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Design and Synthesis of TLR2 and TLR6 Heterodimer Ligands, a Triply Functionalized α-GalCer Derivative for Identifying Proteins Involved in Glycolipid Trafficking, and the Disaccharide of Staphylococcus aureus CP8 Towards a Self-Adjuvanting Vaccine

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A dissertation submitted to the faculty of
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Doctor of Philosophy

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ABSTRACT

Design and Synthesis of TLR2 and TLR6 Heterodimer Ligands, a Triply Functionalized α-GalCer Derivative for Identifying Proteins Involved in Glycolipid Trafficking, and the Disaccharide of *Staphylococcus aureus* CP8 Towards a Self-Adjuvanting Vaccine

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Toll like receptors (TLRs) are found on B cells, macrophages, monocytes, and dendritic cells, and these cells belong to the innate immune system that recognizes antigens and induces multiple cell responses through the release of cytokines. TLR1, TLR2 and TLR6 function as heterodimers, either as TLR1/TLR2 or TLR2/TLR6 to recognize lipopeptides. TLR1/2 dimer activation releases inflammatory cytokines, while TLR2/ TLR6 dimer activation releases immunomodulatory cytokines. Based on the size of the binding pocket between TLR2 and TLR6, it was hypothesized that lipopeptides, such as FSL1, could be simplified while keeping overall activity. FSL1 is a lipopeptide first isolated from *Mycoplasma salivarum* that activates macrophages at picomolar concentrations. It is expected that synthetic lipopeptides mimicking immunostimulatory molecules such as FSL1 will allow development of better ways to stimulate or modulate the immune system. Therefore, novel synthetic TLR2/6 ligands were synthesized replacing the polylysine chain with a polyamine chain showing activation of the immune cells in a manner like FSL1.

Natural killer T-cell (NKT) antigens, such as α-galactosylceramide (α-GalCer), are carried through the body by lipid transfer proteins before they interact with the NKT cells. Not all the proteins involved in glycolipid transportation have been characterized. The synthesis of an α-GalCer analogue, termed CD1d-Triceps was designed to help find additional proteins involved in glycolipid trafficking. CD1d-Triceps has three functionalities: the first is the α-GalCer structure, and the other two are on C6 of the sugar: biotin, which helps tag the molecule for its purification, and a photoactive tag that, upon UV light activation, will cross-link with neighboring proteins.

Antibiotic-resistant strains of *Staphylococcus aureus* (SA) are a growing health problem worldwide. Serotype 5 and 8 are the most common SA pathogens. Loading the serotype 5 or 8 disaccharides onto Qβ-particles that are linked to an NKT cell activator yield a vaccine that is expected to trigger adaptive immunity to the disaccharide. Previous similar studies showed production of antibodies with high affinity against *Streptococcus pyogenes* oligosaccharides in a similar vaccine.

Keywords: immune system, NKT cell, adjuvant, glycolipid, synthesis, organic, chemistry, immunology, trafficking, carbohydrate, TLR2, TLR6, ligand, Q-β particle, *Staphylococcus A*, serotype, disaccharide, vaccine

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Chapter 1: Synthesis and evaluation of TLR2 and TLR6 ligands

1.1 Introduction

Physical as well as immunological barriers protect our bodies against pathogens. Immunological responses to a pathogen typically involve both adaptive and innate immunity.[1] Innate immunity recognizes a pathogen via pattern-recognition receptors (PPRs. These PRRs are found on the surface of dendritic cells and other immune cells. Toll-like receptors (TLRs are PRRs that recognize specific pathogen-associated molecular patterns (PAMPs signaling an immune response so that our bodies can recognize and attack the pathogen. TLRs trigger an immune response via MyD88 dependent or independent signaling pathways, which lead to cytokine release.[2] Inappropriate production of certain cytokines can result in disease.[3] TLR agonists and antagonists are potential therapeutic candidates to help alleviate symptoms of various diseases associated with immune system disorders. Additionally, they are potential candidates as adjuvants due to their ability to trigger immune responses. Because TLR2 appears to recognize the greatest variety of agonists due to its collaboration with either TLR1 or TLR6, is a suitable TLR for which agonist and antagonist molecules may be created. The purpose of our work was finding better TLR2/6 ligands that trigger the immune response towards an immunomodulatory response rather than an immune stimulant cytokine release. By learning the requirements of TLR2/6 binding pocket and the corresponding agonists, better molecules could be synthesized, and immunomodulation be triggered by these agonists.

1.2 Toll Like Receptors

1.2.1 Structure

TLRs are type I membrane glycoproteins and consist of extracellular, transmembrane and intracellular signaling domains. The two main domains of TLRs are LRR (leucine rich repeats and the TIR (Toll-IL-1 receptor domain (Figure 1. TLRs are part of the innate immune system. Innate immunity works in minutes to hours and triggers a response that relies on cytokines; on the other hand, adaptive immunity takes from seven to ten days to be effective and depends on cytotoxic T cells maturation and B cell production of antibodies with high affinity to antigens.[2, 4] Rock et al. found the mammalian homologue of Toll receptor found in the genes of *Drosophilla melanogaster* related to immune protection against fungal infection; the homologue proteins in humans were therefore called Toll-like receptors and were found in monocytes, macrophages, dendritic cells, T cells, and other immune cells.[5] Later, Medzhitov et al.[6] worked with TLRs to elucidate their signaling pathway in mammalian cells, which was found to signal parallel to the signaling pathway induced by the IL-1 receptor (IL-1R. This pathway results in NF-kB translocation to the nucleus, which results in transcription of proinflammatory cytokines.[5, 7] The LRR domain of TLRs create a "horseshoe" like structure.

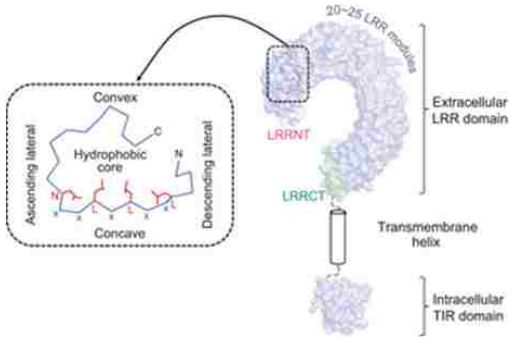


Figure 1. Overall structure of a Toll-like receptor (TLR). The extracellular and intracellular domains of TLR4 are in blue.[8]

1.2.2 Classification

There are twelve TLRs in mice (TLRs 1-9 and 11-13 and ten in humans (TLR1-10 all found in immune cells.[2-3] All TLRs can be classified according to their localization and also the PAMP they recognize. TLRs that are expressed on the cell surface include TLR1, TLR2, TLR4 (they recognize LPS from Gram-negative bacteria, TLR5 (recognizes flagellin, and TLR6. TLRs that are present only on intracellular vesicles such as the endoplasmic reticulum, endosomes, or lysosomes include TLR3 (recognizes double stranded RNA, TLR7, TLR8 and TLR9 (recognize bacterial or viral nucleic acid and synthetic viral compounds.[1-2, 9-10] TLR1, TLR2, and TLR6 work as heterodimers: TLR1 and TLR2 recognize triacyl lipopeptides, TLR6 and TLR2 recognize diacylated lipopeptides.

1.2.3 TLR signaling pathways

TLR transmembrane and intracellular domains contain a toll-interleukin I (toll/IL-I receptor (TIR-I required for their signaling.[2] TLR2 signals through the MyD88-dependent (myeloid differentiation factor-88) pathway that activates NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells and subsequently induces genes encoding inflammatory cytokines.[1] The signaling pathway is not completely understood, but many adapter proteins and cascade components have been characterized.

After TLR2 is activated, its TIR domain associates with MyD88, which then is followed by recruitment of IL-1 receptor-associated kinase 1 and 4 (IRAK. IRAK4 is activated by phosphorylation and subsequently it associates with TRAF6 (tumor necrosis factor receptor (TNFR-associated factor, which mediates cytokine signaling pathways. TIR-domain-containing

adaptor protein (TIRAP, or MyD88-adaptor-like-protein (MAL are also essential for MyD88-dependent signaling of TLR2 and TLR4.[11] TOLLIP (Toll-interacting protein has been shown to also associate directly with the cytoplasmic TIR domain of TLR2 and TLR4, and to inhibit TLR-mediated cellular responses by suppressing the phosphorylation and kinase activity of IRAK1.[12] Thus, TOLLIP has a regulatory function in TLR signaling cascade. The final signaling step is when NF-κB is activated through phosphorylation and proteolysis of the IκB. The activation of NF-κB results in releasing of NF-κB factor and transcription of genes that encode for cytokines.[11-12]

1.2.4 TLR2, TLR1 and TLR6

TLR2 PAMPs include, but are not limited to, lipopeptides derived from bacteria, peptidoglycan or lipoteichoic acid from gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, and virus.[13] TLR2 recognizes the greatest variety of agonists due to its ability to form heterodimers with TLR1 and TLR6 respectively. TLR2/TLR1 heterodimer recognizes triacylated lipopeptides, whereas TLR2/TLR6 heterodimer recognizes diacylated lipopeptides.[1] The convex face of LRR domain of TLR2 has a hydrophobic channel that interacts with the two lipid chains of the lipopeptide. In the case of triacylated lipopeptide, the third lipid chain interacts with the hydrophobic channel of TLR1, thus stabilizing the heterodimer TLR1/TLR2 (Figure 2. This is not the case for diacylated lipopeptides since the TLR6 hydrophobic pocket is blocked by two phenylalanines. The absence of the hydrophobic pocket on TLR6 is replaced by a greater TLR2/TLR6 interaction, which makes the heterodimer more stable through hydrogen bonding. TLR2-TLR1 and TLR2-TLR6 heterodimers share an m-shaped structure.[14] Jie-Oh Lee et al.[15] used a synthetic, diacylated lipopeptide Pam_2CSK_4, and triacylated Pam_3CSK_4, known to mimic TLR2,

TLR1 and TLR6 binding and published crystal structures of TLR2/TLR6 diacylated lipopeptides followed by TLR1/TLR2 crystal structures.[14-15]

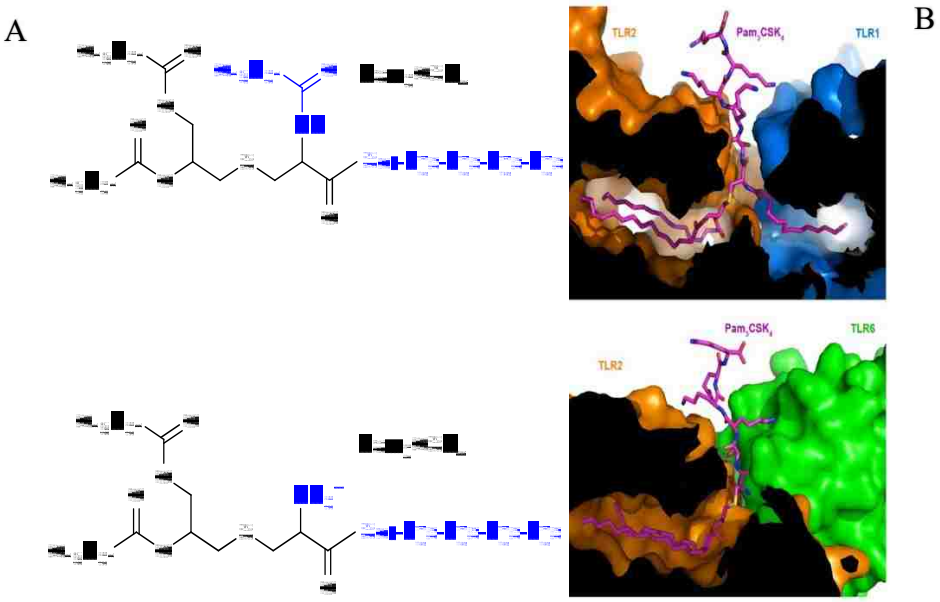


Figure 2 A: Structures of synthetic TLR2/6 ligands;[15] **B:** (right shows the crystal structures of the binding of TLR2/6 ligands Pam_3CSK_4 and Pam_2CSK_4 with TLR2/1 and TLR2/6 heterodimers respectively.[16]

1.2.5 TLR2 and diseases

The main function of TLRs is to induce the establishment of adaptive immunity and production of inflammatory cytokines. Said cytokines activate surrounding cells to produce chemokines and adhesion molecules that help inflammatory cells fight infection.[2] Out of all the types of TLRs, much attention has been paid to TLR2 in terms of disease pathogenesis. TLR2 has been implicated in diseases such as rheumatoid arthritis (RA,[17] type I diabetes (T1D,[18] inflammatory bowel disease (IBD, UC and CD,[19] psoriasis, asthma, allergies, and atherosclerosis.[20]

1.2.6 Agonists and antagonists

Because cytokines serve as molecular messengers between cells, autoimmune diseases can be triggered when there is overproduction or inappropriate production of certain cytokines by the body.[3] This shows the need to synthesize TLR2 agonists (for use as adjuvants in vaccines and antagonists (for therapeutic purposes.[21] Synthetic TLR2 antagonists may block the inflammatory response that is present in many diseases where immune disorders have developed.[3] TLR agonists and antagonists would modulate activity of TLRs and other signaling molecules which would have an impact in the immune therapy of allergy, autoimmunity, transplantation, and cancer. The purpose of our research was to understand better the requirements of the agonists to bind in the TLR2/6 pocket and to trigger immune modulation cytokines.[22-24]

It has been demonstrated that TLR2/1 activates cytokine production of IL-2, or IL-6, which stimulate dendritic cells towards pro-inflammatory T regulation resulting in cellular immunity. On the other hand, stimulation of TLR2/6 results in a humoral immunity through the release of IL-10 mostly which is an anti-inflammatory cytokine. These results show that TLR2 can produce either pro or anti-inflammatory responses in the immune system. The synthesis of stable TLR2/6 ligands require only a diacylated or small chain on the lipopeptide instead of a triacylated lipopeptide. The aims of our research was to synthesize molecules that would stabilize the TLR2/6 heterodimer to produce TH2 response and synthesis of IL-10 and IL-12.[25]

1.2.7 Synthetic routes to obtain TLR2 agonists and antagonists

Two well-known TLR2/6 agonists are MALP-2 derived from *Mycoplasma fermentans*[26] and FSL-1 from *Mycoplasma salivarum.*[27] One of the first synthesized mimics of these agonists was published by J.W. Metzger in 1991.[28] All synthetic TLR2/6 agonists contain two ester-linked fatty acids bound to a glyceryl cysteine, a free amine, and a peptide portion (Figure 3. Synthesis of TLR2/1 ligand functionalized the amine of the cysteine by acetylation giving a third acyl chain that occupied the pocket on TLR 1 for the heterodimer formation as was observed previously with Pam_3CSK_4. The activity of synthetic TLR2/6 ligands is usually measured against FSL-1 since it is higher than that of MALP-2, the measurement used for the activity of these compounds is the IL-10 released by the cells after treatment with either compound.[26]

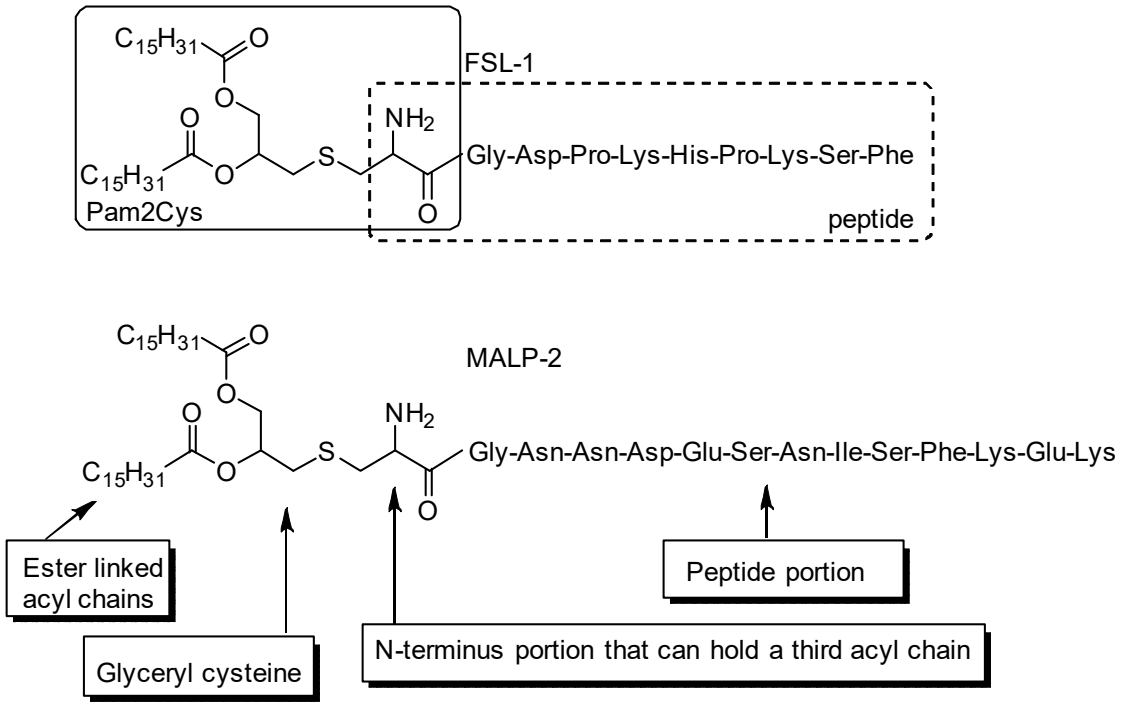


Figure 3. Structures of FSL-1 and MALP-2, TLR2 natural immune-stimulators.

For our synthetic ligand we kept the part of the ligands that have a direct interaction in the pockets of TLR 2 which is the glycerol with the two ester linked acyl chains. Because the amine is the

closest to the pocket of TLR 6 blocked by the two phenylalanines we decided to add different acyl chains to investigate the size of that pocket; and because the peptide chain is not directly involved in the recognition of the ligand by the receptors we decided to change the peptide chain for a synthetic polyamine chain that will keep the overall size of the molecule and most important the overall polarity (Figure 4).

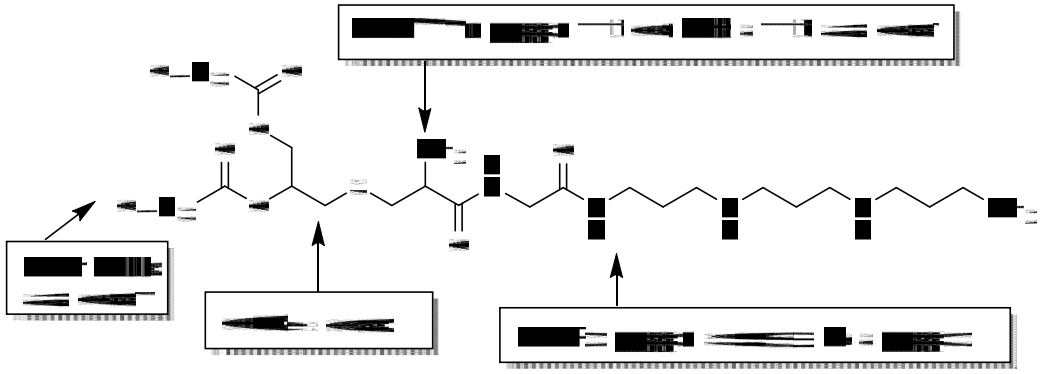


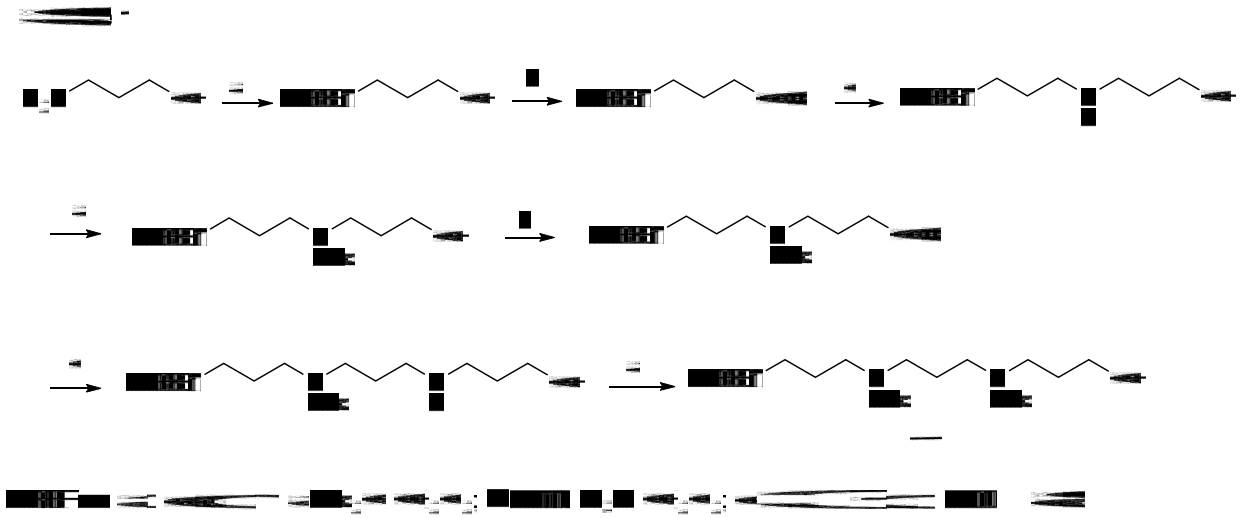
Figure 4. Synthetic TLR2/6 ligand structure.

1.3 Synthesis

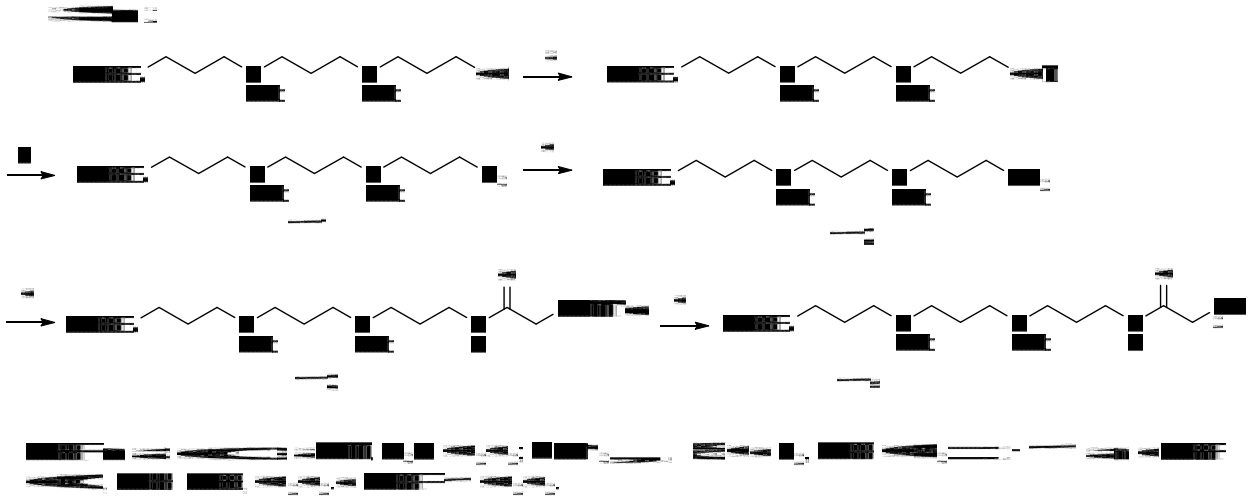
Because most synthetic TLR2 ligands contain two esters linked to a peptide chain by a cysteine and a glyceryl molecule, the compounds we synthesized were called LPMs (Lipid-Peptide Mimetics the lipid part represents the acyl chains attached to the cysteine and the polyamine chain mimics the peptide found in the original TLR ligands. LPMs consisted of two main building blocks: the polyamine chain that replaces the peptide chain of the original TLR ligand (such as FSL-1, and the diacylated cysteine derivative. Because we were trying to study the size of the pocket between TLR 2 and TLR6 the free amine was left intact as a first approach and acyl chains of increasing size were linked later to study how big the chain could be without interfering with the heterodimer TLR2/6 formation.

1.3.1 Synthesis of the polyamine chain

The polyamine chain was obtained by repeating units of 3-amino-propanol. The amine of 3-amino-propanol was first Boc protected, followed by mesylation of the corresponding hydroxyl group and nucleophilic substitution by another 3-amino-propanol molecule thus elongating the chain (Scheme 1. The resulting secondary amine was protected with Boc anhydride and the corresponding terminal hydroxyl activated again by mesylation, and substitution by another molecule of 3-amino-propanol. At the end of the synthesis, there were three molecules of 3-amino-propanol linked together in compound **1.10**.



The hydroxyl was once more activated by mesylation but this time substituted by sodium azide to give **1.11**. Hydrogenation of **1.11** gave compound **1.12**. This amine was linked by a peptide bond formation to FMOC protected glycine which elongates the chain a little further and increases the solubility (Scheme 2). Once the amine was deprotected with piperidine, it was ready to be coupled to the mono-, di-, or tri-acylated piece of the molecule by peptide bond synthesis.

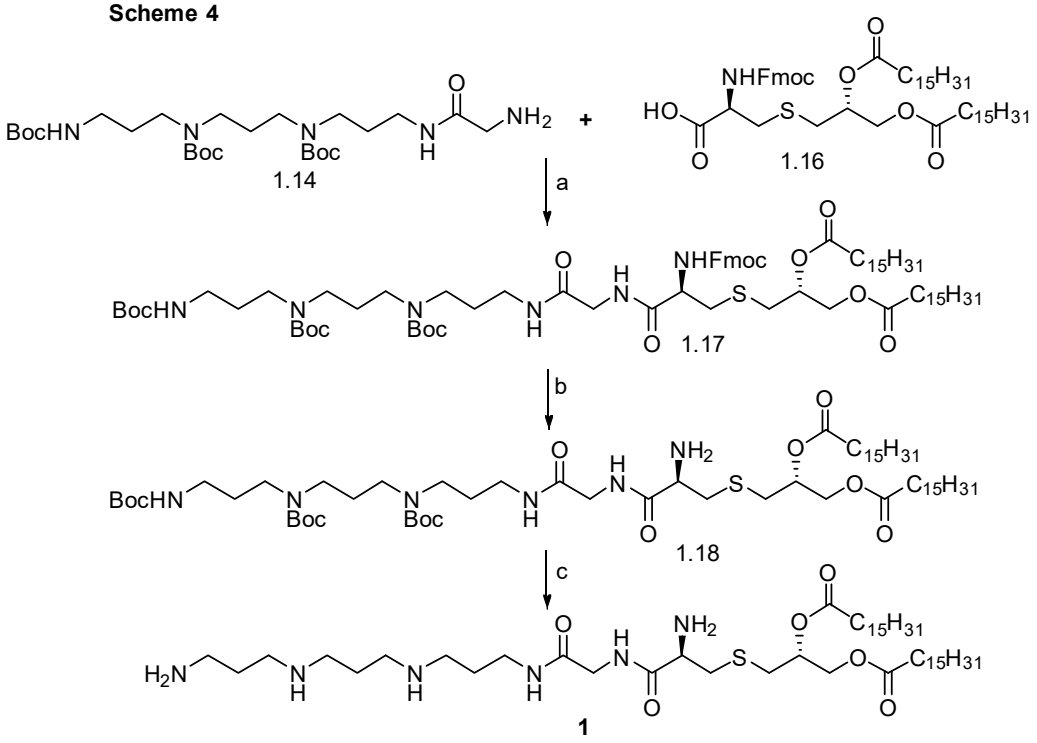


1.3.2 Synthesis of the cysteine derivative

The synthesis of di or tri-acylated block started with N-α;N-α-Bis-Fmoc-L-cysteine bis(tert-butyl ester disulfide. The disulfide bond was cleaved and later the thiol attacked (S or (R-glycidol to give **1.15**. The diol could produce the monoacylated compound, or diacylated using palmitoyl chloride with or without DMAP to form the esters. The tert-butyl ester used to protect the acid was cleaved under acidic conditions using trifluoroacetic acid giving compound **1.16**. Studies have shown that the minimal requirements for the activation of TLR2 is the cysteine and the acyl chains, therefore the synthetic ligand could be simplified to a polyamine chain without decreasing the activity, the size of the chain was kept close to the original amino acid chain to maintain the solubility of the molecule (Scheme 3.[29]

Compound **1.17** was obtained forming the peptide bond between amine **1.14** and acid **1.16** with the help of the carbodiimide EDCI and HOBT. The FMOC protected amine was deprotected with piperidine to be later functionalized. **1** was obtained after global Boc deprotection with HCl in Dioxane (Scheme 4).

Scheme 4



Reagents: **a)**HOBt, EDCI, CH_2Cl_2; **b)**Piperidine, CH_2Cl_2; **c)**HCl in 1,4-dioxane, CH_2Cl_2.

Amine **1.18** was functionalized with acetyl, benzoyl or isobutyryl chloride affording the desired compounds followed by global deprotection with hydrochloric acid in Dioxane (Figure 5).

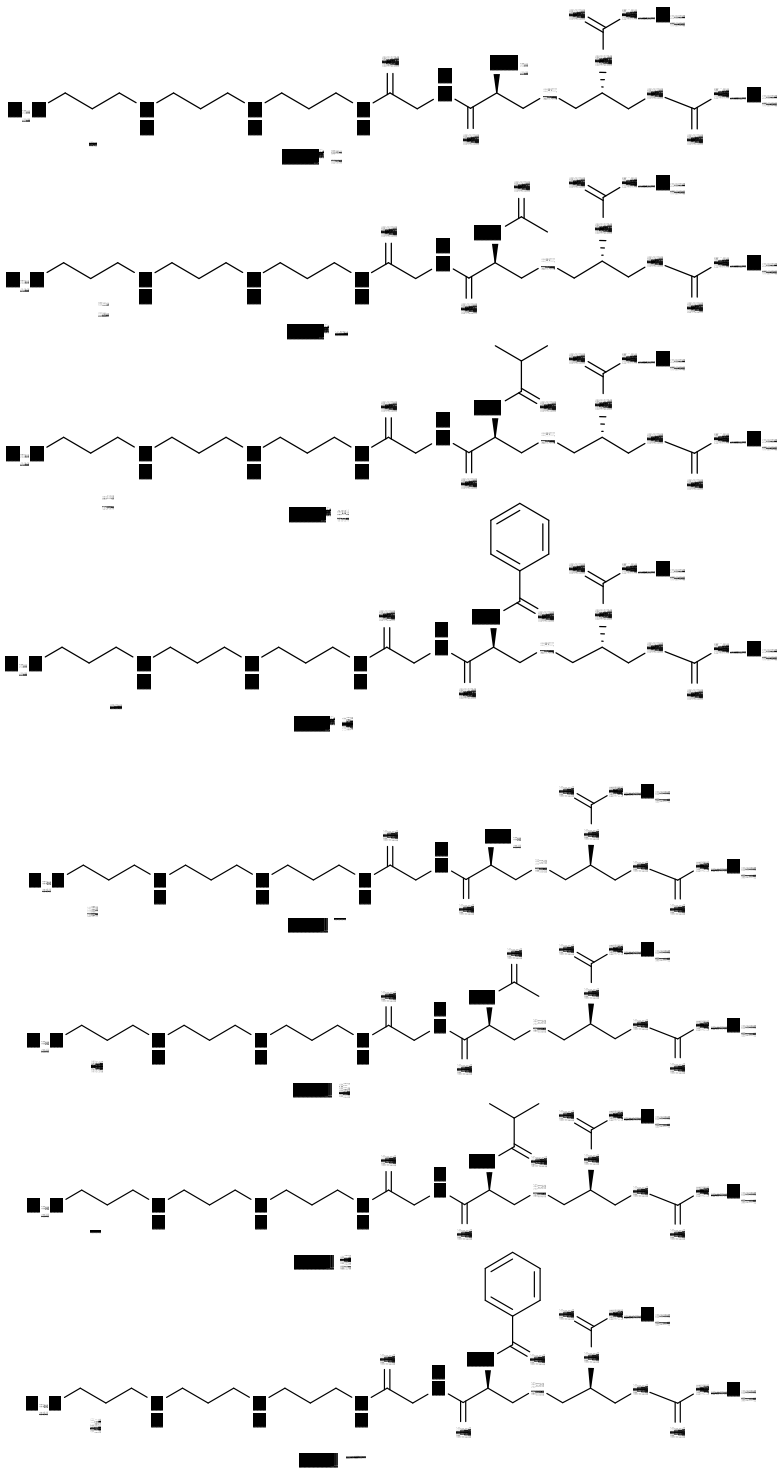
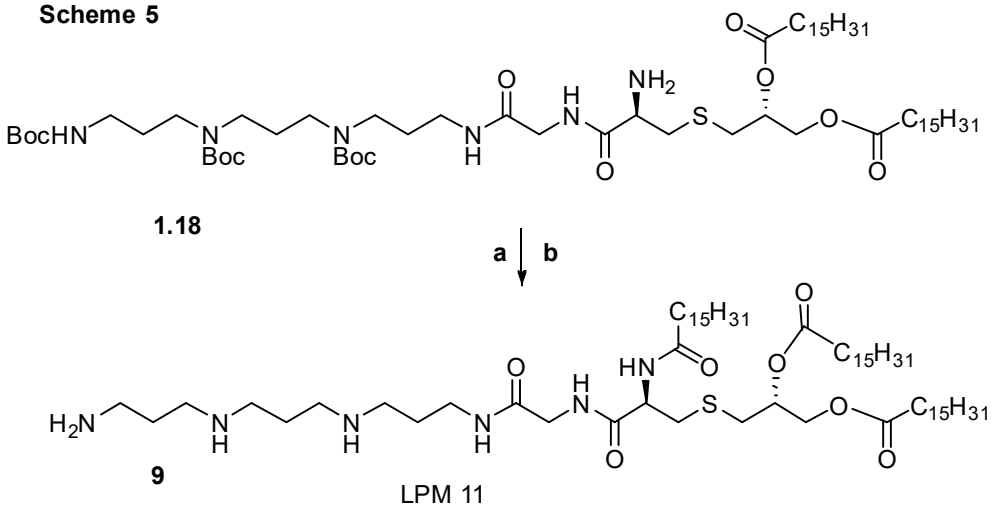


Figure 5. Structure of LPM compounds synthesized.

1.3.3 Synthesis of the TLR2/6 variants

From compound **1.18** the amine was acylated with palmitoyl chloride to give triacylated compound derivative (Scheme 5.

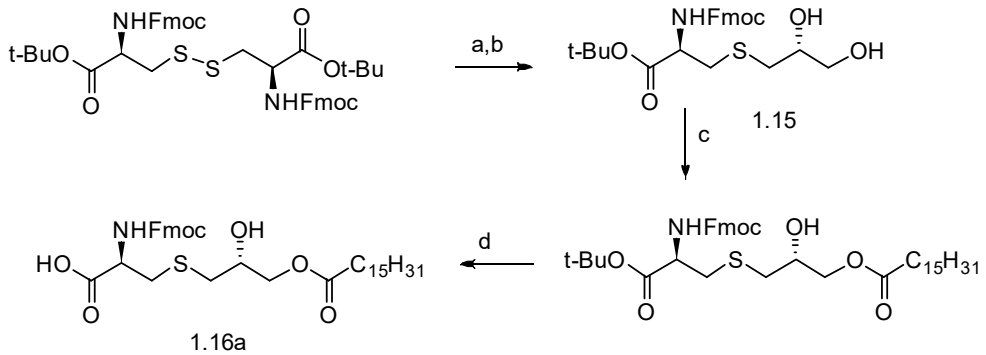
Scheme 5



Reagents: **a)** Palmytoyl Chloride, CH_2Cl_2,Et_3N; **b)**HCl in dioxane, CH_2Cl_2.

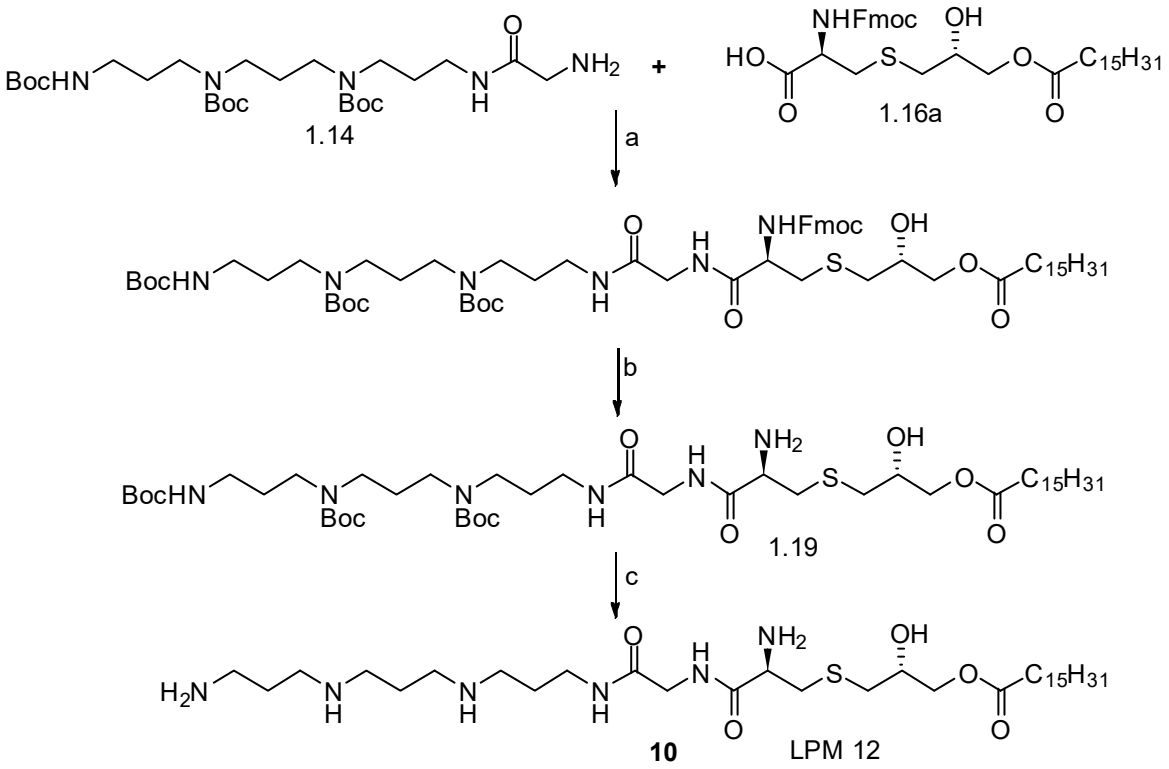
To form the mono-acylated compound the reaction of the glycidol to form the diol was done in the same way that the diacylated compound but without using DMAP, this formed only the acylated molecule on the primary alcohol and the secondary alcohol was unreacted (Scheme 6 and 7).

Scheme 6



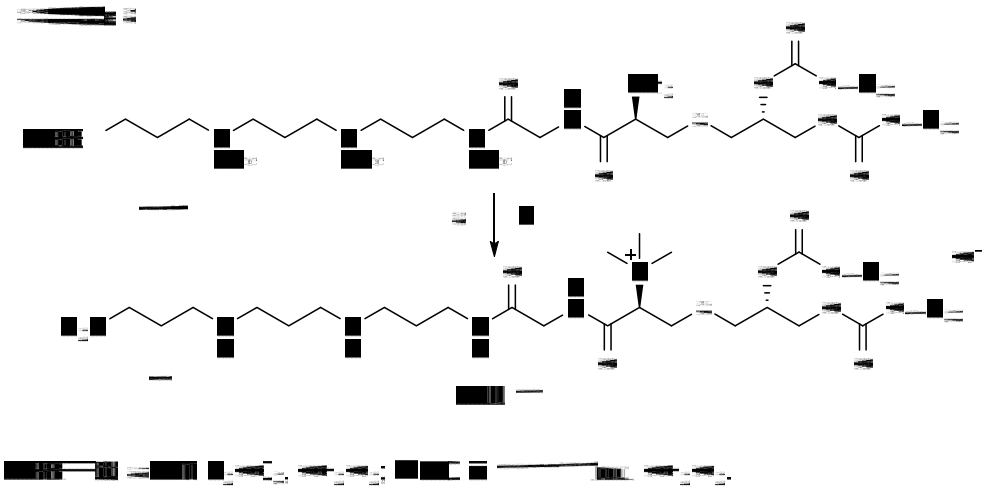
Reagents and conditions: **a)**Zn/HCl/H_2SO_4; **b)** R-glycidol, 5h, r.t; **c)**Palmitoyl-Cl, Et_3N, CH_2Cl_2, CH_2Cl_2; **d)**TFA 50% in CH_2Cl_2.

Scheme 7



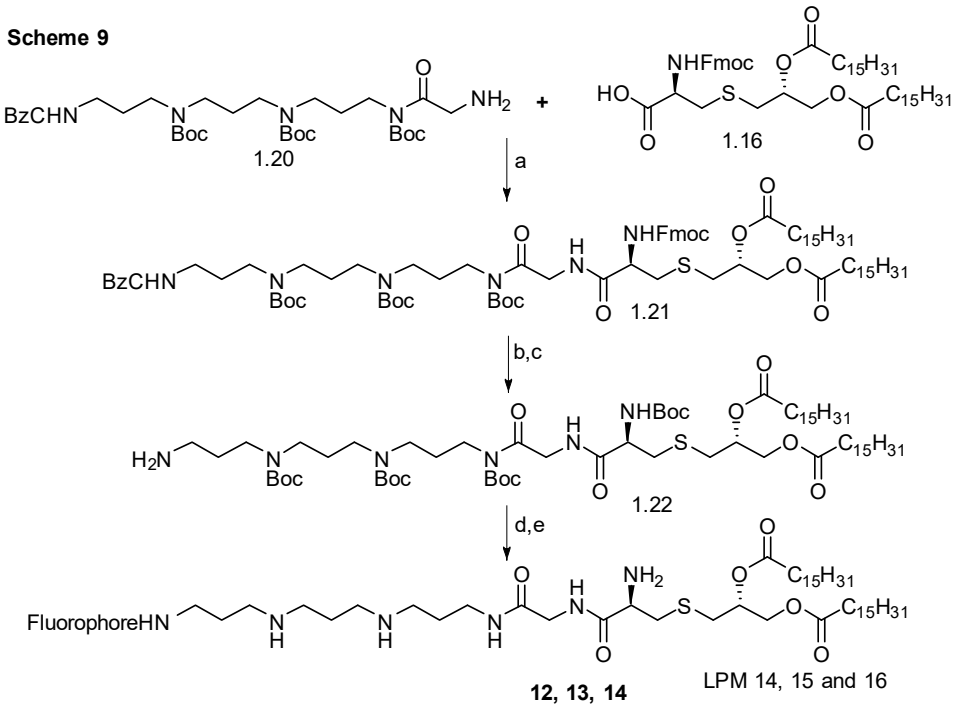
Reagents: **a)**HOBt, EDCI, CH_2Cl_2; **b)**Piperidine, CH_2Cl_2; **c)**HCl in 1,4-dioxane, CH_2Cl_2.

Because the functionalized amine of the synthetic LPMs lays close to the two phenylalanine bases that block the pocket of TLR6, we hypothesized that a positively charged amine would be able to coordinate with the negatively charged rings as a cation-π ring interaction.[30] We functionalized the amine of intermediate **1.18** reacting it with methyl iodide to form the salt, and later global deprotected to obtain compound **11** as shown in Scheme 8.



The final approach to understand the activation of TLR2/TLR6 ligands included the synthesis of the diacylated and triacylated fluorophore derivatives; These were synthesized in a very similar way, with the first amine of the chain protected with CBZ instead of Boc. This gave intermediate **1.20**, which was deprotected in the final steps of the synthesis to obtain the desired compounds as shown on Scheme 9.

Scheme 9



Reagents: **a)**HOBt, EDCI, CH_2Cl_2; **b)**Piperidine, CH_2Cl_2; **c)**Boc_2O, CH_2Cl_2;**d)**Fluorophore-Cl, Et_3N, CH_2Cl_2; **e)**HCl in 1,4-dioxane, CH_2Cl_2.

1.4 Results and discussion

The LPMs synthesized were sent to our collaborator Sangman Kim at the University of Chicago Medical Center - DDRCC (Digestive Diseases Research Core Center under the supervision of Dr. Bana Jabri. Sangman Kim used dendritic cells to measure the TLR responses through IL-10 release. He isolated splenic APCs from WT BL/6 or TLR6-/- mice using OptiPrep density gradient following with the treatment of the APCs (1 million cells per well with known TLR2/6 ligand FSL1 (100 ng/mL, or synthetic ligands (200 or 2000 ng/mL. These were incubated at 37 °C for 24 and 48 h, and later ELISA was used to measure IL-10 levels. From the results, we learned that the bigger the group on the amine of the cysteine, the less was able to fit in the hydrophobic pocket of TLR6. The best stimulation happened when the stereocenter of the glycidol is R. Therefore, following LPM 9, the monoacylated, triacylated, positively charged amine, and fluorophores were synthesized with R-glycidol. LPM1 and LPM2 were previously synthesized by Dr. Yanshu Feng;[31] their activity is not as high because the group found on the amine is bulky and aromatic. LPM3, LPM4 and LPM7 induced similar levels of IL-10 as known TLR2/6 ligand FSL1 (mostly LPM3 and LPM7, which are the LPMs synthesized with both R and S- glycidol respectively and with a free amine moiety (Figure 6. Subsequently, LPM4 and LPM8 respond somewhat but less than FSL1, and LPM6 has some induction (Figure 7. Unfortunately, LPM5, LPM9 and LPM10 have almost no IL-10 induction (Figure 8. The acyl groups found in these molecules are bigger and bulkier, which corresponds to a less active ligand towards TLR2/TLR6 activation. This decrease in activation correlates to the pocket on TLR6 not being big enough for those acyl chains to fit, and therefore the LPMs are not able to activate the heterodimer.

The synthetic LPMs differ from the FLS1 ($Pam_2CysSKKK$ structure in that the amino acid next to cysteine is glycine rather than serine. As described by Dr. Feng, the poly-lysine chain was

replaced by a polyamine to keep the polarity and the chain in place.[29] There is evidence that the role of the lysine chain is to provide solubility to the lipid. The other advantage that a polyamine chain provides is the stability against proteolytic degradation. The graphs show the results from LPM 1 and 2 from Dr. Feng and my synthetic ligands,[31] the levels of IL-10 production were normalized to the concentration of FLS1 used to treat APCs (positive control).

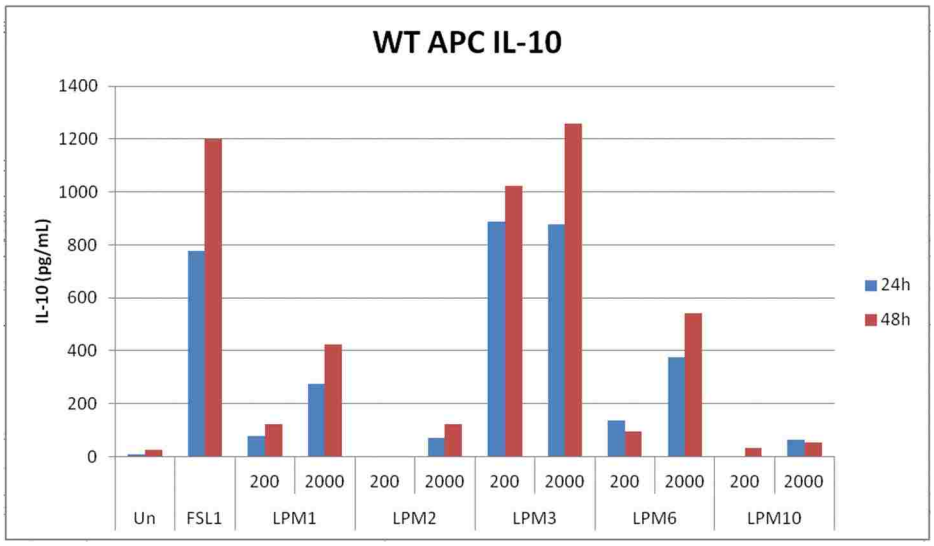


Figure 6: Wild type APC IL-10 production graph normalized to FSL1 production.

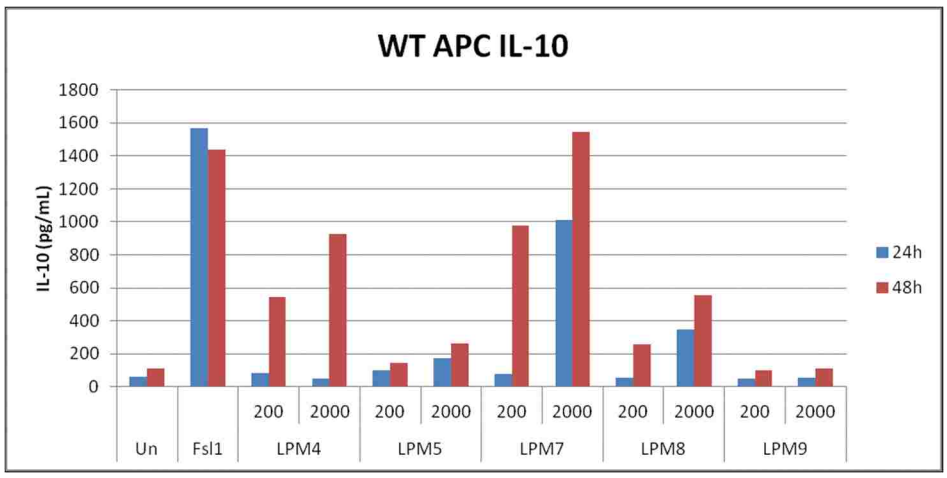


Figure 7: Wild type APC IL-10 production with treatment of LPMs.

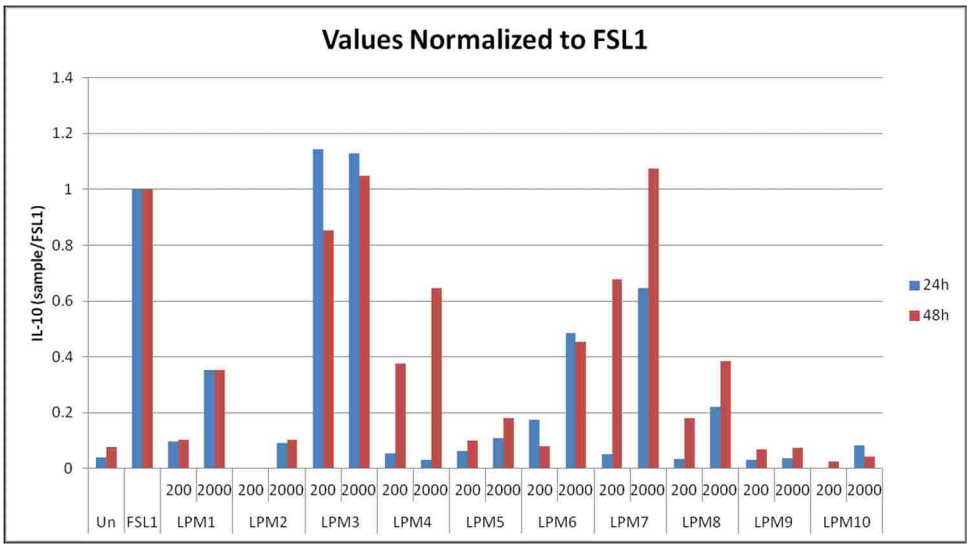


Figure 8: Normalized values of IL-10 production from APCs cells treated with FSL1 and synthetic TLR2/6 ligands.

1.5 Conclusions

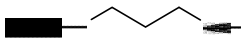
There are two possible outcomes when TLR 2 is activated. One is activation by triacylated lipopeptides of heterodimer TLR2/1, triggering an immune stimulant response with application in vaccines, and perhaps one of the most successful medical interventions against infectious disease.[32] Due to their ability to activate antigen presenting cells (APCs and recognize a greater variety of PAMPs, TLR2/1 agonists can be excellent adjuvants.[2-3, 22]

The second outcome is done by diacylated lipopeptides triggering the activation of heterodimer TLR2/6 towards an anti-inflammatory response. This was the purpose of our research, in other words, the therapeutic applications that good TLR2/6 ligands could have a tremendous impact blocking inflammatory responses present in many diseases such as allergy, autoimmunity, transplantation, and cancer.[22-24]

From the results we obtained, we conclude that the bigger the group on the amine of the cysteine the lesser could fit in the hydrophobic pocket of TLR6. The best stimulation happened when the stereocenter of the glycidol was R, with a free amine and started decreasing as the group bonded by amide bond into that amine increased in size. This activity was noticed by the increased activity of the free amine followed by the acylated amine, isobutyrylated, and finally the benzoylated towards stimulation to the APCs.

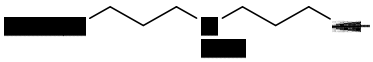
Further experiments could be made by synthesizing more TLR2/6 ligands substituting the Nitrogen atom for other elements that could lead to a better interaction with the phenylalanines found blocking the pocket of TLR6. Depending on the results from the positively charged amine, explore other atoms with a positive charge to increase the cation-π ring interaction.[30]

1.6 Experimental Procedures



1.1

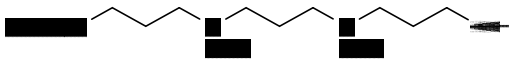
To a round bottom flask (RBF) with 3-amino-1-propanol (20 g, 267 mmol) in DCM (300 mL) was added Boc_2O (69.8 g, 320 mmol) slowly. The solution was stirred overnight, then concentrated under vacuum, purified through column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes). Compound **1.1** was obtained (43 g) in 91 % yield. δ 7.26 (s), 5.02 (br,1H), 3.63-3.60 (m,2H), 3.52 (br,1H), 3.25-3.21 (m,2H), 1.66-1.61 (m,2H), 1.41 (s,9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 157.06, 79.56, 59.14, 37.16, 32.80, 28.42; TOF-MS (ESI) calculated for $C_8H_{17}NO_3$ $[M+H]^+$: 177.1315, found: 177.1219.



1.2

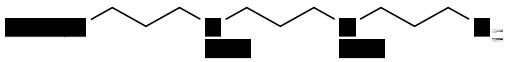
To a 0 °C solution of **1.1** (15.16 g, 86.62 mmol) in DCM (80 mL) was added triethylamine (13.12 g, 129.94 mmol) and methane sulfonyl chloride (11.9 g, 103.95 mmol) slowly. The solution was allowed to warm to room temperature and monitored by thin liquid chromatography (TLC). The reaction was quenched with saturated solution of $NaHCO_3$ (50 mL). The compound was extracted with DCM (100 mL) and the combined organic layers were dried over Na_2SO_4. The solvent was evaporated under vacuum to give a colorless oil. This oil was dissolved in 3-amino-1-propanol (20 mL) and stirred at 80 °C for 2 hrs. After starting material was consumed, H_2O (200 mL) was added to the reaction followed by extraction with DCM (100 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under vacuum. The crude was dissolved in DCM (100 mL), Boc anhydride (14.72 g, 67.54 mmol) was added, and the reaction was stirred overnight. Following evaporation of the solvent, the compound was purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes). Compound **1.2** was obtained (22 g) in 78 %

yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.26 (s), 5.26 (br,1H), 4.69 (br,1H), 3.56-3.53 (m,2H), 3.39-3.36 (m,2H), 3.23-3.19 (m,2H), 3.15-3.10 (m,2H), 1.73-1.67 (m,4H), 1.49 (s,9H), 1.45 (s,9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 163.85, 158.80, 83.08, 80.67, 59.79, 50.79, 46.74, 45.32, 39.54, 37.13, 28.34, 26.66; TOF-MS (ESI) calculated for $C_{16}H_{32}N_2O_5$ $[M+H]^+$: 333.2384, found: 333.2336



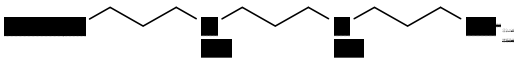
1.3

To a 0 °C solution of **1.2** (10 g, 30.12 mmol) in DCM (100 mL) was added triethylamine (4.56 g, 45.18 mmol) and methane sulfonyl chloride (4.14g, 36.14 mmol) slowly. The solution was warmed to room temperature and then, the reaction was quenched with saturated solution of $NaHCO_3$ (50 mL). The aqueous layer was washed three times with DCM (100 mL), the combined organic layers were dried over Na_2SO_4, and the solvent was evaporated under vacuum to give a colorless, crude oil. The crude was dissolved in 3-amino-1-propanol (20 mL) and stirred at 80 °C for 2 hrs. H_2O (200 mL) was added to the reaction and the mixture was washed three times with DCM (100 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under vacuum. The crude was dissolved in DCM (80 mL), Boc anhydride (7.63 g, 35 mmol) was added, and the reaction was stirred overnight. The solvent was evaporated under vacuum and the compound was purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes). Compound **1.3** was obtained (11.8 g) in 80 % yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.26 (s), 5.27 (br,1H), 4.74 (br,1H), 3.53 (s,2H), 3.36 (s,2H), 3.23 (s,2H), 3.11 (s,4H), 1.64 (s,4H), 1.44 (s,27H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 156.96, 156.44, 156.42, 69.42, 58.23, 44.37, 43.83, 43.02, 37.01, 29.71, 28.94, 28.75, 28.41; TOF-MS (ESI) calculated for $C_{24}H_{47}N_3O_7$ $[M+H]^+$: 490.3487, found: 490.3511.



1.4

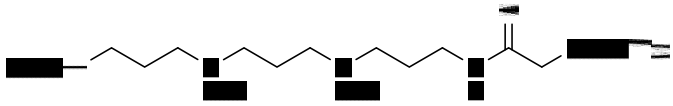
To a 0 °C solution of **1.3** (7.1 g, 14.5 mmol) in DCM (50 mL), was added triethylamine (2.2 g, 21.73 mmol) and methane sulfonyl chloride (1.99 g, 17.38 mmol) dropwise. The solution was warmed to room temperature and monitored by TLC. After completion, the reaction was quenched with a saturated solution of $NaHCO_3$ (50 mL). The aqueous layer was washed three times with DCM (100 mL), and the combined layers were dried over Na_2SO_4. The solvent was evaporated under vacuum to give a colorless, crude oil. The crude was dissolved in DMSO (40 mL), NaN_3 (2.43 g, 43.46 mmol) was added and the mixture was stirred at 80 °C for 2 hrs. The reaction was cooled down and H_2O (150 mL) was added. The compound was extracted with DCM and the combined organic layers were dried over Na_2SO_4. The solvent was evaporated under vacuum and the compound was purified by column chromatography (SiO_2, 1:4 Ethyl acetate: Hexanes). Compound **1.4** was obtained (4.8 g) in 65 % yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.26 (s), 5.29 (s,1H), 3.26-3.17 (m,6H), 3.14-3.05 (m,6H), 1.77-1.71 (m,4H), 1.67-1.62 (m,2H), 1.47 (s,18H), 1.43 (s,9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 156.88, 155.15, 80.12, 79.70, 58.23, 49.01, 44.54, 42.61, 30.56, 28.41, 28.38, 27.84; TOF-MS (ESI) calculated for $C_{24}H_{46}N_6O_6$ $[M+H]^+$: 515.3552, found: 515.3581



1.5

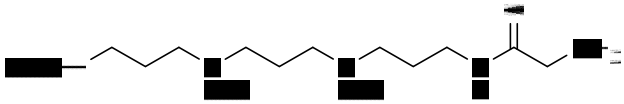
To a solution of **1.4** (4.8 g, 9.4 mmol) in $CHCl_3$ (10 mL) and MeOH (5 mL), was added 10 mol % of Pd/C (0.48 g). The reaction was set up at 1 atm. pressure of H_2, and was stirred at room temperature overnight. The reaction was monitored by TLC and mass spectrometry (MS). Once complete, the reaction was filtered over celite to separate the catalyst Pd/C, and the celite was

rinsed with DCM (100 mL) and with 10 % MeOH in DCM (100 mL). The mixture was concentrated under vacuum and purified by column chromatography (SiO_2, 5 % MeOH in DCM). Intermediate **1.5** was obtained (2.2 g) in 49 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.27 (s), 5.27 (s, 1H) 3.26-3.17 (m,6H), 3.14-3.05 (m,6H), 1.77-1.71 (m,4H), 1.67-1.62 (m,2H), 1.47 (s,18H), 1.43 (s,9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 161.36, 156.00, 79.65, 79.64, 79.04, 77.35, 77.09, 76.84, 46.40, 44.20, 37.68, 37.33, 31.16, 28.38, 28.37; TOF-MS (ESI) calculated for $C_{24}H_{48}N_4O_6$ $[M+H]^+$: 489.3647, found: 489.3713



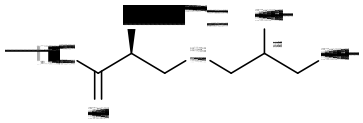
1.6

To a solution of FMOC-Glycine (1.64 g, 5.52 mmol) in DCM (50 mL) was added EDCI (1.05 g, 5.52 mmol) and HOBT (75 mg, 5.52 mmol). The solution was stirred for 45 minutes. **1.5** (2.25 g, 4.6 mmol) was dissolved in DCM (10 mL), this solution was added to the activated acid, and the mixture was stirred overnight. The reaction was concentrated under vacuum and the intermediate was purified by column chromatography (SiO_2, 5 % MeOH in DCM). Compound **1.6** was obtained (2.6 g) in 74 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.76 (d, J = 7.6 Hz, 2H), 7.61 (d, J = 7.5 Hz, 2H), 7.39 (dd, J = 7.5 Hz, 2H), 7.31 (dd, J = 7.4 Hz, 2H), 4.41 (d, J = 7.1 Hz, 2H), 4.23 (t, J = 7.2 Hz, 1H), 3.89 (s, 2H), 3.25 (s, 6H), 3.11 (s, 6H), 1.74 (m, 1H), 1.66 (s, 4H), 1.46 (s, 9H), 1.44 (s, 9H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.11, 156.77, 156.28, 143.59, 141.24, 127.93, 126.93, 125.06, 119.89, 79.55, 76.97, 47.36, 45.09, 44.83, 28.66, 28.17; TOF-MS (ESI) calculated for $C_{41}H_{63}N_5O_9$ $[M+H]^+$: 768.4542, found: 768.4527



1.7

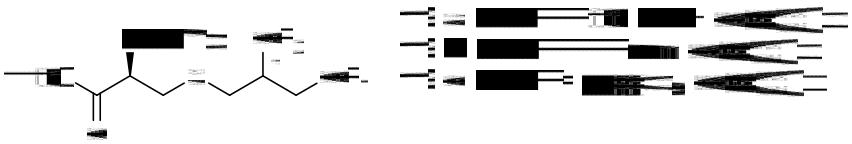
To a solution of **1.6** (2.61 g, 3.4 mmol) in DCM (5 mL) was added piperidine (2 mL) and the mixture was allowed to stir overnight. The solvent was evaporated under vacuum, and the product was purified by column chromatography (SiO_2, 5 % MeOH in DCM). Amine **1.7** was obtained (1 g) in 58 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.72 (s, NH), 5.28 (s, NH), 3.47 (s, 2H), 3.18 (s, 4H), 3.09 (t, *J*=6.3Hz, 6H), 1.84 (t, *J*=5.8Hz, 2H), 1.72-1.64 (m, 2H), 1.63-1.56 (m, 4H), 1.37 (s, 27H).); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 156.77, 156.28, 169.11, 80.00, 73.75, 55.99, 44.47, 43.86, 37.43, 35.85, 28.04, 27.27, 22.18; TOF-MS (ESI) calculated for $C_{26}H_{51}N_5O_7$ $[M+H]^+$: 546.3861, found: 546.3915



1.8

To a solution of N-α;N-α-Bis-Fmoc-L-Cysteine bis(tert-butyl ester) disulfide (2 g, 2.5 mmol) in DCM (15 mL) was added activated zinc powder (1g of Zn previously reacted with HCl, decanted, and dried under vacuum), and a freshly prepared mixture of MeOH, concentrated H_2SO_4 and concentrated HCl (100:1:7, 10 mL). After 15 minutes or vigorous stirring, either (R) or (S)-glycidol (370 mg, 5 mmol) was added into the reaction and allowed to react for 5 hours. Half the solvent was evaporated under vacuum, the reaction was filtered, and saturated solution of $KHSO_4$ (2 mL) was added. The reaction was washed with DCM (30 mL), the combined organic layers were dried over Na_2SO_4 to afford compound **1.8**. Compound **1.8a (R-glycidol)** and **1.8b (S-glycidol)** were purified and characterized separately through column chromatography (SiO_2, 1:2

Ethyl acetate: Hexanes). Intermediate **1.8** was obtained (1 g) in 81 % yield. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.80 (d, *J* = 8.0 Hz, 2H), 7.62 (d, *J* = 7.7 Hz, 2H), 7.43 (dd, *J* = 7.5 Hz, 2H), 7.29 (dd, 2H), 6.60 (s, 1H), 5.76 (d, *J* = 7.9 Hz, 1H), 4.71 (s, 1H), 4.58 (s, 1H), 4.42 (d, *J* = 9.8 Hz, 1H), 4.26 (dd, *J* = 7.7 Hz, 2H), 3.24 (s, 1H), 3.04 (dd, *J* = 10.9 Hz, 1H), 1.52 (s, 9H), 1.35 (s, 2H), 1.33 (s, 2H); ^{13}C NMR (75 MHz, cdcl$_3$) δ 181.95, 156.27, 143.71, 141.30, 127.78, 127.12, 125.15, 120.01, 115.15, 77.48, 77.06, 76.63, 67.14, 47.19, 31.07, 30.85, 29.81, 28.94, 28.05, 27.36; TOF-MS (ESI) calculated for $C_{25}H_{31}NO_6S$ $[M+H]^+$:474.1945 $[M+NH4]^+$:491.2171, found: 474.2091 and 491.2184



1.9 a-c

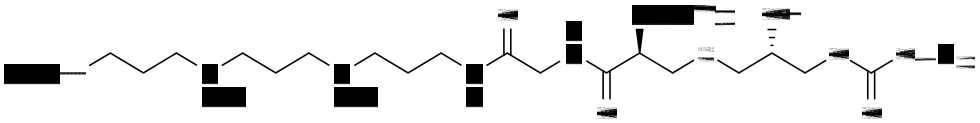
A series of compounds were obtained from compound **1.8a** and **1.8b** either by acylation of one or two of the hydroxyls with palmitoyl chloride. Compound **1.8a** gave monoacylated adducts **1.9a** and diacylated **1.9b**, compound **1.8b** gave **1.9c** diacylated adduct (the S monoacylated was not synthesized because R stereocenter was more active than S). To a 0 °C solution of the diol (959 mg, 2.025 mmol) in DCM (5 mL), was added triethylamine (300 mg, 3 mmol) and palmitoyl chloride dropwise (660 mg, 2.4 mmol). The reaction was stirred at room temperature and monitored by TLC and MS. After the reaction was complete, a saturated solution of $NaHCO_3$ (30mL) was added. The aqueous layer was extracted three times with DCM, and the combined organic layers were dried over Na_2SO_4, followed by evaporation of the solvent under vacuum. The crude was purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes) to give a white powder (1.7 g) in 86 % yield. **1.9a (monoacyl)** ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.77 (d, *J*=8Hz, 2H), 7.61 (d, *J*=7.5Hz, 2H), 7.41 (dd, *J*=7.5Hz, 2H), 7.33 (dd, *J*=7Hz, 2H), 5.75 (br, 1H),

4.53 (d, *J*=7.2Hz, 2H), 4.42-4.37 (m, 2H), 4.24 (dd, *J*=7Hz, 1H), 4.17 (dd, *J*=11.5Hz 1H), 4.10 (dd, *J*=12Hz, 1H), 3.97 (br, 1H), 3.01-2.95 (m, 2H), 2.79 (dd, *J*=7.5Hz, 1H), 2.68 (dd, *J*=7.5Hz, 1H), 2.37-2.33 (m, 2H), 1.61 (t, *J*=7.5Hz, 2H), 1.58 (s, 9H), 1,29 (s, 26H), 0,88 (t, *J*=7Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 174.11, 156.16, 144.03, 141.54, 127.98, 127.33, 125.38, 120.24, 83.413, 68.86, 67.45, 66.87, 54.81, 47.35, 37.43, 36.19, 34.35, 32.16, 29.93, 29.71, 29.60, 29.51, 29.38, 28.24, 25.13, 22.94, 14.37; TOF-MS (ESI) calculated for **1.9a** $C_{41}H_{65}N_2O_7S$ [M^-+$NH4^+$]: 729.4512, found: 729.4487; **1.9b/c (diacyl)** ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.76 (d, *J*=7.5Hz, 2H), 7.62 (d, *J*=7.5Hz, 2H), 7.39 (dd, *J*=7Hz, 2H), 7.31 (dd, *J*=7.5Hz, 2H), 5.67 (br, 1H), 4.51 (dd, *J*=7Hz, 1H), 4.39-4.33 (m, 3H), 4.24 (dd, *J*=7Hz, 1H), 4.15 (dd, *J*=6Hz 1H), 3.09-2.99 (m, 2H), 2.79-2.76 (m, 2H), 2.28 (t, *J*=7.5Hz, 4H), 1.69-1.57 (m, 4H), 1.49 (s, 9H), 1,29 (s, 48H), 0,88 (t, *J* =7 Hz, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 168.55, 168.22, 164.69, 150.94, 139.07, 136.53, 122.95, 122.31, 120.41, 115.22, 78.27, 65.55, 62.47, 58.67, 49.55, 30.67-27.16 (5), 24.94-24.60 (4), 24.53-17.93 (6), 9.29; TOF-MS (ESI) calculated for **1.9b/c** $C_{57}H_{95}N_2O_8S$ [M^-+NH_4^+]: 967.6809, found: 967.6755.

1.10 a-c

To a solution of compound **1.9 a-c** (1.26 g, 1.72 mmol of 1.9a, 1.68 g, 1.73 mmol of **1.9b** or **c**) in DCM (5 mL) was added the same volume of acid TFA (3-5 mL) and the solution was stirred at room temperature overnight. Following the evaporation of the solvent and acid under vacuum, the compound was purified by column chromatography (SiO_2, 5 % MeOH in DCM). Compounds **1.10a, b** and **c** were obtained (1.06 g, 1.1g, and 1.3 g respectively) in 79 % average yield. **1.10a (monoacyl)** ^{1}H NMR (500 MHz, Chloroform-*d*) ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.76 (d, *J*

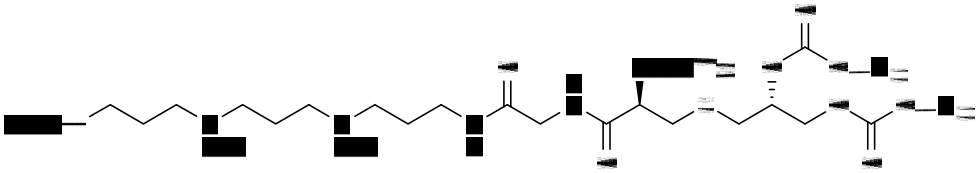
= 7.6 Hz, 2H), 7.59 (d, *J* = 7.5, 2H), 7.39 (dd, *J* = 7.5 Hz, 2H), 7.310 (dd, *J* = 7.4 Hz, 2H), 5.97 (s, NH), 4.65 (d, *J* = 7.4 Hz, 1H), 4.40 (d, *J* = 7.2 Hz, 2H), 4.22 (t, *J* = 7.1 Hz, 1H), 4.17-4.14 (m, 2H), 4.09 (t, *J* = 5.5 Hz, 1H), 3.98 (s, 1H), 3.08 (s, 2H), 2.83-2.76 (m, 2H), 2.70-2.64 (m, 2H), 2.31 (t, *J* = 7.6 Hz, 2H), 1.59 (s, 2H), 1.24 (s, 28H), 0.87 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 181.04, 164.58, 159.10, 143.59, 127.77, 127.10, 125.08, 120.01, 86.56, 73.87, 66.82, 47.06, 34.11, 31.92, 29.69, 29.65, 29.61, 29.47, 29.36, 29.26, 29.13, 24.86, 22.68, 14.12; TOF-MS (ESI) calculated for **1.10a** $C_{37}H_{57}N_2O_7S$ [M^-+NH_4^+]: 673.3886, found: 673.4002; **1.10b/c (diacyl)** ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.61 (d, *J* = 6.7 Hz, 2H), 7.39 (dd, *J* = 7.6 Hz, 2H), 7.31 (dd, *J* = 7.5 Hz, 2H), 5.83 (d, *J* = 7.9 Hz, NH), 5.21-5.13 (m, 1H), 4.71-4.66 (m, 1H), 4.43-4.32 (m, 3H), 4.25 (d, *J* = 7.1 Hz, 1H), 4.14 (dd, *J* = 12.1, 6.2 Hz, 1H), 3.14 (dd, *J* = 14.0, 5.1 Hz, 1H), 3.10 (dd, J= 14.2, 5.6 Hz, 1H), 2.85-2.71 (m, 2H), 2.33-2.25 (m. 4H), 1.59 (s, 4H), 1.25 (s, 48H), 0.88 (t, *J* = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.54, 173.31, 144.06, 143.81, 141.58, 141.29, 127.85, 127.34, 125.26, 120.15, 83.09, 70.64, 67.54, 54.43, 47.44, 35.36, 34.16, 33.31, 31.92, 29.59, 29.22, 28.12, 24.96, 22.73; TOF-MS (ESI) calculated for **1.10b/c** $C_{53}H_{87}N_2O_8S$ [M^-+NH_4^+]: 911.6183, found: 911.6190.



1.11 a

To a solution of **1.10a** (30 mg, 0.044 mmol) in DCM (10 mL) were added EDCI (9 mg, 0.05 mmol) and HOBT (6 mg, 0.05 mmol). The reaction was stirred for 45 minutes. **1.7** (27 mg, 0.05 mmol) was added, suspended in DCM (3 mL); the reaction was stirred overnight. The reaction was concentrated under vacuum and purified by column chromatography (SiO_2. 1:2 Ethyl acetate: Hexanes). Compound **1.11a** was obtained (33 mg) as a white solid in 63 % yield. ^{1}H NMR (500

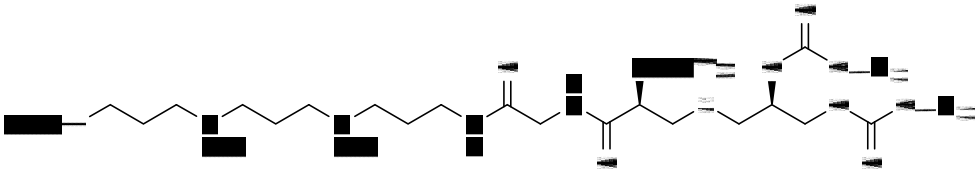
MHz, Chloroform-*d*) δ 7.76 (d, *J*=7.6Hz, 2H), 7.61 (d, *J*=7.5Hz, 2H), 7.44 (br, 1NH), 7.39 (dd, *J*=7.5Hz, 2H), 7.35 (br, 1NH), 7.43 (br, 1NH) 7.31 (dd, *J*=7Hz, 2H), 6.35 (d, *J* = 7.5 Hz, NH), 5.70 (d, *J* = 7.9 Hz, 1H), 4.65 (s, 1H), 4.44-4.33 (m, 2H), 4.24-4.07 (m, 2H), 4.01-3.84 (m, 2H), 3.28-3.04 (m, 10H), 2.33-2.28 (m, 2H), 1.74-1.56 (m, 4H), 1.48 (s, 9H), 1.42 (s, 18H), 1.27-1.23 (s, 24H), 0.88 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 178.36, 177.84, 175.07, 174.25, 172.66, 171.11, 166.37, 125.02, 128.48, 128.06, 127.75, 127.09, 119.98, 83.66, 77.26, 77.01, 76.75, 66.96, 34.12, 31.93, 29.70, 29.66, 29.62, 29.48, 29.36, 29.28, 29.14, 28.43, 24.86, 22.69, 14.12; TOF-MS (ESI) calculated for $C_{63}H_{106}N_7O_{13}S$ $[M^-+NH_4^+]^+$: 1200.7564, found: 1200.7612



1.11 b

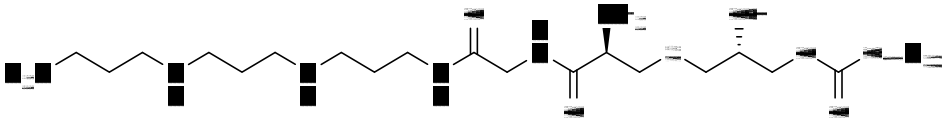
To a solution of **1.10b** (330 mg, 0.37 mmol) in DCM (30 mL) were added EDCI (84 mg, 0.44 mmol) and HOBT (59 mg, 0.44 mmol). The reaction was stirred for 45 minutes and added **1.7** (0.22 g, 0.44 mmol) dissolved in DCM (3 mL). After stirring overnight, the reaction was concentrated under vacuum and the product purified through column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes). Compound **1.11b** was obtained (280 mg) in 53 % yield (SiO_2). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.39 (dd, *J* = 7.5 Hz, 2H), 7.30 (dd, *J* = 7.5 Hz, 2H), 7,18 (br, 1H), 7.12 (d, , *J*= 8.3 Hz, 1H), 6.82 (d, , *J* = 8Hz, 1H), 5.60 (s, 1H), 5.19 (s, 1H), 4.47-4.28 (m, 4H), 4.22 (dd, *J* = 6.9 Hz, 1H), 4.15 (dd, *J* =11.9, 6.0 Hz, 1H), 3.86-3.79 (m, 1H), 3.24 (br, 6H), 3.09 (br, 6H), 2.97-2.87 (m, 1H), 2.77 (s, 1H), 2.29 (td, *J* = 7.4Hz, 4H), 1.78-1.70 (m, 4H), 1.68-1.55 (m, 6H), 1.45 (s, 9H), 1.43 (s, 9H), 1.41 (s, 9H), 1.24 (s, 48H), 0.87 (t, *J* = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.11, 156.77, 156.28, 127.93,

127.31, 124.26, 120.23, 96.46, 95.00, 93.08, 66.55, 43.81, 34.31, 32.15, 29.93, 29.74, 29.59, 29.37, 28.66, 25.09, 22.92, 16.10, 14.34; TOF-MS (ESI) calculated for $C_{79}H_{136}N_7O_{14}S$ $[M+H]^+$: 1438.9866, found: 1438.9733



1.11 c

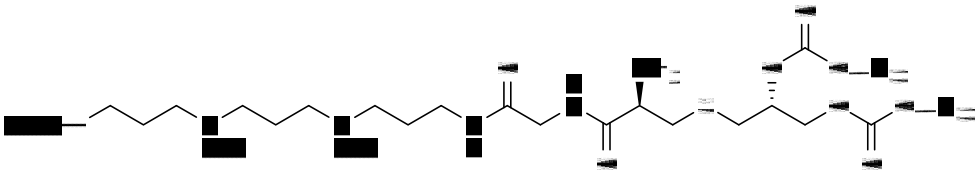
To a solution of **1.10c** (427 mg, 0.48 mmol) in DCM (10 mL) were added EDCI (109 mg, 0.57 mmol) and HOBT (78 mg, 0.57 mmol). The reaction was stirred for 45 minutes and added **1.7** (312 mg, 0.57 mmol) dissolved in DCM (3 mL). After stirring overnight, the reaction was concentrated under vacuum and purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes). Compound **1.11c** was obtained (424 mg) as a white solid in 63 % yield. TOF-MS (ESI) calculated for $C_{79}H_{136}N_7O_{14}S$ $[M+H]^+$: 1438.9866, found: 1438.9733



1.12

To a solution of **1.11a** (18 mg, 0.02 mmol) in DCM (5 mL) was added piperidine (3 mL) and stirred at room temperature overnight, followed by evaporation of the solvent under vacuum. The free amine was then purified through column chromatography (SiO_2, 5 % MeOH in DCM), and obtained a solid in 67 % yield (12 mg, 0.012 mmol). The amine was dissolved in DCM (2 mL) and added 4M HCl in Dioxane (2 mL), the reaction was stirred for 5 minutes and concentrated under vacuum. Compound **1.12** was obtained as a salt (6.6 mg, 0.01 mmol) in 83 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.36 (br, 1H), 9.11 (dd, J = 12.4, 6.6 Hz, 1H), 8.53 (br, 1H), 8.27 (t, J =

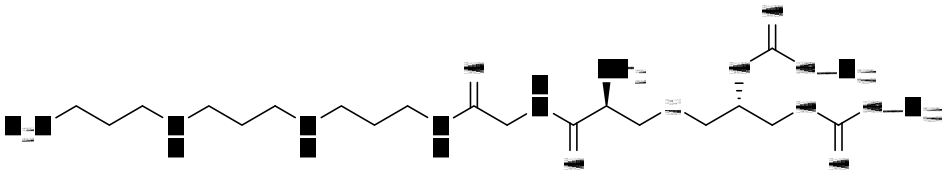
5.9 Hz, 1H), 8.16 (s, 1H), 5.14 (br, 1H), 4.05-3.99 (m, 2H), 3.97 (d, 2H), 3.82 (ddd, *J* = 14.1, 12.0, 5.7 Hz, 1H), 3.63 (dd, *J* = 16.9, 5.2 Hz, 1H), 3.15 (dd, *J* = 6.5 Hz, 2H), 3.02-2.95 (m, 6H), 2.93-2.83 (m, 6H), 2.69 (dd, *J* = 13.7, 5.0 Hz, 1H), 2.61 (dd, *J* = 13.6, 7.0 Hz, 1H), 2.31-2.25 (m, 2H), 2.09-2.04 (m, 2H), 1.99 (t, *J* = 7.4 Hz, 2H), 1.82-1.76 (m, 2H), 1.52-1.45(m, 2H), 1.21 (s, 24H), 0.82 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-*d*$_6$) δ 173.33, 168.72, 168.30, 68.32, 66.93, 52.43, 44.96, 44.41, 44.39, 44.33, 42.87, 40.46, 40.38, 40.29, 40.21, 40.12, 40.05, 39.96, 39.79, 39.62, 39.46, 36.54, 36.03, 35.55, 33.95, 33.86, 33.28, 31.73, 29.48, 29.44, 29.35, 29.17, 29.14, 29.12, 28.92, 28.80, 26.05, 24.93, 24.85, 24.07, 22.65, 22.53, 14.41; TOF-MS (ESI) calculated for $C_{48}H_{93}N_6O_{11}S$ $[M+H]^+$: 961.6618, found: 961.6627 and final product of $C_{33}H_{69}N_6O_5S$ $[M+H]^+$: 661.5045, found: 661.5063.



