SYNTHESIS OF MODIFIED RIBOSE SPIN-LABEL

by

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Abstract

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Transfer ribonucleic acids (tRNAs) from all organisms contain modified nucleosides, which are derived from four different nucleosides. Research has indicated that tRNA modifications affect translation and various metabolic pathways. Among these modifications, some of them need more than one enzyme to produce specific nucleosides. Researchers are interested in investigating the fundamental mechanisms and function of post-transcriptional modifying enzymes. A number of these enzymes are metal-dependent, such as MiaB and MiaE. MiaE enzyme (2-methylthio-N^6-isopentenyl-adenosine(37)-tRNA monooxygenase) stereospecifically hydroxylates an adeonosine derivative at the 37^{th} position of tRNA, $ms^2i^6A_{37}$, to complete the hypermodification of 2-methylthio-N^6-(4-hydroxyisopentenyl)-adenosine ($ms^2io^6A_{37}$). Several studies showed that the ms^2io^6A37 modification of tRNA seems to influence the central metabolic pathways. Therefore, in order to study the biological activity of iron-dependent enzymes, such as MiaE, or other enzymes with radical mechanisms. We planned to synthesize spin-labeled analogs of $ms^2io^6A_{37}$, the substrate of MiaE. Chapter 1 describes the background of tRNA modifications and the MiaA, MiaB, and MiaE pathway for A_{37} modification in bacterial tRNA. Chapter 2 shows different strategies to synthesize the

desired nucleoside spin probe. Chapter 3 proposes several reactions to improve this synthesis in the future. Last, chapter 4 provides the experimental details used in this project to help other chemists to investigate the modified ribose spin-label chemistry.

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List of Abbreviations

°C	Degrees Celsius
Ado	5′-deoxyadenosyl radical
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMAPP	Dimethylallyl pyrophosphate
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EPR	Electron paramagnetic resonance
g	Gram(s)
HYSCORE	Hyperfine Sub-level Correlation
Hz	Hertz (cycles per second)
i^6A_{37}	N^6-isopentenyl-adenosine
J	Coupling constant (NMR)
MiaA	Diphosphate:tRNA dimethylallyltransferase
MiaE	2-methylthio-N^6-isopentenyl-adenosine(37)-tRNA monooxygenase
mmol	millimole
mol	Mole(s)
$ms^2io^6A_{37}$	2-methylthio-N^6-(4-hydroxyisopentenyl)-adenosine
MTTases	Methylthiotransferases
NaAsc	Sodium ascorbate
NMR	Nuclear magnetic resonance
Ph	Phenyl
ppm	Parts per million

rt	Room temperature
SAM	*S*-adenosylmethionine
TEA	Triethylamine
tRNA	Transfer ribonucleic acid
UV	Ultraviolet

Chapter 1

Introduction

Transfer RNA (tRNA) from all three domains-Archaea, Bacteria, and Eucarya contains several specifically modified nucleosides. These modifications are created on the four normal nucleosides: adenosine (A), guanosine (G), uridine (U), and cytosine (C). Currently, over 100 different modified nucleosides have been identified on tRNAs. Although scientists do not understand the full function of these modified nucleosides, they know that these modifications improve the fidelity of the ribosomal machinery, often by maintaining the reading frame and by enhancing the efficiency of tRNA in decoding the genetic message. For instance, modified tRNA display an increased rate of tRNA selection at the A-site, and enhanced fitness in the P-site. Reading frame errors are harmful for organisms because these errors lead to incorrect protein synthesis or incomplete peptides. In general, evidence shows that the degree of tRNA modification affects translation, central metabolism, intermediary metabolism, thiamine metabolism, and bacterial virulence.[1, 9]

The wobble position (position 34) and position 37, which is near to the anticodon, are frequently modified. [9] These modifications of tRNA are related to enzyme, substrate, and energy regulation. Molecular genetics and structural biology also point out that modifications of anticodon of tRNA help to control gene expression.[2, 4]

Some modifications require a variety of enzymes to produce a specific nucleoside. Figure 1-1 shows a biological pathway to synthesize a modified nucleoside at position 37 of tRNAs.[3] The final modified nucleoside product is 2-methylthio-N^6-(4-hydroxyisopentenyl)-adenosine ($ms^2io^6A_{37}$). Researchers discovered that the modified nucleoside ms^2io^6A occurs in several gram negative, pathogenic, facultative anaerobic bacteria (e.g., the tRNAs of *Klebsiella pneumoniae, Serratia marcescens, Pseudomonas aeruginosa, Citrobacterfreundii,* and *Enterobacter cloacae*).[6] Similar to other A_{37} modifications, $ms^2io^6A_{37}$ improved the efficiency of translation. With in complete modification or loss of the methylthio group at A_{37}, the efficiency of

the tRNA was decreased and the codon context sensitivity of tRNAs was increased. [3] By 1998, it was generally hypothesized that the modification of position 37 was a regulator related to amino acid biosynthesis, iron uptake, and aerobiosis.[2, 4]

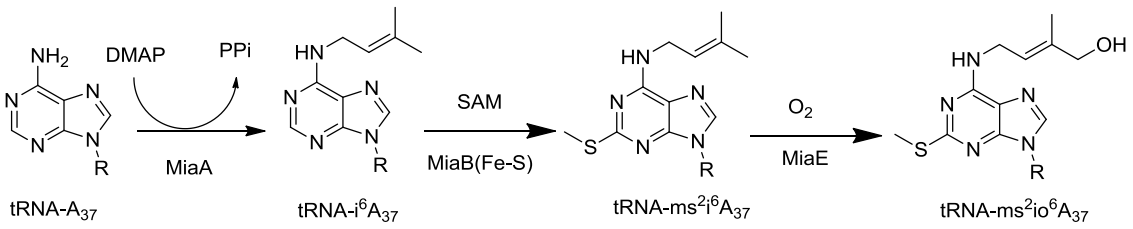


Figure 1-1 Biosynthetic pathway for $ms^2io^6A_{37}$.

Focusing on the MiaA-E biosynthetic pathway, it is observed that the first reaction (see figure 1-1) is an alkylation. In this step, diphosphate:tRNA dimethylallyltransferase (MiaA) catalyzes the substitution of the exocyclic amine of A_{37} by the dimethylallyl group from dimethylallyl pyrophosphate (DMAPP) to yield N^6-isopentenyladenosine (i^6A_{37}). [5] The lack of this modification induced several dramatic effects on gene regulation and cell physiology.[3] For instance, isopentylation improved the binding ability of tRNA to the ribosome, with increased stacking interactions in the codon-anticodon complex. In other words, this modification weakened A-U base bond pairing between A_{36} and the uridine of the codon. Additionally, studies indicated that the mutation of MiaA influences the expression of genes, related to amino acid biosynthesis, bacterial virulence, aromatic amino acid uptake, and growth on citric acid cycle intermediates.[5]

The function of MiaB is to modify N^6-isopentenyladenosine (i^6A) to 2-methylthio-N^6 isopentenyladenosine (ms^2i^6A) in tRNA. This second modification performs a C-H insertion of a sulfur atom into the 2-carbon, followed by an additional methylation of the thiol. Methylthiotransferases (MTTases) similar to MiaB are enzymes, which perform both radical-*S*-adenosylmethionine (SAM) sulfur insertion and SAM-dependent methylation. Two known MTTases have been found, typified by MiaB.[8] The detailed molecular mechanisms by which MTTases function are not entirely understand. Specially, how MTTases convert C-H bonds to

C-S-H functional groups, yielding other by-products, such as biotin and lipoic acid.[7] Several enzymes were discovered to perform sulfur-insertion reactions, such as MiaB, CDKAK1, and RimO. They also contain critical iron-sulfur [4Fe-4S] cluster, in addition to the SAM coenzyme. In the radical-SAM enzyme superfamily, which these belong to, unique free-radicals are generated from a [4Fe-4S] cluster chelated by three cysteines from a conserved CX3CX2C sequence.[7] In some cases, the Radical-SAM cluster cleaves SAM into the 5′-deoxyadenosyl radical (Ado•) and methionine. The 5′-deoxyadenosyl radical was responsible for H-atom abstraction at the C2-position of i^6A_{37}. By this way, the MiaB converts a specific C-H bond to a C-S bond.[7, 8]

MiaB modification showed decreased mutations (GC-to-TA transversions) in *Escherichia coli* with methylthiolation from i^6A to $ms^2i^6A_{37}$. Also, the deficiency of ms^2 modification of ms^2io^6A accelerated the synthesis of aromatic amino acids and decreased the efficiency of the corresponding tRNAs. Mutant MiaB influenced the entire biosynthetic pathway that generates 2-methylthio-N^6-(4-hydroxyisopentenyl)-adenosine ($ms^2io^6A_{37}$), the presence of the 2-methylthio group of $ms^2i^6A_{37}$ was an important signal for MiaE-mediated hydroxylation of i^6A_{37} to io^6A_{37}. This methylthiolation was even sensitive to the cellular concentrations of methionine and iron.[6]

The final enzyme of this series is MiaE (2-methylthio-N^6-isopentenyl-adenosine(37)-tRNA monooxygenase), which performs the hydroxylation of $ms^2i^6A_{37}$ to yield 2-methylthio-N^6-(4-hydroxyisopentenyl)-adenosine ($ms^2io^6A_{37}$).[5, 9] The hydroxylation of ms^2i^6A was discovered to be dependent on the presence of molecular oxygen. However, the hydroxylase was also presented under anaerobic conditions. This led to the hypothesis that, $ms^2io^6A_{37}$ regulates aerobiosis.[5, 10] Based on several experiments, the $ms^2io^6A_{37}$ modification of tRNA seemed to influence the central metabolic pathways. Mutants of MiaE were tested for their relative growth on different types of carbon sources. The results of these experiments showed that MiaE mutants were unable to grow on the dicarboxylic acids of the citric acid cycle under aerobic condition. Also, the organisms did not generate citric acid cycle intermediates succinate,

fumarate, and malate, when MiaE was mutated and non-functoinal. [6, 9, 10] Therefore, it was speculated that the uptake of succinate, fumarate, and malate depended on the modification at position 37 of those tRNAs. The bacteria only grew on these materials if the isopentenyl group was hydroxylated by MiaE.[9,10]

The gene of the hydroxylase MiaE, which modifies the nucleoside at position 37 was identified in the beginning of 1990s. However, there was no fascinating breakthrough on this subject until 2007 by Dr. Fontecave's team. They analyzed the MiaE protein by a combination of UV-visible spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, Mössbauer and Hyperfine Sub-level Correlation (HYSCORE). Thus, they established that the MiaE protein was a member of the carboxylate-bridged non-heme diiron enzymes. MiaE was the first monooxygenase discovered to be involved in modification of tRNAs.[9] The diiron center in this type of enzyme can achieve various forms, either the +2 or the +3 state and the active oxidant could be a variety of oxygenated species involving one or both iron centers. Interestingly and most challenging to the investigation of this enzyme, these diiron enzymes existed in a mixture of forms. However, Dr. Fontecave's team figured out that the diiron center had three major forms: (I) a hydroxo-bridged Fe(III)–OH–Fe(III) center, characterized by Mössbauer spectroscopic; (II) an oxo-bridged Fe(III)–O–Fe(III) center, characterized by the UV-visible spectrum and the Mössbauer spectrum; (III) a hydroxo-bridged Fe(II)–OH–Fe(III) mixed-valent center. Additionally, they showed that each iron was bounded by a nitrogen atom. [9] Though these structures were detected by various spectroscopic methods, the active form was still unknown (hydroxo- or oxo-bridged), because MiaE has a mixture of diiron sites.

The interaction of three important enzymes, which contributed to modification of nucleosides, prompted us to investigate tools that could probe the active site of MiaB or MiaE, both believed to undergo redox state changes, as exciting topic for investigation. Following our initial work to understand the stereospecificity of MiaE through small molecule investigations, we hypothesized that spin-labeled analogues of $ms^2io^6A_{37}$ could ultimately aid in our understanding

of the active species in MiaE. A triazole modified ribose spin-label was then targeted for synthesis as a probe or substrate of MiaE. A general method for the preparation of nucleoside based spin probes was desired so that a single route could be applied to the investigation of a broad range of nucleoside modifying enzymes. These molecules can be used in anisotropy experiments as well in more elaborate experiments that use electron-electron interactions through Electron paramagnetic resonance experiments to monitor the interaction of enzyme and substrate. The following chapters describe various pathways to synthesize the desired spin-labeled product used to investigate the biological activity of MiaE.

Chapter 2

Synthesis of triazole modified ribose spin-label

2-1 Introduction.

Synthesis of nucleosides or nucleotide analogs is a popular topic, since nucleosides and nucleotides play an important role in much of the fundamental cellular metabolism. In addition to being major building blocks for biopolymers, they act as regulators for metabolite carriers, energy donors, secondary messengers, and co-factors. (Some common structures are shown in Figure 2-1) Specifically, the nucleosides and nucleotide analogs influence, not only nucleic acid synthesis, but also protein synthesis, glycan synthesis, and glycoprotein synthesis. Therefore, the analogs of nucleosides or nucleotides can be used to investigate antibacterial, antifungal, antitumor, antiviral, herbicidal, insecticidal, and immunomodulating issues. Moreover, analogs can be classified by their structure: (1) base analogs; (2) simple nucleosides; (3) acyl and glycosyl nucleosides; and (4) nucleotides.[11, 12]

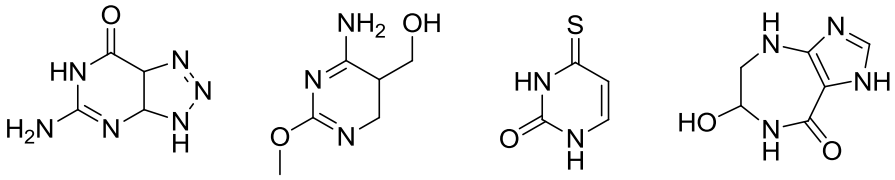
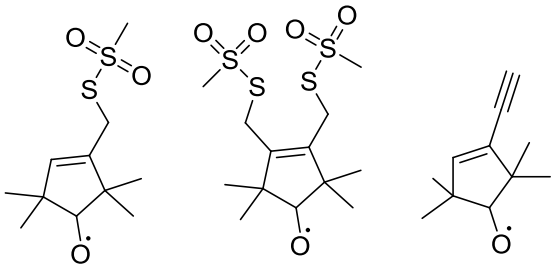


Figure 2-1 Base analogs.

Electron paramagnetic resonance (EPR) is a powerful method for investigating paramagnetic centers in biological systems. Although most biopolymers don't have natural paramagnetic centers, stable paramagnetic spin labels, such as nitroxides, can be attached on the biopolymers to study their structure, function, and biomolecular interactions by EPR. EPR is a fascinating technique because it can also be used to detect the differences in local conformational mobility, while ignoring diamagnetic background. On the contrary, X-ray diffraction and NMR will be confined by some conditions if they are used for studying properties

of biomolecules.[13, 15] Besides, EPR can be utilized to very large molecules such as RNA-protein complexes. Outstandingly, EPR can be applied to measure long range interactions of two paramagnetic species up to 100 Å apart.[13, 16] This can be useful for investigating some classes of metal-dependent enzymes, such as those described in Chapter 1. Also, EPR can determine solvent accessibility of individual residues and measure dynamics over a range of timescales.[15] There are several types of applications shown above and we should not be surprised that EPR techniques are used increasingly in the study of biological research. Some common building blocks for these molecules are shown in Figure 2-2.



MTSL RX TPA

Figure 2-2

2-2 Retrosynthesis of the triazole modified ribose spin-label.

The purpose of this thesis was to synthesize a triazole modified ribose spin-label with the intention of inserting a variety of natural and non-natural bases (Adenine, Guanine, Thymine, Cytosine, Uracil) to our spin label via Vorbrüggen coupling (or some similar method) to provide an array of tools for testing biological systems. The initial synthetic strategy led by Dr. Andra Corder, attempted to make the 5'-azido modified ribose and nitroxide spin-label separately. The plan was to use Huisgen-cycloadditions or click chemistry to couple both of the compounds. However, after trying several cycloaddition trials, the yields were found to be insufficient for general use. For many reasons, we hypothesized that the radical of the spin-label would affect

the cycloaddition and limit the reasonable set of conditions that could lead to efficient cycloadditions. This approach would mean that the common use of Cu(II) pre-catalysts could not be used to make the triazole system, as a reducing agent would be necessary to reduce Cu(II) to Cu(I). The reducing reactant was likely being non-compatible with the nitroxide spin label. Hence, the synthetic strategy was changed to improve overall yield and avoid chemoselectivity issues with the radical. The new strategy was to oxidize the spin-label precursor, a tetramethylpyrroline, after the [3+2] cycloaddition to link the spin-label to the ribose subunit. In Figure 2-3, displays the plan to couple the propargyl chemical with azido-ribose by [3+2] cycloaddition. Then, different kinds of peptide coupling reagents would be tested to combine the carboxylic acid spin-label with triazole ribose. This strategy was hoped to improve the overall yield and ease of accessibility to this class of spin-labeled ribose building block for nucleoside analogs.

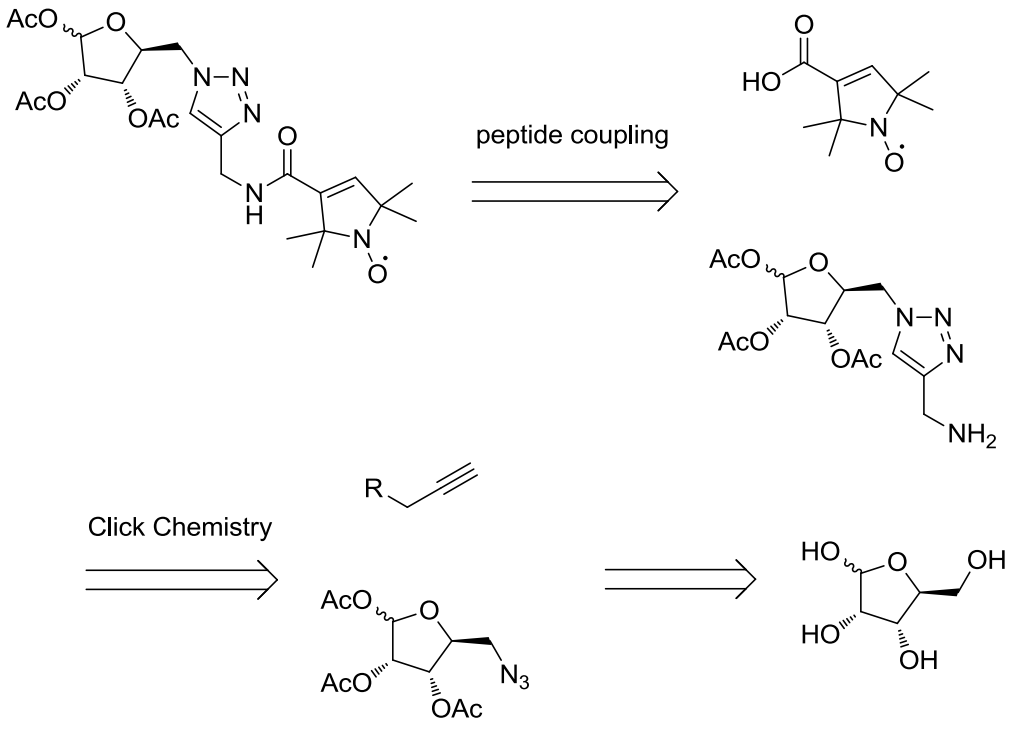


Figure 2-3 Retrosynthesis of modified ribose spin-label

2-3 Materials and Methods

The path A: synthesis of propynyl pyrrole

In the synthetic path A (Figure 2-4), the synthesis of the propynyl pyrrole (2.6) was planned. The propynyl group could be utilized to react with azido-ribose by click chemistry to gain the initial target. First, commercially available 2,2,6,6-tetramethyl-4-piperidone (2.1) was dibrominated by bromine in acidic conditions. 5-dibromo-2,2,6,6-tetramethyl-4-piperidone hydrobromide (2.2) was easily obtained by this method.[25] Second, compound 2.2 was treated under basic NaOMe/MeOH conditions. NaOMe/MeOH conditions were generated in situ by addition of sodium to methanol. This base performed the Favorskii rearrangement to afford the corresponding α,β-unsaturated ester (2.3). The rearrangement could be carried out with other alkoxides to produce numerous esters in good yield.[17] Saponification converted the α,β-unsaturated ester (2.3) to α,β-unsaturated carboxylic acid (2.4).[25] Unfortunately, compound 2.4 was highly water-soluble, as might be expected. Several solvents were investigated for the extraction of this material from aqueous conditions. Finally, warm ethyl acetate was used to extract this material from the aqueous layer after pH adjustment, but the carboxylic acid (2.4) was difficult to obtain in good yields and purity. The aqueous layer was then dried and $LiAlH_4$ was added directly to reduce the carboxylic acid (2.4) to afford α,β-unsaturated alcohol (2.5). Following multiple issues with purification of both 2.4 or 2.5, which could be obtained in crude mixtures, this pathway was abandoned.

