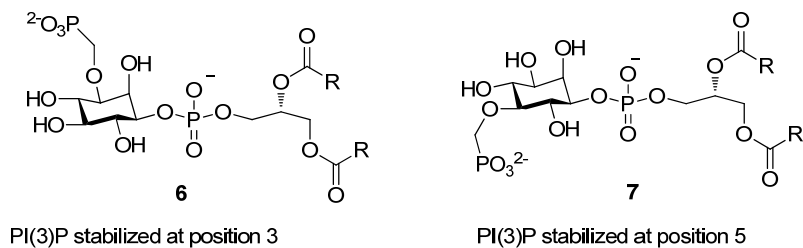
Phosphorothioate analogues of LPA and cPA



Phosphorothioate analogues of PI(3,4,5)P₃



Methylene Phosphonate analogues of PIP_ns



Fuoro and difluoromethylene phosphonates of LPA and cPA

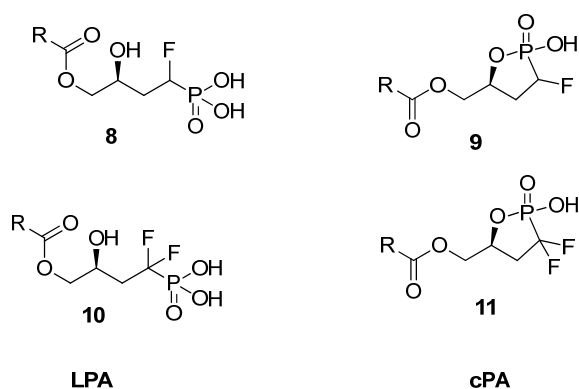


Figure 1.7. Various metabolically stabilized lipid analogues.

They observed that while the 3-, and 5- stabilized phosphorothioate showed equivalent binding to unmodified PI(3,4,5)P₃, the 3,4,5 stabilized phosphorothioate showed reduced binding affinities. This was mainly attributed to the modified pK_a and decreased hydrogen-bonding ability of phosphorothioate compared to phosphate.³⁹

Another phosphomimetic group used to replace the natural phosphates is the phosphonate group. Phosphonate analogues are formed by replacing the bridging oxygen or one of the phosphate oxygens with methylene or methyl groups. (Figure 1.6.) Prestwich and co-workers have synthesized methylphosphonate analogues as potentially useful phosphatase-resistant ligands for various PIP_ns, such as phosphatidylinositol 3-phosphate PI(3)P **6**,⁴⁰ phosphatidylinositol 5-phosphate PI(5)P **7**,⁴¹ and phosphatidylinositol 3,4,5-trisphosphate PI(3,4,5)P₃.⁴² Prestwich and co-workers^{36b} studied the binding affinities of different PI(3,4,5)P₃ and observed that these compounds also exhibited weaker binding to receptor domains when compared to natural PI(3,4,5)P₃. This was attributed to the modified second pK_a value of phosphonates compared to natural phosphates.

In order to improve the binding affinities of phosphonates, fluoromethylene phosphonates can also be used to replace the phosphate group. Fluoromethylene phosphonates have a second pK_a value similar to that of natural phosphonate because of the presence of the electron- withdrawing fluorine group that decreases the pK_a.⁴³ The fluoromethylene phosphonate group has also been successfully incorporated in PIP_ns, PA and LPA to produce biologically active analogues. Fluoromethylene phosphonates of LPA and cPA have been synthesized, and their agonist and antagonist

activity studied.⁴³ (Figure 1.7.) The acyclic α -fluoromethylene phosphonate of LPA **8** was found to be a potent LPA 3 agonist, while its cyclic analogue **9** (cPA) was found to be a selective LPA_{1/3} antagonist. Also, upon cyclization to **11** the inactive acyclic α,α -difluoromethylene phosphonate **10** became a weak LPA _{1/2/3} agonist. Prestwich and co-workers⁴⁴ have also synthesized monofluorinated and difluorinated PA phosphonate analogues that are resistant to the phosphatase activity and have been found to stimulate mTOR signaling pathways similar to PA in quiescent HEK 293 cells.

Due to the crucial role that protein–lipid binding interactions play in various physiological and pathological processes, it is very important to study these interactions at the molecular level. Synthetic probes are useful tools that can be employed for labeling and identifying various protein receptors involved in disease onset. Although a wide variety of metabolically stabilized lipid analogues have been synthesized, their activity has not yet been characterized and quantified in detail. So, there is a great need to synthesize metabolically stabilized analogues of various signaling lipids in order to properly characterize and compare the binding affinities and activity of these analogues. Synthesis of metabolically stabilized lipid analogue would help us in understanding protein–lipid interaction in the biological systems and in trying to find answers to the myriad of questions pertaining to these systems.

Chapter 2: Design and Synthesis of Metabolically Stabilized Phosphatidic Acid Analogues

Background and significance

Phosphatidic acid (PA) is an important signaling lipid that is present in small amounts in cell membranes but plays a crucial role in various physiological and pathological processes. Some important physiological functions include regulation of protein and lipid phosphorylation,^{45,46} membrane trafficking,^{47,6} and signal transduction.⁴⁸ PA can act as a site-specific ligand that is specifically recognized by various protein receptors and plays an important role in cell signaling by regulating the function and subcellular localization of bound proteins. PA binds certain protein receptors including Raf-1 kinase⁴⁹ and certain isoforms of the protein kinase C (PKC) family, such as PKC α ⁵⁰ and PKC ϵ ⁵¹ that have been known to play vital roles in carcinogenesis. As such, it is very important to study protein–PA binding interactions at the molecular level.

The chemical structure of PA consists of glycerol, to which two acyl chains and an anionic phosphate group are attached at the *sn*-1, *sn*-2 and *sn*-3 positions, respectively. (Figure 2.1.) It is this phosphate headgroup that is the defining feature of PA and sets it apart from other phospholipids. B. de Kruijff and co-workers⁵² have presented an electrostatic hydrogen bond switch mechanism to explain the specificity of PA toward binding. They showed that the monoprotonated PA is initially stabilized by an

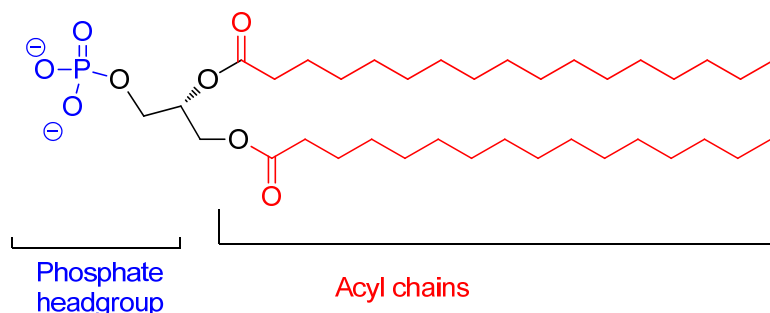


Figure 2.1. General structure of phosphatidic acid.

intramolecular hydrogen bond. The proteins and peptides having basic residues specifically associate to the membrane via electrostatic attraction. This is followed by hydrogen-bond formation between the receptor and PA headgroup, resulting in full phosphate deprotonation (-2 charge). This charge enhances association and provides binding specificity. The shape of PA is also believed to contribute to binding specificity. At physiological pH, PA exists as a cone-shaped structure. This results in the placement of the phosphate headgroup in the mildly polar interfacial region.⁵³ This is believed to enhance the penetration of hydrophobic residues into the membrane, resulting in improved binding. Thus, the specific placement of PA in the membrane and the ionization properties of this phosphomonoester headgroup result in the specificity of PA–protein interaction.

Recent studies have suggested that PA directly regulates the activities of some important signaling molecules, such as mTOR,⁵⁴ Ras,⁵⁵ neurogranin,⁵⁶ and enzymes like PIP₅-kinase,⁵⁷ suggesting that PA is an important signaling lipid in cells. Many of these functions are mediated by the direct interaction of PA with specific target

proteins. There has been a considerable amount of interest in identifying proteins that interact with PA. A number of PA-binding proteins have been identified, which include protein kinases,⁵⁷ protein phosphatases,^{58,59} and cAMP-specific phosphodiesterases.^{60,61}

Interestingly, when one compares the PA binding region of the receptor proteins, no consensus sequence common to the PA binding proteins can be found except for one feature that is present in most PA-binding domains, which is the presence of basic amino acid residues^{8,14} such as arginine, lysine or tryptophan that specifically bind to PA. Since structural approaches have not been very successful in the discovery of PA-binding receptors and the PA binding domains, synthetic approaches can play a crucial role in PA–protein binding interactions. Here, synthetic probes can be used for gaining important information about the precise spatial and temporal regulation of PA signaling. Incorporation of the reporter molecules with the lipid probes would facilitate in mapping the protein receptors and the binding domains that specifically bind to PA. This could possibly lead to the identification of some type of common sequence homology in the binding domains. It will also allow us to understand how PA-binding proteins specifically recognize PA amongst other anionic lipids.

Towards this goal, a large number of PA probes have been synthesized so far. Ktistakis and co-workers^{62,63} have synthesized analogues of PA and immobilized them onto a solid support by means of an amino function attached at the end of the acyl tail at the *sn*-1 position of the glycerol moiety. PA-modified resin was then used as affinity matrices, to purify and identify a number of known proteins as well as some novel

proteins that bound specifically to PA. Nemoz and co-workers⁶⁴ have synthesized PA analogues bearing a photoactivable group, 4-azidotetrafluorobenzylacyl either at the *sn*-1 or *sn*-2 position of the fatty acyl chain. Upon UV irradiation, these polyfluorinated aryl azides can specifically and covalently link to the neighboring amino acid residues on the phosphodiesterases (PDE) peptide chain resulting in covalent labeling of the PDE enzymes. The analogues so obtained retain the ability of PA to activate the cAMP-hydrolyzing enzyme PDE4D3 to varying degrees. Neef and Schultz³⁰ recently developed a bifunctional PA probe with cyclooctyne incorporated at the end of the acyl tail at the *sn*-1 position. Once the probe was incorporated into living cells, the cyclooctyne was used to attach a fluorophore to the PA probe by means of copper-free click chemistry resulting in the labeling of the PA probe within the cell.

Metabolically Stabilized PA Analogues

Cellular PA may be produced and degraded by several enzymes present in the living systems. These enzymes are subject to complex and tight regulation, and the timing of their production regulates the potential pathway that will be followed for PA production and degradation. PA can be formed by various pathways, such as hydrolysis of membrane phospholipids by PLD; phosphorylation of DAG by DGK; acyl transferase, to LPA; and from G3P and DHAP. (Figure 2.2.) PLD and DGK are the two principal enzymes that produce signaling PA.^{65,66}

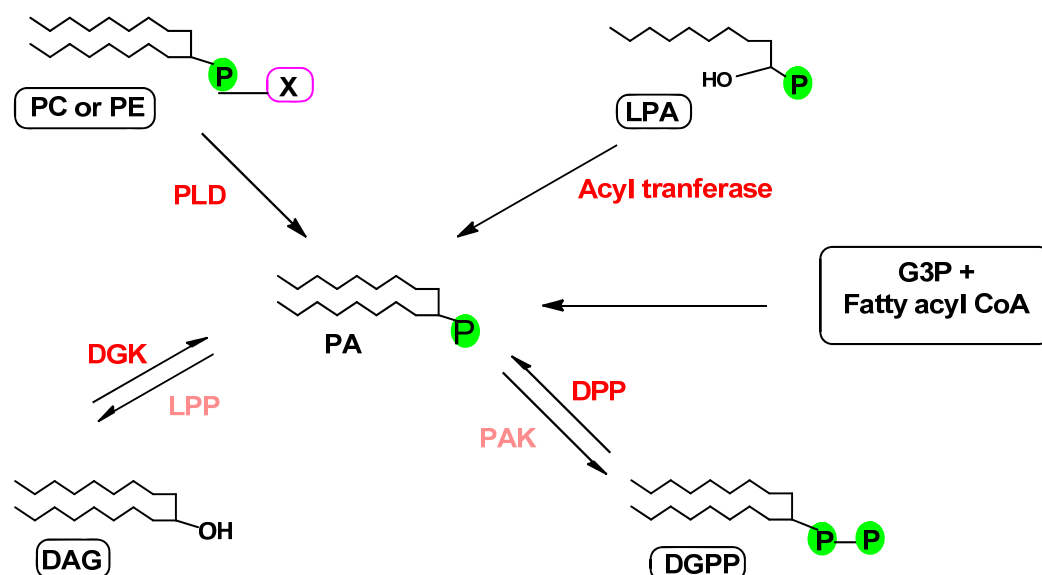


Figure 2.2. Synthesis and degradation of cellular PA by various enzymes.⁶

PA is known to be metabolically labile and is susceptible to hydrolysis by several enzymes that can hydrolyze the phosphate group and the ester groups present in PA. It is possible to make PA metabolically stabilized in order to make it enzymatically resistant and to prolong its biological activity. This can be achieved by (a) the replacement of the acyl ester lipid chains with alkyl ether chains,⁶⁷ and (b) by the strategic replacement of the phosphate group at the *sn*-3 position into non-hydrolyzable phosphomimetic groups.^{44,68} The common types of phosphomimetic functional groups that are currently being used include phosphonates (methylene phosphonate, fluoromethylene phosphonate, phosphonothioate) and the phosphorothioates.

There has been great interest in synthesizing metabolically stabilized lipid analogues of PA that provide it enzymatic resistance without greatly affecting its binding

properties. Interest in enzymatically resistant lipid analogues has risen substantially with the synthesis of a large number of metabolically stabilized lipid analogues for various signaling lipids, such as PIP_{ns},^{36,36b} cPA,^{37,69} and LPA.^{37,38} Recently, this approach has been extended to synthesizing metabolically stabilized PA analogues. In this regard, Prestwich and co-workers⁴⁴ have synthesized monofluorinated and difluorinated PA phosphonate analogues that are resistant to the phosphatase activity and have been found to stimulate mTOR signaling pathways similar to PA in quiescent HEK 293 cells. The difluoromethylene PA analogue was also found to act as an inhibitor of LPP1 activity, potentiate platelet aggregation and shape change responses to LPA and amplify LPA production by agonist-simulated platelets.⁶⁸

In conclusion, PA is an important signaling lipid and plays key roles in cell growth and protein trafficking via protein binding. Therefore, PA–protein binding interactions need to be probed further in order to discover PA binding receptors and to understand the specificity of these receptors towards PA. As such chemical probes would play a crucial role in understanding PA–protein interactions at the molecular level.

Synthesis of Stabilized PA Lipid Analogues

Our strategy for synthesizing metabolically stabilized PA analogues started with the introduction of a phosphorothioate in place of a phosphate group at the *sn*-3 position. Phosphorothioate groups have previously been shown to be resistant to phosphate hydrolysis with minimal effect on binding properties.³⁶ The only modification involved in the synthesis of phosphorothioates analogues of PA is the introduction of a

protected phosphorothioate rather than a protected phosphate, such that the same synthetic route used for the synthesis of regular PA probes can be utilized. The strategy adopted by our group for the PA lipid probe design involves the synthesis of modular PA scaffolds bearing an azide group at the *sn*-1 position, which can be functionalized with various reporter groups at a late stage via click chemistry to access a range of reporter group tagged lipid probes. We attempted to make use of the same strategy for the synthesis of phosphorothioate analogues of PA. This involved the synthesis of a modular PA scaffold having a protected phosphorothiolates group at the *sn*-3 position and an azide tag at the *sn*-1 position that was used to attach a reporter tag via bioorthogonal azide–alkyne click reaction. The fluorophores help in studying protein–lipid binding interactions by acting as Förster resonance energy transfer (FRET) pairs. FRET is a radiationless energy transfer from an excited donor molecule to an acceptor molecule through long-range (10–100 Å) dipole–dipole couplings.⁷⁰ Therefore, for FRET to occur, the donor and acceptor should be present in close proximity as it is highly dependent upon the distance between them. The closer the donor is to the acceptor, the more FRET is observed and vice versa. Thus, FRET is a useful technique for detecting changes in proximity between the donor and acceptor.

The final synthesis, shown in figure **2.3.**, first involved the protection of the diol of the commercially available diethyl L-tartrate **12** using cyclopentylidene to give **13**. The esters were then reduced using lithium aluminum hydride to form **14**.⁷¹ Compound **14**

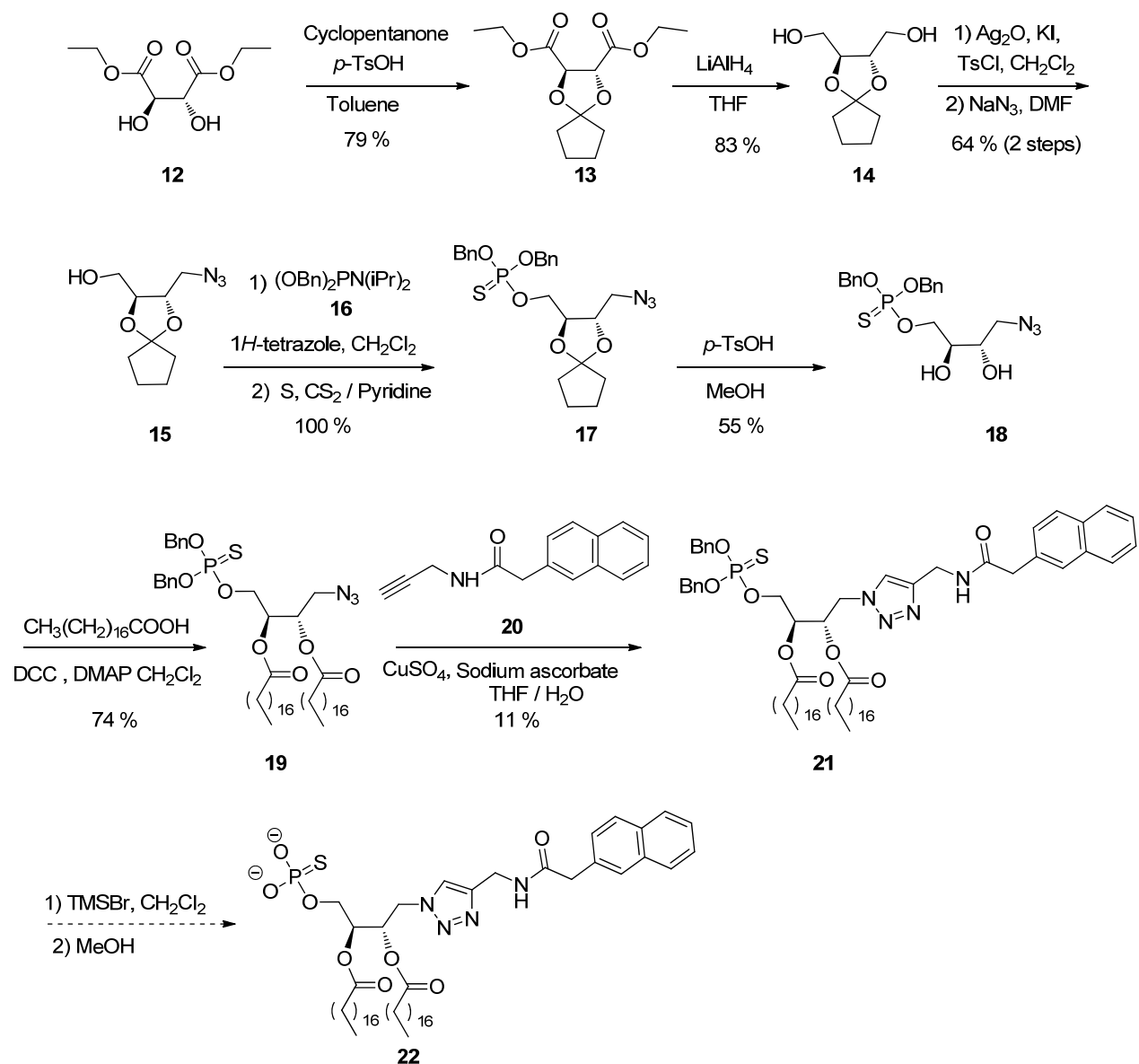


Figure 2.3. Synthesis of phosphorothioate-stabilized PA probe attached to a fluorescent tag.

was then selectively mono-tosylated, followed by its substitution with azide^{71b, 72} to furnish **15**. This was followed by the installation of a protected phosphorothioate group at the *sn*-3 using dibenzyl diisopropylphosphoramidite **16** and sulfur to afford **17**.⁷³ The cyclopentylidene acetal was then removed under acidic conditions using *p*-toluenesulfonic acid to yield diol **18**. The ester tails were then coupled on to the diol to furnish protected phosphorothioates PA scaffold **19**.

Once the protected PA scaffold **19** was synthesized, we now sought to install a reporter group to the core scaffold utilizing the bioorthogonal click reaction between the azide-tagged PA scaffold and naphthyl alkyne **20** in order to obtain a phosphorothioate PA probe. We encountered problems with the click reaction, which proceeded in very low yields. We unsuccessfully attempted to improve the yield either by using a polytriazole ligand (synthesized by Meng M. Rowland), known to act as a copper(I)-stabilizing ligand in catalysis and protect it from oxidation and disproportionation, while enhancing its catalytic activity,⁷⁴ or by using copper fluoride as an alternative reagent for the click reaction. We then went back to the original conditions employed for the click reaction. After functionalizing the protected phosphorothioate PA scaffold with a fluorophore to yield **21**, we then tried to deprotect the benzyl groups on the protected phosphorothioate moiety with bromotrimethylsilane (TMSBr) to produce PA probe **22**. In the case of phosphorothioates, it was observed that deprotection by TMSBr never went to completion even after prolonged reaction times. There have been other similar reports where it has been observed that TMSBr is not a good deprotecting reagent for the benzyl groups in case of the phosphorothioates.^{36a}

These problems made us rethink our strategy for synthesizing metabolically stabilized PA probes. Instead of focusing on one kind of phosphomimetic group, which would require that each probe be synthesized in an individual manner, we started out with designing a synthetic strategy that would allow us to access a wide range of metabolically stabilized PA probes from a common scaffold. Many metabolically stabilized lipid analogues have been synthesized, but their activity and binding properties have not yet been quantified.

In order to study and compare various phosphomimetic groups, we first synthesized a very simple DAG scaffold without an azide tag, which was then derivatized to form various PA analogues. This was done to simplify the synthesis of the PA analogues. We also replaced the ester-linked acyl tails of PA with the ether-linked tails. This change solved some of the problems that we had faced while designing a modular approach for PA probe synthesis that involved installing a phosphate group onto the core DAG scaffold at a late stage of synthesis. Earlier, in our lab, attempts have been made based on this approach, but these were not successful because of the *sn*-2 to *sn*-3 acyl migration that was observed in the final deprotection step of the DAG synthesis and while installing the phosphate head groups.^{26,25} (Figure 2.4.) The migration is known to be catalyzed via both acid and base mechanisms, as well as being facilitated by prolonged exposure to silica gel.⁷⁵ Also, since the removal of common lipid protecting groups such as PMB and TBDPS occur via basic mechanisms, it further promotes the migration.

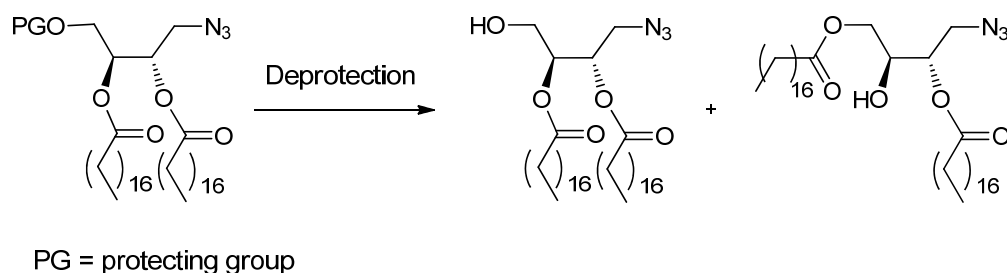


Figure 2.4. Migration of the *sn*-2 acyl chain upon removal of the *sn*-3 protecting group in an azide tagged DAG scaffold.

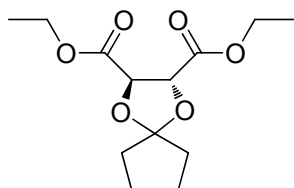
Using a new approach that involves replacing of acyl tails with the ether tails, the problem of acyl migration can be altogether avoided. This approach allows us to access a wide range of PA probes simply by derivatizing the DAG having ether tails at a later stage with various metabolically stabilized phosphomimetic groups such as methylene phosphonates, fluoromethylene phosphonates, and phosphorothioates. Another advantage of using the ether tails instead of acyl tails is that they make the PA analogues resistant to lipases that specifically hydrolyze the esters of the acyl tails.

As show in Figure 2.5., the synthesis of DAG scaffold **27** with ether tails begins with the protection of the hydroxyl group of commercially available (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol **23**, with a *p*-methoxybenzyl (PMB) group to obtain **24**. Next, the acetonide group is deprotected under acidic conditions to give **25**. The ether tails were then introduced using 1-bromooctadecane and tetrabutylammonium iodide (TBAI) as a catalyst yielding **26**.⁷⁶

were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using a Bruker AC250 spectrometer updated with a TecMag data collection system, as a Varian Mercury 300 spectrometer. Mass spectra were obtained with a JEOL DART-AccuTOF spectrometer with high-resolution capabilities. Optical rotation values were obtained using a Perkin-Elmer 241 polarimeter.

Synthetic Procedures

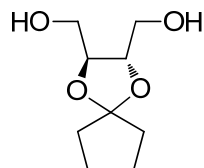
(2*R*,3*R*)-diethyl 1,4-dioxaspiro[4.4]nonane-2,3-dicarboxylate (13**).**



Diethyl L-tartrate **12** (3.37 mL, 19.6 mmol) was dissolved in toluene (130 mL). To this solution was added cyclopentanone (8.7 mL, 98 mmol) and *p*-toluenesulfonic acid (373 mg, 1.96 mmol). A Dean–Stark trap was attached and the reaction was heated at 150 °C overnight. Solid sodium bicarbonate (329 mg, 20%) was added and stirring was continued for 15 min. The reaction mixture was filtered and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution with 0–20% ethyl acetate to hexanes afforded **13** as a yellow oil (3.89 g, 73%).

Characterizations matched those reported in the literature.²⁵

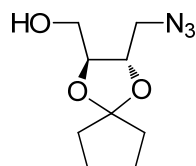
(2S,3S)-1,4-dioxaspiro[4.4]nonane-2,3-diyl dimethanol (14).



Diester **13** (3.1 g, 11.5 mmol), dissolved in anhydrous THF (10 mL), was added dropwise to a solution of lithium aluminum hydride (875 mg, 23.3 mmol) in anhydrous THF (20 mL) at 0 °C under nitrogen. After the addition was complete, stirring was continued for 1 h at 0 °C and then at room temperature for an additional 1 h. The solution was cooled to 0 °C and to it was slowly added, water (1.34 mL), 10% NaOH (1.34 mL), and water (2.7 mL) to quench the reaction. After addition, stirring was continued for 30 min at room temperature. Anhydrous magnesium sulfate was added and stirring was continued for another 30 min. The solution was filtered and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution with 0–10% methanol to dichloromethane afforded **14** as a clear oil (1.79 g, 83%).

Characterizations matched those reported in the literature.²⁵

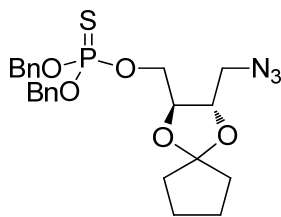
((2S,3S)-3-azidomethyl)-1,4-dioxaspiro[4.4]nonan-2-yl)methanol (15).



