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Characterization of Two Radical *S*-Adenosyl-L-methionine Enzymes in the Biosynthesis of Aminoglycosides

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Characterization of Two Radical *S*-Adenosyl-L-methionine Enzymes in the Biosynthesis of Aminoglycosides

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Dedication

To my parents and parents-in-law for their incessant support and love

and

To my beloved wife, Ji Eun, and my lovely son, Art, for completing me

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Characterization of Two Radical *S*-Adenosyl-L-methionine Enzymes in the Biosynthesis of Aminoglycosides

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The University of Texas at Austin, 2016

Supervisor: Hung-wen Liu

Biosynthetic studies of natural products are essential to the discovery and development of new drugs, because by understanding biosynthetic pathways and the enzymes that characterize them, new pathways can be engineered for the production of new compounds with improved clinical properties. Aminoglycosides have traditionally been used as important antibiotics, but resistance against aminoglycosides is well known. This has resulted in a need to better understand the biosynthesis of aminoglycosides. Besides the practical value, the discovery of new enzymes with unprecedented functions in the biosynthesis of aminoglycosides can expand our knowledge and advance our understanding of enzyme catalysis.

The work described in this dissertation focuses on the *in vitro* characterization of two radical *S*-adenosyl-L-methionine (SAM) enzymes in the biosynthesis of aminoglycosides. First, GenK is a cobalamin (Cbl)-dependent radical SAM enzyme that is responsible for catalyzing the methylation of gentamicin X_2 to produce G418. *In vitro* assays of purified and reconstituted GenK from *Micromonospora echinospora* showed that this enzyme is a radical SAM enzyme with one [4Fe-4S] cluster. Assays of GenK

with SAM, gentamicin X_2 and Cbl confirmed that the methylation reaction occurs at an unactivated carbon during gentamicin biosynthesis. Isotope labeling experiments strongly suggested that SAM is the preliminary methyl donor to cobalamin, followed by secondary transfer from Me-Cbl to gentamicin X_2. It was demonstrated that GenK also accepts alternative aminoglycoside substrates besides gentamicin X_2. Second, three possible mechanisms for the GenK reaction were suggested and tested. Experiments investigating the stoichiometry of the GenK reaction revealed 5'-deoxyadenosine (5'-dAdo), *S*-adenoxylhomocysteine (SAH), and G418 were produced in equal proportion and one-to-one with each methylation reaction catalyzed by GenK. The experiment with labeled substrates indicated that the 6'-pro-*R*-hydrogen atom of gentamicin X_2 is abstracted by 5'-dAdo• and that methylation occurs with retention of configuration at C6'. Several substrate derivatives were synthesized to investigate the manner of methyl transfer from cobalamin to the substrate. Finally, the *in vitro* characterization of AprD4 and AprD3 in the biosynthesis of C3'-deoxyaminoglycoside was also conducted. Purified and reconstituted AprD4 from *Streptomyces tenebrarius* is a radical SAM enzyme, catalyzing homolysis of SAM to 5'-dAdo in the presence of paromamine to produce 4-oxolividamine. AprD3 from the same strain is shown to be a dehydrogenase acting as the reductase counterpart to AprD4 to catalyze the reduction of 4-oxolividamine to generate lividamine. The experiments with labeled compounds confirmed the regiochemistry of hydrogen atom abstraction by 5'-dAdo• and the stereochemical course of C3'-deoxygenation of paromamine.

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1.1. AMINOGLYCOSIDE BIOSYNTHESIS

1.1.1. Introduction

Aminoglycosides belong to a long-standing and important class of antibiotics.[1-2] Waksman reported the first aminoglycoside streptomycin from the soil bacteria, *Streptomyces griseus*, in 1943 and showed that it possesses antituberculosis activity. [3] However, antibiotic resistance against aminoglycosides has been more common, and this has led investigators to search for new aminoglycosides. Consequently, several aminoglycosides like gentamicin,[4] tobramycin,[5] apramycin,[6] lividomycin[7] and others[8] were subsequently discovered and shown to be clinically useful in the treatment of infections by Gram-negative and selective Gram-positive bacterial, fungi, and *Pseudomonas* spp. Also semisynthetic derivatives like dibekacin,[9] amikacin,[10] arbekacin,[11] isepamicin,[12] and netilmicin[13] were introduced during the 1970s when modifications of aminoglycosides such as *N*-acetylation, *O*-phosphorylation, and *O*-adenylation were recognized as resistance mechanisms that reduce the affinity of the drugs for rRNAs.[3]

The mode of action of aminoglycoside antibiotics has been thoroughly studied.[3] Aminoglycosides contain several hydroxyl and amino groups that are important for the binding interactions with bacterial ribosomal RNAs. Such binary complex formation prevents translocation or proofreading during translation resulting in the death of the bacteria.[14] In general, the amino groups of aminoglycosides are positively charged at

neutral pH and thus interact favorably with the negatively charged phosphate backbone of RNAs. Also crystal structure analysis of rRNA complexes with aminoglycoside antibiotics showed the specific interactions that promote binding of aminoglycosides to RNAs.[15-16] Although most aminoglycosides have different binding interactions, they all adversely affect translation. The details as to how their effects on translation lead to the bacterial death are not clear. One possibility is that aminoglycosides combine with ribosomal RNA leading to misread proteins.[17-18] These abnormal proteins combine with the inner membrane and lead to destabilization. Another possibility is that aminoglycosides inhibit ribosomal activity.[19] Another model proposes that binding with aminoglycosides perturbs metabolism and respiration of bacteria which results in oxidative stress due to the increased formation of highly toxic hydroxyl radicals and superoxide production.[20]

1.1.2. Aminoglycoside Structure

The general structure of aminoglycosides consists of an aminocyclitol linked to at least one amino sugar. Aminoglycosides thus contain a number of free hydroxyl groups and at least two amino groups. Based on the structure of the aminocyclitol, the aminoglycosides can be divided into four different groups: a) *myo*-inositol-derived, b) 2-deoxy-*scyllo*-inose-derived, c) 2-*epi*-5-*epi*-valiolone-derived, and d) cyclopentitol-derived aminoglycosides (Figure 1-1). Figure 1-1 shows examples of theses aminoglycosides.[21]

myo-Inositol-derived aminoglycosides include streptomycin, bluensomycin, spectinomycin, the fortimicins, and kasugamycin. These molecules play critical roles in many cellular processes and are involved in cell wall biogenesis, stress response, and signal transduction pathways.[22-24] These aminoglycosides are derived from *myo*-inositol

1-phosphate (MIP) which is biosynthesized from D-glucose 6-phosphate (G6P) by *myo*-inositol-1-phosphate synthase found in both prokaryote and eukaryotes.[25] MIP is converted into aminoinositols via a series of reactions catalyzed by phosphatases, dehydrogenases, and aminotransferases.

2-Deoxy-*scyllo*-inose-derived aminoglycosides commonly contain 2-deoxystreptamine (2-DOS) as a core scaffold and are decorated with several monosaccharides at the C4, C5 and C6 positions. These aminoglycosides include the kanamycins, neomycins, gentamicins, apramycin and butirosins. They are biosynthetically derived from paromamine. Clinically important aminoglycosides in this group are further subclassified according to the substitution pattern of DOS with aminosugars. Thus, neomycin and paromomycin are 4,5-disubstituted aminoglycosides, whereas gentamicin, kanamycin, and tobramycin are 4,6-disubstituted aminoglycosides. The details regarding the biosynthesis of 2-DOS containing aminoglycosides are described below.

The third class of aminoglycosides is characterized by a C_7N aminocyclitol core structure. These aminoglycosides include acarbose, the oligostatins, the trestatins, validamycin A, pyralomycin 1a, and cetoniacytone.[21] Their chemical structures commonly contain an unsaturated aminocyclitol unit, valienamine, although some compounds contain modified forms of valienamine. The biosynthetic origin of valienamine in these aminoglycosides was established to be 2-*epi*-5-*epi*-valiolone by isotope-labeling experiments.[26-28]

The final class of aminoglycosides is relatively rare in nature. These aminoglycosides include pactamycin,[29] an antibiotic antitumor agent from *Streptomyces pactum*, and allosamidin, a chitinase inhibitor isolated from *Streptomyces* sp. No 1713.[30] These aminoglycosides contain a unique cyclopentitol moiety. Pactamicin consists of

three cyclic units, the cyclopentitol, a 6-methylsalicyclic acid functionality, and an *m*-aminoacetophenone moiety which are derived from different biosynthetic pathways. The cyclopentitol moiety is derived from glucose, while the other two are generated via polyketide synthase (PKS) chemistry and a pathway reminiscent of shikimate biosynthesis.[31] Allosamidin has a distinctive structure consisting of a cyclopentitol moiety called allosamizoline and two monosaccharide units of *N*-acetyl-D-allosamine. Cyclopentitol moieties are also found in carbocyclic nucleotides like neplanocin A, aristeromycin, adecypenol, queuosine, and epoxyqueuosine, and bacterial derived hopanoids.[21]

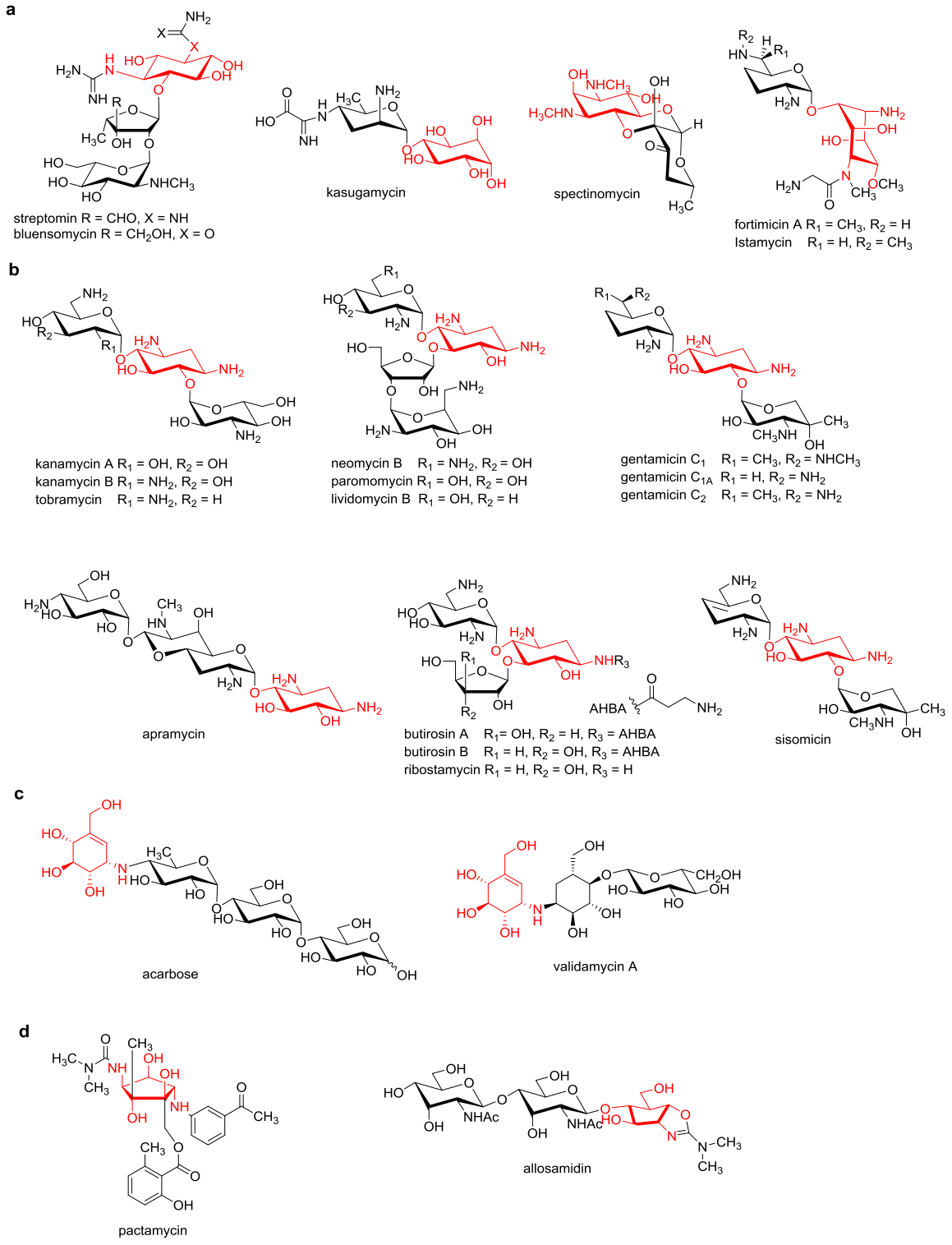


Figure 1-1. Structures of selected aminoglycoside antibiotics

1.1.3. Biosynthesis of Aminoglycosides

This section focuses on the biosynthesis of aminoglycosides and 2-DOS. Biosynthetic studies of other aminoglycosides are covered by several reviews.[8,21,32] Many laboratories are involved to characterize the biosynthetic genes and enzymes for aminoglycoside biosynthesis and their biotechnological applications. As aminoglycoside biosynthetic genes are often reported with different names, the set of gene annotations use by Piepersberg[8] and Eguchi[33] will also be used consistently here.

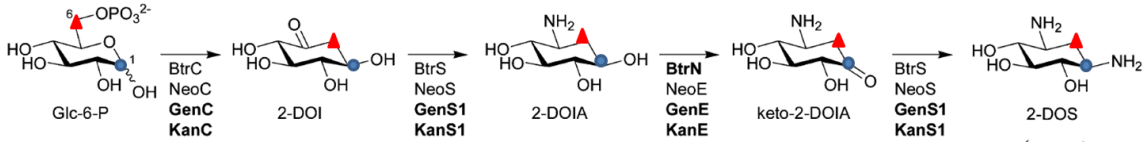
Early feeding experiments with radioisotope- and stable-isotope primary metabolites such as glucose and glucosamine showed that the majority of aminoglycoside components are derived from sugars.[34] Mutant studies were also used to elucidate the structures of biosynthetic intermediates[35] and to determine the order of the biosynthetic steps.[36] However, the mechanisms of the enzymatic reactions and the timing of modifications in the biosynthesis of aminoglycosides were not fully elucidated until now.

The first genetic analysis of aminoglycoside biosynthesis was performed in 1987 and only a portion of the complete gene cluster for streptomycin was identified.[37] Several biosynthetic genes located both up- and downstream of the identified portion were discovered later using hybridization studies, and determining the nucleotide sequences.[38] Since then, analyses of more than 30 aminoglycoside biosynthetic gene clusters have been accomplished and deposited in the public gene data bank. The discovery of 2-deoxy-*scyllo*-inose (2-DOS) synthases from the butirosin producing strain *Bacillus circulans* in 1999[39] boosted the identification of additional 2-DOS-containing aminoglycoside biosynthetic gene clusters such as butirosin, neomycin, ribostamycin, paromamine, lividomycin, kanamycin, tobramycin, gentamicin, sisomycin, istamycin, apramycin and hygromycin B.

Comparison of the gene clusters has led to speculation that some conserved biosynthetic enzymes encoded in different aminoglycoside gene clusters are responsible for the biosynthesis of the common intermediates. There are also many cases of gene clusters encoding aminoglycosides that have mixed structures of other aminoglycosides, such as gentamicin gene cluster that shares modification patterns and subunits with kanamycin and fortimicin gene cluster. Under such situation, the information from biosynthetic gene cluster from one aminoglycoside predicts the modification of the intermediates step by step in the biosynthetic pathway of another aminoglycoside with similar gene cluster.

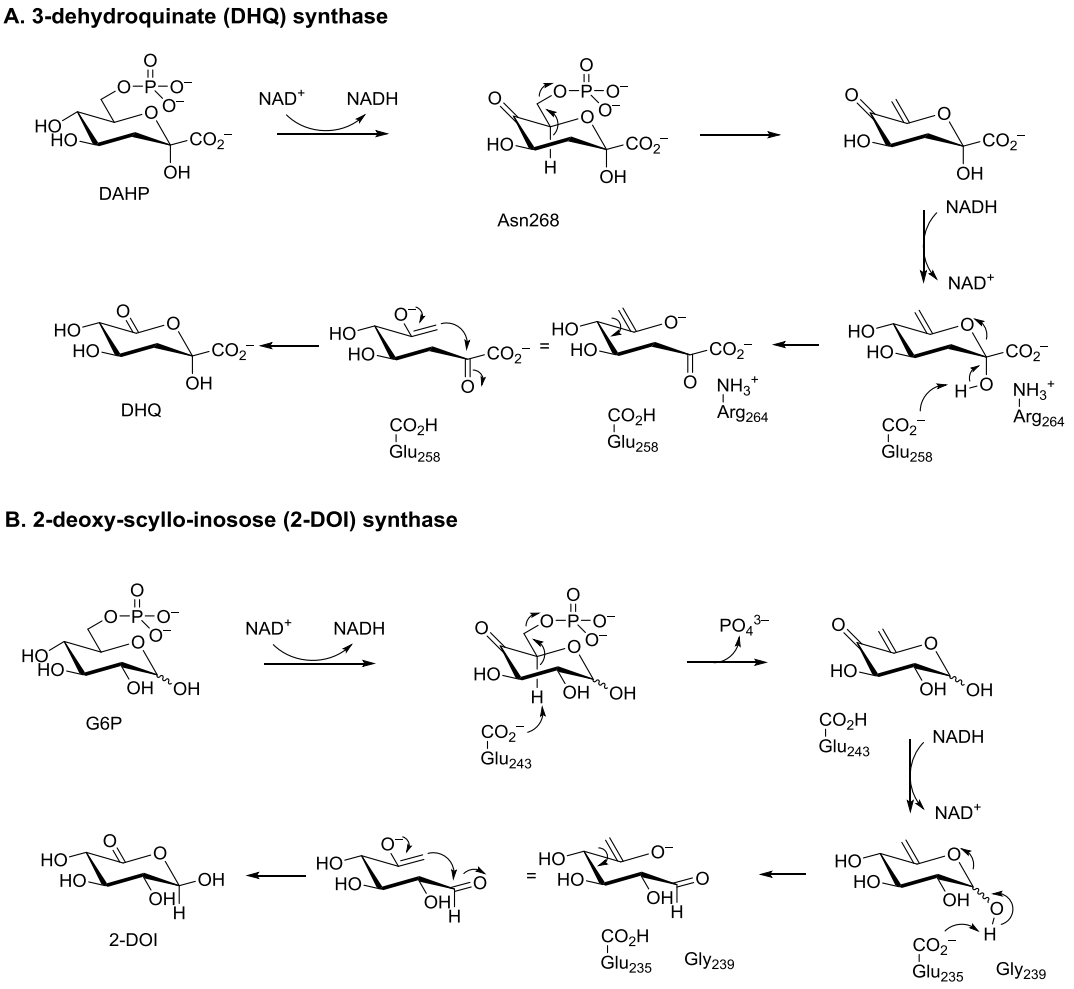
1.1.3.1. Biosynthesis of 2-Deoxystreptamine (DOS)

2-DOS, also known as 1,3-diamino-4,5,6-trihydroxycyclohexane, is found in the majority of aminoglycosides as a central moiety and plays a critical role in their biological function by interacting directly with the 16S ribosomal RNA subunit.[1,40-41] The biosynthesis of 2-DOS has been investigated in detail.[42-44] Early studies using ^{13}C-labeled substrate in neomycin isolated from the culture of *Streptomyces fradiae* demonstrated that 2-DOS originates from D-glucose, with C-1 and C-2 carbons of 2-DOS corresponding respectively to the C-1 and C-6 carbons of glucose (Scheme 1-1).[45] The nitrogen from glucosamine is not incorporated into 2-DOS.[34] Experiments with 6,6-dideuterated glucose in ribostamycin from *Streptomyces ribosidificus* demonstrated that both deuteriums at C-6 are retained in 2-DOS and rearrangement proceeded with retention of stereochemistry.[46-47] Labeling experiments showed that the C-4 proton of glucose is lost during the biosynthesis of 2-DOS.[48-49] The *in vitro* assay with cell-free extracts of *S. fradiae* demonstrated that the conversion of glucose-6-phsophate to 2-DOS is NAD^+-dependent.[50-51]



Scheme 1-1. Biosynthesis of 2-DOS

BtrC, 2-deoxy-*scyllo*-inosose (2-DOI) synthase, was purified from the butirosin-producing bacterium *Bacillus circulans*, and shown to be dependent on both cobalt and NAD^+.[52-54] Enzymes homologous to BtrC have been identified in all 2-DOS-containing aminoglycoside biosynthetic pathways (Scheme 1-1).[55] The mechanism of BtrC was proposed based on its X-ray crystal structure and compared to that of 5-dehydroquinate (DHQ) synthase, because they have moderately similar amino acid sequences (26–34%) (Scheme 1-2).[56] The two enzymes have different conserved amino acids at positions 243 and 239: asparagine and arginine in DHQ vs glutamate and glycine in 2-DOI synthase. Also 2-DOI synthase has an additional conserved glutamate residue at 235. In the DHQ synthase reaction (Scheme 1-2), oxidation at C4 occurs in the first step. Asn268 is not directly involved in the abstraction of a proton at C5, but the substrate phosphate group can abstract a proton to provide an α,β-unsaturated ketone intermediate.[57] After reduction of the carbonyl group at C-4 and ring opening of the hemi acetal, the stereoselective intramolecular aldol condensation gives DHQ. The proposed mechanism of BtrC catalysis is similar to that of DHQ synthase. The difference between them is the acid-base chemistry during proton abstraction at C5 (the second step).[58]



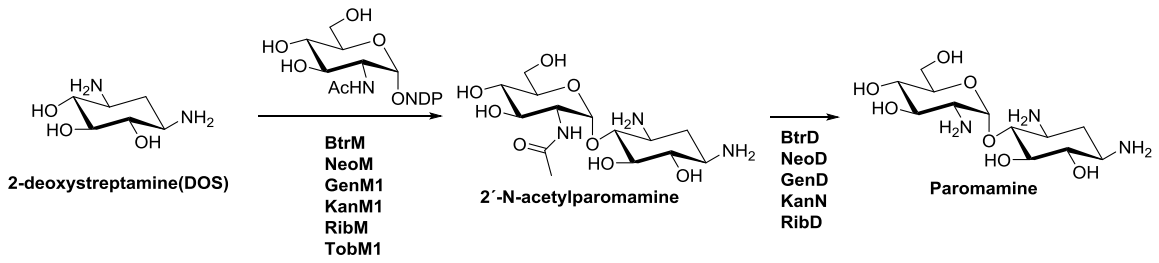
Scheme 1-2. Proposed mechanisms of DHQ synthase and 2-DOI synthases. The amino acid position numbers derived from *Aspergillus nidulans* DHQ and BtrC from 2-DOI synthase are indicated.

The conversion of 2-DOI to 2-deoxy-*scyllo*-inosamine (2-DOIA) is catalyzed by a PLP-dependent aminotransferase BtrS.[30] This enzyme was also found to catalyze the conversion of 2-deoxy-3-keto-*scyllo*-inosamine (keto-2-DOIA) to 2-deoxystreptamine (2-DOS), suggesting that this enzyme play a dual role. This phenomenon is also found in the biosynthesis of other aminoglycosides including neomycin, gentamicin, and kanamycin.[26]

The next step from 2-DOIA to keto-2-DOIA is catalyzed by the radical SAM enzyme BtrN (this enzyme is described in detail in Section 1.3.1).[29] During 2-DOS biosynthesis, only the butirosin biosynthetic pathway involves a radical SAM enzyme for oxidation of hydroxyl group at C1. Other biosynthetic pathways involve the $NADP^+$-dependent enzymes for this step.

1.1.3.2. Biosynthesis of Paromamine

Paromamine is the most common intermediate during biosynthesis of the 2-DOS-based aminoglycosides besides neamine. The biosynthesis of paromamine consists of two steps: glycosylation of 2-DOS with *N*-acetylglucosamine (GlcNAc) and deacetylation (Scheme 1-3). BtrM identified in the butirosin biosynthetic gene cluster and its homologues, NeoD in neomycin biosynthesis, GenM1 in gentamicin biosynthesis, KanM1 in kanamycin biosynthesis, RibM in ribostamycin biosynthesis, and TobM1 in tobramycin biosynthesis, were suggested to catalyze transfer of NDP-D-glucosamine to 2-DOS.[55] It was demonstrated with cell-free extracts of *E. coli* expressing *neoM* that NeoM catalyzes the glycosylation reaction.[59] The function of BtrD in the butirosin gene cluster is to catalyze 2'-*N*-acetylparomamine deacetylation, and was shown by studying the recombinant BtrD and 2'-*N*-acetylparomamine.[60] Also GenD in gentamicin, KanN in kanamycin, and RibD in ribostamycin were demonstrated to be 2'-*N*-acetylparomamine deacetylases by heterologous expression of the respective genes to produce paromamine in *S. venezuelae*.[60]



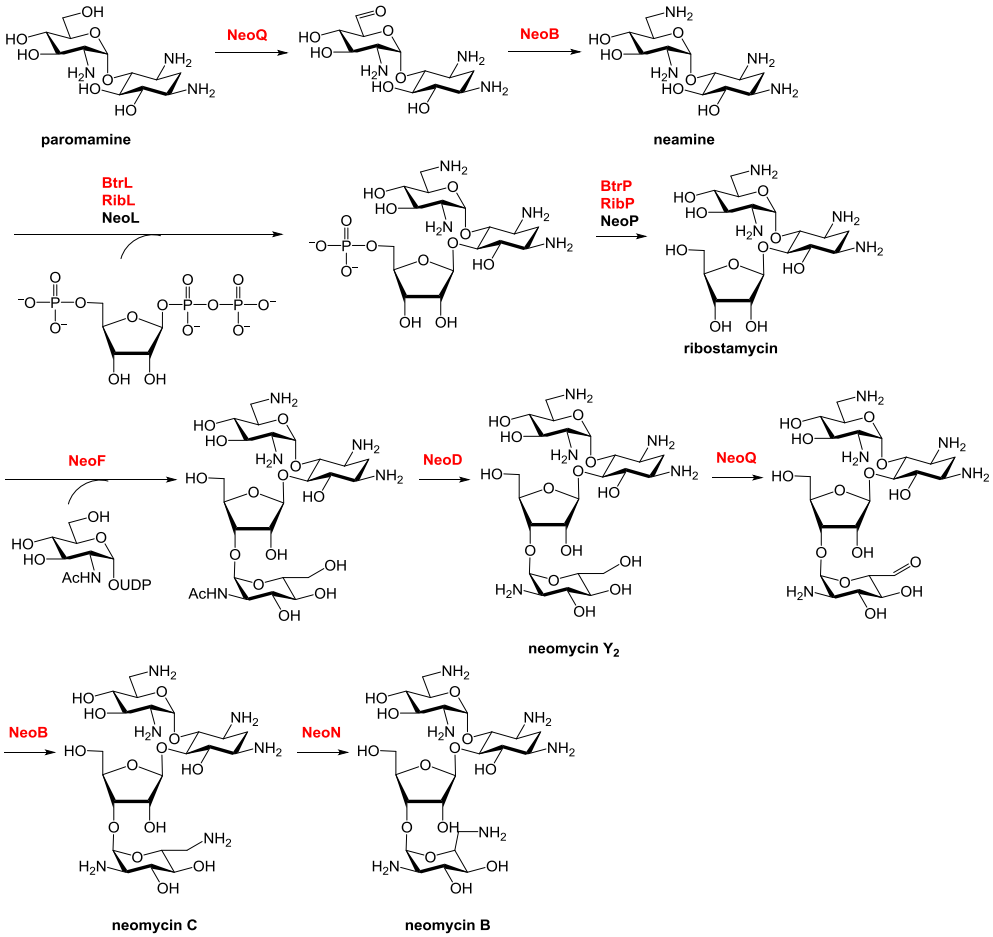
Scheme 1-3. Biosynthesis of paromamine from 2-DOS

1.1.3.3. Biosynthesis of Ribostamycin, Neomycin C, and Neomycin B

Ribostamycin is an intermediate in the production of 4,5-disubstituted aminoglycosides, including butirosin and neomycin. The transformation of paromamine to neamine is catalyzed by the FAD-dependent dehydrogenase NeoQ and the PLP-dependent aminotransferase NeoB (Scheme 1-4).[61] The formation of ribostamycin, a common intermediate fin the biosynthesis of neomycins and butirosin, from neamine occurs in two steps: ribosylation by BtrL using 5-phosphoribosyl-1-pyrophosphate (PRPP) as a ribosyl donor to form 5"-phosphoribostamycin and subsequent hydrolysis by BtrP.[62] BtrL and BtrP also catalyze the ribosylation of paromamine to provide 6'-deamino-6'-hydroxyribostamycin.[62] RibL and RibP in the ribostamycin producer *S. ribosidificus* have the same functions as BtrL and BtrP.[63]

The last glycosylation in neomycin biosynthesis to attach the fourth ring is catalyzed by NeoF which transfers UDP-*N*-acetylglucosamine onto ribostamicin to generate 2"-*N*-acetyl-neomycin Y_2.[59] The function of NeoF was also confirmed by an *in vivo* in-frame gene deletion of *neoF* in *S. fradiae*, resulting in the accumulation of ribostamycin.[64] The deacetylation of 2"-*N*-acetyl-neomycin Y_2 to provide neomycin Y_2 is catalyzed by NeoD, which also recognizes the pseudodisaccharide 2-acetylparomamine (Scheme 1-3 and Scheme 1-4).[59] In the gene cluster of neomycin, only one BtrD-homologous deacetylase, NeoD, is present. From the result that the *neoD S. fradiae*

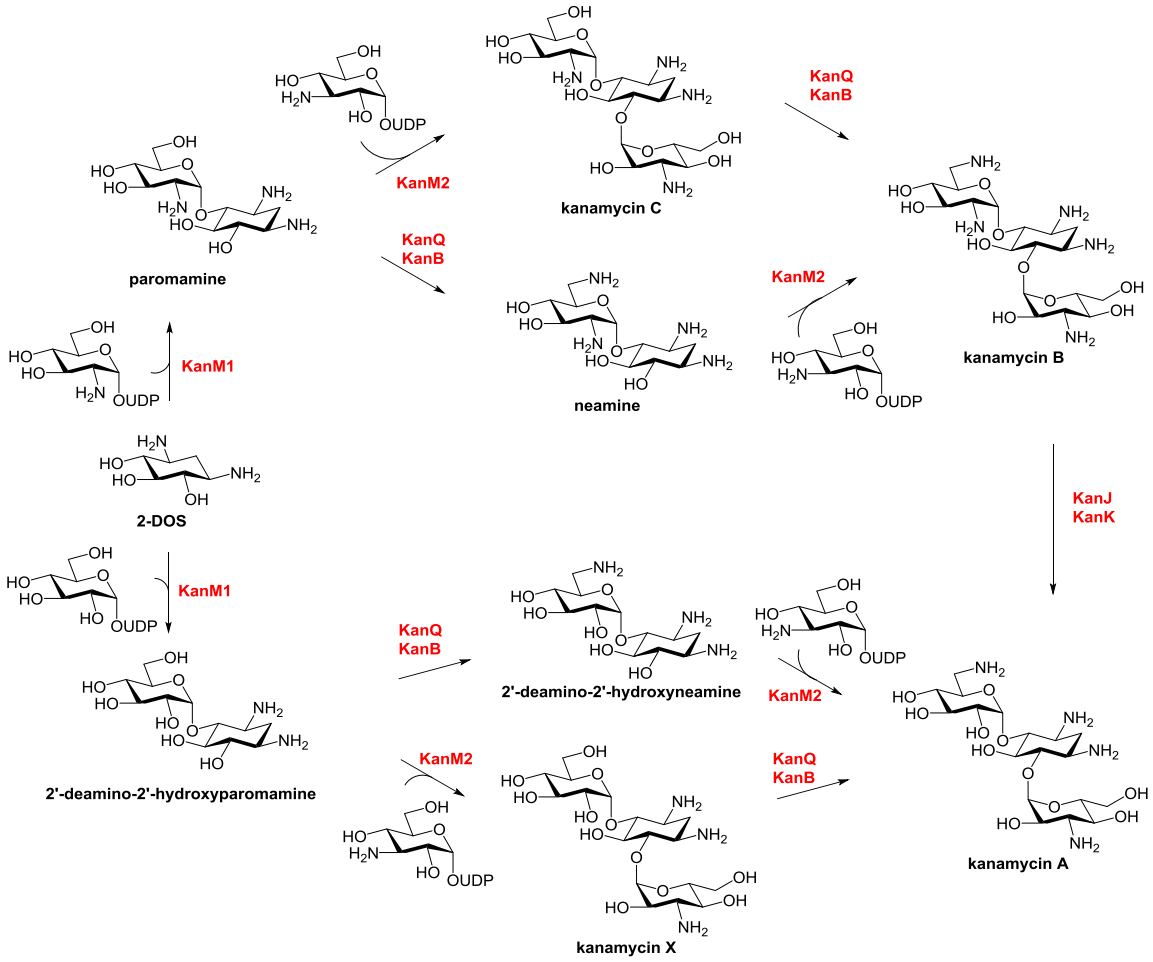
mutant still produces neomycin from neamine, the second deacetylase in *S. fradiae* should exist for the deacetylation of tetrapseudosaccharide.[64] The aminotransfer at C6" is catalyzed by two enzymes, the dehydrogenase NeoQ and aminotransferase NeoB.[65-66] NeoQ and NeoB can also recognize the pseudodisaccharide paromamine to provide neamine (Scheme 1-5).[67] The final epimerization is catalyzed by a radical SAM enzyme NeoN. NeoN is described in detail in section 1.3.2.



Scheme 1-4. Biosynthetic pathway for neomycin B

1.1.3.4. Biosynthesis of Kanamycins

Compared to 4,5-disubstituted aminoglycosides such as ribostamycin and neomycin, kanamycins isolated from *S. kanamyceticus* are 4,6-disubstituted pseudotrisaccharides with a 3-aminosugar moiety at the C6-hydroxyl group of 2-DOS. The Yoon group proposed parallel biosynthetic pathways based on heterologous gene expression of kanamycin genes in *S. venezuelae* (Scheme 1-5).[68] KanM1 is able to accept both UDP-glucosamine and UDP-glucose as the glycosyl donor to provide respectively paromamine and 2'-deamino-2'-hydroxyparomamine.[68] The glycosyltransferase KanM2 can use four different substrates such as paromamine, neamine, 2'-deamino-2'-hydroxyparomamine, and 2'-deamino-2'-hydroxyneamine as glycosyl acceptors. Also KanM2 can accept two glycosyl donors such as UDP-glucose and UDP-kanosamine (the biosynthetic step using UDP-glucose is not shown in Scheme 1-5. After UDP-glucose is accepted by KanM2 to give 3'-deamino-3'-hydroxykanamycin C,[69] subsequent amino group introduction at C3" to produce kanamycin C is unclear even though KanD2 and KanS2 are assigned to this step from the gene analysis of kanamycin biosynthesis). The dehydrogenase KanQ and aminotransferase KanB also show substrate flexibility. They accept both pseudodisaccharides such as paromamine and 2'-deamino-2'-hydroxyparomamine as well as pseudotrisaccharides such as kanamycin C and kanamycin X.[68] An α-ketoglutarate-dependent non-heme iron dioxygenase, KanJ, and a oxidoreductase, KanK, catalyze the deamination of kanamycin B to kanamycin A.[70] KanJ catalyzes the hydroxylation at C2' to provide a hemiaminal intermediate which is converted into 2'-oxokanamycin B by the release of ammonia. Then a NADH-dependent reductase KanK reduces the carbonyl group of 2'-oxokanamycin B to produce kanamycin A.



Scheme 1-5. Biosynthetic pathway for kanamycin A

1.1.3.5. Biosynthesis of Gentamicins

Gentamicins are aminoglycoside antibiotics produced by *Micromonospora echinispora*.[4] Gentamicins are composed of a 2-deoxystraptamine (2-DOS) core substituted at C4 with a purpurosamine moiety and at C-6 with a garosamine moiety. Biosynthetic studies of gentamicin had been carried out involving isolation of gentamicin-related compounds from the wild type producers as well as mutants to identify the biosynthetic intermediates. The major components which compose

approximately 80% of gentamicin include gentamicin C_1, C_{1a} and C_2, and the minor ones include gentamicin A, A_1, A_2, A_3, B, X_2,[71-73] and antibiotics JI-20A and JI-20B.[74] Using these compounds, Tilley *et al* carried out biosynthetic studies of gentamicin and showed interconversion of gentamicin-related components using biological catalysts and extracts.[75] They used *M. purpurea* Paro 346, a mutant that produces paromamine but none of the gentamicin C's. Since gentamicin C_{1a} was not converted into gentamicin C_2 or gentamicin C_1, the *C*-methyl transferase responsible for methylation at the C6' position apparently could not recognize gentamicin C_{1a} as a substrate. Using the mutant, they also showed the transformation of JI-20A to gentamicin C_{1a} and gentamicin C_{2b} whereas JI-20B and G418 were converted to gentamicin C_2 and gentamicin C_1. These results supported the hypothesis that two different pathways are operant for the production of different gentamicin C's. Furthermore, gentamicin A and gentamicin X_2 were transformed by the mutant into gentamicin C_{1a}, gentamicin C_2 and gentamicin C_1, thus it appears that these species serve as precursors to the gentamicins C's before the branch point. From these data, a biosynthetic pathway was proposed (Scheme 1-6).

The gene cluster of gentamicin was sequenced by the Sohng group[76] and Wellington group[77] in 2004 and the Piepersberg group[8] in 2006, all from *Micromonospora echinospora* ATCC 15835 (Figure 1-2). Homology search results for each genes in the cluster are summarized in Table 1-1. The majority of the genes in the gentamicin gene cluster show high similarity with those in the fortimicin,[8] sisomicin,[78] Frankia Ccl3,[33] and istamicin[33] clusters (structures are shown in Figure 1-1). GenC, GenS1, GenE, GenM1, and GenD were assigned to be responsible for paromamine formation, similar to the butirosin biosynthetic pathway (Scheme 1-2 and Scheme 1-3). GenD1, GenD2, GenX, GenU, GenV, GenN, GenS2, and GenM2 were expected to be involved in the biosynthesis of the garosamine moiety. GenM2, GenD2, and GenS2 were

expected to be responsible for xylose transfer. GenD1 and GenN were estimated to be involved in the modification of the xylose moiety. GenK, GenB2, GenB4 and GenG are conserved in the gentamicin and fortimicin biosynthetic gene clusters. GenK may catalyze *C*-methylation of the purpurosamine moiety. GenH, GenI, GenQ, and GenB1 (or GenB3) are conserved in the gentamicin, sisomycin, fortimicin, and istamycin biosynthetic gene clusters. They seem to be responsible for the biosynthesis of the common characters of the purpurosamine moiety. GenX, GenU and GenV remain uncharacterized.

Another interesting finding is that the gentamicin, fortimicin, and Frankia clusters have queuosine biosynthetic genes such as GenO, GenW, GenA, and GenF, and a radical SAM enzyme like GenF. Blast search against known *Streptomyces* genomes (*S. griseus*, *S. avermitilis*, *S. coelicolor*, and *S. acabiei*) shows *Streptomyces* does not have queuosine biosynthetic genes. Furthermore, the Flankia CCl3 aminoglycoside (fortimicin) biosynthetic gene cluster has queuosine biosynthetic genes (Table 1-1). Considering queuosine biosynthetic genes are missing in known genomes of hi-GC Gram-positive bacteria, all gentamicin/fortimicin clusters have a set of genes for queuosine biosynthesis (QueD, QueE, QueC, QueF, and TGT) in the cluster. Moreover, other aminoglycoside biosynthetic gene clusters do not have those homologues. Since these genes are not present in the istamycin cluster, it is less likely that those genes are important for 3,4-dideoxygenation steps. One possible scenario is that homologues of QueD, QueE, QueC, and QueF produce preA1 and the TGT homologue catalyzes the transfer of proQ1 to rRNA for gentamicin/fortimicin self-resistance mechanism.

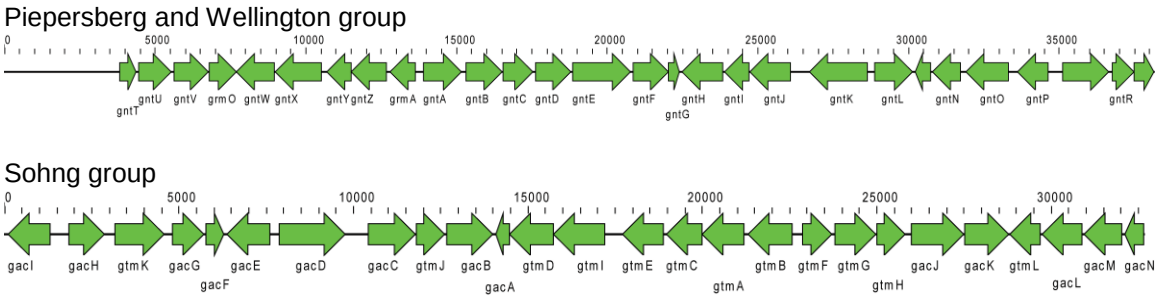


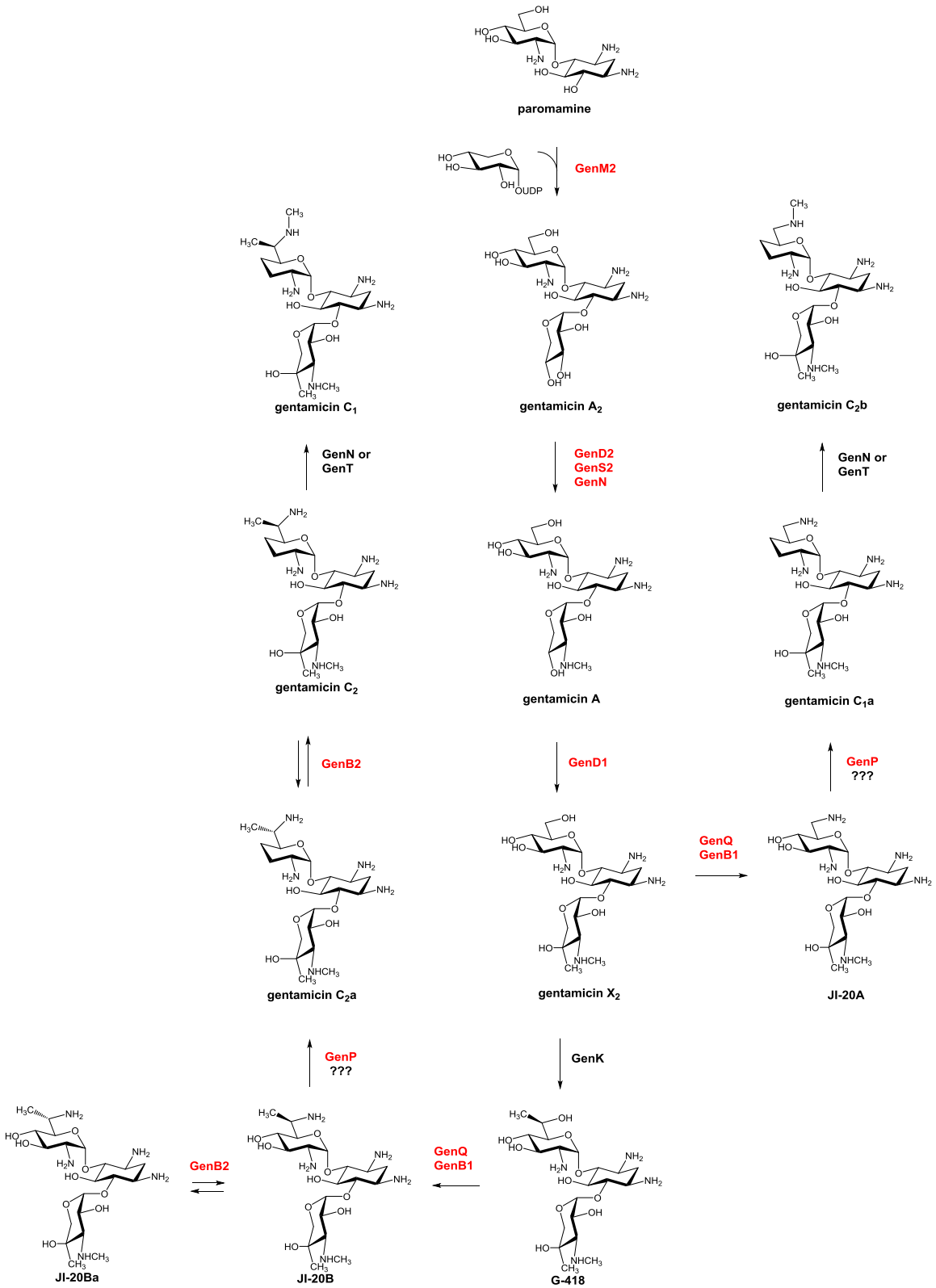
Figure 1-2. Gene cluster of gentamicin

Proposed Function	Piepers-berg	Welling-ton	Sohng	Siso-micin cluster	Forti-micin cluster	Frankia Ccl3 cluster	Ista-mycin cluster
hypothetical protein		GntT	GacN				
putative Trp-tRNA ligase		GntU	GacM				
queuosine/archaeosine tRNA-ribosyltransferase	GenO	GntV	GacL	Sis3	FosA	3378	
putative ribosomal methyltransferase	GmrB	GrmO	GacL	Sis4			
gentamicin (hexosaminyl-6-) aminotransferase	GenB1	GntW	GacK	Sis5 Sis18	ForB	3369	IstB
	GenB2	GntL	GacE				
	GenB3	GntJ	GacB				
	GenB4	GntH	GacC				
hexose-6'-dehydrogenase	GenQ	GntX	GacJ	Sis6	ForQ	3360	IstQ
SDR/putative gentamicin oxidoreductase	GenD3	GntY	GtmH	Sis7			
(*N*-acetyl)hexos-aminyltransferase	GenM1	GntZ	GtmG	Sis8	ForM	3362	IstM
putative ribosomal methyltransferase	GmrA	GrmA	GtmF	Sis9			

putative *L*-glutamine:ketocyclitol aminotransferase	GenS1	GntA	GtmB	Sis10 Sis15	ForS	3363	IstS
	GenS2	GntF	GtmD				
2-deoxy-*scyllo*-inose synthase	GenC	GntB	GtmA	Sis11			IstC
NAD(P) dependent oxidoreductase	GenD2	GntC	GtmC	Sis12			
gentamicin glycosyltransferase II	GenM2	GntD	GtmE	Sis13			
B12 radical SAM dependent oxidoreductase/ methyltransferase	GenD1	GntE	GtmI	Sis14			
GTP cyclohydrolase /NADPH dependent 7-cyclo-7-deazaguanine reductase	GenW	GntG	GacA	Sis16	FosC	3383	
biosynthetic aminoglycoside 3'-	GenP	GntI	GtmJ	Sis17	ForP	3368	
B12 radical SAM dependent *C*-methyltransferase	GenK	GntK	GtmJ		ForK	3370	
hypothetical protein	GenX	GntM	GacF	Sis19			
hypothetical protein	GenU	GntN	GacG	Sis20			
deoxyinosamine dehydrogenase	GenE	GntP	GacH	Sis22	ForE	3364	IstE
putative cation antiporter	GenY	GntQ	GacI	Sis23	ForY		
ATP utilize enzyme (QueC)	GenA	GntR		Sis24	FosE	3379	
radical SAM enzyme (QueE)	GenF	GntS		Sis25	FosF	3380	
Que homologue	GenG				FosG	3381	
export protein	GenV GenH GenI	GntO	GtmK	Sis21 Sis26	ForI ForH ForJ ForV	3359 3358	IstI IstH IstJ
SAM-dependent methyltransferase	GenT			Sis28	ForT	3373	

putative *N*-acetylhexosaminyl deacetylase	GenD		GtmM	Sis29	ForD	3367	IstD
putative gentamicin methyltransferase	GenN			Sis30			
hypothetical					ForW	3361	IstW
putative fortimicin *O*-methyltransferase					ForO	3365	IstO
radical SAM family protein					ForL2	3366	IstL2
fortimicin A *N*-fortimidoyltransferase					ForZ	3371	IstZ
putative fortimicin production protein					ForX	3376	IstX
putative myo-inositol 3-dehydrogenase					ForG	3374	
fortimicin *N*-methyltransferase					ForN	3373	IstN
inositol-1-monophosphatase					ForA		
radical SAM protein					ForL	3357	IstL
						3375	IstL3

Table 1-1. Gene analysis of gentamicin


paromamine
GenM2
gentamicin C1
gentamicin A2
gentamicin C2b
GenN or GenT
GenD2 GenS2 GenN
GenN or GenT
gentamicin C2
gentamicin A
gentamicin C1a
GenB2
GenD1
GenP ???
gentamicin C2a
gentamicin X2
GenQ GenB1
JI-20A
GenP ???
GenK
JI-20Ba
GenB2
JI-20B
GenQ GenB1
G-418

Scheme 1-6. Biosynthetic pathway for gentamicins. The activity of the enzymes with red colors has already been demonstrated.

Many biotransformations in the biosynthetic pathway of gentamicin have been demonstrated. The Yoon group confirmed that GenM2 is required for the glycosylation that forms the pseudotrisaccharide from paromamine and UDP-xylose.[79] They used an engineered paromamine-producing strain of *S. venezuelae* to generate gentamicin A_2. However, it was noted that the amount of gentamicin A_2 produced with *S. venezuelae*/pYJ505 was 4.5 μg/L, and could only be detected by LC-MS analysis. This was mainly due to limited amount of NDP-xylose present in *S. venezuelae* host cells. When cell free extracts of *S. venezuelae*/pYJ505 were supplied with UDP-xylose, gentamicin A_2 production was enhanced up to 8-fold, supporting that the glycosyl donor is UDP-xylose. Two oxidation and amination reactions occur in the biosynthetic pathway of gentamicins. One is the conversion of gentamicin A_2 to gentamicin A, and the other is the conversion of gentamicin X_2 to JI-20A or G-418 to JI-20B. Leadlay and Sun *et al* demonstrated through gene mutation experiments that the first oxidation and amination are catalyzed respectively by GenD2 and GenS2,[80] and the second transformations are catalyzed by GenB1 and GenQ.[81] For the two *N*-methyltransfer reactions from gentamicin A2 to gentamicin A and from gentamicin C_{1a} to gentamicin C_{2b} or from gentamicin C2 to gentamicin C1, only the methylation reaction at N3" has been established to be catalyzed by GenN.[80,82] Two *C*-methyltransfer reactions have now been shown to be catalyzed by cobalamin-dependent radical SAM enzymes (GenD1 and GenK). GenD1 catalyzes the methylation at C4" of garosamine,[80] and is described in detail in Sections 2.1 and 2.2. GenK catalyzes the methylation at C6' and is discussed in Chapters 2 and 3. Two epimerization reactions are also required for gentamicin biosynthesis: the first is from JI-20B to JI-20Ba,[81] and the second is from gentamicin C2a

to gentamicin C2.[83] These two epimerizations are both catalyzed only by GenB2 and are reversible.

The remaining uncharacterized steps are the deoxygenation at C3' and C4' and *N*-methylation at the end to complete the biosynthesis of gentamicins. While GenP is known to catalyze phosphorylation of the hydroxyl group at C3',[84] the remaining enzymatic reactions are enigmatic. From comparison of the genes in the gentamicin and fortimicin clusters, several genes could be involved in the dideoxygenation reaction, such as GenV, GenH, and GenI. These genes have been assigned as membrane associated exporters. However, these proteins do not resemble membrane proteins, and homologues of these enzymes are not present in any other organisms based on BLAST analysis.

1.1.3.6. Biosynthesis of Other Aminoglycosides

Biosynthetic studies of other aminoglycosides with 2-DOS like tobramycin, apramycin, and lividomycin have not been successful so far. Conserved biosynthetic enzymes are expected to be responsible for the construction of the common biosynthetic intermediate, 2-DOS and paromamine based on the gene analysis.[8,33]

Tobramycin (3'-deoxykanamycin B) has been identified from two strains, *Streptoalloteichus hindustanus* and *Streptomyces tenebrarius* (Figure 1-1).[8] Both microorganisms produce apramycin as well, indicating that the biosynthesis of tobramycin and apramycin share some biosynthetic genes. The biosynthesis of tobramycin includes deoxygenation of paromamine or kanamycin C. Comparison of the tobramycin/apramycin, kanamycin and lividomycin biosynthetic gene clusters has suggested that AprD3 and AprD4 catalyze deoxygenation reaction during the biosynthesis of tobramycin and apramycin.[8,33] AprD3 and AprD4 are described in detail in Chapter 4.

1.2. RADICAL *S*-ADENOSYL-L-METHIONINE (SAM) ENZYME

S-Adenosyl-L-methionine (SAM) is a versatile compound in nature. This compound is primarily known for its electrophilic methyl that is used to methylate nucleotides, amino acids, or other molecules containing sulfur, amino, and hydroxyl nucleophiles. However, in the early 1970s, lysine 2,3-aminomutase is shown to use SAM in a novel way.[85-86] This enzyme is sensitive to air and its activity depends on the presence of dithionite and SAM. Further studies showed that the 5'-deoxyadenosyl moiety of SAM is homolytically cleaved and the resulting 5'-deoxyadenosyl radical facilitates hydrogen abstraction from the substrate of lysine 2,3-aminomutase,[87] which is similar to the reactions catalyzed by adenosylcobalamin-dependent mutases using the 5'-deoxyadenosyl radical. EPR analysis revealed the presence of a [4Fe-4S] cluster.[88-89] Studies of several other enzymes such as pyruvate formate-lyase activase,[90-94] biotin synthase,[91] and ribonucleotide triphosphate reductase III,[95-96] led to the characterization of a new class of enzymes that utilize a reduced iron-sulfur cluster and SAM to initiate their catalyses. This class enzymes, which has grown to approximately 64000 based on bioinformatics analysis,[97] are across all phylogenetic kingdoms and are now referred as the radical SAM superfamily.[98]

Radical SAM enzymes have a characteristic Cx_3Cx_2C sequence motif conserved in most of superfamily members, although enzymes exhibiting variations of this motif are also known (see in Section 4.1). In the active site of a typical radical SAM enzyme, the three cysteine residues coordinate three of the four irons of a [4Fe-4S] cluster, and one of the four iron atoms is coordinated by the amino and carboxylate moieties of SAM (Figure 1-3).[99-100] Reducing agents such as flavodoxin or dithionite can provide one electron to reduce the $[4Fe\text{-}4S]^{2+}$ cluster to generate the catalytically active $[4Fe\text{-}4S]^{+}$. The reduced

$[4Fe\text{-}4S]^{+}$ cluster can reduce SAM leading to homolysis of the S-C5' bond to give the a 5'-deoxyadenosyl radical (5'-dAdo•) and methionine. The 5'-dAdo• s then used to abstract a hydrogen atom from the substrate in a stereo- and regiospecific manner to generate a substrate radical which can undergo further reaction such as methylation, sulfur insertion, oxidation, dehydrogenation, methylthiolation, and complex carbon skeleton rearrangements.[101] In most radical SAM enzymes, SAM is consumed during catalysis, and methionine and 5'-dAdo along with the product are produced in a 1:1:1 ratio (pathway A in Figure 1-3). However, when SAM is used catalytically, the product radical abstracts a hydrogen atom from 5'-dAdo to afford the product and 5'-dAdo•. The latter recombines with methionine to regenerate SAM (pathway B in Figure 1-3).

A second auxiliary iron-sulfur cluster has been found in many radical SAM enzymes. In some cases, the second cluster is involved in the reaction (Figure 1-4). For example, in the biosynthesis of biotin, one sulfur atom in the second cluster is inserted between C6 and C9 of dethiobiotin to generate biotin (Figure 1-4A).[101] In the cases of MiaB and RimO, the nucleophilic persulfide-bound moiety of the second cluster accepts the methyl group from SAM (Figure 1-4B).[102] The resulting methylated sulfur atom of the polysulfide chain reacts with the substrate radical to yield the methylthiolated product and a [4Fe-4S] cluster with a terminal persulfide. HydG has another auxiliary [4Fe-4S] cluster coordinated with a $Cx_2Cx_{22}C$ motif (Figure 1-4C).[103] A tyrosine bound to an auxiliary *C*-terminal [4Fe-4S] cluster reacts with 5'-dAdo• generated by SAM and an *N*-terminal [4Fe-4S] cluster to provide a tyrosine radical. Heterolytic cleavage of this tyrosine radical at the Cα–Cβ bond forms a transient 4-oxidobenzyl (4OB) radical and the dehydroglycine bond to the C-terminal [4Fe-4S] cluster, which has been detected by EPR spectroscopy.[104] Other secondary [4Fe-4S] clusters are expected to act as electron donors.[105]

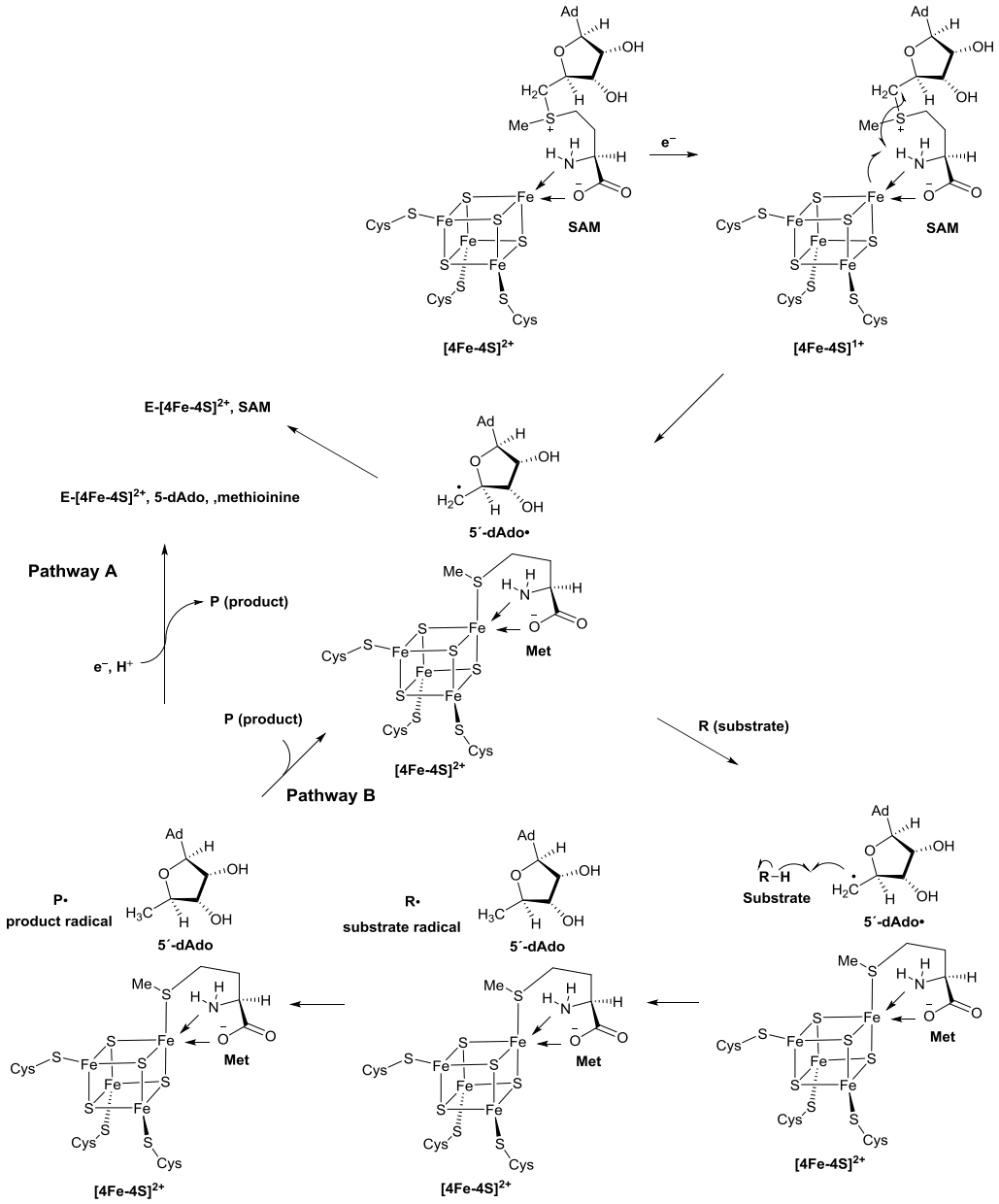


Figure 1-3. Mechanism for radical SAM cleavage

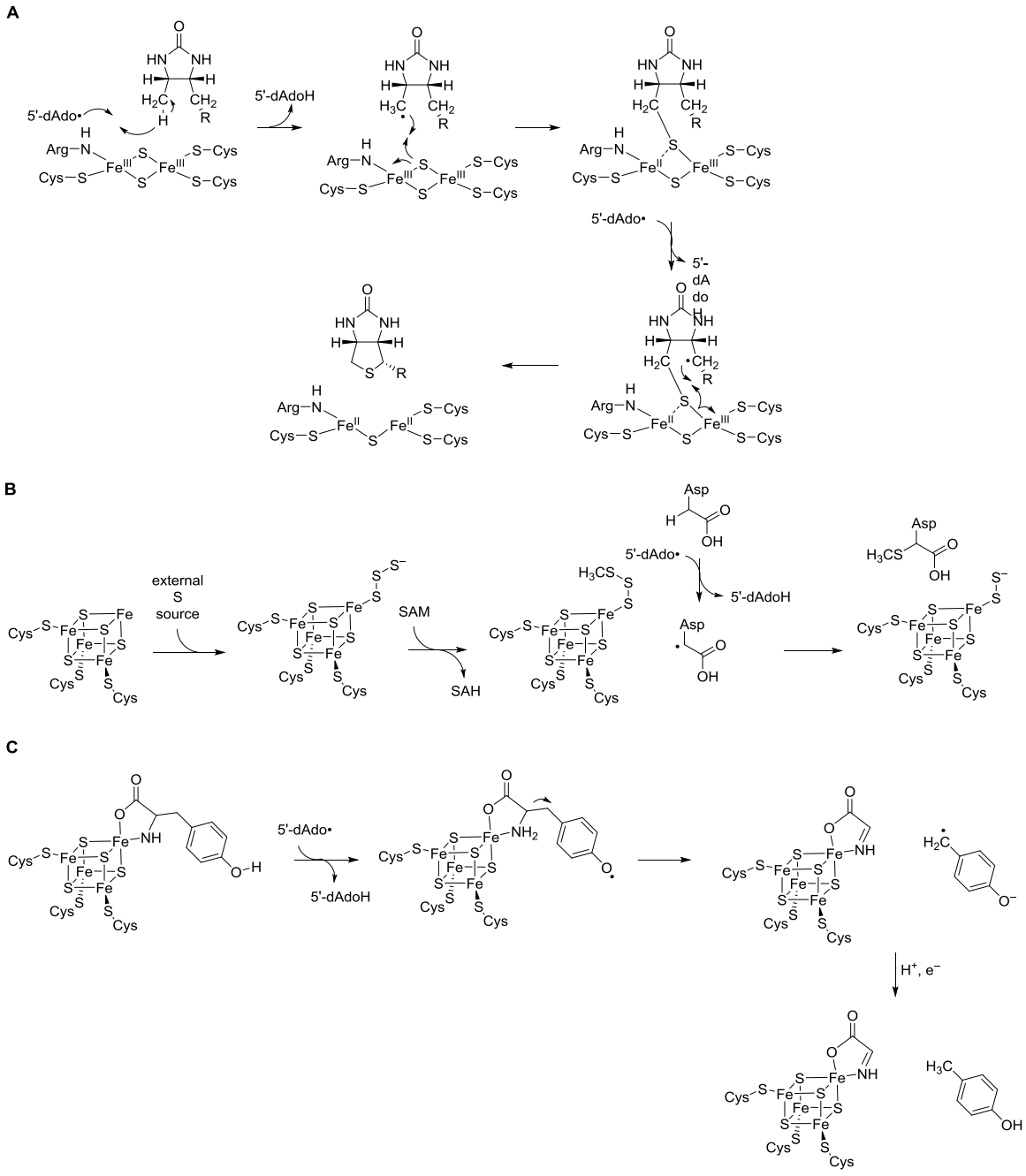


Figure 1-4. Roles of the secondary [4Fe-4S] clusters in selected radical SAM enzymes. A) BioB in biotin biosynthesis, B) RimO in the methylthiolation of aspartate89 of the ribosomal S12 protein, C) HydG in the biosynthesis of the H-cluster of the [FeFe]-hydrogenase

1.3. RADICAL SAM ENZYMES IN AMINOGLYCOSIDE BIOSYNTHESIS

In the biosynthetic pathways of aminoglycosides, several enzymes have been assigned as radical SAM enzymes such as BtrN in the butirosin pathway, NeoN in the neomycin pathway, RibN in the ribostamycin pathway, ParN in the paromomycin pathway, LivN and LivW in the lividomycin pathway, GenD1, GenK and GenF in the gentamicin pathway, IstL2, IstL, and IstL3 in the istamycin pathway, ForK, FosF, ForL2, and FosL in the fortimicin pathway, AprD4 in the apramycin/tobramycin pathway, HygY in the hygromycin pathway, and SpcY in the spectinomycin pathway,[8,33] but only a few radical SAM enzymes (i.e. BtrN, NeoN, and GenD1) in aminoglycoside biosynthesis have been characterized *in vitro*. GenD1 is discussed in detail in Section 2.1 and Section 3.1. The *in vitro* characterizations of GenK and AprD4 are presented in this dissertation in detail.

1.3.1. BtrN

During the biosynthesis of 2-deoxystreptamine (2-DOS) in the butirosin producer *B. circulans*, BtrN catalyzes the oxidation of the alcohol at C1 of 2-deoxy-*scyllo*-inosamine (2-DOIA) to form keto-2-deoxy-*scyllo*-inosamine (keto-2-DOIA) (another named 3-amino-2,3-dideoxy-*scyllo*-inosose (amino-DOI)) under anaerobic conditions (Scheme 1-1).[106] From analysis of the butirosin biosynthetic gene cluster, BtrN has been shown to possess canonical the CxxxCxxC motif in its primary sequence, indicating that this enzyme is a radical SAM enzyme.[106] Electron paramagnetic resonance (EPR) studies performed at 50 °K with reconstituted BtrN but without SAM and 2-DOIA showed characteristic *g* values of 1.92 and 2.04 for the [4Fe-4S] cluster of radical SAM enzymes.

These *g* values can be compared with reconstituted BtrN in the presence of dithionite and SAM (*g* = 1.83, 1.99) or with additional SAM and 2-DOIA (*g* = 1.87, 1.96, 2.05).[107]

Gene analysis of BtrN showed the existence of eight cysteine residues, suggesting that a second [4Fe-4S] cluster may exist.[108] Mutagenesis studies indicated that reconstituted BtrN contains two [4Fe-4S] clusters. When the three cysteine residues of the CxxxCxxC motif were changed to alanine, the enzyme was found to still contain a single [4Fe-4S] cluster based on iron/sulfur titration and Mössbauer spectroscopy.[108] Generation of variants in which the remaining cysteine residues were changed to alanines showed that the C69A mutant provided similar activity as the wild type, but the C235A mutant displayed reduced solubility and low activity. Mutation of Cys169, C187 and C232 produced insoluble proteins. These results led to the proposal that the three latter residues serve as ligands to bind the second [4Fe-4S] cluster.

Kinetics analysis revealed that BtrN proceeds with an ordered mechanism because uncompetitive substrate inhibition was observed with amino-DOI, but not with SAM.[106] In BtrN catalysis, one equivalent of SAM versus 2-DOIA is consumed and an equal number of moles of 5'-dAdo, methionine, and amino-DOI are generated. Assays of BtrN with [3-^{2}H]-2-DOIA generated a mixture of unlabeled, monodeuterated, and dideuterated 5'-dAdo, while incubation of nonlabeled 2-DOIA in the deuterium buffer did not provide incorporation of deuterium at all. These results indicate that 5'-dAdo radical abstracts a hydrogen atom from the C3 position of 2-DOIA.

Determination of the X-ray structure of this enzyme with and without substrate further clarified the mechanism and role of the auxiliary cluster.[109] The auxiliary cluster is fully ligated both in the presence and in the absence of the substrate, which excludes the possibility that this cluster coordinates the substrate. In addition, 2-DOIA is bound in a hydrophilic pocket between SAM and the auxiliary clusters. Based on the observation

that 2-DOIA binds in an equatorial chair conformation and hydrogen bonding interactions with the functional group of the substrate, the auxiliary cluster in BtrN is suggested to function as an electron acceptor during the dehydrogenation reaction. Also the putative base, Arg152, involved in the BtrN catalysis was identified. Formation of the α-hydroxyalkyl radical after H-atom abstraction by 5'-dAdo radical promotes deprotonation of the C3-hydroxy functional group by decreasing its pK_a. The proposed mechanism of the BtrN reaction is shown in Figure 1-5.

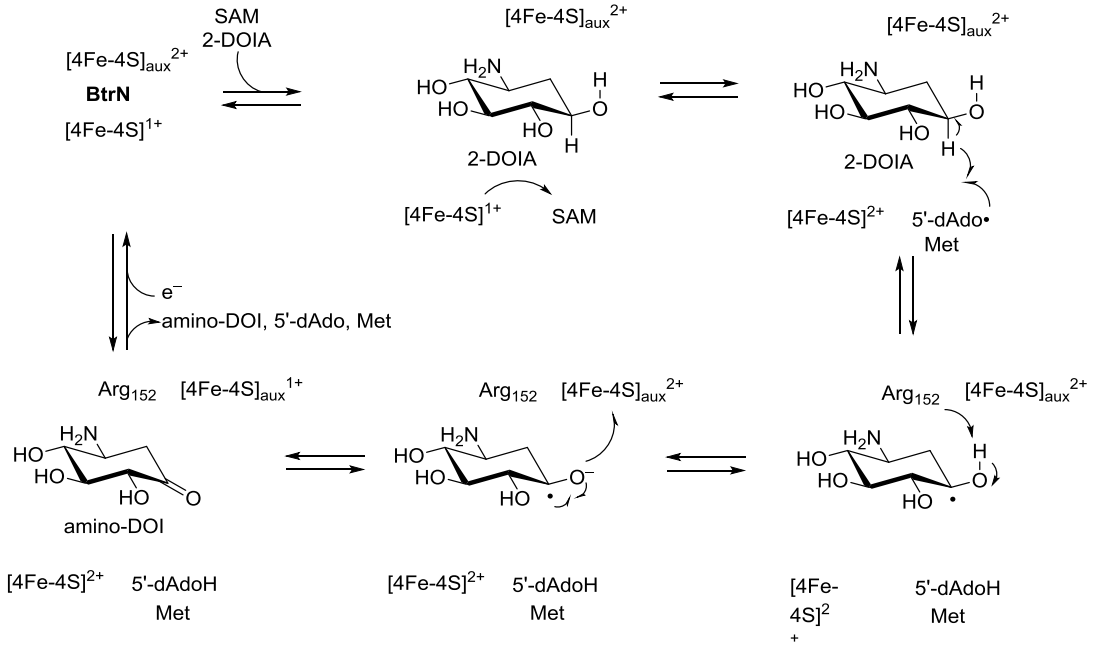


Figure 1-5. Proposed mechanism of the BtrN reaction

The substrate flexibility of BtrN was examined using several cyclitol and sugar compounds (Figure 1-6).[106] Only three substrate analogues such as DOS, *scyllo*-inositol, and *myo*-inositol showed activity with BtrN in terms of the formation of 5'-dAdo. These results indicate that BtrN exhibits strict specificity for cyclitols and not for sugars.

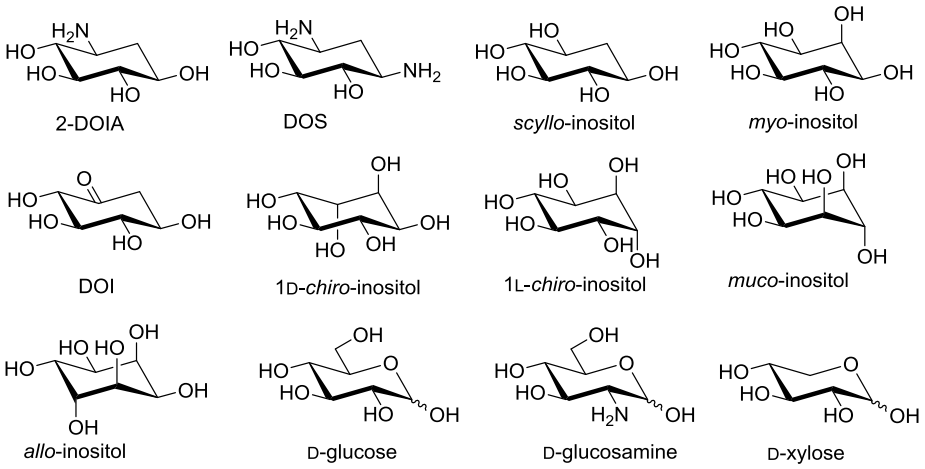
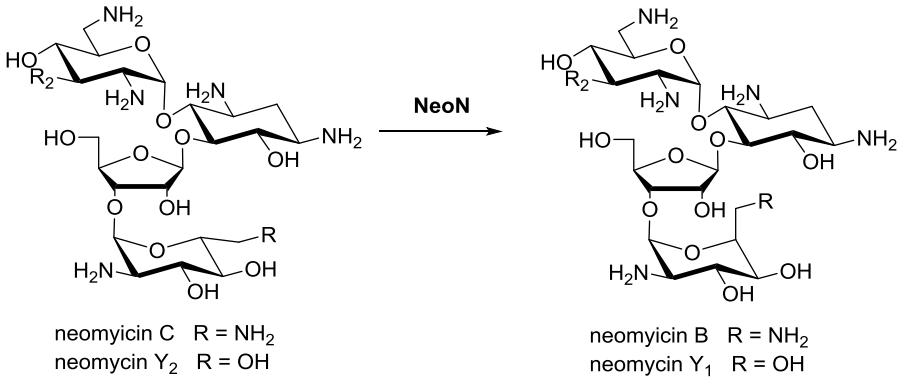


Figure 1-6. Substrate specificity of BtrN

1.3.2. NeoN

In the neomycin biosynthetic pathway, NeoN was assigned to catalyze the epimerization at C5''' of neomycin C to generate neomycin B.[8,33] ParN in paromomycin, and LivN in lividomycin are also assigned to have analogous functions because they have similar structures (Figure 1-1). These enzymes contain the canonical CxxxCxxC motif that is present in most radical SAM enzymes. The reconstituted and reduced NeoN with SAM and neomycin C under anaerobic conditions provided neomycin B.[110] NeoN also catalyzes the conversion of neomycin Y_2 to neomycin Y_1, indicating that the amino group at C6''' is not necessarily involved in substrate recognition (Scheme 1-7). In the NeoN catalytic cycle, SAM is not utilized catalytically based on the observation that one equivalent each of SAM and the substrate are consumed, while equal amounts of 5'-dAdo and neomycin B are generated.[110]



Scheme 1-7. Epimerization of NeoN

The NeoN reaction carried out in a buffer prepared with deuterium oxide was analyzed by LC-ESI-MS and NMR, demonstrating the incorporation of a single deuterium atom at the C-5''' of neomycin B. Mutation studies of NeoN with a triple cysteine mutant for the radical SAM motif (C26/30/33A) did not show epimerization activity, indicating that these cysteine residues are critical for catalysis. The assay of NeoN with C249A provided a new product, a five-membered carbocyclic structure with an aminomethyl group confirmed by 2D NMR analysis (Figure 1-7B). This result suggests that the C249 thiol provides the hydrogen atom at C5''' position in which 5'-dAdo• abstracts to complete the epimerization of neomycin C. EPR spectroscopic studies performed at 10K with reduced and reconstituted NeoN showed characteristic *g* values of 2.03 and 1.92. However, in the presence of SAM, the *g* values changed to 2.00, 1.90, and 1.86, indicating the coordination of SAM to the $[4Fe\text{-}4S]^{+}$ cluster. Addition of neomycin C did not affect the shape of the signal and this result suggests that neomycin C does not coordinate directly with the $[4Fe\text{-}4S]^{+}$ cluster even though the substrate may be located close to the cluster. EPR analysis with the reduced C249A variant, neomycin C and SAM at 10 K revealed almost the same signals compared to the wild type, but at 50 K a

hyperfine-coupled EPR signal was observed. This observation indicated trapping of an organic radical generated at the C-5''' position of neomycin.

The iron and sulfur atom contents in NeoN were estimated respectively at 6.6 ± 0.9 and 5.8 ± 0.9 equivalents per protein monomer, which indicates that NeoN has a second [4Fe-4S] cluster.[107] UV spectroscopic analysis of the C26/30/33A mutants showed an absorption around 420 nm, which is characteristic for [4Fe-4S] clusters. Other mutation studies showed that the C226/246/271/274A mutants led to self-degradation of NeoN during protein purification. Sequence alignment of NeoN with BtrN[109] and anaerobic sulfatase maturating enzyme (anSME)[111] indicated that a putative second [4Fe-4S] cluster might coordinate a set of C226, C247, C271, and C274. Also it is also expected that C249 is located close to the second [4Fe-4S] cluster and might be able to interact with C247. From these analyses, catalytic mechanism was proposed for NeoN as shown in Figure 1-7A.

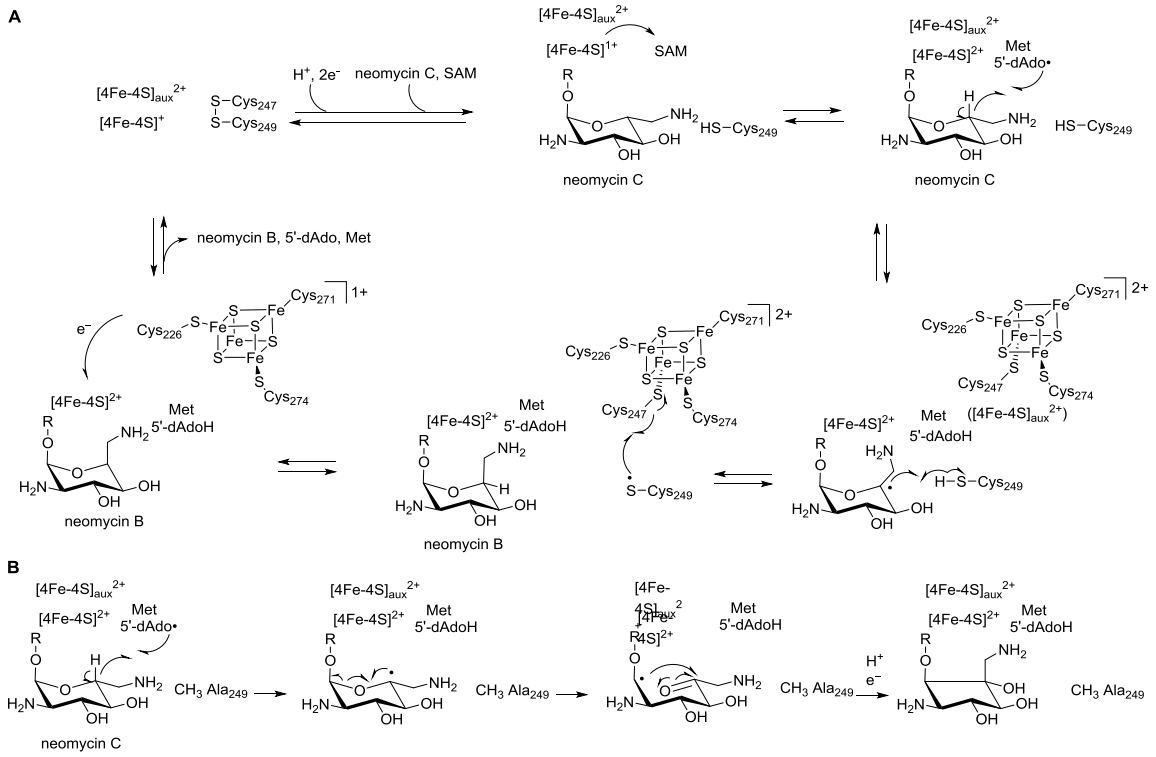


Figure 1-7. NeoN catalysis. A) Proposed mechanism of NeoN reaction. B) Reaction of C249A mutant of NeoN with neomycin C

1.4. DISSERTATION STATEMENT

Biosynthetic studies of natural products have become essential to the discovery and development of new drugs, because understanding the biosynthetic pathways and the chemical mechanisms of each enzyme in the pathways can facilitate efforts in pathway engineering to produce new compounds with improved clinical properties. Aminoglycosides are well known and important antibiotics, but antibiotic resistance to aminoglycosides is increasing. This has led to renewed interest in the biosynthesis of

aminoglycosides. Radical SAM enzymes catalyze novel and miscellaneous reactions in the biosynthesis of natural products. Even though several enzymes in the biosynthesis of aminoglycosides are assigned to be radical SAM enzymes based on the gene analysis, only a few radical SAM enzymes have been characterized *in vitro*. This dissertation focuses on the *in vitro* characterization of two radical SAM enzymes in the biosynthesis of aminoglycosides.

Chapter 2 describes the *in vitro* characterization of a Cbl-dependent radical SAM methyltransferase, GenK. This enzyme catalyzes the methylation of an unactivated sp^3 carbon of gentamicin X_2 to produce G418 during the biosynthesis of gentamicin. Experiments to investigate the stoichiometry of GenK reaction showed that an equal number of equivalents of 5'-dAdo, SAH, and G418 are produced. Isotope-labeling experiments demonstrated that the *S*-methyl group from SAM is transferred via Cbl to the aminoglycoside product during the course of the reaction. Experiments with other aminoglycosides in the biosynthetic pathway of gentamicin demonstrated the promiscuity of GenK substrate recognition.

Chapter 3 describes efforts to elucidate the mechanism of the GenK reaction. From the results of Chapter 2, three mechanisms are proposed and examined using chemically synthesized substrate analogs. The GenK assays with stereospecifically labeled (*R*)- and (*S*)-[6'-^{2}H]-gentamicin X_2 demonstrated the stereochemical course of the hydrogen atom abstraction by 5'-Ado• and the overall stereochemistry of the GenK catalyzed reaction. Various substrate derivatives of gentamicin X_2 modified at C6' were tested to investigate the manner of methyl transfer from methylcobalamin to the substrate to produce the methylated product.

Chapter 4 describes the *in vitro* characterization of a radical SAM enzyme AprD4 and its reductase partner AprD3. AprD4/D3 from *Streptomyces tenebrarius* were purified

following heterologous expression in *E. coli* and examined *in vitro*. The C3'-deoxygenation reaction of paromamine catalyzed by AprD4/D3 was confirmed by comparison of chemically synthesized lividamine. Mass spectroscopy experiments confirmed the function of AprD4 by the detection of 4'-oxolividamine. Experiments with isotope-labeled substrates showed the position of hydrogen atom abstraction by 5'-dAdo• and stereochemical course of proton transfer at C3'-deoxygenation of paromamine.

Chapter 2: Characterization of GenK, a Cobalamin-Dependent Radical SAM Methyltransferase Enzyme

2.1. INTRODUCTION

Methylation is a recurrent and significant reaction in biological processes.[112] Various biological molecules including natural products, metabolites, and macromolecules such as nucleic acids, proteins, and polysaccharides can all serve as substrates in methylation reaction whereupon their biological properties may be modified. For example, DNA methylation is a critical feature in the control of gene expression as methylated DNA sequences are subjected to transcriptional repression.[113] One of the key molecules that serves as methyl donor in these reactions is *S*-adenosyl-L-methionine (SAM).[114] In such reactions, attack at the electrophilic methyl group of SAM by a nucleophile such as amino, alkoxide and thiolate functionality of the substrate results in methyl transfer to the substrate and concomitant production of *S*-adenosyl-L-homocysteine (SAH). However, a number of methyltransferases that are mechanistically unique compared the standard paradigm involving Lewis acid-base chemistry have recently been described.[115] The catalytic cycles of these enzymes instead involve radical chemistry and result in the methylation of non-nucleophilic carbon or phosphrous centers.[116] These enzymes are members of radical SAM superfamily and thus catalyze the reductive homolysis of SAM via single electron transfer form an active site [4Fe-4S] cluster to produce methionine and a 5'-deoxyadenosyl radical (5'-dAdo•) that serves as a radical initiator in the subsequent transformation.[117]

In a recent review, Booker *et al*[116] divided the radical SAM methyltransferase (RSMT) into four classes (i.e. classes A, B, C, and D) organized by protein architecture, expected enzymatic mechanism, and cofactor dependence (Table 2-1). Catalysis by class A RSMTs involves the generation of a methylene radical on two strictly conserved cysteines and results in the methylation of sp^2-hybridized carbon centers. Representative enzymes in this class are RlmN[118-120] and Cfr[121-126], which contribute to the modification of rRNA in various organisms. Class B RSMTs are characterized by distinctive protein architecture that includes a cobalamin-binding domain at the *N*-terminus and a radical SAM domain at the *C*-terminus. These enzymes methylate unactivated sp^2- or sp^3-hybrized carbons or phosphinates. While class B is the largest and most diverse to date, only five enzymes (TsrM[127-129], Fom3[130-131], GenD1[81], ThnK[132] and PhpK[133-135]) have been studied *in vitro*. These enzymes are described in detail below. Class C RSMTs demonstrate significant sequence similarity with coproporpyphyrinogen III oxidase (HemN) and methylate sp^2-hybridized carbons. These enzymes do not have the two conserved cysteines that are characteristic of the class A enzymes and are frequently part of the biosynthetic pathways responsible for complex secondary metabolites with antitumor and antibiotic behavior such as nosiheptide (NosN[136] and NocN[137]), yatakemycin (YtkY[138-139]) and jawsamycin (Jaw5[140-141]). Finally, in the more recently discovered class D radical SAM methyltransferases, which includes MJ0619.[142] Methylenetetrahydrofolate is proposed to serve as the methyl donor rather than SAM.

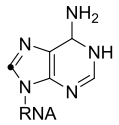
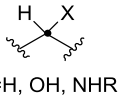
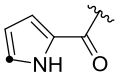
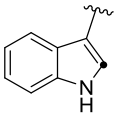
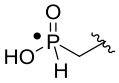
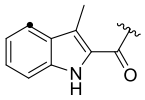
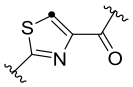
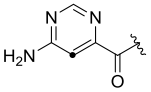
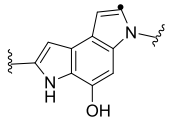
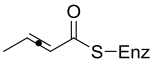
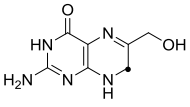
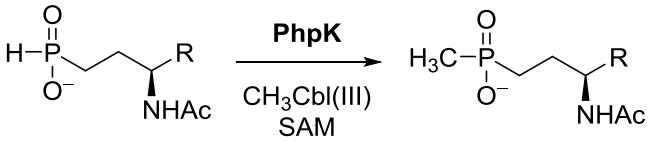
Class	Enzyme	Function	Substrate
A	RlmN Cfr	rRNA and tRNA modification rRNA modification	
B	Fom3 Fms7 GenD1 and GenK ThnK, L, and P PacJ, N, and O Cndl Swb9 HpnP BchQ, BchR PoyB and C	Forsfomycin biosynthesis Fortimicin A biosynthesis Gentamicin biosynthesis Thienamycin biosynthesis Pactamycin biosynthesis Chondrochlorens biosynthesis Quinomycin biosynthesis 2-Methylhopanoid biosynthesis Chlorophyll biosynthesis Polytheonamide biosynthesis	
	CloN6 CouN6	Clorobiocin biosynthesis Cooumermycin biosynthesis	
	TsrM SioT	Thiostrepton biosynthesis Siomycin biosynthesis	
	BcpD/PhpK	Bialaphos biosynthesis	
C	NosN NocN	Nosiheptide biosynthesis Nocathiacin biosynthesis	
	TpdI TpdL and U	Thiomuracin biosynthesis GE2270 biosynthesis	
	Blm-Orf8 Tlm-Orf11 Zpm-Orf26	Bleomycin biosynthesis Tallysomycin biosynthesis Zorbamycin biosynthesis	
	YtkT	Yatakemycin biosynthesis	
	Jaw5	Jawsamycin biosynthesis	
D	MJ0619	Methanopterin modification	

Table 2-1. The four classes of radical SAM methylases[5]

Among the four classes of radical SAM methylases, class B is of particular interest, because it includes GenK, which is the topic of this chapter. Other members of this class include PhpK, TsrM, Fom3, GenD1, and ThnK.

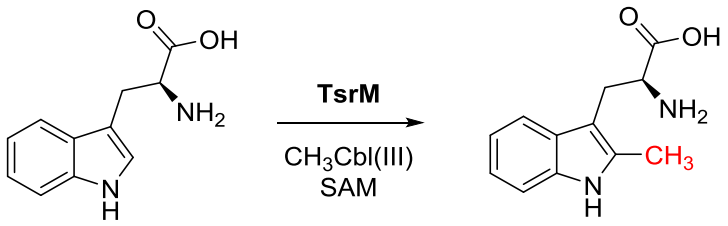
PhpK is isolated from the phosalacin producer *Kitasatospora phosalacinea,* and was the first class B RSMT to be purified and characterized *in vitro*.[133] PhpK catalyzes the methylation of the phosphinate group of 2-acetylamino-4-hydroxyphosphinyl butanoate (*N*-acetyldemethylphosphinothricin, NAcDMPT) to produce 2-acetylamino-4-hydroxymethylphosphinylbutanoate (*N*-acetylphosphinothricin, NAcPT) which possesses the only carbon-phosphorus-carbon linkage currently known in nature (Scheme 2-1). PhpK overexpressed from *E. coli* was not soluble and found only in inclusion bodies. After refolding and purification of the enzyme by anionic exchange chromatography, its Fe/S cluster was anaerobically reconstituted. Reconstituted PhpK coordinates a single [4Fe-4S] cluster, which was confirmed by EPR spectroscopy at 10 K. Assays containing reconstituted PhpK, SAM, methylcobalamin, NAcDMPT and dithionite enable observation of formation of NAcPT. Two dimensional ^{1}H-^{31}P gradient heteronuclear single-quantum correlation (gHSQC) spectroscopy displayed a strong H-P cross-peak indicative a methylated phosphorus center. However, the NAcPT-associated gHSQC cross peak was not found in the absence of dithionite, which is required for the reduction of the [4Fe-4S] cluster. When $^{13}CH_3$-*methyl*-Cbl(III) was used in PhpK reactions, H-P cross peaks from the passive couplings of ^{1}H and ^{31}P to the attached ^{13}C nuclei were found. This experiment demonstrated that MeCbl(III) is a methyl source for NAcPT.



R = COOH, L-Ala-L-Ala, or L-Ala-L-Leu

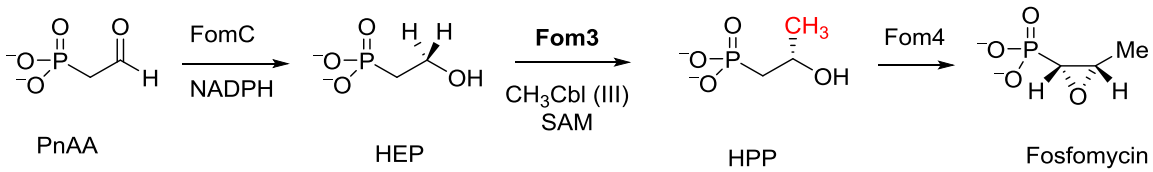
Scheme 2-1. P-Methyl transfer reactions catalyzed by PhpK

TsrM from *Streptomyces laurentii* catalyzes the methylation of sp^2-hybridized C2 carbon in tryptophan to produce 2-methyltryptophan during the biosynthesis of thiostrepton A (Scheme 2-2).[127-129] This reaction is the first step in the conversion of tryptophan to the quinaldic acid moiety of thiostrepton A. Early feeding experiments using chirally labeled methionine showed that the methyl group is transferred with net retention of configuration. This observation suggested a double displacement mechanism that involves two S_N2 type reactions, which differs from the typical methionine-dependent methylations.[143] Berteau *et al* purified TsrM as a strep-tag fusion protein in *E. coli*. The purified protein has one mol of iron and one mol of sulfur per polypeptide.[127] When the reconstituted enzyme was incubated in the presence of tryptophan, SAM and sodium dithionite, there was no detectable formation of 5'-dAdo, which is a typical harbinger of radical-SAM chemistry. However, after methylcobalamin was included in the previous assay mixture, methyltryptophan and SAH were produced in a constant ratio. Furthermore, isotope-tracer experiments demonstrated that the methyl group ultimately comes from SAM while cobalamin acts as an intermediate methyl carrier. When 11 tryptophan derivatives with different substitution at C5 (5-Me-, 5-OH-, 5-F-Trp) or without the α-carboxy group of tryptophan or with other changes were evaluated as potential substrates for TsrM, all compounds that were substituted on the indole ring were found to be susceptible to the methyl transfer reaction.[128]



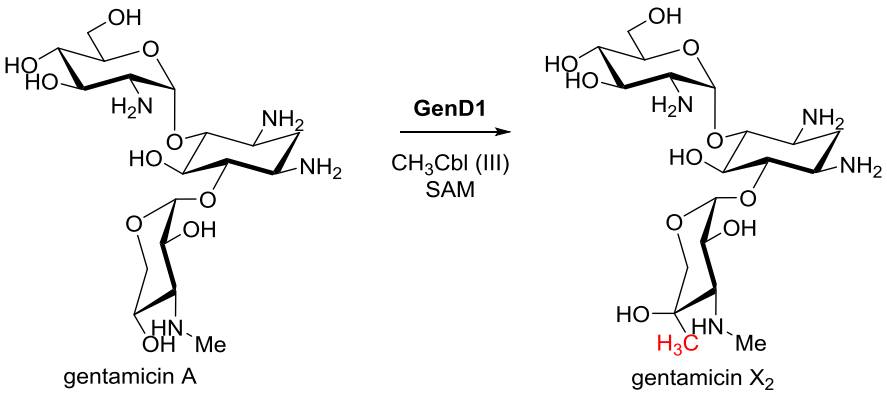
Scheme 2-2. Methylation of tryptophan catalyzed by TsrM

Fom3 isolated from *Streptomyces wedmorensis* catalyzes methylation of 2-hydroxyethylphosphonate (2-HEP) to produce 2-hydroxypropylphosphonate (2-HPP) in the biosynthetic pathway of fosfomycin (Scheme 2-3).[131] Early mutation studies showed that a mutant strain with a block in the vitamin B_{12} biosynthetic pathway could not produce fosfomycin, but converted HPP to fosfomycin.[144] Feeding experiments with ^{14}C-labled MeCbl and this mutant showed the formation of ^{14}C-labeld fosfomycin from HPP.[145] Isotope labeling experiments indicated that methyl transfer occurs at pro-(*R*) hydrogen position.[146] An *in vitro* study of wild type (WT) Fom3 was conducted by Wang *et al*.[130] WT Fom3 overexpressed from *E. coli* was not soluble and found exclusively in the inclusion bodies. After refolding, however, the enzyme could be isolated and reconstituted with additional ion and sulfide. N-terminal hexahistidine tagged WT His-Fom3 was also prepared. WT Fom3 has a single [4Fe-4S] cluster and His_6-Fom3 has four [4Fe-4S] clusters. In low-temperature EPR experiments, WT Fom3 and His_6-Fom3 showed signals of the [4Fe-4S] cluster. Cysteines 282, 286, and 289 in the conserved radical SAM CX_3CX_2C motif were shown to be essential for cluster binding by mutation studies. ^{31}P NMR spectroscopic data with His-Fom3, dithionite, 2-HEP, and MeCbl exhibited signals corresponding to HPP. However, no product formation has been detected due to the instability of the enzyme.



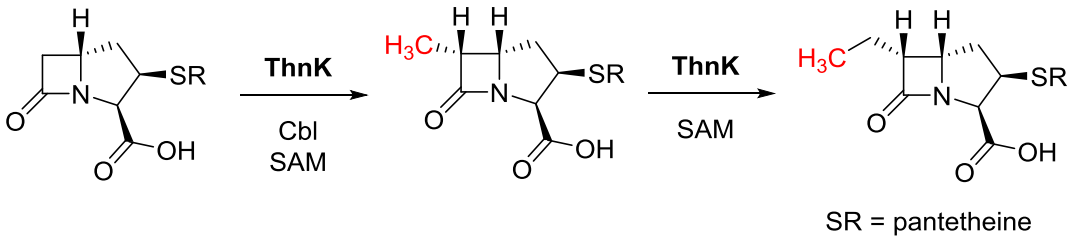
Scheme 2-3. Fosfomycin biosynthetic pathway

GenD1 is another putative radical SAM enzyme in the gentamicin biosynthetic pathway.[81] GenD1 catalyzes the *C*-methylation at C-4" of gentamicin A to produce gentamicin X_2 (Scheme 2-4). GenD1 has a noncanonical Cx_4Cx_2C motif instead of the highly conserved Cx_3Cx_2C binding motif for the [4Fe-4S] cluster found in most radical SAM enzymes. *In vitro* studies of GenD1 were conducted by Leadlay and Sun *et al*.[81] In contrast to other radical SAM methyltransferase, GenD1 could be expressed and isolated as the *N*-His_6-tagged protein in *E. coli* in soluble form and in excellent yield. After reconstitution of the iron-sulfur cluster, assays with GenA, SAM, dithiothreitol (DTT), methylcobalamin, methyl viologen and NADPH showed production of $GenX_2$ and 5'-deoxyadenosine (5'-dAdo) which were confirmed by HPLC and mass spectroscopy. As expected, no activity was observed with non-reconstituted enzyme or under aerobic conditions.



Scheme 2-4. GenD1 catalyzed methylation reaction

Townsend *et al* found that ThnK from *Streptomyces cattleya* in the biosynthetic pathway of thienamycin catalyzes sequential methylations to build out the C6-ethyl side chain in a stereocontrolled manner (Scheme 2-5).[132] ThnK was expressed with a *C*-terminal His_6-tag in *E. coli* Rosetta 2(DE3) and enzyme production was conducted in ethanolamine-M9 medium, which facilitates uptake of externally supplied HOCbl into *E. coli*. Without further reconstitution of iron and sulfur, the as-isolated ThnK has 7.4 ± 1.4 equivalents of iron and 3.7 ± 0.8 equivalents of sulfide per polypeptide. Assays of ThnK with SAM, methyl viologen, NADPH and (*2R*,*3R*,*5R*)-carbapenam showed the formation of 5'-deoxyadenosine (5'-dAdo), *S*-adenosylhomocysteine (SAH) and an ethylated product. Labeling experiments and mass spectrometry using *S*-adenosyl-[CD_3]-*methyl*-methionine (d_3-SAM) demonstrated an M+3 *m/z* shift for the methylated product and an M+5 *m/z* shift for the twice-methylated product, consistent with the formation of an ethyl side chain at C6 of the substrate by successive methyl transfers.



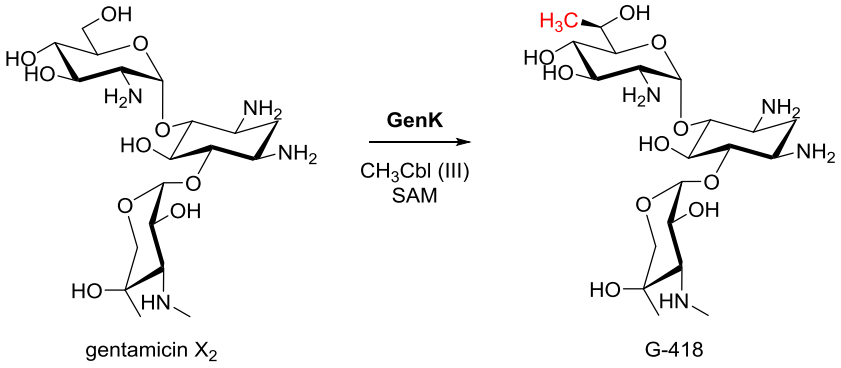
Scheme 2-5. Double methylation catalyzed by ThnK

The class B radical SAM methyltransferases characterized *in vitro* so far are summarized in Table 2-2. TsrM and GenD1 gave different products from SAM. TsrM gave only SAH, not 5'-dAdo, but GenD1 gave opposite results. Most enzymes used MeCbl or HOCbl, indicating that Cbl plays a role as an a methyl carrier for the methyl transfer process. Reducing systems required to reduce the [4Fe-4S] cluster to the active +1 redox state were different. PhpK and Fom3 were reduced by dithionite. The GenD1 and ThnK reactions used methyl viologen (MV) or benzyl viologen (BV) and NADPH. However, TsrM used dithiothreitol (DTT) as the sole reductant.

Enzymes	PhpK	TsrM	Fom3	GenD1	ThnK
Fe S	5.9 4.4	5.0 ± 0.3 3.0 ± 0.2	5.7 ± 0.7 7.5 ± 1.0	No information	7.4 ± 1.4 3.7 ± 0.8
Substrate	P	sp^2 C	sp^3 C	sp^3 C	sp^3 C
SAM amount used	2-5	1	0.5-1	4	1
5'-dAdo formation	No information	No	No information	Yes	Yes
SAH formation	No information	Yes	No information	No	Yes
Cbl used	MeCbl	MeCbl HOCbl CNCbl AdoCbl	MeCbl	MeCbl HOCbl	MeCbl HOCbl
Reducing systems	dithionite	DTT	dithionite	NADPH/MV NADPH/BV	NADPH/MV

Table 2-2. Class B radical SAM methyltransferses characterized *in vitro.*

The biosynthesis of gentamicins is already described in the Chapter 1. Sequence analysis of the gentamicin C_1 biosynthetic gene cluster suggested that the methylation step at C-6' of gentamicin X_2 ($GenX_2$) to yield the clinically useful geneticin (G418)[75, 147] is likely catalyzed by a Cbl-dependent radical SAM enzyme, GenK (Scheme 2-6).[77] GenK belongs to class B of the radical SAM dependent methyltransferases. Recently gene mutation experiments showed that knock-out of the genK gene blocked the production of gentamicin C_1 or C_2, while production of gentamicin C_{1a} was increased significantly.[148] The *vitro* activity of GenK and results of product stoichiometry, mechanism of methyl transfer, and alternative substrates are reported herein.



Scheme 2-6. GenK catalysis

2.2. EXPERIMENTAL PROCEDURES

2.2.1. Materials and Equipment

All chemicals were purchased from Fisher Science (Pittsburgh, PA) or Sigma-Aldrich (St. Louise, MO) and used without further purification unless otherwise specified. DNA gel extraction and spin minipreps were obtained from Qiagen (Valencia, CA). All reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Amicon and Microcon YM-10 filtration products were purchased from Bio-Rad (Hercules, CA) and Millipore (Billerica, MA), respectively. *Escherichia coli* DH5*a* cells were bought from Bethesda Research Laboratories (Muskegon, MI). The vector pET24b (+) and enzyme KOD DNA polymerase were purchased from Novagen (Madison, WI). DNA modifying enzymes (for restriction digestion and ligation), PCR primers, and the overexpression host *E. coli* BL21 star (DE3) were acquired from Invitrogen (Carlsbad, CA) and New England Biolabs (NEB, Beverly, MA). Luria Bertani

(LB) media are products of Difco (Detroit, MI) or Fisher Scientific (Pittsburgh, PA). Pre-stained protein markers were purchased from NEB. Protein concentrations were determined by Bradford Assay[149] using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using SDS-PAGE. The general methods and protocols for recombinant DNA manipulations were as described by Sambrook *et al*.[150] DNA sequencing was performed at the Core Facilities of the Institute of Cellular and Molecular Biology, University of Texas at Austin. All reactions involving GenK were performed in a Coy Anaerobic Chamber (glovebox) under an atmosphere of approximately 97.5% N_2 and 2.5% H_2 with less than 1 ppm O_2. All solutions used for GenK assays were deaerated by bubbling with nitrogen gas before they were transferred into the glovebox. The solvents in the glove box were stirred open to the anaerobic atmosphere overnight to allow equilibration before use. Gentamicin X_2, G-418, and GenA were purchased from TOKU-E (Bellingham, WA). Tetrahydrofuran (THF) was distilled from sodium benzophenone and dichloromethane (CH_2Cl_2) was distilled over calcium hydride under a nitrogen atmosphere. Other anhydrous solvents were purchased from Acros Organics (Pittsburgh, PA). Analytic thin layer chromatography (TLC) was performed on pre-coated TLC glass plates (silica gel, grade 60, F254, 0.25 mm layer thickness) purchased from EMD Chemicals (Madison, WI). Flash column chromatography was carried out on silica gel (230-400 mesh, grade 60) from Sorbent Technologies (Atlanta, GA) by eluting with the specified solvents. ^1H and ^{13}C NMR spectra were recorded at 400, 500, 600 MHz and 100, 125, 150 MHz with a Varian Gemini Spectrometer. Chemical shifts are reported as parts per million (ppm) relative to deuteriochloroform ($CDCl_3$), 7.26 ppm for ^1H NMR and 77.16 ppm for ^{13}C NMR, respectively. Visualization in TLC was effected with *p*-phosphoryl molybdic acid

solution. MS analyses were carried out at the Mass Spectrometry and Proteomics Facility of the Department of Chemistry, University of Texas at Austin.

2.2.2. Cloning and Expression of *Micromonospora echinospora* GenK

The gentamicin producer *M. echinospora* (NRRL 2953) was obtained from the Agricultural Research Service of the US Department of Agriculture as a lyophilized sample. It was inoculated into 10 mL of tryptic soybroth (TSB) starter culture,[151] which was incubated overnight at 30 °C with shaking at 250 rpm. The following day, 0.1 mL of starter culture was inoculated into 10 mL of fresh TSB, and the resulting culture was inoculated at 30 °C for 72 hr. Chromosomal DNA was extracted from 1.5 mL of this culture using a Qiagen DNeasy tissue kit. The *genK* gene was PCR amplified from the isolated *M. echinospora* DNA with the following primers:

5′-CAACATATGAACGCGCTGGTGGCAGC (forward),

5′-TAACGAATTCAGTGGGAAACCGCCTCGG (reverse),

and cloned into a pCR:blunt vector (Invitrogen) prior to excision with *NdeI* and *EcoRI* and ligation into the pET24 vector (Invitrogen). The resulting expression plasmid, pET24:*genK*, was used to transform *E. coli* Rosetta DE3 cells (Novagen) for expression of native and, recombinant GenK. Transformants were grown in 3 L of LB medium at 18 °C with shaking at 200 rpm. GenK expression was induced with 1 mM IPTG when the cells had reached an optical density of 0.6 at 600 nm. Cells were harvested by centrifugation 20 h after induction (5000 × *g*, 15 min), resuspended in 50 mM Tris•HCl buffer (pH 8.0) containing 10% glycerol, and 1 mM PMSF, and lysed by sonication. Cell lysate was centrifuged at 25,000 × *g* for 30 min to pellet the insoluble material. Preliminary protein expression analysis using SDS-PAGE revealed that GenK, although abundant, was expressed exclusively as inclusion bodies. Therefore, the resulting

supernatant was discarded and the pelleted inclusion bodies were washed three times in lysis buffer and dissolved in 20 mL of 50 mM Tris•HCl buffer (pH 8.0) containing 20% glycerol, 1 mM PMSF and 5 M urea. This material was centrifuged at 4,000 × *g* for 10 min to remove insoluble material.

2.2.3. Refolding of GenK from Inclusion Bodies and Reconstitution with Iron and Sulfide

The solublized GenK inclusion bodies, processed as described above, were added dropwise to 200 mL of refolding buffer composed of 50 mM Tris•HCl (pH 8.0), 1 mM $MgSO_4$, 5 mM reduced glutathione, 0.5 mM glutathione disulfide, 5 mM DTT, and 10% glycerol. This mixture was kept refrigerated for 24 h with gentle stirring and was then concentrated to 7 mL in an Amicon YM-10 centrifugal concentrator. The protein was dialyzed overnight against 800 mL of 50 mM Tris•HCl (pH 8.0) containing 0.1 M NaCl, 1 mM DTT, and 20% glycerol. Afterward, the protein was centrifuged to remove precipitated material and the supernatant was reconstituted with iron and sulfide in an anaerobic glovebox according to an established procedure.[152] Iron content in the reconstituted GenK was assessed using the ferrozine assay[153] and the sulfide content was determined by the method devised by Helmut Bienert.[154] The iron and sulfide content reported in the chapter is an average of three measurements.

2.2.4. Enzymatic Synthesis and Purification of *S*-Adenosyl-L-Methionine (SAM)

The gene encoding *E. coli* SAM synthetase (*metK*) was cloned into pET28b(+) and the resulting construct was used to transform BL21(DE3) for expression of recombinant His_6-tagged protein. An incubation of 6 L of *E. coli* containing pET28b(+):*metK* in LB media was induced with 0.1 mM IPTG when the culture had

reached an optical density of 0.5 at 600 nm, overexpressed overnight at 25 °C, harvested by centrifugation (5000 × *g*, 15 min), and resuspended in 100 mL of 50 mM Tris•HCl (pH 8.0), 1 M NaCl, 1 mM β-mercaptoethanol, 10 mM imidazole, and 10% glycerol (lysis buffer). Resuspended cells were lysed by sonication and the lysate was centrifuged at 27,200 × *g* for 20 min. Cleared lysate was mixed with 10 mL of Ni^{2+}-nitrilotriacetic acid (NTA) resin (Qiagen) pre-equilibrated with wash buffer (lysis buffer containing 20 mM imidazole) for 90 min. The Ni^{2+}-NTA was poured into a column casing and rinsed with 100 mL of the wash buffer. MetK was eluted by the addition of elution buffer (lysis buffer containing 0.25 M imidazole). The pooled MetK was dialyzed against a 1 L solution of 50 mM Tris•HCl (pH 8.0), 0.15 M NaCl, 1 mM β-mercaptoethanol, and 10% glycerol. The final yield of MetK was 60 mL of 9.5 mg/mL. MetK was used to prepare SAM enzymatically from L-methionine and ATP and purified as described previously.[155] $^{13}CD_3$-*methyl*-SAM was prepared from $^{13}CD_3$-*methyl*-L-methionine (purchased from SigmaAldrich) using the same procedure. The purity of the enzymatically prepared SAM was assessed by high performance liquid chromatography (HPLC) using a Dionex CarboPac PA1 (4 × 250 mm) column with an isocratic 0.5 M ammonium acetate elution at 1 mL/min. The purified SAM was lyophilized, resuspended in 2 mL H_2O, divided into 50 μL aliquots, and stored at –80 °C until use.

2.2.5. GenK Activity Assays

All GenK activity assays were conducted at ambient temperature under anaerobic conditions in a Coy anaerobic chamber. The reaction mixture contained the following: 50 mM Tris•HCl (pH 8.0), 10 mM dithiothreitol (DTT), 1 mM methyl viologen (MV), 4 mM nicotinamide adenine dinucleotide phosphate (NADPH), 4 mM enzymatically prepared SAM, 1 mM gentamicin X_2 ($GenX_2$) (**7**), 1 mM cobalamin (Cbl), and 5 μM

GenK (unless otherwise noted). Assay reactions were initiated by the addition of $GenX_2$, and ran for 8 h unless otherwise specified. Reaction aliquots designated for HPLC analysis of adenosylated products, *S*-adenosylhomocysteine (SAH) and 5'-deoxyadenosine (5′-dAdo), were quenched by adding a volume of 30% trichloroacetic acid (TCA) equal to one tenth of the analyte volume. Reaction aliquots designated for analysis of aminoglycosides (30 μL) were quenched with a mixture containing 112 μL of methanol, 6 μL of 0.5 M NaOH, and 1.8 μL of 8.4 M 1-fluoro-2,4-dinitrobenzene (FDNB) to give final concentrations of 20 mM NaOH, 75% methanol, and 0.1 M FDNB. These were heated at 80 °C for 5 min to promote derivatization of primary amines with FDNB so they could be detected by UV absorbance at 340 nm.[156] Reaction aliquots analyzed by mass spectroscopy were quenched by passage through YM-10 Microcon centrifugal filters to remove GenK.

2.2.6. HPLC Detection of SAH and 5'-dAdo

A 25 μL aliquot of a TCA-quenched GenK reaction described above was analyzed by HPLC equipped with a Varian Microsorb-MV 100-5 C18 (4.6 × 250 mm) column pre-equilibrated in H_2O containing 0.1% trifluoroacetic acid (solvent A). After sample loading, the column was eluted with a linear gradient from 0–20% acetonitrile containing 0.1% trifluoroacetic acid (solvent B) over 30 min to resolve 5′-dAdo and SAH. The UV detector was set at 260 nm. A blend of authentic, commercially obtained 5′-dAdo and SAH was injected as a retention time standard for each. Quantitation of 5′-dAdo and SAH produced during assays was achieved by comparison of analyte peak areas with those of 5′-dAdo and SAH standards of known concentration.

2.2.7. HPLC Detection of Aminoglycoside

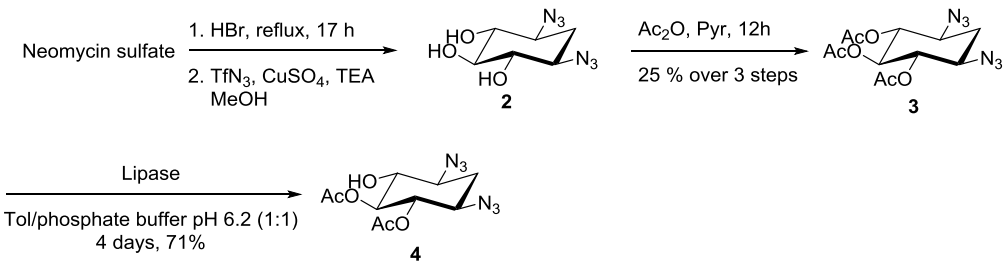
A 10 μL aliquot of a FDNB-derivatized GenK assay mixtures was prepared as described in Section **2.2.5,** loaded onto a Varian Microsorb-MV 100-5 C18 (4.6 × 250 mm) column and eluted with a gradient from 60:40 → 50:50 water:acetonitrile over 20 min. The UV detector was set at 340 nm. A blend of authentic, commercially obtained $GenX_2$ and G418 was injected as a retention time standard for each. Quantitation of the conversion of $GenX_2$ to G418 was achieved by comparing the peak areas for each.

2.2.8. Mass Spectroscopic Analysis of GenK Activity

Assay mixtures were diluted in methanol and infused directly into an Agilent 6530 Accurate Mass QTOF-MS at 0.15 mL/min. The analyte was ionized by electrospray ionization and detected in positive ion mode.

2.2.9. Synthesis of 2-Steptamine Derivative (4).

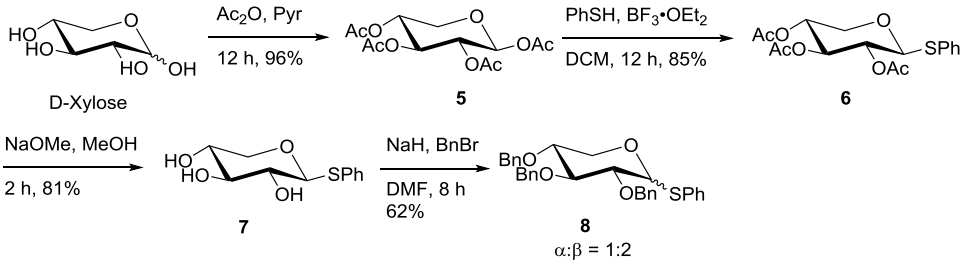
The overall synthetic scheme is shown in Scheme 2-7. Compound **4** was prepared as previously described.[157]



Scheme 2-7. Synthetic scheme for the preparation of 5,6-di-*O*-acetyl-1,3-diazido-1,2,3-trideoxy-myo-inositol (**4**).

2.2.10. Synthesis of Xylose Derivative (8)

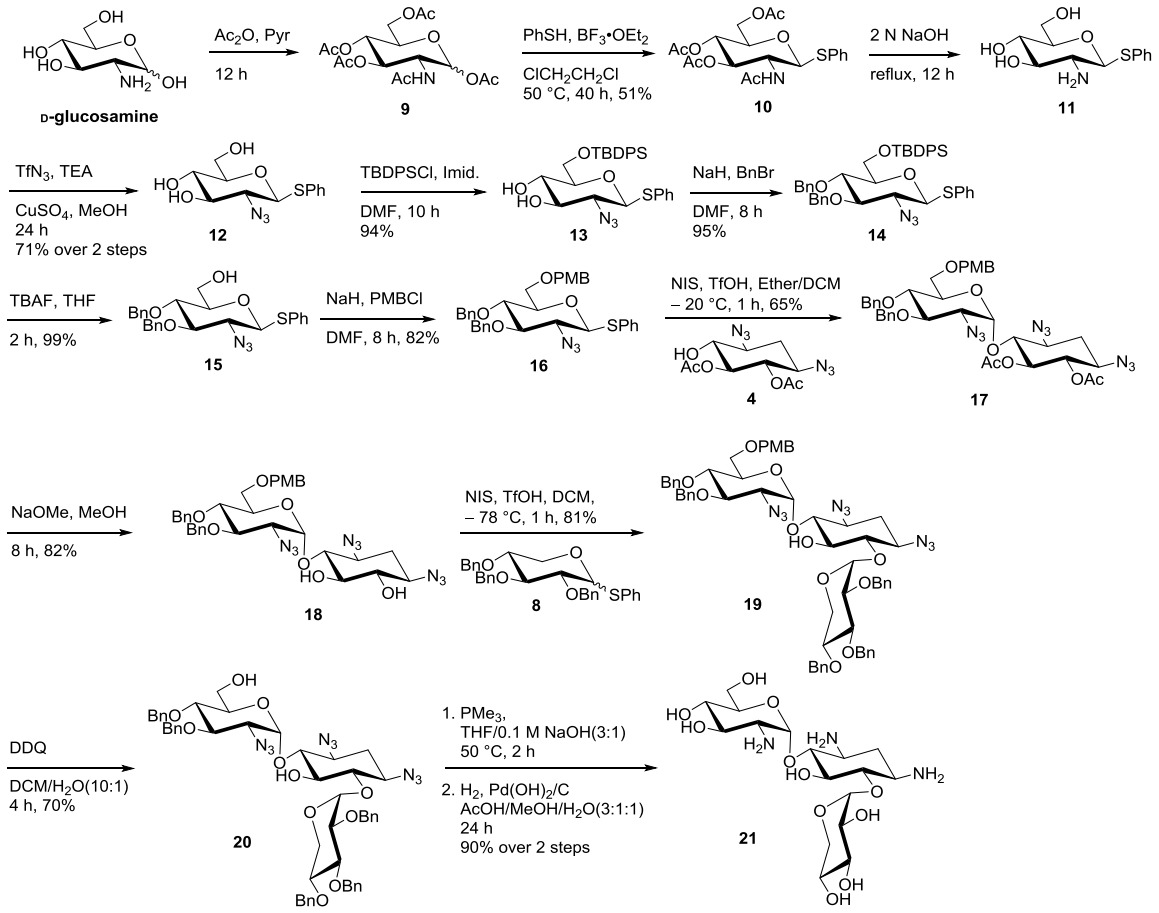
The overall synthetic scheme is shown in Scheme 2-8. Compound **8** was prepared as previously described.[158]



Scheme 2-8. Synthetic scheme for the preparation of (*3R, 3S, 5R*)-3,4,5-tris(benzyloxy)-2-(phenylthio)tetrahydro-2*H*-pyran (**8**).

2.2.11. Synthesis of Gentamicin A_2

The overall synthetic scheme is shown in Scheme 2-9.



Scheme 2-9. Synthetic scheme for the preparation of gentamicin A_2.

(*2R,3S,4R,5R,6S*)-5-azido-2-(((tert-butyldiphenylsilyl)oxy)methyl)-6-(phenylthio)tetrahydro-2*H*-pyran-3,4-diol (13): Compound **12** was prepared as previously described.[159] To a solution of compound **12** (2.0 g, 6.73 mmol) in anhydrous DMF (60 mL), imidazole (0.916 g, 13.45 mmol) was added. The mixture was stirred for 30 min at room temperature. The mixture was cooled to 0 °C and *tert*-butyldiphenylchlorosilane (2.1 mL, 8.07 mmol) was added. The reaction was then allowed to stand for 10 h at room temperature. The mixture was treated with methanol (10 mL) at 0 °C and all the solvent was evaporated under reduced pressure. The residue was diluted with dichloromethane (80 mL) and washed with saturated aqueous solution of ammonium chloride (70 mL).

The aqueous layer was extracted with dichloromethane (70 mL × 2). The combined organic extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 1:1) to afford compound **13** (3.4 g, 94 %) as a colorless oil. All NMR spectra were consistent with previously reported data.[160] HRMS (ESI, positive) calculated for $C_{28}H_{33}N_3O_4SSi^+$ $[M + Na]^+$ 558.1853, found 558.1870.

(((*2R,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-yl)methoxy)(tert-butyl)diphenylsilane (14): Sodium hydride (60% dispersion in mineral oil, 0.558 g, 13.96 mmol) and benzyl bromide (1.96 mL, 16.5 mmol) was added to a solution of compound **13** (3.4 g, 6.35 mmol) in DMF (60 mL) at 0 °C. After stirring the mixture for 8 h at room temperature, methanol (20 mL) was added at 0 °C. The mixture was stirred for an additional 20 min before concentration under reduced pressure. The residue was diluted with dichloromethane (80 mL) and washed with water (60 mL) and brine, dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude compound was purified by flash column chromatography (Hex/EtOAc = 10:1) to give compound **14** (4.311 g, 95%) as a clear oil. All NMR spectra were consistent with previously reported data.[160] HRMS (ESI, positive) calculated for $C_{42}H_{45}N_3O_4SSi^+$ $[M + Na]^+$ 739.2792, found 738.2793.

((*2R,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-yl)methanol (15): Tetrabutylammonium fluoride (1 M solution in THF, 12.04 mL, 12. 04 mmol) was added to a solution of compound **14** (4.311 g, 6.02 mmol) in THF (60 mL). The reaction mixture was stirred at room temperature for 2 h, and quenched by adding a saturated solution of aqueous ammonium chloride (40 mL). The aqueous layer was extracted with ethyl acetate (60 mL × 2). The combined organic extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced

pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to give compound **15** (2.847 g, 99%) as a white solid. All NMR spectra were consistent with previously reported data.[161] HRMS (ESI, positive) calculated for $C_{42}H_{45}N_3O_4SSi^+$ $[M + Na]^+$ 739.2792, found 738.2793.

(*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)-2-(phenylthio)tetrahydro-2*H*-pyran (16): sodium hydride (60% in mineral oil, 0.289 g, 7.23 mmol) and *p*-methoxybenzyl chloride (0.98 mL, 7.23 mmol) were added to a solution of compound **15** (2.847 g, 5.96 mmol) in anhydrous DMF (60 mL) at 0 °C. The reaction mixture was stirred at room temperature for 8 h. The mixture was treated with methanol (10 mL) at 0 °C and all the solvent was evaporated under reduced pressure. The residue was diluted with dichloromethane (60 mL) and washed with water (40 mL). The aqueous layer was extracted with dichloromethane (60 mL × 2), and the combined organic extracts were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 9:1) to afford compound **16** (2.951 g, 82%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68–7.64 (m, 2H, Ph), 7.40–7.29 (m, 13H, Ph), 7.26–7.23 (m, 2H, Ph), 6.95–6.90 (m, 2H, Ph), 4.90 (dd, *J* = 14.0, 10.6 Hz, 2H, PMB), 4.83 (d, *J* = 10.9 Hz, 1H, Bn), 4.62 (d, *J* = 10.9 Hz, 1H, Bn), 4.61 (d, *J* = 11.6 Hz, 1H, Bn), 4.52 (d, *J* = 11.6 Hz, 1H, Bn), 4.47 (d, *J* = 10.1 Hz, 1H, H-1), 3.84 (s, 3H, -OMe), 3.82–3.74 (m, 2H, H-6, H-6'), 3.66 (dd, *J* = 9.8, 8.9 Hz, 1H, H-4), 3.56 (t, *J* = 9.1 Hz, 1H, H-3), 3.53–3.49 (m, 1H, H-5), 3.41 (dd, *J* = 10.1, 9.2 Hz, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.3, 134.9, 137.7, 133.6, 131.4, 130.3, 129.34, 129.03, 128.55, 128.48, 128.35, 128.30, 128.22, 128.03, 127.90, 127.86, 113.8, 86.0, 85.1, 79.4, 77.61, 75.88, 75.04, 73.1, 68.4, 55.3. HRMS (ESI, positive) calculated for $C_{42}H_{45}N_3O_4SSi^+$ $[M + Na]^+$ 739.2792, found 738.2793.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (17) : Compound **4** (2.023 g, 6.78 mmol) and compound **16** (4.865g, 8.139 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **4** and **16** in anhydrous diethyl ether (60 mL) and anhydrous dichloromethane (20 mL) was then added to preactivated 4 Å molecular sieves. After stirring for 30 min at room temperature, the mixture was cooled to –40 °C. *N*-Iodosuccinimide (3.968 g, 17.64 mmol) was added, and the reaction mixture was stirred for 20 min. Trifluoromethanesulfonic acid (0.30 mL, 3.39 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. Sodium bisulfite (1 g), sodium bicarbonate (1 g), and water (10 mL) were added at 0 °C, and the mixture was stirred for 10 min at room temperature. The reaction mixture was diluted with dichloromethane (50 mL), filtered through a Celite pad, and washed with a saturated solution of aqueous sodium bicarbonate (100 mL). The aqueous layers were extracted with dichloromethane (100 mL × 3), and the combined organic phase was washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **17** (3.465 g, 65%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33–7.23 (m, 12H, Ph), 7.13–7.11 (m, 2H, Ph), 6.85–6.83 (m, 2H, Ph), 5.15 (t, *J* = 9.8 Hz, 1H, H-5), 5.14 (d, *J* = 3.9 Hz, 1H, H-1'), 4.93 (t, *J* = 10.0 Hz, 1H, H-6), 4.85 (dd, *J* = 30.6, 10.6 Hz, 2H, PMB), 4.77 (d, *J* = 10.8 Hz, 1H, Bn), 4.61 (d, *J* = 11.8 Hz, 1H, Bn), 4.48 (d, *J* = 10.85 Hz, 1H, Bn), 4.41 (d, *J* = 11.8 Hz, 1H, Bn), 4.15 (td, *J* = 8.1, 2.4 Hz, 1H, H-5'), 3.95 (dd, *J* = 10.5, 9.0 Hz, 1H, H-3'), 3.81 (dd, *J* = 10.8, 2.8 Hz, 1H, H-6'), 3.77 (dd, *J* = 9.9, 9.3 Hz, 1H, H-4'), 3.76 (s, 3H, -OCH_3), 3.67–3.59 (m, 3H, H-1, H-6', H-4), 3.43–3.38 (m, 1H, H-3), 3.36 (dd, *J* = 10.5, 3.8 Hz, 1H, H-2'), 2.40 (dt, *J* = 13.4, 4.6 Hz, 1H,

H-2), 2.094 (s, 3H, -OAc), 2.085 (s, 3H, -OAc), 1.60 (q, J = 12.5 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.8, 169.5, 159.3, 137.9, 137.8, 129.8, 129.7, 128.45, 128.38, 127.97, 127.86, 127.75, 127.67, 113.8, 99.3, 79.7, 78.29, 78.11, 75.3, 74.9, 74.1, 73.6, 73.2, 71.7, 67.4, 63.1, 58.7, 57.7, 55.2, 31.8, 20.64, 20.57. HRMS (ESI, positive) calculated for $C_{42}H_{45}N_3O_4SSi^+$ $[M + Na]^+$ 739.2792, found 738.2793.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((2S,3R,4R,5S,6R)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diol (18) : Sodium methoxide (4.37 M in methanol, 0.19 mL, 8.14 mmol) was added dropwise to a solution of compound **17** (3.2 g, 4.072 mmol) in methanol (40 mL). The reaction mixture was stirred at room temperature for 8 h and then neutralized with Amberlite IR-120 (H^+ form) resin, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **18** (2.347 g, 82%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.24 (m, 10H, Ph), 7.13–7.11 (m, 2H, Ph), 6.84–6.82 (m, 2H, Ph), 5.14 (d, J = 3.7 Hz, 1H, H-1'), 4.88 (dd, J = 37.2, 10.6 Hz, 2H, PMB), 4.75 (d, J = 10.8 Hz, 1H, Bn), 4.60 (d, J = 11.8 Hz, 1H, Bn), 4.48 (d, J = 10.8 Hz, 1H, Bn), 4.40 (d, J = 11.8 Hz, 1H, Bn), 4.21 (s, 1H, OH), 4.08 (td, J = 8.0, 2.6 Hz, 1H, H-5'), 3.96 (dd, J = 10.2, 9.1 Hz, 1H, H-3'), 3.78 (dd, J = 10.7, 3.2 Hz, 1H, H-6'), 3.75 (s, 3H, OCH_3), 3.75 (m, 1H, H-4'), 3.66–3.63 (m, 2H, H-2', H-6'), 3.50–3.38 (m, 3H, H-4, H-5, H-1), 3.28–3.22 (m, 2H, H-3, H-6), 2.33–2.27 (m, 1H, H-2), 1.53–1.46 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.3, 137.8, 137.5, 129.74, 129.70, 128.49, 128.43, 128.05, 127.98, 127.85, 127.72, 113.8, 99.6, 84.0, 80.9, 78.1, 75.82, 75.63, 75.32, 74.97, 73.2, 71.6, 67.5, 64.3, 59.6, 58.7, 55.2, 32.1. HRMS (ESI, positive) calculated for $C_{42}H_{45}N_3O_4SSi^+$ $[M + Na]^+$ 739.2792, found 738.2793.

(*1S,2R,3S,5R,6S*)-3,5-diazido-2-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)-6-(((*2R,3R,4S,5R*)-3,4,5-

tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexan-1-ol (19): Compound **8** (2.352 g, 4.59 mmol) and compound **18** (2.925 g, 4.171 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **8** and **18** in anhydrous dichloromethane (50 mL) was added to a preactivated 4 Å molecular sieves and *N*-iodosuccinimide (1.502 g, 6.67 mmol). After stirring for 30 min at room temperature, the mixture was cooled to –78 °C. Trifluoromethanesulfonic acid (0.11 mL, 1.25 mmol) was slowly added, and the reaction was warmed to 0 °C slowly. The reaction was quenched by adding solid sodium sulfite (0.5 g), sodium bicarbonate (0.5 g) and water (100 mL) at 0 °C. The reaction mixture was diluted with dichloromethane, filtered, and washed with a saturated solution of sodium bicarbonate and brine. The residue was dried over sodium sulfate, filtered, concentrated in reduced pressure, and purified by flash column chromatography on silica gel (Hex:EtOAc = 3:2) to give compound **19** (3.746 g, 81%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44–7.24 (m, 25H, Ph), 7.14 (m, 2H, Ph), 6.89–6.80 (m, 2H, Ph), 5.47 (d, *J* = 3.7 Hz, 1H, H-1'), 5.08 (d, *J* = 3.6 Hz, 1H, H-1''), 4.95–4.84 (m, 4H, Bn), 4.81–4.72 (m, 4H, Bn), 4.63 (dd, *J* = 13.7, 11.7 Hz, 2H, Bn), 4.49 (d, *J* = 10.9 Hz, 1H, Bn), 4.42 (d, *J* = 11.8 Hz, 1H, Bn), 4.37 (d, *J* = 2.1 Hz, 1H, OH), 4.16–4.10 (m, 1H, H-5'), 3.97 (dtd, *J* = 18.4, 9.6, 9.2, 6.9 Hz, 3H, H-5, H-3', H-3''), 3.84–3.69 (m, 6H, H-5'', H-4', H-6', OMe), 3.65 (ddd, *J* = 9.1, 5.3, 2.2 Hz, 2H, H-5, H-6'), 3.62–3.54 (m, 1H, H-4''), 3.54–3.48 (m, 3H, H-1, H-2', H-2''), 3.43–3.36 (m, 1H, H-4), 3.36–3.27 (m, 2, H-3, H-6), 2.40–2.31 (m, 1H, H-2), 1.54 (q, *J* = 12.7 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.40, 138.89, 138.21, 138.17, 138.14, 137.97, 129.99, 129.82, 128.61, 128.59, 128.56, 128.52, 128.49, 128.40, 128.20, 128.17, 128.15, 128.11, 128.05, 128.01, 127.98, 127.92, 127.85, 127.79, 127.73, 113.89, 99.11, 98.21, 83.41, 81.60, 80.90, 80.52, 79.28, 78.18, 77.75, 75.76, 75.64, 75.25, 75.02, 73.77, 73.72, 73.28, 71.54, 67.68, 63.88, 61.23, 59.81, 58.83, 55.34, 32.39, 29.84.

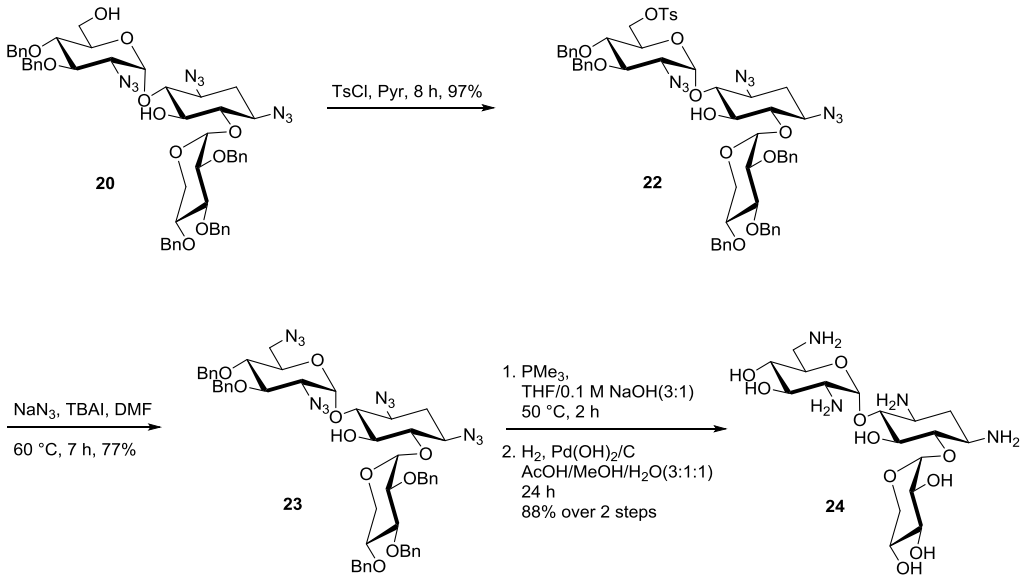
HRMS (ESI, positive) calculated for $C_{60}H_{65}N_9O_{12}^+$ [*M* + *Na*]$^+$ 1126.4645, found 1126.4643.

(*1S,2R,3S,5R,6S*)-3,5-diazido-2-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-6-(((*2R,3R,4S,5R*)-3,4,5-tris(benzyloxy-)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexan-1-ol (20): 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1.183 g, 5.21 mmol) was added to a solution of compound **19** (3.746g, 2.47 mmol) in dichloromethane (50 mL) and water (5 mL). The reaction mixture was stirred for 4 h at room temperature, and then quenched by adding a saturated solution of aqueous sodium bicarbonate (30 mL). The aqueous layer was extracted with dichloromethane (40 mL × 3), and the combined organic phase was washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) and recrystallization with Hex/EtOAc (10:1) to give compound **20** (2.330 g, 70%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58–7.27 (m, 25H, Bn), 5.50 (d, *J* = 3.7 Hz, 1H, H-1'), 5.07 (d, *J* = 3.6 Hz, 1H, H-1''), 5.00–4.85 (m, 5H, Bn), 4.84–4.70 (m, 4H, Bn), 4.66 (d, *J* = 11.5 Hz, 1H, Bn), 4.44 (d, *J* = 2.1 Hz, 1H, OH), 4.10–4.02 (m, 2H, H-3', H-5'), 4.01–3.91 (m, 2H, H-3'', H-5''), 3.87 (dd, *J* = 12.2, 2.5 Hz, 1H, H-6'), 3.80 (dd, *J* = 12.3, 3.4 Hz, 1H, H-6'), 3.75 (dd, *J* = 11.1, 5.8 Hz, 1H, H-5''), 3.69 (m, 2H, H-4', H-5'), 3.61 (ddd, *J* = 10.5, 8.6, 5.6 Hz, 1H, H-4''), 3.53 (m, 2H, H-2'', H-1), 3.44 (dd, *J* = 10.3, 3.7 Hz, 1H, H-2'), 3.36 (m, 3H, H-4, H-3, H-6), 2.36 (dt, *J* = 13.0, 4.3 Hz, 1H, H-2), 1.53 (q, *J* = 12.5 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.82, 138.15, 138.11, 138.00, 137.89, 128.58, 128.54, 128.50, 128.41, 128.16, 128.07, 128.03, 127.98, 127.95, 127.92, 127.67, 98.76, 98.20, 83.90, 81.05, 80.74, 79.21, 77.83, 77.60, 75.63, 75.58, 75.08, 73.67, 73.65, 72.18, 63.79, 61.36, 61.27, 59.60, 58.94, 32.36. HRMS (ESI, positive) calculated for $C_{52}H_{57}N_9O_{11}^+$ [*M* + *Na*]$^+$ 1006.4070, found 1006.4076.

Gentamicin A_2 (21): Trimethylphosphine (1 M solution in THF, 1.96 mL, 1.96 mmol) was added to a solution of compound **20** (0.476 g, 0.50 mmol) in THF (12 mL) and 0.1 M NaOH (3 mL), and the reaction mixture was stirred at 50 °C for 2 h. After cooling to room temperature, the solution was evaporated under reduced pressure. After added in a mixture of acetic acid (3 mL), water (9 mL) and methanol (9 mL), the solution was deaerated by evacuating the air in the flask and refilling it with argon five times. Palladium hydroxide on carbon (20%, Degussa type, 0.2 g) was added and the solution was charged with hydrogen gas. The reaction mixture was then stirred at room temperature under hydrogen gas for 24 h. The solution was then filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (0-5% concentrated ammonium hydroxide in water), acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **21** (0.204 g, 90%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.61 (d, J = 4.0 Hz, 1H, H-1'), 4.96 (d, J = 2.5 Hz, 1H, H-1"), 3.89–3.82 (m, 3H, H-3', H-5', H-6'), 3.81–3.75 (m, 2H, H-4', H-5), 3.72–3.63 (m, 4H, H-5", H-6', H-5", H-3"), 3.63–3.48 (m, 5H, H-6, H-2", H-4", H-1, H-3'), 3.46–3.42 (m, 1H, H-4), 3.42–3.38 (m, 1H, H-2'), 2.47 (dt, J = 12.6, 4.3 Hz, 1H, H-2), 1.85 (q, J = 12.7 Hz, 1H, H-2). ^{13}C NMR (125 MHz, D_2O) δ 101.8, 97.1, 83.4, 80.4, 73.72, 73.61, 72.91, 69.36, 68.99, 68.88, 62.4, 60. 4, 53.9, 49.6, 48.7, 27.9 HRMS (ESI, positive) calculated for $C_{17}H_{33}N_3O_{11}^+$ [M + Na]$^+$ 478.2007, found 478.2012.

2.2.12. Synthesis of 6'-Amino-6'-dehydroxy-gentamicin A_2

The overall synthetic scheme is shown in Scheme 2-10.



Scheme 2-10. Synthetic scheme for the preparation of 6'-amino-6'-deoxy-gentamicin A_2.

((*2R,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(((*1R,2S,3S,4R,6S*)-4,6-diazido-2-hydroxy-3-(((*2R,3R,4S,5R*)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)cyclo-hexyl)oxy)tetrahydro-2*H*-pyran-2-yl)methyl 4-methylbenzenesulfonate (22): *p*-Toluenesulfonyl chloride (0.417 g, 2.19 mmol) was added to a solution of compound **21** (0.420 g, 0.44 mmol) in pyridine (20 mL) at 0 °C. The reaction mixture was stirred at room temperature for 8 h. Methanol (5 mL) was then added to the solution at 0 °C, and the mixture was stirred at room temperature for 30 min. The solution was concentrated under reduced pressure, and co-evaporated with toluene twice. The residue was diluted with dichloromethane (30 mL), and washed with a saturated solution of aqueous sodium bicarbonate (20 mL). The aqueous layer was extracted with dichloromethane (30 mL × 2), and the combined organic phase was washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to give the tosylate **22** (0.481 g, 97%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84–7.78 (m, 2H, Bn),

7.44–7.27 (m, 25H, Bn), 7.22 (dd, *J* = 7.5, 2.0 Hz, 2H, Bn), 5.46 (d, *J* = 3.7 Hz, 1H, H-1'), 5.04 (d, *J* = 3.5 Hz, 1H, H-1''), 4.95–4.87 (m, 3H, Bn), 4.86 (d, *J* = 5.3 Hz, 1H, Bn), 4.82 (d, *J* = 6.2 Hz, 1H, Bn), 4.81–4.73 (m, 3H, Bn), 4.65 (d, *J* = 11.5 Hz, 1H, Bn), 4.54 (d, *J* = 10.8 Hz, 1H, Bn), 4.41–4.32 (m, 2H, H-6', H-6'), 4.31–4.17 (m, 2H, H-5'', H-5'), 4.04–3.87 (m, 3H, H-3'', H-3', H-5''), 3.75 (dd, *J* = 11.2, 5.7 Hz, 1H, H-5''), 3.70–3.56 (m, 3H, H-4', H-4'', H-5), 3.56–3.46 (m, 2H, H-2'', H-1), 3.40–3.26 (m, 4H, H-2', H-4, H-3, H-6), 2.42 (s, 3H, Ts), 2.34 (dt, *J* = 13.4, 4.3 Hz, 1H, H-2), 1.50 (q, *J* = 12.5 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 144.90, 138.77, 138.11, 138.09, 138.07, 137.70, 137.60, 132.93, 129.85, 128.52, 128.51, 128.48, 128.38, 128.37, 128.04, 128.02, 128.00, 127.97, 127.94, 127.92, 127.89, 127.82, 127.80, 127.64, 98.43, 98.26, 84.28, 80.67, 80.51, 80.11, 79.14, 77.51, 77.36, 77.31, 77.10, 77.06, 76.85, 76.81, 75.60, 75.50, 75.28, 75.05, 73.68, 73.64, 69.68, 68.21, 63.47, 61.29, 59.51, 58.68, 32.20, 21.66. HRMS (ESI, positive) calculated for $C_{59}H_{63}N_9O_{13}S^+$ $[M + Na]^+$ 1160.4158, found 1160.4164.

(*1S,2R,3S,5R,6S*)-3,5-diazido-2-(((*2R,3R,4R,5R,6R*)-3-azido-6-(azidomethyl)-4,5-bis-(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)-6-(((*2R,3R,4S,5R*)-3,4,5-tris(benzyloxy)-tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexan-1-ol (23): Sodium azide (0.137 g, 2.11 mmol) and tetrabutylammonium iodide (1.5 mg, 4.3 mmol) was added to a solution of the previously synthesized tosylate **22** (0.481 g, 0.42 mmol) in DMF (20 mL). The reaction mixture was stirred at 60 °C for 7 h. After cooling to room temperature, the reaction solution was evaporated under reduced pressure, and the residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 5:1) to give compound **23** (0.327 g, 77%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.28 (m, 25H, Bn), 5.53 (d, *J* = 3.7 Hz, 1H, H-1'), 5.02 (d, *J* = 3.5 Hz, 1H, H-1''), 4.93–4.71 (m, 8H, Bn), 4.66 (m, 2H, Bn), 4.37 (d, *J* = 2.1 Hz, 1H, OH), 4.19 (ddd, *J* = 10.0, 4.4, 2.5 Hz, 1H, H-5'), 3.99 (dd, *J* = 10.4, 8.8 Hz, 1H, H-3'), 3.97–3.92 (m, 1H, H-3''), 3.92-3.86 (m, 1H, H-5''), 3.72 (dd, *J*

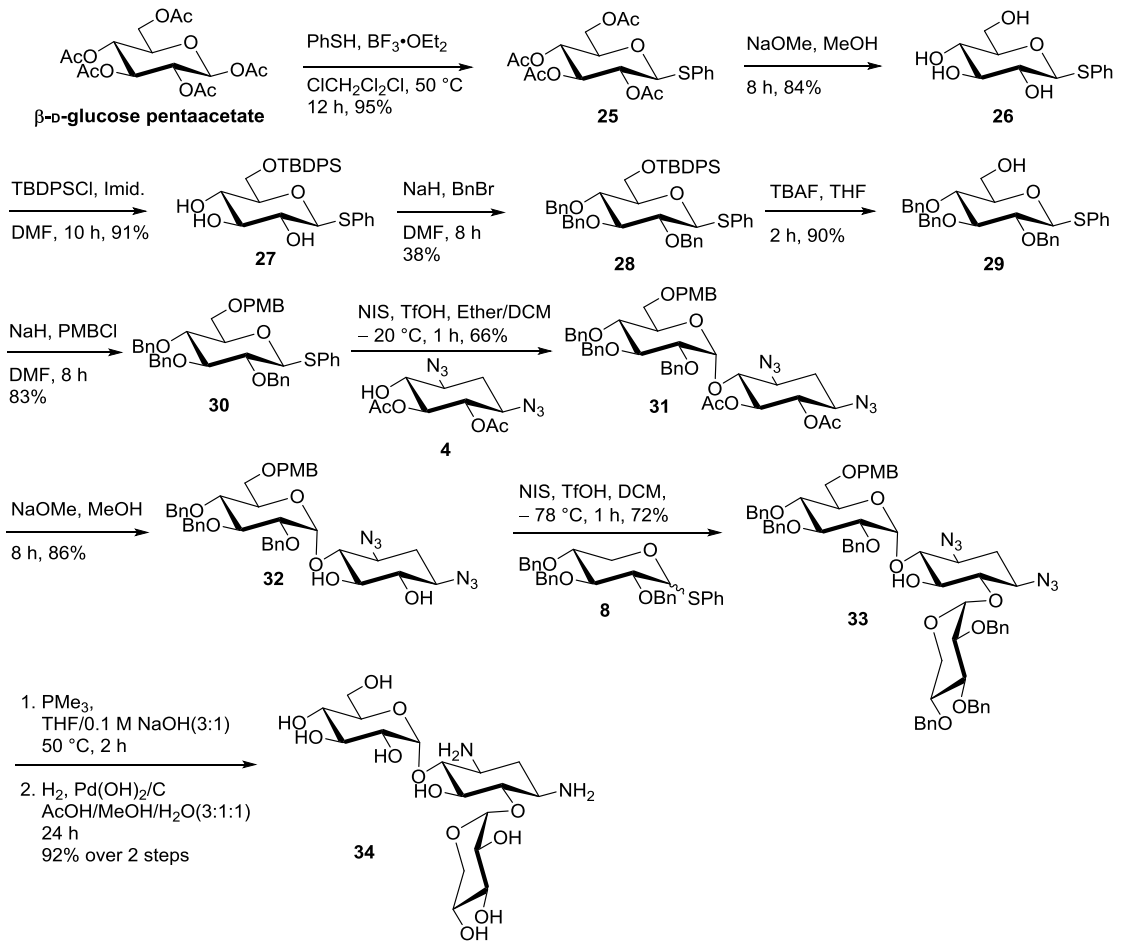
= 11.1, 5.6 Hz, 1H, H-5"), 3.66 (td, *J* = 9.0, 2.0 Hz, 1H, H-5), 3.61-3.47 (m, 5H, H-4, H-4', H-4", H-2", H-1), 3.44 (dd, *J* = 6.9, 3.5 Hz, 1H, H-2'), 3.42-3.33 (m, 3H, H-6', H-6', H-3), 3.30 (t, *J* = 9.5 Hz, 1H, H-6), 2.35 (dt, *J* = 13.2, 4.4, 1H, H-2), 1.54–1.48 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.74, 138.07, 138.03, 137.70, 128.52, 128.50, 128.48, 128.34, 128.06, 128.01, 127.99, 127.98, 127.95, 127.89, 127.87, 127.80, 127.60, 98.44, 98.27, 84.26, 80.65, 80.64, 80.10, 79.11, 78.65, 77.47, 75.58, 75.54, 75.33, 75.17, 73.64, 73.63, 71.07, 63.66, 61.28, 59.54, 58.81, 51.04, 32.30. HRMS (ESI, positive) calculated for $C_{52}H_{56}N_{12}O_{10}^{+}$ $[M + Na]^+$ 1031.4135, found 1031.4142.

6'-amino-6'-dehydroxy-GenA$_2$ (24): Trimethylphosphine (1 M solution in THF, 1.29 mL, 1.29 mmol) was added to a solution of compound **23** (0.327 g, 0.324 mmol) in THF (12 mL) and 0.1 M NaOH (4 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated *in vacuo*. The material was dissolved in a mixture of acetic acid (3 mL), water (9 mL) and methanol (9 mL). The solution was deaerated by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 hr. The solution was filtered through Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (5-10% concentrated ammonium hydroxide in water), acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **24** (0.13 g, 88%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 6.02 (d, *J* = 3.9 Hz, 1H, H-1'), 5.07 (d, *J* = 2.5 Hz, 1H, H-1"), 4.12–4.01 (m, 3H, H-5', H-3', H-5), 3.93 (t, *J* = 9.1 Hz, 1H, H-4'), 3.85–3.50 (m, 8H, H-5", H-3", H-5", H-6, H-2", H-4", H1, H3), 3.60–3.50 (m, 3H, H-6', H-4, H-2'), 3.36 (dd, *J* = 13.6, 6.7 Hz, 1H, H-6'), 2.65–2.56 (m, 1H, H-2), 2.01 (q, *J* = 12.7 Hz, 1H, H-2). ^{13}C NMR (125 MHz,

D_2O) δ 101.9, 96.2, 83.6, 77.51, 74.11, 72.88, 71.63, 70.58, 69.2, 68.83, 68.19, 62.5, 53.4, 49.7, 48.2, 40.2, 27.9. HRMS (ESI, positive) calculated for $C_{17}H_{34}N_4O_{10}^+$ [*M* + *Na*]$^+$ 477.2167, found 477.2167.

2.2.13. Synthesis of 2'-Deamino-2'-hydroxy-gentamicin A_2

The overall synthetic scheme is shown in Scheme 2-11.



Scheme 2-11. Synthetic scheme for the preparation of 2'-deamino-2'-hydroxy-GenA_2.

(*2R,3R,4S,5R,6S*)-2-(acetoxymethyl)-6-(phenylthio)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (27): Compound **26** was prepared as previously described.[162] Imidazole (4.167

g, 36.72 mmol) and *tert*-butyldiphenylsilyl chloride (5.73 mL, 22.03 mmol) was added to a solution of compound **26** (5.00 g,18.36 mmol) in dry DMF (150 mL) at 0 °C, and the reaction mixture was stirred at room temperature for 12 h. The solution was cooled to 0 °C, and methanol (30 mL) was added to quench the reaction. The mixture was concentrated and diluted with dichloromethane (100 mL). The solution was then washed with a saturated solution of aqueous ammonium chloride (80 mL). The aqueous wash was then extracted with dichloromethane (100 mL × 2). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 1:2) to give compound **27** (8.533 g, 91 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75–7.70 (m, 4H, Ph), 7.56–7.52 (m, 2H, Ph), 7.46–7.35 (m, 6H, Ph), 7.27–7.21 (m, 3H, Ph), 4.53 (d, J = 9.7 Hz, 1H, C-1), 4.00–3.88 (m, 2H, C-6, C-6), 3.69–3.56 (m, 2H, C-3, C-4), 3.50–3.42 (m, 1H, C-5), 3.36 (t, J = 8.8 Hz, 1H, C-2), 1.07 (s, 9H, TBDPS). ^{13}C NMR (125 MHz, $CDCl_3$) δ 135.65, 135.60, 132.91, 132.80, 132.53, 131.91, 129.87, 129.86, 129.00, 128.00, 127.82, 127.81, 87.86, 79.01, 77.78, 71.62, 71.14, 64.32, 26.81, 19.23. HRMS (ESI, positive) calculated for $C_{28}H_{34}O_5SSi^+$ $[M + Na]^+$ 533.1788, found 533.1796.

(*2R,3S,4S,5R,6S*)-2-(hydroxymethyl)-6-(phenylthio)tetrahydro-2*H*-pyran-3,4,5-triol (28): Sodium hydride (60% dispersion in mineral oil, 1.039 g, 25.96 mmol) and benzyl bromide (3.11 mL, 25.96 mmol) was added to a solution of compound **27** (3.4 g, 6.35 mmol) in DMF (40 mL) at 0 °C. After stirring the mixture for 8 h at room temperature, methanol (40 mL) was added at 0 °C. The mixture was stirred for an additional 20 min before concentration under reduced pressure. The residue was diluted with dichloromethane (100 mL) and washed with water (80 mL). The remaining aqueous layer was extracted with dichloromethane (80 mL × 2). The combined organic layers were

washed with brine, dried over magnesium sulfate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (Hex/EtOAc = 10:1) to give compound **28** (4.311 g, 95%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.91–7.85 (m, 2H, Ph), 7.84–7.79 (m, 2H, Ph), 7.74–7.67 (m, 2H, Ph), 7.54–7.21 (m, 24H, Ph), 5.04–4.94 (m, 4H, Bn), 4.84 (d, *J* = 10.3 Hz, 1H, Bn), 4.80 (d, *J* = 10.3 Hz, 2H, H-1, Bn), 4.10 (dd, *J* = 11.4, 1.8 Hz, 1H, H-6), 4.04 (dd, *J* = 11.4, 3.7 Hz, 1H, H-6), 3.91 (t, *J* = 9.4 Hz, 1H, H-4), 3.83 (t, *J* = 9.0 Hz, 1H, H-3), 3.66 (dd, *J* = 9.8, 8.7 Hz, 1H , H-2), 3.51 (ddd, *J* = 9.6, 3.7, 1.8 Hz, 1H, H-5), 1.20 (s, 9H, TBDPS). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.43, 138.25, 138.16, 135.96, 135.71, 134.25, 133.54, 132.97, 131.74, 129.76, 129.72, 129.00, 128.59, 128.52, 128.51, 128.24, 128.06, 128.05, 127.95, 127.94, 127.92, 127.88, 127.85, 127.77, 127.34, 87.58, 86.96, 80.87, 80.05, 77.51, 77.40, 77.14, 76.89, 76.10, 75.49, 75.23, 62.76, 26.97, 19.40. HRMS (ESI, positive) calculated for $C_{49}H_{52}O_5SSi^+$ [*M* + *Na*]$^+$ 803.3197, found 803.3204.

(*2R,3S,4S,5R,6S*)-2-(((tert-butyldiphenylsilyl)oxy)methyl)-6-(phenylthio)tetrahydro-2*H*-pyran-3,4,5-triol (29): Tetrabutylammonium fluoride (1.0 M solution in THF, 25.6 mL) was added to a solution of compound **28** (10 g, 12.8 mmol) in THF (120 mL) was added and the reaction mixture was stirred at room temperature for 2 hr. The reaction was quenched by adding a saturated solution of aqueous ammonium chloride (80 mL) at 0 °C. The aqueous layer was extracted with ethyl acetate (80 mL × 2). The combined organic layers were dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **29** (6.253 g, 90 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57–7.50 (m, 2h, Ph), 7.43–7.38 (m, 2H, Ph), 7.38–7.27 (m, 16H, Ph), 4.97–4.84 (m, 4H, Bn), 4.78 (d, *J* = 10.2 Hz, 1H, Bn), 4.74 (d, *J* = 9.9 Hz, 1H, H-1), 4.67 (d, *J* = 11.0 Hz, 1H Bn), 3.89 (dd, *J* = 12.0, 2.7 Hz, 1H, H-6), 3.80–3.66 (m, 2H, H-3, H-

6), 3.60 (t, *J* = 9.4 Hz, 1H, H-4), 3.51 (dd, *J* = 9.8, 8.7 Hz, 1H, H-2), 3.41 (ddd, *J* = 9.8, 4.9, 2.7 Hz ,1H, H-5). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.31, 137.90, 137.84, 133.48, 131.85, 129.06, 128.54, 128.49, 128.46, 128.23, 128.05, 128.05, 127.99, 127.93, 127.81, 127.77, 127.70, 87.55, 86.57, 81.12, 79.33, 77.60, 75.85, 75.57, 75.14, 62.15. HRMS (ESI, positive) calculated for $C_{33}H_{34}O_5S^+$ $[M + Na]^+$ 565.2019, found 565.2040.

***tert*-butyldiphenyl(((2R,3R,4S,5R,6S)-3,4,5-tris(benzyloxy)-6-(phenylthio)tetrahydr-o-2*H*-pyran-2-yl)methoxy)silane (30)**: Sodium hydride (60% dispersion in mineral oil, 0.552 g, 13.8 mmol) was added to a solution of compound **29** (6.24 g, 11.5 mmol) in dry DMF (100 mL) at 0 °C, and the reaction mixture was stirred at room temperature for 30 min. The solution was cooled to 0 °C, and *p*-methoxybenzyl chloride (1.87 mL, 13.8 mmol) was added. The reaction mixture was stirred at room temperature for 8 h, quenched by adding methanol (30 mL) at 0 °C, and concentrated under reduced pressure. The residue was diluted with dichloromethane (80 mL) and washed with a saturated solution of aqueous ammonium chloride (60 mL). The remaining aqueous solution was extracted with dichloromethane (60 mL × 2). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to provide compound **30** (6.326 g, 83%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73–7.62 (m, 2H, Ph), 7.52–7.22 (m, 20H, Ph), 7.01–6.88 (m, 2H, Ph), 5.02–4.97 (m, 2H, Bn), 4.94 (d, *J* = 11.0 Hz, 1H, Bn), 4.91 (d, *J* = 10.9 Hz, 1H, Bn), 4.83 (d, *J* = 10.3 Hz, 1H, Bn, 4.77 (d, *J* = 9.8 Hz, 1H, H-1), 4.70–4.62 (m, 2H, Bn), 4.56 (d, *J* = 11.6 Hz, 1H, Bn), 3.86 (s, 4H, -OMe, H-6), 3.83–3.77 (m, 2H, H-3, H-6), 3.74 (t, *J* = 9.3 Hz, 1H, H-4), 3.65–3.56 (m, 2H, H-2, H-5). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.25, 138.50, 138.15, 138.13, 134.00, 131.95, 130.41, 129.45, 128.98, 128.53, 128.50, 128.48, 128.30, 127.99, 127.94, 127.86, 127.78, 127.48, 113.83, 87.56, 86.82, 80.94, 79.19, 77.90, 75.89, 75.50,

75.10, 73.14, 68.69, 55.33. HRMS (ESI, positive) calculated for $C_{41}H_{42}O_6S^+$ $[M + Na]^+$ 685.2594, found 685.2607.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4S,5R,6R*)-3,4,5-tris(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (31): Compound **4** (2.283 g, 7.65 mmol) and compound **30** (6.088 g, 9.18 mmol) were co-evaporated from dry toluene three times under reduced pressure and further dried under high vacuum 12 h. A solution of compound 4 and 29 in anhydrous diethyl ether (90 mL) and anhydrous dichloromethane (30 mL) was added to preactivated 4 Å molecular sieves. After stirring for 30 min at room temperature, the mixture was cooled to –40 °C. *N*-Iodosuccinimide (4.478 g, 19.9 mmol) was added, and the reaction mixture was stirred for 20 min. Trifluoromethanesulfonic acid (0.34 mL, 3.83 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. The reaction solution was diluted with dichloromethane, and filtered through a Celite pad. The filtered solution was washed with 10% aqueous sodium bisulfite solution (80 mL), and a saturated aqueous solution of sodium bicarbonate solution (80 mL). The combined aqueous solution was extracted with dichloromethane (100 mL x 2). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **31** (4.3 g, 66%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.22 (m, 15H, Ph), 7.14–7.09 (m, 2H, Ph), 6.88–6.81 (m, 2H, Ph), 5.23–5.15 (m, 2H, H-5), 4.95 (t, *J* = 10.0 Hz, 1H, H-6, H-1'), 4.85 (d, *J* = 10.8 Hz, 1H, Bn), 4.82–4.77 (m, 2H, Bn), 4.71 (d, *J* = 11.8 Hz, 1H, Bn), 4.65 (d, *J* = 11.8 Hz, 1H, Bn), 4.59 (d, *J* = 11.8 Hz, 1H, Bn), 4.43 (d, *J* = 11.0 Hz, 1H, Bn), 4.39 (d, *J* = 11.8 Hz, 1H, Bn), 4.06 (dt, *J* = 10.1, 2.5 Hz, 1H, H-5'), 3.99–3.92 (m, 1H, H-3'), 3.79–3.71 (m, 4H, H-6'), 3.68 (dd, *J* = 10.1, 9.1 Hz, 1H, H-4'), 3.66–3.60 (m, 2H, H-6', H-4), 3.60–3.47 (m, 3H, H-

3, H-2', H-1), 2.35 (dt, *J* = 13.4, 4.5 Hz, 1H, H-2), 2.09 (s, 3H, OAc), 1.87 (s, 3H), OAc), 1.53 (dt, *J* = 13.5, 12.5 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.97, 169.74, 159.40, 138.75, 138.53, 137.98, 130.05, 129.84, 128.61, 128.50, 128.39, 128.24, 128.05, 127.73, 127.72, 127.66, 113.89, 98.10, 81.79, 79.71, 77.72, 77.62, 77.36, 75.77, 74.97, 74.21, 73.76, 73.50, 73.24, 71.55, 67.73, 60.13, 57.92, 55.34, 32.55, 20.90, 20.75. HRMS (ESI, positive) calculated for $C_{45}H_{50}N_6O_{11}^+$ $[M + Na]^+$ 873.3430, found 873.3441.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4S,5R,6R*)-3,4,5-tris(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diol (32): Sodium methoxide (5.4 M solution in methanol, 0.19 mL, 1.01 mmol) was added to a solution of compound **31** (4.3 g, 5.05 mmol) in methanol (50 mL), stirred at room temperature for 8 h, and neutralized with Amberlite IR-120 (H^+ form). The mixture was filtered through a Celite pad, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc =1:1) to give compound **32** (3.139 g, 81 %) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.23 (m, 15H, Ph), 7.12–7.07 (m, 2H, Ph), 6.86–6.79 (m, 2H, Ph), 4.94 (d, *J* = 3.8 Hz, 1H, H-1'), 4.91 (d, *J* = 2.8 Hz, 2H, Bn), 4.88 (d, *J* = 11.7 Hz, 1H, Bn), 4.79–4.70 (m, 3H, Bn), 4.60 (d, *J* = 11.9 Hz, 1H, Bn), 4.44 (d, *J* = 10.6 Hz, 1H, Bn), 4.38 (d, *J* = 11.8 Hz, 1H), 4.05–3.98 (m, 2H, H-5', H-3'), 3.80–3.68 (m, 5H, OMe, H-6', H-4'), 3.66–3.59 (m, 2H, H-6', H-2'), 3.48–3.36 (m, 3H, H-1, H-5, H-6), 3.28–3.13 (m, 2H, H-3, H-4), 2.27 (dt, *J* = 13.3, 4.4 Hz, 1H, H-2), 1.46 (dt, *J* = 13.4, 12.1 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.29, 138.43, 138.03, 136.84, 129.81, 129.79, 128.70, 128.53, 128.48, 128.43, 128.36, 127.79, 127.73, 127.71, 127.66, 113.76, 101.39, 85.45, 82.38, 79.13, 77.71, 75.57, 75.55, 75.39, 75.05, 74.58, 73.17, 71.46, 67.52, 59.67, 59.43, 55.20, 32.34. HRMS (ESI, positive) calculated for $C_{41}H_{46}N_6O_9^+$ $[M + Na]^+$ 789.3218, found 789.3232.