1.13

To a solution of **1.11b** (161 mg, 0.011 mmol) in DCM (5 mL) was added piperidine (3 mL) and stirred at room temperature overnight. **1.13** was purified through column chromatography (SiO_2, 5 % MeOH in DCM), and obtained as a white solid in 72 % yield (95 mg, 8 μmol). The amine would undergo global deprotection to give R-amine LPM3 or be functionalized to give the derivatives products of R-glycidol LPM4 (acetyl), LPM5 (Isobutyryl), and LPM6 (benzoyl). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.24 (br. s, 2H), 8.08 (s, 1H), 7.05 (d, *J* = 11.1 Hz, 1H), 6.84 (s, 1H), 5.18 (dq, *J* = 6.6, 3.5 Hz, H), 4.65 (s, 1H), 4.34 (dd, *J* = 12.0, 3.0 Hz, 1H), 4.16 (dd, *J* = 12.0, 6.2 Hz, 1H), 4.04 (dd, *J* = 16.5, 6.4 Hz, 1H), 3.76 (d, *J* = 16.3 Hz, 1H), 3.37 (s, 1H), 3.27-3.14 (m, 8H), 3.06-3.02 (m, 4H), 2.88-2.81 (m, 2H), 2.80-2.72 (m, 2H), 2.33-2.25 (m, 6H), 2.03-

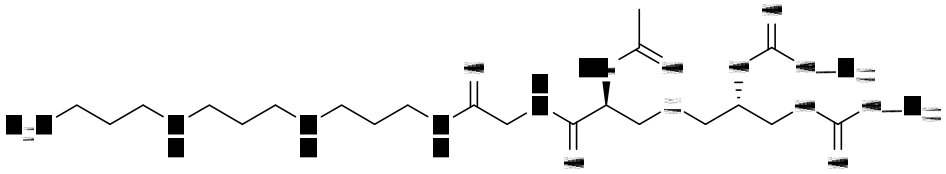
2.01 (m, 2H), 1.83 (s, 2H), 1.75 (s, 2H), 1.61 (s, 6H), 1.45 (s, 9H), 1.45 (s, 18H), 1.25 (s, 48H), 0.87 (t, J = 6.8 Hz, 6H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 173.73, 173.61, 173.55, 173.15, 171.54, 171.33, 169.67, 76.74, 63.77, 36.27, 34.37, 34.10, 31.91, 29.71, 29.68, 29.65, 29.56, 29.54, 29.51, 29.39, 29.36, 29.33, 29.31, 29.14, 28.46, 28.39, 25.53, 24.94, 24.87, 22.68, 14.11; TOF-MS (ESI) calculated for $C_{64}H_{123}N_6O_{12}S$ $[M+H]^+$: 1199.8914, found: 1199.8904.



1.14

Compound **1.13** (22 mg, 18 μmol) was dissolved in DCM (2 mL), 4M HCl in Dioxane (2 mL) was added, the reaction was stirred for 30 minutes. Following concentration by rotary evaporation, the mixture was further dried under high vacuum. No purification was performed after global deprotection. Compound **1.14** was obtained as a white salt (13 mg, 14 μmol) in 81 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.21 (s, 1H), 8.99 (s, 2H), 8.42 (s, 1H), 8.26 (s, 1H), 7.99 (s, 1H), 5.14 (d, J = 7.1 Hz, 1H), 4.27 (dd, J = 12.1, 2.8 Hz, 1H), 4.12 (dd, J = 12.0, 7.1 Hz, 1H), 3.85 (dd, J = 16.6, 6.3 Hz, 1H), 3.70 (dd, J = 5.7 Hz, 1H), 3.66 (dd, 1H), 3.48 (d, J = 4.9 Hz, 1H), 3.15 (t, J = 6.6 Hz, 2H), 3.03-2.94 (m, 6H), 2.92-2.86 (m, 6H), 2.82 (dd, J = 14.2, 8.1 Hz, 1H), 2.73 (dd, J = 14.1, 7.7 Hz, 1H), 2.28-2.24 (m, 4H), 2.04 (t, J = 7.4 Hz, 2H), 1.96 (t, J = 7.6 Hz, 2H), 1.80 (t, J = 7.4 Hz, 2H), 1.52-1.46 (m, 4H), 1.22 (s, 48H), 0.84 (t, J = 6.7 Hz, 6H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 174.31, 173.34, 173.04, 168.65, 70.28, 66.69, 65.25, 63.56, 54.41, 51.38, 44.83, 42.89, 38.26, 37.61, 34.25, 34.13, 34.09, 34.07, 31.89, 29.67, 29.65, 29.63, 29.61, 29.58, 29.56, 29.47, 29.44, 29.42, 29.32, 29.28, 29.26, 29.24, 29.22, 29.13, 29.11, 29.09, 28.51, 28.43, 28.41, 27.32,

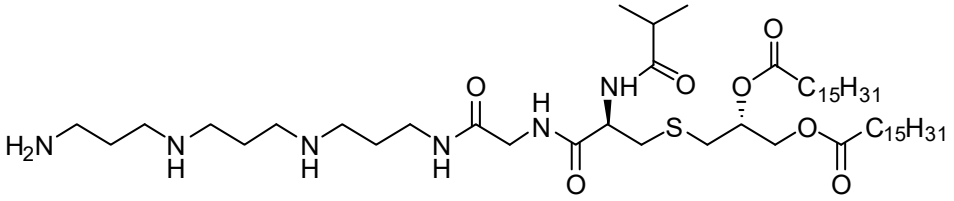
24.94, 24.90, 24.88, 24.86, 22.65, 14.08; TOF-MS (ESI) calculated for $C_{49}H_{99}N_6O_6S$ $[M+H]^+$: 899.7341, found: 899.7353.



1.15

To a solution of **1.13** (24 mg, 20 μmol) in DCM (5 mL) were added triethylamine (3 mg, 24 μmol) and acetyl chloride (19 mg, 24 μmol) dropwise. The reaction was stirred at room temperature and monitored by TLC and MS. After the reaction was complete, a saturated solution of $NaHCO_3$ (30 mL) was added and the aqueous layer was washed three times with DCM. The combined organic layers were dried over Na_2SO_4, followed by evaporation of the solvent under vacuum to give a clear, crude solid that was purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes). The protected intermediate was obtained (18 mg, 15 μmol) in 74 % yield. The purified intermediate was dissolved in DCM (2 mL), 4M HCl in Dioxane (2 mL) was added and stirred for 30 minutes. Following concentration by rotary evaporation, the mixture was further dried under high vacuum. No purification was performed after global deprotection. Compound **1.15** was obtained (8 mg, 9 μmol) in 60 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.21 (s, 1H), 8.93 (s, 1H), 8.39 (s, 1H), 8.28 (d, J = 7.0 Hz, 1H), 8.01 (s, 2H), 7.91 (d, J = 5.9 Hz, 1H), 5.08 (s, 1H), 4.38-4.33 (m, 1H), 4.28-4.25 (d, J = 11.9 Hz, 1H), 4.12-4.06 (m, 1H), 3.95 (dd, J = 11.8, 5.3 Hz, 1H), 3.68 (dd, J = 16.3, 6.4 Hz, 1H), 3.63 (dd, J = 11.0, 5.5 Hz, 1H), 3.55 (s, 2H), 3.15 (d, J = 6.2 Hz, 2H), 3.02-2.95 (m, 8H), 2.93-2.82 (m, 8H), 2.73-2.61 (m, 4H), 2.53 (s, 2H), 2.30-2.23 (m, 2H), 2.06-2.00 (m, 2H), 1.99-1.93 (m,2H), 1.87 (s, 3H), 1.79-1.73 (m, 2H), 1.53-1.43 (m, 2H), 1.22 (s, 48H), 0.84 (t, J = 6.7 Hz, 6H); ^{13}C NMR (126 MHz, dmso) δ 172.98, 170.30, 169.37,

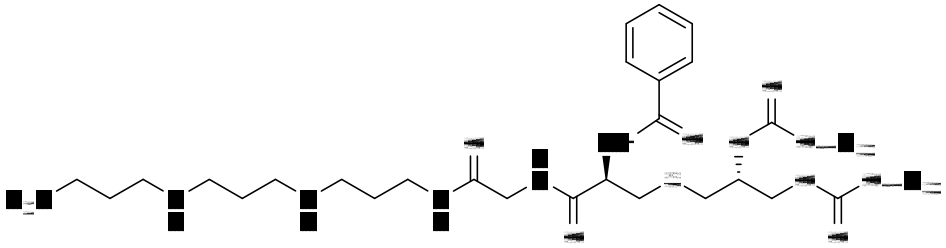
113.46, 55.37, 44.41, 40.55, 40.46, 40.38, 40.29, 40.22, 40.13, 40.05, 39.96, 39.79, 39.62, 39.46, 36.61, 34.02, 33.85, 31.75, 29.51, 29.49, 29.47, 29.39, 29.21, 29.19, 29.16, 28.89, 28.84, 24.94, 24.86, 22.55, 14.40; TOF-MS (ESI) calculated for $C_{66}H_{125}N_6O_{13}S$ $[M+H]^+$: 1241.9020, found: 1241.9136, as well as $C_{66}H_{128}N_7O_{13}S$ $[M^-+NH_4^+]$: 1258.9291, found: 1258.9407 and final product of $C_{51}H_{101}N_6O_7S$ $[M+H]^+$: 941.7447, found: 941.7525.



1.16

To a solution of **1.13** (56 mg, 47 µmol) in DCM (5 mL) were added triethylamine (6 mg, 56 µmol) and isobutyryl chloride (6 mg, 56 µmol) dropwise. The reaction was stirred at room temperature and monitored by TLC and MS. After consumption of the starting material, the reaction was quenched by adding saturated solution of $NaHCO_3$ (30 mL). The aqueous layer was washed three times with DCM and the organic layers were dried over Na_2SO_4 followed by evaporation of the solvent under vacuum to give a clear, crude solid. The crude product was purified by column chromatography (SiO_2, 3 % MeOH in DCM), and obtained (39 mg, 30 µmol) in 65 % yield. The intermediate was dissolved in DCM (2 mL), 4M HCl in Dioxane (2 mL) was added, and the reaction was stirred for 30 minutes. Following concentration by rotary evaporation, the mixture was further dried under high vacuum. No purification was performed after the deprotection. Compound **1.16** was obtained (22 mg, 24 µmol) in 80 %. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.15 (s, 1H), 8.87 (s, 1H), 8.31 (t, J = 5.7 Hz, 1H), 8.15 (d, J = 7.8 Hz, 1H), 7.97 (s, 2H), 5.10-5.05 (m, 1H), 4.41-4.34 (m, 1H), 4.26 (dd, J = 12.1, 2.9 Hz, 1H), 4.08 (dd, J = 11.9, 6.8 Hz, 1H), 3.73 (dd, J = 16.6, 6.0 Hz, 1H), 3.61 (dd, J = 16.6, 5.4 Hz, 1H), 3.13-3.09 (m, 2H), 3.00-2.92 (m, 6H), 2.88-

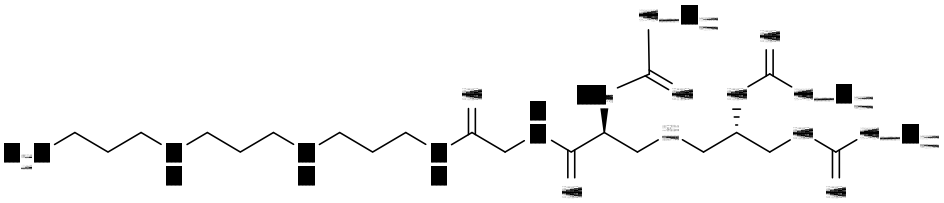
2.83 (m, 6H), 2.79 (dd, J = 13.8, 5.7 Hz, 1H), 2.25 (t, J = 7.5 Hz, 1H), 2.05-1.98 (m, 2H), 1.94-1.90 (m, 2H), 1.78-1.73 (m, 2H), 1.52-1.44 (m, 2H), 1.21 (s, 48H), 1.01 (d, J = 6.8 Hz, 3H), 0.99 (d, J = 6.8 Hz, 3H), 0.83 (t, J = 6.8 Hz, 6H); ^{13}C NMR (126 MHz, dmso) δ 182.72, 173.01, 171.01, 169.17, 160.09, 66.79, 55.37, 44.37, 40.55, 40.46, 40.38, 40.29, 40.22, 40.12, 40.05, 39.96, 39.79, 39.62, 39.46, 36.59, 31.74, 29.51, 29.48, 29.16, 28.84, 24.17, 22.54, 14.40; TOF-MS (ESI) calculated for $C_{68}H_{129}N_6O_{13}S$ $[M+H]^+$: 1269.9333 as well as $C_{68}H_{132}N_7O_{13}S$ $[M^-+NH_4^+]$: 1286.9604, found: 1286.9622 and final product of $C_{53}H_{105}N_6O_7S$ $[M+H]^+$: 969.7760, found: 969.7659.



1.17

To a 0 °C solution of **1.13** (46 mg, 38 μmol) in DCM (5 mL) were added triethylamine (8 mg, 77 μmol) and benzoyl chloride (7 mg, 46 μmol) dropwise. The reaction was stirred until room temperature and monitored by TLC and MS, then quenched by adding saturated solution of $NaHCO_3$ (30 mL). The aqueous layer was washed three times with DCM, the combined organic layers were dried over Na_2SO_4, and the solvent was evaporated by rotovaporization to give a crude solid. The solid was purified by column chromatography (SiO_2, 6:1 Ethyl acetate: Hexanes), and obtained 32 mg (25 μmol) in 65 % yield. The intermediate was then dissolved in DCM (2 mL), 4M HCl in Dioxane (2 mL) was added, and the reaction was stirred for 30 minutes. Following concentration by rotary evaporation, the mixture was further dried under high vacuum. No purification was performed after global deprotection. Compound **1.17** was obtained as a white

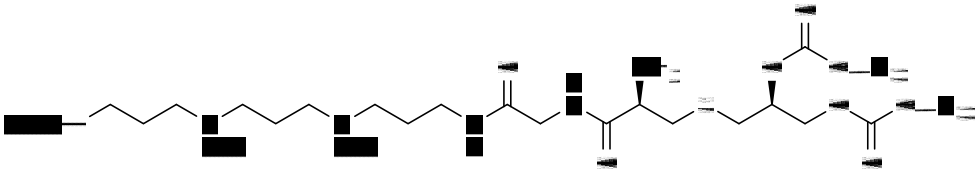
solid in 86 % yield (21 mg, 21 μmol). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.26 (s, 1H), 8.99 (s, 1H), 8.78 (dd, *J* = 17.9, 7.9 Hz, 1H), 8.42 (d, *J* = 5.6 Hz, 1H), 8.05 (s, 2H), 7.93 (d, *J* = 7.5 Hz, 2H), 7.54 (dd, *J* = 7.4 Hz, 2H), 7.47 (dd, *J* = 7.6 Hz, 2H), 5.11 (s, 1H), 4.62 (s, 1H), 4.28 (dd, *J* = 11.9 Hz, 1H), 4.09 (dd, *J* = 12.4, 6.7 Hz, 1H), 3.76 (dd, *J* = 16.7, 5.1 Hz, 1H), 3.62 (dd, *J* = 16.6, 5.4 Hz, 1H), 3.19-3.13 (m, 2H), 3.11-3.03 (m, 1H), 3.01-2.94 (m, 8H), 2.93-2.82 (m, 6H), 2.78-2.67 (m, 2H), 2.27-2.19 (m, 4H), 2.07-1.93 (m, 4H), 1.82-1.75 (m, 2H), 1.51-1.43 (m, 4H), 1.21 (s, 48H), 0.83 (t, *J* = 6.7 Hz, 6H); ^{13}C NMR (126 MHz, dmso) δ 172.96, 172.66, 171.17, 169.41, 167.14, 128.67, 128.05, 127.78, 45.04, 44.37, 42.80, 36.59, 36.14, 34.02, 33.85, 31.74, 29.50, 29.46, 29.36, 29.17, 29.15, 28.87, 28.83, 26.23, 24.91, 24.85, 24.15, 22.75, 22.54, 14.38; TOF-MS (ESI) calculated for $C_{71}H_{127}N_6O_{13}S$ $[M+H]^+$: 1303.9176 as well as $C_{71}H_{130}N_7O_{13}S$ $[M^-+NH_4^+]$: 1320.9447, found: 1320.9483 and final product of $C_{56}H_{103}N_6O_7S$ $[M+H]^+$: 1003.7603, found: 1003.7745.



1.18

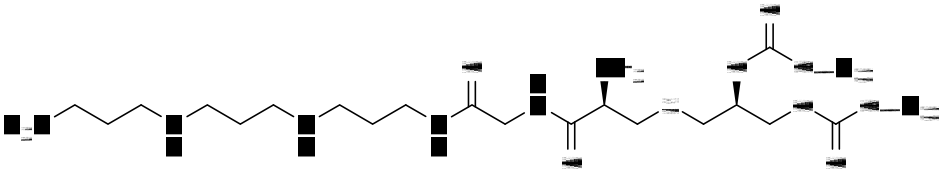
To a 0 °C solution of **1.13** (20 mg, 17 μmol) in DCM (5 mL) were added triethylamine (3 mg, 33 μmol) and palmitoyl chloride (6 mg, 20 μmol) dropwise. The reaction was stirred at room temperature and monitored by TLC and MS. Once complete, the reaction was quenched by adding saturated solution of $NaHCO_3$ (15 mL). The aqueous layer was washed three times with DCM and the combined organic layers were dried over Na_2SO_4. The solution was concentrated under vacuum to give a clear, crude solid that was purified by column chromatography (SiO_2, 8:1 Ethyl acetate: Hexanes), and obtained (16 mg, 11 μmol) in 66 % yield. The pure intermediate was

dissolved in DCM (2 mL) and 4M HCl in Dioxane (2 mL) was added. After 30 minutes, the solution was concentrated by rotary evaporation, and dried under high vacuum. No purification was performed after global deprotection. Compound **1.18** was obtained in 82 % yield (10 mg, 9 μmol). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.22 (s, 2H), 7.17 (s, 1H), 6.70 (s, 1H), 5.25-5.16 (m, 1H), 4.61 (s, 1H), 4.32 (dd, *J* = 12.0, 3.4 Hz, 1H), 4.15 (dd, *J* = 11.9, 6.2 Hz, 1H), 3.99 (dd, *J* = 17.0, 5.8 Hz, 1H), 3.85 (dd, *J* = 16.9 Hz, 1H), 3.28-3.17 (m, 6H), 3.15-3.06 (m, 10H), 3.03-2.90 (m, 2H), 2.81-2.72 (m, 2H), 2.31 (tt, *J* = 7.8 Hz, 4H), 2.24 (t, *J* = 7.7 Hz, 2H), 1.73 (tt, *J* = 7.6 Hz, 2H), 1.70-1.56 (m, 14H), 1.48-1.37 (m, 27H), 1.25 (s, 72H), 0.88 (t, *J* = 6.8 Hz, 9H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 173.56, 173.50, 173.40, 170.81, 170.17, 168.35, 156.05, 156.37, 70.19, 63.66, 47.01, 44.93, 43.22, 36.31, 34.33, 34.22, 34.06, 33.12, 31.90, 29.68, 29.66, 29.64, 29.52, 29.51, 29.49, 29.36, 29.34, 29.32, 29.30, 29.28, 29.17, 29.13, 29.12, 28.45, 28.41, 25.51, 24.92, 24.89, 24.85, 22.66, 14.08; **1.18b** deprotected triacylated: ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 9.21 (s, 2H), 8.99 (s, 1H), 8.42 (s, 1H), 8.25 (s, 2H), 8.00 (s, 1H), 5.31 (s, 1H), 5.14 (d, *J* = 7.1 Hz, 1H), 4.28 (dd, *J* = 12.1, 2.8 Hz, 1H), 4.12 (dd, *J* = 12.0, 7.1 Hz, 1H), 4.05-4.00 (m, 1H), 3.86 (dd, *J* = 16.6, 6.3 Hz, 1H), 3.71-3.63 (m, 4H), 3.18-3.13 (m, 4H), 3.04-2.97 (m, 8H), 2.92-2.86 (m, 8H), 2.81 (dd, *J* = 14.2, 8.0 Hz, 1H), 2.73 (dd, *J* = 14.1, 7.7 Hz, 1H), 2.30-2.22 (m, 6H), 2.07-2.00 (m, 4H), 1.99-1.92 (m, 4H), 1.80-1.75 (m, 4H), 1.53-1.44 (m, 6H), 1.22 (s, 72H), 0.84 (t, *J* = 6.7 Hz, 9H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 173.71, 173.37, 173.08, 168.65, 156.51, 79.90, 70.24, 66.49, 63.55, 54.37, 53.42, 44.72, 42.81, 38.22, 34.76, 34.25, 34.07, 32.57, 31.91, 29.68, 29.65, 29.64, 29.62, 29.48, 29.34, 29.29, 29.28, 29.12, 29.09, 28.43, 28.41, 27.26, 24.90, 24.86, 22.67, 14.11; TOF-MS (ESI) calculated for $C_{80}H_{153}N_6O_{13}S$ $[M+H]^+$: 1438.1211, as well as $C_{80}H_{156}N_7O_{13}S$ $[M^-+NH_4^+]$: 1455.1482, found: 1455.1498 and fragment 0.5M/Z: 727.6019 and final product of $C_{65}H_{129}N_6O_7S$ $[M+H]^+$: 1137.9638, found: 1137.9891.



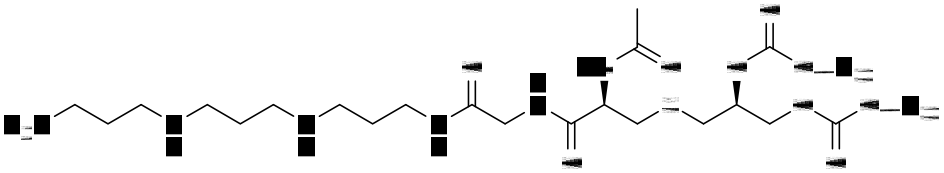
1.19

To a solution of **1.11c** (167 mg, 0.15 mmol) in DCM (5 mL) was added piperidine (3 mL), and the solution was stirred overnight. The free amine **1.19** was purified by column chromatography (SiO_2, 5 % MeOH in DCM), and obtained as a solid in 68 % yield (116 mg, 0.09 mmol). Then, amine would be either globally deprotected to give the S-amine LPM7 or further functionalized to give the acylated products from S-glycidol LPM8 (acetyl), LPM9 (Isobutyryl), or LPM10 (benzoyl). $C_{64}H_{123}N_6O_{12}S$ $[M+H]^+$: 1199.8914, found: 1199.8915.



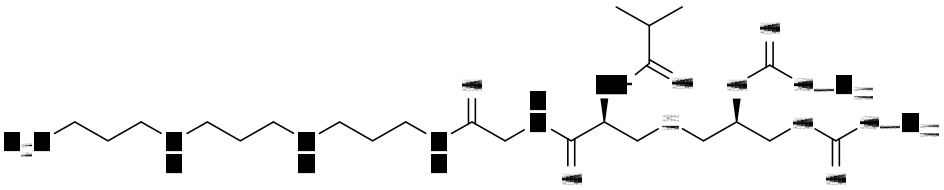
1.20

Product **1.20** was obtained by global deprotection of **1.19** by dissolving it (7 mg, 6 μmol) in DCM (2mL), adding 4M HCl in Dioxane (2mL) and stirring for 30 minutes. The compound was concentrated by rotary evaporation and further dried under vacuum. No purification was performed after global deprotection. Compound **1.20** was obtained (4 mg, 5 μmol) in 79 % yield; TOF-MS (ESI) calculated for $C_{49}H_{99}N_6O_6S$ $[M+H]^+$: 899.7341, found: 899.7521.



1.21

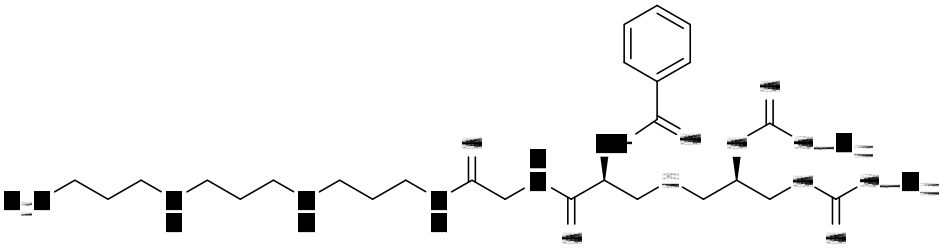
To a 0 °C solution of **1.19** (31 mg, 26 μmol) in DCM (5 mL) was added triethylamine (6 mg, 52 μmol) and acetyl chloride (3 mg, 31 μmol) dropwise. The reaction was stirred at room temperature and monitored by TLC and MS. Once complete, the reaction was quenched by adding saturated solution of $NaHCO_3$ (30 mL). The aqueous layer was washed three times with DCM, and the combined organic layers dried over Na_2SO_4, the solvent was evaporated under vacuum to give a clear solid. The crude solid was purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes). Protected intermediate was obtained (15 mg, 12 μmol) in 46 % yield. The pure intermediate was deprotected by dissolving it in DCM (2 mL), adding 4M HCl in Dioxane (2 mL), and stirring for 30 minutes. The reaction was then concentrated through rotovaporization and under vacuum. No purification was performed after global deprotection. Compound **1.21** was obtained (9 mg, 10 μmol) in 89 %. TOF-MS (ESI) calculated for $C_{66}H_{125}N_6O_{13}S$ $[M+H]^+$: 1241.9020, found: 1241.9027, as well as $C_{66}H_{128}N_7O_{13}S$ $[M^-+NH_4^+]$: 1258.9291, found: 1258.9240 and final product of $C_{51}H_{101}N_6O_7S$ $[M+H]^+$: 941.7447, found: 941.7793.



1.22

To a 0 °C solution of **1.19** (38 mg, 32 μmol) in DCM (5 mL) was added triethylamine (7 mg, 64 μmol) and isobutyryl chloride (4 mg, 38 μmol) dropwise. The reaction was stirred at room

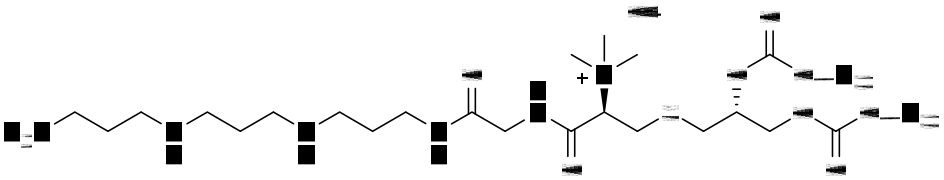
temperature and monitored by TLC and MS. Once complete, the reaction was quenched by adding saturated solution of $NaHCO_3$ (30 mL). The aqueous layer was washed three times with DCM and the combined organic layers dried over Na_2SO_4. The solvents were evaporated under vacuum to give a clear, crude solid that was purified by column chromatography (SiO_2, 3 % MeOH in DCM), and obtained 28 mg (22 μmol) in 69 % yield. The purified intermediate was deprotected by dissolving it in DCM (2 mL), adding 4M HCl in Dioxane (2 mL), and stirring for 30 minutes. The solution was concentrated through rotovaporization, and further dried under vacuum. No purification was performed after global deprotection. Compound **1.22** was obtained (16 mg, 17 μmol) in 78 % yield. TOF-MS (ESI) calculated for $C_{68}H_{129}N_6O_{13}S$ $[M+H]^+$: 1269.9333 as well as $C_{68}H_{132}N_7O_{13}S$ $[M^-+NH_4^+]$: 1286.9604, found: 1286.9643 and final product of $C_{53}H_{105}N_6O_7S$ $[M+H]^+$: 969.7760, found: 969.8118.



1.23

To a 0 °C solution of **1.19** (40 mg, 34 μmol) in DCM (5 mL) was added triethylamine (7 mg, 67 μmol) and benzoyl chloride (6 mg, 40 μmol) dropwise. The reaction was stirred and monitored by TLC and MS. After completion of the reaction, saturated solution of $NaHCO_3$ (30 mL) was added. The aqueous layer was washed three times with DCM, and the combined organic layers were dried over Na_2SO_4. The solvent was evaporated and the colorless, crude solid was purified by column chromatography (SiO_2, 6:1 Ethyl acetate: Hexanes), and 24 mg (18 μmol) of intermediate were obtained in 53 % yield. The pure intermediate was dissolved in DCM (2 mL),

4M HCl in Dioxane (2 mL) was added, and the solution was stirred for 30 minutes. The solution was concentrated by rotary evaporation, and further dried under vacuum. No purification was performed after global deprotection. Compound **1.23** was obtained (15 mg, 15 μmol) in 81 % yield. TOF-MS (ESI) calculated for $C_{71}H_{127}N_6O_{13}S$ $[M+H]^+$: 1303.9176 as well as $C_{71}H_{130}N_7O_{13}S$ $[M^- +NH_4^+]$: 1320.9447, found: 1320.9621 and final product of $C_{56}H_{103}N_6O_7S$ $[M+H]^+$: 1003.7603, found: 1003.7938.



1.24

To a solution of **1.13** (21 mg, 18 μmol) in MeI (7 mL) was added saturated solution of Na_2CO_3 in Acetonitrile (5 mL). The reaction was stirred and monitored by TLC and MS. After completion of the reaction, the solvents were evaporated to give a colorless, crude solid. The compound was purified by column chromatography (SiO_2, 5 % MeOH in DCM), and protected compound was obtained (6 mg, 5 μmol) in 27 % yield. The purified intermediate was dissolved in DCM (2 mL), 4M HCl in Dioxane (2 mL) was added, and the solution stirred for 30 minutes. The solution was concentrated under rotovaporization, and the product was further dried under vacuum. No purification was performed after global deprotection. Compound **1.24** was obtained (3 mg, 4 μmol) in 74 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.28 (s, 3H), 9.06 (s, 1H), 8.29 (s, 1H), 8.02 (s, 1H), 5.12-5.06 (m, 1H), 4.42 (dd, J = 11.9 Hz, 1H), 4.28 (dd, J = 11.9, 2.6 Hz, 1H), 4.12 (dd, J = 12.1, 7.1 Hz, 1H), 3.95 (dd, J = 16.4, 6.0 Hz, 1H), 3.70 (dd, J = 16.4, 5.1 Hz, 1H), 3.17 (s, 9H), 3.00-2.93 (m, 6H), 2.92-2.84 (m, 6H), 2.82-2.75 (m, 2H), 2.30-2.22 (m, 4H), 2.07-2.01 (m, 2H), 1.98-1.93 (m, 2H), 1.80 -1.74 (m, 2H), 1.53-1.44 (m, 4H), 1.21 (s, 48H), 0.83 (t, J = 6.7 Hz, 6H);

^{13}C NMR (126 MHz, dmso) δ 173.34, 168.72, 168.68, 168.30, 68.32, 66.94, 52.43, 44.96, 44.41, 44.39, 44.33, 42.87, 40.46, 40.38, 40.29, 40.22, 40.13, 40.05, 39.96, 39.79, 39.63, 39.46, 36.54, 36.03, 35.55, 33.95, 33.86, 33.28, 31.73, 29.49, 29.45, 29.35, 29.17, 29.14, 29.12, 28.93, 28.80, 26.05, 24.94, 24.85, 24.08, 22.65, 22.54, 14.41; TOF-MS (ESI) calculated for $C_{67}H_{129}N_6O_{12}S$ $[M+H]^+$: 1241.9384, found: 1241.9490, and final product of $C_{52}H_{105}N_6O_6S$ $[M+H]^+$: 941.7811, found: 941.7800.

Chapter 2: Synthesis and evaluation of triply functionalized α-GalCer derivative for identifying proteins involved in glycolipid trafficking

2.1 Introduction

Since the isolation of α-galactosylceramides (αGCs) among other agelasphins (glycolipids from the marine sponge, *Agelas mauritianus*,[33] many studies have been conducted to understand their role in the stimulation of natural killer T (NKT) cells.[34] An αGC known as KRN 7000 or α-GalCer (Figure 2.1), has been the most used glycolipid in the study of stimulation of NKT cells. Other variants (for example, see Figure 2.1 have also been prepared. αGCs activate NKT cells when presented by CD1d on the surfaces of dendritic and B cells, macrophages and monocytes. Glycolipids are first bound by CD1d, then they are presented to NKT cells through a CD1d-glycolipid-T cell receptor complex (Figure 2.2.

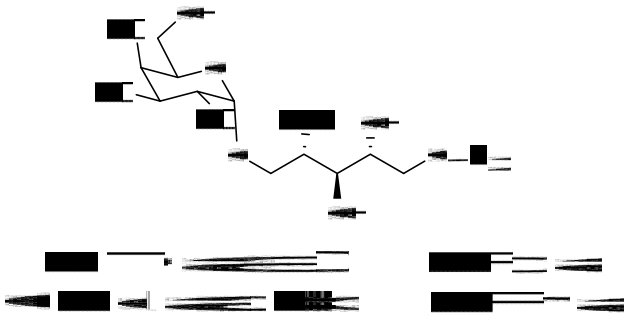


Figure 2.1 KRN7000 and the iNKT cell activator backbone of our CD1d-Triceps molecule.

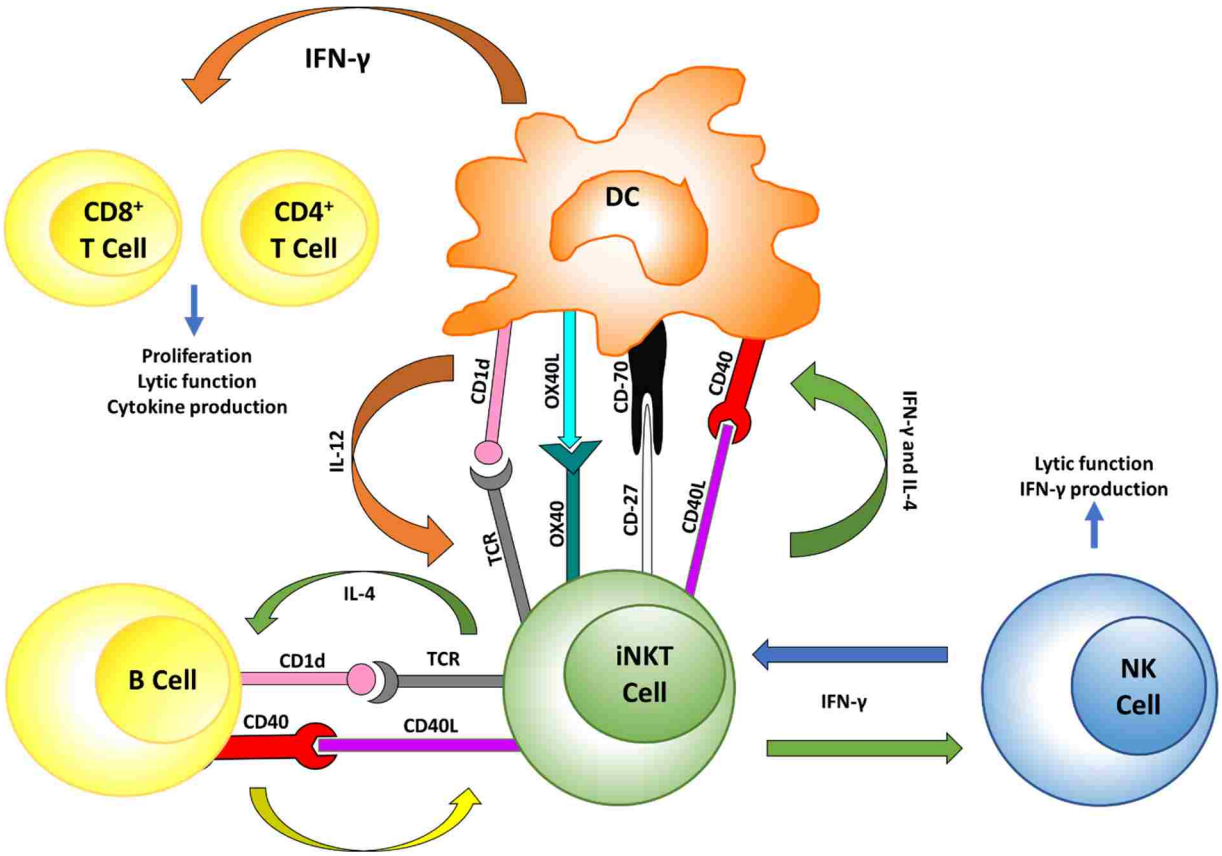


Figure 2.2 CD1d-Glycolipid-TCR complex. NKT cells modulate the innate and adaptive immune responses. iNKT cells activating T cells, dendritic cells, and NK cells. Activation signals can be received through cell surface receptors, such as the TCR recognizing CD1d, costimulatory receptors (CD40, CD70), or cytokines. Adapted from Cerundolo et al.[35-36]

Since glycolipids are not very water soluble, there must be proteins involved in their transportation and trafficking; molecules involved in that have not been fully identified. Multiple lipid transport proteins have been identified that are involved in moving NKT cell glycolipid antigens from blood to cells that present the glycolipid and within the cell. Studies have proved that lipid transfer proteins (LTPs) such as saponins (SAP), ganglioside monosialic 2 (G_{M2}), Niemann-Pick disease type C1 receptor (NPC1), Scavenger receptors (SRs) such as SRA, SRB1, and cluster differentiation 36 (CD36), and lipoproteins such as low density lipoprotein receptor (LDLR) are required for the uptake pathways of NKT cell responses and even though many receptors have

been identified for the recognition of NKT cell activators, the recognition of NKT cell activators by any given receptor does not reflect its ability to transport it or deliver it toward the CD1d-loading compartment.[37-39]

Frei et al. was the first group to publish the synthesis of a trifunctional chemoproteomics reagent (called TriCEPS), it included three active sites: an amine-reactive functionality (to bind to proteins), a protected aldehyde reactive functionality (to covalently bind to nearby receptors), and a biotin group (for purification purposes).[40]

Here we report the synthesis of an αGC analogue, which we also called "CD1d-Triceps," due to its triple functionality. CD1d-Triceps includes an NKT cell stimulator that binds to known receptor CD1d, a biotin tag for facile purification and detection of the compound, and a photoactive tag that, upon UV light activation, would cross-link to any neighboring proteins thus helping us study the proteins involved in glycolipid transportation (Figure 2.3). We expect CD1d-Triceps to bind to CD1-d in iNKT cells, then cross-link to any nearby proteins and later purify the resulting molecules by ligand exchange chromatography.

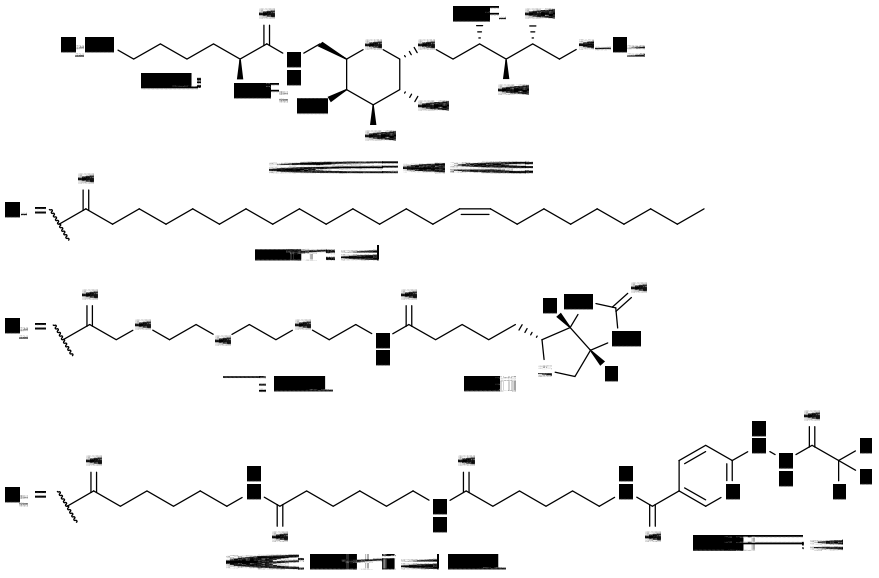


Figure 2.3 Structure of CD1d-Triceps depicting its triple functionality.

2.1.1 Cell proteins involved in transfer and trafficking

Lipids are integral membrane components and are involved in regulation of lipid metabolism. Many LTPs localized in two organelles at the same time may form shuttles, bridges or tubes that link donor and acceptor compartments.[41] Understanding the exact function of these proteins and their conformational change on lipid or glycolipid recognition and metabolism will allow for opportunity to synthetically regulate the presentation of lipid antigens, this will be crucial to improve the design of lipid vaccines, adjuvants, or in the case of NKT cell activation, the immunotherapy of cancer.[37, 39] iNKT cell depend on endosomal trafficking of CD1d for natural antigen recognition; this is mainly controlled by cytoplasmic tail-encoded tyrosine-containing motifs binding adaptor protein 2 and 3 complexes (AP-2 and AP-3.[42-43]

SAPs and G_{M2} are involved in the presentation of ligands by CD1d in iNKT cells during antigen presentation and lipid metabolism. Teyton et al. researched $SAP^{-/-}$ and $CD1d^{-/-}$ mice compared to wild-type mice cells and he found that in the absence of prosaposin (glycoprotein precursor or SAP A, B, C, or D iNKT cell do not develop correctly. He also shared that colocalization of CD1d and saposin occur in the subcellular compartment where glycolipid processing and loading occur, the interaction of CD1d-αGalCer is highly lipid head group-dependent, and that the defective presentation of glycolipid antigens by $SAP^{-/-}$ cells may result from single or combined defects in lipid processing, loading, and unloading.[37]

NPC1 is a membrane protein involved in intracellular cholesterol and glycosphingolipids (GSLs trafficking, encoded by the NPC1 gene, mutations on it have been linked to obesity, and Alzheimer's disease.[44-45] Because CD1 family of glycoproteins specialize in recognizing and presenting self and external glycolipids, we hypothesized that there is a relation between the preexisting proteins involved in the general lipid metabolism and the uptake and trafficking of

lipids from antigens loading to CD1 molecules. Bendelac et al. found *in vitro* NPC1$^{-/-}$ mice lacked a major population of CD1-d restricted T cells comparable to CD1d$^{-/-}$ mice; *in vivo* NPC1$^{-/-}$ mice were unable to clear *Sphingomonas* intravenous infection. They also studied the internalization of BODIPY-labeled GSL ligand of NKT cells and found no internalization in cells that lacked NCP1. Although the precise function of NPC1 in lipid transport is still unclear, there is a close relationship of it and the ability to internalize GSL, antigens and other important NKT cell activators.[38]

CD1d antigen selection and presentation is very complex but it was recently demonstrated to be linked to an important class of PRRs called SRs. SRA, SRB1, and CD36 target lipids toward CD1d antigen presentation, antigens such as αGalCer and bacterial GSLs require antigen uptake via SRA but not LDLR. These studies were done by Teyton et al. using several synthetic NKT cell agonists and studying their uptake by LDLR$^{-/-}$, SRA$^{-/-}$, SRB1$^{-/-}$ and CD36$^{-/-}$ DCs. Their results concluded that different cell surface receptors target lipid antigens for CD1d presentation, influencing NKT cell response and demonstrated the specific uptake is influenced by the structural characteristics of the antigen. They also characterize SRs as innate immune receptors that contribute to the recognition, clearance and effect in the selection of lipid antigens for CD1d antigen presentation.[39]

2.1.2 Identification of membrane receptors

Cell membranes separate the interior from their external environment; biological membranes are composed by two layers of lipids, mostly phospholipids with their hydrophobic tail regions inward and both polar regions facing intracellular and extracellular faces. This forms semipermeable membranes; different molecules may diffuse by passive or active transportation or by other transportations mediated by proteins.[46] Membrane proteins are hydrophobic and less soluble than cytosolic and intraorganellar proteins.[47] Many technologies have been used to analyze

biomolecules, specifically protein-protein interactions (PPI) since these are essential to all cellular functions.[48] Most PPI studies include binary technologies and they mostly involve at least one specific interaction and another step that identifies the co-purifying proteins by mass spectrometry (MS). These methodologies though effective, produce complex data that usually requires a long time to process.[49] Cross-linking technologies complement the MS methods and enhance the detection of weak and transient interactions, they involve covalent linking of proteins and other molecules. Choosing the proper linker is important, as well as the length between the active sites, linkers long enough yield to higher sensitivities, smaller linkers can elucidate close contacts, but results are often sparse. If the linker is specific, it will produce less linked peptides and will then generate less signals, if the sites are unspecific, it allows higher sensitivity, easier detection, but the amount of links will increase that subsequent analysis will be more complex.[48]

Formaldehyde is the smallest cross-linker used in PPI studies, mainly analytical/cell biology techniques, analysis of proteins with formaldehyde and MS had helped localize protein expression and location within cells, tissues, and organs.[50] Reversible cross-link immuno-precipitation (ReCLIP) is another example of cross-linking technology, the use of thiol-cleavable linkers allowed immunoprecipitation of proteins and facilitated data analysis. DSP and DTME are chosen for ReCLIP because they are permeable and cross-link with cell complexes prior to cell-lysis and the cross-link can be cleaved by a reducing agent (Figure 2.4).[51]

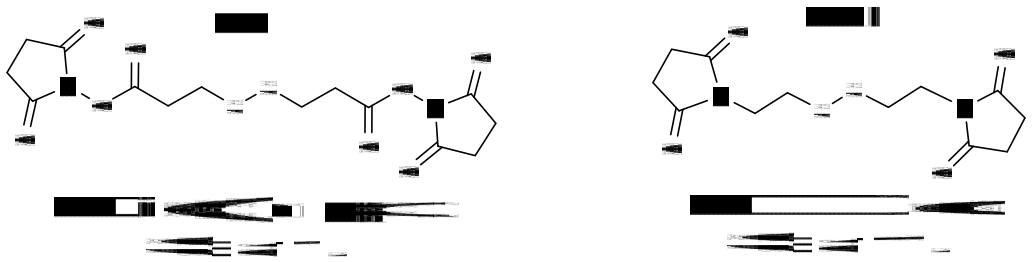


Figure 2.4 DSP reacts with primary amines, forming cross-links between lysine residues or interacting proteins. DTME reacts with sulfhydryl groups and forms cross-links between cysteine residues of interacting proteins.[51]

Ligand-receptor capture-trifunctional chemoproteomics reagents (LRC-TriCEPS) uses a similar approach of cross-linking.[40] LRC-TriCEPS is a biocompatible chemoproteomic reagent (Figure 2.5).

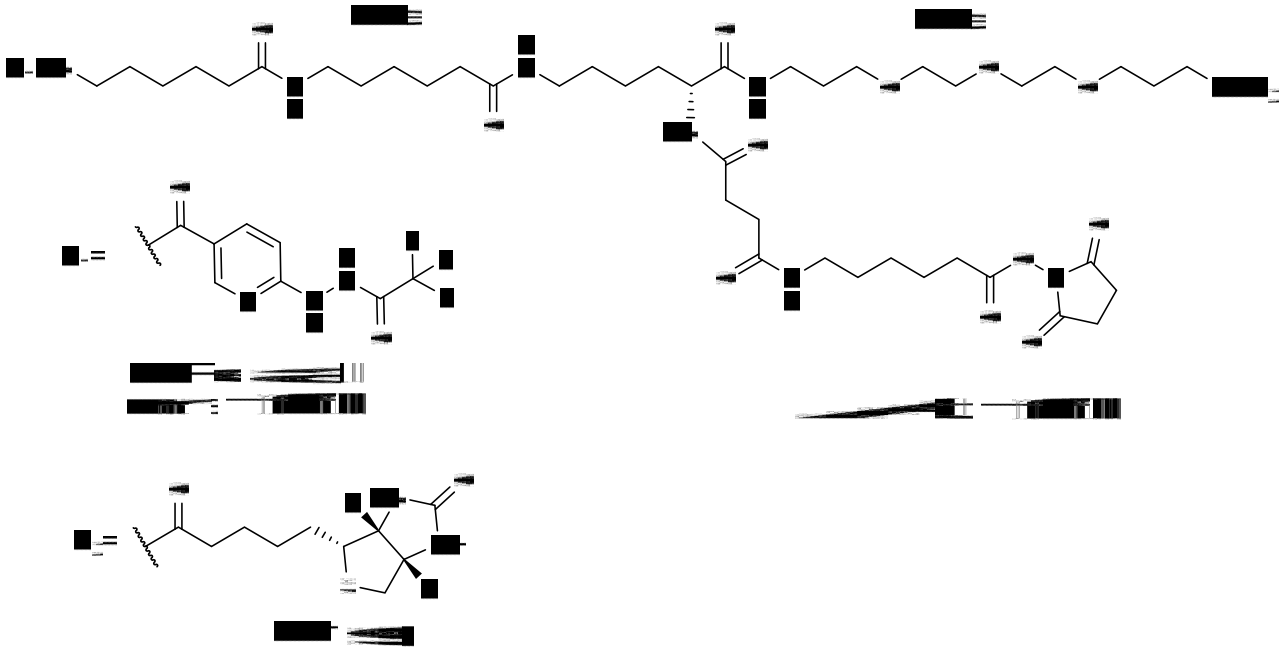


Figure 2.5 TriCEPS structure with functionalities to capture glycopeptides.[40]

They incorporated three independent functionalities: an N-hydroxysuccinimide ester for conjugating the reagent to a *ligand* containing a free amino group, taking into account that most proteins have primary amines to react with the ester and covalently link to it; a protected hydrazine that reacts with aldehydes in carbohydrates on glycoprotein receptors. This condensation reaction is viable with carbohydrate-derived aldehydes under essentially neutral conditions without prior removal of the protection group. Due to the reactivity of hydrazine unwanted reactions are limited at the same time the interaction ligand-receptor is happening. The biotin group was added for the affinity purification of the captured glycopeptides through mass spectrometric analyses. After receptor-capture reaction and cell lysis took place, TriCEPS-labeled peptides were *isolated* using streptavidin beads.[40] Studies of ligand-based receptor interaction are possible using TriCEPS

molecules, this approach was validated by characterizing well known receptor ligands such as insulin, transferrin, and epidermal growth factor.[40, 52] A small modi fication of a TriCEPS molecule includes click chemistry-based to capture ligand-receptor interactions at physiological pH, this method was called hydrazone-azide containing tri-functional compound-based ligand receptor capture (HATRIC-LRC; it used an acetone-protected hydrazone, an azide for the click chemistry, and the succinimidyl ester for ligand interaction; successful identifying ligands binding folate.[53]

2.2 NKT cells and CD1d receptor

2.2.1 NKT cell and function in the immune system

Invariant natural killer T (iNKT cells are a subgroup of NKT cells (a subset of mature T cells that express natural killer NK cell marker, and that develop in the thymus. iNKT cells are also known as type-I-NKT cells and Vα14i NKT cells, and constitute less than 1 % of total T lymphocytes, yet they represent a critical influence on a the variety of immune responses and pathologic conditions.[54-55] In contrast with other T cells that recognize peptide antigens, iNKT cells recognize glycolipid antigens presented by the major histocompatibility complex (MHC class 1-like molecule CD1d, a nonclassical class 1 like antigen-presenting molecule (APM.[56-60] Similar to T cells, NKT cells have a T cell receptor (TCR that differentiate upon stimulation into various subclasses, this process starts with production of interleukin-4 (IL-4, interferon-γ (IFN-γ), or tumor-necrosis factor (TNF).[61-62] There are two CD1d-dependent control points for NKT cell development.[63] One is in the thymus and controls the selection of cells. The second is either in the thymus or periphery and controls the transition of the receptors expressed on the cells towards maturation.[64]

iNKT cells develop and are selected in the thymus. During thymic selection the CD1d expression by iNKT thymocytes and the presence of endogenous antigens generates the positive selection of functional cells.[65] On the contrary, if TCR does not bind to the endogenous ligand, the T cell population is clonally deleted.[66] Many groups have researched the selection process. Kronenberg and coworkers proved that not only glycosphingolipids acted as endogenous antigens, but other molecules could potentially activate iNKT cells towards maturation.[67] Recognition of endogenous antigens by iNKT cells is important to control immune responses. When this process is not optimum, autoimmune diseases, cancer, and other diseases are present. Although several endogenous antigens for iNKT cells have been proposed, it is not fully understood how the production of endogenous antigens and subsequent activation of iNKT cells is regulated.[59]

iNKT cells differ from other NKT cells in the recognition of αGCs when presented by CD1d molecules.[68] Other NKT cells use molecules as endogenous antigen, such as sulfatides[69] and β-glucosylceramides (Figure 2.6).[70] Their functionality is still not well known, but they may play a unique regulatory role in autoimmunity, tumor immunity, and infection.[71] iNKT cells are particularly autoreactive. Even without an external antigen, iNKT cells can respond to CD1d-expressing antigen presenting cells (APCs). This autoreactivity has great implications in regulating immune responses, including cancer, autoimmunity, graft rejection, graft-versus-host disease, and even in controlling the metabolism.[59]

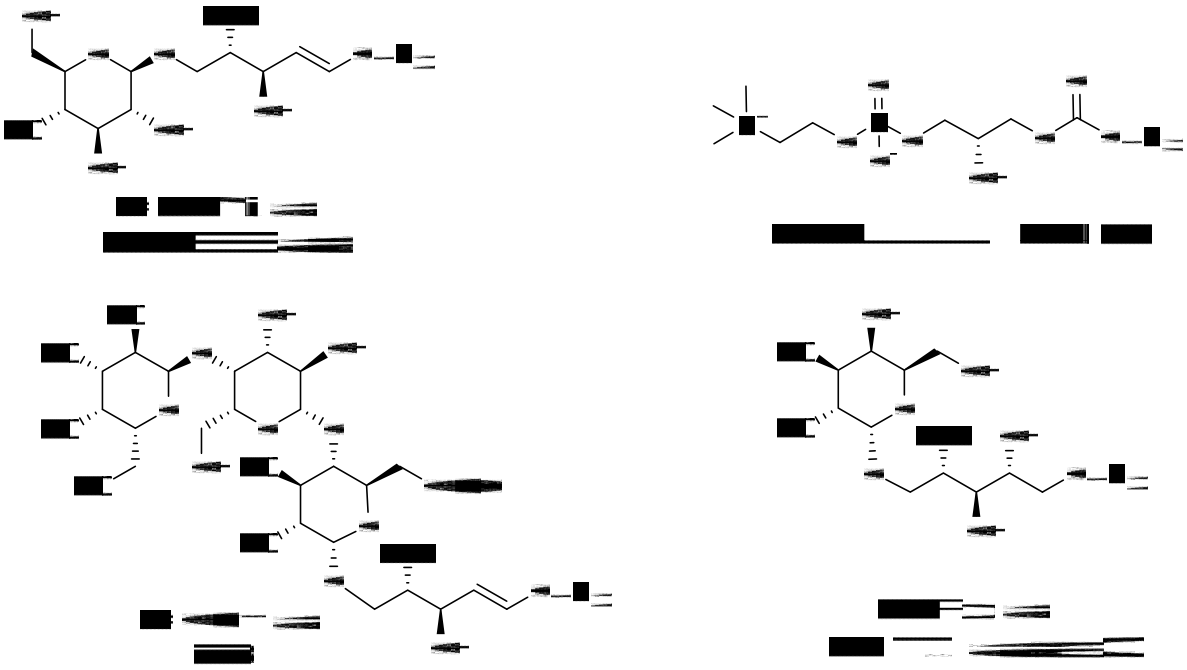


Figure 2.6 Representative NKT cell antigens.

2.2.2 CD1d Receptor and α-GalCer

CD1 molecules are a family of β-microglobulin associated glycoproteins that present lipids and glycolipids at the cell surface for recognition by TCR.[72] CD1d has a deep, narrow, and hydrophobic antigen-binding groove that is optimum for the presentation of lipid antigens, mostly glycolipids.[73] Although there are other NKT cell antigens such as isoglobotrihexosylceramide (iGb3,[74] lysophosphotidylcholine (LPC),[75] pLPE and pLPA,[76] the most used is α-GalCer (Figure 2.6).[54] α-GalCer or KRN7000 is a synthetic glycosphingolipid (GSL, originally isolated from a marine sponge, it has a sugar α linked to the ceramide lipid, the α linkage is critical for recognition by TCR, β-linked GSLs are abundant components of the mammalian body and are not usually antigenic, it is the most studied NKT cell activator and thought to be the endogenous antigen.[58]

2.2.3 Studies on α-GalCer derivatives and functionalities

α-GalCer has two limitations: it induces both immune modulating (IL-4) and stimulating (IFN-γ) cytokines (which may counteract one another and it can stimulate iNKT cells to an extent that reaches cell inactivation, which has urged for research to find more effective iNKT cell antigens.[77-78] Many publications report modifications on α-GalCer and their corresponding activity towards NKT cell activation. The modifications include different lipid chains on the ceramide,[79-82] as well as different modifications on the sugar.[83]

It has been shown through structure activity relationships studies and by X-Ray crystallography of CD1d and α-GalCer, that position 6 of the sugar does not play an important role in the interaction with the receptor and its modification is not relevant to its activity.[84-86] Furthermore, our lab previously published that using nervonic acid for the ramification of the sphingosine chain increased the stimulation properties of α-GalCer. Therefore, our NKT cell activator piece of the CD1d-Triceps was made with nervonic acid.[87]

Several groups have synthesized molecules in aims to elucidate the mechanism of α-GalCer activation and the mechanism of other molecules that are involved in NKT cell activation including trafficking of the glycolipids before the CD1d recognition.[88]

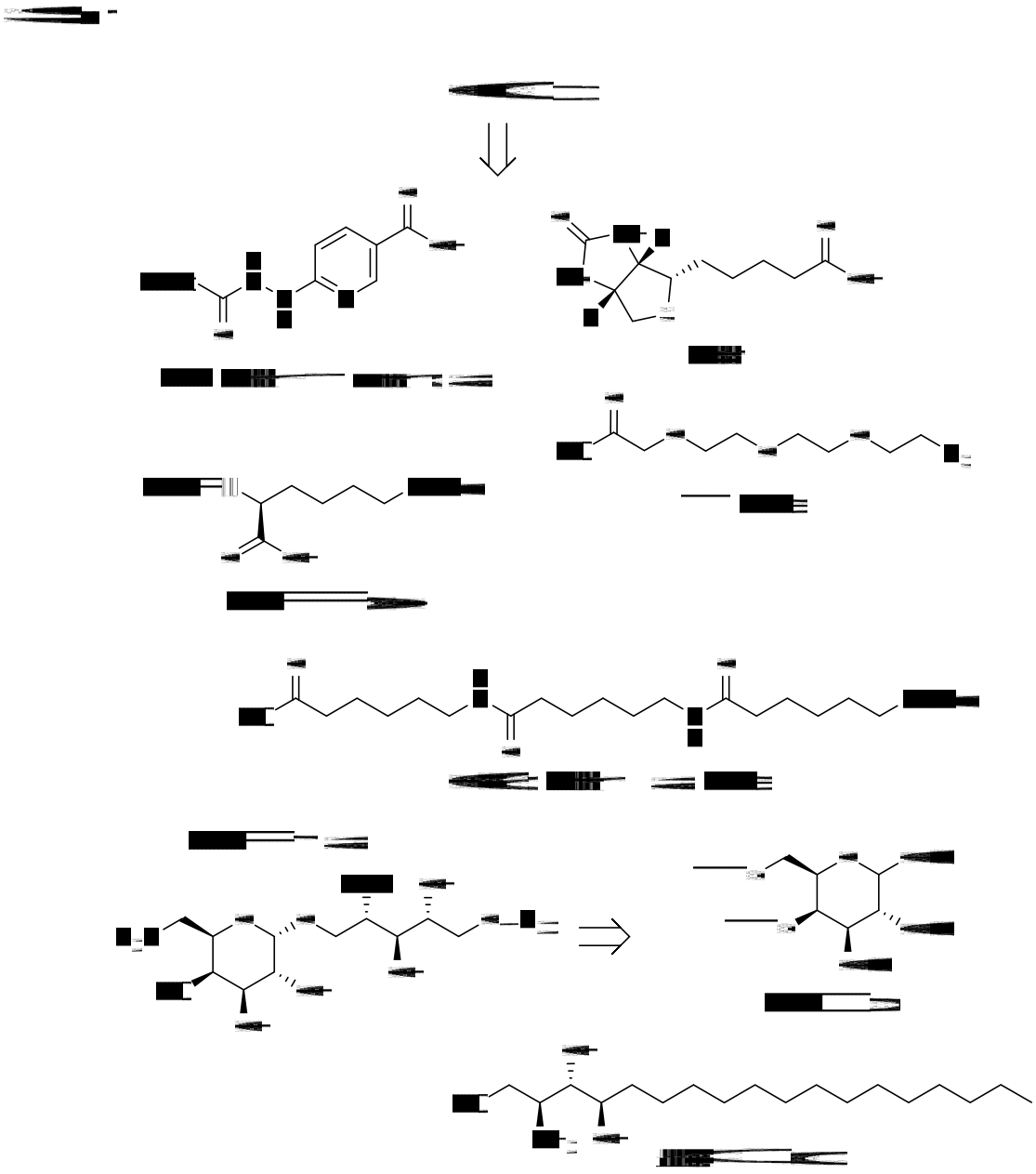
2.2.4 α-GalCer derivative, our approach

The modification of the 6'-hydroxyl to an amine group allowed attachment of different moieties into the α-GalCer parent compound. Fluorophores like dansyl, biotin, and others had been attached to the α-GalCer parent compound through this modification without interfering with NKT cell activation.[89-91] Although activation towards immune response has improved,[92] there is still research to be done to find the best activation for adaptive immunity.[83, 93-95]

We hypothesized that by attaching a Lysine and two linkers, the solubility of the α-GalCer derivative would be preserved,[87] biotin would help with compound purification,[89] and the trifluoroacetyl-HYNIC[96] tag, under biological conditions, would lose the trifluoroacetyl group and link to any proteins close by. These proteins are likely to be involved in the transportation of glycolipids until their recognition by the immune cells. This triple functionality of CD1d-Triceps would help increase the knowledge of the different proteins involved in glycolipid trafficking and lipid transfer.

2.3 Synthesis

Our synthesis started with the α-GalCer intermediate, to which linkers were attached. The TEG linker was eventually functionalized with a biotin molecule, while the photo-active site was attached to the 6-aminohexanoic acid linker. Both linkers were added unto the NKT cell activator through an orthogonally protected Lysine (Scheme 1):



2.3.1 Synthesis of the α-GalCer intermediate:

We first used Liu Yang's synthesis[97] to obtained our NKT cell activator starting from D-Galactose and phytosphingosine and reduced the intermediate using Birch reduction. However, Yang's approach did not provide enough α-GalCer intermediate to complete the CD1d-Triceps. We changed our synthetic route and started with BOC protection of the phytosphingosine, eventually attaching the nervonic acid to a more advanced intermediate (Figure 2.7).[98] This approach was very versatile because other acids could be used instead of nervonic acid.

The best method to purify Boc-phytosphingosine was recrystallization, this avoids the steps of peracetylation and global deprotection required by Liu Yang's synthesis. Once the BOC phytosphingosine intermediate **2.1** was obtained, the primary alcohol was selectively protected with TDS followed by acetylation of the secondary alcohols that gave compound **2.2**. Selective deprotection of TDS group gave the desired intermediate primary alcohol **2.3** (Scheme 2) ready to couple to the D-Gal TMS protected compound **2.4** (Scheme 3).

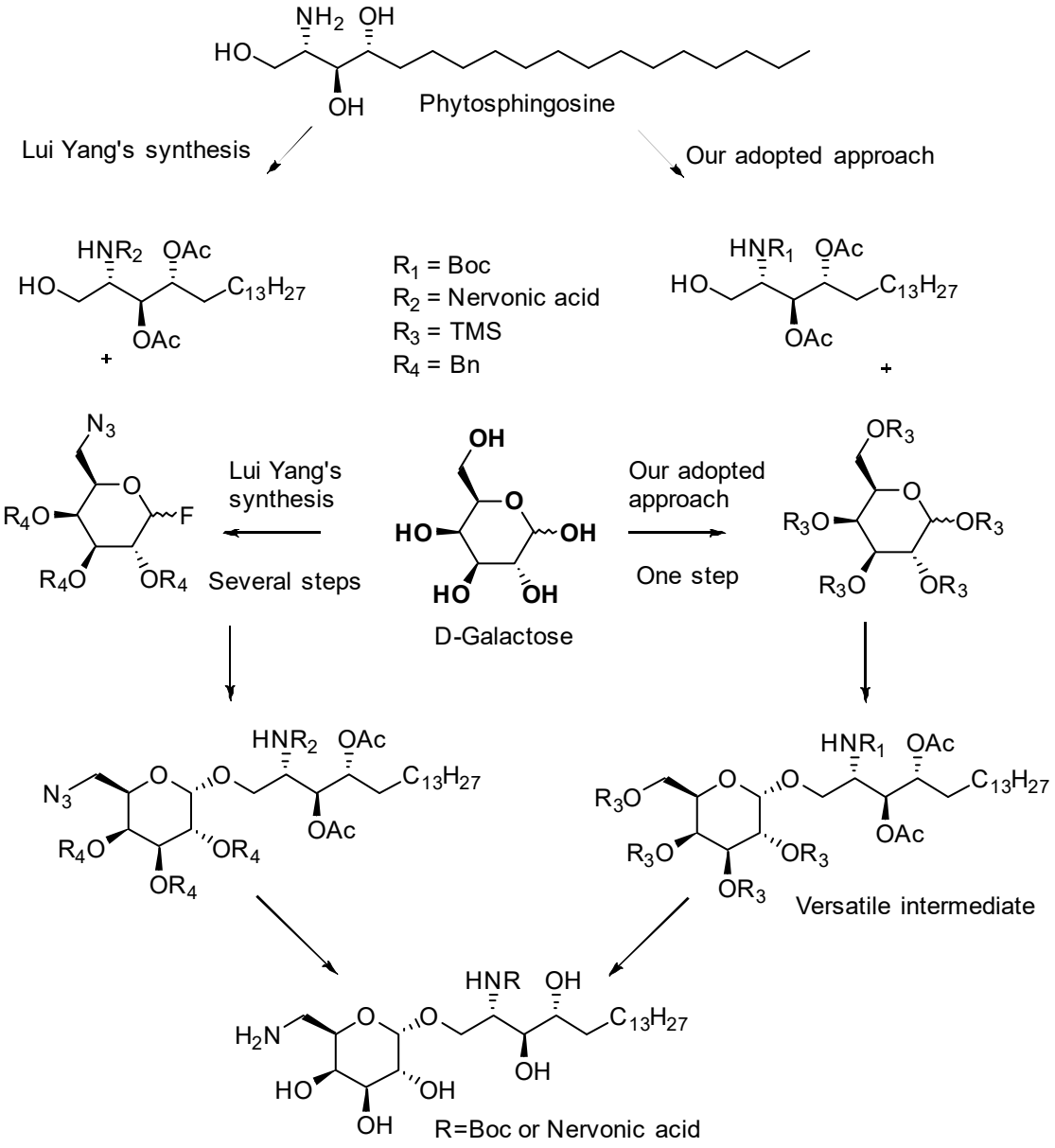
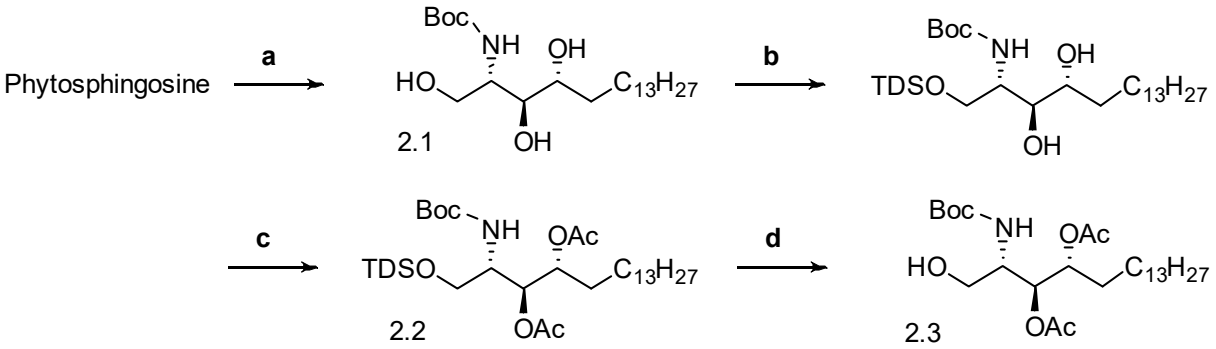


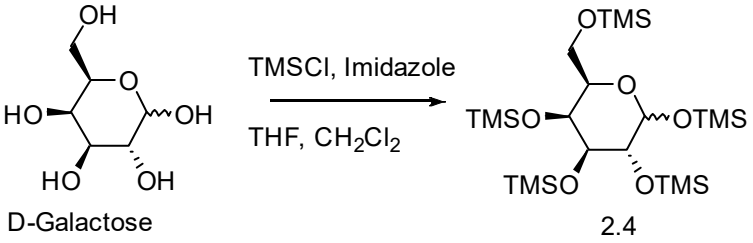
Figure 2.7 Synthesis of versatile intermediate towards the preparation of the CD1d-Triceps.

Scheme 2



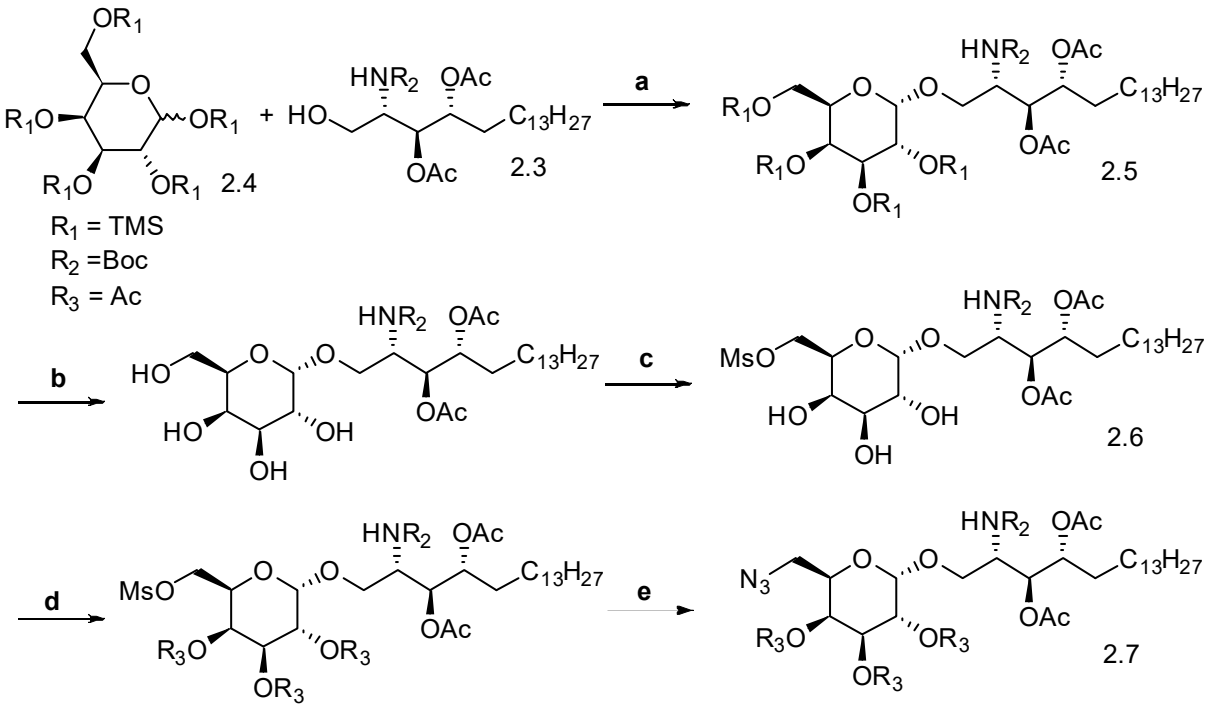
Reagents and conditions: **a)** Boc_2O, CH_2Cl_2, Et_3N;**b)** TDSCl, Et_3N, Pyr.0°C;
c) Ac_2O, DMAP; **d)**HF, ACN, CH_2Cl_2.

Scheme 3



Several coupling methods, including the Schmidt coupling were tested to afford the desired compound **2.5**, but the Gervay-Hague method[99] proved to be the most convenient towards alpha adduct formation (Scheme 4). The fact that only one step was needed to prepare the sugar **2.4** was another advantage of this route. Although putting an azide on the sixth position of the sugar during the beginning steps of the synthesis was practical, the azide on the sixth position of the sugar interfered with the electronic activation of the sugar towards the coupling with the primary alcohol acceptor **2.4**. If used in the coupling, the azide did not form compound **2.5**.[98]

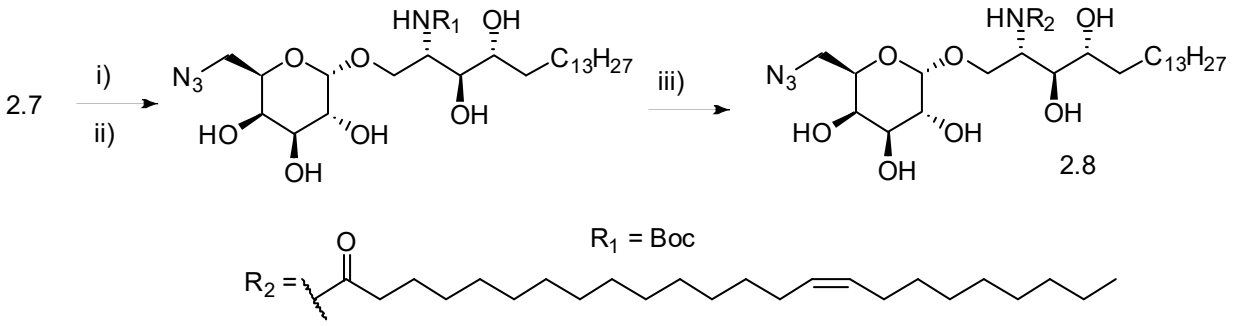
Scheme 4



Reagents and conditions: **a)** TMSI. Benzene, DIPEA, MS3Å, 65°C; **b)** Amberlyte, MeOH; **c)** MsCl, Et_3N, CH_2Cl_2, 0°C; **d)** Ac_2O, DMAP, CH_2Cl_2; **e)** NaN_3, DMSO, 80°C.

α-GalCer intermediate **2.9** amine was obtained by deprotecting the TMS group of compound **2.5** using Amberlyte avoiding heating the reaction or the BOC could be cleaved as well. Although all the alcohols of the sugar were deprotected, mesylation of the primary alcohol was achieved using kinetic conditions, once the mesylation was complete, peracetylation of the secondary alcohols gave compound **2.6**. Nucleophilic attack to the mesylated alcohol with sodium azide gave compound **2.7** (Scheme 4). Global deprotection of the alcohols and BOC deprotection of the amine, allowed the peptide bond formation with nervonic acid on the phytosphingosine and compound **2.8** was obtained.

Scheme 5



Reagents and conditions: **i)** NaOMe, MeOH; **ii)** HCl in Dioxane 4M, CH_2Cl_2;
iii) Nervonic acid, HOBT, EDCI, CH_2Cl_2.

This approach allowed the synthesis of a versatile intermediate **2.8**, it could afford different products changing the acyl chain.

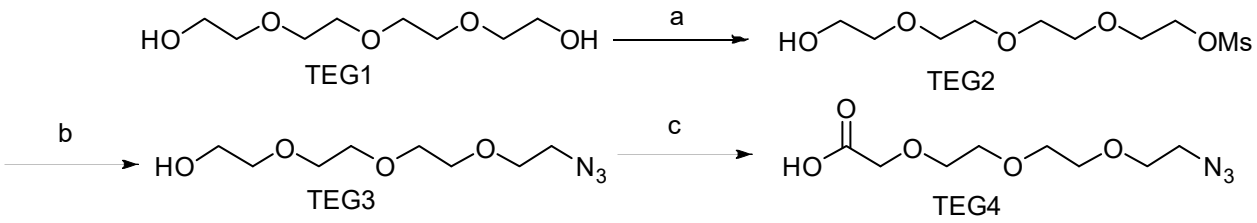
2.3.2 Synthesis of the linkers:

The linkers were selected in hopes of keeping the solubility of the molecule and simultaneously giving enough room to connect to the proteins related to the trafficking of the NKT cell activators. Thus, the linker for the biotin moiety was a TEG-3-linker and the linker for the photoactive site was a polyamide chain of repeating units of 6-aminohexanoic acid.

2.3.2.1 Synthesis of the TEG linker:

The synthesis of the TEG-3 linker started with a chain of tetraethylene glycol **TEG1**. Selective mesylation of one of the alcohols followed by sodium azide nucleophilic attack gave intermediate **TEG3**. Once the azide was in place, the oxidation of the alcohol using Jones reagent gave the desired carboxylic acid linker **TEG4** (Scheme 6).

Scheme 6

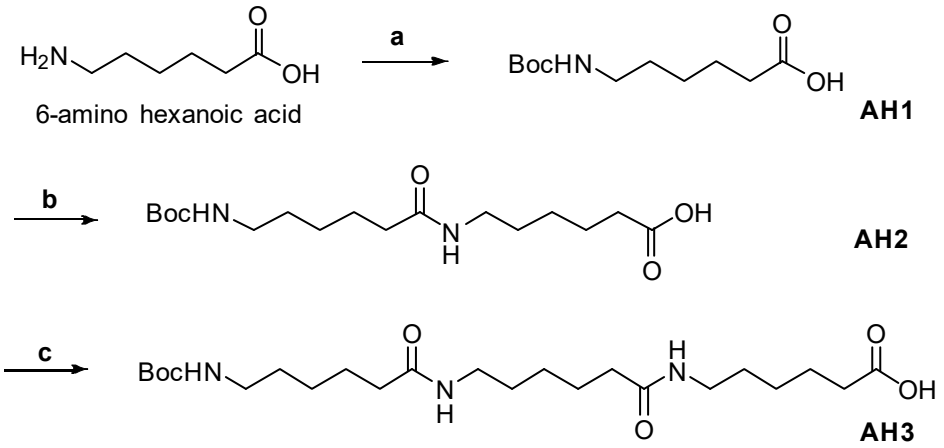


Reagents and conditions: **a)**MsCl, Et_3N, DCM; **b)**NaN_3, DMSO, 80°C; **c)**Jones reagent (CrO_3, H_2SO_4).