Diol **14** (1.77 g, 9.50 mmol) was dissolved in dichloromethane (20 mL). To the solution was added silver(I)oxide (3.30 g, 14.2 mmol), tosyl chloride (1.9 g, 10.1 mmol) and potassium iodide (315 mg, 1.92 mmol). The resulting solution was stirred at room temperature for 2 h and then filtered through a plug of silica gel with 100% ethyl acetate as the eluant. The filtrate was concentrated under reduced pressure, and then dissolved in DMF (95 mL). To the stirring solution was added sodium azide (1.55 g, 23.7 mmol). The solution was stirred at 85 °C overnight, concentrated, extracted with chloroform (2 x 100 mL), dried with magnesium sulfate, and concentrated under reduced pressure. Column chromatography with silica gel and 50% ethyl acetate to hexanes as eluant afforded **15** as a clear oil (1.30 g, 64%).

Characterizations matched those reported in the literature.²⁵

O-(((2S,3S)-3-(azidomethyl)-1,4-dioxaspiro[4.4]nonan-2-yl)methyl) O,O-dibenzyl phosphorothioate (17).

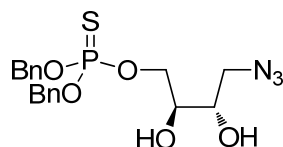


Azido alcohol **15** (114 mg, 0.53 mmol) was dissolved in anhydrous dichloromethane (4 mL). 1*H*-tetrazole (3.56 mL, 1.60 mmol, 0.45 M stock) was added and the solution was cooled to 0 °C under nitrogen. Dibenzyl diisopropylphosphoramidite **16**, (193 μ L, 0.588 mmol) was then added dropwise. Stirring was continued for 10 min at 0 °C and then at

room temperature for 2 h. The reaction mixture was cooled to 0 °C, and sulfur (69 mg, 2.13 mmol), carbon disulfide (90 μ L) and pyridine (90 μ L) were added and stirring was continued for 24 h. The reaction was extracted with dichloromethane, dried with magnesium sulfate, filtered and concentrated under reduced pressure. Column chromatography with silica gel and 40% ethyl acetate to hexanes as eluant afforded **17** as a pale-yellow oil (267 mg, 100%).

^1H NMR (300 MHz, CDCl_3) δ 7.39–7.30 (m, 10H), 5.14–4.99 (m, 4H), 4.14–3.87 (m, 4H), 3.42–3.35 (m, 1H), 3.22–3.15 (m, 1H), 1.83–1.64 (m, 8H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 512.13850, found: 512.14184.

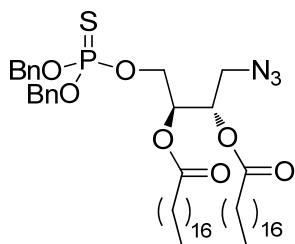
***O*-((2*S*,3*S*)-4-azido-2,3-dihydroxybutyl) *O*,*O*-dibenzyl phosphorothioate (**18**).**



Compound **17** (46 mg, 0.093 mmol) was dissolved in methanol (1 mL). With stirring, *p*-toluenesulfonic acid (12 mg, 0.06 mmol) was added. The solution was stirred at room temp overnight. The reaction was quenched with saturated sodium bicarbonate, extracted with chloroform (2 x 100 mL), dried with magnesium sulfate, filtered and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution with 10–50% ethyl acetate to hexanes afforded **18** as a yellow oil (22 mg, 55%).

^1H NMR (300 MHz, CDCl_3) δ 7.37–7.31 (m, 10H), 5.10–4.96 (m, 4H), 4.01–3.96 (m, 2H), 3.66–3.59 (m, 2H), 3.32–3.12 (m, 4H); HRMS $[\text{M} - \text{N}_2 + \text{H}]^+$ calcd: 396.10346, found: 396.10496.

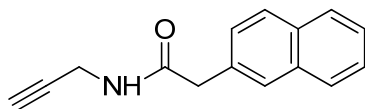
(2S,3S)-1-azido-4-((bis(benzyloxy)phosphorothioyl)oxy)butane-2,3-diyl distearate (19).



Stearic acid (294 mg, 1.034 mmol) was dissolved in dichloromethane (5 mL). To the stirring solution was added *N,N*-dicyclohexylcarbodiimide (213 mg, 1.03 mmol) and 4-dimethylaminopyridine (42 mg, 0.34 mmol) and stirring was continued for 15 min. A white precipitate formed to which was added diol **18** (127 mg, 0.35 mmol) and the solution was stirred at room temperature overnight. The reaction was filtered and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution with 10–25% ethyl acetate to hexanes afforded **19** as a white solid (820 mg, 74%).

^1H NMR (300 MHz, CDCl_3) δ 7.35–7.31 (m, 10H), 5.07–5.02 (m, 4H), 4.27–4.02 (m, 2H), 2.30–2.21 (m, 4H), 1.70–1.50 (m, 4H), 1.36–1.16 (*b s*, 56H), 0.87 (m, 6H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 978.61348, found: 978.61792.

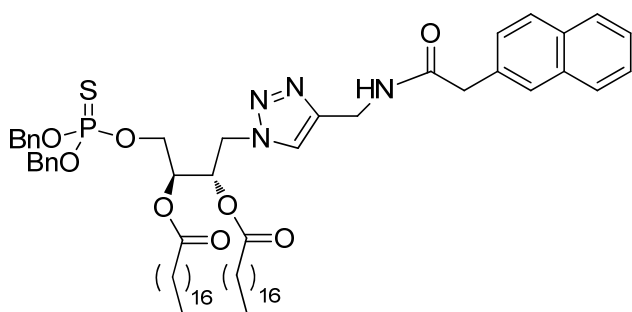
naphthyl alkyne (**20**).



2-Naphthylacetic acid (100 mg, 0.537 mmol), *N,N*-dicyclohexylcarbodiimide (166.2 mg, 0.806 mmol), 4-dimethylaminopyridine, (32.8 mg, 0.269 mmol) and propargylamine (44 μ L, 0.644 mmol) were dissolved in dichloromethane (6 mL). The reaction was stirred at room temperature in the dark overnight. The reaction was extracted with dichloromethane and water and the organic layer was dried with magnesium sulfate, filtered, and the solvent was removed by rotary evaporation. Column chromatography with silica gel and a gradient elution with 25–50% ethyl acetate to hexanes afforded **20** as a white solid (95 mg, 79%).

Characterizations matched the literature.²⁵

naphthyl phosphorothiotriester PA (**21**).

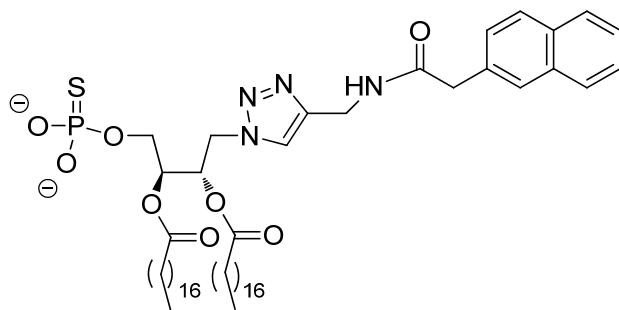


Compound **19** (25 mg, 0.026 mmol) and naphthyl alkyne **20** (5.83 mg, 0.026 mmol) were dissolved in THF (0.7 mL). Copper sulfate pentahydrate (20 mg, 0.078 mmol) and

sodium ascorbate (31 mg, 0.156 mmol) were added along with H₂O (0.3 mL). The solution was stirred at room temperature overnight. Then the solvent was removed under reduced pressure. Column chromatography with silica gel and a gradient solvent system of 20–50% ethyl acetate to hexanes afforded **21** as a white solid (12 mg, 11%).

¹H NMR (300 MHz, CDCl₃) δ 7.76–7.70 (m, 3H), 7.62 (s, 1H), 7.42–7.36 (m, 3H), 7.26 (m, 10H), 6.05–6.01 (m, 1H), 5.35–5.30 (m, 1H), 5.00–4.96 (m, 5H), 4.33–4.35 (m, 4H), 4.09–4.05 (m, 2H), 3.65 (s, 2H), 2.23–2.13 (m, 2H), 1.61–1.40 (m, 4H), 1.18 (b s, 56H), 0.81 (t, *J* = 6.49 Hz, 6H); MALDI–HRMS [*M* + Na]⁺ calcd: 1201.71319, found: 1201.70253.

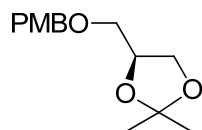
naphthyl phosphorothioate PA (**22**).



Naphthyl phosphotriester **21** (27 mg, 0.029 mmol), was dissolved in dichloromethane (0.7 mL) at 0 °C under argon. Bromotrimethylsilane (387 μL) was added and stirring was continued at room temperature for 2 h. Solvent was removed under reduced pressure and the residue was placed on a high vacuum line to remove any residual solvents. Due to the amphipathic nature of the phosphorothiotriesters, the integration

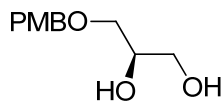
values in the proton NMR were close to actual values but did not always correspond to the exact value.

(S)-4-((4-methoxybenzyloxy)methyl)-2,2-dimethyl-1,3-dioxolane (24).



Sodium hydride (0.256 g, 0.006 mol) dissolved in *N,N*-dimethylformamide (30 mL) was taken in a round bottom flask under nitrogen and cooled to 0 °C. (S)-(2,2-dimethyl-1,3-dioxolan-4-yl)methanol **23** (0.5 mL, 0.004 mol) dissolved in DMF (10 mL) was added dropwise into the reaction mixture over a period of 30 min. The reaction was stirred at 0 °C for 1 h and then *p*-methoxybenzyl chloride (PMBCl, 813 μ L, 0.006 mol) was added to the solution at 0 °C and stirred at room temperature overnight. The reaction was quenched with water and extracted with dichloromethane and water (2 x 150 mL), the organic layer was dried over magnesium sulfate, filtered, and the solvent was removed under reduced pressure. The next step was set up without further purification.

(R)-3-((4-methoxybenzyl)oxy)propane-1,2-diol (25).

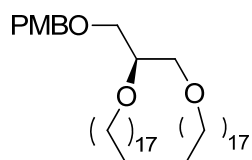


(S)-4-((4-methoxybenzyloxy)methyl)-2,2-dimethyl-1,3-dioxalane **24**, (1.5 g, 5.95 mmol) was dissolved in methanol (40mL) and *p*-toluenesulfonic acid (128 mg, 0.67 mmol) was

added to the reaction mixture and stirred at room temperature overnight. The reaction was then quenched with saturated sodium bicarbonate followed by extraction with dichloromethane and water (3 x 100 mL). Column chromatography with silica gel and a gradient elution with 2–4% of methanol to dichloromethane afforded **25** as a yellow oil (1.2 g, 39% over 2 steps)

Characterizations matched those reported in the literature.⁷⁷

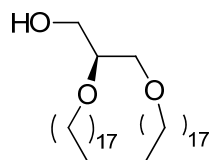
(*R*)-1-((2,3-bis(octadecyloxy)propoxy)methyl)-4-methoxybenzene (26).



Compound **25** (222 mg, 1.05 mmol) was dissolved in dry benzene (4 mL) followed by the addition of 1-bromooctadecane (1.09 mg, 3.13 mmol), potassium hydroxide (237 mg, 4.22 mmol) and a catalytic amount of tetrabutylammonium iodide (TBAI). The reaction was refluxed overnight at 105 °C and a Dean–Stark trap was used to collect water formed in the reaction. Column chromatography with silica gel and a gradient elution with 0–10% of ethyl acetate to hexanes afforded **26** as a white solid (552 mg, 74%).

¹H NMR (300 MHz, CDCl₃) δ 7.30–7.22 (m, 2H), 6.89–6.84 (m, 2H), 4.45 (d, *J* = 15.2 Hz, 2H), 3.80 (s, 3H), 3.61–3.36 (m, 9H), 1.62–1.49 (m, 4H), 1.38–1.17 (*b* s, 60H), 0.88 (t, *J* = 6.6 Hz, 6H); MALDI–HRMS [*M* + Na]⁺ calcd: 739.65803, found: 739.65863.

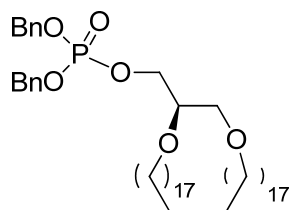
(S)-2,3-bis(octadecyloxy)propan-1-ol (27).



Compound **26** (550 mg, 0.76 mmol) was dissolved in dichloromethane (20 mL), and water (0.2 mL) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (812 mg, 3.83 mmol) were added, and the reaction mixture was stirred overnight. Sodium bicarbonate was added to quench the reaction followed by extraction with dichloromethane and water (2 x 150 mL). Column chromatography with silica gel and a gradient elution with 10–50% of ethyl acetate to hexanes afforded **27** as a white solid (552 mg, 74%).

^1H NMR (300 MHz, CDCl_3) δ 4.23–4.05 (m, 9H), 2.50 (*b* s, 1H), 2.34 (t, J = 7.5 Hz, 4H), 1.69–1.55 (m, 4H), 1.38–1.17 (*b* s, 60H), 0.88 (t, J = 5.9 Hz, 6H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 619.60052, found: 619.60398.

(R)-dibenzyl (2,3-bis(octadecyloxy)propyl) phosphate (28a).



Compound **27** (50 mg, 0.08 mmol) was dissolved in anhydrous dichloromethane/THF (1:1) under nitrogen and cooled to 0°C. To the stirring mixture was added 1*H*-tetrazole (0.56 mL, 0.25 mmol, 0.45 M stock). Dibenzyl diisopropylphosphoramidite **16** (600 μL ,

1.8 mmol) was then added dropwise. Stirring was continued for 30 min at 0 °C and then at room temperature for 1 h. At this point the reaction mixture was again cooled to 0° C and *m*-chloroperoxybenzoic acid (26 mg, 0.15 mmol, 57% purity) was added and stirring was continued for 1.5 h at room temperature. The reaction was quenched with the saturated sodium bicarbonate, extracted with dichloromethane and water (2 x 150 mL), dried with magnesium sulfate, filtered and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution with 10–20% of ethyl acetate to hexanes afforded **10** (35 mg, 45%).

The integrations values for the ¹H NMR spectrum were close but did not completely match the actual values. This was due to the inability to separate starting material, product and phosphoramidite reagent using column chromatography, since they had same polarities.

Chapter 3: Design and Synthesis of Azide-Labeled Diacylglycerol (DAG) Analogues with Ether Chains

Background and significance

Diacylglycerol is an important signaling lipid consisting of a glycerol molecule in which two hydroxyl groups have been replaced by two fatty acids at the *sn*-1 and *sn*-2 position through ester bonds. (Figure 3.1.) Despite its simple structure, DAG is involved in important physiological and pathological processes. DAG is an important lipid intermediate in the biosynthesis and metabolism of various lipids⁷⁸ and also acts as a second messenger in cell signaling.^{79,80} Malfunctions in DAG production and degradation have been linked to the onset of various diseases, such as cancer⁸⁰ and diabetes.⁸¹

Nishizuka and co-workers⁸⁰ discovered protein kinase C (PKC) as the first receptor for DAG. Since then at least eleven different isozymes of protein kinase C (PKC) have been reported as DAG-binding receptors, thereby greatly increasing the number of proteins known to be modulated by direct interaction with DAG. PKCs have long been linked to tumorigenesis because of the central role they play in controlling proliferation, differentiation and apoptosis of different cell types.^{82,83,84}

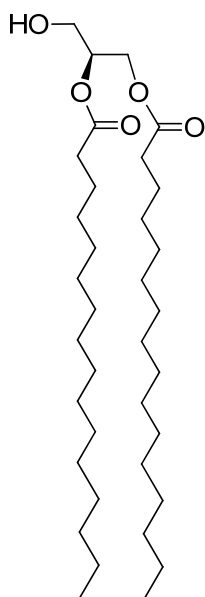


Figure 3.1. Structure of diacylglycerol molecule (DAG).

PKCs are comprised of a family of serine/threonine kinases that are identified by the presence of two C1 domains, which form the DAG-binding modules. In addition to the two C1 domains, PKC enzymes also contain a C2 domain that recognizes anionic phospholipids on the membrane surface and are attracted to it through electrostatic interactions. Studying PKC binding interactions with DAG is complicated by factors including the fact that PKCs contain multiple binding domains that yield multivalent membrane association, different domains having varying DAG binding affinities and various isoenzymes of the PKC family show different specificities and affinities towards DAG. These subtle differences in lipid binding are known to significantly affect the regulation of protein function in biological pathways. As a result, characterization of these binding events becomes necessary.

Although PKC is one of the the most recognizable targets of DAG, other DAG receptors have also been reported. Recent studies have revealed that DAG also activates several other protein receptors. These include chimaerins,⁸⁵ Ras guanyl-releasing proteins (RasGRPs), Munc13,⁸⁶ protein kinase D (PKD),⁸⁷ and DAG kinases. This clearly shows that cellular responses that are attributed to DAG can no longer be attributed solely to PKCs, which further highlights the complexity involved in studying the role of DAG as a signaling lipid in various cellular responses.

Synthesis of DAG-based probes will allow for investigations of the biological activity at the molecular level and will help in understanding the role that DAG plays as a second messenger and as an important intermediate in lipid metabolism. Towards this goal, various probes of DAG have been synthesized. Stahelin et al.⁸⁸ have employed short-chained DAG and phorbol analogues in order to compare their binding affinities towards C1A and C1B domains of PKC ϵ . They observed that the C1A domain of PKC ϵ has a stronger binding affinity towards DAG, while the C1B has a higher binding affinity towards phorbol esters. In another study, Ananthanarayannan et al.⁷⁹ investigated the binding affinities of short chain DAG analogues toward C1A and C1B domains of PKC α . They showed that the DAG analogues showed a high affinity for the C1A domain while no considerable affinity was observed for the C1B domain of PKC α . These studies also indicated that DAG analogs do not necessarily require the membrane for binding with proteins and hence can be employed for binding studies without the need of the membrane.

Synthesis DAG Analogues with ether tails

Following the initial success of replacing the ester tails with ether tails in the DAG scaffold utilized for synthesizing PA analogues, we have attempted to extend this approach to develop the azide-tagged DAG scaffold with ether tails at the *sn*-1 and *sn*-2 position. For this we modify the synthetic scheme such that an azide tag can be introduced in place of a hydrogen at the *sn*-1 position, and ether tails can be introduced at the *sn*-1 and *sn*-2 positions. (Figure 3.2.) The ether tails are used to avoid the migration of the *sn*-2 acyl chain to *sn*-3 position, which is known to become more prominent with the introduction of the azidomethyl moiety at the headgroup.²⁶ It also avoids hydrolysis of acyl chains by various enzymes present in biological systems.

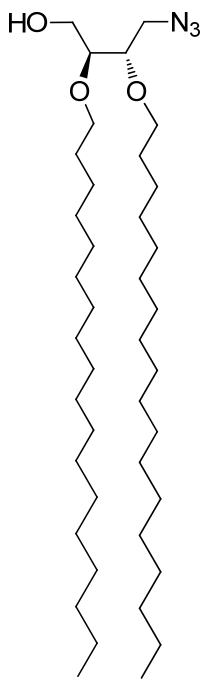


Figure 3.2. Structure of azide-tagged DAG scaffold with ether chains, **42**.

Although synthetic lipid probes have been utilized to study protein–lipid binding events, these investigations are often limited due to the absence of a modular approach, therefore requiring that each probe be synthesized in an individual manner. This has imposed limitations on the range of probes that can be developed for various studies.

Our lab has successfully designed a DAG scaffold containing an azide tag at the *sn*-1 position which can be functionalized at a late stage of the synthesis with various reporter groups in order to obtain a wide array of DAG-based probes.²⁶ The azide tag is placed at the *sn*-1 position as it has been found to be the most beneficial location for attaching the reporter molecule, since it places the reporter molecule close enough to the head group to allow for sensitive detection of binding, yet far enough so as not to interfere with the actual binding motif. We also made use of the same strategy and placed the azide tag at the *sn*-1 position for derivatization. The azide moiety has the advantage of being small in size and possesses bioorthogonal reactivity via 1,3-dipolar cycloadditions (“click” chemistry)^{15,89} or the Staudinger ligation.^{90,91} Since both approaches are highly selective and do not interfere with functionalities that exist in biological systems, they can be used for protein labeling and detection in complex biological samples. We will employ click chemistry to incorporate a variety of reporter molecules such as fluorophores and photoaffinity labels onto the core DAG scaffold structure. (Figure 3.3.)

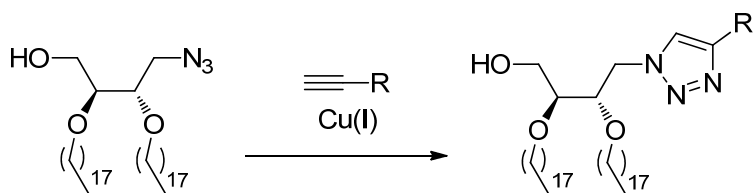


Figure 3.3. Use of click chemistry to attach reporter tags to the DAG scaffold.

One particular aim of this project was to develop bifunctional lipid probes for rapid identification of receptors that bind to DAG via ABPP. The synthesis of such probes involves the introduction of two groups into the lipid structure—a cross-linking group such as a photoaffinity tag that selectively cross links to the receptor protein once the binding has occurred, as well as a secondary handle, usually an azide or alkyne, that is either used to selectively purify, identify and characterize the bound proteins by means of affinity purification or is used to visualize the proteins that have bound to DAG. (Figure 3.4.)

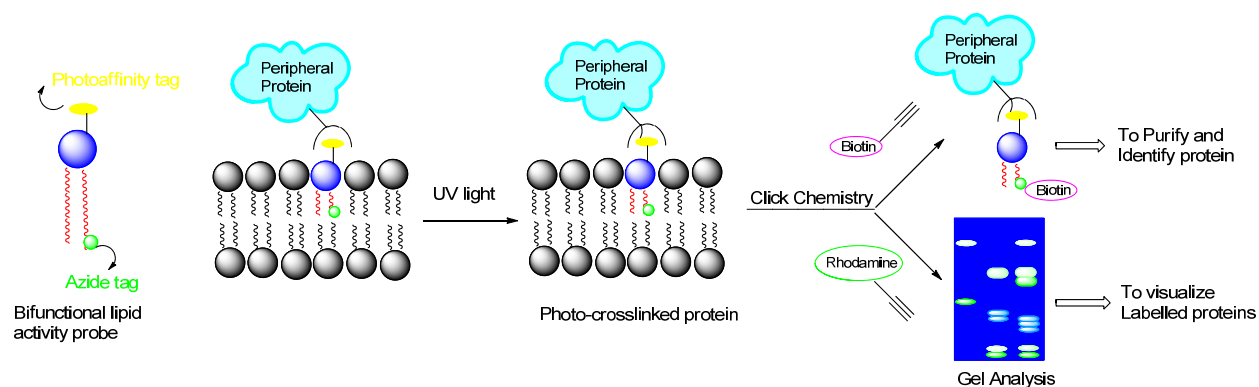


Figure 3.4. An example of Activity Based Protein Profiling (ABPP) using a bifunctional probe to purify or visualize proteins bound to the lipid probe.

We have attempted to develop a bifunctional probe for ABPP that includes both a photoaffinity tag, benzophenone for photo-cross-linking, and an alkyne moiety as a secondary handle for purifying cross-linked proteins. (Figure 3.5.) The photoaffinity group used should be stable enough to avoid non-specific labeling, but reactive enough to modify residues of a bound protein. The role of a photoaffinity group is to label the proteins by photo-cross-linking to them. This aids in mapping the location of binding domains of the receptor proteins and in characterizing the protein–lipid binding interactions. For example, de Kroon and co-workers have developed an activity based probe derived from the structure of PC that contains a secondary azide tag for chemical modification and detection after binding.⁹² Picq and co-workers developed a photoaffinity-tagged PA probe that was used in the covalent labeling of type 4 cAMP-phosphodiesterases (PDE4).⁹³

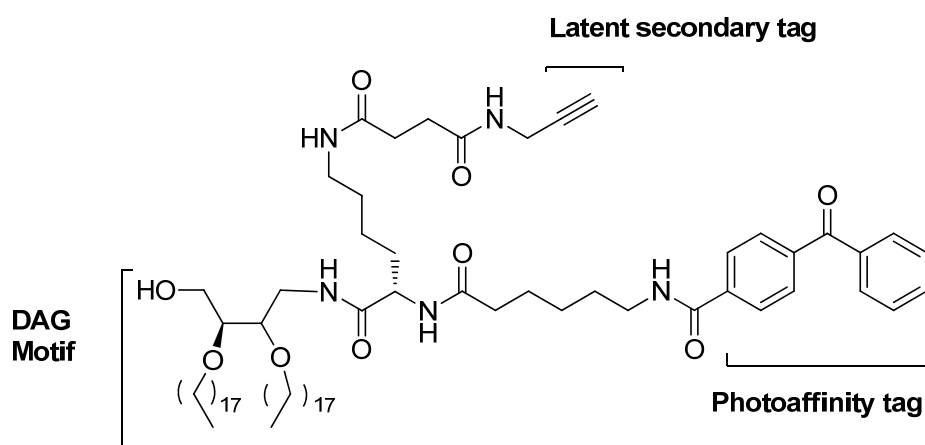
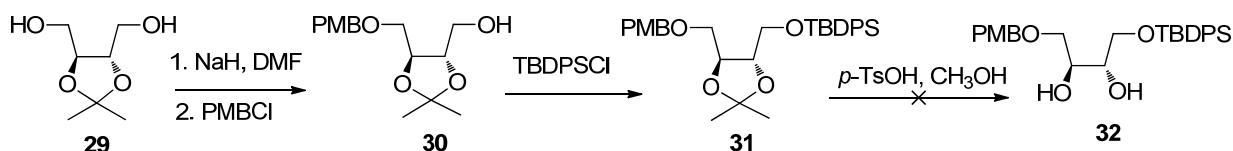


Figure 3.5. A bifunctional lipid probe.

The initial synthetic scheme, as shown in scheme A of figure 3.6., involved protecting the two alcohols of compound **29** with a *p*-methoxybenzyl (PMB) and a *tert*-butyldiphenylsilyl (TBDPS) group, respectively, to obtain **31**. Two different protecting groups were utilized in order to selectively modify the protected alcohol at the *sn*-1, position into an azidomethyl group and to protect the alcohol at the *sn*-3 position respectively. This was followed by the deprotection of the acetal group. Meng M. Rowland from our lab had been working on similar chemistry and had observed that the TBDPS group was removed during the deprotection of the acetal group. As a result, the synthetic scheme was modified (Scheme B, Figure 3.6.) so as to deprotect the acetal group first and then employ a selective protection of the primary alcohol of **33** with a TBDPS group. Once compound **32** was obtained, the next step involved installing the ether tails, utilizing potassium hydroxide (KOH) as a base to yield **34**.

