Spring 2016

Synthesis of phosphoantigens and chiral trisphosphonates

Rebekah Ruth Shippy
University of Iowa

SYNTHESIS OF PHOSPHOANTIGENS AND CHIRAL TRISPHOSPHONATES

by

Rebekah Ruth Shippy

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Chemistry
in the Graduate College of
The University of Iowa

May 2016

Thesis Supervisor: Professor David F. Wiemer

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Rebekah Ruth Shippy

has been approved by the Examining Committee for
the thesis requirement for the Doctor of Philosophy degree
in Chemistry at the May 2016 graduation.

Thesis Committee:

David F. Wiemer, Thesis Supervisor

F. Christopher Pigge

Daniel M. Quinn

Elizabeth A. Stone

Andrew J. Wiemer

To my parents and sister

Science and religion are not at odds. Science is simply too young to understand.

Dan Brown
Angels and Demons

ACKNOWLEDGEMENTS

First and foremost, I would like to that my Lord and Savior Jesus Christ who has allowed me to pursue my academic, recreational, and musical passions. God has given me the strength and will to make my dreams come true and He has given me life on this wonderful planet.

I would like to thank my Ph.D. advisor Professor David Wiemer who allowed me to join his research group and gave me opportunities to grow and increase my knowledge of chemistry. His direction and support have allowed me to advance as a scientist. His mentorship has allowed me to grow both as a chemist and an individual. He has been kind and compassionate, and pushed me to achieve things I never believed I was capable of doing. Even though the Cardinals will always be better than the Brewers, I will miss the many baseball discussions we have over the years. Dr. Wiemer's constant encouragement will remain with me for the rest of my life. I could not have asked for a better advisor both scientifically and personally.

I am also very grateful for members of the Wiemer group, both past and present. I felt welcome to the group from the moment I arrived. The encouragement and support in learning new techniques, discussing possible solutions, preparation for my examinations, and friendships over the last 6 years has been greatly appreciated. I am thankful for Dr. Xiang Zhou who encouraged me to think critically and helped me to begin my path of obtaining my Ph.D. and Dr. Natalie Ulrich who helped me edit and helped me think critically through my comprehensive exam documents and seminar topic. I am thankful to Dr. Rocky Barney who provided helpful intermediates and I am thankful for passing along to me his knowledge of phosphorus chemistry. I am grateful for Dr. John Kodet and Dr.

Alyssa Hartung for their patience and continual knowledge whenever I had questions or needed a second opinion. I would like to thank Ben Foust who tested my patience in both an academic and research setting. He showed me I could learn from my students to mature into a stronger teacher and mentor. It has been a joy watching him grow in his scientific knowledge and I will appreciate his friendship which has blossomed into long lasting relationship. I am grateful to Robert Matthiesen and Chloe Schroeder for their willingness to appreciate the little things every day, including hiding a troll around the lab only to stumble upon it unexpectedly. I would like to thank Dr. Veronica Wills and Kevyn Ricossa for long nights and weekends we spend studying for classes and comps in the chemistry building. Also I will cherish the friendships with Dr. Veronica Wills, Kevyn Ricossa, and Dr. Alyssa Hartung for the endless number of walks to Starbucks in order to obtain coffee to rejuvenate our minds, even in cold Iowa winters.

I would like to express my appreciation for the collaborators, Dr. Andrew Wiemer, as well as Dr. Chia-Hung Christine Hsiao, Ashley Kilcollins, Jin Li, and Olga Vinogradova, at the University of Connecticut, for providing biological data for the phosphoantigen analogues described in this thesis. I am grateful to my committee members, Dr. Daniel Quinn, Dr. F. Christopher Pigge, and Dr. Elizabeth Stone for allowing me to speak with them about possible solutions to problems that have arisen throughout my Ph.D. career. I appreciate the helpfulness of the past and present chemistry staff members including Sharon Robertson, Janet Kugley, Lindsey Elliot, Tim Koon, Benjamin Revis, Frank Turner, Dr. Lynn Teesch, and Vic Parcell for their advice and help in their respectful fields. Financial support provided by NIH, the Ralph Shriner Graduate fellowship, and the McCloskey Fellowship Fund are gratefully acknowledged.

Last but not least, I extend my sincere gratitude to my family and friends for their encouragement and patience as I pursue my chemistry degree. I am grateful for the long lasting friendships with Abby Miller, Matt Miller, and James Arnett whose friendship grows more and more each day. I will forever remember the dinners, movie nights, and fro-yo trips with Dr. Caitlin Runne-Janzcy, Allison Songstad, Katie Cranston, and Simone Cox. I want to thank my trivia team and softball friends, Heather Krammer, Ericka Larson, Kelly Crawford, Adele Vanarsdale, Dan Cushman, Michael Kerns, Bernie Schieltz, Joel Martinek and many more for their encouragement and laughter throughout my time here at the University of Iowa. I would like to thank my better half, Matthew McConnell, who has stood by my side through the ups and downs. He has remained my rock through all the difficult times and I will love and cherish him deeply. Throughout my life, my sister Sarah has been my best friend through the difficult and wonderful times. I always appreciate my time and experiences with her. Finally, my parents have always been supportive, encouraging, and loving throughout my entire life. They have inspired me to live my life to the fullest every day even through difficult times. I love you more than you could possibly imagine and could not have achieve my goals without your guidance from day to day!

ABSTRACT

Phosphorus is an element that is essential for life, and is used in the synthesis of many proteins, carbohydrates and nucleic acids. Phosphorus often exists in the form of phosphate when found in biological systems. Clinical development of possible pharmaceutical agents has used phosphorus in the form of phosphonates to increase the metabolic stability of the potential drug. Some of these phosphonates target the isoprenoid biosynthetic pathway (IBP). The IBP plays an important role in the synthesis of cholesterol and in other aspects of cellular metabolism. The enzymes of the IBP have been the target of possible therapeutic agents for treatment of multiple diseases, including cancer. Often these phosphonic acids are masked by an enzymatically cleavable group in order to increase their bioavailability and activity.

Phosphoantigens are small organophosphorus molecules that stimulate the expansion of Vγ9Vδ2 T-cells which detect and eliminate infected cells. Both natural and non-natural phosphoantigens have exhibited a wide range of effective concentrations (EC_{50}) for γδ T-cells. The most potent phosphoantigen is *E*-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP), which is an intermediate in the bacterial IBP. Nanomolar concentration of this compound stimulate T-cell proliferation. While HMBPP is highly potent, it undergoes rapid decomposition when injected into the blood stream. Synthesis of more stable phosphonate analogues can lead to better activity for expansion of the γδ T-cell population. Further increased activity was observed in T-cell assays after masking the phosphonic acids to increase the bioavailability of the active phosphoantigen.

Because some phosphoantigens showed strong activity with masked phosphonic acids, families of phosphonate analogues now have been prepared. Most use selenium dioxide mediated oxidation to incorporate the terminal alcohol and ester exchange to provide prodrugs to study the structure-activity-relationships. The biological activity of these compounds has been investigated and several new phosphoantigens were shown to be strong activators of γδ T-cells. Furthermore, the phosphoantigens have been shown to bind to the protein butyrophilin 3A1 (BTN3A1) at an intracellular domain. A second family of phosphoantigen derivatives, masked by a new fluorescent cell-cleavable ester, were prepared and tested by our collaborators to explore the compound's activity and to investigate the mechanism of action.

Finally, a new class of phosphorus compounds, alkyl 1, 1, 1-trisphosphonates, has been studied to obtain salts that might be biologically active. Trisphosphonates contain a unique arrangement of phosphonate groups on a single carbon and could provide charge states unseen in the more traditional bisphosphonates. A general route to asymmetric trisphosphonates through a step-wise phosphonylation of each phosphonate has been developed. Selective phosphonate ester cleavage would allow for the ability to obtain a variety of charge states and possible biological activity.

PUBLIC ABSTRACT

Phosphorus is an element that is essential for life, and is found in proteins, carbohydrates and nucleic acids (DNA). The metabolically more stable phosphonates may represent a more attractive template for pharmaceutical agents, and phosphonates which target the isoprenoid biosynthetic pathway (IBP) are known. This pathway has been the target of clinical drugs used to treat multiple diseases including cancer. Often these phosphonic acids are masked by cell-cleavable groups in order to increase their bioavailability and activity.

My research has focused on the synthesis of phosphonates that stimulate immune cells to detect and eliminate infections. Synthesis of phosphonate analogues of natural phosphoantigens determined that they show biological activity, specifically the ability to expand immune cell populations. Families of phosphonate analogues now have been prepared to study how different structures affect their biological activity. One family of phosphonate derivatives, masked by a new pH-sensitive, fluorescent, cell-cleavage ester, was prepared and tested as a biological probe.

A relatively new class of phosphorus compounds that has yet to be biologically explored would be the trisphosphonates. Trisphosphonates contain three phosphorus groups on a single carbon. Selective preparation of various trisphosphonate salts may lead to biological activity. The preparation of these molecules will be examined in detail.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4-HBA	para-hydroxybenzoic acid
15C5	15-crown-5
16C6	16-crown-6
ACN	acetonitrile
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
br	broad (NMR)
Bu	butyl
BRSM	based on recovered starting material
C	celsius
calcd	calculated
CCOM	coumarin carboxyl oxy methyl
d	doublet (NMR)
DABCO	1, 4 diazabicyclo[2.2.2]octane
DMAPP	dimethylallyl diphosphate
DNA	deoxyribonucleic Acid
DMF	dimethylformamide
dt	doublet of triplets (NMR)
EC_{50}	half maximal effective concentration
EI	electron impact
ESI	electrospray ionization
Et	ethyl
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
eq	equivalents
FDPS	farnesyldiphosphate synthase
FPP	farnesyldiphosphate

FTase	farnesyltransferase
g	gram
GGDPS	geranylgeranyl diphosphate synthase
GGPP	geranylgeranyldiphosphate
GGTase	gernaylgeranyltransferase
GPP	geranyldiphosphate
h	hour
HMG-CoA	3-hydroxyl-3-methylglutaryl coenzyme A
HRMS	high resolution mass spectroscopy
Hz	hertz
IL2	interleukin 2
IPP	isopentyldiphosphate
iPr	isopropyl
J	coupling constant (NMR)
K562	human leukemia cell line
KHMDS	potassium hexamethyldisilyl azide
LDA	lithium diisopropyl amide
m	multiplet (NMR)
min	minutes
M	molar
Me	methyl
MeOH	methanol
mg	milligram
mL	milliliter
μM	micromolar
mmol	millimole
nM	nanomolar
n–BuLi	*n*–butyl lithium
Ms	methanesulfonyl
MsCl	methanesulfonyl chloride
m/z	mass/charge ratio

NMR	nuclear magnetic resonance
Piv	pivaloyl
PivCl	trimethylacetylchloride
POM	pivaloyloxymethyl
POMCl	chloromethyl pivalate
PPi	diphosphate
ppm	parts per million
q	quartet (NMR)
rt	room temperature
s	singlet (NMR)
SAR	structure-activity relationship
SeO_2	selenium dioxide
SQS	squalene synthase
t	triplet (NMR)
td	triplet of doublets (NMR)
tt	triplet of triplets (NMR)
TEA	triethylamine
TEMBP	tetraethyl methylenebisphosphonate
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMS	trimethylsilyl
TMSBr	trimethylsilyl bromide

CHAPTER 1

A BRIEF OVERVIEW OF PHOSPHONATES AS DIPHOSPHATE MIMICS AND PHOSPHONATE PRODRUGS

Jacob Moleschott once wrote, "Without phosphorus there would be no thought."[1] Phosphorus is a vital element required for life that is used for numerous processes in the body, along with carbon, hydrogen, nitrogen, oxygen, and sulfur. These six elements are found in all living organisms and are utilized for metabolic and structural purposes in biological systems. Phosphorus, in the form of phosphates, is indispensable for the formation of proteins, carbohydrates, and nucleic acids. This theory was challenged in 2011, when a bacterial species was reported to be able to survive in environments with a high concentration of arsenic. It was proposed that these bacteria substitute arsenic for phosphorus in order to grow. However, it has since been shown that these bacteria lack the ability to grow in phosphorus-diminished environments.[2]

Diphosphates are naturally occurring organophosphorus compounds utilized as intermediates for a myriad of pathways in the body. The most fundamental diphosphate maybe formed through hydrolysis of adenosine triphosphate (ATP, **1**) to adenosine monophosphate (AMP, **2**) and diphosphate (PPi, **3**) (Figure 1). The hydrolysis of the phosphate bond releases energy to promote other enzymatic processes. Utilizing the enzyme ATP synthase, this process can be reversed to form ATP, which can be metabolized again in a continuous cyclic process. With the liberation of PPi, further hydrolysis to phosphate allows the phosphorylation of biological substrates in multiple pathways, including the isoprenoid biosynthetic pathway.[3]

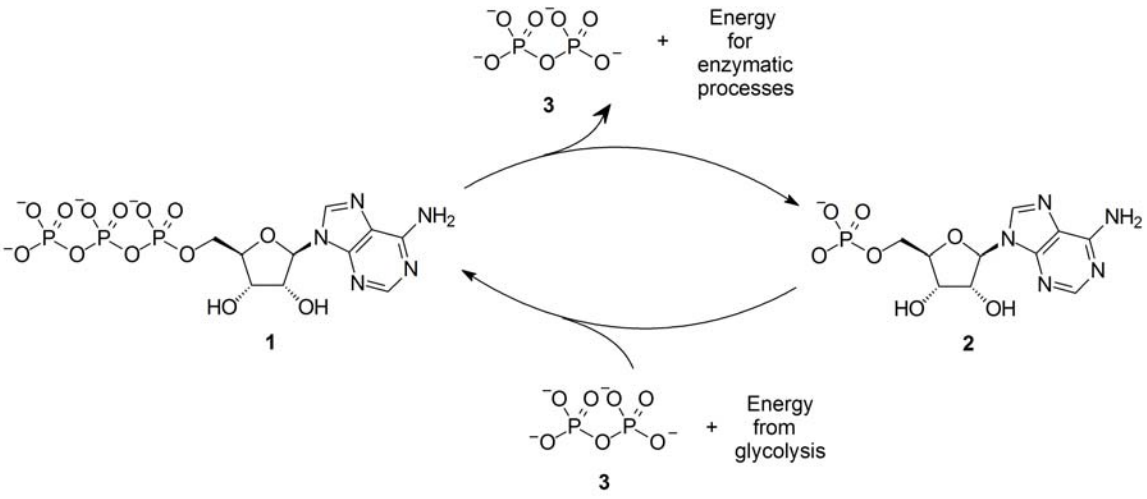


Figure 1. Hydrolysis of ATP

The isoprenoid biosynthetic pathway (IBP, Figure 2) contains numerous diphosphates and is responsible for the biosynthesis of cholesterol. Furthermore, through protein prenylation it allows vesicle trafficking and other essential aspects of cellular metabolism. As shown in Figure 2, the IBP has an important branch point where the bacterial pathway intersects the mammalian pathway. The common intermediate is isopentenyl diphosphate (IPP), which contains the smallest isoprene carbon skeleton, consisting of five carbons and a diphosphate head group. IPP is synthesized differently in mammalian and bacterial cells. In mammalian cells, 3-hydroxy-3-methylglutaryl-CoA (HMN-CoA) is converted to mevalonic acid by the enzyme HMG-CoA reductase. HMG-CoA reductase can be inhibited by statins, which are often used clinically to reduce cholesterol production. In the IBP mevalonic acid is converted to IPP through the intermediate mevalonate-5-phosphate. However, the formation of IPP occurs through a different pathway in bacterial cells. Several enzymes catalyze the conversion of pyruvate to the cyclic diphosphate 2-methyl-D-erythritol-2, 4-cyclodiphosphate and then to its final form, *E*-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP). HMBPP is reduced further by HMBPP reductase to form IPP, where bacterial isoprenoid biosynthesis intersects the mammalian pathway.[4, 5]

In the mammalian pathway, farnesyl diphosphate synthase (FDPS) mediates formation of the 15-carbon farnesyl diphosphate (FPP) by catalyzing the condensation of two molecules of IPP and one molecule of DMAPP. FPP represents another important branch point in the mammalian pathway, promoting the formation of cholesterol through squalene synthase and protein prenylation by farnesyl transferase (FTase). This allows post-translational modifications to Ras and RhoB proteins by transferring a 15-carbon isoprene unit to a cysteine residue, promoting signal transduction and vesicle trafficking. Through a reaction mediated by the enzyme squalene synthase, two equivalents of FPP can undergo a head to head condensation and thereby form squalene. Squalene is modified through multiple steps in order to generate cholesterol along with other steroids. FPP can be extended further through addition of another IPP unit to form the 20-carbon unit geranylgeranyl diphosphate (GGPP) through catalysis by geranylgeranyl diphosphate synthase (GDPS). GGPP is utilized as a substrate of geranylgeranyl transferase I and II for post-translational modifications to RhoA, Rap1a, and Rab proteins by transfer of a geranylgeranyl isoprene unit to the appropriate cysteine moiety.[6, 7]

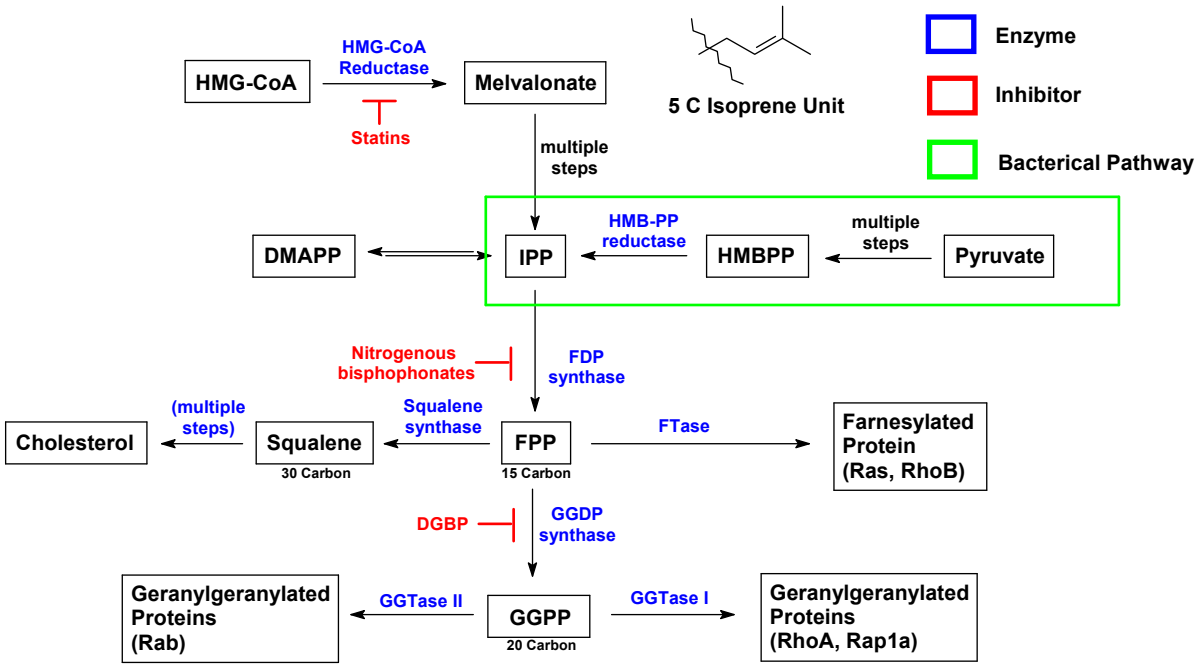


Figure 2. Isoprenoid biosynthetic pathway

The IBP is a target for numerous pharmaceutical agents used to treat of a variety of diseases, due to its roles in metabolism, signal transduction, and vesicle trafficking. One of the most important families of IBP inhibitors is the statins. Statins inhibit HMG CoA reductase in the mevalonate pathway, and are commonly administered because they reduce cholesterol with high efficacy and few side-effects.[8] Clinical trials show that statins decrease the risk of heart disease, cancer, and other ailments.[9] By inhibiting HMG CoA reductase, statins also decrease the concentration of key intermediates in the IBP needed for post-translational modifications of Ras superfamily proteins. In 1960, scientists uncovered mutations in the Ras DNA sequence and correlated these mutations to human cancers. It is hypothesized that 20% of all human cancers are associated with at least one harmful mutation in the Ras family, which makes the IBP pathway a highly desirable target for chemotherapeutics.[7]

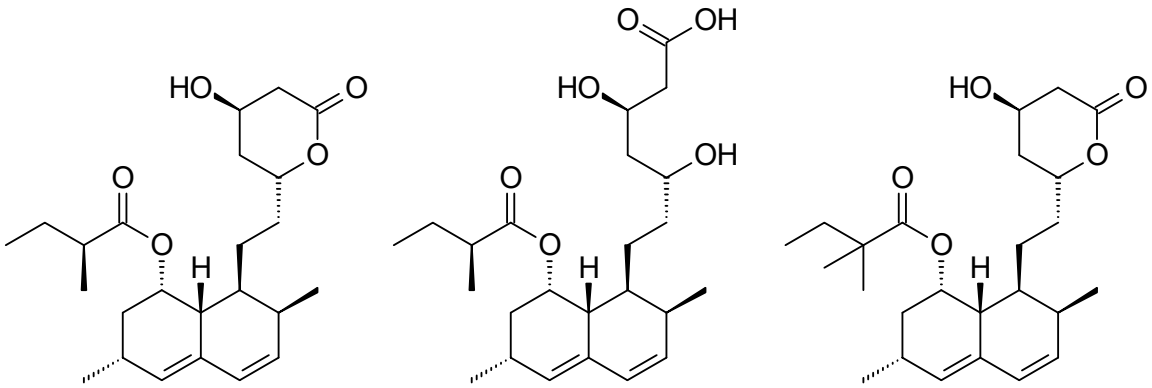
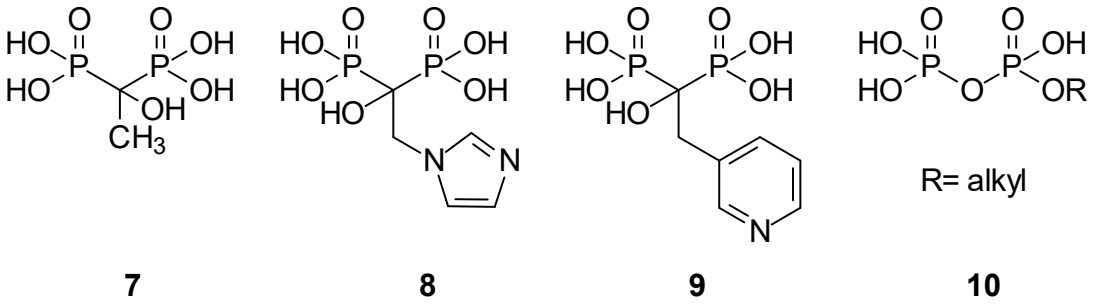


Figure 3. Representative inhibitors of HMG-CoA reductase

Geminal bisphosphonates are often used as candidates to inhibit IBP enzymes for chemotherapeutics, as well as to target other ailments related to this pathway. The parent geminal bisphosphonate contains two phosphonate groups attached to a central carbon and often exists as the phosphonic acid and its salt. They carry three negative charges at

physiological pH.[10] These bisphophonates are often viewed as mimetics of diphosphate substrates, where two phosphorus atoms are linked by an oxygen (Figure 4).

In order to inhibit the desired enzyme, a pharmaceutical must transit cell membrane via passive diffusion. One of the first identified bisphosphonates was etidronate (**7**), which was synthesized over 100 years ago.[11] Nitrogenous bisphosphonates, such as zolendronate (**8**) and risedronate (**9**), are used clinically for the treatment of multiple myeloma and bone diseases by disrupting protein prenylation through the inhibition of FDPS.[5, 6] These compounds contain a geminal diphosphonate, a heteroaromatic ring, and an alpha hydroxyl group. By inhibiting FDPS, these bisphosphonates cause an increase in IPP while decreasing the concentration of FPP and GGPP and ultimately prenylated proteins.



7 **8** **9** **10**

Figure 4. Phosphonate mimics of phosphates

Passive diffusion of a highly ionizable agent does not allow for facile transport across the cell membrane. Bisphosphates have an extremely low bioavailability due to the difficultly that highly charged pharmaceuticals face to cross the cell membrane and move into the cell.[11] To promote diffusion, medicinal chemists have developed moieties to mask functional groups that are highly polar and readily ionized. When these moieties are combined with possible pharmaceuticals they are called prodrugs. These prodrugs often rely upon non-specific esterases or other enzymatic processes to liberate the active pharmacophore intracellularly (Figure 5). Once liberated from the prodrug moiety, the

active pharmaceutical becomes charged and can undergo limited passive diffusion out of the cell. Phosphonate salts often use prodrug moieties originally developed for carboxylic acid-containing pharmaceuticals.[10, 12]

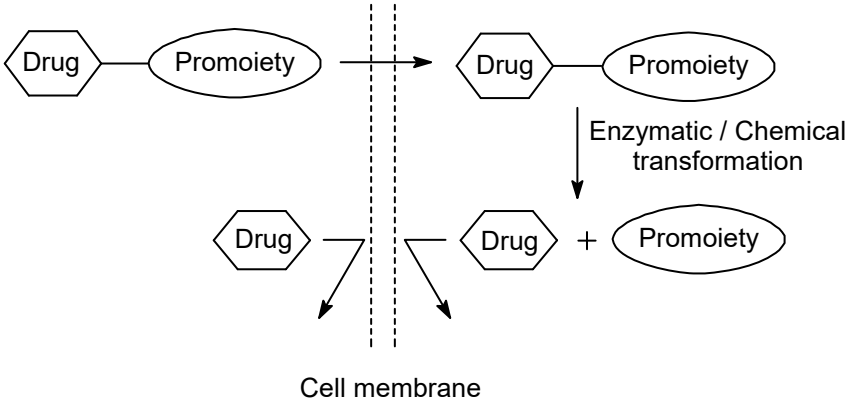
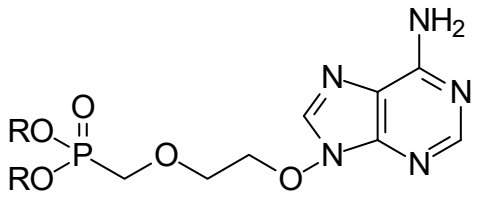


Figure 5. The prodrug concept

In addition to concealing ionizable functional groups, prodrugs can also increase bioavailability. Serafinowska and co-workers examined the ability of various promoieties to increase the bioavailability of a known drug.[13] Using 9-[2-(phosphonomethoxy)ethoxy]adenine, Serafinowska installed various promoieties to the phosphonic acid functionality and monitored the blood concentration of the diester, monoester, and free acids after administration to mice.[13] They found that the diesters were readily absorbed after oral administration and hydrolysis by non-specific esterases, making them readily available when compared to the free acids. [10]



11

R =	Bioavailability of the acid
-H	2 %
$-CH_2CO_2C(CH_3)_3$	30 %
$-CH(CH_3)CO_2C(CH_3)_3$	74 %

Table 1. Bioavailability of various promoieties[13]

It has been known that prodrug moieties increase activity, as compared to the original agent.[10] One example of a pharmaceutical compound that exhibits these traits is adefovir (**15**). Adefovir is a carbocyclic nucleoside phosphonic diacid that serves as an antiviral agent and is used against human immunodeficiency virus (HIV), hepatitis B, herpes simplex, and other viruses. Adefovir inhibits reverse transcriptase and viral replication by inhibiting the first phosphorylation event in DNA replication, thus terminating the growing chain. However, the low bioavailability of the active drug required the synthesis of prodrug analogues. When evaluating the antiviral activity of these modified drugs, the bis(pivaloyloxymethyl) (POM) analogue (Hepsera™, **16**) increased biological response when compared to adefovir. [14] Hepsera™ is rapidly converted to adefovir by a non-specific esterase and subsequent chemical decomposition, which liberates pivalic acid and formaldehyde. Some studies have investigated the ability of bisphosphonate prodrugs to increase both activity and bioavailability. [10, 15]

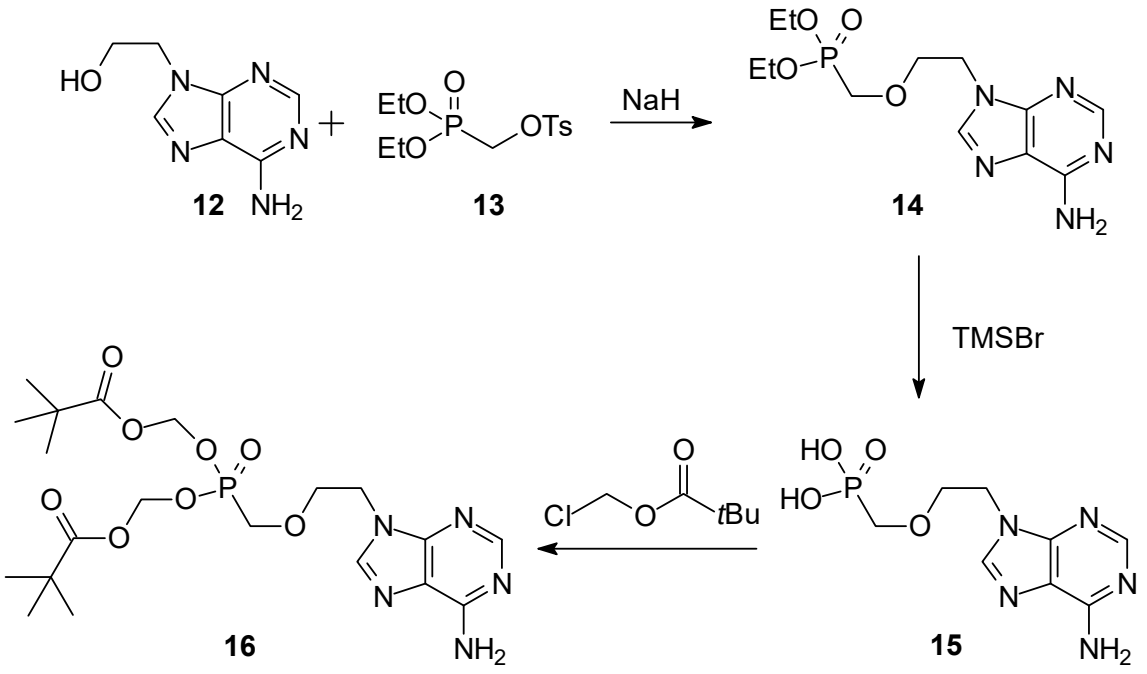


Figure 6. Synthesis of Adefovir and Hepsera™

The Wiemer group has prepared multiple mono- and dialkyl isoprenoid bisphosphonates both as disodium salts and diPOM protected phosphonates. Bisphosphonates **17-21** were synthesized by alkylation of the parent bisphosphonate, tetramethyl methylene bisphosphonate, and found to inhibit enzymes along the IBP. Their studies showed that digeranyl bisphosphonate inhibits GGDPS selectively.[16] While the tetrasodium salt showed activity in enzyme assays, further modifications increased activity in cellular assays. While the sodium salts were able to inhibit GGDPS, difficulties arose because of limited diffusion across the cell membrane. Synthesis of an inhibitor prodrug was achieved by the treatment of tetramethyl methylene bisphosphonate with sodium iodide and chloromethyl pivalate to obtain the tetraPOM mono- and dialkyl bisphosphonates. [17]

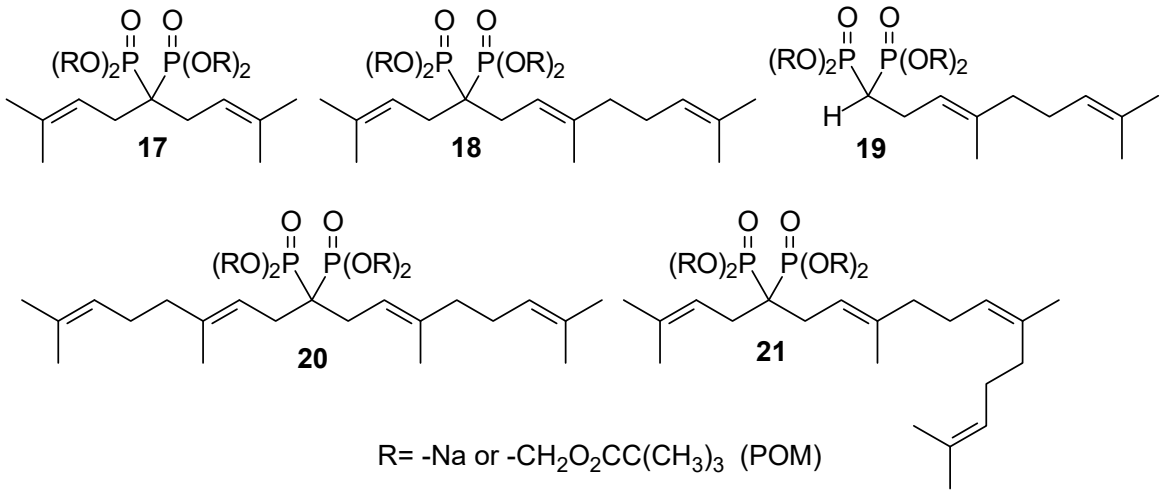


Figure 7. Mono- and dialkyl isoprenoid bisphosphonates

Recently, phosphonates also have been investigated for their activity as phosphoantigens. Phosphoantigens are small molecules containing phosphorus that stimulate Vγ9Vδ2 T-cells. Both Vγ9Vδ2 and αβ T-cells are immune cells that detect and eliminate infections, but each responds to different antigens. Unlike γδ T-cells, the αβ T-cells are stimulated by peptide antigens and account for approximately 90% of blood T-cells. The γδ T-cells account for a small portion of whole blood T-cells. Both natural and non-natural phosphoantigens have been shown to activate γδ T-cells. Natural phosphoantigens originate from the bacterial IBP. The most potent natural antigen known is HMBPP (**22**), the last unique intermediate in bacterial metabolism before it is reduced to IPP (**23**).[18] When HMBPP is introduced to the human blood stream, it undergoes rapid decomposition, making it a poor potential pharmaceutical. At high concentrations, IPP acts as a γδ T-cell agonist by upregulating these cells to respond to infections. The bromohydrin diphosphate of IPP (BrHPP, **24**) once was considered the most promising γδ T-cell simulant. However, it failed to produce the desired response in phase II clinical trials for treatment of leukemia. Decomposition occurred within several minutes and biological response peaked after a week. Several non-natural phosphonates, diphosphates, and diphosphonates have been explored as potential γδ T-cell stimulators. Although the detailed mechanism of γδ T-cell activation has been elusive, these studies suggested that

phosphoantigens bind to the BTN3A1 protein. Recently, the transmembrane protein butyrophilin 3A1 (BTN3A1) was identified and characterized, and its role in γδ T-cells has been investigated. [19]

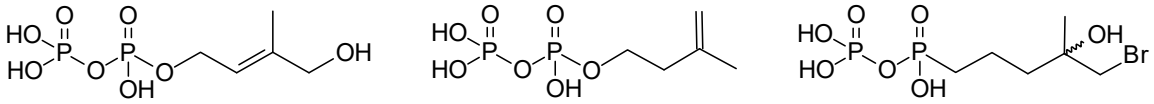
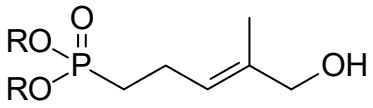


Figure 8. Natural and non-natural phosphoantigens

Recently, POM protected compounds have been used to elucidate some aspects of the mechanism of γδ T-cell stimulation. The Wiemer groups reported the synthesis and biological activity of a POM protected phosphoantigen (**25c**).[19] Both the disodium salt and the diPOM protected phosphonates were synthesized and evaluated for their biological activity. The diPOM phosphonate proved to be the more potent phosphoantigen, with an effective concentration (EC_{50}) of 0.0054 μM, whereas the disodium salt was much less effective (EC_{50} = 4.0 μM, Table 1). This evaluation and subsequent NMR studies lead to the conclusion that these phosphoantigens bind to the intracellular domain of the BTN3A1 protein. This was verified by Hsiao and co-workers through NMR binding studies using the purified intracellular and extracellular domains of BTN3A1, along with the disodium salt (compound **25b**) and HMBPP.[19] These studies showed that the compounds were binding to the intracellular domain of BTN3A1 to induce stimulation.[19]



Compound	R=	EC_{50} (μM)
25a	$-CH_3$	> 10
25b	-Na	4.0
25c	-POM	0.0054

Table 2. Prodrug phosphoantigen activity

Despite these advances in our understanding of phosphoantigens and their prodrugs, further aspects of both phosphoantigen structure and various prodrug forms should be explored. In the following chapters, investigations into the synthesis of mono- and diphosphonate prodrugs and the biological activity of several novel phosphonates, along with a new method for a preparation of chiral prodrug trisphosphonate, will be presented.

CHAPTER 2

SYNTHESIS AND BIOLOGICAL ACTIVITY OF ETHER PHOSPHOANTIGENS

As described in the previous chapter, phosphoantigens are small phosphorous containing compounds that activate γδ T-cells. Along with several other cell types, γδ T-cells contribute to the innate and adaptive immune responses to infectious disease.[20] Innate immunity responds to features present in different pathogens whereas adaptive immunity responds to precise features in a given pathogen. Infections secrete pathogen-associated molecular patterns (PAMPs) allowing them to be recognized by dendritic cells.[21] After interaction with PAMPs, dendritic cells migrate to the lymph nodes were they encounter two species of lymphocytes, B-cells and T-cells.[20] Upon interaction with an activated dendritic cell, B- and T-cells are retained in the lymph node in order to proliferate.[22] These activated B- and T-cells migrate from the lymph nodes to the blood stream. There, B lymphocytes proliferate into antibody-producing cells upon exposure to foreign antigens while T-cells engage in a variety of processes, including activation of macrophages, destruction of infected cells, and bridging a gap between the innate and adaptive immune systems.

Dendritic cells often use two ways to induce stimulation of T-cells and to elicit a T-cell response called co-stimulation.[23] The first effect can occur when dendritic cells, expressing an antigen-containing major histocompatibility complex protein, bind with T-cell receptors. This activation coincides with a second stimulation of proteins on the T-cells by their corresponding proteins on the stimulating cell.[20] When this co-stimulation occurs, it provides protective immunity along with tumor immunity in certain cases. However in the presence of allergens or newly incorporated organs, co-stimulation can lead to an allergic response or rejection of the new transplant.[22] This traditional model for T-cell activation is dependent upon the availability of key structural features on the cell membrane but it is not the only pathway for T-cell stimulation.[5]

After stimulation, T lymphocytes mediate cellular immunity and become activated into multiple different classes. T-cells differentiate into five types: helper, cytotoxic, natural killer, αβ and γδ T-cells.[24, 25] These are characterized based on the T-cell receptors found on the outer membrane. The γδ T-cells are often located in the blood stream, thymus, liver, spleen, and dermis. In order for stimulation to occur, γδ T-cell receptors can bind to antigens which are not bound by the major histocompatibility complex, unlike the traditional model previously presented. Therefore, γδ T-cells carry out their immune response in the presence of the antigen.[21] Because they are not restricted by the requirement for the major histocompatibility complex, they have the ability to recognize a larger subset of possible antigens, including both peptides and non-peptides.[26] This ability allows γδ T-cells to serve as a first line of defense upon interaction with an antigen from a foreign cell, a process known as immune-surveillance.[27] The γδ T-cells have a diversity of different functions, including cell signaling to regulate immune and non-immune cells. They present antigens to activate other T-cells, trigger dendritic cell activation, and help B-cells to produce and regulate key antibodies needed for immunity.[22] Use of γδ T-cells has been investigated for their anti-tumor potential. Due to their role in immune-surveillance, clinical studies have shown the ability of γδ T-cells to infiltrate different tumor cell lines when observed *in vitro*.[21] These studies show no deleterious effects resulting from γδ T-cells acting on normal cells. Furthermore, increased concentrations of γδ T-cells have been observed in survivors of leukemia after bone marrow transplants.[22]

The Vγ9Vδ2 T-cells are a small subset of γδ T-cells found in the peripheral blood, and encompass only about 2-5% of all T-cells.[21, 26] The Vγ9Vδ2 T-cells exist only in humans and other primates.[28] It has been shown that Vγ9Vδ2 T-cells can be activated and have cytotoxic effects on bacteria and parasites, including *M. tuberculosis* and *P. falciparum*.[26] The antigens these T-cells respond to are natural phosphoantigens secreted by bacteria or parasites at pico- to nanomolar concentrations. This allows expansion of T-cells in the blood from 2-5% of total T-cell population up to 90% in certain cases.[26]

Recently, several advancements have been made in determining the mechanism of activation for Vγ9Vδ2 T-cells. It has been found that the protein butyrophilin 3A1 (BTN3A1) plays a pivotal role in T-cell activation. This stimulation occurs through phosphoantigen induced reaction with the intracellular domain of BTN3A1 However HMBPP does not bind to the other butyrophilin proteins found in Vγ9Vδ2 T-cells.[26] Further investigations into the exact binding domain and mechanism of action are still needed.

There are two kinds of phosphoantigens: indirect and direct phosphoantigens. Indirect phosphoantigens cause an increase IPP, a direct activator, by inhibiting enzymes in the IBP. Nitrogenous bisphosphonates have been shown to elevate the cellular concentration of IPP which in turn results in stimulation of the Vγ9Vδ2 T-cells.[5] This type of stimulation has off target effects which can cause other complications. These nitrogenous bisphosphonates inhibit FDPS preventing IPP from being consumed to synthesize FPP (Figure 9). After this increase in IPP, HMG-CoA reductase can slow down its production of mevalonate, eventually leading to a decrease in IPP concentrations. Other compounds could be envisioned as indirect phosphoantigen targeting other enzymes in the IBP, but not all inhibitors of the IBP can function as indirect phosphoantigens.[5] As one example, if GGDPS is inhibited, the increased concentration of FPP downregulates the production of mevalonate.

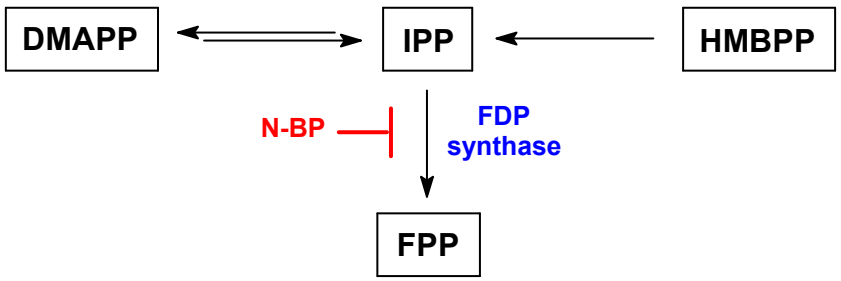


Figure 9. Indirect activators

Direct phosphoantigens stimulate Vγ9Vδ2 T-cells by binding to the BTN3A1 protein. Of the direct phosphoantigens, there are a few naturally occurring compounds derived from intermediates in non-mevalonate IBP, and several have been synthesized including one proceeding to clinical trials. The most potent natural phosphoantigen is HMBPP with an EC_{50} of 0.39 nM. It is an intermediate in the bacterial IBP but, while it is very potent, it lacks the desired pharmacokinetics to be utilized clinically without modifications.[19] At high concentrations, IPP stimulates Vγ9Vδ2 T-cells with an EC_{50} of 81000 nM.[18] In an effort to elucidate more details of the structure activity relationship (SAR), synthetic phosphoantigens have been prepared. The bromohydrin BrHPP (**24**), initially appeared promising. However activity peaks after one week possibly due to cellular exhaustion due to repeated stimulation. Boëdec and coworkers began to investigate the SAR of phosphoantigens by synthesizing an assortment of different phosphonates (**26-31**).[18] When these compounds were evaluated for Vγ9Vδ2 T-cells stimulation, the importance of the olefin stereochemistry was uncovered. The *Z*-olefins were found to stimulate Vγ9Vδ2 T-cells but only at much higher concentration then the *E*-olefins. The *Z*-HMBPP analogue (**27**) was a less effective simulator then the naturally occurring *E*-HMBPP (**22**) with an EC_{50} value of 252 nM. A similar effect was also seen in the diphosphonate derivatives **26** and **28** where there was a 900-fold increase in activity with the *E*-diphosphonate **26** over the *Z*-isomer.[18] The phosphonates displayed the same effect of the olefin stereochemistry as the HMBPP and diphosphonate analogues. The regiochemistry of the olefinic methyl group also had an impact on the ability to stimulate these T-cells. After changing the substitution pattern of the olefin, there was a decrease in activity from an EC_{50} value of 78 μM for compound **31** to greater than 100 μM for compound **29**.

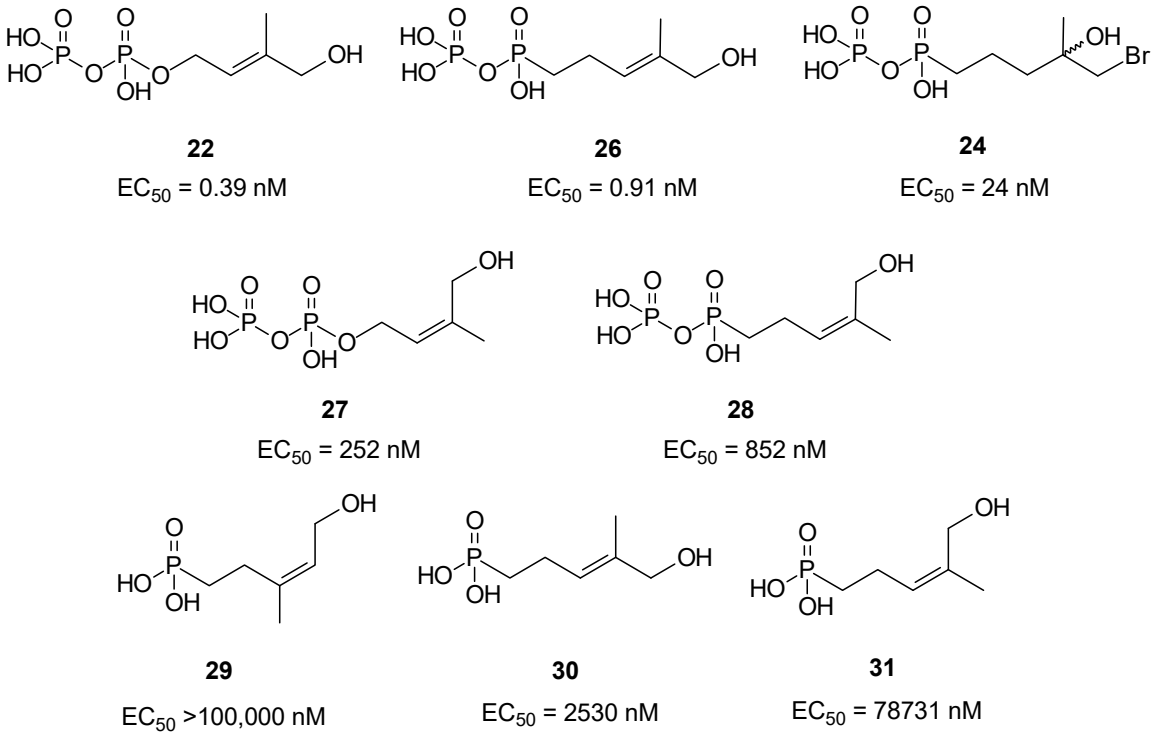


Figure 10. Synthetic phosphoantigens [18]

Synthesis of a monoPOM phosphoantigen

In an effort to develop novel phosphoantigens, Hsiao and co-workers investigated the use of a prodrug phosphoantigen to elucidate the mechanism of action.[19] As stated above, BTN3A1 is stimulated by a phosphoantigen through binding at its intracellular domain. When evaluating the compounds made by Boëdec and co-workers, the phosphonate salts have to cross through the cell membrane in order to bind the intracellular domain and to stimulate T-cells.[18] As discussed in Chapter 1, prodrugs have shown increased activity and bioavailability of phosphonate pharmaceutics by masking the highly charged polar head group. Analysis of the synthetic phosphoantigens prepared to date shows that few modifications have been made to the phosphonic acid portion of these compounds. Masking the polar phosphonate allows facile diffusion of the phosphoantigen prodrug through the cell membrane, which can be followed by cleavage by non-specific

esterases (Figure 11). Through this approach, the desired phosphoantigen would be able to bind to the intracellular domain and would maintain substantial concentration due to limited diffusion out of the cell. With the phosphoantigen **25** as a test case, this salt was protected as the diPOM derivative and this change increased the activity dramatically as shown in Table 2, page 11. The phosphonate **25,** as opposed to HMBPP, has increased stability without a substantial loss in activity. With the increase in activity, further investigation into the SAR was desired in order to determine the optimum characterictics of prodrug phosphoantigens. While prodrugs are common in phosphorous-containing compounds, most of the phosphonate derivatives have both acidic positions protected with labile groups. Phosphonate prodrugs with one labile group and one simple alkyl ester are less common.

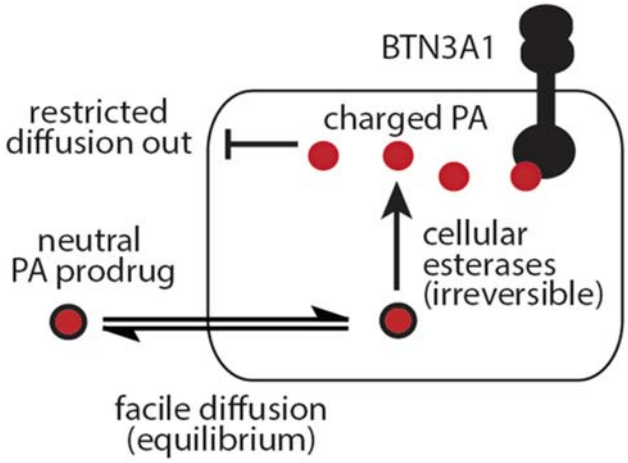


Figure 11. Phosphoantigen model [19]

The initial synthesis of the mono ester phosphonate prodrug **36** began with commercially available dimethyl methylphosphonate. After treatment with LDA, the resulting anion was allowed to react with prenyl bromide to afford the desired dimethyl homoprenylphosphonate **33**. Installation of the prodrug moiety is traditionally achieved through reaction of a methyl ester with NaI and choromethyl pivalate highly concentrated in acetonitrile. When a reaction concentration of 0.1 M was used, the di-POM **34** and the

mono-methyl mono-POM product **35** were easily obtained. The mono-methyl ester **35** was isolated as a racemic mixture. The mono-POM compound **35** was then converted to the desired phosphoantigen through allylic oxidation. Reaction with catalytic selenium dioxide along with stoichiometric *t*-butyl hydroperoxide (*t*BuOOH) was used to install the gamma hydroxyl functionality. The desired product was isolated with the essential *E*-olefin stereochemistry in 43% yield based on recovered starting material (BRSM).

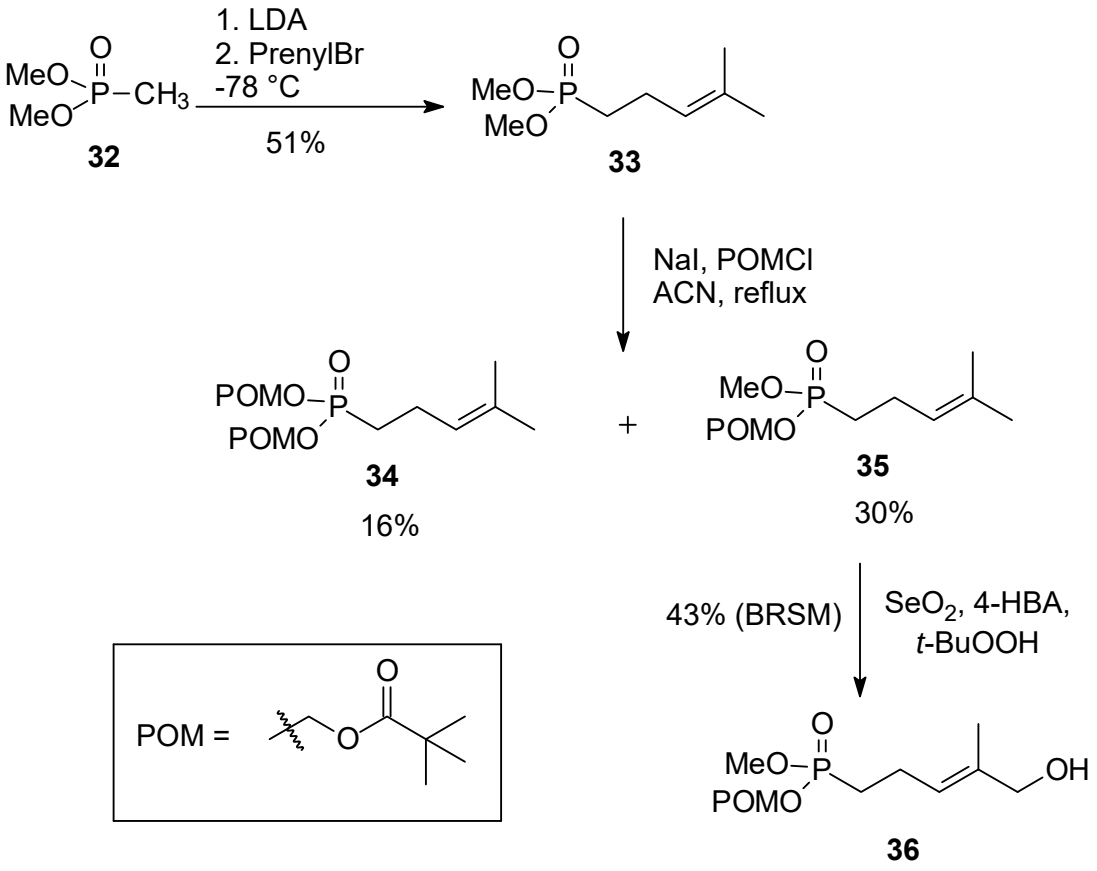
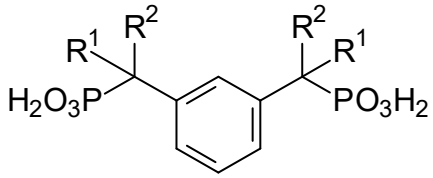


Figure 12. Sythesis of mono prodrug phosphoantigen[19]

Synthesis of ether phosphoantigens

The synthesis of an ether phosphoantigen was desired for further elucidation of the SAR. In naturally occurring phosphoantigens, the diphosphate head group is attached to the isoprenoid through an ester linkage. When this oxygen is formally replaced by carbon, the acidity of the phosphonic acid is diminished.[29] Introduction of an oxygen substituent on that carbon would result in an inductive electron withdrawing effect from the phosphonate head group while maintaining the phosphonate stability. Electron withdrawing groups have been used to decrease the pKa values of the phosphonic acids.[29] This has been shown with a phosphoglycerate kinase inhibitor (**36**) synthesized by Blackburn and co-workers.[29] Not only was there a decrease in the pKa values, but Blackburn also noticed an increase in the activity from an IC_{50} of 200 μM to 0.96 μM after installation of an alpha difluoro group.[29] When a hydroxyl group was added to the alpha position there was an increase in activity and decrease pKa values as well.



36

R^1	R^2	IC_{50} (μM)	pKa_3	pKa_4
H	H	200	7.11	7.71
H	OH	150	6.78	7.23
H	F	1.3	6.01	6.37
F	F	0.96	-	-

Table 3. Phosphoglycerate kinase inhibitors[29]

The synthesis of the ether series of potential phosphoantigens began from dimethyl phosphonate and paraformaldehyde (after thermal cracking). Addition of triethylamine (NEt_3) to the reaction mixture afforded dimethyl hydroxylmethylphosphonate (**38**). The dimethyl ester was necessary to allow eventual installation of the POM functionality. With the dimethyl hydroxylmethylphosphonate in hand, alcohol **38** was allowed to react with sodium hydride followed by the addition of prenyl bromide to give the ether phosphonate **39**. Upon treatment with NaI and choromethyl pivalate, both the di- and mono-POM ethers, compounds **40** and **41**, were obtained and they were easily separated.

The allylic hydroxyl groups were introduced by treatment with catalytic SeO_2. This reaction is believed to preceed first through coordination to the olefin, then a [2,3]-sigmatropic rearrangement of an allylic seleninic acid intermediate, followed by a retro-ene reaction to restore the olefin and give the desired *E*-stereochemistry. Although the yield was lower during the synthesis of both compounds **42** and **43**, this could be due to coordination of SeO_2 to the ether oxygen. In recent literature, installation of the gamma hydroxyl group with SeO_2 was achieved in 38% yield when using a stoichiometric amount.[30] Use of a catalytic amount allows for use of a smaller quantities of SeO_2, simplifies purification, and limits production of elemental selenium. However, coordination with the ether oxygen could compete with the productive forward reaction to obtain the gamma hydroxyl group.

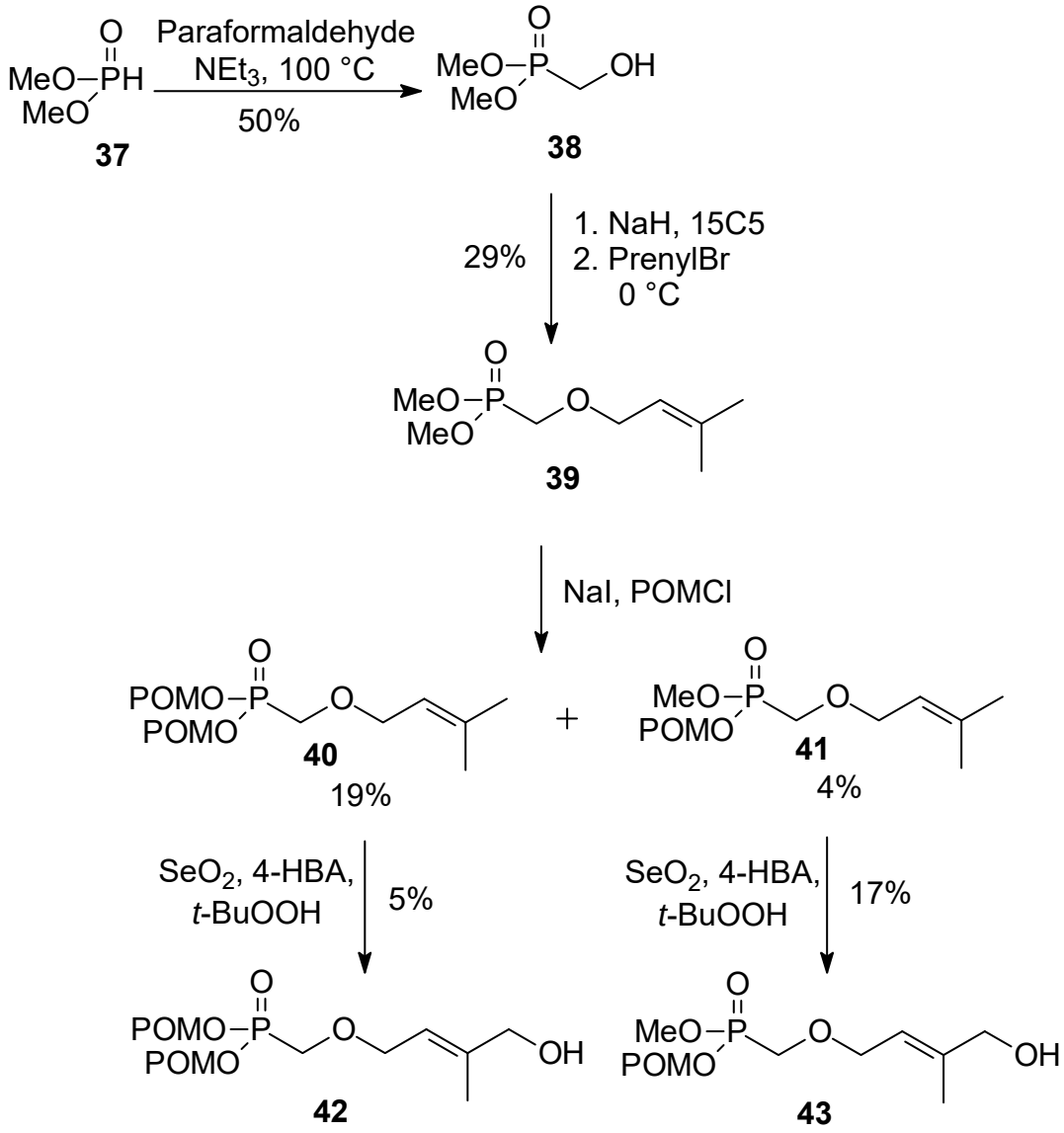


Figure 13. Synthesis of ether phosphoantigens

Biological results

The new potential phosphoantigens presented in this chapter were tested by our collaborators for their ability to stimulate Vγ9Vδ2 T-cells from human blood donors (Figure 14). As discussed earlier, ways to increase the bioavailability of these compounds and increased understanding of the mechanism of action using novel Vγ9Vδ2 T-cells

stimulants were our goals. With the new phosphoantigens in hand, the compounds were allowed to stimulate Vγ9Vδ2 T-cells along with co-stimulation with interleukin 2 (IL-2) to observe a population increase. When comparing the activity to known phosphoantigens, the natural occurring HMBPP (**22**) had the greatest activity with an EC_{50} value of 0.00051 μM. [19]

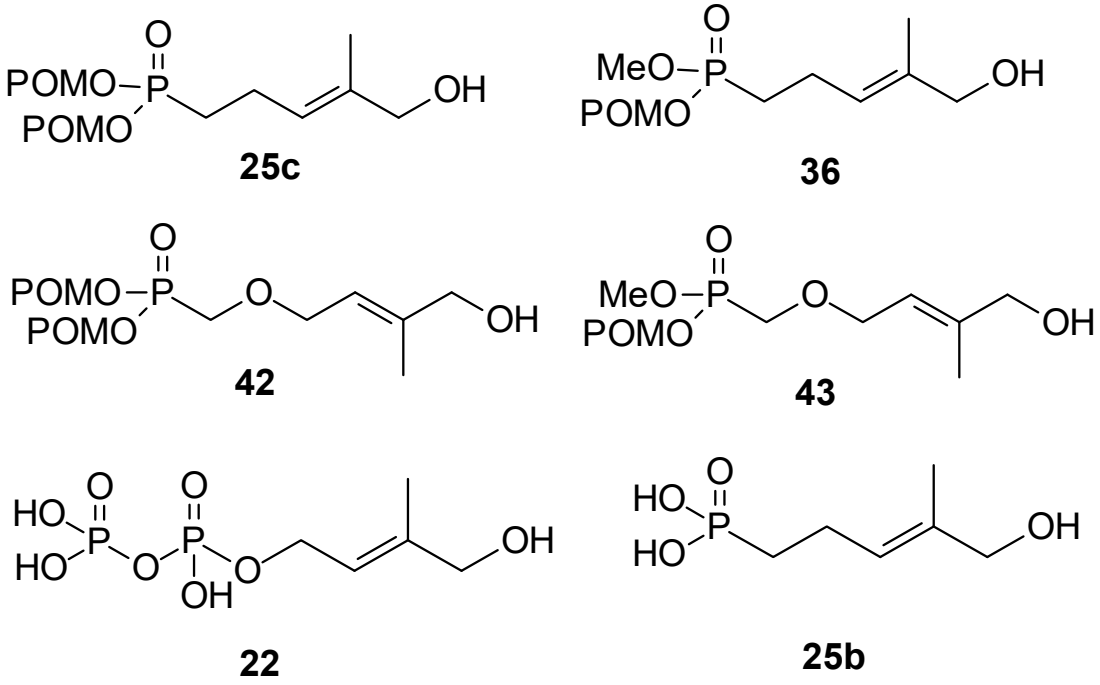


Figure 14. Assayed phosphoantigens

In order to study further the SAR of these analogues, the monomethyl esters (**36**, **43**) and bisPOM (**42**) prodrugs were compared in the ether and alkyl series. When comparing the monoPOM prodrugs to the diPOM prodrugs, the bisPOM compounds expanded the Vγ9Vδ2 T-cell population to a larger extent than did the monomethyl ester compounds for both the ether and alkyl series (Table 4). The monoPOM compound **36** showed weak ability to expand Vγ9Vδ2 T-cell populations when compared to the full prodrug. Monomethylester **43** exhibited a minimal ability to stimulate a Vγ9Vδ2 T-cell population.[31] The mono-prodrug **36** demonstrated weaker activity then its di-prodrug counterpart **25c**. The stimulation data indicated a facile diffusion through the cell

membrane and showed increased activity of prodrugs **25c** and **36** when compared the parent disodium salt, phosphoantigen **25b**. However, the monoPOM phosphoantigens were isolated as a racemic mixture. It has been shown in recent literature that chirality at phosphorus can have an effect on the activity of prodrugs even though that chirality is lost upon hydrolysis of the prodrug.[32] This is most likely due to the chiral nature of the non-specific esterases which are needed to release the active agents. The rate of prodrug cleavage could have an effect on the release rate of the active species, affecting the activity. This might be worth further investigation if the activity were greater.

Compound	EC_{50} (µM)	Magnitude less active than HMBPP (22)
22	0.00051	1.0
25c	0.0054	10
36	0.50	980
42	10	19,600
43	>100	>190,000
25b	4.0	7,800

Table 4. Expansion of Vγ9Vδ2 T-cells [31]

When comparing the alkyl compound versus the ether series, a substantial decrease in activity was observed upon insertion of the ether. Along with addition of the ether, this added atom also increases the length between the phosphorus head group and the isoprene olefin. This increase in length by a formed insertion of the ether oxygen could be the cause of the decreased activity in Vγ9Vδ2 T-cell stimulation. While electron withdrawing effects have been seen in phosphonic acids, the effects of these groups on neutral prodrugs species is not well studied.

In conclusion, a new family of novel phosphoantigens was made readily available using an ester exchange with chloromethyl pivalate and NaI followed by SeO_2 meditated allylic oxidation. These new compounds were tested by our collaborators for their ability to stimulate Vγ9Vδ2 T-cell. The use of mono methyl prodrug **36** reinforces the theory that prodrugs increase the bioavailability and the activity when compared to the disodium salt or the phosphonic acid. However, insertion of an oxygen between the isoprenoid olefin and the phosphonate head group also caused a decrease in stimulation of the Vγ9Vδ2 T-cell population.

CHAPTER 3

SYNTHESIS AND BIOLOGICAL ACTIVITY OF FLUORESCENT PRODRUG PHOSPHOANTIGENS

As discussed in Chapter 2, phosphoantigens bind to an intracellular domain of the BTN3A1 protein. Even though binding studies have shown the ability of these phosphorus containing molecules to stimulate Vγ9Vδ2 T-cells, this still remains a highly contested topic.[25, 33] Over the years, numerous different techniques have been used to elucidate biological mechanisms, including radiolabeling and fluorescence. With the small phosphoantigen pharmacophore, use of either strategy potentially would be viable. However, the ideal molecule to illuminate a more detailed mechanism and determine cellular uptake would not affect the biological activity of the potential probe. With radiolabeled compounds, the essential skeleton could be preserved, but specialized equipment is needed for synthesis whereas fluorescent molecules can be prepared with standard materials.[34] Fluorescent bisphosphonates have been shown to inhibit GGDPS along with revealing the internalization of the compounds. Through use of anthranilic acid labeled isoprenoids, Maalouf and co-workers were able to observe the internalization and localization of digeranyl bisphosphonate analogues by means of fluorescent imaging.[35] This ability allows the visualization of these compounds in live cells without the use of radioactive materials or the need for specialized equipment. Through a parallel avenue, fluorescent phosphoantigens could be envisioned.

Upon addition of a fluorescent group to a phosphoantigen, any significant structural change in the pharmacophore could decrease the activity or change the toxicity of the modified probe. The use of fluorescent probes to determine biological mechanism is well practiced. However these probes are often large organic molecules which could change biological activity when installed on the small phosphoantigen skeleton. Because we have shown that use of a prodrug increased the cell uptake and activity, synthesis of a fluorescent cell cleavable phosphonate prodrug would allow observation of the internalization of the compounds without affecting activity. However, the choice of a fluorescent component to

attach is extensive. Synthesis of a chloromethyl ester of 7-methoxycoumarin-3-carboxylic acid would allow quantification of cellular uptake by observing a decrease in the fluorescence of the media. This might be accompanied by an increase in fluorescence within the cell if fluorescence persists upon esterase mediated hydrolysis, or it might result in disappearance of fluorescence if the coumarin undergoes metabolic breakdown or ionization at physiological pH. Using the known pivalate oxymethyl group as a model, derivatization of the carboxylic acid moiety could be seen through formation of a chloromethyl ester of the coumarin carboxylic acid, similar to that in chloromethyl pivalate. Upon interaction with a non-specific esterase, the fluorescent prodrug might undergo cleavage liberating formaldehyde, the phosphoantigen pharmacophore, and a coumarin carboxylate. With this strategy in mind, development of a novel promoiety would allow the establishment of the first fluorescent prodrug.

