Design and Synthesis of ZMPSTE24 Inhibitors as Senescence Inducing Chemotherapeutic Agents

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Abstract

Zinc metalloprotease STE24 (ZMPSTE24) is a human transmembrane zinc metalloprotease, located in the nuclear membrane. It is responsible for maturation of farnesylated prelamin A into mature lamin A, which is a structural component of the nuclear lamina. Biochemical studies have indicated a relationship between accumulation of prelamin A (either by mutations which make prelamin A impossible to be processed or by a lack of ZMPSTE24 activity) and cell senescence. There is precedence of prelamin A accumulation-induced senescence having antitumor properties. Currently there are no robust senescence inducing compounds, so the promising pathway of cancer treatment by induction of cellular senescence is underexplored.

ZMPSTE24 inhibitors were synthesized in order to induce prelamin A accumulation. These inhibitors were based on HIV PR inhibitor lopinavir which is also a weak ZMPSTE24 inhibitor. Three classes of inhibitors were synthesized: hydroxyethylene, β-hydroxyl amide and phosphinate based inhibitors and were tested by collaborators on cancer cell lines. Some of the compounds had a stronger impact than lopinavir on prelamin A accumulation in cells and antiproliferative effect on cancer cells. Additionally, a derivative of the phosphinate based inhibitor was modified to contain a photolabile moiety in order to be used as a molecular biology tool. Lastly, a farnesyl based, azide-containing and cysteine-linked component for the proposed enzymatic assay was synthesized.

Résumé

La zinc métalloprotéase STE24 (ZMPSTE24) est une zinc métalloprotéase transmembranaire humaine située dans la membrane nucléaire. Il provoque la maturation de la prelamine A farnésylée en lamine A mature qui est une composante structurelle de la lamine nucléaire. Des études biochimiques ont demontré un lien entre l' accumulation de la prelamine A (soit en raison des mutations qui empêchent la prelamine A d'être transformée soit en raison d'une inactivité du ZMPSTE24) et la sénescence cellulaire. Il a déjà été démontré que la sénescence provoquée par l'accumulation de la prelamine a des propriétés anti-tumorales. Il n'existe actuellement aucun composé robuste pouvant provoquer l'induction de la sénescence; par conséquent la voie prometteuse d'un traitement du cancer par l'induction de la sénescence cellulaire demeure inexploée.

Des inhibiteurs du ZMPSTE24 ont été synthetisés afin de provoquer l'accumulation de la prelamine A. Ces inhibiteurs ont été basés sur l'inhibiteur lopinavir HIV PR qui est également un faible inhibiteur du ZMPSTE24. Trois classes d'inhibiteurs ont été synthétisées: des composés de l' hydroxyethylene, du β-hydroxyl amide et de la phosphinate. Ils ont été testés par des collaborateurs sur des lignées de cellules cancéreuses. Certains des composés ont eu un impact plus important que le lopinavir sur l'accumulation de la prelamine A dans les cellules et ont reduit la proliferation des cellules cancéreuses. De plus, un dérivé de l'inhibiteur composé de phosphinate a été modifié pour contenir une fraction photolabile et être utilisé comme un outil de biologie moléculaire. Enfin, un composé de farnesyl, contenant de l'azoture et lié à des cystéines a été synthetisé pour effectuer le test enzymatique proposé.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ACN	Acetonitrile
AcOH	Acetic acid
ADMET	Absorption, distribution, metabolism, excretion, toxicity
Boc	*tert*-Butoxycarbonyl
Bn	Benzyl
CBz	Carboxybenzyl
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
DBU	1,8-Diazabicycloundec-7-ene
DCM	Dichloromethane
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
Et_3N	Triethylamine
EtOAc	Ethyl acetate
EtOH	Ethanol
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Hex	Hexanes
HGPS	Hutchinson-Gilford progeria syndrome
HIV	Human Immunodeficiency Virus
HMDS	Hexamethyldisilazane
HOBt	Hydroxybenzotriazole

HPAF	Human pancreatic adenocarcinoma
IC_{50}	Half maximal inhibitory concentration
ICMT	Isoprenylcysteine carboxyl methyltransferase
iPrOH	Isopropanol
K_i	Inhibitor constant
LDA	Lithium diisopropylamide
LiOH	Lithium hydroxide
MeOH	Methanol
MsOH	Methanesulfonic acid
MMP	Matrix metalloproteases
$NaBH_4$	Sodium borohydride
nBuLi	n-Butyllithium
PPh_3	Triphenylphosphine
RB	Retinoblastoma
$SOCl_2$	Thionyl chloride
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLN	Thermolysin
U2OS	Human bone osteosarcoma epithelial cells
ZMPSTE24	Zinc metalloprotease STE24

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Chapter 1. Introduction

1.1 Biological background

1.1.1 Proteases and their value as drug targets

Proteases are enzymes that catalyze the breakdown of proteins by hydrolyzing their amide bonds. The various proteases are structurally and catalytically diverse enzymes, which share the common ability to hydrolyze peptide bonds. The complete set of proteases produced by human cells, called the degradome, consists of at least 569 proteases, distributed into five classes: 194 metalloproteases, 176 serine, 150 cysteine, 28 threonine and 21 aspartic proteases.[1] Proteases play a key role in a large number of physiological processes such as cell-cycle progression, cell proliferation, DNA replication and cell death. It is only natural, then, that they have been considered as promising targets for drug discovery, since there is a plethora of diseases where inappropriate proteolysis has been found to play a major role. Amongst them, a few major diseases stand out that can be treated with an inhibitor of either a mammalian protease (e.g. hypertension) or an inhibitor of a virally-encoded target (e.g., HIV or hepatitis C infections),[2] thus demonstrating that the targeting of proteases is a valid approach for drug discovery.

Targeting proteases to treat hypertension and heart failure is considered a definite success story, with billions of dollars in annual drug sales. The renin-angiotensin-aldosterone system (RAAS) is the most well-known regulator of blood pressure. Renin cleaves the protein angiotensinogen to produce angiotensin I and subsequently the angiotensin converting enzyme (ACE) catalyzes the conversion of the inactive decapeptide angiotensin I to the biologically active octapeptide angiotensin II. This octapeptide affects most of the biological functions of RAAS, thus making the zinc metalloprotease ACE a very promising target. Indeed, a total of 17 ACE inhibitors

have been developed for clinical use for the treatment of hypertension, heart failure, diabetic nephropathy and/or left ventricular dysfunction.[3]

The treatment of AIDS is another field where HIV protease inhibitors have been successfully used as a component of the combination therapy known as highly active antiretroviral therapy (HAART). Ten HIV protease inhibitors have been approved by the FDA, with the most recent one being darunavir, approved in 2006. These protease inhibitors have been a staple for the treatment of AIDS from their introduction in 1995.[4] Today, HIV protease inhibitors hold an 8% share of the HIV drug market, with the market leader of the protease inhibitors, darunavir, has achieved sales of $1.89 billion.[5]

In the field of the hepatitis C virus (HCV) treatment, one of the key targets exploited is the NS3/4A protease. Pioneering work published in 2003 communicated the first NS3/4A protease inhibitor for which antiviral effect in humans was observed[6] and so far 5-6 NS3/4A protease inhibitors have reached the market.[7] Indicative of the value of protease inhibitors in the treatment of HCV is telaprevir, which became the fastest drug to reach a billion dollars in sales in 2012.[8]

In conclusion, a significant and ever growing number of diseases have been successfully treated with a plethora of protease inhibitors and currently there are many more protease inhibitors in late stages of clinical development.[5,8]

1.1.2 Zinc metalloproteases

Metalloproteases are among the protease family in which the nucleophilic attack on the peptide bond is mediated by an activated water molecule. This water molecule is usually activated by a divalent zinc cation, which is coordinated to several amino acid ligands, most often three residues.[9] The zinc ion in the catalytic site generally exhibits a distorted tetrahedral geometry, with

the three O/N/S ligands bound to it and the fourth ligand being a water molecule that constitutes the activated nucleophile. Depending on the protease, this water molecule can be either involved in the catalytic process as a hydroxide ion or can be polarized by the neighboring amino acids of the active site. In addition, the water molecule can be displaced by a substrate which directly coordinates to the zinc.[10a] An explanation for the propensity of zinc to coordinate water at the zinc containing catalytic sites is that it transfers little charge to the zinc cation (Zn^{2+}) and it is less bulky than protein ligands, enabling zinc to serve as a Lewis acid in catalysis.[11] The most common zinc binding motif is the His-Glu-Xaa-Xaa-His (HEXXH) motif. Xaa can be any hydrophobic amino acids or threonine. This motif occurs on an α helix, so that the zinc binding residues His, His and Glu can be brought together in the correct alignment.

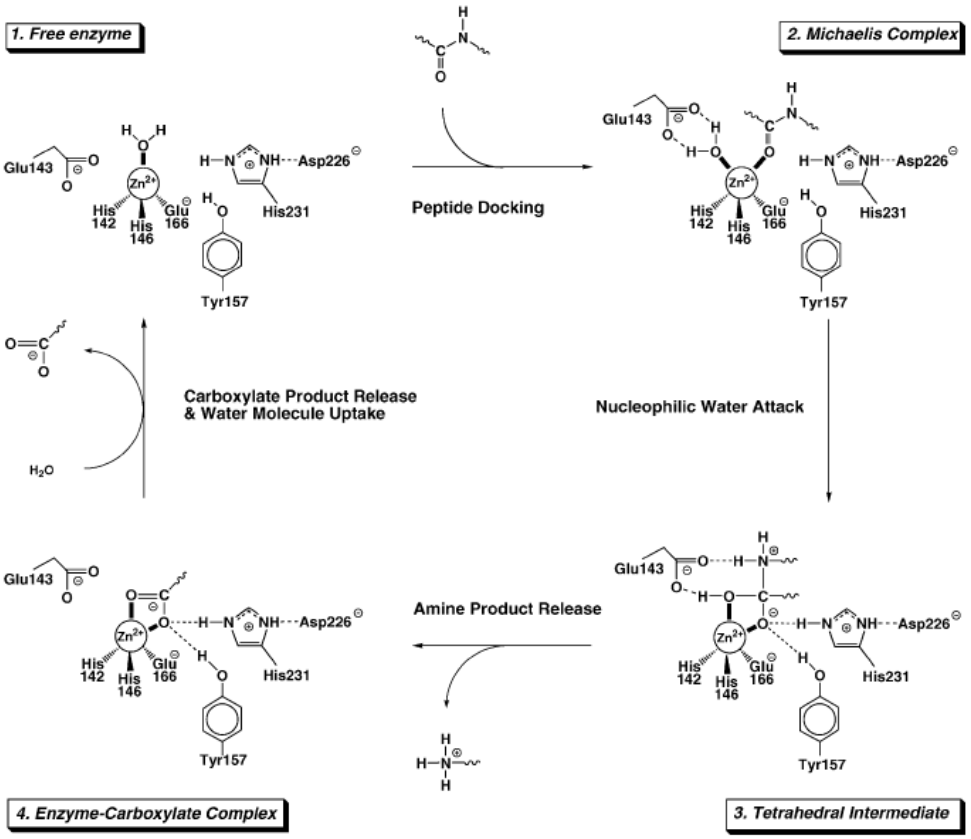


Figure 1. Catalytic cycle for thermolysin, a HEXXH motif containing zinc metalloprotease.[10b]

The zinc cation itself has a remarkably adaptable coordination sphere and if it retains a positive charge after ligating to protein side chains it can act as a Lewis acid in catalysis. It is suggested to be preferred by the metalloproteases since its filled d-shell does not give it oxidation/reduction properties, so it is a stable metal ion species in the biological medium.[9]

Since zinc metalloproteases are ubiquitous in the body, it is logical that they have been considered as potential targets for drug development. There is research precedent spanning decades that should be taken into consideration when discussing the subject of zinc metalloproteases in drug discovery. Angiotensin converting enzyme, as mentioned briefly above and the family of matrix metalloproteases (MMP) are by far the most extensively studied targets.[12] In the case of ACE, 13 inhibitors have been approved for clinical use in the last 20 years, with several others in clinical trials (Fig. 1). A common feature in all of these inhibitors is a metal chelating group which can be a phosphinate, a thiol or a carboxylate that binds to the zinc moiety of the active site.

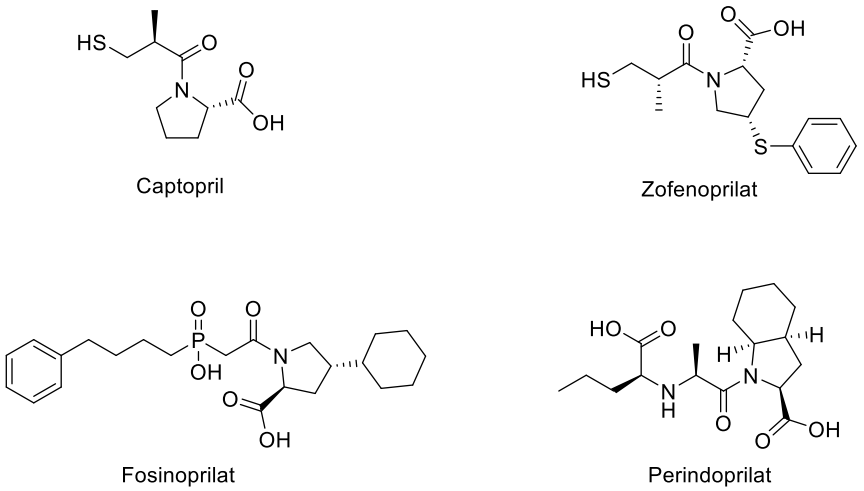


Figure 2. Chemical structures of clinically used ACE inhibitors, containing zinc binding groups.

This interaction offers strong binding activity, with IC_{50} values in the low nanomolar range.[2,13]

As the success of the ACE inhibitors demonstrates, designing inhibitors containing a zinc binding structural motif is a valid strategy. On the other hand, the high affinity does not come without certain liabilities, which are not always easy to overcome. The first one is the highly polar character of the zinc binding moieties, such as a phosphinate, which could be detrimental to the cell-membrane permeability and oral bioavailability of the inhibitors. Indeed, all but three of the ACE inhibitors are administered orally as ester prodrugs.[2] While not trivial, the bioavailability problem pales in comparison to the selectivity issue that plagues the MMP drug development efforts. The matrix metalloprotease family contains at least 23 zinc endopeptidases that degrade both matrix and nonmatrix proteins and therefore play a key role in morphogenesis and tissue remodeling. They are an important drug discovery target, as their abnormal expression contributes to pathological processes, such as arthritis and cancer.[12] Encouraging preclinical results in various cancer models led to clinical trials for a large number of MMP inhibitors but they were all discontinued due to severe side effects and/or no major clinical benefit.[2] Broad MMP inhibition is observed when zinc-binding moieties are used as part of the inhibitor structure, consequently, alternative binding sites were targeted, which provided some compounds of interest but eventually also failed to reach the market.[14]

In conclusion, zinc metalloproteases have been extensively studied as drug targets and have the potential of leading to the development of pharmaceutically importance drugs. A strong zinc binding moiety is regularly used to increase the affinity of the inhibitor, but this can come at a cost of the desirable physicochemical properties and a dramatic loss in target selectivity.

1.1.3 ZMPSTE24

Zinc metalloprotease STE24 (ZMPSTE24) is a mammalian enzyme located in the inner nuclear membrane and is essential for the maturation of lamin A from prelamin A. Lamin A is a

component of the nuclear lamina, a fibrillary network located at the inner side of the nuclear membrane that has both a structural and a functional role. ZMPSTE24 adopts a seven transmembrane α-helical barrel structure which encloses a large chamber that spans the membrane (Fig. 2). The size of this cavity is sufficient to contain a 10-kD protein or 450 water molecules. A possible role for this cavity is suggested by the consumption of a water molecule during the proteolysis, since the maximization of the effective concentration of water may promote the proteolytic cleavage.[15] The zinc metalloprotease domain is located on the nucleoplasmic side of the membrane and resembles the active site of thermolysin, a well-studied bacterial zinc metalloprotease. The seven transmembrane α-helixes are antiparallel in orientation and enclose a large chamber to which access from the nucleoplasm and the membrane is allowed by four fenestrations. The catalytic site itself lies at the apex of the chamber, on the nucleoplasmic side of the membrane. As expected for a zinc metalloprotease, the Zn^{+2} ion is coordinated by three residues, two His and one Glu side chain, so it contains the HEXXH motif in its active site.[16]

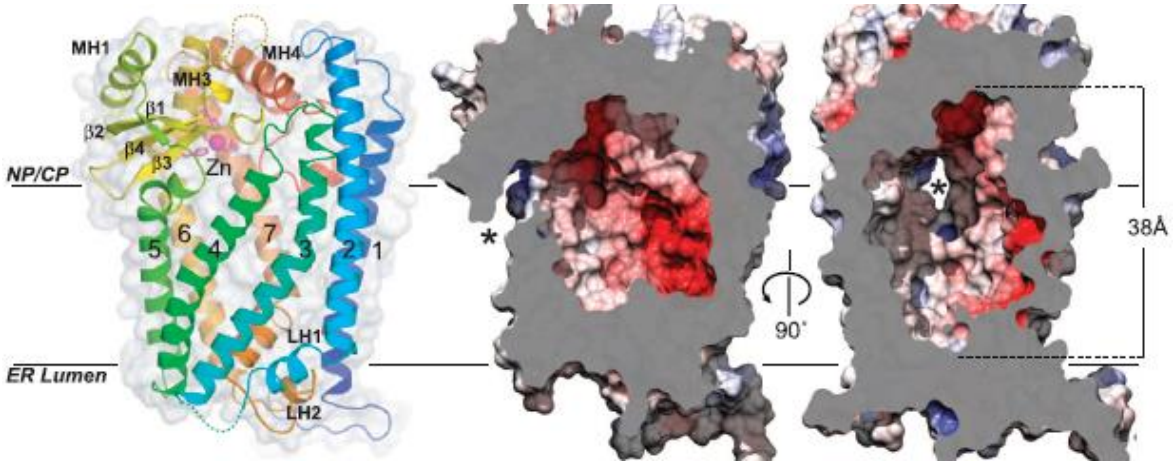


Figure 3. Structure of ZMPSTE24.[16]

ZMPSTE24 performs two cleavages steps in the processing of prelamin A to lamin A (Fig. 3). Prelamin A is subjected to a series of posttranslational modifications: a) it is farnesylated at a cysteine residue near its carboxyl-terminus. ; b) an endoproteolytic cleavage at its C- terminus takes place by the action of either ZMPSTE24 or CAAX prenyl protease 2 (or more commonly called RCE1)), removing three amino acid residues; c) the C-terminal cysteine is capped with a methyl group by isoprenylcysteine carboxyl methyltransferase (ICMT); and d) the modified prelamin A is cleaved by ZMPSTE24, removing another15 amino acids from its carboxyl terminus and producing the mature lamin A (Fig. 3).[17,18] Currently, it is believed that this final transformation can only be performed by ZMPSTE24. Consistent with this hypothesis, studies with in ZMPSTE24 knockout mice, have showed defective prelamin A processing and the symptoms of progeria, a disease caused by accumulation of prelamin A.[19]

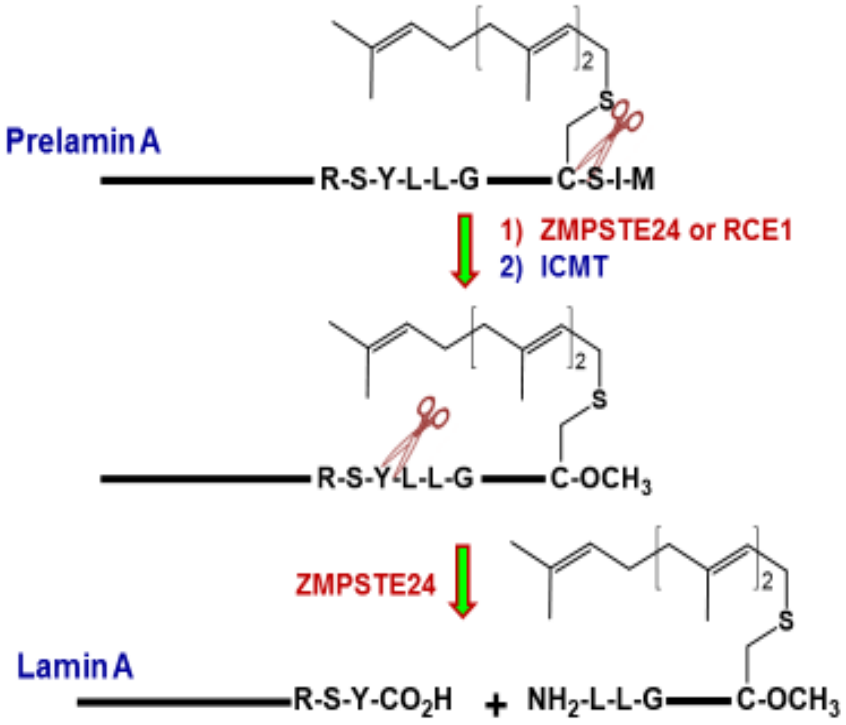


Figure 4. Posttranslational processing of prelamin A to lamin A.

1.1.4 Prelamin A accumulation and progeria

Hutchinson-Gilford progeria syndrome (HGPS, also commonly known as Progeria) is a disease with the phenotypic features of premature aging. On the cellular level it is characterized by premature cell senescence (persistent cytostasis) and the median age of death is 13.4 years of age.[20] It is caused by mutations in the gene which encodes prelamin A (LMNA). This truncated prelamin A retains the final CAAX motif, so it can undergo the first three steps of maturation (like the wild-type prelamin A) but it has lost the cleavage site for the final step, so further processing is blocked and consequently, the peptide it remains prenylated. [17,18,21] The details and effects of prelamin A accumulation have been extensively studied, with Zmptste24 deficient mice being the preferred model for replicating the pathophysiology of progeria. Parallels can be drawn between ZMPSTE24 deficiency and defective prelamin A lacking a cleavage site, since both result in accumulation of prelamin A.

The effects of prelamin A accumulation on the morphology of mammalian cells have been studies extensively. For example, when green fluorescent protein-tagged wild-type prelamin A was expressed in normal fibroblasts and cancer cells, the nuclei were observed to have the expected oval shape. In contrast, the transient expression of green fluorescent protein-tagged defective prelamin A in the same cells caused abnormalities in the nuclear morphology, similar to those seen in HGPS fibroblasts. The most commonly seen morphological change is the development of multinucleated cells with thick regions in the nuclear lamina and nuclear aggregates in the periphery of the nucleus. It was proposed that the defective prelamin A (retaining its farnesyl group) accumulates in the region of the nuclear lamina and has deleterious consequences for the

cell and the organism.[22] These morphological changes result is the induction of cellular senescence, although the direct mechanism has not yet been identified.[23]

There is concrete evidence that it is the accumulation of prelamin A and not the lack of mature lamin A which causes the physiological symptoms of progeria. Lamin C can replace lamin A in lamin C-only expressing mice. Lamin C is a component of the nuclear lamina, homologous to lamin A. These lamin C-only mice are entirely healthy and indistinguishable from wild-type mice and the nuclei of their cells appeared to be only very slightly altered. At the same time, the treatment of ZMPSTE24 deficient cells with a prelamin A-specific antisense oligonucleotide resulted in a significant reduction of prelamin A levels, as well as frequency of morphologically misshapen nuclei.[24]

1.1.5 ZMPSTE24, senescence and cancer

The development of ZMPSTE24 inhibitors is the focus of this project, since the potential therapeutic benefits of inducing cell senescence in the treatment of cancer can be significant. The antitumor effects of senescence (induced by prelamin A accumulation) can be seen qualitatively by the fact that despite accelerated aging HGPS is rarely accompanied by cancer development.[23] The direct correlation between prelamin A accumulation and resistance to tumor invasiveness was established in a study of ZMPSTE24 mosaic mice (complete knockdown of prelamin A expression leads to animals that have an extremely short life span and consequently, are not useful in biochemical studies).[25] In the study with the ZMPSTE24 mosaic mice, half of the cells of the mice expressed ZMPSTE24 while the other half did not, resulting in mice which had normal phenotypic characteristics despite the increased levels of prelamin A in half of their cells. While the increased prelamin A concentration in some of the mice's cells did not affect tumor initiation, it significantly

decreased cancer invasion. The only two carcinomas that were infiltrating (6% of the total carcinomas induced) in the mosaic mice were found to lack prelamin A staining, meaning there was no prelamin A accumulation in the cancer cells themselves. In comparison, in the control mice (i.e. those expressing normal levels of ZMPSTE24), 39% of the induced carcinomas were highly invasive.[25] In a very recent study another important antitumor property of senescent cells was communicated. The prelamin A induced senescence was shown to affect cancer cells that lack p53 or RB tumor suppressor genes as much as in normal (p53 expressing) tumor cells. These two genes are commonly found to be inactivated or mutated in advanced tumors and lead to drug resistance to most currently available chemotherapeutic agents.[23] Additionally, an increase in nucleus stiffness in cells with accumulated prelamin A was shown to decrease the 3D migration ability of lung carcinoma cells. A decrease in the ability of tumor cells to migrate and thus to metastasize is of paramount importance in any chemotherapy regiment. [26] Therefore, it is currently assumed that prelamin A accumulation in cancer cells will induce senescence and block the migration of cells.

Although the potential of therapy-induced senescence can only be realized conclusively during clinical trials, the collective biochemical knowledge suggests that this approach may be associated with reduced toxicity-related side effects and increased tumor-specific immune activity. Additionally, the obliteration of sensitive tumor cells by cytotoxic chemotherapy may result in the rapid proliferation of resistant clones, a weakness which could be overcome by changing approach from cytotoxic to cytostatic cells that can be eliminated by the immune system. In terms of maintaining quality and quantity of life of cancer patients, therapy-induced senescence could be a great improvement over classic cytotoxic approaches. Unfortunately, amongst other issues plaguing the research about senescence as a novel way to combat cancer, there is currently a lack of robust senescence inducing agents.[27] The goal of this project is the development of potent

ZMPSTE24 inhibitors that can act as senescence inducing agents through the accumulation of prelamin A. The goal is to reduce cancer cell proliferation by the irreversible arrest of the cell cycle and also limit the tumor cells ability to migrate, thus reducing their ability to metastasize.

1.2 Chemistry background

1.2.1 Inhibitors of ZMPSTE24

An investigation into the causes of the side effects induced by drugs inhibiting the HIV protease (PR) enzyme led to the discovery that some HIV PR inhibitors, such as lopinavir and tipranavir (Fig. 4), are also weak inhibitors of the ZMPSTE24 protease. Prelamin A accumulation was observed in fibroblasts upon exposure to HIV PR inhibitors and IC_{50} values of 18.4 ± 4.6 μM and 1.2 ± 0.4 μM for lopinavir and tipranavir, respectively, for inhibiting ZMPSATE24 were reported.[28] However, the absolute numbers of these values need to be taken as an estimate, since the pure ZMPSTE24 enzyme was not used, instead the IC_{50} values were estimated from some down-stream biochemical events. Nonetheless, the modest potency of these inhibitors against ZMPSTE24 led us to believe that such compounds could be used as "hits" in initiating structure-activity relationship (SAR) studies on ZMPSTE24 inhibitors. The lopinavir scaffold was favored over that of tipranavir due to its significantly more accessible synthesis. The choice of lopinavir as the hit compound upon which the design of novel ZMPSTE24 inhibitors was based comes with additional advantages. Firstly, since lopinavir has passed clinical trials and entered the market as an oral drug, lopinavir has a desirable absorption, distribution, metabolism, excretion and toxicity profile (ADMET). Moreover, its synthesis is well established and robust enough to be performed in an industrial scale. Finally, there is a wealth of information on peptidomimetic inhibitors of

aspartic and zinc proteases that can be exploited to aid in the design of potent ZMPSTE24 inhibitors.

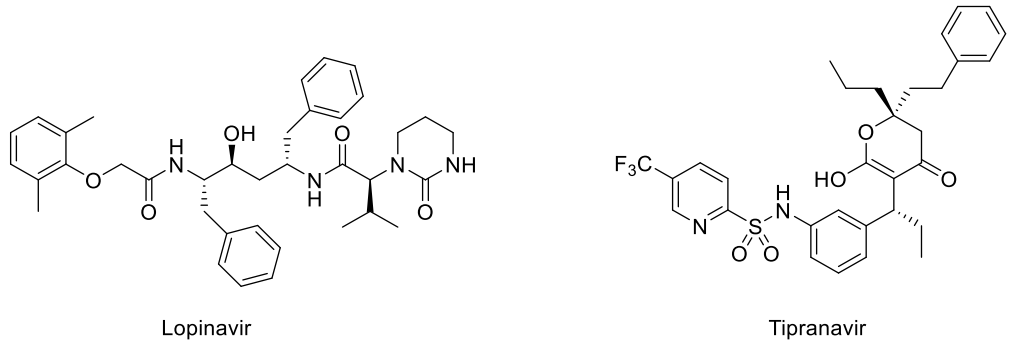


Figure 5. Structures of HIV protease inhibitors lopinavir and tipranavir

It is not surprising that lopinavir and tipranavir can also inhibit proteases other than the intended protease target in spite the fact that the HIV PR is an aspartic protease, much different in its active site to ZMPSTE24, which is a zinc metalloprotease. The hydroxyethylene moiety of lopinavir coordinates to the two catalytic Asp residues of HIV PR,[29] but it is likely also weakly chelating the zinc ion of ZMPSTE24.Additionally, the fact that a moderate inhibitory activity is observed in the absence of a strong zinc binding moiety suggests that lopinavir may bind into the active site of ZMPSTE24 with modest affinity. Based on this hypothesis, two distinct avenues of research were explored in the effort to develop more potent and selective inhibitors of ZMPSTE24. Based on the structure of lopinavir, our initial approach involved the synthesis of hydroxyethylene analogues with more optimized interactions between the inhibitor and the enzyme's active site. Additionally, a search was undertaken for replacing the hydroxyl moiety of these compounds with a more potent zinc binding motif, in order to significantly increase the binding affinity of these inhibitors.

1.2.2 Hydroxyethylene peptidomimetics

Peptidomimetics are bioisosteres of small protein-like chains whose pharmacophore mimics that of a natural peptide and have been used extensively in drug discovery. Converting amides into other functional groups allows peptidomimetics to circumvent some of the problems associated with natural peptides, including their proteolytic instability and poor oral bioavailability. Between the mid-80s and the mid-90s a large number of hydroxyethylene dipeptides were synthesized in the course of developing HIV PR inhibitors.[31] For example, the core of lopinavir is formed by a hydroxyethylene transition state bioisostere (Fig. 5). This scaffold has been extensively studied by academic and industrial research groups since it is used in the synthesis of aspartic protease inhibitors, such as HIV PR and β-secretase.[29,30]

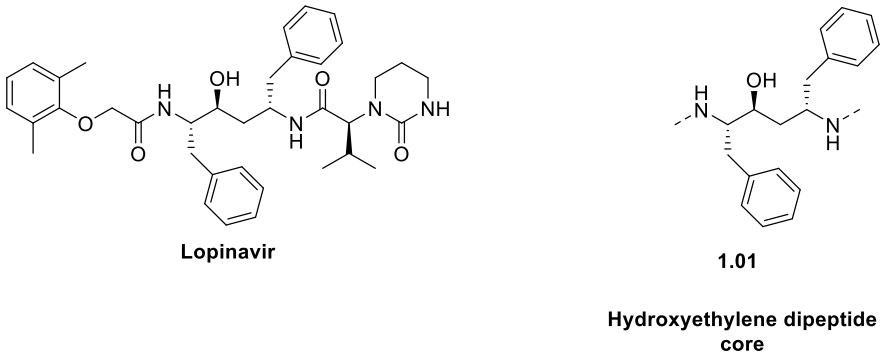


Figure 6. The hydroxyethylene core of lopinavir.

Originally the hydroxyethylene dipeptide **1.01** was accessible through a modified Curtius rearrangement of acid **1.02**, by using diphenyl phosphoryl azide. The acid **1.02** was produced by

the saponification of key lactone **1.03** (Fig. 6), a synthetic intermediate to which most hydroxyethylene isostere converge.[32]

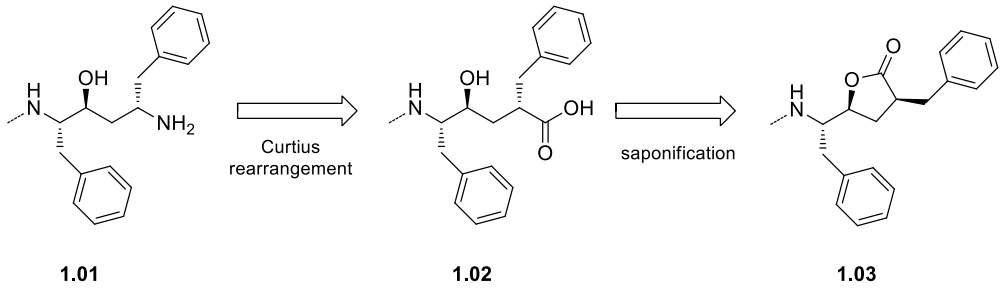


Figure 7. Synthesis of **1.01** through key lactone **1.03**.

The first synthesis in which access to all isomers of **1.03** was achieved was an important milestone (Fig. 7). In this synthesis, lactone **1.03** came from the diethyl benzylmalonate adduct **1.04** after its saponification and decarboxylation. The substrate for this nucleophilic addition was the key intermediate cyclopropane **1.05** which was accessible by a Corey Chaykovsky epoxidation of aldehyde **1.06** by addition of dimethylsulfonium methylide. Aldehyde **1.06** was produced from the reduction of L-phenylalanine. However, this synthesis is not stereoselective and the various diastereomers have to be isolated at each step they are produced by column chromatography, resulting in a low overall yield.[33]

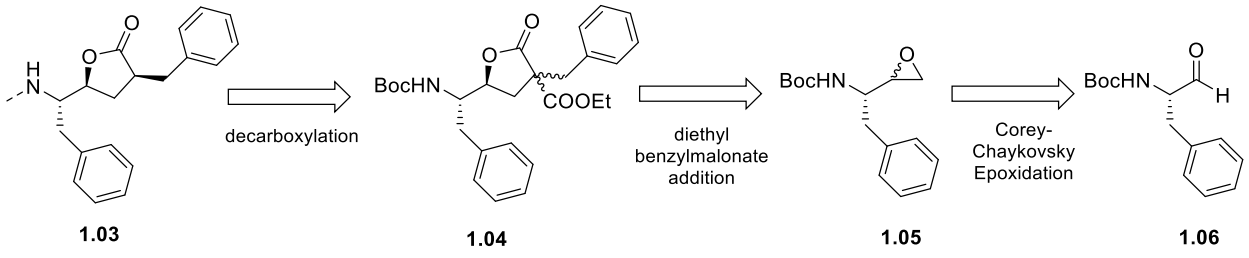


Figure 8. Epoxide opening route to **1.03**.

An improved synthesis, providing access to a larger substrate scope is shown in Figure 8. In this synthesis, the olefin **1.07** was used as the precursor to lactone **1.03**, to which various nucleophiles can be added by a conjugate addition reaction. The desired phenylalanine adduct **1.03** was prepared by addition of cuprate $Ph_2CuCNLi$, followed by chromatographic separation of the desired diastereomer. Intermediate **1.07** was accessible by heat induced cyclization of the hydroxyamide **1.08**. In turn the key intermediate **1.08** was produced by the addition of dilithiated *N*-methylmethacrylamide in the presence of chlorotitanium triisopropoxide to the L-phenylalanine derived aldehyde **1.06**. The drawback of this synthesis is once again the lack of stereocontrol.[34]

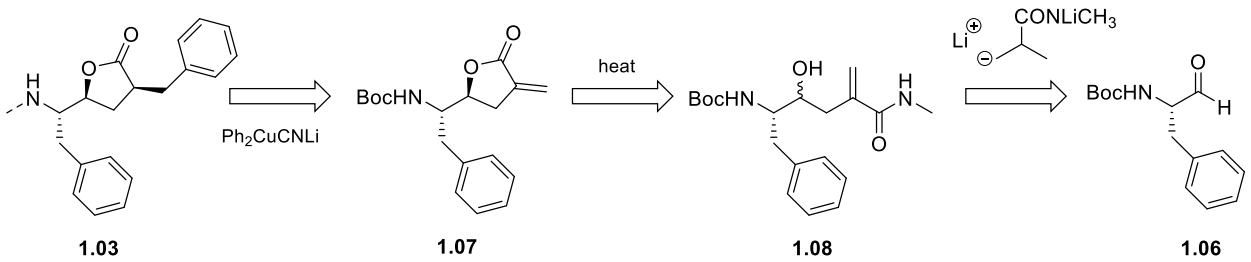


Figure 9. Hydroxyamide route to **1.03**.

The first stereocontrolled synthesis was published by Merck and although it avoided the waste of large amounts of material, it involved additional steps (Fig. 9). Target lactone **1.03** was obtained by an epimerization of intermediate **1.09** in a three step process. The undesired diastereomer **1.09** was treated with LDA, followed by PhSeSePh, in order to provide the phenyl selenide, which was subsequently dehydroselenylated and the resulting olefin was hydrogenated with Pd/C.[32] An addition of benzyl iodide to the HMDS produced enolate of lactone **1.10** produced **1.09**. Access to **1.10** was provided by azide **1.11**, through an oxidation of the methoxy group with

m-CPBA and a catalytic hydrogenation of the azide moiety in the presence of Boc anhydride. The key intermediate which preceded **1.11** was the epoxide **1.12**. A regiospecific attack of BnMgBr afforded an alcohol intermediate upon opening of the epoxide, which underwent a Mitsunobu reaction with the azide as the nucleophile in the form of diphenyl phosphoryl azide. Access to **1.12** was provided by intermediate **1.13** by hydrogenation of the olefin, followed by deprotection of the acetonide with aqueous acetic acid. The stereoselective epoxide formation was achieved by a tosylation of the primary alcohol with p-toluenesulfonyl chloride and subsequent treatment with sodium methoxide. The intermediate **1.13** was accessed in two steps from acetonide **1.14**. Firstly, a modified Appel reaction to chlorinate the free alcohol, with a subsequent reduction of the resulting furanosyl chloride with lithium in ammonia furnished the elimination product at the C-2 position. Secondly, a Ferrier rearrangement (which is a nucleophilic substitution followed by an allylic shift) in methanol and p-toluenesulfonic acid resulted in further dehydroxylation and the introduction of the methoxy group at the C-2 position. Lastly, **1.14** was produced in one steps from D-mannose by addition of dilute sulfuric acid in acetone.[35]

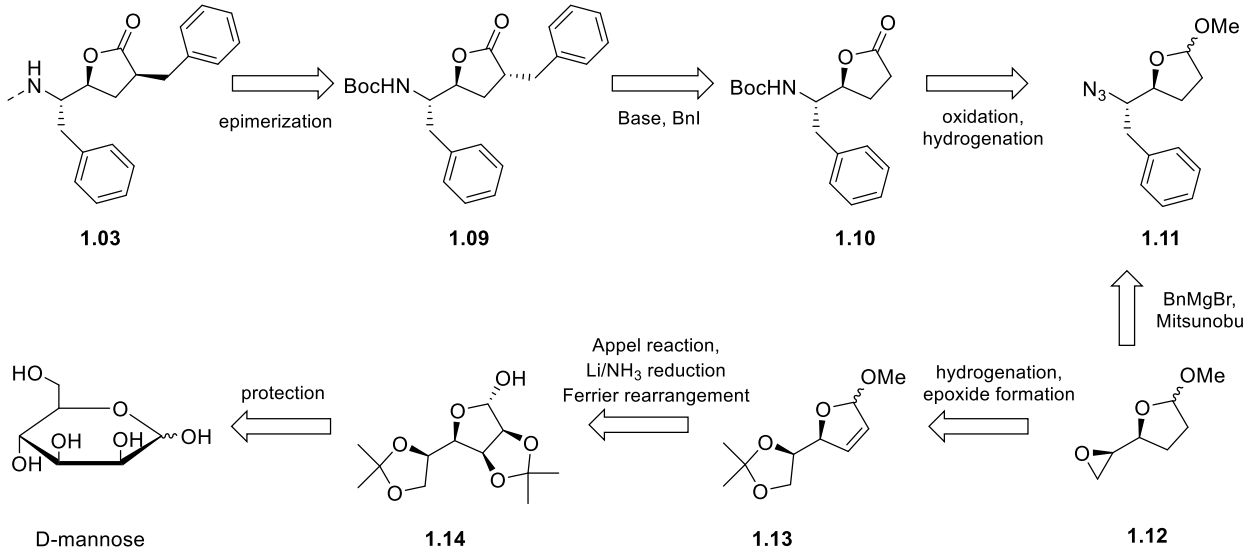


Figure 10. D-mannose based route to **1.03**.

This last synthesis did manage to tackle this diastereoselectivity issue, but required a prohibitively large number of steps to be considered practical. A breakthrough came with a synthetic methodology published by Abbott, the company that developed lopinavir (Fig. 10).[36,37] This synthesis deviates from the previous routes in that it uses L-phenylalanine as the optically pure and inexpensive starting material to produce the desired hydroxyethylene dipeptide **1.01** without going through the lactone intermediate **1.03** (Fig. 6).

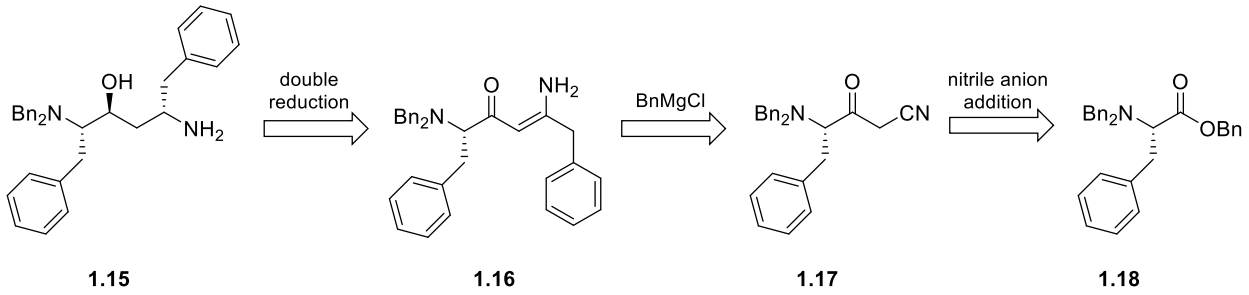


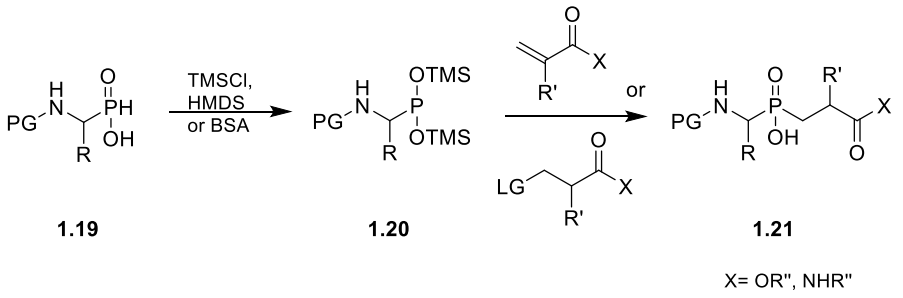
Figure 11. Abbott route to hydroxyethylene dipeptide **1.15**.

The benzyl protected hydroxyethylene dipeptide **1.15** (Fig. 10) was obtained by a key double reduction, starting with sodium borohydride and methanesulfonic acid in THF/iPrOH to reduce the enamine, followed by sodium borohydride and trifluoroacetic acid in the same reaction to reduce the ketone moiety of the enaminone precursor **1.16**. This precursor could be easily obtained from the nitrile **1.17** by an addition of BnMgCl. This nitrile was accessible through an addition of a nitrile anion to the tribenzylated **1.18**, a compound which can be easily produced from L-phenylalanine. This remarkably straightforward synthesis afforded the desired

diastereomerically enriched intermediate **1.15** without any need for chromatographic purifications of the intermediates, in just 4 steps and in good yield.[36,37]

1.2.3 Phosphinic peptidomimetics

The predominant strategy for the preparation of the pseudodipeptide core is the addition of amino-protected aminophosphorus species to carbon electrophiles. There has been a considerable amount of research done, spurred by the development of robust methodologies for the synthesis of the aminophosphorus analogues of all natural aminoacids.[38] The amino acid analogues **1.19** can be activated by application of a silylating agent to generate the trivalent nucleophilic phosphorus species **1.20** (Fig. 11). This intermediate (**1.20**) can attack an electrophile, which can either be an *α,β*-unsaturated ester or an electrophile activated by a leaving group at the *β* position of the ester, thus producing the pseudodipeptide **1.21**.[39]



1.19 **1.20** **1.21**

Figure 12. Synthetic route toward pseudodipeptide core **1.21**.

The accessibility of enantiomerically enriched α-aminophosphinic acids of type **1.19** with a variety of R groups enabled the synthetic approach shown in Figure 11. The synthesis of **1.19** was initiated with a three component condensation of an appropriate aldehyde,

diphenylmethylamine (DPM-NH_2) and hypophosphorous acid, producing the DPM protected aminophosphinic acid **1.22** (Fig. 12). A deprotection under strongly acidic conditions releases the salt of the free amine, which was neutralized with propylene oxide and was subsequently protected with the more convenient Cbz group, affording the racemic product **1.19**. At this point in the synthesis, a resolution with chiral α-methyl benzylamines was possible, in order to obtain the two enantiomers at a high enantiomeric excess.[38]

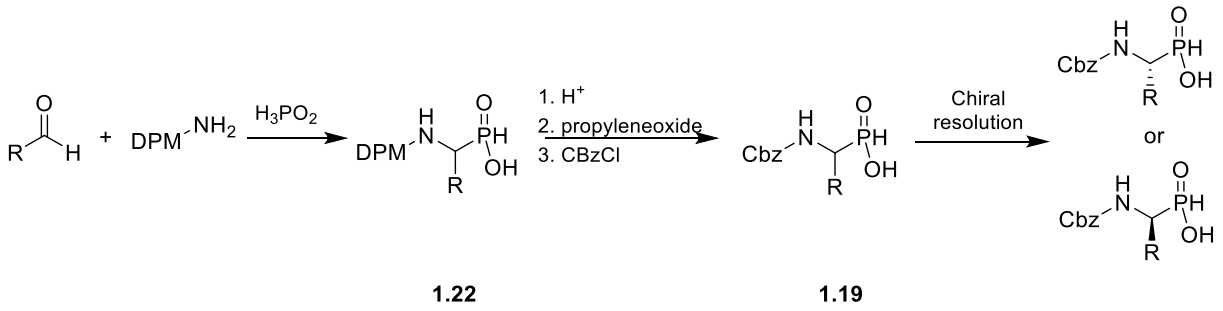


Figure 13. Synthesis of α-aminophosphinic acid **1.19**.

The above synthetic procedure can be used to generate large amounts of starting aminophosphinic acid **1.19**. However, there are a few drawbacks to this methodology, including the need for an exchange of protecting groups and the loss of a large amount of the undesired enantiomer in the chiral resolution. Various methodologies which aim to alleviate these problems have been published. An example of a synthesis which avoids the usage of the DPM protecting group is shown in the synthesis of methionine analogue **1.25** (Fig. 13). Acrolein was used as the starting material and methanethiol was added, to give the aldehyde **1.23**. Hydroxylamine hydrochloride in pyridine was used to generate the oxime **1.24**, which underwent a condensation with anhydrous

hypophosphorous acid, yielding the methionine analogue **1.25**.[40]

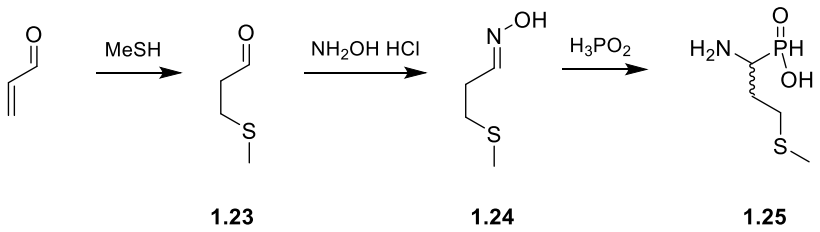


Figure 14. Synthesis of methionine analogue **1.25**.

The first synthetic procedure with a broad substrate scope took advantage of the chiral *tert*-butanesulfinamide auxiliaries, also known as Ellman's auxiliaries (Fig. 14). The chiral sulfonamide **1.26** was prepared by condensation of 2-methylpropane-2-sulfinamide and an aldehyde by titanium isopropoxide or $CuSO_4$. A reaction with diethoxymethylphosphinate **1.27** and rubidium carbonate provides the sulfinamide protected product **1.28** at moderate to high yields. Subsequently, a three step deprotection with HCl in water, then in methanol and finally treatment of the resulting salt with propylene oxide results in the desired product **1.29**. The enatiomeric ratio of the products ranges from 69:31 to 99:1, depending on the substrate.[41] Intermediate **1.29** was then protected and converted to the desired starting material **1.19**. It is noteworthy that a catalytic and enantioselective synthesis for the preparation of α-aminophosphinic acid, such as **1.19**, has not been report (this is the goal of another research project in our group).

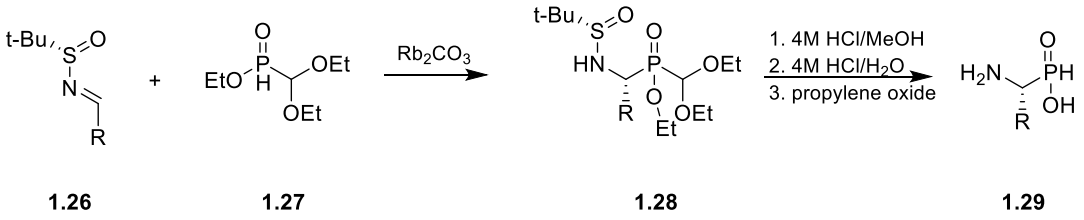


Figure 15. Usage of Ellman's auxiliaries in the diastereoselective synthesis of **1.29**.

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Chapter 2. Design and synthesis of ZMPSTE24 inhibitors

2.1 Synthesis of hydroxyl containing ZMPSTE24 inhibitors

None of the work presented in this chapter has been published. All the synthesis work was performed by myself.

2.1.1 Preface

Various hydroxyethylene peptidomimetics were designed and synthesized in our group. The ability of these compounds to block prelamin A processing and proliferation of cancer cells was evaluated.

2.1.2 Design and synthesis of hydroxyethylene analogues

Previous effort from our group produced compounds that show increased prelamin A accumulation in cancer cells and blocked their proliferation. This improvement in activity came from the substitution of the cyclic urea moiety of lopinavir with various aromatic and heterocyclic substituents, with the more active compounds inducing approximately a two-fold higher prelamin A accumulation, as compared to lopinavir. In an effort to further explore the role of the N-terminal moiety (i.e. R_1; Fig. 15), as well as that of the isopropyl side chain at R_2 (originally present in lopinavir), analogues with other side chains were synthesized (Fig. 15, Compounds **2.01**, **2.02**, **2.03**).

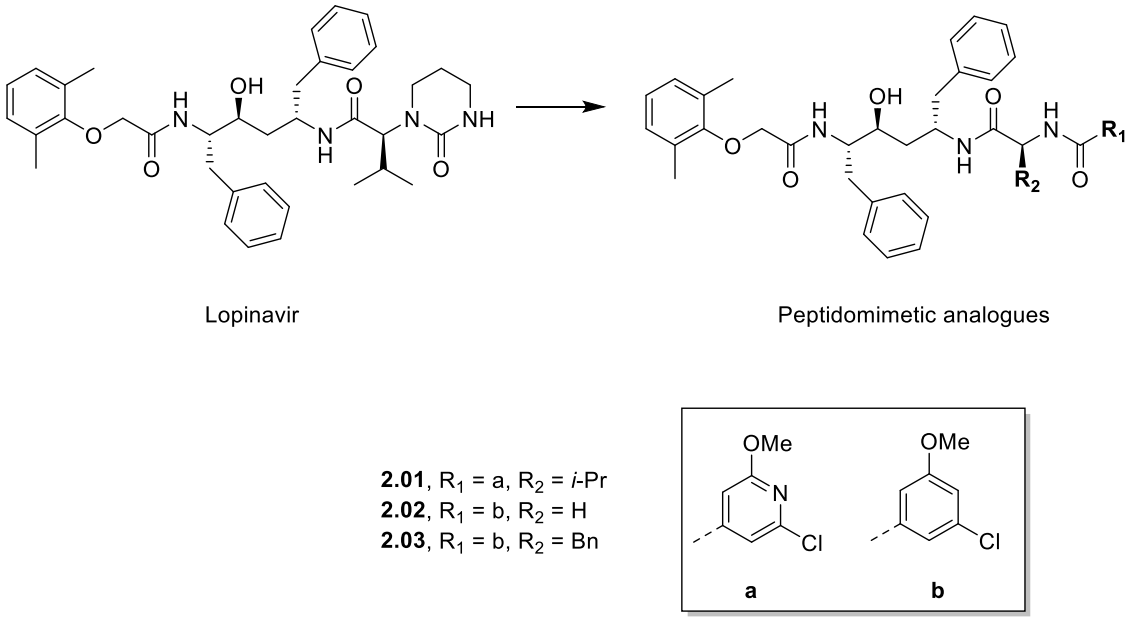
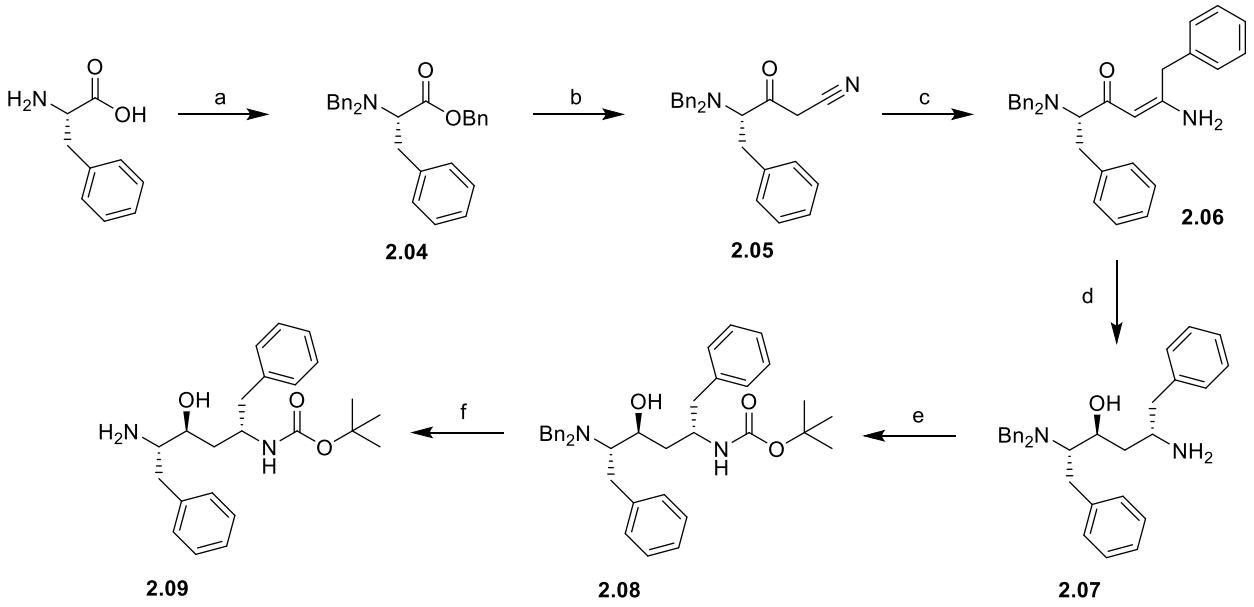


Figure 16. Target peptidomimetic analogues of lopinavir.