Scheme A



Scheme B

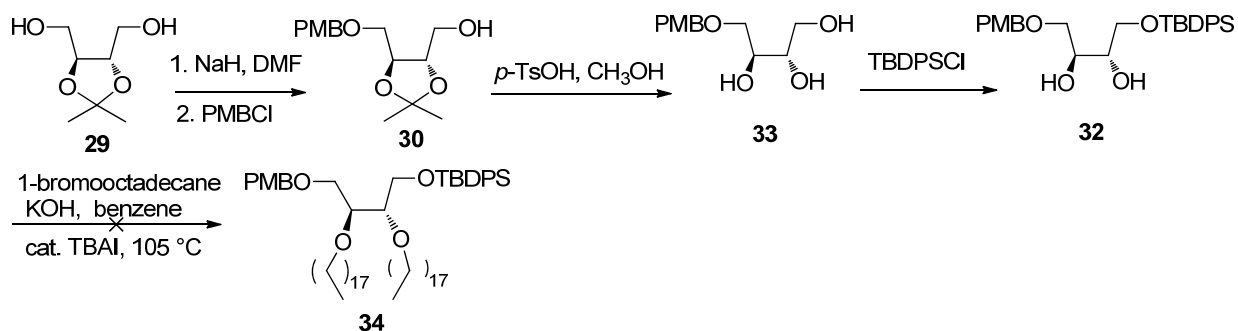


Figure 3.6. Alternate synthetic routes followed for the synthesis of azide-tagged DAG scaffold.

The basic conditions required for introducing the ether tails led to the removal of the TBDPS group. Since there are not many protection groups that can survive such basic conditions (potassium hydroxide), the PMB protecting group was selected to protect both the alcohols, thus introducing the ether chains before differentiating the two hydroxymethyl moieties.

The final synthetic scheme shown in figure 3.7. began with commercially available diethyl L-tartrate **35** and involved acetonide protection of diol **35** to furnish **36**. The esters were then reduced using lithium aluminum hydride to yield diol **29**. The alcohols of compound **29** were then protected with *p*-methoxybenzyl (PMB) protecting groups followed by the removal of the acetonide protecting group, which afforded diol **38**. The ether tails were now introduced using 1-bromooctadecane and tetrabutylammonium iodide (TBAI) as catalyst yielding **39**.⁷⁶ Oxidative removal of the PMB protecting groups then afforded **40**. Monotosylation of compound **40** furnished **41**. The integration values in the ¹H NMR spectrum were close but did not exactly match the expected values. There is a possibility that we might be getting a cyclized product in addition to the actual product. Next, **41** was directly converted to the azide tagged DAG scaffold **42**. Once the DAG scaffold is obtained it can then be used to attach various reporter groups such as fluorophores or affinity tags, or it can be reduced to an amine, which can then be reacted with the a y-shaped handle having a benzophenone and an alkyne tag to form the bifunctional probe **54**.

The synthesis of the y-shaped handle is shown in figure **3.8**. and began with the coupling of 4-benzoylbenzoic acid to methyl-6-aminohexanoate hydrochloride to yield the ester **45**. The methyl ester group of **45** was then hydrolyzed using 2 M sodium hydroxide and methanol to yield acid **46**. After the cBz deprotection of Lysine N-cBz N-Boc to yield the free amine **48**, it was then coupled to the acid **46**, which afforded compound **49**. This was followed by the deprotection of the Boc group of compound **49** to produce the corresponding free amine **50**, which was then coupled to the acid with an alkyne handle **51** to yield compound **52**. Next, the hydrolysis of methyl ester of **52** using 2 M sodium hydroxide yielded the acid **53** having a benzophenone and an alkyne tag. The acid **53** formed can then be coupled to the DAG scaffold having an amine at the *sn*-1 position **43** to form the bifunctional lipid probe **54**. (Figure **3.9**.)

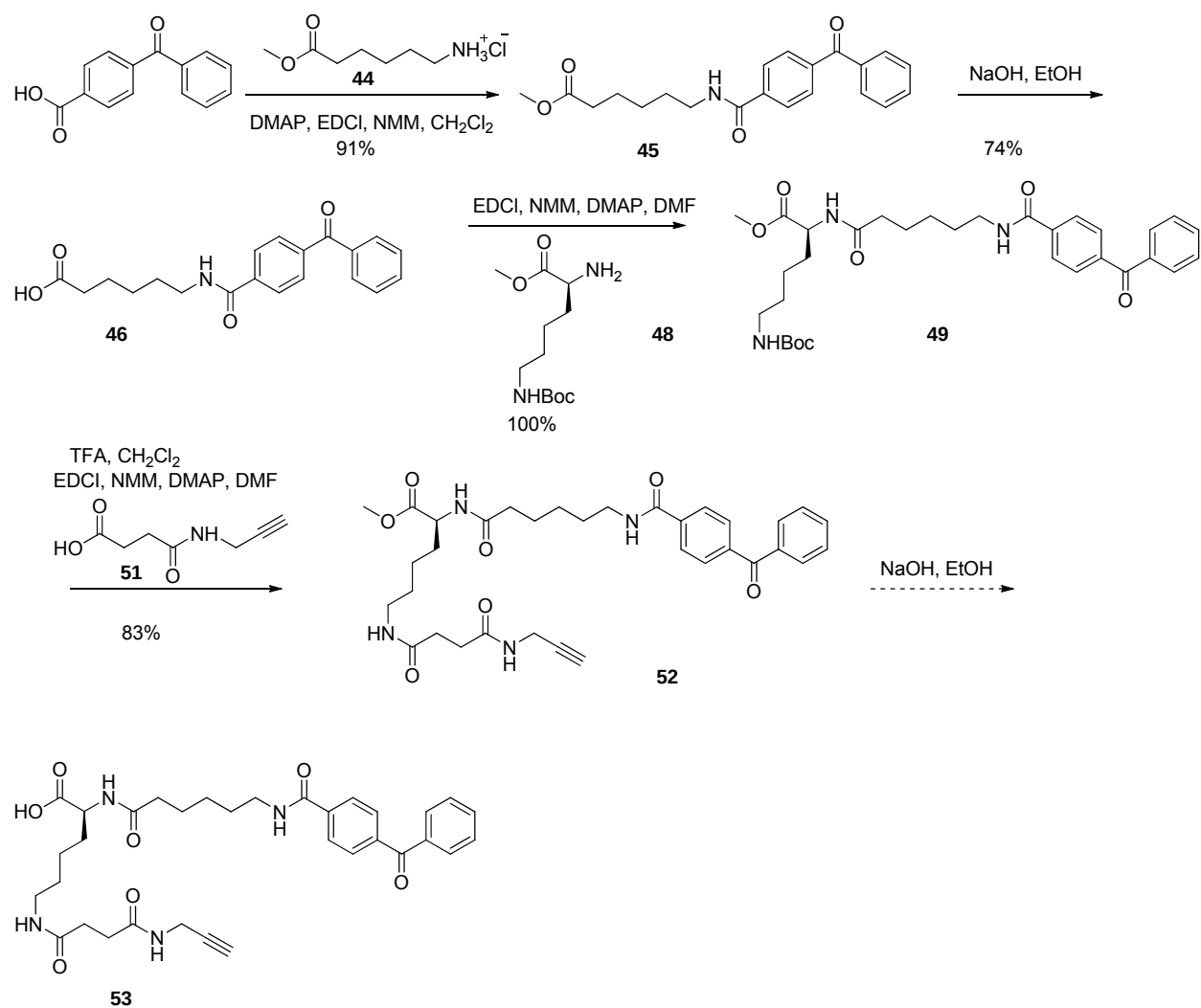


Figure 3.8. Synthetic scheme for y-shaped handle having benzophenone as photoaffinity tag and an alkyne tag.

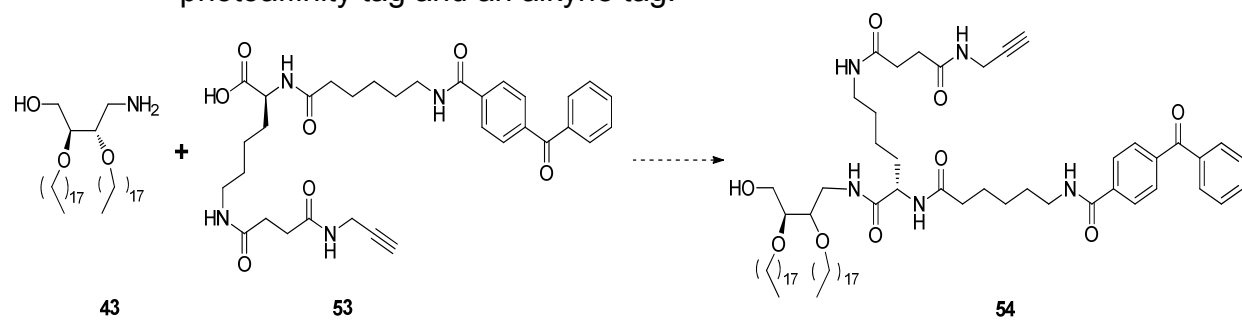


Figure 3.9. Coupling of DAG scaffold with amine at the *sn*-1 position, **43** to the acid having an affinity group and an alkyne handle, **53** to form bifunctional lipid probe **54**.

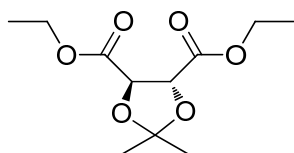
Experimental

General Considerations

All reagents were purchased from Aldrich or Acros and used as received. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using a Bruker AC250 spectrometer updated with a TecMag data collection system and a Varian Mercury 300 spectrometer. Mass spectra were obtained with the JEOL DART-AccuTOF spectrometer with high-resolution capabilities. Optical rotation values were obtained using a Perkin-Elmer 241 polarimeter.

Synthetic Procedures

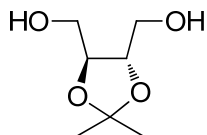
(4R,5R)-diethyl 2,2-dimethyl-1,3-dioxolane-4,5-dicarboxylate (36).



In a flame-dried flask diethyl L-tartrate **35** (6.76 mL, 39.5 mmol) was dissolved in benzene followed by the addition of *p*-toluenesulfonic acid (128 mg, 0.672 mmol). To the stirring solution was added 2,2-dimethoxypropane (6.03 mL, 48.6 mmol). The solution was refluxed at 80 °C for 3 h and the water formed in the reaction was collected in a Dean–Stark trap. After cooling the solution to room temperature, sodium carbonate

(200 mg, 1.89 mmol) was added and stirring was continued for 2 h. The solution was filtered and concentrated under reduced pressure. The next step was set up without further purification.

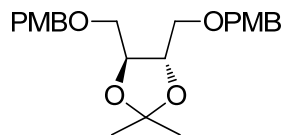
((4S,5S)-2,2-dimethyl-1,3-dioxolane-4,5-diyl)dimethanol (29**).**



A solution of lithium aluminum hydride (3.0 g, 79 mmol) was dissolved in anhydrous THF (70 mL) and cooled to 0 °C and kept under nitrogen. The crude mixture from above step was dissolved in anhydrous THF (20 mL) and added dropwise to the reaction over a period of 30 min. The reaction mixture was stirred at 0 °C for 1 h, then at room temperature for 1 h, and was then cooled back to 0 °C at which point the reaction was quenched by the dropwise addition of 3 mL water, 3 mL of 10% NaOH, and 9 mL more of water. Stirring was continued for 30 min followed by the addition of anhydrous magnesium sulfate and stirring was continued for an additional 1 h. The solution was filtered and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution with 3 to 5% methanol to dichloromethane afforded **29** as a clear oil (3.8 g, 63% over 2 steps).

Characterizations matched those reported in the literature.⁹⁴

((4S,5S)-4,5-bis(((4-methoxybenzyl)oxy)methyl)-2,2-dimethyl-1,3-dioxolane (37).



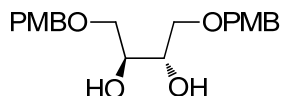
Sodium hydride (0.548 g, 0.024 mmol) dissolved in *N,N*-dimethylformamide (50 mL) was taken in a round bottom flask under nitrogen and cooled to 0 °C. Diol **29** (4.4 g, 0.024 mmol) dissolved in DMF (10 mL) was added dropwise into the reaction mixture over a period of 30 min. The reaction was stirred at 0 °C for 1 h and then *p*-methoxybenzyl chloride (PMBCl, 1.9 mL, 0.024 mol) was added to the solution at 0 °C and stirred at room temperature overnight. The reaction was quenched with water and extracted with dichloromethane and water, the organic layer was dried over magnesium sulfate, filtered and the solvent was removed under reduced pressure. This formed mono PMB protected alcohol.

The reaction was set up again to form the di-PMB protected product. Sodium hydride (0.548 g, 0.024 mmol) dissolved in *N,N*-dimethylformamide (50 mL) was taken in a round bottom flask under nitrogen and cooled to 0 °C. The mono PMB protected alcohol from last step was dissolved in DMF (10 mL) and added dropwise into the reaction mixture over a period of 30 min. The reaction was stirred at 0 °C for 1 h and then *p*-methoxybenzyl chloride (PMBCl, 1.9 mL, 0.024 mol) was added to the solution at 0 °C and stirred at room temperature overnight. The reaction was quenched with water and extracted with dichloromethane and water, the organic layer

was dried over magnesium sulfate, filtered and the solvent was removed under reduced pressure to afford diPMB diol **37** as yellow oil.

The next step was set up without further purification.

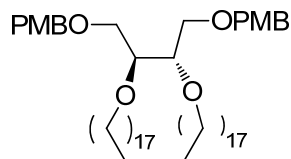
(2S,3S)-1,4-bis((4-methoxybenzyl)oxy)butane-2,3-diol (38).



Compound **37** from last step was dissolved in methanol (40 mL) and *p*-toluenesulfonic acid (1.9 g, 3.67 mmol) was added and the reaction mixture was stirred at room temperature overnight. The reaction was then quenched with saturated sodium bicarbonate followed by extraction with dichloromethane and water. Column chromatography with silica gel and gradient elution with 2-4% methanol to dichloromethane afforded **38** as a yellow solid (2.6 g, 27% over 2 steps).

^1H NMR (300 MHz, CDCl_3) δ 7.28–7.14 (m, 4H), 6.92–6.77 (m, 4H), 4.52–4.35 (m, 4H), 3.87–3.71 (m, 8H), 3.61–3.42 (m, 4H), 3.10–2.96 (s, 2H), HRMS $[\text{M} + \text{H}]^+$ calcd: 362.17294, found 363.18421

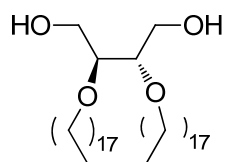
4,4'((((2S,3S)2,3bis(octadecyloxy)butane1,4diyl)bis(oxy))bis(methylene))bis(methoxybenzene) (39).



Compound **38** (130 mg, 0.34 mmol) was dissolved in dry benzene (4mL) followed by the addition of 1-bromooctadecane (359 mg, 1.08 mmol), potassium hydroxide (81 mg, 1.44 mmol) and a catalytic amount of tetrabutylammonium iodide (TBAI). The reaction was refluxed overnight at 105 °C and a Dean–Stark trap was used to collect water formed in the reaction. Column chromatography with silica gel and a gradient elution with 0–10% ethyl acetate to hexanes afforded **39** as a white solid (241 mg, 77%).

^1H NMR (300 MHz, CDCl_3) δ 7.23 (d, J = 8.60 Hz, 4H), 6.85 (d, J = 8.65 Hz, 4H), 4.46–4.40 (m, 4H), 3.82–3.76 (m, 6H), 3.66–3.54 (m, 6H), 3.54–3.38 (m, 4H), 1.60–1.46 (m, 4H), 1.38–1.14 (*b* s, 60 H), 0.88 (t, J = 6.88 Hz, 6H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 889.72611, found: 889.72091.

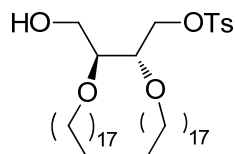
(2S,3S)-2,3-bis(octadecyloxy)butane-1,4-diol (40).



Compound **39** (875 mg, 1.01 mmol) was dissolved in dichloromethane (32 mL) and water (3.2 mL), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (812 mg, 3.83 mmol) was added, and the reaction mixture was stirred overnight. Sodium bicarbonate was added to quench the reaction followed by extraction with dichloromethane and water (2 x 100 mL). Column chromatography with silica gel and a gradient elution with 10–50% ethyl acetate to hexanes afforded **40** as a white solid (2752 mg, 44%).

^1H NMR (300 MHz, CDCl_3) δ 3.93–3.45 (m, 10H), 2.49 (s, 2H), 1.73–1.45 (m, 4H), 1.47–1.13 (b s, 60H), 0.88 (t, J = 6.6 Hz, 6H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 649.61108, found: 649.61458.

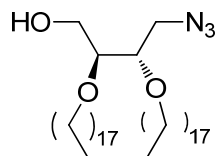
(2S,3S)-4-hydroxy-2,3-bis(octadecyloxy)butyl 4-methylbenzenesulfonate (41).



Compound **40** (56 mg, 0.09 mmol) was suspended in anhydrous dichloromethane (10 mL). With stirring was added silver(I)oxide (34 mg, 0.15 mmol), tosyl chloride (20 mg, 0.11 mmol), and crushed potassium iodide (32 mg, 0.19 mmol). The reaction was stirred at room temperature overnight. Column chromatography with silica gel and a gradient elution with 10–35% ethyl acetate to hexanes afforded **41** as a white solid (16 mg, 23%).

^1H NMR (300 MHz, CDCl_3) δ 7.86–7.47 (m, 2H), 7.30–7.40 (m, 2H), 4.37–3.99 (m, 1H), 3.98–3.86 (m, 1H), 3.83–3.66 (m, 2H), 3.60–3.46 (m, 6H), 2.49 (m, 3H), 2.17 (m, 1H), 1.66–1.41 (m, 4H), 1.25 (b s, 60 H), 0.85 (m, 6H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 803.61993, found: 803.61438.

(2S,3S)-4-azido-2,3-bis(octadecyloxy)butan-1-ol (42).

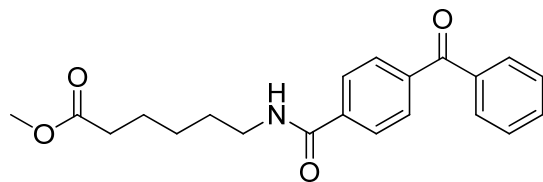


Compound **41** (16 mg, 0.02 mmol) was dissolved in ethyl acetate (20 mL) and sodium azide (40 mg, 0.19 mmol) was added. The reaction was refluxed at 85 °C overnight. The solvent was concentrated under reduced pressure. Column chromatography with silica gel and a gradient elution from 15–50% ethyl acetate to hexanes afforded **42** as a colorless oil (12 mg, 80%).

^1H NMR (300 MHz, CDCl_3) δ 4.01–4.85 (m, 3H), 3.79–3.71 (m, 1H), 3.51–3.40 (m, 3H), 1.71–1.47 (m, 6H), 1.42–0.98 (*b* s, 66H), 0.88 (t, J = 6.7 Hz, 6H)

Due to the low solubility of the product, the integration values in the ^1H NMR spectrum were close but did not exactly match the expected values.

methyl 6-(4-benzoylbenzamido)hexanoate (45).

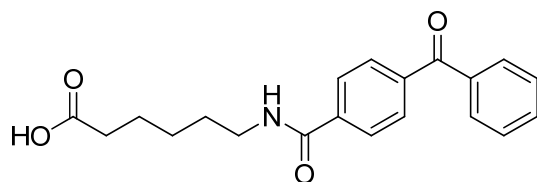


4-Benzoylbenzoic acid (0.9 g, 3.98 mmol) was dissolved in dichloromethane (70 mL) and to the stirring solution was added 4-dimethylaminopyridine (0.63 g, 5.17 mmol) under nitrogen. To a solution of methyl 6-aminohexanoate hydrochloride (0.72 g, 0.817 mmol) in dichloromethane (70 mL) was added *N*-methylmorpholine (4.35 mL, 39.8 mmol). Both the solutions were combined and EDCI (0.99 g, 10.34 mmol) was added to the reaction mixture. The reaction was stirred at room temperature overnight. Column

chromatography on silica gel and a gradient elution with 25–50% ethyl acetate to hexanes afforded **45** (1.29 g, 91%).

^1H NMR (300 MHz, CDCl_3) δ 7.95–7.89 (m, 2H), 7.81–7.73 (m, 4H), 7.68–7.54 (m, 1H), 7.51–7.43 (m, 2H), 7.10 (t, J = 5.67, 1H), 3.64 (s, 3H), 3.56–3.27 (m, 2H), 2.31 (t, J = 7.36, 2H), 1.75–1.56 (m, 4H), 1.47–1.37 (m, 2H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 376.1525, found: 376.1548

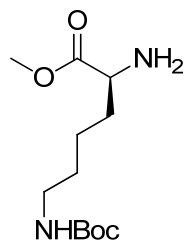
6-(4-benzoylbenzamido)hexanoic acid (46).



The ester **45** (303 mg, 0.598 mmol) was dissolved in methanol (16 mL) and 2 M sodium hydroxide (16 mL) was added. The solution was stirred at room temperature overnight. Solvent was removed and the crude product was extracted with dichloromethane and 2 M hydrochloric acid (2 x 100 mL), dried with magnesium sulfate, and concentrated under reduced pressure. Column chromatography with silica gel and a gradient solvent system of 50% ethyl acetate/hexanes afforded the acid **46** (351 mg, 74%).

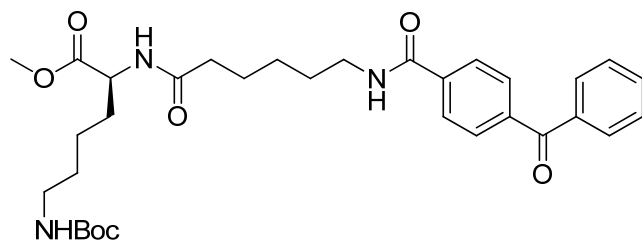
^1H NMR (300 MHz, CDCl_3) δ 7.89–7.76 (m, 6H), 7.64–7.56 (m, 1H), 6.51–6.45 (m, 2H), 7.09–7.11 (m, 1H), 3.53–3.46 (m, 2H), 2.39 (t, J = 7.11, 2H), 1.75–1.62 (m, 4H), 1.51–1.42 (m, 2H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 362.1368, found: 362.1334.

(S)-methyl 2-amino-6-((tert-butoxycarbonyl)amino)hexanoate (48).



Commercially available cBz-protected amine (1.38 g, 3.49 mmol) was dissolved in methanol (38 mL), and 10% palladium/carbon (138 mg) by weight was added. The mixture was placed under hydrogen and stirred for 24 h at room temperature. The reaction was filtered over Celite and the filtrate was concentrated under reduced pressure to afford the amine **48**. The next step was set up without further purification.

(S)-methyl 2-(6-(4-benzoylbenzamido)hexanamido)-6-((tert-butoxycarbonyl)amino)hexanoate (49).

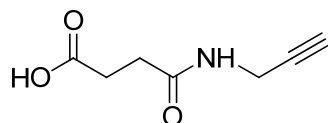


The amine (352 mg, 1.36 mmol) from the last step was dissolved in *N,N*-dimethylformamide (4 mL). To the stirring solution were added *N*-methylmorpholine (595 μ L, 5.4 mmol) under nitrogen. To the acid **46** (460 mg, 1.36 mmol) in *N,N*-dimethylformamide (4 mL) was added 4-dimethylaminopyridine (497 mg, 4.07 mmol). Both the solutions were combined and EDCI (780 mg, 4.07 mmol) was added. The

reaction was stirred at room temperature for 48h. Column chromatography with silica gel with gradient solvent system of 100% ethyl acetate/hexanes afforded **49** (800 mg, 100%).

^1H NMR (300 MHz, CDCl_3) δ 7.84–7.74 (m, 2H), 7.74–7.62 (m, 4H), 7.57–7.42 (m, 1H), 7.41–7.33 (m, 2H), 6.63 (s, 1H), 6.08 (m, 1H), 4.60–4.34 (m, 2H), 3.57 (s, 3H), 3.49–3.29 (m, 2H), 3.03–2.87 (m, 2H), 2.15 (m, 2H), 1.68–1.45 (m, 8H), 1.37–1.24 (m, 11H), 1.23–1.07 (m, 2H); MALDI–HRMS $[\text{M} + \text{Na}]^+$ calcd: 604.2999, 604.2945.

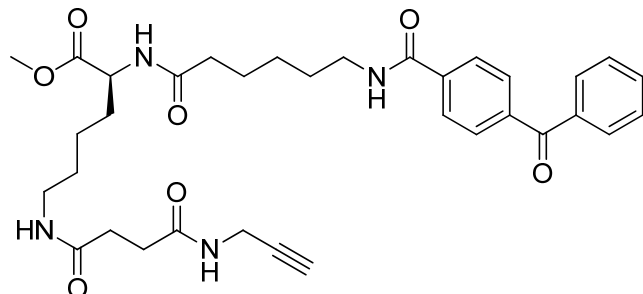
4-oxo-4-(prop-2-yn-1-ylamino)butanoic acid (51).



Propargylamine (400 μL , 5.83 mmol) was dissolved in acetonitrile/*N,N*-dimethylformamide (10 mL/10 mL) at 0 $^{\circ}\text{C}$ under nitrogen. Succinic anhydride (584 mg, 5.83 mmol) dissolved in acetonitrile (20 mL) was added to the stirring mixture. The reaction mixture was warmed to room temperature and run overnight at room temperature. The solvent was removed, the solid was washed with hexanes (2 x 100 mL), and the solvent removed under reduced pressure to afford alkyne **51**.

Characterizations matched those reported in the literature.⁷⁷

(S)-methyl2-(6-(4-benzoylbenzamido)hexanamido)-6-(4-oxo-4-(prop-2-yn-1-yl-amino)butanamido)hexanoate (52).



Compound **49** (343 mg, 0.71 mmol) was dissolved in dichloromethane (6.0 mL). Trifluoroacetic acid (6.0 mL) was added, and the solution was stirred at room temperature overnight. The solvent was removed under reduced pressure and placed under vacuum for 24 h.

Next, the crude product (459 mg, 0.77 mmol) was dissolved in *N,N*-dimethylformamide (4 mL). To the stirring solution were added *N,N*-methylmorpholine (850 μ L, 7.71 mmol) under nitrogen. To compound **51** (120 mg, 0.77 mmol) in *N*-dimethylformamide (4 mL) was added 4-dimethylaminopyridine (141 mg, 1.16 mmol). Both the solutions were combined and EDCI (221 mg, 1.16 mmol) was added. The reaction was stirred at room temperature for 48 h. Column chromatography with silica gel and a gradient solvent system of 1–5% methanol/dichloromethane afforded **52** (316 mg, 83%).

^1H NMR (250 MHz, CDCl_3) δ 7.98–7.94 (m, 2H), 7.84–7.79 (m, 4H), 7.76–7.60 (m, 1H), 7.54–7.48 (m, 2H), 7.72 (m, 1H), 6.98 (m, 1H), 6.71–6.64 (m, 2H), 4.58–4.49 (m, 1H), 3.02–3.99 (m, 2H), 3.70 (s, 3H), 3.54–3.46 (m, 2H), 3.30–3.13 (m, 2H), 2.60–2.45 (m,

4H), 2.33–2.28 (m, 2H), 2.05 (s, 1H), 1.81–1.66 (m, 4H), 1.56–1.33 (m, 8H); MALDI–HRMS $[M + Na]^+$ calcd: 641.2951, 641.2967.