2.3.2.2 Synthesis of the 6-aminohexanoic acid linker:

The synthesis of the 6-aminohexanoic acid linker started with BOC protection of 6-aminohexanoic acid that gave compound **AH1**. Once **AH1** was obtained, activation with HOBT and EDCI in DCM and another equivalent of 6-aminohexanoic acid elongated the chain and gave compound **AH2**. Without purification the acid was activated with more HOBT, EDCI, and one more equivalent of 6-aminohexanoic acid was added to obtain intermediate **AH3** (Scheme 7). After both amide bonds were formed, the linker was purified and used for the total synthesis of CD1d-Triceps.

Scheme 7

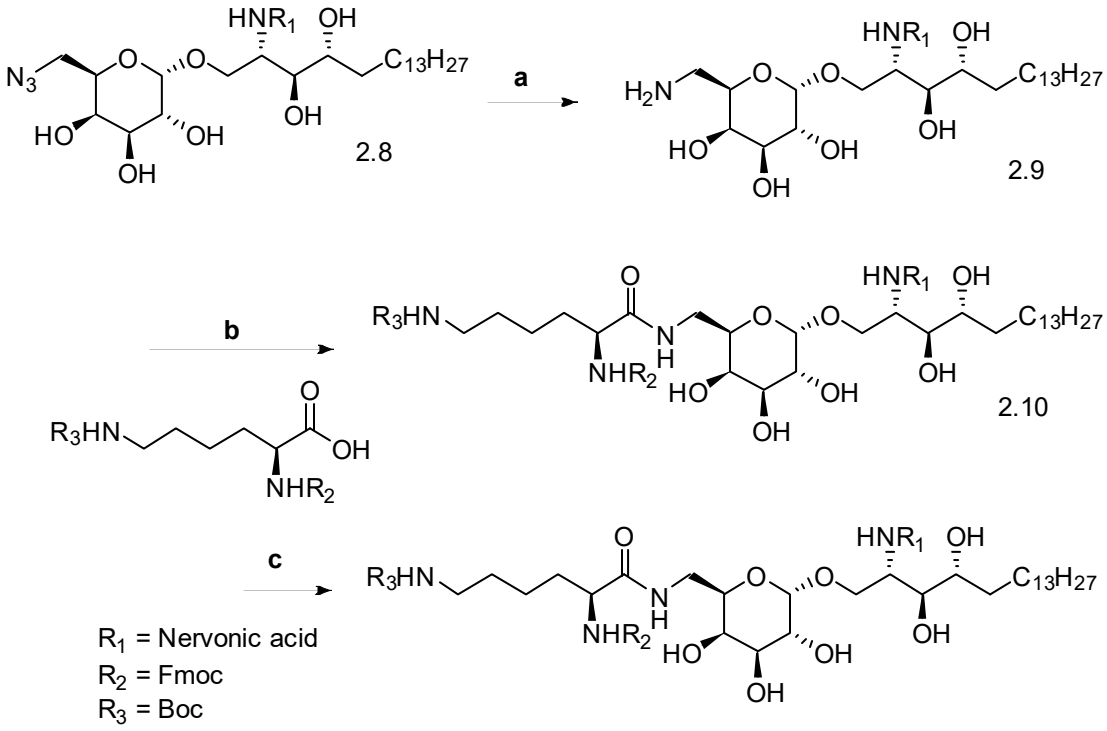


Reagents and conditions: **a)**Boc_2O, DCM, Et_3N; **b)** EDCI, HOBT, 6-NH_2hexanoic acid, DCM; **c)** EDCI, HOBT, 6-NH_2hexanoic acid, DCM.

2.3.3 Synthesis of the CD1d-Triceps:

As shown in scheme 8, once the nervonic acid was installed on the phytosphingosine chain, the azide on the sixth position of the sugar was reduced and gave intermediate **2.9.** Compound **2.9** was coupled to an FMOC-lysine(BOC) and gave compound **2.10** (Scheme 8) . The two orthogonal protecting groups on the Lysine were very important towards a convergent synthesis as the BOC protecting group could be cleaved under acidic conditions and the FMOC under basic conditions.

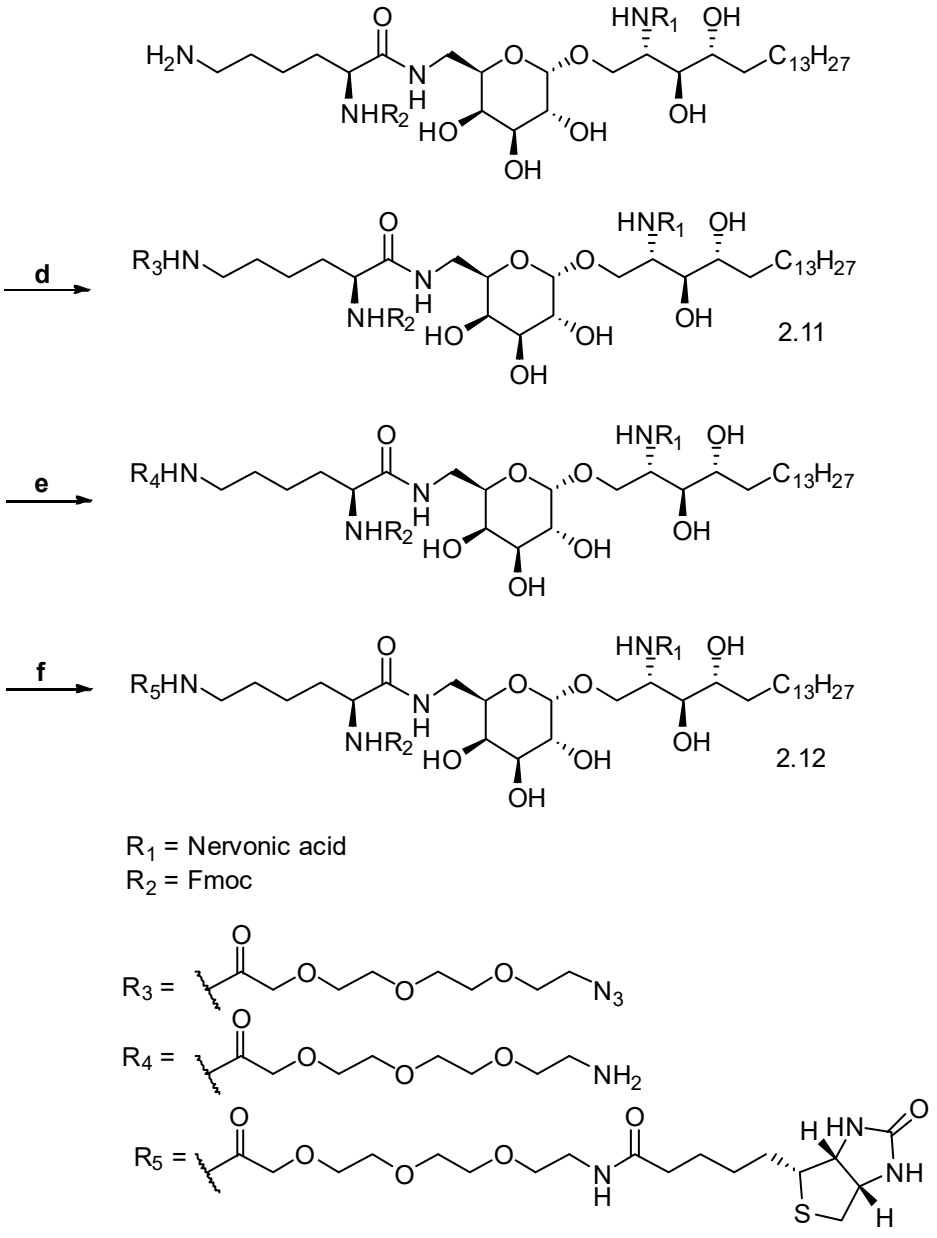
Scheme 8



Reagents and conditions: **a)** Zn, HCl, MeOH:THF; **b)** HOBT, EDCI, CH_2Cl_2; **c)** 4M HCl in Dioxane, CH_2Cl_2.

Boc deprotection on the Lysine of compound **2.10** gave a free amine that was attached to the TEG linker **TEG4**, and gave compound **2.11**. Reduction of the azide on compound **2.11** afforded the amine able to form a peptide bond with biotin and gave compound **2.12**. The peptide bond was installed by activation with HOBT and EDCI or using the already activated biotin as a succinimidyl ester (Scheme 9).

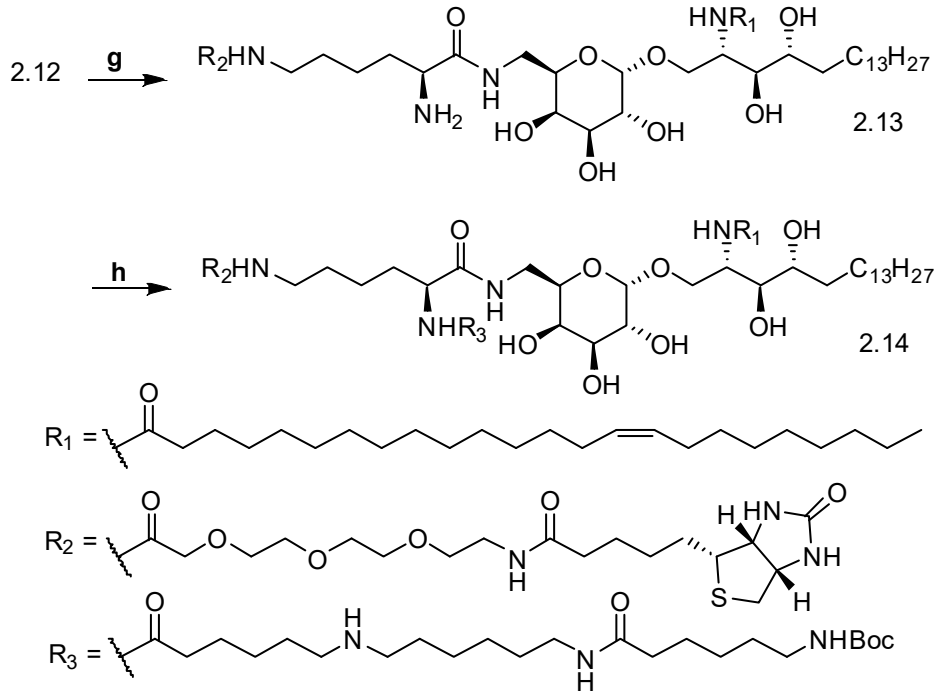
Scheme 9



Reagents and conditions: **d)** TEGlinker, HOBT, EDCI, CH_2Cl_2; **e)** Zn, HCl, MeOH:THF; **f)** Biotin, EDCI, HOBT, CH_2Cl_2.

FMOC deprotection of compound **2.12** gave an amine that was coupled to the acid of the 6-aminohexanoic acid linker **AH3**, which gave compound **2.14** (Scheme 10). Boc deprotected intermediate **2.14**, was linked to the photoactive site by a peptide bond.

Scheme 10



Reagents and conditions: **g)** Et_3N, CH_2Cl_2; **h)** 6-amino hexanoic acid linker, HOBT, EDCI, CH_2Cl_2.

Once the BOC-6-hydrazinonicotinidic acid moiety was attached and formed compound **2.15**, the BOC was cleaved with TFA, both acid and anhydride, this not only cleaved the tert-butyl group but added the trifluoroacetyl amide. In other words, the BOC protecting group was replaced by a trifluoroacetyl amide, this gave CD1d-Triceps **2.16** (Scheme 11).


i

2.4 Results and discussion

Luc Teyton and his associates in La Jolla immunologically evaluated the CD1d-Triceps molecule. They incubated rat basophilic leukemia (RBL) cells in a dish with the probe, washed and imaged with streptavidin-Alexa 400 at time 0 and after 60 minutes chase (to see the amount of internalization). Figure **2.8** shows RBL cells incubated and imaged in the start of the experiment and shows some cells with the compound on the surface of them, Figure **2.9** shows some internalization of the compound after an hour of incubation. These results show the compound working as it was expected, with some internalization after 60 minutes of incubation.

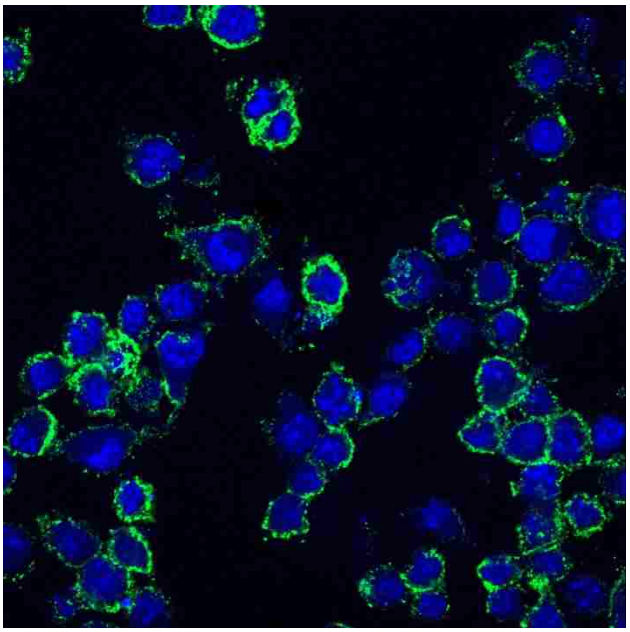


Figure 2.8 CD1d-Triceps labeling 60', 100mM SURFACE, 63x mag.

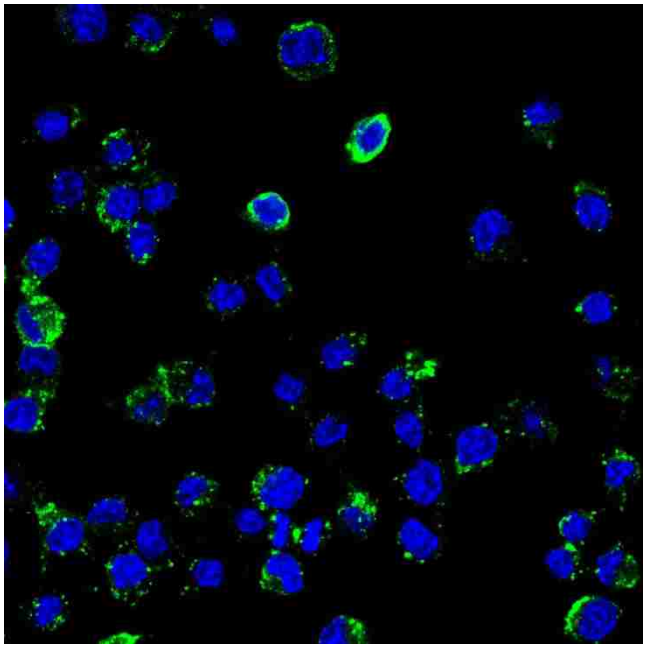
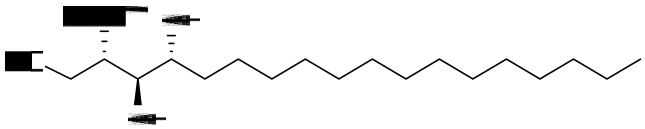


Figure 2.9 CD1d-Triceps labeling 60', 100mM, Chase 60 minutes, 63x mag.

2.5 Conclusions

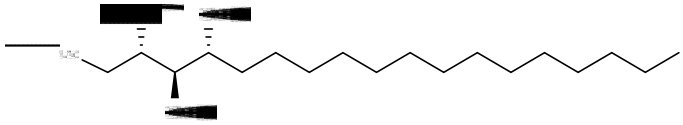
We have successfully synthetized an analogue of KRN7000 linked to a cross-linking moiety and a biotin molecule to facilitate glycolipid transport studies *in vivo*. Preliminary results from our collaborators, who performed the *in vitro* and *in vivo* studies, show that they were able to use our synthesized compound CD1d-Triceps to observe glycolipid transport and localization on the membrane of basophils, supporting the hypothesis that our strategy will advance the understanding of glycolipid trafficking and lipid transfer mediated immune responses. Until this point, *in vivo* studies of glycolipids have been complicated, leading many scientists to search for effective immune regulator α-GalCer analogues through trial and error. Understanding the lipid transfer proteins involved in the trafficking of α-GalCer will help us synthesize compounds with more specific activity.

2.6 Experimental procedures



2.1

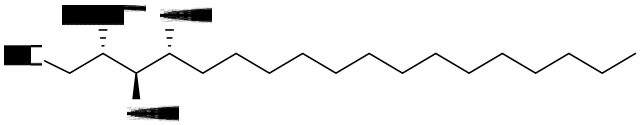
A flask containing phytosphingosine (30 g, 94.6 mmol) in DCM (300 mL) was added Boc_2O (24.75 g, 113.56 mmol) and Triethylamine (3mL). The solution was stirred overnight, concentrated under vacuum, and purified by recrystallization from Ethyl acetate, obtaining 39.45 g of intermediate as white crystals in 99 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.44 (d, *J* = 8.3 Hz, 1H), 3.91-3.85 (m, 1H), 3.85-3.81 (m, 2H), 3.73 (dd, *J* = 10.9, 5.2 Hz, 1H), 3.68-3.61 (m, 3H), 3.37 (br, 1H), 2.02 (br, 1H), 1.71-1.65 (m, 1H), 1.54-1.47 (m, 2H), 1.45 (s, 9H), 1.26 (s, 22H), 0.88 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 156.47, 80.11, 77.27, 77.02, 76.76, 76.15, 72.92, 61.92, 52.93, 33.07, 31.92, 29.71, 29.69, 29.67, 29.66, 29.64, 29.37, 28.36, 27.72, 27.41, 25.93, 22.69, 14.12; TOF-MS (ESI) calculated for $C_{23}H_{48}NO_5[M+H]^+$: 418.3527, found: 418.3450.



2.2

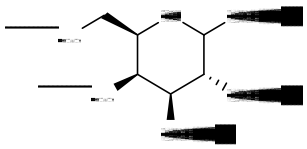
To a solution of carbamate **2.1** (39.45 g, 94.6 mmol) in pyridine, was added TDSCl (20.30 g, 113.56 mmol). After TLC showed the reaction was complete, Ac_2O was added in excess, as well as, a catalytic amount of DMAP. The solution was stirred for another hour, and the pyridine was extracted out with an acidic workup of 5 % HCl and DCM. The organic phase was neutralized

with $NaHCO_3$ and washed three times with DCM (150 mL). The organic fractions were combined and dried over Na_2SO_4, concentrated under vacuum, and purified through column chromatography (SiO_2, 3 % Ethyl acetate in Hexanes), giving 47.5 g of compound **2.2** in 78 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.19 (d, *J* = 9.5, 2.6 Hz, 1H), 5.02 (d, *J* = 10.4 Hz, 1H), 4.91 (d, *J* = 9.9 Hz, 1H), 3.84 (t, *J* = 10.0 Hz, 1H), 3.57 (d, *J* = 2.9 Hz, 2H), 2.06 (s, 3H), 2.01 (s, 3H), 1.74-1.68 (m, 1H), 1.59 (qq, *J* = 13.8, 7.3 Hz, 2H), 1.44 (s, 9H), 1.24 (s, 22H), 0.87 (s, 6H), 0.86 (s, 3H), 0.82 (s, 6H), 0.06 (s, 3H), 0.04 (s, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 73.29, 71.45, 61.44, 51.04, 34.49, 29.59, 28.34, 27.49, 27.48, 20.87, 20.80, 20.25, 18.22, 13.98, -3.85, -3.92; TOF-MS (ESI) calculated for $C_{35}H_{70}NO_7Si$ $[M+H]^+$: 644.4916, found: 644.5027.



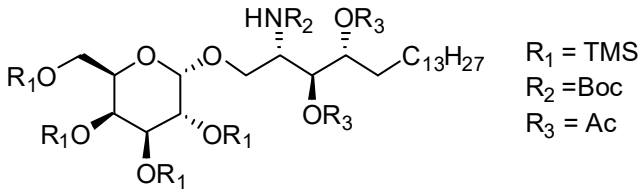
2.3

To a plastic container with Acetonitrile (150 mL) was added a concentrated solution of **2.3** (10 g, 15.53 mmol) in DCM (5 mL) and increasing volumes of HF (5 mL). The reaction was monitored by TLC until almost all the starting material was consumed and little to no byproduct was formed. The HF was neutralized with $NaHCO_3$, and the compound was extracted with DCM and concentrated under vacuum with careful monitoring of the temperature. The intermediate was used in the following step without purification. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.22 (d, *J* = 9.7 Hz, 1H), 5.10-5.02 (m, 2H), 3.83-3.78 (m, 1H), 3.62-3.54 (m, 2H), 2.43 (br, 1H), 2.13 (s, 3H), 2.04 (s, 3H), 1.78-1.72 (m, 1H), 1.70-1.62 (m, 1H), 1.44 (s, 9H), 1.25 (s, 22H), 0.87 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 171.50, 170.76, 155.44, 79.93, 73.03, 72.90, 61.66, 51.09, 31.92, 29.69, 29.68, 29.65, 29.63, 29.56, 29.35, 29.21, 28.29, 27.96, 25.61, 22.69, 21.03, 20.91, 14.12; TOF-MS (ESI) calculated for $C_{27}H_{51}NO_7$ $[M+H]^+$: 501.3660, found: 501.3286.



2.4

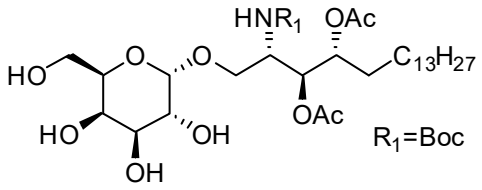
To a 0 °C solution of D-galactose (10 g, 55.5 mmol) in (1:1) DCM and THF (200 mL) was added imidazole (45.34 g, 666 mmol). Trimethylsilyl chloride (36.18 g, 333 mmol) was added slowly, and the reaction was allowed to come to room temperature. The reaction was monitored by TLC and, after completion, the solvent was evaporated under vacuum and the product purified through column chromatography (SiO_2, 1:8 Ethyl acetate: Hexanes), obtaining 22 g of **2.4** in 76 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.05 (d, *J* = 2.2 Hz, 1H), 3.92-3.89 (m, 2H), 3.82 (dd, *J* = 1.7 Hz, 2H), 3.63 (dd, *J* = 9.6, 7.9 Hz, 1H), 3.54 (dd, *J* = 9.6, 5.7 Hz, 1H), 0.14 (s, 27H), 0.12 (s, 9H), 0.11 (s, 9H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 94.57, 72.28, 71.09, 70.45, 69.94, 61.18, 0.64, 0.46, 0.30, 0.18, -0.49; TOF-MS (ESI) calculated for $C_{21}H_{53}O_6Si_5[M+H]^+$: 541.2683, $C_{24}H_{57}N_2O_6Si_5$ $[M+Imid.]^{2+}$: 609.3052, found: (plus imidazole) 609.3174.



2.5

To a nitrogen flushed RBF with **2.4** (17 g, 13.14 mmol) was added TMSI (6.916 g, 34.5 mmol) and Benzene. The reaction was stirred for 4 hours, after which the solvent was evaporated under vacuum, and the crude was kept under nitrogen. In a separate flask, a suspension of compound **2.3** (9.44 g, 18.8 mmol) in benzene (50 mL), was added TBAI (n-Bu$_4$NI) (23.08 g, 62.85 mmol), DIPEA (i-Pr$_2$NEt) (8.77 g, 62.28 mmol) and 28 g of molecular sieves. The suspension was stirred at room temperature for 5 minutes. To the solution of **2.3** was added very slowly the solution of

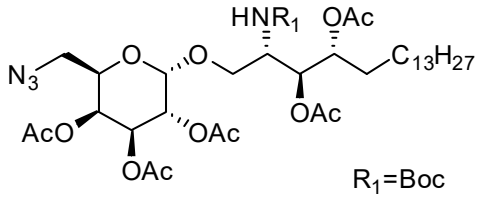
the glycosyl iodide intermediate. The mixture was stirred overnight at reflux temperature of 65 °C. After refluxing, the reaction was filtered through celite. The filtrate was concentrated under vacuum, and the product was purified by basified triethylamine column chromatography (SiO_2, 1:8 Ethyl acetate: Hexanes), giving 5.4 g of compound in 43 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.52 (d, *J* = 9.1 Hz, 1H), 5.21 (dd, *J* = 8.7, 3.0 Hz, 1H), 5.07 (d, *J* = 10.4 Hz, 1H), 4.64 (d, *J* = 3.5 Hz, 1H), 3.99-3.93 (m, 1H), 3.90 (s, 1H), 3.88 (d, *J* = 3.5 Hz, 1H), 3.80-3.75 (m, 2H), 3.66 (dd, *J* = 11.0, 3.4 Hz, 1H), 3.62-3.56 (m, 3H), 2.06 (s, 3H), 2.01 (s, 3H), 1.93 (d, *J* = 2.0 Hz, 1H), 1.74-1.68 (m, 1H), 1.64-1.56 (m, 1H), 1.45 (s, 9H), 1.25 (s, 22H), 0.88 (t, *J* = 6.9 Hz, 3H), 0.16 (s, 9H), 0.14 (s, 9H), 0.13 (s, 9H), 0.12 (s, 9H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 170.58, 169.63, 155.47, 101.39, 94.49, 79.53, 73.33, 73.07, 72.19, 71.81, 70.62, 70.27, 69.68, 69.51, 69.32, 68.37, 63.30, 61.17, 50.10, 31.92, 29.69, 29.65, 29.63, 29.58, 29.36, 29.30, 28.36, 28.22, 27.77, 25.63, 22.69, 22.54, 21.02, 20.92, 20.20, 18.48, 14.12, 1.01, 0.85, 0.73, 0.70, 0.61, 0.49, 0.42, 0.39, 0.29, 0.26, 0.16, -0.08, -0.29, -0.52, -0.76, -0.78; TOF-MS (ESI) calculated for $C_{45}H_{94}NO_{12}Si_4$ $[M+H]^+$: 952.5848, $C_{45}H_{97}N_2O_{12}Si_4$ $[M+NH_4]^+$: 969.6119, found: 952.5936, and 969.6216.



2.6

To a solution of compound **2.5** (4g, 4.2 mmol) in MeOH (10 mL) was added a scoop of Amberlyte IR120 H-form beads, which was stirred for 30 minutes. The complete deprotection of the TMS group of the sugar was confirmed by MS, and the compound was concentrated under low heat and carried on without purification. To a 0 °C solution of deprotected compound **2.5** (1.39 g, 2.11 mmol) in DCM (20 mL) was added triethylamine (382 mg, 3.79 mmol) and a portion of the methane sulfonyl chloride (265 mg, 2.326 mmol) was added very slowly. The reaction was closely

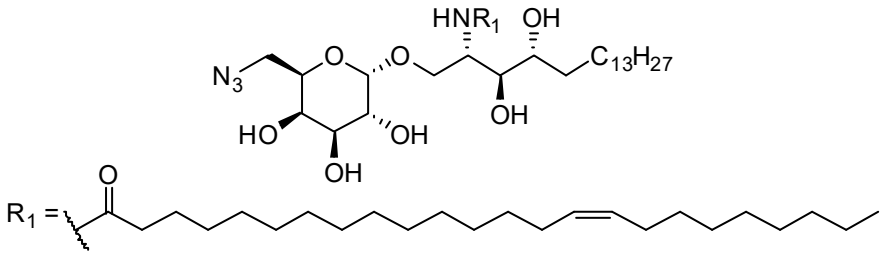
monitored by TLC, once spot of the mono-mesylated compound was observed, the reaction was quenched with saturated solution of $NaHCO_3$ and the aqueous phase washed with DCM. The combined organic layers were dried over Na_2SO_4 and the product was purified by column chromatography (SiO_2, 5 % MeOH in DCM), obtaining 1.46 g of **2.6** in 46 % yield. ^1H NMR (500 MHz, Chloroform-*d*) δ 6.26 (d, *J* = 9.0 Hz, 1H), 6.09 (d, *J* = 9.6 Hz, 1H), 5.25 (d, *J* = 8.0 Hz, 1H), 5.02 (br, 1H), 4.93 (dd, *J* = 10.4 Hz, 1H), 4.76 (d, *J* = 3.2 Hz, 1H), 4.63 (br, 1H), 4.31 (s, 1H), 4.04 (s, 1H), 3.94 (s, 1H), 3.86-3.64 (m, 4H), 3.53 (d, *J* = 8.8 Hz, 1H), 2.07 (s, 3H), 1.98 (s, 3H), 1.72-1.64 (m, 1H), 1.60-1.53 (m, 1H), 1.40 (s, 9H), 1.21 (s, 22H), 0.84 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$ from HSQC and HMBC) δ 171.45, 171.23, 100.59, 73.59, 73.44, 71.50, 70.60, 69.75, 68.18, 61.97, 49.46, 31.51, 29.26, 28.14, 27.33, 27.27, 20.78, 20.56; TOF-MS (ESI) calculated for deprotected compound $C_{33}H_{62}NO_{12}$ $[M+H]^+$: 664.4267, found: 664.4921, and for mesylated compound $C_{34}H_{67}N_2O_{14}S$ $[M+NH_4]^+$: 759.4309, found: 759.4418.



2.7

To a solution of compound **2.6** (1.022 g, 1.38 mmol) in DCM (50 mL) was added an excess amount of Ac_2O (5 mL) and DMAP (30 mg). After stirring for 1 hr, the reaction was quenched with $NaHCO_3$, and the fully protected intermediate was extracted out with DCM. The combined organic layers were dried over Na_2SO_4, the solvent was evaporated under vacuum and the remnant was used for the following step without purification. The fully protected syrup 2.6 (1.61 g, 1.85 mmol) was dissolved in DMSO (20 mL), and NaN_3 (355 mg, 5.45 mmol) was added. The reaction was refluxed at 80 °C overnight and was later quenched with H_2O. The aqueous phase was extracted

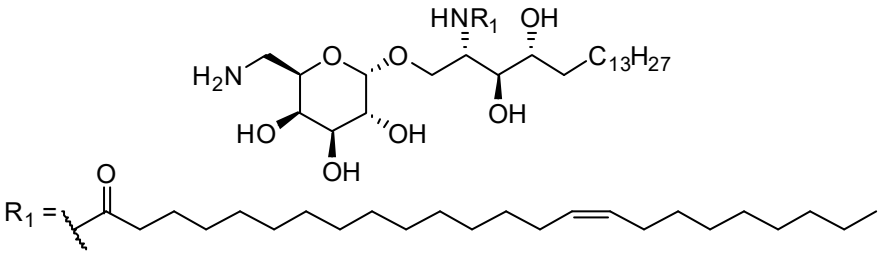
with DCM, the combined organic layers were dried over Na_2SO_4, and the compound was purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes), obtaining 827 mg of **2.7** in 72 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.36 (d, *J* = 3.4 Hz, 1H), 5.22 (dd, *J* = 10.9, 3.4 Hz, 1H), 5.15 (dd, *J* = 9.7, 2.7 Hz, 1H), 5.07 (dd, *J* = 12.0, 2.5 Hz, 1H), 4.94 (dt, *J* = 10.8, 2.6 Hz, 1H), 4.88 (d, *J* = 3.7 Hz, 1H), 4.06 (dd, *J* = 8.3, 4.3 Hz, 1H), 3.99-3.93 (m, 1H), 3.70 (dd, *J* = 10.9, 2.9 Hz, 1H), 3.39 (dd, *J* = 12.8, 8.6 Hz, 2H), 3.10 (dd, *J* = 12.8, 4.2 Hz, 1H), 2.08 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.93 (s, 3H), 1.92 (s, 3H), 1.67-1.64 (m, 1H), 1.59-1.52 (m, 1H), 1.41 (s, 9H), 1.19 (s, 22H), 0.81 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 170.68, 170.57, 170.06, 169.84, 169.66, 155.01, 97.13, 80.08, 73.08, 71.15, 69.40, 68.65, 68.16, 67.80, 67.51, 67.33, 50.62, 49.58, 31.83, 29.60, 29.59, 29.56, 29.53, 29.46, 29.45, 29.26, 29.12, 28.23, 27.47, 25.56, 22.73, 22.60, 20.85, 20.66, 20.51, 20.50, 14.03; TOF-MS (ESI) calculated for fully protected mesylated intermediate $C_{40}H_{73}N_2O_{17}S$ $[M+NH_4]^+$: 885.4624, found: 885.4686 and azide compound $C_{39}H_{70}N_5O_{14}$ $[M+NH_4]^+$: 832.4914, found: 832.4 994.



2.8

To a solution of compound **2.7** (164 mg, 0.202 mmol) in 50 mL of MeOH was added a catalytic amount of NaOMe in MeOH. The reaction was stirred for 20 minutes and then quenched with 4M HCl in Dioxane (10 mL). The fully deprotected intermediate was concentrated under vacuum and kept under vacuum while the acid was activated. To a solution of nervonic acid (86 mg, 0.242 mmol) in DCM (10 mL) was added HOBT (33 mg, 0.242 mmol) and EDCI (46 mg, 0.242 mmol), and stirred for 35 minutes. After stirring, the amine was dissolved in DCM (5 mL),

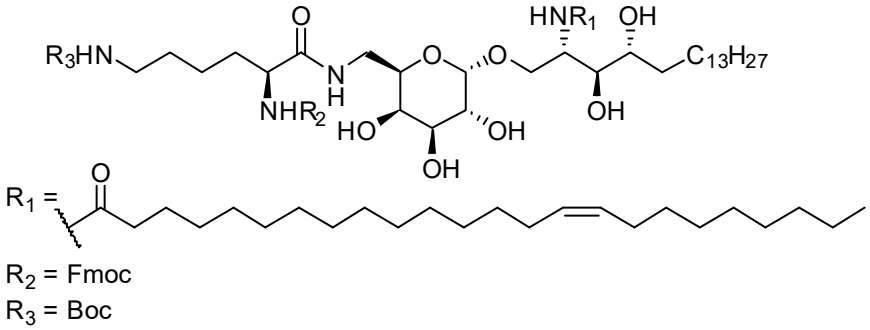
neutralized with triethylamine (3-5 mL), and added into the activated acid. The solution was stirred overnight. The reaction was concentrated under vacuum and the compound was purified by column chromatography (SiO_2, 5 % MeOH in DCM), obtaining 115 mg of **2.8** in 68 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.61 (d, J = 9.0 Hz, 1H), 5.33-5.28 (m, 2H), 4.72 (d, J = 3.2 Hz, 1H), 4.66 (t, J = 5.6 Hz, 2H), 4.59 (d, J = 6.0 Hz, 1H), 4.43 (d, J = 7.4 Hz, 1H), 4.24 (d, J = 7.0 Hz, 1H), 4.00-3.94 (m, 1H), 3.82 (dd, J = 8.9, 4.0 Hz, 1H), 3.66 (dd, J = 10.2, 4.6 Hz, 1H), 3.62-3.58 (m, 1H), 3.57-3.55 (m, 2H), 3.54 (dd, J = 10.2, 3.6 Hz, 1H), 3.49 (dd, J = 10.6, 3.9 Hz, 1H), 3.46-3.42 (m, 1H), 3.39-3.35 (m, 1H), 3.18 (dd, J = 12.8, 4.0 Hz, 1H), 2.08-2.03 (m, 2H), 1.99-1.94 (m, 4H), 1.49-1.40 (m, 4H), 1.22 (s, 58H), 0.84 (t, J = 6.7 Hz, 6H); ^{13}C NMR (126 MHz, dmso) δ 171.99, 130.08, 130.06, 99.78, 73.94, 71.43, 70.99, 70.38, 70.01, 69.80, 69.42, 68.65, 67.14, 51.57, 50.05, 44.30, 39.90, 39.82, 39.65, 39.48, 35.91, 31.77, 31.75, 31.69, 29.69, 29.64, 29.60, 29.57, 29.55, 29.53, 29.51, 29.48, 29.44, 29.37, 29.32, 29.30, 29.20, 29.17, 29.15, 29.05, 29.02, 27.01, 26.99, 25.88, 25.85, 22.56, 14.39; TOF-MS (ESI) calculated for $C_{48}H_{93}N_4O_8$ $[M+H]^+$: 853.6988, found: 853.6991.



2.9

To a solution of compound **2.8** (180 mg, 0.211 mmol) in 1:1 MeOH and THF (50 mL) was added Zn metal mesh 20 (0.5g), concentrated HCl (1 mL), and acetic acid (1 mL). The reaction was sonicated for 15 minutes and monitored by TLC. If the reaction was not complete after 15 minutes, more acid was added. Upon completion, the solution was separated from the metal in a new vessel and concentrated under vacuum. The amine was kept under vacuum until the acid was activated

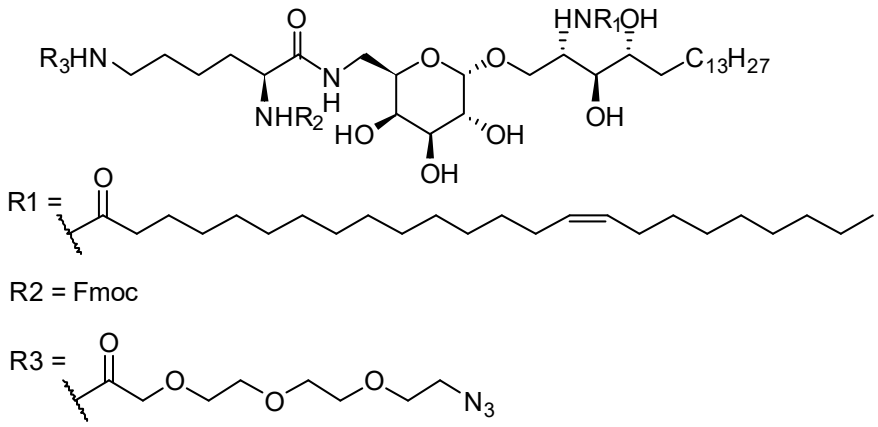
without purification. TOF-MS (ESI) calculated for $C_{48}H_{95}N_2O_8$ $[M+H]^+$: 827.7083, found: 827.7081.



2.10

Lysine NH(Fmoc)-Lys(Boc)COOH (198 mg, 0.422 mmol) was dissolved in DCM (5 mL). HOBT (57 mg, 0.422 mmol) and EDCI (81 mg, 0.422 mmol) were added and the reaction was stirred for 45 minutes. After that, compound **2.9** (206 mg, 0.25 mmol) was dissolved in DCM (10 mL), basified with triethylamine (2 mL), and was added into the Lysine mixture. The reaction was stirred overnight and monitored by TLC. The solvent was evaporated under vacuum and the compound was purified by column chromatography (SiO_2, 10 % MeOH in DCM), obtaining 169 mg of **2.10** in 53 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.90-7.83 (m, 2H), 7.75-7.70 (m, 1H), 7.63 (dd, J = 9.5 Hz, 1H), 7.46 (dd, J = 8.0 Hz, 1H), 7.41-7.36 (m, 2H), 7.33-7.28 (m, 1H), 6.74 (s, 1H), 5.29 (s, 2H), 4.73-4.67 (m, 1H), 4.63-4.56 (m, 1H), 4.54 (dd, J = 10.2 Hz, 1H), 4.29-4.19 (m, 3H), 3.98-3.88 (m, 2H), 3.74 (dd, J = 7.8 Hz, 1H), 3.64 (s, 2H), 3.58 (s, 1H), 3.55-3.44 (m, 3H), 3.40-3.29 (m, 5H), 3.18 (s, 1H), 2.87 (s, 1H), 2.09-2.00 (m, 2H), 1.98-1.88 (m, 4H), 1.34 (s, 9H), 1.20 (s, 62H), 0.83 (t, J = 6.4 Hz, 6H); ^{13}C NMR (126 MHz, dmso) δ 172.73, 172.10, 171.98, 170.20, 156.39, 155.99, 144.47, 144.16, 143.03, 141.17, 141.14, 139.88, 137.88, 132.19, 130.02, 130.00, 129.62, 129.37, 128.01, 127.73, 127.45, 125.87, 125.79, 121.83, 120.48, 110.18, 99.83, 99.68, 77.71, 73.80, 73.68, 71.44, 70.99, 70.00, 69.84, 69.70, 69.42, 69.21, 68.95, 68.66, 66.76, 66.15, 64.94, 55.220, 50.30, 50.08, 47.11, 44.30, 38.24, 35.96, 32.06, 31.79, 31.77, 31.53,

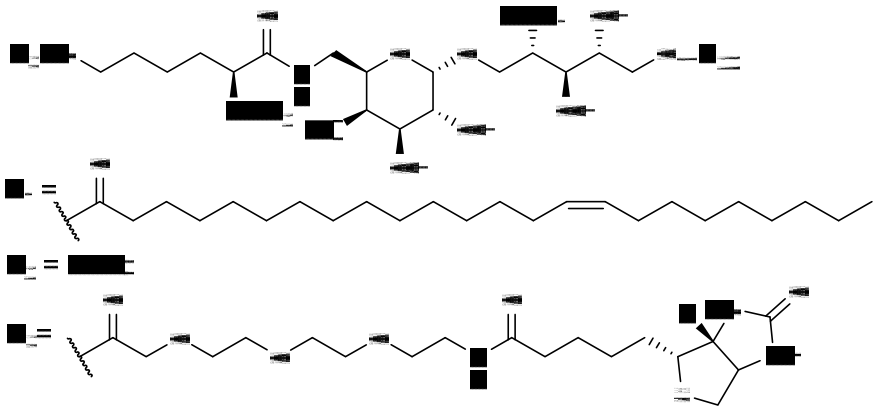
31.14, 29.77, 29.75, 29.73, 29.68, 29.63, 29.60, 29.56, 29.53, 29.50, 29.36, 29.32, 29.22, 29.19, 29.14, 29.07, 29.06, 28.97, 28.70, 27.01, 26.30, 25.95, 25.87, 23.52, 23.35, 22.86, 22.57, 20.97, 14.36, 14.35, 13.94; TOF-MS (ESI) calculated for $C_{74}H_{125}N_4O_{13}$ $[M+H]^+$: 1277.9238, found: 1277.9201.



2.11

To a solution of functionalized tetra ethylene glycol linker (8 mg, 0.033 mmol) in DCM (10 mL) was added HOBT (4 mg, 0.033 mmol), EDCI (6 mg, 0.033 mmol), and the reaction was stirred for 45 minutes. While the acid was reacting, compound **2.10** (35 mg, 0.027 mmol) was dissolved in DCM (2 mL), 4M HCl in Dioxane (2 mL) was added, and the reaction was stirred for 10 minutes until completion via TLC. The free amine was concentrated under vacuum and further dried under high vacuum, then dissolved in DCM (5 mL), basified with triethylamine (2 mL), and added to the activated acid reaction. The amine and the activated acid were stirred overnight or until completion of the reaction. The solvent was evaporated under vacuum, and the compound was purified by column chromatography (SiO_2, 10 % MeOH in DCM), obtaining 19 mg of **2.11** in 52 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.71 (d, *J* = 7.5 Hz, 2H), 7.57 (dd, *J* = 7.9 Hz, 2H), 7.34 (dd, *J* = 7.4 Hz, 2H), 7.25 (d, *J* = 7.6 Hz, 2H), 5.30-5.26 (m, 2H), 4.83 (dd, *J* = 4.0 Hz, 1H), 4.80 (dd, *J* = 3.8 Hz, 1H), 4.38-4.29 (m, 2H), 4.17 (dd, *J* = 7.1 Hz, 1H), 4.12 (dd, *J* = 5.1 Hz, 1H), 4.04 (dd, *J*

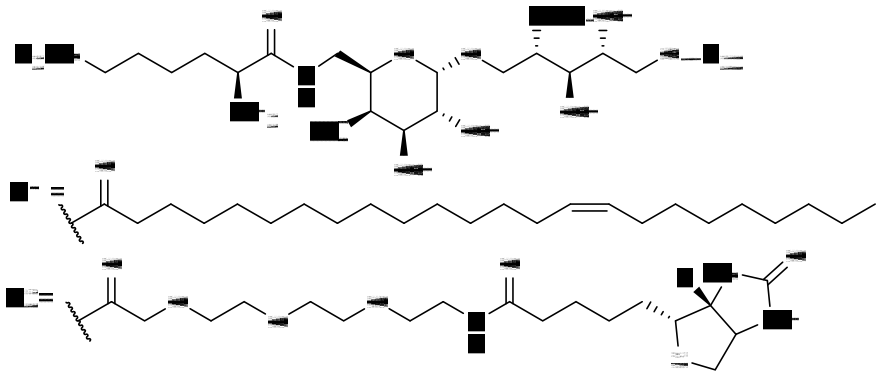
= 8.6, 5.2 Hz, 1H), 3.95-3.88 (m, 3H), 3.80 (dd, *J* = 10.6, 5.1 Hz, 1H), 3.73-3.68 (m, 2H), 3.66-3.61 (m, 2H), 3.61 (s, 4H), 3.51-3.43 (m, 2H), 3.32 (d, *J* = 3.7 Hz, 2H), 3.26 (s, 14H), 2.16-2.08 (m, 2H), 1.97-1.92 (m, 2H), 1.88 (s, 1H), 1.61-1.40 (m, 4H), 1.20 (s, 62H), 0.82 (t, *J* = 6.8 Hz, 6H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 174.29, 163.81, 156.67, 156.25, 143.65, 141.21, 129.82, 127.68, 127.02, 124.98, 119.90, 99.36, 70.72, 70.42, 70.36, 70.17, 70.10, 69.91, 50.53, 49.61, 49.44, 49.27, 49.09, 49.06, 48.92, 48.75, 48.58, 47.05, 36.44, 31.83, 31.81, 29.71, 29.67, 29.63, 29.58, 29.51, 29.42, 29.33, 29.27, 29.22, 28.89, 27.11, 25.76, 22.59, 13.97; TOF-MS (ESI) calculated for the deprotected compound $C_{69}H_{117}N_4O_{11}$ $[M+H]^+$: 1177.8713, found: 1177.8685; and for the coupled compound $C_{77}H_{130}N_7O_{15}$ $[M+H]^+$: 1392.9619, found: 1392.9427, and $C_{77}H_{133}N_8O_{15}$ $[M+NH_4]^+$: 1409.9885, found: 1409.9815.



2.12

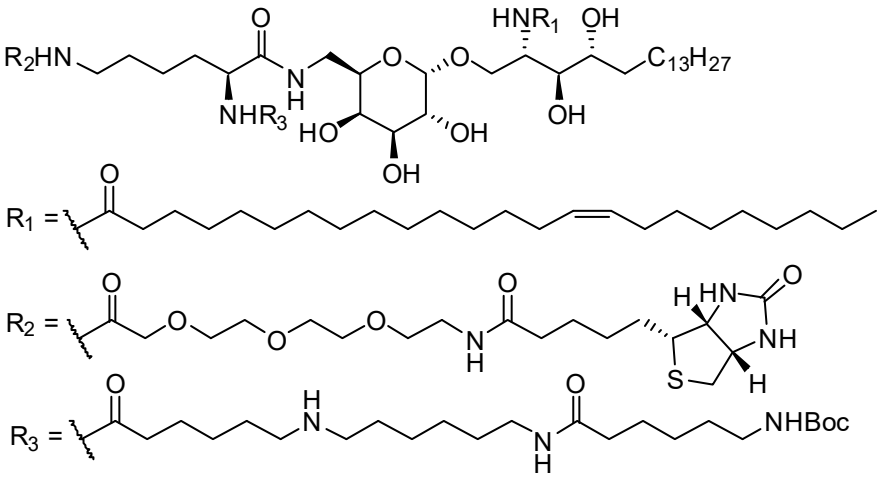
To a solution of biotin (34 mg, 0.014 mmol) in DMF (5 mL) was added HOBT (19 mg, 0.014 mmol) and EDCI (26 mg, 0.014 mmol), and the reaction was stirred for 30 to 45 minutes. While the acid was activating, compound **2.11** (16 mg, 0.011 mmol) was dissolved in 1:1 MeOH and THF (20 mL), and Zn metal mesh 20 (0.5g) and concentrated HCl (2mL) were added until bubbles appeared. The reaction was sonicated and monitored by TLC until completion. After the reaction was complete, the solution was separated from the metal into a new vessel, concentrated

by rotary evaporation, and further dried under vacuum before being carried over to the next step without purification. The amine was dissolved in DMF (5 mL), triethylamine (2 mL) was added, and then the solution was added to the activated biotin reaction, and the mixture was stirred overnight. After the reaction was complete, the solvent was evaporated under vacuum and the compound was purified by column chromatography (SiO_2, 10 % MeOH in DCM with 1 % NH_4OH), obtaining 9 mg of intermediate **2.12** in 41 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.92 (d, J = 8.4 Hz, 2H), 7.87 (d, J = 7.5 Hz, 1H), 7.85-7.80 (m, 1H), 7.76-7.71 (m, 1H), 7.63 (d, J = 8.3 Hz, 2H), 7.45 (dd, J = 7.6 Hz, 2H), 7.35 (dd, J = 7.6 Hz, 2H), 6.43 (s, 1H), 6.35 (s, 1H), 5.30-5.28 (m, 1H), 4.71 (s, 1H), 4.61 (s, 1H), 4.53 (s, 1H), 4.38 (s, 1H), 4.31-4.26 (m, 2H), 4.24 (dd, J = 12.6, 7.0 Hz, 1H), 4.22-4.19 (m, 2H), 4.14-4.09 (m, 2H), 3.98-3.89 (m, 2H), 3.84 (s, 1H), 3.66-3.59 (m, 1H), 3.57 (s, 2H), 3.54-3.46 (m, 6H), 3.39-3.28 (m, 8H), 3.28-3.20 (m, 4H), 3.19-3.12 (m, 2H), 3.11-3.01 (m, 4H), 2.81 (dd, J = 12.5, 5.1 Hz, 1H), 2.56 (dd, J = 12.5, 3.5 Hz, 1H), 2.33-2.26 (m, 3H), 2.19 (t, J = 7.5 Hz, 1H), 2.08-2.00 (m, 2H), 1.98-1.92 (m, 2H), 1.63-1.55 (m, 4H), 1.54-1.36 (m, 10H), 1.21 (s, 62H), 0.81 (t, J = 6.7 Hz, 6H); ^{13}C NMR (126 MHz, cd_3od) δ 174.37, 129.82, 128.13, 126.26, 125.77, 117.37, 110.83, 99.44, 74.56, 71.98, 70.73, 70.44, 70.38, 70.21, 70.15, 70.07, 69.93, 69.84, 69.13, 68.75, 50.55, 50.49, 50.28, 46.13, 45.30, 40.07, 39.00, 38.43, 36.45, 36.10, 35.97, 35.64, 31.84, 31.82, 29.79, 29.71, 29.67, 29.65, 29.64, 29.62, 29.58, 29.53, 29.42, 29.35, 29.28, 29.26, 29.23, 29.21, 28.98, 28.72, 28.24, 27.65, 27.12, 27.10, 26.17, 25.82, 25.34, 25.22, 25.10, 22.93, 22.59, 21.39, 13.96, 8.45; TOF-MS (ESI) calculated for reduced compound $C_{77}H_{132}N_5O_{15}$ $[M+H]^+$: 1366.9714, found: 1366.9702 and coupled $C_{87}H_{146}N_7O_{17}S$ $[M+H]^+$: 1593.0490, found: 1593.0502.



2.13

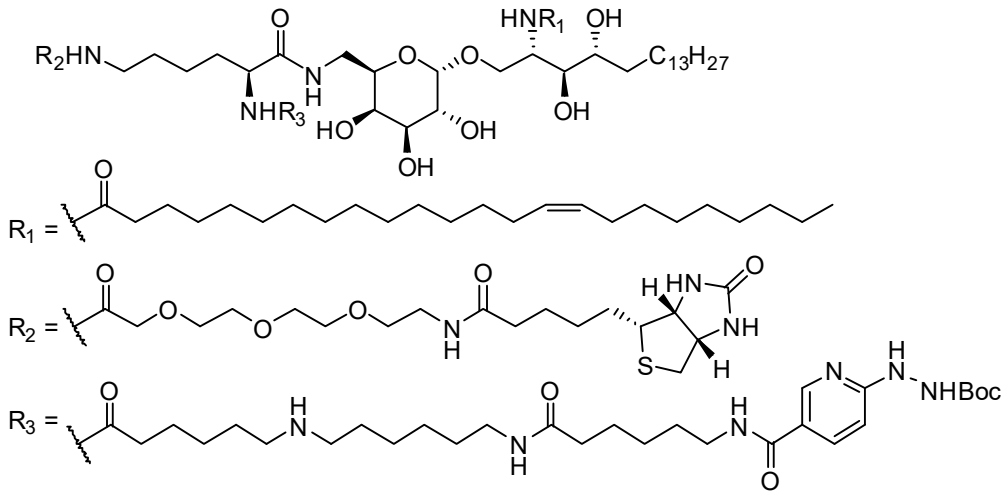
To a solution of FMOC-protected compound **2.12** (80 mg, 0.05 mmol) was added triethylamine (5 mL), the solution was stirred overnight and monitored by TLC and MS. More triethylamine was added until full deprotection was achieved. After the reaction was complete, the compound was concentrated under vacuum and carried over to the next step without purification. TOF-MS (ESI) calculated for $C_{72}H_{136}N_7O_{15}S$ $[M+H]^+$: 1370.9810, found: 1370.9845.



2.14

To a solution of the 6-aminocaproic acid linker (13 mg, 0.024 mmol) in DCM (5 mL) was added HOBT (4 mg, 0.024 mmol), and EDCI (6 mg, 0.024 mmol), and the solution was stirred for 45

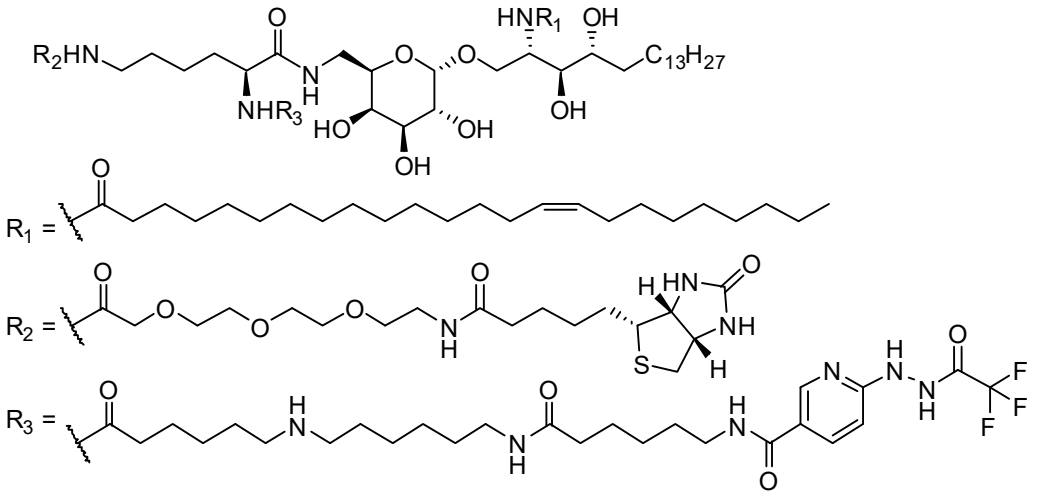
minutes. Deprotected compound **2.13** (27 mg, 0.02 mmol) was dissolved in DCM (3 mL) and added to the activated 6-aminocaproic acid linker and allowed to stir overnight. After the reaction was complete, the solvent was evaporated under vacuum and the desired compound was purified by column chromatography (SiO_2, 15 % MeOH in DCM with 1 % of NH_4OH), obtaining 9 mg of **2.14** in 26 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.41 (d, J = 8.7 Hz, 0H), 8.09 (br, 1H), 8.02 (br, 1H), 7.89 (br, 1H), 7.75 (d, 1H), 7.72 (br, 1H), 7.64 (d, J = 8.2 Hz, 1H), 7.52 (d, J = 7.8 Hz, 1H), 7.17-7.11 (m, 1H), 6.81-6.71 (m, 2H), 6.43 (s, 4H), 6.38 (s, 4H), 5.30 (t, J = 4.9 Hz, 1H), 4.74 (s, 1H), 4.68 (s, 1H), 4.62 (s, 1H), 4.45 (s, 1H), 4.29 (dd, J = 7.7, 5.0 Hz, 1H), 4.27-4.62 (m, 2H), 4.14-4.10 (m, 6H), 3.94 (s, 1H), 3.84 (s, 2H), 3.65-3.58 (m, 2H), 3.54 (s, 4H), 3.52-3.48 (m, 4H), 3.44-3.39 (m, 8H), 3.38-3.28 (m, 20H), 3.20-3.16 (m, 4H), 3.11-3.02 (m, 4H), 3.01-2.94 (m, 4H), 2.93-2.77 (m, 6H), 2.57 (d, J = 12.4 Hz, 4H), 2.23-2.14 (m, 6H), 2.13-2.08 (m, 4H), 2.07-2.03 (m, 4H), 2.02-1.97 (m, 6H), 1.96-1.92 (m, 2H), 1.64-1.55 (m, 4H), 1.53-1.39 (m, 2H), 1.35 (s, 9H), 1.21 (s, 72H), 0.83 (t, J = 6.8 Hz, 6H); ^{13}C NMR (126 MHz, dmso) δ 172.71, 172.61, 172.27, 169.47, 163.18, 156.01, 143.31, 130.07, 127.85, 123.09, 118.57, 111.88, 99.75, 77.71, 70.95, 70.64, 70.40, 70.17, 70.01, 69.61, 69.03, 68.89, 61.51, 61.49, 59.66, 59.65, 55.88, 49.02, 45.76, 41.65, 38.86, 38.76, 38.40, 35.97, 35.83, 35.55, 34.32, 32.01, 31.77, 31.74, 29.75, 29.64, 29.60, 29.57, 29.53, 29.50, 29.47, 29.41, 29.32, 29.28, 29.19, 29.16, 29.04, 29.02, 28.72, 28.65, 28.63, 28.52, 28.49, 27.00, 26.62, 26.47, 25.86, 25.71, 25.54, 25.49, 25.43, 25.14, 23.35, 22.56, 14.39, 11.41; TOF-MS (ESI) calculated for $C_{95}H_{178}N_{10}O_{20}S$ $[M+H]^{2+}$: 1811.2929, found: 1811.3057.



2.15

To a solution of 6-Boc-hydrazynonicotinic acid (2 mg, 9 μmol) in DCM (5 mL) was added HOBT (1 mg, 9 μmol) and EDCI (2 mg, 9 μmol), and the reaction was stirred for 45 minutes. Compound **2.14** (14 mg, 7 μmol) was dissolved in DCM (1 mL) and 4M HCl in Dioxane (1 mL) was added. The reaction was stirred for 10 minutes and monitored by TLC and MS until completion. Once the amine was deprotected, the solvent was evaporated by rotary evaporation and further dried under vacuum. To the deprotected amine dissolved in DCM (5 mL) added triethylamine (1 mL) and then added to the activated acid and stirred overnight. After the reaction was complete, the solvent was evaporated under vacuum and the compound was purified by column chromatography (SiO_2, 15 % MeOH in DCM with 1 % NH_4OH). We obtained 5 mg of intermediate **2.15** in 36 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.48 (s, 1H), 7.96 (d, *J* = 8.8 Hz, 1H), 6.67 (d, *J* = 8.8 Hz, 1H), 5.33-5.25 (m, 2H), 4.79 (d, *J* = 3.7 Hz, 1H), 4.44 (dt, *J* = 8.1, 4.4 Hz, 1H), 4.24-4.22 (m, 1H), 4.19 (t, *J* = 7.0 Hz, 1H), 4.10 (d, *J* = 4.4 Hz, 1H), 3.94 (d, *J* = 3.4 Hz, 2H), 3.78 (dd, *J* = 10.7, 4.7 Hz, 1H), 3.71 (dd, *J* = 9.9, 4.0 Hz, 3H), 3.65 (d, *J* = 3.1 Hz, 1H), 3.62 (s, 4H), 3.59 (s, 4H), 3.57 (s, 1H), 3.49 (s, 6H), 3.38 (s, 22H), 3.24-3.14 (m, 4H), 3.11-3.06 (m, 6H), 2.84 (dd, *J* = 12.8, 4.8 Hz, 1H), 2.66 (dd, *J* = 12.9, 4.4 Hz, 1H), 2.18-2.05 (m, 10H), 2.07 (d, *J* = 7.3 Hz, 1H), 1.96-1.92

(m, 6H), 1.71-1.49 (m, 6H), 1.42-1.39 (m, 2H), 1.33 (s, 9H), 1.18 (s, 72H), 0.81 (t, J = 6.8 Hz, 6H); ^{13}C NMR (126 MHz, cd$_3$od) δ 183.57, 180.69, 168.27, 167.53, 129.81, 126.25, 124.20, 124.13, 117.77, 111.27, 110.56, 101.60, 87.34, 79.87, 65.22, 60.13, 55.87, 55.60, 54.68, 49.19, 49.01, 48.85, 48.67, 48.50, 48.33, 48.16, 46.96, 43.55, 38.99, 36.90, 35.55, 34.75, 31.81, 29.64, 29.40, 29.27, 28.73, 27.95, 29.29, 27.08, 26.43, 26.17, 25.20, 22.66, 22.56, 14.96, 13.86, 7.14; TOF-MS (ESI) calculated for $C_{101}H_{183}N_{13}O_{21}S$ $[M+H]^{2+}$: 1946.3361, found: 1946.3817, and $C_{101}H_{186}N_{14}O_{21}S$ $[M+H]^{2+}$: 1963.3627, found: 1963.8590.



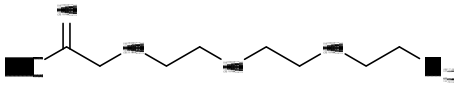
2.16

Compound **2.15** (5 mg, 3 μmol) was dissolved in DCM (2 mL) and 4M HCl in Dioxane (1 mL) was added. After the deprotection was shown to be complete by TLC and MS, added TFA$_{anhydride}$ (0.59 μL, 4.24 μmol) and DIPEA (1 mg, 5.66 μmol). The reaction was stirred overnight and the solvents were evaporated under vacuum. The compound was purified by column chromatography (SiO_2, 15 % MeOH in DCM), obtaining 1 mg of CD1d-Triceps in 15 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.61 – 8.26 (m, 1H), 7.81 (s, 1H), 7.72 (s, 1H), 7.39 (s, 1H), 7.31 (s, 1H), 7.21 (s, 1H), 6.98 (d, J = 11.7 Hz, 1H), 6.80 (s, 1H), 6.76 – 6.59 (m, 1H), 6.55 (s, 1H), 6.42 (s, 1H), 6.36

(s, 1H), 5.80 (d, J = 8.3 Hz, 1H), 5.31-5.28 (m, 1H), 5.01 (s, 1H), 4.68 (s, 1H), 4.64 (s, 1H), 4.55 (s, 1H), 4.48 (s, 1H), 4.40 (s, 1H), 4.29 (dd, J = 6.3 Hz, 1H), 4.12 (dd, J = 6.4 Hz, 1H), 3.94 (s, 2H), 3.84 (s, 1H), 3.63-3.46 (m, 4H), 3.23-3.12 (m, 4H), 3.100-3.02 (m, 2H), 2.99-2.95 (m, 2H), 2.82-2.75 (m, 4H), 2.56 (d, J = 12.6 Hz, 1H), 2.22-2.14 (m, 2H), 2.11-1.98 (m, 4H), 1.98 (s, 8H), 1.59 (s, 4H), 1.47-1.44 (m, 10H), 1.34 (dd, J = 10.2, 4.5 Hz, 2H), 1.22 (s, 72H), 0.83 (d, J = 7.3 Hz, 6H); TOF-MS (ESI) calculated for $C_{98}H_{172}F_3N_{13}O_{20}S$ $[M+H]^+$: 1940.2514, found: 1940.2529.

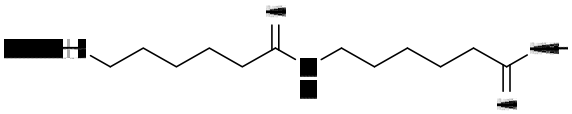
TEG 3

To a 0 °C solution of TEG (10 g, 0.0515 moles) in DCM (10 mL) was added triethylamine (8.6 mL, 0.0618 moles), and very slowly, methane sulfonyl chloride (4 mL, 0.515 mol) was added. The reaction was stirred to room temperature and the mesylated compound was extracted with DCM. The organic layers were dried over Na_2SO_4 and concentrated under vacuum; the intermediate TEG2 was carried over without purification. To TEG 2 was added NaN_3 (6.695 g, 0.103 moles) and the solution was stirred at 80 °C for 2 hours until TEG 3 was obtained. Since this reaction did not use solvents, the compound was directly purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes), obtaining 5.5 g (23 mmol) of **TEG 3** in 45 % yield. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 3.78-3.73 (m, 2H), 3.70 (s, 10H), 3.64 (t, J = 5.4 Hz, 2H), 3.42 (t, J = 5.1 Hz, 2H), 2.54 (s, 1H); ^{13}C NMR (from HSQC) δ 72.50, 70.54, 61.73, 50.67; TOF-MS (ESI) calculated for $C_8H_{17}N_3O_4$ $[M+HH_4]^+$: 237.1563, found: 237.1572.



TEG 4

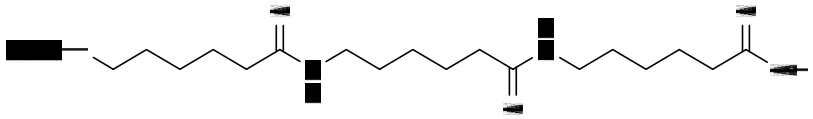
To a solution of **TEG 3** (5.49 g, 0.023 moles) in acetone (5 mL), was added Jones' reagent and sonicated. The reaction was monitored by TLC until TEG 3 was completely consumed. TEG 4 was purified using flash column chromatography by increments of MeOH concentration in DCM (0,1,3,5 and 10 % of MeOH in DCM), obtaining 1.3 g (5.5 mmol) of **TEG 4** in 24 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.00 (br, 1H), 4.12 (q, *J* = 7.1 Hz, 2H), 3.68 (d, *J* = 1.7 Hz, 8H), 3.40 (t, *J* = 5.1 Hz, 2H), 2.04 (s, 2H), 1.25 (t, *J* = 7.1 Hz, 2H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 177.00, 69.47, 69.13, 58.99, 58.86, 58.36, 42.54, 42.01, 40.12, 35.46, 34.94, 22.53, 22.45, 21.81, 21.77; TOF-MS (ESI) calculated for $C_8H_{15}N_3O_5$ $[M]^-$: 232.0939, found: 232.4889.



AH2

6-aminohexanoic acid (3g, 22.8 mmol) was dissolved in DCM (20 mL) and Boc_2O (6 g, 27.5 mmol) was added. The reaction was stirred until protection of the amine was complete. The molecule was activated by dissolving it in DCM (20 mL), adding EDCI (3.5 g, 25.95 mmol) and HOBT (5 g, 25.95 mmol). The reaction was stirred for 30 minutes, 6-aminohexanoic acid (3.4 g, 25.95 mmol) was added, and the mixture was stirred overnight. The intermediate was purified by column chromatography (SiO_2, 5 % MeOH in DCM), obtaining 4.17g of compound **AH2** in 53 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.74 (t, *J* = 5.8 Hz, 1H), 2.99 (dd, *J* = 6.6 Hz, 2H), 2.87 (dd, *J* = 6.6 Hz, 2H), 2.17 (t, *J* = 7.4 Hz, 2H), 2.01 (t, *J* = 7.4 Hz, 2H), 1.90 (s, 1H), 1.52-1.37 (m, 10H), 1.36 (s, 9H), 1.21 (t, *J* = 8.1 Hz, 4H); ^{13}C NMR (126 MHz, dmso) δ 226.39, 177.98, 174.90, 172.48, 172.32, 156.01, 128.26, 127.77, 124.94, 119.58, 110.03, 108.56, 77.72, 38.68, 35.84,

34.05, 29.75, 29.35, 28.69, 26.42, 25.53, 24.66, 21.47. TOF-MS (ESI) calculated for $C_{17}H_{32}N_2O_5$ $[M+HH_4]^+$: 344.2617, found: 344.4830



AH3

To a solution of **AH2** (4.2 g, 12 mmol) in DCM (20 mL) was added HOBT (2.8 g, 14.5 mmol), and EDCI (1.9 g, 14.5 mmol), and stirred for 30 minutes. 6-aminohexanoic acid (1.9 g, 14.5 mol) was added to the activated acid, and was stirred overnight. The desired linker AH3 was purified by column chromatography (SiO_2, 5 % MeOH in DCM), obtaining 2.35g in 43 % yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.17 (br, 1H), 7.71 (br, 2H), 6.75 (t, J = 5.8 Hz, 1H), 2.99 (t, J = 7.8, 7.3, 3.4 Hz, 6H), 2.86 (dd, J = 6.7 Hz, 6H), 2.17 (t, J = 7.4 Hz, 2H), 2.01 (t, J = 7.5, 4H), 1.52 – 1.42 (m, 6H), 1.35 (s, 9H), 1.20 (d, J = 7.3 Hz, 4H); ^{13}C NMR (126 MHz, dmso) δ 226.35, 177.92, 176.98, 174.89, 172.47, 172.26, 156.01, 128.25, 77.72, 38.75, 38.67, 35.84, 35.82, 34.05, 29.75, 29.43, 29.36, 28.71, 26.58, 26.46, 26.43, 25.54, 25.52, 24.66, 21.49; TOF-MS (ESI) calculated for $C_{23}H_{43}N_3O_6$ $[M+H]^+$: 258.6195, found: 458.2682.

Chapter 3: Synthesis and evaluation of SA8 disaccharide vaccine

3.1 Introduction

The emergence of bacteria resistant to antibiotics has become a major public concern around the world.[100] In developing countries, incidence of *Staphylococcus aureus* (SA) infection is highest in children under one year of age, and healthy populations can carry resistant bacteria without having signs of infection.[101] In the United States, SA infection accounts for approximately 300,000 hospitalizations per year, of which about 20 % are nosocomial infections.[102-103] Because antibiotics are of limited use due to development of resistance, a potent vaccine against multi-drug resistant SA is urgently needed.[104] Conjugate vaccine StaphVAX was developed using two capsular polysaccharides (CP5 and CP8) coupled to a carrier protein. This vaccine efficiently promoted a Th2 response but was found ineffective in protecting patients in end-stage renal hemodialysis, likely because the elicited CD4T cell help was not optimal since the vaccine targeted anti-protein B cells more than anti-glycan B cell responses.[104-106] Another approach was the preparation of the human immunoglobulin (IgG) Veronate, which contained high levels of SA surface adhesins (ClfA and SdrG) antibodies. Although Veronate made it into phase III testing, it failed to show clinical benefits in neonates.[107] Pharmaceutical companies are continuing to work on developing vaccines against SA; Merk worked on vaccine V710 that used a SA cell surface localized iron-regulated protein. However, it did not continue after phase II and III clinical trials because it did not show a statistically significant benefit in the outcomes of vaccine recipients versus placebo recipients.[108]

We have worked to prepare a carbohydrate-based vaccine for SA, giving the immune system a highly specific SA moiety for recognition. We are employing a virus-like particle (VLP or Qβ-particle) as a carrier, a pure synthetic disaccharide from SA, and an NKT cell activator as adjuvant. Long-term protection from SA infection requires effective reactivation of cells upon subsequent microbial exposure or persistence of vaccine antibodies; therefore, induction of immune memory is key when designing a vaccine. The goal of activating both B cells and T cell responses is an approach to induce high-affinity antibodies and immune memory by increasing the T cell dependence of the anti-glycan reponse.[109] Since capsular polysaccharides are not very good at triggering an immune response, the α-GalCer molecule is included as an NKT cell adjuvant to activate immune responses to the conjugate vaccines, enhancing immune response to the glycan.[110]

3.2 *Staphylococcus aureus*

SA is a Gram-positive coccus measuring 0.1 to 1.0 μm in diameter, that grows in pairs, clusters, or chains. At least 30 species of staphylococci have been identified by biochemical analysis and by DNA-DNA hybridization. Eleven of these can be isolated from humans as commensals. SA and *Staphylococcus epidermidis* are found in nose and skin and are common commensals that have the greatest pathogenic potential.[111] SA is notorious for causing boils, furuncles, styes, impetigo and other superficial skin infections in humans and may also cause more serious infections, mostly nosocomial, such as pneumonia, deep abscesses, osteomyelitis, endocarditis, phlebitis, mastitis and meningitis, particularly in persons debilitated by chronic illness, traumatic injury, burns or immunosuppression.[112]

3.2.1 Clinical importance

The prevalence of nosocomial methicillin resistant SA (MRSA) infections is an alarming situation in the country.[113-114] More than 50 % of patients in intensive care units get a nosocomial MRSA infection.[115] Factors associated with nosocomial acquisition of MRSA include prolonged hospitalization, especially in intensive care units, and with premature babies who are at risk of acquiring MRSA induced bacteremia or meningitis.[116-117] Other factors include prolonged antibiotic therapy or surgical procedures.[118] Studies have also shown that MSRA infections are acquired by close interaction of individuals who may carry the MSRA strain, such as in the case of military personnel, athletes, inmates or children in daycare centers.[113, 119-120]

3.2.2 Sugar vaccines and our approach

Most vaccines aiming to promote SA immunity use polysaccharide fragments and a protein carrier.[105] Nabi pharmaceuticals, Novartis, GlaxoSmithKline, Merk, Pfizer, and others have developed vaccines targeting SA. Potential vaccines have elicited good levels of Th2 responses in clinical trials, but none has been as potent as desired. Some of those vaccines include StaphVAX (Nabi Pharmaceuticals), StaphCombo (Novartis), PentaStaph (GSK), V710 (Merk and Intercell), and SA3Ag (Pfizer and Inhibitex).[106, 108, 121] As Patti expressed, SA vaccines successfully tested on a small set of recipients are not representative of vaccine efficacy; SA vaccines should include more than one antigen, and the biological role of cell-mediated immune responses should be considered.[104] Some vaccines trigger immune responses but fail to cause dendritic cell maturation and generate memory response in B cells. Vaccines that only contain an antigen specific to a pathogen, are recognized by B cells but fail to recruit T cell help to undergo full maturation. This is partially achieved when vaccines employ a protein carrier; the peptides bind to MHC class II

later presented at high density at the surface of that same B cell to CD4T helper cells. The generation of mature high-affinity anti-glycan producing B cells is proportional to the amount of peptide presented to the cognate CD4T cells. We propose that linking a synthetic sugar antigen (specific immunogen) to a Q-β-particle (used as a carrier), with an NKT cell activator (as adjuvant) triggers an immune response leading to production of high-affinity antibodies that recognize either CP5 or CP8 of SA (Figure 3.1) and memory responses among B cells and possibly T cells.[122]

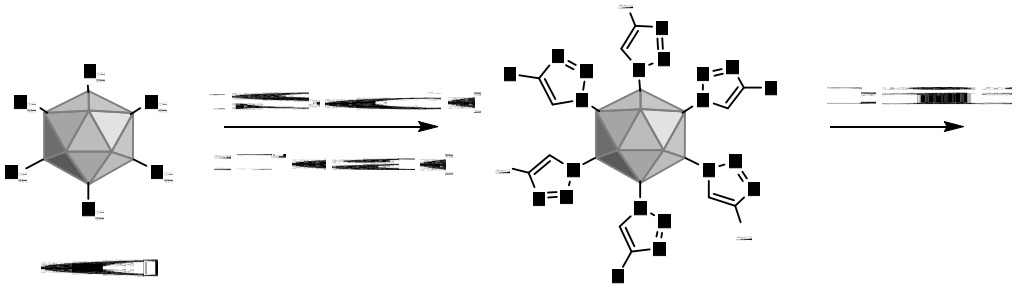


Figure 3.1 Our approach to synthesize a sugar antigen of SA linked to a Q-β-particle.

Our group previously synthesized the repeated glycan unit from serotype 14 of *S. pneumoniae* (SP) and attached the glycan to a Q-β-particle, a nanoparticle made of 180 repetitive units, derived from the 132-amino acid Q-β-phage capsid protein assembled in a virus-like particle (VLP) of 30 nm. The Q-β-particle has bacterial RNA packed inside, the size of the particle is optimal for lymphatic transport and dendritic cell uptake, and the amino acid sequence could be modified to introduce unique conjugation sites for chemical attachment of linkers, adjuvants, and glycan immunogens. Mice showed good immune response to this vaccine. Purified antibodies produced as result of vaccination recognized the sugars of SP with very high affinity and no cross-reactivity to mammalian glycans. Thus, the vaccine triggered the immune response, affinity maturation, and memory of B cells (Figure 3.2).[123-124]

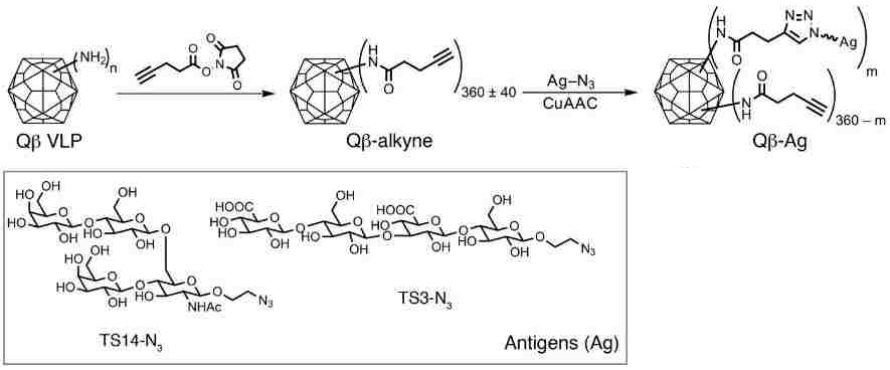


Figure 3.2 Synthesis of Q-β-tetrasaccharide conjugates. Synthesis of the conjugate antigen (Ag) of SP, m is number of antigens per particle (180 CP subunits).[123]

3.2.3 Cellular recognition and glycan display

From previous studies with NKT cells and CD1d activation, we know that the TCR of T cells can recognize glycolipids presented by CD1d molecules.[68, 77, 125] CD1d presents lipids, not peptides unless they are highly hydrophobic, to the TCR of iNKT cells because the groove on CD1d is narrower, deeper, and more hydrophobic than other MHC class-I and II binding grooves.[68, 126-128] When presented to iNKT cells, lipids, glycans, or glycolipids, stimulate B-cells and T-cells by releasing cytokines (such as IL-4 or IFNγ) that modulate or stimulate the immune system.[36, 56, 60, 129-132] Our studies have shown that endogenous αGCs activate NKT cells through CD1d both in mice and humans.[124, 129] Therefore, we anticipated that α-GalCer derivatives, such as that in Figure 3.3, would activate T cells.

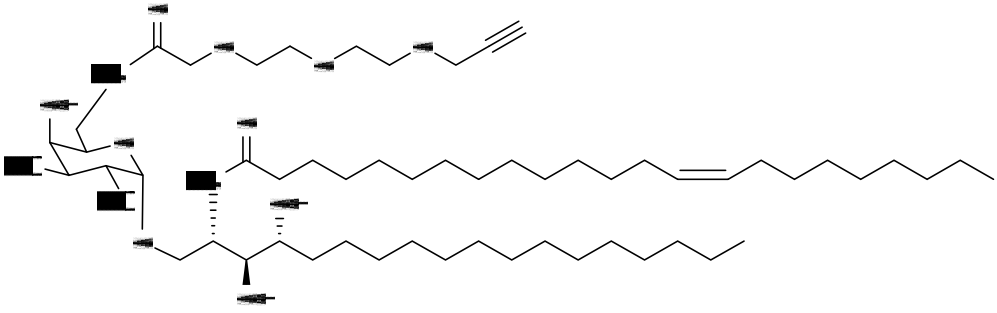


Figure 3.3 NKT cell activator linked to the Q-β-particle with a synthetic glycan of SA.

