

**Phosphatidylinositol 3,4,5–trisphosphate Probe Synthesis and Microarray Protein Binding
Studies to Investigate Multivalency and Detect Proteins Upregulated in Cancer**

A Thesis presented for the
Masters of Science Degree
The University of Tennessee, Knoxville

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August 2013

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Acknowledgements

I would first like to thank my wife for all of her endurance, resolution, and help not only making it through graduate school but helping me to be a better person. I would also like to thank Dr. Best for all of his help and guidance throughout my graduate career. I would also like to thank my committee members Dr. Baker and Dr. Long for their understanding and patience with me. I would also like to thank all of my lab mates especially Sammy Eni Eni for being a true friend in many different circumstances. Lastly I would say that it is truly possible for anyone to achieve their dreams if they set their mind to it.

Abstract

Protein–lipid binding interactions control many processes at the cellular level. Many of these biological events between proteins and lipids are often associated with signaling pathway diseases such as cancer and diabetes. Therefore, increasing our knowledge of protein–lipid binding interactions will provide a better understanding of the signaling pathways of living systems. Pursuing this further, the phosphatidylinositol polyphosphates (PIPs) are an important family of signaling lipids that control key biological processes. Thus, the work described in this thesis was aimed at developing methods to validate these types of lipid–protein binding interactions. The goals of these projects were to synthesize the PIP compound and investigate protein lipid interactions with this compound and others already synthesized using microarray analysis via a pin printer. The results at this juncture in chapter 1 discuss how we were able to achieve successful printing of our target compound as well as determine that binding was occurring. Due to the nature of the microarray we were not able to determine K_d [surface dissociation constant] values for protein–lipid binding interactions due to the fact of not having enough positive data in a singular test event.

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Chapter 1: Design, Synthesis, and Studies of Phosphatidylinositol 3,4,5–trisphosphate Probe

1.1 Background and Significance of Phospholipids in Biological Processes

Phospholipids are a specific class of lipids that are major components of cell membranes and other lipid bilayers. Individual Phospholipids differ based on their headgroup, and biologically relevant examples include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylinositol (PI). They are all comprised of a triacylglycerol backbone with two of the alcohols containing fatty acid chains and the third comprised of a functional headgroup.

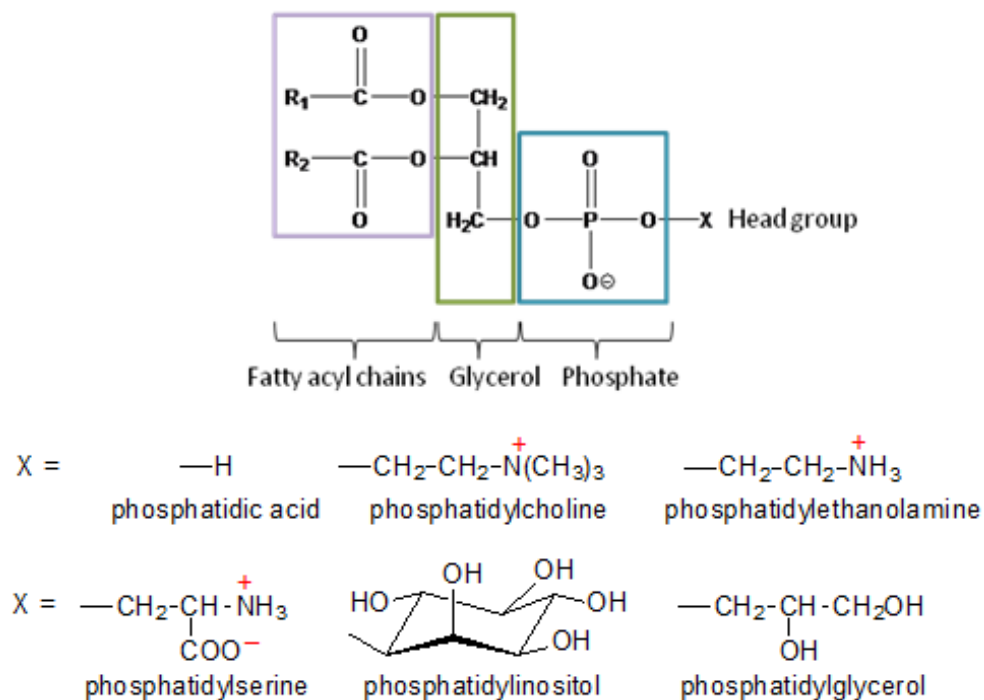


Figure 1.1 Physical Structure of Phospholipids

In general, all of the phospholipids play important roles of various aptitudes in biological systems with our interest lying specifically with the phosphatidylinositol polyphosphate (PIPs) and how they interact with different proteins. The PIPs are often site-specific in their interactions, which enforce membrane-association of protein binding partners. There are a total of seven naturally occurring PIPn isomers, each of which has some manner of regulation in different pathways. The difference between each of the PIP isomers varies only in the phosphorylation pattern of the core headgroup which consists of D-*myo*-inositol ring, with every combination of the 3, 4 or 5 positions being phosphorylated and present in nature.

These are all D-*myo*-inositols due to the IUPAC systematic naming of cyclitols. Absolute configuration is determined by making a vertical Fischer-Tollens projection of the compound, with C-1 at the top and with C-2 and C-3 on the front edge of the ring. Following this further, the configuration is designated D if the lowest numbered carbon bearing a hydroxyl group projects to the right, and as L if pointing to the left.

Many of the pathways of proteins that interact with PIPs are significantly mutation-prone/aberrant in disease. For example, diabetes and cancer metastasis are both associated with upregulation of PIP₃. Thus, learning about the types and multitude of binding interactions with the target lipids will help develop diagnostic methods for detecting processes that are upregulated in disease. The mechanisms by which PIPs control disease-related processes are complex in nature. PI3K¹ and phosphatase tensin homolog (PTEN)² are among the most heavily mutated oncogenes and tumor suppressors in cancer. Hyper-activation of this signaling pathway has been revealed to create cell stresses and apoptosis.³ Moreover, the deregulation of this pathway stimulates tumor suppression.⁴

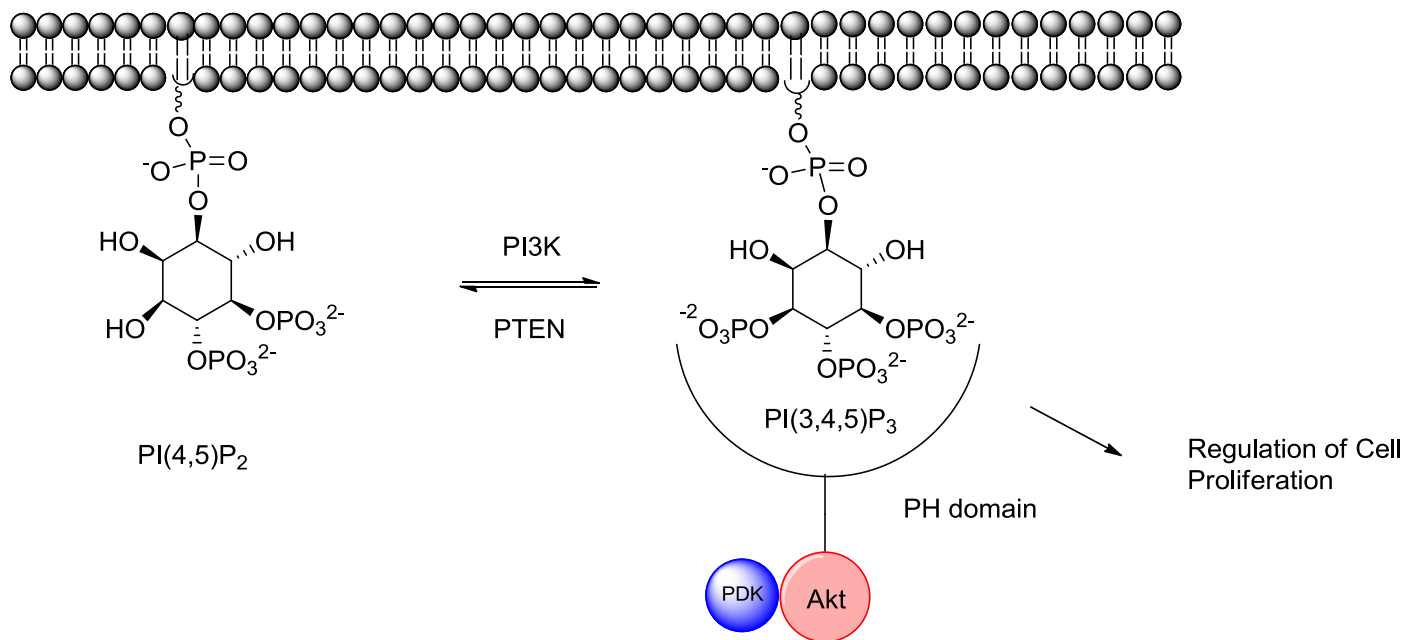


Figure 1.2: The PI3K/Akt pathway: PI3K phosphorylates PIP₂ to PIP₃ which recruits Akt and PDK to the cell membrane. PDK then activates Akt by phosphorylation, which activates many downstream pathways that control cell growth. The reversible reaction takes place with PTEN⁵ converting PIP₃ back to PIP₂. PI3K and PTEN are commonly mutated in cancer.

Following this further, blocking the production of the inositol pyrophosphate IP₇, a well known small molecule involved in signaling pathways,⁶ facilitates the recruitment of Akt by PIP₃, and has a key role in glucose homeostasis, which serves to protect mice against obesity and insulin resistance,⁷ thereby promoting cell growth and survival. After being recruited to the membrane by PIP₃, the activation of Akt by kinases such as protein dependant kinase (PDK)

deters cell death processes by supporting cell growth. In addition, this activation of Akt helps to promote protein synthesis pathways enabling Akt to continue downstream to promote healthy cell life. We can see the many cellular signaling events such as cell proliferation, transformation, cell shape, motility, insulin action, and the altering of glucose transport all involve PIP_3 .⁸

The first goal of the work described in this thesis was to synthesize an amine conjugated phosphatidylinositol 3,4,5–trisphosphate (PIP_3) probe scheme 1.1 (compound **19**) that can be exploited for microarray analysis. Our second goal was to investigate multivalent protein binding studies with a phosphatidylinositol 4,5–bisphosphate (PIP_2) probe and profilin. Human profilin 1 protein is a known tumor suppressor that is involved in regulation of the cytoskeleton.⁹ We sought to measure binding affinities of profilin for PIP_2 as a function of ligand density to understand the effect of multivalency detection from cancer cell extracts.

1.2 Microarray Analysis of Binding between PIPs and Proteins

Microarray analysis of protein–lipid binding interactions is beneficial due to its high-throughput nature. Previous studies by our group showed the ability to detect protein–lipid binding on microarray slides.¹⁰ Grid A was printed at a constant concentration of 500 μM PIP_3 while grid B was printed at varying concentrations from 0 to 500 μM left to right of PIP_3 . Both grids were incubated with purified Akt PH domain at a constant concentration of 1.1 μM and protein binding was detected using fluorescent antibodies. These results prove the high affinity of PIP_3 binding to Akt.

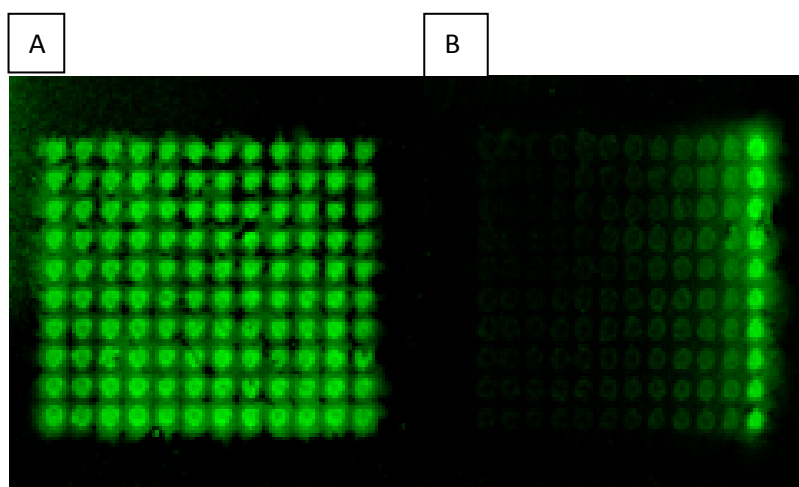
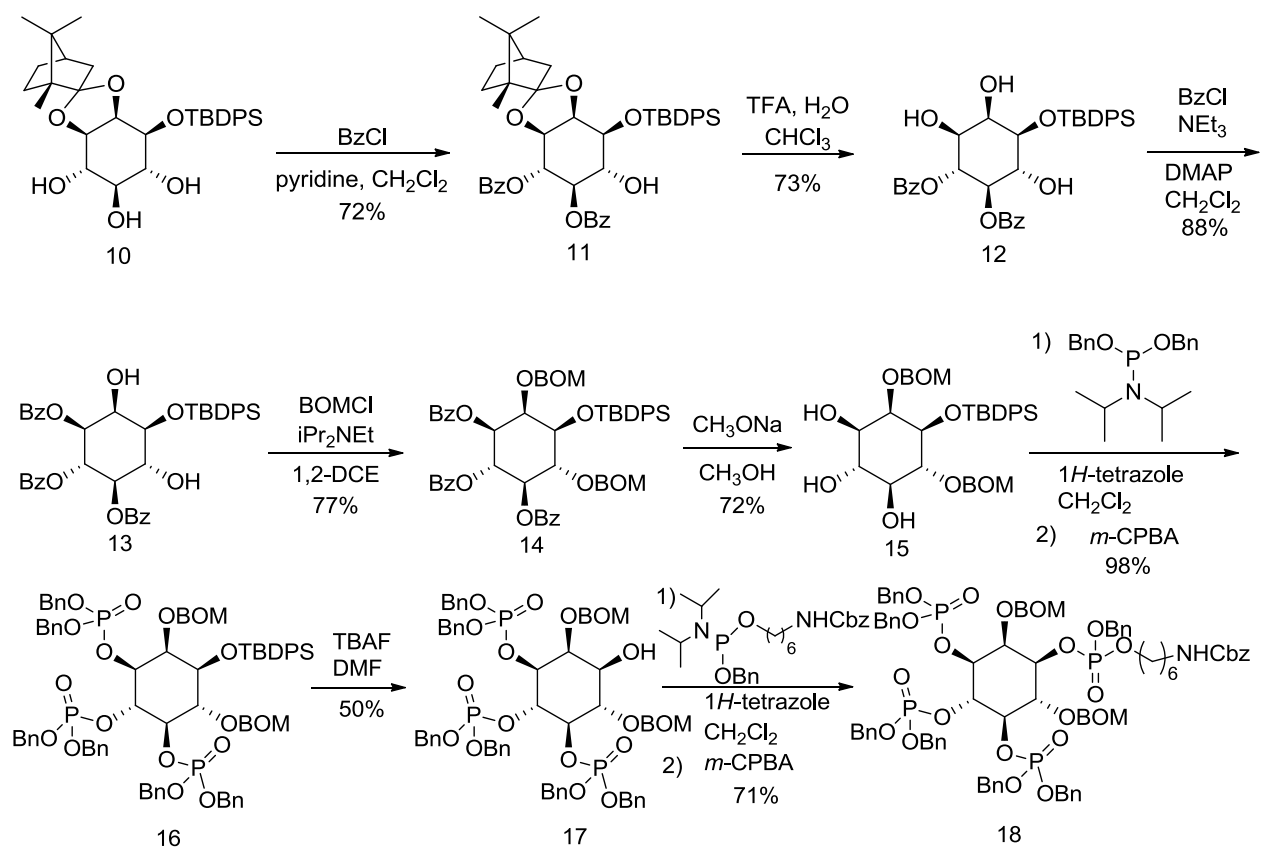
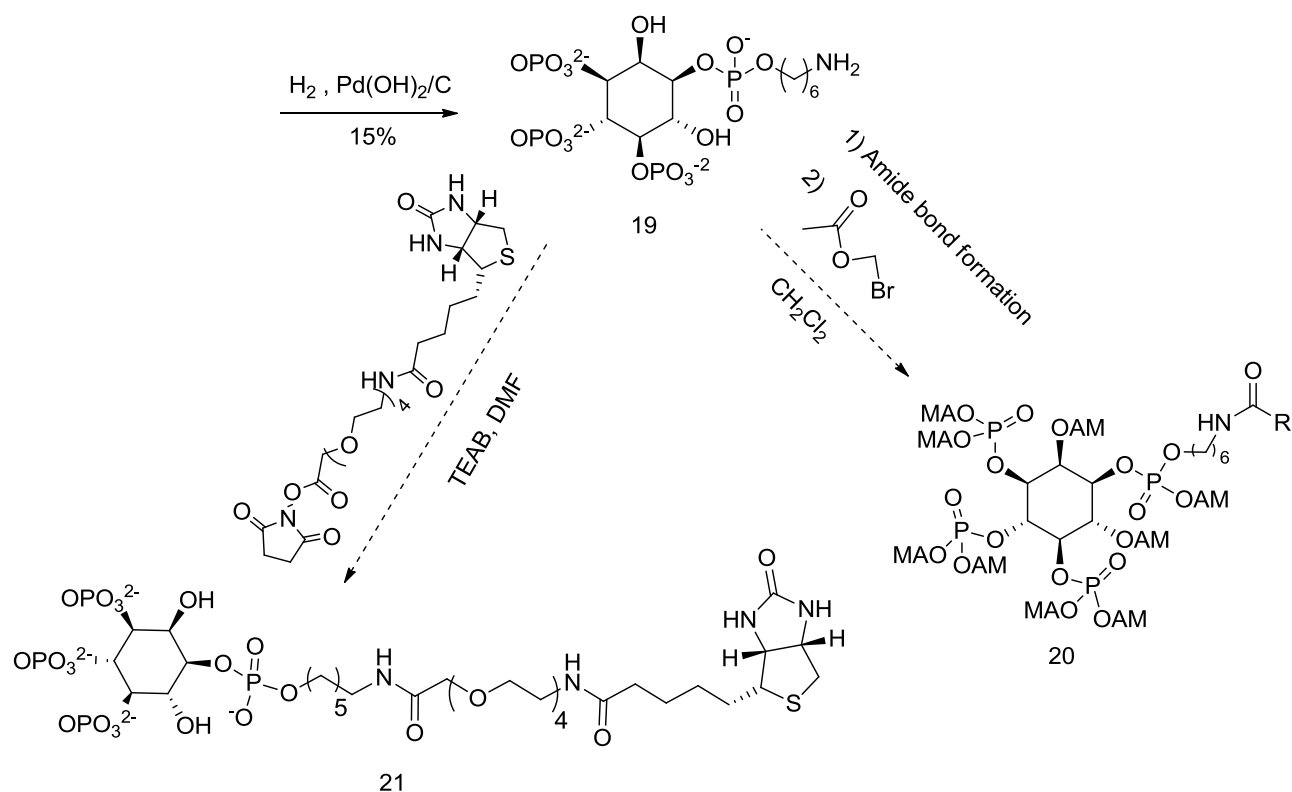


Figure 1.3: Microarray: Previous results from our group from microarray analysis of PIP_3 –Akt binding¹⁰. This established proof of concept for our microarray system, which we extended via the current studies.

Effectively designing PIP probes will enhance the opportunity to successfully study the protein–lipid multivalent binding interactions of profilin and univalent binding of Akt and other PIP binding proteins. We have developed biotinylated PIP headgroups such as **21** for microarray analysis. Certain proteins, including Akt and Profilin, bind predominantly to the phosphorylated inositol headgroup (S-type proteins), while others interact with the interfacial membrane region (I-type proteins), and lastly a third binding mode involves substantial dissemination of protein into the hydrophobic membrane core (H-type proteins). Previous studies have shown PIP₂ and PIP₃ to be headgroup-driven binding lipids (S-type), with electrostatic attraction primarily driving the interaction of the protein with the phosphorylated *myo*-inositol headgroup.^{11,12,13} Thus, simplified PIP probes lacking the glycerolipid backbone are effective for studies.

Scheme 1.1: Synthetic Scheme of PIP₃ Analogs





Scheme 1.1

Using a previously reported procedure^{14,4} the synthesis of PIP₃ probe **21** began with TBDPS-camphor protected triol **10**, which was protected with benzoyl groups to access **11**. The camphor protecting group of **11** was then removed to provide **12**. Next, a third benzoyl group was introduced at the 3 position to yield **13**. The diol **13** was protected at the 2 and 6 positions with benzyloxymethyl groups generating **14**. Next, the benzoyl groups of **14** were removed producing the triol **15**. The triol **15** was then phosphorylated at the 3, 4 and 5 positions to yield compound **16**. Continuing further, the trisphosphate **16** was deprotected at the 1 position

providing **17**. Next, the alcohol **17** was phosphorylated yielding the fully protected PIP₃ probe **18**. Compound **18** was globally deprotected to yield **19**. The amine **19** can have a biotin tag attached in one step to yield **21** for microarray studies which had previously been made by Meng in our group, or **19** can be exploited via amide bond formation and then protected to yield **20**. The tail of **19** contains an amine that can be derivitized to an amide for labeling with different photoaffinity tags for covalent labeling of proteins. The yields obtained thus far match the reported literature fairly closely except step **16** to **17**. It is hypothesized that this is due to TBAF being hygroscopic in nature and moisture being absorbed over time.

Microarray technology is on the forefront of this investigation with its ability to manipulate and control ligand presentation on surfaces, and since small amounts of lipid and protein are used while allowing for maximal data output. The strategy was to incubate and bind the PIP biotinylated headgroup or the amine headgroup onto streptavidin-coated glass slides or epoxide-coated glass slides, followed by thorough buffer washes. Next, the ligand was incubated with purified profilin followed by further buffer washes to remove unbound protein. An anti-profilin rabbit-conjugated goat anti-rabbit Cy3 antibody was then used to label the profilin, followed by further washing. Finally, fluorescence-based detection at 532 nm using a slide scanner was performed to transduce the presence of bound protein.