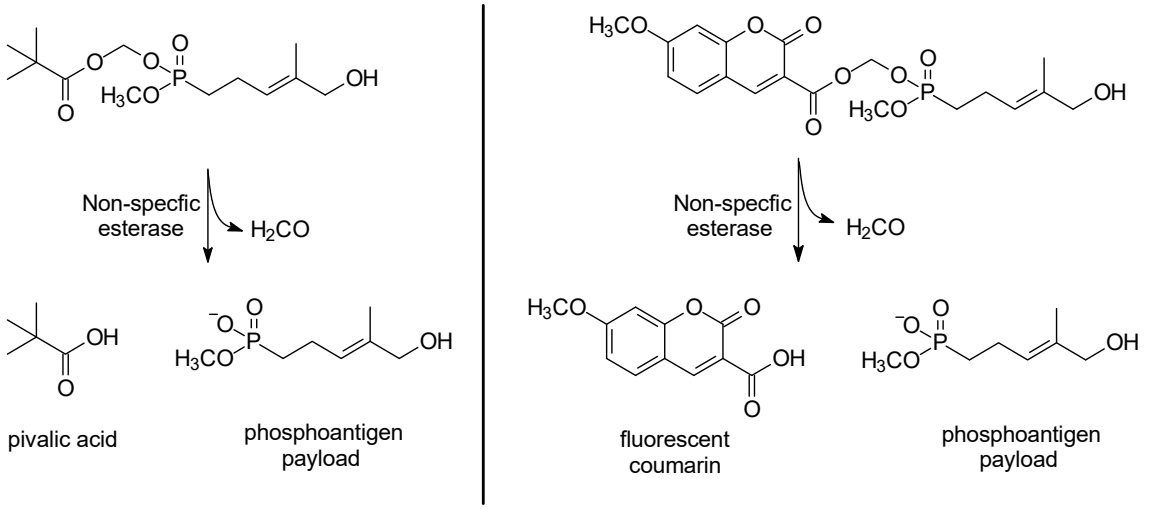


Figure 15. Design of a coumarin prodrug

Synthesis of a fluorescent phosphoantigen prodrug

The incorporation of the coumarin ester could be envisioned through use of a chloromethyl coumarin ester similar to the chloromethyl pivalate. As discussed in Chapter 2, the addition of the prodrug to the phosphonate **33** could be seen in an analogous fashion to introduction of the POM prodrug to the phosphonate. The coumarin-carboxylate

oxymethyl ester (CCOM) groups could be prepared from the commercial Meldrum's acid, benzaldehyde **44** and the known phosphoantigen **25c**. Therefore, the synthesis of a fluorescent analogue could be envisioned through an analogous route to the diPOM phosphonate **25c**.

The synthesis began with the Knoevenagel condensation of Meldrum's acid and 2-hydroxy-4-methyoxybenzaldehyde (**44**), followed by cyclization to form the coumarin **46**.[36] With the carboxylic acid in hand, formation of the chloromethyl ester was next step. The original formation of the chloromethyl pivalate was achieved through use of pivaloyl chloride in a reaction with formaldehyde and catalytic zinc chloride at 100 °C.[37] However in recent literature, generation of chloromethyl esters has been achieved in high yields from the carboxylic acid by use of chloromethyl chlorosulfate at ambient temperatures in a biphasic system.[38] Through reaction with chloromethyl chlorosulfate, the reaction could proceed from the carboxylic acid without the formation of the highly reactive acid chloride. Therefore the carboxylic acid was treated with KOH and chloromethyl chlorosulfate to generate the chloromethyl ester **47** in an exothermic reaction. The reaction was allowed to stir for 5 days, but the desired product was isolated in only 52% yield from the biphasic system. When coumarin **47** was prepared using 3 equivalents of chloromethyl chlorosulfate and the reaction was allowed to stir for 2 days, the desired chloromethyl coumarin was isolated in a more respectable 80% yield after purification via column chromatography.

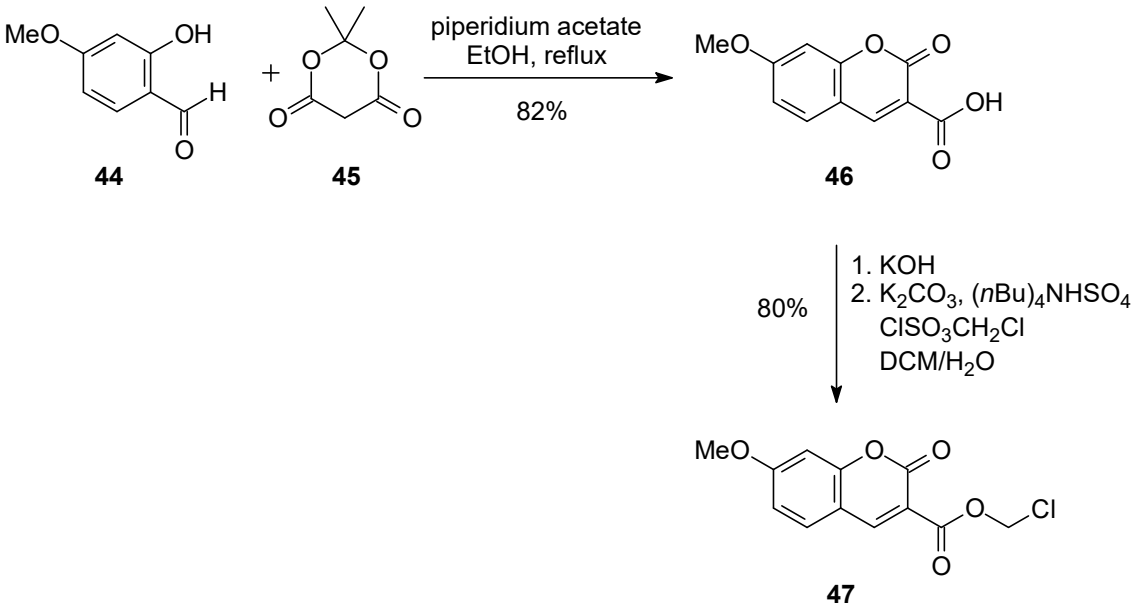


Figure 16. Preparation of coumarin chloromethyl ester **47**

With the coumarin ester **47** in hand, treatment of dimethyl homoprenylphosphonate **33** with NaI and ester **47** was attempted to obtain the diCCOM protected ester, through conditions similar to those used for the POM installation. However, only starting material was recovered. When this strategy proved problematic, introduction of a single fluorescent group to this phosphoantigen was attempted, a change in strategy which would also simplify future fluorescent data. To obtain the desired fluorescent phosphoantigen through an ammonium phosphonate salt, dimethyl homoprenylphosphonate was allowed to react with 1, 4-diazabicyclo[2.2.2]octane (DABCO). The mono ammonium salt **48** was produced after reaction in refluxing acetonitrile overnight, in a manner similar to the debenzylation of phosphonates shown by Rejman *et al*.[39] The phosphonate salt **48** was treated with NaI and compound **47** to form the mono methyl mono CCOM phosphonate **49**. The next step was to introduce the allylic alcohol through SeO_2 oxidation. When compound **49** was allowed to react with SeO_2, *t*-BuOOH, and catalytic *para*-hydroxybenzoic acid (4-HBA), the reaction provided the over oxidized α, β unsaturated aldehyde. Attempts to reduce the aldehyde **50** by treatment with diisobutylaluminium hydride (DIBAL) failed to give the desired allylic alcohol.

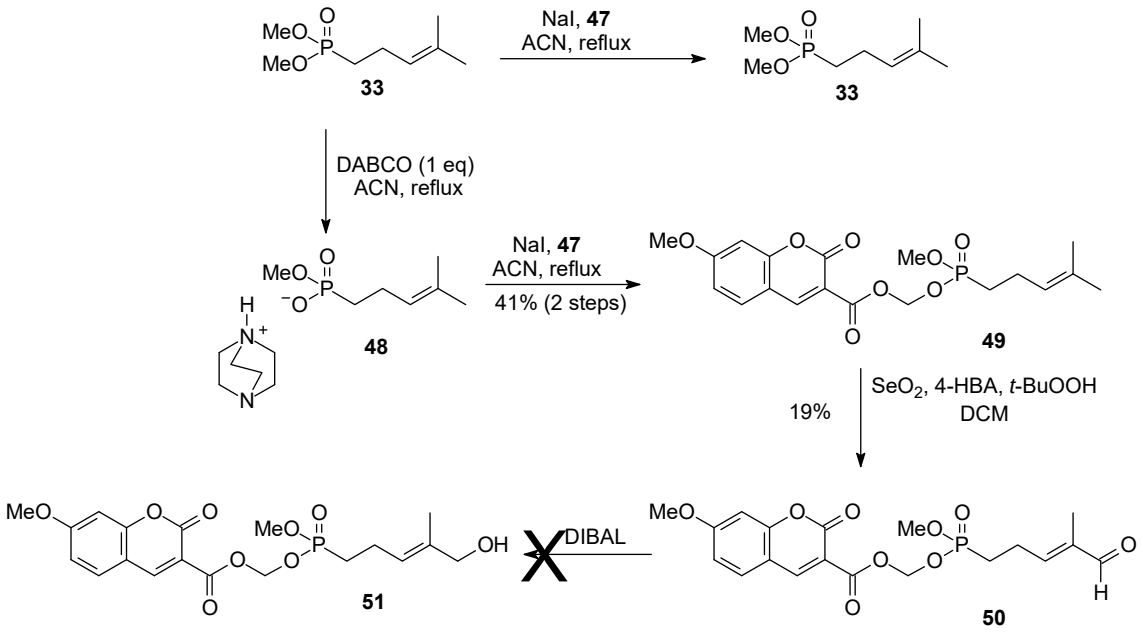


Figure 17. Formation of α, β unsaturated aldehyde

Because the activity of the unsaturated aldehyde was unknown, a new route was needed in order to incorporate the desired gamma hydroxyl functionality. By rearranging the order of reactions in the sequence, the desired mono CCOM prodrug could be envisioned through allylic oxidation followed by addition of the coumarin ester through the ammonium salt. Using literature procedures, the dimethyl homoprenylphosphonate **33** was allowed to react with SeO_2, 4-HBA, and *t*-BuOOH, followed by reduction of any potential aldehyde by treatment with DIBAL to give the desired *E* alcohol **52**.[19] The dimethyl phosphonate **52** then was allowed to react with DABCO in refluxing acetonitrile to form the mono ammonium salt **53**. Formation of the salt was monitored by ^{31}P NMR spectroscopy in order to follow the reaction progress, which was complete after 3 days. Upon formation of the salt **53**, the chloromethyl coumarin **47** was allowed to react with NaI and the ammonium salt **52** in order to form the *in stiu* Finkelstein product, followed by conversion to the phosphonic ester **54** in modest yield.

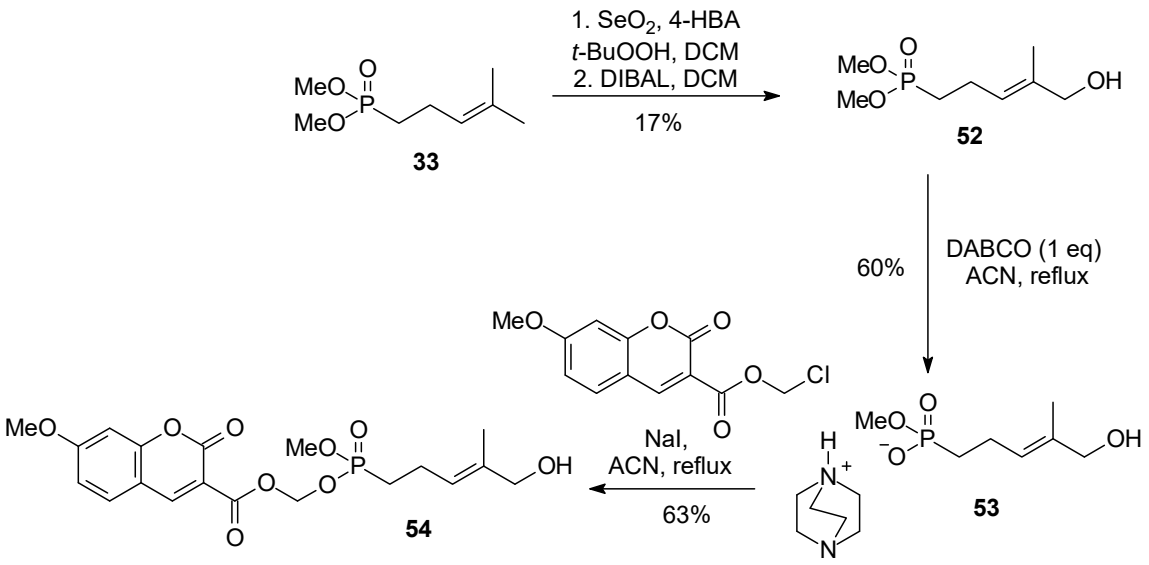


Figure 18. Formation the mono CCOM ester **54** [40]

With the desired mono CCOM ester **54** in hand, the fluorescence spectra of both the ester **54** and the carboxylic acid **46** were obtained at a concentration of 1.0 mM. Both compounds were dissolved in dichloromethane to the desired concentration. The excitation spectra of both compounds were obtained and both compounds had an excitation maximum at 397 nm. Using an excitation of 397 nm, the emission spectra were taken for both compounds, and both show a Rayleigh scattering from the excitation. When comparing the prodrug to the possible metabolic product as shown in Figure 19, the carboxylic acid displays a red shift of 13 nm from 487 nm to 500 nm with approximately the same intensities when observed in this organic solvent.

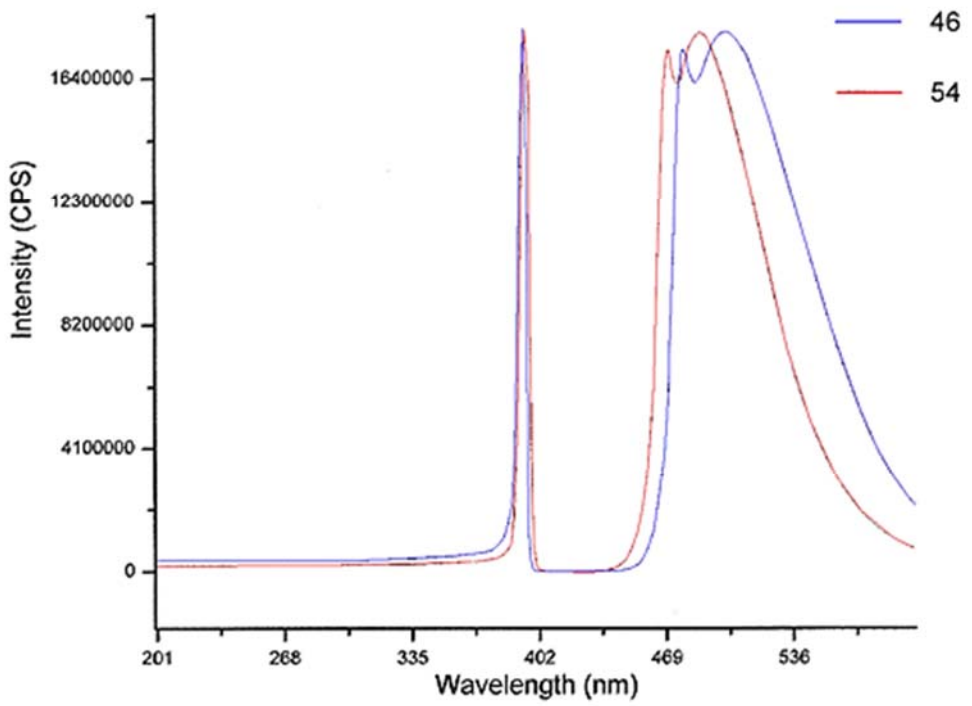


Figure 19. Emission spectra of compounds **46** and **54** in DCM

Synthesis of the potentially more potent bisprodrug also was investigated. As discussed in Chapter 2, the diPOM prodrugs were shown to be more effective at stimulating Vγ9Vδ2 T-cells then the mono prodrug counterparts. While synthesis of a bisCCOM phosphoantigen could be imagined, interpretation of the fluorescent data might be more complex than the mono CCOM counterpart. A bis CCOM prodrug may be twice as intense but it might be more difficult to track then a single CCOM ester. Therefore, synthesis of a mono POM mono CCOM compound was investigated. The triPOM mono ammonium salt of a bisphosphonate has been reported by Troutmen to occur upon treatment of a tetraPOM bisphosphonate with DABCO.[41] Deprotection of only one POM group was observed when the tetraPOM compound was allowed to react in acetonitrile at reflux. Using a parallel idea, DABCO might also be used with a diPOM monophosphonate to obtain the mono ammonium salt. With this in mind, dimethyl homoprenylphosphonate was allowed to react with chloromethyl pivalate in the presence of NaI, affording the desired bisPOM homoprenyl phosphonate **34** in moderate yield. Using a parallel approach to the preparation

of the mono CCOM phosphoantigen, oxidation of compound **34** was achieved through use of SeO_2, 4-HBA, and *t*BuOOH to afford the gamma hydroxy phosphonate **25c**. Phosphonate **25c** was treated with DABCO to provide the ammonium salt **55** in modest yield.

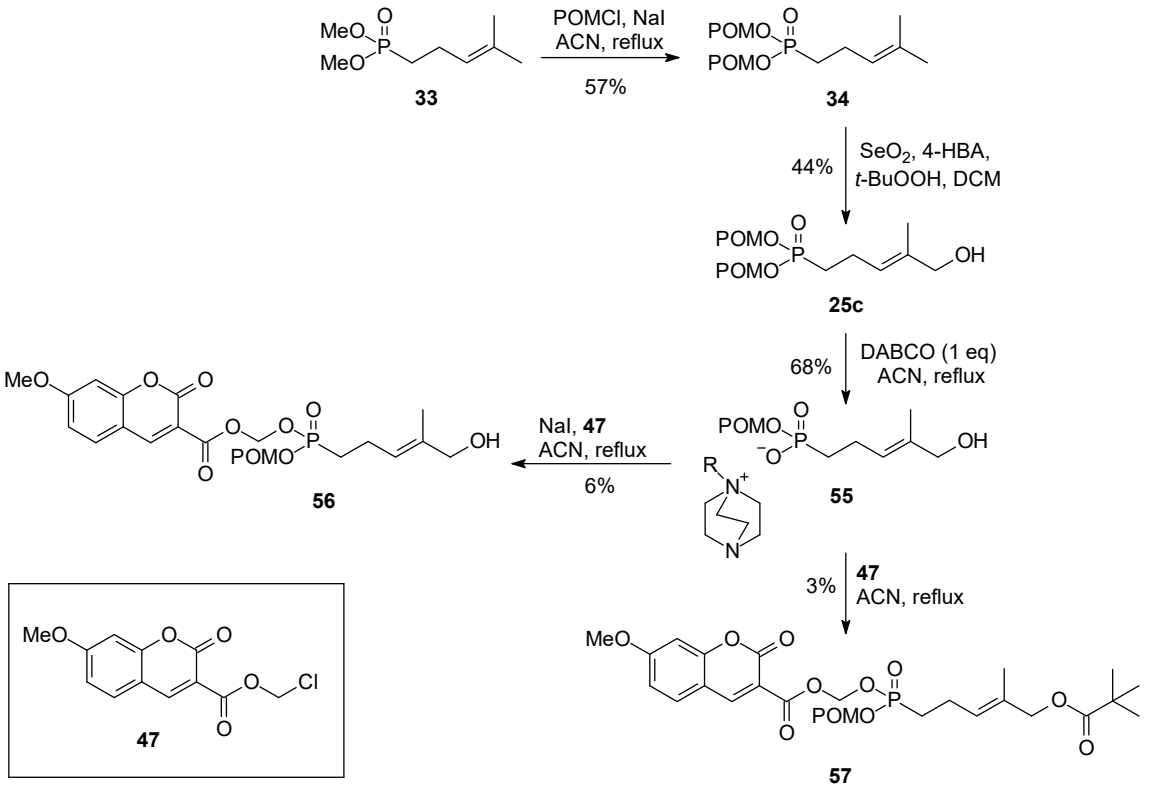


Figure 20. Attempts to for the asymmetric prodrug **56**

Once the ammonium salt was prepared, incorporation of the CCOM ester could easily be anticipated using a reaction sequence parallel to that described in Figure 18. However, when the salt **55** was treated with NaI and the chloromethyl ester **47**, the mono CCOM mono POM product **56** was obtain only in low yield. Upon thin layer chromatography (TLC) analysis, multiple compounds could be detected making isolation difficult. The phosphonate **56** was isolated as a mixture with unknown impurities. These impurities could have been due to the presence of NaI in the reaction. Phosphonic ester cleavage has been observed through use of NaI, and this cleavage could result in hydrolysis

of either the POM or CCOM ester allowing for formation of a complex reaction mixture.[42] With the CCOM and NaI reaction providing a complex mixture, the reaction was attempted without the addition of NaI. Sodium iodide is used in order to obtain the iodomethyl pivalate through the *in stiu* Finkelstein reaction. POM ester have been prepared from the chloromethyl pivalate through the phosphonic acid.[43] Due to the presence of the more nucleophilic ammonium salt, the formation of the iodide might not be necessary so attempts to form the CCOM ester without addition of NaI were undertaken. However upon TLC analysis, numerous by-products were observed and the pivalate ester **57** was obtained only in low yield, suggesting that the possible counterion of the phosphonic salt was complicating the reaction. While the use of the chloromethyl coumarin supplied the CCOM ester, the chloride gave a lower yield than that obtained through the corresponding iodide. After these complications were recognized, a new approach was investigated.

Because NaI and the ammonium counter ion were causing a complex mixture of products, the Finkelstein reaction was performed separately to alleviate the formation of side products. The chloromethyl coumarin **47** was allowed to react with NaI in anhydrous acetone to form the iodomethyl coumarin **58** in reasonable yield. The bisPOM homoprenyl phosphonate **25c** was treated with DABCO until full conversion to the mono salt was observed by ^{31}P NMR spectroscopy. After one week, full conversion was found and the product was treated with Dowex resin (Na^+ form) to obtain the mono sodium mono POM phosphonate. The phosphonate was isolated as the gamma pivalate ester, as indicated by two separate *t*-butyl esters resonances in both the ^{1}H and ^{13}C NMR spectra. While the free gamma hydroxyl group originally was desired, pivalate esters have been used to mask alcohol functionality in pharmaceutics to allow facile diffusion across the cell membrane. The mono CCOM mono POM ester **57** then became available by addition of the iodomethyl coumarin **58** in acetonitrile at reflux.

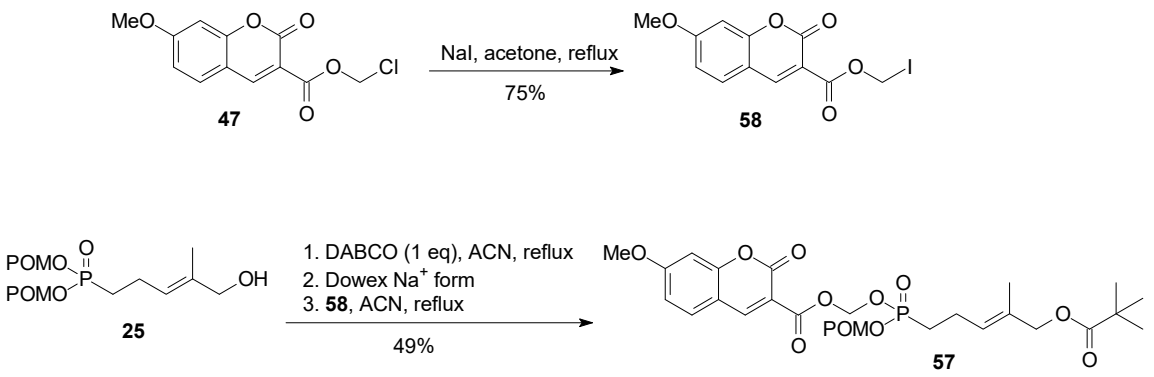


Figure 21. Formation of phosphonate **57**

As discussed in Chapter 2, the stereochemistry of phosphonate prodrugs can have an effect on the activity of the pharmacophore, primarily due to differential rates of ester cleavage of one enantiomer over another. Unlike the POM prodrugs, both the mono CCOM phosphonate **54** and the mono CCOM mono POM phosphonates **56** and **57** were synthesized without control over the stereocenter at phosphorus. This could affect the rates of ester cleavage and in turn impact the activity. However, even though there may be concern about these racemic mixtures, these new compounds were tested for their potential biological activity.

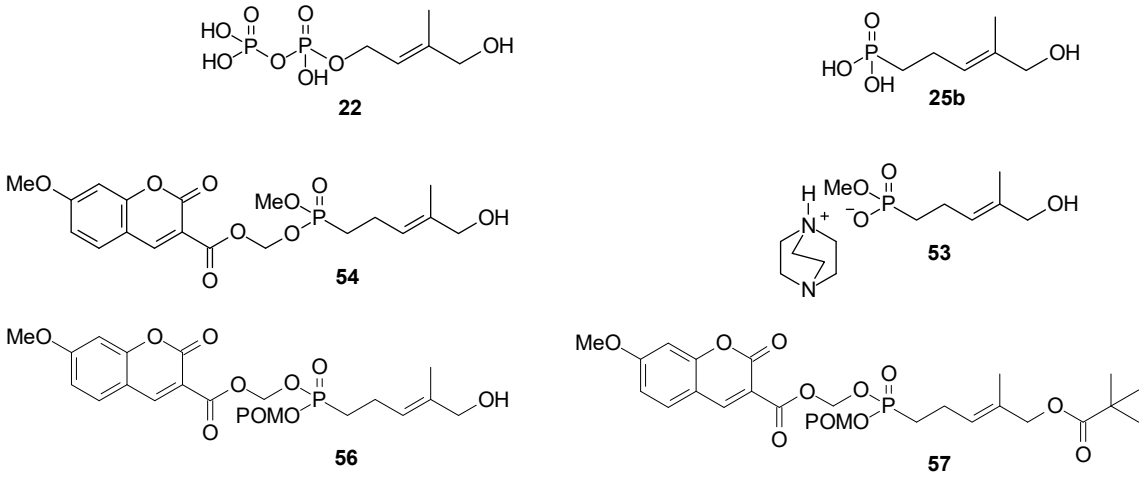


Figure 22. Assayed fluorescent analogues **54**, **56**, and **57**

Biological results

With the coumarin phosphonic esters **54**, **56**, and **57** described above in hand, our collaborators subjected these possible prodrugs to a variety of bioassays. As discussed earlier, our interest was in ways to monitor the uptake and cleavage of these possible phosphoantigens through fluorescence detection. With the novel nature of the CCOM ester, the first assessment that needed to be evaluated was the capacity for non-specific esterases to cleave the CCOM ester and liberate the carboxylic acid **46** and formaldehyde. Treatment of compounds **46**, **54**, and **56** with phosphate buffer solution (PBS) showed these compounds were stable for at least 60 minutes. However, when these compounds were treated with plasma containing non-specific esterases, significant degradation of the phosphonate esters occurred within 5 minutes, with corresponding half-lives of 6 minutes and 125 minutes for compounds **54** and **56** respectively. While this study showed that the coumarin phosphonic ester is functioning as a prodrug, the very rapid rate of cleavage for phosphonate **54** could possibly decrease the ability of this mono CCOM ester to survive long enough to diffuse across the cell membrane.

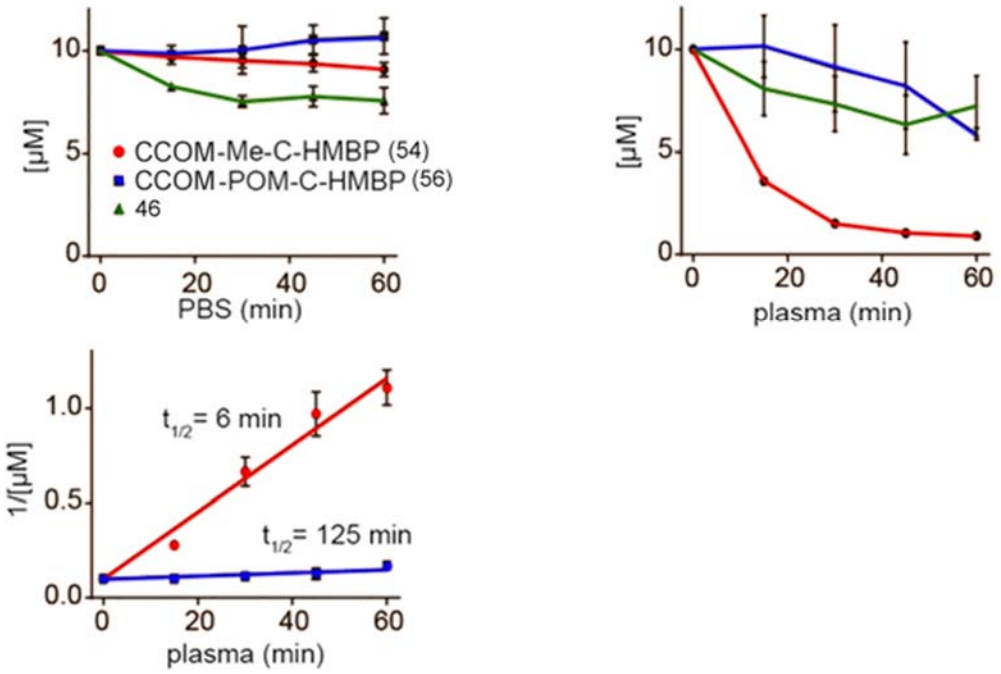


Figure 23. Half-life of CCOM ester cleavage [40, 44]

Once CCOM phosphonate esters were shown to be cleavable by non-specific esterases, these new compounds also were examined for their ability to stimulate Vγ9Vδ2 T-cells. The most active phosphoantigen still proved to be the natural HMBPP. Upon examination of the di-prodrugs, the mono CCOM mono POM phosphonates **56** and **57** showed similar activity to that of the known prodrug phosphoantigen **25c**, with EC_{50} values of 3.9 nM and 4.7 nM, respectively. When evaluated against multiple cell lines for potential cytotoxic effects, these compounds showed acute toxicity beginning at 33 μM. This offers a large therapeutic window for dosing in animal or even human studies. Upon evaluation of the mono CCOM phosphonate **54**, it was shown to stimulate Vγ9Vδ2 T-cells with an EC_{50} of 18 nM. When the mono ammonium salt was tested for its ability to stimulate proliferation, salt **53** exhibited a similar phenomenon to what was observed in Chapter 2. While stimulation was observed, this compound was far less potent then its prodrug analogues, probably due to difficulty reaching the intracellular binding site of the BTN3A1 protein. The mono CCOM phosphoantigen **53** displayed a more effective activity then its POM phosphoantigen counterpart. This switch of prodrug functionality might support the

hypothesis that POM prodrugs are undergoing cleavage before entering the cell. Exploration of alternative prodrugs could give more insight into this possibility.

Compound	EC_{50}
22	0.51 nM
25c	5.4 nM
56	3.9 nM
57	4.7 nM
54	18 nM
53	23000 nM

Table 5. Stimulation Vγ9Vδ2 T-cells of novel phosphonates [40, 44]

With the ability of these phosphonates to stimulate Vγ9Vδ2 T-cells established, the fluorescence was investigated in a cellular system by our collaborators. When the CCOM phosphonate esters **56** and **54** or carboxylic acid **46** were dissolved in PBS buffer, the mono CCOM phosphonate **54** exhibited moderate fluorescence emission which decreased substantially when observing the coumarin **46**. In order to obtain a fluorescent coumarin compound, there needs to be both an electron donation and an electron withdrawing effect across the aromatic system. When coumarin **46** is added to PBS, the carboxylic acid undergoes deprotonation to the carboxylate which has limited fluorescence, and further reduction of the fluorescence intensity was observed when added to plasma. Even though fluorescence intensity was decreased, the phosphonate ester could be observed to undergo cleavage to the phosphonic acid and carboxylic acid **46** by fluorescence spectroscopy.

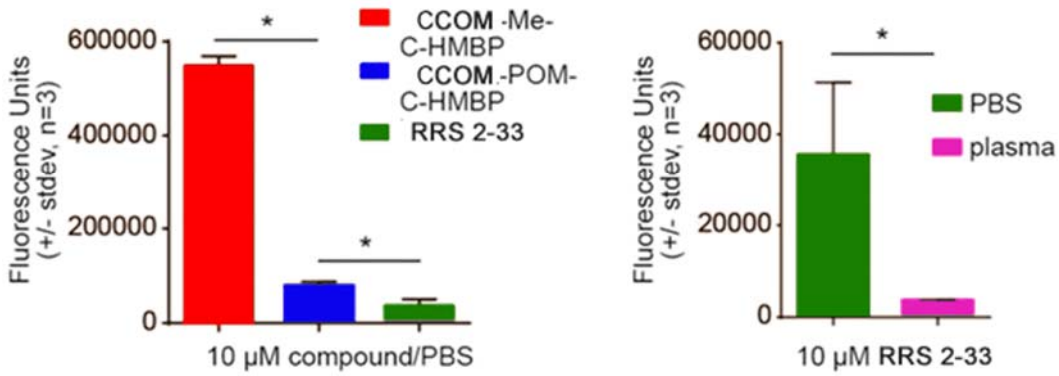


Figure 24. Fluorescence decrease upon addition to PBS and plasma [40, 44]

With the loss of fluorescence observed upon deprotonation of the carboxylic acid, full spectra of coumarin **47** at varied pH would offer a possible alternative wavelength to observe the fluorescence emission. Using a similar methodology as presented before, the fluorescence emission spectrum was obtained of coumarin **46** with a concentration of 0.2 µM in PBS buffer at pH values of 2 and 7. When analysis of the spectrum at pH of 7 was obtained, there was a decrease in fluorescence intensity at a wave length of 400 nm. The pH was then adjusted to 2 and the emission spectrum increased an intensity with a slight red shift of 13 nm. When our collaborators decreased the pH of a similar solution with the concentration of 0.1 mM in citrate buffer, they observed a parallel effect using a different excitation wavelength. In both cases, fluorescence was regained after acidification of the solution, demonstrating that after formation of the carboxylate at physiological pH of approximately 7, the fluorescence signal decreases substantially. While this decrease in fluorescence is observed using a different buffer, the prodrug forms maintain their fluorescence signal allowing for a pH-sensitive fluorescence prodrug. The rate of ester cleavage can be monitored through observation of the decrease in fluorescent signal.

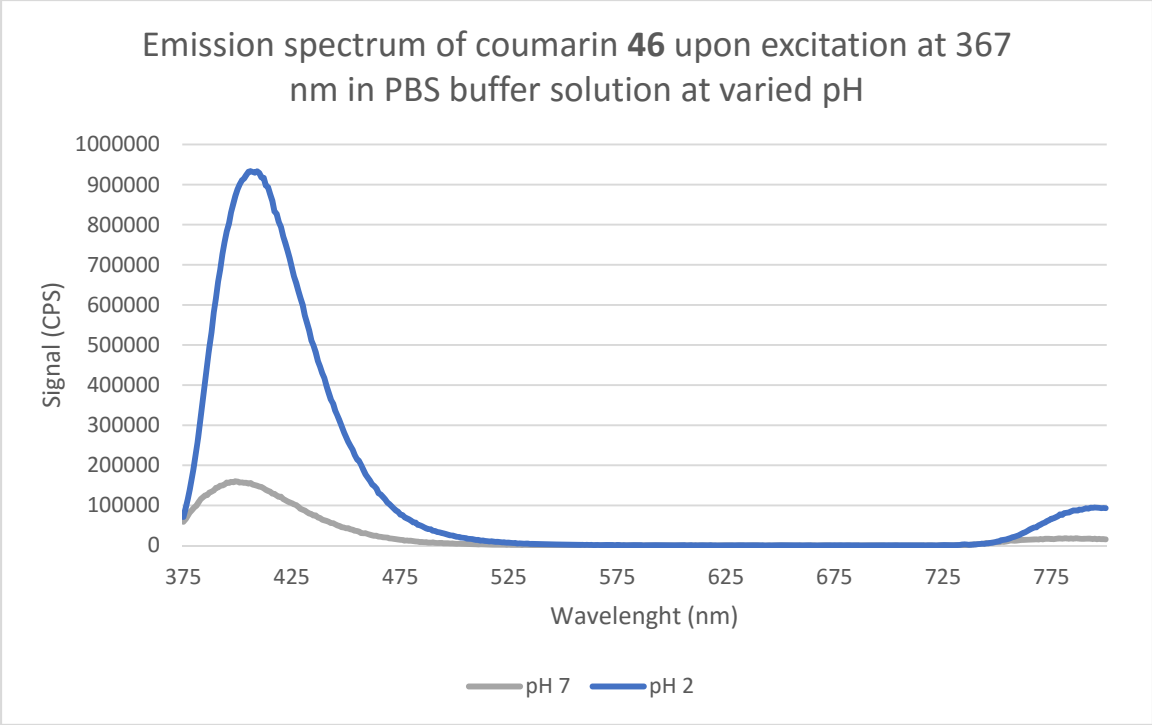


Figure 25. Fluorescence spectra at varied pH values

Synthesis of a gamma protected phosphoantigen

While compound **57** still functioned as a potent phosphoantigen functionality, other modifications of this alcohol have been minimal. The activity of phosphonate **57** was equivalent to that of the earlier phosphoantigen **25c**, even with the pivalate-protected allylic alcohol. Previous derivatives involved the formal removal of the allylic alcohol, and resulted in considerable reduction in Vγ9Vδ2 T-cells stimulation. Pivalate esters have been used in pharmaceutical drugs to mask hydroxyl groups.[45] With the pivalate-protected hydroxyl compound (**57**) showing γδ T-cell stimulation, synthesis of a phosphonic acid derivative with a protected hydroxyl group would allow for a second point of comparison. Starting with dimethyl homoprenyl phosphonate **33**, allylic oxidation provided the gamma hydroxy phosphonate **52**. Standard protection was accomplished using pivaloyl chloride and pyridine to incorporate the pivalate ester. Hydrolysis of the methyl phosphonic ester was possible under McKenna conditions. Upon treatment with bromotrimethylsilane followed by treatment with NaOH, cleavage occurred to generate the sodium salt.

Unfortunately upon further treatment with NaOH, the pivalate ester also underwent cleavage to provide the free gamma hydroxyl group of compound **60**.

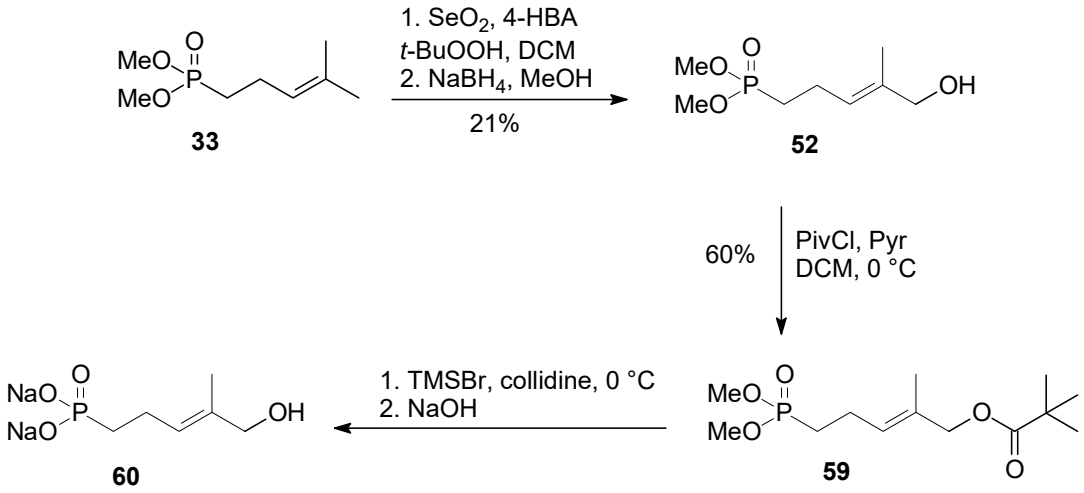


Figure 26. Phosphonic salt of gamma protected hydroxyl

To conclude, a new fluorescent chloromethyl ester has been prepared and shown to allow preparation of fluorescent prodrugs. These CCOM prodrugs have a pH-dependent fluorescence. Using the substructure of the known oxymethyl pivalate, a coumarin carboxylate oxymethyl phosphonate ester was prepared from the ammonium salts. These derivatives were tested by our collaborators to determine their ability to stimulate proliferation of Vγ9Vδ2 T-cells. Non-specific esterases have shown the capacity to cleave the bulky coumarin esters, and these prodrugs maintain activity with limited toxic effects. While fluorescence decreases upon cellular ester hydrolysis, this allows the rate of cleavage to be measured as well as determination of the concentration of the prodrug phosphoantigen still remaining versus actual pharmacophore. Therefore, synthesis of other fluorescent coumarins could be investigated to optimize the fluorescent properties based on the substructure of the new CCOM ester phosphoantigens.

CHAPTER 4

SYNTHESIS AND BIOLOGICAL ACTIVITY OF PHOSPHINOPHOSPHONATE PHOSPHOANTIGENS

As discussed in the previous chapters, development of novel phosphoantigens is desirable for potential pharmaceutics as well as for investigations into the mechanism of action. To this point, the synthesized phosphoantigens from our program have been phosphonates containing only one phosphorus. However, all of the synthetic phosphonates are less potent then the natural HMBPP. Boëdec and co-workers investigated the value of a second phosphorus by developing a diphosphonic acid.[18] When the diphosphonic acid **26** was allowed to stimulate Vγ9Vδ2 T-cells, an increase in potency was observed when compared to the phosphonate phosphoantigen **25b**, with EC_{50} = 0.91 nM versus 2530 nM.[5] The activity of this diphosphonic acid suggests that the use of a second phosphorus group increases the activity when compared to other phosphonates. Similar activity was observed with diphosphonate analogues of DMAPP and IPP. Where HMBPP was more potent than its diphosphonate mimic, the DMAPP and IPP derivatives showed greater efficacy then the naturally occurring diphosphates.[46] While less potent then the naturally occurring HMBPP, these compounds also present an increase in stability while maintaining similar activity profiles.

When Hsiao and co-workers studied phosphoantigen prodrugs, an increase in the activity was observed. This strategy, masking the charged phosphonate, allowed facile diffusion across the cell membrane and binding to the intracellular domain of the BTN3A1 protein. When compared to the corresponding phosphonic acid, the activity increased 500-fold when a prodrug was used. Upon consideration of diphosphorus compounds, analogues of HMBPP and compound **26** could be envisioned as prodrugs to increase the activity and bioavailability. While protecting HMBPP as the oxymethyl pivalate ester might appear to be ideal, this approach would lead to a highly unstable anhydride. This potential analogue of HMBPP would be highly susceptible to nucleophilic attack by water and thus not an

attractive prodrug. The same complication could be envisioned for a protected diphosphonate analogue. Where a protected methylene bisphosphonate analogue of compound **60** would be easily accessible, it has been observed that similar prodrug phosphonates are water sensitive and undergo decomposition after a few days even when stored at low temperatures.[41] This decomposition could be seen from a rearrangement using the lone pair electrons located on the phosphoryl oxygen in a S_N2' like attack at the isoprene olefin. Another possible mechanism for decomposition could be seen in a similar fashion after hydrolysis of the POM ester. To circumvent the potential stability issues, the phosphinophosphonate substructure was viewed as a superior approach for protected analogues. Because several intermediates of the IBP act as phosphoantigens, synthesis of the protected analogue using a phosphinophosphonate skeleton potentially could yield a series of novel T-cell stimulants. Synthesizing protected analogues of HMBPP, DMAPP and IPP, followed by evaluation and investigation into the biological implications, could possibly clarify the importance of a second phosphorus moiety to activation T-cells.

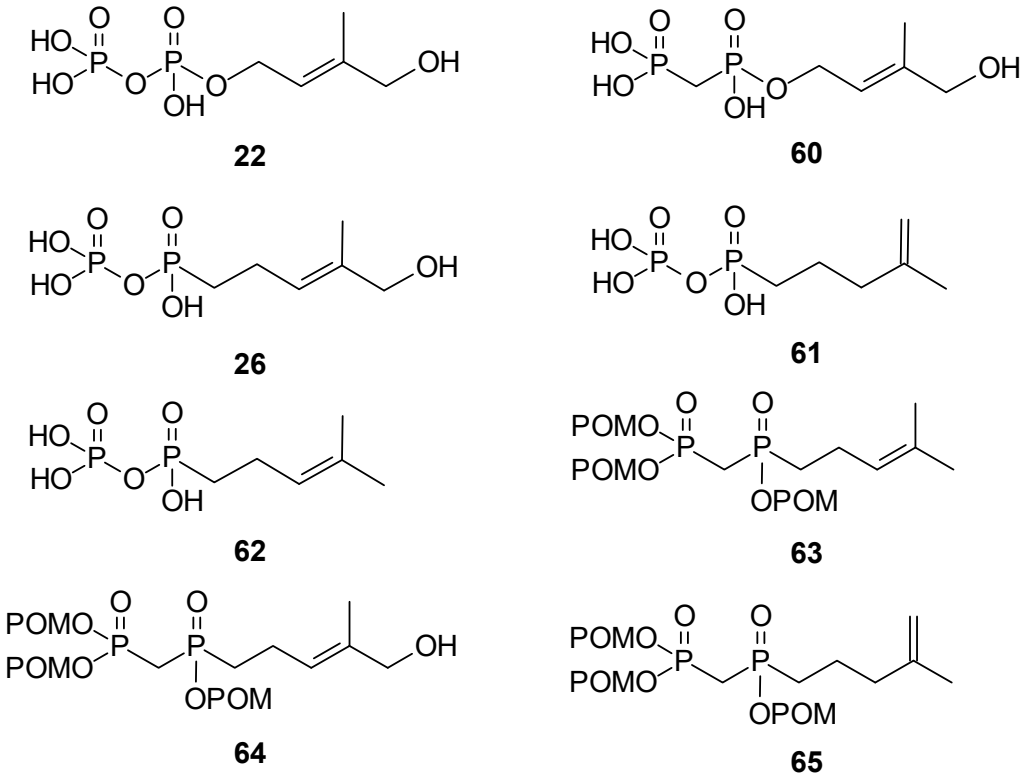


Figure 27. Phosphoantigens containing two phosphorus atoms

Synthesis of phosphinophosphonate phosphoantigens

Using an alkylation methodology similar to the dianion formation from ethyl acetoacetate,[47] a parallel synthesis might be employed for formation of the phosphinophosphonates. Using conditions established by Savignac, a self-condensation of dimethyl methylphosphonate could occur by nucleophilic addition of the anionic phosphonate **32**, displacement of methanol, and formation of the desired phosphinophosphonate **66**.[48] After the self-condensation, phosphonate **66** could be treated with two equivalents of strong base follow by the addition of prenyl bromide to obtain the phosphinophosphonate **66**. Savignac and co-workers noted that upon warming from -78 °C, dimerization occurred after 5 min at 0 °C. [48] When these same conditions were explored in an effort to obtain compound **66**, the self-condensation product was not observed and

only starting material was obtained. With the parent phosphinophosphonate undetected by this method, a new route was needed in order to obtain the desired phosphoantigens.

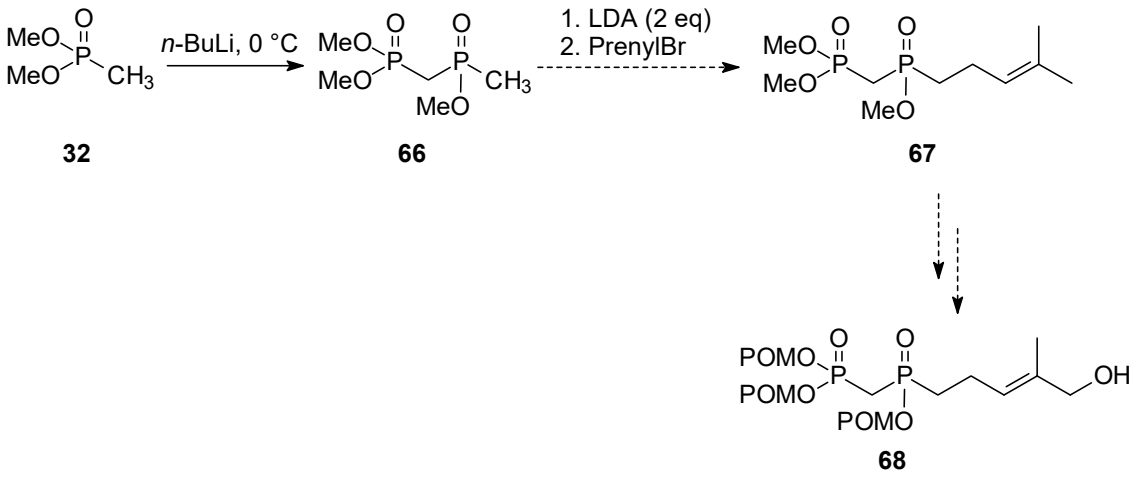


Figure 28. Self-condensation to phosphinophosphonate **66**

The POM protected DMAPP and HMBPP phosphinophosphonates could be envisioned to arise through parallel series of reactions. Using the dimethyl homoprenylphosphonate **33**, the phosphinophosphonate can be generated through the corresponding phosphonic acid chloride and the anion of dimethyl methylphosphonate. Through methodology similar to that used in the previous chapter, the desired chloride could be prepared from the corresponding ammonium salt upon treatment of dimethyl homoprenylphosphonate with DABCO. For the actual synthesis, dimethyl methylphosphonate was allowed to react with lithium diisopropylamide (LDA). However, investigation of the ^{31}P NMR spectrum of the reaction mixture showed formation of numerous by-products. This myriad of products suggests that formation of a diisopropyl phosphonamide could have occurred through reaction of the phosphonic acid chloride **69** with diisopropyl amine. In any event with numerous by-products observed, an alternative methodology to obtain the phosphinophosphonate through chloride **69** became attractive.

Recent literature indicated that dimethyl methylphosphonate could be easily converted to the chloride when allowed to react with oxalyl chloride and catalytic DMF at ambient temperture.[49] Treatment of dimethyl homoprenylphosphonate **33** with oxalyl chloride afforded the desired phosphonic acid chloride, which was used without further purification because of its highly reactive nature. Dimethyl methylphosphonate then was allowed to react with *n*-butyl lithium. After formation of the anion, addition of chlorophosphonate **69** to the anion of dimethyl methylphosphonate afforded the desired phosphinophosphonate **67** in modest yield. With the trimethyl ester **67** in hand, allylic oxidation provided the trimethyl ester **70**, an analogue of HMBPP. Formation of this phosphinophosphonate was confirmed through analysis of the ^{13}C NMR spectrum where the alpha carbon showed splitting by two different phosphorus atoms. This carbon was split into a doublet of doublets with coupling constants of 135.6 Hz and 76.5 Hz. Trimethyl ester phosphonate **67** also could be treated with NaI and POMCl, which allowed the formation of prodrug **71** as the only isolable product. The POM ester **71** was treated with SeO_2, *t*BuOOH, and *para*-hydroxybenzoic acid to provide the desired *E*-olefin **71** with no observable over-oxidation to the aldehyde.

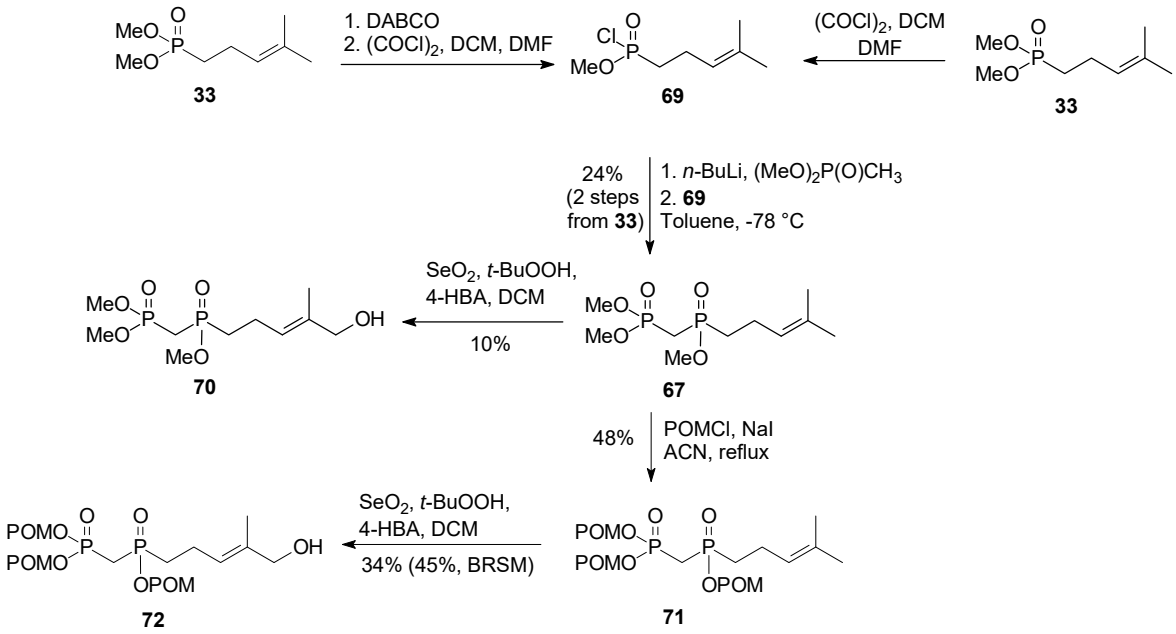
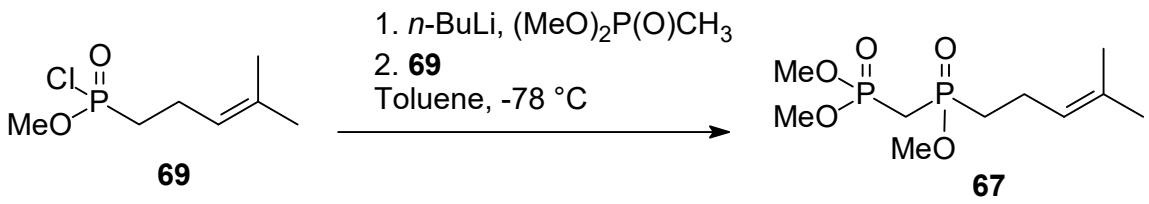


Figure 29. Ester analogues of HMBPP and DMAPP

In the formation of compound **67**, the alpha protons between the two phosphorus atoms are significantly acidic due to the stability of the conjugate anion which is stabilized through both phosphoryl functionalities. Upon formation of the desired phosphinophosphonate, any remaining anion of dimethyl methyl phosphonate is quenched due to the acidic nature of the alpha protons. However, use of 2 equivalents of the resulting anion resulted in a 24% yield of the phosphinophosphonate **67**. With this low yielding step so early in the sequence, optimization proved to be especially important in order to have a viable pathway to the desired targets (Table 6). When 4.7 equivalents of dimethyl methylphosphonate and *n*-BuLi were used and the resulting anion was transferred via cannula into a highly concentrated solution of chlorophosphonate **69** the desired product was obtained in a 58% yield, albeit still as a racemic mixture.



69 **67**

Trial	Scale (mmol)	[mM]	Dimethyl methyl phosphonate (eq)	*n*-BuLi (eq)	Yield
1	2.47	0.10	2	2	24%
2	5.86	0.20	3	3	31%
3	5.06	0.20	3	3	41%
4	3.17	0.10	4.7	4.7	58%

Table 6. Optimization of the phosphinophosphonate skeleton

Due to the novel nature of the phosphinophosphonate substructure, the corresponding salts also were desired in order to test the ability of these compounds to stimulate Vγ9Vδ2 T-cells. Along with Vγ9Vδ2 proliferation, the phosphonic acid salt can be utilized for binding studies with the BTN3A1 protein. With the trimethyl phosphonates **67** and **70** in hand, the tri-sodium salts **73** and **74** were made available by hydrolysis utilizing conditions reported by McKenna, which involve the use of TMSBr and collidine, followed by treatment with NaOH.[50] After concentration, the product was isolated as a white solid along with an impurity lacking carbon, hydrogen and phosphorus. Traditionally isolation of the sodium salt is achieved through precipitation using anhydrous acetone.[51] When such a precipitation was attempted in this case, there was no precipitate observed. This may be due to the highly oxygenated product displaying significant solubility in both acetone and water. Using conditions reported by Boëdec, the desired tri-sodium salts were isolated by dissolving the initial solid in a minimum amount of water. After addition of an anhydrous mixture of isopropyl alcohol and acetonitrile (50/50), the impurity forms a precipitate which was easily removed through filtration.[18] Using this alternative

precipitation procedure, the small phosphoantigens **73** and **74** were able to be purified. The lower yield obtained for compound **74** may simply be a consequence of its greater water solubility.

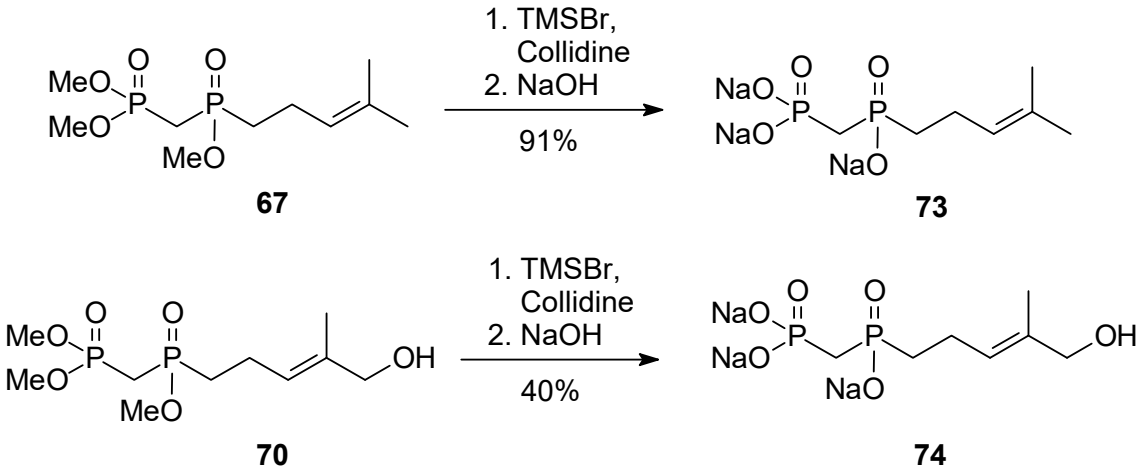


Figure 30. Phosphinophosphonic acid of HMBPP and DMAPP

Biological results

The phosphoantigens described in the chapter were assayed by our collaborators at the University of Connecticut for their ability to stimulate Vγ9Vδ2 T-cells compared to the activity of known phosphoantigens. We were especially interested in the activity of the phosphinophosphonate as the prodrug based on the increased potency observed from the alkyl phosphonate prodrug discussed in Chapter 2.[19] While the alkyl phosphonate prodrug **25c** showed a large increase upon masking the ionizable phosphonic acid, evaluation of diphosphonate or diphosphonate protected analogues has not been reported. Analysis of both the prodrugs and phosphonic acids would show the importance of a second phosphorus atom to the activity. The results of the stimulation assays are summarized in Table 7 below.

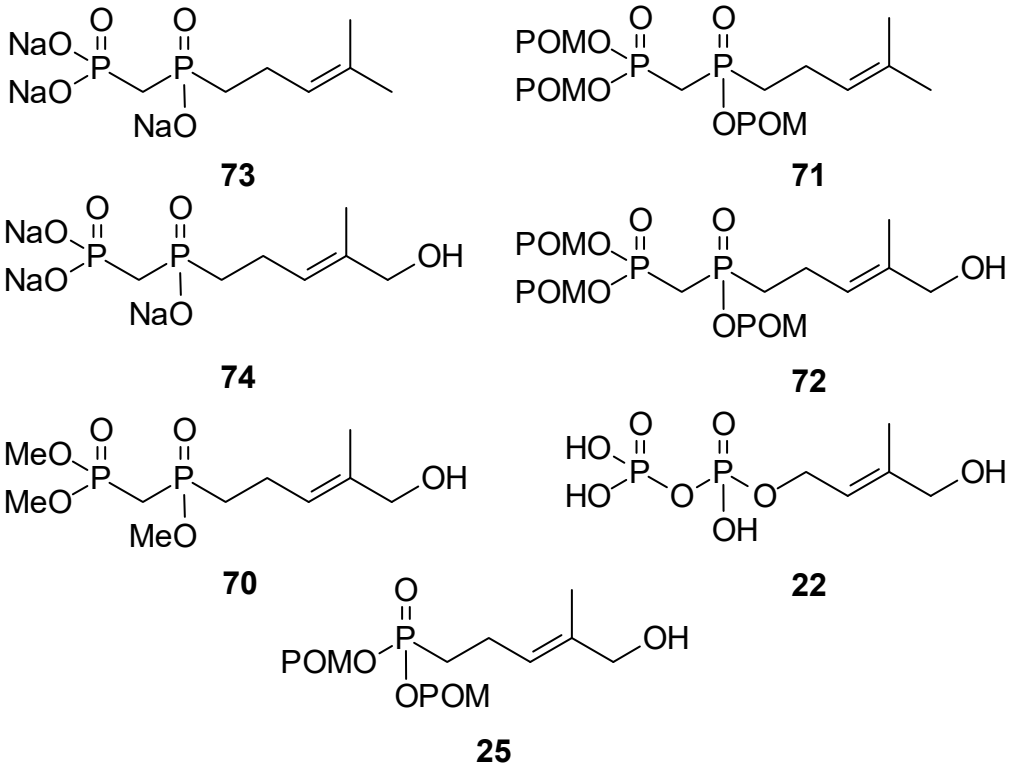


Figure 31. Assayed phosphinophosphonate analogues

Compound	Expansion of Vγ9Vδ2 T-cells EC_{50} (μM)	Toxicity towards Vγ9Vδ2 T-cells IC_{50} (μM)	Lysis of K562 cells by simulated Vγ9Vδ2 T-cells EC_{50} (μM)	Toxicity towards K562 cells IC_{50} (μM)
22	0.00051	0.50	0.0016	>100
25c	0.0054	0.60	0.0024	>100
74	26	>100	30	ND
72	0.041	3.5	0.28	3.4
70	>100	>100	>100	ND
71	>100	>100	7.3	9.7
73	>100	>100	>100	ND

Table 7. Vγ9Vδ2 T-cells proliferation and lysis assays [19, 31]
ND = Not Determined

Our collaborators found that only the hydrolysable phosphinophosphonates containing gamma hydroxyl groups stimulated expansion of the Vγ9Vδ2 T-cells (compounds **72** and **74**). When the phosphonate ester was not readily hydrolyzed or there was the lack of allylic alcohol, stimulation was not observed at concentrations less than 100 μM. As expected, of the new compounds the HMBPP analogue **72** showed the greatest stimulation of Vγ9Vδ2 T-cells with an EC_{50} of 0.041 μM. When compared to the phosphinophosphonate salt **74**, with an EC_{50} of 26 μM, there is a 600-fold increase in stimulation when masking the charged phosphonic acid moiety, similar to the effects discussed in Chapter 2. Phosphonate **72** showed toxicity against Vγ9Vδ2 T-cells at a concentration of 3.5 μM with an 85-fold difference between stimulation versus toxicity to the cells. This difference indicates a significant therapeutic window for a potential use as a Vγ9Vδ2 T-cells agonist. With the trimethyl ester **71** at concentrations of up to 100 μM, no observable expansion of Vγ9Vδ2 T-cells was noted. Compounds **71** and **73**, analogues of DMAPP also, were investigated for their ability to stimulate proliferation of Vγ9Vδ2 T-

cells in the absence of an allylic alcohol. In both cases, these compounds were inactive up to 100 μM concentrations, reaffirming the importance of the gamma hydroxyl functionality on the ability of potential phosphoantigens to stimulate Vγ9Vδ2 T-cells expansion.

These new compounds (**70** – **74**) also were examined for their ability to stimulate Vγ9Vδ2 T-cells and the capacity of those T-cells to lyse leukemia cells (K562 cells). Again the most active agonist is HMBPP followed closely by the diPOM phosphonate **25c**, with EC_{50} values of 0.0016 μM and 0.0024 μM respectively in this assay. Upon examination of the phosphinophosphonate derivatives by our collaborators, the POM-protected compound **72** had a stronger activity than that of its phosphinophosphonate salt **74**, with activities of 0.28 μM and 30 μM respectively.[31] By protecting the phosphonic acids as prodrugs, these examples have shown that the prodrug moieties do indeed increase the effectiveness for these compounds, probably by increasing their ability to cross the cell membrane. However these assays revealed that while the allylic alcohol is important in order to have potentially viable agents with low EC_{50} values, the triPOM compound showed mild activity in this lysis assay at 7.3 μM. While there was no observable activity in the stimulation assay, this result shows that while compound **71** lacks the gamma hydroxyl functionality it still must stimulate Vγ9Vδ2 T-cells, triggering a cascading event leading to the lysis of leukemia cells. This confirms the already existing literature that IPP stimulates Vγ9Vδ2 T-cells proliferation in high concentrations.[5] Both IPP and phosphonate **71** lack the allylic alcohol but are still able to stimulate with low activity. However due to the high concentration required for activity, these phosphoantigens would not be highly attractive as potential pharmaceutics. Along with the ability of these compounds to stimulate Vγ9Vδ2 T-cells, these compounds tend to be more toxic in relation to their activity. This increase in toxicity could be due to the inability of biological components to metabolize the phosphinophosphonates. While the use of carbon-phosphorus bonds increases the stability, this seems to come with a cost of increased toxicity in both the expansion and lysis assays.

The phosphonate salts **73** and **74** were allowed to bind to the BTN3A1 protein along with HMBPP in NMR experiments conducted by our collaborators.[31] When these sodium salts allowed to interact with the protein, they had lower binding affinities then the naturally occuring phosphoantigen with a K_d of 139.6 μM for compound **74** versus a K_d of 1.5 μM for HMBPP. In these binding experiments, showed that examination of phosphonate **73**, which lacks the gamma hydroxyl functionality, it displayed a binding affinity that proved to be less than that of either HMBPP or compound **74**. However, both of these phosphinophosphonates have shown a notable increase in binding affinity for the BTN3A1 protein when compared to IPP. These compounds were analyzed for their kintic properties based on their binding affinities. When this was done, the proliferation activity did not match that of the binding data when using a single BTN3A1 protein. However when a homodimer model of the desired protein was considered, the binding data aligned with the stimulation data (Figure 32). While this can support the formation of a homodimer bound with a phosphoantigen in the mechanism, the creation of this dimer is as yet unproven and further investigations is still needed.

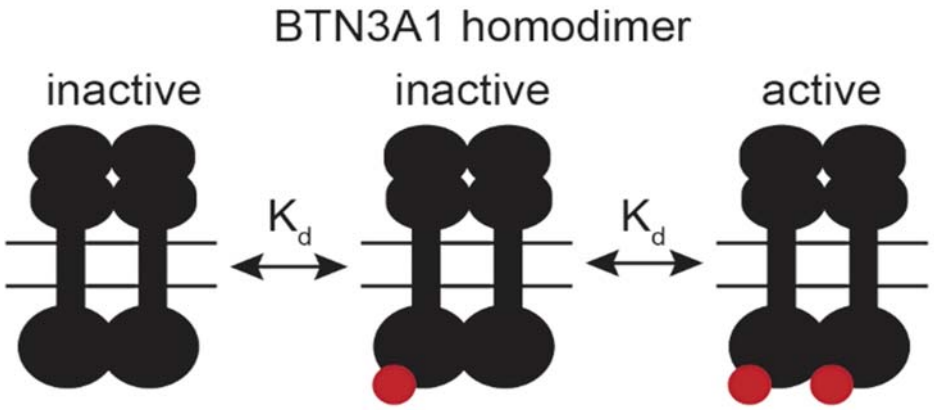


Figure 32. Binding model of phosphoantigens to the BTN3A1 protein[31]

While these derivatives were not as potent as we had hoped, they do offer insight into what could be occurring within the cell. While these compounds were active in Vγ9Vδ2 T-cells stimulation, their potency still was unable to match that of the phosphonate **25c** or the diphosphonate **26**. It could be that the second phosphorus is a needed feature to

maintain strong activity. Alternatively, while the phosphonate **25c** only contains one phosphorus, addition of a phosphate might occur after diffusion into the cell and ester cleavage. With multiple cellular machinery available for phosphorylation, formation of the diphosphonate *in vivo* could be readily envisioned. However, the activity does not correspond to that of the previously discussed diphosphonate **26**. Further investigations would need to be done in order to confirm this possible phosphorylation event.

Synthesis of IPP phosphinophosphonate analogue

While these preceding analogues allowed elucidation of some key mechanistic characteristics related to phosphoantigens and Vγ9Vδ2 T-cells stimulation by our collaborators, synthesis of an IPP derivative would be another important target. With the binding data that was determined, the IPP analogue could have a stronger binding affinity than the naturally occurring agonist. Synthesis of this IPP analogue and comparison of its activity to the natural IPP also could point to the importance of a second phosphorus to the SAR.

The IPP phosphinophosphonate might be prepared from the known iodide **76**. Therefore, the synthesis of IPP phosphinophosphonate analogue was conducted via a methodology corresponding to that described earlier. The primary alcohol 3-methyl-3-buten-1-ol was allowed to react with triphenylphosphine, imidazole, and iodine to afford the desired primary iodide **76** with triphenylphosphine oxide as a side product. When attempting to isolate the iodide **76**, a mixture of the desired product and the phosphine oxide was isolated via column chromatography. Attempts to purify the iodide by distillation resulted only in decomposition. This decomposition could be due a possible polymerization or through elimination of hydrogen iodide to form isoprene. An alternative approach was investigated to form the primary iodide by reaction of alcohol **75** with TsCl, followed by treatment with NaI. Again, attempts to isolate the iodide also proved problematic due to its low volatility and rapid decomposition.