(*1R,2R,3S,5R,6S*)-3,5-diazido-2-(((*2S,3R,4S,5R,6R*)-3,4,5-tris(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)-6-(((*2R,3R,4S,5R*)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexan-1-ol (33): Compound **8** (2.668 g, 5.210 mmol) and compound **32** (3.629 g, 4.73 mmol) were co-evaporated from dry toluene under reduced pressure three times and further dried under high vacuum for 8 hr. A solution of compound **8** and **32** in anhydrous dichloromethane (150 mL) was added to preactivated 4 Å molecular sieves and *N*-iodosuccinimide (1.704 g, 7.57 mmol). After stirring for 30 min at room temperature, the reaction mixture was cooled to –78 °C. Trifluoromethanesulfonic acid (0.13 mL, 1.42 mmol) was added dropwise, and the reaction was warmed to 0 °C slowly. The reaction was quenched by adding solid sodium sulfite (0.5 g), sodium bicarbonate (0.5 g), and a few drops of water. The reaction mixture was diluted with dichloromethane (150 mL), filtered, and washed with a saturated aqueous solution of sodium bicarbonate (100 mL × 2) and brine. The residue was dried over sodium sulfate, filtered, concentrated under reduced pressure, and purified by flash column chromatography on silica gel (Hex:EtOAc = 3:2) to give compound **32** (3.621 g, 81%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44–7.27 (m, 29H, Ph), 7.22–7.18 (m, 1H, Ph), 7.14 (m, 2H, Ph), 6.90–6.81 (m, 2H, Ph), 5.32 (d, *J* = 3.7 Hz, 1H, H-1'), 5.18 (d, *J* = 3.6 Hz, 1H, H-1''), 4.96 (dd, *J* = 11.0, 7.6 Hz, 2H, Bn), 4.90 (d, *J* = 11.0 Hz, 2H, Bn), 4.80 (td, *J* = 12.3, 9.1 Hz, 6H, Bn), 4.65 (dd, *J* = 13.4, 11.7 Hz, 2H, Bn), 4.59–4.53 (m, 1H, OH), 4.48 (d, *J* = 10.7 Hz, 1H, Bn), 4.43 (d, *J* = 11.8 Hz, 1H, Bn), 4.13 (dt, *J* = 10.1, 2.5 Hz, 1H, H-5'), 4.07–3.98 (m, 2H, H-3', H-5''), 3.95 (dd, *J* = 9.6, 8.2 Hz, 1H, H-3''), 3.86–3.78 (m, 1H, H-6'), 3.78–3.71 (m, 4H, H-4', OMe), 3.71–3.63 (m, 3H, H-5, H-2', H-6'), 3.63–3.51 (m, 4H, H-5'', H-4'', H-2'', H-1), 3.42–3.35 (m, 1H, H-6), 3.35–3.25 (m, 2H, H-4, H-3), 2.36 (dt, *J* = 12.9, 4.3 Hz, 1H, H-2), 1.57 (q, *J* = 12.5 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.27, 138.87, 138.70, 138.32, 138.24, 138.15, 137.34,

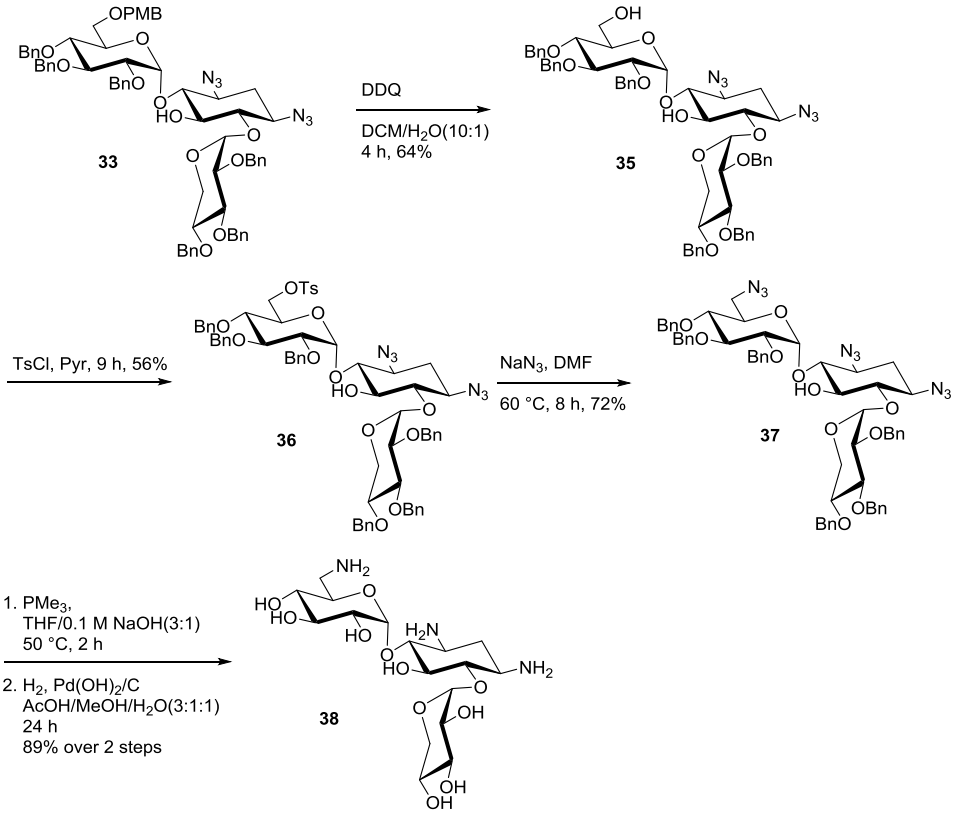
129.96, 129.76, 128.55, 128.42, 128.41, 128.38, 128.35, 128.33, 128.30, 128.08, 128.00, 127.95, 127.81, 127.78, 127.74, 127.65, 127.60, 127.57, 113.77, 99.95, 98.03, 83.58, 82.07, 81.67, 80.99, 79.87, 79.37, 77.88, 77.66, 75.63, 75.57, 75.02, 74.76, 74.14, 73.60, 73.42, 73.14, 71.22, 67.70, 60.86, 60.13, 58.83, 55.18, 32.29. HRMS (ESI, positive) calculated for $C_{67}H_{72}N_6O_{13}^+$ $[M + Na]^+$ 1191.5050, found 1191.5050.

2'-deamino-2'-hydroxy-gentamicin A_2 (34): Trimethylphosphine (1 M solution in THF, 0.08 mL, 0.80 mmol) was added to a solution of compound **33** (0.466 g, 0.39 mmol) in THF (9 mL) and 0.1 M NaOH (3 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic acid (3 mL), water (9 mL) and methanol (9 mL). The solution was deaerated by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 hr. The solution was then filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (1-5% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **34** (0.167 g, 92%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.59 (d, J = 3.9 Hz, 1H, H-1'), 5.04 (d, J = 3.0 Hz, 1H, H-1''), 4.05 (ddd, J = 10.1, 8.3, 3.2 Hz, 1H, H-5'), 4.00–3.90 (m, 2H, H-5, H-3''), 3.80 (t, J = 9.5 Hz, 1H, H-3'), 3.76–3.55 (m, 9H, H-4'', H-2', H-4, H-1, H-5'', H-6, H-5'', H-2'', H-3), 3.47 (dd, J = 13.4, 3.2 Hz, 1H, H-6'), 3.40 (t, J = 9.5 Hz, 1H, H-4'), 3.20 (dd, J = 13.3, 8.3 Hz, 1H, H-6'), 2.57 (dt, J = 12.6, 4.2 Hz, 1H, H-2), 1.96 (q, J = 12.4 Hz, 1H, H-2). ^{13}C NMR (125 MHz, D_2O) δ 101.84, 95.44, 83.82, 78.05,

72.95, 72.11, 71.98, 71.73, 70.80, 70.68, 68.90, 68.63, 62.20, 49.95, 47.54, 40.30, 27.52. HRMS (ESI, positive) calculated for $C_{17}H_{32}N_2O_{12}^+$ $[M + Na]^+$ 479.1847, found 479.1854.

2.2.14. Synthesis of 2'-Deamino-2'-hydroxy-6'-amino-6'-dehydroxy-gentamicin A_2

The overall synthetic scheme is shown in Scheme 2-12.



Scheme 2-12. Synthetic scheme for the preparation of 2'-deamino-2'-hydroxy-6'-amino-6'-dehydroxy-gentamicin A_2.

(*1R,2R,3S,5R,6S*)-3,5-diazido-2-(((*2S,3R,4S,5R,6R*)-3,4,5-tris(benzyloxy)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-6-(((*2R,3R,4S,5R*)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexan-1-ol (35): 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (0.636 g, 2.802 mmol) was added to a solution of compound **33** (2.184 g,

1.87 mmol) in dichloromethane (40 mL) and water (4 mL). The reaction mixture was stirred for 4 h at room temperature and quenched by adding a saturated aqueous solution of sodium bicarbonate (40 mL). The aqueous layer was extracted with dichloromethane (60 mL × 3), and the combined organic phase was washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **35** (1.251 g, 64%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36–7.27 (m, 29H, Ph), 7.21–7.16 (m, 1H, Ph), 5.27 (d, *J* = 3.7 Hz, 1H, H-1'), 5.12 (d, *J* = 3.6 Hz, 1H, H-1''), 5.00–4.82 (m, 6H, Bn), 4.82 – 4.72 (m, 6H, Bn), 4.66 (dd, *J* = 23.4, 11.3 Hz, 2H, Bn), 4.07–3.95 (m, 3H, H-5', H-3', H-5''), 3.91 (dd, *J* = 9.6, 8.2 Hz, 1H, H-3''), 3.84 (dd, *J* = 11.9, 2.6 Hz, 1H, H-6'), 3.76 (dd, *J* = 12.0, 3.6 Hz, 1H, H-6'), 3.64–3.49 (m, 7H, H-5, H-4, H-4'', H-2', H-5'', H-1, H-2''), 3.39–3.23 (m, 3H, H-6, H-3, H-4), 2.37 (dt, *J* = 13.3, 4.4 Hz, 1H, H-2), 1.54 (q, *J* = 12.5 Hz, 2H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.79, 138.58, 138.25, 138.09, 138.06, 137.29, 128.56, 128.49, 128.43, 128.39, 128.34, 128.11, 128.01, 128.00, 127.94, 127.89, 127.81, 127.79, 127.75, 127.64, 127.58, 99.57, 98.17, 83.23, 82.06, 81.87, 80.88, 79.95, 79.27, 77.75, 77.22, 75.61, 75.11, 74.84, 74.12, 73.61, 73.46, 71.78, 61.64, 60.95, 60.02, 58.88, 32.34. HRMS (ESI, positive) calculated for $C_{59}H_{64}N_6O_{12}^{+}$ $[M + Na]^{+}$ 1071.4474, found 1071.4477.

(*1R,2R,3S,5R,6S*)-3,5-diazido-2-(((*2R,3R,4S,5R,6R*)-6-(azidomethyl)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)-6-(((*2R,3R,4S,5R*)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexan-1-ol (36): *p*-Toluenesulfonyl chloride (1.137 g, 5.96 mmol) was added to a solution of compound **35** (1.251 g, 1.19 mmol) in pyridine (30 mL) at 0 °C. The reaction mixture was stirred at room temperature for 9 hr. After the addition of methanol (10 mL) at 0 °C, the mixture was stirred at room temperature for 30 min. The solution was concentrated under reduced pressure, and co-evaporated with

toluene twice. The residue was diluted with dichloromethane (50 mL), and washed with a saturated aqueous solution of sodium bicarbonate (40 mL). The aqueous layer was extracted with dichloromethane (50 mL × 2), and the combined organic phase was washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to give tosylate **36** (0.766 g, 56%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.2 Hz, 2H, Ph), 7.41–7.27 (m, 29H, Ph), 7.23–7.13 (m, 3H, Ph), 5.27 (d, *J* = 3.6 Hz, 1H, H-1'), 5.08 (d, *J* = 3.6 Hz, 1H, H-1''), 4.93 (dd, *J* = 20.2, 11.0 Hz, 2H, Bn), 4.88–4.79 (m, 3H, Bn), 4.79–4.70 (m, 5H, Bn), 4.63 (d, *J* = 11.5 Hz, 1H, Bn), 4.48 (d, *J* = 10.6 Hz, 1H, Bn), 4.43 (s, 1H, OH), 4.35 (dd, *J* = 10.6, 3.2 Hz, 1H, H-6'), 4.21 (dd, *J* = 10.6, 2.1 Hz, 1H, H-6'), 4.15 (dt, *J* = 10.1, 2.6 Hz, 1H, H-5'), 4.03–3.83 (m, 3H, H-5'', H-3', H-3''), 3.63–3.46 (m, 7H, H-5, H-4'', H-4', H-5'', H-2', H-1, H-2''), 3.36–3.23 (m, 3H, H-6, H-3, H-4), 2.40 (s, 3H, Me), 2.37–2.26 (m, 1H, H-2), 1.54–1.42 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 144.81, 138.77, 138.47, 138.21, 138.08, 137.78, 137,26, 132.84, 129.80, 128.55, 128.43, 128.40, 128.34, 128.29, 128.09, 128.04, 127.99, 127.93, 127.90, 127.82, 127.81, 127.64, 127.59, 99.02, 98.29, 82.72, 82.43, 81.68, 80.80, 79.64, 79.23, 77.64, 77.22, 76.83, 75.60, 75.55, 75.07, 74.95, 73.89, 73.62, 73.49, 69.43, 68.38, 61.02, 59.91, 58.66, 32.18, 21.64. HRMS (ESI, positive) calculated for $C_{66}H_{70}N_6O_{14}S^+$ $[M + Na]^+$ 1225.4563, found 1225.4569.

(1R,2R,3S,5R,6S)-3,5-diazido-2-(((2R,3R,4S,5R,6R)-6-(azidomethyl)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)-6-(((2R,3R,4S,5R)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexan-1-ol (37): Sodium azide (0.215 g, 3.30 mmol) and tetrabutylammonium iodide (0.005 g, 0.013 mmol) was added to a solution of the previously synthesized tosylate **36** (0.795 g, 0.66 mmol) in DMF (20 mL). The reaction mixture was stirred at 60 °C for 8 h. After cooling to room temperature, the reaction

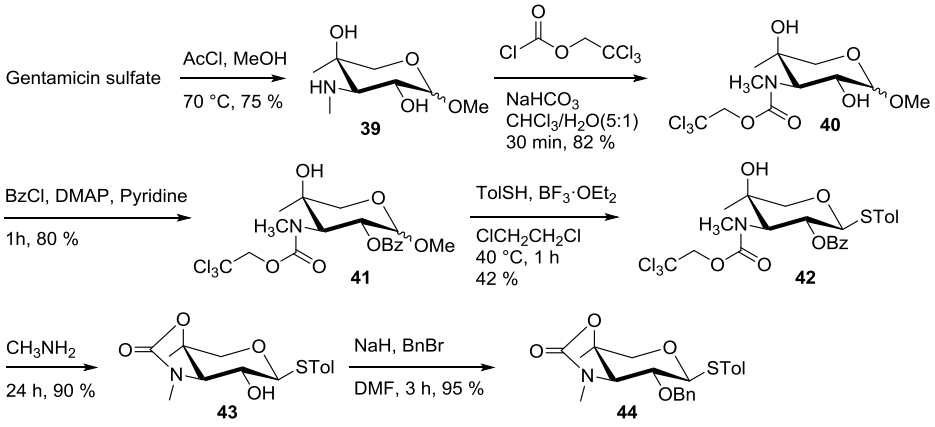
solution was evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to give compound **37** (0.511 g, 72%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.27 (m, 29H, Ph), 7.23–7.17 (m, 1H, Ph), 5.39 (d, *J* = 3.7 Hz, 1H, H-1'), 5.10 (d, *J* = 3.6 Hz, 1H, H-1"), 4.98 (d, *J* = 10.9 Hz, 1H, Bn), 4.92 (d, *J* = 11.1 Hz, 2H, Bn), 4.86 (d, *J* = 10.9 Hz, 2H, Bn), 4.82–4.73 (m, 5H, Bn), 4.64 (dd, *J* = 11.3, 6.1 Hz, 2H, Bn), 4.47 (d, *J* = 1.8 Hz, 1H, OH), 4.18 (ddd, *J* = 10.0, 4.3, 2.5 Hz, 1H, H-5'), 4.02 (t, *J* = 9.4 Hz, 1H, H-3'), 3.98–3.86 (m, 2H, H-5", H-3"), 3.66–3.50 (m, 8H, H-5, H-2', H-5", H-4", H-6', H-4', H-1, H-2"), 3.44 (dd, *J* = 13.2, 4.3 Hz, 1H, H-6'), 3.40–3.31 (m, 3H, H-6, H-3, H-4), 2.38 (dt, *J* = 13.5, 4.0 Hz, 1H, H-2), 1.63–1.46 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.79, 138.52, 138.23, 138.10, 137.95, 137.36, 128.55, 128.49, 128.44, 128.42, 128.36, 128.29, 128.08, 128.01, 127.94, 127.93, 127.82, 127.75, 127.67, 127.60, 98.96, 98.33, 82.91, 82.44, 81.69, 80.79, 79.96, 79.25, 78.18, 77.63, 77.24, 75.61, 75.21, 75.04, 73.91, 73.62, 73.49, 70.76, 61.05, 59.94, 58.82, 51.26, 32.31. HRMS (ESI, positive) calculated for $C_{59}H_{63}N_9O_{11}^{+}$ $[M + Na]^{+}$ 1096.4539, found 1096.4550.

2'-deamino-2'-hydroxy-6'-amino-6'-deoxy-gentamicin A_2 (38): Trimethylphosphine (1 M solution in THF, 1.40 mL, 1.40 mmol) was added to a solution of compound **37** (0.5 g, 0.465 mmol) in THF (9 mL) and 0.1 M NaOH (3 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic acid (3 mL), water (9 mL) and methanol (9 mL). The solution was deaerated by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.2 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 hr. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced

pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (5-10% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **38** (0.188 g, 89%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.62 (d, *J* = 3.8 Hz, 1H, H-1'), 5.07 (d, *J* = 3.2 Hz, 1H, H-1''), 4.08 (ddd, *J* = 10.0, 8.3, 3.2 Hz, 1H, H-5'), 4.02–3.95 (m, 2H, H-5, H-3''), 3.86–3.81 (m, 1H, H-3'), 3.81–3.55 (m, 9H, H-4'', H-5'', H-4, H-5'', H-1, H-6, H-2', H-2'', H-3), 3.50 (dd, *J* = 13.3, 3.2 Hz, 1H, H-6'), 3.47 – 3.38 (m, 1H, H-4'), 3.23 (dd, *J* = 13.4, 8.3 Hz, 1H, H-6'), 2.64 – 2.55 (m, 1H, H-2), 1.99 (q, *J* = 12.6 Hz, 1H, H-2). ^{13}C NMR (125 MHz, D_2O) δ 101.84, 95.47, 83.82, 78.07, 72.97, 72.13, 72.02, 71.76, 70.84, 70.71, 68.93, 68.65, 62.23, 49.98, 47.59, 40.34, 27.54. HRMS (ESI, positive) calculated for $C_{17}H_{33}N_3O_{11}^+$ $[M + Na]^+$ 478.2007, found 478.2009.

2.2.15. Synthesis of Garosamine Derivative (44)

The overall synthetic scheme is shown in Scheme 2-13.

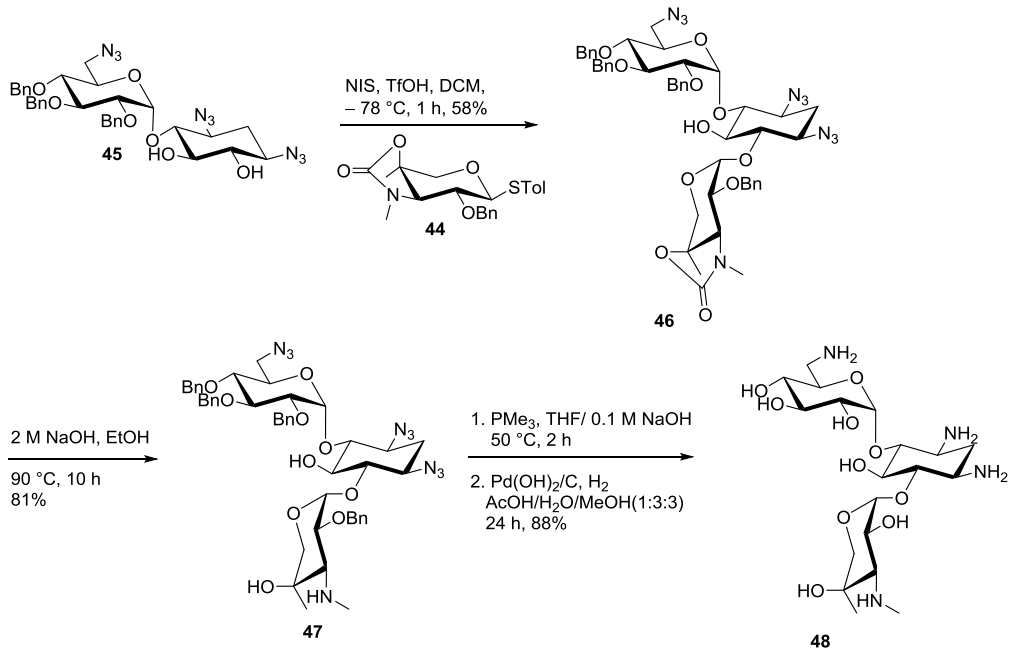


Scheme 2-13. Synthetic scheme for the preparation of garosamine derivative (**44**).

(*3aR,6S,7R,7aR*)-7-(benzyloxy)-1,3a-dimethyl-6-(*p*-tolylthio)hexahydro-2*H*-pyrano-[4,3-d]oxazol-2-one (44): Compound **43** was prepared as previously described.[163] Sodium hydride (60% in mineral oil, 1.022 g, 25.56 mmol) was added to a solution of compound **43** (7.19 g, 23.24 mmol) in DMF (200 mL) at 0 °C under a nitrogen atmosphere. The reaction mixture was stirred for 30 min at room temperature. After the reaction solution was cooled to 0 °C, benzyl bromide (3.32 mL, 27.88 mmol) was added, and the mixture was stirred at room temperature for 3 h. Methanol (30 mL) was added at 0 °C to quench the reaction. After all solvents were evaporated under reduced pressure, the residue was diluted with dichloromethane (200 mL) and water (150 mL). The aqueous layer was extracted with dichloromethane (150 mL × 3). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound 44 (8.81 g, 95%) as a clear solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.46–7.28 (m, 7H, Ph), 7.13 (d, *J* = 7.7 Hz, 2H, Ph), 5.26 (d, *J* = 3.3 Hz, 1H, H-1), 4.84 (d, *J* = 11.7 Hz, 1H, Bn), 4.54 (d, *J* = 11.2 Hz, 1H, Bn), 4.16 (d, *J* = 12.3 Hz, 1H, H-5), 3.81 (t, *J* = 3.6 Hz, 1H, H-2), 3.61 (d, *J* = 12.3 Hz, 1H, H-5), 3.44 (d, *J* = 4.1 Hz, 1H, H-3), 2.86 (s, 3H, NMe), 2.34 (s, 3H, PhMe), 1.52 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.59, 138.10, 137.00, 132.25, 130.34, 129.97, 128.69, 128.29, 127.84, 85.96, 75.97, 75.59, 72.93, 66.46, 64.25, 30.36, 24.09, 21.20. HRMS (ESI, positive) calculated for $C_{22}H_{25}NO_4S^+$ $[M + Na]^+$ 422.1397, found 422.1411.

2.2.16. Synthesis of Gentamicin B

The overall synthetic scheme is shown in Scheme 2-14.



Scheme 2.14. Synthetic scheme for the preparation of gentamicin B.

(*3aR,6R,7R,7aR*)-7-(benzyloxy)-6-(((*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2R,3R,4S,5R,6R*)-6-(azidomethyl)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-1,3a-dimethylhexahydro-2*H*-pyrano[4,3-d]oxazol-2-one (46): Compound **45** was prepared as previously described.[157] Compound **44** (0.263 g, 0.659 mmol) and compound **45** (0.402 g, 0.599 mmol) were co-evaporated from dry toluene under reduced pressure three times and further dried under high vacuum for overnight. A solution of compound **44** and **45** in anhydrous dichloromethane (15 mL) was added to preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.216 g, 0.959 mmol) were added. After stirring for 30 min at room temperature, the reaction mixture was cooled to –78 °C. Trifluoromethanesulfonic acid (0.016 mL, 0.179 mmol) was added dropwise over 10 min, and the reaction was warmed to 0 °C slowly. The reaction was quenched by adding solid sodium sulfite (0.2 g), sodium bicarbonate (0.2 g), and a few drops of water. The reaction mixture was diluted with dichloromethane (20 mL), filtered, and washed

with a saturated aqueous solution of sodium bicarbonate (60 mL × 2) and brine. The residue was dried over sodium sulfate, filtered, concentrated in reduced pressure, and purified by flash column chromatography on silica gel (Hex:EtOAc = 3:2) to give compound **46** (0.329 g, 58%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.28 (m, 20H, Ph), 5.43 (d, *J* = 3.7 Hz, 1H, H-1'), 5.18 (d, *J* = 3.3 Hz, 1H, H-1''), 4.99 (d, *J* = 10.9 Hz, 1H, Bn), 4.94–4.85 (m, 3H, Bn), 4.85–4.76 (m, 2H, Bn), 4.64 (d, *J* = 11.0 Hz, 1H, Bn), 4.59 (d, *J* = 11.5 Hz, 1H, Bn), 4.38 (d, *J* = 1.6 Hz, 1H, OH), 4.22–4.09 (m, 2H, H-5', H-5''), 4.03 (t, *J* = 9.3 Hz, 1H, H-3'), 3.86 (dd, *J* = 5.4, 3.3 Hz, 1H, H-2''), 3.71–3.59 (m, 3H, H-2', H-5, H-5''), 3.58–3.36 (m, 8H, H-6', H-4', H-1, H-6, H-3'', H-6, H-4, H-3), 2.86 (s, 3H, NMe), 2.36 (dt, *J* = 13.2, 4.1 Hz, 1H, H-2), 1.56 (q, *J* = 12.2 Hz, 1H, H-2), 1.30 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.36, 138.55, 138.00, 137.53, 137.38, 128.68, 128.58, 128.57, 128.53, 128.28, 128.23, 128.15, 128.00, 127.93, 127.82, 127.77, 98.89, 96.15, 82.72, 82.47, 81.77, 80.07, 78.30, 77.57, 75.69, 75.29, 74.83, 74.68, 73.90, 70.87, 65.59, 62.77, 60.01, 58.81, 51.32, 32.12, 30.47, 23.33. HRMS (ESI, positive) calculated for $C_{48}H_{54}N_{10}O_{11}^{+}$ $[M + Na]^{+}$ 969.3866, found 969.3864.

(*3R,4R,5R,6R*)-5-(benzyloxy)-6-(((*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2R,3R,4S,5R,6R*)-6-(azidomethyl)-3,4,5-tris(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-3-methyl-4-(methylamino)tetrahydro-2*H*-pyran-3-ol (47): To a solution of compound **46** (0.292 g, 0.309 mmol) in absolute ethanol (5 mL) was added 2N NaOH (2 mL). The reaction mixture was stirred at 90 ℃ for 10 h, and cooled to room temperature. The solution was diluted with ethyl acetate (20 mL) and washed with H_2O (20 mL). The aqueous layer was extracted with ethyl acetate (20 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (4% MeOH in DCM) to give compound **47** (0.231 g, 81%)

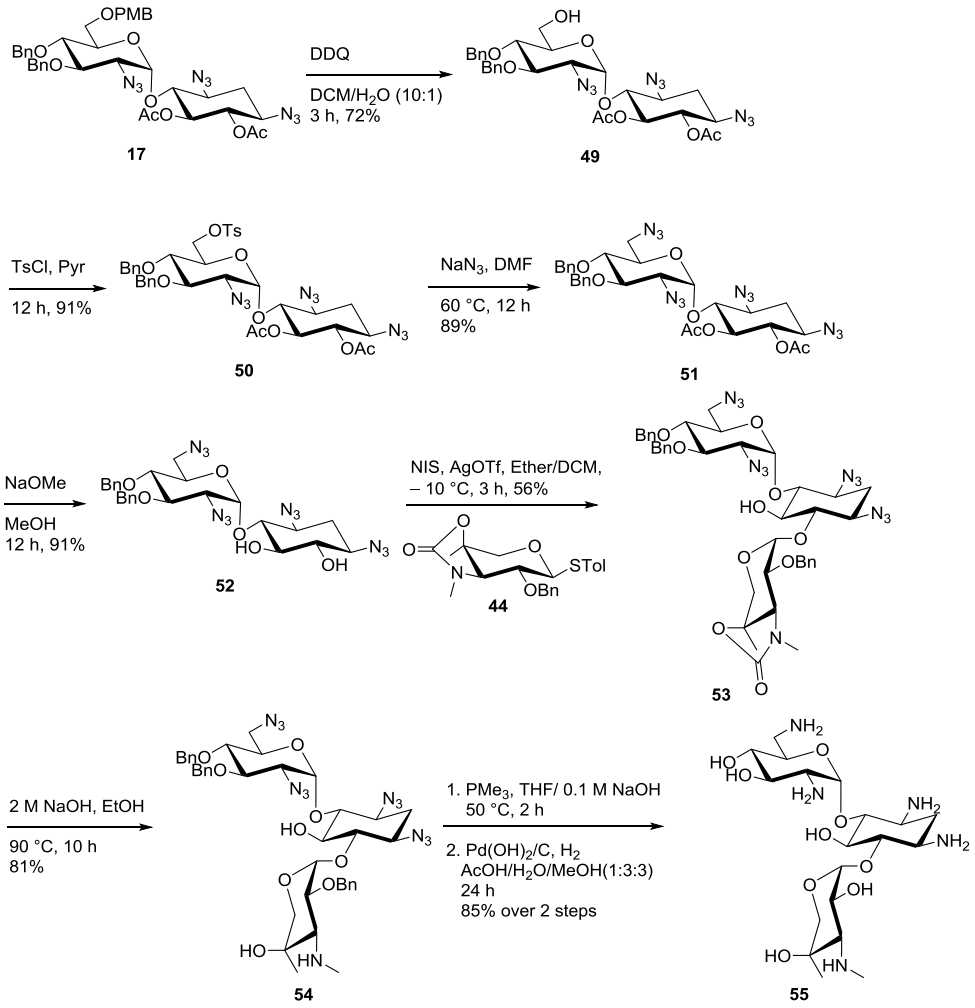
as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.30 (m, 20H, Ph), 5.42 (d, *J* = 3.7 Hz, 1H, H-1'), 5.31 (d, *J* = 3.4 Hz, 1H, H-1''), 5.00 (d, *J* = 11.0 Hz, 1H, Bn), 4.94 (d, *J* = 11.0 Hz, 1H, Bn), 4.89 (d, *J* = 11.0 Hz, 1H, Bn), 4.86–4.76 (m, 3H, Bn), 4.66 (d, *J* = 11.0 Hz, 1H, Bn), 4.56 (s, 1H, OH), 4.50 (d, *J* = 11.8 Hz, 1H, Bn), 4.21 (ddd, *J* = 10.0, 4.3, 2.5 Hz, 1H, H-5'), 4.05 (t, *J* = 9.3 Hz, 1H, H-3'), 3.91 (d, *J* = 12.3 Hz, 1H, H-5''), 3.70–3.49 (m, 6H, H-5, H-2', H-6', H-4', H-2'', H-1), 3.48–3.32 (m, 6H, H-6', H-3'', H-4, H-6, H-3, H-5''), 2.64 (d, *J* = 10.3 Hz, 1H, NH), 2.53 (s, 3H, NMe), 2.40–2.35 (m, 1H, H-2), 1.66–1.52 (m, 1H, H-2), 1.11 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.50, 137.95, 137.77, 137.44, 128.65, 128.52, 128.47, 128.18, 128.13, 128.11, 128.09, 127.95, 127.76, 127.71, 99.06, 96.92, 82.63, 82.45, 81.75, 80.13, 78.27, 76.04, 75.63, 75.24, 75.19, 73.98, 72.19, 70.98, 70.82, 66.25, 63.01, 60.03, 58.83, 51.27, 38.67, 32.21, 24.57. HRMS (ESI, positive) calculated for $C_{47}H_{56}N_{10}O_{10}^{+}$ $[M + Na]^{+}$ 943.4073, found 943.4062.

Gentamicin B (48): Trimethylphosphine (1 M solution in THF, 1.22 mL, 1.22 mmol) was added to a solution of compound **47** (0.188 g, 0.204 mmol) in THF (6 mL) and 0.1 M NaOH (2 mL) was added. The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic acid (1.5 mL), water (4.5 mL) and methanol (4.5 mL). The solution was deaerated by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 hr. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^{+} form) (5-10% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **48**

(0.086 g, 88%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.58 (d, *J* = 3.9 Hz, 1H, H-1'), 5.12 (d, *J* = 3.7 Hz, 1H, H-1''), 4.23 (dd, *J* = 10.9, 3.7 Hz, 1H, H-2''), 4.09–3.99 (m, 2H, H-5', H-5''), 3.98–3.90 (m, 2H, H-5, H-4), 3.82–3.73 (m, 2H, H-3', H-6), 3.69 (dd, *J* = 9.9, 3.9 Hz, 1H, H-2'), 3.67–3.58 (m, 2H, H-1, H-3), 3.52 (dd, *J* = 25.2, 11.8 Hz, 2H, H-3'' H-5''), 3.46 (dd, *J* = 13.4, 3.3 Hz, 1H, H-6'), 3.40 (dd, *J* = 10.0, 9.0 Hz, 1H, H-4'), 3.20 (dd, *J* = 13.4, 8.0 Hz, 1H, H-6'), 2.94 (s, 3H, NMe), 2.57 (dt, *J* = 12.5, 4.2 Hz, 1H, H-2), 1.95 (q, *J* = 12.5 Hz, 1H, H-2), 1.36 (s, 3H, Me). ^{13}C NMR (125 MHz, D_2O) δ 101.14, 95.76, 83.79, 78.19, 72.28, 72.07, 70.69, 70.65, 69.85, 68.64, 67.68, 66.27, 63.24, 49.72, 47.52, 40.22, 34.42, 27.50, 20.85. HRMS (ESI, positive) calculated for $C_{19}H_{38}N_4O_{10}^{+}$ $[M + Na]^{+}$ 505.2480, found 505.2480.

2.2.17. Synthesis of JI-20A

The overall synthetic scheme is shown in Scheme 2-15.



Scheme 2.15. Synthetic scheme for the preparation of JI-20A.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (49): 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (0.251 g, 1.109 mmol) was added to a stirring solution of compound **17** (0.726 g, 0.925 mmol) in dichloromethane (20 mL) and water (2 mL). The reaction mixture was stirred at room temperature for 3 h and quenched by adding a saturated aqueous solution of sodium bicarbonate (40 mL). The aqueous solution was extracted with dichloromethane (50 mL × 3), and the combined organic

layers were washed with brine. The organic layers were dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to provide compound **49** (0.442 g, 72%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42–7.26 (m, 10H, Ph), 5.14 (t, *J* = 9.8 Hz, 1H, H-5), 5.08 (d, *J* = 3.8 Hz, 1H, H-1'), 4.97–4.81 (m, 4H, H-6, Bn), 4.72 (d, *J* = 11.0 Hz, 1H, Bn), 4.08 (dt, *J* = 10.1, 2.9 Hz, 1H, H-5'), 3.98 (dd, *J* = 10.4, 8.9 Hz, 1H, H-3'), 3.88–3.76 (m, 2H, H-6', H-6'), 3.68 (dd, *J* = 10.1, 8.9 Hz, 1H, H-4'), 3.64–3.54 (m, 2H, H-1, H-4), 3.40 (ddd, *J* = 12.4, 9.9, 4.6 Hz, 1H, H-3), 3.32 (dd, *J* = 10.4, 3.8 Hz, 1H, H-2'), 2.36 (dt, *J* = 13.3, 4.6 Hz, 1H, H-2), 2.09 (s, 6H, OAc), 1.58 (dt, *J* = 13.3, 12.5 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.93, 169.58, 137.77, 137.71, 128.59, 128.53, 128.06, 128.05, 127.98, 127.94, 99.48, 79.53, 78.67, 77.74, 75.41, 75.08, 74.07, 73.43, 72.33, 63.23, 61.10, 58.70, 57.63, 31.78, 20.67, 20.60. HRMS (ESI, positive) calculated for $C_{30}H_{35}N_9O_9^+$ $[M + Na]^+$ 688.2450, found 688.2466.

(*1S,2S,3R,4S,6R)*-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((tosyloxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (50): *p*-Toluenesulfonyl chloride (0.304 g, 1.595 mmol) was added to a solution of compound **49** (0.213 g, 0.319 mmol) in pyridine (20 mL) at 0 °C, and the reaction mixture was stirred at room temperature for 15 h, and methanol (5 mL) was added at 0 °C to quench the reaction. The reaction solution was concentrated under reduced pressure, and coevaporated with toluene twice. The residue was diluted with dichloromethane (50 mL) and washed with a saturated aqueous solution of ammonium chloride (40 mL × 3). The organic layer was washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to provide compound **50** (0.238 g, 91%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80–7.78 (m, 2H, Ph), 7.35–7.30 (m,

10H, Ph), 7.21–7.15 (m, 2H, Ph), 5.11 (t, *J* = 9.7 Hz, 1H, H-5), 5.03 (d, *J* = 3.6 Hz, 1H, H-1'), 4.90 (t, *J* = 9.8 Hz, 1H, H-6), 4.87–4.81 (m, 2H, 2×Bn), 4.80 (d, *J* = 10.6 Hz, 1H, Bn), 4.51 (d, *J* = 10.7 Hz, 1H, Bn), 4.35 (dd, *J* = 10.8, 3.0 Hz, 1H, H-6'), 4.23–4.19 (m, 2H, H-5', H-6), 3.93 (dd, *J* = 10.4, 8.8 Hz, 1H, H-3'), 3.65–3.58 (m, 2H, H-4', H-1), 3.55 (t, *J* = 9.6 Hz, 1H, H-4), 3.44–3.34 (m, 1H, H-3), 3.28 (dd, *J* = 10.4, 3.6 Hz, 1H, H-2'), 2.41 (s, 3H, Me), 2.39–2.32 (m, 1H, H-2), 2.08 (s, 3H, OAc), 2.08 (s, 3H, OAc), 1.61–1.52 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.95(OC(=O)), 169.57 (O(C=O)), 145.11 (Ph), 137.61 (Ph), 137.47 (Ph), 132.88 (Ph), 129.99 (Ph), 128.63 (Ph), 128.62 (Ph), 128.13 (Ph), 128.10 (Ph), 128.01 (Ph), 127.95 (Ph), 99.18 (C-1'), 79.58 (C-3'), 78.36 (C-4), 77.50 (C-4'), 75.46 (Bn), 75.26 (Bn), 74.17 (C-6), 73.52 (C-5), 69.98 (C-5'), 68.02 (C-6'), 63.06 (C-2'), 58.61 (C-3), 57.77 (C-1), 31.81 (C-2), 21.77 (Me), 20.76 (Ac), 20.70 (Ac). HRMS (ESI, positive) calculated for $C_{37}H_{41}N_9O_{11}S^+$ $[M + Na]^+$ 842.2538, found 842.2529.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2R,3R,4R,5R,6R*)-3-azido-6-(azidomethyl)-4,5-bis-(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (51): Sodium azide (0.155 g, 2.385 mmol) and tetrabutylammonium iodide (0.02 g, 0.059 mmol) were added to a stirring solution of compound **50** (0.925 g, 1.192 mmol) in DMF (30 mL). The reaction mixture was stirred at 60 °C for 12 h, and cooled to room temperature before concentration under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to provide compound **51** (0.734 g, 89%) as a clear liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39–7.26 (m, 10H, Ph), 5.15 (t, *J* = 9.8 Hz, 1H, H-5), 5.11 (d, *J* = 3.8 Hz, 1H, H-1'), 4.93 (t, *J* = 10.0 Hz, 1H, H-6), 4.90–4.81 (m, 3H, Bn), 4.62 (d, *J* = 11.1 Hz, 1H, Bn), 4.23 (dt, *J* = 10.0, 3.3 Hz, 1H, H-5'), 3.95 (dd, *J* = 10.4, 8.8 Hz, 1H, H-3'), 3.67–3.55 (m, 3H, H-4', H-1, H-4), 3.55–3.49 (m, 1H, H-6'), 3.48–3.37 (m, 2H, H-6', H-3), 3.34 (dd, *J* = 10.4, 3.8 Hz, 1H, H-2'), 2.42

(dt, *J* = 13.4, 4.6 Hz, 1H, H-2), 2.09 (d, *J* = 2.7 Hz, 6H, OAc), 1.69–1.57 (m, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.00, 169.67, 137.67, 128.72, 128.67, 128.24, 128.15, 127.97, 99.27, 79.62, 99.25, 79.62, 78.80, 78.60, 75.57, 75.42, 74.24, 73.60, 71.53, 63.29, 58.79, 57.83, 50.94, 31.97, 20.81, 20.73. HRMS (ESI, positive) calculated for $C_{30}H_{34}N_{12}O_8^+$ $[M + Na]^+$ 713.2515., found 713.2510.

(1S,2R,3R,4S,6R)-4,6-diazido-3-(((2R,3R,4R,5R,6R)-3-azido-6-(azidomethyl)-4,5-bis(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diol (52): Sodium methoxide (5 M solution in methanol, 0.05mL, 0.241 mmol) was added to a solution of **51** (0.834 g, 1.205 mmol) in methanol. The reaction mixture was stirred at room temperature for 12 h. The basic solution was neutralized with Amberlite IR-120 resin (H^+ form), filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **52** (0.665 g, 91%) as a clear liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39–7.26 (m, 10H, Bn), 5.17 (d, *J* = 3.7 Hz, 1H, H-1'), 4.95–4.85 (m, 3H, Bn), 4.64 (d, *J* = 11.0 Hz, 1H, Bn), 4.16–4.08 (m, 1H, H-5'), 4.06 (d, *J* = 2.1 Hz, 1H, OH), 3.99 (dd, *J* = 10.2, 8.9 Hz, 1H, H-3'), 3.67–3.59 (m, 2H, OH, H-6'), 3.56 (dd, *J* = 13.3, 2.5 Hz, 1H, H-5), 3.53–3.38 (m, 4H, H-5, H-6', H-1, H-4), 3.32 – 3.25 (m, 2H, H-6, H-3), 2.37–2.28 (m, 1H, H-2), 1.55–1.44 (m, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.60, 137.46, 128.74, 128.69, 128.27, 128.24, 128.21, 127.94, 99.56, 84.04, 80.94, 78.80, 76.08, 75.87, 75.46, 75.43, 71.50, 64.37, 59.81, 58.93, 51.04, 32.17. HRMS (ESI, positive) calculated for $C_{26}H_{30}N_{12}O_6^+$ $[M + Na]^+$ 629.2303, found 629.2311.

(*3aR,6R,7R,7aR*)-7-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2R,3R,4R,5R,6-R*)-3-azido-6-(azidomethyl)-4,5-bis(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-1,3a-dimethylhexahydro-2*H*-pyrano[4,3-d]oxazol-2-one (53): Compound **44** (0.550 g, 1.378 mmol) and compound **52** (0.698 g, 1.148 mmol) were co-

evaporated with dry toluene under reduced pressure three times and further dried under high vacuum for overnight. A solution of compound **44** and **52** in anhydrous ether (16 mL) and anhydrous dichloromethane (4 mL) was added to a preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.5167 g, 2.297 mmol). After stirring for 30 min at room temperature, the reaction mixture was cooled to –48 °C. Silver trifluoromethanesulfonate (0.590 g, 2.297 mmol) was added, and the reaction was warmed to –10 °C to stand for 3 h. The reaction mixture was diluted with ethyl acetate (20 mL) and filtered through a Celite pad. The solution was washed with 10% aqueous solution of sodium bisulfite (50 mL) and a saturated aqueous solution of sodium bicarbonate (50 mL). The combined aqueous layers were extracted with ethyl acetate (50 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 1.2 : 1) to provide compound **53** (0.567g, 56%) as a clear liquid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.41–7.26 (m, 15H, Ph), 5.62 (d, *J* = 3.8 Hz, 1H, H-1'), 5.02 (d, *J* = 3.2 Hz, 1H, H-1''), 4.94–4.84 (m, 4H, Bn), 4.62 (dd, *J* = 13.2, 11.4 Hz, 2H, Bn), 4.23–4.15 (m, 2H, H-5', H-5''), 4.00 (dd, *J* = 10.3, 8.9 Hz, 1H, H-3'), 3.95 (dd, *J* = 4.3, 3.1 Hz, 1H, H-2''), 3.80 (d, *J* = 12.4 Hz, 1H, H-5''), 3.68 (td, *J* = 8.9, 1.5 Hz, 1H, H-5), 3.57 (dd, *J* = 10.0, 8.8 Hz, 1H, H-4'), 3.53 (dd, *J* = 13.2, 2.5 Hz, 1H, H-6'), 3.51–3.44 (m, 3H, H-1, H-4, H-3''), 3.46–3.35 (m, 4H, H-2', H-6, H-6', H-3), 2.83 (s, 3H, NMe), 2.33 (dt, *J* = 13.3, 4.4 Hz, 1H, H-2), 1.57–1.45 (m, 1H, H-2), 1.41 (s, 3H,Me). ^{13}C NMR (150 MHz, $CDCl_3$) δ 157.27, 137.85, 137.83, 137.42, 128.75, 128.66, 128.63, 128.42, 128.16, 128.13, 128.07, 128.04, 127.95, 98.29, 97.12, 84.86, 80.32, 80.14, 78.79, 75.62, 75.31, 75.20, 74.69, 73.35, 71.21, 67.00, 63.67, 63.10, 59.80, 58.85, 51.15, 32.16, 30.32, 23.67. HRMS (ESI, positive) calculated for $C_{41}H_{47}N_{13}O_{10}^{+}$ $[M + Na]^{+}$ 904.3461, found 904.2459.

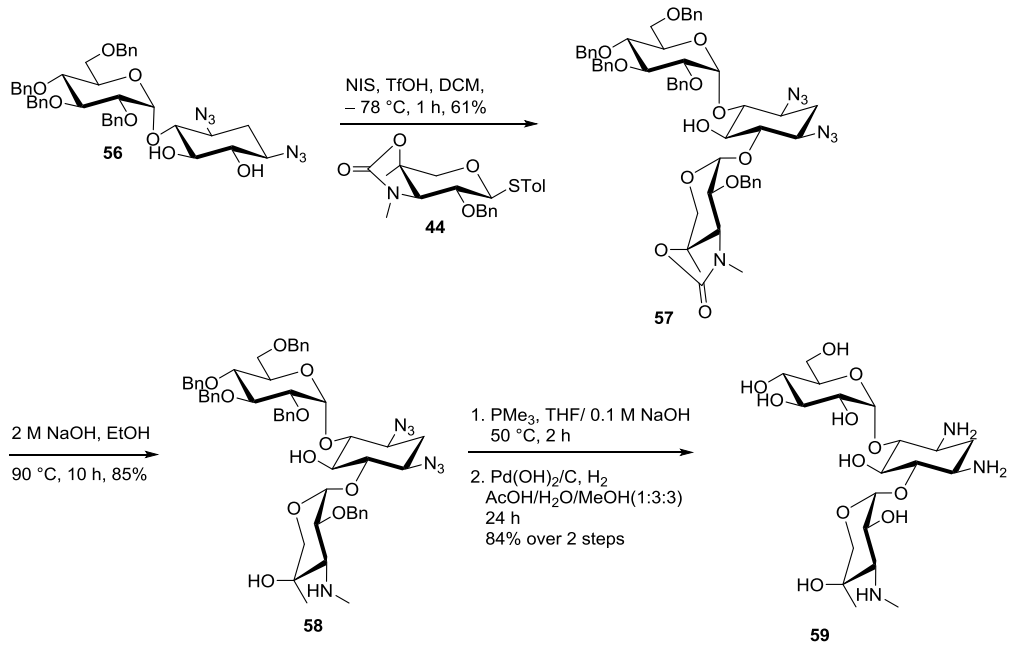
(*3R,4R,5R,6R*)-5-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2R,3R,4R,5R,6R*)-3-azido-6-(azidomethyl)-4,5-bis(benzyloxy)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-3-methyl-4-(methylamino)tetrahydro-2*H*-pyran-3-ol (54): A 2 N aqueous sodium hydroxide solution (4 mL) was added to a solution of compound **53** (0.4385 g, 0.497 mmol) in ethanol (12 mL). The reaction mixture was stirred at 90 ℃ for 12 h, and cooled to room temperature. The solution was diluted with ethyl acetate (20 mL) and washed with water (20 mL). The residue was washed with brine, dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel (4% methanol in dichloromethane) to give compound **54** (0.344 g, 81%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41–7.27 (m, 15H), 5.57 (d, *J* = 3.8 Hz, 1H, H-1'), 5.20 (d, *J* = 3.4 Hz, 1H, H-1''), 4.91–4.84 (m, 3H, Bn), 4.78 (d, *J* = 11.7 Hz, 1H, Bn), 4.63 (d, *J* = 11.2 Hz, 1H, Bn), 4.52 (d, *J* = 11.7 Hz, 1H, Bn), 4.45 (d, *J* = 1.9 Hz, 1H, OH), 4.20 (ddd, *J* = 10.0, 4.4, 2.5 Hz, 1H, H-5'), 3.98 (dd, *J* = 10.3, 8.8 Hz, 1H, H-3'), 3.89 (d, *J* = 12.2 Hz, 1H, H-5''), 3.73–3.64 (m, 1H, H-5), 3.62 (m, 1H, H-2''), 3.59–3.46 (m, 3H, H-4', H-5'', H-6'), 3.46–3.32 (m, 4H, H-2', H-6', H-4, H-3), 3.30 (t, *J* = 9.4 Hz, 1H, H-6), 2.69 (d, *J* = 10.3 Hz, 1H, H-3''), 2.54 (s, 3H, NMe), 2.38–2.29 (m, 1H, H-2), 1.52 (q, *J* = 12.7 Hz, 1H, H-2), 1.19 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.85, 137.84, 137.78, 128.82, 128.69, 128.65, 128.38, 128.28, 128.18, 128.16, 128.11, 127.97, 98.54, 97.40, 84.79, 80.65, 80.24, 78.81, 76.13, 75.85, 75.67, 75.35, 72.65, 71.23, 71.04, 66.92, 63.76, 63.47, 59.71, 59.01, 51.20, 38.63, 32.37, 25.25. HRMS (ESI, positive) calculated for $C_{40}H_{49}N_{13}O_9^+$ $[M + Na]^+$ 878.3668, found 878.3649.

JI-20A (55): Trimethylphosphine (1 M solution in THF, 1.08 mL, 1.08 mmol) was added to a solution of compound **54** (0.231 g, 0.270 mmol) in THF (9 mL) and 0.1 M NaOH (3 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic

acid (2 mL), water (6 mL) and methanol (6 mL). The solution was degassed by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (5-10% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **55** (0.110 g, 85%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.99 (d, *J* = 3.9 Hz, 1H, H-1'), 5.14 (d, *J* = 3.7 Hz, 1H, H-1"), 4.25 (dd, *J* = 10.9, 3.7 Hz, 1H, H-2"), 4.10–4.02 (m, 4H, H-4, H-5', H-3', H-5"), 3.93 (t, *J* = 9.1 Hz, 1H, H-5), 3.88–3.81 (m, 1H, H-6), 3.72–3.59 (m, 2H, H-1, H-3), 3.59–3.47 (m, 5H, H-3", H-4', H-5", H-2', H-6'), 3.32 (dd, *J* = 13.6, 6.9 Hz, 1H, H-6'), 2.95 (s, 3H, NMe), 2.57 (dt, *J* = 12.6, 4.3 Hz, 1H, H-2), 2.07–1.90 (m, 1H, H-2), 1.38 (s, 3H, Me). ^{13}C NMR (125 MHz, D_2O) δ 101.12, 95.90, 83.63, 77.54, 74.22, 70.60, 69.85, 69.13, 68.19, 67.85, 66.17, 63.17, 53.43, 49.57, 48.20, 40.07, 34.47, 28.00, 20.90. HRMS (ESI, positive) calculated for $C_{19}H_{39}N_5O_9^+$ $[M + Na]^+$ 504.2640, found 504.2639.

2.2.18. Synthesis of 2'-Deamino-2'-hydroxy-gentamicin X_2

The overall synthetic scheme is shown in Scheme 2-16.



Scheme 2.16. Synthetic scheme for the preparation of 2'-deamino-2'-hydroxy-GenX_2.

(*3aR,6R,7R,7aR*)-7-(benzyloxy)-6-(((*1S,2R,3R,4S,6R*)-4,6-diazido-2-hydroxy-3-(((*2S,-3R,4S,5R,6R*)-3,4,5-tris(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2*H*-pyran-2-yl-)oxy)cyclohexyl)oxy)-1,3a-dimethylhexahydro-2*H*-pyrano[4,3-d]oxazol-2-one (57): Compound **56** was prepared as previously described.[157] Compound **44** (0.4478 g, 1.121 mmol) and compound **56** (0.688 g, 0.934 mmol) were co-evaporated with dry toluene under reduced pressure three times and further dried under high vacuum overnight. A solution of compound **44** and **56** in anhydrous ether (12 mL) and anhydrous dichloromethane (3 mL) was added to preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.4204 g, 1.868 mmol). After stirring for 30 min at room temperature, the reaction mixture was cooled to –40 °C, silver trifluoromethanesulfonate (0.480 g, 1.868 mmol) was added, and the reaction was warmed to –10 °C to stand for 3 h. The reaction mixture was diluted with ethyl acetate (20 mL) and filtered through a Celite pad. The solution was washed with a 10% aqueous solution of sodium bisulfite (50 mL) and a

saturated aqueous solution of sodium bicarbonate solution (50 mL). The combined aqueous layers were extracted with ethyl acetate (50 mL × 3), and the combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 1.5 : 1) to provide compound **57** (0.576g, 61%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.26 (m, 23H, Ph), 7.14–7.12 (m, 2H, Ph), 5.31 (d, *J* = 3.6 Hz, 1H, H-1'), 5.24 (d, J = 3.7 Hz, 1H, H-1''), 4.95 (d, *J* = 10.9 Hz, 1H, Bn), 4.91–4.85 (m, 2H, 2×Bn), 4.82 (d, *J* = 11.1 Hz, 2H, 2×Bn), 4.76 (d, *J* = 11.4 Hz, 1H, Bn), 4.65 (d, *J* = 12.1 Hz, 1H, Bn), 4.57 (d, *J* = 11.3 Hz, 1H, Bn), 4.49 (dd, *J* = 22.7, 10.9 Hz, 3H, 2×Bn, OH), 4.17 (d, *J* = 12.8 Hz, 1H, H-5''), 4.11–4.07 (m, 1H, H-5'), 4.02 (t, *J* = 9.4 Hz, 1H, H-3'), 3.83–3.80 (m, 2H, H-6', H-2''), 3.74 (dd, *J* = 10.1, 9.0 Hz, 1H, H-4'), 3.69 (dd, *J* = 10.6, 1.9 Hz, 1H, H-6'), 3.67–3.56 (m, 3H, H-2', H-5, H-5''), 3.52–3.40 (m, 3H, H-1, H-6, H-3''), 3.35–3.29 (m, 2H, H-4, H-3), 2.87 (s, 3H, NMe), 2.34 (d, *J* = 14.5 Hz, 1H, H-2), 1.58–1.51 (m, 1H, H-2), 1.26 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.50 (N(C=O)O), 138.69 (Ph), 138.28 (Ph), 138.04 (Ph), 137.50 (Ph), 137.41 (Ph), 128.71 (Ph), 128.63 (Ph), 128.55 (Ph), 128.49 (Ph), 128.32 (Ph), 128.29 (Ph), 128.23 (Ph), 128.11 (Ph), 127.99 (Ph), 127.97 (Ph), 127.84 (Ph), 127.82 (Ph), 127.81 (Ph), 127.77 (Ph), 100.00 (C-1'), 95.75 (C-1''), 83.63 (C-4), 82.20 (C-3'), 81.61 (C-6), 80.04 (C-2'), 77.88 (C-4'), 77.83 (C-4''), 75.72 (Bn), 75.43 (C-2''), 75.20 (Bn), 74.69 (C-5), 74.24 (Bn), 73.69 (Bn), 73.63 (Bn), 71.39 (C-5'), 68.36 (C-6'), 65.07 (C-5''), 62.76 (C-3''), 60.22 (C-1), 58.91 (C-3), 32.19 (C-2), 30.70 (NMe), 23.31 (Me). HRMS (ESI, positive) calculated for $C_{55}H_{61}N_7O_{12}^{+}$ $[M + Na]^{+}$ 1034.4270, found 1034.4252.

(*3R,4R,5R,6R*)-5-(benzyloxy)-6-(((*1S,2R,3R,4S,6R*)-4,6-diazido-2-hydroxy-3-*(((2S,3-R,4S,5R,6R*)-3,4,5-tris(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2*H*-pyran-2-yl)-oxy)cyclohexyl)oxy)-3-methyl-4-(methylamino)tetrahydro-2*H*-pyran-3-ol (58): A

solution of 2 N aqueous sodium hydroxide (5 mL) was added to a solution of compound **57** (0.446 g, 0.441 mmol) in ethanol (15 mL). The reaction mixture was stirred at 90 ℃ for 10 h, and cooled to room temperature. The solution was diluted with ethyl acetate (25 mL) and washed with water (25 mL). The residue was washed with brine, dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel (4% methanol in dichloromethane) to give compound **58** (0.369 g, 85%) as a clear oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38–7.24 (m, 23H, Ph), 7.13–7.12(m, 2H, Ph), 5.33 (d, *J* = 3.5 Hz, 1H, H-1''), 5.27 (d, *J* = 3.7 Hz, 1H, H-1'), 4.94 (d, *J* = 11.0 Hz, 1H, Bn), 4.88 (d, *J* = 11.0 Hz, 1H, Bn), 4.85–4.79 (m, 3H, Bn), 4.75 (d, *J* = 11.3 Hz, 1H, Bn), 4.65 (d, *J* = 12.2 Hz, 1H, Bn), 4.53 (d, *J* = 1.7 Hz, 1H, OH), 4.50 (dd, *J* = 13.3, 11.4 Hz, 2H, Bn), 4.45 (d, *J* = 11.9 Hz, 1H, Bn), 4.10 (dt, *J* = 10.3, 2.5 Hz, 1H, H-5'), 4.02 (dd, *J* = 9.8, 9.1 Hz, 1H, H-3'), 3.94 (d, *J* = 12.4 Hz, 1H, H-5''), 3.81 (dd, *J* = 10.7, 3.1 Hz, 1H, H-6'), 3.74 (dd, *J* = 10.2, 9.0 Hz, 1H, H-4'), 3.69 (dd, *J* = 10.7, 2.1 Hz, 1H, H-6'), 3.64 (dd, *J* = 9.8, 3.7 Hz, 1H, H-2'), 3.62–3.57 (m, 1H, H-5), 3.56–3.47 (m, 2H, H-1, H-2''), 3.39 (t, *J* = 9.5 Hz, 1H, H-4), 3.31–3.27 (m, 3H, H-6, H-3, H-5''), 2.59 (d, *J* = 10.4 Hz, 1H, H-3''), 2.49 (s, 3H, NMe), 2.36 (dt, *J* = 12.9, 4.3 Hz, 1H, H-2), 1.56 (q, *J* = 12.6 Hz, 1H, H-2), 1.05 (s, 3H, Me). ^{13}C NMR (150 MHz, $CDCl_3$) δ 137.89, 128.79, 128.65, 128.58, 128.57, 128.52, 128.33, 128.30, 128.17, 128.14, 128.00, 127.86, 127.79, 100.26, 96.70, 83.98, 82.29, 81.14, 80.23, 77.90, 77.32, 76.13, 75.75, 75.24, 75.01, 74.43, 73.73, 72.23, 71.42, 71.15, 68.41, 66.15, 63.10, 60.42, 58.99, 32.37, 24.77. HRMS (ESI, positive) calculated for $C_{54}H_{63}N_7O_{11}^+$ $[M + H]^+$ 986.4658, found 986.4660.

2'-deamino-2'-hydroxy-gentamicin X_2 (59): Trimethylphosphine (1 M solution in THF, 0.66 mL, 0.66 mmol) was added to a solution of compound **58** (0.164 g, 0.166 mmol) in THF (6 mL) and 0.1 M NaOH (2 mL). The reaction mixture was stirred at 50 °C for 2 h,

cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic acid (1 mL), water (3 mL) and methanol (3 mL). The solution was deaerated by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.05 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (1-5% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **59** (0.067 g, 84%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.41 (d, *J* = 4.0 Hz, 1H, H-1'), 5.10 (d, *J* = 3.8 Hz, 1H, H-1''), 4.22 (dd, *J* = 10.9, 3.7 Hz, 1H, H-2''), 4.02 (d, *J* = 12.9 Hz, 1H, H-5''), 3.96–3.88 (m, 2H, H-6', H-5), 3.88–3.80 (m, 2H, H-5', H-6), 3.80–3.73 (m, 2H, H-3', H-4), 3.73–3.66 (m, 2H, H-2', H-6'), 3.66–3.57 (m, 2H, H-1, H-3), 3.54 (d, *J* = 10.8 Hz, 1H, H-3''), 3.49 (d, *J* = 12.9 Hz, 1H, H-5''), 3.39 (dd, *J* = 10.1, 9.0 Hz, 1H, H-4'), 2.93 (s, 3H, NMe), 2.55 (dt, *J* = 12.6, 4.3 Hz, 1H, H-2), 1.93 (q, *J* = 12.6 Hz, 1H, H-2), 1.35 (s, 3H, Me). ^{13}C NMR (125 MHz, D_2O) δ 100.99, 98.48, 83.06, 80.71, 73.06, 72.90, 72.50, 71.09, 69.88, 69.47, 67.60, 66.30, 63.30, 60.64, 49.71, 48.30, 34.46, 27.62, 20.87. HRMS (ESI, positive) calculated for $C_{19}H_{37}N_3O_{11}^+$ $[M + Na]^+$ 506.2320, found 506.2328.

2.3. RESULTS AND DISCUSSION

2.3.1. Gene Analysis

GenK is assigned to be a Cbl-dependent radical SAM enzyme based on gene analysis of *Micromonospora echinospora*.[77] A sequence analysis and comparison with other putative Cbl-dependent radical SAM sp^3-carbon methyltransferases is shown in Figure 2-1. The absence of a conserved histidine residue within the Cbl-binding domain indicates that the corrin cobalt is probably coordinated to dimethylbenzimidazole. This is in contrast to methionine synthase, methyl malonyl-CoA mutase, glutamate mutase and others in which histidine in a conserved $DXHX_2G$ motif serves as the lower axial ligand for the corrinoid cobalt.[121] The sequence alignment also suggests that Cbl-dependent radical SAM sp^3 *C*-methyltransferases harbor a single [4Fe-4S] cluster per monomer as only the cysteine residues comprising the CX_3CX_2C motif (highlighted in grey) are universally conserved in this class of enzymes.

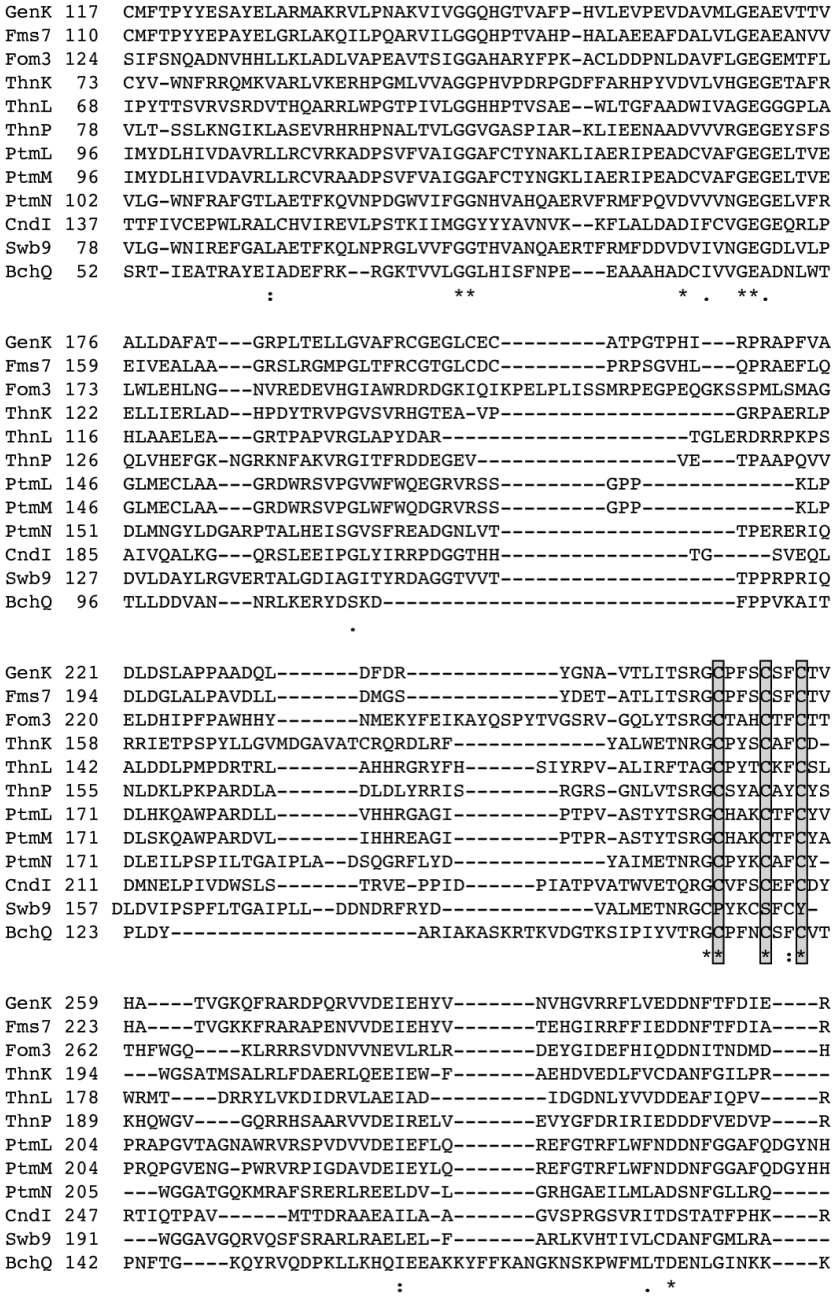


Figure 2-1. Partial amino acid sequence alignment of demonstrated and putative Cbl-dependent radical SAM sp^3-carbon methyltransferases. GenK (gi|85814024) from *Micromonospora echinospora*, Fms7 (gi|1125024) from *Micromonospora olivasterospora*, Fom3 (gi|196166519) from *Streptomyces fradiae*, ThnK (gi|30577688), ThnL (gi|30577689), and ThnP (gi|30577693) from *Streptomyces cattleya,* PtmL (gi|212379254), PtmM (gi|212379255), and PtmN (gi|212379256) from *Streptomyces pactum*, CndI (gi|223940938) from *Chondromyces crocatus*, Swb9 (gi|283131230) from

Streptomyces sp. SNA15896, and BchQ (gi|21674591) from *Chlorobium tepidum* TLS are involved in the biosynthesis of gentamicin, fortimicin, fosfomycin, thienamycin, pactamycin, chondrochlorens, quinomycin, 2-methylhopanoid, and bacteriochlorophyll biosynthesis, respectively.

2.3.2. Purification of GenK

The *genK* gene was amplified from the genomic DNA of *M. echinospora* using polymerase chain reaction (PCR) and was cloned into a pET24 vector. The recombinant *genK* was heterologously overexpressed in *Escherichia coli*. Although the expression of GenK was quite good, the protein was obtained only as insoluble inclusion bodies. It was therefore subjected to denaturation with 5 M urea and the refolding protocol described in Section 2.2.3. The refolded GenK was then incubated with $FeCl_3$ and Na_2S under anaerobic conditions in order to reconstituted [4Fe-4S] cluster unless otherwise stated. In some cases, MeCbl was added during reconstitution. Unbound iron and sulfide was removed by gel filtration. The purity of refolded, reconstituted GenK was assessed by SDS-PAGE (Figure 2-2).

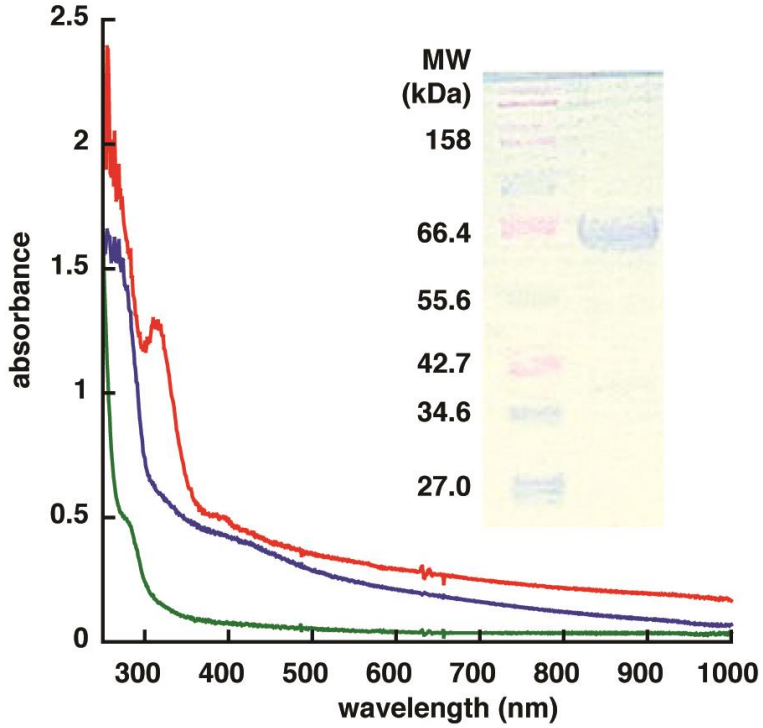


Figure 2-2. UV-visible absorbance spectra and SDS PAGE of isolated GenK. The green, blue, and red spectra correspond to as-isolated GenK (8 mM), reconstituted GenK (15 mM), and reconstituted GenK (15 mM) treated with 1 mM sodium dithionite. The bleaching of the absorbance shoulder at 420 nm is characteristic of a bound iron-sulfur cluster. The calculated molecular weight of GenK is 69.3 kDa.

The UV-visible spectrum of reconstituted GenK and the bleaching of the characteristic absorbance shoulder at 420 nm in the presence of sodium dithionite are consistent with the presence of a bound [4Fe-4S] cluster. Further analysis revealed that 3.5 ± 0.9 equivalents of iron and 3.8 ± 1.1 equivalents of sulfide are bound to each GenK monomer, implying that GenK contains a single [4Fe-4S] cluster as predicted by the amino acid sequence alignment.

2.3.3. *In vitro* Activity Assays of GenK

The activity of GenK was assayed using 1 mM commercially available Gentamicin X_2 as the substrate in 50 mM Tris•HCl buffer (pH 8.0) containing 10 mM DTT, 4 mM SAM, and 1 mM cobalamin (Cbl). NADPH (4 mM) and methyl viologen (MV, 1mM) were employed as the electron source and mediator, respectively, to reduce the GenK [4Fe-4S] cluster to the active +1 redox state. Methylcobalamin (MeCbl) or hydroxocobalamin (HOCbl) were usually present in the GenK assay, but other cobalamins also supported GenK activity. The incubation time was usually 8 h, however, assays to check for the presence of active enzyme or assess cobalamin dependency had different times as discussed below. The assay concentration of GenK was typically 0.01 mM unless otherwise states.

2.3.4. Products of GenK Catalysis

The GenK-catalyzed reaction was monitored using reversed-phased HPLC and two different elution programs: one to detect the formation of 5'-dAdo and SAH and another to detect the conversion of $GenX_2$ to G418. As aminoglycosides do not have any UV-active chromophore, the amino moieties of $GenX_2$ and G418 were derivatized with 1-fluoro-2,4-dinitorobenzene (FDNB) allowing detection at 340 nm. HPLC traces monitoring the formation of the conversion of $GenX_2$ to G418 catalyzed by GenK are shown in Figure 2-3. HPLC traces monitoring the formation of 5'-dAdo and SAH formation as co-product during turnover are shown in Figure 2-4. Control assays without cobalamin, without substrate, without enzyme, or with non-reconstituted enzyme were also conducted. In the presence of either MeCbl or HOCbl (but not without Cbl), GenK catalyzes methylation of $GenX_2$ to produce G418 (traces b and c in Figure 2-3) as well as the formation of 5'-Ado and SAH from SAM (traces b and c in Figure 2-4). Reconstitution of GenK with iron and sulfide is required to obtain activity (trace h in

Figure 2-3 and Figure 2-4). The presence of SAH in assays without GenK indicates that a notable extent of Cbl methylation takes place non-enzymatically under the conditions employed even when MeCbl is used as substrate (traces d, e, and f in Figure 2-4).[164] This suggests that the C-Co bond of MeCbl is prone to non-enzymatic cleavage during the assay (see Section 3.3.4).

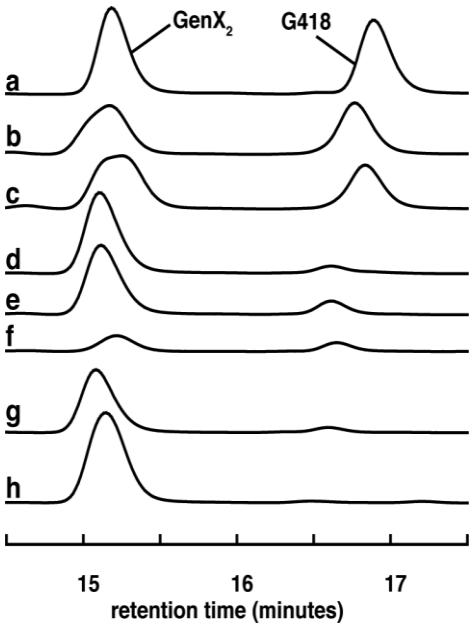


Figure 2-3. HPLC traces showing conversion of $GenX_2$ to G418 in the presence of GenK. The small peaks at ~16.6 min in traces d-g and ~15.4 min in trace f are unrelated to product and substrate. Trace (a) is a standard composed of derivatized, authentic $GenX_2$ and G418, reaction mixtures. The other HPLC traces correspond to reaction mixtures containing (b) MeCbl, (c) HOCbl, (d) MeCbl but no GenK, (e) HOCbl but no GenK, (f) MeCbl but no $GenX_2$, (g) no Cbl, (h) MeCbl and non-reconstituted GenK.

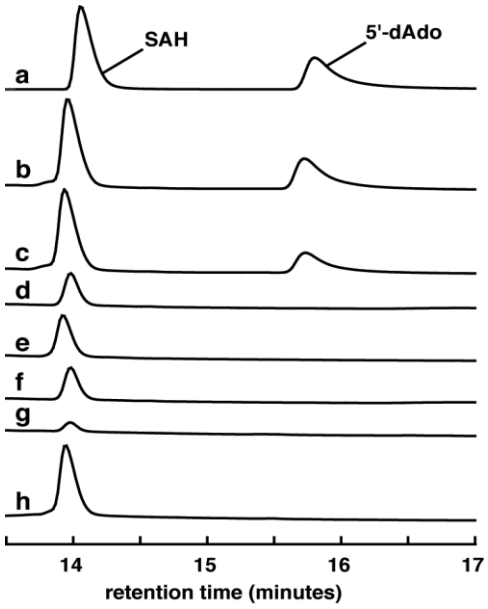


Figure 2-4. HPLC traces showing the production of both 5′-dAdo and SAH during the GenK reaction. Trace (a) is a standard composed of authentic 5′-dAdo and SAH. The other HPLC traces correspond to reaction mixtures containing (b) MeCbl, (c) HOCbl, (d) MeCbl but no GenK, (e) HOCbl but no GenK, (f) MeCbl but no GenX_2, (g) no Cbl, (h) MeCbl and non-reconstituted GenK. Detector was set at 260 nm.

2.3.5. Reducing Systems for GenK

In order to check which reducing systems can reduce the $[4Fe\text{-}4S]^{2+}$ cluster of GenK to active $[4Fe\text{-}4S]^{1+}$ state, various reducing systems including *E. coli* flavodoxin (fld)/flavodoxin reductase (fpr)/NADPH, methyl viologen (MV)/NADPH, benzyl viologen (BV)/ NADPH, dithionite, dithionite/MV, and dithionite/BV were investigated (Figure 2-5). The reaction was monitored by the formation of SAH and 5'-dAdo (Although SAH is present in all traces, separate experiments demonstrated that it is formed non-enzymatically due to the methylation of reduced cobalamin under the reaction conditions employed). Among the experimental conditions, only MV and NADPH or the flavodoxin/flavodoxin reductase/NADPH (fpr/fld/NADPH) system were

capable of activating GenK. Although dithionite is commonly used to activate radical SAM enzymes for *in vitro* studies, it is no effective for GenK. Neither NADPH and benzyl viologen (BV), dithionite and MV, nor dithionite and BV are capable of activating GenK. The coordination of SO_2^- to the corrinoid cobalt may compromise the ability of dithionite to support multiple turnovers with Cbl-dependent radical SAM enzymes.

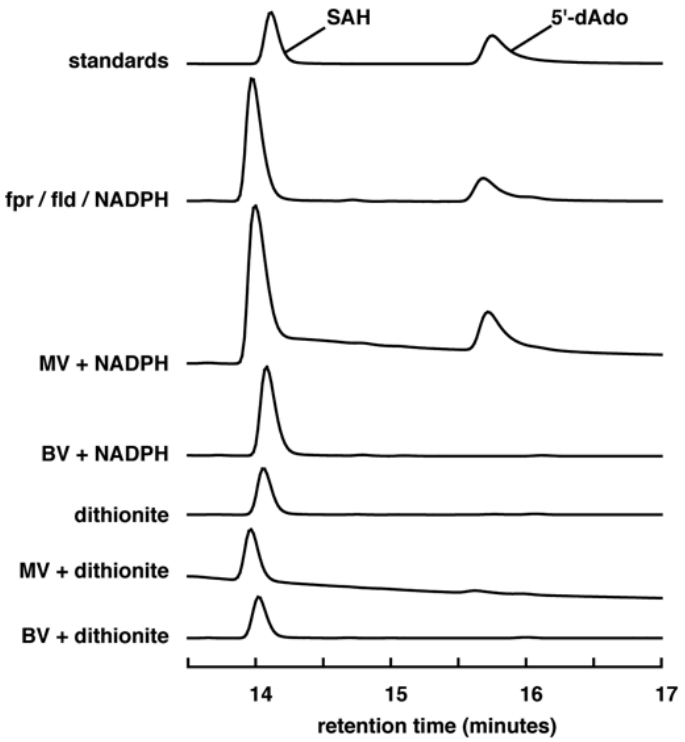


Figure 2-5. Effectiveness of various reducing systems for the activation of GenK. HPLC traces with UV detection at 260 nm for observation of SAH and 5′-dAdo are shown.

2.3.6. Stoichiometry of Products Catalyzed by GenK

In order to measure the quantity of 5'-dAdo and SAH produced by GenK, assays with different concentrations of the enzyme (2, 4, 6, 8, and 10 μM) over a time course (8, 12, 16, and 20 h) were conducted. Control reactions without GenK were used to assess the quantity of non-enzymatically produced SAH and this amount was subtracted out of

the enzyme assay results. The results indicate that that 5'-dAdo and SAH are produced in equal proportions (Figure 2-6). Also they show a linear relationship between the enzyme concentration and extent of product formation. Based on 8 h incubation, the average rate of both 5′-dAdo and SAH production was estimated to be approximately 0.02 min^{-1}.

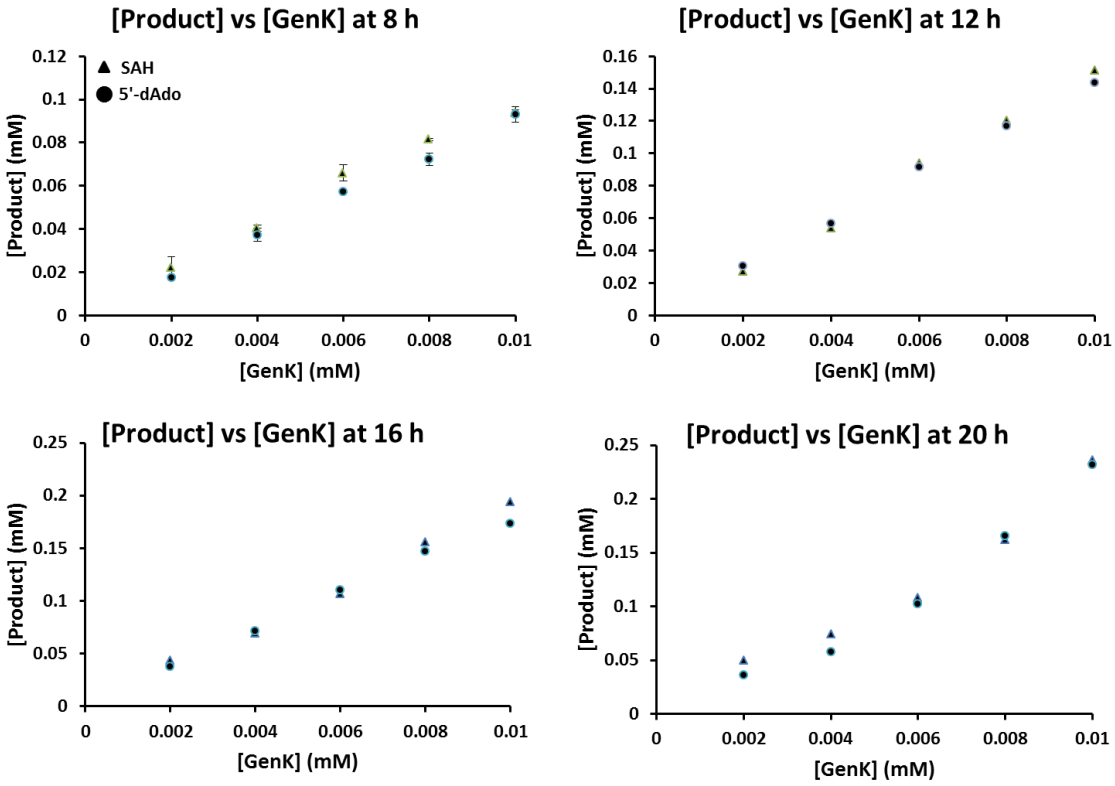


Figure 2-6. 5'-dAdo and SAH production vs GenK concentration at different times.

Figure 2-7 indicates the stoichiometry of product formation at 0.01 mM GenK over a time course. This figure also demonstrates that 5'-dAdo and SAH are produced in equal proportion. The relative amounts of 5'-dAdo and G-418 produced by GenK reaction were also measured. Quantification of 5'-dAdo from trace b and c in figure 2-4, and G418 in traces b and c in Figure 2-3, gives 0.49, 0.32, 0.49, and 0.40 mM, respectively,

demonstrating that 5'-dAdo and G418 are produced at a ratio close to 1:1. Combined with the previous data, 5'-dAdo, SAH and G418 are each produced in equivalent amounts.

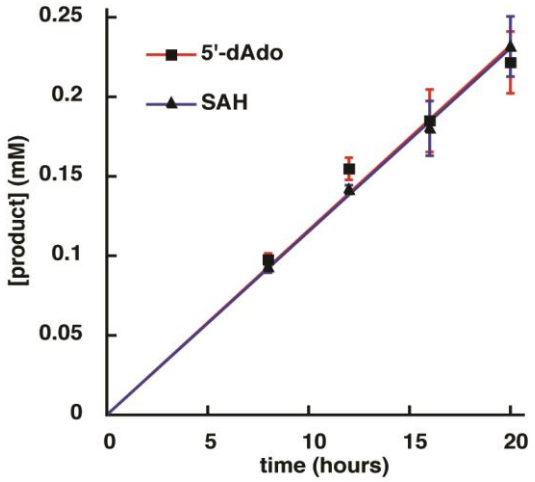


Figure 2-7. Stoichiometry of 5'-dAdo and SAH production during the GenK reaction

2.3.7. Methyl Transfer Manner of GenK Using Labeled SAM

In order to further investigate the role of cobalamin and the origin of the methyl group during the methyl transfer reaction, assays were conducted in the presence of either HOCbl or MeCbl and unlabeled SAM or $^{13}CD_3$-*methyl*-SAM followed by mass spectroscopic analysis of G418 and Cbl. The results are shown in Figures 2-8, 2-9, and 2-10. Figure 2-8 shows the mass spectra limited to the regions for substrate and product. And Figure 2-9 and Figure 2-10 display mass regions for the $[M + 2H]^{2+}$ and $[M + H]^{+}$ ions, respectively, of the cobalamin complexes. Mass spectra of cobalamin complexes appeared clearly $[M + 2H]^{2+}$ region rather than $[M + H]^{+}$ region. When unlabeled SAM was used as a substrate in the GenK reaction with HOCbl (trace a in Figure 2-8, 2-9 and 2-10) or MeCbl (trace e in Figure 2-8, 2-9 and 2-10), unlabeled G418 ($[M + H]^{+}$ *m/z*

497.28), $MeCbl^{2+}$ ($[M + H]^{2+}$ *m/z* 672.80), and $MeCbl^{+}$ ($[M + H]^{+}$ *m/z* 1344.58) were detected in the mass spectra. When $^{13}CD^{3}$-*methyl*-SAM was used with HOCbl, $^{13}CD_3$-labeled products were detected in both G418 ($[M + H]^{+}$ *m/z* 501.30) (trace b in Figure 2.8) and Cbl ($[M + H]^{2+}$ *m/z* 674.81, $[M + H]^{+}$ *m/z* 1348.57) (trace b in Figure 2-9 and 2-10). When $^{13}CD_3$-*methyl*-SAM was used with MeCbl (f), both $^{13}CD_3$-labeled and unlabeled G418 (trace f in Figure 2-8) and $^{13}CD_3$-labeled Cbl and unlabeled Cbl (trace f in Figure 2-9 and 2-10) were detected. As control reactions, assays without GenK were conducted (c, d, g, and h in Figure 2-8, 2-9 and 2-10). As expected, G418 was not obtained at all (trace c, d, g, and h in Figure 2-8), and $^{13}CD_3$-labelled or unlabeled MeCbl or HOCbl appeared (trace c, d, g, and h in Figure 2-9 and 2-10). These observations indicate that methylcobalamin can be generated from SAM and cobalamin and with or without the presence of the enzyme. Second, SAM is the methyl source for methylcobalamin is SAM, that is, the S-methyl substituent in SAM is transferred to Cbl. Third, combined with the previous results that methylation at $GenX_2$ does not occur without cobalamin (trace g in Figure 2-3 and Figure 2-4), the methyl group cannot be directly transferred from SAM to substrate to generate product. Fourth, the methyl group of product is from methylcobalamin because methylcobalamin was generated and the methyl group was transferred to $GenX_2$ to generate G418 when hydroxocobalamin instead of methylcobalamin was used in the GenK assay (trace a in Figure 2-8, 2-9, and 2-10). Altogether, methyl group is transferred from SAM to Cbl, and subsequently to $GenX_2$ to give G418.

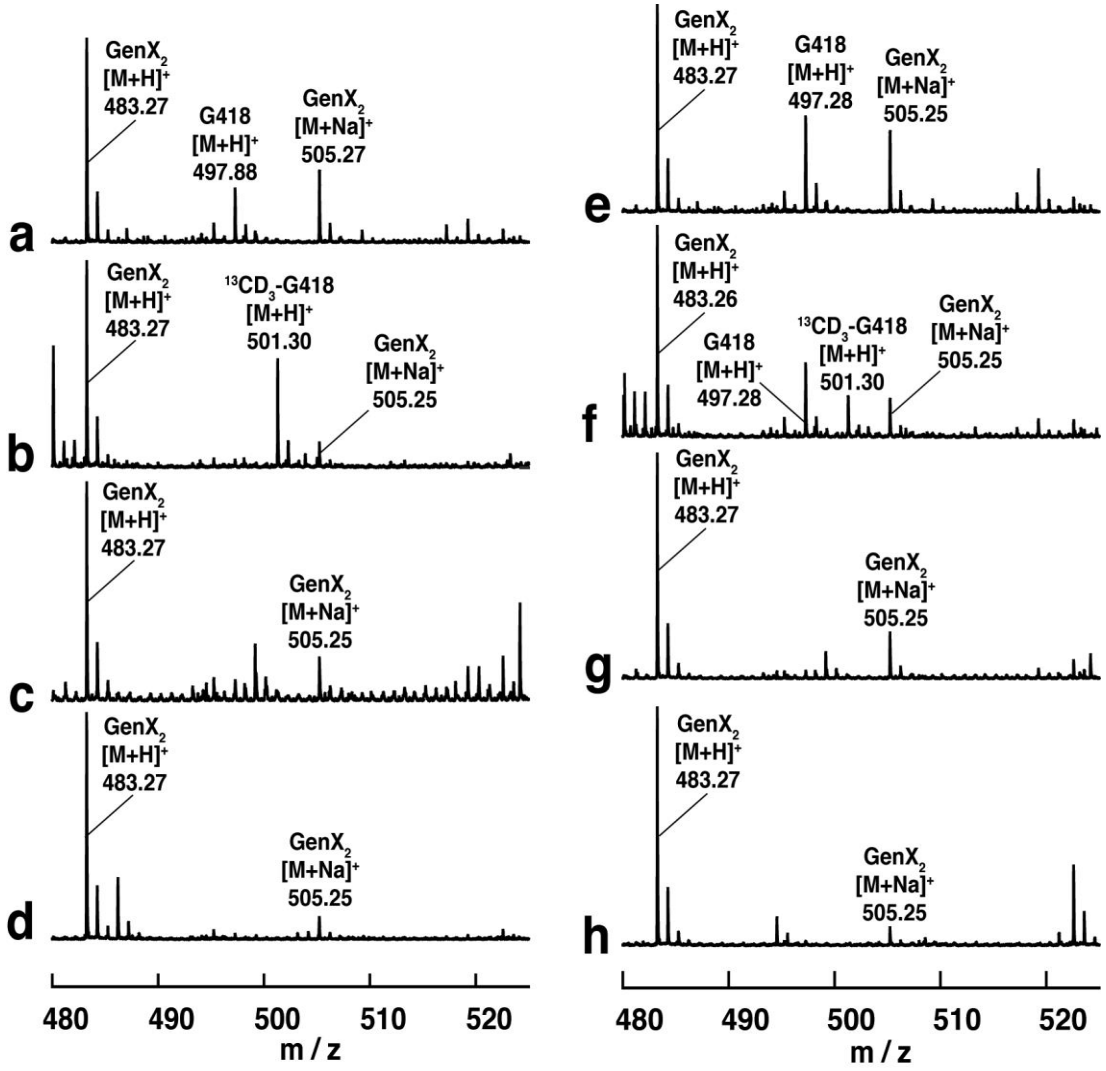


Figure 2-8. Mass spectra of $GenX_2$ and G418. The spectra correspond to assays with (a) HOCbl and unlabeled SAM, (b) HOCbl and $^{13}CD_3$-*methyl*-SAM, (c) HOCbl and unlabeled SAM without GenK, (d) HOCbl and $^{13}CD_3$-*methyl*-SAM without GenK, (e) MeCbl and unlabeled SAM, (f) MeCbl and $^{13}CD_3$-*methyl*-SAM, (g) MeCbl and unlabeled SAM without GenK, (h) MeCbl and $^{13}CD_3$-*methyl*-SAM without GenK.

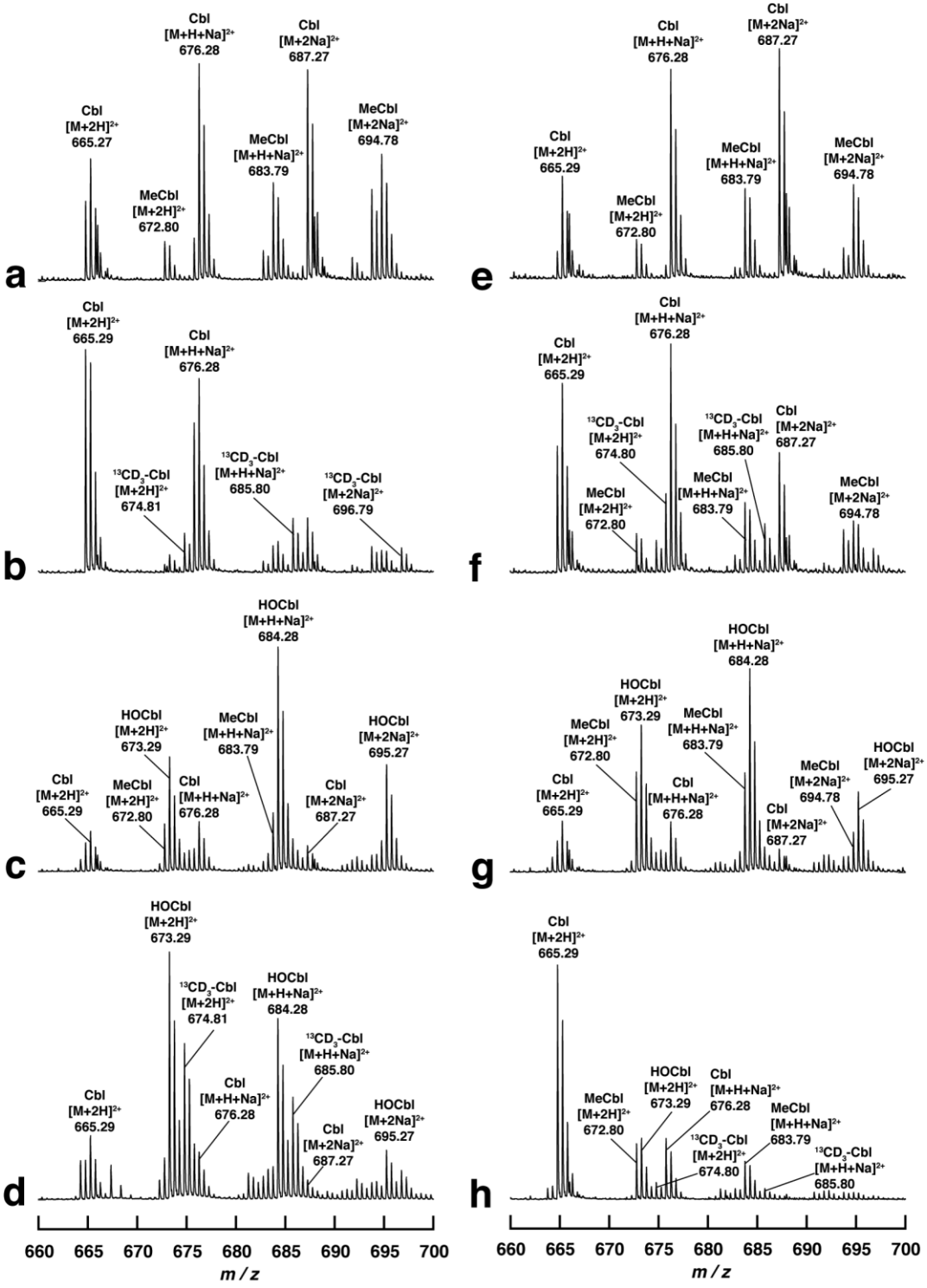


Figure 2-9. Mass spectra of the Cbl $[M + H]^{2+}$ region. Traces are described in the legend of Figure 2-10.

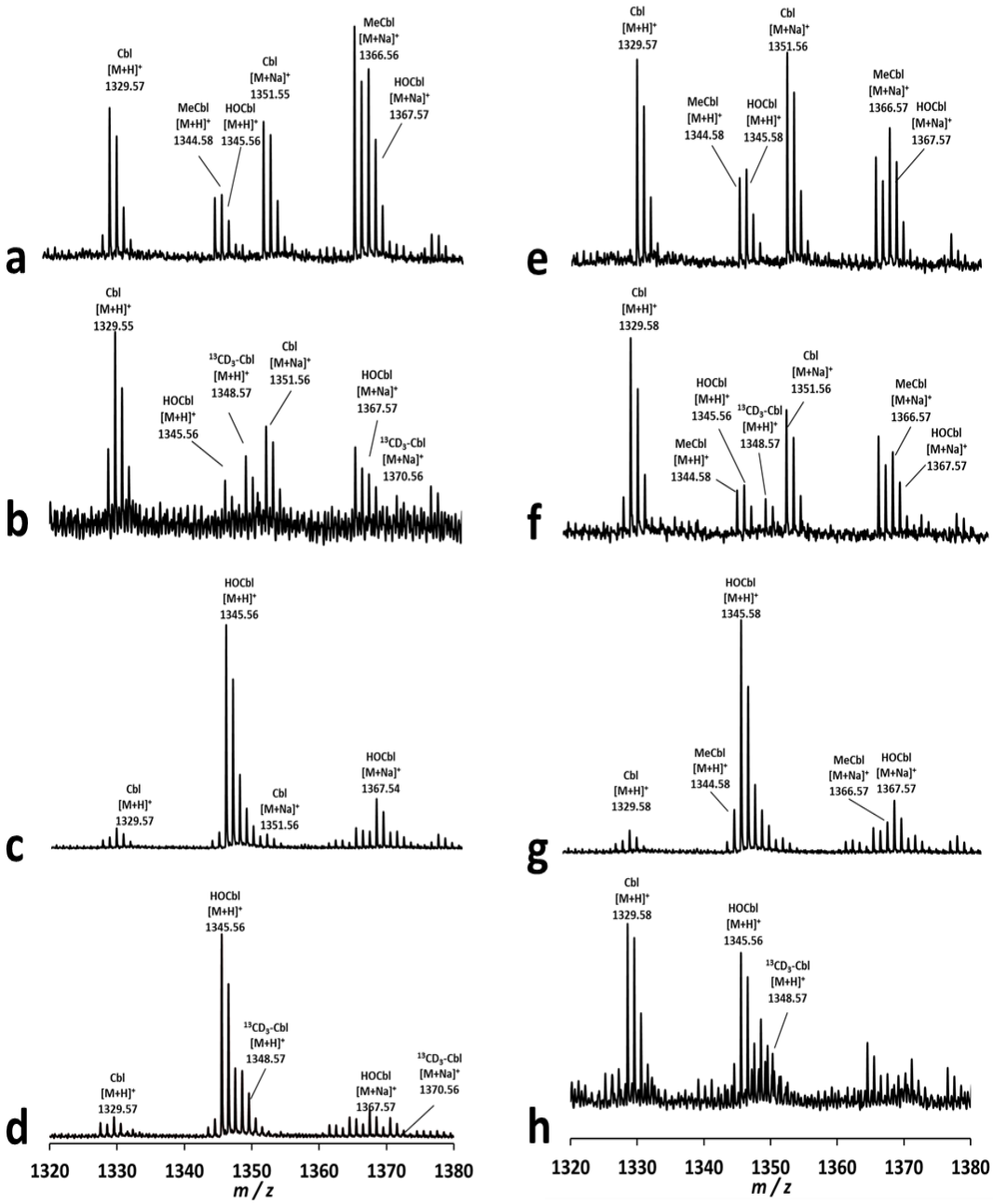


Figure 2-10. Mass spectra of the Cbl $[M + H]^+$ region. Traces are described in the legend of Figure 2-10.

2.3.8. Cobalamin Dependence of GenK Activity

In order to test whether any form of cobalamin will support GenK activity or only methylcobalamin is required for methylation reaction, methyl-, hydroxo-, cyano-, and adenosyl-cobalamin were considered in the assay. The formation of 5'-dAdo and SAH were monitored by HPLC (Figure 2-11). Assays with different types of cobalamin showed the formation of 5'-dAdo and SAH (traces c, d, e, and f in Figure 2-8). Combined with the previous results in Section 2.3.4 which the formation of 5'-dAdo is related to methylation on $GenX_2$ (Figure 2-3 and 2-4), these results indicates that any of these complexes can support GenK activity. However, there was no observable formation of 5'-dAdo and SAH in absence of any cobalamin (trace b in Figure 2-11).

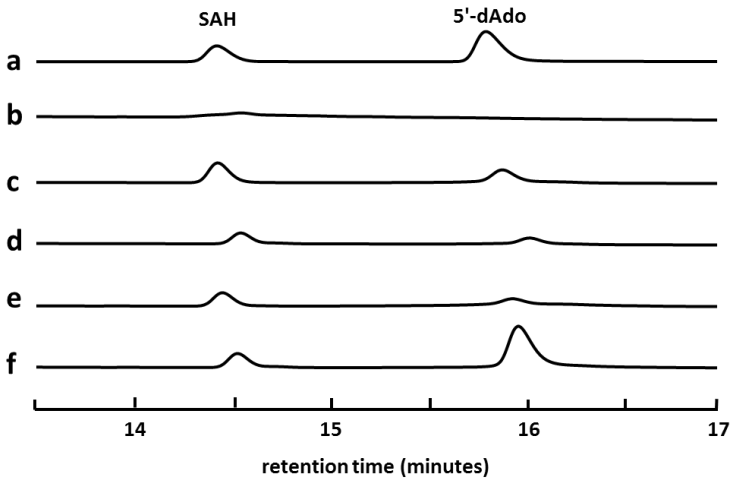


Figure 2-11. HPLC traces of GenK reaction in the presence of cobalamin. Trace (a) is a standard composed of authentic 5'-dAdo and SAH. The other HPLC traces correspond to reaction mixtures containing (b) no Cbl, (c) MeCbl, (d) HOCbl, (e) CNCbl, and (f) AdoCbl. Detector was set at 260 nm.

In order to check whether cobalamin is a cofactor and MeCbl is regenerated via methylation by SAM, assays with different concentrations of MeCbl were conducted at

various time points, 4, 8, 12 and 16 h (Figure 2-12). Considered that the formations of a product, G418, and 5'-dAdo are related (Section 2.3.6), G418 production is nearly maximized in the presence of any cobalamin (as same amount as the substrate $GenX_2$). This observation shows that cobalamin is used as a co-substrate for the GenK reaction.

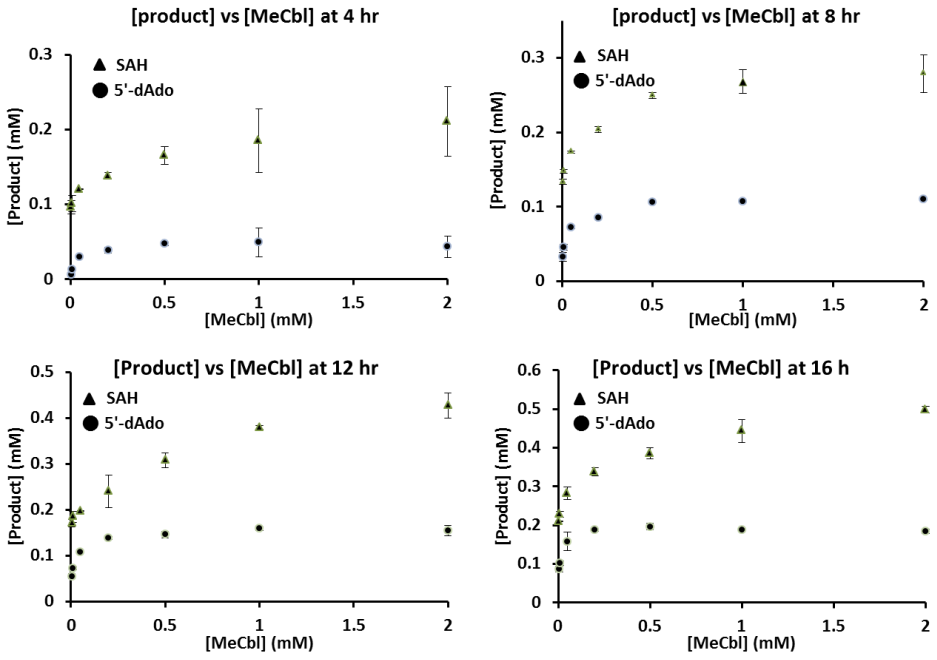


Figure 2-12. GenK activity vs concentrations of [MeCbl].

2.3.9. Syntheses of Putative GenK Substrates

As shown in Scheme 2-9 in section 2.2.11 and Scheme 2-10 in section 2.2.12, syntheses of gentamicin A_2 and 6'-amino-6'-dehydroxy-gentamicin A_2 began from D-glucosamine. Acetylation of free alcohols and amino groups with acetic anhydride on pyridine, thiophenyl group introduction using boron trifluoride diethyletherate and basic hydrolysis with 2 N aqueous sodium hydroxide solution gave the triol **11**. Azide

interconversion from the amine used the diazotransfer reaction with triflic azide and copper sulfate in methanol and dichloromethane.[165] The primary alcohol was selectively protected with *tert*-butyl diphenylsilyl (TBDPS) group and secondary alcohols were protected with benzyl group. TBDPS group removal using tetrabutyl ammonium fluoride and *p*-methoxybenzyl (PMB) group protection on the primary alcohol gave compound **16**. Coupling with 2-streptamine derivative **4** with *N*-iodosuccinimide and trifluoromethanesulfonic acid in ether and dichloromethane at –20 °C gave pseudodisaccharide **17**. After basic hydrolysis of the acetyl group using sodium methoxide, another coupling reaction with the xylose derivative **8** provided the pseudotrisaccharide **19**. PMB group deprotection using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone gave the alcohol **20**. The azide groups were converted to amines using the Staudinger reaction.[157] Final deprotection of benzyl group by hydrolysis provided gentamicin A_2 in 2.5% yield over 14 steps.

From the alcohol **20**, an azide group was introduced via S_N2 type reaction using sodium azide and tetrabutylammonium iodide after tosylate group was introduced. Staudinger reaction and hydrogenation gave 6'-amino-6'-dehydroxy-gentamicin A_2 in 66% yield over 4 steps from the previous synthesized compound **20**.

As shown in Scheme 2-11 in Section 2.2.13 and Scheme 2-12 in Section 2.2.14, the syntheses of 2'-deamino-2'-hydroxy-gentamicin A_2 and 2'-deamino-2'-hydroxy-6'-amino-6'-dehydroxy-gentamicin A_2 started from β-D-glucose pentaacetate. The following steps in the syntheses were similar to the syntheses of gentamicin A_2 and 6'-amino-6'-dehydroxy-gentamicin A_2. The synthesis of 2'-deamino-2'-hydroxy-gentamicin A_2 was finished in 7.8% yield over 11 steps. The synthesis of 2'-deamino-2'-hydroxy-6'-amino-6'-dehydroxy-gentamicin A_2 was accomplished with 23% yield over 5 steps from the previously synthesized compound **33**.

As shown in Scheme 2-13 in Section 2.2.15, the synthesis of garosamine derivative **44** began from acidic hydrolysis using anhydrous hydrochloric acid (acetyl chloride in methanol) of gentamicin sulfate (a mixture of gentamicin C_{1A}, gentamicin C_1 and gentamicin C_2). The secondary amine was selectively protected with trichloroethyloxycarbonyl (Troc) group. Selective benzoylation of the secondary alcohol and replacement of the anomeric methoxy group with thioglycoside followed. Oxazolidinone ring formation via treatment with methylamine (33% $MeNH_2$ in EtOH) resulted in simultaneous removal of the benzoate to provide the compound **43**. Benzyl protection with benzyl bromide and sodium hydroxide in DMF gave the oxazolidinone donor **44** in 17.7% yield over 6 steps.

As shown in Scheme 2-14 in Section 2.2.16, the synthesis of gentamicin B started from the previously synthesized compound **45**. After coupling reaction with garosamine derivative **44** and *N*-iodosuccinimide and trifluoromethanesulfonic acid in dichloromethane under –78 °C, basic hydrolysis using 2 N aqueous sodium hydroxide to remove oxazolidinone ring followed. Staudinger reaction and hydrogenation gave gentamicin B in 41.3% yield over 4 steps.

As shown in Scheme 2-15 in Section 2.2.17, the synthesis of JI-20A started from pseudodisaccharide **17**. After PMB deprotection using DDQ, azide introduction, basic hydrolysis, coupling with garosamine derivative **44** with *N*-iodosuccinimide and silver trifluoromethanesulfonate in ether and dichloromethane at –10 °C gave pseudotrisaccharide **53**. Basic hydrolysis and final deprotections of azide and benzyl groups using the Staudinger reaction and hydrogenation furnished JI-20A in 21.2% yield over 8 steps.

As shown in Scheme 2-16 in Section 2.2.18, the synthesis of 2'-deamino-2'-hydroxy-gentamicin X_2 began from previously synthesized pseudodisaccharide **56**. The

following steps followed the same reactions as the synthesis of gentamicin B. The synthesis of 2'-deamino-2'-hydroxy-gentamicin X_2 was finished in 43.6% over 4 steps.

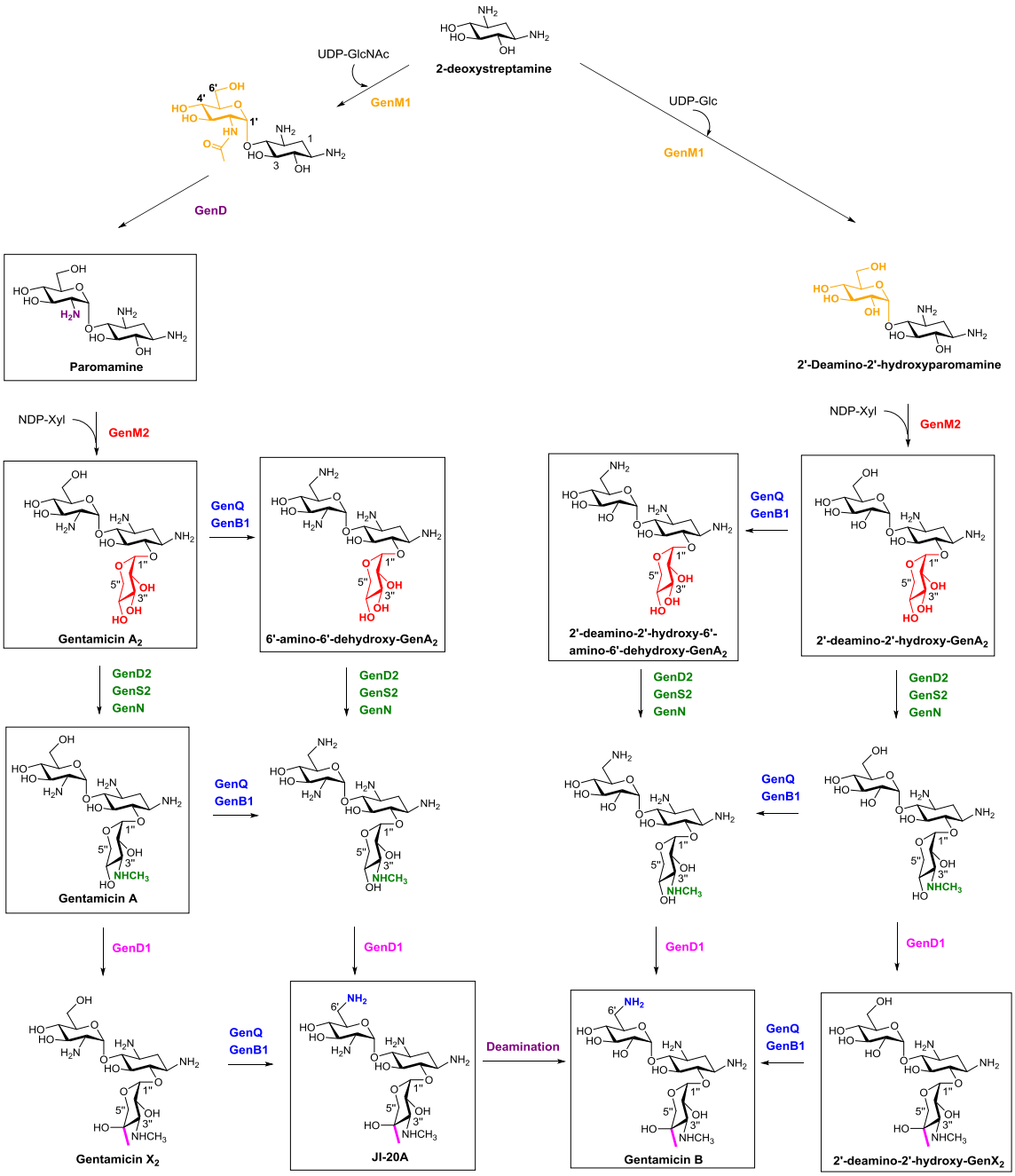


Figure 2-13. Early biosynthetic pathway for gentamicins.

2.3.10. Substrates Catalyzed by GenK

Members of class B RSMTs such as TsrM[128] and ThnK[132] can accept other compounds besides the intermediates in their biosynthetic pathway. Also previous experiments using mutation and reconstitution for target genes showed that GenK may catalyze methylation with gentamicin A_2 and gentamicin A.[81]

In order to probe the substrate specificity of GenK catalysis, nine intermediates from earlier steps in the biosynthetic pathway for gentamicin, paromamine, gentamicin A_2, 6'-amino-6'-dehydroxy-gentamicin A_2, 2'-deamino-2'-hydroxy-6'-amino-6'-dehydroxy-gentamicin A_2, 2'-deamino-2'-hydroxy-gentamicin A_2, gentamicin A, JI-20A, gentamicin B, 2'-deamino-2'-hydroxy-gentamicin X_2 (boxed compounds in Figure 2-13), and neamine were selected. Paromamine[166] and neamine[167] were obtained by acidic hydrolysis of paromomycin and neomycin.

GenK assays with various substrate homologues were conducted with 0.02 mM of GenK and 1 mM of substrate for 18 h (other conditions were the same as before). Formation of 5'-dAdo was followed by HPLC to check whether 5'-dAdo• can abstract a hydrogen atom from the putative substrates (Figure 2-14). HPLC traces of the incubation using pseudodisaccharides, paromamine and neamine, in the GenK assay did not show the appearance of the 5'-dAdo peak, indicating that GenK does not accept disaccharide as a substrate (trace b and c in Figure 2-14). GenK assays with eight pseudotrisaccharides showed the formation of 5'-dAdo, even though the majority of these compounds gave only small amounts of 5'-dAdo detected by HPLC (traces d–m in Figure 2-15). The relative proportions of 5'-dAdo formation compared to consumption of GenX_2 were 3.0% (gentamicin A_2), 6.5% (6'-amino-6'-dehydroxy-gentamicin A_2), 2.1% (2'-deamino-2'-hydroxy-6'-amino-6'-dehydroxy-gentamicin A_2), 7.6% (2'-deamino-2'-hydroxy-gentamicin A_2), 80.8% (gentamicin A), 29.0% (JI-20A), 3.1% (gentamicin B), and 6.5%

(2'-deamino-2'-hydroxy-gentamicin X_2). Except gentamicin A and JI-20A, other compounds showed very slow formation of 5'-dAdo.

Mass spectroscopic data shown in Figure 2-15 indicated that all eight compounds were converted to their methylated products by GenK. Even though the observed molecular ion peaks of these methylated products were weak, their masses were confirmed by HRMS (gentamicin A_2: calc. $[M + H]^+$ 470.2344, observed 470.2340, 6'-amino-6'-dehydroxy-gentamicin A_2: calc. $[M + H]^+$ 469.2504, observed 469.2502, 2'-deamino-2'-hydroxy-6'-amino-6'-dehydroxy-gentamicin A_2: calc. $[M + H]^+$ 471.2185, observed 471.2163, 2'-deamino-2'-hydroxy-gentamicin A_2: calc. $[M + H]^+$ 470.2344, observed 470.2343, Gentamicin A: calc. $[M + Na]^+$ 505.2480, observed 505.2491, JI-20A: calc. $[M + Na]^+$ 518.2796, observed 518.2794, gentamicin B: calc. $[M + H]^+$ 497.2817, observed 497.2816, 2-deamino-2'-hydroxy-gentamicin X_2: calc. $[M + H]^+$ 498. 2657, observed 497.2654). These data indicate that GenK can accept a variety of alternative substrates but substitutions at C2' or C6', or garosamine moiety have significant influence on GenK activity. GenX_2 remains the substrate-choice for GenK.

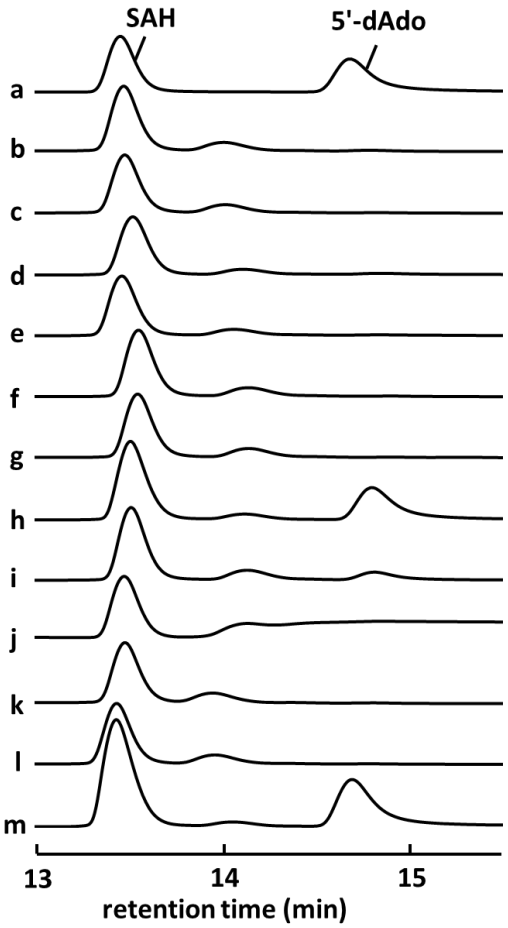


Figure 2-14. HPLC traces of GenK reaction with the following putative substrates; a) is corresponding to SAH and 5'-dAdo standard b) paromamine, c) neamine, d) $GenA_2$, e) 6'-amino-6'-dehydroxy-$GenA_2$, f) 2'-deamino-2'-hydroxy-6'-amino-6'-dehydroxy-$GenA_2$, g) 2'-deamino-2'-hydroxy-$GenA_2$, h) GenA, i) JI-20A, j) GenB, k) 2'-deamino-2'-hydroxy-$GenX_2$, l) without any substrate, and m) $GenX_2$..

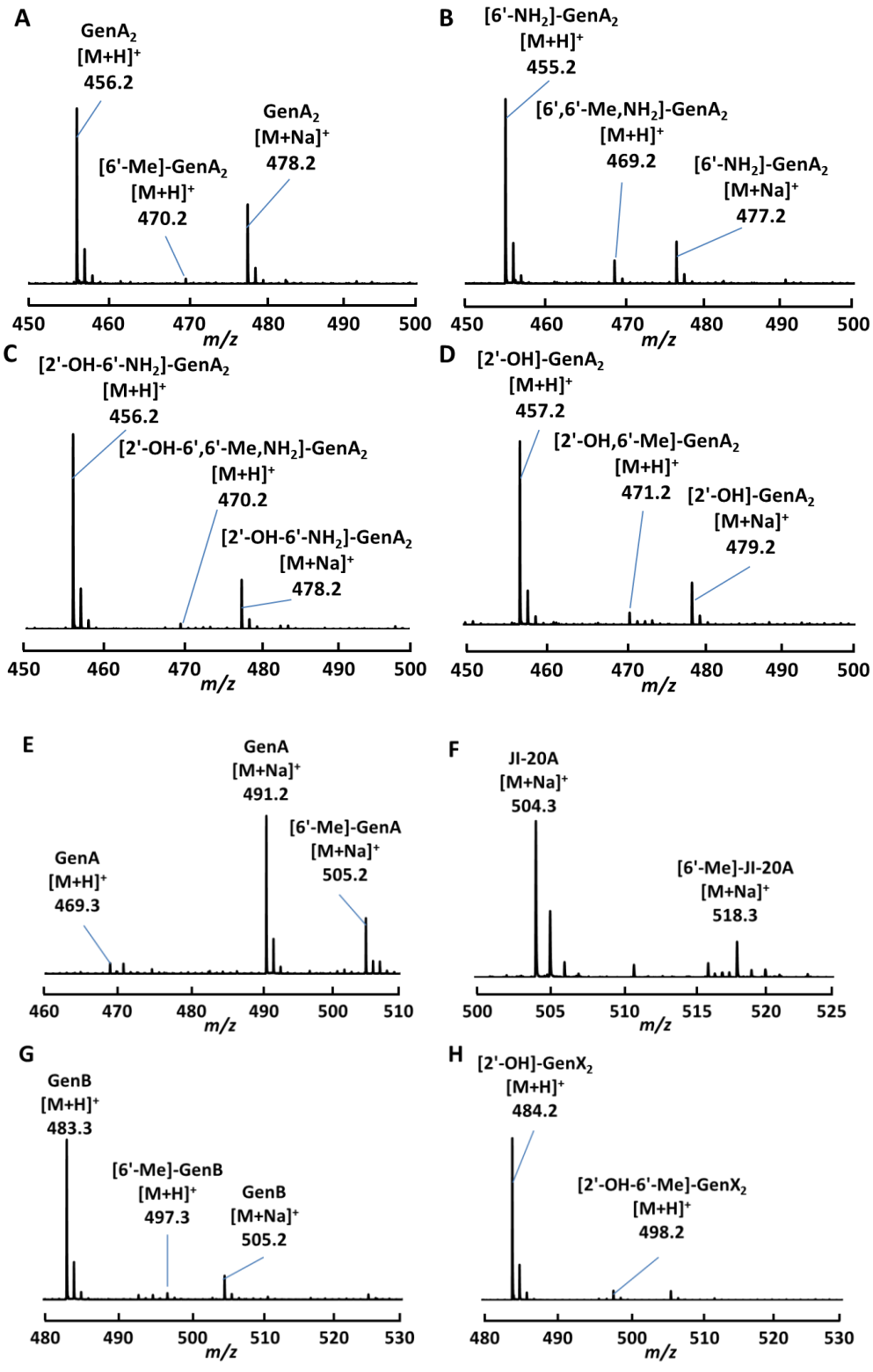

A
GenA2
[M+H]+
456.2
GenA2
[M+Na]+
478.2
[6'-Me]-GenA2
[M+H]+
470.2
450 460 470 480 490 500
m/z
B
[6'-NH2]-GenA2
[M+H]+
455.2
[6',6'-Me,NH2]-GenA2
[M+H]+
469.2
[6'-NH2]-GenA2
[M+Na]+
477.2
450 460 470 480 490 500
m/z
C
[2'-OH-6'-NH2]-GenA2
[M+H]+
456.2
[2'-OH-6',6'-Me,NH2]-GenA2
[M+H]+
470.2
[2'-OH-6'-NH2]-GenA2
[M+Na]+
478.2
450 460 470 480 490 500
m/z
D
[2'-OH]-GenA2
[M+H]+
457.2
[2'-OH,6'-Me]-GenA2
[M+H]+
471.2
[2'-OH]-GenA2
[M+Na]+
479.2
450 460 470 480 490 500
m/z
E
GenA
[M+Na]+
491.2
[6'-Me]-GenA
[M+Na]+
505.2
GenA
[M+H]+
469.3
460 470 480 490 500 510
m/z
F
JI-20A
[M+Na]+
504.3
[6'-Me]-JI-20A
[M+Na]+
518.3
500 505 510 515 520 525
m/z
G
GenB
[M+H]+
483.3
[6'-Me]-GenB
[M+H]+
497.3
GenB
[M+Na]+
505.2
480 490 500 510 520 530
m/z
H
[2'-OH]-GenX2
[M+H]+
484.2
[2'-OH-6'-Me]-GenX2
[M+H]+
498.2
480 490 500 510 520 530
m/z

Figure 2-15. Mass spectroscopic data of GenK reaction with A) $GenA_2$, B) 6'-amino-6'-dehydroxy-$GenA_2$, C) 2'-deamino-2'-hydroxy-6'-amino-6'-dehydroxy-$GenA_2$, D) 2'-deamino-2'-hydroxy-$GenA_2$, E) GenA, F) JI-20A, G) GenB, H) 2'-deamino-2'-hydroxy-$GenX_2$.

2.4. CONCLUSION

The existence of cobalamin (Cbl)-dependent enzymes that are members of the radical *S*-adenosyl-L-methionine (SAM) superfamily was previous predicted on the basis of bioinformatics analysis. A number of these are Cbl-dependent methyltransferases. In this Chapter, *in vitro* activity of GenK, a Cbl-dependent radical SAM enzyme that methylates an unactivated sp^3 carbon during the biosynthesis of gentamicin, an aminoglycoside antibiotic, was demonstrated. Experiments to investigate the stoichiometry of the GenK reaction revealed that 1 equiv each of 5'-deoxyadenosine and *S*-adenosyl-homocysteine are produced for each methylation reaction catalyzed by GenK. Furthermore, isotope-labeling experiments demonstrated that the *S*-methyl group from SAM is transferred to Cbl and the aminoglycoside product during the course of the reaction. The catalytic cycle of GenK, and likely other Cbl-dependent radical SAM methyltransferases, involves the consumptions of two SAM molecules and two reducing equivalents. The first molecule of SAM acts as the source of 5'-dAdo•, which abstracts a hydrogen atom from C6' of $GenX_2$ to give 5'-dAdo and the $GenX_2$ radical. The second SAM is used to methylate Cbl(I) during turnover to regenerate Me-Cbl for a subsequent round of catalysis. GenK does not only catalyze methylation of $GenX_2$, but also catalyzes the methylation of many aminoglycoside intermediates in the biosynthetic pathway of gentamicin even though the methylation rate is slower compared to the original substrate, $GenX_2$. These results demonstrate the promiscuity of GenK catalysis. Efforts to further

elucidate the chemical mechanism of this intriguing enzyme will be discussed in Chapter 3. Although it is a technical challenge to study Cbl-dependent radical SAM enzymes, this emerging class of biocatalysts offers considerable potential for the discovery of new and unprecedented enzyme chemistry.

Chapter 3: Mechanistic Studies of GenK

3.1. INTRODUCTION

Most methylation reactions in nature involve the transfer of the *S*-adenosyl-L-methionine (SAM) (**1**) methyl group to the target molecule through an S_N2 type reaction with a carbon, nitrogen, oxygen or sulfur anion serving as the nucleophile.[168] However, cases of enzyme-catalyzed methylation reaction involving a single electron transfer mechanism, referred to as radical SAM methyltransferases, are increasing.[115] In the introduction to Chapter 2, several examples of these enzymes were examined. The methyl transfer reaction for most radical SAM methyltransferases is initiated by single electron transfer from the reduced ion-sulfur cluster to the positively charged sulfonium moiety of SAM accompanied by homolysis of the C5'-S bond to generate methionine (**2**) and a 5'-deoxyadenosyl radical (5'-dAdo•) (**3**) (Figure 3-1).[101] This 5'-dAdo radical can then induce the subsequent methylation reaction by abstracting a hydrogen atom from a substrate to generate substrate radical, which then reacts with the methyl donor.[101]

The class B radical SAM methyltransferases contain a cobalamin (Cbl)-binding domain at the *N*-terminus and a characteristic Cx_3Cx_2C or similar sequence at the *C*-terminus, which are important for their activities.[115] Members of this class whose activity has been demonstrated *in vitro* include PhpK,[133-135] TsrM,[127-129] Fom3,[130-131] GenD1,[81] and ThnK.[132] Recent research has provided some insights into their mechanisms.

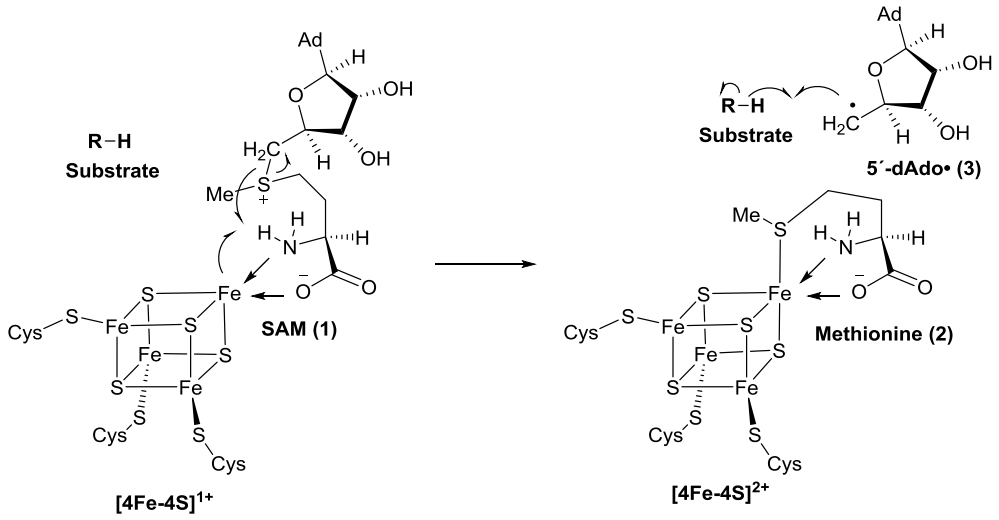


Figure 3-1. Initial reaction of radical SAM enzymes.

The mechanism of PhpK, the *P*-methyltransferase responsible for the formation of the second C-P bond in L-phosphinotricin, was explored by Wang *et al*.[133-135] They used a two-dimensional ^{1}H-^{31}P gradient heteronuclear single quantum correlation (gHSQC) nuclear magnetic resonance (NMR) experiment to determine that methylcobalamin (MeCbl) is a direct methyl donor.[133] They also performed a ^{13}C-^{31}P multiple quantum ^{1}H-^{13}C-^{31}P(HCP) experiment in ^{1}H-^{31}P two-dimensional mode directly on a PhpK-catalyzed reaction mixture using $^{13}CH_3$-labeled methylcobalamin as the methyl group donor. The observation of a 3:1:1:3 mutiplet in the spectrum suggested direct bond formation between $^{13}CH_3$ and ^{31}P.[135] These results indicated that the primary methyl donor is MeCbl in the reaction. However, they did not report the isolation of 5'-deoxyadenosine (5'-dAdo) (**4**) and/or other SAM cleavage products. Figure 3-2 shows two proposed mechanisms for PhpK catalysis.[135]

In Figure 3-2A, after hydrogen abstraction by 5'-dAdo•, the substrate radical obtains a methyl radical from MeCbl to yield the methylated product. The oxidation state of cobalamin is changed from Cbl(III) to Cbl(II). In Figure 3-2B, after reductive cleavage of SAM followed by reduction of phosphinate radical with one electron and one proton, a nucleophilic attack by the phosphorus anion on MeCbl(III) provides the methylated intermediate and Cbl(I). A final deprotonation reaction furnishes the product.

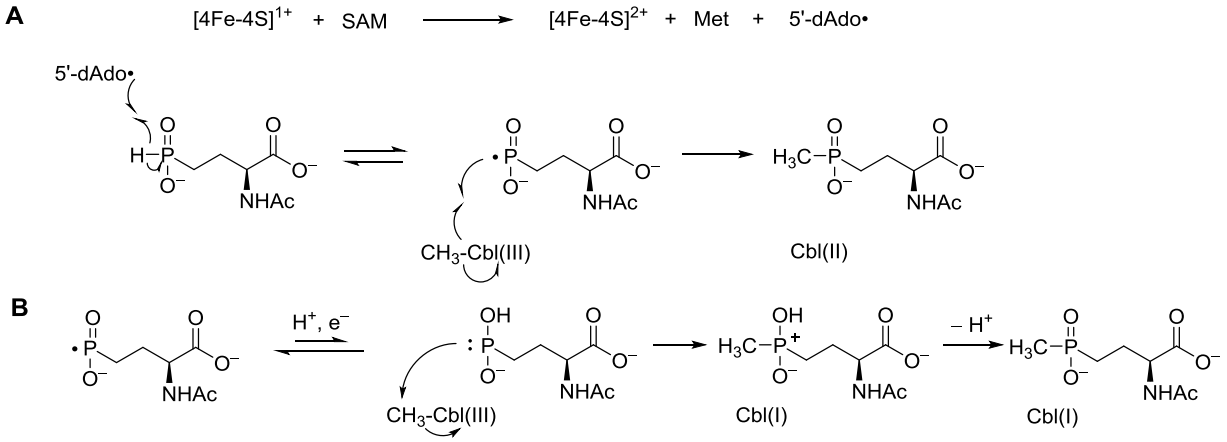


Figure 3-2. Proposed mechanisms for PhpK catalysis.

The mechanistic studies of tryptophan methyltransferase TsrM, involved in the biosynthesis of the thiopeptide antibiotic thiostrepton, have been done by Berteau *et al.*[127-128] Isotope-labeling experiments using [methyl-$^2H_3$]-SAM as the substrate showed the generation of [methyl-$^2H_3$]-2-methyltryptophan as a product, even when unlabeled MeCbl was used. These results suggest that the enzyme initially transfers a methyl group from SAM to cobalamin in the reaction. UV-visible spectroscopic monitoring of the reaction showed a slight increase in absorption between 400 and 476 nm and a decrease at 520 nm, which are indicative of the formation of Cbl(II). Unlike a number of radical

SAM enzymes, the TsrM reaction did not produce 5'-dAdo and did not require an external electron donor like dithionite. A mutant TsrM which lacks the canonical CxxxCxxC motif did not catalyze methylation of tryptophan, but it produced large amounts of *S*-adenosylhomocysteine (SAH) (**5**) and MeCbl. These results indicate that radical SAM chemistry was not required to initiate the methyl transfer reaction. The proposed mechanism for TsrM is shown in Figure 3-3.[127] Following transfer of the methyl radical to C2 of tryptophan, deprotonation occurs to give the observed methylated product. A one-electron transfer from the radical intermediate to the $[4Fe\text{-}4S]^{2+}$ cluster is proposed, and the electron is then transferred from the reduced $[4Fe\text{-}4S]^{1+}$ cluster to Cbl(II) to generate Cbl(I). The generated Cbl(I) attacks the electrophilic methyl group of SAM to regenerate MeCbl and form SAH (**5**).

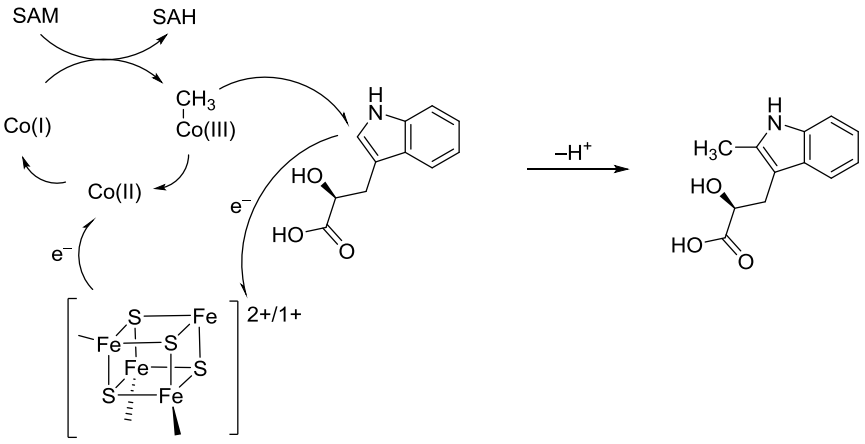


Figure 3-3. Proposed mechanism for TsrM catalysis.

Mechanistic studies concerning Fom3, a putative Cbl-dependent radical SAM enzyme which catalyzes the methylation of 2-hydroxyethylphosphonate (2-HEP) to produce 2-hydroxypropylphosphonate (2-HPP), have not yielded much insight. Wang *et*

al demonstrated through spectroscopic evidence using electron paramagnetic resonance (EPR) that Fom3 catalyzes methyl group transfer to 2-HEP.[130] The proposed mechanism of Fom3 is shown in Figure 3-4. Fom3 uses radical SAM chemistry to generate 5'-dAdo• (**3**) which abstracts a hydrogen atom from the C-2 position of 2-HEP to produce 5'-dAdo (**4**) and a substrate radical. The C-2 substrate radical reacts with MeCbl(III) to yield 2-HPP and Cbl(II).

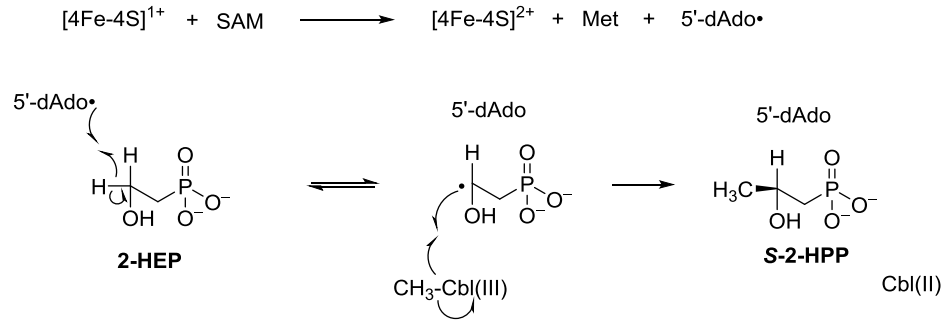


Figure 3-4. Hypothesized mechanism for Fom3.

The catalytic mechanism of GenD1, a Cbl-dependent methyltransferase which uses gentamicin A (GenA) to generate gentamicin X_2 (GenX_2) in the biosynthetic pathway of gentamicin C_1, has not yet been proposed. Leadlay and Sun *et al* detected the formation of the product and 5'-dAdo using reconstituted GenD1.[81] One result they found was that assays using hydroxocobalamin (HOCbl) instead of MeCbl also showed GenD1 activity, presumably demonstrating that MeCbl is regenerated by a methyltransfer process during catalysis. However, the formation of SAH was not observed. The mechanism of GenD1 proposed in Figure 3-5 is based on these results.

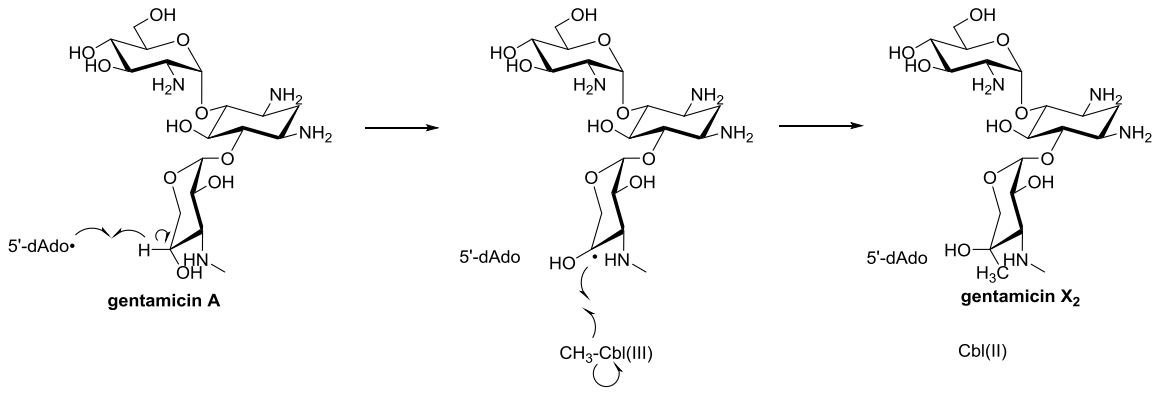


Figure 3.5. Proposed mechanism of GenD1.

ThnK, a Cbl-dependent methyltransferase which catalyzes the methylation required to form the C6-ethyl side chain in a stereocontrolled manner in thienamycin biosynthesis, was studied by Townsend *et al*.[132] They found near-equimolar amounts of SAH (**5**) and 5'-dAdo (**4**) during ThnK catalysis. Early labeling studies showed that the C-6 ethyl side chain of thienamycin originates from methionine.[169] An experiment using a chiral [^{1}H, ^{2}H, ^{3}H-methyl]-L-methionine showed that the absolute configuration of the terminal methyl in the C6 side chain is retained.[169] This result is consistent with an overall methyl transfer processes involving two inversions and a MeCbl intermediate. The proposed mechanism of ThnK is shown in Figure 3.6.

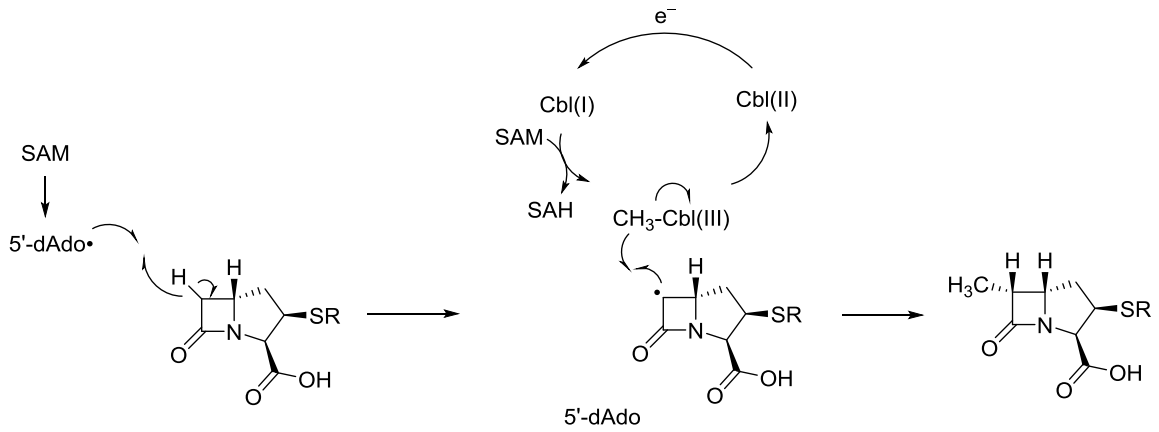


Figure 3-6. Proposed mechanism of ThnK.

Class B radical SAM methyltransferases potentially utilizes two molecules of SAM. The 5'-dAdo• (**3**) from one SAM initiates the methylation reaction, and the other SAM acts as the methyl source. Cobalamin is an intermediate methyl group carrier between SAM and the substrate. The general mechanism of class B radical SAM methyltransferases is shown in Figure 3-7.

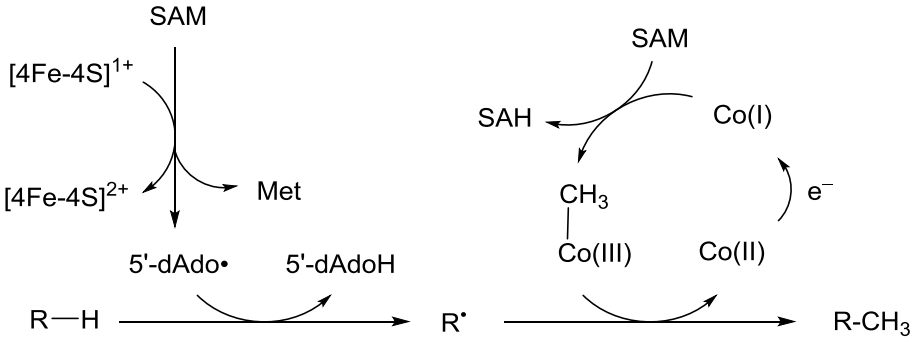


Figure 3-7. Proposed mechanism of Class B radical SAM methyltransferases.

Several mechanisms can be envisioned for the GenK-catalyzed conversion of $GenX_2$ (**6**) to G418 (**7**). In this chapter, possible GenK reaction mechanisms are proposed,

and several attempts to elucidate the chemical mechanism of this enzyme are reported. These experiments involve analysis by UV-visible spectroscopy, assays using isotope-labeled compounds, and mechanistic investigation using various substrate analogs.

3.2. Experimental Procedures

3.2.1. Materials and Equipment

All chemicals were purchased from Fisher Science (Pittsburgh, PA) or Sigma-Aldrich (St. Louise, MO) and used without further purification unless otherwise specified. DNA gel extraction and spin minipreps were obtained from Qiagen (Valencia, CA). All reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Amicon and Microcon YM-10 filtration products were purchased from Bio-Rad (Hercules, CA) and Millipore (Billerica, MA), respectively. *Escherichia coli* DH5*a* cells were bought from Bethesda Research Laboratories (Muskegon, MI). The vector pET24b (+) and enzyme KOD DNA polymerase were purchased from Novagen (Madison, WI). DNA modifying enzymes (for restriction digestion and ligation), PCR primers, and the overexpression host *E. coli* BL21 star (DE3) were acquired from Invitrogen (Carlsbad, CA) and New England Biolabs (NEB, Beverly, MA). Luria Bertani (LB) media are products of Difco (Detroit, MI) or Fisher Scienctific (Pittsburgh, PA). Pre-stained protein markers were purchased from NEB. Protein concentrations were determined by Bradford Assay[149] using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using SDS-PAGE. The general methods and protocols for recombinant DNA manipulations are as described by Sambrook *et al*.[150] DNA sequencing was performed at the Core Facilities of the Institute of Cellular and Molecular Biology, University of Texas at Austin. All

reactions involving GenK were performed in a Coy Anaerobic Chamber (glovebox) under an atmosphere of approximately 97.5% N_2 and 2.5% H_2 with less than 1 ppm O_2. All solvents used for GenK assays were deaerated by bubbling with nitrogen gas before they were transferred into the glovebox. The solvents in the glove box were stirred open to the anaerobic atmosphere overnight to allow equilibration before use. Gentamicin X_2, G-418, and GenA were purchased from TOKU-E (Bellingham, WA). Tetrahydrofuran (THF) was distilled from sodium benzophenone and dichloromethane (CH_2Cl_2) was distilled from calcium hydride under a nitrogen atmosphere. Other anhydrous solvents were purchased from Acros Organics (Pittsburgh, PA). Analytic thin layer chromatography (TLC) was performed on pre-coated TLC glass plates (Silica gel, grade 60, F254, 0.25 mm layer thickness) purchased from EMD chemicals (Madison, WI). Flash column chromatography was carried out on silica gel (230-400 mesh, grade 60) from Sorbent Technologies (Atlanta, GA) by eluting with the specific solvents. ^1H and ^{13}C NMR spectra were recorded at 400, 500, 600 MHz and 100, 125, 150 MHz with a Varian Gemini spectrometer. Chemical shifts are reported as parts per million (ppm) relative to the deuteriochloroform ($CDCl_3$), 7.26 ppm for ^1H NMR and 77.16 ppm for ^{13}C NMR, respectively. Visualization was effected with *p*-phosphoryl molybdic acid solution. The MS analyses were carried out at the Mass Spectrometry and Proteomics Facility of the Department of Chemistry, University of Texas at Austin.

3.2.2. Cloning, Expression, Refolding and Reconstitution of GenK

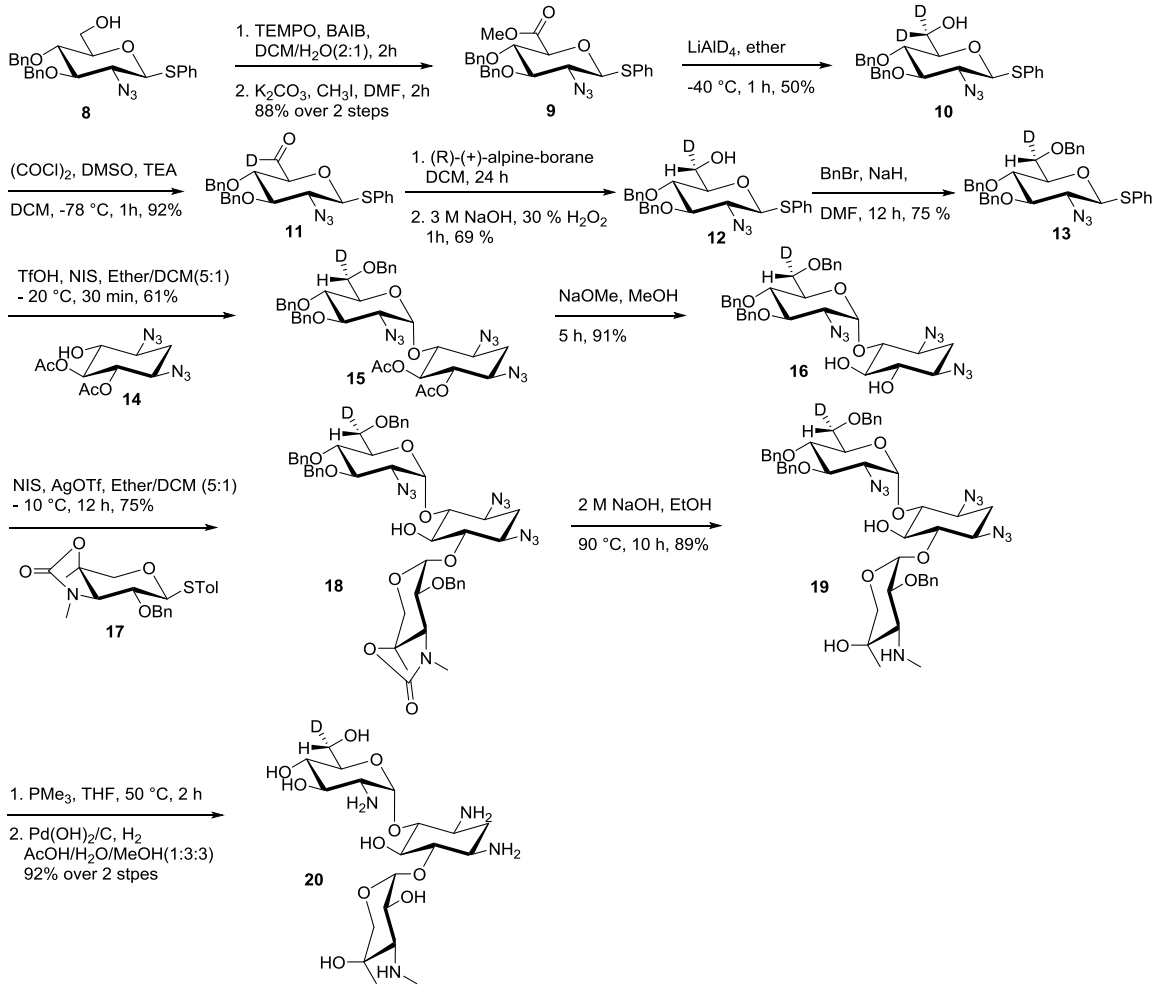
Cloning, expression, refolding and reconstitution of GenK were identical to the previous methods in Sections 2.2.2 and 2.2.3.

3.2.3. GenK Activity Assays, HPLC detection of SAH, 5'-dAdo, Aminoglycosides, and Mass Spectroscopic Analysis of GenK Activity

GenK activity assays, HPLC detection of SAH, 5'-dAdo and aminoglycosides, and mass spectroscopic analysis of GenK activity were conducted by following the previous methods outlined in Sections 2.2.5, 2.2.6, 2.2.7, and 2.2.8.

3.2.4. Synthesis of [6'*S*-^{2}H]-Gentamicin X_2 (20)

The overall synthetic scheme is shown in Scheme 3-1.



Scheme 3-1. Synthetic scheme for the preparation of [6'*S*-^{2}H]-GenX$_2$ (**20**).

Methyl(*2S,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-carboxylate (9): (2,2,6,6,-Tetramethylpiperidin-1-yl)oxyl (TEMPO) (0.025 g, 0.16 mmol) and (diacetoxyiodo)benzene (0.646 g, 2.01 mmol) were added to a solution of compound **8** (0.384 g, 0.80 mol) in dichloromethane (10 mL) and water (10 mL). The reaction mixture was stirred at room temperature for 2 h, and 10% aqueous solution of sodium sulfite (30 mL) was added to quench the reaction. The two layers were separated, and the aqueous layer was extracted with ethyl acetate (30 mL × 3). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude acid was dissolved in dimethylformamide (20 mL). Potassium carbonate (0.333 g, 2.41 mmol) and iodomethane (0.10 mL, 1.61 mmol) were added to the solution. The reaction mixture was stirred at room temperature for 12 h, and concentrated under reduced pressure. The residue was diluted with dichloromethane (30 mL) and washed with saturated aqueous solution of ammonium chloride (30 mL × 3). The combined aqueous solution was extracted with dichloromethane (40 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1 ~ 2:1) to provide compound **9** (0.357 g, 88%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71–7.63 (m, 2H, Ph), 7.45–7.33 (m, 11H, Ph), 7.32–7.29 (m, 2H, Ph), 4.91 (d, *J* = 1.3 Hz, 2H, 2×Bn), 4.82 (d, *J* = 10.9 Hz, 1H, Bn), 4.66 (d, *J* = 10.9 Hz, 1H, Bn), 4.50 (d, *J* = 10.1 Hz, 1H, C-1), 3.97 (d, *J* = 9.8 Hz, 1H, H-5), 3.82 (dd, *J* = 9.8, 9.0 Hz, 1H, H-4), 3.79 (s, 3H, OMe), 3.59 (t, *J* = 9.2 Hz, 1H, H-3), 3.43 (dd, *J* = 10.1, 9.3 Hz, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.29, 137.54, 137.40, 133.84, 130.57, 129.15, 128.70, 128.53, 128.46, 128.22, 128.10, 127.98, 127.87, 86.64, 84.17,

79.08, 78.06, 75.92, 75.09, 64.59, 52.57. HRMS (ESI, positive) calculated for $C_{27}H_{27}N_3O_5S^+$ $[M + Na]^+$ 528.1564, found 528.1564.

((*2R,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-yl)methan-d2-ol (10): Lithium aluminum deuteride (0.315 g, 7.52 mmol) was added to a solution of compound **9** (2.923 g, 5.78 mmol) in tetrahydrofuran (50 mL) at – 78 °C. The reaction mixture was stirred for 1 h and ethyl acetate (20 mL) was added. The solution was warmed to room temperature and water (10 mL) was added to quench the reaction. Diethyl ether (20 mL) and 10% aqueous solution of sodium hydroxide (5 mL) were added. The mixtures were filtered through a Celite pad and washed with diethyl ether (50 mL). The filtered solution was dried over sodium sulfate, filtered again, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to provide compound **10** (1.385 g, 50%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58–7.49 (m, 2H, Ph), 7.38–7.27 (m, 13H, Ph), 4.91–4.79 (m, 3H, 3×Bn), 4.64 (d, J = 11.0 Hz, 1H, Bn), 4.46 (dd, J = 10.2, 1H, H-1'), 3.58–3.46 (m, 2H, H-4, H-5), 3.40–3.29 (m, 2H, H-2, H-3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.75, 137.63, 133.64, 131.17, 129.29, 128.72, 128.69, 128.36, 128.22, 128.07, 86.23, 85.06, 79.61, 76.06, 75.27, 65.41. HRMS (ESI, positive) calculated for $C_{26}H_{25}D_2N_3O_4S^+$ $[M + Na]^+$ 502.1740, found 502.1741.

(*2S,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-carbaldehyde-d (11): Dimethyl sulfoxide (8.04 mL, 57.72 mmol) was added dropwise to a solution of oxalyl chloride (1.48 mL, 17.31 mmol) in dichloromethane (80 mL) at –78 °C. After stirring for 15 min, compound **10** (5.534 g, 11.54 mmol) in dichloromethane (30 mL) was added dropwise. After stirring for 45 min, trimethylamine (8.04 mL, 57.72 mmol) was added slowly. After stirring for 20 min, the solution was warmed to room temperature, and the reaction mixture was stirred for additional 1 h. The reaction was

quenched by adding H_2O (100 mL). The aqueous layer was extracted with dichloromethane (80 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was not purified and used for the next reaction.

(*S*)-((*2R,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-yl)methan-d-ol (12): Compound **11** was dissolved in dichloromethane (120 mL). (*R*)-Alpine-Borane® (0.5 M solution in THF, 25.39 mL, 12.70 mmol) was added to the stirring solution and the reaction mixture was stirred for 24 h. Acetaldehyde (20 mL) was added to the solution and the mixture was stirred for 1 h. The solution was concentrated under reduced pressure. The residue was diluted with THF (110 mL) and 3 N aqueous solution of sodium hydroxide (80 mL) and 30% hydrogen peroxide (80 mL) was added at 0 °C. The solution was stirred for 1 h, and concentrated under reduced pressure until all organic solvents disappeared. The remaining aqueous residue was extracted with ethyl acetate (100 mL × 3). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to provide compound **12** (3.809 g, 69%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57–7.52 (m, 2H,, Ph), 7.39–7.27 (m, 13H, Ph), 4.91–4.81 (m, 3H, 3×Bn), 4.64 (d, J = 11.0 Hz, 1H, Bn), 4.46 (d, J = 10.1 Hz, 1H, H-1), 3.67 (d, J = 4.7 Hz, 1H, H-6'), 3.57–3.47 (m, 2H, H-4, H-5), 3.41–3.29 (m, 2H, H-2, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.78, 137.67, 133.64, 131.23, 129.29, 128.71, 128.69, 128.36, 128.21, 128.08, 86.28, 85.09, 79.71, 75.27, 65.47. HRMS (ESI, positive) calculated for $C_{26}H_{26}DN_3O_4S^+$ $[M + Na]^+$ 501.1677, found 501.1679.

(*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((*S*)-(benzyloxy)methyl-d)-2-(phenylthio)tetrahydro-2*H*-pyran (13): Sodium hydride (60% dispersion in mineral oil, 0.177

g, 4.20 mmol) was added to a solution of compound **12** (1.321 g, 2.80 mmol) in dimethylformamide (30 mL) at 0 °C. The solution was warmed to room temperature, and the reaction mixture was stirred for 30 min. The temperature was cooled to 0 °C, and benzyl bromide (0.49 mL, 4.20 mmol) was added. The reaction mixture was stirred at room temperature for 13 h. At 0 °C, methanol (10 mL) was added slowly and the reaction solution was concentrated under reduced pressure. The residue was diluted with dichloromethane (70 mL), and washed with water (70 mL × 2). The combined aqueous layers were extracted with dichloromethane (70 mL × 2). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 15:1) to provide compound **13** (1.194 g, 75%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64–7.60 (m, 2H, Ph), 7.40–7.16 (m, 18H, Ph), 4.89–4.82 (m, 2H, 2×Bn), 4.80 (d, *J* = 10.9 Hz, 1H, Bn), 4.65–4.57 (m, 2H, 2×Bn), 4.55 (d, *J* = 12.0 Hz, 1H, Bn), 4.43 (d, *J* = 10.1 Hz, 1H, H-1), 3.73 (d, *J* = 4.3 Hz, 1H, H-6), 3.62 (dd, *J* = 9.7, 9.0 Hz, 1H, H-4), 3.52 (t, *J* = 9.1 Hz, 1H, H-3), 3.48 (dd, *J* = 9.7, 4.4 Hz, 1H, H-5), 3.36 (dd, *J* = 10.1, 9.2 Hz, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.33, 137.98, 137.74, 133.75, 131.30, 129.10, 128.63, 128.58, 128.48, 128.31, 128.12, 128.00, 127.95, 127.72, 127.68, 86.07, 85.19, 79.40, 77.65, 75.99, 75.15, 73.51, 65.19. HRMS (ESI, positive) calculated for $C_{33}H_{32}DN_3O_4S^+$ $[M + Na]^+$ 591.2147, found 591.2148.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((*S*)-(benzyloxy)methyl-d)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (15): Compound **13** (0.812 g, 1.43 mmol) and compound **14** (0.327 g, 1.10 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **13** and **14** in anhydrous diethyl ether (15 mL) and anhydrous dichloromethane (3 mL) was then added to preactivated 4 Å molecular sieves.