Chapter 4: Design and Synthesis of Metabolically Stabilized Phosphatidylinositol phosphate (PIP_n) Analogues

Background and significance

Phosphatidylinositol phosphates (PtdInsP_ns or PIP_ns) are phosphorylated derivatives of *myo*-inositol belonging to an important class of membrane signaling lipids.^{95,96} There are seven isomers of the PIP_n family differing only in the degree of phosphorylation of the hydroxyl groups at the 3-, 4- and 5-positions of the *myo*-inositol rings. The role of PIP_ns as signaling lipids results from their ability to bind to receptors called peripheral proteins through non-covalent binding, resulting in the anchoring of the protein receptors onto the cell membrane surface. These binding events are known to regulate many important physiological pathways, generally through the regulation of protein function and subcellular localization. They are known to play a crucial roles in numerous cellular functions such as vesicle trafficking, apoptosis, cell proliferation and metabolism.^{95,96,49a} Furthermore, misregulation of signaling pathways due to defects in binding and lipid composition results in a range of disease conditions, such as cancer, leukemia, diabetes, and Alzheimer's.^{97,98}

Despite the significance of PIP_n–protein binding, many of these binding events are poorly understood due to the complex nature of these interactions. The subtle differences in the structure of various isomers of PIP_ns can result in different binding affinities and specificities of these isomers towards the receptors. There are certain receptors that bind with high affinity towards a particular PIP_n isomer, while others appear to be more promiscuous. The PIP_ns are also known to bind to a wide array of protein-binding domains, such as the PH,⁹ PX, FYVE, ENTH, ANTH, FERM, Tubby, and PROPPIN¹² domains.^{10, 13,14} Variations exist even within the domain families, particularly

with the PH domains, where investigations have indicated that there are over 250 PH domains in humans. While all the PH domains are currently not believed to bind to PIP_ns, different PH domains have been shown to target different PIP_n isomers.¹⁴ In addition, the structural requirements for recognition also vary among receptors. There are certain receptors that require the presence of the membrane for binding because the receptor inserts a hydrophobic sequence into the hydrophobic membrane core, there are other receptors that show high affinity for isolated PIP_ns headgroups.^{2,99} In such cases, binding is primarily driven by electrostatic attraction to the *myo*-inositol headgroup, with little-to-no dependence on the membrane environment. All these factors make studying PIP_n–protein binding interactions challenging, primarily due to their complex structure and even more complex ways in which they interact with proteins.

Amongst various PIP_ns, PI(3,4,5)P₃ is an important signaling lipid found in higher eukaryotic cells.¹⁰⁰ PI(3,4,5)P₃ is known to play a central role in a plethora of downstream cellular processes. These signaling events include cell proliferation and transformation,¹⁰¹ cell shape and motility,¹⁰² insulin action and alteration of glucose transport.¹⁰³ An important example involves the conversion of PI (4,5)P₂ to PI(3,4,5)₃, by means of the forward and reverse reactions catalyzed by phosphoinositide 3-kinase (PI3K)⁹⁸ and the phosphatase PTEN,¹⁰⁴ respectively. These conversions are known to control the activity of an important protein Akt (protein kinase B). Upon binding to PI(3,4,5)P₃, Akt is activated via phosphorylation, which results in the regulation of numerous downstream processes such as cell apoptosis and cell survival. For this

reason, PI(3,4,5)P₃ production must be tightly regulated to avoid abnormal cell growth. The importance of this transformation becomes more clear from the fact that PI3K¹⁰⁵ and PTEN¹⁰⁶ are amongst the most commonly mutated enzyme and tumor suppressor, respectively, in tumorigenesis. In order to understand the role PI(3,4,5)P₃ plays in the signaling pathways and its effect on downstream processes, it is necessary to study PI(3,4,5)P₃–protein binding interactions at the molecular level.

In order to understand these binding interactions, synthetic probes can be used to identify the structure of the natural biomolecules, to detect PIP_n activity by the utilizing reporter-derivatized probes and to identify receptors that bind to PIP_ns by developing affinity probes. Due to the presence of various isomers of PIP_ns in nature, a modular approach that allows derivatization at a late stage from a common scaffold in order to obtain several PIP_n isomer probes would be beneficial. Furthermore, chemical probe synthesis will allow access to analogs with different structural features, such as those that consist of isolated lipid headgroups as well as others those that contain the full glycerolipid backbone.

Metabolically Stabilized Analogs of PIP_ns

There has been great interest in developing metabolically stable analogs of PIP_ns that are resistant to enzymatic modification and retain the biological activity of the natural compound. The primary focus has been on stabilizing the phosphate groups

present in various PIP_ns with phosphomimetic groups that are resistant to phosphate hydrolysis.

The initial approach to generating metabolically stabilized PIP_ns involved the introduction of phosphorothioates in place of phosphate moieties. Potter and co-workers synthesized the first metabolically stabilized PIP_n probe, Ins(1,4,5)PS₃³⁹ **55**, (Figure 4.1.) Since then, numerous phosphorothioate derivatives of different PIP_ns have been synthesized. The disadvantage of using phosphorothioate derivatives is that they have reduced binding affinities compared to their natural analogues.⁴¹ This was shown by Prestwich and co-workers, who synthesized an analogue of PI(3)P containing a phosphorothioate group in place of the 3-phosphate group. The decrease in binding affinity has been mainly attributed to the modified pK_a and hydrogen-bonding ability of phosphorothioate compared to phosphate.^{39,107} Prestwich and co-workers also developed PI(3,4,5)P₃ analogue **56** stabilized at the 3-position to specifically instill resistance to the 3-phosphatase PTEN. This involved replacing the 3-phosphate group with a phosphorothioate moiety.¹⁰⁷ The resulting analogs exhibited similar and even slightly higher activity in the sodium transport assay compared to the unmodified lipid. Prestwich and co-workers also synthesized

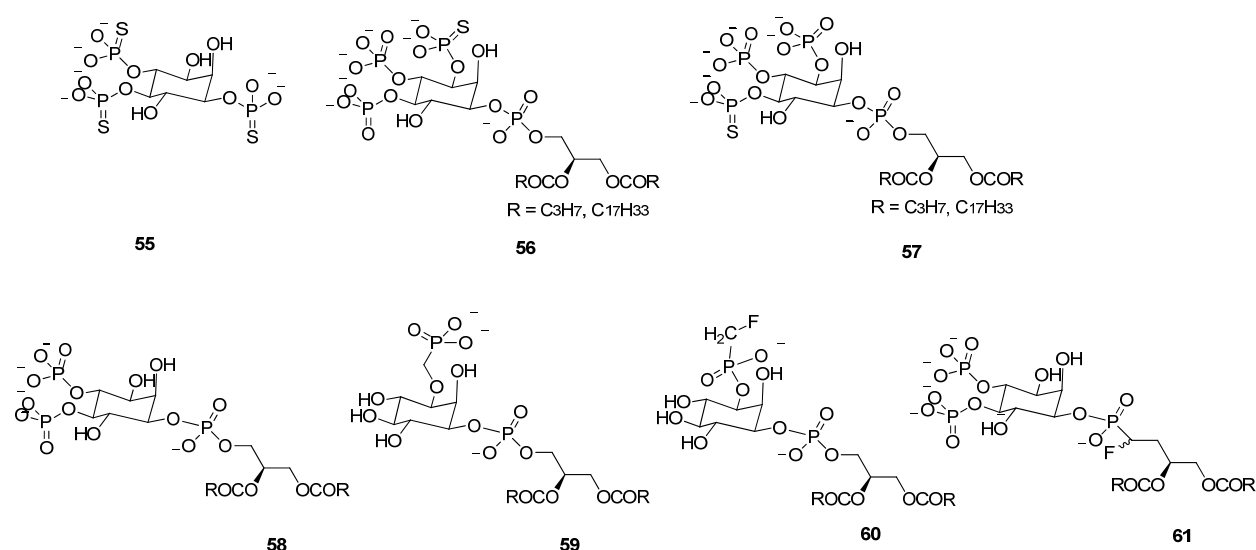


Figure 4.1. Various metabolically stabilized PIP_ns analogues.

PI(3,4,5)P₃ analogs stabilized at the 5-position, **57** to instill resistance for the 5-phosphatases SHIP1 and SHIP2. The analogue exhibited binding affinity to the PH domain of Grp1 comparable to unmodified PIP₃ analogue.^{36b}

Phosphonate moieties can also be used to provide enzymatic resistance to PIP_ns. Prestwich and co-workers have synthesized a derivative of PI(4,5)P₂ **58**, where they used a phosphonate linkage instead of a phosphodiester group to attach *myo*-inositol with the lipid backbone.¹⁰⁸ They have also synthesized a metabolically stabilized PI(3)P analogue where they replaced phosphate moieties on the *myo*-inositol headgroups with a methylene phosphonate **59**.⁴¹ Methylene phosphonates also exhibited weaker binding affinities compared to natural PIP_ns, which is mainly due to the higher second pK_a of a phosphonate group compared to that of phosphate.

In order to improve the binding affinity of the phosphomimetic groups toward the receptors, recently fluoromethylene phosphonate have been utilized to replace the phosphate in PIP_ns. The electron withdrawing nature of the fluorine group decreases the pK_a to match the second pK_a of the natural phosphate.⁴³ Prestwich and co-workers have successfully incorporated the fluoromethylene phosphonate group into the *myo*-inositol headgroup of PI(3)P analog **60** and into the phosphodiester of PI(4,5)P₂ **61**,¹⁰⁹ In terms of binding affinities, fluoromethylene group might seem most suited for synthesizing metabolically stabilized analogs. However, the optimal approach may depend upon the particular system being studied.

Synthesis of Metabolically Stabilized PI(3,4,5)P₃ Analogue

Our strategy for synthesizing metabolically stabilized PI(3,4,5)P₃ analogue involved the incorporation of phosphorothioate groups in place of the phosphate groups at the 1, 2 and 3 position of the *myo*-inositol ring. For this, we utilized the same modular synthetic route used by our lab for the synthesis of regular PI(3,4,5)P₃ and all other PIP_ns analogues. This involves the synthesis of a modular PIP_n headgroup scaffold tethered to an amino group that can then be conveniently derivetized by coupling chemistry at a later stage to form a range of functionalized headgroup analogs for use as probes.

The synthetic route to a headgroup phosphorothiorate analogue corresponding to PI(3,4,5)P₃ **76** attached to an amino group is detailed in figure 4.2. and first involves the synthesis of intermediate **68**, from *myo*-inositol, as described previously by Bruzik and co-workers.¹¹⁰ In 2003, they reported an efficient synthesis of all seven PIP_n isomers,

which started with the synthesis of stereospecific *myo*-inositol-camphor acetal product **64** through the reaction of *myo*-inositol **62** with D-camphor dimethyl acetal **71**. The products so formed were selectively crystallized to obtain the **64** having required stereochemistry. Next, a *tert*-butyldiphenylsilyl (TBDPS) group was introduced onto **64** to yield **65** with high regioselectivity. The compound **65** was then used to synthesize PI(3,4,5)P₃ precursor **68**, containing benzoyl groups at the position 3,4, and 5 of the *myo*-inositol structure. This involved the introduction of benzoyl groups at position 4 and 5 of **65** to produce **66**. Next, the acetal group was deprotected followed by the introduction of a third benzoyl group at position 3, to obtain the precursor **68**. The remaining two hydroxyl groups were then protected with BOM groups to generate **69**. This was followed by benzoyl deprotection to obtain **70**. Next, phosphoramidite chemistry was used to introduce phosphorothioate moieties at position 3, 4, and 5 to afford **73**. We obtained very low yields while attempting to install the phosphorothioate groups utilizing dibenzyl diisopropylphosphoramidite **72** and sulfur. We also attempted to utilize phenylacetaldisulfide instead of sulfur to obtain **73**, but the reaction did not occur. We then went back to the original conditions, and next carried out silyl deprotection by tetra-*n*-butylammonium fluoride to produce **74** which also gave low yields.

Since low yields were obtained using phosphorothioate chemistry compared to the original synthesis utilizing phosphate groups, in the future, alternate routes will be explored for developing metabolically stabilized PIP_ns.

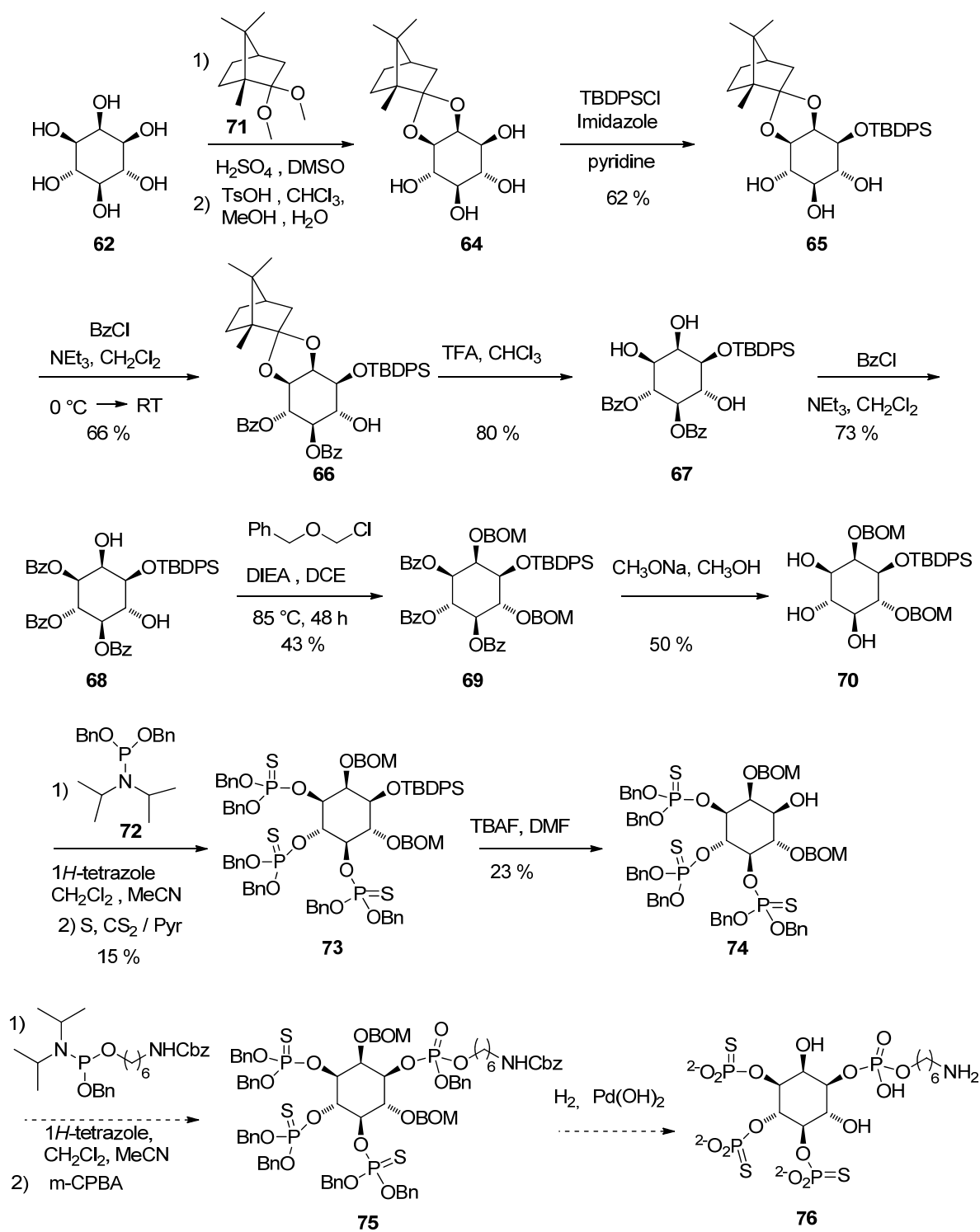


Figure 4.2. Synthetic scheme for phosphorothioate PI(3,4,5)P₃ headgroup analogue.

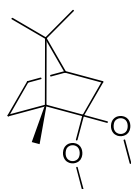
Experimental

General Considerations

All reagents were purchased from Aldrich, Acro. and used as received. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230 – 400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using a Bruker AC250 spectrometer updated with a TecMag data collection system, a Varian Mercury 300 spectrometer. Mass spectra were obtained with JEOL DART-AccuTOF spectrometer with high resolution capabilities. Optical rotation values were obtained using a Perkin-Elmer 241 polarimeter.

Synthetic Procedures

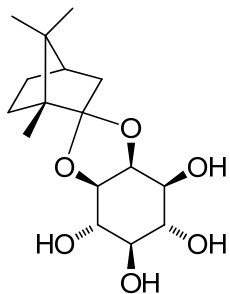
(1R,4R)-2,2-dimethoxy-1,7,7-trimethylbicyclo[2.2.1]heptanes (71).



To a dry flask was added D-camphor (32.6 g, 0.21 mol), dry methanol (33 mL), trimethylorthoformate (165 mL, 1.51 mol) and concentrated sulfuric acid (0.32 mL, catalytic amount) The mixture was stirred at room temperature for 48 h, neutralized with sodium methoxide and concentrated. The resulting residue was dissolved in toluene, the precipitated sodium sulfate was filtered off, and the filtrate was concentrated to give

D-camphor dimethyl acetate **71**. The crude product was used in the next step without further purification.

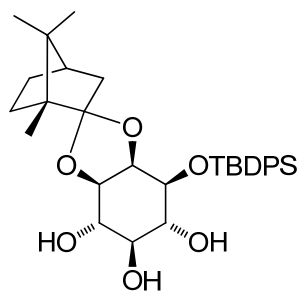
(1'R,2R,3aR,4S,4'R,5R,6R,7S,7aS)-1',7',7'-trimethylhexahydrospirobenzo[d][1,3]dioxole-2,2'-bicyclo[2.2.1]heptane]-4,5,6,7-tetraol (64**).**



The crude from the above step was dissolved in dimethylsulfoxide (184 mL) and *myo*-inositol (16.86 g, 93.6 mmol) was added followed by sulfuric acid (0.95 mL) addition. The resulting mixture was stirred for 3 h at 75 °C, neutralized with triethylamine and concentrated. To the obtained residue was added dimethylsulfoxide to a total weight of 82 g, as well as chloroform (285 mL), methanol (17.9 mL), water (5.7 mL), and *p*-toluenesulfonic acid (63 mg). The mixture was stirred overnight, neutralized with triethylamine, filtered and washed with CHCl₃ to give crude product. Recrystallization from MeOH (0.1% triethylamine) gave pure product **64** as white solid.

Characterization matched the literature.¹¹¹

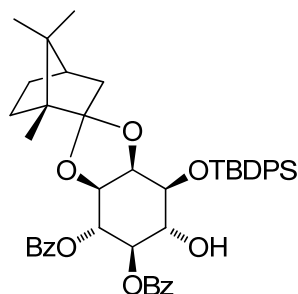
(1'R,2R,3aR,4S,4'R,5R,6S,7S,7aR)-4-((tert-butyldiphenylsilyl)oxy)-1',7',7'-trimethyl-hexahydrospiro[benzo[d][1,3]dioxole-2,2'-bicyclo[2.2.1]heptane]-5,6,7-triol (65**).**



A solution of tetrol **64** (1.56 g, 4.96 mmol) and imidazole (473 mg, 6.95 mmol) in pyridine (17 mL) was treated with *tert*-butyl diphenylchlorosilane (TBDPSCI) (1.3 mL, 6.95 mmol) at -10 °C. Then the reaction was warmed to 15 °C and stirred overnight. The mixture was concentrated and extracted with ethyl acetate from water. The organic layer was dried over magnesium sulfate and the solvent was removed by rotary evaporation. Column chromatography with silica gel and elution from 20 : 1 to 10 : 1, CH₂Cl₂ / CH₃OH afforded triol **65** (1.7 gm, 62 %) as a white foam.

Characterization matched literature.¹¹¹

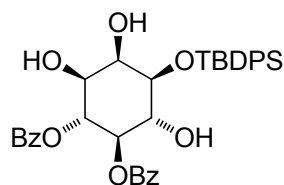
(1'R,2R,3aR,4S,4'R,5R,6R,7R,7aS)-4-((*tert*-butyldiphenylsilyl)oxy)-5-hydroxy-1',7',7'-trimethylhexahydrospiro[benzo[d][1,3]dioxole-2,2'-bicyclo[2.2.1]heptane]-6,7-diyl dibenzoate (66**).**



To a stirring solution of the triol **65** (1.6 g, 2.9 mmol) in pyridine (15.3 mL) and dichloromethane (2.7 mL) under nitrogen at -40 °C, was added benzoyl chloride (0.7 mL, 6.12 mmol) dropwise, then the mixture was slowly warmed to room temperature for about 5 h. The mixture was concentrated and extracted with ethyl acetate and water. The organic layer was dried with magnesium sulfate and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution from 15 : 1 to 10 : 1 of hexanes to acetone afforded alcohol **66** (1.4 g, 66%) as a white foam.

Characterization matched literature.¹¹⁰

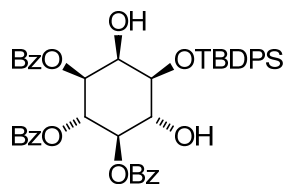
(1R,2R,3R,4R,5R,6S)-4-((tert-butyldiphenylsilyl)oxy)-3,5,6-trihydroxycyclohexane-1,2-diyl dibenzoate (67).



To a stirring solution of the alcohol **66** (1.05 g, 1.38 mmol) in chloroform (10 mL) and water (0.37 mL), was added trifluoroacetic acid (1.07 mL, 13.9 mmol). The mixture was stirred at room temperature overnight, neutralized using triethylamine and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution from 30 : 1 to 20 : 1 of dichloromethane to methanol, afforded the triol **67** (627 mg, 80%) as a white foam.

Characterization matched the literature.¹¹⁰

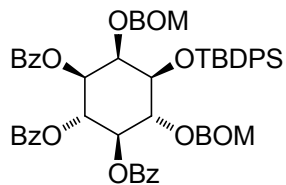
(1R,2R,3S,4R,5R,6R)-5-((tert-butyldiphenylsilyl)oxy)-4,6-dihydroxycyclohexane-1,2,3-triyl tribenzoate (68)



To a solution of the diol **67** (877 mg, 1.40 mmol) in dichloromethane (15 mL) under nitrogen, was added 4-dimethylaminopyridine (DMAP) (171mg, 1.39 mmol) and triethyl amine (0.59mL, 4.19 mmol) The reaction was cooled to 0 °C followed by the addition of benzoyl chloride (0.161 mL, 1.40 mmol). The reaction was run at 0 °C for 10 min and then at room temperature overnight. Solvent was removed under reduced pressure and column chromatography with silica gel and a gradient elution from 5:1 to 1:1 hexanes to acetone afforded alcohol **68** (1.02g, 73%)

Characterization matched the literature.¹¹⁰

(1R,2R,3S,4R,5R,6R)-5-((tert-butyldiphenylsilyl)oxy)-4,6-dihydroxycyclo -1,2,3-triyl tribenzoate (69).

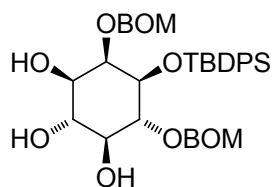


To a solution of the diol **68** (120mg, 0.16 mmol) in dichloroethane (5.5 mL) under nitrogen, was added diisopropylethylamine (217 µL, 1.31 mmol) and benzyl

chloromethylether (182 μ L, 1.31 mmol). The solution was refluxed for 24 h then cooled to room temperature. Another portion of diisopropylethylamine (217 μ L, 1.31 mmol) and benzyl chloromethylether (182 μ L, 1.31 mmol) were added, and the solution was refluxed for another 24 h. The solution was then extracted with dichloromethane and water. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. Column chromatography with silica gel and gradient elution from 10:1 hexanes to acetone, afforded the product **69** (69 mg, 43%) as a light yellow solid.

Characterization matched literature.¹¹²

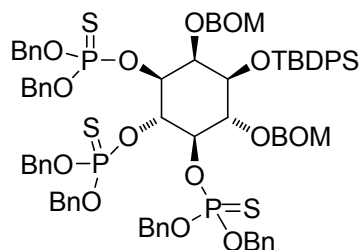
(1R,2S,3S,4R,5S,6R)-4,6-bis((benzyloxy)methoxy)-5-((tert-butyldiphenylsilyl)oxy)cyclohexane-1,2,3-triol (70).



To a solution of the fully protected inositol **69** (1.03 g, 1.06 mmol) in methanol (40 mL) was added 2M sodium hydroxide solution (16 mL), the solution was then stirred at room temperature overnight. The solution was filtered, and the solvent removed under reduced pressure. The residue was extracted with dichloromethane and water and the organic layer was dried over magnesium sulfate. Evaporation of solvent under reduced pressure and column chromatography with silica gel and gradient elution from 6 : 1 to 3 : 1 of hexanes to acetone, afforded the diol **70** (870 mg, 52%) as a light yellow gum.

Characterization matched the literature.¹¹²

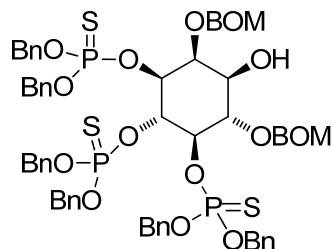
O,O,O',O',O'',O''-hexabenzylO,O',O''-((1R,2S,3S,4S,5S,6S)-4,6bis((benzyloxy) methoxy)-5-((tertbutyldiphenylsilyl)oxy)cyclohexane1,2,3triyl)triphosphorothioate (73).



Compound **70** (80 mg, 0.60 mmol) was dissolved in anhydrous dichloromethane (12 mL). 1*H*-tetrazole (11.8 mL, 5.32 mmol, 0.45 M stock) was added and the solution was cooled to 0 °C under nitrogen. Dibenzyl diisopropylphosphoramidite **72** (1.25 mL, 0.588 mmol) was then added dropwise. Stirring was continued for 10 min at 0 °C and then at room temperature for overnight. The reaction mixture was cooled to 0 °C and sulfur (233 mg, 7.26 mmol), carbon disulfide (314 μ L) and pyridine (314 μ L) were added and stirring was continued for 24 h. The reaction was extracted with dichloromethane, dried with magnesium sulfate, and concentrated under reduced pressure. Column chromatography with silica gel and gradient elution from 50:1 hexanes to acetone to 100 % acetone afforded **73** as a pale yellow oil (124 mg, 15%).

¹H NMR (300 MHz, CDCl₃) δ 7.80–7.69 (m, 4H), 7.41–7.09 (m, 51H), 5.16–4.86 (m, 12 H), 4.82–4.93 (m, 10H), 4.93–4.37 (m, 12 H), 4.33–4.18 (m, 1H), 4.11–4.02 (m, 3H), 3.47(s, 1H), 1.13–1.02 (m, 11H).

O,O,O',O',O'',O''-hexabenzylO,O',O''-((1R,2S,3S,4S,5S,6S)-4,6-bis((benzyloxy) methoxy)-5-hydroxycyclohexane-1,2,3-triyl) triphosphorothioate (74).



Compound **73** (53mg, 0.03 mmol) was dissolved in tetrahydrofuran and tetra-*n*-butylammonium fluoride (TBAF) (110mg, 0.34 mmol) was added. The reaction was stirred at room temperature for 48 h. Solvent was removed and column chromatography with silica gel and gradient elution from 20% to 50 % acetone to hexanes afforded **74** as a pale yellow oil (124 mg, 15%).

^1H NMR (300 MHz, CDCl_3) δ 7.38–7.09 (m, 40H), 5.43–5.30 (m, 1H), 5.24–4.93 (m, 10H), 4.93–4.82 (m, 3H), 4.80–4.67 (m, 2H), 4.67–4.62 (m, 1H), 4.62–4.58 (m, 1H), 4.56–4.41 (m, 3H), 4.38–4.23 (m, 1H), 4.29–4.22 (m, 1H), 3.91–3.79 (m, 1H), 3.58–3.49 (m, 1H).