It is important to run control experiments to establish binding involved with the protein target. These controls are used to demonstrate that labeling of profilin involves specific binding and that the Cy3 tagged antibody is crucial for detection of fluorescence. Studies with profilin

have demonstrated that it fails to bind monovalently to headgroups at physiologically relevant concentrations but has a high affinity for multivalent binding of phosphoinositide headgroups, promoting the need to understand this interaction at the molecular level.¹⁵

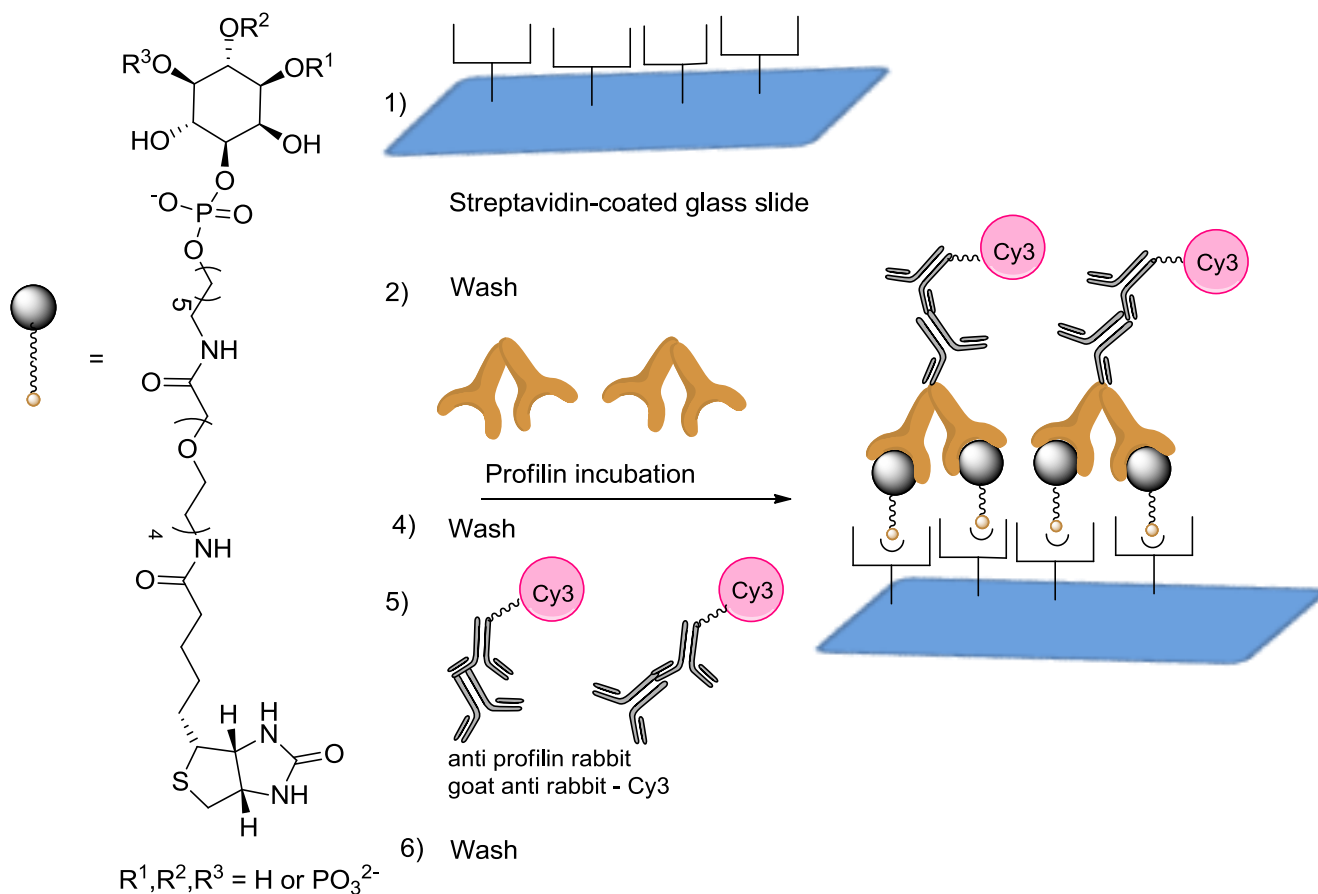


Figure 1.4: Microarray-based assay for the detection of receptor–PIP headgroup binding interactions

The goal of these studies is to determine the effect of density on protein–lipid binding in these studies to understand how this regulates binding affinity. Thus, determination of binding affinity with changing different ligand densities will need to be found for various concentrations of proteins. The signal intensity in an array depends on the surface density¹⁶, so it will be essential to normalize PIP concentrations prior to printing the microarray. Determination of the dissociation constant will be performed by printing increasing concentration of PIP onto each grid of the glass slide. The difference between each grid will be the amount of protein incubated onto each grid. Therefore, by measuring the increase in incubated protein concentration versus the fluorescence from the resulting protein binding, we will obtain Langmuir isotherms for each ligand concentration, as Wong and associates have previously shown with carbohydrate–protein binding.¹⁶

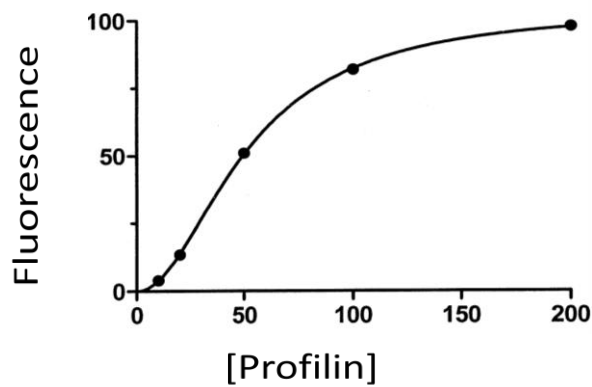
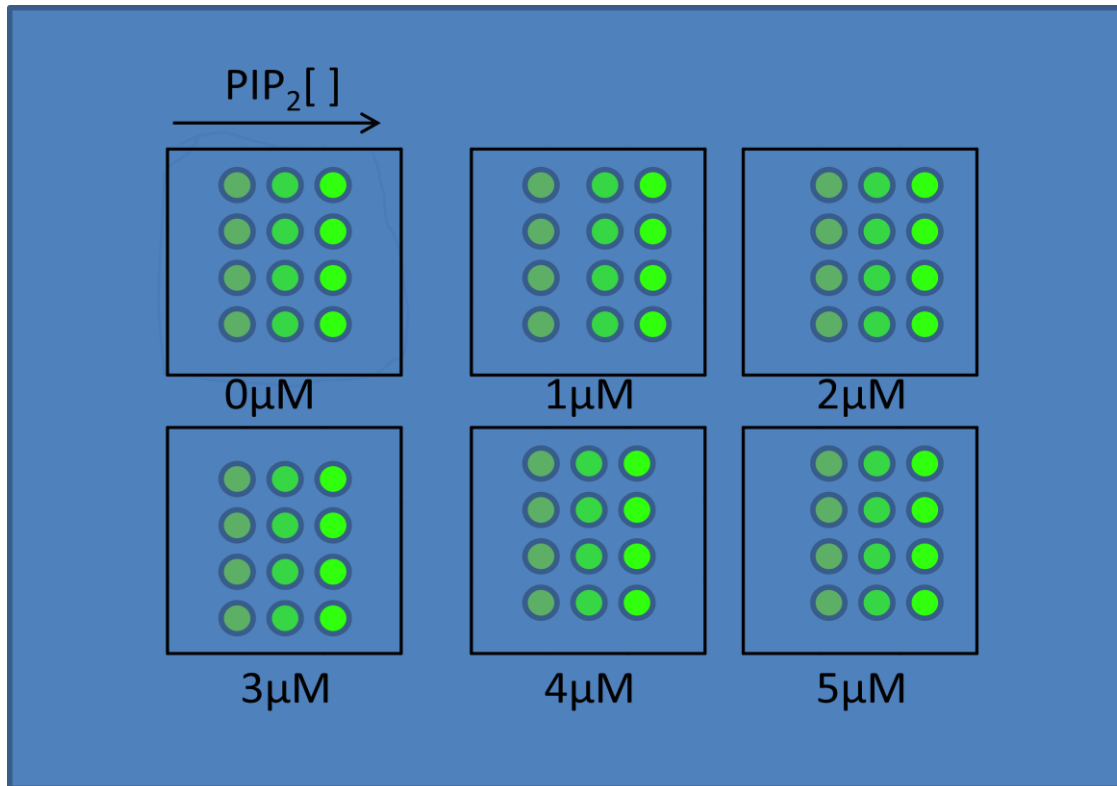


Figure 1.5: Multivalent Density Studies: Example of increasing [PIP₂] left to right within each grid while increasing [Profilin] for separate grids. This allows for multiple K_d affinity curves to be determined. K_d can then be plotted versus [PIP₂] on the surface.

In previous carbohydrate–protein multivalency studies, both a threshold type of binding, in which the binding significantly increased after a certain density of ligand, and a gradient type of binding, in which increasing the density of the ligand continually reduced the dissociation constant, have been observed.^{16,17} This allowed determination of the amount of ligand needed to produce maximal amount of fluorescent response. In our studies, we have taken a similar approach to this prior work. We sought to print multiple grids with varying concentration of ligand, and then fluorescence detection using a slide scanner was pursued to determine relative intensity versus concentration, in order to calculate K_d .

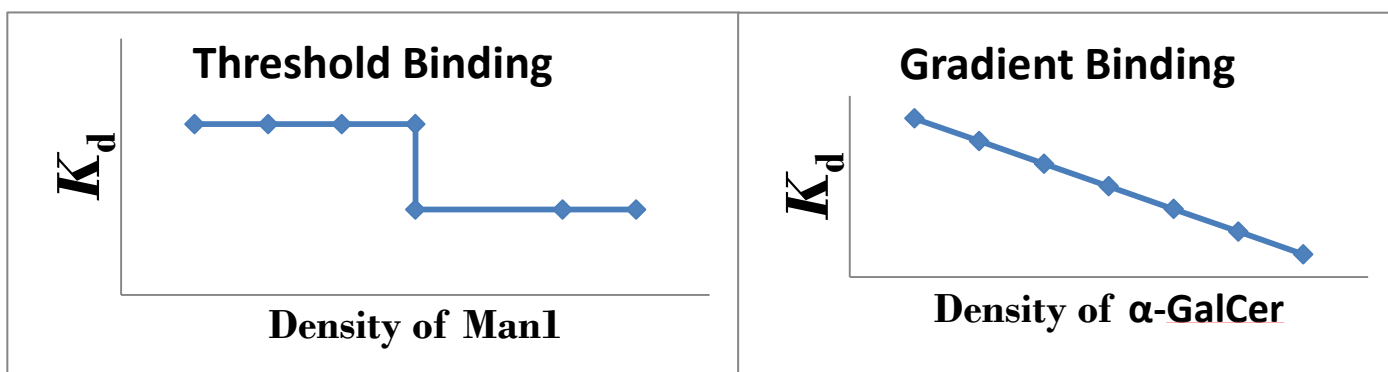


Figure 1.6: Examples of the effects of ligand clustering on dissociation constant from prior carbohydrate studies.^{16,17}

It should be noted that we hoped to see a point in the concentration of PIP₂–profilin binding where the value of K_d significantly increased at a low concentration of PIP₂. Profilin

binding should be significantly affected by multivalency since this protein is believed to bind in a ~6:1 ratio of PIP to profilin, as determined by sedimentation equilibrium experiments.¹⁵ If we have too little concentration of PIP₂ on the glass slide we would have seen a significant decrease in fluorescence since it has been shown that profilin does not bind univalently. Finally, this would have allowed us to determine the average space between each PIP₂ bound to the glass slide using known equations.¹⁶

1.3 Results and Discussion

As previously mentioned above, PIPs interact with a vast amount of proteins. Here, we describe the efficacy of microarray for using PIP probes as lipid mimics to detect certain protein binding affinities. A previously synthesized PIP₂-amine linked probe was printed onto epoxide-functionalized glass slides purchased from Schott Nexterion®.

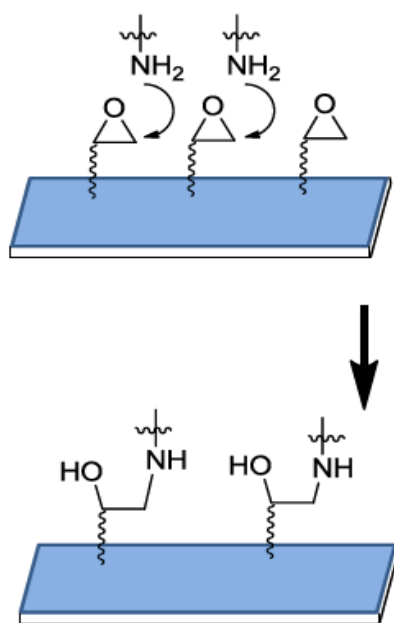


Figure 1.7: Immobilization of primary amine to epoxy coated slides.

The amine of the PIP probe cleaves open the epoxide on the slide to form a covalent bond when immobilized with the print buffer (Arrayit Protein Printing Buffer 2X®). On the glass slide 16 supergrids are usually printed as described in figure 1.5 with varying concentrations

of PIP probe within each supergrid. This is followed by incubation, washing, blocking of unreacted slide, followed by incubation of protein.

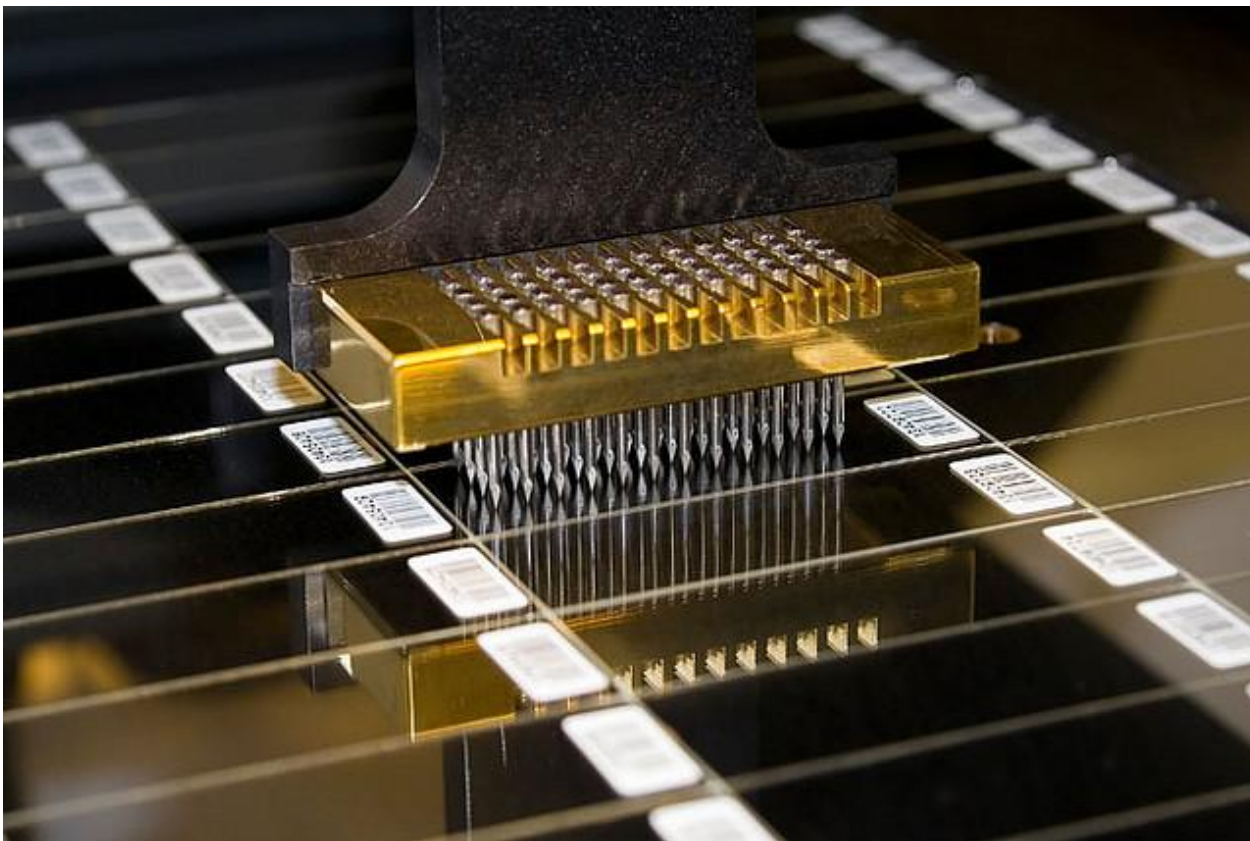


Figure 1.8: Robotic pin printer with 32 pins

Using the SDDC-2 robotic printer from ESI, PIP₂ probe was printed onto the epoxide-functionalized slides. The pin has an uptake volume of 0.25µL/sample, a tip opening of 75 µm, and delivery volume of 600 pL. Several different buffer types and concentrations were used when spotting. (TRIS) tris(hydroxymethyl)aminomethane buffer was used first at a

concentration of 20mM, pH 8.0, and .05% tween additive. One of the first issues of the printing process was repeatable solid dots as shown in figure 1.3. After experimentation of differing concentrations up to 200mM TRIS it was determined replicable spots were not achievable with TRIS. Solvation and replication of dots was achieved with the use of Arrayit Print Buffer. No further modification was made to print buffer after purchase. After incubation overnight, the slides were washed with TRIS buffer to remove any unbound probe providing reasonable results as shown in Figure 1.9-1.10.

To ensure specific interactions between probe and protein the slide needed to be blocked after ligand immobilization. Blocking conditions were varied with ethanolamine, diethylamine, and superbloc. High fluorescence background was still seen with ethanolamine concentrations of 20-200 mM, so subsequent assays were used with 200 mM diethylamine and superbloc as both achieved similar results. After one hour of blocking, the slide was washed 3 times with TRIS buffer to remove any traces of block buffer and dried. The supergrids printed with PIP probe were then incubated in a solution of Profilin protein that ranged from 0 μ g to 100 μ g in TRIS buffer for one hour. This was followed by washing 3 times with TRIS buffer to remove unbound protein. Following this, the supergrids were incubated with goat-anti-Profilin antibody for one hour followed by washing 3 times with TRIS buffer to remove unbound antibody. Lastly, the supergrids were incubated with rabbit-anti-goat Cy3 tagged antibody for one hour followed by one wash with TRIS buffer and washed two times with H₂O. The slide was then dried over nitrogen and scanned for fluorescence using slide scanner.

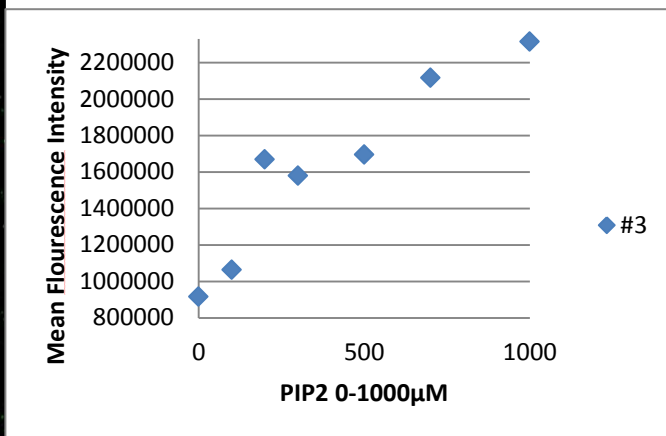
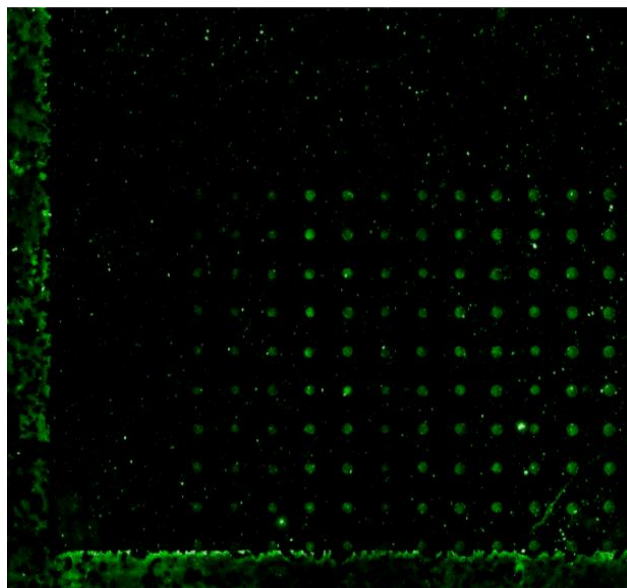


Figure 1.9: Results of 0-1000 μ M PIP₂ probe incubated with Superblock, 25 μ g solution of Profilin, 10 μ g AP, and 5 μ g Cy3 antibody

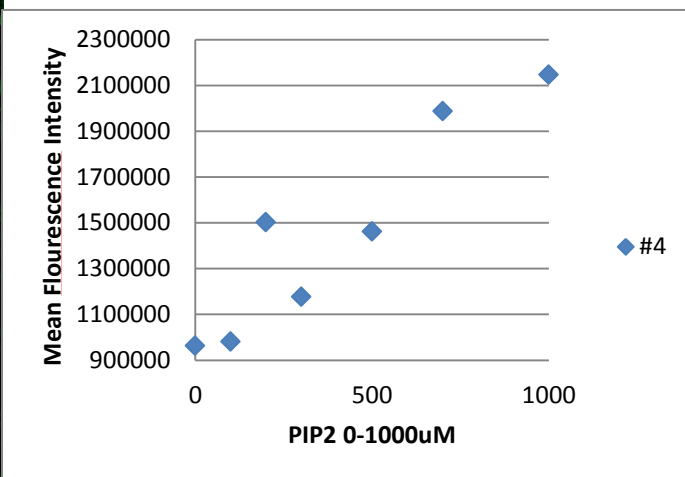
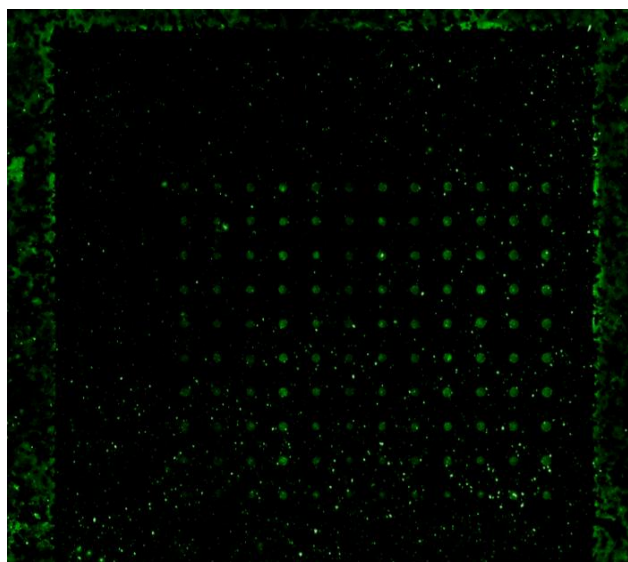


Figure 1.10: Results of 0-1000 μ M PIP₂ probe incubated with Superblock, 20 μ g solution of Profilin, 10 μ g AP, and 5 μ g Cy3 antibody

From the previous figures 1.9-1.110 we are able to see that there is binding occurring between ligand and protein. As the concentration of ligand is increased on the supergrid while keeping the same amount of profilin on that supergrid we could see that binding was increasing up to 1000 μM ligand concentration. We were able to successfully use microarray techniques to show that binding with Profilin and the PIP_2 probe can occur on epoxide functionalized glass slides. The success of printing seemed to be a good portion of the challenging aspect in the microarray analysis. Ultimately, we printed with the Arrayit Printing Buffer achieving the most successful results. Blocking conditions of the unreacted slide surface was initially unsuccessful at lower concentrations of diethylamine below 90 mM. Further optimization of the conditions employed is necessary to achieve a full slide of supergrids with varying concentrations of Profilin.

Even though the previously mentioned assays yielded a substantial start to the studies, further optimization requirements must be employed. A control experiment below was setup with Atto 550®, a fluorescent molecule attached to biotin. This was printed from concentrations of 0-500 μM as indicated in figures 1.11-1.12. This showed that using our Arrayit buffer worked and reestablished proof of concept of the experiment. The one issue with the control experiment is that the same amount of compound was printed in each row, which should have led to same amount of mean fluorescence for each row. This inconsistency is one of the obstacles that we were unable to be overcome in the printing process of our ligand.

Undoubtedly the largest issue with our microarray work is inconsistency. At first the

printing of the supergrids and amount of solution that repetitively printed was difficult. There were many variables alone for just this issue, for example, the pin may not have picked up any solution or not deposited the solution. Secondly the pins need to be cleaned in a specific manner in between each super grid printing and this could lead to minor changes in the amount of solution picked up or spotted in the next round of printing. Thirdly the pin opening is quite small and extensively fragile and proved on more than one occasion to close up and bend calling for a need to replace pins, which are quite expensive.

Data was analyzed the GenePix® 4000B microarray scanner from Axon instruments, of which the fluorescence intensities were quantified. The scanner has a 532 nm wavelength laser, which would scan the slide and show images of the supergrids. The control in the experiments was the spot of print buffer which contained no PIP₂ probe. The spots that had uncharacteristic results as either lower or higher fluorescence were discarded from the analysis. Ultimately the main goal was to print a slide such as in figure 1.13 and obtain mean fluorescence of all of the grids which was never accomplished to date.