After stirring for 30 min at room temperature, the mixture was cooled to –40 °C. *N*-Iodosuccinimide (0.641 g, 2.85 mmol) was added, and the reaction mixture was stirred for 20 min. Trifluoromethanesulfonic acid (0.07 mL, 0.77 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. The reaction solution was diluted with ether and filtered. The residue was washed with 10% aqueous solution of sodium bisulfite (20 mL) and saturated aqueous solution of sodium bicarbonate (20 mL). The combined aqueous solutions were extracted with dichloromethane (30 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to provide compound **15** (0.506 g, 61%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46–7.26 (m, 13H, Ph), 7.18–7.15 (m, 2H, Ph), 5.19–5.10 (m, 2H, H-1', H-5), 4.92–4.88 (dd, *J* = 19.7, 10.1 Hz, 2H, Bn, H-6), 4.86–4.77 (m, 2H, 2×Bn), 4.65 (d, *J* = 12.1 Hz, 1H, Bn), 4.55 (d, *J* = 10.9 Hz, 1H, Bn), 4.50 (d, *J* = 12.1 Hz, 1H, Bn), 4.16 (dd, *J* = 10.1, 2.9 Hz, 1H, H-5'), 3.96 (dd, *J* = 10.4, 8.9 Hz, 1H, H-3'), 3.85–3.73 (m, 2H, H-6', H-4'), 3.69–3.54 (m, 2H, H-4, H-1), 3.45–3.31 (m, 2H, H-3, H-2'), 2.39 (dt, *J* = 13.3, 4.6 Hz, 1H, H-2), 2.09 (s, 3H, OAc), 2.09 (s, 3H, OAc), 1.59 (q, *J* = 12.7 Hz, 1H, H-2'). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.99, 169.65, 137.97, 137.89, 137.85, 128.58, 128.54, 128.52, 128.11, 128.02, 127.92, 127.87, 127.84, 99.48, 79.68, 78.39, 78.20, 75.47, 75.10, 74.20, 73.66, 71.71, 63.22, 58.75, 57.80, 31.90, 20.77, 20.70. HRMS (ESI, positive) calculated for $C_{37}H_{40}DN_9O_9^+$ $[M + Na]^+$ 779.2982, found 779.2980.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((*S*)-(benzyloxy)methyl-d)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diol (16): Sodium methoxide (4.37 M in methanol, 0.06 mL, 0.24 mmol) was added dropwise to a solution of compound **15** (0.610 g, 0.81 mmol) in methanol (9 mL) and tetrahydrofuran

(1 mL). The reaction mixture was stirred at room temperature for 6 h and then neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **16** (0.493 g, 91%) as a pale yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38–7.27 (m, 13H, Ph), 7.21–7.12 (m, 2H, Ph), 5.17 (d, *J* = 3.6 Hz, 1H, H-1'), 4.93 (d, *J* = 10.6 Hz, 1H, Bn), 4.86 (d, *J* = 10.7 Hz, 1H, Bn), 4.79 (d, *J* = 10.8 Hz, 1H, Bn), 4.64 (d, *J* = 12.0 Hz, 1H, Bn), 4.55 (d, *J* = 10.9 Hz, 1H, Bn), 4.50 (d, *J* = 12.0 Hz, 1H, Bn), 4.10 (dd, *J* = 10.1, 3.3 Hz, 1H, H-5'), 3.99 (dd, *J* = 10.2, 9.0 Hz, 1H, H-3'), 3.81–3.76 (m, 2H, H-6', H-4'), 3.64 (dd, *J* = 10.2, 3.6 Hz, 1H, H-2'), 3.52–3.46 (m, 1H, H-5), 3.44 (t, *J* = 9.1 Hz, 1H, H-6), 3.42–3.37 (m, 1H, H-1), 3.30–3.22 (m, 2H, H-4, H-3), 2.29 (dt, *J* = 12.9, 4.0 Hz, 1H, H-2), 1.54–1.44 (m, 1H, H-2). ^{13}C NMR (150 MHz, $CDCl_3$) δ 137.91 (Ph), 137.87 (Ph), 137.68 (Ph), 128.62 (Ph), 128.59 (Ph), 128.54 (Ph), 128.19 (Ph), 128.11 (Ph), 128.08 (Ph), 128.00 (Ph), 127.91 (Ph), 127.89 (Ph), 99.66 (C-1'), 83.98 (C-4), 81.08 (C-3'), 78.29 (C-4'), 76.01 (C-5), 75.77 (Bn), 75.47 (C-6), 75.15 (Bn), 73.71 (Bn), 71.77 (C-5'), 64.40 (C-2'), 59.78 (C-1), 58.90 (C-3), 32.22 (C-2). HRMS (ESI, positive) calculated for $C_{33}H_{36}DN_9O_7^+$ $[M + Na]^+$ 695.2771, found 695.2768.

(*3aR,6R,7R,7aR*)-7-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6-R*)-3-azido-4,5-bis(benzyloxy)-6-((*S*)-(benzyloxy)methyl-d)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-1,3a-dimethylhexahydro-2*H*-pyrano[4,3-d]oxazol-2-one (18): Compound **16** (0.369 g, 0.549 mmol) and compound **17** (0.263 g, 0.659 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **16** and **17** in anhydrous diethyl ether (5 mL) and anhydrous dichloromethane (1 mL) was added to a preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.247 g, 1.098 mmol). After stirring 30 min at room temperature,

the mixture was cooled to –40 °C. Silver trifluoromethanesulfonate (0.282 g, 1.098 mmol) was added, and the reaction was warmed to –10 °C to stand for 12 h. The reaction mixture was diluted with ethyl acetate (20 mL) and filtered through a Celite pad. The solution was washed with 10% aqueous solution of sodium bisulfite (20 mL) and saturated aqueous solution of sodium bicarbonate (20 mL). The combined aqueous layers were extracted with ethyl acetate (50 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 1.5 : 1) to provide compound **18** (0.390 g, 75%) as a clear liquid. ^{1}H NMR ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.25 (m, 18 H, Bn), 7.21–7.13 (m, 2H, Bn), 5.57 (d, *J* = 3.8 Hz, 1H, H-1'), 5.05 (d, *J* = 3.2 Hz, 1H, H-1''), 4.90–4.88 (m, 3H, Bn), 4.80 (d, *J* = 10.9 Hz, 1H, Bn), 4.63 (d, *J* = 12.1 Hz, 1H, Bn), 4.59 (d, *J* = 11.6 Hz, 1H, Bn), 4.55 (d, *J* = 11.0 Hz, 1H, Bn), 4.49 (d, *J* = 12.1 Hz, 1H, Bn), 4.24–4.09 (m, 3H, H-5'', H-5', OH), 4.00 (t, *J* = 9.6 Hz, 1H, H-3'), 3.93 (t, *J* = 3.9 Hz, 1H, H-2''), 3.84–3.71 (m, 4H, H-6', H-6, H-5'', H-4'), 3.66 (t, *J* = 9.0 Hz, 1H, H-5), 3.50–3.43 (m, 4H, H-4, H-1, H-3'', H-2'), 3.41–3.26 (m, 2H, H-6, H-3), 2.83 (s, 3H, NMe), 2.30 (dt, *J* = 13.4, 4.6 Hz, 1H, H-2), 1.48 (d, *J* = 12.7 Hz, 1H, H-2), 1.38 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.22, 138.07, 137.98, 137.93, 137.37, 128.62, 128.47, 128.41, 128.25, 128.04, 127.92, 127.89, 127.86, 127.73, 127.70, 98.60, 96.71, 84.06, 80.62, 80.26, 78.14, 77.24, 75.44, 75.02, 74.95, 74.38, 73.55, 73.50, 71.39, 66.54, 63.64, 62.87, 59.75, 58.72, 32.01, 30.24, 23.47. HRMS (ESI, positive) calculated for $C_{48}H_{53}DN_{10}O_{11}^{+}$ $[M + Na]^{+}$ 970.3929, found 970.3925.

(*3R,4R,5R,6R*)-5-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((*S*)-(benzyloxy)methyl-d)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-3-methyl-4-(methylamino)tetrahydro-2*H*-pyran-3-ol (19): A 2 N aqueous solution of sodium hydroxide (4 mL) was added to a solution of

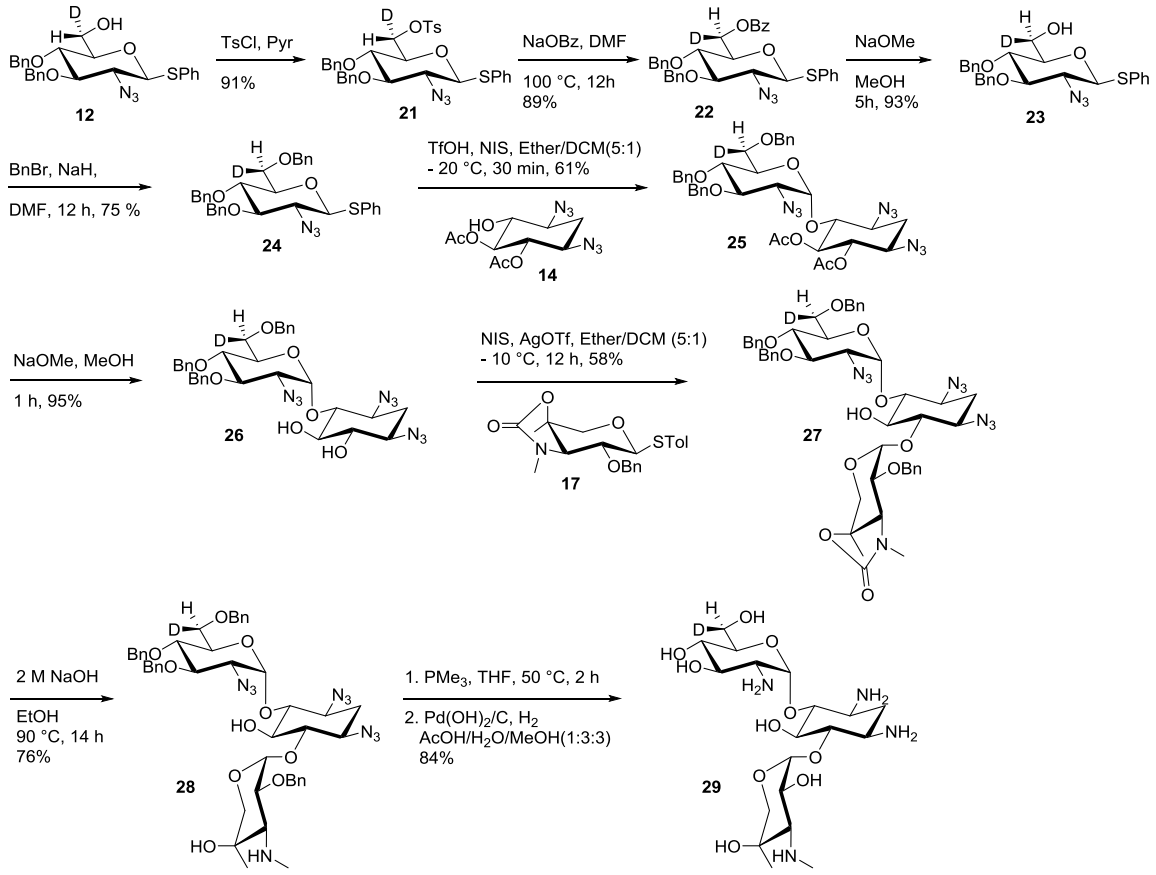
compound **11** (0.302 g, 0.329 mmol) in ethanol (12 mL). The reaction mixture was stirred at 90 °C for 12 h, and cooled to room temperature. The solution was diluted with ethyl acetate (15 mL) and washed with water (15 mL). The residue was washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (5% methanol in dichloromethane) to provide compound **19** (0.261 g, 89%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44–7.23 (m, 18H, Bn), 7.18–7.16 (m, 2H, Bn), 5.52 (d, *J* = 3.7 Hz, 1H, H-1'), 5.25 (d, *J* = 3.3 Hz, 1H, H-1"), 4.93–4.84 (m, 2H, Bn), 4.81 (dd, *J* = 11.3, 2.8 Hz, 2H, Bn), 4.65 (d, *J* = 12.1 Hz, 1H, Bn), 4.55 (d, *J* = 10.9 Hz, 1H, Bn), 4.52 (d, *J* = 6.3 Hz, 1H, Bn), 4.50 (d, *J* = 6.6 Hz, 1H, Bn), 4.15 (dd, *J* = 10.1, 3.2 Hz, 1H, H-5'), 4.00 (dd, *J* = 10.3, 8.9 Hz, 1H, H-3'), 3.95 (d, *J* = 12.3 Hz, 1H, H-5"), 3.84–3.72 (m, 2H, H-6', H-4'), 3.68 (t, *J* = 9.0 Hz, 1H, H-5), 3.61 (dd, *J* = 10.2, 3.4 Hz, 1H, H-2"), 3.56–3.46 (m, 3H, H-5", H-1, H-2'), 3.42 (dd, *J* = 10.0, 8.9 Hz, 1H, H-4), 3.38–3.25 (m, 2H, H-3, H-6), 2.68 (d, *J* = 10.3 Hz, 1H, H-3"), 2.54 (s, 3H, NMe), 2.34 (dt, *J* = 13.2, 4.5 Hz, 1H, H-2), 1.52 (q, *J* = 12.7 Hz, 1H, H-2), 1.18 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.11, 138.02, 137.96, 137.81, 128.74, 128.56, 128.51, 128.48, 128.28, 128.22, 128.13, 128.00, 127.97, 127.85, 127.83, 127.81, 98.97, 97.11, 83.77, 81.23, 80.47, 78.20, 76.14, 75.61, 75.59, 75.06, 73.61, 72.46, 71.49, 71.05, 66.68, 63.81, 63.27, 59.79, 58.90, 38.67, 32.29, 24.55. HRMS (ESI, positive) calculated for $C_{47}H_{55}DN_{10}O_{10}^{+}$ $[M + Na]^{+}$ 944.4136, found 944.4142.

[6'*S*-^{2}H]-Gentamicin X$_2$ (20): Trimethylphosphine (1 M solution in THF, 0.34 mL, 0.34 mmol) was added to a solution of compound **19** (0.63 mg, 0.068 mmol) in THF (3 mL) and 0.1 M aqueous solution of sodium hydroxide (1 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic acid (0.5 mL), water (1.5 mL),

and methanol (1.5 mL). The solution was deaerated by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.05 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (5-10% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **20** (0.03 g, 92%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.75 (d, *J* = 4.0 Hz, 1H, H-1'), 5.17 (d, *J* = 3.7 Hz, 1H, H-1''), 4.26 (dd, *J* = 11.0, 3.7 Hz, 1H, H-2''), 4.07 (d, *J* = 12.8 Hz, 1H, H-5''), 4.02–3.95 (m, 2H, H-3', H-4), 3.89–3.76 (m, 4H, H-4', H-5, H-6', H-6), 3.70–3.60 (m, 2H, H-1, H-3), 3.59–3.46 (m, 4H, H-2', H-3', H-5', H-5'''), 2.96 (s, 3H, NMe), 2.60 (dt, *J* = 12.7, 4.3 Hz, 1H, H-2), 2.00 (q, *J* = 12.6 Hz, 1H, H-2), 1.39 (s, 3H. Me). ^{13}C NMR (125 MHz, D_2O) δ 101.03, 96.88, 83.33, 80.11, 73.75, 73.57, 69.88, 69.35, 68.93, 67.77, 66.20, 63.26, 53.93, 49.50, 48.65, 34.50, 27.79, 20.90. HRMS (ESI, positive) calculated for $C_{19}H_{37}DN_4O_{10}^+$ $[M + H]^+$ 484.2723, found 484.2723.

3.2.5. Synthesis of [6'*R*-^{2}H]-Gentamicin X_2 (29)

The overall synthetic scheme is shown in Scheme 3-2.



Scheme 3-2. Synthetic scheme for the preparation of [6'*R*-^{2}H]-GenX$_2$ (**29**).

(*S*)-((*2R,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-yl)methyl-d 4-methylbenzenesulfonate (21): *p*-Toluenesulfonyl chloride (0.457 g, 2.40 mmol) was added to a solution of **12** (0.574 g, 1.20 mmol) in pyridine (12 mL). The reaction mixture was stirred at room temperature for 24 h, and concentrated under reduced pressure. The residue was diluted with dichloromethane (40 mL) and washed with saturated aqueous solution of ammonium chloride (30 mL). The remaining aqueous layer was washed with dichloromethane (30 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel

(Hex/EtOAc = 4:1) to provide compound **21** (0.689 g, 91%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81–7.79 (m, 2H, Ph), 7.58–7.45 (m, 2H, Ph), 7.41–7.24 (m, 13H, Ph), 7.20–7.18 (m, 2H, Ph), 4.86 (d, *J* = 10.6 Hz, 1H, Bn), 4.83–4.73 (m, 2H, 2×Bn), 4.50 (d, *J* = 10.7 Hz, 1H, Bn), 4.33 (d, *J* = 10.1 Hz, 1H, H-1), 4.15 (d, *J* = 4.0 Hz, 1H, H-6), 3.50–3.38 (m, 3H, H-3, H-4, H-5), 3.25 (t, *J* = 9.5 Hz, 1H, H-2'), 2.40 (s, 3H, OMe). ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.15, 137.50, 137.39, 134.03, 132.93, 130.69, 130.05, 129.17, 128.70, 128.27, 128.16, 128.12, 85.97, 84.98, 76.04, 75.28, 64.98, 21.79. HRMS (ESI, positive) calculated for $C_{33}H_{32}DN_3O_6S_2^+$ $[M + Na]^+$ 655.1766, found 655.1764.

(*R*)-((*2R,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-yl)methyl-d benzoate (22): Sodium benzoate (0.388 g, 3.40 mmol) was added to a solution of compound **21** (0.717 g, 1.13 mmol) in dimethylformamide (11mL). The reaction mixture was stirred at 100 °C for 12 h, cooled to room temperature, and concentrated under reduced pressure. The residue was diluted with dichloromethane (40 mL) and washed with water (30 mL × 3). The combined aqueous layers were extracted with dichloromethane (40 mL × 3). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to provide compound **22** (0.582 g, 89%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05–7.99 (m, 2H, Ph), 7.67–7.59 (m, 1H, Ph), 7.59–7.53 (m, 2H, Ph), 7.50–7.45 (m, 2H, Ph), 7.40–7.22 (m, 10H, Ph), 7.17–7.10 (m, 2H, Ph), 4.92 (d, *J* = 10.3 Hz, 1H, Bn), 4.86 (d, *J* = 10.2 Hz, 2H, Bn), 4.69 (d, *J* = 2.0 Hz, 1H, H-6), 4.61 (d, *J* = 10.9 Hz, 1H, Bn), 4.45 (d, *J* = 10.1 Hz, 1H, H-1), 3.70–3.53 (m, 3H, H-3, H-4, H-5), 3.40–3.29 (m, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.10, 137.41, 137.38, 134.30, 133.37, 130.47, 129.91, 129.86, 129.04, 128.77, 128.71, 128.69, 128.58, 128.50, 128.35,

128.28, 128.16, 85.72, 85.21, 77.27, 77.22, 76.26, 75.35, 64.99. HRMS (ESI, positive) calculated for $C_{33}H_{30}DN_3O_5S^+$ $[M + Na]^+$ 605.1939, found 605.1947.

(*R*)-((*2R,3S,4R,5R,6S*)-5-azido-3,4-bis(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-2-yl)methan-d-ol (23): 2 N aqueous solution of sodium hydroxide (2.52 mL, 5.03 mmol) was added to a solution of **22** (0.733 g, 1.26 mmol) in methanol (12 mL). The reaction mixture was stirred at room temperature for 5 h, neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **23** (0.560 g, 93%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57–7.52 (m, 2H, Ph), 7.37–7.27 (m, 13H), 4.91–4.81 (m, 3H, 3×Bn), 4.64 (d, *J* = 11.0 Hz, 1H, Bn), 4.46 (d, *J* = 10.2 Hz, 1H, H-1), 3.86 (d, *J* = 2.6 Hz, 1H, H-6), 3.57–3.49 (m, 2H, H-4, H-5), 3.39 – 3.29 (m, 2H, H-2, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.79, 137.68, 133.64, 131.24, 129.29, 128.71, 128.69, 128.36, 128.21, 128.07, 86.29, 85.09, 75.27, 65.47. HRMS (ESI, positive) calculated for $C_{26}H_{26}DN_3O_4S^+$ $[M + Na]^+$ 501.1677, found 501.1679.

(*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((*R*)-(benzyloxy)methyl-d)-2-(phenylthio)tetrahydro-2*H*-pyran (24): Sodium hydride (60% dispersion in mineral oil, 0.072 g, 1.71 mmol) was added to a solution of compound **23** in dimethylformamide (9 mL) at 0 °C. The reaction mixture was stirred at room temperature for 30 min. Benzyl bromide (0.20 mL, 1.71 mmol) was added at 0 °C, and the reaction mixture was stirred at room temperature for 12 h. At 0 °C, methanol (5 mL) was added slowly, and the solution was concentrated under reduced pressure. The residue was diluted with dichloromethane (50 mL) and washed with water (30 mL × 2). The combined aqueous layers were extracted with dichloromethane (40 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The

crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 15:1) to provide compound **24** (0.364 g, 75%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67–7.59 (m, 2H, Ph), 7.39–7.19 (m, 18H, Ph), 4.90–4.82 (m, 2H, 2×Bn), 4.80 (d, *J* = 10.9 Hz, 1H, Bn), 4.66–4.58 (m, 2H, Bn), 4.55 (d, *J* = 12.0 Hz, 1H, Bn, 4.43 (d, *J* = 10.1 Hz, 1H, H-1), 3.77 (d, *J* = 1.9 Hz, 1H, H-6), 3.63 (dd, *J* = 9.7, 9.0 Hz, 1H, H-4), 3.52 (t, *J* = 9.1 Hz, 1H, H-3), 3.48 (dd, *J* = 9.7, 1.9 Hz, 1H, H-5), 3.36 (dd, *J* = 10.1, 9.3 Hz, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.32, 137.98, 137.72, 133.74, 131.28, 129.08, 128.62, 128.57, 128.47, 128.30, 128.11, 127.98, 127.93, 127.71, 127.67, 86.06, 85.18, 79.39, 77.64, 75.98, 75.14, 73.51, 65.17. HRMS (ESI, positive) calculated for $C_{33}H_{32}DN_3O_4S^+$ $[M + Na]^+$ 591.2147, found 591.2146.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((*R*)-(benzyloxy)methyl-d)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (25): Compound **24** (0.956 g, 1.68 mmol) and compound **14** (0.385 g, 1.29 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **24** and **14** in anhydrous diethyl ether (15 mL) and anhydrous dichloromethane (3 mL) was then added to preactivated 4 Å molecular sieves. After stirring for 30 min at room temperature, the mixture was cooled to –40 °C. *N*-Iodosuccinimide (0.756 g, 3.36 mmol) was added, and the reaction mixture was stirred for 30 min. Trifluoromethanesulfonic acid (0.08 mL, 0.90 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. The reaction solution was diluted with ether (15 mL) and filtered. The residue was washed with 10% aqueous solution of sodium bisulfite (15 mL) and saturated aqueous solution of sodium bicarbonate (15 mL). The combined aqueous solutions were extracted with dichloromethane (40 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue

was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to provide compound **25** (0.595 g, 61%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43–7.27 (m, 13H, Ph), 7.18–7.15 (m, 2H, Ph), 5.21–5.09 (m, 2H, H-1', H-5), 4.95–4.88 (dd, *J* = 20.3, 10.3 Hz, 2H, Bn, H-6), 4.85–4.77 (m, 2H, 2×Bn), 4.65 (d, *J* = 12.1 Hz, 1H, Bn), 4.54 (d, *J* = 10.9 Hz, 1H, Bn), 4.52–4.46 (m, 1H, Bn), 4.16 (dd, *J* = 10.1, 2.0 Hz, 1H, H-5'), 3.96 (dd, *J* = 10.4, 8.9 Hz, 1H, H-3'), 3.79 (dd, *J* = 10.1, 9.0 Hz, 1H, H-4'), 3.70–3.56 (m, 3H, H-4, H-1, H-6'), 3.46–3.40 (m, 1H, H-3), 3.36 (dd, *J* = 10.4, 3.9 Hz, 1H, H-2'), 2.39 (dt, *J* = 13.4, 4.6 Hz, 1H, H-2), 2.09 (s, 3H, OAc), 2.08 (s, 3H, OAc), 1.59 (q, *J* = 12.7 Hz, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.99, 169.66, 138.00, 137.91, 137.86, 128.59, 128.55, 128.53, 128.12, 128.03, 127.93, 127.89, 127.84, 99.49, 79.70, 75.47, 75.11, 74.21, 73.68, 73.64, 71.73, 63.24, 58.77, 57.81, 31.92, 20.78, 20.71. HRMS (ESI, positive) calculated for $C_{37}H_{40}DN_9O_9^+$ $[M + Na]^+$ 779.2982, found 779.2996.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((*R*-)-(benzyloxy)methyl-d)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diol (26): Sodium methoxide (4.37 M in methanol, 0.08 mL, 0.35 mmol) was added dropwise to a solution of compound **25** (0.874 g, 1.15 mmol) in methanol (10 mL). The reaction mixture was stirred at room temperature for 1 h and then neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **26** (0.738 g, 95%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.28 (m, 13H, Ph), 7.21–7.15 (m, 2H, Ph), 5.22 (d, *J* = 3.7 Hz, 1H, H-1'), 4.94 (d, *J* = 10.7 Hz, 1H, Bn), 4.87 (d, *J* = 10.7 Hz, 1H, Bn), 4.81 (d, *J* = 10.9 Hz, 1H, Bn), 4.66 (d, *J* = 12.1 Hz, 1H, Bn), 4.57 (d, *J* = 10.9 Hz, 1H, Bn), 4.51 (d, *J* = 12.0 Hz, 1H, Bn), 4.12 (dd, *J* = 10.0, 2.1 Hz, 1H, H-5'), 4.01 (dd, *J* = 10.2, 9.0 Hz, 1H, H-3'), 3.80 (dd, *J* = 10.1, 9.0 Hz, 1H, H-4'), 3.69 (d, *J* = 2.0 Hz, 1H, H-6'), 3.63 (dd, *J* = 10.2, 3.6 Hz, 1H, H-2'),

3.50 (t, J = 8.7 Hz, 1H, H-5), 3.47–3.35 (m, 2H, H-6, H-1), 3.34–3.22 (m, 2H, H-4, H-3), 2.28 (dt, J = 12.9, 4.2 Hz, 1H, H-2), 1.48 (dt, J = 13.0, 11.7 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.92 (Ph), 137.86 (Ph), 137.69 (Ph), 128.57 (Ph), 128.54 (Ph), 128.50 (Ph), 128.15 (Ph), 128.04 (Ph), 127.93 (Ph), 127.87 (Ph), 127.83 (Ph), 99.51 (C-1'), 83.56 (C-4), 80.96 (C-3'), 78.27 (C-4'), 75.69 (C-5), 75.46 (C-6), 75.09 (Bn), 73.65 (Bn), 71.70 (C-5'), 67.90 (C-6'), 64.30 (C-2'), 59.77 (C-1), 58.89 (C-3), 32.15 (C-2). HRMS (ESI, positive) calculated for $C_{33}H_{36}DN_9O_7^+$ $[M + Na]^+$ 695.2771, found 695.2770.

(*3aR,6R,7R,7aR*)-7-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6-R*)-3-azido-4,5-bis(benzyloxy)-6-((*R*)-(benzyloxy)methyl-d)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-1,3a-dimethylhexahydro-2*H*-pyrano[4,3-d]oxazol-2-one (27): Compound **26** (0.721 g, 1.07 mmol) and compound **17** (0.471 g, 1.18 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **26** and **17** in anhydrous diethyl ether (10 mL) and anhydrous dichloromethane (2 mL) was added to a preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.482 g, 2.14 mmol). After stirring 30 min at room temperature, the mixture was cooled to –40 °C. Silver trifluoromethanesulfonate (0.551 g, 2.14 mmol) was added, and the reaction was warmed to –10 °C to stand for 3 h. The reaction mixture was diluted with ethyl acetate (10 mL) and filtered through a Celite pad. The solution was washed with 10% aqueous solution of sodium bisulfite (15 mL) and saturated aqueous solution of sodium bicarbonate (15 mL). The combined aqueous layers were extracted with ethyl acetate (20 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to provide compound **27** (0.589 g, 58%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.26 (m, 18H, Bn), 7.19–7.14 (m, 2H, Bn), 5.57 (d, J = 3.7 Hz, 1H, H-1'), 5.06 (d, J = 3.2 Hz,

1H, H-1''), 4.92–4.87 (m, 3H, Bn), 4.81 (d, *J* = 10.9 Hz, 1H, Bn), 4.65 (d, *J* = 12.1 Hz, 1H, Bn), 4.61 (d, *J* = 11.6 Hz, 1H, Bn), 4.55 (d, *J* = 11.0 Hz, 1H, Bn), 4.50 (d, *J* = 12.2 Hz, 1H, Bn), 4.24–4.12 (m, 3H, H-5'', H-5', OH), 4.00 (dd, *J* = 10.3, 8.9 Hz, 1H, H-3'), 3.94 (dd, *J* = 4.5, 3.2 Hz, 1H, H-2''), 3.85–3.73 (m, 3H, H-6, H-5'', H-4'), 3.70–3.63 (m, 2H, H-5, H-6'), 3.48 (ddd, *J* = 8.7, 5.7, 4.1 Hz, 4H, H-4, H-1, H-3'', H-2'), 3.45–3.37 (m, H, H-6), 3.37–3.29 (m, 1H, H-3), 2.84 (s, 3H, NMe), 2.32 (dt, *J* = 13.2, 4.5 Hz, 1H, H-2), 1.56–1.44 (m, 1H, H-2), 1.40 (s, 3H, Me). ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.35, 138.13, 138.03, 137.98, 137.41, 128.74, 128.59, 128.53, 128.39, 128.16, 128.03, 128.00, 127.86, 98.77, 96.81, 84.15, 80.85, 80.41, 78.21, 77.35, 75.60, 75.09, 74.50, 73.69, 73.63, 71.50, 66.64, 63.75, 63.01, 59.88, 58.79, 32.14, 30.37, 23.60. HRMS (ESI, positive) calculated for $C_{48}H_{53}DN_{10}O_{11}^{+}$ $[M + Na]^+$ 970.3929, found 970.3925.

(*3R,4R,5R,6R*)-5-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((*R*)-(benzyloxy)methyl-d)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-3-methyl-4-(methylamino)tetrahydro-2*H*-pyran-3-ol (28): A 2 N aqueous solution of sodium hydroxide (10 mL) was added to a solution of compound **27** (0.592 g, 0.625 mmol) in ethanol (30 mL). The reaction mixture was stirred at 90 °C for 14 h, and cooled to room temperature. The solution was diluted with ethyl acetate (30 mL) and washed with water (30 mL). The residue was washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (5% methanol in dichloromethane) to provide compound **28** (0.437 g, 76%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41–7.24 (m,18, Bn), 7.17–7.15 (m, 2H, Bn), 5.51 (d, *J* = 3.7 Hz, 1H, H-1'), 5.25 (d, *J* = 3.3 Hz, 1H, H-1''), 4.88 (d, *J* = 3.3 Hz, 2H, Bn), 4.83–4.76 (m, 2H, Bn), 4.65 (d, *J* = 12.1 Hz, 1H, Bn), 4.56–4.47 (m, 3H, Bn), 4.42 (s, 1H, OH), 4.13 (dd, *J* = 10.1, 2.1 Hz, 1H, H-5'), 3.99 (dd, *J* = 10.3, 9.0 Hz, 1H, H-3'), 3.94 (d, *J* = 12.3 Hz, 1H,

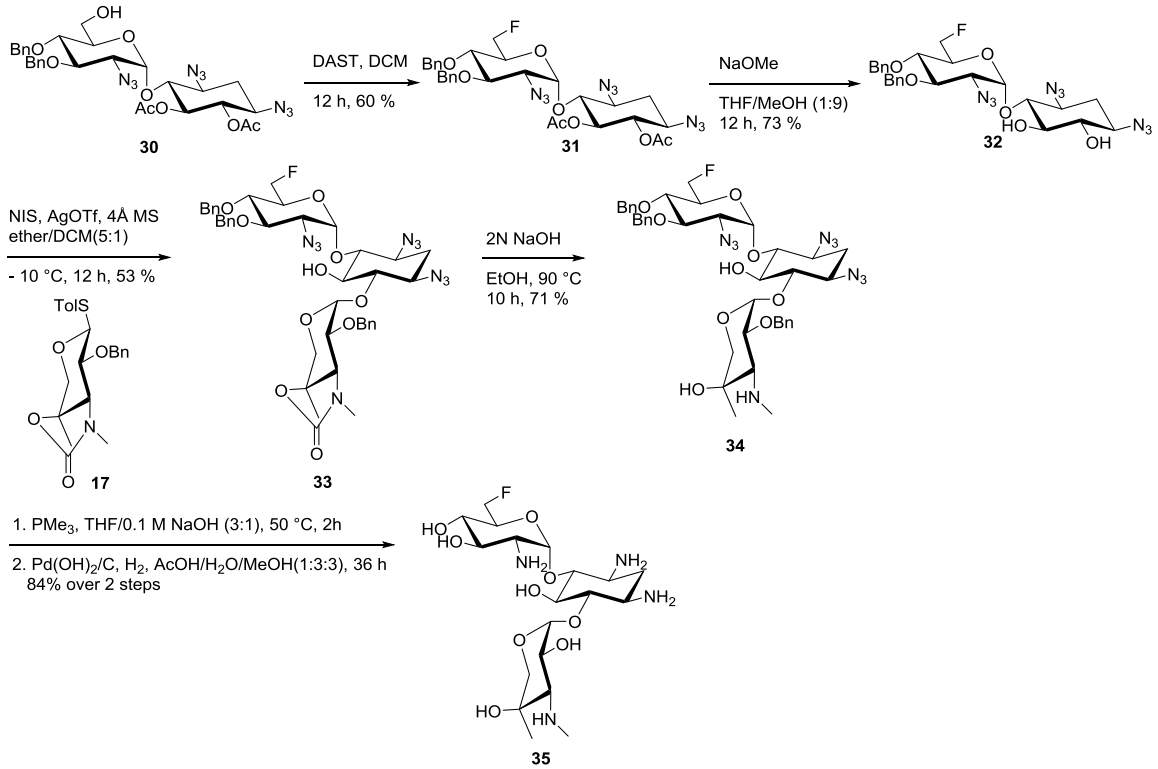
H-5''), 3.77 (dd, *J* = 10.1, 8.9 Hz, 1H, H-4'), 3.70–3.64 (m, 3H, H-5, H-6', H-4'), 3.64–3.58 (m, 1H, H-2''), 3.56–3.45 (m, 3H, H-1, H-5'', H-2'), 3.41 (dd, *J* = 10.1, 8.9 Hz, 1H, H-4), 3.37–3.28 (m, 2H, H-6, H-3), 2.68 (d, *J* = 10.3 Hz, 1H, H-3''), 2.54 (s, 3H, NMe), 2.34 (dt, *J* = 13.2, 4.4 Hz, 1H, H-2), 1.51 (q, *J* = 12.7 Hz, 1H, H-2), 1.18 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.14, 138.04, 137.98, 137.81, 128.77, 128.60, 128.54, 128.52, 128.32, 128.27, 128.16, 128.03, 128.00, 127.89, 127.85, 99.02, 97.11, 83.75, 81.30, 80.51, 78.23, 77.36, 76.13, 75.64, 75.62, 75.09, 73.66, 72.50, 71.53, 71.07, 63.85, 63.35, 59.83, 58.92, 38.66, 32.32, 24.54. HRMS (ESI, positive) calculated for $C_{47}H_{55}DN_{10}O_{11}^{+}$ $[M+Na]^{+}$ 944.4136, found 944.4106.

[6'*R*-^{2}H]-Gentamicin X_2 (29): Trimethylphosphine (1 M solution in THF, 0.84 mL, 0.84 mmol) was added to a solution of compound **28** (0.129 mg, 0.14 mmol) in THF (4.8 mL) and 0.1 M aqueous solution of sodium hydroxide (1.6 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic acid (1.2 mL), water (3.6 mL) and methanol (3.6 mL). The solution was degassed by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^{+} form) (5% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **29** (0.057 g, 84%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.70 (d, *J* = 4.0 Hz, 1H, H-1'), 5.13 (d, *J* = 3.7 Hz, 1H, H-1''), 4.24 (dd, *J* = 10.9, 3.7 Hz, 1H, H-2''), 4.04 (d, *J* = 12.8 Hz, 1H, H-5''), 3.99–3.90 (m, 3H, H-6', H-3', H-4),

3.90–3.79 (m, 3H, H-4', H-5, H-6), 3.68–3.58 (m, 2H, H-1, H-3), 3.57–3.44 (m, 4H, H-5", H-3", H-5', H-2'), 2.94 (s, 3H, NMe), 2.57 (dt, J = 12.6, 4.3 Hz, 1H, H-2), 1.95 (q, J = 12.7 Hz, 1H, H-2), 1.37 (s, 3H, Me). ^{13}C NMR (125 MHz, D_2O) δ 101.05, 97.01, 83.36, 80.22, 73.69, 73.59, 69.83, 69.27, 68.92, 67.78, 66.17, 63.20, 53.86, 49.43, 48.57, 34.45, 27.81, 20.87. HRMS (ESI, positive) calculated for $C_{19}H_{37}DN_4O_{10}^+$ $[M + H]^+$ 484.2723, found 484.2725.

3.2.6. Synthesis of [6'-F]-gentamicin X_2 (35)

The overall synthetic scheme is shown in Scheme 3-3.



Scheme 3-3. Synthetic scheme for the preparation of [6'-F]-GenX_2 (**35**)

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6S*)-3-azido-4,5-bis(benzyloxy)-6-(fluoromethyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (31): Diethylaminosulfur trifluoride (DAST) (0.20 mL, 1.53 mmol) was added to a solution of compound **30** (0.511 g, 0.767 mmol) in dichloromethane (10 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 12 h. The reaction was quenched by adding saturated aqueous solution of sodium bicarbonate (20 mL). The aqueous layer was extracted with dichloromethane (30 mL × 3). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to provide compound **31** (0.307 g, 60%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.34 (m, 10H, Ph), 5.19–5.15 (m, 2H, H-1', H-5), 4.99–4.87 (m, 4H, H-6, 3×Bn), 4.82–4.55 (m, 3H, Bn, H-6', H-6'), 4.22 (dd, *J* = 31.2, 10.2 Hz, 1H, H-5'), 4.02 (t, *J* = 9.6 Hz, 1H, H-3'), 3.72 (t, *J* = 9.6 Hz, 1H, H-4'), 3.66 (t, *J* = 9.8 Hz, 1H, H-4), 3.64–3.54 (m, 1H, H-1), 3.41–3.36 (m, 2H, H-2', H-3), 2.35 (dt, *J* = 13.4, 4.7 Hz, 1H, H-2), 2.12 (s, 1H, Ac), 2.09 (s, 1H, Ac), 1.62 (q, *J* = 12.7 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.75 (Ac), 169.46 (Ac), 137.59 (Ph), 137.52 (Ph), 128.50 (Ph), 128.45 (PH), 127.98 (Ph), 127.96 (Ph), 127.92 (Ph), 127.78 (Ph), 99.29 (C-1'), 81.43 (d, *J* = 173.4, C-6'), 79.44 (C-3'), 78.40 (C-4), 77.30 (d, *J* = 5.1 Hz, C-4'), 75.29 (Bn), 75.17 (Bn), 73.94 (C-6), 73.31 (C-5), 71.08 (d, *J* = 18.2 Hz, C-5'), 62.93 (C-2'), 58.42 (C-3), 57.53(C-1), 31.44 (C-2), 20.52 (OAc), 20.48 (OAc). ^{19}F NMR (375 MHz, $CDCl_3$) δ -234.52 (td, *J* = 47.7, 31.3 Hz). HRMS (ESI, positive) calculated for $C_{30}H_{34}FN_9O_8^+$ [*M* + *Na*]$^+$ 690.2407, found 690.2414.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6S*)-3-azido-4,5-bis(benzyloxy)-6-(fluoromethyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diol (32): Sodium methoxide (4.37 M in methanol, 0.7 mL, 0.33 mmol) was added to a solution of compound **31**

(0.735 g, 1.10 mmol) in tetrahydrofuran (2 mL) and methanol (18 mL). The reaction mixture was stirred at room temperature for 12 h, neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **32** (0.426 g, 73%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.30 (m, 10H, Bn), 5.17 (d, *J* = 3.7 Hz, 1H, H-1'), 4.95 (d, *J* = 10.6 Hz, 1H, Bn), 4.91–4.86 (m, 2H, Bn), 4.76–4.52 (m, 3H, Bn, H-6', H-6'), 4.18–3.97 (m, 2H, H-5', H-3'), 3.71 (dd, *J* = 10.2, 9.0 Hz, 1H, H-4'), 3.63 (dd, *J* = 10.2, 3.6 Hz, 1H, H-2'), 3.55–3.36 (m, 3H, H-4, H-5, H-1), 3.32–3.19 (m, 2H, H-6, H-3), 3.03 (s, 1H, OH), 2.31 (dt, *J* = 12.9, 3.9 Hz, 1H, H-2), 1.58–1.43 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.59 (Ph), 137.49 (Ph), 128.73 (Ph), 128.67 (Ph), 128.23 (Ph), 128.21 (Ph), 127.93 (Ph), 99.77 (C-1'), 84.09 (C-6), 81.63 (d, *J* = 172.1, C-6'), 80.94 (C-3'), 77.46 (d, *J* = 5.4, C-4'), 76.07 (Bn), 75.87 (C-4), 75.43 (Bn, C-5), 71.35 (d, *J* = 18.2, C-5'), 64.28 (C-2'), 59.78 (C-1), 58.91 (C-3), 32.12 (C-2). ^{19}F NMR (470 MHz, $CDCl_3$) δ -235.11 (td, *J* = 47.8, 30.9 Hz). HRMS (ESI, positive) calculated for $C_{26}H_{30}FN_9O_6^+$ $[M + Na]^+$ 606.2195, found 606.2202.

(*3aR,6R,7R,7aR*)-7-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6-S*)-3-azido-4,5-bis(benzyloxy)-6-(fluoromethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-1,3a-dimethylhexahydro-2*H*-pyrano[4,3-d]oxazol-2-one (33): Compound **32** (0.543 g, 0.808 mmol) and compound **17** (0.355 g, 0.888 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **32** and **17** in anhydrous diethyl ether (10 mL) and anhydrous dichloromethane (2 mL) was added to a preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.364 g, 1.616 mmol). After stirring 30 min at room temperature, the mixture was cooled to –40 °C. Silver trifluoromethanesulfonate (0.415 g, 1.616 mmol) was added, and the reaction was warmed to –10 °C to stand for 3 h. The reaction mixture

was diluted with ethyl acetate (10 mL) and filtered through a Celite pad. The solution was washed with 10% aqueous solution of sodium bisulfite (20 mL) and saturated aqueous solution of sodium bicarbonate (25 mL). The combined aqueous layers were extracted with ethyl acetate (40 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to provide compound **33** (0.365 g, 53%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.28 (m, 15H, Ph), 5.60 (d, *J* = 3.8 Hz, 1H, H-1'), 5.03 (d, *J* = 3.2 Hz, 1H, H-1''), 4.92–4.87 (m, 4H, 4×Bn), 4.74–4.52 (m, 4H, 2×Bn, H-6', H-6''), 4.22–4.08 (m, 3H, H-5'', OH, H-5'), 4.03 (dd, *J* = 10.3, 8.9 Hz, 1H, H-3'), 3.95 (dd, *J* = 4.4, 3.1 Hz, 1H, H-2''), 3.80 (d, *J* = 12.4 Hz, 1H, H-5''), 3.70–3.65 (m, 2H, H-4', H-5), 3.51–3.38 (m, 5H, H-1, H-3'', H-4, H-2', H-6), 3.35 (ddd, *J* = 12.4, 10.0, 4.4 Hz, 1H, H-3), 2.83 (s, 3H, NMe), 2.33 (dt, *J* = 13.2, 4.4 Hz, 1H, H-2), 1.51 (q, *J* = 12.6 Hz, 1H, H-2), 1.41 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.28 (N(C(=O)O), 137.85 (Ph), 137.82 (Ph), 137.41 (Ph), 128.73 (Ph), 128.64 (Ph), 128.61 (Ph), 128.39 (Ph), 128.15 (Ph), 128.08 (Ph), 128.05 (Ph), 128.02 (Ph), 127.91 (Ph), 98.54 (C-1'), 97.00 (C-2''), 84.60 (C-6), 81.76 (d, *J* = 173.3 Hz, C-6'), 80.50 (C-4), 80.15 (C-3'), 77.36 (C-4'), 77.21 (C-4''), 75.63 (Bn), 75.33 (Bn), 75.12 (C-5), 74.63 (Bn), 73.42 (C-2''), 71.06 (d, *J* = 18.2 Hz, C-5'), 66.87 (C-5''), 63.58 (C-2'), 63.04 (C-3''), 59.78 (C-1), 58.77 (C-3), 32.08 (C-2), 30.32 (NMe), 23.63 (Me). ^{19}F NMR (375 MHz, $CDCl_3$) δ -234.59 (td, *J* = 47.8, 30.7 Hz). HRMS (ESI, positive) calculated for $C_{41}H_{47}FN_{10}O_{10}^{+}$ $[M + Na]^{+}$ 881.3353, found 881.3368.

(*3R,4R,5R,6R*)-5-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6S*)-3-azido-4,5-bis(benzyloxy)-6-(fluoromethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-3-methyl-4-(methylamino)tetrahydro-2*H*-pyran-3-ol (34): A 2 N aqueous solution of sodium hydroxide (5 mL) was added to a solution of compound **33**

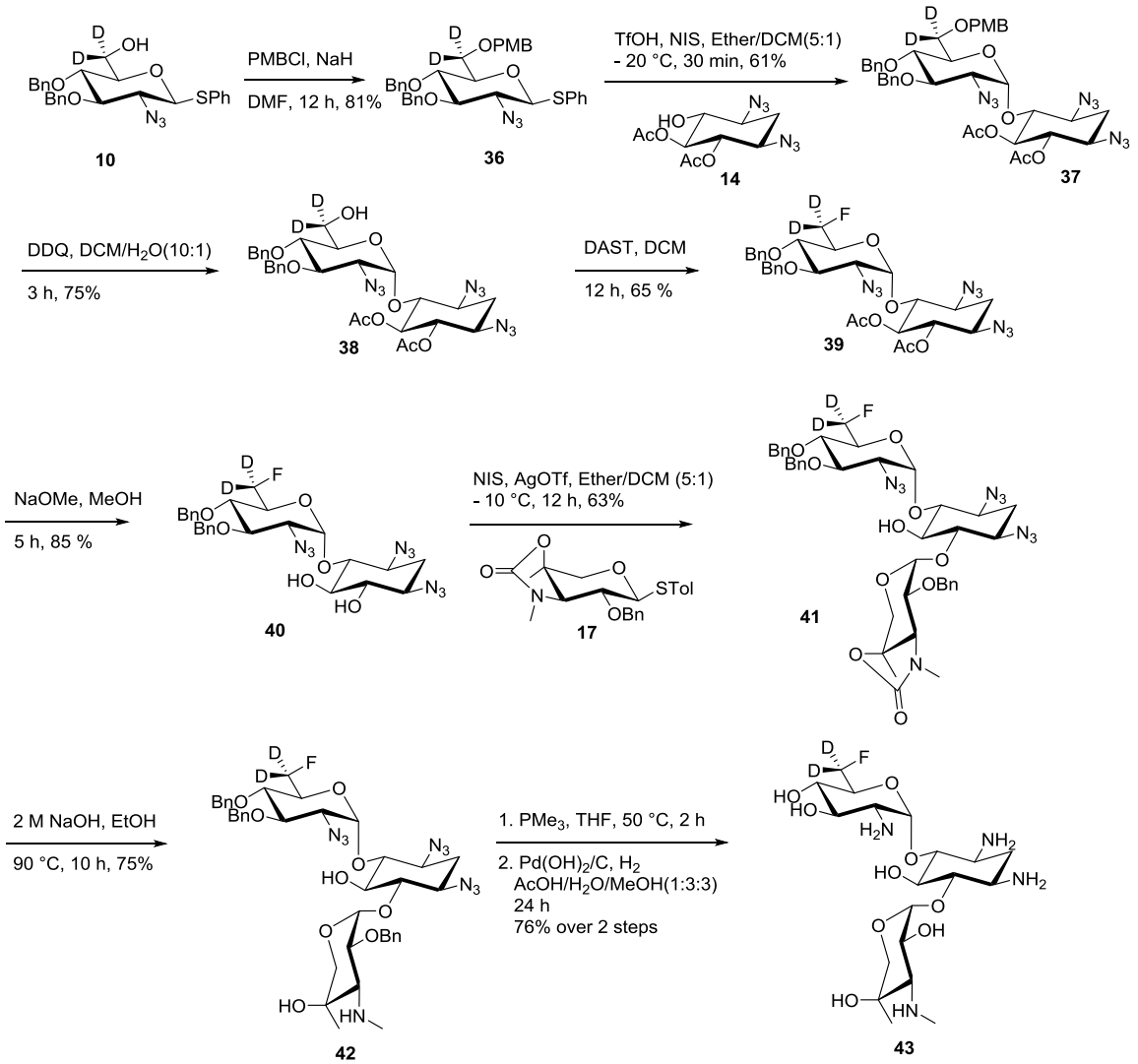
(0.365 g, 0.425 mmol) in ethanol (15 mL). The reaction mixture was stirred at 90 °C for 10 h, and cooled to room temperature. The solution was diluted with ethyl acetate (20 mL) and washed with water (20 mL). The residue was washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (5–8% methanol in dichloromethane) to provide compound **34** (0.251 g, 71%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.30 (m, 15H, Ph), 5.56 (d, *J* = 3.7 Hz, 1H, H-1'), 5.23 (d, *J* = 3.3 Hz, 1H, H-1''), 4.89 (d, *J* = 8.7 Hz, 3H, 3×Bn), 4.78 (d, *J* = 11.6 Hz, 1H, Bn), 4.75–4.64 (m, 2H, Bn H-6'), 4.63–4.50 (m, 2H, Bn H-6'), 4.20–4.09 (m, 1H, H-5'), 4.03 (dd, *J* = 10.3, 9.0 Hz, 1H, H-3'), 3.92 (d, *J* = 12.2 Hz, 1H, H-5''), 3.74–3.64 (m, 3H, H-2'', H-5, H-4'), 3.57–3.47 (m, 2H, H-5'', H-1), 3.47–3.39 (m, 2H, H-2', H-4), 3.39–3.27 (m, 2H, H-3, H-6), 2.72 (d, *J* = 10.3 Hz, 1H, H-3''), 2.56 (s, 3H, NMe), 2.34 (dt, *J* = 13.2, 4.5 Hz, 1H, H-2), 1.52 (q, *J* = 12.7 Hz, 1H, H-2), 1.21 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.80 (Ph), 137.78 (Ph), 137.74 (Ph), 128.72 (Ph), 128.63 (Ph), 128.59 (Ph), 128.28 (Ph), 128.23 (Ph), 128.14 (Ph), 128.08 (Ph), 128.04 (Ph), 127.90 (Ph), 98.76 (C-1'), 97.27 (C-1''), 84.42 (C-6), 81.74 (d, *J* = 173.3 Hz, C-6'), 80.81 (C-4), 80.21 (C-3'), 77.37 (C-4'), 77.33 (C-4''), 76.12 (C-2''), 75.71 (C-5), 75.63 (Bn), 75.32 (Bn), 72.54 (Bn), 71.04 (d, *J* = 18.1 Hz, C-5'), 67.07 (C-5''), 63.59 (C-2'), 63.44 (C-3''), 59.65 (C-1), 58.86 (C-3), 38.41 (C-2), 32.21 (NMe), 24.26 (Me). ^{19}F NMR (376 MHz, Chloroform-*d*) δ -234.58 (td, *J* = 47.7, 30.8 Hz). HRMS (ESI, positive) calculated for $C_{40}H_{49}FN_{10}O_9^+$ $[M + Na]^+$ 855.3560, found 855.3570.

[6'-F]-gentamicin X_2 (35): Trimethylphosphine (1 M solution in THF, 1.89 mL, 1.89 mmol) was added to a solution of compound **34** (0.165 mg, 0.198 mmol) in THF (3 mL) and 0.1 M aqueous solution of NaOH (1 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The

residue was dissolved in a mixture of acetic acid (2 mL), water (6 mL) and methanol (6 mL). The solution was degassed by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (5% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **35** (0.080 g, 84%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.79 (d, *J* = 4.0 Hz, 1H, H-1'), 5.14 (d, *J* = 3.7 Hz, 1H, H-1''), 4.85–4.82 (m, 1H, H-6'), 4.74–4.67 (m, 1H, H-6'), 4.25 (dd, *J* = 10.9, 3.7 Hz, 1H, H-2''), 4.11–4.01 (m, 2H, H-5', H-5''), 4.00–3.95 (m, 2H, H-3', H-4), 3.90 (t, *J* = 9.0 Hz, 1H, H-5), 3.83 (dd, *J* = 10.2, 8.9 Hz, 1H, H-6), 3.69–3.59 (m, 3H, H-1, H-3, H-4'), 3.56 (d, *J* = 10.9 Hz, 1H, H-3''), 3.54 (d, *J* = 12.8 Hz, 1H, H-5''), 3.51 (dd, *J* = 10.9, 4.0 Hz, 1H, H-2'), 2.94 (s, 3H, NMe), 2.58 (dt, *J* = 12.6, 4.2 Hz, 1H, H-2), 1.96 (q, *J* = 12.6 Hz, 1H, H-2), 1.38 (s, 3H, Me). ^{13}C NMR (125 MHz, D_2O) δ 101.07 (C-1''), 96.85 (C-1'), 83.43 (C-6), 82.21 (d, *J* = 167.3 Hz, C-6'), 79.52 (C-4), 73.84 (C-5), 72.22 (d, *J* = 17.6 Hz, C-5'), 69.85 (C-4''), 68.77 (C-3'), 68.19 (d, *J* = 7.2 Hz, C-4'), 67.83 (C-5''), 66.17 (C-2''), 63.19 (C-3''), 53.70 (C-2'), 49.46 (C-1), 48.37 (C-3), 34.48 (NMe), 27.80 (C-2), 20.90 (Me). ^{19}F NMR (565 MHz, D_2O) δ -233.97 (td, *J* = 47.1, 25.3 Hz). HRMS (ESI, positive) calculated for $C_{19}H_{37}FN_4O_9^+$ $[M + H]^+$ 485.2617, found 485.2628.

3.2.7. Synthesis of [6',6' 6'-^{2}H, ^{2}H,F]-Gentamicin X_2 (43)

The overall synthetic scheme is shown in Scheme 3-4.



Scheme 3-4. Synthetic scheme for the preparation of [6', 6', 6'-^{2}H, ^{2}H,F]-GenX$_2$ (**43**).

(*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl-d2)-2-(phenylthio)tetrahydro-2*H*-pyran (36): Sodium hydride (60% dispersion in mineral oil, 0.125 g, 2.97 mmol) and 4-methoxybenzyl chloride (0.47 mL, 3.43 mmol) were added to a solution of compound **10** (1.096 g, 2.29 mmol) in dimethylformamide (20 mL) at 0 °C. The reaction mixture was stirred at room temperature for 12 h, and concentrated.

The residue was diluted with dichloromethane (80 mL) and washed with water (60 mL). The aqueous layer was extracted with dichloromethane (50 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 10:1) to give compound **36** (1.109 g, 81%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68–7.63 (m, 2H, Ph), 7.40–7.28 (m, 13H, Ph), 7.25–7.21 (m, 2H, Ph), 6.94–6.88 (m, 2H, Ph), 4.93–4.85 (m, 2H, PMB), 4.82 (d, *J* = 10.9 Hz, 1H, Bn), 4.61 (d, *J* = 11.3 Hz, 2H, Bn), 4.51 (d, *J* = 11.6 Hz, 1H), Bn, 4.46 (d, *J* = 10.1 Hz, 1H, H-1), 3.83 (s, 3H, -OMe), 3.64 (dd, *J* = 9.7, 8.9 Hz, 1H, H-4), 3.56 (d, *J* = 9.2 Hz, 1H), H-3, 3.49 (d, *J* = 9.6 Hz, 1H, H-5), 3.40 (dd, *J* = 10.1, 9.2 Hz, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.29, 137.97, 137.73, 133.64, 131.39, 130.36, 129.37, 129.07, 128.58, 128.52, 128.40, 128.26, 128.07, 127.94, 127.90, 113.86, 86.09, 85.14, 79.35, 77.63, 75.92, 75.07, 73.12, 65.19, 55.34. HRMS (ESI, positive) calculated for $C_{34}H_{33}D_2N_3O_5S^+$ $[M + Na]^+$ 622.2315, found 622.2317.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl-d2)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (37): Compound **36** (1.040 g, 1.73 mmol) and compound **14** (0.431 g, 1.44 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **36** and **14** in anhydrous diethyl ether (18 mL) and anhydrous dichloromethane (6 mL) was then added to preactivated 4 Å molecular sieves. After stirring for 30 min at room temperature, the mixture was cooled to –40 °C. *N*-Iodosuccinimide (0.845 g, 3.75 mmol) was added, and the reaction mixture was stirred for 30 min. Trifluoromethanesulfonic acid (0.08 mL, 0.87 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. The reaction solution was diluted with ether and filtered. The residue was washed with 10% aqueous solution

of sodium bisulfite (20 mL) and saturated aqueous solution of sodium bicarbonate (20 mL). The combined aqueous solutions were extracted with dichloromethane (40 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to provide compound **37** (0.682 g, 60%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41–7.23 (m, 12H, Ph), 7.15–7.13 (m, 2H, Ph), 6.86–6.84 (m, 2H, Ph), 5.18–5.11 (m, 2H, H-5, H-1'), 4.94 (t, *J* = 10.0 Hz, 1H, H-6), 4.89 (d, *J* = 10.5 Hz, 1H, PMB), 4.83 (d, *J* = 10.7 Hz, 1H, PMB), 4.77 (d, *J* = 10.9 Hz, 1H, Bn), 4.61 (d, *J* = 11.8 Hz, 1H, Bn), 4.49 (d, *J* = 10.9 Hz, 1H, Bn), 4.42 (d, *J* = 11.8 Hz, 1H, Bn), 4.15 (d, *J* = 10.1 Hz, 1H, H-5'), 3.96 (dd, *J* = 10.5, 8.9 Hz, 1H, H-3'), 3.84–3.72 (m, 4H, -OCH', H-4'), 3.68–3.56 (m, 2H, H-1, H-4), 3.46–3.33 (m, 2H, H-3, H-2'), 2.40 (dt, *J* = 13.3, 4.5 Hz, 1H, H-2), 2.09 (d, *J* = 3.6 Hz, 6H, OAc), 1.60 (q, *J* = 12.6 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.86, 169.52, 159.31, 137.92, 137.80, 129.82, 129.67, 128.48, 128.47, 128.45, 128.39, 128.38, 127.99, 127.88, 127.77, 127.68, 127.63, 113.80, 99.40, 79.56, 78.28, 78.08, 75.33, 74.93, 74.11, 73.54, 73.15, 71.55, 63.12, 58.68, 57.71, 55.21, 31.82, 20.66, 20.60. HRMS (ESI, positive) calculated for $C_{38}H_{41}D_2N_9O_{10}^+$ $[M + Na]^+$ 810.3151, found 810.3144.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(hydroxymethyl-d2)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (38): 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (0.250 g, 1.10 mmol) was added to a solution of compound **37** (0.5784 g, 0.734 mmol) in dichloromethane (20 mL) and water (2 mL). The reaction mixture was stirred for 4 h at room temperature and quenched by adding saturated aqueous solution of sodium bicarbonate (20 mL). The aqueous layer was extracted with dichloromethane (30 mL × 3), and the combined organic phase was washed with brine, dried over magnesium sulfate, filtered, and concentrated under

reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **38** (0.367 g, 75%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.28 (m, 10H, Ph), 5.14 (t, *J* = 9.8 Hz, 1H, H-5), 5.08 (d, *J* = 3.9 Hz, 1H, H-1'), 4.92 (dd, *J* = 14.0, 10.3 Hz, 2H, H-6, Bn), 4.89–4.82 (m, 2H, Bn), 4.70 (d, *J* = 11.1 Hz, 1H, Bn), 4.06 (dd, *J* = 10.1, 0.5 Hz, 1H, H-5'), 3.98 (dd, *J* = 10.4, 8.9 Hz, 1H, H-3'), 3.68–3.57 (m, 3H, H-4', H-1, H-4), 3.42 (ddd, *J* = 12.4, 9.9, 4.6 Hz, 1H, H-3), 3.32 (dd, *J* = 10.4, 3.8 Hz, 1H, H-2'), 2.40 (dt, *J* = 13.4, 4.6 Hz, 1H, H-2), 2.09 (s, 6H, OAc), 1.59 (dt, *J* = 13.5, 12.5 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.02, 169.65, 137.85, 137.80, 128.70, 128.63, 128.17, 128.16, 128.08, 128.06, 99.58, 79.67, 78.81, 77.83, 75.55, 75.21, 74.23, 73.58, 72.30, 63.41, 58.90, 57.80, 32.02, 20.80, 20.72. HRMS (ESI, positive) calculated for $C_{30}H_{33}D_2N_9O_9^+$ $[M + Na]^+$ 690.2575, found 690.2578.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6S*)-3-azido-4,5-bis(benzyloxy)-6-(fluoromethyl-d2)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (39): Diethylaminosulfur trifluoride (DAST) (0.104 mL, 0.786 mmol) was added to a solution of compound **38** (0.262 g, 0.393 mmol) in dichloromethane (5 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 12 h. The reaction was quenched by adding saturated aqueous solution of sodium bicarbonate (10 mL). The aqueous layer was extracted with dichloromethane (20 mL × 3). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to provide compound **39** (0.171 g, 65%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.28 (m, 10H, Bn), 5.19–5.09 (m, 2H, H-1', H-5), 4.98–4.82 (m, 4H, H-6, 3×Bn), 4.67 (d, *J* = 11.0 Hz, 1H, Bn), 4.16 (dd, *J* = 31.2, 10.2 Hz, 1H, H-5'), 4.02–3.94 (m, 1H, H-3'), 3.72–3.58 (m, 3H, H-4', H-4, H-1), 3.41 (ddd, *J* = 12.4, 10.0,

4.5 Hz, 1H, H-2'), 3.35 (dd, *J* = 10.4, 3.8 Hz, 1H, H-3), 2.40 (dt, *J* = 13.4, 4.6 Hz, 1H, H-2), 2.09 (d, *J* = 0.8 Hz, 6H, OAc), 1.61 (q, *J* = 12.7 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.96 (Ac), 169.63 (Ac), 137.70 (Ph), 137.65 (Ph), 128.69 (Ph), 128.63 (Ph), 128.17 (Ph), 128.12 (Ph), 128.11 (Ph), 127.93 (Ph), 99.47 (C-1'), 79.61 (C-3'), 78.60 (C-4), 77.48 (C-4') , 75.56 (Bn), 75.40 (Bn), 74.19 (C-6), 73.55 (C-5), 71.20 (d, *J* = 18.2 Hz, C-5'), 63.19 (C-2'), 58.72 (C-3), 57.79 (C-1), 31.86 (C-2), 20.77 (Ac), 20.70 (Ac). ^{19}F NMR (375 MHz, $CDCl_3$) δ -236.30 (d, *J* = 31.3 Hz). HRMS (ESI, positive) calculated for $C_{30}H_{32}D_2FN_9O_8^+$ $[M + Na]^+$ 692.2532, found 692.2525.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6S*)-3-azido-4,5-bis(benzyloxy)-6-(fluoromethyl-d2)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diol (40): Sodium methoxide (4.37 M in methanol, 0.004 mL, 0.019 mmol) was added to a solution of compound **39** (0.043 g, 0.065 mmol) in tetrahydrofuran (0.5 mL) and methanol (4.5 mL). The reaction mixture was stirred at room temperature for 5 h, neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **40** (0.033 g, 85%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41–7.29 (m, 10H, Bn), 5.19 (d, *J* = 3.7 Hz, 1H, H-1'), 4.95 (d, *J* = 10.6 Hz, 1H, Bn), 4.92–4.85 (m, 2H, Bn), 4.69 (d, *J* = 10.9 Hz, 1H, Bn), 4.17 (s, 1H, OH), 4.13–3.98 (m, 2H, H-5', H-3'), 3.71 (dd, *J* = 10.2, 9.0 Hz, 1H, H-4'), 3.63 (dd, *J* = 10.2, 3.6 Hz, 1H, H-2'), 3.55–3.36 (m, 3H, H-4, H-5, H-1), 3.35–3.22 (m, 2H), H-6, H-3, 3.17 (s, 1H, OH), 2.31 (dt, *J* = 12.9, 4.0 Hz, 1H, H-2), 1.57–1.42 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.58 (Ph), 137.48 (Ph), 128.71 (Ph), 128.65 (Ph), 128.20 (PH), 128.19 (Ph), 127.92 (Ph), 99.73 (C-1'), 83.97 (C-6), 80.90 (C-3'), 77.45 (C-4'), 76.09 (Bn), 75.84 (C-4), 75.42 (Bn), 75.40 (C-5), 71.21 (d, *J* = 18.1 Hz, C-5'), 64.24 (C-2'), 59.78 (C-1), 58.90 (C-3), 32.10 (C-2).

^{19}F NMR (375 MHz, $CDCl_3$) δ -235.84 (d, *J* = 31.1 Hz). HRMS (ESI, positive) calculated for $C_{26}H_{28}D_2FN_9O_6^+$ $[M + Na]^+$ 608.2321, found 608.2313.

(*3aR,6R,7R,7aR*)-7-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((2S,3R,4R,5S,6-S)-3-azido-4,5-bis(benzyloxy)-6-(fluoromethyl-d2)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-1,3a-dimethylhexahydro-2*H*-pyrano[4,3-d]oxazol-2-one (41): Compound **40** (0.418 g, 0.714 mmol) and compound **17** (0.342 g, 0.856 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **40** and **17** in anhydrous diethyl ether (10 mL) and anhydrous dichloromethane (2 mL) was added to a preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.321 g, 1.427 mmol). After stirring 30 min at room temperature, the mixture was cooled to –40 °C. Silver trifluoromethanesulfonate (0.367 g, 1.427 mmol) was added, and the reaction was warmed to –10 °C to stand for 12 h. The reaction mixture was diluted with ethyl acetate (20 mL) and filtered through a Celite pad. The solution was washed with 10% aqueous solution of sodium bisulfite (20 mL) and saturated aqueous solution of sodium bicarbonate (20 mL). The combined aqueous layers were extracted with ethyl acetate (30 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to provide compound **41** (0.387 g, 63%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.29 (m, 15H, Ph), 5.60 (d, *J* = 3.7 Hz, 1H, H-1'), 5.03 (d, *J* = 3.2 Hz, 1H, H-1''), 4.92–4.86 (m, 4H, 4×Bn), 4.68 (d, *J* = 11.1 Hz, 1H, Bn), 4.61 (d, *J* = 11.6 Hz, 1H, Bn), 4.22–4.17 (m, 2H, H-5'',OH), 4.13 (dd, *J* = 30.6, 10.2 Hz, 1H, H-5'), 4.07–4.00 (m, 1H, H-3'), 3.95 (dd, *J* = 4.4, 3.2 Hz, 1H, H-2''), 3.80 (d, *J* = 12.4 Hz, 1H, H-5''), 3.73–3.63 (m, 2H, H-4', H-5), 3.53–3.38 (m, 5H, H-1, H-3'', H-4, H-2', H-6), 3.35 (ddd, *J* = 12.4, 10.0, 4.4 Hz, 1H, H-3), 2.83 (s, 3H, NMe), 2.33 (dt, *J* = 13.2, 4.5 Hz, 1H. H-2), 1.51 (q, *J*

= 12.6 Hz, 1H, H-2), 1.41 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.28 (N(C(=O)O), 137.85 (Ph), 137.82 (Ph), 137.41 (Ph), 128.73 (Ph), 128.64 (Ph), 128.61 (Ph), 128.39 (Ph), 128.15 (Ph), 128.08 (Ph), 128.05 (Ph), 128.02 (Ph), 128.01 (Ph), 127.91 (Ph), 98.54 (C-1'), 97.00 (C-1''), 84.60 (C-6), 80.50 (C-4), 80.15 (C-3'), 77.34 (C-4'), 77.22 (C-4''), 75.63 (Bn), 75.32 (Bn), 75.12 (C-5), 74.62 (Bn), 73.41 (C-2''), 70.93 (d, *J* = 18.2 Hz, C-5'), 66.87 (C-5''), 63.57 (C-2''), 63.03 (C-3''), 59.78 (C-1), 58.76 (C-3), 32.08 (C-2), 30.32 (NMe), 23.63 (Me). HRMS (ESI, positive) calculated for $C_{41}H_{45}D_2FN_{10}O_{10}^+$ $[M + H]^+$ 883.3478, found 883.3499.

(*3R,4R,5R,6R*)-5-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6S*)-3-azido-4,5-bis(benzyloxy)-6-(fluoromethyl-d2)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-3-methyl-4-(methylamino)tetrahydro-2*H*-pyran-3-ol (42): A 2 N aqueous solution of sodium hydroxide (5 mL) was added to a solution of compound **41** (0.303 g, 0.352 mmol) in ethanol (15 mL). The reaction mixture was stirred at 90 °C for 10 h, and cooled to room temperature. The solution was diluted with ethyl acetate (15 mL) and washed with water (15 mL). The residue was washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (5–6% methanol in dichloromethane) to provide compound **42** (0.221 g, 75%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.30 (m, 15H, Ph), 5.55 (d, *J* = 3.7 Hz, 1H, H-1'), 5.22 (d, *J* = 3.3 Hz, 1H, H-1''), 4.89 (d, *J* = 8.5 Hz, 3H, 3×Bn), 4.79 (d, *J* = 11.7 Hz, 1H, Bn), 4.67 (d, *J* = 11.0 Hz, 1H, Bn), 4.51 (d, *J* = 11.7 Hz, 1H, Bn), 4.13 (dd, *J* = 30.7, 10.2 Hz, 1H, H-5'), 4.02 (dd, *J* = 10.2, 9.0 Hz, 1H, H-3'), 3.91 (d, *J* = 12.3 Hz, 1H, H-5''), 3.72–3.58 (m, 3H, H-5, H-2'', H-4), 3.56–3.48 (m, 2H, H-5'', H-1), 3.47–3.39 (m, 2H, H-2', H-4), 3.37–3.26 (m, 2H, H-3, H-6), 2.68 (d, *J* = 10.3 Hz, 1H, H-3''), 2.53 (s, 3H, NMe), 2.34 (dt, *J* = 13.2, 4.5 Hz, 1H, H-2), 1.52 (q, *J* = 12.7 Hz, 1H, H-2), 1.18 (s, 3H, Me). ^{13}C NMR (125 MHz,

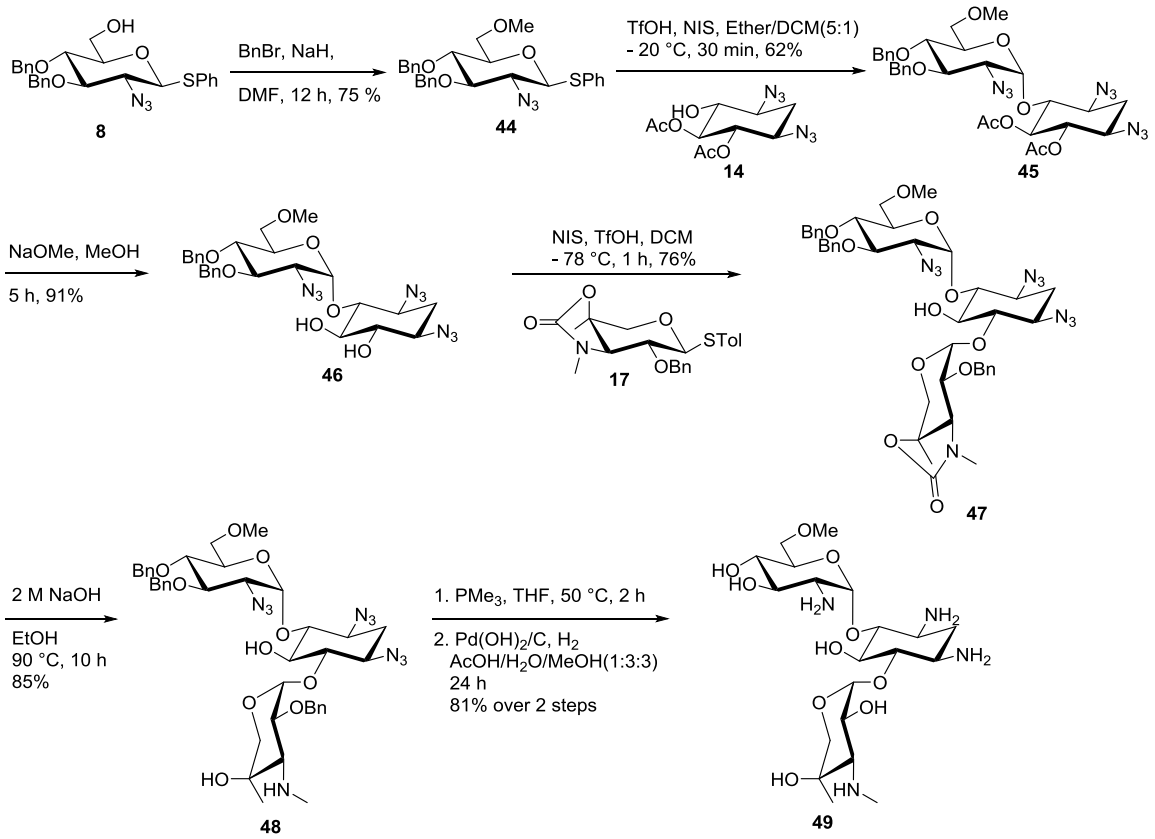
$CDCl_3$) δ 137.82 (Ph), 137.80 (Ph), 137.79 (Ph), 128.76 (Ph), 128.66 (Ph), 128.62 (Ph), 128.31 (Ph), 128.22 (Ph), 128.16 (Ph), 128.11 (Ph), 128.07 (Ph), 127.92 (Ph), 98.78 (C-1'), 97.30 (C-1''), 84.38 (C-6), 80.87 (C-4), 80.24 (C-3'), 77.34 (C-4'), 76.14 (C-2''), 75.71 (C-5), 75.67 (Bn), 75.34 (Bn), 72.54 (Bn), 70.87 (d, J = 18.3, C-5'), 66.80 (C-5''), 63.63 (C-2'), 63.30 (C-3''), 59.69 (C-1), 58.88 (C-3), 38.66 (C-2), 32.26 (NMe), 24.53 (Me). ^{19}F NMR (375 MHz, $CDCl_3$) δ -235.80 (d, J = 30.7 Hz). HRMS (ESI, positive) calculated for $C_{40}H_{47}D_2FN_{10}O_9^+$ $[M + H]^+$ 837.3686, found 857.3686.

[6',6',6'-^{2}H, ^{2}H,F]-gentamicin X_2 (43): Trimethylphosphine (1 M solution in THF, 0.8 mL, 0.789 mmol) was added to a solution of compound **42** (0.109 mg, 0.132 mmol) in THF (4 mL) and 0.1 M aqueous solution of NaOH (1.3 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic acid (2 mL), water (6 mL) and methanol (6 mL). The solution was degassed by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.05 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (5% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **43** (0.049 g, 76%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.79 (d, J = 4.0 Hz, 1H, H-1'), 5.14 (d, J = 3.6 Hz, 1H, H-1''), 4.25 (dd, J = 10.9, 3.7 Hz, 1H, H-2''), 4.09–4.02 (m, 2H, H-5', H-5''), 4.01–3.96 (m, 2H, H-3', H-4), 3.90 (t, J = 9.1 Hz, 1H, H-5), 3.84 (dd, J = 10.2, 8.9 Hz, 1H, H-6), 3.69–3.59 (m, 3H, H-1, H-3, H-4'), 3.56 (d, J = 11.0 ppm, 1H, H-3''), 3.55 (d, J = 12.9 Hz, 1H, H-5''), 3.51 (dd, J = 10.9, 4.1 Hz, 1H, H-

2'), 2.95 (s, 3H, NMe), 2.59 (dt, *J* = 12.5, 4.2 Hz, 1H, H-2), 1.97 (q, *J* = 12.6 Hz, 1H, H-2), 1.38 (d, *J* = 1.5 Hz, 3H, Me). ^{13}C NMR (125 MHz, D_2O) δ 101.08 (C-1"), 96.85 (C-1'), 83.44 (C-6), 79.52 (C-4), 73.84 (C-5), 72.10 (d, *J* = 17.8 Hz, C-5'), 69.85 (C-4"), 68.77 (C-3'), 68.17 (d, J = 7.4 Hz, C-4'), 66.17 (C-2"), 63.19 (C-3"), 53.69 (C-2'), 49.46 (C-1), 48.36 (C-3), 34.47 (NMe), 27.81 (C-2), 20.89 (Me). ^{19}F NMR (565 MHz, D_2O) δ -234.65 (d, *J* = 25.0 Hz). HRMS (ESI, positive) calculated for $C_{19}H_{35}D_2FN_4O_9^+$ $[M + H]^+$ 487.2743, found 487.2742.

3.2.8. Synthesis of [6'-OMe]-Gentamicin X_2 (49)

The overall synthetic scheme is shown in Scheme 3-5.



Scheme 3-5. Synthetic scheme for the preparation of [6'-OMe]-GenX_2 (**49**).

(*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(methoxymethyl)-2-(phenylthio)-tetr-ahydro-2*H*-pyran (44): Sodium hydride (60% dispersion in mineral oil, 0.267 g, 6.34 mmol) was added to a solution of compound **8** (1.515 g, 3.17 mmol) in dimethylformamide (30 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h. Iodomethane (0..40 mL, 6.34 mmol) was added at 0 °C and the reaction mixture was stirred at room temperature for 12 h. At 0 °C, methanol (10 mL) was added slowly and the reaction mixture was stirred at room temperature for additional 30 min. After being concentrated under reduced pressure, the mixture was diluted with dichloromethane (70 mL) and water (70 mL). The aqueous layer was extracted with dichloromethane (60 mL × 3). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to give compound **44** (1.17 g, 75%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75–7.70 (m, 2H, Ph), 7.48–7.36 (m, 13H, Ph), 5.00–4.89 (m, 3H, 3×Bn), 4.75 (d, J = 11.0 Hz, 1H, Bn), 4.53 (d, J = 10.2 Hz, 1H, H-1), 3.75 (d, J = 3.1 Hz, 2H, H-6), 3.71 (t, J = 9.4 Hz, 1H, H-4), 3.62 (t, J = 9.1 Hz, 1H, H-3), 3.54–3.45 (m, 5H, H-5, OMe, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.89 (Ph), 137.60 (Ph), 133.25 (Ph), 131.51 (Ph), 128.90 (Ph), 128.40 (Ph), 128.39 (Ph), 128.16 (Ph), 128.06 (Ph), 127.87 (Ph), 127.80 (Ph), 127.72 (Ph), 86.17 (C-1), 84.93(C-3), 79.16 (C-5), 77.37 (C-4), 75.68 (Bn), 74.87 (Bn), 70.96 (C-6), 65.11 (C-2), 59.30 (OMe). HRMS (ESI, positive) calculated for $C_{27}H_{29}N_3O_4S^+$ $[M + Na]^+$ 514.1771, found 514.1784.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(methoxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (45): Compound **44** (1.413 g, 2.83 mmol) and compound **14** (0.714 g, 2.39 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **44** and **14** in anhydrous diethyl ether (30 mL) and anhydrous dichloromethane (10 mL) was then added to preactivated 4 Å molecular sieves. After stirring for 30 min at room temperature, the mixture was cooled to –40 °C. *N*-Iodosuccinimide (1.401 g, 6.23 mmol) was added, and the reaction mixture was stirred for 30 min. Trifluoromethanesulfonic acid (0.11 mL, 0.120 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. The reaction solution was diluted with diethyl ether (40 mL) and filtered. The residue was washed with 10% aqueous solution of sodium bisulfite (50 mL) and saturated aqueous solution of sodium bicarbonate (50 mL). The combined aqueous solutions were extracted with dichloromethane (60 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to provide compound **45** (1.009 g, 62%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.27 (m, 10, Ph), 5.18–5.10 (m, 2H, H-5, H-1'), 4.98–4.88 (m, 2H, Bn, H-6), 4.88–4.80 (m, 2H, 2×Bn), 4.66 (d, *J* = 11.0 Hz, 1H, Bn), 4.13 (dt, *J* = 10.2, 2.3 Hz, 1H, H-5'), 3.96 (dd, *J* = 10.4, 8.9 Hz, 1H, H-3'), 3.79–3.69 (m, 2H, H-4', H-6'), 3.67–3.55 (m, 3H, H-4, H-1, H-6'), 3.46–3.32 (m, 5H, H-3, OMe, H-2'), 2.39 (dt, *J* = 13.4, 4.6 Hz, 1H, H-2), 2.09 (s, 3H, OAc), 2.08 (s, 3H, OAc), 1.60 (q, *J* = 12.8 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.88 (OAc), 169.56 (OAc), 138.02 (Ph), 137.84 (Ph), 128.55 (Ph), 128.50 (Ph), 128.03 (Ph), 127.93 (Ph), 127.92 (Ph), 127.84 (Ph), 99.51 (C-1'), 79.56 (C-3'), 78.45 (C-4), 78.08 (C-4'), 75.35 (Bn), 75.09 (Bn), 74.13 (C-6), 73.55 (C-5), 71.57 (C-5'), 70.36 (C-6'), 63.15

(C-2'), 59.26 (OMe), 58.70 (C-3), 57.73 (C-1), 31.82 (C-2), 20.67(OAc), 20.62 (OAc). HRMS (ESI, positive) calculated for $C_{31}H_{37}N_9O_9^+$ $[M + Na]^+$ 702.2606, found 702.2604.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(methoxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)cyclohexane-1,2-diol (46): Sodium methoxide (4.37 M in methanol, 0.04 mL, 0.086 mmol) was added to a solution of compound **45** (0.585 g, 0.861 mmol) in methanol (20 mL). The reaction mixture was stirred at room temperature for 5 h, neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 1:1) to give compound **46** (0.468 g, 91%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.29 (m, 10H, Ph), 5.16 (d, *J* = 3.7 Hz, 1H, H-1'), 4.94 (d, *J* = 10.6 Hz, 1H, Bn), 4.86 (d, *J* = 10.6 Hz, 1H, Bn), 4.85 (d, *J* = 11.0 Hz, 1H, Bn), 4.66 (d, *J* = 11.0 Hz, 1H, Bn), 4.25 (d, *J* = 2.3 Hz, 1H, OH), 4.07 (ddd, *J* = 10.1, 2.9, 1.9 Hz, 1H, H-5'), 3.99 (dd, *J* = 10.2, 9.0 Hz, 1H, H-3', H-3'), 3.75 (dd, *J* = 10.1, 9.0 Hz, 1H, H-4'), 3.71 (dd, *J* = 10.7, 3.2 Hz, 1H, H-6'), 3.63 (dd, *J* = 10.2, 3.7 Hz, 1H, H-2', H-2'), 3.59 (dd, *J* = 10.7, 2.0 Hz, 1H, H-6'), 3.52–3.38 (m, 3H, H-5, H-6, H-1), 3.37 (s, 3H, OMe), 3.28–3.25 (m, 3H, H-3, H-4, OH), 2.33–2.25 (m, 1H, H-2), 1.56 – 1.43 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.98 (Ph), 137.67 (Ph), 128.63 (Ph), 128.57 (Ph), 128.16 (Ph), 128.05 (Ph), 128.02 (Ph), 127.89 (Ph), 99.74 (C-1'), 83.96 (C-4), 80.98 (C-3'), 78.15 (C-4'), 76.05 (C-5), 75.70 (Bn), 75.42 (C-6), 75.17 (Bn), 71.60 (C-5'), 70.56 (C-6'), 64.31 (C-2'), 59.75 (C-1), 59.32 (OMe), 58.92 (C-3), 32.18 (C-2). HRMS (ESI, positive) calculated for $C_{27}H_{33}N_9O_7^+$ $[M + Na]^+$ 618.2395, found 618.2396.

(*3aR,6R,7R,7aR*)-7-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6-R*)-3-azido-4,5-bis(benzyloxy)-6-(methoxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-1,3a-dimethylhexahydro-2*H*-pyrano[4,3-d]oxazol-2-one

(47): Compound **46** (0.432 g, 0.726 mmol) and compound **17** (0.319 g, 0.798 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **46** and **17** in anhydrous dichloromethane (10 mL) was added to a preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.261 g, 1.161 mmol). After stirring 30 min at room temperature, the mixture was cooled to –78 °C. Trifluoromethanesulfonic acid (0.02 mL, 0.217 mmol) was added slowly, and the reaction was warmed to 0 °C during 1 h. Sodium sulfite (0.1 g), sodium bicarbonate (0.1 g), and few drops of water were added to the reaction solution. The reaction mixture was diluted with dichloromethane (20 mL) and filtered through a Celite pad. The solution was washed with saturated aqueous solution of sodium bicarbonate (30 mL). The combined aqueous layers were extracted with dichloromethane (30 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 1:1) to provide compound **47** (0.480 g, 76%) as a clear solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.29 (m, 15H, Ph), 5.53 (d, *J* = 3.7 Hz, 1H, H-1'), 5.07 (d, *J* = 3.2 Hz, 1H, H-1''), 4.93–4.83 (m, 4H, 4×Bn), 4.66 (d, *J* = 11.1 Hz, 1H, Bn), 4.60 (d, *J* = 11.6 Hz, 1H, Bn), 4.21 (d, *J* = 12.4 Hz, 1H, H-5''), 4.17 (d, *J* = 1.6 Hz, 1H, OH), 4.13–4.07 (m, 1H, H-5'), 4.00 (dd, *J* = 10.3, 9.0 Hz, 1H, H-3'), 3.93 (dd, *J* = 4.6, 3.2 Hz, 1H, H-2''), 3.79 (d, *J* = 12.6 Hz, 1H, H-5''), 3.76–3.63 (m, 3H, H-4', H-6', H-5), 3.58 (dd, *J* = 10.6, 2.0 Hz, 1H, H-6'), 3.53–3.39 (m, 5H, H-1, H-3'', H-6, H-2', H-4), 3.40–3.30 (m, 4H, OMe, H-3), 2.84 (s, 3H, NMe), 2.33 (dt, *J* = 13.2, 4.5 Hz, 1H, H-2), 1.60–1.44 (m, 1H, H-2), 1.40 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.30 (N(C=O)O), 138.20 (Ph), 138.00(Ph), 137.41 (Ph), 128.70 (Ph), 128.57 (Ph), 128.53 (Ph), 128.34 (Ph), 128.11 (Ph), 128.00 (Ph), 127.93 (Ph), 127.91 (Ph), 127.88 (Ph), 98.83 (C-1'), 96.77 (C-1''), 84.07 (C-6), 80.97 (C-4), 80.29 (C-3'), 78.12 (C-4'), 77.30 (C-4''), 75.51 (Bn), 75.11

(Bn), 75.05 (C-5), 74.45 (Bn), 73.70 (C-2''), 71.37 (C-5'), 70.66 (C-6'), 66.59 (C-5''), 63.71 (C-3''), 62.98 (C-2'), 59.86 (C-1), 59.32 (OMe), 58.77 (C-3), 32.11 (C-2), 30.33 (NMe), 23.56 (Me). HRMS (ESI, positive) calculated for $C_{42}H_{50}N_{10}O_{11}^{+}$ $[M + Na]^{+}$ 893.3553, found 893.3556.

(*3R,4R,5R,6R*)-5-(benzyloxy)-6-(((*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(methoxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-2-hydroxycyclohexyl)oxy)-3-methyl-4-(methylamino)tetrahydro-2*H*-pyran-3-ol (48): A 2 N aqueous solution of sodium hydroxide (8 mL) was added to a solution of compound **47** (0.431 g, 0.495 mmol) in ethanol (20 mL). The reaction mixture was stirred at 90 °C for 10 h, and cooled to room temperature. The solution was diluted with ethyl acetate (20 mL) and washed with water (20 mL). The residue was washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (5% methanol in dichloromethane) to provide compound **48** (0.355 g, 85%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.29 (m, 15H, Ph), 5.48 (d, *J* = 3.8 Hz, 1H, H-1'), 5.25 (d, *J* = 3.4 Hz, 1H, H-1''), 4.90 (d, *J* = 10.7 Hz, 1H, Bn), 4.86 (d, *J* = 10.8 Hz, 1H, Bn), 4.85 (d, *J* = 11.1 Hz, 1H, Bn) 4.80 (d, *J* = 11.7 Hz, 1H, Bn), 4.65 (d, *J* = 11.1 Hz, 1H), 4.49 (d, *J* = 11.8 Hz, 1H, Bn), 4.45–4.39 (m, 1H, OH), 4.10 (dt, *J* = 10.1, 2.5 Hz, 1H, H-5'), 3.99 (dd, *J* = 10.3, 9.0 Hz, 1H, H-3'), 3.94 (d, *J* = 12.3 Hz, 1H, H-5''), 3.77–3.71 (m, 1H, H-4'), 3.71–3.63 (m, 2H, H-6', H-5), 3.60–3.49 (m, 4H, H-2'', H-6', H-5'', H-1), 3.46 (dd, *J* = 10.3, 3.8 Hz, 1H, H-2'), 3.40 (dd, *J* = 10.1, 8.8 Hz, 1H, H-4), 3.37–3.30 (m, 5H, OMe, H-6, H-3), 2.66 (d, *J* = 10.3 Hz, 1H, H-3''), 2.52 (s, 3H, NMe), 2.35 (dt, *J* = 13.1, 4.4 Hz, 1H, H-2), 1.53 (dt, *J* = 13.2, 12.4 Hz, 1H, H-2), 1.16 (s, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.21 (Ph), 137.99 (Ph), 137.82 (Ph), 128.76 (Ph), 128.61 (Ph), 128.57 (Ph), 128.31 (Ph), 128.23 (Ph), 128.15 (Ph), 127.98 (Ph), 127.96 (Ph), 127.91 (Ph), 99.10 (C-

1'), 97.11 (C-1''), 83.66 (C-6), 81.46 (C-4), 80.43 (C-3'), 78.14 (C-4'), 76.17 (C-2''), 75.58 (C-5, Bn), 75.15 (Bn), 72.47 (Bn), 71.39 (C-5'), 71.08 (C-4''), 70.69 (C-6'), 66.64 (C-5''), 63.81 (C-2'), 63.24 (C-3''), 59.84 (C-1), 59.35 (OMe), 58.90 (C-3), 38.73 (NMe), 32.32 (C-2), 24.63 (Me). HRMS (ESI, positive) calculated for $C_{41}H_{52}N_{10}O_{10}^{+}$ $[M + Na]^{+}$ 867.3760, found 867.3764.

[6'-OMe]-Gentamicin X_2 (49): Trimethylphosphine (1 M solution in THF, 1.01 mL, 1.01 mmol) was added to a solution of compound **48** (0.143 mg, 0.169 mmol) in THF (6 mL) and 0.1 M aqueous solution of sodium hydroxide (2 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in a mixture of acetic acid (2 mL), water (6 mL) and methanol (6 mL). The solution was deaerated by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^{+} form) (5% concentrated ammonium hydroxide in water), concentrated under reduced pressure, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **49** (0.068 g, 81%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.63 (d, J = 4.0 Hz, 1H, H-1'), 5.12 (d, J = 3.7 Hz, 1H, C-1''), 4.22 (dd, J = 10.9, 3.7 Hz, 1H, H-2''), 4.04 (d, J = 12.8 Hz, 1H, H-5''), 3.98–3.94 (m, 1H, H-5'), 3.94–3.88 (m, 1H, H-3'), 3.86–3.82 (m, 2H, H-4, H-5), 3.82–3.75 (m, 2H, H-6', H-6), 3.66 (dd, J = 10.9, 6.6 Hz, 1H, H-6'), 3.62–3.47 (m, 5H, H-1, H-3'', H-5'', H-4', H-3), 3.44 (dd, J = 10.8, 4.0 Hz, 1H, H-2'), 3.41 (s, 3H, OMe), 2.93 (s, 3H, NMe), 2.50 (dt, J = 12.9, 4.3 Hz, 1H, H-2), 1.86 (q, J = 12.6 Hz, 1H, H-2), 1.36 (s, 3H, Me). ^{13}C NMR (125 MHz, D_2O) δ 100.90 (C-1''), 97.13

(C-1'), 83.66 (C-6), 81.34 (C-4), 73.73 (C-5), 72.22 (C-5'), 70.75 (C-6'), 69.84 (C-4''), 69.34 (C-4'), 69.13 (C-3'), 67.71 (C-5''), 66.19 (C-2''), 63.26 (C-3''), 58.72 (OMe), 53.94 (C-2'), 49.58 (C-1), 48.60 (C-3), 34.51 (NMe), 28.68 (C-2), 20.89 (Me). HRMS (ESI, positive) calculated for $C_{20}H_{40}N_4O_{10}^+$ $[M + Na]^+$ 519.2637, found 519.2642.

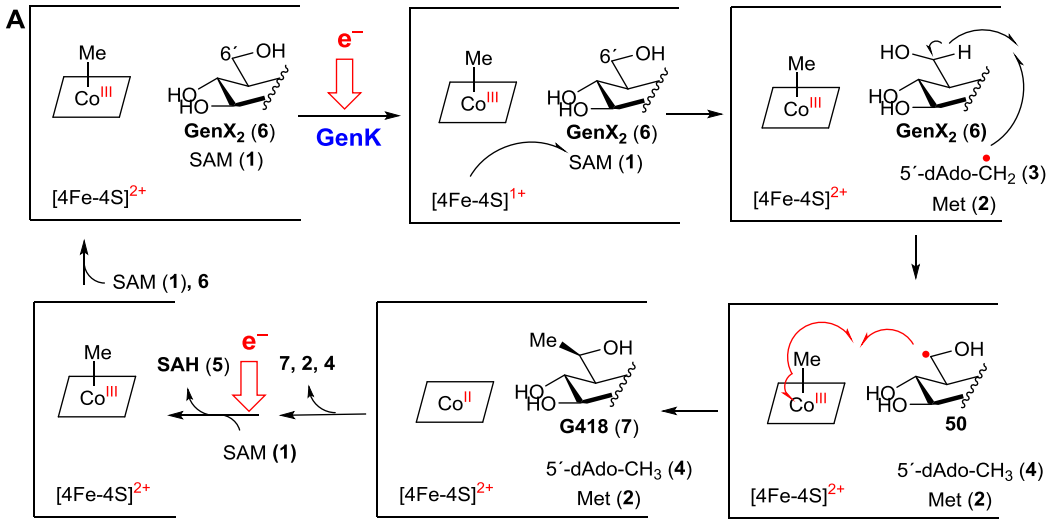
3.3. RESULTS AND DISCUSSION

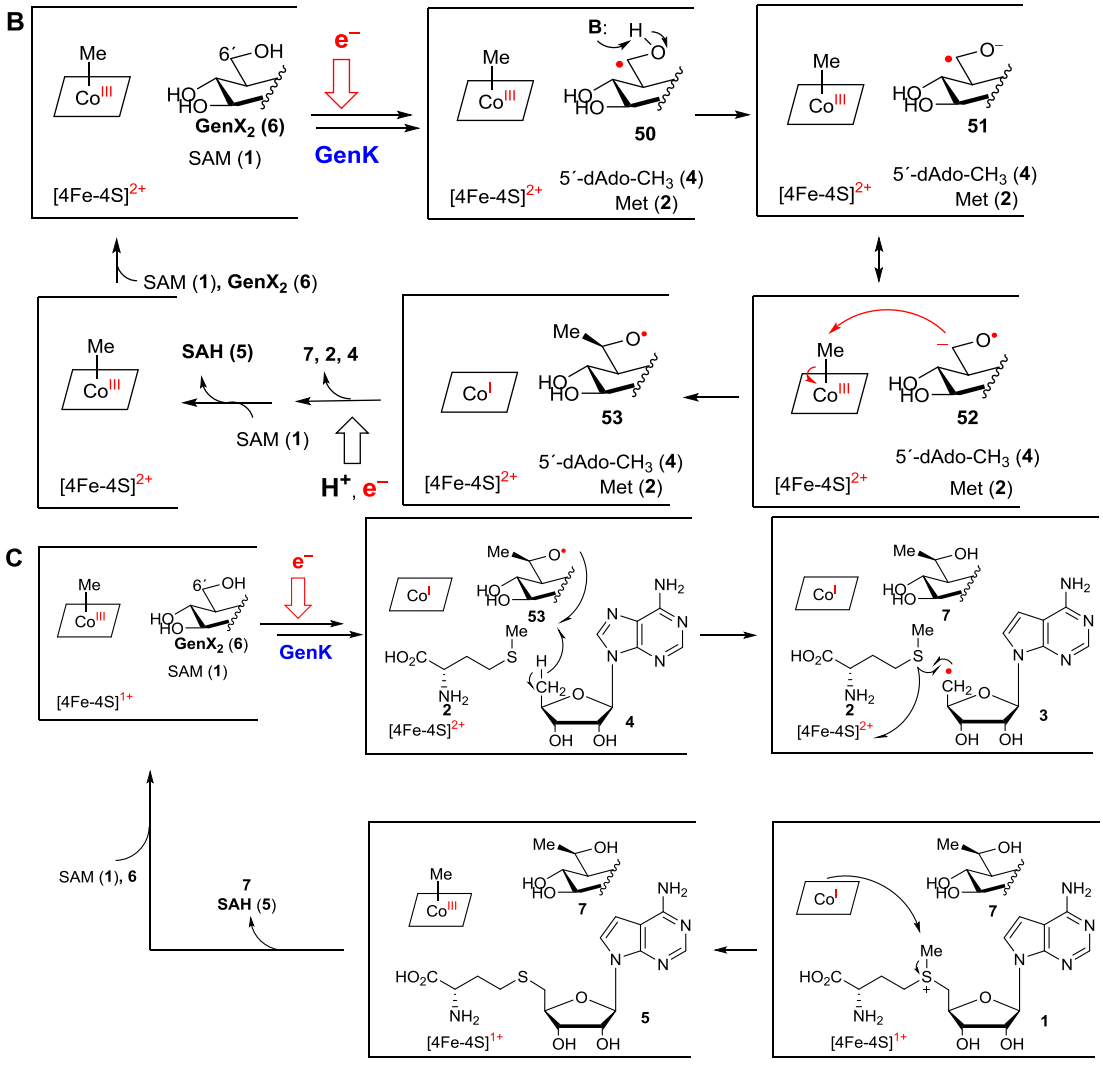
3.3.1. Proposed Mechanism

As discussed in Chapter 2, three mechanisms have been proposed for the GenK catalyzed methylation of $GenX_2$ (**6**) to form G418 (**7**) (Scheme 3-6). In the first mechanism (Scheme 3-6A), the 5'-dAdo• (**3**) is generated via reductive cleavage of SAM (**1**) and abstracts a hydrogen atom from C-6' of the substrate (**6**). The resulting radical (**50**) then accepts a methyl radical from Me-Cbl to generate Cbl(II) and the G418 product (**7**). A similar mechanism has been proposed for most class B radical SAM methyltransferases, such as Fom3,[130] GenD1,[81] and ThnK[132] as mentioned in Section 3.1. In this mechanism, MeCbl could be regenerated after each turnover via reduction of Cbl(II) to Cbl(I), followed by methylation by SAM (**1**).

Given that the transfer of a methyl radical is heretofore unprecedented among the Cbl-dependent methyltransferases, mechanisms involving the transfer of a methyl cation from Me-Cbl were also considered (Scheme 3-6B, C). The early steps of these alternative mechanism are identical to those of Scheme 3-6A. However, after hydrogen atom abstraction, the radical intermediate (**50**) is deprotonated to form a ketyl radical **51**/**52**, similar to those in the reactions catalyzed by DesII[152, 170–176] and (*R*)-2-hydroxyacyl-CoA dehydratase.[177–178] Nucleophilic attack by the ketyl radical anion (**52**) on Me-Cbl leads to the transfer of an electrophilic methyl cation, yielding a Cbl(I) intermediate (as in

traditional Me-Cbl chemistry)[179-180], and an alkoxy product radical (**53**). The alkoxy radical (**53**) could then be quenched by an external electron and a proton (Scheme 3-6B), or via hydrogen atom transfer from 5'-dAdo (**4**) (Scheme 3-6C). In this latter mechanism, SAM is regenerated while restoring the [4Fe-4S] cluster to the +1 redox state. The resultant SAM (**1**) could then methylate Cbl(I), releasing SAH (**5**) as the sole adenosylated product. Thus, in this mechanism, the input of exogenous electrons is not required and only a single equivalent of SAM (**1**) is consumed during each catalytic cycle. This is in contrast to mechanisms A and B, in which two equivalents of SAM (**1**) (in addition to two reducing equivalents) are consumed for each methyl transfer event catalyzed by GenK.





Scheme 3-6. Possible GenK reaction mechanisms. (A) $GenX_2$ radical (**50**) is quenched by the transfer of methyl radical from Me-Cbl(III) to give Cbl(II), which is reduced to Cbl(I) in order to accept a new methyl group from SAM (**1**). (B) Transfer of methyl cation to $GenX_2$ ketyl radical (**52**) followed by reduction and protonation of product radical (**53**). (C) Product radical (**53**) is quenched by a hydrogen from 5'-dAdo (**4**) to give 5'-dAdo• (**3**), which can be re-incorporated into SAM (**1**). In this mechanism, a single equivalent of SAM (**1**) can serve as a source of both 5'-dAdo• (**3**) and CH_3^+.

3.3.2. Syntheses of [6'*S*-^{2}H]-$GenX_2$ (20) and [6'*R*-^{2}H]-$GenX_2$ (29)

To characterize the initial hydrogen abstraction by 5'-dAdo• (**3**) and determine the stereochemistry of the methyltransfer reaction from $GenX_2$ (**6**) to produce G418 (**7**), two isotope-labeled compounds, [6'*S*-^{2}H]-$GenX_2$ (**20**) and [6'*R*-^{2}H]-$GenX_2$ (**29**), were examined. In the mechanism proposed for GenK in Section 3.3.1, hydrogen atom abstraction by 5'-dAdo• (**3**) occurs at the C6' position of $GenX_2$ (**6**). The syntheses of the two stereochemically labeled compounds began from the alcohol (**8**) which was used in the synthesis of gentamicin A_2 in Section 2.2.11. Scheme 3-1 in Section 3.2.4 and Scheme 3-2 in Section 3.2.5 show the overall schemes to synthesize [6'*S*-^{2}H]-$GenX_2$ (**20**) and [6'*R*-^{2}H]-$GenX_2$ (**29**) respectively. Oxidation using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and (diacetoxyiodo)benzene (BAIB) generated 6'-carboxylic acid,[181] and the following methylation reaction with iodomethane and mildly basic potassium carbonate converted the carboxylic acid to a methyl ester to provide compound **9**. The C6'-dideuterated alcohol (**10**) was then generated by the hydride reduction of the ester (**9**) with lithium aluminum tetradeuteride. The resulting dideuterated O-6 primary alcohol of compound **10** was oxidized to an aldehyde with one deuterium by Swern oxidation. Selective reduction with (*R*)-(+)-Alpine-Borane® gave [6'-*S*-^{2}H]-substrate (**12**).[176] The synthesis of [6'-*R*-^{2}H]-substrate (**23**) was attempted using (*S*)-(–)-Alpine-Borane®, however the diastereomer, [6'-*S*-^{2}H]-substrate (**12**), was also produced. In order to produce one stereospecific compound with (*R*) stereochemistry, an S_N2 type conversion was used instead to invert the (*S*)-isomer. From compound **12**, the formation of tosylate, followed by nucleophilic benzoate substitution, and basic hydrolysis with sodium methoxide was performed to get [6'-*R*-^{2}H]-substrate (**23**). The benzyl protection reaction on the primary alcohol gave tribenzylated compounds, **13** and **24**. NMR spectra in Figure 3-8 shows tribenzylated compounds, **13** and **24**, with varying stereochemisty. The compound **12** in Figure 3-8A was obtained from using (*R*)-(+)-Alpine-Borane®.

NMR spectrum displays only one stereoisomer (doublet at 3.72 ppm (J = 4.4 Hz) for the pro-(*S*) proton). The compounds in Figure 3-8B was acquired using (*S*)-(–)-Alphine-Borane®, and two diastereomers were found (doublet in 3.76 ppm (J = 1.9 Hz) for the pro-(*R*) proton and 3.72 ppm (J = 4.4 Hz) for the pro-(*S*) proton) with a ratio of *S* isomer/*R* isomer = 0.65/ 0.23 based on NMR integration. The compound in Figure 3-8C was obtained by the stereochemical inversion through S_N2 reaction, and its NMR spectrum indicates the presence of only a single diastereomer (doublet at 3.75 ppm (J = 1.9 Hz) having the pro-(*R*) proton at C6'.

After coupling to the 2-strepatamine derivative **14** and basic hydrolysis with sodium methoxide, another coupling reaction of compounds **16** and **26** with the garosamine derivative **17** gave pseudotrisaccharides **19** and **27** respectively. Basic hydrolysis, and a final deprotection reaction using a Staudinger reaction and hydrogenation using palladium hydroxide on carbon gave [6'*S*-^{2}H]-GenX_2 (**20**) and [6'*R*-^{2}H]-GenX_2 (**29**). The synthesis of [6'*S*-^{2}H]-GenX_2 (**20**) was completed with a 7.1% yield over 10 steps from the known compound **8**. The synthesis of [6'-*R*-^{2}H]-GenX_2 (**29**) was completed with a 3.4% yield over 13 steps from the intermediate **12** in the synthesis of [6'*S*-^{2}H]-GenX_2 (**20**).

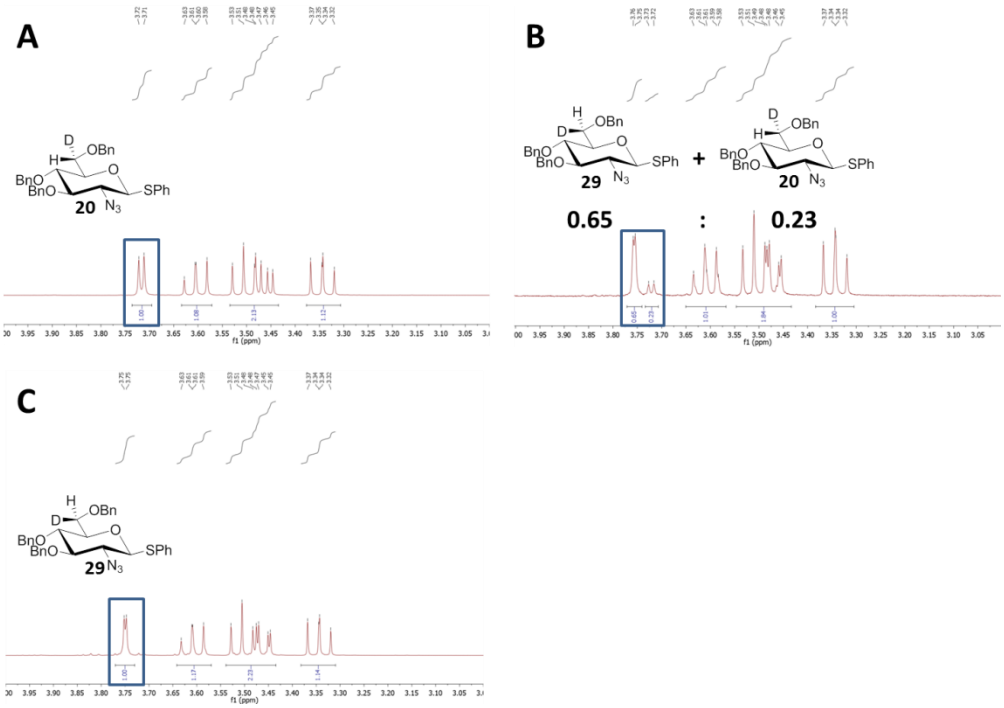


Figure 3-8. NMR spectra of two labeled compounds, **20** and **29** obtained from A) (*R*)-(+)-Alpine-Borane®, B) (*S*)-(–)-Alphine-Borane®, and C) (*R*)-(+)-Alpine-Borane® and stereochemical inversion through S_N2 reaction.

3.3.3. Stereospecific Hydrogen Abstraction

GenK assays with 6'-deuterated compounds, **20** and **29**, were conducted to determine the stereochemical course of the hydrogen abstraction step. Assay conditions are the same as the previously described GenK assays. Three substrates, $GenX_2$ (**6**), [6'*S*-^{2}H]-$GenX_2$ (**20**) and [6'*R*-^{2}H]-$GenX_2$ (**29**) were used in the assays. Detection of 5'-dAdo (**4**) by mass spectroscopy was conducted after collecting the corresponding fractions by HPLC (Figure 3-9).

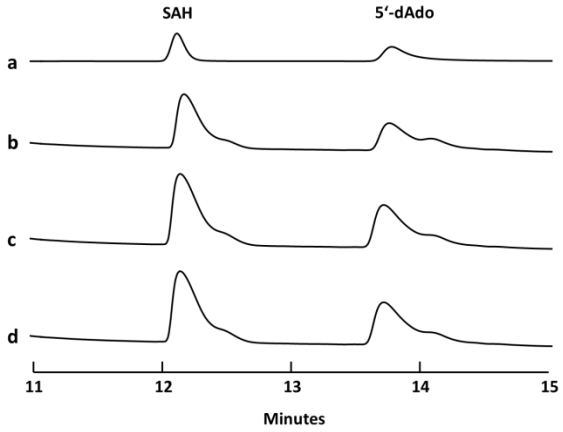


Figure 3-9. HPLC traces showing the production of 5'-dAdo (**4**) and SAH (**5**) during GenK assays with 6'-labeled compounds. Trace (a) a standard composed of authentic 5'-dAdo (**4**) and SAH (**5**). The remaining traces result from reaction mixtures containing (b) $GenX_2$ (**6**), (c) [6'*S*-^{2}H]-$GenX_2$ (**20**), and (c) [6'*R*-^{2}H]-$GenX_2$ (**29**)

Figure 3-10 shows the mass spectroscopic data used to identify 5'-dAdo (**4**; *m*/*z* calc. 252.2 $[M + H]^+$) when the three previously mentioned substrates were used ($GenX_2$ (**6**) in 3-10A, [6'*S*-^{2}H]-$GenX_2$ (**20**) in 3-10B, and [6'*R*-^{2}H]-$GenX_2$ (**21**) in 3-10C). When $GenX_2$ (**6**) and [6'*S*-^{2}H]-$GenX_2$ (**20**) were used for the GenK reaction, an *m*/*z* 252 peak was observed. However, when [6'*R*-^{2}H]-$GenX_2$ (**29**) was used, a m/z 253 peak was identified, indicating that deuterium from the substrate is incorporated into 5'-dAdo• (**3**) to form the deuterated 5'-dAdo (**54**; calc. $[M + H]^+$ 253.2 *m/z*). The small peak around 252 in Figure 3-10C might be due to uncoupled quenching of one electron reduction followed by protonation. These results demonstrate that the pro-*R* hydrogen at the C6' position of the substrate is abstracted by 5'-dAdo• (**4**) in the beginning of the GenK reaction.

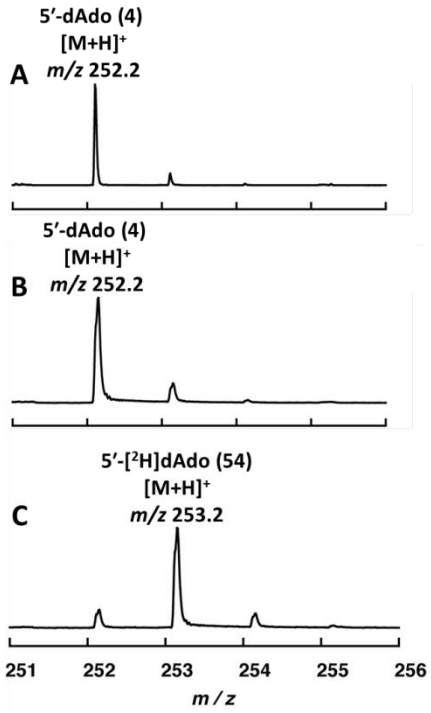


Figure 3-10. Electrospray ionization (ESI) mass data of resulting 5'-dAdo (**4**) generated in the assays of GenK with A) $GenX_2$ (**6**), B) [6'*S*-^{2}H]-$GenX_2$ (**20**), and C) [6'*R*-^{2}H]-$GenX_2$ (**29**).

In order to examine mechanism C outlined in Section 3.3.1 where SAM is regenerated and SAH is released at the end of each catalytic cycle, the reaction mixture was analyzed by ESIMS to check for SAH (**5**; calc. $[M + H]^+ = 385.1$ *m*/*z*)and SAM (**1**; calc. $[M + H]^+ = 399.1$ *m*/*z*) in the assays using $GenX_2$ (**6**), [6'*S*-^{2}H]-$GenX_2$ (**20**), and [6'*R*-^{2}H]-$GenX_2$ (**29**) as substrates (Figures 3-11 and 3-12). All three mass spectra appear to be the same in Figures 3-11 and 3-12, and there is no deuterium incorporation in SAH (**5**) and SAM (**1**). These results combined with the observation that 5'-dAdo (**4**), SAH (**5**), and G418 (**7**) are each produced in equivalent amounts (See Section 2.3.6) indicate that mechanism C can be ruled out. Two SAM molecules are consumed per reaction. The first molecule of SAM (**1**) acts as the source of the 5'-dAdo• (**3**), which abstracts a hydrogen atom from C-6' of $GenX_2$ (**6**) to give 5'-dAdo (**4**) and the $GenX_2$ radical (**3**). The second

SAM (**1**) is used to re-methylate Cbl(I) during turnover to regenerate Me-Cbl for the subsequent round of catalysis.

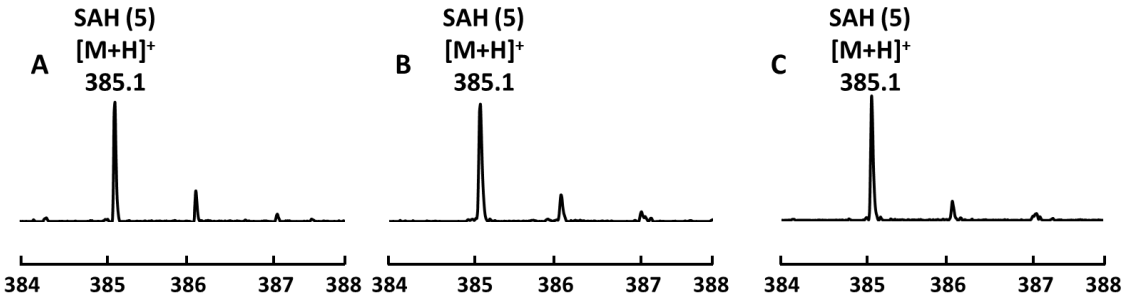


Figure 3-11. ESI Mass data of resulting SAH (**5**; calc. $[M + H]^+ = 385.1$ *m/z*) in the assays using A) $GenX_2$ (**6**), B) [6'*S*-^{2}H]-$GenX_2$ (**20**), and C) [6'*R*-^{2}H]-$GenX_2$ (**29**).

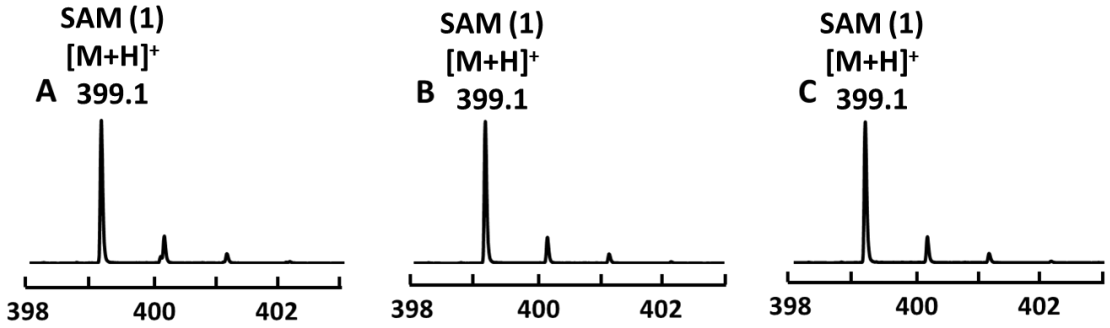


Figure 3-12. ESI mass data of resulting SAM (**1**; calc. $[M + H]^+ = 399.1$ *m/z*) in the assays using A) $GenX_2$ (**6**), B) [6'*S*-^{2}H]-$GenX_2$ (**20**), and C) [6'*R*-^{2}H]-$GenX_2$ (**29**).

Figure 3-13 shows mass spectra of the product in the assays using $GenX_2$ (**6**), [6'*S*-^{2}H]-$GenX_2$ (**20**), and [6'*R*-^{2}H]-$GenX_2$ (**29**). When unlabeled substrate (**6**; $[M + H]^+ = 483.27$ *m/z*) and [6'*R*-^{2}H]-$GenX_2$ (**29**; $[M + H]^+ = 484.28$ *m/z*) were used, unlabeled G418 (**7**; $[M + H]^+ = 497.27$ *m/z*) was produced as a result of GenK catalysis. However, when

[6'*S*-^{2}H]-GenX_2 (**20**) was utilized as a substrate, labeled G418 (**55**; $[M + H]^+$ = 498.28 *m/z*) was detected.

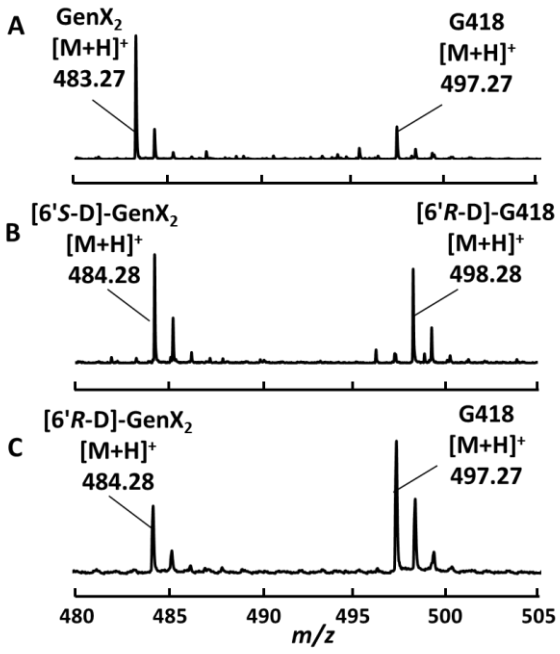


Figure 3-13. ESI mass data of resulting G418 in the assays using A) GenX_2 (**6**), B) [6'*S*-^{2}H]-GenX_2 (**20**), and C) [6'*R*-^{2}H]-GenX_2 (**29**).

In addition, products in the assays using GenX_2 (**6**), [6'*S*-^{2}H]-GenX_2 (**20**), and [6'*R*-^{2}H]-GenX_2 (**29**) were collected by HPLC after derivatizations with 1-fluoro-2,4-dinitrobenzene (FDNB) to add a UV-active chromophore which would otherwise be absent in the aminoglycosides. FDNB-derivatized G418 (**56**; $[M + H]^+$ = 995.3 *m/z*) was detected at $[M+H]^+$ 995.3 *m/z* when unlabeled GenX_2 (**6**) and [6'*R*-^{2}H]-GenX_2 (**29**) were used (Figure 3-14A). However, FDNB-derivatized G418 with deuterium (**57**; $[M+H]^+$ 996.4 *m/z*) was produced when [6'*S*-^{2}H]-GenX_2 (**20**) was treated in the assay. These results also show that deuterium in [6'*R*-^{2}H]-GenX_2 (**29**) was incorporated into 5'-dAdo

(**4**) and the methyl group from Me-Cbl was transferred at C6' position with retention of configuration. As expected, in the GenK reaction with [6'*S*-^{2}H]-GenX$_2$ (**20**), deuterium was retained in the product, producing deuterated G418 (**55**).

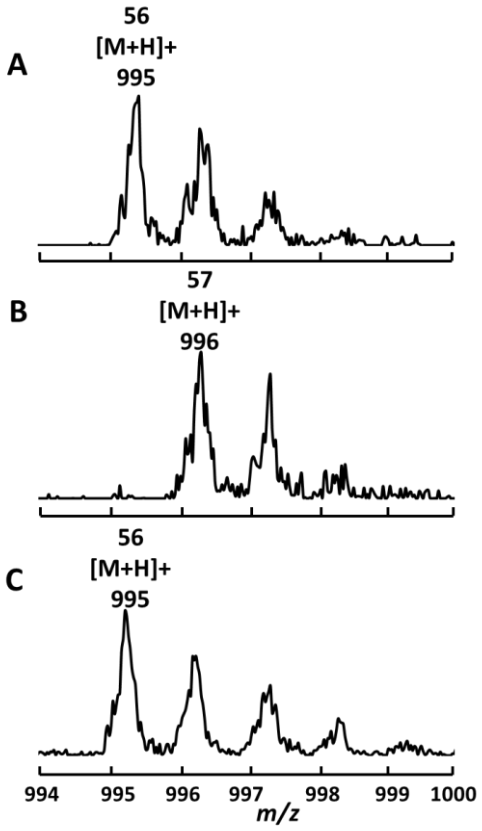


Figure 3-14. Chemical ionization (CI) mass data of 1-fluoro-2,4-dinitrobenzene (FDNB)-derivatized G418 in the assays using A) GenX$_2$(**6**), B) [6'*S*-^{2}H]-GenX$_2$ (**20**), and C) [6'*R*-^{2}H]-GenX$_2$ (**29**).

Combined with the results concerning 5'-dAdo (**4**) formation in the assays using [6'*S*-^{2}H]-GenX$_2$ (**20**), and [6'*R*-^{2}H]-GenX$_2$ (**29**) and the absolute stereochemistry of G418 (**7**) obtained by X-ray crystallography,[14] it can be concluded that the pro-*R* hydrogen at the C6' position of GenX$_2$ (**6**) is abstracted by 5'-dAdo• (**3**) and without any rotation, the

methyl group is transferred from Me-Cbl to the exact same position of pro-*R* hydrogen. The overall stereochemistry in GenK catalysis is maintained from the substrate to the product.

3.3.4. UV-Vis Spectroscopic Experiments

In order to investigate the redox states of cobalamin during GenK catalysis, the reaction was monitored by UV-visible spectroscopy under anaerobic conditions for 1 h and the spectrum of the reaction mixture was recorded every 5 min. (Figure 3-15). This experiment was conducted using this assay conditions containing GenK (0.005 mM), DTT (1 mM), MeCbl (0.1 mM), NADPH (0.4 mM), MV (0.1 mM), SAM (0.4 mM), $GenX_2$ (**6**) (0.1 mM) in Tris·HCl buffer (5 mM; pH 8.0) under anaerobic condition. The spectra in Figure 3-15 correspond to assays resulting from (A) the reaction mixture, (B) no $GenX_2$ (**6**), (C) no GenK, and (D) no GenK and no SAM. In Figure 3-15A, the peak around 520 nm corresponding to Cbl(III)[183] was found to decrease, while the peak around 400 nm (Cbl(I))[184] increased. In Figures 3-15B and 3-15C, a clear shift from ~520 nm to ~476 nm and an increase in the absorbance between 400 nm and 450 nm were observed. Both are characteristics of MeCbl(III) conversion into Cbl(II).[184] It was also noted that without SAM or GenK (Figure 3-15D), the decrease of the peak at ~520 nm was not as significant as those observed in other spectra, but increases of the peak around 476 and 400 nm were detected, indicating that Cbl(II) and Cbl(I) were generated from MeCbl in the presence of the reducing system (methyl viologen and NADPH). Thus, based on the combined spectroscopic data, it can be concluded that the Me-Cbl(III) bond of methylcobalamin is easily cleaved in the presence of SAM and MV/NADPH to give Cbl(I) regardless whether GenK is present or absent. However, in the absence of SAM, the cleavage rate of Me-Cbl bond was much slower when compared to other conditions.

The formation of Cbl(II) is not obvious in the presence of GenK and $GenX_2$ (**6**). The peak around 476 nm was not obvious in the assay of all reaction mixtures (Figure 3-15A). In the absence of substrate, SAM, or GenK, the peak about 476 nm corresponding to Cbl(II) was discerned clearly (Figures 3-15B, 3-15C, and 3-15D). In the presence of GenK and $GenX_2$ (Figure 3-15A), Cbl(II) might exist too transiently to be detected by UV-vis spectrometry, or Cbl(II) might not exist during the GenK reaction. From UV-vis spectroscopy, it was not possible to differentiate between mechanism A and mechanism B described in Section of 3.3.1.

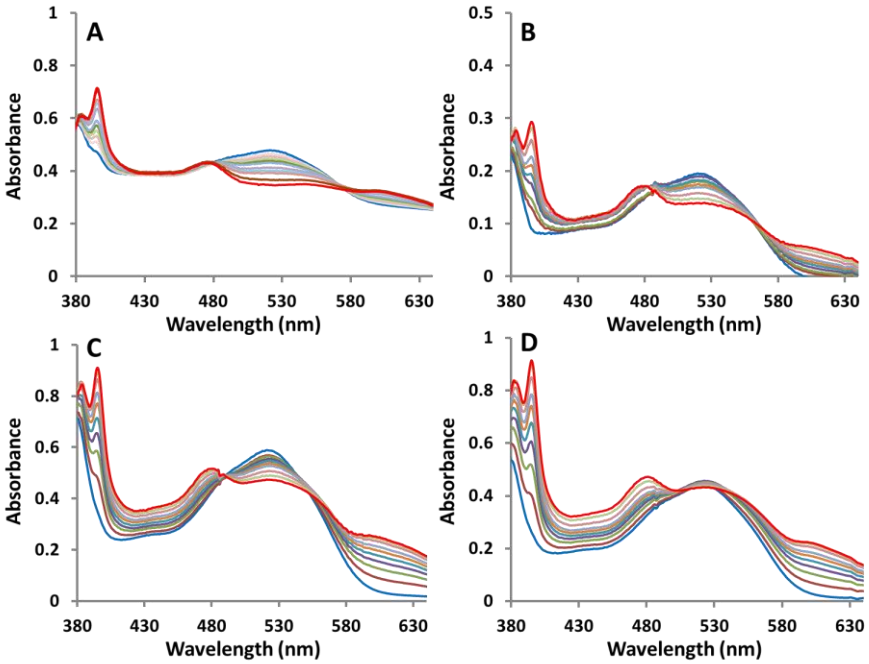


Figure 3-15. UV-visible analysis of GenK assay. The traces (A-D) are from the reaction mixtures containing GenK (0.05 mM), DTT (10 mM), MeCbl (1 mM), NADPH (4 mM), MV (1 mM), SAM (4 mM), $GenX_2$ (1 mM) in Tris·HCl buffer (50 mM; pH 8.0) under anaerobic condition with the following modifications: A) no changes, B) no $GenX_2$, C) no GenK, and D) no GenK and no SAM. Measurements were performed every 5 min from 0 min to 30 min and every 10 min from 30 min to 60 min (from blue to red).

3.3.5. Syntheses of [6'-F]-GenX$_2$ (35), [6',6',6'-^{2}H,^{2}H,F]-GenX$_2$ (43), and [6'-OMe]-GenX$_2$ (49)

To distinguish between mechanisms A and B in Section 3.3.1, [6'-F]-GenX$_2$ (**35**) and [6',6',6'-^{2}H,^{2}H,F]-GenX$_2$ (**43**) were designed as a probe because an alkoxy radical could not be generated from [6'-F]-GenX$_2$ (**35**) and the GenK reaction could only proceed via mechanism A if GenK can accept it as a substrate. Using [6',6',6'-^{2}H,^{2}H,F]-GenX$_2$ (**43**) as a probe may give clues to distinguish the mechanism of the reaction. The synthesis of [6'-F]-GenX$_2$ (**35**) began with the alcohol **30** which was used for the synthesis of JI-20A in Section 2.2.17. The fluoro group was introduced through an S_N2 type reaction using diethylaminosulfur trifluoride (DAST) in dichloromethane to provide compound **31**. After basic hydrolysis using sodium methoxide, coupling of compound **32** with garosamine derivative **17** gave the pseudotrisaccharide **33**. After basic hydrolysis of the carbamate, final deprotection using the Staudinger reaction, and hydrogenation with palladium hydroxide on carbon, [6'-F]-GenX$_2$ (**35**) was obtained with a 14% yield over 6 steps from the previous synthesized compound **30**. The synthesis of [6',6',6'-^{2}H,^{2}H,F]-GenX$_2$ (**43**) was conducted using the similar methods as the synthesis of [6'-F]-GenX$_2$ (**35**) with 7% yield over 9 steps from the previously synthesized dideuterated alcohol **10**.

Another possible substrate to distinguish between mechanisms A and B in the Section 3.3.1 is [6'-OMe]-GenX$_2$ (**49**), because deprotection of the initially formed C6' radical (**50**) by a base cannot occur and thus the alkoxy radical species (**51**) cannot be produced. If the reaction proceeds via mechanism B, no reaction is expected. The synthesis of [6'-OMe]-GenX$_2$ (**49**) began from the alcohol **8** which was prepared in the synthesis of gentamicin A$_2$ in Section 2.2.11. A methoxy group was introduced with sodium hydride and methyl iodide in dimethylformamide (DMF) to provide compound

44. The following steps were similar to the other substrate syntheses. The overall yield of the synthesis was 22% over 7 steps from the previously synthesized compound **8**.

3.3.6. Assays with $GenX_2$ Derivatives

Assays with $GenX_2$ derivatives, [6'-F]-$GenX_2$ (**35**), [6',6',6'-^{2}H,^{2}H,F]-$GenX_2$ (**43**), and [6'-OMe]-$GenX_2$ (**49**) were conducted under the same conditions as before, and 5'-dAdo (**4**) formation was monitored by HPLC as the evidence of radical reaction initiation (Figure 3-16). For comparison, the assay with the original substrate $GenX_2$ (**6**) was also performed. Neither [6'-F]-$GenX_2$ (**35**), [6',6',6'-^{2}H,^{2}H,F]-$GenX_2$ (**43**), nor [6'-OMe]-$GenX_2$ (**49**) showed any reaction with GenK. Different reducing systems like MV/NADPH, BV/NADPH, dithionite, fld/fdr/NADPH, dithionite/MV and dithionite/BV were all tested with [6'-F]-$GenX_2$ (**35**), but none of them resulted in the formation of 5'-dAdo (**4**). These three $GenX_2$ derivatives which were designed as mechanistic probes to determine the mechanism of GenK are not recognized by GenK.

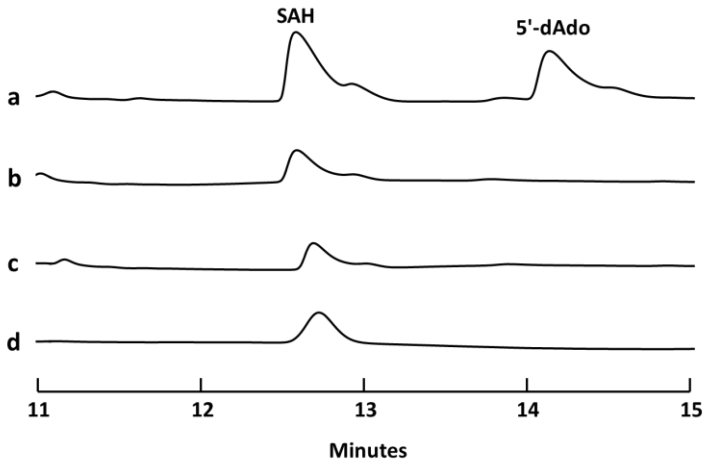


Figure 3-16. HPLC traces with GenK reaction with the following $GenX_2$ derivatives; a) SAH and 5'-dAdo standard, b) [6'-F]-$GenX_2$ (**35**), c) [6',6',6'-^{2}H,^{2}H,F]-$GenX_2$ (**43**), and d) [6'-OMe]-$GenX_2$ (**49**)

Whether [6'-F]-$GenX_2$ (**35**) is an inhibitor for GenK was also tested because it has a similar structure to the natural substrate. If [6'-F]-$GenX_2$ (**35**) could compete with $GenX_2$ (**6**), it may be considered as an inhibitor competing for the active site of the enzyme even though GenK does not catalyze the radical formation reaction. Inhibition assays were conducted under the same conditions as before, but the incubation time was 20 h. When the concentration of $GenX_2$ (**6**) was fixed at 1 mM and lower concentrations of [6'-F]-$GenX_2$ (**35**) were used (0.05, 0.1, 0.2, 0.5, and 1 mM) in the GenK assay, the amounts of 5'-dAdo (**4**) and SAH (**5**) formations were the same as when $GenX_2$ (**6**) was used alone (Figure 3-17). Even in the GenK assays where the enzyme was pre-incubated with [6'-F]-$GenX_2$ (**35**) for 4 h and $GenX_2$ (**6**) was added later, the results were similar as in the absence of pre-incubation time. Experiments with higher concentrations of $GenX_2$ (**6**) compared to [6'-F]-$GenX_2$ (**35**) did not provide any evidences that the fluoride-substrate binds to the active site of GenK and inhibits methylation of the enzyme.

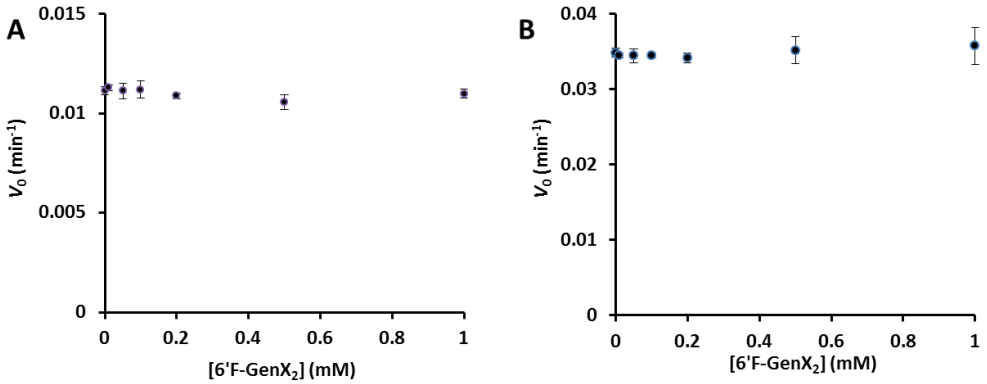


Figure 3-17. Formations of A) 5'-dAdo and B) SAH from the co-incubation of [6'-F]-$GenX_2$ (**35**) and $GenX_2$ (**6**) in the GenK reaction for 20 h. Concentration of $GenX_2$ (6) was fixed as 1 mM, and concentration of [6'-F]-$GenX_2$ (**35**) was changed from 0 to 1 mM.

Assays with excess [6'-F]-GenX_2 (**35**) compared to GenX_2 (**6**) (1 mM) showed that although the fluoro-substrate inhibits the activity of GenK, it is a very weak inhibitor (Figure 3-18). Assuming that it competes with GenX_2 (**6**) for the active site, it does not display a strong affinity. Combined with the results of the [6'-OMe]-GenX_2 (**49**) assay, it may be concluded that the 6'-hydroxy group plays a role in binding GenX_2 to GenK.

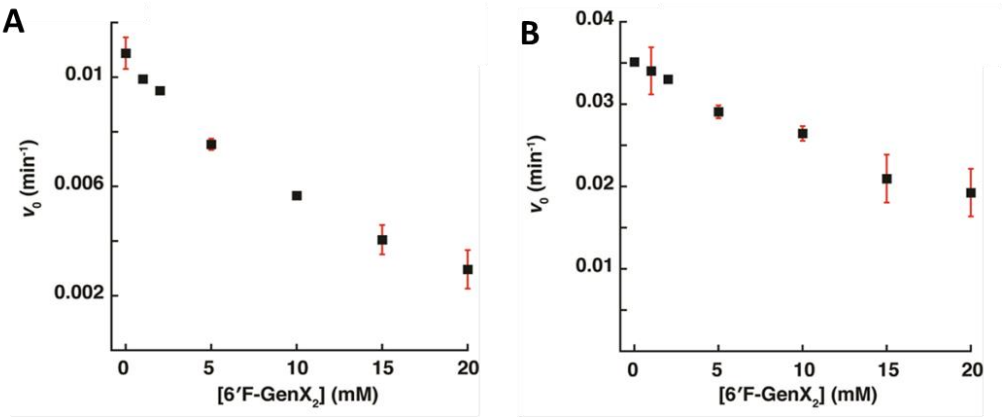


Figure 3-18. Formations of A) 5'-dAdo and B) SAH from the co-incubation of [6'-F]-GenX_2 (**35**) and GenX_2 (**6**) in the GenK reaction for 20 h. Concentration of GenX_2 (6) was fixed as 1 mM, and concentration of [6'-F]-GenX_2 (**35**) was changed from 1 to 20 mM.

3.4. CONCLUSION

The mechanism of methyl transfer by Class B radical SAM methyltransferase has not yet been elucidated. In this chapter, three mechanisms were considered for the GenK-catalyzed conversion of GenX_2 to G418. In the first mechanism, the 5'-dAdo• generated via the reductive cleavage of SAM abstracts a hydrogen atom from C-6' of the substrate.

The resulting substrate radical accepts a methyl radical from Me-Cbl to generate Cbl(II) and the G418 product. Other mechanisms, which involve the transfer of a methyl cation from Me-Cbl, were also considered. After hydrogen atom abstraction, the radical intermediate is deprotonated to form a ketyl radical. Nucleophilic attack by an anion at the Me-Cbl leads to methyl transfer, yielding a Cbl(I) intermediate and an alkoxide product radical. The radical could then be quenched by an external electron and proton or via hydrogen atom transfer from 5'-dAdo. In this latter mechanism, SAM is regenerated while restoring the [4Fe-4S] cluster to the +1 redox state.

The observation that 5'-dAdo, SAH and G418 are each produced in equivalent amounts is consist with mechanisms A and B, but not C, because in mechanisms A and B two equivalents of SAM are consumed for each methyl transfer event (one to initiate the radical reaction and the other as a methyl donor), but in mechanism C the input of exogenous electrons is not required and only a single equivalent of SAM is necessary for each catalytic cycle.

Incorporation of deuterium into 5'-dAdo and loss of deuterium from $GenX_2$ were observed by mass spectra, which showed that the reaction with (*R*)-deuterated $GenX_2$ gave deuterated 5'-dAdo and non-deuterated product, and that the reaction with (*S*)-deuterated $GenX_2$ gave the opposite results. These results indicate that the 6'-pro-*R* hydrogen is abstracted by the 5'-dAdo radical. Combined with the reported structure of G418, it was ascertained that methylation occurs with retention of configuration at C6'.

To distinguish the manner of methyl transfer, three $GenX_2$ derivatives were tested. However, none of them showed turnover upon incubation with GenK. The fluoride substrate did inhibit the activity of GenK very weakly. Assuming that it competes with $GenX_2$ for the active site, then it must not have a strong affinity for the

active site. The 6'-hydroxyl group apparently plays a key role in binding of $GenX_2$ to GenK.

Efforts are currently underway to further elucidate the chemical mechanism of this intriguing enzyme. Another substrate [5'-F]-$GenX_2$ would be a possible substrate to distinguish the two mechanisms. If the methyl transfer reaction proceeds via methyl radical reaction (mechanism A), this compound would produce [5'-F]-G418. However, if an anionic mechanism is operative (mechanism B), and both the leaving group and the anion have an anti-orientation, the product would not contain fluoride because an anion at the C6' position would result in the elimination of fluoride. The synthesis of [5'-F]-$GenX_2$ is currently underway in our group.

Chapter 4: Characterization of the Radical SAM Enzyme AprD4 and Its Reductase Partner AprD3

4.1. INTRODUCTION

The aminoglycosides are an important class of antibiotics for the treatment of Gram-negative bacterial infections.[1-2,185] Their biological activity is a result of their ability to bind the bacterial 30S ribosome and subsequently inhibit protein synthesis. Several years after aminoglycosides were first used to treat human antibacterial chemotherapy, new organisms resistant to these drugs began to appear. The major cause of aminoglycoside resistance is decreased affinity of the drug to the bacterial ribosome. Numerous genetic and biological studies have focused on the mechanisms of bacterial resistance to aminoglycosides.[185-188] Among these mechanisms, enzymatic inactivation of the antibiotic is the most clinically important. Three classes of aminoglycoside-modifying enzymes have been described: the aminoglycoside nucleotidyltransferases (ANTs), the aminoglycoside phosphotransferases (APHs), and the aminoglycoside acetyltransferases (AACs). The regioselective modification site on the targeted aminoglycoside by a specific enzyme is specified in parentheses. For example, AAC(2') is an aminoglycoside acetyltranferase catalyzing the acetylation of hydroxyl group at C2' position.

Aminoglycoside adenyltransferases found in both Gram-negative and Gram-positive bacteria can be either plasmid-encoded or chromosomally encoded. The reaction sites are 2" and 3" for enzymes found in Gram-negative bacteria, and 4', 6, and 9 for those derived from Gram-positive organisms.[189] These enzymes catalyze *O*-adenylation of aminoglycosides using Mg-ATP and in the process generate magnesium chelated

inorganic pyrophosphate (Figure 4-1). Adenylation at the 2" and 4' are clinically the most significant and have been well-studied.

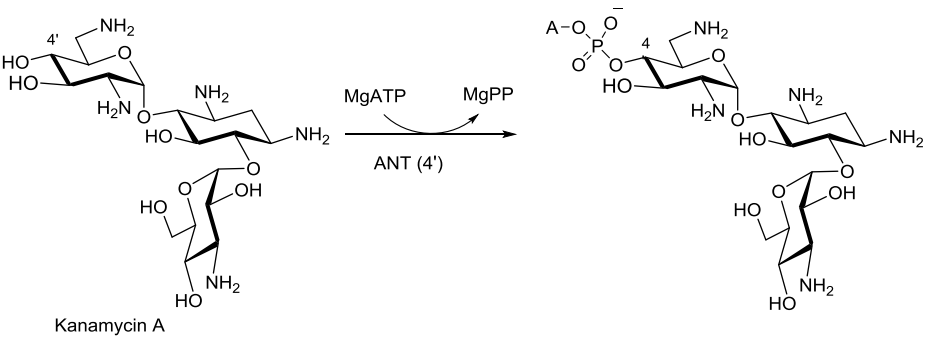


Figure 4-1. 4'-*O*-adenylation of kanamycin A by ANT(4')

The aminoglycoside phosphotransferases represent a large group of aminoglycoside-modifying enzymes that play important roles in the clinical resistance of *Staphylococcal* and *Enterococcal* species to aminoglycosides.[185] They can regioselectively transfer the γ-phosphate of ATP to one of the hydroxyl groups of the aminoglycoside (Figure 4-2). Among them, the plasmid-encoded APH(3')-IIIa enzymes from *Enterococcus faecalis* that have been the focus of biochemical investigations.[190-194]

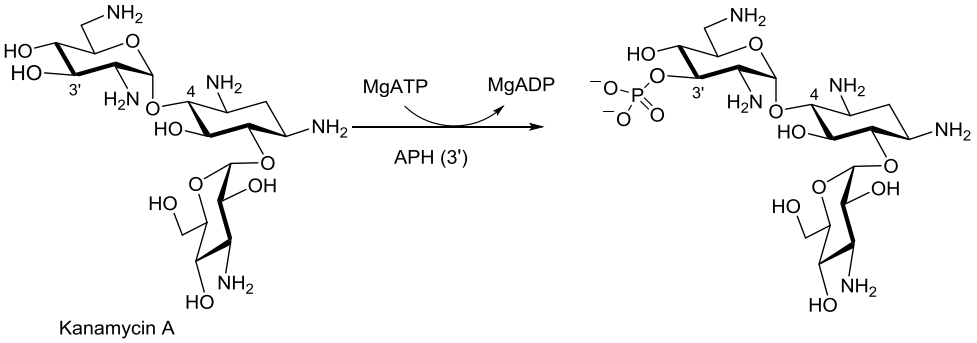


Figure 4-2. 3'-*O*-phosphorylation of kanamycin A by APH(3')

Acetyl coenzyme A-dependent aminoglycoside acetylases (AACs) catalyze *N*-acetylation of the four amino groups of aminoglycosides: the 1- or 3-amino groups of the central 2-deoxystreptamine ring or the 2' or 6'-amino groups of the 6-amino-6-deoxy glucose ring (Figure 4-3). Two types of AAC have been identified in *E. coli* and *Actinomycete* bacterial strains; however, they are not clinically important, because these enzymes are not virulence factors with respect to infection of humans.[195-196]

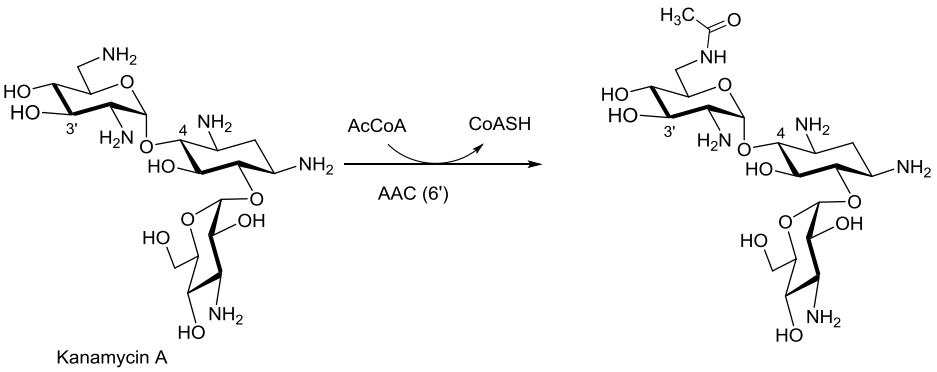


Figure 4-3. 4'-*N*-acetylation of kanamycin A by AAC(6')

A number of aminoglycosides, such as apramycin (**2**),[6] tobramycin (**3**),[197] nebramycin 5' (**4**),[198] and lividomycin B (**5**),[199] possess the 2-deoxystreptamine (2-DOS; **1**) core decorated with a variety of carbohydrates necessary for activity; however, they are characterized by a C3'-deoxyhexose linked to the C4 position of 2-DOS via a glycosidic bond (Figure 4-4). The absence of a C3' hydroxyl group in these aminoglycosides renders them more effective as antimicrobial agents, because they are less susceptible to modification by aminoglycoside phosphotransferase (APH(3')), which is a known mechanism of resistance.[185]

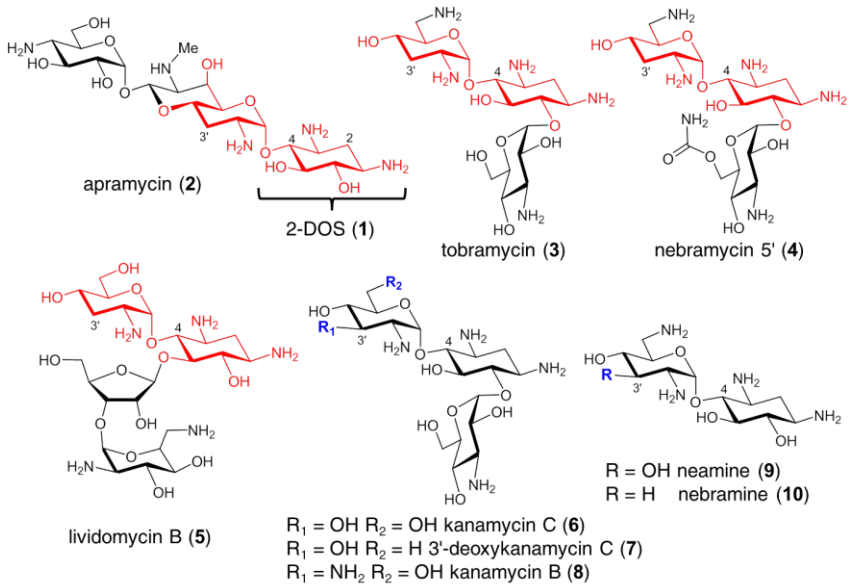


Figure 4-4. Selected examples of aminoglycosides.

Although the biosynthesis of paromamine has been investigated (see Section 1.1.3.2), little is known about the mechanism of C3'-deoxygenation despite its importance to the development and improvement of clinically useful aminoglycoside antibiotics.[200] C3'-deoxygenation of paromamine is similar to the reactions catalyzed by Cbl-dependent dehydratases like diol dehydratases (DD), propanediol hydro-lyase, glycerol dehydratase (GD), and glycerol hydro-lyase, which catalyze the conversion of 1,2-propanediol and other 1,2-diols to the corresponding aldehydes, or deaminases such as ethanolamine ammonia-lyase (EAL) or ethanolamine deaminase which catalyze the conversion of ethanolamine to acetaldehyde in the presence of AdoCbl.[201-202] The mechanism of DDs is shown in Figure 4-5. Two water molecules are bound in the active site of the holoenzyme and are displaced upon substrate binding. The adenosyl radical generated by homolytic cleavage of the carbon-cobalt bond in adenosylcobalamin abstracts a hydrogen atom from the C1 position of the substrate. The resulting substrate radical then undergoes hydroxyl

group migration from C2 to C1, generating a 1,1-diol-2-yl radical. The resulting radical then abstracts a hydrogen atom from the C5' methyl of 5'-deoxyadenosine to form the aldehyde product and adenosyl radical, the latter of which recombines with cob(II)alamin to regenerate AdoCbl. The mechanism of EAL is similar to that of the DDs. One particular thing is that DDs and EAL are not enantioselective: the adenosyl radical can abstract either the pro-*R* or pro-*S* hydrogen from the substrate.[202] Another example of a radical mediated dehydration is the reaction catalyzed by Cbl-independent glycerol dehyratase.[203] In the case of both, Cbl-dependent DDs and Cbl-independent glycerol dehydratase, the radical initiator is typically regenerated.

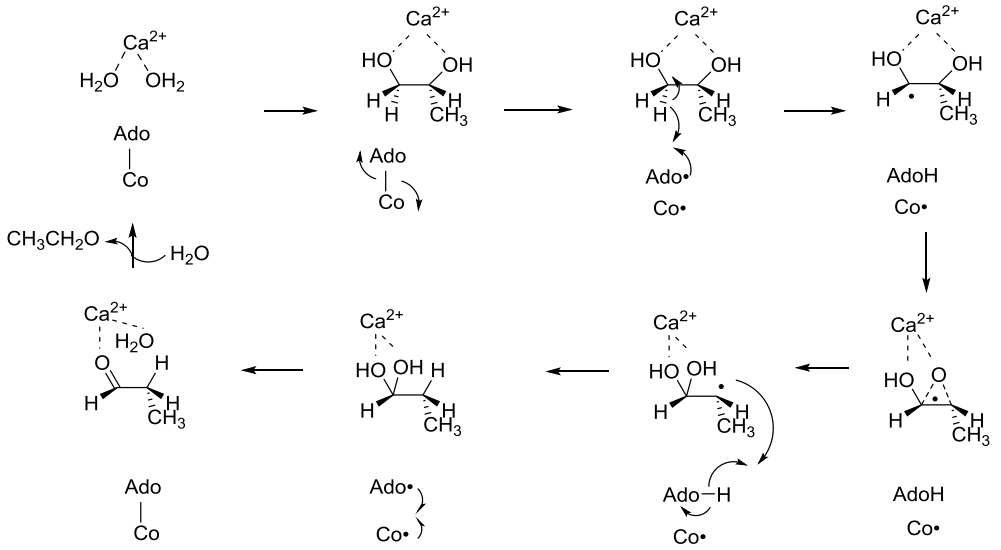
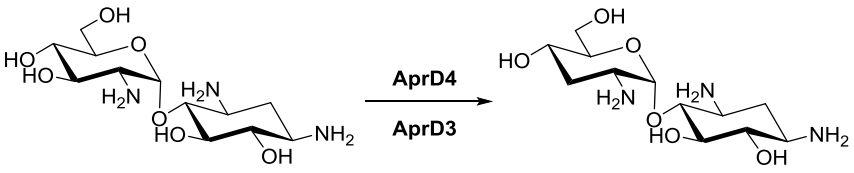


Figure 4-5. Mechanism of the diol dehydratases.

The gene clusters responsible for the biosynthesis of apramycin (**2**), tobramycin (**3**), and lividomycin B (**5**) in *Streptomyces* and *Streptoalloteichius* species have been described,[8,204-206] and significant progress has been made in elucidating the reactions involved in the tobramycin pathway.[55,68] Although no gene product in the tobramycin

cluster has been implicated in catalyzing C3'-deoxygenation,[8,204] gene-knockout studies[207] of the aparamycin and lividomycin B pathway have identified the AprD4/AprD3 and LivW/LivY enzyme pairs as likely candidates for their activity. For example, incorporation of the *aprD4* and *aprD3* genes into the kanamycin-producing strain *S. kanamyceticus* led to the isolation of 3'-deoxykanamycins (such as **7**),[204] which are not normally produced. Likewise previous studies have also shown the conversion of neamine (**9**) to nebramine (**10**) in cell-free extracts of *S. venezuelae* expressing protein AprD4 and AprD3.[204] However, no conversion of kanamycin B (**8**) to tobramycin (**3**) was observed under the same conditions, leading to the suggestion that the substrate for AprD4/AprD3 may be a pseudo-disaccharide (such as **9**) rather than a pseudo-trisaccharide (such as **8**).[204] Since apramycin (**2**) and tobramycin (**3**) are produced together in the same bacterial strains, it is possible that AprD4/AprD3 serves as the C3'-deoxygenation machinery for both pathways.

This chapter describes the purification and biochemical characterization of AprD4 and AprD3 from *S. tenebraris* and demonstrates their ability to catalyze 3'-deoxygenation of paromamine (**11**) to lividamine (**13**) in vitro (Scheme 4-1). Using various paromamine derivatives, the mechanistic details regarding the AprD4-catalyzed conversion of paromamine to 4-oxolividamine (**12**) are considered.



Scheme 4-1. AprD4/AprD3 catalysis.

4.2. EXPERIMENTAL PROCEDURES

4.2.1. Materials and Equipment

All chemicals were purchased from Fisher Science (Pittsburgh, PA) or Sigma-Aldrich (St. Louise, MO) and used without further purification unless otherwise specified. DNA gel extraction and spin miniprep were obtained from Qiagen (Valencia, CA). All reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Amicon and Microcon YM-10 filtration products were purchased from Bio-Rad (Hercules, CA) and Millipore (Billerica, MA), respectively. *Escherichia coli* DH5*a* cells were bought from Bethesda Research Laboratories (Muskegon, MI). The vector pET24b (+) and enzyme KOD DNA polymerase were purchased from Novagen (Madison, WI). DNA modifying enzymes (for restriction digestion and ligation), PCR primers, and the overexpression host *E. coli* BL21 star (DE3) were acquired from Invitrogen (Carlsbad, CA) and New England Biolabs (NEB, Beverly, MA). Luria Bertani (LB) media are products of Difco (Detroit, MI) or Fisher Scienctific (Pittsburgh, PA). Pre-stained protein markers were bought from NEB. Kits for Protein concentrations were determined by Bradford Assay[149] using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using SDS-PAGE. The general methods and protocols for recombinant DNA manipulations were as described by Sambrook *et al*.[150] DNA sequencing was performed at the Core Facilities of the Institute of Cellular and Molecular Biology, University of Texas at Austin. All reactions involving AprD4 were performed in a Coy Anaerobic Chamber (glovebox) under an atmosphere of approximately 97.5% N_2 and 2.5% H_2 with less than 1 ppm O_2. All solvents using AprD4 reaction were deaerated by bubbling with nitrogen gas before they were transferred into the glovebox. The solvents in the glove box were stirred open

to the anaerobic atmosphere overnight to allow equilibration before use. Paromamine (11) was obtained by acidic hydrolysis of paromomycin.[166] SAM was prepared as described in Section 2.2.4. [5-$^2H_2$]-SAM were prepared as previous described.[173] Tetrahydrofuran (THF) was distilled from sodium benzophenone and dichloromethane (CH_2Cl_2) was distilled from calcium hydride under a nitrogen atmosphere. Other anhydrous solvents were purchased from Acros Organics (Pittsburgh, PA). Analytic thin layer chromatography (TLC) was performed on pre-coated TLC glass plates (Silica gel, grade 60, F254, 0.25 mm layer thickness) purchased from EMD chemicals (Madison, WI). Flash column chromatography was carried out on silica gel (230-400 mesh, grade 60) from Sorbent Technologies (Atlanta, GA) by eluting with the specific solvents. ^1H and ^{13}C NMR spectra were recorded at 400, 500, 600 MHz and 100, 125, 150 MHz with a Varian Gemini spectrometer. Chemical shifts are reported as parts per million (ppm) from ppm relative to the deuteriochloroform ($CDCl_3$), 7.26 ppm for ^1H NMR and 77.16 ppm for ^{13}C NMR, respectively. Visualization was effected with *p*-phosphoryl molybdic acid solution. The MS analyses were carried out at the Mass Spectrometry and Proteomics Facility of the Department of Chemistry, University of Texas at Austin.

4.2.2. Cloning and Expression of *Streptomyces tenebrarius* AprD3

The apramycin/tobramycin producer *S. tenebrarius* was obtained from the Agricultural Research Service of the US Department of Agriculture as a lyophilized sample. It was inoculated into 10 mL of tryptic soy broth (TSB) starter culture,[151] and the resulting solution was incubated overnight at 30 °C with shaking at 250 rpm. The following day, 0.1 mL of the starter culture was inoculated into 10 mL of fresh TSB, and the resulting culture was incubated at 30 °C for 72 hr. Chromosomal DNA was extracted from 1.5 mL of this culture using a Qiagen DNeasy tissue kit. The *aprD3* gene was PCR

amplified from the isolated *S. tenebrarius* DNA with the following primers: 5′-CATATGGAGCAACG-GTACGTGCTGGTCACCGGGGCCAGTCGCGGTCTGGG-3' (forward), 5′-AAGCTTCATCACGACGC-CGACCACCGCGGGACGGGGGGGCGG-3' (reverse), and cloned into a pCR:blunt vector (Invitrogen) prior to excision with *NdeI* and *HindIII* ligation into the pET28b(+) vector (Invitrogen). The resulting expression plasmid, pET28b(+):*aprD3*, was used to transform *E. coli* BL21 cells for expression of the native, recombinant AprD3. An overnight culture of *E. coli* transformed with the pET28b:*aprD3* plasmid was grown at 37 °C in LB medium containing 34 g/mL kanamycin, and used to inoculate 6 x 1 L of the same medium in a 100-fold dilution. Then 1 L cultures were incubated at 37 °C until cell had reached an optical density of 0.6 at 600 nm. At which point AprD3 expression was induced by 1mM IPTG. The cells were harvested by centrifugation at 20000 x *g* for 30 min, thawed and suspended in 50 mM EPPS (pH 8.0) with 20 mM imidazole and 300 mM NaCl. After sonication (8 x 1 min), the lysate was spun at 26500 x *g* for 40 min. 1 mM PMSF was added. The supernatant was mixed with 8 mL of Ni-NTA resin at 4 °C. The slurry was transferred to an empty column and washed with 100 mL of 50 mM EPPS buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole. The protein was eluted with 50 mL of 50 mM EPPS buffer (pH 8.0) containing 300 mM NaCl and 300 mM imidazole. The purified protein was dialyzed twice against 1 L of 50 mM EPPS buffer (pH 8.0) containing 300 mL NaCl. The dialyzed protein was frozen with liquid nitrogen and stored at –80 °C until use.

4.2.3. Cloning, Expression and Reconstitution of Streptomyces tenebrarius AprD4

The aprD4 gene was PCR amplified from the isolated *S. tenebrarius* DNA with the following primers: 5'-CATATGCGACGAATGCGGCTCGGCACGGTAC-3' (forward), 5'-AAGCTTCATCAGGCG-TCACCGGTCGACCAGGCGTG-3' (reverse),

and cloned into a pCR:blunt vector (Invitrogen) prior to excision with *NdeI* and *HindIII* and ligation into the pET28 vector (Invitrogen). The resulting expression plasmid, pET28:*aprD4*, was used to transform *E. coli* Rosetta DE3 (Novagen) for expression of native, recombinant AprD4. Transformants were grown in 6 x 1 L of LB medium with 34 g/mL kanamycin and 68 g/mL chloramphenicol at 37 °C with shaking at 200 rpm. AprD4 expression was induced with 1 mM IPTG when the cells had reached an optical density of 0.6 at 600 nm. Cells were harvested by centrifugation at 4500 x *g* for 20 min, thawed and suspended in 50 mM Tris buffer (pH 8.0, HCl) with 20 mM imidazole. After sonication (8 x 1 min), the lysate was spun at 26500 x *g* for 30 min. Lysate was loaded on a Ni-NTA column (8 mL) pre-equilibrated with 50 mM Tris buffer (pH 8.0, HCl) and 20 mM imidazole. After washing with 10 mL of 50 mM Tris buffer (pH 8.0, HCl) and 20 mM imidazole, the column was developed with 8 mL of 50 mM Tris buffer (pH 8.0, HCl) and 40 mM imidazole, 8 mL of 50 mM Tris buffer (pH 8.0, HCl) and 100 mM imidazole, and 8 mL of 50 mM Tris buffer (pH 8.0, HCl) and 300 mM imidazole. The fractions were collected and centrifuged with a YM-10 filter at 4500 × *g* for 1 min. The protein was dialyzed twice against 1 L of 50 mM Tris buffer (pH 8.0, HCl). The dialyzed protein was frozen with liquid nitrogen and stored at –80 °C until reconstitution with iron and sulfur.