These target compounds necessitated the synthesis of the hydroxyethylene dipeptide core **2.07** (Scheme 1), which was achieved following the procedure previously published by Abbott.[1] Initially, L-phenylalanine was tribenzylated with benzyl bromide to give the ester **2.04** which underwent an addition reaction with an acetonitrile anion, to form the β-keto nitrile **2.05**. The subsequent addition of excess BnMgCl produced the key intermediate enaminone **2.06**. Intermediate **2.06** was exposed to a double reduction with $NaBH_4$ in the presence of methanesulfonic acid for the first step, and then with trifluoroacetic acid for the second step. The major diastereomer produced was isolated by flash column chromatography and its ^1H NMR spectrum was consistent with that reported nu Abbott.[1] Subsequently, protection of the amine group with Boc anhydride afforded **2.08** which was debenzylated by hydrogenolysis using 20% Pd/C, to provide the desired amine **2.09** in good yield.

Scheme 1. Synthesis of the hydroxyethylene backbone.

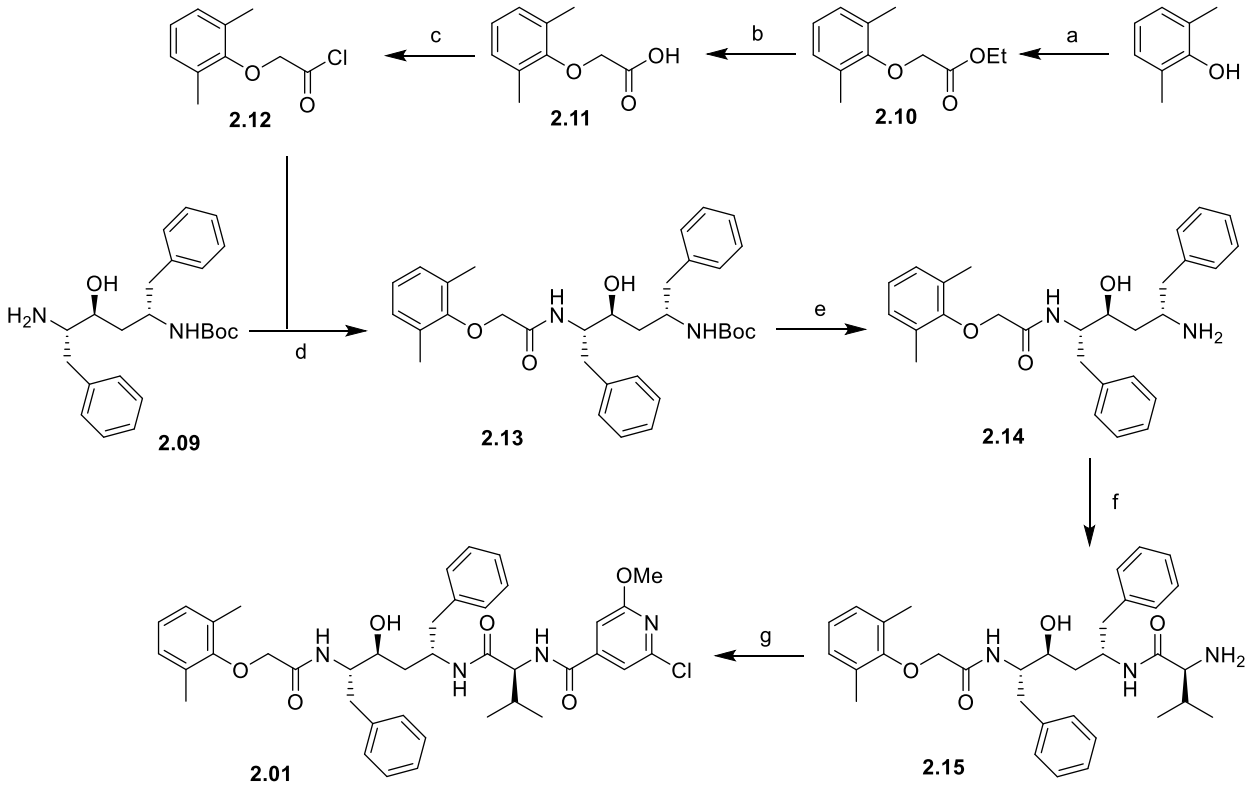


Reagents: (a) BnBr, K_2CO_3, H_2O (96%); (b) CH_3CN, $NaNH_2$ (66%); (c) BnMgCl, THF (83%); (d) i. $NaBH_4$, MsOH, THF/iPrOH, ii. $NaBH_4$, TFA, THF/iPrOH (45%); (e) $(Boc)_2O$, Et_3N, CH_2Cl_2 (98%); (f) $Pd(OH)_2/C$ 20%, MeOH (81%).

The coupling partner for the amine **2.09** was produced in three steps from 2,6-dimethylxylenol (Scheme 2). Initially, a cesium carbonate promoted nucleophilic attack on ethyl bromoacetate gave rise to ester **2.10**, which was hydrolyzed with excess lithium hydroxide. The resulting acid **2.11**. The acyl chloride **2.12** was freshly prepared with thionyl chloride before coupling with the amine **2.09**. This reaction proceeded smoothly, giving rise to **2.13** in good purity and yield; intermediate **2.13** was used as such in the subsequent steps without further purification.[2] Carbamate **2.13** was then treated with TFA to obtain the key intermediate **2.14**. The following addition of L-valine-N-carboxyanhydride to **2.14** produced the amine **2.15**, with no observed

epimerization observed under our reaction conditions, as determined by reversed phase HPLC-MS analysis. The amine **2.15** was coupled to various carboxylic acids using standard peptide coupling conditions, with HBTU as the coupling reagent.[3] This overall reaction scheme lead to the preparation of the peptidomimetic inhibitor **2.01** (Scheme 2).

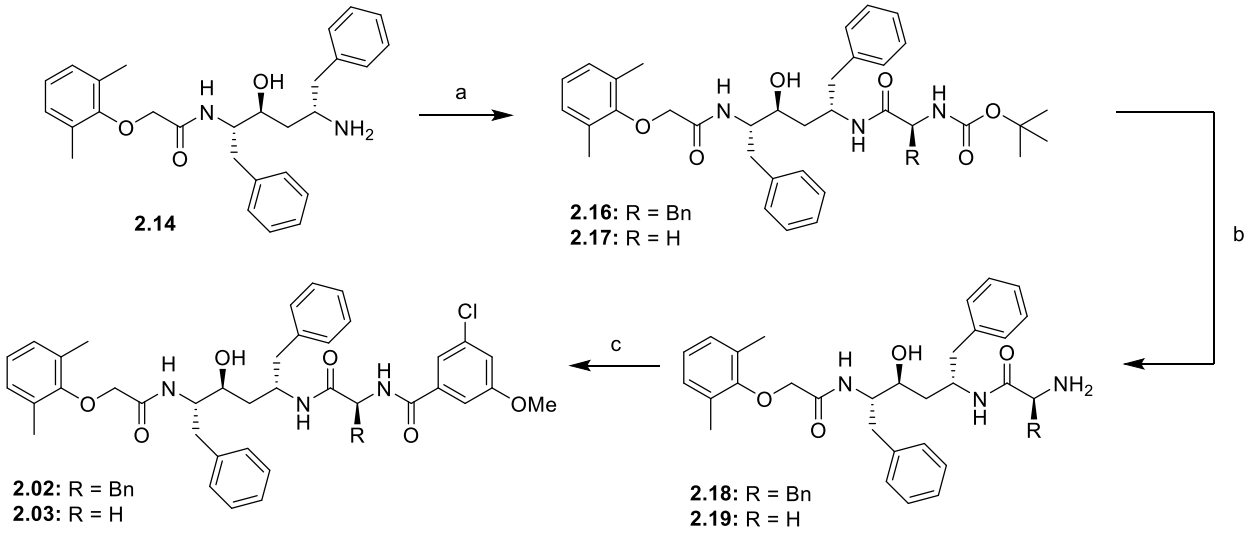
Scheme 2. Synthesis of the hydroxyethylene inhibitor **2.01**.



Reagents: (a) ethyl bromoacetate, Cs_2CO_3, 1,4-dioxane (96%); (b) LiOH, MeOH/H_2O (93%); (c) $SOCl_2$, DMF, EtOAc; (d) $NaHCO_3$, EtOAc/H_2O (82%); (e) TFA, CH_2Cl_2 (97%); (f) L-valine-N-carboxyanhydride, Et_3N, CH_2Cl_2 (95%); (g) 2-Chloro-6-methoxypyridine-4-carboxylic acid, HBTU, DIPEA, DMF (68%).

The key intermediate **2.14** was also used for the synthesis of the two analogues in which the R moiety is replaced with –H or benzyl (Scheme 3, compounds **2.02** and **2.03**). Boc-glycine and Boc-L-phenylalanine were coupled with the backbone **2.14** using EDC and HOBt as the coupling reagents, affording carbamates **2.16** and **2.17** (Scheme 3). After deprotection with trifluoroacetic acid in dichloromethane, the free amines **2.18** and **2.198** were coupled with 3-chloro-5-methoxybenzoic acid using HBTU in the presence of Hünig's base, affording the final compounds **2.02** and **2.03**.

Scheme 3. Synthesis of glycine and phenylalanine containing inhibitors **2.02** and **2.03**.



Reagents: (a) HOBt, EDC, DIPEA, THF, Boc-L-Phenylalanine for **2.16** (71%), Boc-Glycine for **2.17** (62%); (b) TFA, CH_2Cl_2 (87% for **2.18**, 85% for **2.19**); (c) 3-chloro-5-methoxybenzoic acid, HBTU, DIPEA, DMF (67% for **2.02**, 40% for **2.03**).

2.1.3 Design and synthesis of *β*-hydroxy amide analogues

An effort to increase the potency of our inhibitors through retention of the hydroxyl group and a simultaneous addition of an interaction with the catalytic zinc ion of ZMPSTE24 was undertaken. It was presumed that *β*-hydroxy amide backbone may be capable of chelating to the zinc cation as shown in Figure 16. The six-membered chair-like transition state of the Reformatsky reaction, held together by a zinc ion, which is coordinated between two oxygen atoms, represents an example of a zinc ion chelating to a *β*-hydroxy esters (Fig. 16).[4,5] This moiety also exists in natural products which exhibit potent cytotoxic activity against cancer cells, yet the mechanism by which this is achieved is still unknown.[6] Certain compounds based on a *β*-hydroxy amide scaffold were synthesized, such as analogue **2.44**.

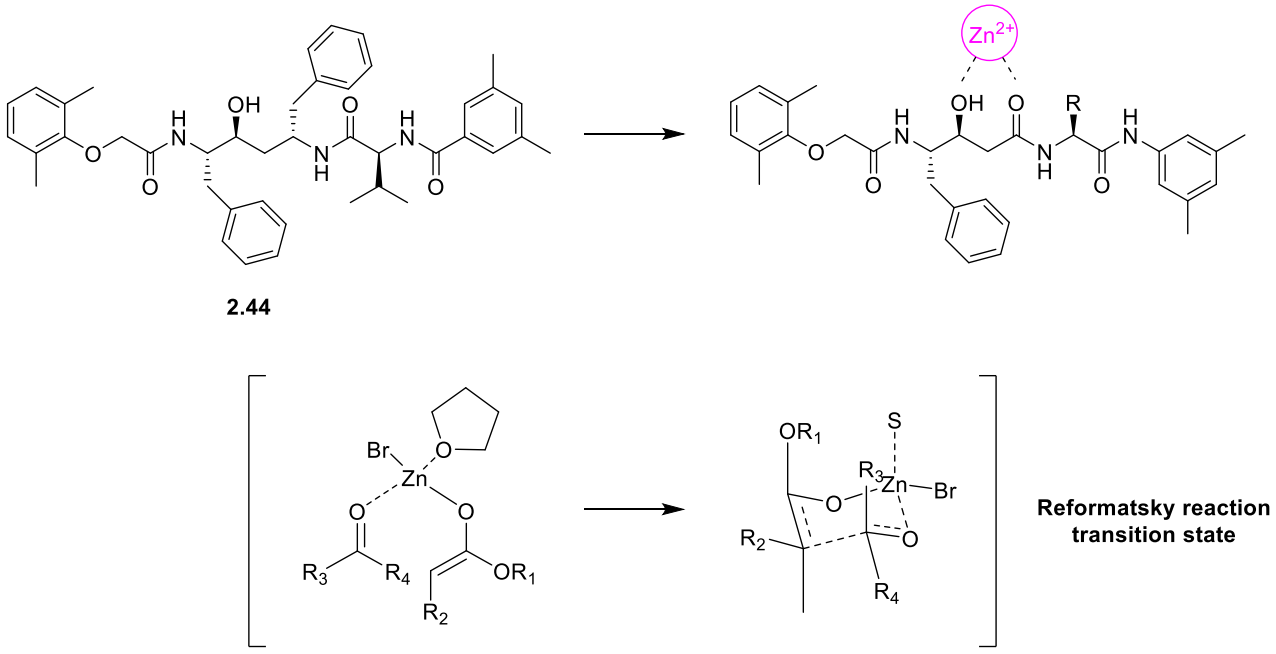
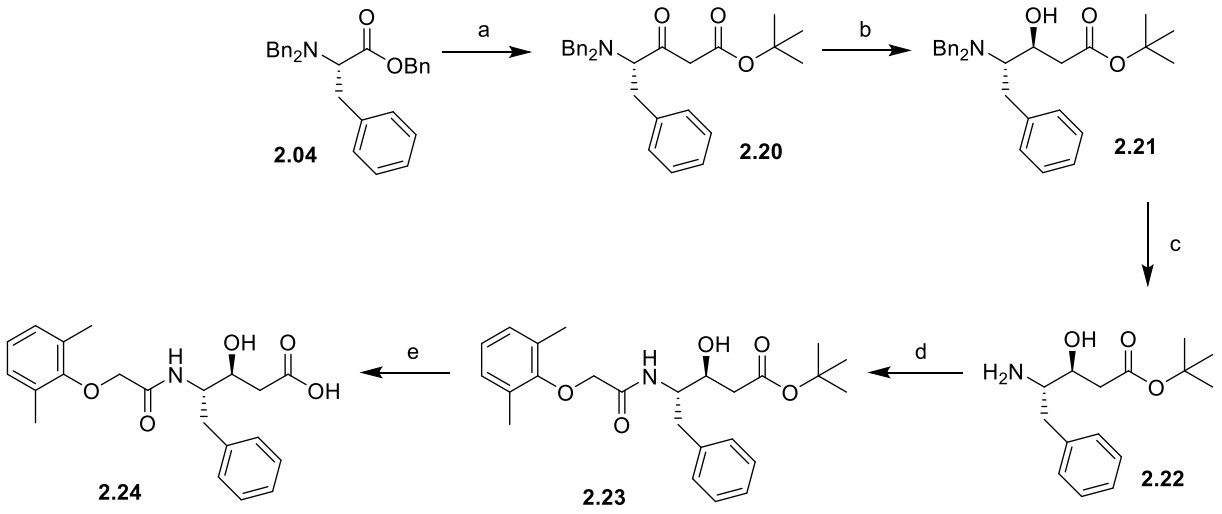


Figure 17. Change from hydroxyethylene to *β*-hydroxy amide backbone and Zn^{2+} bound in Reformatsky reaction's transition state.[7]

Tribenzylated phenylalanine **2.04** was used as the starting material (Scheme 1) and was converted to rester **2.20** via a cross-Claisen condensation with *tert*-butyl acetate in the presence of LDA (Scheme 4).[8] The subsequent reduction of the ketone with $NaBH_4$ in methanol proceeded with good diastereoselectivity (15:1 as determined by isolated yields after flash column chromatography), providing alcohol **2.21**. The absolute configuration of product **2.21** was confirmed by comparing its ^1H NMR spectrum to the ^1H NMR spectrum of the same product from a natural product synthesis.[9] A hydrogenolysis with Pd/C 10% furnished the deprotected amine **2.22**,[9] which was coupled with 2-(2,6-dimethylphenoxy) acetyl chloride under the same conditions as those used in the hydroxyethylene analogue synthesis. Lastly, TFA was used for the removal of the *t*-butyl ester of **2.23**, providing the free acid **2.24**.

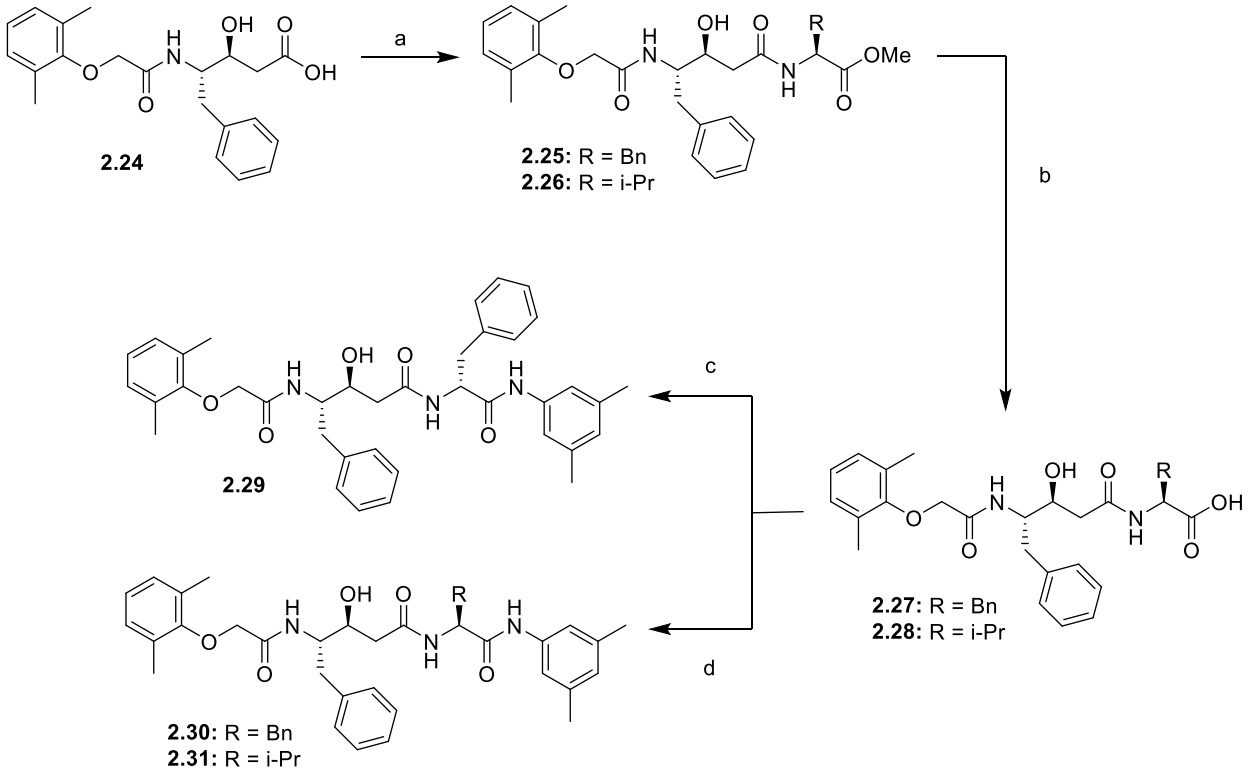
Scheme 4. Synthesis of β-hydroxy ester backbone.



Reagents: (a) t-butyl acetate, LDA, THF (93%); (b) $NaBH_4$, MeOH (80%); (c) Pd/C 10%, MeOH (95%); (d) 2-(2,6-dimethylphenoxy)acetyl chloride (**2.12**), $NaHCO_3$, $EtOAc/H_2O$ (98%); (e) TFA, CH_2Cl_2 (99%).

Subsequently, intermediate **2.24** was coupled with L-phenylalanine and L-valine using HBTU and HOBt, affording the methylesters **2.25** and **2.26**, which were hydrolyzed in the presence of aqueous NaOH. in methanol to acids **2.27** and **2.28** (Scheme 5). Finally, these intermediates were coupled with 3,5-dimethyaniline, using TBTU as the coupling reagent, however this step was plagued by a significant epimerization. While the optimal conditions were investigated, diastereomer **2.29** was isolated by reversed phase HPLC from the 1:1 mixture of products of the reaction under initial conditions. Eventually, change of the reaction conditions from TBTU/DIPEA to HATU/ $NaHCO_3$, yielded the desired products with 90% d.e. (as estimated by HPLC-MS). These crude reaction mixtures were purified by reversed phase HPLC, to give compounds **2.30** and **2.31** (Scheme 5).

Scheme 5. Synthesis of β-hydroxy amide final compounds.



Reagents: (a) HBTU, HOBt, DIPEA, CH_2Cl_2, L-Phenylalanine methyl ester HCl for **2.25** (81%), L-Valine methyl ester HCl for **2.26** (61%); (b) NaOH 1N, MeOH (94% for **2.27**, 74% for **2.28**); (c) 3,5-dimethylaniline, TBTU, DIPEA, DMF (24%); (d) 3,5-dimethylaniline, HATU, $NaHCO_3$, DCM/DMF 3:1 (42% for **2.30**, 29% for **2.31**).

2.2 Synthesis of phosphinate containing ZMPSTE24 inhibitors

2.2.1 Preface

Phosphinate peptidomimetic inhibitors were previously synthesized in our group and shown to inhibit ZMPSTE24. The structure of these compounds was inspired by literature reports of inhibitor targeting the functionally related thermolysin enzyme. Based on this background information, a diazirine containing inhibitor retaining as much of the structure of the previously synthesized phosphinate compounds compound was also synthesized, in order to be used as a photoaffinity label.

2.2.2 Design and synthesis of phosphinate inhibitors

Thermolysin (TLN) is a bacterial zinc metalloprotease which has been used as a model zinc metalloprotease for the understanding of ligand-enzyme binding of functionally-related proteases of pharmaceutical value.[10,11] The morphology of the active site of ZMPSTE24 was recently determined with the aid of the very well characterized active site of thermolysin. In this publication, the resemblance between the active sites of the two proteases is highlighted both in terms of the catalytic residues and in the hydrophobic subpockets adjacent to the active site, which determine substrate specificity.[12] Superimposition of STE24p's (the saccharomyces ortholog of ZMPSTE24) active site with that of TLN suggested significant similarities (Fig. 17).[14] However, the co-crystal structure of a short peptide bound to ZMPSTE24 was of very low resolution and consequently, conclusive predictions cannot be drawn about the ligand-ZMPTE24 interactions (Fig. 17).[13] Nonetheless, we decided to adopt the zinc binding moiety found in the TLN inhibitors in the design of our ZMPSTE24 inhibitors. Although the currently available crystallographic

evidence does not permit clear parallels to be drawn between the active sites of these two enzymes, the phosphinic inhibitor **2.32** was also found to exhibit good potency in blocking prelamin A maturation in our cell-based assays

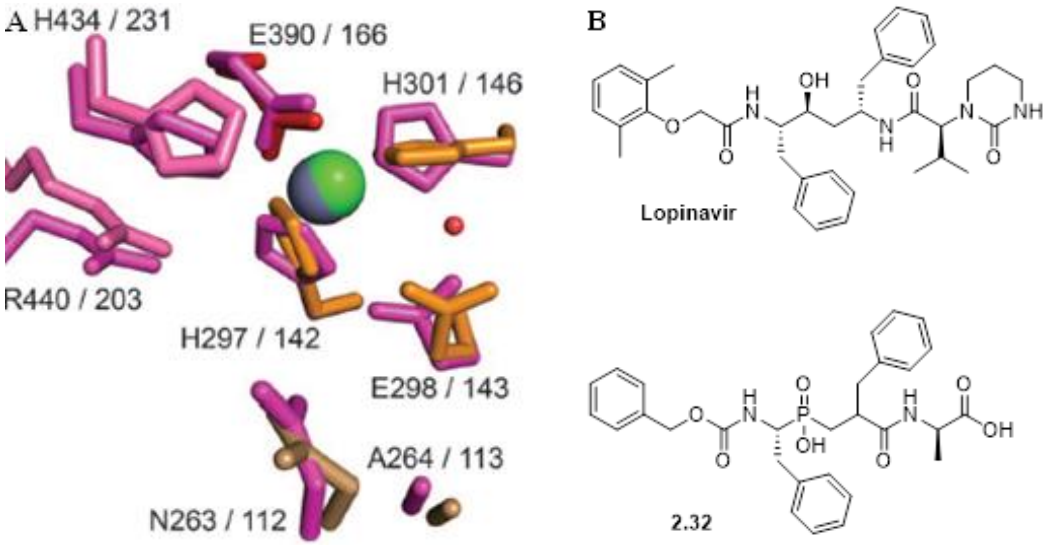
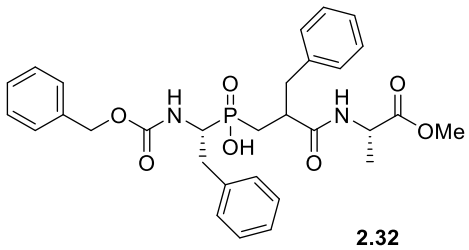


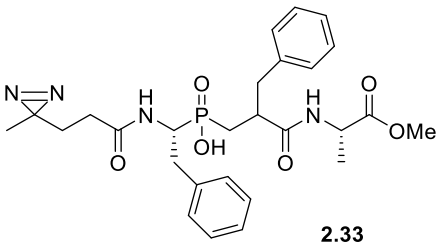
Figure 18. (**A**) Superimposition of STE24p's and TLN's active sites.[13] (**B**) Structures of lopinavir and TLN inhibitor **2.32**.

Based on the literature, the alanine residue present in inhibitor **2.32** is reported to result in the most potent TLN inhibitors out of the hydrophobic amino acids tested at this position.[15] In addition to inhibitor **2.32**, the diazirine analogue **2.33**, presumed to be a suicidal inhibitor of ZMPSTE24was synthesized (Fig. 18). Inhibitor **2.33** is expected to provide insights about the selectivity of this family of compounds in inhibiting ZMPSTE24 in cells. The proposed use of inhibitor **2.33** is the treatment of ZMPSTE24 expressing cells with it and the subsequent irradiation of the cells by UV light to induce the irreversible cross linking of the inhibitor with the enzyme. Lysis of the cells, purification by gel electrophoresis of ZMPSTE24 (which is now linked to **2.33**)

and analysis by MS/MS is expected to demonstrate the binding of the inhibitor to the enzyme. This is necessary because there is currently no evidence that the tested compounds are indeed inhibitors of ZMPSTE24.



2.32

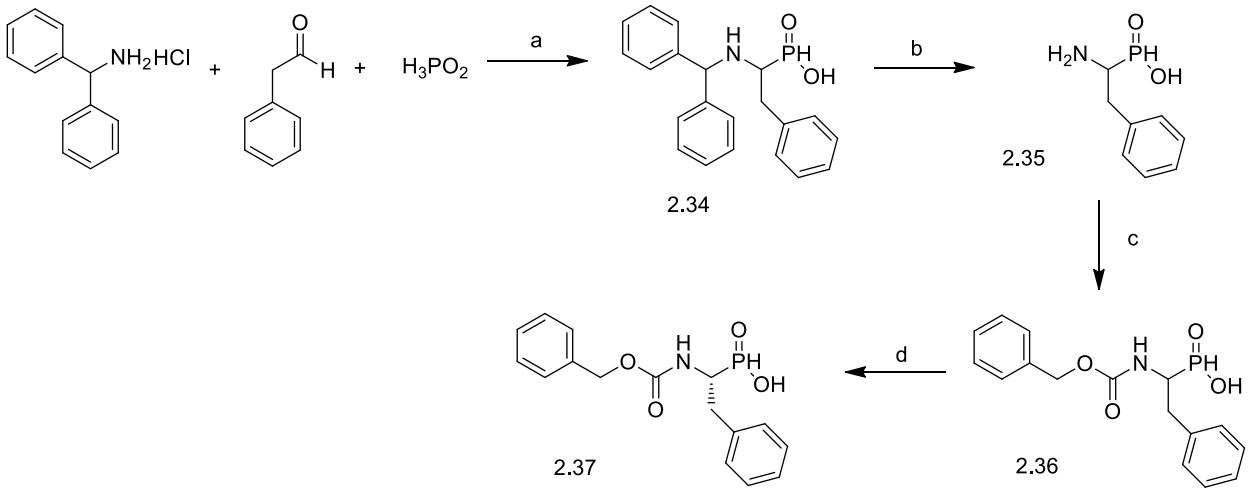


2.33

Figure 19. Target phosphinate inhibitors.

The phosphinic acid **2.37** was prepared as previously reported and summarized in Scheme 6.[16] Initially, a three component condensation of diphenylmethanamine hydrochloride, phenyl acetaldehyde and hypophosphorous acid afforded the N-protected phosphinic acid **2.34**, which was subsequently deprotected with concentrated aqueous HBr in reflux. After work-up, the product salt was neutralized by treatment with propylene oxide, affording the aminophosphinic acid **2.35** in good to excellent yields. The careful addition of benzyl chloroformate in water, with the reaction's pH being kept at the range of 9-10, produced the racemic Cbz protected aminophosphinic acid **2.36**. Subsequently, formation of a salt with the readily available R-(+)-methylbenzylamine allows the separation of the desired enantiomer **2.37** by a series of two recrystallizations and a subsequent treatment with HCl to remove the amine as its hydrochloric salt.[16] The optical purity of the aminophosphinic acid **2.37** was determined by comparison of the optical rotation value taken to that reported in the literature.[17]

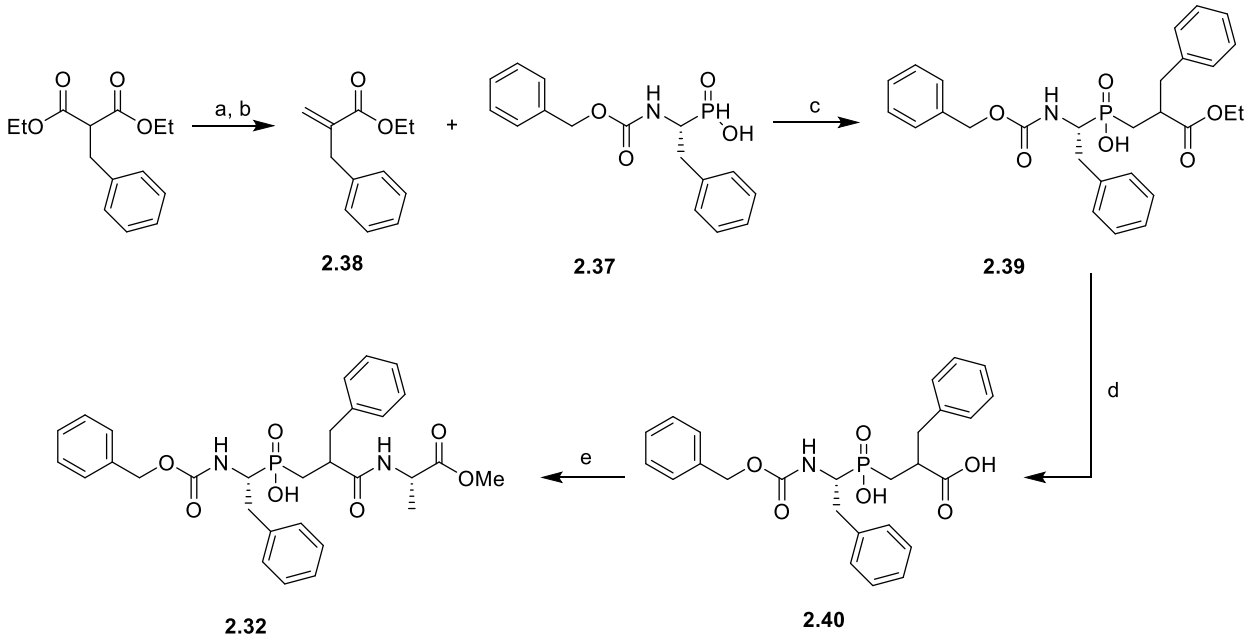
Scheme 6. Synthesis of the aminophosphinic acid **2.37**.



Reagents: (a) EtOH : H_2O (76%); (b) 48% aq. HBr, propylene oxide (90%); (c) CbzCl, NaOH, H_2O (91%); (d) R-(+)-methylbenzylamine, EtOH, HCl (41%).

The acrylate **2.38** was prepared in two steps from diethyl malonate, by a saponification with potassium hydroxide, followed by treatment with aqueous formaldehyde and triethylamine (Scheme 7). Next, a key phospha-Michael addition was performed, with the reactive trivalent phosphorus species generated by heating with neat hexamethyldisilazane (HMDS) to produce phosphinate **2.39**. ^{31}P-NMR indicated that a single set of diastereomers is generated, suggesting that the aminophosphinic acid **2.37** was enantiomerically enriched; however chiral HPLC with authentic standards was not used in order to confirm the enantiomeric excess. The carboxylic acid **2.40** was then generated by saponification with sodium hydroxide and peptide coupling reaction with alanine hydrochloride methyl ester was performed, using EDC and HOBt as the coupling reagents.[18] After purification by C18 reversed phase HPLC, the target phosphinate inhibitor **2.32** was obtained.

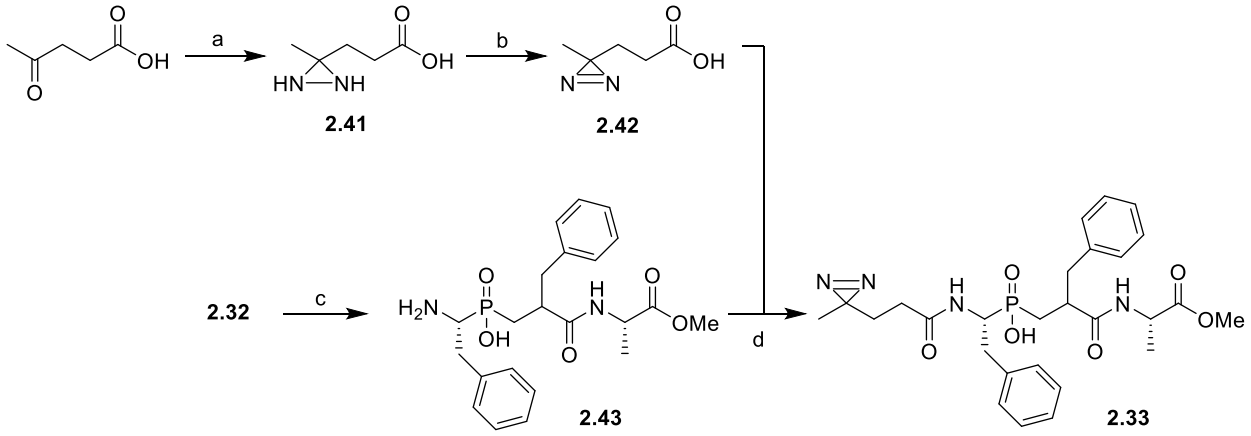
Scheme 7. Synthesis of the phosphinate inhibitor **2.32**.



Reagents: (a) KOH, EtOH; (b) Et_3NH, 37% aq. CH_2O (68% over two steps); (c) HMDS, 110°C (73%); (d) NaOH, EtOH, H_2O (84%); (e) alanine methylester hydrochloride, EDC, HOBt, DIPEA, CH_2Cl_2 (84%).

The CBz protecting group of **2.32** was removed by hydrogenation with 10% Pd/C and a few drops of acetic acid, affording the free amine **2.43** (Scheme 8).[19] Treatment of levulinic acid with hydroxylamine-O-sulfonic acid and ammonia in methanol produced diaziridine **2.41**, which was then oxidized with iodine, in the presence of triethylamine, to furnish the diazirine **2.42**, as previously reported.[20] Intermediate **2.42** was coupled to fragment **2.43**, under standard peptide coupling conditions with EDC and HOBt, to give the photolabile moiety containing inhibitor **2.33** which was purified by C18 reversed phase HPLC.

Scheme 8. Synthesis of the suicide inhibitor **2.33**.



Reagents: (a) Hydroxylamine-O-sulfonic acid, NH_3, MeOH (97%); (b) I_2, Et_3N, MeOH (46%); (c) Pd/C 10%, AcOH, MeOH (45%); (d) EDC, HOBt, DIPEA, CH_2Cl_2 (14%).

2.3 Preliminary Biological evaluation

All of the inhibitors synthesized in our group were tested by Prof. Gerardo Ferbeyre's group in Département de biochimie of Université de Montréal.

The ability of our compounds to induce prelamin A accumulation was assessed in various cancer cell lines. This cell-based assay involves to use of western blotting with a prelamin A-specific antibody and a tubulin specific antibody. After the treatment of the cells with 10 μM of each inhibitor for 24 h and their subsequent lysis, the levels of prelamin A and tubulin were estimated. Tubulin is used as a control protein, a protein whose expression in a cells should be independent of ZMPSTE24 inhibition. The ratio between the amount of tubulin and that of

prelamin A in cells treated with only buffer, as opposed to those treated with an inhibitor allows an assessment of the effect of each compound on prelamin A accumulation. The ratio of prelamin A to tubulin of untreated cells is set as 1. It must be noted that the standard deviation of this assay has not yet been determined. The antiproliferative effect of the compounds was tested on the U2OS (osteosarcoma) cell line by their treatment with 10 μM of each compound for 7 days. At the end of that time period the effectiveness of each compound was determined by a comparison of the cell count of the treated cells with the cell count of cells treated with only buffer. The structures of the compounds tested are shown in Figure 19 and the biological results in Figures 20-23.

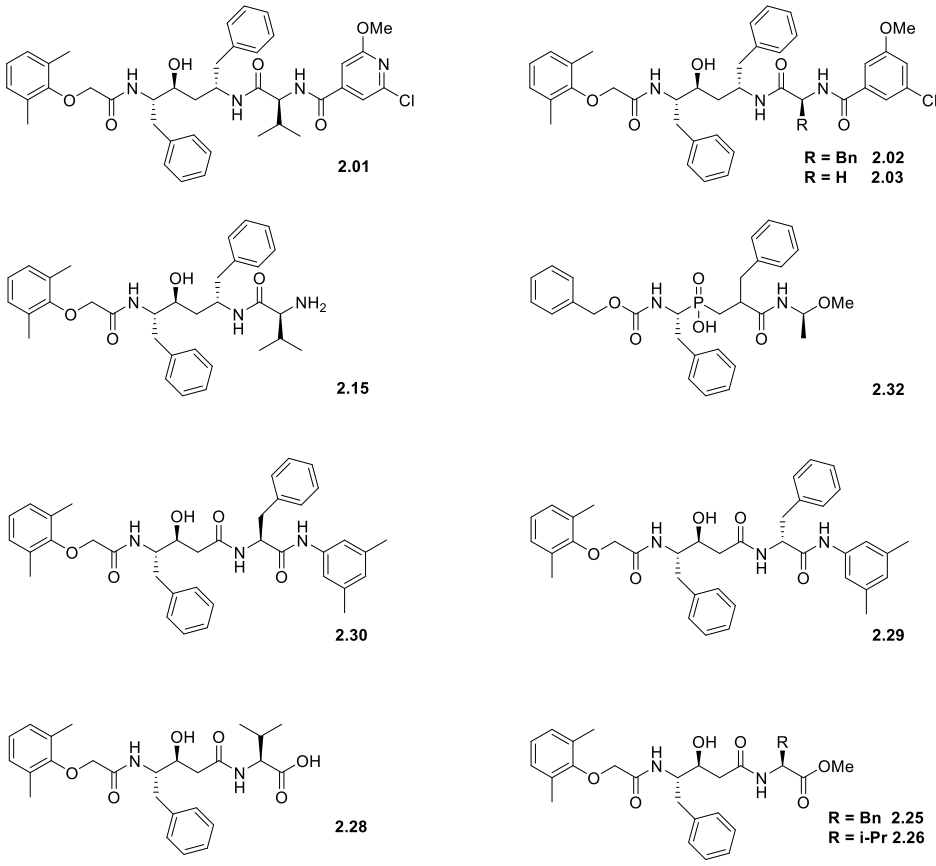


Figure 20. Structures of synthesized inhibitors.

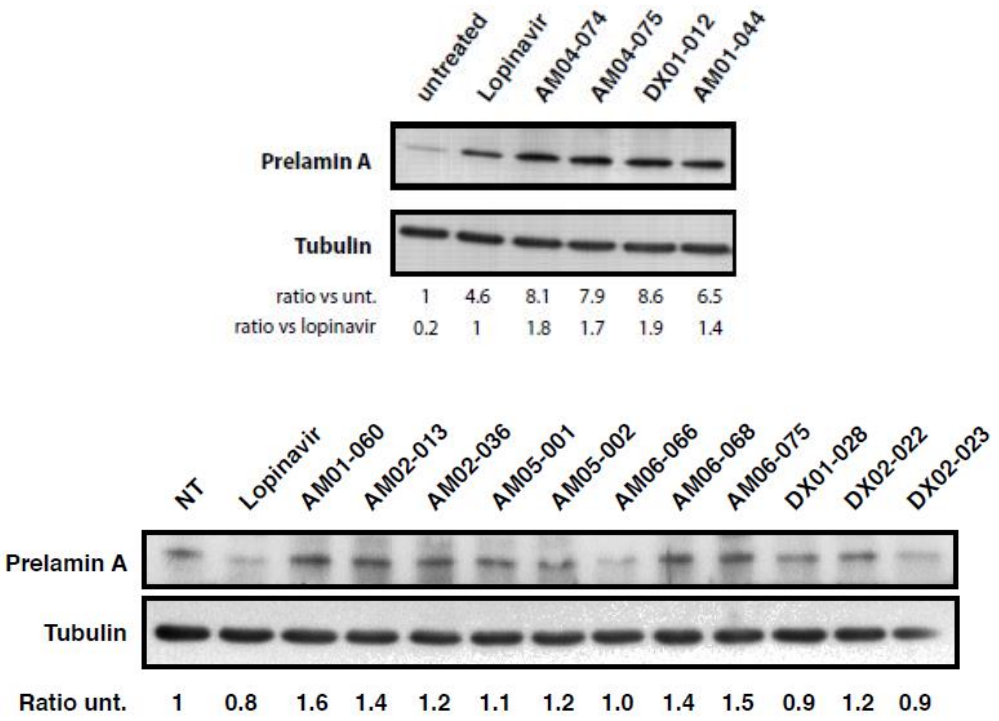


Figure 21. Prelamin A accumulation in U2OS cells induced by the hydroxyethylene analogues (DX01-012 = **2.15**, DX01-028 = **2.01**, DX02-022 = **2.02**, DX02-023 = **2.03**).

Out of the hydroxyethylene based compounds, the lopinavir fragment **2.15** seems to be the most effective at inducing prelamin A accumulation (Fig. 20), as it is about 2-fold more potent than lopinavir. The three analogues **2.01**, **2.02**, **2.03** were not found to be increasing the prelamin content of the treated cells. On the cell proliferation assays on U2OS cells of the hydroxyethylene based compounds (Fig. 21), **2.03** and the lopinavir fragment **2.01** seem to be the ones with significantly better antiproliferation activity than lopinavir. Compound **2.02** treated cells proliferated no differently than the non-treated cells.

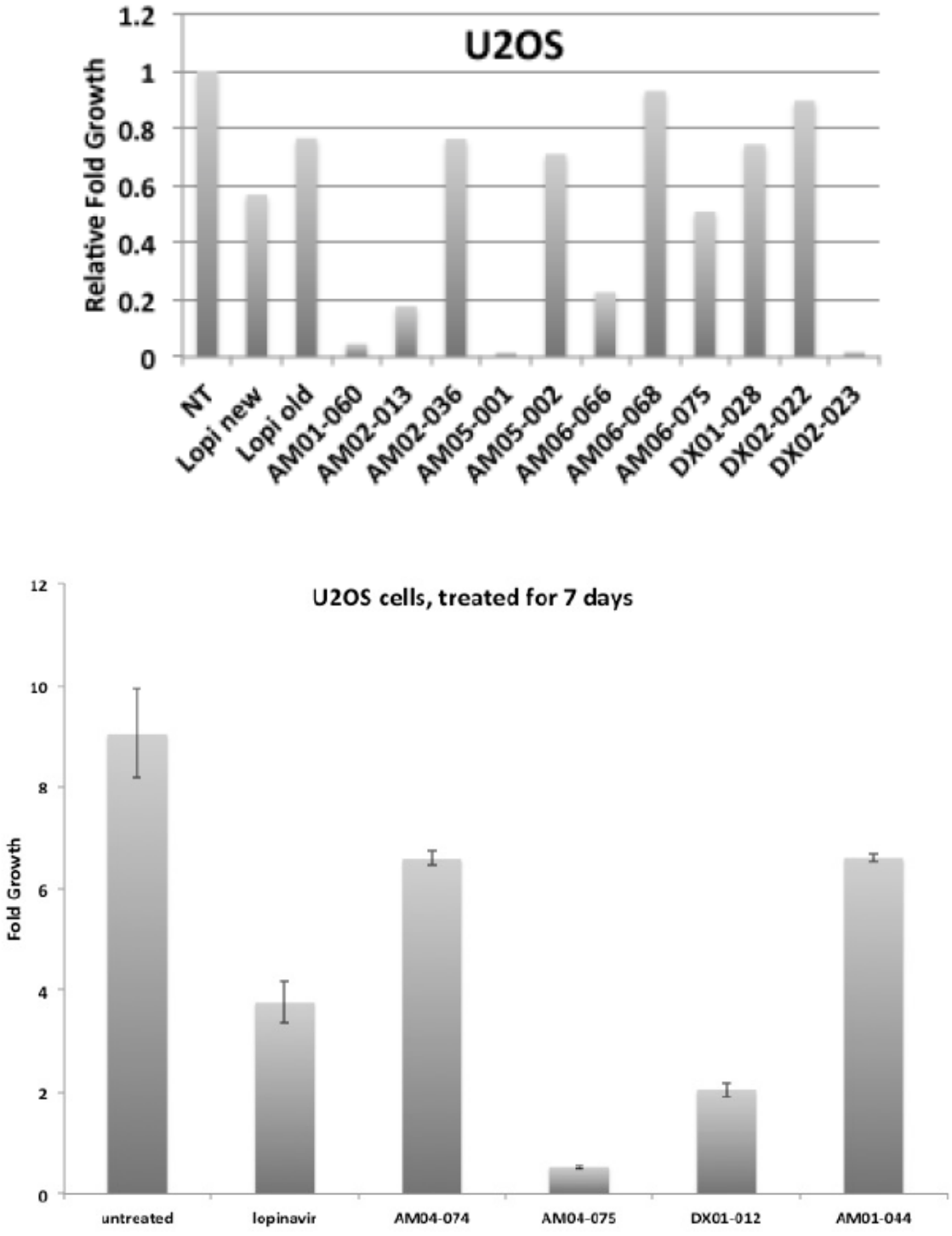


Figure 22. Antiproliferation effect of hydroxyethylene inhibitors on osteosarcoma (U2OS) cells (DX01-012 = **2.15**, DX01-028 = **2.01**, DX02-022 = **2.02**, DX03-023 = **2.03**).

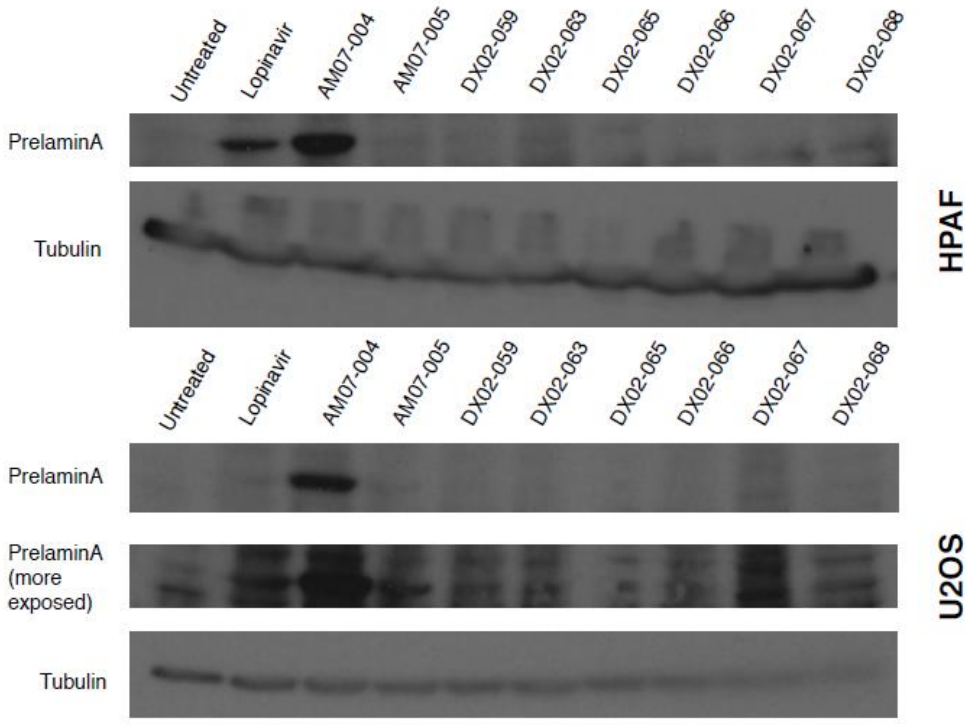


Figure 23. Prelamin A accumulation in U2OS cells induced by the β-hydroxy amide and phosphinate analogues (DX02-063 = **2.25**, DX02-065 = **2.26**, DX02-059 = **2.28**, DX02-068 = **2.29**, DX02-067 = **2.30**, AM07-004 = **2.32**).

None of the *β*-hydroxy amide analogues showed significant prelamin A accumulation in osteosarcoma (U2OS) and human pancreatic adenocarcinoma (HPAF) cells (Fig. 22). On the other hand, the phosphinate **2.32** had a significantly stronger effect on prelamin A accumulation than lopinavir on these cell lines. Since the effects of inhibitor **2.32** on U2OS cells was impressive, its ability to block prelamin A accumulation in other cancer cell lines was also examined. As seen in Figure 23, significant inhibition of prelamin A processing was also observed in the human pancreatic adenocarcinoma cells SW1990, but the effect was much less prominent in human colon

carcinoma cells HCT-116, human pancreatic adenocarcinoma cells HPAFII and pancreatic ductal cell carcinoma cells Kp4.

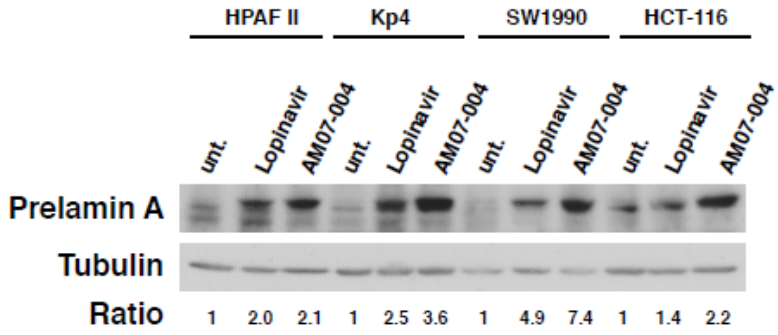


Figure 24. Prelamin A accumulation in various cell lines induced by the phosphinate analogue **2.32** (AM07-004 = **2.32**).

2.4 Experimental

All compounds were purified by normal phase flash column chromatography on silica gel either manually or by using a CombiFlash instrument and a solvent gradient from as indicated. The homogeneity of all final compounds was confirmed to ≥93% by reverse-phase HPLC. HPLC analysis was performed using a Waters ALLIANCE instrument (e2695 with 2489 UV detector and 3100 mass spectrometer). Each final compound was characterized by ^{1}H, ^{13}C, and ^{31}P NMR (where applicable) and ESI-MS. Chemical shifts (δ) are reported in ppm relative to the internal deuterated solvent (^{1}H, ^{13}C) or external H_3PO_4 (^{31}P δ 0.00). Method (homogeneity analysis using a Waters Atlantis T3 C18 5 μm column): solvent A, H_2O, 0.1% formic acid; solvent B, CH_3CN, 0.1% formic acid; mobile phase, linear gradient from 95%A and 5%B to 1%A and 99%B in 10 min, then 5 min at 99% B; flow rate, 1 mL/min.

Benzyl dibenzyl-L-phenylalaninate (2.04): To a homogenous solution of L-phenylalanine (2.50 g, 13.95 mmol), K_2CO_3 (6.75 g, 48.83 mmol) in H_2O was added benzyl bromide (5.5 mL, 46.03 mmol). The mixture was refluxed overnight and the completion of the reaction was confirmed by TLC (PE/EtOAc 8:1, Rf = 0.5). The reaction was cooled to RT, Hexanes (13 mL) and water (8 mL) were added to the mixture and the organic phase was separated and washed twice with water methanol (2:1 v/v, 8 mL). The organic phase was then dried over $MgSO_4$, filtered and concentrated under reduced pressure to give the desired product as a yellow oil (5.84 g, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.06 (dd, J = 14.0, 8.1 Hz, 1H), 3.20 (dd, J = 14.0, 7.4 Hz, 1H), 3.60 (d, J = 14.0 Hz, 2H), 3.78 (t, J = 7.7 Hz, 1H), 3.98 (d, J = 14.0 Hz, 2H), 5.18 (d, J = 12.2 Hz, 1H), 5.30 (d, J = 19.2 Hz, 1H), 7.06 (dd, J = 6.5, 3.0 Hz, 2H), 7.21 – 7.17 (m, 4H), 7.24 (dd, J = 8.7, 3.1 Hz, 3H), 7.31 – 7.26 (m, 5H), 7.47 – 7.35 (m, 6H).

(S)-4-(Dibenzylamino)-3-oxo-5-phenylpentanenitrile (2.05): Sodium amide was weighed into a flask, the air was removed under vacuum and was replaced with Ar. To the flash dry THF was added (70 mL). The dispersion was cooled to -45 ^{o}C and acetonitrile was added dropwise. This solution was stirred at the same temperature for 15 min. A solution of benzyl dibenzyl-L-phenylalaninate in dry THF (60 mL) was added dropwise and the resulting mixture was stirred at -45 ^{o}C and under Ar for 1.5 h, before quenching with a solution of 25% (w/v) aq. citric acid (150 mL). The organic phase was separated and washed twice with brine, dried over $MgSO_4$ and concentrated under reduced pressure. 1-Butanol (25 mL) was added to the oily residue, the mixture was sonicated for 15 min, the butanol was filtered off and the resulting off yellow solid was dried under vacuum (66% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.98 (dd, J = 13.4, 3.3 Hz, 1H), 3.04 (d, J = 19.6 Hz, 1H), 3.21 (dd, J = 13.4, 9.6 Hz, 1H), 3.53 (dd, J = 9.6, 3.4 Hz, 1H), 3.57 (d, J = 13.4 Hz, 2H), 3.89-3.82 (m, 3H), 7.17 – 7.14 (m, 2H), 7.20 (dt, J = 4.7, 1.8 Hz, 1H), 7.28 (dt, J = 15.7, 2.8 Hz, 8H), 7.39 – 7.33 (m, 4H).

(S, Z)-5-Amino-2-(dibenzylamino)-1,6-diphenylhex-4-en-3-one (2.06). To a solution of (S)-4-(dibenzylamino)-3-oxo-5-phenylpentanenitrile (2.50g, 6.78 mmol, 1.00 eq.) in 50 mL of dry THF at 0^{o}C was added a 2M solution of benzylmagnesium chloride (3.07 g, 20.35 mmol, 3.00 eq.) in THF. The solution was warmed to RT and was stirred overnight. The reaction was cooled to 0^{o}C and was quenched by a slow addition of 10% w/v aqueous citric acid solution (50 mL). The organic layer was separated and washed twice with 17% w/v aqueous NaCl solution (2x25 m), was dried over MgSO4, filtered and concentrated under vacuum, Ethanol (10 mL) was added to the residue, the mixture was sonicated for 15 min, filtered and dried to give the product as a white solid (83% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.96 (dd, J = 13.7, 6.3 Hz, 1H), 3.14 (dd, J = 13.8, 7.8 Hz,

1H), 3.43-3.53 (m, 3H), 3.63 (d, *J* = 14.0 Hz, 2H), 3.79 (d, *J* = 14.0 Hz, 2H), 4.92 (brs, 1H), 5.09 (s, 1H), 7.10-7.41 (m, 20 H), 9.79 (s, 1H).