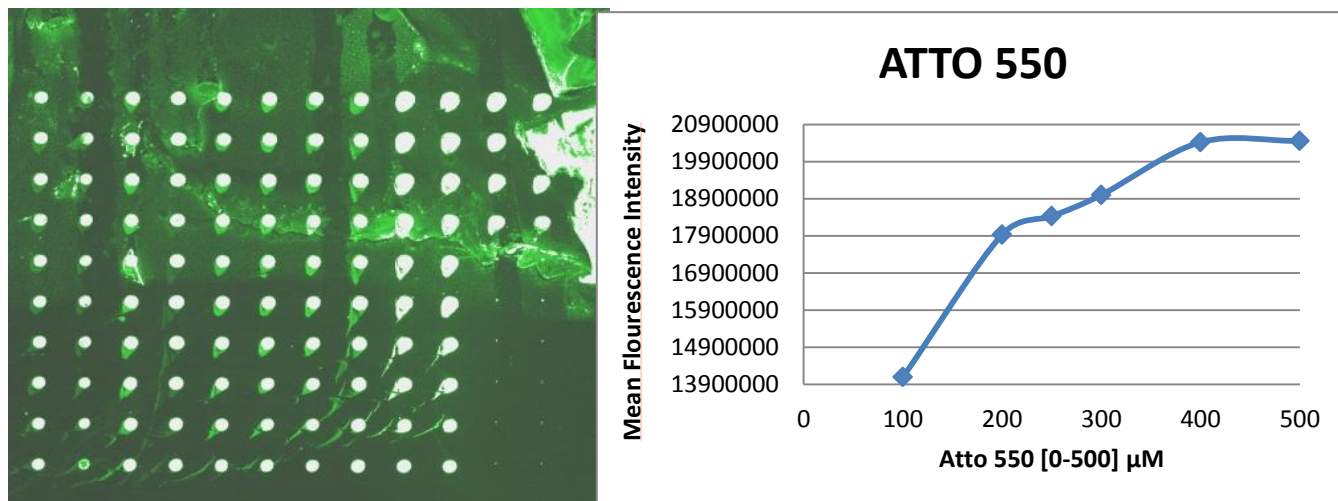


Figure 1.11: Results of 0-500 μM Atto 550 first grid

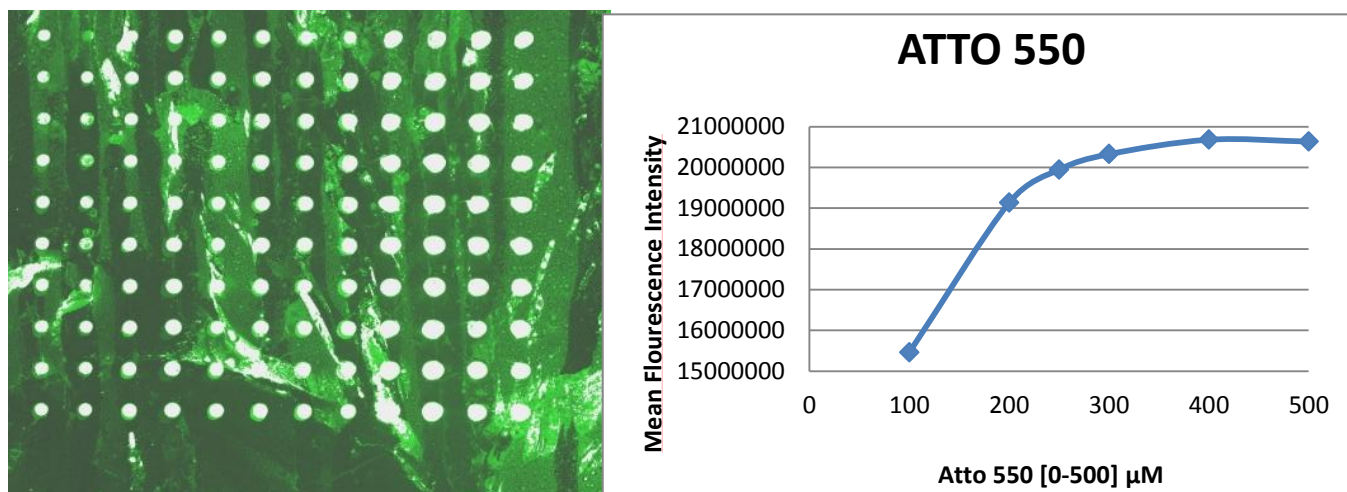


Figure 1.12: Results of 0-500 μM Atto 550 second grid

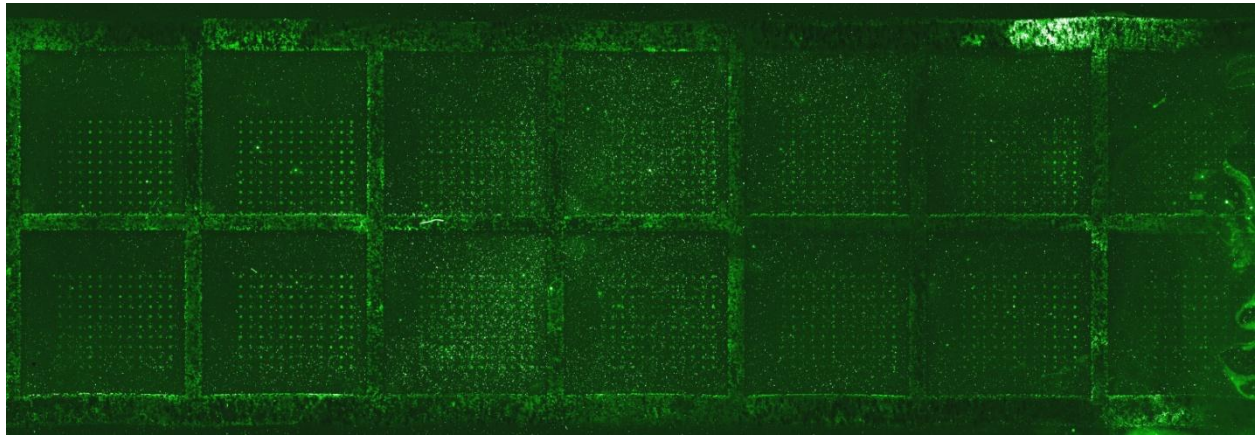


Figure 1.13: Results of 0-1000 μM PIP_2

Chapter 2: Design and Synthesis of Inositol

Hexaphosphate probe

2.1 Background and Significance of Inositol Polyphosphates in

Biological Processes

Inositol polyphosphates, or (IPs), are a diverse family of eukaryotic signaling molecules that control many key cellular processes. Due to the numerous isomers that exist of IPs and their interconnection, identifying individual pathways with different protein binding partners have elucidated scientists to present day. In terms of biosynthesis the lipid PIP_2 acts as the precursor to IP_3 specifically showing the complex nature of small molecules in the cellular environment.¹⁸

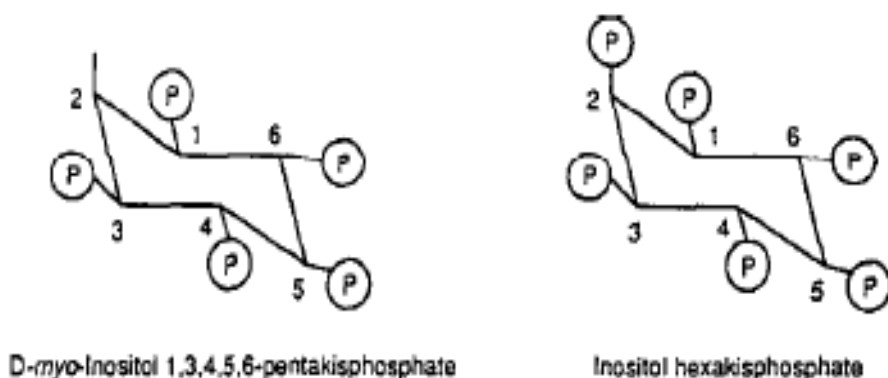


Figure 2.1: Structures of IP_5 and IP_6 . Circled P = phosphate.¹⁸

Strategies have emerged for the synthesis of chemically modified IPs for the determination of such binding activities. All of the IPs have the *myo*-inositol core with different phosphorylation patterns about the cyclohexane ring. The most abundant IPs are *myo*-inositol (1,2,3,4,5,6)IP₆, “phytate” as commonly referred, IP₆ and *myo*-inositol (1,3,4,5,6)IP₅, IP₅, both of which tend to have concentrations generally between 10-100 μ M in the cell.⁶ IP₆ and IP₅ are involved in phosphatase and kinase regulation as well as calcium channel aggregations.¹⁹

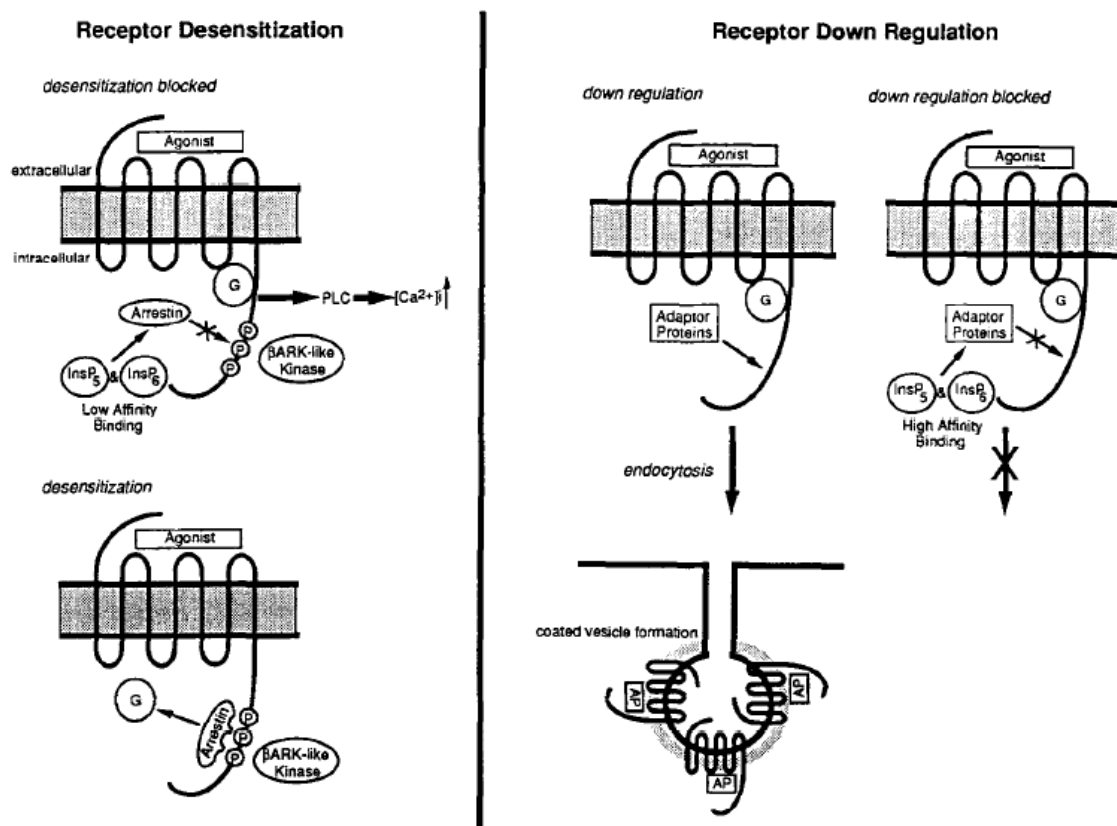


Figure 2.2: Possible roles of IP₅ and IP₆ in the mechanisms of G-protein coupled receptor desensitization and down-regulation¹⁸

The negatively charged phosphate groups on IP₆ provide aptitude to chelate divalent and trivalent cations.¹⁸ IP₆ is a beneficial dietary component found in many plant sources.²⁰ Studies have been suggestive of the importance of various cancers and their associations with dietary intake. The lack of dietary fiber, certain vegetables, and total dietary fat could lead to a role in the etiology of the cancer.²⁰ This can be related to the amount of particular small molecules that play crucial roles in protein pathways.

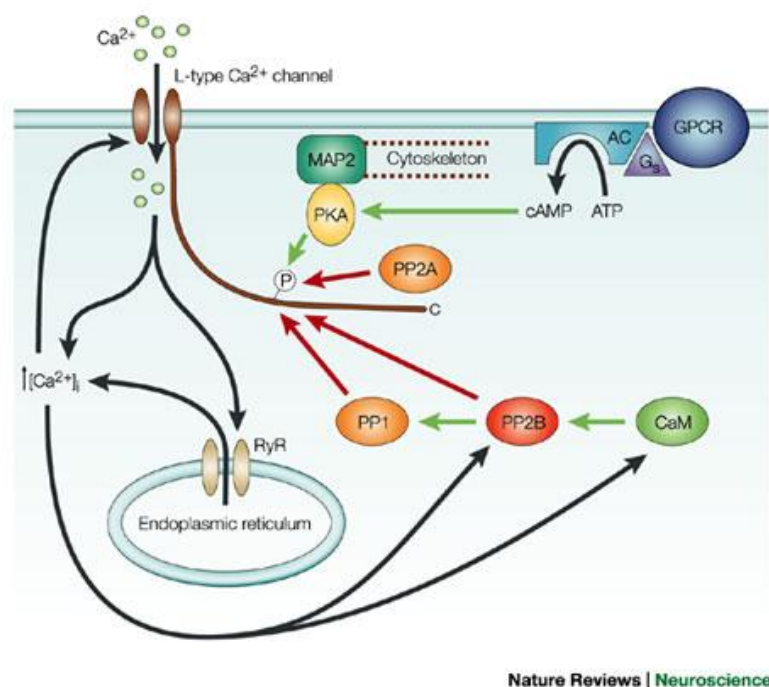


Figure 2.3: Example of a Calcium channel L- type. Possible roles of IP₅ and IP₆ in the mechanisms of G-coupled receptor desensitization and down-regulation.²¹

IP₅, which lacks a phosphate group at the only axial position on the inositol ring, has a

variety of roles in cell proliferation,²² as well as being involved with competition of kinases and phosphatases such as Akt,²³ and PDK1,²⁴ and their respective phosphoinositide targets.

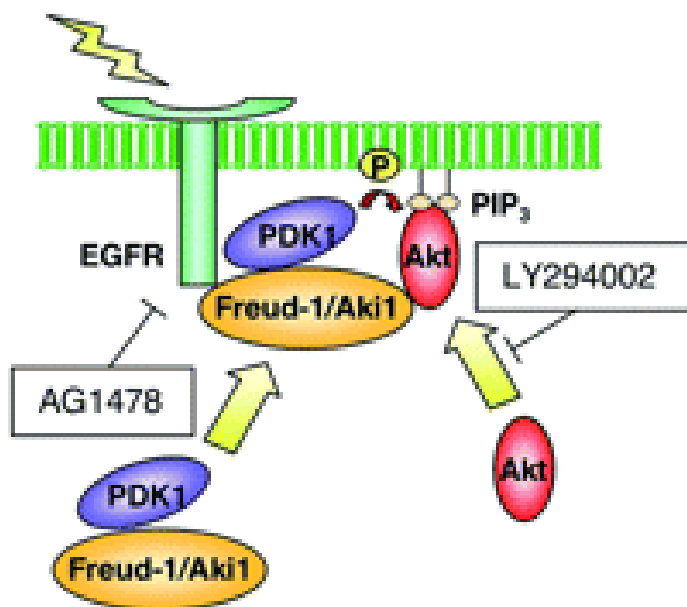
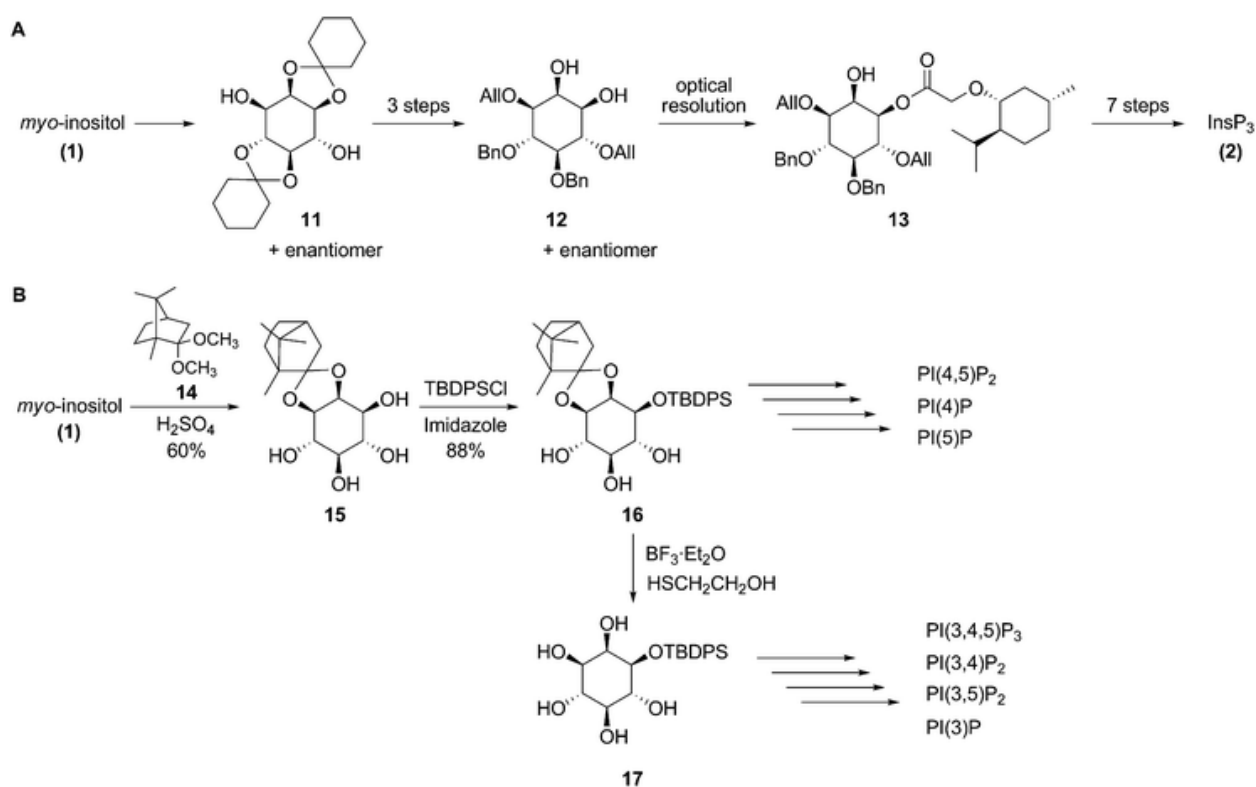


Figure 2.4: PDK1 involvement in activation cycle of Akt. PI3K mediates membrane recruitment of Akt and PDK1, resulting in PDK1-induced Akt phosphorylation and activation.²⁵

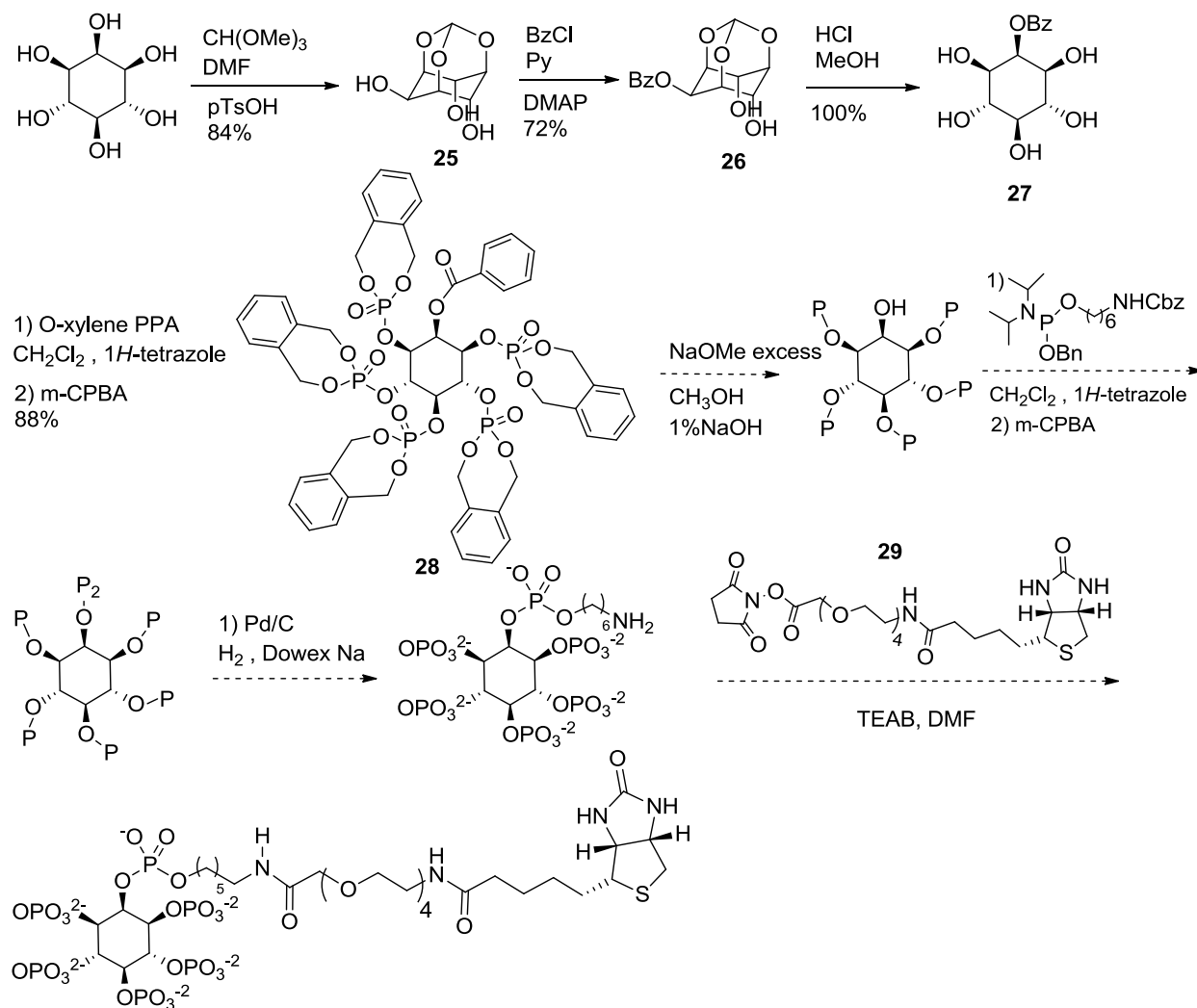
Effectively designing IP probes will enhance the opportunity to successfully study the protein–IP binding interactions. There are 63 possible variations of IP structure of which only half or so have been identified to be involved in biological pathways.⁶ Elucidation of the specific processes that the IPs are involved in could promote a much better understanding of disease. A necessity to overcome when associating the synthesis of IPs is awareness of phosphorylation at specific positions about the inositol ring. There has been a great deal of research into synthetic

strategies so only a few routes of recent strategy will be covered.^{6,19,26} Of those cited most notable are IP₃, IP₇, IP₅, and IP₆ have been of interest due to the amount of biological signaling events they have a role in. Our interest was to make an IP₆-biotin molecule to purify protein targets by affinity chromatography. Previous examples using IP₇ showed positive results that biological activity between target molecule and proteins.²⁶



Scheme 2.1: Synthetic approaches to the desymmetrization of *myo*-inositol using resolution steps to produce IPs and PIPs.⁶

2.2 Results and Discussion



Scheme 2.2: Synthetic scheme of IP₆ analog. P = phosphate

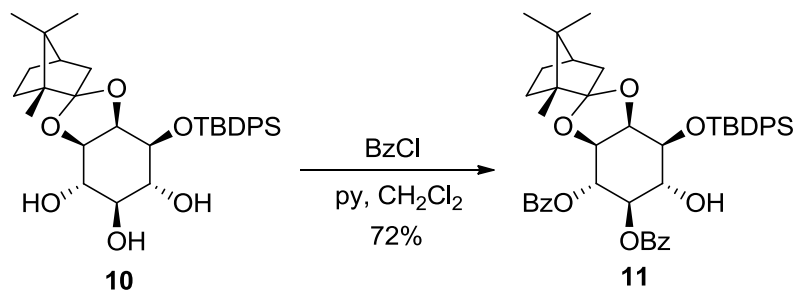
The approach to the biotin linked IP₆ analog began with *myo*-inositol which was first protected with trimethylorthoformate to produce **25**.²⁷ Compound **25** was protected at the 2

position with a benzoyl group to access **26**.²⁸ The orthoformate protecting group was then removed to provide **27**.²⁸ Next, an *o*-xylene phosphoramidite (PPA) protecting group was introduced at the five free hydroxyl positions to yield **28**.^{29,26} We next attempted to deprotect **28** selectively at the 2 position with a multitude of different reagents to generate **29**. In this approach it was first attempted to use 1% NaOH and excess NaOMe for which all the xylene PPA groups were removed. Following further, it was then attempted to try varying amounts of 1%-10% NaOH for deprotection. This resulted in either starting material being recovered or removal of the xylene PPA groups. Subsequently, it was then attempted to reflux with 5% NaOH to which removal of the xylene groups happened again. After that, a different approach using saturated NH₃/MeOH and stirring for varying amounts of time from 2 hours to 4 days resulting in recovery of starting material again. With the aim to remove this protecting benzoyl group it was then attempted to reflux with saturated NH₃/MeOH to which the removal of the xylene PPA groups happened again.

It has become evident with support from literature that the xylene protecting groups are not of sufficient stability to withstand the conditions for removal of the benzoyl group as well as evidence that sterics play a crucial role about the inositol ring.²⁶ One approach would be to protect at the 4 and 6 positions on the inositol ring after introduction of the benzoyl protecting group. Silyl groups could be advantageous in this effort as they are known to resist being removed by acid. The next step would be to deprotect the orthoformate group and yield a full silyl protected moiety which would be much less bulky. Additionally it could be conceived that protection of compound **27** with five silyl protecting groups directly to achieve a less hindered molecule.

Experimental

1D-4,5-*O*-(*tert*-Butyldiphenylsilyl)-2,3-*O*-(D-1',7',7'-trimethyl[2.2.1]bicyclohept-2'ylidene)-*myo*-inositol (**10**)

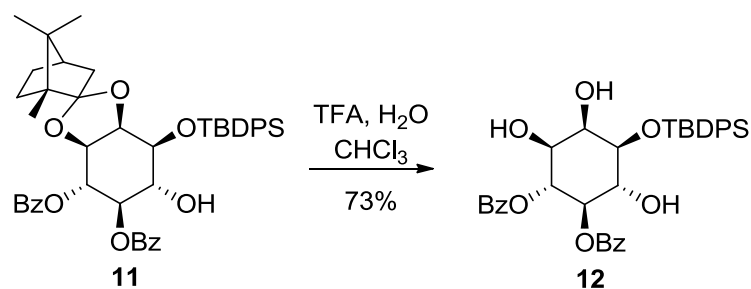


To the stirred solution of triol (**10**) (1.345g, 2.43 mmol) in pyridine (15 mL) and methylene chloride (2.5 mL) at -40 °C was added slowly benzoyl chloride (535μL, 5.10 mmol). The mixture was slowly warmed-up to room temperature and solvent removed by evaporation. The crude product was dissolved in ethyl acetate, washed with water and chromatographed on silica gel (hexane/acetone gradient) to afford pure (**11**) (1.33 g, 72%) as a colorless solid.

Characterization matched prior literature.¹⁴

¹H NMR (CDCl₃) δ 0.90 (s, 3H), 1.11 (s, 9H), 1.13 (m, 7H), 1.31 (d, J = 12.9 Hz, 1H), 1.43 (d tr, J = 7.8, 3.9 Hz, 1H), 1.69 (m, 1H), 1.72 (m, 1H), 1.80 (m, 1H), 1.90 (m, 1H), 4.02 (tr, J = 6.7 Hz, 1H), 4.09 (m, 2H), 5.13 (tr, J = 8.5 Hz, 1H), 5.63 (m, 1H), 7.25-7.50 (m, 12H), 7.70-7.96 (m, 8H).

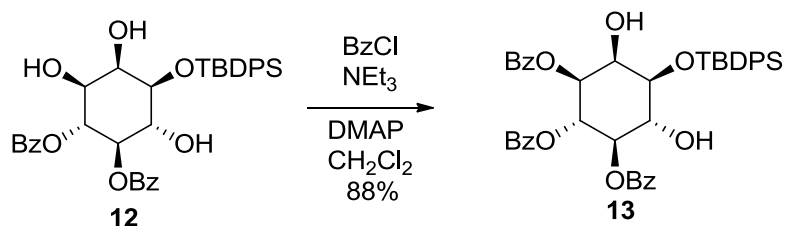
1D-4,5-*O*-(*tert*-Butyldiphenylsilyl)-*myo*-inositol (**12**)



The solution of the alcohol (**11**) (830 mg, 1.1 mmol) in chloroform (15 mL) was treated with trifluoroacetic acid (3.15 mL) containing 5% v/v water. After 18 h at room temperature, the solvent was removed and the remaining trifluoroacetic acid was neutralized with triethylamine (0.7 mL). Chromatography on silica gel (hexane/acetone gradient) afforded pure (**12**) (479 mg, 73%) as a white foam. Characterization matched prior literature.¹⁴

^1H NMR (CD_3OD) δ 1.13 (s, 9H), 3.51 (dd, $J = 10.0, 2.4$ Hz, 1H), 3.81 (tr, $J = 2.64$ Hz, 1H), 4.30 (tr, $J = 9.6$ Hz, 1H), 5.20 (tr, $J = 9.8$ Hz, 1H), 5.71 (tr, $J = 10.0$ Hz, 1H), 7.29-7.51 (m, 12H), 7.82-7.91 (m, 8H).