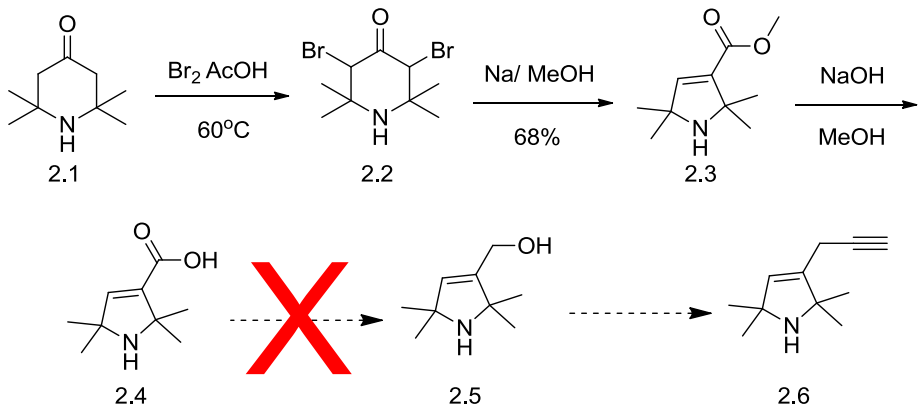


Figure 2-4 Path A : Synthesis of propynyl pyrrole

Path B: Synthesis of propynyl pyrrole.

After path A to synthesize the target compound 2.6 failed to yield efficiently pure and convenient material, an attempt to reduce the polarity of the compound intermediates material was made to aid in purification from aqueous systems. Thus, a protection of the secondary amine was planned (Figure 2-5). In this method, the protecting group not only avoided the compound dissolving in water, but also prevented the amine from participating in unwanted side reactions. Starting from commercially available compound 2.1, the previously mentioned bromination was completed. Then, the Favorskii reaction in sodium methoxide generated the ester 2.3. Diverging from the previous unprotected approach, di-*tert*-butyl dicarbonate was utilized in the presence of triethylamine and DMAP to protect the secondary amine.[30] The tetramethyl substitution of the pyrroline greatly hindered the di-*tert*-butyl dicarbonate to approach the secondary amine. Yields of this protection reaction could not be increased above 35%, even with use of different solvent conditions and at elevated temperature. Then, the ester 2.7 was reduced to the alcohol 2.8 by preparation of $LiBH_4$ by use of LiCl and $NaBH_4$ in ethanol.[26] Unfortunately, this reduction reaction did not go to completion. Only around 20% of the product was observed. With the low yielding transformations and the need for three more

steps to get the final product 2.6, this approach was abandoned for a system that would avoid these issues.

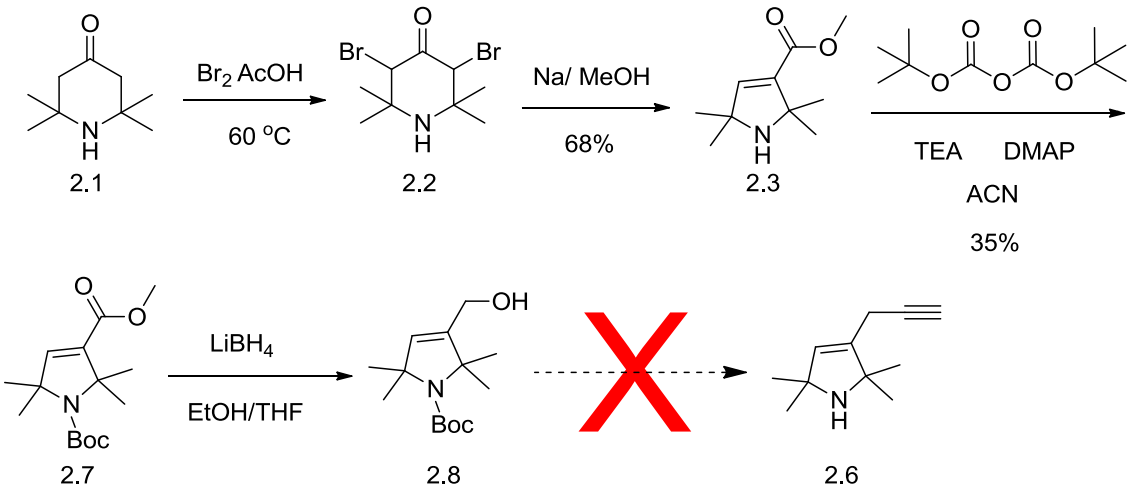


Figure 2-5 Path B : Synthesis of propynyl pyrrole.

Path C: Synthesis of modified ribose.

In path C, the preparation of pyrrole derivatives was put off to a later stage of the synthesis. *D*-ribose was modified to be a suitable compound for coupling with the nitroxide or nitroxide precursor. The 5'-hydroxyl group was selectively protected by trityl chloride and triethylamine in DMF. Then, the remaining hydroxyl groups of trityl ether (2.10) were protected by acetic anhydride in pyridine. Pyridine was not easily removed. Hence, pyridine was evaporated by repeated evaporation of toluene. The organic layer was also washed with 10% $CuSO_4$ solution. Finally, after flash chromatography, all of the pyridine in this reaction was removed. The 5'-position was then selectively deprotected under acidic conditions to yield the triacetyl alcohol (2.12). In this deprotection reaction, Dr. Corder initially tested a number of conditions, trying to increase the yield. The formic acid/ether mixture condition was continued from these early studies. Unfortunately, small amounts of the formylated ribose were found in this reaction. Acetic acid (80%) was also used under high temperature to attempt to removal of the trityl group,

but the results were not better. Therefore, the initial formic acid conditions were used, even with lower than desired yield, ~64%. In the fourth step, the primary alcohol (2.12) was mesylated by methanesulfonyl chloride in dry DCM. Normally, tosylation would be considered to be the first option to generate a good leaving group for the next SN2 reaction. However, tosylation was not suitable for this primary alcohol (2.12). The yield of the tosylation reaction was only 34%. Finally, generation of the the mesylate of compound 2.13 was used to install the azido-ribose (2.14) with sodium azide treatment. [18] A variety of conditions were evaluated in an attempt to increase the yield of this nucleophilic substitution (Table 2-1). After testing several conditions, DMF was found to be the best solvent system used under mild temperatures generating the better yields.[27]

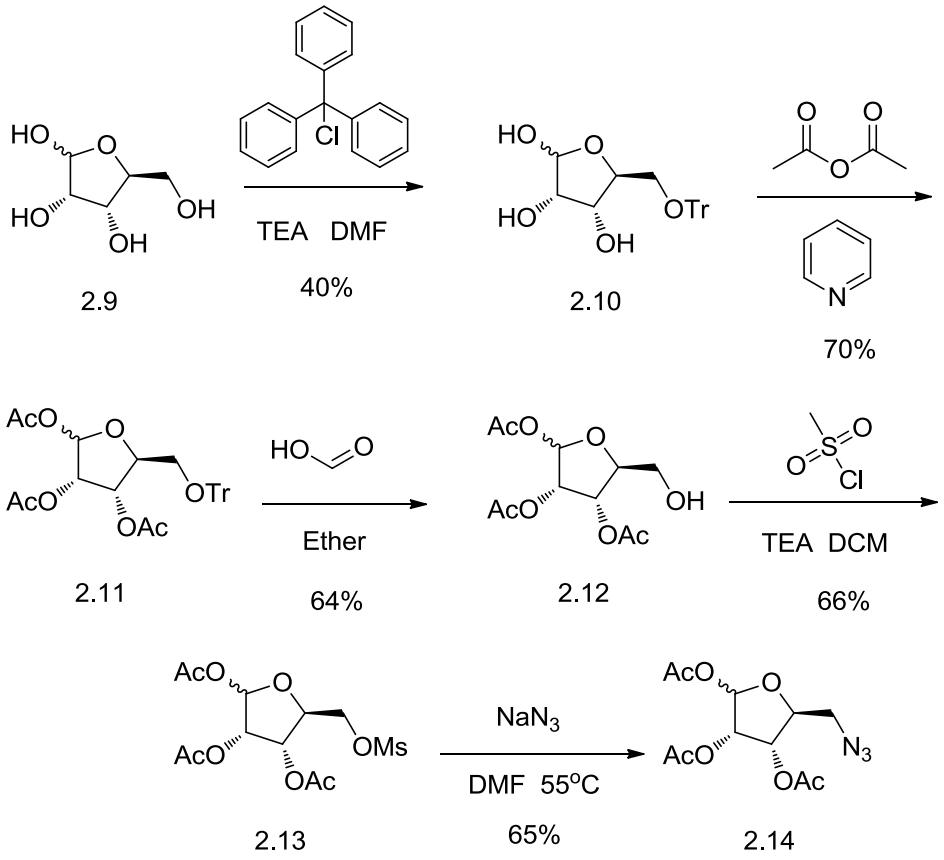


Figure 2-6 Path C : Synthesis of 5'-azido ribose

Table 2-1 Nucleophilic substitution of mesylated ribose

Conditions	Time (h)	Temperature (^{o}C)	Yield (%)
DMF(0.6 M)	18	90	10
DMSO(0.3 M)	18	90	10
DMSO(1.0 M)	18	65	40
DFM(1.0 M)	18	55	65

Synthesis of triazole modified ribose.

To date, the Huisgen 1,3-dipolar cycloaddition between a terminal alkyne and an azide (Figure 2-7) has rapidly become a popular reaction. The robust 1,2,3-triazole compound is generated conveniently. The cycloaddition was first reported by Dimroth at the beginning of the 20th century, but the potential of this reaction and mechanism of this cycloaddition were not fully understood until the 1960s by Huisgen et al.[19]

The cycloaddition was useful because alkyne and azide compounds could be incorporated into different kinds of substituents. However, this reaction suffered from a lack of selectivity to generate a mixture of the 1,4- and the 1,5- disubstituted triazoles. Besides, this transformation needed heating and long reaction times to complete this cycloaddition and the two regioisomers were not easy to be separated by using column chromatography. Many conditions were tested for controlling the regioselectivity without much breakthrough until the discovery of the copper(I)-catalyzed reaction in 2002. The copper(I)-catalyzed condition was able to accelerate this cycloaddition and made this reaction be worked under room temperature or with only moderate heating. Not to mention the copper catalyst generally yielded one of the two regioisomers, namely the 1,4-disubstituted 1,2.3-triazole.[20]

The basic catalytic system for the Huisgen-cycloaddition utilized copper (II) salts (e.g.,

copper sulfate pentahydrate or copper acetate) with sodium ascorbate. The reducing agent reduced copper (II) to copper (I) to enhance the capability of the catalytic species. A mixture of *tert*-butanol and water was often used as solvent. Under the Cu(II) pre-catalyst conditions, it was not necessary to use a base to generate the copper acetylide species. When the cycloaddition was not working in aqueous conditions, several copper(I) salts(e.g., CuI, $CuBr(PPh_3)$) could be utilized in organic solvents(e.g., tetrahydrofuran, toluene, dichloromethane, acetonitrile) and required a nitrogen base(e.g., TEA, DIPEA).[21]

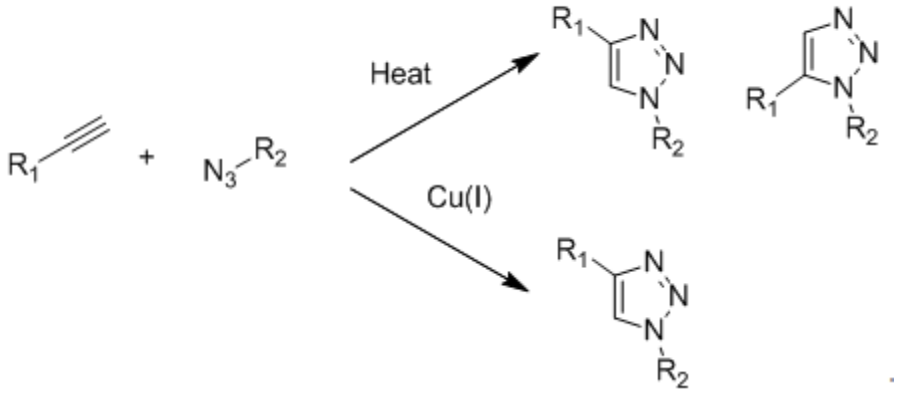
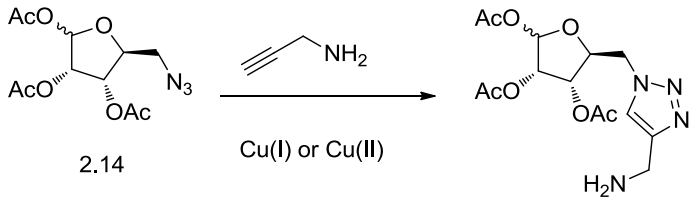


Figure 2-7 The 1,3-dipolar cycloaddition between azides and alkynes under thermal or Cu(I) catalyzed conditions.

Since our early efforts couldn't make the alkynyl pyrrole for the cycloaddition with azido-ribose, direct coupling of the azido-ribose with the alkyne derivate was tried. Then, triazole derivative could be utilized to react with the spin-label. Propargylamine was proposed to be an efficient solution to linking the two groups together, as the resulting amine group could couple with the carboxylic pyrrole spin-label in the next step. The azido-ribose and propargylamine were tested for cycloaddition under varying ligation conditions. (Table 2-2) In the beginning, Cu(II) with the reducing agent was attempted in aqueous condition.[22] However, after running this reaction for several hours, only a small conversion of starting material to the triazole

compound was observed. Thus, a variety of reactions were performed (Table 2-2). Different solvent conditions were tried, such as mixing the organic solvent with water or alcohol, Cu(I) was attempted directly. Finally, heat was increased within reactions that were not initially productive.[23, 24, 28, 29] Eventually, it was discovered that some product was generated in good yields with the uncatalyzed conditions at high temperature in DMF. At this point, a 37% yield was a welcome result. However, it was difficult to purify this product. The triazole amine and propargylamine polarity created an issue for separating the materials by column chromatography. Thus, the amine group of propargyamine was protected. Through this way, the compound could be more easily isolated and purified after cycloaddition. Two different amine protecting reactions were tried.

Table 2-2 1,3-dipolar cycloaddition trials



2.14 2.15

Conditions	Catalysts	NaAsc	Temperature(^{o}C)	Time(h)	Yield(%)
tBuOH/H_2O 1:1 0.5 M	20% $Cu(OAc)_2$	0.4 equiv	rt	16	<10
tBuOH/H_2O 1:1 0.4 M	2% $CuSO_4 \cdot 5H_2O$	0.2 equiv	rt	16	<10
THF:DIPEA 2:1 0.5 M	5% CuCl		rt	16	<10
$CHCl_3$/EtOH/H_2O 9:1:1 0.5 M	5% $CuSO_4 \cdot 5H_2O$	0.2 equiv	rt	16	<10
DMSO:H_2O 9:1 0.5 M	5% $CuSO_4 \cdot 5H_2O$	0.2 equiv	65	16	<10
tBuOH/H_2O 1:1 0.5 M	10% $CuSO_4 \cdot 5H_2O$	0.25 equiv	60	16	<10
THF 0.5 M 2 equiv DIPEA	5% CuI		50	16	<10
Toluene 0.3 M 3 equiv DIPEA	10% CuI		40	16	<10
Toluene 0.5 M			85	16	<10
DMF 0.3 M			100	16	37
neat			110	16	<10

Figure 2.8 shows the successful protection of propargylamine. *N*-Boc protection was attempted first. Initially, an excess of triethylamine was used with the dicarbonate to protect the propargylamine.[30] However, it was discovered that there were many by-products resulting in 35% yield. Also, this reaction couldn't run under room temperature for several hours. The product and starting material were not stable in the room temperature. Therefore, the amount of triethylamine, dicarbonate and reaction time were reduces in a second set of trials. No by-products were observed under these more mild conditions and the yield was raised from 35 % to 66%. Satisfyingly, the second protection strategy that was attempted to protect amine was successful. During nosyl protection of the amine, the yield was 74%.[31] The reaction was also easily followed by TLC and purified.

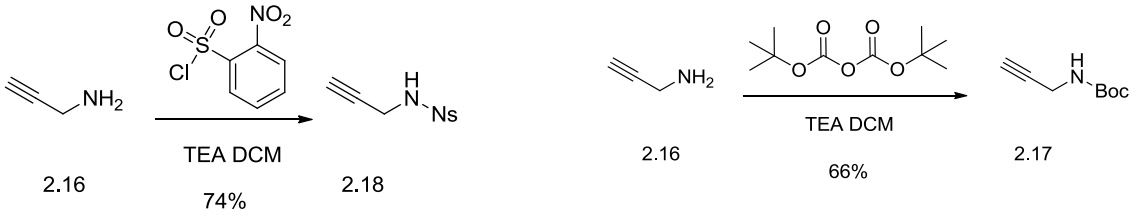


Figure 2-8 Synthesis of protected propargylamine.