Reconstitution followed the previously published procedure.[152] Approximately 3–5 mg of AprD4 was thawed, and transferred into a conical vial. The vial was transferred to the glovebox. The enzyme was diluted with 20 mM NH_4HCO_3 buffer (pH 7.8) containing 5 mM DTT to a volume of ca. 2 mL and gently stirred at ca. 10 °C in an open vial for ca. 2 h in order to equilibrate with the anaerobic atmosphere. The solution was made 1.88 mM in dithiothreitol and allowed to stir for 15 min. To this solution was slowly added 42 μL of a 50 mM solution of $Fe(NH_4)_2(SO_4)_2$ in anaerobic water over 10

min. After 10 min, 42 μL of a 50 mM solution of Na_2S in anaerobic buffer containing 20 mM NH_4HCO_3 (pH 7.8) and 5 mM DTT was added over 10 min. The solution was gently stirred at ca. 10 °C for 2 hr. The reconstituted enzyme was loaded onto a 50 mL Sephadex G-25 column pre-equilibrated with anaerobic buffer containing 20 mM NH_4HCO_3 (pH 7.8) and 5 mM DTT, and the column was eluted with the same buffer. Protein fractions were collected, concentrated to approximately 100 μM of enzyme and stored in the glovebox at ca. 10 °C until use. Iron content in the reconstituted AprD4 was assessed using the ferrozine assay,[153] and the sulfide content was determined by the method devised by Helmut Bienert.[154] The iron and sulfide content reported in the text is an average of three measurements.

4.2.4. AprD4 Activity Assays and AprD3 Activity Assays

The reaction mixture contained the following: 50 mM NH_4HCO_3 (pH 7.8), 10 mM DTT, 1 mM paromamine (**11**), 2 mM enzymatically prepared SAM, 2 mM sodium dithionite, and 0.01 mM AprD4. Assays were initiated by the addition of AprD4, incubated for 8 h at room temperature, and quenched by passage through YM-10 Microcon centrifugal filters to remove enzymes. The filtrate of each sample was frozen and stored at –80 °C until analysis of HPLC and mass spectroscopy.

To find proper reducing systems for the AprD4 reaction, all reactions were treated with 1 mM of the chemical reductants being tested. When flavodoxin and flavodoxin reductase from *E. coli* (obtained as previously described[170]) were used as the reducing system, the protein concentration was 0.1 mM.

To an assay mixture of AprD4, 0.01 mM AprD3 and 1 mM NAD(P)H were added. Assays were incubated for 1 h at room temperature, quenched by passage through

YM-10 Microcon centrifugal filters to remove enzymes. The filtrate of each sample was frozen and stored at –80 °C until analysis of HPLC and mass spectroscopy.

4.2.5. HPLC Detection of 5'-Deoxyadenosine (5'-dAdo)

A 20 μL aliquot of the quenched AprD3/AprD4 reaction or AprD4 reaction described above was analyzed using HPLC equipped with a Varian Microsorb-MV 100-5 C18 (4.6 x 250 nm) column pre-equilibrated in H_2O containing 0.1% trifluoroacetic acid (solvent A). After sample loading, the column was eluted with a linear gradient from 0–20% acetonitrile containing 0.1% trifluoroacetic acid (Solvent B) over 30 min. The UV detector was set at 260 nm. An authentic, commercially obtained 5'-dAdo was injected as a retention time standard for each. The stoichiometric ratio reported in the text is an average of three measurements.

4.2.6. HPLC Detection of AprD4/AprD3 Reaction Product

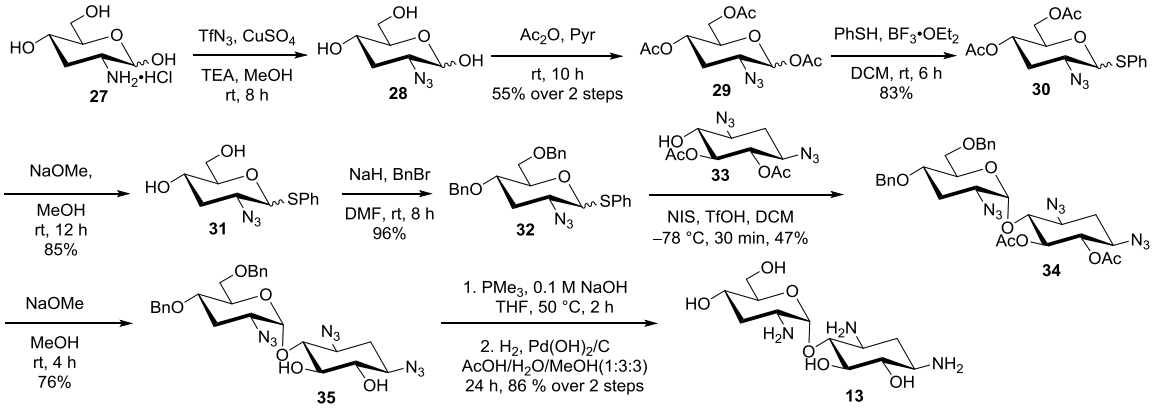
A 10 L aliquot of the quenched AprD4/AprD3 reaction or the AprD4 reaction was analyzed using HPLC equipped with a Varian Microsorb-MV 100-5 C18 (4.6 x 250 nm) and eluted with H_2O containing 0.4% trifluoroacetic acid for 15 min. The elution was monitored by Corona® charged aerosol detector. An authentic paromamine was injected as a retention time standard.

4.2.7. Mass Spectroscopic Analysis of AprD3/AprD4 Activity and AprD4 Activity

Assay mixtures were diluted in water and injected directly into an Agilent 6350 Accurate Mass QTOF-MS at 0.15 mL/min. The analyte was ionized by electrospray ionization and detected in positive ion mode.

4.2.8. Synthesis of Lividamine

The overall synthetic scheme is shown in Scheme 4-2.



Scheme 4-2. Synthetic scheme for the preparation of lividamine.

(*3R,5S,6R*)-6-(acetoxymethyl)-3-azidotetrahydro-2H-pyran-2,5-diyl diacetate (29): Compound **27** was prepared as previously described.[209] Copper sulfate (0.158 g, 1.01 mmol), triethylamine (14.09 mL, 101.09 mmol), and triflic azide (0.4 M solution in dichloromethane, 80 mL) was added to a solution of compound **27** (2.0181 g, 10.11 mmol) in methanol (60 mL) at 0 °C. The reaction mixtures were stirred at room temperature for 24 h, and concentrated *in vacuo*. The residue was dissolved in acetonitrile and washed with water. The remaining aqueous layer was extracted with acetonitrile five times, and the combined organic layers were dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was filtered by short column filled with silica gel by 20% methanol in dichloromethane. Acetic anhydride (50 mL) was added to a solution of the crude compound **28** in pyridine (50 mL) at 0 °C. The reaction mixture was stirred at room temperature for 12 h, and concentrated. The residue was diluted with dichloromethane and washed with saturated aqueous solution of sodium bicarbonate (50

mL) two times. The organic layer was dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **29** (1.75 g, 55% over two steps) as a light yellow liquid. ^{1}H NMR (500 MHz, $CDCl_3$) α isomer δ 6.18 (d, *J* = 3.3 Hz, 1H, H-1), 4.88 (ddd, *J* = 13.2, 8.3, 4.9 Hz, 1H, H-4), 4.29–4.24 (m, 1H, H-6), 4.12–4.09 (m, 1H, H-6), 4.0 (ddd, *J* = 10.3, 4.5, 2.3, 1H, H-5), 3.54–3.50 (m, 1H, H-2), 2.48 (dt, *J* = 11.5, 4.8 Hz, H-3), 2.21 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.05–1.98 (m, 1H, H-3). β isomer δ 5.53 (d, *J* = 8.4 Hz, H-1), 4.80 (ddd, *J* = 12.9, 8.1, 4.9 Hz, H-4), 4.29–4.24 (m, 1H, H-6), 4.16–4.12 (m, 1H, H-6), 3.79 (ddd, *J* = 10.0, 5.2, 2.6 Hz, 1H, H-5), 3.60 (ddd, *J* = 12.8, 7.9, 5.0 Hz, 1H, H-2), 2.55 (dt, *J* = 12.6, 4.9 Hz, 1H, H-3), 2.19 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.07 (s, 3H, OAc), 1.59–1.51 (m, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) α isomer δ 170.71 (OAc), 169.58 (OAc), 168.77 (OAc), 89.6 (C-1), 69.9 (C-5), 65.4 (C-4), 61.9 (C-6), 55.3 (C-2), 28.5 (C-3), 21.05 (OAc), 20.89 (OAc, 20.74 (OAc). β isomer δ 170.71 (OAc), 169.69 (OAc), 168.93 (OAc), 94.7 (C-1), 75.8 (C-5), 65.2 (C-4), 61.9 (C-6), 57.6 (C-2), 33.2 (C-3), 21.03 (OAc), 20.87 (OAc), 20.76 (OAc). HRMS (ESI, positive ion mode) calculated for $C_{12}H_{17}N_3O_7^+$ $[M + Na]^+$ 338.0959, found 338.0960.

((*2R,3S,5R*)-3-acetoxy-5-azido-6-(phenylthio)tetrahydro-2H-pyran-2-yl)methyl acetate (30): Boron trifluoride etherate (ca. 48%, 0.99 mL, 2.99 mmol) was added to a solution of compound **29** (0.787 g, 2.50 mmol) and thiophenol (0.51 mL, 4.99 mmol) in dichloromethane (40 mL) at 0 °C under nitrogen atmosphere, and the reaction mixture was stirred at room temperature for 6 h. The reaction was quenched by the addition of saturated aqueous solution of sodium bicarbonate (20 mL) at 0 °C, and the aqueous layer was extracted by dichloromethane (40 mL × 3). The combined organic layers were dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue

was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to provide compound **30** (α/β = 4:1, 0.759 g, 83%) as a clear liquid. ^{1}H NMR (500 MHz, $CDCl_3$) α isomer δ 7.53–7.51 (m, 2H, Ph), 7.33–7.28 (m, 3H, Ph), 5.53 (d, *J* = 4.6 Hz, 1H, H-1), 4.82 (ddd, *J* = 13.1, 8.1, 4.9 Hz, 1H, H-4), 4.49 (ddd, *J* = 10.0, 5.6, 2.3 Hz, 1H, H-5), 4.27 (dd, *J* = 12.2, 5.6 Hz, 1H, H-6), 4.11 (dd, *J* = 12.2, 2.3 Hz, 1H, H-6), 4.0 (ddd, *J* = 12.7, 5.1, 4.3 Hz, 1H, H-2), 2.45–2.41 (m, 1H, H-3), 2.08 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.9–1.82 (m, 1H, H-3). β isomer δ 7.59–7.57 (m, 2H, Ph), 7.30–7.27 (m, 3H, Ph), 4.70 (ddd, *J* = 12.9, 8.2, 4.8 Hz, 1H, H-4), 4.46 (d, *J* = 8.9 Hz, 1H, H-1), 4.24–4.16 (m, 2H, H-6, H-6), 3.62 (ddd, *J* = 9.9, 5.4, 2.7 Hz, 1H, H-5), 3.42 (ddd, *J* = 12.3, 8.4, 4.9 Hz,1H, H-2), 2.60 (dt, *J* = 12.4, 4.9 Hz, 1H, H-3), 2.07 (s, 3H, OAc), 2.04 (s, 3H, OAc), 1.55 (q, *J* = 11.8 Hz, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) α isomer δ 170.60 (OAc), 169.72 (OAc), 132.91 (Ph), 132.32 (Ph), 129.13 (Ph), 127.85 (Ph), 87.94 (C-1), 68.71 (C-5), 66.11 (C-4), 62.42 (C-6), 57.23 (C-2), 30.64 (C-3), 20.91 (OAc), 20.71 (OAc). β isomer δ 170.62 (OAc), 169.64 (OAc), 133.43 (Ph), 131.36 (Ph), 128.92 (Ph), 128.38 (Ph), 88.18 (C-1), 77.87 (C-5), 65.76 (C-4), 62.66 (C-6), 57.88 (C-2), 35.47 (C-3), 20.86 (OAc), 20.76 (OAc). HRMS (ESI, positive) calculated for $C_{27}H_{27}N_3O_5S^+$ $[M + Na]^+$ 528.1569, found 528.1559.

(*2R,3S,5R*)-5-azido-2-(hydroxymethyl)-6-(phenylthio)tetrahydro-2H-pyran-3-ol (31): Sodium methoxide (4.35 M in methanol, 95.4 μL, 0.42 mmol) was added to a solution of compound **30** (0.759 g, 2.08 mmol) in methanol (40 mL). The reaction mixture was stirred at room temperature for 10 h, and neutralized with Amberlite IR-120 (H^+ form). The mixture was filtered through a Celite pad, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 1:2) to give compound **31** (α/β mixtures, 0.450 g, 85%) as a white solid. As time goes, β isomer converted into α isomer. ^{1}H NMR (400 MHz, $CDCl_3$) α isomer δ 7.55–7.50 (m,

2H, Ph), 7.35–7.28 (m, 3H, Ph), 5.49 (d, *J* = 5.0 Hz, 1H, H-1), 4.09 (m, 1H, H-4), 3.91–3.77 (m, 3H, H-2, H-6, H-5), 2.33–2.28 (m, 1H, H-3), 1.91–1.82 (m, 1H, H-3). β isomer δ 7.55–7.50 (m, 2H, Ph), 7.35–7.28 (m, 3H, Ph), 4.50 (d, *J* = 9.9 Hz, 1H, H-1), 3.91–3.77 (m, 2H, H-4, H-6), 3.74–3.68 (m, 1H, H-4), 3.39–3.33 (m, 1H, H-2), 3.32–3.27 (m, 1H, H-5), 2.50 (dt, *J* = 12.5, 4.7 Hz, 1H, H-3), 1.59 (q, *J* = 11.6 Hz, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) α isomer δ 133.21 (Ph), 132.51 (Ph), 129.16 (Ph), 127.84 (Ph), 88.2 (C-1), 72.9 (C-4), 65.84 (C-5), 62.5 (C-6), 57.8 (C-2), 33.7 (C-3). β isomer δ 133.06 (Ph), 132.83 (Ph), 129.08 (Ph), 128.26 (Ph), 88.2 (C-1), 81.9 (C-5), 65.4 (C-4), 62.7 (C-6), 58.6 (C-2), 33.9 (C-3). HRMS (ESI, positive ion mode) calculated for $C_{12}H_{15}N_3O_3S^+$ [*M* + *Na*]$^+$ 304.0726, found 304.0726.

(*3R,5S,6R*)-3-azido-5-(benzyloxy)-6-((benzyloxy)methyl)-2-(phenylthio)tetrahydro-2-H-pyran (32): Sodium hydride (60% dispersion in mineral oil, 0.165 g, 3.91 mmol) was added to a solution of compound **31** (0.458 g, 1.63 mmol) in dry dimethylformamide (25 mL) under nitrogen gas at 0 °C. The reaction mixture was stirred at room temperature for 30 min. The reaction solution was cooled to 0 °C and benzyl bromide (0.48 mL) was added. The reaction mixture was stirred at room temperature for 8 h. After adding methanol (5 mL) at 0 °C, the solution was concentrated under reduced pressure. The residue was diluted with dichloromethane (30 mL) and washed with water (20 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 5:1) to give compound **32** (α/β mixtures, 0.721 g, 96%) as a clear liquid. ^{1}H NMR (400 MHz, $CDCl_3$) α isomer δ 7.56–7.53 (m, 2H, Ph), 7.36–7.29 (m, 7H, Ph),7.29–7.25 (m, 6H), 5.55 (d, *J* = 5.1 Hz, 1H, H-1), 4.62 (d, *J* = 12.0 Hz, 1H, Bn), 4.62 (d, *J* = 11.2 Hz, 1H, Bn), 4.48 (d, *J* = 12 Hz, 1H, Bn), 4.46 (d, *J* = 11.5 Hz, 1H, Bn), 4.35–4.31 (m, 1H, H-5), 3.90–3.81 (m, 2H, H-2, H-6), 3.74–3.68 (m, 2H, H-6, H-4), 2.46–4.40 (m, 1H, H-3),

1.89–1.80 (m, 1H, H-3). β isomer δ 7.62–7.60 (m, 2H, Bn), 7.36–7.24 (m, 13H, Bn), 4.62 (d, *J* = 12.1 Hz, 1H, Bn), 4.58 (d, *J* = 11.6 Hz, 1H, Bn), 4.56 (d, *J* = 11.9 Hz, 1H, Bn), 4.50 (d, *J* = 9.9 Hz, 1H, H-1), 4.45 (d, *J* = 11.5 Hz, 1H, Bn), 3.82 (dd, *J* = 10.9, 1.8 Hz, 1H, H-6), 3.73 (dd, *J* = 10.9, 4.5 Hz, 1H, H-6), 3.59–3.48 (m, 2H, H-4, H-5), 3.35 (ddd, *J* = 13.3, 8.4, 4.9 Hz, 1H, H-2), 2.61 (dt, *J* = 12.4, 4.5 Hz, 1H, H-3), 1.61–1.52 (m, 1H, H-3). ^{13}C NMR (125 MHz, CD_3Cl_3) α isomer δ 138.03 (Ph), 137.71 (Ph), 133.63 (Ph), 132.24 (Ph), 128.99 (Ph), 128.33 (Ph), 127.90 (Ph), 127.81 (Ph), 127.77 (Ph), 127.65 (Ph), 127.51 (Ph), 88.5 (C-1), 73.4 (Bn), 71.62 (C-5), 71.48 (C-4), 71.34 (Bn), 68.49 (C-6), 57.8 (C-2), 31.0 (C-3). β isomer δ 138.35 (Ph), 137.72 (Ph), 132.89 (Ph), 132.19 (Ph), 128.87 (Ph), 128.44 (Ph), 128.31 (Ph), 127.88 (Ph), 127.72 (Ph), 127.59 (Ph), 127.53 (Ph), 88.26 (C-1), 81.36 (C-5), 73.41 (Bn), 71.46 (Bn), 71.21 (C-4), 69.1 (C-6), 58.48 (C-2), 35.96 (C-3). HRMS (ESI, positive) calculated for $C_{26}H_{27}N_3O_3S^+$ $[M + Na]^+$ 484.1665, found 484.1669.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,5S,6R*)-3-azido-5-(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)cyclohexane-1,2-diyl diacetate (34): Compound **33** (0.244 g, 0.821 mmol) and compound **32** (0.417 g, 0.903 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **33** and compound **32** in anhydrous dichloromethane (10 mL) was added to a preactivated 4 Å molecular sieves and *N*-iodosuccinimide (0.295 g, 1.31 mmol). After stirring for 20 min at room temperature, the mixture was cooled to –78 °C. Trifluoromethanesulfonic acid (21 μL, 0.246 mmol) was slowly added, and the reaction was warmed to 0 °C slowly. The reaction was quenched by adding solid sodium sulfite (0.3 g), sodium bicarbonate (0.3 g) and a few drops of water. The color of solution turned to clear from dark yellow. The reaction mixture was diluted with dichloromethane, filtered, and washed with saturated solution of sodium bicarbonate and brine. The residue

was dried over magnesium sulfate, filtered, concentrated under reduced pressure, and purified by flash column chromatography on silica gel (Hex:EtOAc =3:1) to give compound **34** as a clear oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34–7.26 (m, 8H, Bn), 7.23–7.21 (m, 2H, Bn), 5.14 (t, *J* = 9.8 Hz, 1H, H-5), 5.07 (d, *J* = 3.5 Hz, 1H, H-1'), 4.92 (t, *J* = 10.0 Hz, 1H, H-6), 4.64 (d, *J* = 12.2 Hz, 1H, Bn), 4.59 (d, *J* =11.4 Hz, 1H, Bn), 4.50 (d, *J* = 12.2 Hz, 1H, Bn), 4.43 (d, *J* = 11.3 Hz, 1H, Bn), 4.08 (ddd, *J* = 9.9, 3.0, 2.1 Hz, 1H, H-5'), 3.83 (dd, *J* = 10.7, 3.1 Hz, 1H, H-6'), 3.73–3.69 (m, 2H, H-4, H-4'), 3.66 (dd, *J* = 10.7, 2.0 Hz, 1H, H-6'), 3.62 (ddd, *J* = 12.5, 10.0, 4.5 Hz, 1H, H-1), 3.43 (ddd. *J* = 12.4, 9.9, 4.6 Hz, 1H, H-3), 3.10 (ddd, *J* = 13.1, 4.5, 3.6 Hz, 1H, H-2'), 2.38 (dt, *J* = 13.4, 4.6 Hz, 1H, C-2), 2.32 (dt, *J* = 11.5, 4.5 Hz, 1H, C-3'), 2.08 (s, 3H, OAc), 2.07 (s, 3H, OAc), 1.98 (dt, *J* = 13.2, 11.3 Hz, 1H, C-3'), 1.58 (dt, *J* = 13.4, 12.5 Hz, 1H, C-2). ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.82 (OAc), 169.48 (OAc), 138.04 (Ph), 137.81 (Ph), 128.39 (Ph), 128.34 (Ph), 127.84 (Ph), 127.73 (Ph), 127.65 (Ph), 98.5 (C-1'), 77.7 (C-4), 74.15 (C-6), 73.84 (C-5), 73.53 (Bn), 71.63 (C-5'), 71.30 (Bn), 71.13 (C-4'), 68.05 (C-6'), 58.75 (C-3), 57.72 (C-1), 56.09 (C-2'), 31.82 (C-2), 27.83 (C-3'), 20.67 (OAc), 20.55 (OAc). HRMS (ESI, positive) calculated for $C_{30}H_{35}N_9O_8^+$ $[M + Na]^+$ 672.2501, found 672.2515.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,5S,6R*)-3-azido-5-(benzyloxy)-6-((benzyloxy-)methyl)tetrahydro-2H-pyran-2-yl)oxy)cyclohexane-1,2-diol (35): Sodium methoxide (4.35 M in methanol, 7.8 mL, 0.034 mmol) was added to a solution of compound **34** (0.221 g, 0.341 mmol) in methanol (10 mL). The reaction mixture was stirred at room temperature for 4 h, and neutralized with Amberlite IR-120 (H^+ form) resin. The mixture was filtered through a Celite pad, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **35** (0.146 g, 76%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.28 (m, 8H, Bn), 7.24–7.21 (m, 2H, Bn), 5.20 (d, *J* = 3.5 Hz, 1H, H-1'), 4.64 (d, *J* = 12.0 Hz, 1H,

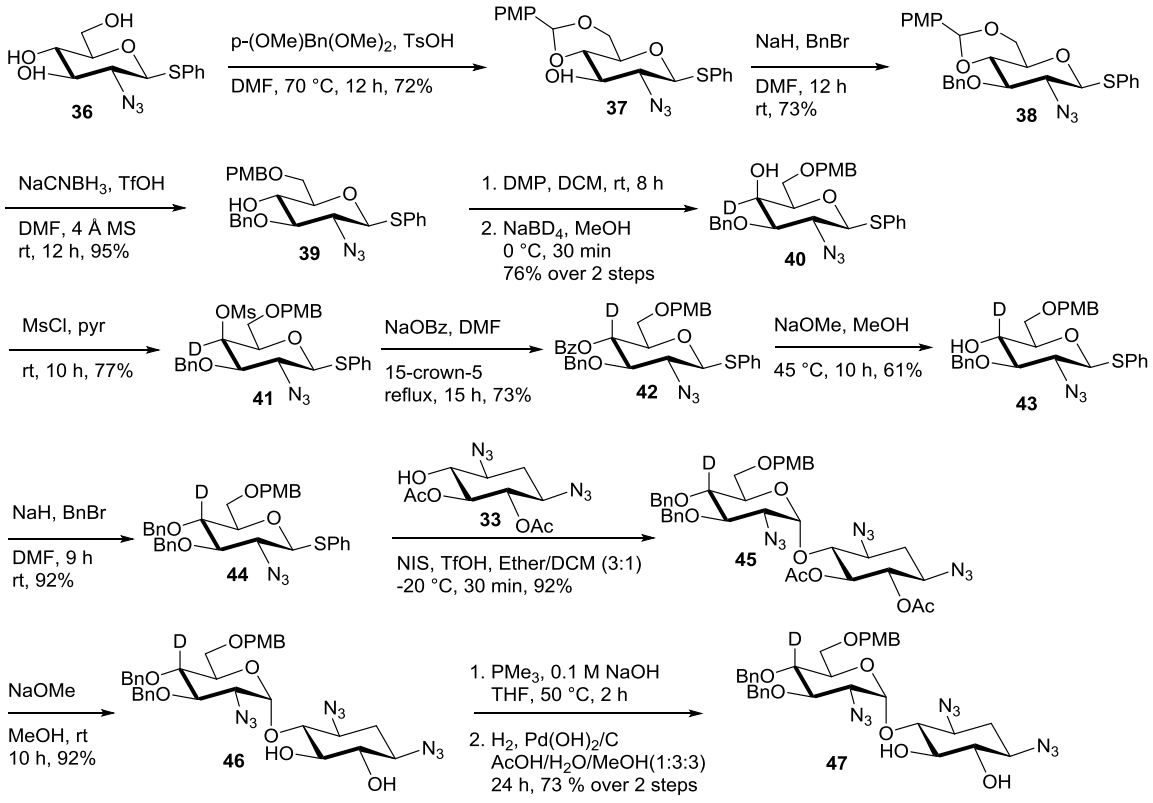
Bn), 4.60 (d, *J* = 11.4 Hz, 1H, Bn), 4.50 (d, *J* = 11.4 Hz, 1H, Bn), 4.43 (d, *J* = 12.1 Hz, 1H, Bn), 4.07 (ddd, *J* = 9.9, 3.8, 2.00 Hz, 1H, H-5'), 3.95 (d, *J* = 3.0 Hz, 1H, OH), 3.80 (dd, *J* = 10.6, 3.8 Hz, 1H, H-6'), 3.75–3.62 (m, 2H, H-4', H-6'), 3.57–3.43 (m, 2H, C-2', H-5), 3.43–3.35 (m, 3H, H-1,H-4, H-6), 3.31 (ddd, *J* = 12.3, 9.9, 4.5 Hz, 1H, H-3), 3.13 (s, 1H, OH), 2.38 (ddt, *J* = 11.6, 4.6 Hz, 1H, H-3'), 2.28 (dt, *J* = 13.3, 4.2 Hz, 1H, H-2), 2.00 (dt, *J* = 12.9, 11.3 Hz, 1H, H-3'), 1.53–1.36 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.87 (Ph), 137.76 (Ph), 128.45 (Ph), 128.39 (Ph), 127.97 (Ph), 127.88 (Ph), 127.76 (Ph), 97.59 (C-1'), 82.0 (C-6), 75.98 (C-5), 75.56 (C-4), 73.56 (Bn), 71.40 (C-5), 71.30 (Bn), 71.18 (C-4'), 68.37 (C-6'), 59.65 (C-1), 58.76 (C-3), 57.58 (C-2'), 32.09 (C-3'), 28.57 (C-2). HRMS (ESI, positive ion mode) calculated for $C_{26}H_{31}N_9O_6^+$ $[M + Na]^+$ 588.2290, found 588.2294.

Lividamine (13): Trimethylphosphine (1 M solution in THF, 1.12 mL, 1.123 mmol) was added to a solution of compound **35** (0.105 g, 0.187 mmol) in THF (6 mL) and 0.1 M NaOH (2 mL). The reaction mixture was stirred at 50 °C for 2 h, cooled to room temperature, and concentrated *in vacuo*. The residue was dissolved in a mixture of acetic acid (1 mL), water (3 mL) and methanol (3 mL). The solution was deaerated by evacuating the air inside of the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen gas. The reaction mixture was stirred at room temperature under hydrogen gas for 24 hr. The solution was filtered through a Celite pad, washed with water and methanol, and concentrated under reduced pressure. The residue was purified by chromatography on Amberlite CG-50 (NH_4^+ form) resin (5-10% concentrated ammonium hydroxide in water), acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **13** as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.51 (d, *J* = 3.5 Hz, 1H, H-1'), 3.95–3.88 (m, 2H, H-5, H-5'), 3.84 (ddd, J = 9.3, 7.1, 2.5 Hz, 1H, H-6'), 3.80–

3.65 (m, 4H, H-6', H-2', H-6, H-4'), 3.65–3.57 (m, 2H, H-4, H-1), 3.38 (ddd, J = 12.5, 10.2, 4.3 Hz, 1H, H-2), 2.54 (dt, *J* = 12.7, 4.3 Hz, 1H, H-2), 2.31 (dt, *J* = 12.3, 4.4 Hz, 1H, H-3'), 1.99 (td, J = 12.0, 10.0 Hz, 1H, H-3'), 1.91 (q, *J* = 12.6 Hz, 1H, H-2). ^{13}C NMR (125 MHz, D_2O) δ 95.3, 80.2, 75.13, 74.54, 72.26, 63.16, 60.04, 49.61, 48.86, 48.10, 29.76, 28.17. HRMS (ESI, positive) calculated for $C_{12}H_{25}N_3O_6^+$ $[M + Na]^+$ 330.1636, found 330.1645.

4.2.9. Synthesis of [4'-^{2}H]-Paromamine (47)

The overall synthetic scheme is shown in Scheme 4-3.



Scheme 4-3. Synthetic scheme for the preparation of [4'-^{2}H]-paromamine.

(*4aR,6S,7R,8R,8aS*)-7-azido-2-(4-methoxyphenyl)-6-(phenylthio)hexahydropyrano-[3,2-d][1,3]dioxin-8-ol (37): Compound **36** was prepared as previously described.[159] Compound **36** (12.88 g, 43.32 mmol) and anisaldehyde dimethyl acetal (9.14 mL, 53.72 mmol) were dissolved in dimethylformamide (200 mL). *p*-Toluenesulfonic acid monohydrate (0.745 g, 4.33 mmol) was added and the flask was attached to a rotary evaporator, rotated, evacuated, and lowered into a water bath at approximately 60 °C to remove the methanol, which was formed during the reaction. After 1 h, triethylamine (10 mL) was added and all solvent was removed by evaporation. The residue was diluted with ethyl acetate (200 mL) and saturated aqueous solution of sodium bicarbonate (100 mL). The aqueous layer was extracted with dichloromethane (3 × 200 mL). The combined organic extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (Hex/EtOAc = 2:1) to afford compound **37** (13.50 g, 72%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61–7.54 (m, 2H, Ph), 7.40–7.26 (m, 5H, Ph), 6.92–6.87 (m, 2H, Ph), 5.49 (s, 1H, PMPCH), 4.55 (d, *J* = 10.2 Hz, 1H, H-1), 4.36 (dd, *J* = 10.2, 4.1 Hz, 1H, H-4), 3.80 (s, 3H, OMe), 3.79–3.73 (m, 2H, 2×H-6), 3.50–3.43 (m, 2H, H-5, H-3), 3.36 (dd, *J* = 10.1, 9.0 Hz, 1H, H-2), 2.68 (d, *J* = 2.7 Hz, 1H, OH). ^{13}C NMR (150 MHz, CD_3OD) δ 161.7, 134.9, 132.5, 130.8, 130.6, 130.0, 129.2, 115.2, 103.2, 88.0, 81.6, 75.2, 71.7, 69.7, 66.6, 56.7. HRMS (ESI, positive ion mode) calculated for $C_{20}H_{21}N_3O_5S^+$ $[M + Na]^+$ 438.1100, found 438.1093.

(*4aR,6S,7R,8R,8aS*)-7-azido-8-(benzyloxy)-2-(4-methoxyphenyl)-6-(phenylthio)hexa-hydropyrano[3,2-d][1,3]dioxine (38): Sodium hydride (60% dispersion in mineral oil, 0.945g, 23.62 mmol) was added to a solution of compound **37** (7.55 g, 18.17 mmol) in anhydrous dimethylformamide (120 mL) at 0 °C under nitrogen atmosphere. The mixture was warmed to room temperature and was stirred for 30 min. The mixture was cooled to

0 °C and benzyl bromide (2.81 mL, 23.62 mmol) was added dropwise. The reaction was then allowed to stand for 12 h at room temperature. The mixture was treated with methanol at 0 °C and all the solvent was evaporated under reduced pressure. The residue was diluted with dichloromethane (200 mL) and washed with saturated aqueous solution of sodium bicarbonate (100 mL). The aqueous layer was extracted with dichloromethane (3 × 100 mL). The combined organic extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to afford compound **38** (6.68 g, 73 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58–7.56 (m, 2H, Ph), 7.42–7.40 (m, 2H, Ph), 7.39–7.30 (m, 8H, Ph), 6.96–6.91 (m, 2H, Ph), 5.54 (s, 1H, PMPCH), 4.92 (d, *J* = 10.9 Hz, 1H, $PhCH_2$), 4.79 (d, *J* = 11.0 Hz, 1H, $PhCH_2$), 4.50 (d, *J* = 10.2, 1H, H-1), 4.38 (dd, *J* = 10.5, 5.5 Hz, 1H, H-4), 3.83 (s, 3H, OCH_3), 3.78 (t, *J* = 10.3 Hz, 1H, H-3), 3.65–3.61 (m, 2H, H-6), 3.47–3.43 (m, 1H, H-5), 3.37 (dd, *J* = 10.3, 8.8 Hz, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.24, 137.70, 134.01, 130.77, 129.67, 129.23, 128.83, 128.55, 128.54, 128.43, 128.11, 127.40, 127.40, 113.78, 101.37, 86.73, 81.36, 81.08, 75.29, 70.63, 68.56, 64.81, 55.42. HRMS (ESI, positive) calculated for $C_{27}H_{27}N_3O_5S^+$ $[M + Na]^+$ 528.1569, found 528.1559.

(*2R,3S,4R,5R,6S*)-5-azido-4-(benzyloxy)-2-(((4-methoxybenzyl)oxy)methyl)-6-(phenylthio)tetrahydro-2H-pyran-3-ol (39): A mixture of compound **38** (4.544 g, 8.99 mmol), sodium cyanoborohydride (4.518 g, 71.91 mmol), and freshly activated 4 Å molecular sieve (1 g) in dry dimethylformamide (90 mL) was stirred at room temperature under nitrogen for 30 min. Trifluoroacetic acid (6.87 mL, 89.88 mmol) was added dropwise at 0 °C. After stirring at room temperature for 12 h, the reaction mixture was filtered through a Celite pad and poured into ice-cold saturated aqueous solution of sodium bicarbonate (100 mL). The mixture was diluted with ethyl acetate (200 mL),

washed with saturated aqueous solution of sodium bicarbonate (50 mL), brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to give compound **39** (4.326 g, 95%) as a clear oil. Compound **39** was unstable, only mass spectra were measured. HRMS (ESI, positive) calculated for $C_{27}H_{29}N_3O_5S^+$ $[M + Na]^+$ 530.1726, found 530.1718.

(*2R,3R,4R,5R,6S*)-5-azido-4-(benzyloxy)-2-(((4-methoxybenzyl)oxy)methyl)-6-(phenylthio)tetrahydro-2H-pyran-3-d-3-ol (40): Dess-Martin periodinane (7.229 g, 17.04 mmol) was added to a mixture of compound **39** (4.326 g, 8.52 mmol) in dry dichloromethane (100 mL). After stirring for 12 h under nitrogen gas at room temperature, mixtures (60 mL) of 10% aqueous solution of sodium thiosulfate and saturated aqueous solution of sodium bicarbonate (1:1) were added to the reaction mixture. The reaction was allowed to stir for an additional 30 min. The organic layer was washed with aqueous solution of sodium thiosulfate/sodium bicarbonate (1:3) (3 × 60 mL) and then brine, dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The crude ketone compound was dissolved in anhydrous methanol (50 mL) and dry dichloromethane (50 mL). Sodium borodeuteride (0.428 g, 10.23 mmol) was added portionwise to this solution 0 °C. After stirring for 10 min at 0 °C, the reaction mixture was stirred at room temperature for an additional 3 h. The reaction was quenched by the addition of saturated aqueous solution of ammonium chloride (50 mL), and the aqueous layer was extracted with dichloromethane. The combined organic layers were dried over magnesium sulfate, filtered, and concentrated under reduced pressure. Purification by flash column chromatography on silica gel (Hex/EtOAc = 3:1) gave compound **40** (3.294 g, 76%) as a white solid.

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.60– 7.57 (m, 2H, Ph), 7.39– 7.32 (m, 4H, PH, 7.28–7.23 (m, 6H, PMP, Ph)), 6.89–6.85 (m, 2H, Ph), 4.69 (d, *J* = 11.5 Hz, 1H, Bn), 4.65 (d, *J* = 11.5 Hz, 1H, Bn), 4.52–4.47 (m, 2H, PMP), 4.37 (d, *J* = 10.2 Hz, 1H, H-1), 3.79 (s, 3H, OMe), 3.77–3.70 (m, 2H, 2×H-6), 3.64 (dd, *J* = 10.2, 9.5 Hz, 1H, H-2), 3.52 (t, *J* = 5.7 Hz, 1H, H-5), 3.37 (d, *J* = 9.5 Hz, 1H, H-3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.43, 137.08, 133.20, 131.78, 129.97, 129.55, 129.05, 128.71, 128.34, 128.23, 128.14, 113.95, 86.39, 81.12, 73.49, 72.01, 69.08, 61.08, 55.38. HRMS (ESI, positive) calculated for $C_{27}H_{28}DN_3O_5S^+$ $[M + Na]^+$ 531.1788, found 531.1780.

(*2R,3R,4R,5R,6S*)-5-azido-4-(benzyloxy)-2-(((4-methoxybenzyl)oxy)methyl)-6-(phenylthio)tetrahydro-2H-pyran-3-yl-3-d methanesulfonate (41): Methanesulfonyl chloride (1.77 mL, 22.93 mmol) and dimethylaminopyridine (0.028 g, 0.23 mmol) was added to a solution of compound **40** (2.332 g, 4.59 mmol) in anhydrous pyridine (40 mL) at 0 °C. The reaction mixture was stirred at room temperature for 10 h. Methanol (10 mL) was added to quench the reaction at 0 °C. The mixture was concentrated under reduced pressure and co-evaporated with toluene (3 × 30 mL). The residue was diluted with dichloromethane (50 mL) and water (50 mL). The aqueous layer was extracted with dichloromethane (3 × 50 mL), dried over magnesium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to afford compound **41** (2.081g, 77%) as a light yellowish oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60–7.57 (m, 2H, Ph), 7.42–7.30 (m, 10H, Ph), 6.91–6.89 (m, 2H, Ph), 4.81 (d, *J* = 10.6, 1H, Bn), 4.58 (dd, *J* = 10.7, 2.0 Hz, 2H, PMP), 4.43 (d, *J* = 10.8 Hz, 1H, Bn), 4.41 (d, *J* = 9.56 Hz, 1H, H-1) 3.81 (s, 3H, OCH_3), 3.75–3.69 (m, 3H, 2×H-6, H-3), 3.54–3.44 (m, 2H, H-2, H-5), 2.92 (s, 3H, SCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.51,

136.40, 133.52, 131.26, 130.03, 129.77, 129.06, 128.72, 128.66, 128.54, 128.50, 113.95, 86.39, 79.36, 75.56, 73.60, 72.81, 67.51, 61.14, 55.37, 39.16. HRMS (ESI, positive) calculated for $C_{28}H_{30}DN_3O_7S_2^+$ $[M + Na]^+$ 609.1564, found 609.1559.

(*2R,3S,4R,5R,6S*)-5-azido-4-(benzyloxy)-2-(((4-methoxybenzyl)oxy)methyl)-6-(phenylthio)tetrahydro-2H-pyran-3-yl-3-d benzoate (42): Sodium benzoate (1.214 g, 10.64 mmol) and 15-crown-5 (50 μL) were added to a solution of compound **41** (2.081 g, 3.55 mmol) in anhydrous dimethylformamide (DMF, 100 mL). The reaction mixture was heated to reflux for 15 h. The solvent was evaporated under reduced pressure, and the residue was diluted with dichloromethane (50 mL) and water (50 mL). The aqueous layer was extracted with dichloromethane (3 × 50 mL), dried over magnesium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to give compound **42** (1.316 g, 60 %) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.97–7.95 (m, 2H, Bz), 7.61–7.59 (m, 3H, Ph, Bz), 7.45–7.43 (m, 2H, Bz), 7.32–7.29 (m, 3H, Ph), 7.16–7.11 (m, 7H, PMB, Bn), 6.77–6.75 (m, 2H, PMB), 4.72 (d, *J* = 10.8 Hz, Bn), 4.58 (d, *J* = 10.8 Hz, Bn), 4.51 (d, *J* = 10.8 Hz, 1H, H-1), 4.41 (m, 2H, PMB), 3.76 (s, 3H, OCH_3), 3.72 (m, 1H, H-6), 3.66 (d, *J* = 9.3 Hz, 1H, H-3), 3.59 (m, 2H, H-5, H-6), 3.47 (dd, *J* = 10.1, 9.2 Hz, 1H, H-2). ^{13}C NMR (150 MHz, $CDCl_3$) δ 165.31, 159.24, 137.09, 133.63, 133.51, 131.17, 130.00, 129.88, 129.51, 129.45, 129.20, 128.61, 128.59, 128.43, 128.37, 128.05, 113.78, 86.25, 82.48, 78.07, 75.51, 73.32, 69.23, 64.93, 55.35. HRMS (ESI, positive) calculated for $C_{34}H_{32}DN_3O_6S^+$ $[M + Na]^+$ 632.2051, found 635.2013.

(*2R,3S,4R,5R,6S*)-5-azido-4-(benzyloxy)-2-(((4-methoxybenzyl)oxy)methyl)-6-(phenylthio)tetrahydro-2H-pyran-3-d-3-ol (43): Sodium methoxide (4.37 M in methanol, 0.18 mL, 0.78 mmol) was added dropwise to a solution of compound **42** (2.399 g, 3.92 mmol) in anhydrous methanol (10 mL) and anhydrous dichloromethane (40 mL). The

reaction mixture was stirred at 45 °C for 10 h. After cooling down to room temperature, the reaction mixture was neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated. The residue was purified by flash column chromatography (Hex/EtOAc = 3:1) on silica gel to give compound **43** (1.215 g, 61%) as a clear oil. Compound **34** was unstable so it was characterized only by mass spectroscopy. HRMS (ESI, positive) calculated for $C_{27}H_{28}DN_3O_5S^+$ $[M + Na]^+$ 531.1788, found 531.1781.

(*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)-2-(phenylthio)tetrahydro-2H-pyran-5-d (44): Sodium hydride (60% dispersion in mineral oil, 0.215g, 5.39 mmol) was added portionwise to a solution of compound **43** (2.283 g, 4.49 mmol) in anhydrous DMF (50 mL) at 0 °C under nitrogen atmosphere. The mixture was warmed to room temperature and stirred for 30 min. The solution was cooled to 0 °C and benzyl bromide (0.64 mL, 5.39 mmol) was added dropwise. The reaction was continued with stirring for 9 h at room temperature. The mixture was quenched with methanol at 0 °C and the solvent was evaporated *in vacuo*. The residue was diluted with dichloromethane (100 mL) and washed with saturated aqueous solution of sodium bicarbonate (50 mL). The aqueous layer was repeatedly extracted with dichloromethane (3 × 50 mL). The combined extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to provide compound **44** (2.208 g, 82 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70–7.68 (m, 2H, Ph), 7.41–7.33 (m, 13H, Ph), 7.28–7.27 (m, 2H, Ph), 6.96–6.94 (m, 2H, Ph), 4.95–4.90 (m, 2H, Bn), 4.86 (d, *J* = 10.9 Hz, 1H, Bn), 4.66–4.63 (m, 2H, PMB), 4.55 (d, *J* = 11.6 Hz, 1H, Bn), 4.50 (d, *J* = 10.1 Hz, 1H, H-1), 3.86 (s, 3H, OCH_3), 3.84-3.78 (m, 2H, H-6), 3.58 (d, *J* = 9.5 Hz, 1H, H-3), 3.53 (dd, *J* = 4.12, 2.1 Hz, 1H, H-5), 3.44 (t, *J* = 9.7, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.3, 138.0, 137.7, 131.4, 130.3, 129.4,

129.0, 128.6, 128.5, 128,4, 128.2, 128.1, 127.92, 127.88, 113.9, 86.1, 85.1, 79.4, 75.9, 75.0, 73.2, 68.4, 65.2, 55.3. HRMS (ESI, positive) calculated for $C_{34}H_{34}DN_3O_5S^+$ [*M* + *Na*]$^+$ 621.2258, found 621.2250.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2H-pyran-2-yl-5-d)oxy)cyclohexane-1,2-diyl diacetate (45): Compound **44** (2.208 g, 3.69 mmol) and compound **33** (0.916 g, 3.07 mmol) were co-evaporated from dry toluene (500 mL) three times and further dried under high vacuum overnight. A solution of compound **44** and **33** in anhydrous diethyl ether (30 mL) and anhydrous dichloromethane (10 mL) was added to a preactivated 4 Å MS (3 g). The mixture was stirred for 30 min at room temperature, and cooled to –40 °C. *N*-Iodosuccinimide (1.797 g, 7.99 mmol) was added, and the reaction mixture was stirred for 20 min. Trifluoromethanesulfonic acid (136 μL, 1.53 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. The reaction mixture was diluted with dichloromethane (100 mL), filtered through a Celite pad, and washed with 10% aqueous solution of sodium bisulfite (100 mL) and saturated aqueous solution of sodium bicarbonate (100 mL). The aqueous layers were extracted with dichloromethane (3 × 50 mL). The combined organic phase was washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **45** (2.212g, 92%) as a sticky liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33–7.23 (m, 12H, Ph), 7.13–7.11 (m, 2H, Ph), 6.83 (d, *J* = 8.4 Hz, 2H, Ph), 5.14 (t, *J* = 9.2 Hz, 1H, H-5), 5.13 (d, *J* = 4 Hz, 1H, H-1'), 4.92 (t, *J* = 10.0 Hz, 1H, H-6), 4.84 (dd, *J* = 25.0, 10.5 Hz, 2H, PMB), 4.75 (d, *J* = 10.8 Hz, 1H, Bn), 4.60 (d, *J* = 11.6 Hz, 1H, Bn), 4.47 (d, *J* = 11.2 Hz, 1H, Bn), 4.40 (d, *J* = 11.6 Hz, 1H, Bn), 4.13 (s, 1H, H-5'), 3.93 (d, *J* = 10.4 Hz, 1H, H-3'), 3.80 (dd, *J* = 10.6, 2.0 Hz, 1H, H-6'), 3.75 (s, 3H, -OCH_3), 3.66–3.58 (m,

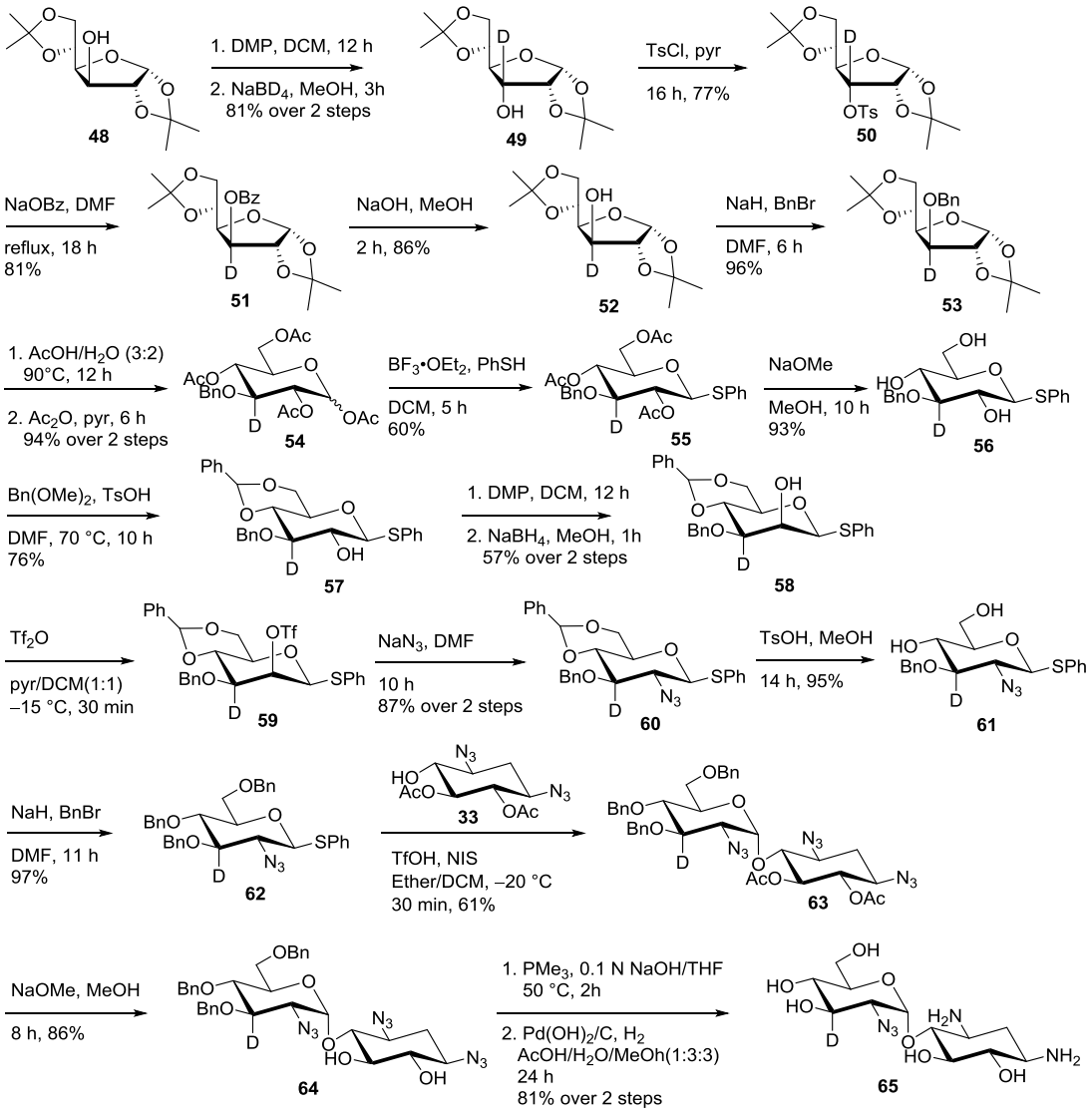
3H, H-1, H-6', H-4), 3.43–3.37 (m, 1H, H-3), 3.35 (dd, *J* = 10.2, 3.6 Hz, 1H, H-2'), 2.40 (dt, *J* = 13.6, 4.4 Hz, 1H, H-2), 1.585 (q, *J* = 12.8 Hz, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.95, 169.61, 159.43, 138.05, 137.92, 129.92, 129.77, 128.56, 128.49, 128.08, 127.97, 127.86, 127.77, 113.92, 99.50, 79.60, 78.40, 75.42, 74.97, 74.23, 73.67, 73.32, 71.72, 67.52, 63.25, 58.81, 57.83, 55.32, 31.94, 20.75, 20.68. HRMS (ESI, positive) calculated for $C_{38}H_{42}DN_9O_{10}^+$ $[M + Na]^+$ 809.3093, found 809.3079.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2H-pyran-2-yl-5-d)oxy)cyclohexane-1,2-diol (46): Sodium methoxide (4.37 M in methanol, 64 μL, 0.28 mmol) was added dropwise to a solution of compound **45** (2.196 g, 2.79 mmol) in methanol (30 mL) and dichloromethane (10 mL). The reaction mixture was stirred at room temperature for 10 h. The reaction mixture was neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **46** (1.215 g, 92%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.23 (m, 10H, Ph), 7.14–7.10 (m, 2H, Ph), 6.85–6.81 (m, 2H, Ph), 5.13 (d, *J* = 3.2 Hz, 1H, H-1), 4.88 (dd, *J* = 31.2, 10.8 Hz, 2H, PMB), 4.75 (d, *J* = 10.4 Hz, 1H, Bn), 4.60 (d, *J* = 12.0 Hz, 1H, Bn), 4.48 (d, *J* = 10.8 Hz, 1H, Bn), 4.40 (d, *J* = 11.6 Hz, 1H, Bn), 4.19 (s, 1H, OH), 4.07 (t, *J* = 2.0, 1H, H-5'), 3.96 (d, *J* = 10.4 Hz, 1H, H-3'), 3.78 (dd, *J* = 10.6, 2.8 Hz, 1H, H-6'), 3.75 (s, 3H, OCH_3), 3.66–3.62 (m, 2H, H-2', H-6'), 3.49–3.38 (m, 3H, H-4, H-5, H-1), 3.25 (dd, *J* = 5.6, 2.8 Hz, 2H, H-3, H-6), 2.33–2.28 (m, 1H, H-2), 1.49–1.45 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.45, 137.90, 137.67, 129.87, 129.83, 128.62, 128.56, 128.18, 128.11, 127.98, 127.85, 113.92, 99.71, 84.12, 81.03, 75.98, 75.76, 75.46, 75.04, 73.35, 71.73, 67.61, 64.38, 59.76, 58.88, 55.34, 32.23. HRMS (ESI, positive) calculated for $C_{34}H_{38}DN_9O_8^+$ $[M + Na]^+$ 725.2882, found 725.2876.

[4'-^{2}H]-Paromamine (47): Trimethylphosphine (1 M solution in THF, 2.21 mL) was added to a solution of compound **46** (0.259 g, 0.37 mmol) in THF (9 mL) and 0.1 M NaOH (3 mL). The reaction mixture was stirred at 50 °C for 2 h. After cooling down to room temperature, the solution was evaporated under reduced pressure. The material was dissolved in a mixture of acetic acid (2 mL), water (6 mL) and methanol (6 mL). The solution was deaerated by evacuating the air in the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.095 g) was added and the solution was charged with hydrogen. The reaction mixture was stirred at room temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad and concentrated. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (2.5% concentrated ammonium hydroxide in water), concentrated, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **47** (0.086 g, 73%) as an oily solid. ^{1}H NMR (400 MHz, D_2O) δ 5.65 (d, J = 4.0 Hz, 1H, H-1'). 3.96–3.79 (m, 4H, H-3', H-5, H-6', H-5'), 3.75–3.72 (m, 1H, H-6'), 3.67 (dd, J = 12.0, 6.5 Hz, 1H, H-6), 3.61–3.55 (m, 2H, H-1, H-4), 3.45 (dd, J = 12.2, 6.6, 1H, H-2'), 3.37–3.31 (m, 1H, H-3), 2.50 (dt, J = 12.6, 4.3 Hz, 1H, H-2), 1.86 (q, J = 12.6 Hz, 1H, H-2) . ^{13}C NMR (150 MHz, D_2O) δ 97.1 (C-1'), 80.4 (C-5), 74.7 (C-6), 73.6 (C-5'), 72.3 (C-4), 69.0 (C-4'), 60.3 (C-6'), 54.0 (C-2'), 49.6 (C-3), 48.8 (C-1), 28.3 (C-2). HRMS (ESI, positive) calculated for $C_{12}H_{24}DN_3O_7^+$ $[M + H]^+$ 325.1829, found 325.1834.

4.2.10. Synthesis of [3'-^{2}H]-Paromamine (65)

The overall synthetic scheme is shown in Scheme 4-4. Yu-Cheng Yeh synthesized this compound.



Scheme 4-4. Synthetic scheme for the preparation of [3'-^{2}H]-paromamine.

(*3aR,5S,6S*)-5-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)-2,2-dimethyltetrahydrofuro[2,3-d][1,3]dioxol-6-d-6-ol (52): Compound **51** was prepared as previously described.[170] Compound **51** (1.059 g, 2.90 mmol) was dissolved in 5% solution of sodium hydroxide in methanol (0.1 M, 29 mL), and the reaction mixture was stirred for 2 h. The solvent was removed under reduced pressure, and the residue was dissolved in dichloromethane (40

mL). The organic layer was washed with water (40 mL × 2), brined, dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 1:1) to give compound **52** (0.652 g, 86%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.95 (d, *J* = 3.6 Hz, 1H, H-1), 4.53 (d, *J* = 3.7 Hz, 1H, H-2), 4.34 (ddd, *J* = 7.6, 6.2, 5.4 Hz, 1H, H-5), 4.17 (dd, *J* = 8.6, 6.2 Hz, 1H, H-6), 4.07 (d, *J* = 7.6 Hz, 1H, H-4), 3.98 (dd, *J* = 8.6, 5.4 Hz, 1H, H-6), 2.48 (s, 1H, OH), 1.50 (d, *J* = 0.7 Hz, 3H, Me), 1.44 (d, *J* = 0.7 Hz, 3H, Me), 1.36 (d, *J* = 0.7 Hz, 3H, Me), 1.32 (d, *J* = 0.7 Hz, 3H, Me). ^{13}C NMR (125 MHz, $CDCl_3$) δ 111.99(acetal), 109.82 (acetal), 105.47 (C-1), 85.18 (C-2), 81.21 (C-4), 73.71 (C-5), 67.83 (C-6), 27.01 (Me), 26.92 (Me), 26.34 (Me), 25.29 (Me). HRMS (ESI, positive) calculated for $C_{12}H_{19}DO_6^+$ $[M + Na]^+$ 284.1215, found 284.1215.

(*3aR,5R,6S*)-6-(benzyloxy)-5-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)-2,2-dimethyltetra-hydrofuro[2,3-d][1,3]dioxole-6-d (53): Sodium hydride (60% dispersion in mineral oil, 1.15 g, 28.70 mmol) was added to a solution of compound **52** (6.25 g, 23.92 mmol) in dimethylformamide (205 mL) at 0 °C. The reaction mixture was stirred at room temperature for 30 min. Benzyl bromide (3.5 mL, 28.70 mmol) was added at 0 °C, and the reaction mixture was stirred for 15 h. Methanol (10 mL) was added slowly at 0 °C. The reaction solution was concentrated under reduced pressure. The residue was diluted with dichloromethane (200 mL), and washed with water (150 mL × 2). The combined aqueous layers were extracted with dichloromethane (200 mL × 2). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude compound was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to provide compound **53** (8.047 g, 96%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38–7.28 (m, 5H, Ph), 5.90 (d, *J* = 3.7 Hz, 1H), 4.72–4.61 (m, 2H, 2×Bn), 4.58 (d, *J* = 3.7 Hz, 1H, H-2), 4.37 (dt, *J* = 7.7, 6.0

Hz, 1H, H-5), 4.17–4.08 (m, 2H, H-6, H-4), 4.05–3.94 (m, 1H, H-6), 1.49 (d, *J* = 0.6 Hz, 3H, OAc), 1.43 (d, *J* = 0.7 Hz, 3H, OAc), 1.37 (d, *J* = 0.7 Hz, 3H, OAc), 1.31 (s, 3H, OAc). HRMS (ESI, positive) calculated for $C_{19}H_{25}DO_6^+$ $[M + Na]^+$ 374.1684, found 374.1690.

(*3R,4S,5R,6R*)-6-(acetoxymethyl)-4-(benzyloxy)tetrahydro-2*H*-pyran-2,3,5-triyl-4-d triacetate (54): Compound **53** (8.047 g, 22.90 mmol) was dissolved in acetic acid (69 mL) and H_2O (46 mL), and the reaction mixture was stirred at 90 °C for 12 h. The reaction solution was evaporated under reduced pressure and co-evaporated with toluene three times. The residue was dissolved in pyridine (0.4 M, 57 mL) and acetic anhydride (0.4 M, 57 mL) was added at 0 °C. The reaction mixture was stirred at room temperature for 6 h. The reaction solution was evaporated under reduced pressure and co-evaporated with toluene three times. The residue was dissolved in ethyl acetate (100 mL) and washed with saturated aqueous solution of sodium bicarbonate (60 mL × 2) and brine. The organic layer was dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 1:1) to provide compound **54** (9.47 g, 94%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) α isomer δ 7.36–7.21 (m, 5H, Ph), 6.32 (d, *J* = 3.6 Hz, 1H), 5.19 – 5.12 (m, 1H, H-4), 5.06 (d, *J* = 3.7 Hz, 1H, H-2), 4.71 (d, *J* = 11.8 Hz, 1H, Bn), 4.65–4.56 (m, 1H), Bn, 4.21–4.18 (m, 1H, H-6), 4.10–4.05 (m, 1H, H-6), 4.02 (ddd, *J* = 10.3, 4.5, 2.4 Hz, 1H, H-5), 2.17 (s, 3H, OAc), 2.08 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.97 (s, 3H). β isomer δ 7.36–7.21 (m, 5H, Ph), 5.66 (d, *J* = 8.2 Hz, 1H, H-1), 5.19–5.12 (m, 2H, H-2, H-4), 4.65–4.56 (m, 2H, 2×Bn), 4.21 (ddd, *J* = 12.5, 9.9, 4.7 Hz, 1H, H-6), 4.08 (td, *J* = 12.4, 2.4 Hz, 1H, H-6), 3.74 (ddt, *J* = 10.1, 4.9, 2.5 Hz, 1H, H-5), 2.10 (s, 3H, OAc), 2.07 (s, 3H, OAc), 1.98 (s, 6H, 2×OAc). ^{13}C (125 MHz, $CDCl_3$) α isomer 170.75 (O(C=O)), 169.61 (O(C=O)), 169.29 (O(C=O)), 168.79 (O(C=O)), 137.99 (Ph), 128.49 (Ph), 127.87

(Ph), 127.55 (Ph), 89.54 (C-1), 74.77 (Bn), 71.50 (C-2), 70.32 (C-5), 69.07 (C-4), 61.87 (C-6), 20.93 (OAc), 20.76 (OAc), 20.74 (OAc), 20.62 (OAc). β isomer 170.72 (O(C=O)), 169.32 (O(C=O)), 169.24 (O(C=O)), 169.11 (O(C=O)), 137.63 (Ph), 128.53 (Ph), 127.99 (Ph), 127.83 (Ph), 92.04(C-1), 74.18 (Bn), 73.04 (C-5), 71.55 (C-2), 69.07 (C-4), 61.87 (C-6), 20.89 (OAc), 20.77 (OAc), 20.76 (OAc), 20.73 (OAc). HRMS (ESI, positive) calculated for $C_{21}H_{25}DO_{10}^{+}$ $[M + Na]^{+}$ 462.1481, found 462.1487.

(*2R,3R,4S,5R,6S*)-2-(acetoxymethyl)-4-(benzyloxy)-6-(phenylthio)tetrahydro-2*H*-pyran-3,5-diyl-4-d diacetate (55): Thiophenol (2.43 mL, 23.7 mmol) and boron trifluoride diethyl etherate (6.3 mL, 23.7 mmol) were added to a solution of compound **54** (9.47 g, 21.55 mmol) in dichloromethane (215 mL) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 5 h. The reaction mixture was quenched by adding saturated aqueous solution of sodium bicarbonate (150 mL) at 0 °C. The aqueous layer was extracted with dichloromethane (100 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to provide compound **55** (6.35 g, 60%) as a clear solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52–7.45 (m, 2H, Ph), 7.34–7.25 (m, 6H, Ph), 7.23–7.19 (m, 2H, Ph), 5.08–5.04 (m, 2H, H-4, H-2), 4.64 (d, J = 10.0 Hz, 1H, H-1), 4.63–4.57 (m, 2H, 2×Bn), 4.19–4.15 (m, 2H, 2×H-6), 3.63 (ddd, J = 10.1, 5.1, 3.2 Hz, 1H, H-5), 2.07 (s, 3H, OAc), 2.04 (s, 3H, OAc), 1.96 (s, 3H, OAc). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.76 (O(C=O)), 169.44 (O(C=O)), 169.31 (O(C=O)), 137.80 (Ph), 132.71 (Ph), 132.63 (Ph), 128.96 (Ph), 128.57 (Ph), 128.16 (Ph), 127.99 (Ph), 127.88 (Ph), 86.34 (C-1), 76.21 (C-5), 74.27 (Bn), 71.41 (C-2), 69.69 (C-4), 62.67 (C-6), 21.06 (OAc), 20.87 (OAc), 20.84 (OAc). HRMS (ESI, positive) calculated for $C_{25}H_{27}DO_8S^{+}$ $[M + Na]^{+}$ 512.1460, found 512.1465.

(*2R,3R,4S,5R,6S*)-4-(benzyloxy)-2-(hydroxymethyl)-6-(phenylthio)tetrahydro-2*H*-pyran-4-d-3,5-diol (56): Sodium methoxide (5.4 M solution in methanol, 0.24 mL, 1.29 mmol) was added to a solution of compound **55** (6.35 g, 12.97 mmol) in methanol (130 mL). The reaction mixture was stirred for 10 h, neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (EtOAc/DCM = 1:1) to provide compound **56** (4.37 g, 93%) as a white solid. No characterization of this compound was conducted.

(*4aR,6S,7R,8R,8aR*)-8-(benzyloxy)-2-phenyl-6-(phenylthio)hexahydropyrano[3,2-d][1,3]dioxin-8-d-7-ol (57): p-Toluenesulfonic acid monohydrate (0.247 g, 1.30 mmol) and compound **56** (4.72 g, 12.99 mmol) was co-evaporated from dry toluene (30 mL) three times and further dried under high vacuum overnight. Compound **56** and p-toluenesulfonic acid were dissolved in dimethylformamide, and benzaldehyde dimethyl acetal (3.90 mL, 26 mmol) was added. The reaction mixture stirred at 70 °C for 10 h under house vacuum. The solvent was removed under reduced pressure. The residue was diluted with dichloromethane (70 mL), and washed with saturated aqueous solution of sodium bicarbonate (60 mL × 2). The combined aqueous layers were extracted with dichloromethane (70 mL × 2). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel (Hex:EtOAc = 1:1) to provide compound **57** (4.44 g, 76%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.56–7.53 (m, 2H, Ph), 7.51–7.47 (m, 2H, Ph), 7.40–7.35 (m, 6H, Ph), 7.35–7.31 (m, 5H, Ph), 5.58 (s, 1H, PhC<u>H</u>O_2), 4.96 (d, J = 11.5 Hz, 1H, Bn), 4.80 (d, J = 11.6 Hz, 1H, Bn), 4.64 (d, J = 9.8 Hz, 1H, H-1), 4.39 (dd, J = 10.5, 5.0 Hz, 1H, H-6), 3.80 (t, J = 10.3 Hz, 1H, H-6), 3.66

(d, J = 9.4 Hz, 1H, H-4), 3.58–3.49 (m, 2H, H-2, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.30 (Ph), 137.31 (Ph), 133.32 (Ph), 131.44 (Ph), 129.17 (Ph), 129.15 (Ph), 128.60 (Ph), 128.50 (Ph), 128.39 (Ph), 128.25 (Ph), 128.02 (Ph), 126.13 (Ph), 101.40 (Ph$\underline{C}HO_2$), 88.59 (C-1), 81.17 (C-4), 74.91 (Bn), 72.31 (C-2), 70.87 (C-5), 68.76 (C-6). HRMS (ESI, positive) calculated for $C_{26}H_{25}DO_5S^+$ $[M + Na]^+$ 474.1456, found 474.1459.

(*4aR,6S,7S,8R,8aR*)-8-(benzyloxy)-2-phenyl-6-(phenylthio)hexahydropyrano[3,2-d][1,3]dioxin-8-d-7-ol (58): Dess-Martin periodinane (8.341 g, 19.66 mmol) was added to a solution of compound **57** (4.44 g, 9.83 mmol) in dichloromethane (98 mL). The reaction mixture was stirred for 12 h. Saturated aqueous solutions of sodium sulfite (50 mL) and sodium bicarbonate (50 mL) were added, and the reaction mixture was stirred for additional 30 min. The organic layer was washed with saturated aqueous solutions of sodium sulfite (30 mL) and sodium bicarbonate (90 mL) three timed. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated. The resulting ketone was dissolved in methanol (98 mL), and cooled to 0 °C. Sodium borohydride (0.470 g, 11.80 mmol) was added portionwise during 10 min. The reaction mixture was stirred at 0 °C for 10 min and at room temperature for 1 h. The reaction was quenched by adding saturated aqueous solution of ammonium chloride (50 mL). The solvent was evaporated under reduced pressure. The residue was dissolved in dichloromethane (100 mL) and washed with water (100 mL). The aqueous layer was extracted with dichloromethane (120 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel (EtOAc/DCM = 1:9) to give compound **58** (2.51 g, 57%) as a white solid. Compound **58** was unstable, only ^{1}H NMR and mass spectra were measured. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55–7.45 (m, 4H, Ph), 7.43–7.26 (m, 11H, Ph), 5.62 (s, 1H, PhC$\underline{H}O_2$), 4.93–4.81 (m, 2H, H-1, Bn), 4.76 (d, J =

12.1 Hz, 1H, Bn), 4.38–4.28 (m, 2H, H-6, H-2), 4.17 (d, J = 9.4 Hz, 1H, H-4), 3.93 (t, J = 10.3 Hz, 1H, H-6), 3.49–3.36 (m, 1H, H-5), 2.82 (d, J = 1.8 Hz, 1H, OH). HRMS (ESI, positive) calculated for $C_{26}H_{25}DO_5S^+$ $[M + Na]^+$ 474.1456, found 474.1458.

(*4aR,6S,7S,8S,8aR*)-8-(benzyloxy)-2-phenyl-6-(phenylthio)hexahydropyrano[3,2-d][1,3]dioxin-7-yl-8-d trifluoromethanesulfonate (59): Trifluoromethanesulfonic anhydride (4.69 mL, 27.9 mmol) was added to a solution of compound **58** (2.52 g, 5.58 mmol) in dry pyridine (28 mL) and dry dichloromethane (28 mL) at –15 °C. The reaction mixture was warmed to room temperature, and diluted with ethyl acetate (50 mL). The reaction was quenched by adding few drops of water. The reaction solution was washed with saturated aqueous solutions of ammonium chloride (40 mL) and sodium bicarbonate (40 mL). The organic layer was washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was used in next step without further purification.

(*4aR,6S,7R,8R,8aS*)-7-azido-8-(benzyloxy)-2-phenyl-6-(phenylthio)hexahydro-pyrano[3,2-d][1,3]dioxine-8-d (60): Sodium azide (1.814 g, 27.9 mmol) was added to a solution of the crude compound **59** in dimethylformamide (56 mL) at 0 °C. The reaction mixture was stirred at room temperature for 10 h, and diluted with ethyl acetate (50 mL). The organic layer was washed with water (40 mL × 3). The combined aqueous layers were extracted with ethyl acetate (60 mL × 2). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 4:1) to provide compound **60** (2.32 g, 87%) as a clear oil. Compound **60** was unstable, only ^{1}H NMR and mass spectra were measured. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60–7.54 (m, 2H, Ph), 7.50–7.44 (m, 2H, Ph), 7.42–7.28 (m, 11H, Ph), 5.57 (s, 1H, PhC<u>H</u>O_2), 4.91 (d, J = 10.9 Hz, 1H, Bn), 4.78 (d, J = 10.9 Hz, 1H, Bn), 4.49 (d, J =

10.2 Hz, 1H, H-1), 4.39 (dd, *J* = 10.5, 5.0 Hz, 1H, H-6), 3.84–3.75 (m, 1H, H-6), 3.63 (d, *J* = 9.4 Hz, 1H, H-4), 3.46 (ddd, *J* = 10.0, 9.4, 5.0 Hz, 1H, H-5), 3.36 (d, *J* = 10.3 Hz, 1H, H-2). HRMS (ESI, positive) calculated for $C_{26}H_{24}DN_3O_5S^+$ $[M + Na]^+$ 499.1521, found 499.1518.

(*2R,3S,4R,5R,6S*)-5-azido-4-(benzyloxy)-2-(hydroxymethyl)-6-(phenylthio)tetra-hydro-2*H*-pyran-4-d-3-ol (61): p-toluenesulfonic acid monohydrate (0.046 g, 0.242 mmol) was added to a solution of compound **60** (1.154 g, 2.42 mmol) in methanol (24 mL). The reaction mixture was stirred for 14 h. The solvent was removed under reduced pressure, and the residue was diluted with ethyl acetate (25 mL). The organic layer was washed with saturated aqueous solution of sodium bicarbonate (20 mL × 2). The combined aqueous layers were extracted with ethyl acetate (30 mL × 2). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (Hex/EtOAc = 1:1) to give compound **61** (0.895 g, 95%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57–7.51 (m, 2H, Ph), 7.39–7.35 (m, 4H, Ph), 7.34–7.32 (m, 4H, Ph), 4.95 (d, *J* = 11.2 Hz, 1H, Bn), 4.76 (d, *J* = 11.3 Hz, 1H, Bn), 4.48 (d, *J* = 10.1 Hz, 1H, H-1), 3.87 (dd, *J* = 12.0, 3.3 Hz, 1H, H-6), 3.76 (dd, *J* = 12.0, 4.9 Hz, 1H, H-6), 3.54 (d, *J* = 9.7 Hz, 1H, H-4), 3.32 (ddd, *J* = 10.1, 5.0, 3.6 Hz, 2H, H-5, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.87 (Ph), 133.44 (Ph), 131.34 (Ph), 129.27 (Ph), 128.89 (Ph), 128.65 (Ph), 128.41 (Ph), 128.29 (Ph), 86.56 (C-1), 79.48 (C-5), 75.58 (Bn), 70.31 (C-4), 65.00 (C-2), 62.57 (C-6). HRMS (ESI, positive) calculated for $C_{19}H_{20}DN_3O_4S^+$ $[M + Na]^+$ 411.1208, found 411.1212.

(*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-(phenylthio)-tetrahydro-2*H*-pyran-4-d (62): Sodium hydride (60% dispersion in mineral oil, 0.216 g, 5.41 mmol) was added to a solution of compound **61** (0.841 g, 2.16 mmol) in

dimethylformamide (22 mL) at 0 °C. The reaction mixture was stirred at room temperature for 30 min. Benzyl bromide (0.65 mL, 5.41 mmol) was added at 0 °C, and the reaction mixture was stirred for 11 h. Methanol (5 mL) was added slowly at 0 °C. The reaction solution was concentrated under reduced pressure. The residue was diluted with dichloromethane (30 mL), and washed with water (20 mL × 3). The combined aqueous layers were extracted with dichloromethane (30 mL × 3). The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude compound was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to provide compound **62** (1.189 g, 97%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.62–7.58 (m, 2H, Ph), 7.36–7.23 (m, 16H, Ph), 7.22–7.18 (m, 2H, Ph), 4.86 (d, *J* = 10.5 Hz, 1H, Bn), 4.82 (d, *J* = 10.6 Hz, 1H, Bn), 4.78 (d, *J* = 10.9 Hz, 1H, Bn), 4.61 (d, *J* = 12.0 Hz, 1H, Bn), 4.58 (d, *J* = 10.9 Hz, 1H, Bn), 4.54 (d, *J* = 12.0 Hz, 1H, Bn), 4.41 (d, *J* = 10.2 Hz, 1H, H-1), 3.77 (dd, *J* = 10.9, 2.0 Hz, 1H, H-6), 3.74 (dd, *J* = 11.0, 4.2 Hz, 1H, H-6), 3.60 (d, *J* = 9.8 Hz, 1H H-4), 3.47 (ddd, *J* = 9.8, 4.2, 2.0 Hz, 1H, H-5), 3.34 (d, *J* = 10.2 Hz, 1H, H-2). ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.33 (Ph), 137.98 (Ph), 137.74 (Ph), 133.76 (Ph), 131.29 (Ph), 129.11 (Ph), 128.65 (Ph), 128.60 (Ph), 128.50 (Ph), 128.48 (Ph), 128.33 (Ph), 128.14 (Ph), 128.02 (Ph), 127.97 (Ph), 127.74 (Ph), 127.70 (Ph), 86.08 (C-1), 79.46 (C-5), 77.60 (C-4), 75.97 (Bn), 75.18 (Bn), 73.57 (Bn), 68.87 (C-6) 65.11 (C-2). HRMS (ESI, positive) calculated for $C_{33}H_{32}DN_3O_4S^+$ $[M + Na]^+$ 591.2147, found 591.2149.

(*1S,2S,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2*H*-pyran-2-yl-4-d)oxy)cyclohexane-1,2-diyl diacetate (63): Compound **33** (0.087 g, 0.293 mmol) and compound **62** (0.200 g, 0.352 mmol) were co-evaporated from dry toluene three times and further dried under high vacuum overnight. A solution of compound **33** and **62** in anhydrous diethyl ether (3 mL) and

anhydrous dichloromethane (1 mL) was then added to preactivated 4 Å molecular sieves. After stirring for 30 min at room temperature, the mixture was cooled to –40 °C. *N*-Iodosuccinimide (0.171 g, 0.762 mmol) was added, and the reaction mixture was stirred for 20 min. Trifluoromethanesulfonic acid (0.013 mL, 0.147 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. Sodium bisulfite (0.1 g), sodium bicarbonate (0.1 g), and water (1 mL) were added at 0 °C, and the mixture was stirred for 10 min at room temperature. The reaction mixture was diluted with dichloromethane (10 mL), filtered through a Celite pad, and washed with a saturated solution of aqueous sodium bicarbonate (15 mL). The aqueous layers were extracted with dichloromethane (15 mL × 3), and the combined organic phase was washed with brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 2:1) to give compound **63** (0.135 g, 61%) as a clear oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.36–7.26 (m, 13H, Ph), 7.18–7.14 (m, 2H, Ph), 5.17–5.11 (m, 2H, H-1', H-5), 4.92 (t, *J* = 10.0 Hz, 1H, H-6), 4.88 (d, *J* = 10.5 Hz, 1H, Bn), 4.82 (d, *J* = 10.7 Hz, 1H, Bn), 4.79 (d, *J* = 10.9 Hz, 1H, Bn), 4.64 (d, *J* = 12.1 Hz, 1H, Bn), 4.54 (d, *J* = 10.9 Hz, 1H, Bn), 4.49 (d, *J* = 12.1 Hz, 1H, Bn), 4.18–4.12 (m, 1H, H-5'), 3.83 (dd, *J* = 10.8, 2.9 Hz, 1H, H-6'), 3.78 (d, *J* = 10.1 Hz, 1H, H-4'), 3.68 (dd, *J* = 10.8, 2.1 Hz, 1H, H-6'), 3.66–3.58 (m, 2H, H-4, H-1), 3.41 (ddd, *J* = 12.5, 10.0, 4.6 Hz, 1H, H-3), 3.36 (d, *J* = 3.8 Hz, 1H, H-2'), 2.39 (dt, *J* = 13.4, 4.5 Hz, 1H, H-2), 2.09 (s, 3H, OAc), 2.08 (s, 3H, OAc), 1.65–1.54 (m, 1H, H-2). ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.99 (O(C=O)), 169.65 (O(C=O)), 138.04 (Ph), 137.94 (Ph), 137.91 (Ph), 128.60 (Ph), 128.56 (Ph), 128.54 (Ph), 128.12 (Ph), 128.04 (Ph), 128.02 (Ph), 127.93 (Ph), 127.90 (Ph), 127.85 (Ph), 99.51 (C-1'), 78.44 (C-4), 78.19 (C-4'), 75.43 (Bn), 75.14 (Bn), 74.24 (C-6), 73.74 (Bn), 73.68 (C-5), 71.83 (C-5'), 68.08 (C-6'), 63.23 (C-2'), 58.82 (C-3), 57.85 (C-1), 31.97 (C-2), 20.79 (OAc), 20.72 (OAc).

HRMS (ESI, positive) calculated for $C_{37}H_{40}DN_9O_9^+$ [*M* + *Na*]$^+$ 779.2982, found 779.3050.

(*1S,2R,3R,4S,6R*)-4,6-diazido-3-(((*2S,3R,4R,5S,6R*)-3-azido-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2*H*-pyran-2-yl-4-d)oxy)cyclohexane-1,2-diol (64): Sodium methoxide (5.4 M in methanol, 0.006 mL, 0.032 mmol) was added dropwise to a solution of compound **63** (0.245 g, 0.324 mmol) in methanol (3.2 mL). The reaction mixture was stirred at room temperature for 8 h and then neutralized with Amberlite IR-120 (H^+ form) resin, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **64** (0.187 g, 86%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.27 (m, 13H, Ph), 7.18–7.12 (m, 2H, Ph), 5.15 (d, *J* = 3.6 Hz, 1H, H-1'), 4.93 (d, *J* = 10.6 Hz, 1H, Bn), 4.85 (d, *J* = 10.6 Hz, 1H, Bn), 4.79 (d, *J* = 10.8 Hz, 1H, Bn), 4.65 (d, *J* = 12.0 Hz, 1H, Bn), 4.54 (d, *J* = 10.8 Hz, 1H, Bn), 4.49 (d, *J* = 12.0 Hz, 1H, Bn), 4.21 (s, 1H, OH), 4.10 (ddd, *J* = 10.1, 3.4, 2.1 Hz, 1H, H-5'), 3.85–3.75 (m, 2H, H-6', H-4'), 3.69 (dd, *J* = 10.8, 2.0 Hz, 1H, H-6'), 3.65 (d, *J* = 3.6 Hz, 1H, H-5), 3.56–3.38 (m, 3H, H-6, H-1, H-4), 3.30–3.22 (m, 2H, H-3, H-2'), 2.37–2.24 (m, 1H, H-2), 1.56–1.41 (m, 1H, H-2). HRMS (ESI, positive) calculated for $C_{33}H_{36}DN_9O_7^+$ [*M* + *Na*]$^+$ 695.2771, found 695.2770.

[3'-^{2}H]-Paromamine (65): Trimethylphosphine (1 M solution in THF, 1.418 mL) was added to a solution of compound **64** (0.159 g, 0.236 mmol) in THF (5.9 mL) and 0.1 M NaOH (2 mL). The reaction mixture was stirred at 50 °C for 2 h. After cooling down to room temperature, the solution was evaporated under reduced pressure. The material was dissolved in a mixture of acetic acid (1.3 mL), water (3.9 mL) and methanol (3.9 mL). The solution was deaerated by evacuating the air in the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.1 g) was added and the solution was charged with hydrogen. The reaction mixture was stirred at room

temperature under hydrogen gas for 24 h. The solution was filtered through a Celite pad and concentrated. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (2.5% concentrated ammonium hydroxide in water), concentrated, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **65** (0.067 g, 81%) as an oily solid. ^{1}H NMR (500 MHz, D_2O) δ 5.72 (d, *J* = 3.9 Hz, 1H, H-1'), 3.98 (dd, *J* = 12.0, 2.2 Hz, 1H, H-6'), 3.95 (dd, *J* = 10.2, 9.1 Hz, 1H, H-5), 3.89 (ddd, *J* = 9.9, 6.6, 2.1 Hz, 1H, H-5'), 3.80 (dd, *J* = 12.1, 6.6 Hz, 1H, H-6'), 3.73 (t, *J* = 9.2 Hz, 1H, H-6), 3.69–3.61 (m, 2H, H-1, H-4), 3.55 (d, *J* = 9.9 Hz, 1H, H-4'), 3.51 (d, *J* = 4.0 Hz, 1H, H-2'), 3.40 (ddd, *J* = 12.4, 10.3, 4.2 Hz, 1H, H-3), 2.57 (dt, *J* = 12.7, 4.3 Hz, 1H, H-2), 1.93 (q, *J* = 12.6 Hz, 1H, H-2). ^{13}C NMR (125 MHz, D_2O) δ 97.07 (C-1'), 80.33 (C-5), 74.69 (C-6), 73.65 (C-5'), 72.33 (C-4), 69.30 (C-4'), 60.36 (C-6'), 53.91 (C-2'), 49.62 (C-3), 48.85 (C-1), 28.27 (C-2). HRMS (ESI, positive) calculated for $C_{12}H_{24}DN_3O_7^+$ $[M + H]^+$ 325.1829, found 325.1824.

4.3. RESULTS AND DISCUSSION

4.3.1. Gene Analysis

Sequence analysis led to the assignment of AprD3 as an NAD-dependent dehydrogenase and AprD4 as a member of the radical *S*-adenosyl-L-methionine (SAM) family of enzymes. The catalytic [4Fe-4S] cluster characteristic of radical SAM enzyme is typically coordinated by a highly conserved CxxxCxxC motif, however, in the case of AprD4, a noncanonical CxxxCxxxC motif is found instead. Recently a number of examples of radical SAM enzymes with different [4Fe-4S]-binding motifs have been reported. PhnJ, which catalyzes cleavage of the C-P bond of phosphonates, has a $Cx_2Cx_{21}Cx_5C$ motif.[209-210] HmdB, which is involved in the biosynthesis of hydrogenase

cofactor, contains a Cx_5Cx_2C cluster-binding motif.[211] QueE participates in the synthesis of 7-carboxy-7-deazaguanine and contains a $Cx_{14}Cx_2C$ cluster-binding motif.[212] ThiC, the thiamine pyrimidine synthase found in plants and bacteria, has a Cx_2Cx_4C motif.[213] Also GenD1 has a Cx_4Cx_2C motif.[81] Although these radical SAM enzymes do indeed exhibit atypical [4Fe-4S] binding motifs, the noncanonical motif of AprD4 still raises questions, as to whether AprD4 operates as a radical SAM enzyme and the mechanism by which C3'-deoxygenation is effected during the biosynthesis of aminoglycosides.

4.3.2. Purification of AprD3 and AprD4

Both the *aprD3* and *aprD4* genes were amplified by polymerase chain reaction from *S. tenebrarius* genomic DNA, cloned in to a pET28 vector, and heterologously overexpressed in *Escherichia coli* (Figure 4-6 for AprD3 and Figure 4-7 for AprD4). The His_6-tagged AprD4 was isolated from cell lysates using a nickel-nitrilotriacetate (Ni-NTA) affinity column and was then subjected to anaerobic incubation with $[Fe(NH)_4(SO_4)_2]$ and Na_2S in a Coy anaerobic chamber with an atmosphere of more than 97% N_2 and 3% H_2 to reconstituted the [4Fe-4S] cluster. Iron[153] and Sulfur analysis[154] of the reconstituted AprD4 revealed the presence of 9.2 ± 0.6 equivalents of iron and 8.2 ± 0.7 equivalents of sulfide per AprD4 monomer. These results indicated the presence of two [4Fe-4S] clusters bound to each AprD4 monomer. Likewise, the UV/Vis absorption spectrum of the reconstituted AprD4 exhibits a shoulder around λ = 420 nm, which is typical of $[4Fe\text{-}4S]^{2+}$-containing proteins.[214-215] Furthermore, addition of sodium dithionite (2mM) to the reconstituted enzyme led to a decrease in the absorbance at λ = 420 nm, implying reduction of the cluster.

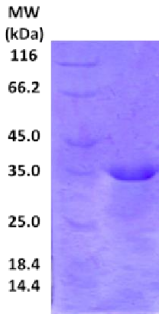


Figure 4-6. SDS PAGE of AprD3. The calculated molecular weight of AprD3 is 29.4 kDa.

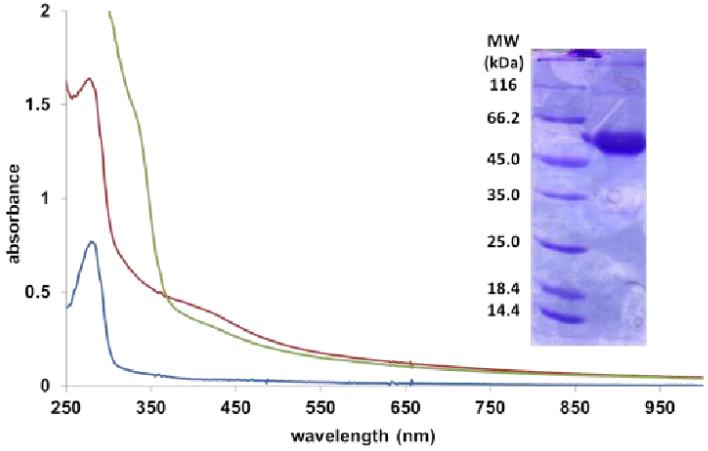


Figure 4-7. UV-visible absorption spectra and SDS PAGE of AprD4. The blue, red, and green spectra correspond to no reconstituted AprD4 (8 mM), reconstituted AprD4 (15 mM), and reconstituted AprD4 (15 mM) treated with 1 mM sodium dithionite. The bleaching of the absorbance shoulder at 420 nm is characteristic of a bound iron-sulfur cluster. The calculated molecular weight of AprD4 is 52.5 kDa.

4.3.3. Synthesis of Lividamine

As shown in Scheme 4-2 in Section 4.2.8, D-lividosamine (**27**) was obtained after four steps from *N*-acetyl-D-glucosamine.[208] *N*-acetyl-D-glucosamine was converted into a furanosyl oxazoline intermediate using dry acetone at reflux. The hydroxyl group at C3

position was removed using Barton-McCombie deoxygenation.[216] Deprotection of oxazoline ring was conducted in 2 N HCl at reflux to give D-lividosamine. After introduction of an azide group at the C2 position and acetylation of the remaining alcohols, a thiophenyl group was incorporated at C1. After changing the protecting group on the alcohols, a coupling reaction with 2-streptamine derivative **33** was used to give pseudo-disaccharide **34**. Basic hydrolysis of the acetyl group with sodium methoxide, Staudinger reaction, and hydrogenation using palladium hydroxide on carbon gave lividamine (**13**) in 11.4% over 9 steps from compound **27**.

4.3.4. *In vitro* AprD3 and AprD4 Assay

Co-incubation of reconstituted AprD4 (0.01 mM) with 1 mM paromamine and 2 mM SAM in the presence of 10 mM dithiothreitol (DTT) and 2 mM dithionite (50 mM NH_4HCO_3 buffer, pH 7.8) at room temperature led to the formation of 5'-dAdo, as detected by monitoring the change in the absorption at $\lambda = 260$ nm (Figure 4-8 , trace b). Although exclusion of dithionite from the reaction did not lead to detectable levels of 5'-dAdo formation, the replacement of dithionite with either methyl viologen and NADPH or the *E. coli* flavodoxin/flavodoxin reductase/NADPH system were effective at doing so (Figure 4-9). In contrast, benzyl viologen with NADPH was not suitable for observing enzyme activity. Likewise, when the incubation was performed without prior reconstitution of the enzyme, no 5'-dAdo was detected (trace e in Figure 4-8). Finally, although uncoupled formation of 5'-dAdo was discernible in the absence of paromamine, the extent of SAM turnover to 5'-dAdo was significantly lower than that of the full reaction (traces d and b in Figure 4-8). These results strongly imply that AprD4 is able to catalyze radical homolysis of SAM consistent with its assignment as a radical SAM enzyme.[101,117]

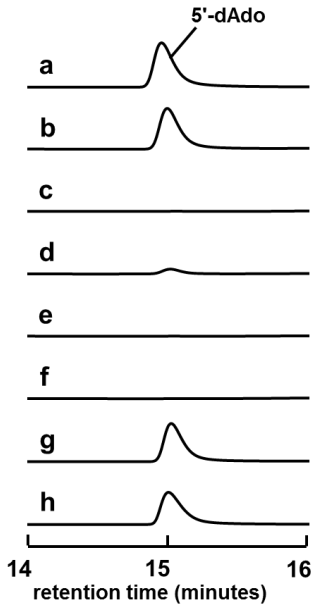


Figure 4-8. HPLC traces of AprD4 reaction mixtures for production of 5'-dAdo observed by UV detection at 260 nm. Trace (a) corresponds to 5'-dAdo. The remaining traces (b-h) are from reaction mixtures containing AprD4 (10 μM), paromamine (1 mM), SAM (2 mM), DTT (10 mM), dithionite (2 mM) in NH_4HCO_3 (50 mM; pH 7.8) buffer with the following modifications: b) no changes, c) no AprD4, d) no paromamine, e) non-reconstituted AprD4, f) no SAM, g) with AprD3 (10 μM) and NADPH (1 mM), and h) with AprD3 (10 μM) and no NADPH.

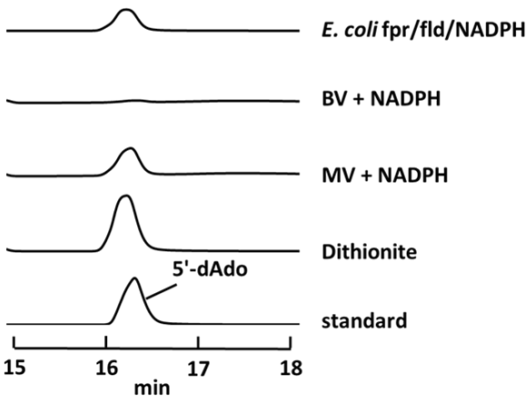


Figure 4-9. Competence of various reducing systems for supporting AprD4 activity. HPLC traces with UV detection at 260 nm showed 5'-dAdo formation.

The AprD4 reaction was monitored by HPLC using a Corona charged aerosol detector (CAD)[217] as well as FDNB derivatization method with UV absorbance detect at 340 nm, because the pseudo-disaccharide substrate paromamine and its products lack a UV-absorbing chromophore (Figure 4-10). Despite the detected conversion of SAM to 5'-dAdo, neither significant consumption of paromamine nor new product formation was noted, unless AprD3 (10 μM) and NADPH (1mM) were also include in the reaction mixture (trace b and g in Figure 4-10). In trace g in Figure 4-10A, a new peak with a retention time of 7.2 min was detected, in addition to the consumption of paromamine. Electrospray ionization mass spectra (ESIMS) of the new product revealed a signal at *m*/*z* 330.1638, consistent with the sodium adduct of lividamine (**13**; calc. 330.1636 [M + Na]$^+$; Figure 4-11, Figure 4-12E). In trace g in Figure 4-10B, a new peak with the same retention time with lividamine standard at 12.9 min was detected. This result demonstrated that the position where the deoxygenation reaction occurs is the C3' of paromamine. These findings led to the hypothesis that AprD4 operates as a radical-mediated dehydratase with AprD3 serving as its reductase partner to effect the overall reduction of paromamine to lividamine (**11**→**12**→**13**).

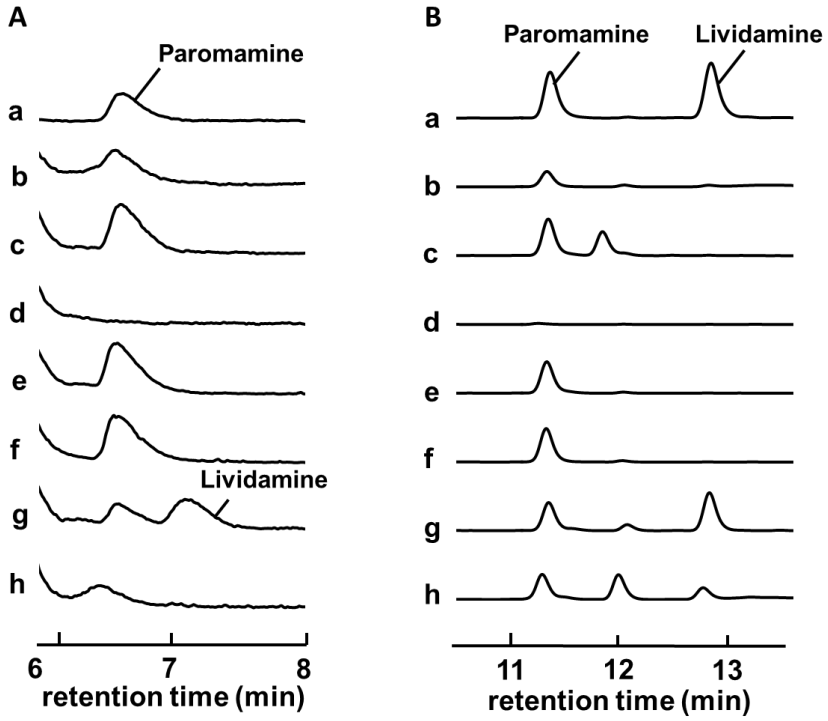


Figure 4-10. HPLC traces of AprD4 reaction mixtures showing the consumption of paromamine, monitored using A) a Corona CAD, and B) UV detection at 340 nm after the assay mixture was subjected to the reaction with 1-fluoro-2,4-dinitrobenzene (FDNB). Traces b–h are as described in the legend of **Figure 4-8**. The small peaks at ~12 min in trace c, g, and h are unrelated to product and substrate.

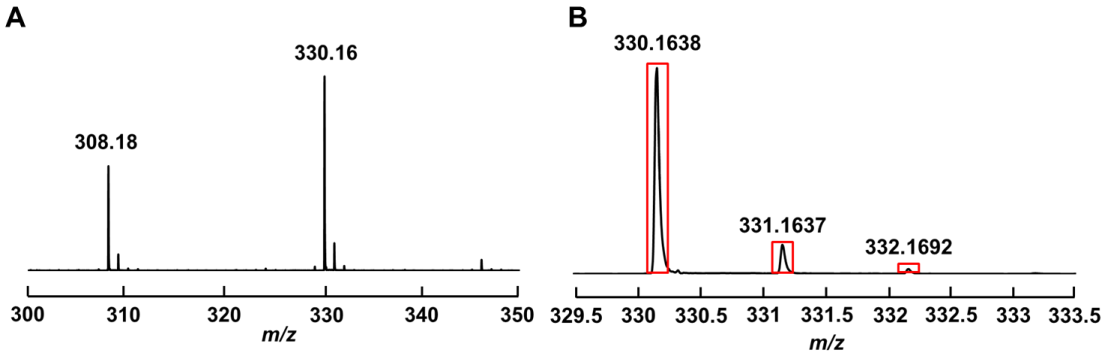


Figure 4-11. (A) Low resolution, and (B) high resolution mass spectra of the collected peak at 7.2 min shown in Figure 4-9A, trace g. (A) LRMS (ESI, positive) calculated for $C_{12}H_{25}N_3O_6^+$ $[M + H]^+$ 308.18 and $[M + Na]^+$ 330.16, observed 308.18 and 330.16,

respectively. (B) HRMS (ESI, positive) calcd $[M + Na]^+$ 330.1636, observed 330.1638. These results were consistent with the chemical structure of lividamine (**13**).

As a first test of this hypothesis, reaction mixtures excluding AprD3 were analyzed by ESIMS to check for low concentrations of the putative dehydration product of paromamine that may have gone undetected by Corona CAD and FDNB derivatization. As shown in Figure 4-12A, a signal was detected at *m/z* 328.15, consistent with the sodium adduct of such an intermediary species (**12**). To further support assignment of this MS signal to the putative ketone intermediate, the AprD4 reaction in the absence of AprD3 was run in the presence of phenylhydrazine (10 mM) for 4 h prior to analysis by ESIMS. In this case, a new signal at *m/z* 418.20 was detected whereas the signal at *m/z* 328.15 was significantly decreased, suggesting formation of a phenylhydrazone derivative (**14**; Figure 4-12B; Scheme 4-6). Moreover, treatment of the AprD4-only reaction with $NaBH_4$ or $NaBD_4$ resulted in a shift of the signal at *m*/*z* 328.15 to 330.17 and 331.17, respectively (Figure 4-12C, D; Scheme 4-6). These results support the hypothesis that AprD4 catalyzes the first of two half-reactions, resulting in the dehydration of paromamine to form a keto intermediate such as **12**.

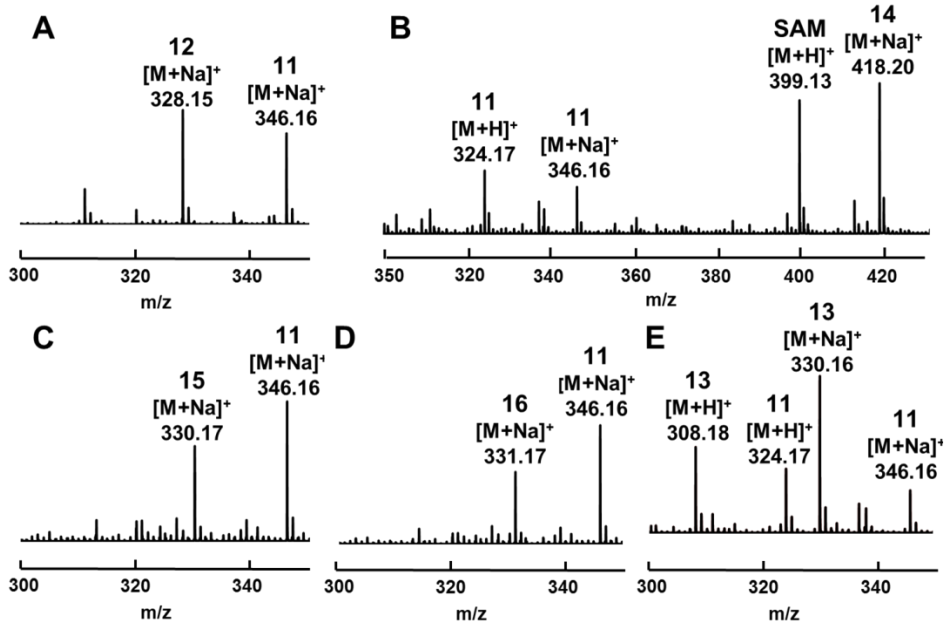
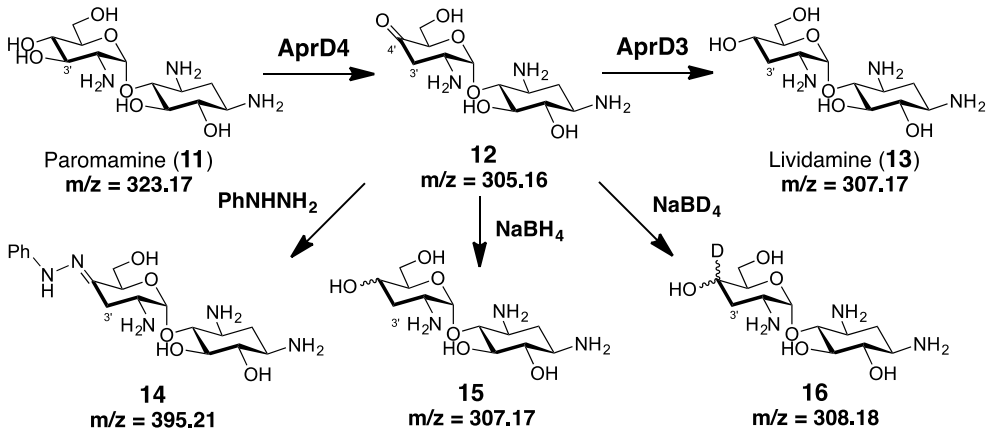


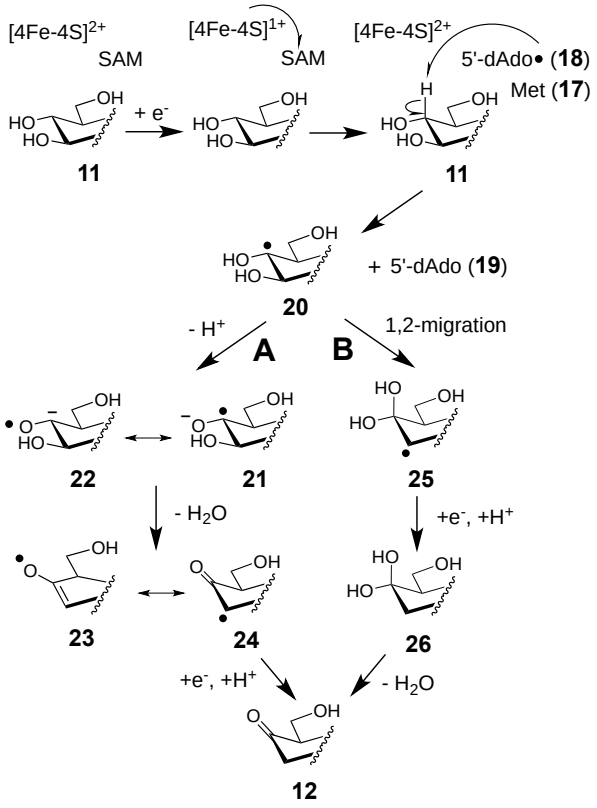
Figure 4-12. Electrospray ionization (ESI) mass spectra of the AprD4 reaction with paromamine (**11**) (A), together with the addition of (B) phenylhydrazine, (C) $NaBH_4$ and (D) $NaBD_4$ as described in the text. (E) ESI mass spectrum of the AprD4/AprD3 co-incubation with paromamine and NADPH.



Scheme 4-6. The reduction of paromamine (**11**) to lividamine (**13**) catalyzed by AprD4 and AprD3. Derivatization reactions carried out in the absence of AprD3 are also shown.

4.3.5. Proposed Mechanism of AprD4 Catalysis

Based on the results in the Section 4.3.4, at least two mechanisms can be envisioned for the AprD4-catalyzed dehydration of paromamine (Scheme 4-7). Consistent with radical SAM chemistry, both mechanisms begin with initial hydrogen-atom abstraction from the C4' position of paromamine (**11**→**20**) by the 5'-dAdo radical (**18**) generated by the reductive homolysis of SAM. The resulting substrate radical (**20**) may then be deprotonated to form a ketyl radical **21**/**22** that subsequently undergoes β-elimination of the 3'-OH group to yield the enol radical **23** (Scheme 4-7, route A). This mechanism is similar to the mechanisms proposed for (*R*)-2-hydroxyacyl-CoA dehydratase[178] and ribonucleotide reductase.[218] One-electron reduction of the resulting enol radical intermediate would then complete the dehydration reaction (**23**/**24**→**12**). Alternatively, the initially formed substrate radical **20** may instead undergo a radical-induced 1,2-hydroxy shift to form the *gem*-diol radical **25** (Scheme 4-7, route B), in direct analogy to the mechanisms of the Cbl-dependent diol-dehydratases and ethanolamine ammonia lyase.[201-202] Following reduction , the *gem*-diol intermediate **26** would then be converted into **12** upon elimination of water.



Scheme 4-7. Proposed mechanisms for the AprD4-catalyzed dehydration of paromamine.

4.3.6. Synthesis of [4′-^{2}H]-Paromamine

As shown in Scheme 4-3 in section 4.2.9, [4′-^{2}H]-paromamine (**47**) was prepared from **36** as summarized below. Intermediate **39** was obtained from **36**[159] via 4,6-diol protection with anisaldehyde dimethyl acetal (**36**→**37**), followed by benzyl protection of the 3-OH group (**37**→**38**) and selective reduction using sodium cyanoborohydride and trifluoroacetic acid.[219] Equatorial introduction of deuterium at C4 was achieved via Dess-Martin oxidation and sodium borodeuteride reduction (**39**→**40**).[174] The stereochemistry at C4 of **40** was inverted in a two-step procedure with sodium benzoate serving as a nucleophile under reflux conditions (**40**→**41**→**42**).[220] Alkaline hydrolysis of the benzoate

protecting group in **42** followed by benzyl protection of the exposed hydroxyl at C4 provided **44**. Intermediate **44** was then coupled with an azide derivative of 2-deoxystreptamine (**33**)[157] in the presence of *N*-iodosuccinimide and trifluoromethanesulfonic acid to provide the pseudodisaccharide **45**. Next, the acetyl protecting groups were removed by alkaline hydrolysis to give **46**, and the azide groups were converted to amines using Staudinger reduction. Final deprotection of benzyl group by hydrogenolysis provided **47** in 7.4% yield over 13 steps.

4.3.7. Synthesis of [3′-^{2}H]-Paromamine

As shown in Scheme 4-4 in Section 4.2.10, the synthesis of [3′-^{2}H]-paromamine (**65**) began from commercially available 1,2,5,6-di-*O*-isopropylidene-α-D-glucofuranose (**48**). Deuterium was incorporated at the C3 position with double inversion of stereochemistry to provide compound **52**. After benzyl protection of the C3 alcohol of compound **52**, furanose was converted into pyranose by acid hydrolysis and the remaining alcohols were acetylated to form the triacetylate **54**. A thiophenyl group was introduced at the C1 position using the Lewis acid boron trifluoride diethyletherate to provide compound **55**. The acetyl group was hydrolyzed by basic hydrolysis using catalytic amounts of sodium methoxide. Selective protection of the C4 and C6 alcohols with benzylidine acetal was conducted to provide the intermediate **57**. An azide group was introduced at C2 again with double inversion of stereochemistry using oxidation, reduction, and S_N2 type reaction to give compound **60**. After acid hydrolysis to remove benzylidine acetal, the free alcohols of compound **61** were protected as benzyl group. Coupling reaction of compound **62** with 2-deoxystreptamine derivative **33**, basic hydrolysis, Staudinger reaction, and hydrogenation gave [3′-^{2}H]-paromamine (**65**) in 3.2 % over 21 steps from compound **48**.

4.3.8. Mechanistic Studies of AprD4 Catalysis

When AprD4 (10 μM) was incubated with [4'-^{2}H]-paromamine (1 mM), SAM (2 mM), and dithionite (2 mM) in NH_4HCO_3 (50 mM; pH 7.8) for 8 h at room temperature, formation of 5'-dAdo was again detected and this time with single-deuterium incorporation beyond natural abundance as determined by ESIMS (**Figure 4-13**). A complimentary assay with unlabeled paromamine (**11**) and AprD4 using the dideuterated [5'-$^2H_2$]-SAM isotopologue[173] in the incubation revealed hydrogen transfer from **11** to [5'-$^2H_2$]- 5'-dAdo• to yield [5'-$^2H_2$]- 5'-dAdo as the product (**Figure 4-14**). These results further substantiated the proposed early steps of AprD4 catalysis shown Scheme 4-7, where AprD4 acts as a radical SAM enzyme that initiates a radical-mediated dehydration of paromamine through hydrogen-atom abstraction from the C4' position.

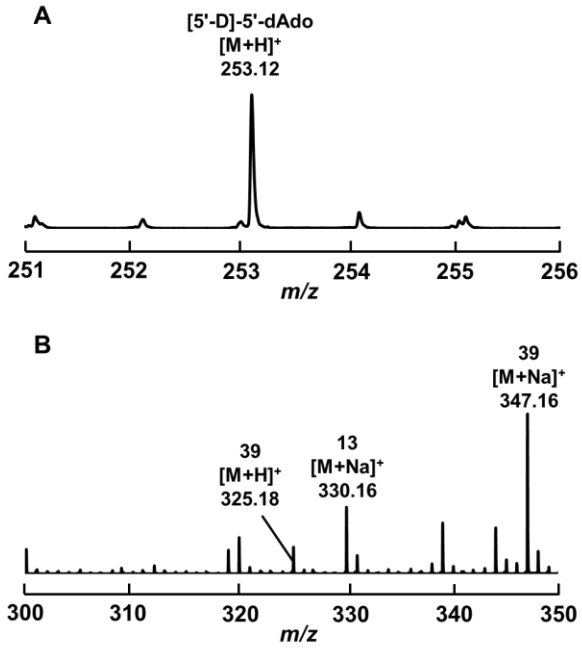


Figure 4-13. Mass spectra of (A) [5'-^{2}H]-5'-dAdo isolated from the incubation of SAM and AprD4/D3 with [4'-^{2}H]-paromamine (**47**). (B) assay mixture showing formation of product (**13**) in the incubation of [4'-^{2}H]-paromamine (**47**) with AprD4/AprD3.

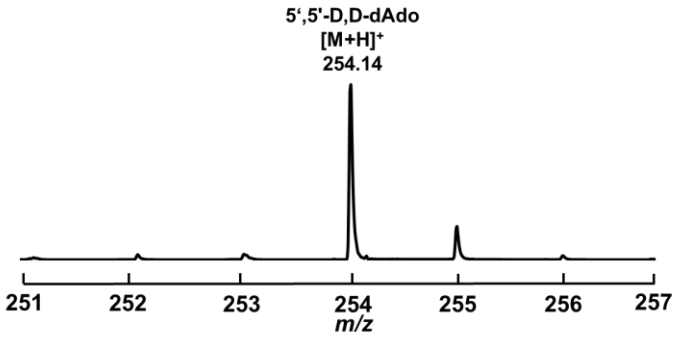


Figure 4-14. Mass spectra of [5'-$^2H_2$]-5'-dAdo produced during the AprD4/AprD3 reaction with [5'-$^2H_2$]-SAM and unlabeled paromamine (**11**).

Proton transfer manner at C3' position from paromamine to lividamine by AprD4 catalysis was determined by ^{1}H NMR spectroscopic analysis with the labeled substrate, [3'-^{2}H]-paromamine. Assays were conducted with 0.06 mM AprD4/D3, 20 mM dithionite, 20 mM SAM, 10 mM NADPH, 100 mM DTT in NH_4HCO_3 buffer (500 mM; pH 7.8) with 10 mM of paromamine, [3'-^{2}H]-paromamine, lividamine, or without substrate. After a 16 h incubation, assay mixtures were analyzed by ^{1}H NMR spectroscopy. Additionally, to confirm AprD4 reaction with [3'-^{2}H]-paromamine, ESIMS of [3'-^{2}H]-lividamine was verified at *m/z* 331.1701 (calc. [*M* + Na]$^+$ 331.1698). Paromamine and lividamine NMR standards in pH 7.8 buffer were prepared for comparison with the ^{1}H NMR spectra. A new peak in the assay with paromamine was observed at approximately 1.7 ppm (Figure 4-15a). In the assay with [3'-^{2}H]-paromamine, this peak was not seen (Figure 4-15c). This peak is also observed in the assays with lividamine and the lividamine standard spectra (Figure 4-15b and 4-15e), but

not without any substrate and paromamine standard (Figure 4-15d and 4-15f). The peak around 1.7 ppm corresponds to the axial hydrogen at C3' of lividamine. These results demonstrated that the axial hydrogen at C3' of paromamine is retained in lividamine during AprD4/D3 catalysis, whereas the equatorial hydroxyl group at C3' of paromamine is replaced with hydrogen.

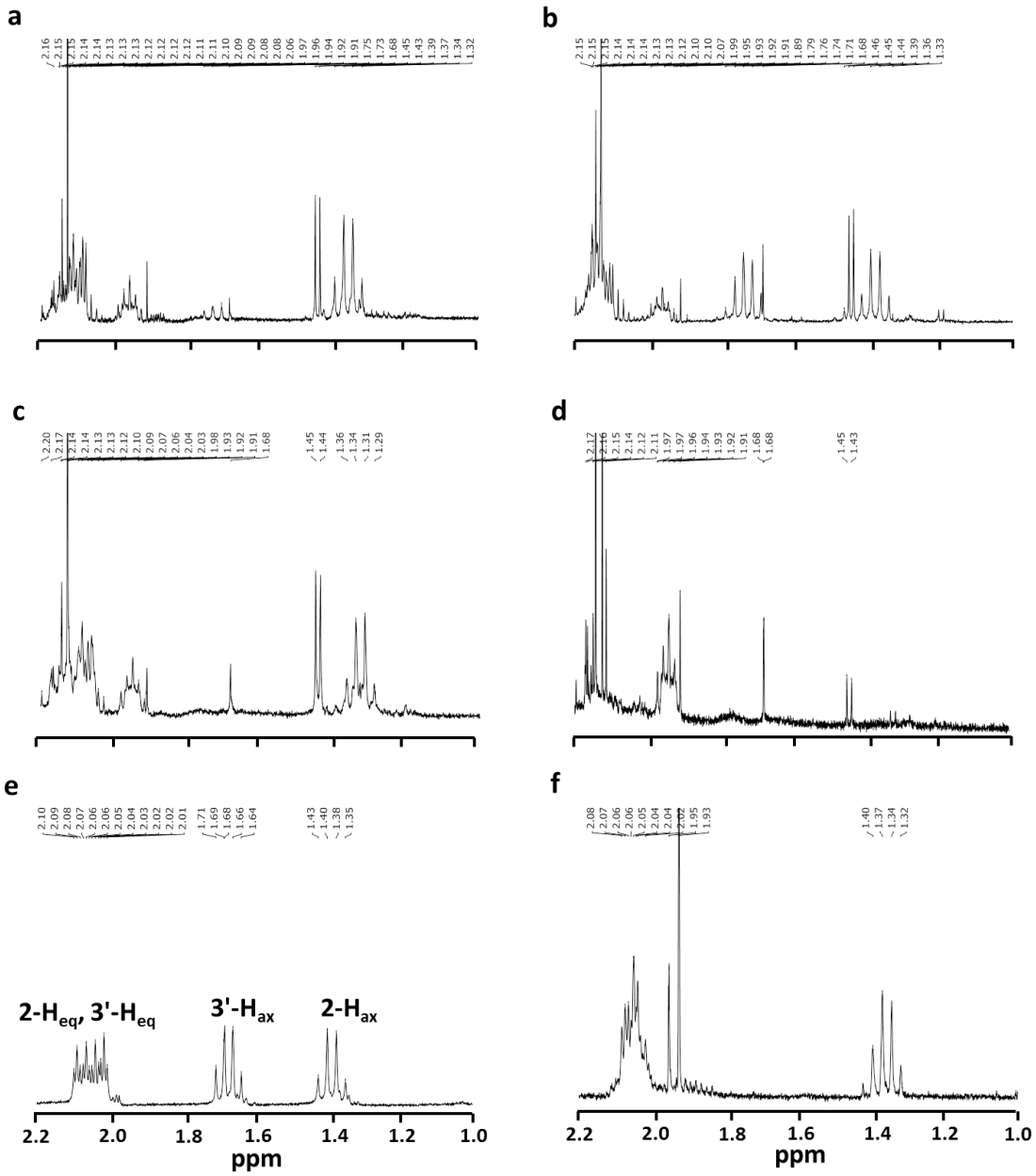


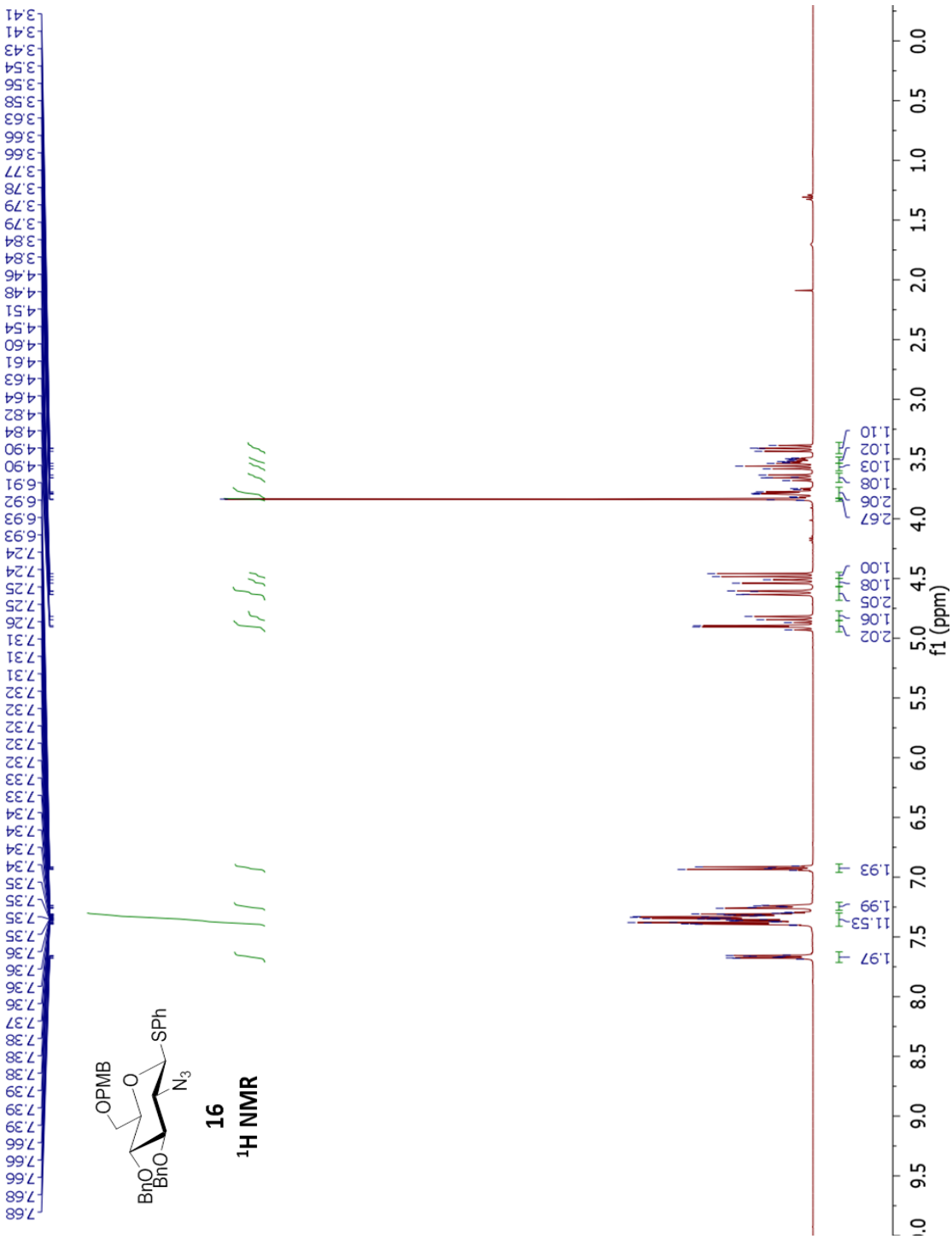
Figure 4-15. ^{1}H NMR spectra of AprD4/D3 reaction with a) paromamine, b) lividamine, c) [3'-^{2}H]-paromamine, d) without any substrate. e) and f) are respectively ^{1}H NMR spectra from the lividamine and paromamine standards at the same pH.

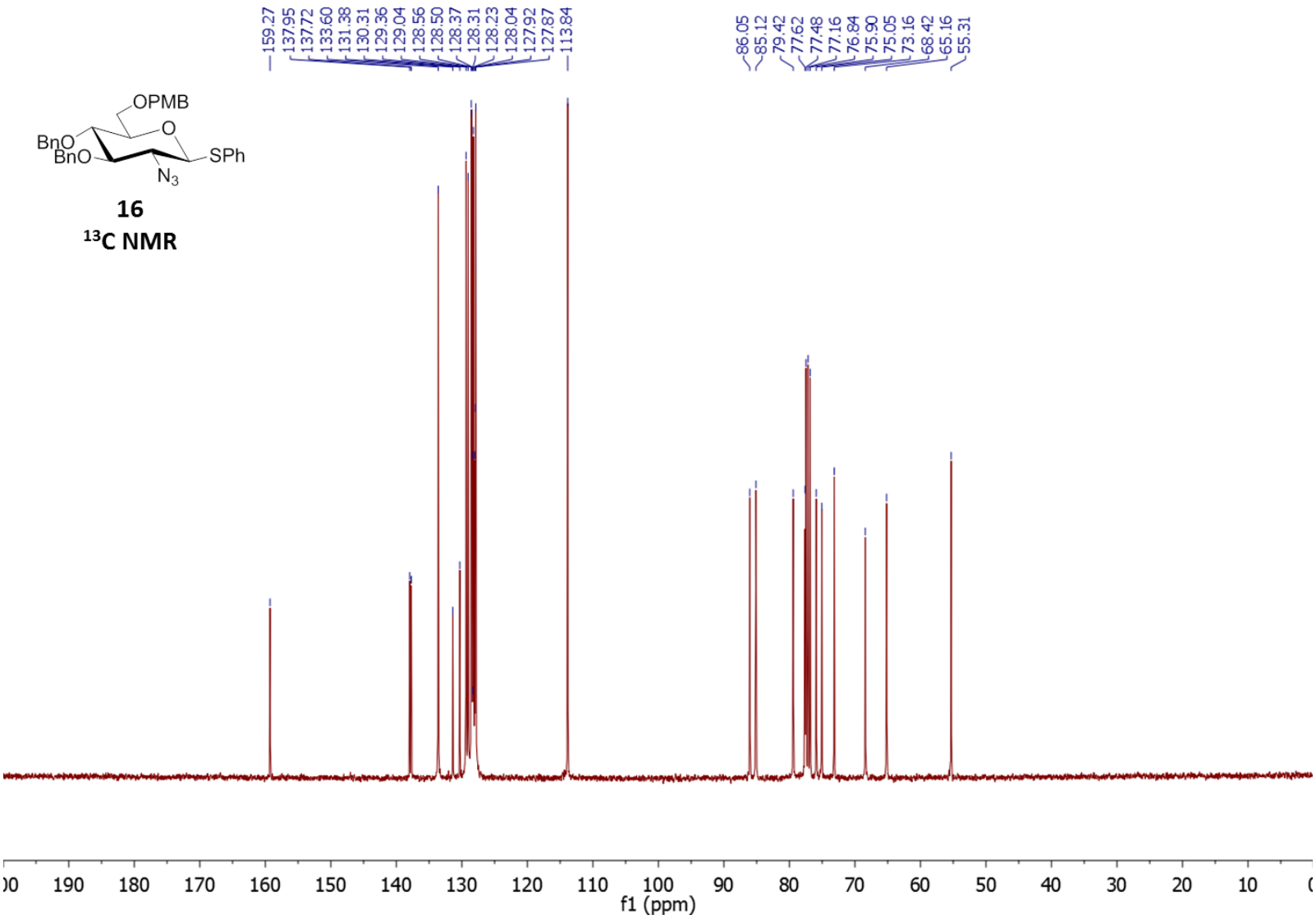
4.4. CONCLUSION

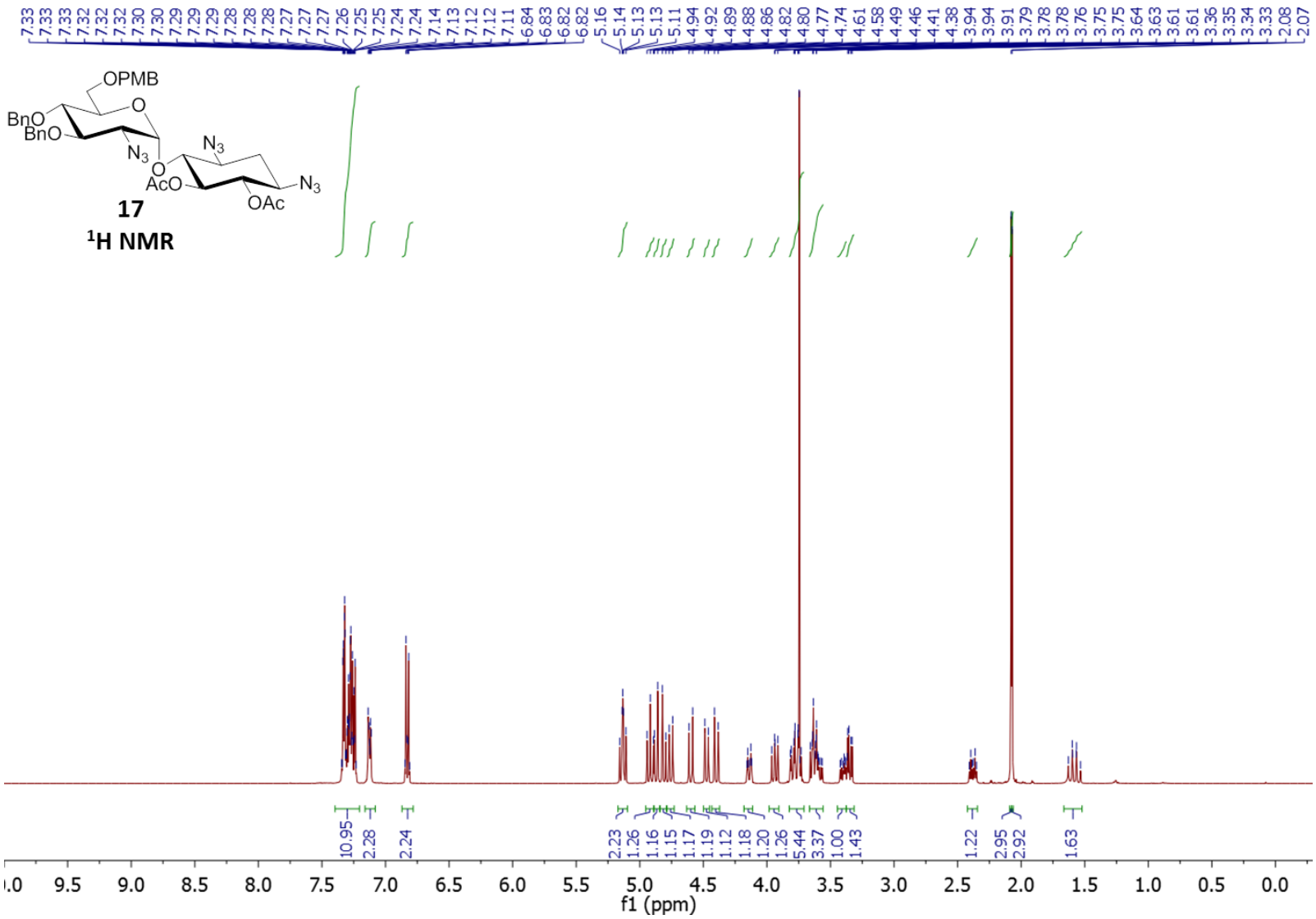
C3'-deoxygenation of aminoglycosides results in their decreased susceptibility to phosphorylation thereby increasing their efficacy as antibiotics. However, the biosynthetic mechanism of C3'-deoxygenation is unknown. To address this issue, the *aprD4* and *aprD3* genes from the apramycin gene cluster in *Streptomyces tenebrarius* were expressed in *E. coli* and the resulting gene products were purified and characterized *in vitro*. AprD4 was shown to be a radical *S*-adenosyl-L-methionine (SAM) enzyme, catalyzing homolysis of SAM to 5'-dAdo in the presence of paromamine. C3'-deoxygenation of paromamine catalyzed by AprD4/D3 was confirmed by comparing the lividamine standard with reaction mixtures treated with FDNB and analyzed by HPLC. [4'-^{2}H]-Paromamine was prepared and used to show that its C4'-H is transferred to 5'-dAdo by AprD4, after which the substrate is dehydrated to a product consistent with 4'-oxolividamine. In contrast, paromamine is reduced to a deoxy product when incubated with AprD4/AprD3/NADPH. The AprD4/D3 assay with [3'-^{2}H]-paromamine showed that axial C3'-H is retained during the reaction, and the equatorial hydroxyl group is replaced with new hydrogen. These results provide evidence for the assignment of functions to the enzymes AprD4 and AprD3 in the biosynthesis of C3'-deoxyaminoglycosides. AprD4 appears to be the first example of a radical SAM diol-dehydratase and catalyzes the radical-mediated dehydration of paromamine. AprD3 is shown to be a dehydrogenase that acts as the reductase counterpart to AprD4 in order to facilitate the net C3'-deoxygenation of the pseudo-disaccharide substrates in the biosynthesis of apramycin and tobramycin. Enzymes LivW and LivY are expected to play analogous roles in the lividomycin biosynthetic pathway.

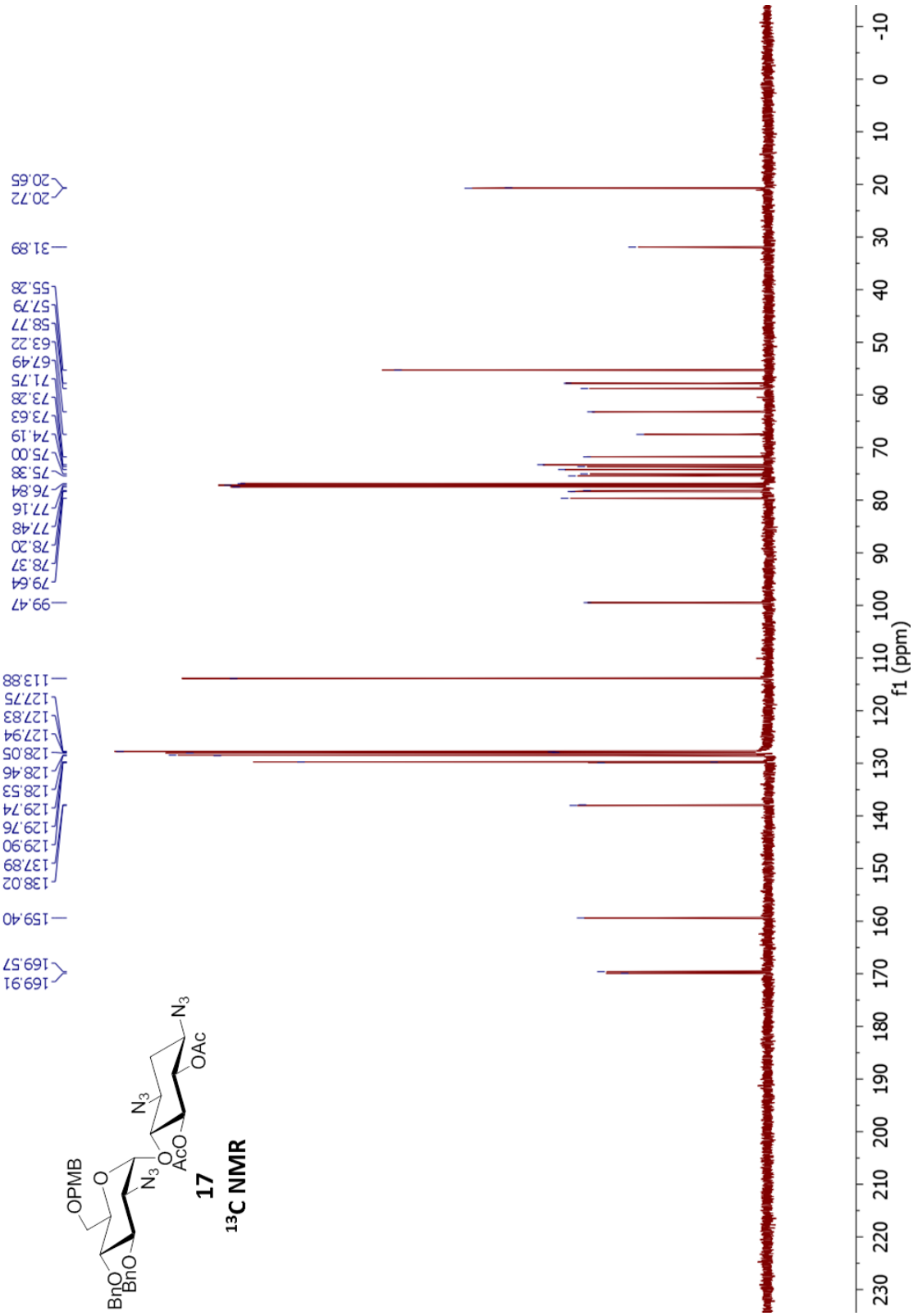
Appendix

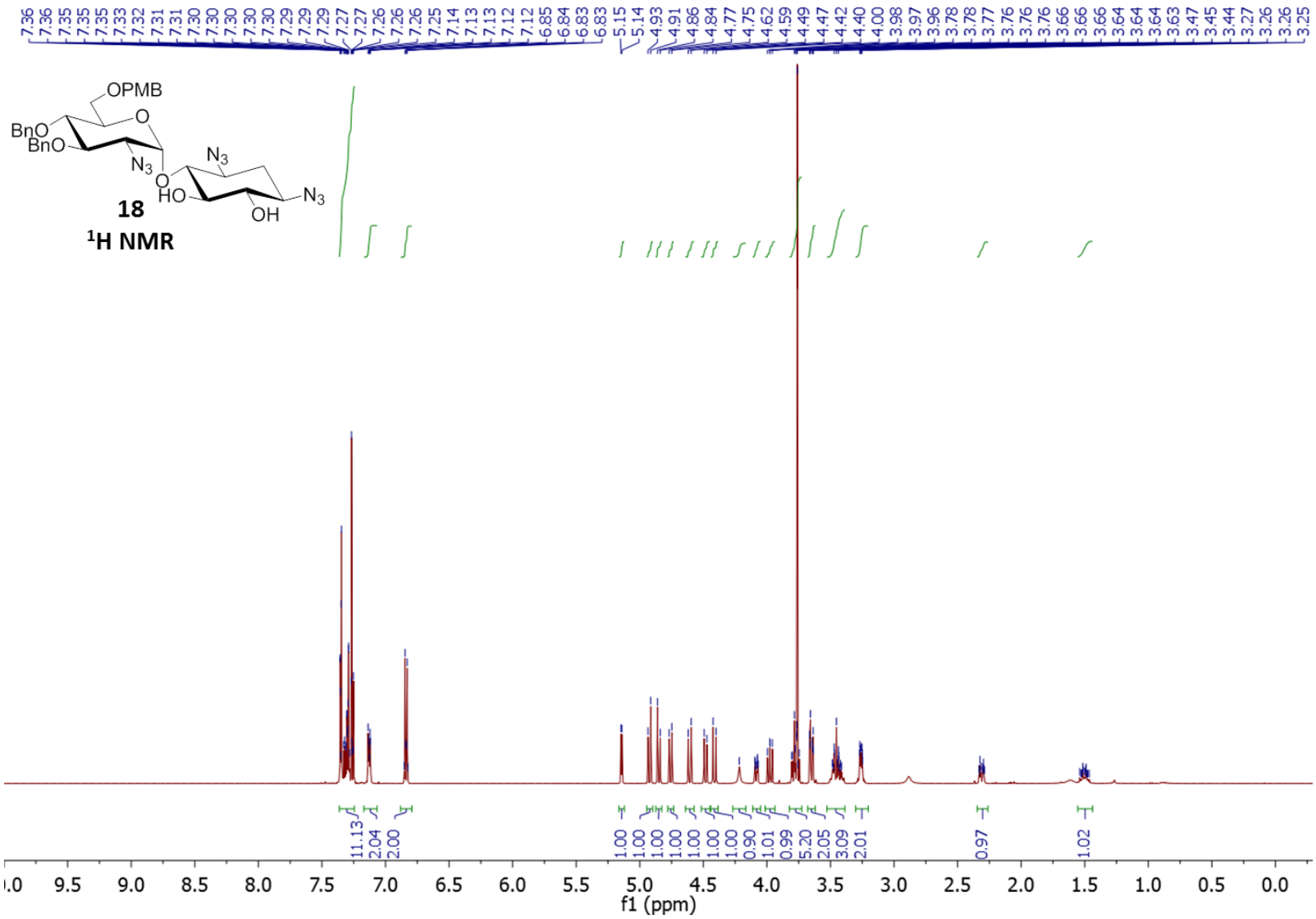
A.1. Spectral Data For Chapter 2

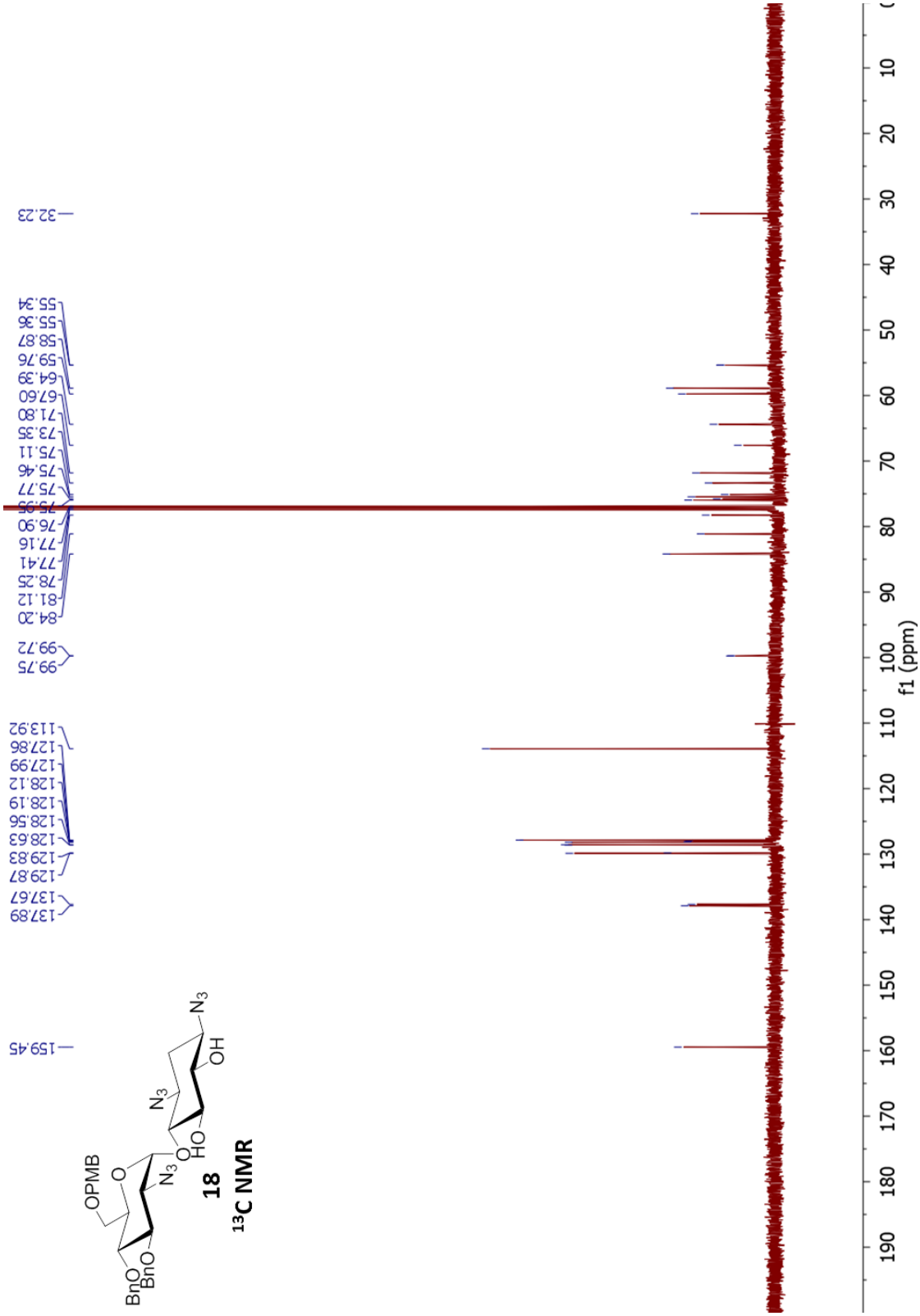


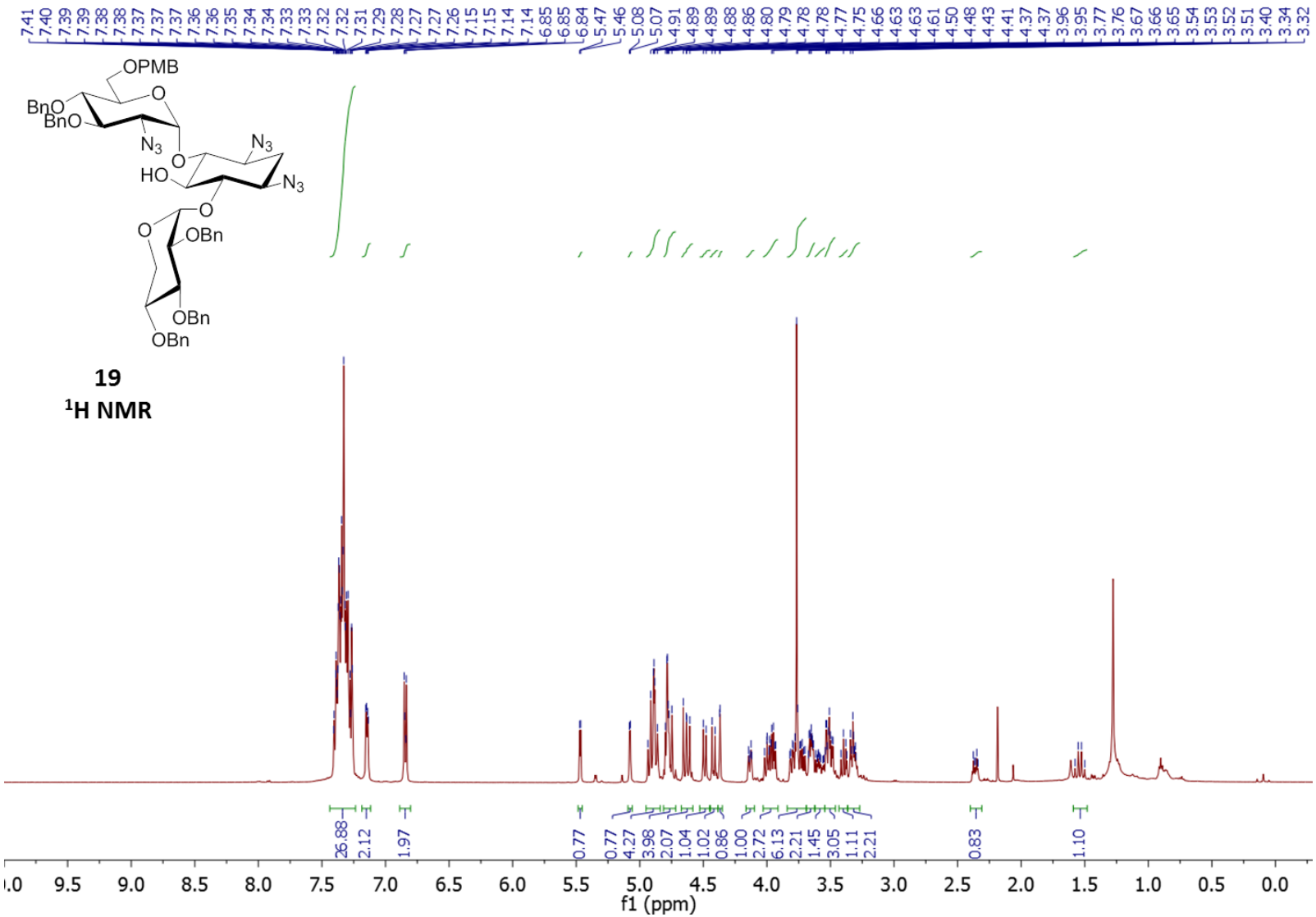

159.27
137.95
137.72
133.60
131.38
130.31
129.36
129.04
128.56
128.50
128.37
128.31
128.23
128.04
127.92
127.87
113.84
86.05
85.12
79.42
77.62
77.48
77.16
76.84
75.90
75.05
73.16
68.42
65.16
55.31
OPMB
O
BnO
BnO
SPh
N3
16
13C NMR
f1 (ppm)

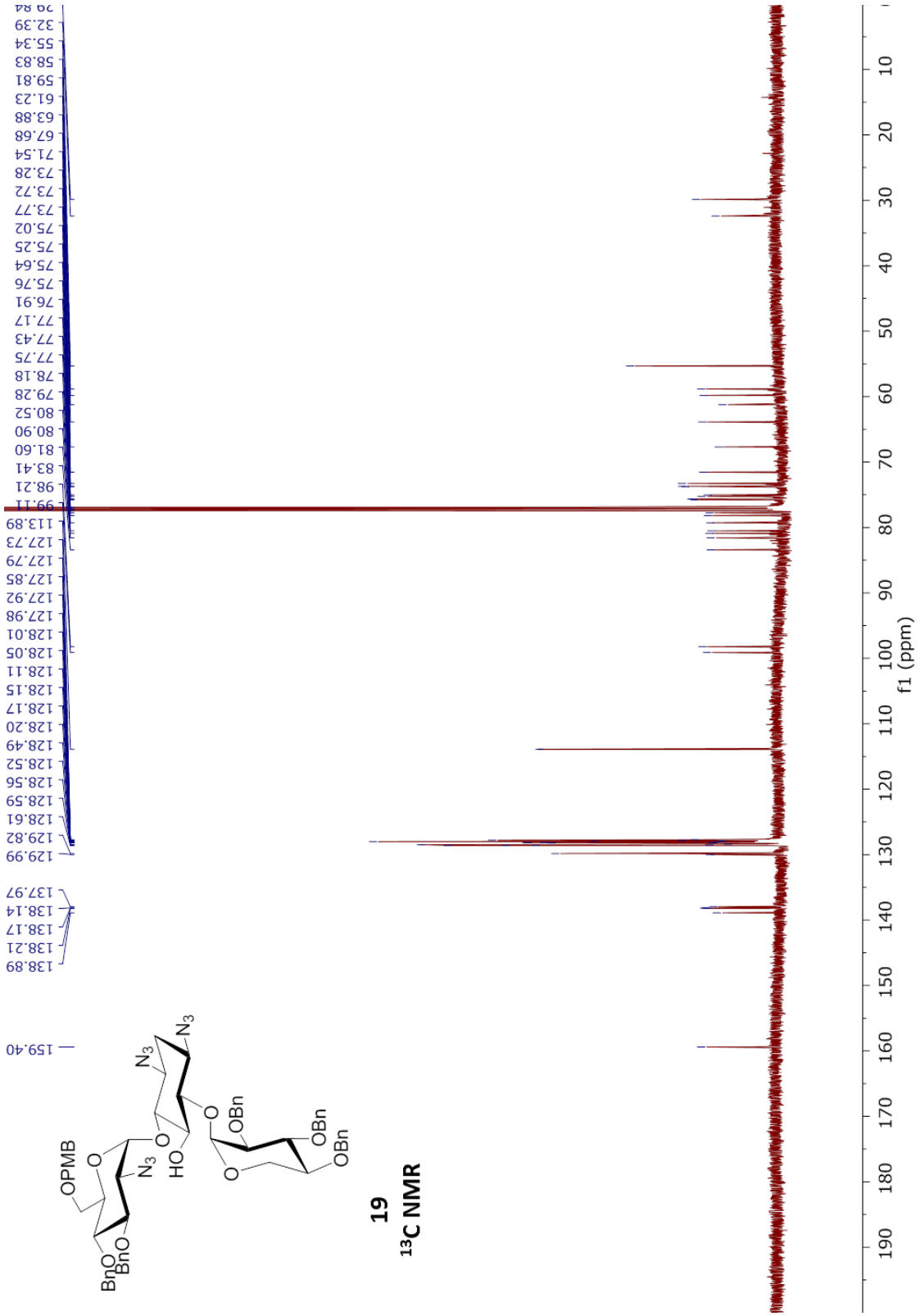

OPMB
BnO
BnO
N3
N3
AcO
OAc
N3
17
1H NMR
f1 (ppm)

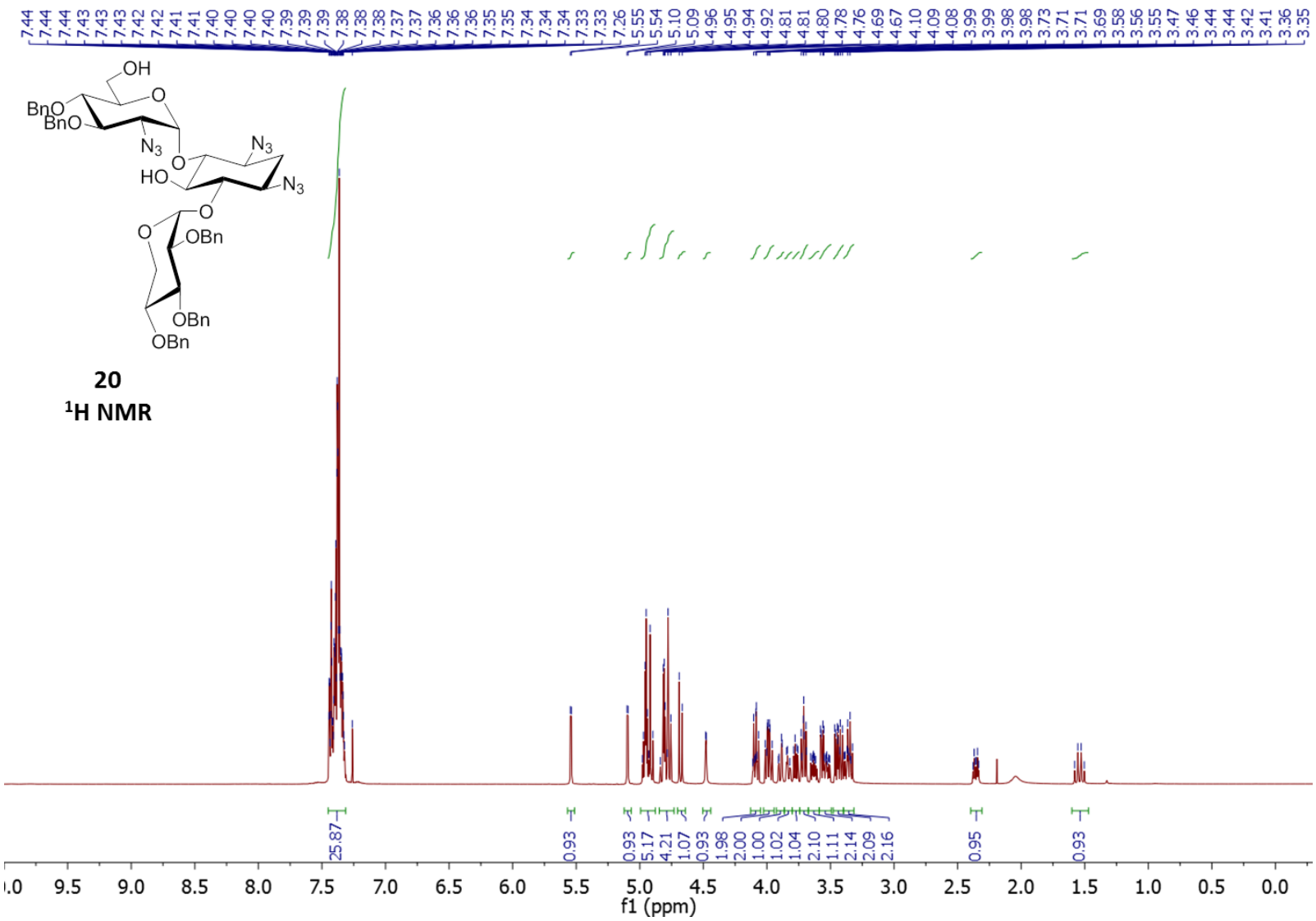

20.72
20.65
31.89
55.28
57.79
58.77
63.22
67.49
71.75
73.28
73.63
74.19
75.00
75.38
76.84
77.16
77.48
78.20
78.37
79.64
99.47
113.88
127.75
127.83
127.94
128.05
128.46
128.53
129.74
129.76
129.90
137.89
138.02
159.40
169.57
169.91
OPMB
BnO
BnO
O
N3
O
AcO
N3
OAc
17
13C NMR
f1 (ppm)

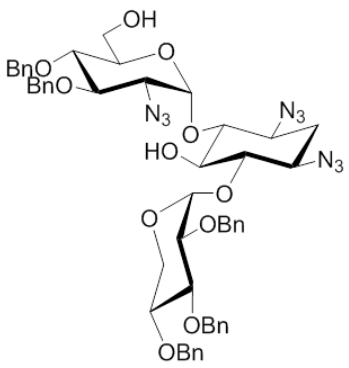

OPMB
BnO
BnO
N3
O
N3
HO
N3
OH
18
1H NMR
f1 (ppm)


OPMB
BnO
BnO
N3
N3
HO
OH
18
13C NMR
159.45
137.89
137.67
129.87
129.83
128.63
128.56
128.19
128.12
127.99
127.86
113.92
99.75
99.72
84.20
81.12
78.25
77.41
77.16
76.90
75.95
75.77
75.46
75.11
73.35
71.80
67.60
64.39
59.76
58.87
55.36
55.34
32.23
f1 (ppm)


OPMB
BnO
BnO
N3
O
HO
N3
N3
O
O
OBn
OBn
OBn
19
1H NMR
f1 (ppm)


OPMB
BnO
BnO
O
N3
O
HO
N3
N3
O
OBn
OBn
OBn
O
19
13C NMR
f1 (ppm)

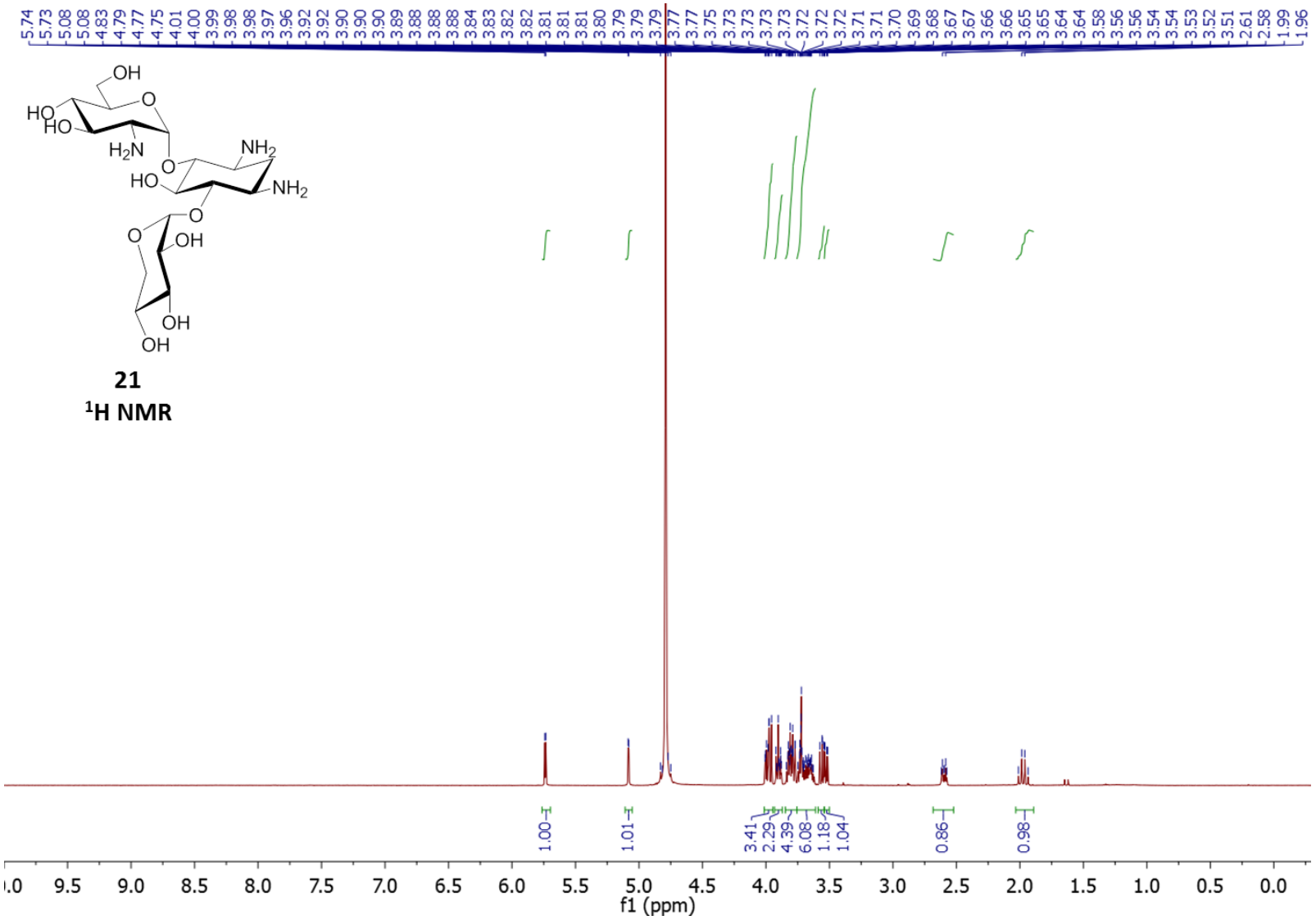

OH
BnO
BnO
O
N3
O
HO
N3
N3
O
O
OBn
OBn
OBn
20
1H NMR
f1 (ppm)

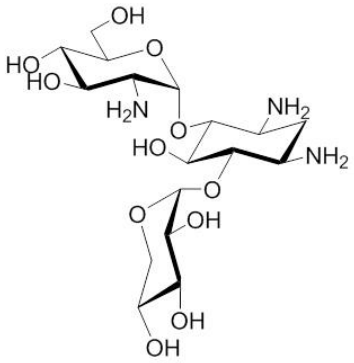

OH
O
BnO
BnO
N3
O
N3
HO
N3
O
O
OBn
OBn
OBn

20

^{13}C NMR

138.80
138.14
138.09
137.99
137.87
128.56
128.53
128.48
128.40
128.15
128.06
128.01
127.96
127.93
127.91
127.65
98.74
98.19
83.89
81.03
80.72
80.17
79.19
77.81
77.59
77.41
77.16
76.91
75.62
75.56
75.29
75.07
73.65
73.64
72.17
63.78
61.34
61.25
59.59
58.92
32.34
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

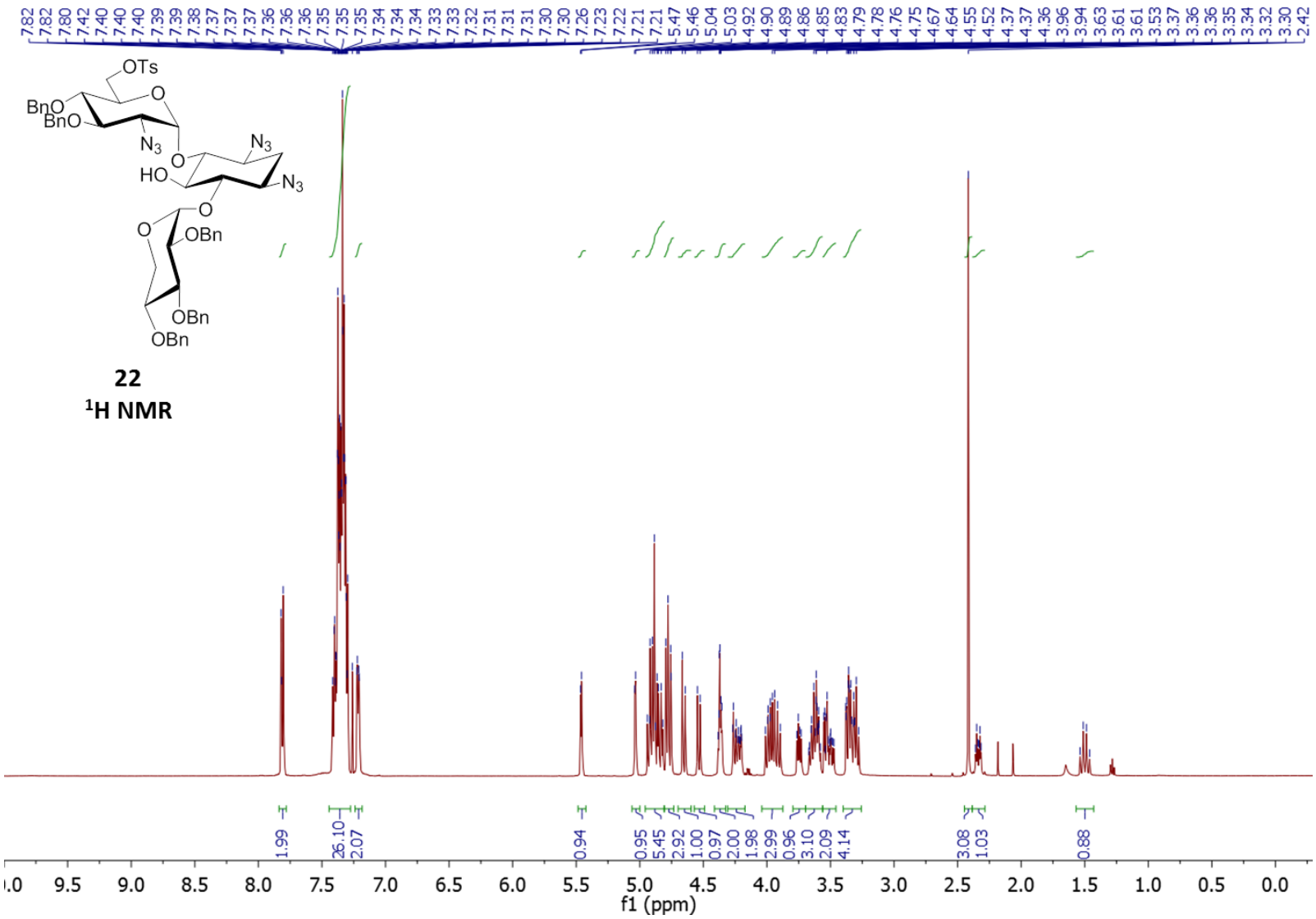

21
1H NMR
f1 (ppm)