(2S,3S,5S)-5-Amino-2-(dibenzylamino)-1,6-diphenylhexan-3-ol (2.07). A suspension of sodium borohydride (2.793 g, 73.84 mmol, 2.45 eq) in THF (140 mL) was cooled to 0 ºC. Methanesulfonic acid (17.754 g, 184.76 mmol, 6.13 eq) was added very slowly followed by the addition of the enaminone DX01-025 (13.883 g, 30.14 mmol, 1.00 eq) in THF (30 mL) and isopropanol (15 mL). The mixture was stirred at 10 ºC overnight. Sodium borohydride (4.561 g, 120.56 mmol, 4.00 eq) and THF (50 mL) were added to a separate flask. After cooling to 0 ºC, trifluoroacetic acid (17.183 g, 150.70 mmol, 5.00 eq), was added slowly. The solution was stirred at 0 ºC for 45 min and then it was added to the previous reaction slowly. This mixture was stirred for 4h at 15 ºC and then quenched with 3N NaOH (100 mL). After adding *tert*-butyl methyl ether (100 mL), the organic layer was separated and was washed with 0.5N NaOH (100 mL), 20% aqueous NH_4Cl (100 mL) and 6% aqueous NaCl (2x100 mL). The organic layer was dried over $MgSO_4$ and concentrated under vacuum. This afforded a mixture of diastereomers (yellow oil) which after separation with flash column chromatography (hexanes:isopropanol:NH_4OH 12:1:0,1), gave the desired isomer (45% yield) as a white solid. ^1H NMR (400 MHz, $CDCl_3$): δ 1.27-1.35 (m, 1H), 1.43-1.57 (m, 1H), 2.63-2.79 (m, 4H), 2.97-3.21 (m, 2H), 3.45 (d, *J* = 13.5 Hz, 2H), 3.66-3.69 (m, 1H), 3.92 (brs, 2H), 4.08 (d, *J* = 13.5 Hz, 2H), 7.08 (d, *J* = 6.9 Hz, 2H), 7.17 (d, *J* = 7.0 Hz, 2H), 7.21-7.36 (m, 16 H).

***tert*-Butyl ((2S,4S,5S)-5-(dibenzylamino)-4-hydroxy-1,6-diphenylhexan-2-yl)carbamate (2.08):** To a solution of (2S,3S,5S)-5-amino-2-(dibenzylamino)-1,6-diphenylhexan-3-ol (1.31 g, 2.89 mmol) in CH_2Cl_2 was added trimethylamine (0.48 mL, 3.46 mmol, 1.20 eq) and the resulting

mixture was cooled to 0°C. Then, a solution of $(Boc)_2O$ (0.76 g, 3.46 mmol, 1.20 eq) in 15 mL of CH_2Cl_2 was added dropwise and the mixture was stirred at the same temperature for 15 min and at room temperature for 1h and 45min. The organic phase was washed with water and brine, dried over $MgSO_4$ and concentrated in vacuum. The residue (off yellow solid) was purified with flash column chromatography (Hexanes/EtOAc 1:0.25) to yield the desired product as a white solid (1.63g, 98% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.21 (m, 1H), 1.43 (s, 9H), 2.61 (dd, *J* = 14.3, 6.7 Hz, 1H), 2.70 (dd, *J* = 13.4, 6.9 Hz, 1H), 2.81 (dd, *J* = 14.6, 6.4 Hz, 2H), 3.08 (dd, *J* = 14.3, 5.9 Hz, 1H), 3.40 (d, *J* = 13.4 Hz, 2H), 3.63 (t, *J* = 7.9 Hz, 1H), 3.82 (dd, *J* = 12.8, 6.4 Hz, 1H), 3.92 (d, *J* = 13.3 Hz, 2H), 4.37 (s, 1H), 4.88 (s, 1H), 7.06 (d, *J* = 6.9 Hz, 2H), 7.13 (d, *J* = 7.0 Hz, 2H), 7.18-7.28 (m, 11H), *7*.32 (dd, J = 14.0, 6.5 Hz, 5H). MS (ESI+) *m/z* 565.22 $[M + H]^+$ for $C_{37}H_{44}N_2O_3$.

***tert*-Butyl ((2S,4S,5S)-5-amino-4-hydroxy-1,6-diphenylhexan-2-yl)carbamate (2.09):** To a solution of tert-butyl ((2S,4S,5S)-5-(dibenzylamino)-4-hydroxy-1,6-diphenylhexan-2-yl)carbamate (815 mg, 1.44 mmol) in MeOH (40 mL) was added 20% $Pd(OH)_2$ and the air was drawn from the flask using vacuum. A balloon with H_2 was added and the mixture was stirred at room temperature for 4 h. The reaction mixture was filtered through celite and washed with methanol. The combined filtrates were concentrated in vacuum, affording the desired amine as a white solid (451 mg, 81% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.26 (s, 9H), 1.63 (t, *J* = 7.3 Hz, 2H), 2.54 (dd, *J* = 10.7, 5.7 Hz, 1H), 2.64 (dd, *J* = 13.4, 7.7 Hz, 1H), 2.73 (dd, *J* = 13.5, 4.4 Hz, 1H), 2.79 (dd, *J* = 13.4, 6.4 Hz, 1H), 3.05 (s, 1H), 1.26 (s, 1H), 3.50 (dd, *J* = 6.5, 4.1 Hz, 1H), 3.75 (dd, *J* = 11.8, 7.3 Hz, 1H), 6.63 (d, *J* = 9.0 Hz, 1H), 7.15 (t, *J* = 8.1 Hz, 3H), 7.26 – 7.18 (m, 5H), 7.31 – 7.26 (m, 2H). MS (ESI+) *m/z* 385.00 $[M + H]^+$ for $C_{23}H_{32}N_2O_3$.

Ethyl 2-(2,6-dimethylphenoxy)acetate (2.10): To a solution of 2,6-dimethylphenol (3.20 g, 26.20 mmol), in 1,4-dioxane (150 mL) was added ethyl bromoacetate (7.24 mL, 65.49 mmol) and cesium carbonate (22.19 g, 68.11 mmol) and the reaction mixture was heated at reflux for 48 h. Then it was cooled to room temperature, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (Hexanes/EtOAc 20:1), providing the desired compound as a colorless oil (5.25 g, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.33 (t, J = 7.1 Hz, 3H), 2.30 (s, 6H), 4.30 (q, J = 7.1 Hz, 2H), 4.40 (s, 2H), 6.94 (dd, J = 8.4, 6.3 Hz, 1H), 7.01 (d, J = 7.6 Hz, 2H).

2-(2,6-Dimethylphenoxy)acetic acid (2.11): To a solution of ethyl 2-(2,6-dimethylphenoxy)acetate (5.25 g, 25.21 mmol) in MeOH (180 mL) and H_2O (135 mL) was added lithium hydroxide (6.04 g, 252.1 mmol) at 0 ^{o}C and the reaction mixture was stirred for 1.5 h at room temperature. The solution was then acidified with 1M HCl until pH = 1and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated to afford the desired compound as a white solid (4.23 g, 93% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.30 (s, 6H), 4.47 (s, 2H), 6.98 (dd, J = 8.7, 5.9 Hz, 1H), 7.06 – 7.02 (m, 2H).

2-(2,6-Dimethylphenoxy)acetyl chloride (2.12): To a solution of 2-(2,6-dimethylphenoxy)acetic acid in ethyl acetate (12 mL), thionyl chloride and three drops of DMF were added and the solution was stirred for 6 h at 55 ^{o}C. The solvent and the excess of thionyl chloride were evaporated under vacuum and the residue was used immediately in the next step.

***tert*-Butyl ((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)carbamate (2.13):** To a solution of *tert*-butyl ((2S,4S,5S)-5-amino-4-hydroxy-1,6-diphenylhexan-2-yl)carbamate in a mixture of EtOAc/H_2O 1:1 (10 mL) was added $NaHCO_3$ and the mixture was stirred at RT until an emulsion was formed. Then, a solution of 2-(2,6-

dimethylphenoxy)acetyl chloride (1.37 mmol) in 5 mL of dry EtOAc was added dropwise and under Ar. When the addition was completed the mixture was stirred for 1h at room temperature. 20 mL of EtOAc were added and the organic phase was separated and washed with saturated aq. $NaHCO_3$ and water, dried over $MgSO_4$ and concentrated under reduced pressure to afford the desired product as a white solid (525 mg, 82% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.42 (s, 9H), 1.76 – 1.66 (m, 2H), 2.20 (s, 6H), 2.84 – 2.74 (m, 2H), 3.00 (d, *J* = 7.5 Hz, 2H), 3.76 (s, 1H), 3.92 (dd, *J* = 12.2, 6.4 Hz, 1H), 4.20 – 4.13 (m, 1H), 4.22 (s, 2H), 1.42 (s, 1H), 6.97 (dd, *J* = 8.5, 6.2 Hz, 1H), 7.06 – 7.01 (m, 2H), 7.15 (d, *J* = 6.9 Hz, 2H), 7.28 – 7.19 (m, 6H), 7.34 – 7.29 (m, 2H). MS (ESI+) *m/z* 547.53 $[M + H]^+$ for $C_{33}H_{42}N_2O_5$.

N-((2S,3S,5S)-5-Amino-3-hydroxy-1,6-diphenylhexan-2-yl)-2-(2,6-dimethylphenoxy)acetamide (2.14): To a solution of *tert*-butyl ((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)carbamate (525 mg, 0.96 mmol) in dry CH_2Cl_2 (35 mL) trifluoroacetic acid (3.3 mL, 43,2 mmol) was added dropwise and the mixture was stirred at RT for 2.5 h. After the consumption of the starting material the solvent was evaporated and the residue was dissolved in EtOAc, washed with saturated aq. $NaHCO_3$, brine, dried over $MgSO_4$ and concentrated under reduced pressure to afford the desired product as a white solid (415 mg, 97% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.74 (brs, 2H), 2.19 (s, 6H), 2.83 (ddd, *J* = 20.9, 11.2, 5.2 Hz, 2H), 2.90 (dd, *J* = 13.7, 7.7 Hz, 1H), 2.99 (dd, *J* = 13.7, 7.4 Hz, 1H), 3.35 (brs, 1H), 3.87 (d, *J* = 8.7 Hz, 1H), 4.13 (d, *J* = 14.8 Hz, 1H), 4.22 (dd, *J* = 14.4, 6.7 Hz, 1H), 4.29 (d, *J* = 14.8 Hz, 1H), 6.96 (dd, *J* = 8.6, 6.0 Hz, 1H), 7.03 – 6.99 (m, 2H), 7.16 (d, *J* = 7.1 Hz, 2H), 7.28 – 7.18 (m, 6H), 7.31 (t, *J* = 7.3 Hz, 2H), 7.39 (d, *J* = 9.5 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.2, 37.1, 37.9, 43.2, 53.7, 54.8, 70.3, 71.6, 124.6, 126.5, 127.1, 128.5, 128.9, 129.1, 129.3, 129.4, 130.5, 136.4, 137.8, 154.3, 169.0. MS (ESI+) *m/z* 447.48 $[M + H]^+$ for $C_{28}H_{34}N_2O_3$.

(S)-2-Amino-N-((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)-3-methylbutanamide (2.15): A solution of L-valine-N-carboxyanhydride (232 mg, 1.50 eq.) in CH_2Cl_2 (3 mL) was added to a solution of N-((2S,3S,5S)-5-amino-3-hydroxy-1,6-diphenylhexan-2-yl)-2-(2,6-dimethylphenoxy)acetamide (1.0 eq.) in CH_2Cl_2 (5 mL) in a salt/ice bath (-10 ^oC) under Ar, followed by Et_3N (1.08 mmol, 1.0 eq.). The mixture was subsequently stirred at the same temperature and under Ar for 4 h. CH_2Cl_2 was added (15 mL) and the organic phase was washed with water (2 x 20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue (white solid) was used for the next step without further purification (95% yield). Reversed phase preparatory HPLC was used to purify the compound sent for biological evaluation. A low quality HPLC trace was obtained due to the low UV absorption of the compound. ^1H NMR (400 MHz, $CDCl_3$): *δ 0.47* (d, *J* = 6.8 Hz, 3H), 0.83 (d, *J* = 7.0 Hz, 3H), 1.73 – 1.66 (m, 1H), 1.78 (dt, *J* = 14.6, 3.8 Hz, 1H), 2.19 (s, 6H), 2.75 (dd, *J* = 13.9, 9.0 Hz, 1H), 2.86 (dd, *J* = 13.9, 5.9 Hz, 1H), 2.99 (qd, *J* = 13.5, 7.6 Hz, 2H), 3.12 (d, *J* = 3.9 Hz, 1H), 3.77 (ddd, *J* = 9.2, 3.5, 2.1 Hz, 1H), 4.15 – 4.06 (m, 1H), 4.27 – 4.15 (m, 3H), 6.95 (dd, *J* = 8.7, 5.9 Hz, 1H), 7.00 (d, *J* = 6.4 Hz, 2H), 7.19 – 7.12 (m, 3H), 7.25 – 7.20 (m, 3H), 7.31 – 7.26 (m, 3H), 7.56 (d, *J* = 7.9 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): *δ* 15.3, 16.3, 19.6, 24.7, 30.3, 36.6, 38.3, 41.9, 42.2, 49.3, 54.6, 59.9, 69.6, 70.3, 124.6, 126.4, 126.7, 128.5, 128.5, 129.1, 129.1, 129.5, 130.5, 137.6, 138.2, 154.4, 168.8. MS (ESI+) *m/z* 547.60 $[M + 2H]^+$ for $C_{33}H_{43}N_3O_4$.

2-Chloro-N-((S)-1-(((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-6-methoxyisonicotinamide (2.01). To (S)-2-amino-N-((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)-3-methylbutanamide (40.0 mg, 0.073 mmol, 1eq), 2-Chloro-6-methoxypyridine-4-carboxylic acid (8.7 mg, 0.077 mmol, 1.05 eq) in 4.0 mL of DMF was added.

Subsequently, HBTU (29.2 mg, 0.077 mmol, 1.05 eq) was added, followed by DIPEA (19.0 mg, 0.147 mmol, 2.00 eq) under Ar, at room temperature. The reaction was then stirred for 60 min. The reaction was quenched with 10 mL of brine and the precipitate was filtered and washed two times with 10 mL of cold H_2O, two times with 10 mL of cold hexanes and 5 mL of cold 4:1 hexanes/EtOAc to yield the product as a white solid (68% yield). ^{1}H NMR (500 MHz, DMSO): δ 0.83 (dd, J = 28.6, 6.5 Hz, 6H), 1.60 – 1.41 (m, 2H), 2.05 – 1.93 (m, 1H), 2.14 (s, 6H), 2.66 (ddd, J = 21.1, 13.5, 8.0 Hz, 2H), 2.85 – 2.74 (m, 1H), 3.71 (dd, J = 12.3, 5.9 Hz, 1H), 3.91 (s, 3H), 4.04 (q, 2H), 4.15-4.28 (m, 3H), 5.05 (d, J = 5.7 Hz, 1H), 6.92-7.49 (m, 16H), 7.98 (t, J = 8.5 Hz, 1H), 8.61 (t, J = 8.9 Hz, 1H). ^{13}C NMR (125wd MHz, DMSO): δ 16.3, 19.3, 19.7, 30.3, 38.3, 39.2, 47.5, 53.1, 54.9, 60.1, 68.6, 70.6, 108.4, 115.2, 124.7, 126.1, 126.4, 128.2, 128.5, 129.3, 129.6, 130.7, 139.1, 139.5, 147.8, 148.1, 154.9, 163.5, 164.3, 167.7, 170.2. MS (ESI+) *m/z* 715.98 $[M + H]^+$ for $C_{40}H_{47}ClN_4O_6$.

***tert*-Butyl ((S)-1-(((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (2.16):** To a solution of N-((2S,3S,5S)-5-amino-3-hydroxy-1,6-diphenylhexan-2-yl)-2-(2,6-dimethylphenoxy)acetamide (50 mg, 0.11 mmol) and Boc-L-Phenylalanine (30 mg, 0.11 mmol) in THF (4 mL) were added HOBt (26 mg, 0.17 mmol), EDC hydrochloride (43 mg, 0.22 mmol) and DIPEA (78 μL, 0.45 mmol). The mixture was stirred for 3 h at RT and then the solvent was evaporated under reduced pressure. The residue was diluted with EtOAc. The organic phase was washed with saturated three times with aq. Na_2CO_3 solution, twice with water, once with brine, dried over $MgSO_4$ and concentrated under reduced pressure. It was purified by flash column chromatography (Hexanes/EtOAc 4:1) to yield the product as a white solid (55.3 mg, 71% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.42 (s, 9H), 1.59 – 1.49 (m, 1H), 1.66 (dt, J = 14.6, 3.8 Hz, 1H), 2.18 (s, 1H), 2.69 – 2.81 (m, 2H), 2.93

(dd, *J* = 7.4, 2.3 Hz, 2H), 2.98 (bs, 2H), 3.60 (d, *J* = 7.6 Hz, 1H), 4.27 – 4.12 (m, 5H), 1.42 (s, 1H), 6.16 (d, J = 7.6 Hz, 1H), 6.97 (dd, *J* = 8.5, 6.1 Hz, 1H), 7.04 (dd, *J* = 13.1, 7.1 Hz, 4H), 7.15 (d, *J* = 9.3 Hz, 1H), 7.22 – 7.17 (m, 3H), 7.28 – 7.22 (m, 6H), 7.34 – 7.29 (m, 4H). MS (ESI+) *m/z* 694.66 $[M + H]^+$ for $C_{42}H_{51}N_3O_6$.

***tert*-Butyl (2-(((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)amino)-2-oxoethyl)carbamate (2.17):** To a solution of N-((2S,3S,5S)-5-amino-3-hydroxy-1,6-diphenylhexan-2-yl)-2-(2,6-dimethylphenoxy)acetamide (40 mg, 0.09 mmol) and Boc-glycine (16 mg, 0.09 mmol) in THF (4 mL) were added HOBt (21 mg, 0.13 mmol), EDC hydrochloride (34 mg, 0.18 mmol) and DIPEA (62 μL, 0.36 mmol). The mixture was stirred for 3 h at RT and then the solvent was evaporated under reduced pressure. The residue was diluted with EtOAc. The organic phase was washed with saturated three times with aq. Na_2CO_3 solution, twice with water, once with brine, dried over $MgSO_4$ and concentrated under reduced pressure. It was purified by flash column chromatography (Hexanes/EtOAc 4:1) to yield the product as a white solid (34 mg, 62% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9H), 1.66 – 1.59 (m, 1H), 1.74 (dt, *J* = 14.5, 4.2 Hz, 1H), 2.17 (s, 6H), 2.86 – 2.72 (m, 2H), 2.96 (d, *J* = 7.6 Hz, 2H), 3.65 (dd, *J* = 5.9, 2.4 Hz, 2H), 3.73 (dd, *J* = 4.6, 2.7 Hz, 1H), 3.86 (d, *J* = 4.3 Hz, 1H), 4.28 – 4.14 (m, 3H), 5.03 (s, 1H), 6.29 (d, *J* = 8.0 Hz, 1H), 6.95 (dd, *J* = 8.7, 5.8 Hz, 1H), 7.02 – 6.98 (m, 2H), 7.10 (d, *J* = 6.8 Hz, 2H), 6.95 (dd, *J* = 8.7, 5.8 Hz, 6H), 7.32 – 7.26 (m, 2H). MS (ESI+) *m/z* 604.58 $[M + H]^+$ for $C_{35}H_{45}N_3O_6$.

(S)-2-Amino-N-((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)-3-phenylpropanamide (2.18): To a solution of N-((2S,3S,5S)-5-amino-3-hydroxy-1,6-diphenylhexan-2-yl)-2-(2,6-dimethylphenoxy)acetamide (55 mg, 0.08 mmol) in dry CH_2Cl_2 (8 mL), trifluoroacetic acid was added dropwise and the mixture was stirred at RT for 4

hours. After the consumption of the starting material the reaction was quenched with a saturated aq. $NaHCO_3$ solution and the organic layer was washed with saturated aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated under reduced pressure to afford the desired product as an off white residue (41 mg, 87% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.68 (dd, *J* = 15.7, 7.8 Hz, 1H), 1.75 (dt, *J* = 14.5, 3.9 Hz, 1H), 2.18 (s, 6H), 2.37 – 2.25 (m, 1H), 2.75 (dd, *J* = 13.8, 7.9 Hz, 1H), 2.82 (dd, *J* = 13.8, 6.4 Hz, 1H), 2.98 (qd, *J* = 13.5, 7.7 Hz, 2H), 3.07 (dd, J = 13.8, 4.0 Hz, 1H), 3.51 – 3.45 (m, 2H), 3.73 (d, *J* = 7.1 Hz, 1H), 4.25 – 4.09 (m, 4H), 6.95 (dd, *J* = 8.5, 6.2 Hz, 1H), 7.00 (d, *J* = 7.3 Hz, 2H), 7.11 (dd, *J* = 11.0, 7.0 Hz, 3H), 7.26 – 7.14 (m, 7H), 7.29 (dt, *J* = 9.6, 5.3 Hz, 5H), 7.43 (d, *J* = 8.2 Hz, 1H). MS (ESI+) *m/z* 594.61 $[M + H]^+$ for $C_{37}H_{43}N_3O_4$.

2-Amino-N-((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)acetamide (2.19): To a solution of *tert*-butyl (2-(((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)amino)-2-oxoethyl)carbamate (34 mg, 0.06 mmol) in dry CH_2Cl_2 (5.5 mL), trifluoroacetic acid was added dropwise and the mixture was stirred at RT for 4 hours. After the consumption of the starting material the reaction was quenched with a saturated aq. $NaHCO_3$ solution and the organic layer was washed with saturated aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated under reduced pressure to afford the desired product as an off white residue (24 mg, 85% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.80 – 1.66 (m, 2H), 2.18 (s, 6H), 2.82 (d, *J* = 7.5 Hz, 2H), 2.98 (d, *J* = 9.0 Hz, 2H), 3.23 (s, 1H), 3.87 – 3.62 (m, 2H), 4.23 – 4.10 (m, 4H), 6.95 (dd, *J* = 8.7, 5.8 Hz, 1H), 7.03 – 6.98 (m, 2H), 7.11 (dd, *J* = 15.9, 6.8 Hz, 2H), 7.26 – 7.16 (m, 5H), 7.29 (dd, *J* = 12.9, 4.7 Hz, 3H), 7.43 (brd, *J* = 7.2 Hz, 1H). MS (ESI+) *m/z* 504.49 $[M + H]^+$ for $C_{30}H_{37}N_3O_4$.

3-Chloro-N-(2-(((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)amino)-2-oxoethyl)-5-methoxybenzamide (2.03): To a solution of 2-

amino-N-((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)acetamide (68 mg, 0.135 mmol, 1.0 eq) in dry DMF (8.0 mL) was added 3-chloro-5-methoxybenzoic acid (26 mg, 1.05 eq) in dry DMF (2.0 mL). Subsequently, HBTU (54 mg, 1.05 eq) was added, followed by DIPEA (47 μL, 2.00 eq) under Ar at RT and the reaction was stirred for 1 h. The reaction was quenched with brine and was extracted with EtOAc (3 x 10 mL), dried over $MgSO_4$, concentrated under reduced pressure and purified using preparatory reversed phase HPLC (36 mg, 40% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.73 – 1.66 (m, 1H), 1.82 – 1.77 (m, 1H), 2.19 (s, 6H), 2.80 (dd, *J* = 13.8, 6.7 Hz, 1H), 2.86 (dd, *J* = 13.8, 6.7 Hz, 1H), 3.02 – 2.98 (m, 3H), 3.78 (brs, 2H), 3.86 (s, 3H), 4.03 – 3.92 (m, 2H), 4.31 – 4.15 (m, 4H), 6.28 (d, *J* = 7.7 Hz, 1H), 6.86 (t, *J* = 5.0 Hz, 1H), 6.97 (dd, *J* = 8.6, 6.0 Hz, 1H), 7.02 (d, *J* = 7.0 Hz, 2H), 7.08 – 7.06 (m, 1H), 7.12 (d, *J* = 6.8 Hz, 2H), 7.27 – 7.18 (m, 9H), 7.30 (d, *J* = 7.3 Hz, 2H), 7.35 (t, *J* = 1.6 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 16.4, 38.1, 38.9, 40.6, 43.2, 47.5, 53.0, 56.3, 68.5, 70.7, 112.5, 117.2, 119.9, 124.7, 126.3, 128.4, 128.4, 129.3, 129.6, 129.7, 130.7, 134.3, 137.4, 139.1, 139.6, 155.0, 160.5, 165.3, 167.7, 168.30. MS (ESI+) *m/z* 672.56 $[M + H]^+$ for $C_{38}H_{42}ClN_3O_6$.

3-Chloro-N-((S)-1-(((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-5-methoxybenzamide (2.02): To a solution of 2-amino-N-((2S,4S,5S)-5-(2-(2,6-dimethylphenoxy)acetamido)-4-hydroxy-1,6-diphenylhexan-2-yl)acetamide (41 mg, 0.07 mmol) in dry DMF (3.0 mL) was added 3-chloro-5-methoxybenzoic acid in dry DMF (1.0 mL). Subsequently, HBTU was added, followed by DIPEA under Ar at RT and the reaction was stirred for 1 h. The reaction was quenched with brine and the precipitate was filtered and washed two times with H_2O (5 mL), two times with hexanes (5 mL), and hexanes/EtOAc 4:1 (3 mL) to yield the product as a white solid (36 mg, 67% yield). ^{1}H NMR

(500 MHz, DMSO-d_6): δ 1.59 – 1.43 (m, 2H), 2.13 (s, 6H), 2.69 (d, *J* = 6.3 Hz, 2H), 2.82 – 2.76 (m, 2H), 2.87 (td, *J* = 14.1, 7.9 Hz, 2H), 3.68 (dd, *J* = 14.0, 6.7 Hz, 1H), 3.81 (s, 3H), 4.05 (q, *J* = 14.4 Hz, 2H), 4.14 (dd, *J* = 14.4, 8.1 Hz, 1H), 4.23 (dd, *J* = 15.4, 7.7 Hz, 1H), 4.62 (td, *J* = 9.1, 5.7 Hz, 1H), 5.03 (d, *J* = 5.9 Hz, 1H), 6.93 (dd, *J* = 8.1, 6.8 Hz, 1H), 7.01 (d, *J* = 7.5 Hz, 2H), 7.19 – 7.07 (m, 9H), 7.25 (dd, *J* = 7.3, 5.9 Hz, 6H), 7.29 (dd, *J* = 8.9, 5.4 Hz, 3H), 7.43 – 7.40 (m, 1H), 7.46 (d, *J* = 9.6 Hz, 1H), 7.94 (d, *J* = 8.4 Hz, 1H), 8.62 (d, *J* = 8.5 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 15.9, 37.3, 37.7, 38.1, 47.1, 55.0, 55.8, 67.7, 70.2, 112.1, 116.7, 119.5, 124.2, 125.8, 125.9, 126.2, 127.9, 128.0, 128.0, 128.8, 129.1, 129.1, 129.3, 130.2, 133.7, 136.8, 138.4, 138.4, 139.0, 154.5, 159.9, 164.4, 167.2, 170.4. MS (ESI+) *m/z* 762.62 $[M + H]^+$ for $C_{45}H_{48}ClN_3O_6$.

***tert*-Butyl (S)-4-(dibenzylamino)-3-oxo-5-phenylpentanoate (2.20):** A solution of *tert*-butyl acetate (6.0 mL, 44.7 mmol) in dry THF (10 mL) was added dropwise to LDA (made by diluting freshly distilled diisopropylamine (5.5 mL, 39.3 mmol) in dry THF (80 mL), cooling to -78^{o}C and adding n-BuLi 2.5M in hexanes (15.4 mL, 38.5 mmol) dropwise and under stirring. The addition was performed over 15 min, with cooling to -45^{o}C. After stirring the solution of the *tert*-butyl acetate and the LDA for 60 min at that temperature, a solution of benzyl dibenzyl-L-phenylalaninate (5.8 g, 13.4 mmol) in dry THF (10 mL) cooled to -45^{o}C was added dropwise over 15 min. The resulting mixture was stirred at the same temperature for 1 h and then poured into a solution of citric acid (16.5g in 50 mL of water) to quench the reaction. The organic layer was separated and the aqueous layer was extracted with ethyl acetate two times. The combined organic layers were washed with 10% aq. citric acid solution (30 mL), saturated aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The product was purified by flash column chromatography (Hexanes/EtOAc 30:1) to afford the product as a white solid (5.5 g, 93% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.26 (s, 9H), 2.96 (dd, *J* = 13.5, 3.8 Hz, 1H), 3.22 (dd, *J* =

13.5, 9.1 Hz, 1H), 3.39 (d, *J* = 15.5 Hz, 1H), 3.57 (dd, *J* = 14.5, 10.0 Hz, 3H), 3.64 (dd, *J* = 9.1, 3.9 Hz, 1H), 3.84 (d, *J* = 13.4 Hz, 2H), 7.28 – 7.15 (m, 7H), 7.37 – 7.29 (m, 8H). MS (ESI-) *m/z* 442.39 $[M - H]^-$ for $C_{29}H_{33}NO_3$.

***tert*-Butyl (3S,4S)-4-(dibenzylamino)-3-hydroxy-5-phenylpentanoate (2.21):** A solution of *tert*-butyl (S)-4-(dibenzylamino)-3-oxo-5-phenylpentanoate (5.52 g, 12.45 mmol) in dry methanol (70 mL) was cooled to -20 ^oC and $NaBH_4$ (1.65 g, 43.59 mmol) was added. After 3.5 h the solution was quenched with H_2O (120 mL) at pH = 5-6 (adjusted by 1M HCl), extracted with EtOAc, washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography (Hexanes/EtOAc 30:1) to give the product as a colorless oil (4.42 g, 80% yield). ^1H NMR (400 MHz, $CDCl_3$): δ 1.40 (s, 9H), 2.05 (dd, *J* = 16.1, 2.5 Hz, 1H), 2.41 (dd, *J* = 16.1, 9.5 Hz, 1H), 2.78 (ddd, *J* = 8.3, 6.6, 5.0 Hz, 1H), 2.87 (dd, *J* = 13.6, 8.3 Hz, 1H), 3.12 (dd, *J* = 13.6, 4.9 Hz, 1H), 3.44 (d, *J* = 13.5 Hz, 2H), 3.95 (ddd, *J* = 9.3, 6.6, 2.5 Hz, 1H), 4.02 (s, 1H), 4.08 (d, *J* = 13.4 Hz, 2H), 7.26 – 7.17 (m, 5H), 7.35 – 7.27 (m, 10H). MS (ESI+) *m/z* 446.47 $[M + H]^+$ for $C_{29}H_{35}NO_3$.

***tert*-Butyl (3S,4S)-4-amino-3-hydroxy-5-phenylpentanoate (2.22):** *tert*-Butyl (3S,4S)-4-(dibenzylamino)-3-hydroxy-5-phenylpentanoate (4.42 g, 9.92 mmol) was transferred in a flash, methanol (250 mL) was added, the flask was purged with argon and then Pd/C 10%, was added (0.26 g, 0.246 mmol). A balloon with H_2 was added and the mixture was stirred overnight at RT. The solution was filtered through a celite pad, then the resulting filtrate was concentrated under reduced pressure to give the product as an off white solid (95% yield). ^1H NMR (400 MHz, $CDCl_3$): δ 1.46 (s, 9H), 2.44 (dd, *J* = 15.8, 3.9 Hz, 1H), 2.62 – 2.51 (m, 2H), 2.93 (td, *J* = 10.4, 5.0 Hz, 2H), 3.87 (dt, *J* = 8.7, 3.9 Hz, 1H), 7.25 – 7.18 (m, 3H), 7.35 – 7.28 (m, 2H). MS (ESI+) *m/z* 266.34 $[M + H]^+$ for $C_{15}H_{23}NO_3$.

***tert*-Butyl (3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoate (2.23):** To a suspension of *tert*-butyl (3S,4S)-4-amino-3-hydroxy-5-phenylpentanoate (2.50 g, 9.422 mmol), in 80 mL of H_2O / EtOAc (1:1), $NaHCO_3$ (3.12 g, 37.69 mmol) was added and the mixture was stirred at RT to form an emulsion. Then, a solution of 2-(2.6-dimethylphenoxy)acetyl chloride (2.1 g, 10.36 mmol) was added dropwise and under Ar. When the addition was completed the mixture was stirred at RT for 1 h. Then EtOAc (40 mL) was added and the organic phase was washed with saturated aq. $NaHCO_3$ and water, dried over $MgSO_4$ and concentrated under reduced pressure to afford the product as a white solid (3.98 g, 98% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9H), 2.23 (s, 6H), 2.36 (dd, J = 17.0, 2.7 Hz, 1H), 2.49 (dd, J = 17.0, 10.4 Hz, 1H), 3.11 – 2.95 (m, 2H), 3.68 (dd, J = 2.6, 1.1 Hz, 1H), 4.07 (ddd, J = 10.3, 4.4, 2.6 Hz, 1H), 4.35 – 4.15 (m, 3H), 6.96 (dd, J = 8.6, 6.1 Hz, 1H), 7.04 – 6.99 (m, 2H), 7.25 – 7.21 (m, 1H), 7.33 – 7.28 (m, 4H). MS (ESI+) *m/z* 428.40 $[M + H]^+$ for $C_{25}H_{33}NO_5$.

(3S,4S)-4-(2-(2,6-Dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoic acid (2.24): To a solution of *tert*-butyl (3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoate (200 mg, 0.468 mmol) in CH_2Cl was added TFA (5 mL). The reaction mixture was stirred at RT for 2 h and the solvent was evaporated. The residue was dissolved in CH_2Cl_2 and concentrated to dryness three times. The residue was used immediately for the next reaction (174 mg, 99% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.21 (s, 6H), 2.56 (dd, J = 17.1, 3.4 Hz, 1H), 2.63 (dd, J = 17.1, 9.6 Hz, 1H), 3.05 (qd, J = 13.7, 7.7 Hz, 2H), 4.17 (ddd, J = 9.5, 3.2, 1.9 Hz, 1H), 4.40 – 4.23 (m, 3H), 6.97 (dd, J = 8.6, 6.0 Hz, 1H), 7.04 – 6.99 (m, 2H), 7.25 – 7.22 (m, 1H), 7.27 (s, 2H), 7.35 – 7.29 (m, 2H), 7.54 (d, J = 9.4 Hz, 1H). MS (ESI+) *m/z* 372.32 $[M + H]^+$ for $C_{21}H_{25}NO_5$.

Methyl ((3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-phenylalaninate (2.25): To a solution of (3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoic acid (184 mg, 0.50 mmol) and (L)-phenylalanine methyl ester hydrochloride (112 mg, 0.52 mmol) in CH_2Cl_2 (7 mL), HBTU (375 mg, 0.99 mmol), HOBt (152 mg, 0.99 mmol) and DIPEA (0.35 mL, 1.98 mmol) were added at RT. The mixture was stirred at that temperature overnight. The solvent was then evaporated under reduced pressure. The residue was dissolved in EtOAc and washed two times with 1M HCl, two times with saturated aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting yellow solid was purified with flash column chromatography (Hexanes/EtOAc 1:1), affording the desired product as a white solid (215 mg, 81% yield). A low quality HPLC trace was obtained due to the low UV absorption of the compound. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.21 (s, 6H), 2.28 (dd, J = 15.3, 3.0 Hz, 1H), 2.40 (dd, J = 15.3, 9.9 Hz, 1H), 3.09 – 2.97 (m, 3H), 3.16 (dd, J = 13.9, 5.7 Hz, 1H), 3.72 (s, 1H), 4.07 (dd, J = 9.8, 1.6 Hz, 1H), 4.23 (ddd, J = 14.9, 13.6, 7.3 Hz, 2H), 6.96 (dd, J = 8.4, 6.3 Hz, 1H), 7.02 (d, J = 7.4 Hz, 2H), 7.09 (d, J = 6.7 Hz, 2H), 7.25 – 7.18 (m, 2H), 7.33 – 7.27 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.3, 37.7, 38.1, 40.0, 52.4, 53.2, 53.8, 68.1, 70.3, 124.7, 126.6, 127.3, 128.6, 128.7, 129.1, 129.2, 129.4, 130.4, 135.6, 137.7, 154.3, 169.1, 171.7, 172.0. MS (ESI+) *m/z* 533.42 $[M + H]^+$ for $C_{31}H_{36}N_2O_6$.

Methyl ((3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-valinate (2.26): To a solution of (3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoic acid (76 mg, 0.21 mmol) and (L)-phenylalanine methyl ester hydrochloride (36 mg, 0.22 mmol) in CH_2Cl_2 (4 mL), HBTU (155 mg, 0.41 mmol), HOBt (63 mg, 0.41 mmol) and DIPEA (0.14 mL, 0.82 mmol) were added at RT. The mixture was stirred at that temperature overnight. The solvent was then evaporated under reduced pressure. The residue was dissolved in

EtOAc and washed two times with 1M HCl, two times with saturated aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting yellow solid was purified with flash column chromatography (Hexanes/EtOAc 1:1), affording the desired product as a white solid (61 mg, 61% yield). A low quality HPLC trace was obtained due to the low UV absorption of the compound. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.93 (dd, J = 13.7, 6.9 Hz, 6H), 2.19 – 2.12 (m, 1H), 2.20 (s, 6H), 2.39 (dd, J = 15.2, 3.4 Hz, 1H), 2.50 (dd, J = 15.2, 9.5 Hz, 1H), 3.05 (qd, J = 13.7, 7.8 Hz, 2H), 3.73 (s, 3H), 4.10 (d, J = 11.4 Hz, 1H), 4.34 – 4.16 (m, 3H), 4.39 (d, J = 2.0 Hz, 1H), 4.51 (dd, J = 8.6, 5.0 Hz, 1H), 6.51 (d, J = 8.6 Hz, 1H), 6.95 (dd, J = 8.4, 6.3 Hz, 1H), 7.01 (d, J = 7.4 Hz, 2H), 7.25 – 7.19 (m, 1H), 7.29 (d, J = 4.4 Hz, 4H), 7.32 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.2, 17.8, 18.9, 31.0, 38.1, 40.2, 52.2, 53.9, 57.2, 68.4, 70.3, 124.7, 126.6, 128.6, 129.1, 129.3, 130.4, 137.4, 154.3, 169.2, 172.1, 172.4. MS (ESI+) *m/z* 485.41 $[M + H]^+$ for $C_{27}H_{36}N_2O_6$.

((3S,4S)-4-(2-(2,6-Dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-phenylalanine (2.27): To a solution of methyl ((3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-phenylalaninate (201 mg, 0.39 mmol) in methanol (6 mL) was added aqueous NaOH 1N (1.93 mL, 1.93 mmol) and the reaction was stirred at RT overnight. When the reaction was complete, the solvent was removed under reduced pressure and the resulting solid was dissolved in H_2O (5 mL). It was acidified with HCL 1N to pH = 1 and the resulting precipitate was filtered and washed with water, affording the desired product as a white solid (189 mg, 94% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.19 (s, 6H), 2.36 (dd, J = 16.3, 10.4 Hz, 1H), 2.52 (dd, J = 16.3, 2.2 Hz, 1H), 3.05 – 2.95 (m, 2H), 3.08 (dd, J = 13.9, 7.3 Hz, 1H), 3.14 (dd, J = 13.8, 5.8 Hz, 1H), 4.08 (d, J = 10.2 Hz, 1H), 4.18 (q, J = 15.3 Hz, 2H), 4.30 (dd, J = 17.6, 9.6 Hz, 1H), 4.75 (dd, J = 12.6, 5.8 Hz, 1H), 6.85 (d, J = 7.0 Hz, 1H), 7.00 (dd, J = 8.9, 5.6 Hz,

1H), 7.12 – 7.02 (m, 7H), 7.25 – 7.18 (m, 3H), 7.31 – 7.26 (m, 2H), 7.41 (d, *J* = 10.1 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.2, 37.7, 37.9, 40.1, 53.4, 53.9, 68.2, 69.8, 125.0, 126.7, 126.9, 128.2, 128.6, 129.2, 129.2, 129.4, 130.4, 136.2, 137.5, 170.2, 172.3, 173.5. MS (ESI+) *m/z* 519.40 $[M + H]^+$ for $C_{30}H_{34}N_2O_6$.

((3S,4S)-4-(2-(2,6-Dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-valine (2.28): To a solution of methyl ((3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-valinate (60 mg, 0.12 mmol) in methanol (3 mL) was added aqueous LiOH 1N (0.14 mL, 0.14 mmol) and the reaction mixture was stirred at RT overnight. Then, aqueous NaOH 1N (5eq) was added to drive the reaction to completion and it was left to stir overnight again. When the reaction was complete, the solvent was removed under reduced pressure and the resulting solid was dissolved in H_2O (5 mL). It was acidified with HCl 1N to pH = 1 and the resulting precipitate was filtered and washed with water, affording the desired product as a white solid (43 mg, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.93 (d, *J* = 6.9 Hz, 6H), 2.24 – 2.15 (m, 7H), 2.46 (dd, *J* = 16.1, 10.4 Hz, 1H), 2.79 (dd, *J* = 16.1, 1.7 Hz, 1H), 3.00 (dd, *J* = 13.9, 8.5 Hz, 1H), 3.11 (dd, *J* = 13.9, 7.1 Hz, 1H), 4.10 (d, *J* = 10.2 Hz, 1H), 4.23 (q, *J* = 15.3 Hz, 2H), 4.33 (dd, *J* = 17.1, 8.9 Hz, 1H), 4.58 (dd, *J* = 8.1, 4.2 Hz, 1H), 6.96 (dd, *J* = 8.9, 5.6 Hz, 1H), 7.02 – 6.98 (m, 2H), 7.25 – 7.15 (m, 2H), 7.32 – 7.27 (m, 3H), 7.40 (d, *J* = 10.0 Hz, 1H). ^{1}C NMR (500 MHz, $CDCl_3$): δ 16.20, 17.79, 18.67, 31.47, 37.66, 40.13, 54.07, 56.82, 68.48, 69.87, 124.89, 126.66, 128.60, 129.15, 129.18, 130.34, 137.51, 154.09, 170.04, 172.85, 174.04. MS (ESI+) *m/z* 471.44 $[M + H]^+$ for $C_{26}H_{34}N_2O_6$.

(3S,4S)-4-(2-(2,6-Dimethylphenoxy)acetamido)-N-((R)-1-((3,5-dimethylphenyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-hydroxy-5-phenylpentanamide (2.29): To ((3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-phenylalanine (10 mg, 0.019

mmol) dissolved in CH_2Cl_2 (500 μL), 3,5-dimethylaniline (2.5 μL, 0.020 mmol) was added. Subsequently TBTU (6.8 mg, 0.021 mmol) was added, followed by HOBt (3.3 mg, 0.021 mmol) and DIPEA (10 μL, 0.058 mmol) at RT. The reaction mixture was stirred at the same temperature for 1 h. The reaction was quenched with brine (10 mL) and the precipitate was filtered and washed two times with H_2O (5 mL), two times with hexanes (5 mL) and a cold mixture of 4:1 hexanes/EtOAc (3 mL). A mixture of epimers (66:33 as determined by analytical reversed phase HPLC) was obtained. This mixture was purified by preparatory reversed phase HPLC to afford the desired compound as a white powder (2.9 mg, 24% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.19 (s, 6H), 2.26 (s, 6H), 2.33 (dd, *J* = 15.2, 3.0 Hz, 1H), 2.45 (dd, *J* = 15.2, 9.8 Hz, 1H), 3.13 – 2.97 (m, 3H), 3.18 (dd, *J* = 13.8, 6.4 Hz, 1H), 4.07 (d, *J* = 9.7 Hz, 1H), 4.20 – 4.15 (m, 2H), 4.27 (d, *J* = 15.0 Hz, 1H), 4.38 (d, *J* = 3.1 Hz, 1H), 4.71 (dd, *J* = 14.3, 7.7 Hz, 1H), 6.49 (d, *J* = 7.6 Hz, 1H), 6.74 (s, 1H), 6.97 – 6.93 (m, 3H), 7.00 (d, *J* = 7.1 Hz, 2H), 7.25 – 7.21 (m, 4H), 7.29 (dd, *J* = 16.5, 7.2 Hz, 7H), 7.40 (s, 1H). MS (ESI+) *m/z* 622.49 $[M + H]^+$ for $C_{38}H_{43}N_3O_5$.

(3S,4S)-4-(2-(2,6-Dimethylphenoxy)acetamido)-N-((S)-1-((3,5-dimethylphenyl)amino)-1-oxo-3-phenylpropan-2-yl)-3-hydroxy-5-phenylpentanamide (2.30): A solution of ((3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-phenylalanine (10 mg, 0.019 mmol) and 3,5-dimethylaniline (2.5 μL, 0.020 mmol) in CH_2Cl_2 (500 μL) was cooled to 0 ^oC. Subsequently, $NaHCO_3$ (1.7 mg, 0.021 mmol) and HATU (7.9 mg, 0.021 mmol) were added at 0 ^oC and the reaction was stirred at that temperature for 1 h. The solvent was evaporated and the residue was dissolved in EtOAc, washed with 1N HCl, saturated aq. $NaHCO_3$ and brine. The organic phase was then dried with $MgSO_4$ to afford the desired product as a mixture of epimers (95:5 as determined by analytical reversed phase HPLC). It was purified by preparatory reversed phase HPLC to afford the desired compound as a white powder (5.5 mg, 42% yield). ^{1}H NMR

(400 MHz, $CDCl_3$): δ 2.19 (s, 6H), 2.25 (s, 6H), 2.33 (dd, *J* = 15.3, 3.4 Hz, 1H), 2.45 (dd, *J* = 15.3, 9.2 Hz, 1H), 3.01 (dd, *J* = 7.7, 1.9 Hz, 2H), 3.14 (d, *J* = 7.2 Hz, 2H), 4.07 (brd, *J* = 4.5 Hz, 2H), 4.29 – 4.14 (m, 3H), 4.72 (q, *J* = 7.3 Hz, 1H), 6.74 (s, 1H), 6.81 (d, *J* = 7.7 Hz, 1H), 7.04 – 6.92 (m, 5H), 7.26 – 7.15 (m, 8H), 7.30 (dd, *J* = 7.8, 5.9 Hz, 3H), 7.64 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.4, 21.5, 38.2, 38.3, 40.6, 41.2, 54.1, 55.6, 68.7, 70.4, 118.0, 128.7, 129.0, 129.3, 129.4, 129.4, 130.5, 136.7, 137.1, 137.7, 138.8, 154.4, 168.8, 169.4, 172.4. MS (ESI+) *m/z* 622.49 $[M + H]^+$ for $C_{38}H_{43}N_3O_5$.

(3S,4S)-4-(2-(2,6-Dimethylphenoxy)acetamido)-N-((S)-1-((3,5-dimethylphenyl)amino)-3-methyl-1-oxobutan-2-yl)-3-hydroxy-5-phenylpentanamide (2.31): A solution of ((3S,4S)-4-(2-(2,6-dimethylphenoxy)acetamido)-3-hydroxy-5-phenylpentanoyl)-L-valine (12 mg, 0.026 mmol) and 3,5-dimethylaniline (3.4 μL 0.027 mmol) in a mixture of DMF / CH_2Cl_2 3:1 (600 μL) was cooled to 0 ^{o}C. Subsequently, $NaHCO_3$ (2.4 mg, 0.028 mmol) and HATU (11 mg, 0.028 mmol) were added at 0 ^{o}C and the reaction was stirred at that temperature for 1 h. The solvent was evaporated and the residue was dissolved in EtOAc, washed with 1N HCl, saturated aq. $NaHCO_3$ and brine. The organic phase was then dried with $MgSO_4$ to afford the desired product as a mixture of epimers (95:5 as determined by analytical reversed phase HPLC). It was purified by preparatory reversed phase HPLC to afford the desired compound as a white powder (4.4 mg, 29% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.01 (dd, *J* = 9.4, 6.8 Hz, 6H), 2.18 (s, 6H), 2.22 (d, *J* = 6.8 Hz, 1H), 2.26 (s, 6H), 2.41 (dd, *J* = 14.9, 4.3 Hz, 1H), 2.51 (dd, *J* = 14.9, 8.9 Hz, 1H), 3.10 – 2.94 (m, 2H), 4.10 (brs, 1H), 4.38 – 4.14 (m, 5H), 6.76 (s, 1H), 7.02 – 6.89 (m, 4H), 7.14 (s, 2H), 7.24 – 7.15 (m, 5H), 7.94 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.4, 18.6, 19.5, 21.5, 31.1, 18.2, 40.8, 54.1, 59.9, 69.1, 70.3, 118.0, 124.9, 126.7, 128.7, 129.2, 129.3, 130.5, 137.4, 137.7, 138.8, 154.2, 169.6, 172.7. MS (ESI+) *m/z* 574.47 $[M + H]^+$ for $C_{34}H_{43}N_3O_5$.

(2-Benzyl-3-(((S)-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)((R)-1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)phosphinic acid (2.32): 2-benzyl-3-(((R)-1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)(hydroxy)phosphoryl)propanoic acid (130 mg, 0.27 mmol) was dissolved in dry CH_2Cl_2 (6 mL) and then DIPEA (0.14 mL, 0.81 mmol), alanine methyl ester hydrochloride (40 mg, 0.28 mmol), HOBt (41 mg, 0.27 mmol) and EDC hydrochloride (207 mg, 1.08 mmol) were added. The mixture was stirred under Ar at RT for 2.5 h. Then, CH_2Cl_2 was added and the organic phase was washed with 1N HCl three times, H_2O once, 1N HCl twice and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Then, a mixture of Et_2O / PE 1:5 was added to the residue (9 mL), the mixture was triturated in the ultrasound for 5 min, the organic phase was decanted and the residue was dried under vacuum to afford the compound as an off white solid (135 mg, 84% yield). A low quality HPLC trace was obtained due to the low UV absorption of the compound. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.22 (d, J = 7.3 Hz, 3H), 1.65 (ddd, J = 15.0, 12.7, 6.6 Hz, 1H), 1.93 – 1.78 (m, 1H), 2.70 (td, J = 13.7, 5.7 Hz, 1H), 2.94 – 2.84 (m, 2H), 3.06 – 3.00 (m, 2H), 3.82 (dd, J = 21.4, 11.8 Hz, 1H), 4.24 – 4.12 (m, 1H), 4.84 (d, J = 13.1 Hz, 1H), 4.94 (d, J = 13.0 Hz, 1H), 7.32 – 6.92 (m, 15H), 7.56 (d, J = 8.8 Hz, 1H), 8.50 (brs, 1H). ^{31}P NMR (202 MHz, DMSO-d_6): δ 42.85. ^{13}C NMR (125 MHz, DMSO-d_6): δ 17.3, 17.4, 21.5, 27.6, 28.3, 33.3, 47.8, 48.1, 52.2, 65.6, 112.2, 114.4, 116.7, 119.0, 125.8, 126.5, 126.6, 127.4, 127.5, 127.9, 128.0, 128.4, 128.5, 128.5, 128.5, 128.6, 128.7, 129.3, 129.4, 129.5, 129.5, 129.6, 137.6, 137.8, 138.9, 139.6, 139.7, 156.4, 158.4, 158.7, 159.0, 159.3, 173.4. MS (ESI+) *m/z* 567.36 $[M + H]^+$ for $C_{30}H_{35}N_2O_7P$.

(1-(Benzhydrylamino)-2-phenylethyl)phosphinic acid (2.34): To a suspension of diphenylmethylamine hydrochloride (6.0 g, 27 mmol) in EtOH / H_2O 9:1 (43 mL) was added a 50% aq. solution of H_3PO_2 (2.9 mL, 27 mmol) and then the reaction mixture was heated at 90 ^{o}C.

At that temperature, 90% phenylacetaldehyde (3.9 mL, 30 mmol) dissolved in EtOH (10 mL) was added over a period of 30 min and the reaction was stirred at that temperature for 5 h, followed by stirring at RT overnight. The precipitate that was formed was filtered off, washed with cold EtOH and Et_2O and dried under vacuum to afford the desired product as a white solid (7.3 g, 76% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.76 – 2.64 (m, 2H), 3.18 – 3.11 (m, 1H), 4.91 (s, 1H), 6.40 (d, *J* = 1.7 Hz, 1H), 7.01 – 6.96 (m, 2H), 7.29 – 7.20 (m, 8H), 7.33 (t, *J* = 7.4 Hz, 2H), 7.45 – 7.37 (m, 3H). ^{31}P NMR (202 MHz, $CDCl_3$): δ 29.83.

(1-Amino-2-phenylethyl)phosphinic acid (2.35): A solution of (1-(benzhydrylamino)-2-phenylethyl)phosphinic acid (7.3 g, 20.8 mmol) in 48% aq. HBr (49 mL, 290 mmol) was heated at 100 ^{o}C for 2.5 h until two distinct phases separated. The mixture was allowed to cool to RT and the volatile material was evaporated under reduced pressure. The oily residue was dissolved in water (75 mL) and extracted with Et_2O (3x35 mL) to remove the side product. Water was evaporated again under reduced pressure and the oily residue of the hydrobromide salt of the desired compound was dissolved in EtOH (30 mL), cooled at 0 ^{o}C and cold propylene oxide was slowly added to neutralize the hydrobromide until precipitation started. The mixture was allowed to stand in the freezer overnight to complete the precipitation. The solid was filtered, washed with EtOH (10 mL) and Et_2O (40 mL) and dried under vacuum to afford the desired compound as a white solid (3.46 g, 90% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.39 – 2.30 (m, 1H), 2.53 (dd, *J* = 9.7, 2.4 Hz, 1H), 2.94 (ddd, *J* = 13.5, 6.9, 3.3 Hz, 1H), 6.11 (s, 0.5H), 7.07 (s, 0.5H), 7.17 – 7.12 (m, 1H), 7.23 (ddd, *J* = 17.9, 7.8, 4.1 Hz, 4H). ^{31}P NMR (202 MHz, DMSO-d_6): δ 25.627.

(1-(((Benzyloxy)carbonyl)amino)-2-phenylethyl)phosphinic acid (2.36): To a suspension of (1-amino-2-phenylethyl)phosphinic acid (2.50 g, 13.5 mmol) was added 4M aqueous NaOH (5.4 mL 21.6 mol). The reaction mixture was brought to 0 ^{o}C and then benzyl chloroformate (2.3 mL, 16.2

mmol) was added over a period of 1 h. The mixture was stirred at the same temperature for 1 h and then at room temperature for 4 h, whilst bringing the pH of the solution to 9-10 by adding a 2M aqueous solution of NaOH. The mixture was then stirred at RT overnight to and then was extracted once with Et_2O. The aqueous phase was acidified by the addition of 6M aq. HCl to pH = 1 and the precipitate was filtered, washed with H_2O, Et_2O and dried overnight under high vacuum to afford the desired compound as a white solid (3.9 g, 91% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.58 (ddd, J = 14.3, 11.9, 7.2 Hz, 1H), 2.97 (ddd, J = 14.1, 4.8, 3.4 Hz, 1H), 3.49 – 3.40 (m, 1H), 4.86 (d, J = 13.0 Hz, 1H), 4.94 (d, J = 13.0 Hz, 1H), 6.31 (s, 1H), 7.20 – 7.13 (m, 3H), 7.24 – 7.20 (m, 4H), 7.33 – 7.24 (m, 3H). ^{31}P NMR (202 MHz, DMSO-d_6): δ 16.75.