References

1. Wiener, M. C.; White, S. H., Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron-diffraction data .3. complete structure. *Biophys. J.* **1992**, 61 (2), 434-447.
2. Cho, W. H.; Stahelin, R. V., Membrane-protein interactions in cell signaling and membrane trafficking. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, 34, 119-151.
3. (a) Exton, J. H., Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **1990**, 265 (1), 1-4; (b) Nishizuka, Y., Intracellular signaling by hydrolysis of phospholipids and activation of protein-kinase-C. *Science* **1992**, 258 (5082), 607-614.
4. Carrasco, S.; Merida, I., Diacylglycerol, when simplicity becomes complex. *Trends Biochem. Sci.* **2007**, 32, 27-36.
5. Colon-Gonzalez, F.; Kazanietz, M. G., C1 domains exposed: From diacylglycerol binding to protein-protein interactions. *Biochim. Biophys. Acta, Mol. Cell. Biol. Lipids* **2006**, 1761 (8), 827-837.
6. Stace, C. L.; Ktistakis, N. T., Phosphatidic acid- and phosphatidylserine-binding proteins. *Biochim. Biophys. Acta, Mol. Cell. Biol. Lipids* **2006**, 1761 (8), 913-926.
7. Cockcroft, S., Phosphatidic acid regulation of phosphatidylinositol 4-phosphate 5-kinases. *Biochim. Biophys. Acta, Mol. Cell. Biol. Lipids* **2009**, 1791 (9), 905-912.
8. Testerink, C.; Munnik, T., Phosphatidic acid: a multifunctional stress signaling lipid in plants. *Trends Plant Sci.* **2005**, 10 (8), 368-375.
9. Lietzke, S. E.; Bose, S.; Cronin, T.; Klarlund, J.; Chawla, A.; Czech, M. P.; Lambright, D. G., Structural basis of 3-phosphoinositide recognition by pleckstrin homology domains. *Mol. Cell* **2000**, 6 (2), 385-394.
10. Prestwich, G. D., Phosphoinositide signaling: From affinity probes to pharmaceutical targets. *Chem. Biol.* **2004**, 11 (5), 619-637.
11. Manna, D.; Bhardwaj, N.; Vora, M. S.; Stahelin, R. V.; Lu, H.; Cho, W. H., Differential roles of phosphatidylserine, PtdIns(4,5)P-2, and PtdIns(3,4,5)P-3 in plasma membrane targeting of C2 domains - Molecular dynamics simulation, membrane binding, and cell translocation studies of the PKC alpha C2 domain. *J. Biol. Chem.* **2008**, 283 (38), 26047-26058.
12. Michell, R. H.; Heath, V. L.; Lemmon, M. A.; Dove, S. K., Phosphatidylinositol 3,5-bisphosphate: metabolism and cellular functions. *Trends Biochem. Sci.* **2006**, 31 (1), 52-63.
13. Kutateladze, T. G.; Lee, S.; Cheever, M.; Overduin, M.; Burd, C., Phosphoinositide recognition domains: Targeting of proteins to membranes. *Abstracts of Papers of the American Chemical Society* **2005**, 229, 274-MEDI.
14. Lemmon, M. A., Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell Biol.* **2008**, 9 (2), 99-111.
15. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click chemistry: Diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed.* **2001**, 40 (11), 2004-2008.
16. Tron, G. C.; Pirali, T.; Billington, R. A.; Canonico, P. L.; Sorba, G.; Genazzani, A. A., Click chemistry reactions in medicinal chemistry: Applications of the 1,3-

- dipolar cycloaddition between azides and alkynes. *Med. Res. Rev.* **2008**, 28 (2), 278-308.
17. Manetsch, R.; Krasinski, A.; Radic, Z.; Raushel, J.; Taylor, P.; Sharpless, K. B.; Kolb, H. C., In situ click chemistry: Enzyme inhibitors made to their own specifications. *J. Am. Chem. Soc.* **2004**, 126 (40), 12809-12818.
 18. Hawker, C. J.; Fokin, V. V.; Finn, M. G.; Sharpless, K. B., Bringing efficiency to materials synthesis: The philosophy of click chemistry. *Aust. J. Chem.* **2007**, 60 (6), 381-383.
 19. Brustad, E.; Bushey, M. L.; Lee, J. W.; Groff, D.; Liu, W.; Schultz, P. G., A Genetically Encoded Boronate-Containing Amino Acid. *Angew. Chem. Int. Ed.* **2008**, 47 (43), 8220-8223.
 20. Tippmann, E. M.; Schultz, P. G., A genetically encoded metallocene containing amino acid. *Tetrahedron* **2007**, 63 (27), 6182-6184.
 21. Vocadlo, D. J.; Hang, H. C.; Kim, E. J.; Hanover, J. A.; Bertozzi, C. R., A chemical approach for identifying O-GlcNAc-modified proteins in cells. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, 100 (16), 9116-9121.
 22. Weisbrod, S. H.; Marx, A., Novel strategies for the site-specific covalent labelling of nucleic acids. *Chem. Commun.* **2008**, (44), 5675-5685.
 23. Seo, T. S.; Li, Z. M.; Ruparel, H.; Ju, J. Y., Click chemistry to construct fluorescent oligonucleotides for DNA sequencing. *J. Org. Chem.* **2003**, 68 (2), 609-612.
 24. Gierlich, J.; Burley, G. A.; Gramlich, P. M. E.; Hammond, D. M.; Carell, T., Click chemistry as a reliable method for the high-density postsynthetic functionalization of alkyne-modified DNA. *Org. Lett.* **2006**, 8 (17), 3639-3642.
 25. Smith, M. D.; Sudhakar, C. G.; Gong, D.; Stahelin, R. V.; Best, M. D., Modular synthesis of biologically active phosphatidic acid probes using click chemistry. *Mol. BioSyst.* **2009**, 5 (9).
 26. Smith, M. D.; Gong, D. H.; Sudhakar, C. G.; Reno, J. C.; Stahelin, R. V.; Best, M. D., Synthesis and convenient functionalization of azide-labeled diacylglycerol analogues for modular access to biologically active lipid probes. *Bioconjugate Chem.* **2008**, 19 (9), 1855-1863.
 27. Saxon, E.; Bertozzi, C. R., Cell surface engineering by a modified Staudinger reaction. *Science* **2000**, 287 (5460), 2007-2010.
 28. (a) Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click chemistry: Diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed.* **2001**, 40 (11), 2004-2021; (b) Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radic, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B., Click chemistry in situ: Acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from an array of building blocks. *Angew. Chem., Int. Ed.* **2002**, 41 (6), 1053-1057; (c) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B., A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed.* **2002**, 41 (14), 2596-2599.

29. (a) Kohn, M.; Breinbauer, R., The Staudinger ligation - A gift to chemical biology'. *Angew. Chem., Int. Ed.* **2004**, *43* (24), 3106-3116; (b) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R., A "traceless" Staudinger ligation for the chemoselective synthesis of amide bonds. *Org. Lett.* **2000**, *2* (14), 2141-2143.
30. Neef, A. B.; Schultz, C., Selective Fluorescence Labeling of Lipids in Living Cells. *Angew. Chem. Int. Ed.* **2009**, *48* (8), 1498-1500.
31. Wright, A. T.; Cravatt, B. F., Chemical proteomic probes for profiling cytochrome p450 activities and drug interactions in vivo. *Chem. Biol.* **2007**, *14*, 1043-1051.
32. Wang, J.; Uttamchandani, M.; Li, J. Q.; Hu, M. Y.; Yao, S. Q., "Click" synthesis of small molecule probes for activity-based fingerprinting of matrix metalloproteases. *Chem. Commun.* **2006**, (36), 3783-3785.
33. Gubbens, J.; Ruijter, E.; de Fays, L. E. V.; Damen, J. M. A.; de Kruijff, B.; Slijper, M.; Rijkers, D. T. S.; Liskamp, R. M. J.; de Kroon, A., Photocrosslinking and Click Chemistry Enable the Specific Detection of Proteins Interacting with Phospholipids at the Membrane Interface. *Chem. Biol.* **2009**, *16* (1), 3-14.
34. Eckstein, F., Nucleoside phosphorothioates. *J. Am. Chem. Soc.* **1966**, *88* (18), 4292-&.
35. Eckstein, F., Nucleoside phosphorothioates. *J. Am. Chem. Soc.* **1970**, *92* (15), 4718-&.
36. (a) Xu, Y.; Lee, S. A.; Kutateladze, T. G.; Sbrissa, D.; Shisheva, A.; Prestwich, G. D., Chemical synthesis and molecular recognition of phosphatase-resistant analogues of phosphatidylinositol-3-phosphate. *J. Am. Chem. Soc.* **2006**, *128* (3), 885-897; (b) Zhang, H. L.; He, J.; Kutateladze, T. G.; Sakai, T.; Sasaki, T.; Markadiou, N.; Erneux, C.; Prestwich, G. D., 5-Stabilized Phosphatidylinositol 3,4,5-Trisphosphate Analogues Bind Grp1 PH, Inhibit Phosphoinositide Phosphatases, and Block Neutrophil Migration. *ChemBioChem* **2010**, *11* (3), 388-395.
37. Rytczak, P.; Koziolkiewicz, M.; Okruszek, A., The chemical synthesis of phosphorothioate and phosphorodithioate analogues of lysophosphatidic acid (LPA) and cyclic phosphatidic acid (CPA). *New J. Chem.* **2010**, *34* (5), 1008-1017.
38. (a) Prestwich, G. D.; Xu, Y.; Qian, L.; Hama, K.; Aoki, J., Synthesis and biological evaluation of metabolically stabilized lysophosphatidic acid analogues. *FASEB J.* **2003**, *17* (5), A994-A994; (b) Xu, Y.; Qian, L.; Prestwich, G. D., Synthesis of alpha-fluorinated phosphonates from alpha-fluorovinylphosphonates: A new route to analogues of lysophosphatidic acid. *Org. Lett.* **2003**, *5* (13), 2267-2270; (c) Xu, Y.; Qian, L.; Pontsler, A. V.; McIntyre, T. M.; Prestwich, G. D., Synthesis of difluoromethyl substituted lysophosphatidic acid analogues. *Tetrahedron* **2004**, *60* (1), 43-49.
39. Hampton, A.; Brox, L. W.; Bayer, M., Analogs of inosine 5'-phosphate with phosphorus - nitrogen and phosphorus - sulfur bonds . binding and kinetic studies with inosine 5'-phosphate dehydrogenase. *Biochemistry* **1969**, *8* (6), 2303-&.

40. Gajewiak, J.; Xu, Y.; Lee, S. A.; Kutateladze, T. G.; Prestwich, G. D., Synthesis and molecular recognition of phosphatidylinositol-3-methylenephosphate. *Org. Lett.* **2006**, 8 (13), 2811-2813.
41. Huang, W.; Zhang, H. L.; Davrazou, F.; Kutateladze, T. G.; Shi, X. B.; Gozani, O.; Prestwich, G. D., Stabilized phosphatidylinositol-5-phosphate analogues as ligands for the nuclear protein ING2: Chemistry, biology, and molecular modeling. *J. Am. Chem. Soc.* **2007**, 129 (20), 6498-6506.
42. Zhang, H. L.; Markadieu, N.; Beauwens, R.; Erneux, C.; Prestwich, G. D., Synthesis and biological activity of PTEN-resistant analogues of phosphatidylinositol 3,4,5-trisphosphate. *J. Am. Chem. Soc.* **2006**, 128, 16464-16465.
43. Prestwich, G. D.; Xu, Y.; Qian, L.; Gajewiak, J.; Jiang, G., New metabolically stabilized analogues of lysophosphatidic acid: agonists, antagonists and enzyme inhibitors. *Biochem. Soc. Trans.* **2005**, 33, 1357-1361.
44. Xu, Y.; Fang, Y. M.; Chen, J.; Prestwich, G. D., Activation of mTOR signaling by novel fluoromethylene phosphonate analogues of phosphatidic acid. *Bioorg. Med. Chem. Lett.* **2004**, 14 (6), 1461-1464.
45. Wang, X. M., Regulatory functions of phospholipase D and phosphatidic acid in plant growth, development, and stress responses. *Plant Physiol.* **2005**, 139 (2), 566-573.
46. Carman, G. M.; Han, G. S., Phosphatidic Acid Phosphatase, a Key Enzyme in the Regulation of Lipid Synthesis. *J. Biol. Chem.* **2009**, 284 (5), 2593-2597.
47. Gerke, V.; Creutz, C. E.; Moss, S. E., Annexins: Linking Ca²⁺ signalling to membrane dynamics. *Nat. Rev. Mol. Cell Biol.* **2005**, 6 (6), 449-461.
48. Wang, X. M.; Devalah, S. P.; Zhang, W. H.; Welti, R., Signaling functions of phosphatidic acid. *Prog. Lipid Res.* **2006**, 45 (3), 250-278.
49. (a) Gupta, S.; Ramjaun, A. R.; Haiko, P.; Wang, Y. H.; Warne, P. H.; Nicke, B.; Nye, E.; Stamp, G.; Alitalo, K.; Downward, J., Binding of Ras to phosphoinositide 3-kinase p110 α is required for Ras-driven tumorigenesis in mice. *Cell* **2007**, 129 (5), 957-968; (b) Wittinghofer, A.; Waldmann, H., Ras - A molecular switch involved in tumor formation. *Angew. Chem. Int. Ed.* **2000**, 39 (23), 4193-4214.
50. Ochoa, W. F.; Corbalan-Garcia, S.; Eritja, R.; Rodriguez-Alfaro, J. A.; Gomez-Fernandez, J. C.; Fita, I.; Verdaguer, N., Additional binding sites for anionic phospholipids and calcium ions in the crystal structures of complexes of the C2 domain of protein kinase C α . *J. Mol. Biol.* **2002**, 320 (2), 277-291.
51. Ochoa, W. F.; Garcia-Garcia, J.; Corbalan-Garcia, I. F. S.; Verdaguer, N.; Gomez-Fernandez, J. C., Structure of the C2 domain from novel protein kinase C ϵ . A membrane binding model for Ca (2⁺)-independent C2 domains. *J. Mol. Biol.* **2001**, 311 (4), 837-849.
52. (a) Kooijman, E. E.; Carter, K. M.; van Laar, E. G.; Chupin, V.; Burger, K. N. J.; de Kruijff, B., What makes the bioactive lipids phosphatidic acid and lysophosphatidic acid so special? *Biochemistry* **2005**, 44 (51), 17007-17015; (b) Kooijman, E. E.; Tieleman, D. P.; Testerink, C.; Munnik, T.; Rijkers, D. T. S.

- Burger, K. N. J.; de Kruijff, B., An electrostatic/hydrogen bond switch as the basis for the specific interaction of phosphatidic acid with proteins. *J. Biol. Chem.* **2007**, *282* (15), 11356-11364.
53. Hanshaw, R. G.; Stahelin, R. V.; Smith, B. D., Noncovalent keystone interactions controlling biomembrane structure. *Chem. Eur. J.* **2008**, *14* (6), 1690-1697.
 54. Fang, Y. M.; Vilella-Bach, M.; Bachmann, R.; Flanigan, A.; Chen, J., Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* **2001**, *294* (5548), 1942-1945.
 55. Zhao, C.; Du, G. W.; Skowronek, K.; Frohman, M. A.; Bar-Sagi, D., Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. *Nat. Cell Biol.* **2007**, *9* (6), 706-U171.
 56. Dominguez-Gonzalez, I.; Vazquez-Cuesta, S. N.; Algaba, A.; Diez-Guerra, F. J., Neurogranin binds to phosphatidic acid and associates to cellular membranes. *Biochem. J.* **2007**, *404*, 31-43.
 57. Moritz, A.; Degraan, P. N. E.; Gispen, W. H.; Wirtz, K. W. A., Phosphatidic-acid is a specific activator of phosphatidylinositol-4-phosphate kinase. *J. Biol. Chem.* **1992**, *267* (11), 7207-7210.
 58. Frank, C.; Keilhack, H.; Opitz, F.; Zschornig, O.; Bohmer, F. D., Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation. *Biochemistry* **1999**, *38* (37), 11993-12002.
 59. Jones, J. A.; Hannun, Y. A., Tight binding inhibition of protein phosphatase-1 by phosphatidic acid - Specificity of inhibition by the phospholipid. *J. Biol. Chem.* **2002**, *277* (18), 15530-15538.
 60. Baillie, G. S.; Huston, E.; Scotland, G.; Hodgkin, M.; Gall, I.; Peden, A. H.; MacKenzie, C.; Houslay, E. S.; Currie, R.; Pettitt, T. R.; Walmsley, A. R.; Wakelam, M. J. O.; Warwicker, J.; Houslay, M. D., TAPAS-1, a novel microdomain within the unique N-terminal region of the PDE4A1 cAMP-specific phosphodiesterase that allows rapid, Ca²⁺-triggered membrane association with selectivity for interaction with phosphatidic acid. *J. Biol. Chem.* **2002**, *277* (31), 28298-28309.
 61. Grange, M.; Sette, C.; Cuomo, M.; Conti, M.; Lagarde, M.; Prigent, A. F.; Nemoz, G., The cAMP-specific phosphodiesterase PDE4D3 is regulated by phosphatidic acid binding - Consequences for cAMP signaling pathway and characterization of a phosphatidic acid binding site. *J. Biol. Chem.* **2000**, *275* (43), 33379-33387.
 62. Manifava, M.; Thuring, J.; Lim, Z. Y.; Packman, L.; Holmes, A. B.; Ktistakis, N. T., Differential binding of traffic-related proteins to phosphatidic acid- or phosphatidylinositol (4,5)-bisphosphate-coupled affinity reagents. *J. Biol. Chem.* **2001**, *276* (12), 8987-8994.
 63. Lim, Z. Y.; Thuring, J. W.; Holmes, A. B.; Manifava, M.; Ktistakis, N. T., Synthesis and biological evaluation of a PtdIns(4,5)P-2 and a phosphatidic acid affinity matrix. *Journal of the Chemical Society-Perkin Transactions 1* **2002**, (8), 1067-1075.

64. Picq, M.; Huang, Y.; Lagarde, M.; Doutheau, A.; Nemoz, G., Synthesis of photoreactive phosphatidic acid analogues displaying activatory properties on cyclic AMP-phosphodiesterases. Photoaffinity labeling of an isoform of phosphodiesterase. *J. Med. Chem.* **2002**, 45 (8), 1678-1685.
65. Wang, X. M., Phospholipase D in hormonal and stress signaling. *Current Opinion in Plant Biology* **2002**, 5 (5), 408-414.
66. Gomez-Merino, F. C.; Brearley, C. A.; Ornatowska, M.; Abdel-Halim, M. E. F.; Zanol, M. I.; Mueller-Roeber, B., AtDGK2, a novel diacylglycerol kinase from *Arabidopsis thaliana*, phosphorylates 1-stearoyl-2-arachidonoyl-sn-glycerol and 1,2-dioleoyl-sn-glycerol and exhibits cold-inducible gene expression. *J. Biol. Chem.* **2004**, 279 (9), 8230-8241.
67. Xu, Y.; Tanaka, M.; Arai, H.; Aoki, J.; Prestwich, G. D., Alkyl lysophosphatidic acid and fluoromethylene phosphonate analogs as metabolically-stabilized agonists for LPA receptors. *Bioorg. Med. Chem. Lett.* **2004**, 14 (21), 5323-5328.
68. Smyth, S. S.; Sciorra, V. A.; Sigal, Y. J.; Pamuklar, Z.; Wang, Z. C.; Xu, Y.; Prestwich, G. D.; Morris, A. J., Lipid phosphate phosphatases regulate lysophosphatidic acid production and signaling in platelets - Studies using chemical inhibitors of lipid phosphate phosphatase activity. *J. Biol. Chem.* **2003**, 278 (44), 43214-43223.
69. Xu, Y.; Jiang, G. W.; Tsukahara, R.; Fujiwara, Y.; Tigyi, G.; Prestwich, G. D., Phosphonothioate and fluoromethylene phosphonate analogues of cyclic phosphatidic acid: Novel antagonists of lysophosphatidic acid receptors. *J. Med. Chem.* **2006**, 49 (17), 5309-5315.
70. (a) Clegg, R. M., Fluorescence resonance energy transfer. *Curr. Opin. Biotechnol.* **1995**, 6, 103-110; (b) Sapsford, K. E.; Berti, L.; Medintz, I. L., Materials for fluorescence resonance energy transfer analysis: Beyond traditional donor-acceptor combinations. *Angew. Chem., Int. Ed.* **2006**, 45 (28), 4562-4588; (c) Wu, P. G.; Brand, L., Resonance energy-transfer: methods and applications. *Anal. Biochem.* **1994**, 218 (1), 1-13.
71. (a) Rzepecki, P. W.; Prestwich, G. D., Synthesis of hybrid lipid probes: Derivatives of phosphatidylethanolamine-extended phosphatidylinositol 4,5-bisphosphate (Pea-PIP₂). *J. Org. Chem.* **2002**, 67 (16), 5454-5460; (b) Smith, M. D.; Gong, D.; Sudhakar, C. G.; Reno, J. C.; Stahelin, R. V.; Best, M. D., Synthesis and convenient functionalization of azide-labeled diacylglycerol analogues for modular access to biologically active lipid probes. *Bioconj. Chem.* **2008**, 19, 1855-1863.
72. Wroblewski, A. E.; Glowacka, I. E., Enantiomerically pure 4-amino-1,2,3-trihydroxybutylphosphonic acids. *Tetrahedron* **2005**, 61 (50), 11930-11938.
73. Qian, L.; Xu, Y.; Hasegawa, Y.; Aoki, J.; Mills, G. B.; Prestwich, G. D., Enantioselective responses to a phosphorothioate analogue of lysophosphatidic acid with LPA(3) receptor-selective agonist activity. *J. Med. Chem.* **2003**, 46 (26), 5575-5578.

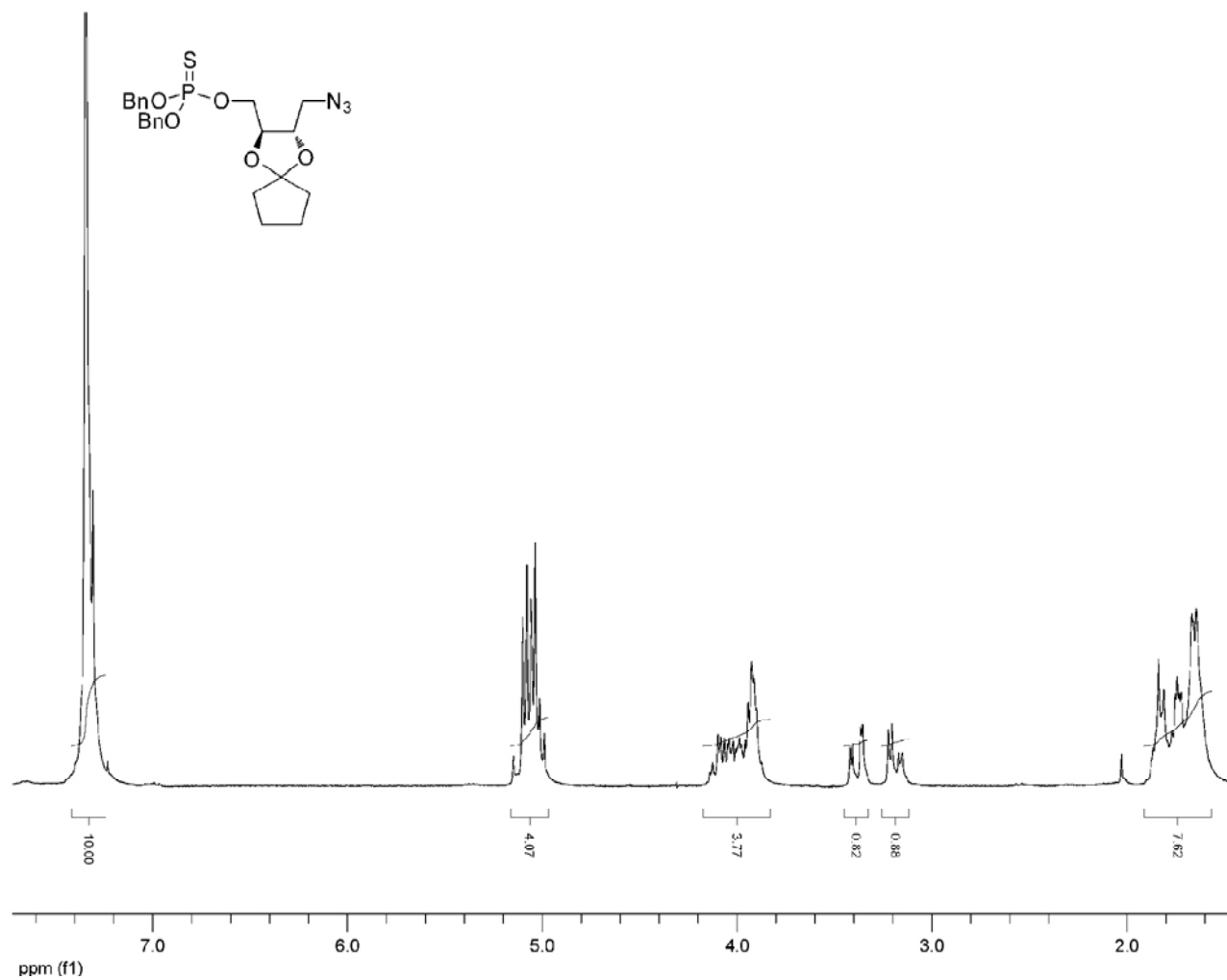
74. Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V., Polytriazoles as copper(I)-stabilizing ligands in catalysis. *Org. Lett.* **2004**, 6 (17), 2853-2855.
75. Kodali, D. R.; Tercyak, A.; Fahey, D. A.; Small, D. M., Acyl migration in 1,2-dipalmitoyl-*sn*-glycerol. *Chem. Phys. Lipids* **1990**, 52 (3-4), 163-170.
76. Bajaj, A.; Paul, B.; Kondaiah, P.; Bhattacharya, S., Structure-activity investigation on the gene transfection properties of cardiolipin mimicking gemini lipid analogues. *Bioconjugate Chem.* **2008**, 19 (6), 1283-1300.
77. Losey, E. A.; Smith, M. D.; Meng, M.; Best, M. D., Microplate-Based Analysis of Protein-Membrane Binding Interactions via Immobilization of Whole Liposomes Containing a Biotinylated Anchor. *Bioconjugate Chem.* **2009**, 20 (2), 376-383.
78. Kanoh, H.; Kai, M.; Wada, I., Molecular properties of enzymes involved in diacylglycerol and phosphatidate metabolism. *J. Lipid Mediators Cell Signalling* **1996**, 14 (1-3), 245-250.
79. Ananthanarayanan, B.; Stahelin, R. V.; Digman, M. A.; Cho, W. H., Activation mechanisms of conventional protein kinase C isoforms are determined by the ligand affinity and conformational flexibility of their C1 domains. *J. Biol. Chem.* **2003**, 278 (47), 46886-46894.
80. Takai, Y.; Kishimoto, A.; Kikkawa, U.; Mori, T.; Nishizuka, Y., Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid dependent protein-kinase system. *Biochem. Biophys. Res. Commun.* **1979**, 91 (4), 1218-1224.
81. Sampson, S. R.; Cooper, D. R., Specific protein kinase C isoforms as transducers and modulators of insulin signaling. *Mol. Genet. Metab.* **2006**, 89 (1-2), 32-47.
82. Griner, E. M.; Kazanietz, M. G., Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Cancer* **2007**, 7 (4), 281-294.
83. Mackay, H. J.; Twelves, C. J., Targeting the protein kinase C family: are we there yet? *Nat. Rev. Cancer* **2008**, 8 (3), 255-255.
84. Gutcher, I.; Webb, P. R.; Anderson, N. G., The isoform-specific regulation of apoptosis by protein kinase C. *Cell. Mol. Life Sci.* **2003**, 60 (6), 1061-1070.
85. Yang, C. F.; Kazanietz, M. G., Chimaerins: GAPs that bridge diacylglycerol signalling and the small G-protein Rac. *Biochem. J.* **2007**, 403, 1-12.
86. Rossner, S.; Fuchsbrunner, K.; Lange-Dohna, C.; Hartlage-Rubsamen, M.; Bigl, V.; Betz, A.; Reim, K.; Brose, N., Munc13-1-mediated vesicle priming contributes to secretory amyloid precursor protein processing. *J. Biol. Chem.* **2004**, 279 (27), 27841-27844.
87. Storz, P.; Doppler, H.; Toker, A., Protein kinase C delta selectively regulates protein kinase D-dependent activation of NF-kappa B in oxidative stress signaling. *Mol. Cell. Biol.* **2004**, 24 (7), 2614-2626.
88. Stahelin, R. V.; Digman, M. A.; Medkova, M.; Ananthanarayanan, B.; Rafter, J. D.; Melowic, H. R.; Cho, W. H., Mechanism of diacylglycerol-induced membrane targeting and activation of protein kinase C delta. *J. Biol. Chem.* **2004**, 279 (28), 29501-29512.