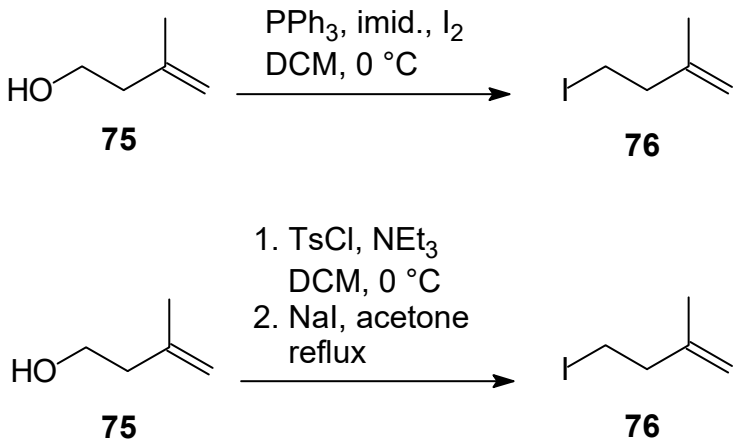


Figure 33. Attempts at formation of the primary iodide

Where the iodide **76** underwent rapid decomposition, recent literature revealed that formation of the primary bromide **77** could be conducted using MsCl, followed by a Finkelstein reaction with LiBr.[52] Through this strategy, the primary bromide **77** was isolated in 51% yield. Using an established route Zgani utilized to form the diphosphonate **26**, the mono phosphonate **78** would be available after generation of the dimethyl methylphosphonate anion. The bromide **77** was added to the phosphonate anion to generate phosphonates **78** and **33** in a 1 to 2 mixture. The reaction did not go to completion after 4 hours and a large amount of starting material still remained.

The formation of phosphonate **33** could be due to a base mediated isomerization to create the more stable alkene. Base isomerization might occur with the addition of base to bromide **77** forming the more stable trisubstituted olefin via an allylic anion. Due to similar pka values, the allylic anion could then propagate the formation of the tri-substituted olefin, increase its concentration, and decrease the amount of terminal alkene present in the reaction mixture. Attempts to prevent this isomerization were explored by changing reaction length and temperature. When the reaction times were shortened, both isomers were observed in low yields and 1:1 ratios. However when the reaction length was

extended, the ratios favored the formation of the trisubstituted olefins. Furthermore, the terminal and trisubstituted olefin were not readily separable by column chromatography. Due to the difficulties in separating mixtures of these isomeric olefins and low yielding steps, a more selective synthetic approach was desirable to obtain the potential phosphoantigen **85**.

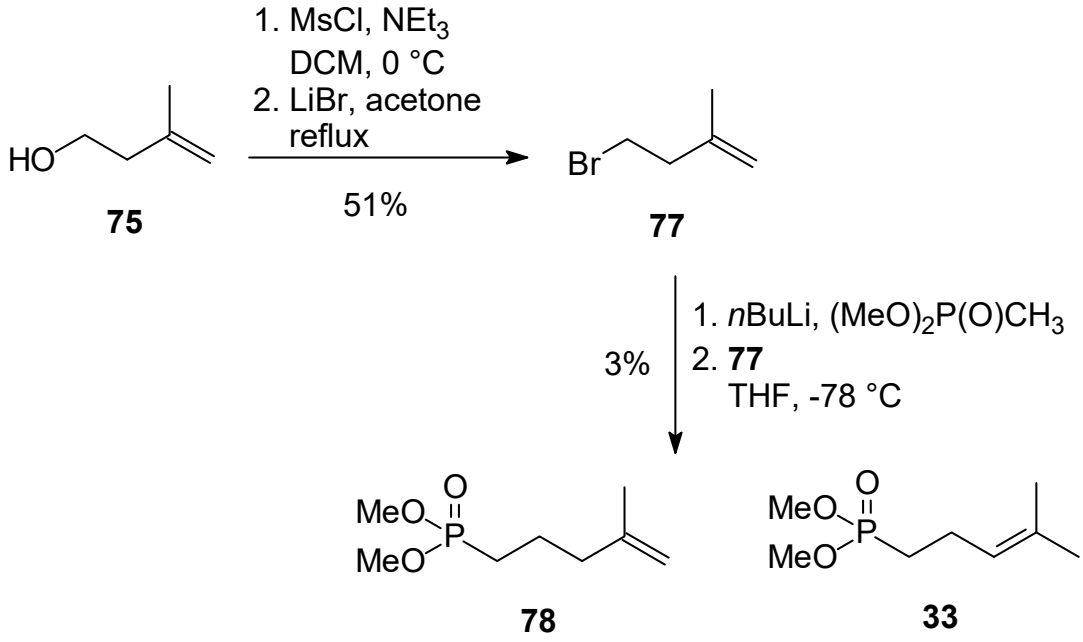


Figure 34. Unexpected rearranged olefin

The second strategy toward the synthesis of the phosphinophosphonate **85** was focused on a 1, 4-addition of dimethyl methylphosphonate anion to methyl vinyl ketone. However, the desired product was not observed and the ^{31}P NMR spectrum showed a single resonance corresponding to starting material.

To avoid the complications arising from the previous routes, yet another strategy was investigated. The alternative approached focused on a Wittig reaction to install the

terminal olefin from levulinic acid. Using methyltriphenylphosphonium bromide, the ylide was formed upon treatment with *n*-BuLi at 0 °C and then levulinic acid was added to excess ylide to generate the carboxylic acid **80**.[53] Isolation of the resulting acid was readily ascertainable by column chromatography with the use of acetic acid to maintain a neutral species. Formation of the alcohol **81** was achieved through treatment of the carboxylic acid **80** with lithium aluminum hydride.[54]

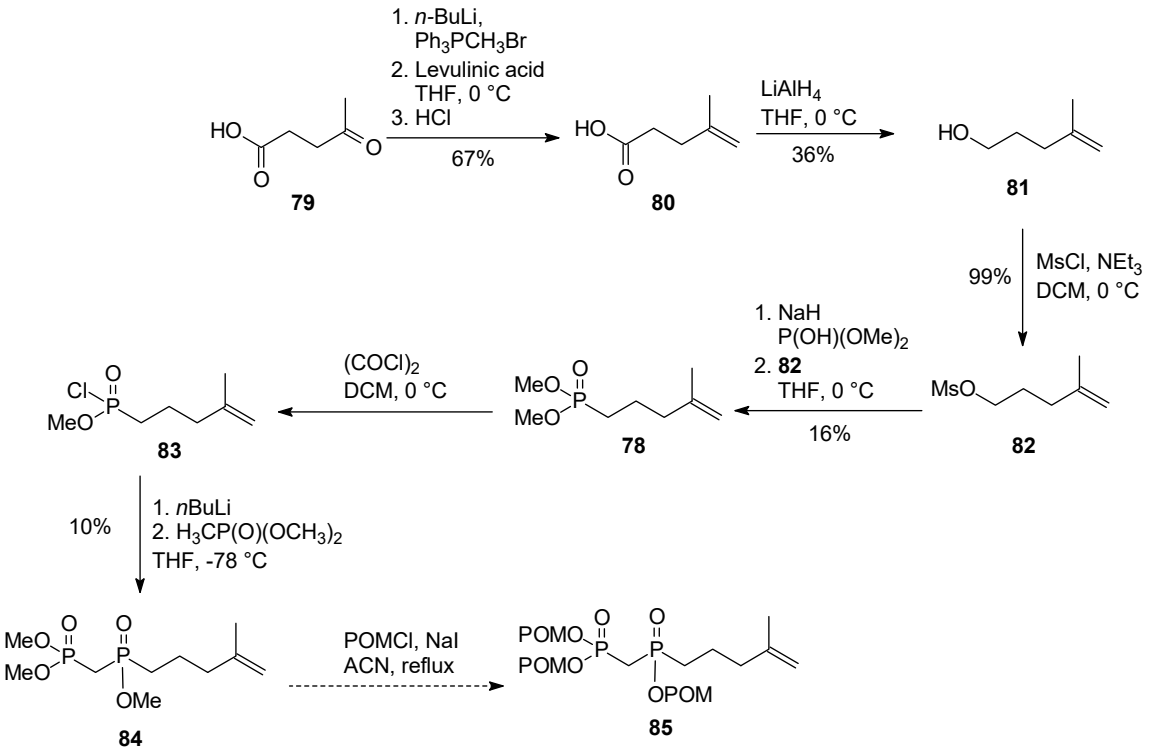
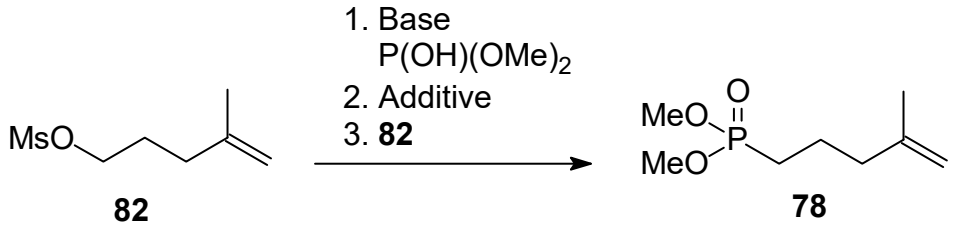


Figure 35. Preparation of phosphinophosphonate **84**

This primary alcohol **81** then was treated with MsCl along with triethyl amine to form the mesylate **82** in near quantitative yields. With the mesylate in hand, Michaelis-Becker style reaction could occur through reaction with dimethyl hydrogen phosphonate, followed by treatment of base. Using established conditions, the dimethyl hydrogen phosphonate was allowed react with NaH, followed by the addition of the mesylate in order to form phosphonate **78** in low yield.[55] Again optimization was needed in order to increase

the formation of desired phosphonate. It was observed upon addition of base, the solubility of the forming anion decreased dramatically in THF. Once this low solubility was recognized, exploration of additives and alternative bases to increase solubility was then undertaken. Use of crown ethers has been common in order to separate ion pairs and facilitate reactivity. When applied to this reaction, solubility seemed to improve slightly with the largest effect occurring with the change in base. While the yields remain low, potential improvements could occur with optimization of solvents. Using a solvent with a high dielectric constant might allow for elevated solvation of the anion, and in turn increase the availability of the anion in solution.



82 **78**

Trial	Base	Additive	Solvent	Time	Temperature	Yield
1	NaH	-	THF	18h	0 °C	16%
2	NaH	15C5	THF	18h	0 °C	12%
3	NaH	15C5	THF	18h	60 °C	Decomposition
4	KHMDS	-	THF	2 days	0 °C	26%
5	KHMDS	16C6	THF	5 days	25 °C	21%

Table 8. Effect of base and additives to increase solubility

Conversion of the phosphonate **78** to the phosphinophosphonate **84** can arise using similar conditions as previously mentioned in the preparation of HMBPP and DMAPP analogues. However, given the susceptibility of the terminal olefin to base induced isomerization, lower equivalents of base were to circumvent the possible rearrangement.

Full conversion of phosphonate **78** after treatment with oxalyl chloride to the desired phosphonic acid chloride was observed. Dimethyl methylphosphonate was allowed to react with *n*-BuLi followed by addition to the chloride **83** to give the desired phosphinophosphonate in low yields along with additional by-products. With its reactive nature and ability to rearrange, care will need to be taken to avoid loss of product during efforts to form the POM analogue.

To conclude, a family of phosphoantigens has been synthesized containing non-hydrolysable phosphorus-carbon bonds. These compounds were readily available after optimization. The phosphinophosphonates were tested by our collaborators to determine their activity as Vγ9Vδ2 T-cells stimulants. The agonist **72** was found to be a moderately active compound although it was still significantly less potent then HMBPP and the known phosphonate prodrug **25c**. These compounds also show the importance of the allylic alcohol in binding to BTN3A1 and the expansion of Vγ9Vδ2 T-cells. The DMAPP analogue has shown a greater activity then IPP in binding to BTN3A1. Therefore, the synthesis of an IPP derivative as a prodrug still should be pursued. Several attempts were investigated toward the synthesis of the IPP phosphinophosphonate **85**, with the most promising avenue arising from levulinic acid. With the installation of the POM functionality along with McKenna hydrolysis, two novel possible phosphoantigens could be obtained in order to further elucidate the complexity of Vγ9Vδ2 T-cells stimulation.

CHAPTER 5

SYNTHESIS OF SYMMETRIC AND CHIRAL ALKYL 1, 1, 1-TRISPHOSPHONATES

While there are a multitude of enzymatic reactions, many of them use phosphorus-containing compounds as substrates. Most biological phosphorus is found as phosphates. Due to the considerable use of phosphorus in the body, synthesis of novel phosphorus-containing compounds has been actively pursued for treatment of a variety of diseases. Phosphonates are attractive for their increased metabolic stability through the carbon-phosphorous bond. However, only certain chemical classes have been employed for medicinal practice, especially phosphonic and bisphosphonic acids. Whether protected through ester linkages or used as the parent phosphonic acids, the most commonly applied phosphonate class would be the bisphosphonates. Recently a class of compounds known as the α-alkyl 1, 1, 1-trisphosphonates has been investigated in chemical space but it has not yet been examined for any biological use.

Substitution at the α–position of the trisphosphonate motif has been reported and includes halogens, aryl groups, amines, and alkyl groups. [56-59] The trisphosphonates were first reported by Gross through conjugate addition of a hydrogen phosphonate to the quinone methide **86** forming the phenol **87**.[60] Further extension of this class involved formation of the methane trisphosphonate from the tetra isopropyl bisphosphonate ester by treatment with base, a chlorophosphite, and oxidation.[60] Modifications of the system allowed synthesis of the halogenated trisphosphonates (**90**).

In 2011, Smits reported synthetic approaches to increase the scope of this functional group. While the simplest trisphosphonate had been prepared by Gross[56] and then by Blackburn,[58] alkylation of the trisphosphonate anion was unsuccessful due to its ability to

function as a strong carbon acid.[61] However, synthesis of alkyl trisphosphonates (**93**) could be accomplished from an alkyl bisphosphonate (**92**), after removal of trace amounts of water, through reaction with base and diethyl chlorophosphite followed by oxidation of the initial P(III) product with either air or hydrogen peroxide.[60] Smits explored these compounds and found that they undergo a variety of chemical reactions reflecting the stability of the alkyl 1, 1, 1-trisphosphonate system. These attempted modifications showed that the trisphosphonates are stable to oxidation, metathesis, hydrogenation, coupling reactions, and hydroboration/oxidation.[61] However, these phosphonate esters are not likely to elicit a biological response. Hydrolysis would be needed to unveil their biological activity.

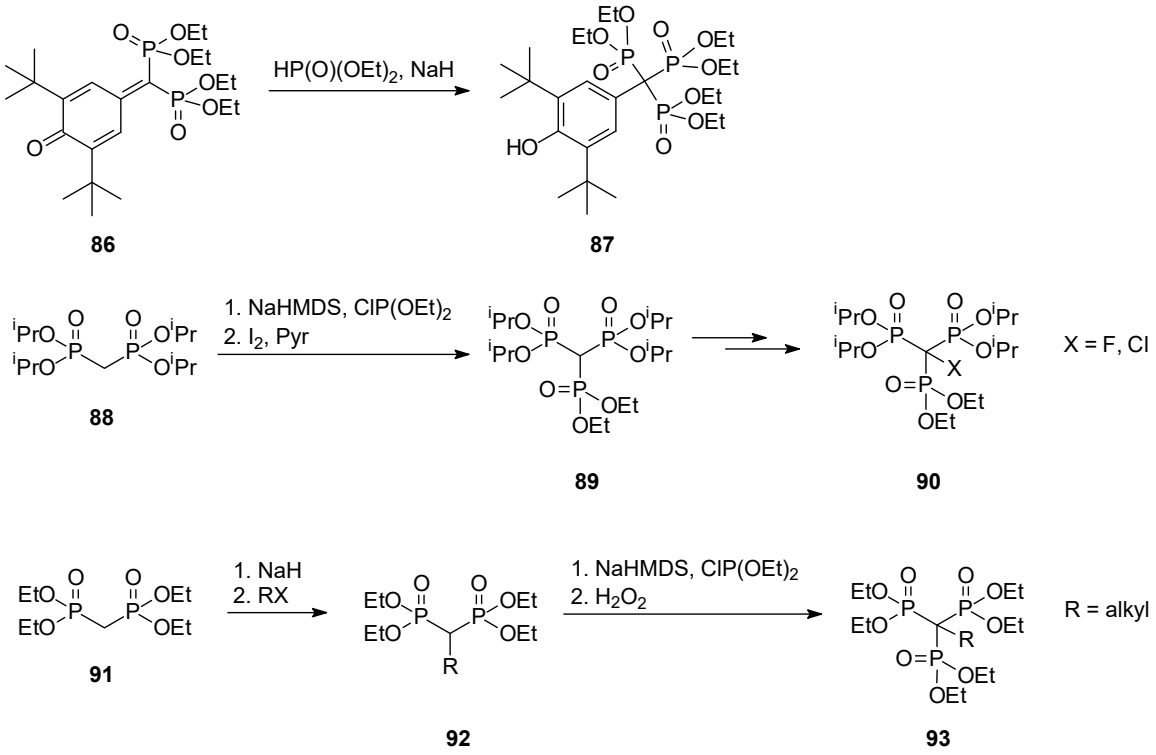


Figure 36. Reported examples of trisphosphonates

Upon hydrolysis, trisphosphonates could undergo cleavage to either the hexa-salt or one of two tri-salt species (**97** or **98**). While the hexa-salt would be chemically viable,

this highly charged species may have difficulty entering the active site of any targeted enzyme because of the high degree of negative charge. A tri-salt species would mimic the natural diphosphate substrates more closely. The tri-salts could serve as potential inhibitors of many enzymes in the isoprenoid biosynthetic pathway. While methodology to synthesize trisphosphonates has existed for some time, cleavage to the phosphonic acids has yet to be explored in detail. Hydrolysis of bisphosphonates has been known for some time. Through the use of TMSBr and NaOH, or upon treatment with HCl, cleavage to the tetra sodium salt has been achieved. However attempted acid hydrolysis of the trisphosphonate by treatment with HCl resulted in decomposition to the bisphosphonate ester.[62] In contrast, formation of the hexa-salt (**96**) was readily achievable through McKenna hydrolysis.[61]

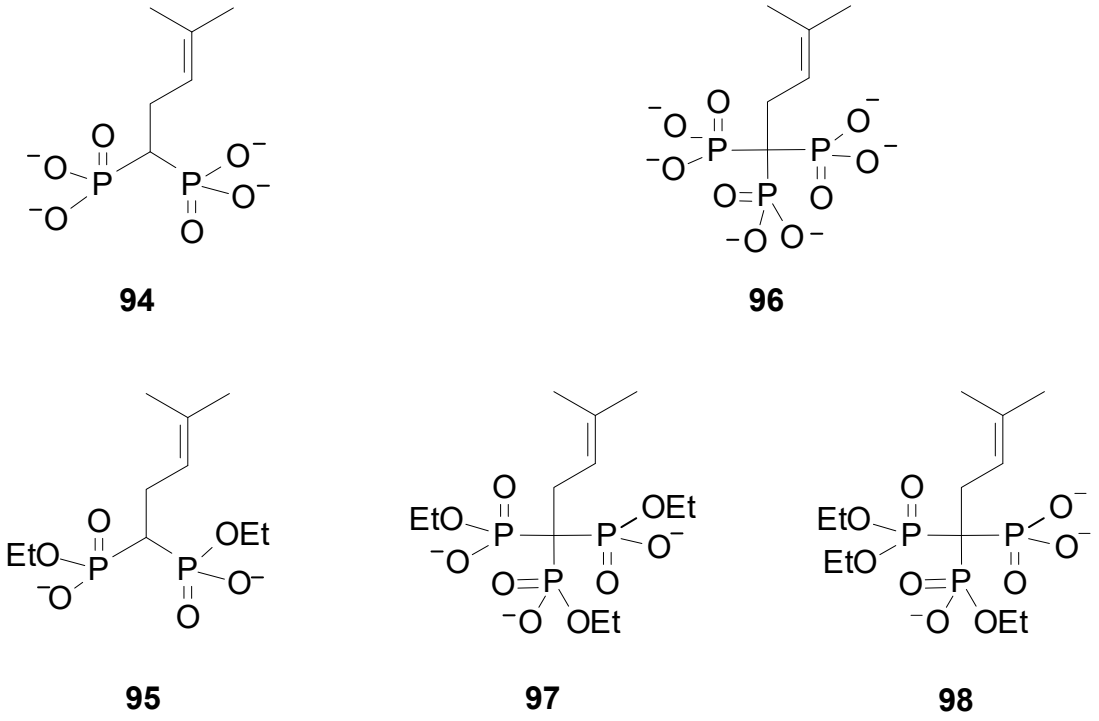


Figure 37. Possible charged phosphonate salts

Formation of a disodium salt is less commonly practiced with bisphosphonates, yet some have been synthesized through treatment of a tetra ester with sodium iodide and a variety of amines including morpholine. Treatment of bisphosphonates with morpholine allows cleavage of two ester linkages in a symmetric fashion through *N*-alkylation. This same process could be applied to the trisphosphonate to provide a structure similar to phosphonate **97** or perhaps even compound **98**. This process was attempted on a geranyltrisphosphonate ester unsuccessfully, even after prolonged reaction periods and addition of heat.

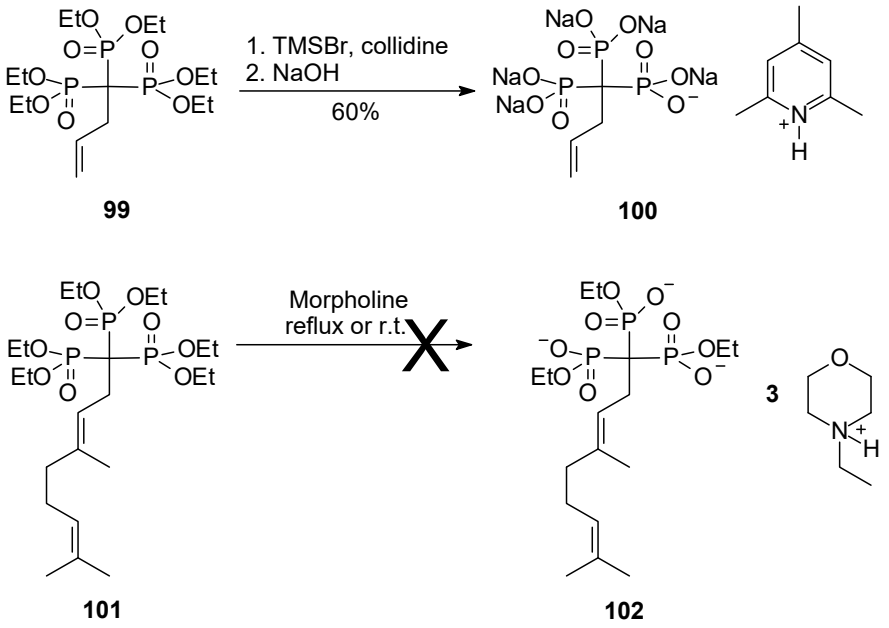


Figure 38. Formation of mixed salts of trisphosphonates

Synthesis of Symmetrical Trisphosphonate salts

Even though hexa-salts of trisphosphonates have been isolated, further investigation into the formation of tri-salts should be explored to increase the chemical

scope of trisphosphonates. Nitrogenous bisphosphonates, such as risedronate, have been used pharmaceutically for treatment of multiple myeloma and as an indirect phosphoantigen as discussed in the previous chapters.[5] Another nitrogenous analogue that has been developed is 2-(3-pyridinyl)-1-hydroxyethylidene-1, 1 phosphonocarboxylic acid (3-PEHPC), which has been shown to inhibit Rab GGTase at high concentrations and may be a potential treatment for multiple myeloma. Both of these classes of compounds target enzymes in the IBP, yet synthesis of a nitrogenous trisphosphonate has not yet been pursued.

The synthesis of the pyridine trisphosphonate **111** began with alkylation of tetraethyl methylenebisphosphonate (**91**) with 3-(chloromethyl)pyridine (**103**). After neutralization of 3-(chloromethyl)pyridine hydrochloride, the bisphosphonate anion was added and the desired pyridyl bisphosphonate was formed in extremely low yields. Upon investigation, polymerization of the chloromethyl pyridine was observed undoubtedly contributing to the low yields. It was imagined the desired reaction could proceed through use of excess base if neutralization of pyridine hydrochloride were followed by alkylation to form compound **104**. When this approach was attempted, an observable color formation was witnessed after partial purification, but only starting material was observed after analysis of the ^{31}P NMR spectrum. An alternative route through a Knoevenagel condensation using 3-pyridine carboxaldehyde was pursued by means of titanium tetrachloride and base.[63] However, using conditions established by Huang resulted in no apparent formation of the desired product even after the reaction was heated.[63]

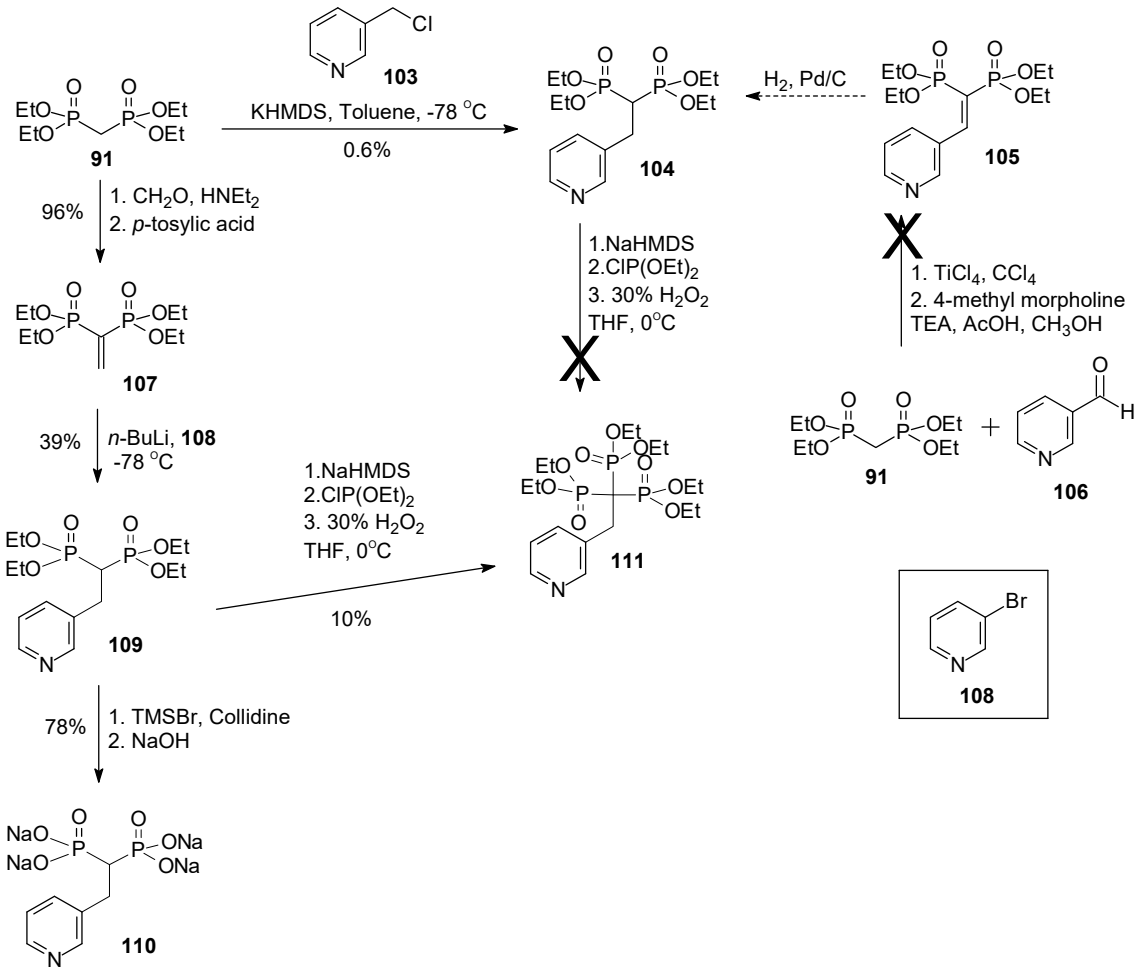


Figure 39. Preparation of pyridine trisphosphonate

After the previous attempts to provide the desired intermediate went unrewarded, the target was achieved by synthesis through a Michael addition of a lithiated pyridine (**108**) to the vinyl bisphosphonate **107**. Preparation of the vinyl bisphosphonate was accomplished through a Knoevenagel condensation of paraformaldehyde (after thermal cracking) with tetraethyl methylenebisphosphonate in nearly quantitative yields. Halogen metal exchange provided the lithiated pyridine derivative of compound **108**. Dropwise addition of the vinyl bisphosphonate at -78 °C to the lithiated anion resulted in formation of the pyridine bisphosphonate **109** in good yield. [64] Bisphosphonates contain a high carbon-to-phosphorus ratio and readily form hydrates, which has been shown to be

problematic when forming the trisphosphonates.[61, 62] After using a drying procedure based upon azeotropic removal of any trace amounts of water with anhydrous benzene, treatment of bisphosphonate **109** with KHMDS and diethyl chlorophosphite followed by oxidation with H_2O_2 provided the desired trisphosphonate in a modest conversation based upon analysis of the ^{31}P NMR spectrum. Upon partial purification using column chromatography, the trisphosphonate **111** was isolated in low yield.

After formation of the pyridine trisphosphonate, methodology to form the symmetrical tri-salt is needed in order to produce an attractive inhibitor. Efforts to produce a tri-salt using conditions developed by Reddy[65] with geranyltrisphosphonate **101**, prepared using the same methodology as in the pyridinetrisphosphonate were pursued. Morpholine, NaOH, and NaI were allowed to react with compound **101** in an attempt to produce a symmetric tri-salt. Analysis of the ^{31}P NMR spectrum revealed four peaks, including remaining starting material. Unfortunately, the other ^{31}P NMR resonances corresponded to geranylbisphosphonate **112** and the partially hydrolyzed phosphates.

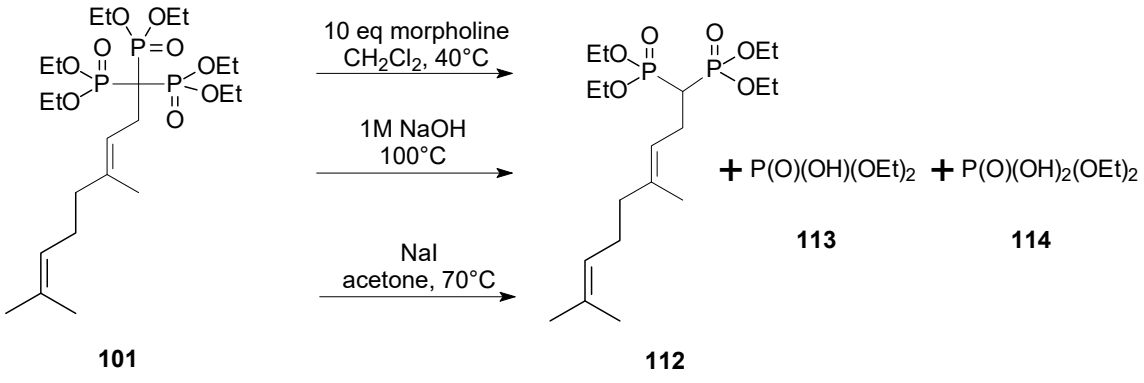


Figure 40. Attempted hydrolysis using NaI and base

In summary synthesis of a pyridinetrisphosphonate was readily accomplished through a Michael addition to a vinyl bisphosphonate followed by treatment with base, and

a subsequent reaction with diethyl chlorophosphite and oxidation. Hydrolysis conditions have been studied using geranyltrisphosphonate. Under conditions established on bisphosphonates, treatment of geranyltrisphosphonates with NaI and morpholine revealed decomposition of the trisphosphonate functionality, even though it has been shown to be stabile to many other reaction conditions. Efforts to make and purify the tri-salts using various reaction conditions are still being investigated. However further attempts to make trisphosphonate salts have led to the development of a new synthetic plan to make chiral trisphosphonate.

Synthesis of a chiral alkyl 1, 1, 1 trisphosphonate

One possible hydrolysis product of an alkyl trisphosphonate would be a chiral tri-salt (**98**) as shown in Figure 37 (page 61). Synthesis of these species could be envisioned through selective ester cleavage by hydrogenolysis, acid, or base, or perhaps even enzymatic hydrolysis. Using various esters, synthesis of a chiral trisphosphonate could be anticipated upon formation through sequential addition of a different phosphonate groups, including adding chirality to the α-carbon and one phosphorus. This could be achieved through the use of an alkyl dichlorophosphite in a reaction with a monophosphonate, followed by treatment with an alcohol and an amine base to esterify the bisphosphonate.

A chiral trisphosphonate also could be seen to arise from phosphorylation with ethyl dichlorophosphite of a bisphosphonate in a similar fashion to the sequence established with the symmetrical trisphosphonates. Initially, investigation using a symmetrical bisphosphonate and ethyl dichlorophosphite was attempted to simplify analysis of the possible products. Treatment of tetraethyl methylenebisphosphonate with NaH and prenyl bromide afforded the prenylbisphosphonate **115**. After azeotropic distillation to remove any residual water, the prenylbisphosphonate was treated with KHMDS followed by addition of ethyl dichlorophosphite, presumably forming a chlorophosphonite intermediate. Conversion to the benzyl ester was attempted using

benzyl alcohol and ammonia followed by oxidation through addition of H_2O_2. Isolation of the product occurred through column chromatography to afford an unexpected phosphonamide **116**.

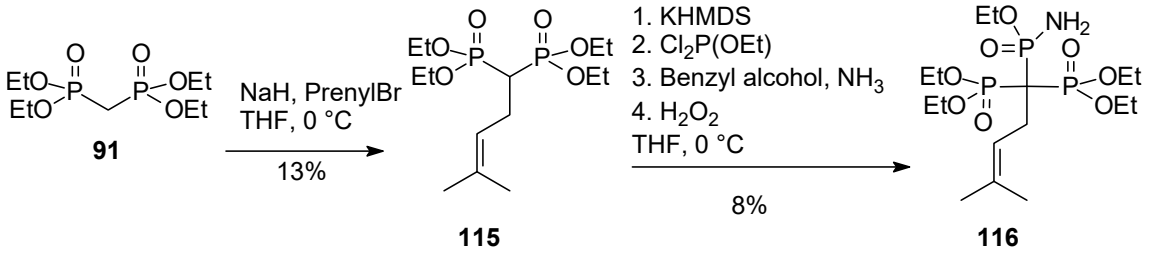


Figure 41. Synthesis of the phosphonamide **116**

The NMR characteristics of the product **116** showed evidence for the formation of this novel trisphosphonate. In the ^{31}P NMR spectrum, two resonances at 26 and 19 ppm were observed with one a triplet and one a doublet with the same coupling constant. Likewise the ^{13}C NMR spectrum exhibited a characteristic splitting pattern for the α-carbon. The resonance corresponding to the α-carbon shifted to 50 ppm and was observed as a triplet of doublets with C-P coupling constants of 119 and 100 Hz. Along with coupling to the phosphonate ester carbons, there was an observable C-P coupling to the olefinic carbon, which appeared as a quartet at 120 ppm with a coupling constant of 6.3 Hz. Analysis of the high resolution mass spectrum confirmed the incorporation of nitrogen in the phosphonamide functionality.

With the ability for phosphorylation to occur with ethyl dichlorophosphite, synthesis of a chiral trisphosphonate was attempted. Dimethyl methylphosphonate **32** was treated with *n*-BuLi to form the anion, followed by addition of prenyl bromide which resulted in the formation of the desired monoalkylated phosphonate **33** and a side product believed to be the dialkylated phosphonate. Upon treatment of this phosphonate with base followed by dropwise addition of diethyl chlorophosphate, phosphorylation was achieved

to form the unsymmetrical diphosphonate **117**. Synthesis of trisphosphonate **118** was attempted by treatment of diphosphonate **117** with KHMDS and ethyl dichlorophosphite followed by oxidation and addition of triethyl amine. After some purification, three new peaks were observed in the ^{31}P NMR spectrum at 15, 18 and 31 ppm. Isolation of the desired product was attempted through column chromatography, but the product was isolated only as a mixture with starting material in low yield. Optimization of this reaction is still needed for successful isolation (Table 9). Using ^{31}P NMR, the reaction was observed by monitoring the progression of product formation. After treatment with base for one hour, ethyl dichlorophosphite was added dropwise and the reaction was allowed to stir overnight. These conditions afforded the chiral trisphosphonate **118** albeit in low conversion.

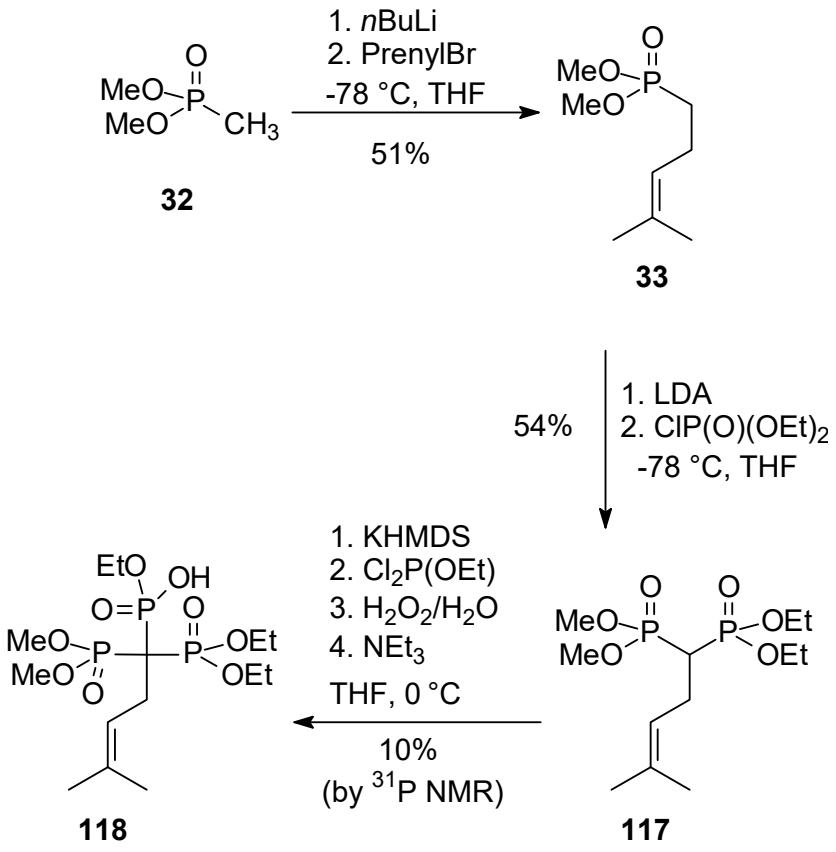


Figure 42. Synthesis of the phosphonic acid **118**

Trial	KHMDS	$Cl_2P(OEt)$	H_2O_2	NEt_3	Product formation
1	30 mins	30 mins	Overnight	2 hrs	Starting material
2	30 mins	1 hr	Overnight	1 hr	Starting material
3	30 mins	Overnight	2 hrs	1 hr	Starting material
4	1 hr	Overnight	2 hrs	2 hrs	10 % by ^{31}P NMR

Table 9. Optimization of trisphosphonate reaction with ethyl dichlorophosphite

After encountering these difficulties in formation of the chiral trisphosphonate through the final phosphonylation, rearrangement of the synthetic steps was explored to incorporate the asymmetric phosphonate earlier. Cleavage of 2-cyanoethyl esters has been observed through treatment of ammonia to obtain the ammonium phosphonate salt. Synthesis of both a di(cyanoethyl) chlorophosphite **120** and phosphate **121** were easily accomplished through treatment of trichlorophosphite with 3-hydroxypropionitrile followed by partial oxidation to obtain a mixture of the P(III) and P(V) oxidation states. These phosphorous compounds were allowed to react with the anion of diethyl phosphonate **123**, which was prepared in turn through alkylation of diethyl methylphosphonate with benzyl bromide.

With the monophosphonate **123** in hand, treatment with base followed by dropwise addition of either the di(cyanoethyl) chlorophosphite or di(cyanoethyl) chlorophosphate failed to provide the desired products. This could be due to the lack of purification of the highly reactive chlorophosphite and chlorophosphate. Conversion of a diisopropyl phosphonamide to alternative phosphonic acid esters could perhaps be accomplished through the desired alcohol and tetrazole. Using the known 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite **126**, synthesis of a chiral bisphosphonate was attempted. The diethyl phosphonate **123** was deprotonated by treatment with KHMDS and

then treated with the chlorophosphite **126**. Treatment of the resulting intermediate with ethanol and tetrazole followed by oxidation would provide the triethyl 2-cyanoethyl bisphosphonate **125**.[66] However after 24 hours, there was no observable formation of the desired product in the ^{31}P NMR spectrum of the reaction mixture.

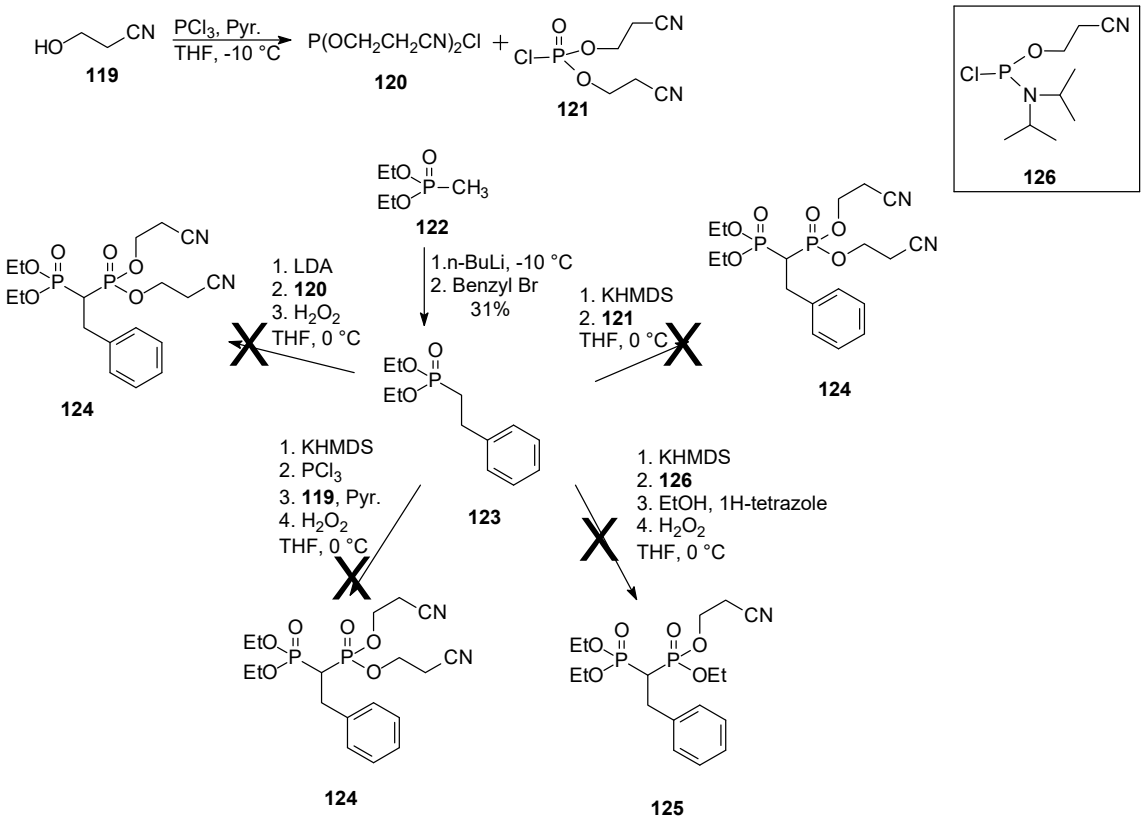


Figure 43. Attempted synthesis of a 2-cyanoethyl ester phosphonate

Given these difficulties in forming a cyanoethyl chiral bisphosphonate, an alternative ester was selected. Using a benzyl ester, cleavage could be envisioned upon treatment with palladium on carbon and hydrogen through hydrogenolysis. To explore this strategy, chloromethyl phosphonic dichloride was allowed to react with benzyl alcohol and triethyl amine resulting in the formation of a precipitate. After filtration, the filtrate was concentrated providing the dibenzyl chloromethylphosphonate **129**. With the dibenzyl

chloromethylphosphonate in hand, formation of a bisphosphonate could be seen through a Michaelis-Arbuzov or Michaelis-Becker reaction. The Michaelis-Arbuzov strategy employs reaction of a trialkyl phosphite with an alkyl halide in order to form a phosphonate. Using a commercially available phosphite and a synthesized dibenzyl methyl phosphite with compound **129**, examination of the reaction mixture by ^{31}P NMR revealed only starting material. When diethyl phosphite was treated with base, followed by dropwise addition to chloromethylphosphonate, a Michaelis-Becker reaction was attempted. After partial purification, analysis of the ^{31}P NMR spectrum showed no evidence for formation of the bisphosphonate.

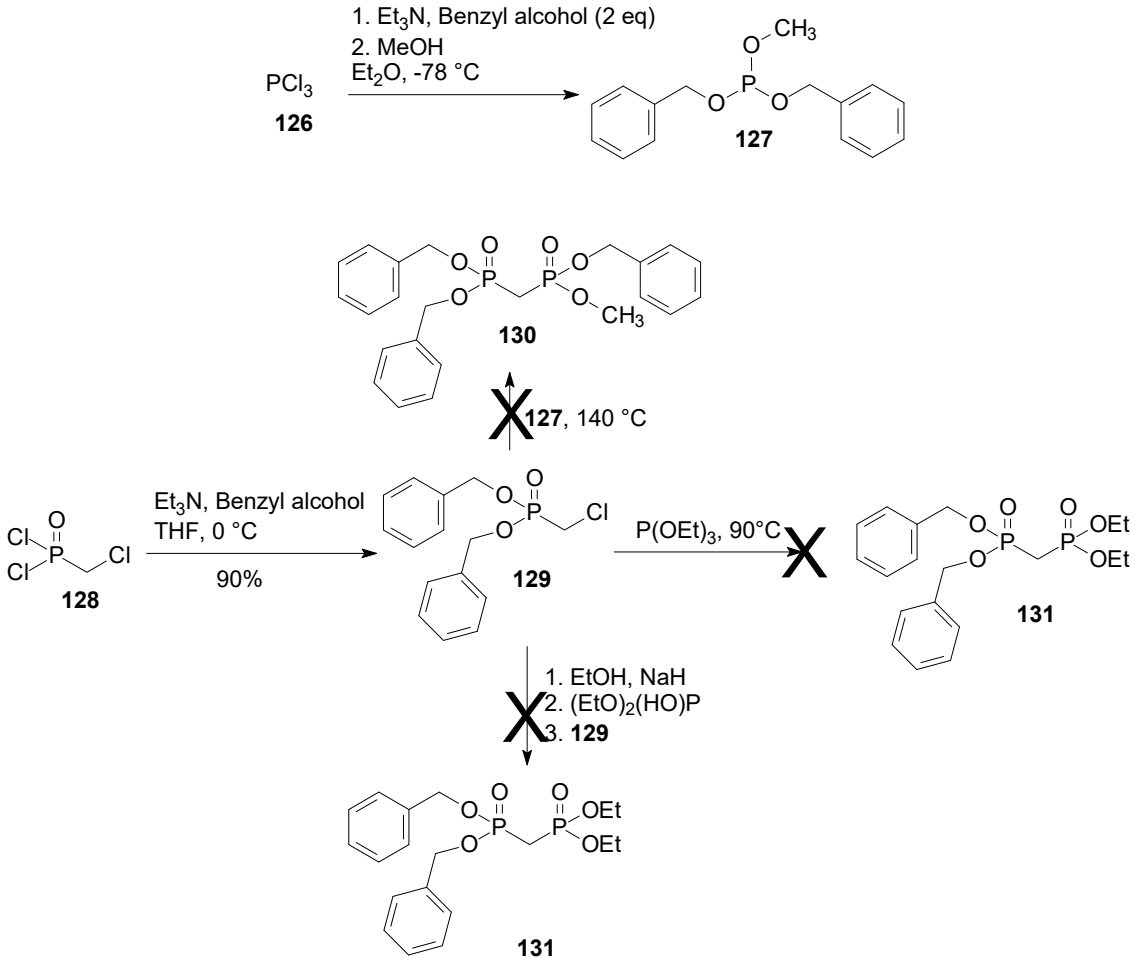


Figure 44. Attempted synthesis of benzyl bisphosphonates

After the previous efforts went unrewarded, synthesis of the parent chiral trisphosphonate was attempted through a one pot phosphonylation of an unsymmetrical bisphosphonate. Using methodology developed by Grison, [67] synthesis of an asymmetric bisphosphonate would occur through phosphonylation of a lithated dimethyl methylphosphonate anion followed by addition to ethyl dichlorophosphate at -78 °C. Displacement of the second chloride would occur through reaction with a nucleophile. Anhydrous methanol was added dropwise in order to form the trimethyl ethyl bisphosphonate **132**. With this bisphosphonate in hand, methodology established by Smits could be used for formation of the parent chiral trisphosphonate **133**.[61] Bisphosphonate **132** was deprotonated upon addition of KHMDS to form a carbanion, to which diethyl chlorophosphite was added. Oxidation of the phosphinite was achieved through use of hydrogen peroxide to obtain the parent chiral trisphosphonate **133** in a low yield.

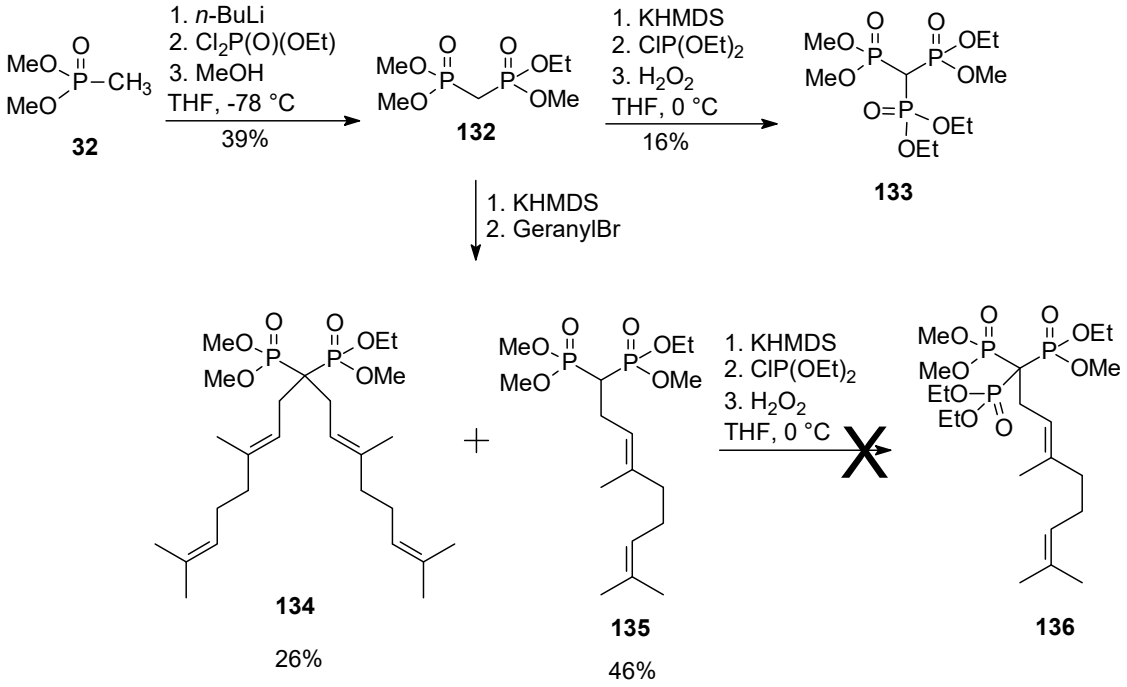


Figure 45. Synthesis of the chiral parent trisphosphonate **133** and related compounds

The NMR characteristics of this novel trisphosphonate supported its formation. The ^{31}P NMR spectrum showed the appearance of three resonances at 17, 15 and 14 ppm with one at a similar shift to the known parent trisphosphonate. In the ^{1}H NMR spectrum, the α-proton appeared as a quartet with a coupling constant of 47 Hz due to the similar (yet different) phosphonate esters. These different esters were observed with different resonances including a doublet at 3.8 ppm and a multiplet at 4.2 ppm. The ^{13}C NMR spectrum also exhibited unique characteristics. Multiple resonances showed complex splitting patterns in the phosphonate ester signals. The resonance attributed to the α-carbon appeared as an apparent quartet with a coupling constant of 248 Hz.

As demonstrated in the literature,[61] attempted alkylation of phosphonate **133** would be unlikely to occur due to the stabilized anion formed after treatment with base. To circumvent this issue, alkylation of the bisphosphonate **132** with various alkyl bromides would provide access to a multitude of bisphosphonates. Treatment of bisphosphonate **132** with KHMDS followed by addition of geranyl bromide afforded a dialkylated bisphosphonate **134** and a diastereomeric mixture of the monoalkylated bisphosphonate **135**. After formation of the bisphosphonate **135**, addition of anhydrous benzene was used for azeotropic distillation followed by treatment with base. The resulting carbanion was then treated with diethyl chlorophosphite followed by oxidation. Analysis of the reaction mixture by ^{31}P NMR showed no significant evidence for the formation of the desired chiral trisphosphonate. However, the reaction was attempted on a 100 mg scale, which would mean the smallest amount of water would prevent the phosphonylation of the bisphosphonate.

While scaling up the previous reaction might result in a decreased amount of water trapped in the bisphosphonate, selective hydrolysis would be unlikely to be achieved using ethyl and methyl esters. Therefore, exploration of another possible ester functionality was needed to allow selective cleavage of the esters. Through use of a “prodrug” strategy, selective cleavage could be achieved through chemical and/or enzymatic means. Troutmen and coworkers were able to desymmetrize a tetraPOM methylene bisphosphonate upon

treatment with DABCO followed by oxalyl chloride to form a phosphonyl chloride. Using this approach would allow the synthesis of an asymmetric bisphosphonate, which could undergo selective ester cleavage by hydrolysis of the acetal linkages.

Synthesis of a POM-protected trisphosphonate could be envisioned through the alkylation of the unsymmetrical POM bisphosphonate followed by phosphonylation in order to prepare the desired trisphosphonate. Treatment of tetramethyl methylene-bisphosphonate with NaI and chloromethyl pivalate resulted in the formation of the tetra protected methylene bisphosphonate **138**. One acetal ester was cleaved using a single equivalent of DABCO, and this reaction was followed by addition of oxalyl chloride. As shown earlier, phosphonyl chlorides are easily converted to esters by addition of an alcohol and an amine base. With the phosphonyl chloride **139** in hand, addition of anhydrous ethanol and triethyl amine resulted in formation of a precipitate, which was removed by filteration, and the filtrate was concentrated to give compound **140**. After using the established drying procedure for removal of the trace amounts of water with anhydrous benzene, the bisphosphonate **140** was treated with KHMDS and diethyl chlorophosphite followed by oxidation with H_2O_2 and the reaction material was quenched with acetic acid. Upon analysis of the ^{31}P NMR spectrum, the reaction mixture showed degradation of the starting material and lack of formation of trisphosphonate **141**. Attempts to alkylate the bisphosphonate **140** through reaction with KHMDS and benzyl bromide also were unsuccessful. Upon investigation of the ^{31}P NMR spectrum of the reaction mixture, only starting material was observed. This could mean that the significant steric interactions limit successful alkylation.

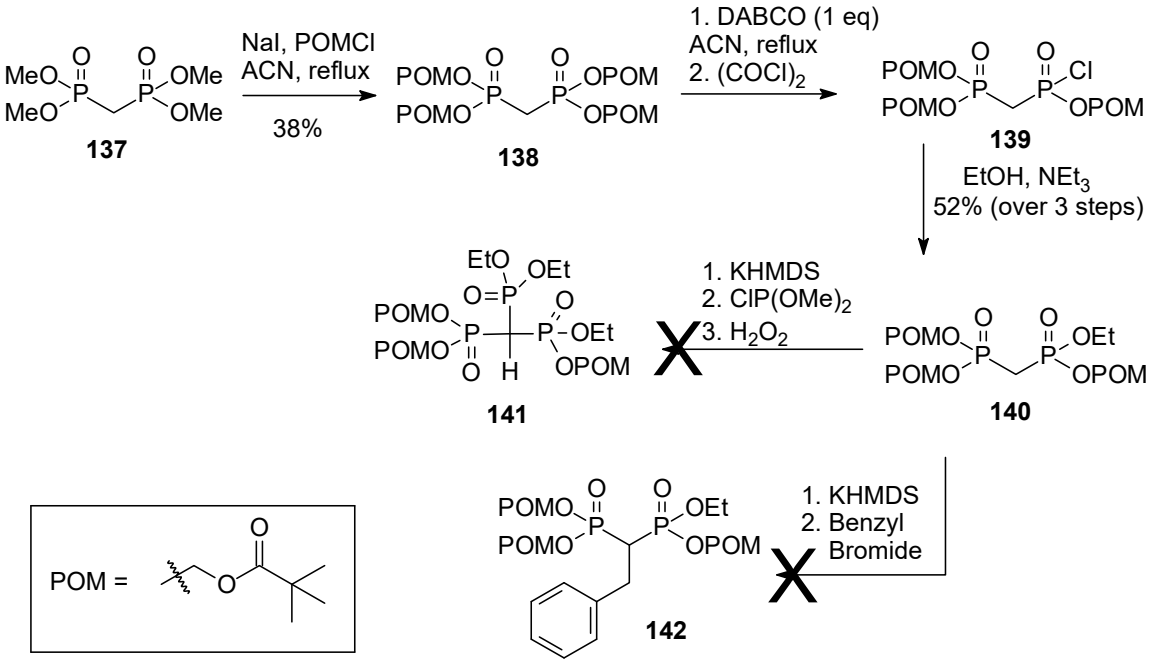


Figure 46. Attempted synthesis of a mixed ester phosphosphonate

Rearrangement of the steps of this reaction sequence might allow formation of the desired chiral trisphosphonate. Therefore tetramethyl methylenebisphosphonate was treated with KHMDS to produce an anion followed by the addition of benzyl bromide to afford benzyl bisphosphonate **143**. Acetal formation through ester exchange, by addition of NaI and chloromethyl pivalate, provided the POM phosphonate ester **144**. Without purification, the acetal ester was allowed to react with a single equivalent of DABCO. Addition of oxalyl chloride allowed the formation of the phosphonyl chloride followed by addition of anhydrous ethanol and triethyl amine to give the compound **146**. Unfortunately upon reaction under conditions utilized by Smits, formation of the trisphosphonate was not observed and only starting material was isolated.[61]

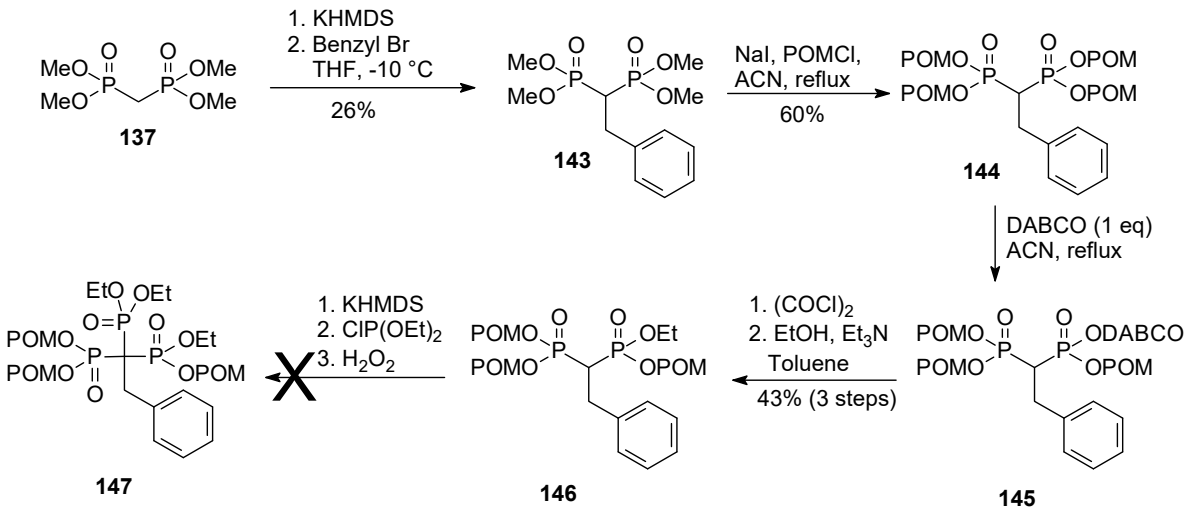


Figure 47. Synthesis of the asymetric bisphosphonate **146**

While previous attempts to synthesis a protected trisphosphonate ester had not proven successful, synthesis of the prodrug trisphosphonate could be accomplished. Using the tetramethyl methylenebisphosphonate **137** as a branch point, conversion to the tetraPOM acetal **149** was easily observed upon treatment with chloromethyl pivalate and NaI. Upon treatment under known conditions,[41] the asymmetric bisphosphonate **150** was readily available from reaction with DABCO followed by treatment with oxalyl chloride. The bisphosphonate **150** then was allowed to undergo reaction with KHMDS, followed by phosphonylation with diethyl chlorophosphite and oxidation. Unfortunately, formation of the trisphosphonate was not observed and the only ^{31}P NMR resonances corresponded to starting material.

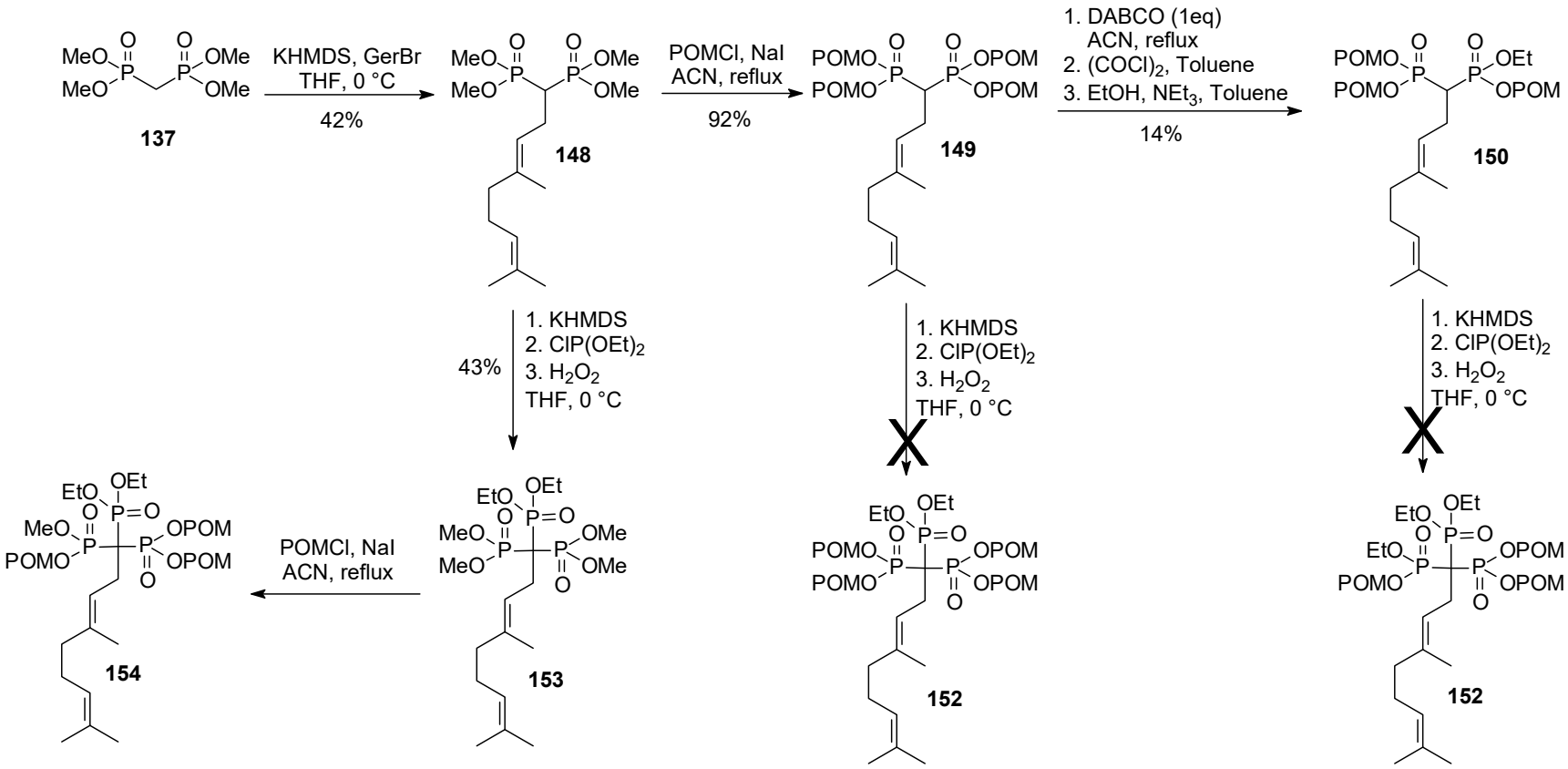


Figure 48. Attempted synthesis of POM geranyltrisphosphonate

Attempts to synthesize trisphosphonate **152** through the tetraPOM ester also were investigated. After azeotropic distillation with anhydrous benzene, phosphonylation was attempted in an argon environment followed by oxidation. However, no observed product was formed and only starting material was isolated. Another effort to synthesize this trisphosphonate used a Schlenk line to limit possible contact with water, yet no formation of product was observed. Synthesis of trisphosphonate **154** was attempted by formation of the trisphosphonate and subsequent installation of the acetal esters. Again using a Schlenk line, azeotropic distillation was first conducted to remove any residual water. Bisphosphonate **148** was treated with KHMDS followed by dropwise addition of diethyl chlorophosphite and subsequent oxidation using H_2O_2 to form the tetramethyl diethyl phosphonate **153**. Using the different rates of ester exchange, POM ester exchange was shown to occur at a faster rate with methyl esters in the model system, tetra ester methylenebisphosphonates. Exchange with the tetramethyl methylenebisphosphonate was evident after 18 hours, while exchange with the tetraethyl methylenebisphosphonate was still incomplete after 1 week under parallel conditions. With the trisphosphonate **143** in hand, it was allowed to react with NaI and chloromethyl pivalate. Upon analysis through liquid chromatography mass spectrometry, the liquid chromatography trace supported formation of the tri-protected POM phosphonate **154** along with the dephosphonylated product **149**.

In conclusion, formation of symmetrical and asymmetrical trisphosphonates was attempted. Methodology to synthesize a family of chiral alkyl 1, 1, 1-trisphosphonates has been developed though the stepwise phosphorylation of alkyl trisphosphonates. The first example of chiral trisphosphonate have been prepared is compound **133**. However this was achieved in low yields, and further exploration to optimize the ester exchange is need to provide a viable method for preparation of chiral trisphosphonates.

CHAPTER 6

SUMMARY AND FUTURE WORK

Several new phosphoantigens have been synthesized and identified as γδ T-cells stimulants. These phosphoantigens have great potential for therapeutic use in expanding the γδ T-cell population. Currently, one γδ T-cell simulant has advanced as far as phase II clinical trials for the treatment of leukemia. In previous research, γδ T-cell have exhibited a response to phosphonic acids, diphosphates, and diphosphonate derivatives. Initially, we were interested in determining how these phosphoantigens simulate γδ T-cell populations by using masked phosphonates. Synthesis of several phosphoantigen prodrugs as pivaloyl oxymethyl ester derivatives has been accomplished, and their activity as T-cell simulants has been established. These POM derivatives have demonstrated activity at nanomolar concentrations for both the bis POM analogue and mono POM mono methyl analogue, which is much greater potency then their phosphonic acids counterparts. The most potent phosphoantigen prodrug, a monoCCOM phosphonate **56**, was able to simulate Vγ9Vδ2 T-cells with an EC_{50} value of 3.9 nM.

Because the previously known phosphoantigens were all phosphonic acids or diphosphate-like derivatives, the SAR of these compounds was pursued as discussed in Chapter 2. These compounds were prepared through allylic oxidation of the protected acetal esters of alkyl and ether phosphoantigens. While phosphonate **36** was shown to stimulate γδ T-cells at high concentrations, this activity might still be increased with the synthesis of an enantiomerically pure version because the rate of ester cleavage might be a function of phosphorus stereochemistry.[68]

The effects of electron withdrawing substituents on phosphoantigens also were explored for their possible impact on γδ T-cell proliferation. Electron withdrawing groups have been shown to decrease the pKa values of some phosphonic acids but the ether series prepare here showed a substantial decrease in activity in our studies. This could be due to the extension of the length between the phosphonate and the gamma hydroxyl

functionality. Keeping the same length while installing an electron withdrawing group, might allow greater activity. Some possibilities are shown in Figure 49.