((R)-1-(((Benzyloxy)carbonyl)amino)-2-phenylethyl)phosphinic acid (2.37): A suspension of (1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)phosphinic acid (3.9 g, 12.2 mmol) in absolute EtOH (20 mL) was heated at reflux until a solution formed. Then, a solution of R-(+)-methylbenzylamine in absolute EtOH (3 mL) was added dropwise at 80 ^{o}C in a period of 10 min and then the mixture was stirred at the same temperature for 30 min and at RT for further 15 min. Then it was left in the freezer overnight. The precipitate was filtered off, washed with cold EtOH (8 mL) and Et_2O (15 mL) and the solid was recrystallized from absolute EtOH (16 mL) and left in the freezer overnight. The salt obtained was filtered off and suspended in 6M aq. HCl (10 mL) and stirred for 2 h. The solid was filtered off, washed with H_2O, Et_2O and dried under vacuum overnight to afford the desired compound as a white solid (0.8 g, 41% yield). ^{1}H NMR (500 MHz, 1% TFA in DMSO-d_6): δ 2.75 (ddd, J = 14.0, 12.3, 8.1 Hz, 1H), 3.01 (ddd, J = 14.1, 4.9, 3.5 Hz, 1H), 3.83 (td, J = 12.3, 3.3 Hz, 1H), 5.01 – 4.87 (m, 2H), 6.35 (s, 0.5H), 7.36 – 7.06 (m, 10H), 7.41 (s, 0.5H), 7.70 (d, J = 9.1 Hz, 1H). ^{31}P NMR (202 MHz, DMSO-d_6): δ 27.11.

2-Benzyl-3-ethoxy-3-oxopropanoic acid: To a solution of diethyl benzylmalonate (6.0 g, 24 mmol) in EtOH (150 mL) was added KOH (1.4 g, 25 mmol) as pellets. The mixture was stirred at RT for 24 h and then the solvent was concentrated under reduced pressure. The residue was dissolved in H_2O, transferred to a separatory funnel and washed twice with Et_2O. The aqueous phase was acidified by using conc. HCl to pH = 1 and extracted with Et_2O twice. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the compound as a colorless oil (4.7 g, 87% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.21 (t, J = 7.1 Hz, 3H), 3.24 (dd, J = 7.7, 2.8 Hz, 2H), 3.70 (t, J = 7.7 Hz, 1H), 4.17 (qd, J = 7.2, 1.3 Hz, 2H), 7.25 – 7.19 (m, 3H), 7.29 (ddd, J = 7.5, 4.4, 1.2 Hz, 2H).

Ethyl 2-benzylacrylate (2.38): 2-benzyl-3-ethoxy-3-oxopropanoic acid (4.7 g, 21.0 mmol) was cooled in an ice bath and then diethylamine (2.3 mL) and 37% aq. formaldehyde solution (2.3 mL) was added over 10 min while stirring. After stirring for 7 h, the mixture was diluted with water and extracted with Et_2O. The organic layer was washed with 2N HCl, saturated aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to dryness to provide the desired product as a colorless oil (3.1 g, 78% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.27 (t, J = 7.1 Hz, 3H), 3.64 (s, 2H), 4.19 (q, J = 7.1 Hz, 2H), 5.45 (q, J = 1.4 Hz, 1H), 6.23 (d, J = 1.3 Hz, 1H), 7.23 – 7.19 (m, 3H), 7.32 – 7.27 (m, 2H).

(2-Benzyl-3-ethoxy-3-oxopropyl)((R)-1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)phosphinic acid (2.39): To a vial containing ((R)-1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)phosphinic acid (140 mg, 0.44 mmol), HMDS (0.46 mL, 2.2 mmol) was added under Ar and the mixture was stirred at 110 ^{o}C for 2 h. After that time the temperature was reduced to 105 ^{o}C and then ethyl 2-benzylacrylate (109 mg, 0.57 mmol) (in a few drops of CH_2Cl_2) was added dropwise and the solution was stirred for an additional 5 h at that temperature. At that point

the temperature was decreased to 70 ^{o}C and absolute EtOH (0.8 mL) was added dropwise and the mixture was left to stir overnight at room temperature. On the next day the mixture was concentrated to dryness and purified by flash column chromatography (CH_3Cl / MeOH / AcOH 9:0.3:0.3) to afford the an off white oil. This oil was co-evaporated with toluene three times to afford the desired product as an off white solid (165 mg, 73% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 1% TFA): δ 1.01 (dt, J = 9.8, 7.1 Hz, 3H), 1.77 (tdd, J = 16.0, 11.8, 5.4 Hz, 1H), 2.07 – 1.95 (m, 1H), 2.72 (ddd, J = 19.0, 12.1, 5.2 Hz, 1H), 2.82 (ddd, J = 17.6, 13.4, 5.0 Hz, 1H), 3.02 – 2.92 (m, 2H), 3.06 (d, J = 14.3 Hz, 1H), 3.99 – 3.84 (m, 3H), 4.98 – 4.83 (m, 2H), 5.73 (s, 1H), 7.14 (dd, J = 7.5, 6.1 Hz, 4H), 7.20 – 7.17 (m, 2H), 7.31 – 7.22 (m, 9H), 7.68 (dd, J = 9.5, 5.8 Hz, 1H). ^{31}P NMR (202 MHz, DMSO-d_6, 1% TFA): δ 44.49, 44.62.

2-Benzyl-3-(((R)-1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)(hydroxy)phosphoryl)propanoic acid (2.40): To a solution of (2-benzyl-3-ethoxy-3-oxopropyl)((R)-1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)phosphinic acid (165 mg, 0.32 mmol) in EtOH (6 mL) was added an aqueous 1N NaOH solution (3.2 mL) dropwise at 0 ^{o}C. Then, the reaction mixture was stirred at room temperature for 48 h. EtOH was evaporated under reduced pressure, water was added and the mixture was washed with Et_2O two times. After acidification with 1N HCl to pH = 1, EtOH was evaporated under high vacuum, EtOAc was added and the organic phase was washed with H_2O and brine, dried over $MgSO_4$, filtered, concentrated and dried under reduced pressure to afford the desired product as a white solid (130 mg, 84% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.81 – 1.65 (m, 1H), 2.00 (ddd, J = 19.1, 13.9, 7.2 Hz, 1H), 2.75 – 2.63 (m, 1H), 2.97 – 2.80 (m, 3H), 3.06 (d, J = 13.8 Hz, 1H), 3.96 – 3.84 (m, 1H), 4.98 – 4.83 (m, 2H), 7.31 – 7.11 (m, 15H), 7.64 (dd, J = 9.4, 4.6 Hz, 1H). ^{31}P NMR (202 MHz, DMSO-d_6): δ 44.95.

3-(3-Methyldiaziridin-3-yl)propanoic acid (2.41): Levulinic acid (1.61 g, 13.8 mmol,) was dissolved in 7 N NH_3 in CH_3OH (13.5 mL, 91.0 mmol). The resulting solution was stirred under Ar on ice for 3 h. A solution of hydroxylamine-O-sulfonic acid (1.80 g, 15.9 mmol) in CH3OH (12 mL) was added dropwise at a rate of 1 drop per second. The reaction mixture was stirred for 20 h and allowed to warm to rt. Ar was bubbled through the solution for 1 h to remove the NH_3 gas. Vacuum filtration and concentration resulted in a yellow oil that was used in the next step immediately and without purification (1.72 g, 97% yield).

3-(3-Methyl-3H-diazirin-3-yl)propanoic acid (2.42): 3-(3-methyldiaziridin-3-yl)propanoic acid (1.72 g, 13.2 mmol) was dissolved in methanol (10 mL) and stirred in ice for 5 min in a tin foil-covered flask. Triethylamine (3.00 mL, 21.5 mmol) was added and allowed to stir for 5 min. Slowly, chips of I_2 were added until the solution remained a brown-red color for longer than 5 min after the last addition. The reaction solution was then diluted with EtOAc and washed with 1N HCl and 10% aq. $Na_2S_2O_3$ until the organic layer was almost colorless. The aqueous layer was further extracted with EtOAc twice, the organic layers were combined, dried over $MgSO_4$ and concentrated under reduced pressure to afford the product as a yellow oil (0.8 g, 46% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.04 (s, 3H), 1.77 – 1.66 (t, J = 6.8 Hz, 2H), 2.28 – 2.20 (t, J = 6.8 Hz, 2H), 11.40 (brs, 1H).

((*R*)-1-Amino-2-phenylethyl)(2-benzyl-3-(((S)-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)phosphinic acid (2.43): Pd/C 10% (0.15 eq.) was added to a solution of (2-benzyl-3-(((S)-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)((R)-1-(((benzyloxy)carbonyl)amino)-2-phenylethyl)phosphinic acid in MeOH and 1 drop of AcOH. The reaction mixture was stirred for 2h at room temperature, under H_2 and then filtered through a pad of celite. The evaporation of solvents afforded the product as an off-white solid. The crude mixture was purified by reversed

phase HLPC to afford the product as a white solid (45% yield). ^{1}H NMR (500 MHz, DMSO-d_6, 1% TFA): δ 1.31 – 1.18 (m, 4H), 1.63 – 1.48 (m, 1H), 2.50 (s, 3H), 2.63 (dd, J = 15.9, 9.3 Hz, 1H), 2.93 – 2.78 (m, 4H), 3.07 – 2.96 (m, 1H), 4.17 (p, J = 7.1 Hz, 1H), 7.37 – 7.10 (m, 10H), 8.93 (d, J = 6.1 Hz, 1H). ^{31}P NMR (202 MHz, DMSO-d_6, 1% TFA): δ 25.66. MS (ESI+) *m/z* 433.27 $[M + H]^+$ for $C_{22}H_{29}N_2O_5P$.

(2-Benzyl-3-(((S)-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)((R)-1-(3-(3-methyl-3H-diazirin-3-yl)propanamido)-2-phenylethyl)phosphinic acid (2.33): ((*R*)-1-amino-2-phenylethyl)(2-benzyl-3-(((S)-1-methoxy-1-oxopropan-2-yl)amino)-3-oxopropyl)phosphinic acid was dissolved in dry CH_2Cl_2 (2 mL) and DIPEA, 3-(3-methyl-3H-diazirin-3-yl)propanoic acid, HOBt and EDC were added. The mixture was stirred at room temperature and under Ar for 2.5 h. Then, CH_2Cl_2 was added (5 mL) and the organic phase was washed with aq. saturated NH_4Cl and brine, dried over $MgSO_4$, filtered and concentrated under vacuum. Then it was purified by reversed phase preparatory HPLC to afford the desired product as a white solid (14% yield). The HPLC trace indicates the presence of two compounds in a single peak due to the presence of the two diastereomers. ^{1}H NMR (500 MHz, DMSO-d_6): δ 0.83 (s, 3H), 1.28 – 1.19 (m, 3H), 1.37 – 1.27 (m, 1H), 1.64 – 1.54 (m, 1H), 1.81 – 1.72 (m, 1H), 1.92 – 1.82 (m, 2H), 2.72 – 2.63 (m, 1H), 2.85 (dd, J = 13.8, 5.9 Hz, 1H), 3.01 – 2.89 (m, 2H), 3.06 (d, J = 14.1 Hz, 1H), 3.56 (s, 3H), 4.26 – 4.11 (m, 2H), 7.30 – 7.05 (m, 10H), 8.27 (d, J = 5.9 Hz, 1H), 8.33 (d, J = 6.4 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 17.3, 19.5, 26.1, 30.0, 30.4, 33.1, 48.1, 52.2, 126.4, 126.5, 128.4, 129.4, 129.4, 129.5, 129.5, 171.0, 173.5. ^{31}P NMR (202 MHz, DMSO-d_6): δ 44.49. MS (ESI+) *m/z* 543.45 $[M + H]^+$ for $C_{27}H_{35}N_4O_6P$.

2.5 References

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Chapter 3. Farnesol derived component for the ZMPSTE24 enzymatic assay

None of the work presented in this chapter has been published. All the synthesis work was performed by myself.

3.1 Preface

An azide containing farnesol derivative was synthesized in order to be used as a key component in an enzymatic assay which will be developed in the future.

3.2 Design of the enzymatic assay

A project revolving around the discovery of potent ZMPSTE24 inhibitors calls for a robust enzymatic assay to be developed. While the IC_{50} of lopinavir and tipranavir against ZMPSTE24 has been estimated,[1] these results were laborious to obtain, used a downstream event to the direct inhibition of ZMPSTE24 and are likely unreliable due to the lack of purity of the enzyme preparation and the high levels of detergent used. Such an assay cannot be applied in a medicinal chemistry project which involves hundreds of compounds to be tested reliably and thus a decision was made to develop our own fluorescence based enzymatic assay.

Since ZMPSTE24 is a membrane-bound enzyme that is nearly impossible to isolate in pure soluble form, the construction of a thermolysin/ZMPSTE24 hybrid is in progress in collaboration with the group of Prof. Albert Berghuis. Dealing with transmembrane enzymes is extremely challenging and while there is recent progress in the field,[2] our strategy avoids the use of the wild-type ZMPSTE24 enzyme. The enzyme thermolysin, a thermostable bacterial peptidase was

chosen, since it is ideally suited for use in an enzymatic assay. In our design, thermolysin will have a few catalytic residues in the active site mutated, in order for it to mimic the ZMPSTE24 active site. The feasibility of the undertaking of generating this chimeric enzyme is suggested by the inherent similarity of those enzymes' active sites and catalytic residues.[3]

The natural substrate of ZMPSTE24 is the farnesylated prelamin A,[4] therefore it was conjectured that a farnesylated oligopeptide whose amino acid sequence is the same as prelamin A's and with the farnesyl attached to the C-terminal cysteine will be a viable choice as the chimeric enzyme's substrate. The assay's design calls for the oligopeptide to be attached to biotin near to its N-terminus and the assay to be run on streptavidin coated plates, so that the oligopeptide will be essentially immobilized on the surface of the wells. Simultaneously, the C-terminal cysteine of the oligopeptide will be farnesylated. The farnesyl, besides aiding in simulating the natural substrate, has an azide attached on it, which will be used to attach a fluorophore by a copper (I) catalyzed 1,2,3-triazole formation,[5] in order to provide the detection signal.

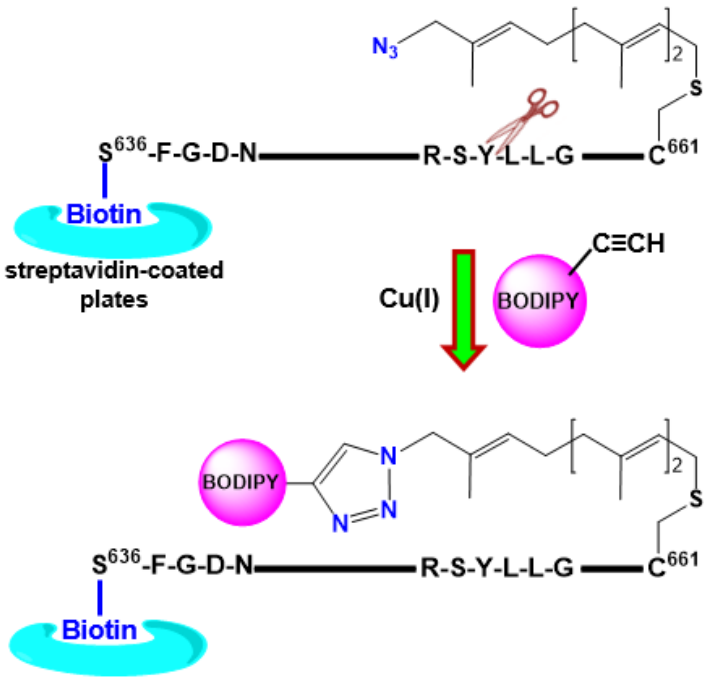


Figure 25. Depiction of the farnesylated oligopeptide for the proposed enzymatic assay, exhibiting the azide containing farnesol derivative and its function in the assay.

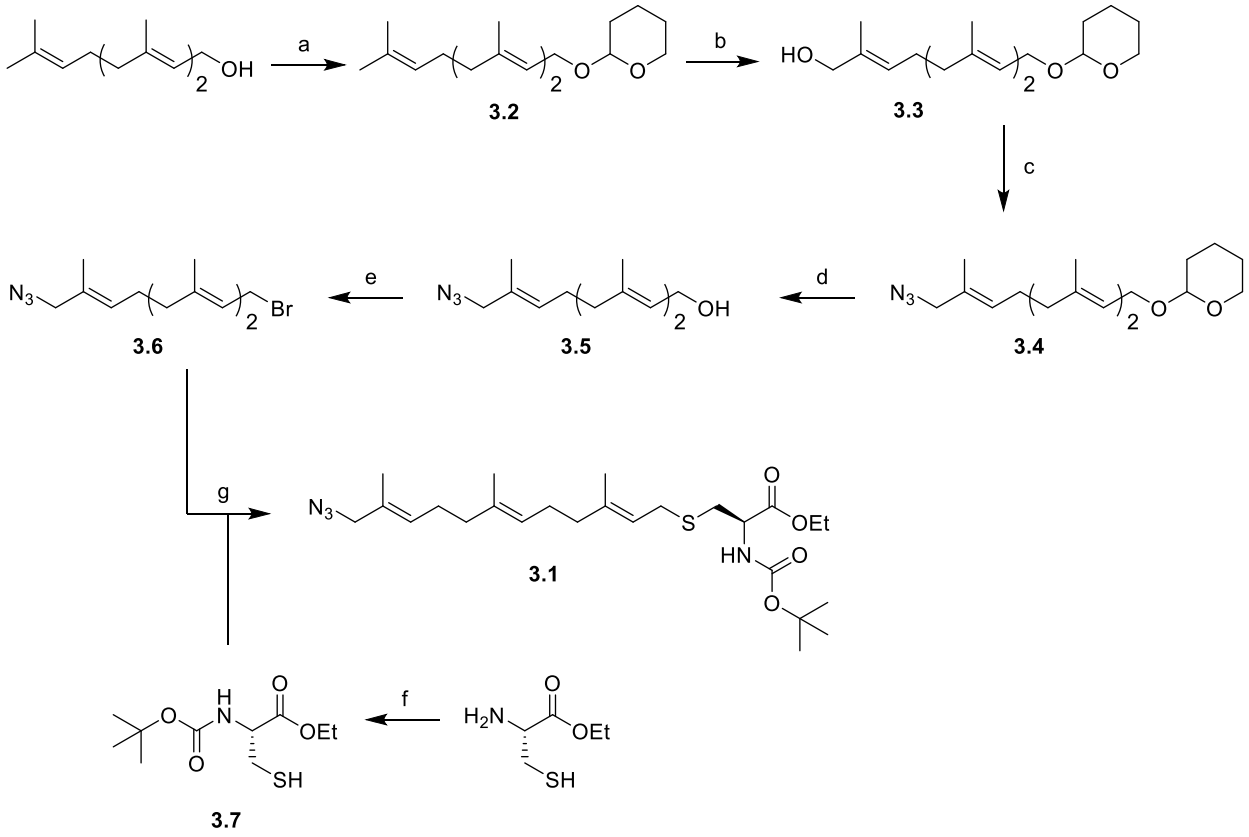
The suggested protocol is as follows: Streptavidin coated plates hold a solution of biotin linked farnesylated oligopeptide. Incubation with the thermolysin/ZMPSTE24 chimeric enzyme in the absence of an inhibitor for an appropriate length of time will allow most of the oligopeptide to be cleaved near its C-terminus, allowing the farnesyl attached azide to be removed by washing the plates. Subsequent introduction of an alkyne containing fluorophore, under click reaction conditions and washing to remove the excess of the non-covalently linked, to the farnesyl-azide, fluorophore will allow the determination of the amount of oligopeptide left intact by measuring the fluorescence intensity. If a ZMPSTE24 inhibitor was to be introduced along with the enzyme, then a smaller fraction of the oligopeptide is expected to be cleaved, thus retaining more of the farnesol-azide linked to the plates and eventually producing stronger fluorescence signal after the

introduction of the fluorophore. This protocol calls for the synthesis of farnesol derivative **3.1**, with an azide linked to the one end and a cysteine to the other, a synthesis which was performed in the context of this thesis.

3.3 Synthesis of farnesol derivative

Farnesol derivatives have been extensively used for chemical biology studies, so there is literature precedent for most of the synthesis. More specifically, the azide functional group, a ubiquitous tool in chemical biology, has been introduced in a farnesol based probe by Distefano and co-workers and their synthesis paved the road to ours (Scheme 9).[6]

Scheme 9. Synthesis of the target azide containing farnesol derivative **3.1**.



Reagents: (a) 3,4-dihydropyran, pyridinium-p-toluenesulfonate, CH_2Cl_2 (94%); (b) SeO_2, *tert*-butyl hydroperoxide 70% aq., salicylic acid, CH_2Cl_2 (17%); (c) diphenyl phosphoryl azide, DBU, CH_2Cl_2 (50%); (d) pyridinium-p-toluenesulfonate, EtOH (86%); (e) CBr_4, PPh_3, Et_2O (43%); (f) di-*tert* butyl dicarbonate, Et_3N, CH_2Cl_2 (84%); (g) aq. NaOH 1N, MeOH (20%).

The synthesis was started with the protection of farnesol as the 2-tetrahydro-2H-pyran **3.2** upon reaction with 3,4-dihydropyran in the presence of catalytic pyridinium-*p*-toluenesulfonate (Scheme 9). The tetrahydropyranyl ether **3.2** was then subjected to an allylic oxidation by catalytic selenium dioxide and salicylic acid, with an excess of 70% aqueous *tert*-butyl hydroperoxide as the oxidant. This reaction afforded the primary alcohol **3.3**, albeit in low isolated yields of about 20%, despite some effort for optimization. This alcohol was converted to the azide **3.4** by treatment with diphenyl phosphoryl azide in moderate yields. Subsequently, a deprotection by catalytic pyridinium-*p*-toluenesulfonate in ethanol afforded the alcohol **3.5** which was then subjected to an Appel reaction with carbon tetrabromide, converting the hydroxyl to the bromide **3.6**. This bromide is quite unstable at room temperature and attempted purification of the reaction mixture by flash column chromatography results in its decomposition of this compound on the column. Consequently, the crude reaction mixture was treated with hexanes to remove most of the triphenyl phosphine oxide and then it was used immediately for the final reaction. An S_N2 condensation of *N*-Boc cysteine methyl ester **3.7** with the bromide gave the desired product **3.1**. Intermediate **3.7** was prepared from commercially available cysteine methyl ester hydrochloride, which was converted to the carbamate with 1 equivalent of di-*tert*-butyl carbonate and 2 equivalents of triethylamine.[7] For the final reaction, a deprotonation of **3.7** with 1 equivalent of sodium hydroxide was performed first and then the crude bromide **3.6** was added and left to stir overnight, furnishing the target compound **3.1** in low yields, but with a straightforward purification by flash column chromatography.

3.4 Experimental

All compounds were purified by normal phase flash column chromatography on silica gel either manually or by using a CombiFlash instrument and a solvent gradient from as indicated. Each final compound was characterized by ^{1}H and ^{13}C NMR and HRMS. Chemical shifts (δ) are reported in ppm relative to the internal deuterated solvent. The high-resolution MS spectra of final products were recorded using electrospray ionization (ESI±) and Fourier transform ion cyclotron resonance mass analyzer (FTMS).

2-(((2E,6E)-3,7,11-Trimethyldodeca-2,6,10-trien-1-yl)oxy)tetrahydro-2H-pyran (3.2): To a solution of farnesol (2.0 g, 9.0 mmol) in dry CH_2Cl_2 (20 mL) was added 3,4-dihydropyran (1.3 mL, 14.4 mmol) and a solution of pyridinium p-toluenesulfonate (113 mg, 0.45 mmol) in dry CH_2Cl_2 (3 mL), at 0 ^{o}C and under Ar. The mixture was stirred overnight at RT. The mixture was evaporated, dissolved in diethyl ether, washed with saturated aq. K_2CO_3, H_2O and brine, dried over MgSO4, filtered and the solvent was evaporated under vacuum. The residue was purified by flash column chromatography (Hexanes/EtOAc 10:1) to afford the desired product as a colorless oil (2.59 g, 94% yield). ^{1}H NMR spectrum is in agreement with published spectra of the title compound.[8] ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.63 – 1.48 (m, 9H), 1.77 – 1.66 (m, 8H), 1.84 (ddd, J = 20.8, 11.0, 3.9 Hz, 1H), 2.00 – 1.94 (m, 2H), 2.15 – 2.02 (m, 7H), 3.55 – 3.48 (m, 1H), 3.90 (ddd, J = 11.1, 7.7, 3.1 Hz, 1H), 4.03 (dd, J = 11.9, 7.5 Hz, 1H), 4.24 (dd, J = 11.9, 6.3 Hz, 1H), 4.65 – 4.60 (m, 1H), 5.15 – 5.04 (m, 2H), 5.36 (t, J = 6.9 Hz, 1H).

(2E,6E,10E)-2,6,10-Trimethyl-12-((tetrahydro-2H-pyran-2-yl)oxy)dodeca-2,6,10-trien-1-ol (3.3): 2-(((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)oxy)tetrahydro-2H-pyran (1.25 g, 4.1 mmol) was dissolved in 10 mL of CH_2Cl_2 and a 70% aqueous solution of *tert*-butyl hydroperoxide (2.2 mL, 16.3 mmol) was added, followed by salicylic acid (56 mg, 0.41 mmol) and selenium

dioxide (23 mg, 0.20 mmol). The solution was stirred for 8h at RT. After that time, the mixture was concentrated under reduced pressure (temperature of the water bath should not exceed 40 ^{o}C), it was co-evaporated three times with toluene to remove all of the TBHP and the resulting residue was dissolved in Et_2O. It was washed witch $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc 4.5:1) to afford the desired product as a colorless oil (216 mg, 17% yield). ^{1}H NMR spectrum is in agreement with published spectra of the title compound.[8] ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.54 (dt, J = 13.1, 6.0 Hz, 4H), 1.60 (s, 3H), 1.67 (t, J = 5.9 Hz, 6H), 1.86 – 1.71 (m, 2H), 2.16 – 1.99 (m, 8H), 3.58 – 3.45 (m, 1H), 3.89 (ddd, J = 11.1, 7.7, 3.1 Hz, 1H), 4.07 – 3.96 (m, 3H), 4.23 (dd, J = 11.9, 6.3 Hz, 1H), 4.67 – 4.58 (m, 1H), 5.12 (dd, J = 11.5, 6.1 Hz, 1H), 5.30 (s, 1H), 5.45 – 5.32 (m, 2H).

2-(((2E,6E,10E)-12-Azido-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)oxy)tetrahydro-2H-pyran (3.4): A solution of (2E,6E,10E)-2,6,10-trimethyl-12-((tetrahydro-2H-pyran-2-yl)oxy)dodeca-2,6,10-trien-1-ol and diphenyl phosphoryl azide in toluene was stirred at 0^{o}C for 30 min. DBU was added and the reaction was stirred for 15 min at 0^{o}C. The ice bath was then removed and the reaction was allowed to warm to room temperature. The progress of the reaction was followed by TLC (hexanes/EtOAc 4:1, R_f = 0.7). The reaction was quenched after 3.5 hours with water, transferred with EtOAc to a separatory funnel and washed with brine two times. Any remaining organic species in the brine were back-extracted 3 times with EtOAc. The organic layers were combined, washed with 10% HCl and dried over $MgSO_4$. The solvent was removed under vacuum and the residue was purified by flash column chromatography (Hexanes/EtOAc 8:1), affording the desired product as an off yellow oil (50% yield). ^{1}H NMR spectrum is in agreement with published spectra of the title compound.[6] ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.58 – 1.49 (m, 4H),

1.61 (t, *J* = 6.5 Hz, 3H), 1.65 – 1.70 (m, 6H), 1.76 – 1.71 (m, 2H), 1.87 – 1.77 (m, 1H), 2.25 – 1.93 (m, 8H), 3.55 – 3.47 (m, 1H), 3.69 – 3.63 (m, 1H), 3.80 (t, *J* = 7.3 Hz, 0.5H), 3.88 (dt, *J* = 7.0, 5.8 Hz, 1H), 4.06 – 3.99 (m, 1H), 4.24 (dd, *J* = 11.9, 6.4 Hz, 1H), 4.64 – 4.61 (m, 1H), 5.04 – 4.93 (m, 1H), 5.16 – 5.09 (m, 1H), 5.36 (t, *J* = 6.9 Hz, 1H), 5.44 – 5.39 (m, 0.5H).

(2E,6E,10E)-12-Azido-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (3.5): 2-(((2E,6E,10E)-12-azido-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)oxy)tetrahydro-2H-pyran was dissolved in absolute EtOH and pyridinium p-toluenesulfonate was added. The reaction was stirred at 60 ^{o}C for 6 h, after which time the solvent was evaporated under reduced pressure and the mixture was purified by flash column chromatography (Hexanes/EtOAc 4:1), affording the desired product as an off yellow oil (86% yield). ^{1}H NMR spectrum is in agreement with published spectra of the title compound.[6] ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.63 – 1.59 (m, 3H), 1.69 (s, 6H), 1.80 – 1.73 (m, 2H), 2.18 – 2.01 (m, 8H), 3.69 – 3.63 (m, 1H), 3.79 (ddd, *J* = 13.0, 7.0, 4.3 Hz, 1H), 4.16 (d, *J* = 6.7 Hz, 2H), 5.01 – 4.93 (m, 1H), 5.17 – 5.10 (m, 1H), 5.49 – 5.39 (m, 2H).

(2E,6E,10E)-1-Azido-12-bromo-2,6,10-trimethyldodeca-2,6,10-triene (3.6): (2E,6E,10E)-12-azido-3,7,11-trimethyldodeca-2,6,10-trien-1-ol and carbon tetrabromide were dissolved in anhydrous Et_2O and a solution of triphenyl phosphine in 2 mL of dry Et_2O was added dropwise and under Ar. The mixture was stirred for 2.5 h at RT. The mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was dissolved in hexane and the mixture was filtered again. The filtrate, was evaporated and used immediately in the next reaction (43% yield).

Ethyl (*tert*-butoxycarbonyl)cysteinate (3.7): L-cysteine methyl ester hydrochloride was dissolved in anhydrous THF at a temperature of 0 ^{o}C and treated with Et_3N under argon. Di-Boc was dissolved in anhydrous THF and added dropwise to the stirring solution of cysteine. The resulting mixture was stirred overnight at room temperature. After addition of water and extraction

with EtOAc, the combined organic phases were washed with brine and dried over anhydrous $MgSO_4$. The organic solvent was evaporated under reduced pressure and the resulting crude produce was purified by flash column chromatography (Hex/EtOAc 4:1), affording the desired product as a colorless oil (84% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.27 (t, J = 7.1 Hz, 3H), 1.42 (s, 9H), 3.00 – 2.90 (m, 2H), 4.27 – 4.15 (m, 2H), 4.57 – 4.51 (m, 1H), 5.42 (d, J = 6.0 Hz, 1H).

Ethyl S-((2E,6E,10E)-12-azido-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)-N-(*tert*-butoxycarbonyl)cysteinate (3.1): To a solution of (2E,6E,10E)-1-azido-12-bromo-2,6,10-trimethyldodeca-2,6,10-triene in MeOH, was added aq. NaOH 1N and after 5 min was added ethyl (*tert*-butoxycarbonyl)cysteinate. The mixture was stirred for 1 h at 0 ^{o}C and at RT overnight. The solvent was evaporated, the residue was dried under vacuum and was purified by flash column chromatography (Hexanes/EtOAc 4:1) to afford the desired product as an off white solid (20% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.45 (s, 9H), 1.57 (s, 2H), 1.63 – 1.58 (m, 3H), 1.70 – 1.65 (m, 5H), 1.79 – 1.72 (m, 2H), 2.18 – 1.95 (m, 8H), 2.85 (dd, J = 13.8, 5.7 Hz, 1H), 2.91 (dd, J = 13.6, 4.7 Hz, 1H), 3.14 (dd, J = 13.1, 7.6 Hz, 1H), 3.20 (dd, J = 13.0, 8.0 Hz, 1H), 3.68 – 3.64 (m, 1H), 3.76 (s, 3H), 3.82 – 3.76 (m, 1H), 4.52 (d, J = 6.7 Hz, 1H), 4.97 (dd, J = 18.0, 10.1 Hz, 1H), 5.14 – 5.07 (m, 1H), 5.21 (t, J = 7.8 Hz, 1H), 5.30 (d, J = 6.5 Hz, 1H), 5.45 – 5.37 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.8, 16.1, 16.3, 16.3, 17.8, 17.9, 26.5, 28.5, 30.2, 30.8, 33.8, 33.9, 36.1, 39.4, 39.7, 39.7, 39.9, 40.0, 52.7, 53.4, 59.5, 59.6, 68.2, 68.3, 80.2, 114.7, 119.8, 119.9, 120.0, 120.1, 124.5, 125.0, 125.2, 125.7, 129.7, 130.1, 130.4, 160.6, 134.2, 134.3, 134.8, 139.9, 140.1, 142.5, 155.5, 171.8. HRMS calculated for $C_{24}H_{40}N_4NaO_4S$ *m/z* 503.2664 [M + Na^+], found 503.2662.

3.5 References

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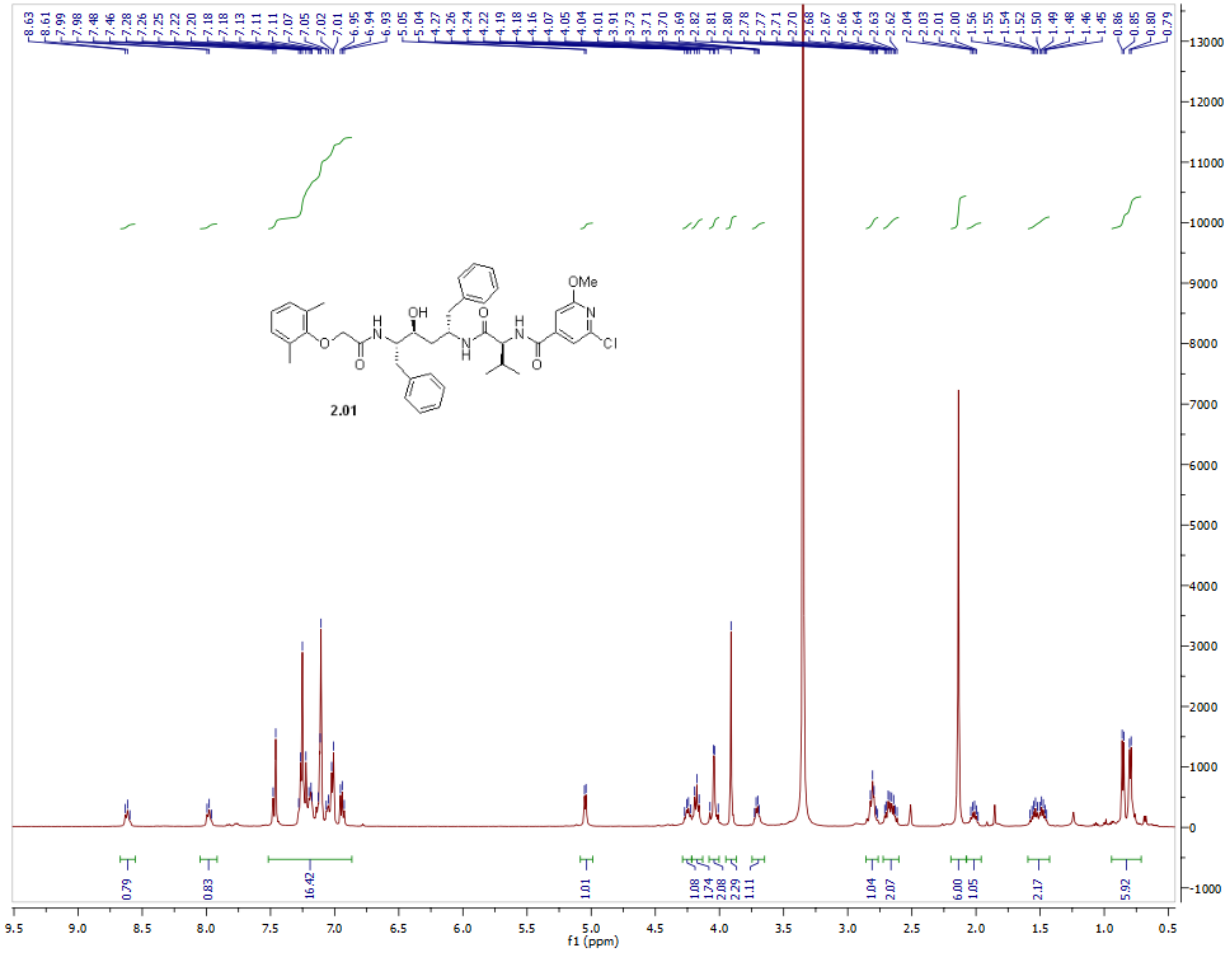
Appendix I