Carbohydrates generally do not bind to MHC molecules and are therefore T cell independent antigens.[68, 133] Historically, capsular polysaccharide vaccines have provided immune responses.[134-135] And these are dependent upon generation and of signals provided by $CD4^+$T cells (T helper lymphocytes or Th cells). These cells are subdivided into T helper 1 (Th1, proinflammatory cytokines such as IFN-γ and TNF-α) and T helper 2 (immunomodulatory cytokines such as IL-4, 5, 10, and 13) responses.[136] The balance of these two responses should be such that they will not counteract but activate the immune system creating a long-lasting effect. Our design is to provide B cells with a simple and pure synthetic antigen linked to a Q-β-particle, and the latter simultaneously linked to an NKT cell activator (α-GalCer) to induce sufficient $CD4^+$T helper cells to generate mature B cells and production of high-affinity antibodies.[56, 137]

TCR of T cells can discriminate between trisaccharide glycolipids (like iGB3 by CD1d) and tetrasaccharide glycolipids (like GM2 by CD1b), but does not accommodate glycans larger than 4 to 6 saccharides.[138] This explains why many vaccines containing polysaccharides are not effective, and suggests the synthetic glycans to be linked to the Q-β-particle should not be longer than six units.[139] Once the cascade of cell activation takes effect after vaccination, we could measure B cell activation by identifying the amount of antibodies expressed on the surface of B cells, their proliferation and maturation into effector cells by measuring immunoglobulin production and specificity towards SA recognition.[140]

3.2.4 Challenges on the synthesis of SA8 disaccharide

The most common clinical isolates of MRSA (including strains resistant to other antibiotics, including vancomycin), are coated by the capsular polysaccharides associated with serotypes 5 and 8 (CP5, CP8), which are repeating trisaccharide fragments of mannose, D-fucose and L-fucose, as shown in Figure 3.4.[106, 141]

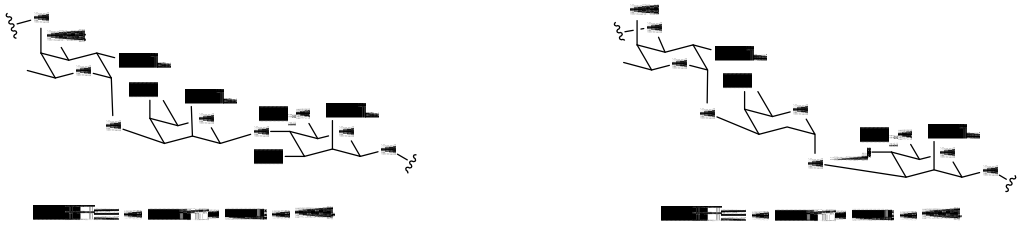


Figure 3.4 Repeating units of CP5 and CP8 of most MRSA clinical isolates.

Due to their unique sugars and linkages, the CP linkages were initially hard to elucidate. Revisions of initially proposed structures using nuclear magnetic resonance techniques led to publication of the correct linkages of CP5 and CP8 (Figure 3.5).[142]

Type5→4)-β-D-ManNAc-(1→4)-α-L-FucNAc(3OAc)-(1→3)-β-D-FucNAc-(1→

Type8→3)-β-D-ManNAc(4OAc)-(1→3)-α-L-FucNAc-(1→3)-α-D-FucNAc-(1→

Figure 3.5 MRSA CP5 and CP8 sugars and linkages.

We hypothesized that synthesizing the repeating unit of the polysaccharide with one extra sugar would be specific and small enough to be recognized by immune cells. Thus, tetrasaccharides were our first goal; however, we experienced problems reducing the azide groups of advanced intermediates. The reason for this complication was an intramolecular cyclization that occurred because of the proximity of the azide on the second carbon of the sugar and the alkyne (Figure 3.6). With small amount of heat or acid, the cyclization occurred, transforming the alkyne into a triazole and destroying the desired functionality of the tetrasaccharides. Though the conditions were not favorable for this ring closure (click chemistry type), the products observed were

triggered due to functional groups proximity and triflic anhydride acidity that could form during the coupling. Our experimental observations were confirmed in a modeling experiment showing the proximity of these functional groups.

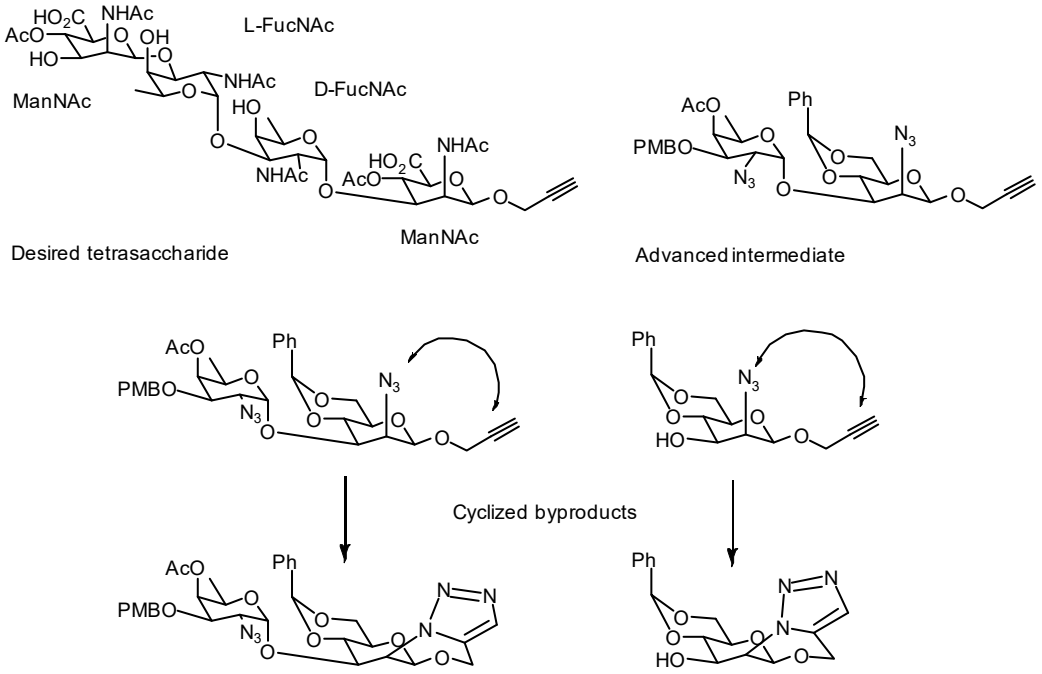


Figure 3.6 Tetrasaccharide, our first desired product and the ring closure formation due to functional groups proximity.

We decided to synthesize the disaccharides shown in Figure 3.7, with the expectation that the disaccharides would provide enough epitope for antibody binding. We also anticipated using the syntheses of these disaccharides will later facilitate synthesis of the tetrasaccharide (Figure 3.6).

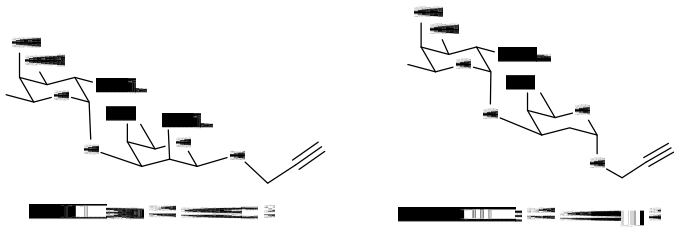


Figure 3.7 Disaccharide of CP5 and CP8.

The synthesis shown in the remainder of this chapter is the SA disaccharide of CP8, containing alpha linked D and L fucose (Figure 3.8).

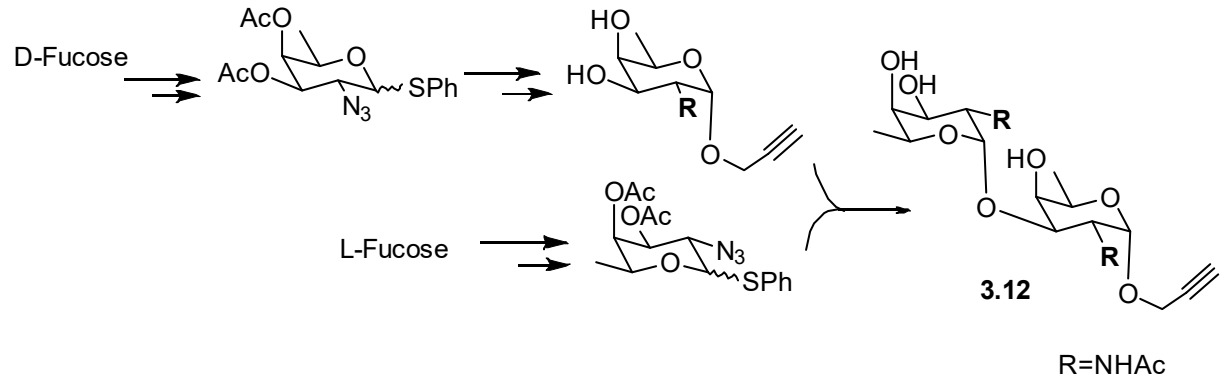


Figure 3.8 Overall synthesis of SA disaccharide of CP8.

3.2.5 PEG(11) biotin

SA8 disaccharide was also synthesized with biotin linked by a PEG (11) molecule. This biotin moiety was required for the purification of B cells that express the disaccharide antibody from sera of vaccinated mice; biotin-avidin association was used to immobilize the disaccharide on a solid support, and the antibodies expressed on B cells bind to the disaccharide of disaccharide-biotin.

3.3 Synthesis

Based on previous syntheses published by Boons and Demchenko,[143-144] we decided to synthesize the disaccharide (1→3)-α-D-FucNAc-β-D-ManNAc-alkyne using the activated thioglycoside as donor and diphenyl sulfoxide and triflic anhydride as promotors to make β linkages of mannose azide sugars as described in the literature.[145] However, mannose β linkages are very unfavored, and after our observations of the intramolecular cyclization we decided to synthesize the disaccharide (1→3)-α-L-FucNAc-α-D-FucNAc- propargyl) (Figure 3.9).[146]

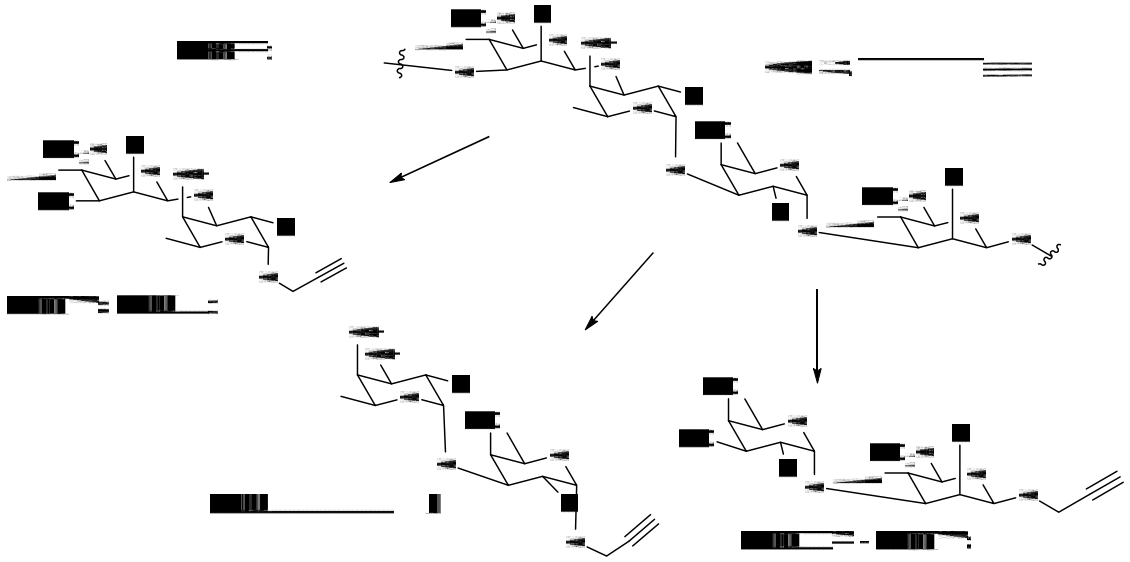
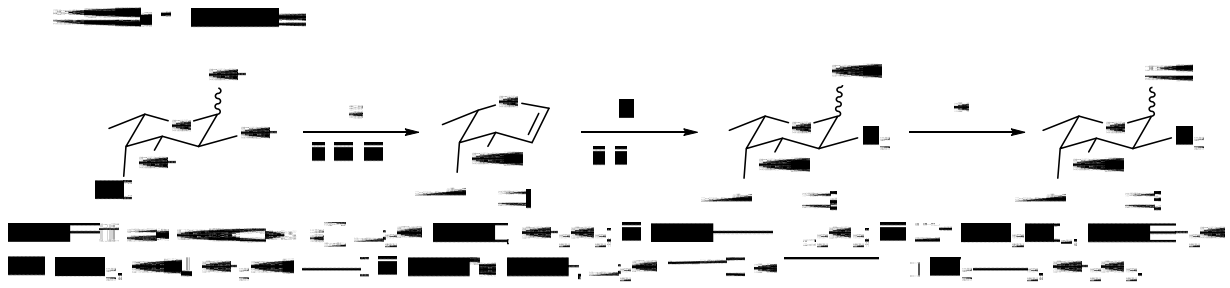


Figure 3.9 CP8 SA tetrasaccharide and the three possible disaccharides.

3.3.1 Synthesis of L-fucose intermediate

Preparation of the L-fucose intermediate started with the formation of alkene **3.1.** We first peracetylated the sugar. Then, using zinc and sodium phosphate monobasic aqueous solution, performed an elimination reaction of the brominated intermediate. Once alkene **3.1** was obtained,

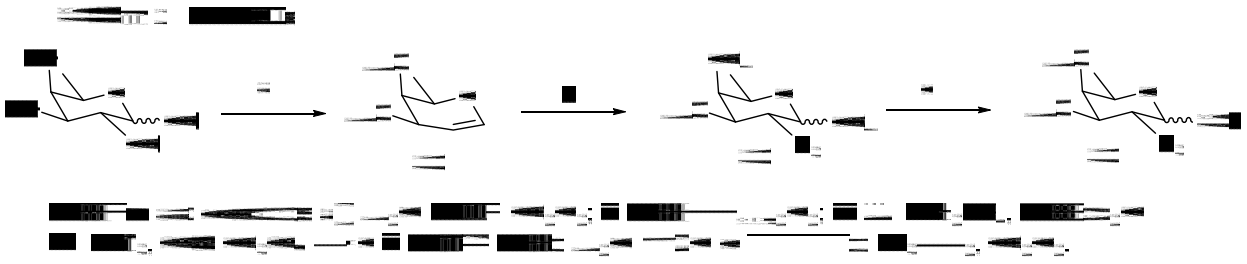
the azide functionality was placed with treatment of ceric ammonium nitrate and sodium azide (Scheme 1).



During hydrolysis, some intermediate **3.2** did not gain the acetyl in the anomeric position. Peracetylation with acetic anhydride solved this problem. Once intermediate **3.2** was obtained, using boron trifluoride in ether as a Lewis acid, activated the same acetyl group for nucleophilic attack by thiophenol, obtaining intermediate **3.3**. This thiophenol intermediate acted as a donor during the coupling with intermediate **3.9**.

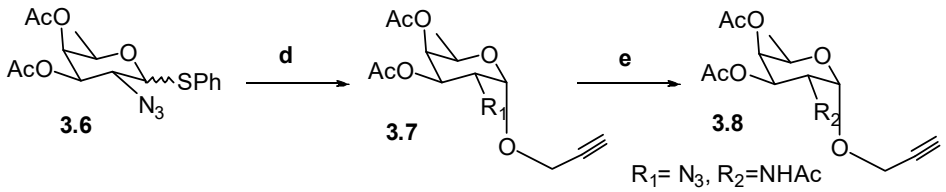
3.3.2 Synthesis of D-fucose intermediate

The synthesis of D-fucose was very similar to the L-fucose, until the thiophenol intermediate **3.6** (Scheme 2).



When intermediate **3.6** was obtained, the thiophenol was attacked by a propargyl alcohol molecule to form intermediate **3.7**. To avoid ring closure formation, the intermediate was reduced and acetylated *in-situ* before moving forward with the synthesis (Scheme 3).

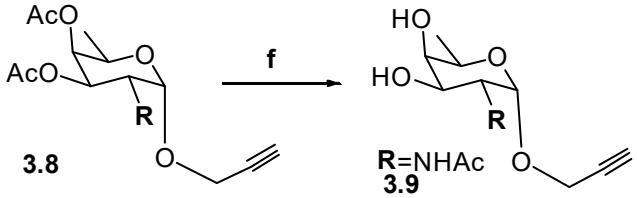
Scheme 3



Reagents and conditions: **d)** NIS, propargyl alcohol, CH_2Cl_2, M.S 3Å, TMSOTf. **e)i.** $\mathbf{H_2S}$ Pyridine, Et_3N, H_2O, 0°C. **ii.** Ac_2O.

Compound **3.7** was obtained mostly as the alpha adduct due to the presence of the azide on carbon 2. Once intermediate **3.7** was reduced and acetylated, the two ester groups of **3.8** were cleaved under basic conditions. Intermediate **3.9** had two hydroxyl groups exposed and ready to act as acceptors during the coupling of L-fucose and D-fucose (Scheme 4).

Scheme 4

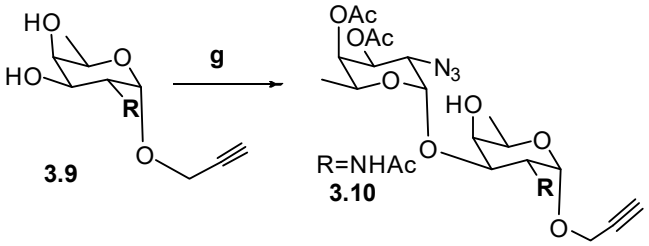


Reagents and conditions: **f)** NaOMe, MeOH.

3.3.3 Synthesis of disaccharide SA8

Once donor **3.3** and acceptor **3.9** were ready for the coupling, alpha linkage was achieved by activating the donor with phenyl sulfoxide and tri-tert-butyl-pyrimidine under basic and dry conditions. Because the acceptor had two hydroxyl groups, Gin conditions[147] were used to afford the desired intermediate through series of kinetic conditions shown in Scheme 5.

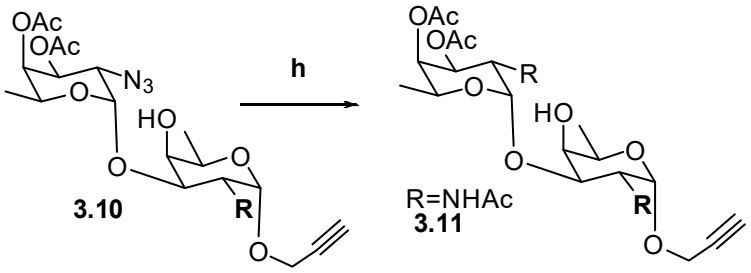
Scheme 5



Reagents and conditions: **g) 3.3**, CH_2Cl_2, M.S 3Å, Ph_2SO, TTBP, Tf_2O, -60°C to -40°C. then at -60°C add **3.9** and let go until reaction is complete.

Because propargyl alcohol would be reduced by either hydrogenation or Zinc mediated reduction, the necessary method for reduction of the azide was using pyridine as the solvent at 0 °C, bubbling hydrogen sulfide, as shown in Scheme 3 and 6. The azide was therefore, transformed into the free amine and acetylated before purification.

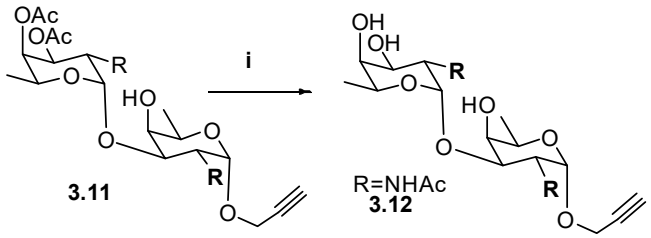
Scheme 6



Reagents and conditions: **h)i.** H_2S, Pyridine, Et3N, H_2O, 0°C. **ii.** Ac_2O.

Once intermediate **3.11** was purified, global deprotection of the hydroxyl groups was performed using sodium methoxide solution in methanol and monitoring by TLC to get the final product **3.12**, disaccharide SA8 or SA disaccharide of CP8 (Scheme 7).

Scheme 7

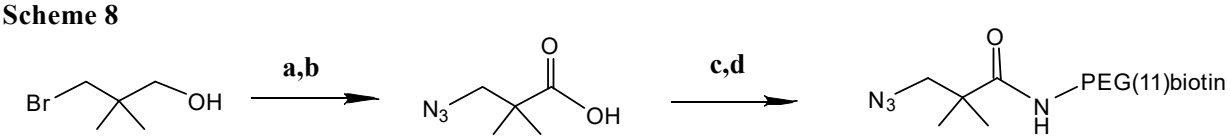


Reagents and conditions: **i)** NaOMe, MeOH.

3.3.4 Synthesis of biotinylated disaccharide

In order to attach the biotin moiety to the disaccharide and simultaneously provide a bulky group near the triazole, we began with 3-bromo-2,2-dimethyl-1-propanol, the methyl substituents were selected in an effort to distinguish the bulkier triazole from the triazole formed when the glycan was attached unto the Q-β particle during click chemistry. First, we acetylated the alcohol, displaced the halide through nucleophilic attack by sodium azide, and removed the acetyl protecting group. Jones oxidation gave acid **3.14**.

Scheme 8



3.13 **3.14** **3.15**

Reagents and Conditions: a) Ac_2O, Et_3N, CH_2Cl_2; **b)** i.NaN_3, DMSO 80°C, ii.NaOMe/MeOH; **c)** Jones oxidation; **d)**PEG11biotin, HOBT, EDCI, DMF.

Acid **3.14** was attached through a peptide bond to give PEG(11)biotin molecule **3.15** (Scheme 8). Compound **3.16** or biotinylated-SA8 was obtained through click chemistry using **3.12** and compound **3.15**.

Scheme 9

3.15 **3.16**

Reagents and Conditions: e) sodium ascorbate, Cu_2SO_4, MeOH,50°C.

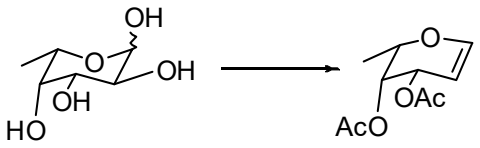
3.4 Results and discussion

Disaccharide SA8 **3.12**, was sent to our collaborator Zinaida Polonskaya at Scripps University in La Jolla California, under the supervision of Dr. Luc Teyton. Zina worked on attaching our compound **3.12** to a Q-β particle expressing azide moieties using click chemistry. The Q-β particle is a bacterial mimic. An NKT cell activator containing an alkyne functional group was also attached to the Q-β particle, this NKT cell activator acts as the adjuvant, and just as described in the previous chapter, our NKT cell activators are potent T cell activators capable of stimulating T cell maturation and B cell antibody production. Once the Q-β particle contained both the disaccharide and the NKT cell activator, was used to vaccinate mice, followed by a 4-week boost vaccination. After 8 weeks, the mice were euthanized, and the sera collected. biotinylated-SA8 was used to pull SA8 disaccharide binding antibodies out of the sera in order to measure vaccine efficacy.

3.5 Conclusions

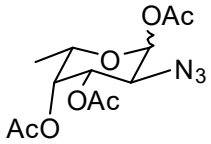
We have synthesized SA disaccharide and biotinylated-SA-disaccharide. Our collaborators at Scripps University attached our disaccharide to a Q-β-particle, immunized mice and quantify the antibodies produced by the mice. Future work will include monoclonal production of the antibodies recognizing the disaccharide, along with measurement of the affinity of the antibodies in context. By attaching an NKT cell activator into the Q-β-particles, we favored high-affinity anti-glycan antibodies production using a synthetic glycan immunogen, an NKT cell activator adjuvant to help B cell maturation and increase somatic hypermutation.

3.6 Experimental procedures



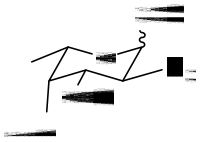
3.1

To a solution of L-fucose (10 g, 60.9 mmol) in acetic anhydride (30 mL) and pyridine (2 mL) was added 2 pellets of DMAP, the reaction was stirred overnight, after which the solution was clear and it was concentrated under reduced pressure to give a clear syrup. The protected L-fucose intermediate was dissolved in DCM (20mL) and HBr in AcOH 33 % solution (20mL) was added. The reaction was stirred for at least 4 hours, and monitored by TLC and MS. The brominated intermediate reaction was quenched with $NaHCO_3$, the aqueous phase was washed with DCM, and the organic layers were combined and dried over Na_2SO_4 and concentrated under vacuum. The brominated intermediate was dissolved in EtOAc (50 mL) and poured into a concentrated solution of NaH_2PO_4 (about 100g in 100 mL) containing Zinc powder (40g). The yellow solution of the brominated compound was stirred vigorously for 30 minutes and monitored by TLC until the reaction was complete. The organic layer was separated, and the aqueous phase was washed twice with EtOAc. The organic phases were combined and dried over Na_2SO_4 and concentrated under vacuum. The desired intermediate was purified by column chromatography (SiO_2, 1:8 Ethyl acetate: Hexanes), obtaining 7.22 g of **3.1** in 56 % yield from L-fucose. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.38 (d, *J* = 6.4 Hz, 1H), 5.52-5.47 (m, 1H), 5.208 (dd, *J* = 4.7, 1.6 Hz, 1H), 4.56 (dd, *J* = 6.3, 2.0 Hz, 1H), 4.15 (dd, *J* = 6.6 Hz, 1H), 2.08 (s, 3H), 1.93 (s, 3H), 1.19 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 184.43, 170.44, 98.27, 77.27, 77.01, 76.76, 69.88, 65.04, 20.89, 16.55; TOF-MS (ESI) calculated for $C_{10}H_{14}O_5$ $[M+NH_4]^+$:232.1185 , found: 232.1246.



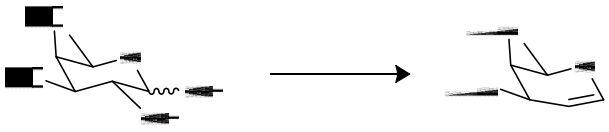
3.2

To a solution of alkene **3.1** (7.22 g, 47.1 mmol) in CH_3CN (30 mL) was added NaN_3 (3.29 g, 50 mmol). The reaction was cooled to -20 °C, after which CAN (55.48 g, 101.2 mmol) was added slowly and allowed to react for 1 hour at the same temperature before slowly heating to room temperature. The reaction was diluted with distilled H_2O and the compound was extracted with Et_2O (3 x 100 mL). The organic fractions were combined and dried under Na_2SO_4 and concentrated under vacuum. The intermediate was dissolved in AcOH (25 mL), NaOAc (4.0 g) and Ac_2O (1 mL) were added, and the reaction was heated at 100 °C for 1 hour. After completion, the reaction was washed with H_2O (30 mL) and the organic layer was quenched with saturated solution of $NaHCO_3$. The aqueous phase was washed again with DCM and the organic fractions were combined and dried over Na_2SO_4. The solvent was evaporated under vacuum and the product was purified through column chromatography (SiO_2, 1:4 Ethyl acetate: Hexanes), obtaining 4.77 g of intermediate **3.2** in 32 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.30 (d, *J* = 3.6 Hz, 1H), 5.36-5.32 (m, 2H), 4.21 (dd, *J* = 6.4 Hz, 1H), 3.91 (dd, *J* = 12.0, 3.7, 1.3 Hz, 1H), 2.19 (s, 3H), 2.17 (s, 3H), 2.08 (s, 3H), 1.16 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 170.35, 169.92, 168.96, 90.69, 70.05, 69.14, 67.22, 56.75, 20.99, 20.69, 20.61, 15.96; TOF-MS (ESI) calculated for $C_{12}H_{18}N_3O_7$ $[M+H]^+$: 316.1139, found: 316.1180.



3.3

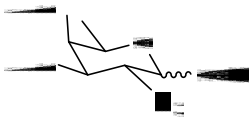
To a solution of Acetylated compound **3.2** (3.95 g, 12.53 mmol) in DCM, were added thiophenol (2.10 mL,18.79 mmol) and $BF_3 \cdot Et_2O$ (3.11 mL, 25.06 mmol), and the solution was stirred overnight. The reaction was quenched with $NaHCO_3$, the aqueous phase was washed with DCM and the organic phase was dried over Na_2SO_4. The solvent was evaporated under vacuum and the compound was purified by column chromatography (SiO_2, 1:6 Ethyl acetate: Hexanes), obtaining 3.85 g of product **3.3** in 84 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.63 (d, *J* = 5.1 Hz, 2dH), 7.48 (dd, *J* = 7.3 Hz, 1H), 7.36-7.33 (m, 2H), 5.65 (d, *J* = 4.7 Hz, 1H), 5.33 (s, 1H), 5.18 (dd, *J* = 11.3, 2.8 Hz, 1H), 4.62 (dd, *J* = 6.7 Hz, 1H), 4.28 (dq, *J* = 11.3, 5.6 Hz, 1H), 2.18 (s, 3H), 2.07 (s, 3H), 1.14 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 170.31, 169.63, 133.33, 132.07, 129.12, 128.94, 127.75, 87.13, 70.56, 70.37, 65.85, 58.15, 20.64, 20.60, 15.84; TOF-MS (ESI) calculated for $C_{16}H_{23}N_4O_5S$ $[M+NH_4]^+$: 383.1389, found: 383.1329 .



3.4

To a solution of D-fucose (10 g, 60.9 mmol) in acetic anhydride (30 mL) and pyridine (2 mL) were added 2 pellets of DMAP, and the reaction was stirred overnight. After completion of the reaction, the solvent was evaporated under vacuum to give the protected acetylated D-fucose intermediate in the form of a syrup. The intermediate was dissolved in DCM (20mL) and HBr in AcOH 33 % solution (20mL) was added, and the reaction was stirred for at least 4 hours. The brominated

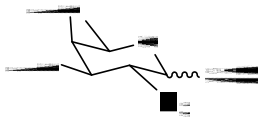
intermediate reaction was quenched with $NaHCO_3$. The aqueous phase was washed with DCM, and the organic layers were dried over Na_2SO_4 and concentrated under vacuum. This yellow syrup was dissolved in EtOAc and poured into an aqueous concentrated solution of NaH_2PO_4 (about 100 g in 100 mL) containing Zinc powder (40 g). The reaction was stirred vigorously for 30 minutes and monitored by TLC until completion. The organic phase was separated, and the aqueous phase was washed with EtOAc. The organic layers were dried over Na_2SO_4 and the solvent was evaporated under vacuum. The compound was purified by column chromatography (SiO_2, 1:8 Ethyl acetate: Hexanes), obtaining 9.56 g of compound **3.4** in 67 % yield from D-fucose. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.68 (d, *J* = 3.9 Hz, 1H), 5.43-5.33 (m, 2H), 5.01 (dd, *J* = 10.6, 3.9 Hz, 1H), 4.39 (dd, *J* = 6.8, 1.3 Hz, 1H), 4.11 (dd, *J* = 7.1 Hz, 1H), 2.16 (s, 3H), 2.10 (s, 3H), 1.21 (d, *J* = 6.6 Hz, 3H); 13C NMR (126 MHz, cdcl3) δ 170.26, 169.79, 89.29, 69.94, 68.38, 20.78, 20.62, 15.54; TOF-MS (ESI) calculated for $C_{10}H_{18}NO_5$ $[M+NH_4]^+$: 232.1185, found: 232.1049.



3.5

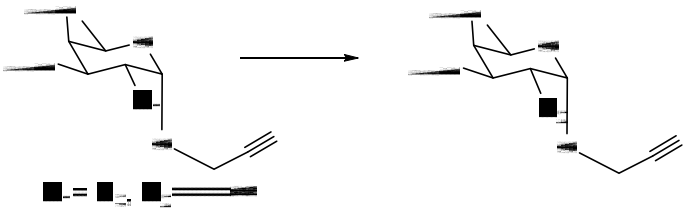
To a solution of alkene **3.4** (8.87 g, 41.44 mmol) in CH_3CN (30 mL) was added NaN_3 (4.04 g, 62.17 mmol), and the reaction was cooled to -20 °C. CAN (67.85 g, 123.76 mmol) was added little by little and the reaction was stirred for 1 hour at the same temperature before slowly warming to room temperature. When the reaction was complete, distilled H_2O was added and the compound was washed with Et_2O (100mL). The organic fractions were combined and dried under Na_2SO_4, and the solvent was evaporated under vacuum. The resulting syrup was dissolved in AcOH (25 mL), NaOAc (4.0g) and Ac_2O (1mL) were added, and the mixture was heated at 100 °C for 1

hour. After the reaction cooled down, distilled H_2O was added, and the organic phase was quenched with concentrated solution of $NaHCO_3$. The compound was extracted with DCM, and the organic layers were combined and dried over Na_2SO_4. The solvent was evaporated under vacuum and the product purified through column chromatography (SiO_2, 1:4 Ethyl acetate: Hexanes), obtaining 4.07 g of intermediate **3.5** in 31 % yield. TOF-MS (ESI) calculated for $C_{12}H_{18}N_3O_7$ $[M+H]^+$: 316.1139, found: 316.1334.



3.6

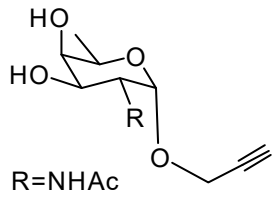
To a solution of D-fucose acetylated compound **3.5** (4.03 g, 12.79 mmol) in DCM (10 mL), were added thiophenol (2.14 mL, 19.19 mmol) and $BF_3 \cdot Et_2O$ (3.17 mL, 38.38 mmol), and the reaction was stirred overnight. The reaction was quenched with $NaHCO_3$, the aqueous phase was washed with DCM, and the organic layers were combined and dried over Na_2SO_4. The compound was purified by column chromatography (SiO_2, 1:6 Ethyl acetate: Hexanes), obtaining 3.83 g of product in 82 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.64 (d, *J* = 4.5 Hz, 2H), 7.49 (dd, *J* = 8.0 Hz, 1H), 7.45-7.28 (m, 2H), 5.21 (d, *J* = 3.3 Hz, 1H), 4.87 (dd, *J* = 10.3, 3.2 Hz, 1H), 4.51 (dd, *J* = 10.1, 1.2 Hz, 1H), 3.79 (dd, *J* = 6.4 Hz, 1H), 3.64 (dq, *J* = 10.1, 1.2 Hz, 1H), 2.13 (s, 3H), 2.04 (s, 3H), 1.24 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 170.34, 169.79, 133.34, 132.07, 131.48, 129.14, 128.95, 128.39, 86.44, 77.28, 77.02, 76.77, 73.10, 69.64, 65.86, 59.31, 20.67, 20.59, 16.60; TOF-MS (ESI) calculated for $C_{16}H_{23}N_4O_5S$ $[M+NH_4]^+$: 383.1389, found: 383.1406 .



3.7 **3.8**

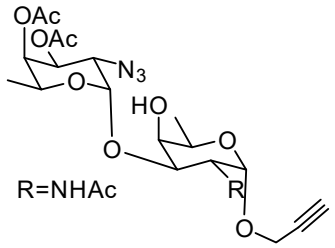
To a flame dried RBF was added compound **3.6** (200 mg, 0.55 mmol) dissolved in dry DCM (2 mL). Propargyl alcohol (36 mg, 0.65 mmol), and molecular sieves 4Å (500 mg) were added and the suspension was stirred at room temperature for one hour. After that, NIS (369 mg, 1.64 mmol) was added dropwise, and the reaction and stirred for ten minutes. The reaction was cooled to -30 °C, and TMSOTf (12 mg, 5.4 μmol) was added dropping on the sides. The reaction was stirred for 30 minutes and monitored by TLC and MS. Once complete, the reaction was quenched with three drops of triethylamine, the molecular sieves were filtered out and saturated solution of $NaHSO_3$ was added. After the aqueous phase was washed with DCM, the organic layers were combined and dried over Na_2SO_4, and the solvent was evaporated with very mild heating under vacuum. The propargyl intermediate **3.7** was dissolved in pyridine (6 mL), triethylamine (2 mL) and H_2O (1 mL). The solution was then cooled to 0 °C, and H_2S gas was bubbled into it with stirring for 5 minutes. After that, it was stirred for at least 3 hours, and closely monitored by MS. After the reaction was done, the mixture was concentrated. Product **3.8** was purified by column chromatography (SiO_2, 1:3 Ethyl acetate: Hexanes), obtaining 80 mg of compound in 45 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.21 (dd, *J* = 3.5 Hz, 1H), 5.19-5.16 (m, 1H), 4.75 (d, *J* = 8.3 Hz, 1H), 4.42 (s, 2H), 3.86 (dq, *J* = 6.4 Hz, 1H), 3.75 (dd, *J* = 9.0 Hz, 1H), 2.47 (s, 1H), 2.19 (s, 3H), 2.01 (s, 6H), 1.22 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 170.75, 170.48, 158.34, 99.24, 78.70, 75.37, 70.97, 69.98, 69.31, 69.173, 55.79, 52.06, 20.77, 20.75, 16.18; TOF-

MS (ESI) calculated for **3.7** $C_{13}H_{21}N_4O_6$ $[M+NH_4]^+$:329.1461, found: 329.1437: for **3.8** $C_{15}H_{21}NO_7$ $[M+H]^+$: 328.1391, found: 328.1481.



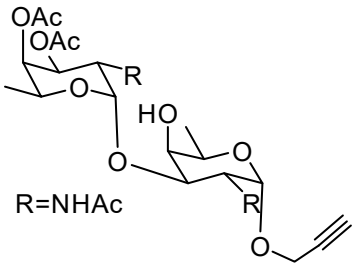
3.9

To a solution of compound **3.8** (80 mg, 0.25 mmol) in MeOH was added NaOMe solution in MeOH (0.5 mL), and the solution was stirred at room temperature for 10 minutes. After that, the reaction was concentrated and the intermediate was purified by column chromatography (SiO_2, 5 % MeOH in DCM), obtaining 15 mg of **3.9** in 76 % yield. ^{1}H NMR (500 MHz, cdcl3 and Methanol-d_4) δ 4.79 (d, J = 3.8 Hz, 1H), 4.14-4.11 (m, 1H), 4.09 (dd, J = 7.3, 2.4 Hz, 2H), 3.90 (s, 2H), 3.82 (q, J = 6.6 Hz, 1H), 3.56 (d, J = 9.2 Hz, 1H), 3.22 (br, 1H), 2.41 (t, J = 2.4 Hz, 1H), 1.89 (s, 3H), 1.14 (d, J = 6.6 Hz, 3H); ^{13}C NMR (126 MHz, cd_3od) δ 172.59, 96.61, 78.80, 74.76, 71.40, 69.51, 66.79, 54.80, 49.62, 22.63, 15.96; TOF-MS (ESI) calculated for deprotected compound $C_{11}H_{17}NO_5$ $[M+H]^+$: 244.1179 found: 244.1193, and $C_{11}H_{17}NNaO_5$ $[M+Na]^+$: 266.0999, found: 266.1020.



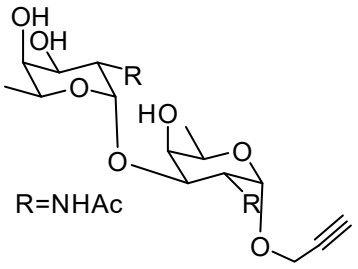
3.10

In a flame dried RBF, compound **3.3** (101 mg, 0.28 mmol), Ph_2SO (75 mg, 0.37 mmol) and TTBP (161 mg, 0.56 mmol) were put under vacuum for 30 minutes. The mixture was then dissolved in dry DCM (3 mL), molecular sieves 4Å (500 mg) were added and allowed to stir for one hour at room temperature. The reaction was then cooled to -60 °C and Tf_2O (3 drops with micropipette) was added dropwise to the reaction while being stirred. The mixture was allowed to warm to -40 °C for 5 minutes, then cooled back down to -60 °C. Compound **3.9** (45.18 mg, 0.19 mmol) dissolved in THF and DCM (1:1, 1 mL) was added, and the reaction was allowed to reach room temperature. The reaction was monitored by MS and quenched with triethylamine (2 drops). The molecular sieves were filtered off, and the mixture was concentrated under vacuum. The crude was purified by column chromatography (SiO_2, 1:2 Ethyl acetate: Hexanes), obtaining 19 mg of **3.10** in 22 % yield. ^{1}H NMR (500 MHz, Chloroform-*d* and Methanol-d_4) δ 5.70 (d, *J* = 8.7 Hz, 1H), 5.35 (dd, *J* = 11.0, 3.3 Hz, 1H), 5.31 (d, *J* = 4.41 Hz, 1H), 5.05 (dd, *J* = 16.2, 3.7 Hz, 2H), 4.56 (ddd, *J* = 10.9, 8.7, 3.8 Hz, 1H), 4.48-4.37 (m, 1H), 4.24 (dd, *J* = 7.0, 2.4 Hz, 2H), 4.00 (dq, *J* = 6.6 Hz, 1H), 3.83 (dd, *J* = 11.0, 3.0 Hz, 1H), 3.75 (dd, *J* = 11.0, 3.6 Hz, 1H), 3.54-3.44 (m, 1H), 3.42 (s, 2H), 2.49-2.40 (m, 1H), 2.27 (s, 1H), 2.17 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 1.62 (s, 2H), 1.30 (d, *J* = 6.6 Hz, 3H), 1.14 (d, *J* = 6.5 Hz, 4H), 0.92-0.80 (m, 2H); ^{13}C NMR (126 MHz, $cdcl_3$) δ 170.45, 170.27, 169.57, 99.04, 96.97, 78.87, 78.60, 77.26, 77.01, 76.75, 74.80, 70.86, 70.62, 70.49, 68.80, 66.33, 65.84, 58.11, 55.17, 47.64, 26.50, 23.35, 20.66, 20.63, 16.24, 16.04; TOF-MS (ESI) calculated for $C_{21}H_{31}N_4O_{10}$ $[M+H]^+$: 499.2035, found: 499.2039.



3.11

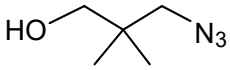
Into a 0 °C solution of compound **3.10** (18 mg, 0.036 mmol), pyridine (3 mL), triethylamine (1 mL) and H_2O (1 mL), was bubbled H_2S gas for 15 minutes and allowed to stir for 3 hours. The reaction was monitored by MS. Once complete, the solvents were evaporated, Ac_2O (2mL) and DMAP (2 pellets) were added, and the reaction was stirred for 1 hour. After the solvents were evaporated, the desired compound was purified by column chromatography (SiO_2, 5 % MeOH in DCM), obtaining 7 mg of **3.11** in 37 % yield. ^{1}H NMR (500 MHz, Chloroform-*d* and Methanol-d_4) δ 6.18 (d, *J* = 9.9 Hz, 1H), 5.81 (d, *J* = 9.7 Hz, 1H), 5.26 (dd, *J* = 11.4, 3.2 Hz, 1H), 5.23 (dd, *J* = 2.9 Hz, 1H), 5.13 (dd, *J* = 11.3, 3.2 Hz, 1H), 4.90 (dd, *J* = 4.0 Hz, 2H), 4.65-4.51 (m, 2H), 4.25 (dd, *J* = 8.4, 2.4 Hz, 2H), 4.23-4.18 (m, 1H), 4.09 (p, *J* = 6.5 Hz, 1H), 4.02-3.95 (m, 1H), 3.74 (dd, *J* = 10.7, 3.1 Hz, 1H), 3.68 (d, *J* = 3.2 Hz, 1H), 3.41 (s, 4H), 2.48 (t, *J* = 2.4 Hz, 1H), 2.18 (s, 3H), 2.08 (s, 3H), 2.03 (s, 3H), 1.97 (s, 3H), 1.31 (d, *J* = 6.6 Hz, 3H), 1.12 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 170.89, 170.72, 161.91, 169.92, 100.54, 96.77, 77.26, 77.01, 76.76, 75.20, 72.46, 71.53, 70.61, 70.27, 69.27, 66.54, 65.58, 54.92, 49.62, 47.51, 46.75, 26.49, 23.55, 23.13, 20.78, 20.75, 20.51, 16.15, 16.13; TOF-MS (ESI) calculated for $C_{23}H_{35}N_2O_{11}$ $[M+H]^+$: 515.2235, found: 515.2270.



3.12 Disaccharide SA8

To a solution of compound **3.11** (7 mg, 10 μmol) in MeOH (2 mL) was added NaOMe solution in MeOH (3 drops) and stirred for 5 minutes. The desired product was purified by column chromatography (SiO_2, 5 % MeOH in DCM), obtaining 3 mg of **3.12** in 51 % yield. ^{1}H NMR (500 MHz, Chloroform-*d* and Methanol-d_4) δ 7.76 (s, 1H), 6.63 (s, 1H), 4.83 (d, 1H), 4.65 (d, 1H), 4.33 (dd, 1H), 4.18 (2H), 3.98 (dd, 1H), 3.95 (dd, 1H), 3.81 (dd, 1H), 3.69 (dd, 1H), 3.64 (1H), 3.53 (1H), 3.49 (1H), 2.49 (1H), 1.81 (3H), 1.23 (3H), 1.12 (d, 3H), 1.07 (d, 3H); ^{13}C NMR (126 MHz, cdcl$_3$ and Methanol-d_4) δ 170.54, 168.85, 100.88, 96.17, 82.39, 77.01, 76.39, 74.84, 72.65, 70.32, 59.01, 55.12, 45.22, 45.14, 45.05, 44.89, 44.72, 44.55, 44.38, 44.22, 28.22, 27.97, 22.00, 21.95, 21.80, 16.85, 16.34; TOF-MS (ESI) calculated for $C_{19}H_{31}N_2O_9$ $[M+H]^+$: 431.2024, found: 431.2048.

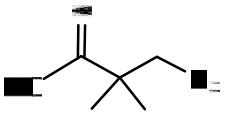
PEG11 and linker



3.13

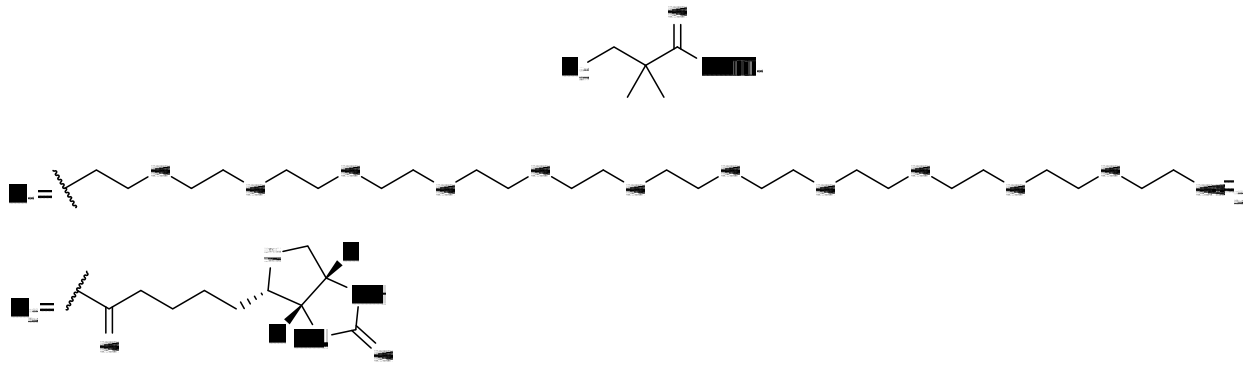
3-Bromo-2,2-dimethyl-1-propanol (1 g, 5.9 mmol) was dissolved in Ac_2O (5 mL) and allowed to react for 1 hour. The reaction was monitored by TLC and then quenched with saturated solution of $NaHCO_3$. The intermediate was extracted with DCM, the organic phase was dried over Na_2SO_4, and the solvent was evaporated under vacuum. The crude was dissolved in DMSO (5 mL) and NaN_3 (1.15 g, 17.7 mmol) was added. The reaction was stirred at 80 °C for 2 hours. Once complete,

the reaction was quenched with H_2O and the compound was extracted with DCM. The organic fractions were combined, dried over Na_2SO_4, and the solvent was evaporated under vacuum. The compound was purified by column chromatography (SiO_2, 1:2 Ethyl acetate: hexanes), obtaining 700 mg **3.13** in 91 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 2.44 (s, 2H), 1.98 (s, 2H), 1.06 (s, 3H), 1.05 (s, 3H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 77.51, 77.25, 76.99, 69.59, 53.84, 31.56, 29.00; TOF-MS (ESI) calculated for $C_5H_{12}N_3O$ $[M+H]^+$: 130.0975. Did not detect in MS.



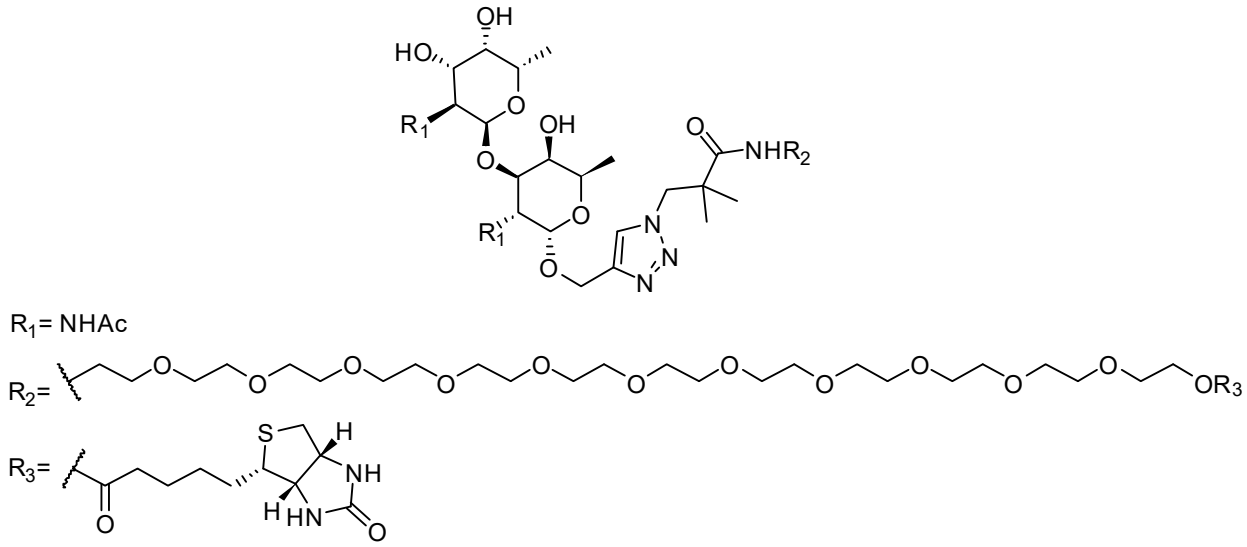
3.14

To a solution of compound **3.13** (700 mg, 5.4 mmol) in acetone (5 mL), was added Jones' reagent slowly. The reaction was sonicated until red color was permanent. After completion, the reaction was concentrated under vacuum and the intermediate was purified through flash column chromatography. The fractions were concentrated under vacuum obtaining 25 mg of the desired intermediate **3.14** in 33 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 12.27 (s, 1H), 3.45 (s, 2H), 1.27 (s, 6H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 182.03, 77.27, 77.01, 76.76, 59.25, 43.49, 22.87; TOF-MS (ESI) calculated for $C_5H_9N_3O_2$ $[M+H]^+$: 144.0768. Did not detect in MS.



3.15

To a solution of compound **3.14** (38 mg, 0.155 mmol) in DMF (5 mL) was added HOBT (21 mg, 0.155 mmol) and EDCI (30 mg, 0.155 mmol) and the reaction was stirred at room temperature for 40 minutes. PEG(11)biotin compound (100 mg, 0.129 mmol) was added into the previous solution and allowed to stir overnight at room temperature. Compound **3.15** (72 mg) was purified by column chromatography (SiO_2, 10 to 15 % MeOH in DCM), obtaining 48 mg (67 μmol) of **3.15** in 52 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.83 (d, *J* = 8.3 Hz, 1H), 7.66 (d, *J* = 8.3 Hz, 1H), 7.34 (d, *J* = 7.3 Hz, 1H), 6.72 (s, 13H), 5.93 (s, 12H), 4.74 (s, 1H), 4.67 (d, *J* = 6.5 Hz, 1H), 3.67-3.61 (m, 42H), 3.55 (t, *J* = 5.1 Hz, 2H), 3.47 – 3.38 (m, 2H), 3.32 (t, *J* = 6.0 Hz, 2H), 3.17 (td, *J* = 7.4, 2.1 Hz, 4H), 2.84 (s, 2H), 2.23 (dt, *J* = 13.3, 7.1 Hz, 2H), 1.19 (s, 6H), 1.09 (t, *J* = 7.2 Hz, 8H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 183.36, 174.79, 159.82, 77.30, 77.05, 76.79, 70.49, 69.88, 55.73, 43.25, 43.06, 36.37, 35.02, 25.65, 23.29, 15.53; TOF-MS (ESI) calculated for $C_{39}H_{72}N_6O_{15}S$ $[M+H]^+$: 896.4776, found: 896.5415.



3.16

To a solution of compound **3.15** (10 mg, 11 μmol) and **3.12** (5 mg, 11 μmol) in MeOH (1 mL) and H_2O (3mL) was added sodium ascorbate ($C_6H_7NaO_6$) (6 mg, 33 μmol) and cupric sulfate (Cu_2SO_4) (4 mg, 22 μmol). The reaction was stirred at 60 °C for 1 hour and closely monitored by MS until the starting material was consumed. The product was first concentrated under vacuum followed by purification by column chromatography (SiO_2, 10 % MeOH in DCM), obtaining 3 mg of **3.16** in 20 % yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.83 (d, *J* = 8.3 Hz, 1H), 7.66 (d, *J* = 8.3 Hz, 1H), 7.34 (d, *J* = 7.3 Hz, 1H), 6.72 (s, 13H), 5.93 (s, 12H), 4.74 (s, 1H), 4.67 (d, *J* = 6.5 Hz, 1H), 3.67-3.61 (m, 42H), 3.55 (t, *J* = 5.1 Hz, 2H), 3.47-3.39 (m, 2H), 3.32 (t, *J* = 6.0 Hz, 2H), 3.17 (td, *J* = 7.4, 2.1 Hz, 4H), 2.84 (s, 2H), 2.23 (dt, *J* = 13.3, 7.1 Hz, 2H), 1.19 (s, 6H), 1.09 (t, *J* = 7.2 Hz, 8H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 183.36, 174.79, 159.82, 77.30, 77.05, 76.79, 70.49, 69.88, 55.73, 43.25, 43.06, 36.37, 35.02, 25.65, 23.29, 15.53; TOF-MS (ESI) calculated for $C_{58}H_{104}N_9O_{24}S$ $[M+NH_4]^+$:1342.6909, found: 1342.6895, and $C_{58}H_{103}N_9NaO_{24}S$ $[M+H]^+$: 1364.6729, found: 1364.6768.

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Appendix A: List of abbreviations

Chapter 1	
PRR	Pathogen Recognition Receptor
TLR	Toll-Like Receptor
PAMP	Pathogen Associated Molecular Pattern
TIR	Toll-IL-1 Receptor
IL	Interleukin
IL-1R	Interleukin 1 Receptor
NK-κB	Nuclear factor Kappa-light-chain-enhancer of activated B cell
LRR	Leucine-Rich Repeat
MyD88	Myeloid Differentiation factor-88
IRAK	IL-1 Receptor Associated Kinase
TRAF	TNFR Associated Factor
TNF	Tumor Necrosis Factor
TNFR	TNF Receptor
TIRAP	TIR-domain-containing Adaptor Protein
MAL	MyD88-Adaptor-Like
TOLLIP	Toll-Interacting Protein
CSK	Cysteine-Serine-Lysine
RA	Rheumatoid Arthritis

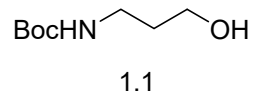
T1D	Type-1-Diabetes
IBD	Inflammatory Bowel Disease
UC	Ulcerative Colitis
CD	Crohn's Disease
MALP-2	Macrophage Activating Lipopeptide 2
FSL-1	Fibroblast-Stimulating Lipopeptide 1
TH	Helper T cell
LPM	Lipid-Peptide Mimetic
FMOC	Fluorenylmethyloxycarbonyl
BOC	Tert-butyloxycarbonyl
DMAP	4-Dimethylaminopyridine
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
HOBT	Hydroxybenzotriazole
HCl	Hydrogen chloride
CBZ	Carboxybenzyl
DDRCC	Digestive Diseases Research Core Center
APC	Antigen Presenting Cell
WT	Wild Type
BL	Black
ELISA	Enzyme-Linked Immunosorbent Assay
RBF	Round-Bottom Flask

DCM	Dichloromethane
Boc_2O	Di-tert-butyl dicarbonate
MeOH	Methanol
DMSO	Dimethyl sulfoxide
TLC	Thin Layer Chromatography
TFA	Trifluoro acetic
SiO_2	Silicon Dioxide
MS	Mass Spectrometry
TOF-MS	Time-of-flight Mass Spectrometry
Ac	Acetyl
Chapter 2	
αGC	α-galactosyl ceramide
NKT	Natural Killer T cell
CD1d	Cluster Differentiation 1 type d
iNKT	Invariant Natural Killer T cell
LTP	Lipid Transfer Protein
SAP	Saponin
G_{M2}	Ganglioside monosialic 2
NCP1	Niemann-Pick Disease type C1
SR	Scavenger Receptor (A or B)
LDLR	Low Density Lipoprotein Receptor

TRICEP	Trifunctional Chemoproteomics Reagent
PPI	Protein-Protein Interactions
Re-CLIP	Reversible Cross-Link Immunoprecipitation
GSL	Glycosphingolipid
DSP	Dithiobis (succimidyl propionate)
DTME	Dithio-bismaleimidoethane
LRC	Ligand Receptor Capture
HATRIC	Hydrazone-Azide containing TRI-funtional Compound
MHC	Major Histocompatibility Complex
APM	Antigen Presenting Molecule
IFN	Interferon
TCR	T-cell Receptor
iGB3	isoglobotrihexosylceramide
LPC	Lysophophotidylcholine
HYNIC	Hydrazinonicotinic acid
TEG	Tetraethylene glycol
PEG	Polyethylene glycol
TMS	Trimethylsilyl in TMSI (iodide), TMSOTf (triflate), TMSCl (chloride)
DIPEA	Diisopropylethylamine
RBL	Rat Basophilic Leukemia cell
TDSCl	Thexyldimethylsilyl Chloride

Chapter 3	
SA	*Staphylococcus aureus*
CP	Capsular Polysaccharide
Ig	Immunoglobulin
VLP	Virus-Like Particle
MRSA	Methicillin-Resistant SA
SP	*Staphylococcus pneumoniae*
Fuc	Fucose
Man	Mannose
CAN	Ceric Ammonium Nitrate
AcOH	Acetic acid
TTBP	2,4,6-Tri-tert-butylpyrimidine

Appendix B: NMR Spectra



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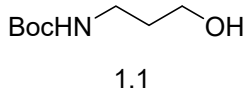

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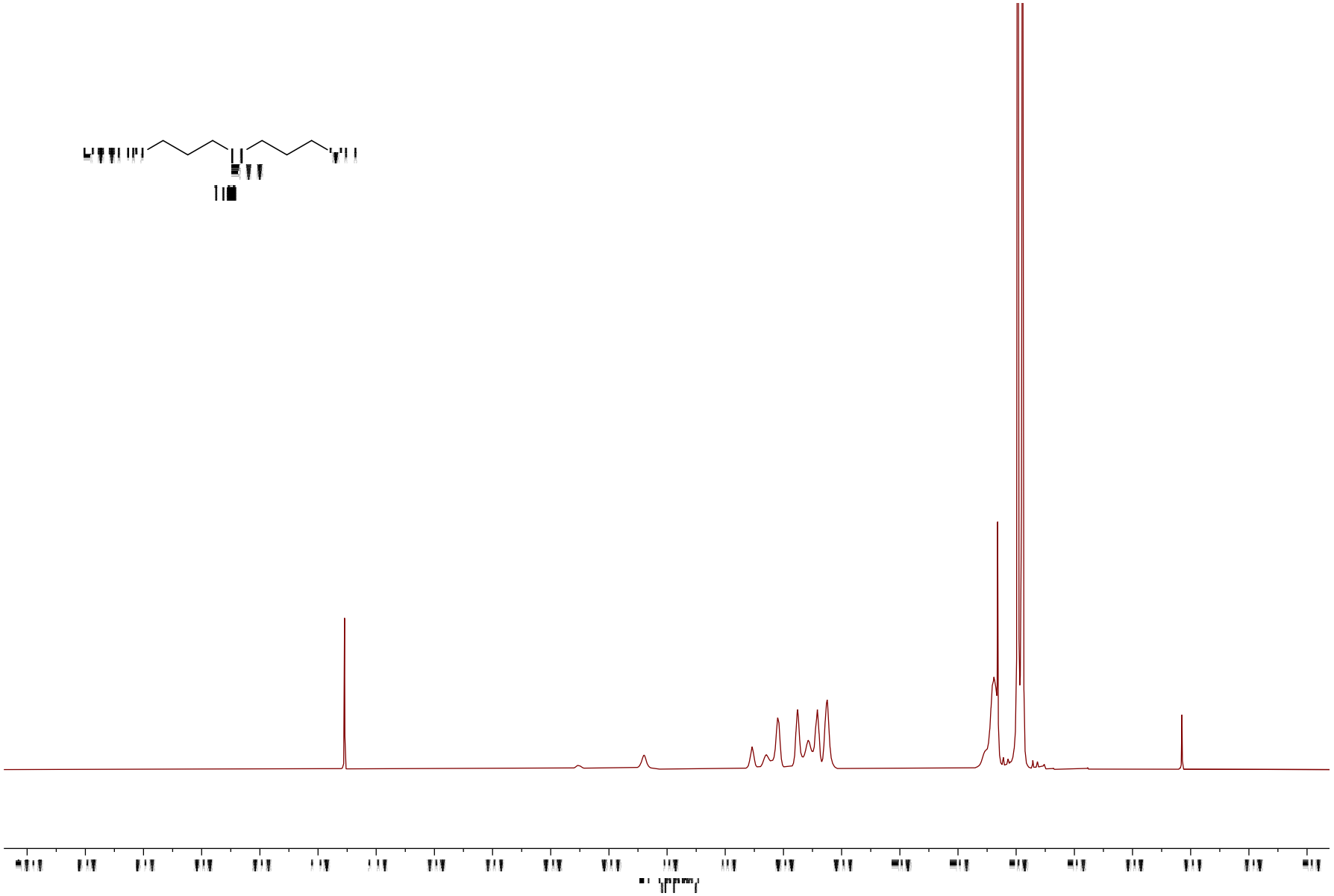