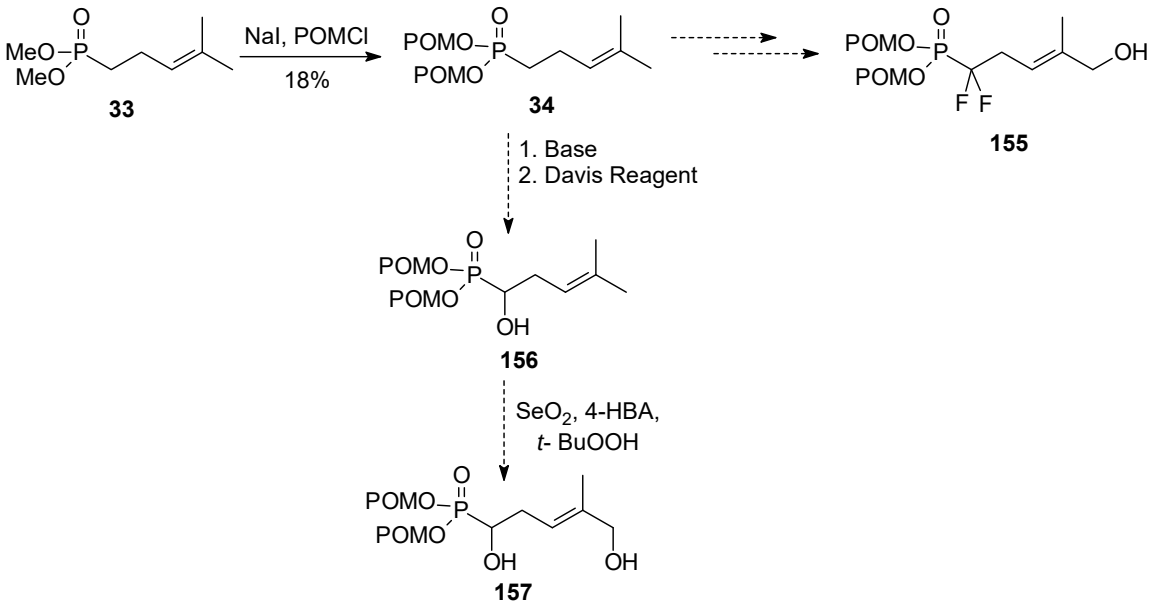


Figure 49. α-Hydroxylation and fluorination of a phosphoantigen

A new set of fluorescent acetal phosphonates was described in Chapter 3. During the synthesis of these derivatives, the new, fluorescent CCOM ester phosphonates were made through reaction of the ammonium salt. The ideal fluorescent analogues should show the same or very similar activity to other prodrug forms. Our collaborators have showed that these new compounds have similar activities to that of the known prodrugs described in Chapter 2, with the lowest EC_{50} = 3.9 nM. Upon exposure to non-specific esterases, this new fluorescence acetal was enzymatically cleaved revealing the active phosphoantigen and a pH sensitive fluorescent coumarin **46**. At physiological pH values, there is a loss of fluorescence at low concentration but upon acidification fluorescence is recovered. This shows that even with a large coumarin oxymethyl ester, cleavage by non-specific esterases can occur and the formation of these new prodrugs is readily achieved from the coumarin carboxylate and chloromethyl chlorosulfate. This new prodrug form also has low toxicity, making it viable option for a possible biological probe. Using this synthesis, other fluorescent coumarin species could be explored in order to enhance the properties with

respect to cellular localization or to further mechanistic studies. With a pH dependent prodrug now available, synthesis of a pH independent prodrug could also be pursued through a conjugate addition of the methyl ketone **160** to extend the chain followed by addition of the chloromethyl group. Modifications to reduce the carboxylic acid to the benzyl alcohol might also maintain fluorescence without decreasing the intensity at physiological pH.

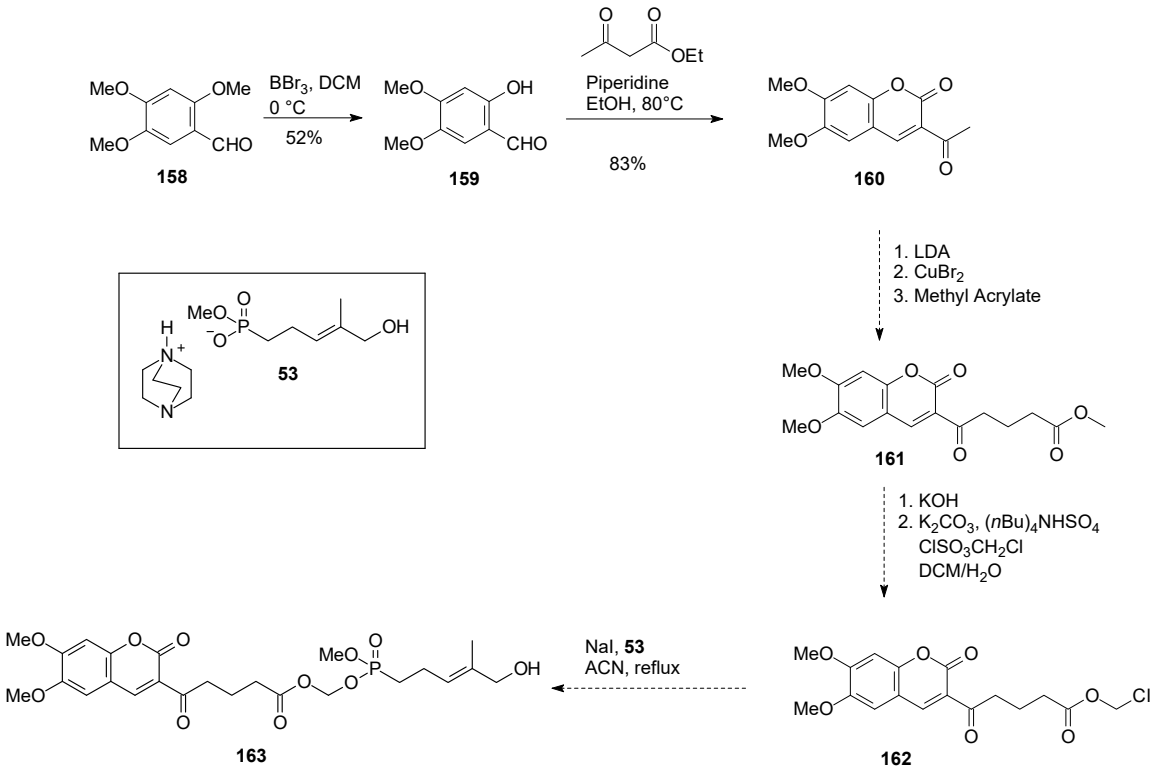


Figure 50. Proposed synthesis of a pH independent prodrug form

Other modifications that were made included protection of the gamma hydroxyl group. Using a pivalate-protected phosphoantigen maintained the potency of this compound with an EC_{50} of 4.7 nM. While complete removal of the gamma hydroxyl group leads to a significant loss of γδ T-cell stimulation, modifications to the gamma hydroxyl group have not been widely investigated. Conversion of the hydroxyl group to a methyl ether and testing the product for T-cell stimulation could determine whether a hydrogen

bond donor or acceptor is needed. This could also lead to investigation of other functional groups that might be tolerated such as an omega aldehyde or carboxylic acid.

A series of phosphinophosphonate phosphoantigens was described in detail in Chapter 4. While the incorporation of prodrugs has increased the activity of the phosphonic acid phosphoantigen, the most potent compound remains HMBPP. Other more potent analogues might involve the incorporation of two phosphorus atoms. During the synthesis of these analogues, conversion of the dimethyl phosphonate to the phosphonyl chloride was achieved in order to form the skeleton of these compounds. Isolation of the phosphonate salts was achieved by addition of anhydrous isopropyl alcohol and acetonitrile to a solution of the compound dissolved in water. While the prodrug forms were more potent than their phosphonic acid counterparts, there was an increased level of toxicity with the phosphinophosphonate. This could be due to the lack of cellular equipment need to metabolize the P-C bonds. While phosphonates increase stability when compared to phosphates, they also could be more difficult to metabolize in cells. These phosphinophosphonates offered insight into how these compounds stimulate γδ T-cells by binding to the intracellular domain and inducing dimerization of the BTN3A1 protein.

Several additional modifications could be done using these compounds as starting materials. As described above, incorporation of an electronegative atom at the α-position could increase the potency. The other modification that could be undertaken is the synthesis of prodrug protected bisphosphonates with a possible isoprenoid side chain as one ester of the phosphonate. While this might circumvent the decomposition of similar compounds as noted in the literature, a late stage incorporation could be envisioned and might allow stimulation data to be collected. Other possible ways to increase the stability would be through the use of a phosphonamide linkage to an isoprenoid side chain.

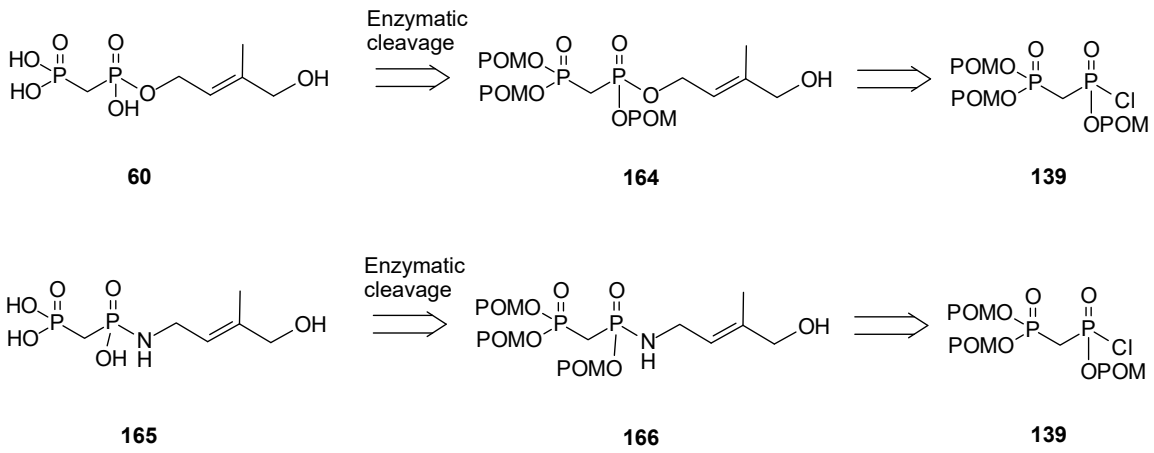


Figure 51. Synthesis of a potential bisphosphonate phosphoantigen

In an effort to increase the chemical space for new bioactive phosphonates, synthesis of symmetric and chiral alkyl 1, 1, 1-trisphosphonates was explored as discussed in detail in Chapter 5. The symmetrical trisphosphonate esters were discussed and a new nitrogenous trisphosphonate was made available through a Michael addition to a vinyl bisphosphonate. Using standard trisphosphonate synthesis conditions, the pyridine trisphosphonate was formed. Investigation of formation of the symmetrical tri-salts was attempted on a geranyltrisphosphonate. Using standard conditions in order form a symmetric bisphosphonate salt, those same condition when applied to this trisphosphonate resulted in decomposition to the bisphosphonate and hydrolysis to phosphate.

Even though difficulties forming symmetric salts were encountered, investigations on the chiral trisphosphonate were begun. While formation of different phosphites was easily achieved, maintaining the chlorophosphites was difficult due to their highly reactive nature. Attempts to form the trisphosphonate by reaction with a dichloro-or trichlorophosphite gave only limited yields. However, formation of the parent asymmetric bisphosphonate was easier to control, allowing use of a commercially available chlorophosphite. With the formation of an asymmetric bisphosphonate, synthesis of the parent trisphosphonate was easily achieved using a Schlenk line to prevent water from interfering with the reaction. While this was also attempted with the POM protected bisphosphonate, only starting material was recovered in this case leading to the belief that

the POM acetal might not be stable to non-nucleophilic base and also may sterically hinder incorporation of the final phosphoryl group. Ester exchange did allow the formation of the desired product along with a decomposed POM geranylbisphosphonate.

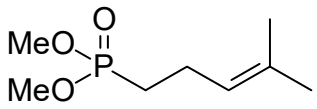
In conclusion, this work reports the synthesis of novel phosphorus containing molecules, including families of phosphoantigens and several trisphosphonates. The γδ T-cells simulation results from our collaborators have revealed some of the new phosphoantigens to be potent agonists of the BTN3A1 protein. These results have directed the synthesis of other potential derivatives in efforts to develop still more active stimulants and elicit the mechanism of action in T-cell simulation. The attempts to synthesize a chiral trisphosphonate have led to a possible route that may allow us to investigate the biological activity of this family of compounds, and provides a foundation to guide the synthesis of analogues of this novel functional group.

CHAPTER 7

EXPERIMENTAL PROCEDURES

General experimental procedures

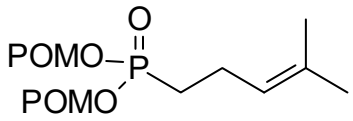
Both diethyl ether (Et_2O) and THF (THF) were freshly distilled from sodium and benzophenonone, while acetonitrile, methylene chloride (CH_2Cl_2), diisopropyl amine, and trimethylamine (Et_3N) were distilled from calcium hydride prior to use. Solutions of *n*–BuLi were purchased from a commercial source and titrated with diphenylacetic acid prior to use. All other reagents and solvents were purchased from commercial sources and used without further purification. All reactions in nonaqueous solvents were conducted in flame-dried glassware under a positive pressure of argon and with magnetic stirring. The NMR spectra were obtained at 300, 400, or 500 MHz for ^1H, and 75, 100, or 125 MHz for ^{13}C, with internal standards of $Si(CH_3)_4$ (^1H, 0.00) or $CDCl_3$ (^1H, 7.27; ^{13}C, 77.0 ppm) for non-aqueous samples or D_2O (^1H, 4.80) and 1,4-dioxane (^{13}C, 66.6 ppm) for aqueous samples. The ^{31}P NMR spectra were obtained at 121, 161, or 202 MHz and were reported in ppm relative to 85% H_3PO_4 (external standard). High resolution mass spectra were obtained at the University of Iowa Mass Spectrometry Facility. Silica gel (60 Å, 0.040 – 0.063 mm) was used for flash chromatography.



Phosphonate 33

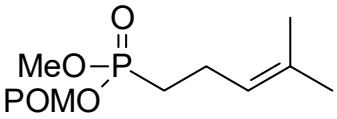
Diisopropyl amine (6.79 mL, 83.0 mmol) was added to THF (250 mL) and the solution was cooled to -78 °C. After *n*-butyl lithium (33.7 mL, 76.1 mmol) was added dropwise, the reaction was allowed to stir for 1 hour. Dimethyl methylphosphonate (7.50 mL, 69.2 mmol) was added dropwise and the reaction mixture was allowed to stir for 1 hour forming a solid. Prenyl bromide (11.3 g, 76.1 mmol) was added dropwise and the resulting solution was allowed to stir overnight. The reaction was quenched by addition of aqueous hydrochloric

acid (1M) and extracted with dichloromethane. The organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (2% EtOH in ether). The desired product was isolated as a clear oil in 51% yield (6.70 g): Both ^{1}H and ^{31}P NMR data were consistent with literature data.[19]



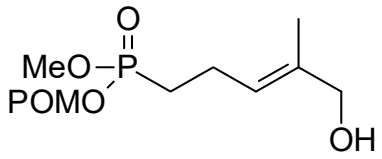
DiPOM phosphonate 34

Dimethyl homoprenylphosphonate **33** (0.99 g, 5.17 mmol), sodium iodide (2.36 mL, 10.6 mmol), and chloromethyl pivalate (2.36 mL, 16.3 mmol) were dissolved in acetonitrile (40 mL) and heated at 80 °C overnight. The solution was quenched by addition of water and extracted with diethyl ether. The organic portions were washed with brine and aqueous sodium thiosulfate, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (20% ether in hexanes). The product phosphonate **34** was isolated as a clear oil in 16% yield (0.33 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.68 (d, J_{PH} = 13.4 Hz, 4H), 5.11-5.04 (m, 1H), 2.35-2.20 (m, 2H), 1.92-1.72 (m, 2H), 1.68 (s, 3H), 1.61 (s, 3H), 1.24 (d, J_{PH} =1.1 Hz, 18H); ^{31}P NMR (121 MHz, $CDCl_3$) +32.7.[19]



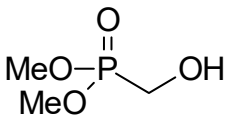
MonoPOM phosphonate 35

The product phosphonate **35** was isolated as a clear oil in 30% yield (0.46 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.67 (d, J_{PH} = 13.4 Hz, 2H), 5.14-5.05 (m, 1H), 3.75 (d, J_{PH} = 11.1 Hz, 3H), 2.36-2.20 (m, 2H), 1.91-1.74 (m, 2H), 1.68 (s, 3H), 1.62 (s, 3H), 1.24 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.6 (s), 134.7 (d, J_{PC} = 1.8 Hz), 122.3 (d, J_{PC} = 17.4 Hz), 81.3 (d, J_{PC} = 5.9 Hz), 51.4 (d, J_{PC} = 7.0 Hz), 38.3 (s), 26.5 (s, 3C), 25.3 (s), 25.0 (d, J_{PC} = 35.1 Hz), 20.2 (d, J_{PC} = 4.8 Hz), 17.3 (s); ^{31}P NMR (121 MHz, $CDCl_3$) +33.7; HRMS (ESI^+) calcd for $C_{13}H_{25}O_5PNa$ [M^+ + Na] 315.1337; found: 315.1337.[19]



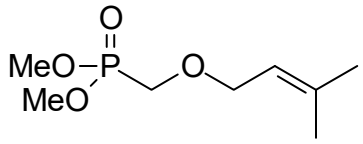
MonoPOM phosphonate 36:

Phosphonate **35** (0.45 g, 1.54 mmol), selenium dioxide (0.09 g, 0.77 mmol), 4-hydroxybenzonic acid (0.02 g, 0.15 mmol), and *t*-butyl hydroperoxide (0.79 mL, 6.15 mmol) were dissolved in dichloromethane (10 mL) and allowed to stirred overnight. The solution was quenched by addition of water and extracted with diethyl ether. The organic portions were washed with brine and aqueous sodium thiosulfate, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (ether). The desired product was isolated as a yellow oil in 32% yield (0.15 g, 43% BRSM): ^{1}H NMR (300 MHz, $CDCl_3$) 5.71-5.61 (m, 2H), 5.44-5.34 (m, 1H), 3.98 (s, 2H), 3.75 (d, J_{PH} = 11.3 Hz, 3H), 2.85 (bs, 1H), 2.38-2.28 (m, 2H), 1.92-1.81 (m, 2H), 1.66 (s, 3H), 1.24 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.98 (s), 136.41 (d, J_{PC} = 1.4 Hz), 122.87 (d, J_{PC} = 16.2 Hz), 81.50 (d, J_{PC} = 6.3 Hz), 67.81 (s), 51.83 (d, J_{PC} = 7.2 Hz), 38.59, 26.75 (s, 3C), 26.62 (d, J_{PC} = 20.3 Hz), 20.28 (d, J_{PC} = 4.8 Hz), 13.52 (s); ^{31}P NMR (121 MHz, $CDCl_3$) +33.5; HRMS (ESI^+) calcd for $C_{13}H_{25}O_6PNa$ [M^+ + Na] 331.1286; found: 331.1291.[19]



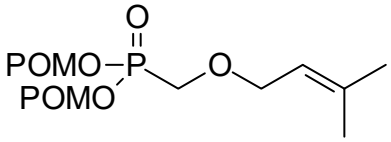
Hydroxymethyl phosphonate 38

Dimethyl phosphonate (9.20 mL, 99.9 mmol), paraformaldehyde (3.00 g, 99.9 mmol), and triethyl amine (1.40 mL, 9.99 mmol) were allowed to reflux at 100 °C for 2 hours. After 2 hours, the reaction mixture was concentrated *in vacuo*. The residue was purified via flash chromatography (ether), and the desired product was isolated as a clear oil in 50% yield (6.95 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.00-3.90 (m, 2H), 3.82 (d, J_{PH} = 10.5 Hz, 6H), 3.54 (bs, 1H); ^{31}P NMR (121 MHz, $CDCl_3$) + 26.3[69]



Ether prenyl phosphonate 39

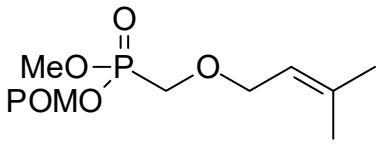
Sodium hydride (0.63 g, 15.70 mmol) was added to THF (100 mL) and the mixture was cooled to 0°C. Dimethyl hydroxymethylphosphonate (**38**, 2.00 g, 14.3 mmol) was added dropwise and the reaction allowed to stir for 30 mins. After 30 mins, prenyl bromide (2.34 g, 15.7 mmol) was added dropwise and the reaction allowed to stir overnight. The solution was quenched by the addition of aqueous ammonium chloride and extracted with ether. The organic portions were dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (5% EtOH in hexanes)[70] and the desired product was isolated as a clear oil in a 29% yield (0.87 g): ^{1}H NMR (500 MHz, D_2O) δ 5.60-5.45 (m, 1H), 4.78-4.72 (m, 6H), 4.16-4.05 (m, 2H), 3.53-3.34 (m, 2H), 1.90-1.80 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.9, 119.8, 69.3 (d, J_{CP} = 12.8 Hz, 2C), 62.5 (d, J_{PC} = 167.6 Hz), 52.9 (d, J_{CP} = 6.5), 25.8, 18.1; ^{31}P NMR (202 MHz, $CDCl_3$) + 24.5; HRMS (ES^+) calcd for $C_8H_{17}O_4PNa$ [M^+ + Na] 231.0762; found; 231.0771.



DiPOM ether phosphonate 40

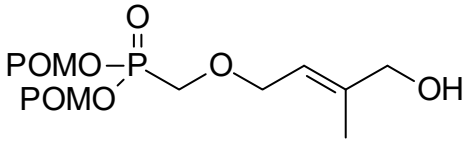
Dimethyl phosphonate **39** (0.83 g, 3.99 mmol), sodium iodide (1.23 g, 8.22 mmol), and chloromethyl pivalate (1.82 mL, 12.6 mmol) were dissolved in acetonitrile (40 mL) and the solution was heat at 80 °C overnight. The solution was quenched by addition of water and extracted with diethyl ether. The organic portions were washed with brine and sodium thiosulfate, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (ether), the product **39** was isolated as a clear oil in 19% yield (0.31 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.71 (d, J_{PH} = 13.2 Hz, 4H), 5.37-5.28 (m, 1H), 4.09 (d, J_{PH} = 7.2 Hz, 2H), 3.70 (d, J = 8.3 Hz, 2H), 1.76 (s, 3H), 1.68 (s, 3H), 1.24 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.8 (2C), 139.0, 119.9, 81.4 (d, J_{PC}

= 6.2 Hz, 2C), 69.1 (d, J_{PC} = 15.4 Hz), 63.3 (d, J_{PC} = 166.6 Hz), 38.5 (2C), 26.6 (6C), 25.6, 17.9; ^{31}P NMR (121 MHz, $CDCl_3$) +22.3; HRMS (ES^+) calcd for $C_{18}H_{33}O_8PNa$ [M^+ + Na] 431.1811; found: 431.1804.



MonoPOM ether phosphonate 41

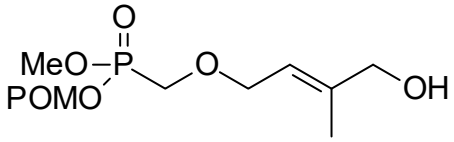
The product **41** was isolated as a clear oil in 4.3% yield (53 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.73-5.64 (m, 2H), 5.33-5.25 (m, 1H), 4.09 (d, J_{PH} =7.2 Hz, 2H), 3.82 (d, J_{PH} = 10.9 Hz, 3H), 3.82 (d, J = 8.6 Hz, 2H), 1.76 (s, 3H), 1.68 (s, 3H), 1.24 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.8, 138.9, 119.7, 81.8 (d, J_{PC} = 6.1 Hz), 69.3 (d, J_{PC} = 13.0 Hz), 63.1 (d, J_{PC} = 166.5 Hz), 52.6 (d, J_{PC} = 7.3 Hz), 38.6, 26.8 (3C), 25.8, 18.0; ^{31}P NMR (121 MHz, $CDCl_3$) + 23.5; HRMS (ES^+) calcd for $C_{13}H_{25}O_6PNa$ [M^++ Na] 331.1286; found: 331.1284.



Allylic alcohol 42

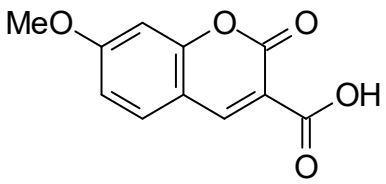
Phosphonate **40** (0.307 g, 0.75 mmol), selenium dioxide (4 mg, 0.38 mmol), 4-hydroxybenzonic acid (0.01 g, 0.08 mmol), and *t*-butyl hydroperoxide (0.49 mL, 3.00 mmol) were dissolved in dichloromethane (5 mL) and the reaction was allowed to stirred overnight. The solution was quenched by addition of water and extracted with diethyl ether. The organic portions were washed with brine and aqueous sodium thiosulfate, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (5% EtOH in ether) to give the desired product as a clear oil in a 5% yield (0.014 g): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.76-5.67 (m, 4H), 5.63-5.57 (m, 1H), 4.19 (d, J_{PH} = 5.9 Hz, 2H), 4.06 (s, 2H), 3.84 (d, J = 7.2 Hz, 2H), 1.72 (s, 3H), 1.25 (s, 18H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 176.9 (2C), 140.9, 119.7, 81.6 (d, J_{PC} = 6.4 Hz, 2C), 77.2, 69.0 (d, J_{PC} = 11.6 Hz), 63.8 (d, J_{PC} = 163.8 Hz), 38.7 (2C), 26.8 (6C), 13.9; ^{31}P NMR

(202 MHz, $CDCl_3$) + 21.9; HRMS (ES^+) calcd for $C_{18}H_{33}O_9PNa$ [M^++ Na] 447.1760; found: 447.1776.



Allylic alcohol 43

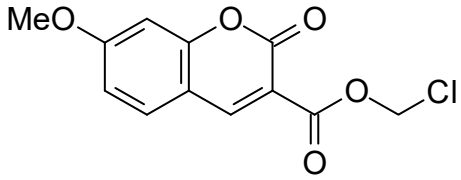
Phosphonate **41** (0.052 g, 0.17 mmol), selenium dioxide (0.009 g, 0.08 mmol), 4-hydroxybenzonic acid (0.002 g, 0.017 mmol), and *t*-butyl hydroperoxide (0.11 mL, 0.68 mmol) were dissolved in dichloromethane (5 mL) and the reaction was allowed to stirred overnight. The solution was quenched by addition of water and extracted with diethyl ether. The organic portions were washed with brine and aqueous sodium thiosulfate, dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (5% EtOH in ether). The desired product was isolated as a clear oil in 17% yield (9 mg): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.77-5.67 (m, 2H), 5.65-5.60 (m, 2H), 4.20 (d, J_{PH} = 6.1 Hz, 2H), 4.07 (s, 2H), 3.86-3.82 (m, 5H), 1.72 (s, 3H), 1.25 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.0, 140.9, 119.8, 81.9 (d, J_{PC} = 6.7 Hz), 77.2, 69.1 (d, J_{PC} = 12.5 Hz), 68.4(d, J_{PC} = 152.8 Hz), 52.8 (d, J_{PC} =7.8 Hz), 38.7, 26.9 (3C), 13.9; ^{31}P NMR (202 MHz, $CDCl_3$) + 22.0; HRMS (ES^+) calcd for $C_{13}H_{25}O_7PNa$ [M^++ Na] 347.1236; found: 347.1235.



Methoxy courmarin 46

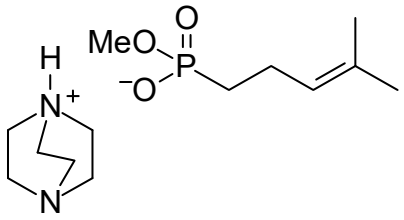
2–Hydroxy–4–methyoxybenzaldehyde (2.02 g, 13.3 mmol), Meldrum's acid (2.11 g, 13.6 mmol) and piperdinium acetate (39 mg, 0.27 mmol) were dissolved in ethanol. The solution was heated at reflux for 2.5 hours. The solution was then cooled to 0 °C forming a precipitate. The precipitate was isolated by filtration and recrystallized from hot ethanol.

The product was filtered to give a light yellow solid in 82% yield (2.4 g): The ^{1}H NMR data was consistent with literature data. [36]



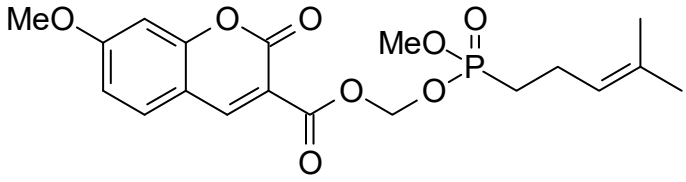
Chloromethyl coumarin 47

7-Methoxy-2-oxo-2H-chromene-3-carboxylic acid (1.10 g, 5.02 mmol) and KOH (0.28 g, 5.02 mmol) were dissolved in ethanol and the solution was heated at reflux for 4 hours. After the solution was concentrated *in vacuo*, the resulting solid was dissolved in water (20 mL) and dichloromethane (20 mL) and cooled to 0 °C. Potassium carbonate (6.93 g, 50.0 mmol), tetrabutylammonium hydrogen sulfate (0.41 g, 1.21 mmol), and chloromethyl chlorosulfate (1.52 mL, 15.1 mmol) were added. The solution was stirred vigorously for 10 mins and then allowed to stir for 48 hr. The organic portion was separated and the aqueous layer was extracted with dichloromethane. The organic portions were combined and washed with brine, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (8% EtOH in dichloromethane) [71] to give the product in 80% yield (1.1 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (s, 1H), 7.55 (d, J = 8.8 Hz, 1H), 6.93 (dd, J = 8.8, 2.4 Hz,1H), 6.83 (d, J = 2.4 Hz, 1H), 5.95 (s, 2H), 3.93 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 165.4, 160.6, 157.3, 155.7, 151.0, 132.0, 113.4, 111.2, 111.0, 100.2, 69.8, 56.2; HRMS(ES^+) calcd for $C_{12}H_9O_5ClNa$ [M^++ Na] 291.0036; found: 291.0038.[40]



Ammonium salt 48

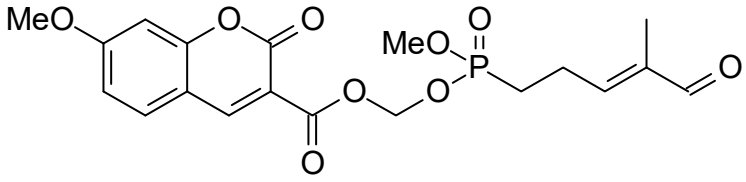
(5-Hydroxy-4-methyl-pent-3-enyl)-phosphonic acid dimethyl ester (**33**) (0.18 g, 0.87 mmol) and 1,4-diazabicyclo [2.2.2] octane (0.09 g, 0.87 mmol) were dissolved in acetonitrile and the reation mixture was heated at reflux overnight. The solution was concentrated in vacuo and used without further purification, giving the desired product as a brown residue in 61% yield (0.17 g): ^{1}H NMR (400 MHz, D_2O) δ 5.44-5.38 (m, 1H), 3.89 (s, 2H), 3.46 (d, J_{PH} = 10.5 Hz, 3H), 3.33–3.30 (m, 6H), 3.15–3.10 (m, 6H), 2.19–2.15 (m, 2H), 1.61–1.53 (m, 5H); ^{13}C NMR (125 MHz, D_2O) δ 135.3, 127.1 (d, J_{PC} = 16.6 Hz), 68.1, 54.6, 54.6, 54.6, 52.2, 52.2, 52.1, 51.6 (d, J_{PC} = 5.4 Hz), 25.8 (d, J_{PC} = 134.0 Hz), 21.8 (d, J_{PC} = 4.8 Hz), 12.9; ^{31}P NMR (161 MHz, $CDCl_3$) + 29.3; HRMS(ES^-) calcd for $C_7H_{14}O_4P$ [M^-- DABCO] 193.0641; found: 193.0630



MonoCCOM phosphonate 49

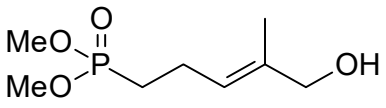
Ammonium salt **48** (170 mg, 0.53 mmol), sodium iodide (0.12 g, 0.80 mmol), and 3-(2-chloro-acetyl)-7-methoxy-chromen-2-one (0.21 g, 0.80 mmol) were dissolved in acetonitrile (5 mL). The solution was heated at reflux overnight, and then quenched by addition of brine and extracted with dichloromethane. The combined organic portions were washed with saturated $Na_2S_2O_3$, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (5% EtOH in dichloromethane) to give the product as a white solid in 63% yield (142 mg): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.59 (s, 1H), 7.51 (d, *J* = 8.6 Hz, 1H), 6.88 (dd, *J* = 8.6 Hz, 2.2 Hz, 1H), 6.78 (d, *J* = 2.2 Hz, 1H), 5.84 (d, J_{PH} = 13.1 Hz, 2H), 5.39–5.37 (m, 1H), 3.93 (s, 2H),

3.89 (s, 3H), 3.74 (d, J_{PH} = 10.9 Hz, 3H), 2.41 (br s, 1H), 2.28–2.35 (m, 2H), 1.86–1.93 (m, 2H), 1.60 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.8, 161.7, 157.8, 156.5, 150.4, 136.4 (d, J_{PC} = 1.5 Hz), 131.1 (d, J_{PC} = 15.9 Hz), 122.5 (d, J_{PC} = 17.2 Hz), 113.9, 112.0, 111.4, 100.3, 82.2 (d, J_{PC} = 6.0 Hz) 67.9, 56.0, 52.0 (d, J_{PC} = 7.1 Hz), 25.5 (d, J_{PC} = 139.4 Hz), 20.3 (d, J_{PC} = 4.9 Hz), 13.5; ^{31}P NMR (202 MHz, $CDCl_3$) + 33.8; HRMS (ES^+) calcd for $C_{19}H_{24}O_9P$ [$(M + H)^+$] 427.1158; found 427.1164.



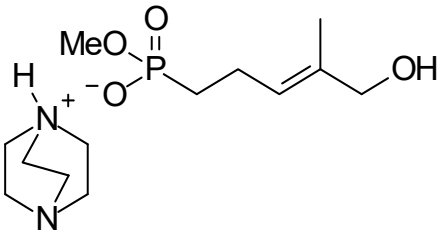
Aldehyde 50

Phosphonate **49** (0.16 g, 0.38 mmol), selenium dioxide (0.042 g, 0.38 mmol), *p*–hydroxybenzonic acid (0.026 g, 0.19 mmol), *tert*–Butyl hydroperoxide (0.58 mL, 1.53 mmol) were added to dichloromethane (5 mL) and the reaction was allowed to stir overnight. The solution was quenched by the addition of brine and extracted with dichloromethane. The organic portions were washed with saturated $Na_2S_2O_3$, dried (Na_2SO_4), and filtered and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (5% EtOH in dichloromethane) and the product was isolated as a white solid in 19% yield (30 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.40 (s, 1H), 8.62 (s, 1H), 7.54 (d, J = 8.8 Hz, 1H), 6.92 (dd, J = 8.8, 2.4 Hz, 1H), 6.82 (d, J = 2.4 Hz, 1H), 6.52-6.46 (m, 1H), 5.95-5.87 (m, 2H), 3.92 (s, 3H), 3.81 (d, J_{PH} = 11.3 Hz, 3H), 2.67 (m, 2H), 2.10 (m, 2H), 1.73 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.9, 165.9, 162.0, 167.9, 156.5, 151.4 (d, J_{PC} = 15.3 Hz), 150.7, 140.0, 131.1, 114.0, 112.1, 111.4, 100.4, 82.6 (d, J_{PC} = 6.1 Hz), 56.1, 52.3 (d, J_{PC} = 7.3 Hz), 24.6 (d, J_{PC} = 141.9 Hz), 21.9 (d, J_{PC} = 4.7 Hz), 9.1; ^{31}P NMR (161 MHz, $CDCl_3$) + 32.4; HRMS (ES^+) calcd for $C_{19}H_{21}O_9PNa$ [M^+ + Na] 447.0821; found: 447.0816.



Allylic alcohol 52

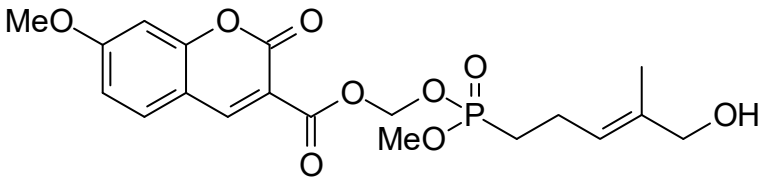
Phosphonate **33** (0.96 g, 4.98 mmol), selenium dioxide (0.28 g, 2.49 mmol), *tert*–butyl hydroperoxide (3.23 mL, 9.95 mmol), and *p*–hydroxybenzonic acid (0.07g, 0.49 mmol) were added to dichloromethane (10 mL) and the solution was allowed to stir overnight. The reaction was quenched by addition of brine and extracted with dichloromethane. The organic portions were washed with saturated $Na_2S_2O_3$, dried (Na_2SO_4), and filtered, and the filtrate was concentrated *in vacuo* and used without further purification. The residue was dissolved in anhydrous dichloromethane and cooled to -78 °C. Diisobutylaluminium hydride (2.22 mL, 12.5 mmol) was added dropwise and the reaction mixture was allowed to stir. After 30 mins, the solution was quenched by addition of aqueous sodium potassium tartrate and allowed to stir overnight. The layers were separated and extracted with dichloromethane. The organic portions were combined, dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (5% EtOH in ether). The product was isolated as a clear oil in 17% yield (0.18 g). The ^{1}H and ^{31}P NMR data were consistent with literature data. [19]



Ammonium salt 53

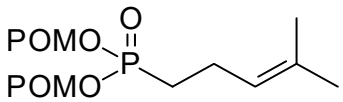
Phosphonate **52** (0.18 g, 0.87 mmol) and 1,4-diazabicyclo[2.2.2]octane (0.09 g, 0.87 mmol) were dissolved in acetonitrile and the reaction mixture was heated at reflux overnight. The solution was concentrated, giving the desired product as a brown residue in 61% yield (0.17 g): ^{1}H NMR (400 MHz, D_2O) δ 5.44-5.38 (m, 1H), 3.89 (s, 2H), 3.46 (d, J_{PH} = 10.5 Hz, 3H), 3.33–3.30 (m, 6H), 3.15–3.10 (m, 6H), 2.19–2.15 (m, 2H), 1.61–1.53 (m, 5H); ^{13}C NMR (125 MHz, D_2O) δ 135.3, 127.1 (d, J_{PC} = 16.6 Hz), 68.1, 54.6, 54.6,

54.6, 52.2, 52.2, 52.1, 51.6 (d, J_{PC} = 5.4 Hz), 25.8 (d, J_{PC} = 134.0 Hz), 21.8 (d, J_{PC} = 4.8 Hz) 12.9; ^{31}P NMR (161 MHz, $CDCl_3$) + 29.3; HRMS(ES^-) calcd for $C_7H_{14}O_4P$ [M^--DABCO] 193.0641; found: 193.0630.[40]



CCOM prodrug 54

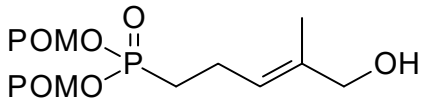
The phosphonic salt **53** (170 mg, 0.53 mmol), sodium iodide (0.12 g, 0.80 mmol), and 7–methoxy–2–oxo–2H–chromene–3–carboxy chloromethyl ester (0.21 g, 0.80 mmol) were dissolved in acetonitrile (5 mL). The solution was heated at reflux overnight, and then quenched by the addition of brine and extracted with dichloromethane. The combined organic portions were washed with saturated $Na_2S_2O_3$, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (5% EtOH in dichloromethane) to give the product was isolated as a white solid in 63% yield (142 mg): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.59 (s, 1H), 7.51 (d, J = 8.6 Hz, 1H), 6.88 (dd, J = 8.6 Hz, 2.2 Hz, 1H), 6.78 (d, J = 2.2 Hz, 1H), 5.84 (d, J_{PH} = 13.1 Hz, 2H), 5.39–5.37 (m, 1H), 3.93 (s, 2H), 3.89 (s, 3H), 3.74 (d, J_{PH} = 10.9 Hz, 3H), 2.41 (br s, 1H), 2.35-2.28 (m, 2H), 1. 93–1.86 (m, 2H), 1.60 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.8, 161.7, 157.8, 156.5, 150.4, 136.4 (d, J_{PC} = 1.5 Hz), 131.1 (d, J_{PC} = 15.9 Hz), 123.1 (d, J_{PC} = 15.8 Hz), 113.9, 112.0, 111.4, 100.3, 82.2 (d, J_{PC} = 6.0 Hz) 67.9, 56.0, 52.0 (d, J_{PC} = 7.1 Hz), 25.5 (d, J_{PC} = 139.4 Hz), 20.3 (d, J_{PC} = 4.9 Hz), 13.5; ^{31}P NMR (202 MHz, $CDCl_3$) + 33.8; HRMS (ES^+) calcd for $C_{19}H_{24}O_9P$ [$(M + H)^+$] 427.1158; found 427.1164.[40]



DiPOM phosphonate 34

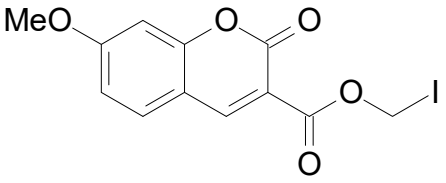
Dimethyl homoprenylphosphonate (1.02 g, 5.29 mmol), sodium iodide (1.63 g, 10.9 mmol), and chloromethyl pivalate (2.43 mL, 16.7 mmol) were dissolved in acetonitrile (5 mL) and the solution was heated at reflux overnight. The reaction was quenched by

addition of water and extracted with diethyl ether. The combined organic portions were washed with $Na_2S_2O_3$, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (1:1 Et_2O/ hexanes) to give the product as a clear oil in 57% yield (1.17 g). Both the ^1H and ^{31}P NMR data were consistent with literature data. [19]



Allylic alcohol 25

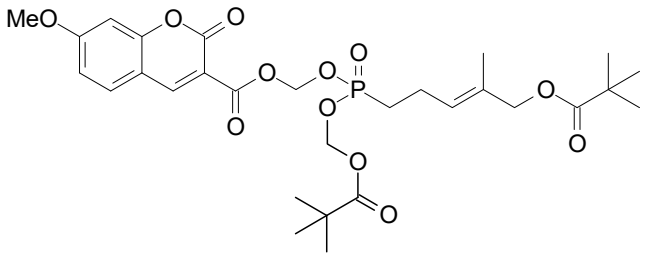
BisPOM phosphonate (60 mg, 1.52 mmol), selenium dioxide (80 mg, 0.76 mmol), *p*–hydroxybenzonic acid (100 mg, 0.76 mmol), *tert*–butylhydroperoxide (0.74 mL, 4.56 mmol) were added to dichloromethane (10 mL). The reaction mixture was cooled to 0 °C and allowed to stir overnight. The reaction was quenched by addition of brine and extracted with dichloromethane. The organic portions were washed with saturated $Na_2S_2O_3$, dried (Na_2SO_4), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (ether) to afford the product as a yellow oil in 44% yield (270 mg). The ^1H and ^{31}P NMR data were consisted with literature data. [19]



Iodomethyl coumarin 58

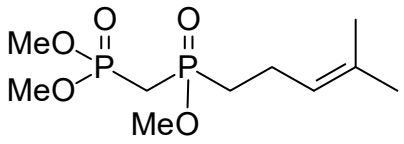
Chloromethyl ester **47** (0.100 g, 0.37 mmol) and sodium iodide (0.17 g, 1.12 mmol) were dissolved in acetone (2 mL) and the reaction mixture was heated at reflux overnight. The reaction mixture was filtered and concentrated giving a white solid. The solid was purified by column chromatography (8% EtOH in dichloromethane) and the product was isolated as a white solid in 75% yield (0.10 g): ^1H NMR (400 MHz, $CDCl_3$) δ 8.55 (s, 1H), 7.53 (d, J = 9.1 Hz, 1H), 6.91 (dd, J = 9.1, 2.5 Hz, 1H), 6.80 (d, J = 2.5 Hz, 1H), 6.14 (s, 2H), 3.92 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.9 (2C), 157.9, 150.5, 131.2, 113.9, 112.2, 111.4

(2C), 100.4, 56.0, 30.8; HRMS (ES^+) calcd for $C_{12}H_9IO_5$ [M^++H] 360.9573; found 360.9588.



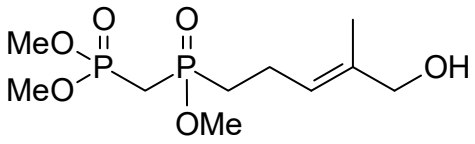
Triprodrug Phosphonate 57

BisPOM phosphonate **25** (66 mg, 0.161 mmol) and 1, 4-diazabicyclo[2.2.2]octane (18 mg, 0.161 mmol) were dissolved in acetonitrile and the reaction mixture was heated at reflux for 4 days until the reaction was complete. The solution was concentrated, giving a brown residue. The residue was purified by ion exchange chromatography (Na^+ form Dowex, H_2O) and the product was isolated as a clear oil and used without further purification. The iodomethyl ester **58** (0.10 g, 1.56 mmol) and phosphonate salt (56 mg, 0.18 mmol) were dissolved in acetonitrile (10 mL) and the reaction was heated at reflux overnight. The reaction mixture was concentrated and purified by column chromatography (8% EtOH in dichloromethane) and the product was isolated as a white solid in 49% yield (49 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (s, 1H), 7.56 (d, J = 8.9 Hz, 1H), 6.92 (dd, J = 8.9 Hz, 2.4 Hz, 1H), 6.83 (d, J = 2.4 Hz, 1H), 5.90–5.87 (m, 2H), 5.76–5.66 (m, 2H), 5.44–5.39 (m, 1H), 4.43 (s, 2H), 3.93 (s, 3H), 2.41–2.31 (m, 2H), 2.01-1.91 (m, 2H),1.65 (s, 3H), 1.58 (br s,1H), 1.25 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.2, 176.9, 165.8, 161.6, 157.9, 156.5, 150.5, 132.2 (d, J_{PC} = 1.9 Hz), 131.2, 126.1 (d, J_{PC} = 19.1), 113.9, 112.2, 111.5, 100.4, 81.8 (d, J_{PC} = 6.8 Hz), 81.4 (d, J_{PC} = 6.8 Hz), 69.2, 56.1, 38.8, 38.7, 27.2 (3C), 26.8 (3C), 26.2 (d, J_{PC} = 140.1 Hz), 20.3 (d, J_{PC} = 4.7 Hz), 13.8; ^{31}P NMR (161 MHz, $CDCl_3$) δ 32.4; HRMS (ES^+) calcd for $C_{29}H_{39}O_{12}PNa$ [M^+ + Na] 633.2077 found 633.2083.



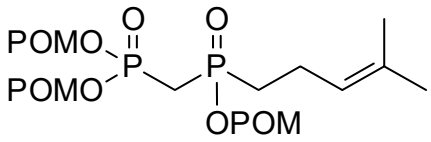
Phosphinophosphonate 67

(4-Methyl-pent-3-enyl)-phosphonic acid dimethyl ester (**33**) (0.59 g, 3.11 mmol) and oxalyl chloride (0.80 mL, 9.35 mmol) were dissolved in anhydrous dichloromethane (20 mL) and dimethylformamide (0.02 mL) and the solution was cooled to 0 °C. After the reaction mixture was stirred overnight, the solution was concentrated *in vacuo* and was used without further purification.[1] To a stirred solution of *n*–BuLi (6.11 mL, 15.2 mmol) in toluene at -78 °C, dimethyl methylphosphonate (1.69 mL, 15.2 mmol) was added dropwise. The resulting solution was allowed to stir for 30 min and methyl homoprenyl phosphonic chloride **69** (3.11 mmol) was added dropwise. The reaction temperature was held at - 78 °C for 1 hour and then allowed to warm unassisted while it stirred overnight. The solution was quenched by addition of aqueous NH_4Cl and extracted with dichloromethane. The combined organic portions were dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (10% EtOH in hexanes) and the product was isolated as a clear oil in 58% yield (0.52 g): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.12–5.06 (m, 1H), 3.78 (d, J_{PH} = 10.1 Hz, 6H), 3.74 (d, J_{PH} = 11.3 Hz, 3H), 2.44–2.34 (m, 2H), 2.33–2.22 (m, 2H), 1.99–1.90 (m, 2H), 1.66 (s, 3H), 1.61 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 133.1 (d, J_{PC} = 1.4 Hz), 122.6 (d, J_{PC} = 15.7 Hz), 52.9 (t, J_{PC} = 6.2 Hz, 2C), 51.4 (d, J_{PC} = 6.8 Hz), 29.2 (d, J_{PC} = 97.9 Hz), 26.2 (dd, J_{PC} = 135.6, 76.5 Hz), 25.5, 20.1 (d, J_{PC} = 4.2 Hz), 17.6; ^{31}P NMR (202 MHz, $CDCl_3$) δ +48.2 (d, J_{PP} = 5.0 Hz), +22.8 (d, J_{PP} = 5.0 Hz); HRMS (ES^+) calcd for $C_{10}H_{23}O_5P_2$ [M^+ +H] 285.1021; found 285.1021.



Trimethyl phosphinophonate 70

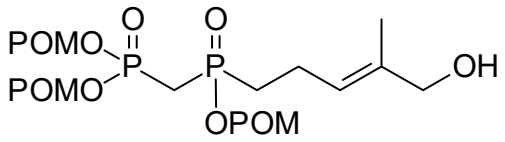
Phosphonate **67** (0.47 g, 1.66 mmol), selenium dioxide (0.14 g, 1.24 mmol), and *tert*-butylhydroperoxide (1.07 mL, 6.64 mmol) were dissolved in CH_2Cl_2 (10 mL) and the solution was allowed to stir overnight. The reaction was quenched by addition of brine and extracted with diethyl ether. The combined organic portions were washed with $Na_2S_2O_3$, dried ($NaSO_4$), and filtered, and the filtrated was concentrated *in vacuo*. The resulting oil was added to a solution of $NaBH_4$ (0.13 g, 3.31 mmol) in methanol (5 mL). After 2 hours, the reaction was concentrated *in vacuo* and then quenched by addition of NH_4Cl and extracted with ether. The combined organic portions were dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (20% EtOH in hexanes) and the product was isolated as a clear oil in 9% yield (42 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.48–5.42 (m, 1H), 3.99 (s, 2H), 3.82 (d, J_{PH} = 11.4 Hz, 3H), 3.81 (d, J_{PH} = 11.4 Hz, 3H), 3.76 (d, J_{PH} = 11.0 Hz, 3H), 2.48–2.34 (m, 2H), 2.09–1.98 (m, 2H), 1.69 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.5 (d, J_{PC} = 1.1 Hz), 123.5 (d, J_{PC} = 14.1 Hz), 68.1, 53.0 (d, J_{PC} = 6.6 Hz, 2C), 51.4 (d, J_{PC} = 6.9 Hz), 28.9 (d, J_{PC} = 98.3 Hz), 26.3 (dd, J_{PC} = 135.5, 75.9 Hz), 19.8 (d, J_{PC} = 4.2 Hz), 13.6; ^{31}P NMR (161 MHz, $CDCl_3$) δ +48.1 (d, J_{PP} = 4.4 Hz, 1P), +22.7 (d, J_{PP} = 4.4 Hz, 1P); HRMS (ES^+) calcd for $C_{10}H_{22}O_6P_2$ [M^+ + Na] 323.0789; found 323.0787.



Prodrug 71

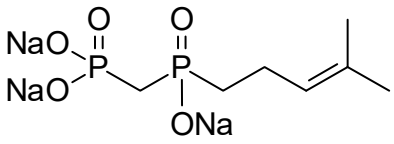
Trimethyl phosphinophosphonate **67** (0.52 g, 1.82 mmol), sodium iodide (1.09 g, 7.30 mmol), and chloromethyl pivalate (1.06 mL, 7.30 mmol) were dissolved in acetonitrile (2 mL) and the solution was heated at reflux overnight. The reaction was quenched by

addition of water and extracted with diethyl ether. The combined organic portions were washed with $Na_2S_2O_3$, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (40% EtOAc in hexanes) and the product was isolated as a clear oil in 48% yield (0.51 g): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.76–5.66 (m, 6H), 5.12–-5.09 (m, 1H), 2.69–-2.52 (m, 2H), 2.40–2.25 (m, 2H), 2.10–1.95 (m, 2H), 1.68 (s, 3H), 1.63 (s, 3H), 1.24 (s, 27H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.0, 176.8 (2C), 133.6, 122.3 (d, J_{PC} = 16.1 Hz), 81.9 (d, J_{PC} =5.6 Hz, 2C), 81.0 (d, J_{PC} = 5.6 Hz), 38.7 (2C), 38.7, 30.3 (d, J_{PC} = 147.5 Hz), 29.8 (dd, J_{PC} = 84.5, 41.0 Hz), 26.9 (3C), 26.8 (6C), 25.6, 19.9 (d, J_{PC} = 3.5 Hz), 17.7; ^{31}P NMR (202 MHz, $CDCl_3$) δ +48.9-49.1 (br s), +19.5 (d, J_{PP} = 3.4 Hz); HRMS (ES^+) calcd for $C_{25}H_{47}O_{11}P_2$ [M^+ + H] 585.2594; found 585.2599.



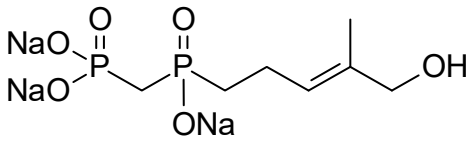
Allylic alcohol 72

Phosphonate **71** (0.51 g, 0.88 mmol), selenium dioxide (49 mg, 0.44 mmol), *p*-hydroxybenzonic acid (60 mg, 0.44 mmol), and *tert*-butylhydroperoxide (0.30 mL, 2.64 mmol) were dissolved in CH_2Cl_2 (5 mL) and the solution was allowed to stir overnight. The reaction was quenched by addition of brine and extracted with diethyl ether. The combined organic portions were washed with $Na_2S_2O_3$, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (5% EtOH in ether) and the product was isolated as a clear oil in 34% yield (179 mg, 45% yield BRSM): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.77–5.63 (m, 6H), 5.45–5.40 (m, 1H), 3.98 (s, 2H), 3.58 (br s, 1H), 2.69–2.50 (m, 2H), 2.46–2.31 (m, 2H), 2.12–2.02 (m, 2H), 1.67 (s, 3H), 1.23 (s, 27H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.0, 176.9 (d, J_{PC} = 4.1 Hz, 2C), 136.8, 123.1 (d, J_{PC} = 14.3 Hz), 81.8 (d, J_{PC} = 5.3 Hz, 2C), 80.9 (d, J_{PC} = 6.3 Hz), 68.2, 38.7–38.6 (m, 3C), 30.0 (d, J_{PC} = 96.7 Hz), 29.1 (dd, J_{PC} = 135.2 Hz, 74.9 Hz), 26.8 (3C), 26.8 (6C), 19.6 (d, J_{PC} = 4.4 Hz), 13.6; ^{31}P NMR (201 MHz, $CDCl_3$) δ +48.4 (d, J_{PP} = 4.0 Hz), +19.3 (d, J_{PP} = 4.0 Hz); HRMS (ES^+) calcd for $C_{25}H_{46}O_{12}P_2$ [M^+ + Na] 623.2362; found 623.2372.



Trisalt 73

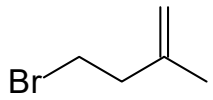
To a solutions of 2, 4, 6-collidine (0.17 mL, 1.31 mmol) in CH_2Cl_2 at 0° C was added TMSBr (0.26 mL, 1.96 mmol) and phosphonate **67** (92 mg, 0.32 mmol) and the solution was allowed to stir overnight. The volatiles were removed and toluene was added then removed *in vacuo*. The resulting residue was treated with NaOH (3 M, 0.33 mL, 0.98 mmol) and the solution was stirred overnight. The reaction mixture was dried on a lyophilizer to obtain a residue which was dissolved in a small amount of water and precipitated by addition of a 2-propanol/ acetonitrile mixture (1:1). The mother liquor was concentrated *in vacuo* to give the desired product as a white solid in 91% yield (91 mg): ^{1}H NMR (400 MHz, D_2O) δ 5.31–5.24 (m, 1H), 2.20 (br s, 2H), 2.00– 1.82(m, 2H), 1.80–1.67 (m, 5H), 1.65 (s, 3H); ^{13}C NMR (100 MHz, D_2O) δ 133.2, 125.1 (d, J_{PC} = 14.3 Hz), 32.2–30.0(m), 24.8, 24.2 (d, J_{PC} = 111.0 Hz), 20.6, 16.9; ^{31}P NMR (161 MHz, D_2O) δ +40.4 (s) +12.6 (s); HRMS (ES^-) calcd for $C_7H_{15}O_{12}P_2$ [M^- - H] 241.0395; found 241.0404.



Allylic alcohol 74

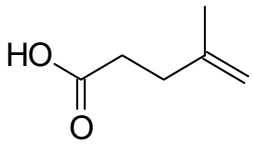
To a solution of 2, 4, 6-collidine (36 μL, 0.27 mmol) in CH_2Cl_2 at 0° C was added TMSBr (54 μL, 0.42 mmol) and phosphonate **70** (21 mg, 0.07 mmol). The solution was allowed to stir overnight at ambient temperture. The volatiles were removed and toluene was added then removed *in vacuo*. The resulting residue was treated with NaOH (1M, 36 μL, 0.27 mmol) and the solution was stirred overnight. The reaction mixture was dried on a lyophilizer to obtain a residue which was dissolved in a small amount of water and precipitated by addition of a 2-propanol/ acetonitrile mixture (1:1). The mother liquor was concentrated *in vacuo* to give the desired product as a white solid in 40% yield (9 mg): ^{1}H NMR (500 MHz, D_2O) δ 5.56–5.51 (m, 1H), 3.99 (s, 2H), 2.35–2.25 (m, 2H), 2.05–1.95

(m, 2H), 1.85–1.75 (m, 2H), 1.70 (s, 3H); ^{13}C NMR (125 MHz, D_2O) δ 133.7, 126.7 (d, J_{PC} = 16.3 Hz), 67.0, 32.3–27.3 (m, 2C), 19.8–19.7 (m), 12.4; ^{31}P NMR (202 MHz, D_2O) δ +39.2, +13.6; HRMS (ES^+) calcd for $C_7H_{15}O_6P_2$ [M^-] 257.0344; found 257.0357.



Bromide 77

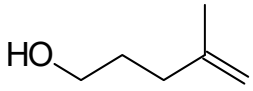
3-Methyl-3-buten-1-ol (10.0 mL, 99.0 mmol) and trimethylamine (21.0 mL, 148.6 mmol) were dissolved in DCM (200 mL) and the resulting solution was cooled to 0 °C. Methanesulfonyl chloride (11.6 mL, 148.6 mmol) was added dropwise and the reaction was allowed to stir for 2 hours. The reaction mixture was quenched with H_2O and extracted with dichloromethane. The combined organic portions were washed with 1 N HCl, brine, and $NaHCO_3$, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The crude reaction mixture was dissolved in anhydrous acetone (60 mL). Oven dried lithium bromide (12.9 g, 148.5 mmol) was added and the reaction mixture was heated at reflux overnight. The reaction was quenched by addition of water, extracted with ether. The combined organic portions were dried ($NaSO_4$), filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (petroleum ether) and the desired product was isolated as a clear oil in 51% yield (7.54 g). The ^{1}H NMR data was consistent with literature data.[72]



Carboxylic acid 80

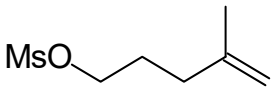
Methyltriphenylphosphonium bromide (22.5 g, 63.0 mmol) was added to a solution of *n*-BuLi (25.2 mL, 63.0 mmol) in THF (270 mL) cooled to -78 °C and the reaction was allowed to stir for an hour. Levulinic acid (2.14 mL, 21 mmol) was added dropwise and allowed to stir overnight. The reaction was quenched with 1M HCl and extracted with ether. The organic portions were combined, dried ($MgSO_4$) and filtered, and the filtrate

was concentrated *in vacuo*. The resulting residue was purified via flash chromatography (15% EtOAc in petroleum ether with 0.1% acetic acid). The desired product was isolated in 67% yield (1.61 g). The ^{1}H NMR spectrum was consistent with literature data. [53]



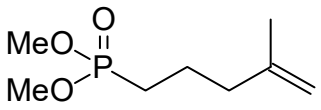
Alcohol 81

Carboxylic acid **80** (3.68 g, 32.2 mmol) was added to a solution of $LiAlH_4$ (2.45 g, 64.5 mmol) in THF (125 mL) cooled to 0 °C and allowed to stir for an hour. The reaction was quenched by the addition of water and aqueous NH_4Cl and extracted with ether. The organic portions were combined, washed with brine, dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The resulting residue was purified via flash chromatography (25% EtOAc in hexanes) and the desired product was isolated in 51% yield (1.65 g). The ^{1}H NMR spectrum was consistent with literature data.[54]



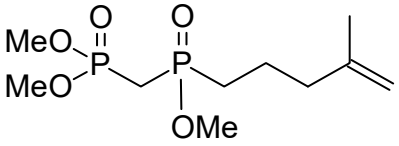
Mesylate 82

Methanesulfonyl chloride (0.26 mL, 3.41 mmol) was added to a solution of alcohol **81** (0.31 g, 3.09 mmol) in dichloromethane (3 mL) cooled to 0 °C and the reaction was allowed to stir for 30 mins. The reaction was quenched by addition of water and extracted with dichloromethane. The organic portions were combined, washed with 1N HCl and saturated $NaHCO_3$, dried ($NaSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The desired product was used without further purification and isolated in a 98% yield (0.54 g). The ^{1}H NMR spectrum was consistent with literature data. [55]



Phosphonate 78

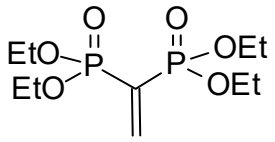
Dimethyl phosphite (0.71 mL, 7.70 mmol) was added to a solution of KHMDS (7.70 mL, 7.70 mmol) cooled to 0 °C and the resulting solution was allowed to stir for an hour. Mesylate **82** (1.06 g, 5.92 mmol) was added dropwise and the reaction was allowed to stir overnight. The reaction was concentrated *in vacuo*, quenched with the addition of brine, and extracted with dichloromethane. The organic portions were combined, dried ($MgSO_4$) and filtered, and the filtrated was concentrated *in vacuo*. The resulting residue was purified by flash chromatography (5% EtOH in ether) and the desired product was isolated in 26% yield (0.300 g). Both the ^1H NMR and ^{31}P NMR spectra were consistent with literature data.[55]



Phosphinophosphonate 84

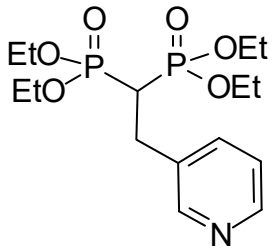
Phosphonate **78** (91 mg, 0.47 mmol) and oxalyl chloride (0.12 mL, 1.42 mmol) were dissolved in anhydrous dichloromethane (1 mL) and dimethylformamide (0.01 mL) and the solution was cooled to 0 °C. After the reaction mixture was stirred overnight, the solution was concentrated *in vacuo* and was used without further purification.[1] To a stirred solution of *n*–BuLi (0.88 mL, 2.22 mmol) in toluene at -78°C, dimethyl methylphosphonate (0.24 mL, 2.22 mmol) was added dropwise. The resulting solution was allowed to stir for 30 mins and phosphonic chloride **83** (0.47 mmol) was added dropwise. The reaction was held at -78°C for 1 hour and then allowed to warm unassisted while it was stirred overnight. The solution was quenched by addition aqueous NH_4Cl and extracted with dichloromethane. The combined organic portions were dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (5% EtOH in ether) and the product was isolated as a clear oil in 9% yield (11 mg): ^1H NMR (400 MHz, $CDCl_3$) δ 4.76 (s, 1H), 4.70 (s, 1H), 3.81 (d, J_{PH} = 11.3 Hz, 6H), 3.77 (d,

J_{PH} = 11.0 Hz, 3H), 2.40 (dd, J_{PH} = 16.0 Hz, J = 2.9 Hz, 1H), 2.38 (dd, J_{PH} = 16.1 Hz, J = 2.9, 1H), 2.12 (t, J = 7.4 Hz, 2H), 1.99-1.88 (m, 2H), 1.82-1.66 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 144.2, 111.1, 53.0 (dd, J_{PC} = 6.3, 3.0 Hz), 51.4 (d, J_{PC} = 6.9 Hz, 2C), 38.2 (d, J_{PC} = 99.5 Hz), 26.2 (dd, J_{PC} = 135.4, 76.3 Hz), 22.1 (d, J_{PC} = 6.3 Hz), 19.4 (d, J_{PC} = 4.2 Hz); ^{31}P NMR (161 MHz, $CDCl_3$) δ + 48.6 (d, J_{PP} = 4.1 Hz, 1P), 22.8 (d, J_{PP} = 4.1 Hz, 1P); HRMS (ES^+) calcd for $C_{10}H_{22}O_5P_2$ [M^+] 284.0942; found .



Bisphosphonate 107

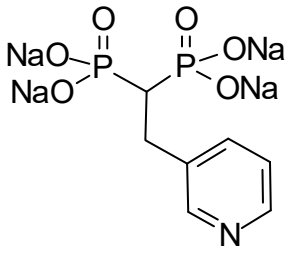
Paraformaldehyde (1.65 g, 52.1 mmol) was added to methanol (30 mL) and the resulting solution was heated to 95 °C. Diethyl amine (1.1 mL, 10.4 mmol) and tetraethylmethylene bisphosphonate (2.6 mL, 10.4 mmol) were added and the reaction mixture was stirred overnight. The solvent was removed *in vacuo* and residue was dissolved in toluene (1 mL) and concentrated *in vacuo*. Toluene was added a dean stark trap and heated to 110 °C. After *p*- toluenesulfonic acid (0.05 g, 0.29 mmol) and toluene were added and stirred overnight. The solvent was removed *in vacuo* giving the desired product in a 96% yield (2.99 g): The ^{1}H and ^{31}P NMR spectra were consistent with literature data.[73]



Pyridine bisphosphonate 109

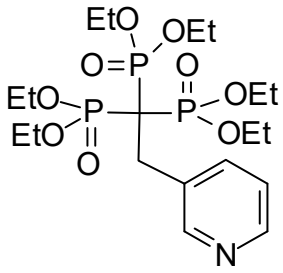
n-Butyl lithium (1.76 mL, 4.33 mmol, 2.5 M in hexane) was added to toluene (15 mL) and the solution was cooled to -78°C. As 3-bromopyridine (0.42 mL, 4.33 mmol) was added dropwise. Upon addition, the mixture was warmed to room temperature and allowed to stir for 30 minutes. The mixture was cooled to -50 °C and phosphonate **107** (1.00 g, 3.33 mmol) was added dropwise. After the resulting mixture was stirred overnight, the reaction was

quenched with NH_4Cl, extracted with diethyl ether, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The resulting residue was purified via flash chromatography (silica gel, 3% EtOH in dichloromethane). The desired product was isolated in 39% yield (0.49 g) and gave the ^{1}H and ^{31}P NMR data consistent with literature data.[64]



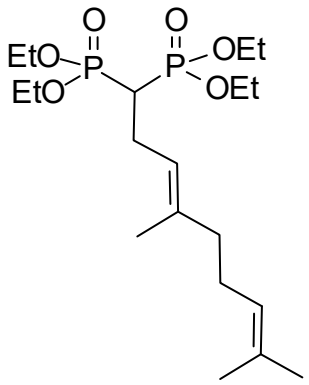
Tetrasodium salt 110

To a solutions of 2, 4, 6-collidine (0.35 mL, 2.64 mmol) in CH_2Cl_2 (5 mL) at 0 ° C was added TMSBr (0.42 mL, 3.16 mmol) and pyridine bisphosphonate **109** (0.22 g, 0.57 mmol). After the solution was allowed to stir overnight, the volatiles were removed and toluene was added then removed *in vacuo*. The resulting residue was treated with NaOH (0.70 mL, 2.11 mmol) and the reaction was stirred overnight. The reaction mixture was dried on a lyophilizer to obtain a residue which was dissolved in a small amount of water and precipitated by addition of acetone, isolated by filtration, and dried. It was then dissolved in water and lyophilized to produce the pure white salt in 78% yield (0.16g). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (s, 1H), 8.28 (d, J = 4.9 Hz, 1H), 7.83 (d, J = 7.5 Hz, 1H), 7.33 (dd, J = 7.5, 4.9 Hz), 3.08 (td, J_{PH} = 15.3 Hz, J = 6.9 Hz, 2H), 2.22-2.00 (m, 1H); ^{31}P NMR (121 MHz, $CDCl_3$) δ + 18.7. [74]



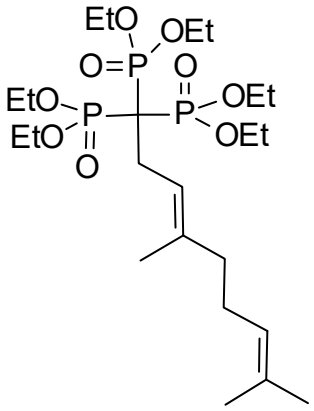
Trisphosphonate 111

Bisphosphonate **109** (0.18 g, 0.49 mmol) was dissolved in benzene (1 mL) and concentrated *in vacuo*. After three such cycles, the residue was dissolved in THF (5 mL) and the solution was cooled to 0 °C. After KHMDS (1.46 mL, 0.73 mmol) was added, the solution was allowed to stir for 30 minutes, after which diethyl chlorophosphite (0.13 mL, 1.00 mmol) was added dropwise. After an additional 10 minutes, excess hydrogen peroxide (0.25 mL, 8.12 mmol) was added dropwise. The solution was allowed to stir overnight then diluted with brine and extracted with dichloromethane. The organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The resulting residue was purified via flash chromatography (silica gel, 0% to 30% EtOH in ethyl acetate) to give the desired product in 10% yield (0.03 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.66 (s, 1H), 8.43 (d, J = 4.8 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H), 7.16 (dd, J = 8.0, 4.8 Hz, 1H), 4.19 (m, 12H), 3.56 (q, J_{PH} = 15.7 Hz, 2H), 1.28 (m, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.9, 148.3, 140.2, 132.8, 122.6, 64.2 (m, 6C), 52.8 (q, J_{PC} = 118.3 Hz, 1C), 33.9 (m, 6C); ^{31}P NMR (121 MHz, $CDCl_3$) + 17.4 ppm. HRMS (ES^+) calcd for $C_{19}H_{37}NO_9P_3$ [M^+ + H] 516.1681; found 516.1687.