NMR spectra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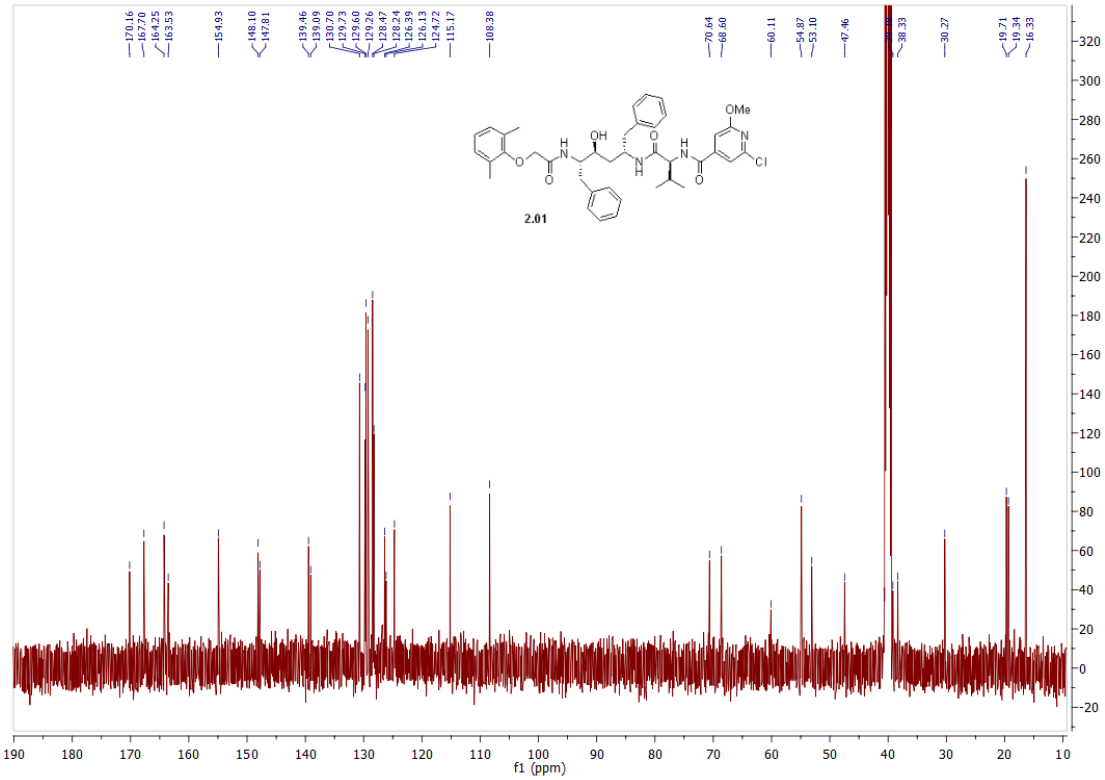
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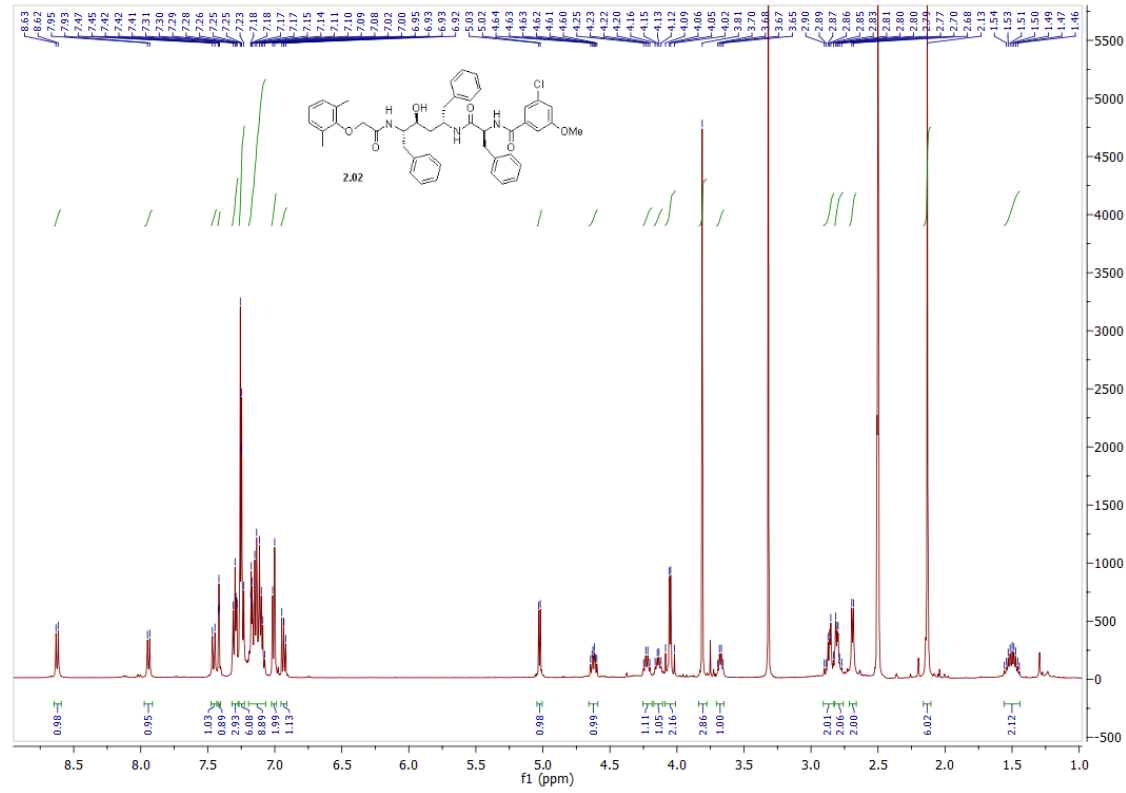
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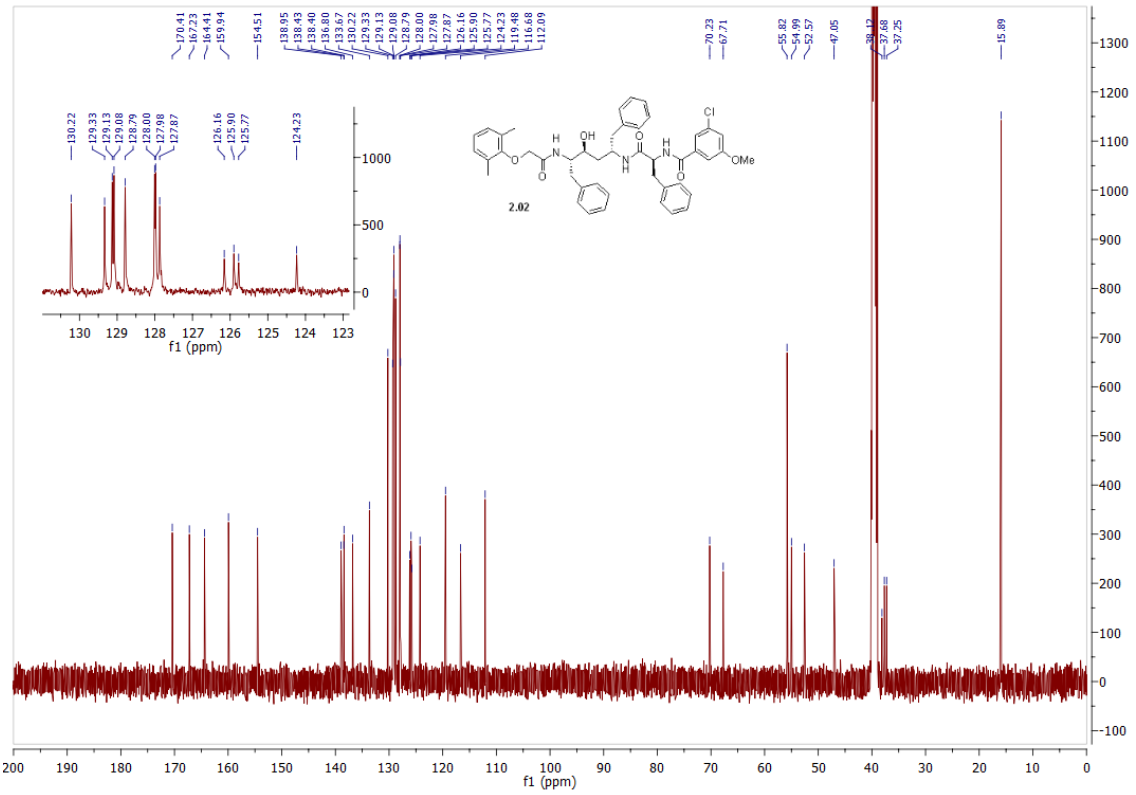
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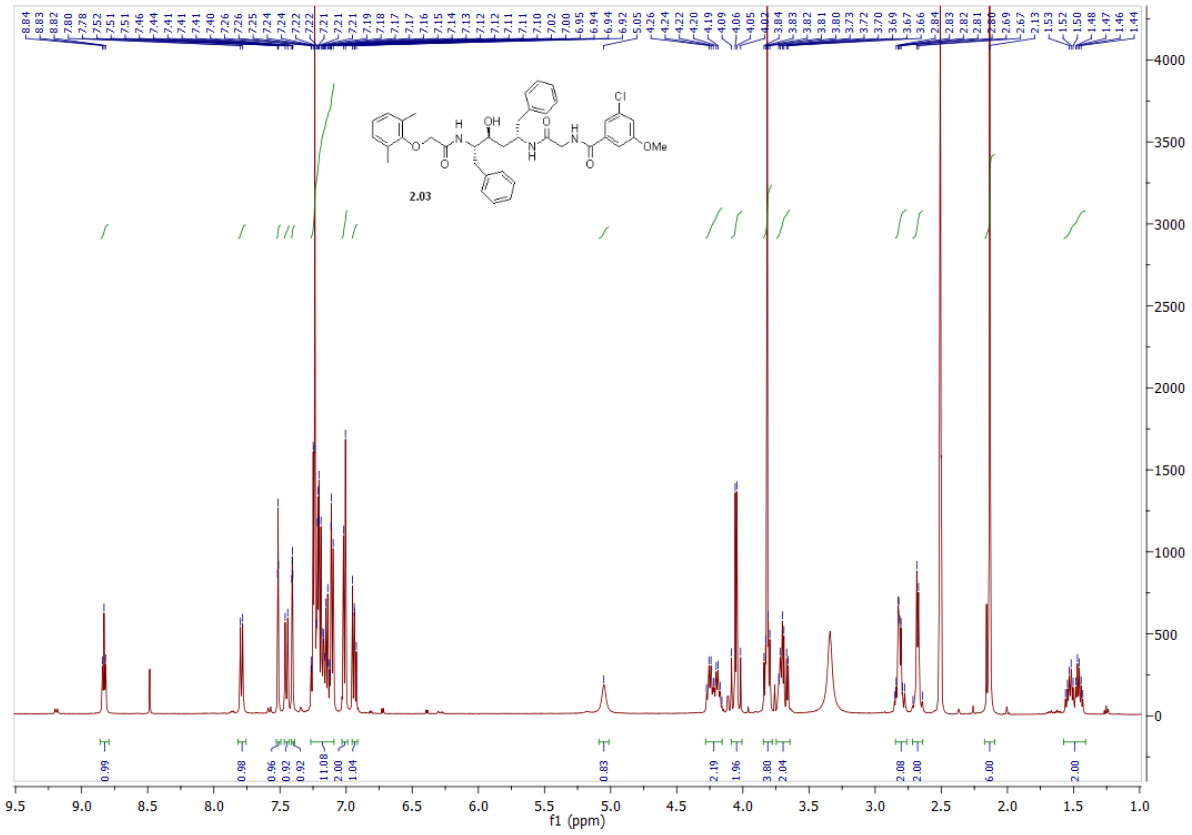
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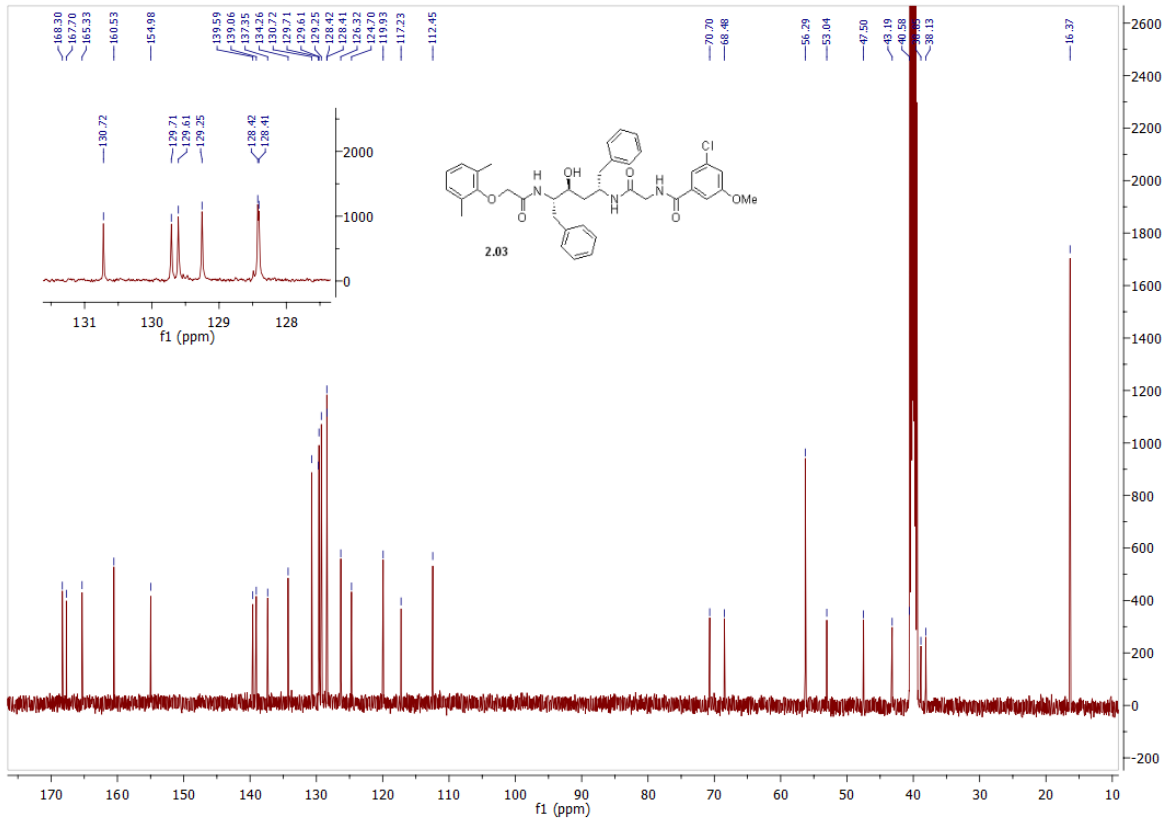
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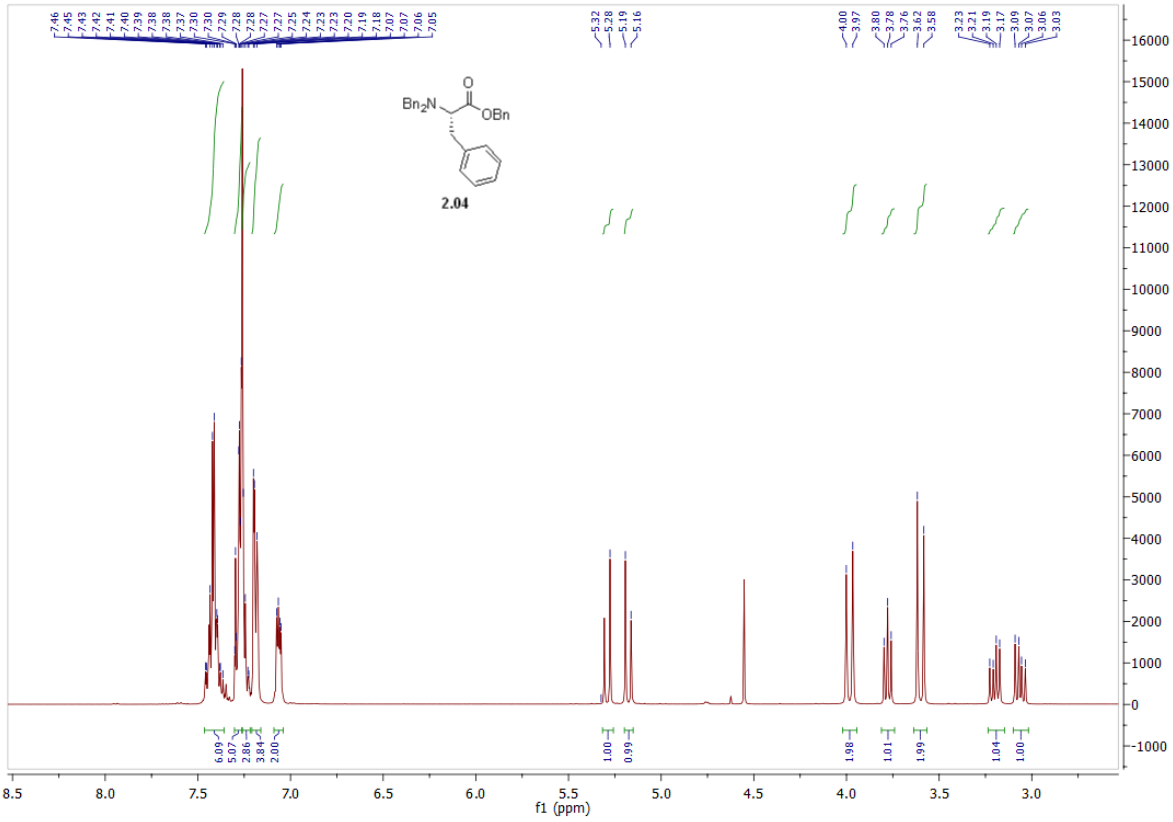
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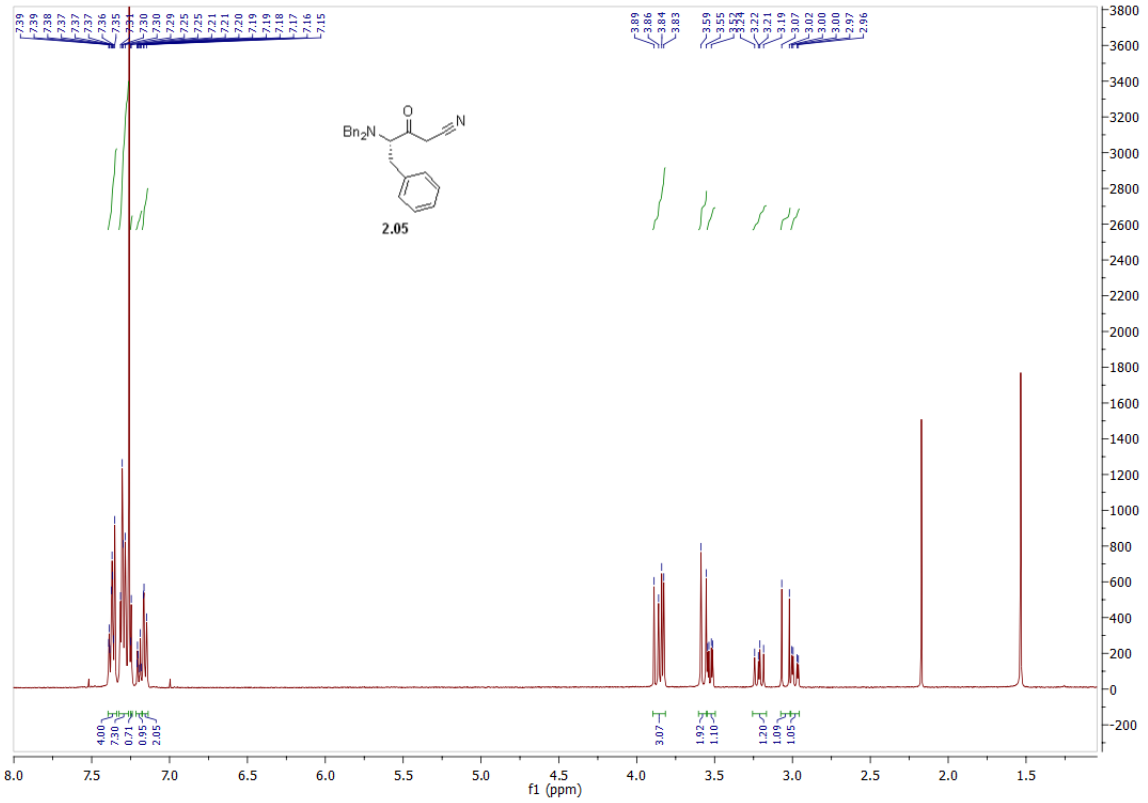
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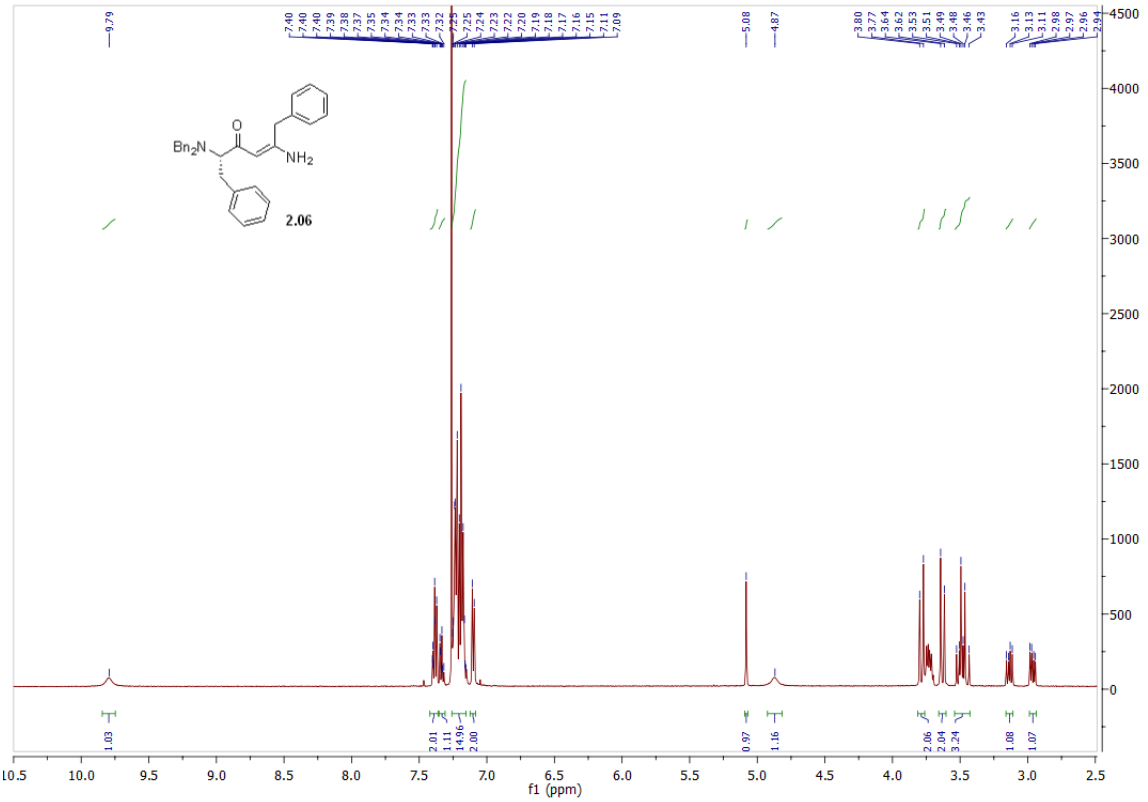
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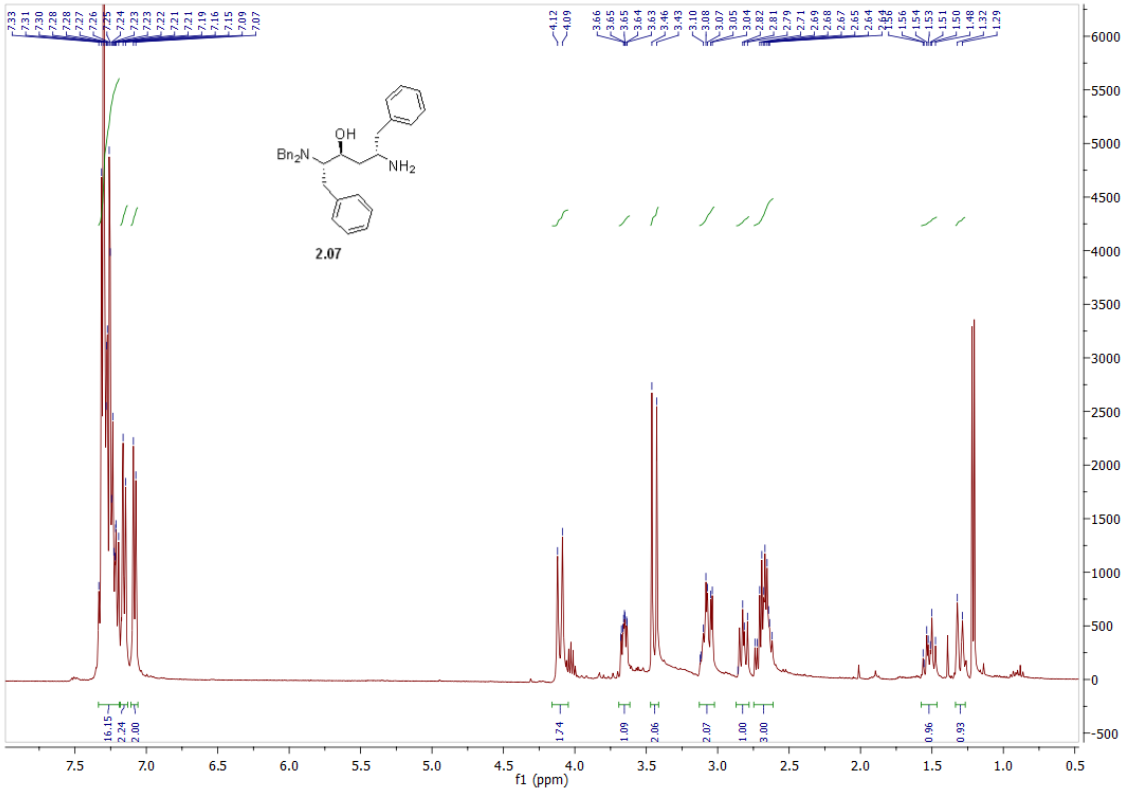
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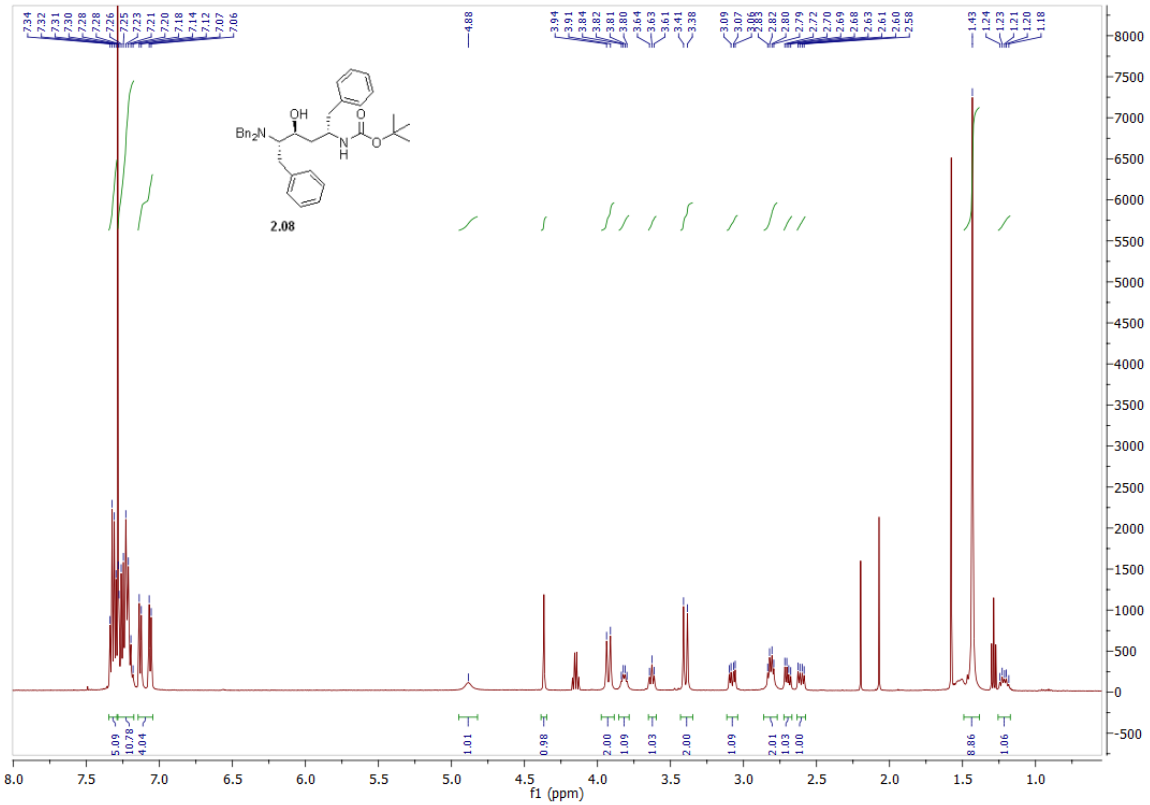
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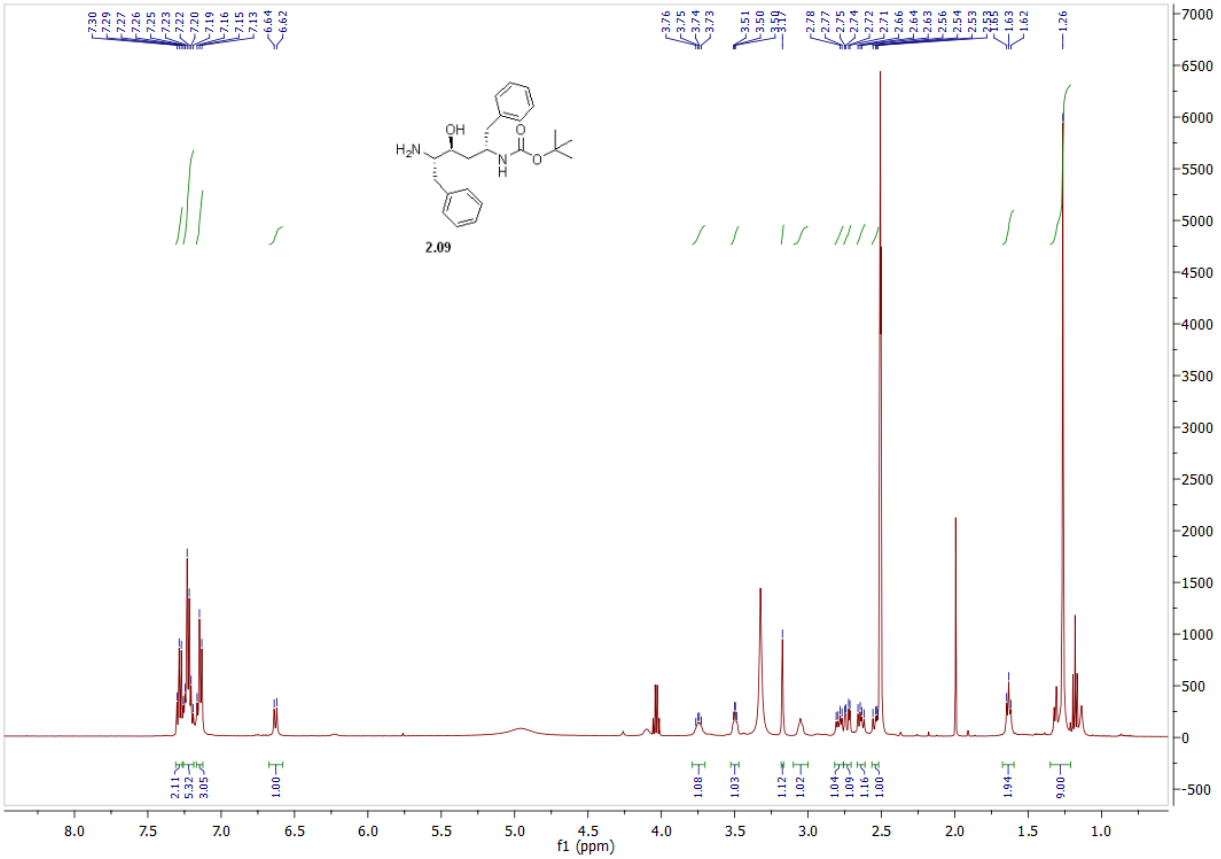
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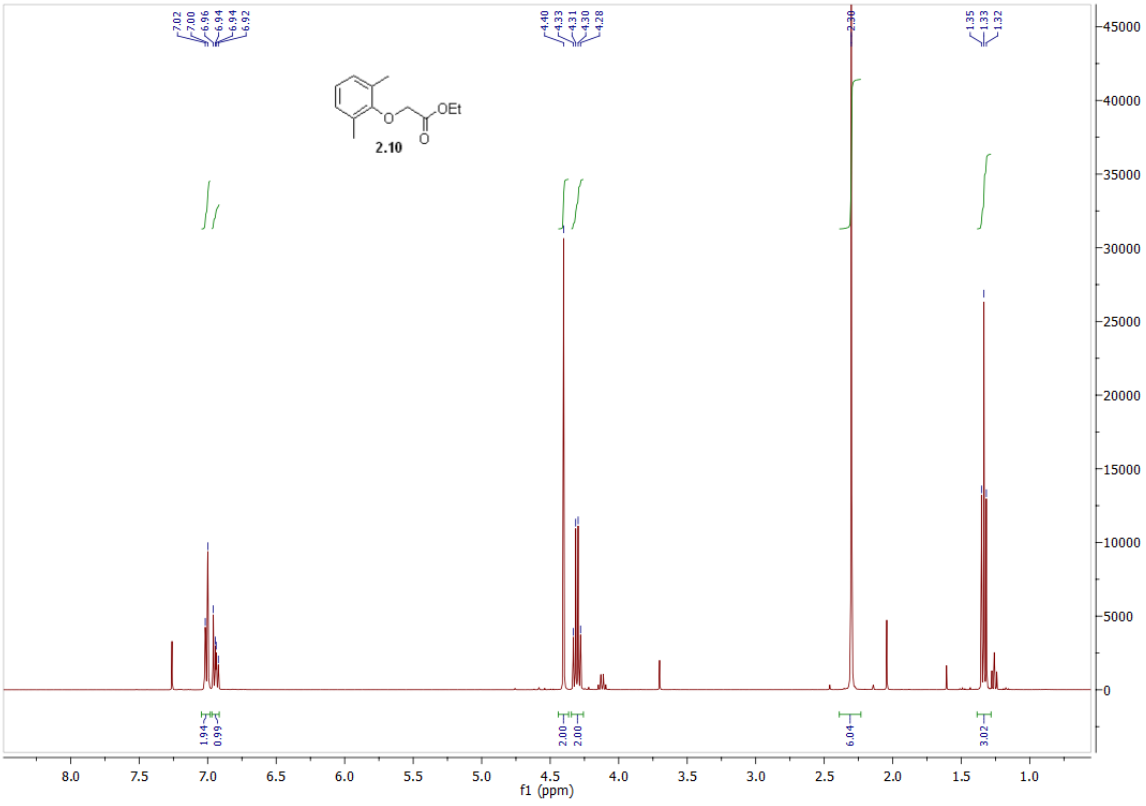
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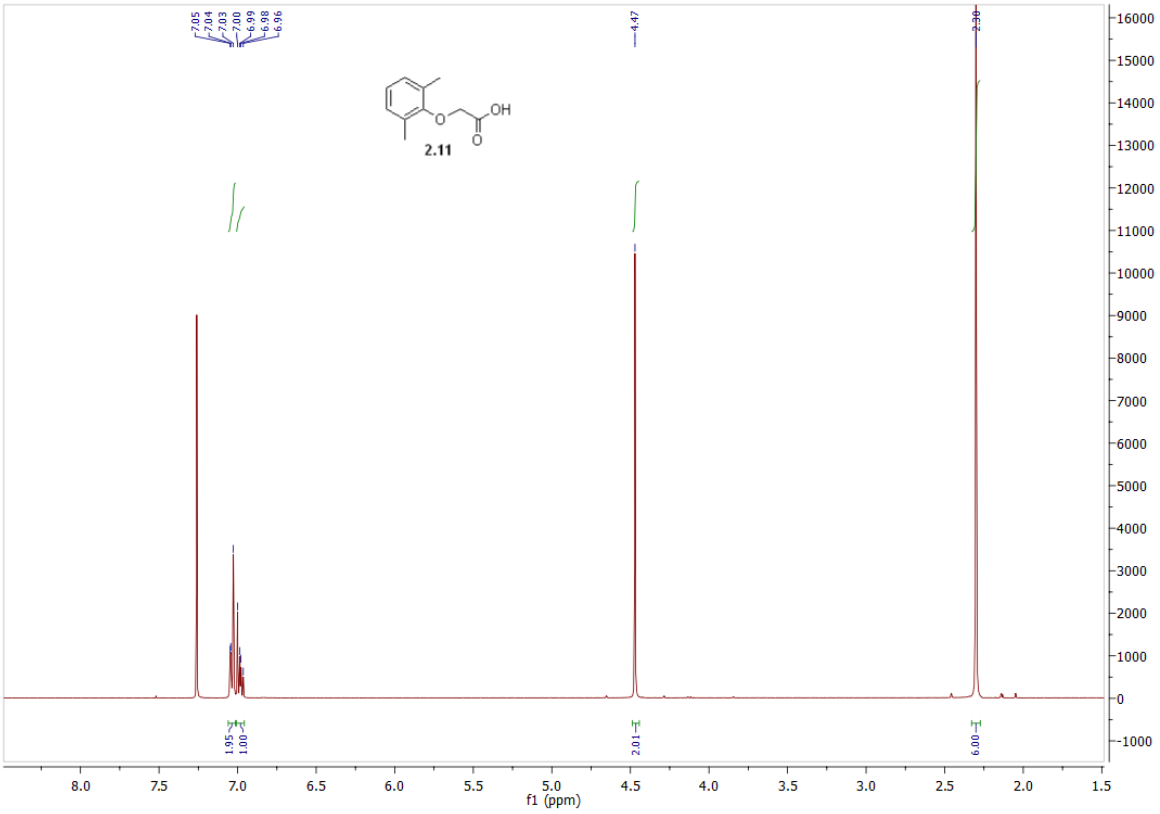
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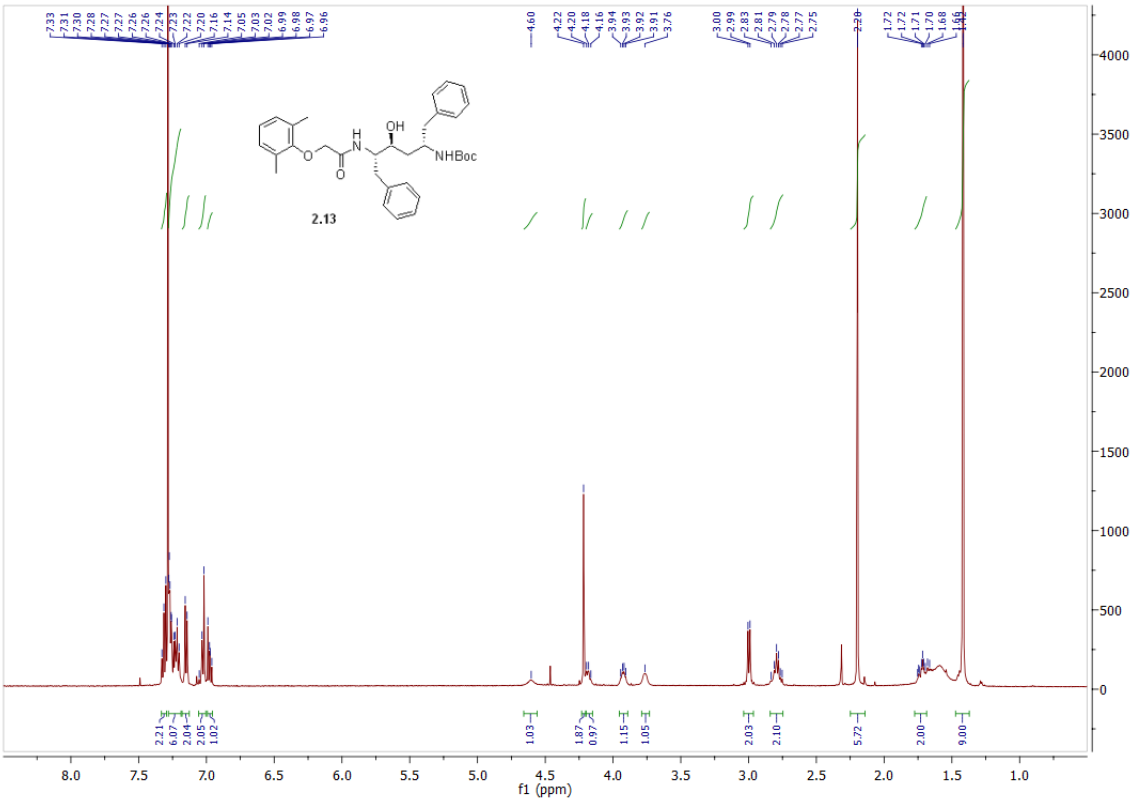
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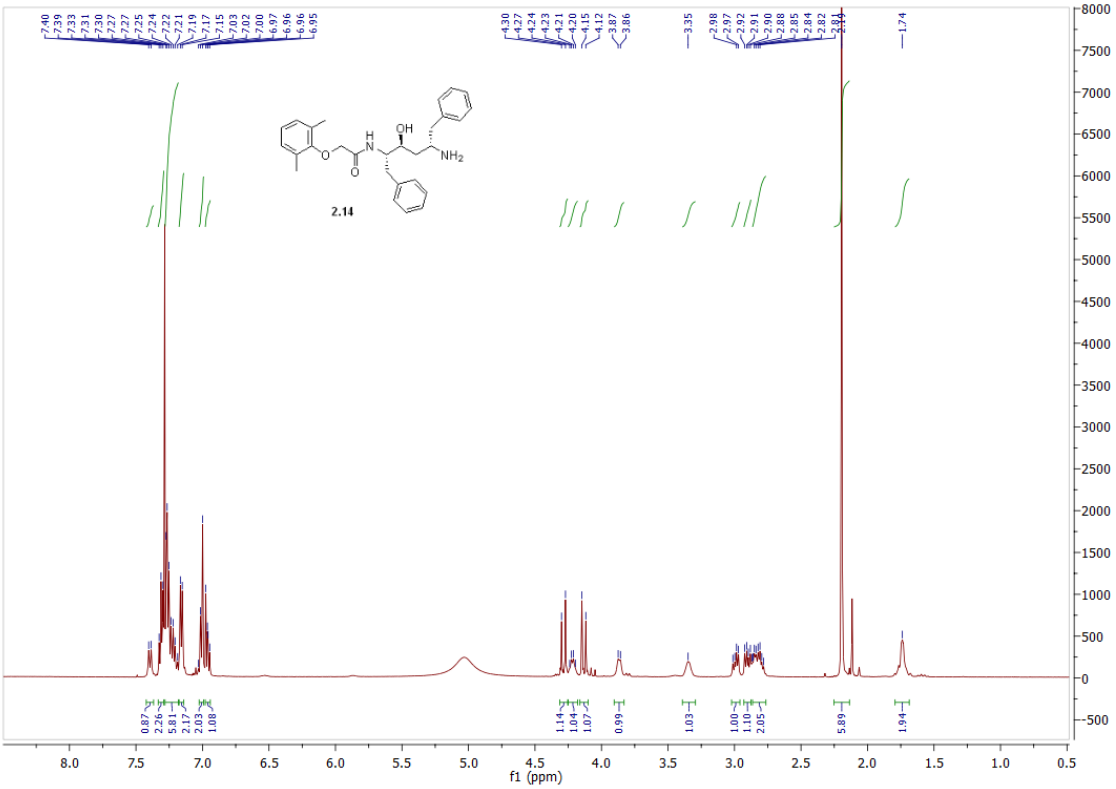
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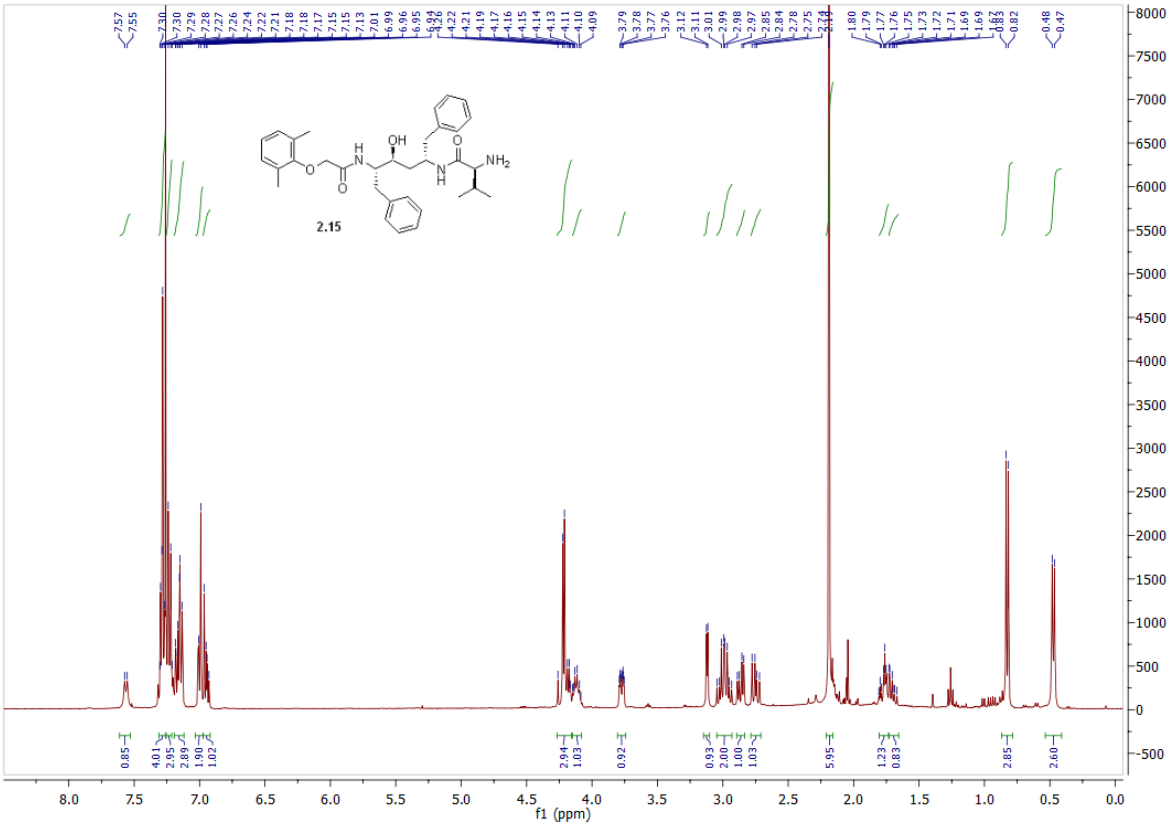
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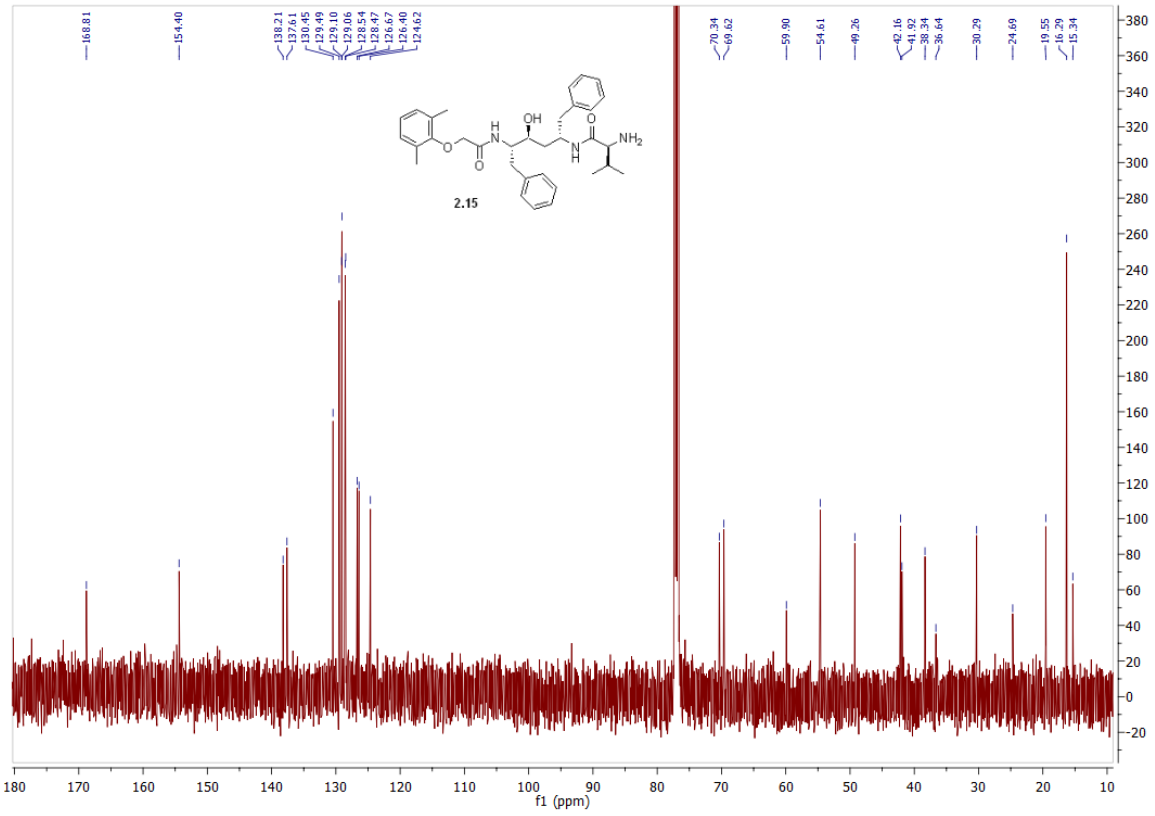
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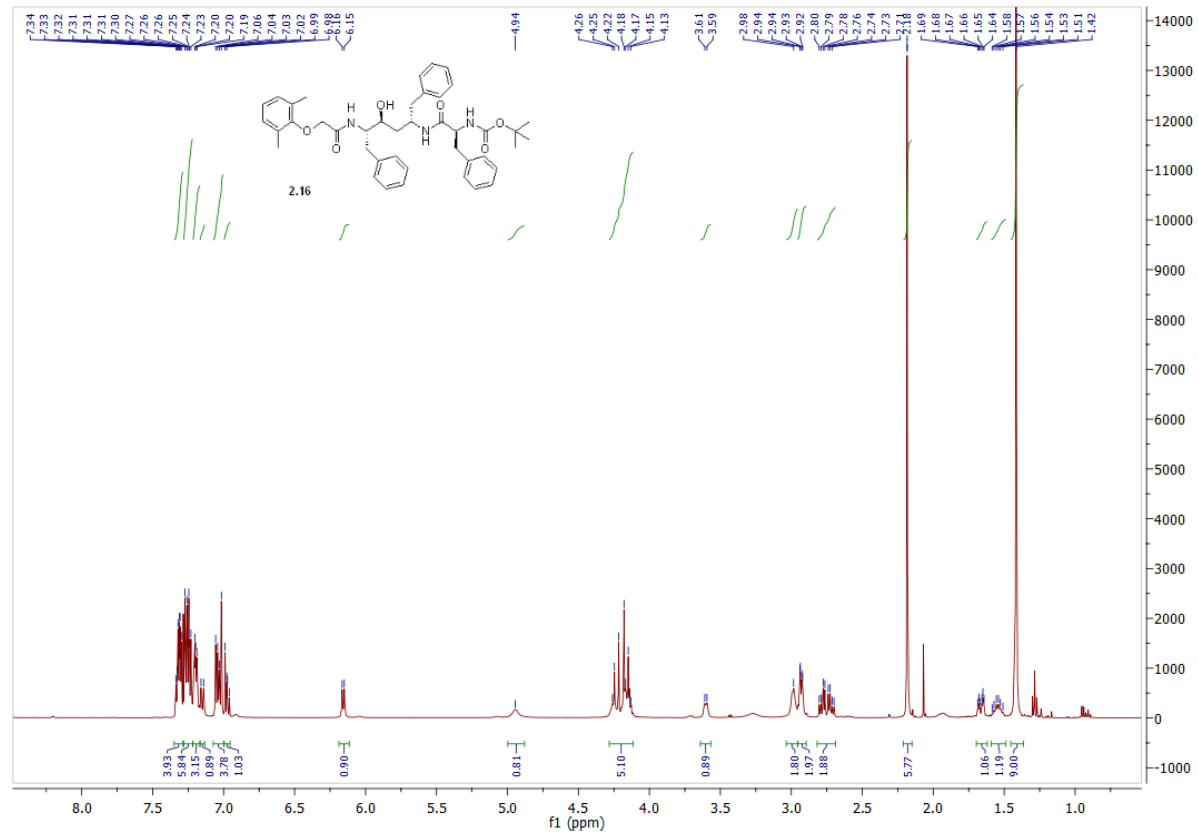
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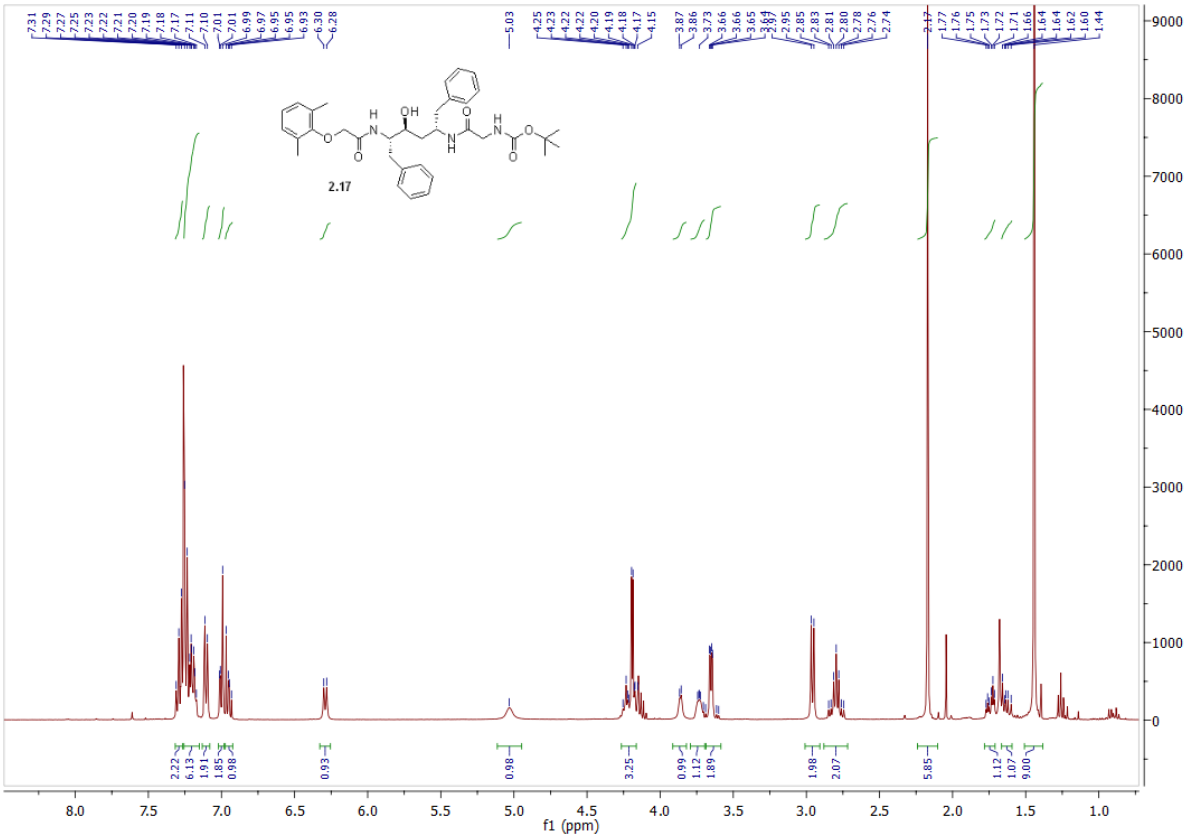
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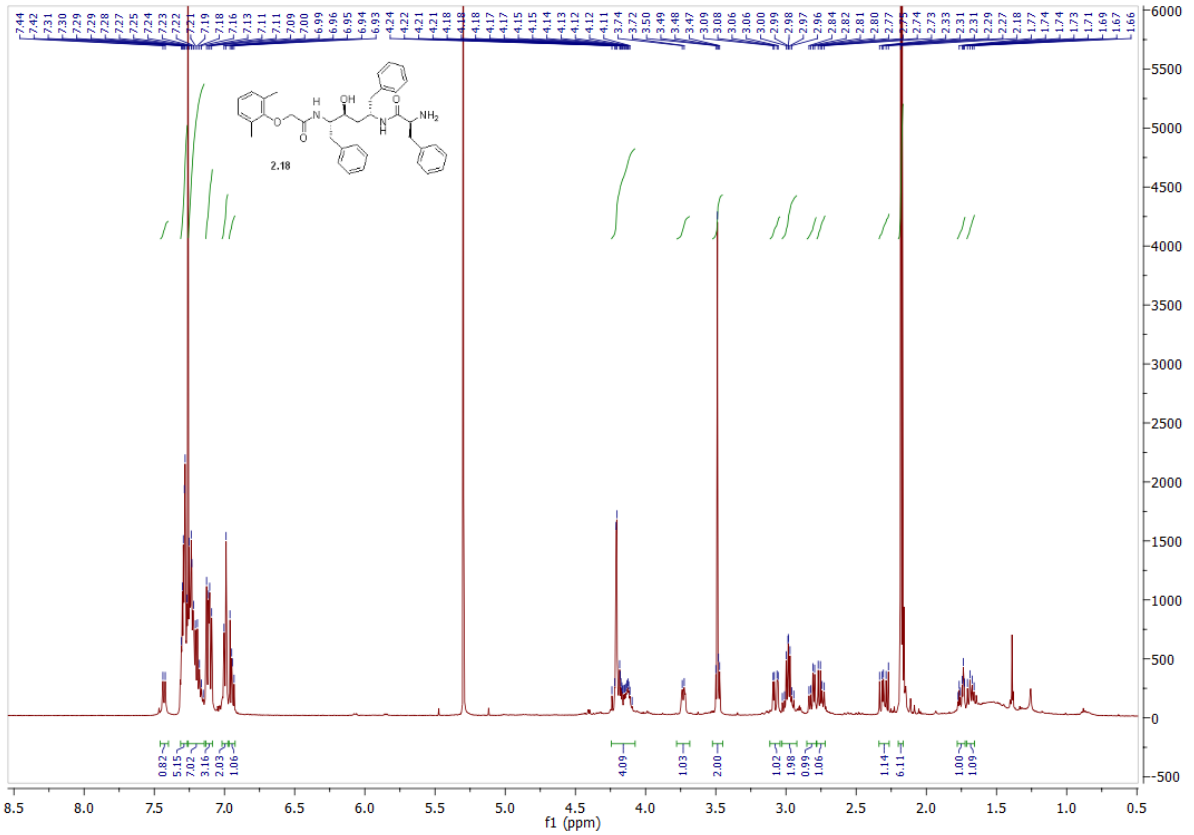
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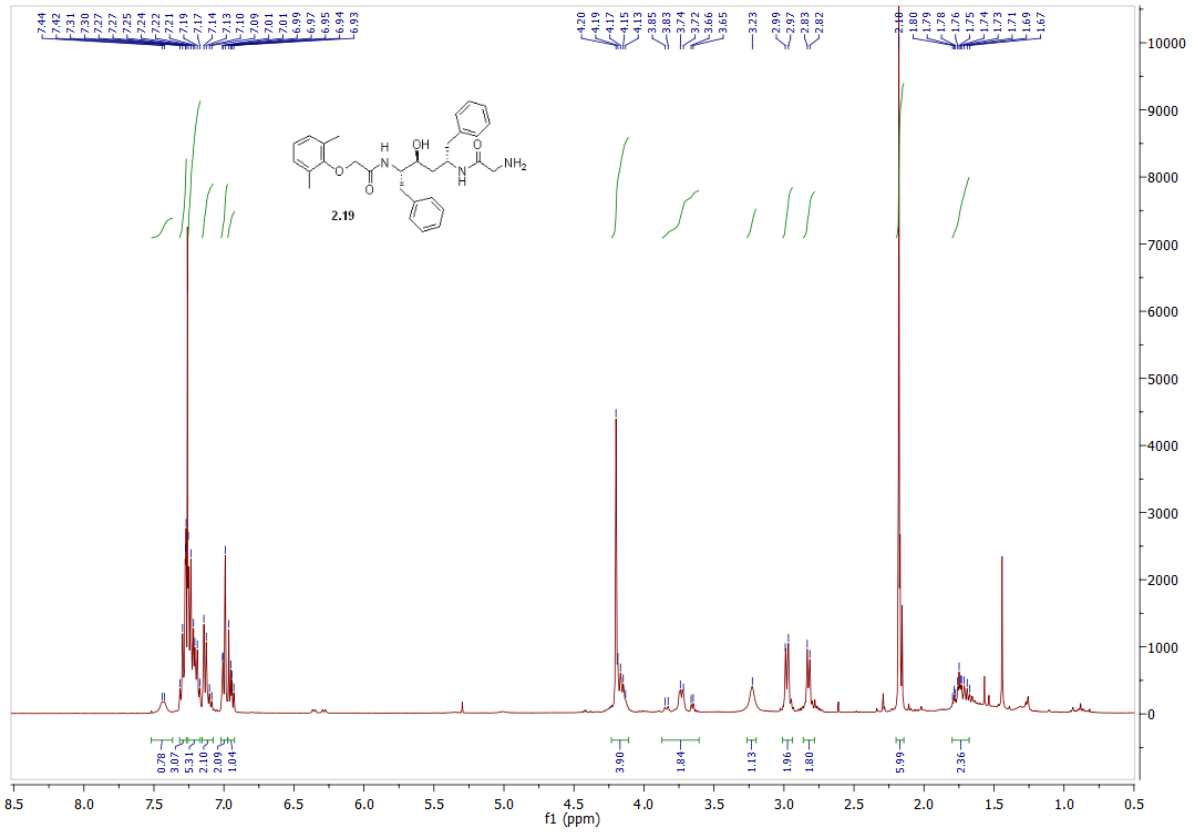
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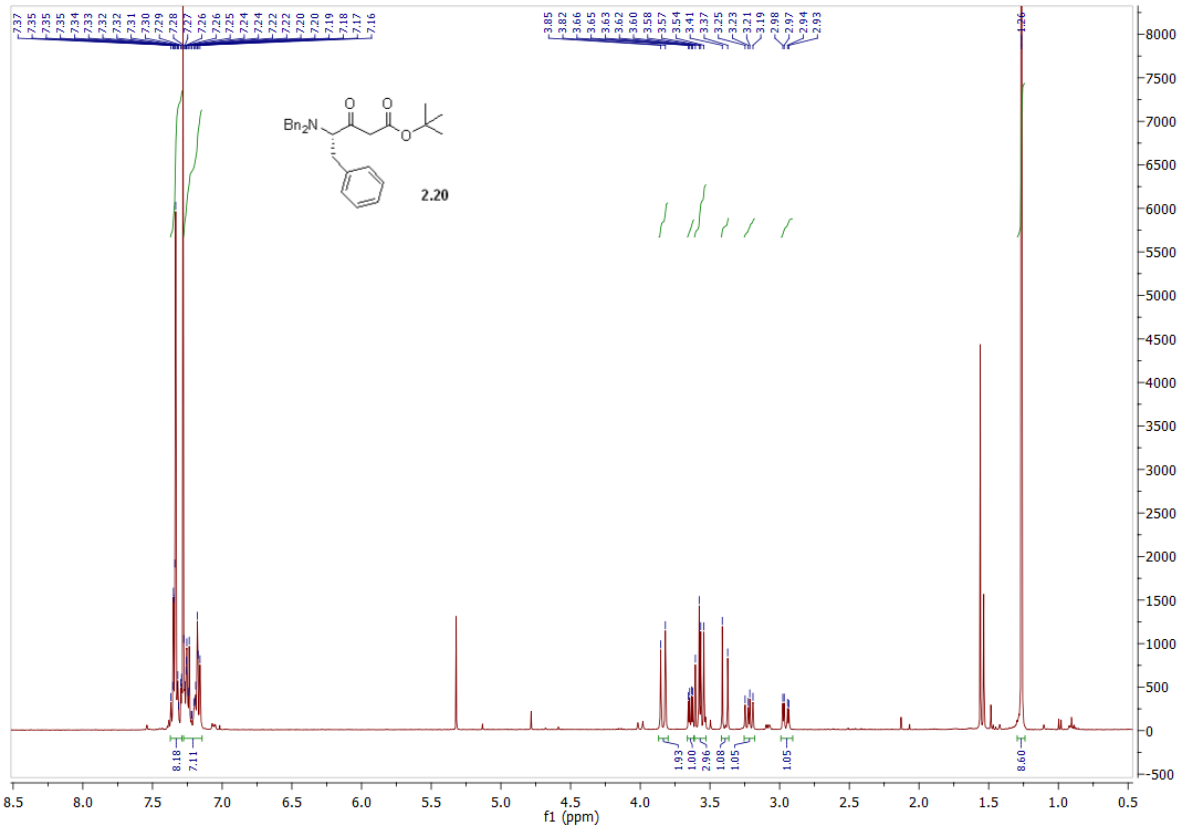
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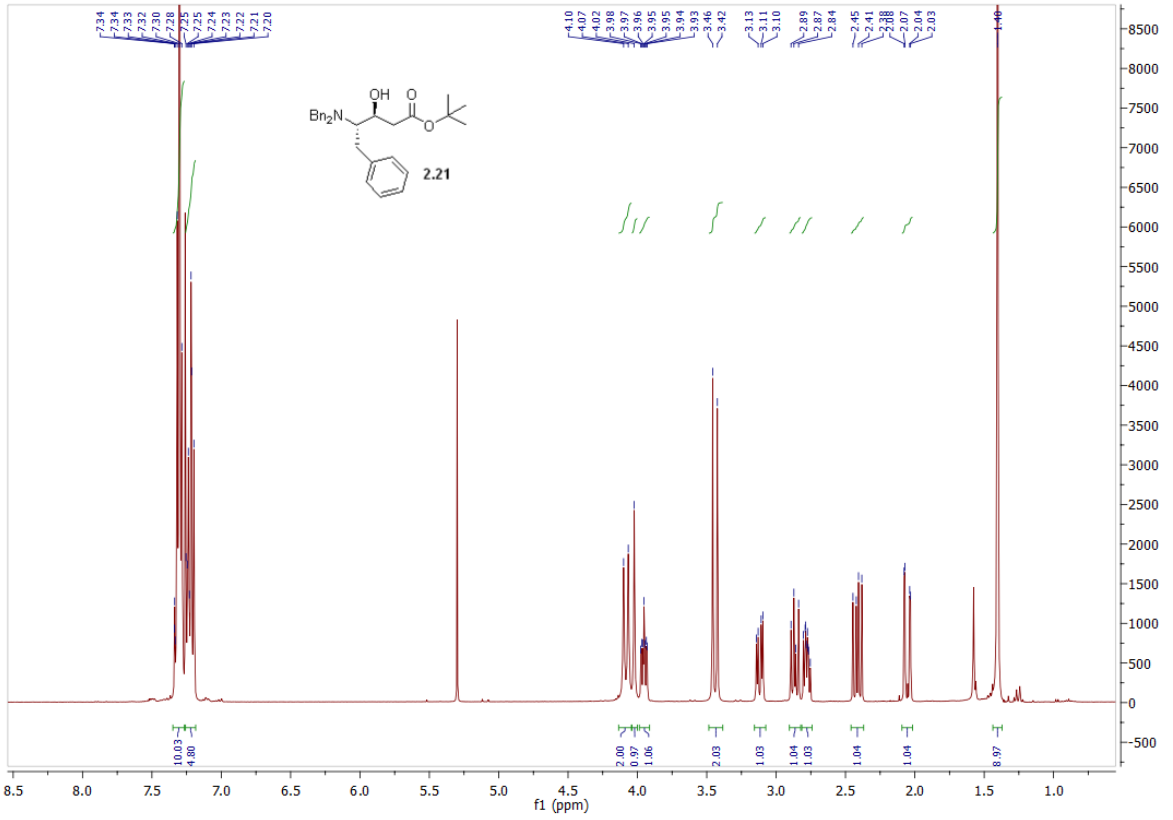
f1 (ppm)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O
Bn_2N
O
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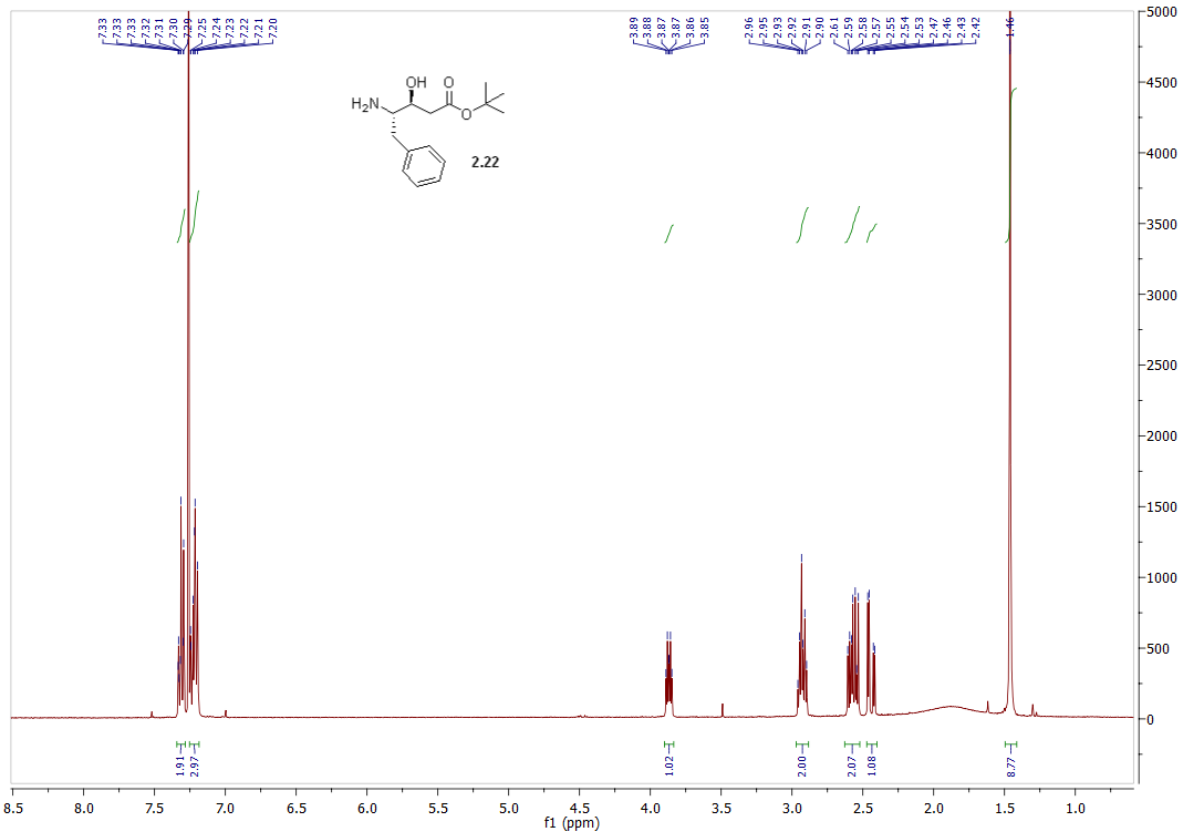
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O
H_2N
O
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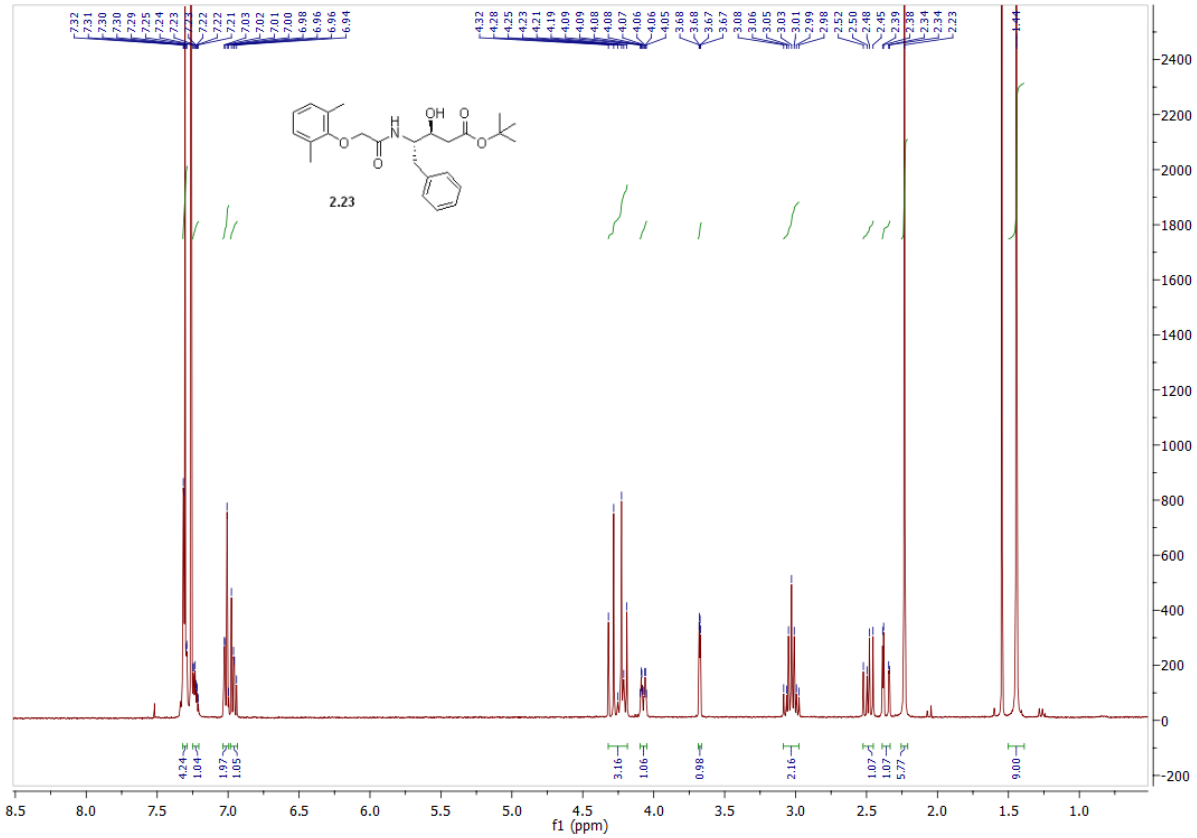
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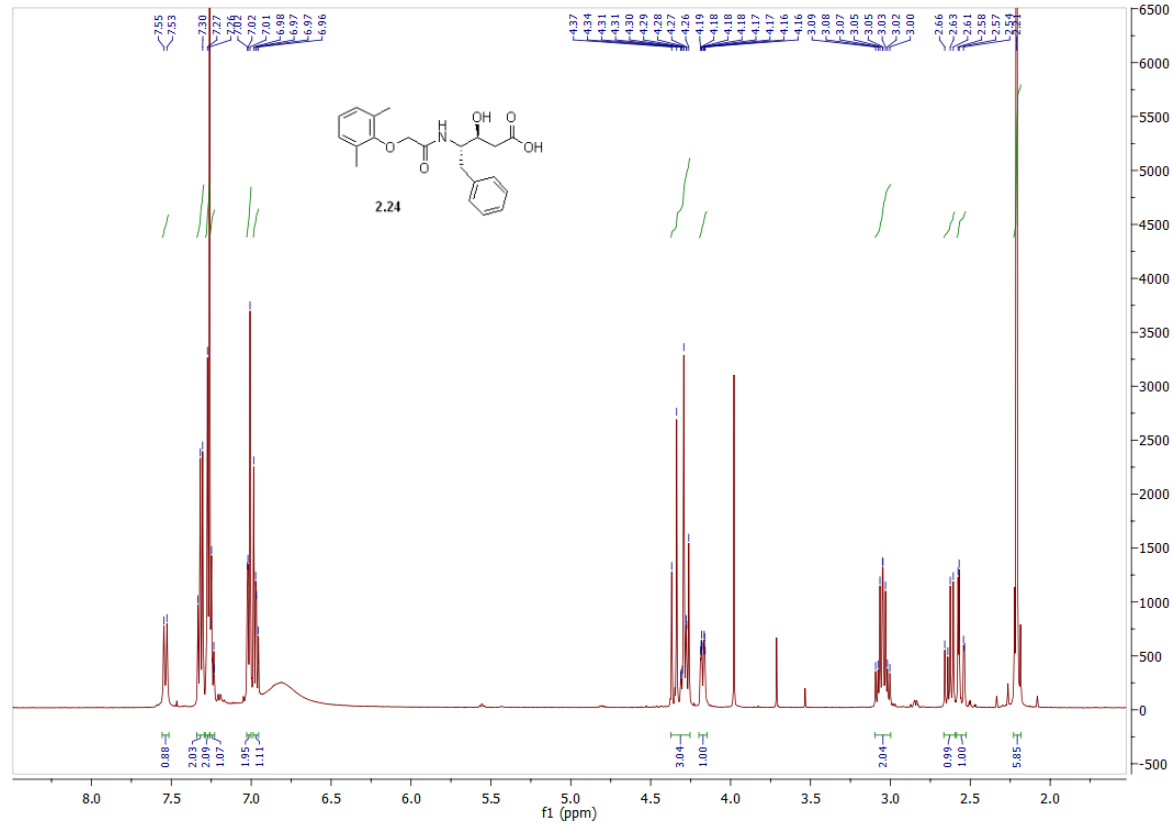
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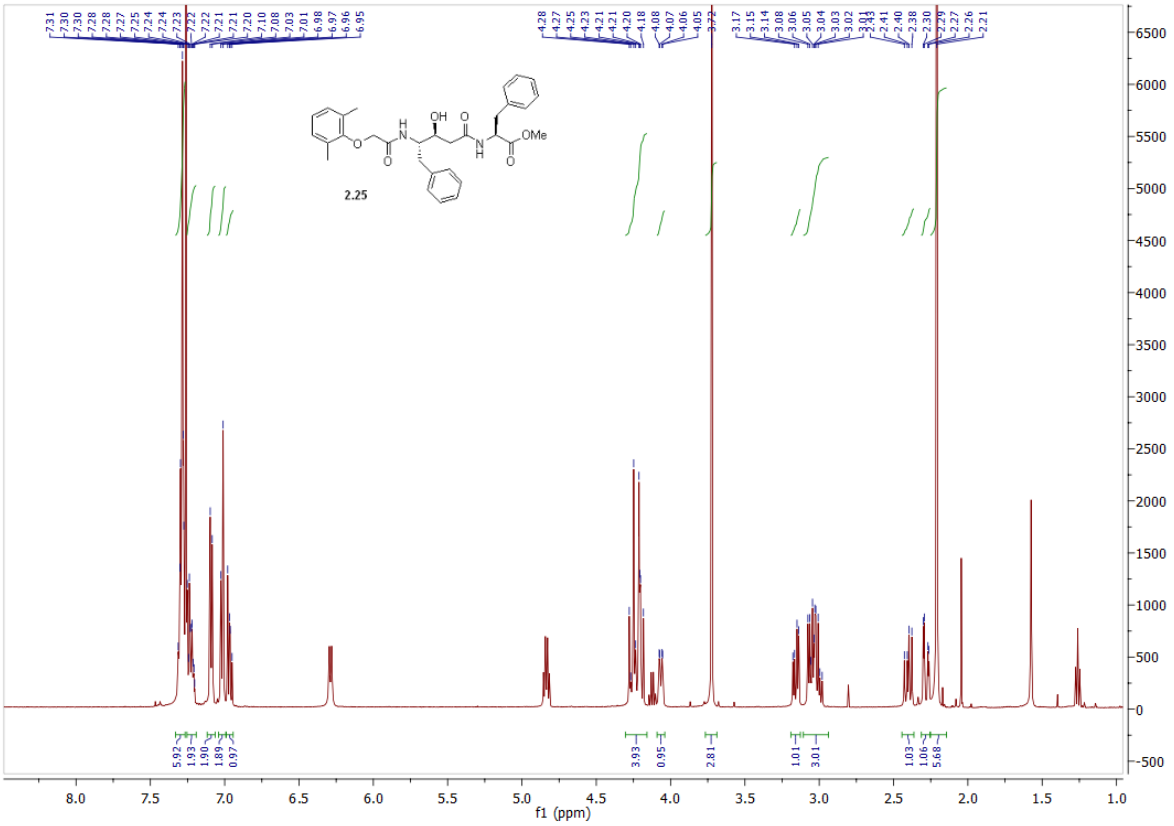
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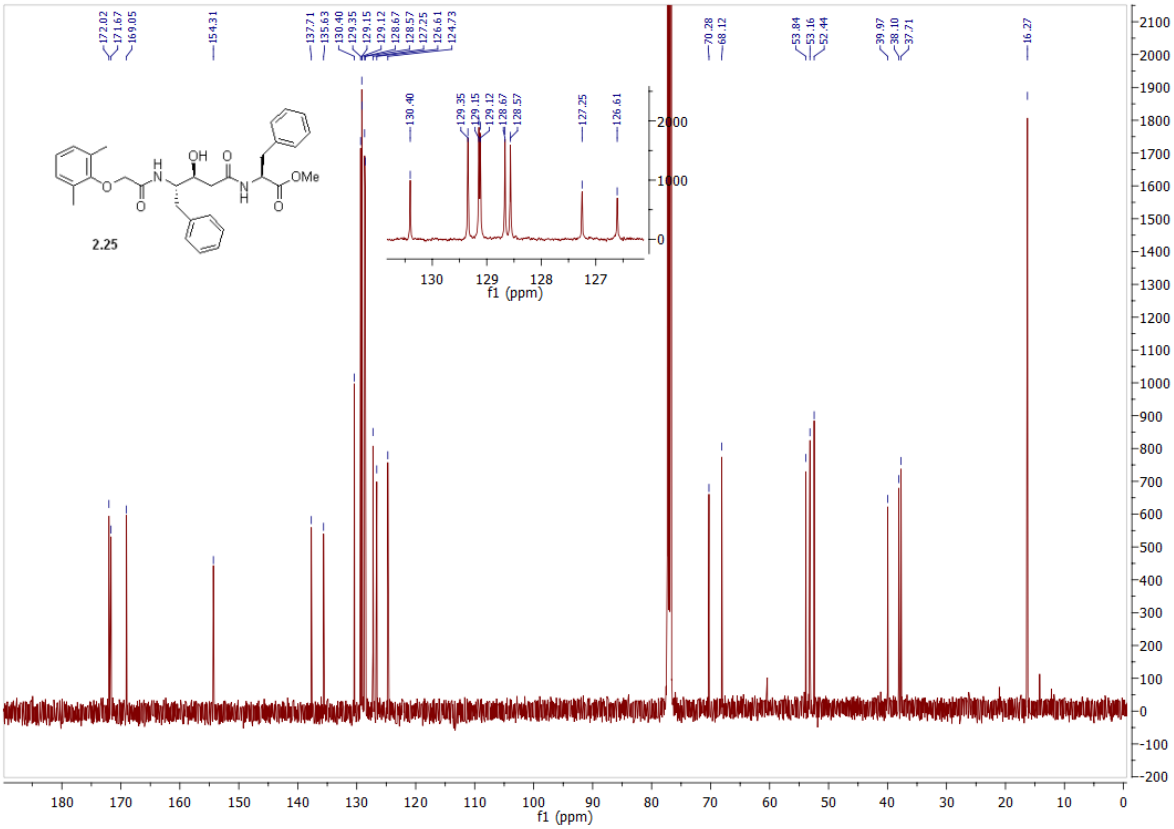
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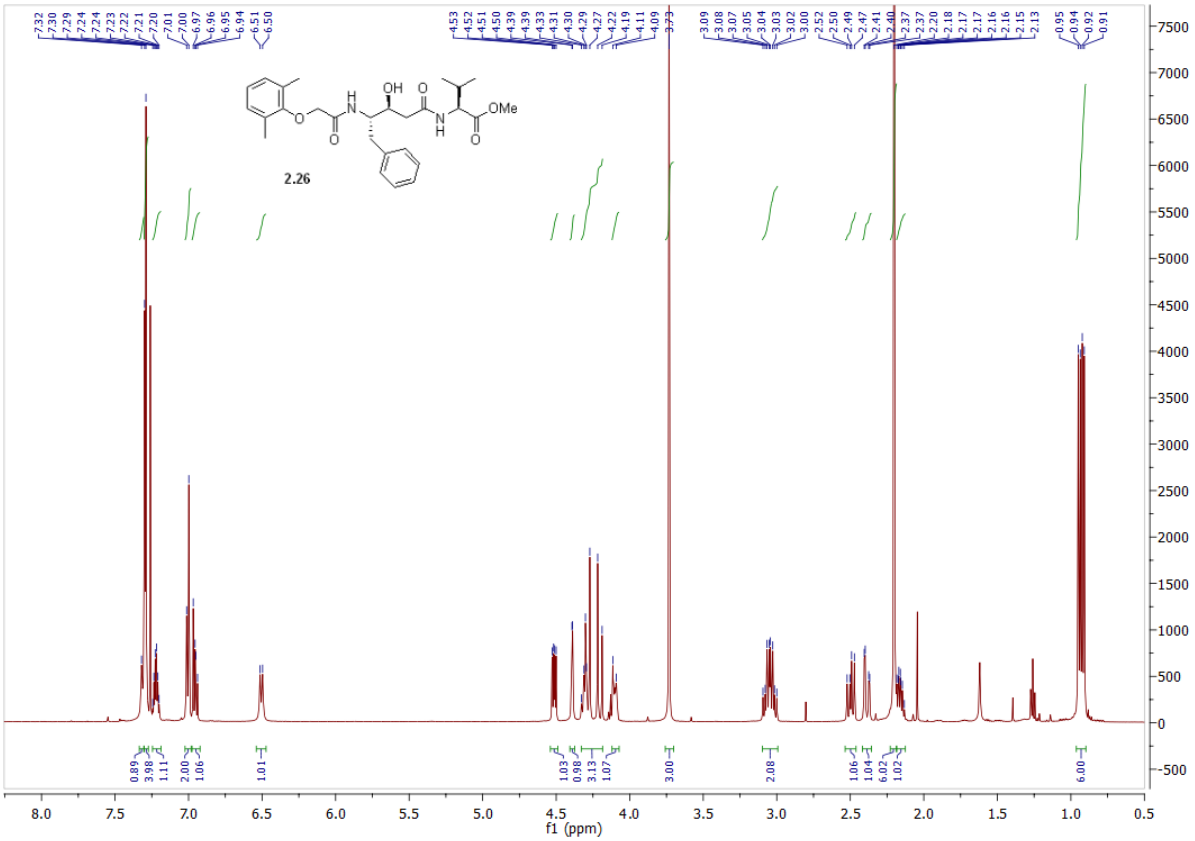
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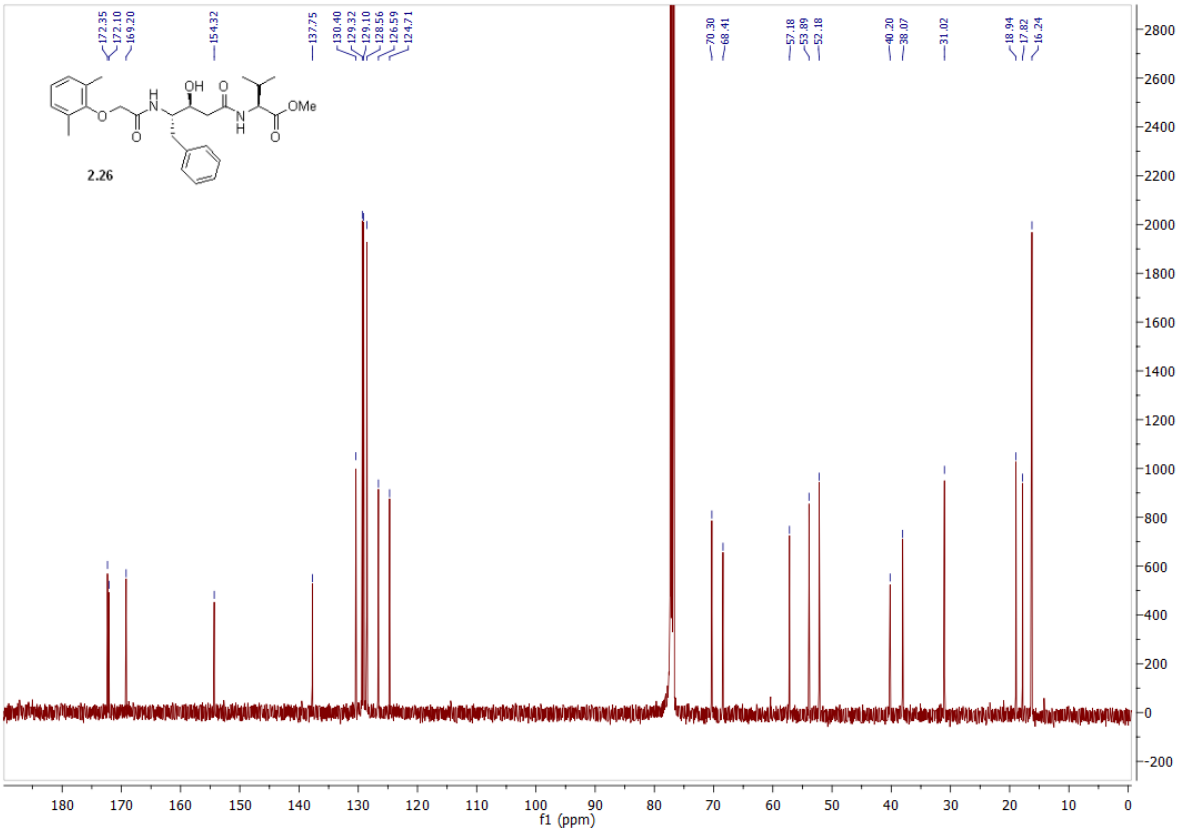
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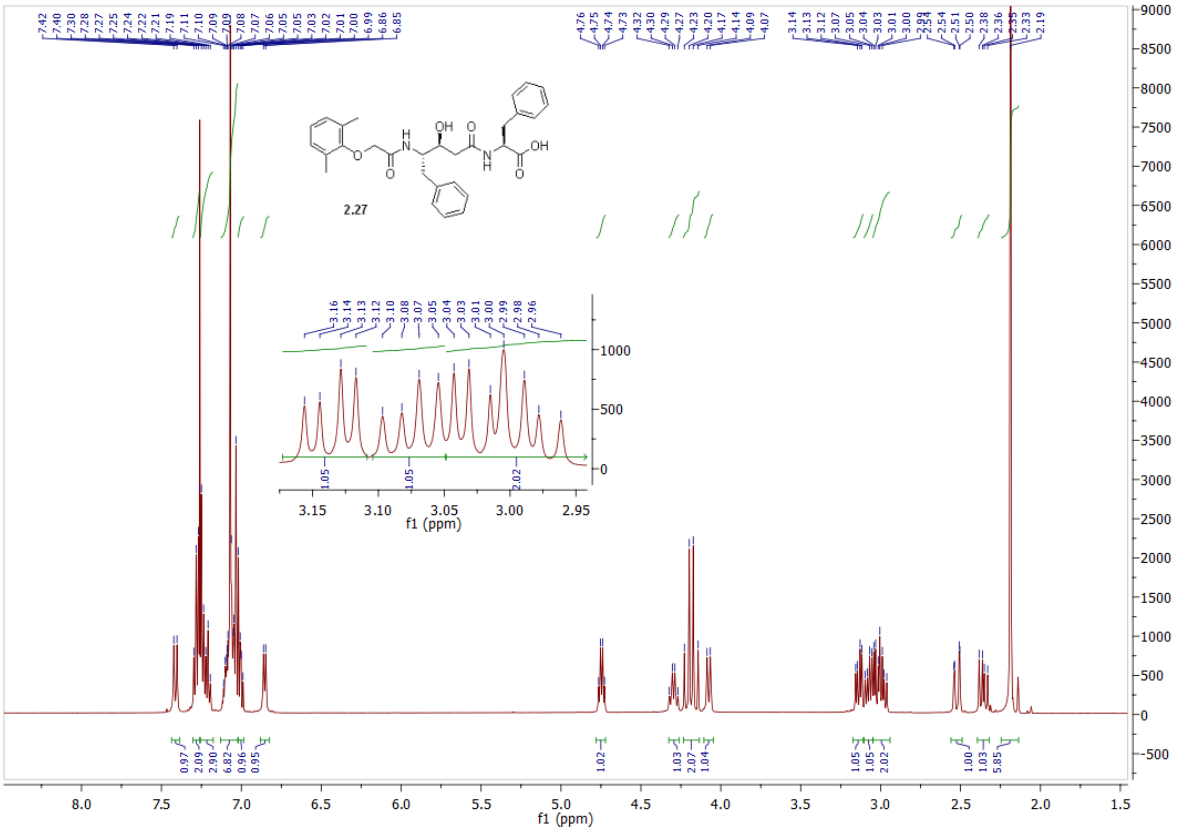
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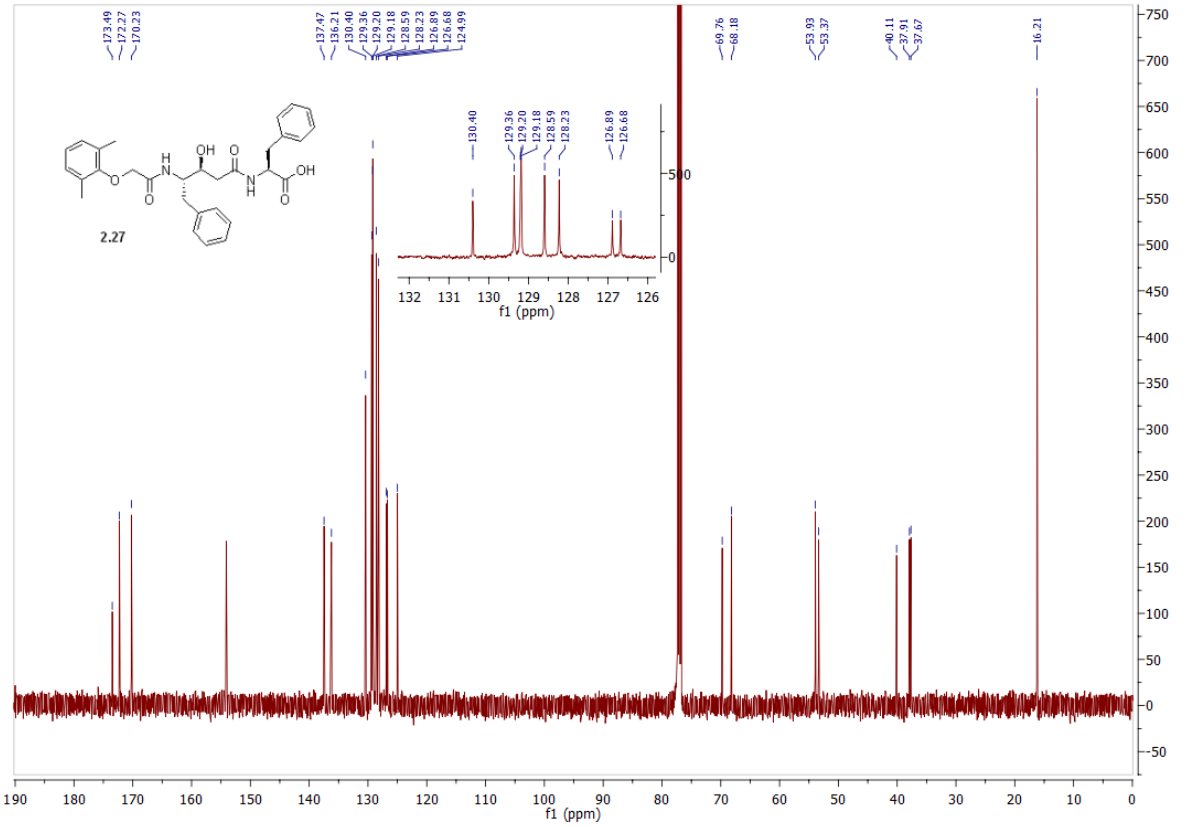
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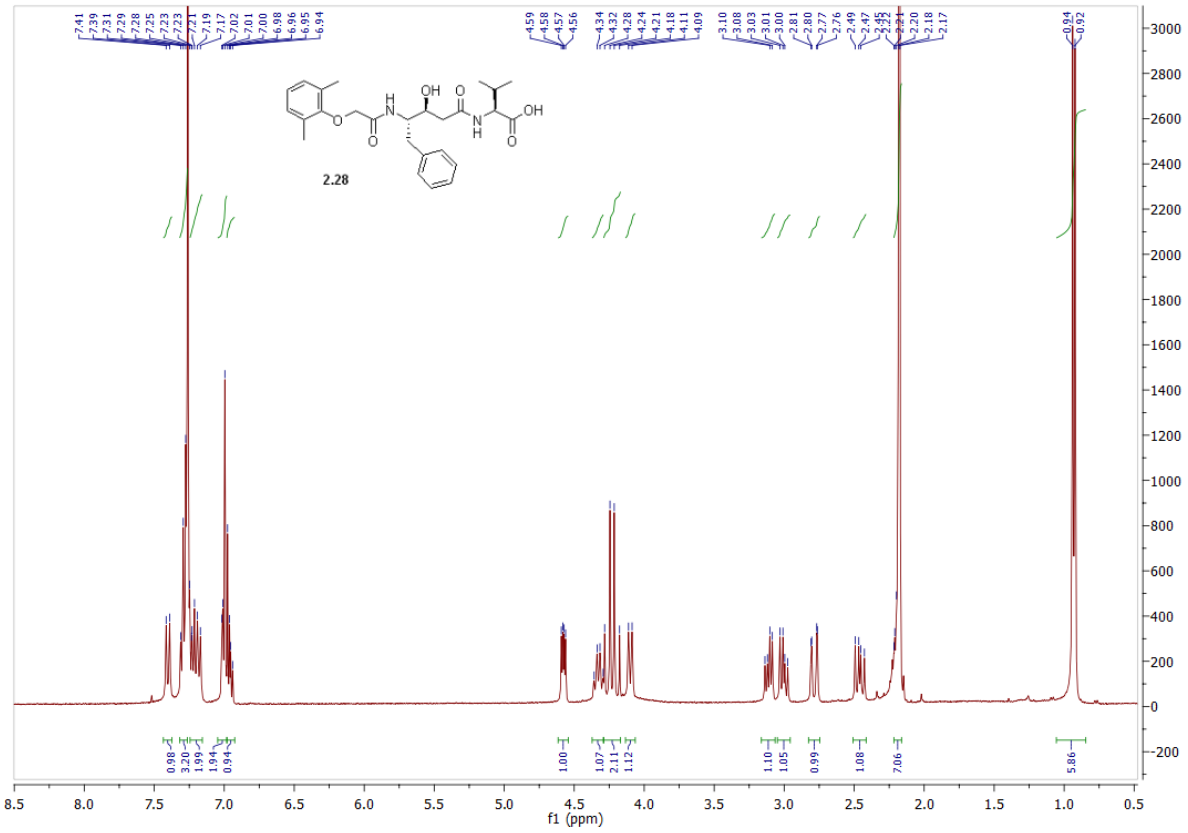
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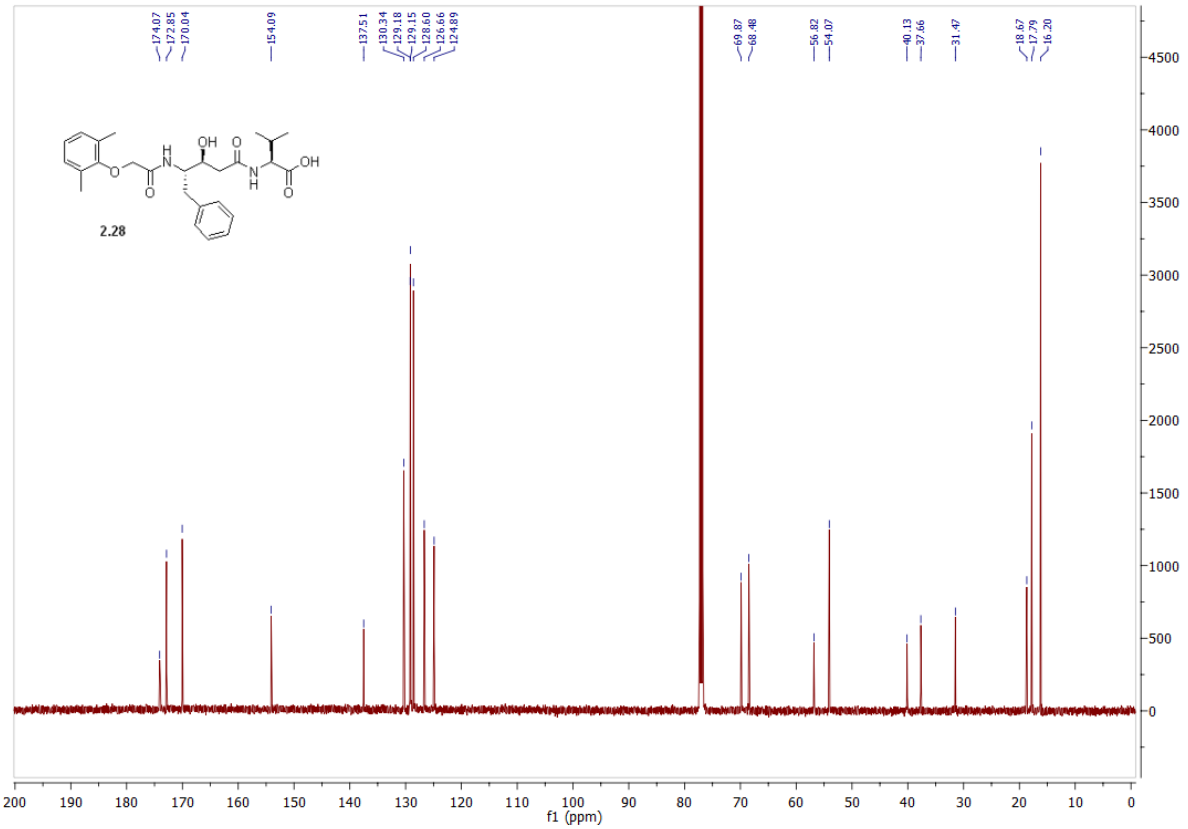
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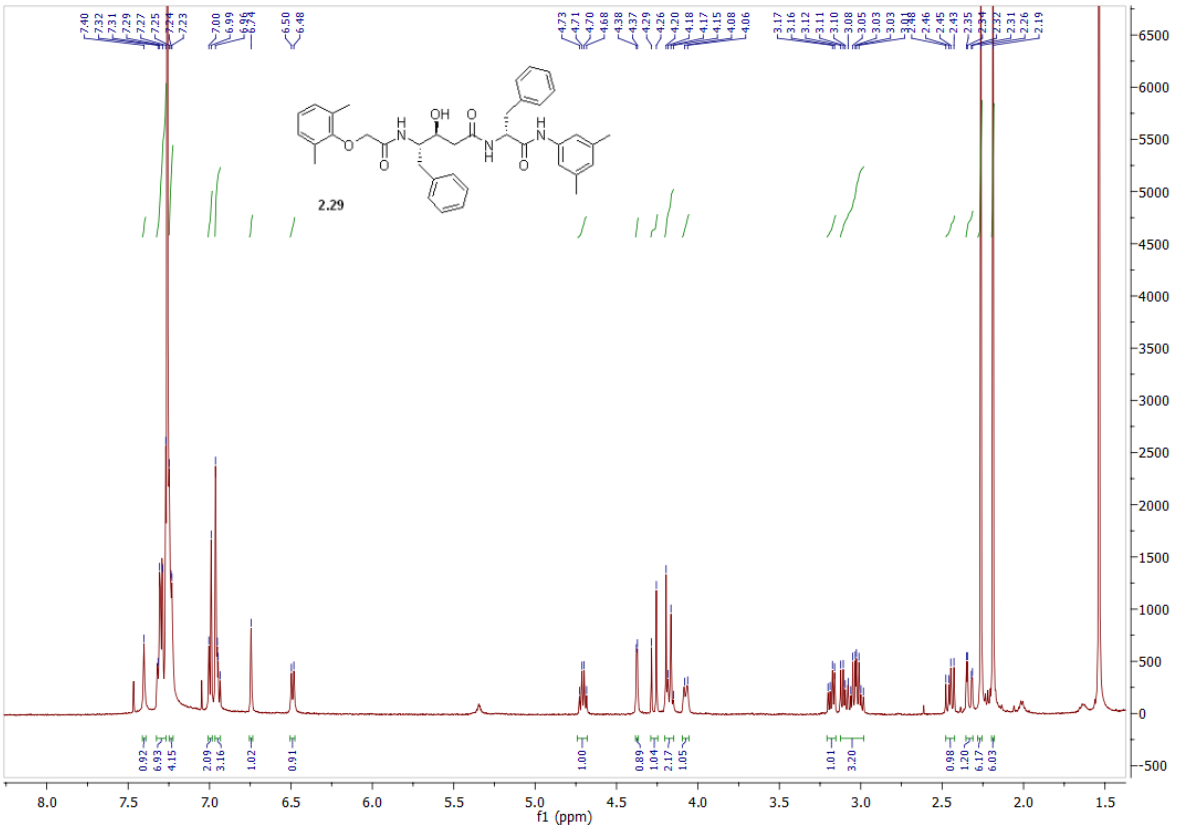
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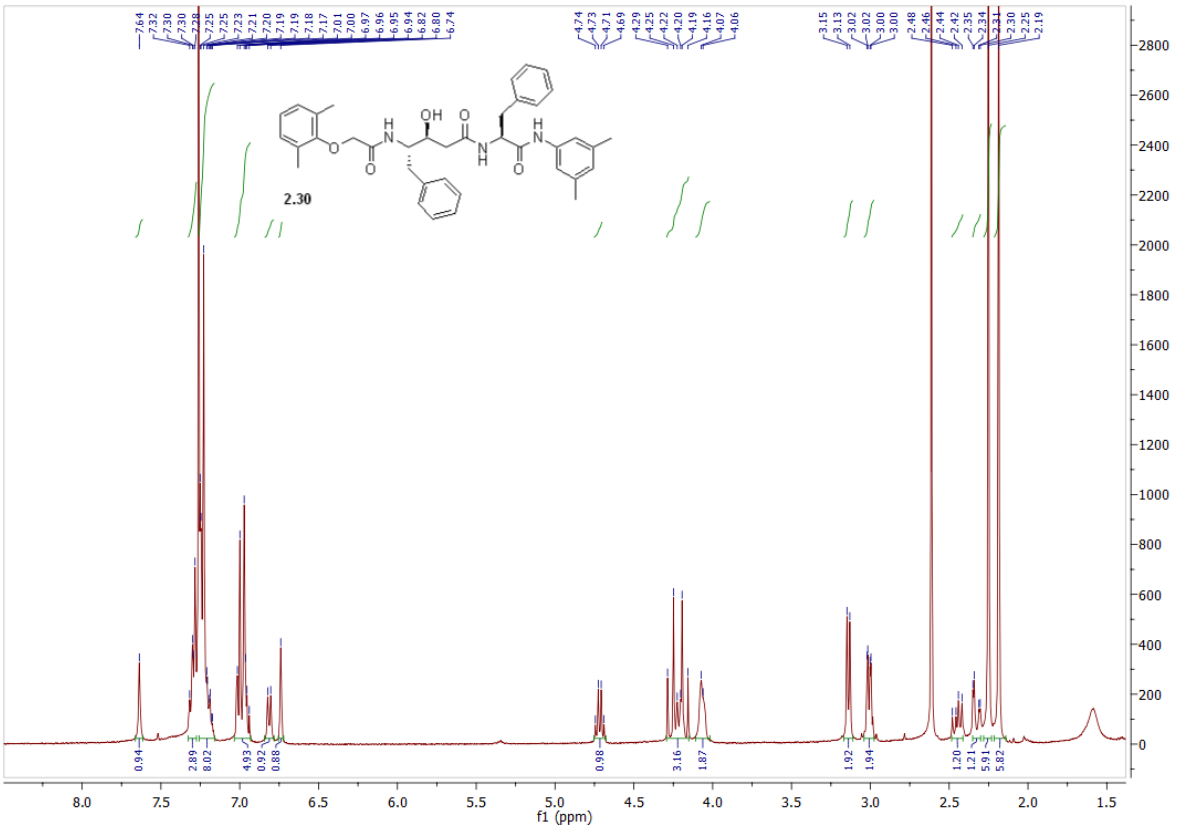
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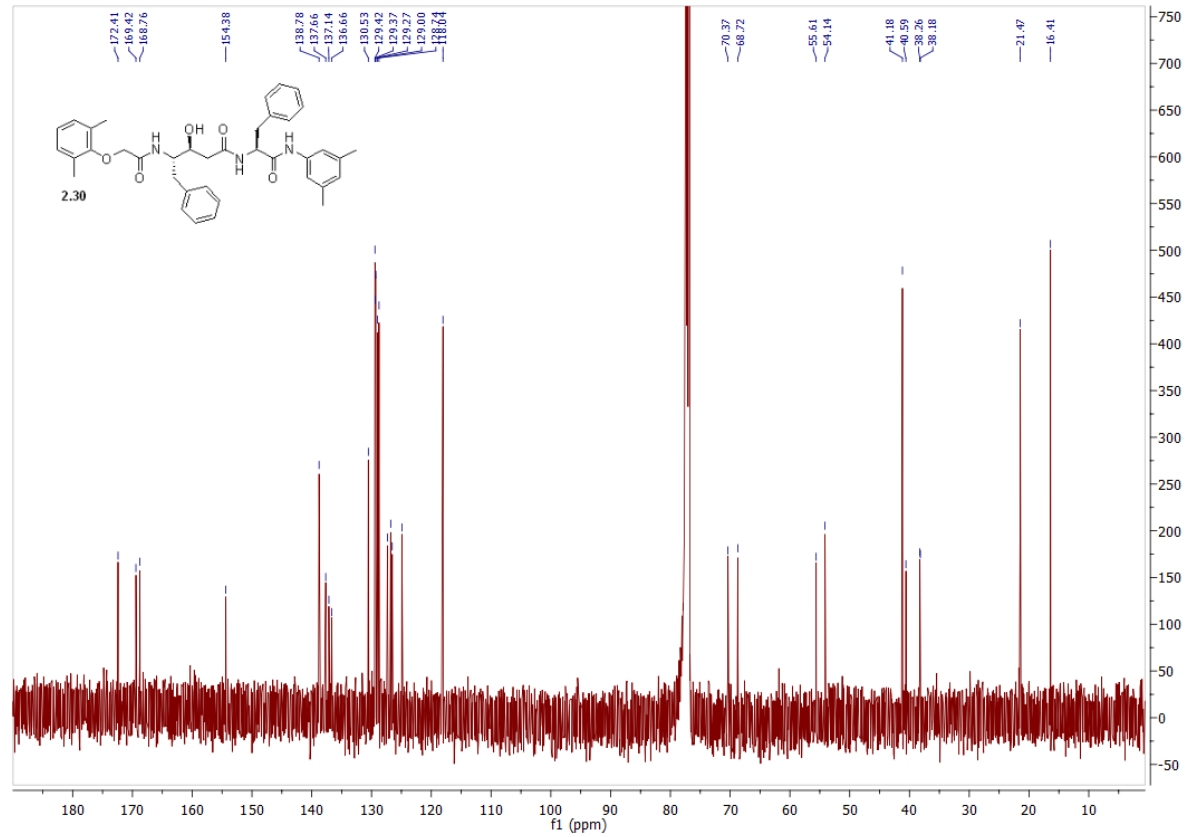
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168.76
154.38
138.78
137.66
137.14
136.66
130.53
129.42
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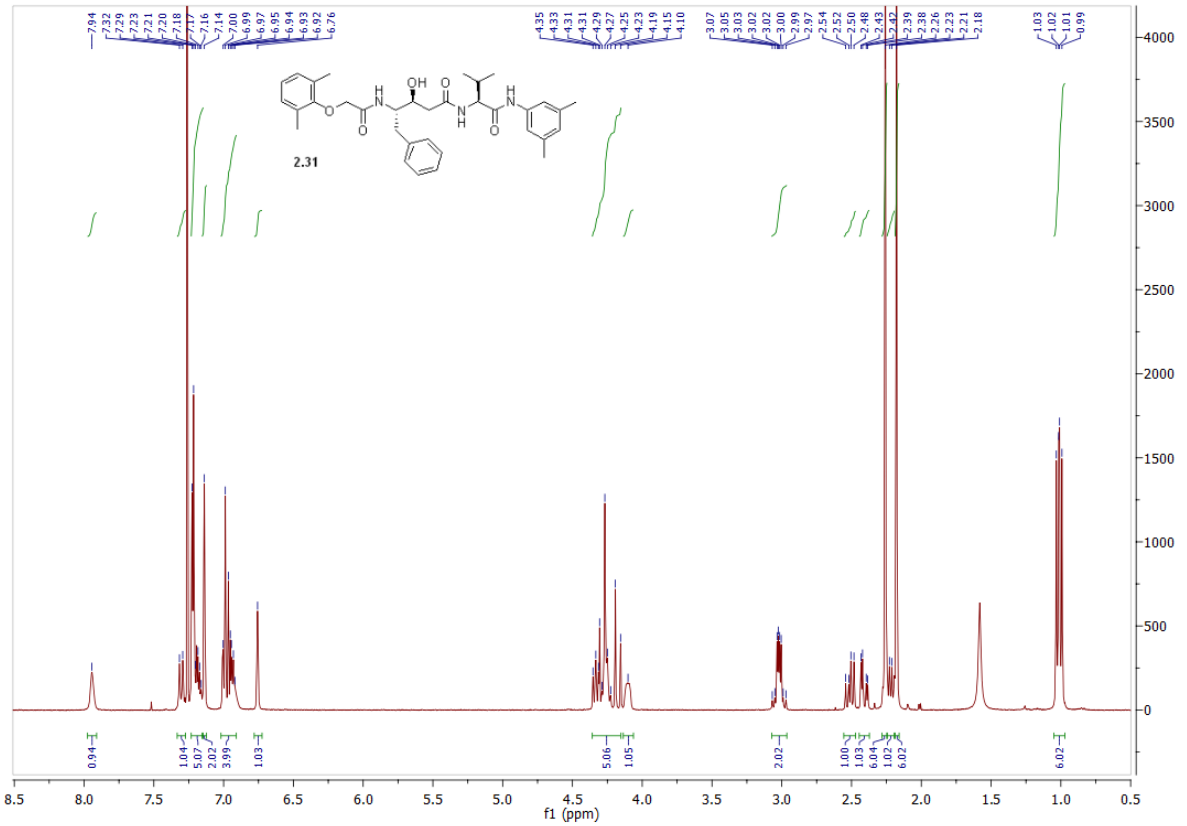
129.27
129.00
128.74
128.04
70.37
68.72
55.61
54.14
41.18