After successfully protecting propargylamine, the *N*-Boc-amine and the *N*-Ns-amine were both used to try the 1,3-dipolar cycloaddition. (Figure 2-9) In both of the reactions, Cu(II) conditions were tried to investigate whether the cycloaddition was worked or not. Both cycloadditions were performed nicely in contrast to the previously poor yields, which were found for the unprotected propargylamine. Though the spin-label was not a participant in this reaction, it was the first successful yield of the 1,3-dipolar cycloaddition for this system. With this achievement, we were encouraged to continue on with this current method.[32]

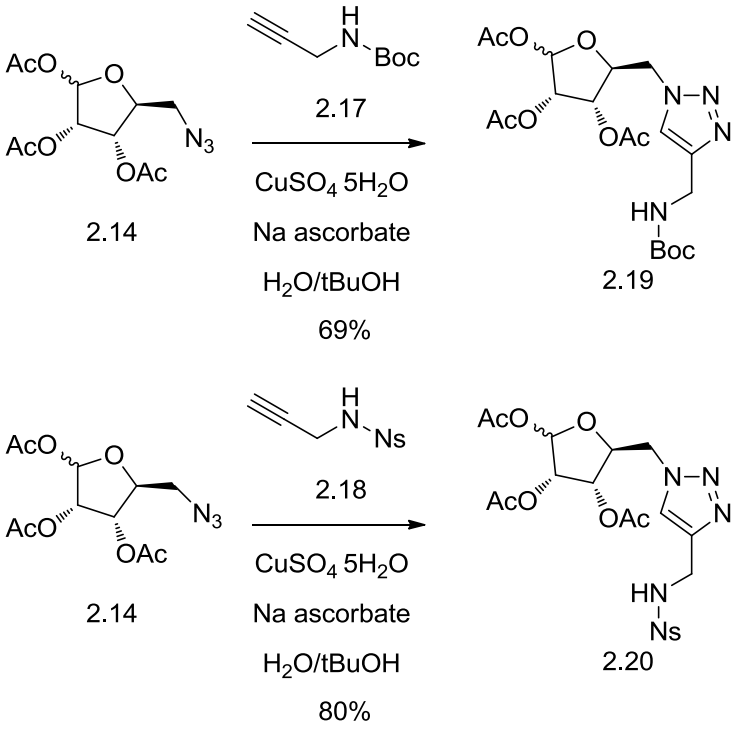


Figure 2-9 Synthesis of triazole protecting amine modified ribose.

Next, amine protecting groups were removed by standard methods to yield common compound 2.15, which would then be utilized to link with the spin-label by various peptide coupling reactions. In first reaction (Figure 2-10), TFA in DCM and HCl in ethyl acetate conditions were both attempted to remove the N-Boc protecting group.[33] The nosyl group in compound 2.20 was removed by standard conditions (thiophenol, K_2CO_3, in acetonitrile) under higher temperature.[34] The conversion of both reactions was promising by TLC analysis. However, compound 2.15 was highly polar and water soluble. At the time of writing this dissertation, purification and extension of this promising pathway was still under investigation and will be discussed in Chapter 3.

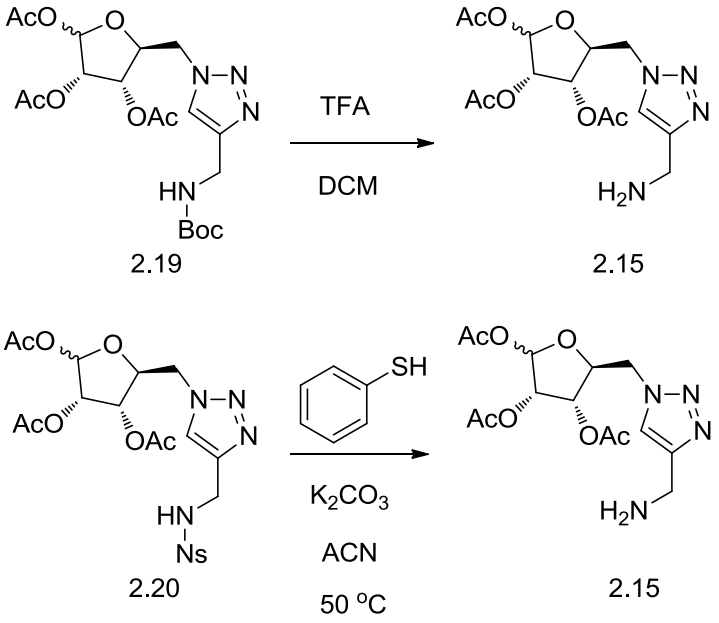


Figure 2-10 Synthesis of triazole amine modified ribose

In order to investigate another method, the carboxylic acid spin-label was prepared first. (Figure 2-11) The starting material, pyrroline ester, was made from commercially available compound 2.1 as described earlier. The oxidation of pyrroline ester was achieved with H_2O_2/Na_2WO_4 in methanol giving the corresponding nitroxide 2.21 with 73% yield. The saponification of nitroxide ester was done nicely by 1 M NaOH in THF and the yield of this reaction was 85%.[35]

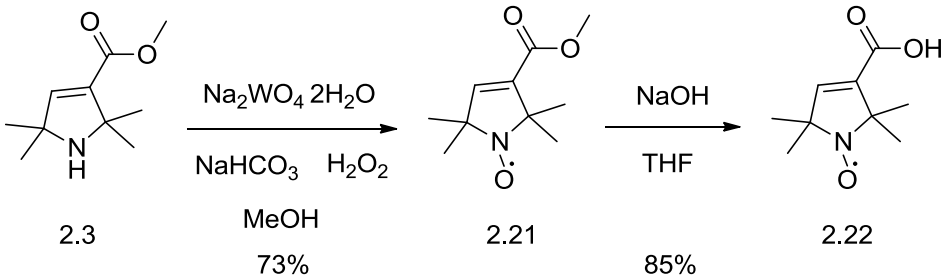


Figure 2-11 Synthesis of carboxylic acid spin-label.

After successfully synthesizing the carboxylic acid nitroxide (2.22), the propargylamine was coupled with the compound 2.22 (Figure 2-12). By this way, the alkyne group of compound 2.23 was poised to perform the cycloaddition with azido-ribose, affording our final target. The yield of this peptide synthesis was interestingly only 41%. Since this reaction was tested one time, it is believed that better conditions could be found to improve the yield in the future.[36]

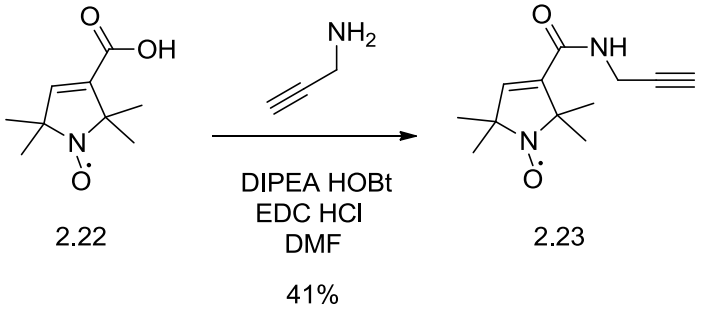


Figure 2-12 Synthesis of propargyl amide nitroxide

In the final step, Cu(I) and Cu(II) catalysts were tested to investigate conditions suitable for cycloaddition. In the CuI condition, the yield was only 45% and some by-products were found, which might influence this reaction. On the contrary, the $CuSO_4$ condition was done nicely and the yield was 75%. (Figure 2-13). This achieved the preparation, although not optimized of our target material.

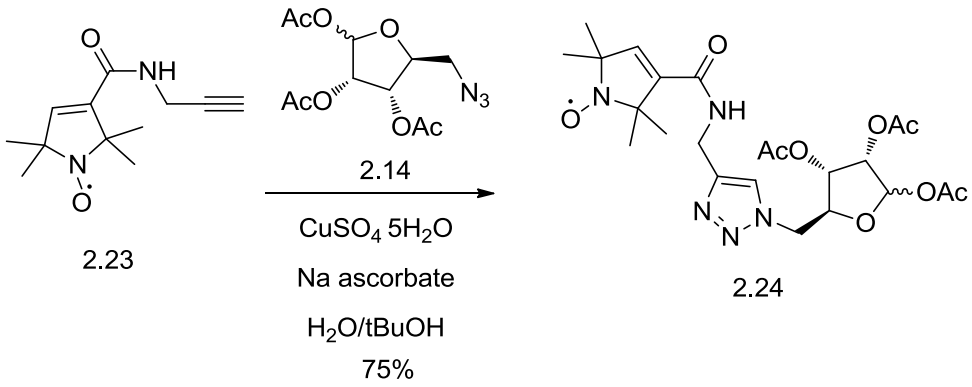


Figure 2-13 Synthesis of triazole modified ribose spin-label.

Chapter 3

Conclusion and Future work

Overall, the final product was successfully prepared with dramatically improved yields of cycloaddition from 5% to 75%. The radical character was proven to not be a major problem to affect the 1,3-dipolar cycloaddition. On one hand, 5 steps were spent to synthesize the azido-ribose (2.14), and the yield of the entire reaction was ~8%. On the other hand, 5 steps were spent to synthesize the propargyl amide nitroxide (2.23), and the yield of total reaction was ~17%. Finally, the convergence of these two pathways resulted in the planned 1,3-dipolar cycloaddition with a 75% yield. Therefore, the target compound was completed in 11 total steps with an overall yield of ~1%.

Even though final product 2.24 was successfully synthesized, it is believed that many reaction can be improved in the future. Furthermore, our work has provided insight for how to best achieve these improved yields. In figure 2-6, the selective deprotection of trityl group should be tried by different acidic condition or ratios of mixtures of solvent and acid. In the peptide coupling reaction, other types of carbodiimde reagents (or acid activating species) as well as addictives can be attempted. For the carbodiimide reagent, *N,N'*-dicyclohexylcarbodimide (DCC) can be attempted; 1-hydroxy-7-azabenzotriazole (HOAt) or 1-hydroxy-6-chlorobenzotriazole (Cl-HOBt) are common addictives: however the nature of these hydroxylamine additives reacting with the spin-label is not entirely known. What's more, in the final reaction (Figure 2-12), the cycloaddition was successfully prepared under a high yield. However, the reducing agent, sodium ascorbate, might reduce the radical of the starting material. In other words, the yield of this reaction might decline due to losing some of the starting material. Thus, we have two options to improve this reaction. On one hand, the equivalents of the reducing agent in comparison to the copper catalyst can be investigated. By this way, additional reducing agent, which might affect the spin-label, can be avoided. However, the reactivity of this cycloaddition might decrease as well. On the other hand, Cu(I) catalysts

can also be investigated through different conditions. Nevertheless, the usages of Cu(I) should certainly be reduce to 5% of the amount of starting material, which might eliminate the problem of acetylenic homocoupling. There is another way to avoid the potential homocoupling of the terminal acetylene species. sterically hindered bases could be employed to stabilize the copper acetylide intermediates and to slow this unwanted side reaction.

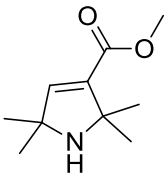
Chapter 4

Experimental section

General Experimental Information.

Reactions requiring anhydrous conditions were performed under an atmosphere of nitrogen or argon in flame or oven-dried glassware. Anhydrous dichloromethane (DCM) was distilled from CaH_2. All other solvents and reagents were purchased from Sigma Aldrich, Acros, and VWR. All reactions were stirred magnetically and monitored by analytical thin layer chromatography using aluminum-backed 0.2 mm silica gel F-254 plates. Detection was performed by UV light (254 nm) and appropriate stains such as phosphomolybdic acid **(**PMA). NMR spectra were recorded on the 300 MHz NMR spectrometer. $_1$H NMR chemical shifts was referenced to $CDCl_3$ (7.26 ppm). $_{13}$C NMR chemical shifts were referenced to $CDCl_3$ (77.23 ppm). Data were reported as follows: chemical shift multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, bs = broad singlet). In order to gain the $_1$ H spectrum of radical compounds, a reducing agent, such as phenylhydrazine, was commonly used to reduce nitroxide radicals to hydroxylamine. Generally, phenylhydrazine (0.1 mmol) was first added to $CDCl_3$ (0.6 mL). Then, the solution was added to approximately 10 mg of the radical compound. $_1$H NMR of phenylhydrazine (300 MHz, $CDCl_3$) 7.25 (2H, m), 6.83 (3H, m), 5.18 (1H, bs), 3.57 (2H, bs).

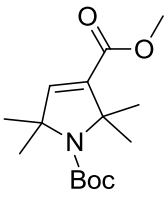
Analysis and characterization of synthesized compounds



2.3

2,2,5,5-Tetramethyl-3-carbmethoxypyrroline. (2.3)

The sodium methoxide was prepared by slowly adding sodium (290 mg, 12.8 mmol) to anhydrous methanol (6.4 mL) at 0 °C . Dibromo-piperidone 2.2 (1.0 g, 3.2 mmol) was added in 5 portions. The reaction mixture was warmed to room temperature and stirred overnight. The water (15 mL) was added to the reaction, and the product was extracted by using ethyl acetate (10 mL x 3). Then, the organic extract was dried with magnesium sulfate and concentrated *in vacuo* to afford 0.4 g yellow liquid (2.2 mmol, 68%). ^{1}H NMR (300 MHz, $CDCl_3$) 6.63 (1H, s), 3.74 (3H, s), 1.40 (6H, s), 1.28 (6H, s). ^{13}C NMR (75 MHz, $CDCl_3$) 164.6, 149.2, 139.1, 65.9, 63.5, 51.4, 30.0, 29.9.

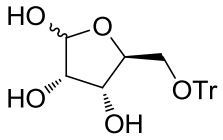


2.7

1-N-Boc-2,2,5,5-Tetramethyl-3-carbmethoxypyrroline. (2.7)

2,2,5,5-Tetramethyl-3-carbmethoxypyrroline 2.3 (110 mg, 0.6 mmol), DMAP (3.67 mg, 0.03 mmol), TEA (0.17 ml, 1.2 mmol) and $(Boc)_2O$ (0.13 g, 0.6 mmol) were added together in acetonitrile (2 ml, 0.3 M) for 4 h. The reaction mixture was heated to 40 ^{o}C. The reaction was cooled to room temperature. The water (15 mL) was added to the reaction, and the product was extracted by using ethyl acetate (10 mL x 3). Then, the organic extract was dried with magnesium sulfate and concentrated *in vacuo* to afford 59.5 mg (0.21 mmol, 35%) ^{1}H NMR

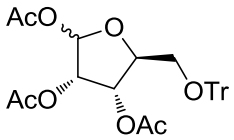
(300 MHz, $CDCl_3$) 6.53 (1H, s), 3.75 (3H, s), 1.70 (3H, s), 1.55 (3H, s), 1.51 (3H, s), 1.51 (6H, s), 1.49 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) 163.0, 145.3, 144.8, 135.8, 134.8, 84.6, 70.3, 69.6, 67.7, 67.1, 51.7, 27.6, 27.2, 25.1, 24.5.



2.10

1,2,3-trihydroxyl-5-*O*-trityl-D-ribofuranose. (2.10)

D-ribose (3.0 g, 20 mmol), trityl chloride (5.8 g, 21 mmol), and triethylamine (5.7 mL, 0.04 mol) were added in dimethylformamide (50 mL). The reaction was allowed to stir at room temperature overnight. The mixture was slowly quenched by adding water (20 mL). The compound of interest was extracted by using DCM (10 mL x 3). The organic extract was washed with brine (10 mL). Then, the organic extract was dried with magnesium sulfate and concentrated *in vacuo*. The crude mixture was purified by flash chromatography (25% ethyl acetate: hexane) to get 3.13 g of a clear viscous liquid (8 mmol, 40%). ^{13}C NMR (125 MHz, $CDCl_3$) 143.7, 128.7, 128.0,127.2, 96.9, 87.0, 83.2, 72.1, 71.8, 64.1.

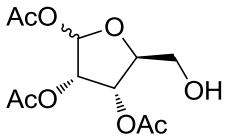


2.11

1,2,3- Tri-O-acetyl-5-O-trityl-D-ribofuranose. (2.11)

1,2,3-trihydroxyl-5-O-trityl-D-ribofuranose 2.10 (3.13 g, 8 mmol) was dissolved in pyridine (8.0 mL) and cooled to 0 °C. Acetic anhydride (5.3 mL) was slowly added in the solution. The reaction was allowed to slowly warm to room temperature and stir for 4 hours. The reaction was concentrated in *vacuo* via azeotroping with toluene (10 mL x 3). The crude product was

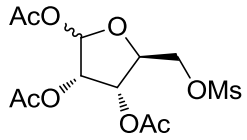
dissolved in DCM, and washed with saturated aqueous sodium bicarbonate (10 mL) and brine (10 mL). Then, the organic extract was washed with saturated $CuSO_4 \cdot 5H_2O$ and concentrated in *vacuo*. The crude mixture was purified by flash chromatography (20% ethyl acetate: hexane) to afforded 2.9 g (5.6 mmol, 70%) 1,2,3- Tri-O-acetyl- 5-O-trityl- D-ribofuranose, a viscous clear liquid. $_1$H NMR (300 MHz, $CDCl_3$) 7.47 (6H, m), 7.26~7.28 (9H, m), 6.21 (1H, s), 5.51 (1H, m), 5.46 (1H, d), 4.33 (1H, m), 3.35 (1H, dd), 3.16 (1H, dd), 2.09 (3H, s), 2.00 (3H, s), 1.98 (3H, s). $_{13}$C NMR (75 MHz, $CDCl_3$) 169.7, 169.6, 169.5, 143.8, 98.4, 86.8, 81.0, 74.4, 70.9, 63.3, 21.2.



2.12

1,2,3- Tri-O-acetyl-5-hydroxyl-D-ribofuranose. (2.12)

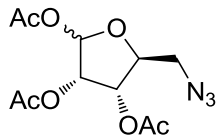
1,2,3- Tri-O-acetyl-5-O-trityl-D-ribofuranose 2.11 (2.9 g, 5.6 mmol) was dissolved in ether (4.5 mL), then cooled to 0 °C. Formic acid (6.7 mL; 3:2 formic acid: ether ratio) was slowly added to the solution. The reaction was allowed to warm to room temperature and stir for 3 hours. The mixture was slowly quenched by adding the water (15 mL). The crude product was then extracted by using ethyl acetate (10 mL x 3), and washed with brine (5 mL). Then, the organic extract was dried with magnesium sulfate and concentrated in *vacuo* The crude mixture was purified by flash chromatography (50% ethyl acetate: hexane) to gain 0.95 g (3.6 mmol, 64%) 1,2,3- Tri-O-acetyl-5-hydroxyl-D-ribofuranose, a clear viscous liquid. $_1$H NMR (300 MHz, $CDCl_3$) 6.14 (1H, s), 5.40 (1H, m), 5.35 (1H, m) 4.24 (1H, m), 3.81(1H, m), 3.65 (1H, m), 2.12 (6H, s), 2.09 (3H, s). $_{13}$C NMR (75 MHz, $CDCl_3$) 170.1,169.6, 169.4, 98.3, 82.4, 74.6, 69.9, 61.9, 21.2, 20.6, 20.6.