Geranyl bisphosphonate 112

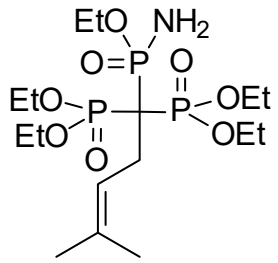
Tetraethyl methylenebisphosphonate (5 g, 17.3 mmol) was dissolved in THF, cooled to 0 °C, and sodium hydride (0.458 g, 19.1 mmol) was added. The mixture was allowed to stir under argon for 1 hour after which geranyl bromide (4.15 g, 19.1 mmol) was added. After the reaction mixture was allowed to stir overnight and warm to room temperature, the reaction was quenched by addition of water and extracted with Et_2O. The organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The resulting residue was purified via flash chromatography (silica gel, 10% EtOH in hexanes), to give the desired product 48 % yield (3.50 g). Both ^{1}H and ^{31}P NMR data were consistent with literature data.[61]



Geranyl trisphosphonate 101

Geranyl bisphosphonate **101** (1.32 g, 3.11 mmol) was dissolved in benzene (1 mL) and the resulting solution was concentrated *in vacuo*. After three such cycles, the residue was dissolved in THF (35 mL) and the solution was cooled to 0 °C. Potassium

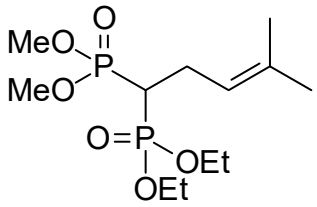
bis(trimethylsilyl)amide in toluene (9.33 mL, 4.66 mmol) was added. The mixture was allowed to stir under argon for 30 minutes after which diethylchlorophosphite was added. The reaction mixture was allowed to stir under argon for 30 minutes after which hydrogen peroxide (1.12 mL, 36.2 mmol) was added and allowed to stir overnight. After the mixture was diluted with brine and extracted with dichloromethane, the organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The resulting residue was purified via flash chromatography (silica gel, 0 to 30% EtOH in ethyl acetate). The desired product was isolated in 22% yield (0.39 g) and gave the ^1H and ^{31}P NMR data consistent with literature data.[61]



Phosphonamide 116

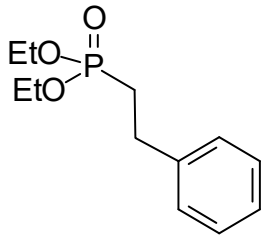
Bisphosphonate **115** (1.00 g, 2.81 mmol) was dissolved in benzene (1 mL) and concentrated *in vacuo*. After three such cycles, the residue was dissolved in THF (50 mL) and the solution was cooled to 0 ºC. KHMDS (4.21 mL, 4.21 mmol) was added and the solution was allowed to stir for 30 minutes, after which ethyl dichlorophosphite (0.64 mL, 5.61 mmol) was added dropwise. After 30 minutes, benzyl alcohol (0.73 mL, 7.02 mmol) and triethylamine (0.98 mL, 7.02 mmol) were added and the solution was allowed to stir overnight. Hydrogen peroxide (1.45 mL, 14.0 mmol) was added dropwise. After 30 minutes, the solution was diluted with brine and extracted with dichloromethane. The organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The resulting residue was purified via flash chromatography (silica gel, 15% EtOH in ethyl acetate), and the desired product was isolated in 8% yield (100 mg): ^1H NMR (300 MHz, $CDCl_3$) δ 5.68-5.58 (m, 1H), 4.21-4.05 (m, 12H), 3.00-2.76 (m, 2H), 1.73 (s, 3H), 1.66 (s, 3H), 1.38-1.25 (m, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 133.3 (s), 120.5 (q, J_{PC} = 6.3 Hz), 64.2-63.2 (m, 3C), 61.6 (d, J_{PC} = 7.0 Hz), 50.8 (td, J_{PC} = 119.4, 99.6 Hz), 29.9 (s), 29.3 (q, J_{PC} = 4.9, 4C), 26.2 (s), 18.1 (s), 16.7-16.5 (m, 2C); ^{31}P NMR

(121 MHz, $CDCl_3$) +26.3 (t, J_{PP} = 5.2 Hz, 1P), +19.1 (d, J_{PP} = 5.2 Hz, 2P); HRMS (ES^+) calcd for $C_{16}H_{36}NO_9P_3$ [M^+ + H] 464.1732; found: 464.1726.



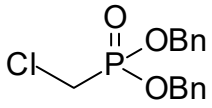
Prenyl diphosphonate 117

Diisopropyl amine (5.27 mL, 68.6 mmol) was added to THF (50 mL) and the solution was cooled to -78 °C. After *n*-butyl lithium (26.6 mL, 66.6 mmol) was added dropwise, the solution was allowed to stir for 30 minutes. Phosphonate **33** (4.00 g, 20.8 mmol) was added dropwise and the solution was stirred for 30 minutes. Diethyl chlorophosphate (10.5 mL, 72.8 mmol) was added and the solution allowed to stir overnight. After the solution was quenched by addition aqueous hydrochloric acid (1M), it was extracted with dichloromethane. The organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (3% EtOH in dichloromethane), to give the desired product as a clear oil in 54% yield (3.69 g): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.15-5.03 (m, 1H), 4.10-3.96 (m, 4H), 3.70-3.61 (m, 6H), 2.57-2.38 (m, 2H), 2.36-2.10 (m, 1H), 1.56 (s, 3H), 1.50 (s, 3H), 1.22-1.16 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 133.8, 122.0 (t, J_{PC} = 7.1 Hz, 1C), 63.0 (dd, J_{PC} = 6.6, 1.6 Hz, 2C), 53.5 (dd, J_{PC} = 14.3, 6.6 Hz, 2C), 37.3 (t, J_{PC} = 133.1 Hz, 1C), 26.1, 24.4 (t, J_{PC} = 4.8 Hz, 2C), 18.1, 16.0 (dd, J_{PC} = 6.2, 1.0 Hz, 1C); ^{31}P NMR (121 MHz, $CDCl_3$) + 26.3 (d, J_{PP} = 1.2 Hz), 23.1 (d, J_{PP} = 1.2 Hz); HRMS (ES^+) calcd for $C_{12}H_{27}O_6P_2$ [M^+ + H] 329.1283; found: 329.1283.



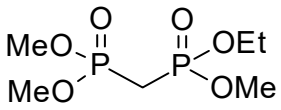
Monophosphonate 123

n-BuLi (27.9 mL, 2.49 M in hexanes) was added diethyl methylphosphonate (4.0 mL, 26.6 mmol) in THF (20 mL) via syringe at -78 °C over a period of 30 min. The solution was stirred at room temperature for 30 min and then benzyl bromide (3.23 mL, 27.2 mmol) was added as a neat liquid at 0 °C. The resulting solution was stirred for 1 h, quenched by the addition of NH_4Cl (sat), and extracted with ether. The organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The resulting oil was purified by flash chromatography (2% methanol in Et_2O) to give the desired product in 31% yield (2.01 g). Both ^1H and ^{31}P NMR data were consistent with literature data.[75]



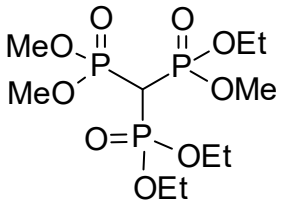
Dibenzyl phosphonate 129

Triethyl amine (4.10 mL, 29.36 mmol) and chloromethyl phosphonic dichloride (1 mL, 9.79 mmol) were added dropwise to THF (50 mL). Benzyl alcohol (2.23 mL, 21.53 mmol) was then added dropwise to the reaction mixture and it was allowed to stir overnight. The precipitate was removed by filtration, and the filtrate was concentration *in vacuo* followed by purification through flash chromatography (1:1 hexanes/ethyl acetate). The desired product was isolated in 89% yield (2.70 g) and gave the ^1H and ^{31}P NMR data consistent with literature data.[76]



Bisphosphonate 132

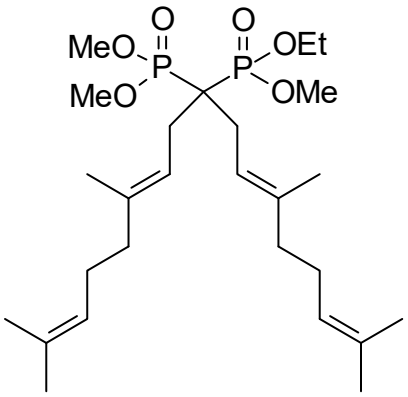
Dimethyl methylphosphonate (4.00 mL, 36.9 mmol) was added dropwise to *n*–butyl lithium (17.5 mL, 36.9 mmol) in THF cooled to –78°C and the reaction mixture was allowed to stir. After 30 minutes, dichloro ethyl phosphate (2.19 mL, 18.5 mmol) was added dropwise to the solution and it was allowed to stir. After 2.5 hours, anhydrous methanol was added dropwise and the reaction mixture was allowed to stir overnight while warming to room temperature. The solution was quenched by addition of water and extracted with $CHCl_2$. The organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The product was isolated as a clear oil in 40% yield (1.79 g) with both the ^{1}H and ^{31}P NMR spectra that consistent with literature data. [67]



Trisphosphonate 133

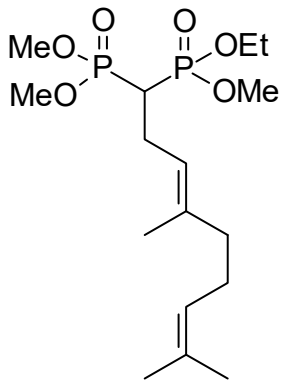
Trimethyl ethyl bisphosphonate **132** (0.35 g, 1.41 mmol) was dissolved in anhydrous benzene and then concentrated *in vacuo* to remove any traces of water. After three such cycles, the residue was dissolved in THF and cooled to 0 °C. KHMDS (2.12 mL, 2.12 mmol) was added dropwise and the reaction mixture was allowed to stir. After 30 mins, diethyl chlorophosphite (0.37 mL, 2.82 mmol) was added dropwise and the reaction mixture was allowed to stir. After 30 mins, hydrogen peroxide (1 mL) was added dropwise and the reaction mixture was allowed to stir for an additional 30 mins. The reaction was quenched by addition of acetate buffer (pH 4.5) and extracted with dichloromethane. The organic portions were combined, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo* and the residue was purified by column chromatography (0 to 30% EtOH in ethyl acetate 2% gradient) and the product was isolated as a clear oil in 16% yield

(84 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.24–4.22 (m, 6H), 3.84 (d, J_{PH} = 11.3 Hz, 9H) 3.28 (q, J_{PH} = 46.9 Hz, 1H), 1.35–1.31 (m, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 63.4– 63.9 (m, 3C), 53.9–54.0 (m, 3C), 39.6 (q, J_{PC} = 248.1 Hz), 16.2 (d, J_{PC} = 6.6 Hz, 3C); ^{31}P NMR (161 NMR, $CDCl_3$) +16.9 (br s), +15.3 (br s), +13.8 (br s); HRMS (ES^+) calcd for $C_{10}H_{24}O_9P_3Na$ [M^+ - H] 405.0609; found: 405.0607.



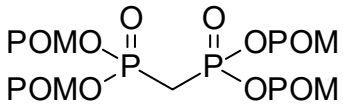
Digeranyl bisphosphonate 134

Trimethyl ethyl methylene bisphosphonate **132** (0.64 g, 2.60 mmol) was dissolved in THF and cooled to 0 °C. KHMDS (2.60 mL, 2.60 mmol) was added dropwise and the reaction mixture was allowed to stir. After 30 mins, geranyl bromide (0.52 mL, 2.60 mmol) was added dropwise and the reaction mixture was allowed to stir overnight. The solution was quenched by addition of aqueous NH_4Cl and extracted with ether. The organic portions were dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (5% EtOH in hexanes) and the product **134** was isolated as a clear oil in a 20% yield (0.14 g): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.37–5. 36 (m, 2H), 5.07– 5.10 (m, 2H), 4.18–4.14 (m, 2H), 3.78–3.75 (m, 9H), 2.60 (td, J_{PH} = 16.4, 7.1 Hz, 4H), 2.07-1.98 (m, 8H), 1.65 (s, 6H), 1.60 (s, 6H), 1.58 (s, 6H), 1.31 (t, J_{PH} = 7.1 Hz, 3Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.5 (d, J_{PC} = 2.9 Hz), 137.3 (t, J_{PC} = 2.5 Hz), 131.3 (d, J_{PC} = 1.3 Hz, 2C), 124.3 (d, J_{PC} =1.3 Hz, 2C), 119.1-118.9 (m, 2C), 62.6–62.5 (m), 53.2–53.3 (m, 3C), 46.3 (t, J_{PC} = 131.6 Hz), 40.0 (2C), 29.1 (t, J_{PC} = 4.3 Hz, 2C), 26.7 (2C), 25.6 (2C), 17.6 (2C), 16.4 (d, J_{PC} = 5.8 Hz, 3C), 16.3; ^{31}P NMR (202 MHz, $CDCl_3$) +29.3 (d, J = 7.0 Hz), +27.7 (d, J = 7.0 Hz); HRMS (ES^+) calcd for $C_{26}H_{49}O_6P_2$ [M^+] 519.3004; found: 519.3008.



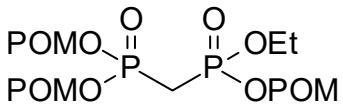
Geranyl bisphosphonate 135

The product **135** was isolated as a clear oil in 37% yield (0.37 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.29–5.28 (m, 1H), 5.10–5.08 (m, 1H), 4.16–4.21 (m, 2H), 3. 83–3.78 (m, 9H), 2.62–2.65 (m, 2H), 2.36 (tt, J_{PH} = 23.7, J = 6.2 Hz, 1H), 2.04 (m, 4H), 1.68 (s, 3H), 1.65 (s, 3H), 1.60 (s, 3H), 1.35 (t, J_{PH} = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 136.9, 131.2, 123.9 (d, J_{PC} = 1.8 Hz) 121.4–121.2 (m), 62.5–62.4 (m), 53.0–52.7 (m, 3C), 39.4, 36.6 (t, J_{PC} =133.1 Hz), 26.3, 25.4, 23.8–23.7 (m), 17.4, 16.2–16.1 (m), 15.8; ^{31}P NMR (161 MHz, $CDCl_3$) +26.2 (d, J = 2.3 Hz), +24.6 (d, J = 2.3 Hz); HRMS (ES^+) calcd for $C_{16}H_{33}O_6P_2$ [M^+] 383.1752; found: 383.1754.



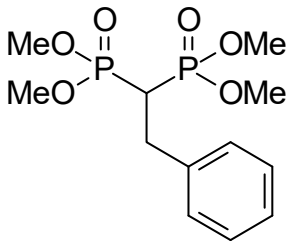
TetraPOM bisphosphonate 138

Tetramethyl methylene bisphosphonate (3.98 mL, 21.5 mmol), sodium iodide (12.9 g, 86.2 mmol), and chloromethyl pivalate (15.6 mL, 107.7 mmol) were dissolved in acetonitrile (40 mL) and the solution heated at 80 °C overnight. The reaction was quenched by addition of water and extracted with diethyl ether. The organic portions were washed with brine and sodium thiosulfate, dried ($MgSO_4$), and filtered and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (30% EtOAc in hexanes), to give the desired product in a 31% yield (4.19 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.69-5.60 (m, 8H), 2.69 (t, J_{PH} = 21.6 Hz, 2H), 1.24 (s, 36H); ^{31}P NMR (162 MHz, $CDCl_3$) + 17.8.[77, 78]



TriPOM ethylbisphosphonate 140

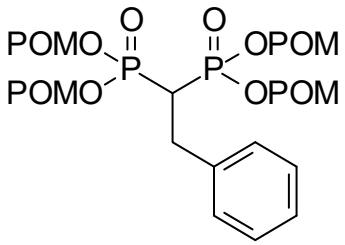
Bisphosphonate **139** (4.19 g, 6.62 mmol) and 1,4-diazabicyclo[2.2.2]octane (0.75 g, 6.62 mmol) were dissolved in acetonitrile (20 mL) and the reaction was heated at 80 °C for 2 hours. The solution was concentrated, anhydrous acetonitrile was added and evaporated 3 times *in vacuo*. Chloride **139** was dissolved in anhydrous toluene (95 mL) and anhydrous dimethylformamide (0.04 mL). Oxalyl chloride (2.56 mL, 29.8 mmol) was added dropwise and the reaction was allowed to stir at room temperature for 45 mins. The solution was filtered through Na_2SO_4 and celite then washed with anhydrous toluene. The organics were then concentrated. The resulting oil was dissolved in anhydrous toluene (20 mL) and cooled to 0°C. Triethyl amine (1.85 mL, 13.2 mmol) and ethanol (0.77 mL, 13.2 mmol) were added dropwise and the reaction was allowed to stir overnight. The solution was filtered through celite and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (30% EtOAc in hexanes). The desired product was isolated in a 52% yield over 3 steps (1.89 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.76-5.66 (m, 6H), 4.29-4.10 (m, 2H), 2.62 (t, J_{PH} = 21.6, 2H), 1.36 (t, J_{PH} = 7.0, 3H), 1.24 (s, 24H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.8, 176.7 (d, J_{PC} = 0.7, 2C), 82.2 (d, J_{PC} = 5.6), 82.0-81.8 (m, 2C), 63.1 (d, J_{PC} = 6.6), 38.7 (3C), 26.8 (3C), 26.7 (6C), 26.6 (t, J_{PC} = 138.5), 16.1 (d, J_{PC} = 6.5); ^{31}P NMR (162 MHz, $CDCl_3$) + 16.9 (d, J_{PP} = 7.7), 16.1 (d, J_{PP} = 7.7); HRMS (ES^+) calcd for $C_{21}H_{40}O_{12}P_2Na$ [M^+] 569.1893; found: 569.1907.



Benzyl bisphosphonate 143

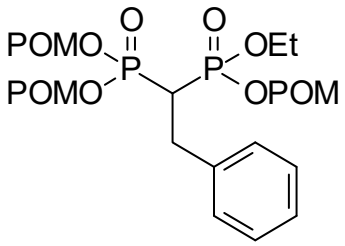
Tetramethyl methylene bisphosphonate (2.4 mL, 13 mmol) was added to a solution of KHMDS (14.3 mL, 14.3 mmol) in THF cooled to 0 °C and the solution was allowed to stir.

After 30 minutes, benzyl bromide (1.74 mL, 14.3 mmol) was added dropwise. After 4 hours, the reaction was quenched by addition of water and extracted with diethyl ether. The organic layers were combind, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (10% EtOH in hexanes) and the desired product was isolated as a clear oil in 26% yield (1.07 g). The ^{1}H and ^{31}P NMR spectra were consistent with literature data. [79]



TetraPOM bisphosphonate 144

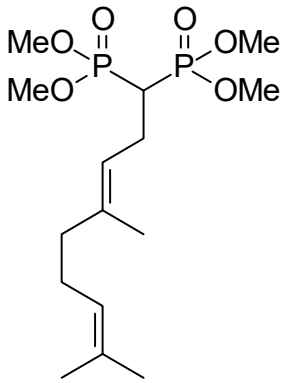
Bisphosphonate **143** (1.07 g, 3.32 mmol), sodium iodide (2.48 g, 16.6 mmol), and chloromethyl pivalate (1.92 mL, 13.3 mmol) were dissolved in acetonitrile (5 mL) and the solution was heated at reflux overnight. The reaction was quenched by addition of water and extracted with diethyl ether. The combined organic portions were washed with brine and $Na_2S_2O_3$, dried ($MgSO_4$), and filtered, and the filtrate was concentrated *in vacuo* to give, the product was isolated as a yellow oil in a 60% yield (1.45 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29–7. 12 (m, 5H), 5.63 (d, J_{PH} =12.7 Hz, 8H), 3.23 (td, J_{PH} = 17.1 Hz, J= 6.7 Hz, 2H), 2.88 (tt, J_{PH} = 24.8 Hz, J= 6.7 Hz, 1H), 1.21 (s, 36H); ^{31}P NMR (161 MHz, $CDCl_3$) +21.2. HRMS (ES^+) calcd for $C_7H_{13}Na_3O_6P_2$ [M^+] xxx; found .



Bisphosphonate 146

Bisphosphonate **144** (1.45 g, 1.97 mmol) and DABCO (0.22 g, 1.97 mmol) were dissolved in acetonitrile (5 mL) and the solution was heated at reflux overnight. The reaction was

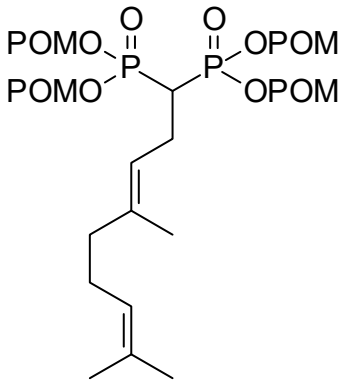
concentrated *in vacuo* and the residue was used without further purification. Phosphonic salt **145** (1.97 mmol) was dissolved in anhydrous acetonitrile and then concentrated *in vacuo* to remove any trace amounts of water. After three cycles, the residue was dissolved in toluene (30 mL) and DMF (0.01 mL). Oxalyl chloride (0.76 mL, 8.88 mmol) was added dropwise and the solution was stirred at room temperature for 1 hour. The reaction mixture was filtered through Na_2SO_4 and the filtrate was concentrated *in vacuo*. The residue was dissolved in toluene (10 mL) and cooled to 0 °C. Triethylamine (0.55 mL, 3.95 mmol) and anhydrous ethanol (0.23 mL, 3.95 mmol) were added dropwise and the solution was allowed to stir overnight. The solution was quenched by addition of aqueous 1N HCl and extracted with diethyl ether. The organic portions were dried (Na_2SO_4), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (1% triethylamine in 1:1 ethyl acetate in hexanes). The desired product was isolated as a yellow oil in 43% yield (0.54 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20-7.18 (m, 5H), 5.60-5.54 (m, 6H), 4.00 (q, J_{PH} = 7.3 Hz, 2H), 3.12-3.05 (m, 2H), 2.85-2.77 (m, 1H), 1.19-1.13 (m, 39H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.5 (2C), 170.8, 138.3-138.2 (m), 128.8 (d, J_{PC} = 2.4 Hz, 2C), 128.2 (s, 2C), 126.2, 82.3-81.6 (m, 3C), 62,7 (dd, J_{PC} = 10.1, 6.7 Hz, 1C), 60.2, 39.6 (t, J_{PC} =134.1), 38.5 (3C), 26.7 (9C), 15.9 (d, J_{PC} = 6.6); ^{31}P NMR (161 MHz, $CDCl_3$) +22.2 (d, J =22.8 Hz), +21.3 (d, J = 22.8 Hz). HRMS (ES^+) calcd for $C_{28}H_{55}OP_2$ [M^+] 668.3066; found .



Geranyl bisphosphonate 148

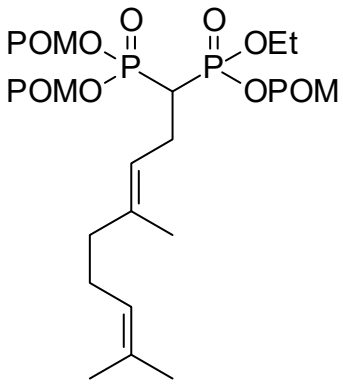
Tetramethyl methylenebisphosphonate (2.4 mL, 13 mmol) was dissolved in THF and the solution was cooled to 0° C. After KHMDS (13 mL, 13 mmol) was added dropwise, the reaction mixture was allowed to stir. After 30 mins, geranyl bromide (2.58 mL, 13 mmol)

was added dropwise as a 1 M solution in THF and the resulting solution was allowed to stir overnight. The reaction was quenched by addition of aqueous NH_4Cl and extracted with ether. The organic portions were dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. After the residue was purified by column chromatography (3% EtOH in dichloromethane), the product was isolated as a clear oil in 33% yield (1.60 g). Both the ^{1}H and ^{31}P NMR data were consistent with literature data.[16]



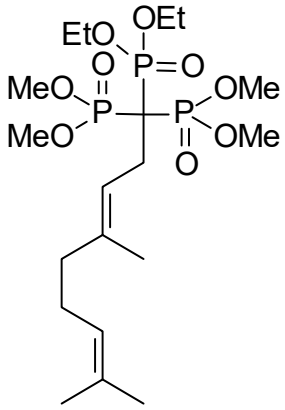
TetraPOM geranylbisphosphonate 149

Tetramethyl geranylbisphosphonate **148** (0.89 g, 2.41 mmol), sodium iodide (1.45 g, 9.64 mmol) and chloromethyl pivalate (1.75 mL, 12.1 mmol) were dissolved in acetonitrile (5 mL) and the solution was heated at reflux overnight. The reaction was quenched by addition of water and extracted with diethyl ether. The organic portions were washed with $Na_2S_2O_3$, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The residue was used without further purification. (1.71 g, 92%). The ^{1}H and ^{31}P NMR data were consistent with literature data.[17]



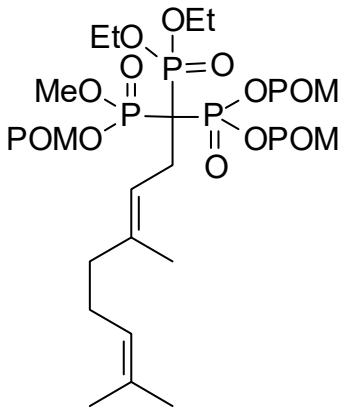
TriPOM bisphosphonate 150

TetraPOM geranyl bisphosphonate **149** (1.49 g, 1.94 mmol) and 1,4–diazabicyclo [2.2.2] octane (0.22 g, 1.94 mmol) were dissolved in acetonitrile (5 mL) and the reaction mixture was heated at reflux overnight. The reaction was concentrated, the residue was dissolved in anhydrous acetonitrile and then concentrated *in vacuo* to remove any traces of water. After three such cycles, the residue was dissolved in toluene (30 mL) and DMF (0.01 mL). Oxalyl chloride (0.75 mL, 8.71 mmol) was added dropwise and the solution was stirred at room temperature for 45 mins. The reaction mixture was filtered through Na_2SO_4 and the filtrated was concentrated *in vacuo*. The residue was dissolved in toluene (10 mL) and cooled to 0 °C. Triethylamine (0.54 mL, 3.87 mmol) and anhydrous ethanol (0.22 mL, 3.87 mmol) were added dropwise and the solution was allowed to stir overnight. The reaction was quenched by addition of water and extracted with diethyl ether. The organic portions were dried (Na_2SO_4), and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via flash chromatography (3% EtOH in dichloromethane) to afford the desired product as a yellow oil in 15% yield (190 mg).[41] ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.71–5.64 (m, 6H), 5.22–5.21 (m, 1H), 5.05–5.04 (m, 1H), 4.17–4.13 (m, 2H), 2.62–2.54 (m, 2H), 2.46 (tt, J_{PH} = 24.2 Hz, J = 6.5 Hz, 1H), 2.04–1.93 (m, 4H), 1.64 (s, 3H), 1.59 (s, 3H), 1.56 (s, 3H), 1.31 (t, J = 7.4 Hz, 3H), 1.21–1.20 (m, 27 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.7–176.6 (m, 3C), 137.7 (d, J_{PC} = 5.3 Hz), 131.4, 123.9, 120.7–120.5 (m), 81.6–82.4 (m, 3C), 62.7 (dd, J_{PC} = 6.7 Hz, 5.6 Hz), 39.6 (3C), 38.6, 38.6 (t, J_{PC} = 133.9 Hz), 26.8–26.7 (m, 9C), 26.5, 25.6, 23.6–23.5 (m), 17.5, 16.1 (dd, J_{PC} = 6.4 Hz, 1.6 Hz), 16.0 (d, J_{PC} = 2.1 Hz); ^{31}P NMR (161 MHz, $CDCl_3$) δ 22.8 (d, J_{PP} = 3.8 Hz), 22.1 (d, J_{PP} = 3.8 Hz); HRMS (ES^+) calcd for $C_{31}H_{56}O_{12}P_2Na$ [$(M+Na)^+$] 705.3145; found 705.3153.



Trisphosphonate 153

Tetramethyl geranylbisphosphonate **148** (0.70 g, 1.91 mmol) was dissolved in anhydrous benzene and then concentrated *in vacuo* to remove any traces of water. After three such cycles, the residue was dissolved in THF and the resulting solution was cooled to 0 °C. After KHMDS (3.81 mL, 3.81 mmol) was added dropwise, the reaction mixture was allowed to stir for an hour, diethyl chlorophosphite (0.50 mL, 3.81 mmol) was added dropwise, and the reaction mixture was allowed to stir overnight. Hydrogen peroxide (1 mL) was added and the reaction mixture was allowed to stir for an hour. The reaction was quenched by addition of brine and extracted with dichloromethane. The organic portions were dried ($MgSO_4$) and filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (0 to 30% EtOH in ethyl acetate) to give the product was isolated as a clear oil in 43% yield (0.42 g): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.53–5.51 (m, 1H), 5.01–4.98 (m, 1H), 4.17–4.07 (m, 4H), 3.73 (d, J_{PH} = 10.2 Hz, 12H), 2.74 (qd, J_{PH} = 16.3 Hz, J = 6.5 Hz, 2H), 1.99-1.89 (m, 4H), 1.55 (s, 3H), 1.52 (s, 3H), 1.47 (s, 3H), 1.23 (t, J = 7.0 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 136.5, 130.9, 123.9, 119.1 (q, J_{PC} = 6.1 Hz), 63.2 (d, J_{PC} = 7.0 Hz, 4C), 53.8–53.7 (m, 2C), 50.1 (q, J_{PC} = 121.6 Hz), 39.7, 28.9 (q, J_{PC} = 5.6 Hz), 26.3, 25.3, 17.3 16.1(d, J_{PC} = 6.1 Hz, 2C), 15.9; ^{31}P NMR (202 Hz, $CDCl_3$) δ + 20.9 (d, J = 6.8 Hz, 2P), + 17.9 (t, J = 6.8 Hz, 1P); HRMS (ES^+) calcd for $C_{19}H_{39}O_9P_3Na$ [M^+] 527.1705; found 527.1713.



Trisphosphonate 154

Tetramethyl diethyltrisphosphonate **153** (121 mg, 0.24 mmol), sodium iodide (107 mg, 0.72 mmol) and chloromethyl pivalate (104 μL, 0.72 mmol) were dissolved in acetonitrile (2 mL) and the solution was heated at reflux for 48 hr. The reaction was quenched by addition of water and extracted with diethyl ether. The organic portions were, dried ($NaSO_4$), and filtered, and the filtrate was concentrated *in vacuo*. The 20 mg of crude reaction mixture was dissolved in 2 mL of acetonitrile to be used in purification by HPLC. An LCMS was obtain and showed three peaks in the LC trace. Using a gradient of acetonitrile in water with 1% formic acid (50% –100 % over 8 mins), the LCMS showed a peak with retention time of 4.26 mins that showed formation of product with a mass of 463.17m/z for a hydrolysis product peak. When repeated without acid, the LCMS showed a peak with retention time of 7.56 mins that showed formation of product with a mass of 827.33 m/z. Further investigation is still on going.