40.59
38.26
38.18
21.47
16.41
OH
2.30
f1 (ppm)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7.17
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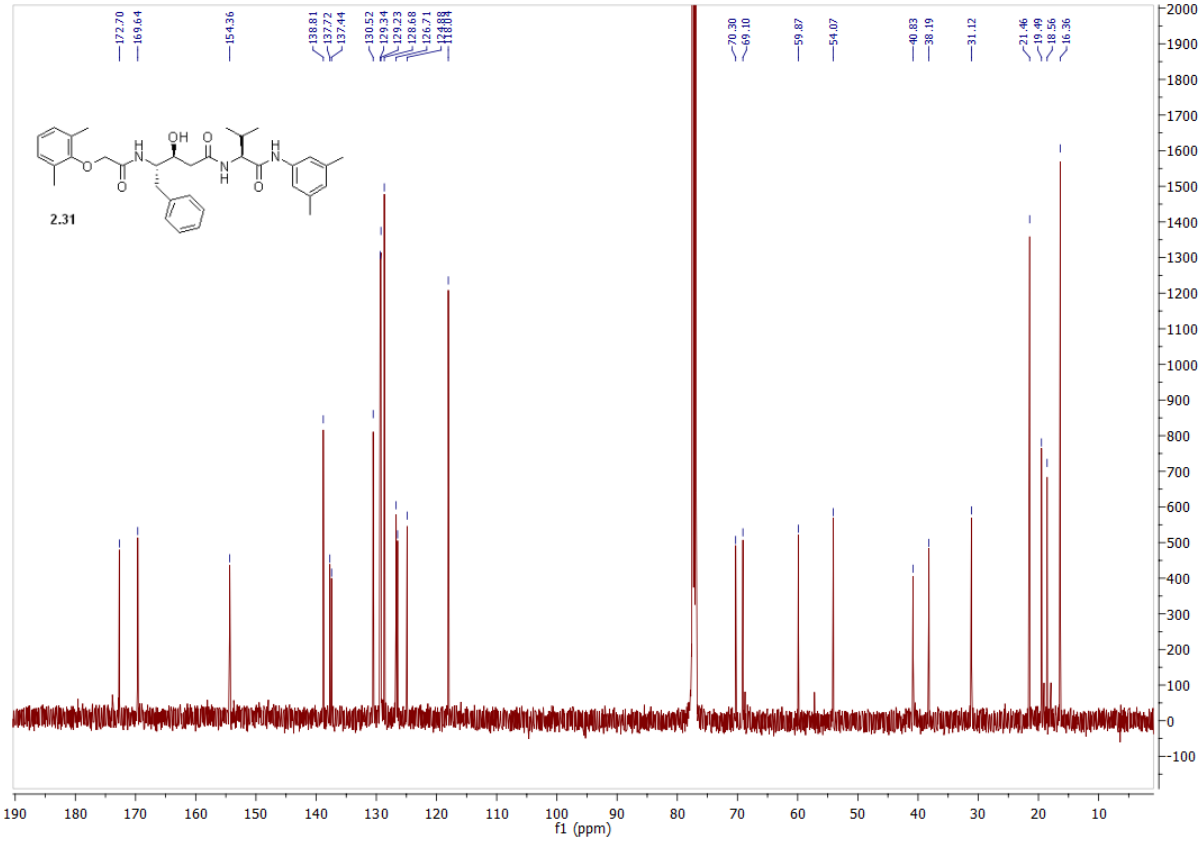
6.95
6.94
6.93
6.92
6.76
4.35
4.33
4.31
4.31
4.29
4.27
4.25
4.23
4.19
4.15
4.10
3.07
3.05
3.03
3.02
3.02
3.00
2.99
2.97
2.54
2.52
2.50
2.48
2.43
2.42
2.39
2.38
2.26
2.23
2.21
2.18
1.03
1.02
1.01
0.99
OH
2.31
0.94
1.04
5.07
2.02
3.99
1.03
5.06
1.05
2.02
1.00
1.03
6.04
1.02
6.02
6.02
f1 (ppm)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128.68
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70.30
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59.87
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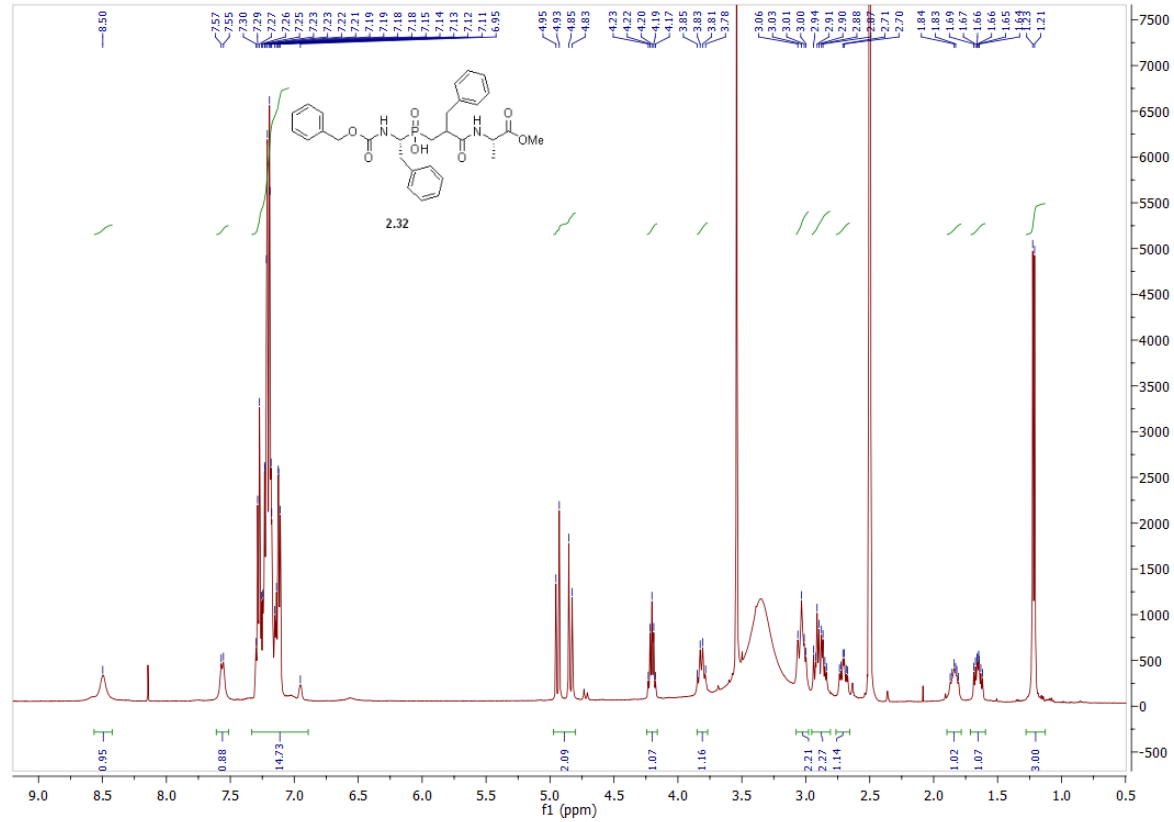
40.83
38.19
31.12
21.46
19.49
18.56
16.36
2.31
f1 (ppm)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7.15
7.14
7.13
7.12
7.11
6.95
4.95
4.93
4.85
4.83
4.23
4.22
4.20
4.19
4.17
3.85
3.83
3.81
3.78
3.06
3.03
3.01
3.00
2.94
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2.90
2.88
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2.70
1.84
1.83
1.69
1.67
1.66
1.66
1.65
1.21
OMe
2.32
0.95
0.88
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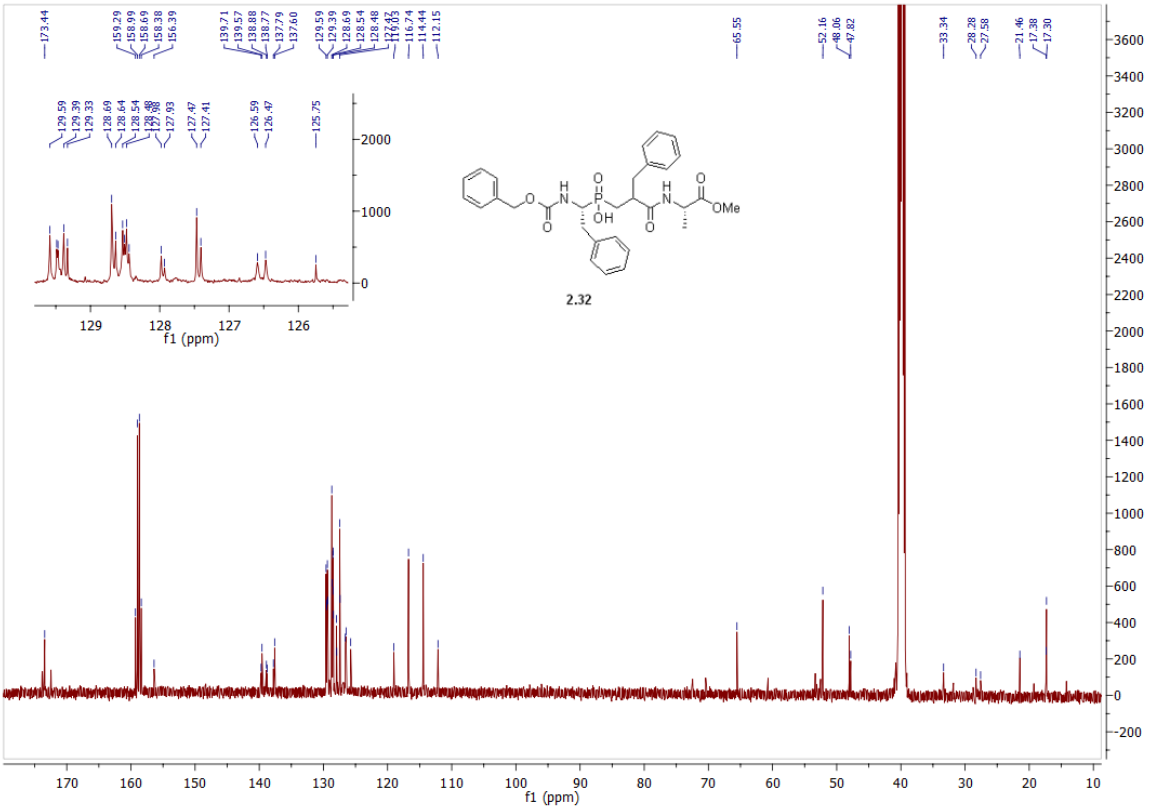
2.21
2.27
1.14
1.02
1.07
3.00
f1 (ppm)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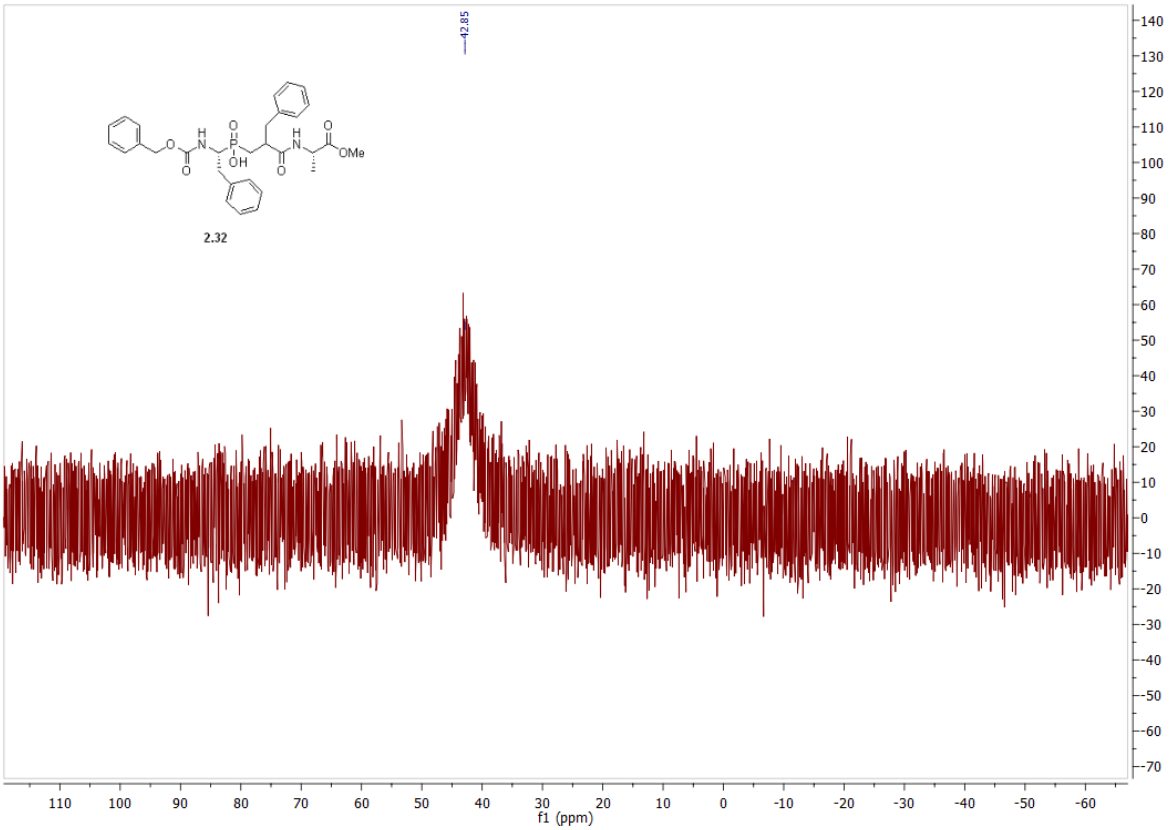
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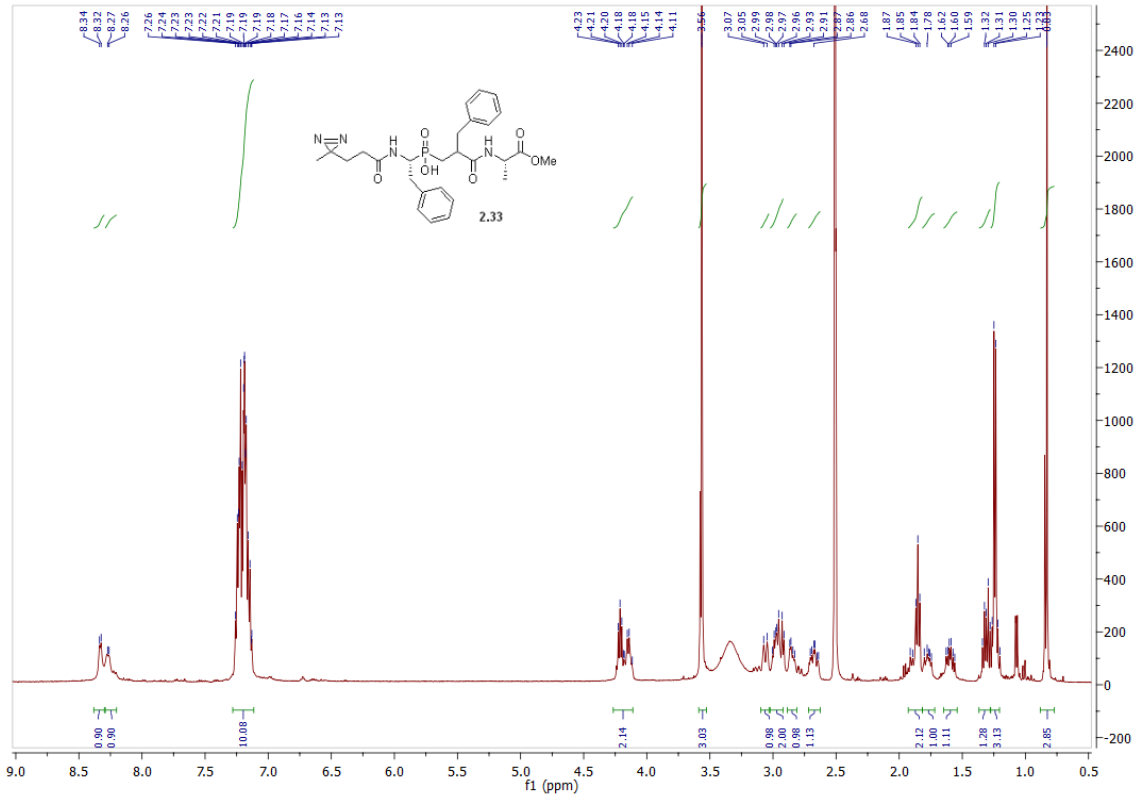
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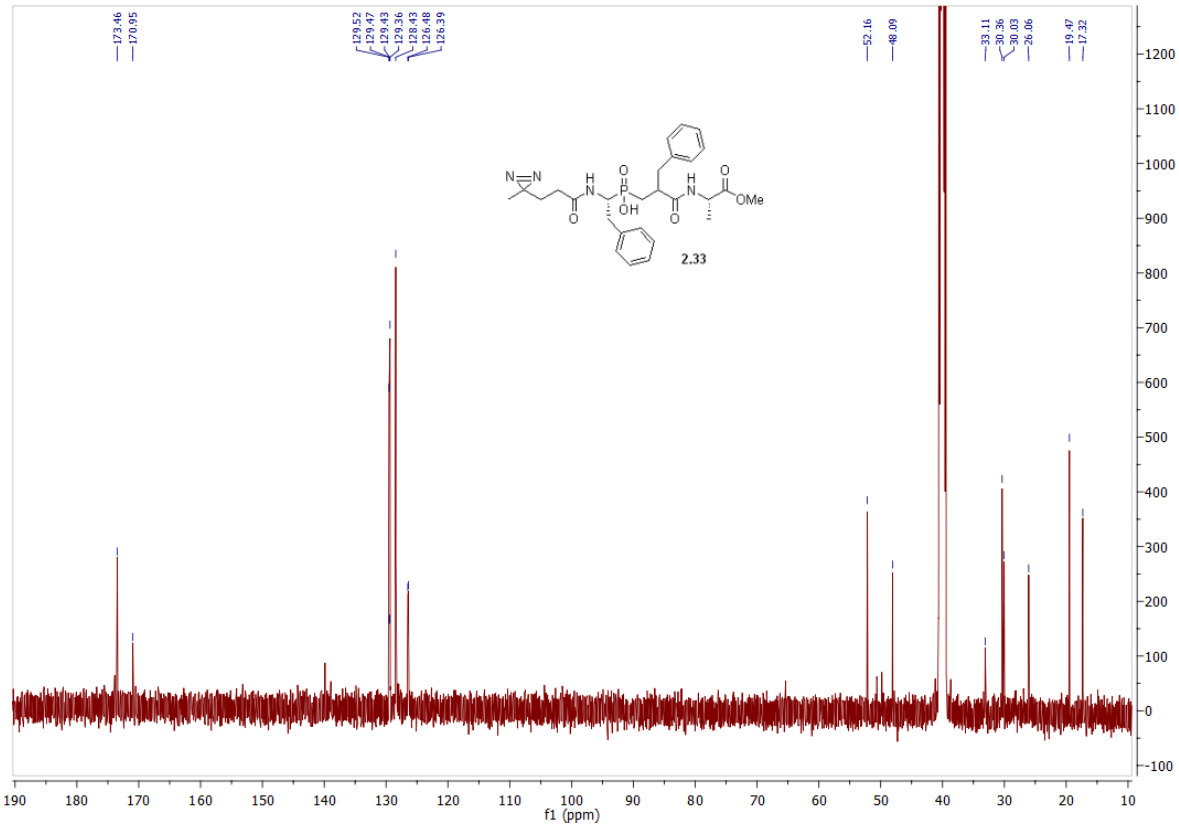
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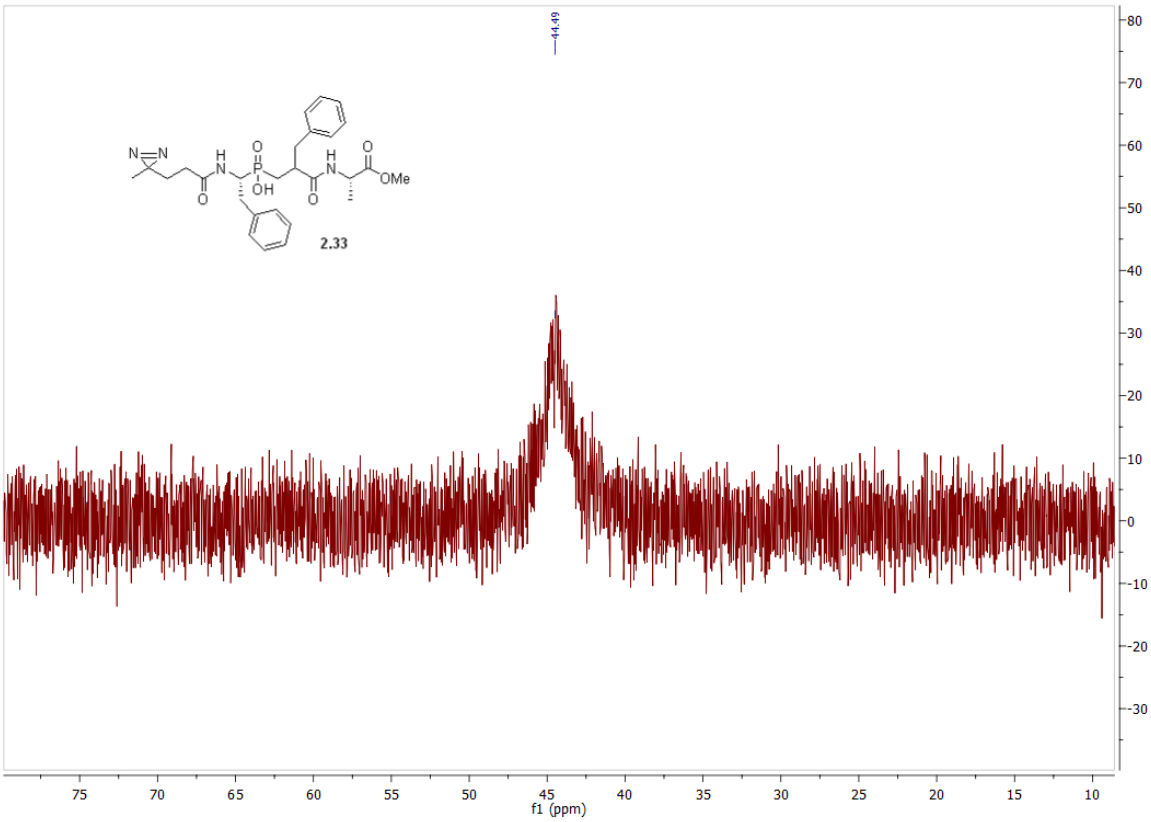
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2.33
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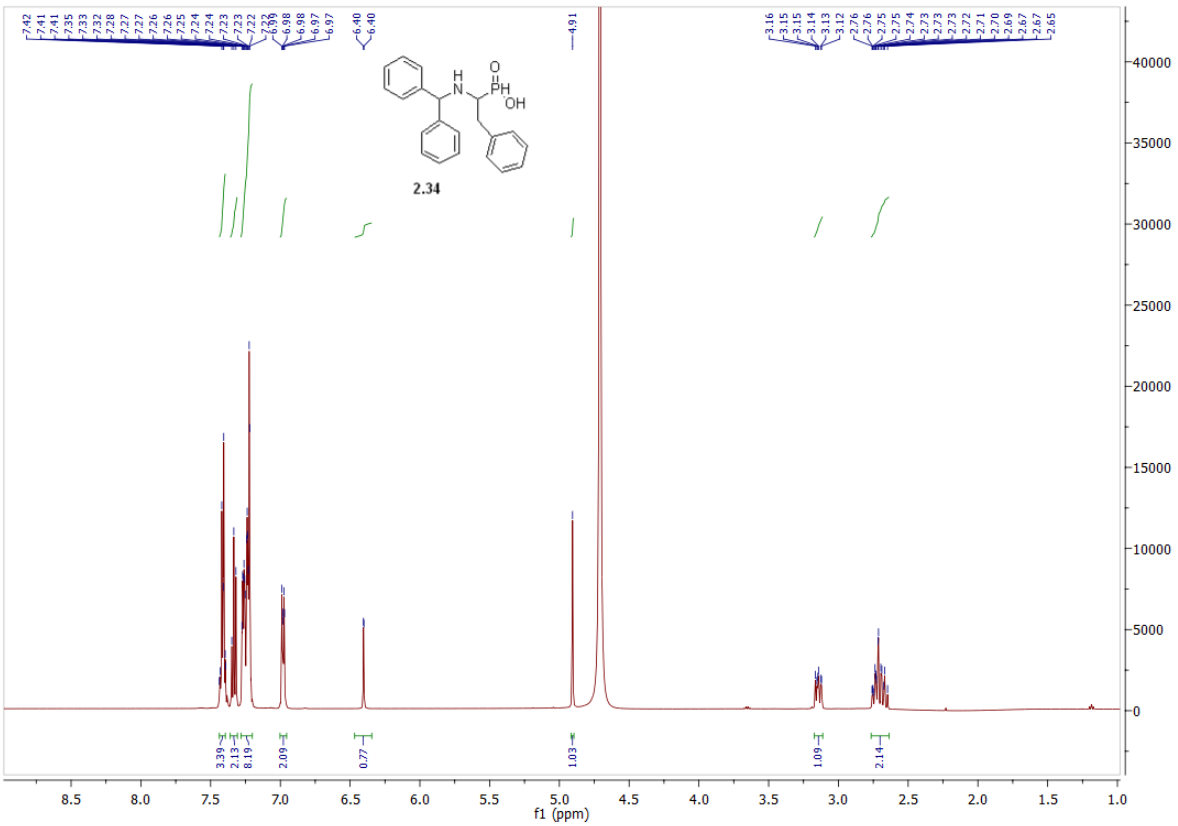
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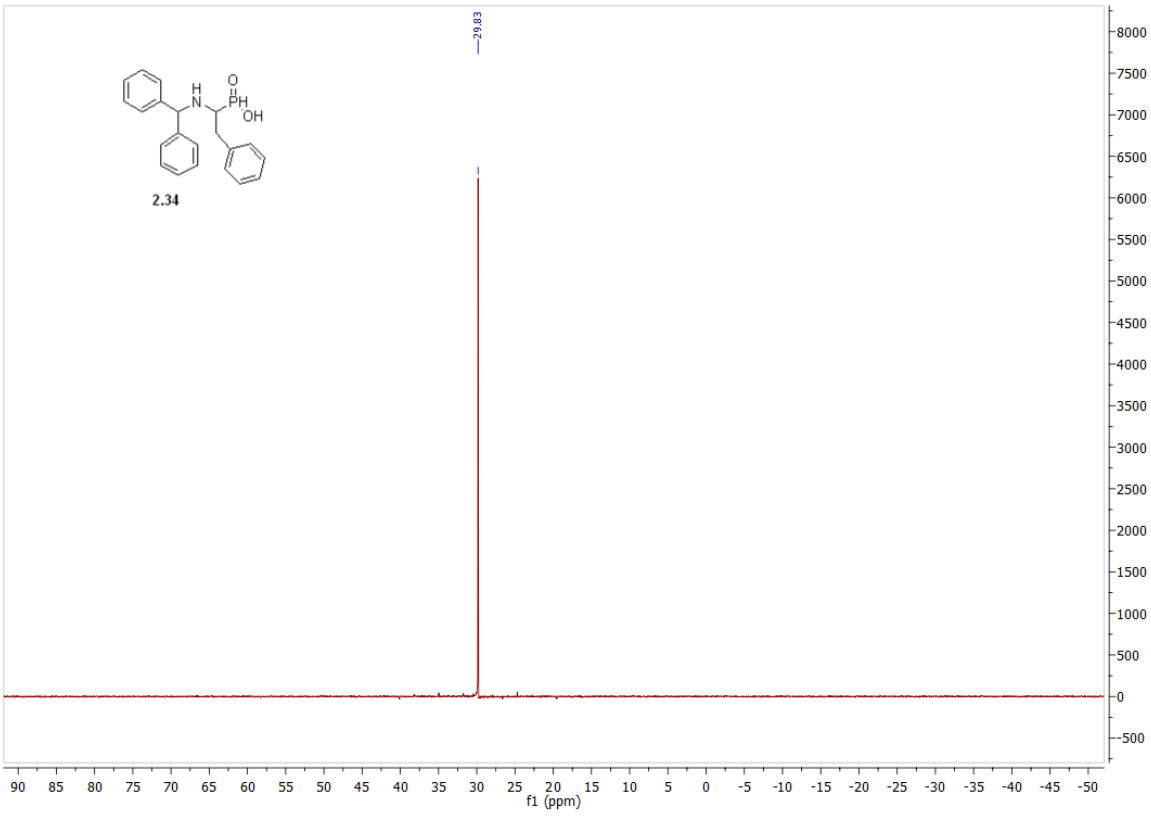
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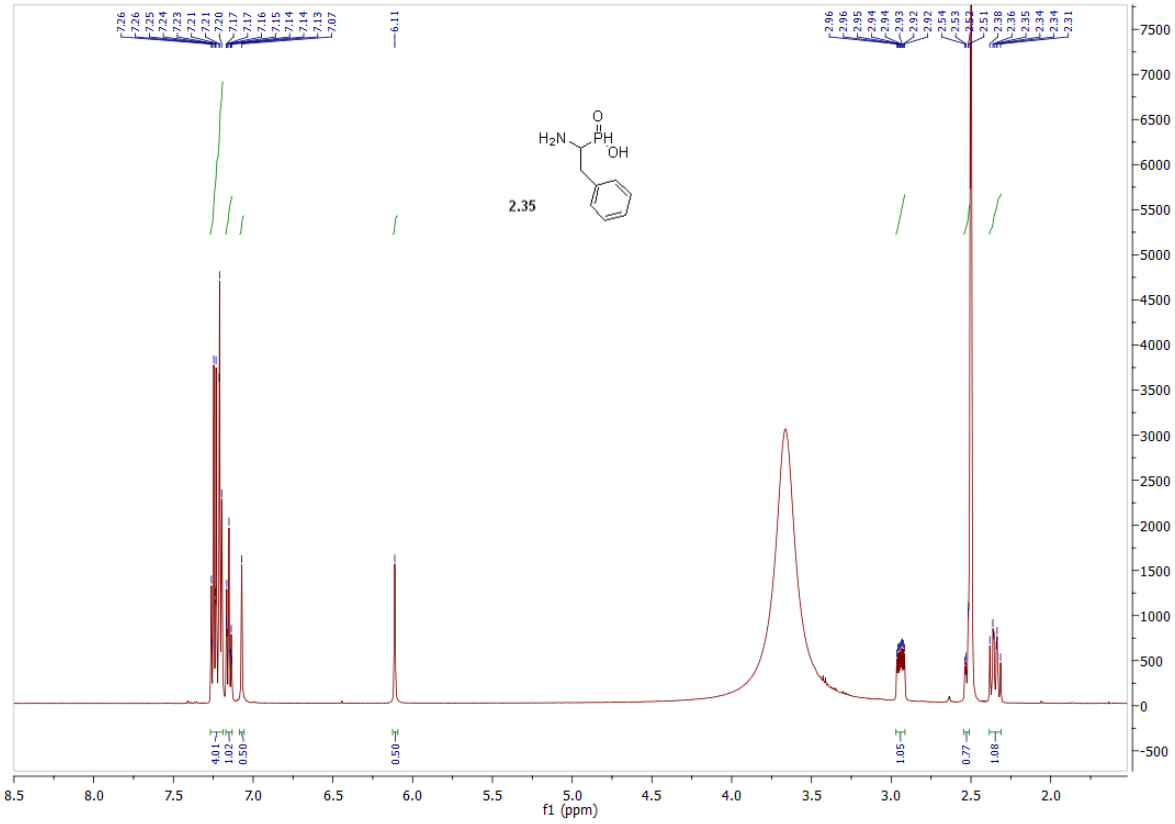
2.34
f1 (ppm)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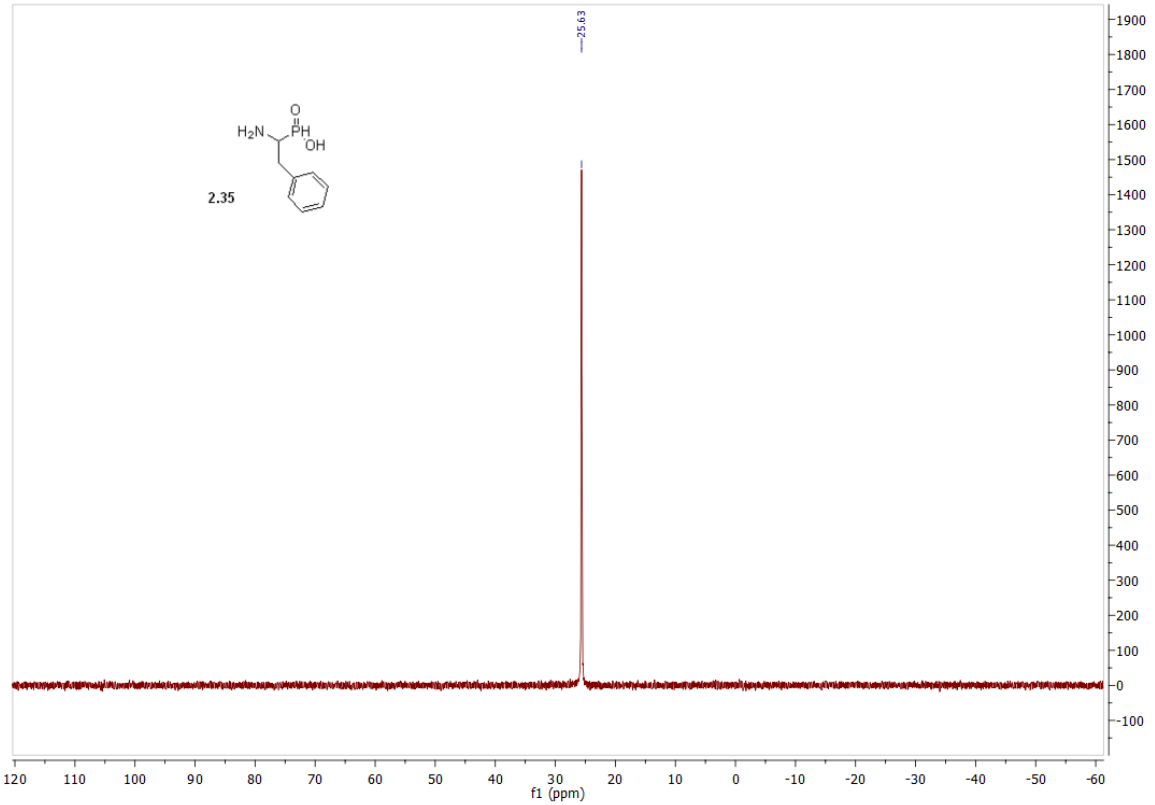
0.50
0.50
1.05
0.77
1.08
f1 (ppm)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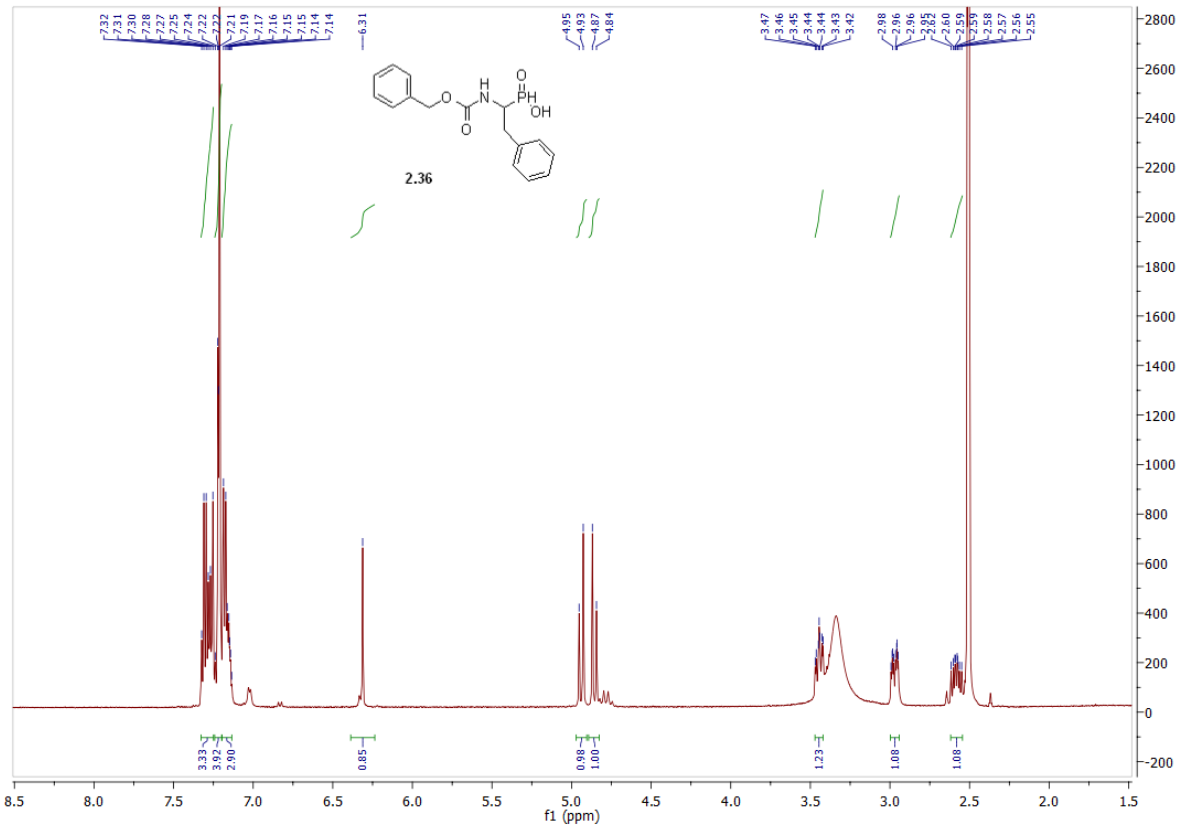
2.35
f1 (ppm)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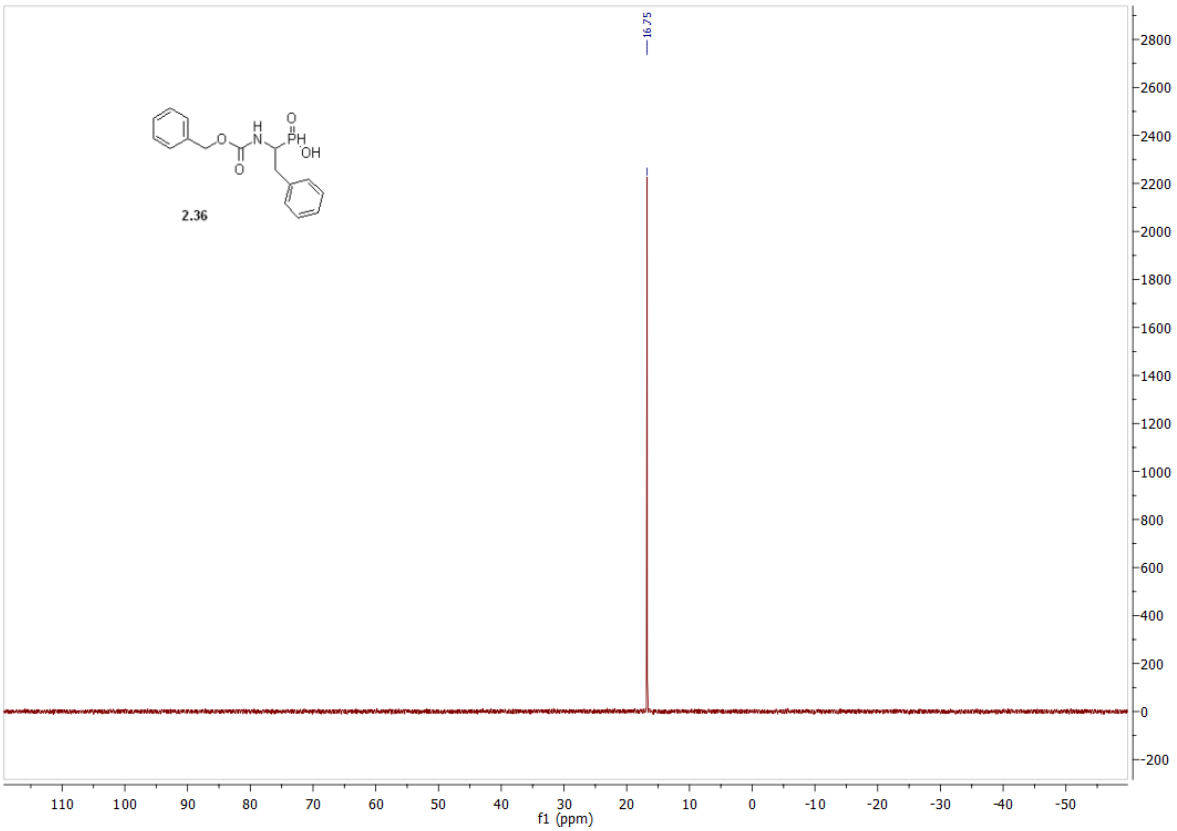
4.87
4.84
2.36
f1 (ppm)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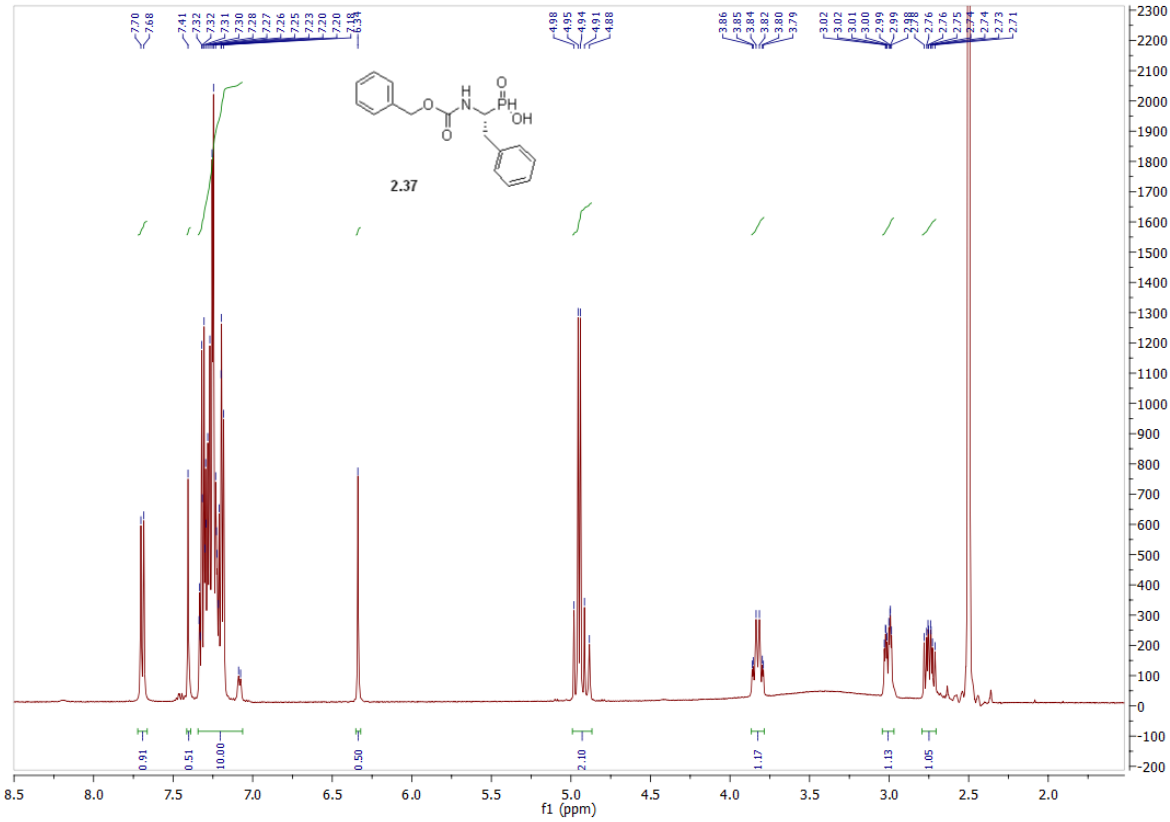
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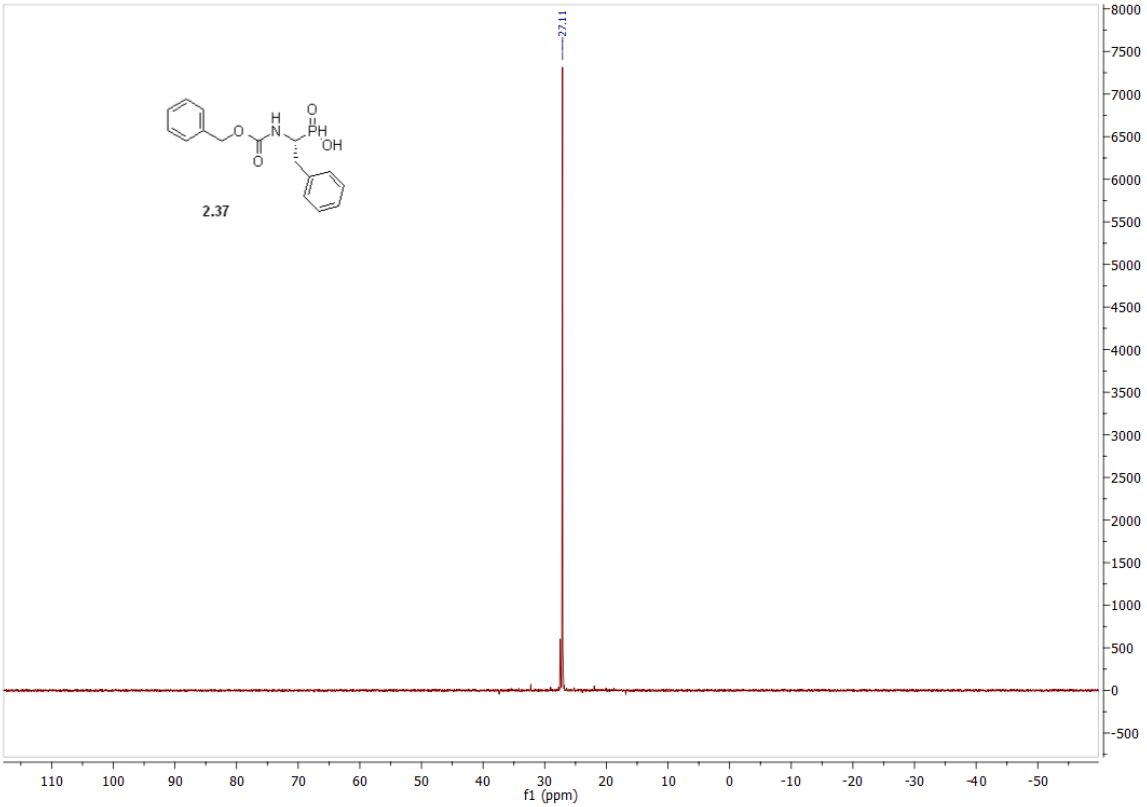
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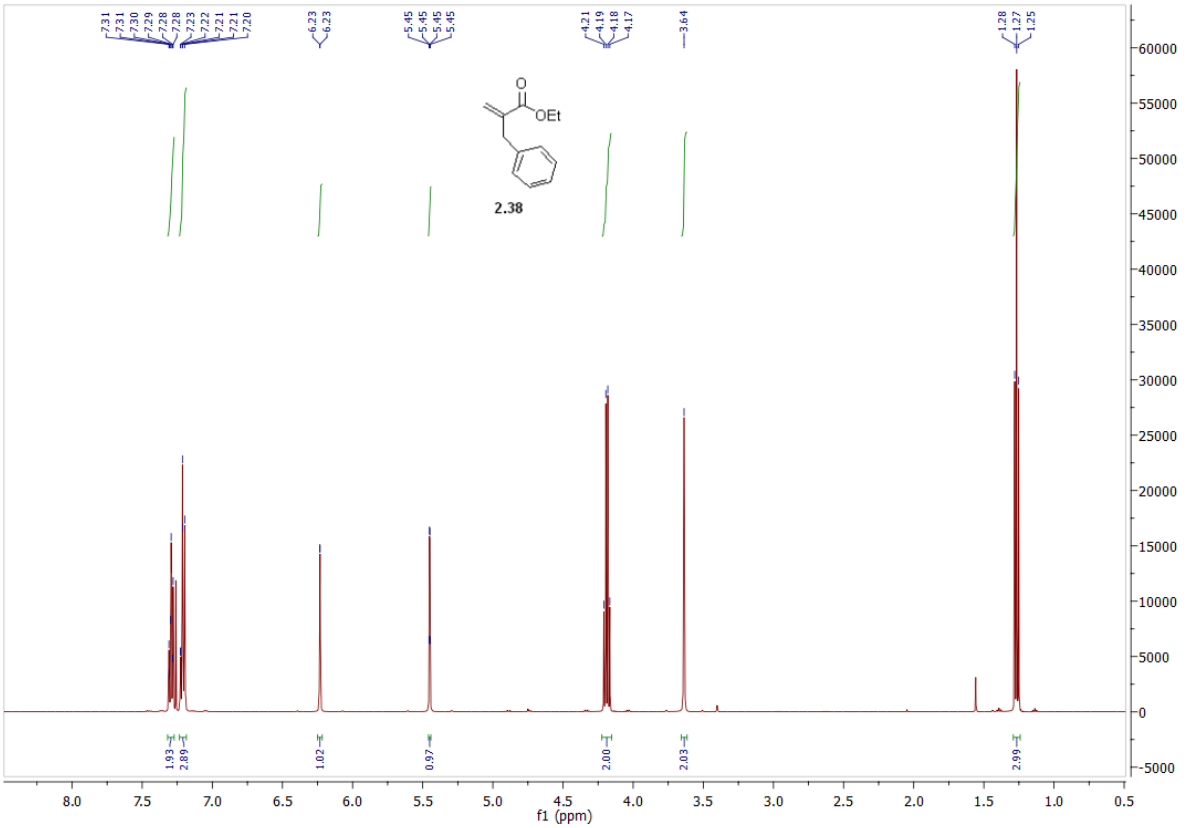
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6.23
6.23
5.45
5.45
5.45
5.45
4.21
4.19
4.18
4.17
3.64
1.28
1.27
1.25
OEt
2.38
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2.89
1.02
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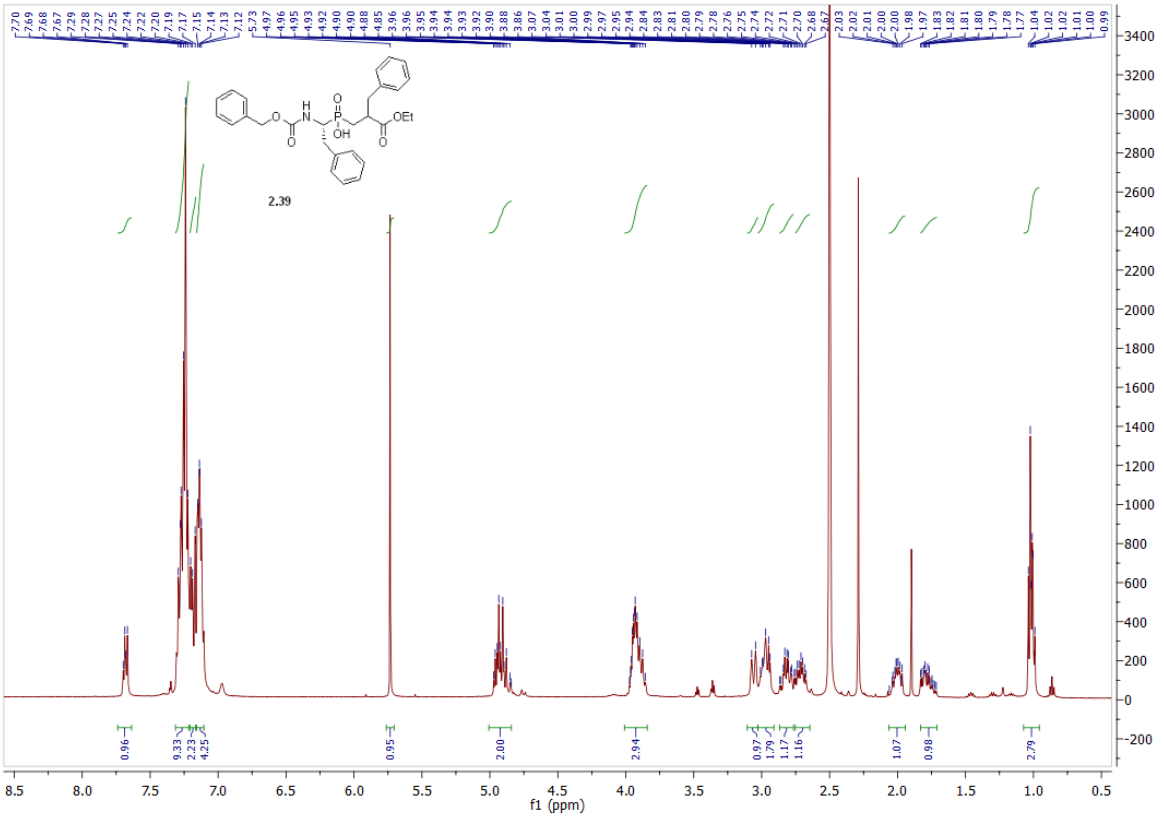
2.00
2.03
2.99
f1 (ppm)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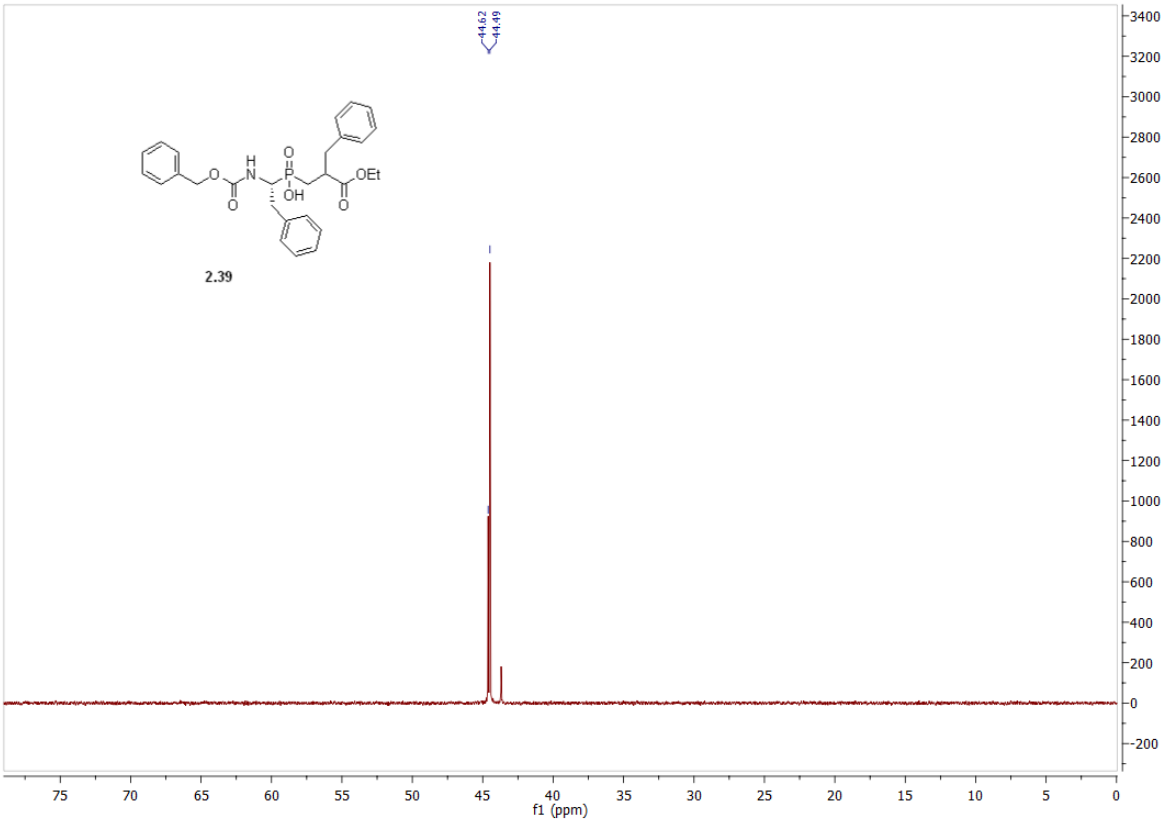
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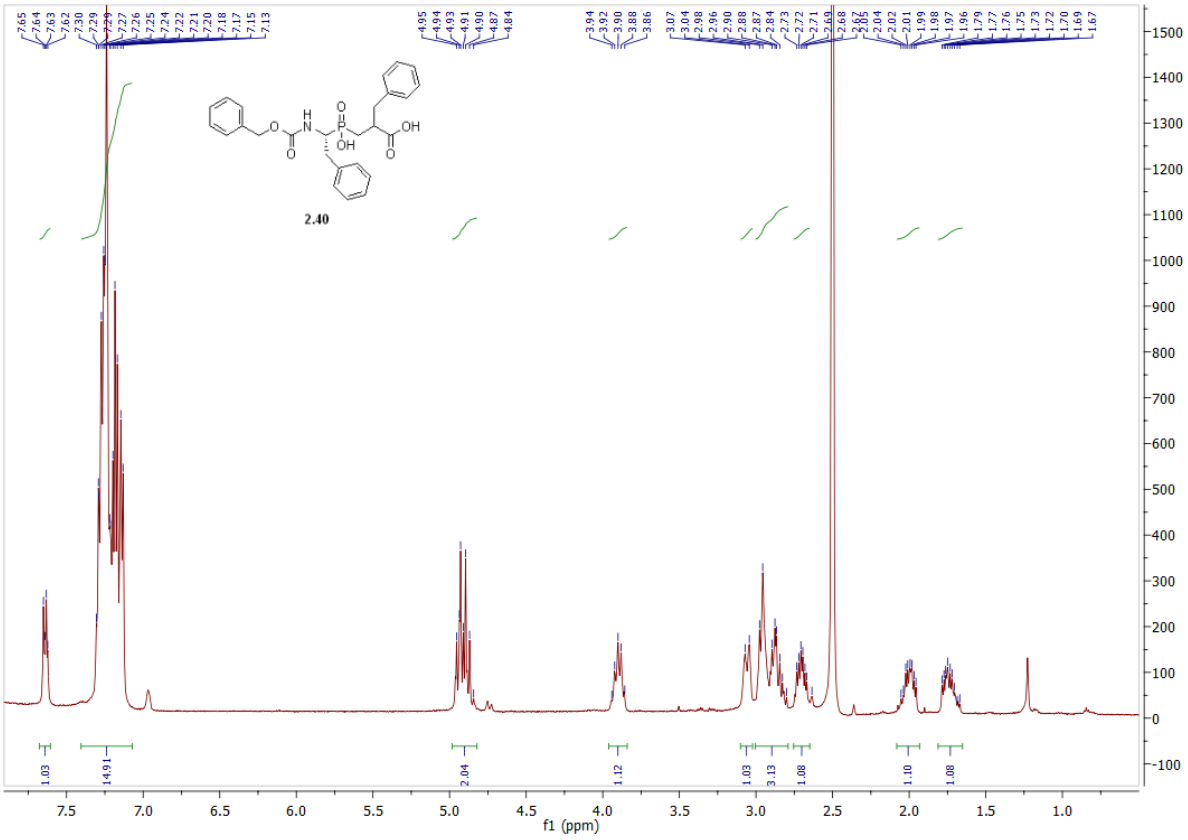
44.49
2.39
f1 (ppm)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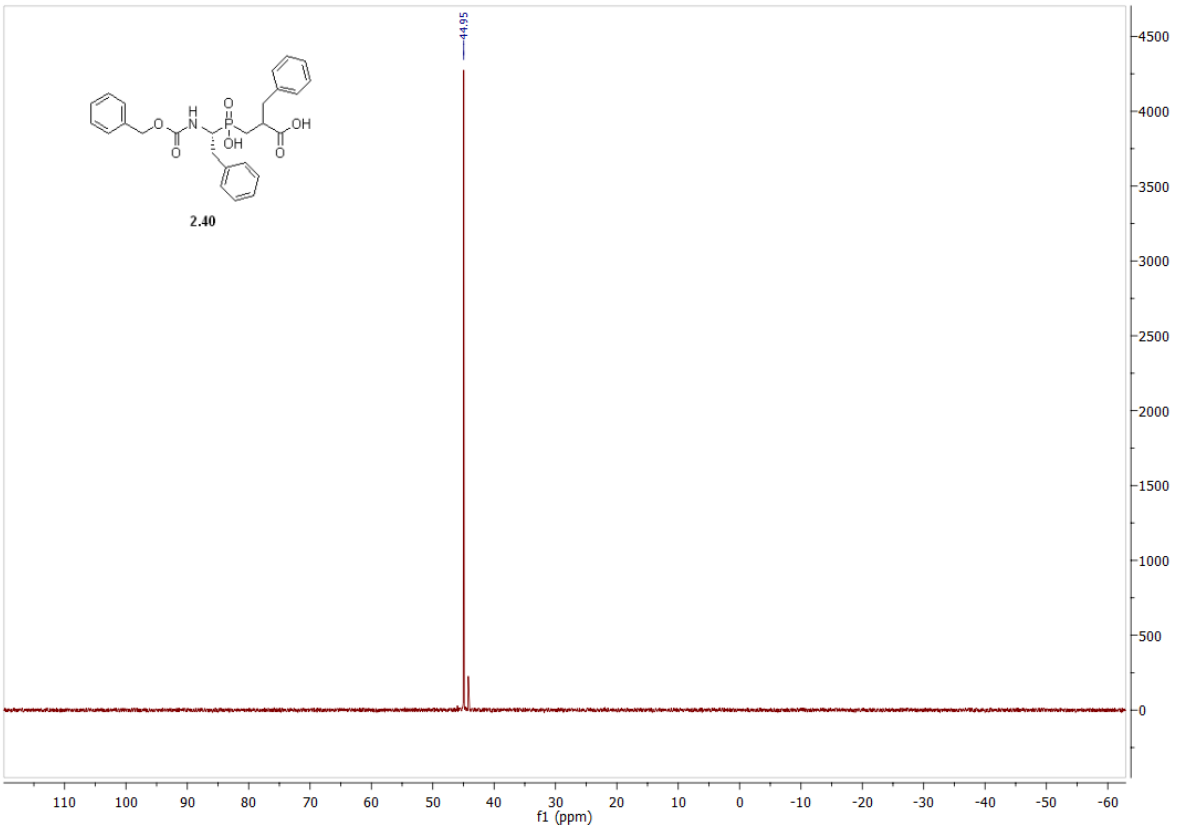
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2.40
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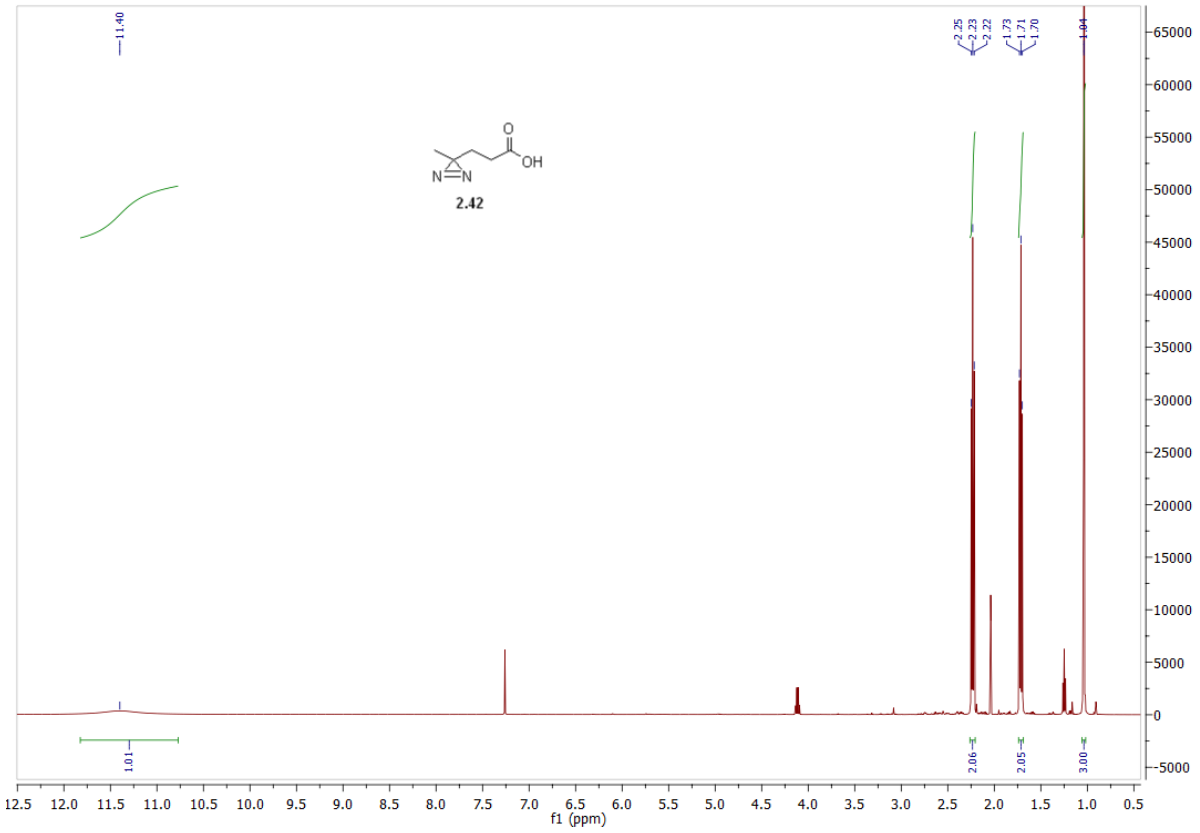
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OH
N
N
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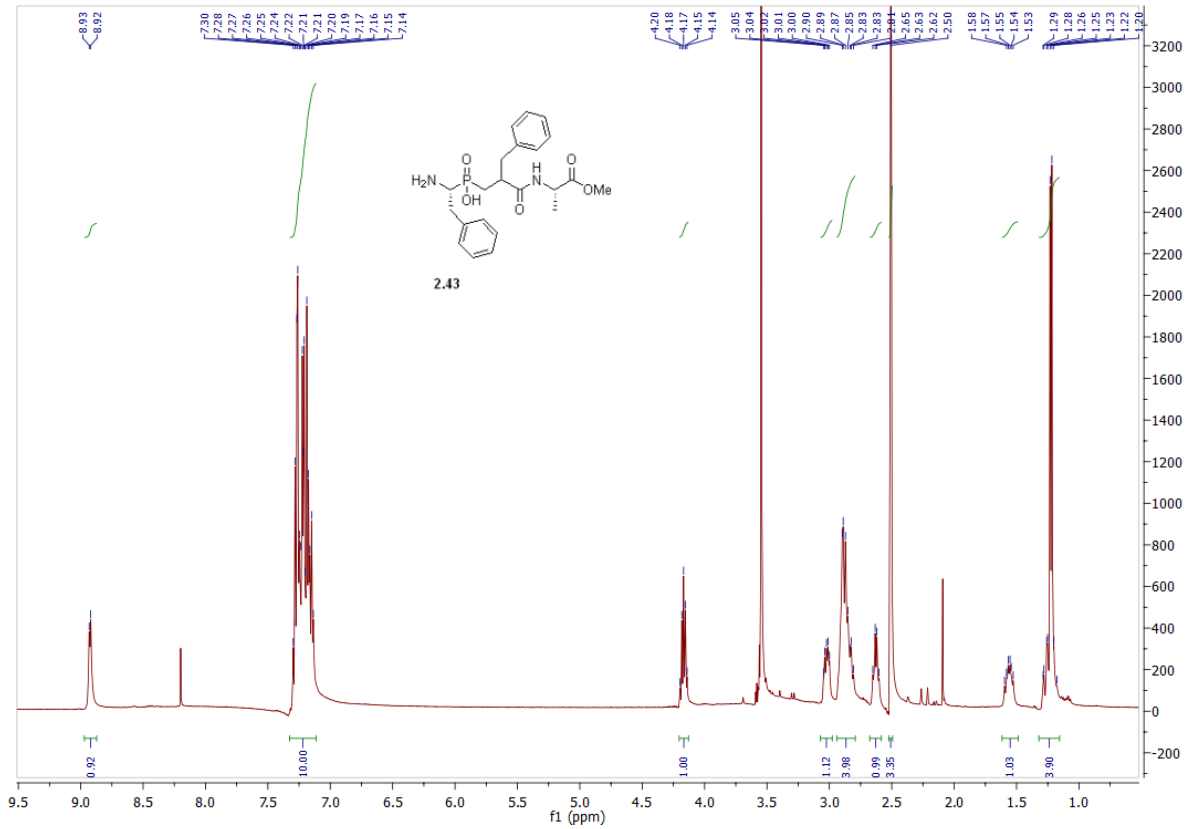
2.06
2.05
3.00
f1 (ppm)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OH
H
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O
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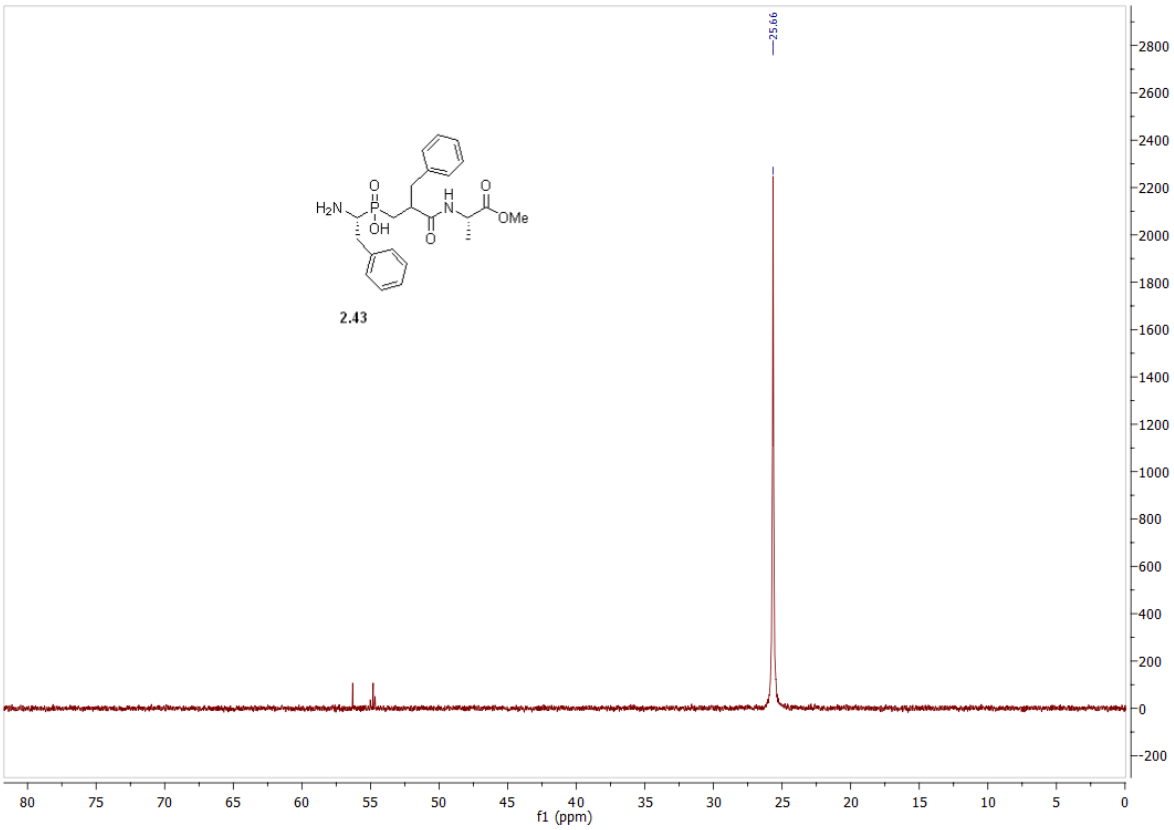
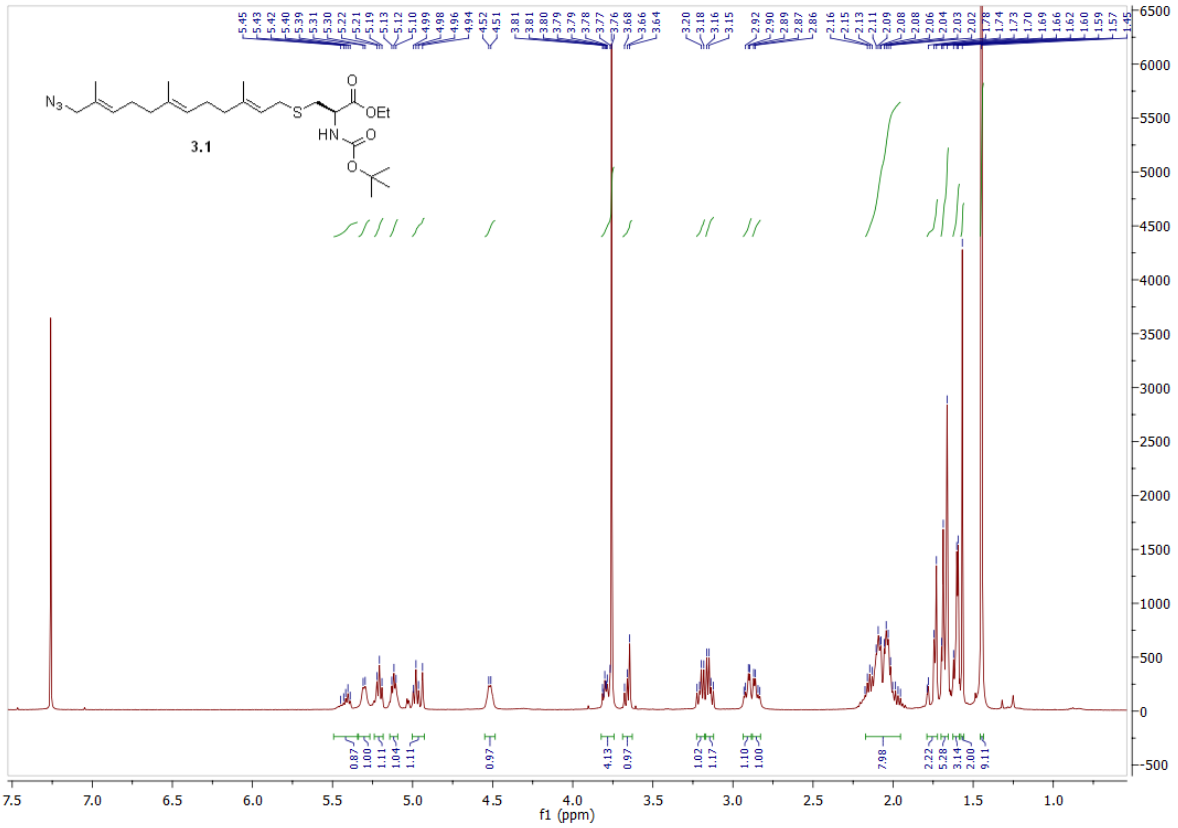
0.99
3.35
1.03
3.90
f1 (ppm)