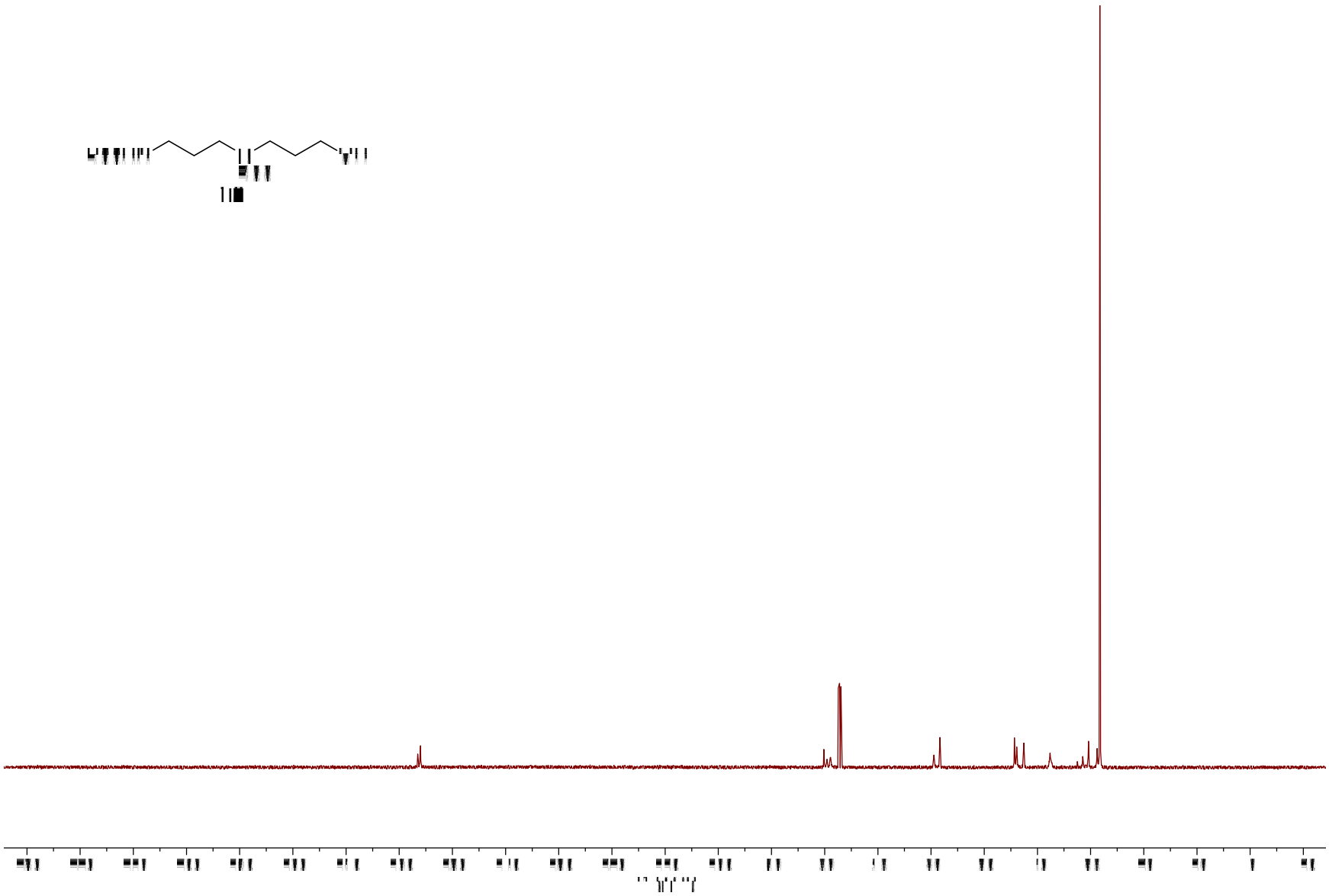

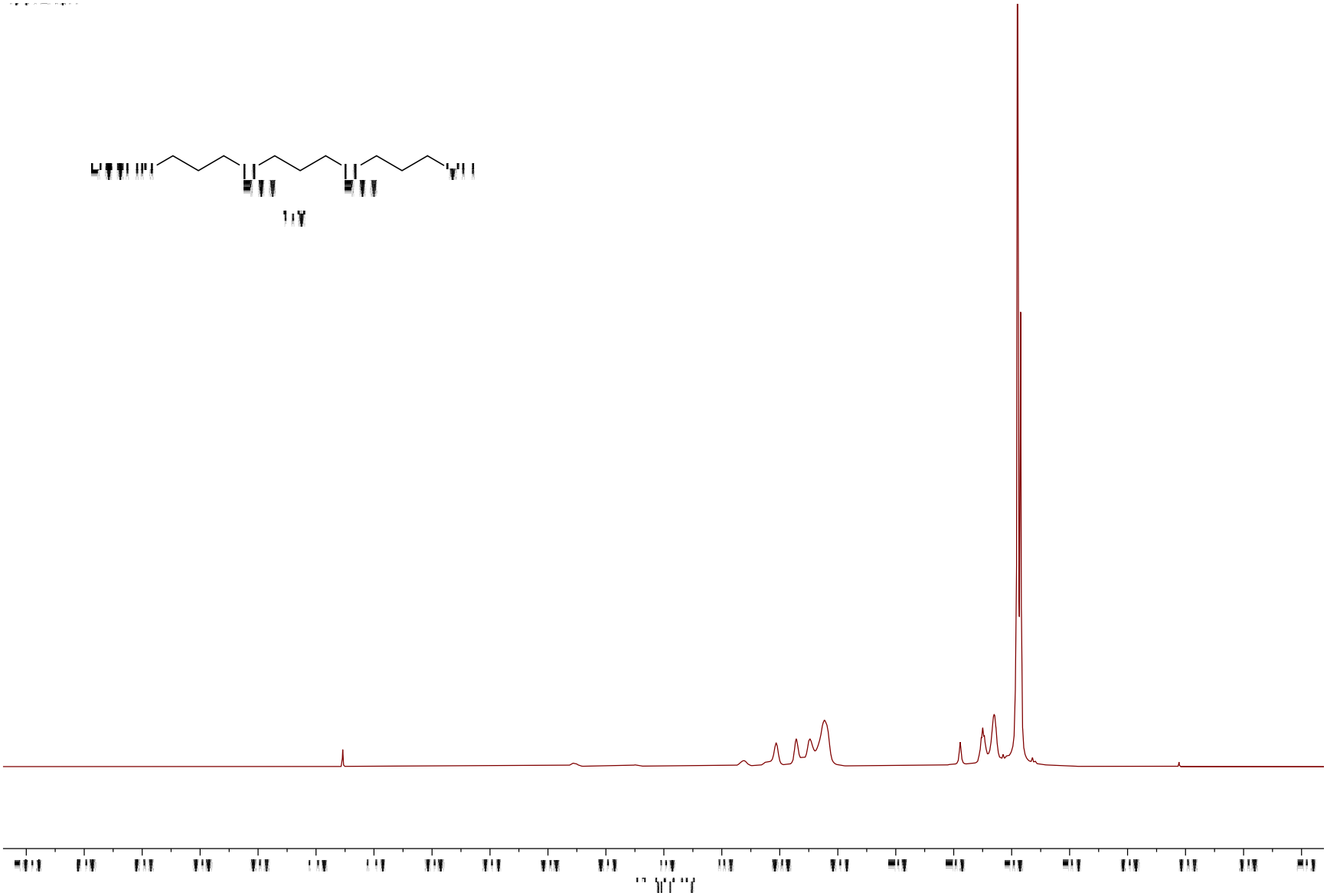

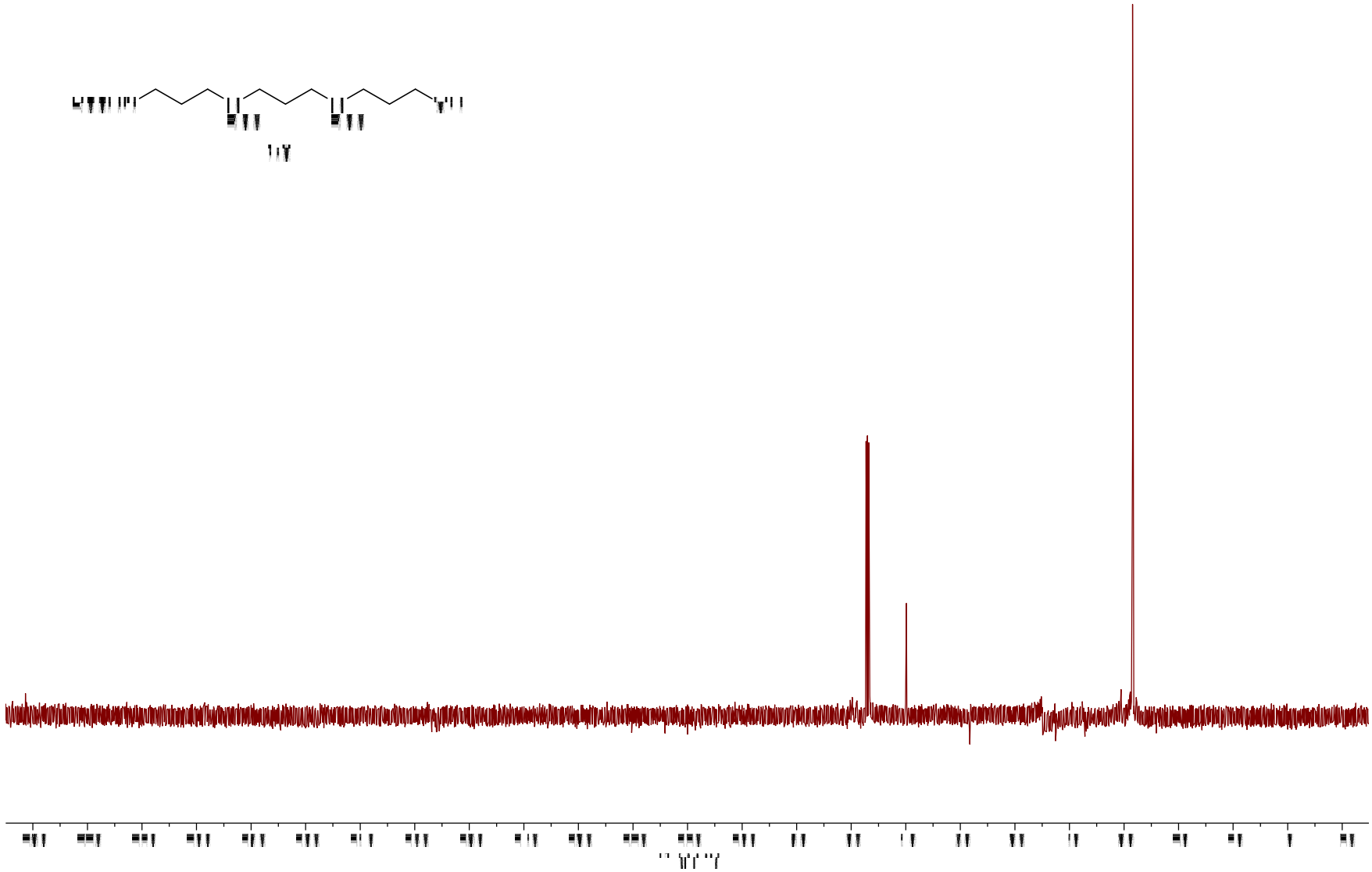

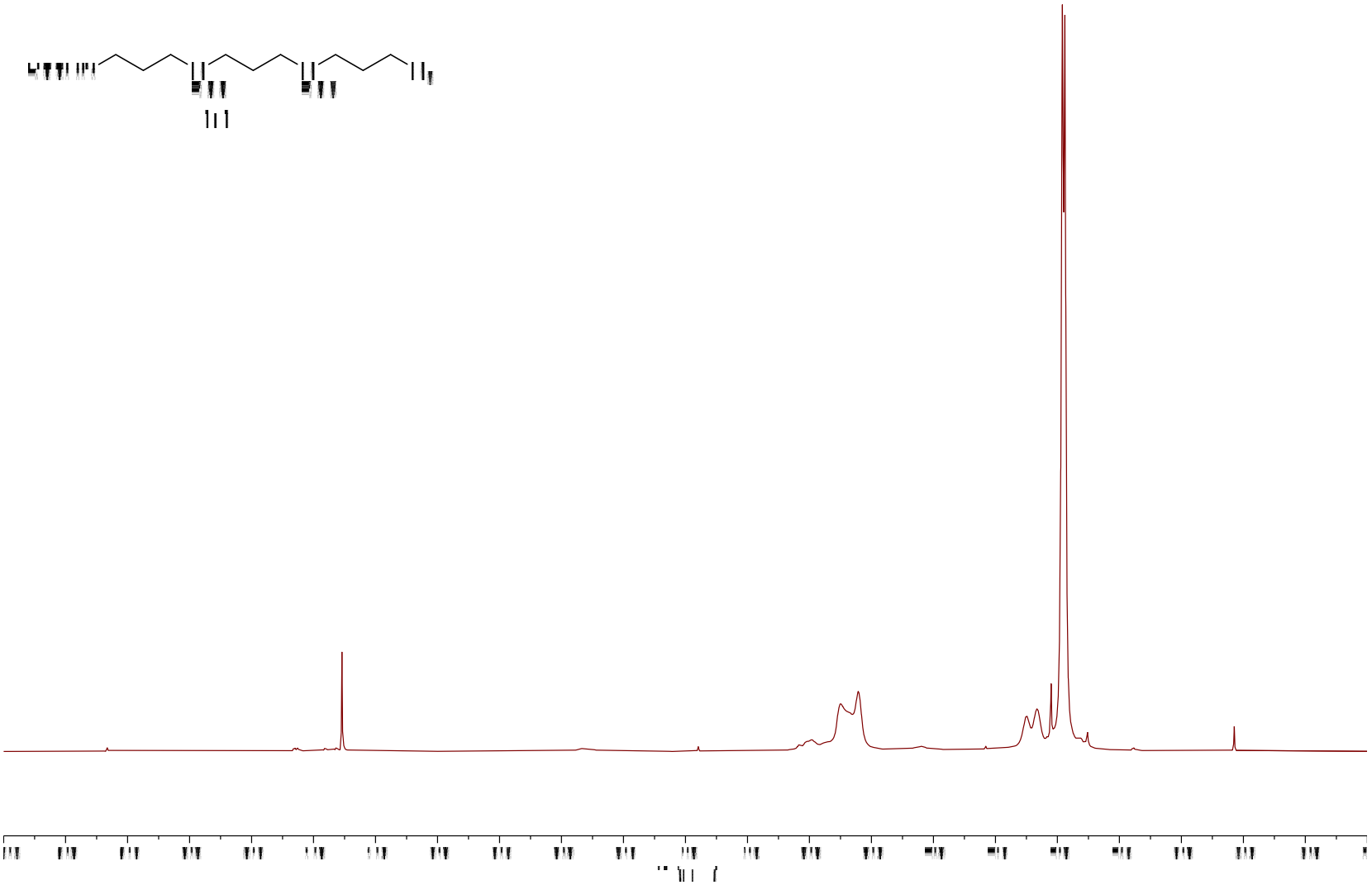

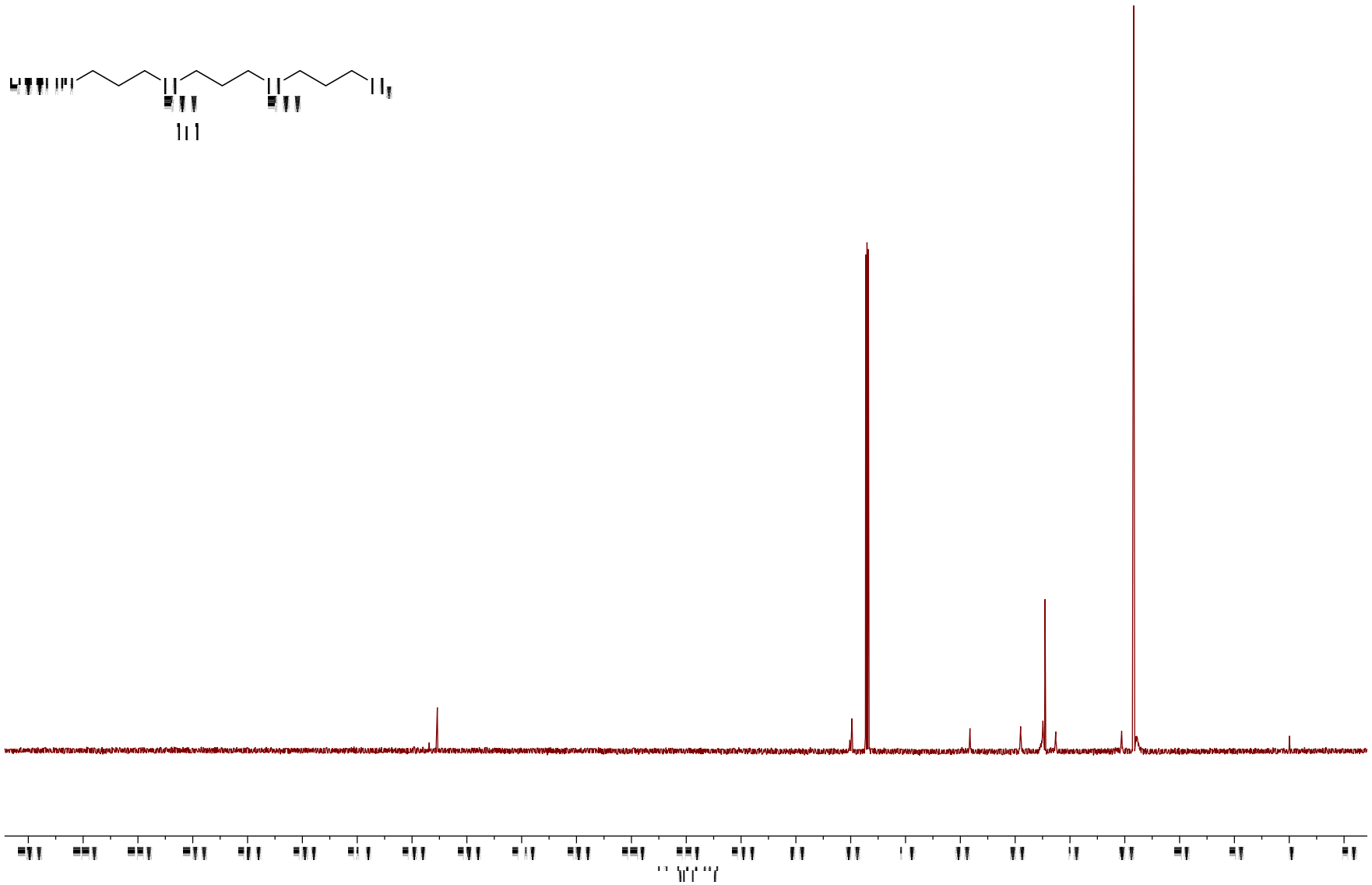

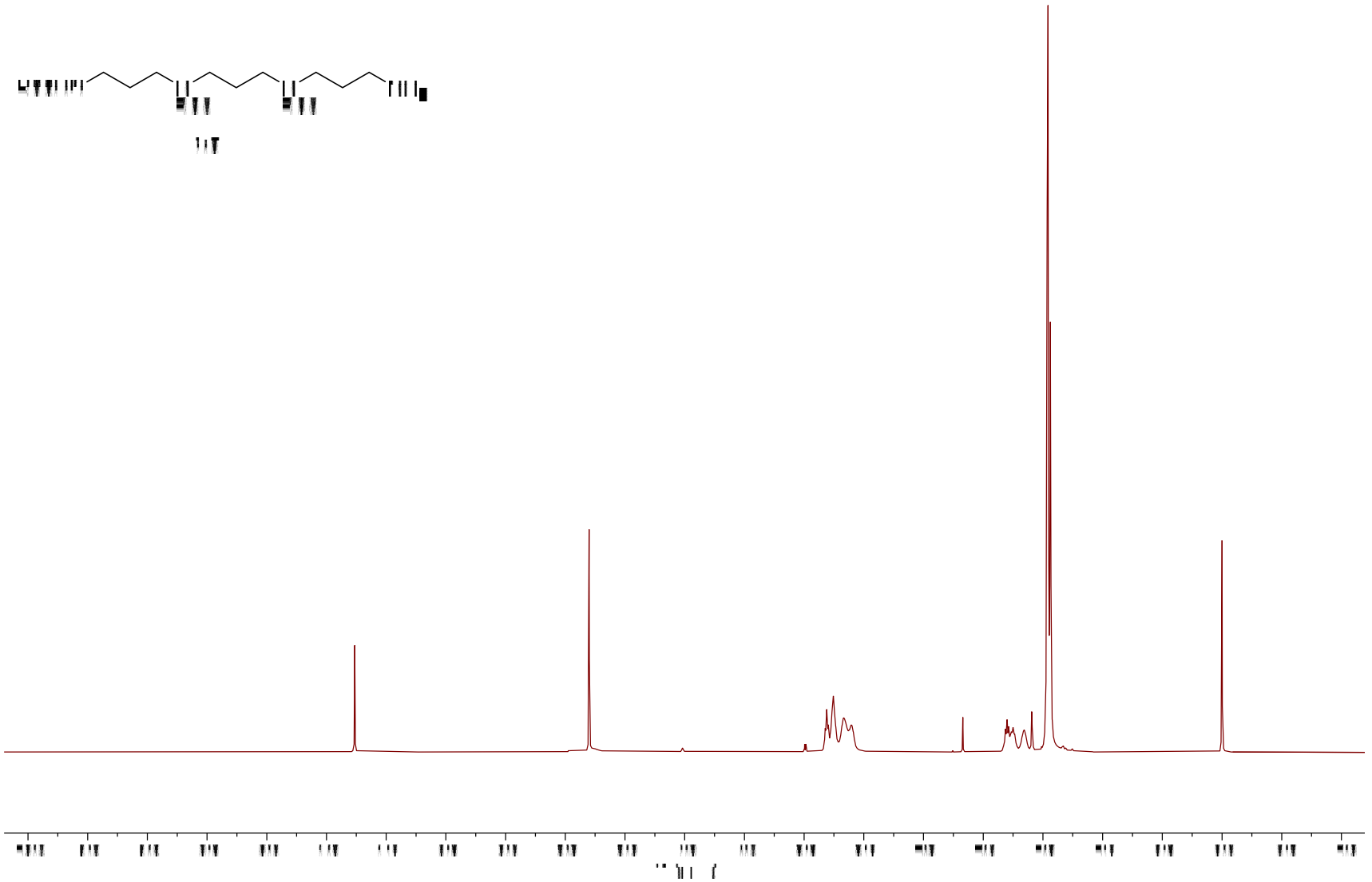

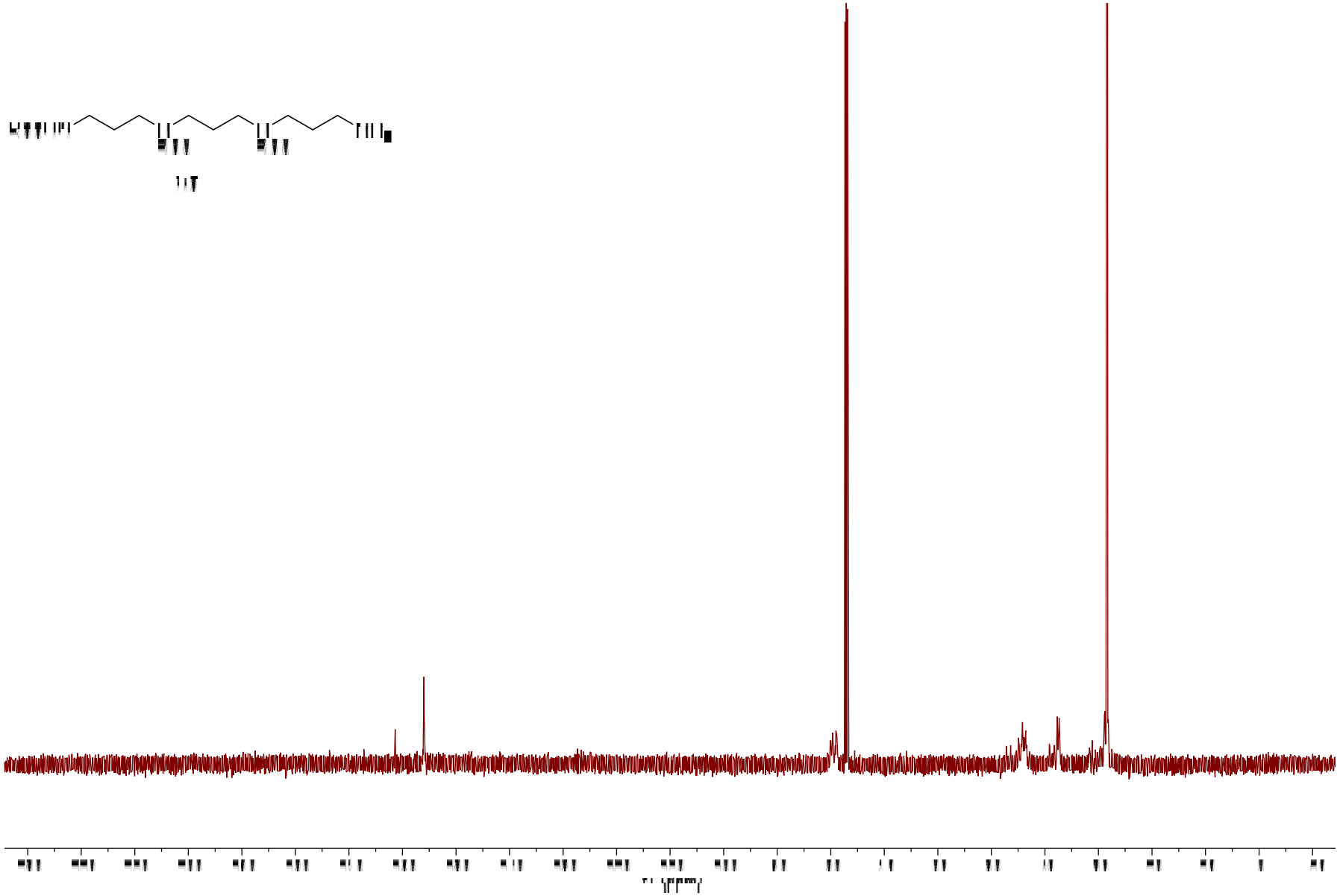

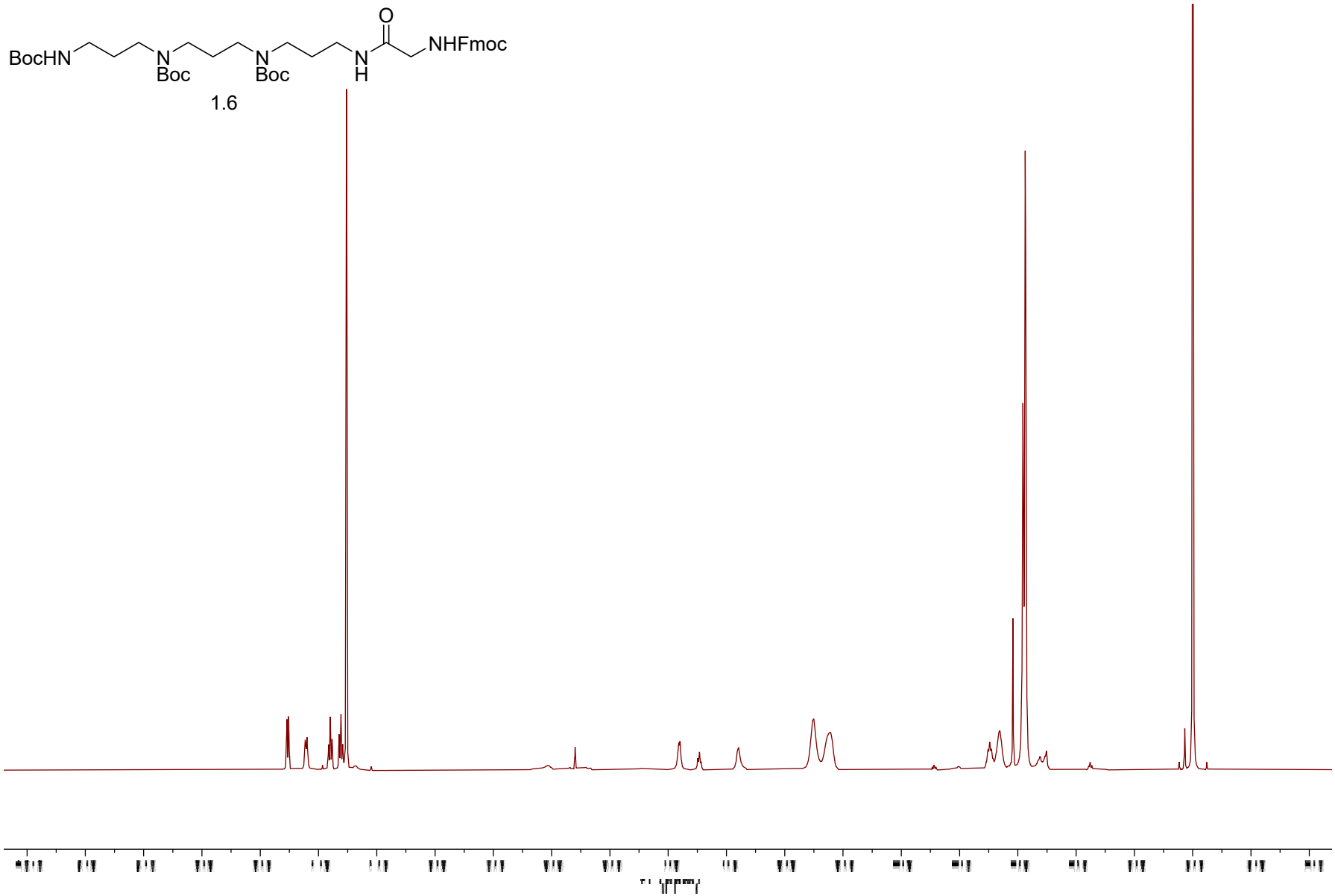
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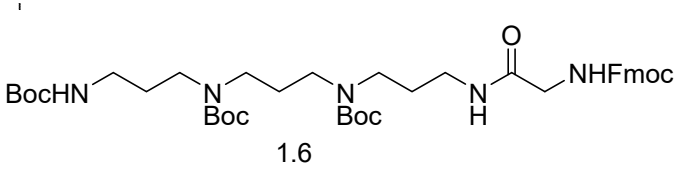

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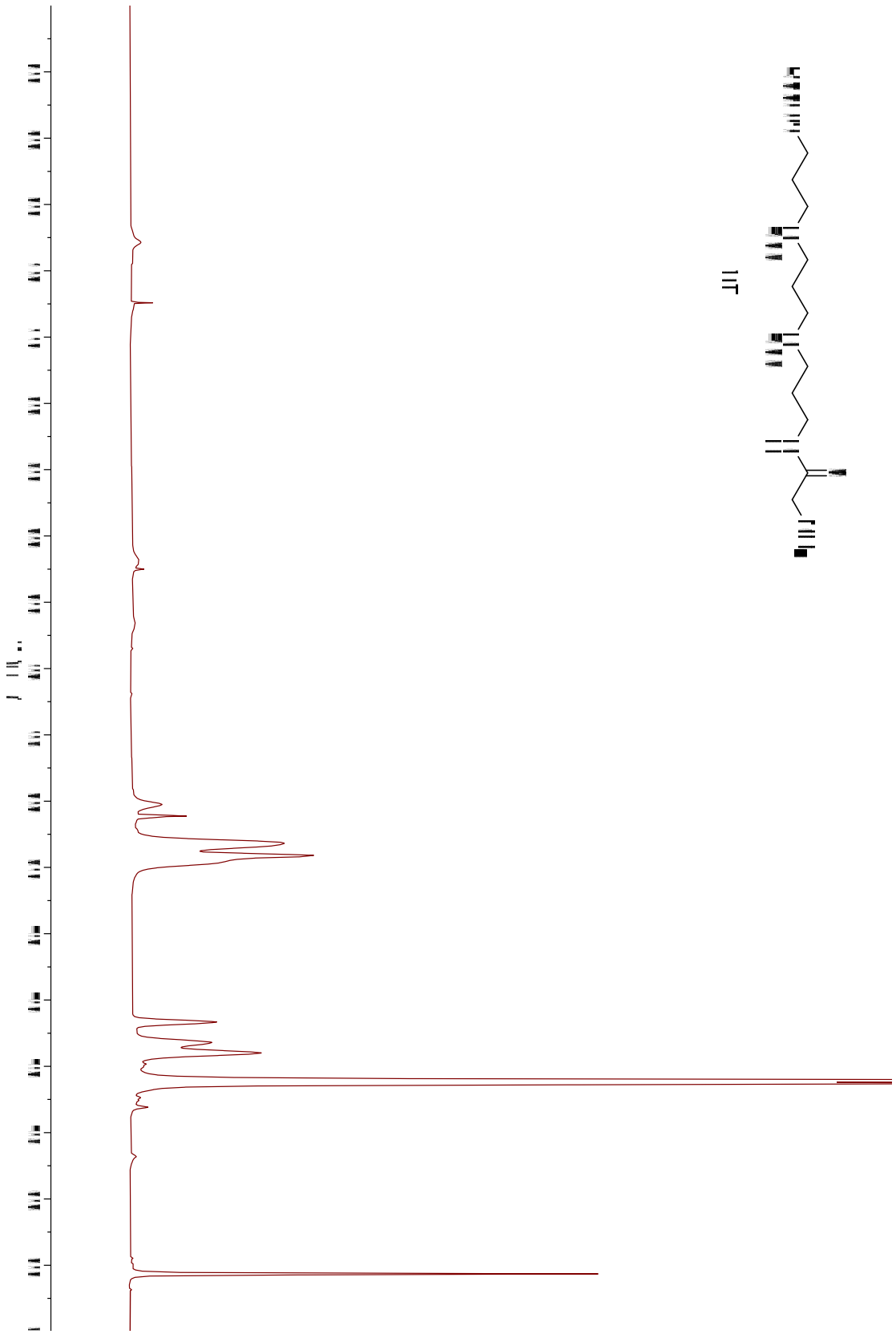

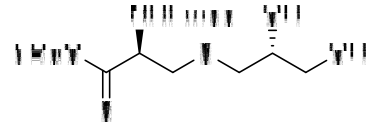

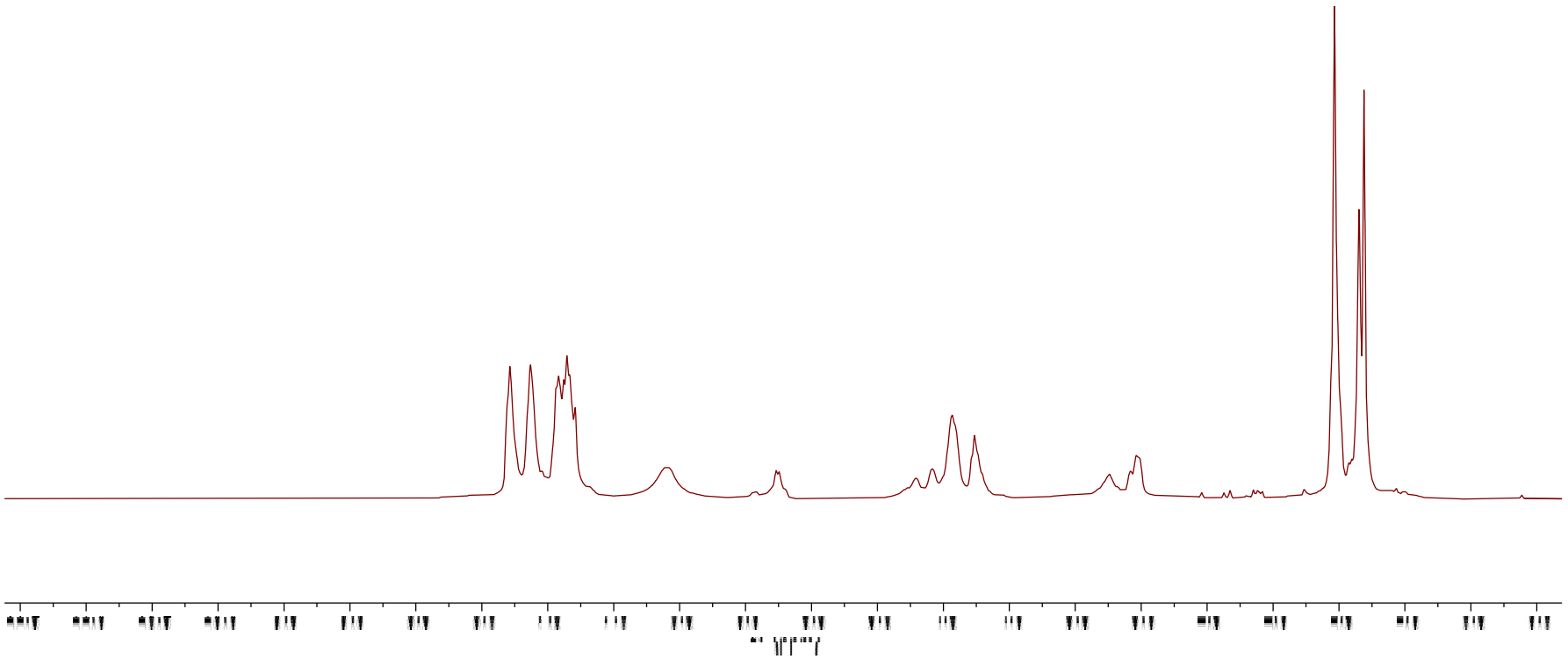

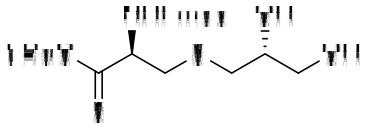

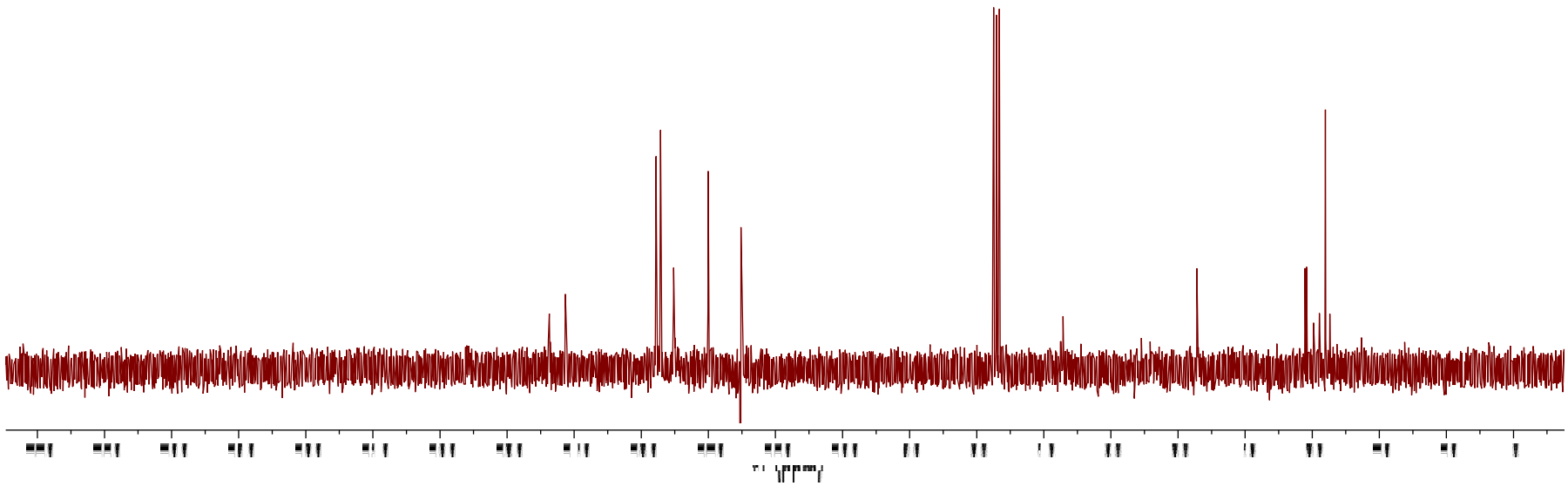

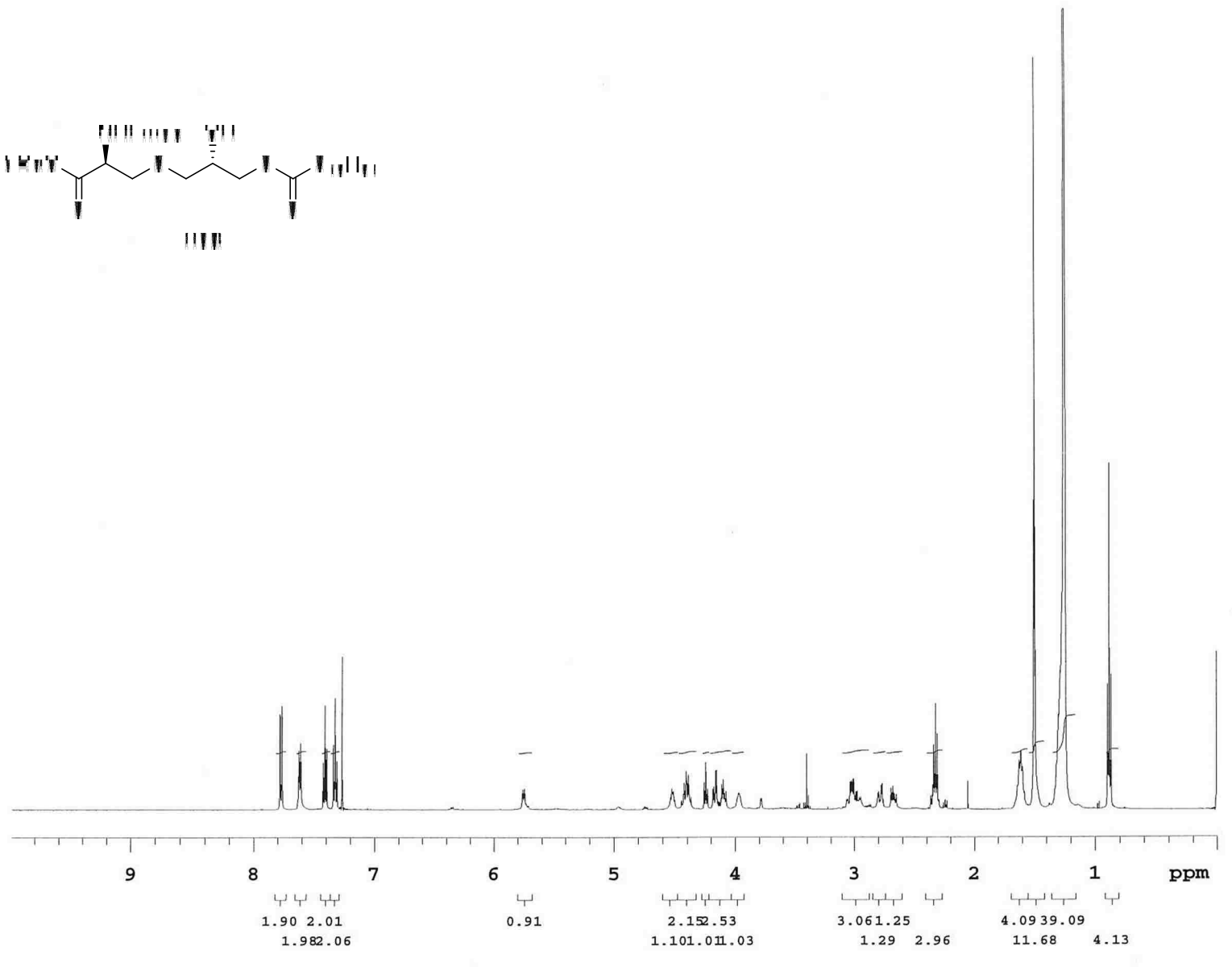
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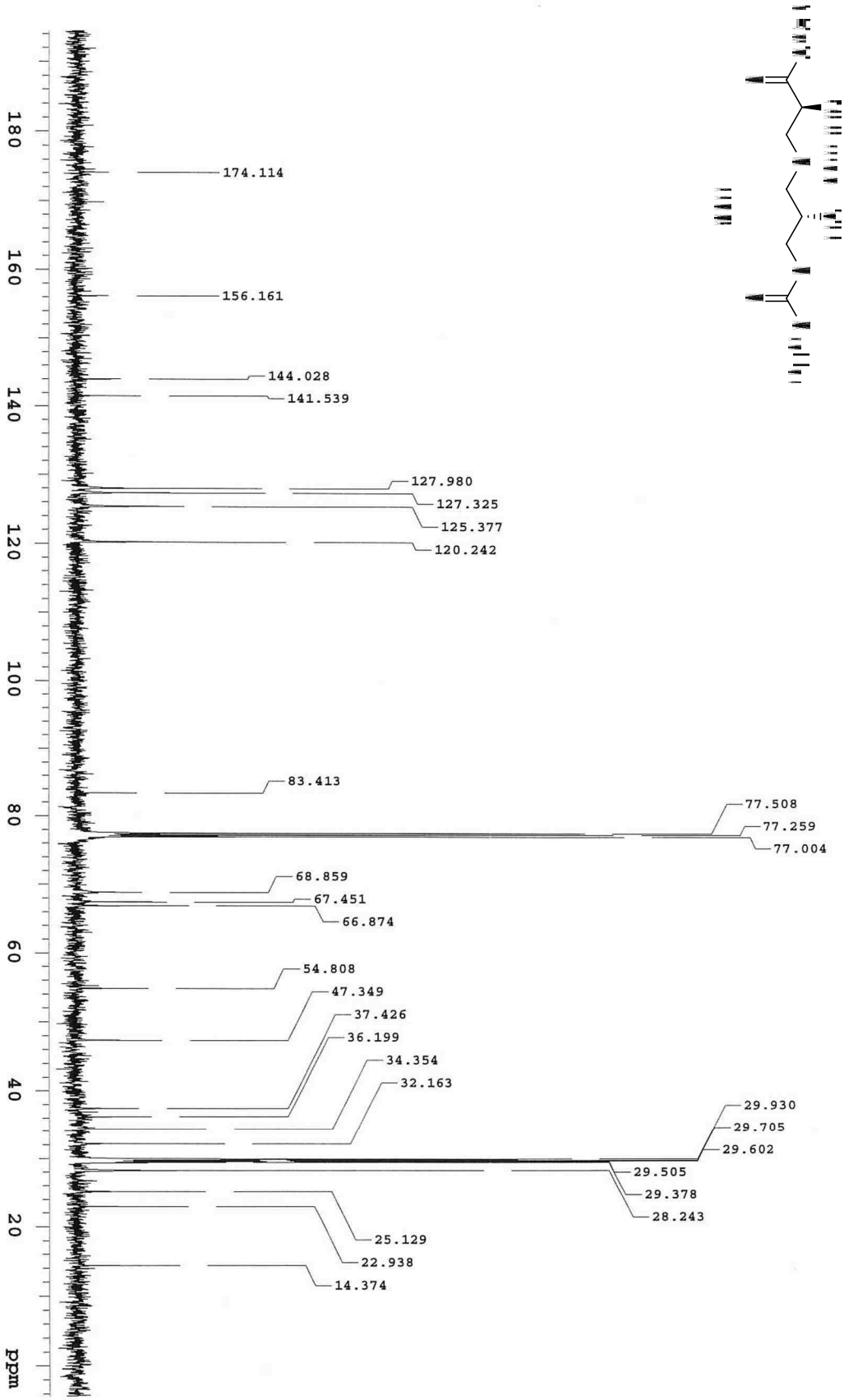
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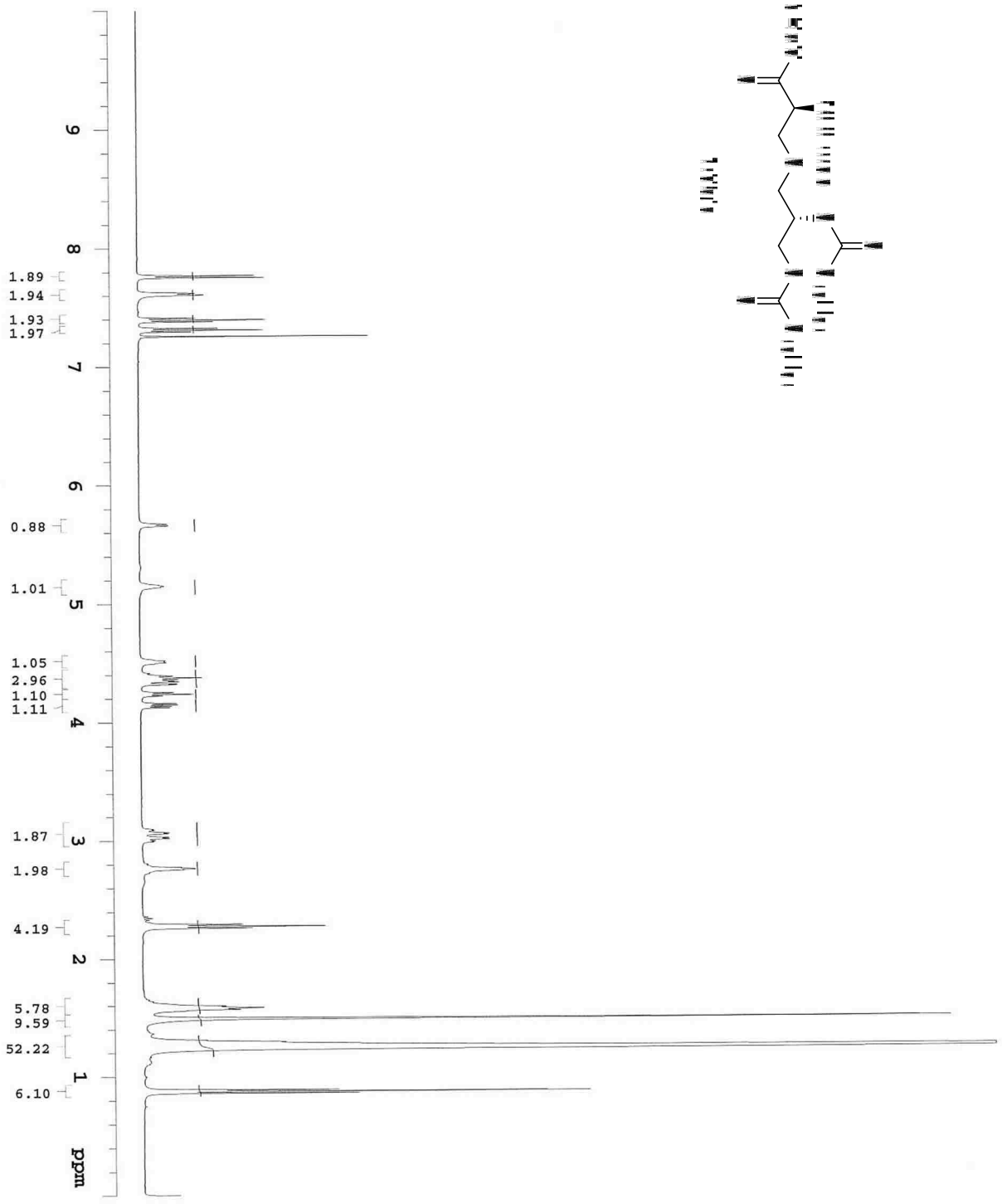
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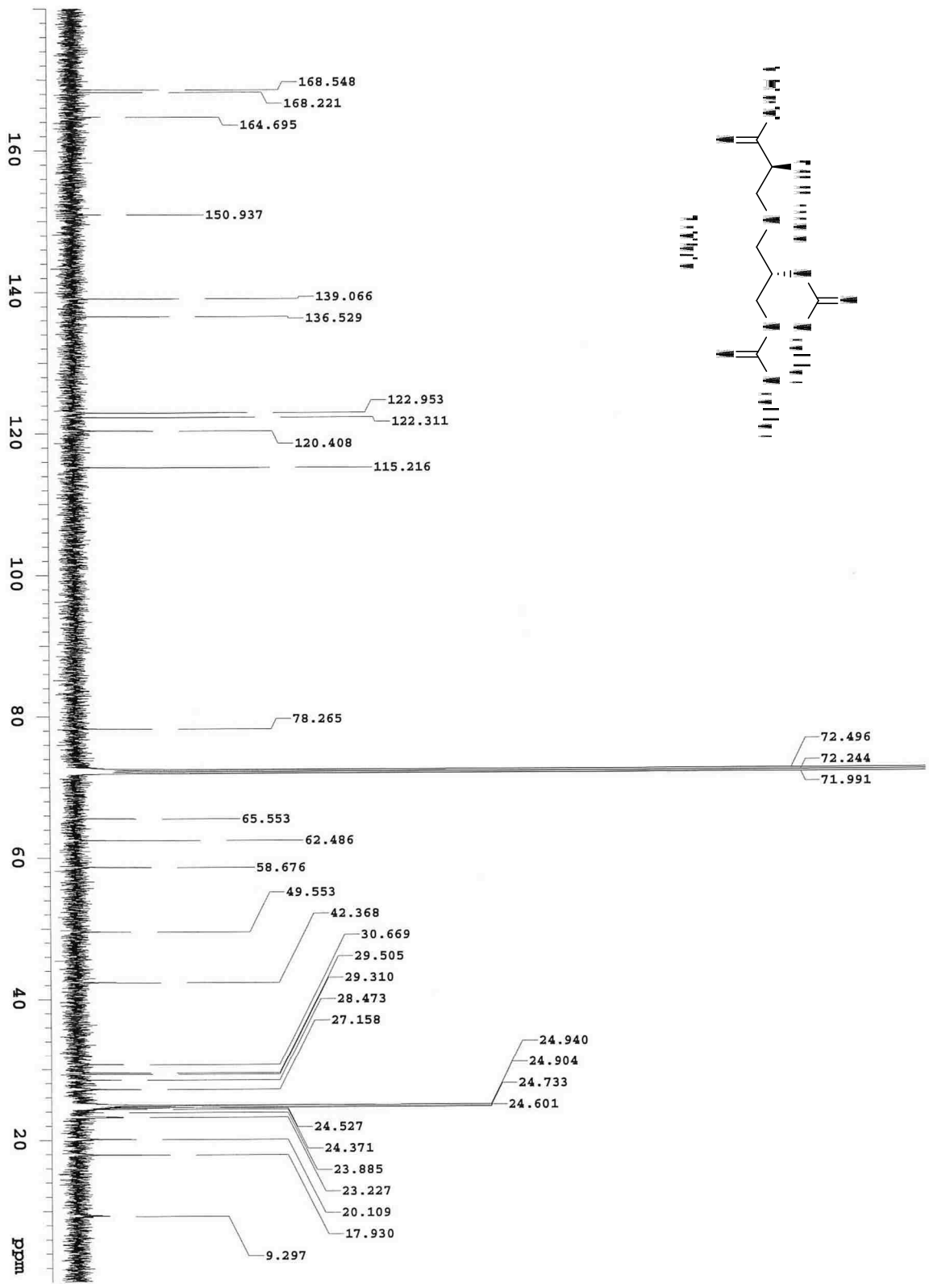
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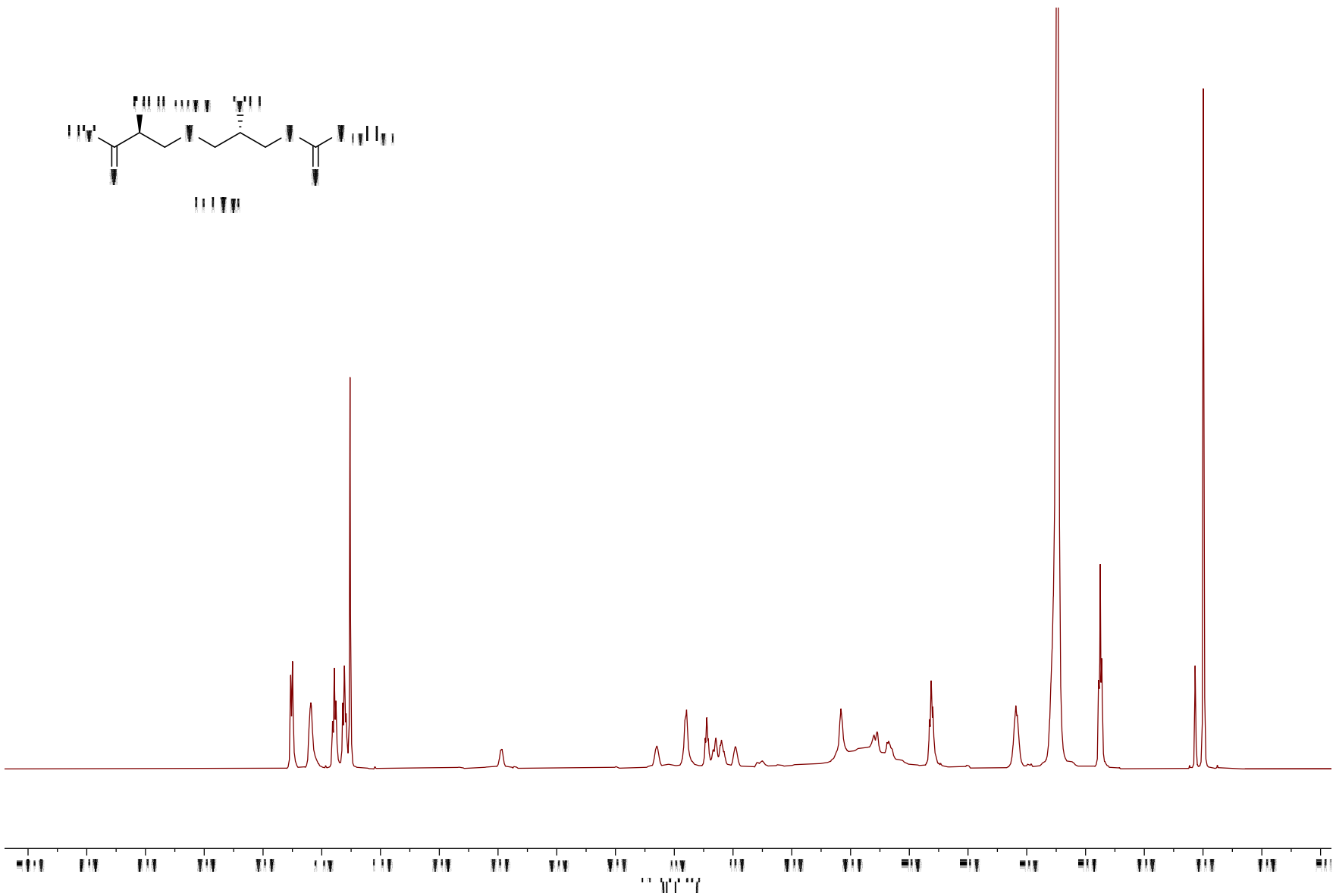

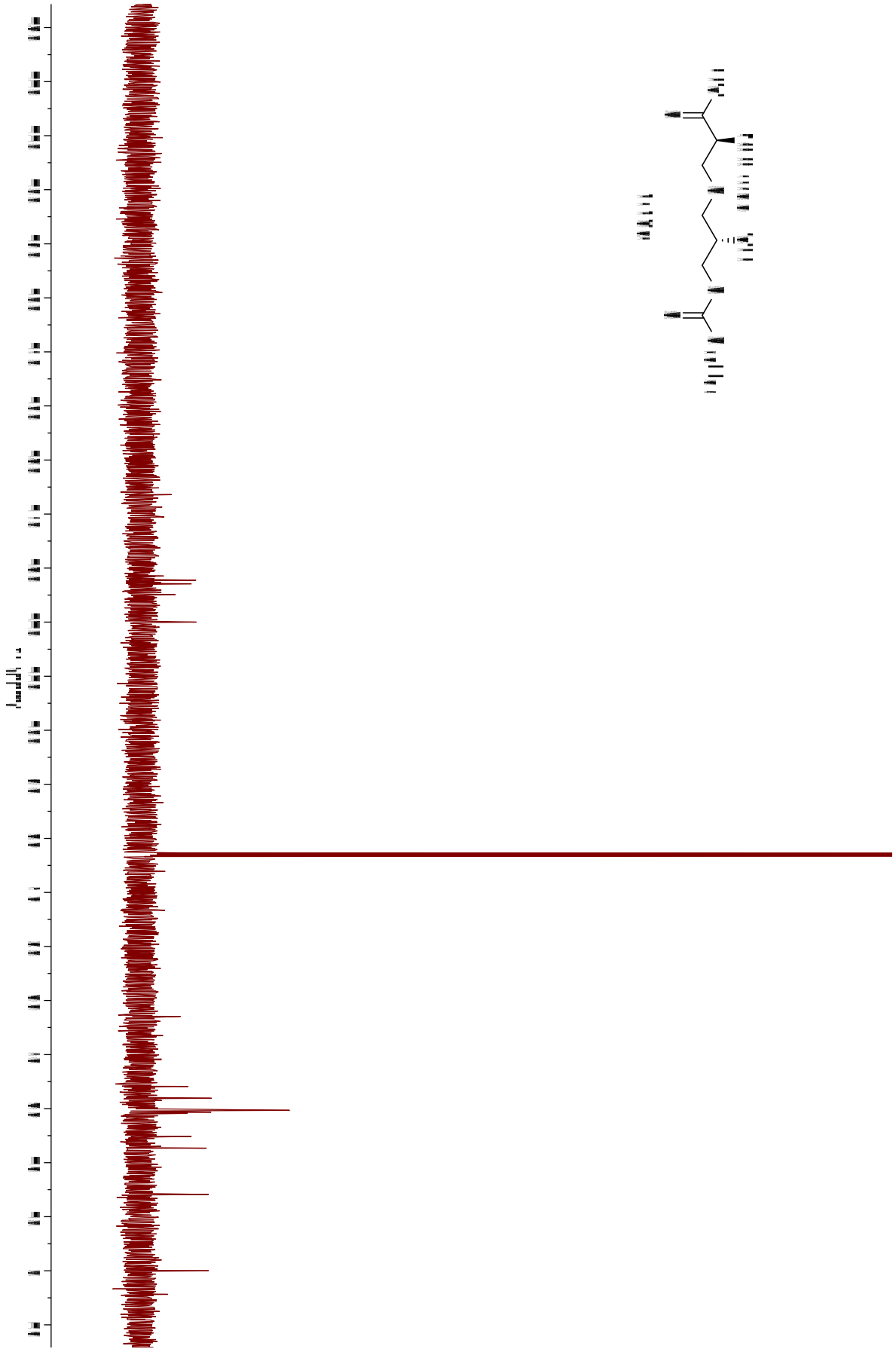

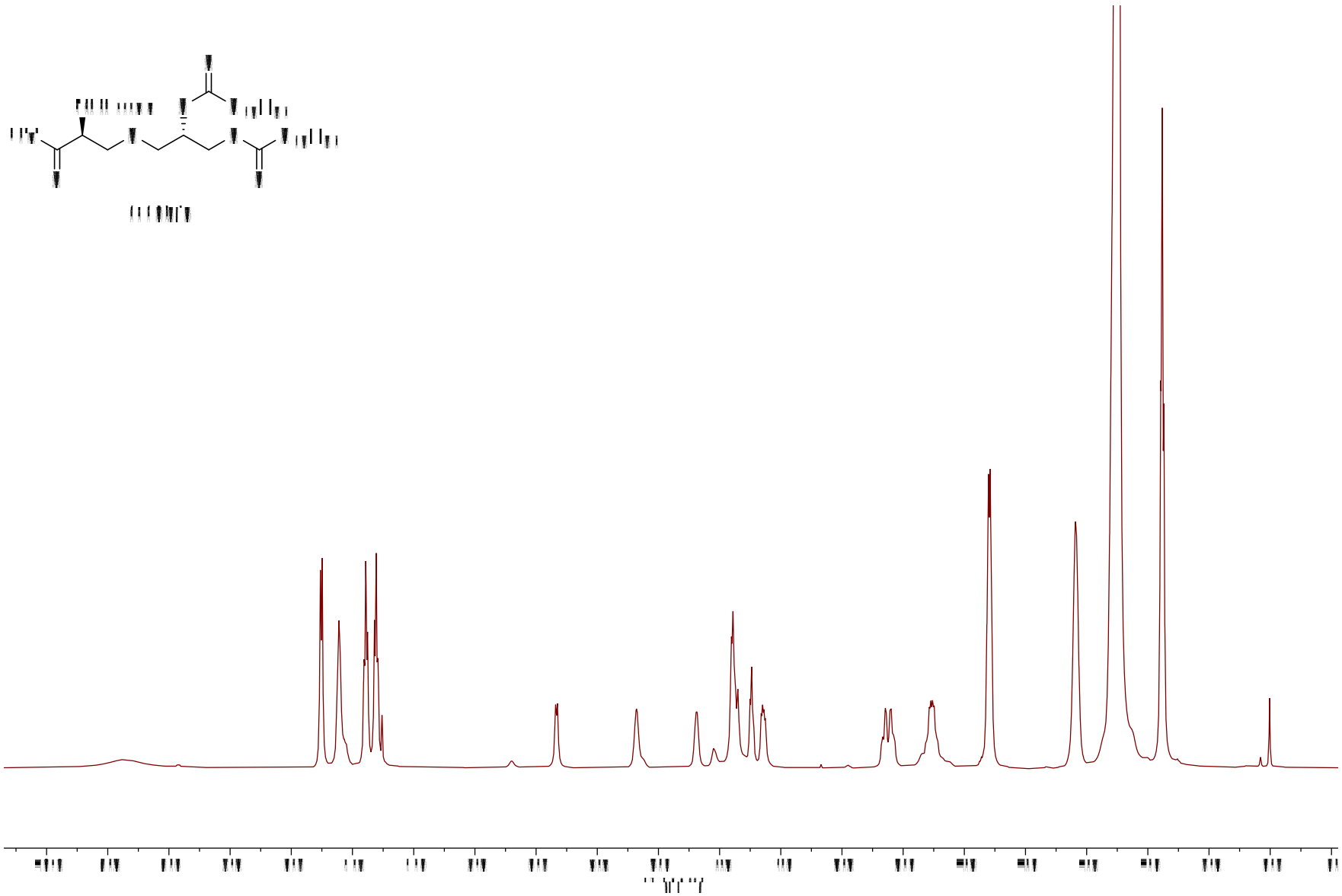

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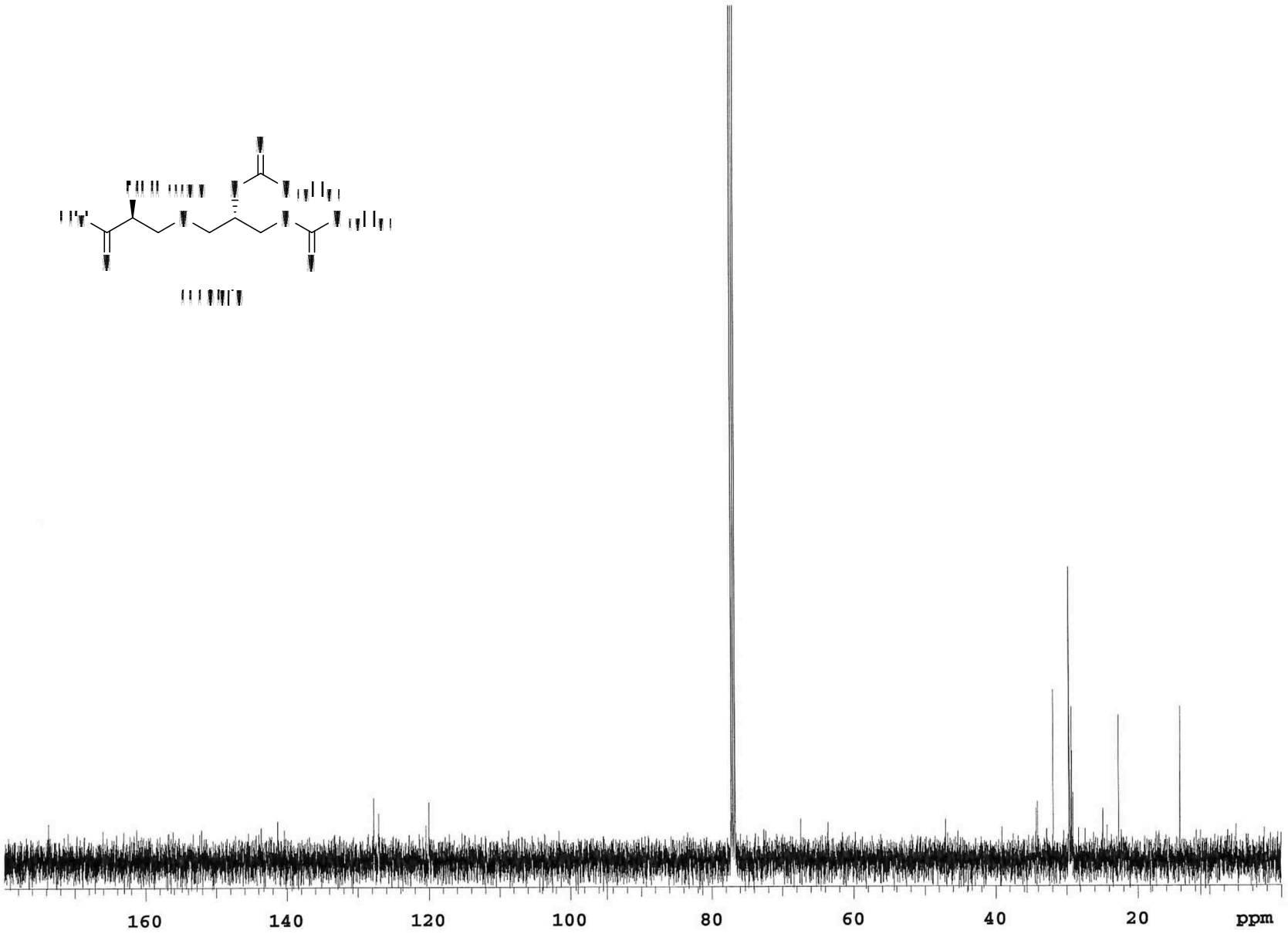
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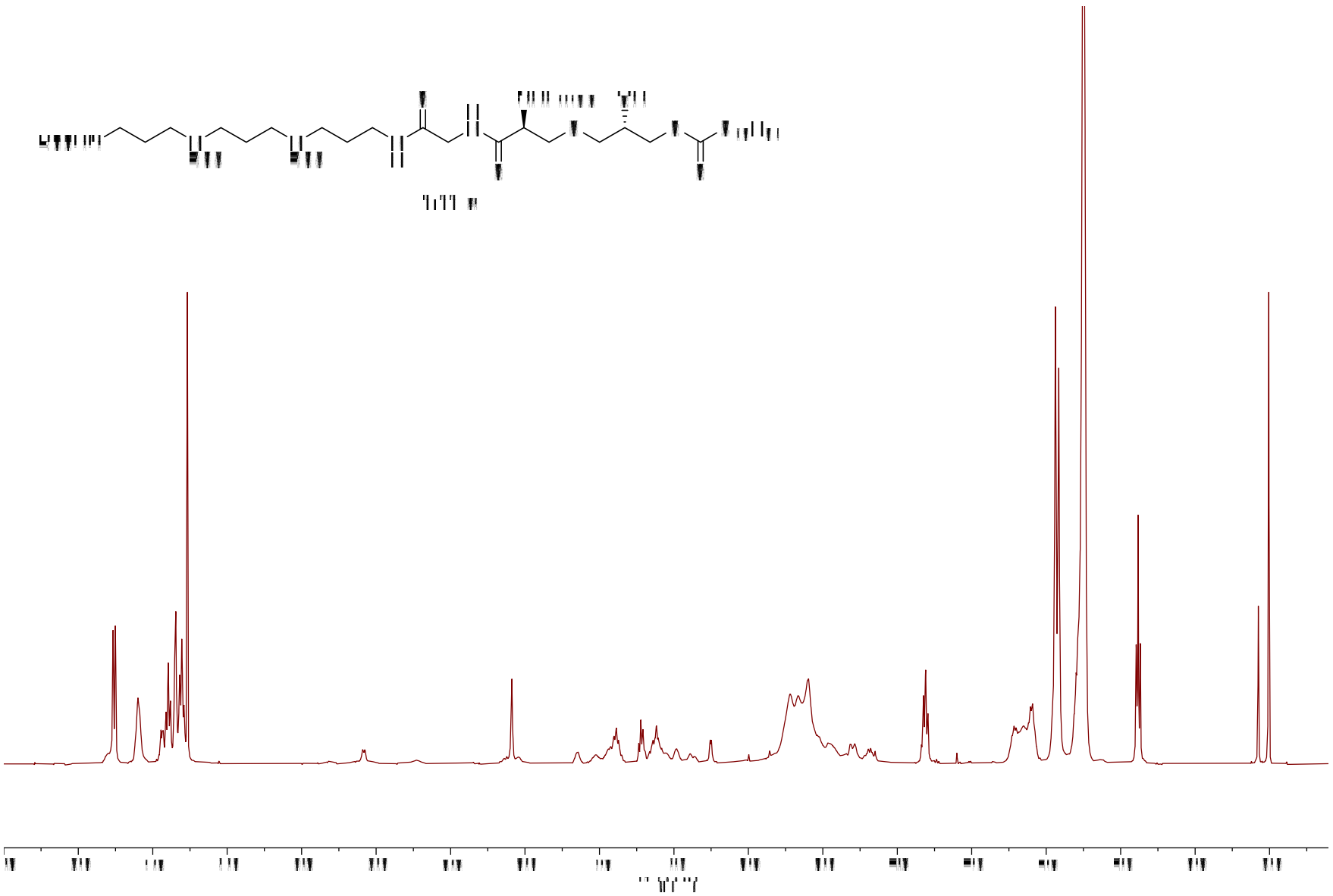

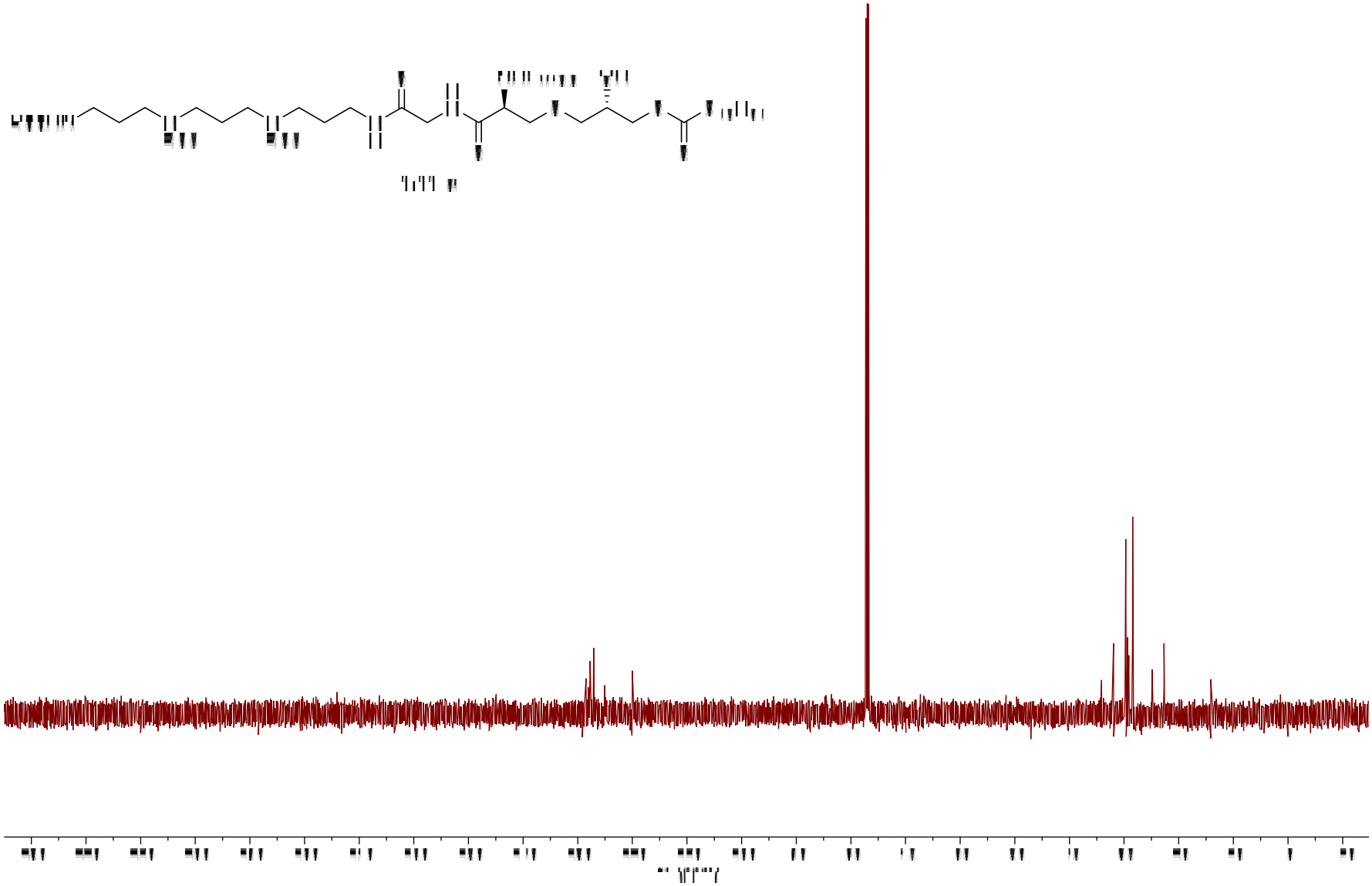

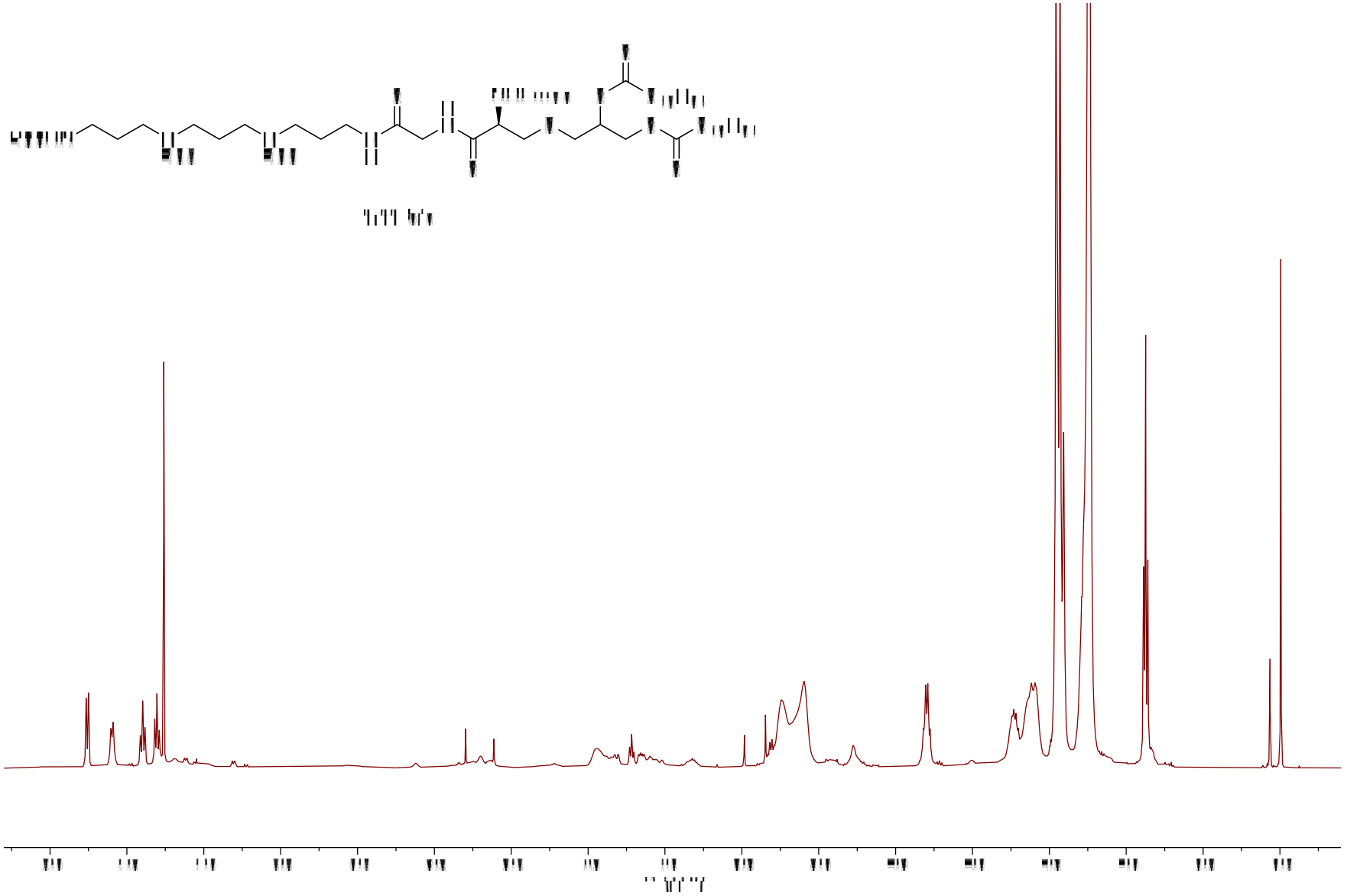

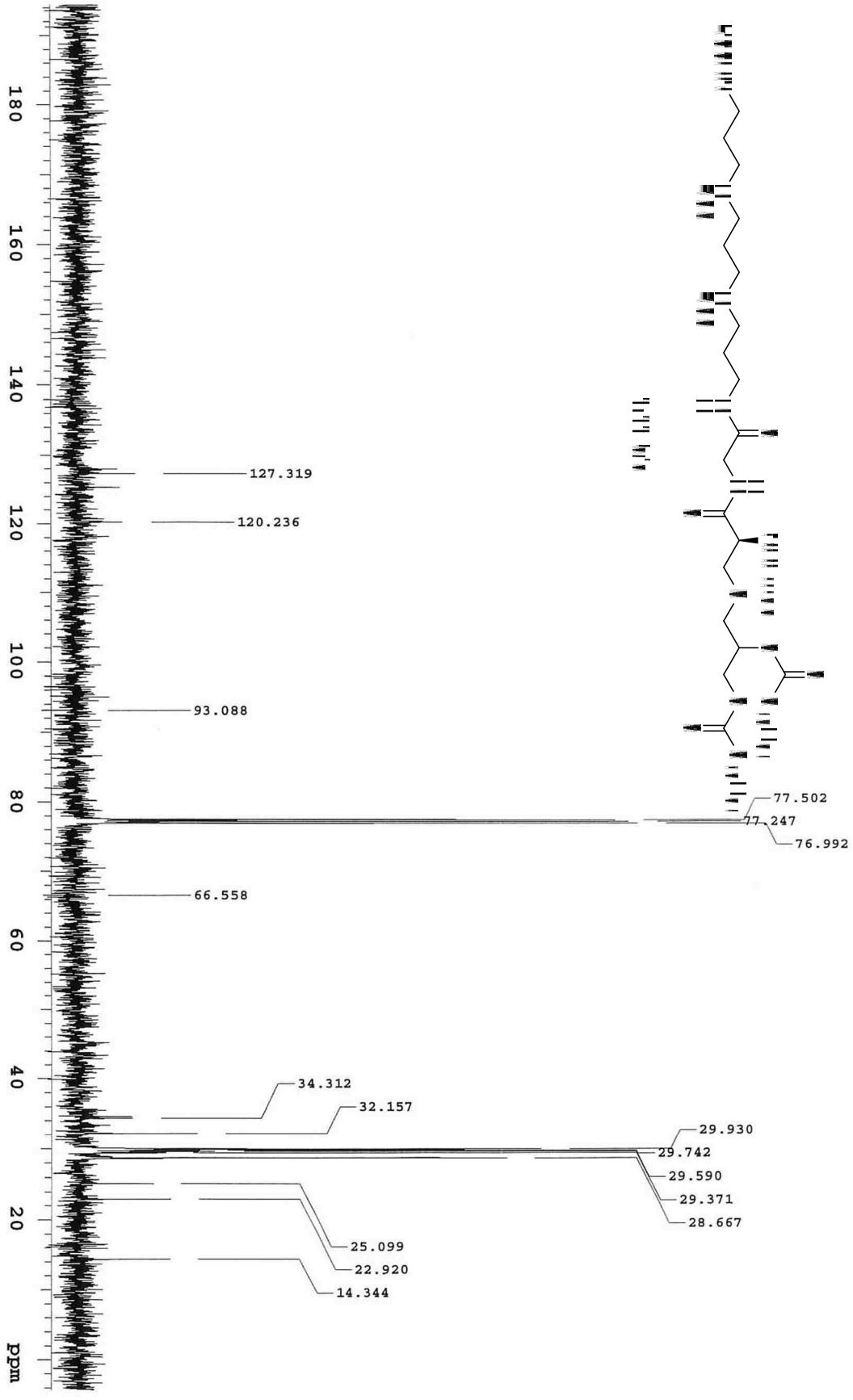
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100
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40
20
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120.236
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77.502
77.247
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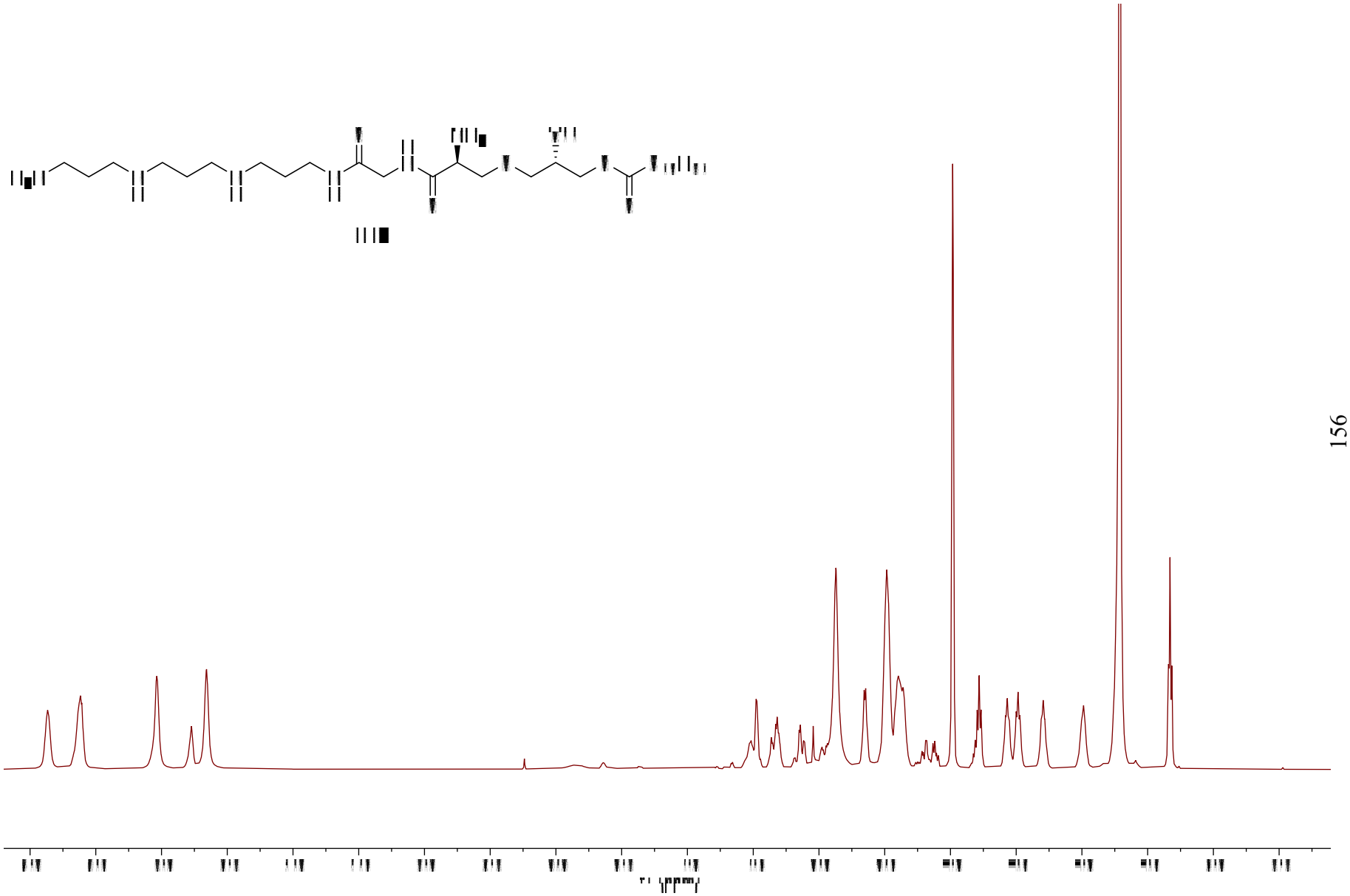
29.742
29.590
29.371
28.667
25.099
22.920
14.344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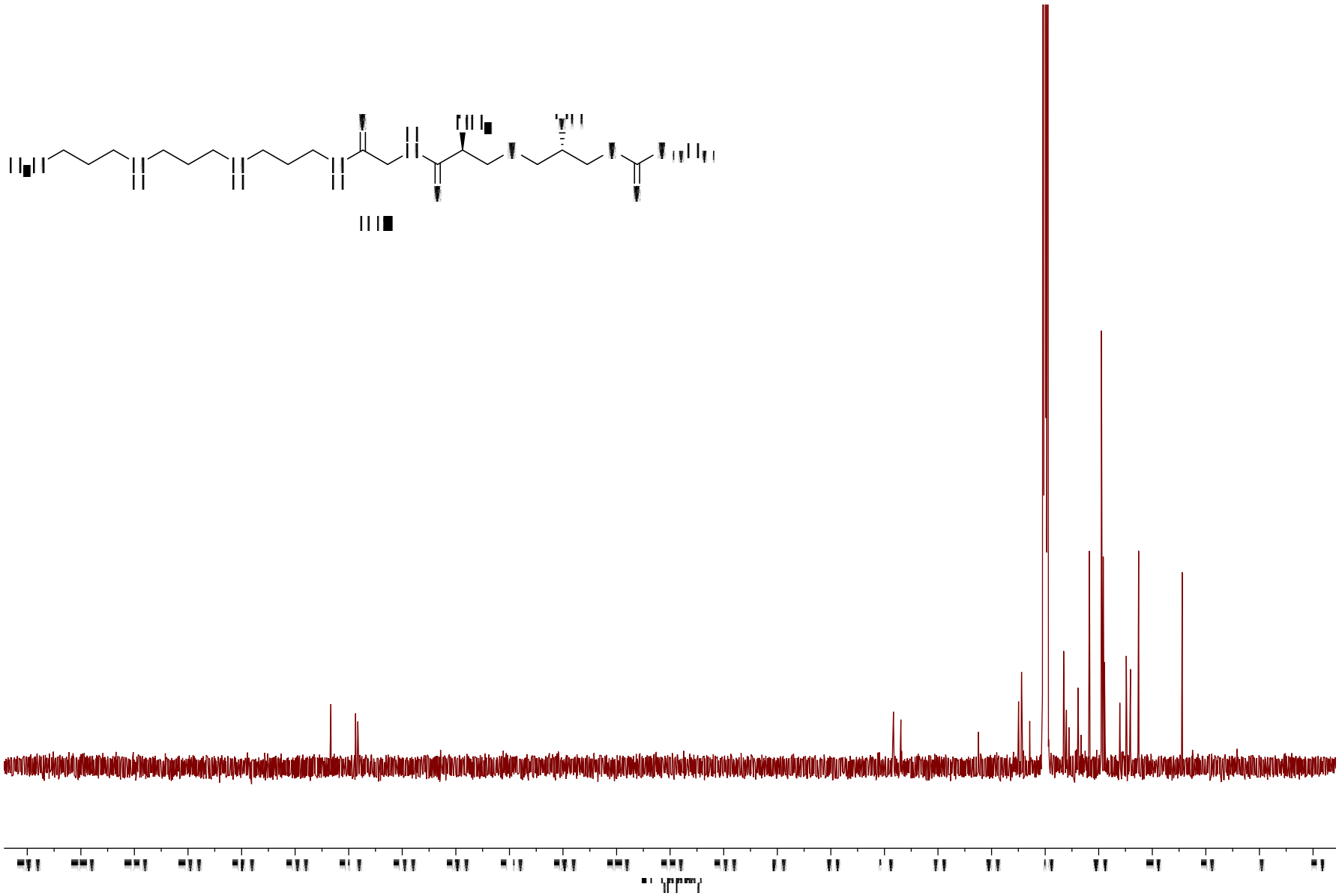

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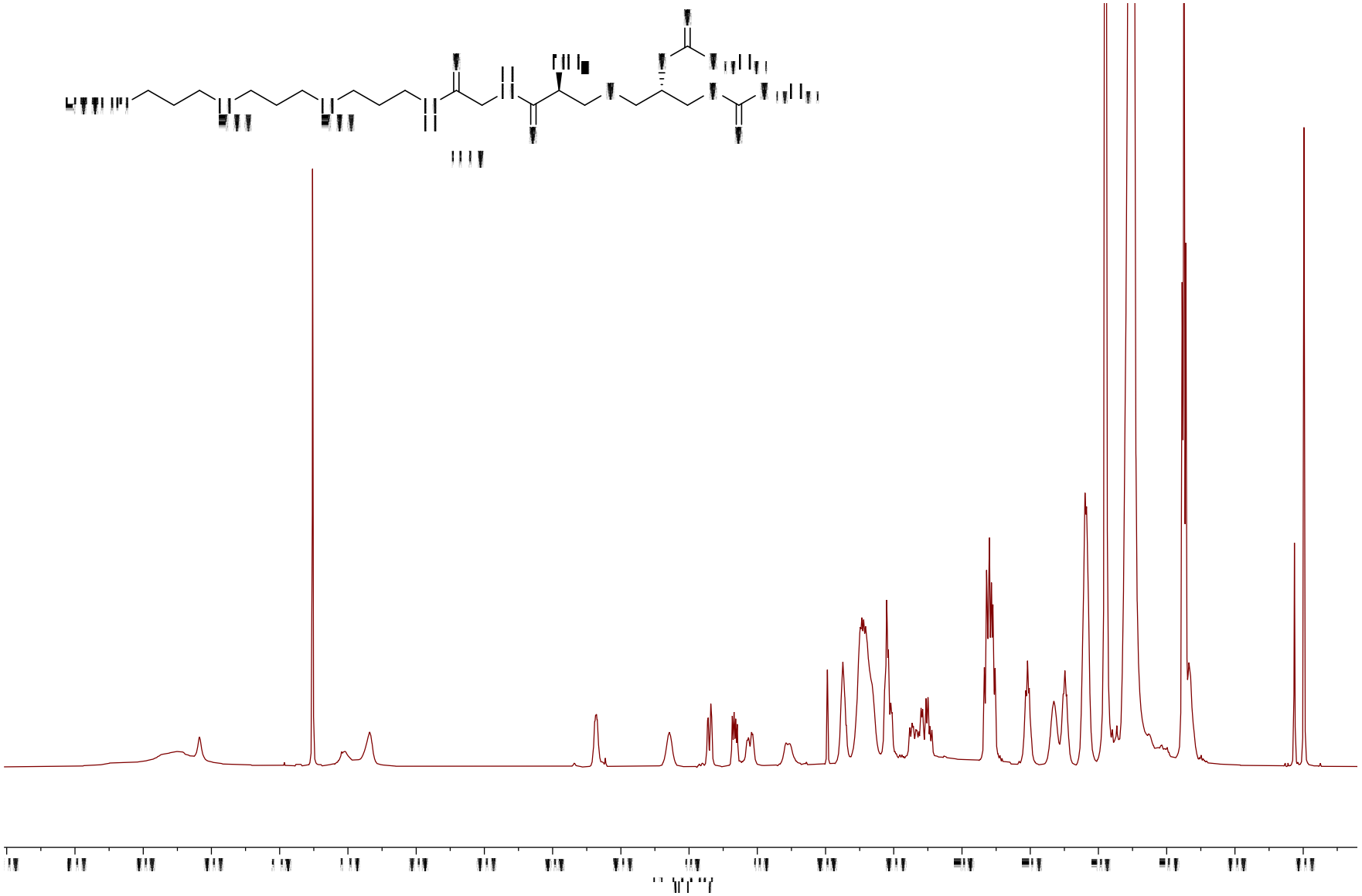