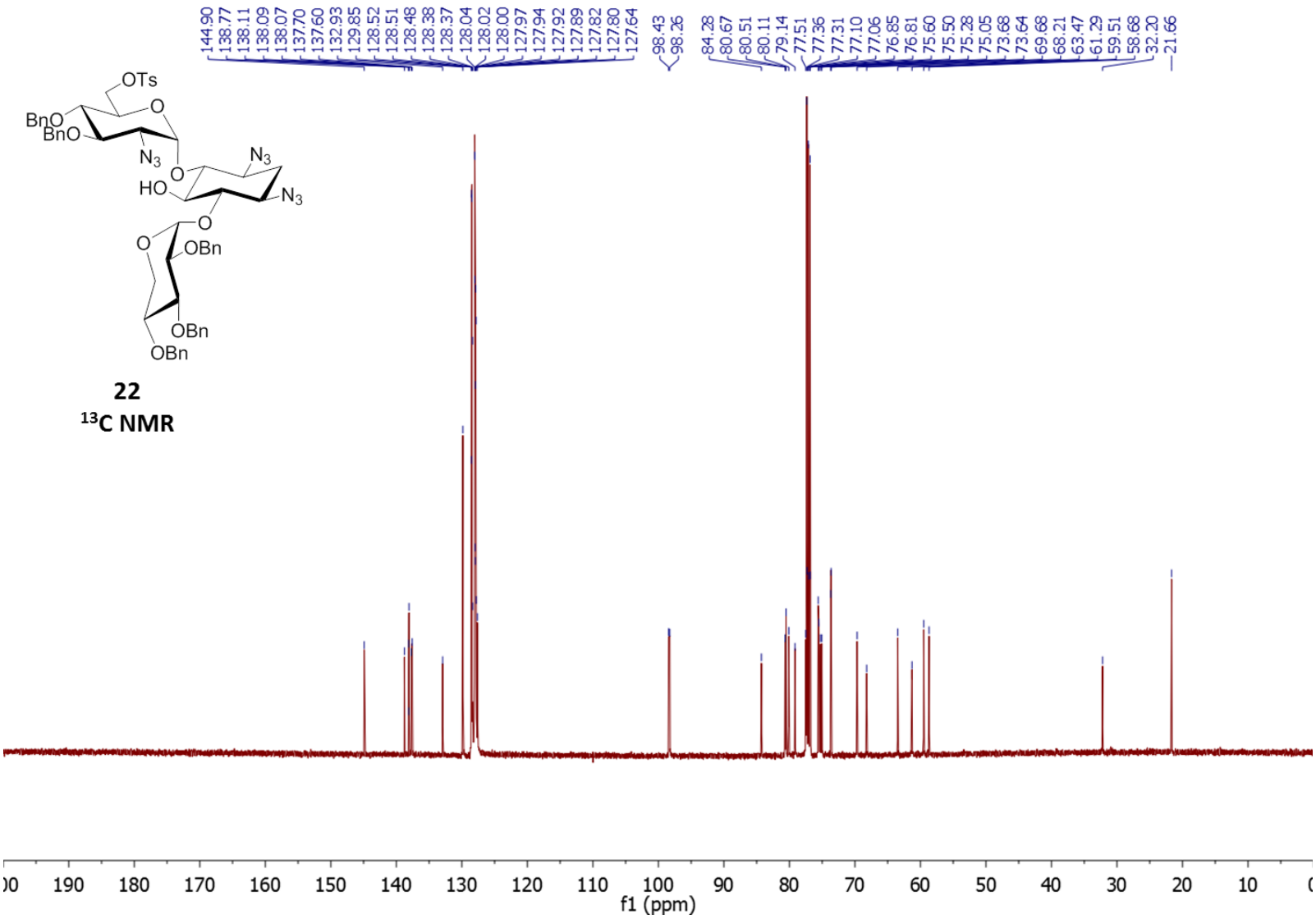

OH
HO
HO
O
H_2N
O
HO
NH_2
NH_2
O
O
OH
OH
OH

21

^{13}C NMR

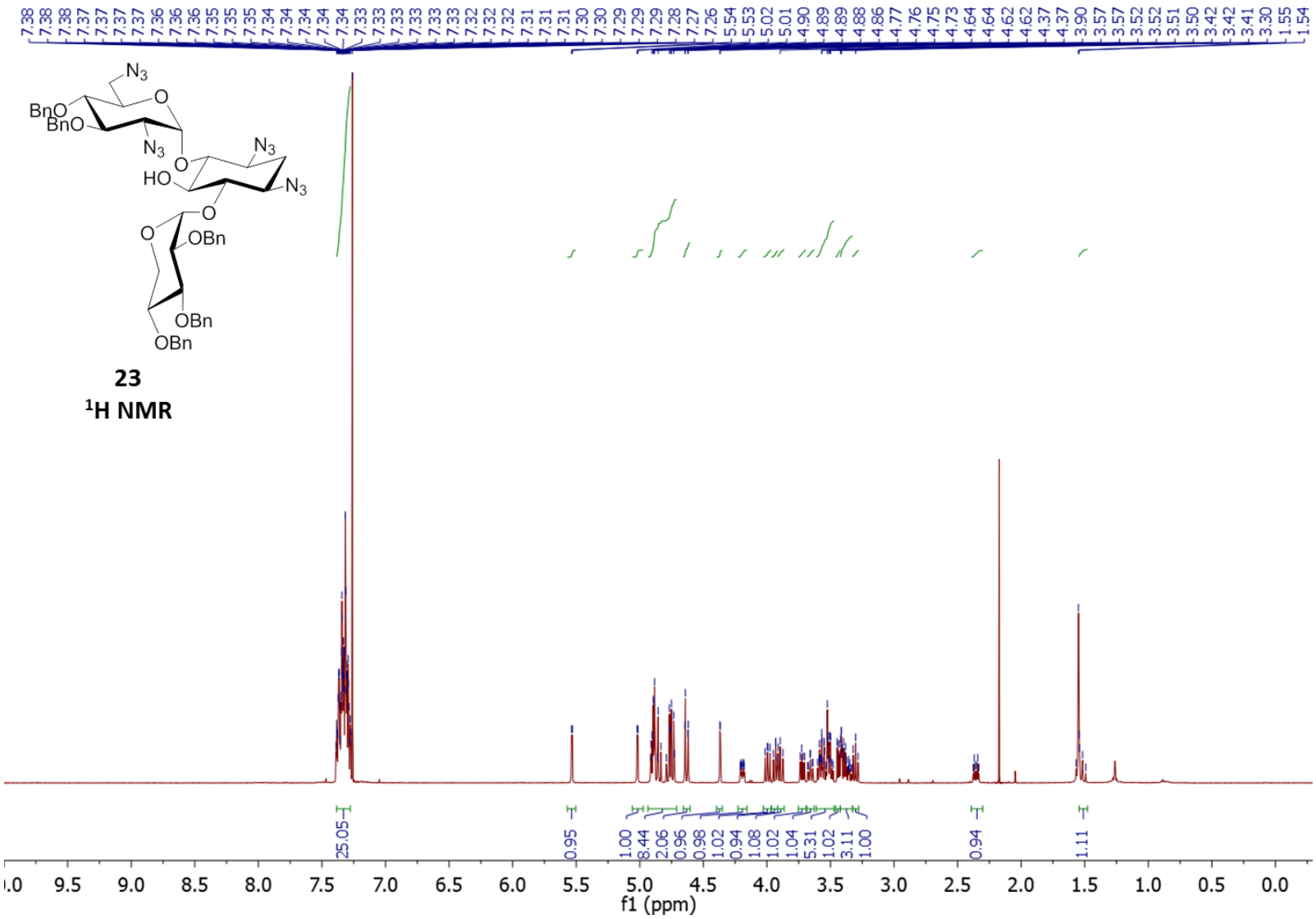
101.80
97.10
83.38
80.40
73.72
73.61
72.91
71.64
69.36
68.99
68.88
62.42
60.36
53.93
49.64
48.68
27.90
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

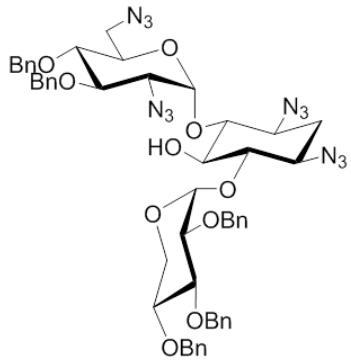

OTs
O
BnO
BnO
N3
O
N3
HO
N3
O
O
OBn
OBn
OBn
22
1H NMR
f1 (ppm)


144.90
138.77
138.11
138.09
138.07
137.70
137.60
132.93
129.85
128.52
128.51
128.48
128.38
128.37
128.04
128.02
128.00
127.97
127.94
127.92
127.89
127.82
127.80
127.64
98.43
98.26
84.28
80.67
80.51
80.11
79.14
77.51
77.36
77.31
77.10
77.06
76.85
76.81
75.60
75.50
75.28
75.05
73.68
73.64
69.68
68.21
63.47
61.29
59.51
58.68
32.20
21.66
OTs
BnO
BnO
N3
O
N3
HO
N3
O
O
OBn
OBn
OBn
f1 (ppm)

22

^{13}C NMR

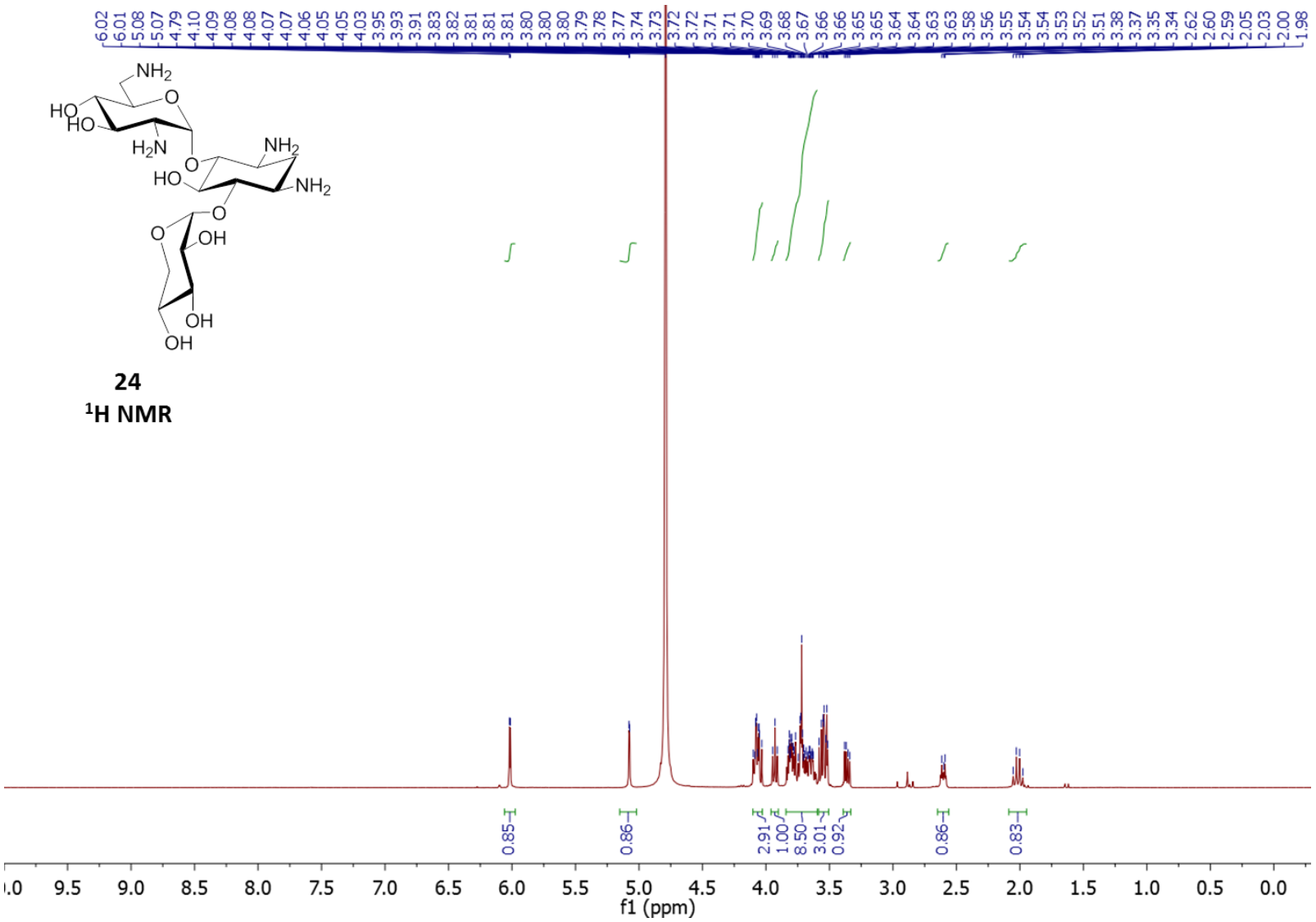

N3
BnO
BnO
N3
O
HO
N3
N3
O
O
OBn
OBn
OBn
23
1H NMR
f1 (ppm)

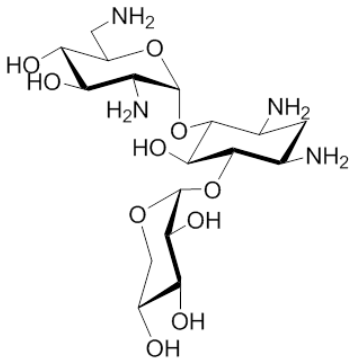

N3
BnO
BnO
O
N3
O
HO
N3
N3
O
O
OBn
OBn
OBn

23

^{13}C NMR

138.74
138.07
138.03
137.70
128.52
128.50
128.48
128.44
128.42
128.34
128.06
128.01
127.99
127.95
127.89
127.87
127.80
127.78
127.60
98.44
98.27
84.26
80.65
80.64
80.10
79.11
78.65
77.47
77.27
77.21
77.17
77.01
76.76
75.58
75.54
75.33
75.17
73.64
73.63
71.07
63.66
61.28
59.54
58.81
51.04
32.30
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

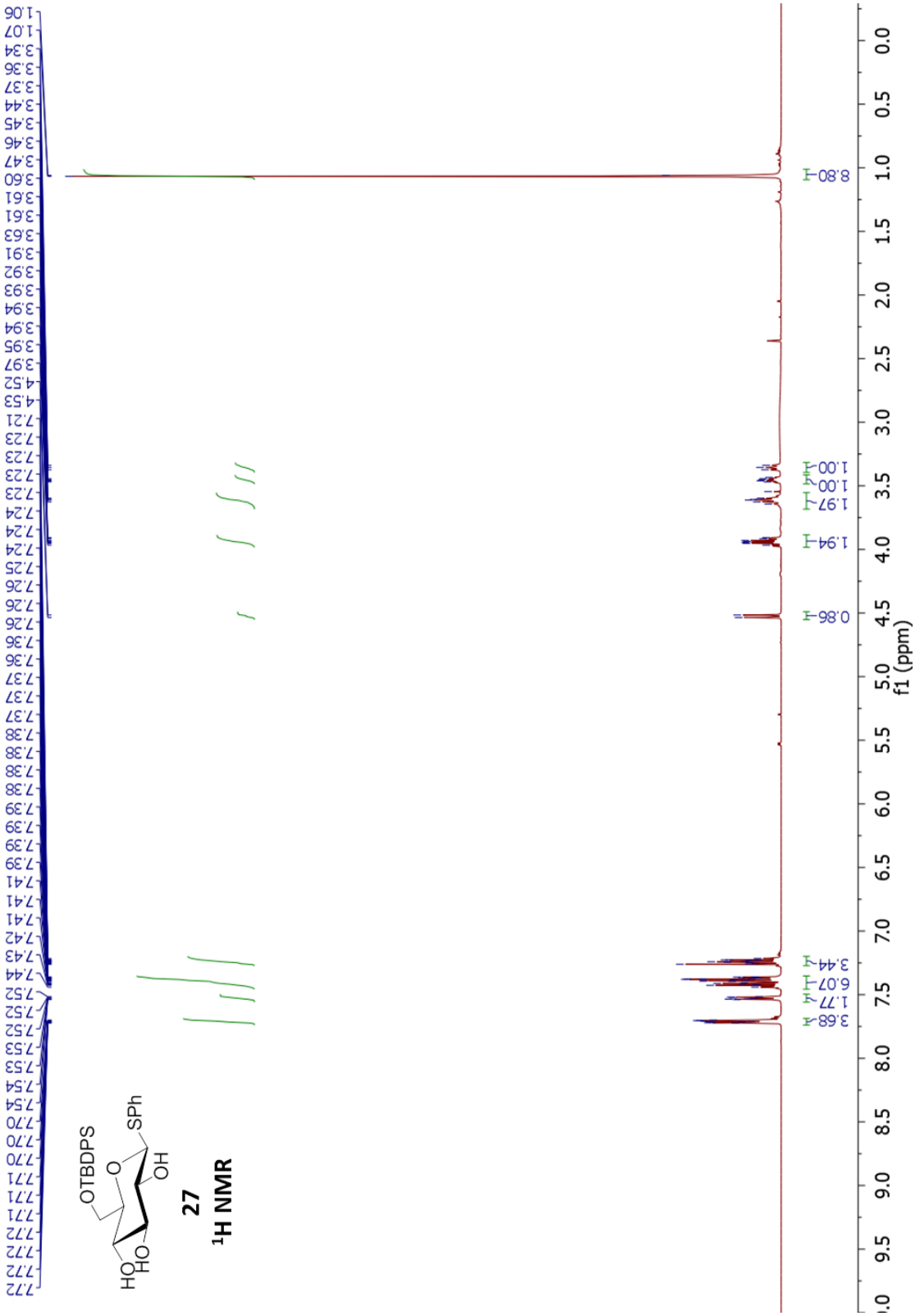

24
1H NMR
f1 (ppm)

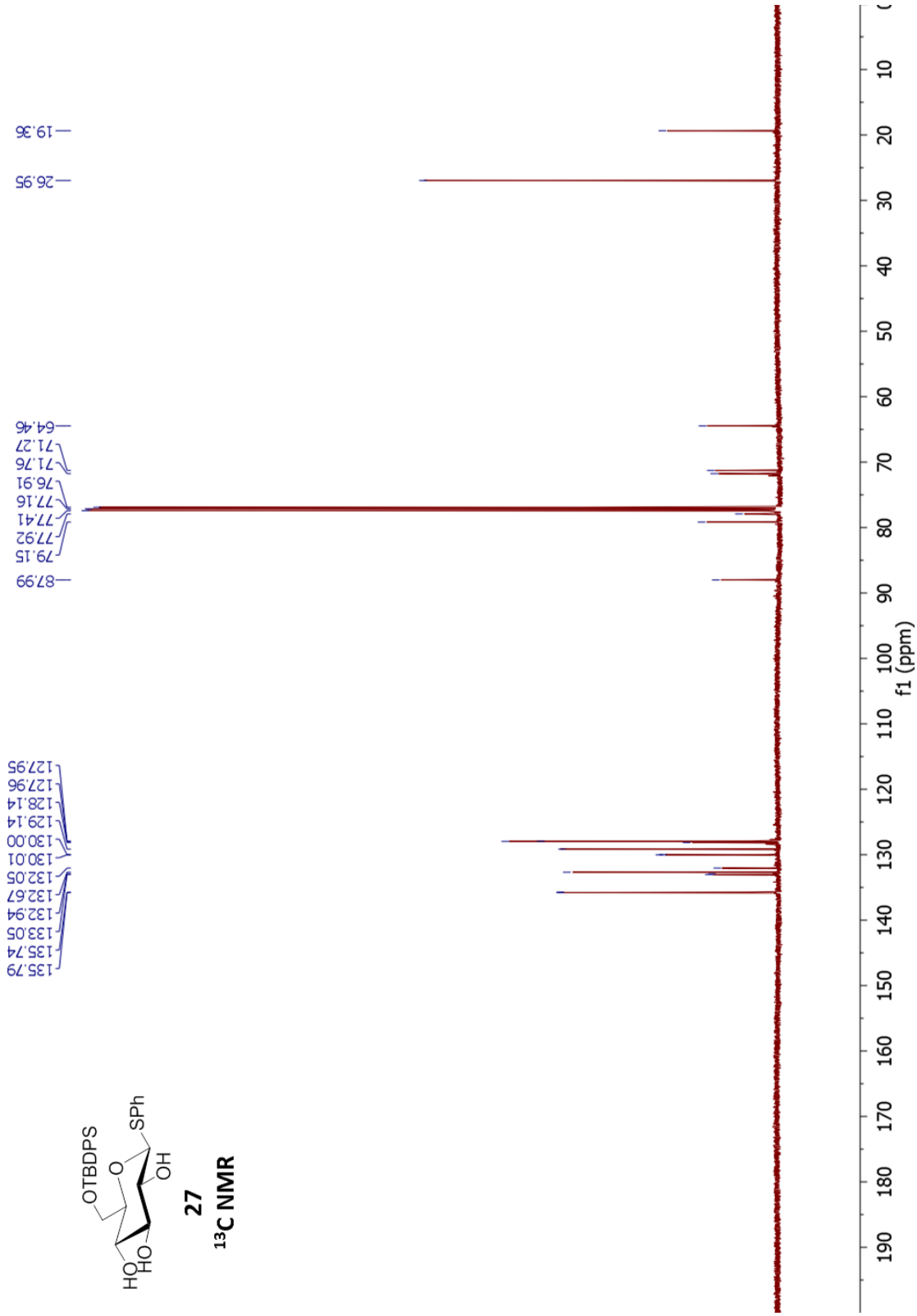

NH2
O
HO
HO
H2N
O
NH2
HO
NH2
O
O
OH
OH
OH

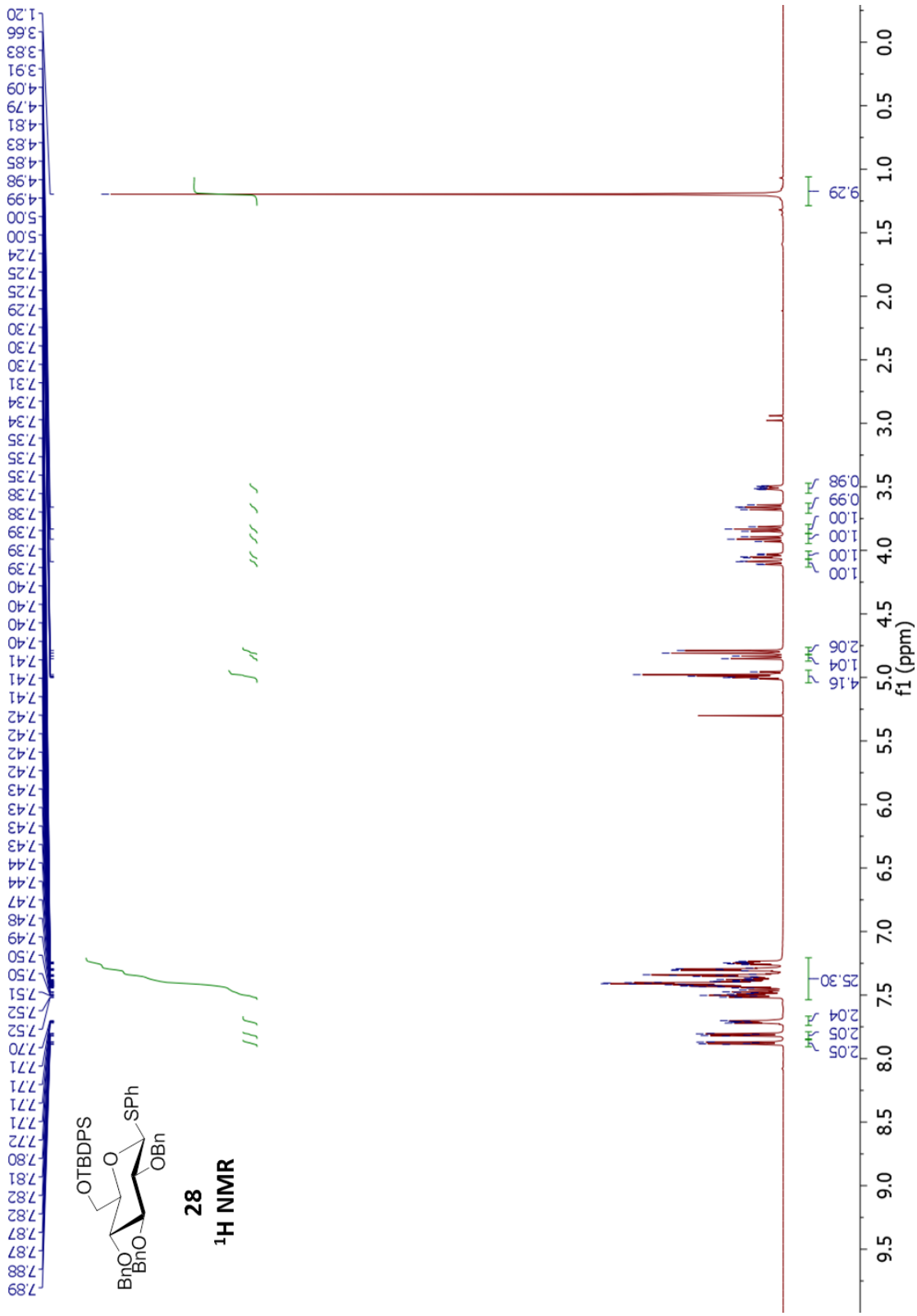
24

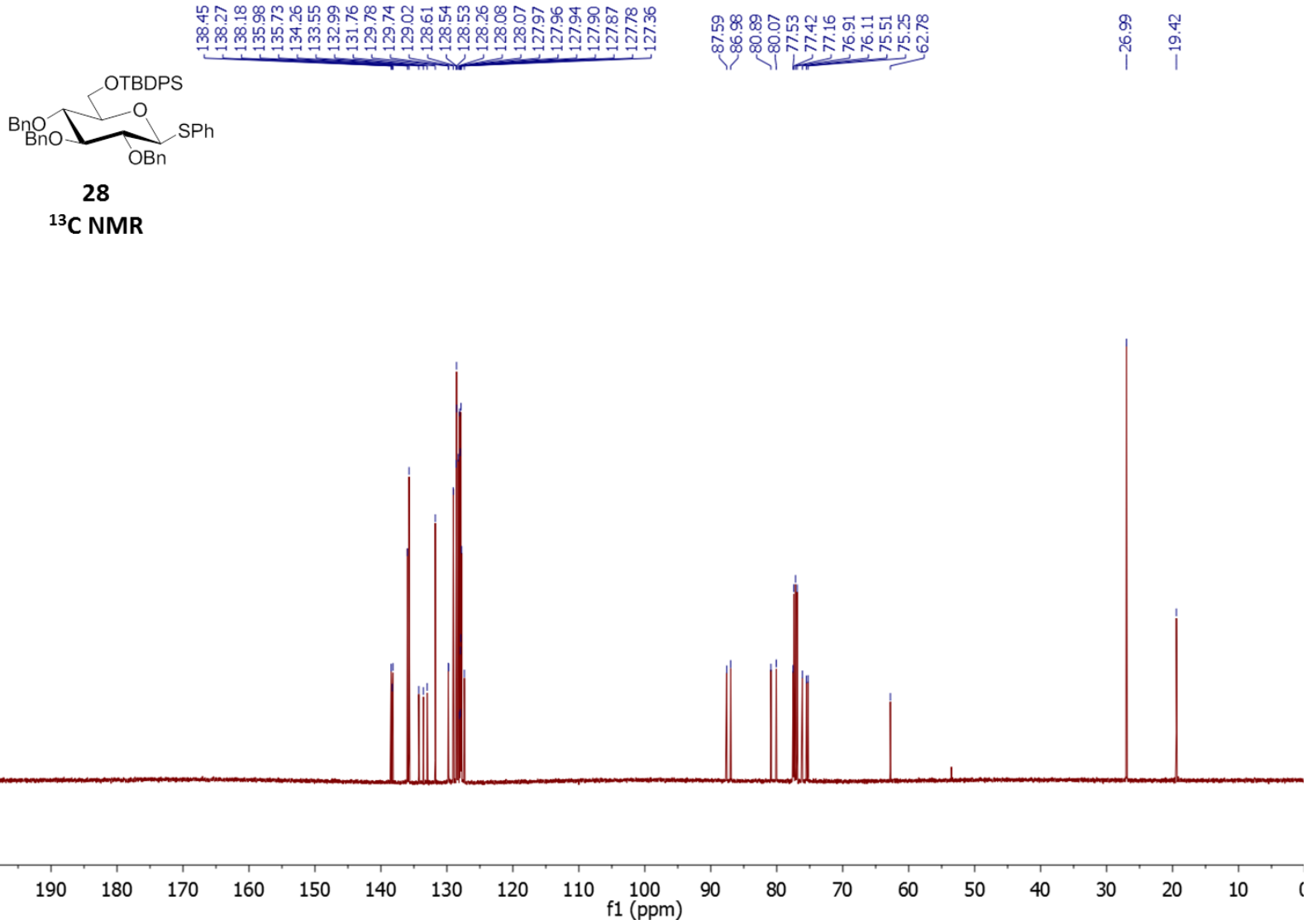
^{13}C NMR

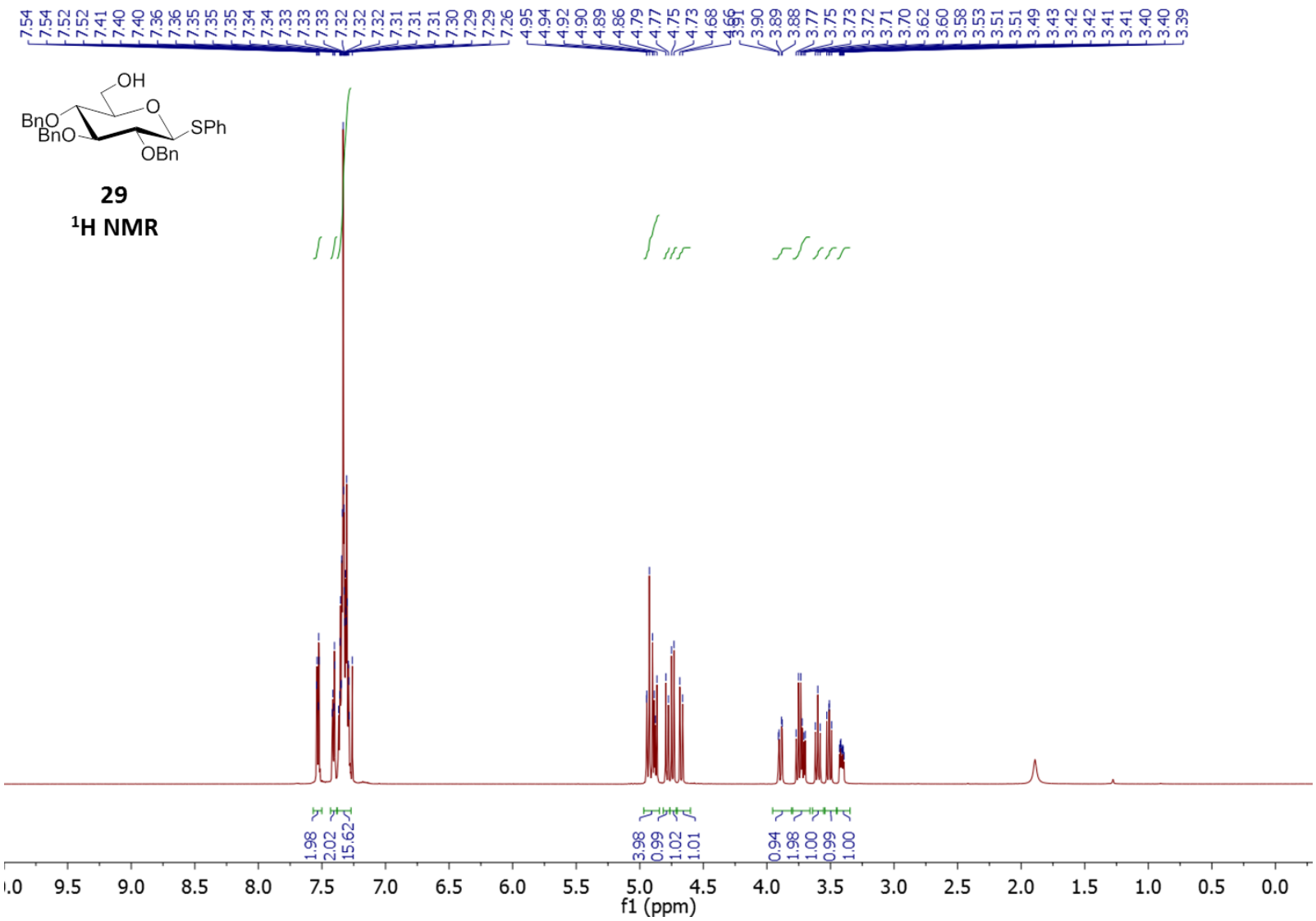
101.92
96.02
83.60
77.51
74.11
72.88
71.63
70.58
69.20
68.83
68.19
62.50
53.43
49.69
48.24
40.06
27.87
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

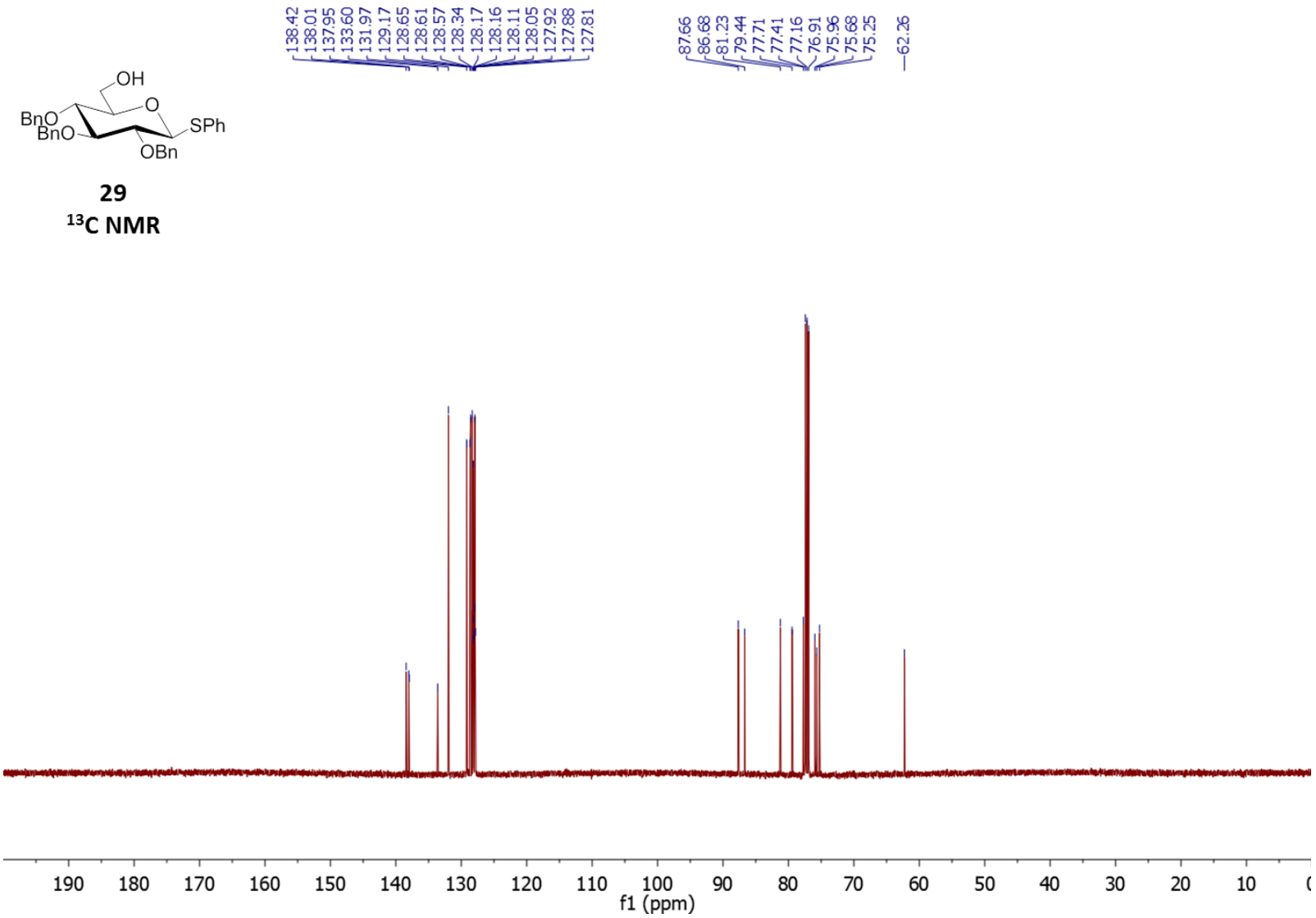

OTBDPS
O
SPh
OH
HO
HO
27
1H NMR
f1 (ppm)


OTBDPS
O
SPh
OH
HO
HO
27
13C NMR
135.79
135.74
133.05
132.94
132.67
132.05
130.01
130.00
129.14
128.14
127.96
127.95
87.99
79.15
77.92
77.41
77.16
76.91
71.76
71.27
64.46
26.95
19.36
f1 (ppm)


OTBDPS
O
SPh
OBn
BnO
BnO
28
^{1}H NMR
f1 (ppm)

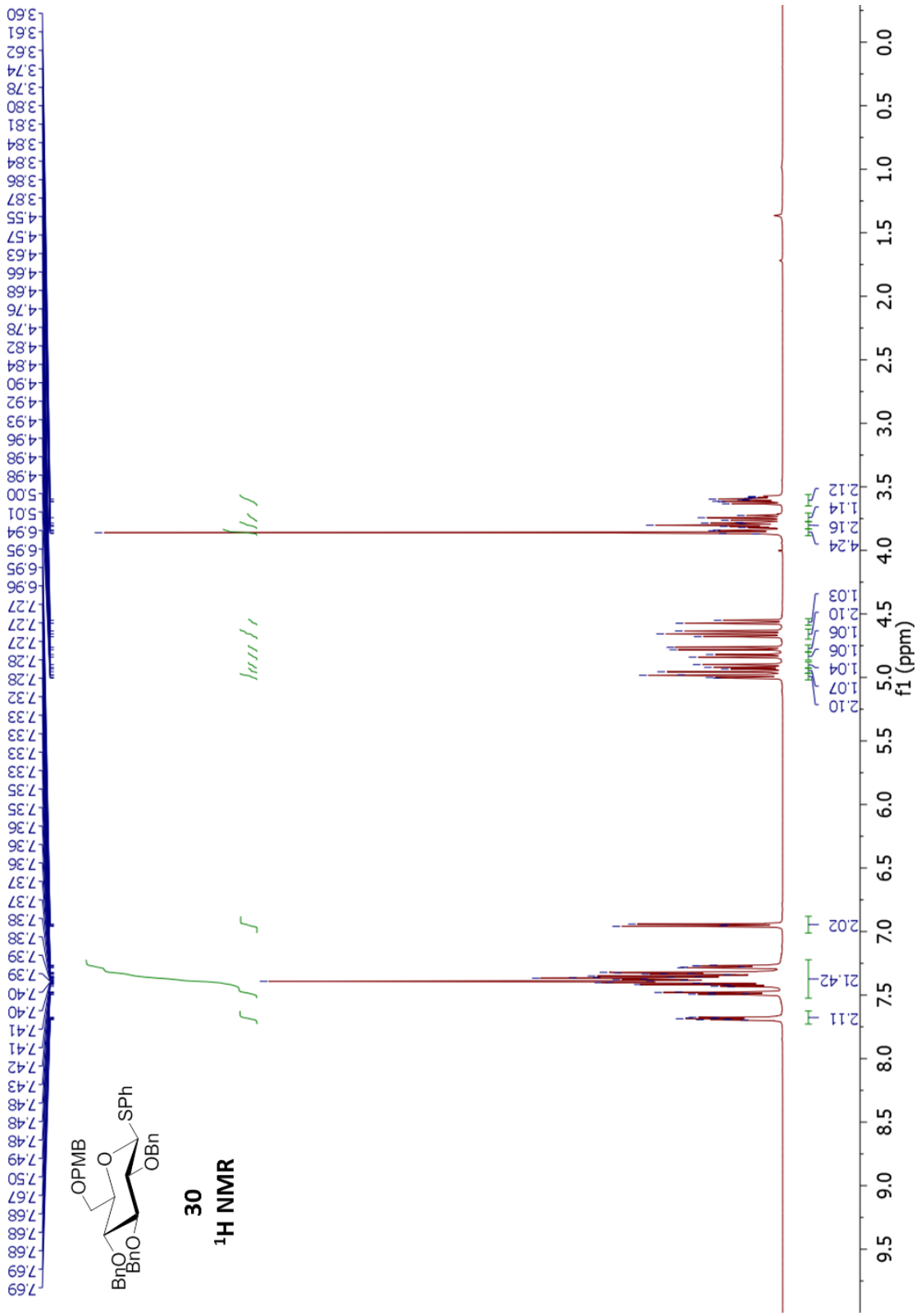

138.45
138.27
138.18
135.98
135.73
134.26
133.55
132.99
131.76
129.78
129.74
129.02
128.61
128.54
128.53
128.26
128.08
128.07
127.97
127.96
127.94
127.90
127.87
127.78
127.36
87.59
86.98
80.89
80.07
77.53
77.42
77.16
76.91
76.11
75.51
75.25
62.78
26.99
19.42
OTBDPS
O
BnO
BnO
SPh
OBn
28
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

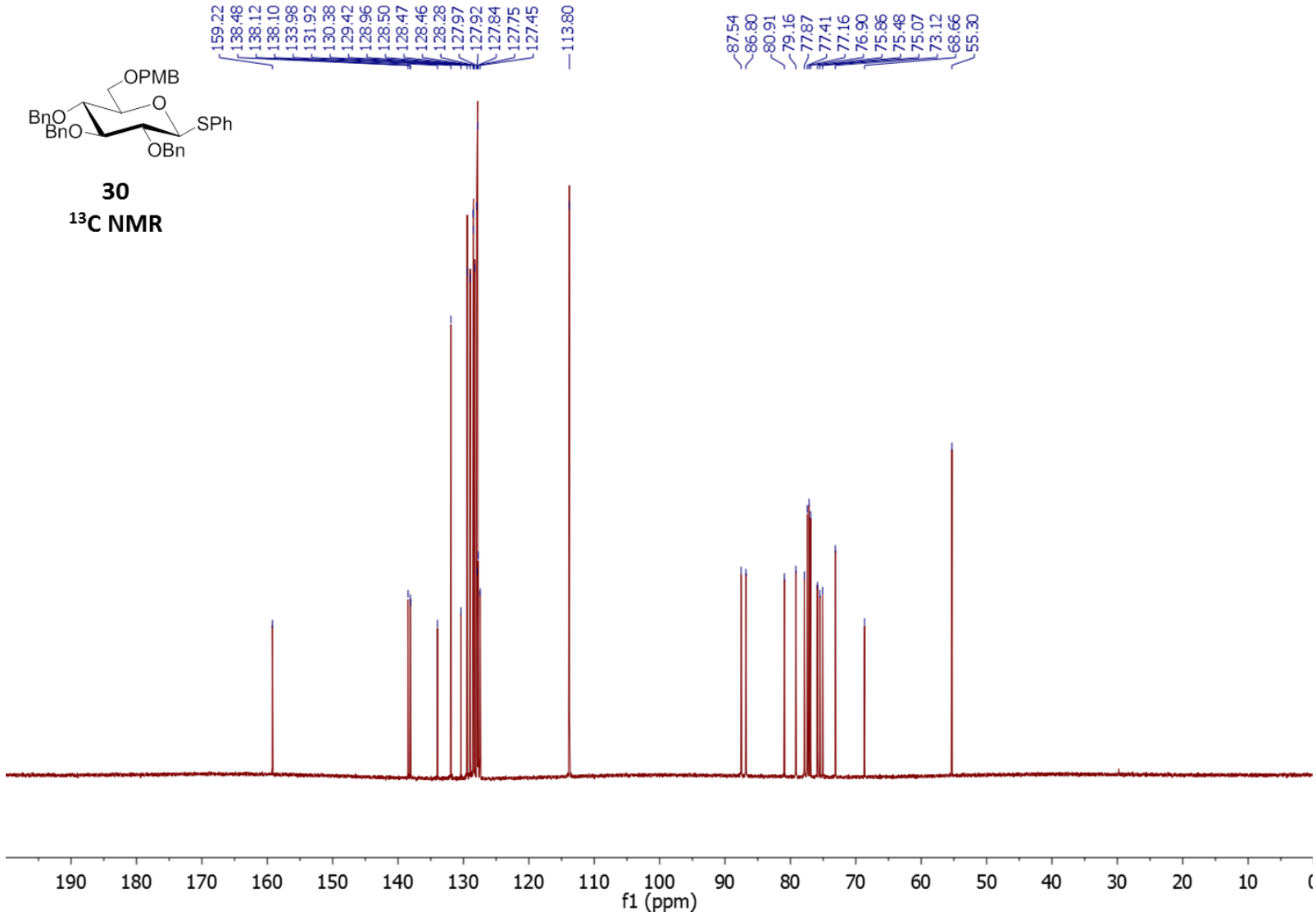

OH
O
BnO
BnO
SPh
OBn
29
1H NMR
f1 (ppm)

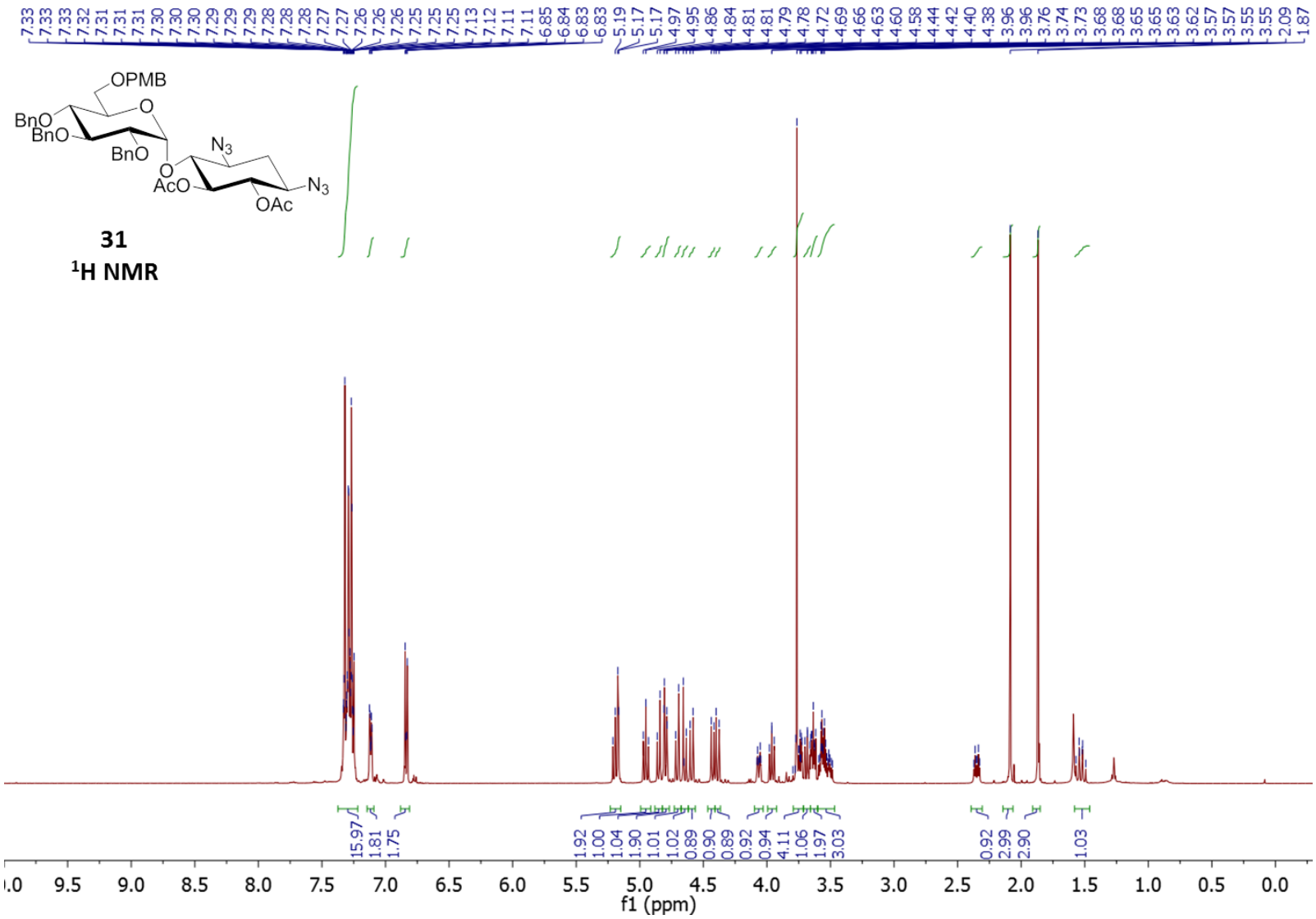

138.42
138.01
137.95
133.60
131.97
129.17
128.65
128.61
128.57
128.34
128.17
128.16
128.11
128.05
127.92
127.88
127.81
87.66
86.68
81.23
79.44
77.71
77.41
77.16
76.91
75.96
75.68
75.25
62.26
OH
O
BnO
BnO
SPh
OBn
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

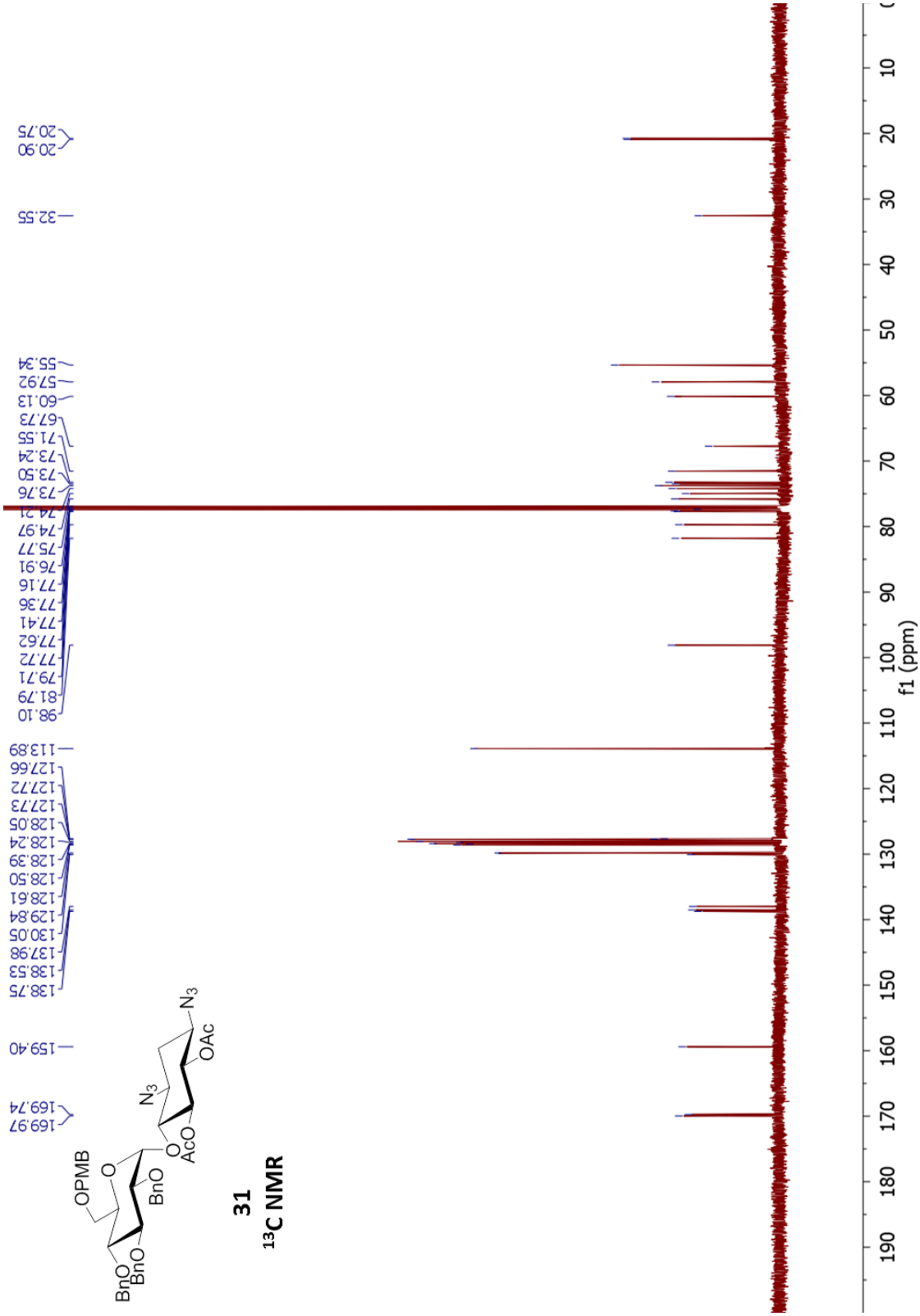
29

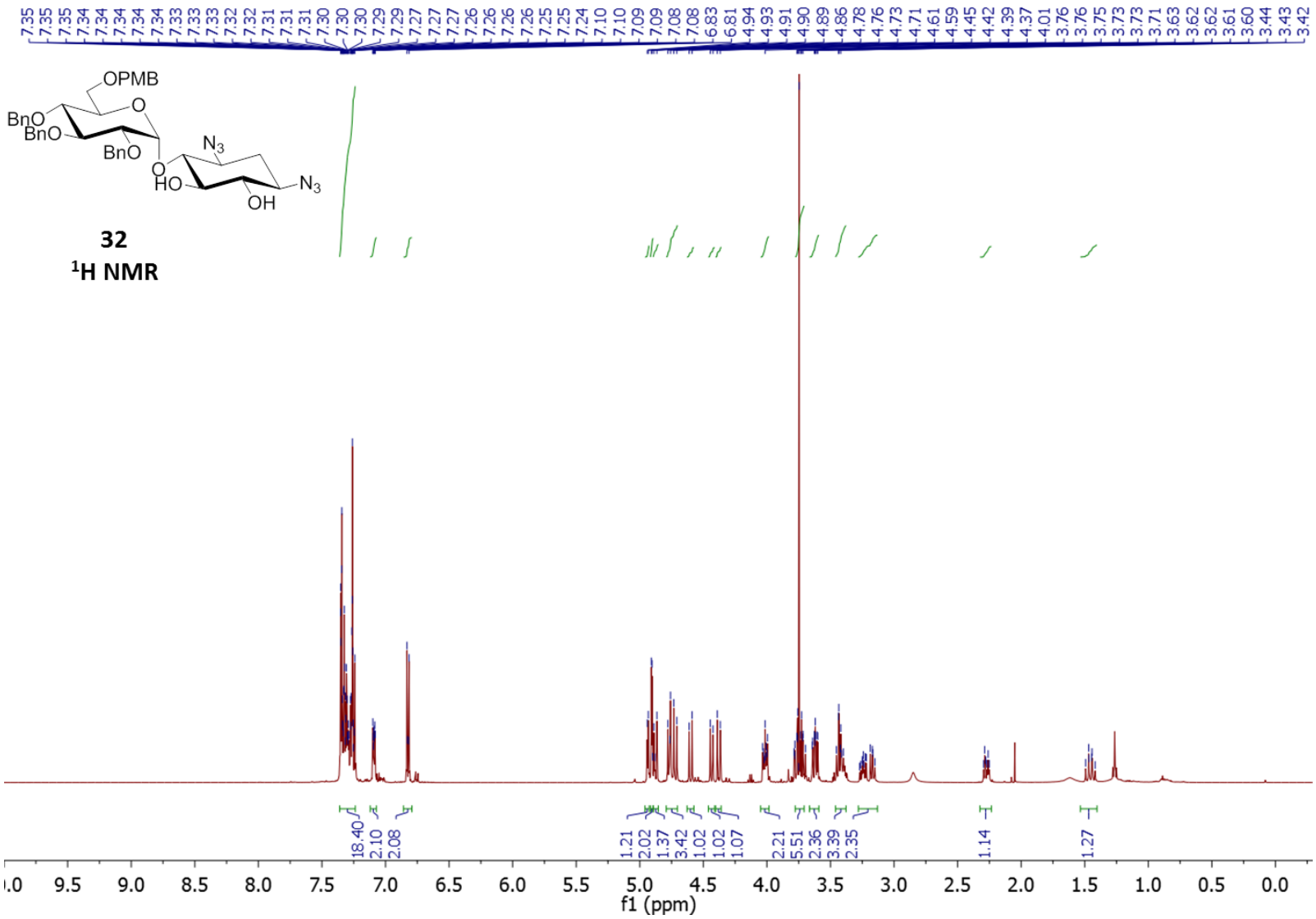
^{13}C NMR

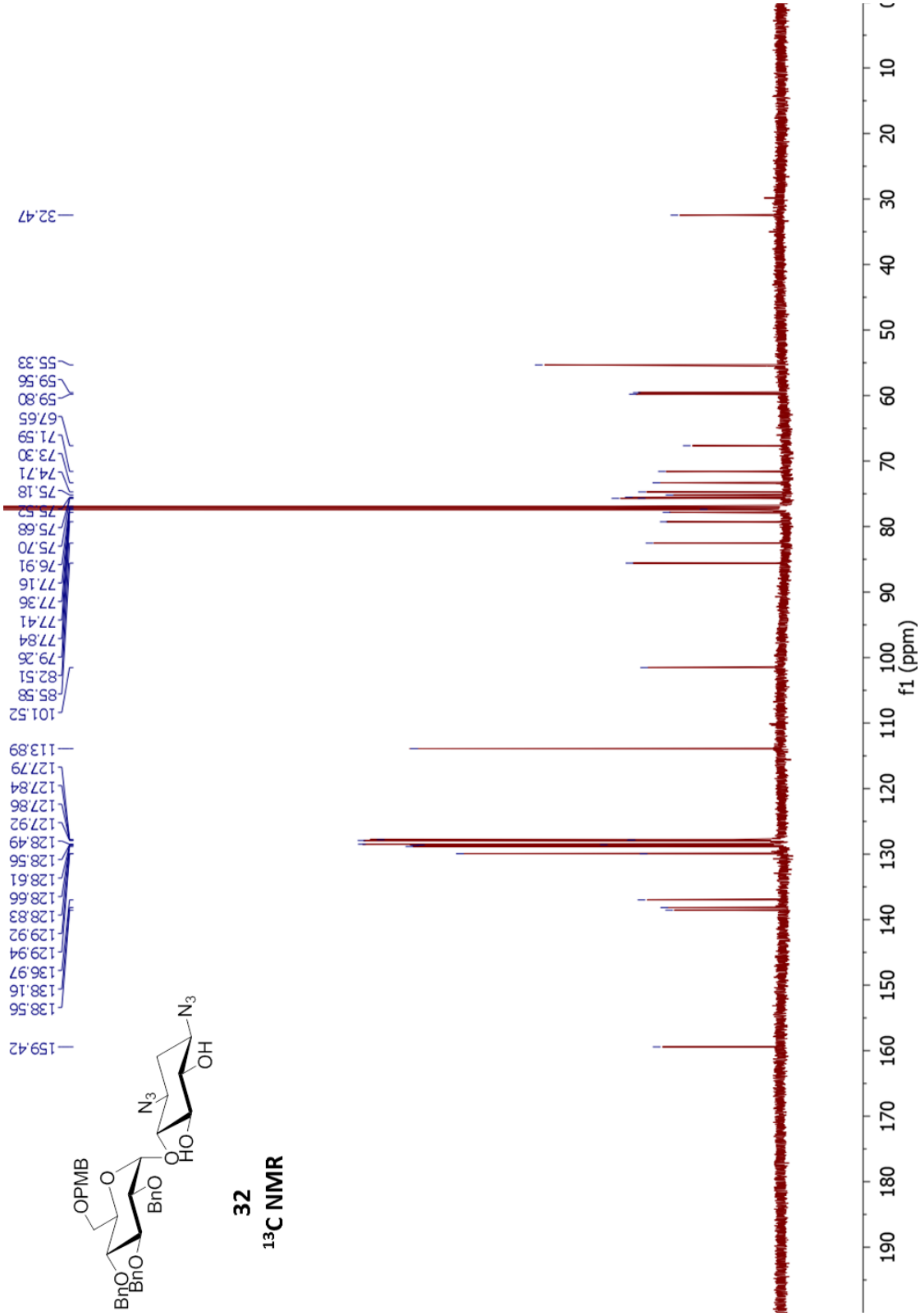

OPMB
O
SPh
OBn
BnO
BnO
30
1H NMR
f1 (ppm)

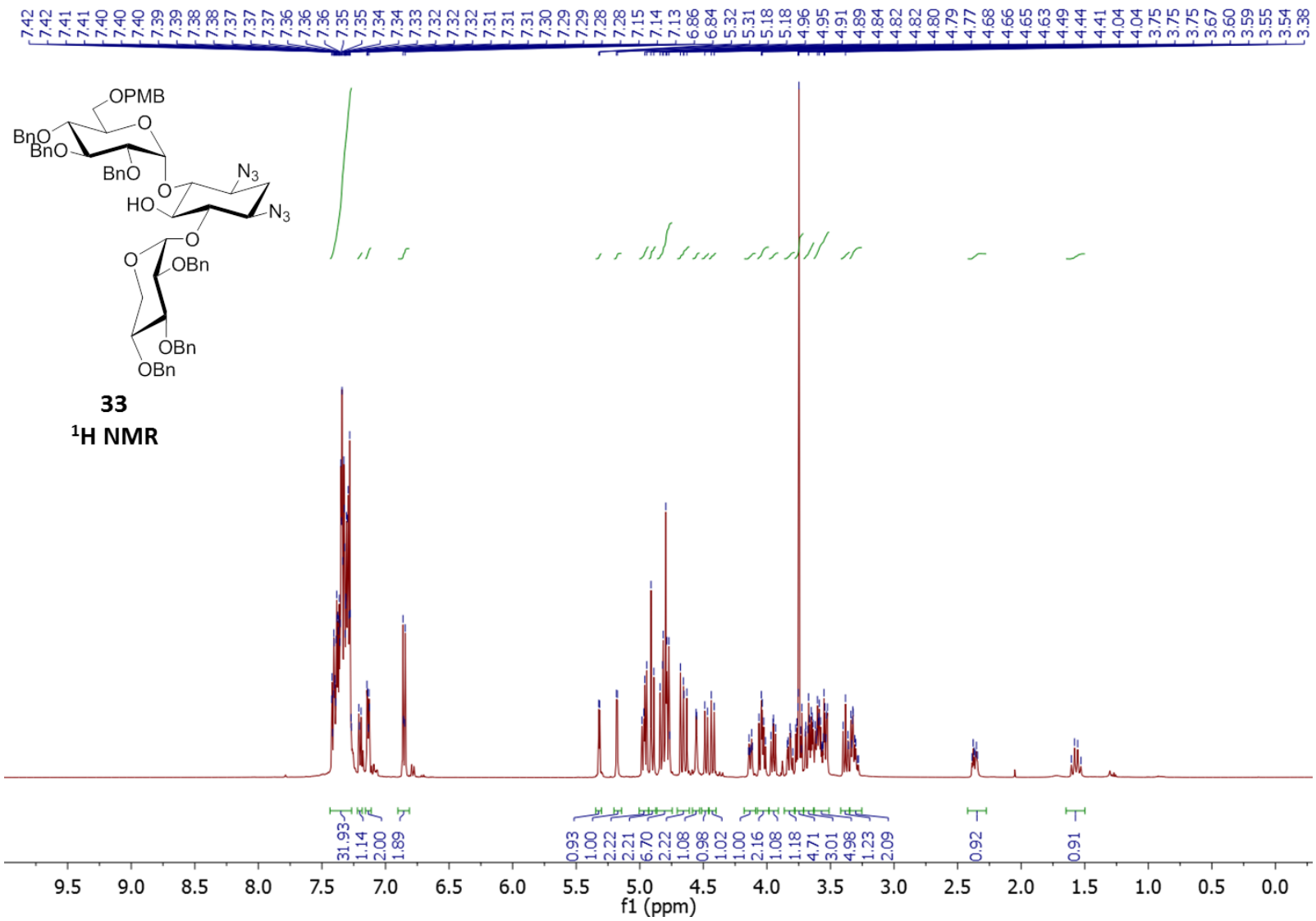

159.22
138.48
138.12
138.10
133.98
131.92
130.38
129.42
128.96
128.50
128.47
128.46
128.28
127.97
127.92
127.84
127.75
127.45
113.80
87.54
86.80
80.91
79.16
77.87
77.41
77.16
76.90
75.86
75.48
75.07
73.12
68.66
55.30
OPMB
O
BnO
BnO
SPh
OBn
30
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

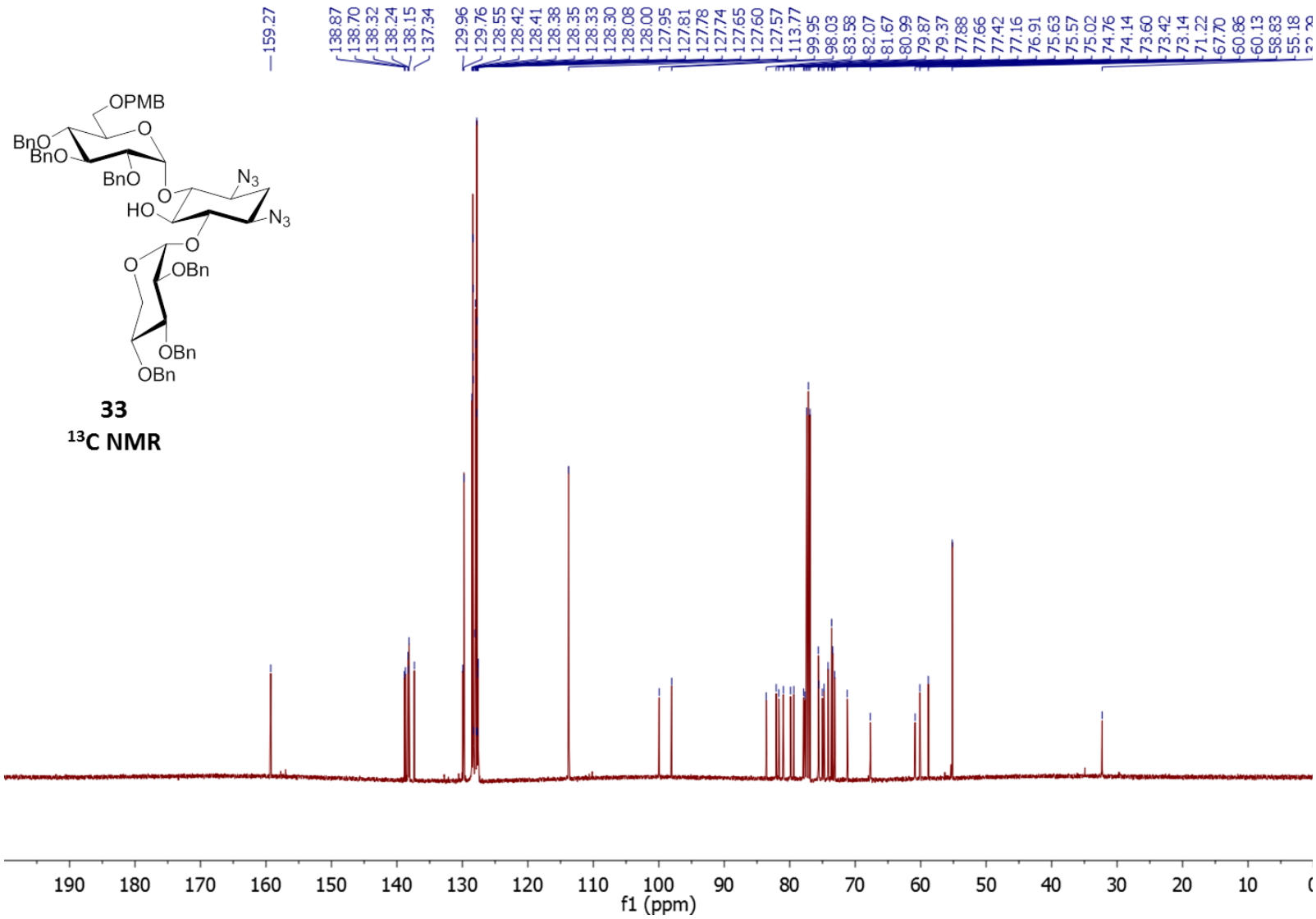

OPMB
BnO
BnO
BnO
O
O
AcO
N3
N3
OAc
31
1H NMR
f1 (ppm)


20.75
20.90
32.55
55.34
57.92
60.13
67.73
71.55
73.24
73.50
73.76
74.21
74.97
75.77
76.91
77.16
77.36
77.41
77.62
77.72
79.71
81.79
98.10
113.89
127.66
127.72
127.73
128.05
128.24
128.39
128.50
128.61
129.84
130.05
137.98
138.53
138.75
159.40
169.74
169.97
OPMB
BnO
BnO
BnO
AcO
OAc
N3
N3
31
13C NMR
f1 (ppm)


OPMB
BnO
BnO
BnO
O
O
HO
N3
N3
OH
32
1H NMR
f1 (ppm)

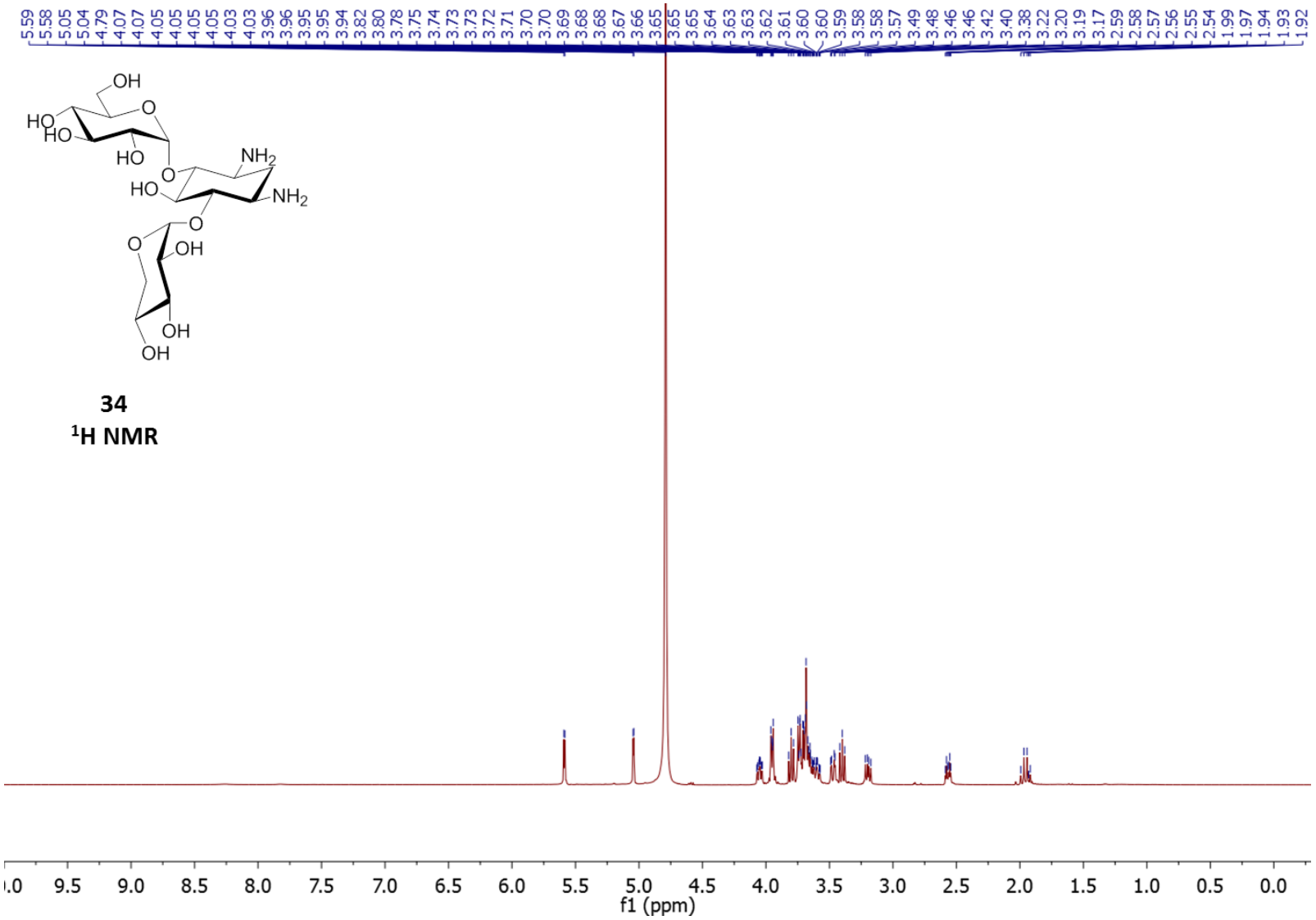

32
13C NMR
OPMB
BnO
BnO
BnO
O
O
HO
N3
N3
OH
32.47
55.33
59.56
59.80
67.65
71.59
73.30
74.71
75.18
75.52
75.68
75.70
76.91
77.16
77.36
77.41
77.84
79.26
82.51
85.58
101.52
113.89
127.79
127.84
127.86
127.92
128.49
128.56
128.61
128.66
128.83
129.92
129.94
136.97
138.16
138.56
159.42
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
f1 (ppm)


OPMB
BnO
BnO
BnO
HO
N3
N3
OBn
OBn
OBn
33
1H NMR
f1 (ppm)


OPMB
BnO
BnO
BnO
O
HO
O
N3
N3
O
O
OBn
OBn
OBn
159.27
138.87
138.70
138.32
138.24
138.15
137.34
129.96
129.76
128.55
128.42
128.41
128.38
128.35
128.33
128.30
128.08
128.00
127.95
127.81
127.78
127.74
127.65
127.60
127.57
113.77
99.95
98.03
83.58
82.07
81.67
80.99
79.87
79.37
77.88
77.66
77.42
77.16
76.91
75.63
75.57
75.02
74.76
74.14
73.60
73.42
73.14
71.22
67.70
60.86
60.13
58.83
55.18
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

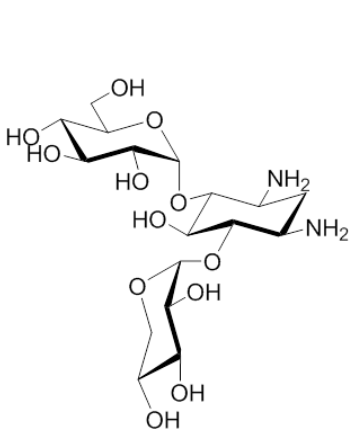
33

^{13}C NMR


5.59 5.58 5.05 5.04 4.79 4.07 4.07 4.05 4.05 4.05 4.05 4.03 4.03 3.96 3.96 3.95 3.95 3.94 3.82 3.80 3.78 3.75 3.74 3.73 3.73 3.72 3.71 3.70 3.70 3.69 3.68 3.68 3.67 3.66 3.65 3.65 3.65 3.64 3.63 3.63 3.62 3.61 3.60 3.60 3.59 3.58 3.58 3.57 3.49 3.48 3.46 3.46 3.42 3.40 3.38 3.22 3.20 3.19 3.17 2.59 2.58 2.57 2.56 2.55 2.54 1.99 1.97 1.94 1.93 1.92
OH
HO
HO
HO
O
O
HO
NH2
NH2
O
O
OH
OH
OH
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

34

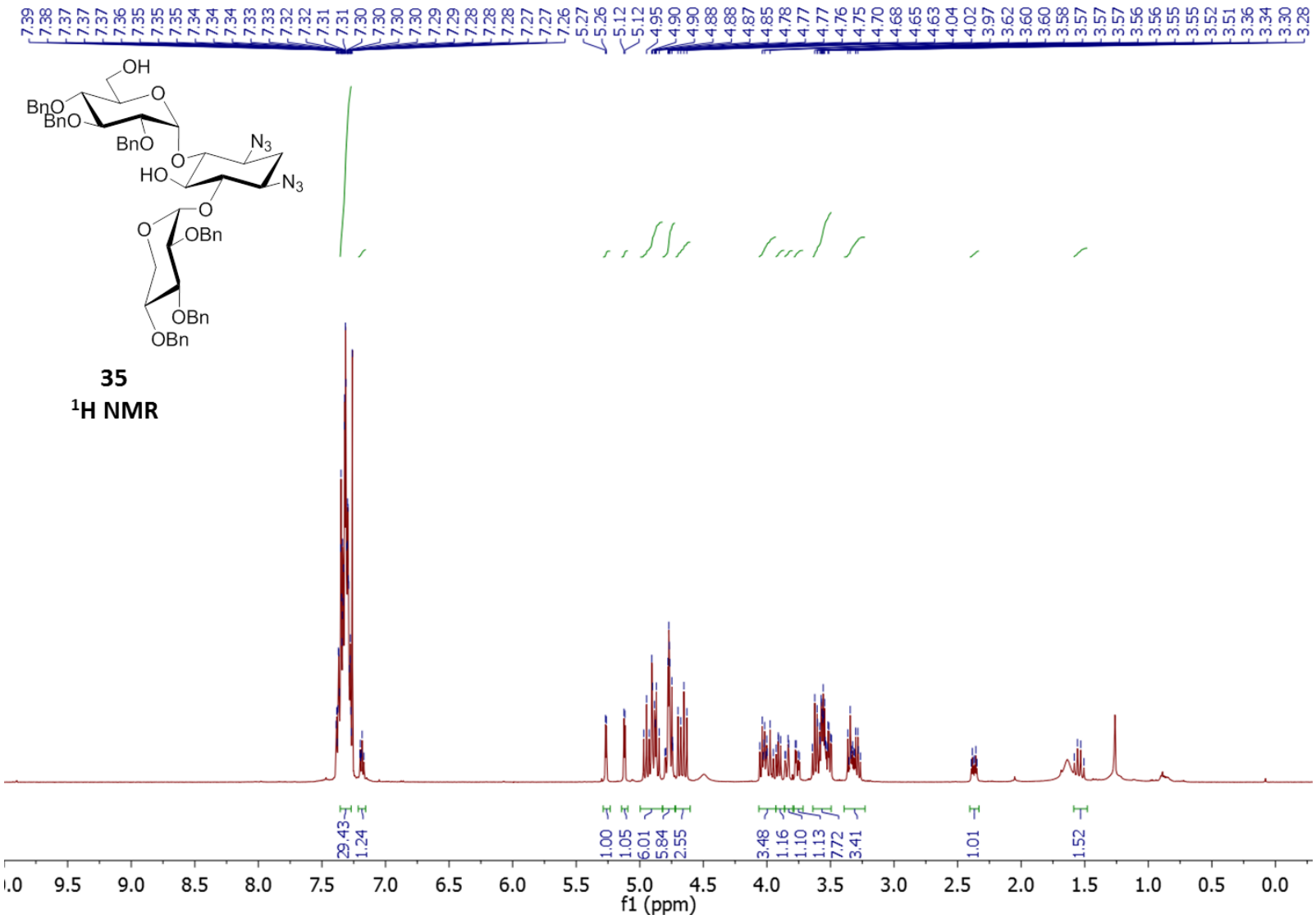
^{1}H NMR

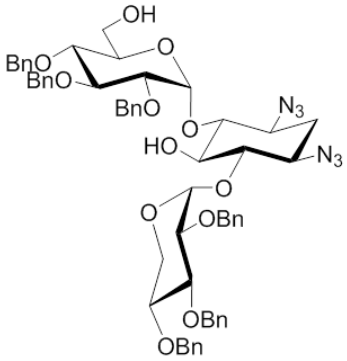

OH
HO
HO
O
HO
NH2
O
HO
NH2
O
O
OH
OH
OH

34

^{13}C NMR

101.84
95.44
83.82
78.05
72.95
72.11
71.98
71.73
70.80
70.68
68.90
68.63
62.20
49.95
47.54
40.30
27.52
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

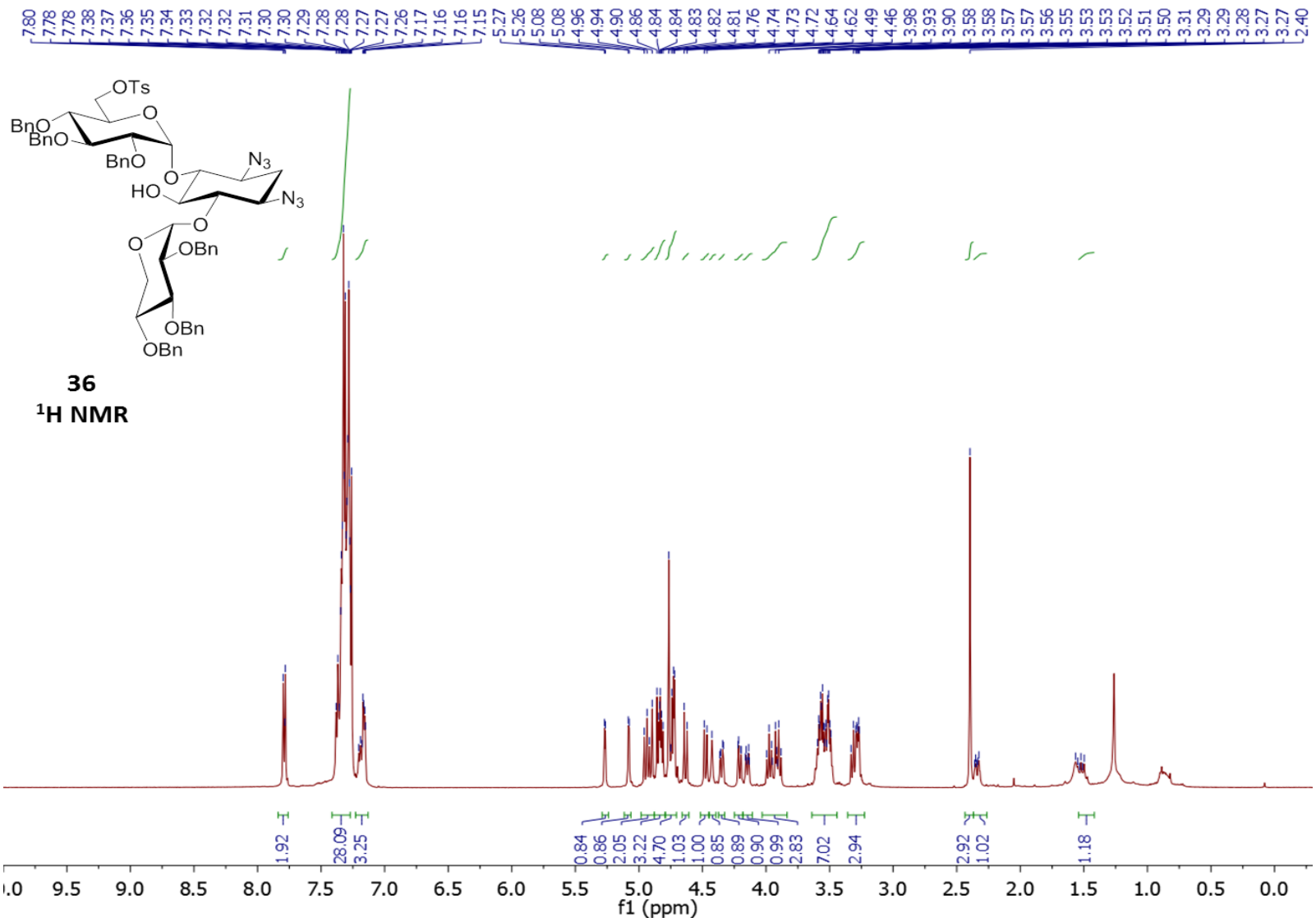

OH
BnO
BnO
BnO
O
O
HO
N3
N3
O
O
OBn
OBn
OBn
35
1H NMR
f1 (ppm)

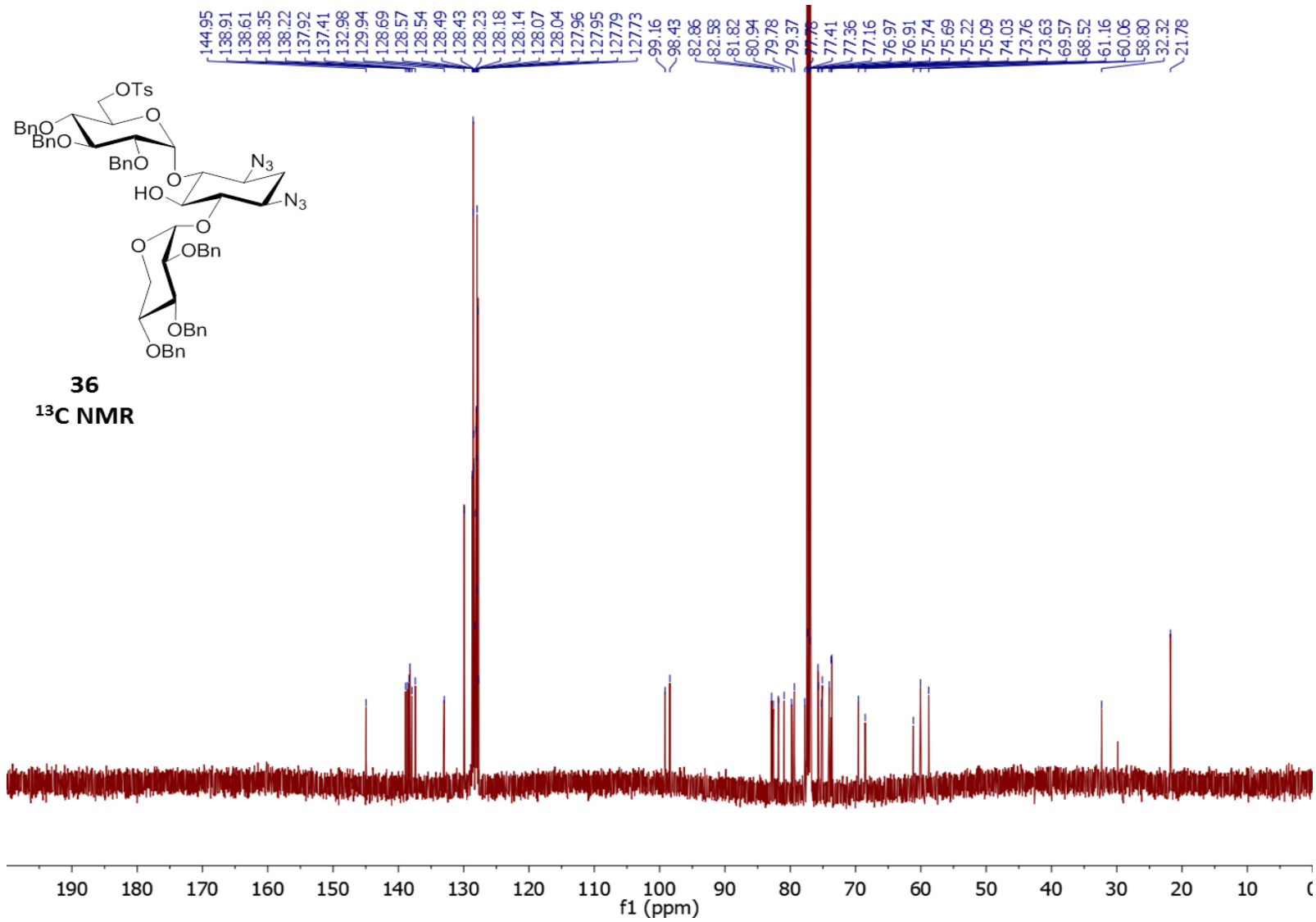

OH
O
BnO
BnO
BnO
O
N3
HO
N3
O
O
OBn
OBn
OBn

35

^{13}C NMR

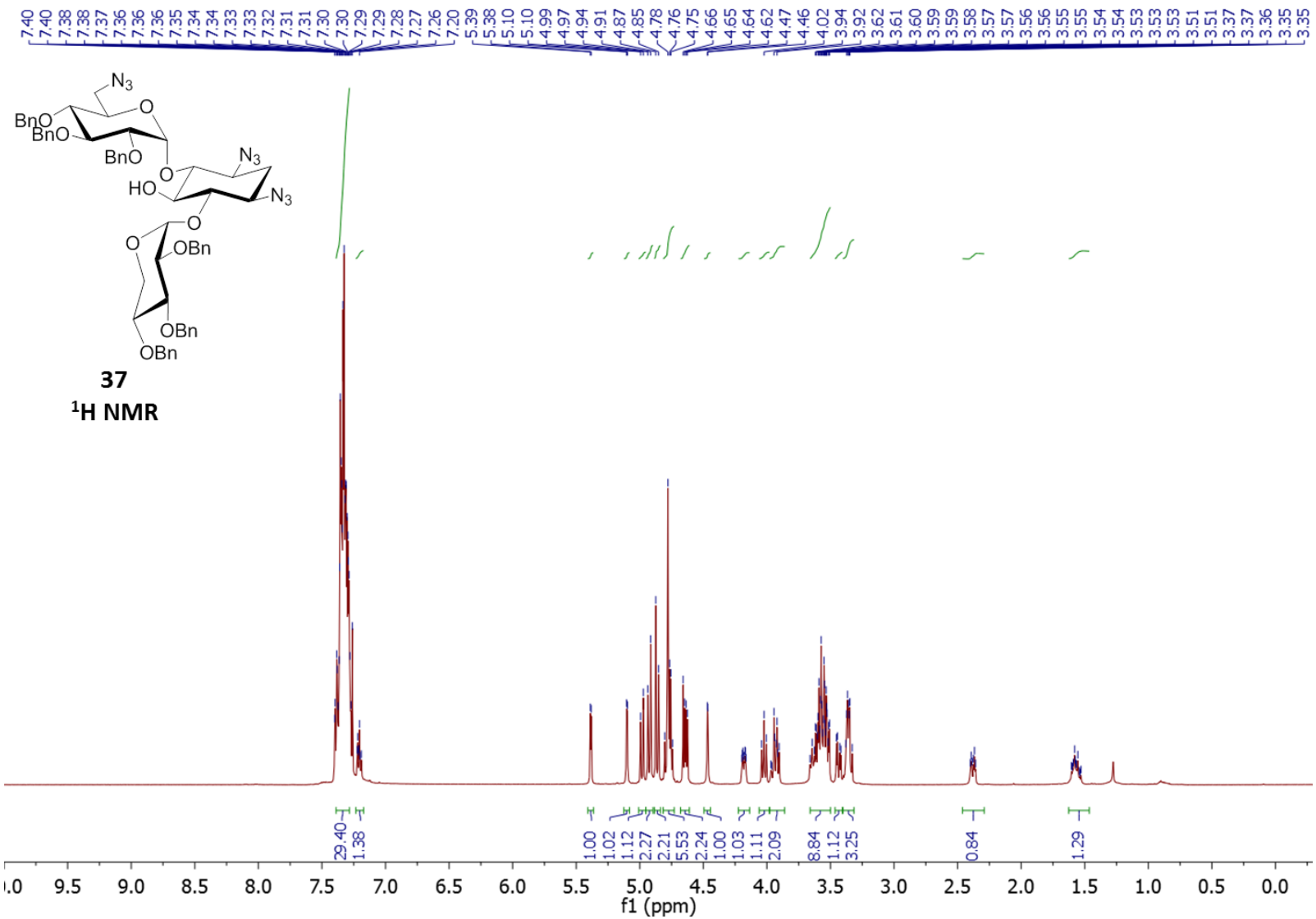
138.93
138.72
138.40
138.23
138.21
137.43
128.70
128.64
128.57
128.54
128.49
128.25
128.16
128.14
128.08
128.03
127.95
127.93
127.89
127.79
127.72
99.71
98.32
83.38
82.20
82.02
81.03
80.09
79.41
77.89
77.45
77.42
77.36
77.16
76.91
75.75
75.25
74.98
74.26
73.76
73.60
71.93
61.78
61.09
60.16
59.02
32.48
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

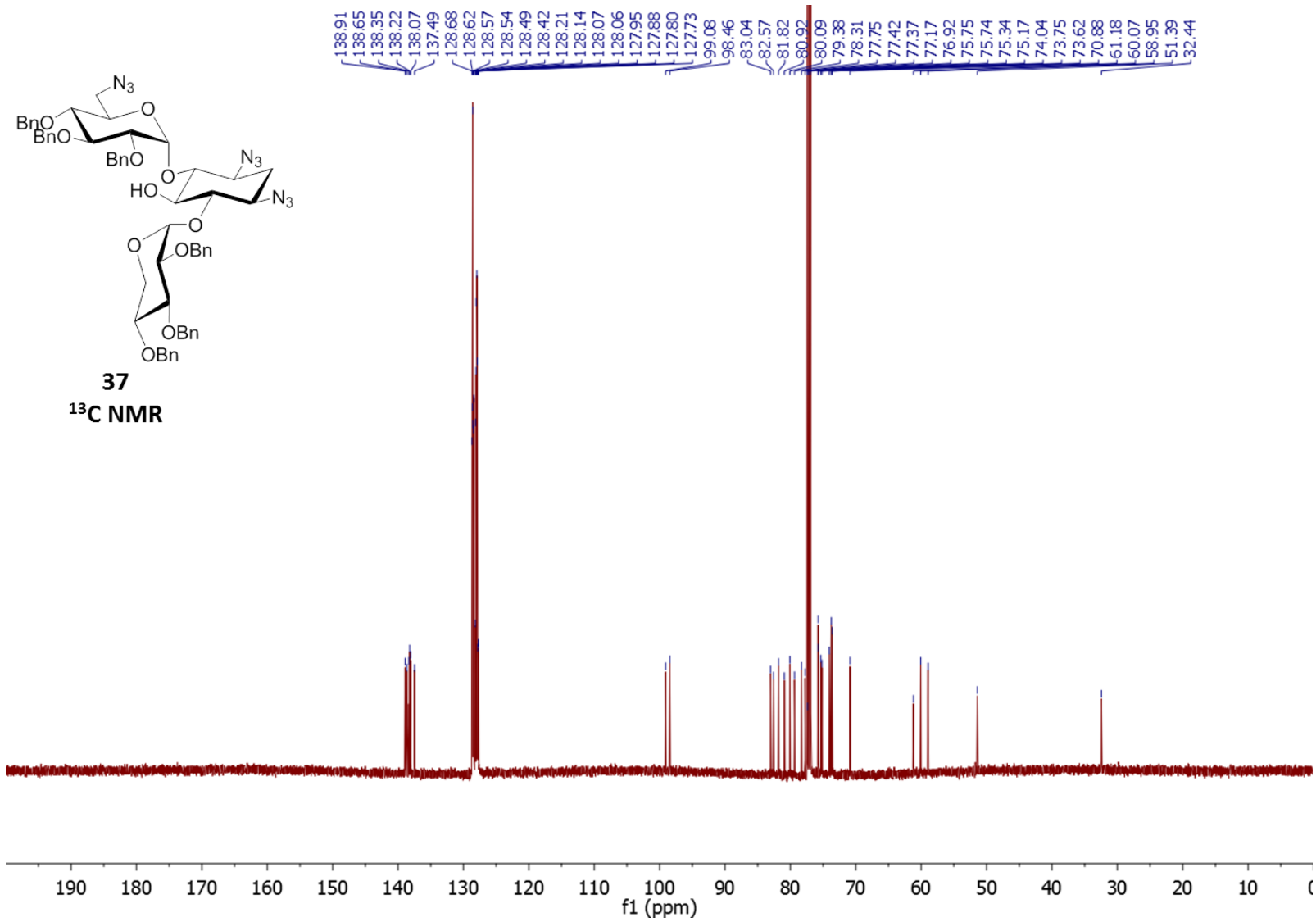

OTs
BnO
BnO
BnO
O
HO
O
N3
N3
O
O
OBn
OBn
OBn
36
1H NMR
f1 (ppm)


144.95
138.91
138.61
138.35
138.22
137.92
137.41
132.98
129.94
128.69
128.57
128.54
128.49
128.43
128.23
128.18
128.14
128.07
128.04
127.96
127.95
127.79
127.73
99.16
98.43
82.86
82.58
81.82
80.94
79.78
79.37
77.78
77.41
77.36
77.16
76.97
76.91
75.74
75.69
75.22
75.09
74.03
73.76
73.63
69.57
68.52
61.16
60.06
58.80
32.32
21.78
OTs
O
BnO
BnO
BnO
O
N3
HO
N3
O
O
OBn
OBn
OBn
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

36

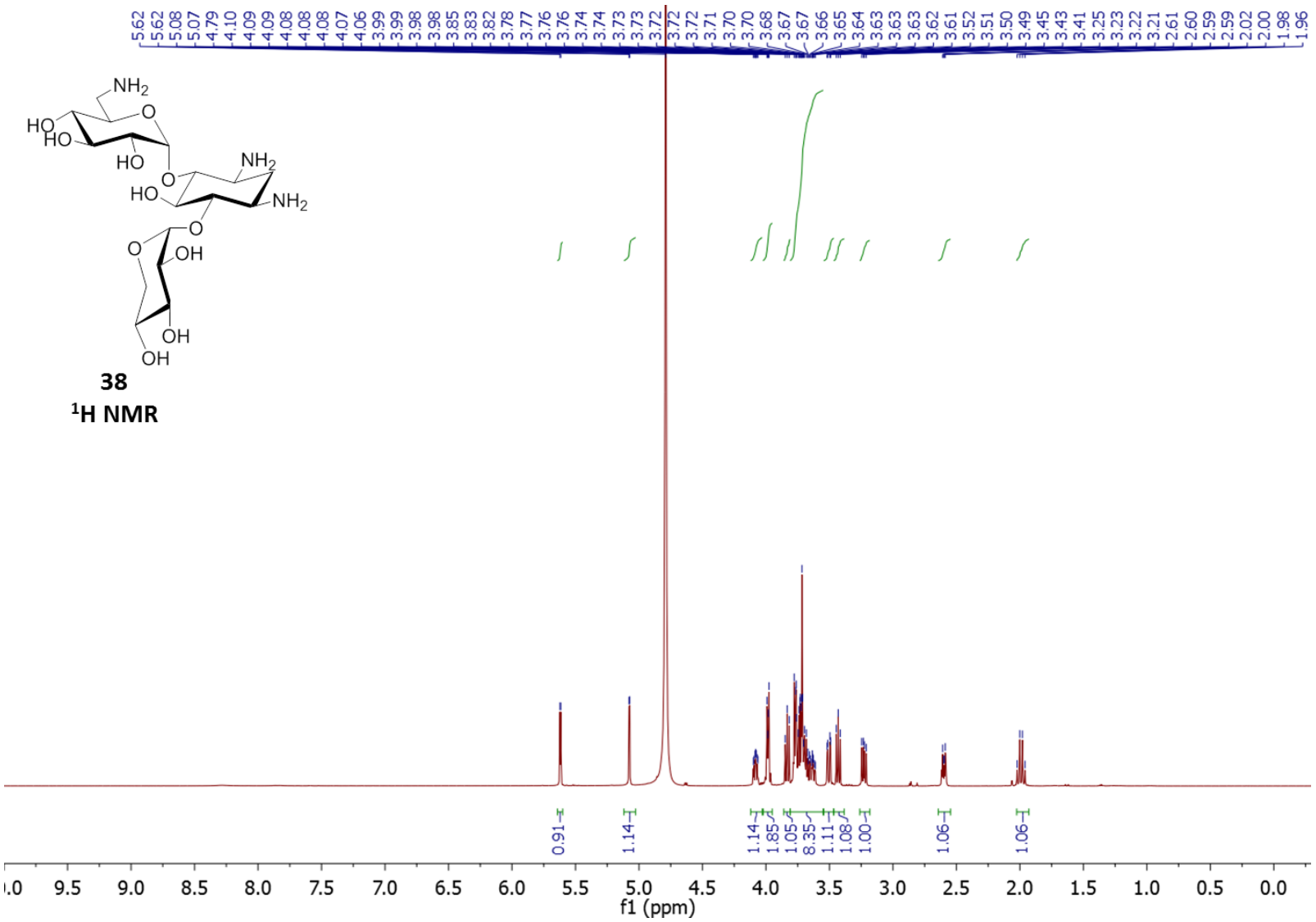
^{13}C NMR

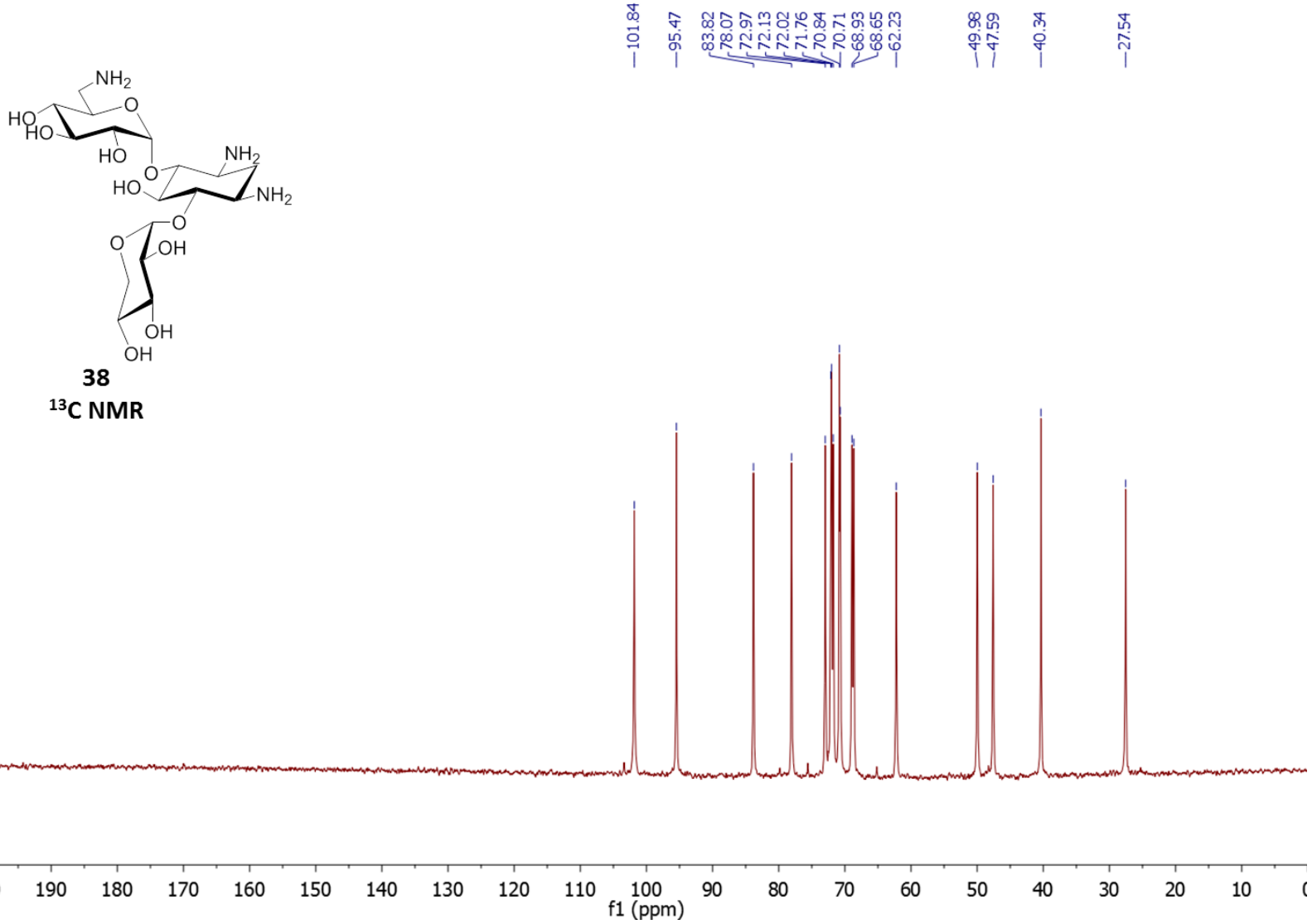

37
1H NMR
f1 (ppm)

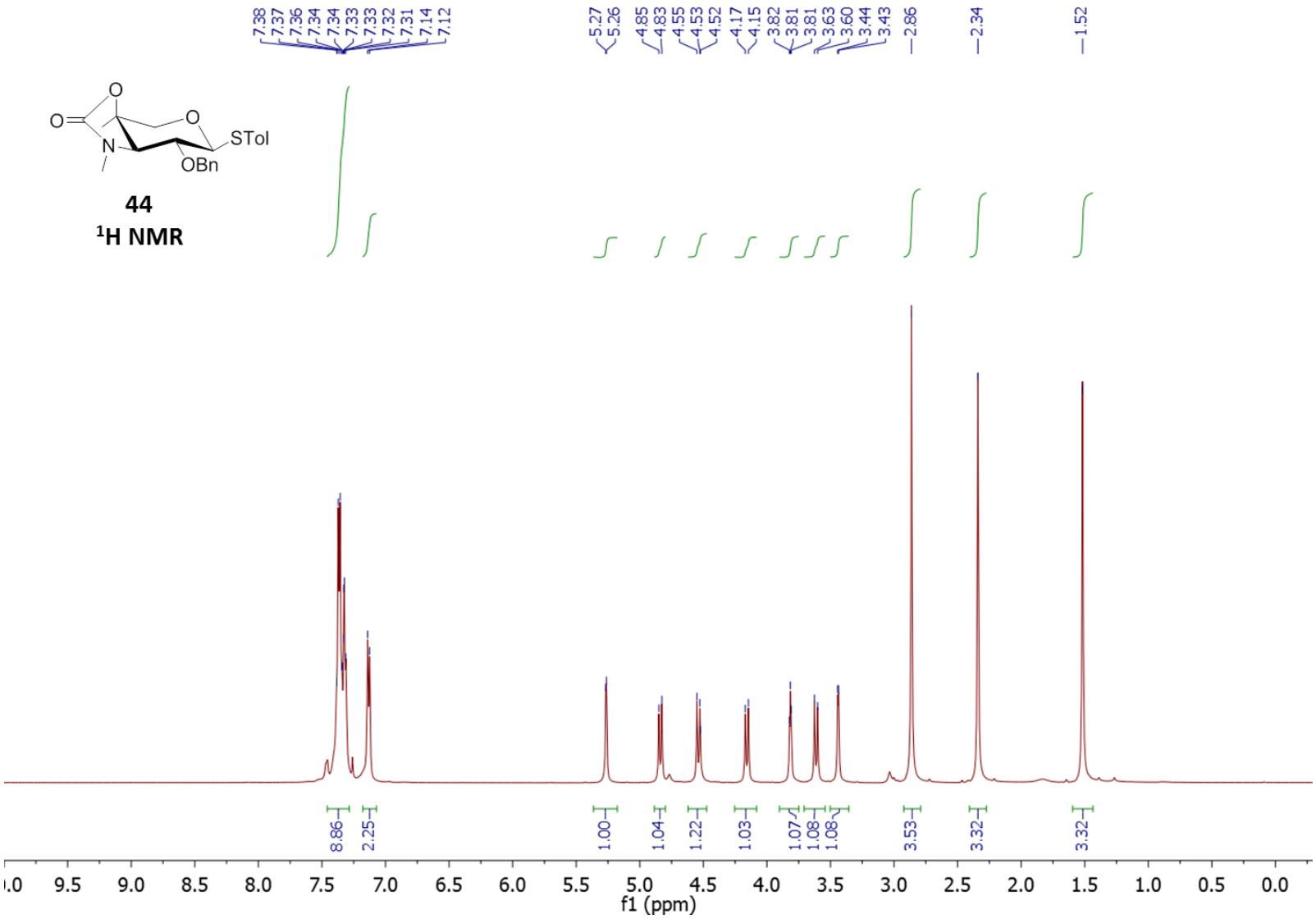

138.91
138.65
138.35
138.22
138.07
137.49
128.68
128.62
128.57
128.54
128.49
128.42
128.21
128.14
128.07
128.06
127.95
127.88
127.80
127.73
99.08
98.46
83.04
82.57
81.82
80.92
80.09
79.38
78.31
77.75
77.42
77.37
77.17
76.92
75.75
75.74
75.34
75.17
74.04
73.75
73.62
70.88
61.18
60.07
58.95
51.39
32.44
N3
BnO
BnO
BnO
O
HO
O
N3
N3
O
O
OBn
OBn
OBn
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

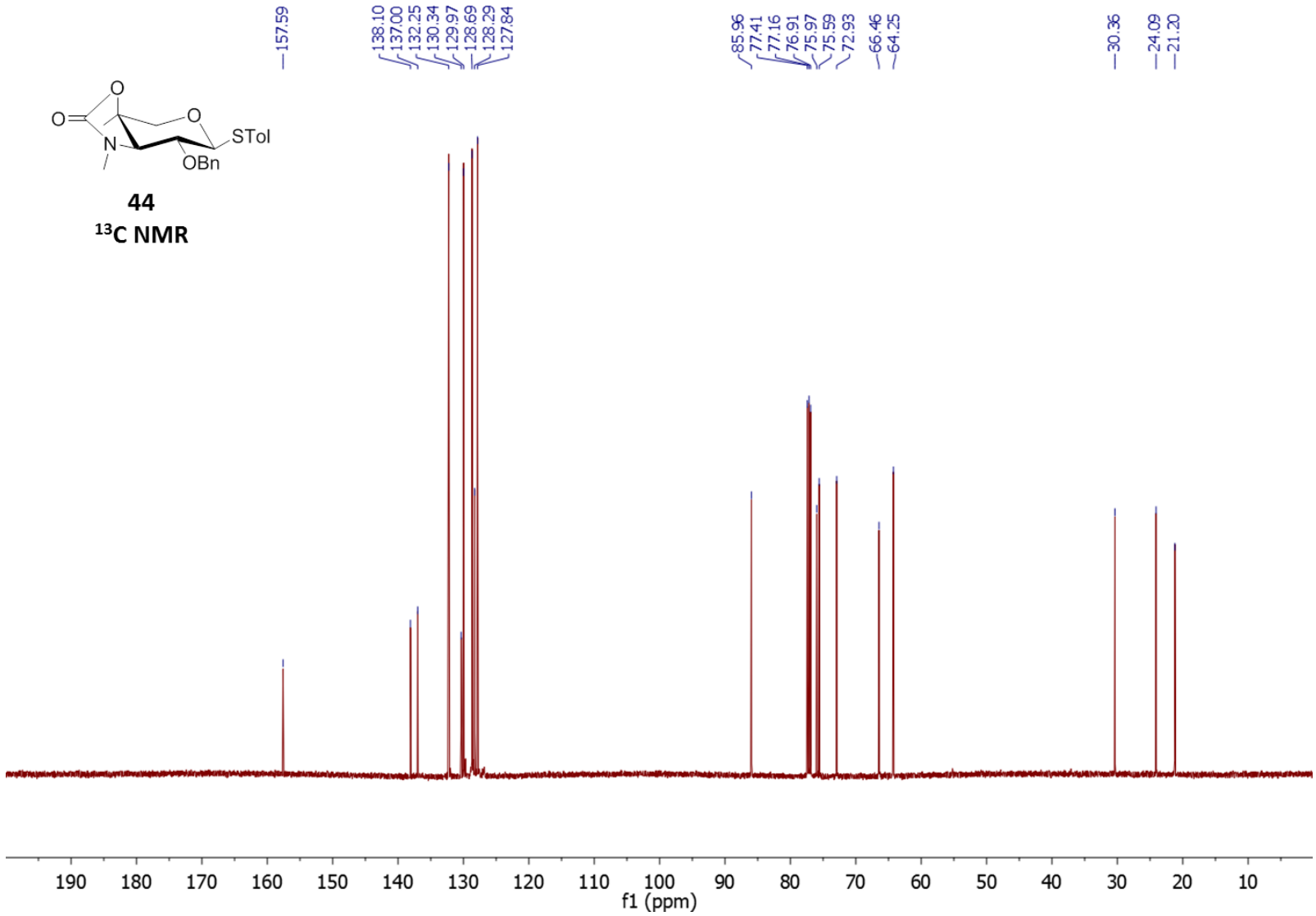
37

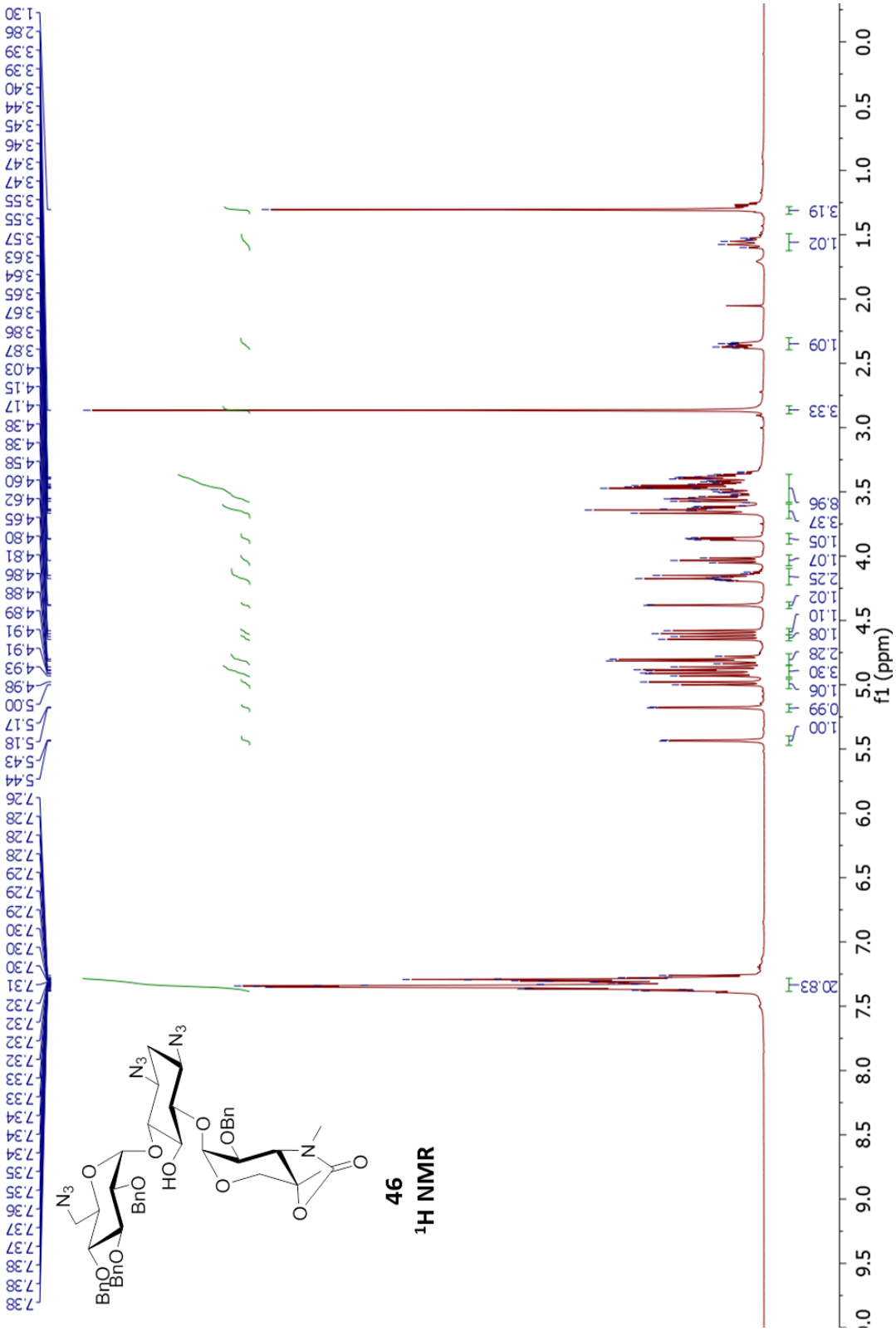
^{13}C NMR

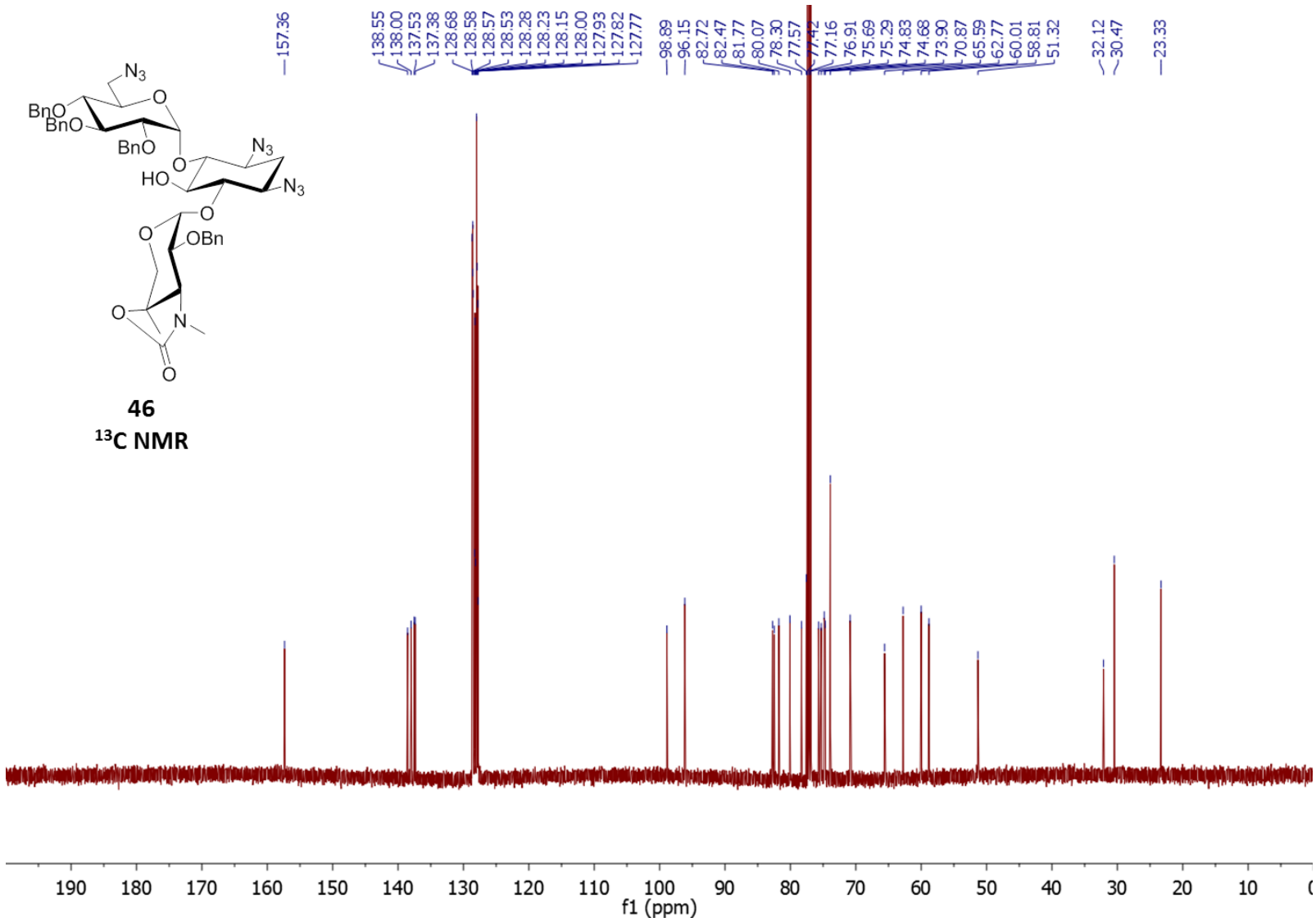

NH2
HO
HO
HO
O
NH2
HO
O
NH2
O
O
OH
OH
OH
38
1H NMR
f1 (ppm)


NH2
HO
HO
HO
O
O
HO
NH2
NH2
O
O
OH
OH
OH
38
13C NMR
101.84
95.47
83.82
78.07
72.97
72.13
72.02
71.76
70.84
70.71
68.93
68.65
62.23
49.98
47.59
40.34
27.54
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


7.38
7.37
7.36
7.34
7.34
7.33
7.33
7.32
7.31
7.14
7.12
5.27
5.26
4.85
4.83
4.55
4.53
4.52
4.17
4.15
3.82
3.81
3.81
3.63
3.60
3.44
3.43
2.86
2.34
1.52
O
O
O
N
STol
OBn
44
1H NMR
8.86
2.25
1.00
1.04
1.22
1.03
1.07
1.08
1.08
3.53
3.32
3.32
f1 (ppm)

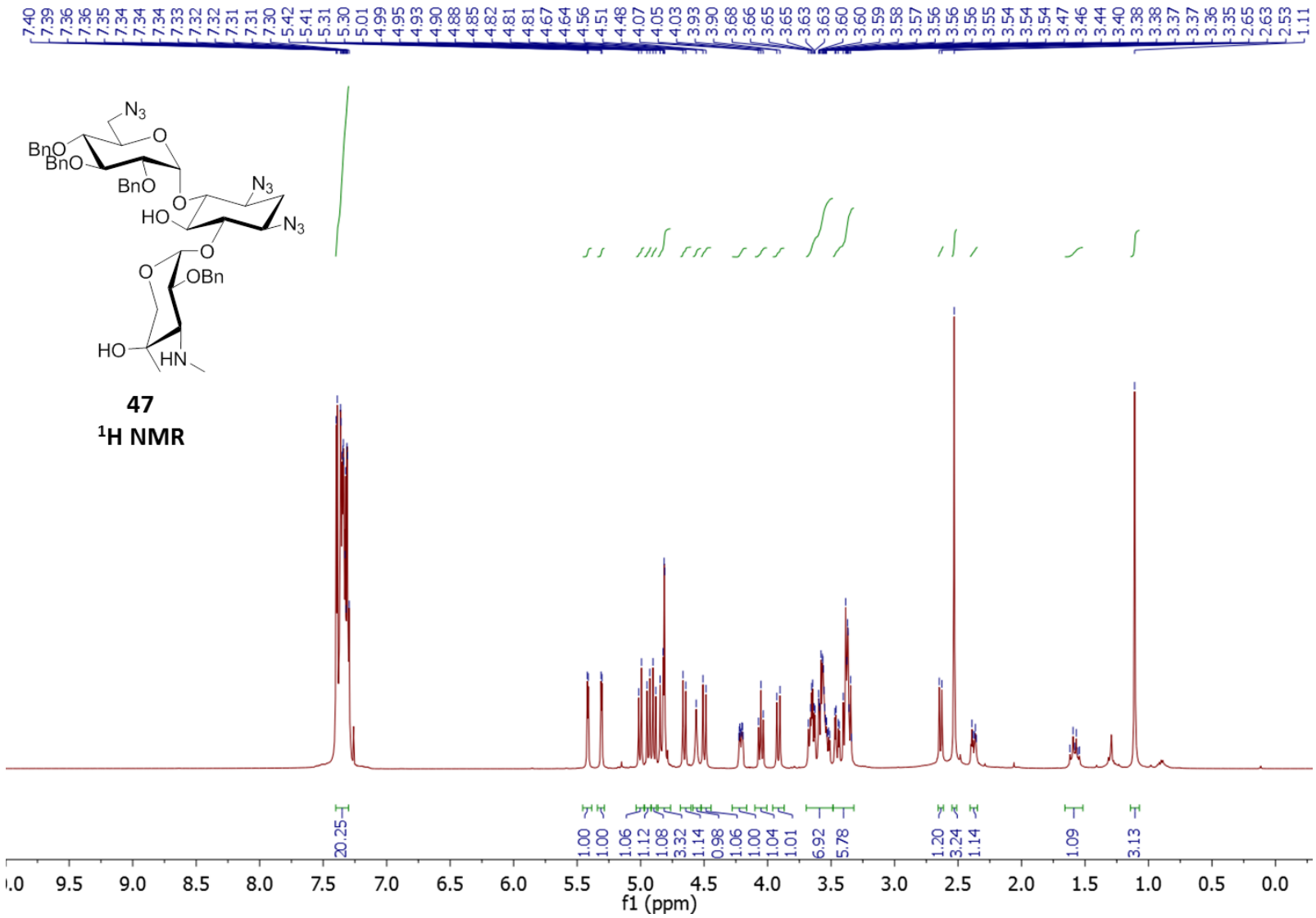

157.59
138.10
137.00
132.25
130.34
129.97
128.69
128.29
127.84
85.96
77.41
77.16
76.91
75.97
75.59
72.93
66.46
64.25
30.36
24.09
21.20
O
O
O
N
STol
OBn
44
13C NMR
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)


46
1H NMR
f1 (ppm)


157.36
138.55
138.00
137.53
137.38
128.68
128.58
128.57
128.53
128.28
128.23
128.15
128.00
127.93
127.82
127.77
98.89
96.15
82.72
82.47
81.77
80.07
78.30
77.57
77.42
77.16
76.91
75.69
75.29
74.83
74.68
73.90
70.87
65.59
62.77
60.01
58.81
51.32
32.12
30.47
23.33
N3
BnO
BnO
BnO
O
N3
HO
N3
O
O
OBn
O
N
O
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

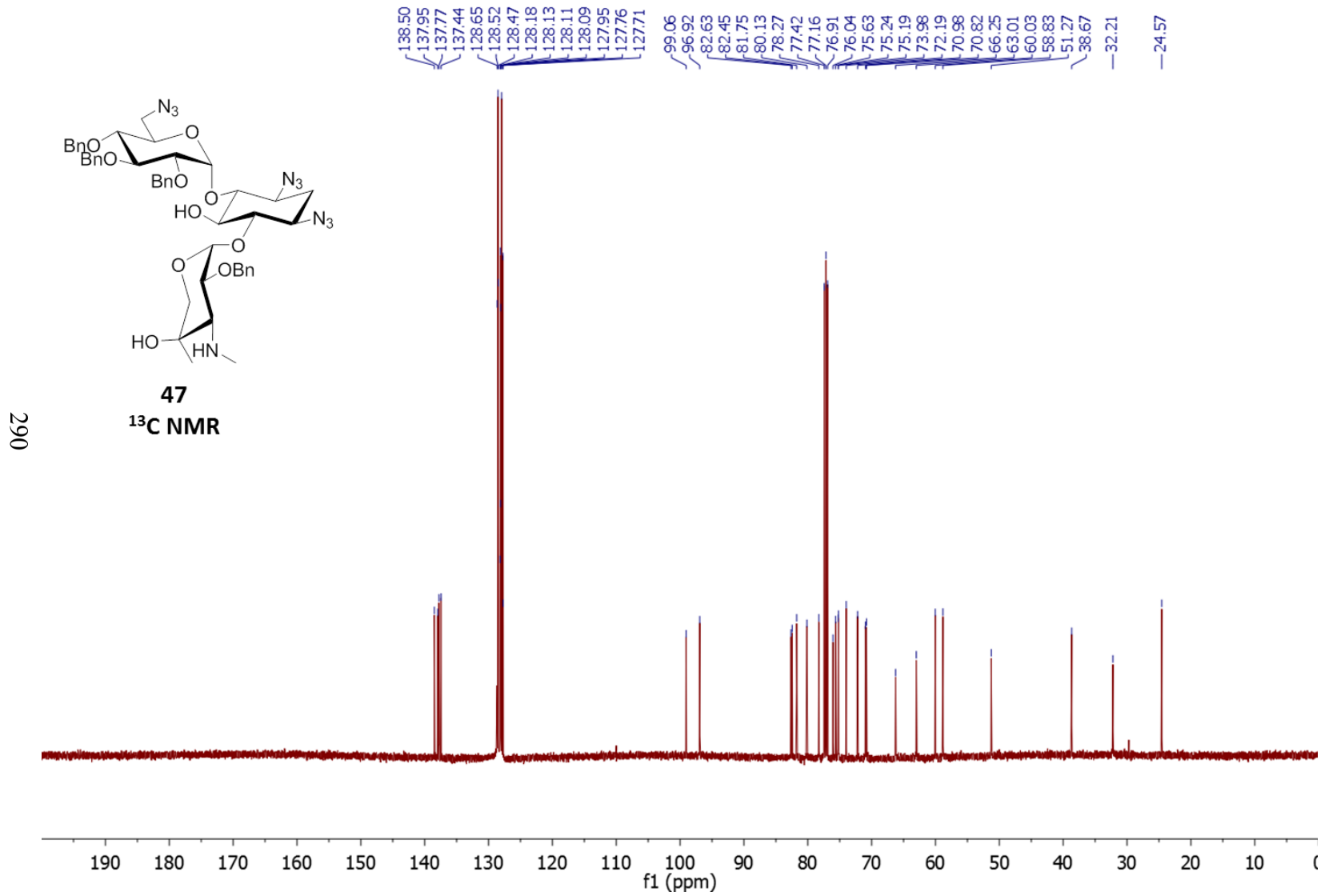
46

^{13}C NMR


7.40 7.39 7.36 7.36 7.35 7.34 7.34 7.34 7.33 7.32 7.32 7.31 7.31 7.30 5.42 5.41 5.31 5.30 5.01 4.99 4.95 4.93 4.90 4.88 4.85 4.82 4.81 4.81 4.67 4.64 4.56 4.51 4.48 4.07 4.05 4.03 3.93 3.90 3.68 3.66 3.65 3.65 3.63 3.63 3.60 3.60 3.59 3.58 3.57 3.56 3.56 3.56 3.55 3.54 3.54 3.54 3.47 3.46 3.44 3.40 3.38 3.38 3.37 3.37 3.36 3.35 2.65 2.63 2.53 1.11
N3
BnO
BnO
O
BnO
HO
O
N3
N3
O
O
OBn
HO
HN
20.25
1.00
1.00
1.06
1.12
1.08
3.32
1.14
0.98
1.06
1.00
1.04
1.01
6.92
5.78
1.20
3.24
1.14
1.09
3.13
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

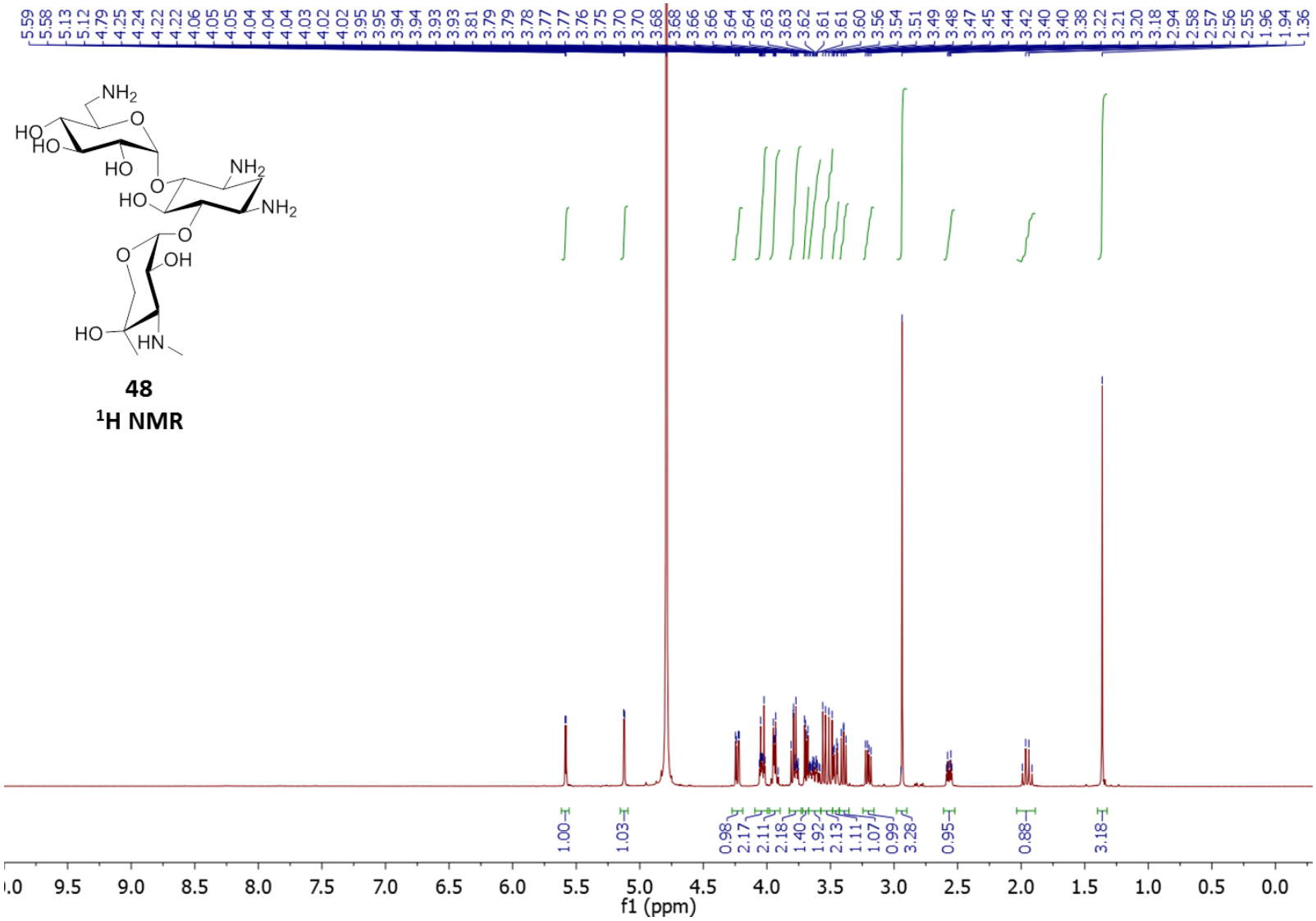
47

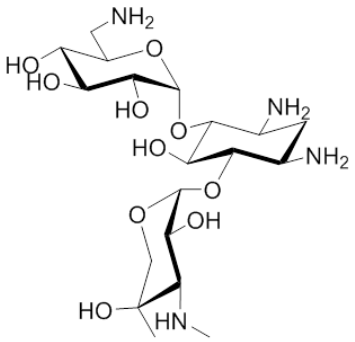
^{1}H NMR


138.50
137.95
137.77
137.44
128.65
128.52
128.47
128.18
128.13
128.11
128.09
127.95
127.76
127.71
99.06
96.92
82.63
82.45
81.75
80.13
78.27
77.42
77.16
76.91
76.04
75.63
75.24
75.19
73.98
72.19
70.98
70.82
66.25
63.01
60.03
58.83
51.27
38.67
32.21
24.57
N3
BnO
BnO
O
BnO
N3
HO
O
N3
O
O
OBn
HO
HN
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

47

^{13}C NMR

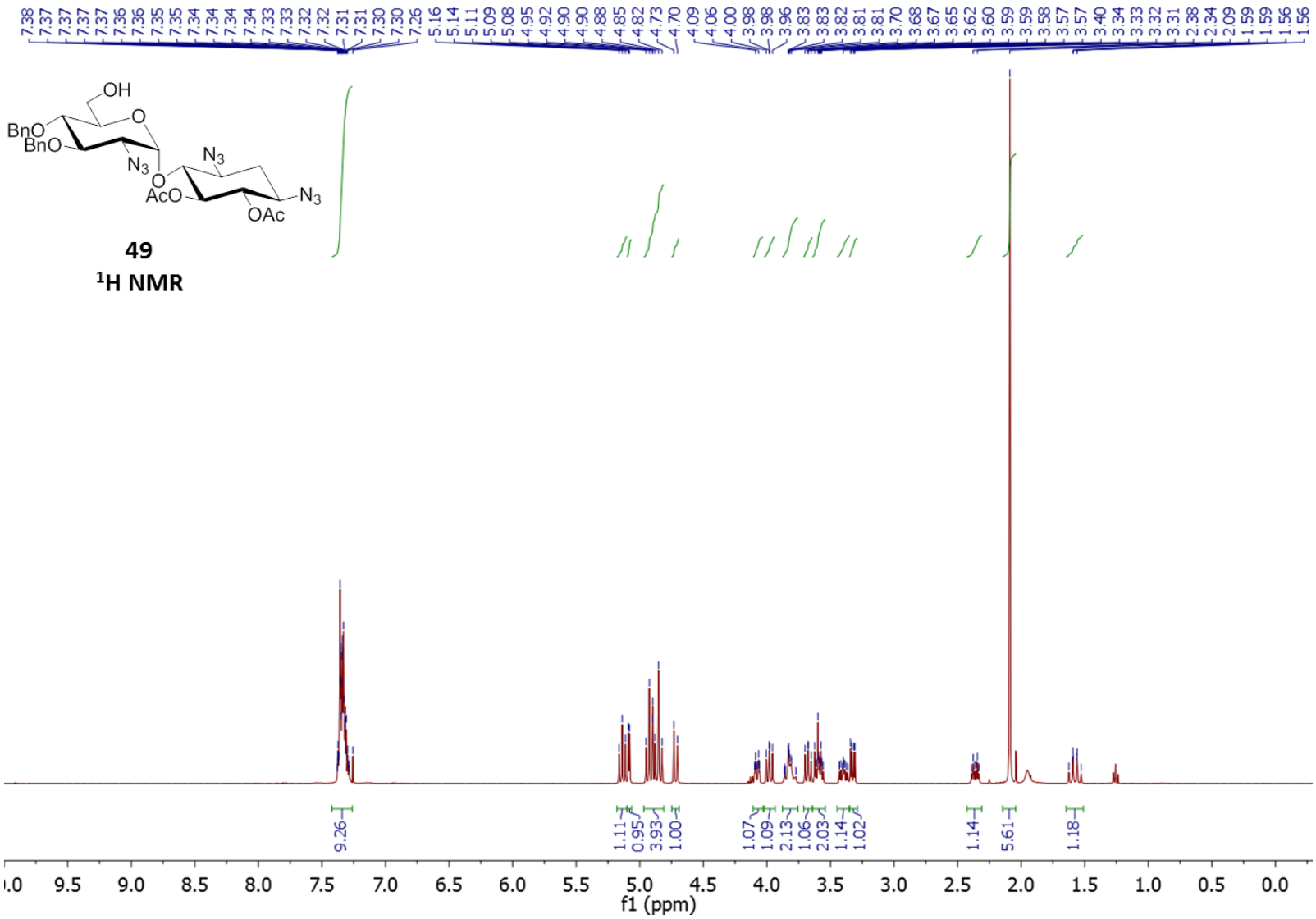

48
1H NMR
f1 (ppm)

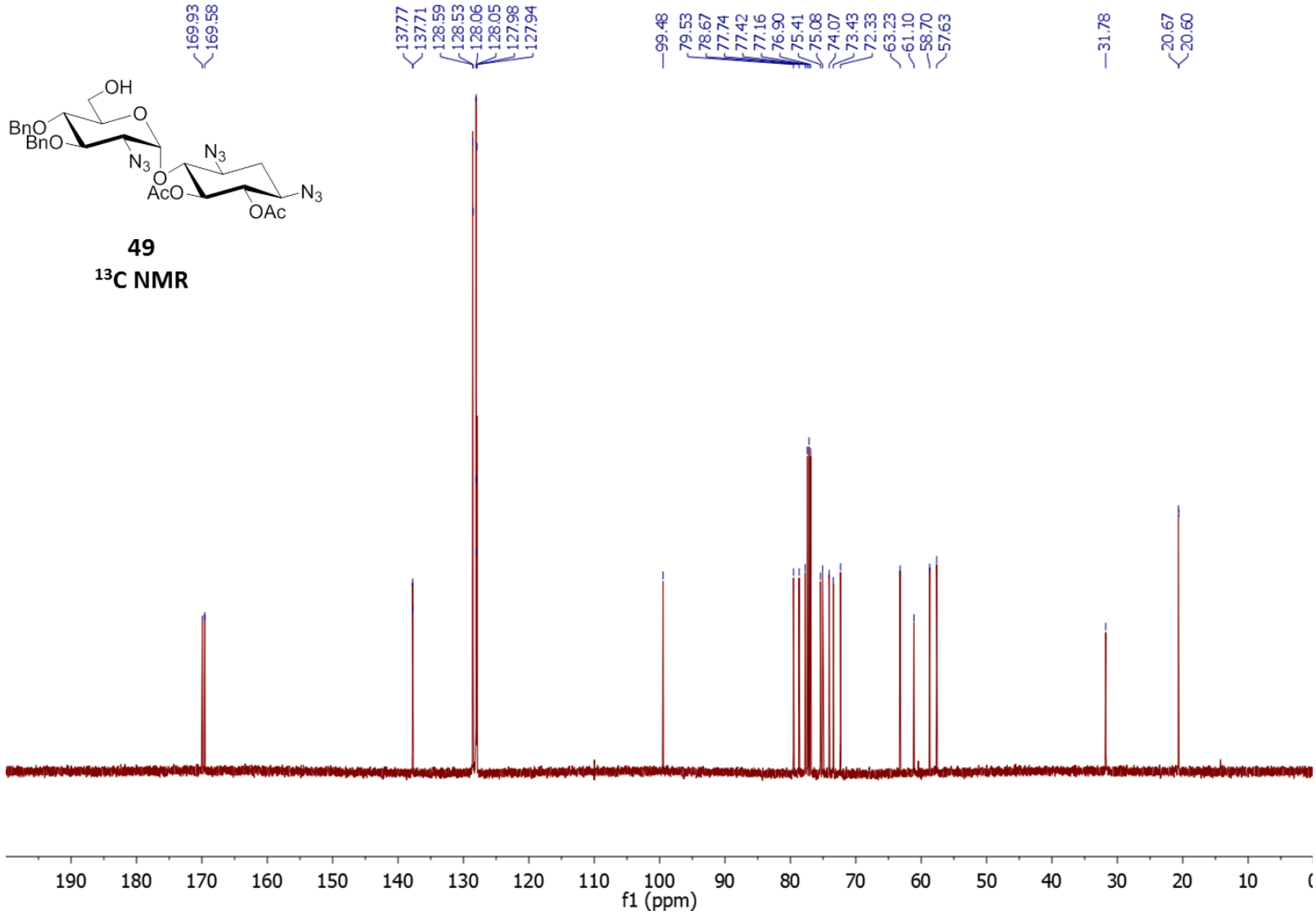

NH2
O
HO
HO
HO
O
NH2
HO
NH2
O
O
OH
HO
HN

48

^{13}C NMR

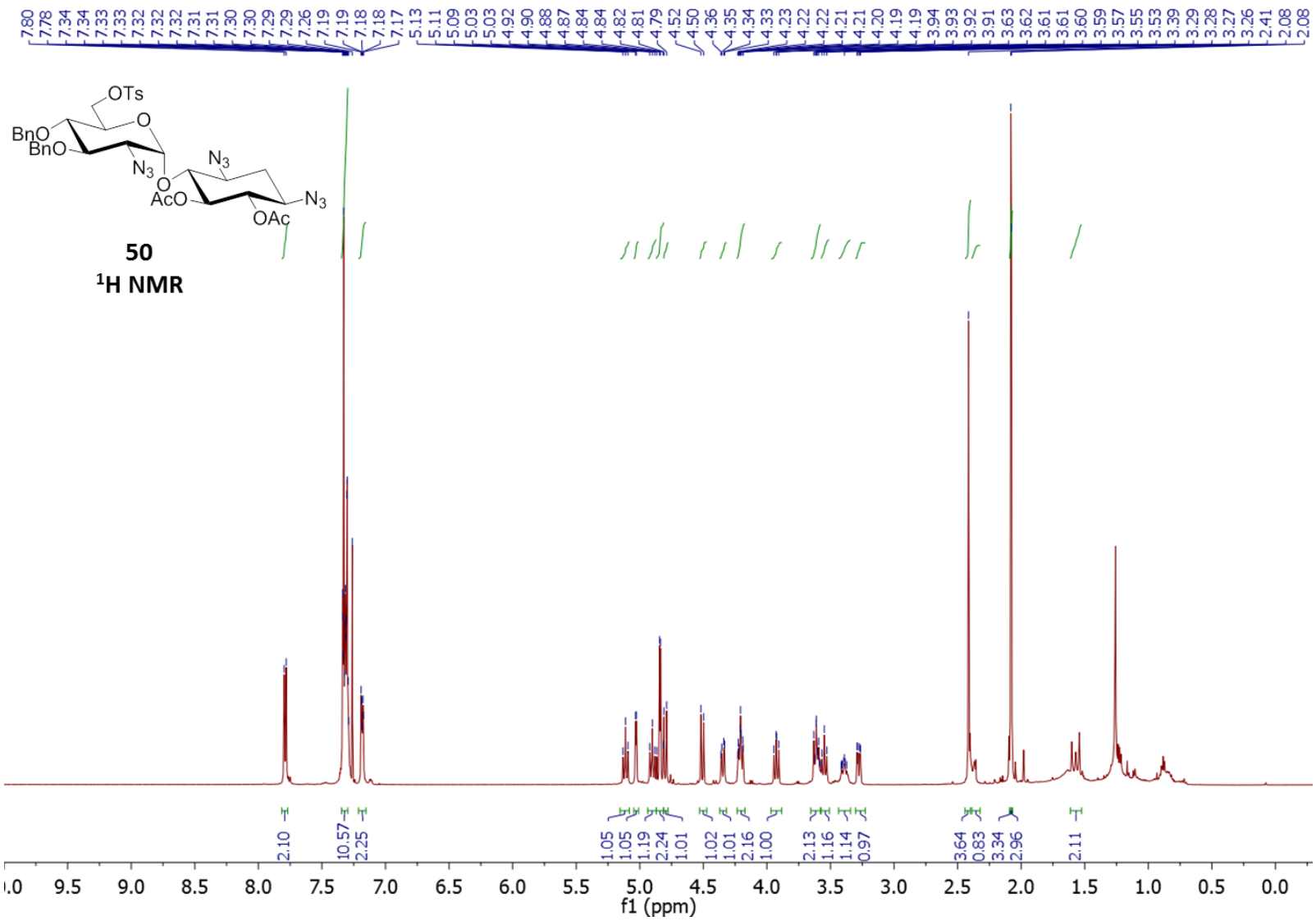
101.14
95.76
83.79
78.19
72.28
72.07
70.69
70.65
69.85
68.64
67.68
66.27
63.24
49.72
47.52
40.22
34.42
27.50
20.85
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

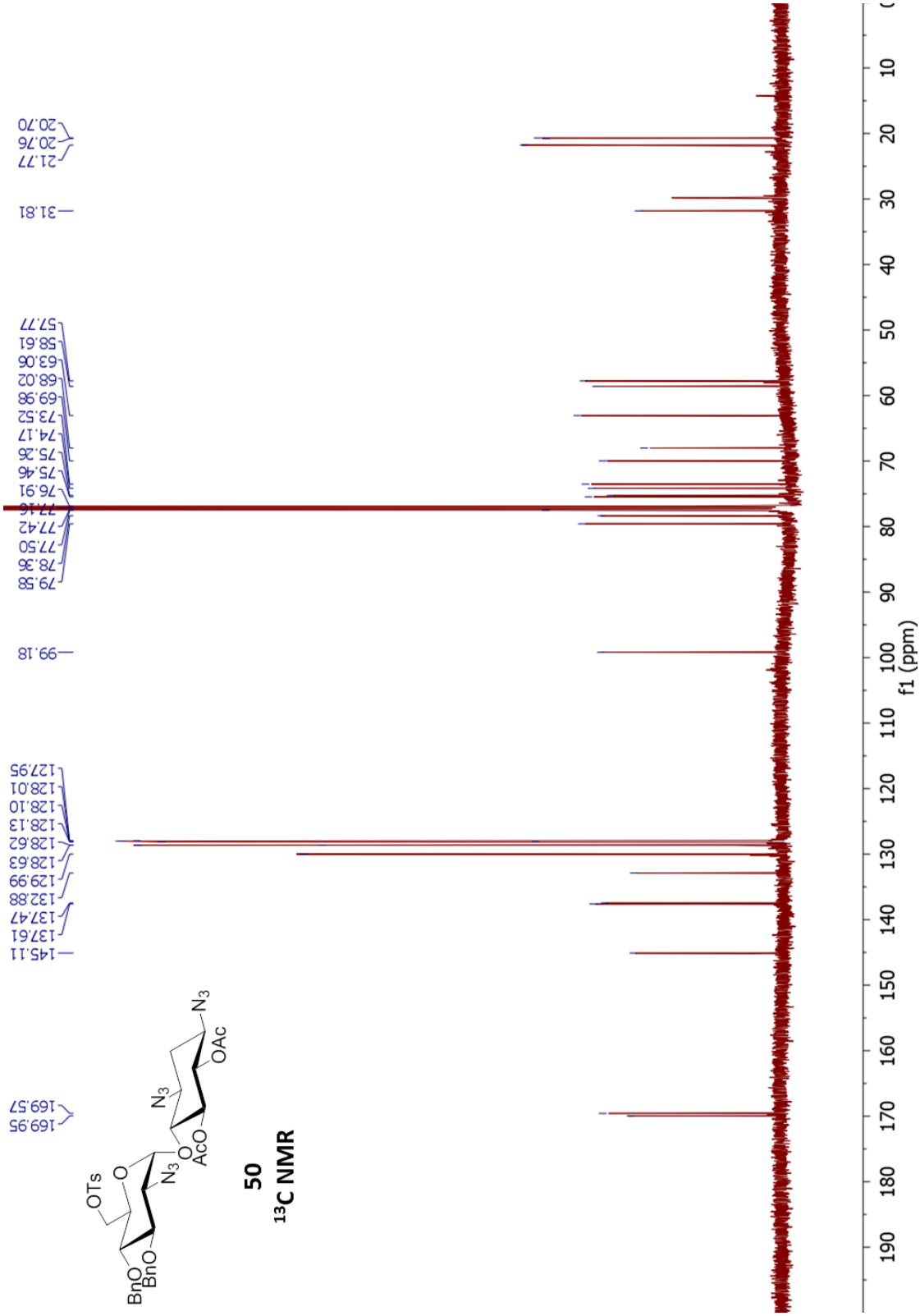

OH
O
BnO
BnO
N3
O
N3
AcO
N3
OAc
49
1H NMR
f1 (ppm)

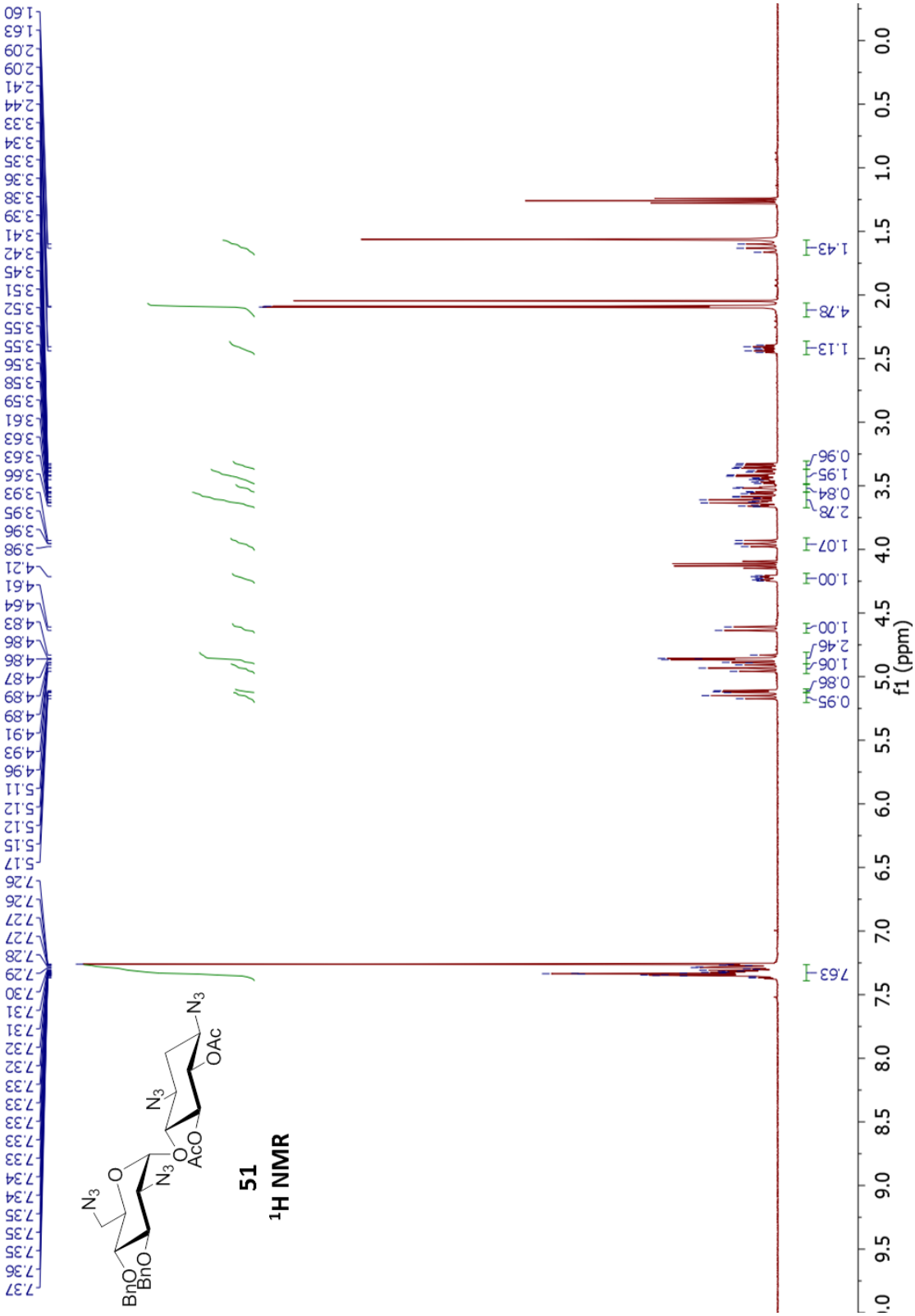

169.93
169.58
137.77
137.71
128.59
128.53
128.06
128.05
127.98
127.94
99.48
79.53
78.67
77.74
77.42
77.16
76.90
75.41
75.08
74.07
73.43
72.33
63.23
61.10
58.70
57.63
31.78
20.67
20.60
OH
O
BnO
BnO
N3
N3
O
AcO
N3
OAc
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

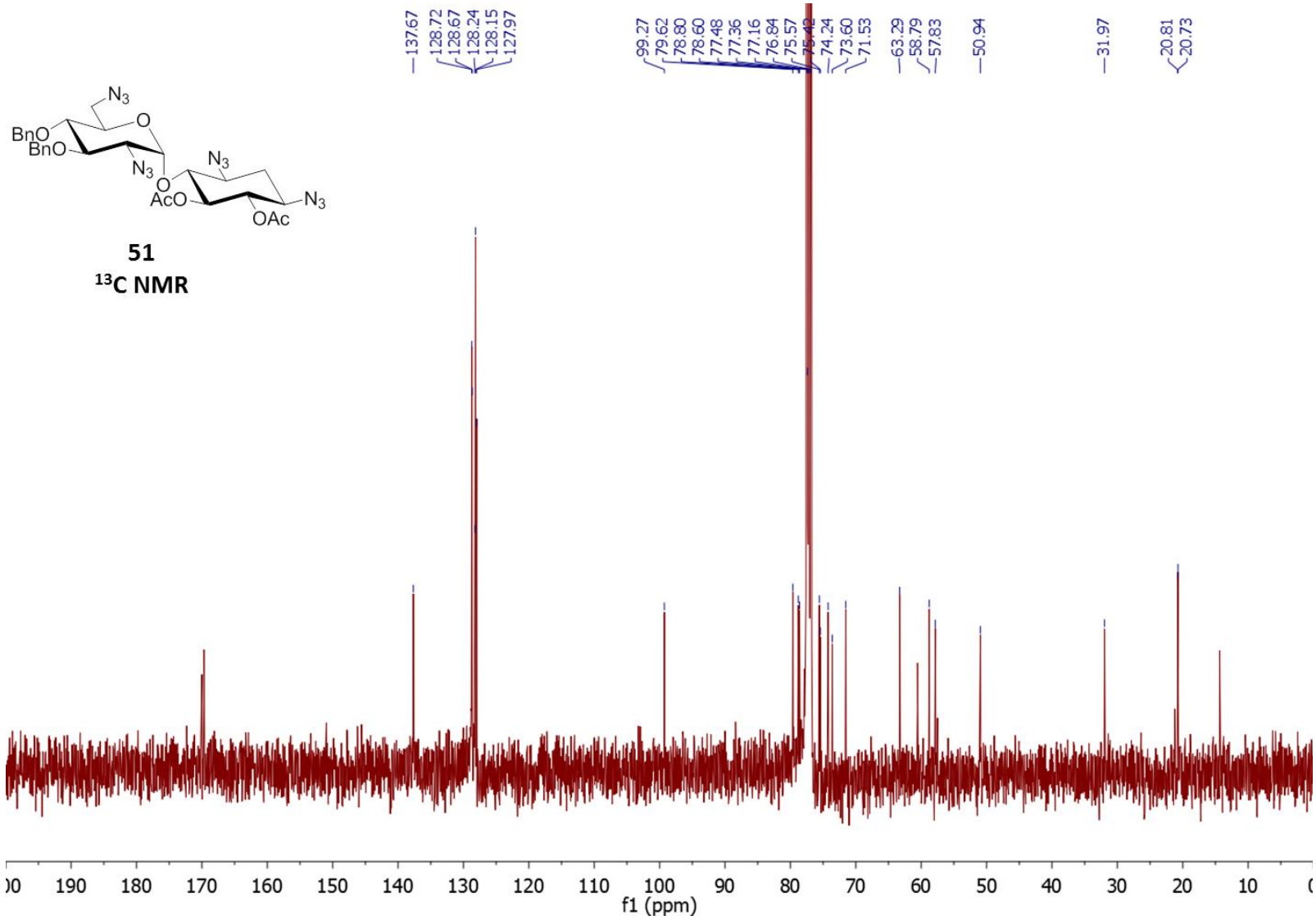
49

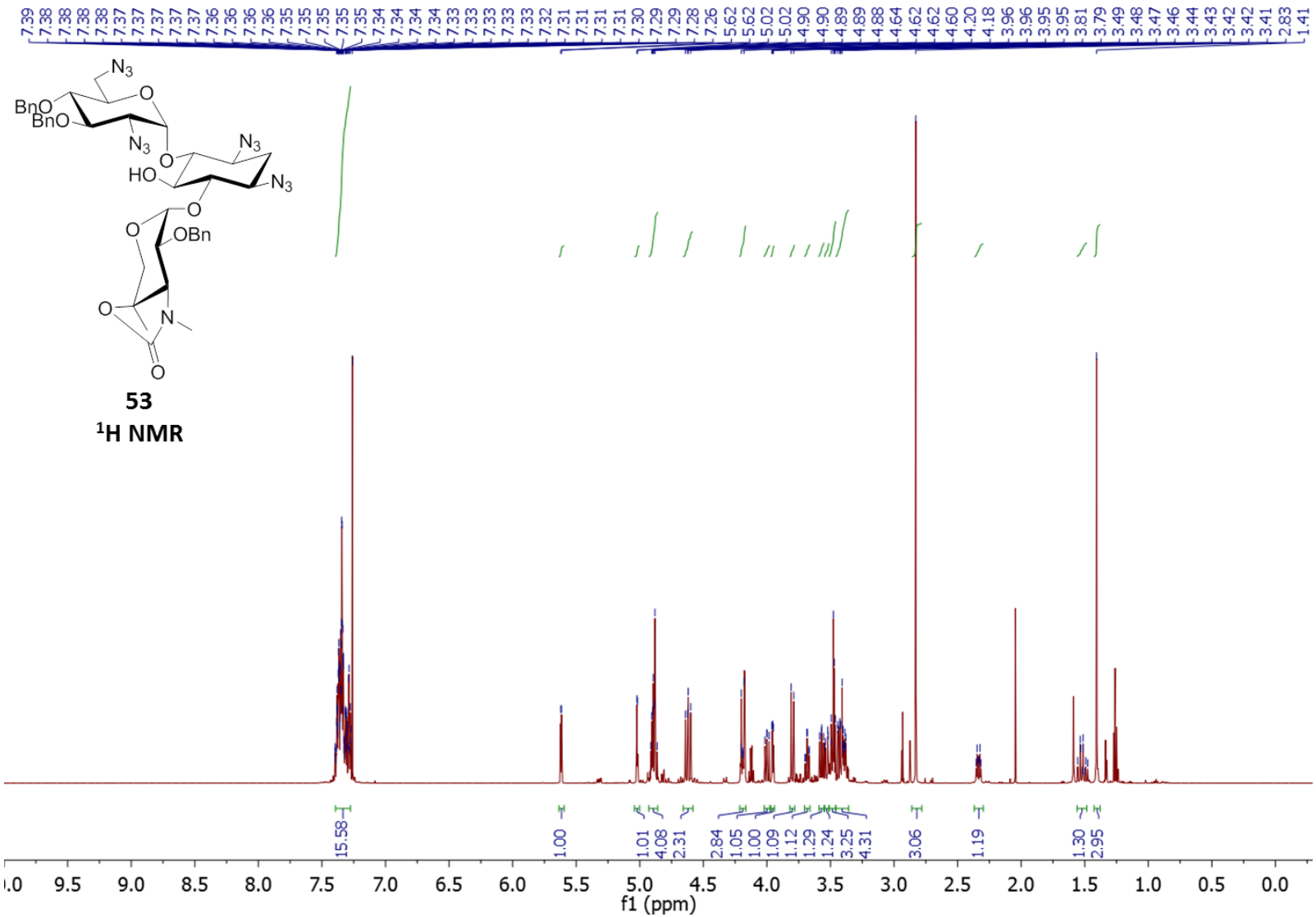
^{13}C NMR


OTs
O
BnO
BnO
N3
O
AcO
N3
N3
OAc
50
1H NMR
f1 (ppm)