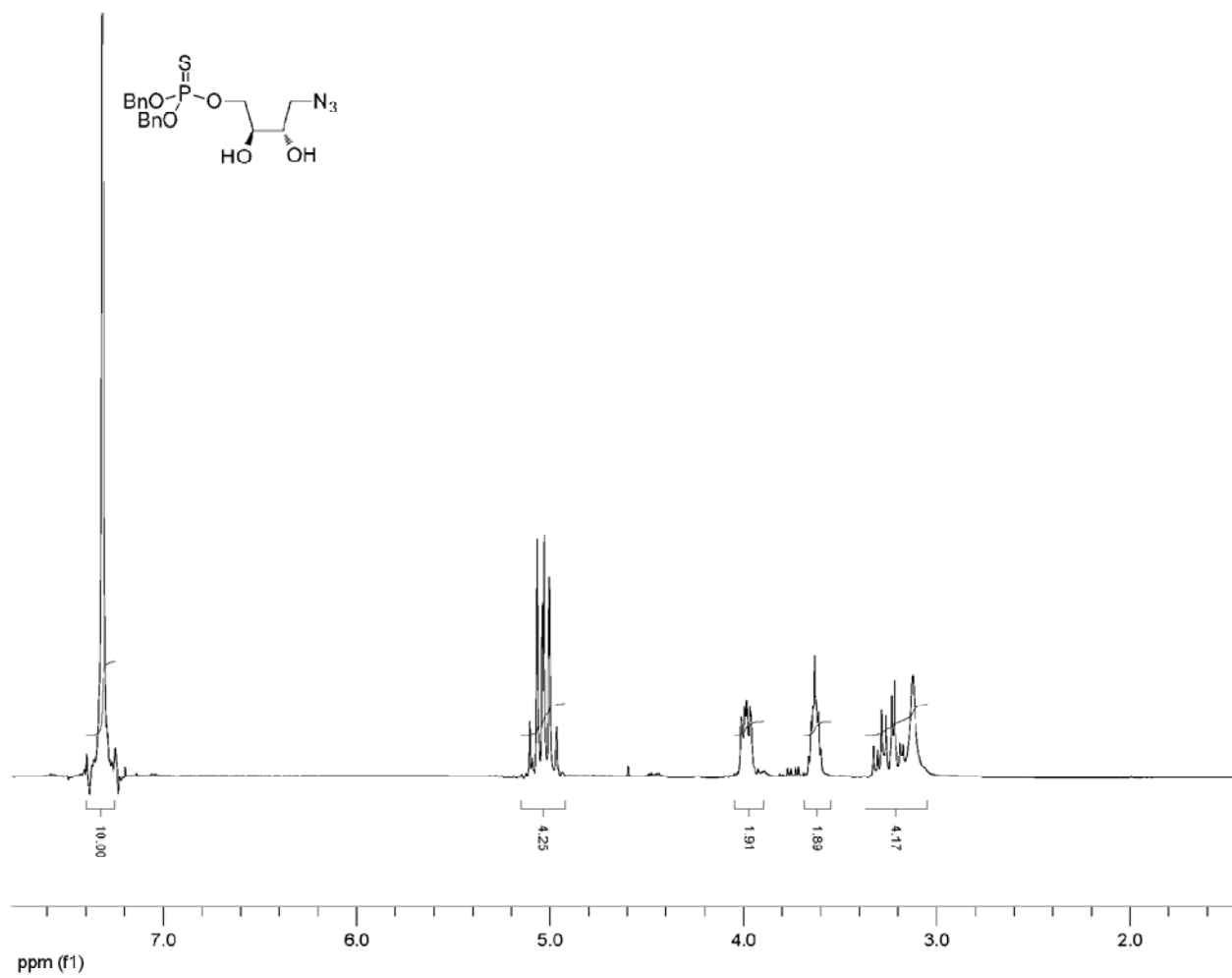
89. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B., A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem. Int. Ed.* **2002**, *41* (14), 2596-+.
90. Kohn, M.; Breinbauer, R., The Staudinger ligation - A gift to chemical biology'. *Angew. Chem. Int. Ed.* **2004**, *43* (24), 3106-3116.
91. Saxon, E.; Armstrong, J. I.; Bertozzi, C. R., A "traceless" Staudinger ligation for the chemoselective synthesis of amide bonds. *Org. Lett.* **2000**, *2* (14), 2141-2143.
92. Gubbens, J.; Ruijter, E.; de Fays, L. E. V.; Damen, J. M. A.; de Kruijff, B.; Slijper, M.; Rijkers, D. T. S.; Liskamp, R. M. J.; de Kroon, A. I. P. M., Photocrosslinking and Click Chemistry Enable the Specific Detection of Proteins Interacting with Phospholipids at the Membrane Interface. *Chem. Biol.* **2009**, *16* (1), 3-14.
93. Picq, M.; Huang, Y.; Lagarde, M.; Doutheau, A.; Nemoz, G., Synthesis of photoreactive phosphatidic acid analogues displaying activatory properties on cyclic AMP-phosphodiesterases. Photoaffinity labeling of an isoform of phosphodiesterase. *J. Med. Chem.* **2002**, *45* (8), 1678-1685.
94. Fernandes, R. A., An efficient synthesis of (-)-posticure: The sex pheromone of *Orgyia postica*. *Eur. J. Org. Chem.* **2007**, (30), 5064-5070.
95. Di Paolo, G.; De Camilli, P., Phosphoinositides in cell regulation and membrane dynamics. *Nature* **2006**, *443* (7112), 651-657.
96. McLaughlin, S.; Wang, J. Y.; Gambhir, A.; Murray, D., PIP2 AND proteins: Interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct.* **2002**, *31*, 151-175.
97. Pendaries, C.; Tronchere, H.; Plantavid, M.; Payrastre, B., Phosphoinositide signaling disorders in human diseases. *FEBS Lett.* **2003**, *546* (1), 25-31.
98. Katso, R.; Okkenhaug, K.; Ahmadi, K.; White, S.; Timms, J.; Waterfield, M. D., Cellular function of phosphoinositide 3-kinases: Implications for development, immunity, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* **2001**, *17*, 615-675.
99. Manna, D.; Albanese, A.; Park, W. S.; Cho, W., Mechanistic basis of differential cellular responses of phosphatidylinositol 3,4-bisphosphate- and phosphatidylinositol 3,4,5-trisphosphate-binding pleckstrin homology domains. *J. Biol. Chem.* **2007**, *282*, 32093-32105.
100. Traynorkaplan, A. E.; Harris, A. L.; Thompson, B. L.; Taylor, P.; Sklar, L. A., An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature* **1988**, *334* (6180), 353-356.
101. Wong, A. J.; Croce, C. M., Oncogenes and Signal-Transduction. *Hospital Practice* **1993**, *28* (7), 128-8.
102. Pinal, N.; Goberdhan, D. C. I.; Collinson, L.; Fujita, Y.; Cox, I. M.; Wilson, C.; Pichaud, F., Regulated and polarized Ptdins(3,4,5)P3 accumulation is essential for apical membrane morphogenesis in photoreceptor epithelial cells (vol 16, pg 140, 2006). *Curr. Biol.* **2006**, *16* (3), 332-332.

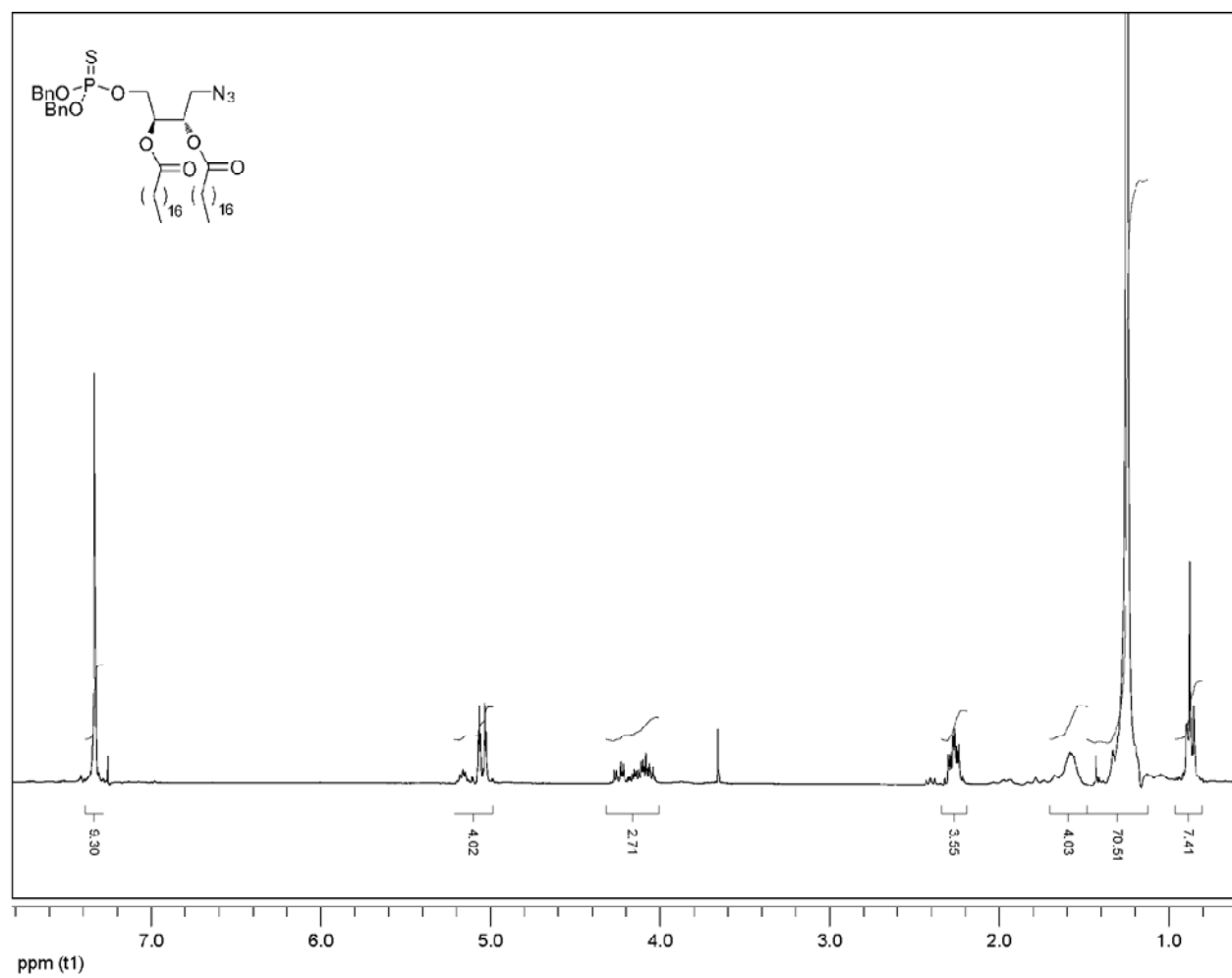
103. Sweeney, G.; Garg, R. R.; Ceddia, R. B.; Li, D. L.; Ishiki, M.; Somwar, R.; Foster, L. J.; Neilsen, P. O.; Prestwich, G. D.; Rudich, A.; Klip, A., Intracellular delivery of phosphatidylinositol (3,4,5)-trisphosphate causes incorporation of glucose transporter 4 into the plasma membrane of muscle and fat cells without increasing glucose uptake. *J. Biol. Chem.* **2004**, 279 (31), 32233-32242.
104. Maehama, T.; Dixon, J. E., The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **1998**, 273 (22), 13375-13378.
105. Samuels, Y.; Wang, Z. H.; Bardelli, A.; Silliman, N.; Ptak, J.; Szabo, S.; Yan, H.; Gazdar, A.; Powell, D. M.; Riggins, G. J.; Willson, J. K. V.; Markowitz, S.; Kinzler, K. W.; Vogelstein, B.; Velculescu, V. E., High frequency of mutations of the PIK3CA gene in human cancers. *Science* **2004**, 304 (5670), 554-554.
106. Chow, L. M. L.; Baker, S. J., PTEN function in normal and neoplastic growth. *Cancer Letters* **2006**, 241 (2), 184-196.
107. Zhang, H.; Xu, Y.; Markadiou, N.; Beauwens, R.; Erneux, C.; Prestwich, G. D., Synthesis and biological activity of phosphatidylinositol-3,4,5-trisphosphorothioate. *Bioorg. Med. Chem. Lett.* **2008**, 18 (2), 762-766.
108. Chen, J.; Prestwich, G. D., Asymmetric synthesis of water-soluble, nonhydrolyzable phosphonate analogue of phosphatidylinositol 4,5-bisphosphate. *J. Org. Chem.* **1998**, 63 (3), 430-431.
109. Zhang, H. L.; Xu, Y.; Zhang, Z.; Liman, E. R.; Prestwich, G. D., Synthesis and biological activity of phospholipase C-resistant analogues of phosphatidylinositol 4,5-bisphosphate. *J. Am. Chem. Soc.* **2006**, 128 (17), 5642-5643.
110. Kubiak, R. J.; Bruzik, K. S., Comprehensive and uniform synthesis of all naturally occurring phosphorylated phosphatidylinositols. *J. Org. Chem.* **2003**, 68 (3), 960-968.
111. Bruzik, K. S.; Tsai, M. D., Efficient and systematic syntheses of enantiomerically pure and regiospecifically protected myoinositols. *J. Am. Chem. Soc.* **1992**, 114 (16), 6361-6374.
112. Gong, D. H.; Bostic, H. E.; Smith, M. D.; Best, M. D., Synthesis of Modular Headgroup Conjugates Corresponding to All Seven Phosphatidylinositol Polyphosphate Isomers for Convenient Probe Generation. *Eur. J. Org. Chem.* **2009**, (24), 4170-4179.

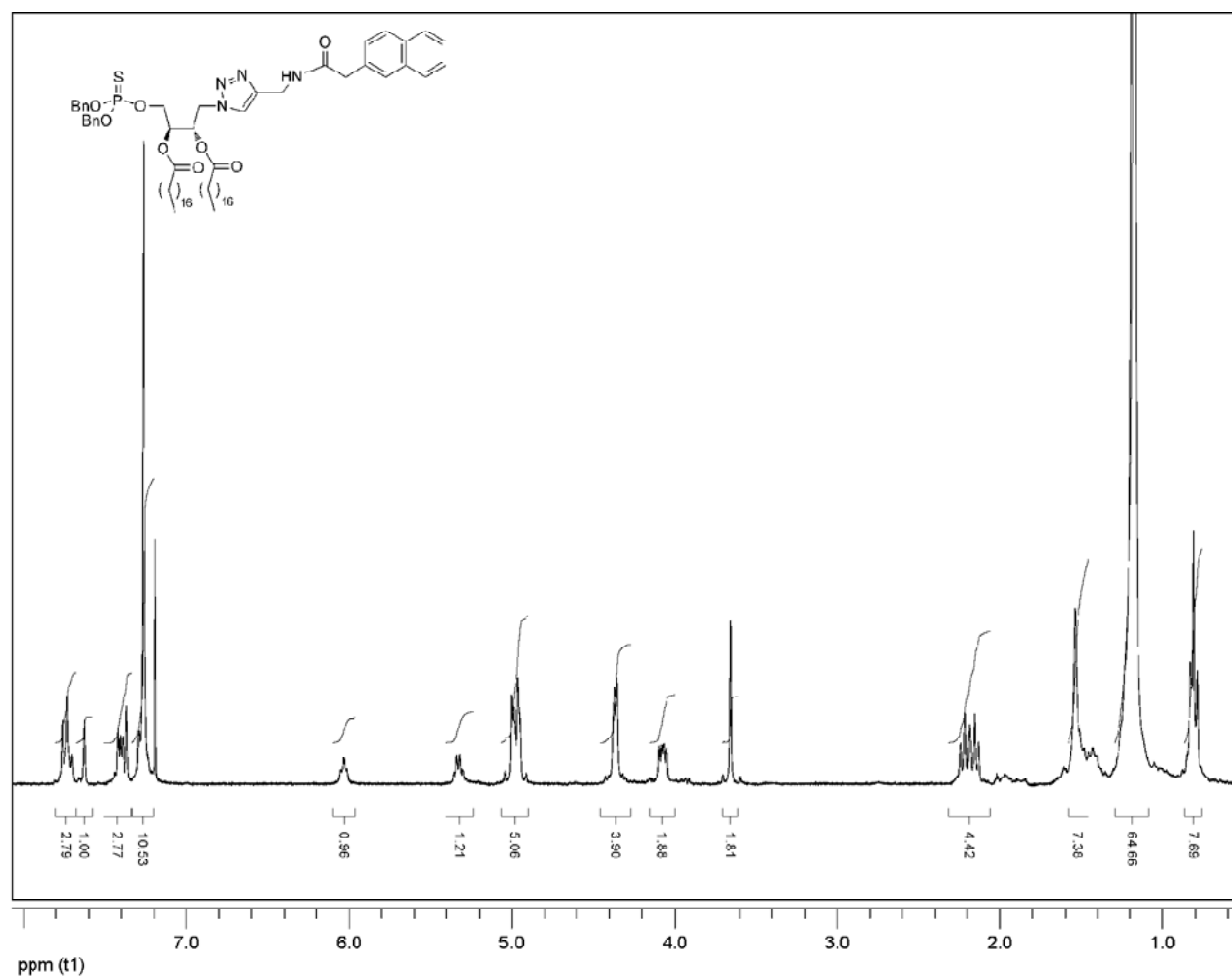
Appendix

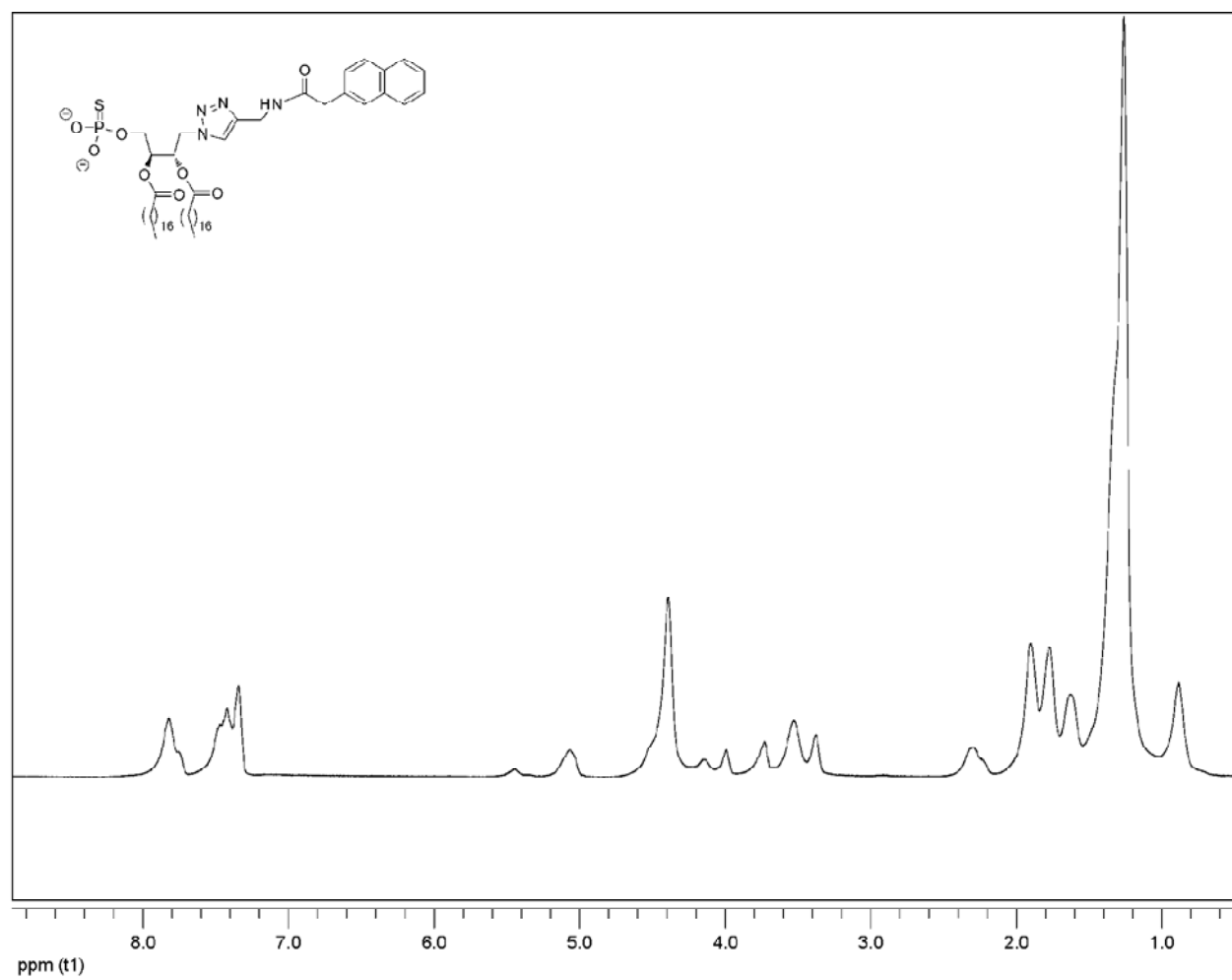
NMR Spectra

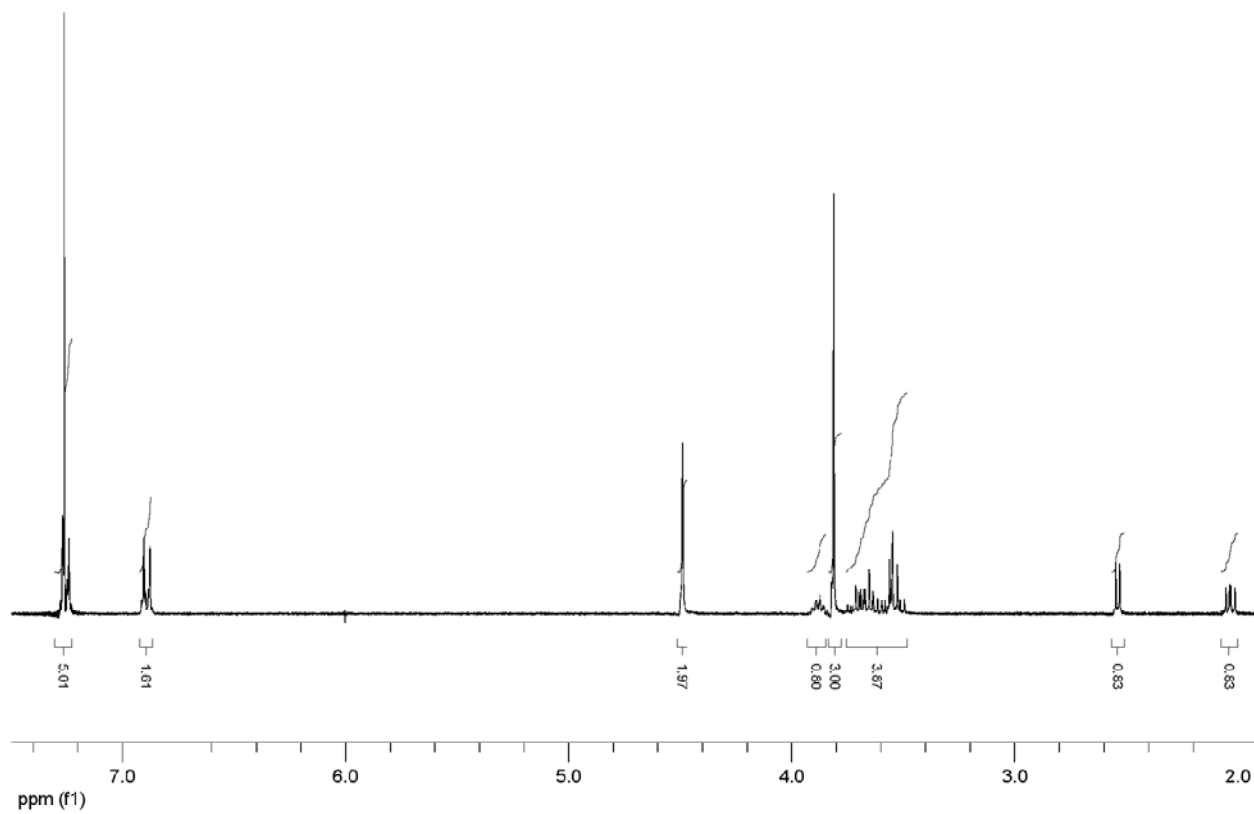
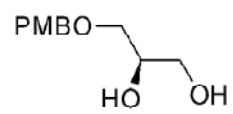