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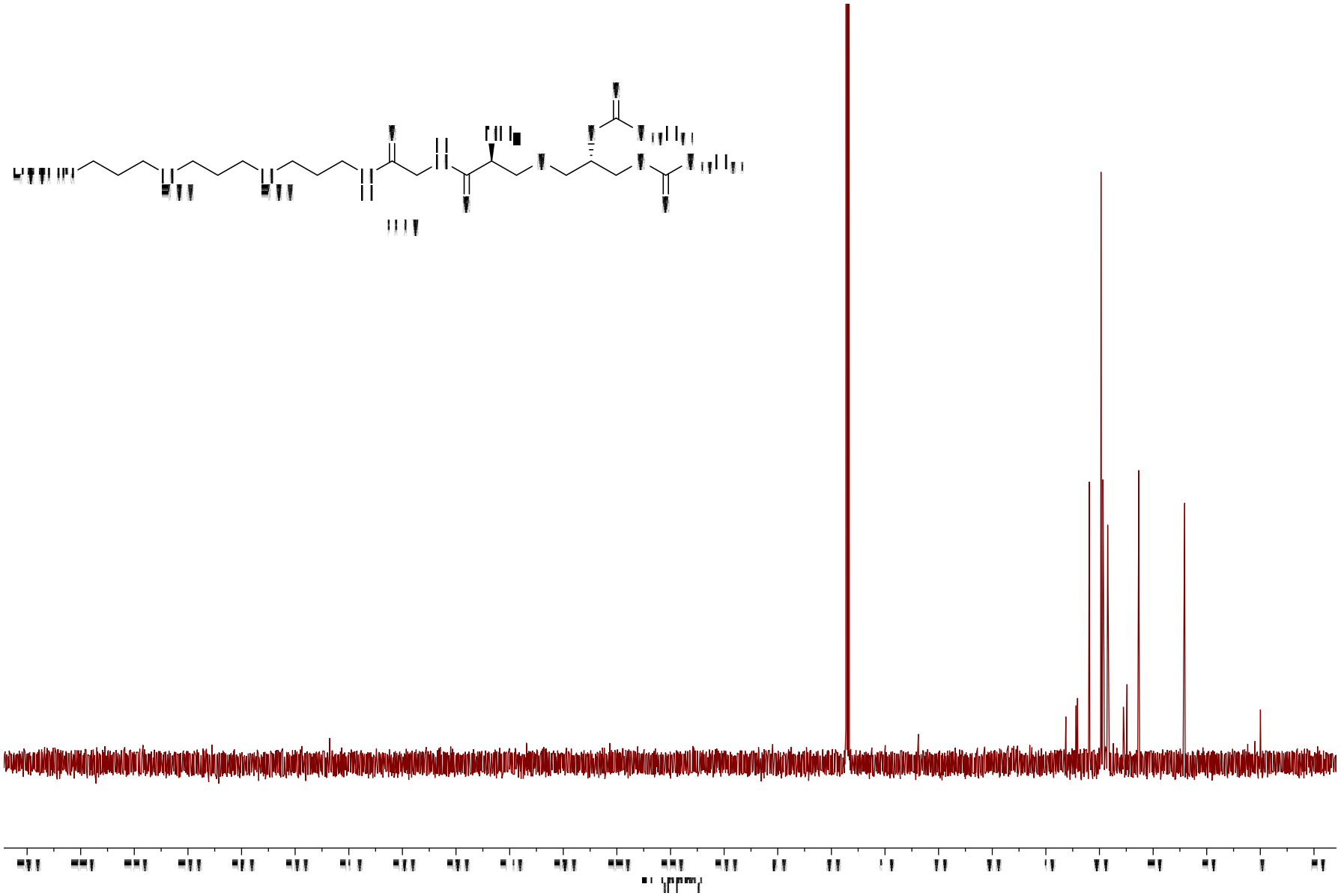

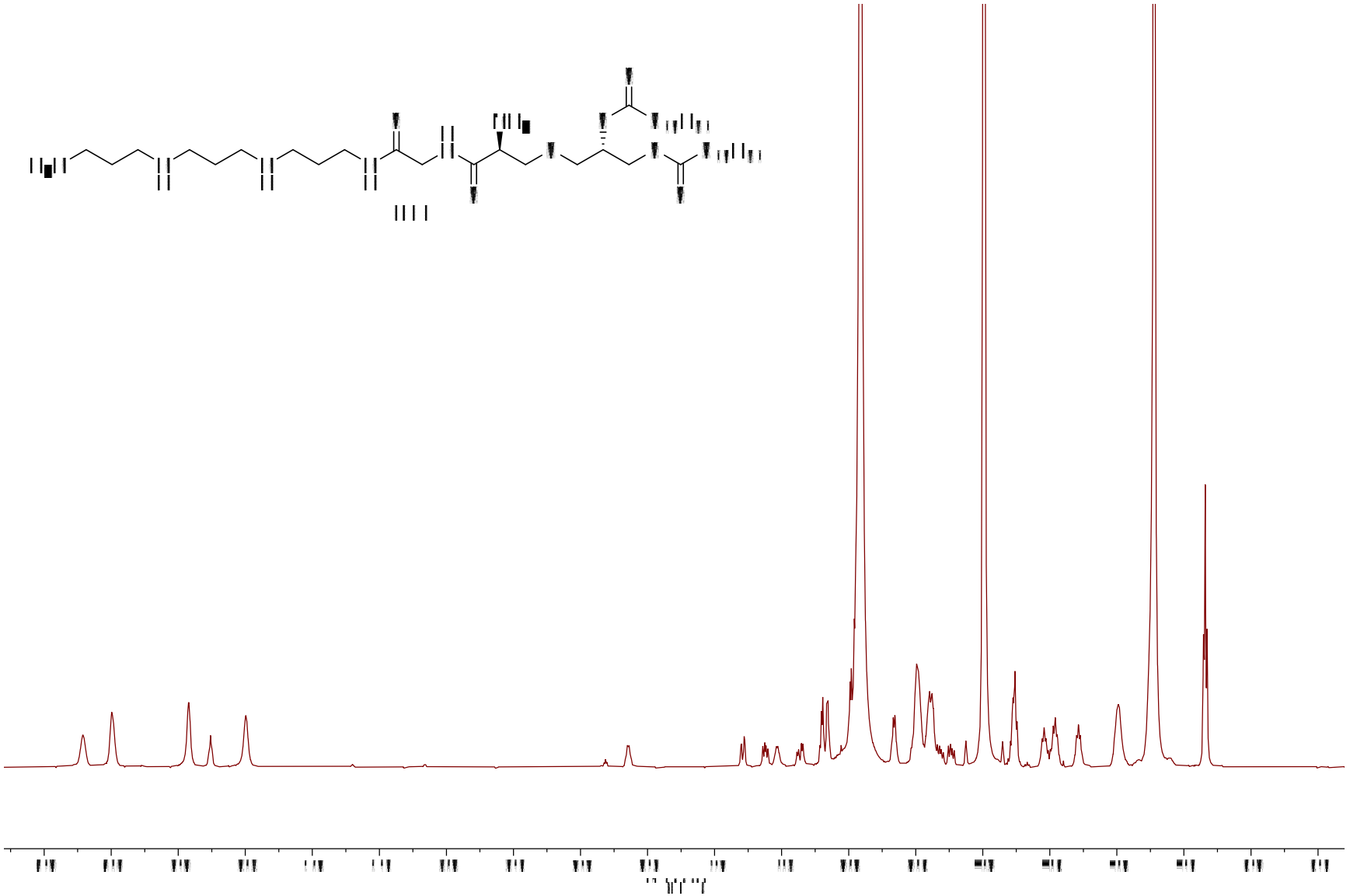

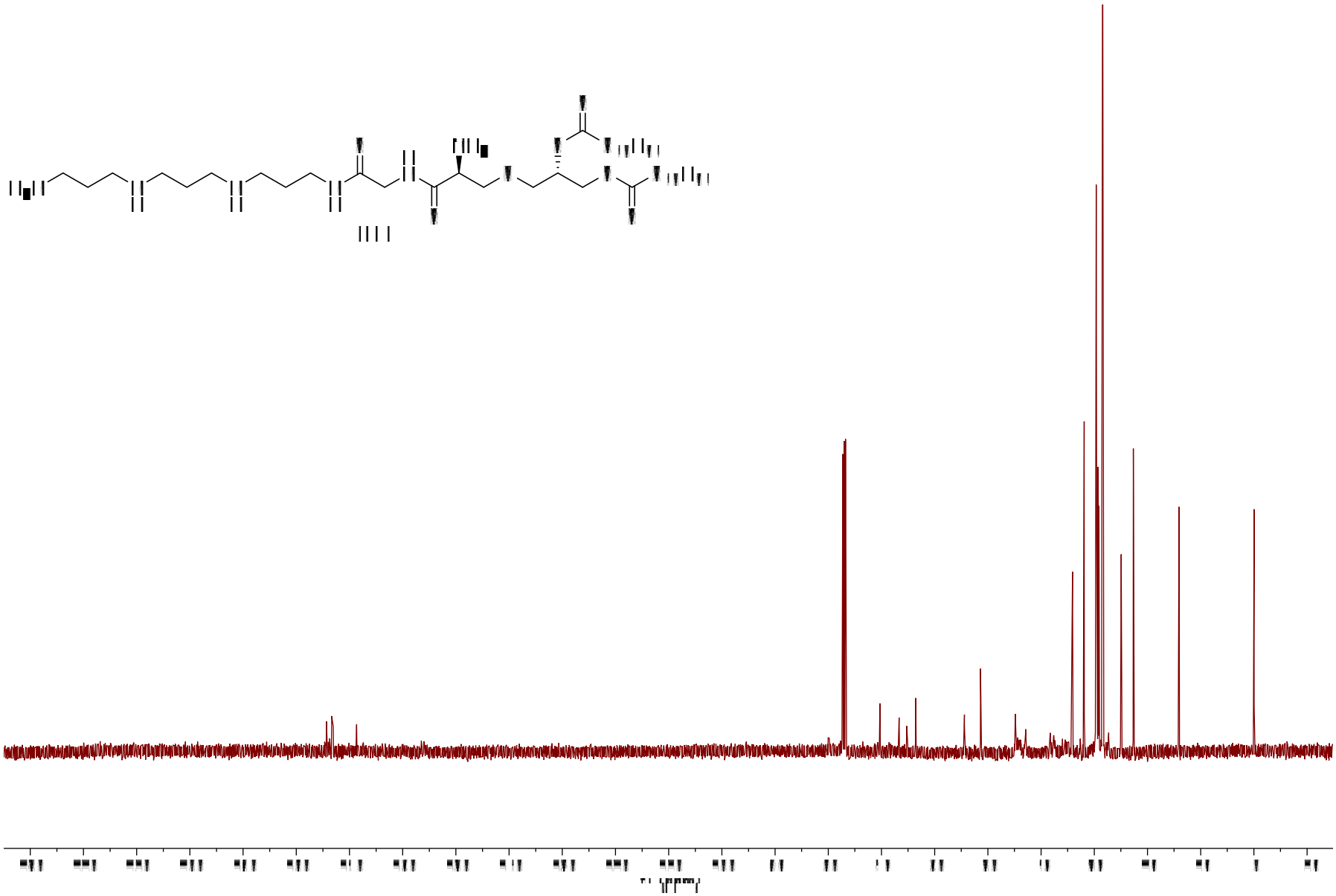

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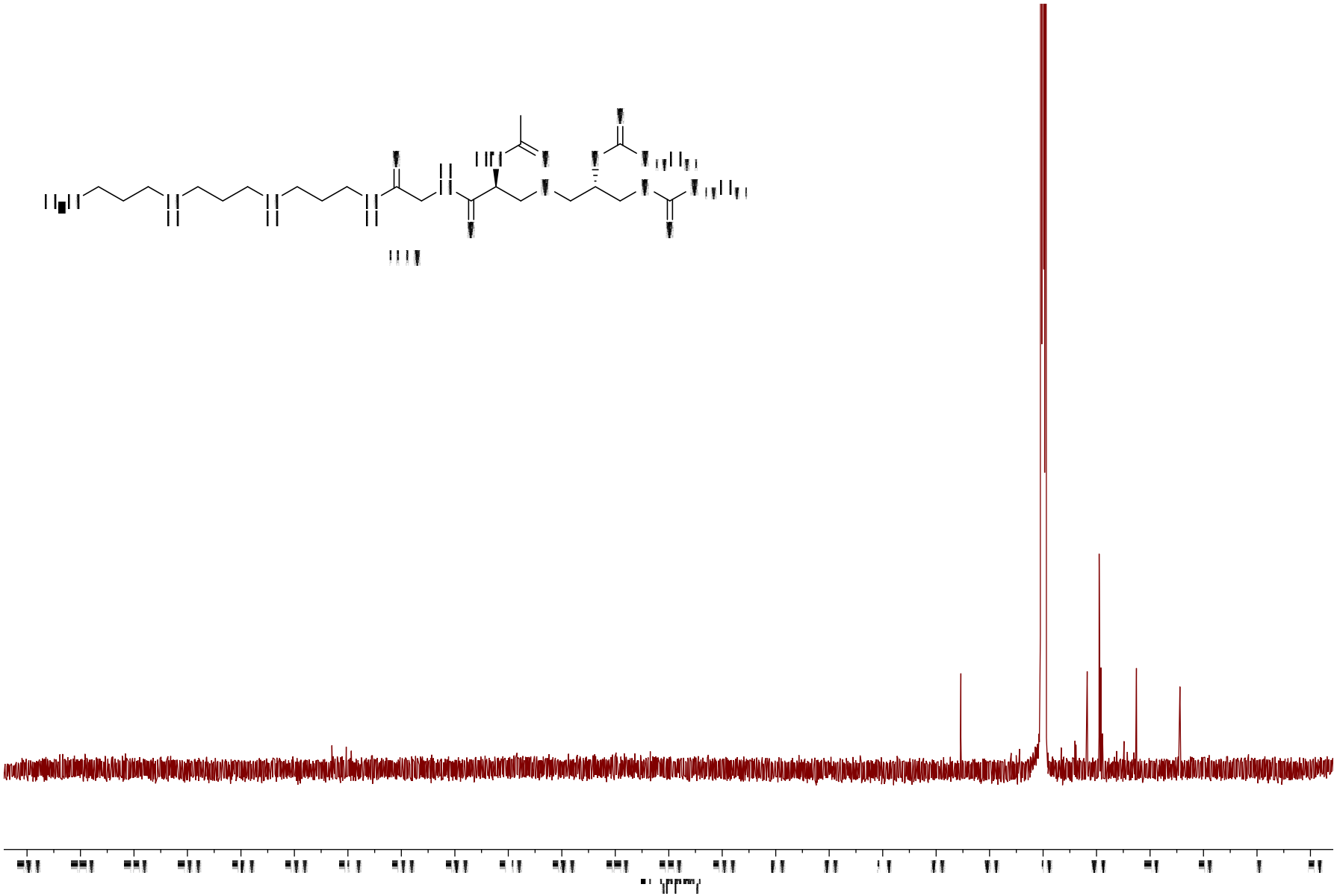

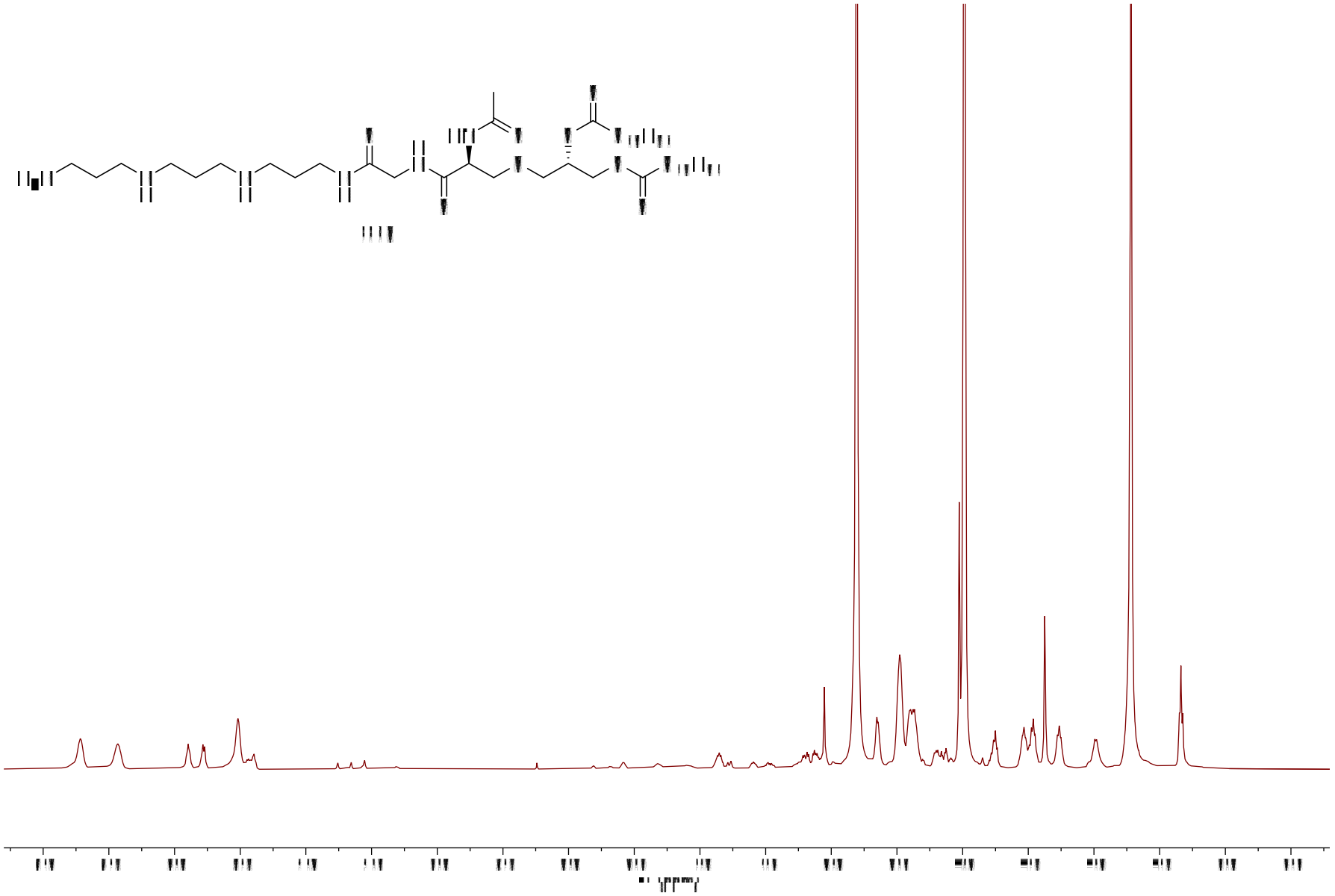

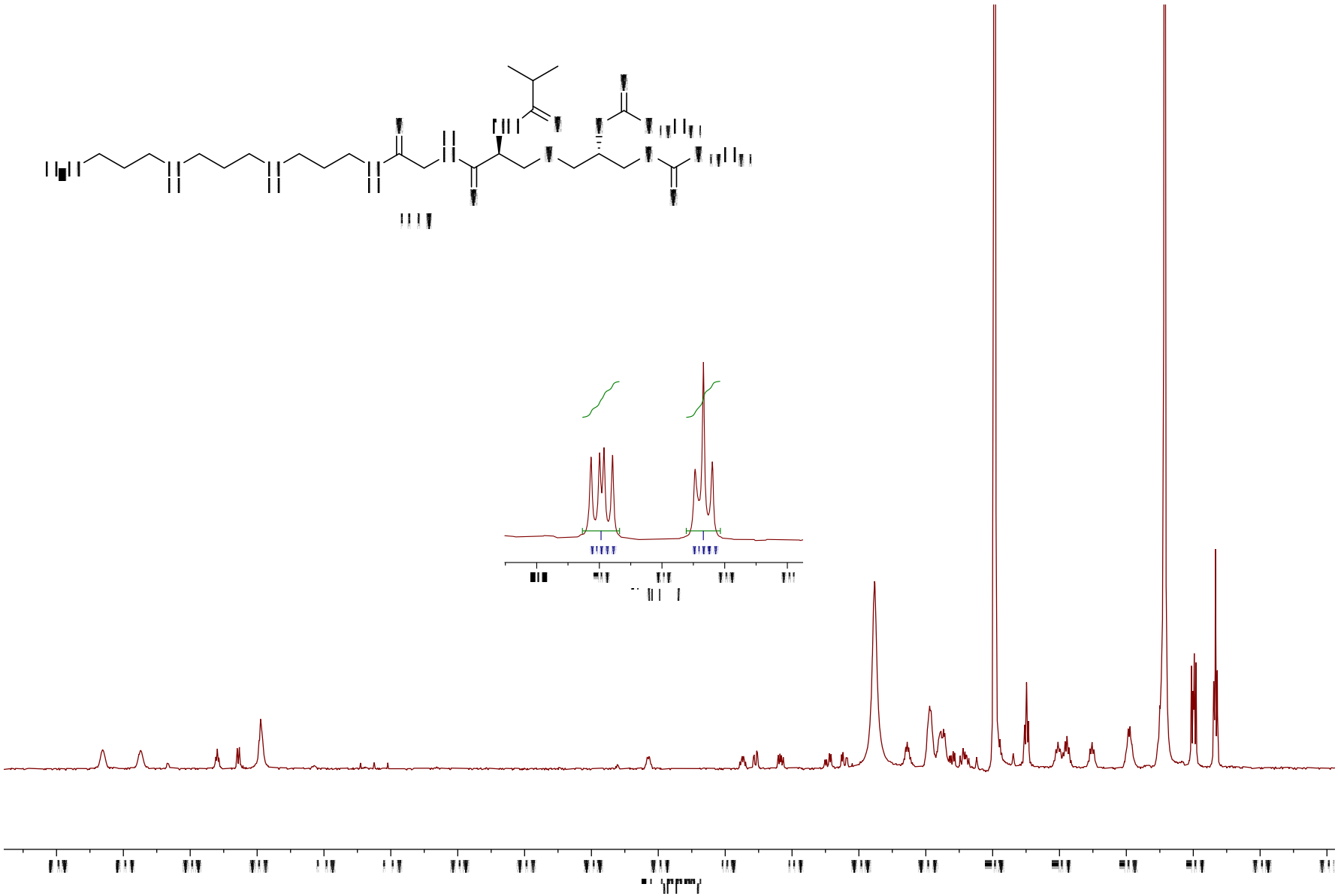

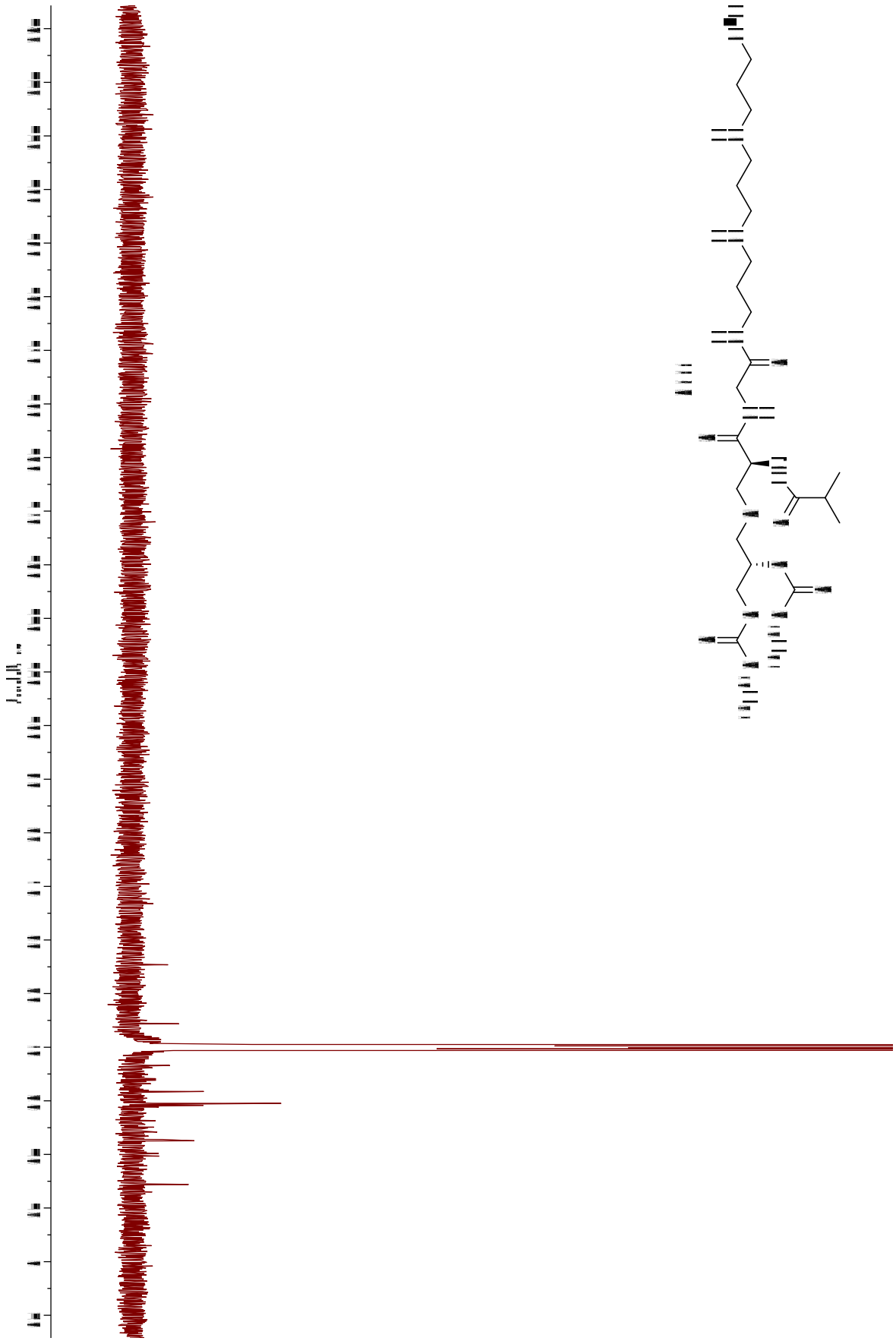

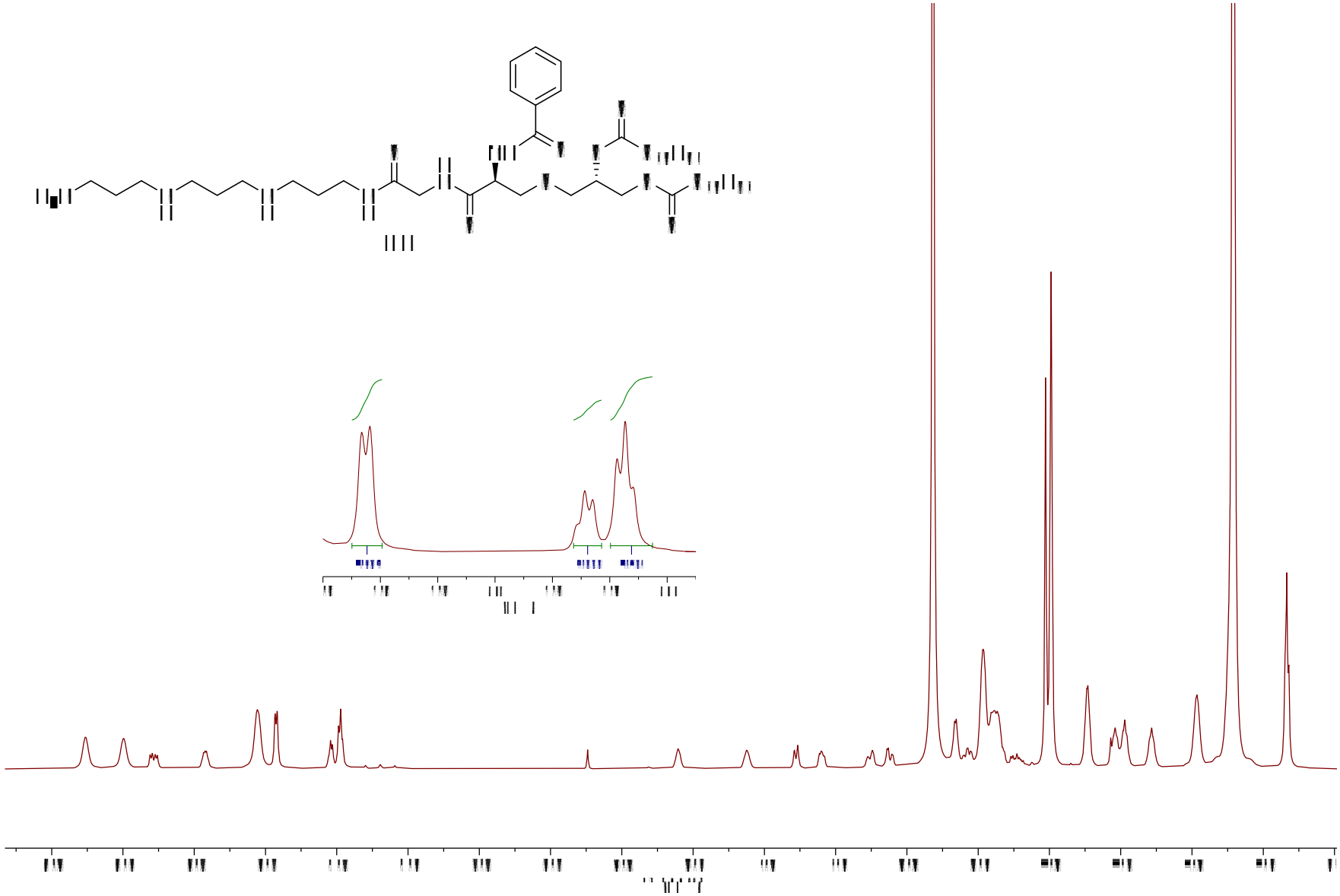

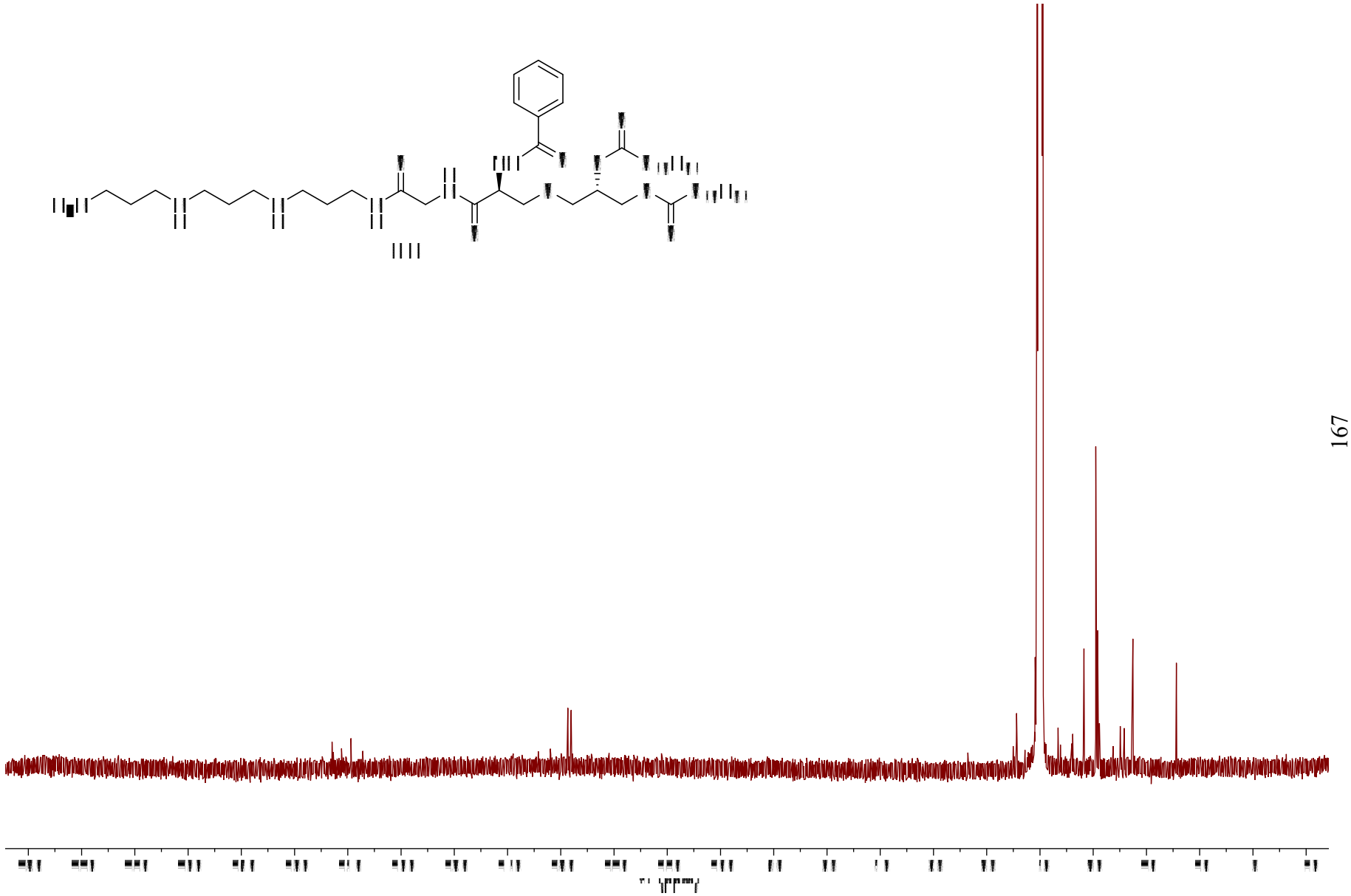

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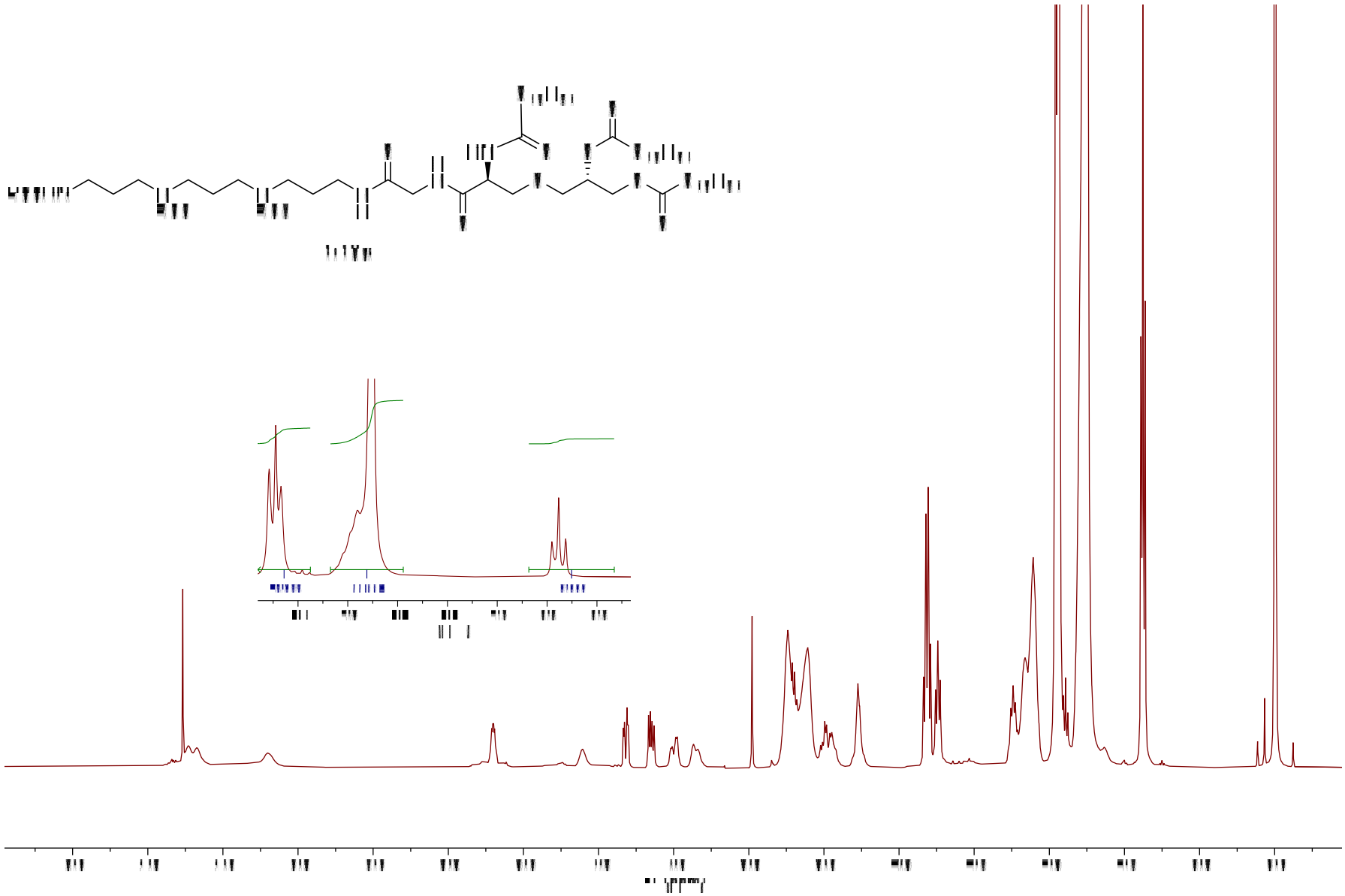

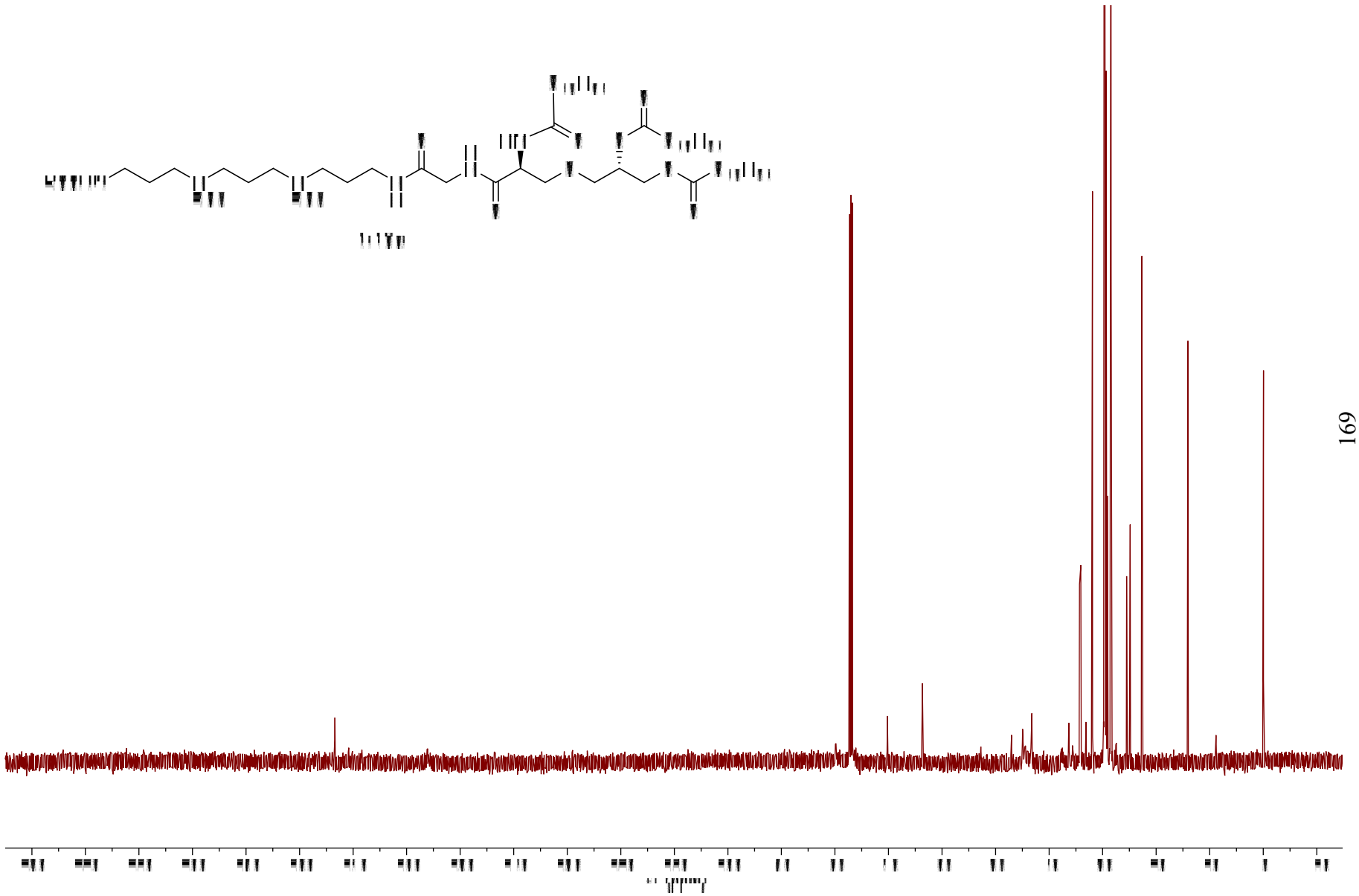
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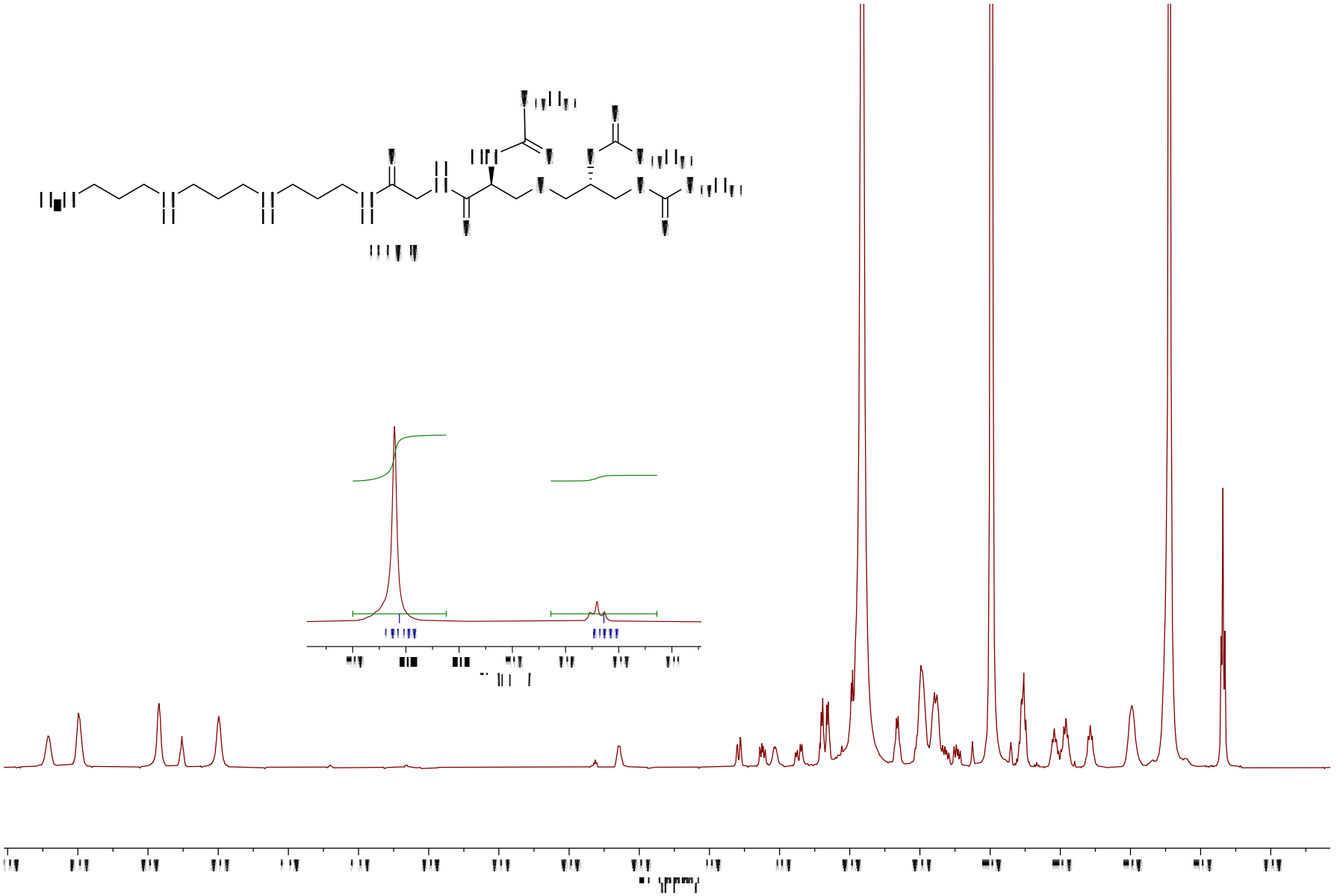

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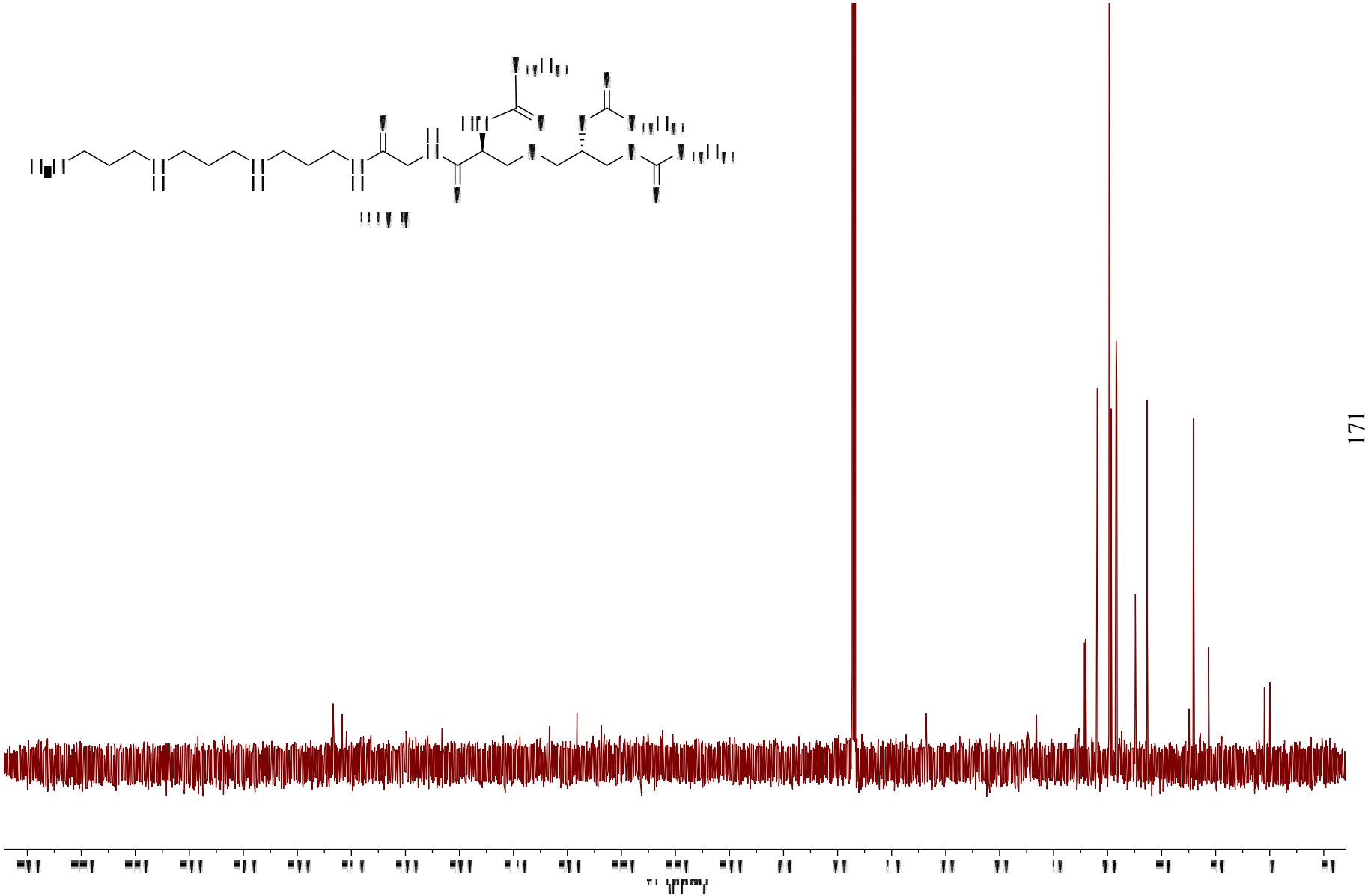

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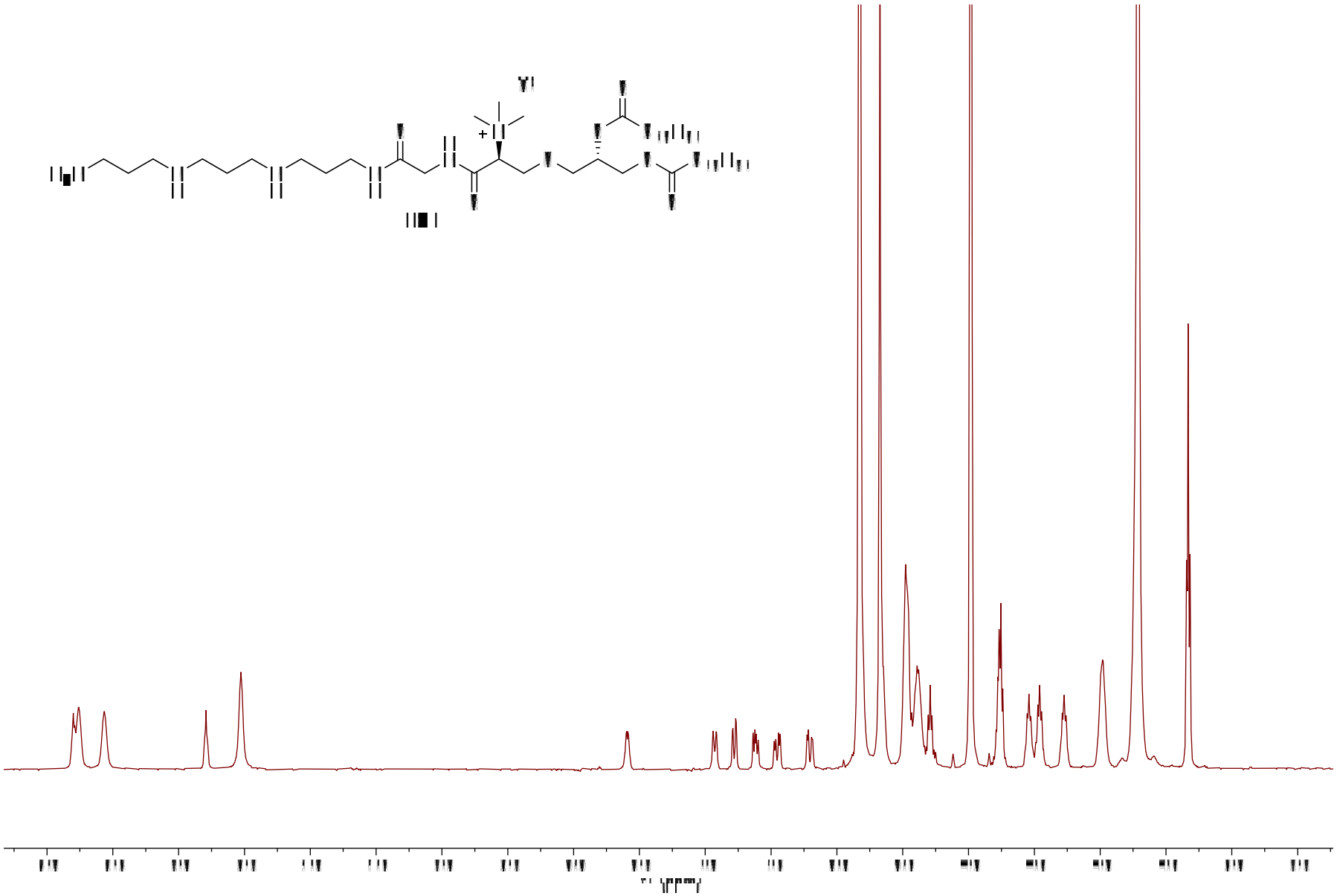

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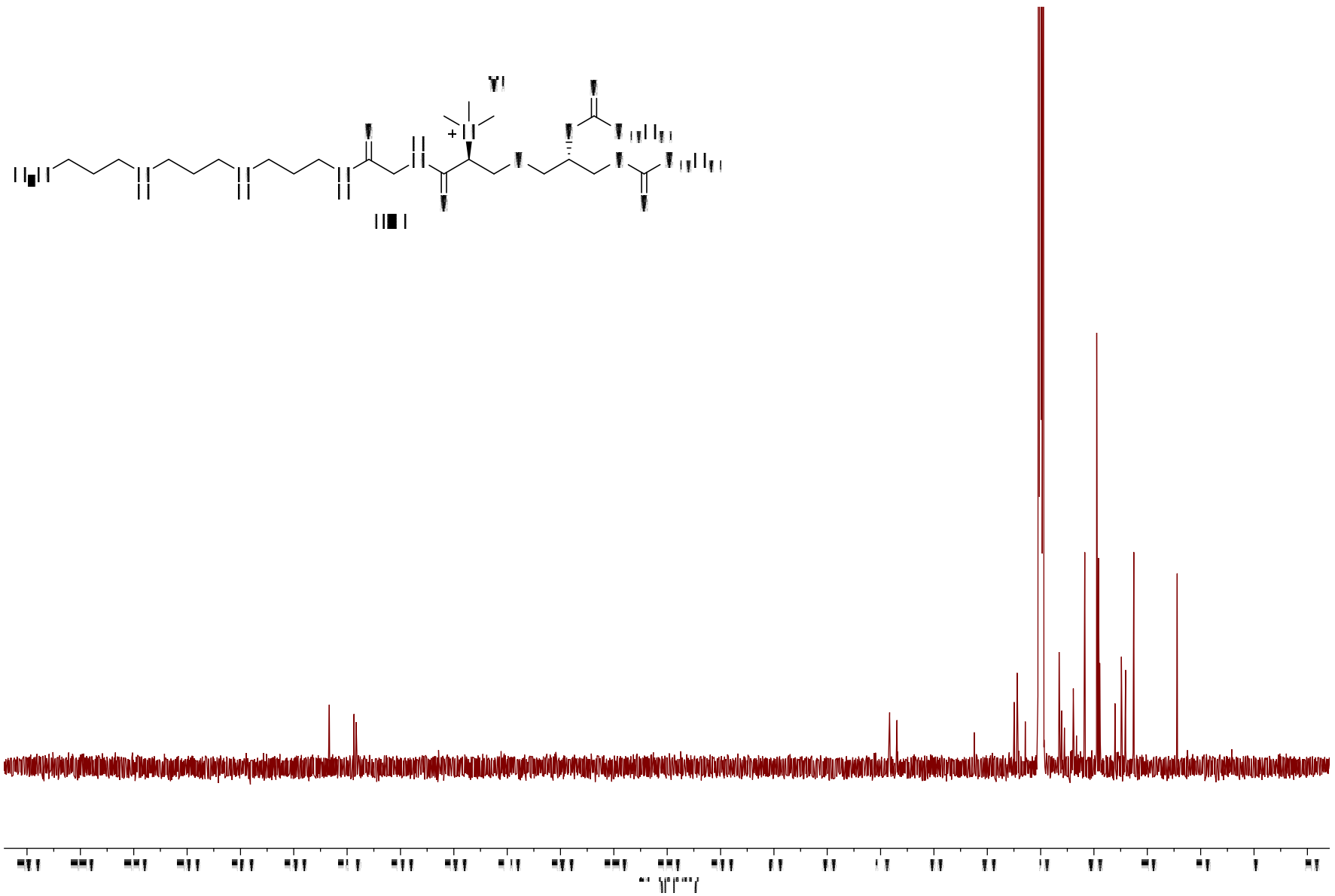

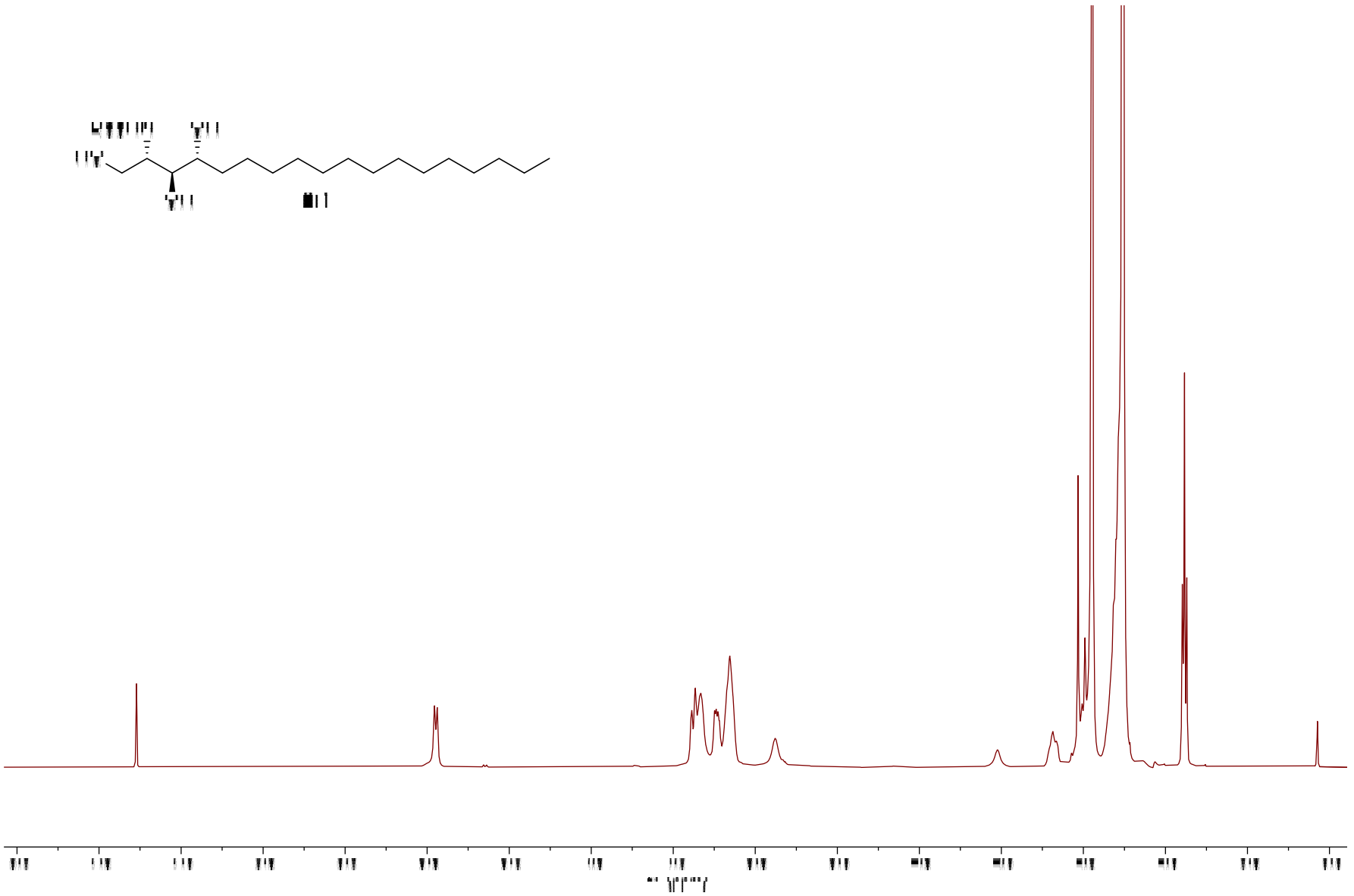

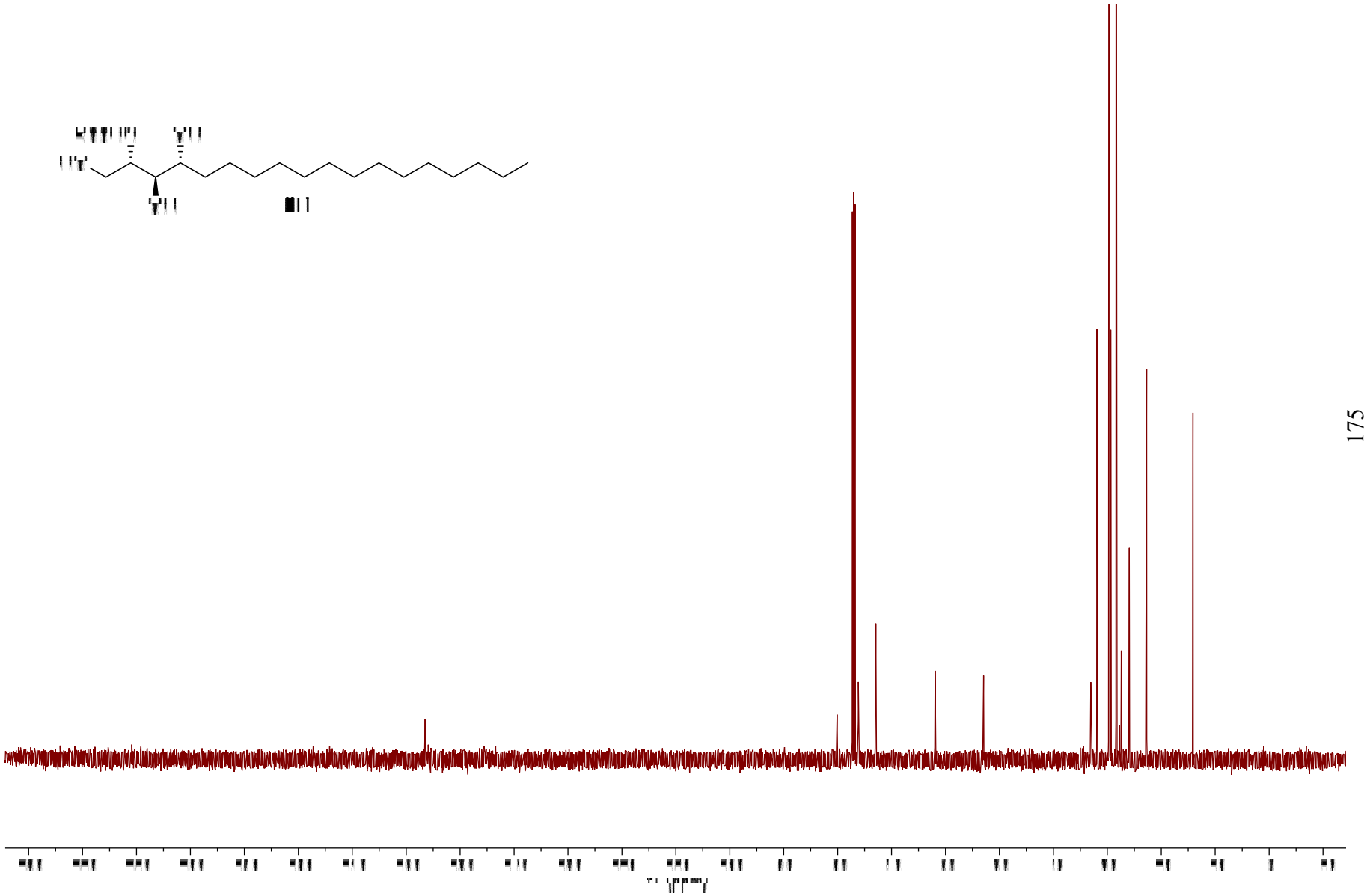
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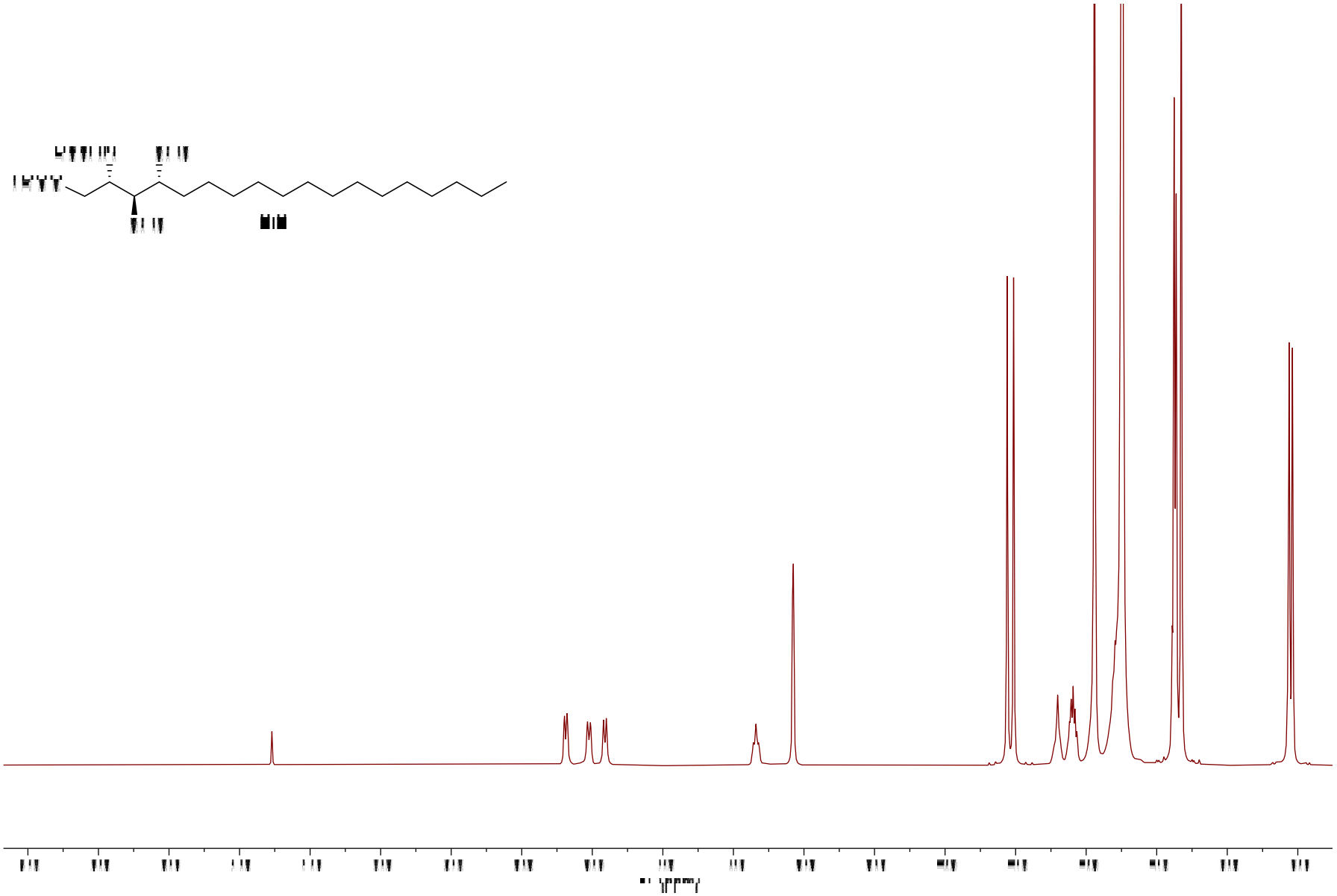
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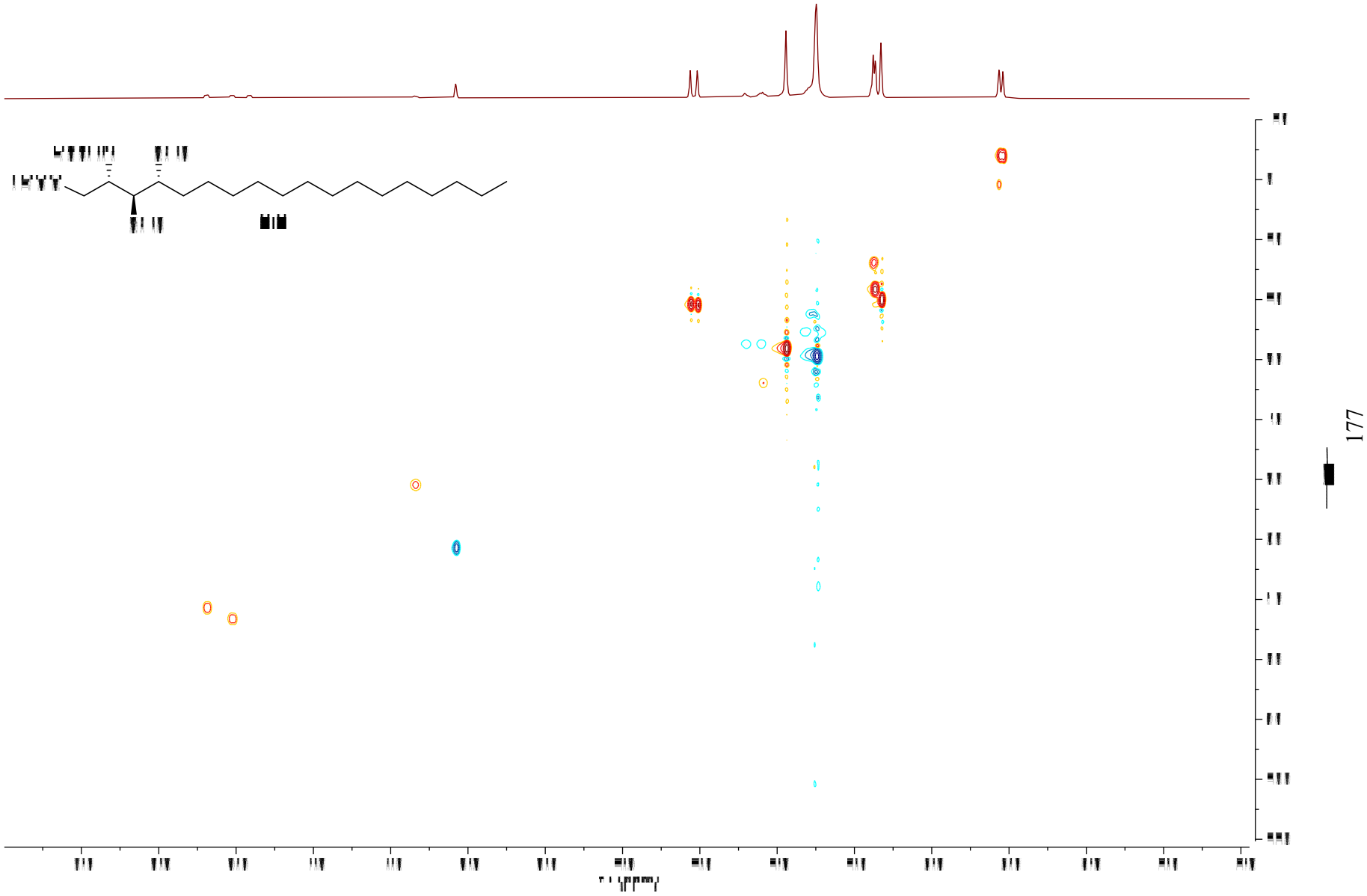
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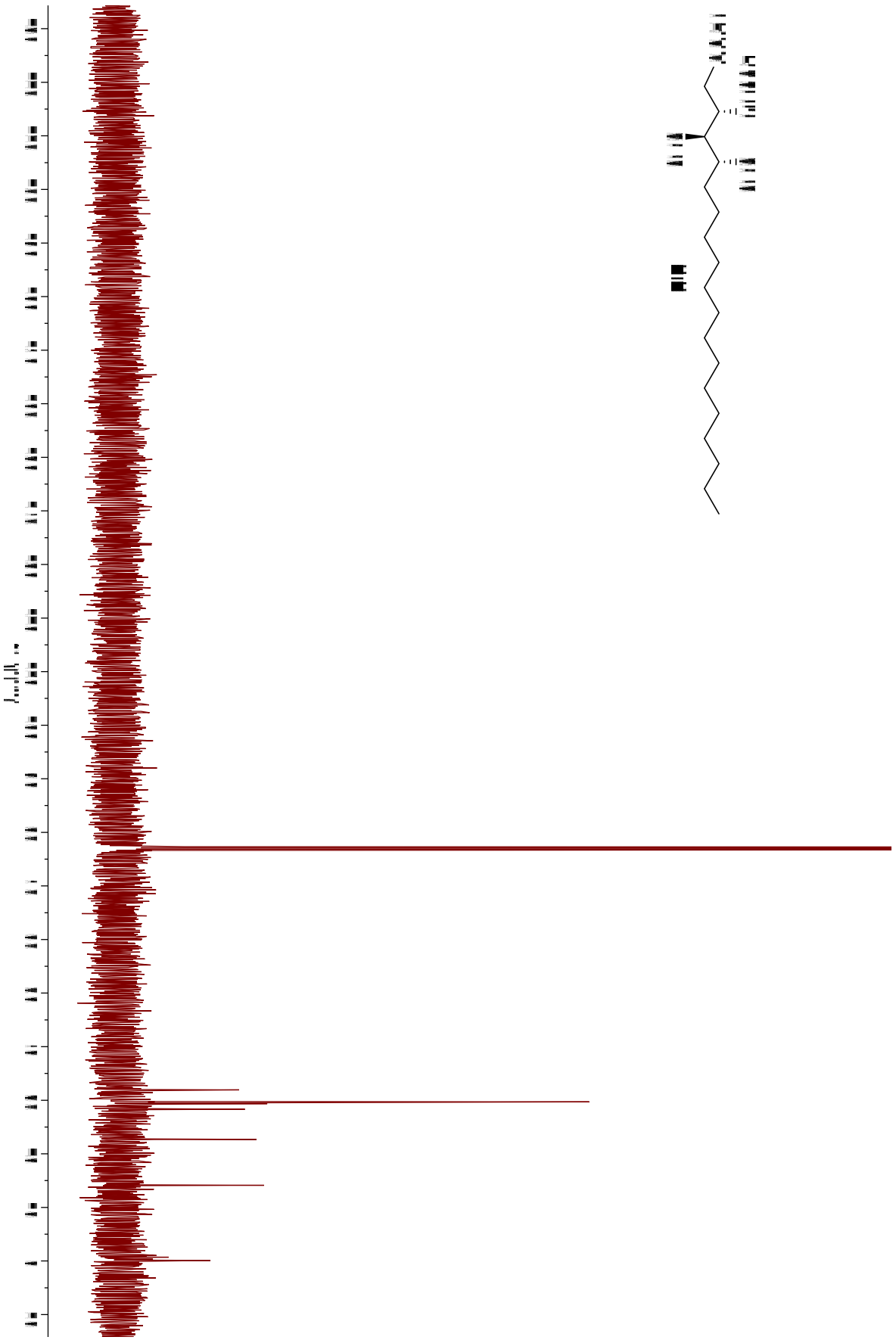

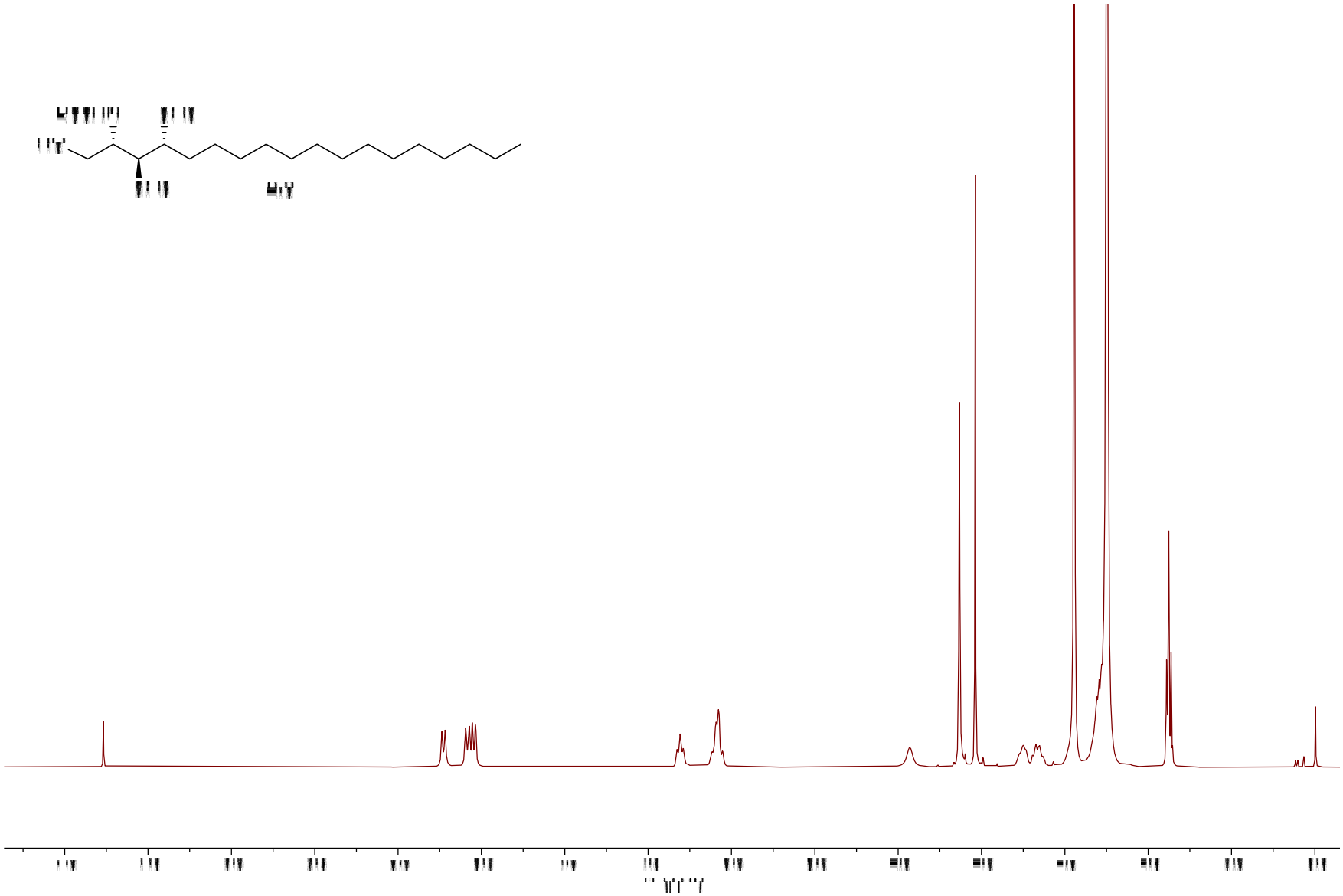

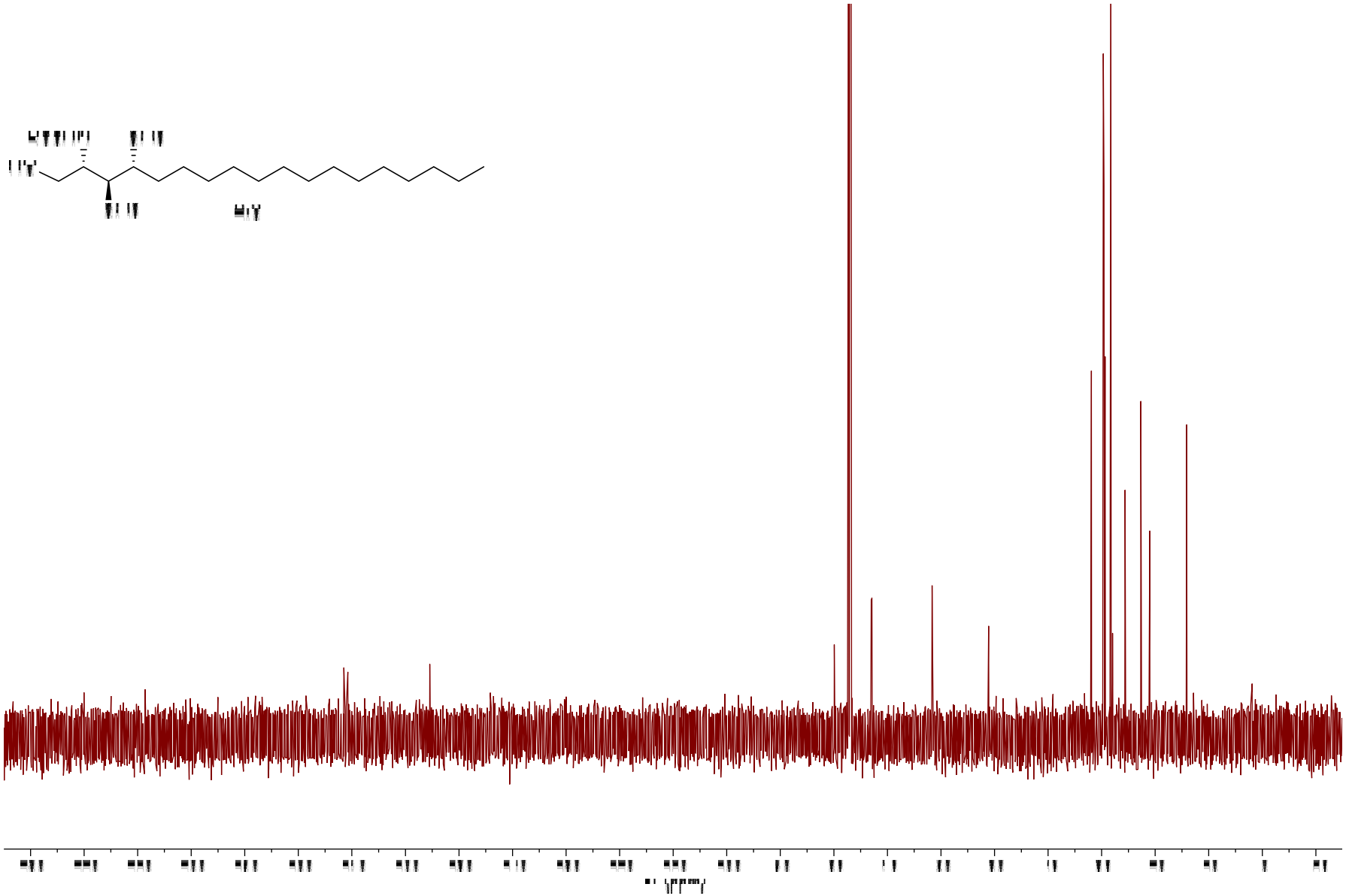
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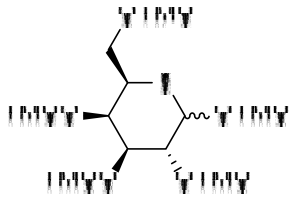