169.95
169.57
145.11
137.61
137.47
132.88
129.99
128.63
128.62
128.13
128.10
128.01
127.95
99.18
79.58
78.36
77.50
77.42
77.16
76.91
75.46
75.26
74.17
73.52
69.98
68.02
63.06
58.61
57.77
31.81
21.77
20.76
20.70
f1 (ppm)
50
13C NMR

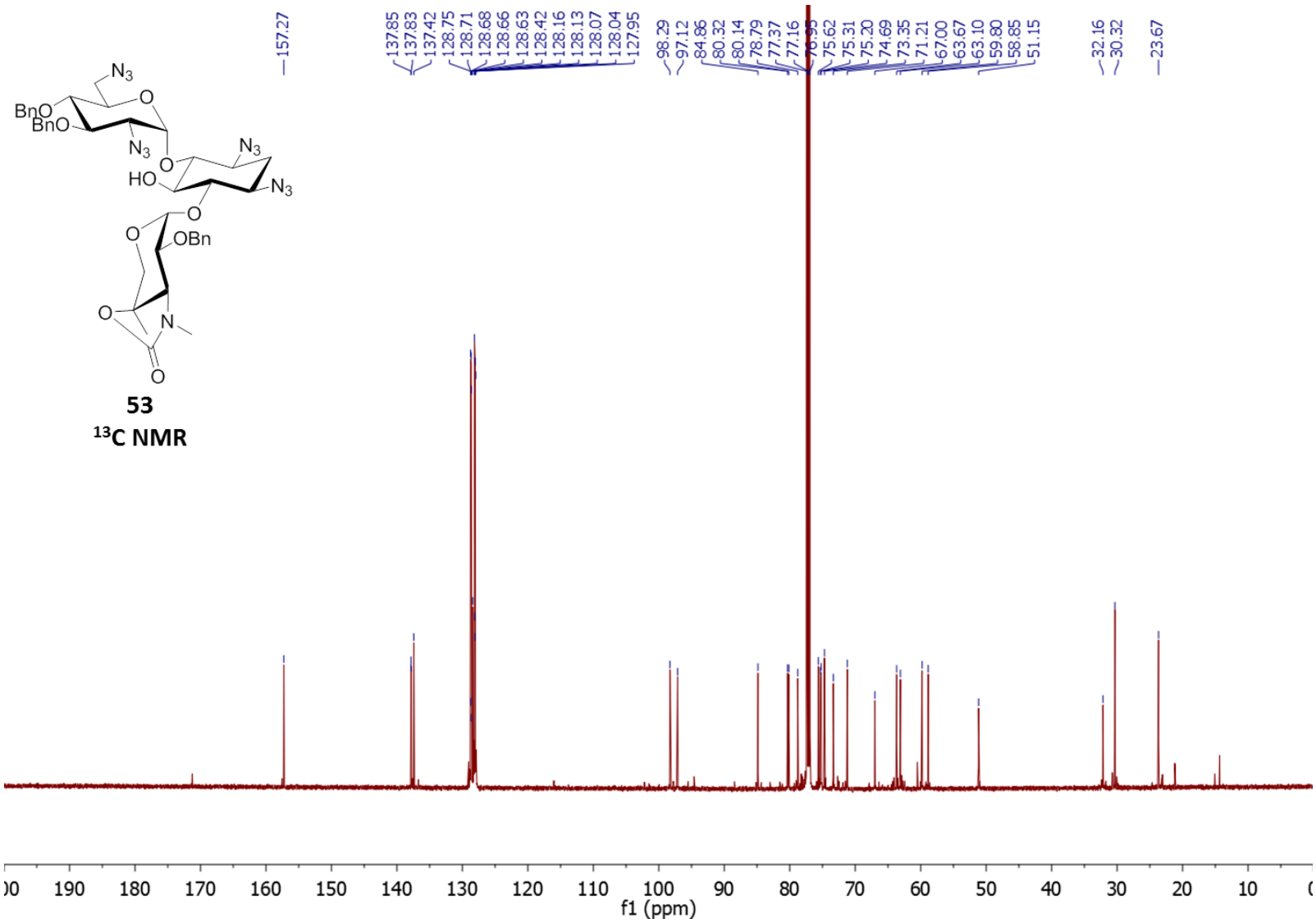

51
^{1}H NMR
f1 (ppm)


137.67
128.72
128.67
128.24
128.15
127.97
99.27
79.62
78.80
78.60
77.48
77.36
77.16
76.84
75.57
74.24
73.60
71.53
63.29
58.79
57.83
50.94
31.97
20.81
20.73
BnO
BnO
N3
O
N3
N3
O
AcO
N3
OAc
51
13C NMR
f1 (ppm)



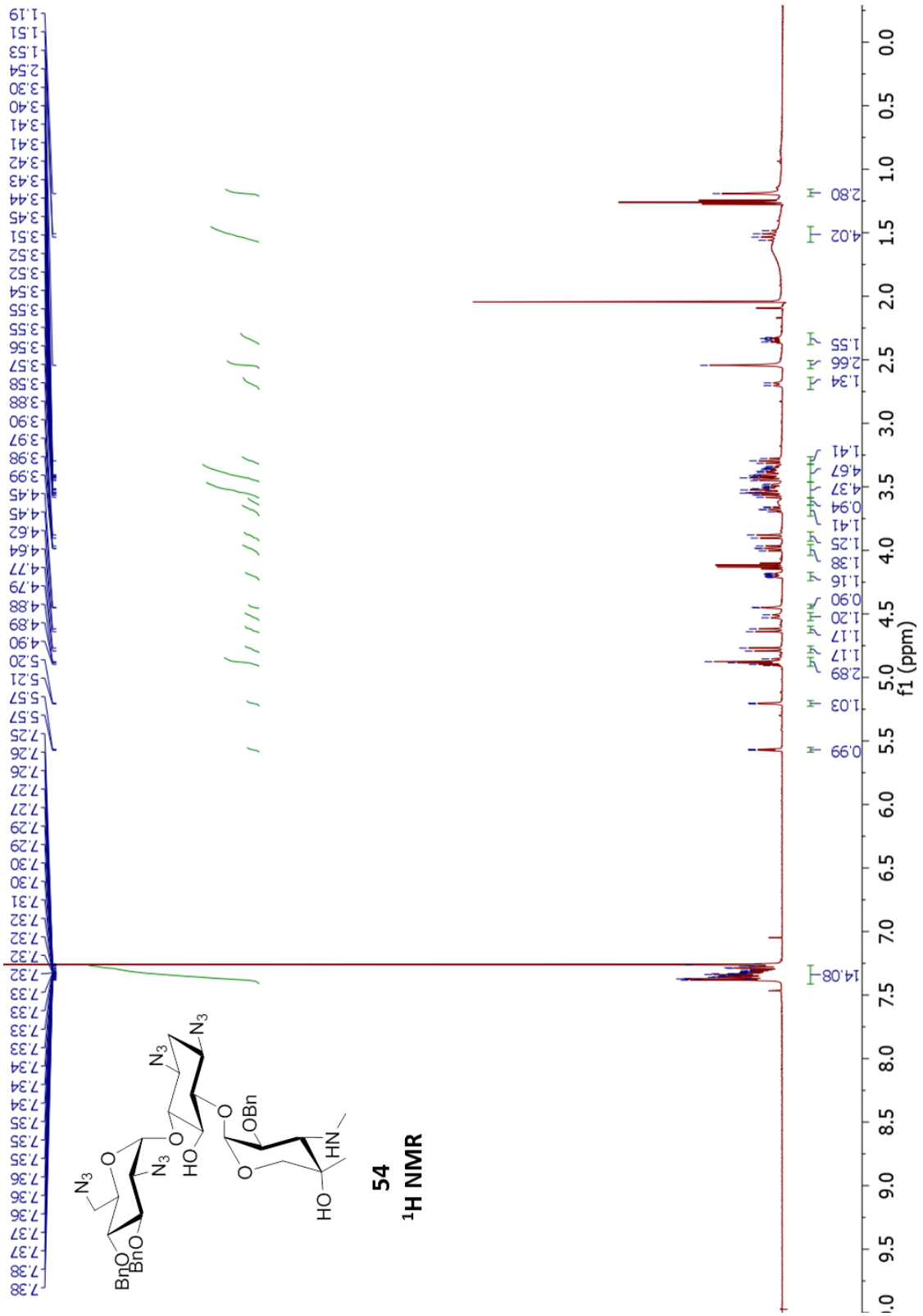
53

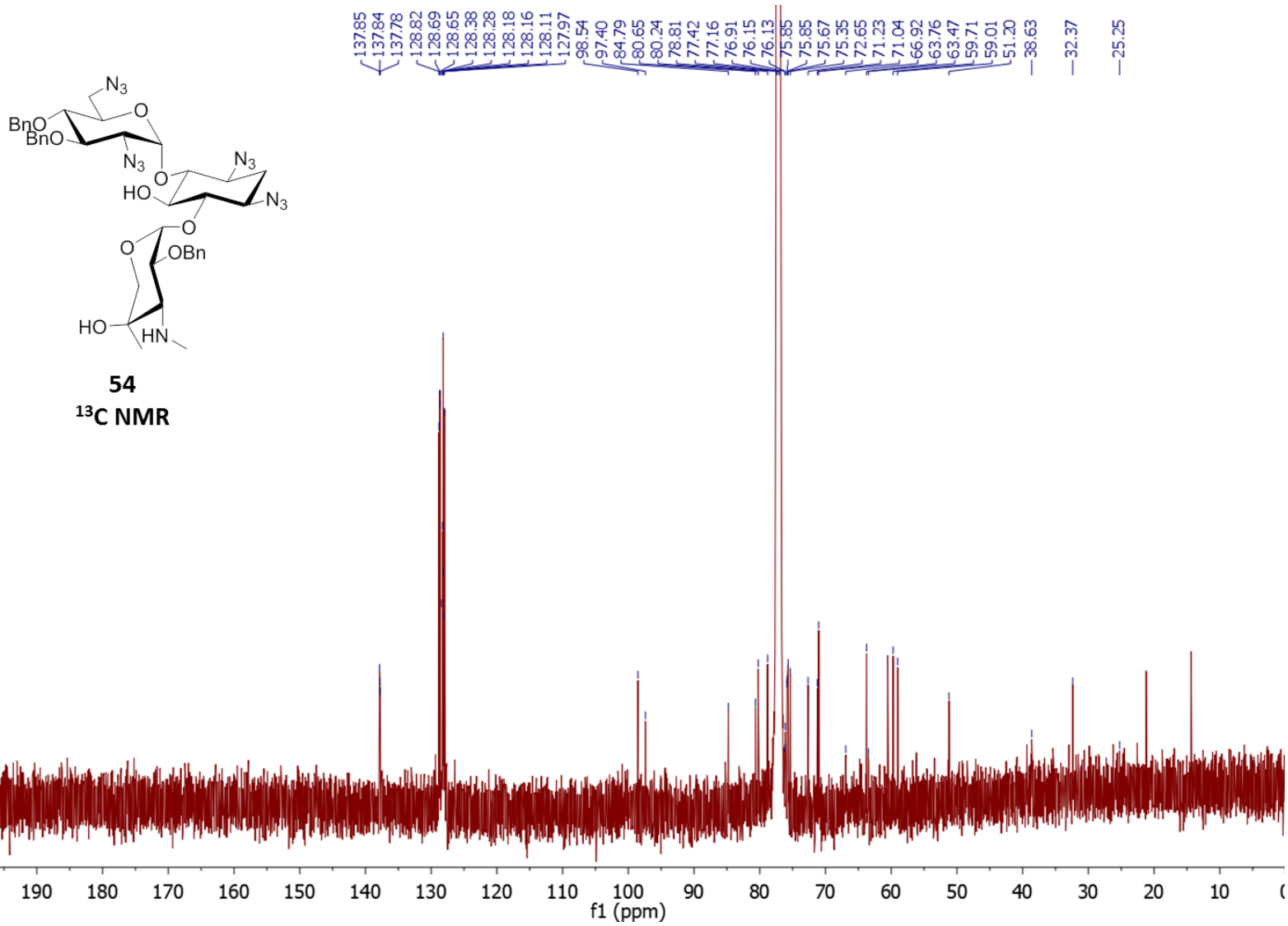
^{1}H NMR


157.27
137.85
137.83
137.42
128.75
128.71
128.68
128.66
128.63
128.42
128.16
128.13
128.07
128.04
127.95
98.29
97.12
84.86
80.32
80.14
78.79
77.37
77.16
76.95
75.62
75.31
75.20
74.69
73.35
71.21
67.00
63.67
63.10
59.80
58.85
51.15
32.16
30.32
23.67
f1 (ppm)

53

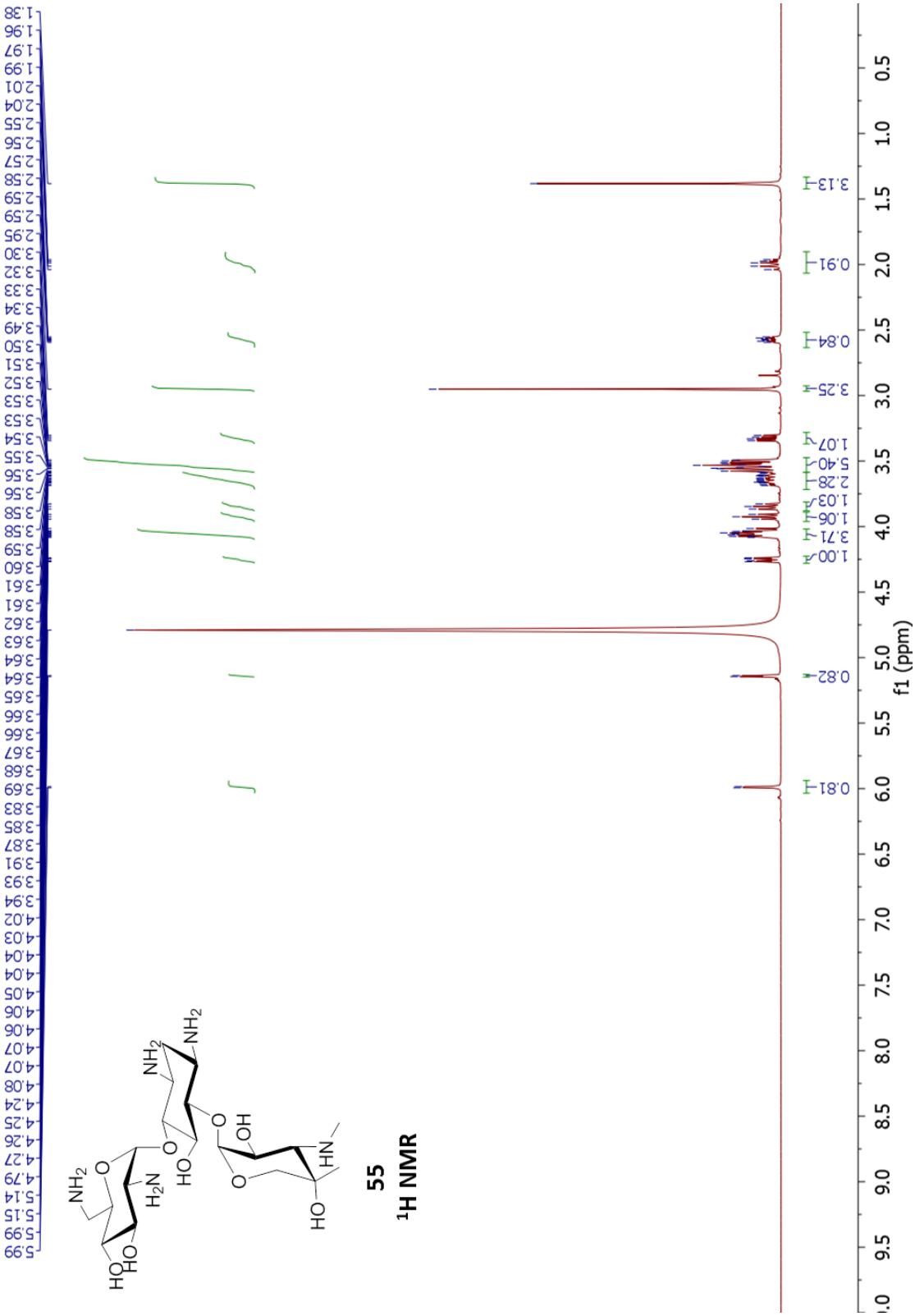
^{13}C NMR

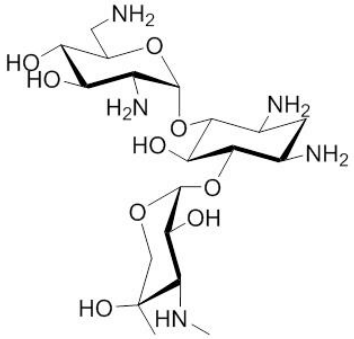

54
^{1}H NMR
f1 (ppm)


137.85
137.84
137.78
128.82
128.69
128.65
128.38
128.28
128.18
128.16
128.11
127.97
98.54
97.40
84.79
80.65
80.24
78.81
77.42
77.16
76.91
76.15
76.13
75.85
75.85
75.67
75.35
72.65
71.23
71.04
66.92
63.76
63.47
59.71
59.01
51.20
38.63
32.37
25.25
f1 (ppm)

54

^{13}C NMR

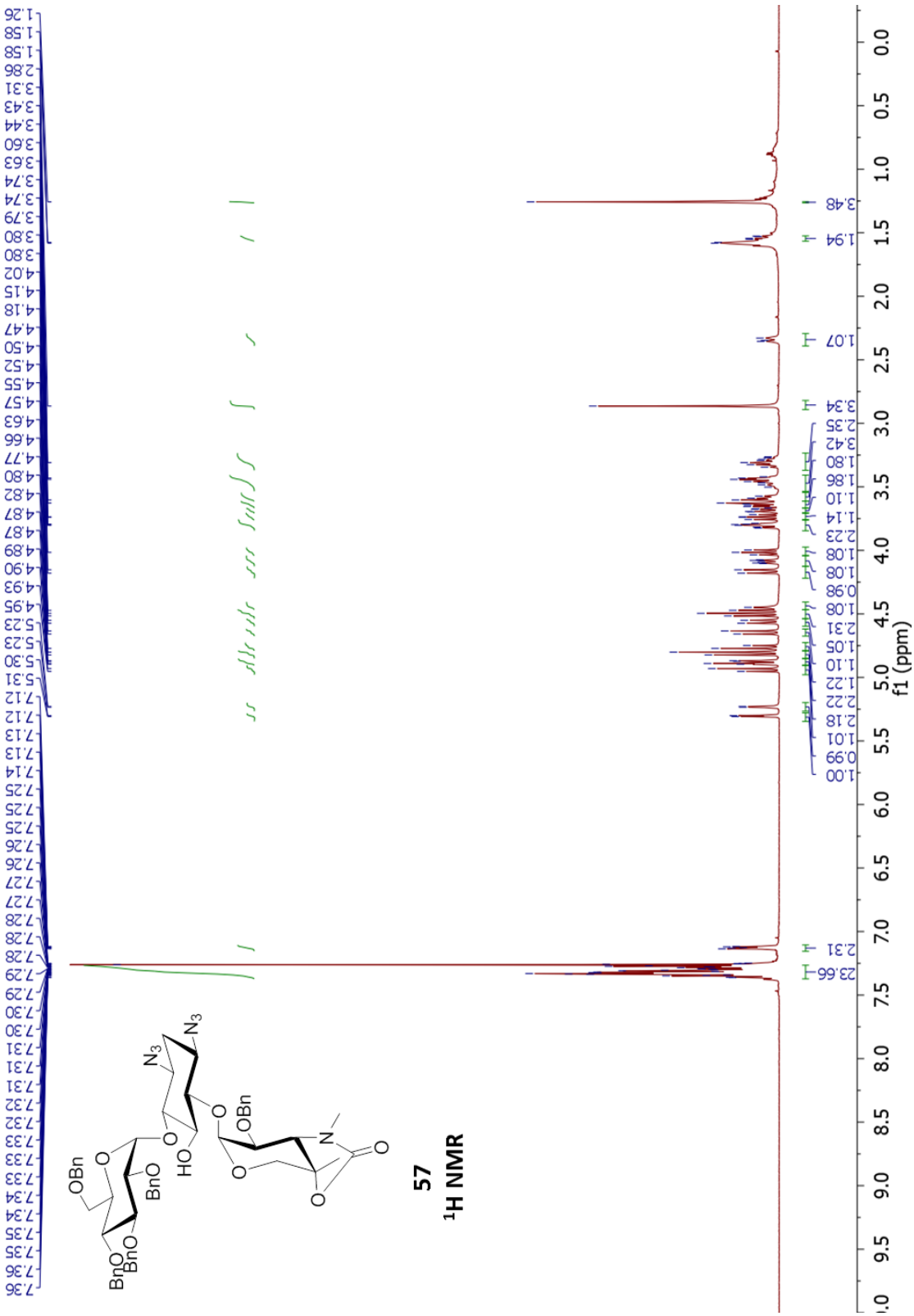

55
^{1}H NMR
f1 (ppm)

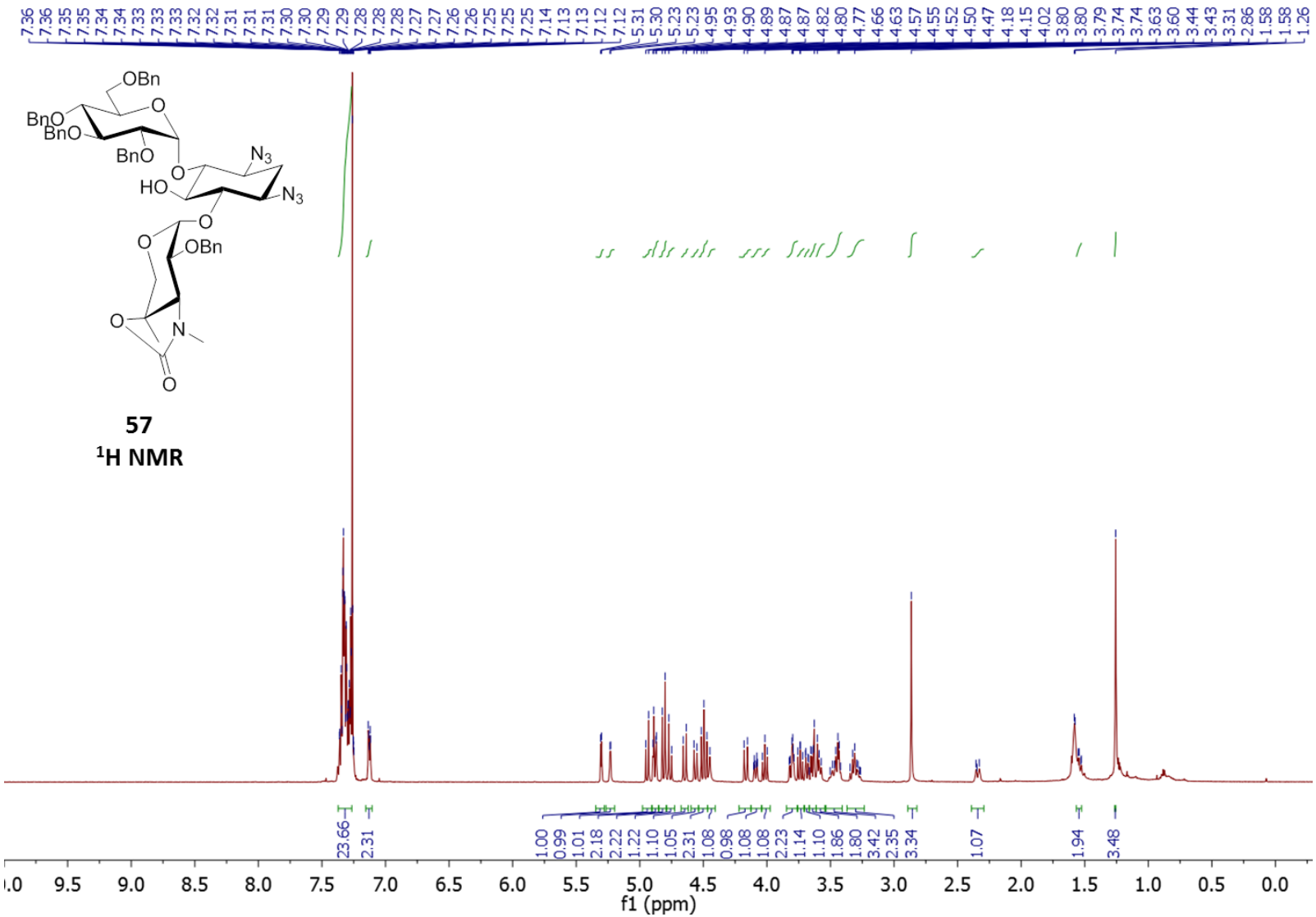

NH2
HO
HO
O
H2N
O
NH2
HO
NH2
O
O
OH
HO
HN

55

^{13}C NMR

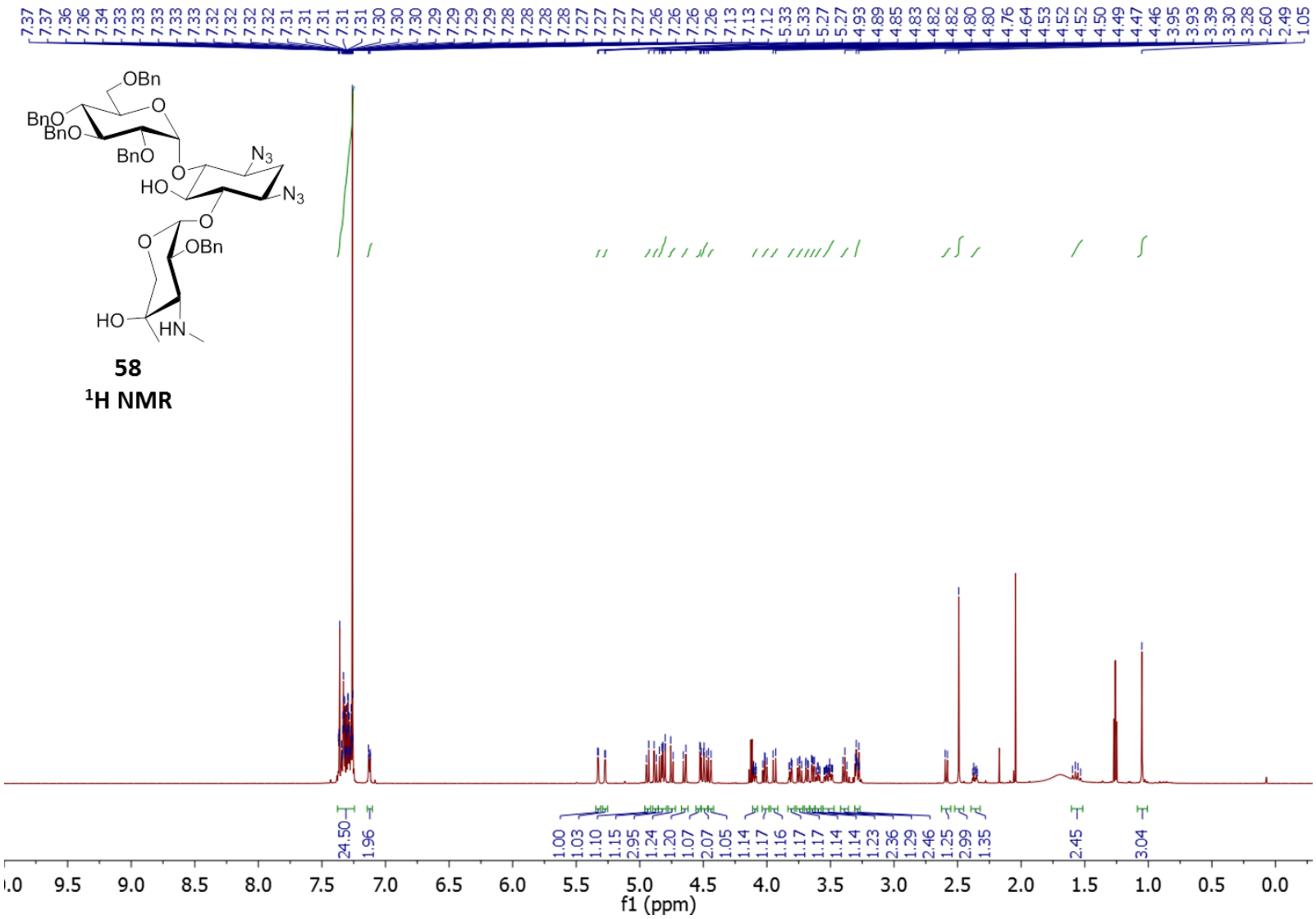
101.12
95.90
83.63
77.54
74.22
70.60
69.85
69.13
68.19
67.85
66.17
63.17
53.43
49.57
48.20
40.07
34.47
28.00
20.90
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

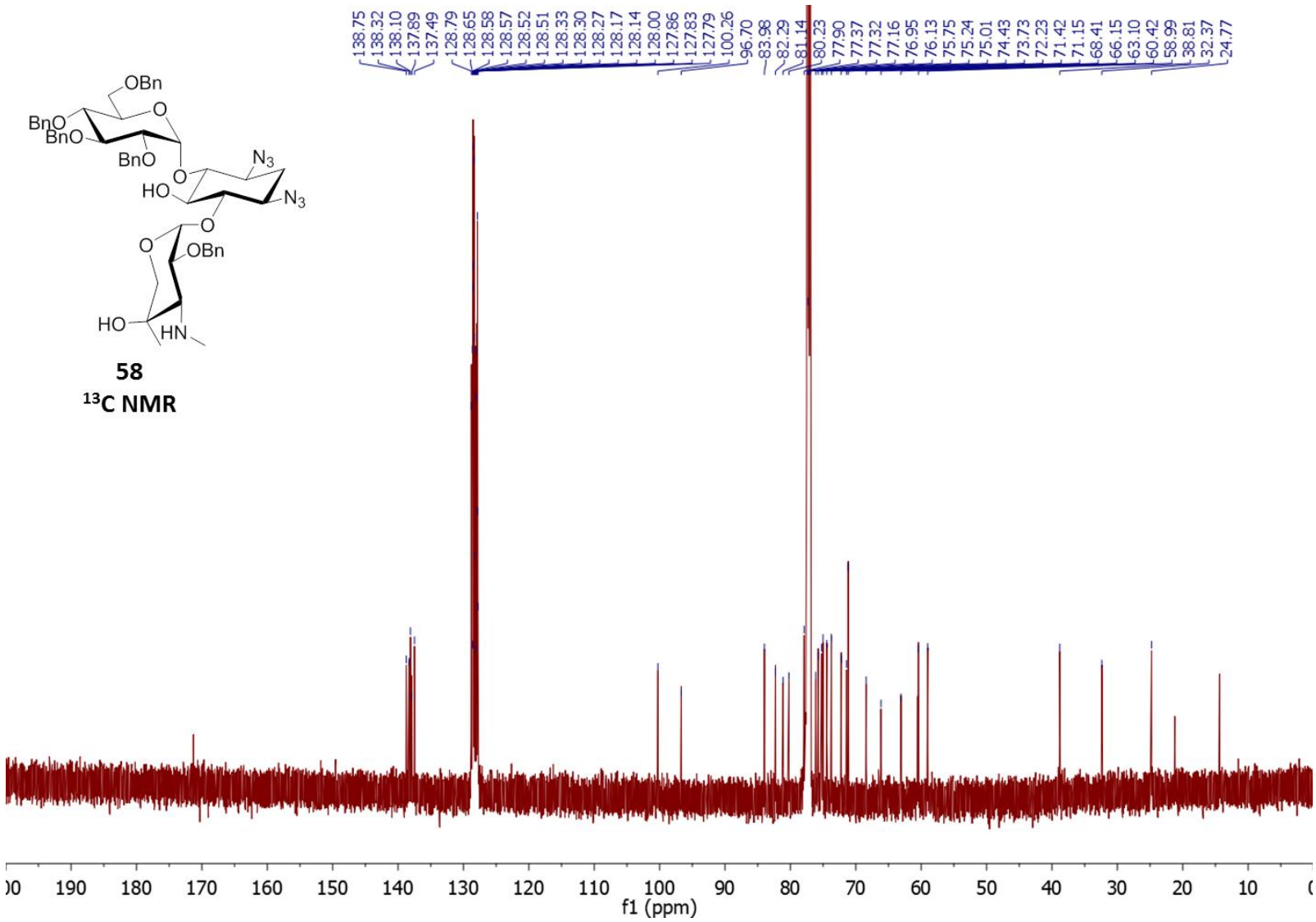

57
^{1}H NMR
f1 (ppm)



57

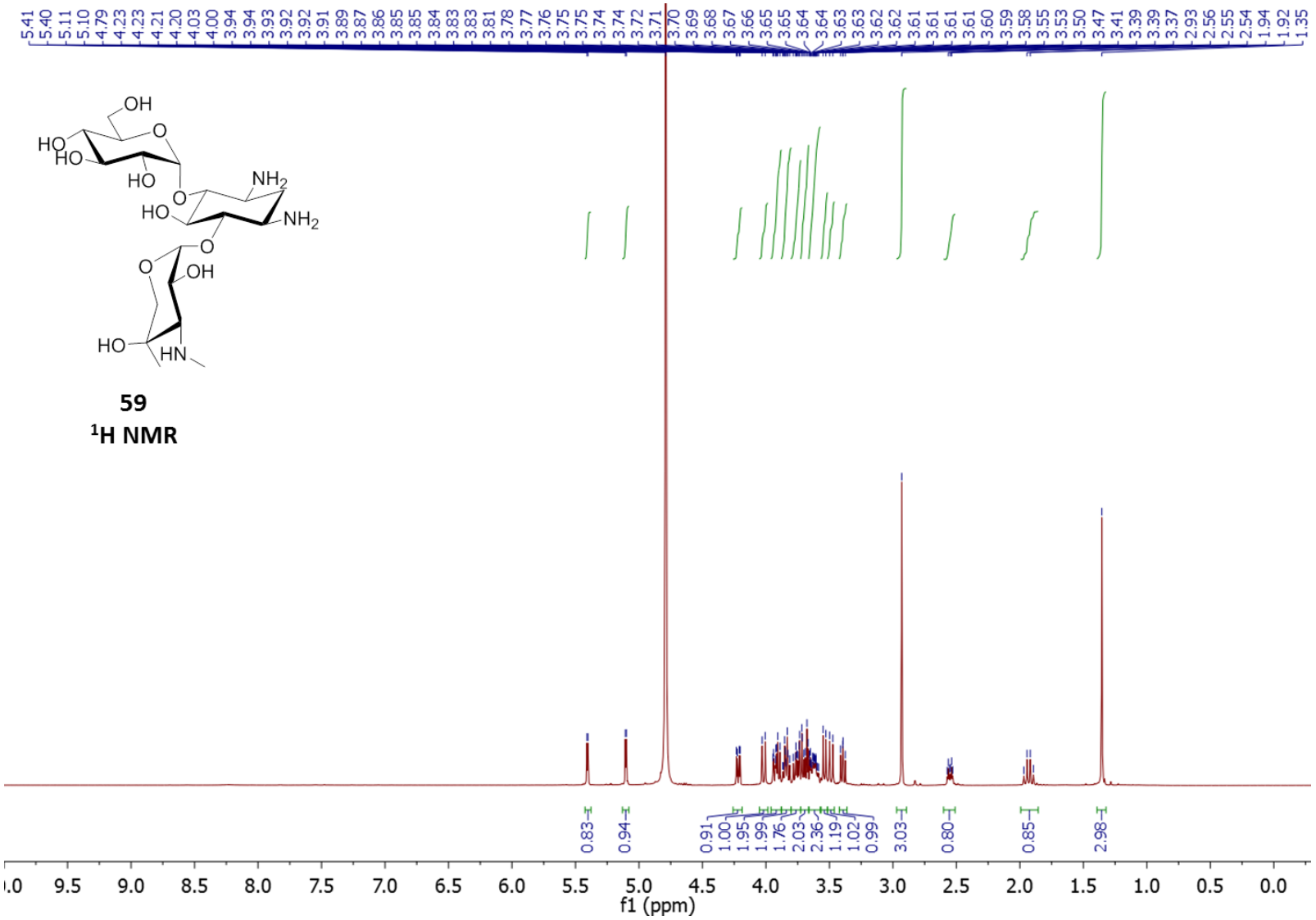
^{1}H NMR

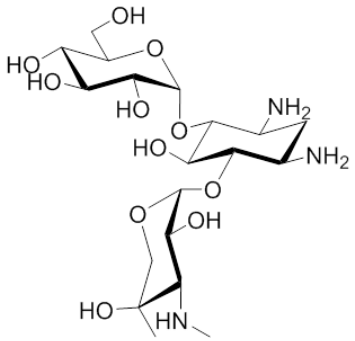

OBn
BnO
BnO
BnO
O
O
HO
N3
N3
O
O
OBn
HO
HN
58
1H NMR
f1 (ppm)


138.75
138.32
138.10
137.89
137.49
128.79
128.65
128.58
128.57
128.52
128.51
128.33
128.30
128.27
128.17
128.14
128.00
127.86
127.83
127.79
100.26
96.70
83.98
82.29
81.14
80.23
77.90
77.37
77.32
77.16
76.95
76.13
75.75
75.24
75.01
74.43
73.73
72.23
71.42
71.15
68.41
66.15
63.10
60.42
58.99
38.81
32.37
24.77
OBn
BnO
BnO
BnO
O
O
HO
N3
N3
O
O
OBn
HO
HN
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

58

^{13}C NMR


OH
HO
HO
O
HO
O
HO
NH2
NH2
O
O
OH
HO
HN
59
1H NMR
f1 (ppm)

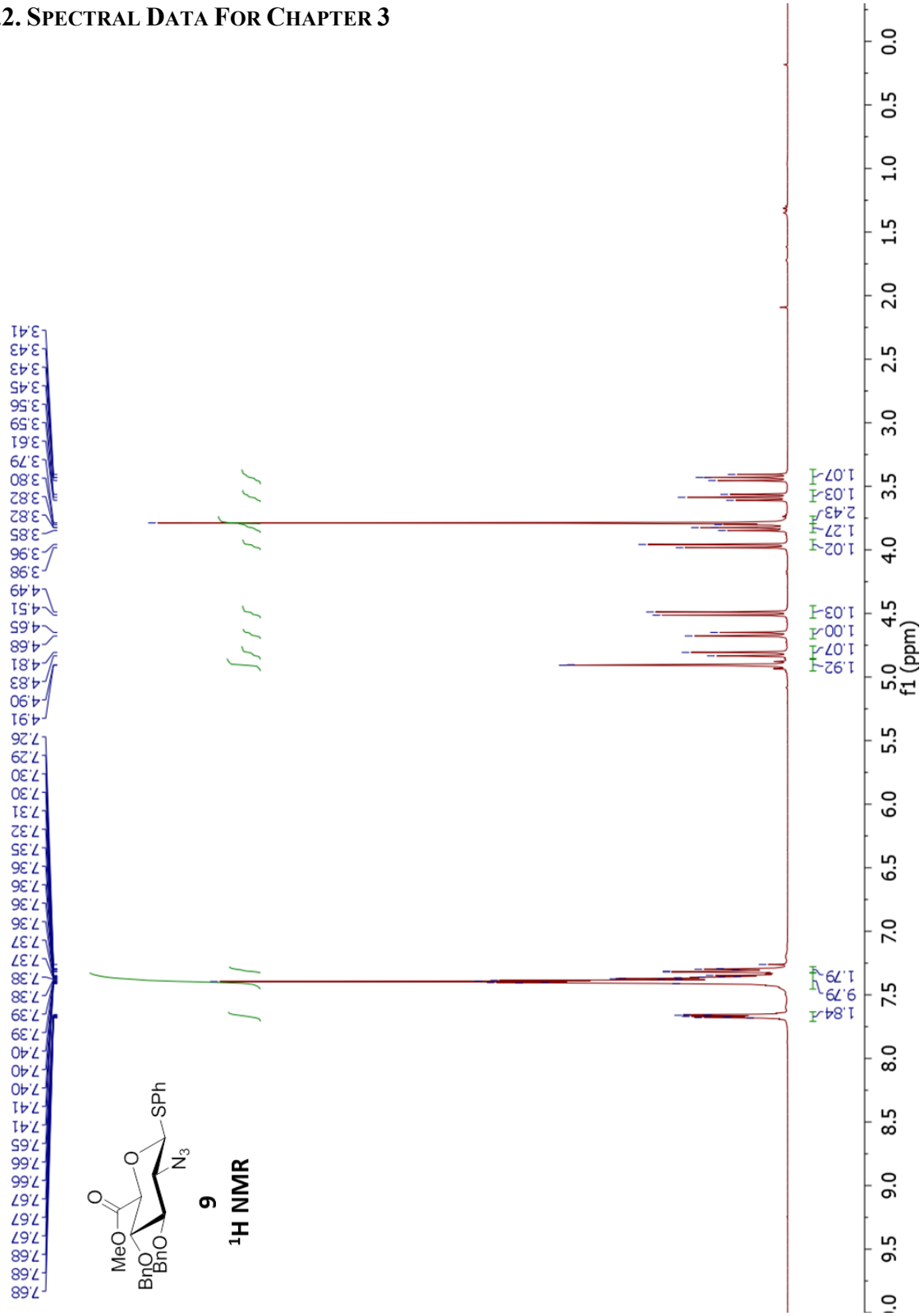

OH
HO
HO
O
HO
O
HO
NH2
NH2
O
O
OH
HO
HN

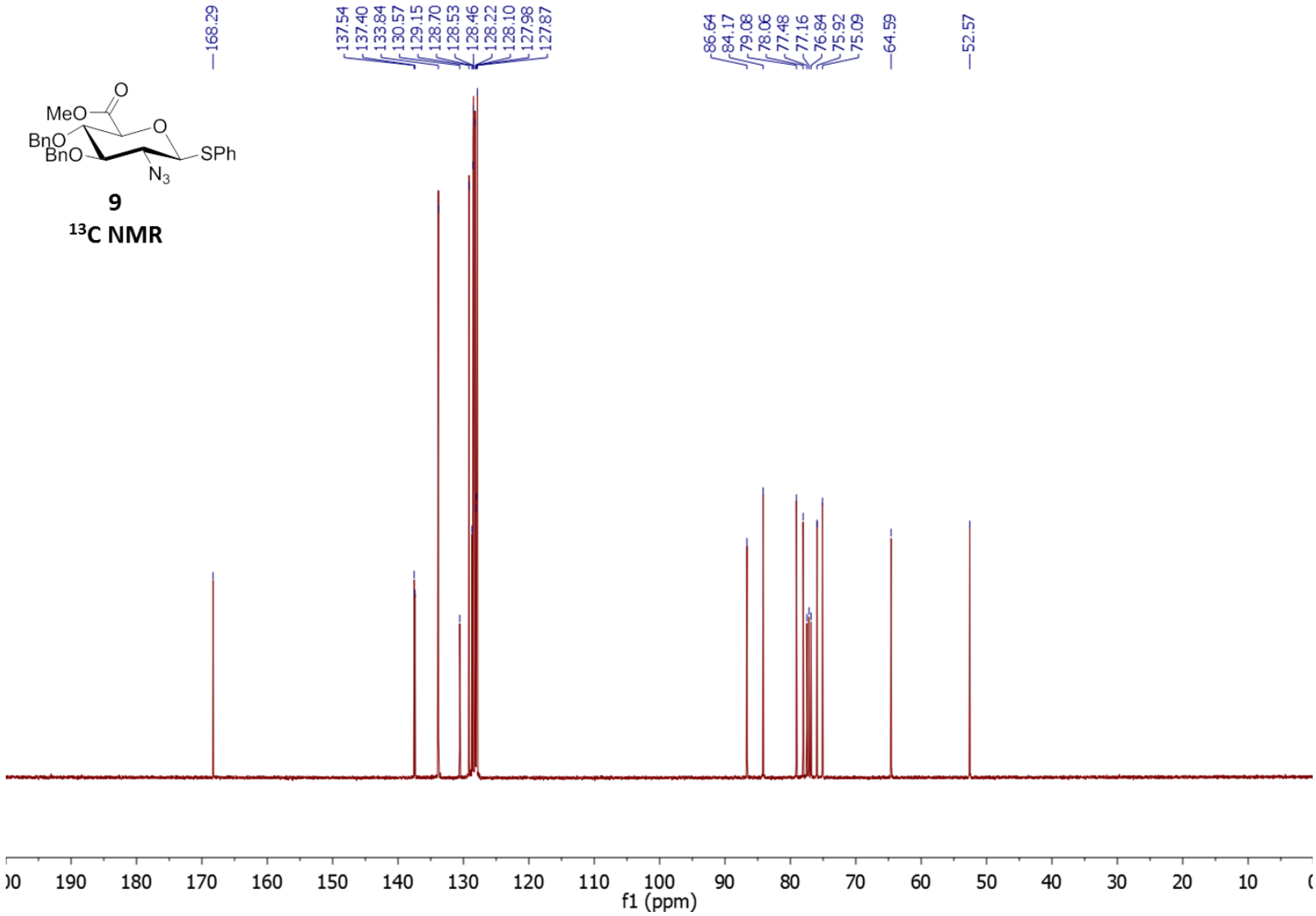
59

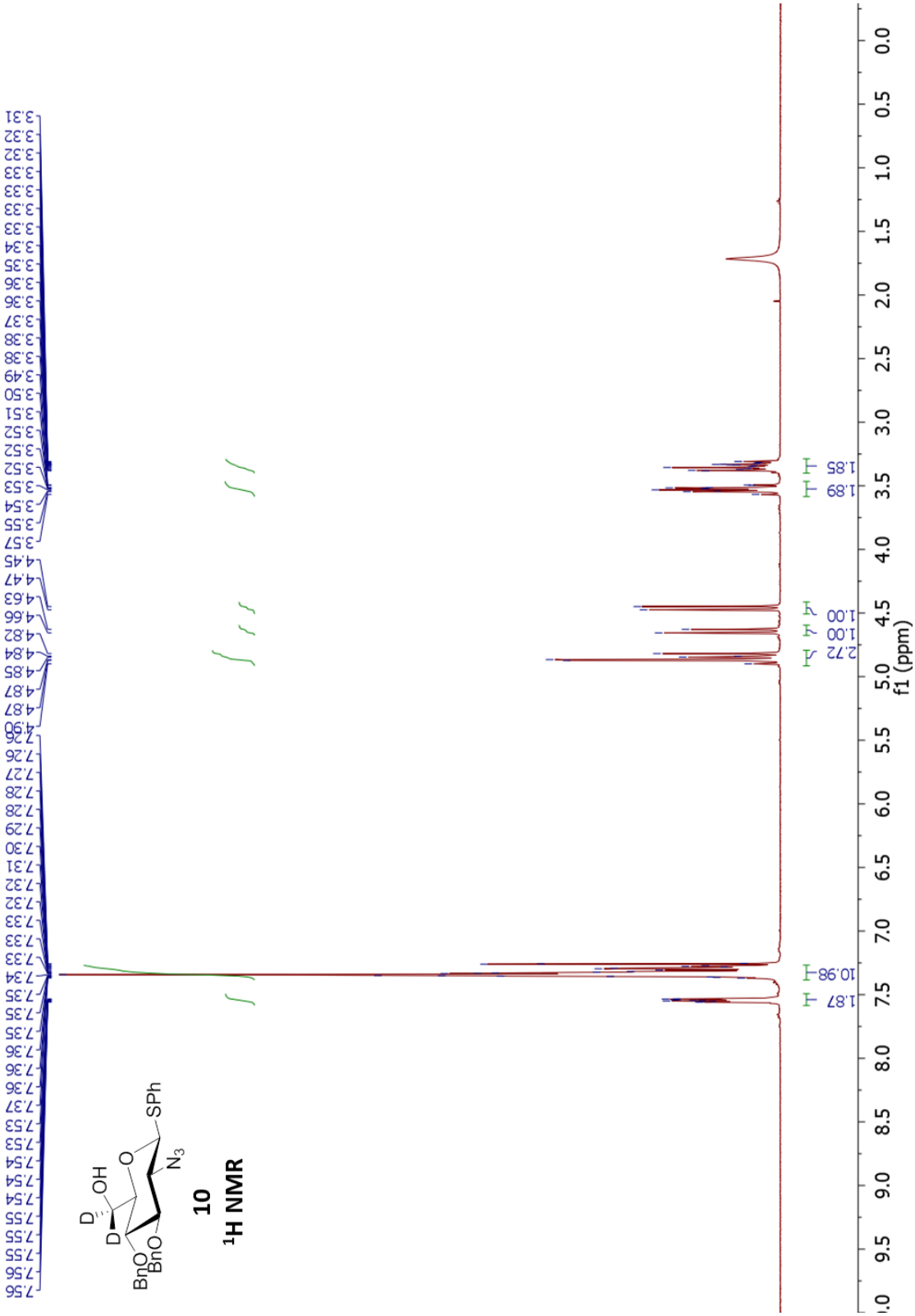
^{13}C NMR

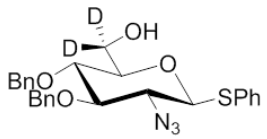
100.99
98.48
83.06
80.71
73.06
72.90
72.50
71.09
69.88
69.47
67.60
66.30
63.30
60.64
49.71
48.30
34.46
27.62
20.87
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

A.2. SPECTRAL DATA FOR CHAPTER 3




168.29
137.54
137.40
133.84
130.57
129.15
128.70
128.53
128.46
128.22
128.10
127.98
127.87
86.64
84.17
79.08
78.06
77.48
77.16
76.84
75.92
75.09
64.59
52.57
MeO
O
O
BnO
BnO
N3
SPh
9
13C NMR
f1 (ppm)

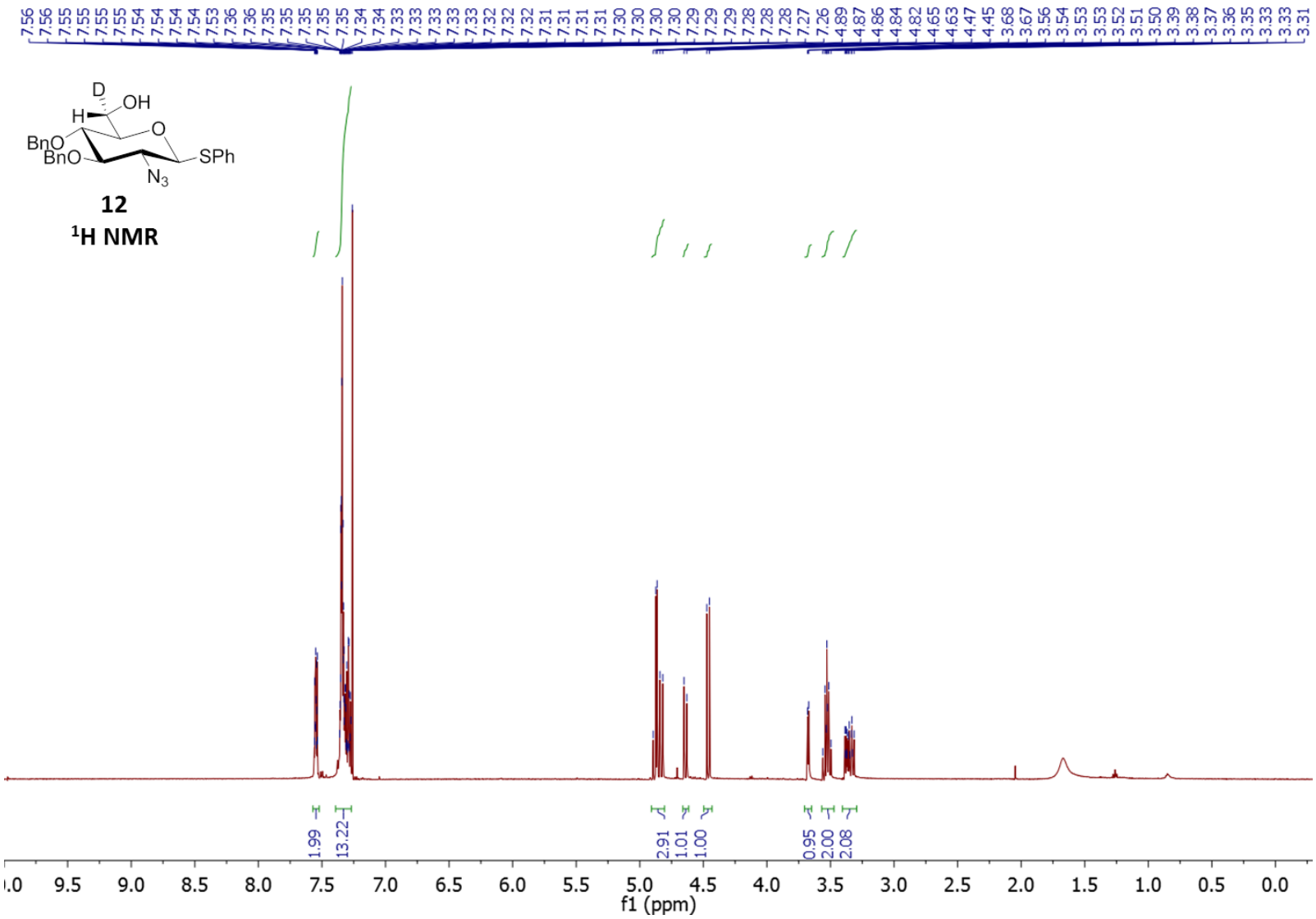

10
^{1}H NMR
f1 (ppm)

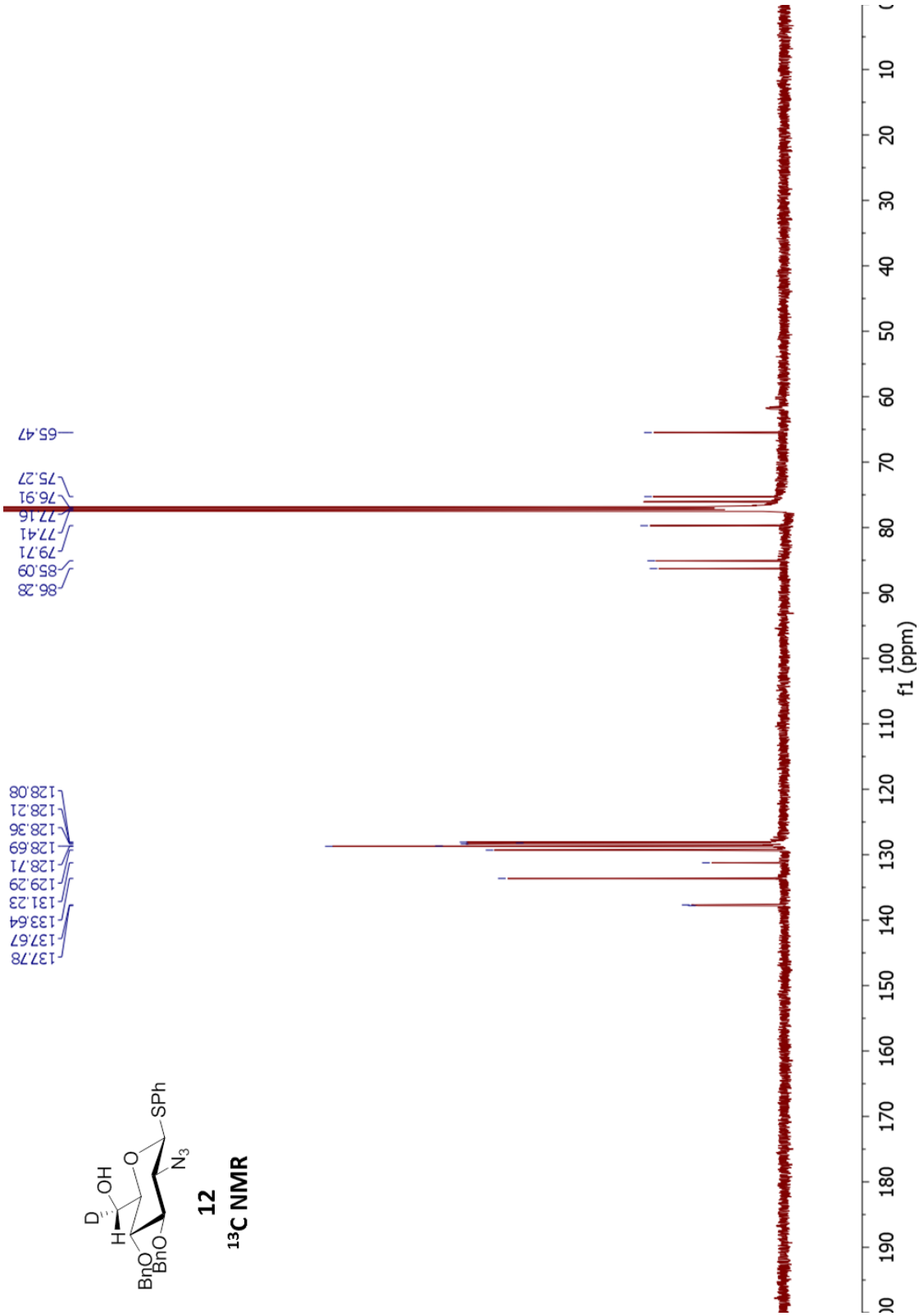

D
OH
D
O
BnO
BnO
SPh
N3

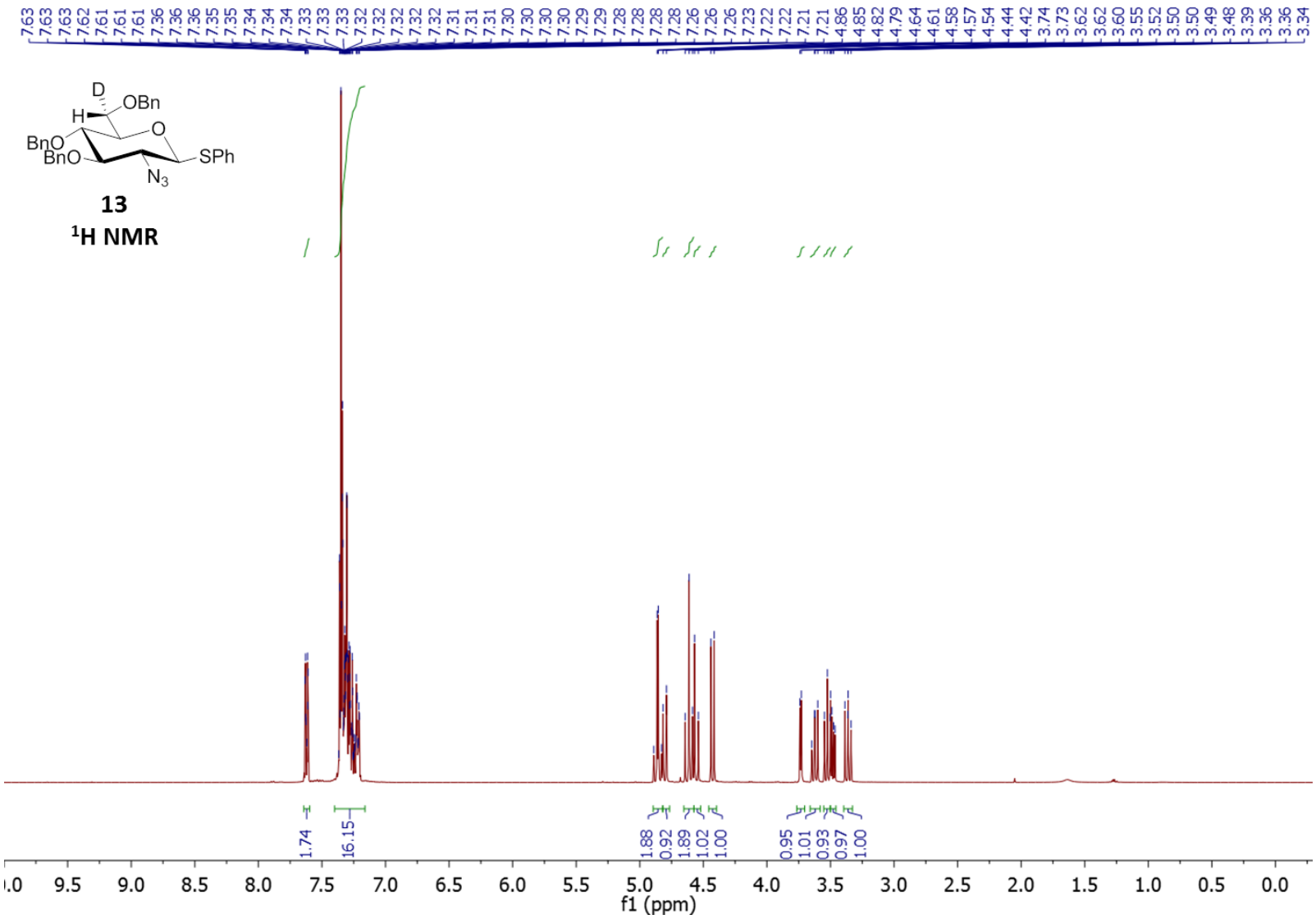
10

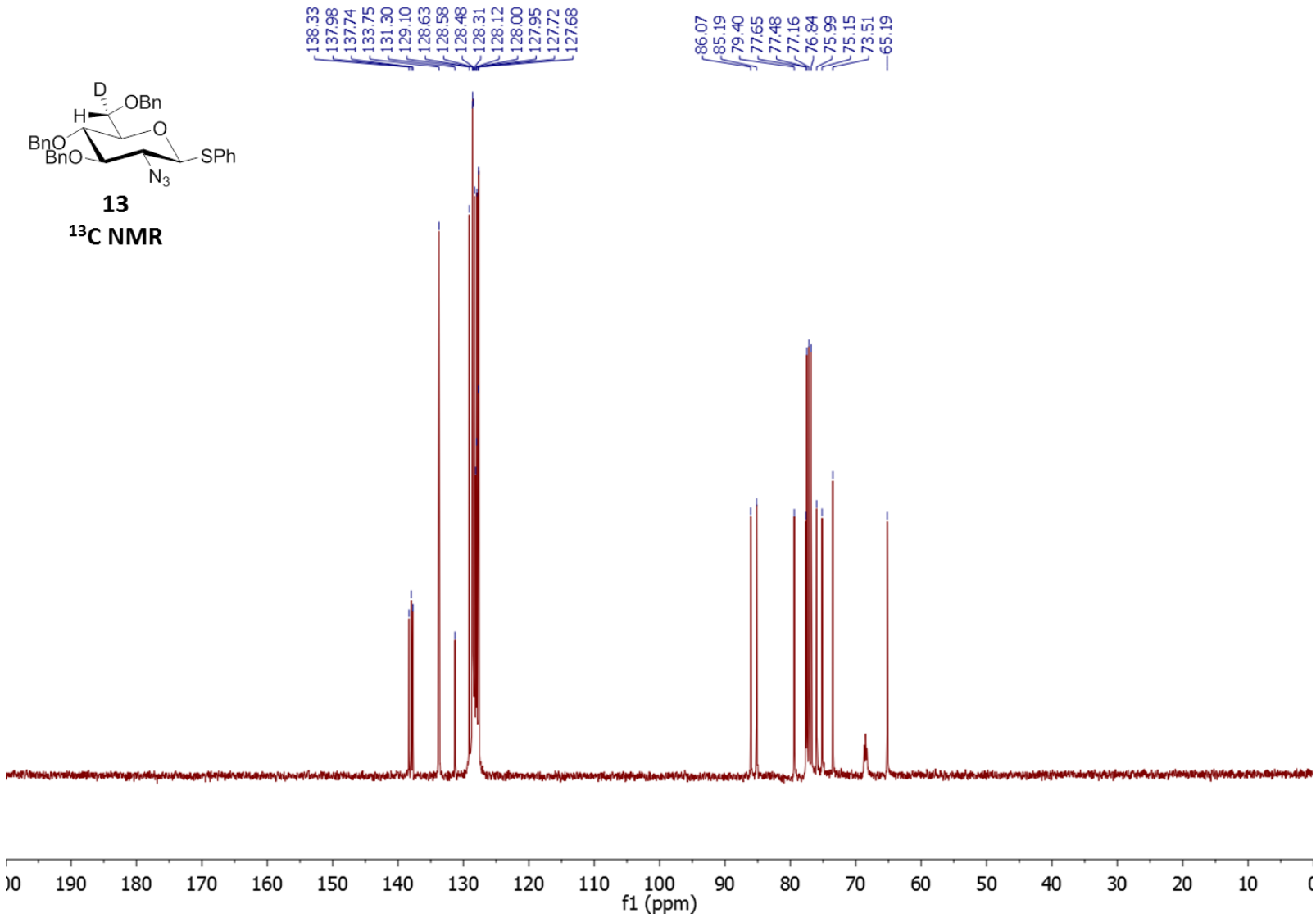
^{13}C NMR

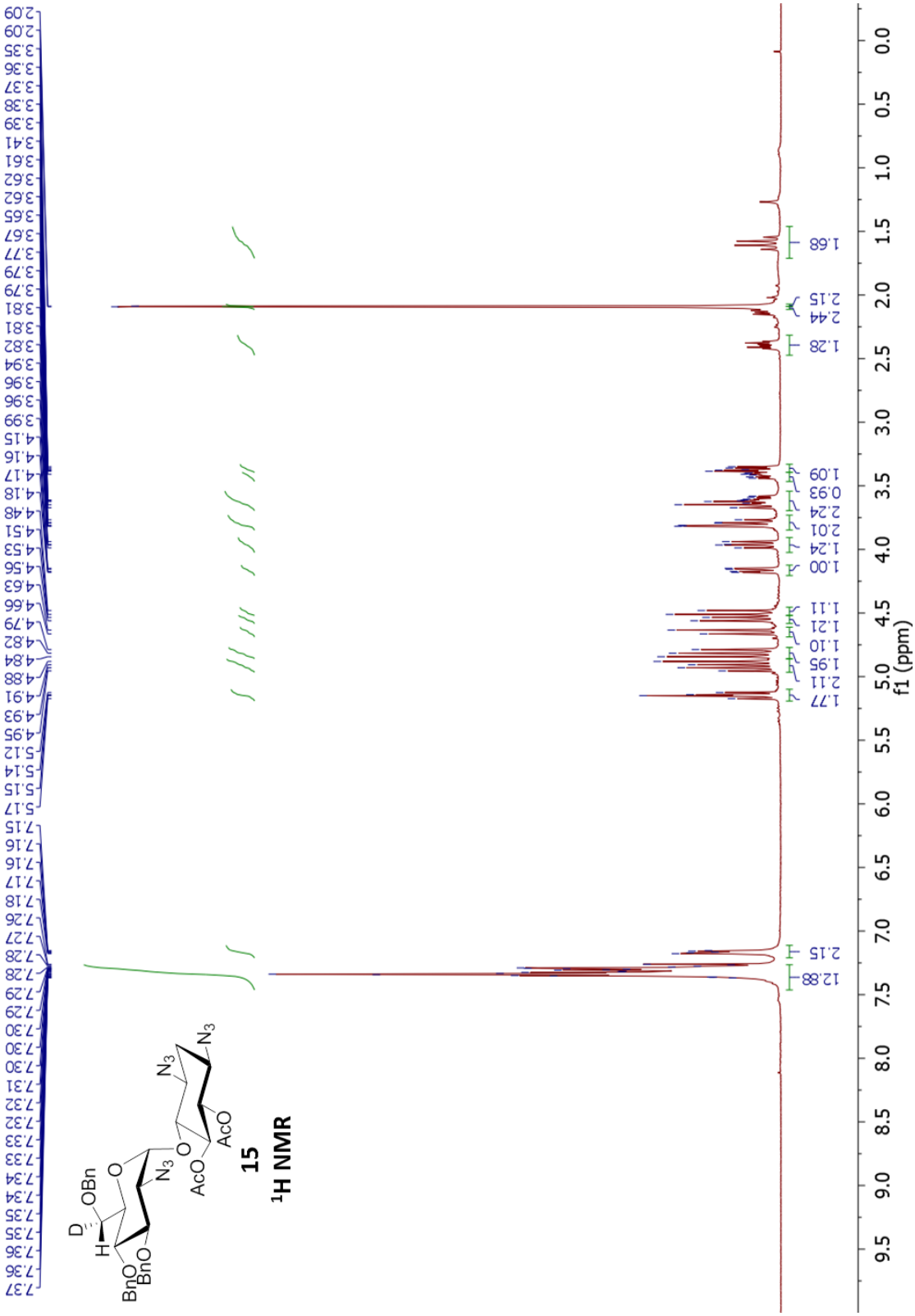
137.75
137.63
133.64
131.17
129.29
128.72
128.69
128.36
128.22
128.07
86.23
85.06
79.61
77.48
77.16
76.84
76.06
75.27
65.41
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

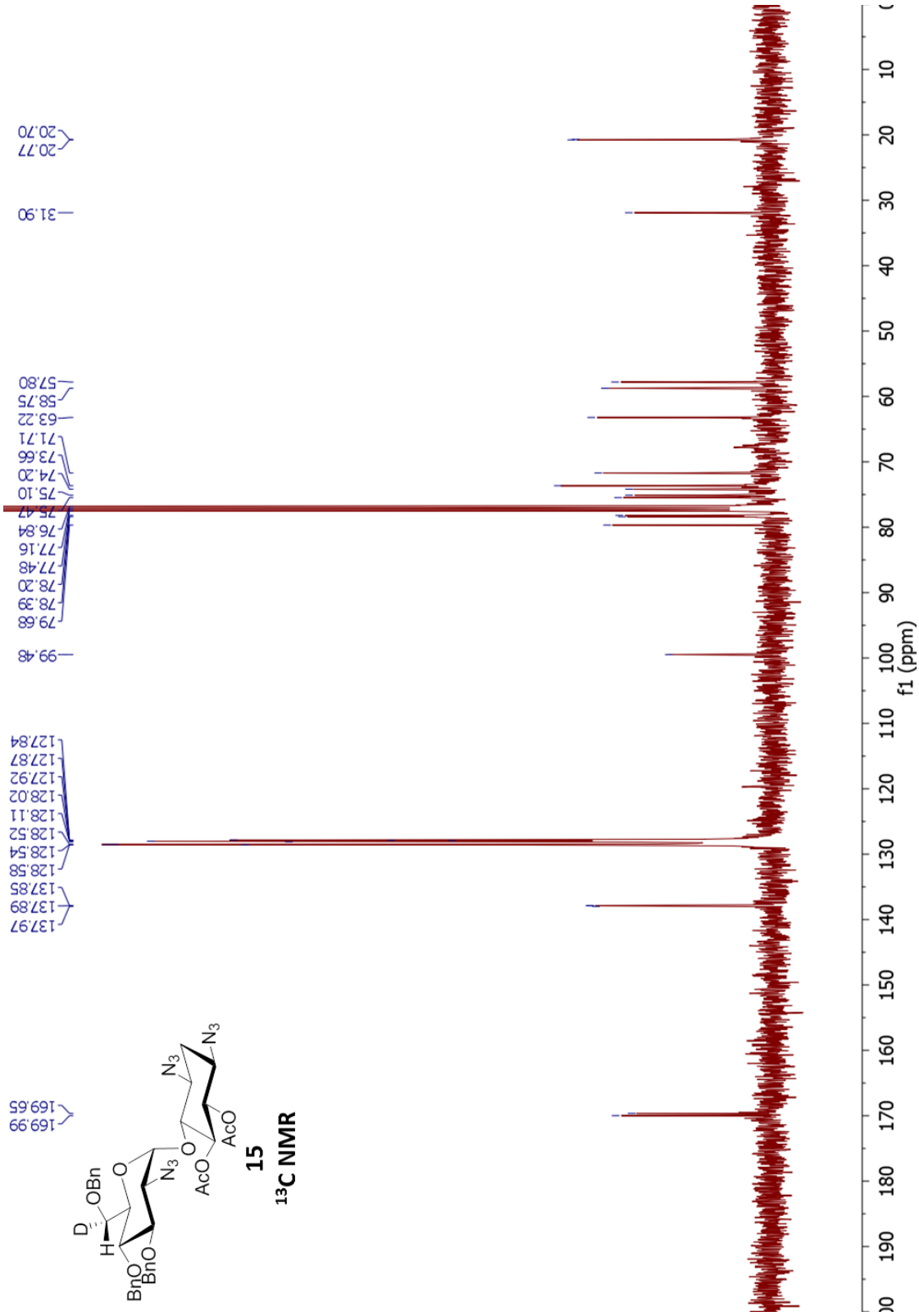

D
OH
H
O
BnO
BnO
SPh
N3
12
1H NMR
1.99
13.22
2.91
1.01
1.00
0.95
2.00
2.08
f1 (ppm)

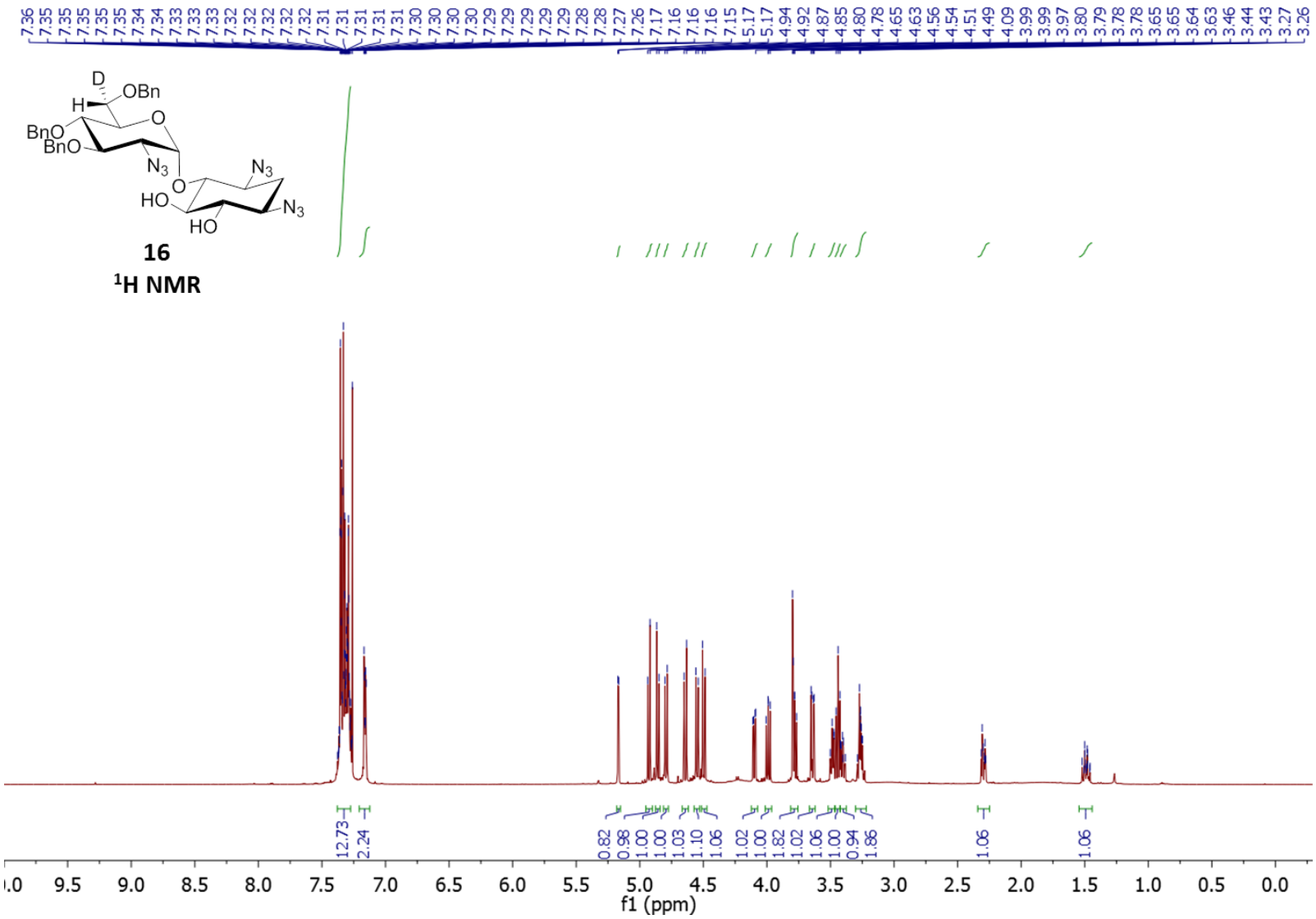

137.78
137.67
133.64
131.23
129.29
128.71
128.69
128.36
128.21
128.08
86.28
85.09
79.71
77.41
77.16
76.91
75.27
65.47
f1 (ppm)
12
13C NMR

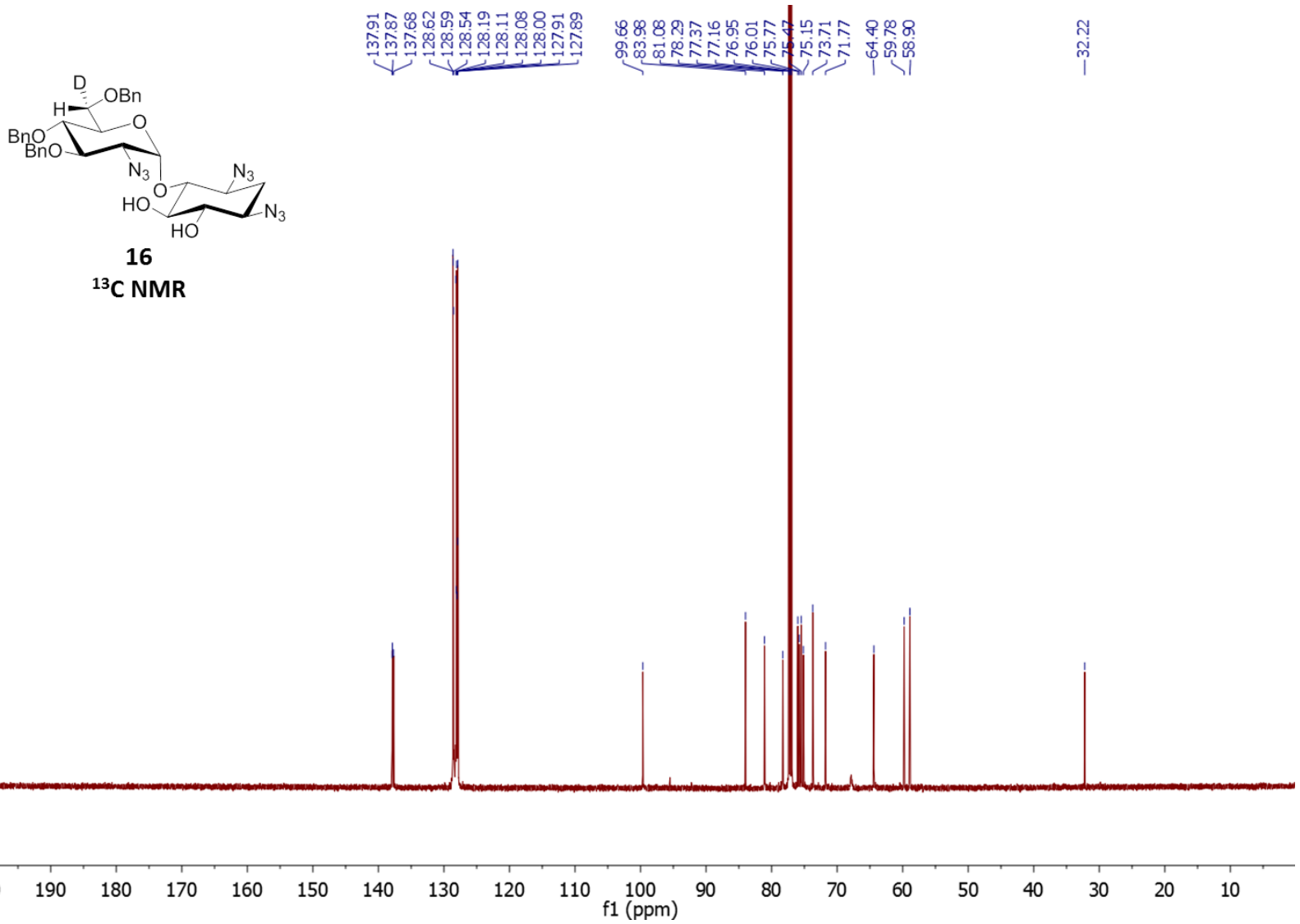

D
OBn
H
O
BnO
BnO
SPh
N3
13
1H NMR
f1 (ppm)

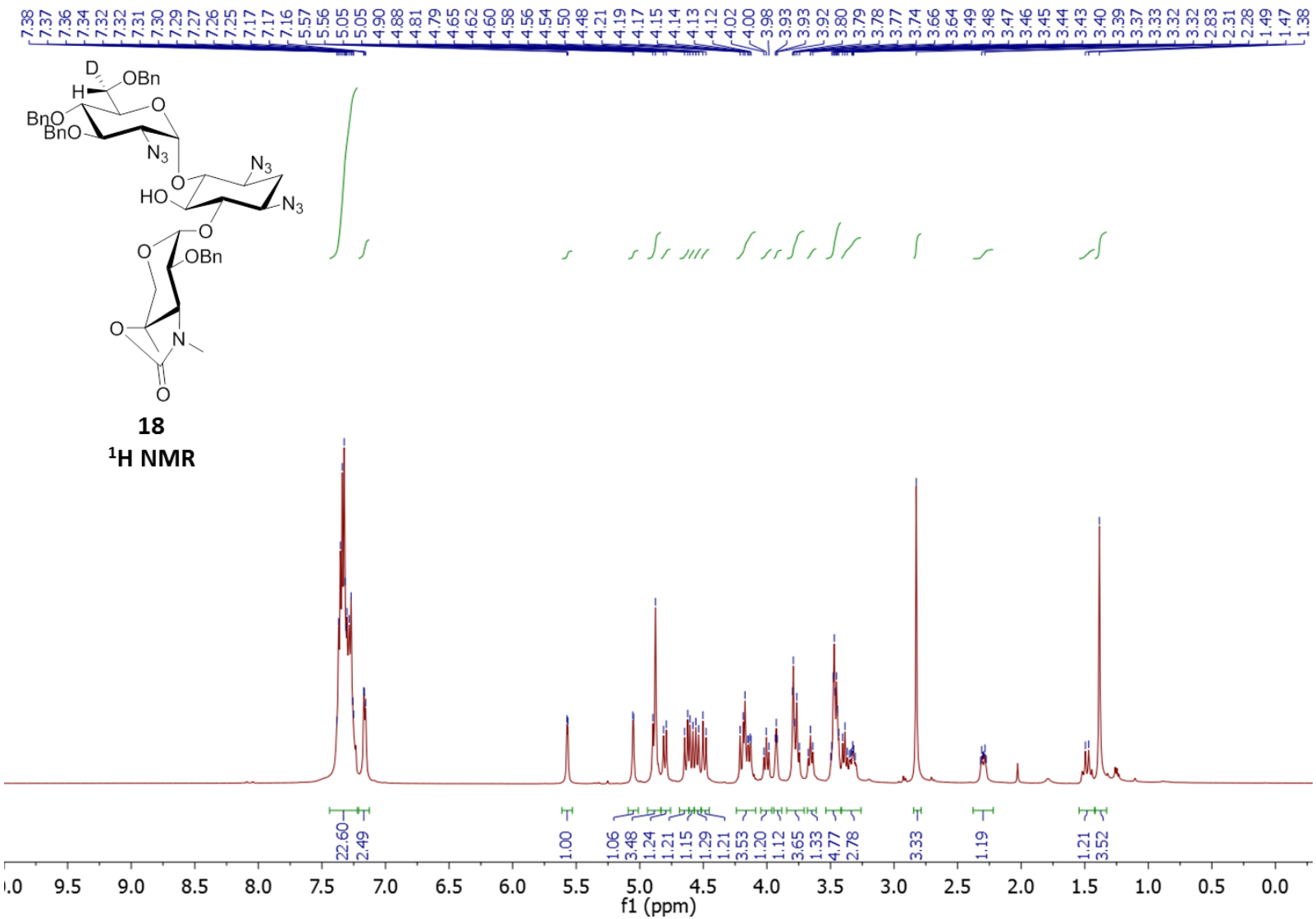

138.33
137.98
137.74
133.75
131.30
129.10
128.63
128.58
128.48
128.31
128.12
128.00
127.95
127.72
127.68
86.07
85.19
79.40
77.65
77.48
77.16
76.84
75.99
75.15
73.51
65.19
D
OBn
H
O
BnO
BnO
SPh
N3
13
13C NMR
f1 (ppm)


15
1H NMR
f1 (ppm)


20.77
20.70
31.90
57.80
58.75
63.22
71.71
73.66
74.20
75.10
75.47
76.84
77.16
77.48
78.20
78.39
79.68
99.48
127.84
127.87
127.92
128.02
128.11
128.52
128.54
128.58
137.85
137.89
137.97
169.65
169.99
BnO
BnO
H
D
OBn
O
N3
O
AcO
AcO
N3
N3
15
13C NMR
f1 (ppm)

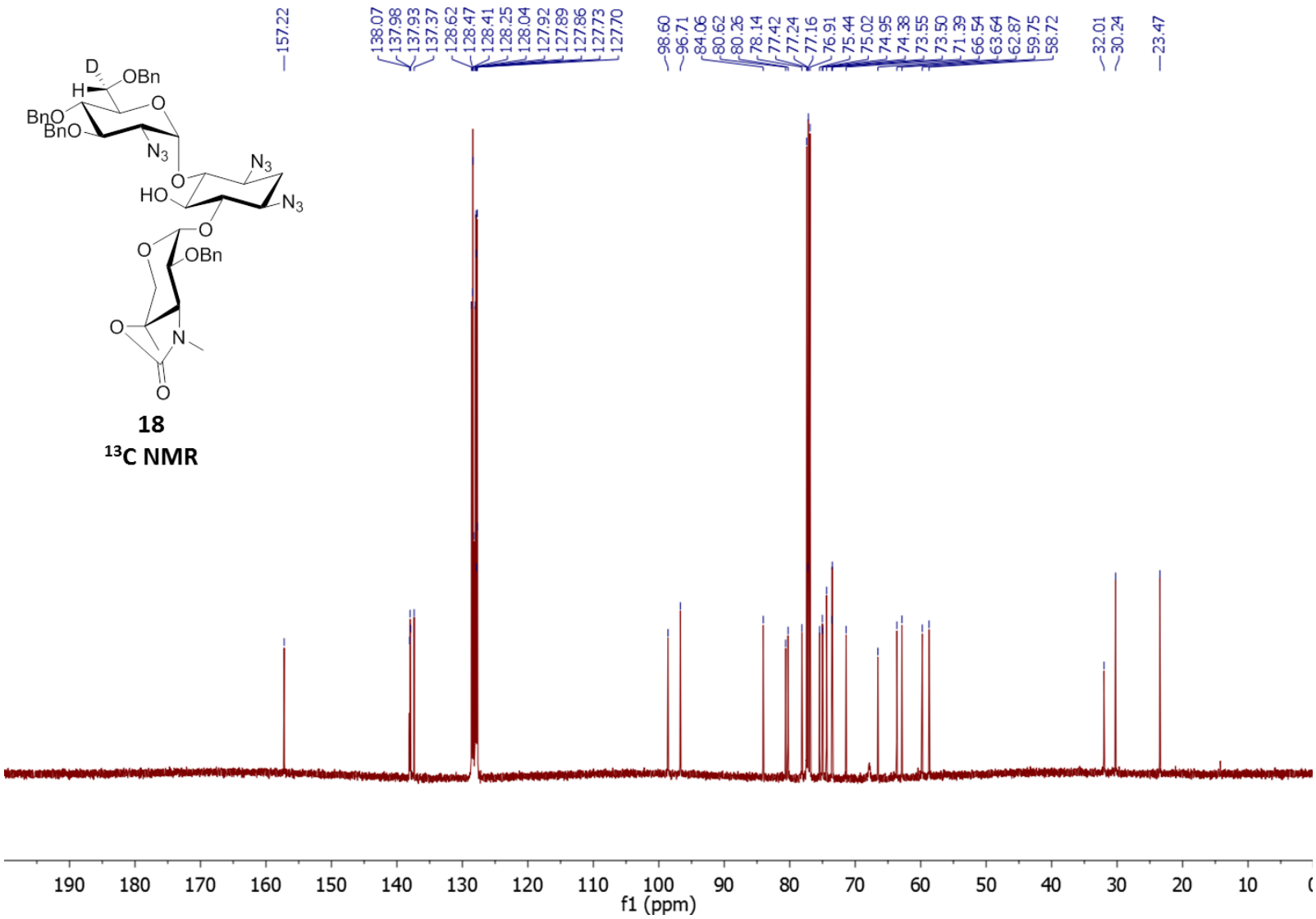

D
OBn
H
O
BnO
BnO
N3
O
N3
HO
N3
HO
16
1H NMR
f1 (ppm)


137.91
137.87
137.68
128.62
128.59
128.54
128.19
128.11
128.08
128.00
127.91
127.89
99.66
83.98
81.08
78.29
77.37
77.16
76.95
76.01
75.77
75.47
75.15
73.71
71.77
64.40
59.78
58.90
32.22
D
OBn
H
O
BnO
BnO
N3
O
N3
HO
N3
HO
16
13C NMR
f1 (ppm)


7.38 7.37 7.36 7.34 7.32 7.32 7.31 7.30 7.29 7.27 7.26 7.25 7.17 7.17 7.16 5.57 5.56 5.05 5.05 4.90 4.88 4.81 4.79 4.65 4.62 4.60 4.58 4.56 4.54 4.50 4.48 4.21 4.19 4.17 4.15 4.14 4.13 4.12 4.02 4.00 3.98 3.93 3.93 3.92 3.80 3.79 3.78 3.77 3.74 3.66 3.64 3.49 3.48 3.47 3.46 3.45 3.44 3.43 3.40 3.39 3.37 3.33 3.32 3.32 2.83 2.31 2.28 1.49 1.47 1.38
D
H
OBn
O
BnO
BnO
N3
N3
O
HO
N3
O
O
OBn
O
N
O
22.60
2.49
1.00
1.06
3.48
1.24
1.21
1.15
1.29
1.21
3.53
1.20
1.12
3.65
1.33
4.77
2.78
3.33
1.19
1.21
3.52
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

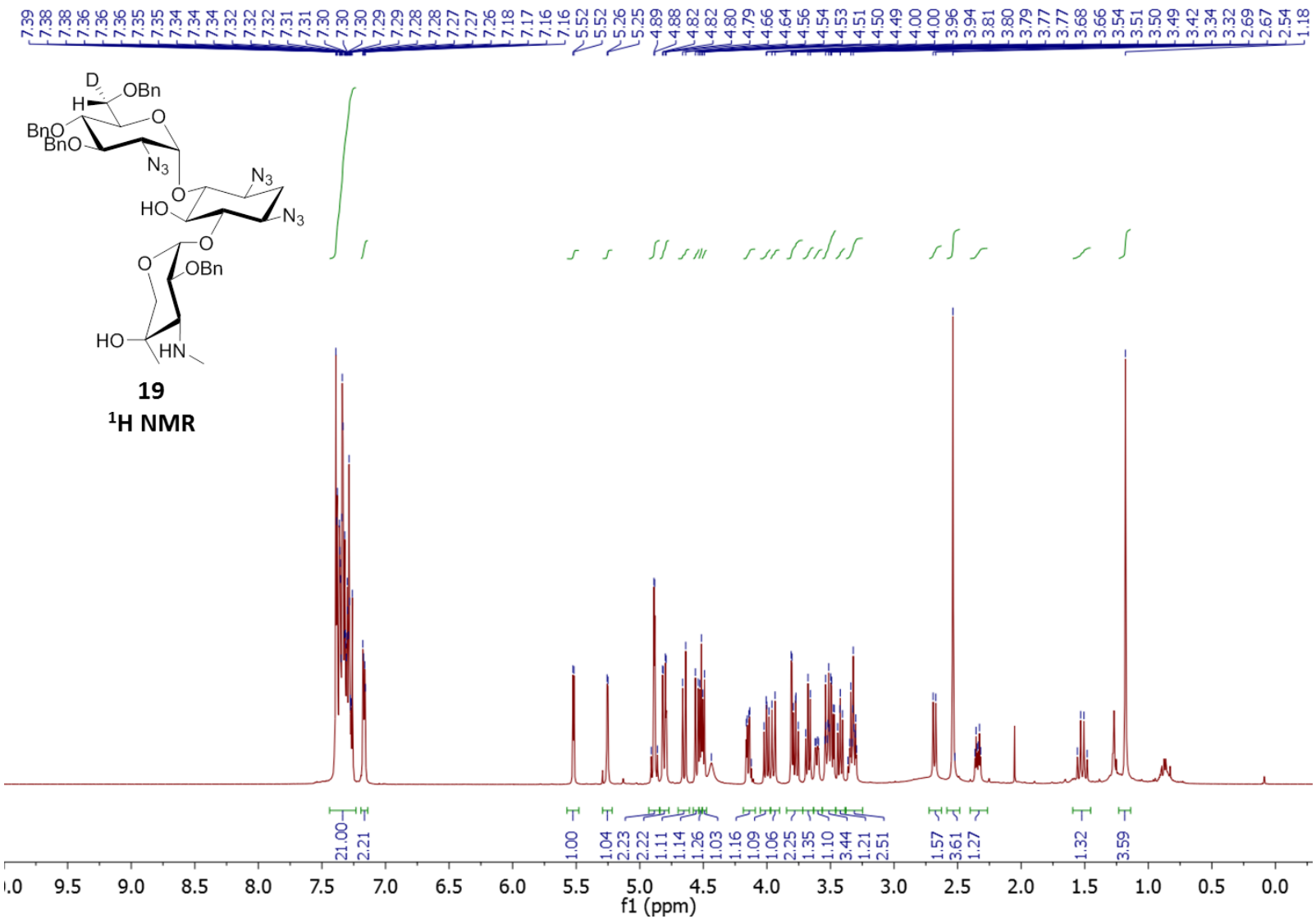
18

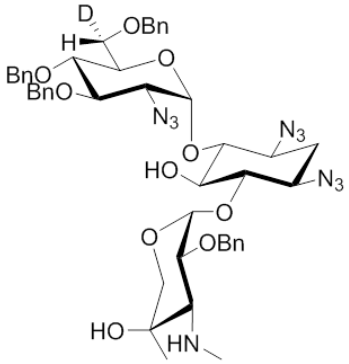
^{1}H NMR


157.22
138.07
137.98
137.93
137.37
128.62
128.47
128.41
128.25
128.04
127.92
127.89
127.86
127.73
127.70
98.60
96.71
84.06
80.62
80.26
78.14
77.42
77.24
77.16
76.91
75.44
75.02
74.95
74.38
73.55
73.50
71.39
66.54
63.64
62.87
59.75
58.72
32.01
30.24
23.47
D
H
OBn
BnO
BnO
O
N3
N3
HO
N3
O
O
OBn
O
O
N
O
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

18

^{13}C NMR

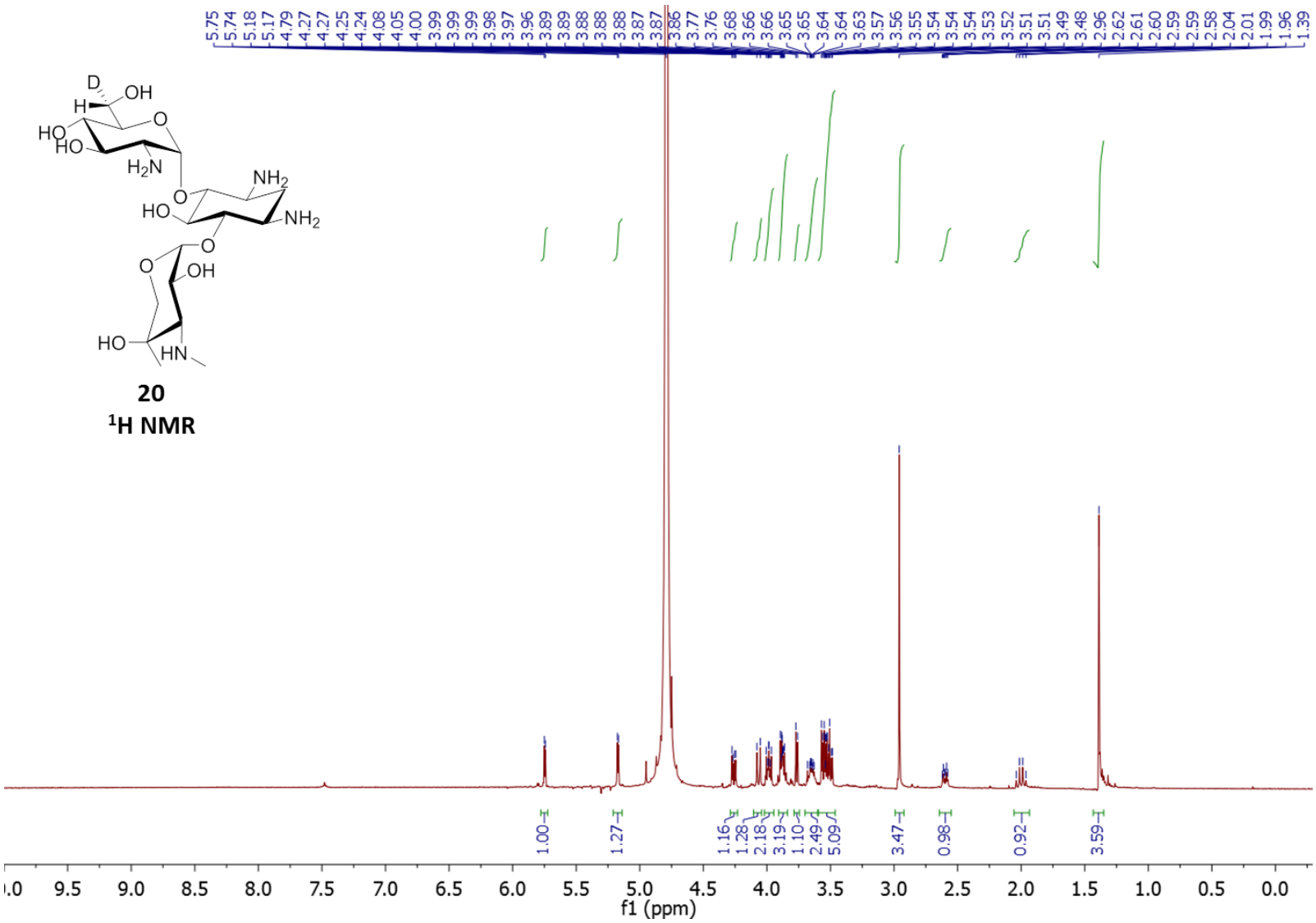

19
1H NMR
f1 (ppm)


D
H
OBn
O
BnO
BnO
N3
O
N3
HO
N3
O
O
OBn
HO
HN

19

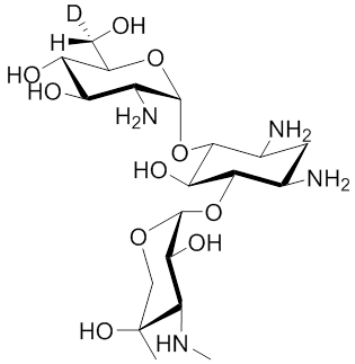
^{13}C NMR

138.11
138.02
137.96
137.81
128.74
128.56
128.51
128.48
128.28
128.22
128.13
128.00
127.97
127.85
127.83
127.81
98.97
97.11
83.77
81.23
80.47
78.20
77.42
77.16
76.91
76.14
75.61
75.59
75.06
73.61
72.46
71.49
71.05
66.68
63.81
63.27
59.79
58.90
38.67
32.29
24.55
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


5.75
5.74
5.18
5.17
4.79
4.27
4.27
4.25
4.24
4.08
4.05
4.00
3.99
3.99
3.99
3.98
3.97
3.96
3.89
3.89
3.88
3.88
3.88
3.87
3.87
3.86
3.77
3.76
3.68
3.66
3.66
3.65
3.65
3.64
3.64
3.63
3.57
3.56
3.55
3.54
3.54
3.54
3.53
3.52
3.51
3.51
3.49
3.48
2.96
2.62
2.61
2.60
2.59
2.59
2.58
2.04
2.01
1.99
1.96
1.39
1.00
1.27
1.16
1.28
2.18
3.19
1.10
2.49
5.09
3.47
0.98
0.92
3.59
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

20

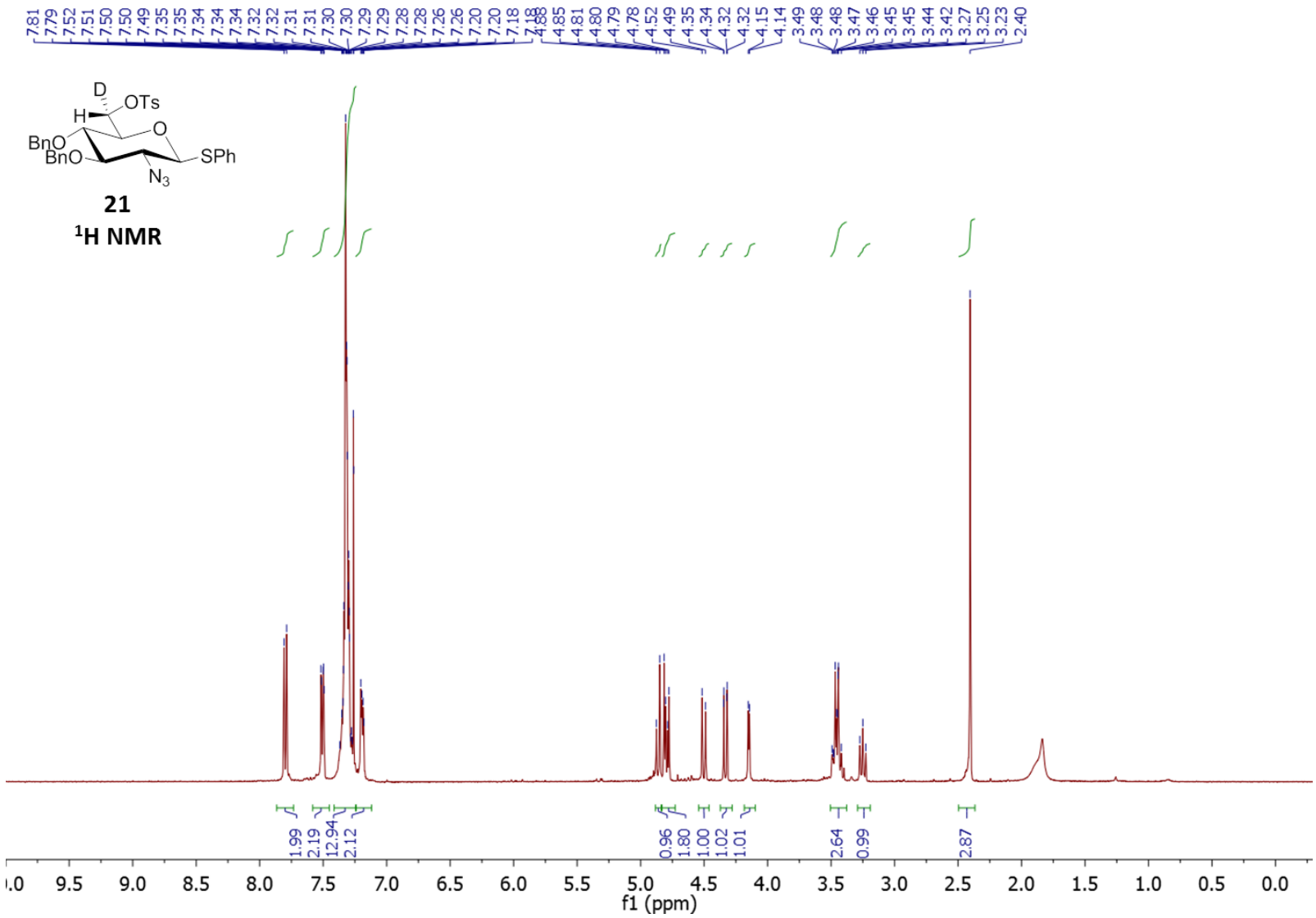
^{1}H NMR

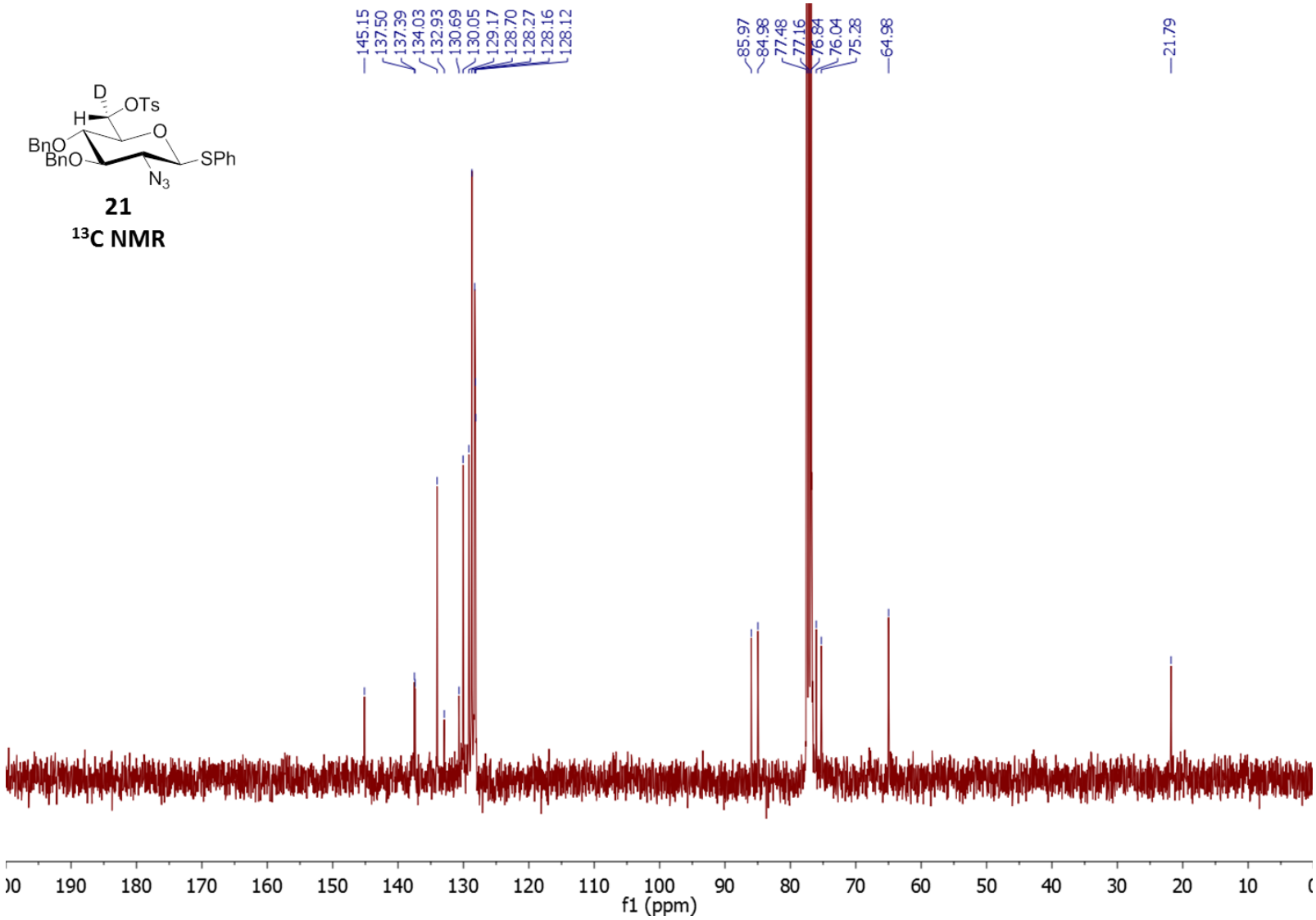

D
H
OH
O
HO
HO
H2N
NH2
O
HO
NH2
O
O
OH
HO
HN

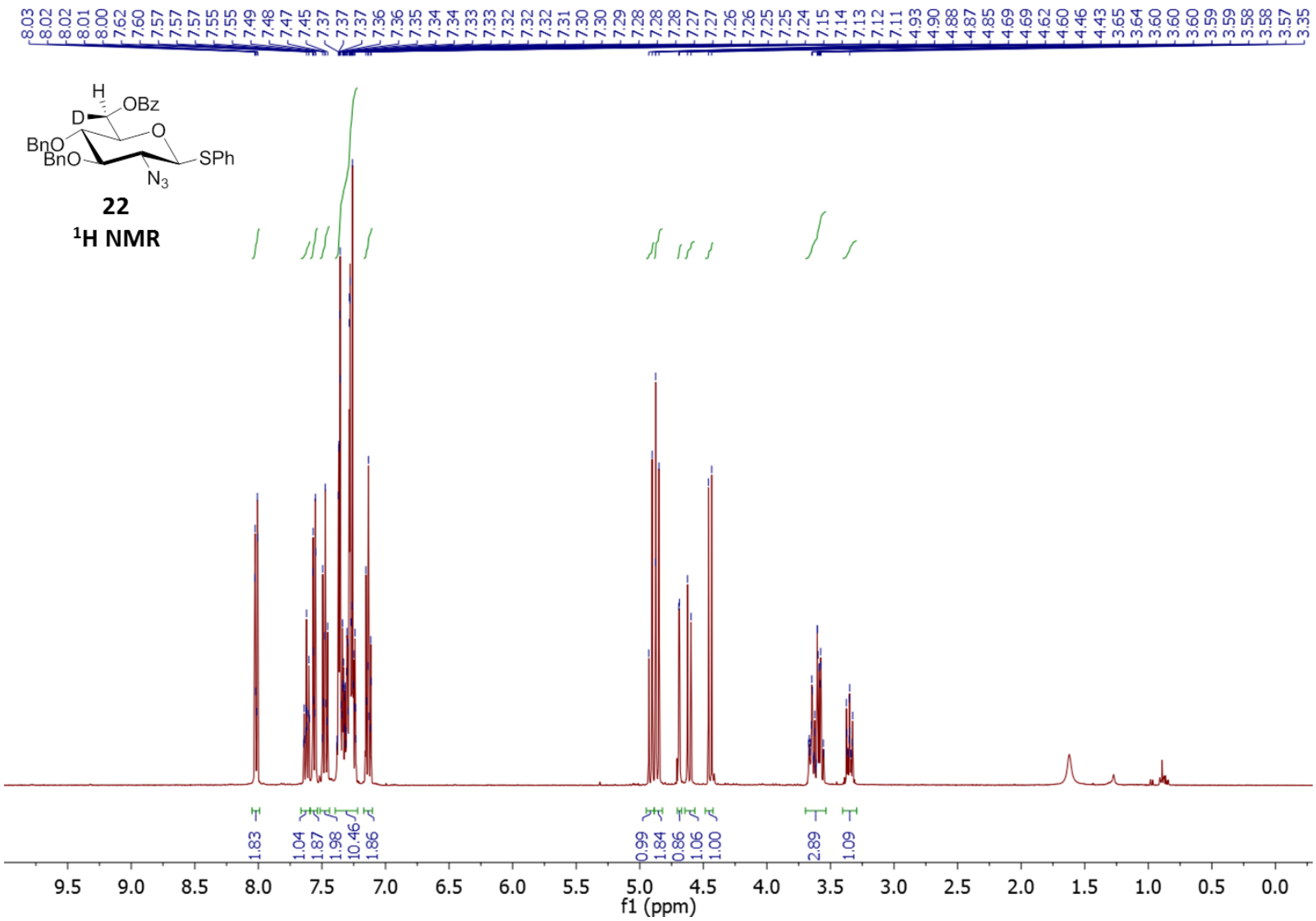
20

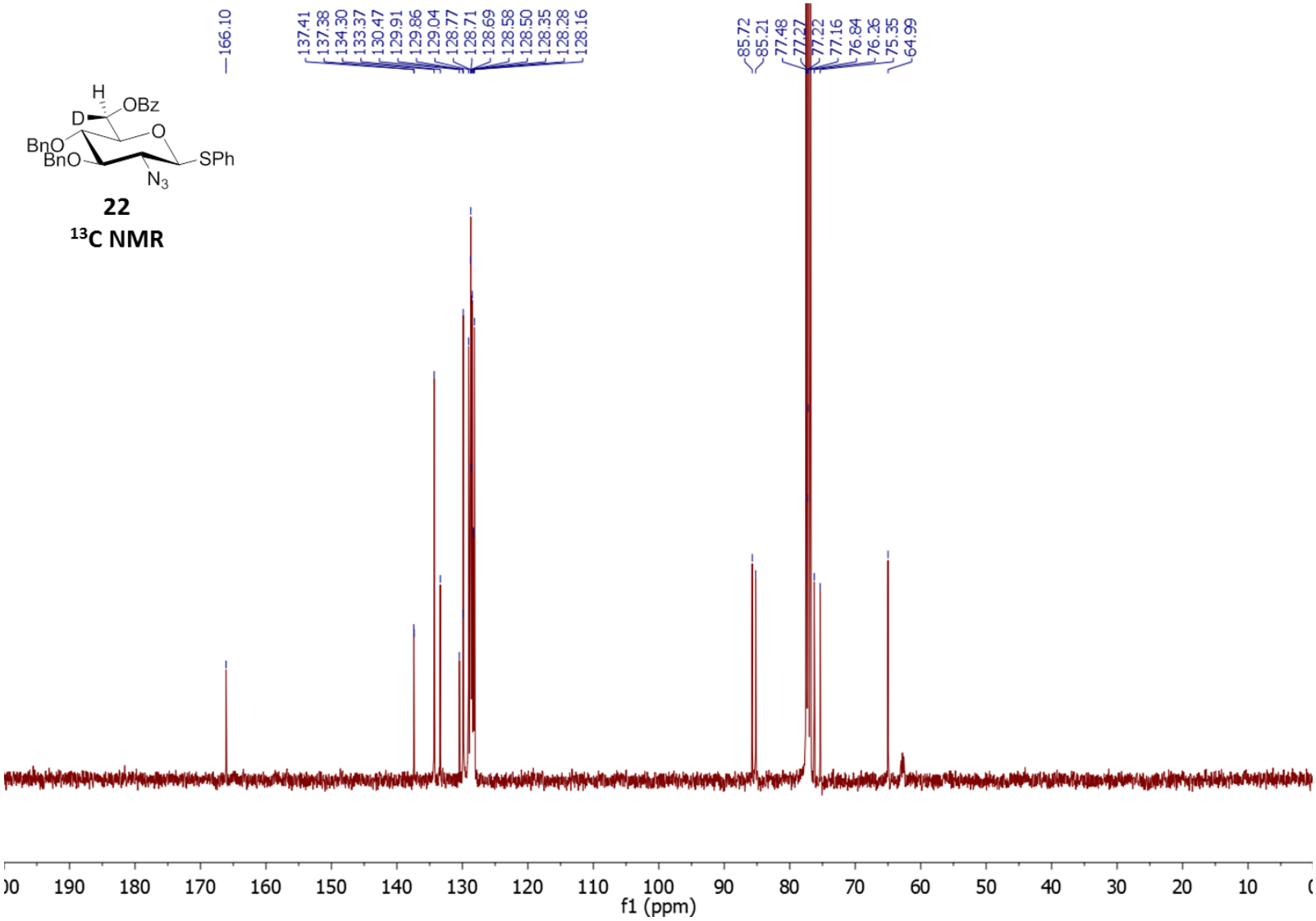
^{13}C NMR

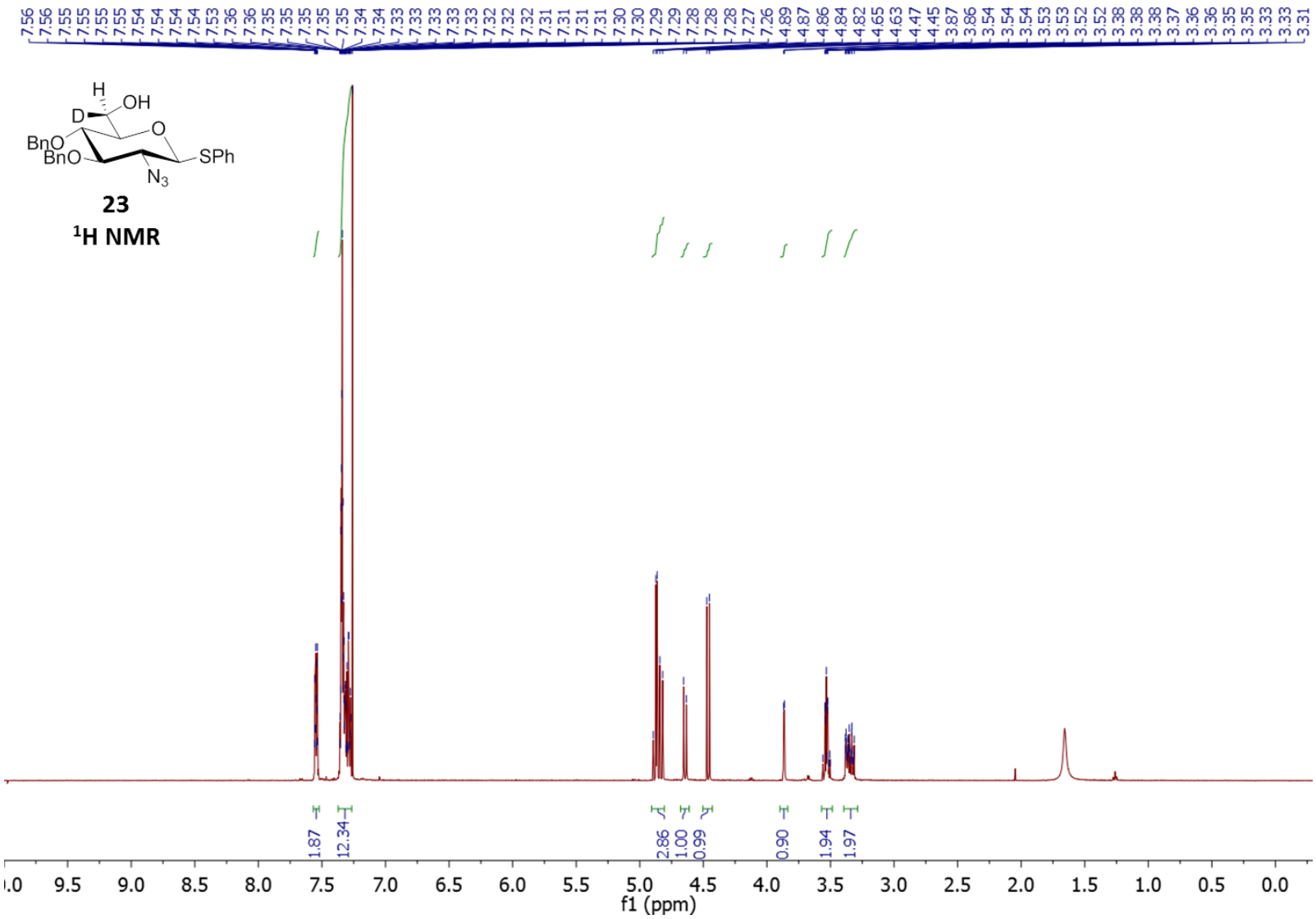
101.03
96.88
83.33
80.11
73.75
73.57
69.88
69.35
68.93
67.77
66.20
63.26
53.93
49.50
48.65
34.50
27.79
20.90
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

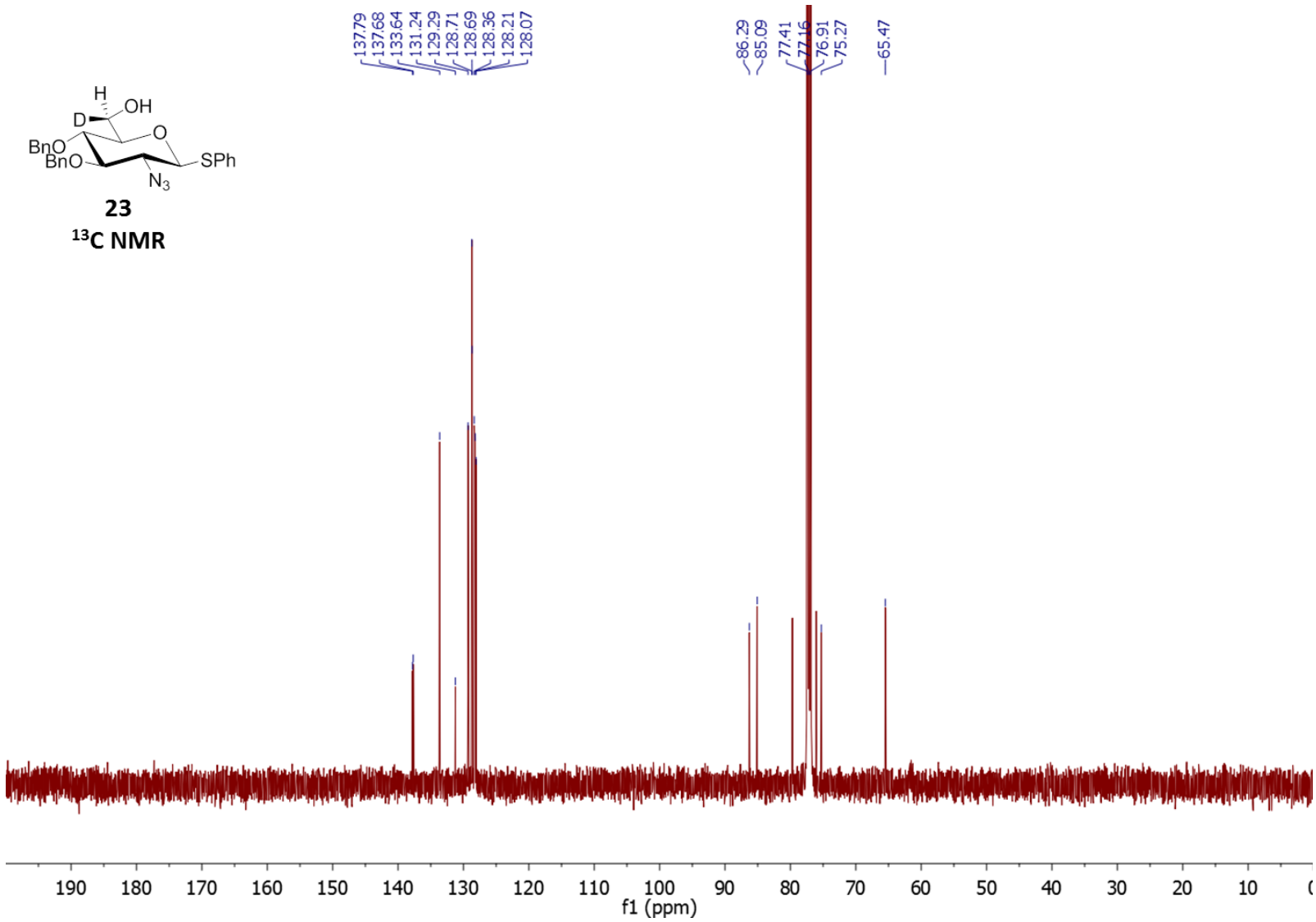

7.81 7.79 7.52 7.51 7.50 7.50 7.49 7.35 7.35 7.34 7.34 7.34 7.32 7.32 7.31 7.31 7.30 7.30 7.29 7.29 7.28 7.28 7.26 7.26 7.20 7.20 7.18 7.18 4.88 4.85 4.81 4.80 4.79 4.78 4.52 4.49 4.35 4.34 4.32 4.32 4.15 4.14 3.49 3.48 3.48 3.47 3.46 3.45 3.45 3.44 3.42 3.27 3.25 3.23 2.40
D
OTs
H
O
BnO
BnO
SPh
N3
21
1H NMR
1.99 2.19 12.94 2.12 0.96 1.80 1.00 1.02 1.01 2.64 0.99 2.87
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

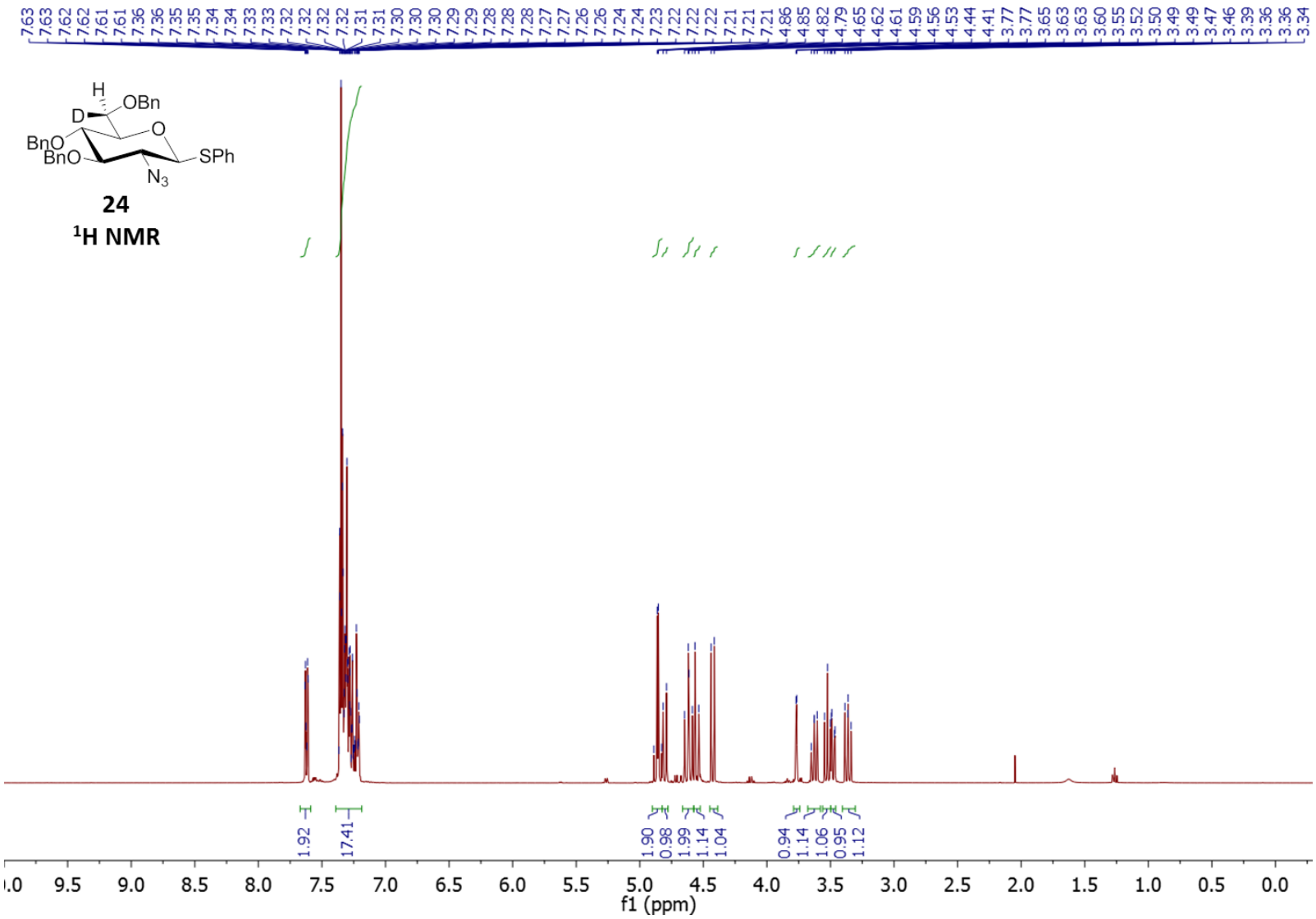

145.15
137.50
137.39
134.03
132.93
130.69
130.05
129.17
128.70
128.27
128.16
128.12
85.97
84.98
77.48
77.16
76.84
76.04
75.28
64.98
21.79
D
OTs
H
O
BnO
BnO
SPh
N3
21
13C NMR
f1 (ppm)

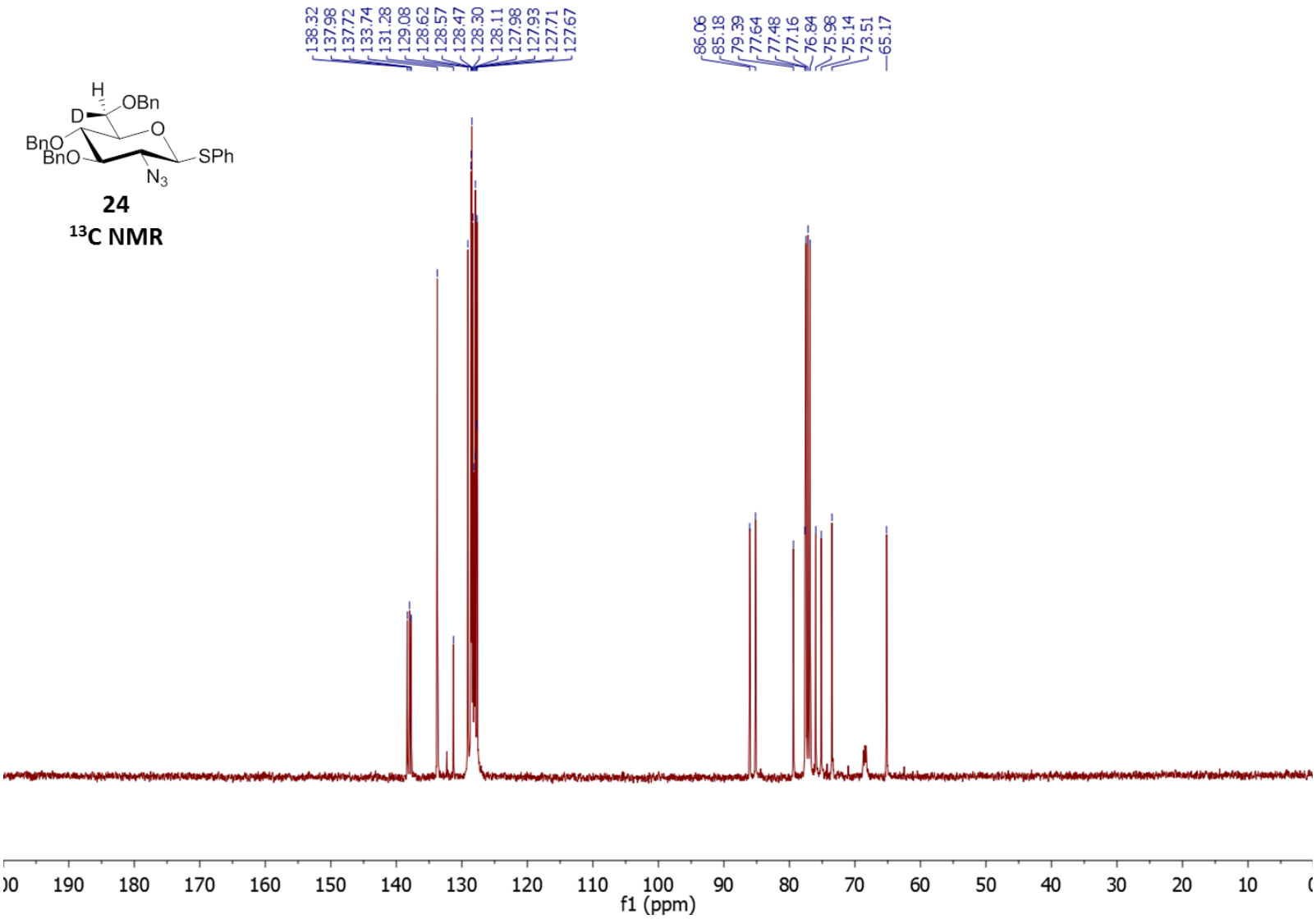

8.03 8.02 8.02 8.01 8.00 7.62 7.60 7.57 7.57 7.57 7.55 7.55 7.49 7.48 7.47 7.45 7.37 7.37 7.37 7.36 7.36 7.35 7.34 7.34 7.33 7.33 7.32 7.32 7.32 7.31 7.30 7.30 7.29 7.28 7.28 7.28 7.27 7.27 7.26 7.26 7.25 7.25 7.24 7.15 7.14 7.13 7.12 7.11 4.93 4.90 4.88 4.87 4.85 4.69 4.69 4.62 4.60 4.46 4.43 3.65 3.64 3.60 3.60 3.60 3.59 3.59 3.58 3.58 3.57 3.35
H
OBz
D
O
BnO
BnO
SPh
N3
22
1H NMR
1.83
1.04
1.87
1.98
10.46
1.86
0.99
1.84
0.86
1.06
1.00
2.89
1.09
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

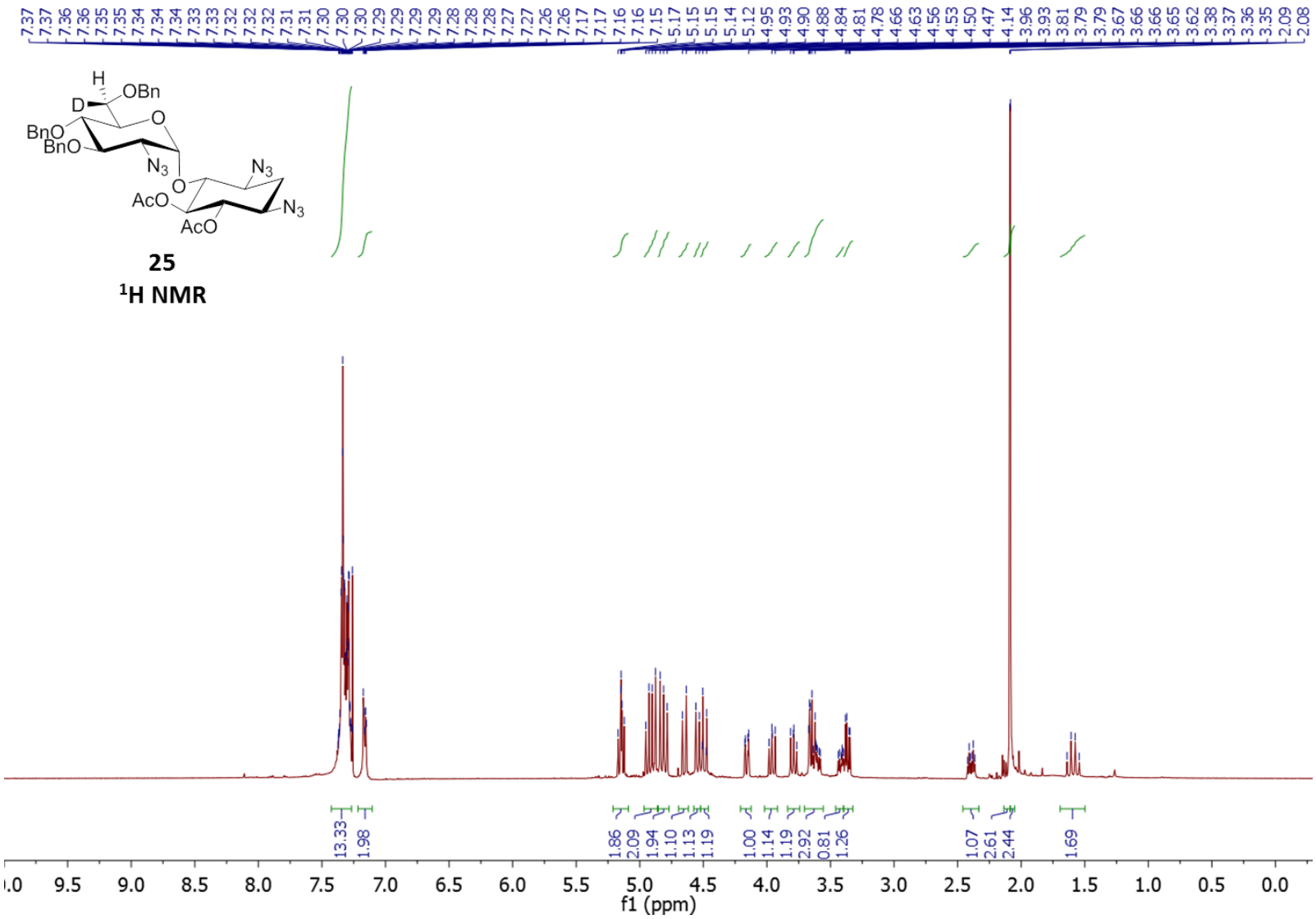

166.10
137.41
137.38
134.30
133.37
130.47
129.91
129.86
129.04
128.77
128.71
128.69
128.58
128.50
128.35
128.28
128.16
85.72
85.21
77.48
77.27
77.22
77.16
76.84
76.26
75.35
64.99
H
OBz
D
O
BnO
BnO
SPh
N3
22
13C NMR
f1 (ppm)

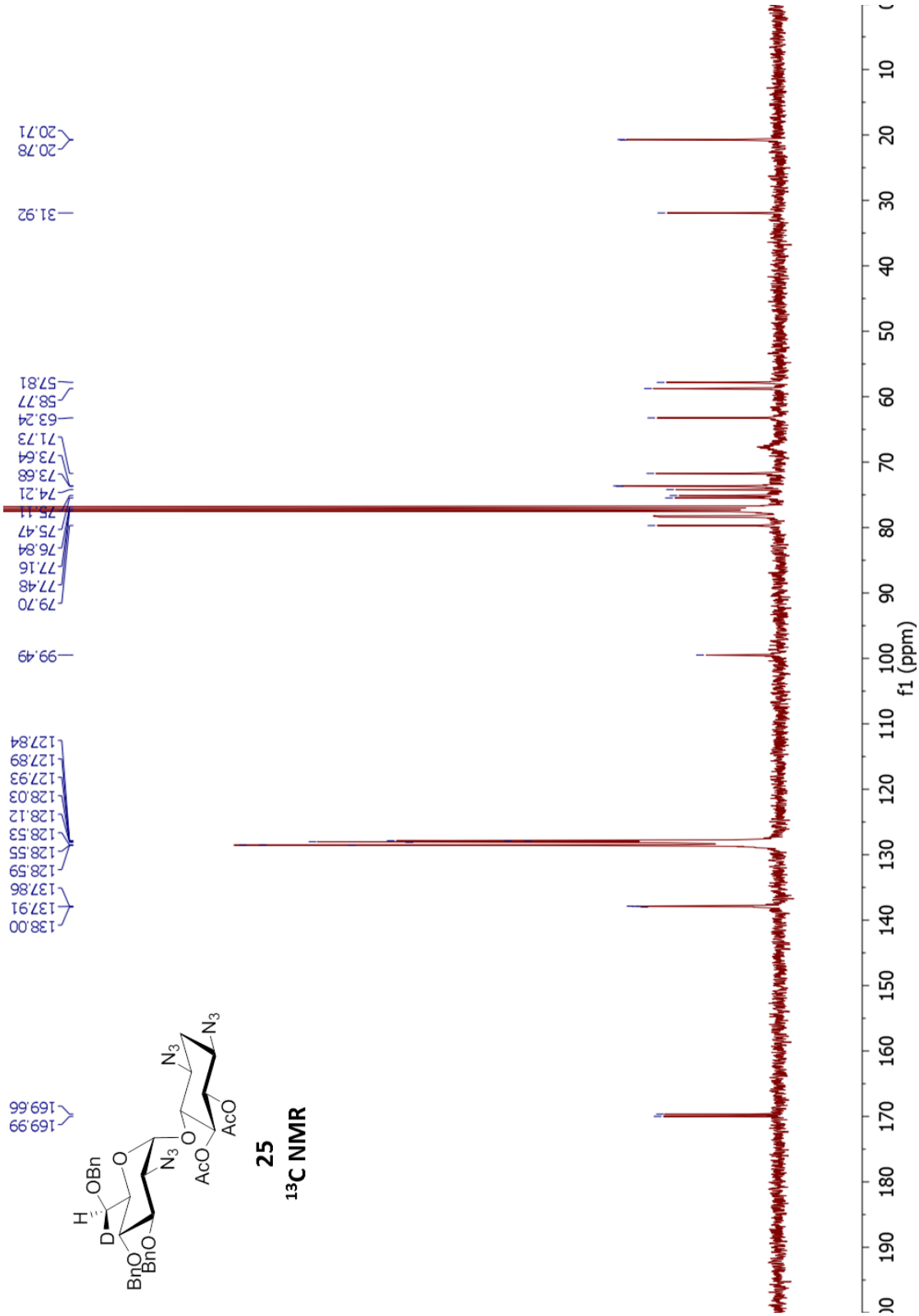

H
OH
D
O
BnO
BnO
SPh
N3
23
1H NMR
1.87
12.34
2.86
1.00
0.99
0.90
1.94
1.97
f1 (ppm)

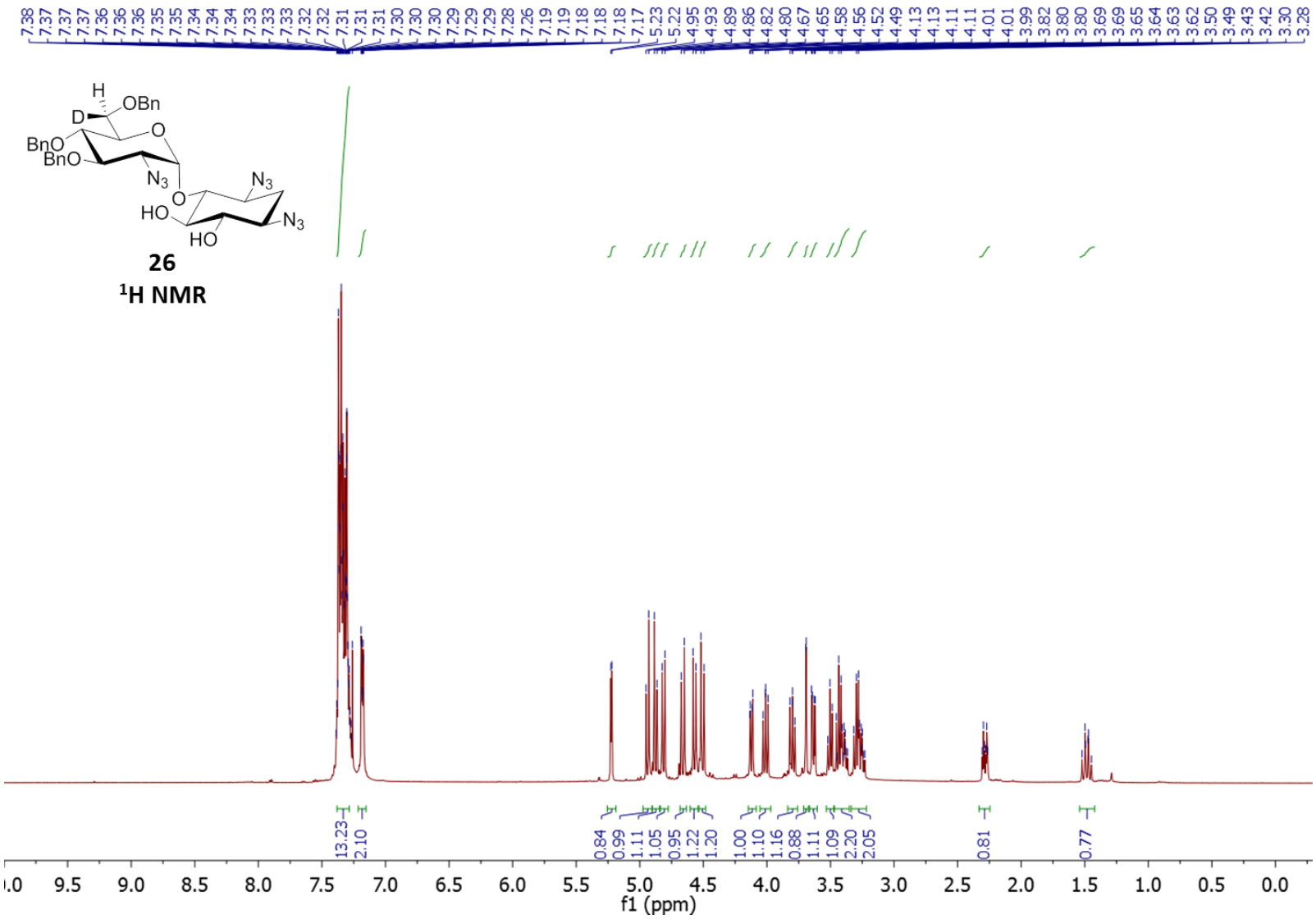

137.79
137.68
133.64
131.24
129.29
128.71
128.69
128.36
128.21
128.07
86.29
85.09
77.41
77.16
76.91
75.27
65.47
H
OH
D
O
BnO
BnO
SPh
N3
23
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

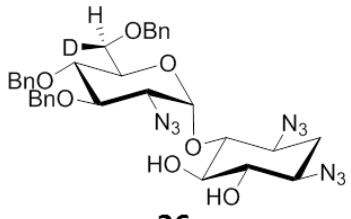

7.63 7.63 7.62 7.62 7.61 7.61 7.36 7.36 7.35 7.35 7.34 7.34 7.33 7.33 7.32 7.32 7.32 7.31 7.31 7.30 7.30 7.30 7.29 7.29 7.28 7.28 7.28 7.27 7.27 7.26 7.26 7.24 7.24 7.23 7.22 7.22 7.22 7.21 7.21 7.21 4.86 4.85 4.82 4.79 4.65 4.62 4.61 4.59 4.56 4.53 4.44 4.41 3.77 3.77 3.65 3.63 3.63 3.60 3.55 3.52 3.50 3.49 3.49 3.47 3.46 3.39 3.36 3.36 3.34
H
OBn
D
O
BnO
BnO
SPh
N3
24
1H NMR
1.92
17.41
1.90
0.98
1.99
1.14
1.04
0.94
1.14
1.06
0.95
1.12
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


138.32
137.98
137.72
133.74
131.28
129.08
128.62
128.57
128.47
128.30
128.11
127.98
127.93
127.71
127.67
86.06
85.18
79.39
77.64
77.48
77.16
76.84
75.98
75.14
73.51
65.17
H
OBn
D
O
BnO
BnO
SPh
N3
24
13C NMR
f1 (ppm)


H
OBn
D
O
BnO
BnO
N3
O
N3
AcO
N3
AcO
25
1H NMR
f1 (ppm)


20.71
20.78
31.92
57.81
58.77
63.24
71.73
73.64
73.68
74.21
75.11
75.47
76.84
77.16
77.48
79.70
99.49
127.84
127.89
127.93
128.03
128.12
128.53
128.55
128.59
137.86
137.91
138.00
169.66
169.99
25
13C NMR
f1 (ppm)

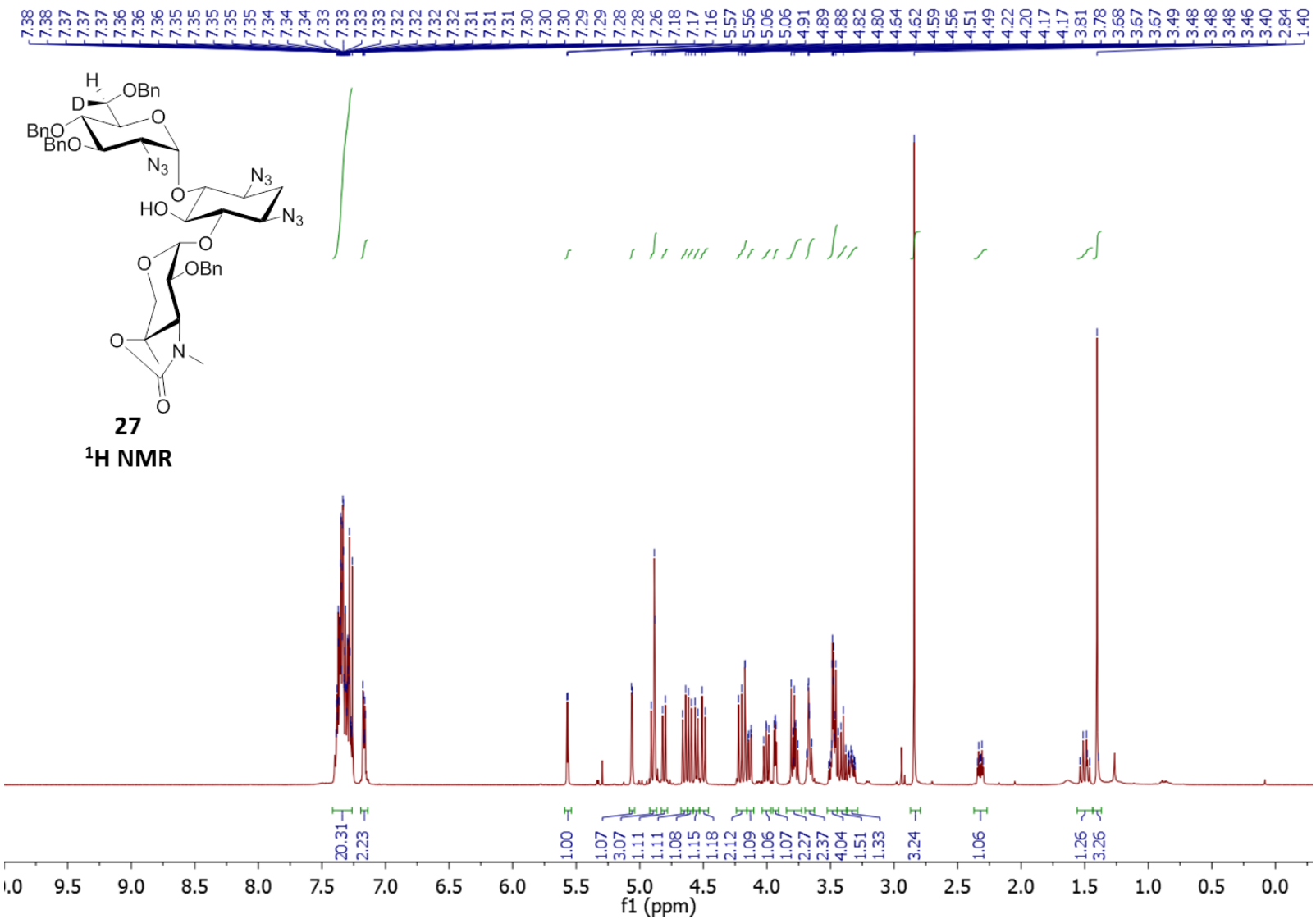

26
1H NMR
f1 (ppm)

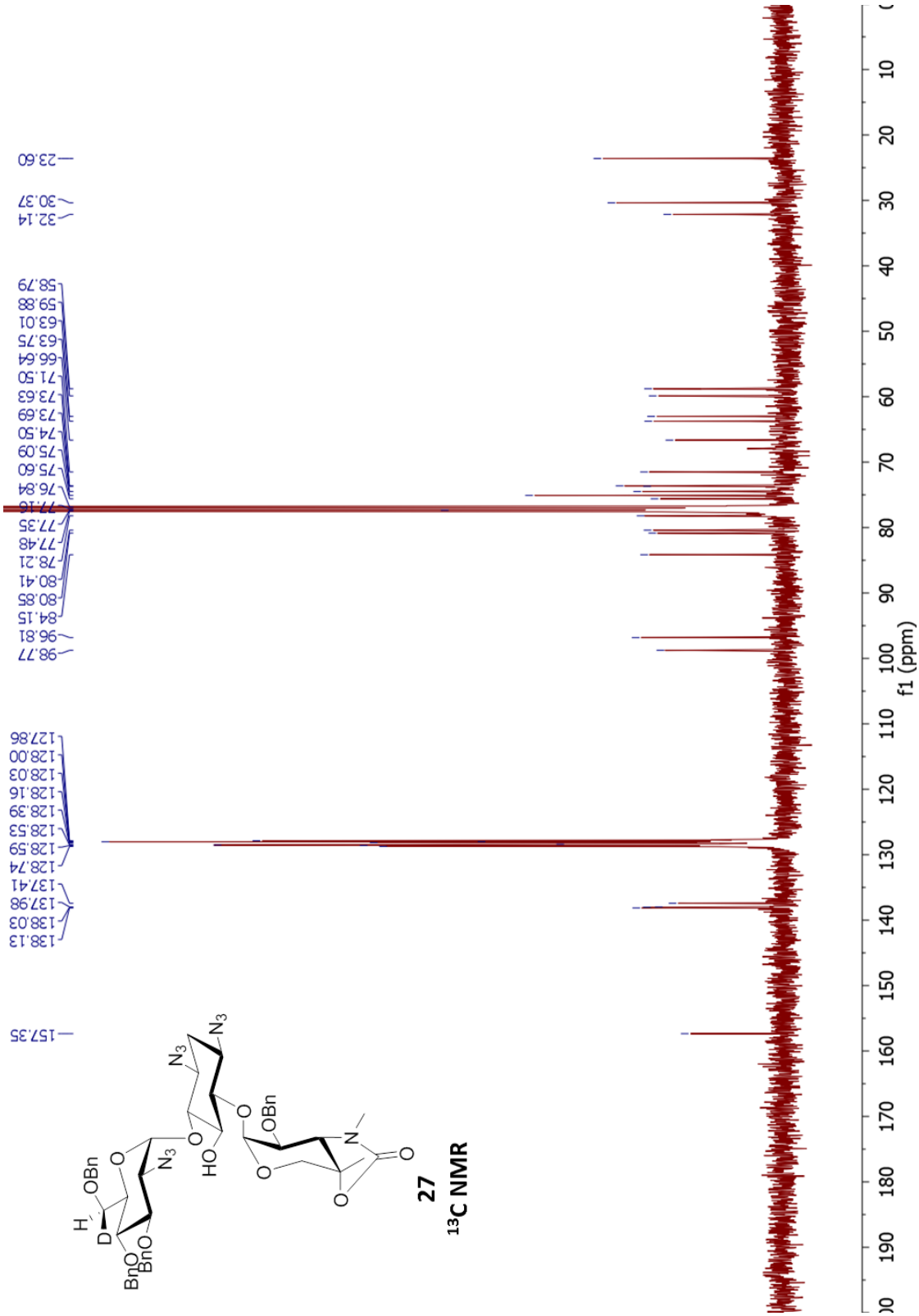

H
OBn
D
O
BnO
BnO
N3
N3
O
HO
N3
HO

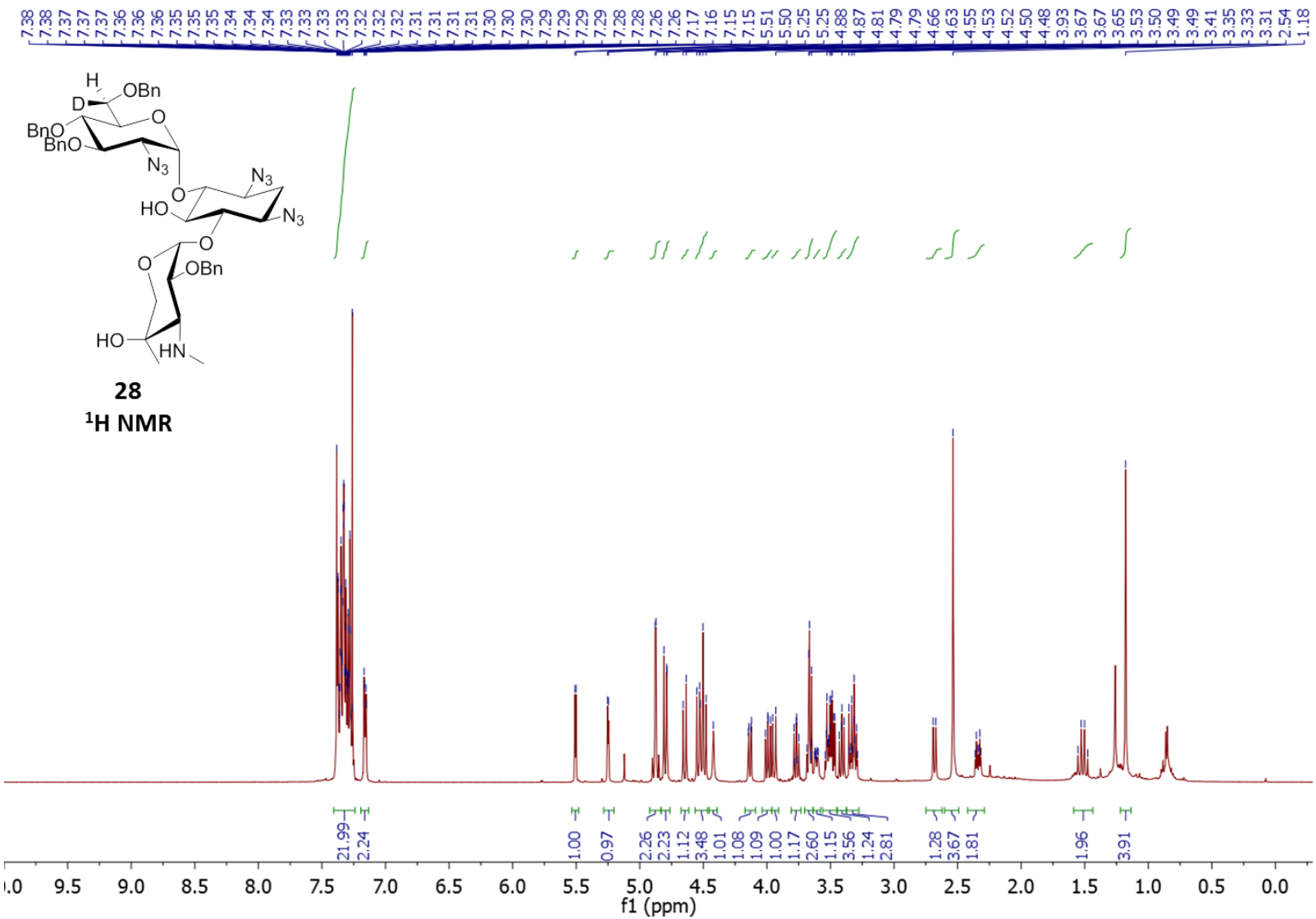
26

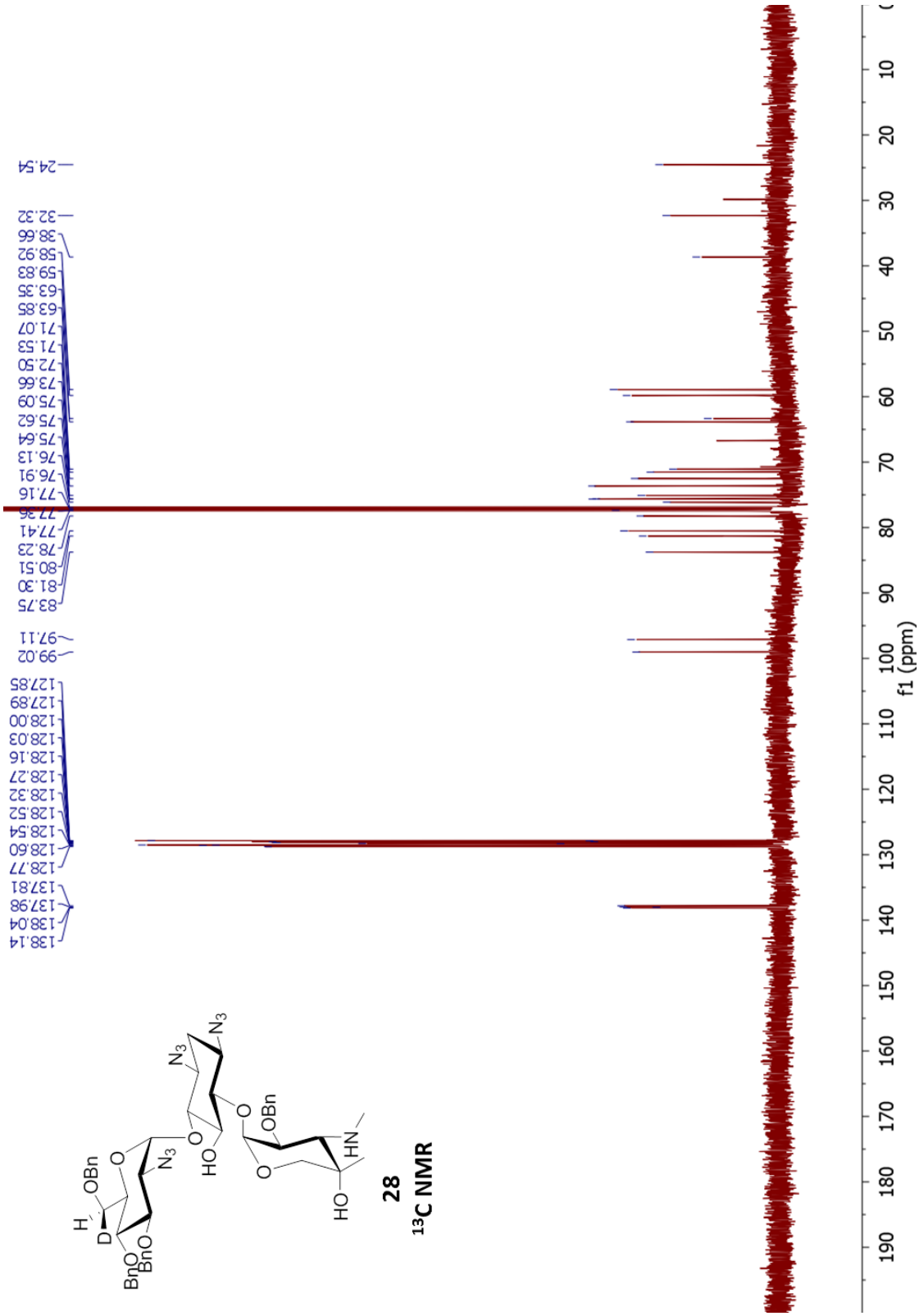
^{13}C NMR

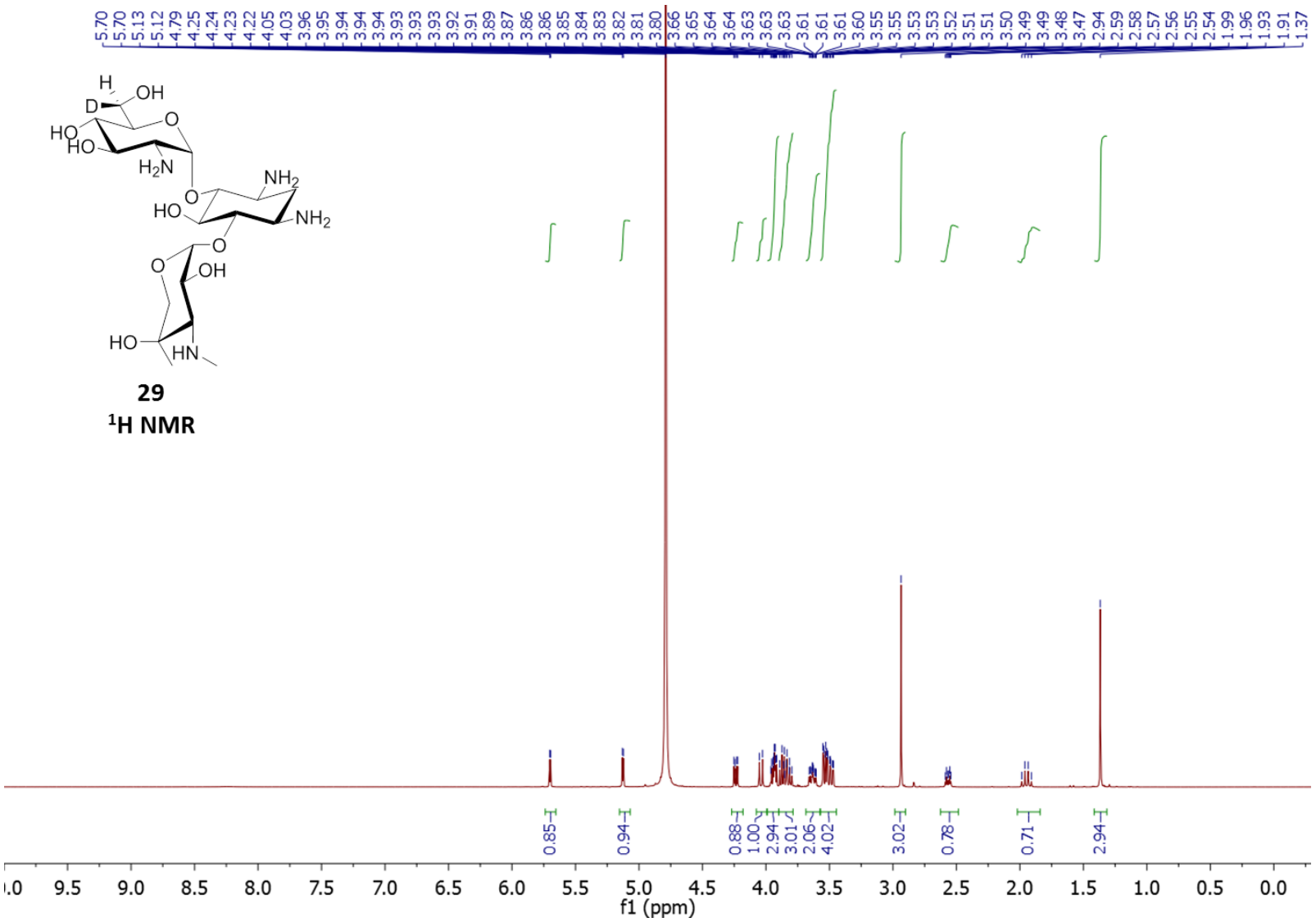
137.92
137.86
137.69
128.57
128.54
128.50
128.15
128.04
127.93
127.87
127.83
99.51
83.56
80.96
78.27
77.41
77.16
76.91
75.69
75.46
75.09
73.65
71.70
67.90
64.30
59.77
58.89
32.15
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