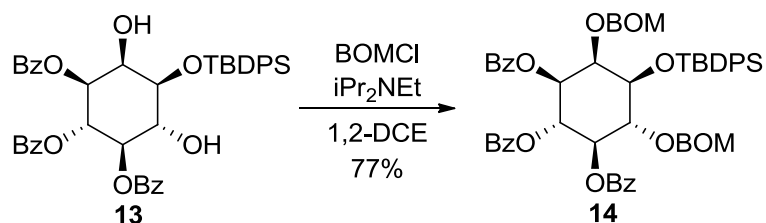
1D-1-*O*-(*tert*-Butyldiphenylsilyl)-3,4,5-*O*-trisbenzoyl-*myo*-inositol (**13**)



The solution of triol (**12**) (277 mg, 0.45 mmol) in dichloromethane (5 mL) was added triethylamine (190 μ L, 1.36 mmol) and 4-dimethylaminopyridine (55 mg, 0.45 mmol) at -40 °C. Benzoyl chloride (72 μ L, 0.68 mmol) was added drop wise and stirred overnight at room temperature. After aqueous workup the mixture was chromatographed on silica gel (hexane/acetone gradient) giving (**13**) (292 mg, 88%) as a white solid. Characterization matched prior literature.³⁰

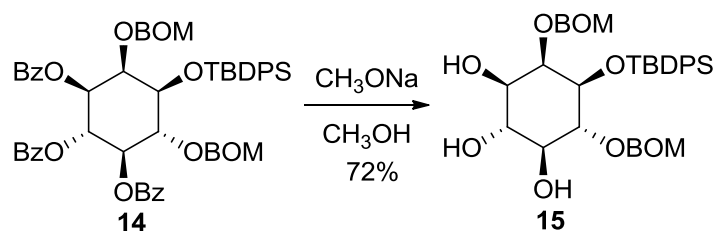
¹H NMR (CDCl₃) δ 8.0-7.25 (m, Ph, 25 H), 6.20 (tr, H-4, J = 10.3 Hz, 1H), 5.38 (tr, H-5, J = 9.9 Hz, 1H), 5.16 (dd, H-3, J = 2.7, 10.4 Hz, 1H), 4.41 (d tr, H-6, J = 9.5, 4.0 Hz, 1H), 4.23 (tr, H-2, J = 2.7 Hz, 1H), 3.90 (dd, H-1, J = 2.7, 9.2 Hz, 1H), 1.13 (9 H, Me).

3,4,5-*O*-Tribenzyl-2,6-*O*-bis(benzyloxymethyl)-1-*O*-(*tert*-butyldiphenylsilyl)-*D*-*myo*-inositol (**14**)



Diisopropylethylamine (1 mL, 6.13 mmol) and dichloroethane (8mL) were added to diol (**13**) (560 mg, 0.766 mmol). Benzyl chloromethyl ether (849 μ L, 6.13 mmol) was added and reaction mixture was stirred at reflux for 24 h. After reaction finished, water was added and extraction with dichloromethane. The mixture was chromatographed on silica gel (hexane/acetone gradient) to yield (**14**) (573 mg, 77%) as a clear solid. Characterizations matched previous reports.⁴

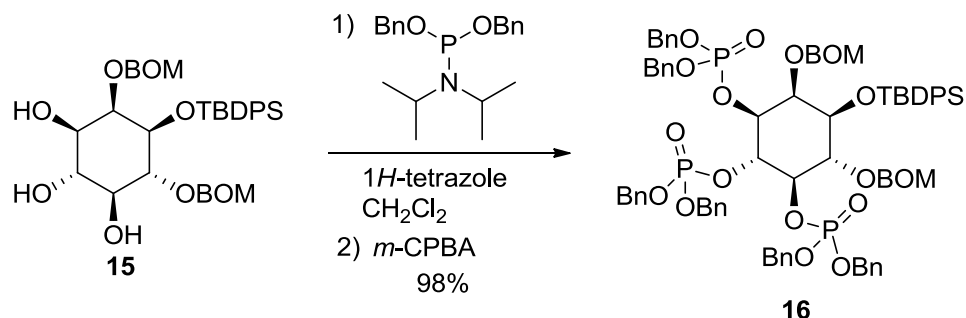
2,6-*O*-Bis(benzyloxymethyl)-1-*O*-(*tert*-butyldiphenylsilyl)-*D*-myoinositol (**15**)



A 1% sodium hydroxide solution (4 mL) and dichloromethane (4 mL) were added to a solution of (**14**) (240 mg, 0.25 mmol) in methanol (8 mL). The mixture was stirred for 18 hours at room temperature. The solvent was removed and the residue purified by chromatography on silica gel (hexane/acetone gradient) to yield (**15**) (99 mg, 72%) as a clear solid. Characterizations matched previous reports.⁴

¹H NMR (CDCl_3) δ 7.68 (t, $J = 7.2$ Hz, 4H), 7.45–7.25 (m, 16 H), 4.94 (d, $J = 6.8$ Hz, 1 H), 4.75–4.65 (m, 3 H), 4.54–4.51 (m, 3 H), 4.42 (d, $J = 11.6$ Hz, 1 H), 4.30 (br. s, 1H), 3.81–3.72 (m, 2 H), 3.66–3.54 (m, 3 H), 3.20–3.13 (m, 2 H), 2.75 (br. s, 1 H), 1.04 (s, 9 H) ppm.

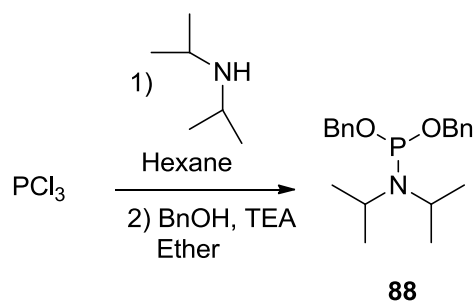
2,6-*O*-Bis(benzyloxymethyl)-1-*O*-(*tert*-butyldiphenylsilyl)-*D*-*myo*-inositol 3,4,5-Tris(dibenzyl phosphate) (**16**)



Dibenzyl *N,N*-diisopropylphosphoramidite (294 μL , 1.22 mmol) was added to a solution of alcohol (**15**) (103 mg, 0.15 mmol) and 1*H*-tetrazole (3.1 mL, 1.4 mmol, 0.45 M in acetonitrile) in dichloromethane (4 mL) under nitrogen, and the mixture was stirred at room temperature for 18 h. The reaction solution was cooled to $-20\text{ }^\circ\text{C}$, and *meta*-chloroperoxybenzoic acid (220 mg, 1.27 mmol) was added. The mixture was warmed to room temperature and stirred for 3 h. The solvent was removed and the residue purified by flash chromatography on silica gel (hexane/acetone gradient) to access (**16**) (210 mg, 98%) as a clear solid. Characterizations matched previous reports.⁴

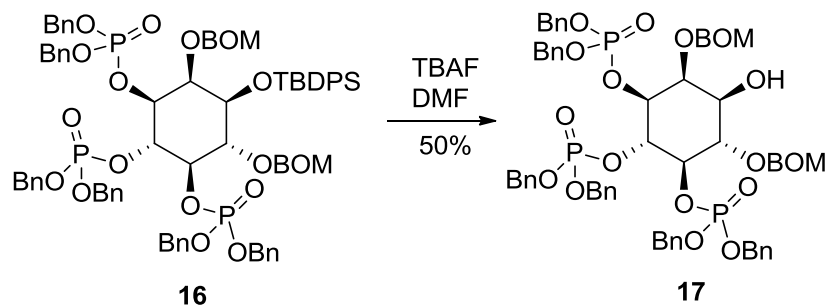
^1H NMR (CDCl_3): δ 7.76–7.67 (m, 4 H), 7.38–7.05 (m, 46 H), 5.15–4.88 (m, 12 H), 4.83–4.64 (m, 7 H), 4.54–4.48 (m, 2 H), 4.37–4.26 (m, 2 H), 4.08 (t, $J = 12\text{ Hz}$, 1 H), 3.98–3.91 (m, 2H), 1.07 (s, 9H) ppm.

Dibenzyl N,N-diisopropylphosphoramidite (**88**)



Diisopropylamine (7.8 mL, 55.8 mmol) was added drop-wise to a solution of phosphorus trichloride (2.6 mL, 26.1 mmol) in (70 mL) hexane at -20°C . After addition the sludge was stirred for 1.5h at room temp. The crude was filtered and washed with hexane and dried. Ether (60 mL) was added to the dried crude at -20°C from which a mixture of benzyl alcohol (5.0 mL, 48.3 mmol), triethylamine (6.7 mL, 48.3 mmol), and ether (60 mL) were added drop wise. After addition the mixture was stirred for 6h and was filtered over ethyl acetate and dried while chilled. The residue was purified by flash chromatography on silica gel over (hexane/ethyl acetate/ triethylamine) to yield pure (**88**) (4.80 g) as a clear liquid. Characterizations matched previous reports.⁴

2,6-*O*-Bis(benzyloxymethyl)-D-*myo*-inositol 3,4,5-Tris(dibenzylphosphate) (**17**)



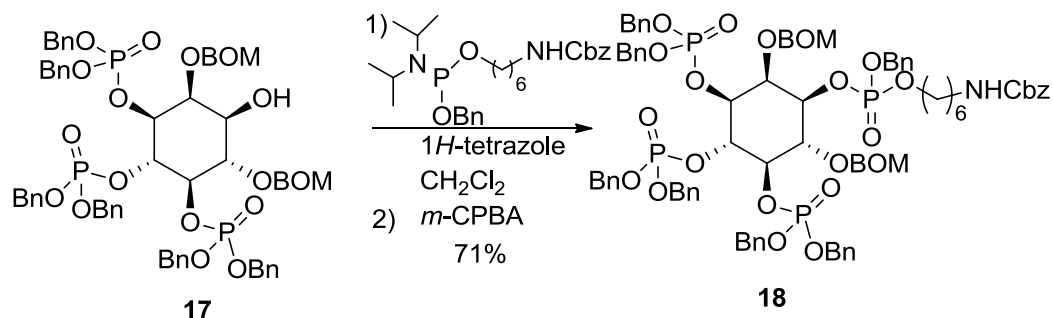
A solution of fully protected inositol (**16**) (225 mg, 0.15 mmol) in tetrahydrofuran (8 mL) was treated with tetrabutylammonium fluoride trihydrate (147 mg, 0.47 mmol) and stirred at room temperature for 18 h. Following concentration, the residue was purified by chromatography on silica gel (hexane/acetone gradient) to give alcohol (**17**) (74 mg, 50%) as a syrup.

Characterizations matched previous reports.⁴

¹H NMR (CDCl₃) δ 7.32–7.15 (m, 40 H), 5.14–4.84 (m, 16 H), 4.70–4.60 (m, 5 H), 4.48–4.40 (m, 3 H), 4.28–4.19 (m, 2 H), 3.85 (t, *J* = 9.4 Hz, 1 H), 3.48–3.44 (m, 1 H) ppm.

Benzyl (6-Benzyloxycarbonylaminoethyl) 2,6-O-Bis(benzyloxymethyl)-D-*myo*-inosit-1-yl

Phosphate 3,4,5-Tris(dibenzyl phosphate) (**18**)

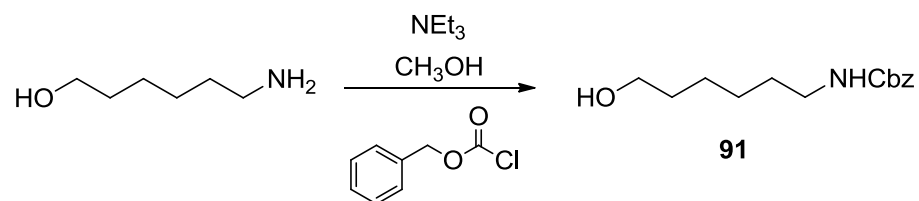


To alcohol (**17**) (74 mg, 0.062 mmol), 1*H*-tetrazole (1.0 mL, 0.45 mmol, 0.45 M in acetonitrile), dichloromethane (15 mL), and the cbz-aminohexanol phosphoramidite (700 mg, 1.54 mmol), were added and the mixture was stirred at room temperature for 18 h. The reaction solution was cooled to $-60\text{ }^{\circ}\text{C}$, and *meta*-chloroperoxybenzoic acid (750 mg, 57–86%) was added. The mixture was stirred at this temperature for 60 min and slowly warmed to room temp. The solution was diluted to 60 mL with dichloromethane, washed with saturated aqueous sodium hydrogen carbonate (30 mL), and the aqueous phase was extracted with dichloromethane (60 mL). The organic phase were dried with magnesium sulfate, filtered, and concentrated.

Purification by chromatography on silica gel (hexane/acetone gradient) was used to afford product (**18**) (70 mg, 71%) as clear syrup. Characterizations matched previous reports.⁴

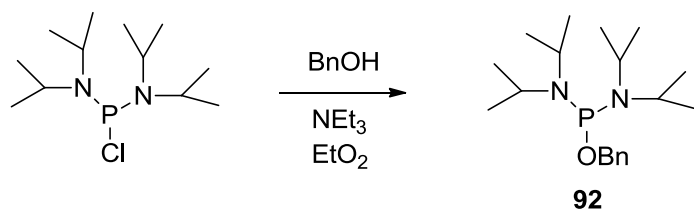
¹H NMR (CDCl₃) δ 7.32–7.17 (m, 50 H), 5.13–4.81 (m, 21 H), 4.76–4.52 (m, 5 H), 4.45–4.17 (m, 4 H), 3.94–3.82 (m, 2 H), 3.09–3.03 (m, 2 H), 1.46–1.18 (m, 8H) ppm.

Cbz-Aminohexanol (**91**)



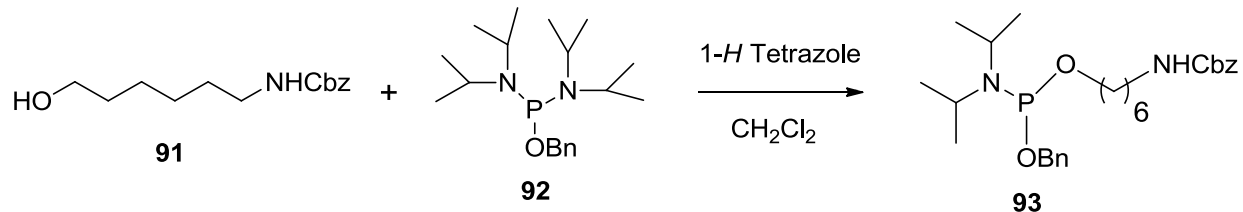
To a mixture of 6 amino-1 hexanol (2.0 g, 1.7 mmol), methanol (10 mL), and triethylamine (5.45 mL, 2.0 mmol) was added drop wise benzyl chloroformate and stirred overnight. The mixture was dried and extracted with dichloromethane (150 mL) and potassium hydrogen sulfate (50 mL). The brown (**91**) (1.5 g, 35%) was carried on without further purification. Characterizations matched previous reports.⁴

Benzyloxybis(diisopropylamino) phosphane (**92**)



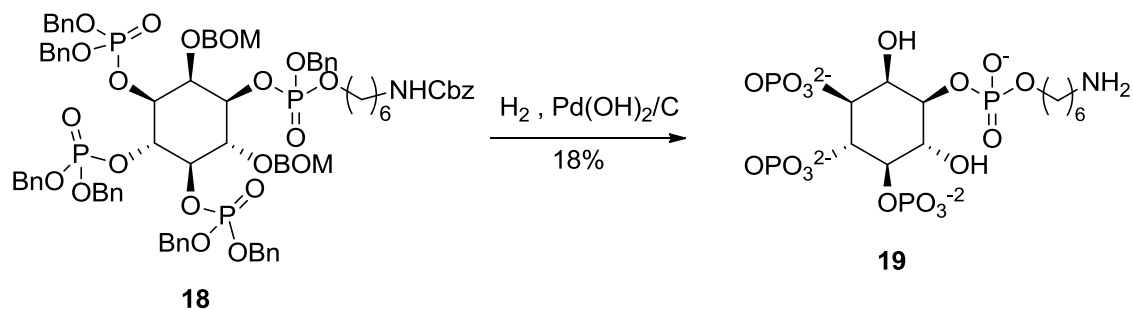
Ether (40 mL) and benzyloxybis(diisopropylamino) phosphane (1.52 g, 8.27 mmol) were mixed at 0 °C. Separately benzyl alcohol (850 μ L, 8.27 mol), triethylamine (1.15 mL, 8.27 mmol), and ether (10 mL) were mixed and added drop wise to phosphine mixture. After stirring for two hours at 0 °C the salt was filtered and purified by column chromatography on silica gel (hexane/triethylamine) to yield (**92**) (1.47 g, 58%) as a clear liquid. Characterizations matched previous reports.⁴

Cbz-Aminohexanol Phosphoramidite (**93**)



1-*H*-tetrazole (4.6 mL of a 0.45 M solution in acetonitrile, 2.07 mmol) and benzyloxybis(diisopropylamino) phosphane (**92**) (1.0 g, 2.96 mmol) were added to a solution of cbz-aminohexanol (**91**) (500 mg, 1.9 mmol) in dichloromethane (30 mL) under nitrogen. This mixture was stirred at room temperature for 2 h, at which point the solution was extracted with dichloromethane (3X80 mL) from saturated sodium hydrogen carbonate (80 mL). The combined organic phases were dried with magnesium sulfate, filtered, and concentrated, and the residue was purified by chromatography on silica gel (hexane/ethyl acetate/triethylamine, 100:10:1). The colorless liquid (**93**) (800 g, 1.7 mmol) was passed on to the next step without further purification.

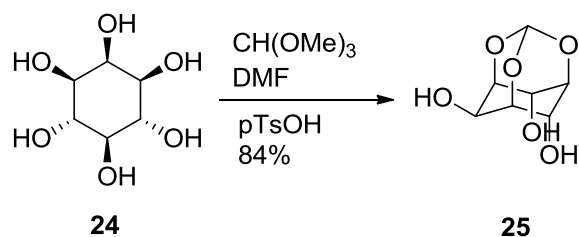
1-(6-Aminohexyl sodium phosphate)-D-*myo*-inositol 3,4,5-Tris(disodium phosphate) (**19**)



Palladium hydroxide (20%) (14 mg) on charcoal was added to a solution of (**18**) (70mg, 0.044 mmol) in methanol (10 mL). The mixture was then stirred at room temperature with 1 atm of hydrogen (balloon) for 4 d. The catalyst was removed by filtration and the residue washed with methanol. The solvent was removed, and the crude product was dissolved in water and stirred with Chelex 100 resin (Sigma, Na⁺ form) for 3 h. The resin was removed by filtration and the filtrate lyophilized to give the corresponding amino conjugate (**19**) (13mg, 18%).

Characterizations matched previous reports.⁴

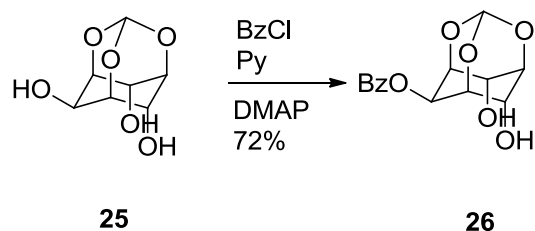
myo-inositol Monoorthoformate (**25**)



To the commercially available (**24**) (10.8 g, 0.06 mmol) was added dimethylformamide (160 mL), *para*-toluenesulfonic acid (3.0 g, 0.016 mmol), and trimethyl-*ortho* formate (12.5 mL, 0.12 mmol). The solution was reflux at 110 °C for 18 hours. After cooling to room temperature sodium hydrogen carbonate (10 mL) was added and stirred for 30 minutes. The crude concentrated, and the residue was purified by chromatography on silica gel (dichloromethane/methanol gradient) to afford the pure (**25**) (9.75 g, 84%) as a white solid. Characterizations matched previous reports.²⁷

¹H NMR (D₂O) δ 4.23 (2 H, m), 4.26 (1 H, m), 4.33 (1 H, m), 4.57 (2 H, t, J = 3.8 Hz), 5.59 (1 H, d, J = 0.8 Hz)

2-*O*-Benzoyl-*myo*-inositol 1,3,5-Orthoformate (**26**)

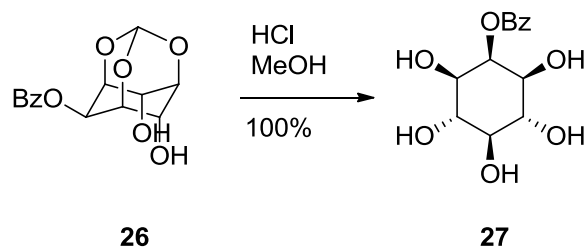


To a solution of (**25**) (1.03 g, 5.4 mmol) in anhydrous pyridine (30 mL) were added benzoyl chloride (0.7 mL, 5.95 mmol) and a catalytic amount of 4-dimethylaminopyridine at 0 °C. The mixture was stirred at room temperature overnight. Ethyl acetate was added to the reaction system and washed with potassium hydrogen sulfate, sodium hydrogen carbonate, and brine. After the solvent was removed, the residue was purified by chromatography on silica gel (ethyl acetate/ hexane gradient) to afford the pure (**26**) (1.16 g, 72%) as a white solid.

Characterizations matched previous reports.²⁸

¹H NMR (CD₃OD/CDCl₃ = 1/9, v,v) δ 4.21 (3 H, m), 4.30 (2 H, m), 5.30 (2 H, m), 7.21 (3 H, m, aromatic), 7.58 (2 H, m, aromatic)

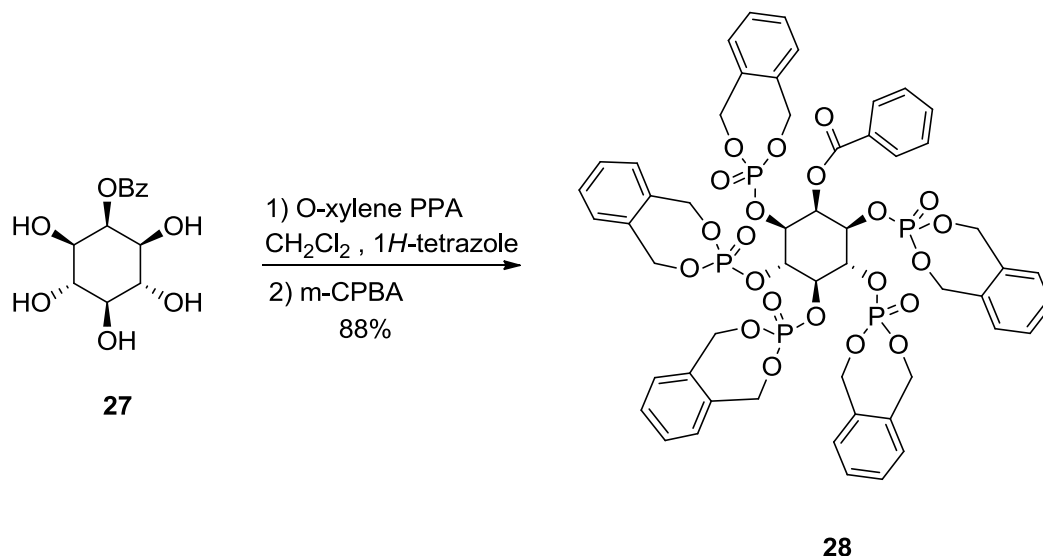
2-*O*-Benzoyl-*myo*-inositol (**27**)



To a solution of (**26**) (250 mg, 0.85 mmol) in methanol (12 mL) was added concentrated hydrochloric acid (6 mL). The mixture was stirred at room temperature for 60 minutes. The solvent was evaporated to dryness. The residue was dried under vacuum to give (**27**) quantitatively. Characterizations matched previous reports.²⁸

¹H NMR (D₂O) δ = 3.26 (1H, t, J = 7.3 Hz), 3.66 (4H, m), 5.55 (1H, s, H-2), 7.41 (2H, t, J = 7.6 Hz, aromatic), 7.56 (1H, t, J = 7.6 Hz, aromatic), 7.91 (2H, d, J = 7.6 Hz, aromatic).