2.13

1,2,3- Tri-O-acetyl-5-O-mesyl-D-ribofuranose. (2.13)

1,2,3- Tri-O-acetyl-5-hydroxyl-D-ribofuranose 2.12 (0.95 g, 3.6 mmol) was dissolved in dry DCM (11.9 mL) and pyridine (0.34 mL), then cooled to 0 °C. Mesyl chloride (0.4 mL, 5.3 mmol) was then added dropwise. The reaction was allowed to warm to room temperature and stir for 3 hours. The mixture was slowly quenched by adding the water (15 mL). The crude product was then extracted by using DCM (10 mL x 3). The organic extract was dried with magnesium sulfate and concentrated in *vacuo* to afford 0.84 g (2.38 mmol, 66%) 1,2,3- Tri-O-acetyl-5-O-mesyl-D-ribofuranose 2.13. $_{1}$H NMR (300 MHz, $CDCl_3$) 6.14 (1H, s), 5.36 (2H, m), 4.43-4.39 (3H, m), 3.05 (3H, s), 2.14 (3H, s), 2.13 (3H, s), 2.11 (3H, s). $_{13}$C NMR (75 MHz, $CDCl_3$) 169.8, 169.5, 169.2, 98.1, 79.2, 74.1, 70.2, 68.8, 37.9, 21.0, 20.6, 20.5.

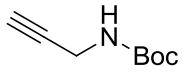


2.14

1,2,3- Tri-O-acetyl-5-azido-D-ribofuranose. (2.14)

1,2,3-Tri-O-acetyl-5-O-mesyl-D-ribofuranose 2.13 (0.75 g, 2.13 mmol) was dissolved in DMF (2.1 mL). Sodium azide (0.4 g, 6.35 mmol) was added, and the reaction was heated to 60 °C for 18 hours. Upon cooling, the reaction was diluted with DCM. The crude product was then extracted with DCM (10 mL x 3). The organic extract was dried with magnesium sulfate and concentrated in *vacuo* to afford 0.29 g (0.95 mmol, 45%) 1,2,3-Tri-O-acetyl-5-azido-D-ribofuranose 2.14. $_{1}$H NMR (300 MHz, $CDCl_3$) 6.15 (1H, s), 5.41-5.33 (2H, m), 4.30 (1H, m), 3.64 (1H, dd), 3.28 (1H, dd), 2.12 (3H, s), 2.11 (3H, s), 2.05 (3H, s). $_{13}$C NMR (75 MHz, $CDCl_3$)

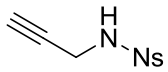
169.8, 169.5, 169.3, 98.1, 80.5, 74.4, 70.4, 51.5, 21.1, 20.6, 20.5.



2.17

N-Boc-propargylamine (2.17)

Propargylamine (200 mg, 3.63 mmol), TEA (0.5 ml, 3.63 mmol) and di-tert-butyl dicarbonate (0.8 g, 3.63 mmol) were added together in DCM (12 ml, 0.3 M). The solution was stirred at the room temperature for 4 h. Water (15 mL) was added to quench the reaction and the crude product was extracted by using DCM (10 mL x 3). Then, the organic extract was dried with magnesium sulfate and concentrated in *vacuo* to afford 370 mg (2.38 mmol, 66%) N-Boc-propargylamine. $_1$H NMR (300 MHz, $CDCl_3$) 4.81 (1H, bs), 3.90 (2H, s), 2.20 (1H, m,), 1.42 (9H, s). $_{13}$C NMR (75 MHz, $CDCl_3$):155.5, 80.5, 71.1, 30.6, 28.3.

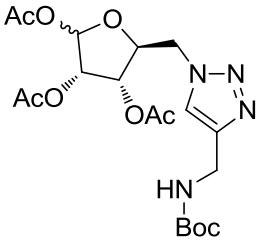


2.18

2-nitro-N-(propynyl)benzenesulfonamide (2.18)

Triethylamine (0.5 ml, 3.6 mmol), DCM (6ml, 0.6 M), and propargylamine (0.23 ml, 3.6 mmol) were added together. The solution was cooled to 0 ^{0}C and then treated with 2-nitrobenzenesulfonyl chloride (0.8 g, 3.6 mmol) slowly over 15 min. The reaction was warmed to room temperature for 3 h, and quenched with water (10 ml). The crude product was extracted by using DCM (10 mL x 3) and washed with brine (10 mL).The organic extract was dried with magnesium sulfate and concentrated in *vacuo* to afford 660 mg (2.75 mmol, 75%) 2-nitro-N-(propynyl)benzenesulfonamide. $_1$H NMR (300 MHz, $CDCl_3$) 8.20 (1H, m), 7.91 (1H, m), 7.72-7.79 (2H, m), 5.70(1H, bs), 4.01(1H, dd),1.97 (1H, t). $_{13}$C NMR (75 MHz, $CDCl_3$):134.0, 133.1,

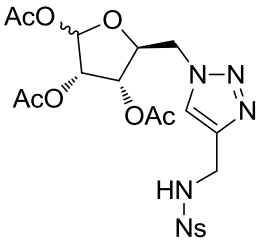
131.7, 73.4, 33.5.



2.19

1,2,3- Tri-O-acetyl-5-triazolyl-methyl-Boc-amine (2.19)

The azido sugar (80 mg, 0.27 mmol) and N-boc-propargylamine (50 mg, 0.32 mmol) were dissolved in a mixed solution of n-BuOH/H_2O (1:1) (0.53 mL, 0.5 M). Sodium ascorbate (9.7 mg, 0.05 mmol) and $CuSO_4 \cdot 5H_2O$ (6.1 mg, 0.025 mmol) were added. The reaction was stirred at room temperature overnight. The crude product was extracted by using ethyl acetate (10 mL x 3). The organic phase was dried with $MgSO_4$ and concentrated under reduced pressure to afford (84 mg, 69%)1,2,3- Tri-O-acetyl-5-triazolyl-methyl-Boc-amine. $_1$H NMR (300 MHz, $CDCl_3$) 7.60 (1H, s), 6.10 (1H, s), 5.22-5.26 (2H, m), 4.99 (1H, b), 4.65 (2H, b), 4.47-4.54 (3H, m), 4.37 (1H, b), 2.08 (3H, s), 2.05 (3H, s), 2.03 (3H, s), 1.50 (3H, s),1.48 (3H, s),1.42 (3H, s).

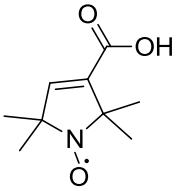


2.20

1,2,3- Tri-O-acetyl-5-triazolyl-methyl-Ns-amine (2.20)

The azido sugar (50 mg, 0.17 mmol) and N-Ns-propargylamine (44 mg, 0.18 mmol) were dissolved in a mixed solution of n-BuOH/H_2O (1:1) (0.53 mL, 0.5 M). Sodium ascorbate (6.6 mg, 0.03 mmol) and $CuSO_4 \cdot 5H_2O$ (4 mg, 0.016 mmol) were added. The reaction was stirred at

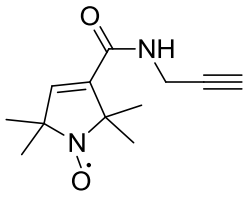
room temperature overnight. The crude product was extracted by using ethyl acetate (10 mL x 3). The organic phase was dried with $MgSO_4$ and concentrated under reduced pressure to afford 71.9 mg (0.13mmol , 80%) 1,2,3- Tri-O-acetyl-5-triazolyl-methyl-Ns-amine. $_1$H NMR (300 MHz, $CDCl_3$) 8.06 (1H, m), 7.82 (1H, m), 7.70-7.72 (2 H, m), 7.65 (1H, s), 6.24 (1H, b), 6.07 (1H, s), 5.10-5.25 (2H, m), 4.66-4.70 (1H, m), 4.60 (1H, m), 4.38-4.41 (3H, m), 2.12 (3H, s), 2.09 (3H, s), 2.02 (3H, s). $_{13}$C NMR (75 MHz, $CDCl_3$):169.9, 169.4, 169.0, 148.0, 143.6, 133.8, 133.6, 133.0, 131.1, 125.5, 124.0, 98.1, 79.4, 73.9, 70.9, 52.2, 31.1, 21.0, 20.6, 20.5.



2.22

2,2,5,5- Tetramethyl-3-carboxypyrroline-1-oxyl (2.22)

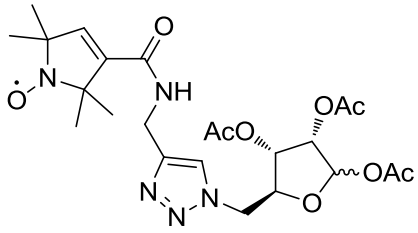
2,2,5,5-Tetramethyl-3-carbmethoxypyrroline-1-oxyl (0.2 g, 1 mmol) was added to 1 M sodium hydroxide (1.5 mL) and THF (2 mL) solution at 0 °C. The reaction was stirred for overnight. The solution was then concentrated *in vacuo* by half, and acidified with 3 M HCl. The product was extracted by ethyl acetate. The resulting filtrate was then concentrated *in vacuo* to give 0.158 g (0.86 mmol, 85%) 2,2,5,5- Tetramethyl-3-carboxypyrroline-1-oxyl. $_1$H NMR (300 MHz, $CDCl_3$) 6.52 (1H, s), 1.56 (6H, s), 1.48 (6H, s).



2.23

2,2,5,5- Tetramethyl-3-pyrroline-1-oxyl-3-propargylamide (2.23)

The EDC · HCl (0.35 g, 1.8 mmol) was added in a mixture of propargylamine (0.116 ml, 1.8 mmol) and HOBt (278 mg, 1.8 mmol) in DMF (9 mL) under an ice bath. Stir the mixture for 15 min, and remove the ice bath. Then, the 2,2,5,5- Tetramethyl-3-carboxypyrroline-1-oxyl (2.22) (278 mg, 1.5 mmol) and DIPEA (0.8 mL, 4.5 mmol). were added into the reaction. The reaction should be run at RT for overnight. The crude product was extracted by ethyl acetate. The resulting filtrate was then concentrated *in vacuo* to give 0.136 g (0.6 mmol, 41%) 2,2,5,5-Tetramethyl-3-pyrroline-1-oxyl-3-propargylamide (2.23). $_1$H NMR (300 MHz, $CDCl_3$) 7.37 (1H, s), 4.14 (2H, m), 2.17 (1H, s), 1.41 (3H, s), 1.32 (3H, s).



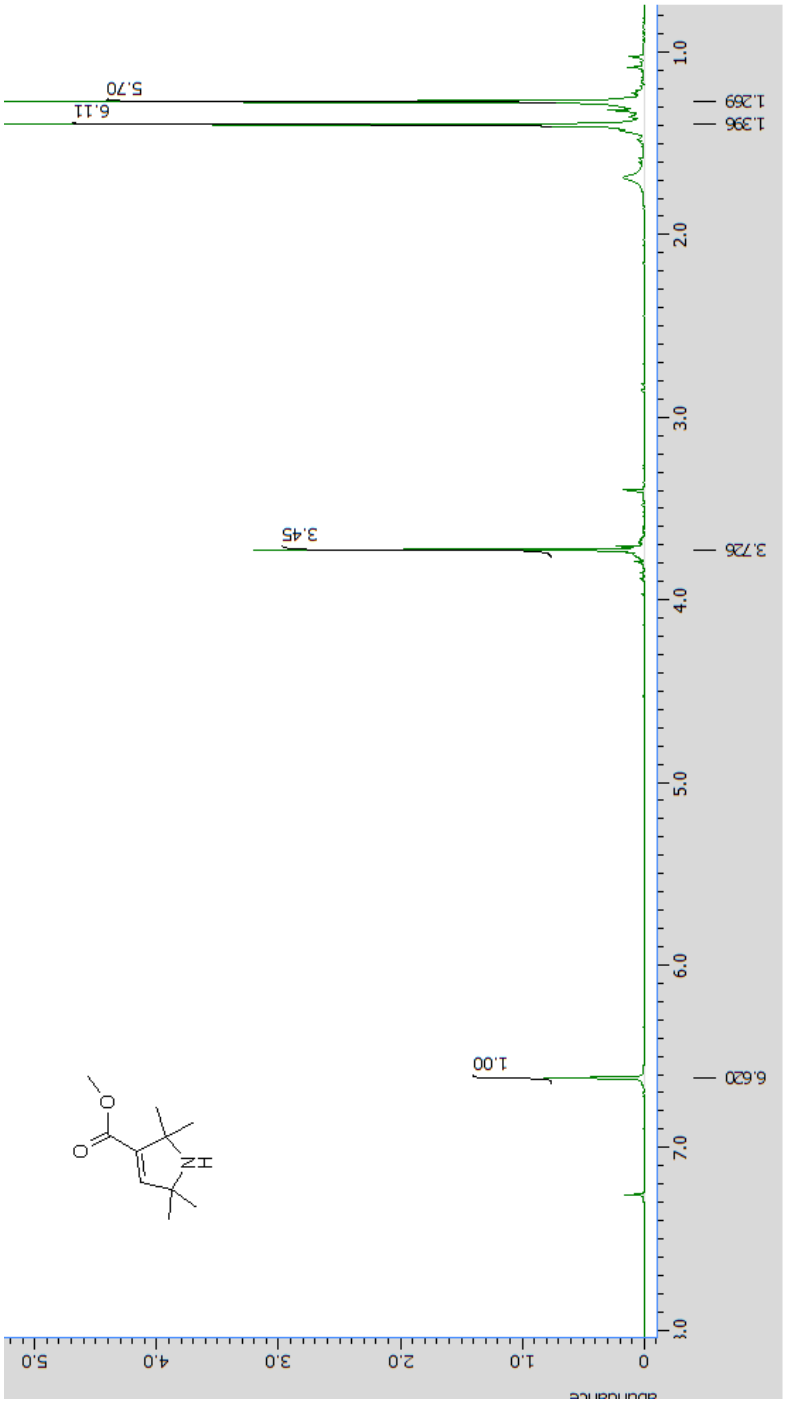
2.24

The azido sugar (40 mg, 0.13 mmol) and 2,2,5,5- Tetramethyl-3-pyrroline-1-oxyl-3-propargylamide (32 mg, 0.15 mmol) were dissolved in a mixed solution of n-BuOH/H_2O (1:1) (0.44 mL, 0.3 M). Sodium ascorbate (3.9 mg, 0.02 mmol) and $CuSO_4 \cdot 5H_2O$ (3.3 mg, 0.13 mmol) were added. The reaction was stirred at room temperature overnight. The crude product was extracted by ethyl acetate. The organic phase was dried with $MgSO_4$, filtered and concentrated under reduced pressure to afford (52 mg, 75%) compound 2.24. $_1$H NMR (300

MHz, $CDCl_3$) 7.67 (1H, s), 7.35 (1H, s), 6.10 (1H, s), 5.28 - 5.21 (2H, m), 4.71 - 4.59 (4H, m), 4.53 - 4.47 (2H, m), 2.10 (3H, s), 2.08 (3H, s), 2.04 (3H, s), 1.74 (6H, s), 1.27 (6H, s). ^{13}C NMR (75 MHz, $CDCl_3$):169.8, 169.4, 168.9,163.9, 144.5,139.2, 128.4, 123.7, 98.2, 79.8, 73.9, 71.6, 71.5, 70.9, 52.2, 34.9, 24.7, 24.5, 21.1, 20.6.