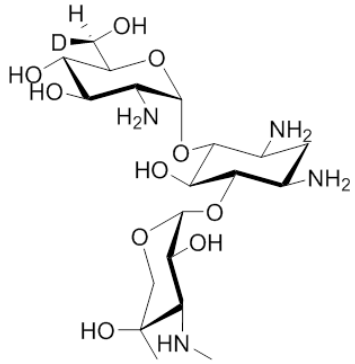

27
1H NMR
f1 (ppm)


157.35
138.13
138.03
137.98
137.41
128.74
128.59
128.53
128.39
128.16
128.03
128.00
127.86
98.77
96.81
84.15
80.85
80.41
78.21
77.48
77.35
77.16
76.84
75.60
75.09
74.50
73.69
73.63
71.50
66.64
63.75
63.01
59.88
58.79
32.14
30.37
23.60
f1 (ppm)
27
13C NMR


28
1H NMR
f1 (ppm)


28
13C NMR
f1 (ppm)

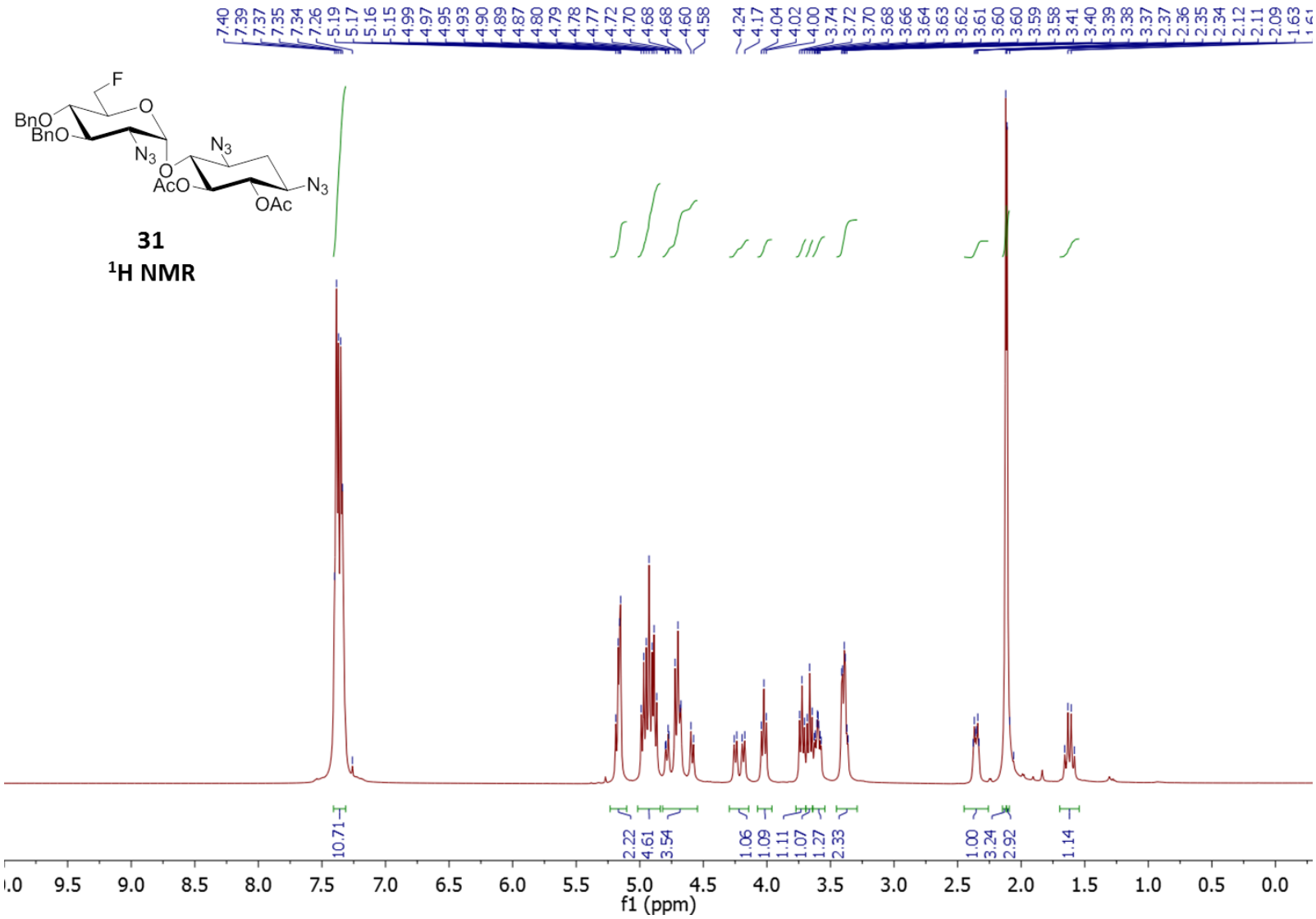

29
1H NMR
f1 (ppm)

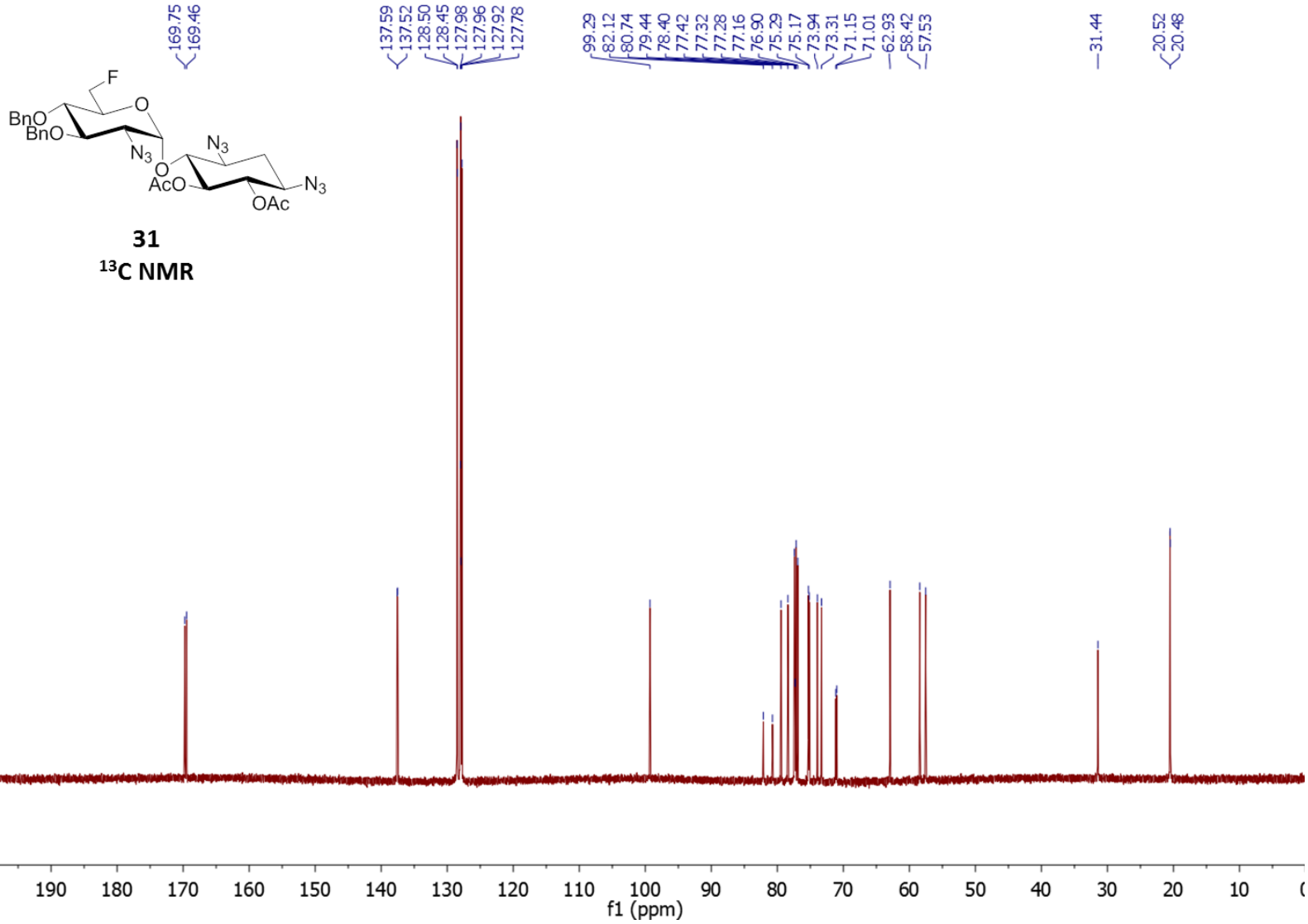

H
D
OH
O
HO
HO
H_2N
NH_2
O
HO
NH_2
O
O
OH
HO
HN

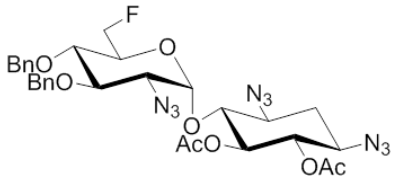
29

^{13}C NMR

101.05
97.01
83.36
80.22
73.69
73.59
69.83
69.27
68.92
67.78
66.17
63.20
53.86
49.43
48.57
34.45
27.81
20.87
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


F
O
BnO
BnO
N3
O
AcO
N3
N3
OAc
31
1H NMR
f1 (ppm)

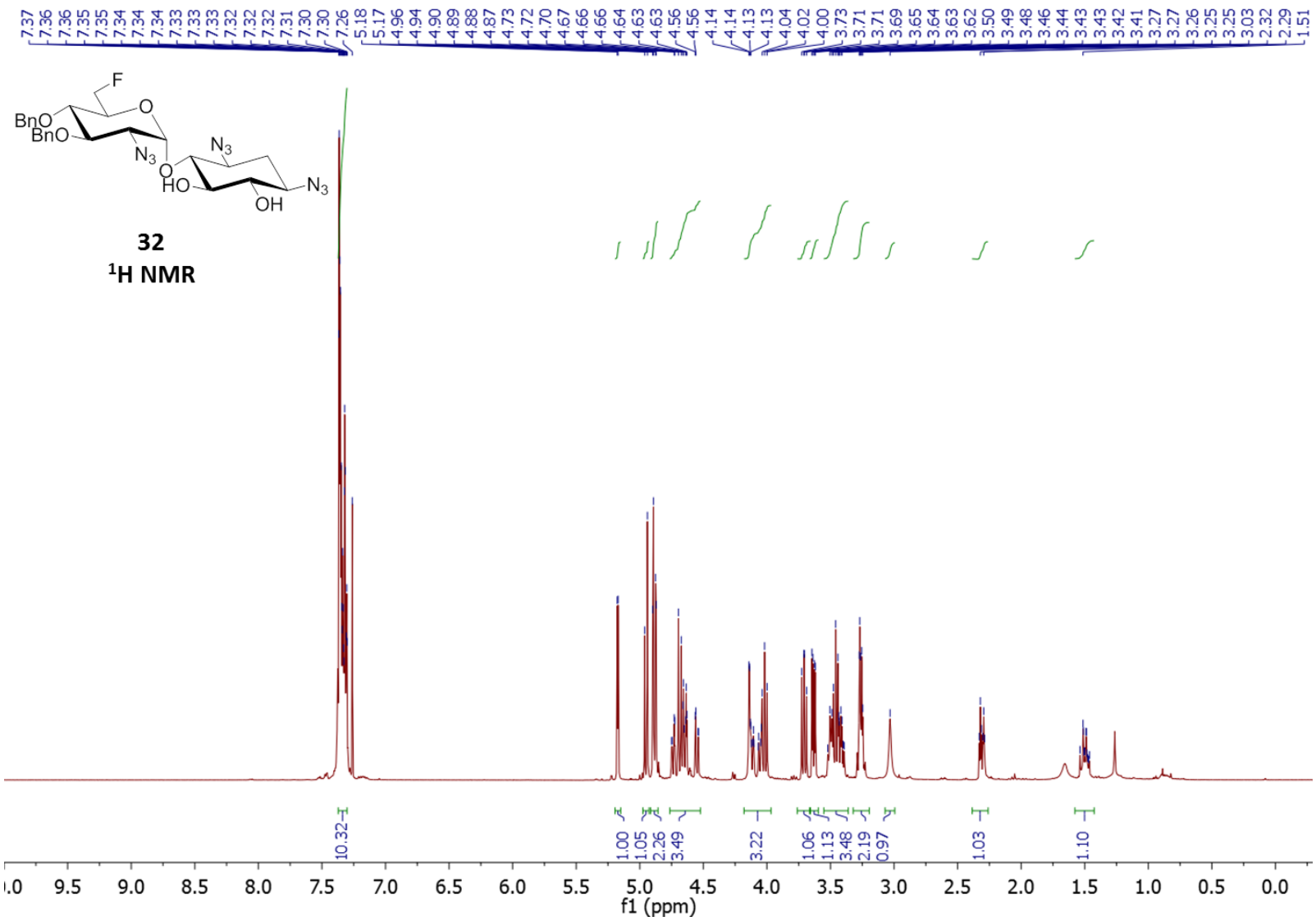

169.75
169.46
137.59
137.52
128.50
128.45
127.98
127.96
127.92
127.78
99.29
82.12
80.74
79.44
78.40
77.42
77.32
77.28
77.16
76.90
75.29
75.17
73.94
73.31
71.15
71.01
62.93
58.42
57.53
31.44
20.52
20.48
F
O
BnO
BnO
N3
O
AcO
N3
N3
OAc
31
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

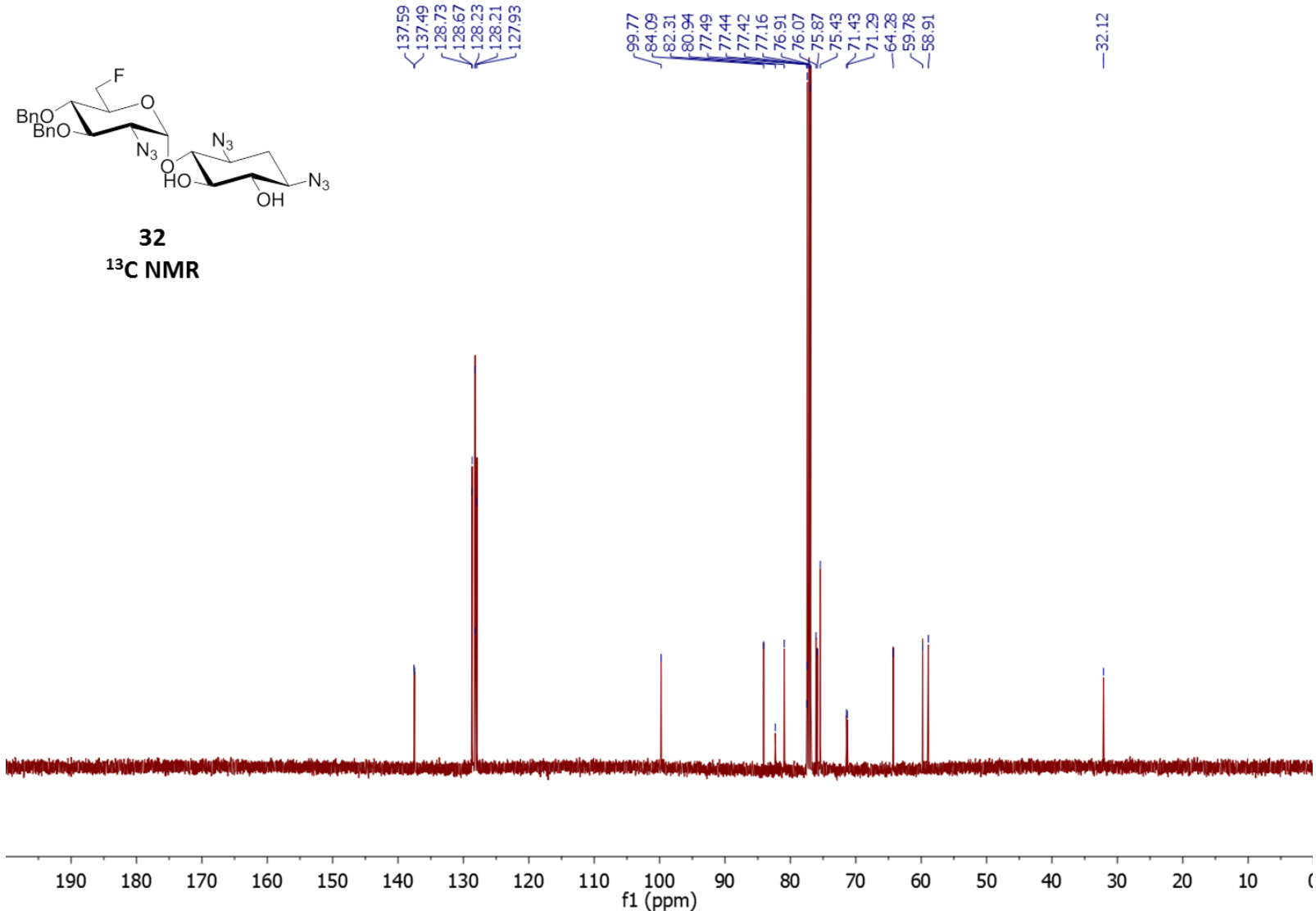

F
O
BnO
BnO
N3
O
N3
AcO
N3
OAc

31

^{19}F NMR

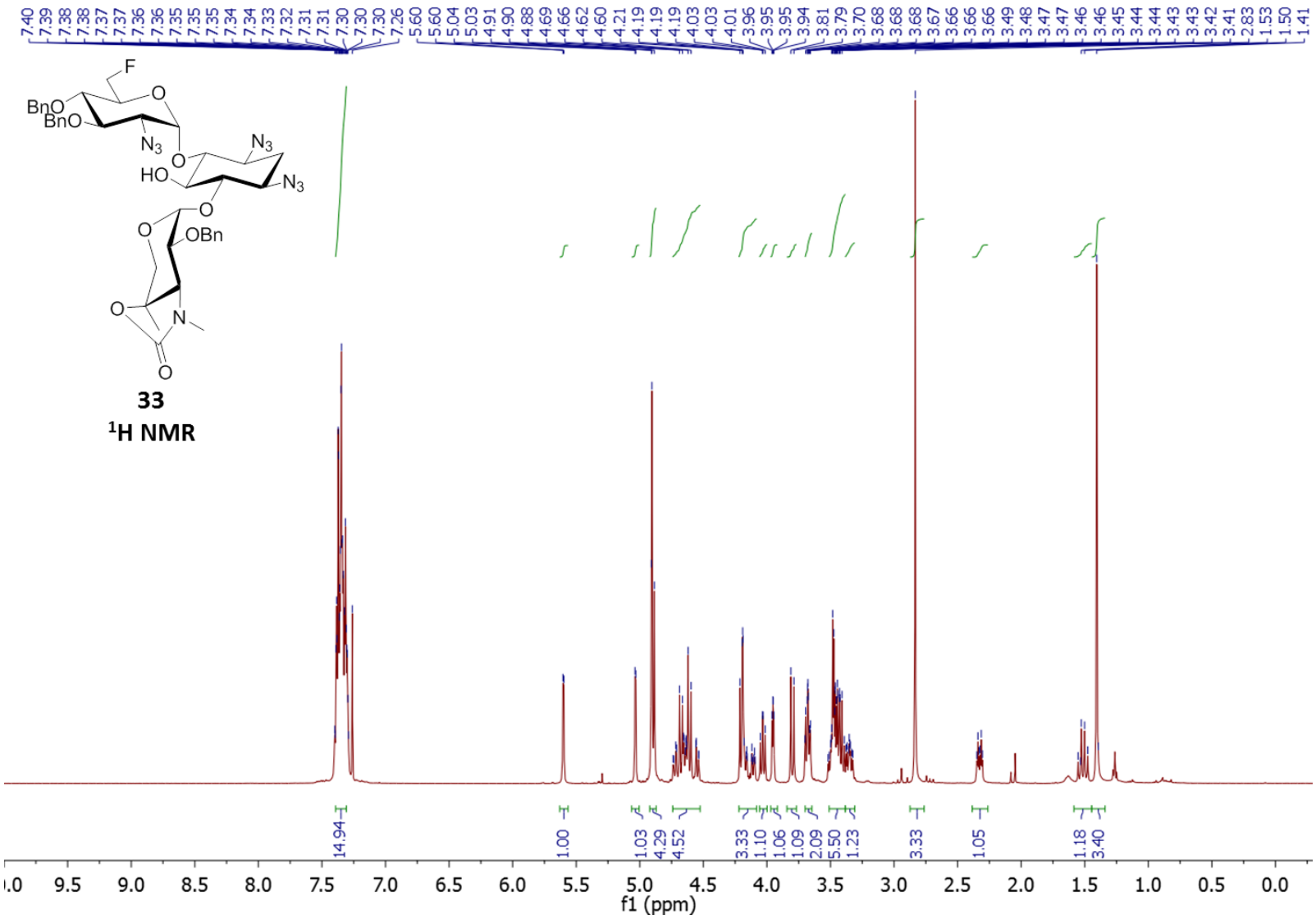
-234.35
-234.43
-234.48
-234.56
-234.61
-234.69
0.0 -230.5 -231.0 -231.5 -232.0 -232.5 -233.0 -233.5 -234.0 -234.5 -235.0 -235.5 -236.0 -236.5 -237.0 -237.5 -238.0 -238.5 -239.0 -239.5 -24
f1 (ppm)

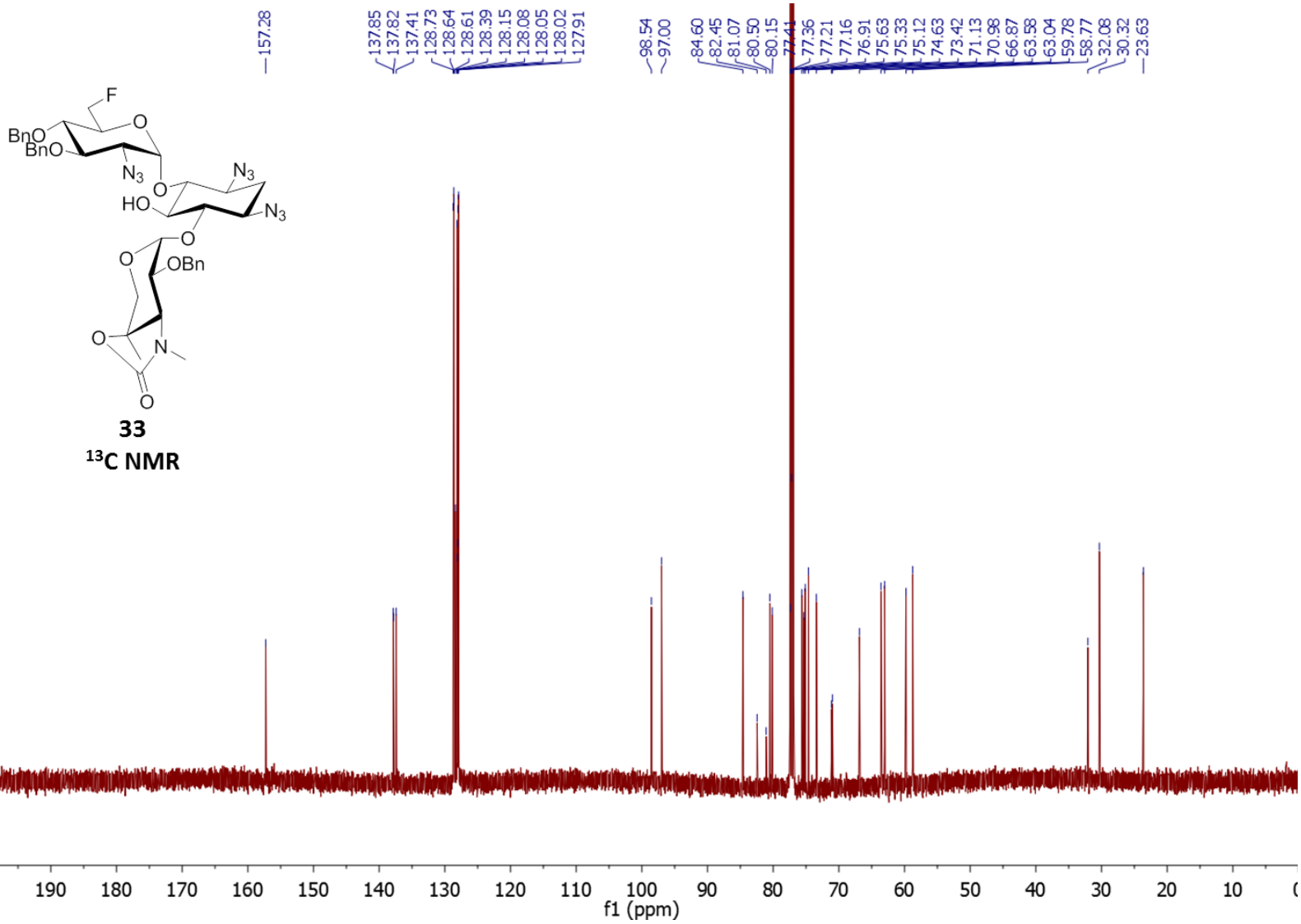

7.37 7.36 7.36 7.35 7.35 7.34 7.34 7.34 7.33 7.33 7.33 7.32 7.32 7.32 7.31 7.30 7.30 7.26 5.18 5.17 4.96 4.94 4.90 4.89 4.88 4.87 4.73 4.72 4.70 4.67 4.66 4.66 4.64 4.63 4.63 4.56 4.56 4.14 4.14 4.13 4.13 4.04 4.02 4.00 3.73 3.71 3.71 3.69 3.65 3.64 3.63 3.62 3.50 3.49 3.48 3.46 3.44 3.43 3.43 3.42 3.41 3.27 3.27 3.26 3.25 3.25 3.03 2.32 2.29 1.51
F
O
BnO
BnO
N3
N3
O
HO
OH
N3
32
1H NMR
10.32
1.00
1.05
2.26
3.49
3.22
1.06
1.13
3.48
2.19
0.97
1.03
1.10
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


137.59
137.49
128.73
128.67
128.23
128.21
127.93
99.77
84.09
82.31
80.94
77.49
77.44
77.42
77.16
76.91
76.07
75.87
75.43
71.43
71.29
64.28
59.78
58.91
32.12
F
O
BnO
BnO
N3
O
N3
HO
OH
N3
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

32

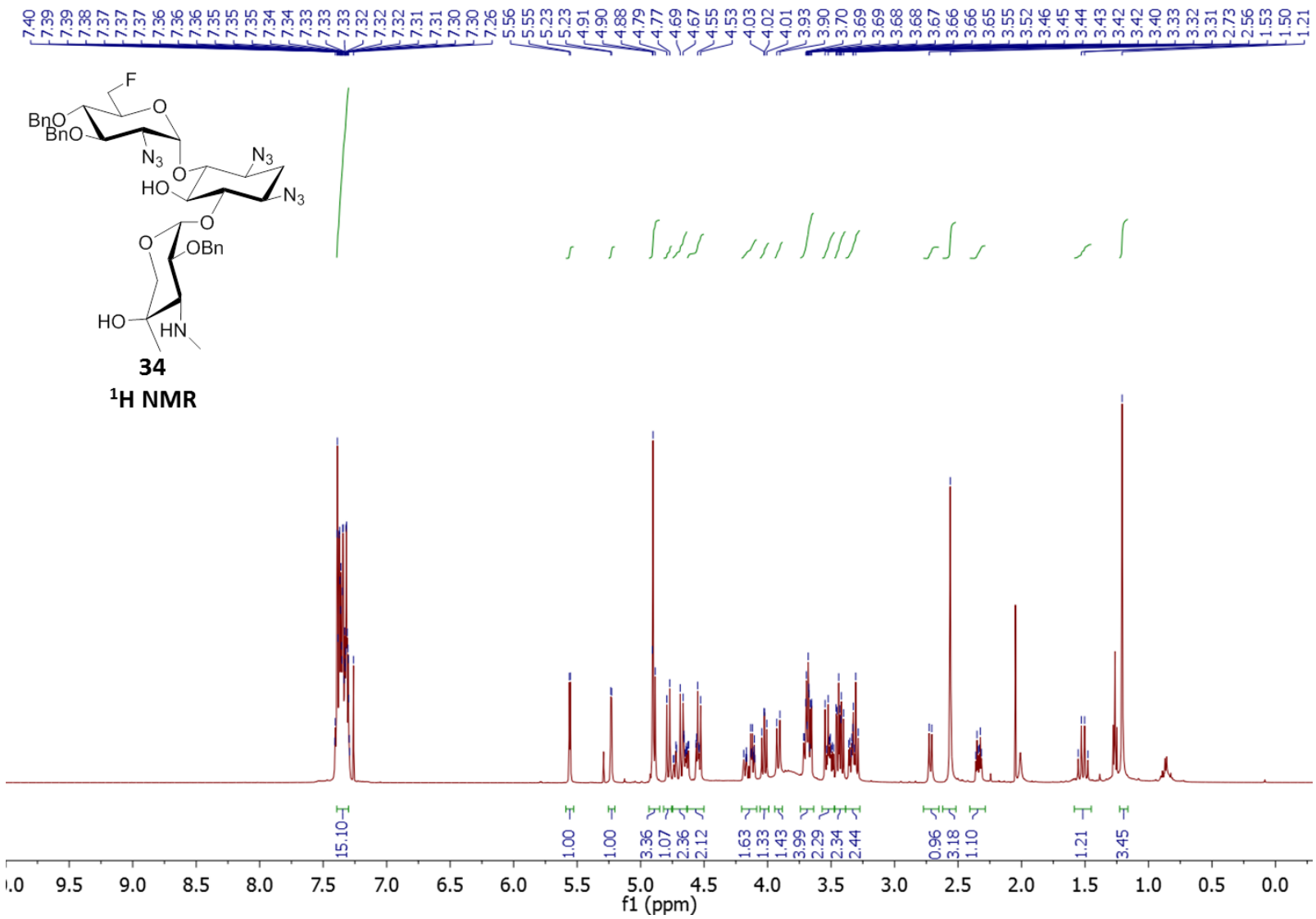
^{13}C NMR

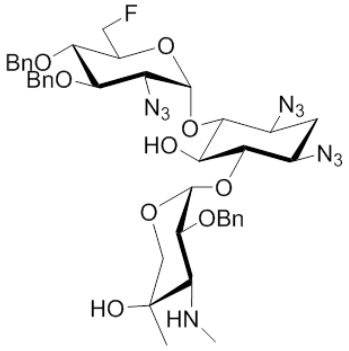

F
BnO
BnO
O
N3
O
HO
N3
N3
O
O
OBn
O
N
O
33
1H NMR
f1 (ppm)


157.28
137.85
137.82
137.41
128.73
128.64
128.61
128.39
128.15
128.08
128.05
128.02
127.91
98.54
97.00
84.60
82.45
81.07
80.50
80.15
77.41
77.36
77.21
77.16
76.91
75.63
75.33
75.12
74.63
73.42
71.13
70.98
66.87
63.58
63.04
59.78
58.77
32.08
30.32
23.63
F
BnO
BnO
O
N3
O
N3
HO
N3
O
O
OBn
O
N
O
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

33

^{13}C NMR

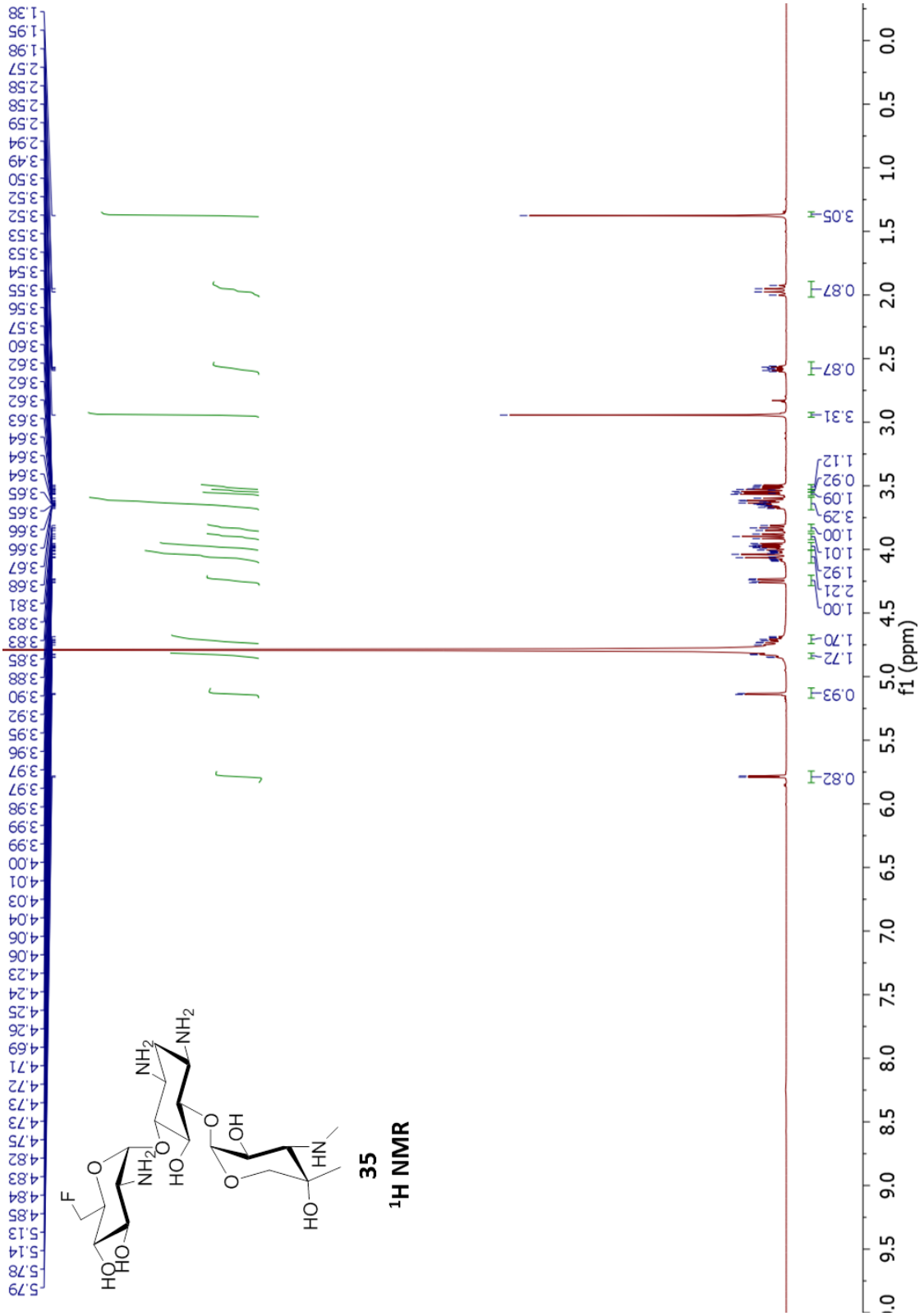

7.40 7.39 7.39 7.38 7.37 7.37 7.37 7.36 7.36 7.36 7.35 7.35 7.35 7.34 7.34 7.33 7.33 7.33 7.32 7.32 7.32 7.31 7.31 7.30 7.30 7.26 5.56 5.55 5.23 4.91 4.90 4.88 4.79 4.77 4.69 4.67 4.55 4.53 4.03 4.02 4.01 3.93 3.90 3.70 3.69 3.69 3.68 3.68 3.67 3.66 3.66 3.65 3.55 3.52 3.46 3.45 3.44 3.43 3.42 3.42 3.40 3.33 3.32 3.31 2.73 2.56 1.53 1.50 1.21
F
BnO
BnO
O
N3
N3
O
HO
N3
O
O
OBn
HO
HN
34
1H NMR
15.10
1.00
1.00
3.36
1.07
2.36
2.12
1.63
1.33
1.43
3.99
2.29
2.34
2.44
0.96
3.18
1.10
1.21
3.45
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

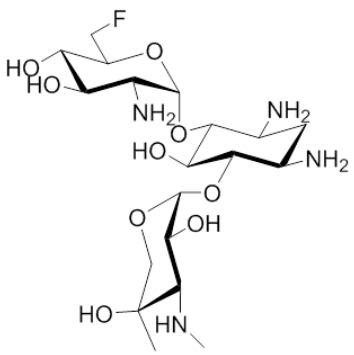

F
BnO
BnO
O
N3
N3
O
HO
N3
O
O
OBn
HO
HN

34

^{13}C NMR

137.80
137.78
137.74
128.72
128.63
128.59
128.28
128.23
128.14
128.13
128.08
128.04
127.90
98.76
97.27
84.42
82.43
81.05
80.81
80.21
77.41
77.37
77.33
77.16
76.90
76.12
75.71
75.63
75.32
72.54
71.12
70.97
70.94
67.07
63.59
63.44
59.65
58.86
38.41
32.21
24.26
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

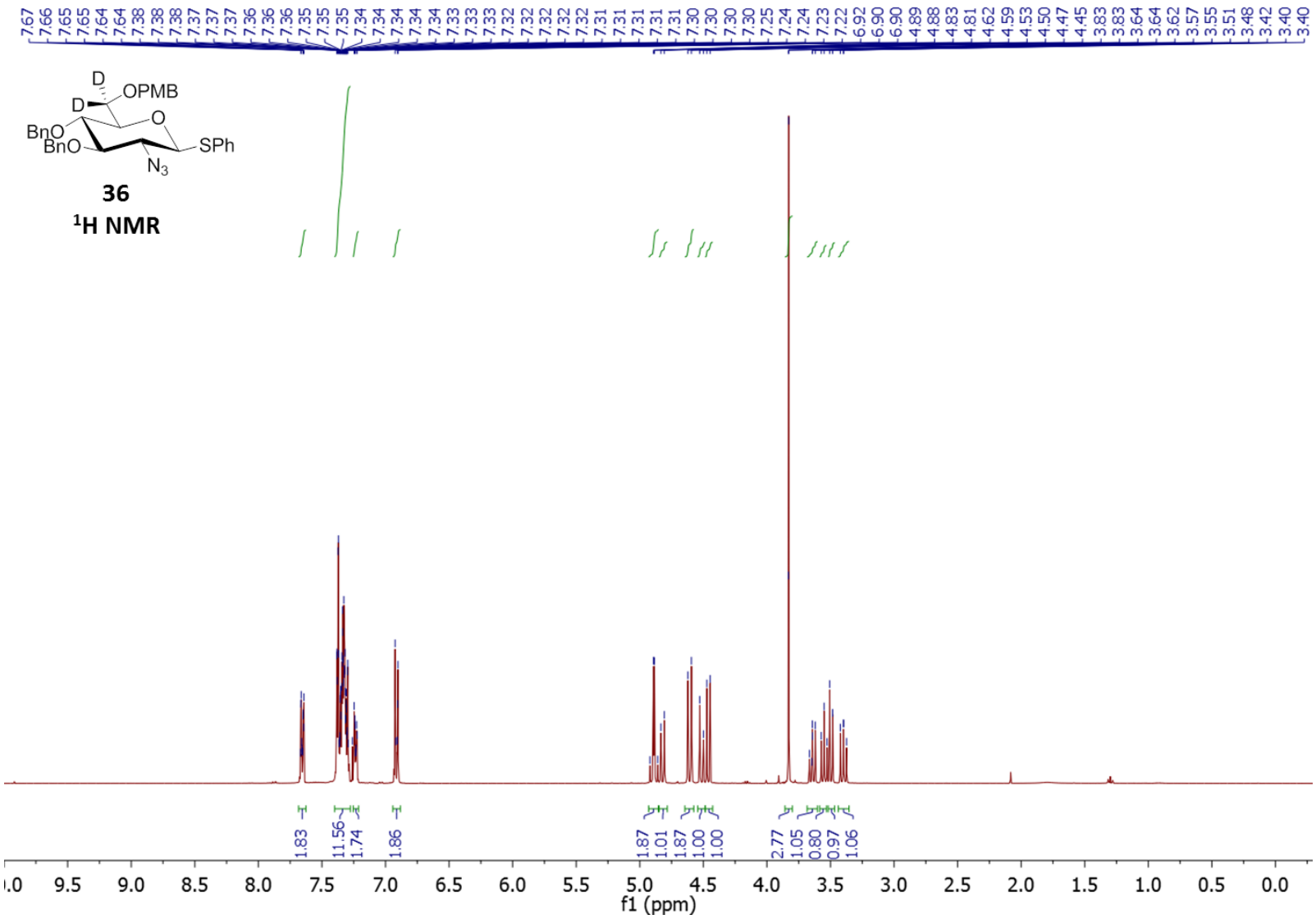

35
^{1}H NMR
f1 (ppm)

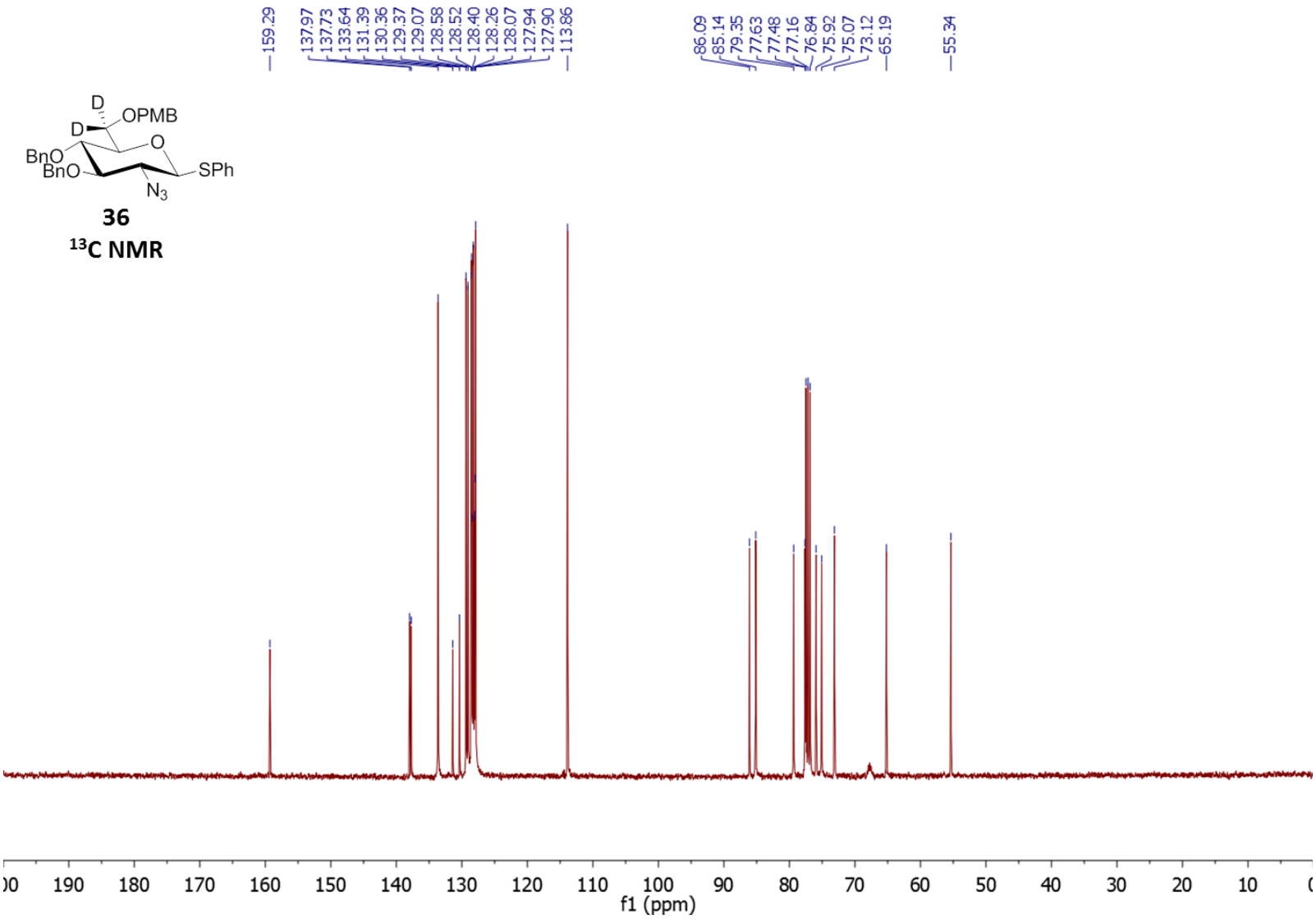

F
HO
HO
O
NH2
NH2
O
HO
NH2
O
O
OH
HO
HN

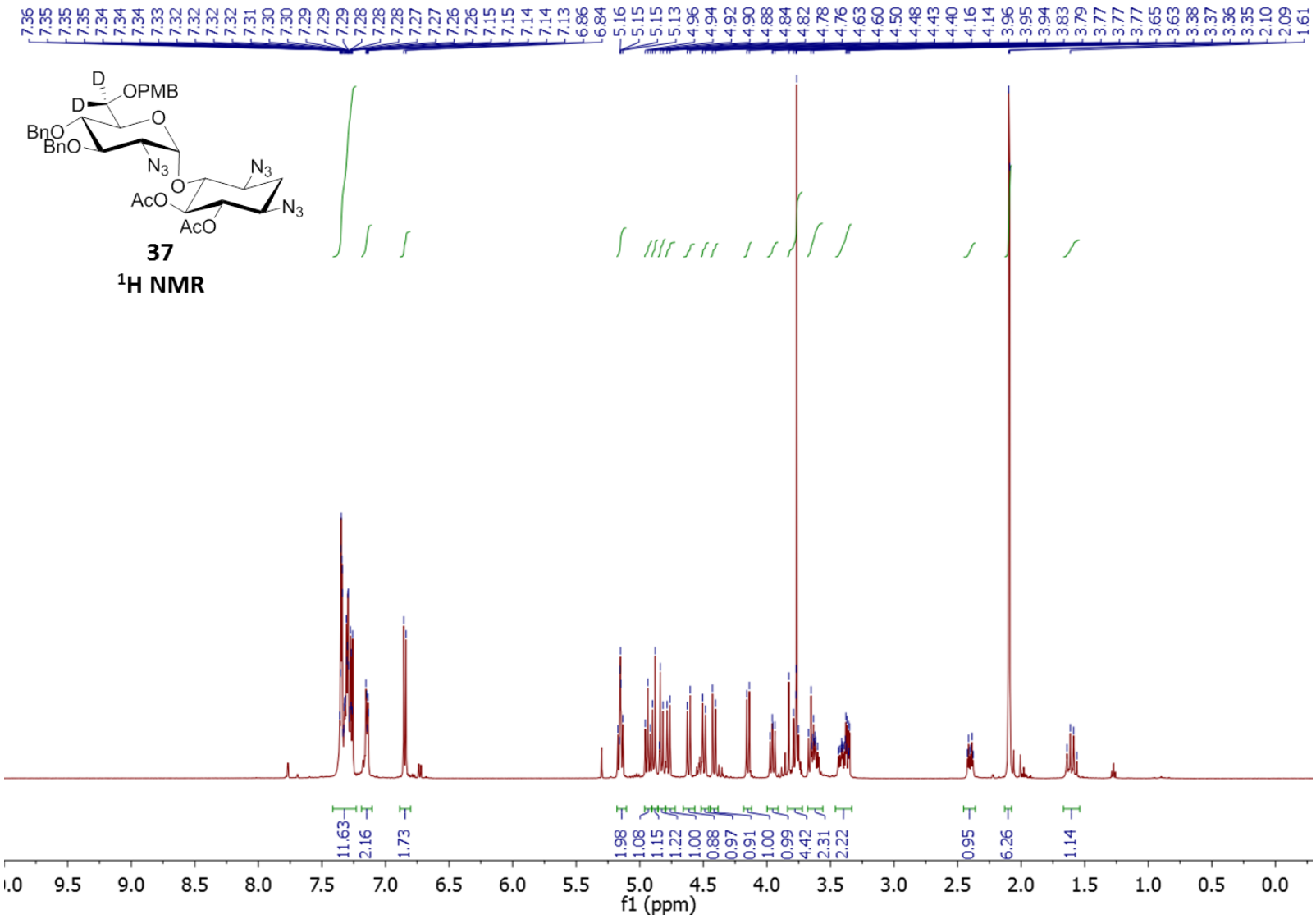
35

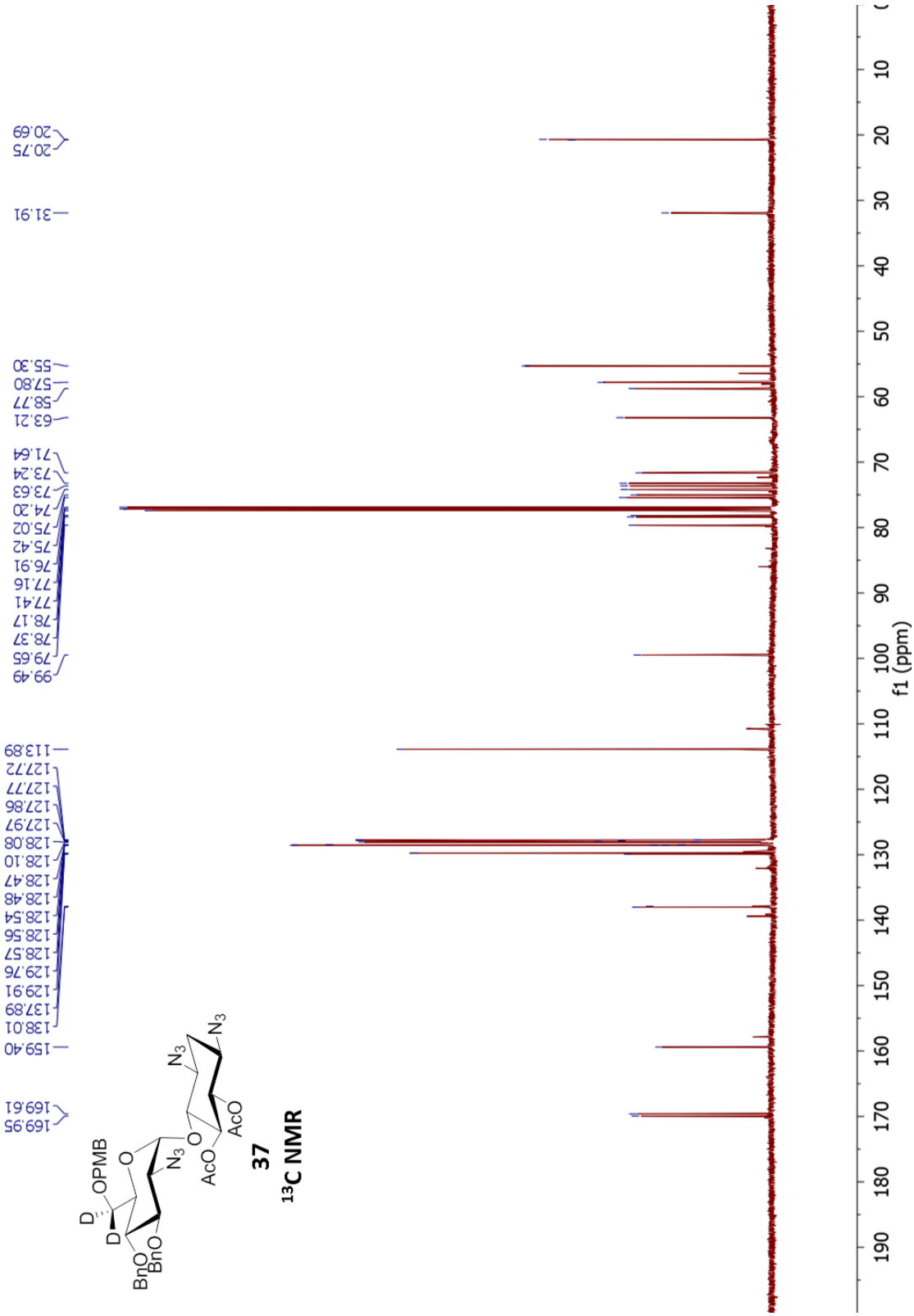
^{13}C NMR

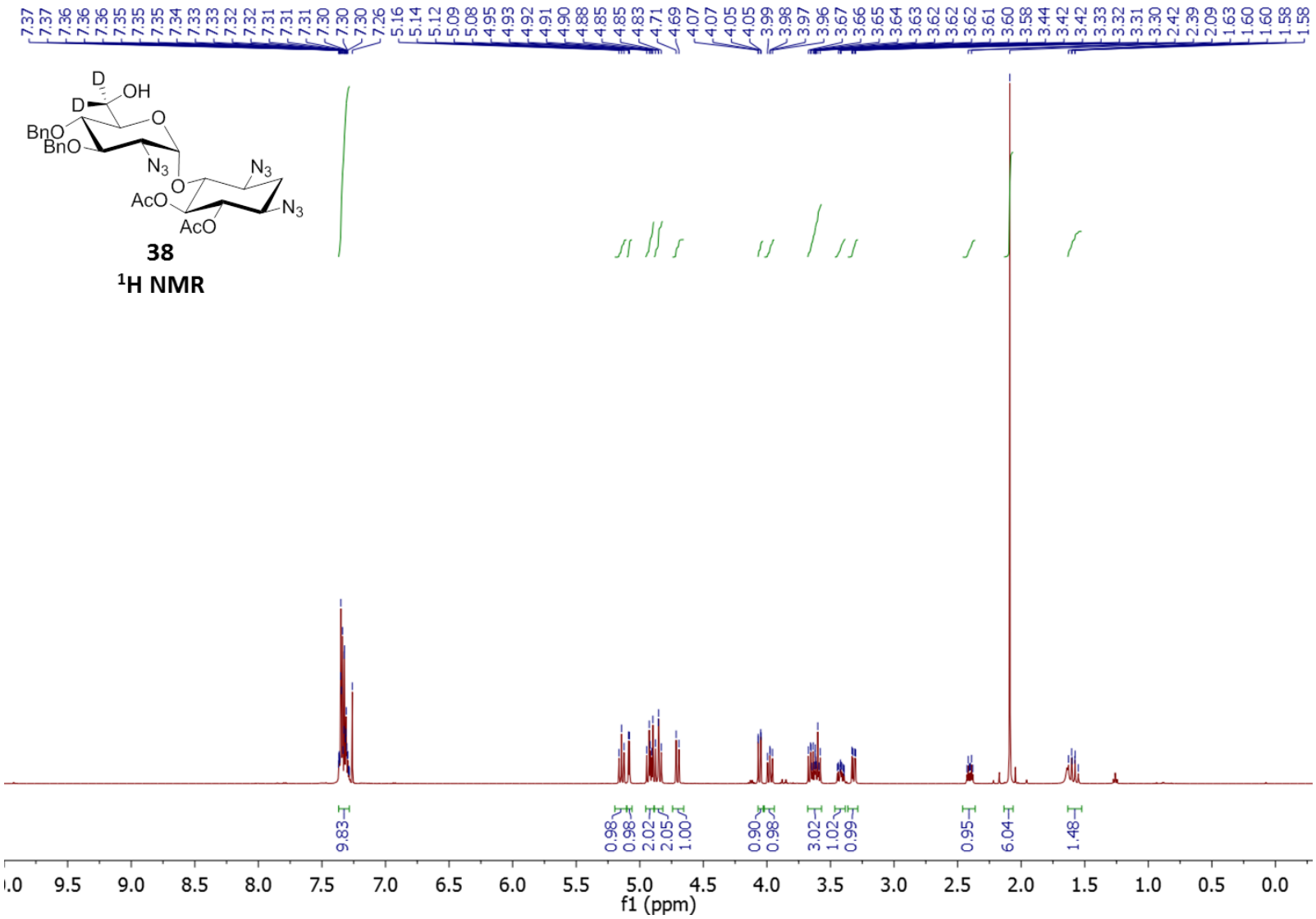
101.07
96.85
83.43
82.87
81.54
79.52
73.84
72.29
72.15
69.85
68.77
68.22
68.16
67.83
66.17
63.19
53.70
49.46
48.37
34.48
27.80
20.90
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

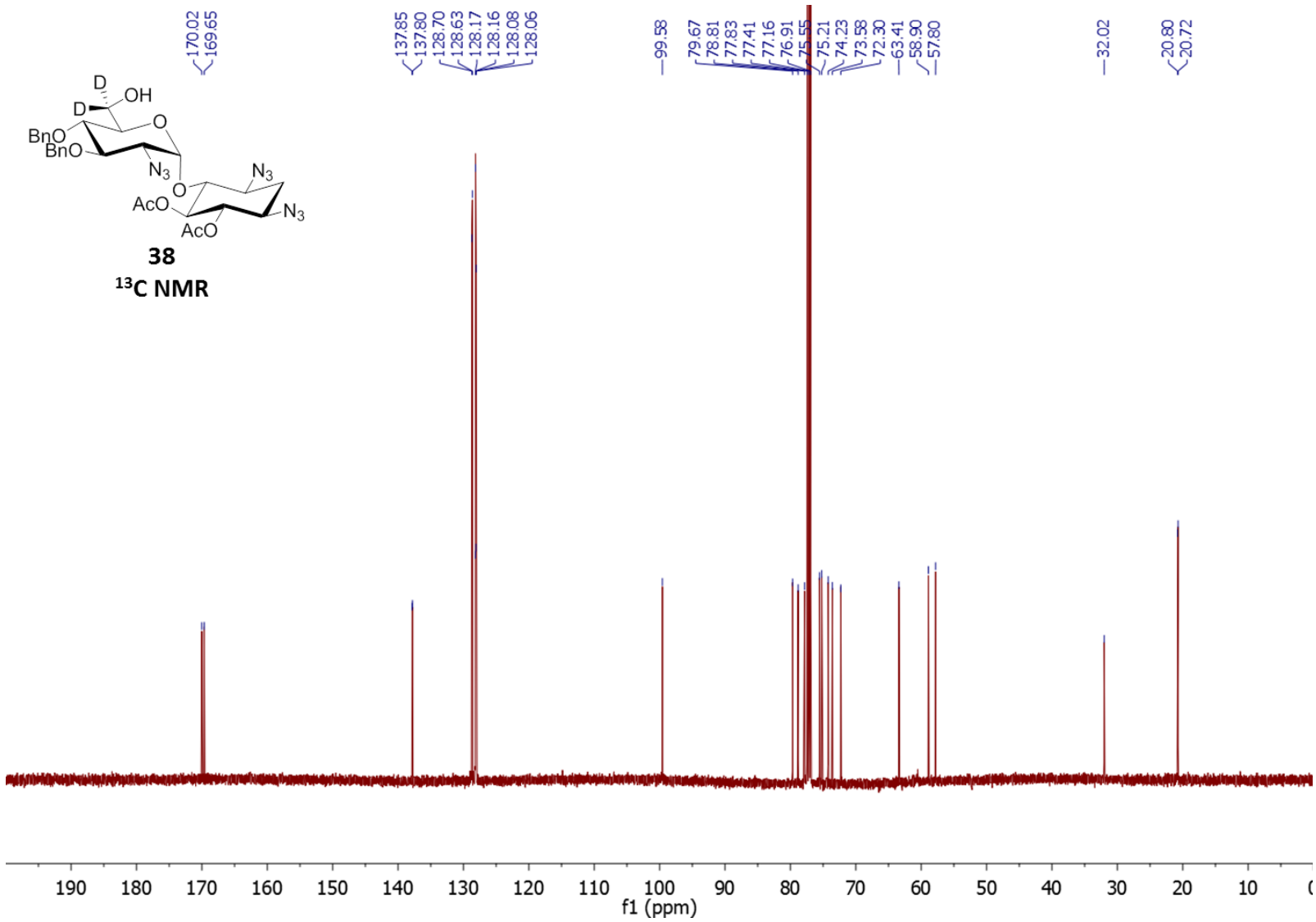

D
OPMB
D
O
BnO
BnO
SPh
N3
36
1H NMR
f1 (ppm)

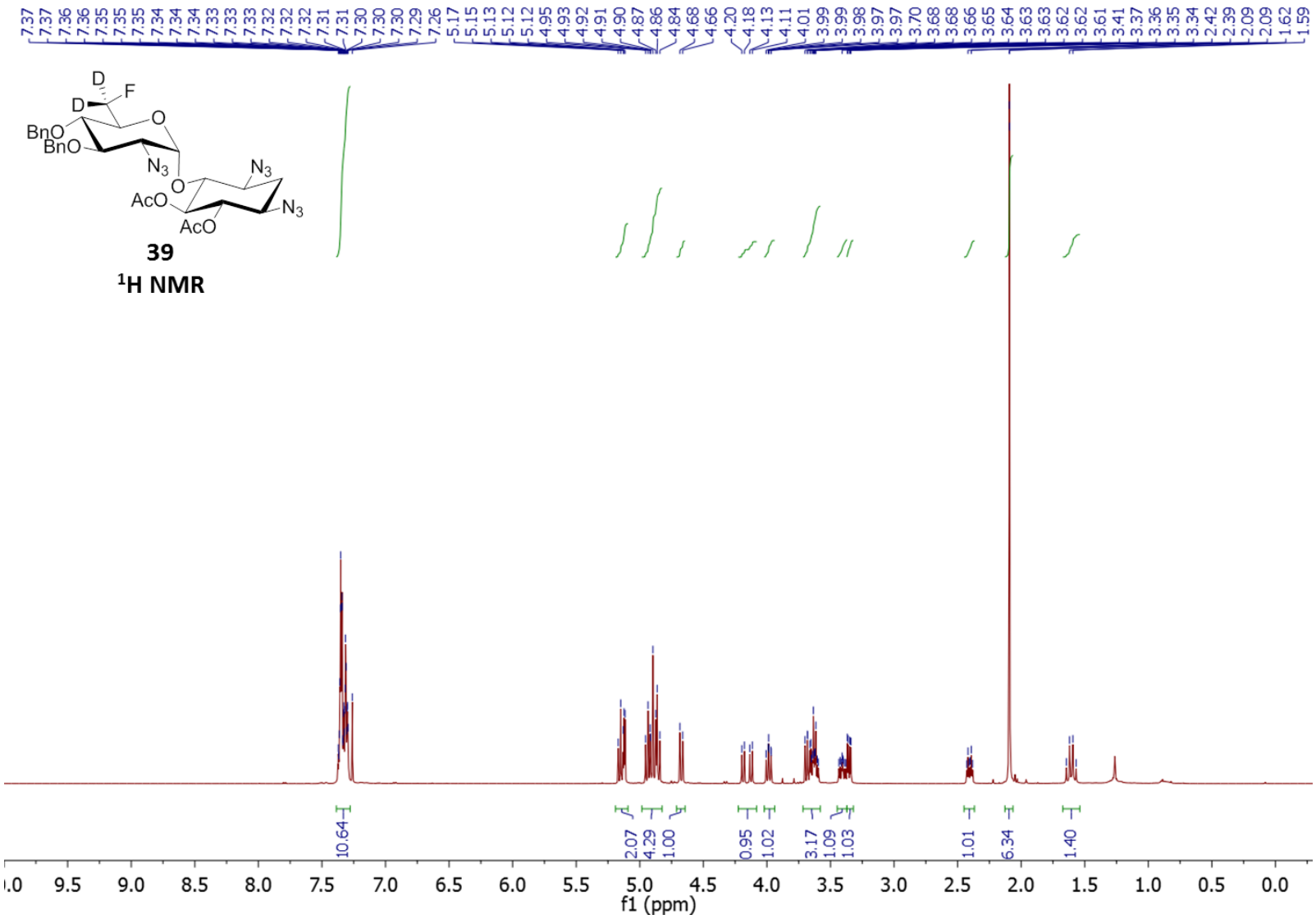

159.29
137.97
137.73
133.64
131.39
130.36
129.37
129.07
128.58
128.52
128.40
128.26
128.07
127.94
127.90
113.86
86.09
85.14
79.35
77.63
77.48
77.16
76.84
75.92
75.07
73.12
65.19
55.34
D
OPMB
D
O
BnO
BnO
SPh
N3
36
13C NMR
f1 (ppm)

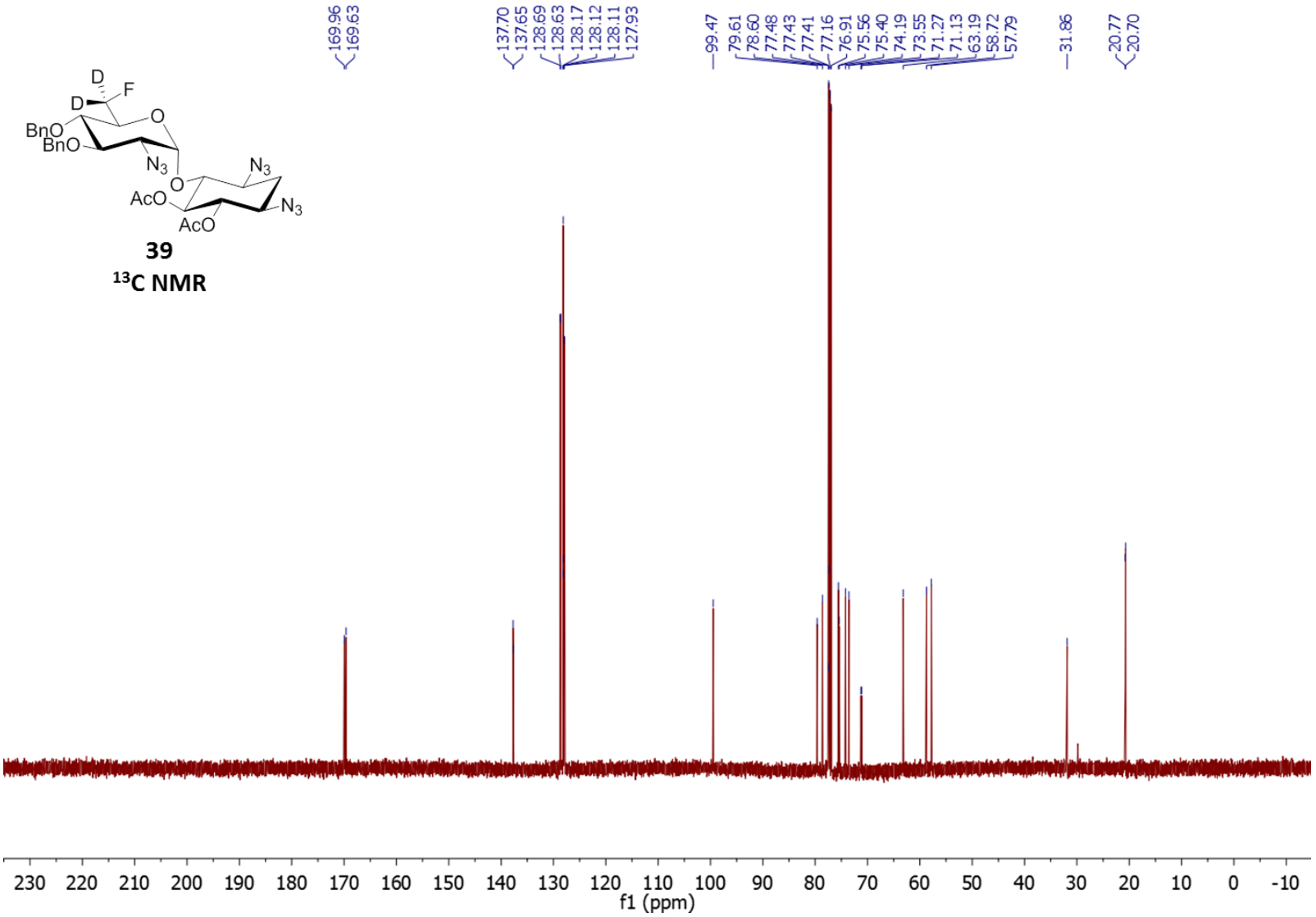

D
OPMB
D
O
BnO
BnO
N3
O
N3
AcO
N3
AcO
37
1H NMR
f1 (ppm)


169.95
169.61
159.40
138.01
137.89
129.91
129.76
128.57
128.56
128.54
128.48
128.47
128.10
128.08
127.97
127.86
127.77
127.72
113.89
99.49
79.65
78.37
78.17
77.41
77.16
76.91
75.42
75.02
74.20
73.63
73.24
71.64
63.21
58.77
57.80
55.30
31.91
20.75
20.69
OPMB
BnO
BnO
AcO
AcO
N3
N3
37
13C NMR
f1 (ppm)


D
OH
D
O
BnO
BnO
N3
O
N3
AcO
N3
AcO
38
1H NMR
f1 (ppm)


170.02
169.65
137.85
137.80
128.70
128.63
128.17
128.16
128.08
128.06
99.58
79.67
78.81
77.83
77.41
77.16
76.91
75.55
75.21
74.23
73.58
72.30
63.41
58.90
57.80
32.02
20.80
20.72
D
OH
D
O
BnO
BnO
N3
O
N3
AcO
N3
AcO
38
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


7.37 7.37 7.36 7.36 7.35 7.35 7.35 7.34 7.34 7.34 7.33 7.33 7.33 7.32 7.32 7.32 7.31 7.31 7.30 7.30 7.30 7.29 7.26 5.17 5.15 5.13 5.12 5.12 4.95 4.93 4.92 4.91 4.90 4.87 4.86 4.84 4.68 4.66 4.20 4.18 4.13 4.11 4.01 3.99 3.99 3.98 3.97 3.97 3.70 3.68 3.68 3.66 3.65 3.64 3.63 3.63 3.62 3.62 3.61 3.41 3.37 3.36 3.35 3.34 2.42 2.39 2.09 2.09 1.62 1.59
D
F
D
O
BnO
BnO
N3
O
N3
AcO
N3
AcO
39
1H NMR
10.64
2.07
4.29
1.00
0.95
1.02
3.17
1.09
1.03
1.01
6.34
1.40
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


169.96
169.63
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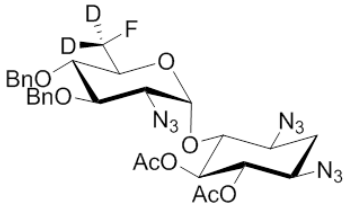
137.65
128.69
128.63
128.17
128.12
128.11
127.93
99.47
79.61
78.60
77.48
77.43
77.41
77.16
76.91
75.56
75.40
74.19
73.55
71.27
71.13
63.19
58.72
57.79
31.86
20.77
20.70
D
F
D
O
BnO
BnO
N3
O
N3
AcO
N3
AcO
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

39

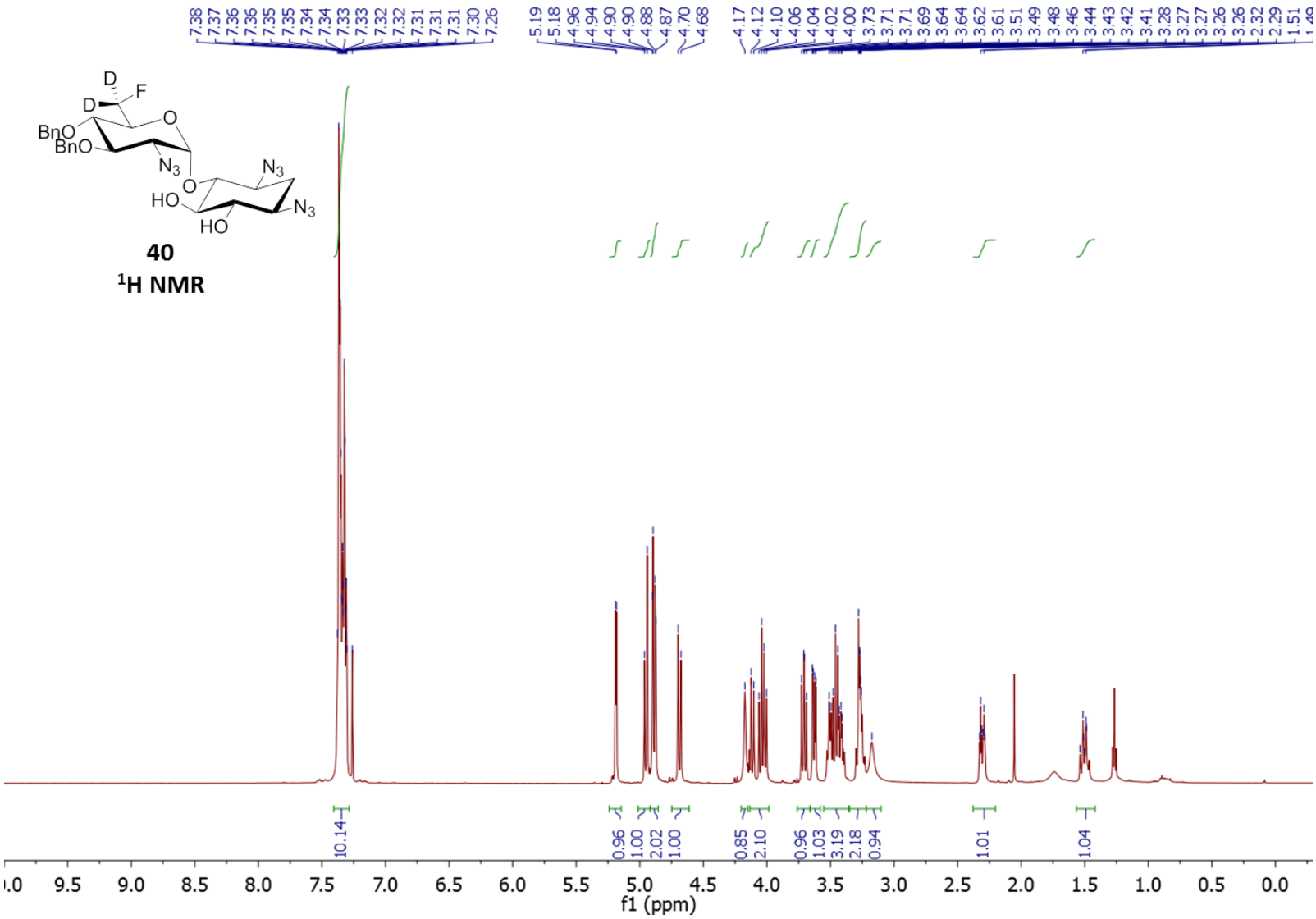
^{13}C NMR

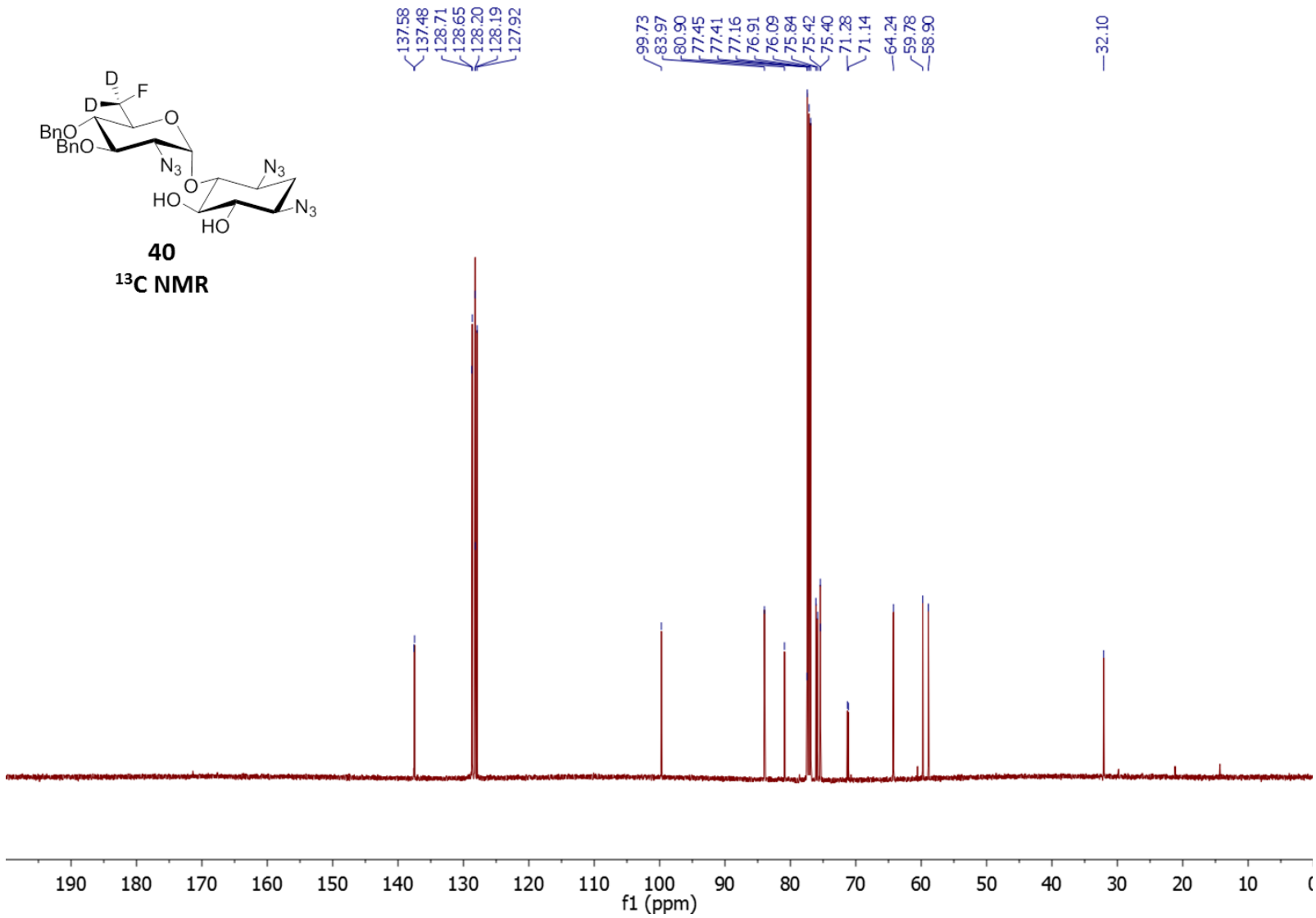

D
F
D
O
BnO
BnO
N3
O
N3
AcO
N3
AcO

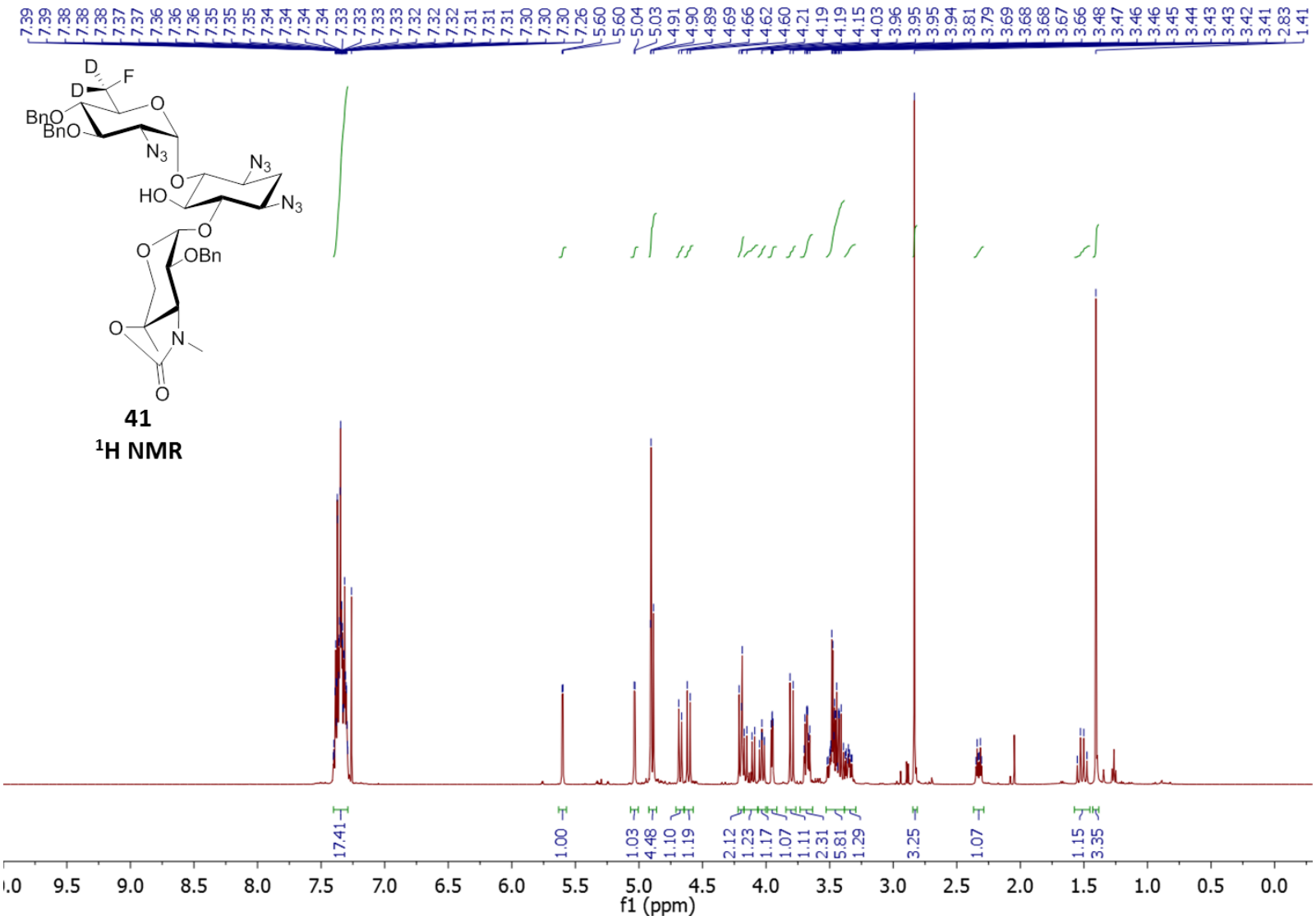
39

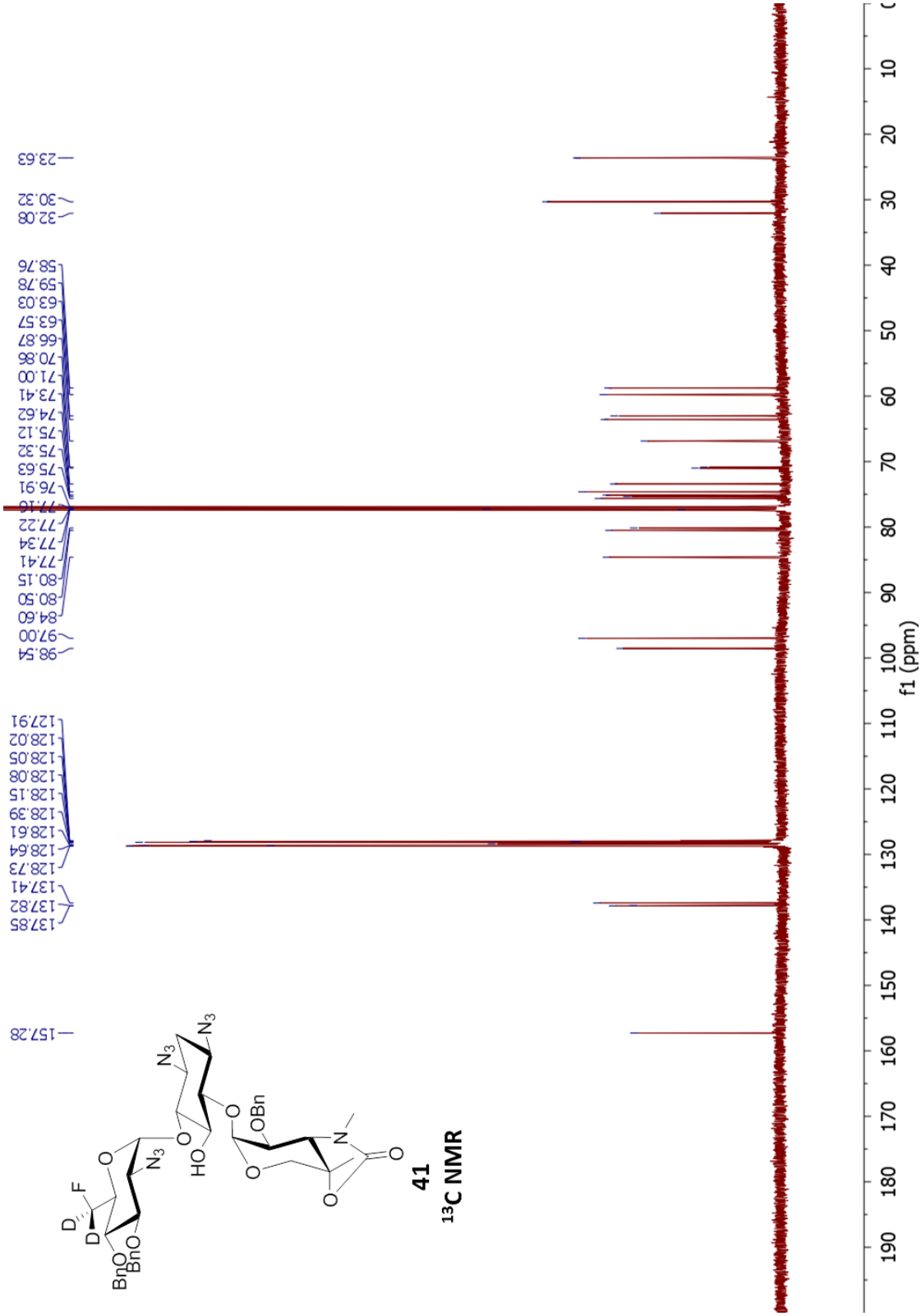
^{19}F NMR

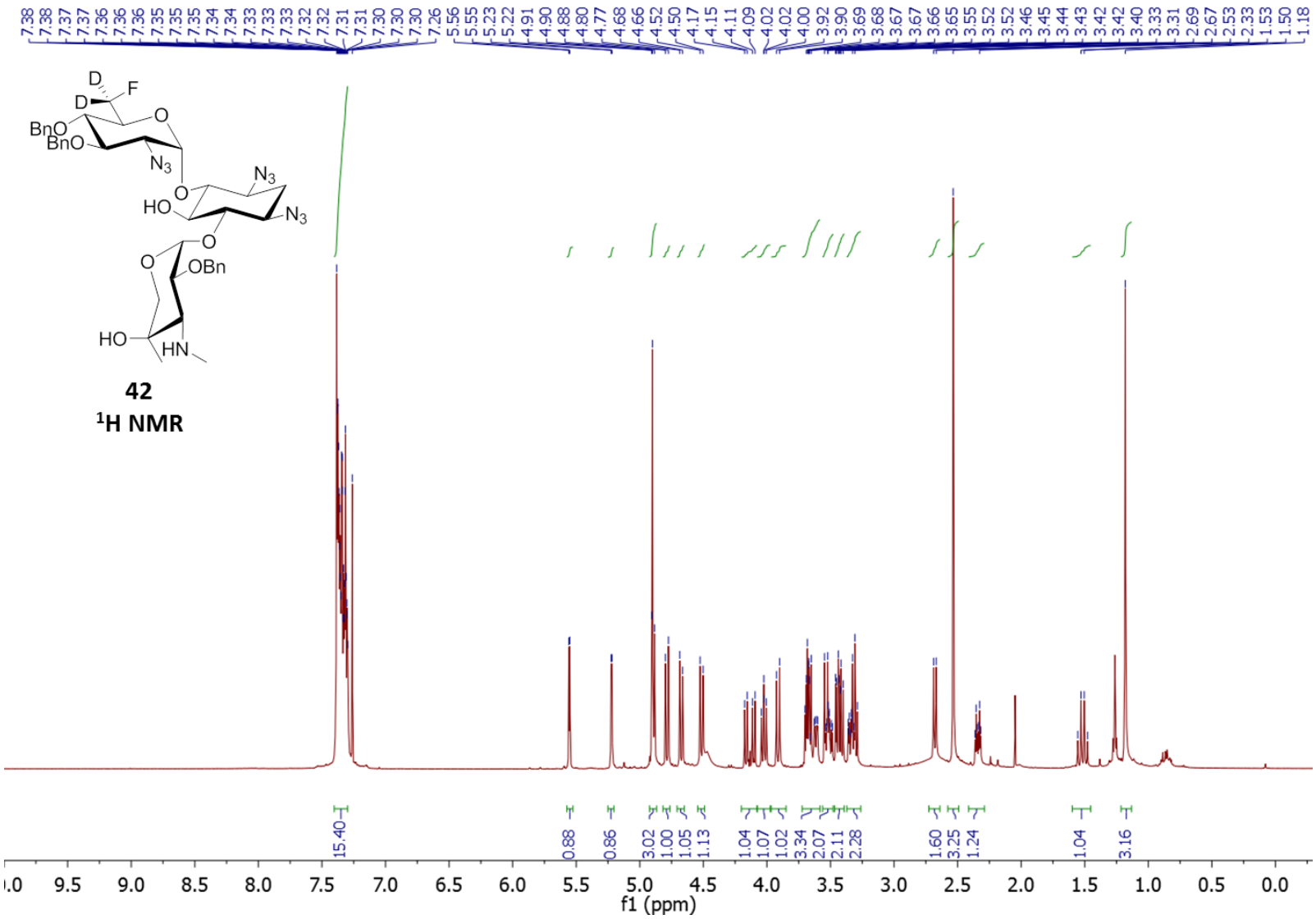
-236.26
-236.34
0.0 -230.5 -231.0 -231.5 -232.0 -232.5 -233.0 -233.5 -234.0 -234.5 -235.0 -235.5 -236.0 -236.5 -237.0 -237.5 -238.0 -238.5 -239.0 -239.5 -24
f1 (ppm)

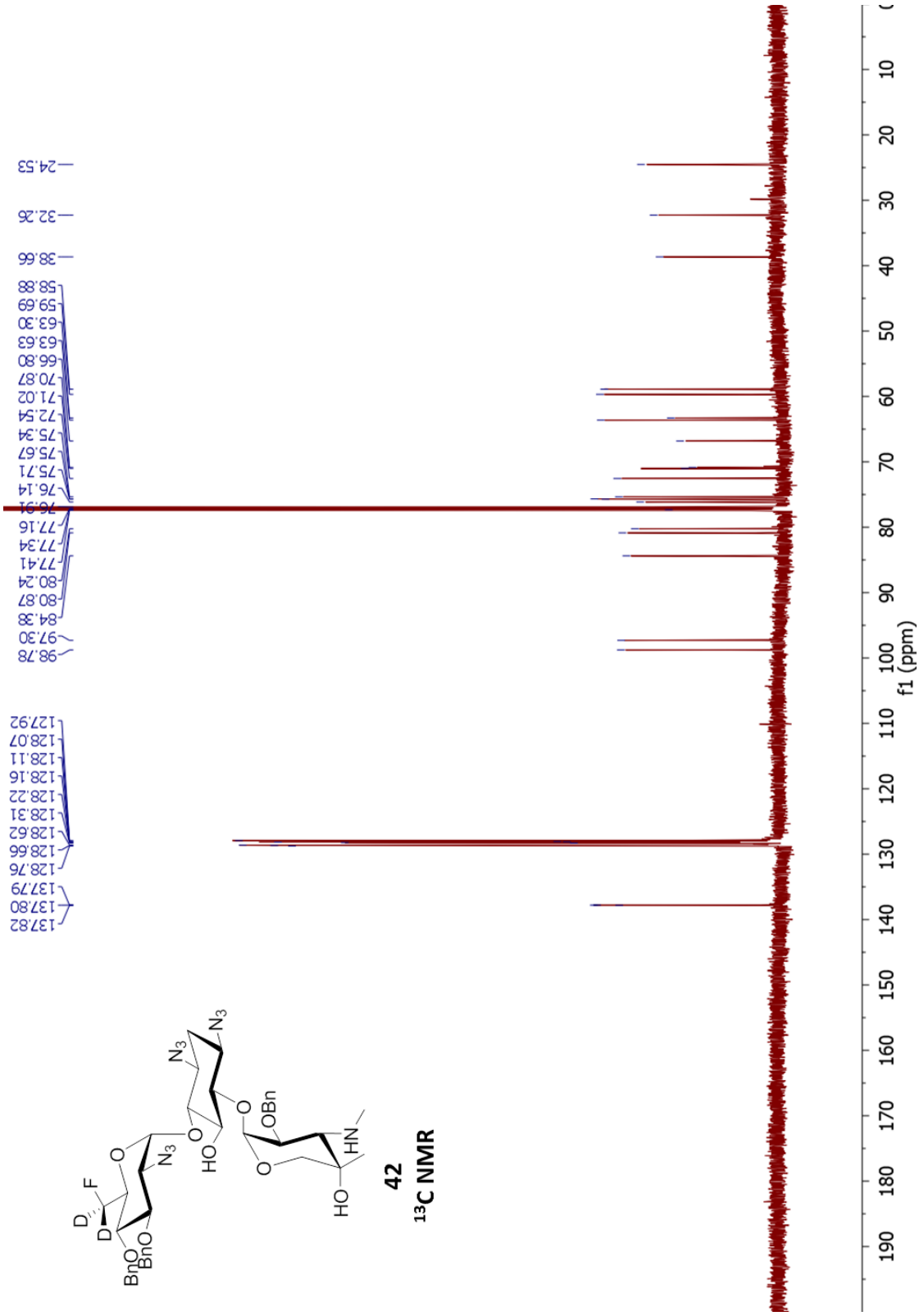

40
1H NMR
f1 (ppm)

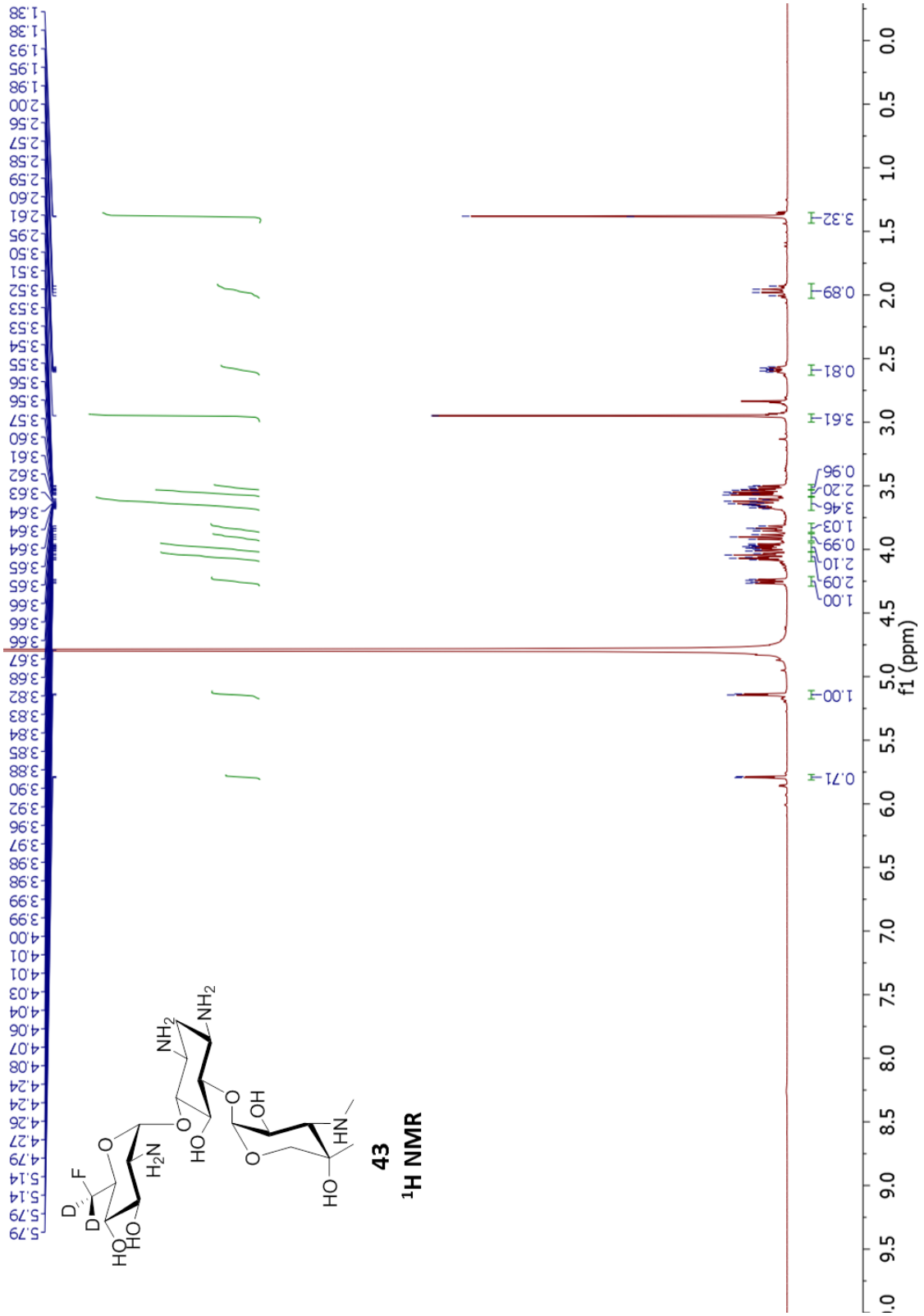

137.58
137.48
128.71
128.65
128.20
128.19
127.92
99.73
83.97
80.90
77.45
77.41
77.16
76.91
76.09
75.84
75.42
75.40
71.28
71.14
64.24
59.78
58.90
32.10
D
F
D
O
BnO
BnO
N3
O
N3
HO
N3
HO
40
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

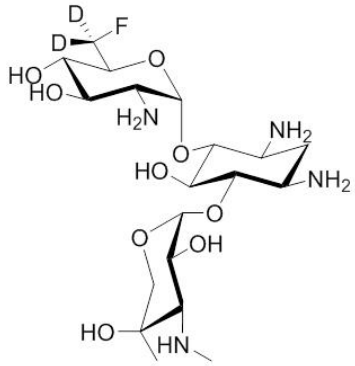

41
1H NMR
f1 (ppm)


157.28
137.85
137.82
137.41
128.73
128.64
128.61
128.39
128.15
128.08
128.05
128.02
127.91
98.54
97.00
84.60
80.50
80.15
77.41
77.34
77.22
77.16
76.91
75.63
75.32
75.12
74.62
73.41
71.00
70.86
66.87
63.57
63.03
59.78
58.76
32.08
30.32
23.63
f1 (ppm)
41
13C NMR


42
1H NMR
f1 (ppm)


42
13C NMR
f1 (ppm)

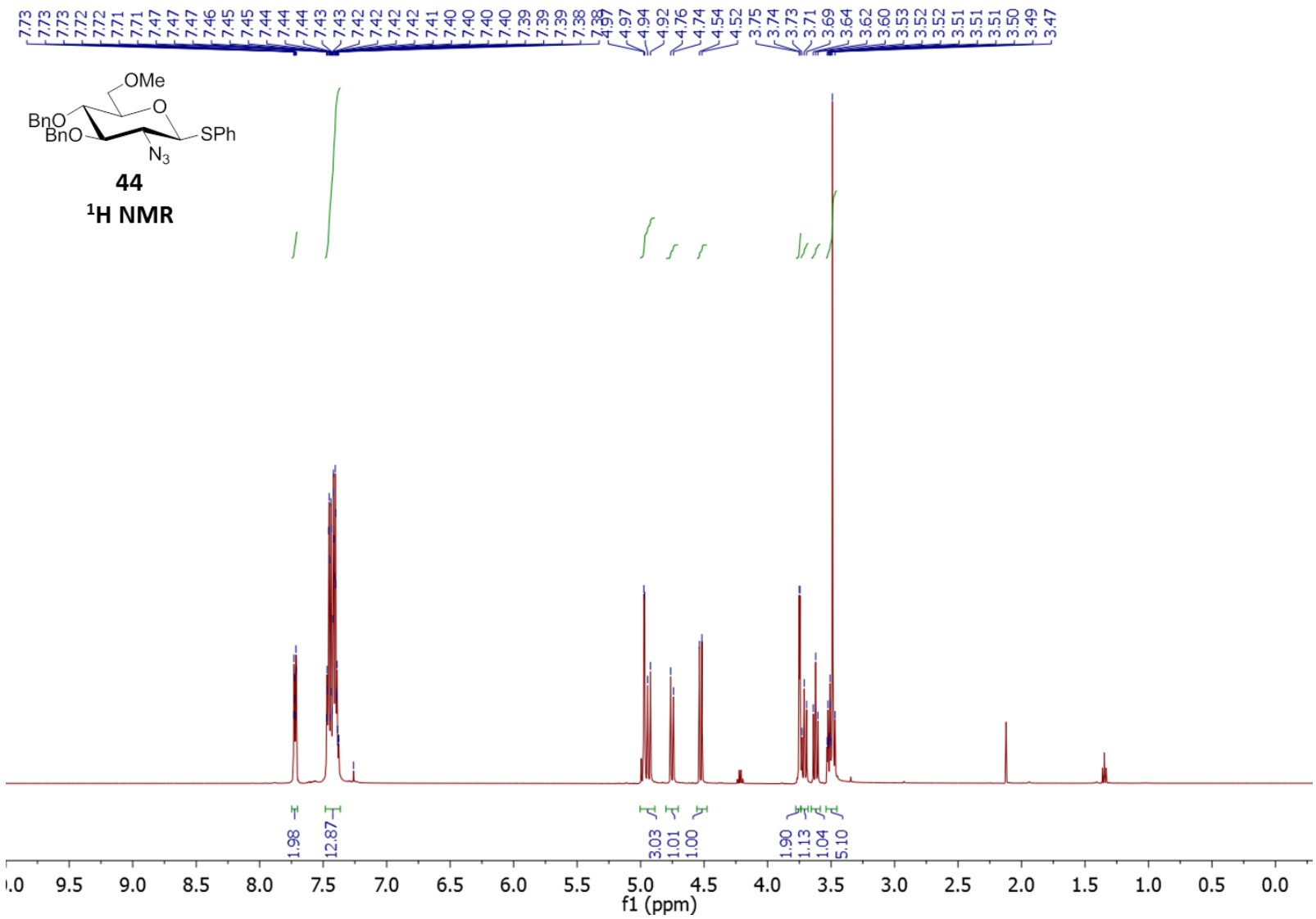

43
^{1}H NMR
f1 (ppm)

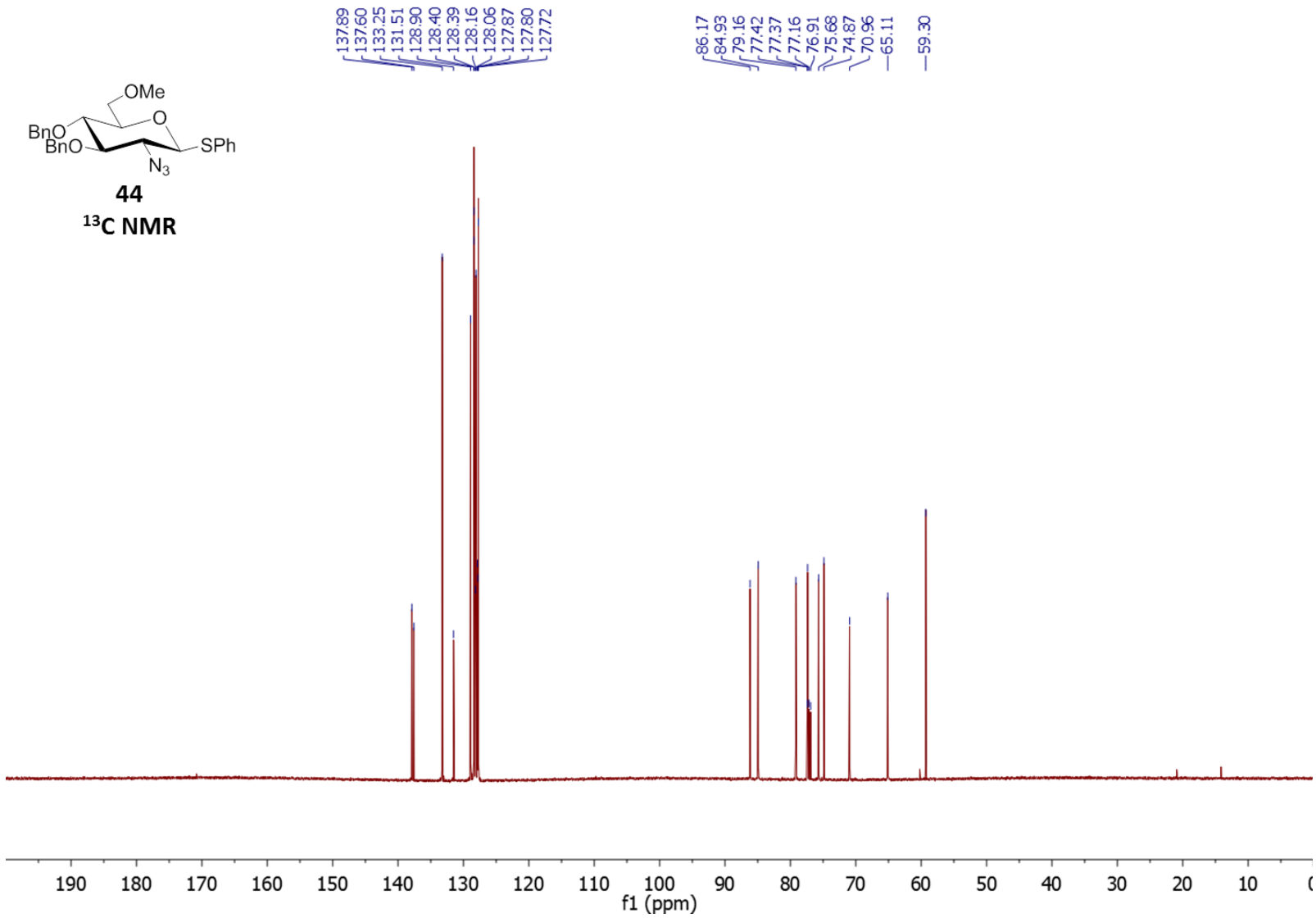

D
F
D
HO
HO
O
H2N
NH2
O
HO
NH2
O
O
OH
O
HO
HN

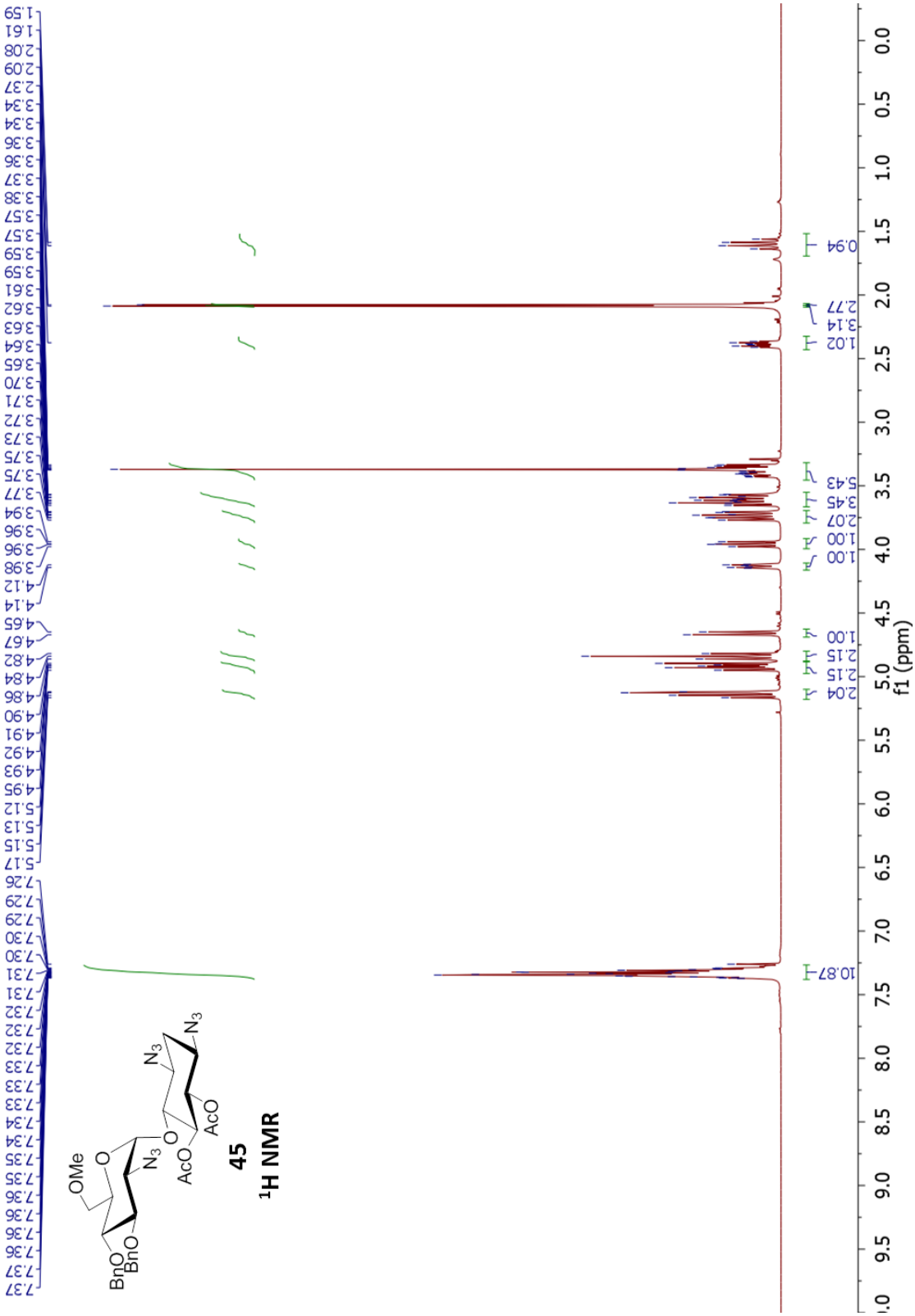
43

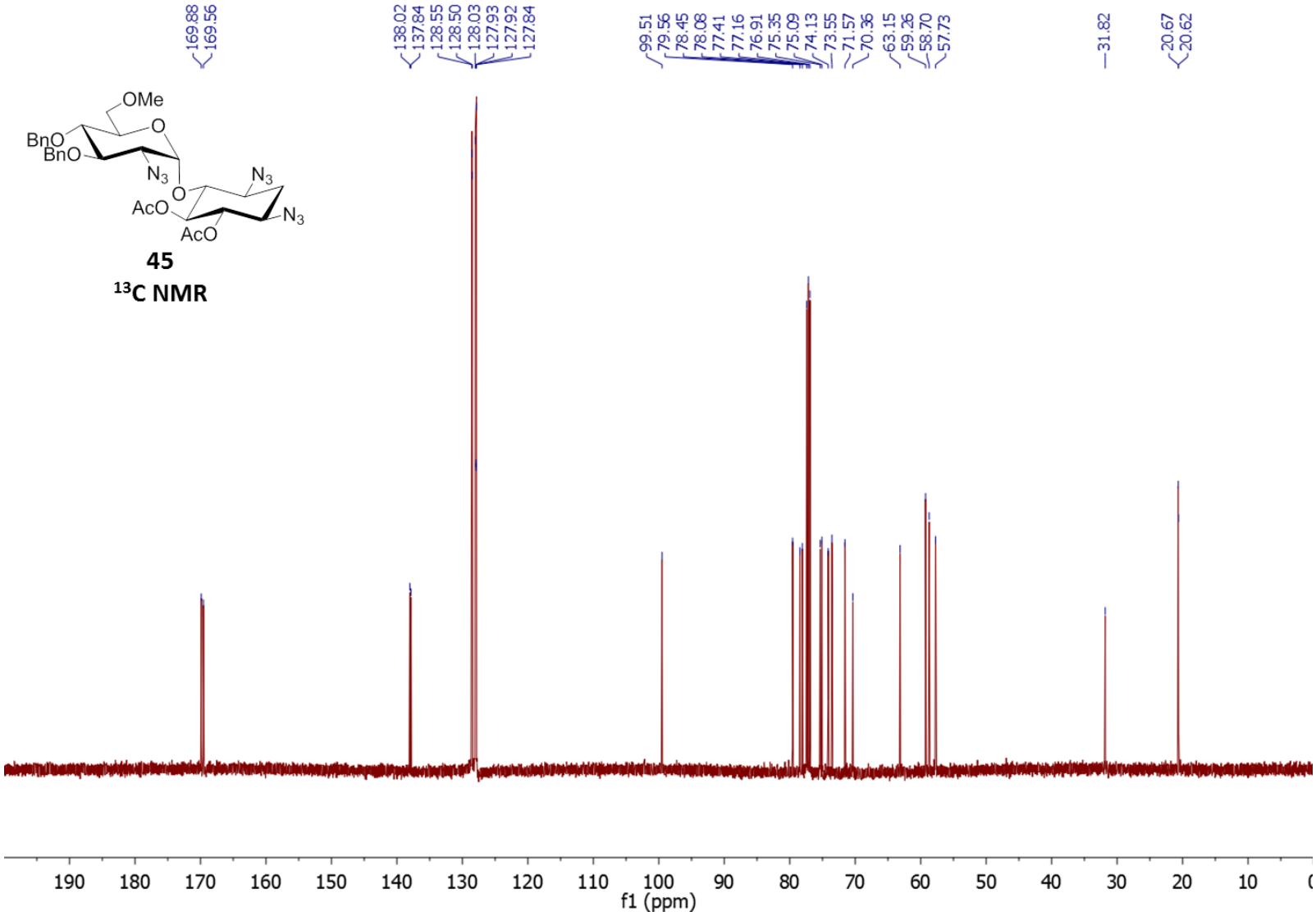
^{13}C NMR

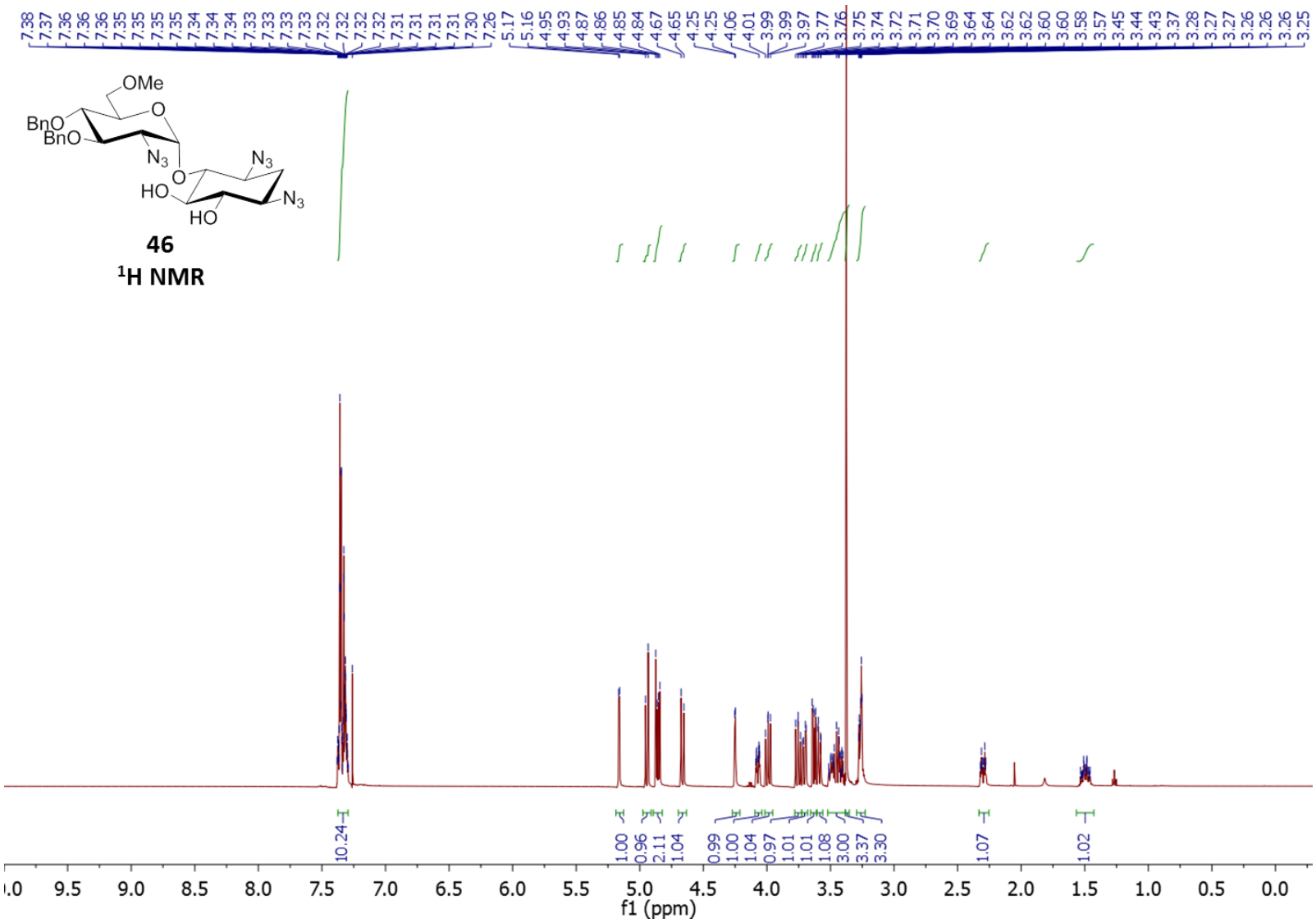
101.08
96.85
83.44
79.52
73.84
72.18
72.03
69.85
68.77
68.20
68.14
66.17
63.19
53.69
49.46
48.36
34.47
27.81
20.89
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

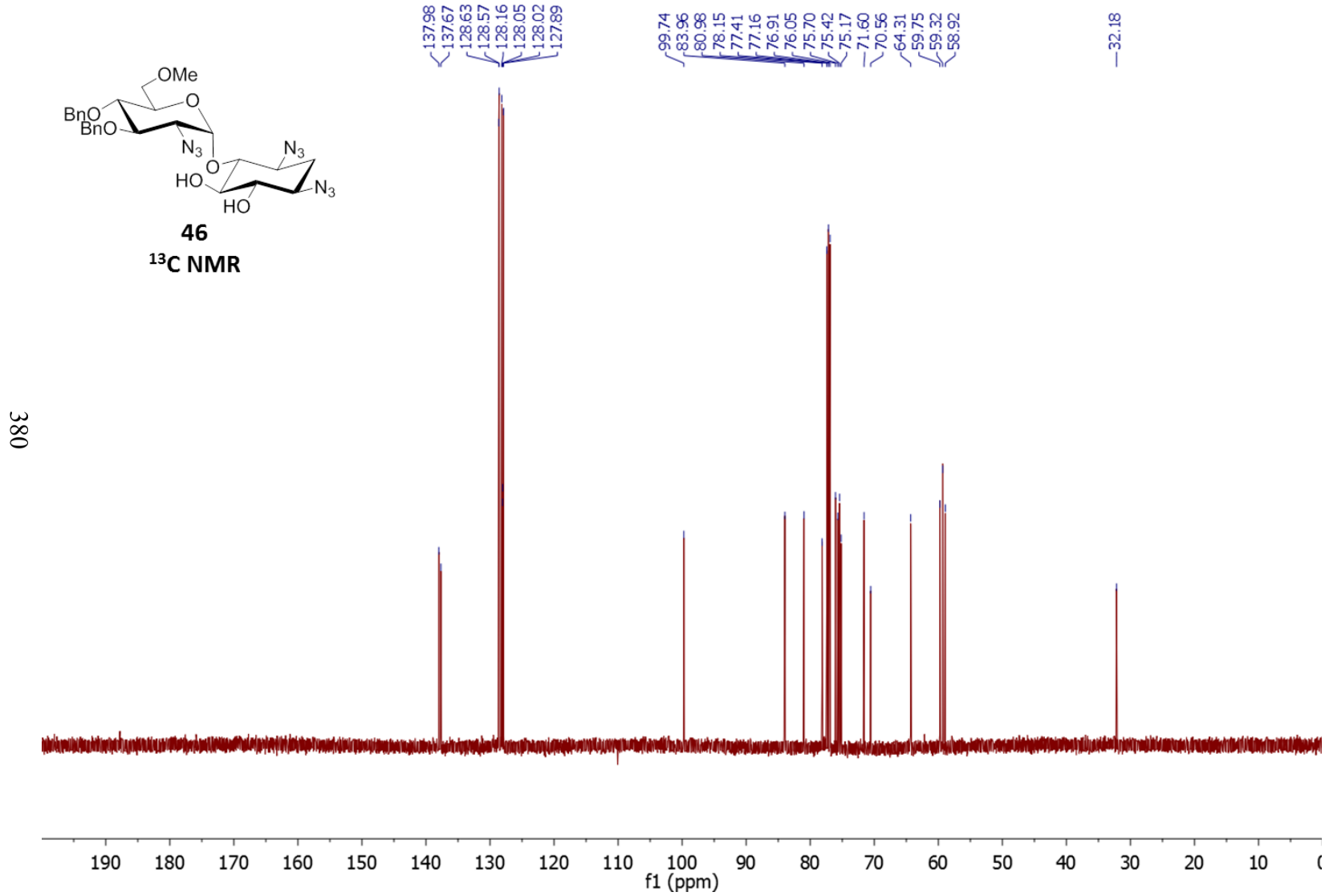

OMe
O
BnO
BnO
SPh
N3
44
1H NMR
f1 (ppm)

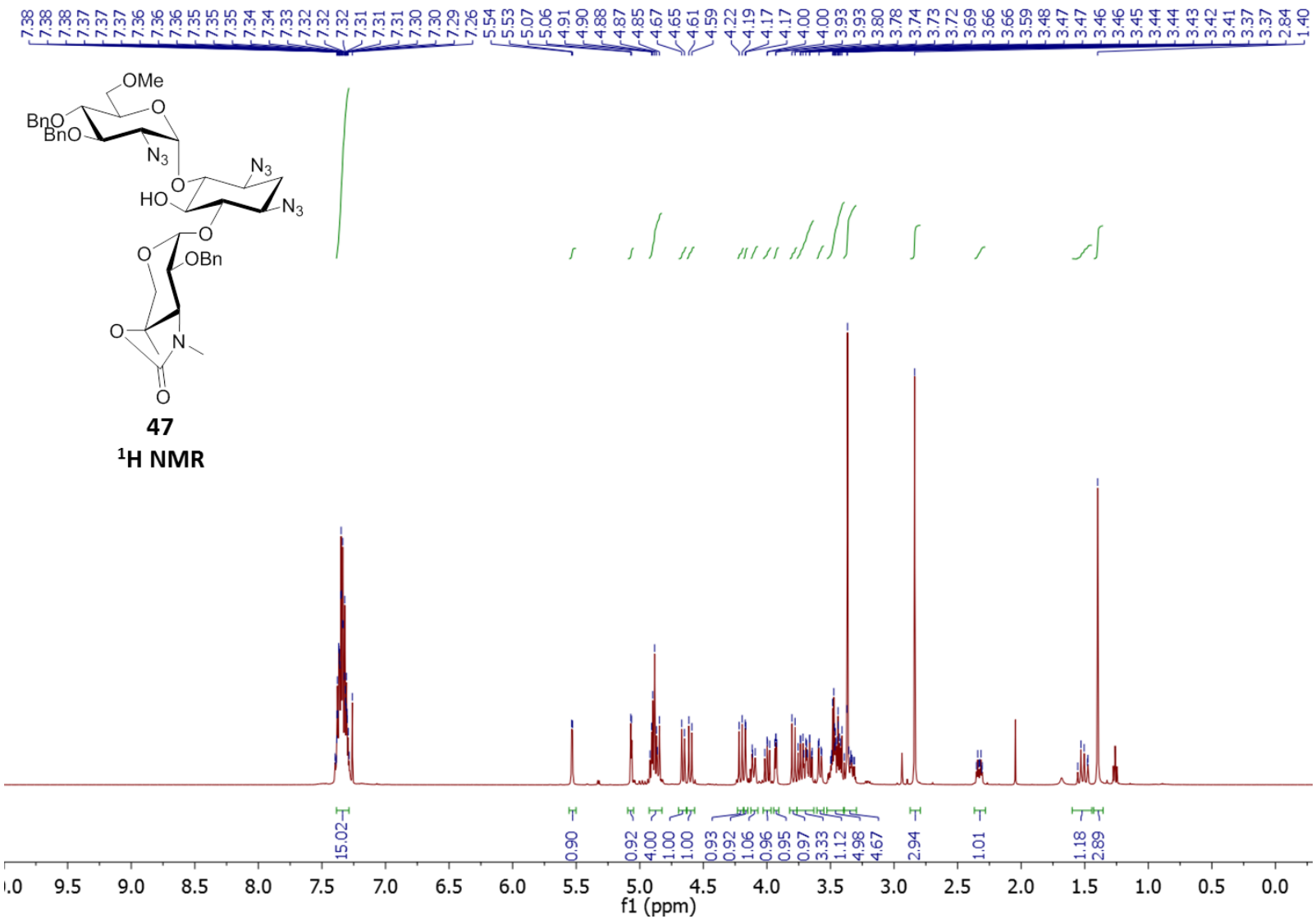

137.89
137.60
133.25
131.51
128.90
128.40
128.39
128.16
128.06
127.87
127.80
127.72
86.17
84.93
79.16
77.42
77.37
77.16
76.91
75.68
74.87
70.96
65.11
59.30
OMe
O
BnO
BnO
SPh
N3
44
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

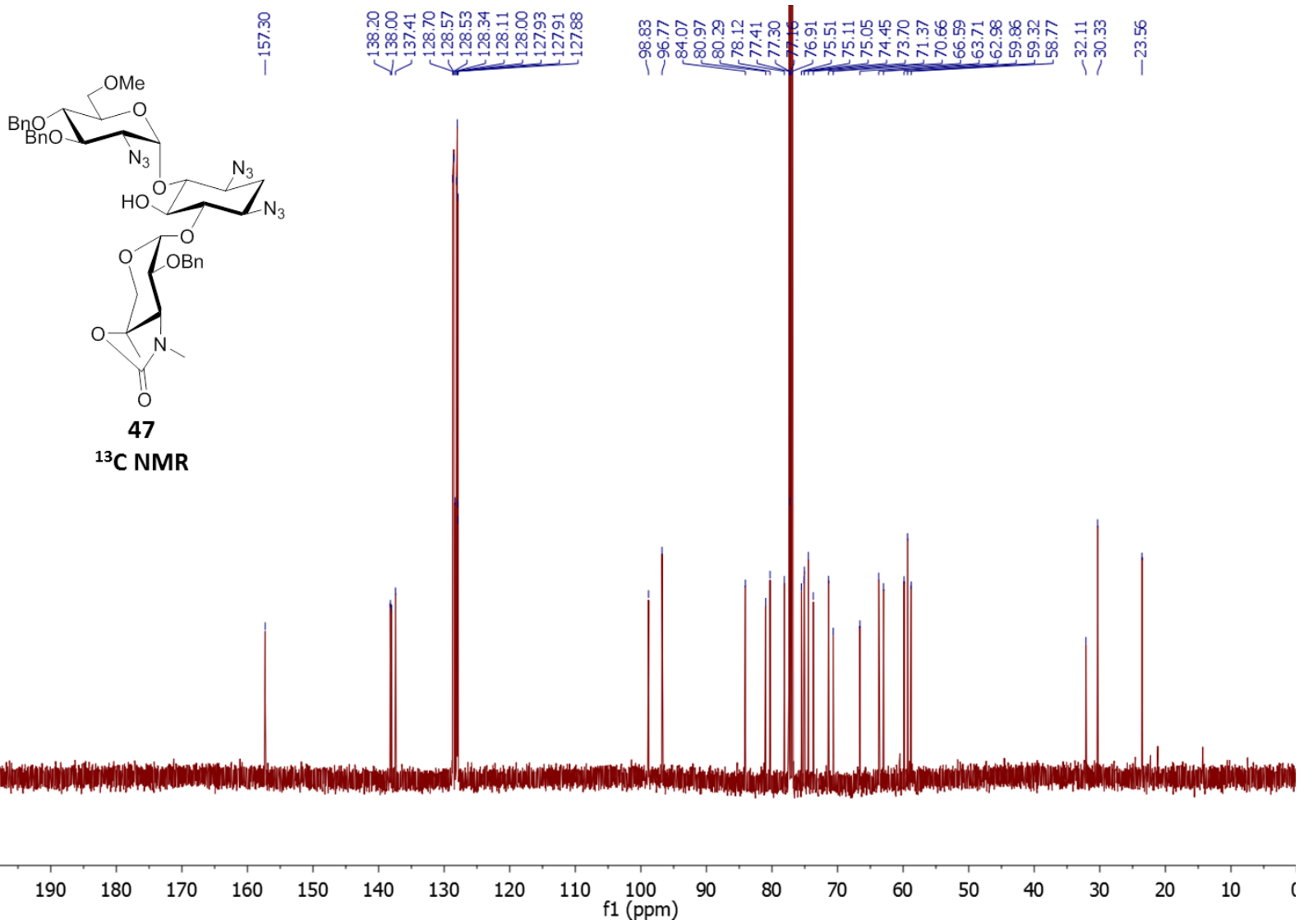

OMe
BnO
BnO
N3
O
AcO
N3
O
AcO
N3
45
1H NMR
f1 (ppm)


169.88
169.56
138.02
137.84
128.55
128.50
128.03
127.93
127.92
127.84
99.51
79.56
78.45
78.08
77.41
77.16
76.91
75.35
75.09
74.13
73.55
71.57
70.36
63.15
59.26
58.70
57.73
31.82
20.67
20.62
OMe
O
BnO
BnO
N3
O
N3
AcO
N3
AcO
45
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


OMe
O
BnO
BnO
N3
O
N3
HO
N3
HO
46
1H NMR
f1 (ppm)

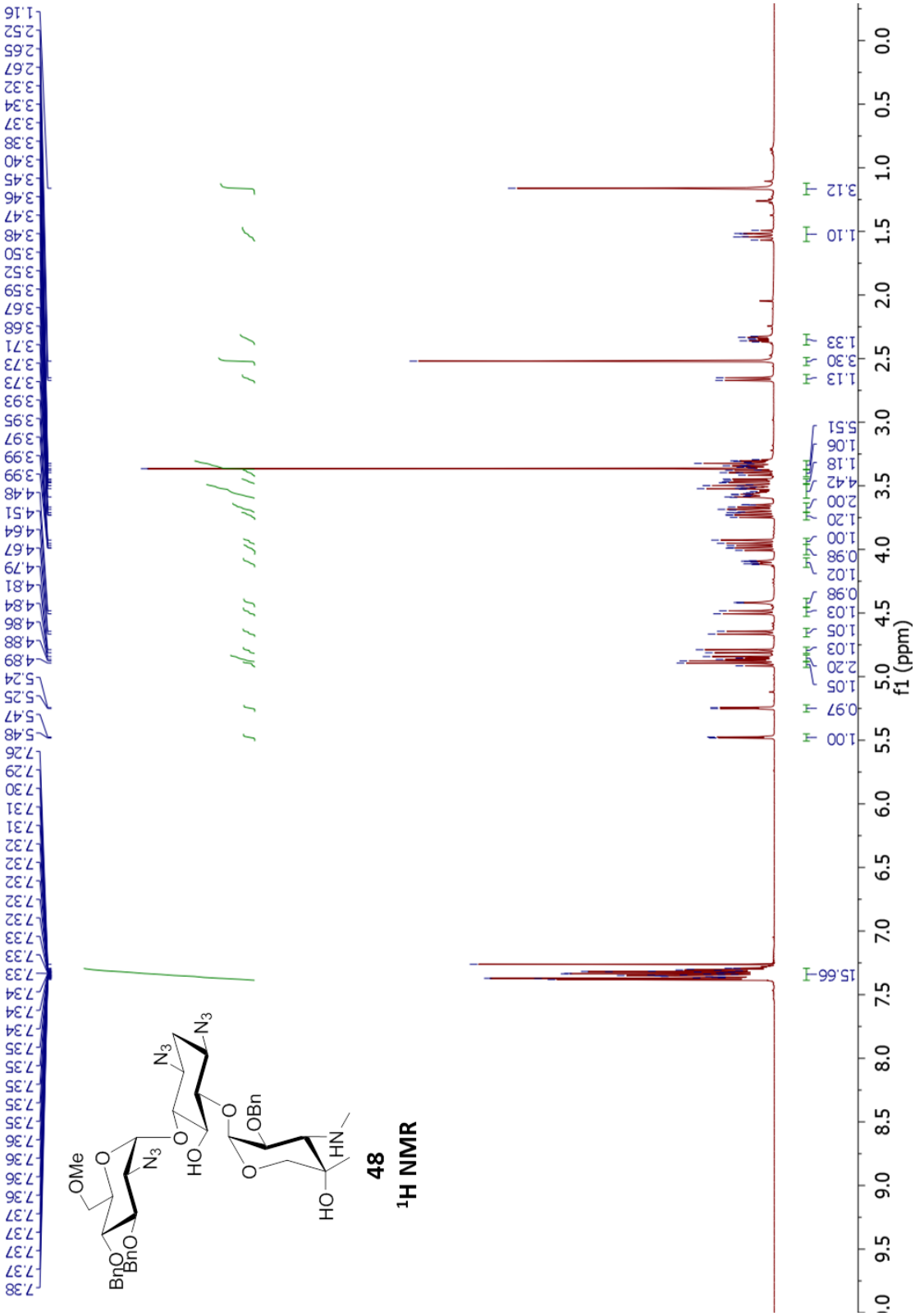

137.98
137.67
128.63
128.57
128.16
128.05
128.02
127.89
99.74
83.96
80.98
78.15
77.41
77.16
76.91
76.05
75.70
75.42
75.17
71.60
70.56
64.31
59.75
59.32
58.92
32.18
OMe
O
BnO
BnO
N3
O
N3
HO
N3
HO
46
13C NMR
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

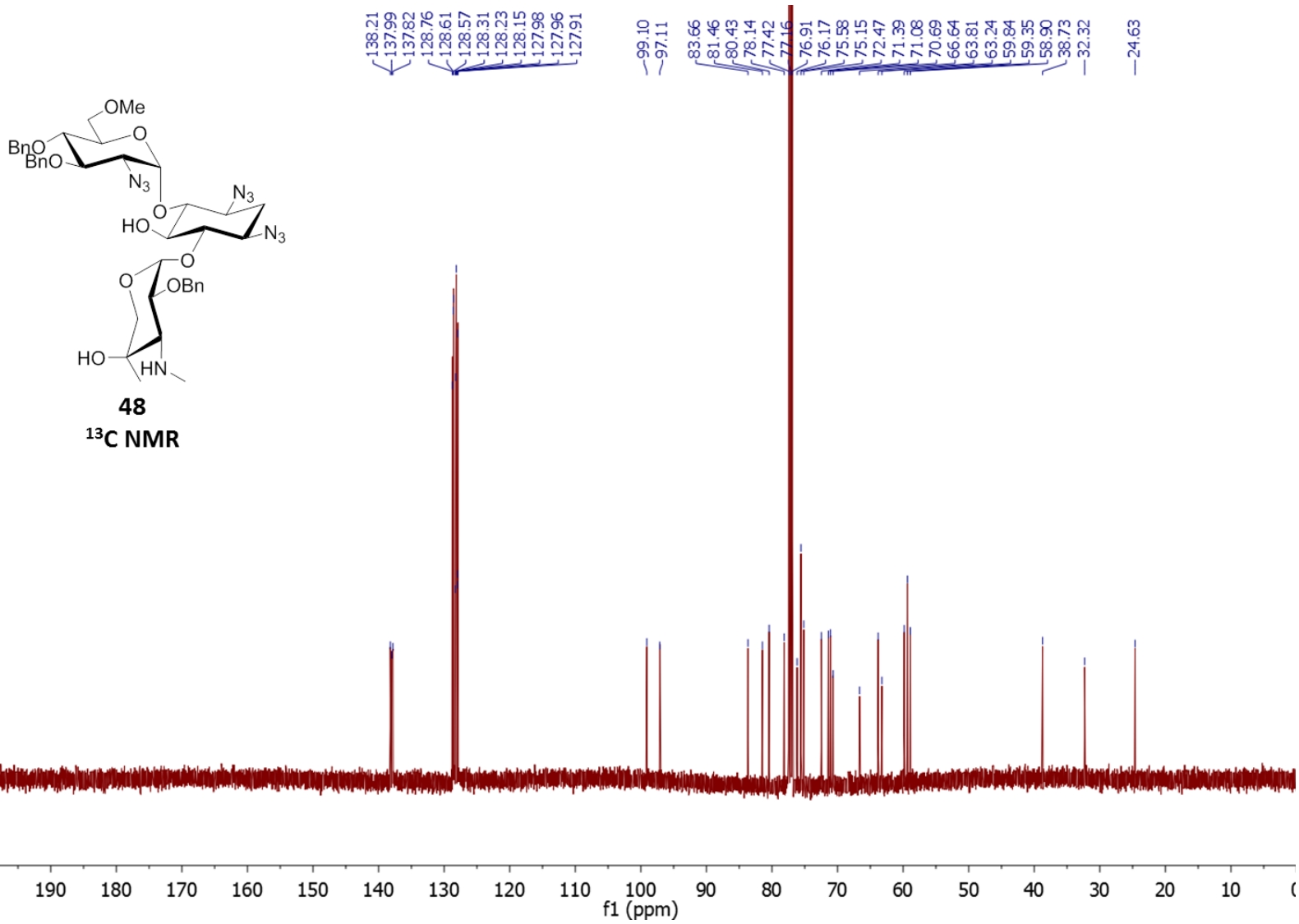

OMe
BnO
BnO
N3
O
N3
HO
N3
O
O
OBn
O
N
O
47
1H NMR
f1 (ppm)


157.30
138.20
138.00
137.41
128.70
128.57
128.53
128.34
128.11
128.00
127.93
127.91
127.88
98.83
96.77
84.07
80.97
80.29
78.12
77.41
77.30
77.16
76.91
75.51
75.11
75.05
74.45
73.70
71.37
70.66
66.59
63.71
62.98
59.86
59.32
58.77
32.11
30.33
23.56
OMe
BnO
BnO
N3
N3
HO
N3
OBn
N
O
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

47

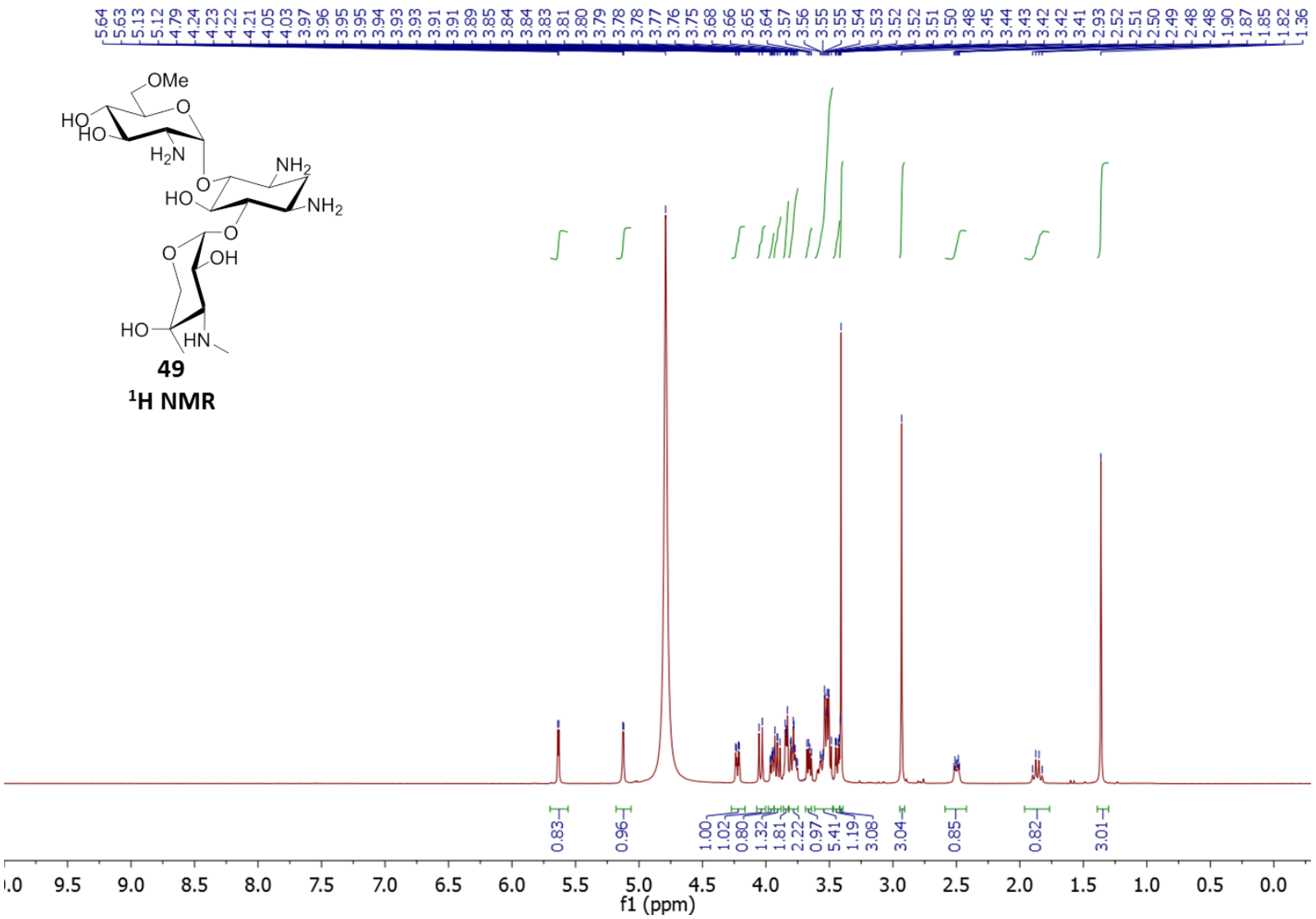
^{13}C NMR

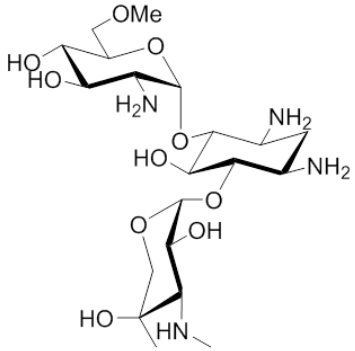

OMe
BnO
BnO
N3
O
HO
N3
N3
O
OBn
O
HN
HO
48
1H NMR
f1 (ppm)


OMe
BnO
BnO
N3
O
N3
HO
N3
O
O
OBn
HO
HN
138.21
137.99
137.82
128.76
128.61
128.57
128.31
128.23
128.15
127.98
127.96
127.91
99.10
97.11
83.66
81.46
80.43
78.14
77.42
77.16
76.91
76.17
75.58
75.15
72.47
71.39
71.08
70.69
66.64
63.81
63.24
59.84
59.35
58.90
38.73
32.32
24.63
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

48

^{13}C NMR


OMe
HO
HO
O
H2N
NH2
HO
O
NH2
O
O
OH
HO
HN
49
1H NMR
f1 (ppm)

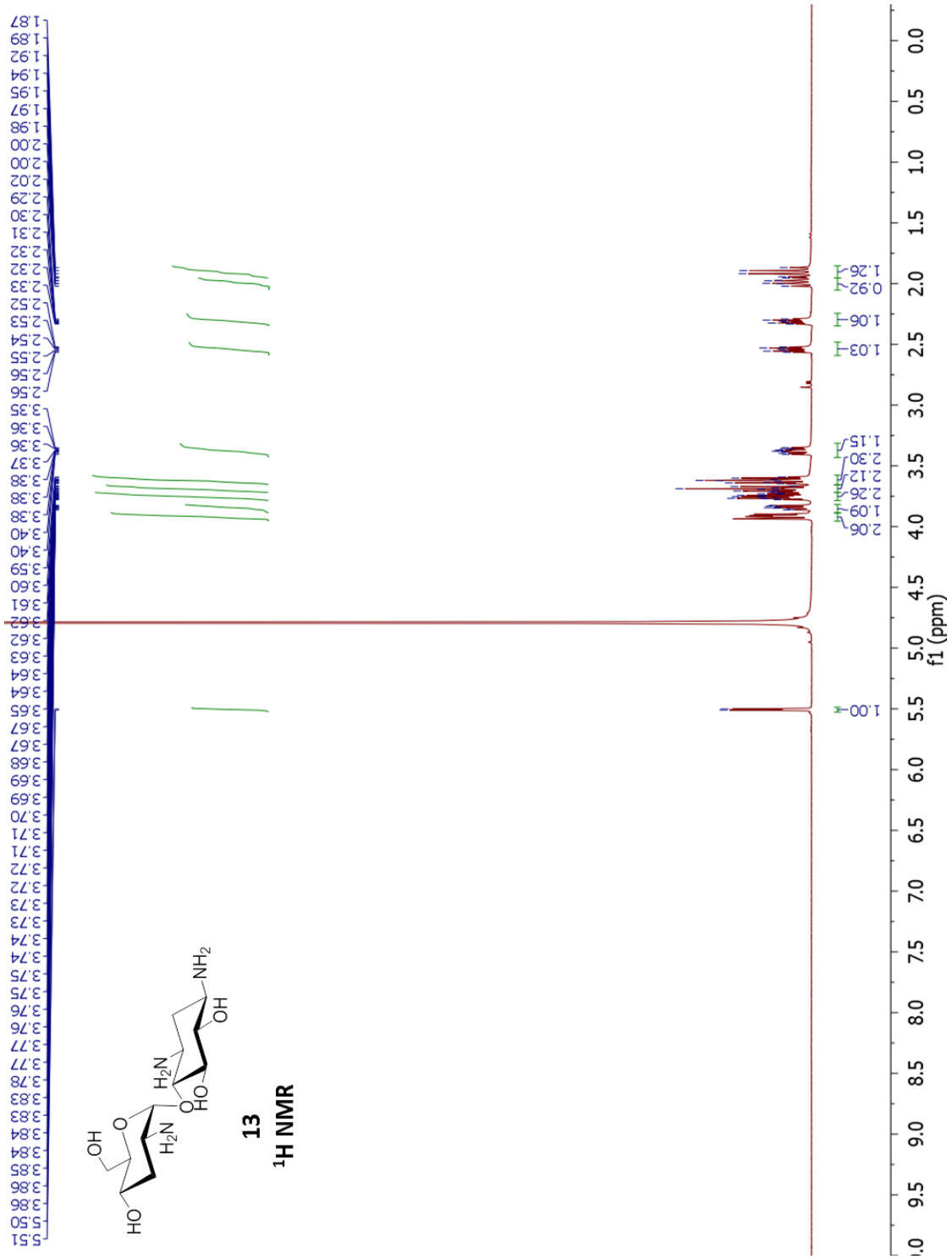

OMe
O
HO
HO
H2N
NH2
O
HO
NH2
O
O
OH
HO
HN

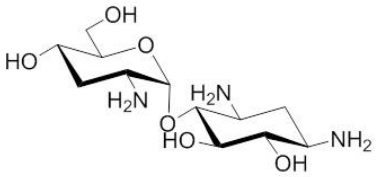
49

^{13}C NMR

100.90
97.13
83.66
81.34
73.73
72.22
70.75
69.84
69.34
69.13
67.71
66.19
63.26
58.72
53.94
49.58
48.60
34.51
28.68
20.89
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

A.3. SPECTRAL DATA FOR CHAPTER 4

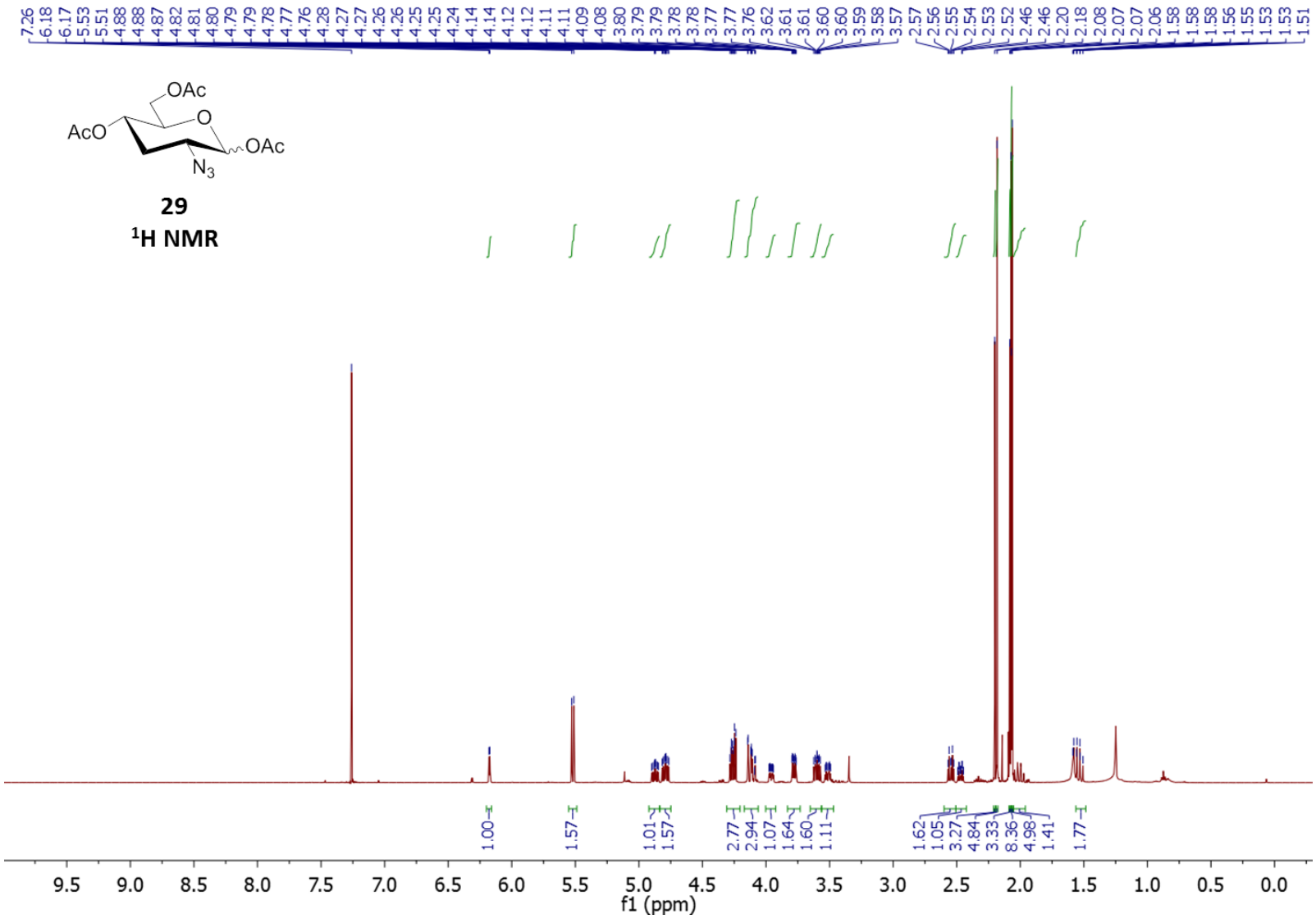


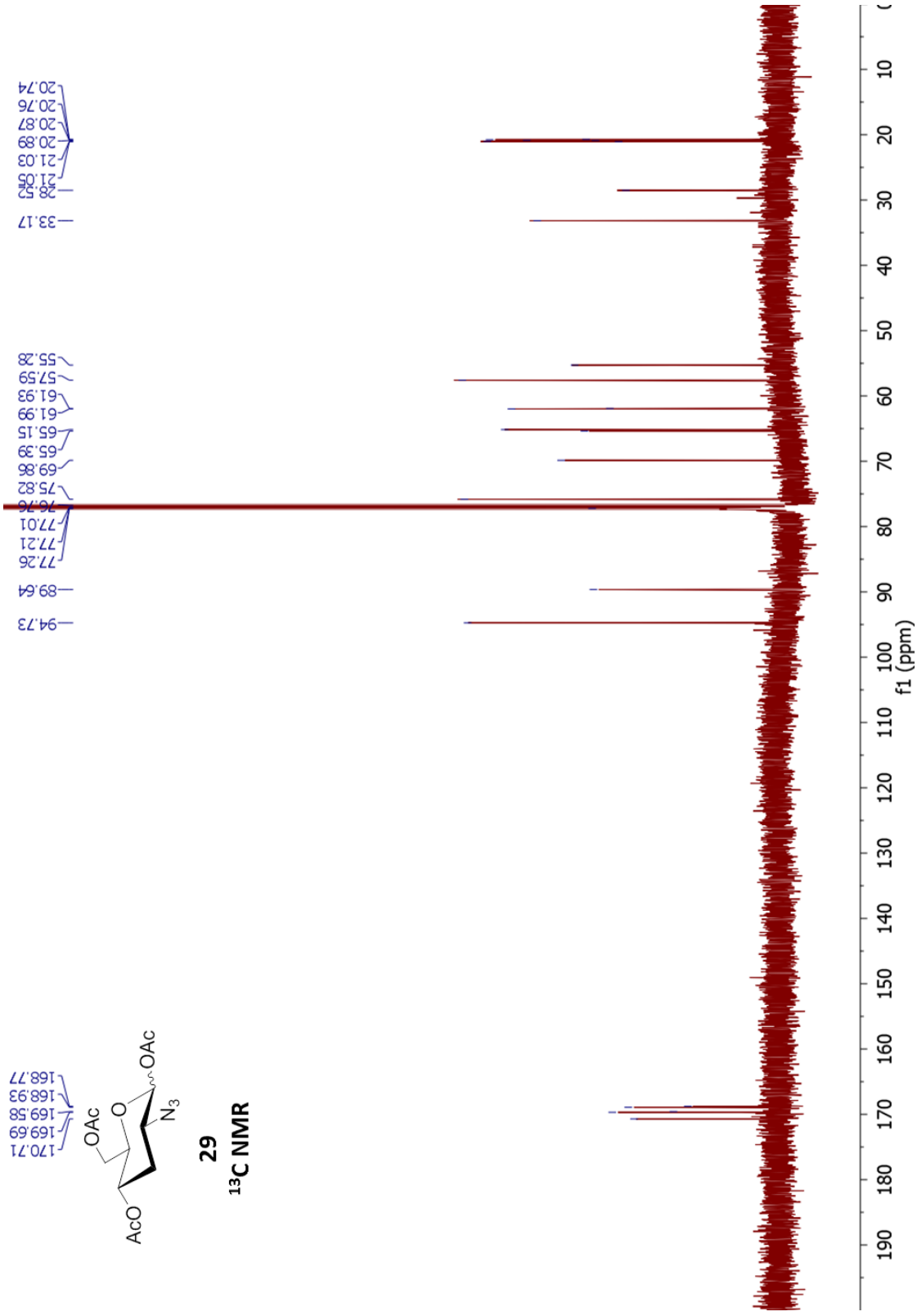

OH
HO
O
H2N
H2N
O
HO
NH2
OH

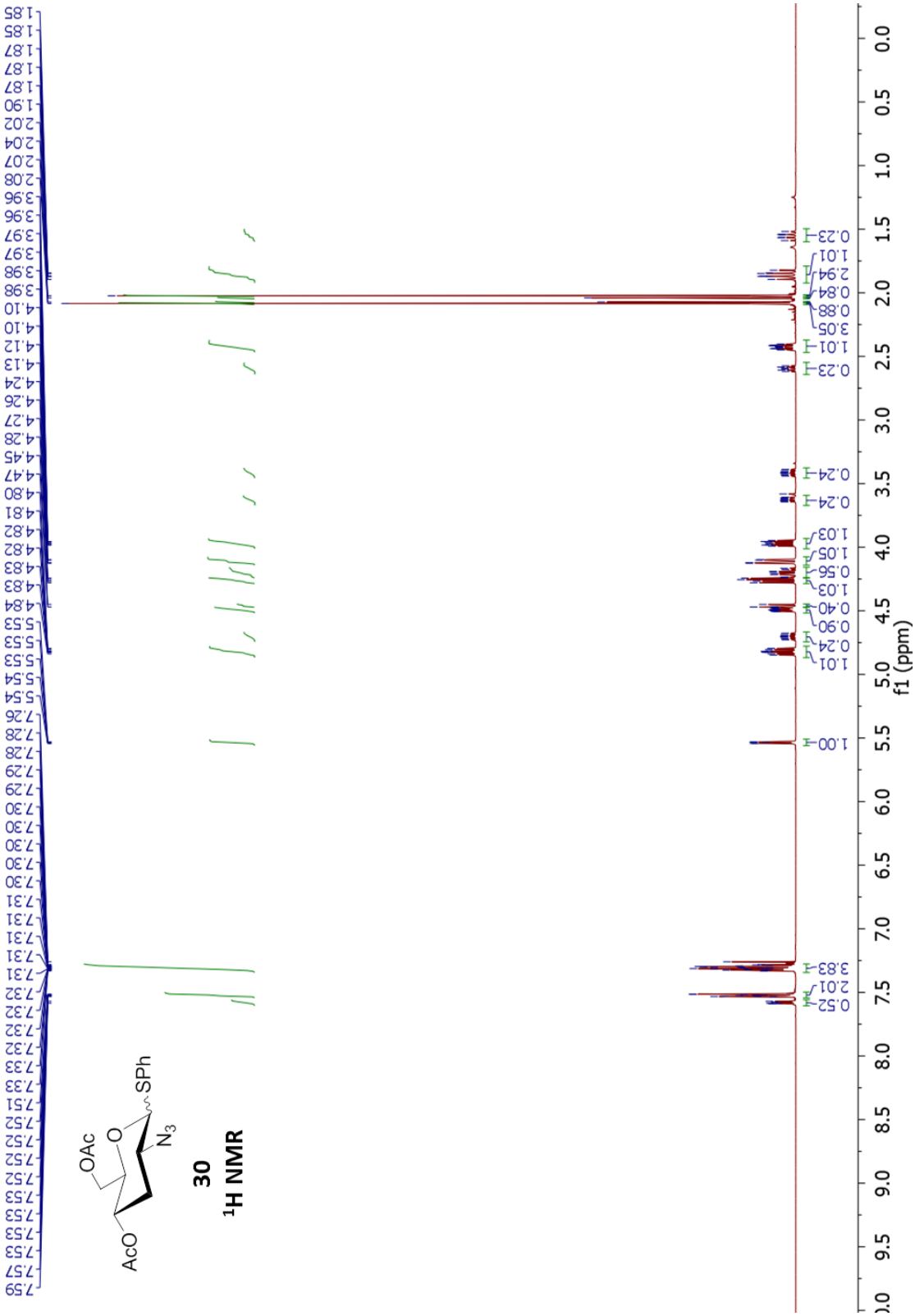
13

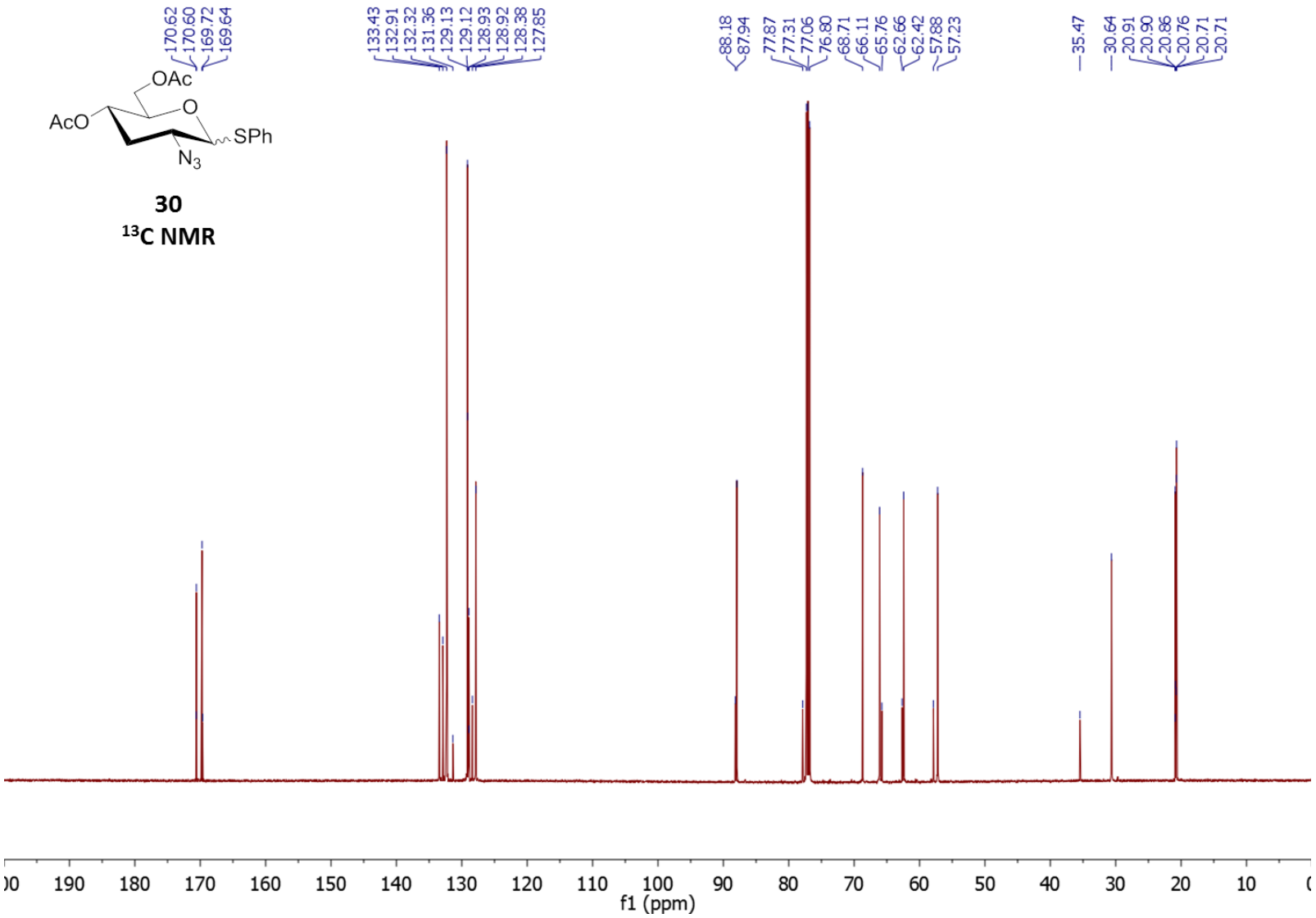
^{13}C NMR

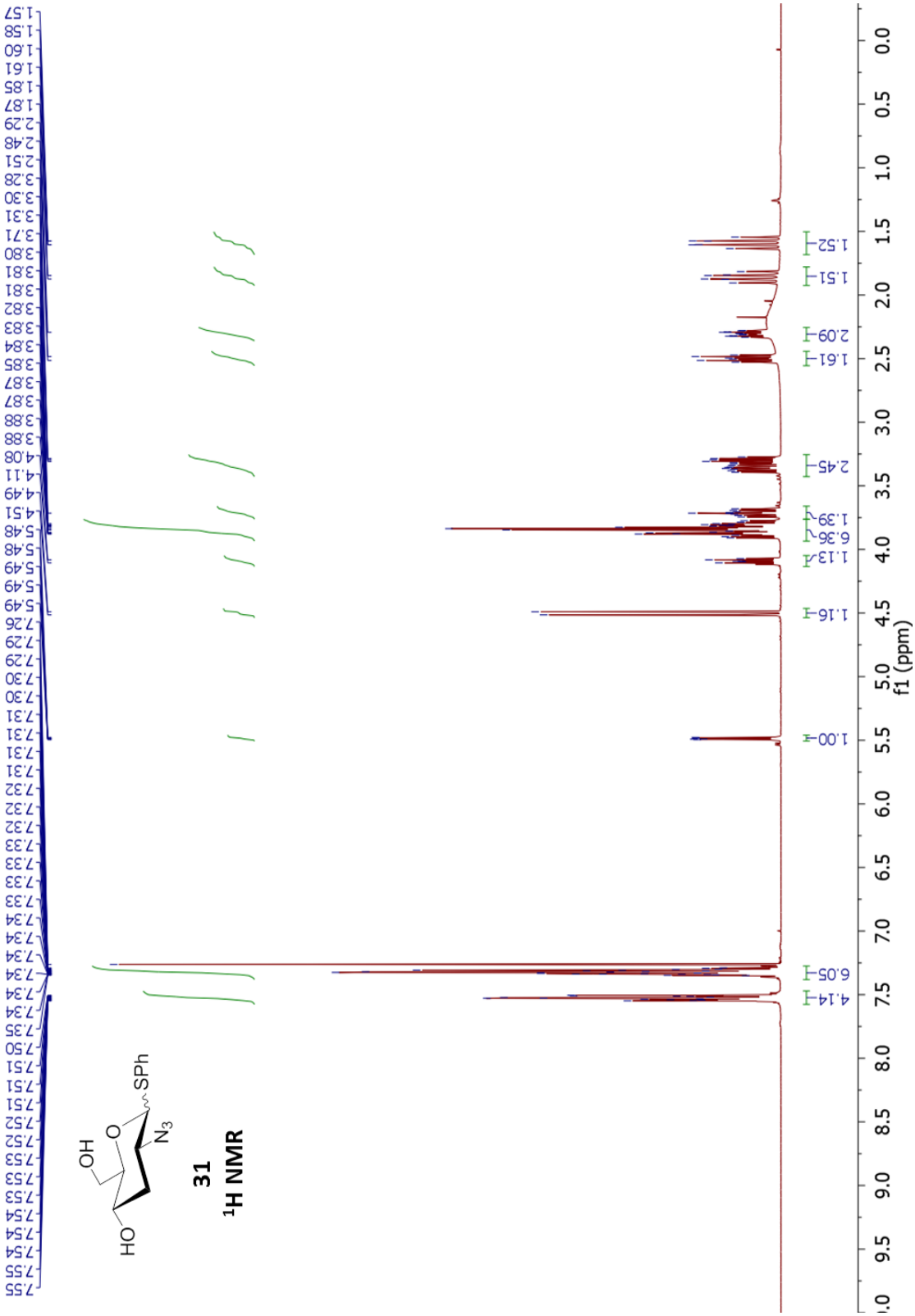
95.30
80.15
75.13
74.54
72.26
63.16
60.04
49.61
48.86
48.10
29.76
28.17
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