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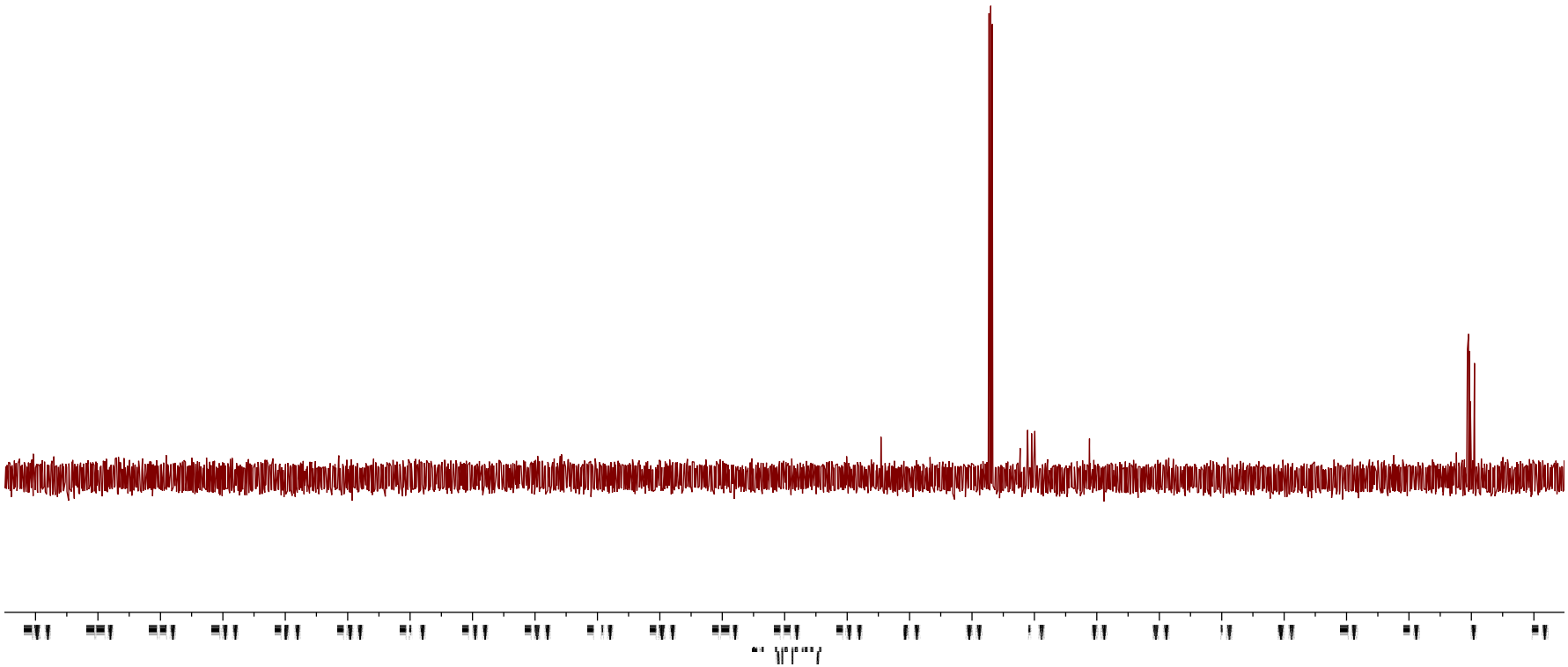

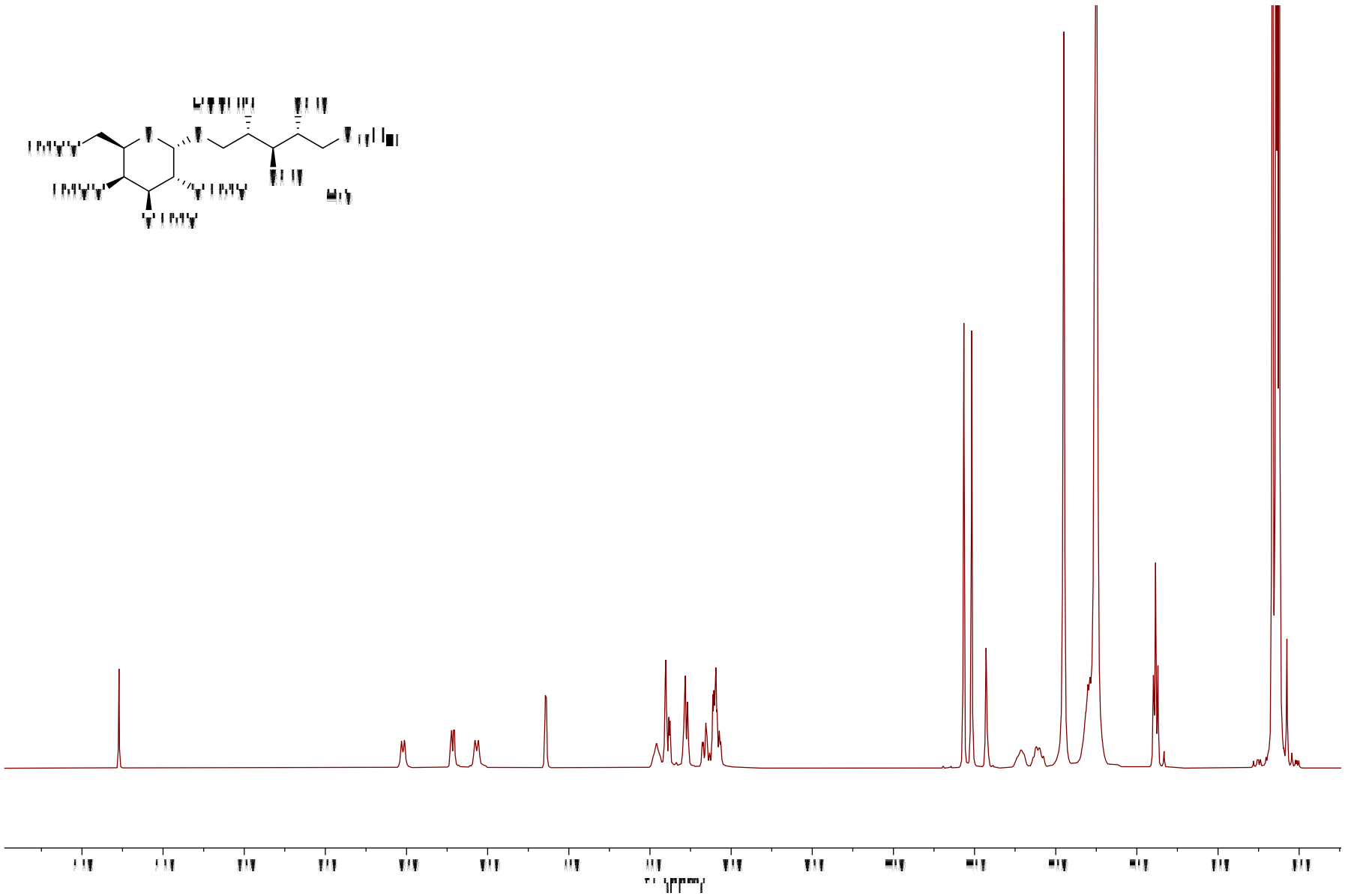
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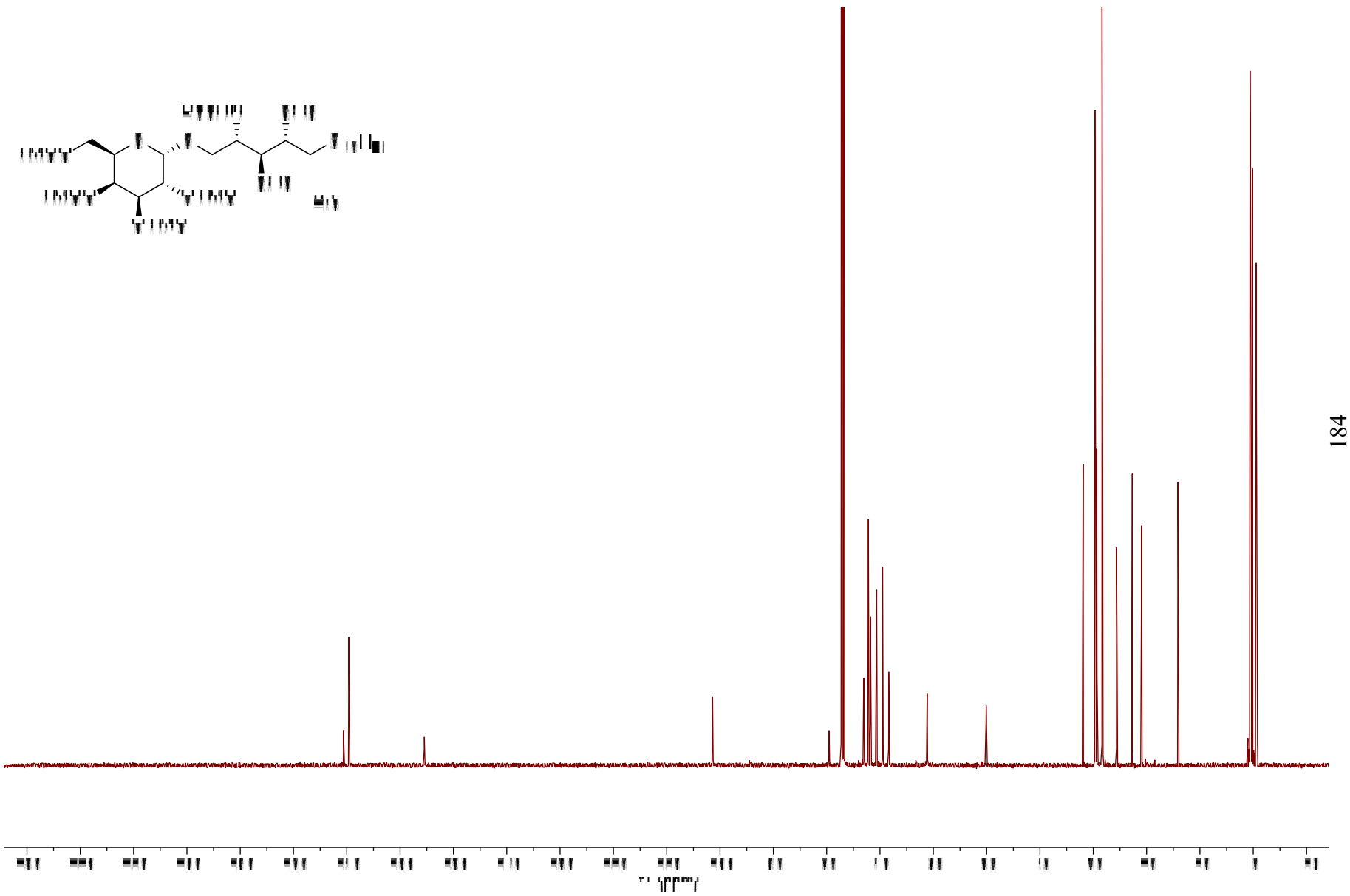
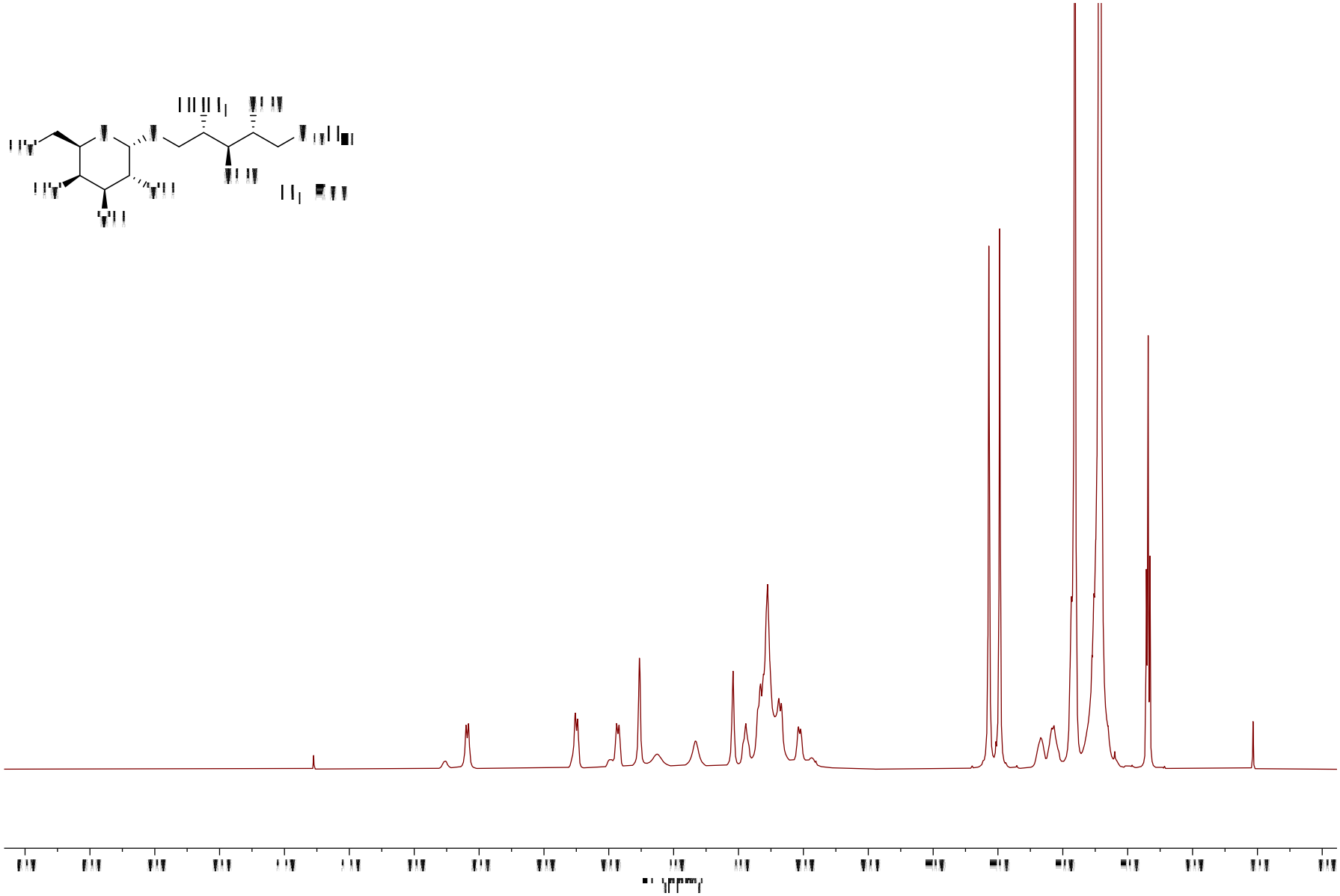


184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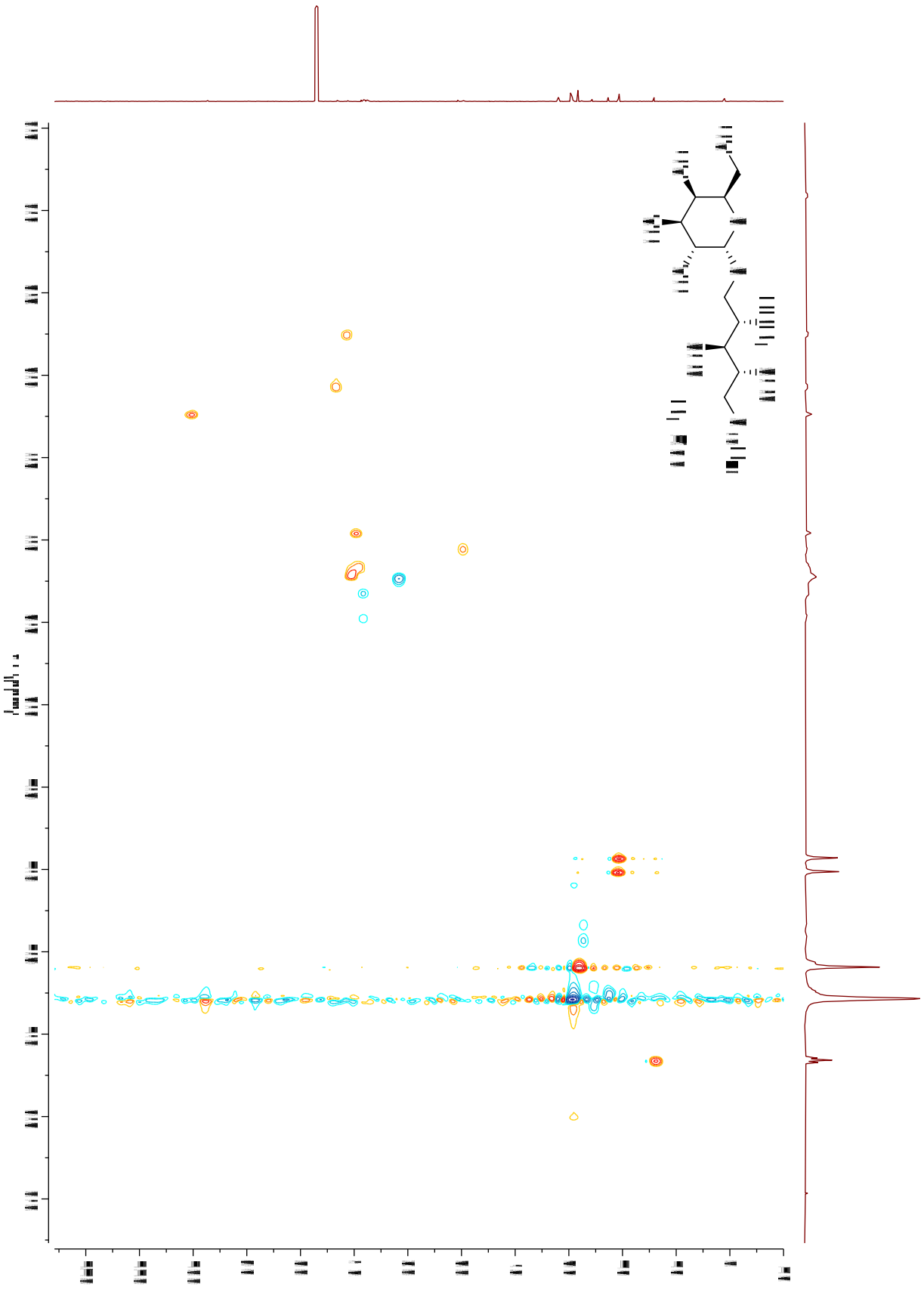
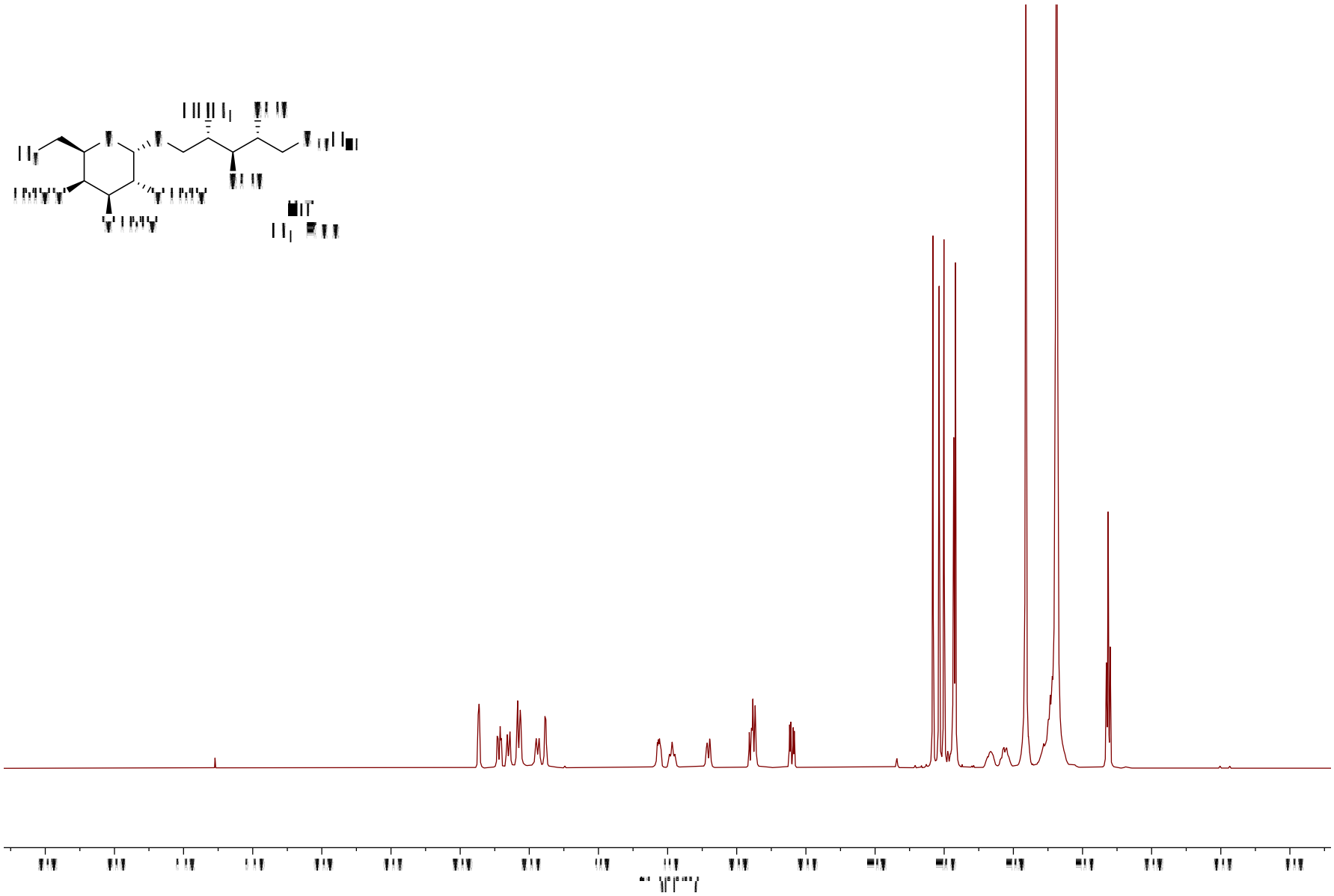
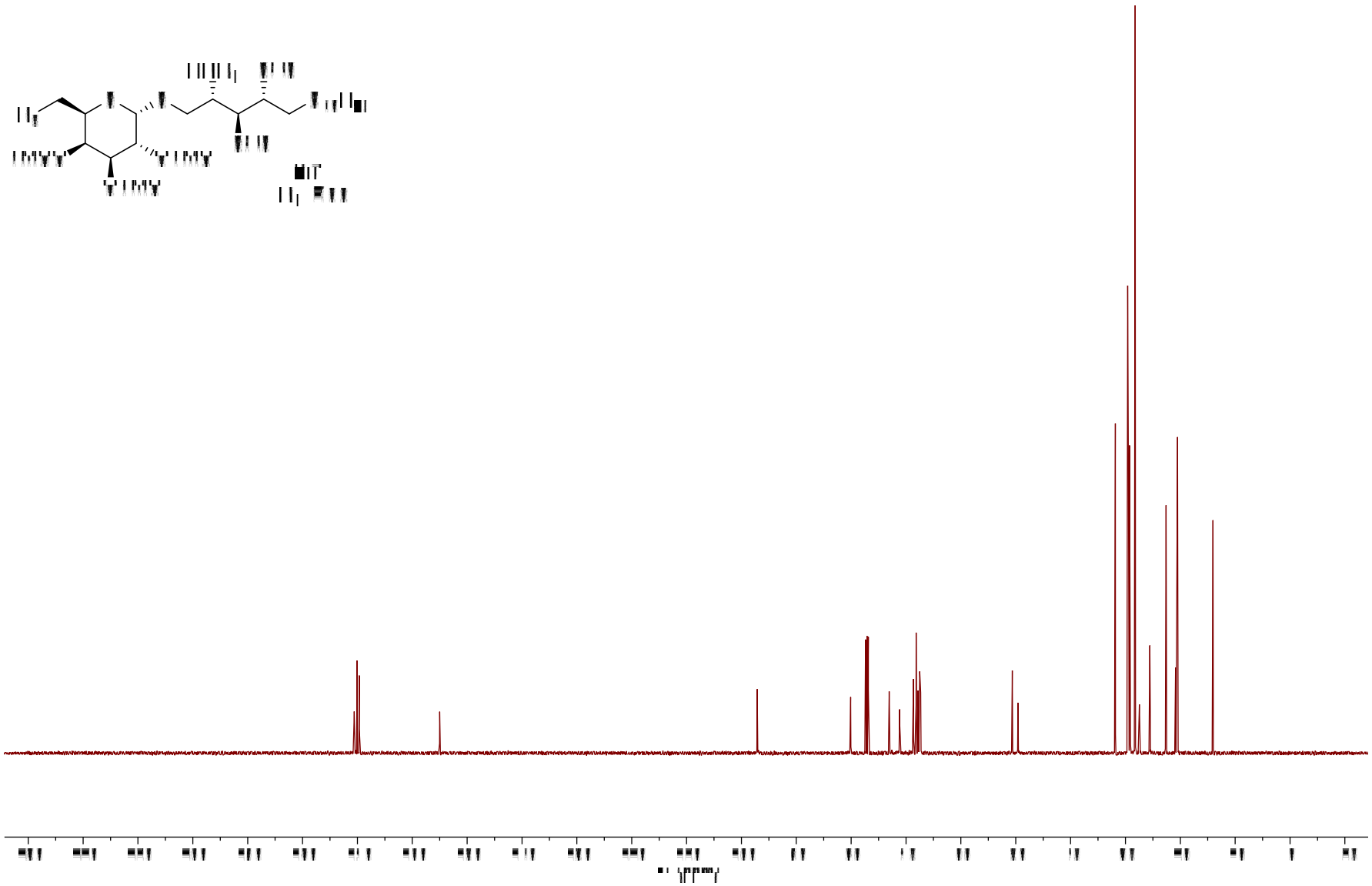
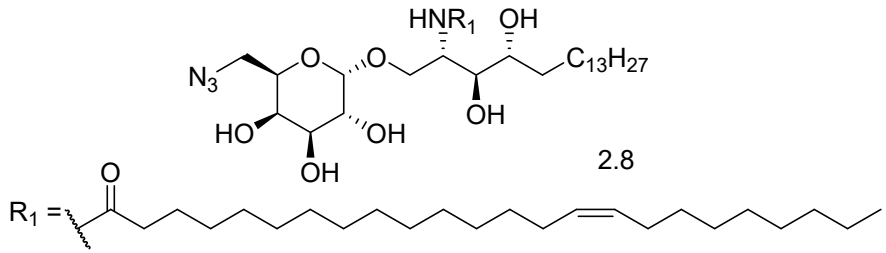




187

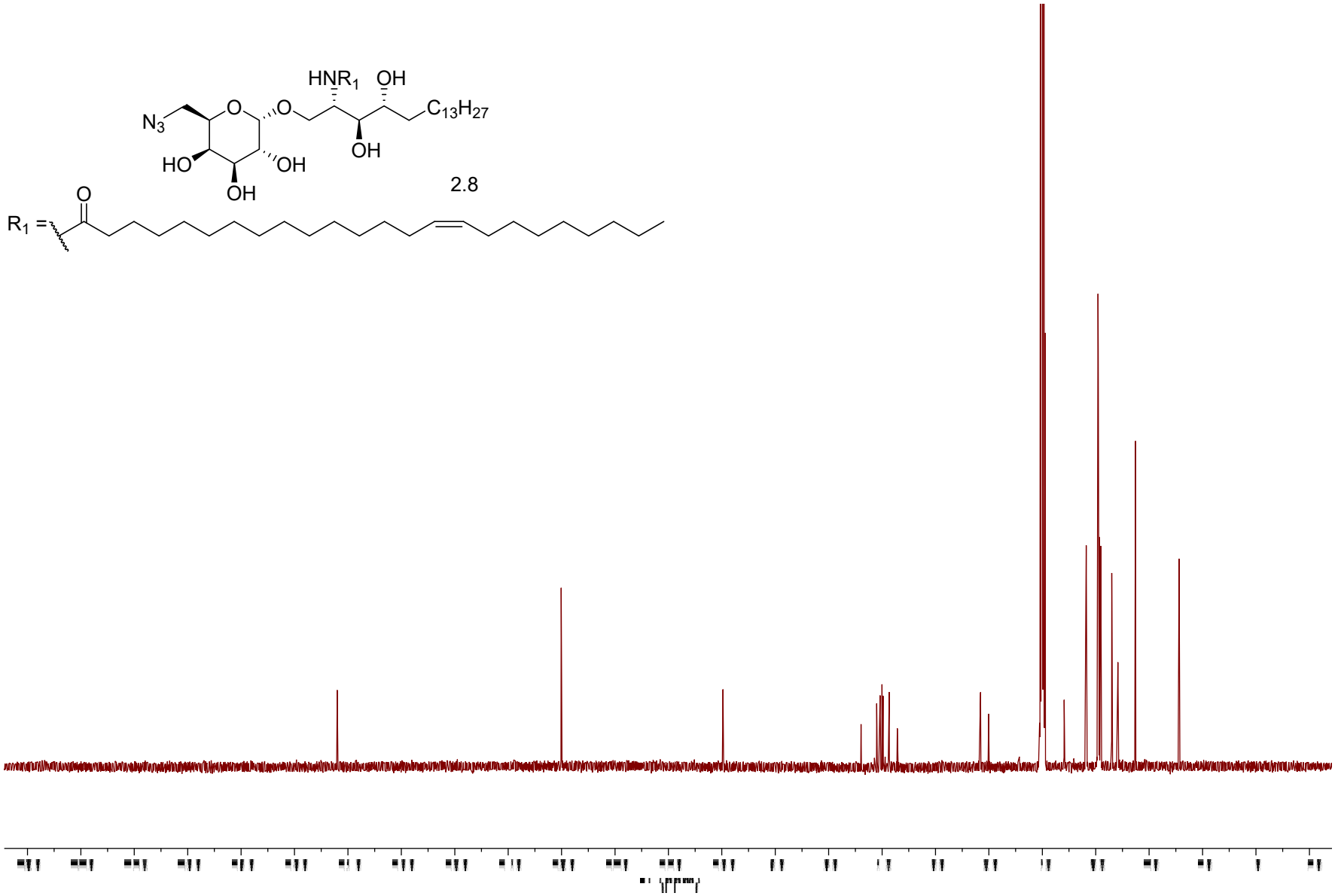


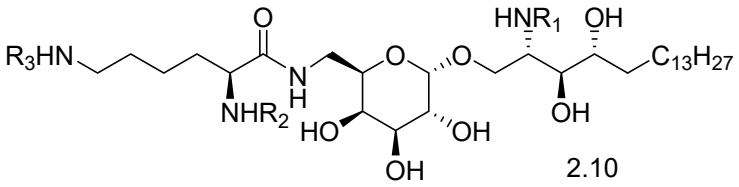
188



2.8

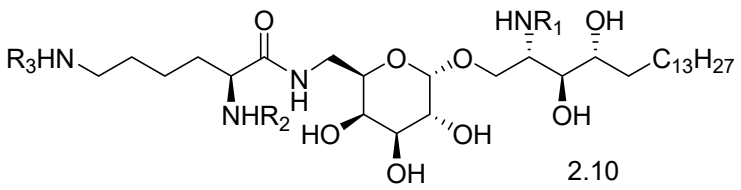
189


HNR1
OH
N3
O
O
C13H27
OH
HO
OH
OH
2.8
O
R1 =



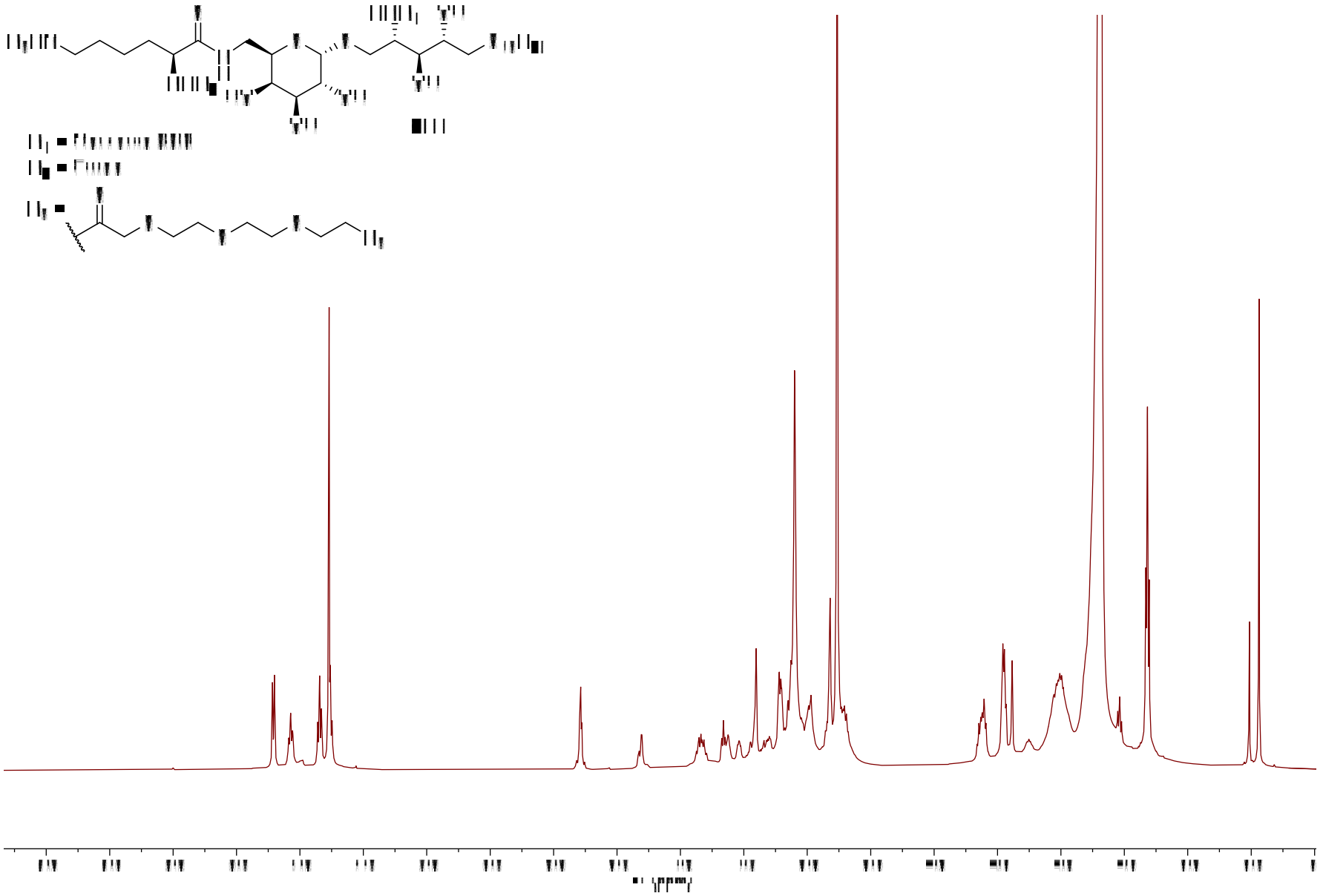
2.10

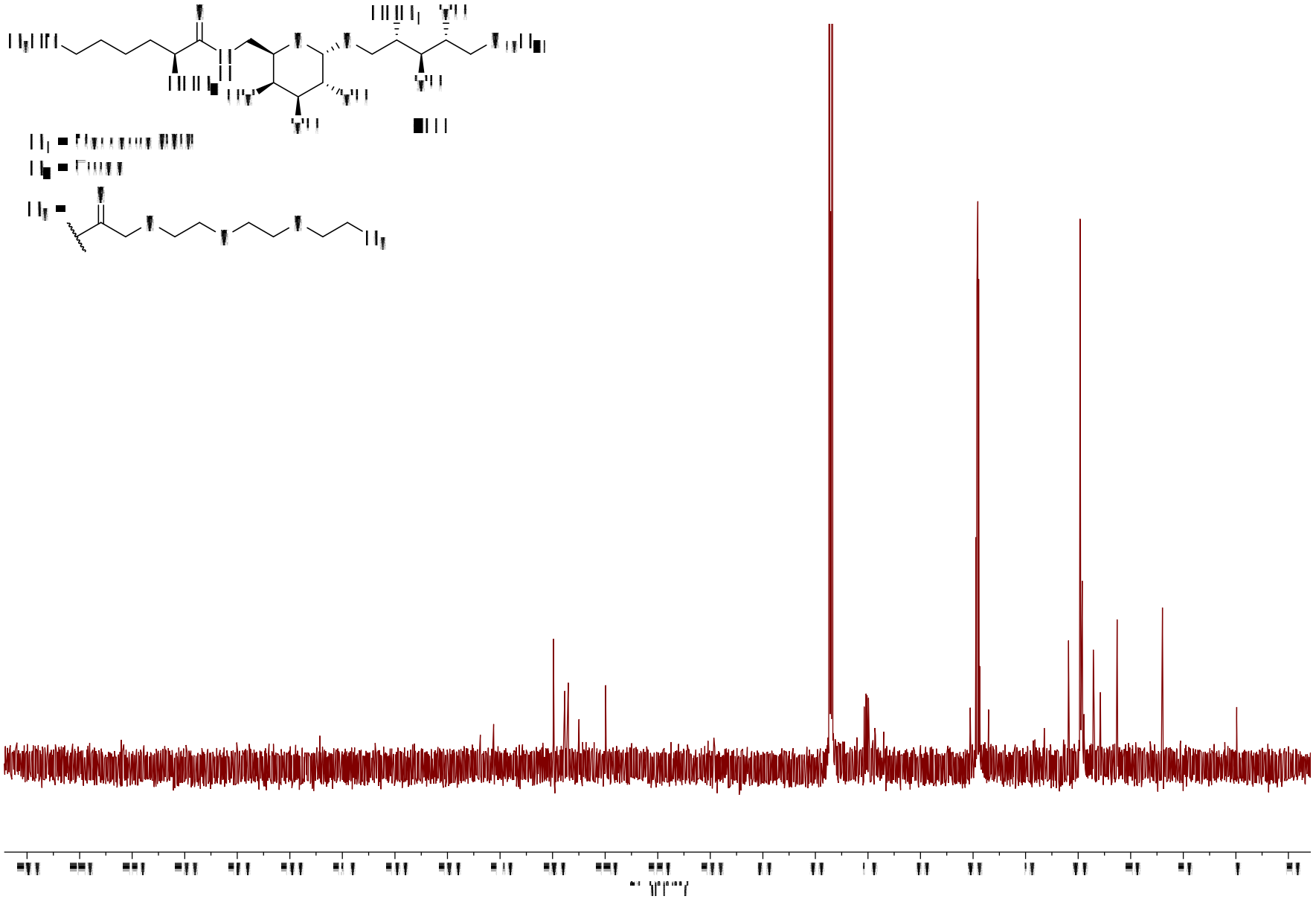
R_1 = Nervonic acid
R_2 = Fmoc
R_3 = Boc

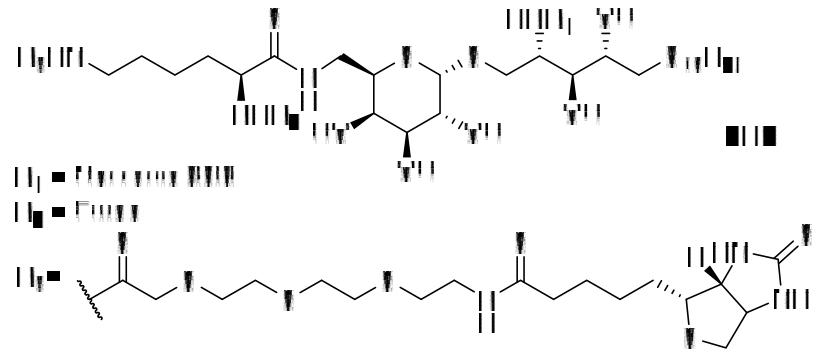


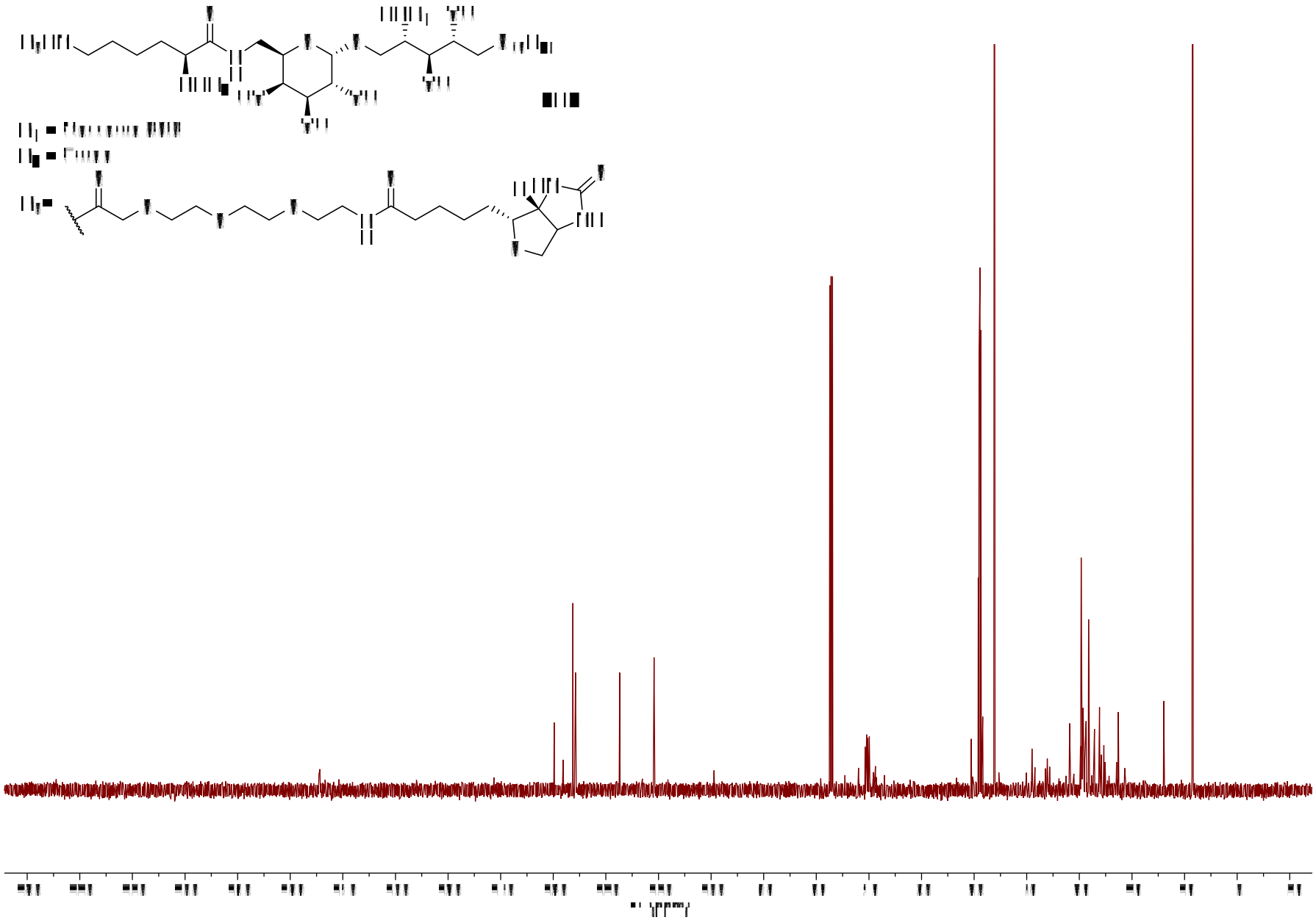
2.10

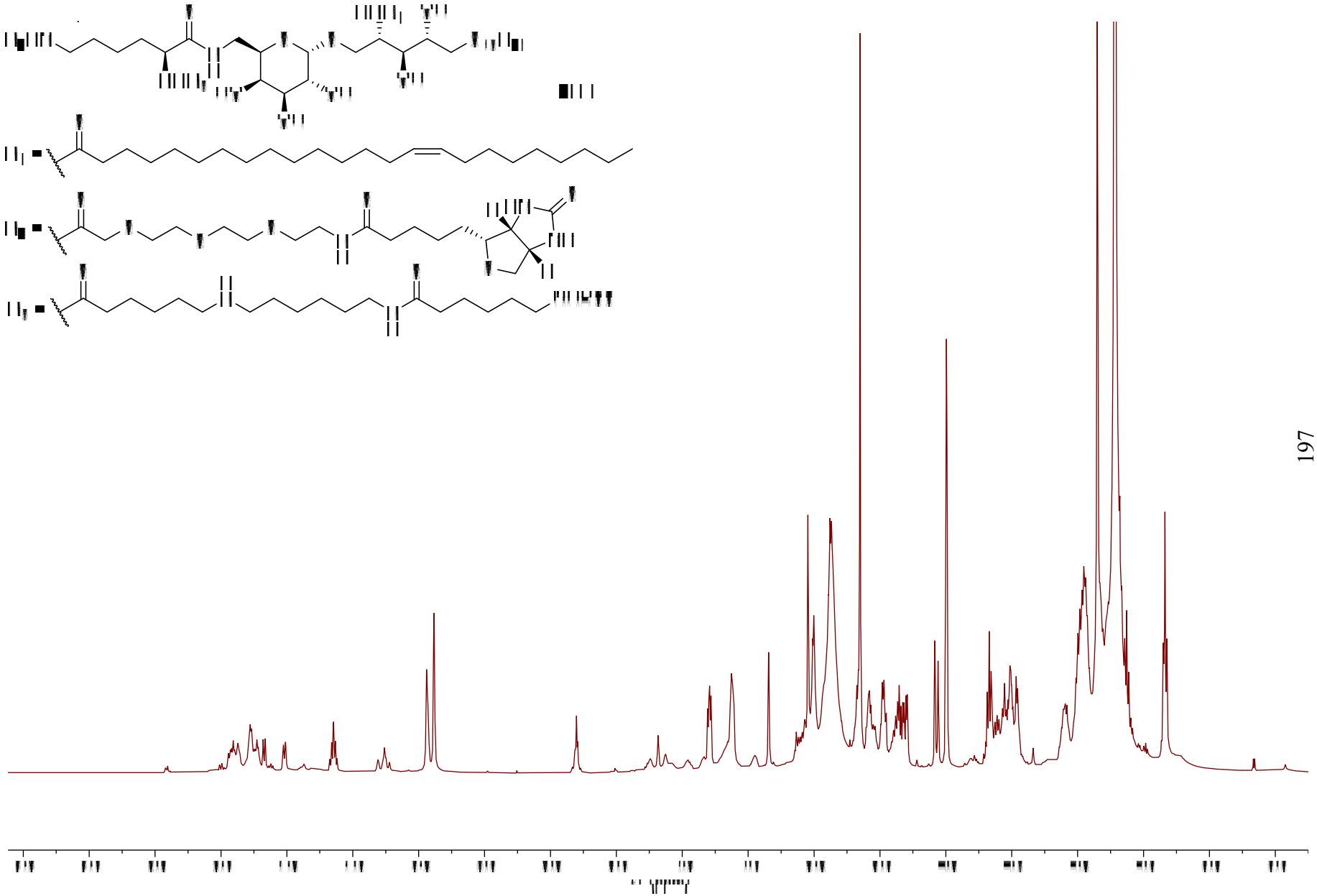
R_1 = Nervonic acid
R_2 = Fmoc
R_3 = Boc

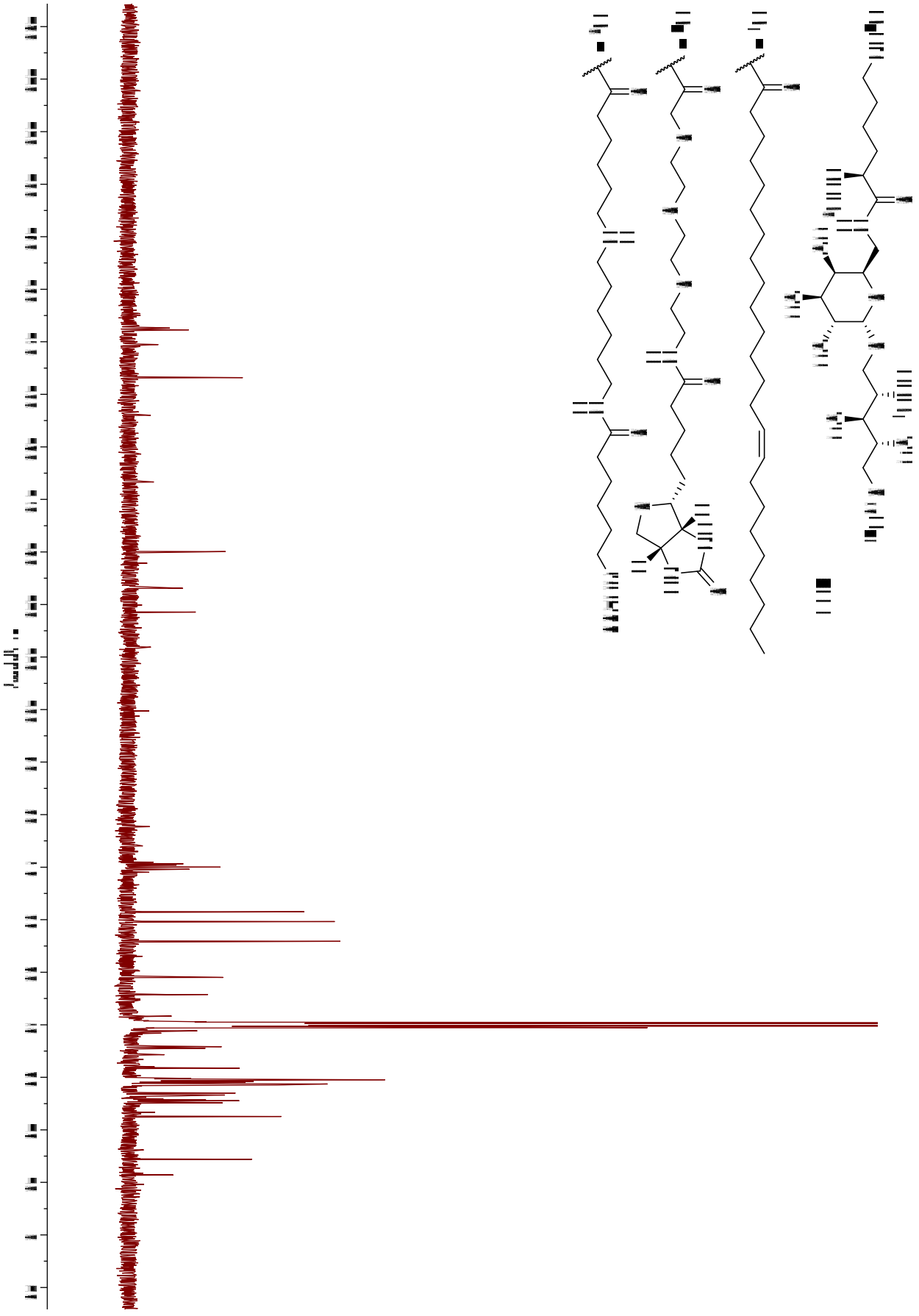


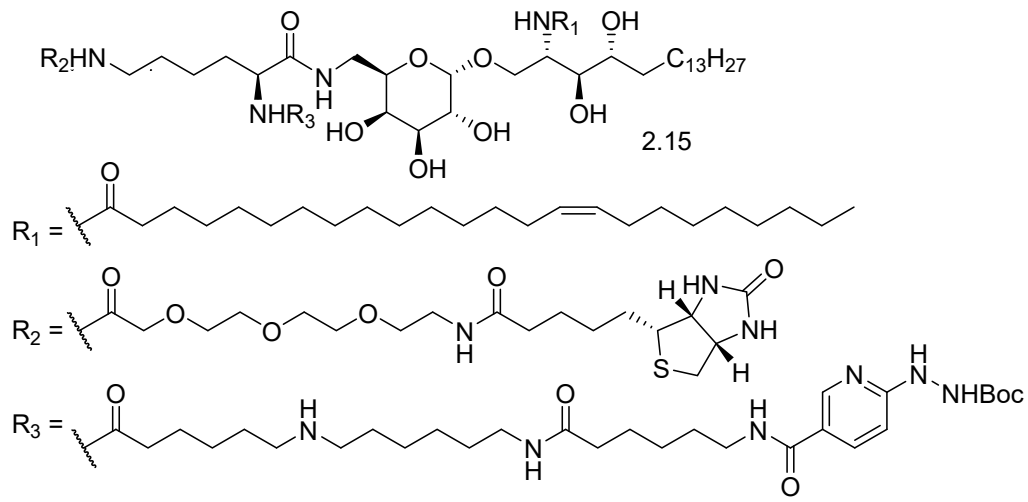


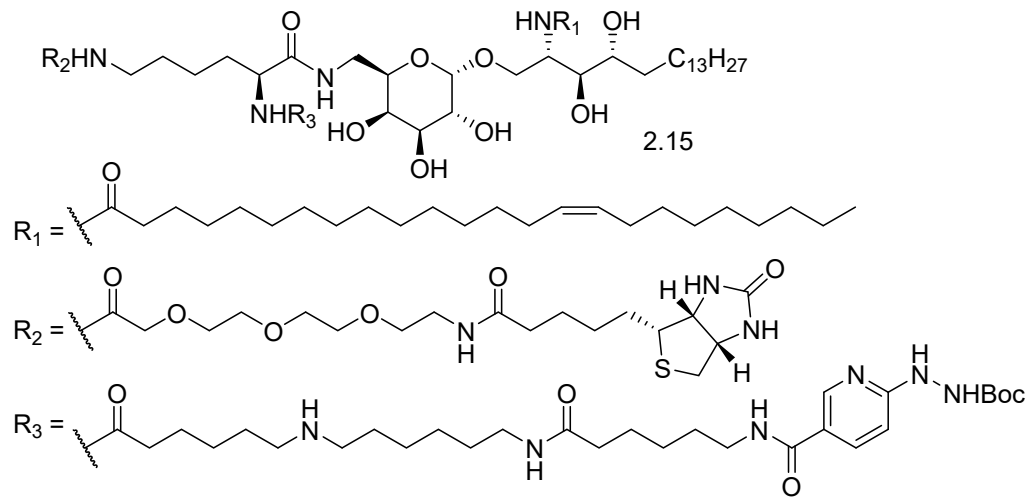




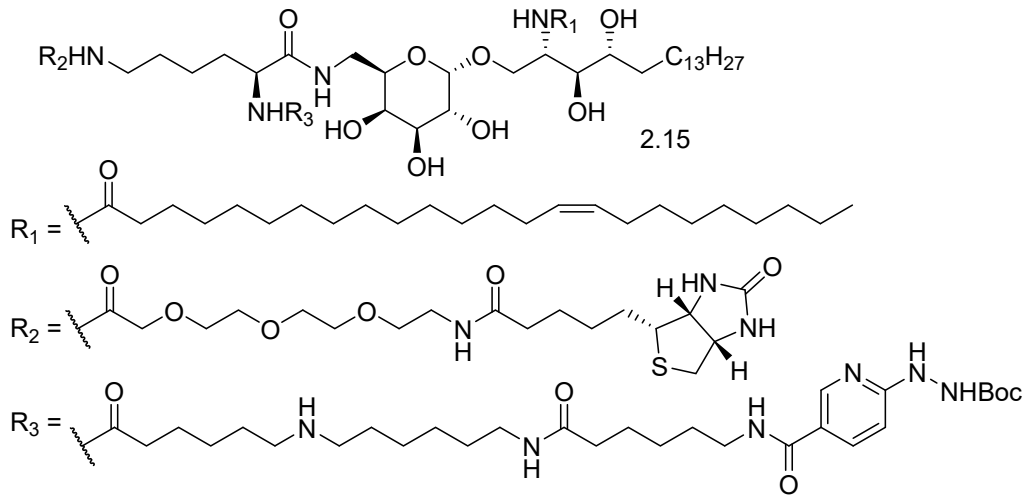

197



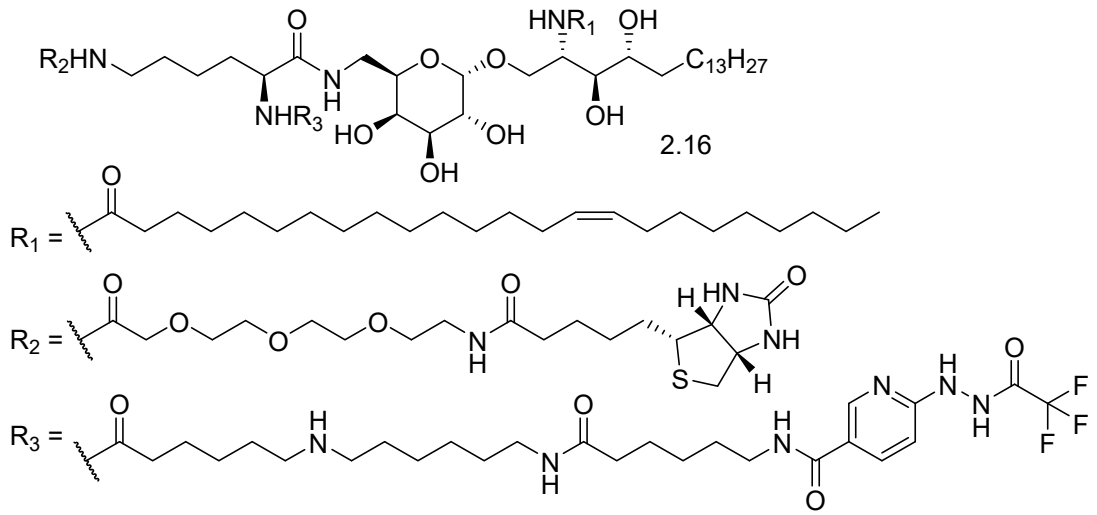

R2HN
O
N
H
NHR3
HO
OH
OH
HNR1
OH
O
O
OH
C13H27
2.15
R1 =
O
R2 =
O
O
O
O
O
N
H
H
HN
O
NH
S
H
R3 =
O
H
N
O
N
H
H
N
N
H
N
NHBoc
O


R2HN
O
N
H
NHR3
HO
O
HNR1
OH
C13H27
OH
OH
OH
2.15
R1 =
R2 =
R3 =
HN
NH
S
H
N
H
N
NHBoc

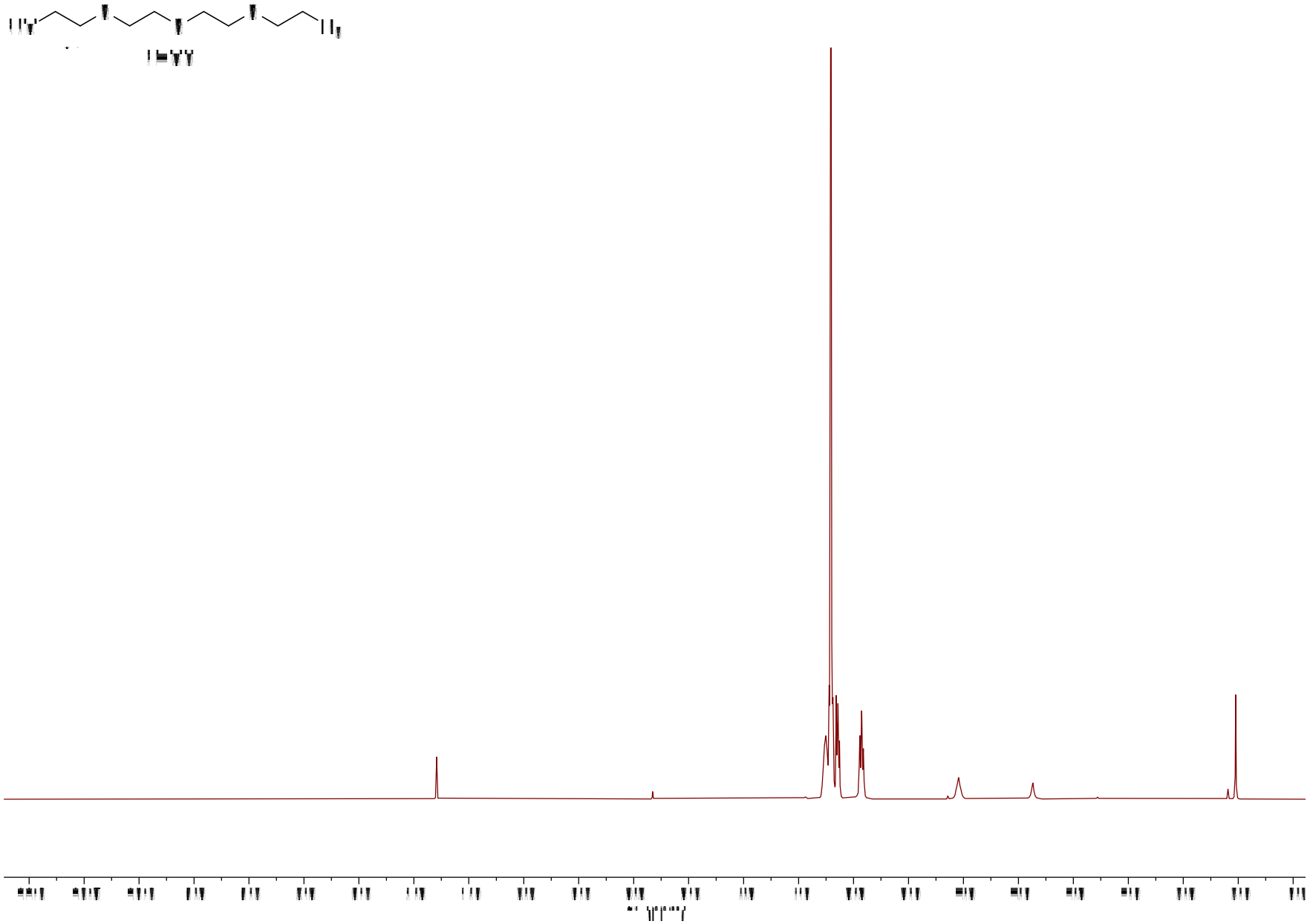
200


R2HN
O
N
H
NHR3
HO
O
OH
OH
O
HNR1
OH
OH
C13H27
2.15
R1 =
O
R2 =
O
O
O
O
N
H
O
H
HN
O
NH
H
S
R3 =
O
H
N
O
N
H
H
N
N
H
N
NHBoc
O

201

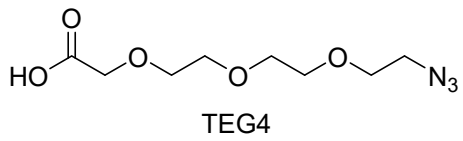


202



203

204



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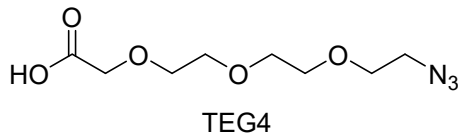

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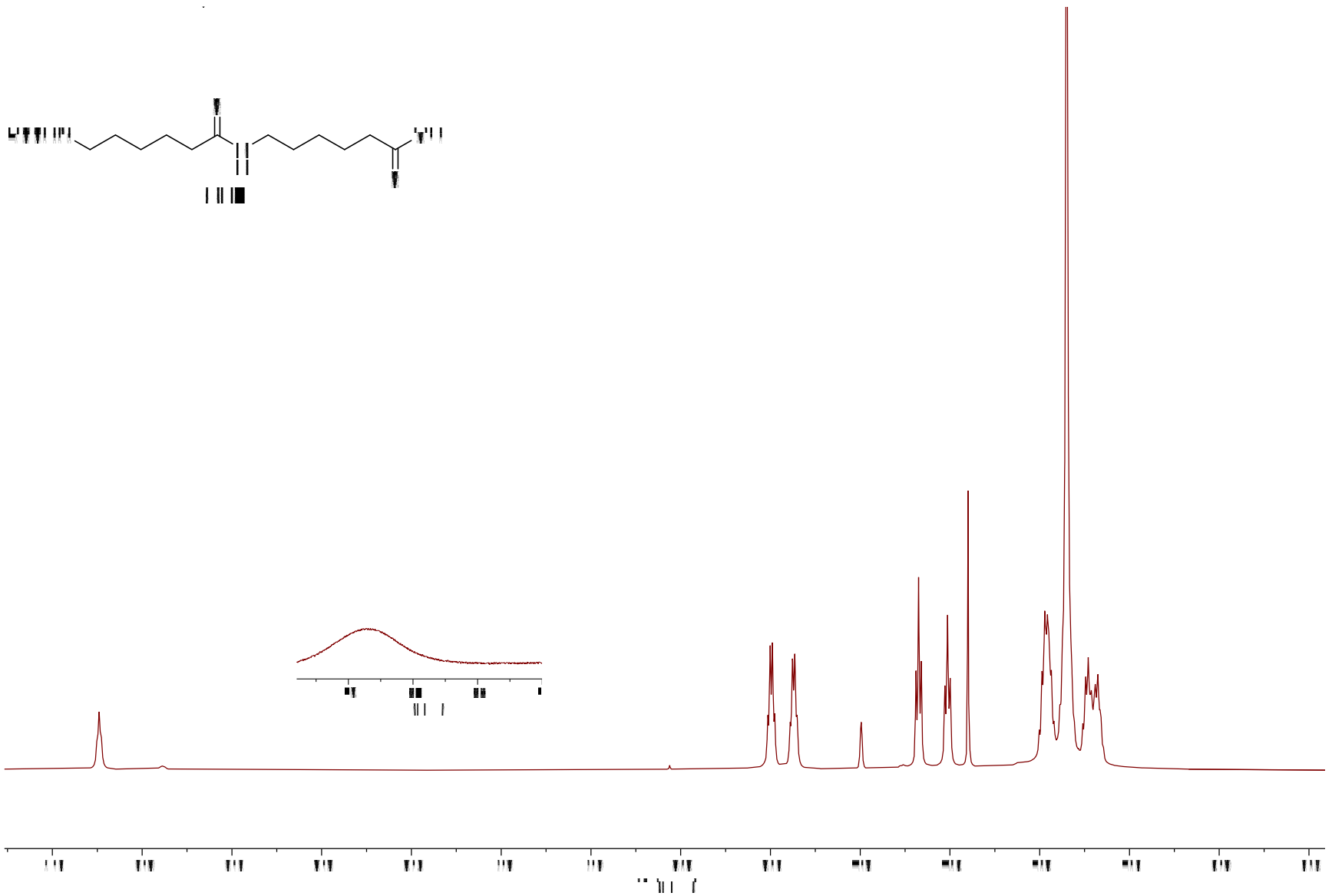

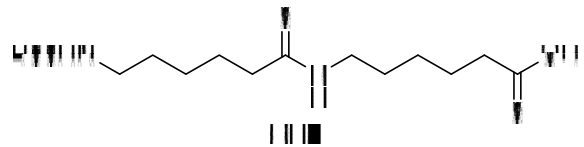

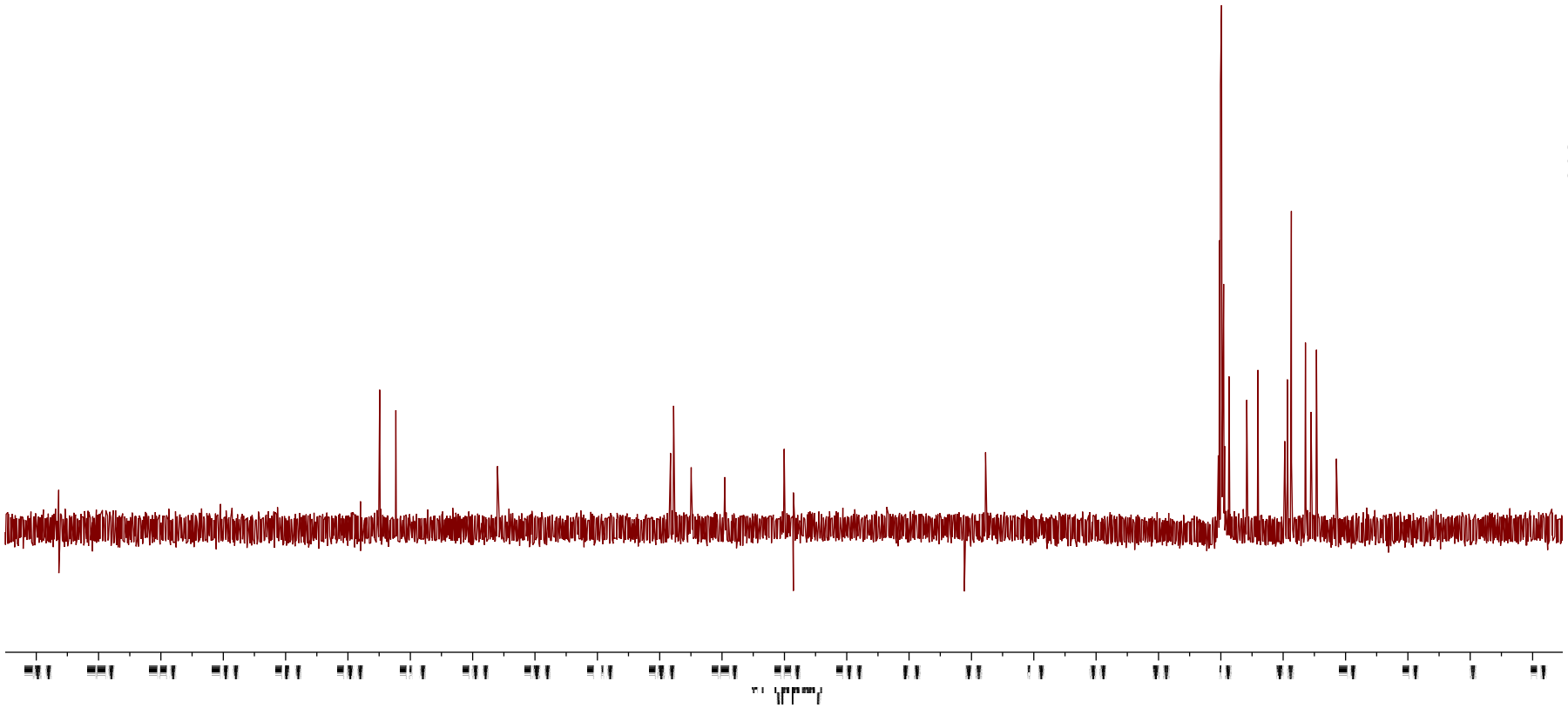

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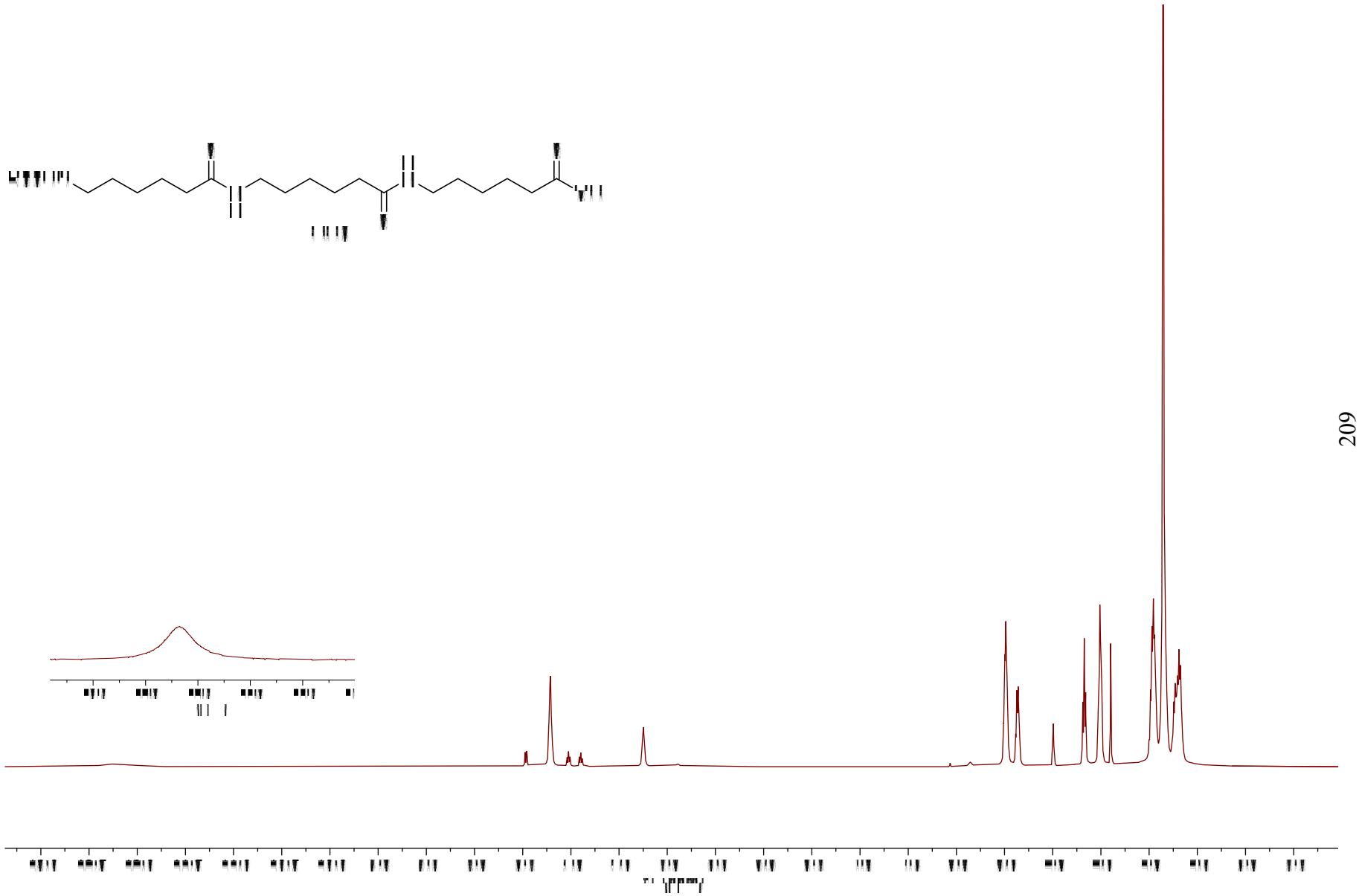

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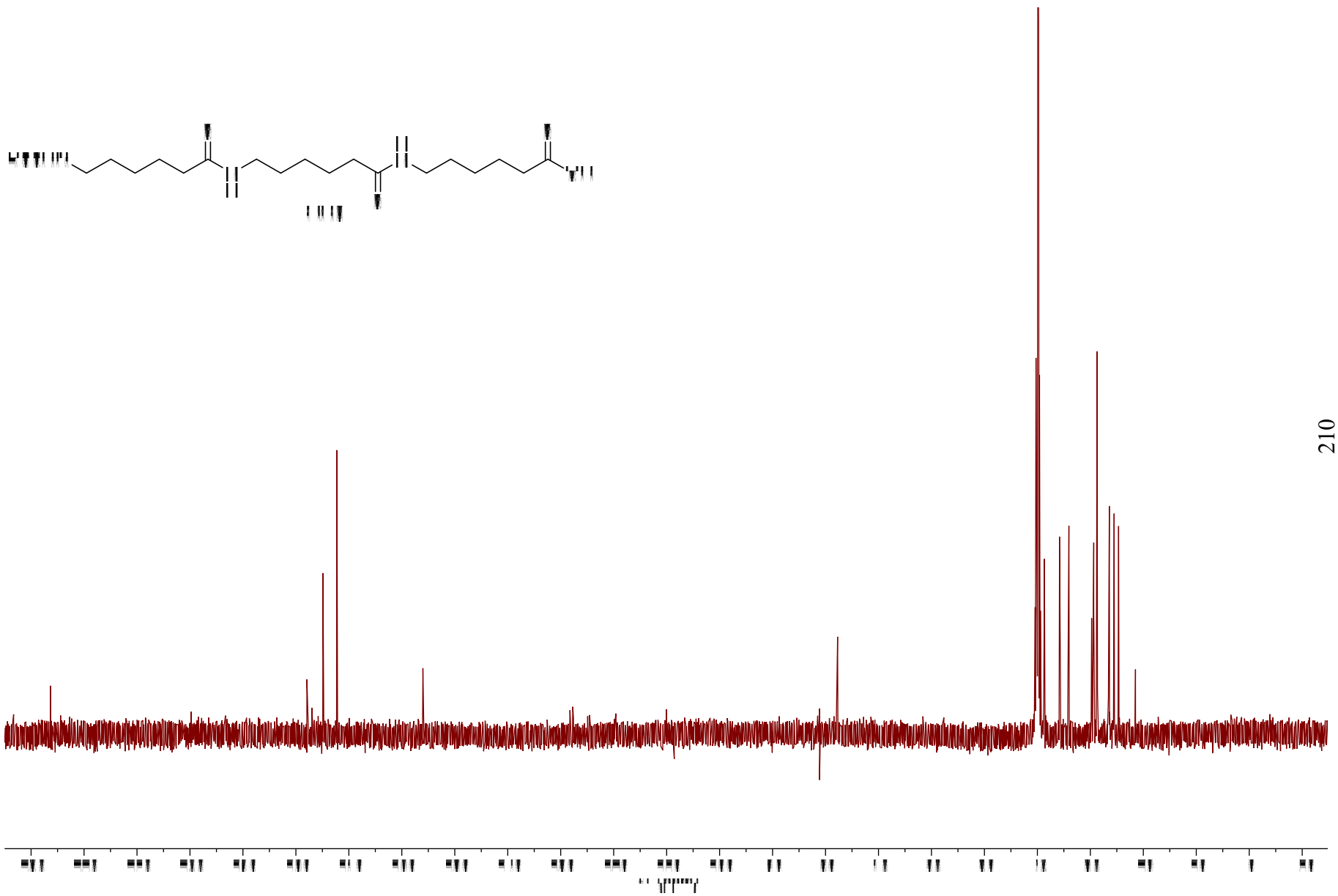
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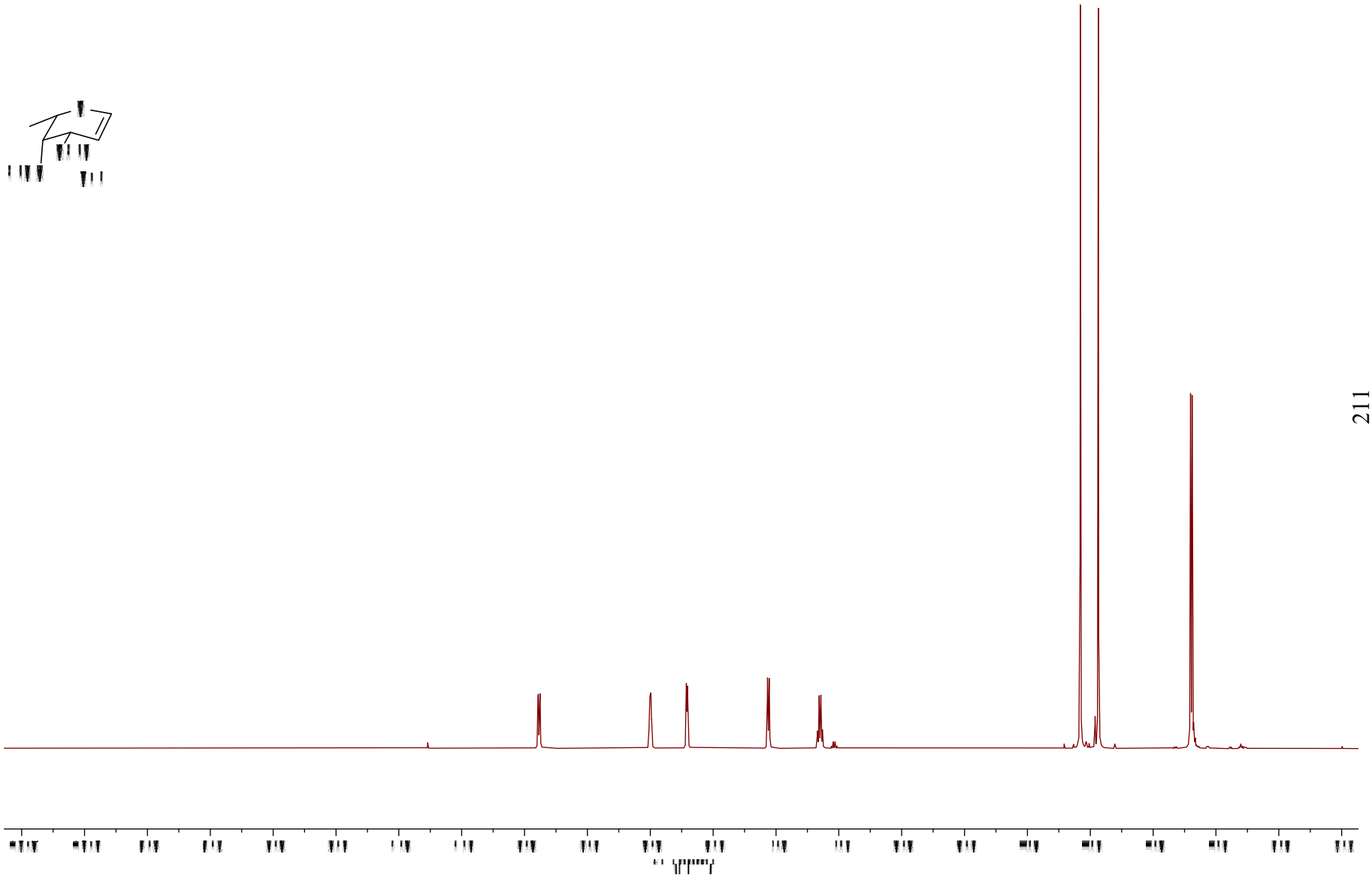
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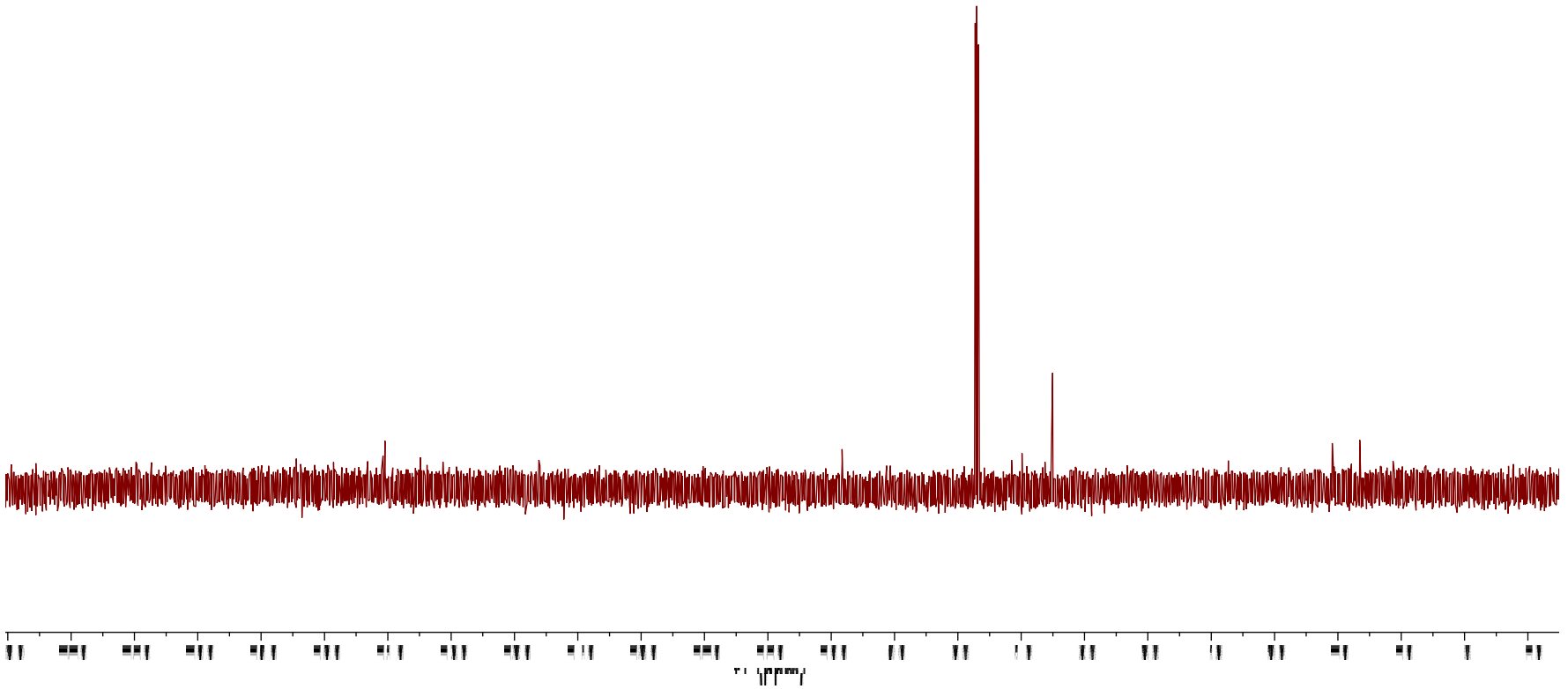