APPENDIX: SELECTED NMR SPECTRA

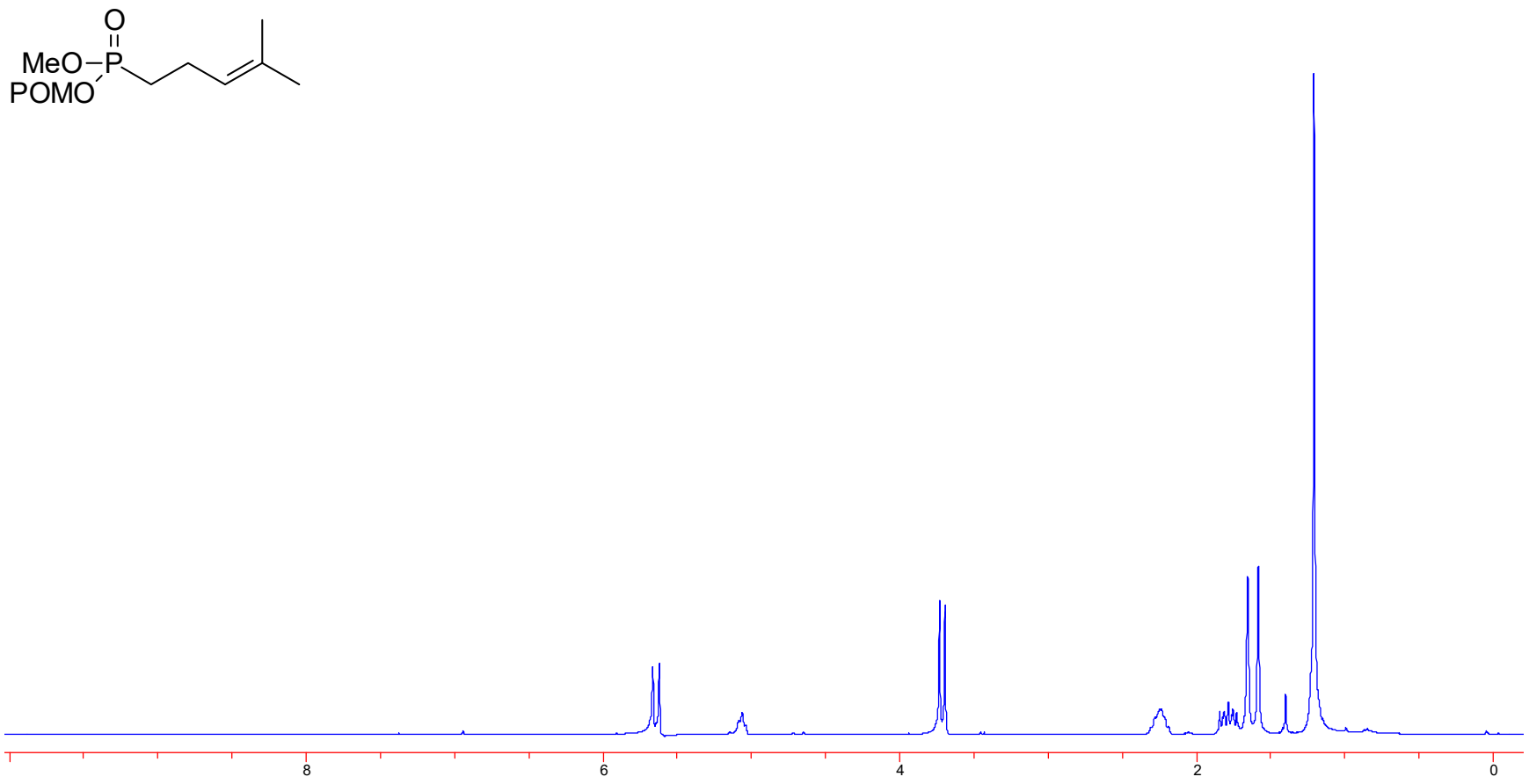


Figure A- 1. ^{1}H NMR spectrum of compound **35**

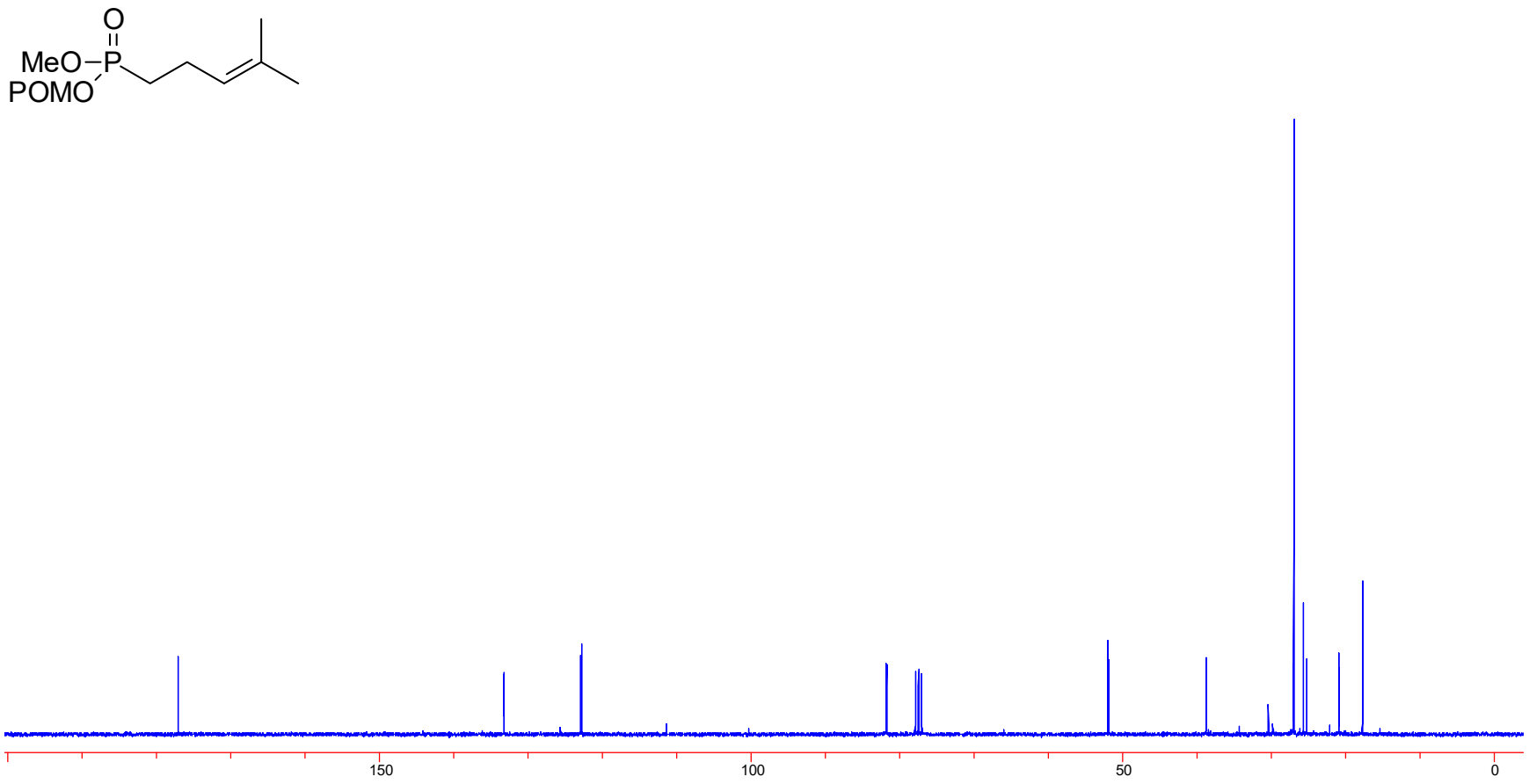


Figure A- 2. ^{13}C NMR spectrum of compound **35**

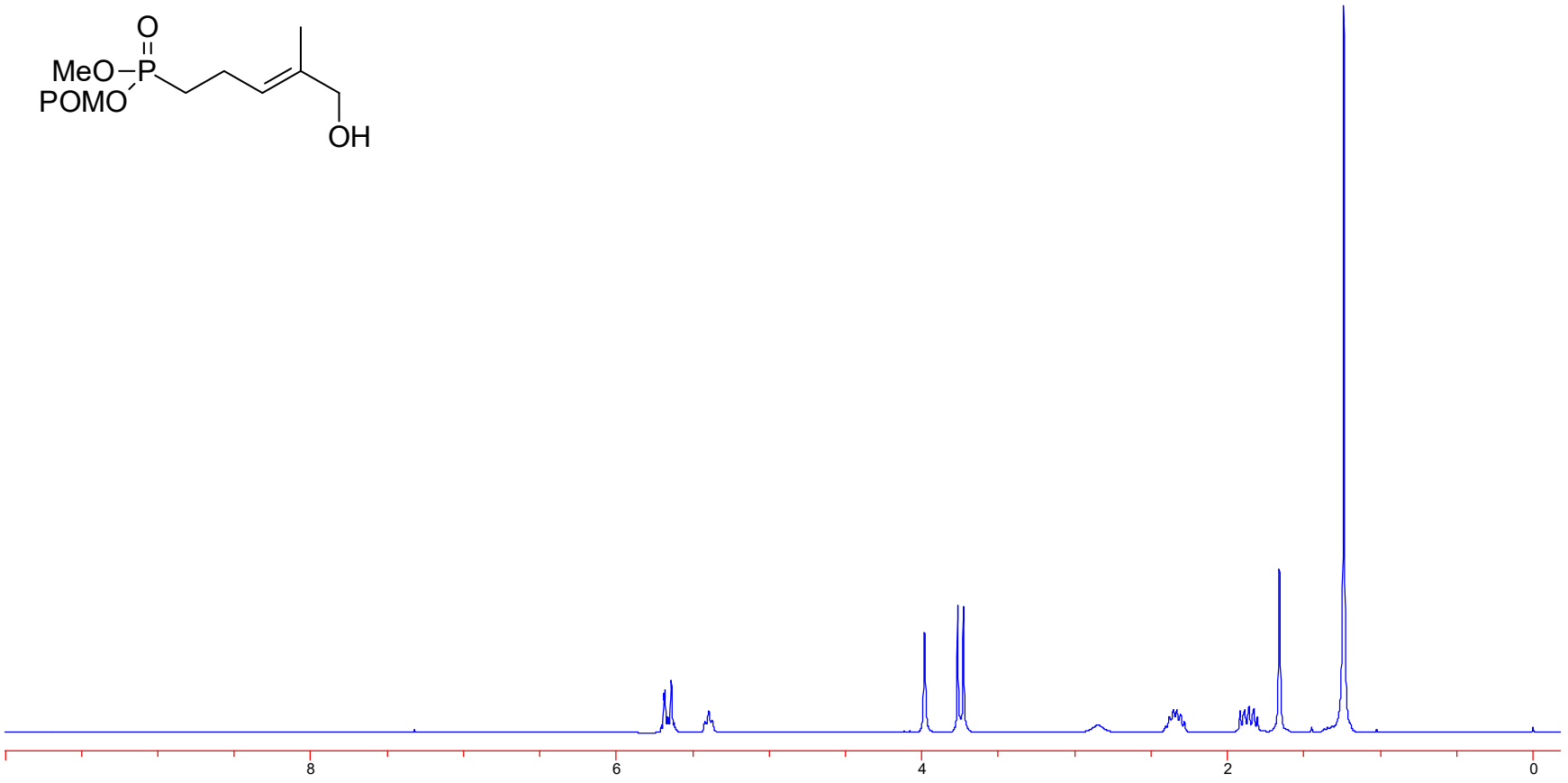


Figure A- 3. ^{1}H NMR spectrum of compound **36**

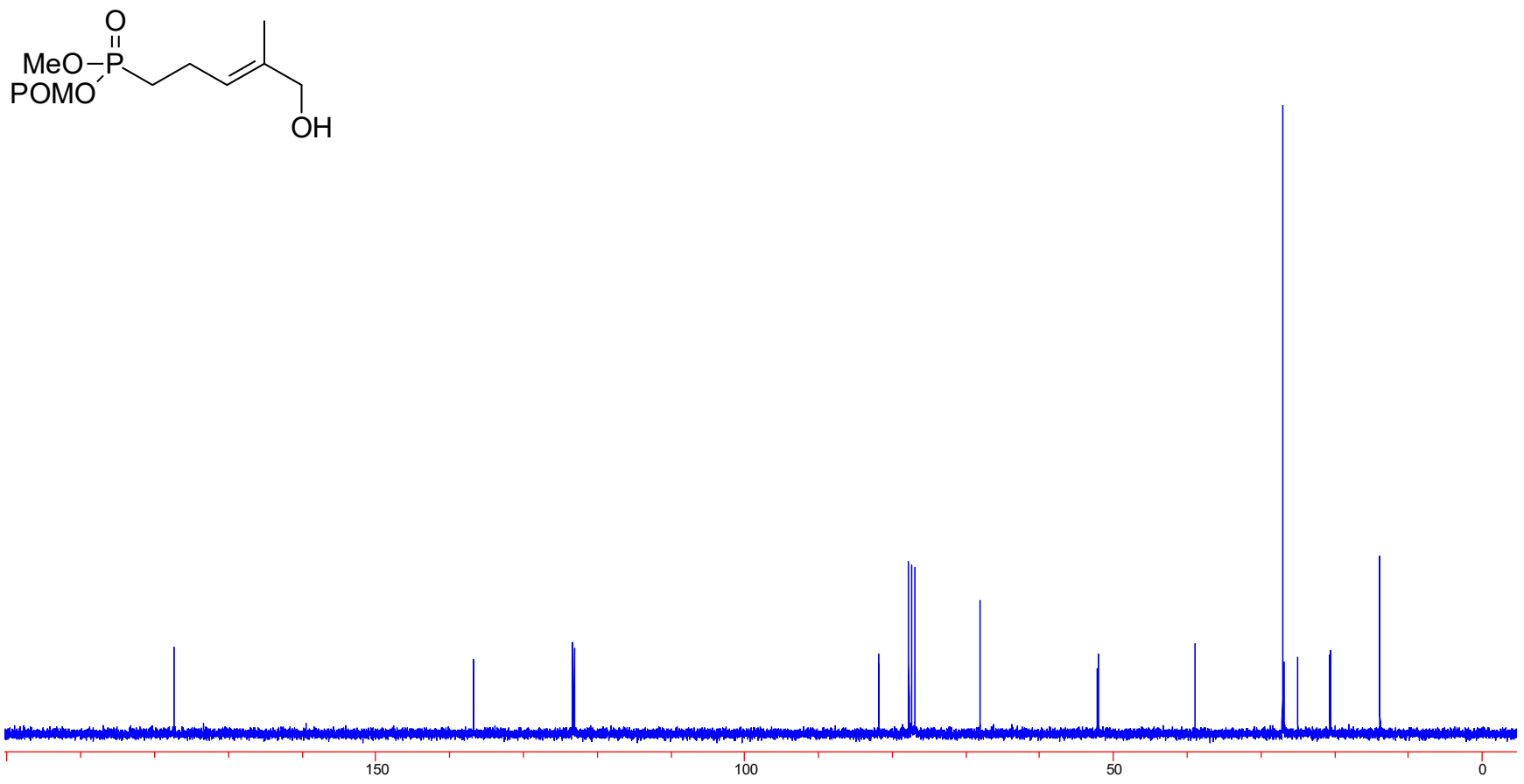


Figure A- 4. ^{13}C NMR spectrum of compound **36**

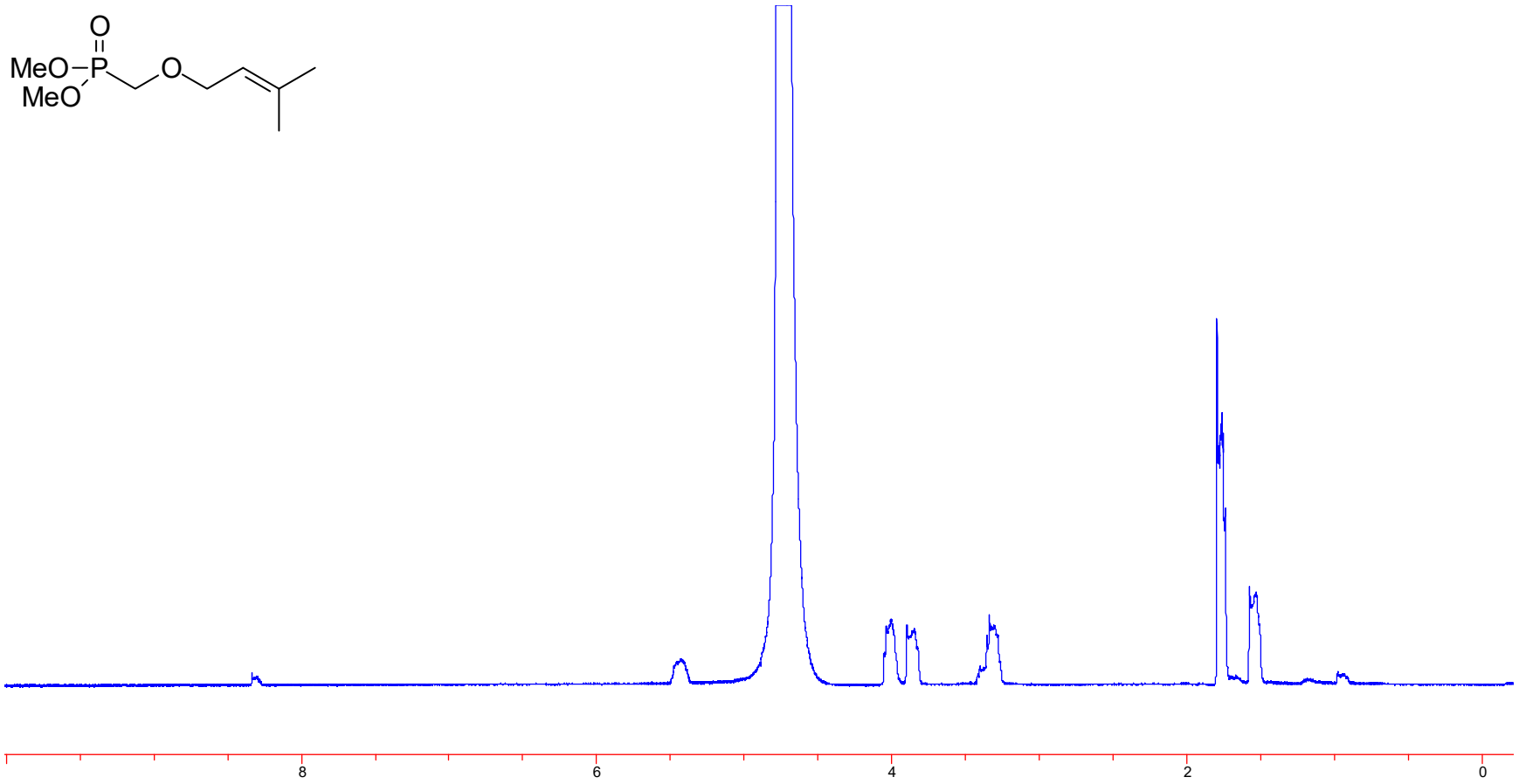


Figure A- 5. ^{1}H NMR spectrum of compound **39**

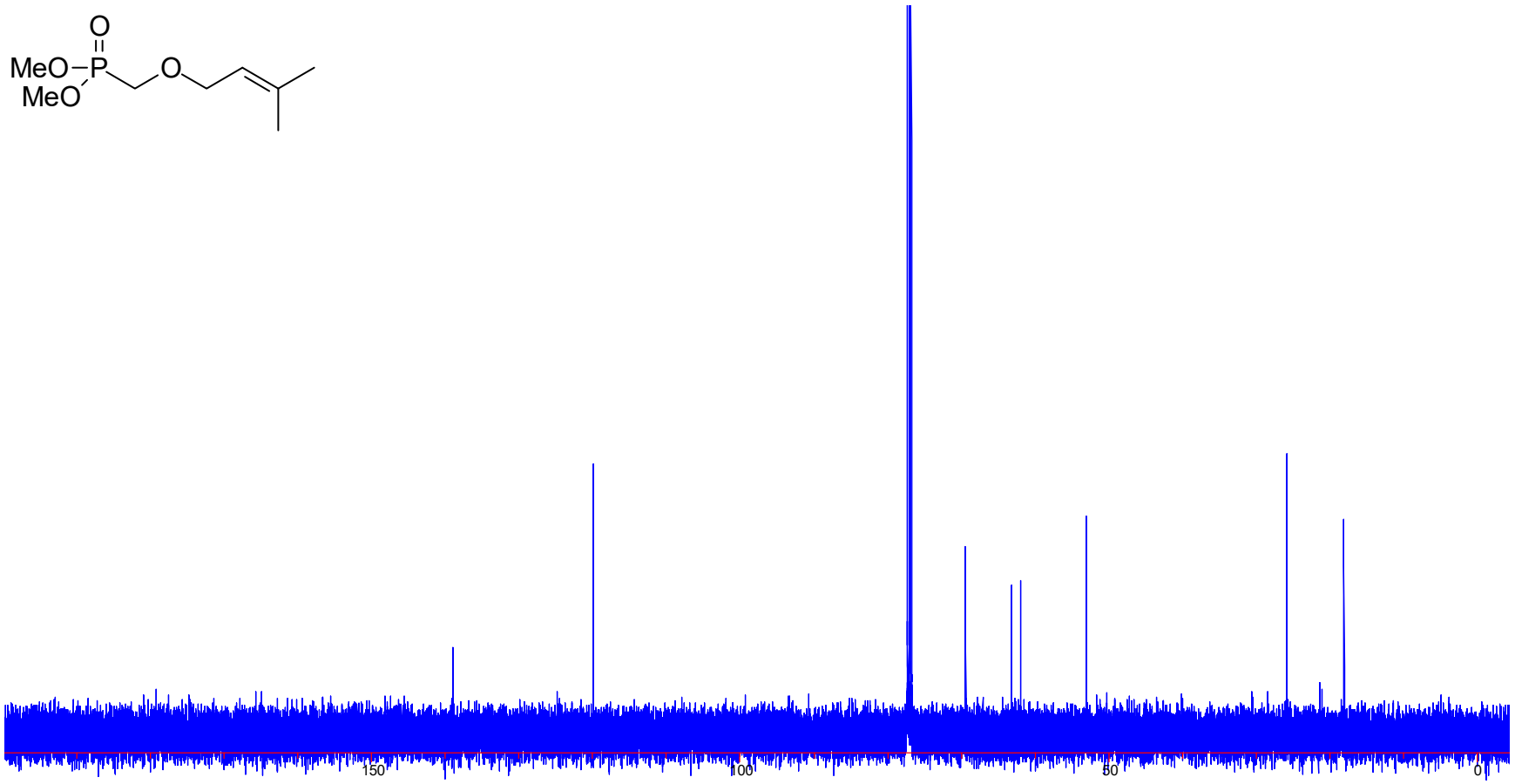


Figure A- 6.^{13}C NMR spectrum of compound **39**

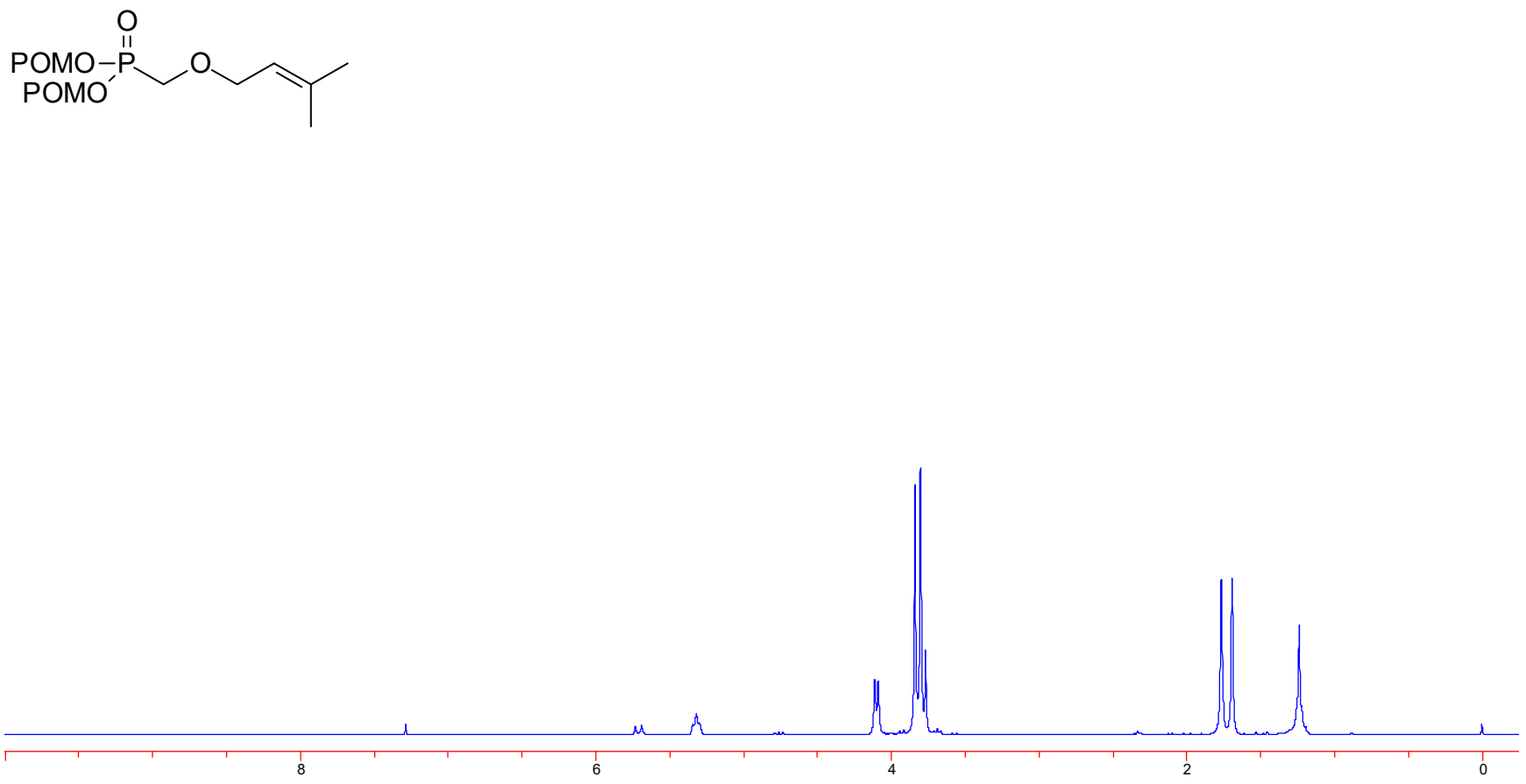


Figure A- 7. ^{1}H NMR spectrum of compound **40**

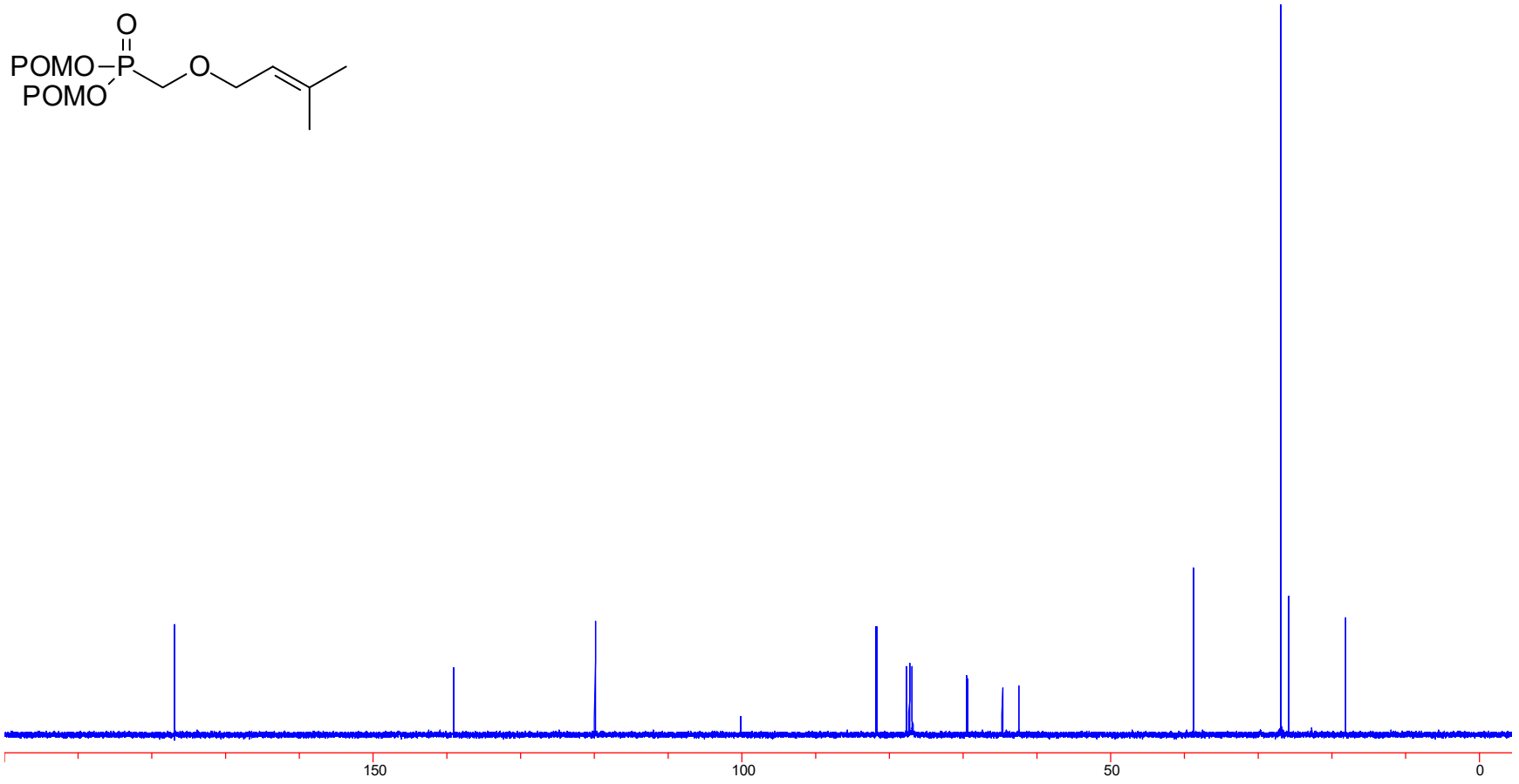


Figure A- 8. ^{13}C NMR spectrum of compound **40**

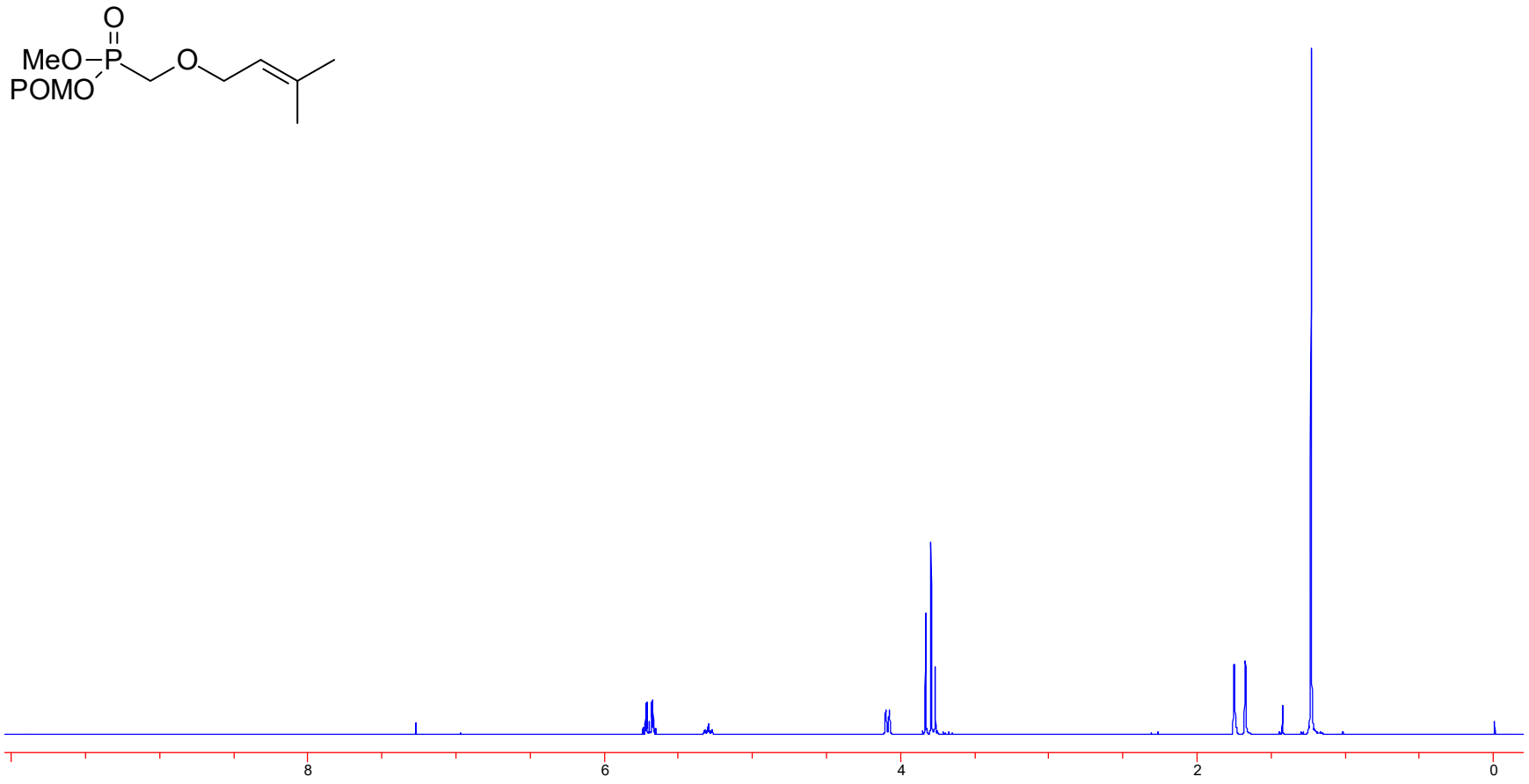


Figure A- 9. ^{1}H NMR spectrum of compound **41**

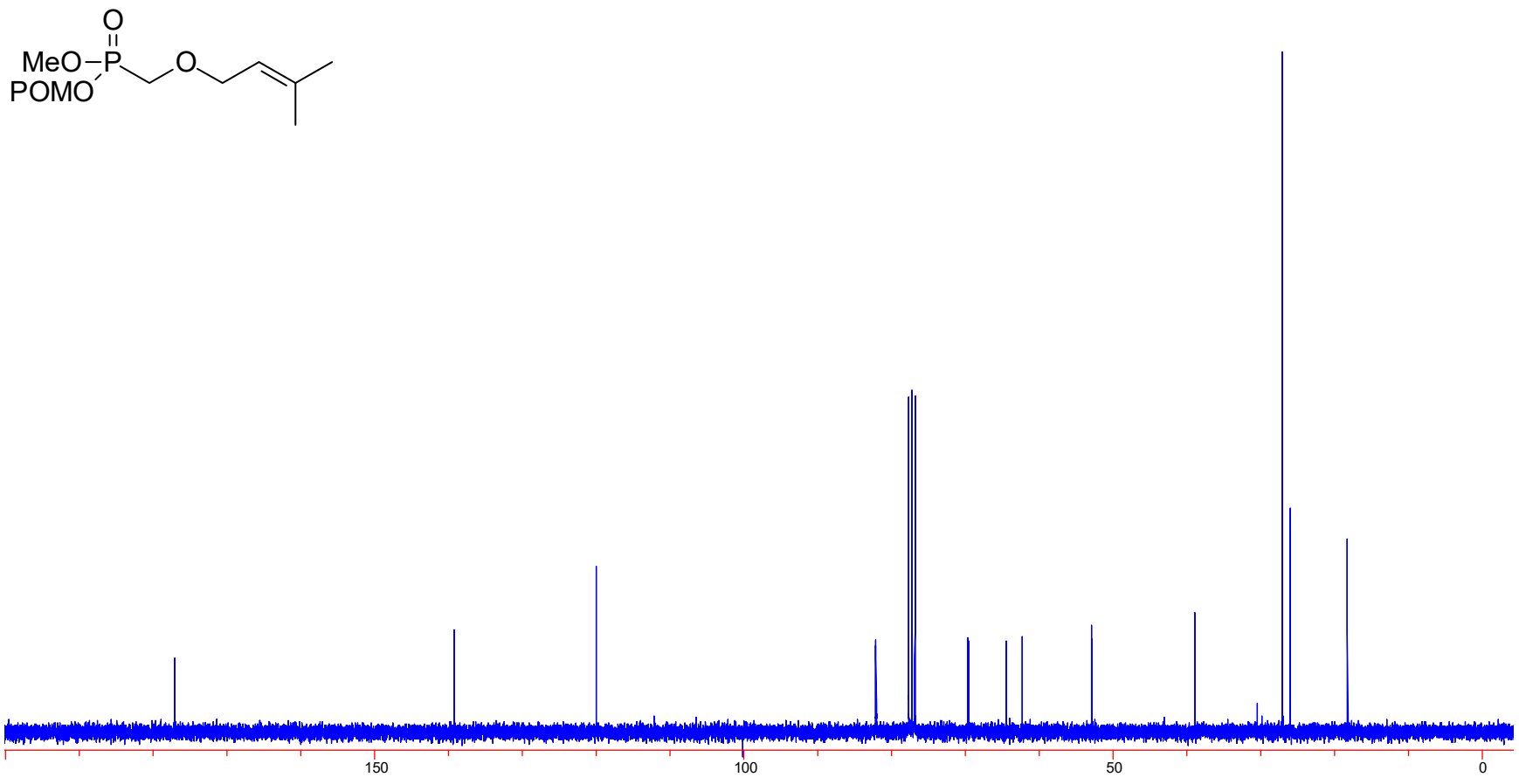


Figure A- 10. ^{13}C NMR spectrum of compound **41**

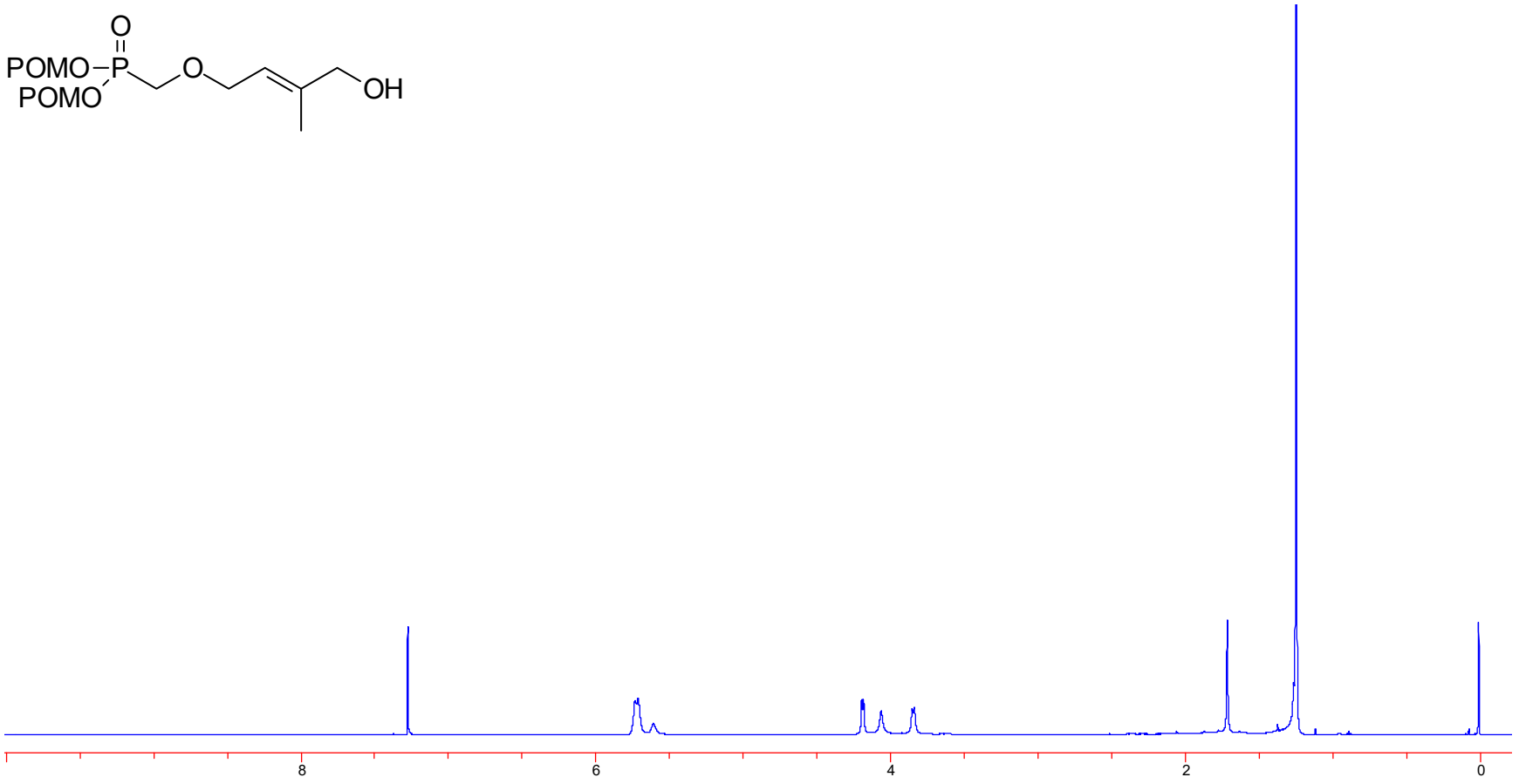


Figure A- 11. ^{1}H NMR spectrum of compound **42**

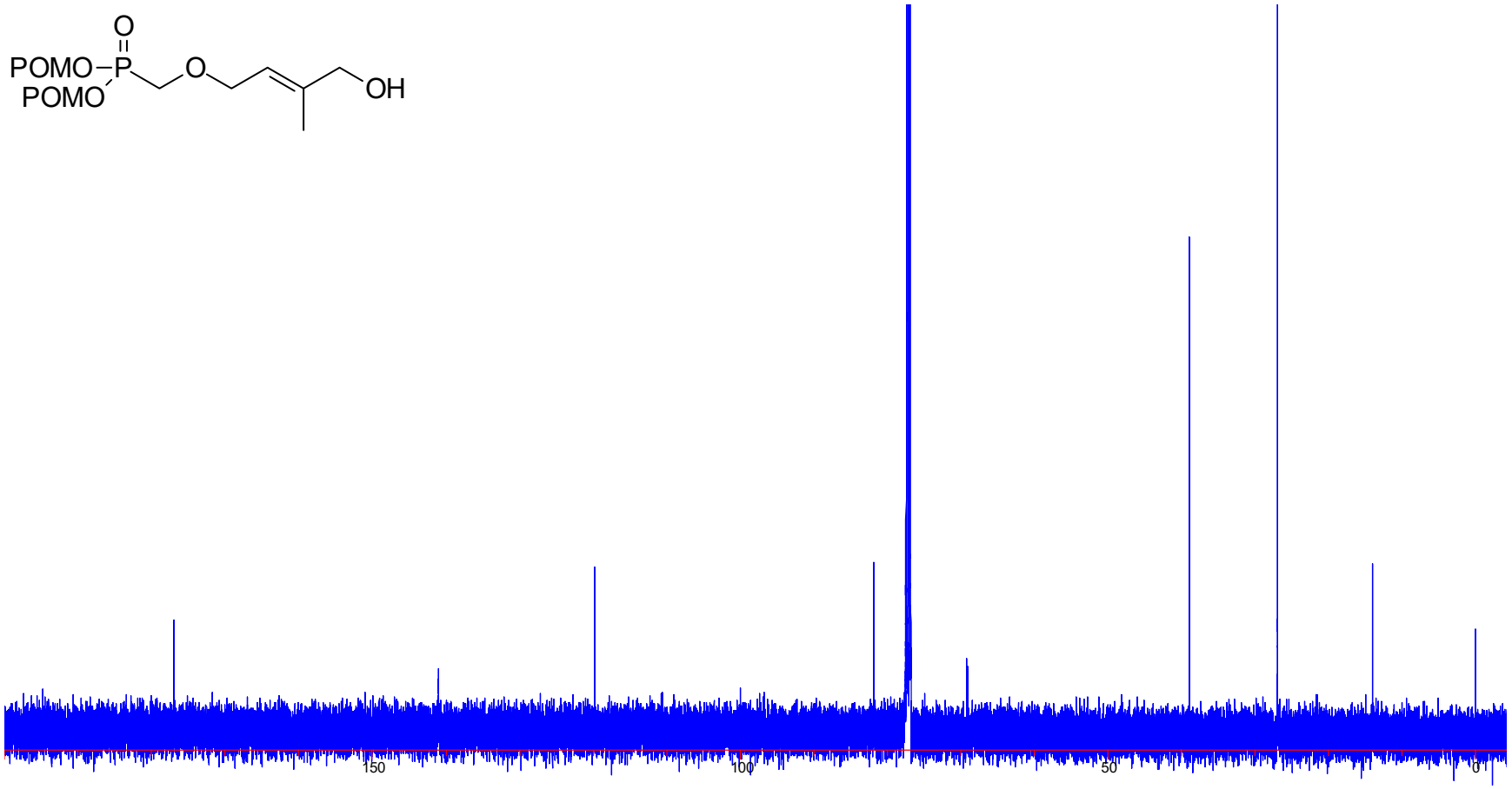


Figure A- 12. ^{13}C NMR spectrum of compound **42**

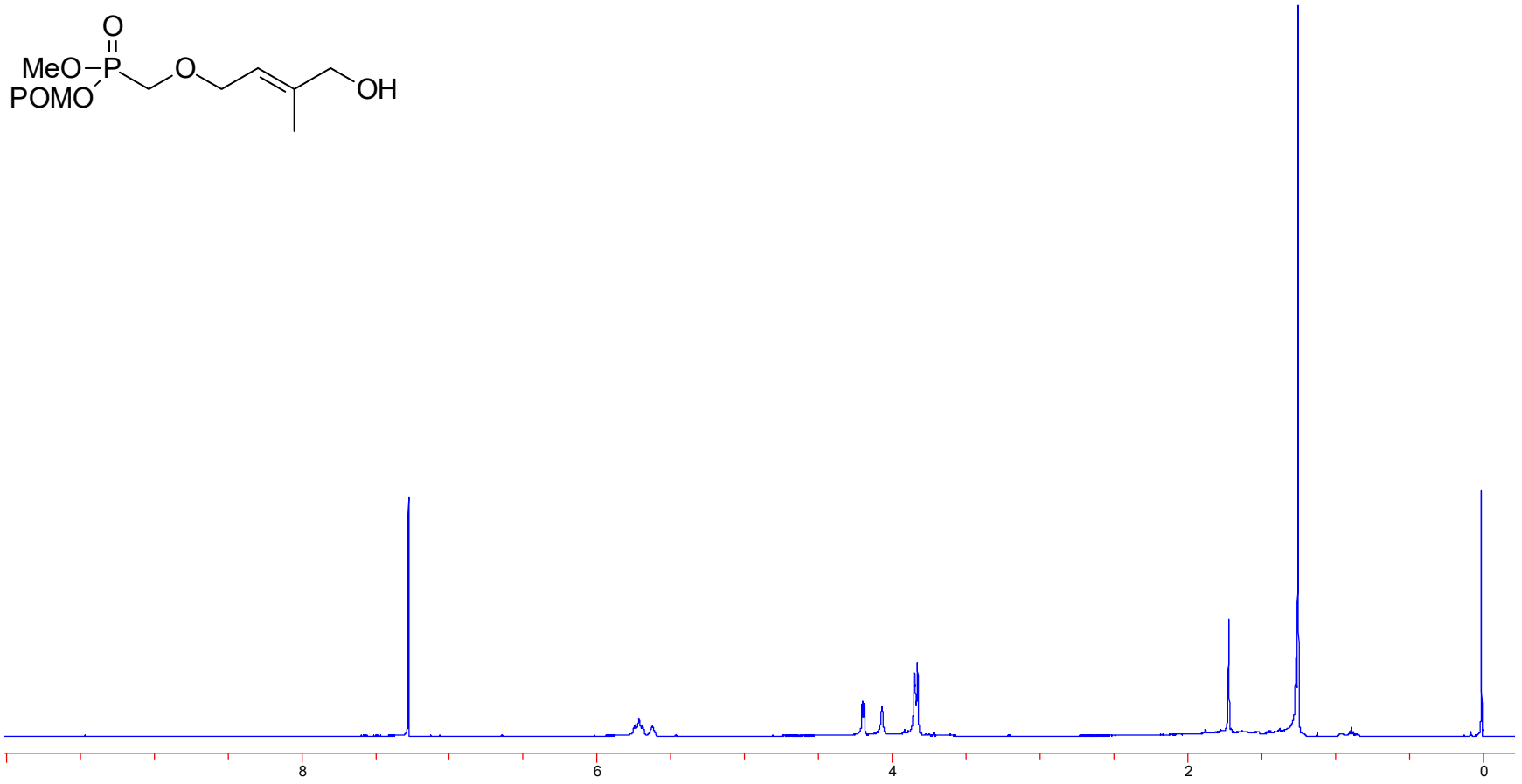


Figure A- 13. ^{1}H NMR spectrum of compound **43**

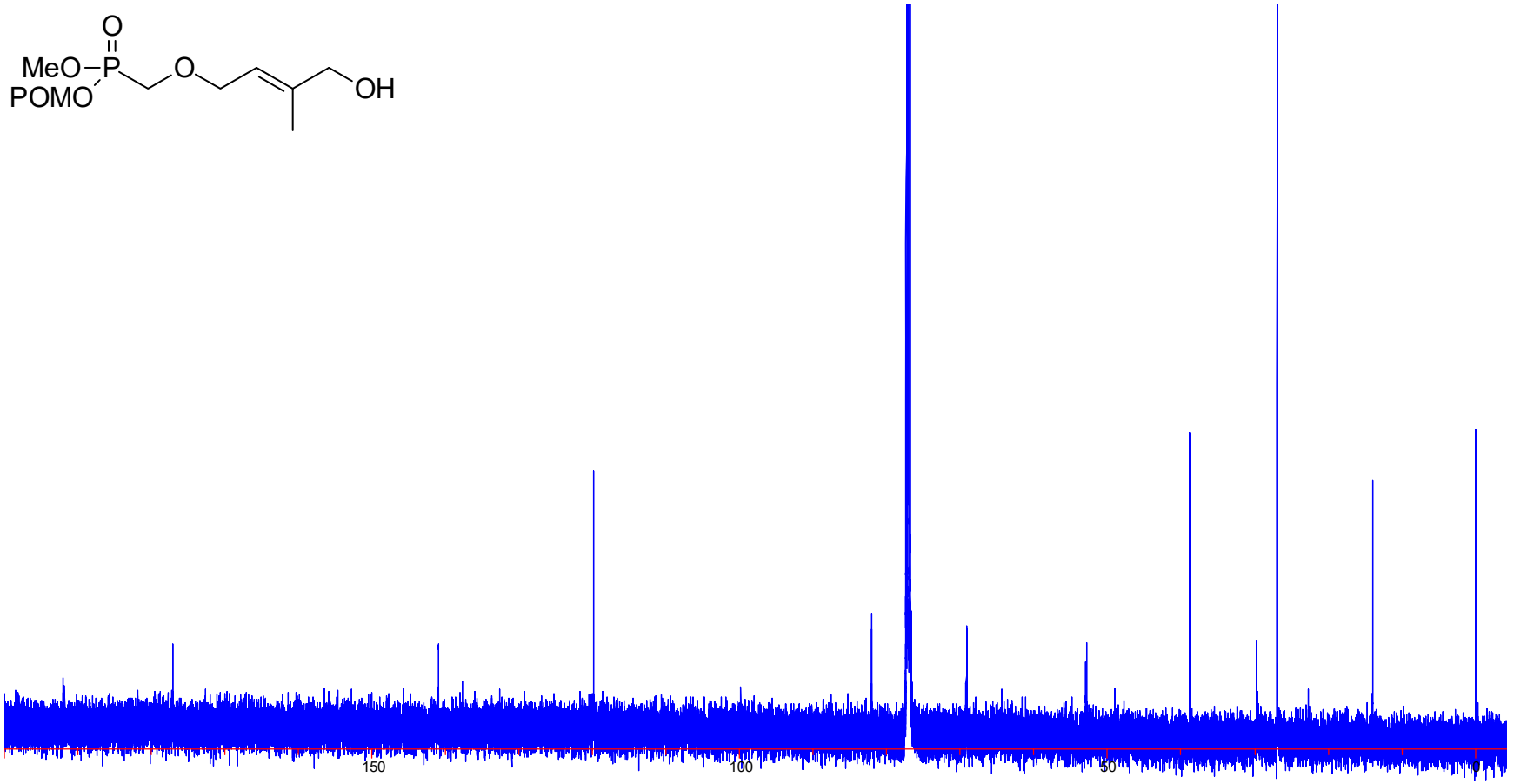


Figure A- 14. ^{13}C NMR spectrum of compound **43**

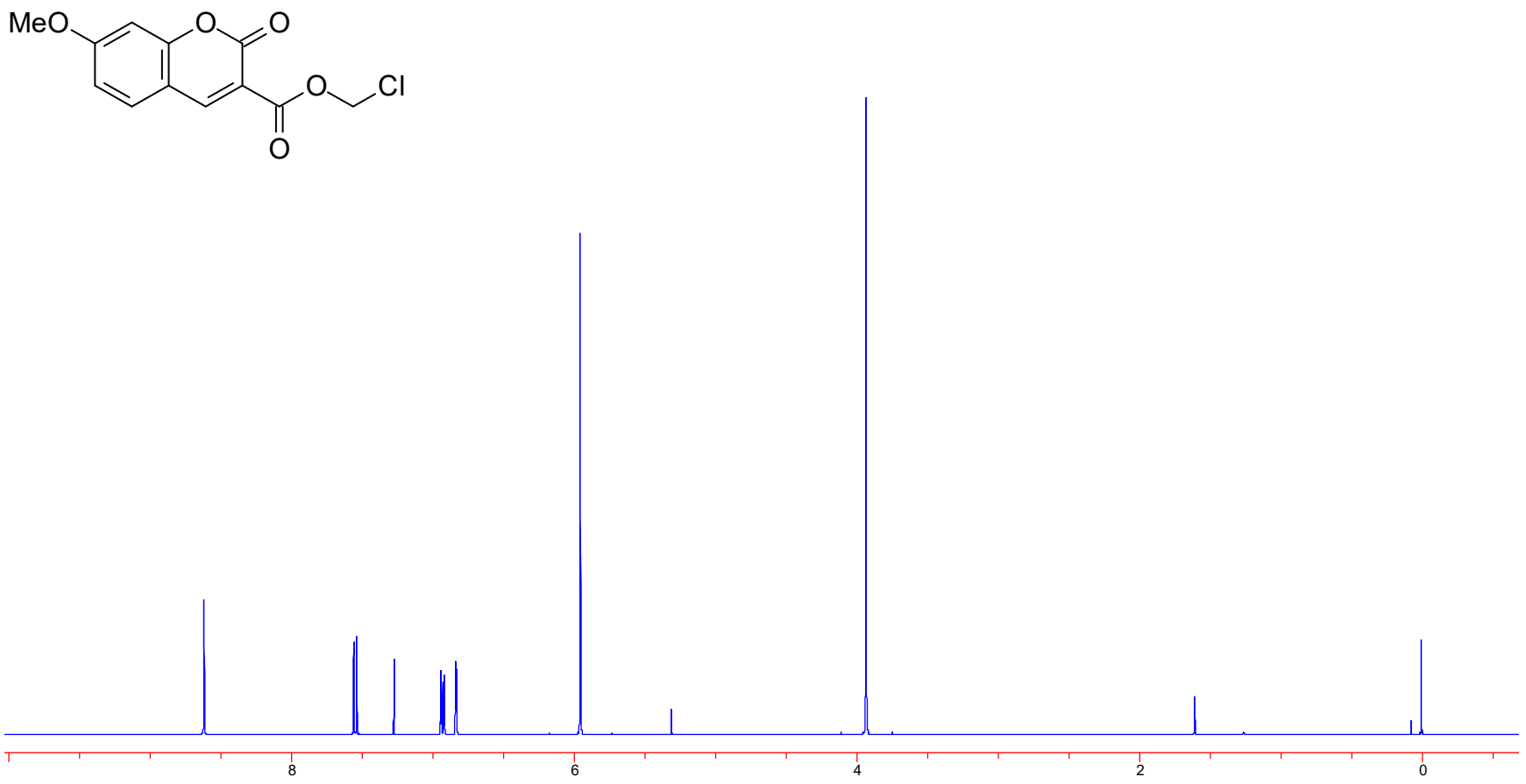


Figure A- 15. ^{1}H NMR spectrum of compound **47**

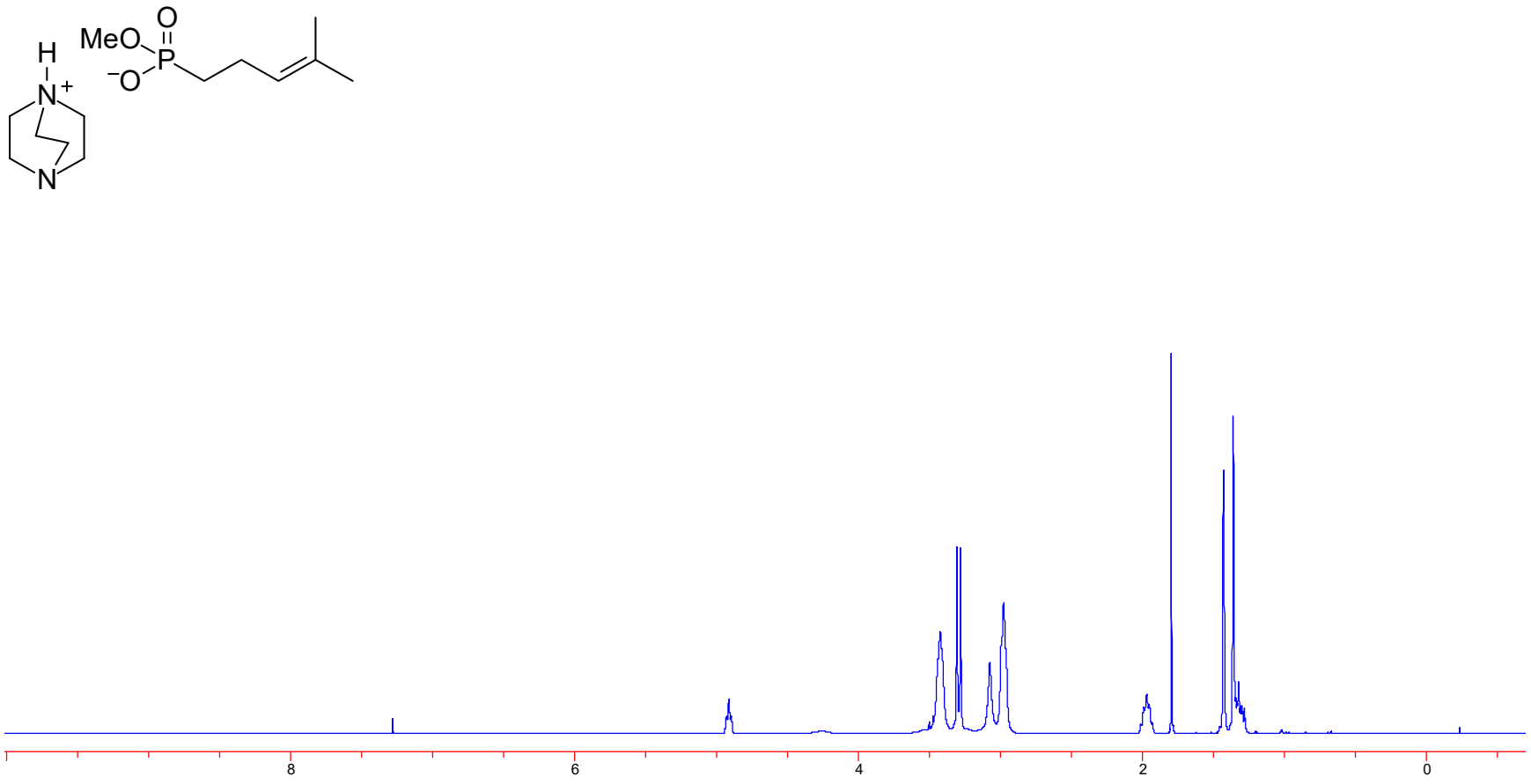


Figure A- 16. ^{1}H NMR spectrum of compound **48**

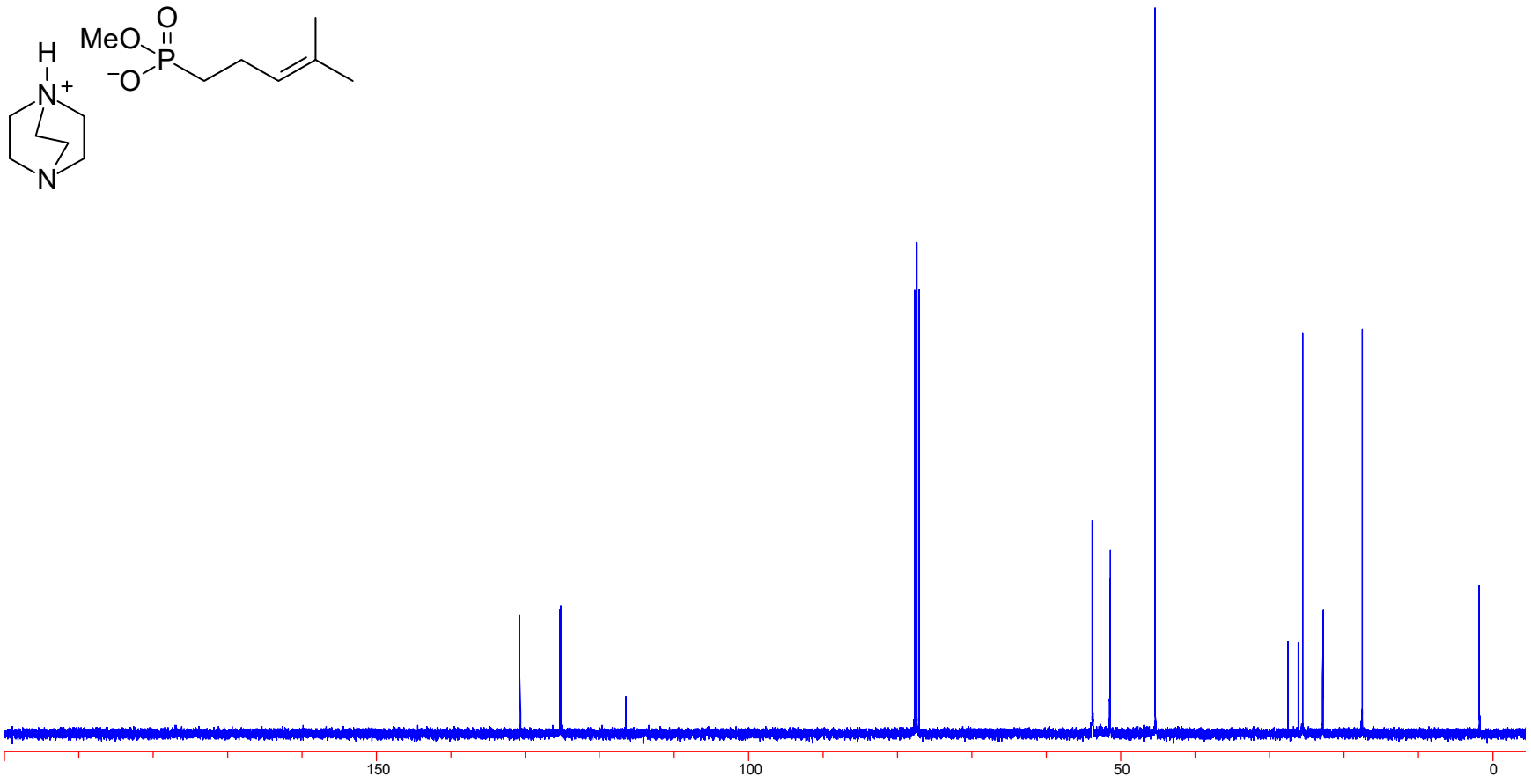


Figure A- 17. ^{13}C NMR spectrum of compound **48**

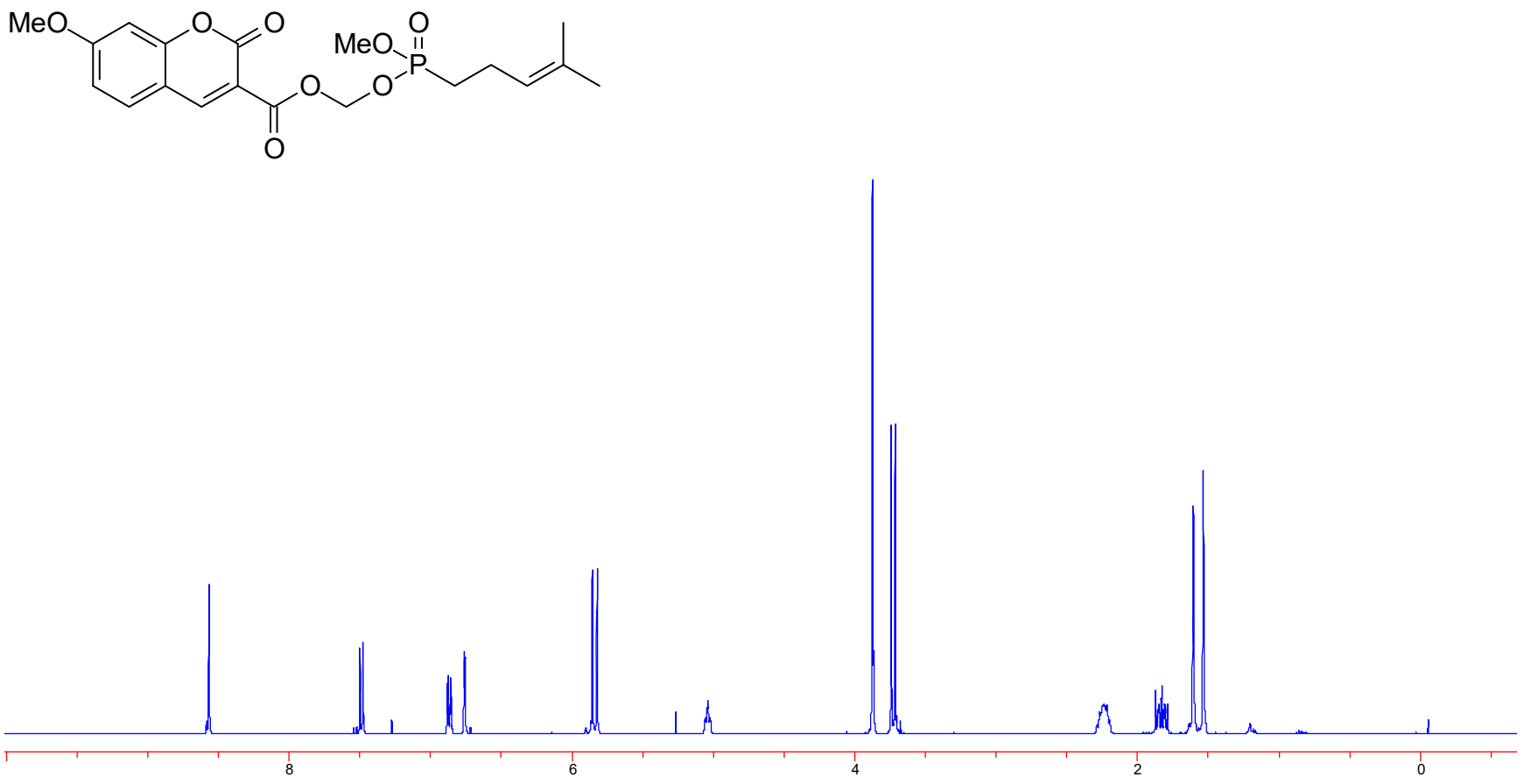


Figure A- 18. ^{1}H NMR spectrum of compound **49**

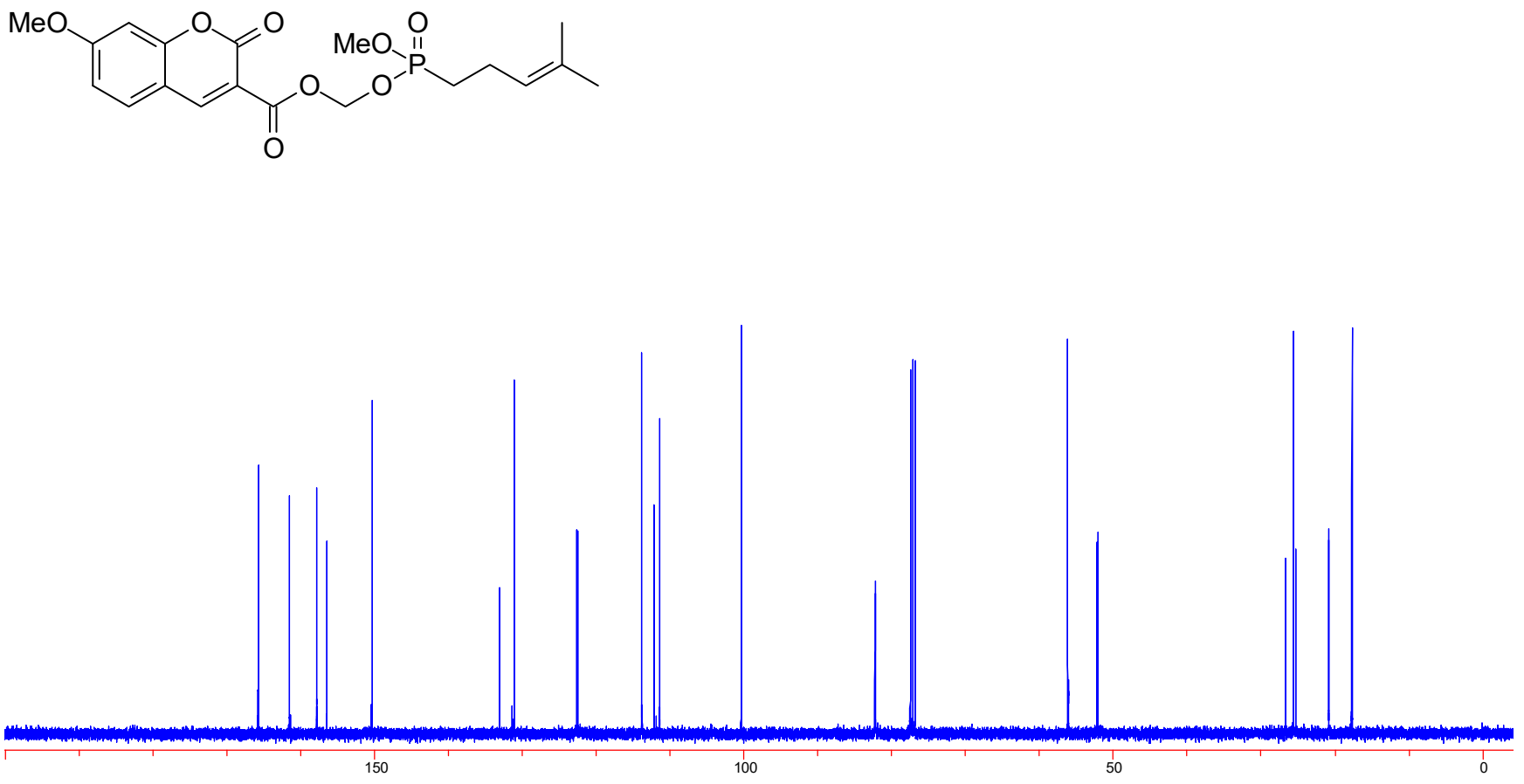


Figure A- 19. ^{13}C NMR spectrum of compound **49**

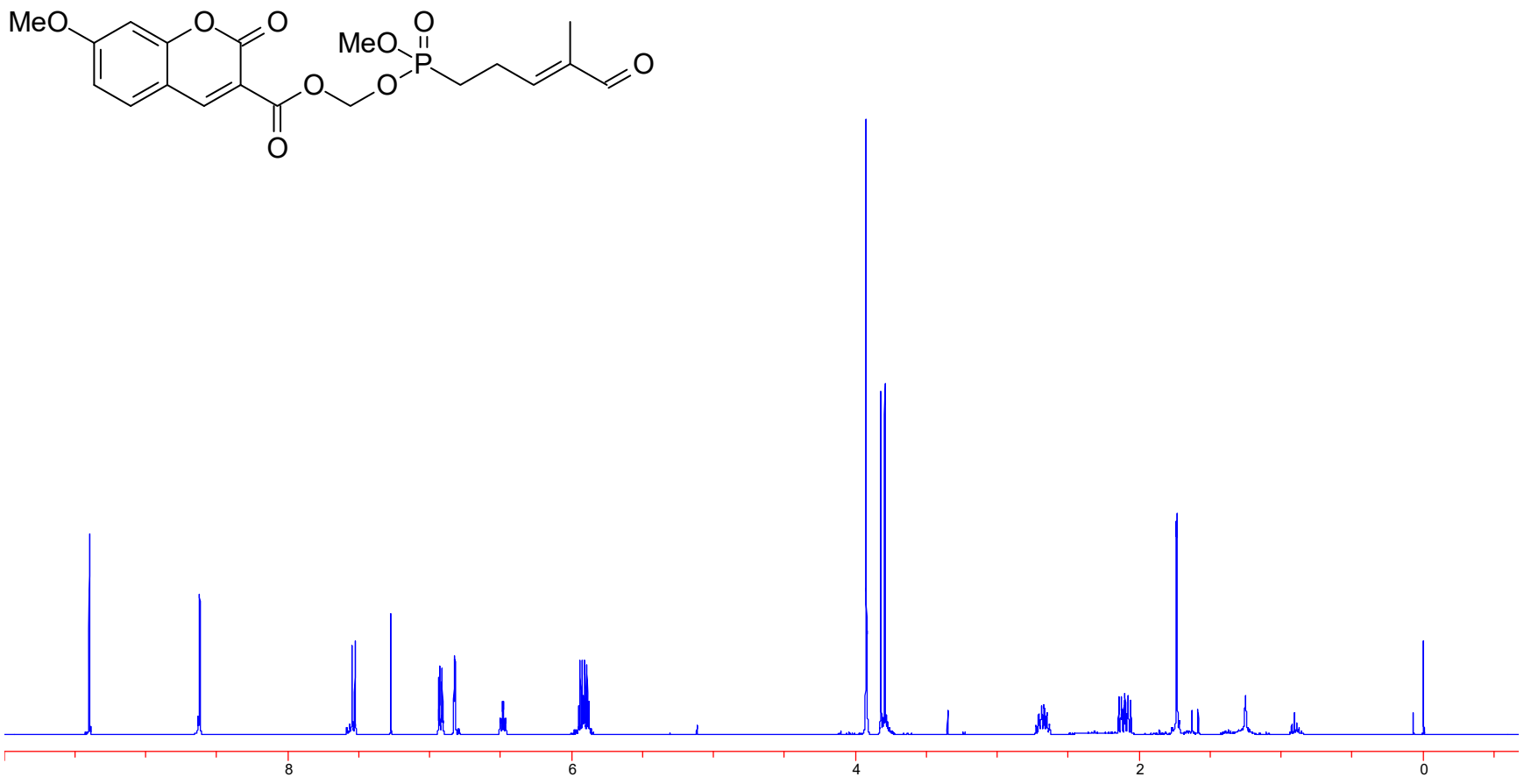


Figure A- 20. ^{1}H NMR spectrum of compound **50**

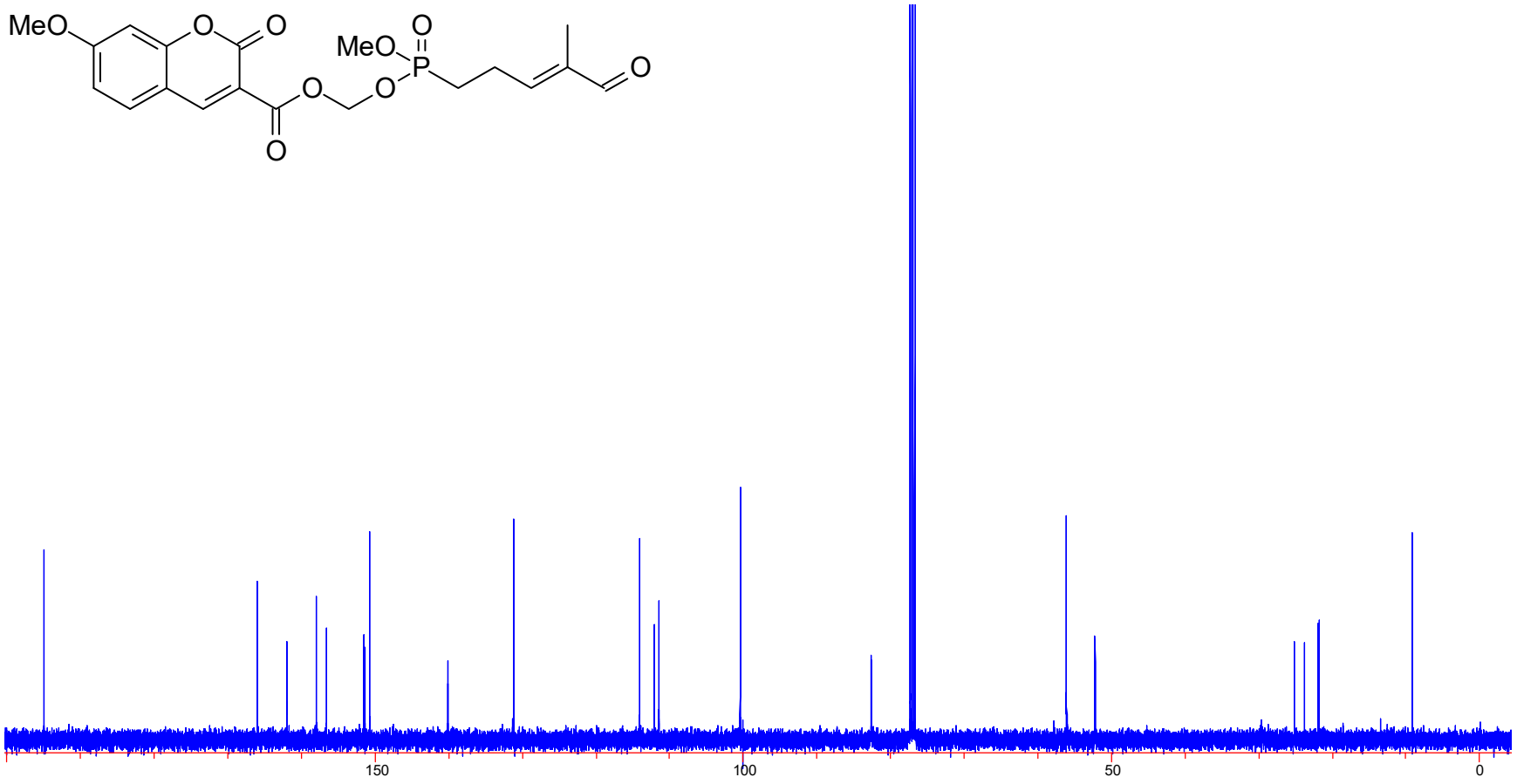


Figure A- 21. ^{13}C NMR spectrum of compound **50**

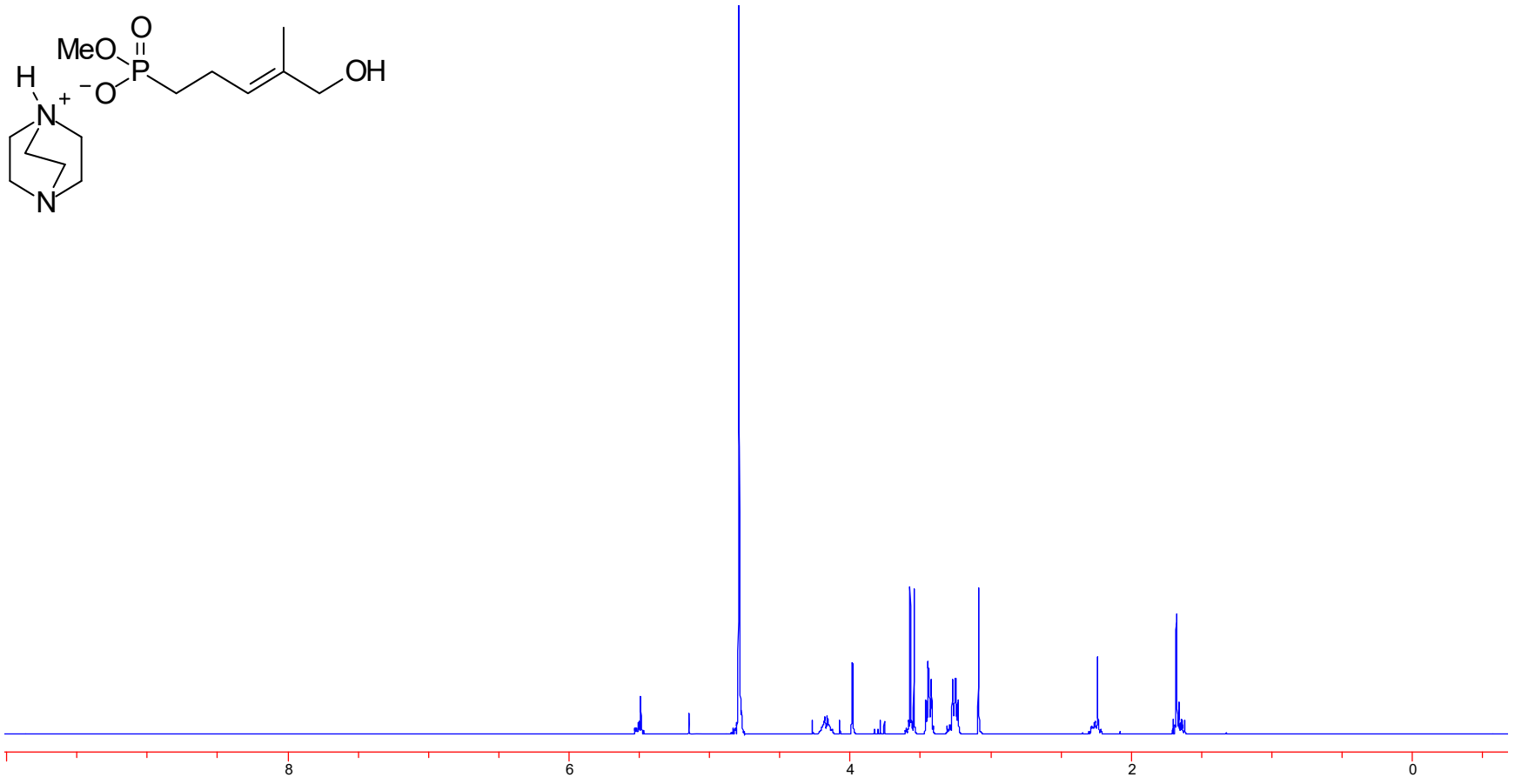


Figure A- 22. ^{1}H NMR spectrum of compound **53**

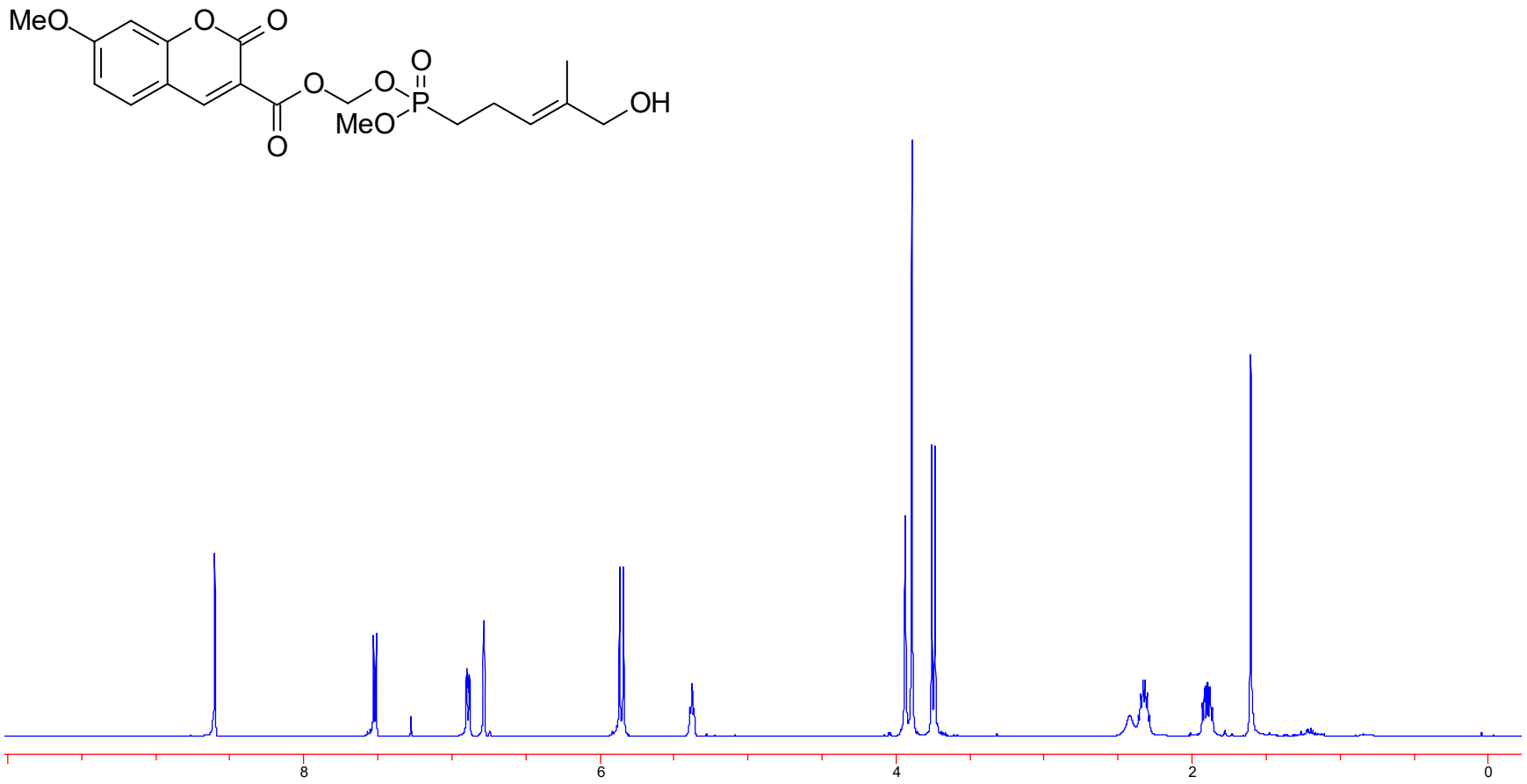


Figure A- 23. ^{1}H NMR spectrum of compound **54**

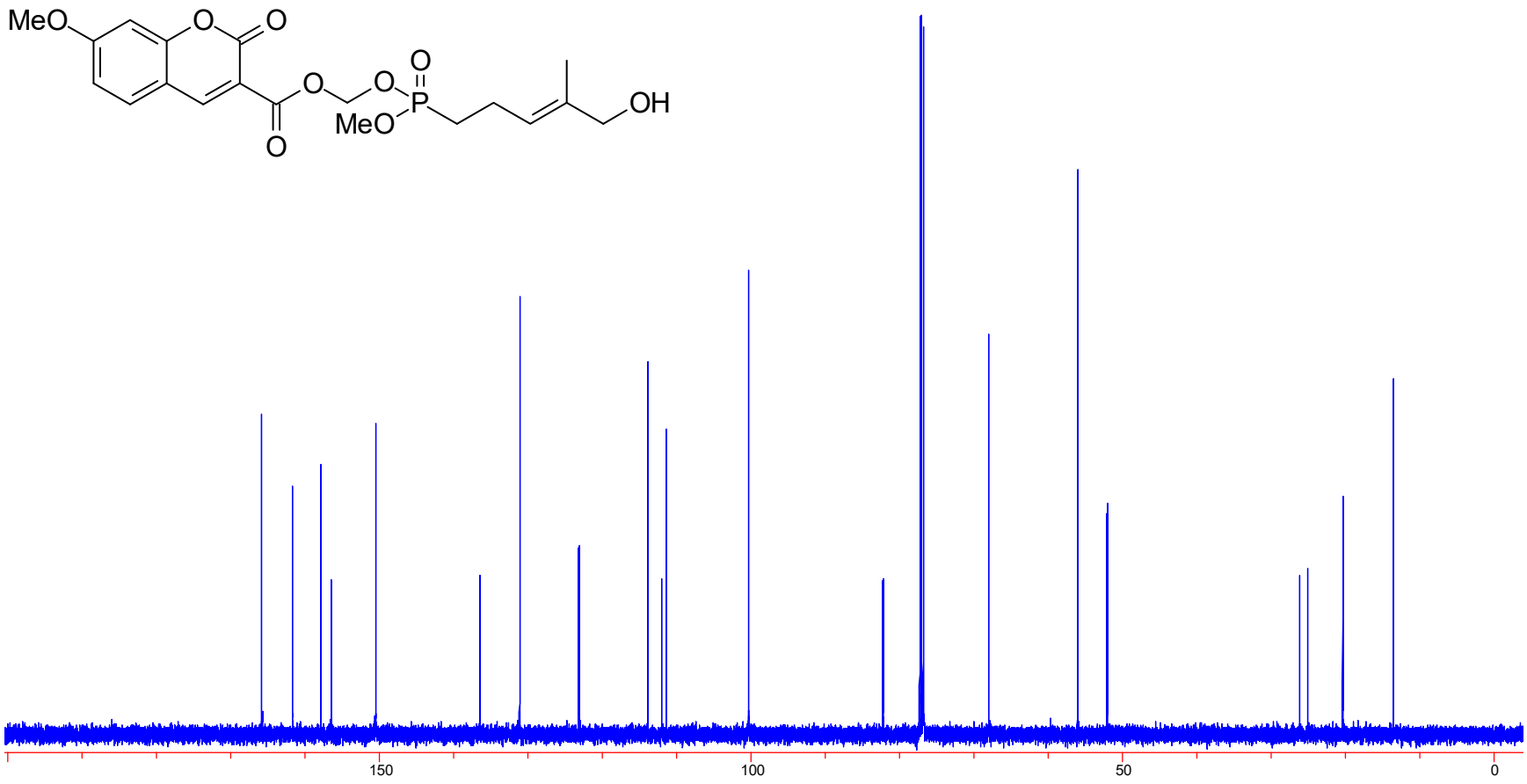


Figure A- 24. ^{13}C NMR spectrum of compound **54**

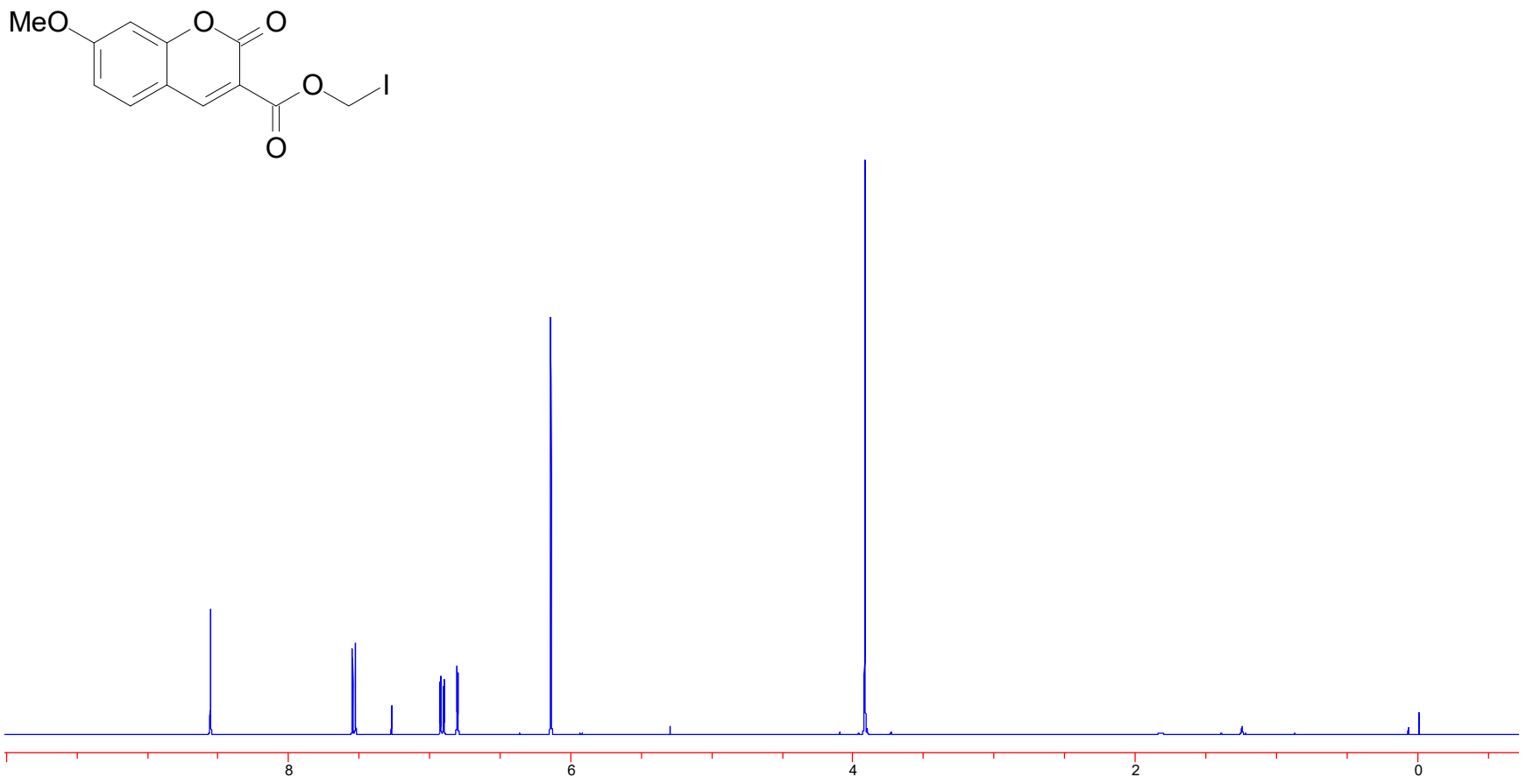


Figure A- 25. ^{1}H NMR spectrum of compound **58**

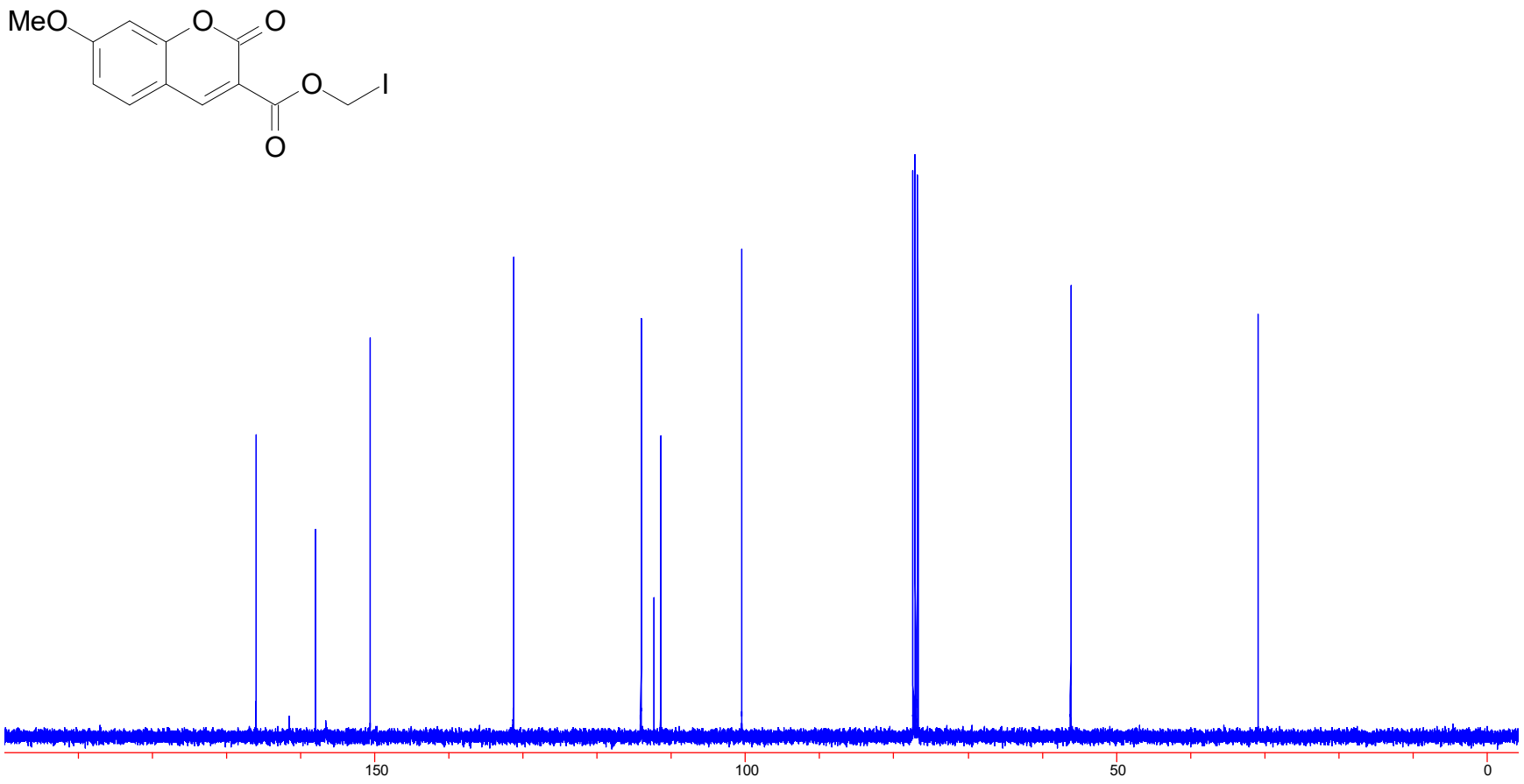


Figure A- 26. ^{13}C NMR spectrum of compound **58**

Figure A- 27. ^{1}H NMR spectrum of compound **57**

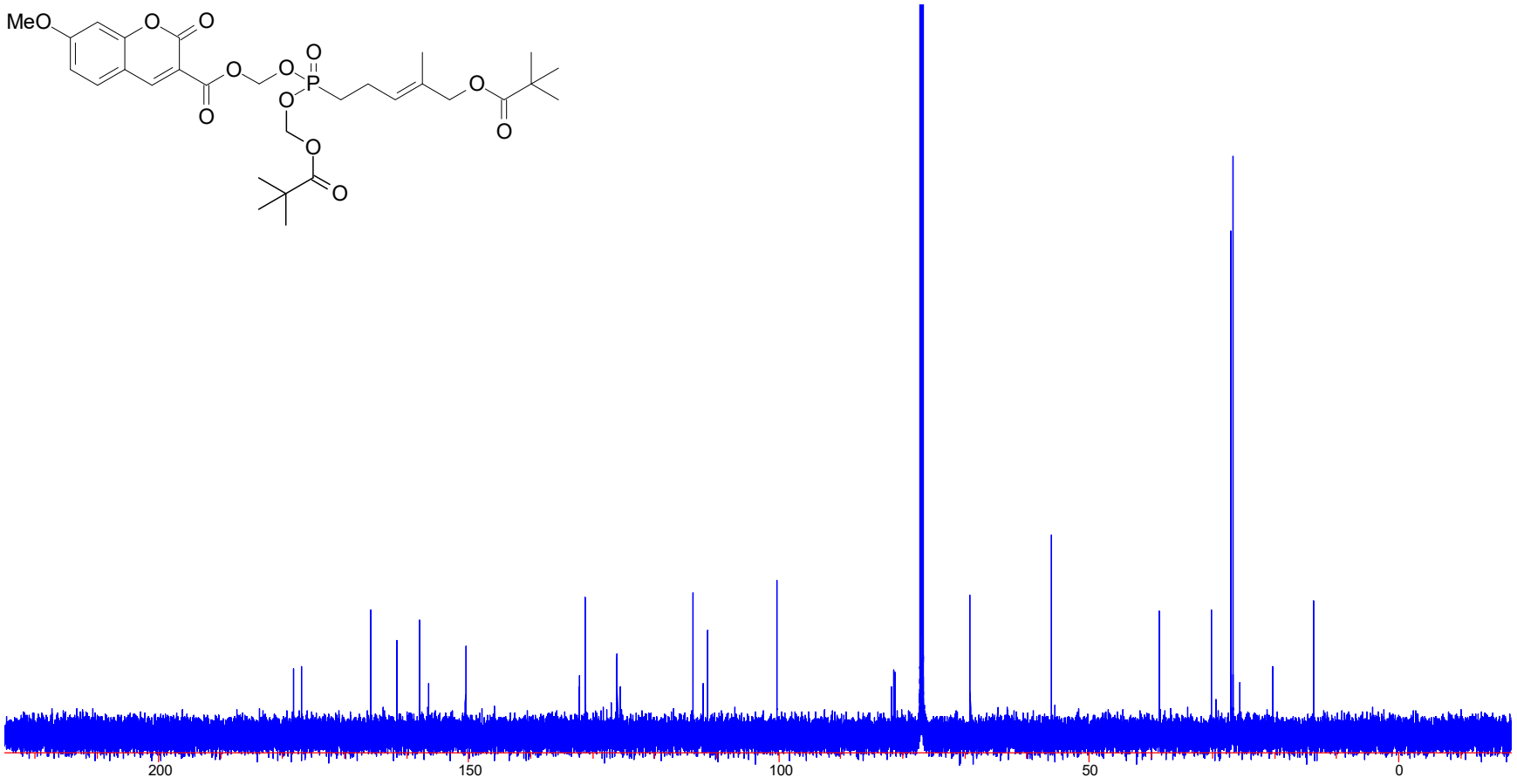


Figure A- 28. ^{13}C NMR spectrum of compound **57**

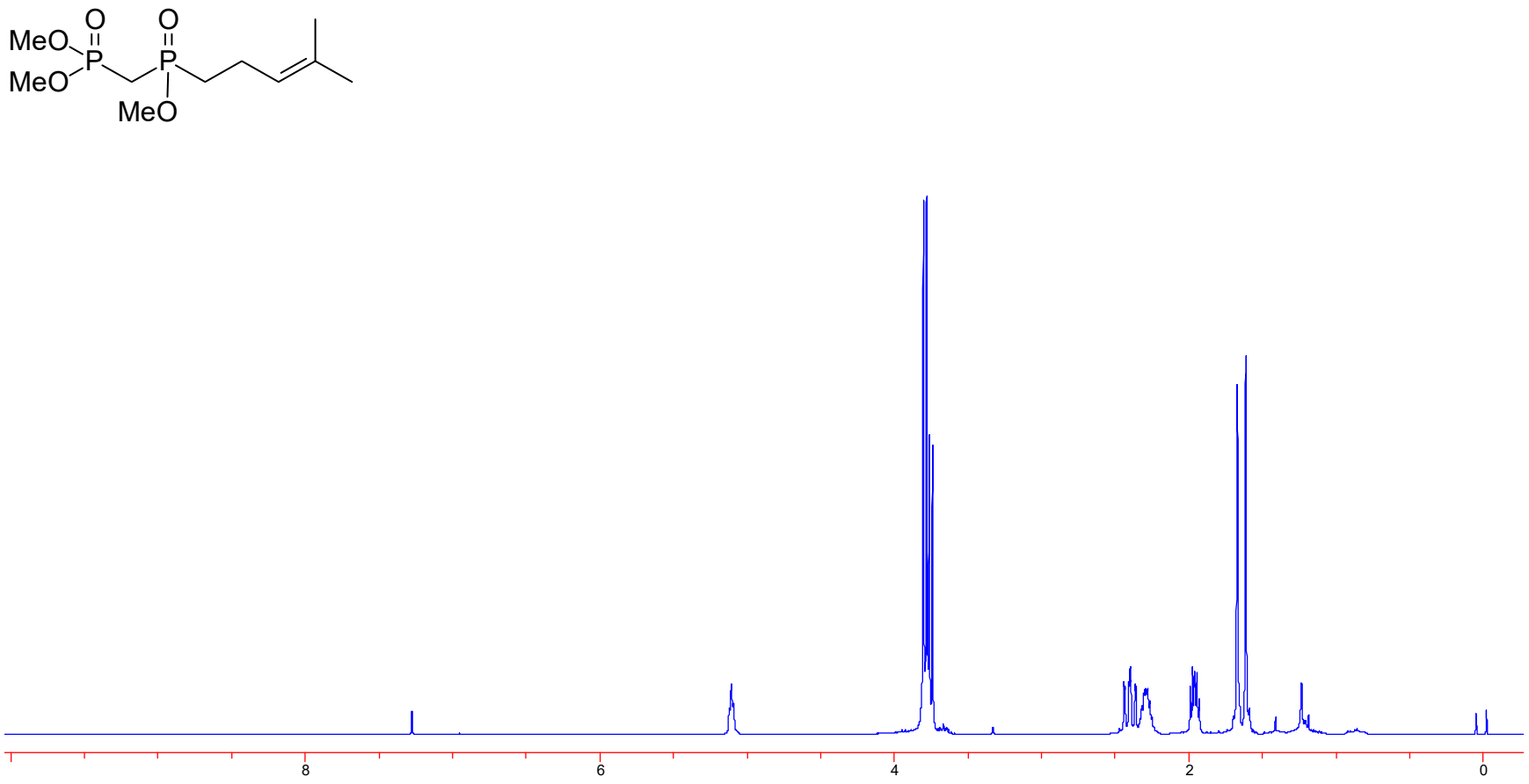


Figure A- 29. ^{1}H NMR spectrum of compound **67**

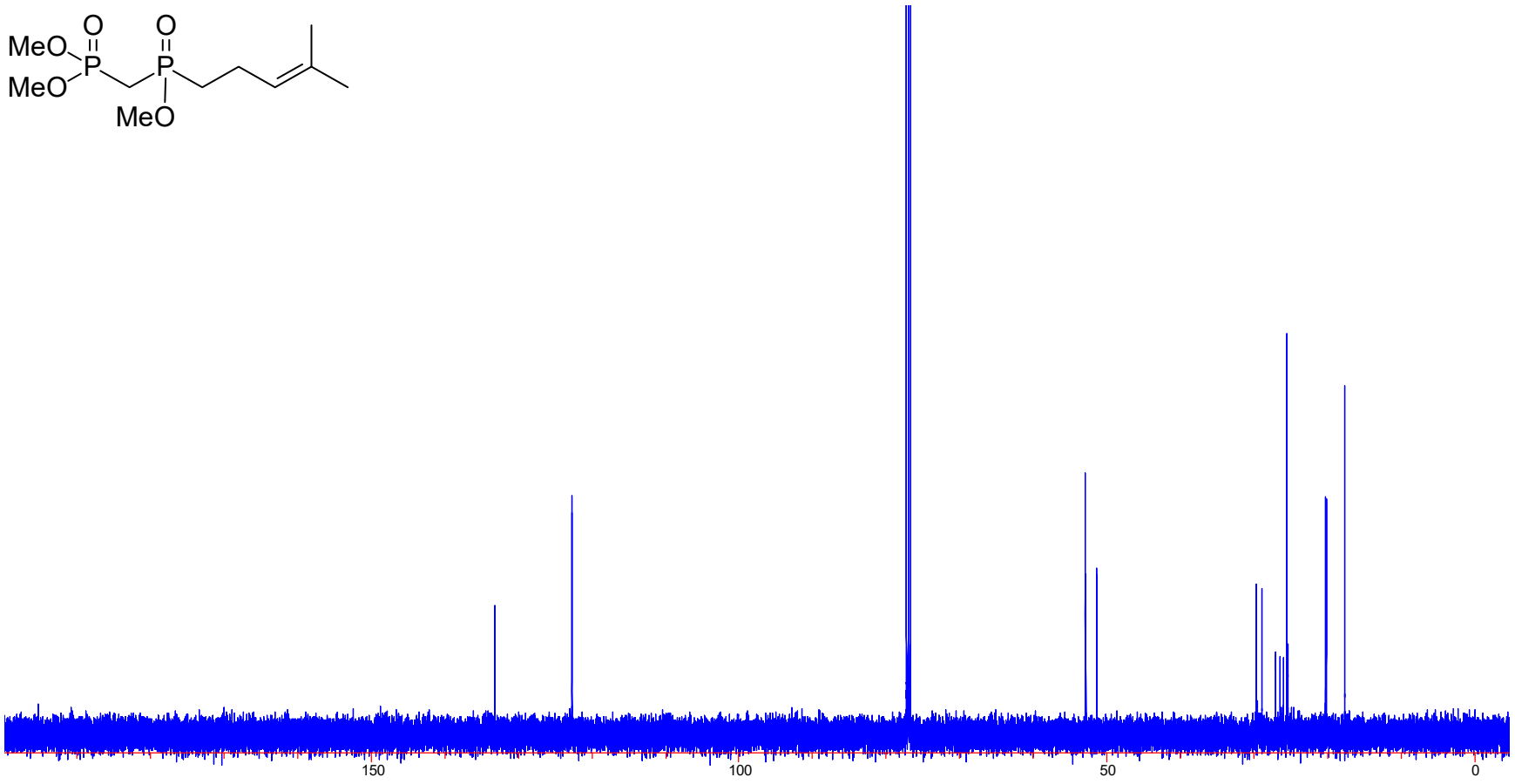
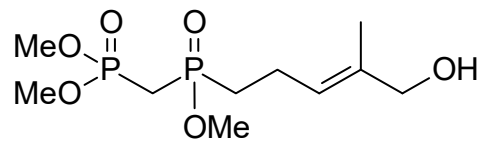