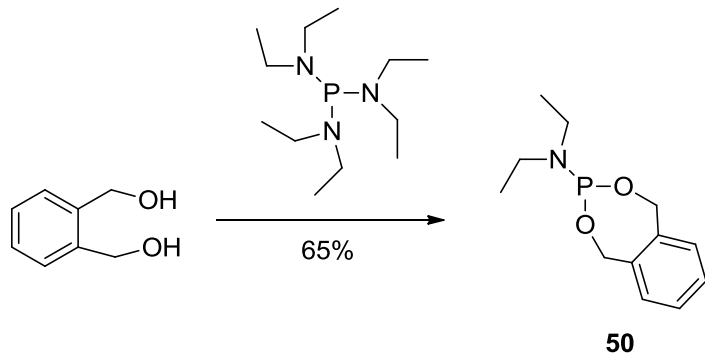
2-*O*-Benzoyl-(1,3,4,5,6)-pentakis(*o*-xylene phosphate)-*myo*-inositol (**28**)



To a solution of (**27**) (10 mg, 0.32 mmol) in dichloromethane (15 mL) at 0 °C were added 1*H*-tetrazole (12.5 mL of a 0.45 M solution in acetonitrile, 5.71 mmol) and phosphine (1.0 mL, 12 eq) and stirred for one hour, then room temp for four hours. The mixture was cooled to -78 °C and oxidized with *meta*-chloroperoxybenzoic acid (1.7 g) for 30 minutes, then room temperature for 45 minutes. Addition of dichloromethane (20 mL) and sodium bisulfate (50 mL) to the reaction mixture and collection of the organic layer with drying over magnesium sulfate. After the solvent was removed, the residue was purified by chromatography on silica gel (ethyl acetate/ hexane gradient) to afford the pure (**28**) (370 mg, 88%) as a white solid. Compounds of similar structure and symmetry were reported in the literature and compared to for analysis.^{29,26}

¹H NMR (CDCl₃) δ = 4.90-5.30 (14H, m), 5.39-5.45 (4H, m), 5.55 (2H), 5.64 (4H, m), 6.4 (1H, s), 7.20-7.50 (21H, m, aromatic), 7.59 (1H, t, aromatic), 8.08 (2H, d).

N, N-Diethylamino-5, 6-benzo-1, 3, 2-dioxaphosphane (**50**)



To the solid 1, 2-benzenedimethanol (4.85 g, 35.0 mmol) was added hexaethyl phosphorus triamide (9.2 mL, 35.0 mmol) and the mixture was condensed at 100 °C without lid until the evolution of diethylamine had ended. The pure (**50**) (5.10 g, 65%) was recovered by vacuum distillation. Characterizations matched previous reports.³¹

¹H NMR (CDCl₃) δ = 1.20 (6H, t), 3.18 (4H, m), 4.90 (2H, q), 5.20 (2H, m), 7.15-7.30 (4H, m, aromatic).

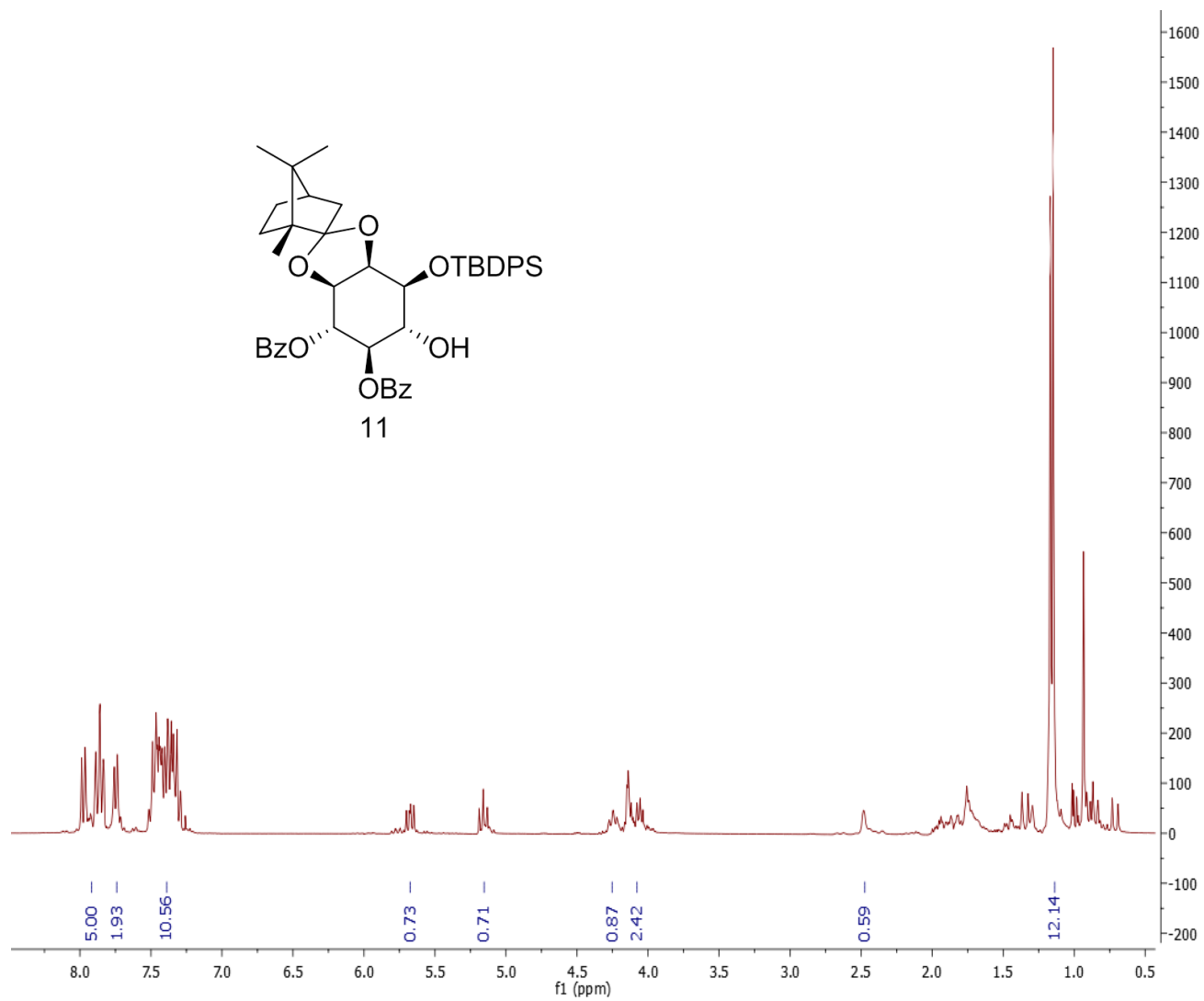
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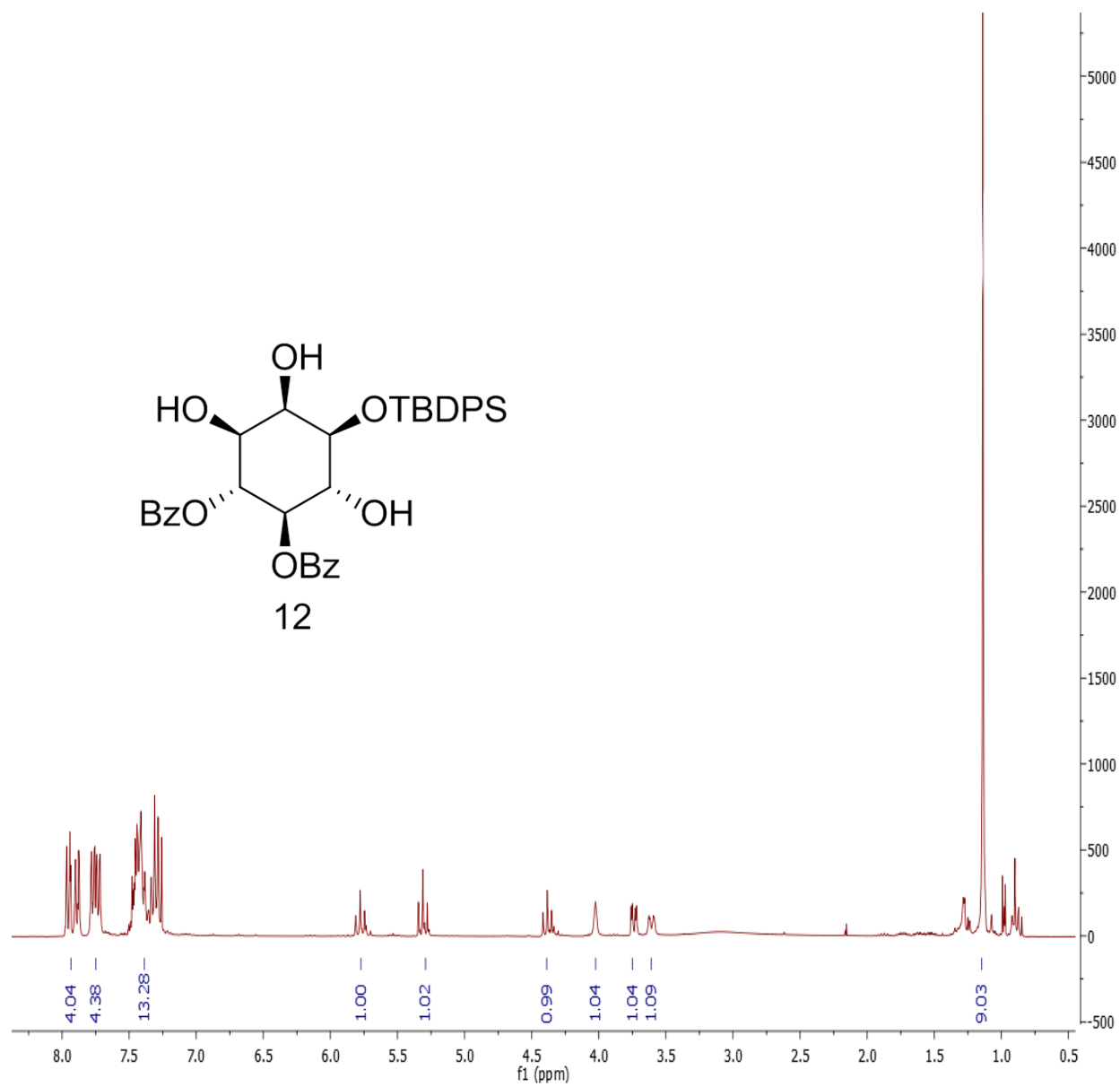
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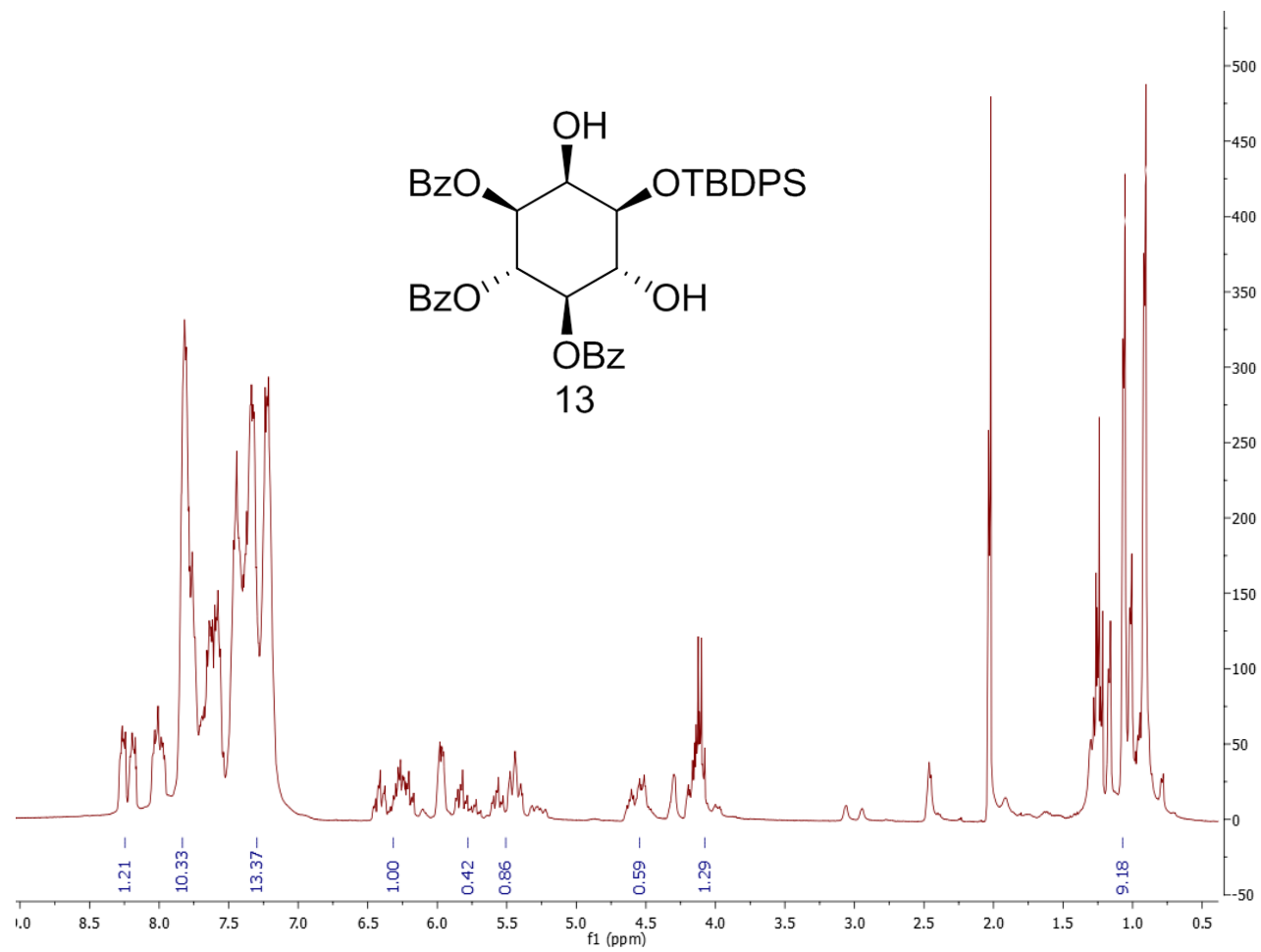
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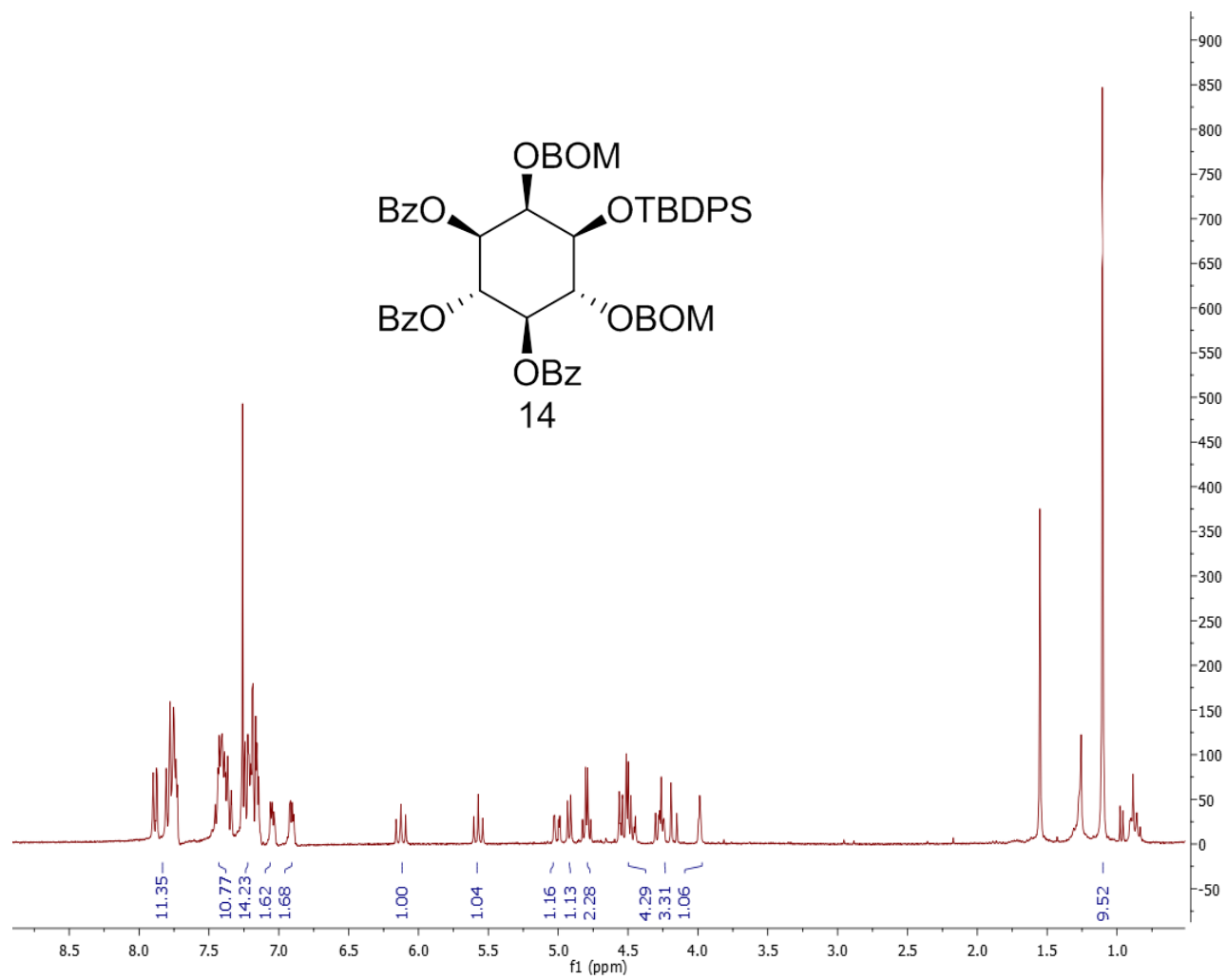
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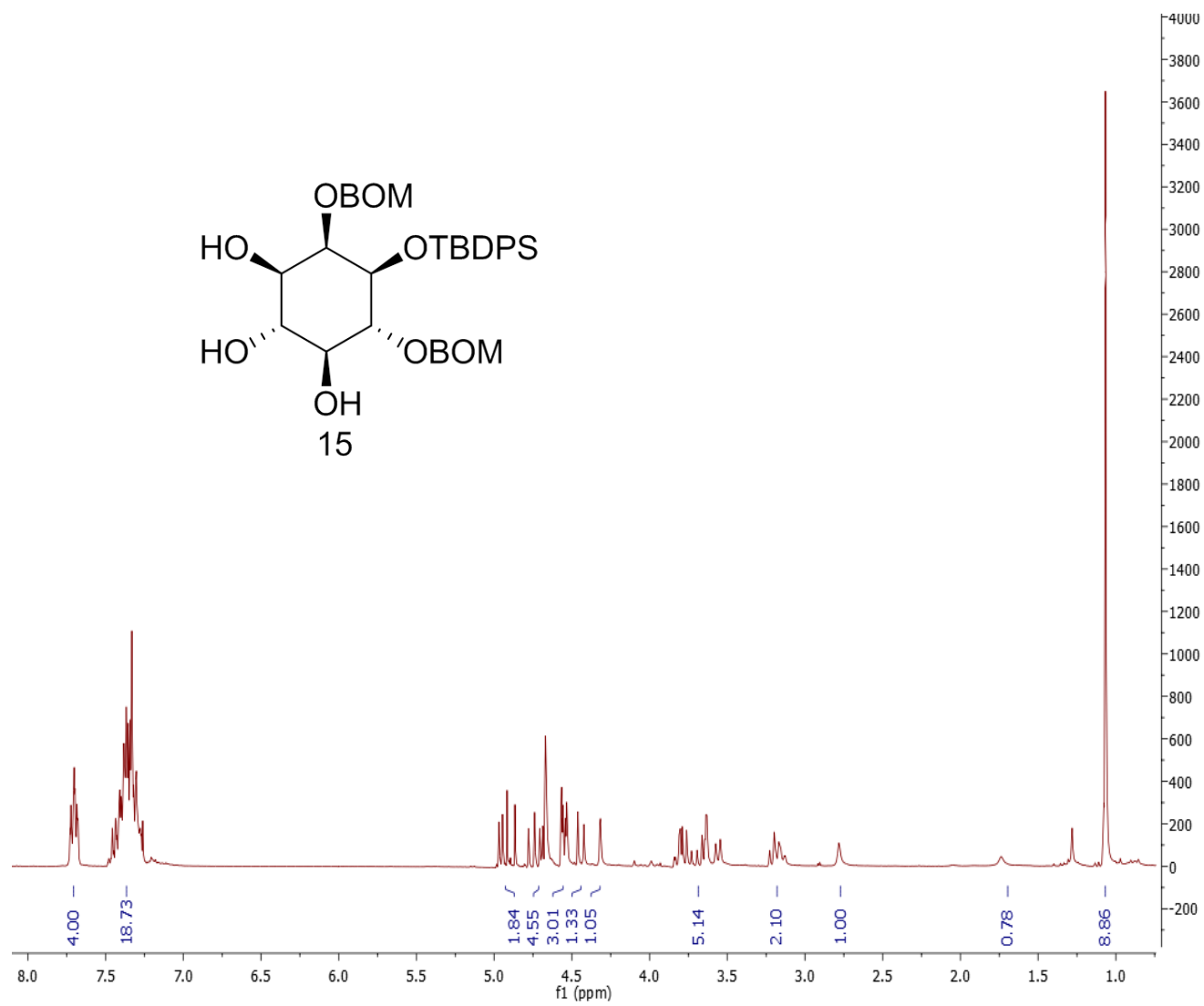
NMR Spectra

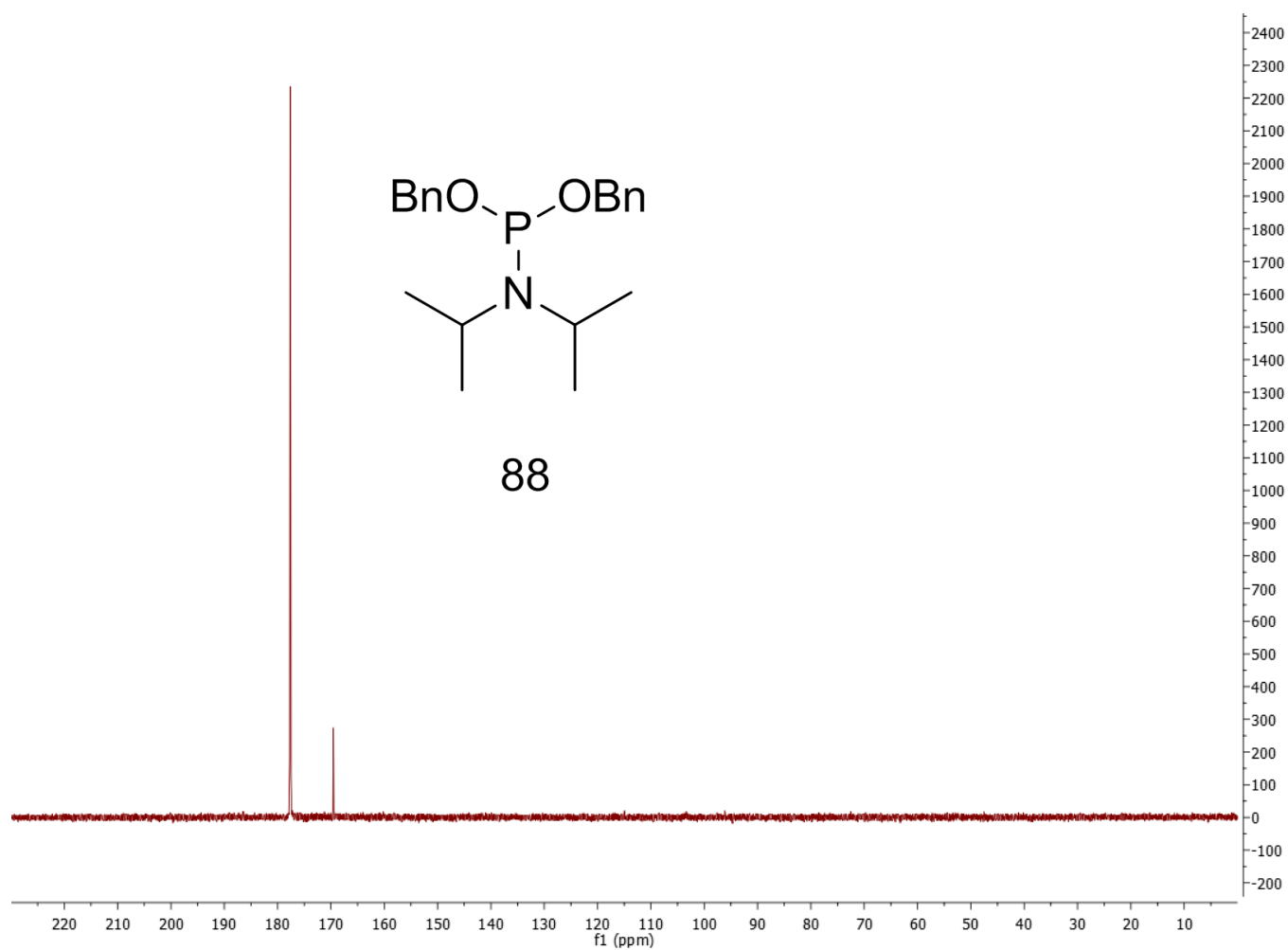


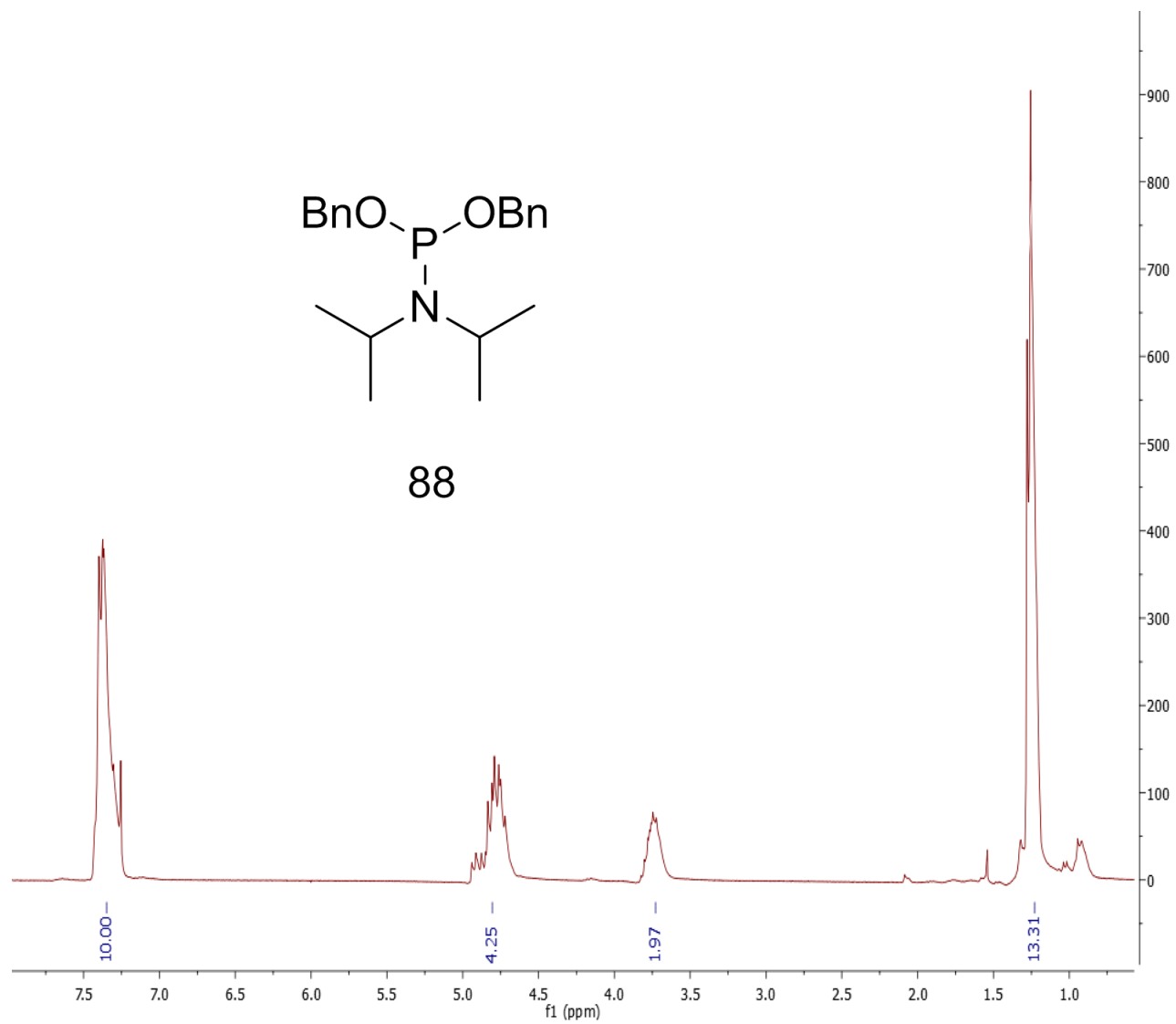
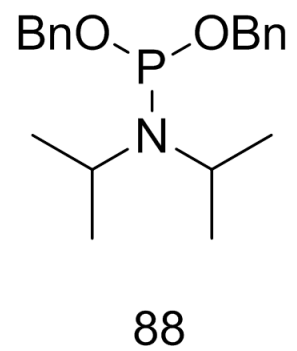