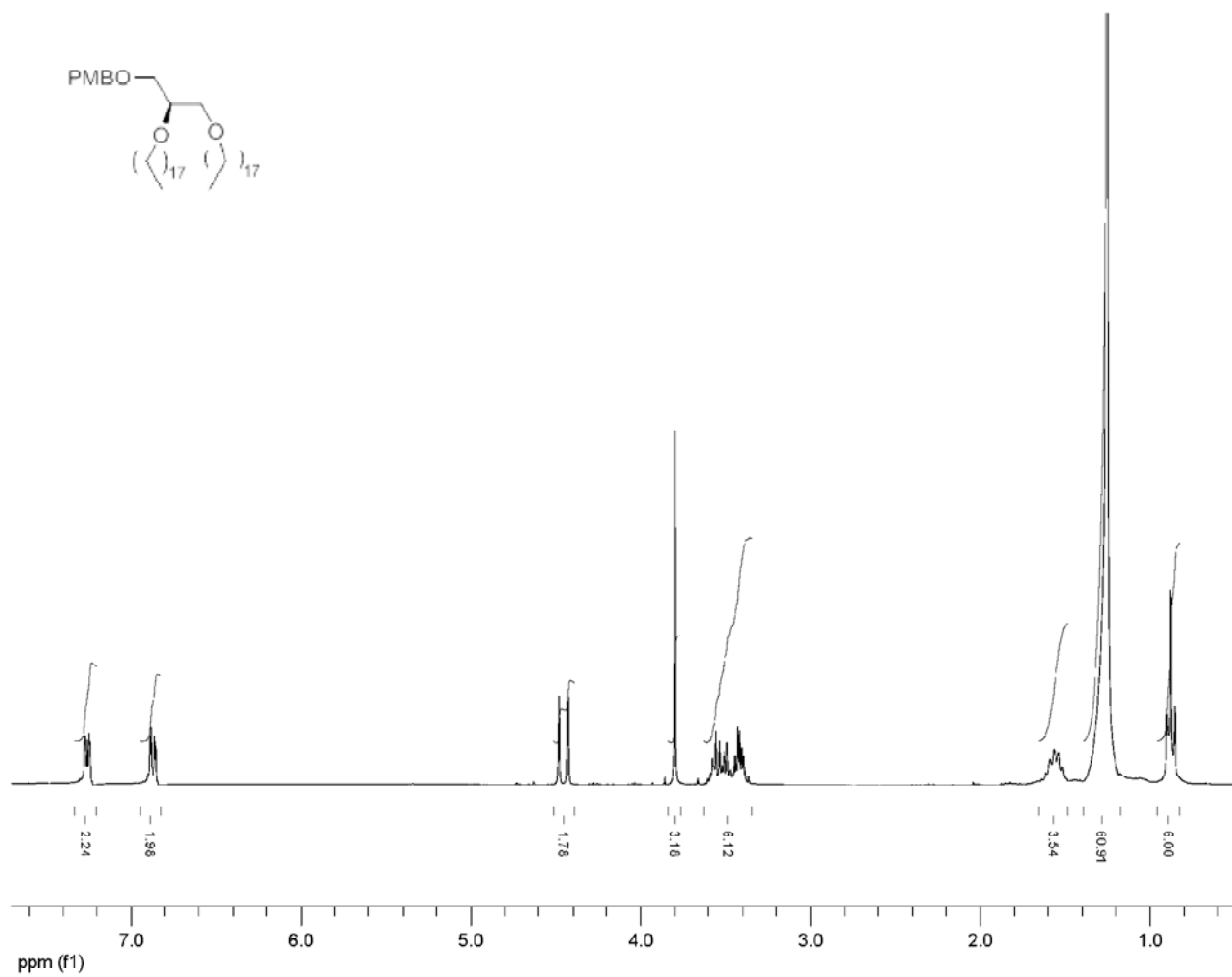


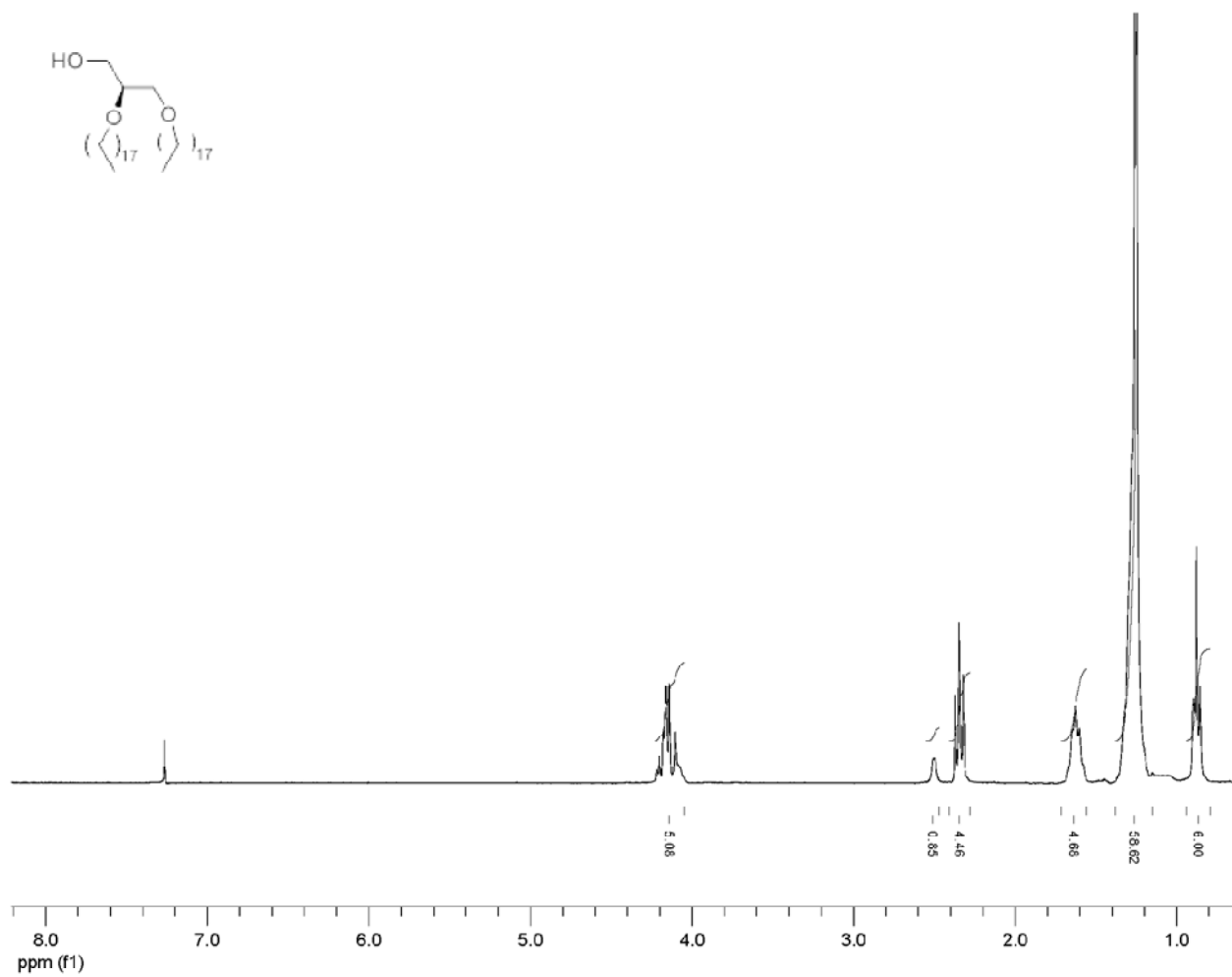


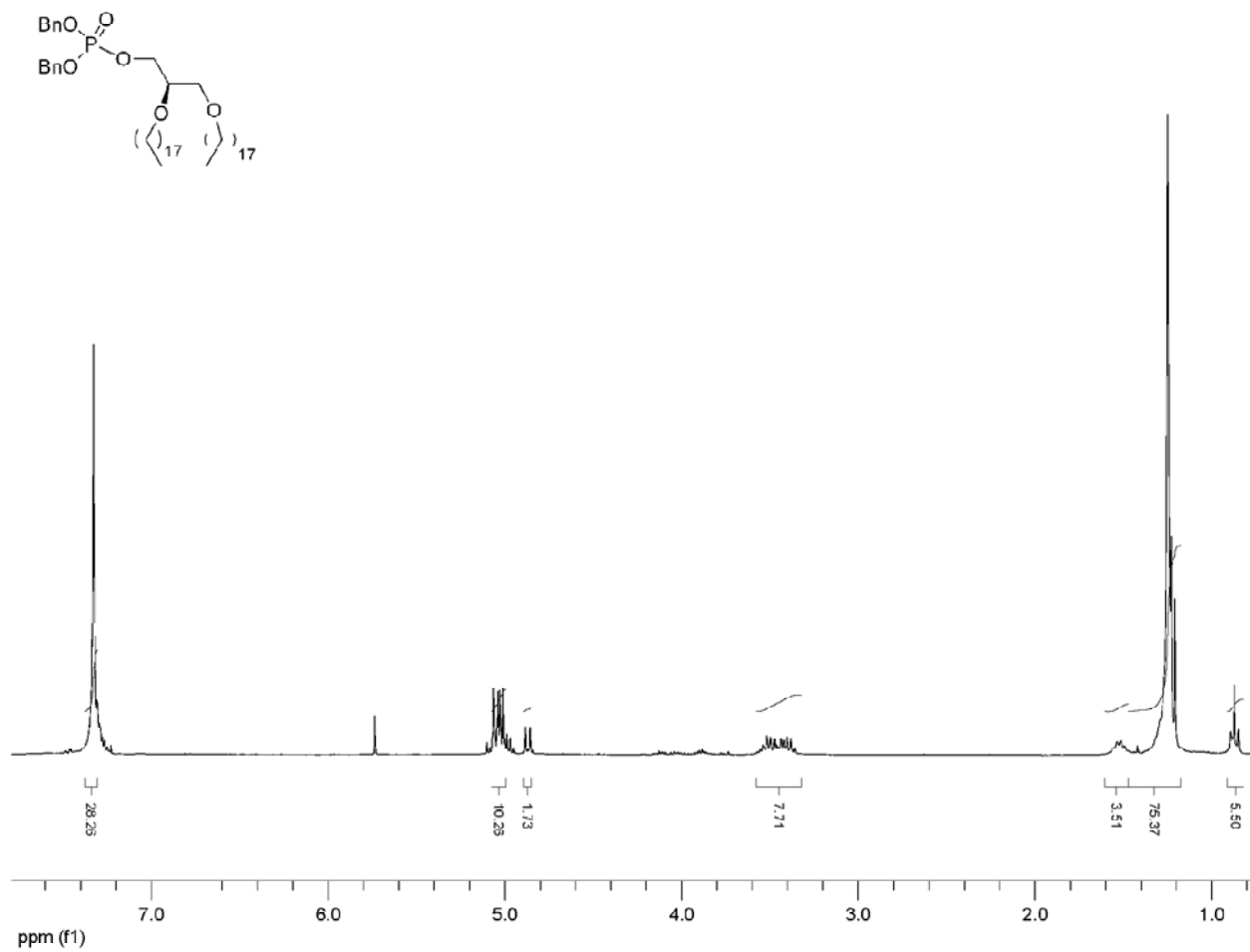


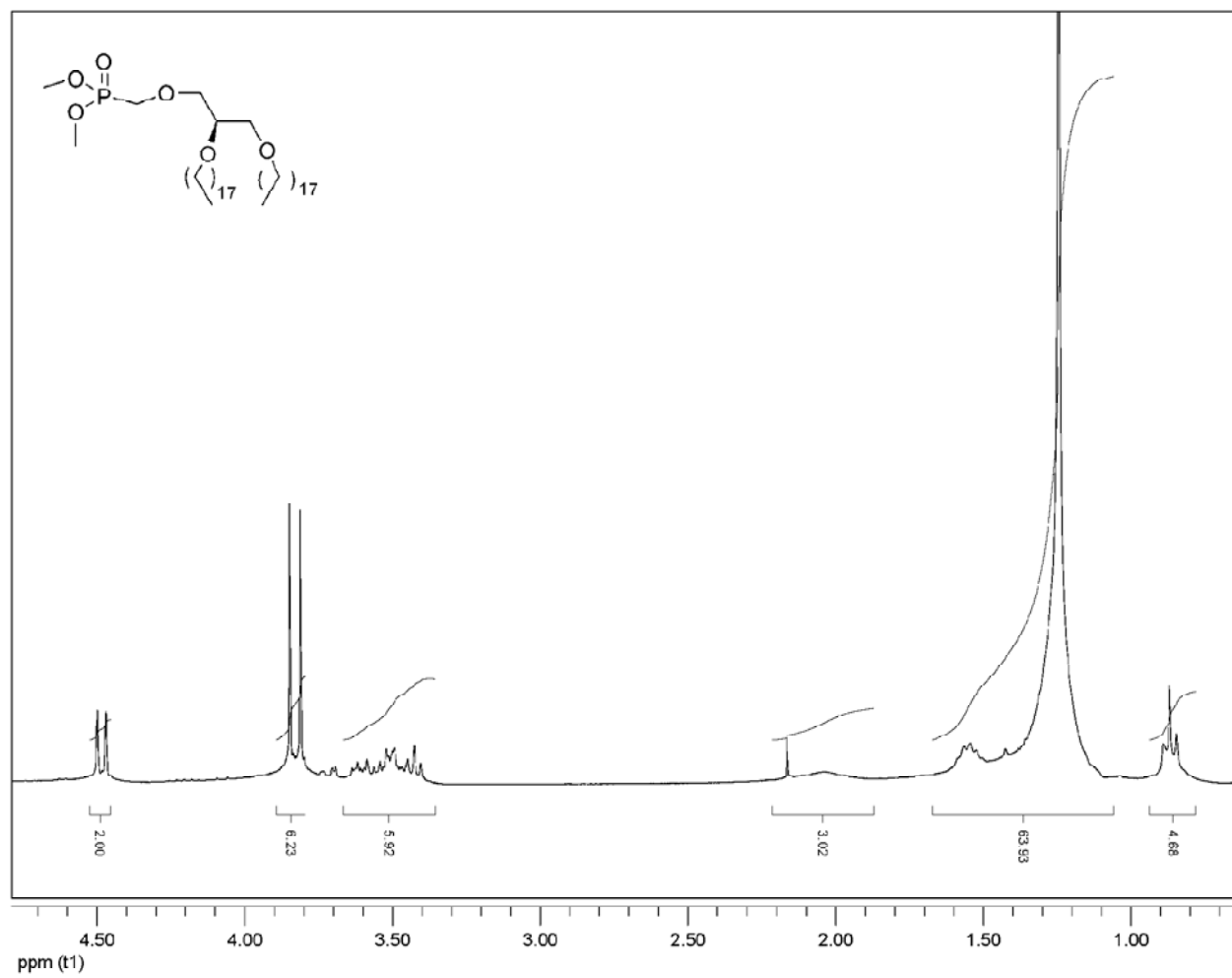


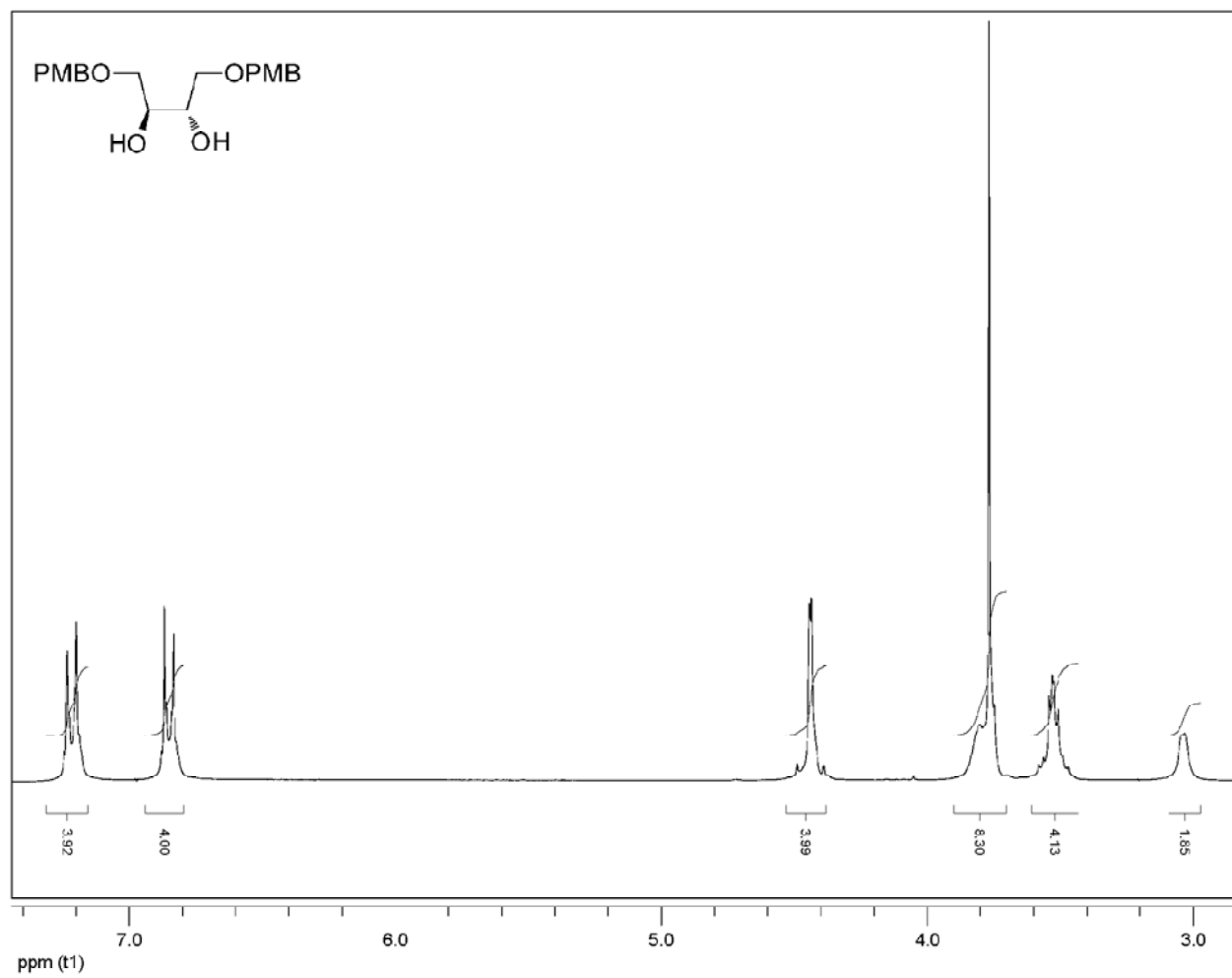


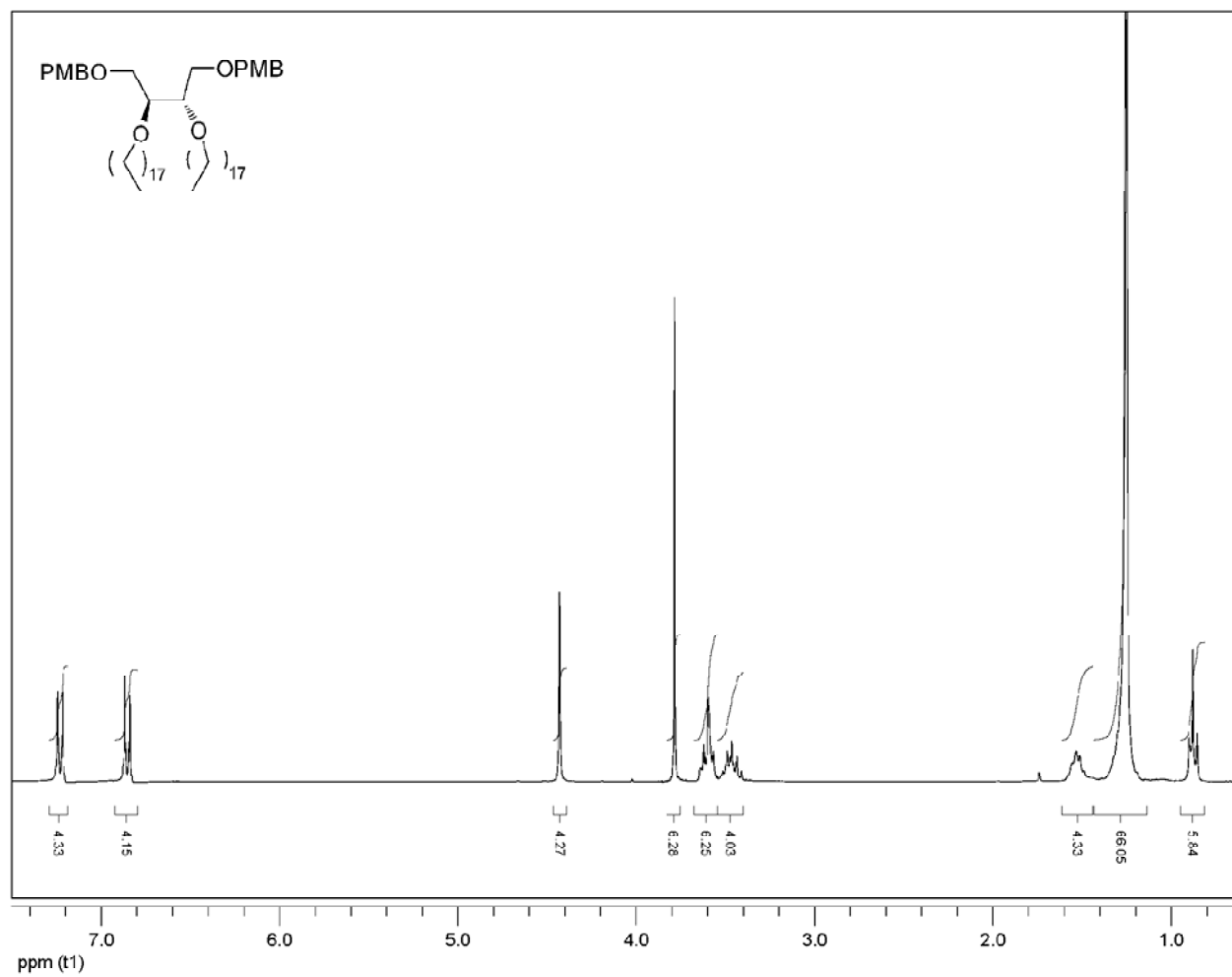


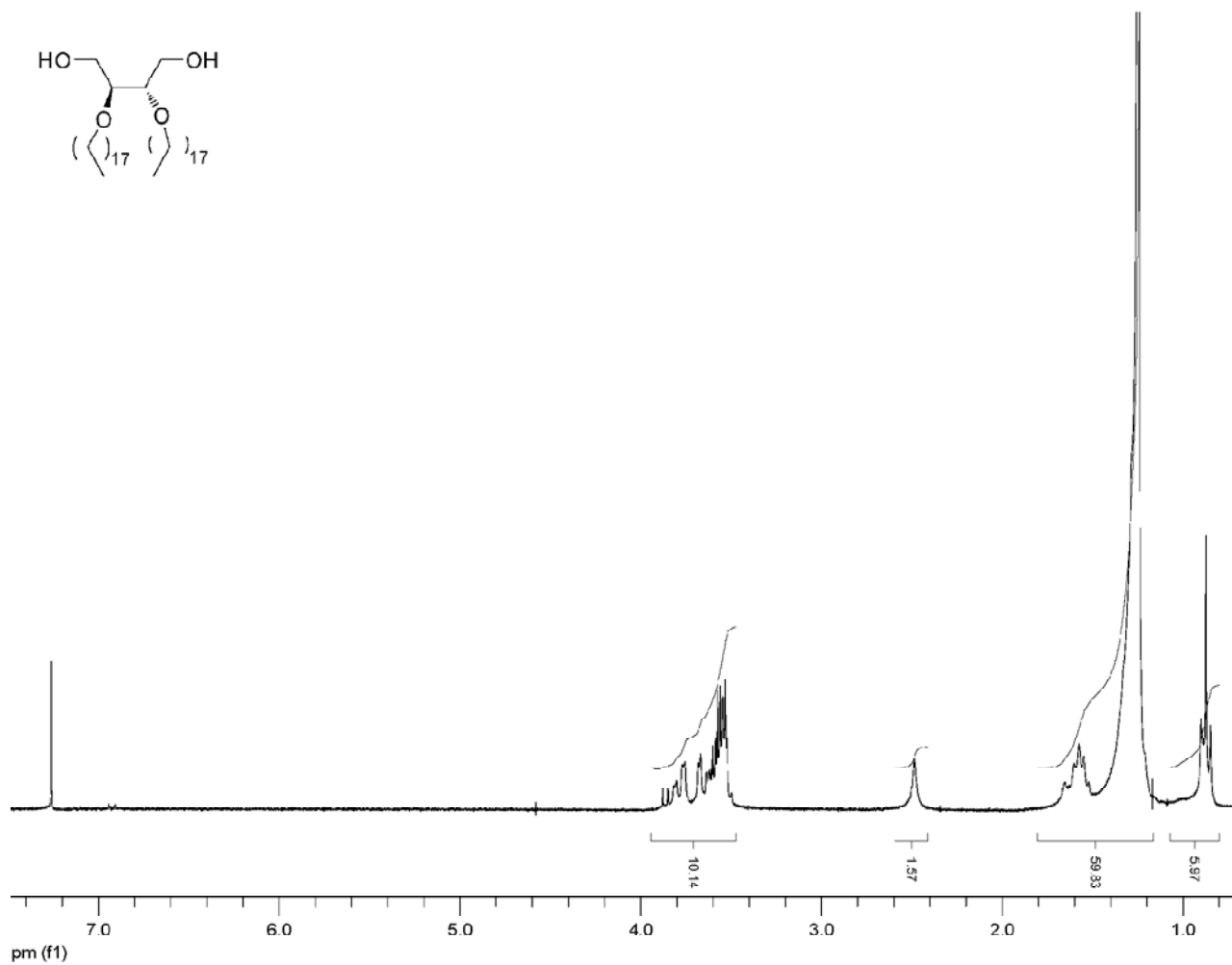
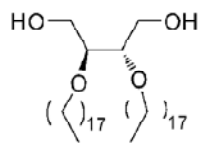