Figure A- 30. ^{13}C NMR spectrum of compound **67**



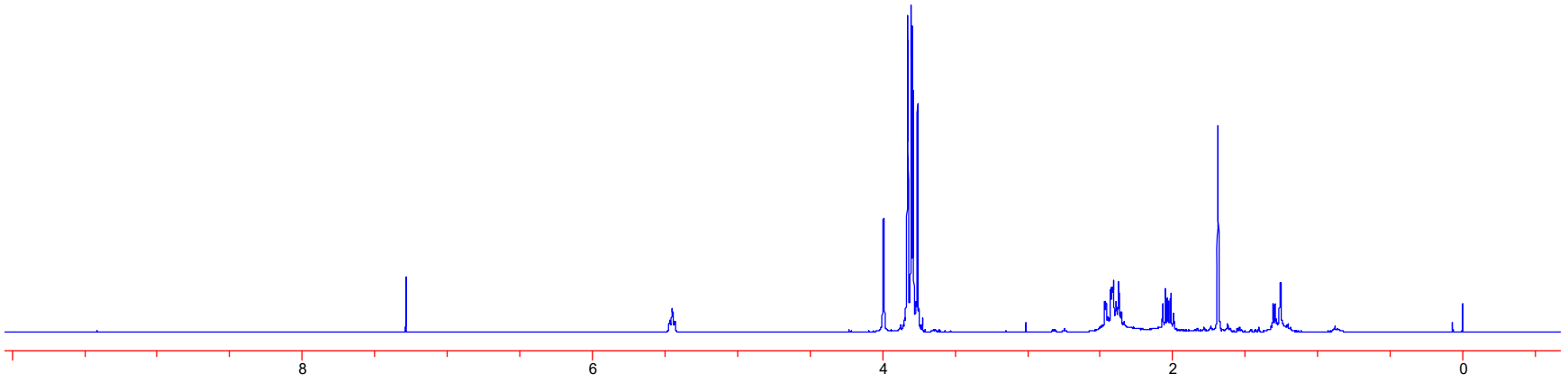


Figure A- 31. ^{1}H NMR spectrum for compound **70**

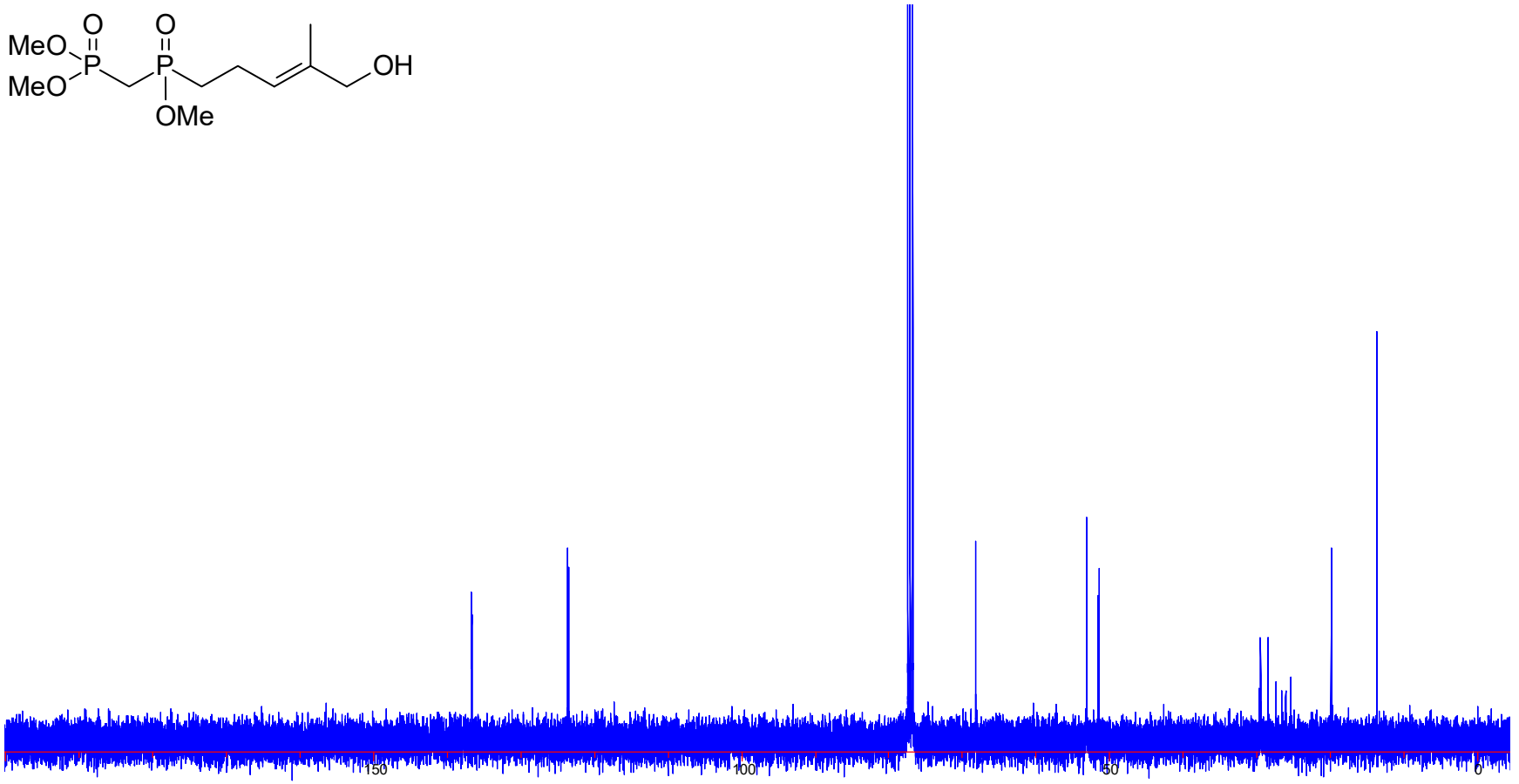


Figure A- 32. ^{13}C NMR spectrum for compound **70**

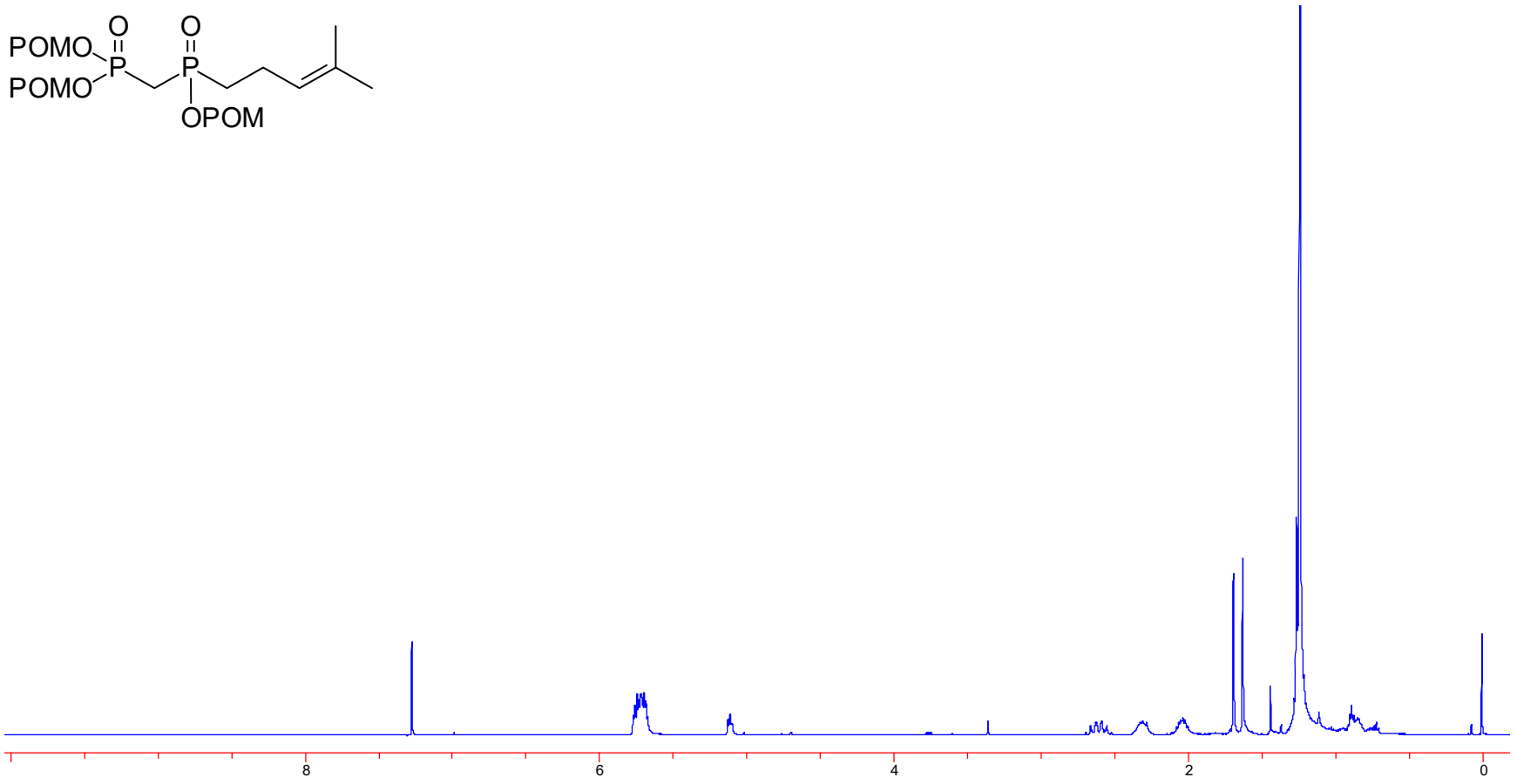


Figure A- 33. ^{1}H NMR spectrum of compound **71**

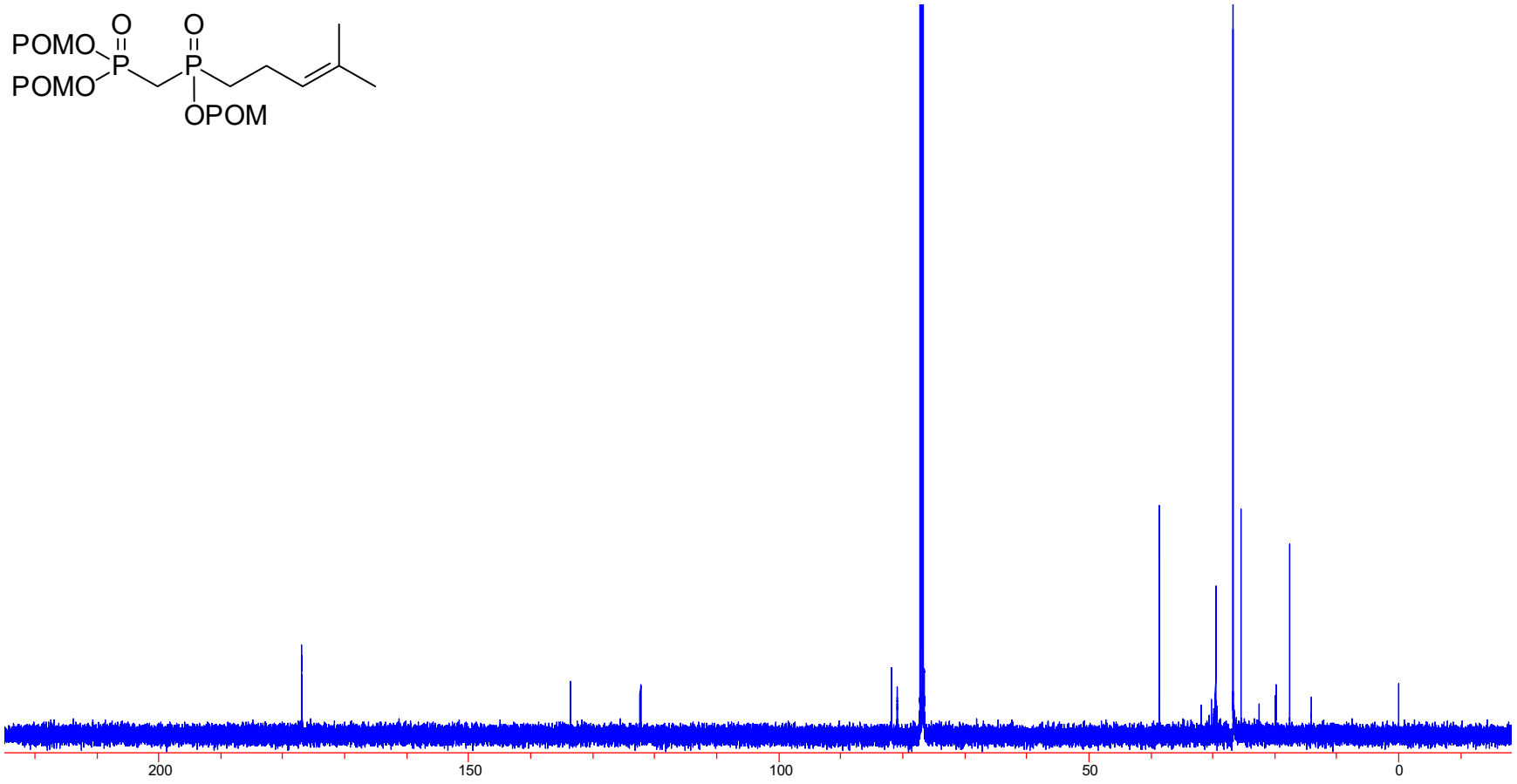


Figure A- 34. ^{13}C NMR spectrum of compound **71**

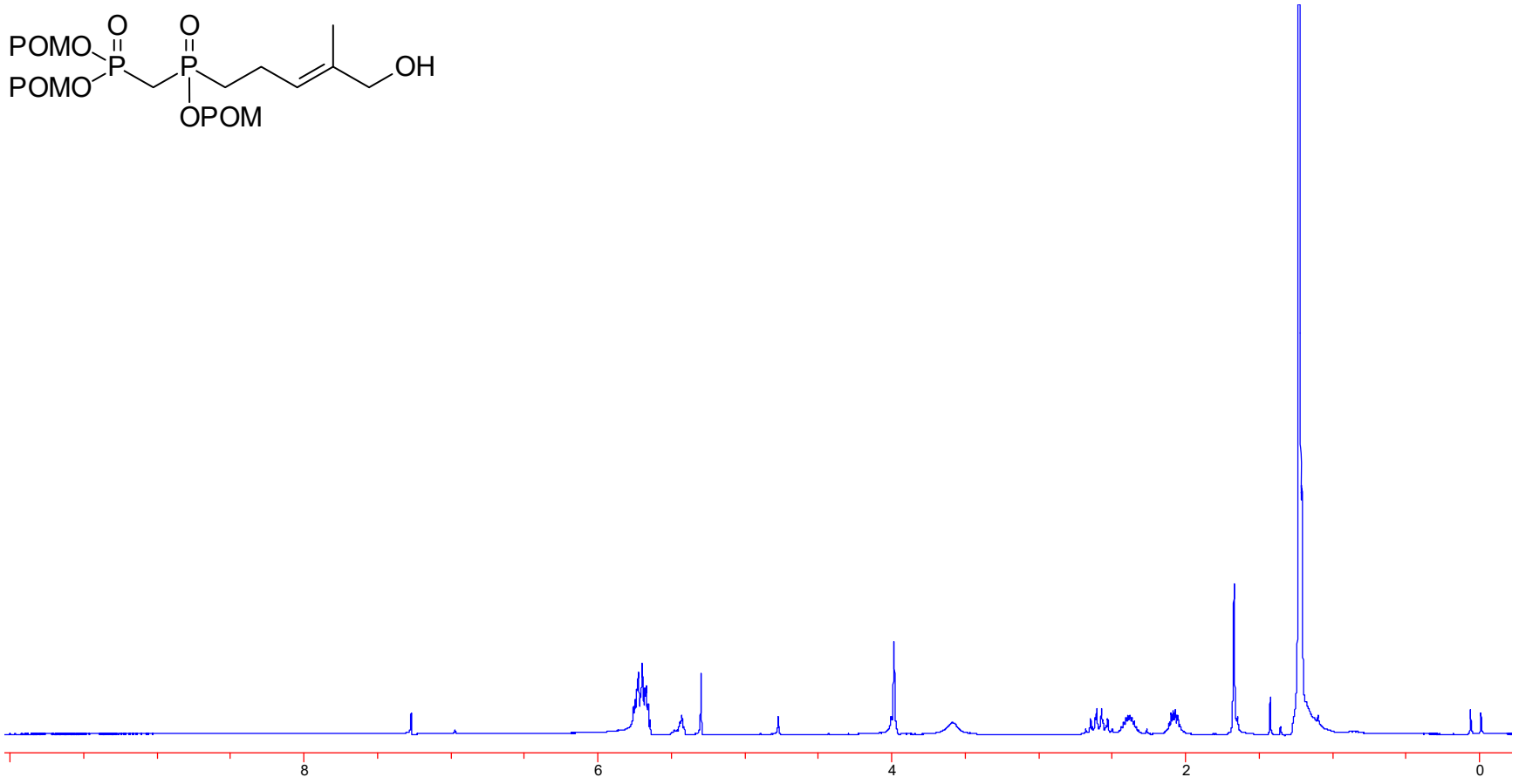


Figure A- 35. ^{1}H NMR spectrum of compound **72**

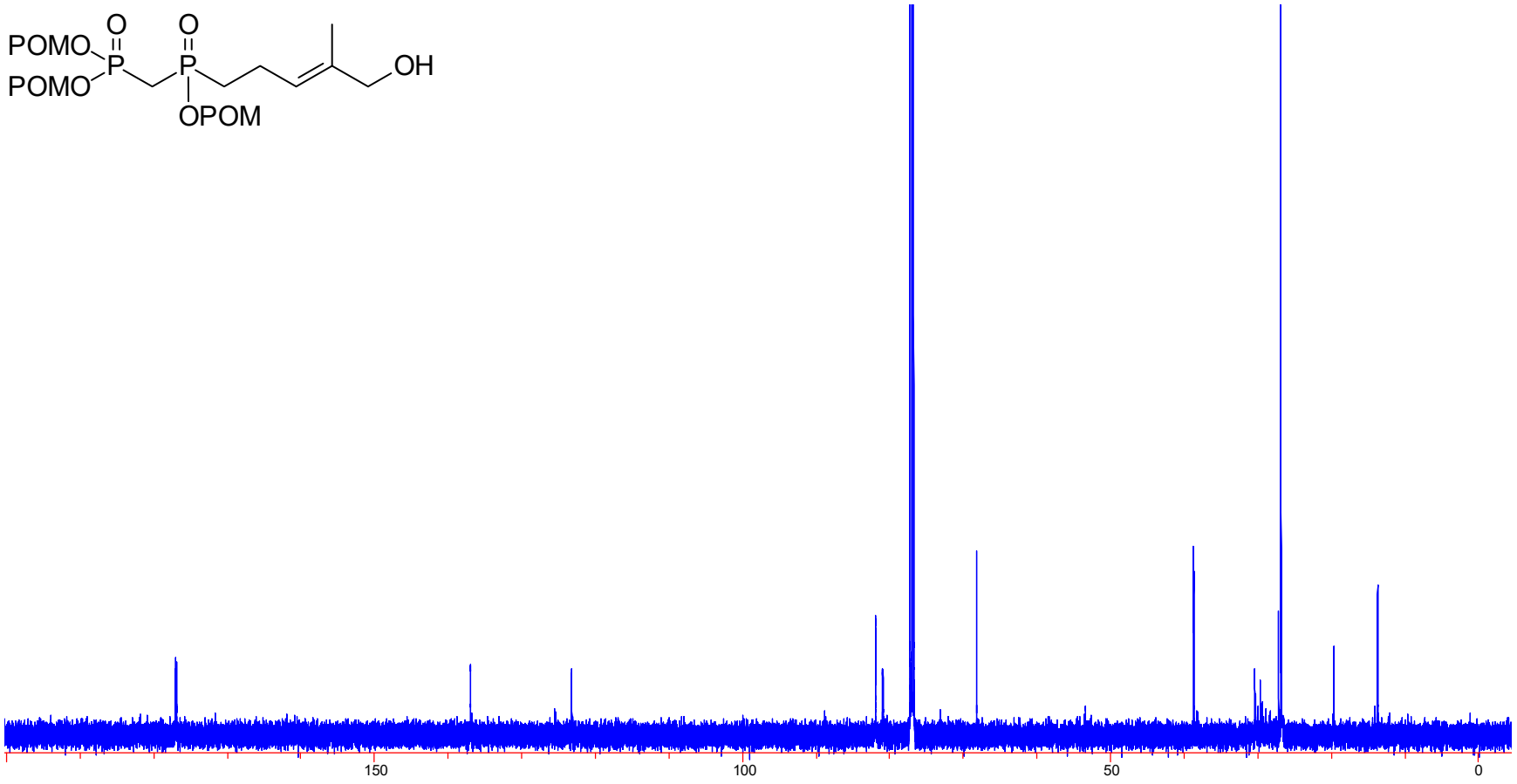


Figure A- 36. ^{13}C NMR spectrum of compound **72**

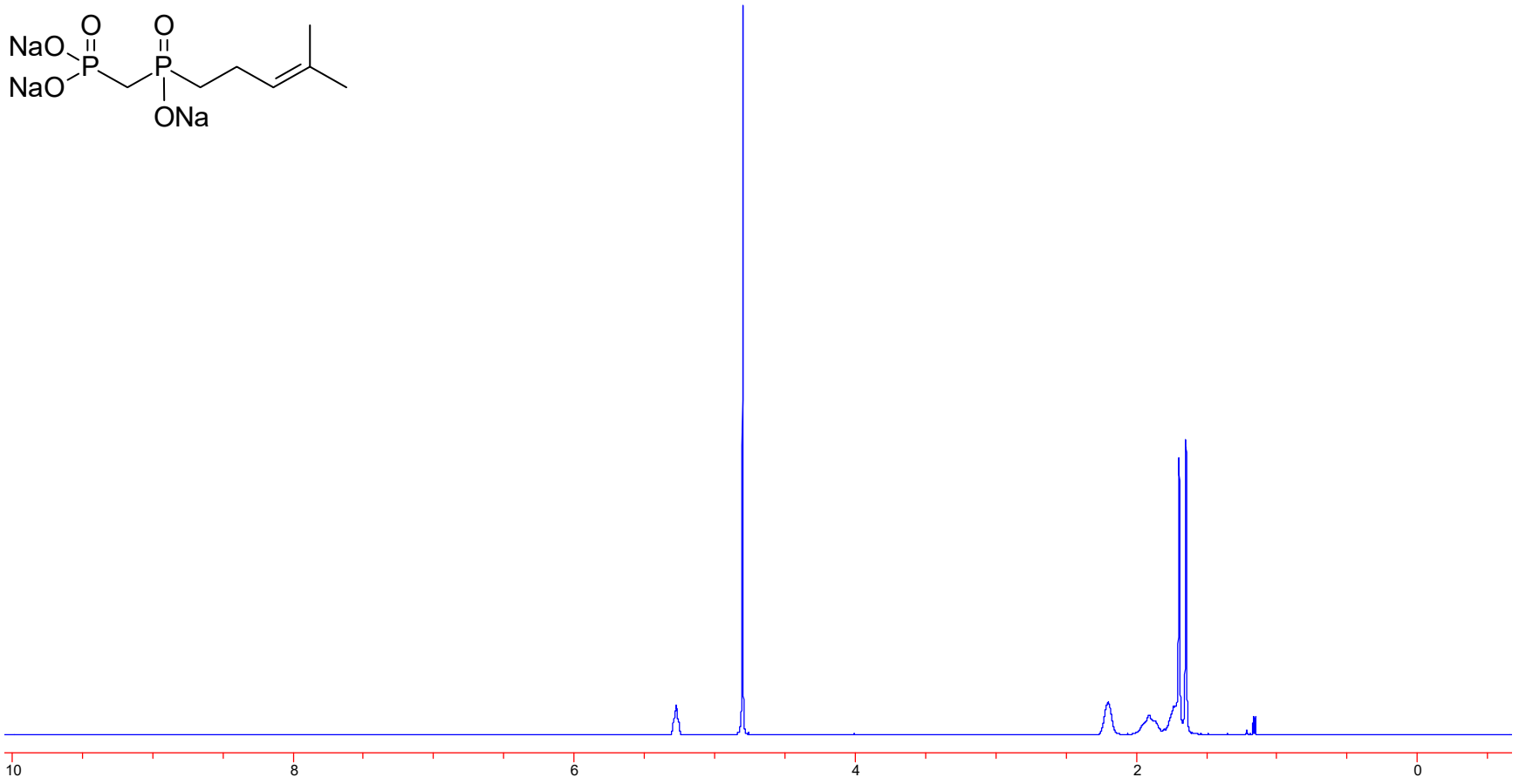


Figure A- 37. ^{1}H NMR spectrum of compound **73**

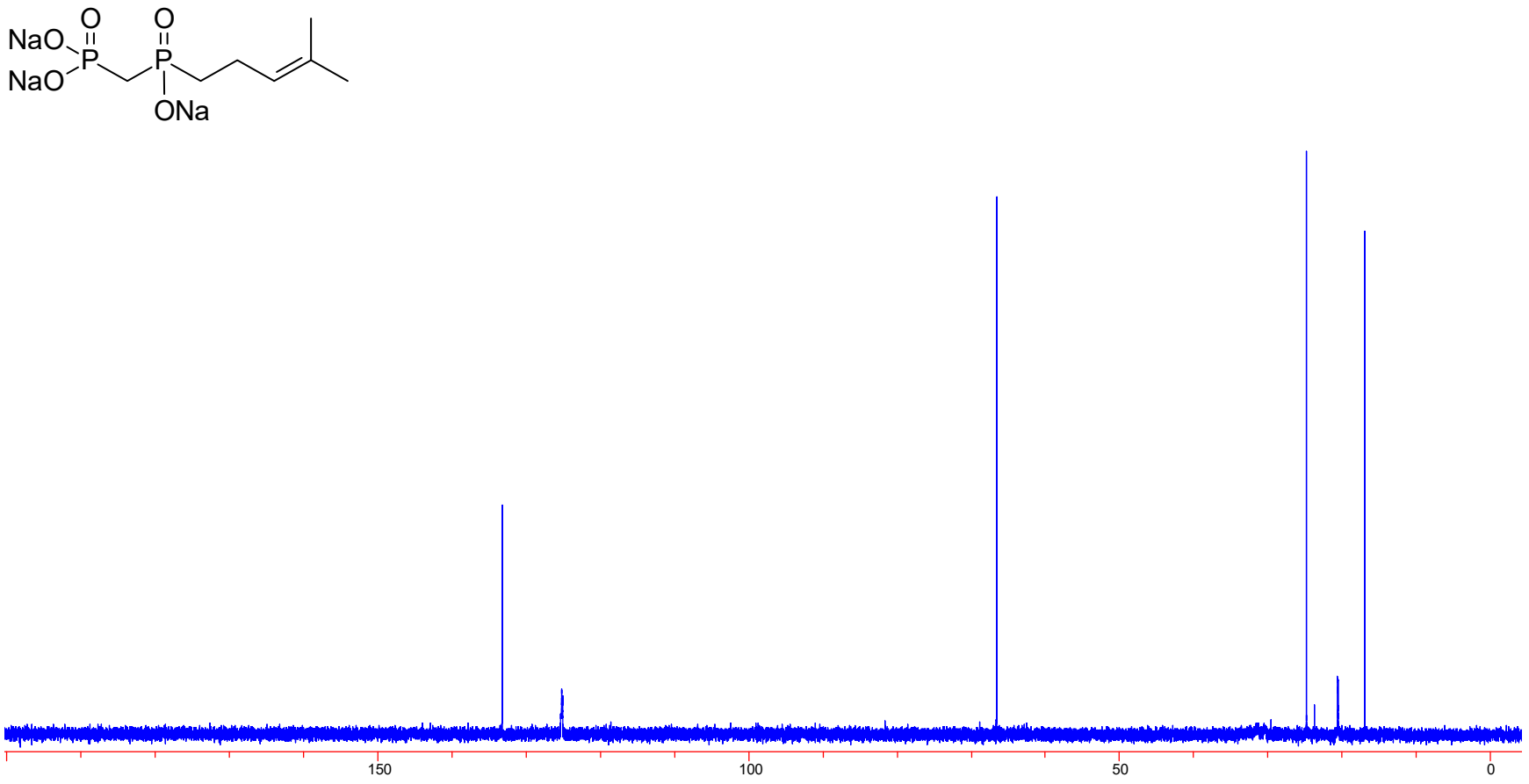


Figure A- 38. ^{13}C NMR spectrum of compound **73**

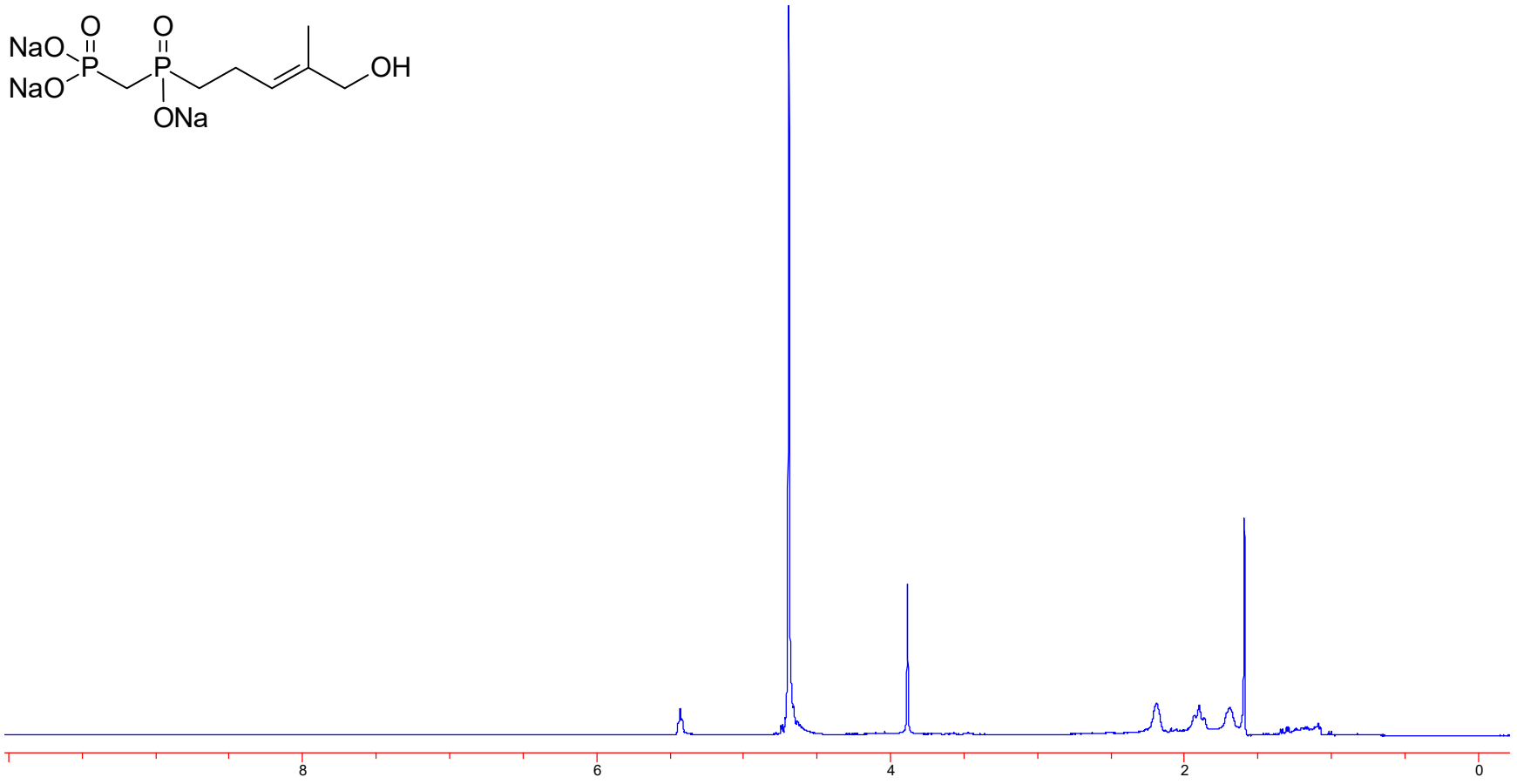


Figure A- 39. ^{1}H NMR spectrum of compound **74**

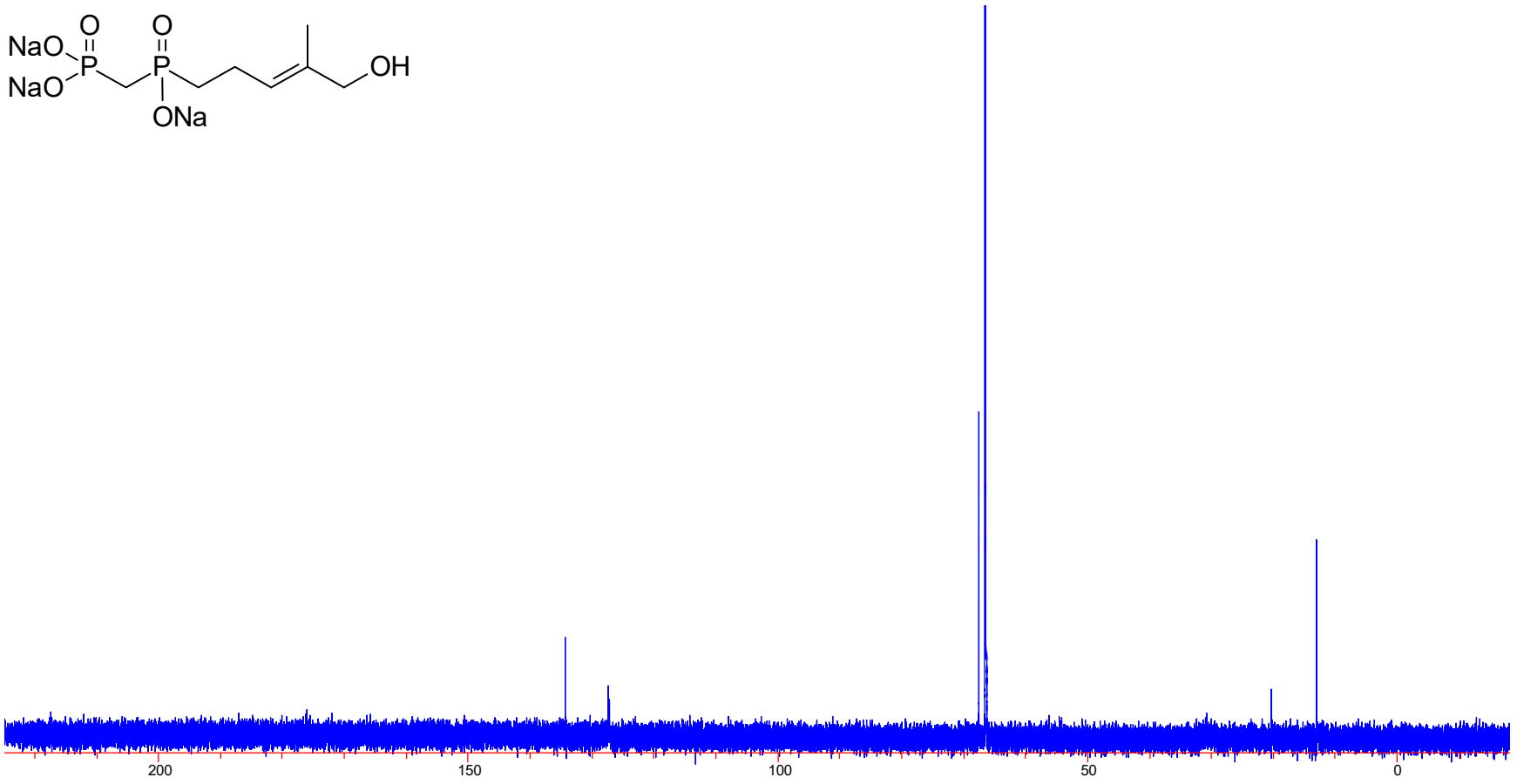


Figure A- 40. ^{13}C NMR spectrum of compound **74**

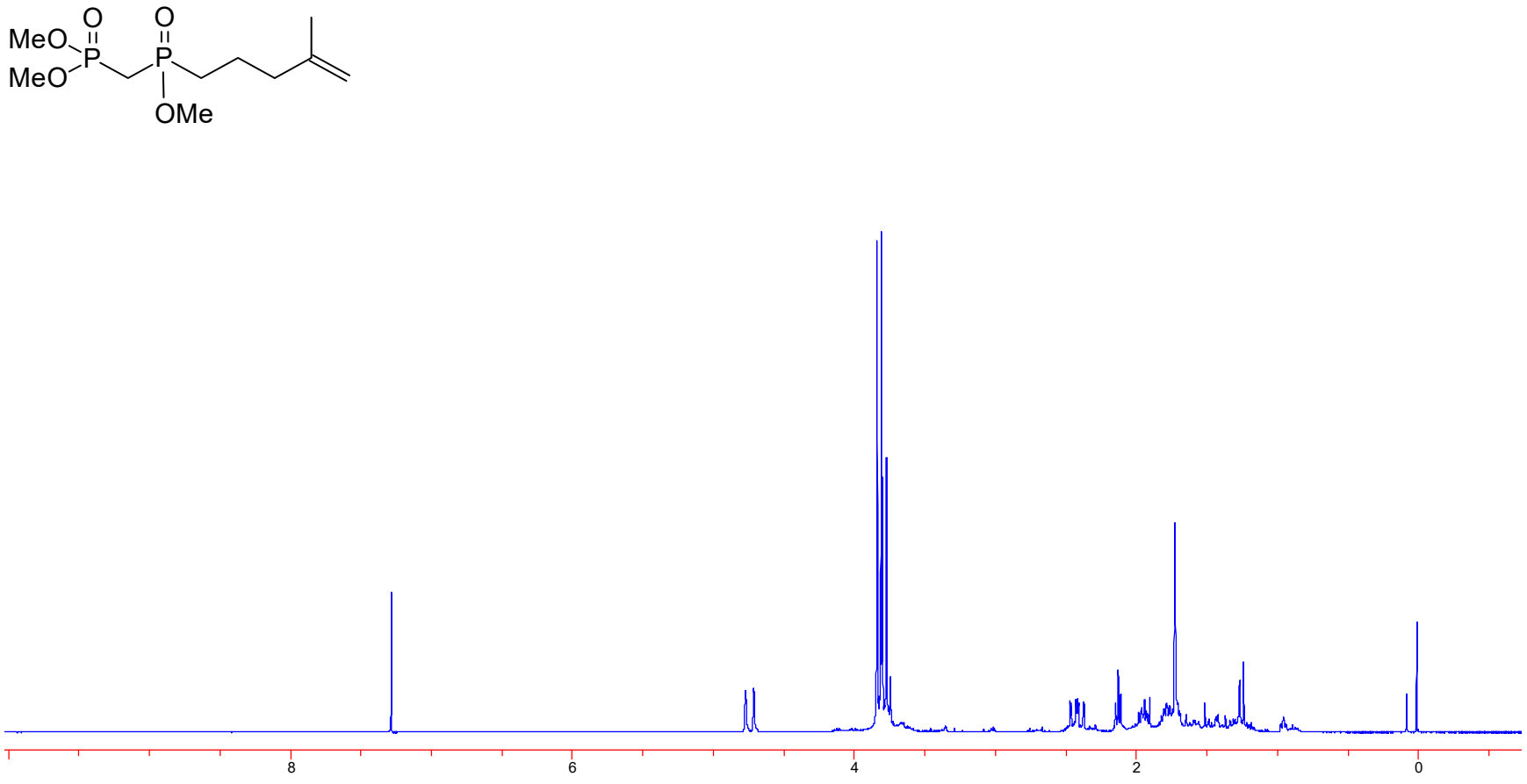


Figure A- 41. ^{1}H NMR spectrum of compound **84**

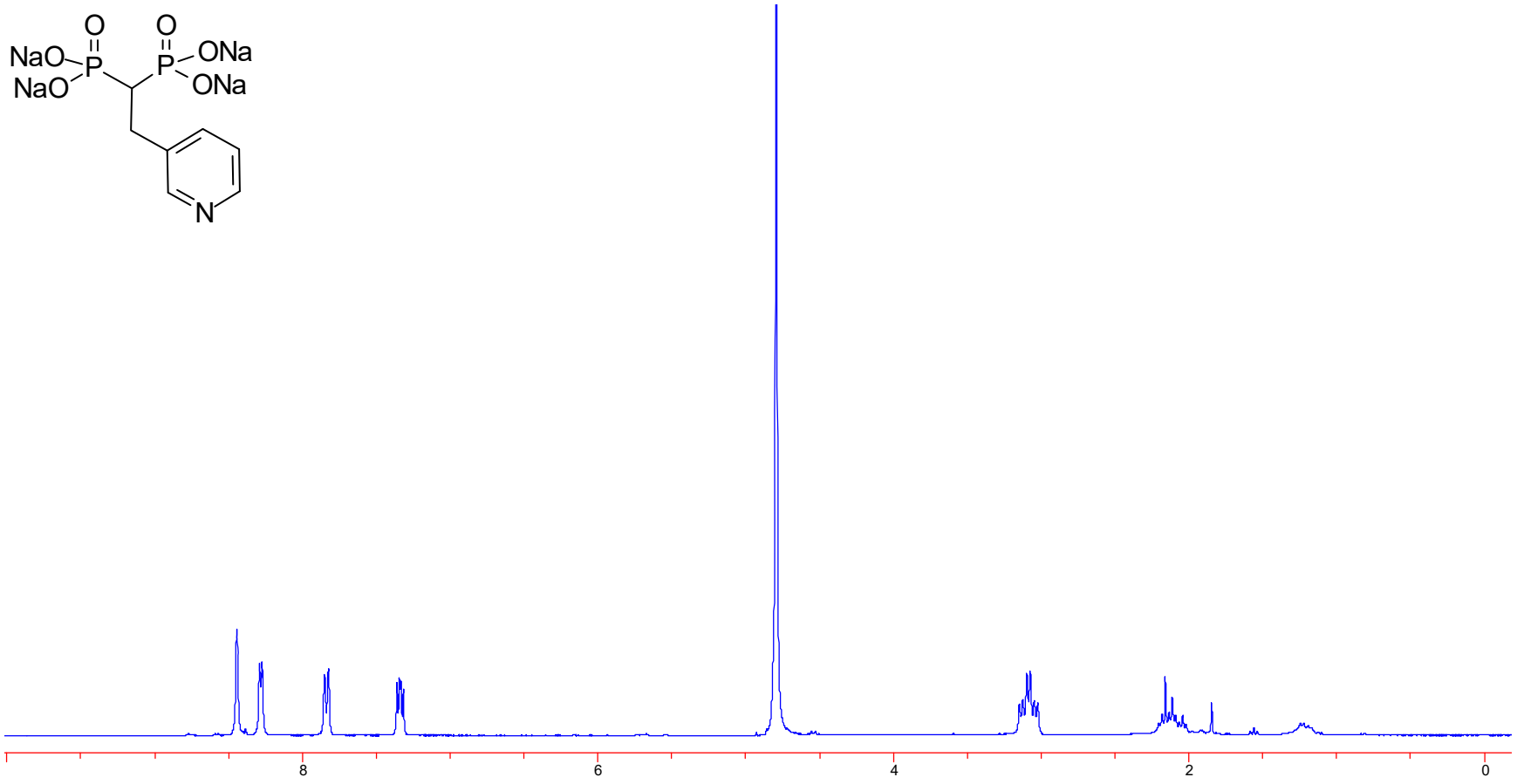


Figure A- 42. ^{1}H NMR spectrum for compound **110**

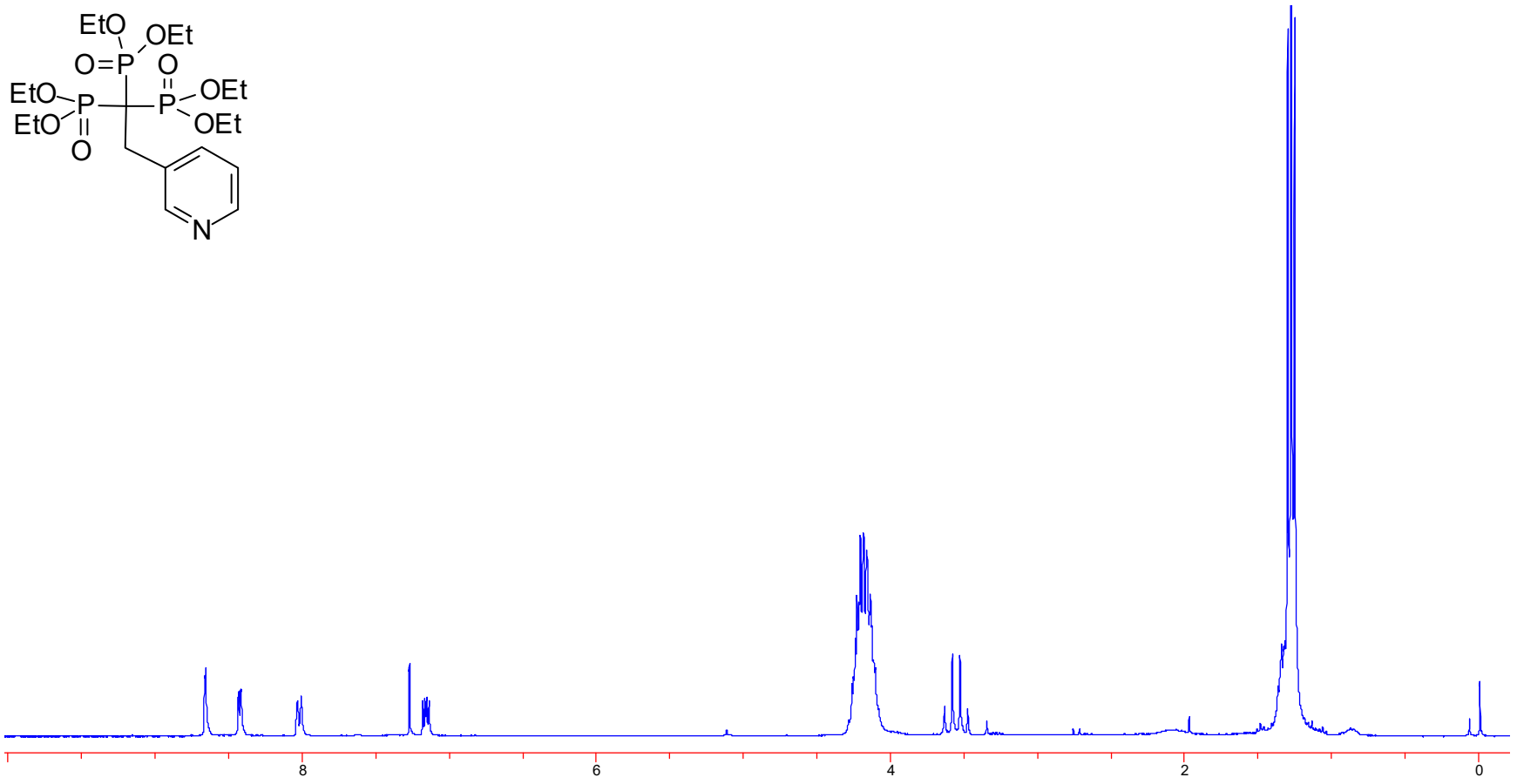


Figure A- 43. ^{1}H NMR spectrum of compound **111**

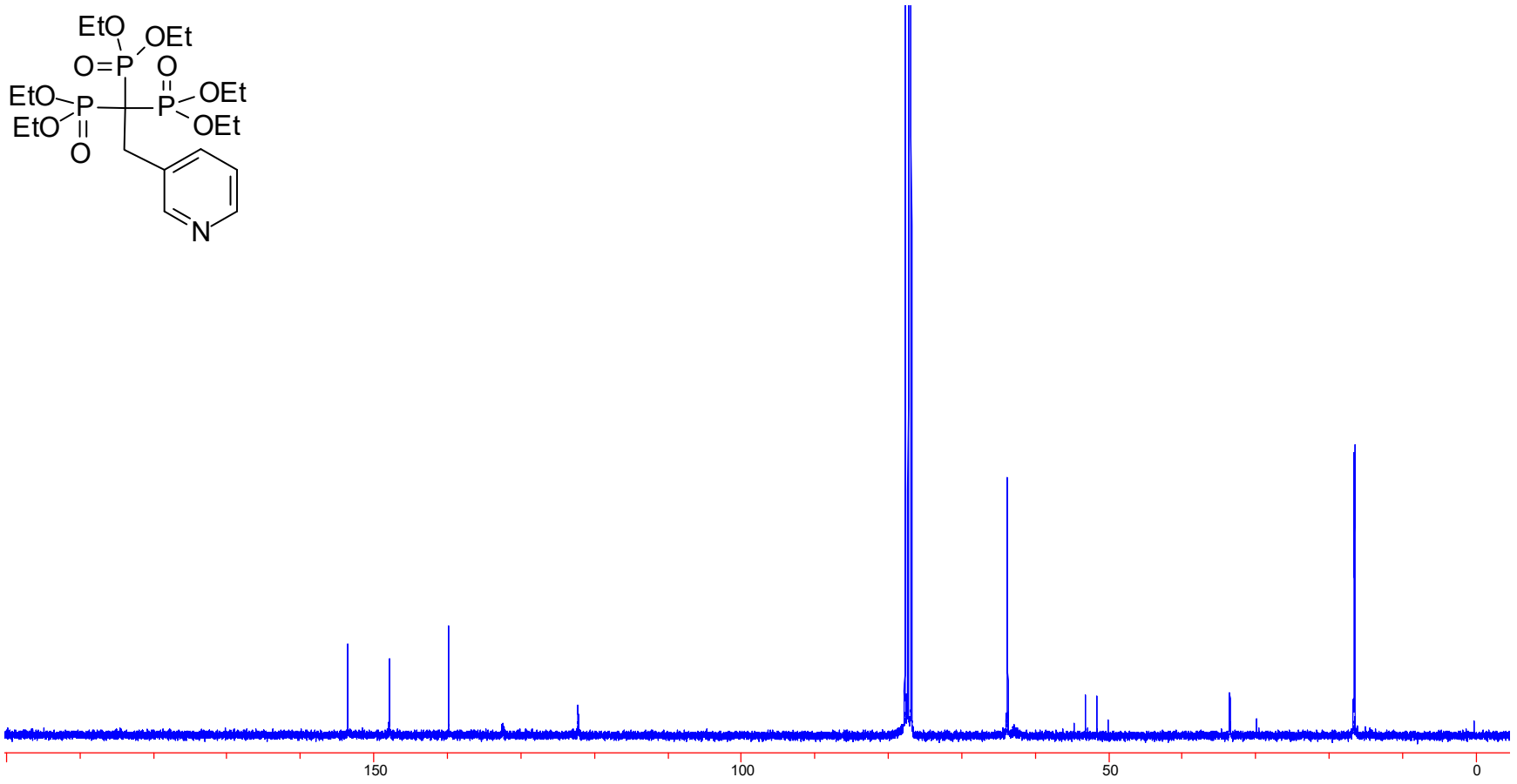


Figure A- 44. ^{13}C NMR spectrum of compound **111**

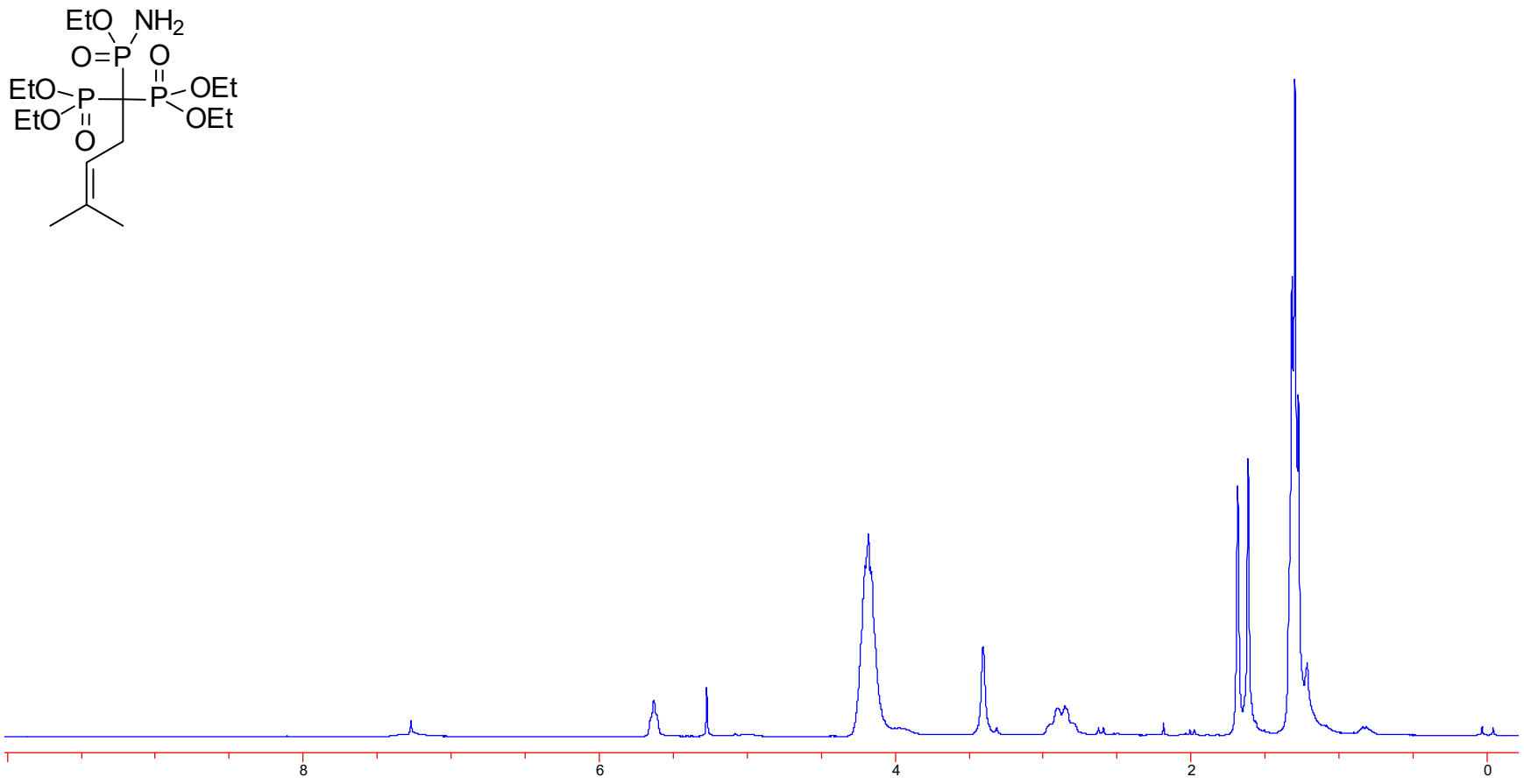


Figure A- 45. ^{1}H NMR spectrum of compound **116**

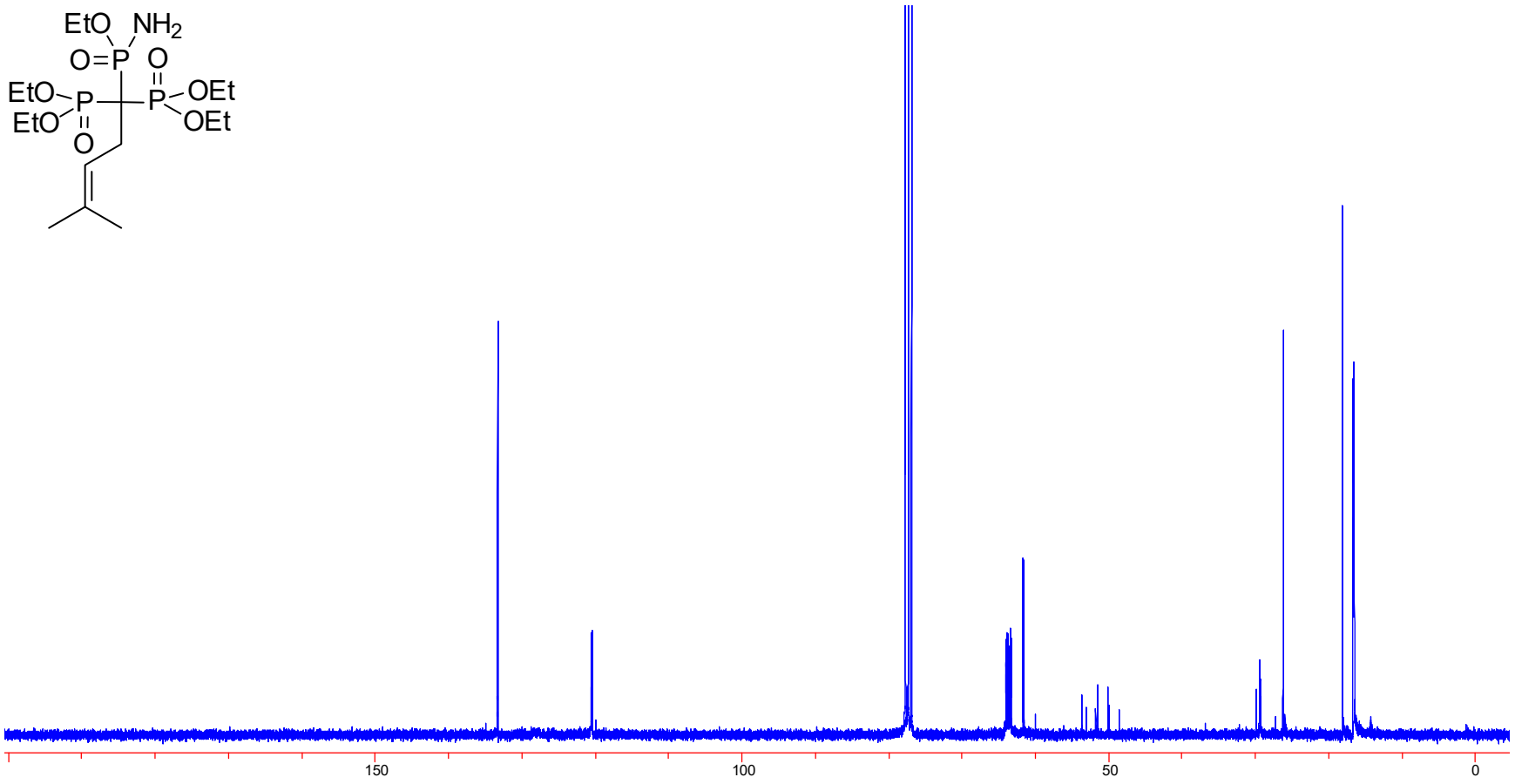


Figure A- 46. ^{13}C NMR spectrum of compound **116**

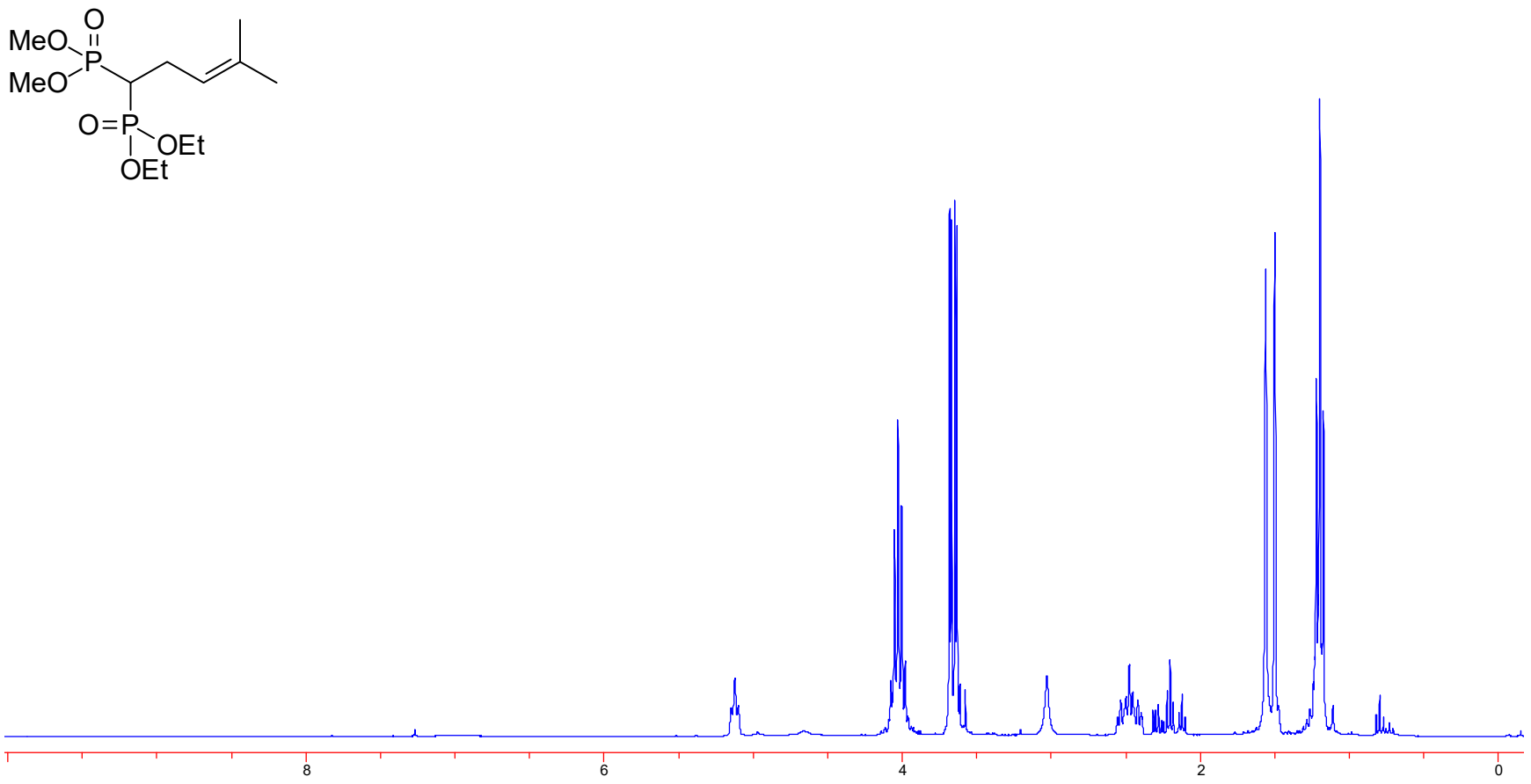


Figure A- 47. ^{1}H NMR spectrum of compound **117**

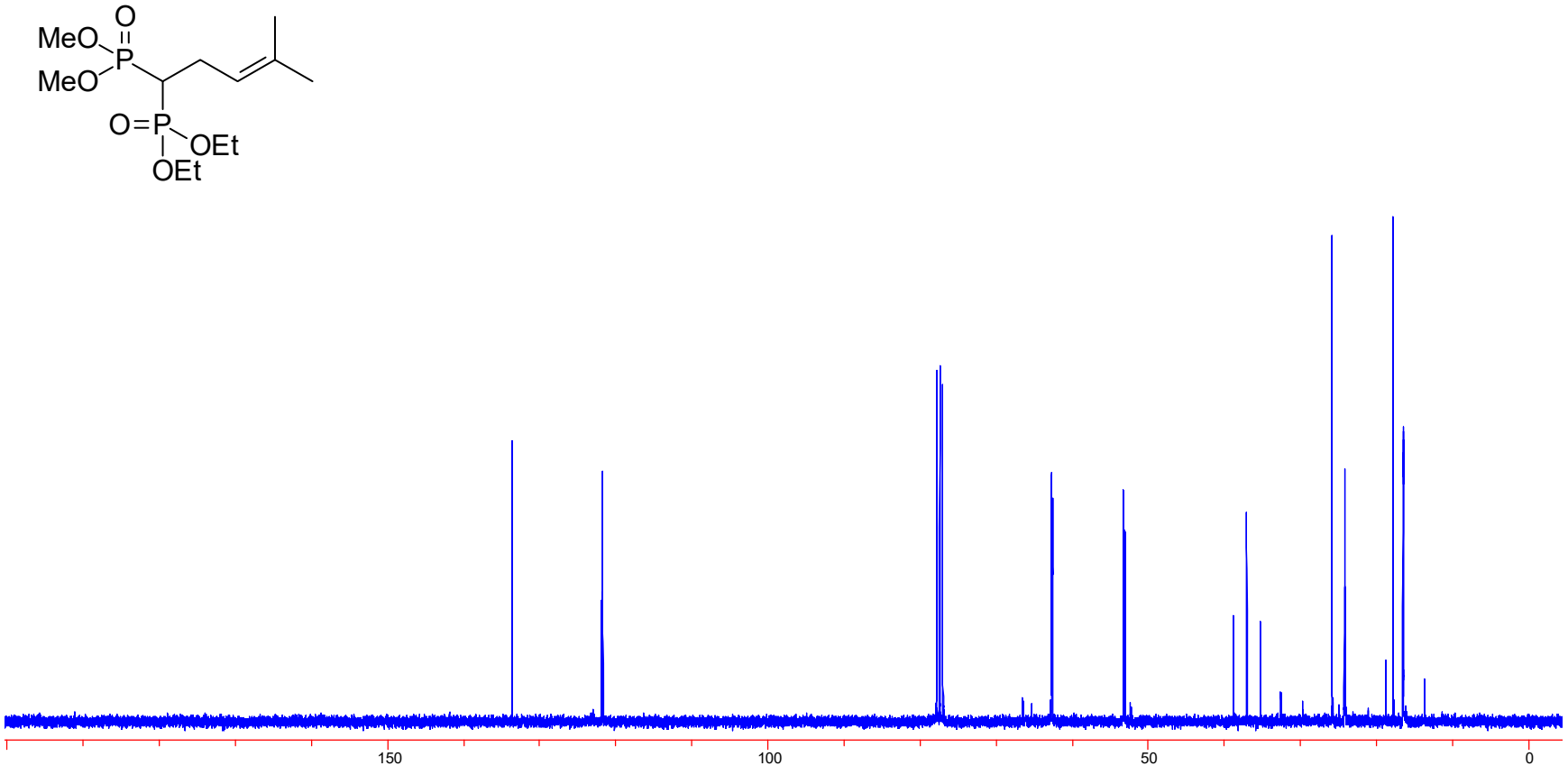


Figure A- 48. ^{13}C NMR spectrum of compound **117**

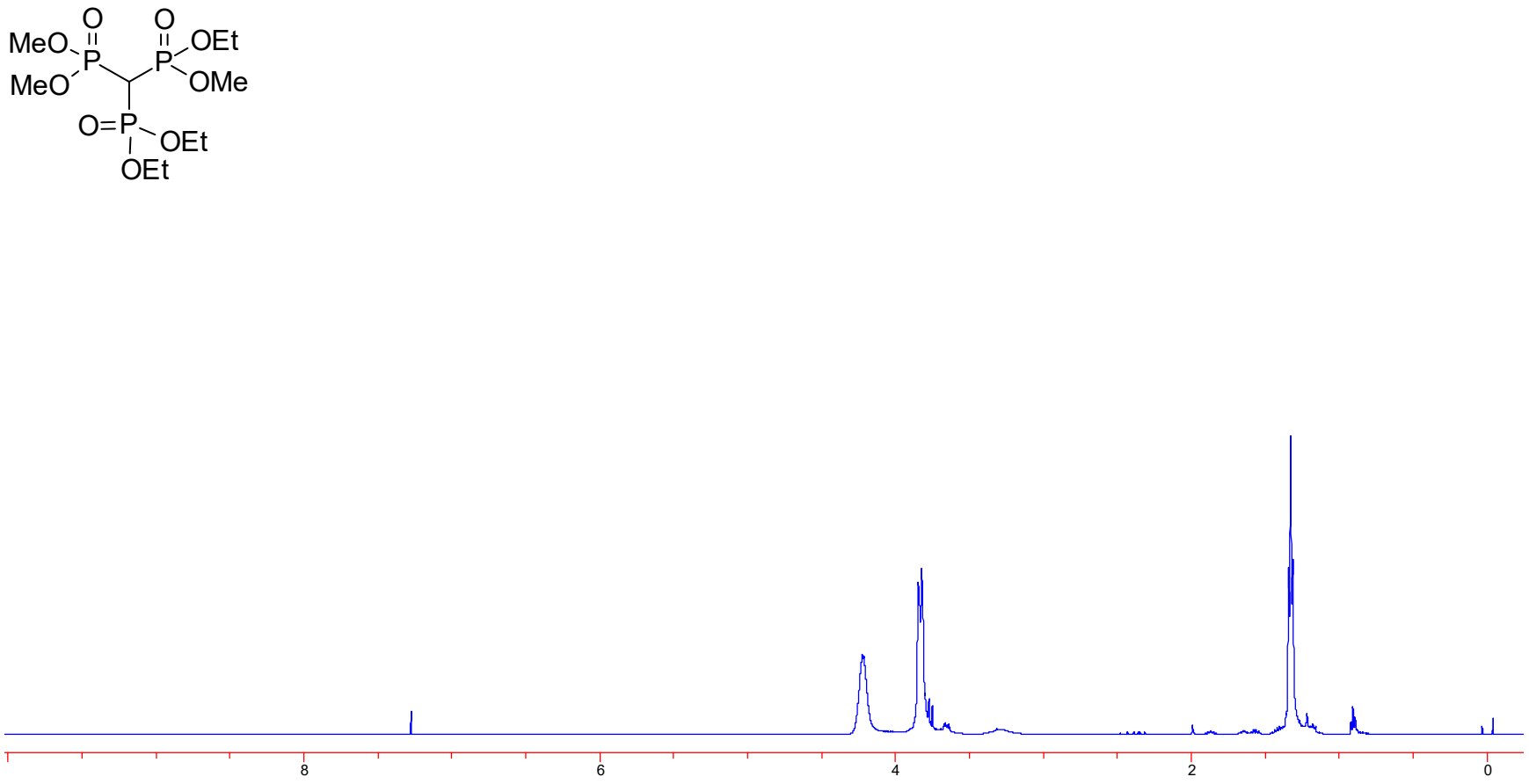


Figure A- 49. ^{1}H NMR spectrum of compound **133**

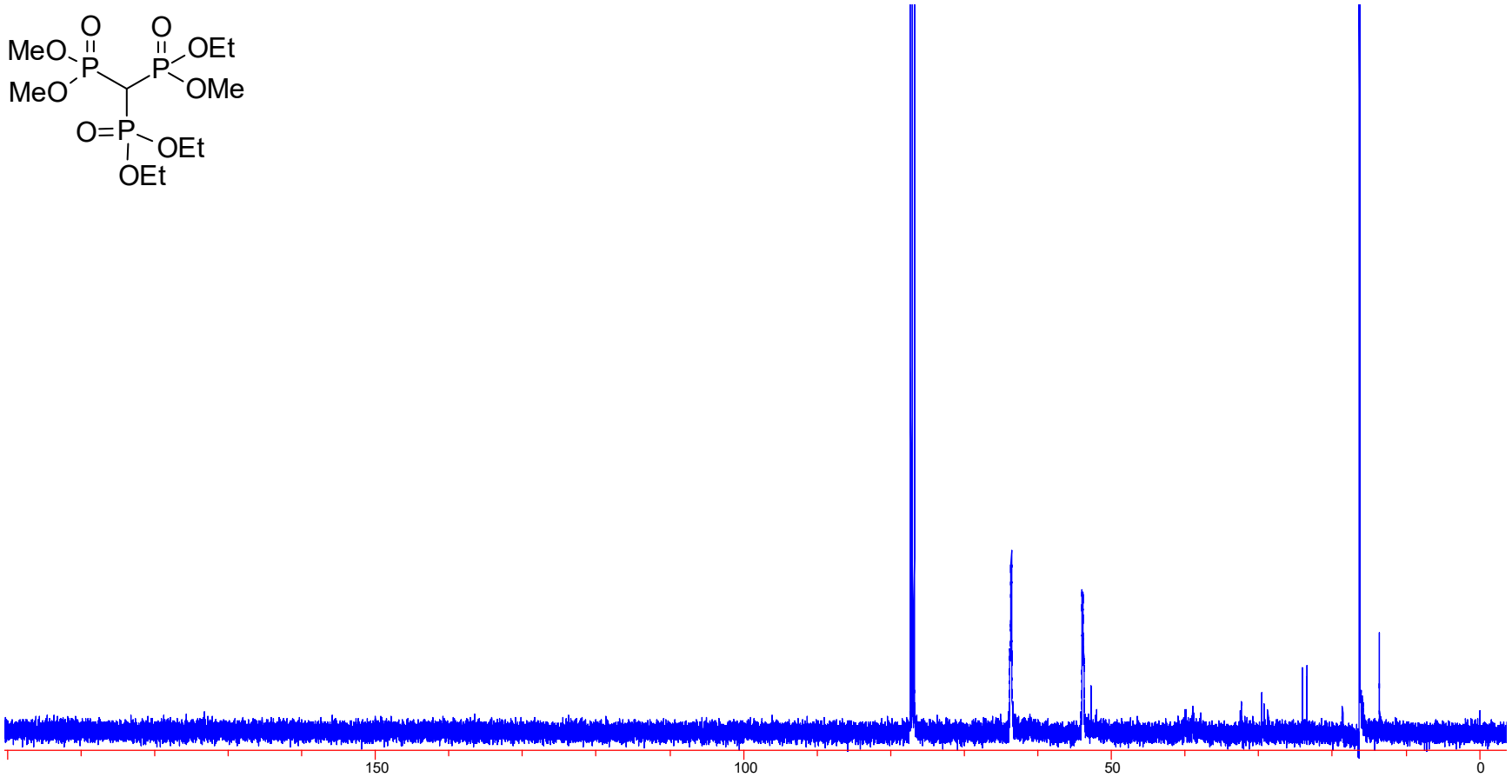


Figure A- 50. ^{13}C NMR spectrum of compound **133**

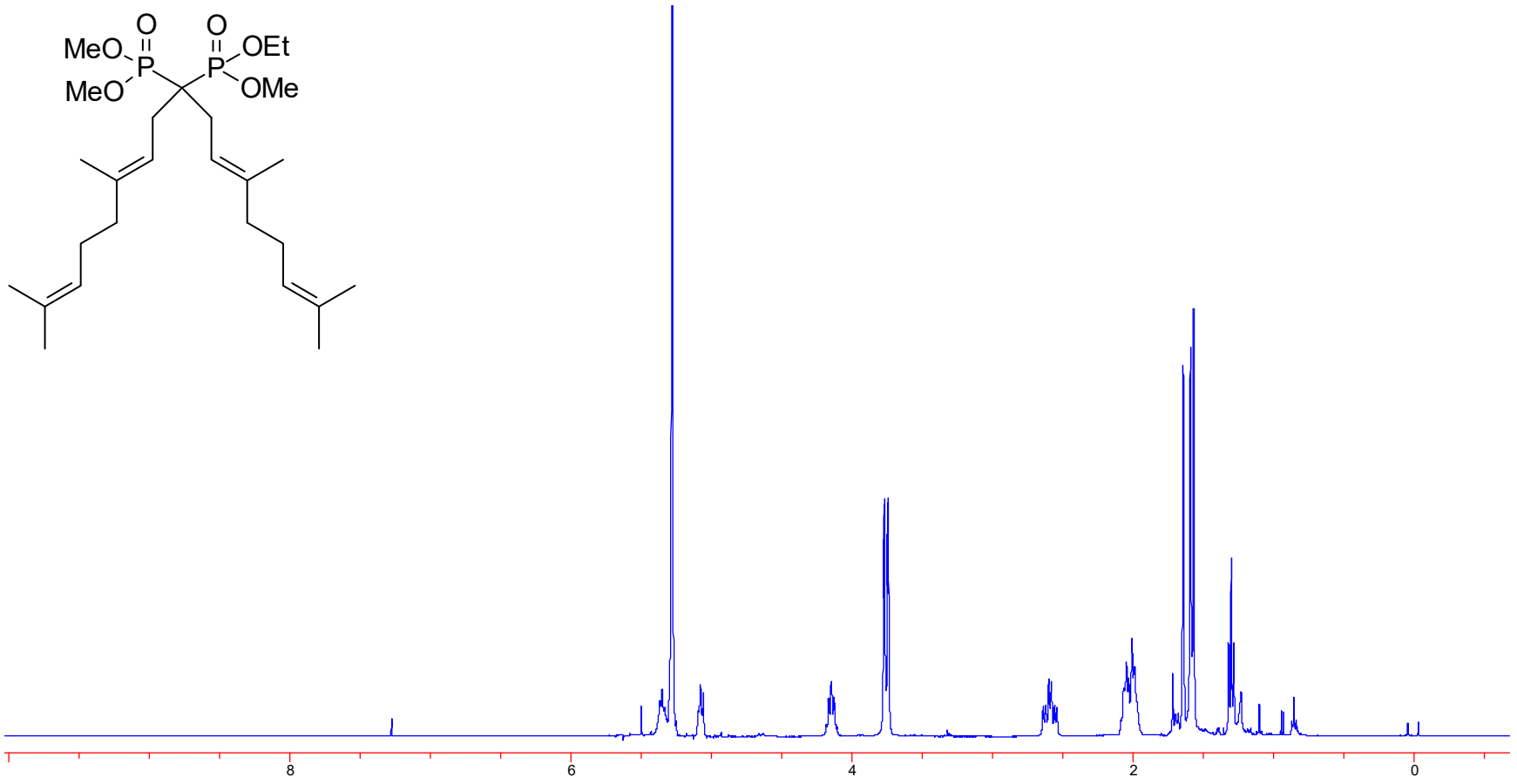


Figure A- 51. ^{1}H NMR spectrum of compound **134**

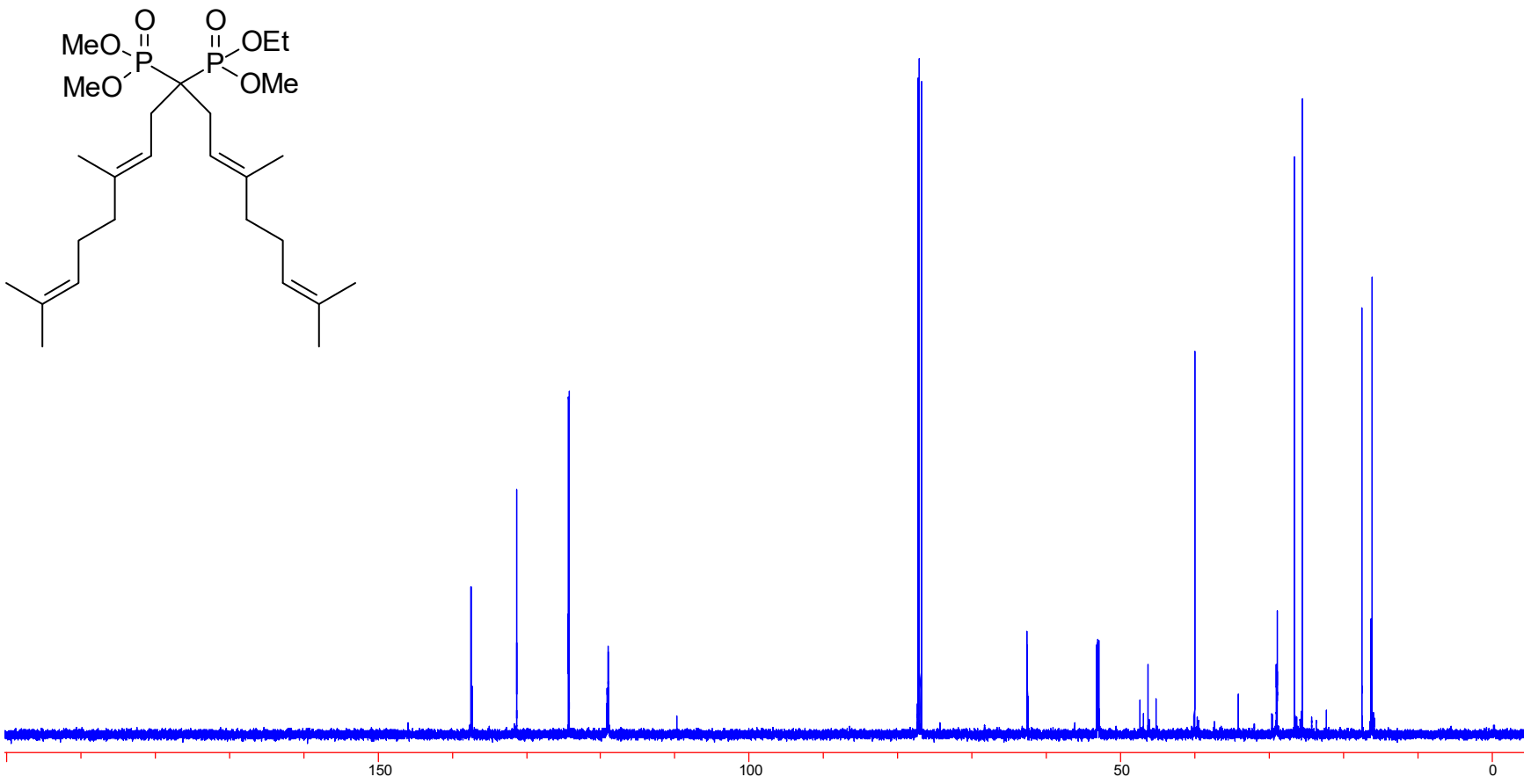


Figure A- 52.^{12}C NMR spectrum of compound **134**

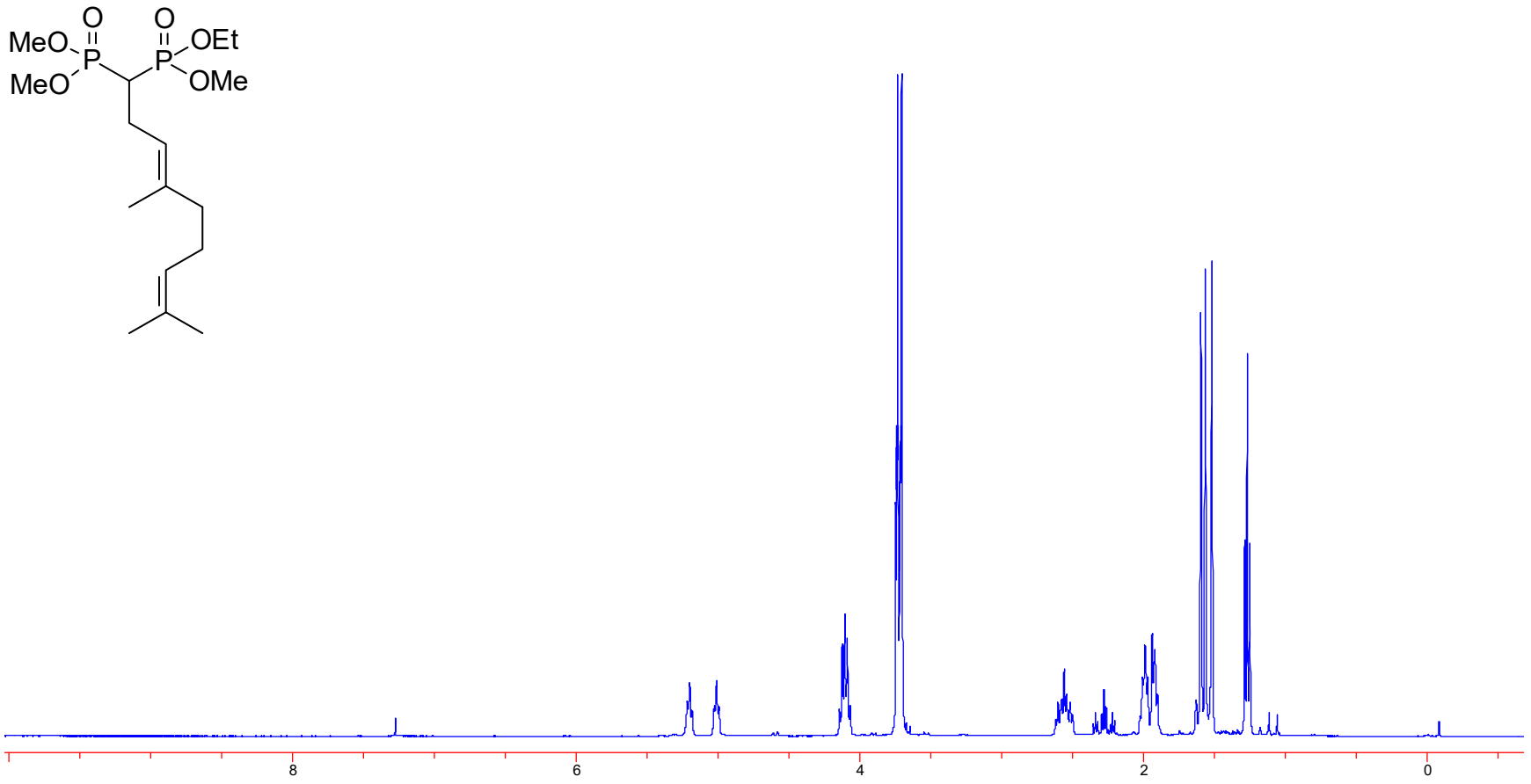


Figure A- 53. ^{1}H NMR spectrum of compound **135**

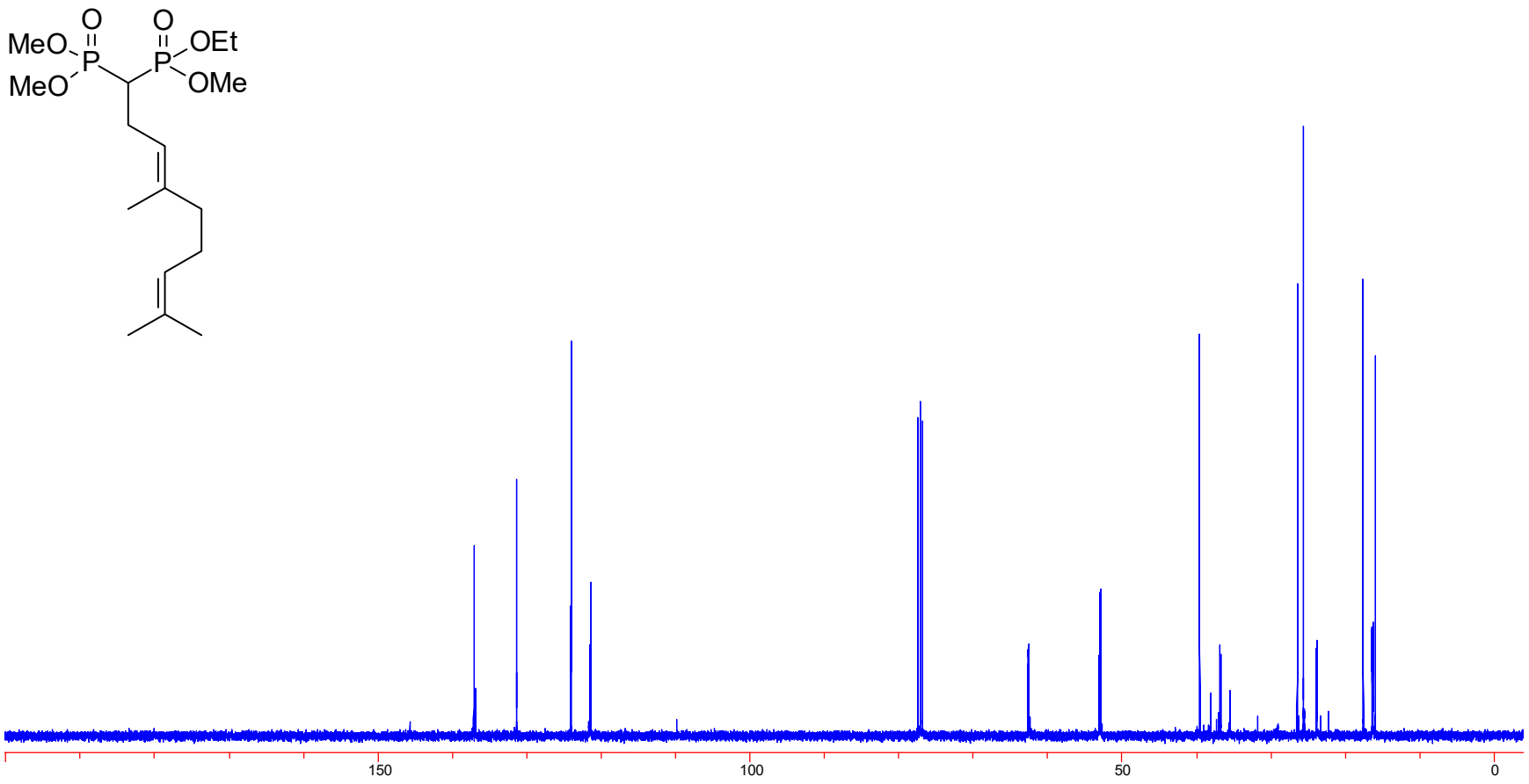


Figure A- 54. ^{13}C NMR spectrum of compound **135**

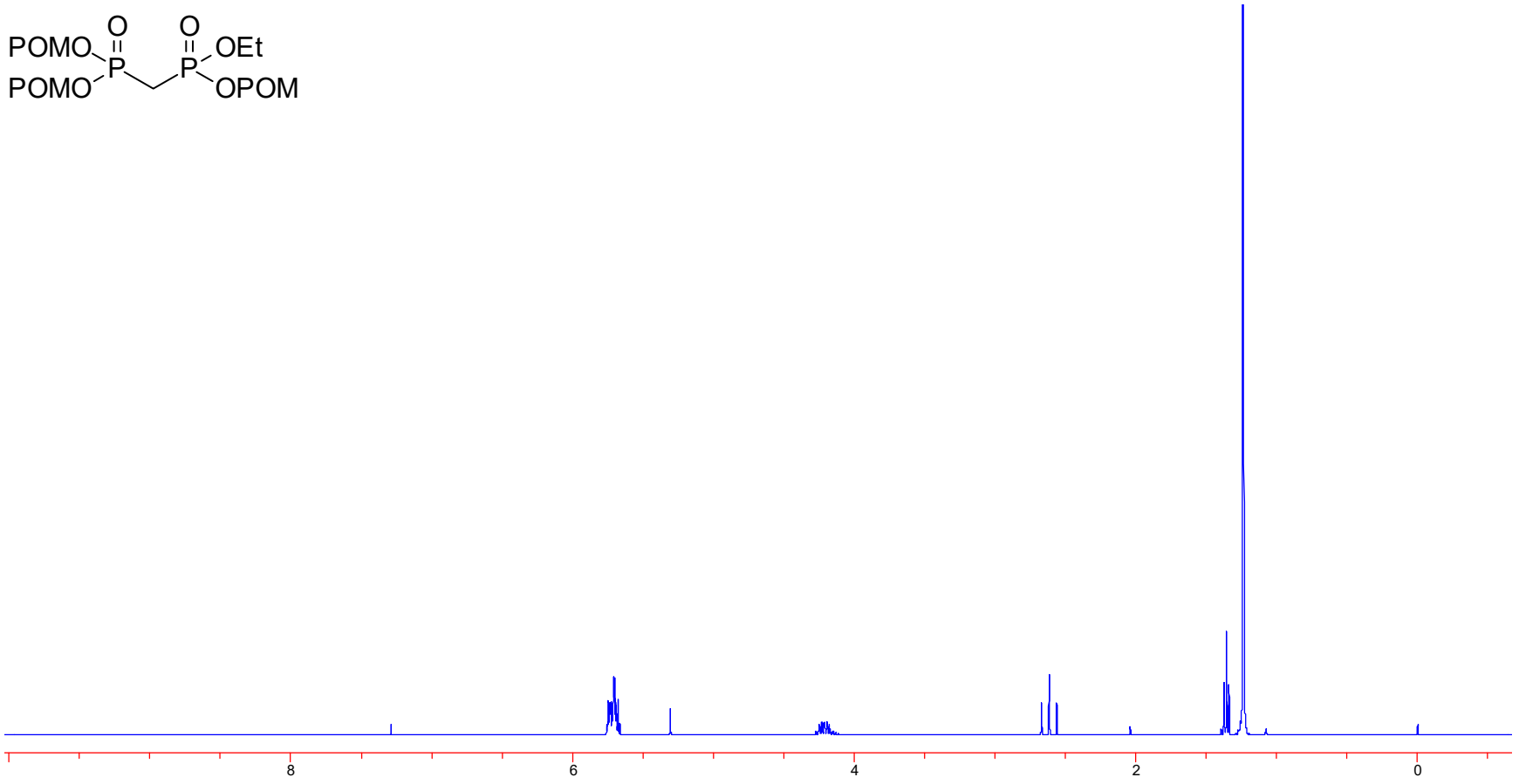


Figure A- 55. ^{1}H NMR spectrum of compound **140**

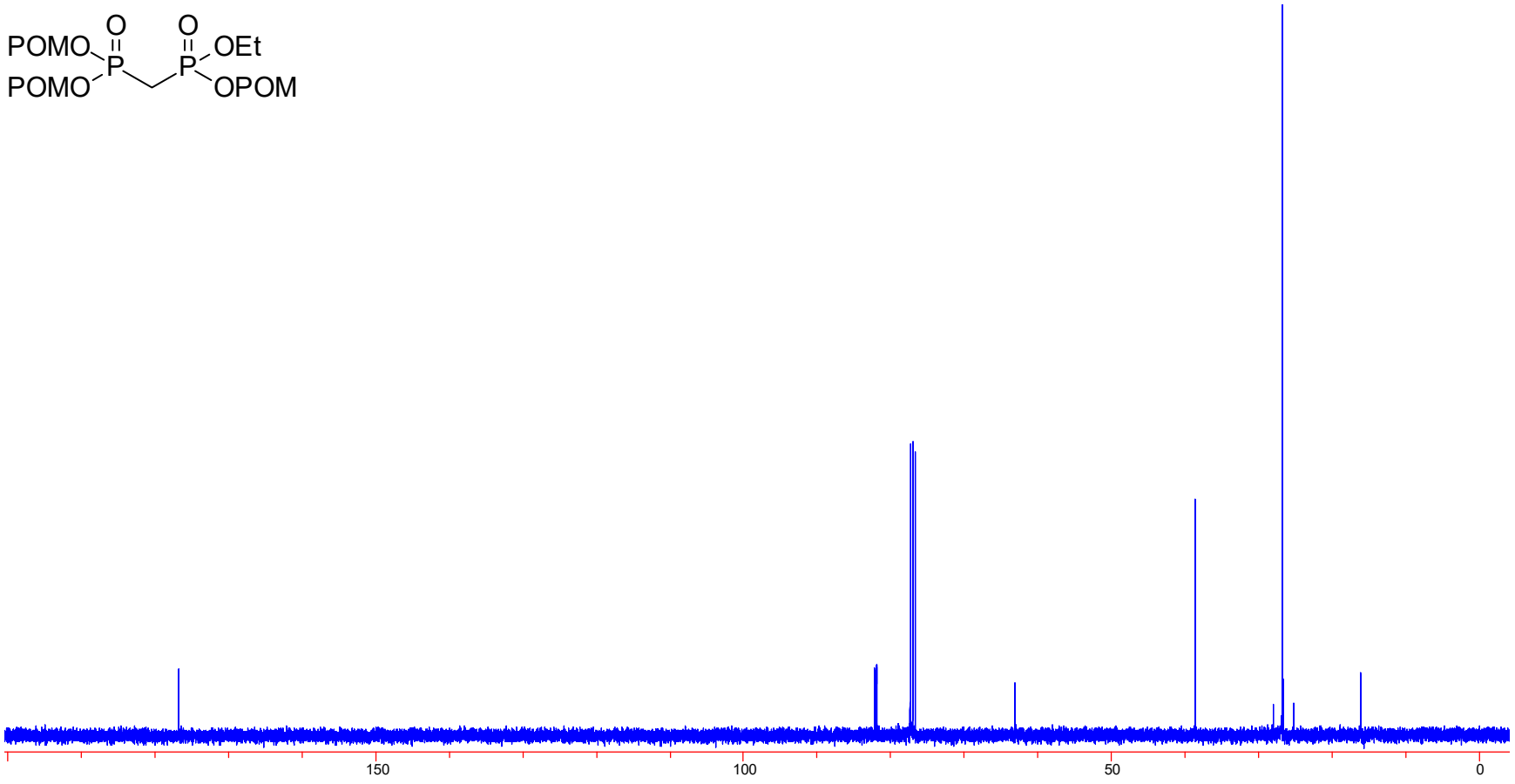


Figure A- 56. ^{13}C NMR spectrum of compound **140**

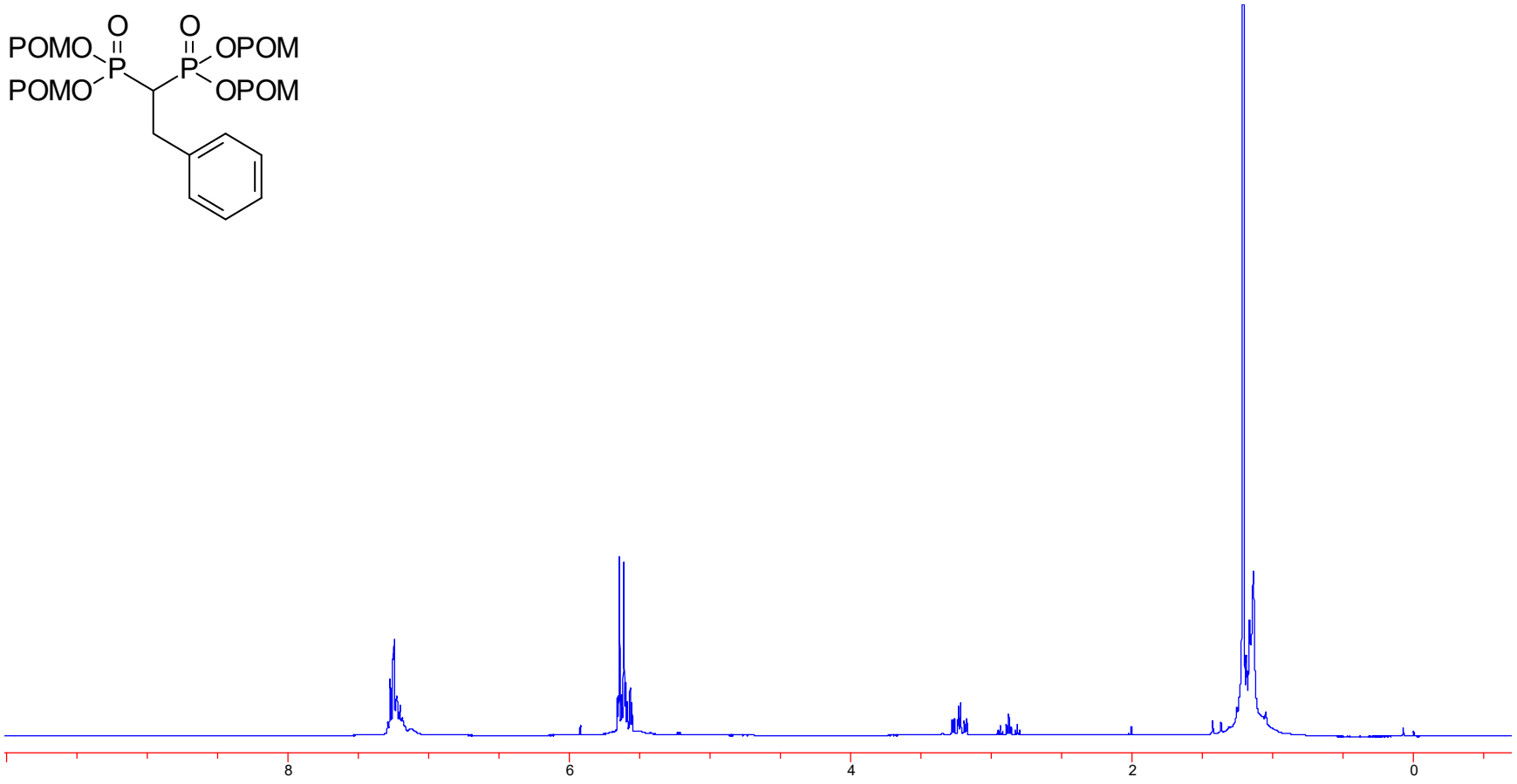


Figure A- 57. ^{1}H NMR spectrum of compound **144**

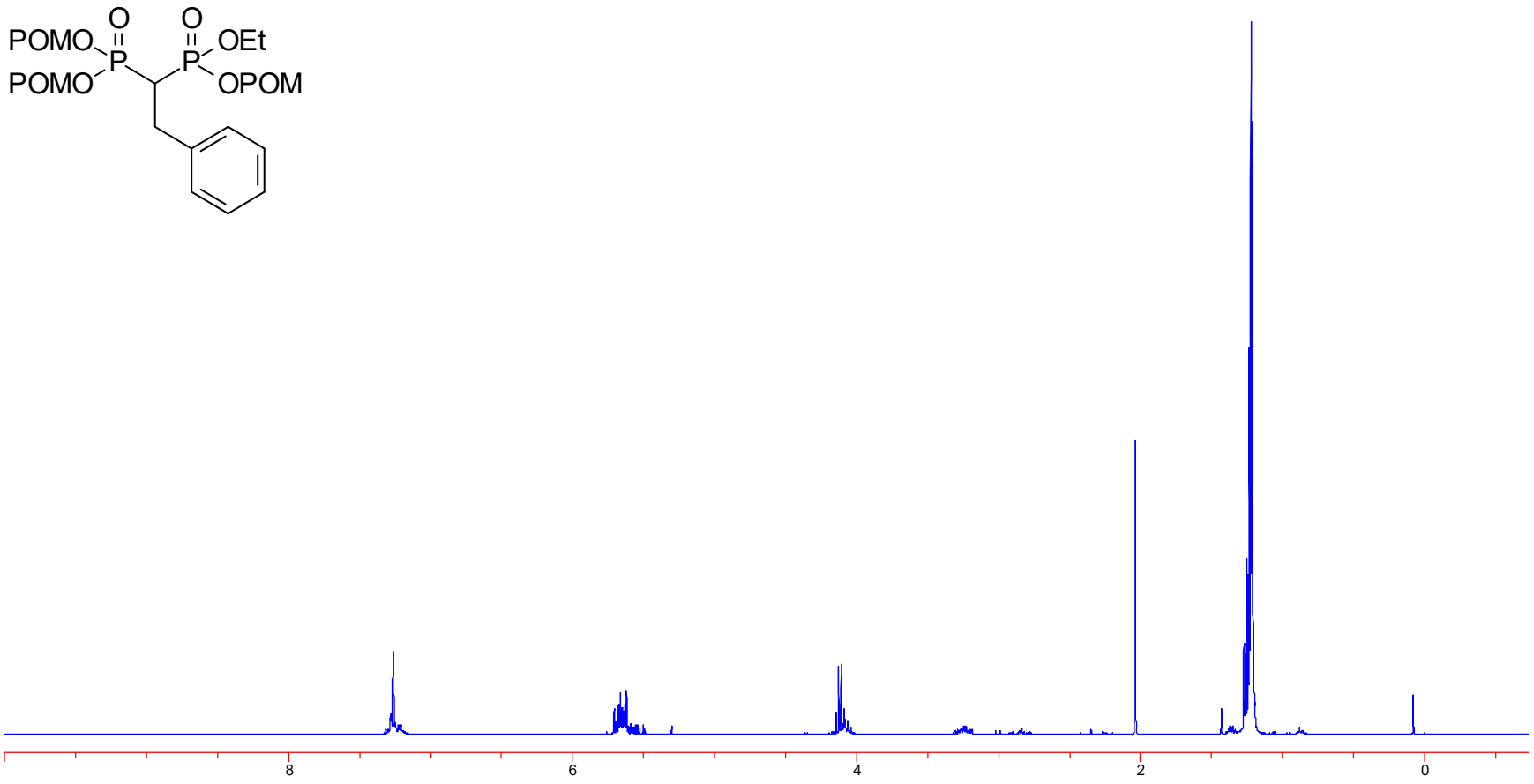


Figure A- 58. ^{1}H NMR spectrum of compound **146**

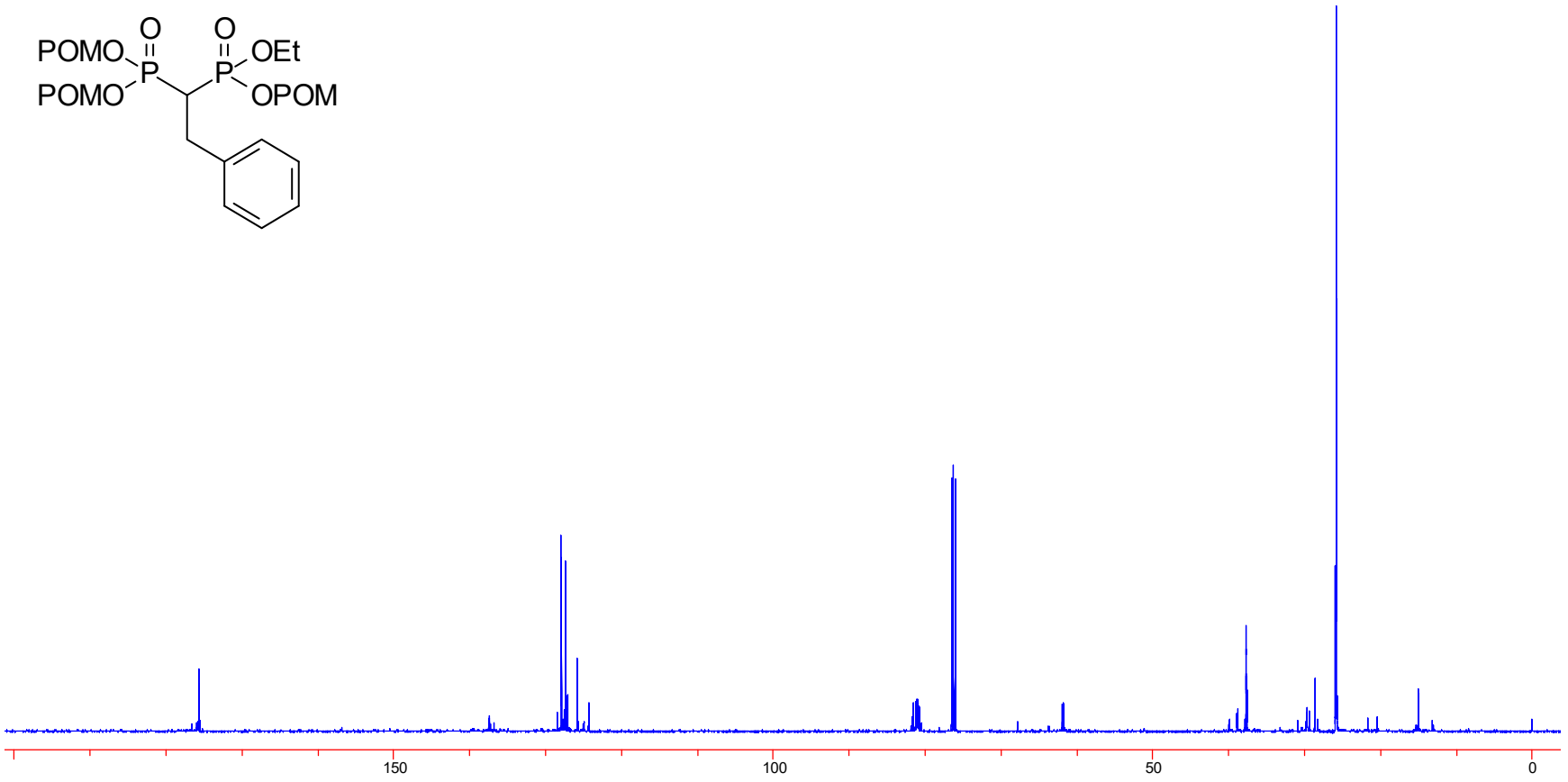


Figure A- 59. ^{13}C NMR spectrum of compound **146**

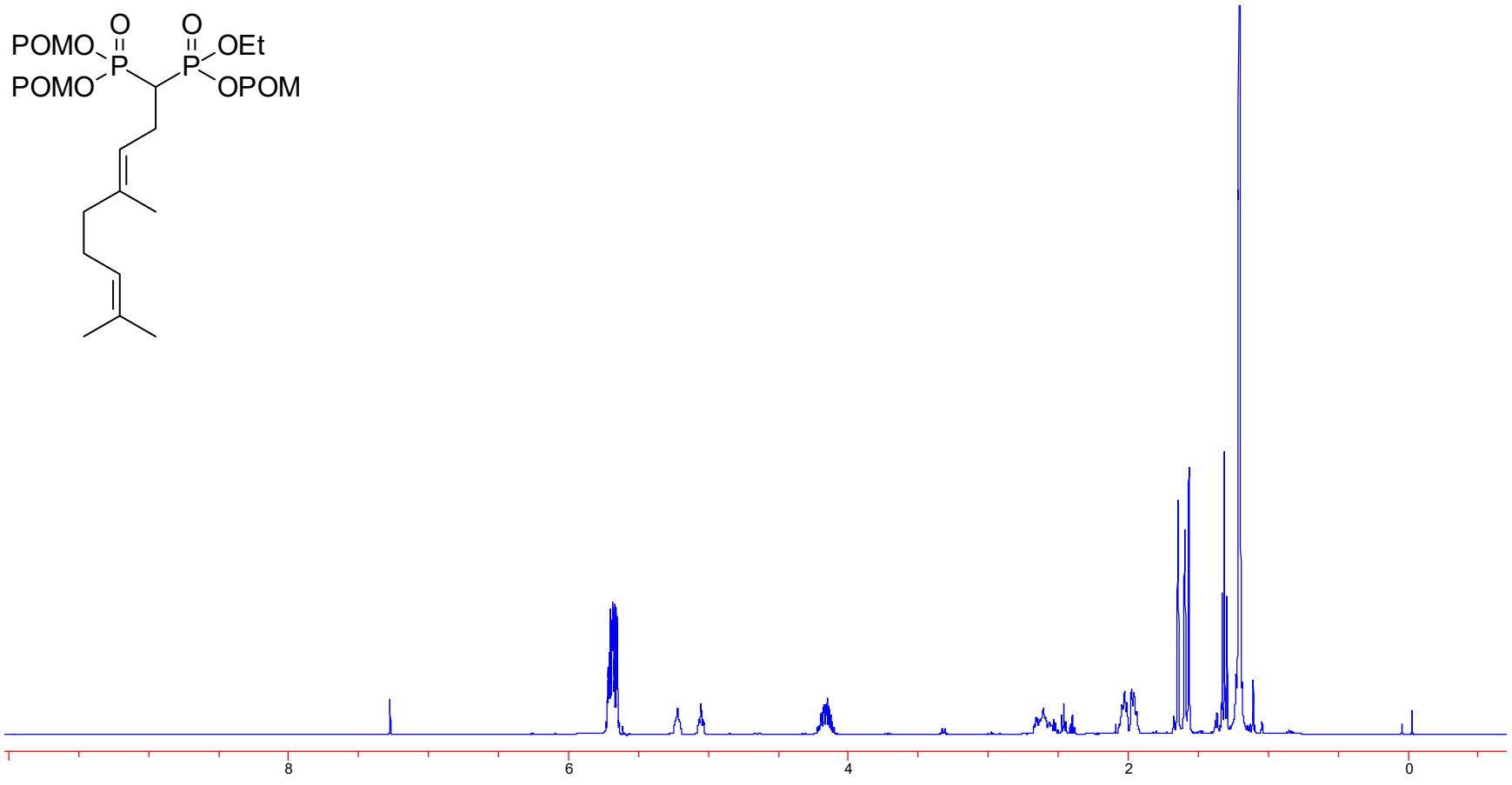


Figure A- 60. ^{1}H NMR spectrum of compound **150**

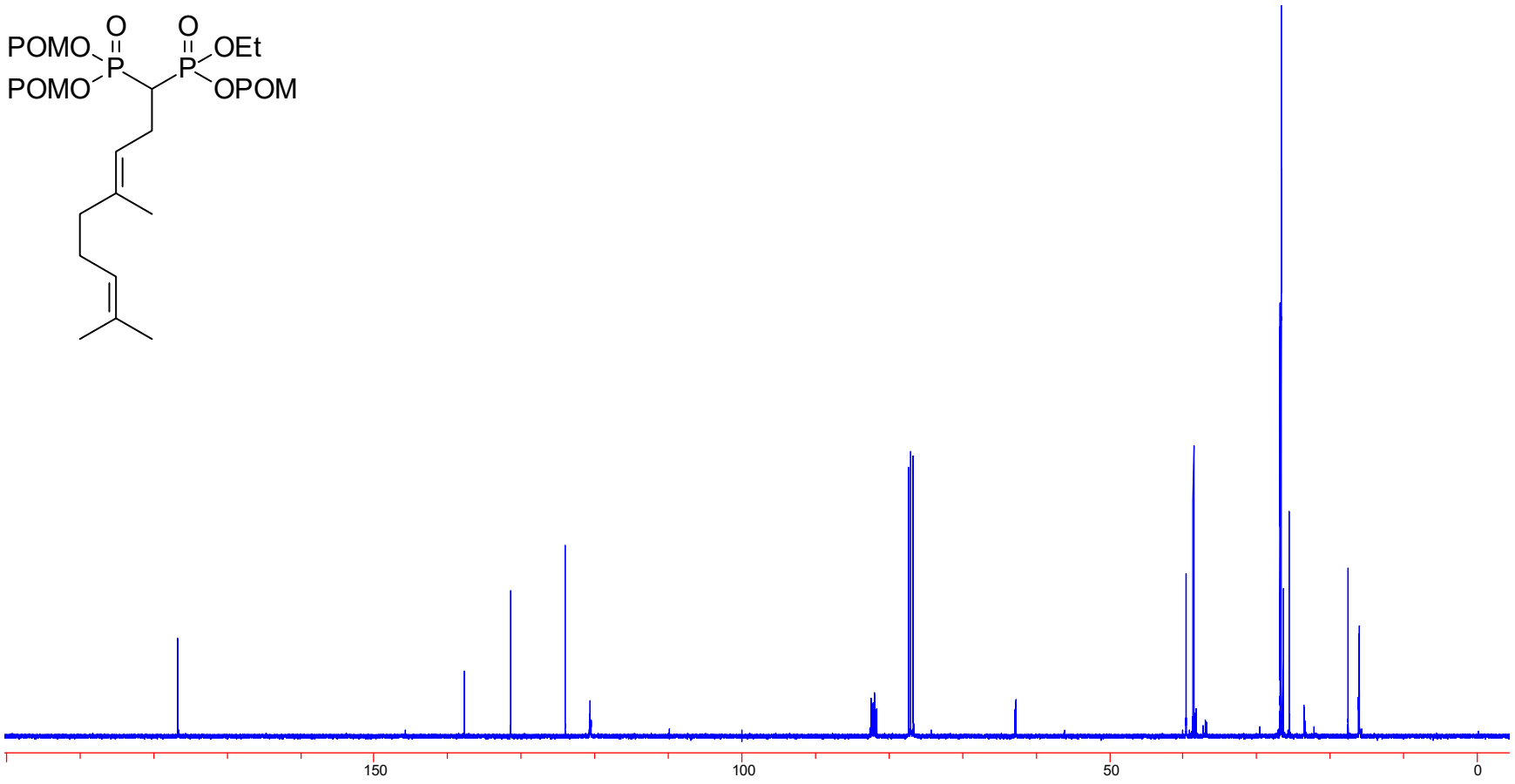


Figure A- 61. ^{13}C NMR spectrum of compound **150**

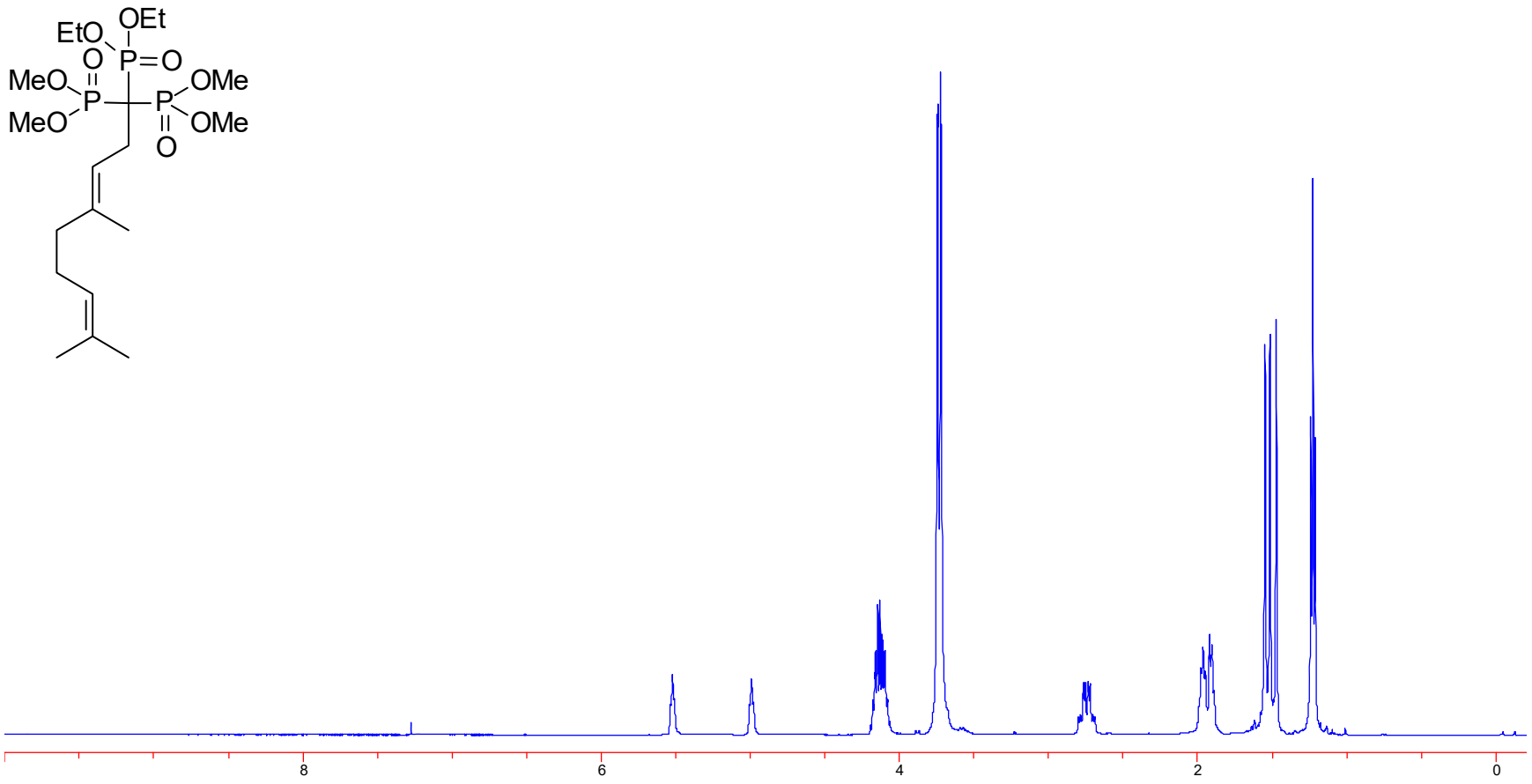


Figure A- 62. ^{1}H NMR spectrum of compound **153**

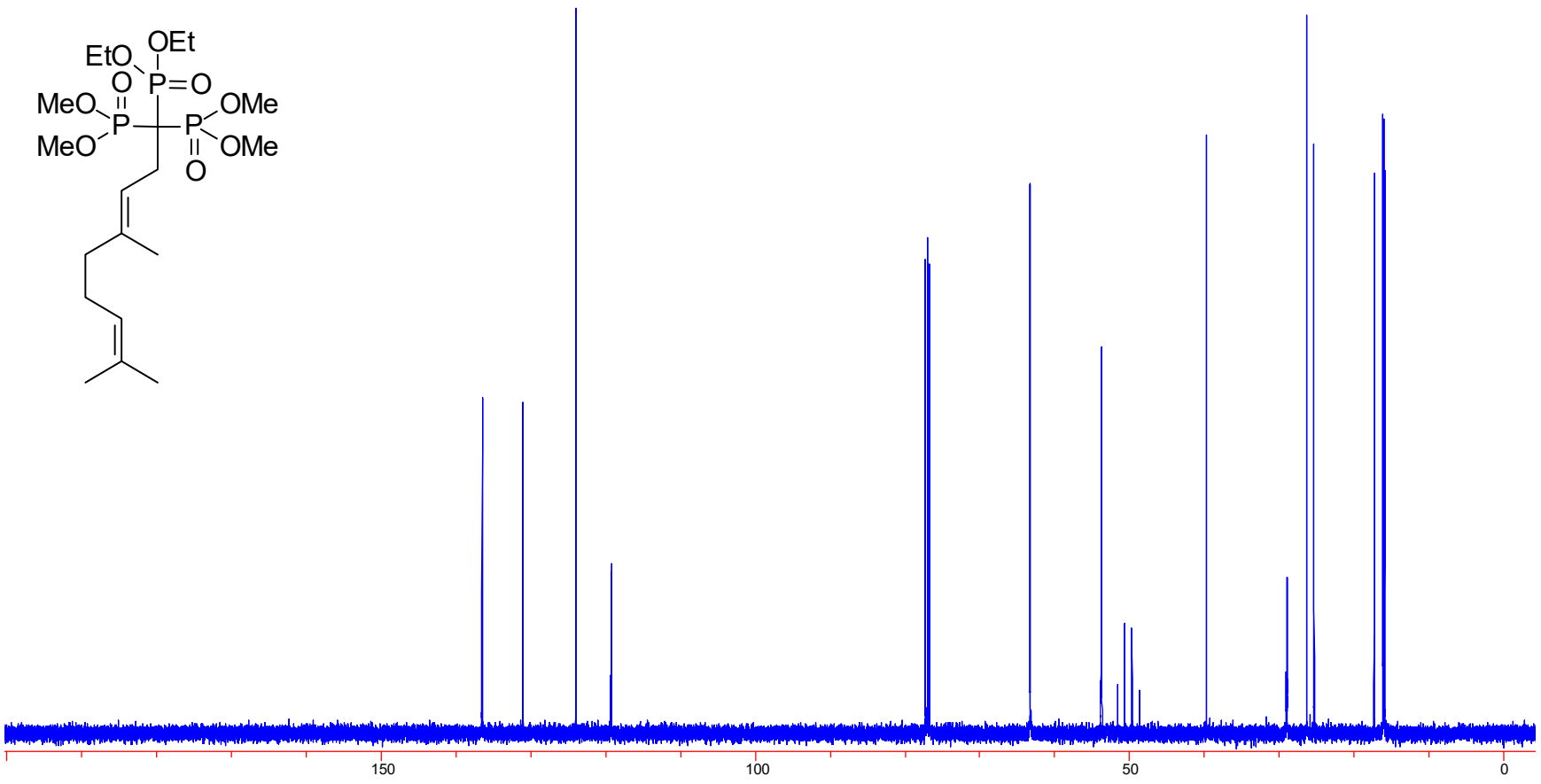


Figure A- 63. ^{13}C NMR spectrum of compound **153**

REFERENCES

1. Moleschott, J. *Lehre der nahrungsmittel. Für das volk*. 2 ed.; F. Enke: Erlangen, 1853; p 256.
2. Erb, T. J.; Kiefer, P.; Hattendorf, B.; Gunther, D.; Vorholt, J. A. *Science* **2012,** *337* (6093), 467-470.
3. Smil, V. *Annu. Rev. Energy Environ.* **2000,** *25*, 53-88.
4. Eisenreich, W.; Bacher, A.; Arigoni, D.; Rohdich, F. *Cell. Mol. Life Sci.* **2004,** *61* (12), 1401-1426.
5. Wiemer, D. F.; Wiemer, A. J. *Biochem. Pharmacol.* **2014,** *89* (3), 301-312.
6. Buhaescu, I.; Izzedine, H. *Clin. Biochem.* **2007,** *40* (9-10), 575-584.
7. Rajalingam, K.; Schreck, R.; Rapp, U. R.; Albert, S. *Biochim. Biophys. Acta.* **2007,** *1773* (8), 1177-1195.
8. Kuoppala, J.; Lamminpaa, A.; Pukkala, E. *Eur. J. Cancer* **2008,** *44* (15), 2122-2132.
9. Osmak, M. *Cancer Lett.* **2012,** *324* (1), 1-12.
10. Stella, V. J.; Borchardt, R. T.; Hageman, M. J.; Oliyai, R.; Maag, H.; Tilley, J. W. Prodrugs Challenges and Rewards Part 1. In *Biotechnology: Pharmaceutical Aspects V* [Online]; p. 1464 http://proxy.lib.uiowa.edu/login?url=http://dx.doi.org/10.1007/978-0-387-49785-3.
11. Russell, R. G. *Bone* **2011,** *49* (1), 2-19.
12. Rautio, J.; Kumpulainen, H.; Heimbach, T.; Oliyai, R.; Oh, D.; Jarvinen, T.; Savolainen, J. *Nat. Rev. Drug Discov.* **2008,** *7* (3), 255-270.
13. Serafinowska, H. T.; Ashton, R. J.; Bailey, S.; Harnden, M. R.; Jackson, S. M.; Sutton, D. *J. Med. Chem.* **1995,** *38* (8), 1372-1379.
14. Roux, L.; Priet, S.; Payrot, N.; Weck, C.; Fournier, M.; Zoulim, F.; Balzarini, J.; Canard, B.; Alvarez, K. *Euro. J. Med. Chem.* **2013,** *63*, 869-881.
15. Committee, A. I. S. *HIV Med.* **2002,** *3* (4), 229-238.
16. Wiemer, A. J.; Yu, J. S.; Lamb, K. M.; Hohl, R. J.; Wiemer, D. F. *Bioorg. Med. Chem.* **2008,** *16* (1), 390-399.

17. Wiemer, A. J.; Yu, J. S.; Shull, L. W.; Barney, R. J.; Wasko, B. M.; Lamb, K. M.; Hohl, R. J.; Wiemer, D. F. *Bioorg. Med. Chem.* **2008,** *16* (7), 3652-3660.

18. Boedec, A.; Sicard, H.; Dessolin, J.; Herbette, G.; Ingoure, S.; Raymond, C.; Belmant, C.; Kraus, J. L. *J. Med. Chem.* **2008,** *51* (6), 1747-1754.

19. Hsiao, C. H. C.; Lin, X. C.; Barney, R. J.; Shippy, R. R.; Li, J.; Vinogradova, O.; Wiemer, D. F.; Wiemer, A. J. *Chem. Biol.* **2014,** *21* (8), 945-954.

20. Abbas, A. K.; Lichtman, A. H.; Pober, J. S. *Cellular and molecular immunology*. Saunders: Philadelphia, 1991; p 417

21. Bonneville, M.; O'Brien, R. L.; Born, W. K. *Nat. Rev. Immunol.* **2010,** *10* (7), 467-478.

22. Wu, Y. L.; Ding, Y. P.; Tanaka, Y.; Shen, L. W.; Wei, C. H.; Minato, N.; Zhang, W. *Int. J. Biol. Sci.* **2014,** *10* (2), 119-135.

23. Yao, S.; Huang, D.; Chen, C. Y.; Halliday, L.; Zeng, G.; Wang, R. C.; Chen, Z. W. *PLoS Pathog.* **2010,** *6* (2).

24. Adams, E. J.; Gu, S.; Luoma, A. M. *Cell Immunol.* **2015,** *296* (1), 31-40.

25. Karunakaran, M. M.; Herrmann, T. *Front Immunol.* **2014,** *5*, 648.

26. Sandstrom, A.; Peigne, C. M.; Leger, A.; Crooks, J. E.; Konczak, F.; Gesnel, M. C.; Breathnach, R.; Bonneville, M.; Scotet, E.; Adams, E. J. *Immunity* **2014,** *40* (4), 490-500.