Appendix A

NMR spectrum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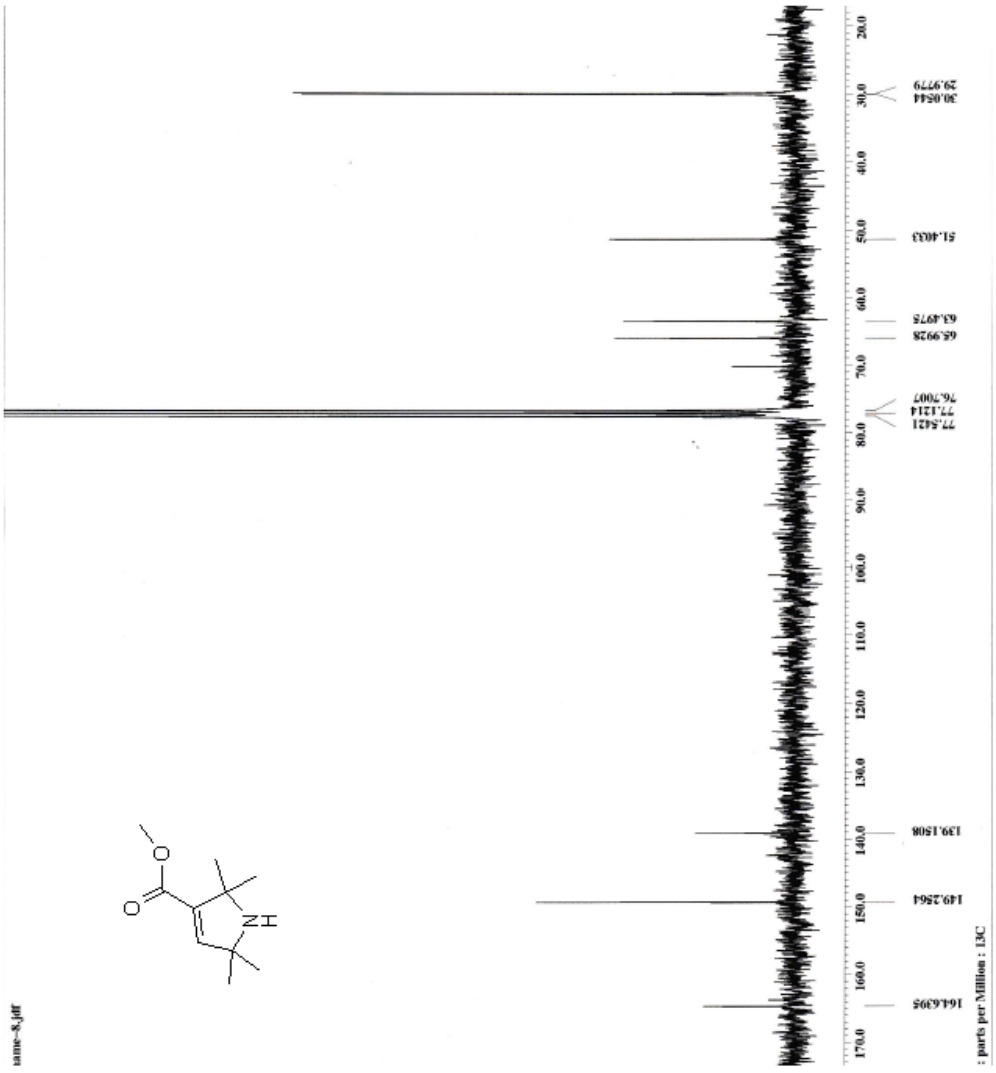
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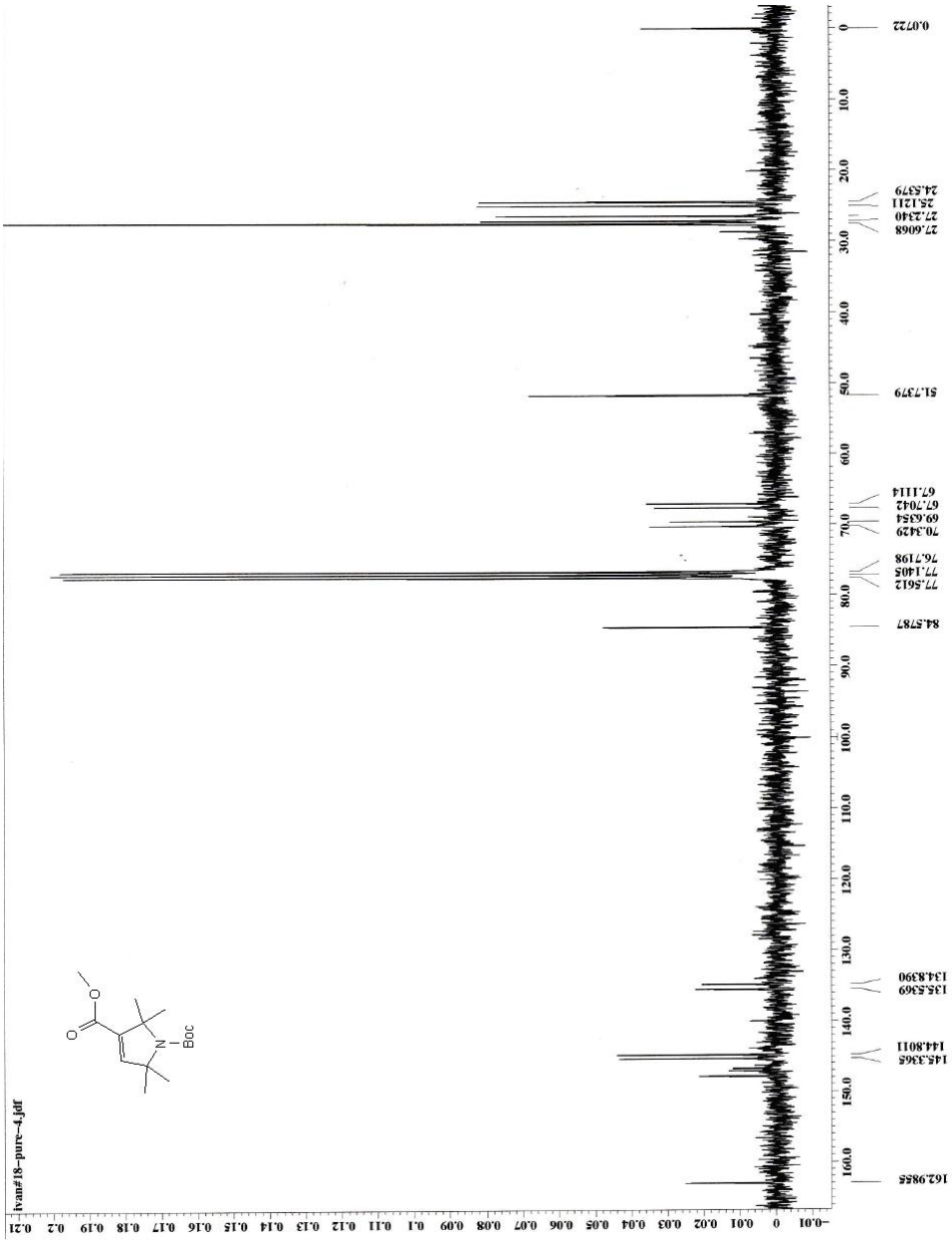
29.9779
: parts per Million : 13C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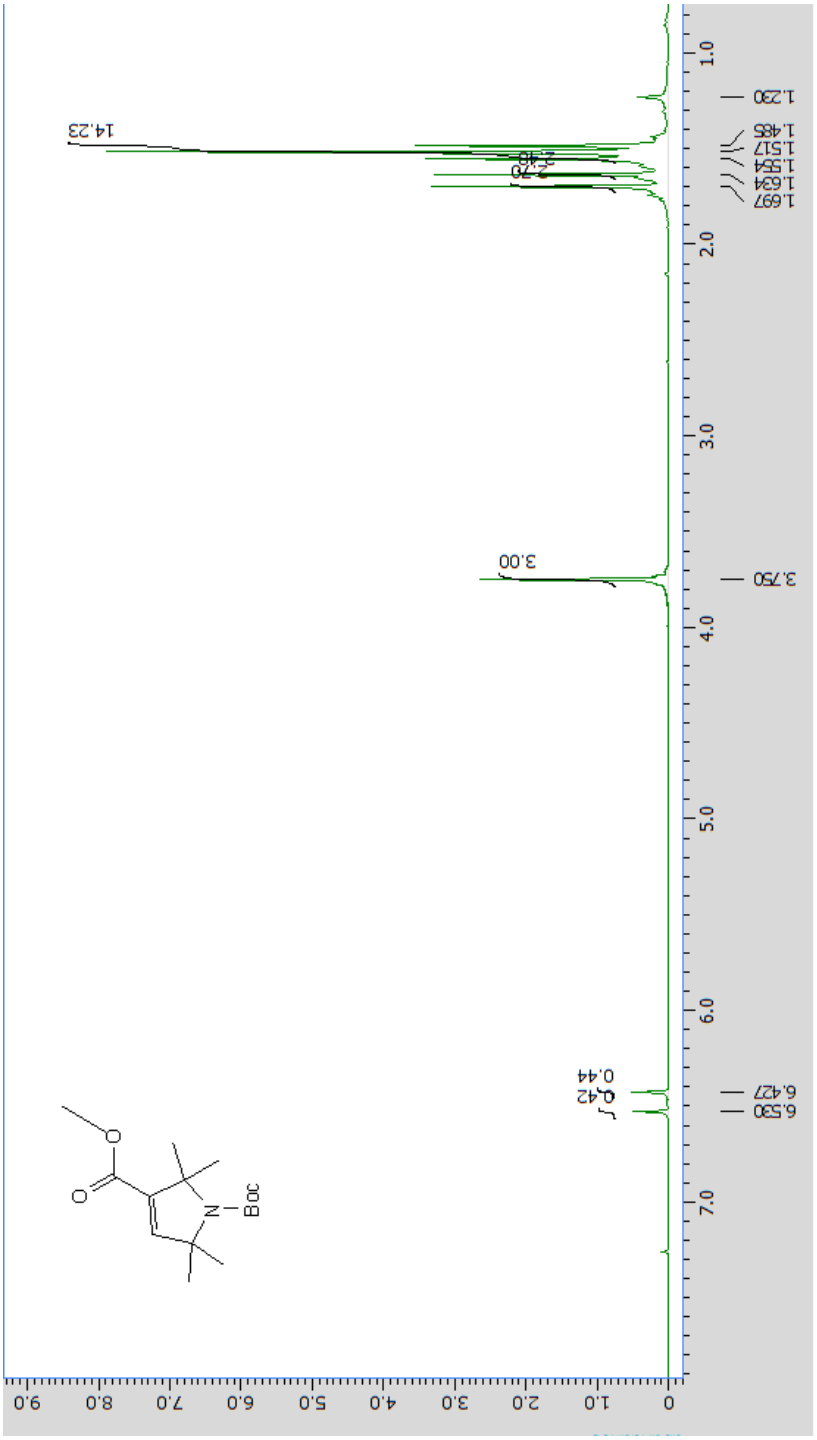
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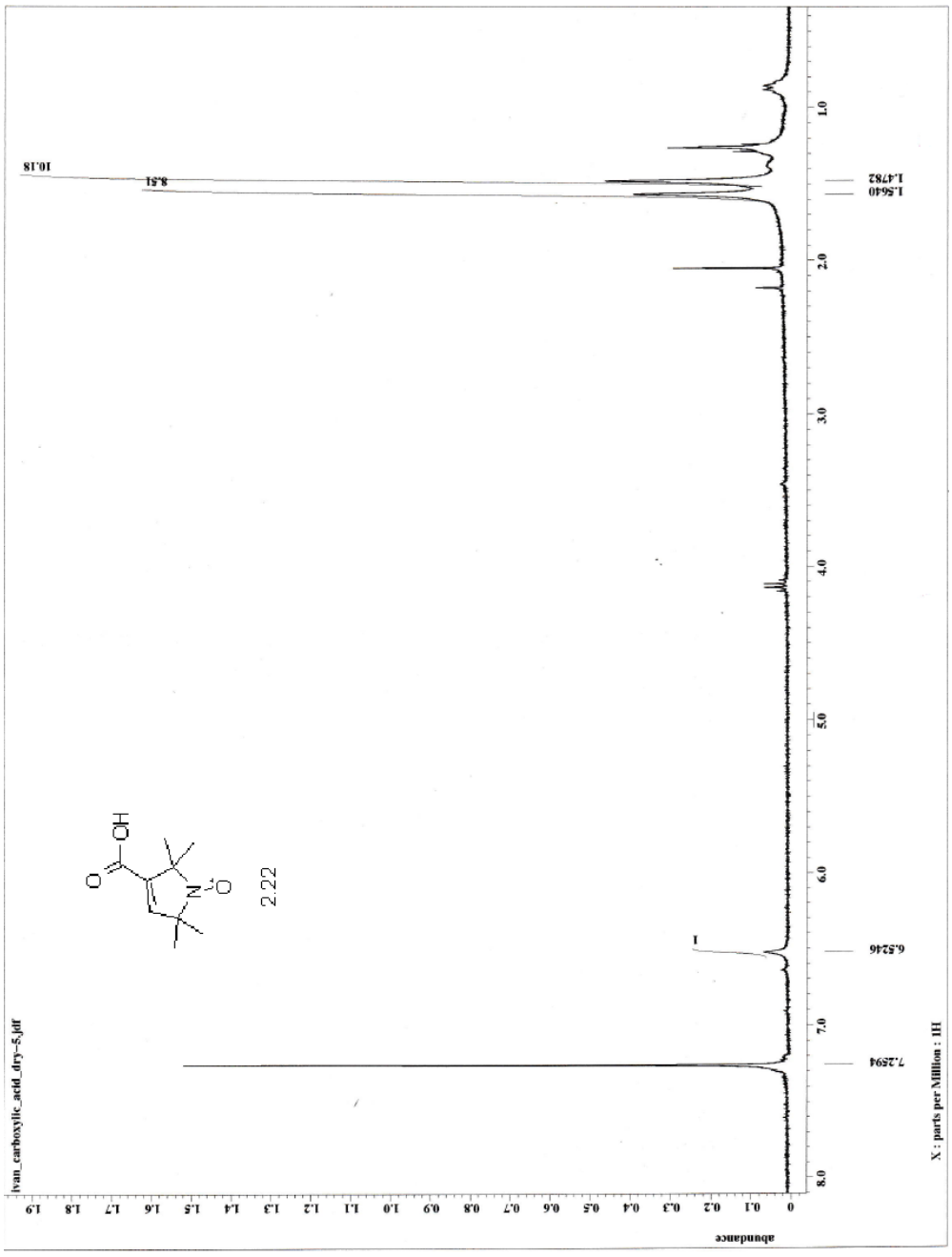
Boc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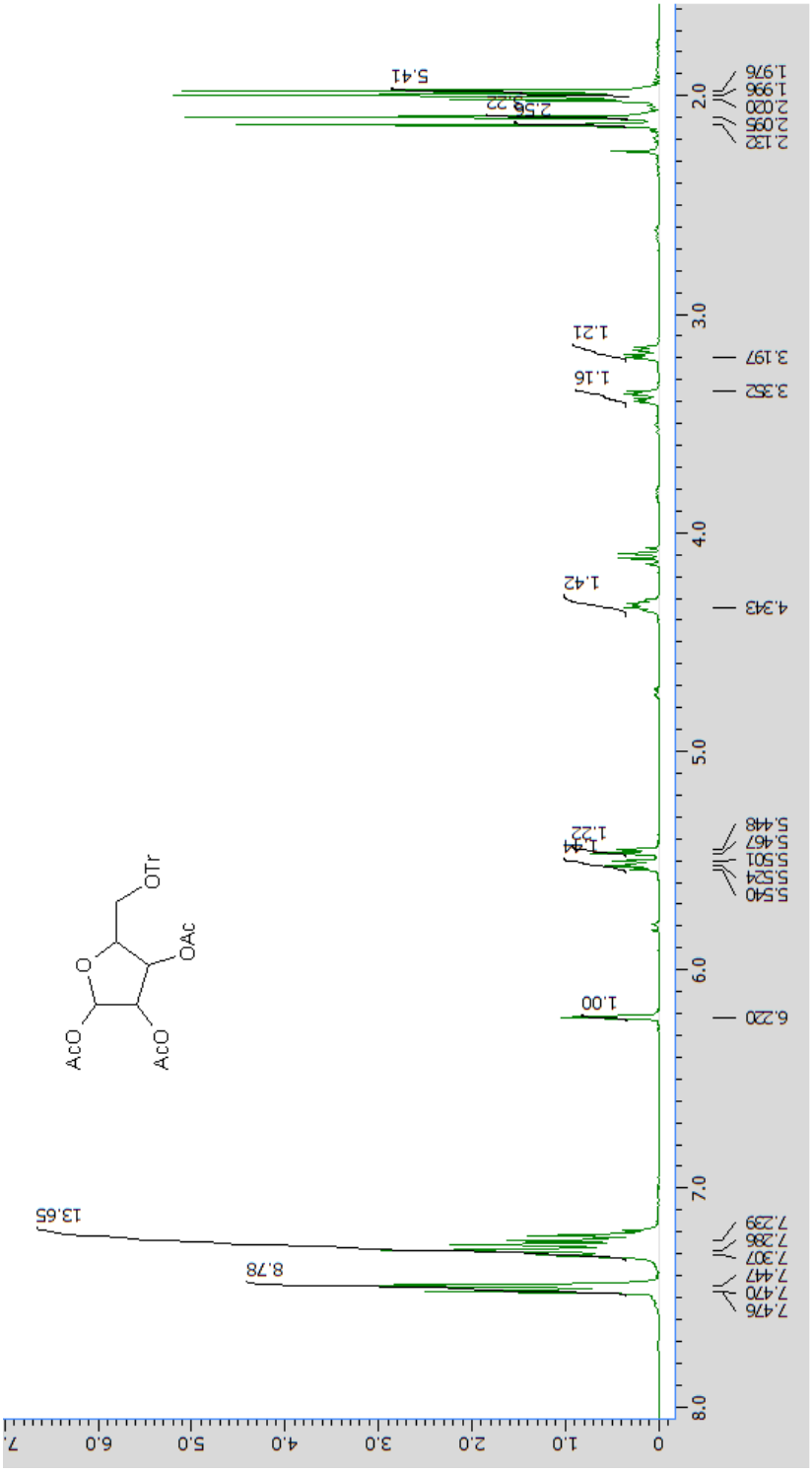
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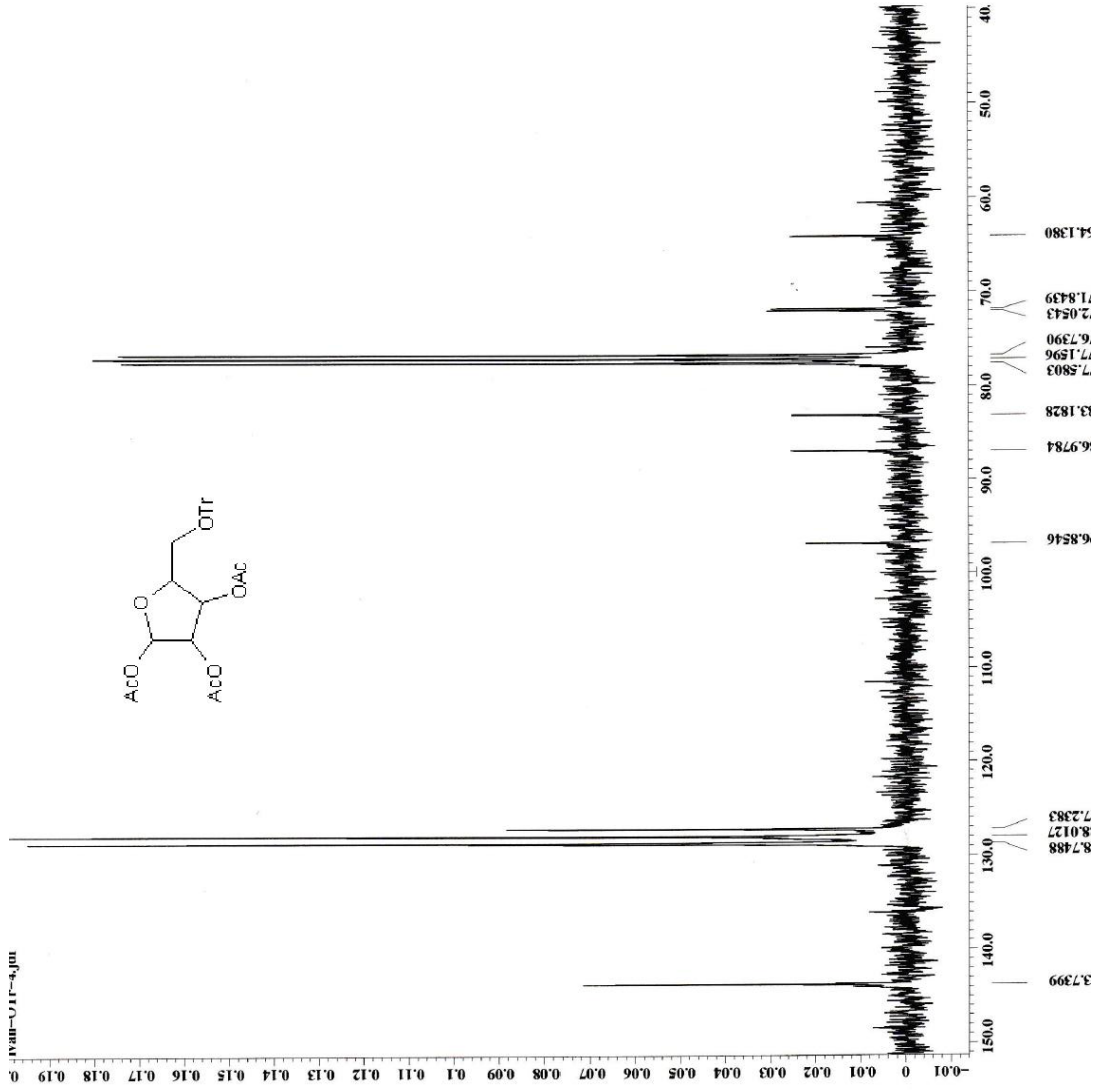
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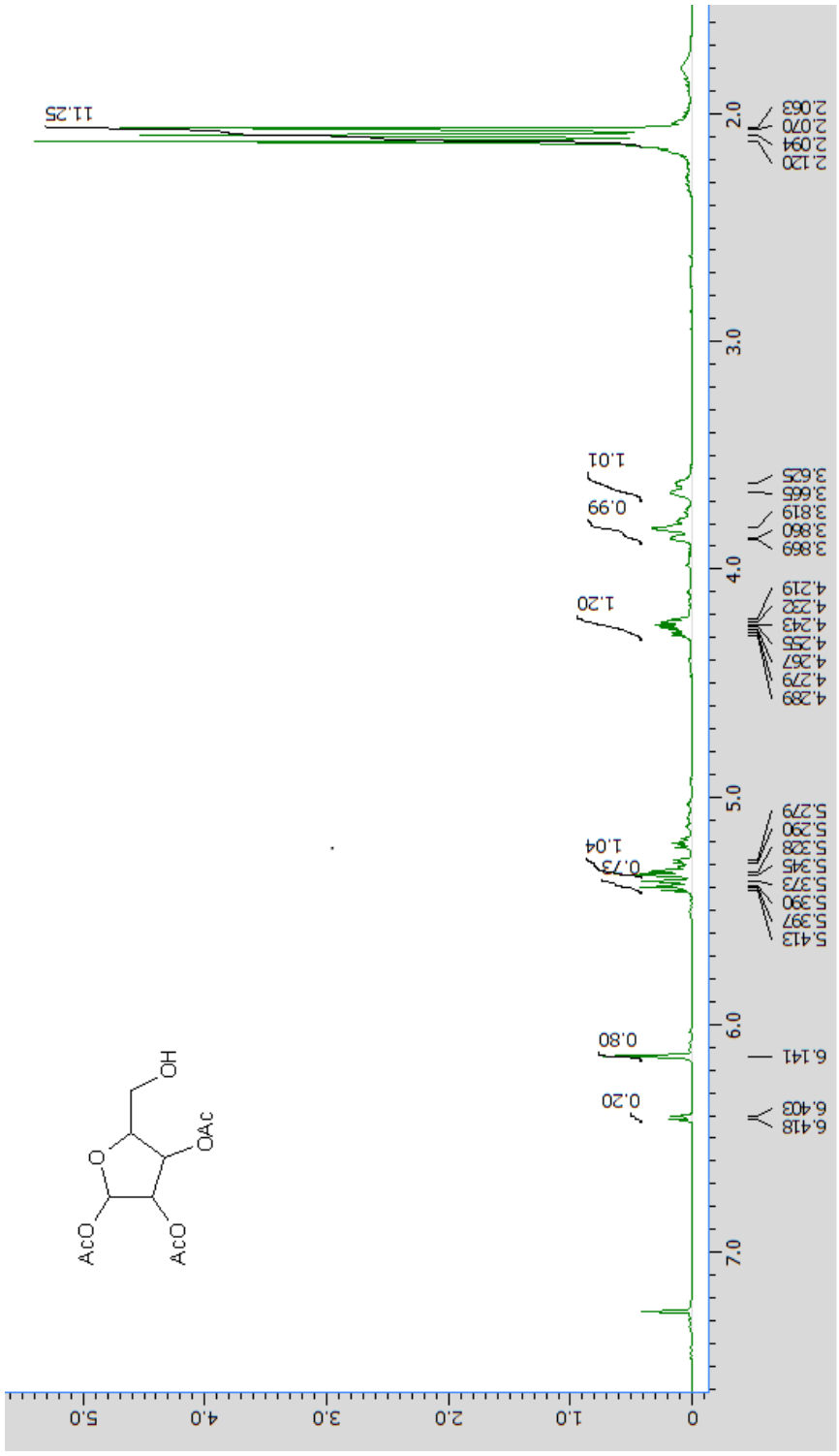