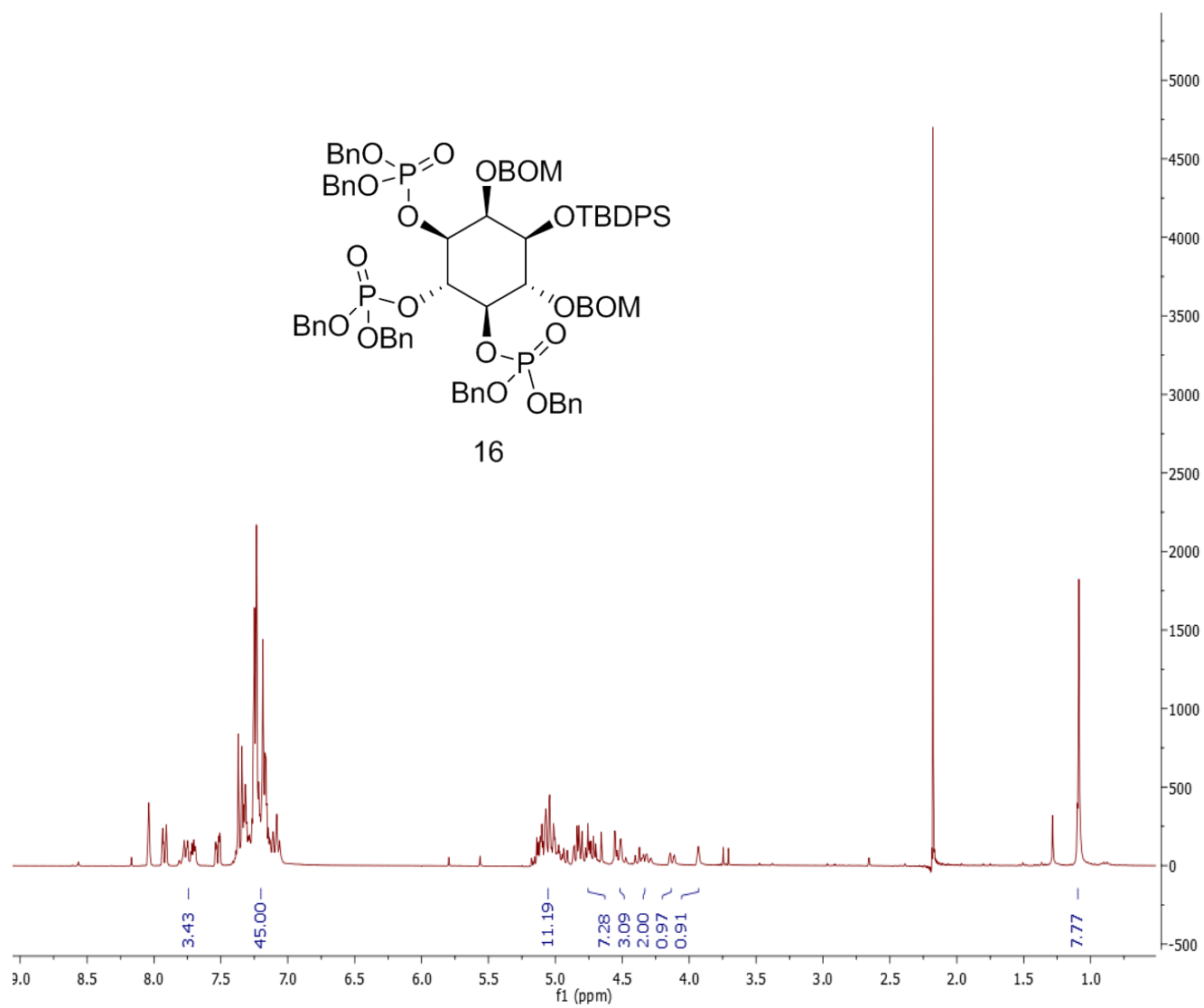


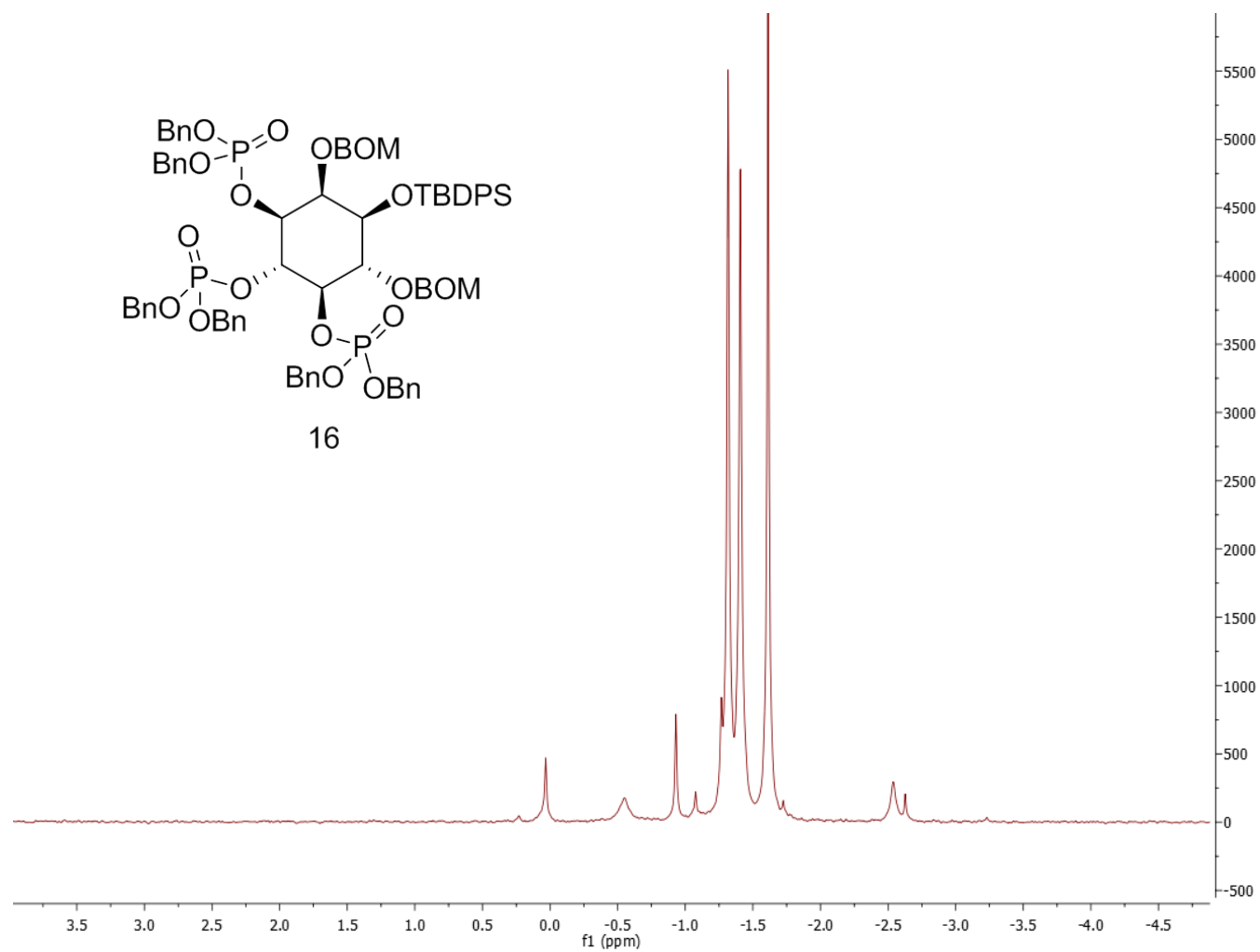


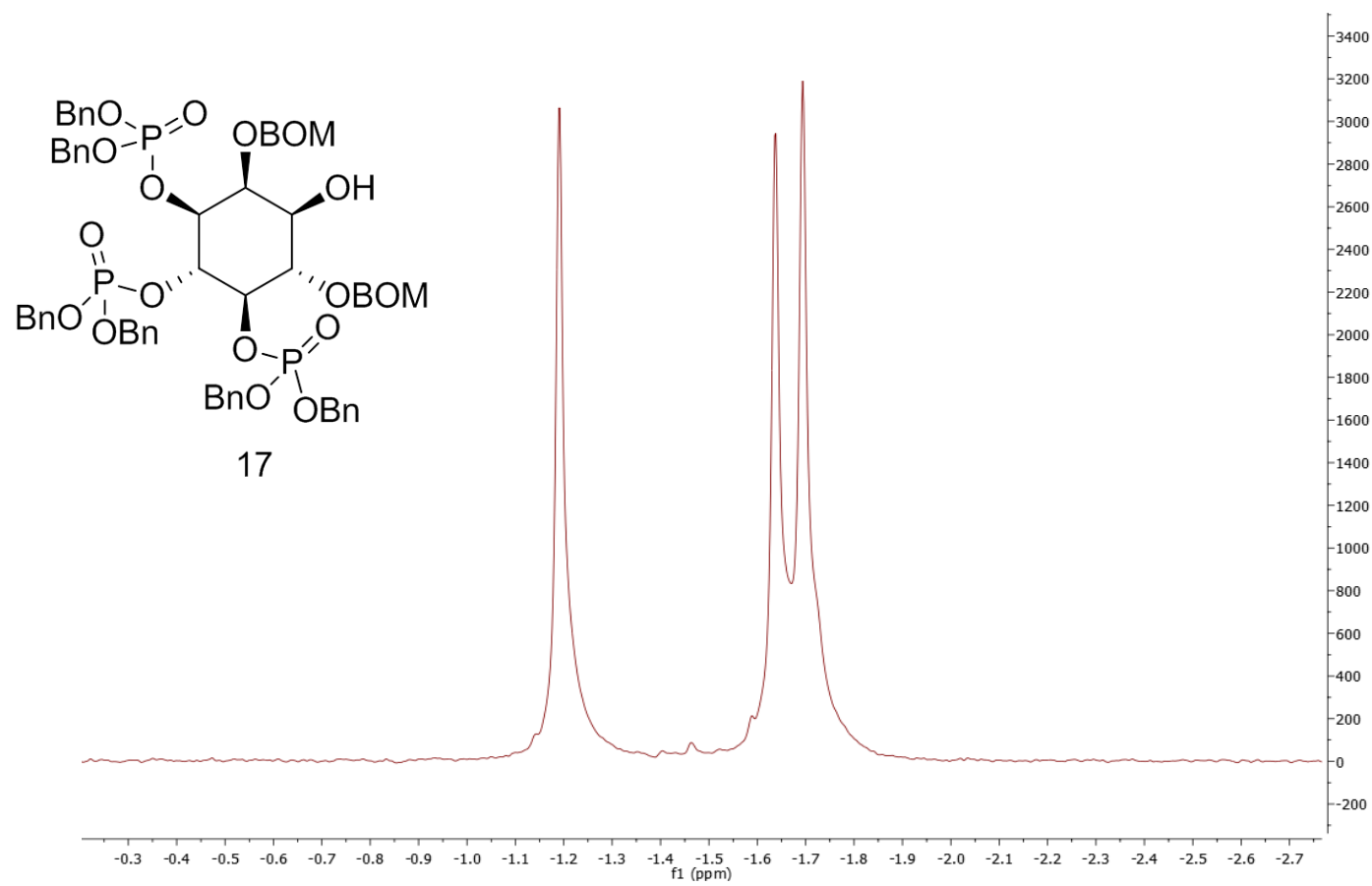


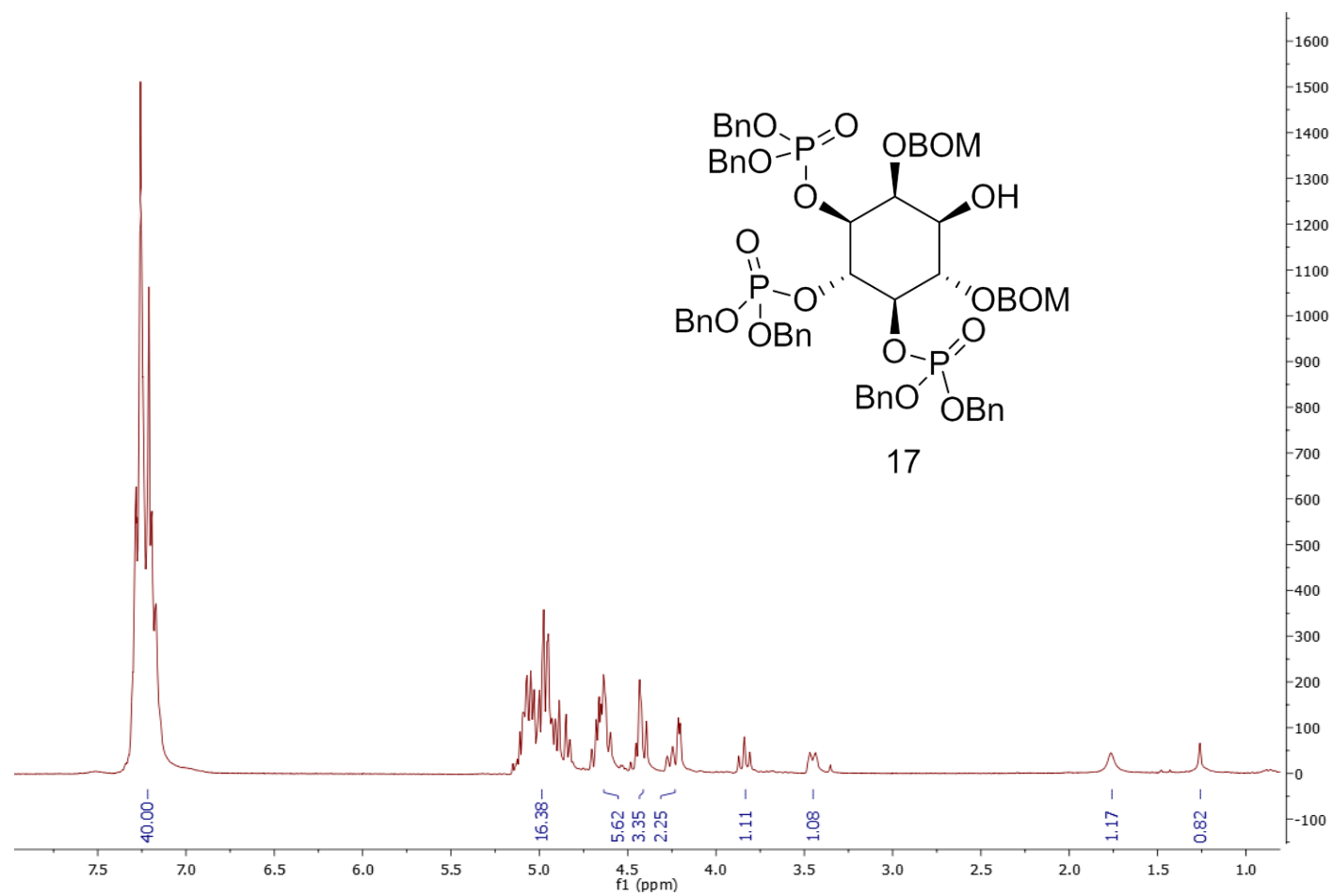


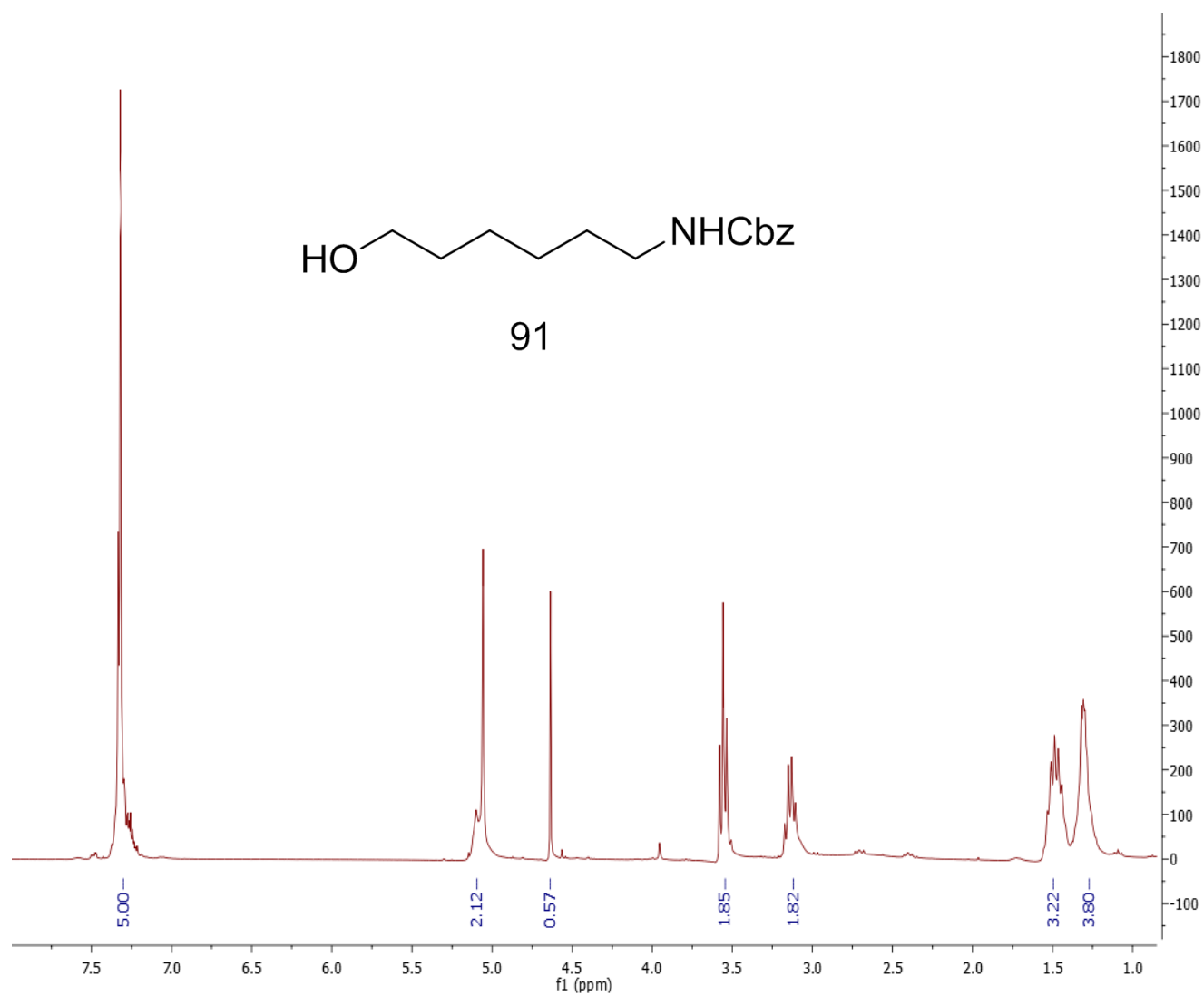