27. Carding, S. R.; Egan, P. J. *Nat. Rev. Immunol.* **2002,** *2* (5), 336-345.

28. Gertner-Dardenne, J.; Castellano, R.; Mamessier, E.; Garbit, S.; Kochbati, E.; Etienne, A.; Charbonnier, A.; Collette, Y.; Vey, N.; Olive, D. *J. Immunol.* **2012,** *188* (9), 4701-4708.

29. Romanenko, V. D.; Kukhar, V. P. *Chem. Rev.* **2006,** *106* (9), 3868-3935.

30. Koolaji, N.; Abu-Mellal, A.; Tran, V. H.; Duke, R. K.; Duke, C. C. *Euro. J. Med. Chem.* **2013,** *63*, 415-422.

31. Lin, X.; Zangari, B.; Hsiao, C. C.; Vinogradova, O.; Wiemer, A. *Unpublished data*.

32. Roman, C. A.; Balzarini, J.; Meier, C. *J. Med. Chem.* **2010,** *53* (21), 7675-7681.

33. Harly, C.; Peigne, C. M.; Scotet, E. *Front. Immunol.* **2014,** *5*, 657.

34. Robbins, B. L.; Srinivas, R. V.; Kim, C.; Bischofberger, N.; Fridland, A. *Antimicrob. Agents Chemother.* **1998,** *42*, 612-617.

35. Maalouf, M. A.; Wiemer, A. J.; Kuder, C. H.; Hohl, R. J.; Wiemer, D. F. *Bioorg. Med. Chem.* **2007,** *15* (5), 1959-1966.

36. Caron, K.; Lachapelle, V.; Keillor, J. W. *Org. Biomol. Chem.* **2011,** *9* (1), 185-197.

37. Rasmusse.M; Leonard, N. J. *J. Am. Chem. Soc.* **1967,** *89* (21), 5439.

38. Ingram, A. M.; Stirling, K.; Faulds, K.; Moore, B. D.; Graham, D. *Org. Biomol. Chem.* **2006,** *4* (15), 2869-2873.

39. Rejman, D.; Olesiak, M.; Chen, L. Q.; Patterson, S. E.; Wilson, D.; Jayaram, H. N.; Hedstrom, L.; Pankiewicz, K. W. *J. Med. Chem.* **2006,** *49* (16), 5018-5022.

40. Wiemer, A. J.; Shippy, R. R.; Kilcollins, A. M.; Li, J.; Hsiao, C. C.; Barney, R. J.; Geng, M. L.; Wiemer, D. F. *Chembiochem* **2015**.

41. Troutman, J. M.; Chehade, K. A. H.; Kiegiel, K.; Andres, D. A.; Spielmann, H. P. *Bioorg. Med. Chem. Lett.* **2004,** *14* (19), 4979-4982.

42. Point, V.; Malla, R. K.; Diomande, S.; Martin, B. P.; Delorme, V.; Carriere, F.; Canaan, S.; Rath, N. P.; Spilling, C. D.; Cavalier, J. F. *J. Med. Chem.* **2012,** *55* (22), 10204-10219.

43. Niemi, R.; Turhanen, P.; Vepsalainen, J.; Taipale, H.; Jarvinen, T. *Eur. J. Pharm. Sci.* **2000,** *11* (2), 173-180.

44. Wiemer, A.; Kilcollins, A. M.; Li, J.; Hsiao, C. C. *Unpublished data*.

45. Maag, H. *Biotechnol Pharm Asp* **2007,** *5*, 3-29.

46. Zgani, I.; Menut, C.; Seman, M.; Gallois, V.; Laffont, V.; Liautard, J.; Liautard, J. P.; Criton, M.; Montero, J. L. *J. Med. Chem.* **2004,** *47* (18), 4600-4612.

47. Fort, Y.; Pete, J. P. *Tetrahedron Lett* **1984,** *25* (2), 215-218.

48. Teulade, M. P.; Savignac, P.; Aboujaoude, E. E.; Collignon, N. *J. Organo. Chem.* **1986,** *312* (3), 283-295.

49. Nemeth, G.; Greff, Z.; Sipos, A.; Varga, Z.; Szekely, R.; Sebestyen, M.; Jaszay, Z.; Beni, S.; Nemes, Z.; Pirat, J. L.; Volle, J. N.; Virieux, D.; Gyuris, A.; Kelemenics, K.; Ay, E.; Minarovits, J.; Szathmary, S.; Keri, G.; Orfi, L. *J. Med. Chem.* **2014,** *57* (10), 3939-3965.

50. Mckenna, C. E.; Higa, M. T.; Cheung, N. H.; Mckenna, M. C. *Tetrahedron Lett* **1977,** (2), 155-158.

51. Zhou, X.; Ferree, S. D.; Wills, V. S.; Born, E. J.; Tong, H.; Wiemer, D. F.; Holstein, S. A. *Bioorg. Med. Chem.* **2014,** *22* (9), 2791-2798.

52. Leach, A. G.; Wang, R. X.; Wohlhieter, G. E.; Khan, S. I.; Jung, M. E.; Houk, K. N. *J. Am. Chem. Soc.* **2003,** *125* (14), 4271-4278.

53. Nicolai, S.; Erard, S.; Gonzalez, D. F.; Waser, J. *Org. Lett.* **2010,** *12* (2), 384-387.

54. Park, H.; Hong, Y. L.; Kim, Y. B.; Choi, T. L. *Org. Lett.* **2010,** *12* (15), 3442-3445.

55. Yokomatsu, T.; Shioya, Y.; Iwasawa, H.; Shibuya, S. *Heterocycles* **1997,** *46*, 463-472.

56. Gross, H.; Cosstisella, B.; Keitel, I.; Ozegowski, S. *Phosphorus, Sulfur, and Silicon* **1993,** *83*, 203-207.

57. Gross, H.; Keitel, I.; Costisella, B.; McKenna, C. E. *Phosphorus, Sulfur, and Silicon* **1991,** *61*, 177-181.

58. Liu, X.; Adam, H.; Blackburn, G. M. *Chem. Commun.* **1998**, 2619-2620.

59. Liu, X.; Brenner, C.; Guranowski, A.; Starzynska, E.; Blackburn, G. M. *Angew. Chem. Int. Ed.* **1999,** *38*, 1244-1247.

60. Romanenko, V. D.; Kukhar, V. P. *Beil. J. Org. Chem.* **2013,** *9*, 991-1001.

61. Smits, J. P.; Wiemer, D. F. *J. Org. Chem.* **2011,** *76* (21), 8807-8813.

62. Smit, J. P. Synthesis and Evaluation of Novel Bis- and Trisphosphonates. The University of Iowa, Iowa City IA, December 2011.

63. Cai, X. F.; Huang, W. X.; Chen, Z. P.; Zhou, Y. G. *Chem. Commun.* **2014,** *50* (67), 9588-9590.

64. Inoue, S.; Okauchi, T.; Minami, T. *Synthesis* **2003,** *13*, 1971-1976.

65. Reddy, G. V.; Jacobs, H. K.; Gopalan, A. S.; Barrans, R. E.; Dietz, M. L.; Stepinski, D. C.; Herlinger, A. W. *Synthetic Commun* **2004,** *34* (2), 331-344.

66. Timmer, M.; Ovaa, H.; Filippov, D.; van der Marel, G.; van Boom, J. *Tetrahedron Lett* **2000,** *41*, 8635-8638.

67. Grison, C.; Coutrot, P.; Joliez, S.; Balas, L. *Synthesis* **1996,** (6), 731-735.

68. Roman, C. A.; Balzarini, J.; Meier, C. *J. Med. Chem.* **2010,** *53* (21), 7675-7681.

69. Maegawa, T.; Otake, K.; Hirosawa, K.; Goto, A.; Fujioka, H. *Org. Lett.* **2012,** *14* (18), 4798-4801.

70. Zhou, X.; Reilly, J. E.; Loerch, K. A.; Hohl, R. J.; Wiemer, D. F. *Beil. J. Org. Chem.* **2014,** *10*, 1645-1650.

71. Ingram, A. M.; Stirling, K.; Faulds, K.; Moore, B. D.; Graham, D. *Org. Biomol. Chem* **2006,** *4* (15), 2869-2873.

72. Dowling, M. S.; Vanderwal, C. D. *J. Org. Chem.* **2010,** *75* (20), 6908-6922.

73. Simoni, D.; Gebbia, N.; Invidiata, F. P.; Eleopra, M.; Marchetti, P.; Rondanin, R.; Baruchello, R.; Provera, S.; Marchioro, C.; Tolomeo, M.; Marinelli, L.; Limongelli, V.; Novellino, E.; Kwaasi, A.; Dunford, J.; Buccheri, S.; Caccamo, N.; Dieli, F. *J. Med. Chem.* **2008,** *51* (21), 6800-6807.

74. Sanders, J. M.; Ghosh, S.; Chan, J. M.; Meints, G.; Wang, H.; Raker, A. M.; Song, Y.; Colantino, A.; Burzynska, A.; Kafarski, P.; Morita, C. T.; Oldfield, E. *J. Med. Chem.* **2004,** *47* (2), 375-384.

75. Russell, G. A.; Ngoviwatchai, P. *J. Org. Chem.* **1989,** *54* (8), 1836-1842.

76. Antczak, M. I.; Montchamp, J. L. *Org. Lett.* **2008,** *10* (5), 977-980.

77. Vepsalainen, J. J. *Tetrahedron Lett* **1999,** *40* (48), 8491-8493.

78. Troutman, J. M.; Chehade, K. A. H.; Kiegiel, K.; Andres, D. A.; Spielmann, H. P. *Bioorg Med Chem Lett* **2004,** *14* (19), 4979-4982.

79. Nguyen, L. M.; Niesor, E.; Bentzen, C. L. *J. Med. Chem.* **1987,** *30* (8), 1426-1433.