25.66
2.43
f1 (ppm)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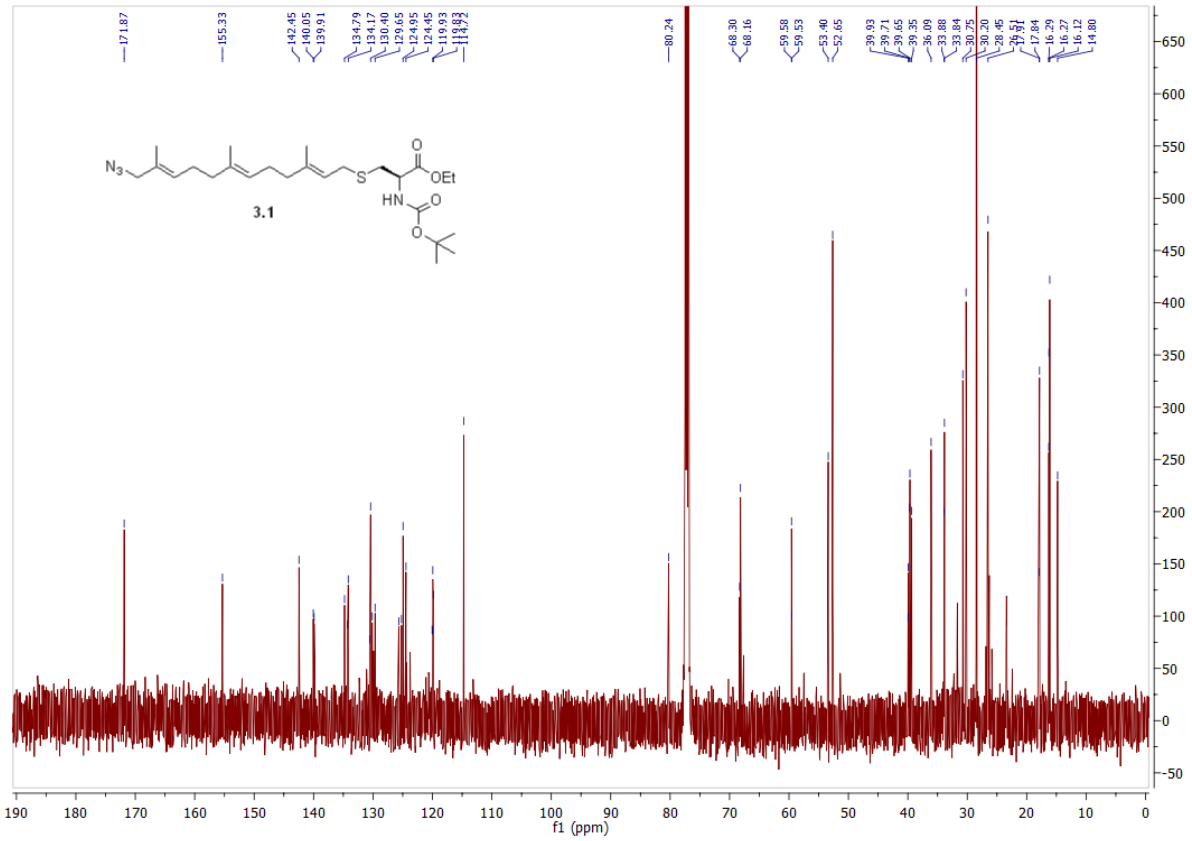
f1 (ppm)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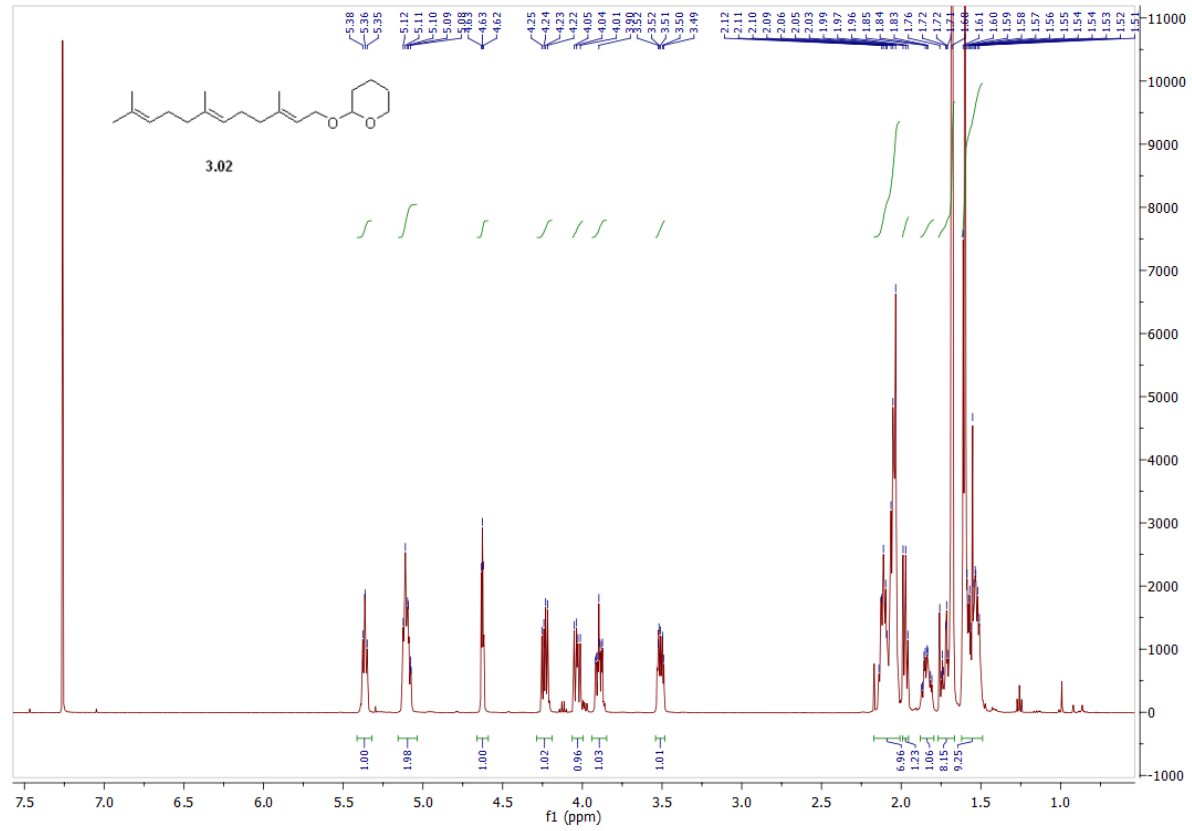
f1 (ppm)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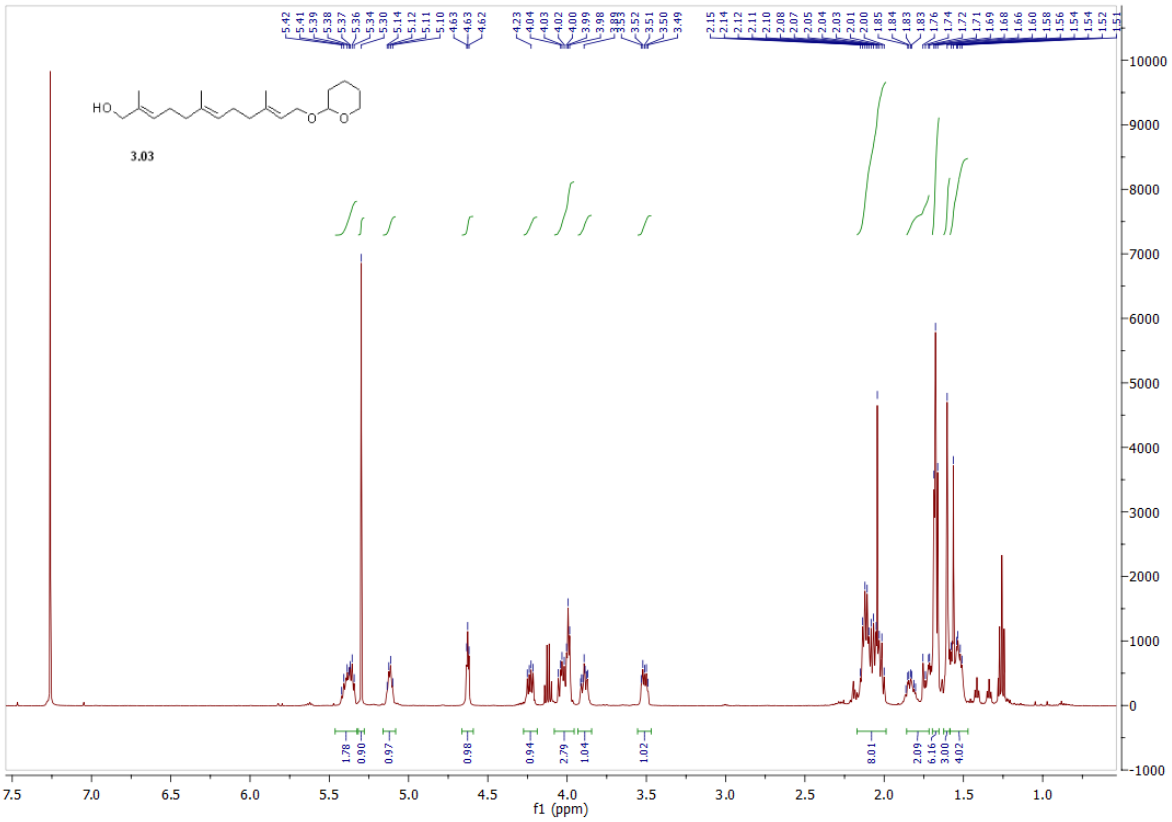
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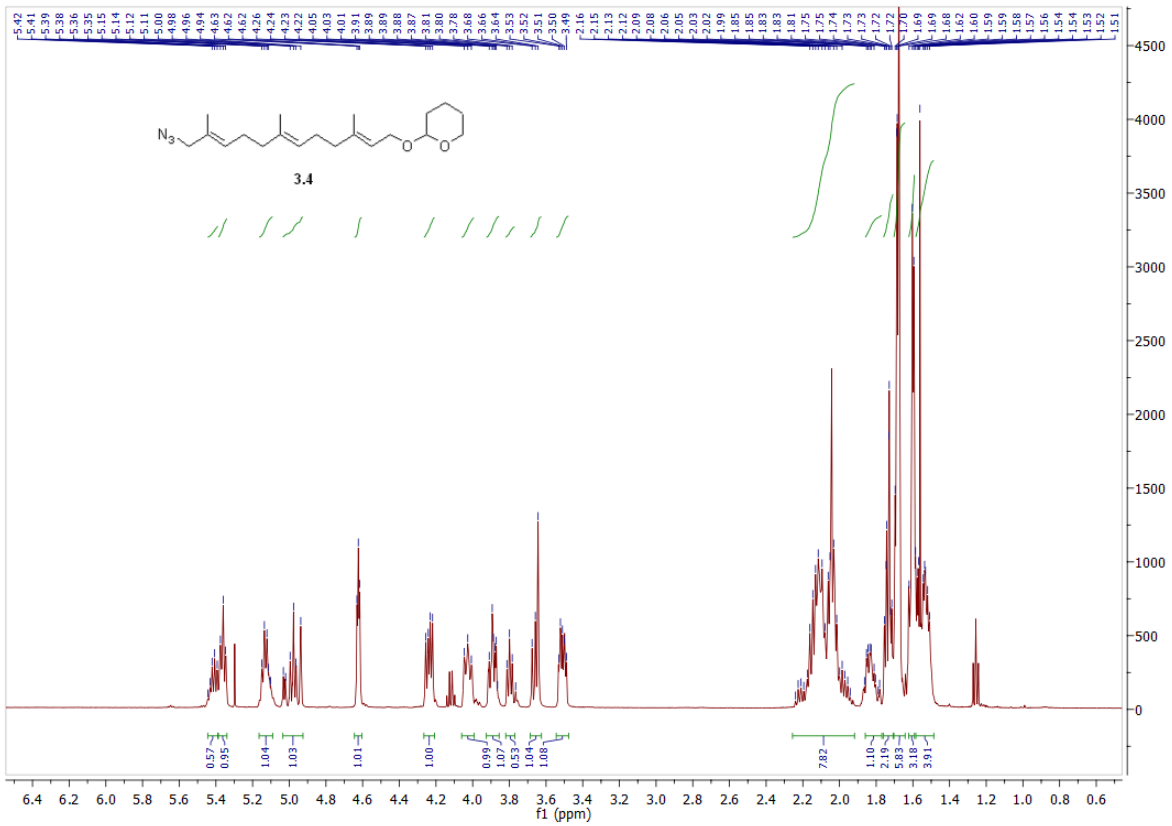
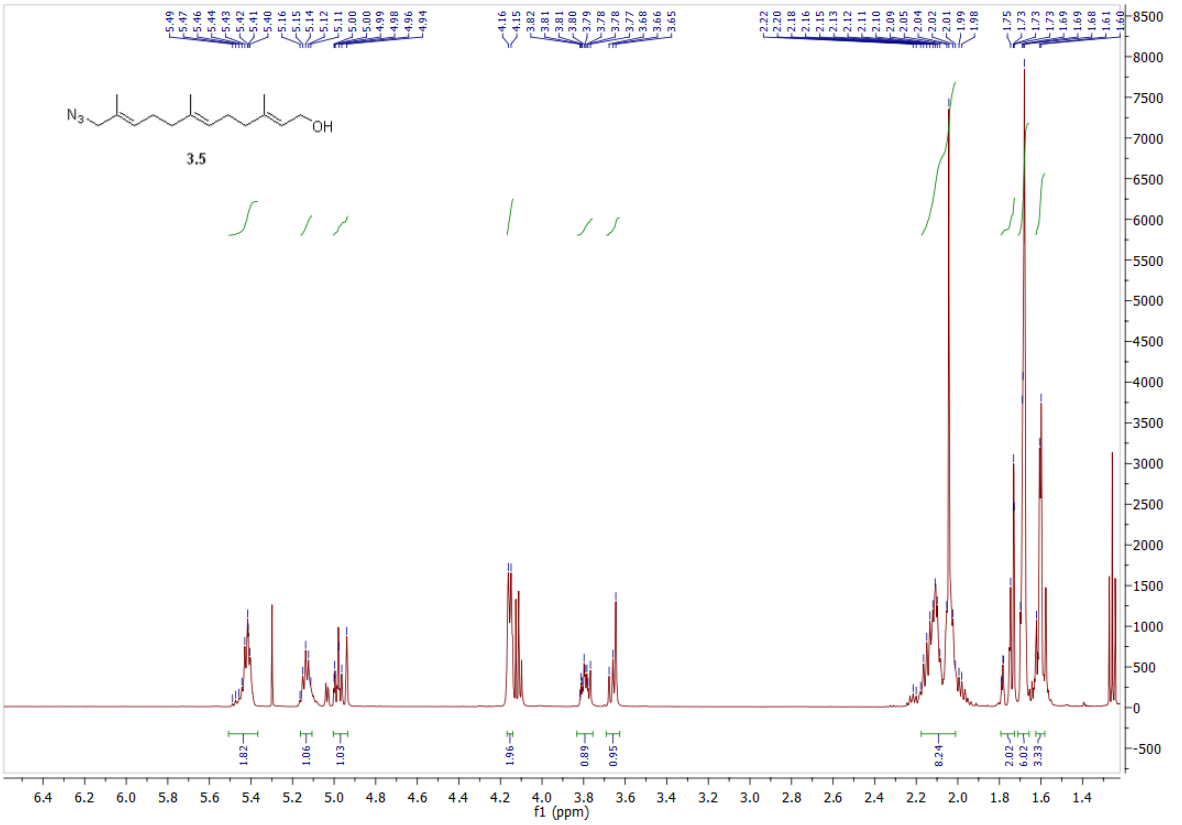
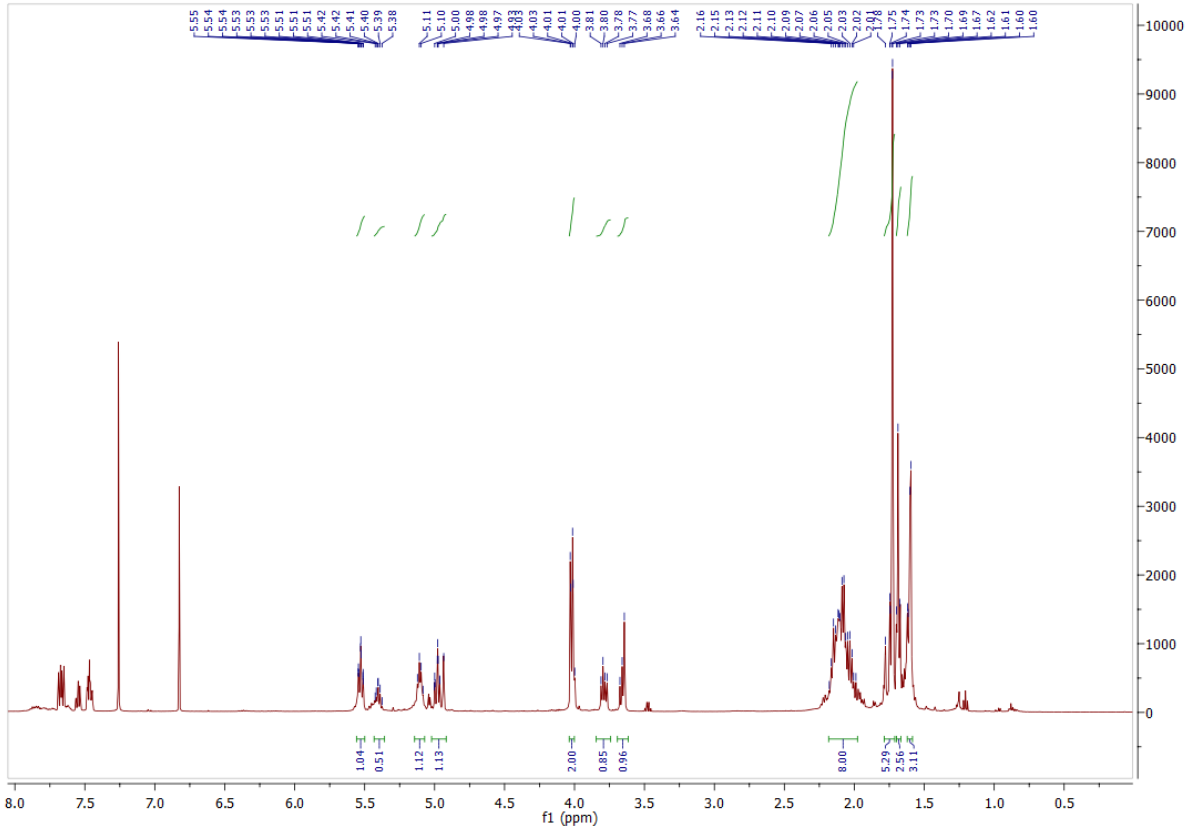
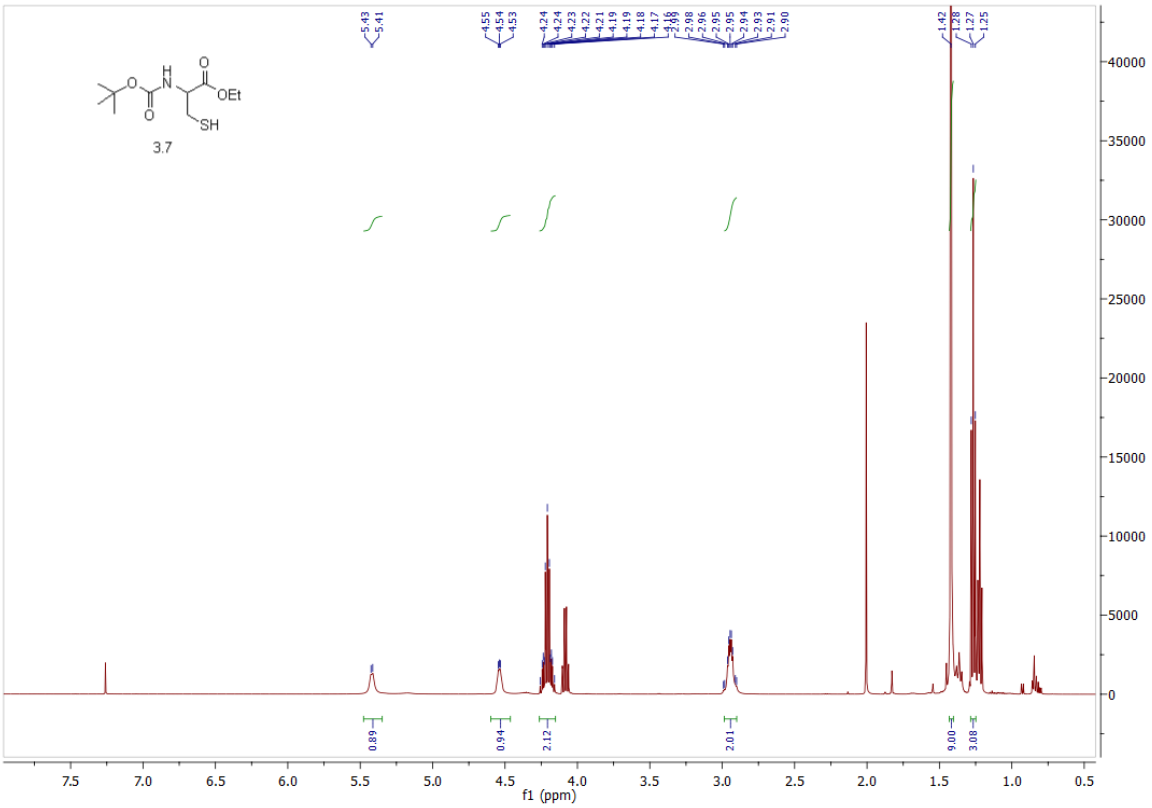
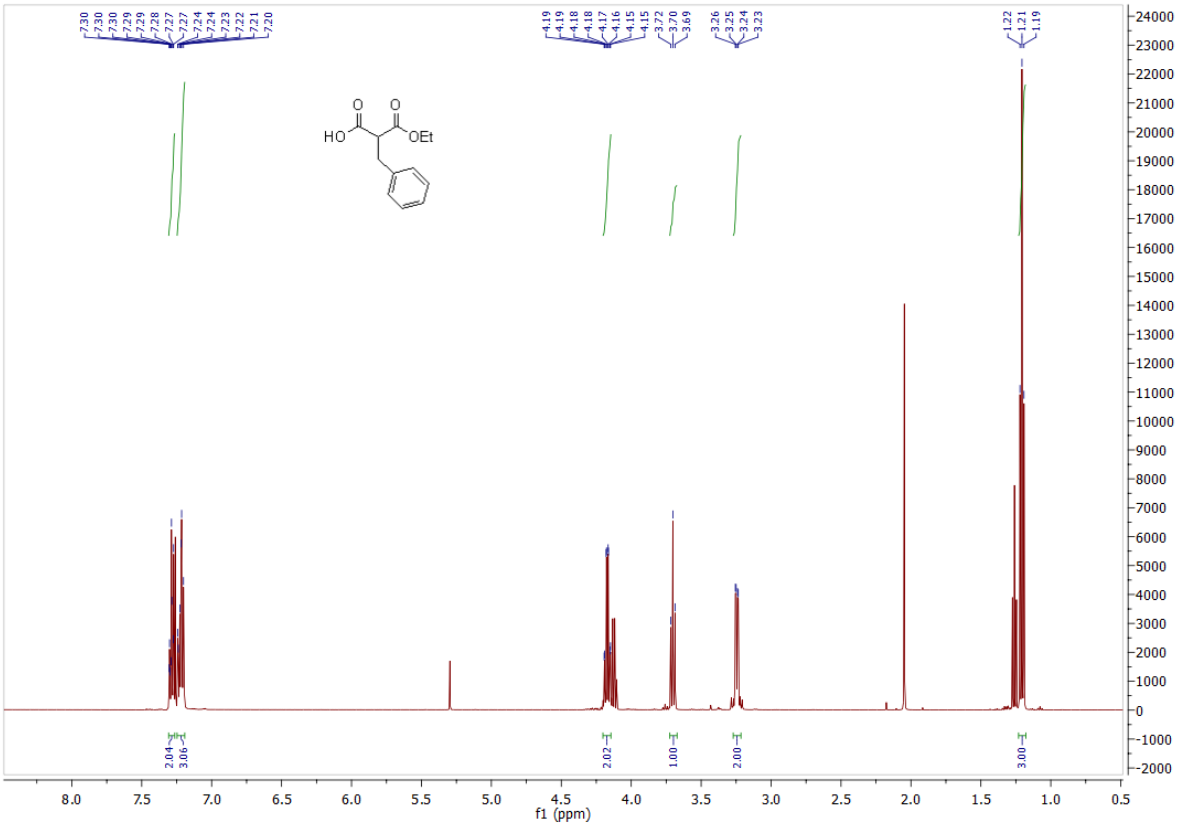
O
O
3.03
f1 (ppm)