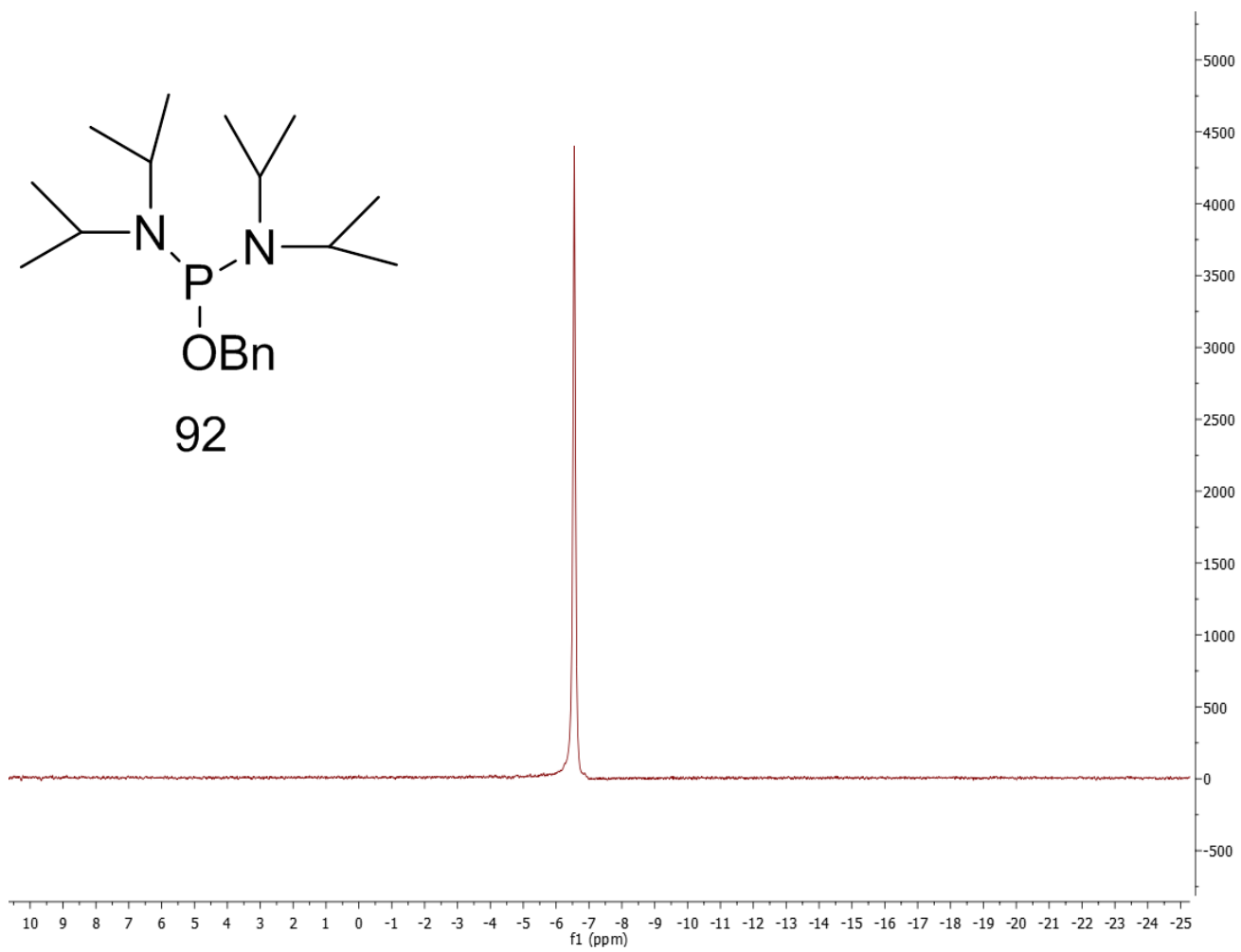


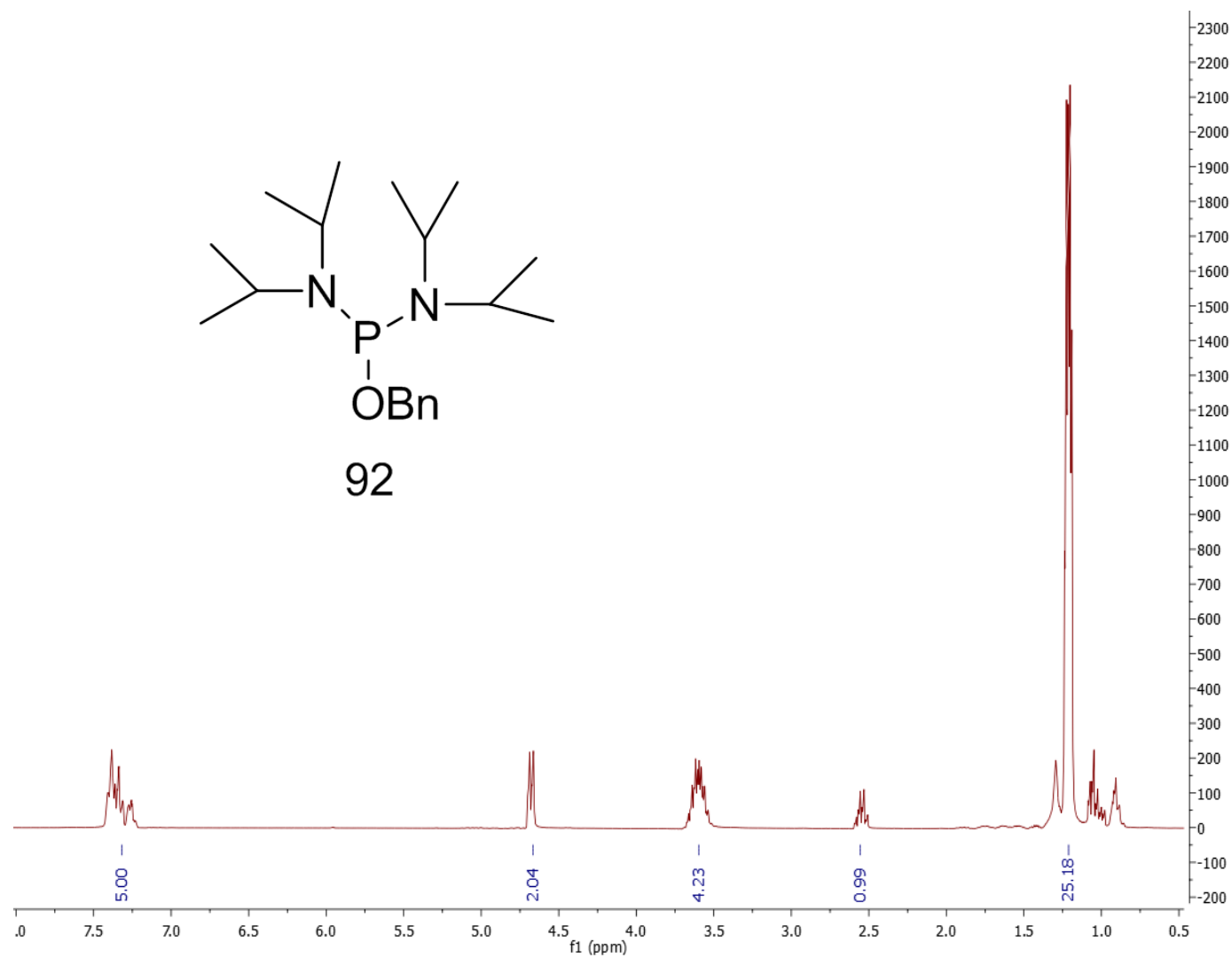


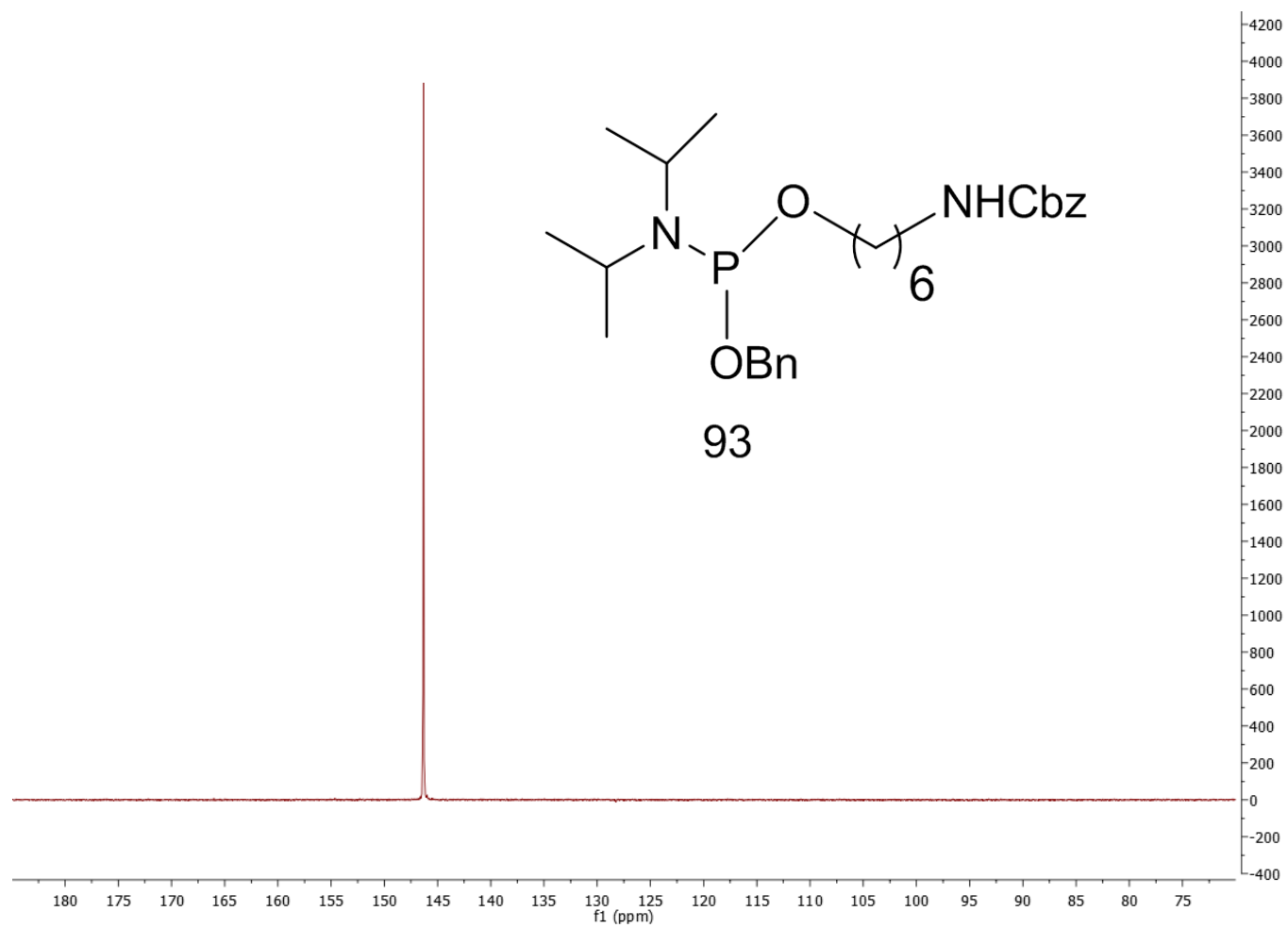


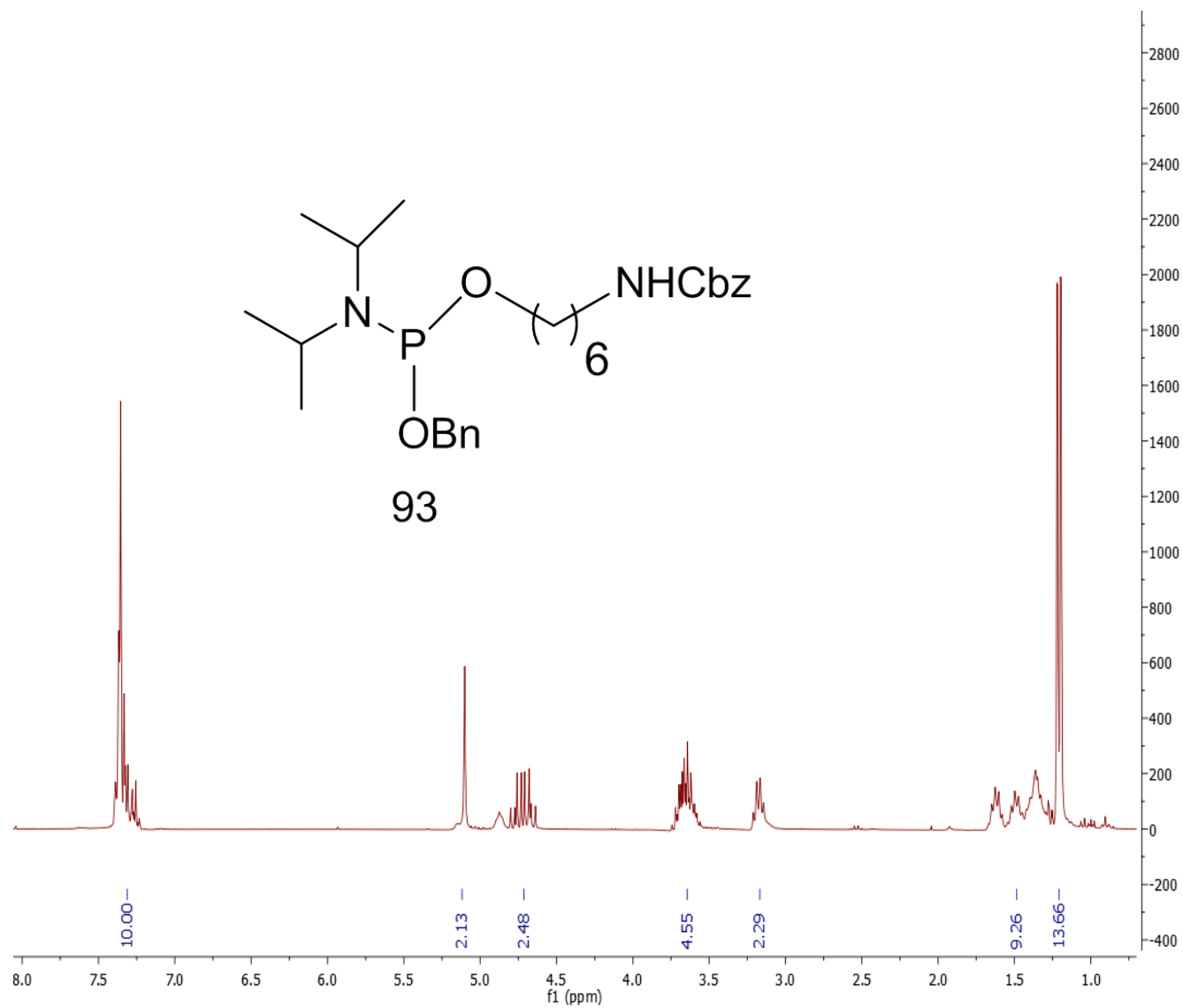


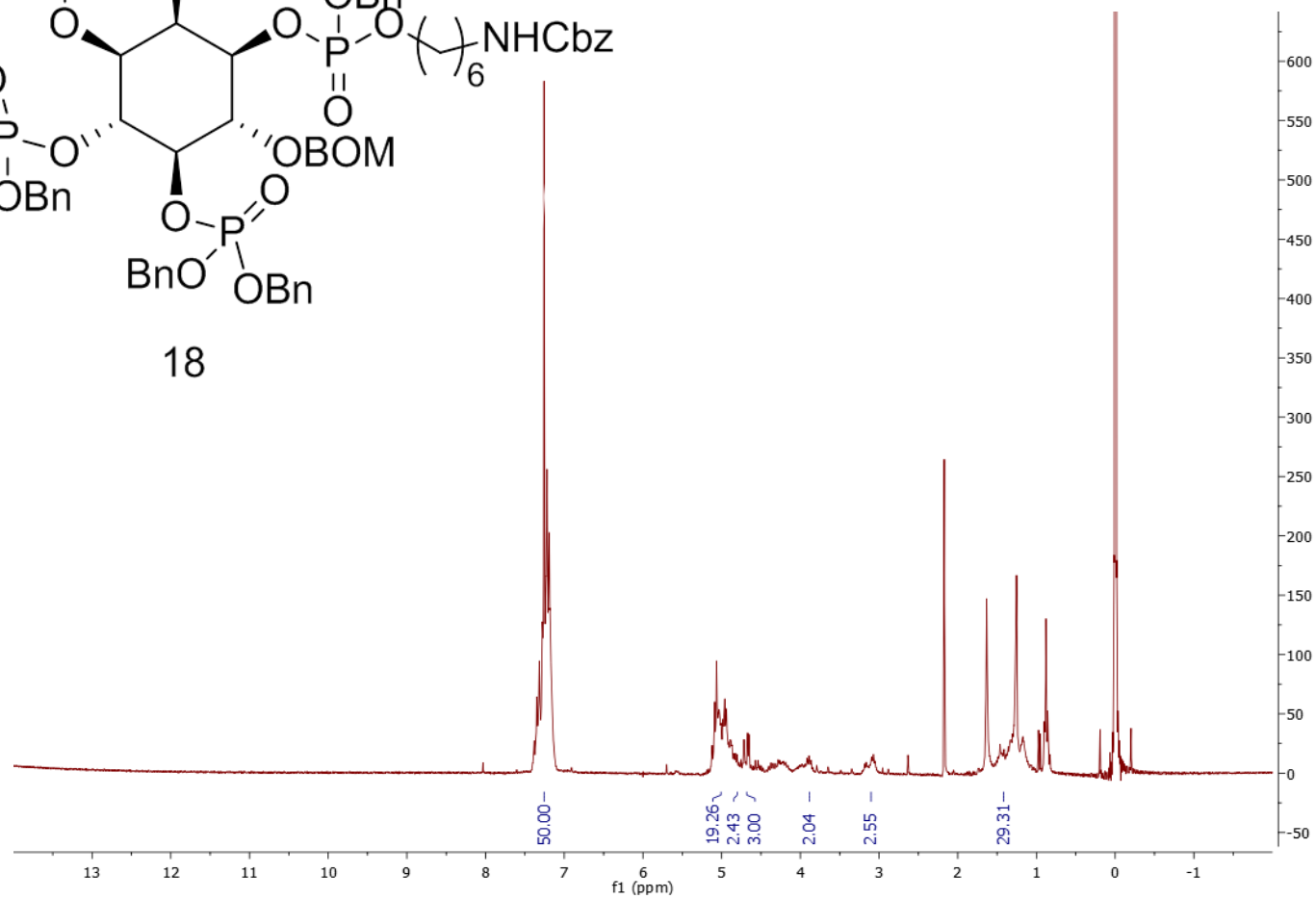
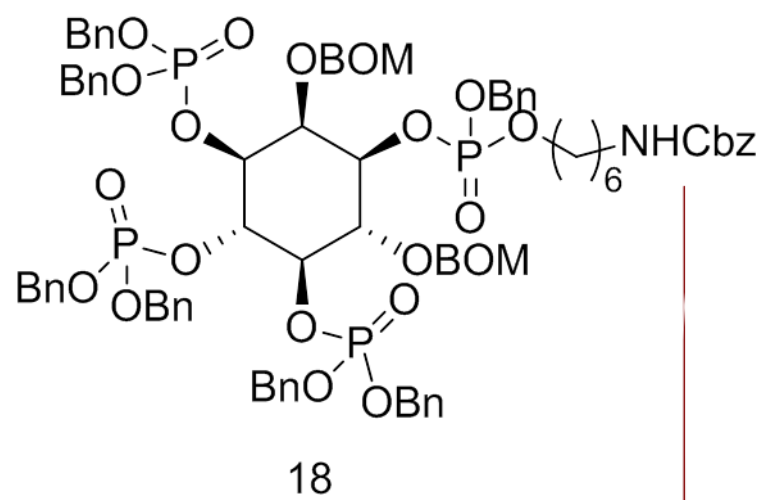