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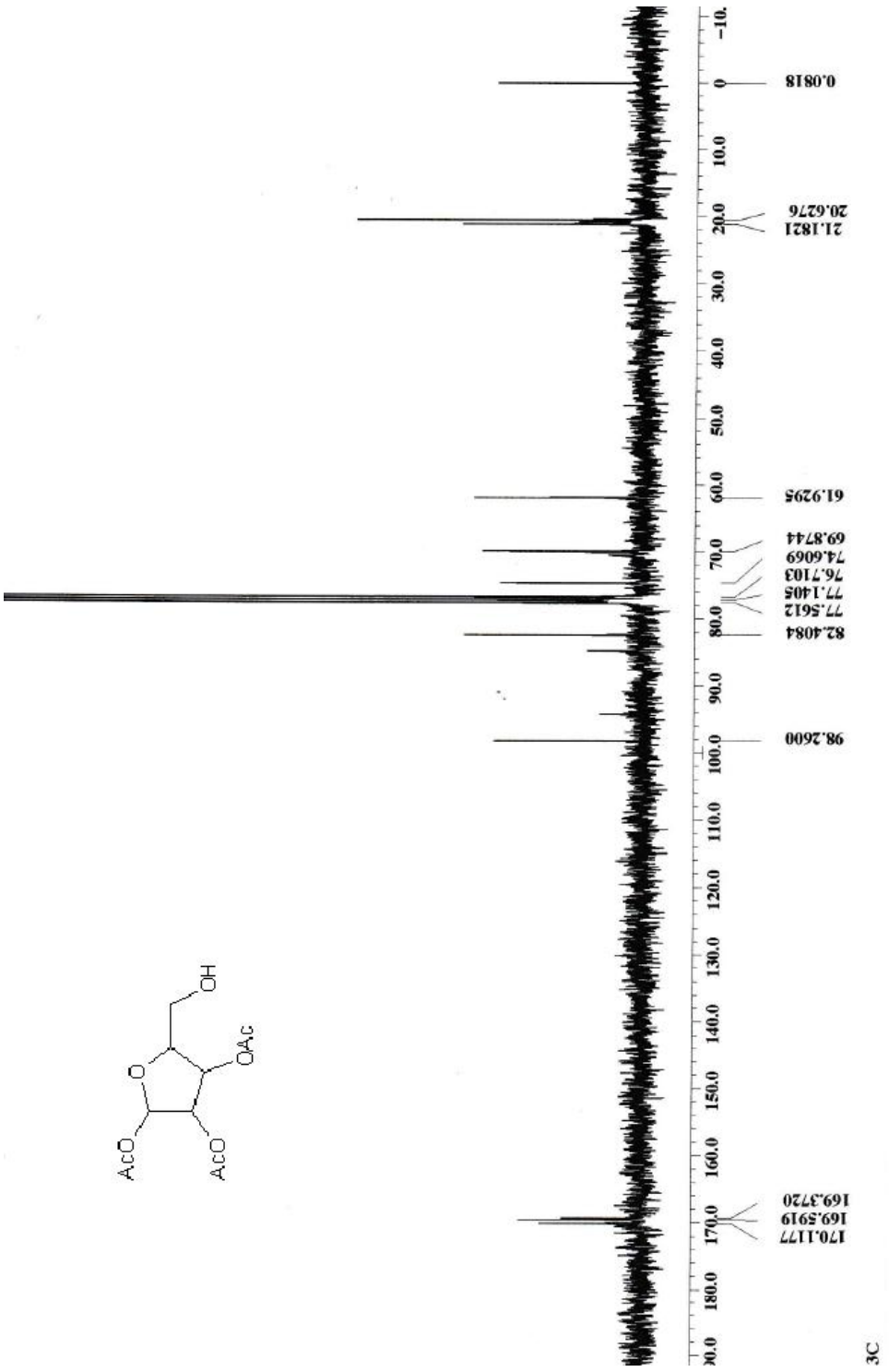
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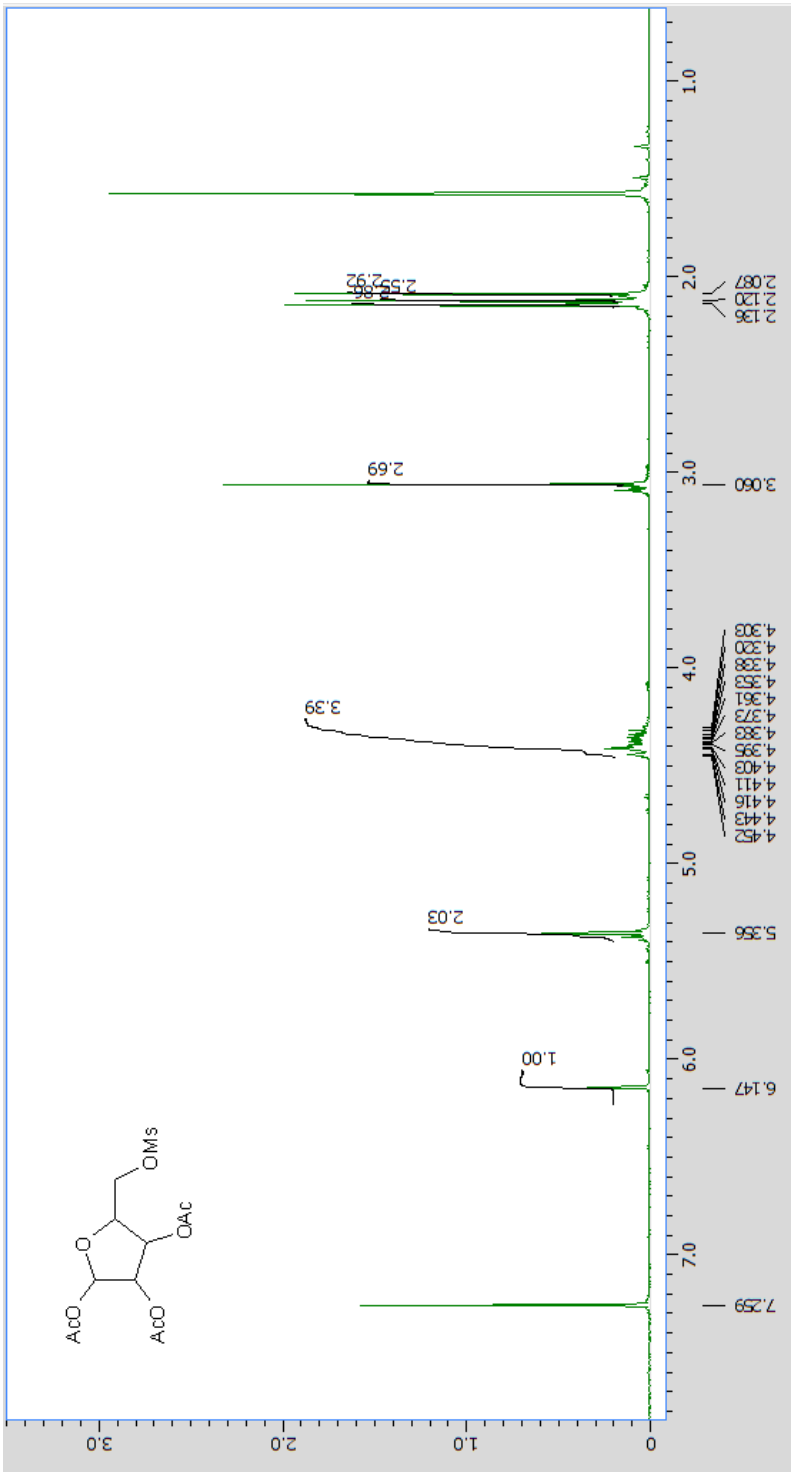
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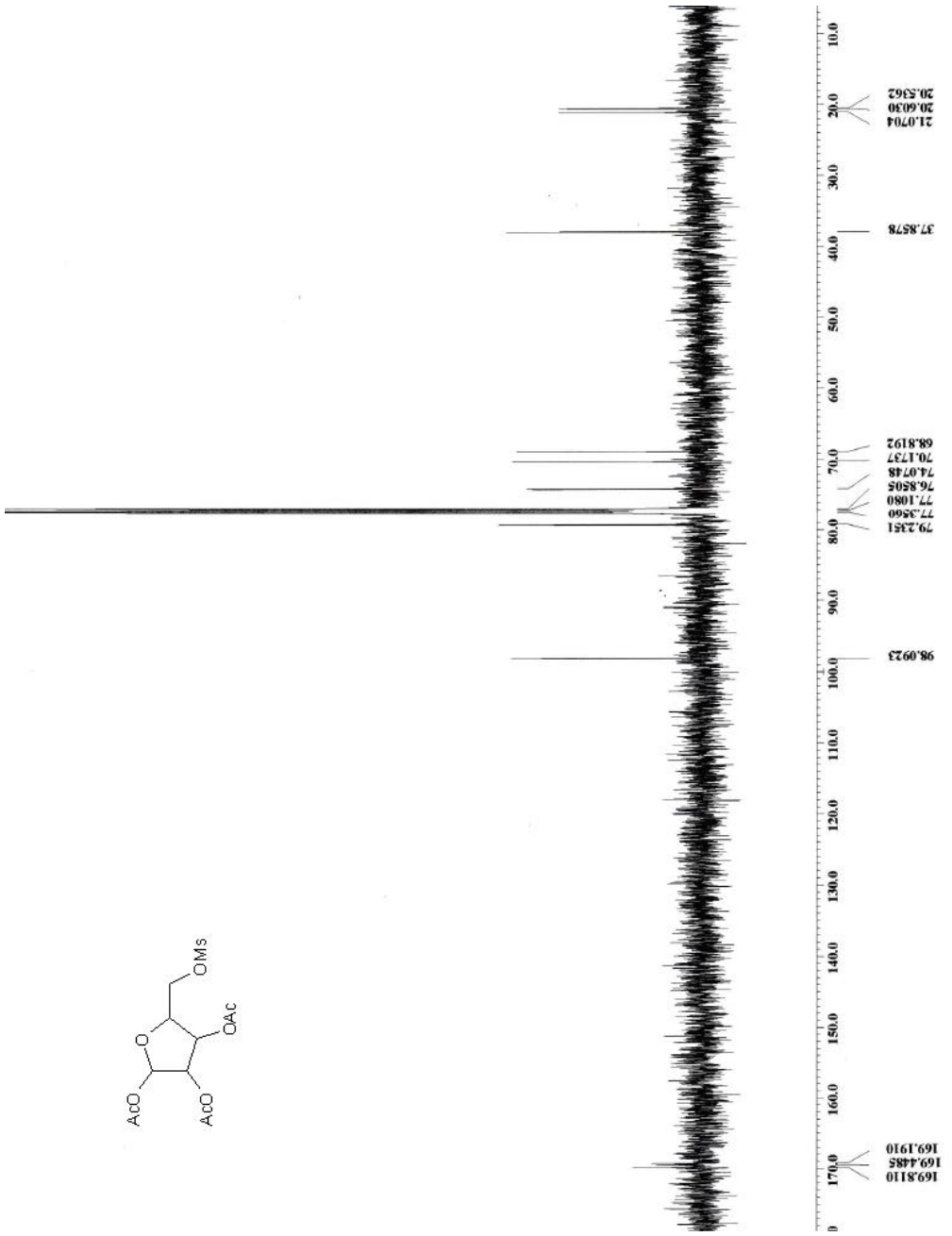
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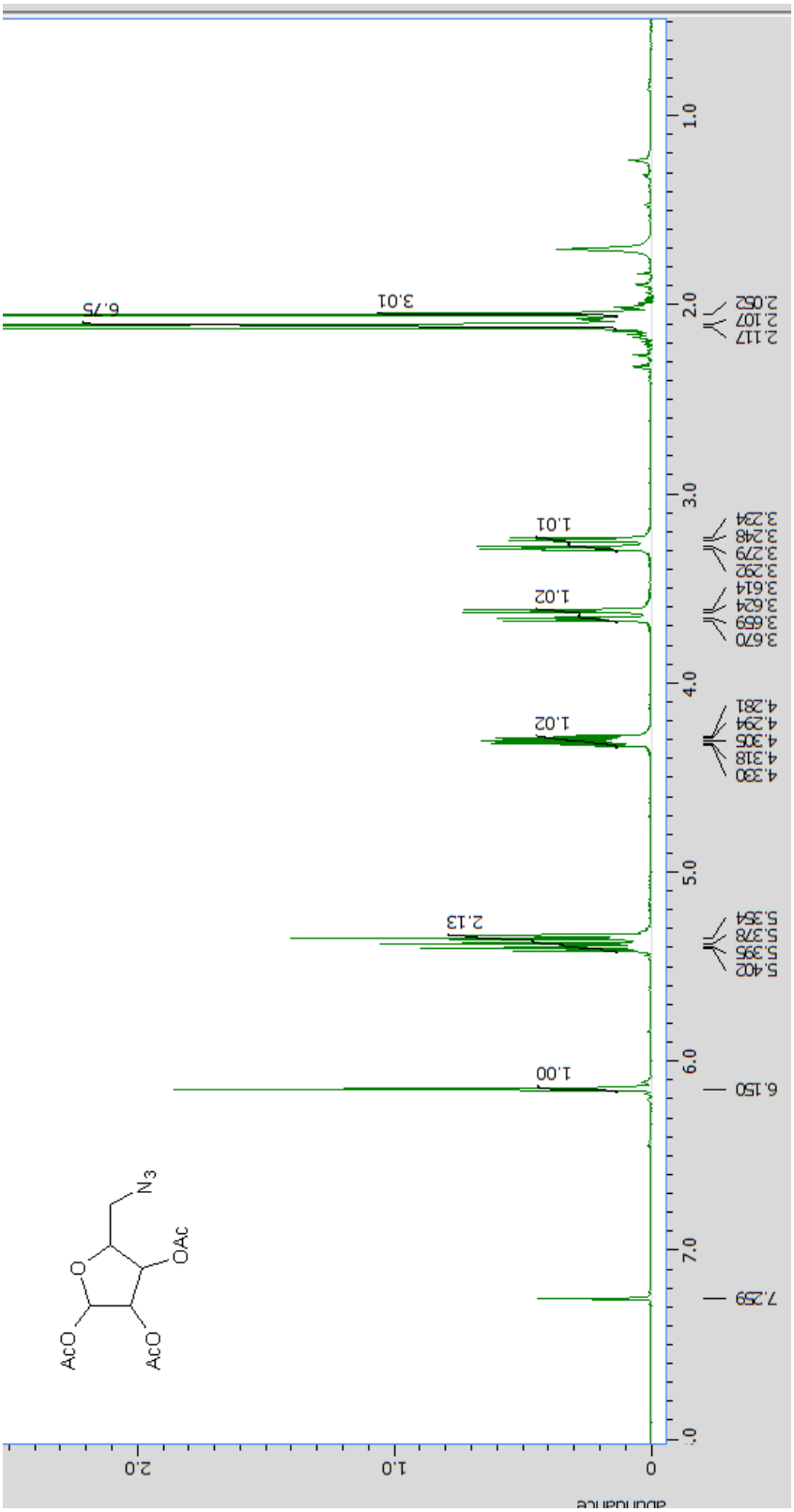
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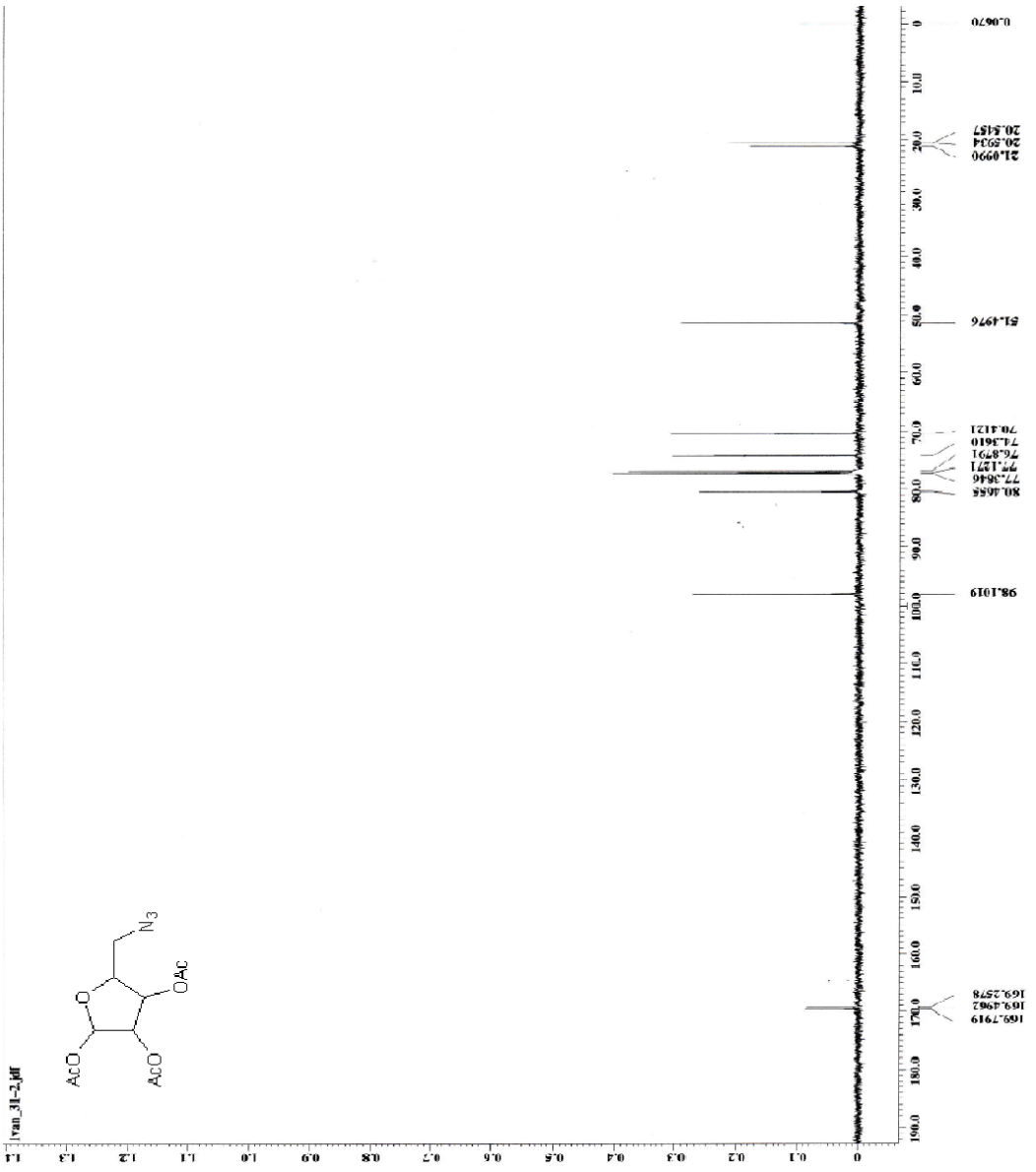
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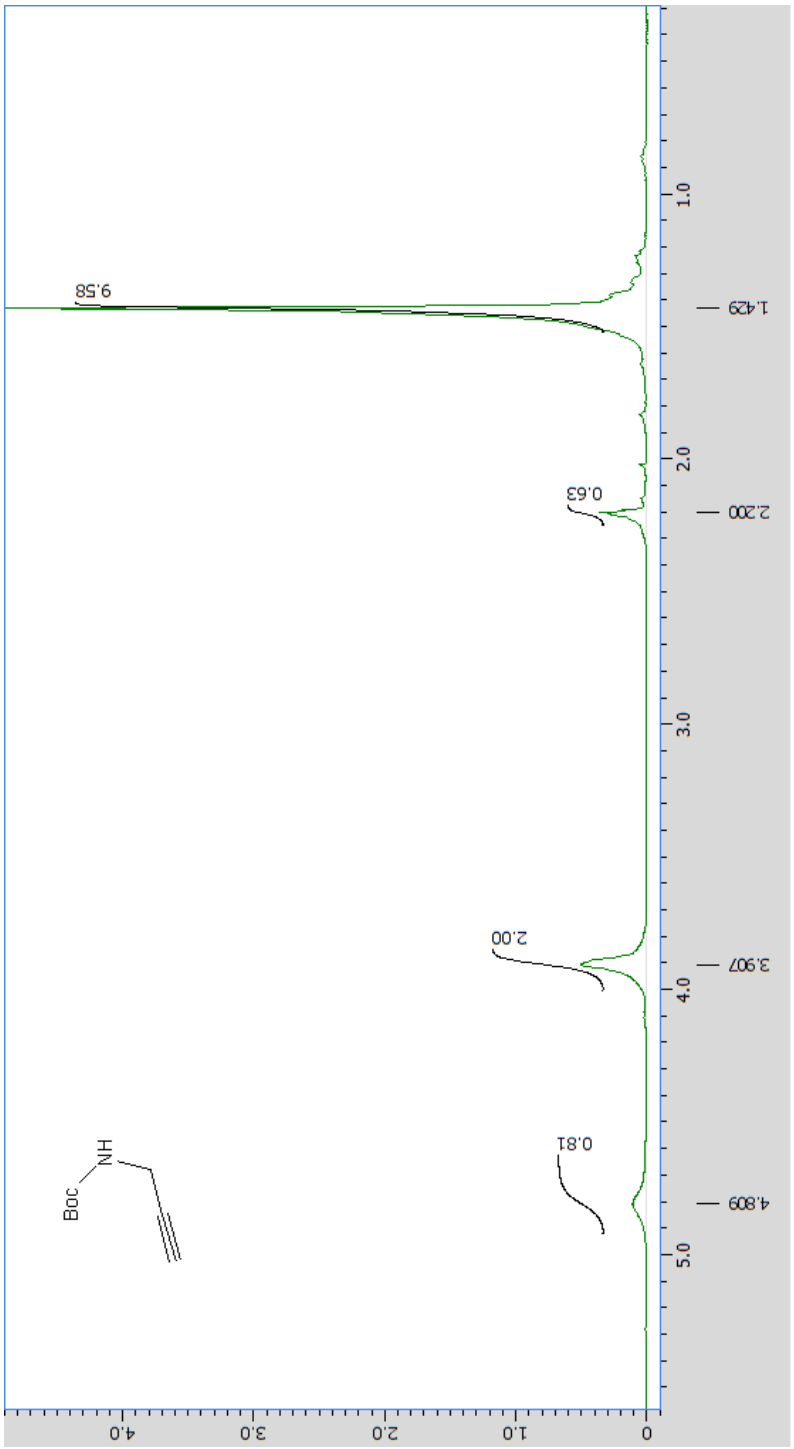
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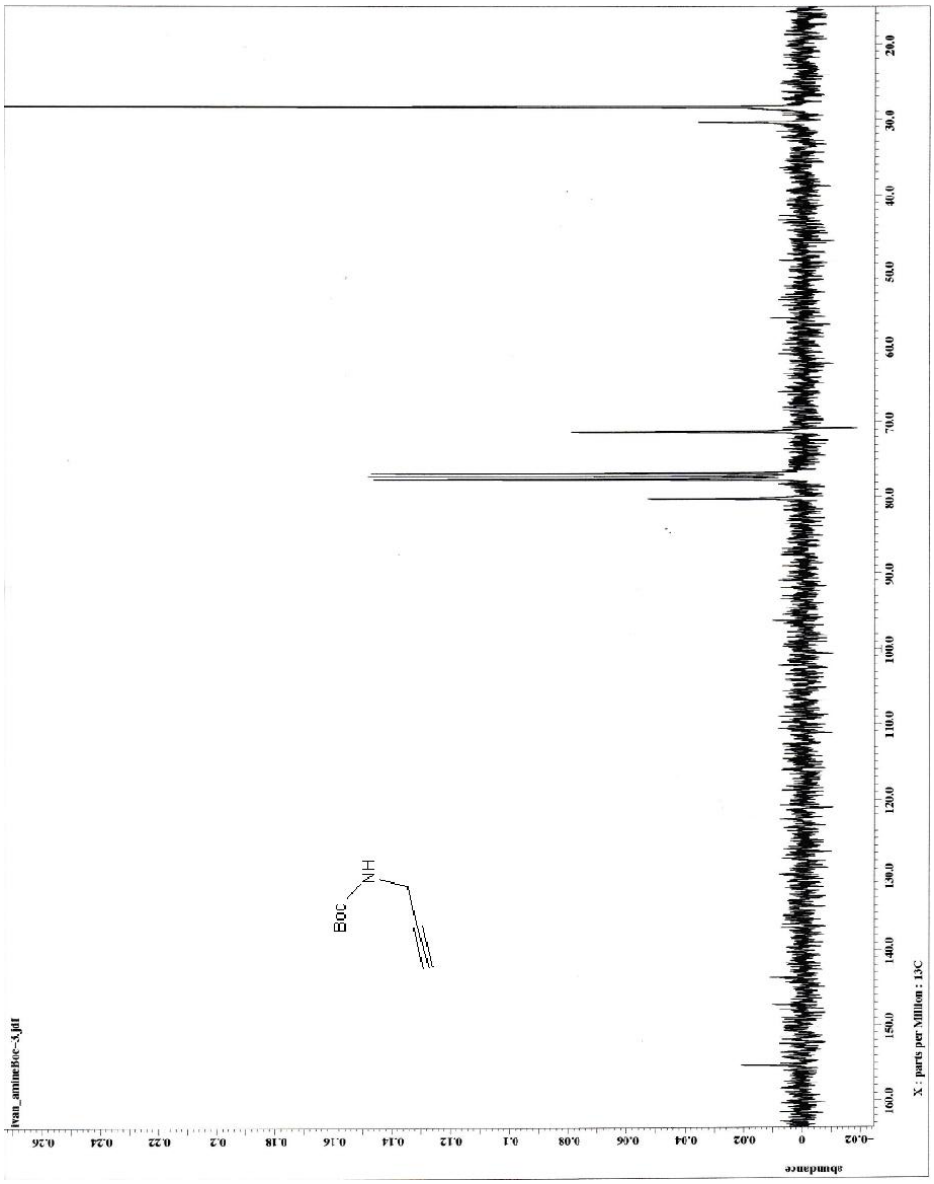