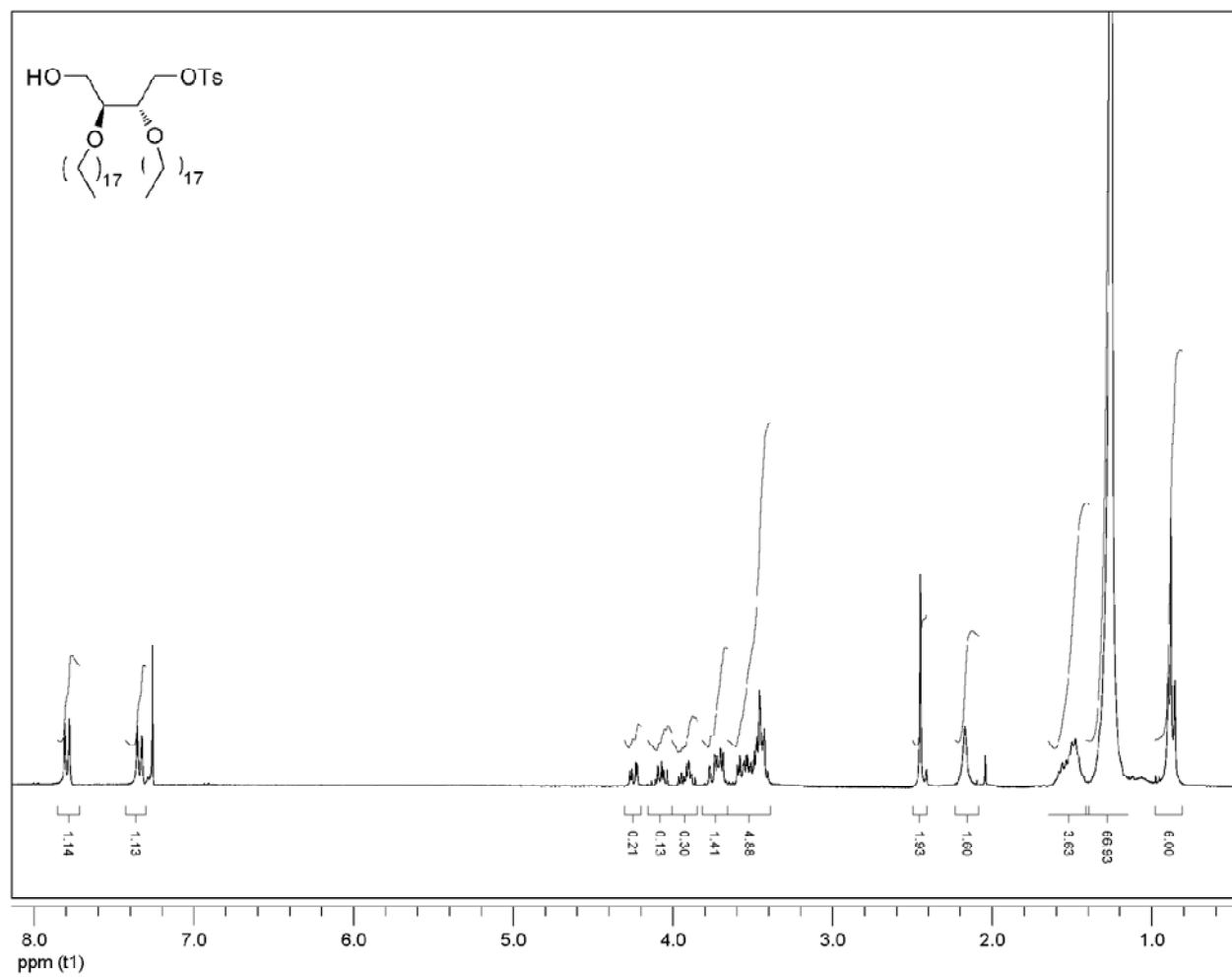


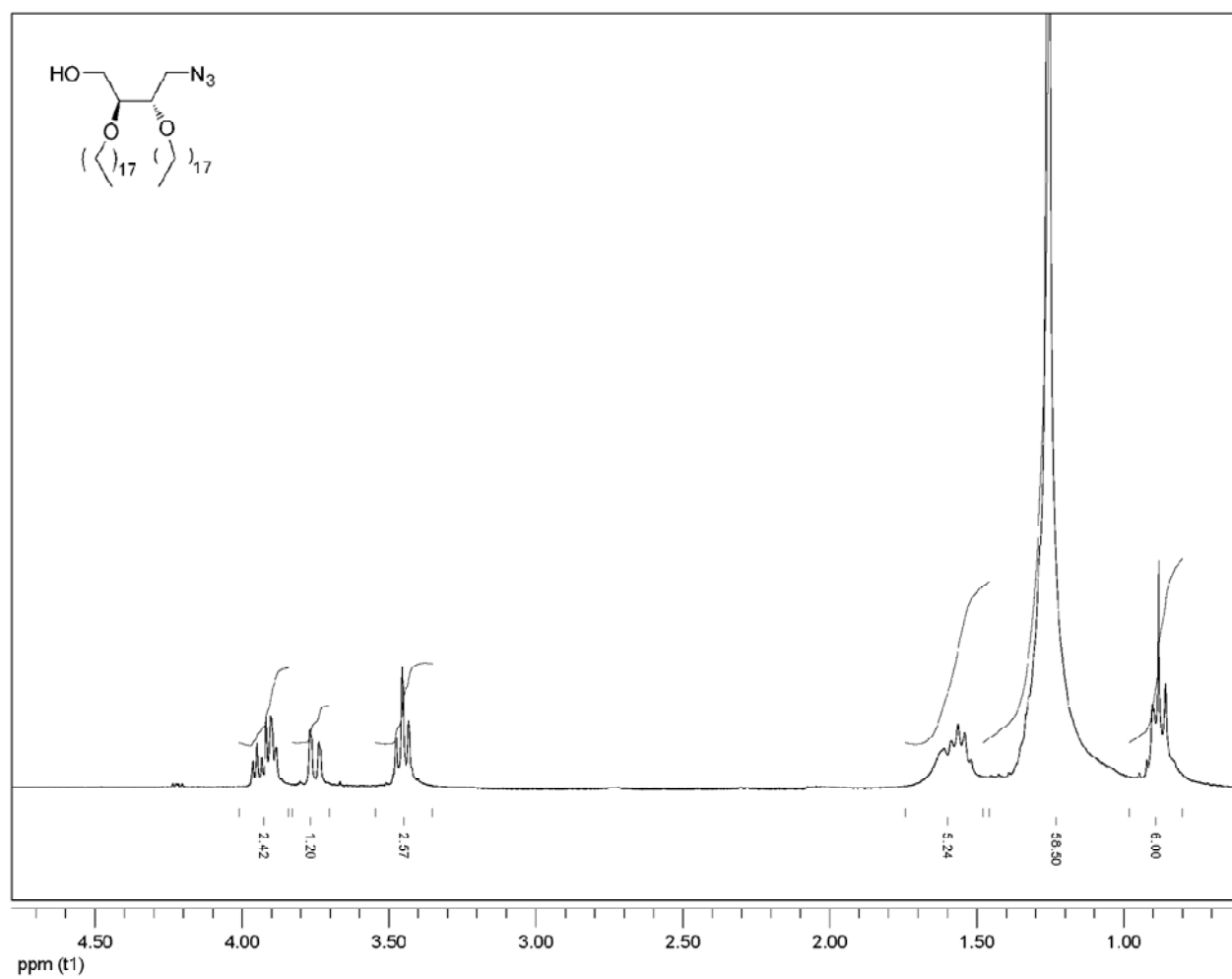


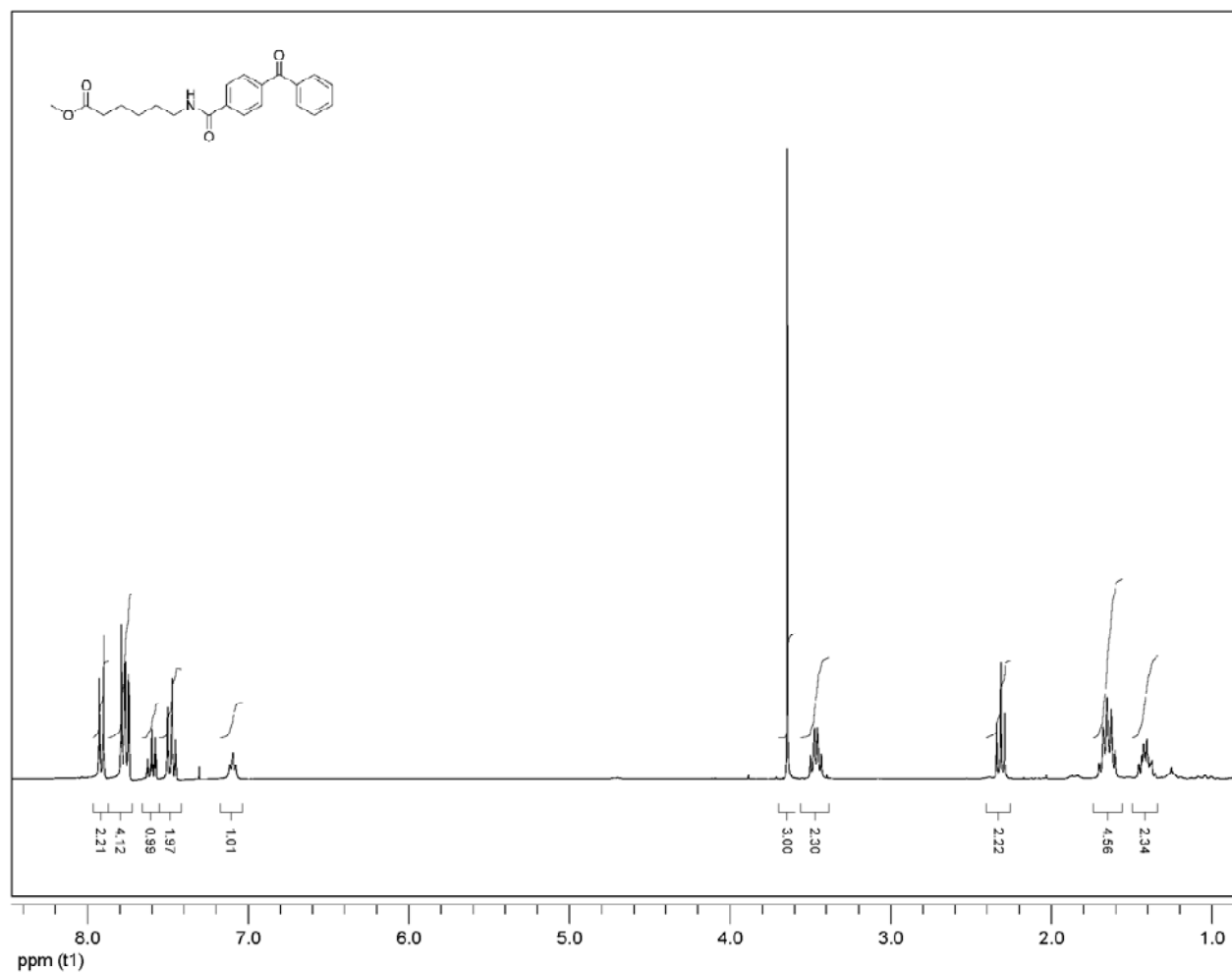


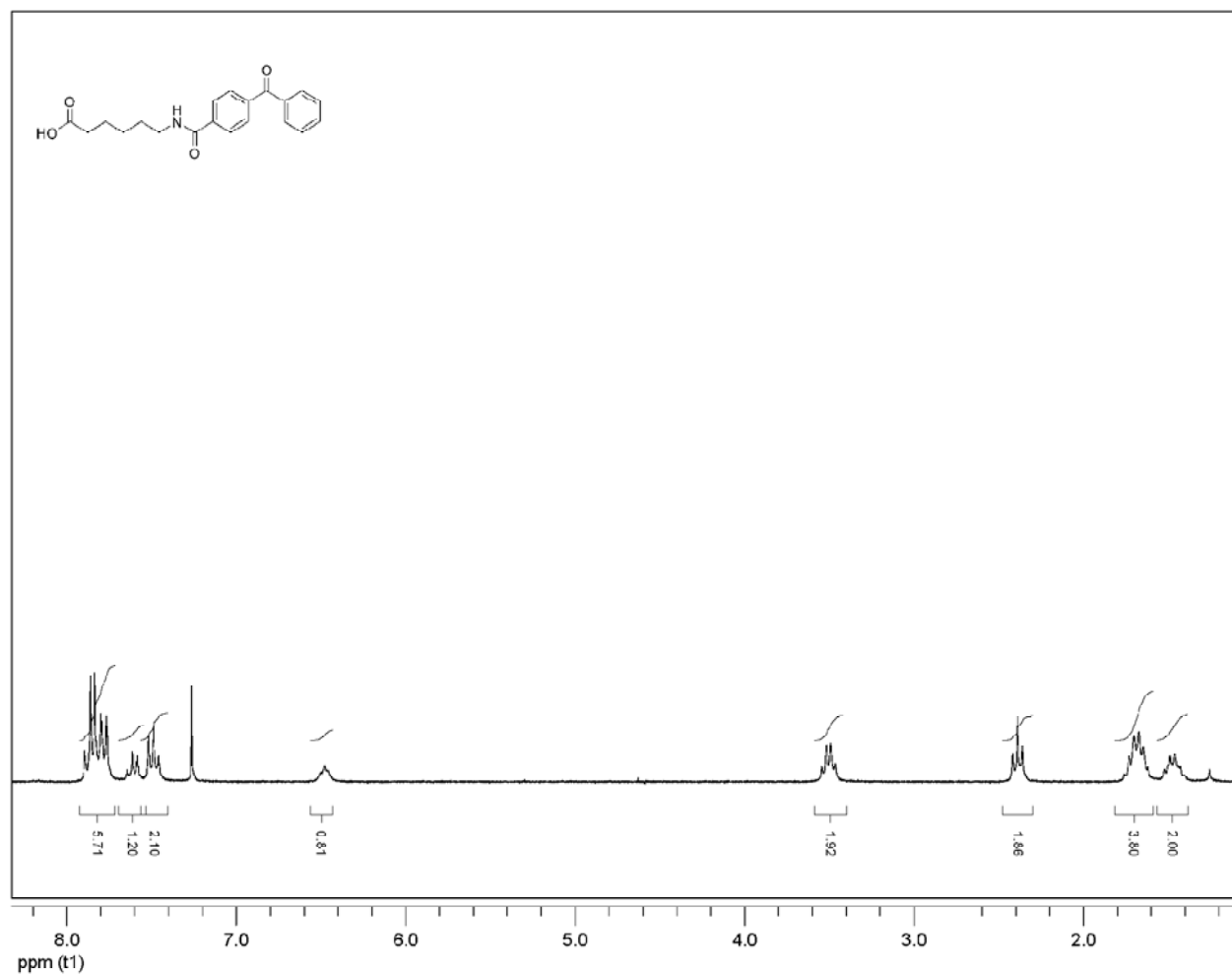


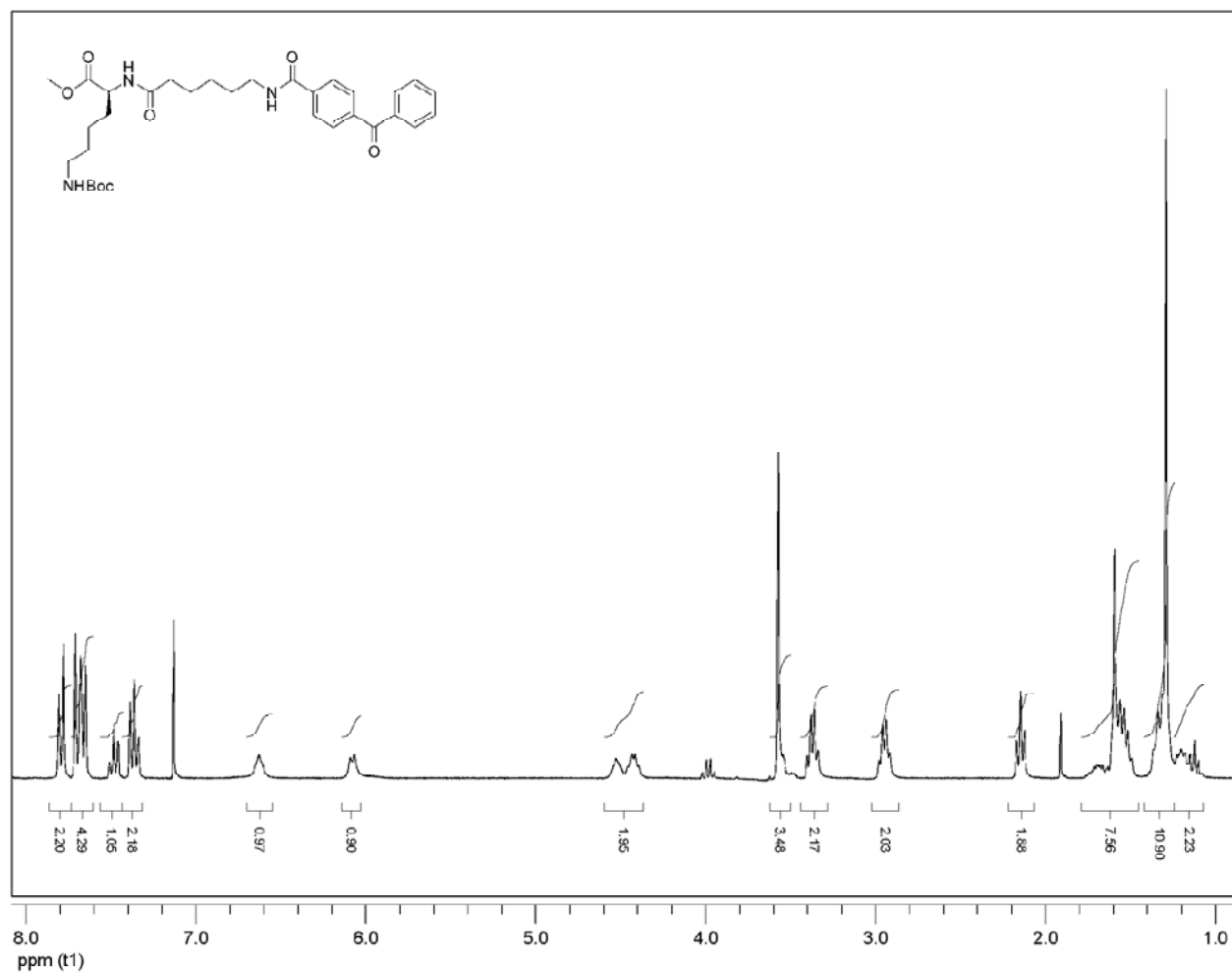


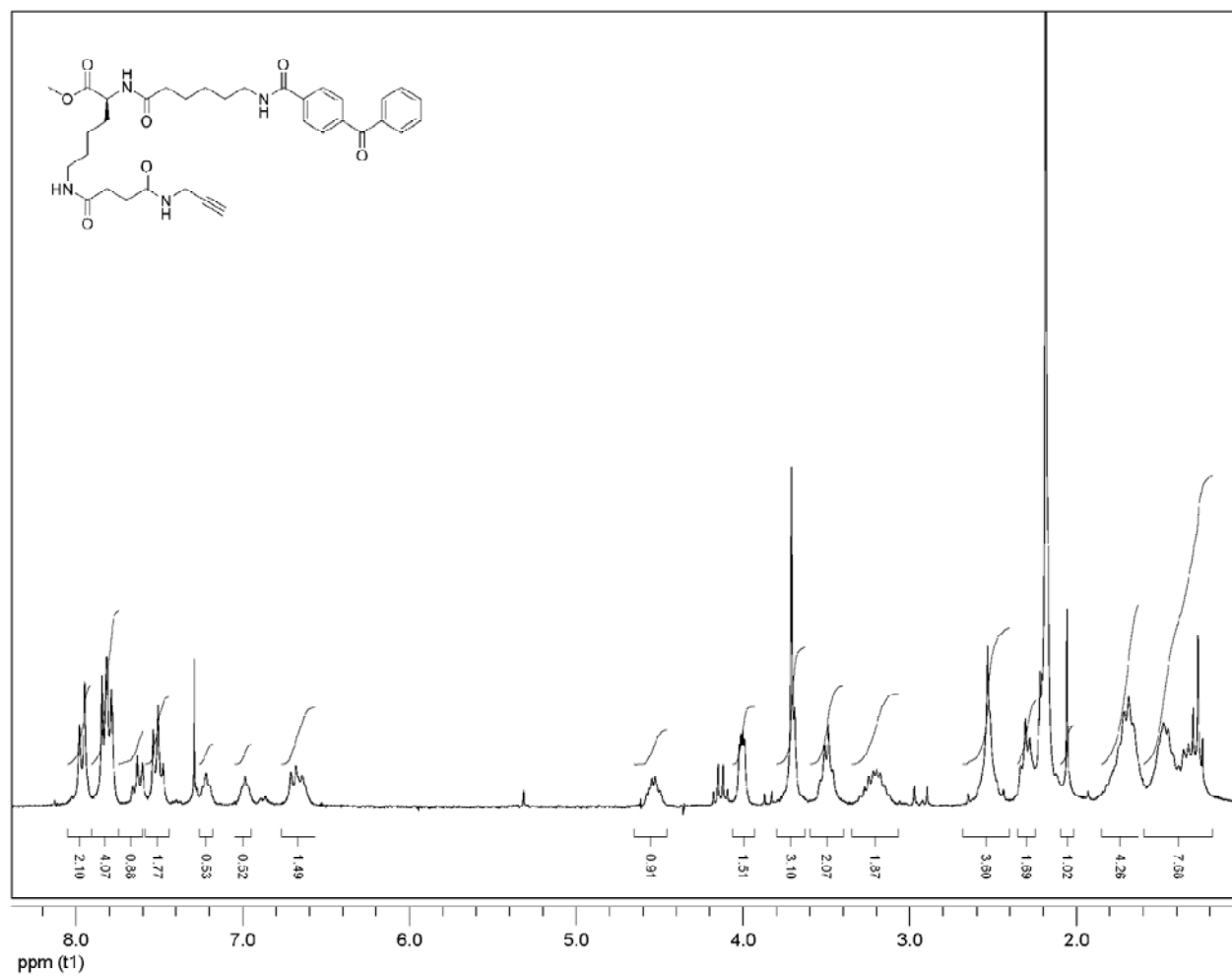


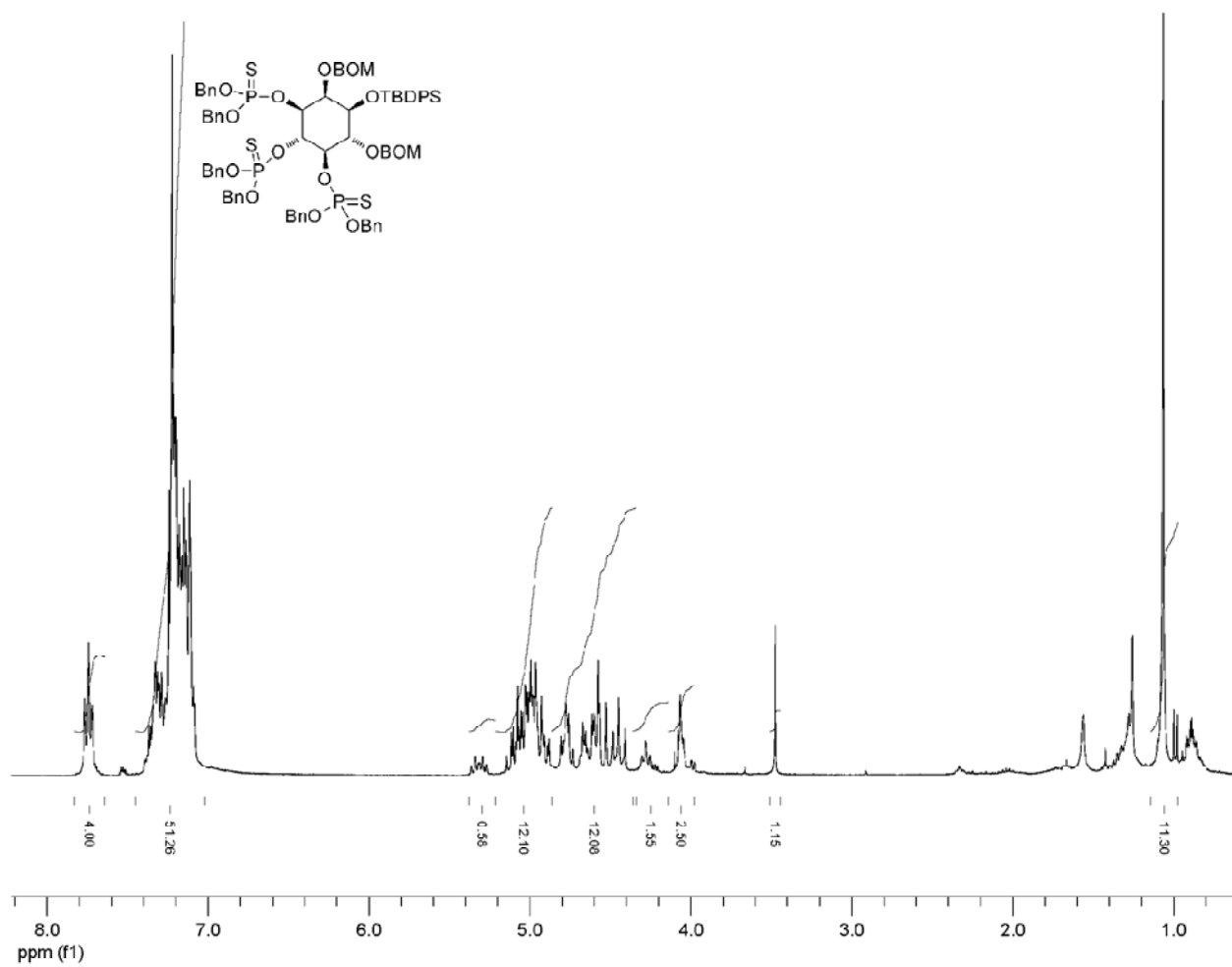


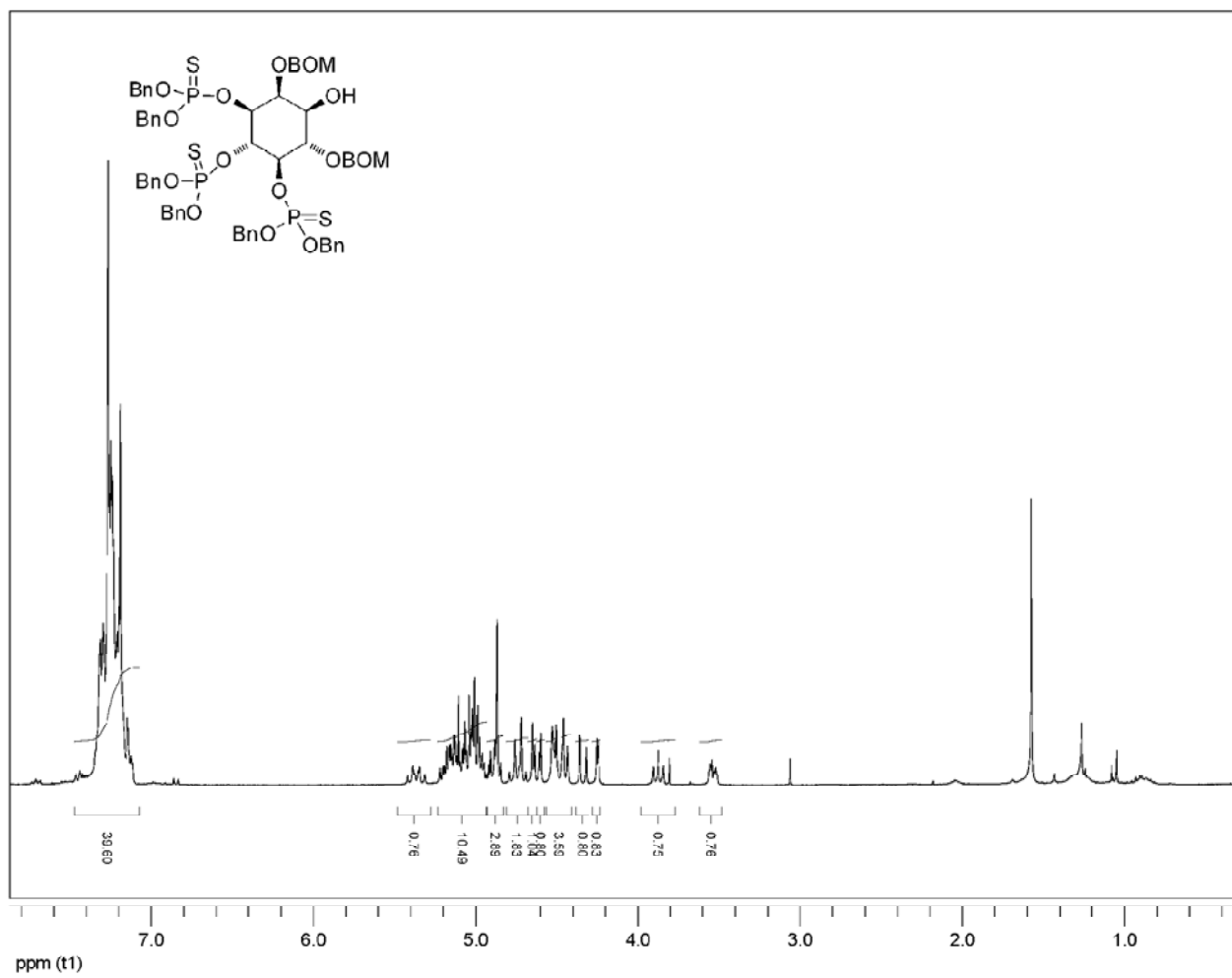












Vita

Ashdeep Kaur Rajpal was born in India in 1985. She completed her B.S. and M.S. degree in chemistry from Panjab University, Chandigarh, India. She attended University of Tennessee, Knoxville and completed her Masters degree in chemistry in May 2011.