N3
O
O
3.4
f1 (ppm)


N3
OH
3.5
f1 (ppm)

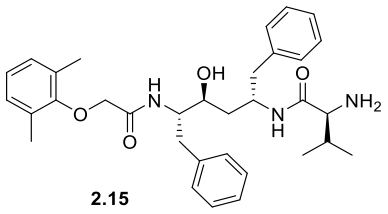

f1 (ppm)


3.7
f1 (ppm)


f1 (ppm)

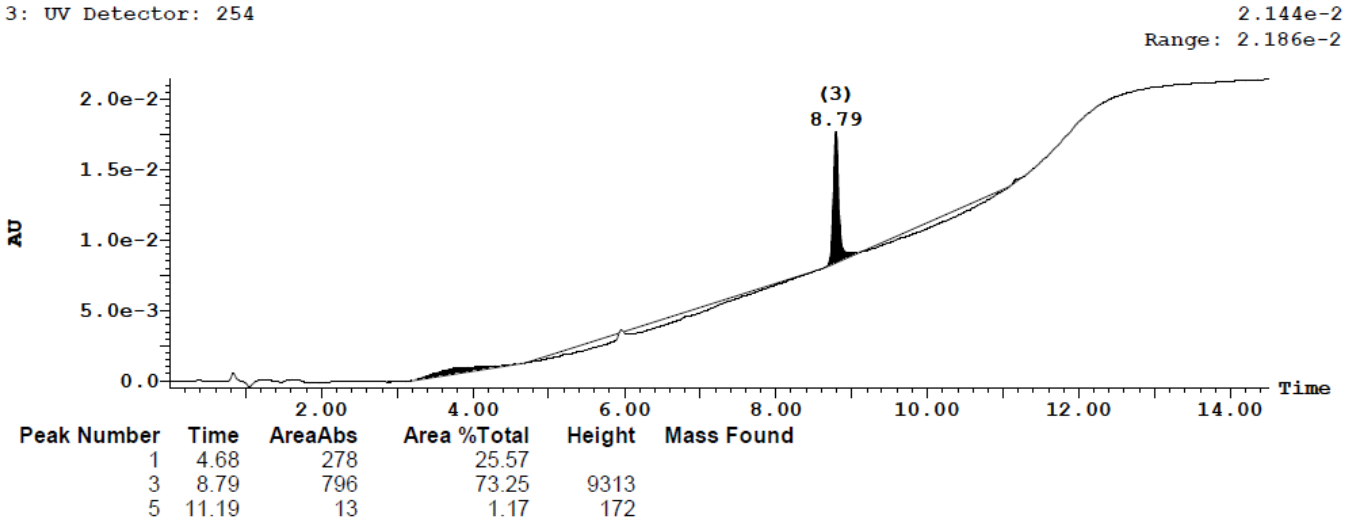
Appendix II

HPLC UV traces of final compounds

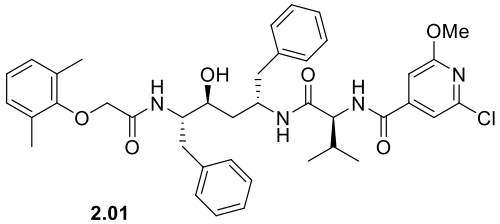


2.15

Sample Report:

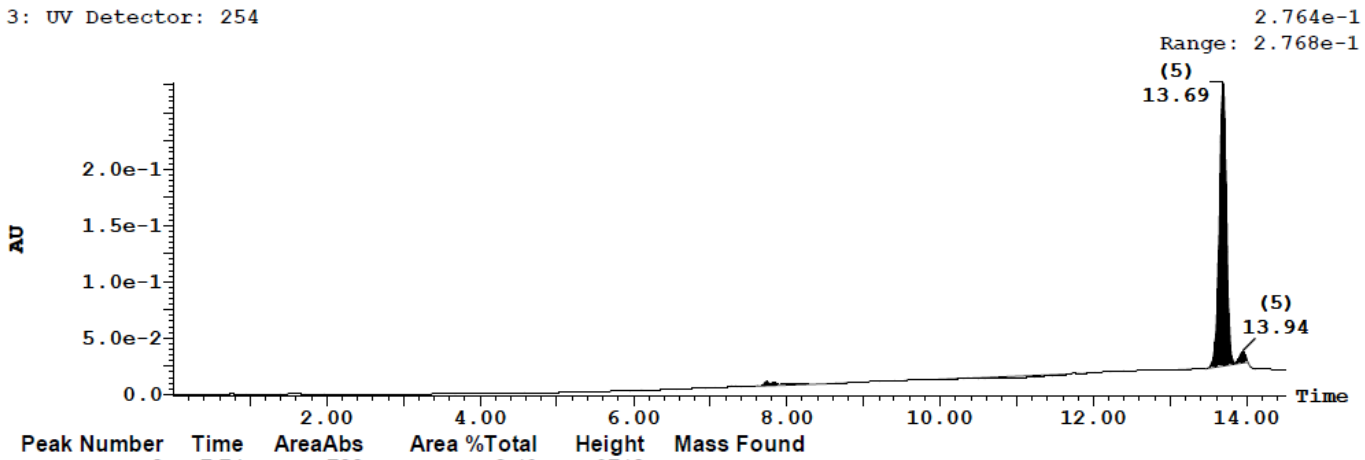


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
1	4.68	278	25.57		
3	8.79	796	73.25	9313	
5	11.19	13	1.17	172	

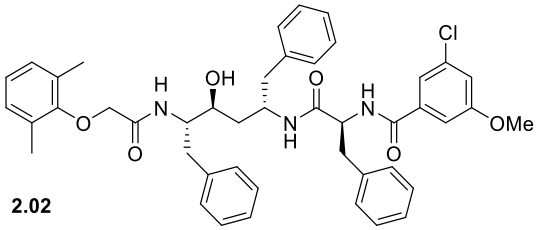


2.01

Sample Report:

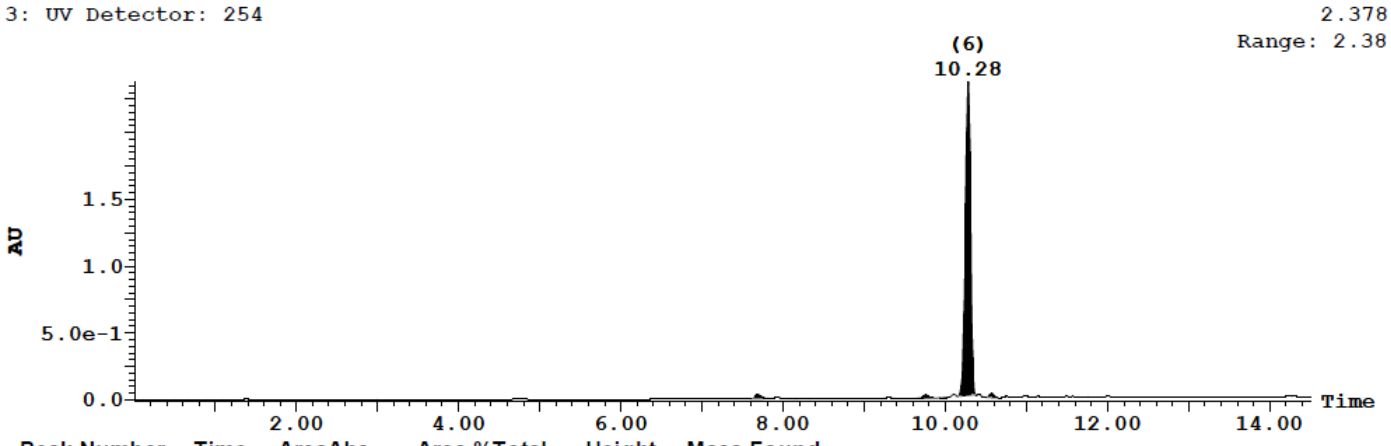


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
2	7.74	702	2.46	3710	
5	13.69	27837	97.54	251306	

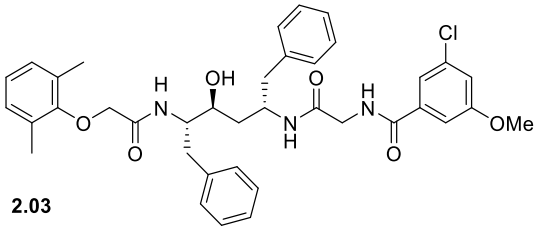


2.02

Sample Report:

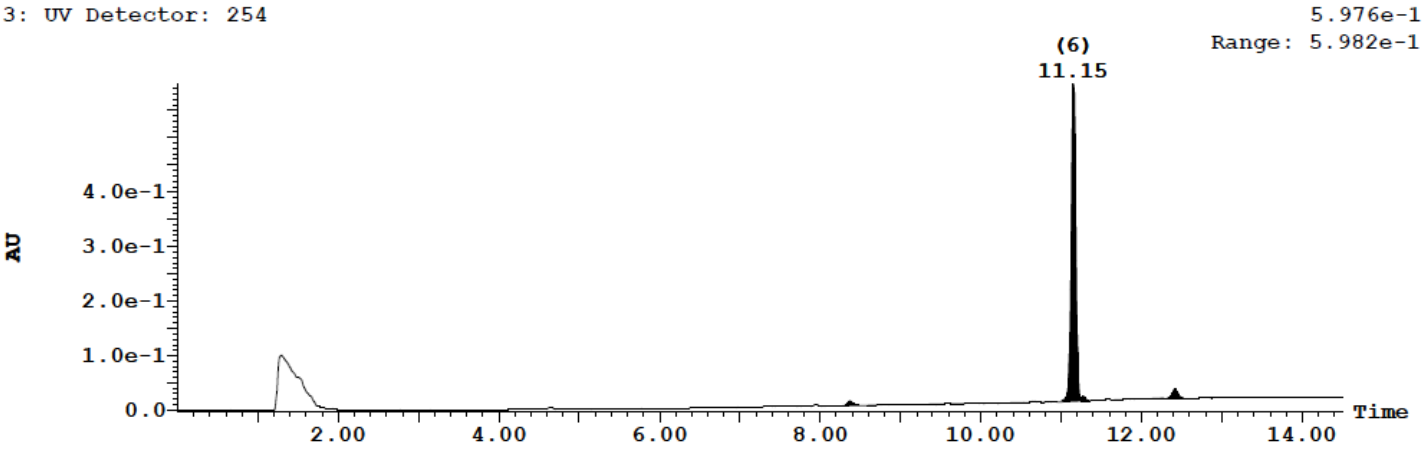


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
2	7.68	2704	1.54	32709	
4	9.76	1832	1.04	24995	
6	10.28	169066	96.33	2345245	
8	10.57	1901	1.08	30117	

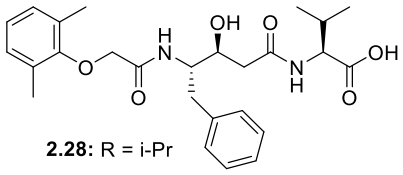


2.03

Sample Report:

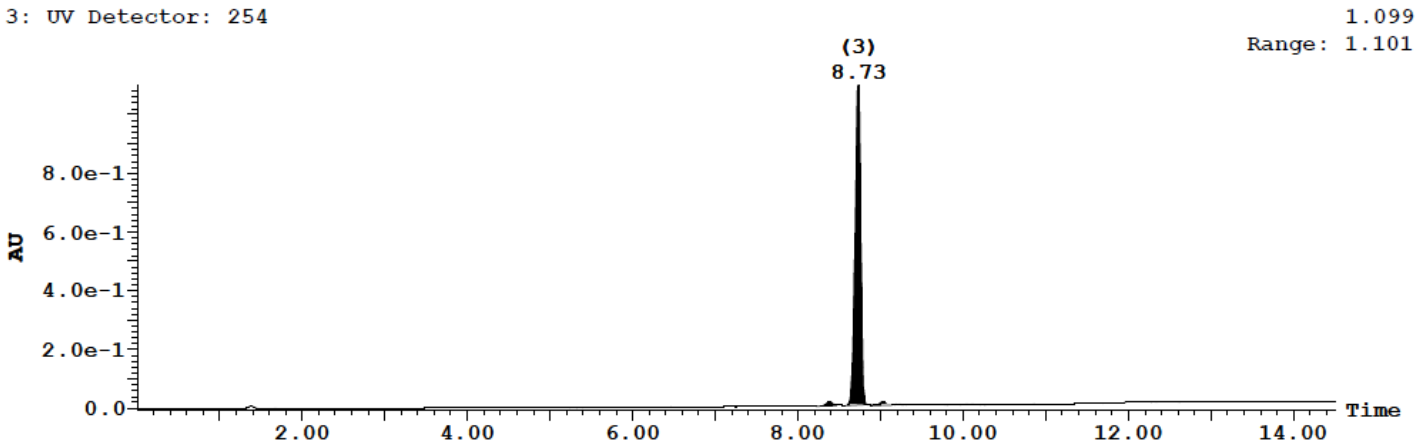


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
4	8.37	793	2.02	8810	
6	11.15	36706	93.49	581658	
7	11.28	453	1.15	7918	
9	12.42	1311	3.34	17015	

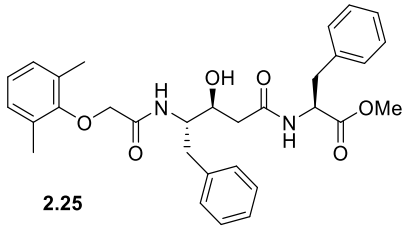


2.28: R = i-Pr

Sample Report:

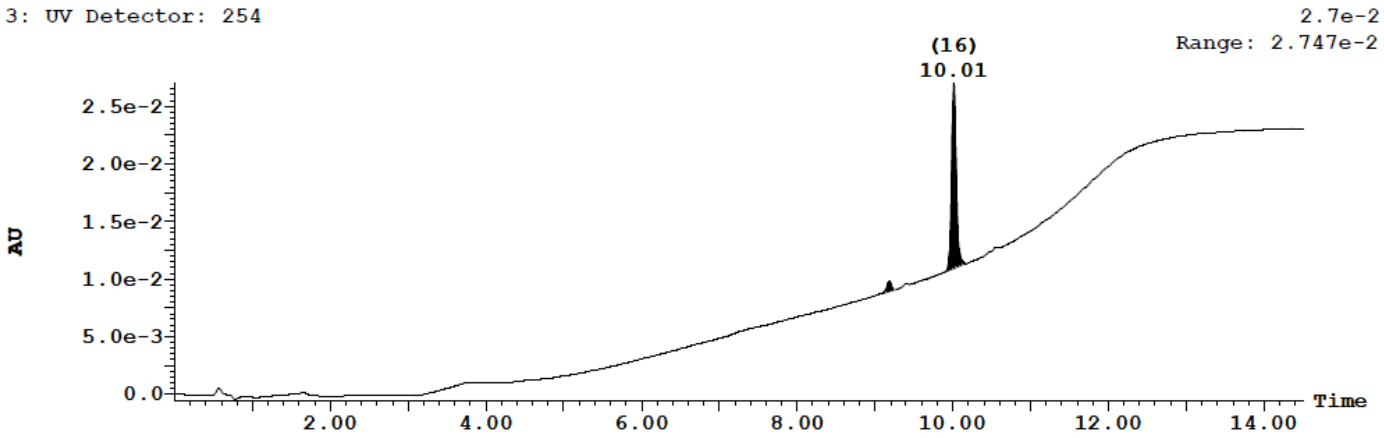


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
2	8.39	958	1.12	12755	
3	8.73	83284	97.82	1086579	
4	9.03	896	1.05	11953	

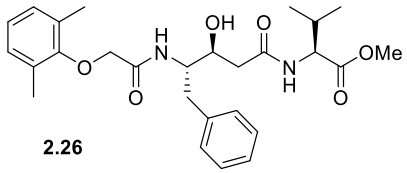


2.25

Sample Report:

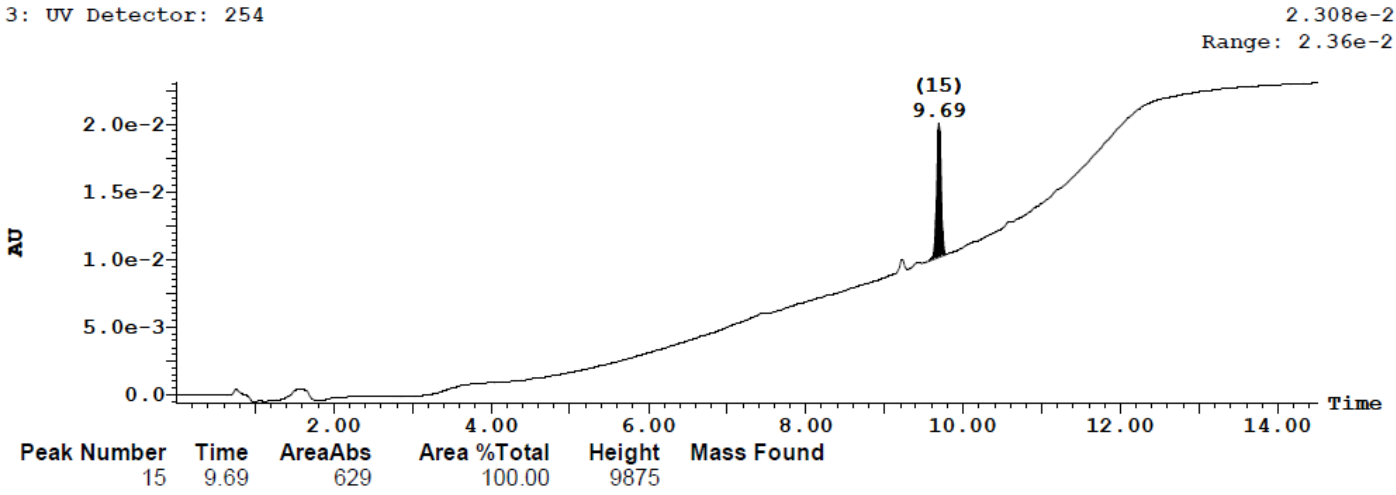


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
15	9.19	61	5.29	886	
16	10.01	1090	94.71	16087	

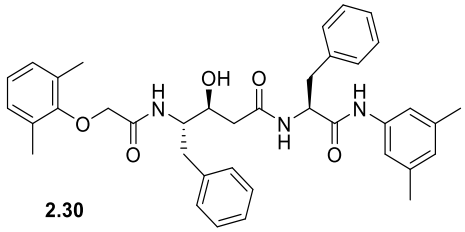


2.26

Sample Report:

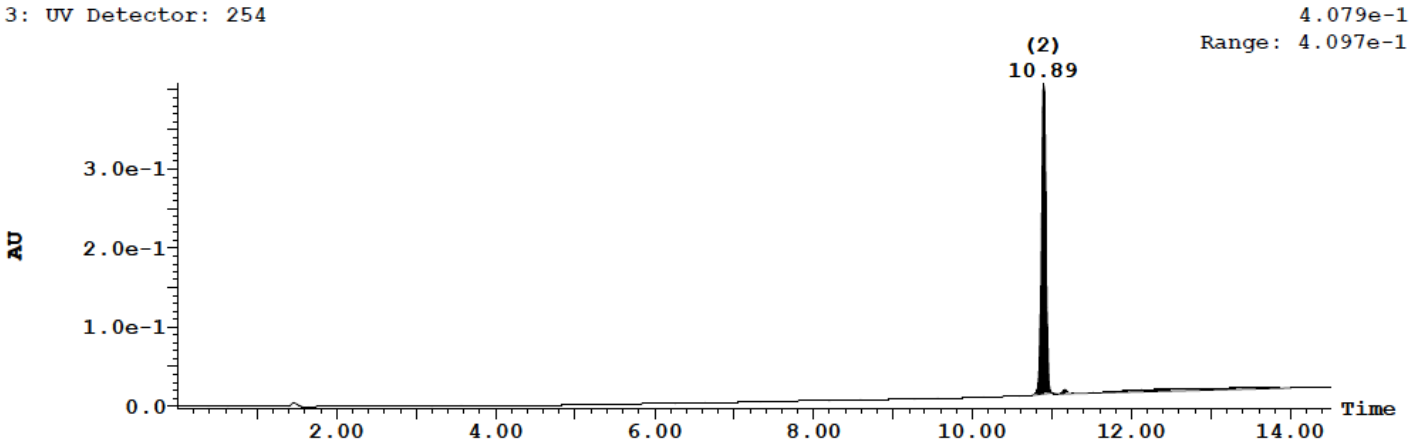


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
15	9.69	629	100.00	9875	

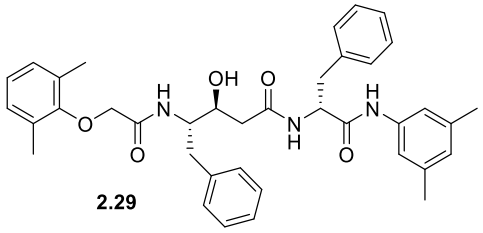


2.30

Sample Report:

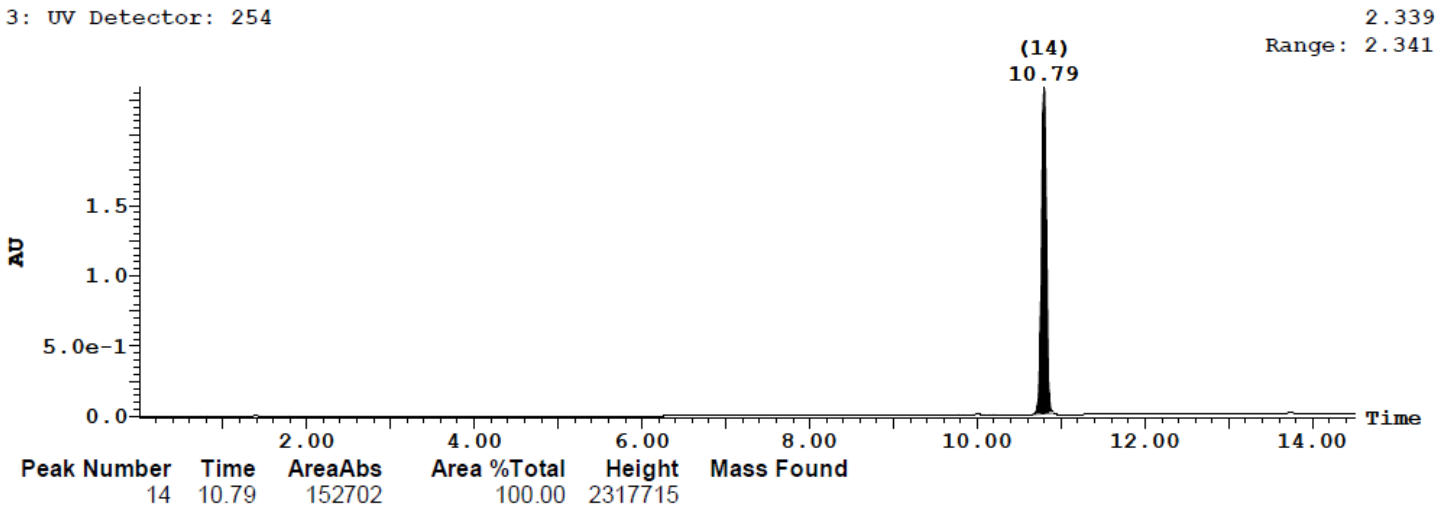


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
2	10.89	24623	83.69	392747	
3	11.16	319	1.09	5209	
4	14.01	4479	15.22		

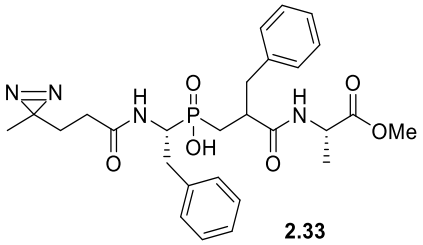


2.29

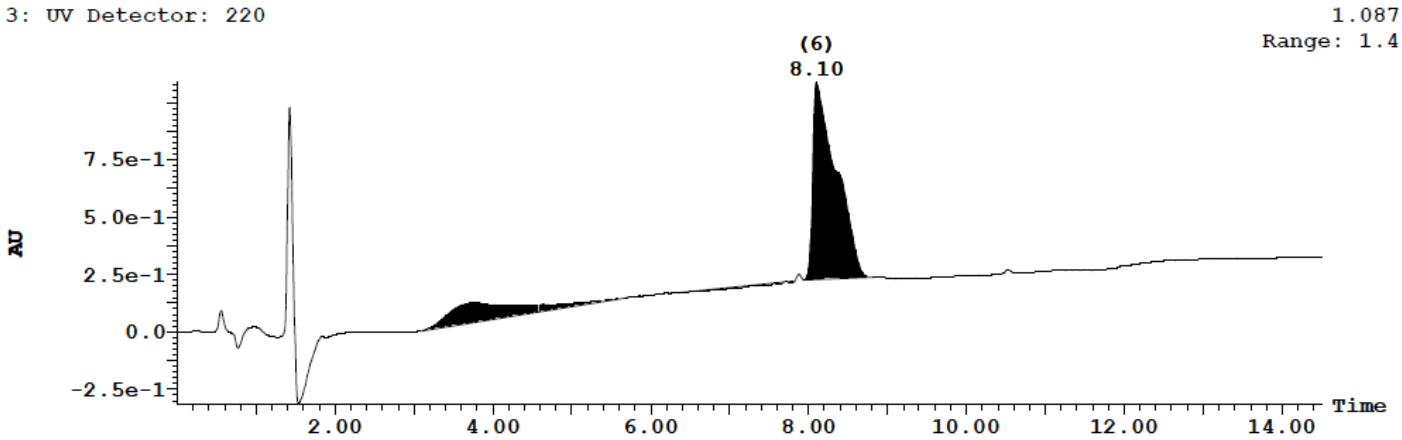
Sample Report:



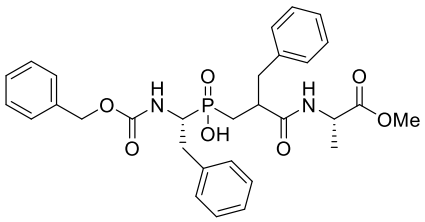
Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
14	10.79	152702	100.00	2317715	



2.33

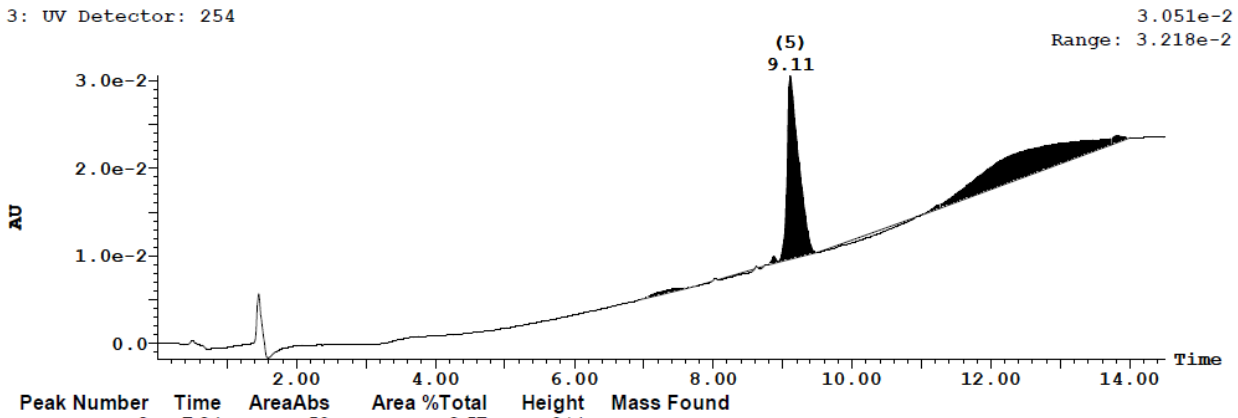


Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
1	3.76	76169	20.08	85858	
3	5.67	13389	3.53		
6	8.10	289862	76.40	858540	



2.32

Sample Report:



Peak Number	Time	AreaAbs	Area %Total	Height	Mass Found
2	7.24	53	0.57	314	
3	7.76	98	1.07		
4	8.88	49	0.53	775	
5	9.11	3964	42.91	20895	
6	11.26	49	0.54	383	
7	13.73	4886	52.90	702	
8	13.82	137	1.48	790	