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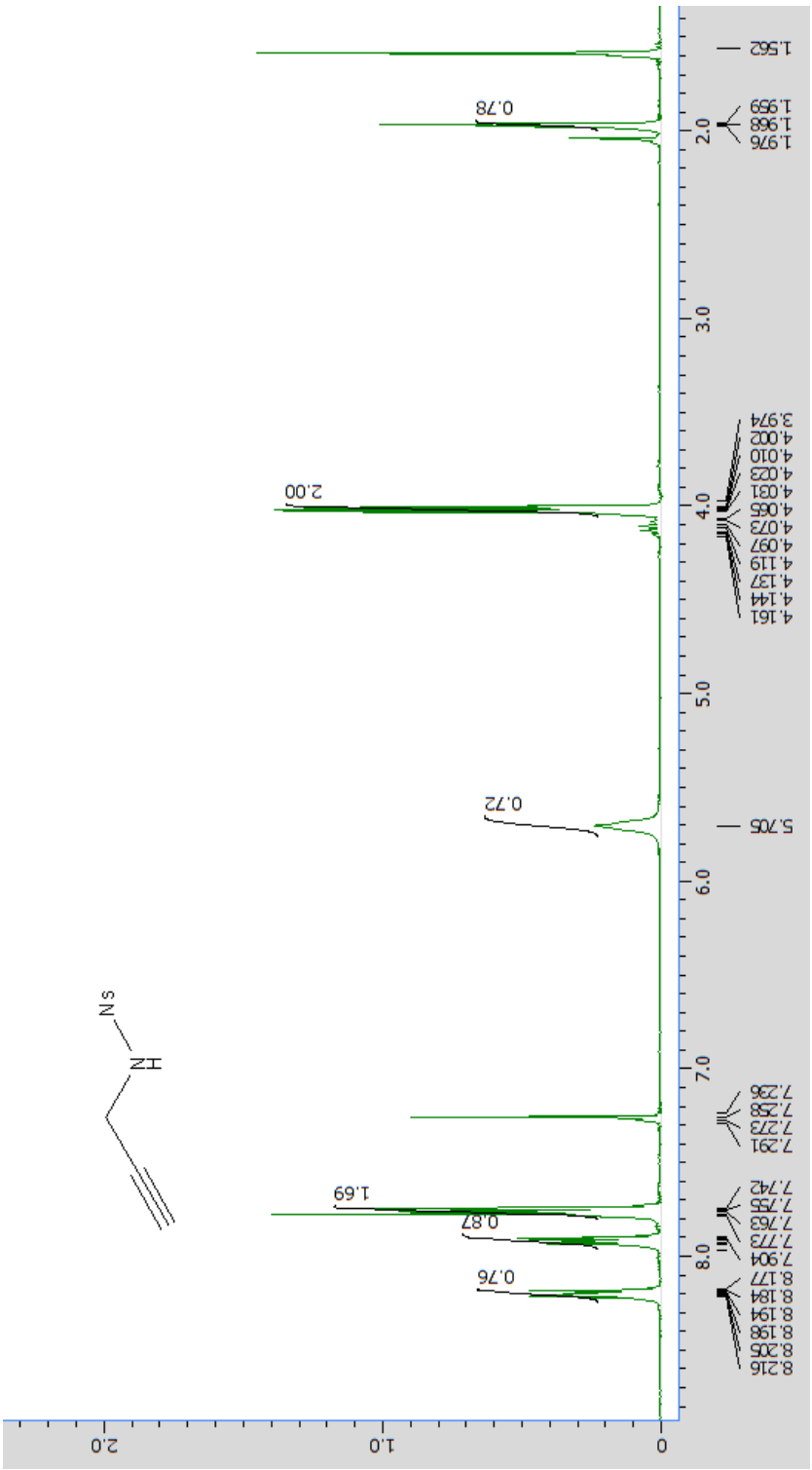
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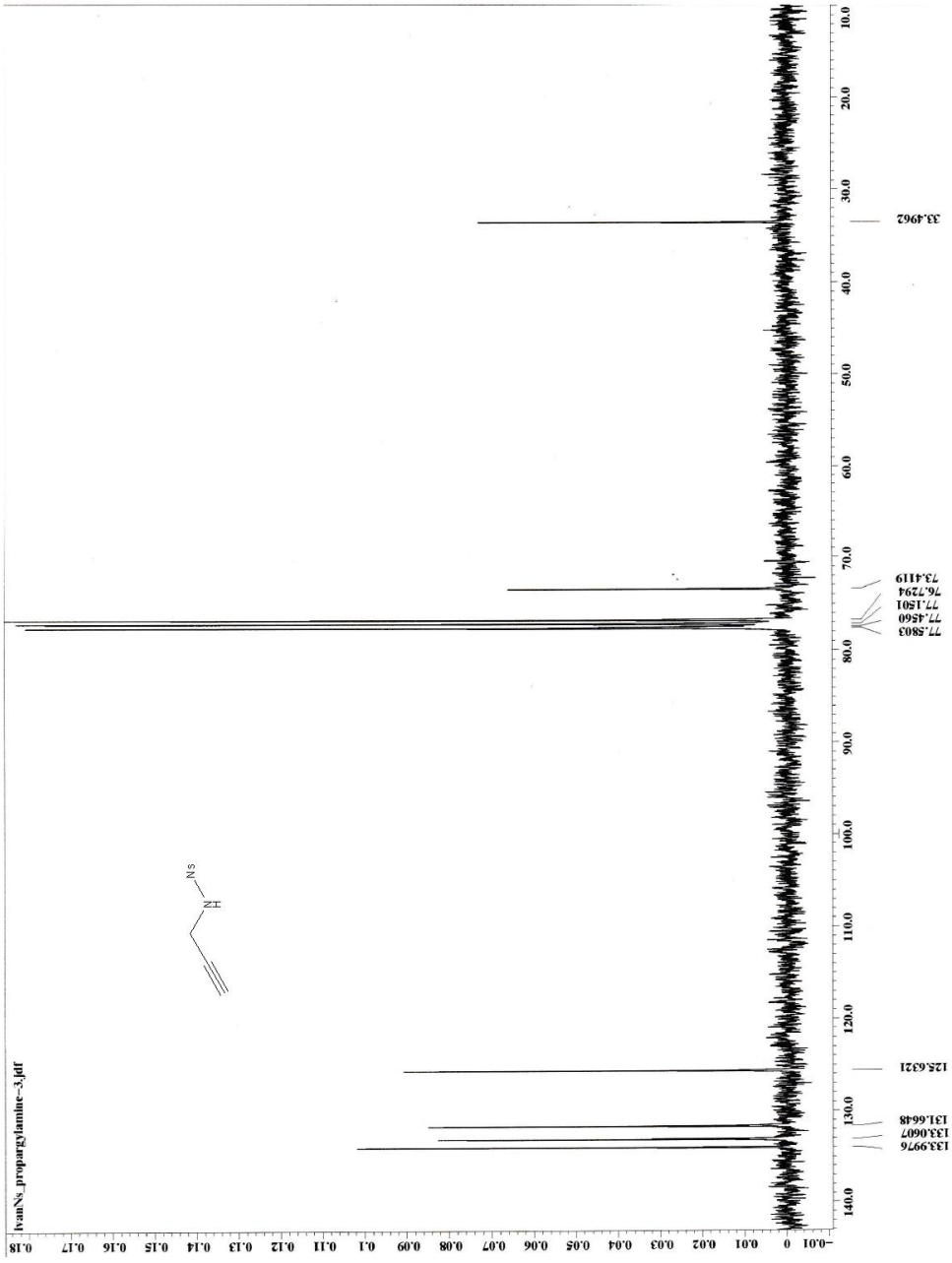
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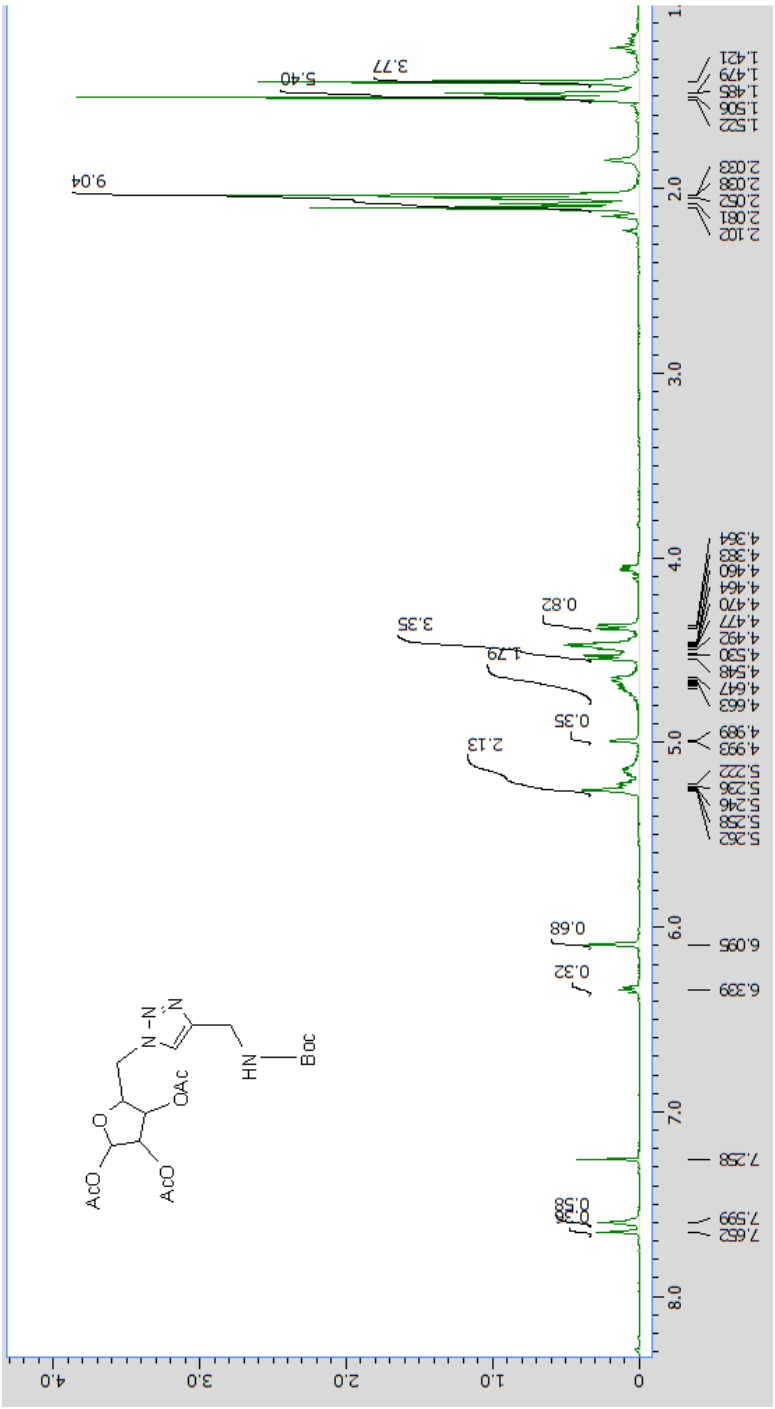
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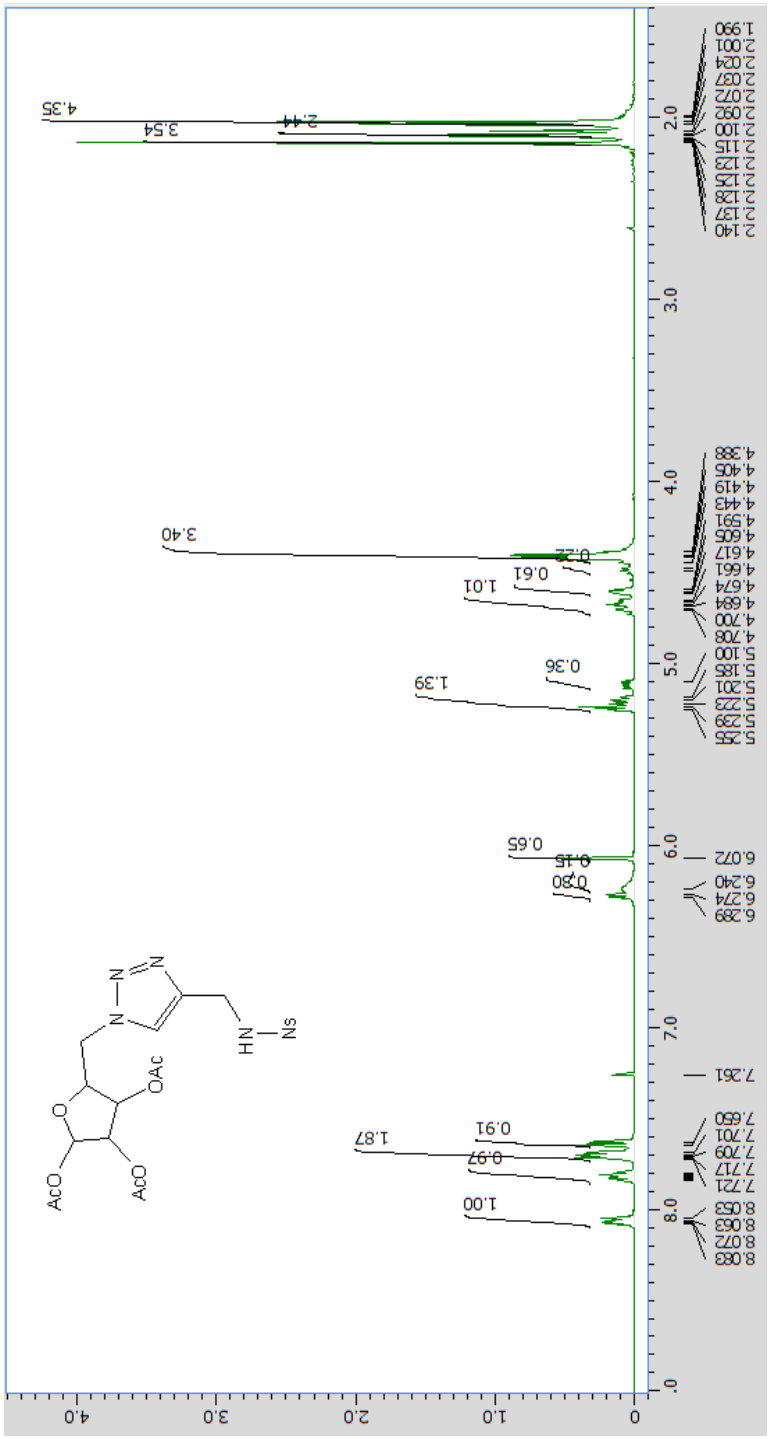
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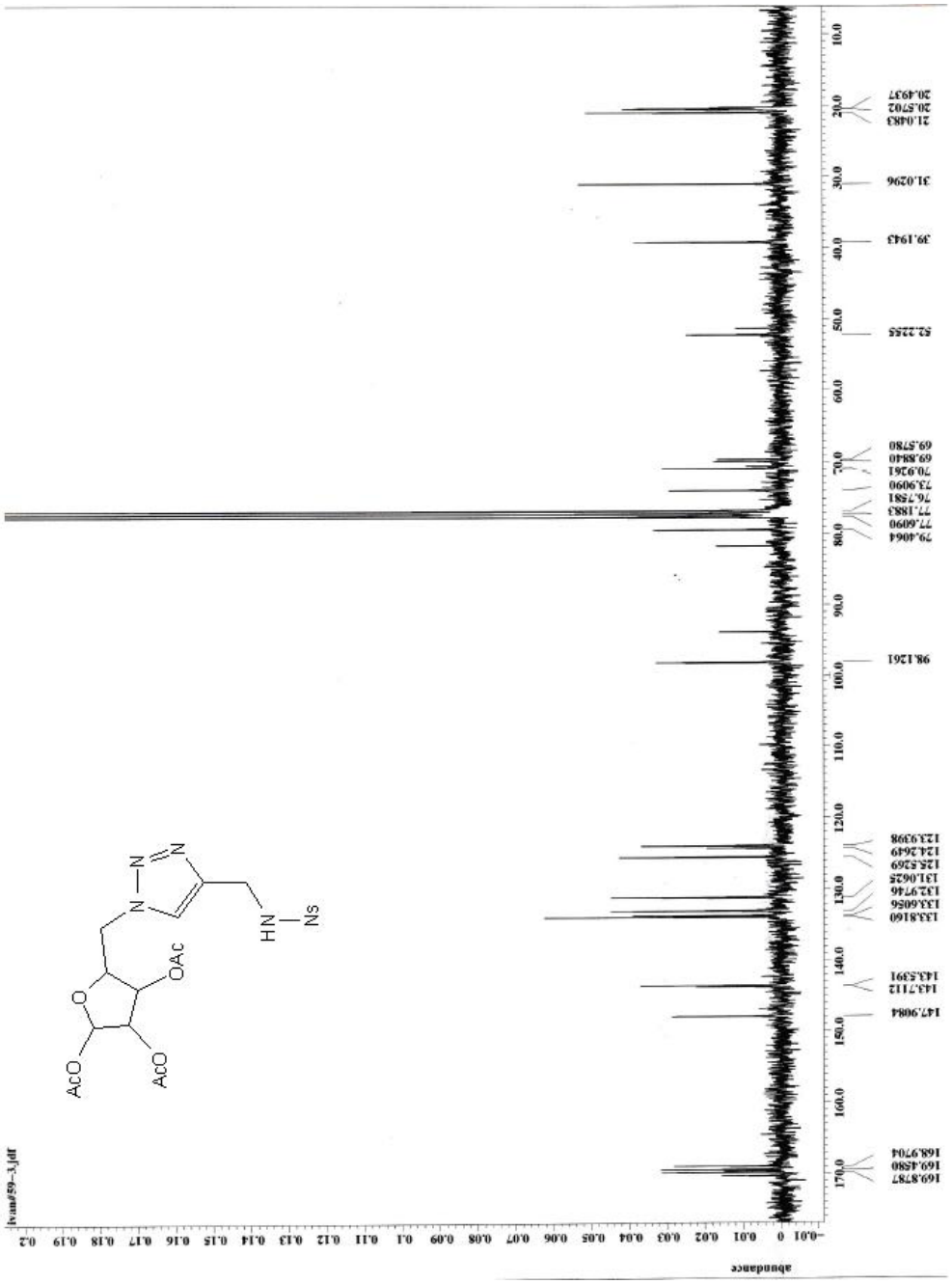
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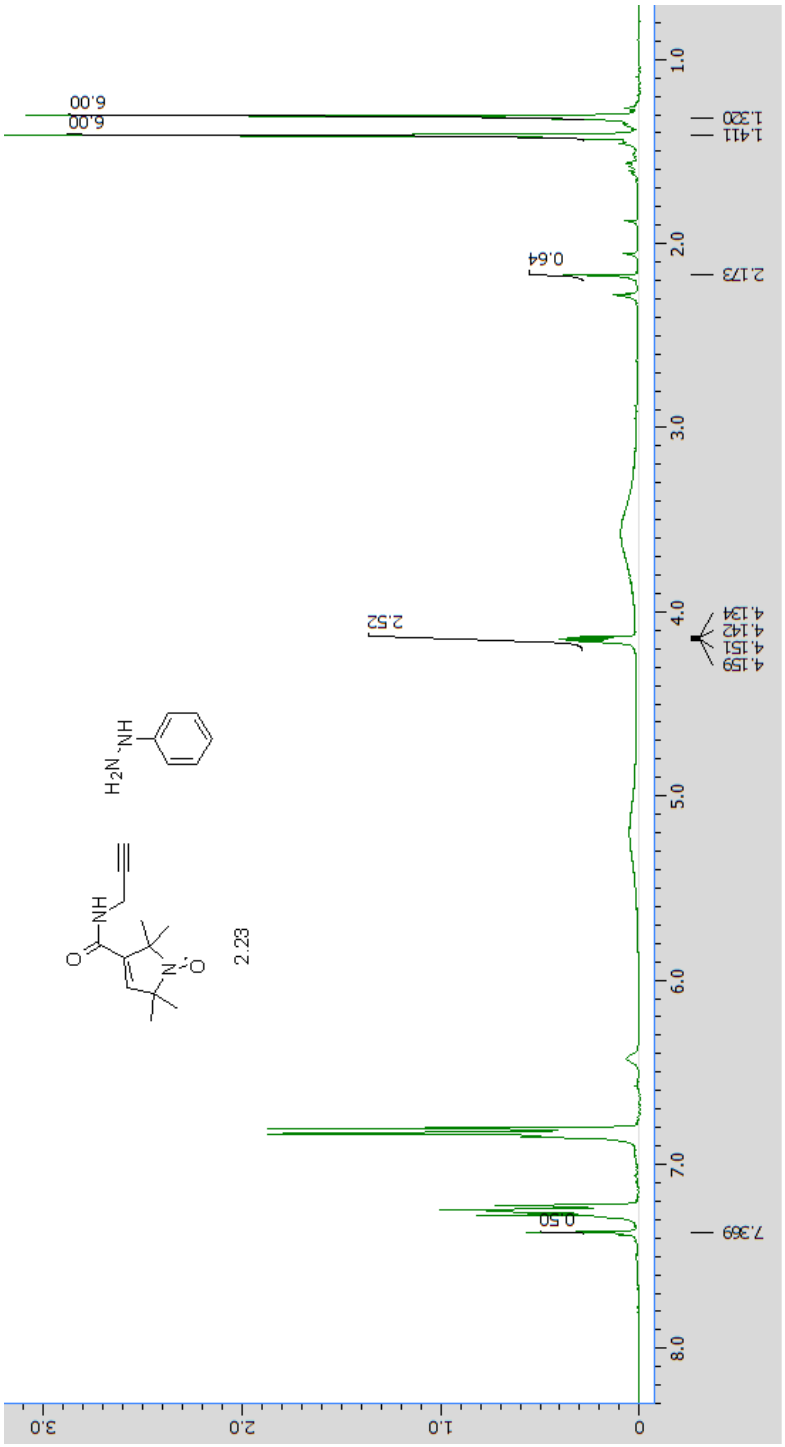
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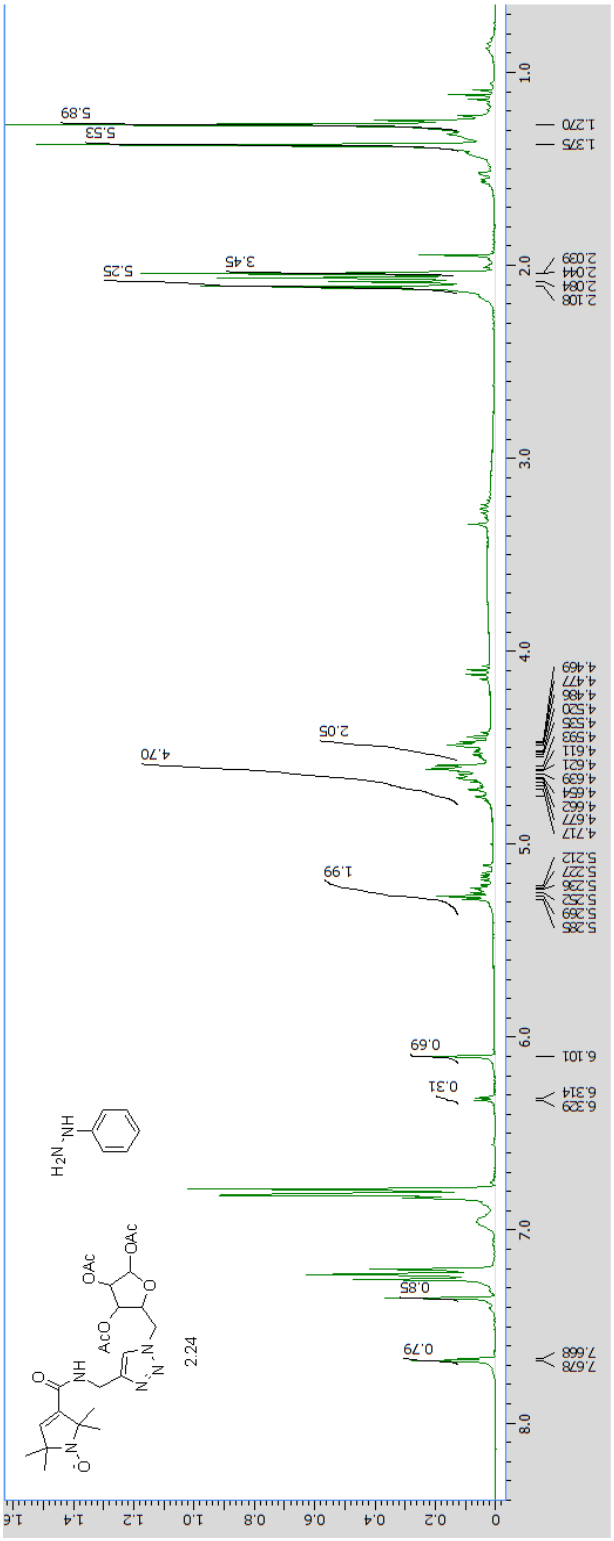