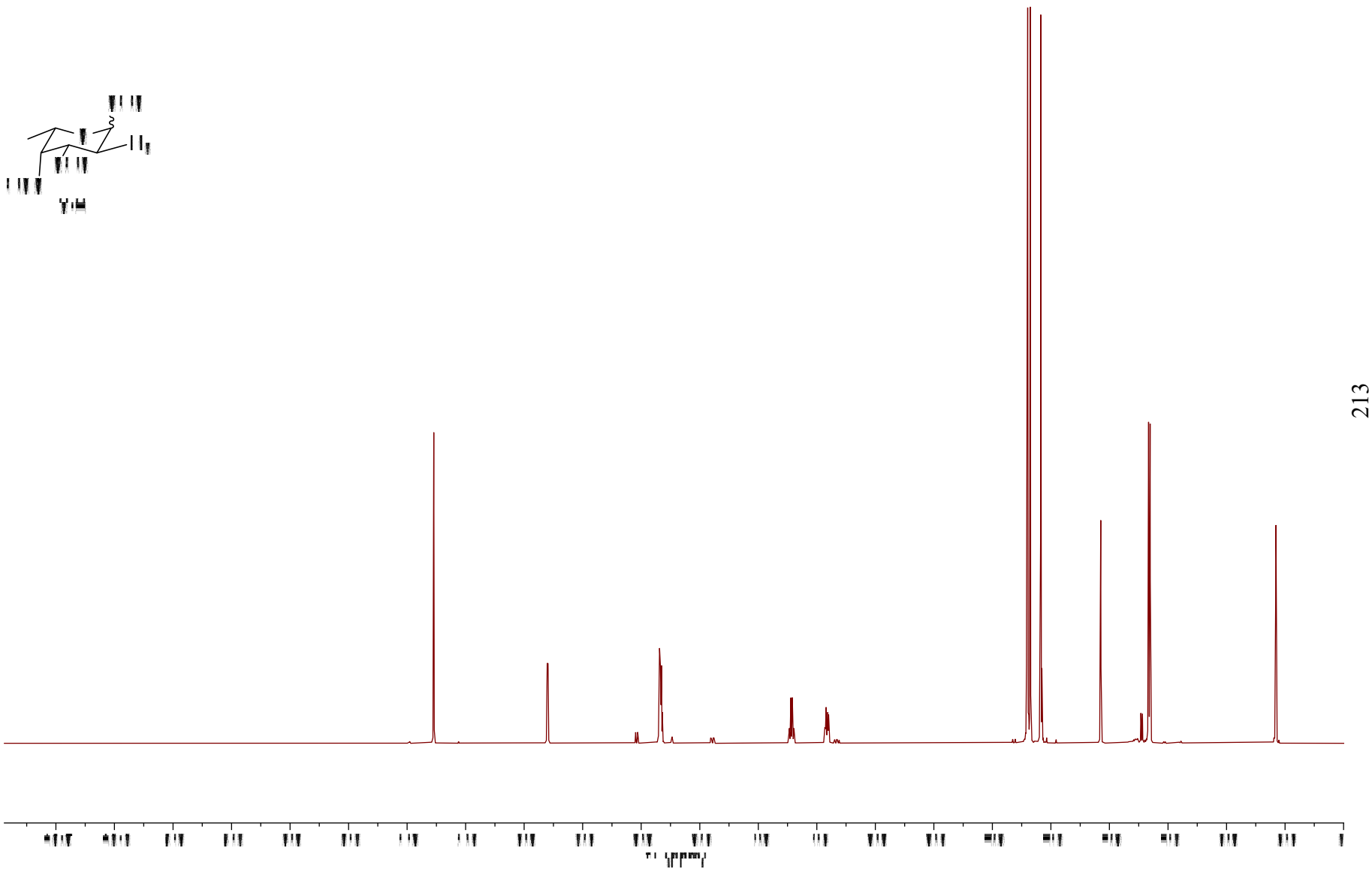

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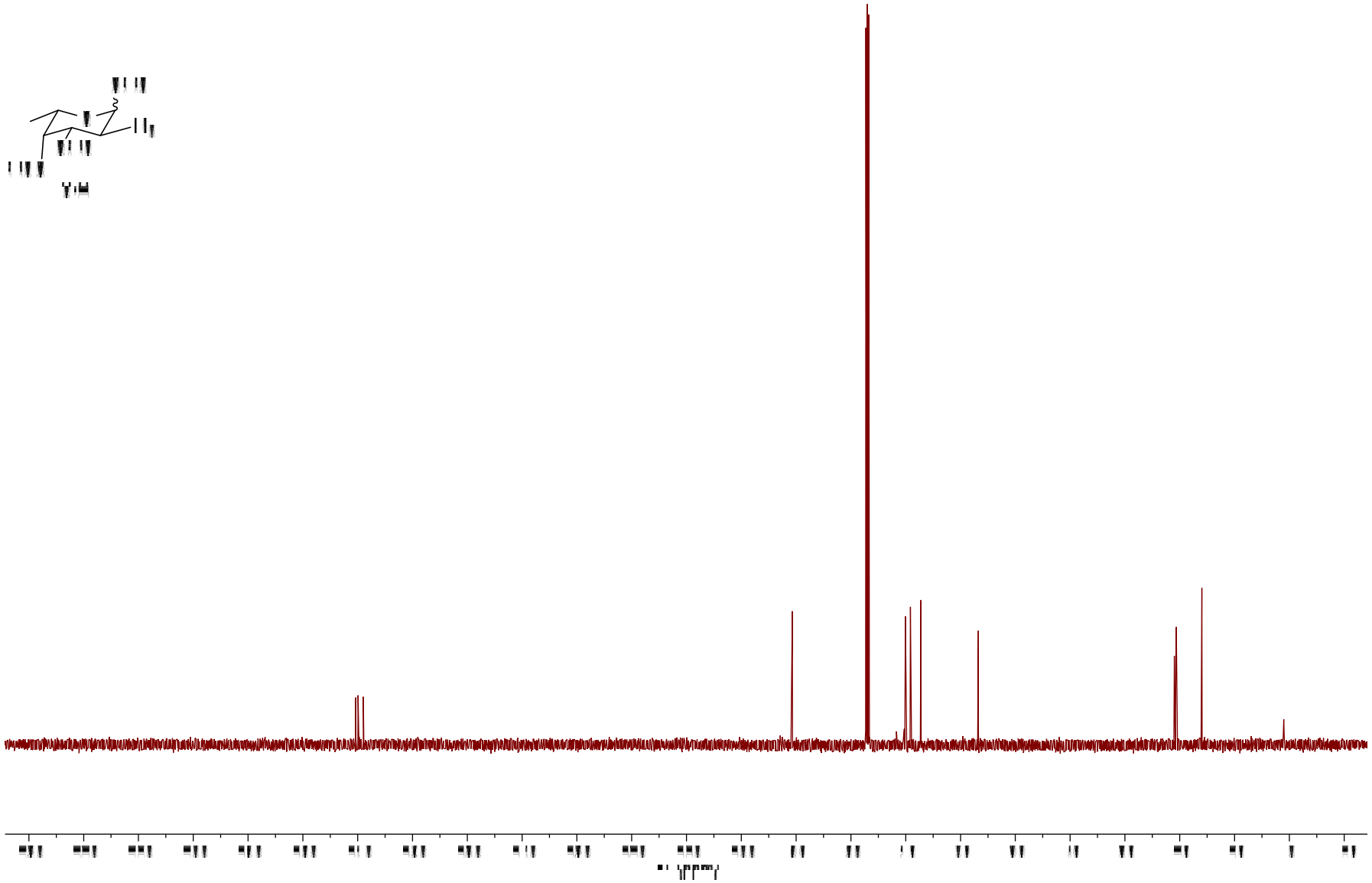

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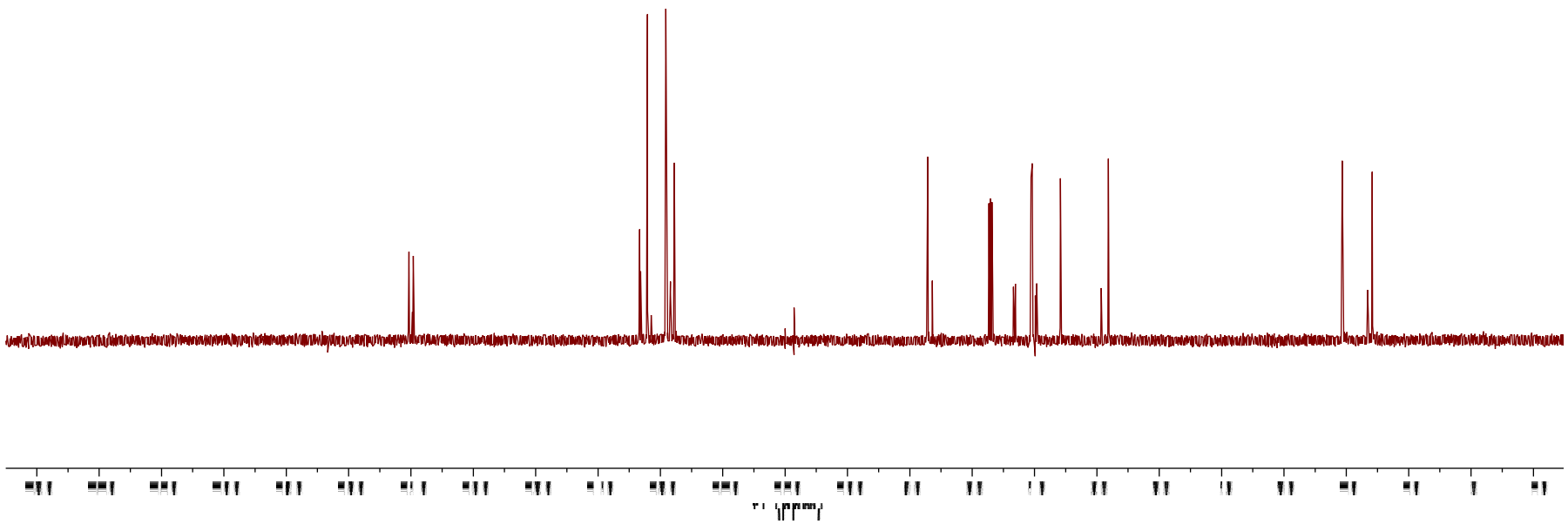

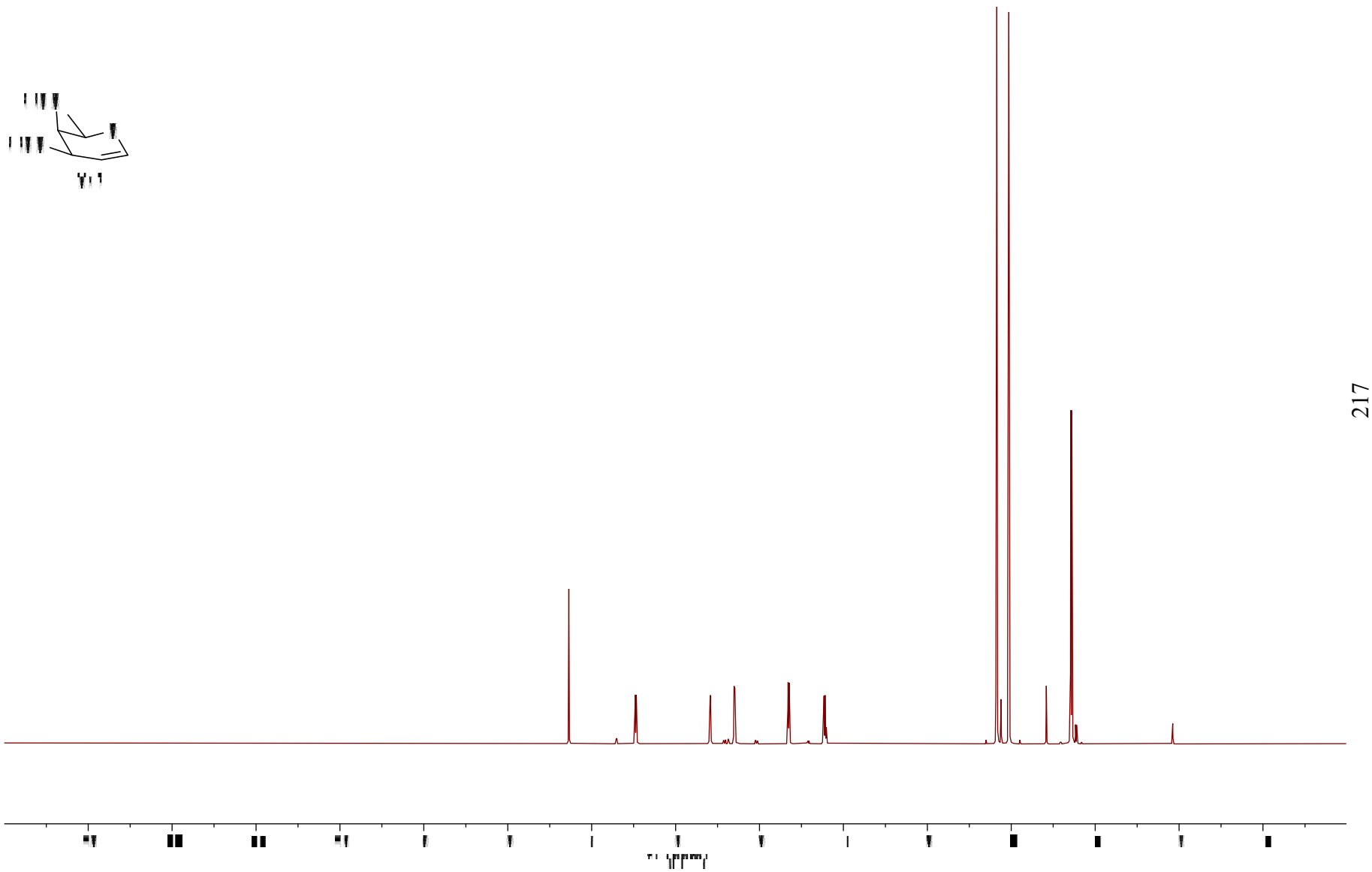
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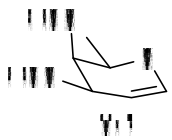

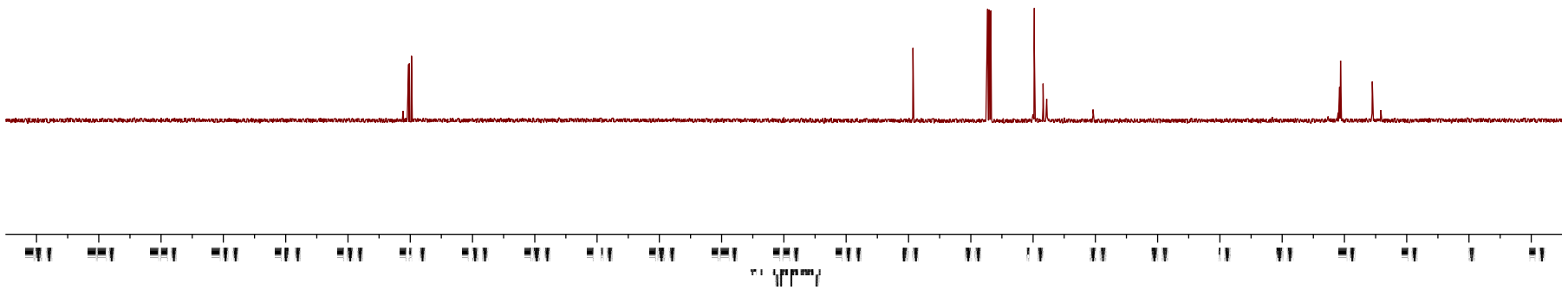

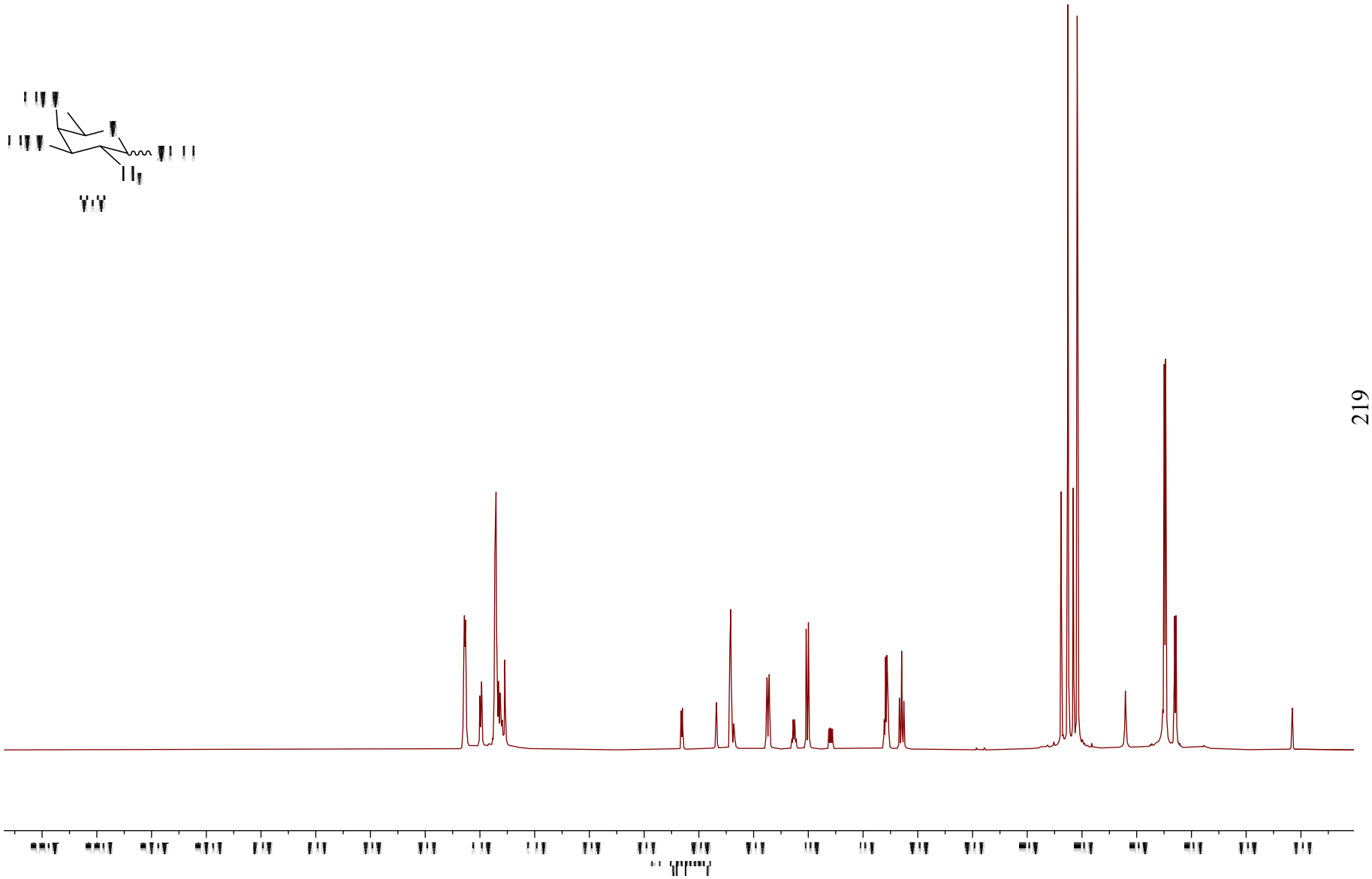
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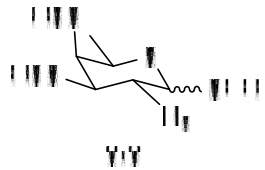

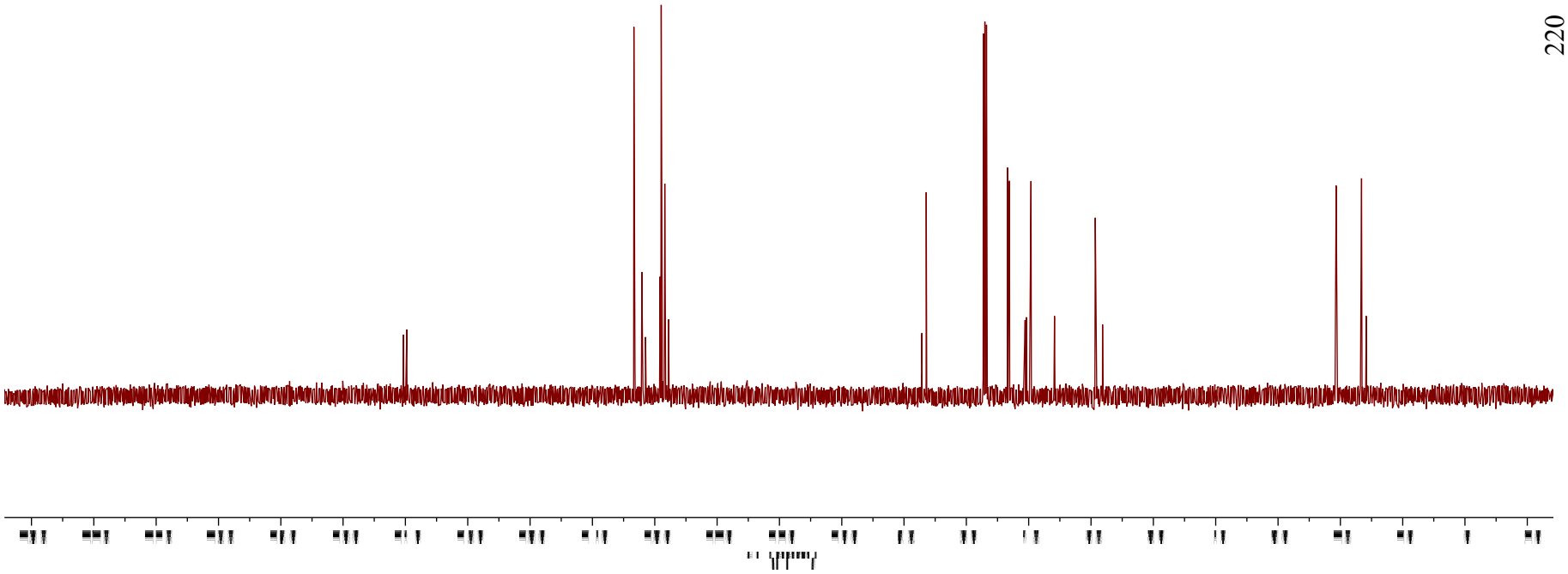
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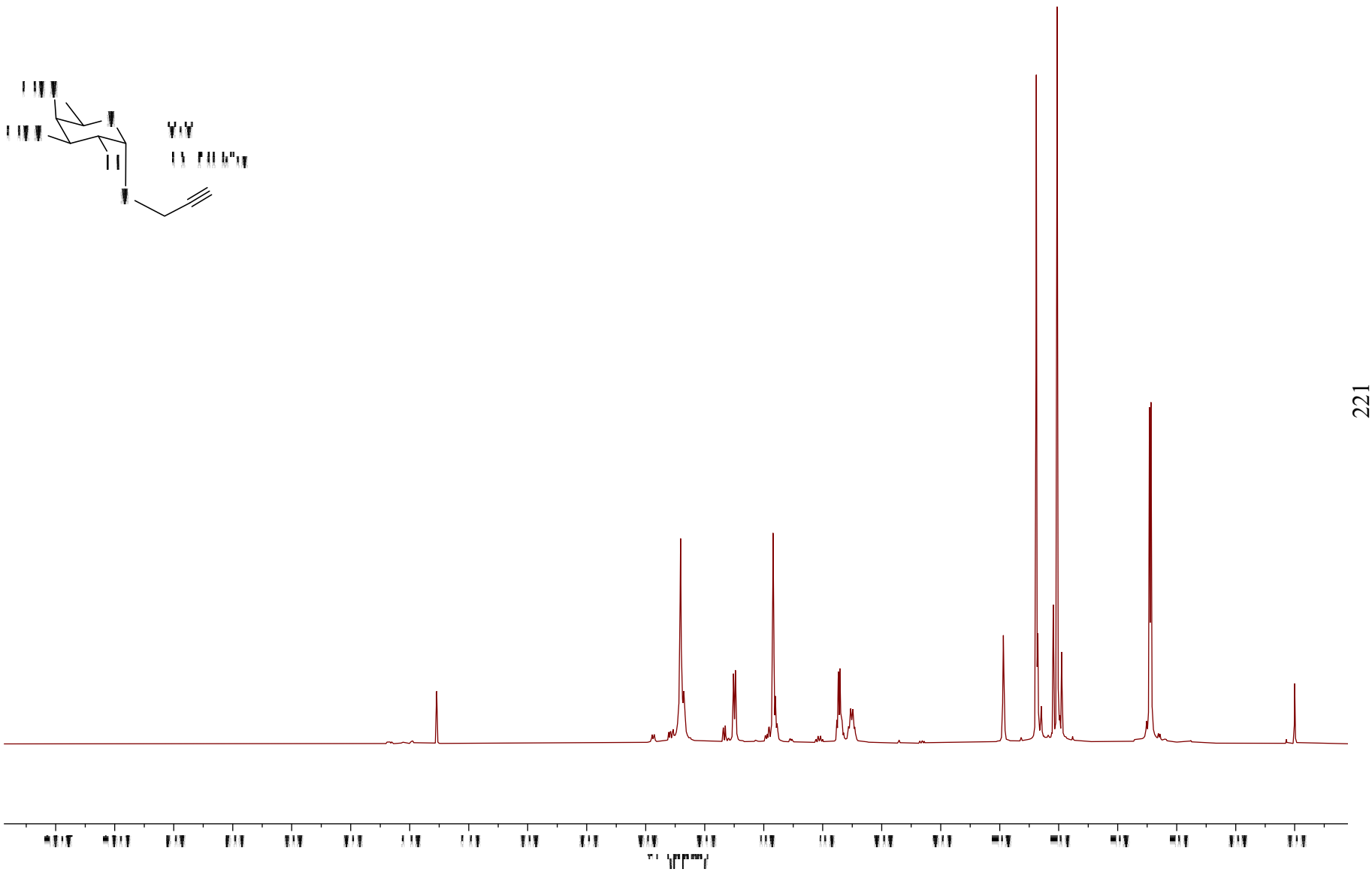
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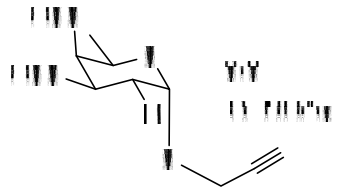

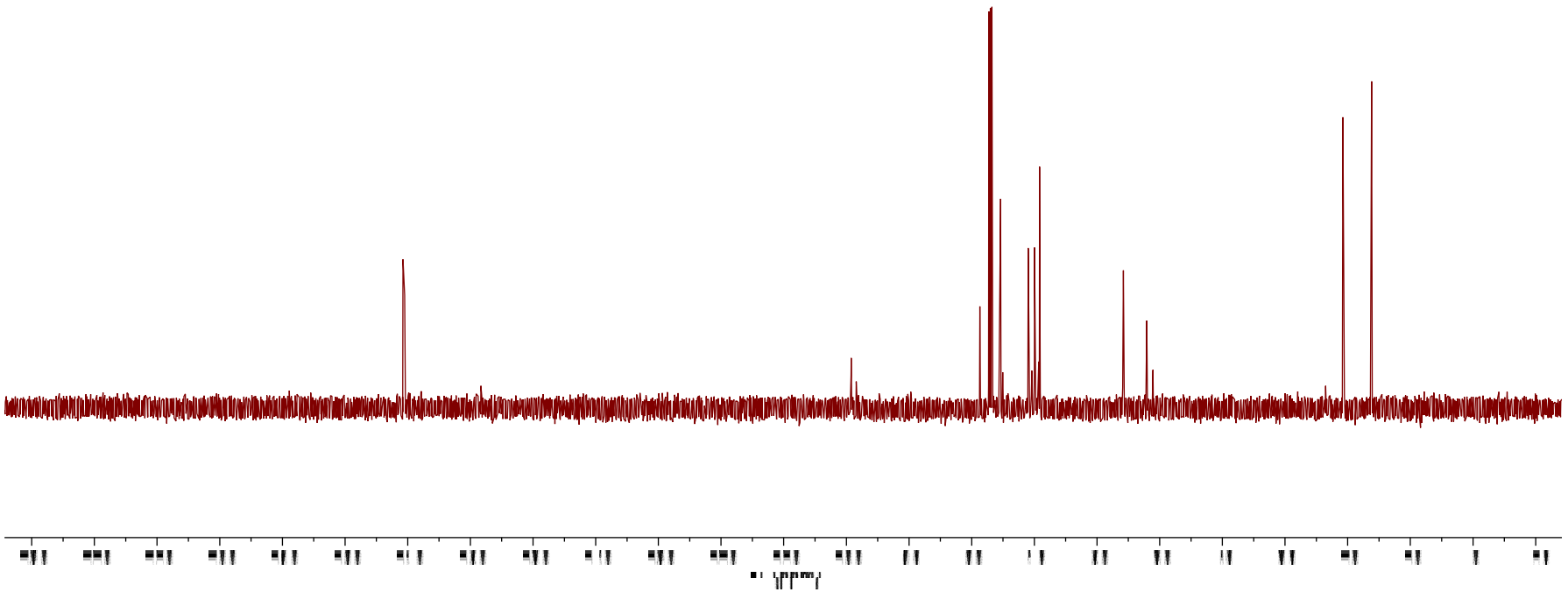

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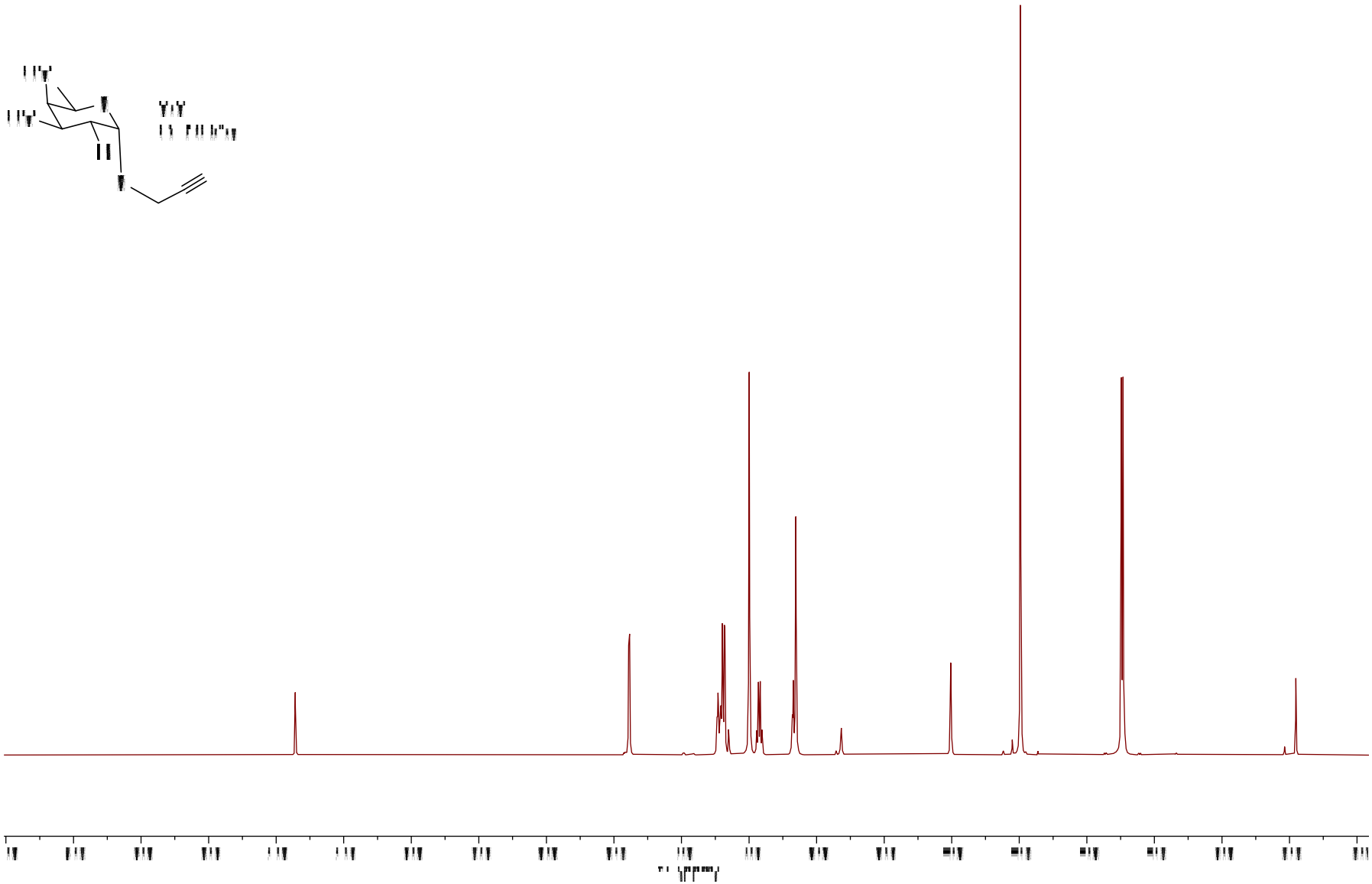
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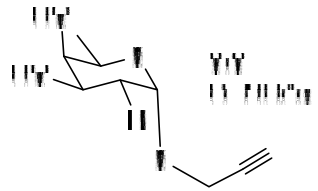

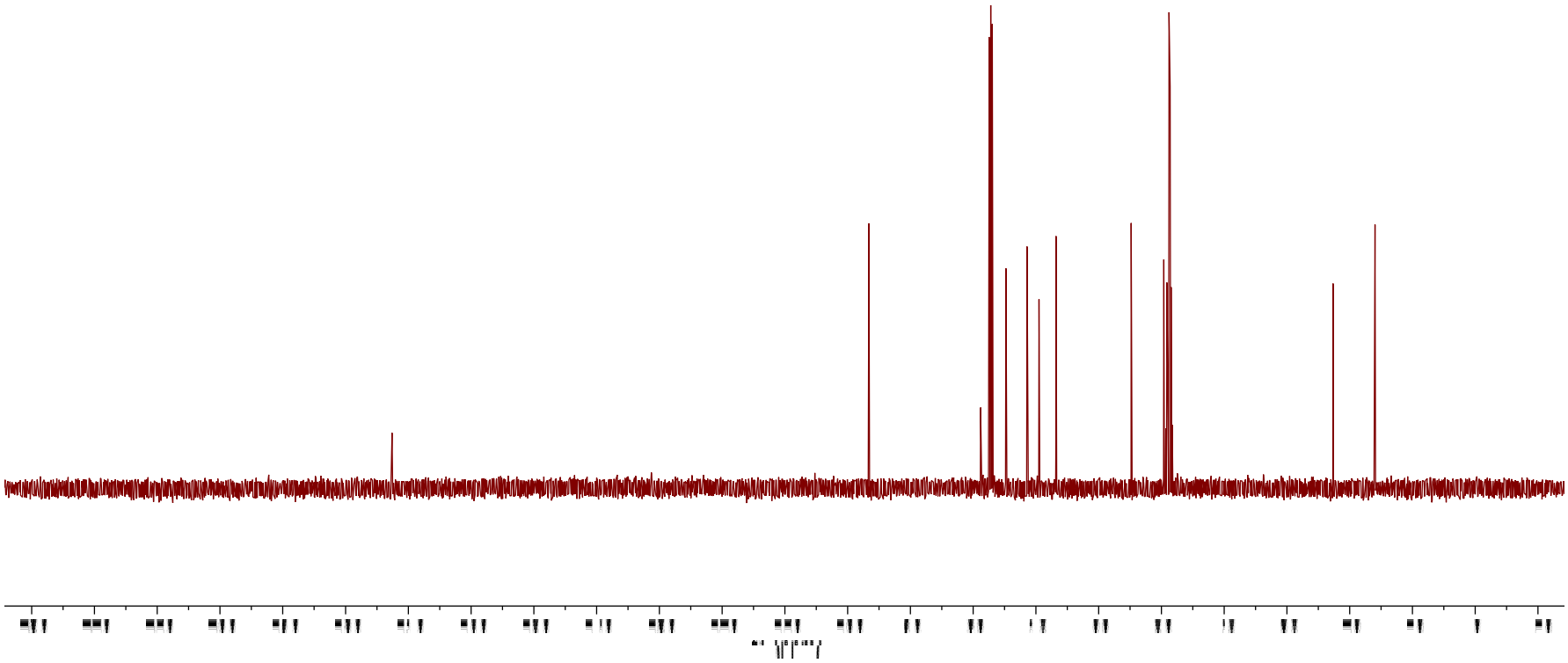

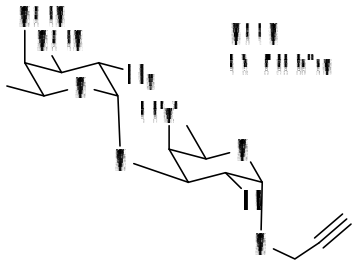

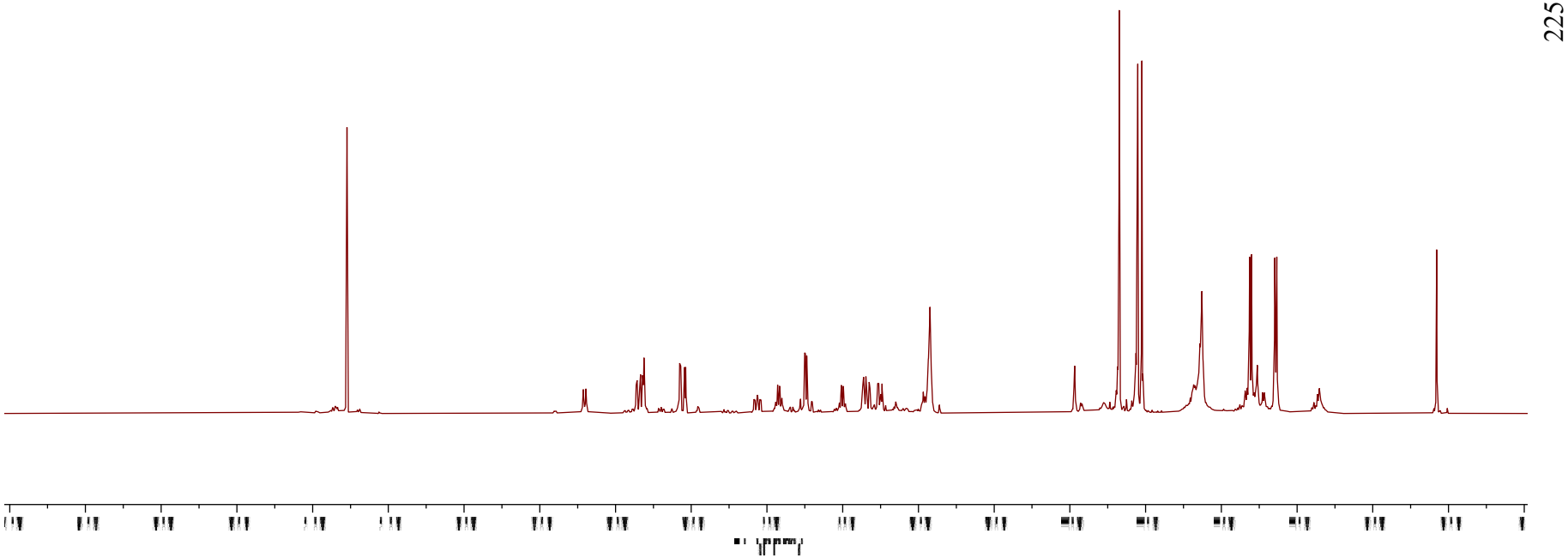

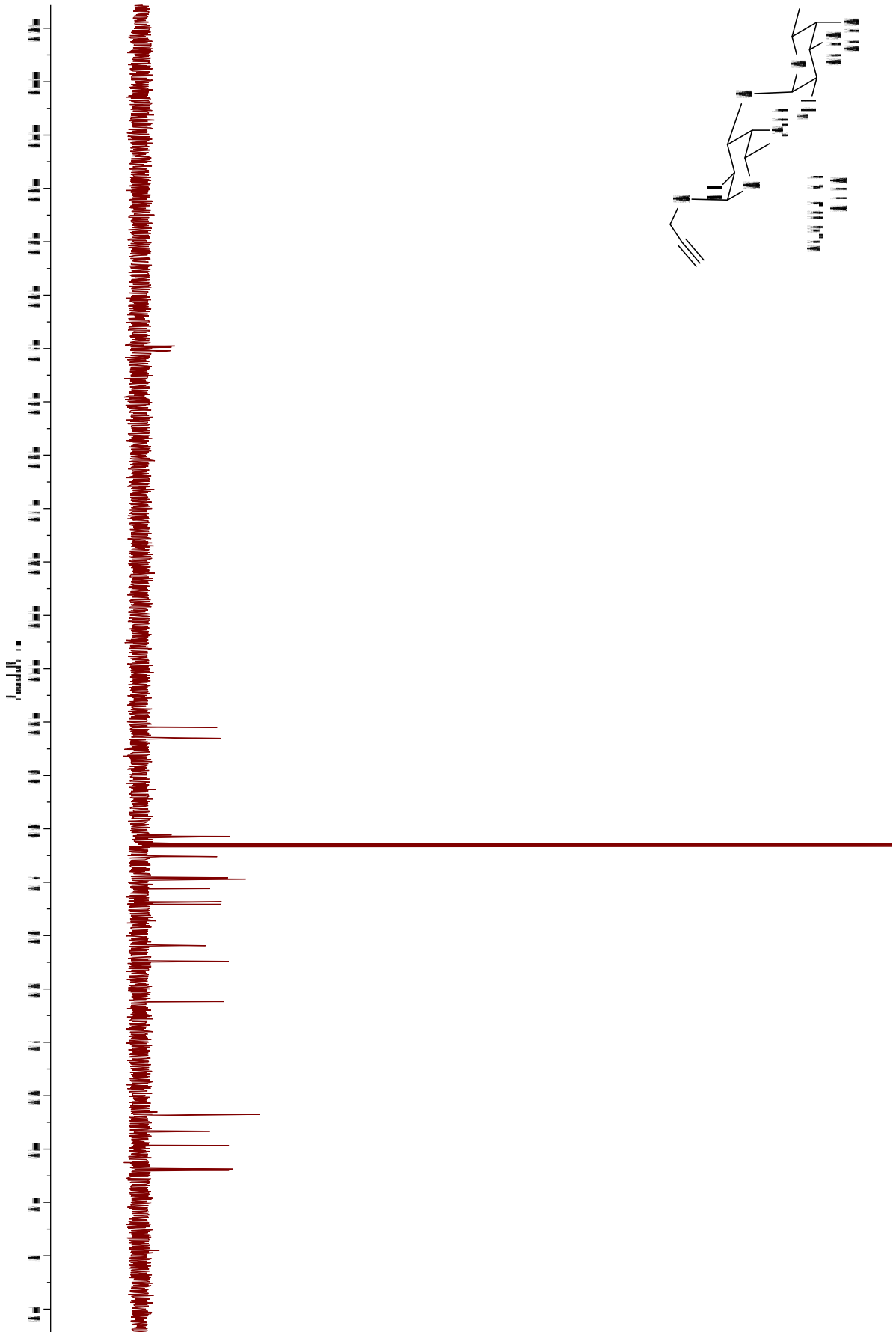

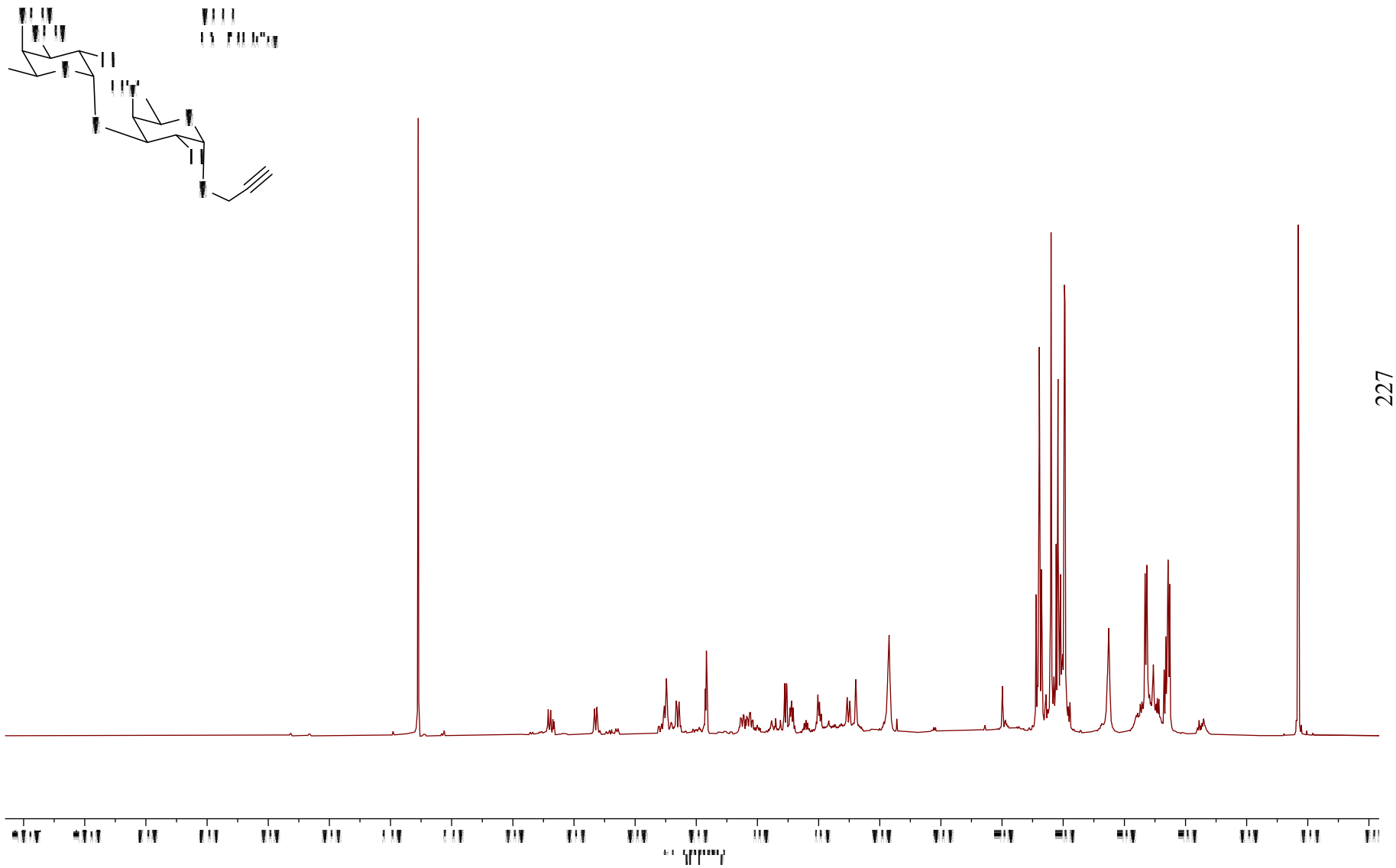
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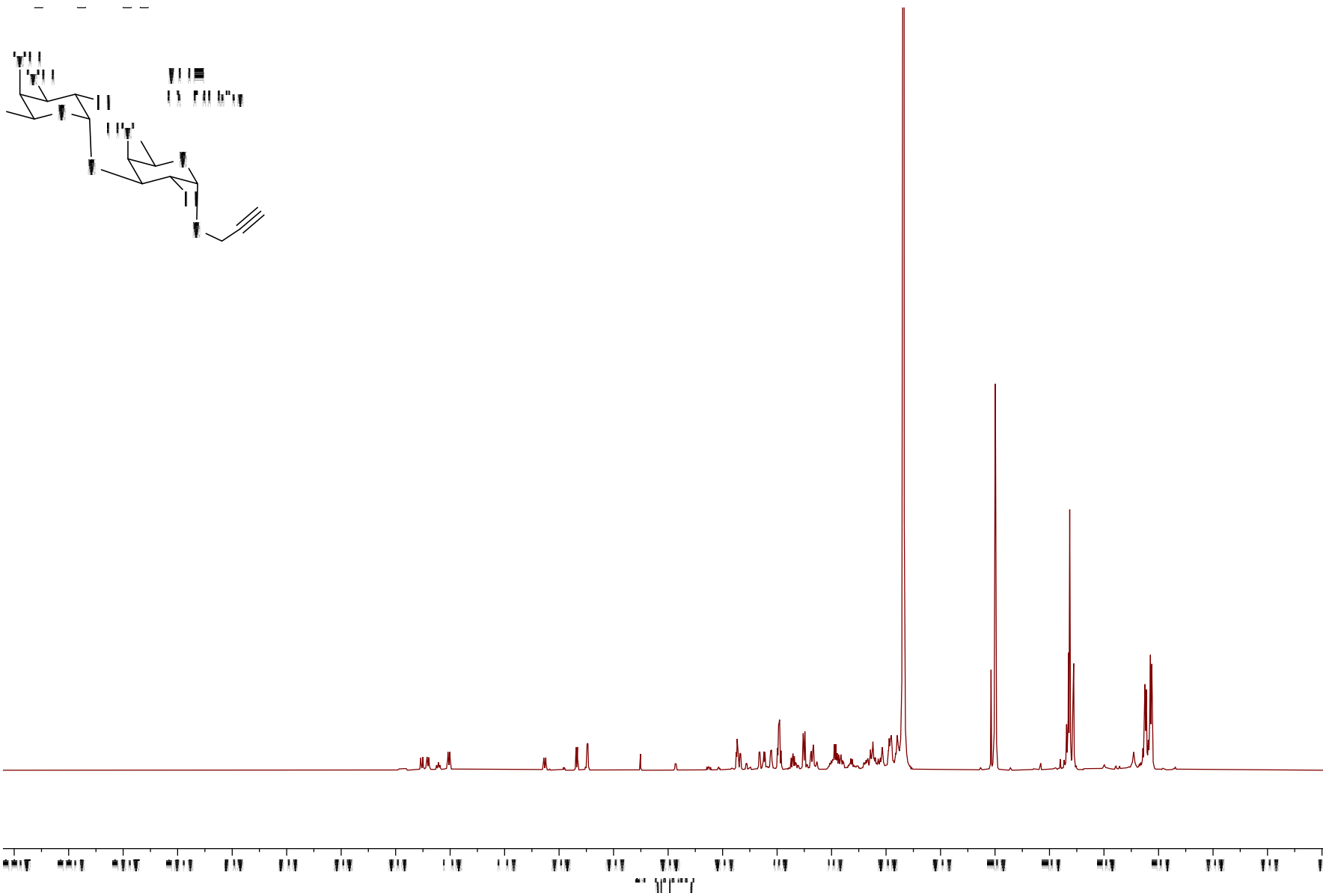

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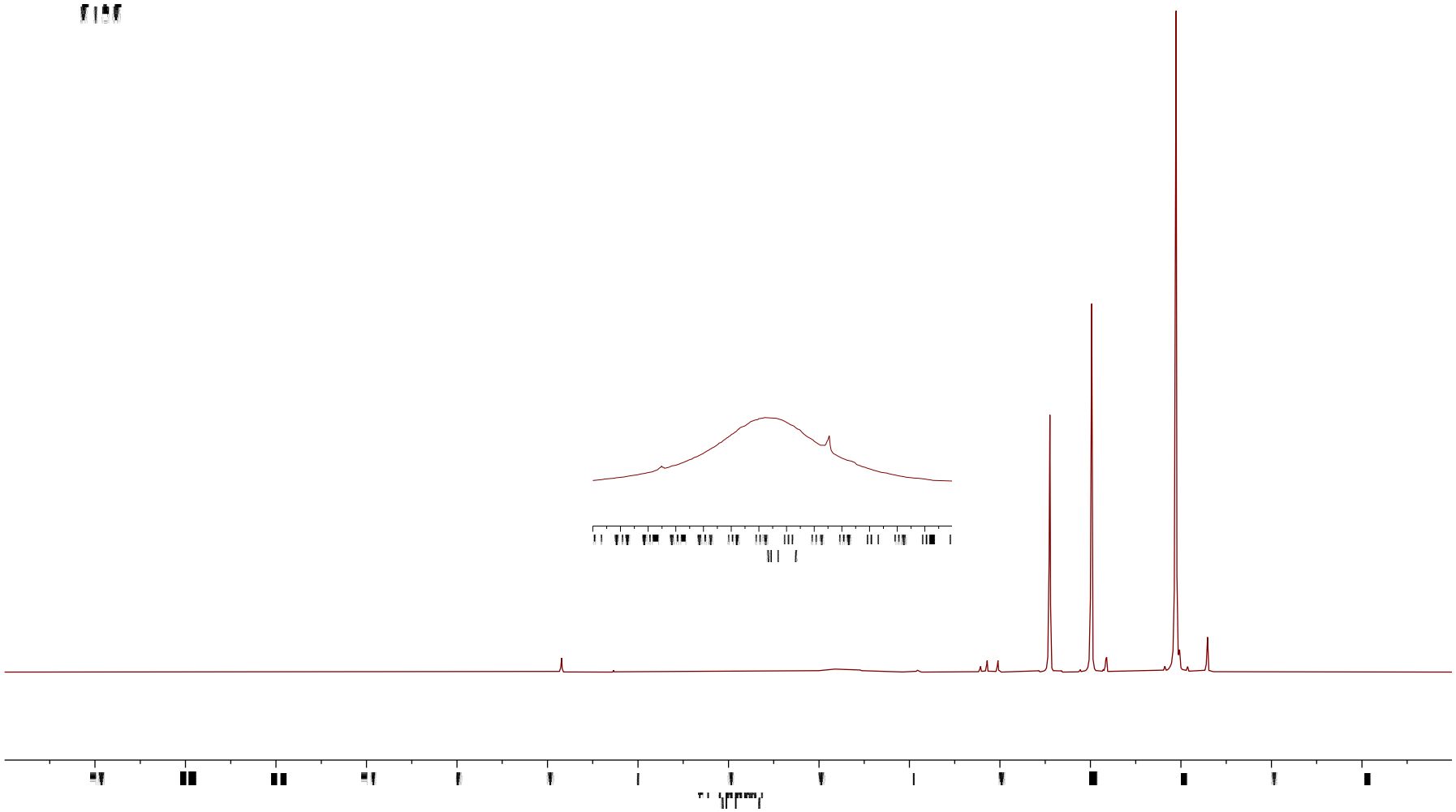

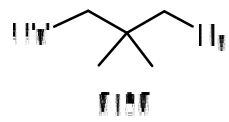

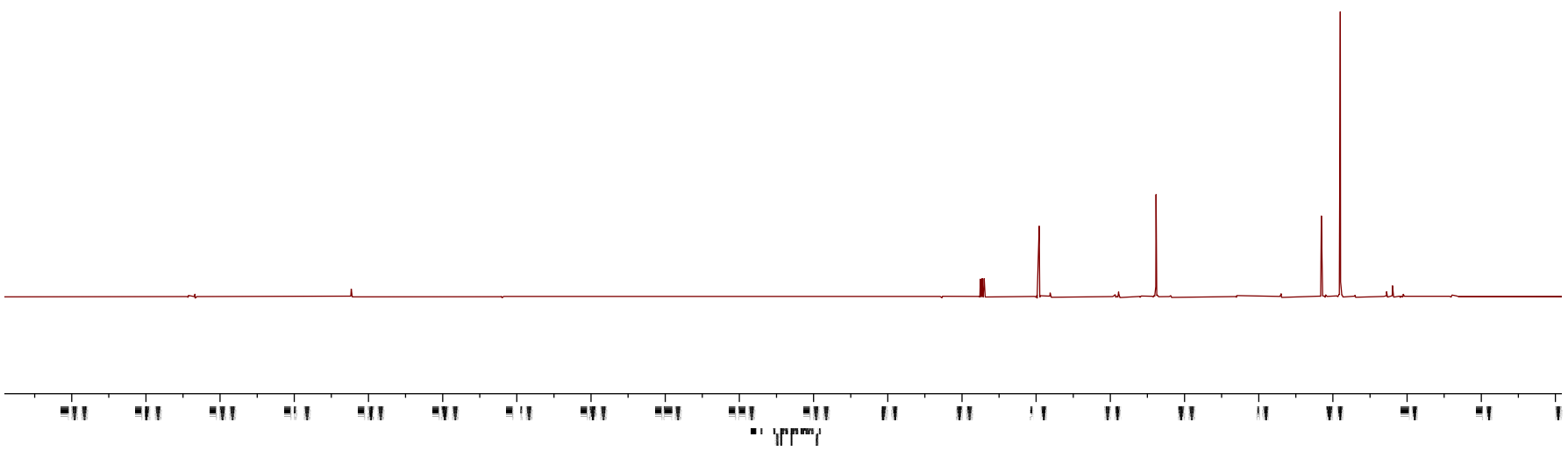

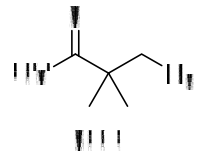

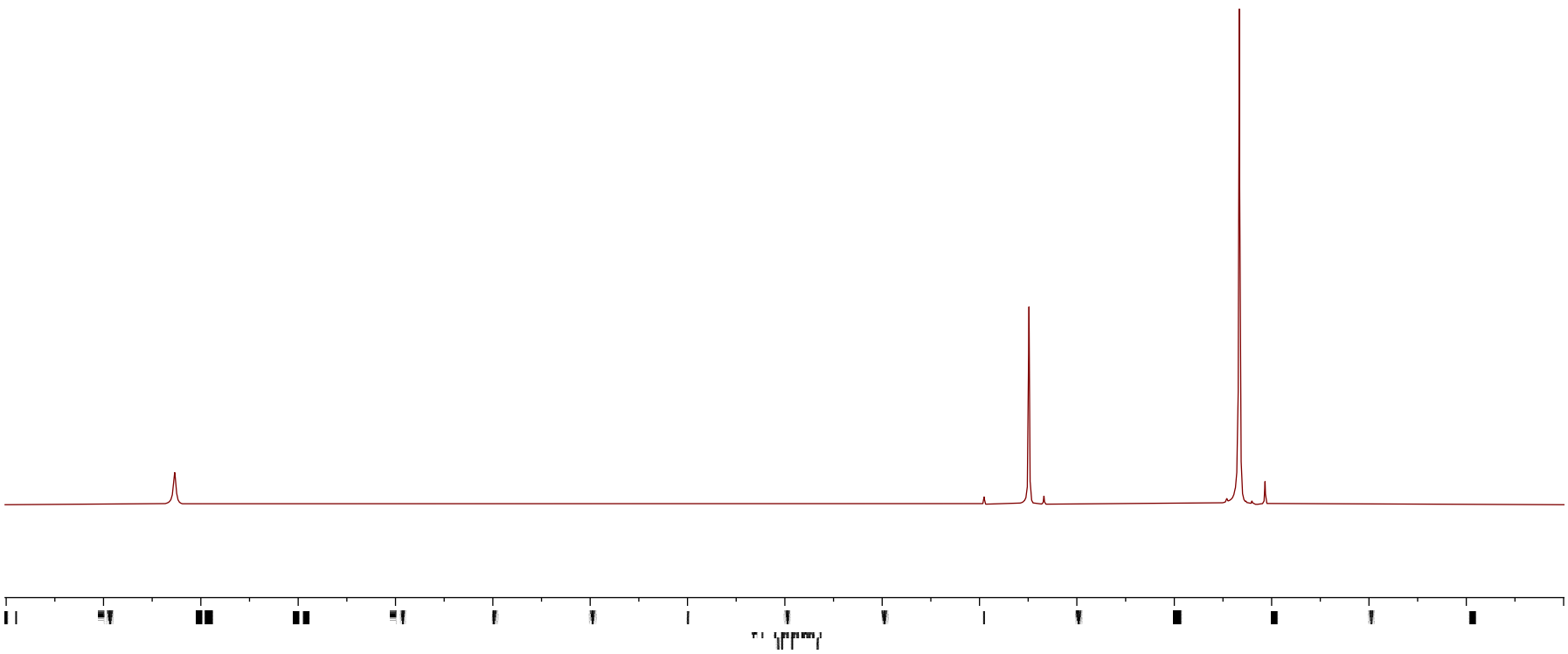

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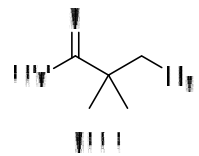

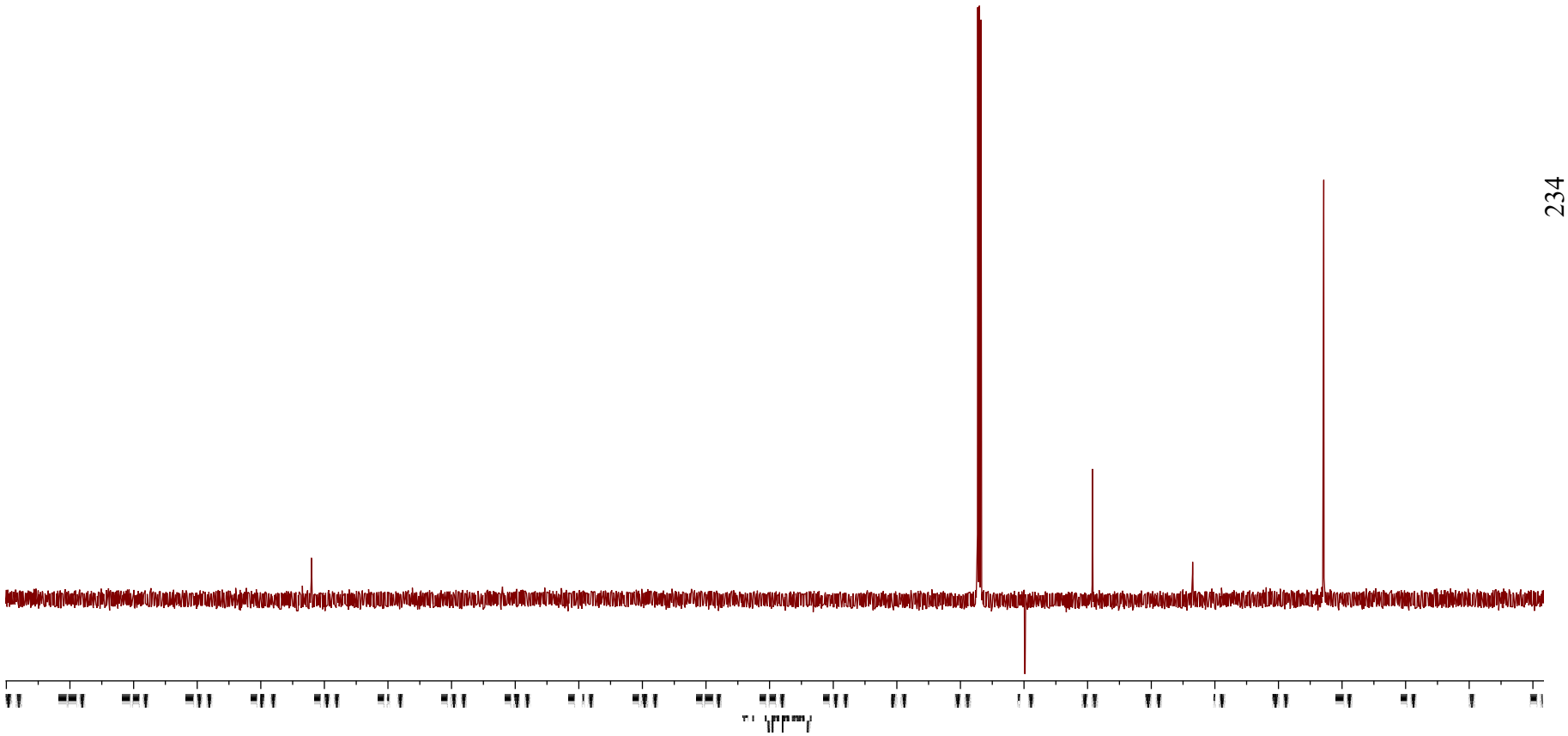

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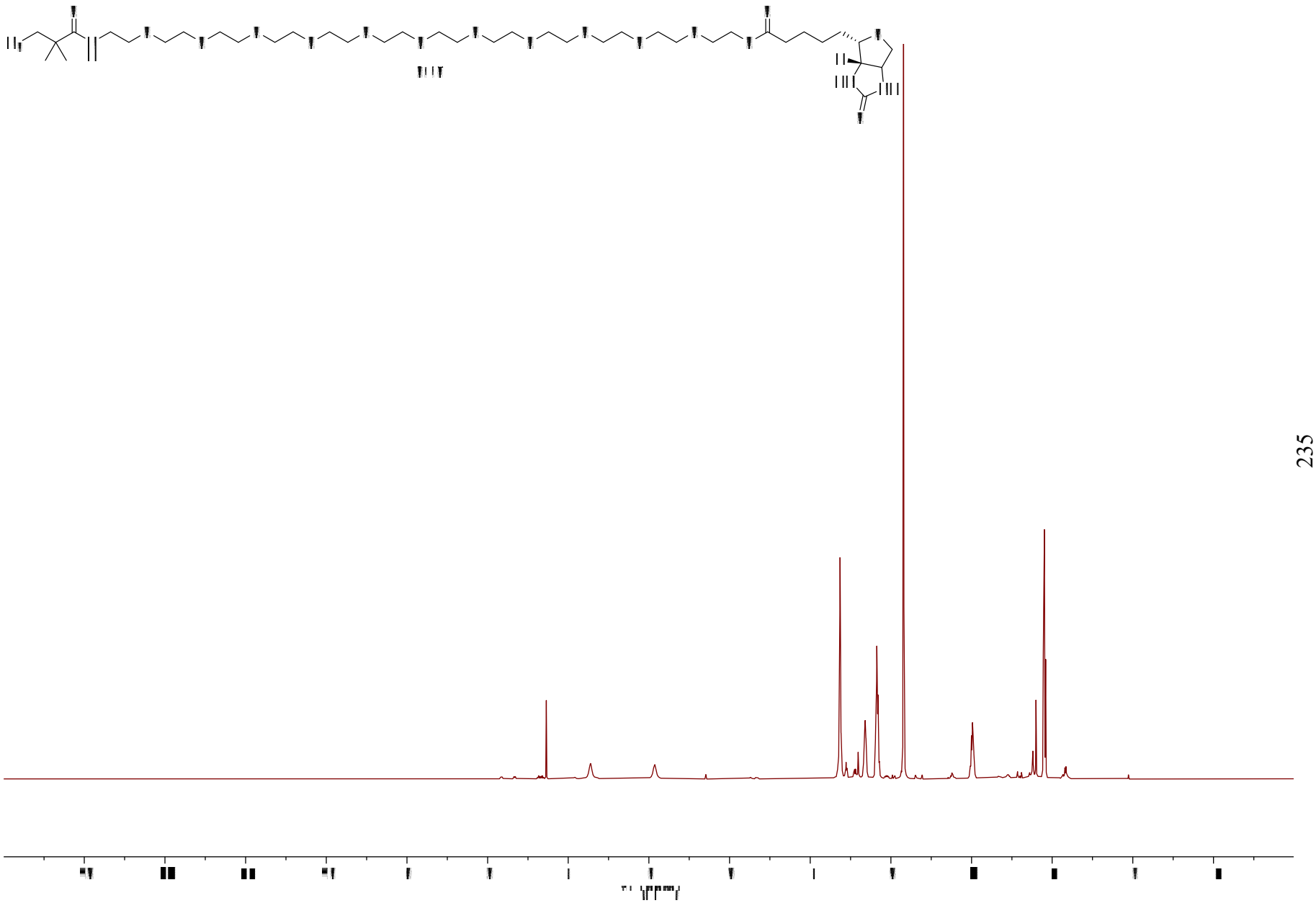

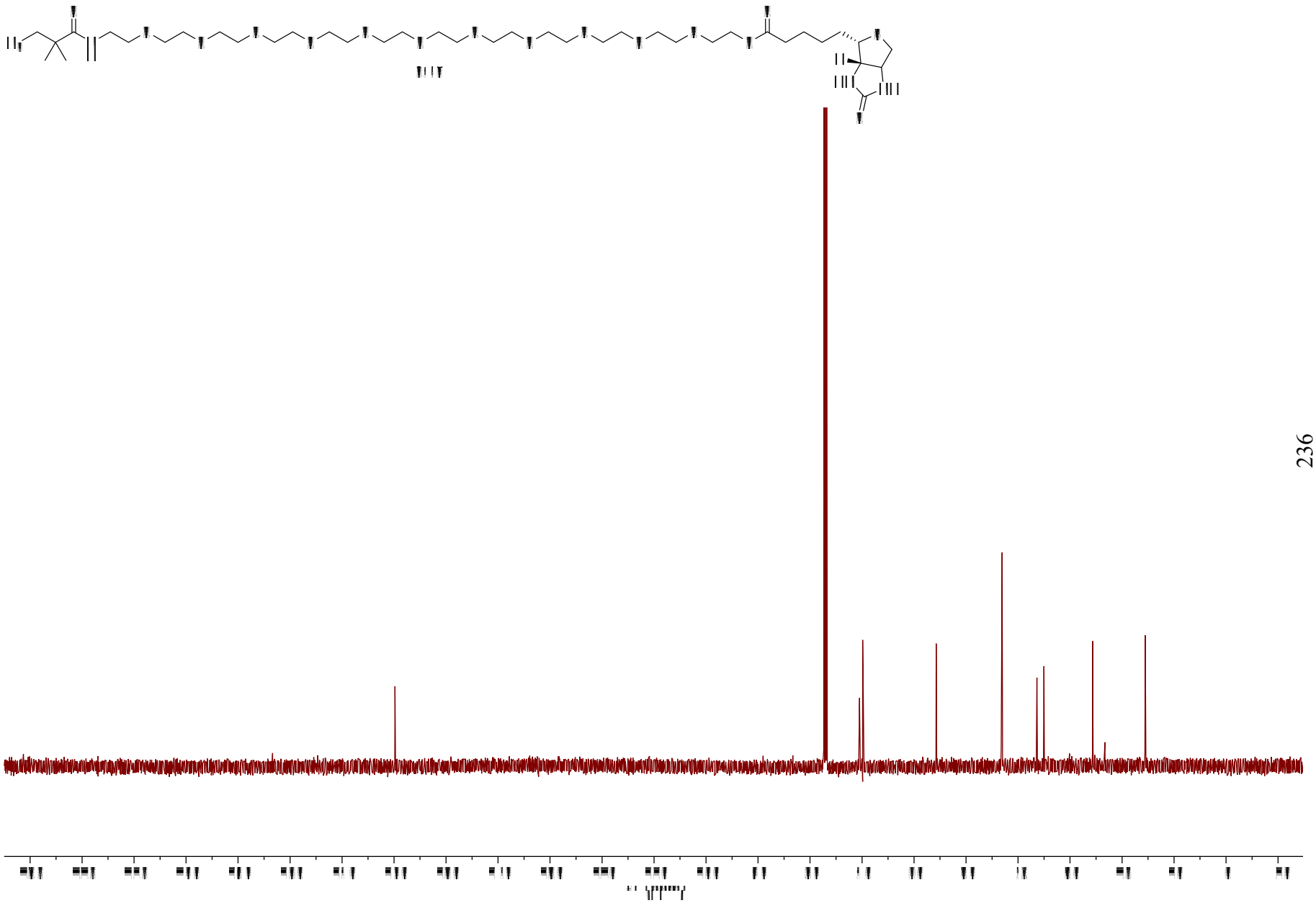

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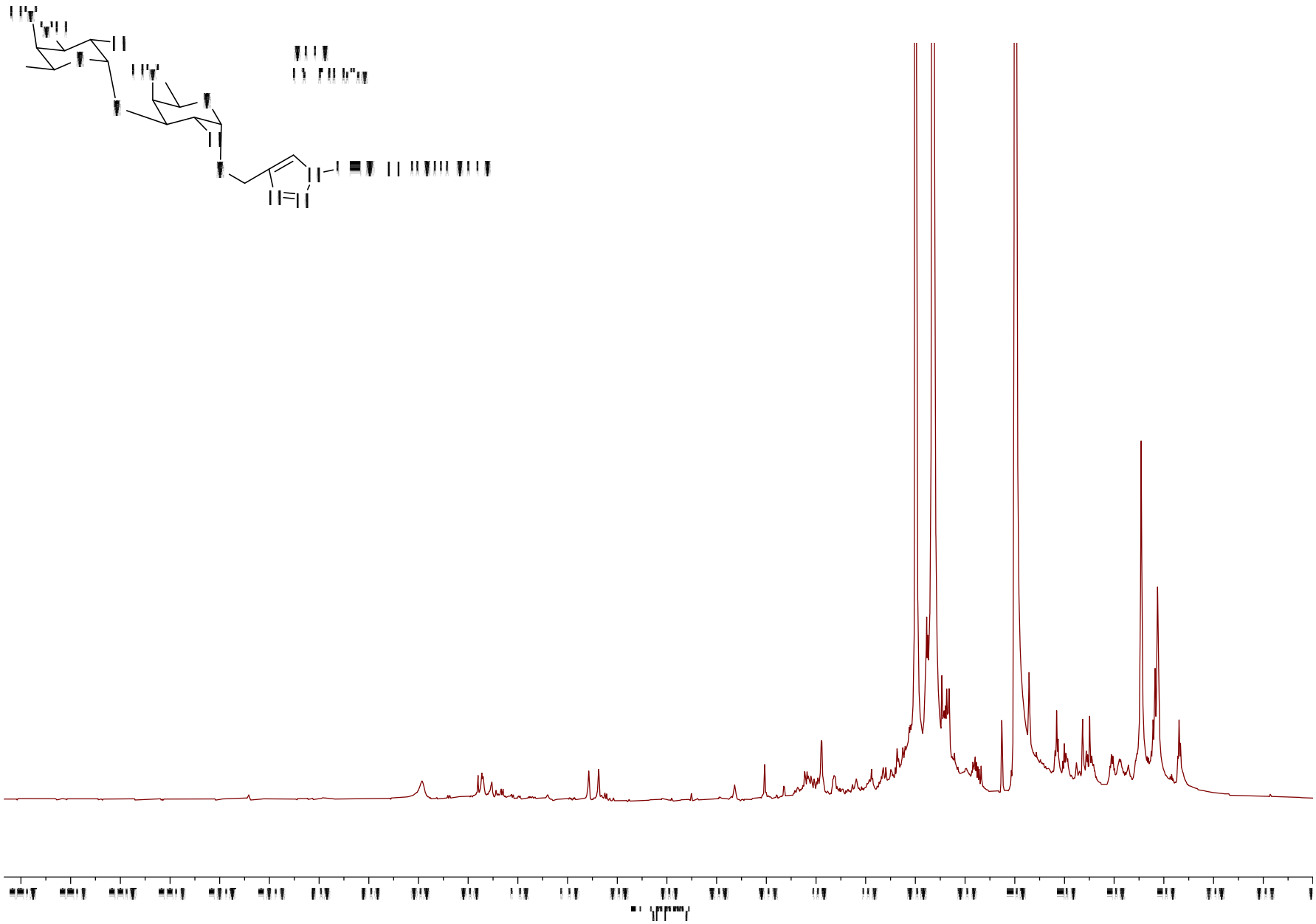
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