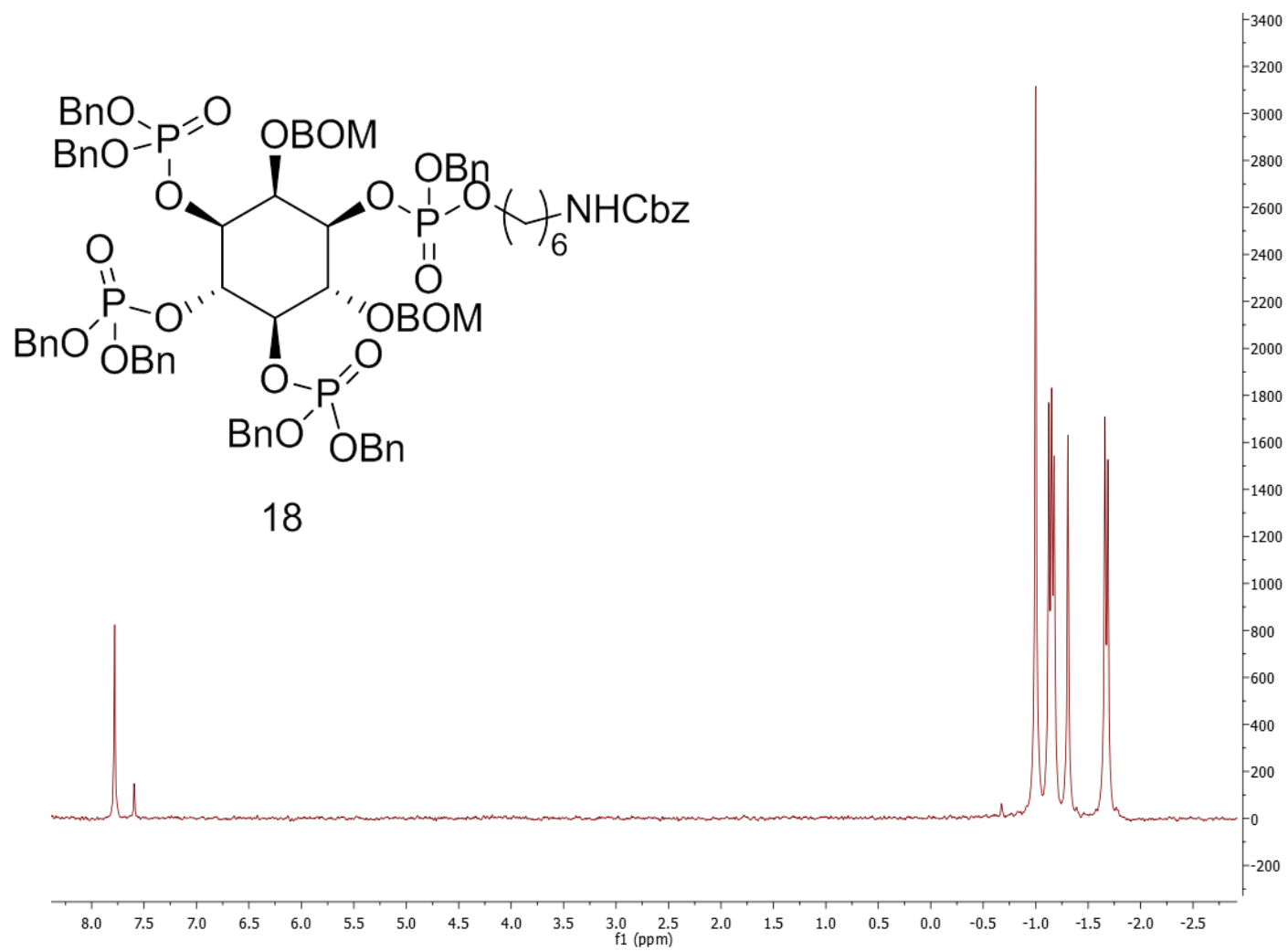


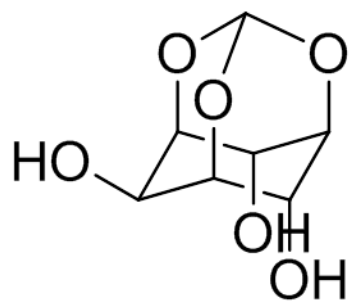




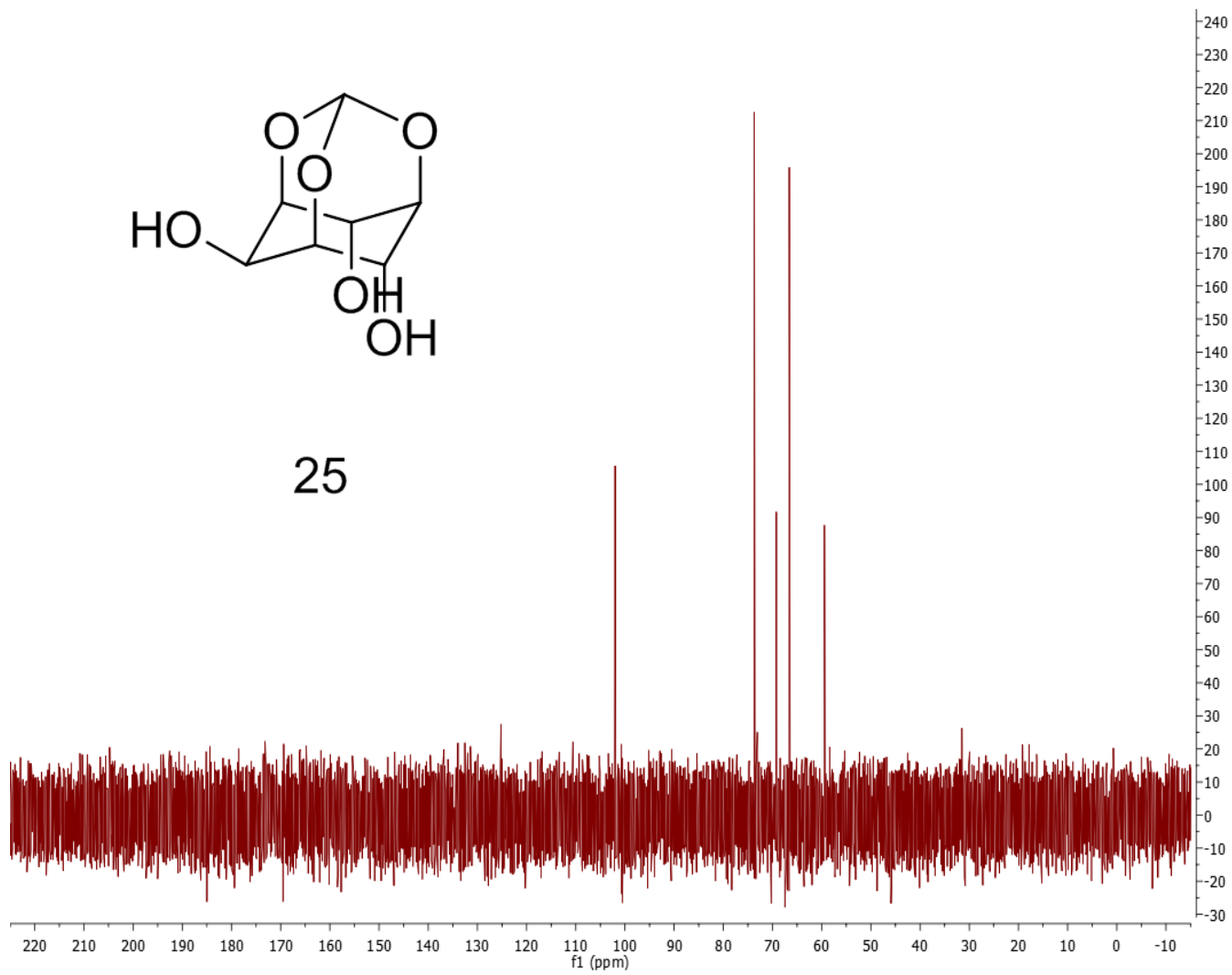


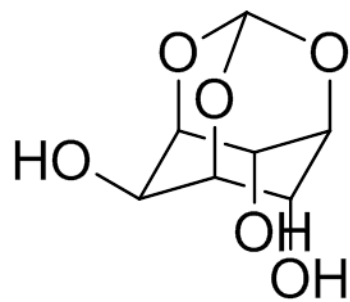




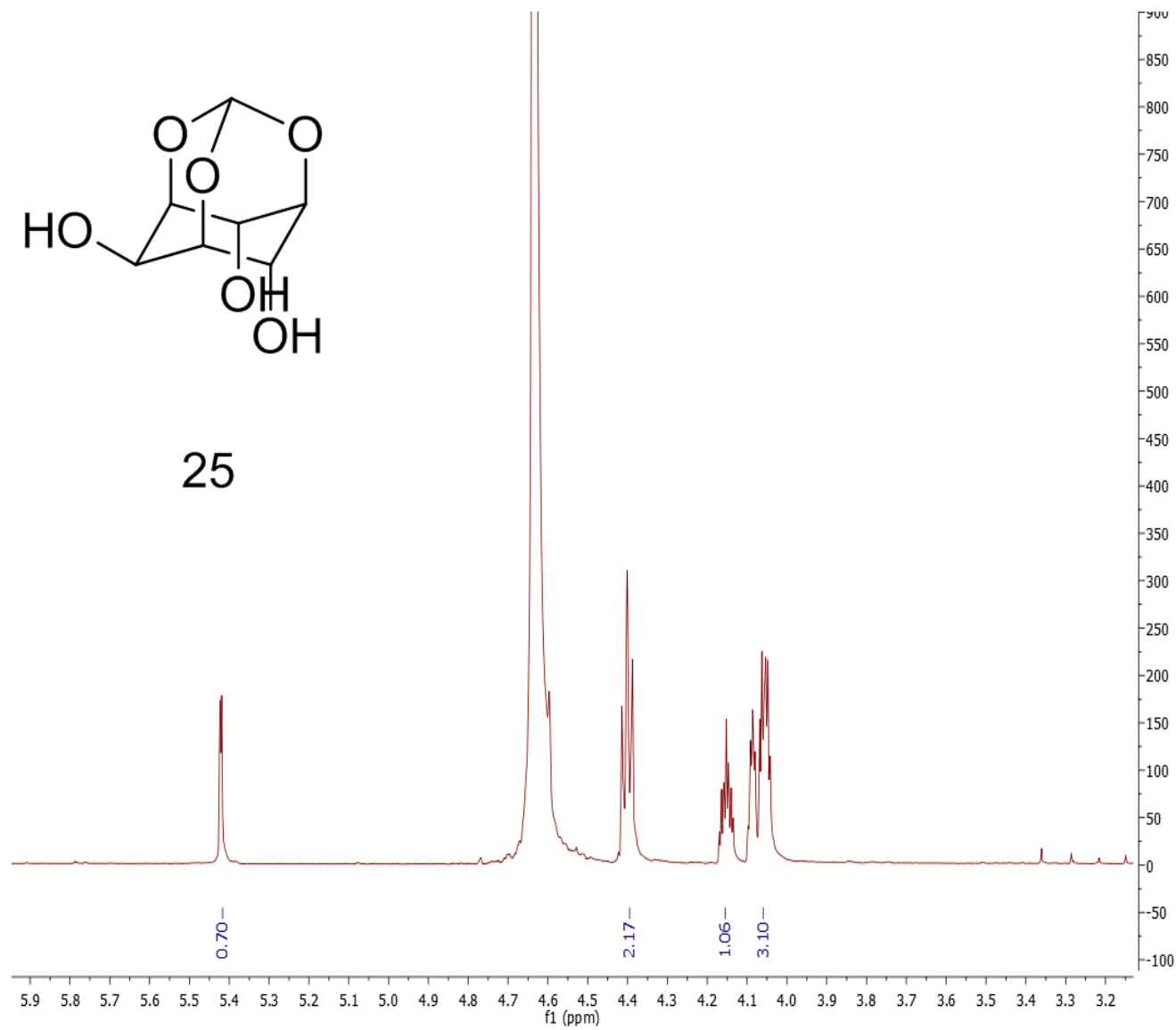


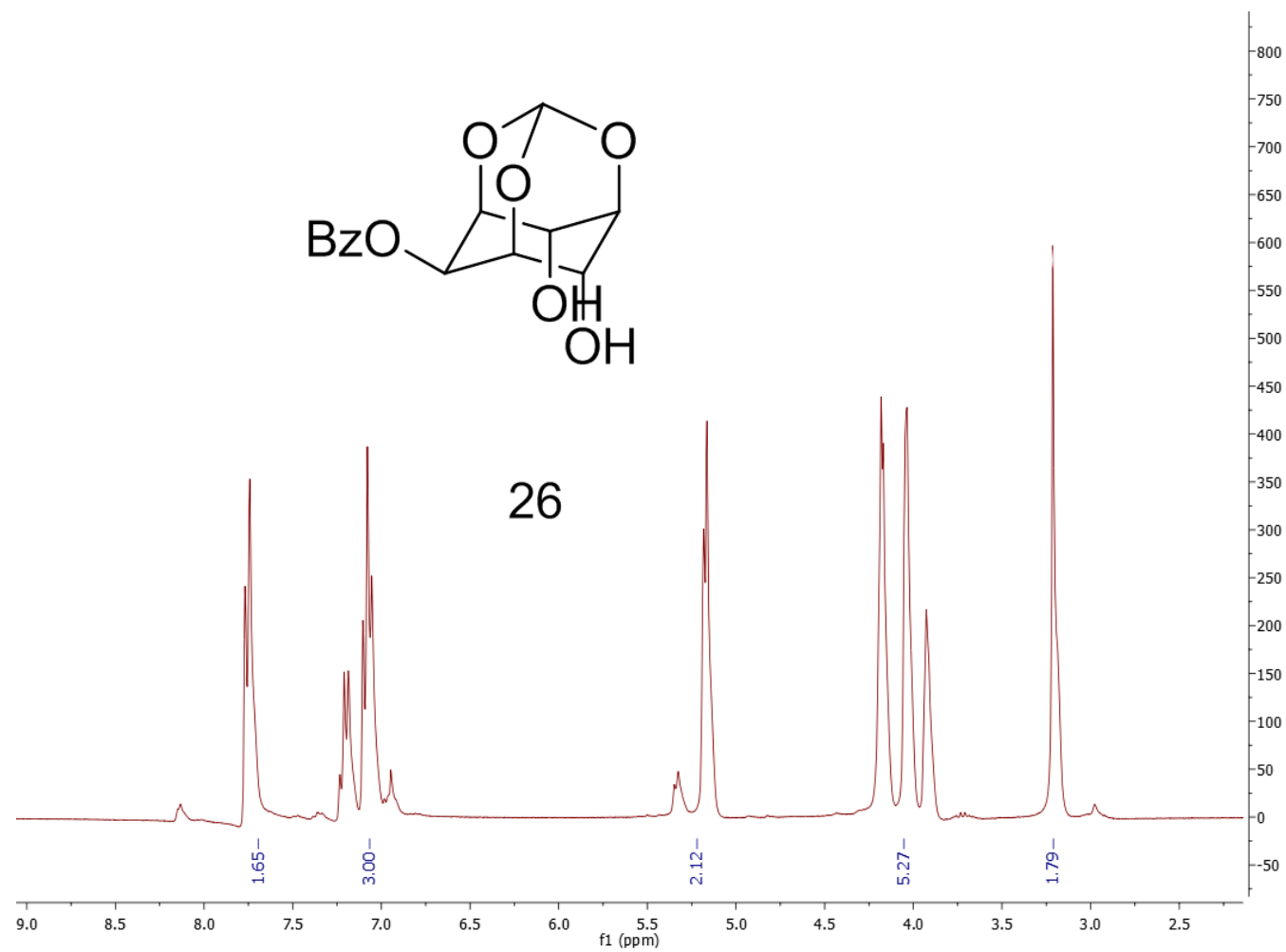
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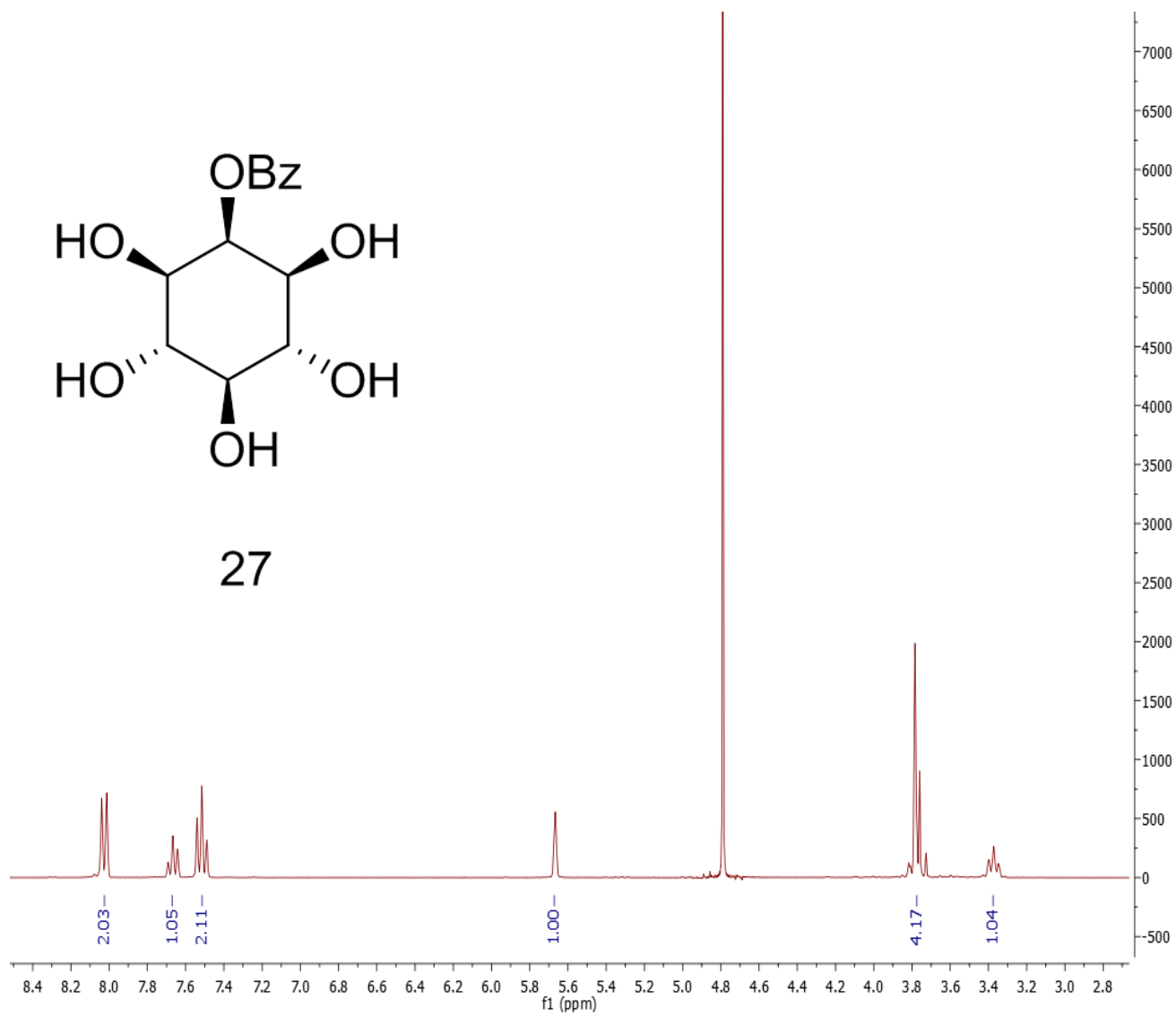


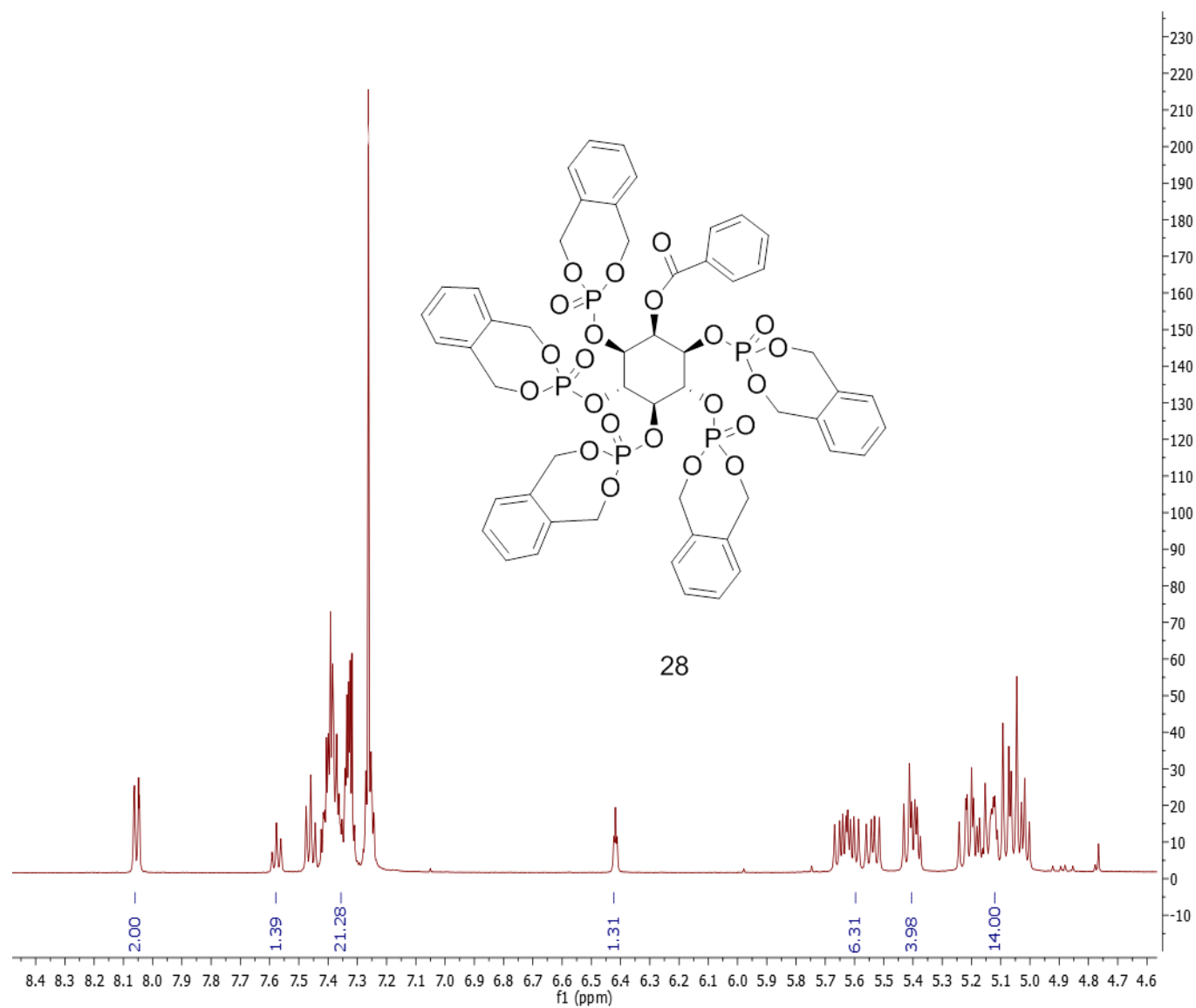


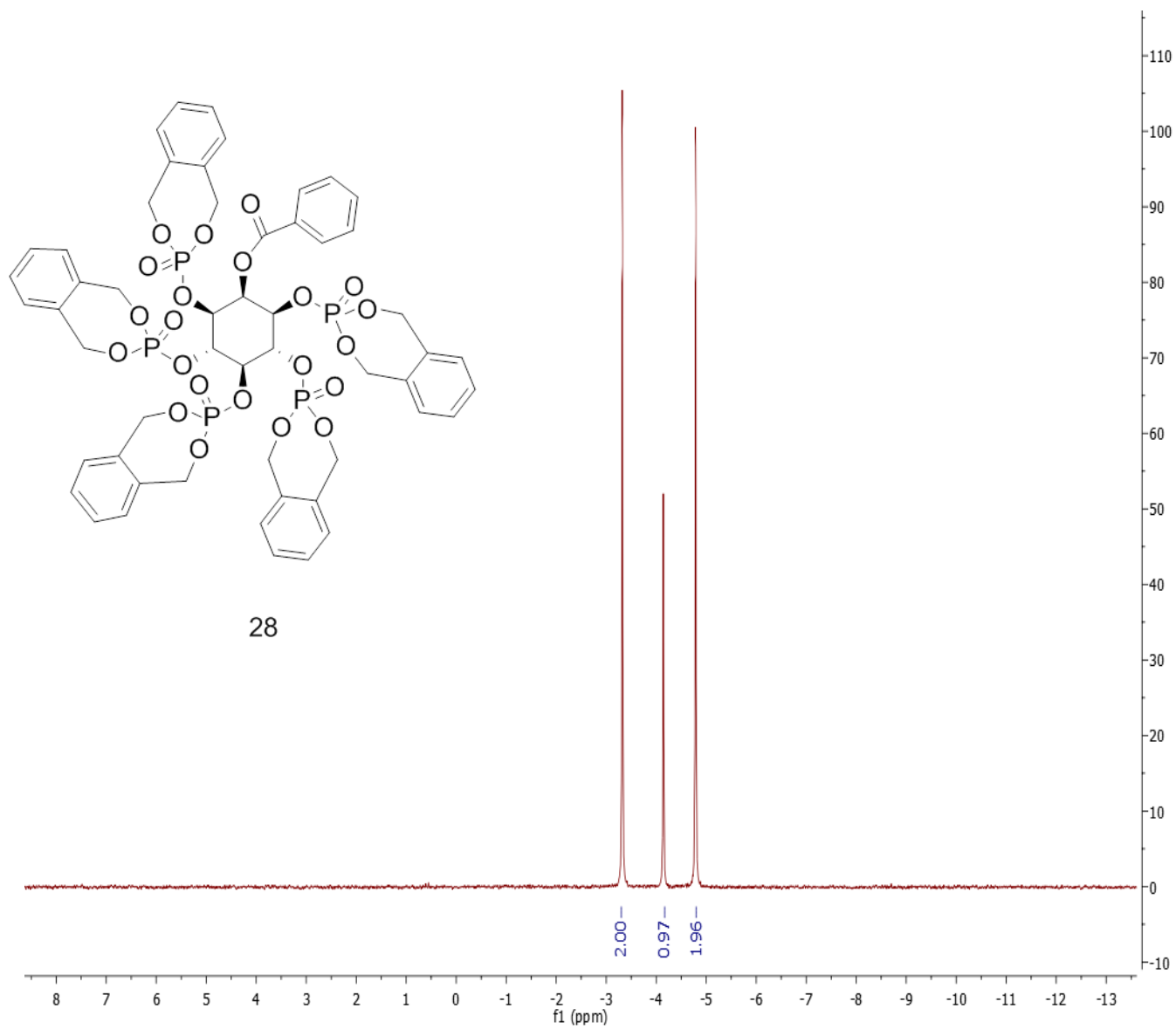
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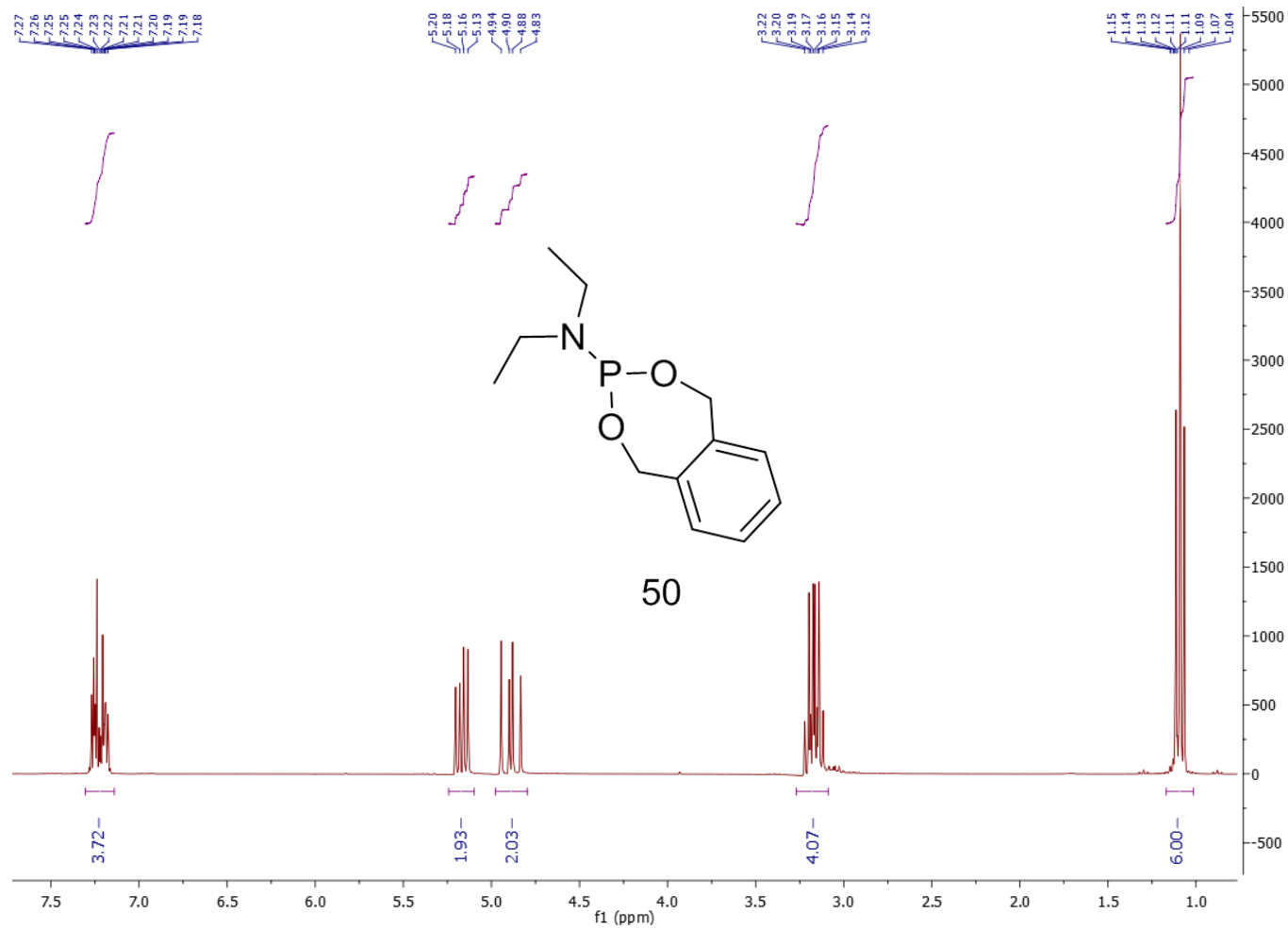


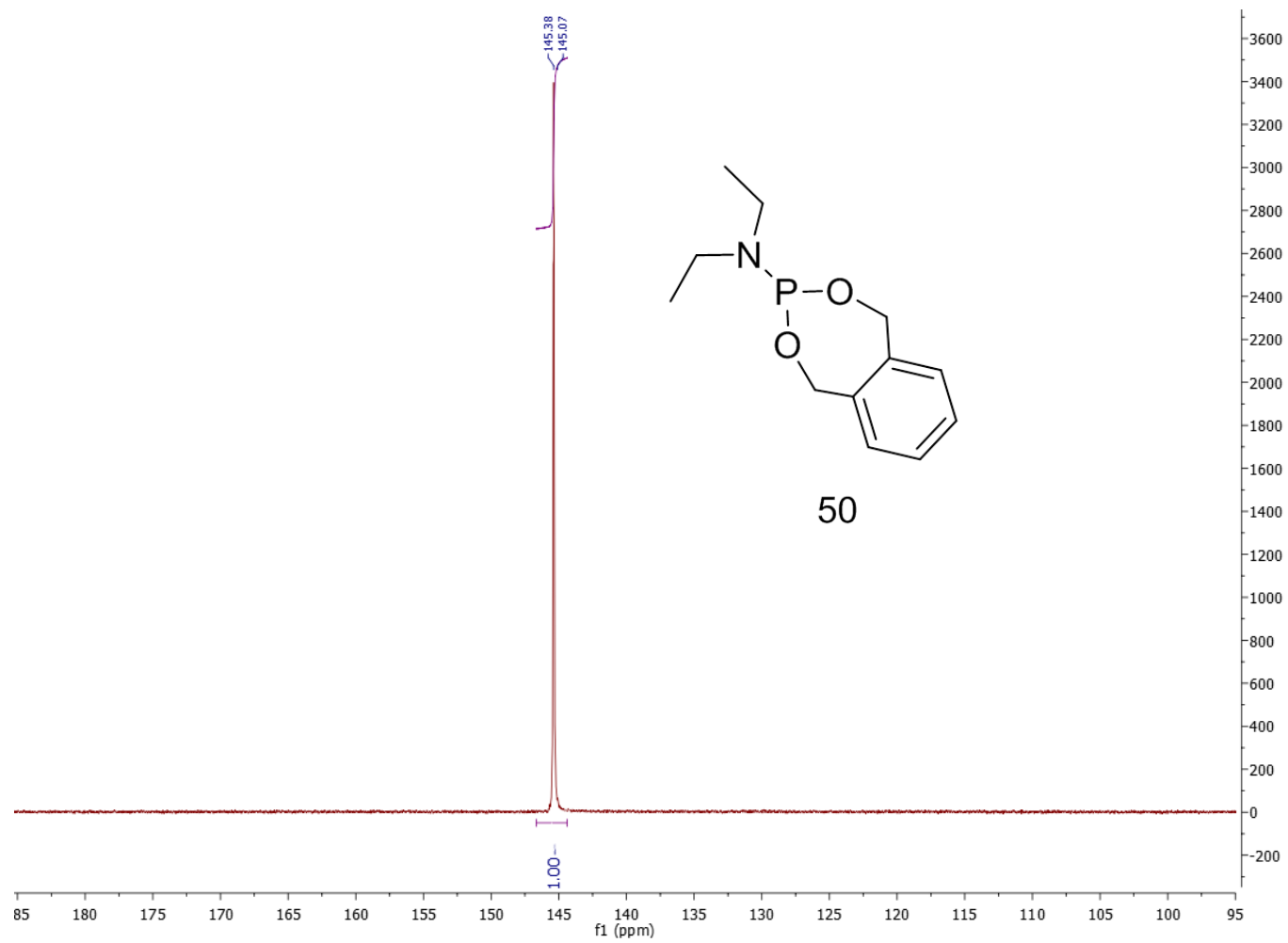












Vita

Benjamin Smith was born in Meadville, Pennsylvania in 1979. He moved to Clarion, Pennsylvania in 2006 to complete his undergraduate education at Clarion University of Pennsylvania. He pursued his graduate career at the University of Tennessee at Knoxville. With the supreme guidance of Dr. Michael Best, Ben completed his Masters degree in Organic Chemistry in August 2013.