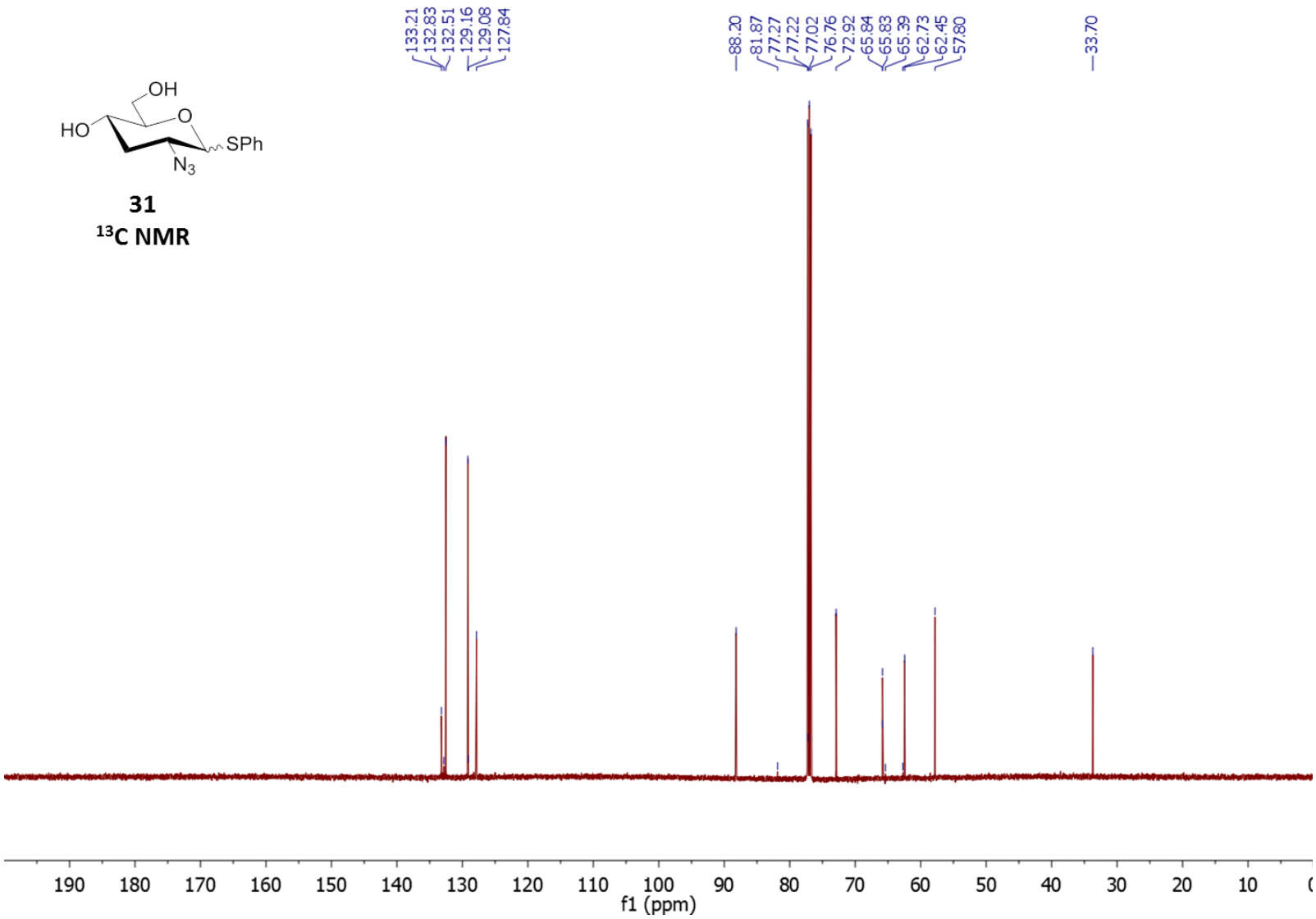

OAc
AcO
O
OAc
N3
29
1H NMR
f1 (ppm)

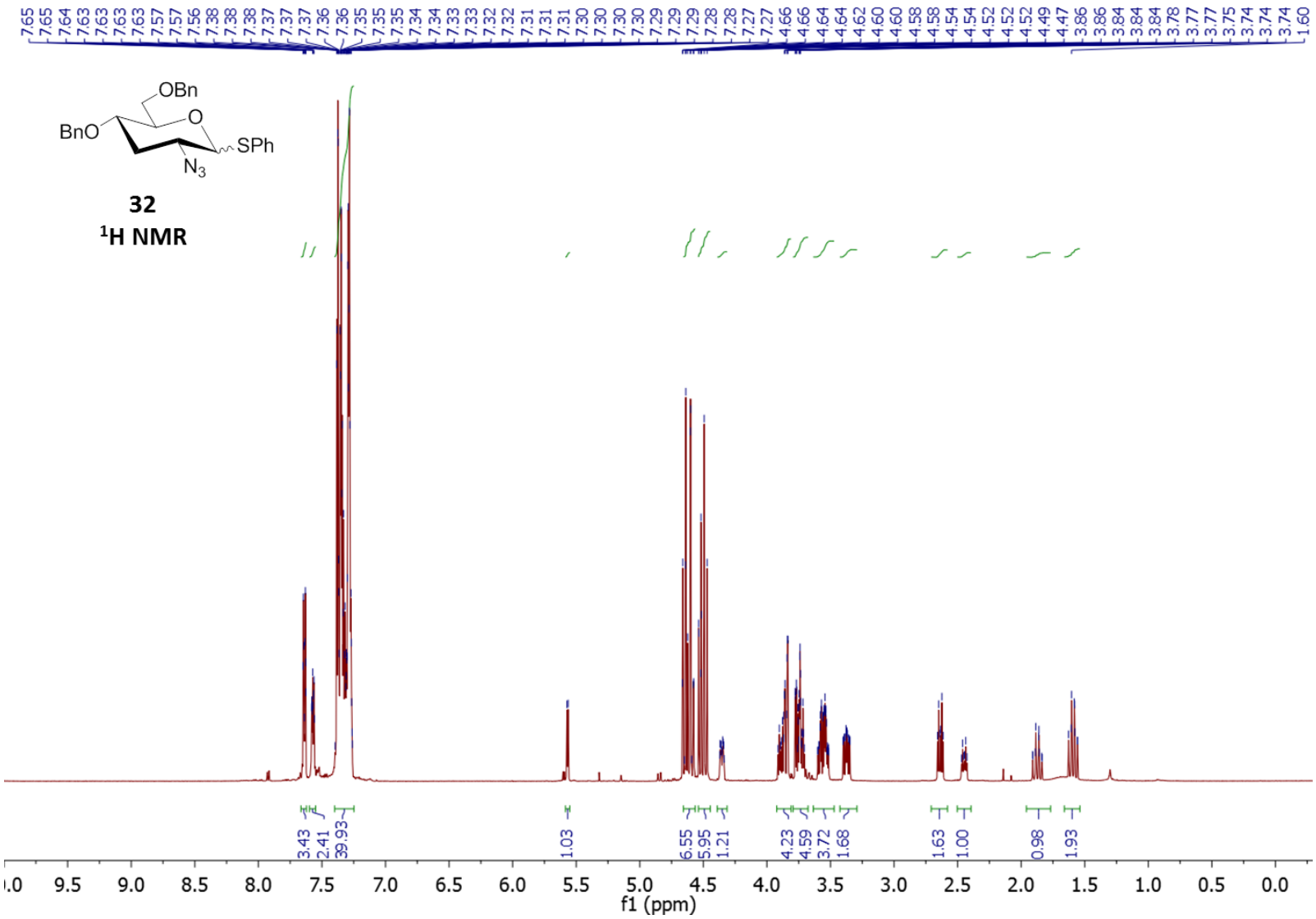

20.74
20.76
20.87
20.89
21.03
21.05
28.52
33.17
55.28
57.59
61.93
61.99
65.15
65.39
69.86
75.82
76.76
77.01
77.21
77.26
89.64
94.73
168.77
168.93
169.58
169.69
170.71
OAc
O
OAc
N3
AcO
29
13C NMR
f1 (ppm)

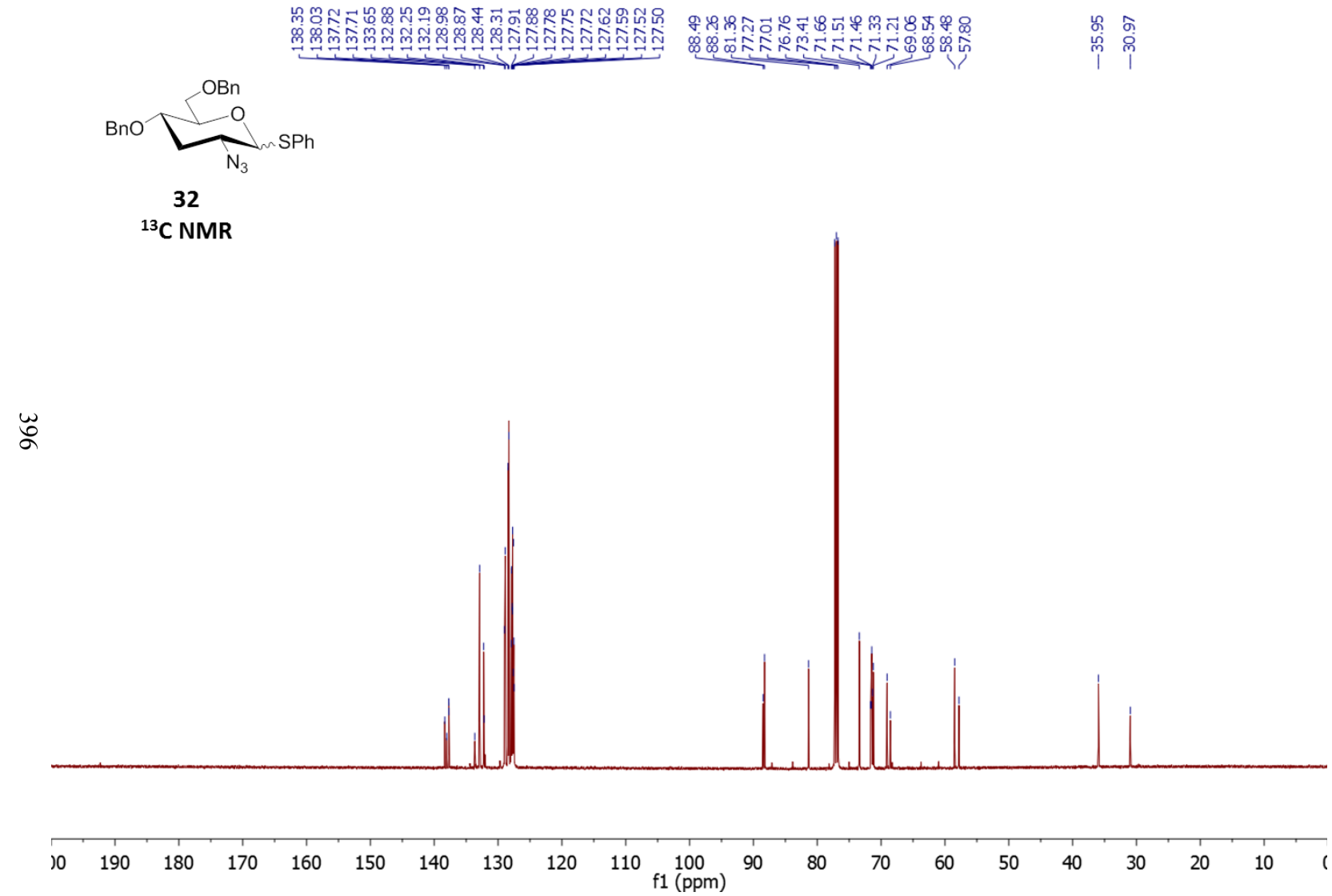

OAc
O
SPh
N3
AcO
30
1H NMR
f1 (ppm)

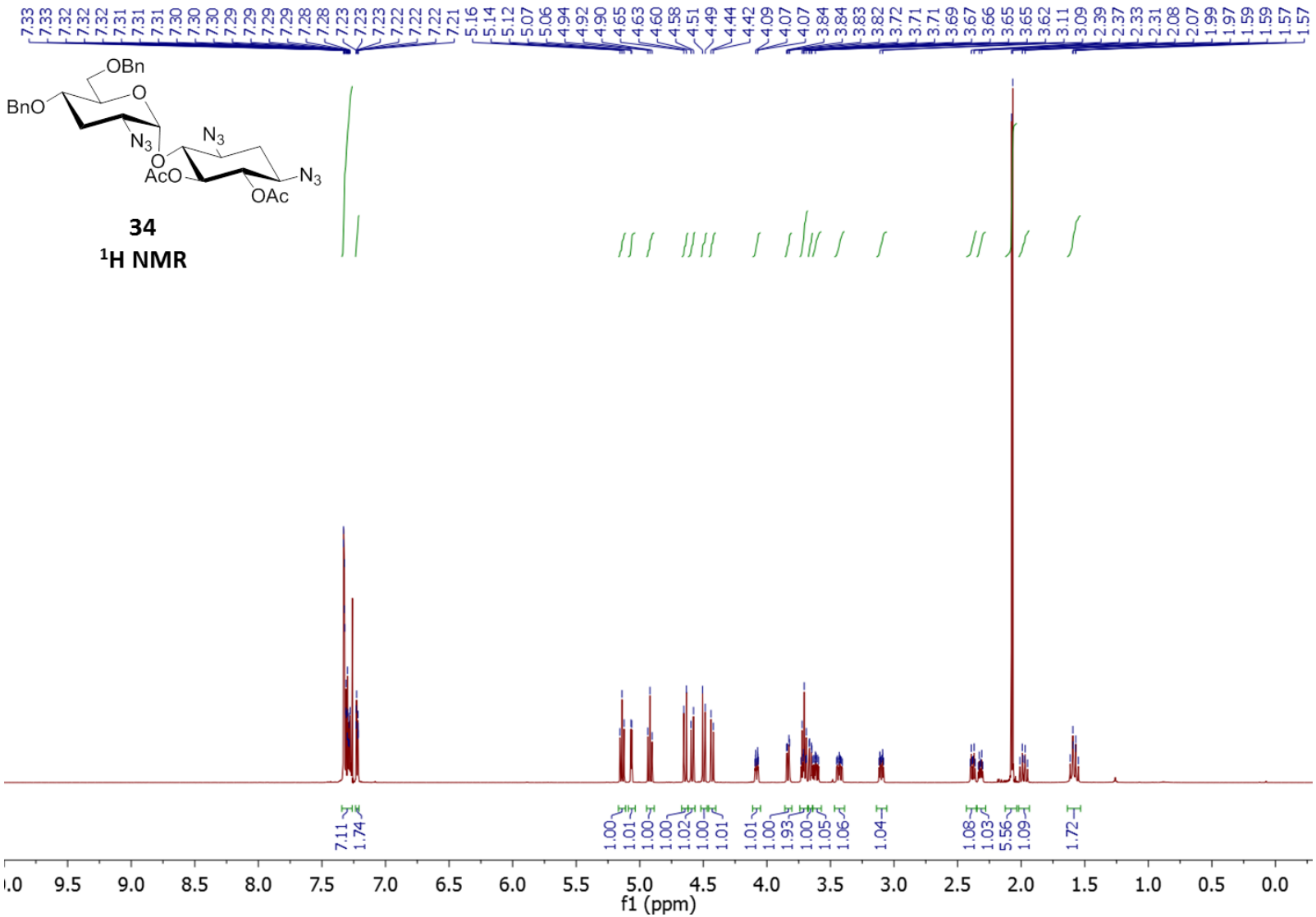

170.62
170.60
169.72
169.64
133.43
132.91
132.32
131.36
129.13
129.12
128.93
128.92
128.38
127.85
88.18
87.94
77.87
77.31
77.06
76.80
68.71
66.11
65.76
62.66
62.42
57.88
57.23
35.47
30.64
20.91
20.90
20.86
20.76
20.71
20.71
OAc
AcO
O
SPh
N3
30
13C NMR
f1 (ppm)

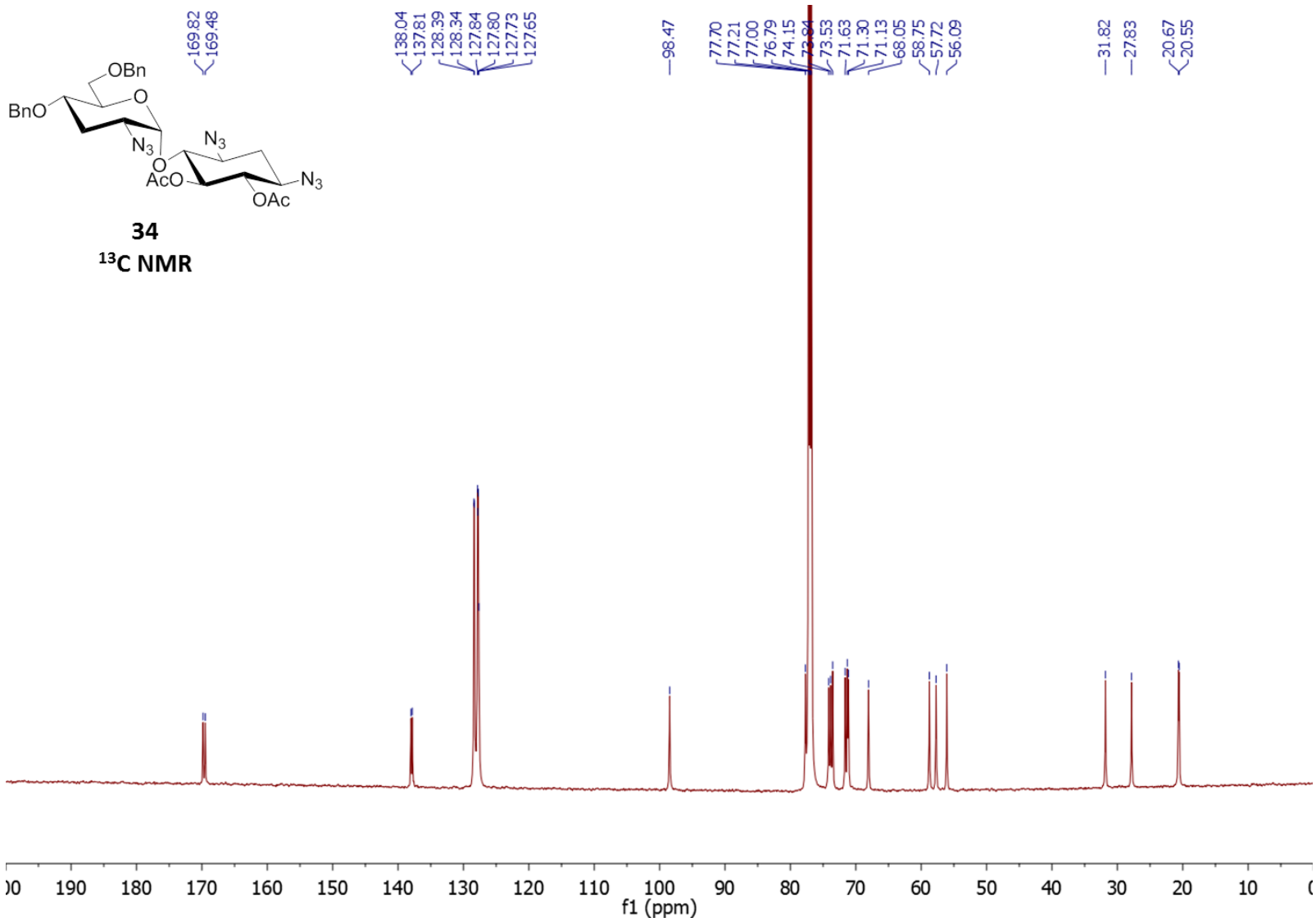

OH
O
SPh
N3
HO
31
1H NMR
f1 (ppm)


OH
HO
O
SPh
N3
31
13C NMR
133.21
132.83
132.51
129.16
129.08
127.84
88.20
81.87
77.27
77.22
77.02
76.76
72.92
65.84
65.83
65.39
62.73
62.45
57.80
33.70
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


OBn
O
BnO
SPh
N3
32
1H NMR
f1 (ppm)

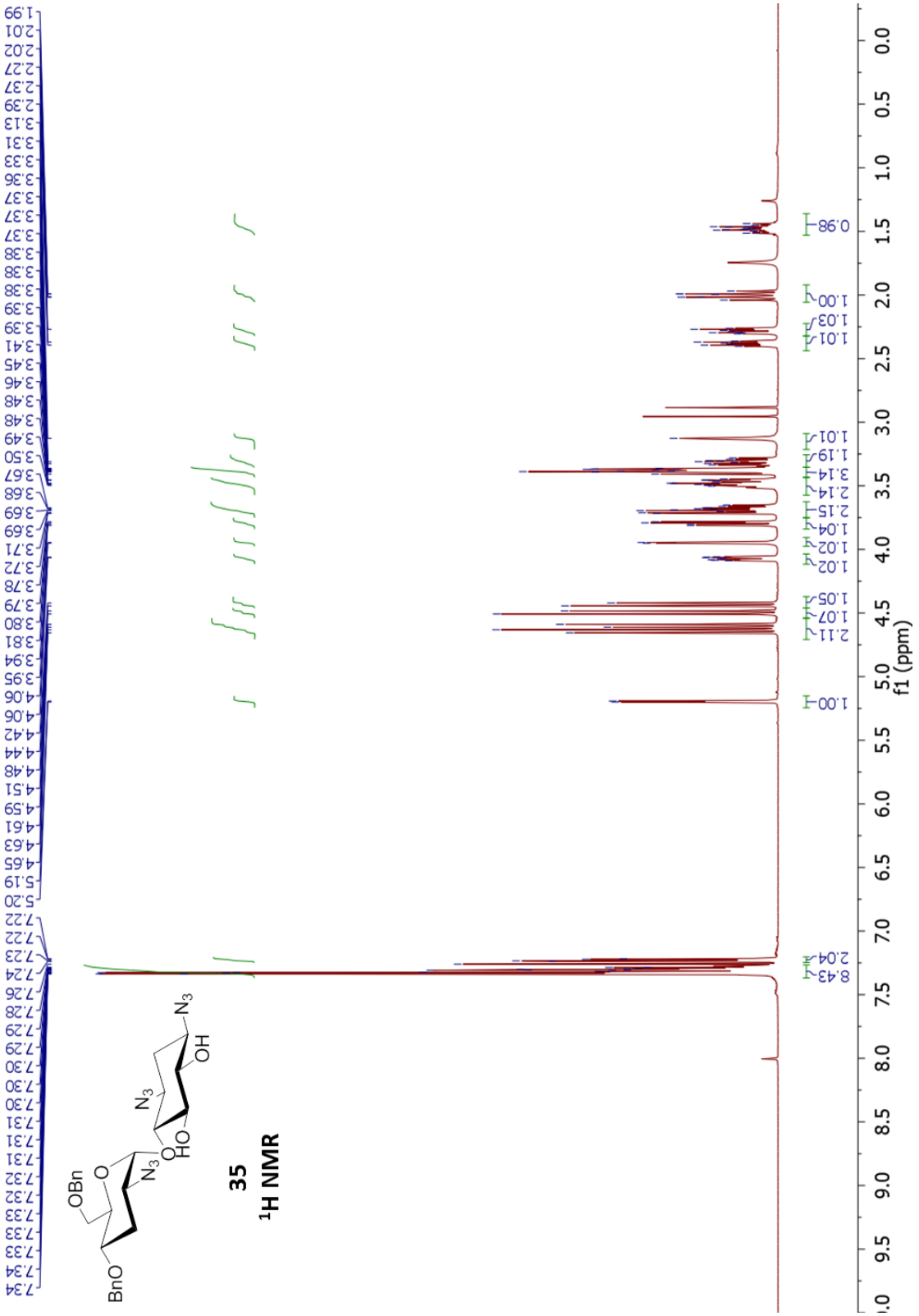

138.35
138.03
137.72
137.71
133.65
132.88
132.25
132.19
128.98
128.87
128.44
128.31
127.91
127.88
127.78
127.75
127.72
127.62
127.59
127.52
127.50
88.49
88.26
81.36
77.27
77.01
76.76
73.41
71.66
71.51
71.46
71.33
71.21
69.06
68.54
58.48
57.80
35.95
30.97
OBn
O
BnO
SPh
N3
32
13C NMR
f1 (ppm)

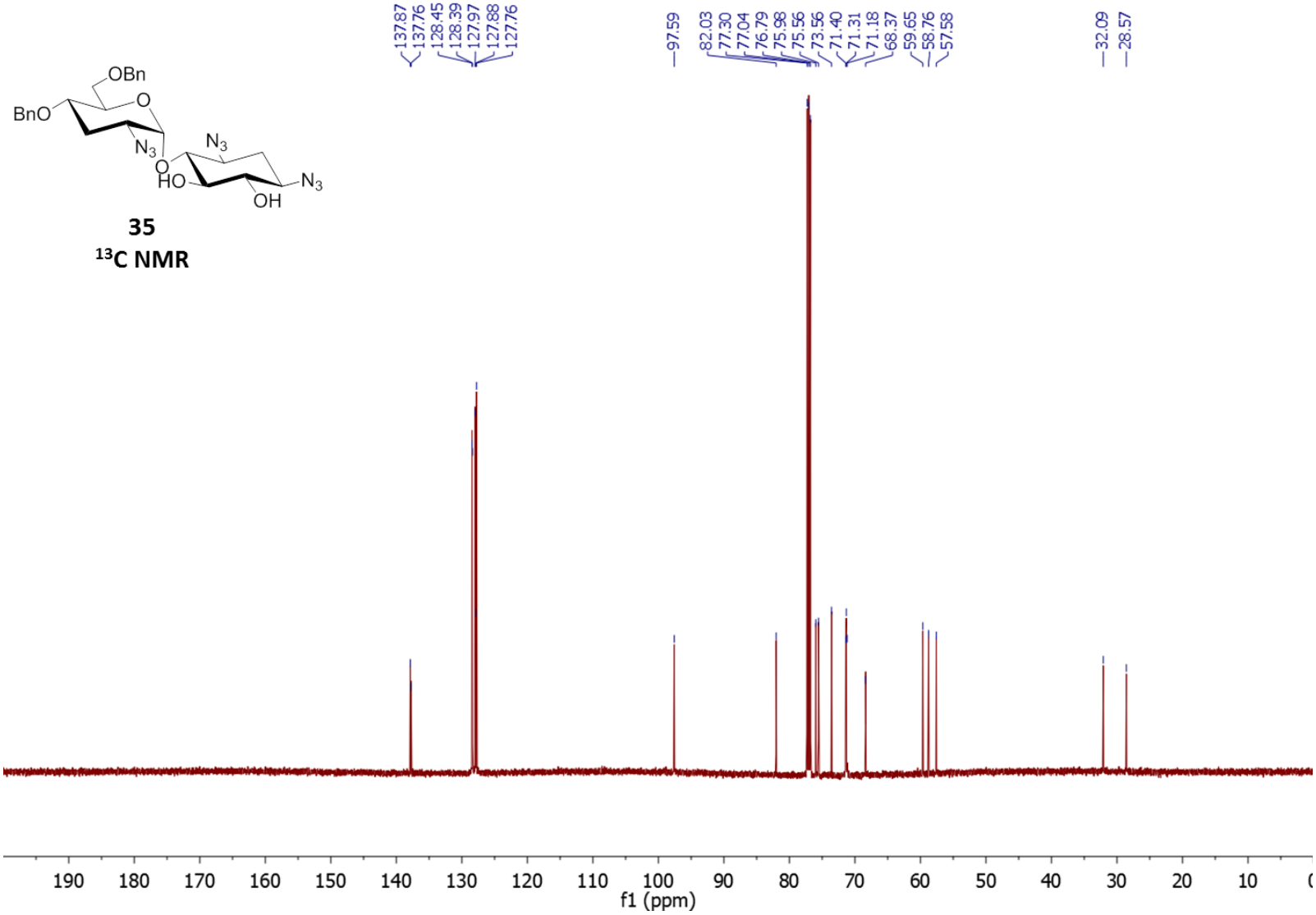

OBn
BnO
O
N3
O
AcO
N3
N3
OAc
34
1H NMR
f1 (ppm)

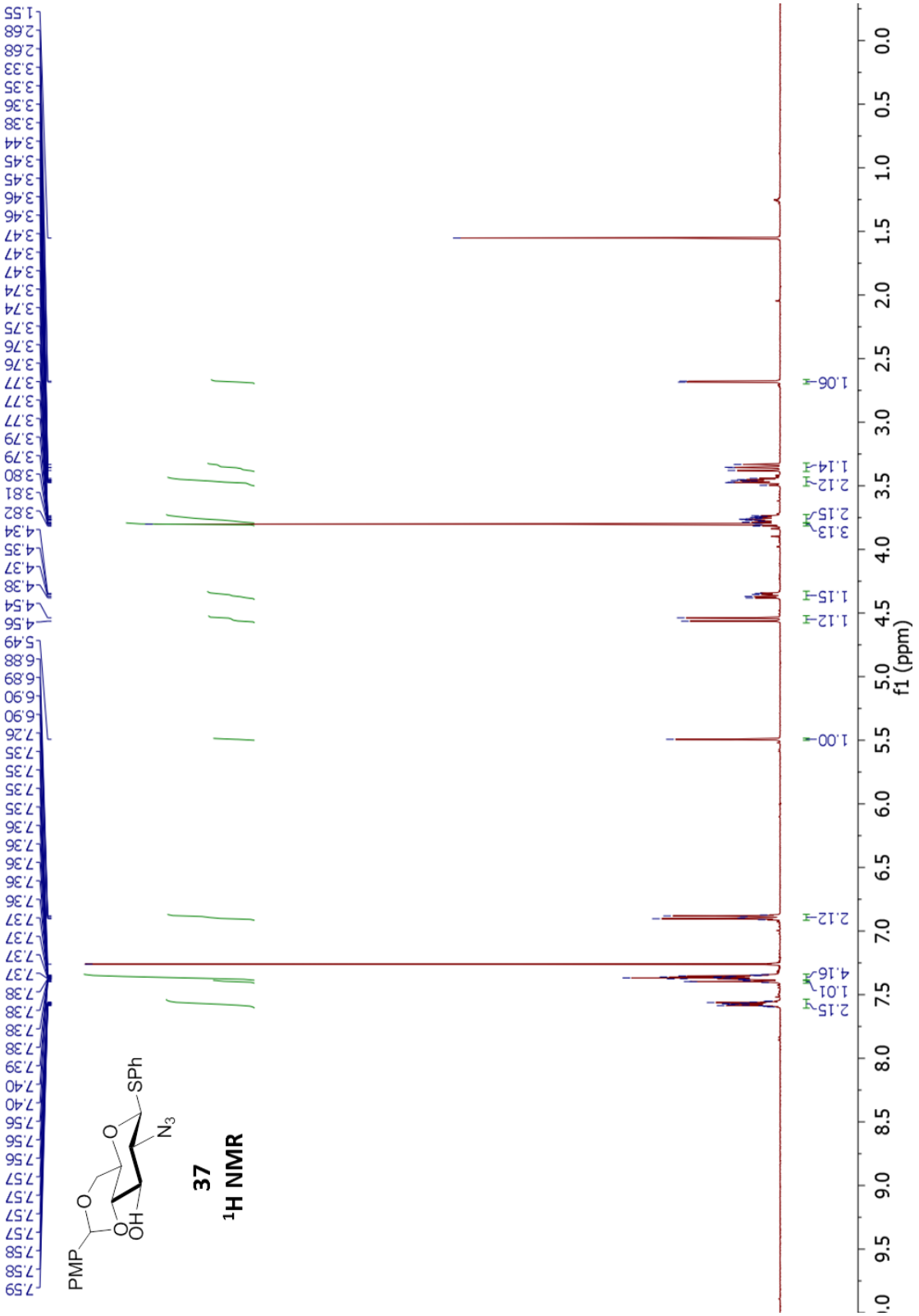

OBn
BnO
O
N3
N3
O
AcO
N3
OAc
169.82
169.48
138.04
137.81
128.39
128.34
127.84
127.80
127.73
127.65
98.47
77.70
77.21
77.00
76.79
74.15
73.84
73.53
71.63
71.30
71.13
68.05
58.75
57.72
56.09
31.82
27.83
20.67
20.55
f1 (ppm)

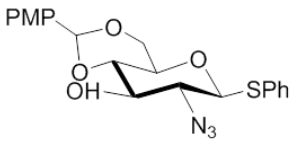
34

^{13}C NMR


BnO
OBn
O
N3
N3
O
HO
OH
N3
35
1H NMR
f1 (ppm)


137.87
137.76
128.45
128.39
127.97
127.88
127.76
97.59
82.03
77.30
77.04
76.79
75.98
75.56
73.56
71.40
71.31
71.18
68.37
59.65
58.76
57.58
32.09
28.57
OBn
BnO
O
N3
N3
O
HO
N3
OH
35
13C NMR
400
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

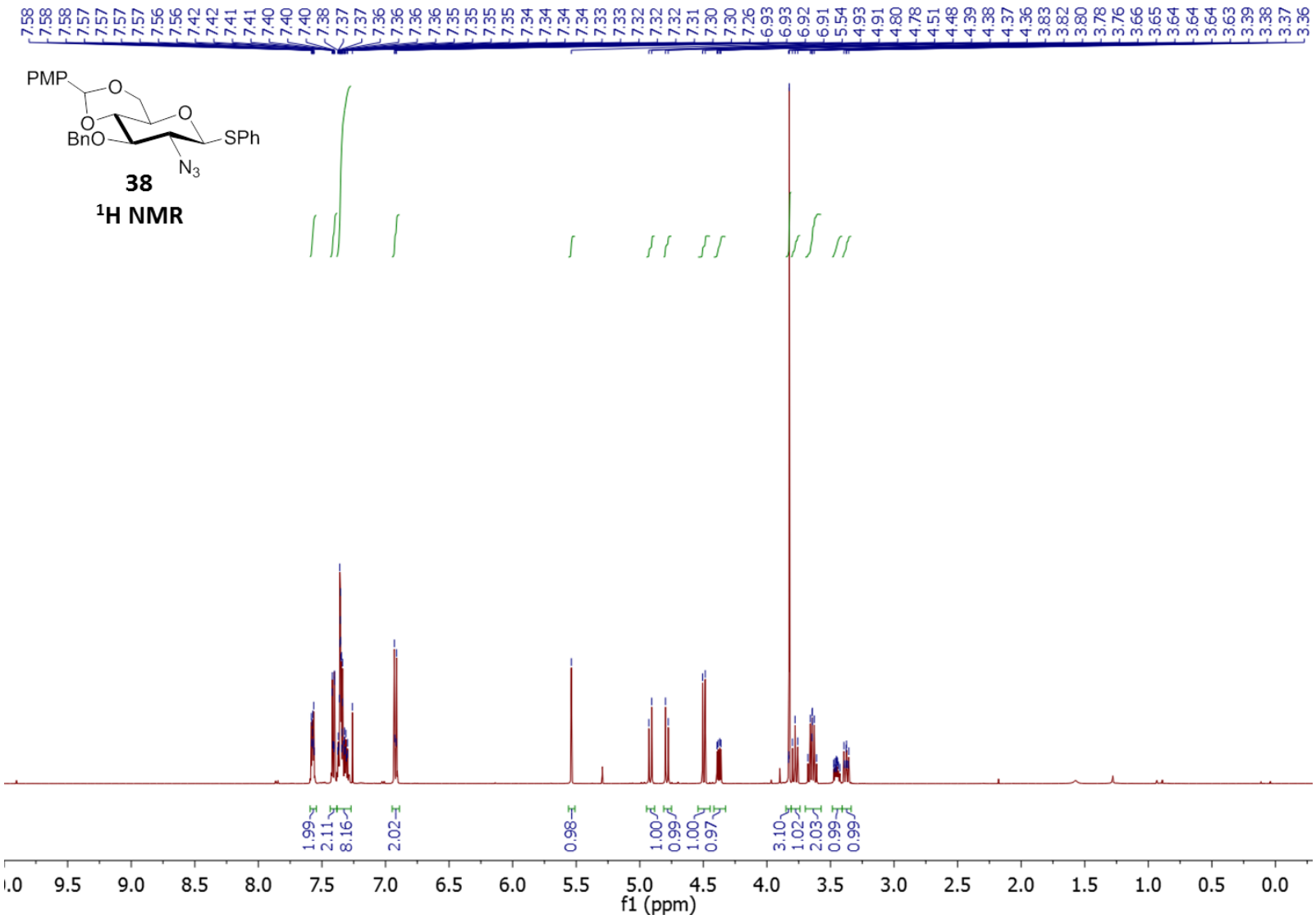

PMP
O
O
O
OH
SPh
N_3
37
^{1}H NMR
1.06
1.14
2.12
2.15
3.13
1.15
1.12
1.00
2.12
4.16
1.01
2.15
f1 (ppm)

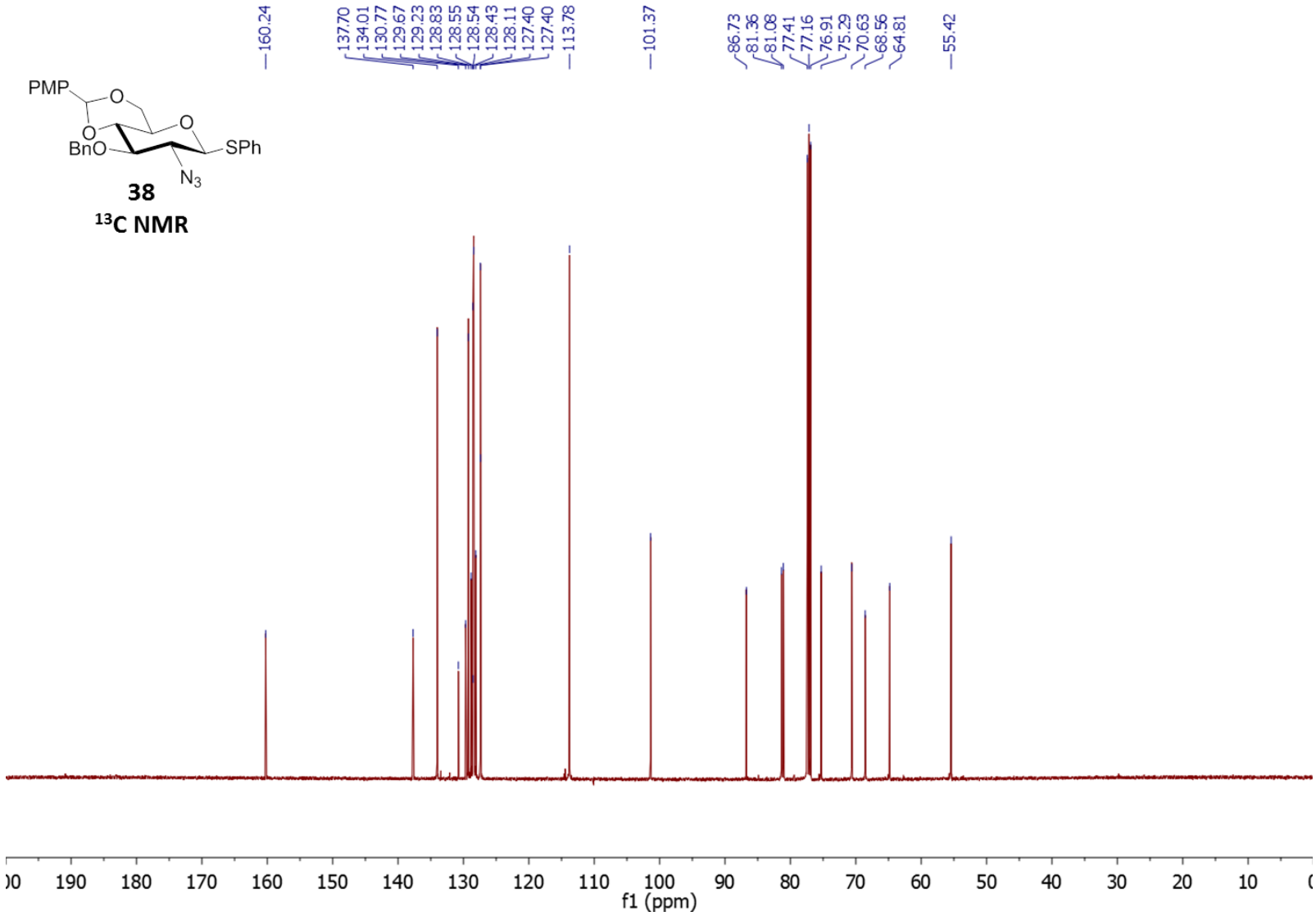

PMP
O
O
O
OH
SPh
N3

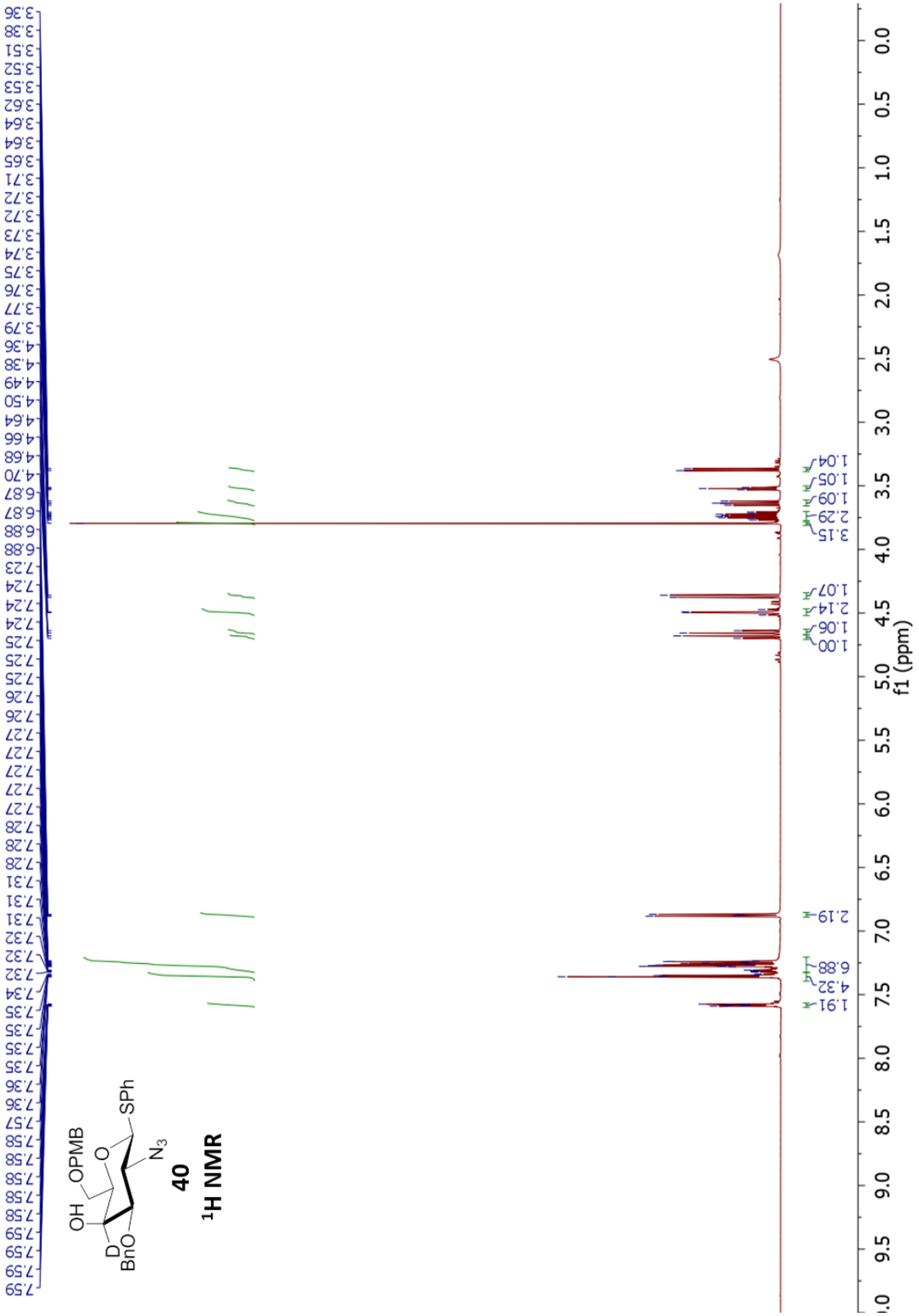
37

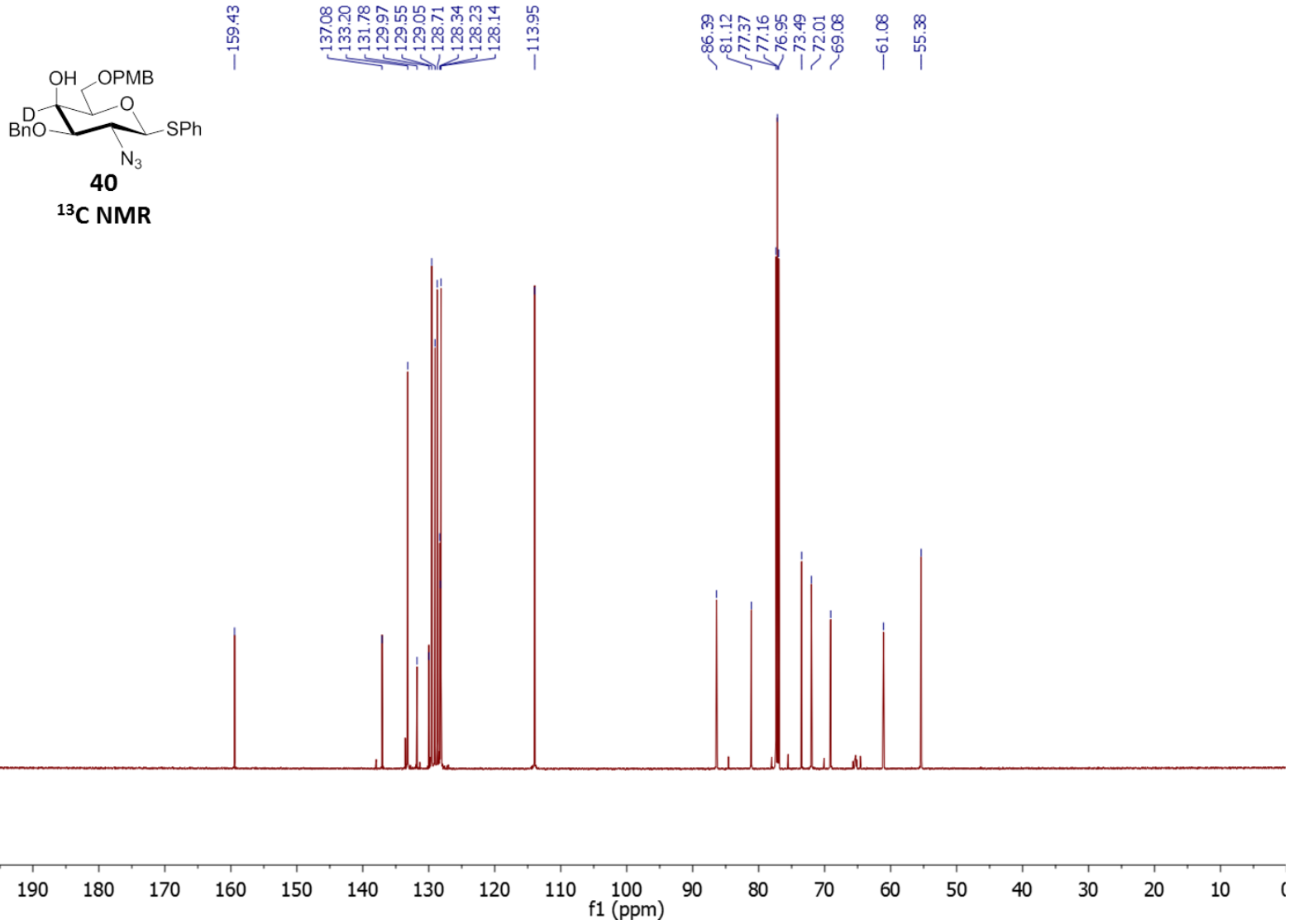
^{13}C NMR

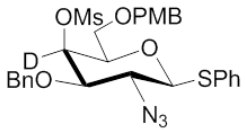
133.79
133.57
130.00
129.02
87.23
82.11
78.40
71.13
67.08
62.65
49.43
49.28
49.14
49.00
48.86
48.72
48.57
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


PMP
O
O
O
BnO
SPh
N3
38
1H NMR
f1 (ppm)


PMP
O
O
O
BnO
SPh
N3
38
13C NMR
160.24
137.70
134.01
130.77
129.67
129.23
128.83
128.55
128.54
128.43
128.11
127.40
127.40
113.78
101.37
86.73
81.36
81.08
77.41
77.16
76.91
75.29
70.63
68.56
64.81
55.42
f1 (ppm)


OH
OPMB
O
SPh
N_3
D
BnO
40
^{1}H NMR
f1 (ppm)

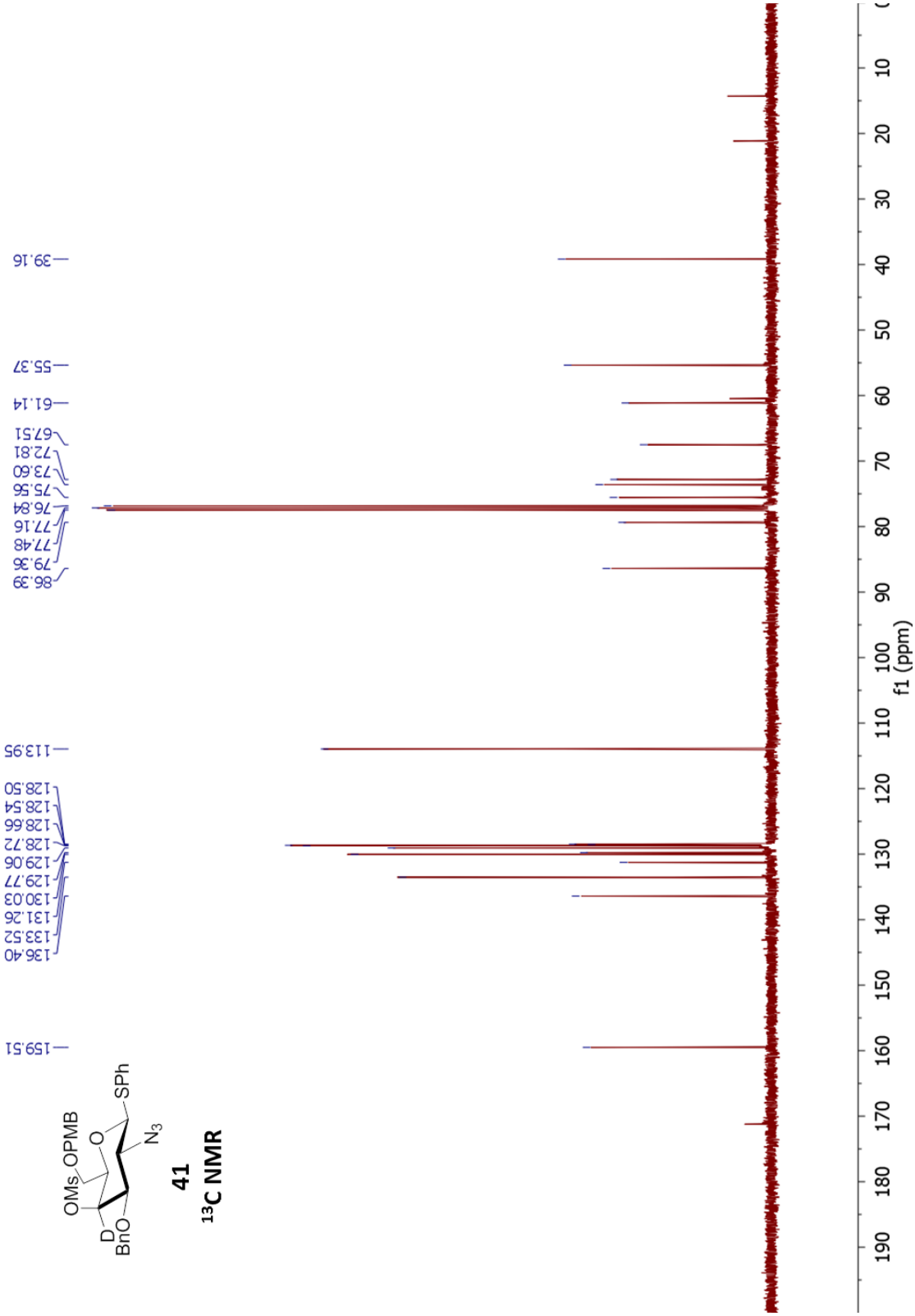

159.43
137.08
133.20
131.78
129.97
129.55
129.05
128.71
128.34
128.23
128.14
113.95
86.39
81.12
77.37
77.16
76.95
73.49
72.01
69.08
61.08
55.38
OH
OPMB
D
BnO
O
SPh
N3
40
13C NMR
f1 (ppm)

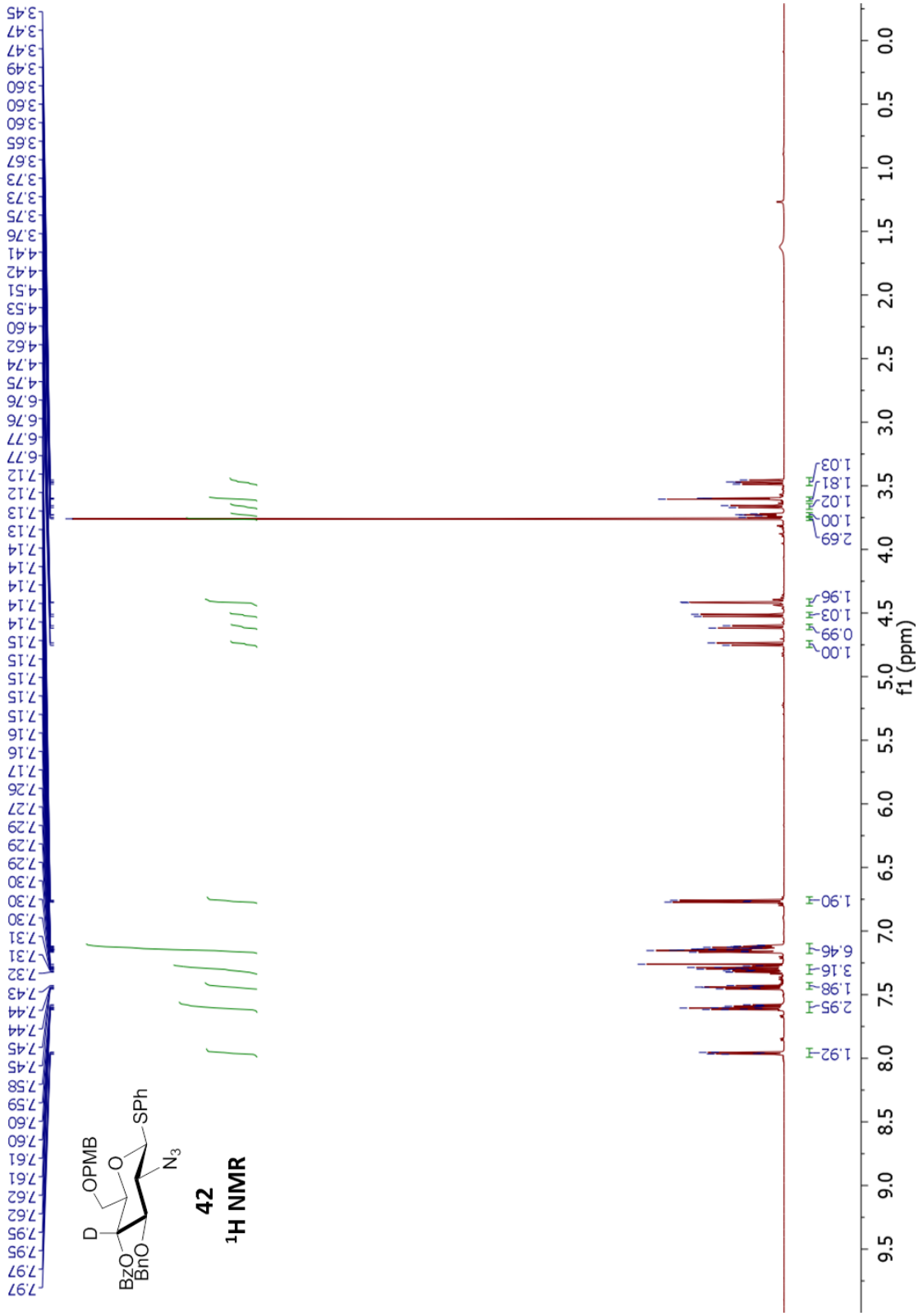

OMs
OPMB
O
D
BnO
SPh
N3

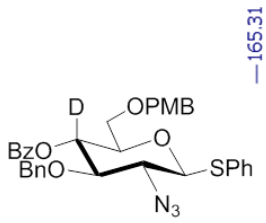
41

^{1}H NMR

4.83
4.80
4.60
4.60
4.58
4.57
4.44
4.43
4.41
4.40
3.81
3.75
3.74
3.72
3.72
3.70
3.69
3.69
3.54
3.52
3.49
3.47
3.45
2.92
2.16
10.46
2.17
1.18
2.19
1.13
1.00
3.28
3.16
2.31
3.07
f1 (ppm)


159.51
136.40
133.52
131.26
130.03
129.77
129.06
128.72
128.66
128.54
128.50
113.95
86.39
79.36
77.48
77.16
76.84
75.56
73.60
72.81
67.51
61.14
55.37
39.16
f1 (ppm)
OMs
OPMB
O
SPh
N3
D
BnO
41
13C NMR

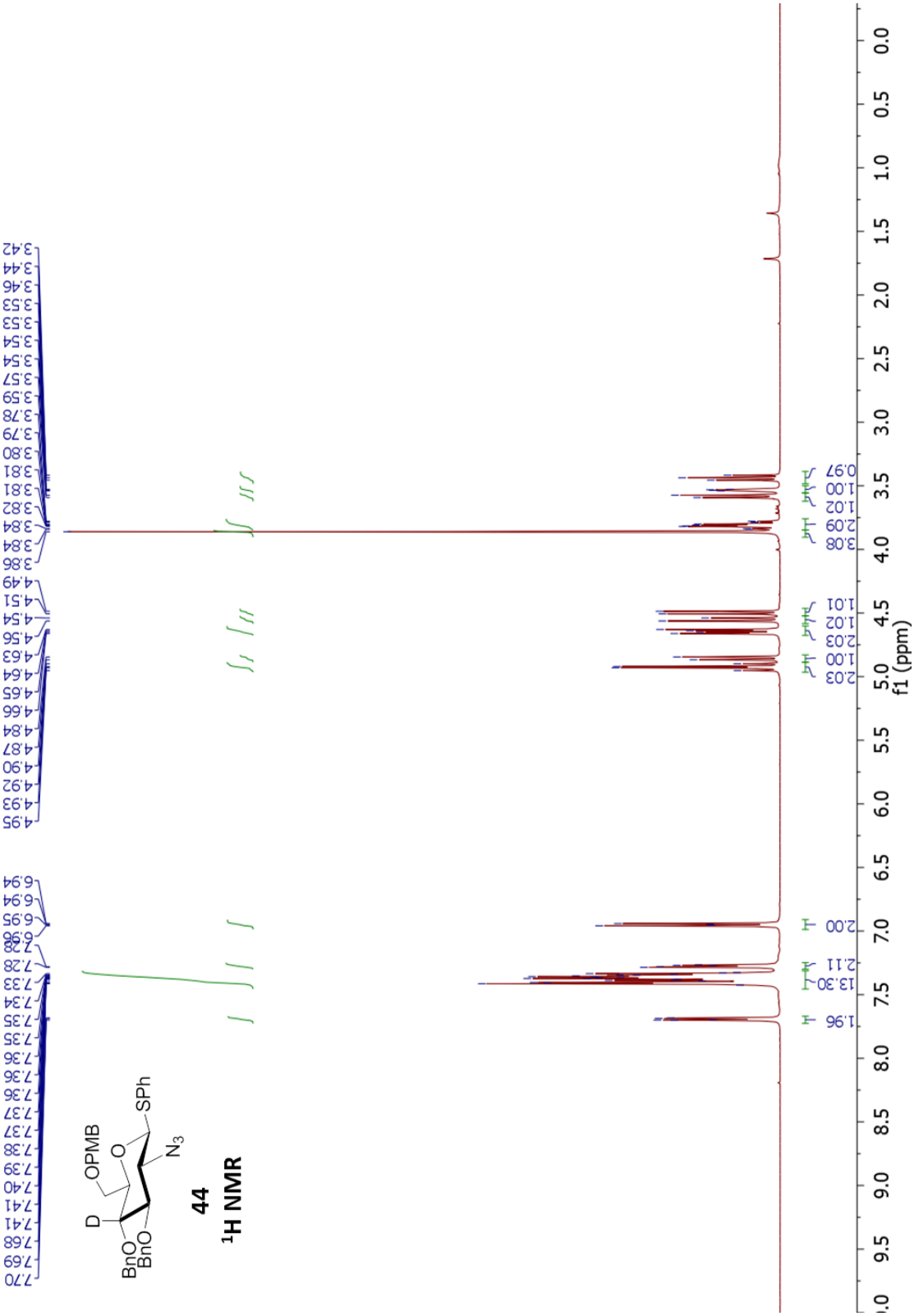

42
^{1}H NMR
D
OPMB
BzO
BnO
O
SPh
N_3
f1 (ppm)

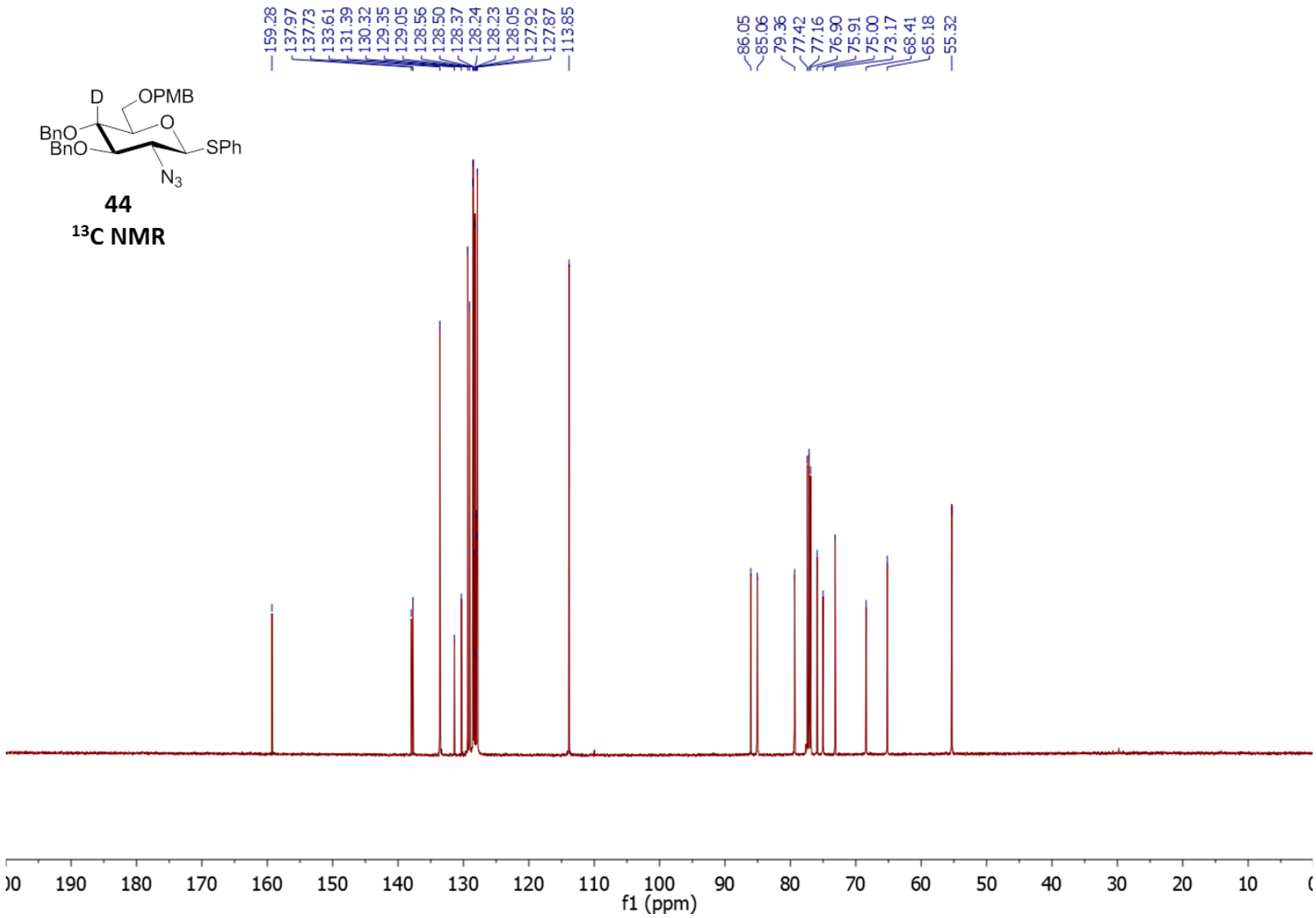

D
OPMB
O
BzO
BnO
SPh
N3

42

^{13}C NMR

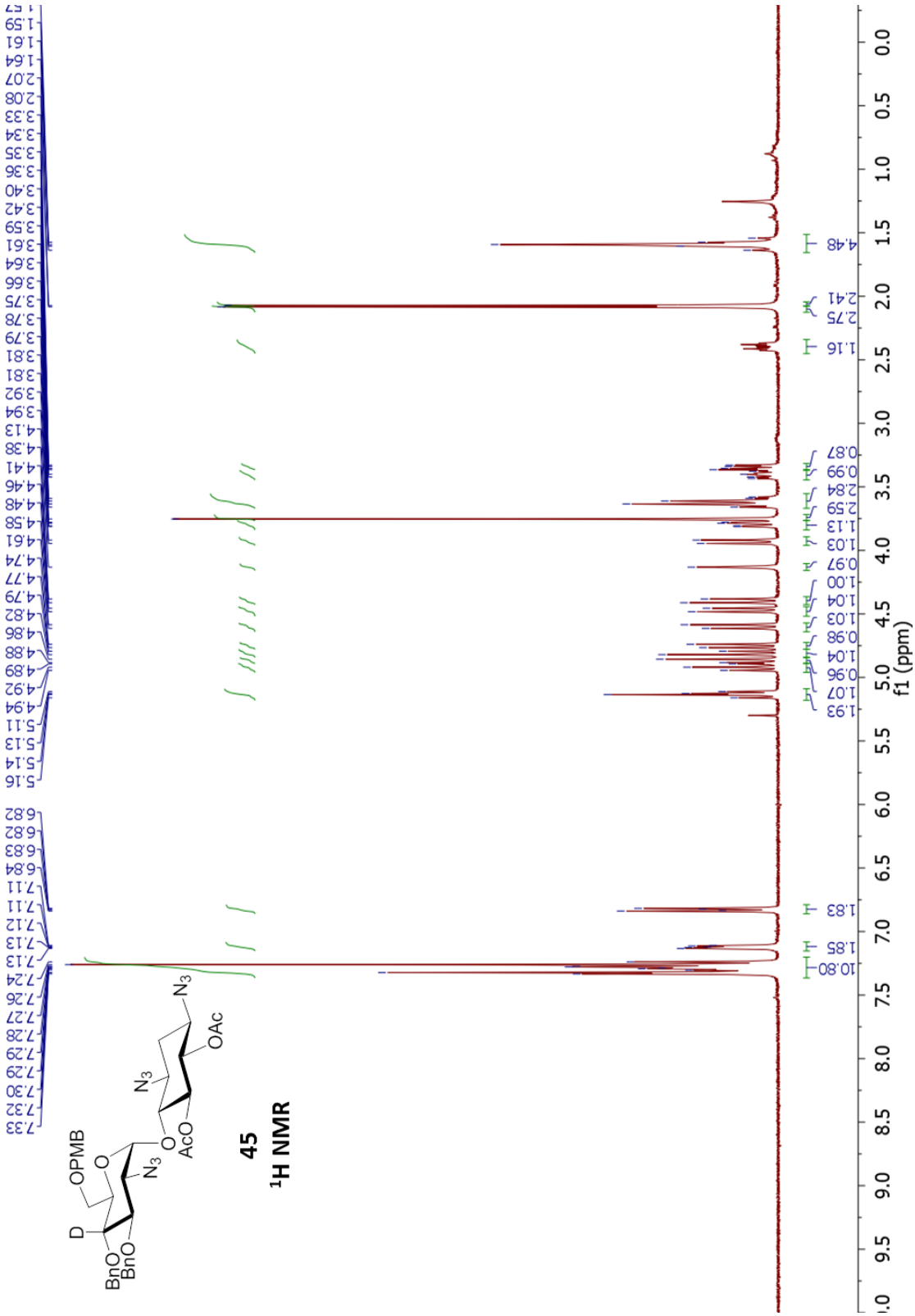
165.31
159.24
137.09
133.63
133.51
131.17
130.00
129.88
129.51
129.45
129.20
128.61
128.59
128.43
128.37
128.05
113.78
86.25
82.48
78.07
77.37
77.16
76.95
75.51
73.32
69.23
64.93
55.35
f1 (ppm)

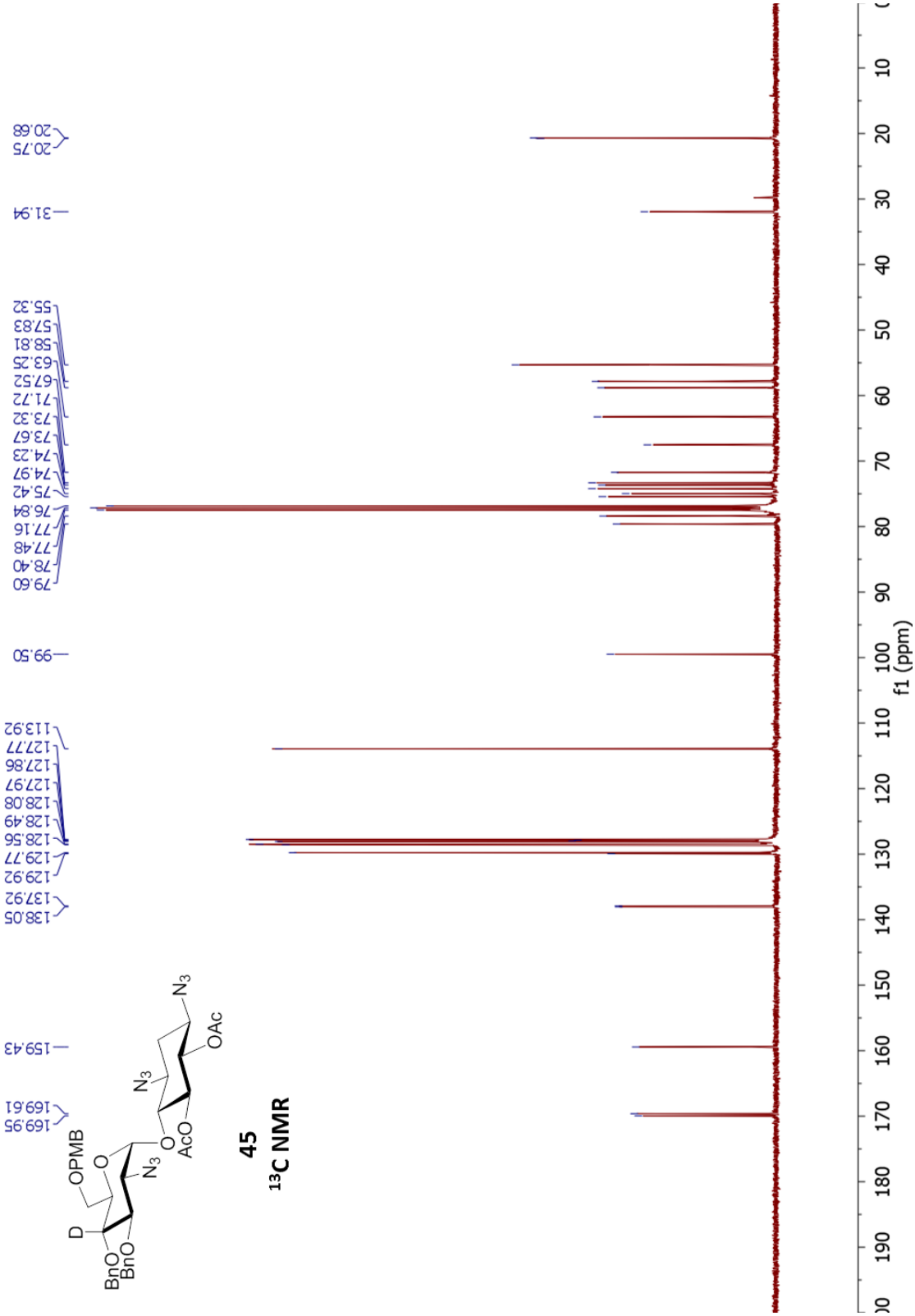

OPMB
O
SPh
D
N3
BnO
BnO
44
1H NMR
f1 (ppm)

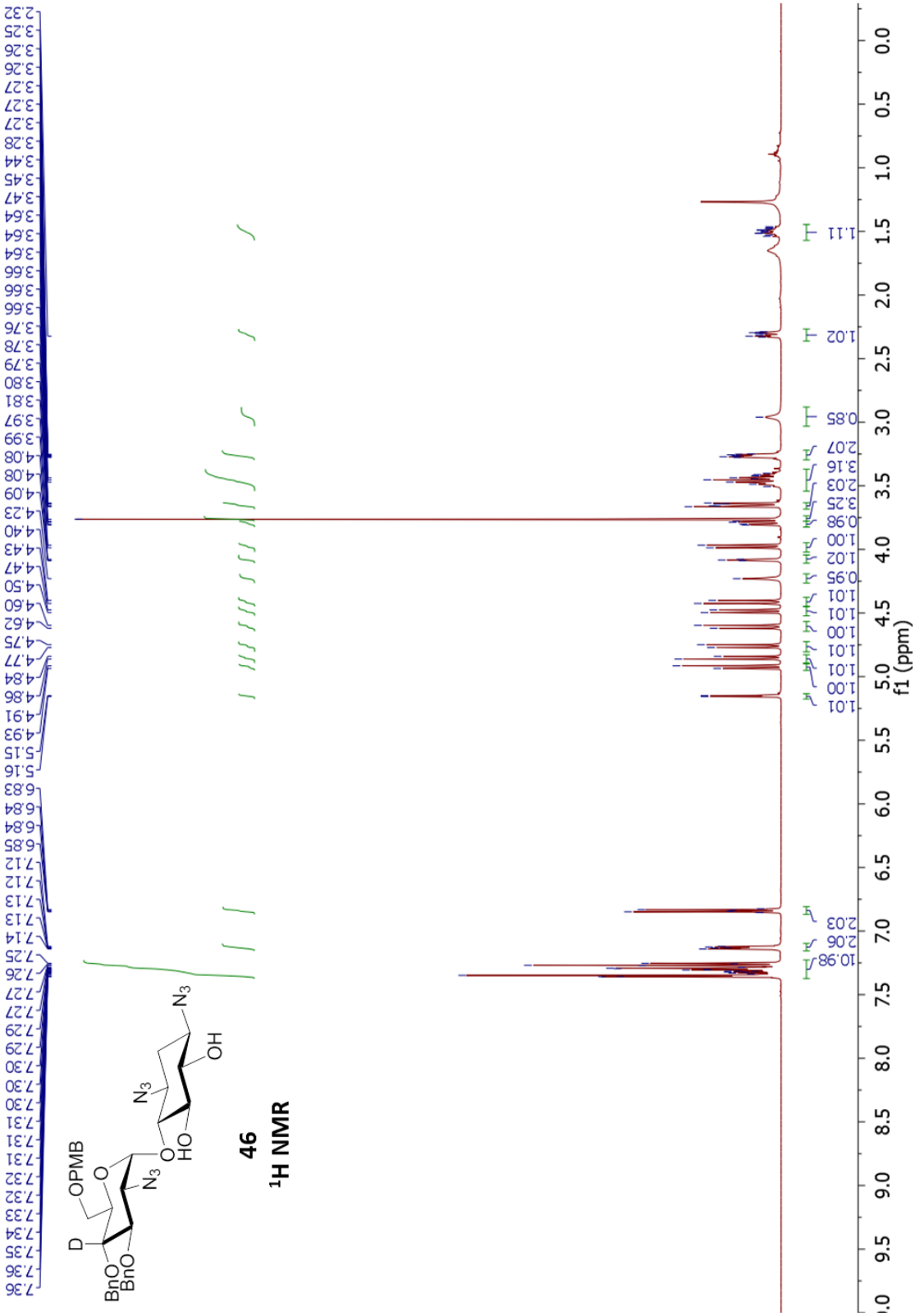

159.28
137.97
137.73
133.61
131.39
130.32
129.35
129.05
128.56
128.50
128.37
128.24
128.23
128.05
127.92
127.87
113.85
86.05
85.06
79.36
77.42
77.16
76.90
75.91
75.00
73.17
68.41
65.18
55.32
D
OPMB
O
BnO
BnO
SPh
N3
f1 (ppm)

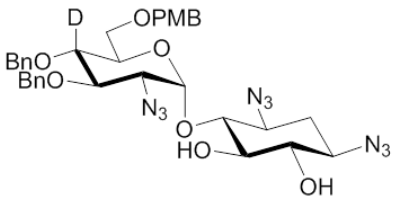
44

^{13}C NMR


OPMB
D
BnO
BnO
O
N3
N3
O
AcO
OAc
N3
45
1H NMR
f1 (ppm)


45
13C NMR
f1 (ppm)

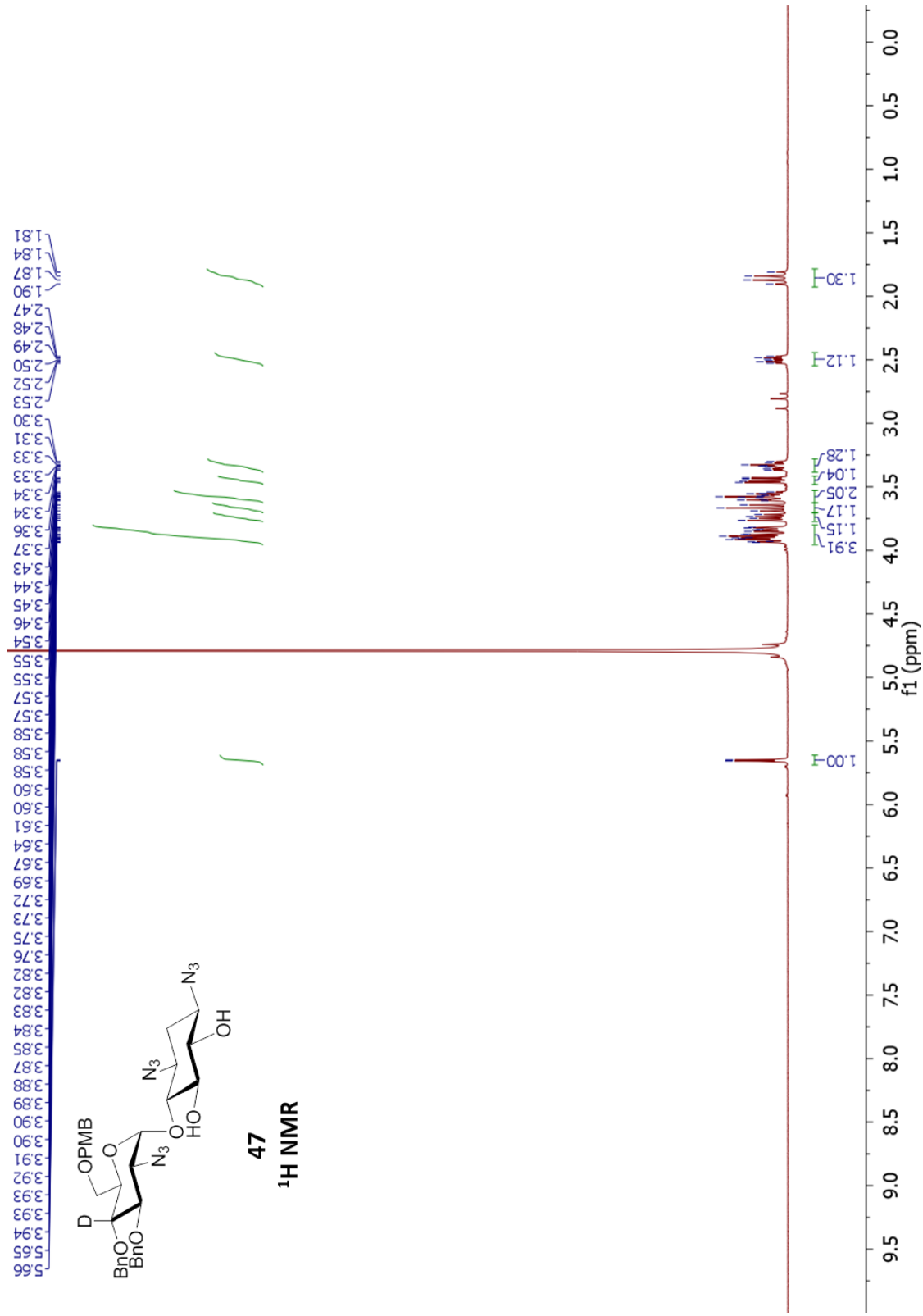

BnO
BnO
D
OPMB
O
N3
O
HO
N3
N3
OH
46
1H NMR
f1 (ppm)

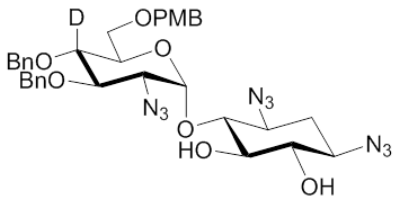

D
OPMB
BnO
BnO
O
N3
O
HO
N3
N3
OH

46

^{13}C NMR

159.45
137.90
137.67
129.87
129.83
128.62
128.56
128.18
128.11
127.98
127.85
113.92
99.71
84.12
81.03
77.41
77.16
76.91
75.98
75.76
75.46
75.04
73.35
71.73
67.61
64.38
59.76
58.88
55.34
32.23
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

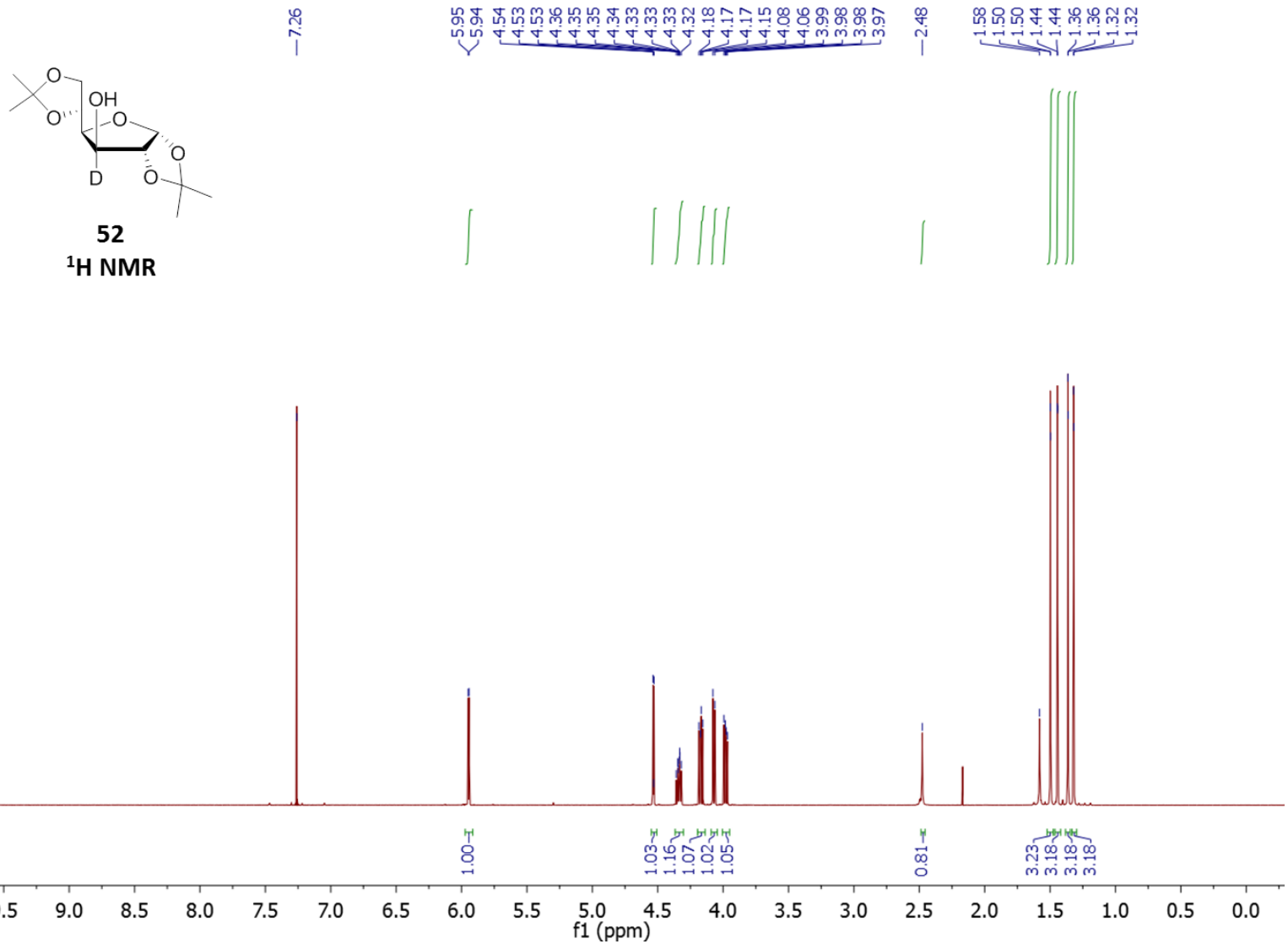

47
^{1}H NMR
f1 (ppm)

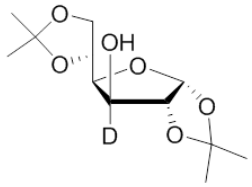

D
OPMB
BnO
BnO
O
N3
N3
O
HO
N3
OH

47

^{13}C NMR

97.10
80.40
74.70
73.59
72.34
68.95
60.33
53.98
49.62
48.84
28.30
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

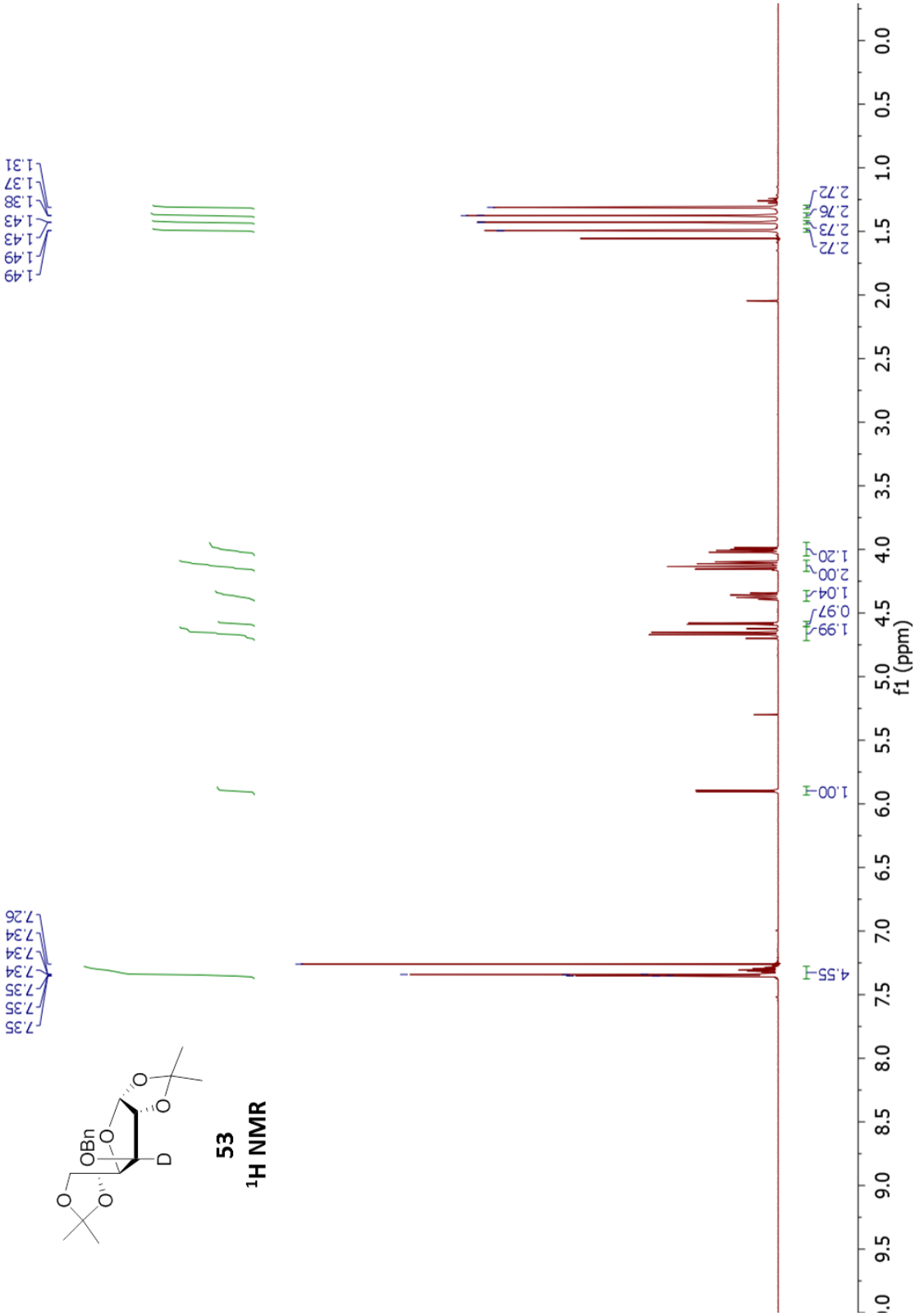

OH
O
O
O
O
O
D
52
1H NMR
7.26
5.95
5.94
4.54
4.53
4.53
4.36
4.35
4.35
4.34
4.33
4.33
4.33
4.32
4.18
4.17
4.17
4.15
4.08
4.06
3.99
3.98
3.98
3.97
2.48
1.58
1.50
1.50
1.44
1.44
1.36
1.36
1.32
1.32
1.00
1.03
1.16
1.07
1.02
1.05
0.81
3.23
3.18
3.18
3.18
f1 (ppm)

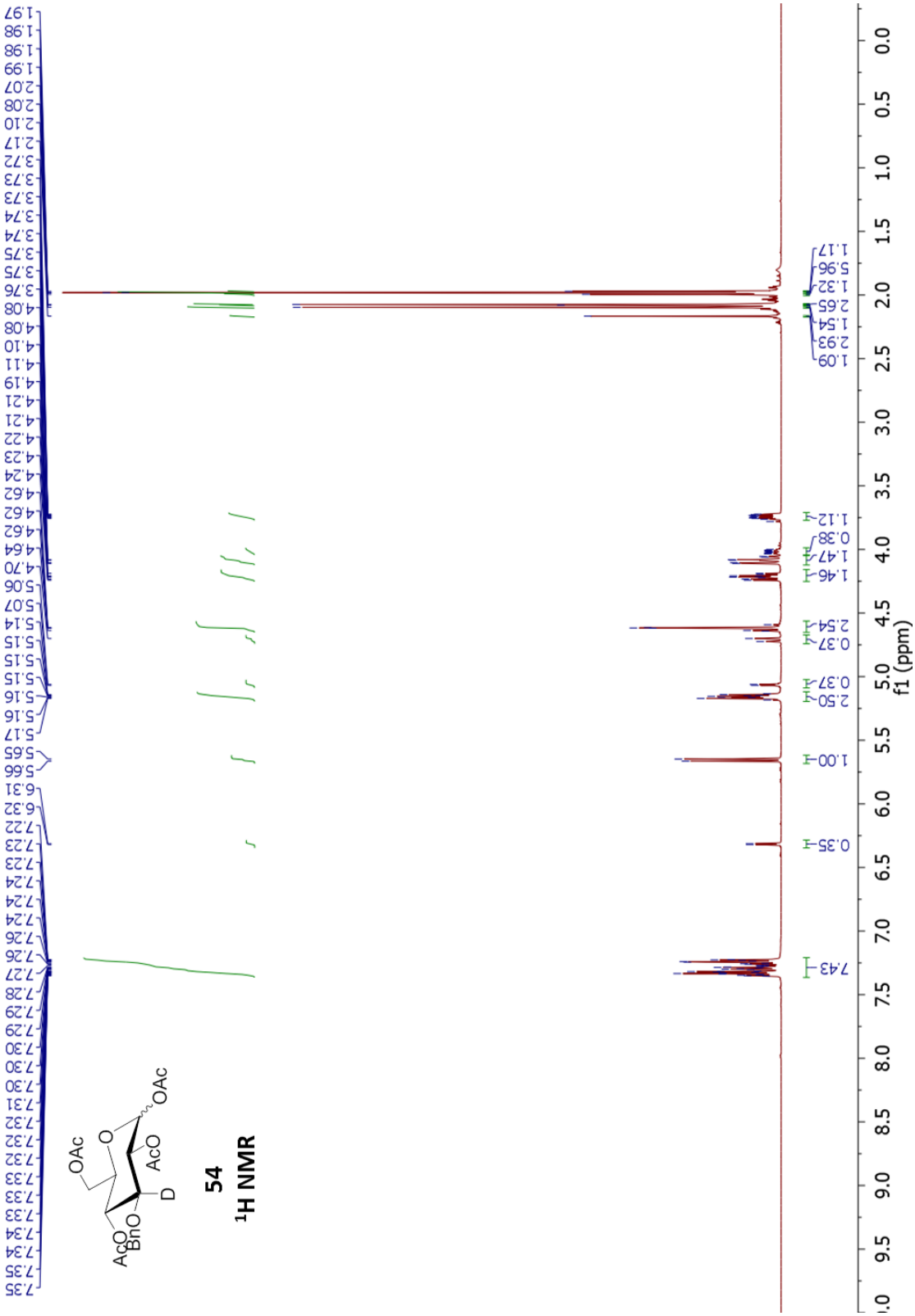

O
OH
O
O
O
D
O

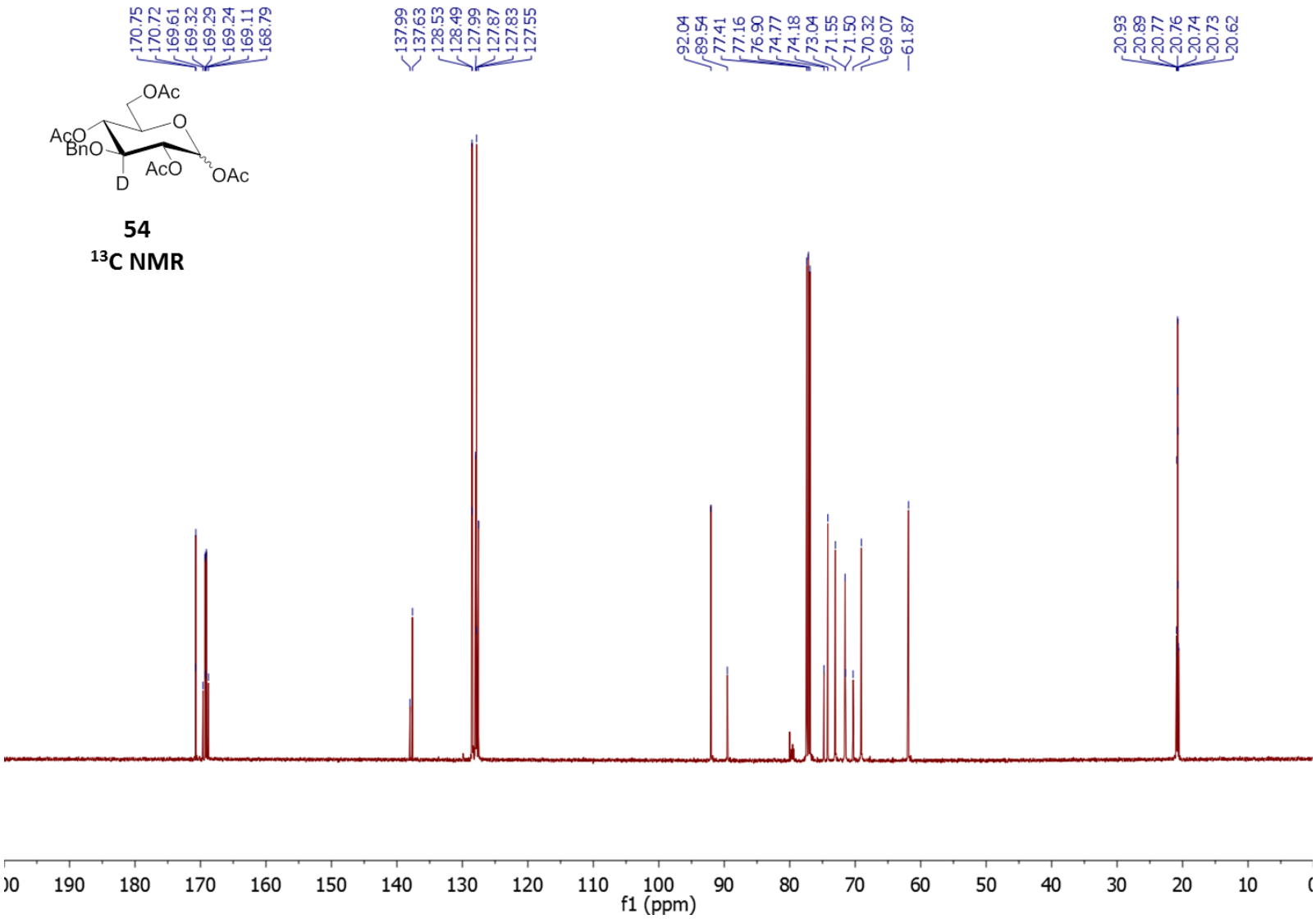
52

^{13}C NMR

111.99
109.82
105.47
85.18
81.21
77.41
77.16
76.91
73.71
67.83
27.01
26.92
26.34
25.29
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

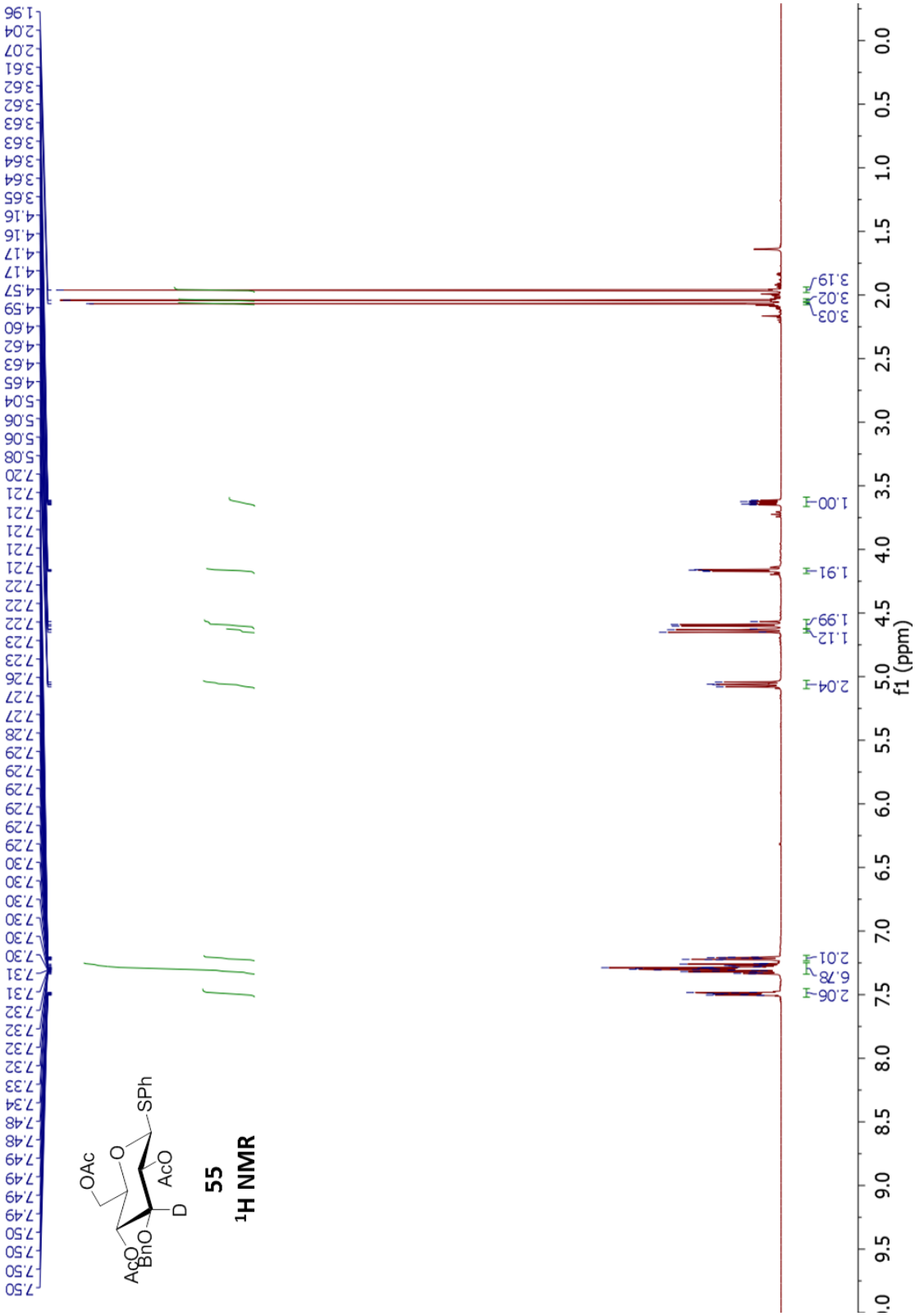

53
^{1}H NMR
OBn
D
7.35
7.35
7.35
7.34
7.34
7.34
7.26
1.49
1.49
1.43
1.43
1.38
1.37
1.31
4.55
1.00
1.99
0.97
1.04
2.00
1.20
2.72
2.73
2.76
2.72
f1 (ppm)

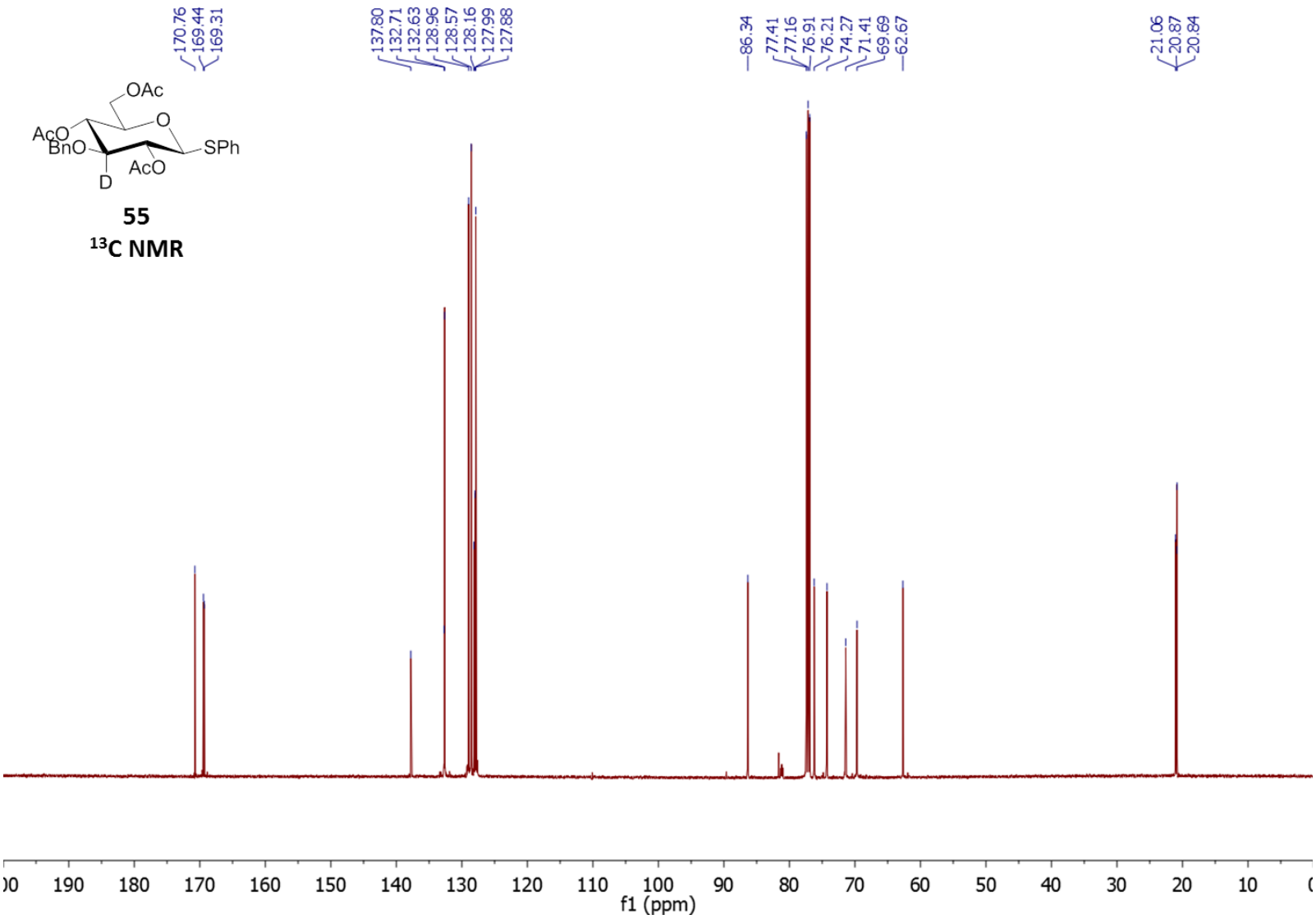

54
^{1}H NMR
OAc
O
OAc
AcO
D
BnO
AcO
f1 (ppm)

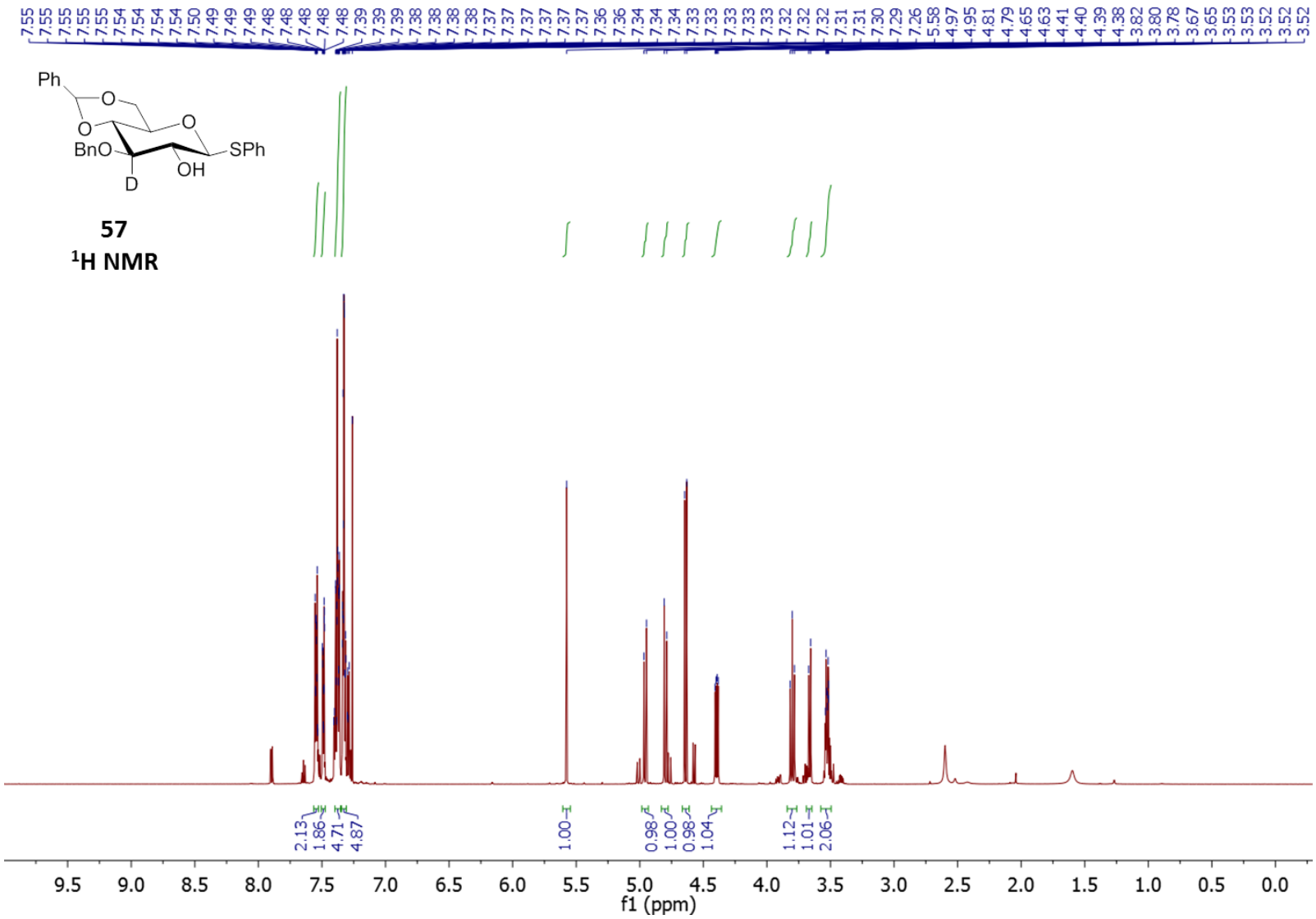

170.75
170.72
169.61
169.32
169.29
169.24
169.11
168.79
137.99
137.63
128.53
128.49
127.99
127.87
127.83
127.55
92.04
89.54
77.41
77.16
76.90
74.77
74.18
73.04
71.55
71.50
70.32
69.07
61.87
20.93
20.89
20.77
20.76
20.74
20.73
20.62
OAc
AcO
BnO
D
AcO
O
OAc
f1 (ppm)

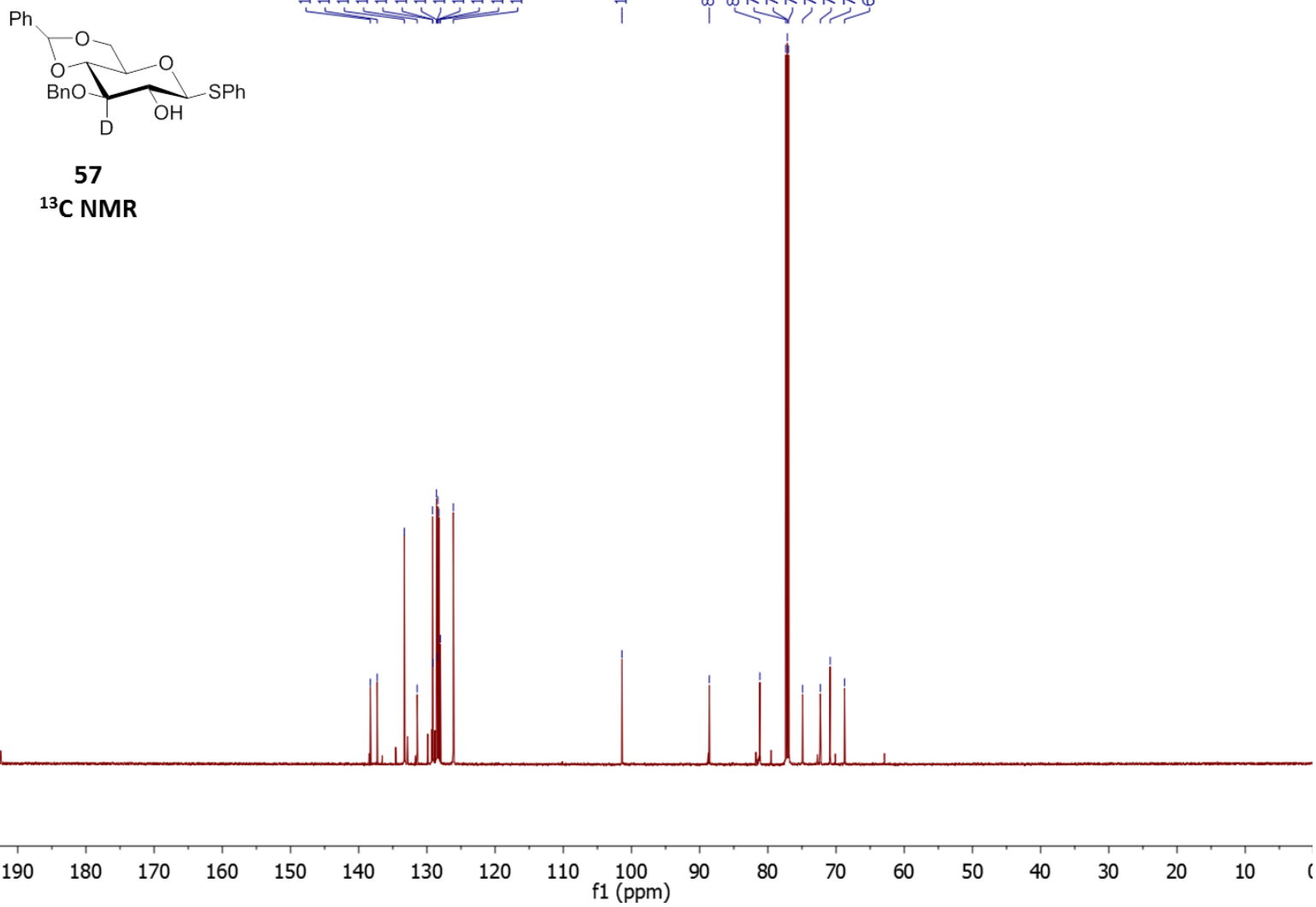
54

^{13}C NMR


55
^{1}H NMR
f1 (ppm)

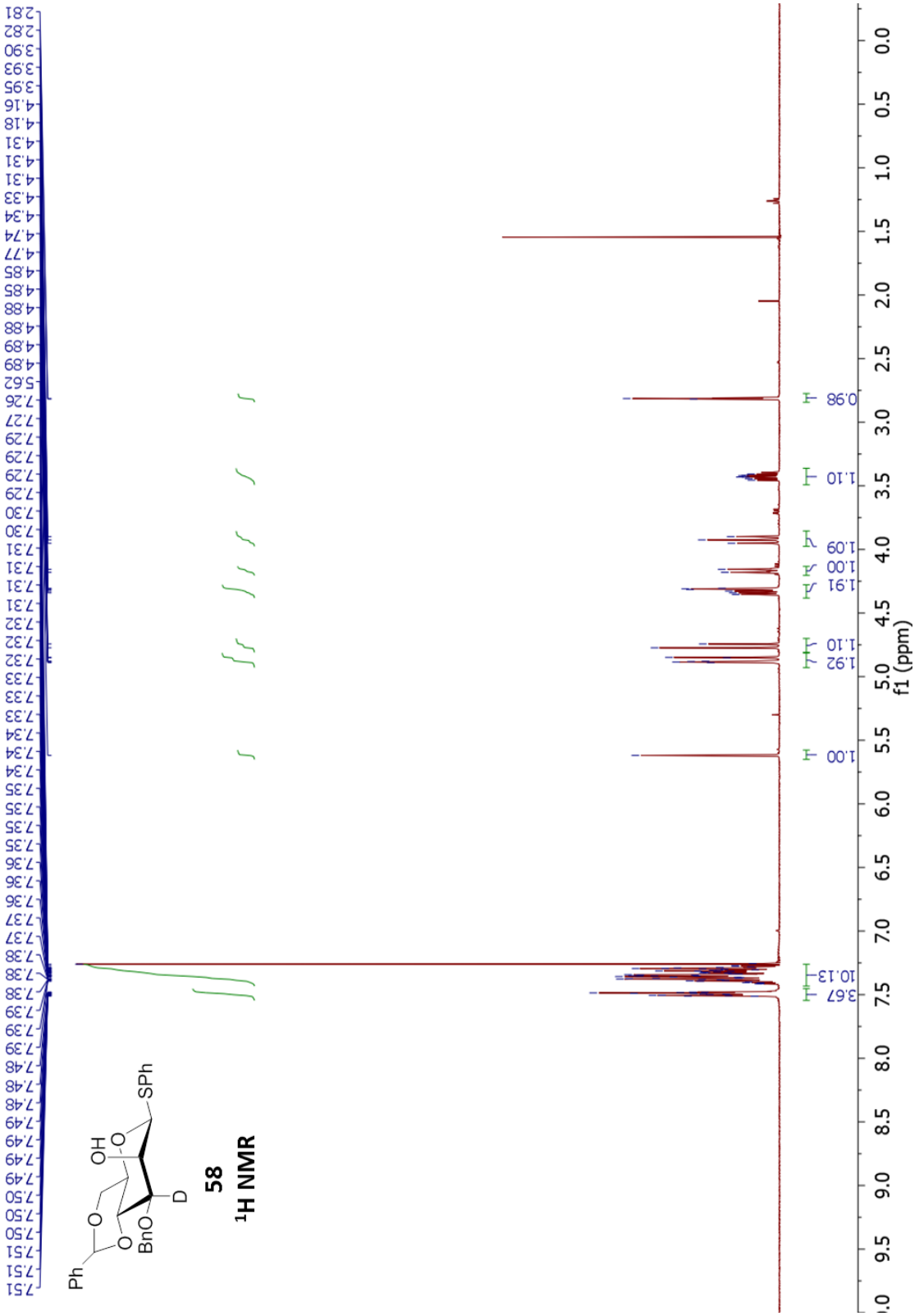

170.76
169.44
169.31
137.80
132.71
132.63
128.96
128.57
128.16
127.99
127.88
86.34
77.41
77.16
76.91
76.21
74.27
71.41
69.69
62.67
21.06
20.87
20.84
OAc
O
AcO
BnO
SPh
AcO
D
55
13C NMR
f1 (ppm)

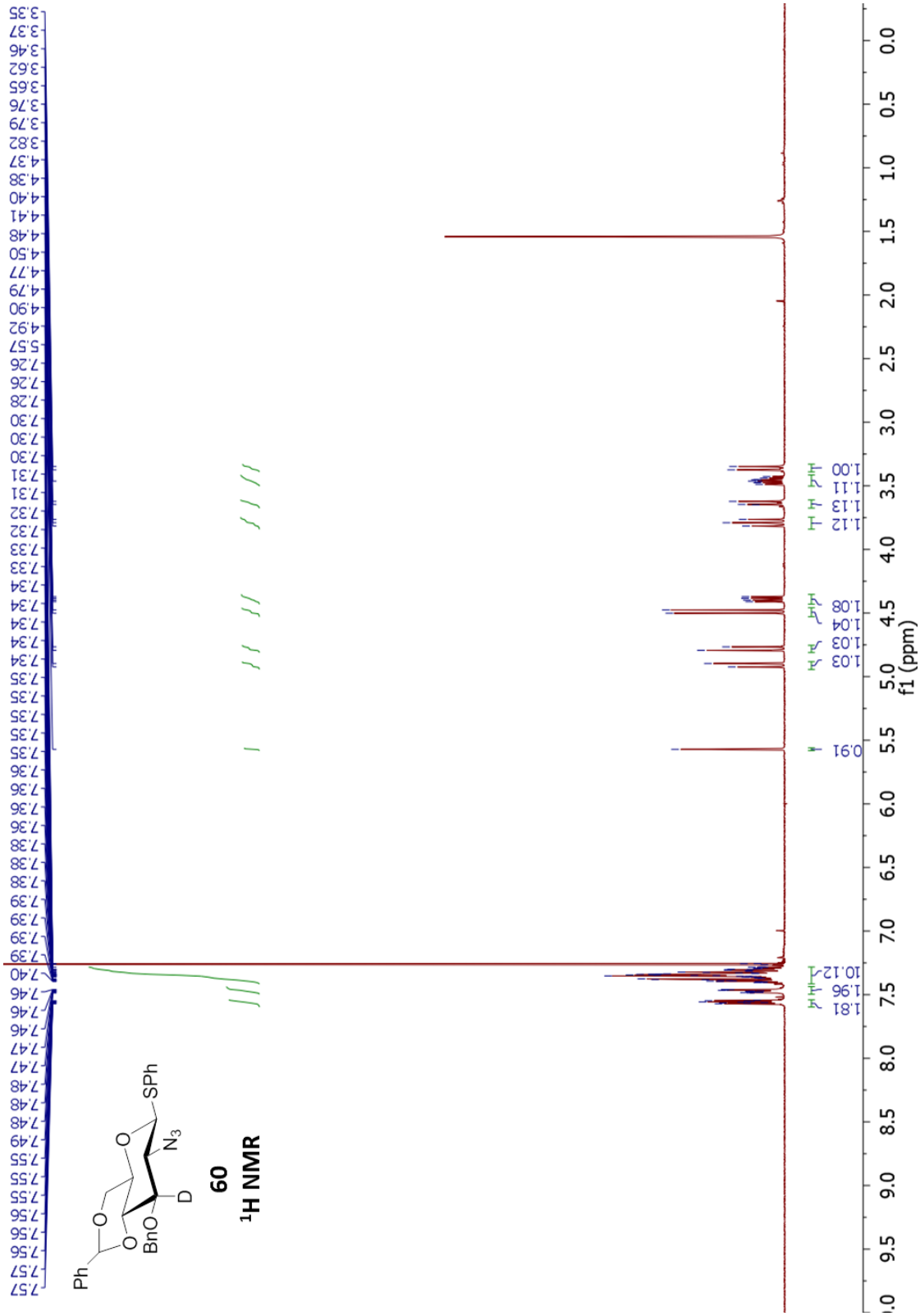

Ph
O
O
O
BnO
SPh
OH
D
57
1H NMR
f1 (ppm)


Ph
O
O
O
BnO
SPh
OH
D
138.30
137.31
133.32
131.44
129.17
129.15
128.60
128.50
128.39
128.25
128.02
126.13
101.40
88.59
81.17
77.37
77.16
76.95
74.91
72.31
70.87
68.76
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

57

^{13}C NMR


Ph
O
O
BnO
OH
O
D
SPh
58
^{1}H NMR
f1 (ppm)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O
BnO
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SPh
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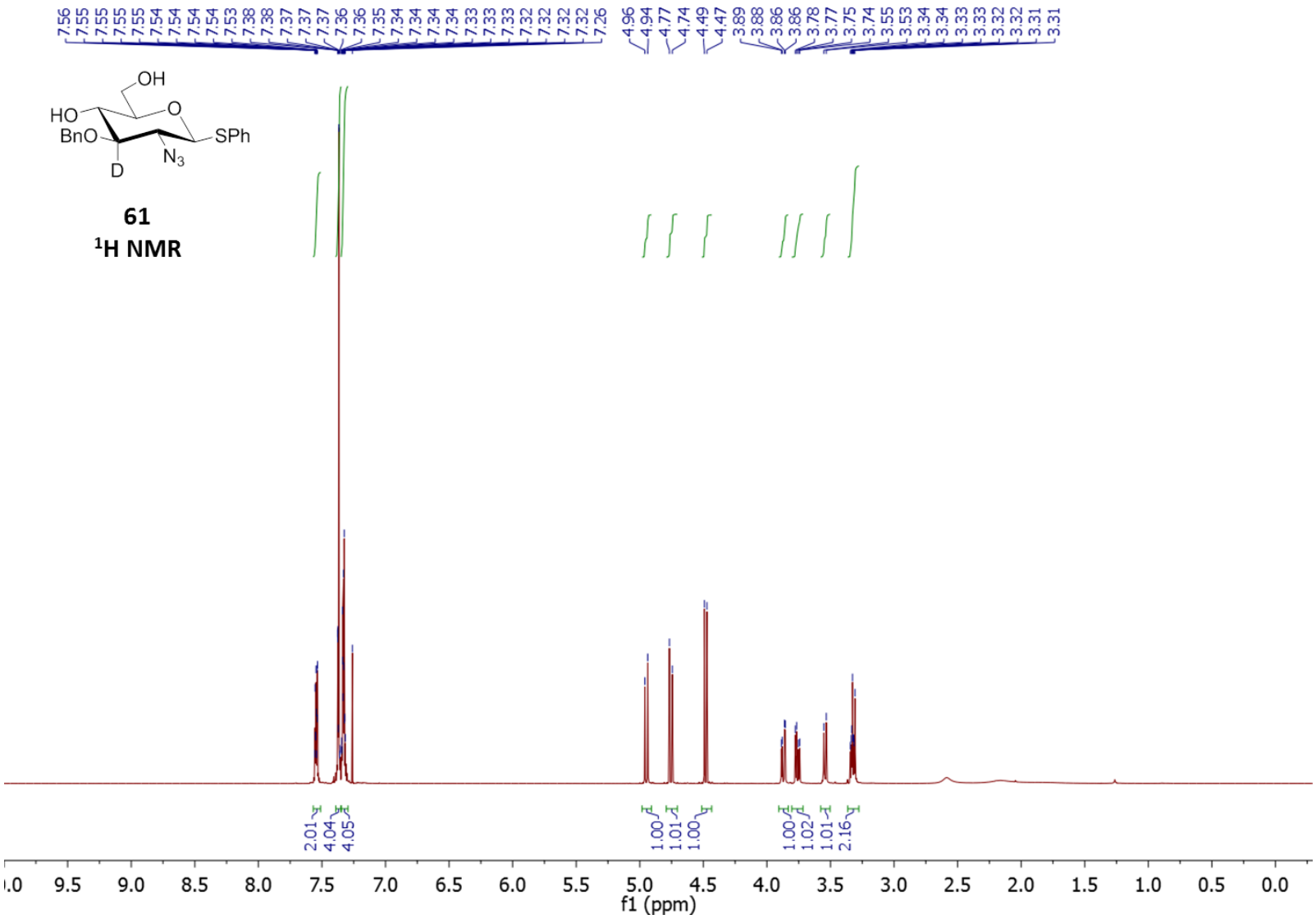
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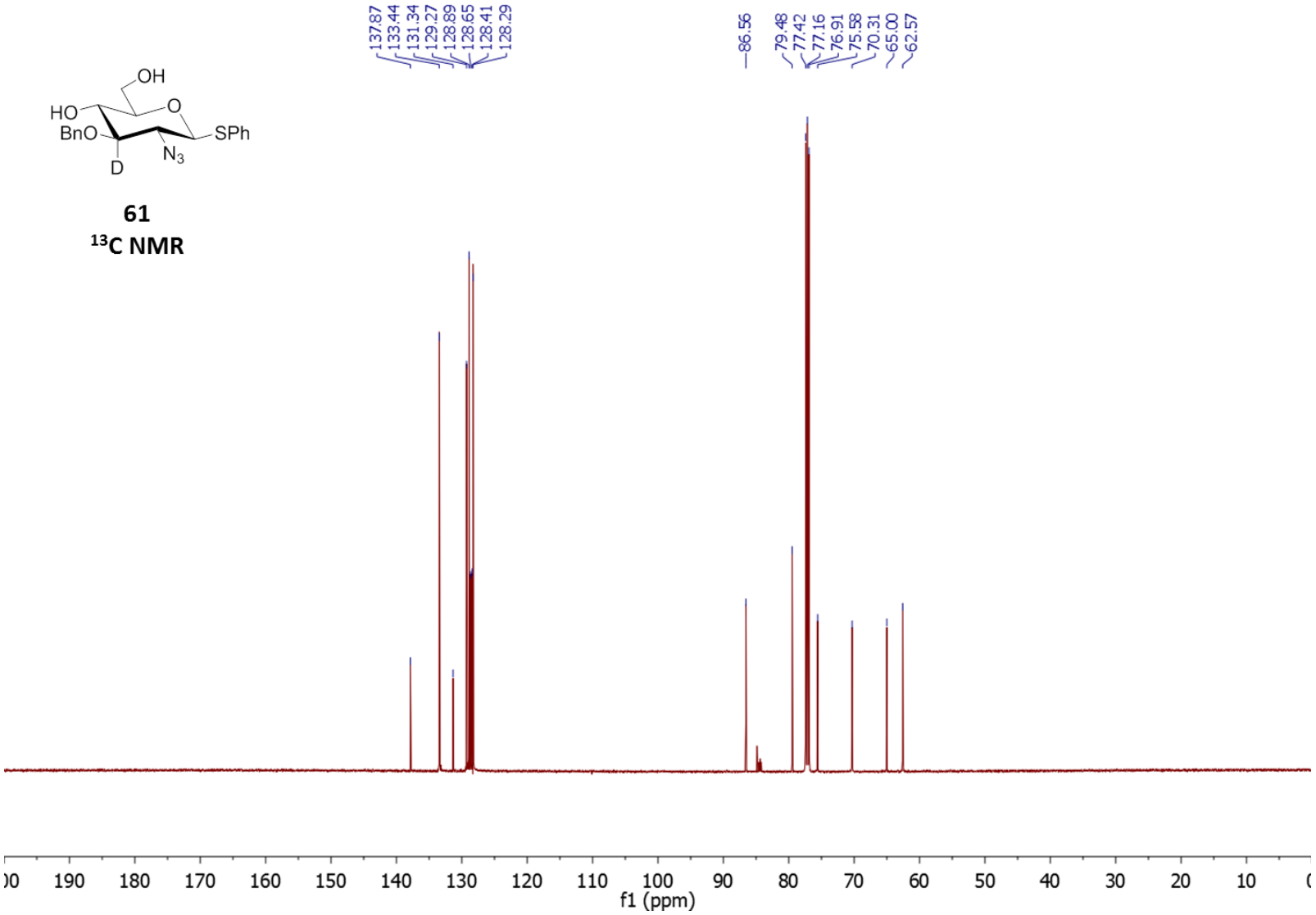
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76.91
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HO
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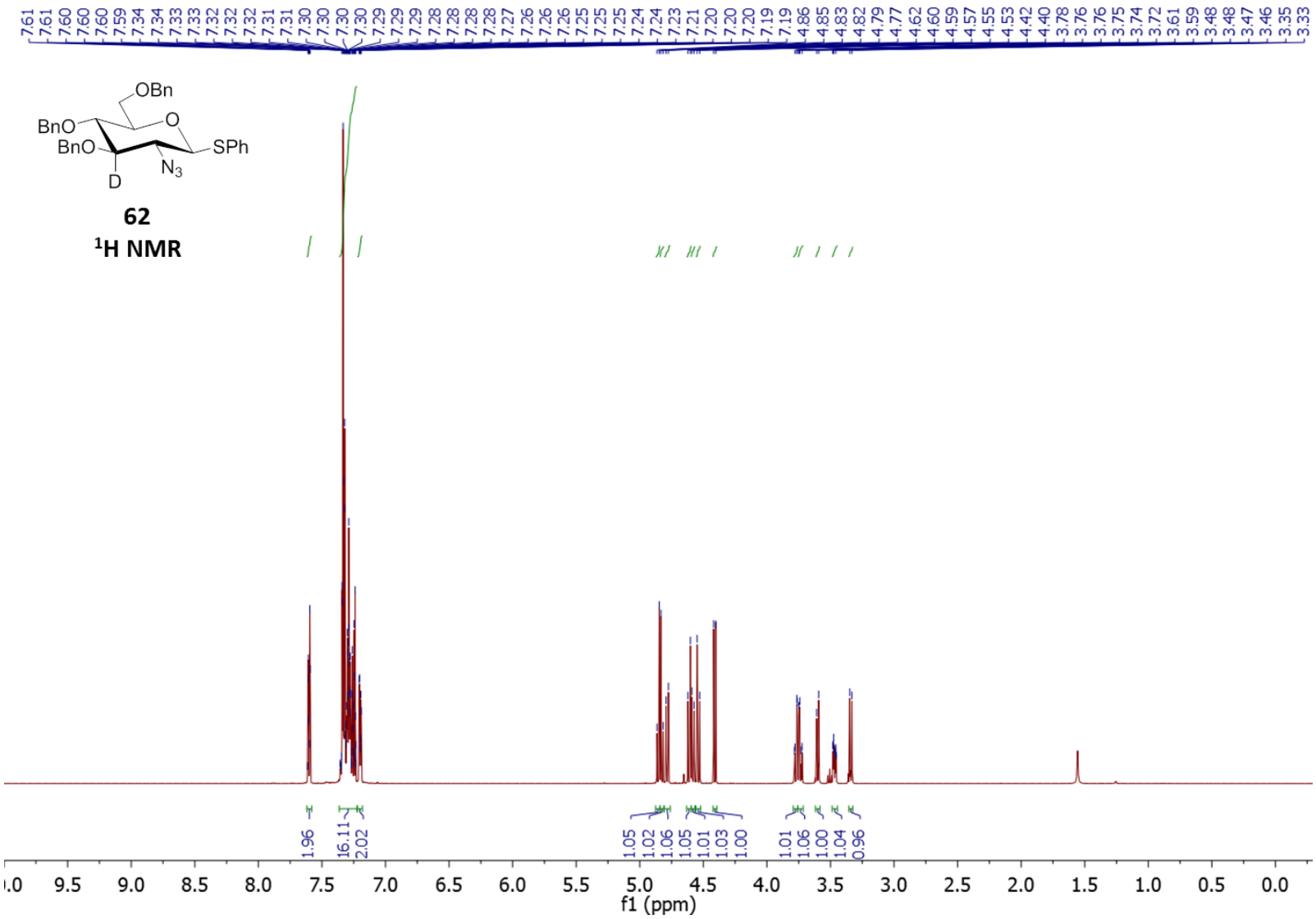
BnO
SPh
N3
D
61
13C NMR
f1 (ppm)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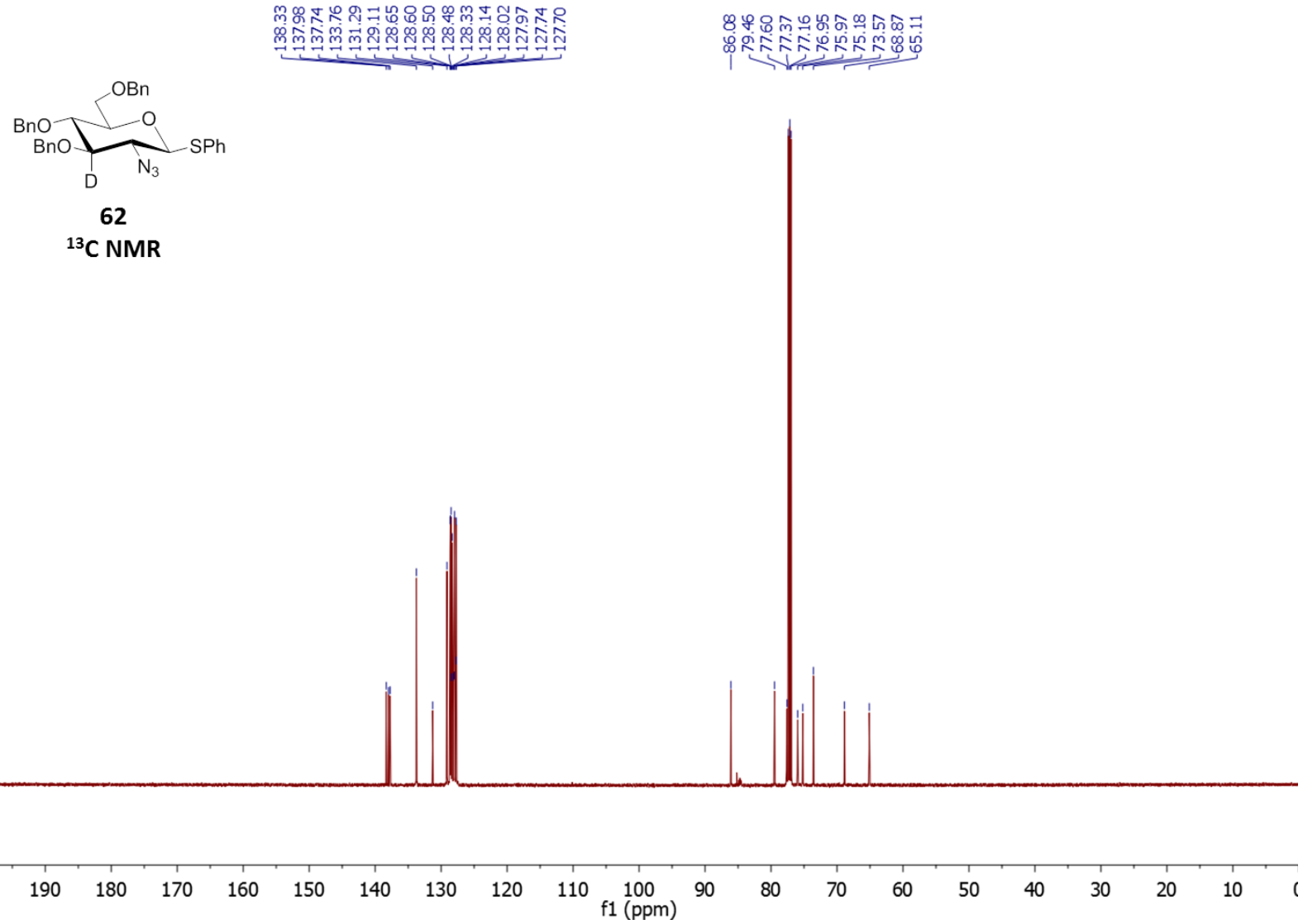
D
62
1H NMR
f1 (ppm)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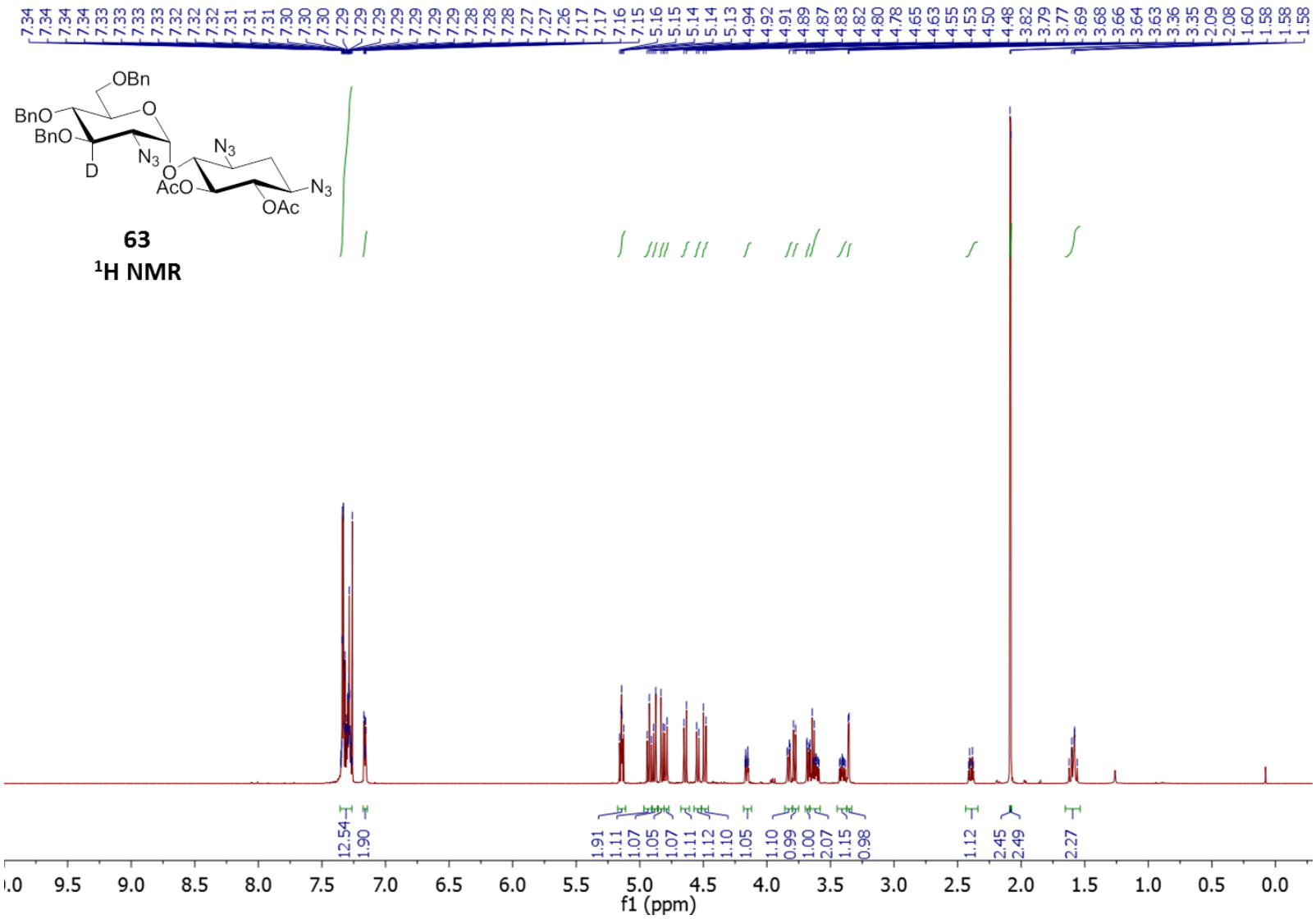
N3
D
f1 (ppm)

62

^{13}C NMR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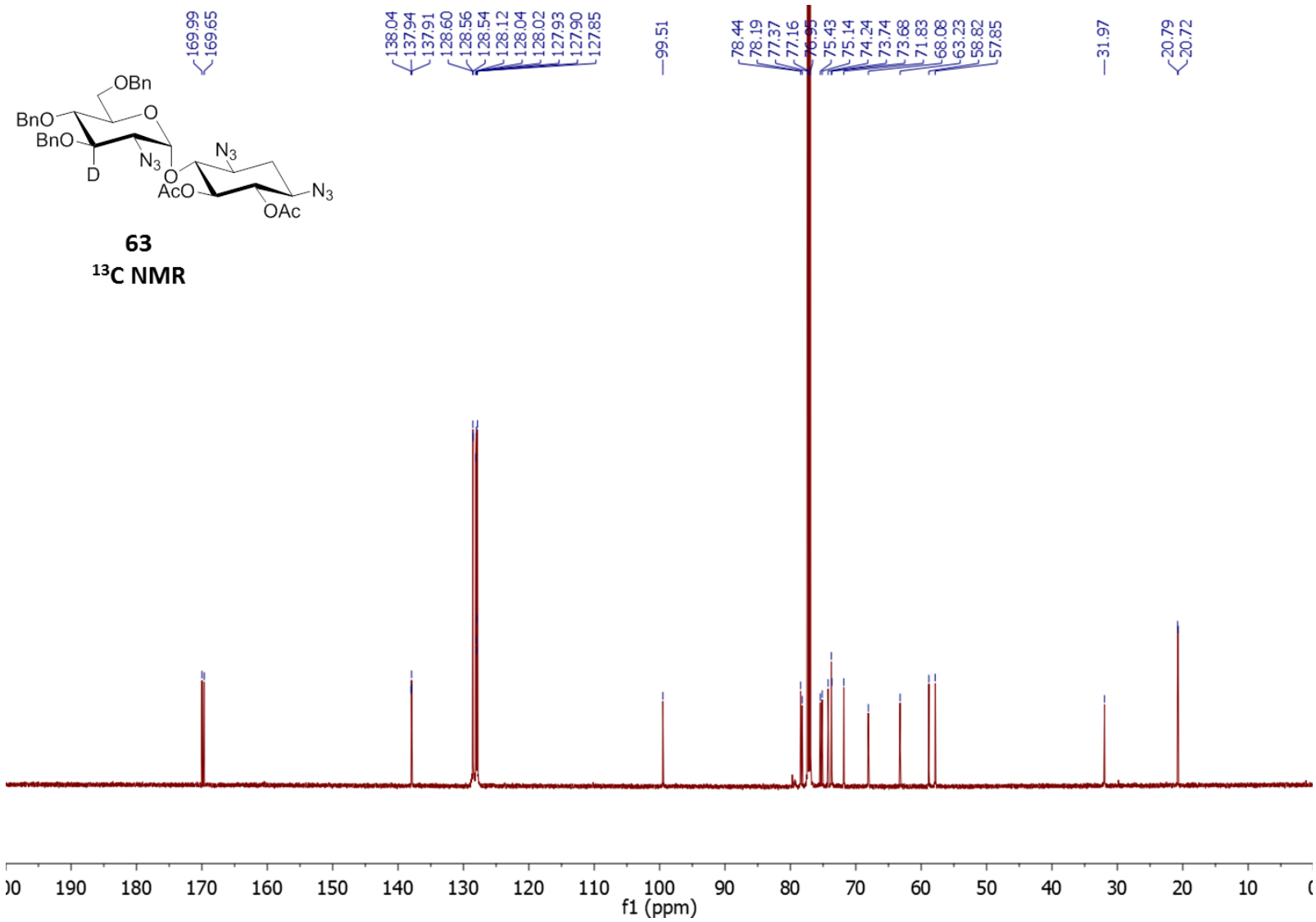
f1 (ppm)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OAc
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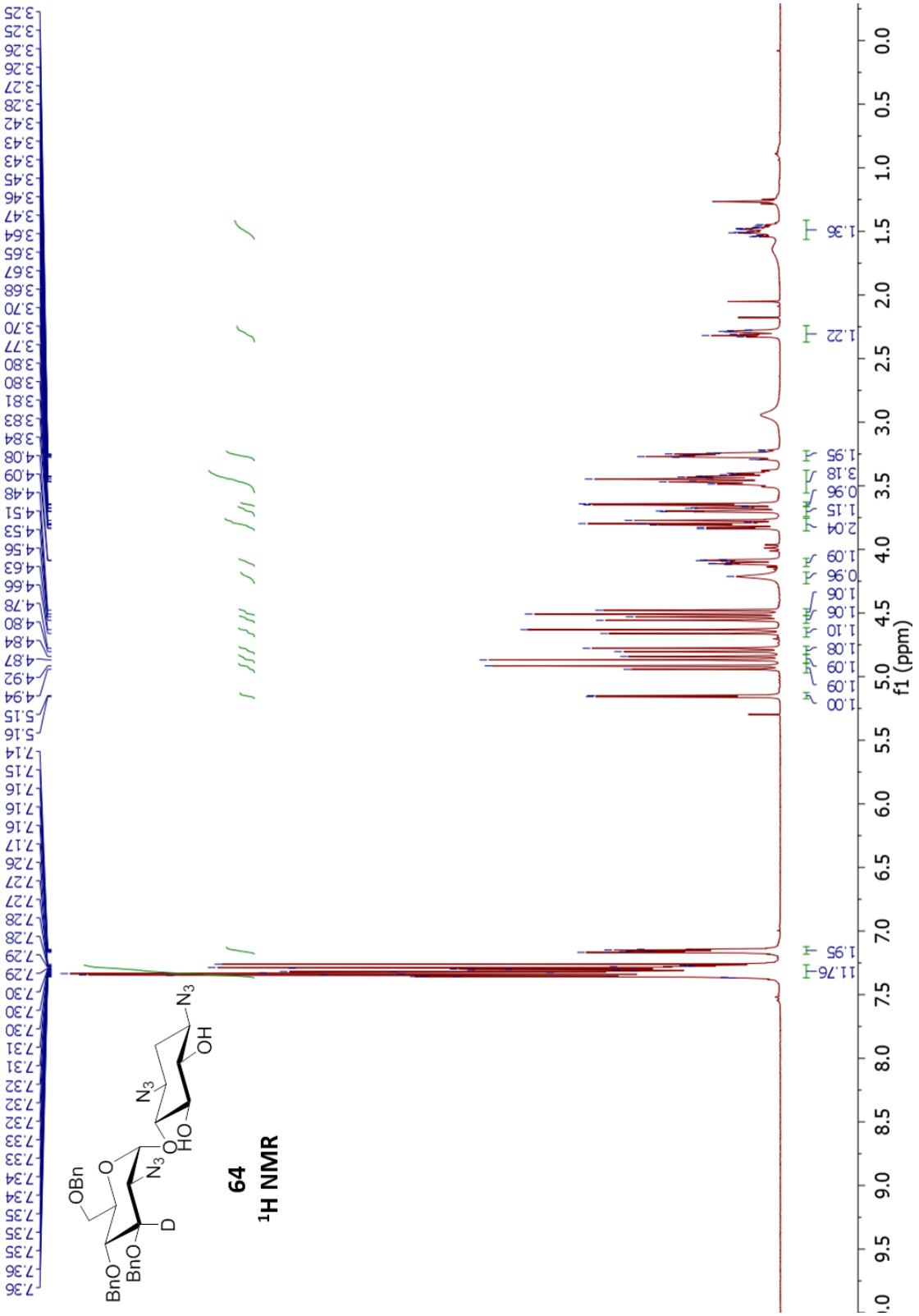
13C NMR
f1 (ppm)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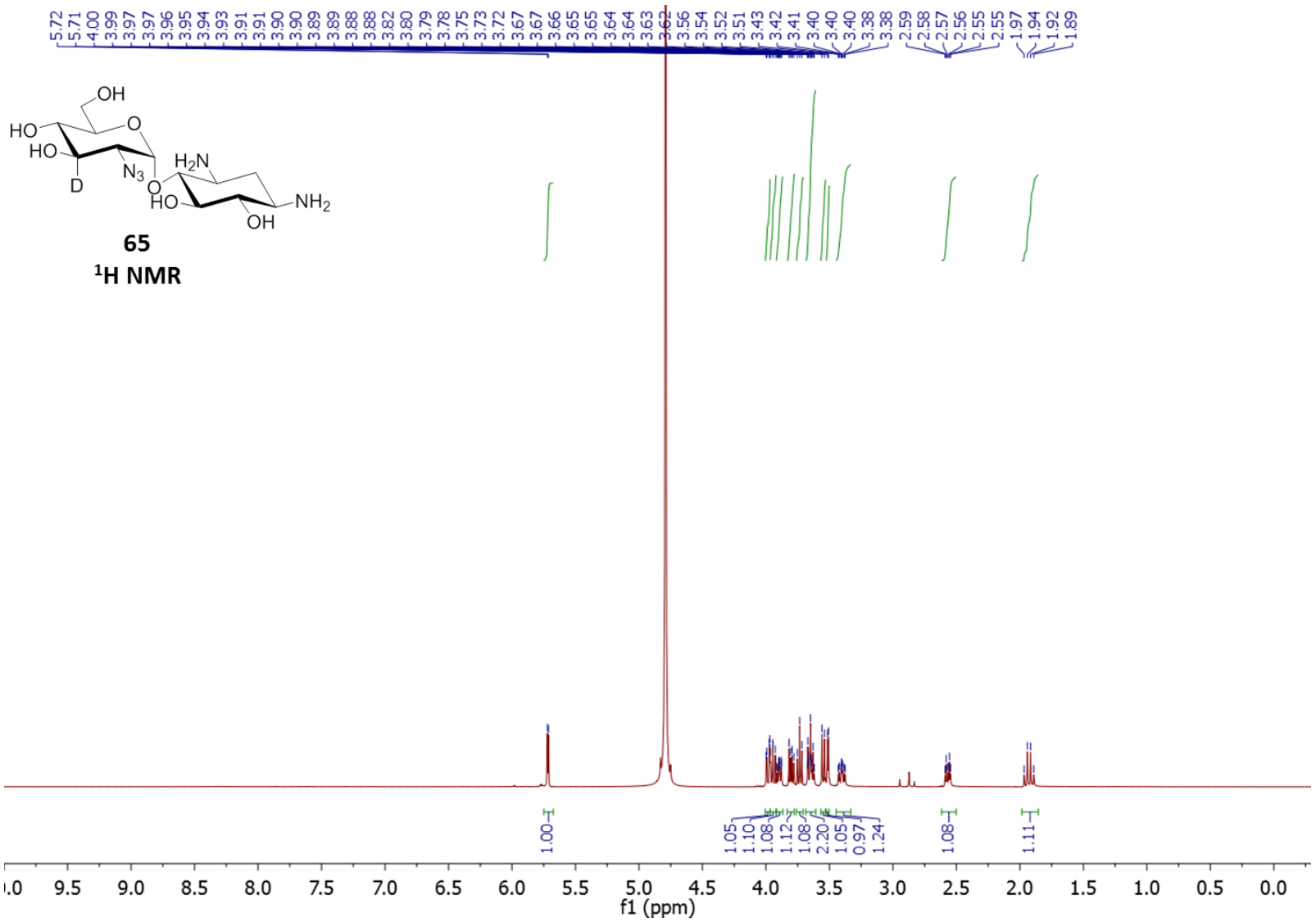
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HO
O
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N3
O
H2N
HO
NH2
OH
1.00
1.05
1.10
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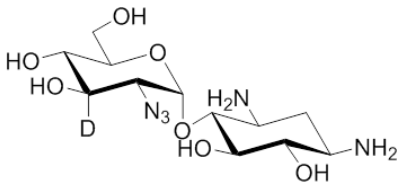
1.08
1.11
f1 (ppm)

65

^{1}H NMR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N3
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HO
NH2
OH

65

^{13}C NMR

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74.69
73.65
72.33
69.30
60.36
53.91
49.62
48.85
28.27
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

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