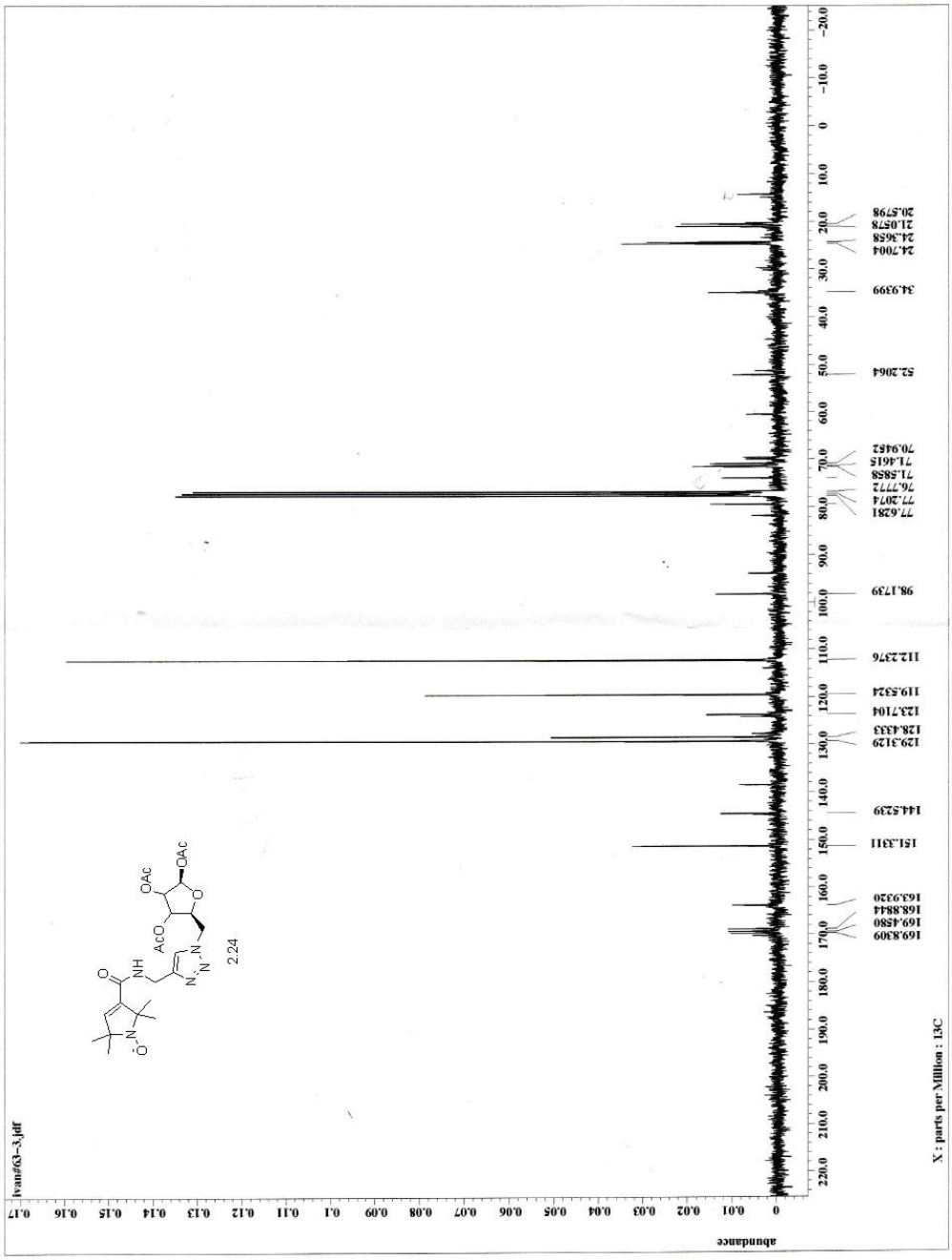
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20.5798
OAc
OAc
AcO
NH
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N
O
N
N
2.24

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